THE DEVELOPMENT OF POTENTIAL MALE CONTRACEPTIVES VIA INHIBITION OF CATSPER AND ALSO GBA2

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Last, but certainly not least is to thank my family. Though diasporic we may be, the bonds of kinship ground me in my roots. Your obvious love is a beacon in any dark day. I love you all.

Dedication

This thesis is dedicated to those who helped me through the years.

They know who they are.

They are cherished above all else.

And they will never, ever read this thesis.

ABSTRACT

The work presented herein constitutes the effort of one graduate student to mimic the efforts of a small biotech company. To this end, success was had on multiple fronts and the author is excited to showcase the data and results obtained on two different projects. Both projects focus on different targets of potential male contraceptives: spermiogenesis and sperm motility. In chapter one a very brief summary and introduction to the field of contraceptive research is presented, serving as an hors d'oeuvre to the main course of chapters two through four.

Chapters two and three detail the efforts towards developing blockers of an intriguing calcium ion channel called CatSper. The proper function of this channel is required for fertilization, as knockout mice are completely infertile with no deleterious phenotypes observed. Inhibitors of this channel are well-suited to applications in non-hormonal male contraception and two approaches towards developing blockers of this channel are described in chapters two and three.

Chapter two discusses the work done towards converting the endogenous activator of the channel, progesterone, into a blocker via systematic modifications to the steroid scaffold This approach yielded three compounds able to block the physiologically relevant openers of the channel and revealed discrepancies between steroidal blockers and classic t-type calcium channel blockers. The discovery, efficacy and discrepancies of and between these compounds is discussed at length.

Chapter three details the hit-to-lead development of two new scaffolds of CatSper blockers found via an HTS campaign which finished in 2012. Matrix chemistry was

utilized to generate libraries of focused compounds. The activity of these compounds in a developed influx assay could lead to further analog generation and eventually two submicromolar blockers of the channel were discovered and characterized. Additionally, the first steps of a fragment-inspired approach towards the development of a second scaffold is described in the latter half of chapter three.

Finally, chapter four discusses the efforts of a separate project, focusing on a class of molecules termed iminosugars, specifically aminocyclopentitols. These compounds strongly modulate glycosphingolipid processing, resulting in infertility from improper spermatogenesis. A series of compounds were synthesized and showed potent, and more importantly, selective inhibition of the enzyme responsible for degradation of these lipids (GBA2). Iminosugars, as a whole, are hindered by species-specific efficacy observations and the compounds synthesized and evaluated as part of Chapter 4 could help to illuminate these discrepancies in the future, should the desire arise to explore these observations further.

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Abbreviations

$[Ca^{2+}]_i$	Intracellular calcium ion concentration
11β-MNT	11β-Methyl-19-nortestosterone
11β-MNTD	11β-Methyl-19-nortestosterone dodecyl carbonate
2-AG	2-Arachidonyl glycerol
ABHD2	α/β-Hydrolase domain-containing protein 2
ADMET	Absorption, Distribution, Metabolism, Excretion, Toxicity
ALDO	Aldosterone
ALH	Amplitude of lateral head displacement
AM	Acetoxymethyl
APCI-TOF	Atmospheric-pressure chemical ionization – time of flight
app	Apparent
BODIPY	4,4-difluoro-4-bora-3a,4a-diaza-s-indacene
BRD4	Bromodomain 4
BRDT	Bromodomain – testes specific
BRSM	By recovered starting material
BSA	Bovine serum albumin
CASA	Computer-aided sperm analysis
CatSper	Cation channel of sperm
CCB	Calcium channel blocker
CDK2	Cyclic-dependent kinase 2
CF_3	Trifluoromethyl

CFTR	Cystic fibrosis transmembrane conductance
CGT	Ceramide-specific glucosyl transferase
CH_3CO_2H	Acetic acid
CI	Confidence interval
C_{max}	Max clearance
DCE	Dichloroethane
DMA	Dimethylandrolone
DMSO	Dimethylsulfoxide
EC_{50}	Effective concentration (to elicit 50% maximal effect)
EDC	Endocrine disrupting compound
EDG	Electron donating group
E_{max}	Effect concentration (to elicit full effect)
EPPIN	Epididymal peptidase inhibitor
EWG	Electron withdrawing group
FDA	Food and Drug Administration
FLIPR	Fluorescence Imaging Plate Reader
FSH	Follicle-stimulating hormone
GBA1	Glucocerebrosidase 1 (lysosomal)
GBA2	Glucosylceramidase 2 (non-lysosomal)
HAM	Hyperactivated motility
HDL	High-density lipoprotein
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HRMS	High resolution mass spectrometry
HTS	High throughput screening
IC_{50}	Inhibitory constant
$I_{catsper}$	CatSper currents
ITDD	Institute for Therapeutics Discovery and Development
IUD	Intrauterine device
K_B/pK_B	Equilibrium constant of binding (and log of this value)
K_i	Inhibitory constant
LC/MS/MS	Liquid chromatography/tandem mass spectrometry
LH	Luteinizing hormone
LHRH	Luteinizing hormone releasing hormone
LIN	Linearity
LNG	Levonorgestrel
lo/lo	Low potassium/ low pH buffer
LRMS	Low resolution mass spectrometry
m	*meta*
Me	Methyl
MeNT	7α-Methyl-19-nortestosterone
MLPCN	Molecular Libraries Probe Production Centers Network
MPA	Medroxyprogesterone acetate
mRNA	Messenger RNA
MUG	4-Methylumbelliferyl β-D-glucopyranoside

MW	Molecular weight
*N*B-DGJ	*n*-Butyl- deoxygalactonojirimycin
*N*B-DNJ	*n*-Butyl- deoxynojirimycin
ND	Not determined
NICHD	*Eunice Kennedy Shiver* National Institute of Child Health and Human Development
NNC	NNC 55-0396
o	*ortho*
OMe	Methoxy
p	*para*
PGE_1	Prostaglandin E_1
π	Pi, hydrophobicity constant
PK	Pharmacokinetics
prog	Progesterone
py	Pyridine
pyr	Pyrimidine
qICP-MS	Quantitative inductively coupled plasma mass spectrometry
qNMR	Quantitative nuclear magnetic resonance
r.t.	Room temperature
RARα	Retinoic acid receptor alpha
RFU	Relative fluorescence units
SAR	Structure activity relationship

SARM	Selective androgen receptor modulator
SD	Standard deviation
SEM	Standard error of the mean
σ	Sigma, substitution (electronic) effect
S_N2	Bimolecular nucleophilic substitution
S_NAr	Nucleophilic aromatic substitution
STAB	Sodium triacetoxyborohydride
$t_{1/2}$	Half life
THDOC	Tetrahydrodeoxycorticosterones
TLC	Thin layer chromatography
TMT	Trimercaptotriazine
UDP-Glucose	Uracil diphosphate glucose
UPLC	Ultra-performance liquid chromatography
V_{AP}	Average path velocity
V_{CL}	Curvilinear velocity
V_{SL}	Straight-line velocity.

Chapter One: Current Scope of Discovery in Male Contraception

Section 1.1 Impact and General Strategies of Research

The nutritional, economic and social consequences of unwanted pregnancies are wide-ranging and a great burden on both families and governments. Every year, worldwide there are more than 500 000 deaths from pregnancy-related complications and this figure does not include morbidities suffered by women from complications during pregnancy and childbirth.[2] Additionally, maternal deaths and morbidities associated with pregnancies and child birth are as high as 1324 per 100 000 births.[3] For these and many other reasons, further control over fertility is needed via novel, safe and effect contraceptive methods.

According to a 2008 report in *Contraception*, just over 50% of pregnancies in the US are unintended.[4] This number is decreasing slowly over time as cultural norms change but still remains around 50%.[5] Given that many women cannot make use of hormonal therapies or IUDs, they must instead rely on their partners for contraception, to which the only reliable methods are vasectomy or condoms, though the efficacy of these methods is questioned.[6] To this end, the need for a greater range of male-specific contraceptive options

Table 1.1. Willingness of men from surveyed countries to utilize a male contraceptive

If available would you be willing to use a new male fertility control?	*GER*	*SWE*	*USA*	*ARG*	*BRA*	*MEX*	*INDON*
Willing	69.0	58.1	49.3	44.4	62.7	65.4	28.5
Uncertain	24.4	17.4	38.4	13.2	12.8	8.9	37.3
Disapproving	6.6	24.4	12.4	42.3	24.5	25.7	34.2

GER = Germany, SWE = Sweden, USA = United States of America, ARG = Argentina, BRA = Brazil, MEX = Mexico and INDON = Indonesia. Data is adapted from reference.[1]

is great. The discovery of new contraceptive methods for men has been hindered by cultural stigmas and social dynamics.[7, 8] Fortunately, it appears this outlook on male fertility control is finally changing with recent polls and press showing an increasing interest from men with respect to male-specific contraceptive methods.[9] An acceptability study of over 9000 men from 9 countries on 4 continents showed that men from all major religions and cultures indicated a willingness to use a male contraceptive if proven safe and reversible.

From a drug development standpoint, the development of an effective, safe and reversible male contraceptive is incredibly challenging. Pregnancy, as a medical condition, is limited to the female half of any partnership. Societal and moral obligations aside, the man in any relationship, however casual or committed, suffers no physical health risks from pregnancy. Mental health of both partners in an unwanted pregnancy is obviously at risk, however it is the female of a couple who carries the child and suffers the physical ailments associated with pregnancy. Because of this, a contraceptive intended for men must be exquisitely safe and reversible, unlike in other morbidities in which the standards for safety can be reduced, given the state of the physical risk to the patient if left untreated. The "patients" taking a male contraceptive therapy are healthy individuals and the course of treatment could be many years, rather than months.[10]

In general, potential male contraceptives target either spermatogenesis or the function of mature spermatozoa.[11] Contraceptives affecting either of these categories exist as hormonal or non-hormonal therapies. Hormonal therapies were first explored in men, given their success in women, however these methods are difficult to achieve success, given the complicated hormonal regulation and serious side effects of testosterone

imbalance.[12] As such, non-hormonal therapies are highly sought after, though it should be noted that recent successes of a synthetic androgen have garnered much attention in the press.[13] This synthetic androgen prodrug successfully reduces levels of testosterone within the testes to levels insufficient to support production of sperm, while the concentrations circulating in the rest of the body remain nominal, circumventing one of the greatest pitfalls of hormonal treatments as discussed below.

Section 1.2 Hormonal Therapies as Male Contraceptives

Hormonal therapies as potential male contraceptives have been the first and longest studied, given their success in women.[14] The studies surrounding hormonal therapies have centered on the inhibition of gonadotropin production and the resulting decrease in the production of testicular testosterone. Testicular testosterone concentrations are typically 40- to 100-fold higher than serum levels.[15] This high concentration is needed to support spermatogenesis and therefore therapies that reduce testicular testosterone levels are able to decrease sperm production. This has been proven to be the case in several independent studies.[16, 17] The problems surrounding these therapies has been an inability to limit the reduction of serum testosterone concentrations, thus avoiding several side effects.[18]

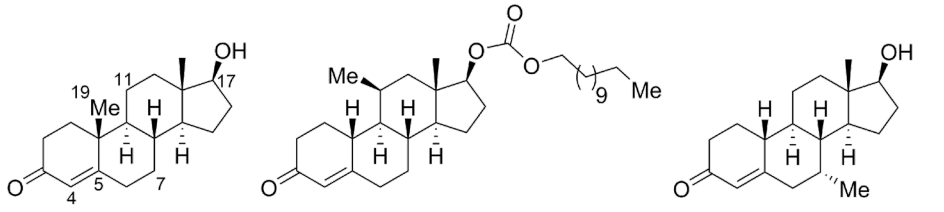
In all studies except the most recent, serum testosterone levels drop sufficiently low to cause a negative feedback to the hypothalamus and pituitary, shutting of secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH). Diminished levels of these hormones reduce testosterone production by the Leydig cells, however concomitant drop in serum testosterone levels causes the frequently observed side-effects such as loss

of libido and muscle mass.[19] If used, these therapies would require androgen replacement therapy to restore these functions.

Progestins have long been used in female contraceptives and are available in a variety of formulations, making them great candidates for potential use in men.[20, 21] This class of hormones has been shown to reduce gonadotropin levels, and by extension, greatly diminish sperm counts.[22] Their efficacy in vivo, however, has been limited and the focus of the field has turned, instead, to androgen-mediated therapies.[23]

The parent androgen testosterone is, itself, not available in an oral dosage form and hepatotoxicity has been observed in orally bioavailable derivatives.[24] Additionally, testosterone, as a drug, displays severe side effects given its inability to selectively modulate its androgenic (sexual) or anabolic (muscle building) effects.[25] As such, synthetic derivatives of testosterone able to selectively modulate these functions of testosterone hold great promise. These selective androgen receptor modulators, or SARMs, have been the pursuit of researchers for over a decade and several synthetic androgens acting as SARMs are currently being tested in human patients, showing promise.[26, 27]

Figure 1.1 shows the structures of two of the most promising synthetic androgens. 11-beta-Methylnortestosterone (11β–MNT) and its undecanoate prodrug (11β–MNTD)



Testosterone | 11β-Methyl-19-nortestosterone dodecylcarbonate | 7α-Methyl-19-nortestosterone

Figure 1.1. Structures of Testosterone and Synthetic Androgens Being Pursued as Hormonal Male Contraceptives.

have shown efficacy in rabbit models and are currently being tested in healthy human males, passing initial safety studies.[28] Another synthetic androgen, 7α-methylnortestosterone (MENT), shows promise since it cannot undergo 5α-reduction and, as such, is less likely to show the same side effects observed with testosterone.[29] However, 7α-MENT shows poor bioavailability and as such needs to be delivered continuously via an implant.[30] A similar application is employed in androgen replacement therapies, and therefore an implant requirement is not problematic. The use of implants is also frequently used in female contraceptives for continuous release.

All androgens discussed thus far act by inhibiting sperm production. While promising as a means to male contraception, is not without its drawbacks. One drawback with respect to these therapies is the time required to sufficiently reduce sperm population.[31] It takes 12 to 16 weeks of treatment to reduce the sperm count to levels deemed contraceptive.[32] This is a nontrivial amount of time for a therapy to take effect. Furthermore, patient compliance would need to be pristine, as several missed administrations could trigger sperm production in Leydig cells, undoing weeks of treatment. This of course assumes oral dosing instead of gel patches or subcutaneous release mechanisms, which apply a constant dose of would-be contraceptive.

Another drawback to this approach is the potential need for regular screening to ensure sperm production is indeed sustained at a low level. Kits exist for at home use that could alleviate this pitfall, however it is nonetheless an extant obstacle. These drawbacks assume that the side effects observed with androgen treatment in the past are indeed just that: in the past.

But the largest drawback for testosterone-modulating contraceptives is that of the side effects observed in men dosed with the hormone. The anabolic effects of testosterone dosing come first to mind in public – changes in behavior and irritable mood dominate the press.[33] However even more serious is the uncertainties surrounding the effects of testosterone therapy on cardiovascular health of men. One study found that testosterone therapy can increase the risk of heath attack, while another showed that it may, in fact, lower the risk of cardiovascular disease.[34] While the cardiovascular side effects are serious and warrant further investigation, other side effects remain including sleep apnea, enlarged prostrate (noncancerous) and blood clotting.[35] For previously mentioned reasoned, these side effects need to be nearly nonexistent for a male contraceptive to be approved by the FDA. As such, the field has largely turned to non-hormonal contraceptive options.

Section 1.3 Nonhormonal Therapies as Male Contraceptives

Given the challenges surrounding hormonal therapies for male contraception, nonhormonal targets have been the subject of intense research in the community. Nonhormonal targets include those effecting sperm production, maturation and or function.[11, 36] While a plethora of targets exist and show promising initial results, the challenge remains to ensure the testes-specificity of these compounds. Additionally, penetration of the blood-testes barrier presents another hurdle.[37] Nonhormonal targets include proteins ranging from kinases to ion channels to nuclear receptors. The diversity of this class of potential therapies has been the source of extensive and multiple reviews and will not be discussed in detail in this thesis, however a brief overview follows.[38-40]

Spermatogenesis is a promising target for contraception, both by hormonal and nonhormonal agents. Inhibitors of proteins involved in meiosis prevent spermatogenesis by reducing the number of germ cells present, as well as their maturation. Kinases are involved in the meiotic process and cyclin-dependent kinase 2 (CDK2) knockout male and female mice are infertile.[41]

Targeting retinoic acid receptor alpha (RARα) has demonstrated efficacy when studied with antagonists thats inhibit spermatogenesis, similar to the aforementioned kinases.[42] Difficulties in the metabolism of these compounds have limited their development, though work is ongoing in several labs.[43] At the later stages of spermatogenesis, the germ cell must interact with the surrounding Sertoli cells as they finish maturation.[44, 45] Several classes of compounds have been developed to inhibit this

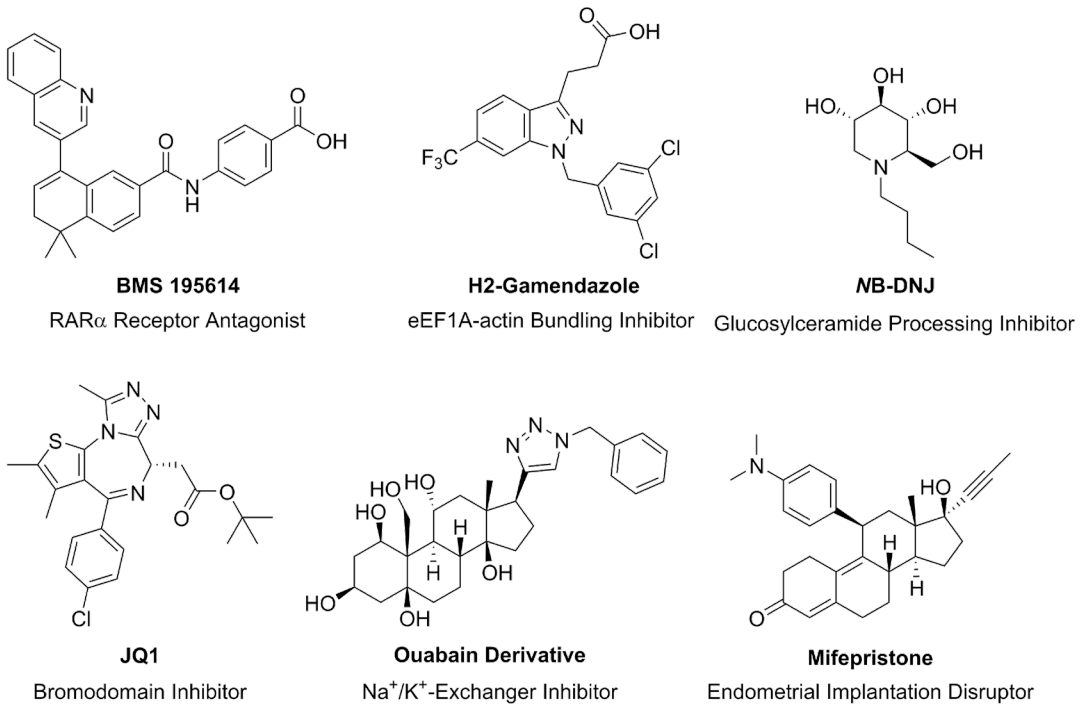


Figure 1.2. Structure and Targets for Studied Nonhormonal Contraceptives Affecting both Spermatogenesis and Sperm Function.

interaction, leading to infertility. Indazole carboxylic acids such as lonidamine,[46] adjudin[44] and gamendazole[47] (Figure 1.2) disrupt Sertoli cell-germ cell interactions.

The second chapter of this thesis pertains to a class of compounds referred to as iminosugars and their ability to inhibit spermatogenesis by modulating glycoprocessing. Specifically, the formation and degradation of glucosylceramide.[48]

This class of compounds reversibly induce infertility in C57BL/6 mice but not in other mice species, rabbits and humans.[49] Further research is required to better understand the physiologically relevant targets that these compounds are inhibiting.

The testes-specific bromodomains (BRDT), an epigenetic regulator, is another target regulating spermatogenesis and is the subject of intense study, though selectivity over analogous, ubiquitous domains will need to be achieved, given the side effects observed with pan bromodomain inhibitors.[50-53]

Affecting sperm function once matured is one of the most prevailing strategies when for non-hormonal male contraception. Most of the targets currently explored have been validated by knockout studies in which the knockout mice showed little effect beyond the desired infertility.[54] Within this class of potential drug targets, ion channels are prominent and include channels specific for calcium[55] and potassium[56], and as well as channels responsible for the regulation of pH, volume and osmolarity (aquaporins).[57-60] Many targets are available for study within this class and noteworthy strides have been made in developing inhibitors for the testis-specific Na,K-α4 ATPase exchanger.[61] Analogs of the cardenolide ouabain have proven to be potent and selective inhibitors of the

testes-specific isoform α4 with in vivo activity that significantly reduces sperm motility, including hyperactivated motility (HAM).[62, 63]

In addition to small molecule inhibition of targets responsible for sperm maturation and function, effort has been put towards the development of a contraceptive vaccine. Immunocontraception involves the use of antigens to target different aspects of gamete production.[38] Vaccines targeting luteinizing hormone releasing hormone (LHRH) have been tested and reduce testosterone production.[64] It remains to be seen if these vaccines would circumvent the issues observed with hormonal therapies. These therapies would not be orally bioavailable and could have difficulty penetrating the blood-testes barrier. Nonetheless, efficacy has been shown in male monkeys after injections with human recombinant epididymal protease inhibitor (EPPIN), a serine protease inhibitor expressed in the testis. Contraceptive vaccines remain an ongoing area of research for several research groups.[65, 66]

Section 1.4 Summary and Outlook

Given recent results from the safety studies for 11β-MNTDC male contraception may be available to men seeking fertility control in the not-so-distant future. Further development of these promising compounds, and indeed, any compound showing efficacy, be it hormonal or nonhormonal, will require the involvement of a pharmaceutical company.[1] This involvement is currently lacking but once a contraceptive has completed the rigid clinical trials required of these potential therapies, involvement of a company is more likely. Men all over the world, from all walks of life are seeking to carry their weight in preventing unplanned pregnancies. The development of a safe, reversible male

contraceptive would be a boon to men and women everywhere and this thesis details efforts on two fronts: aminocyclopentitols as inhibitors of spermatogenesis and CatSper inhibitors which significantly reduce sperm motility.

Chapter 2 Discovery and Characterization of Steroidal Blockers of the CatSper Complex

Section 2.1 CatSper Form and Function

Characterized for the first time in 2001, the cation channel of sperm (CatSper) is the foremost entry point of calcium into mature spermatozoa.[67, 68] CatSper is a tetrameric, strongly voltage-gated ion channel consisting of at least 4 subunits: alpha, beta, delta, gamma and zeta and epsilon, though the latter two have only been shown to exist in mice, currently.[69-72] CatSper is exquisitely testes-specific, demonstrated by Northern blot analysis in which it was shown that target mRNA was only present in tissue isolated from the testes and nowhere else in the body.[68]

Given this specificity, CatSper was immediately implicated in fertility. Mating studies with catsper3$^{-/-}$ mice showed that male mice bearing this knockout were completely infertile with no other observable phenotypes. Furthermore, it was shown that if any of the complex components were knocked out, mice exhibited complete infertility. It should be noted that female mice bearing this knockout were completely homologous to their wildtype counterparts.[55]

The observed infertility was a result of the inability of sperm from knockout mice to achieve hyperactivated motility (HAM). HAM is crucial to successful union between the sperm and egg and is also required for mature spermatozoa to traverse the upper reproductive tract, in which the mucus is quite thick.[73] Frequently sperm become ensconced in the vascular folds of the endometrium and must release themselves when the time comes for the final step of fertilization.[74] The egg itself is coated with a thick polysaccharide coat known as the zona pellucida.[75] This final barrier cannot be penetrated

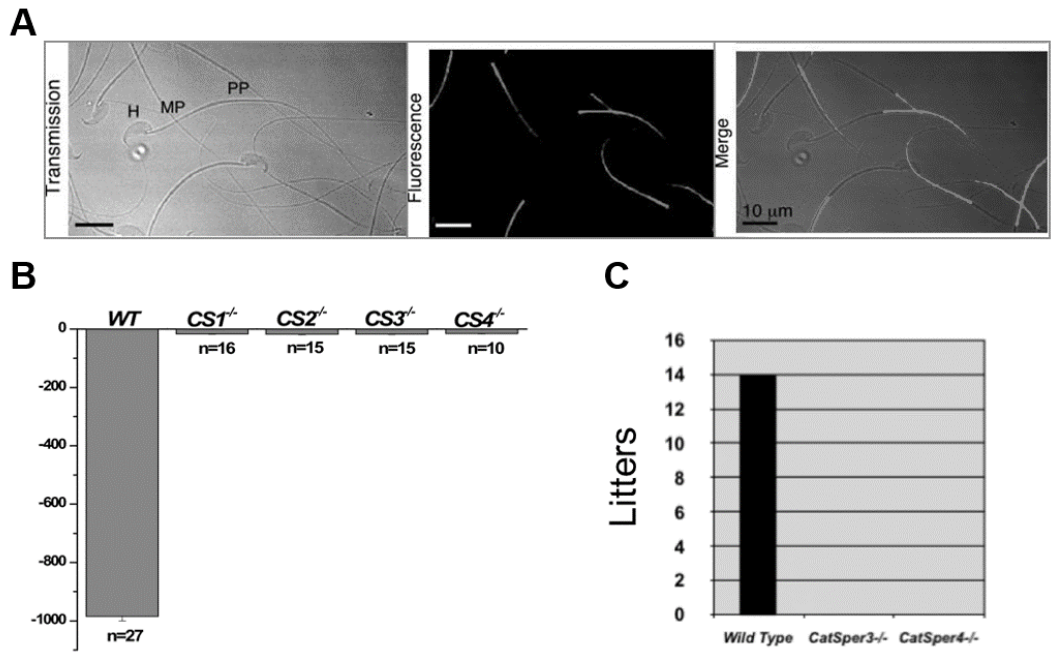


Figure 2.1. The CatSper Complex is Required for Proper Fertilization. (A) Confocal microscopy showing localization of CatSper antibodies to the principal piece of the sperm tail. (B) Electrophysiology experiments demonstrating the need for each α-subunit of the CatSper complex for proper function. (C) Results of mating studies between wildtype female and the respective knockout male mice. Images from 54.

without first achieving HAM.[76] As such, CatSper function is vital to successful fertilization and is therefore considered to be a promising non-hormonal contraceptive target.

Endogenously, calcium influx via CatSper is greatly potentiated by the sex hormone progesterone and prostaglandin E_1 (PGE_1, Figure 2.2).[77, 78] A large bolus of progesterone is released into the upper reproductive tract from the cumulus cells surrounding the ovum upon release of an egg.[79] This increase in progesterone concentration activates CatSper and causes the sperm to release from the endometrium wall and proceed towards the released egg. In certain infertility cases, sperm from otherwise healthy men failed to respond to a progesterone stimulus.[80, 81] Besides giving further credence to

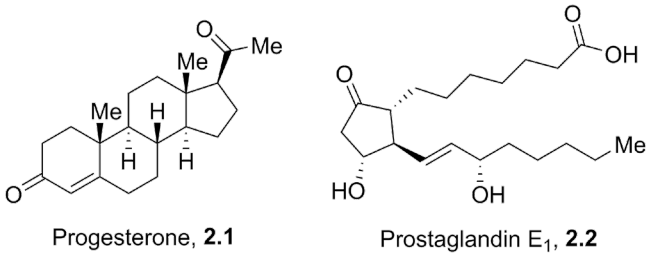


Figure. 2.2. Structures of Progesterone and Prostaglandin E_1 (PGE_1).

CatSper as a promising contraceptive target, this observation also directly validated the hypothesis that compounds able to prevent the progesterone-induced influx of Ca^{2+} from CatSper could serve as contraceptives.

While the actions of progesterone on sperm have been known for many decades, it is only recently that a detailed characterization was achieved. It was shown in the late 80s that progesterone induced calcium influx in sperm, which elicited the aforementioned HAM.[77] In 2011, electrophysiology experiments by two independent groups demonstrated that progesterone activates CatSper.[82, 83] And, in 2016, it was reported that rather than binding to the CatSper complex, progesterone instead binds to a previously orphaned hydrolase called α/β-hydrolase domain containing protein 2 (ABHD2).[84] By binding to and activating this hydrolase, 2-arachidonoyl glycerol (2-AG) concentrations within the sperm plasma membrane drop. Lowering 2-AG concentrations reduces the voltage threshold at which CatSper activation occurs, potentiating the inward flux observed in electrophysiology experiments.[84] What remains to be determined is whether or not the other activator of CatSper, PGE_1 also activates CatSper via the same mechanism. It has been proposed that this is not the case, though the work presented herein will stand in contrast to the prevailing views.

CatSper, a validated target for nonhormonal contraception in men, is the subject of vigorous study in the field. A compound able to selectively block CatSper activation would be an excellent, novel contraceptive method for men. To this end we endeavored to discover new CatSper blockers via two distinct strategies. Detailed in this chapter are the efforts towards modifying one of the endogenous activators, progesterone, in an attempt to change this activator to a blocker of CatSper.

Section 2.2 The SAR of Progesterone-Induced CatSper Activation

Section 2.2.1 Study Goal and Overview

It has been demonstrated that structural modifications to a ligand can lead to drastic changes in its activity at a particular target, such as converting agonists to antagonists.[85] To this end, a series of progesterone analogs were purchased, bearing small structural changes to the parent steroid in order to thoroughly probe the structure-activity relationship (SAR) of this CatSper-activating steroid scaffold as represented by Figure 2.3. Additionally, clinically used progestins and anti-progestins were purchased and tested for their ability to activate CatSper. Since all steroids share similar scaffolds, we investigated additional steroid classes to extend this SAR study.

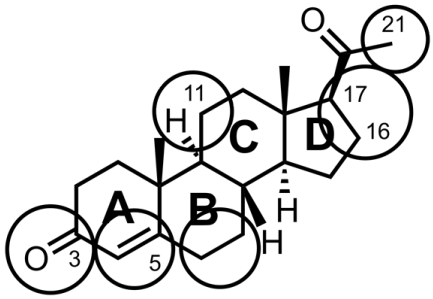


Figure 2.3. Sites of Potential Modification to the Progesterone Scaffold.

Section 2.2.2 Fluorescence Intensity Plate Reader (FLIPR) Assay for the Measurement of Calcium Influx: Development and Validation

My study began by verifying a previously developed fluorescence intensity plate reader (FLIPR) assay by comparing the potency of progesterone to increase $[Ca^{2+}]_i$ in

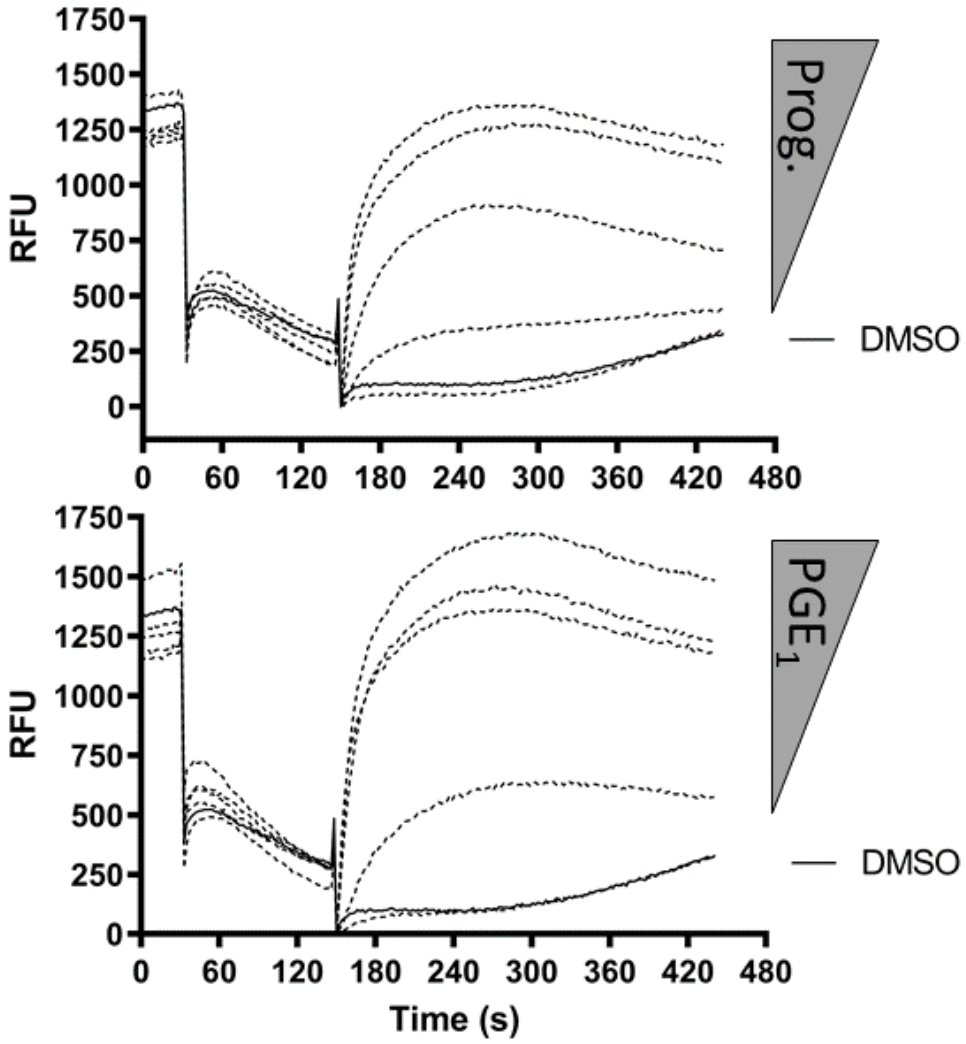


Figure 2.4. Progesterone and PGE_1 Elicit Calcium Influx in Live Human Sperm Cells as Monitored in a Representative FLIPR Experiment. Dotted lines representing increasing concentrations of test compound as shown. DMSO response is shown as solid black line. For both experiments, highest concentration 1 μM (highest RFU) down to 0.1 nM (lowest RFU) in 10X dilutions.

human sperm cells to the previously published patch-clamp and calcium fluorimetry experimental EC_{50} values of 7.7 ± 1.8 and 42 ± 9.3 nM, respectively. [82, 83]

The FLIPR assay consists of loading live human sperm cells with a calcium-selective fluorescent dye called Fluo-4-AM.[86] Selectively fluorescent in the presence of calcium, this dye has long been used to study calcium concentrations in a variety of settings. Live human sperm cells are collected and purified via swim up or centrifugation procedures, loaded with the dye and transferred to the FLIPR instrument where the fluorescence of the cells is monitored in real time. Test compounds to be tested as blockers are added to sperm cells in microtiter plates and given 2 min to bind, after which the opener relevant to the study is added, and the fluorescence of the well is monitored. DMSO

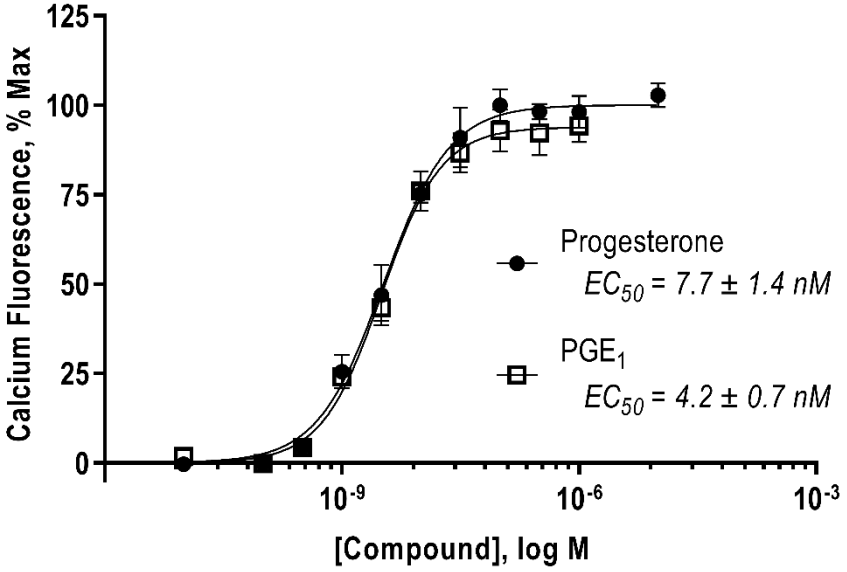


Figure 2.5. Dose-Response Curves Confirming Potencies of Progesterone and PGE_1. The EC_{50} values compare well with previously reported literature values for these compounds. Data are plotted as mean ± standard error, presented as a percent response relative to a saturating dose of progesterone (3 μM). Smooth curves represent the best fits of a nonlinear regression model to the data for each compound. EC_{50} values determined using Prism v6.05.

controls and compounds showing efficacy in the assay show reduced fluorescence as displayed in Figure 2.4.

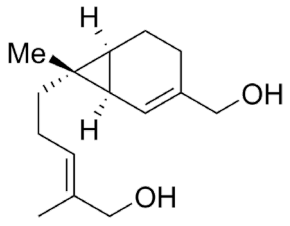
The observed EC_{50} value of 7.7 ± 1.4 nM for progesterone agreed with the previously reported values. Additionally, the EC_{50} value for PGE_1 was confirmed to be 4.2 ± 0.7 nM, which was sufficiently close to those found previously in the literature.

Preliminary dose-response experiments with progesterone indicated a concentration of 3 μM was sufficient to saturate response in the assay, therefore, 3 μM progesterone served as the high control in all subsequent response measurements.

Section 2.2.2.1 The Characterization of l-*Sirenin as a CatSper Activator*

While CatSper channels were originally thought to be animal-specific, it has been shown that a CatSper channel complex is present in the basal fungus *Allomyces macrogynus* (*A. macrogynus*).[87] This fungus produces motile gametes that exhibit chemotaxis towards their female counterparts. This chemotaxis is driven by the sexual pheromone *l*-sirenin (Figure 2.6).[88]

It is not known whether CatSper channels underlie the molecular mechanisms for chemotaxis in *A. macrogynus*, however the mechanism relies on calcium flux.[89] We hypothesized that *l*-sirenin may activate fungal CatSper to enable gamete fertilization.



l-Sirenin, **2.3**

Figure 2.6. The Chemical Structure of *l*-Sirenin.

Since animals and fungi diverged from a common ancestor over 1 billion years ago, a

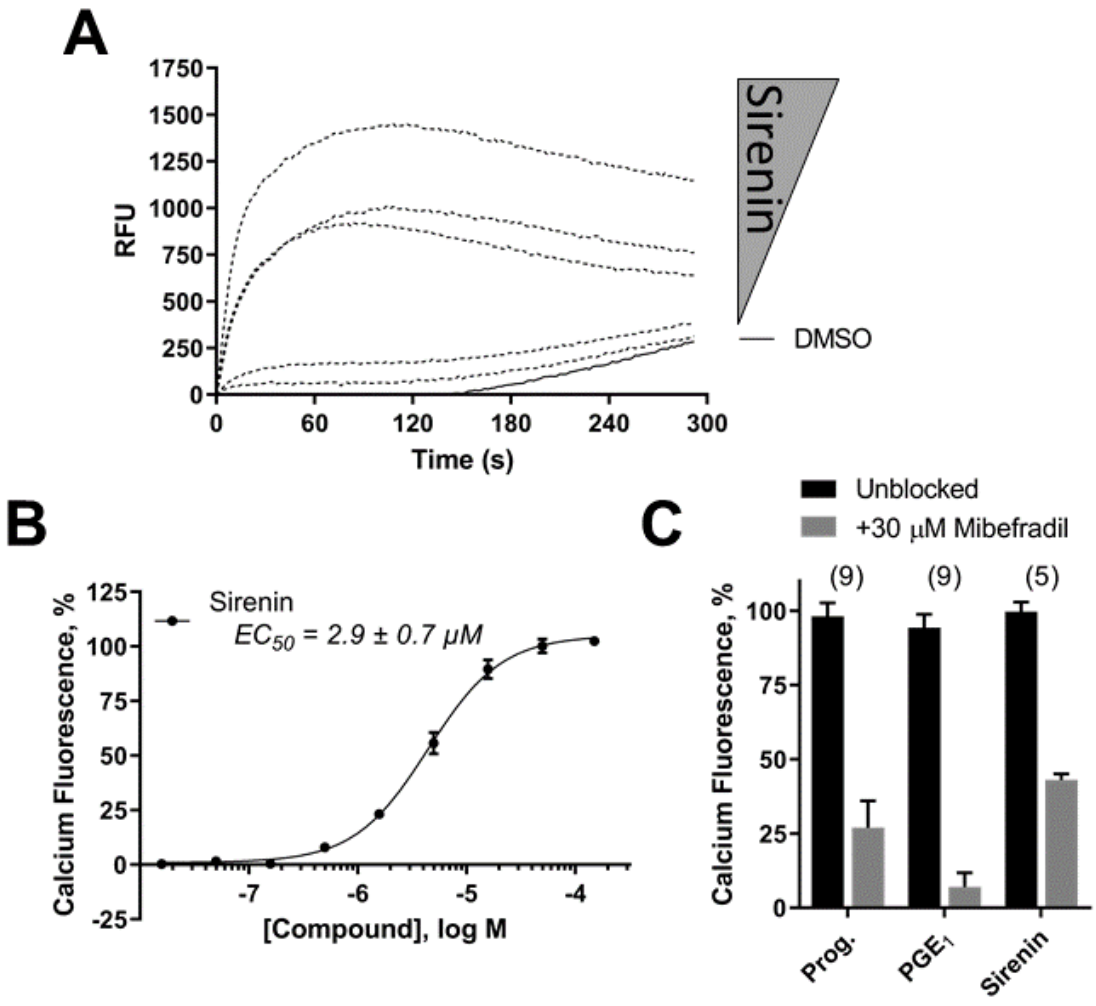


Figure 2.7. Sirenin Activates Catsper in Human Sperm. (A) Raw FLIPR traces showing increases in $[Ca^{2+}]_i$ elicited by increasing concentrations of synthetic sirenin. The sirenin dose-response increases from 10 nM (bottom) to 100 μM (top) by 10X fold increases. (B) Concentration-dependent increases in $[Ca^{2+}]_i$ elicited by sirenin show an EC_{50} value of 2.9 μM ± 0.7 μM. (C) Sirenin elicits the same level of calcium influx as the endogenous activators of CatSper. Treatment concentrations (black) were as follows: sirenin – 30 μM, progesterone and PGE_1 – 1 μM. Pretreatment with 30 μM (grey) reduced the maximum efficacy of all openers tested. Data are plotted as mean ± standard error, presented as a percent response relative to a saturating dose of progesterone (3 μM) and the numbers in parenthesis represent the number of experiments performed. Smooth curves represent the best fits of a nonlinear regression model to the data for each compound. EC50 values determined using Prism v6.05.

compound capable of eliciting a similar response in gametes of both species would serve to demonstrate the high degree of conservation thought to govern most reproductive signaling.[87] As such, we sought to demonstrate that *l*-sirenin could activate human CatSper

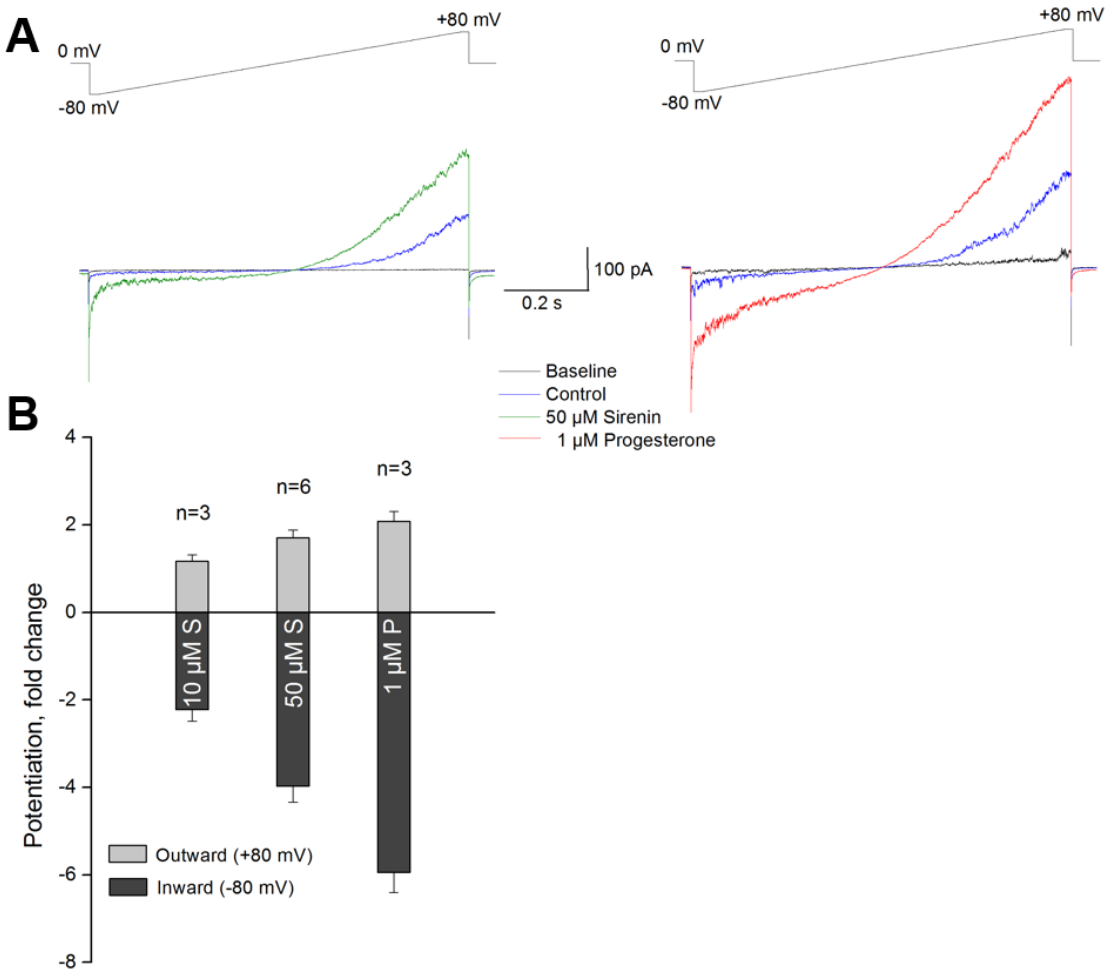


Figure 2.8. Sirenin Increases Intracellular Calcium in Human Sperm through Activation of the CatSper Complex. (A) Representative monovalent $I_{CatSper}$ whole-cell recordings from human spermatozoa in the absence (blue) or presence of test compound. Left panel, 50 µM sirenin (green) or right panel, 1 µM Progesterone (red). B) Averaged amplitudes of $I_{CatSper}$ recorded from human spermatozoa in the presence of indicated test compound. Potentiation was determined by dividing current amplitudes of $I_{CatSper}$ at -80 mV (negative) and +80 mV (positive) by the amplitude of $I_{CatSper}$ in the absence of the corresponding compound from the same cell. Data are represented as mean ± standard error with n indicating the number of individual cells recorded.

and cause calcium influx in healthy human sperm cells via our FLIPR assay and subsequent electrophysiological confirmation.

Sirenin has been an attractive target for the synthetic chemistry community, given its compact, complex structure and several total syntheses have been reported.[90-95] A member of the Georg group, Dr. Syeda, did great work in optimizing the synthesis of this compound, further characterizing the side products of several key reactions and ultimately improving the synthetic route greatly. The detailed of synthesis of sirenin is not discussed here but has been reported.[96] The efficacy of the synthesized sirenin acting at the CatSper complex was measured in the FLIPR assay. When assayed, the synthetic sirenin produced a concentration-dependent rise in $[Ca^{2+}]_i$ in human sperm with an EC_{50} value of 2.9 ± 0.7 μM. Interestingly, though perhaps not unexpectedly, this potency is greatly attenuated compared to the 10 pM concentrations shown to elicit chemotaxis in fungal sperm. This reduced potency between the two species is likely the result of the billions of years of evolution separating the two species.[87] Additionally, the synthetic sirenin was able to elicit a maximum response in the assay, though at a reduced potency. Pretreatment with 30 μM of the known CatSper blocker mibefradil reduced the sirenin-induced activation 55%. These observations indicate that sirenin increases sperm $[Ca^{2+}]_i$ by activating the CatSper complex.

To confirm that the sirenin-mediated rise in $[Ca^{2+}]_i$ resulted from CatSper activation, rather than second messenger pathways or other ion channels present in sperm, whole cell patch-clamp electrophysiology experiments were conducted according to established methods and are shown in Figure 2.8. $I_{CatSper}$ is a pH-sensitive Ca^{2+}-selective

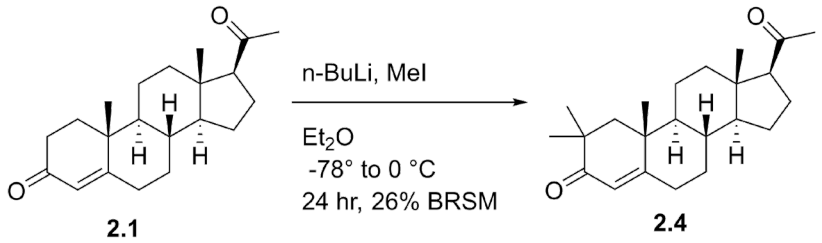
ion current. A step hyperpolarization of the sperm from 0 to – 80 mV followed by a ramp depolarization from -80 to +80 mV elicited an inward current followed by an outward current mediated by CatSper. Both 10 and 50 μM sirenin potentiated both the inward and outward $I_{CatSper}$ currents. This potentiation was blocked by co-application of 30 μM mibefradil, further supporting the conclusion that sirenin-induced calcium flux is the result of CatSper activation and not that of other channels within the sperm.

Section 2.2.3 Various Steroids Effectively Activate CatSper

With the FLIPR assay validated, a general trend of steroid SAR causing calcium influx was sought so that compounds exhibiting limited or no activity in the influx assay could then be tested for their ability to instead block Ca^{2+} influx. While testing each compound, 30 μM mibefradil co-application was performed concomitantly to ensure any observed efficacy was likely due to CatSper activation, given the ability of mibefradil to inhibit CatSper.

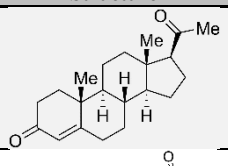
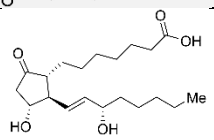
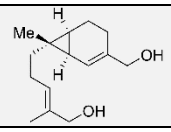
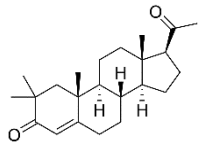
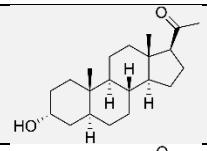
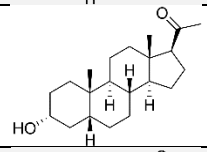
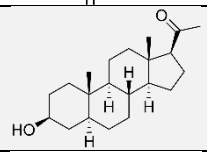
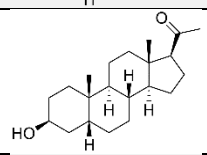
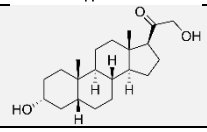
In order to assess the effect of substitutions on the northern half of the A ring, methylation of the C2 position was sought and achieved via enolate chemistry as shown in Scheme 2.1.

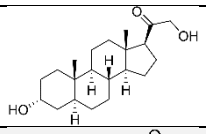
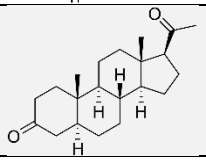
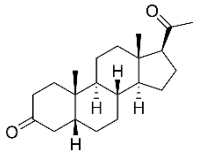
Scheme 2.1. The synthesis of 2,2-Dimethylprogesterone



2,2-Dimethylprogesterone (**2.4**) showed full efficacy when tested in the influx assay with an EC_{50} of 491 nM as shown in Table 2.1.

TABLE 2.1. The ability of various steroids bearing modifications to the A ring and A-B ring fusion to increase $[Ca^{2+}]_i$ in a whole cell calcium influx assay with and without added mibefradil

					+30 μM Mibefradil		
Compound	**Structure**	**EC_{50}, nM[a]**	**E_{max}, %**	***n***	**EC_{50}, nM[a]**	**E_{max}, %**	***n***
2.1		7.7 ± 1.4	100	9	5.1 ± 0.6	31	8
2.2		4.2 ± 0.7	94	9	3.5 ± 1.8	28	8
2.3		2900 ± 700	99	9	3300 ± 1100	45	6
2.4		491 ± 95	98	3	ND	< 10	3
2.5		301 ± 79	90	3	349 ± 38	38	3
2.6		400 ± 18	97	3	291 ± 75	37	3
2.7		77 ± 9	101	3	82 ± 29	48	4
2.8		184 ± 38	107	3	106 ± 25	45	4
2.9		1460 ± 360	107	6	1180 ± 160	17	4

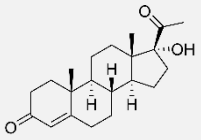
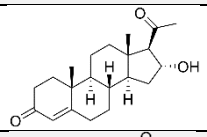
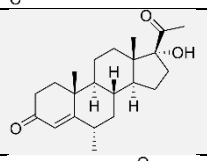
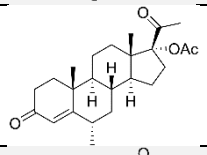
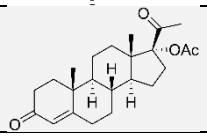
2.10		1060 ± 190	108	6	1190 ± 388	23	4
2.11		77 ± 27	104	5	115 ± 13	46	4
2.12		159 ± 26	92	5	187 ± 45	18	4

[a] EC_{50} values given as the mean ± standard error. ND = not determined

Next, a thorough examination of the individual rings of the progesterone scaffold was undertaken starting with the southern half of the A and B rings and their fusion. In general, modification of the A ring is well-tolerated. Compounds containing a reduced C3 keto group (compounds **2.5**–**2.12**) elicit full response in the influx assay, though at reduced potencies varying from 77 to 400 nM. Furthermore, there appears little preference for the configuration of the A-B ring fusion, as both 5α-dihydroprogesterone (**2.11**) and 5β-dihydroprogesterone (**2.12**) display full activity in the assay and lack a significant difference in their EC_{50} values: 77 and 159 nM, respectively. Indeed, all tested compounds containing either reduced C4 or, as will be seen, substituted C5 positions show no observed preference for a *cis* vs *trans* relationship of the A-B ring fusion. Finally, tetrahydrodeoxycorticosterones (THDOCs) **2.9** and **2.10**, which contain a 21-hydroxyl group in addition to a fully reduced A ring, showed a large drop in potency with EC_{50} values of 1460 and 1060 nM for the 5β- and 5α-THDOC diastereomers, respectively.

Given the attenuated activity of the THDOCs, we next pursued modifications near the D-ring despite the known ability of 17α-hydroxyprogesterone (**2.13**) to evoke calcium influx in sperm.[77] Table 2.2 shows the results of these modifications and Figure 2.9 shows the corresponding dose-response curves. When tested, **2.13** elicited calcium influx with an EC_{50} of 7.2 nM (Figure 2.9, filled squares), equaling the potency of progesterone. Shifting the hydroxyl group to the C16 position resulted in a 10-fold loss of activity observed for 16α-hydroxyprogesterone (**2.14**). Medroxyprogesterone (**2.15**), the 6α-methyl analog of

TABLE 2.2. The effect of C17, C16 and C6 modifications to the progesterone scaffold on the ability to increase $[Ca^{2+}]_i$ in a whole cell calcium influx assay with and without added mibefradil

					+30 μM Mibefradil		
Compound	**Structure**	**EC_{50}, nM[a]**	**E_{max}, %**	***n***	**EC_{50}, nM[a]**	**E_{max}, %**	***n***
2.13		7.2 ± 0.8	117	3	11 ± 2	59	3
2.14		190 ± 40	106	3	220 ± 55	53	4
2.15		480 ± 52	95	3	ND	< 10	3
2.16		> 10 000	ND	3	> 10 000	ND	3
2.17		> 10 000	ND	8	> 10 000	ND	4

[a] EC_{50} values given as the mean ± standard error. ND = not determined

2.13 showed over 50-fold loss of activity (Figure 2.9, filled diamonds). The acetylated analog of **2.15**, medroxyprogesterone acetate (**2.16**, MPA) showed no ability to increase $[Ca^{2+}]_i$ up to 10 μM (Figure 2.9, unfilled squares). Interestingly, acetylation alone of the 17α-hydroxyl group (**2.17**) was sufficient to almost entirely ablate activity (Figure 2.9, filled triangles).

Section 2.2.3.1 Synthesis of Target Steroids

Given the observed lack of activity of compounds **2.16** (MPA) and **2.17** (17α-acetoxyprogesterone) in the influx assay, we endeavored to evaluate 6α-methylprogesterone (**2.18**) to ascertain the effect on activity of B-ring modifications

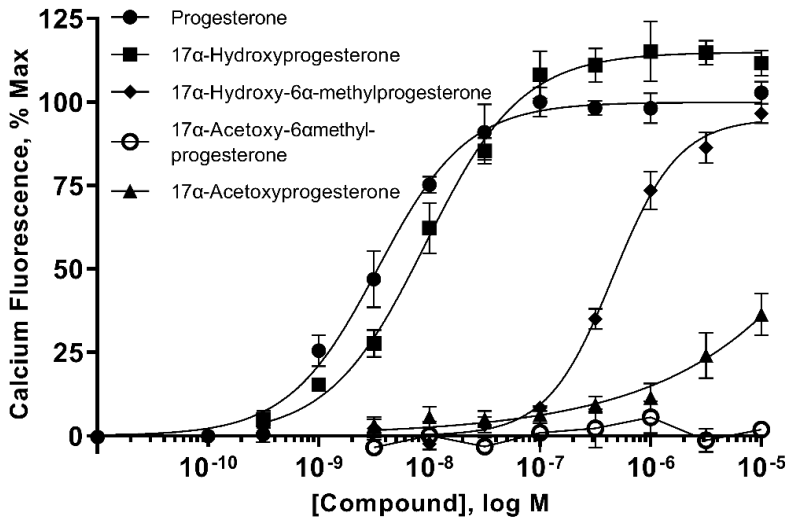
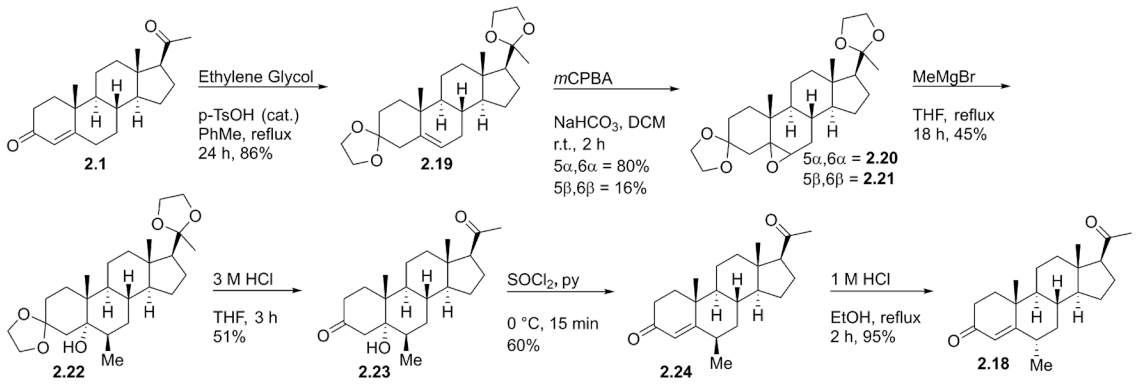


Figure 2.9. Dose-Response Curves Comparing Potencies of Progesterone and Several C17 and/or C6-Modified Analogues in Healthy Human Sperm. Data are plotted as mean ± standard error, presented as a percent response relative to a saturating dose of progesterone (3 μM). The number of separate experiments for individual compounds can be found in Table 2.2 along with EC_{50} and E_{max} values in the presence and absence of mibefradil. Smooth curves represent the best fits of a nonlinear regression model to the data for each compound. No fit was obtained for MPA and instead a connecting line is shown.

without any additional C17 modifications. Synthesis of **2.18** was achieved following previously described literature procedures for C6-modified androstanes and is outlined in Scheme 2.2.[97] The synthesis began via the acid-catalyzed double protection of the C3 and C20 ketones of **2.1** furnishing the corresponding ethylene ketal **2.19**. Epoxidation of **2.19** with *m*-CPBA proceeded in good yield and the two resulting diastereomers were separable by column chromatography, revealing 3,3:20,20-bis(ethylenedioxy)-5α,6α-epoxypregnane (**2.20**) as the more polar product. Conditions yielding β-epoxide **2.21** as the sole product were successful on small scale, but a lack of solubility greatly hampered scale up. Furthermore, while the route to **2.18** could be shortened by alkylation of **2.21**, this β-epoxide proved entirely resistant to ring-opening addition by Grignard reagents or alkyl lithium species no matter the conditions used.[98]

Scheme 2.2 Synthesis of 6α-Methylprogesterone (**2.18**)

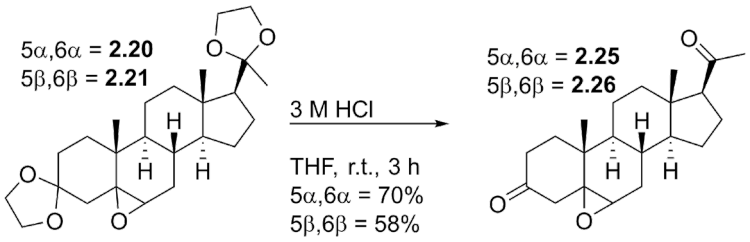


Proceeding towards target molecule **2.18**, Grignard addition into **2.20** proceeded smoothly under refluxing conditions and furnished pregnane **2.22.** Removal of the ketal groups under acidic conditions at elevated temperatures yielded 5α-hydroxy-6β-methylpregnan-3,20-dione (**2.23**). Since ketal deprotection conditions proved insufficient,

dehydration of the 5α-hydroxy was achieved by treatment with thionyl chloride to provide **2.24** in good yield. Finally, epimerization of the C6 position to the thermodynamically favored 6α epimer gave 6α-methylprogesterone (**2.18**) in 6 steps and 7% overall yield.

The epoxidation of **2.19** served as a valuable branching point for the generation of additional analogs of the steroid backbone as shown in Scheme 2.3. Compounds **2.20** and **2.21** were deprotected to give their corresponding epoxyprogesterones **2.25** and **2.26**, respectively. Additionally, each intermediate along the synthetic route was tested in the influx assay and is, as such, included in Table 2.3 which shows the effect of these compounds in the calcium influx assay.

Scheme 2.3 Synthesis of Analogs **2.25** and **2.26**

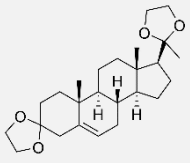
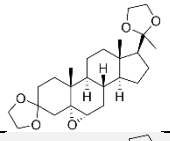
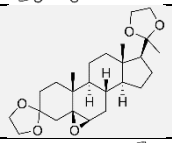
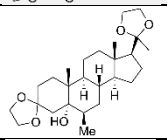
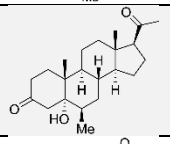
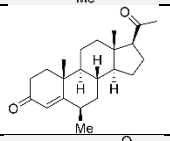
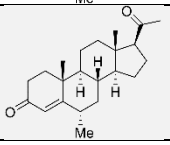


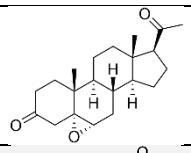
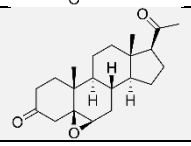
Section 2.2.3.2 Activity of Synthesized Steroids

Interestingly, every compound tested containing ketalized C3 and C20 positions showed no activity in the influx assay (Figure 2.10, open diamonds, representative). The corresponding, ketone-containing epoxides (**2.25** dose-response in Figure 2.10, filled diamonds) showed submicromolar EC_{50} values. The same pattern is not quite observed with 5α-hydroxy-6β-methylprogesterone (**2.23**) and its analogous ketal **2.22** though the compound containing the free ketones is still more potent.

With respect to the C6 methyl substitution, there is only a slight difference in activity between the two diastereomers **2.24** and **2.18** (Figure 2.10, filled squares vs filled triangles). The 6α-methyl substitution found in **2.18** influences activity to a slightly greater extent than its 6β-methyl counterpart in **2.24**. The 6α-methyl modification of **2.18** caused a 93-fold reduction in activity with respect to progesterone activation, a significant decrease if not as significant as that of the 17α-acetoxy modification.

TABLE 2.3. The observed effects on calcium influx with and without added mibefradil of synthesized steroids

					+30 μM Mibefradil		
Compound	**Structure**	**EC_{50}, nM[a]**	**E_{max}, %**	***n***	**EC_{50}, nM[a]**	**E_{max}, %**	***n***
2.19		> 10 000	ND	3	ND	< 10	3
2.20		> 10 000	ND	3	ND	< 10	3
2.21		> 10 000	ND	3	ND	< 10	3
2.22		> 10 000	ND	3	ND	< 10	3
2.23		6390 ± 1210	ND	3	ND	< 10	3
2.24		267 ± 34	99	3	ND	< 10	3
2.18		720 ± 110	110	3	ND	< 10	3

2.25		243 ± 61	98	3	ND	< 10	3
2.26		422 ± 30	86	3	ND	< 10	3

[a] EC_{50} values given as the mean ± standard error. ND = not determined

Section 2.2.4 Clinically Relevant Steroids Elicit Calcium Influx in Human Sperm

Given that MPA, a synthetic progestin, was the only compound tested thus far to show no activity at high concentrations in the influx assay, a series of clinically relevant progestins, anti-progestins and androgens were tested for their ability to increase $[Ca^{2+}]_i$ in human sperm. As shown in Table 2.4, the progestin activity of MPA is likely not the cause

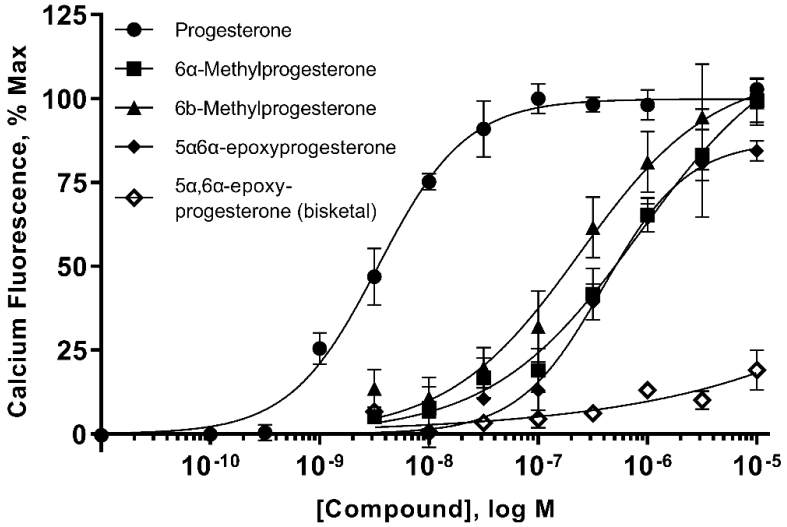
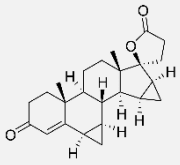
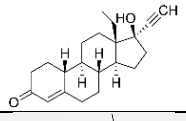
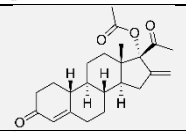
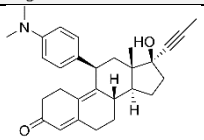
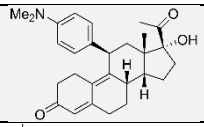
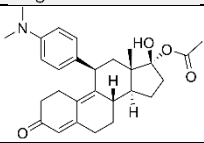


Figure 2.10. Dose-Response Curves Comparing Potencies of Progesterone and Select Synthesized Compounds in Healthy Human Sperm. Data are plotted as mean ± standard error, presented as a percent response relative to a saturating dose of progesterone (3 μM). The number of separate experiments for individual compounds can be found in Table 2.3 along with EC_{50} and E_{max} values in the presence and absence of mibefradil. Smooth curves represent the best fits of a nonlinear regression model to the data for each compound.

TABLE 2.4. The effects of clinically used steroids on calcium influx with and without added mibefradil

					+30 µM Mibefradil		
Compound	**Structure**	**EC_{50}, nM[a]**	**E_{max}, %**	***n***	**EC_{50}, nM[a]**	**E_{max}, %**	***n***
2.27		429 ± 140	104	7	486 ± 180	12	3
2.28		>> 10 000	ND	4	ND	ND	3
2.29		≈ 10 000	ND	4	ND	ND	3
2.30		5730 ± 2030	99	4	ND	< 10	3
2.31		4410 ± 950	106	5	ND	< 10	5
2.32		>>10 000	ND	3	ND	< 10	3

[a] EC_{50} values given as the mean ± standard error. ND = not determined

of the observed lack of activity, since drospirenone (**2.27**, Figure 2.11, filled circles), another synthetic progestin, displayed an EC_{50} of 429 nM in the assay.

However, two other progestins did show little to no ability to cause calcium influx: levonorgestrel (LNG, **2.28**, Figure 2.11, filled squares) and nestorone (**2.29**, Figure 2.11, upright filled triangles). Intriguingly, both LNG and nestorone contain substitutions at the C17 position, bearing an ethynyl and acetoxy group, respectively, adding credence to the previous observations that C17 modifications reduce CatSper activity.

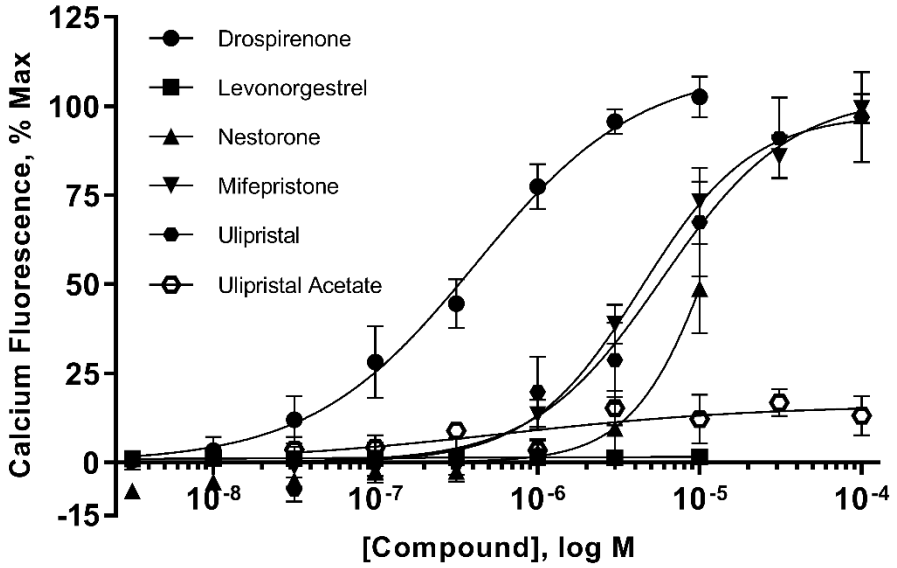
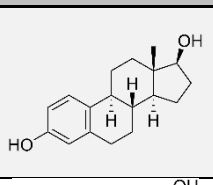
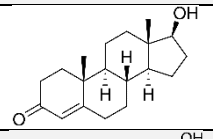
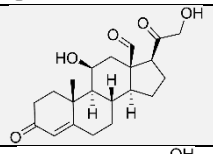
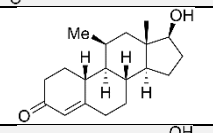
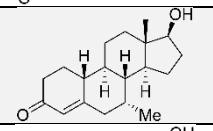
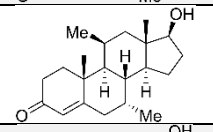
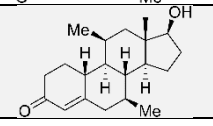


Figure 2.11. Dose-Response Curves Comparing Potencies of Clinically Relevant Progestins and Antiprogestins. Data are plotted as mean ± standard error, presented as a percent response relative to a saturating dose of progesterone (3 μM). The number of separate experiments for individual compounds can be found in Table 2.4 along with EC_{50} and E_{max} values in the presence and absence of mibefradil. Smooth curves represent the best fits of a nonlinear regression model to the data for each compound.

Two anti-progestins, mifepristone (**2.30**, Figure 2.11, downward filled triangles) and ulipristal (**2.31**, Entry 2.29 and Figure 2.11, filled hexagons) were also tested and showed very weak activity in the calcium influx assay with EC_{50} values of 5.7 and 4.4 μM, respectively. Again, following the previously observed trend, the 17α-*O*-acetylated ulipristal analog **2.32** showed decreased activity in the influx assay to >>10 μM (Figure 2.11 unfilled hexagons).

Additionally, as shown in Table 2.5, the parent estrogen, 17β-estradiol (**2.33**, Figure 2.12, circles) was shown to elicit calcium influx in human sperm. Testosterone (**2.34**, Figure 2.12, squares) was shown to cause calcium influx with a potency similar to that of the parent estrogen. Another hormone, aldosterone (ALDO, **2.35**, Figure 2.12, upward

TABLE 2.5. The effects of clinically relevant androgens estrogens and mineralocorticoids on calcium influx both with and without added mibefradil

					+30 μM Mibefradil		
Entry	Compound	EC_{50}, nM[a]	E_{max}, %	*n*	EC_{50}, nM[a]	E_{max}, %	*n*
2.33		404 ± 92	99	4	302 ± 25	44	3
2.34		339 ± 22	98	3	433 ± 49	17	3
2.35		>>10 000	ND	6	ND	< 10	3
2.36		280 ± 36	84	6	344 ± 30	15	3
2.37		437 ± 144	92	5	520 ± 60	43	3
2.38		1890 ± 460	90	9	ND	< 10	3
2.39		4970 ± 600	32	6	4040 ± 30	30	3

[a] EC_{50} values given as the mean ± standard error. ND = not determined

triangles) was completely inactive in the influx assay. While not bearing any direct C17 modifications, steric hindrance by the close proximity of the C18 aldehyde and the C21 hydroxyl group found in the molecule could be responsible for this lack of activity.

Given the activity of testosterone, several synthetic androgens were tested and shown to cause a calcium influx. 11β-Methyl-19-nortestosterone (11β-MNT, **2.36**, Figure

2.12, diamonds) was the most potent androgen at 280 nM. Its close analog 7α-methyl-19-nortestosterone (7α-MNT, **2.37**) shows similar activity. Interestingly, both 7α,11β-dimethylandrolone (**2.38**) and its diastereomer 7β,11β-dimethylandrolone (**2.39**, Figure 2.12, hexagons) showed comparatively diminished potencies at 1890 and 4970 nM, respectively. Intriguingly, 7β,11β-dimethylandrolone exhibited a maximum effect (E_{max}) of only 32%, the only such partial activator seen in all of the tested compounds.

Section 2.3 Characterization of T-type Calcium Blockers Inhibiting CatSper

Section 2.3.1 Mibefradil Blocks All Known CatSper Openers

As noted previously, in Tables 2.1 – 2.5, 30 μM of the t-type calcium channel blocker (CCB) mibefradil (**2.40**) was included in all influx assays to prove that the induced

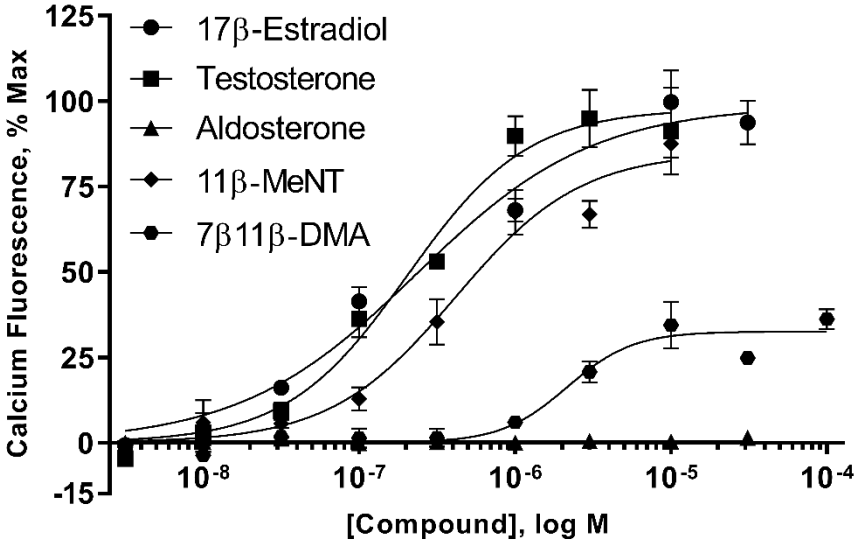


Figure 2.12. Dose-Response Curves Comparing Potencies of 17β-Estradiol, Testosterone, Aldosterone and Representative Synthetic Androgens. Data are plotted as mean ± standard error, presented as a percent response relative to a saturating dose of progesterone (3 μM). The number of separate experiments for individual compounds can be found in Table 2.5 along with EC_{50} and E_{max} values in the presence and absence of mibefradil. Smooth curves represent the best fits of a nonlinear regression model to the data for each compound.

influx was indeed from CatSper activation, as it has long been known that mibefradil blocks

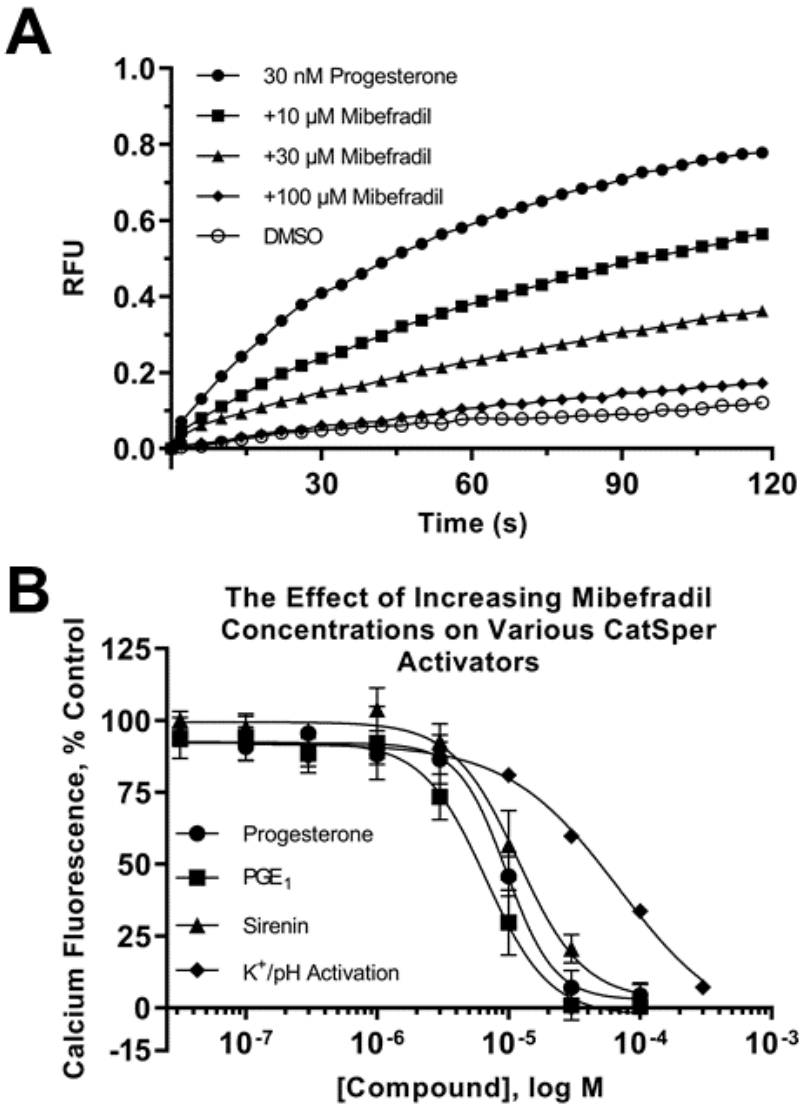
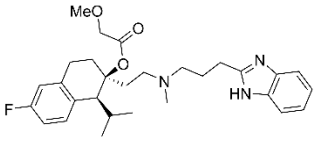
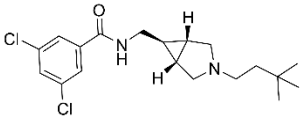


Figure 2.13. Inhibition of Progesterone-, PGE_1-, *l*-Sirenin and K+-Induced Calcium Influx by Mibefradil. (A) Representative raw FLIPR traces showing dose-dependent reduction of progesterone CatSper activation. Cells from DMSO group received neither blocker nor progesterone. Data normalized to experiment-specific high (3 µM progesterone). (B) Dose-response inhibition curves showing potency of mibefradil against progesterone-, PGE_1-, sirenin-, or K+-induced calcium influx. In all experiments, the concentration of opener used corresponded to an EC_{80} dose as determined from uninhibited dose-response experiments (see text). The data are plotted as the mean ± the standard error with the number of repeated experiments and IC_{50} values given in Table 2.6. Smooth curves represent the best fits of a nonlinear regression model to the data for each compound.

CatSper currents.[99-101] We sought to validate the influx assay with respect to blocking CatSper currents with a control compound before continuing on with testing the inactive steroids for their ability to block CatSper currents.

During the course of the influx assays, the EC_{80} doses for progesterone, PGE_1 and sirenin were determined from their nonlinear regressions. These values are 30 nM, 10 nM and 1 μM, respectively. These concentrations were used when studying the ability of compounds to block each respective opener. CatSper can also be artificially opened by introducing the cells to a high pH/ high K^+ environment.[56] The concentration of K^+ used in these experiments was 140 mM.

TABLE 2.6. Inhibition by mibefradil and ML218 of various modes of CatSper activation

Compound	Structure	Activator	IC_{50}, μM[a]	Hill Slope[b]	*n*
2.40		*Progesterone*	7.5 ± 1.3	–2.3	8
		PGE_1	5.8 ± 1.2	–2.6	8
		l-Sirenin	13 ± 4	–1.7	6
		K^+/pH	18 ± 2	–1.7	8
2.41		*Progesterone*	9.9 ± 0.9	–2.0	5
		PGE_1	15 ± 5	–3.6	3
		l-Sirenin	14 ± 3	–2.2	3
		K^+/pH	11 ± 3	–2.1	7

[a] IC50 values given as the mean ± standard error. [b] Hill slope for each compound is given. Each opener was dosed at a constant EC_{80} concentration corresponding to concentrations listed in the text.

As shown in Figure 2.13 (A) mibefradil displayed a dose-dependent inhibition of progesterone-induced calcium influx in the FLIPR assay, as expected. Shown in Table 2.6 and Figure 2.13 (B), mibefradil inhibits the progesterone-induced influx in the FLIPR assay with an IC_{50} value of 7.5 ± 1.3 μM. This value is higher than that observed in electrophysiology experiments in which 1 μM mibefradil was sufficient to ablate all CatSper currents. This trend of compounds appearing less potent in the FLIPR assay when compared to other assays was observed throughout the course of this study. Not unexpectedly, mibefradil was also able to completely block the PGE_1-, sirenin, and high K^+/pH-induced calcium influx, also shown in Table 2.6.

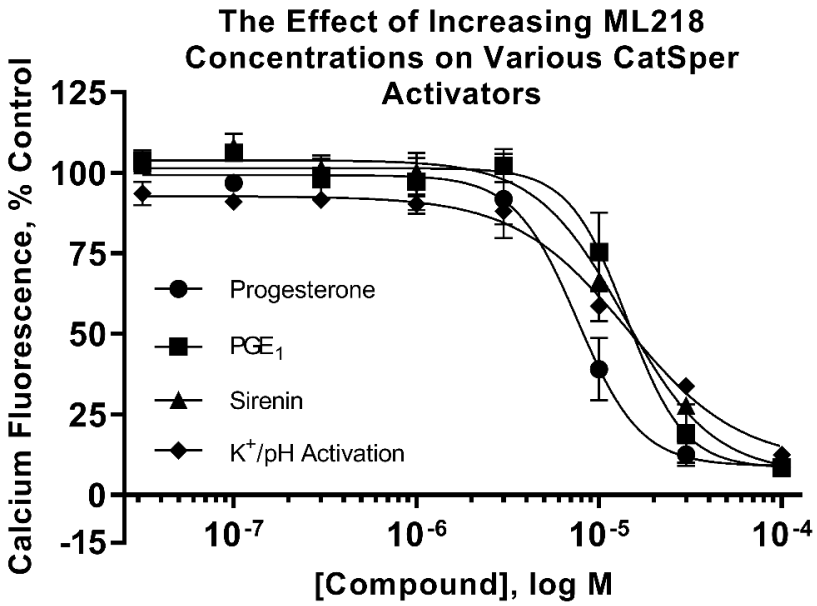


Figure 2.14. Dose-Response Inhibition Curves Showing the Potency of ML218 Against Progesterone-, PGE1-, l-Sirenin-, or K+-Induced Calcium Influx**.** In all experiments, the concentration of opener used corresponded to an EC_{80} dose (see text). The data are plotted as the mean ± the standard error with the number of repeated experiments and IC_{50} values given in Table 2.6. Smooth curves represent the best fits of a nonlinear regression model to the data for each compound.

Section 2.3.2 ML218 also Blocks All Known CatSper Openers

There have been reports in the past that mibefradil itself causes calcium influx above 30 μM in human sperm cells.[101] While no mibefradil-induced calcium influx was observed in any assay described thus far, another structural class of t-type CCBs capable of blocking CatSper activation would be a useful compound to the scientific community. To this end we tested whether ML218 (**2.41**), a t-type CCB that was originally developed as a central nervous system probe molecule as an initiative of the molecular libraries production center network (MLPCN), is able to block calcium influx caused by CatSper.[102] Originally developed for $Ca_v3.2$ and $Ca_v3.3$ channels, showing sub-micromolar activity, we anticipated this compound might prove to be more potent that the established control compounds.

While ML218 can completely inhibit the progesterone-induced calcium influx observed in the FLIPR assay, its potency of 9.9 ± 0.9 μM (Table 2.6 and Figure 2.14) is no higher than mibefradil.

Like mibefradil, ML218 was also able to completely block the signal generated by PGE_1, sirenin, and high K^+/pH (Figure 2.14) with IC_{50} values given in Table 2.6. In all cases, IC_{50} values between ML218 and mibefradil were not significantly different for any method of activation and ML218 did not provoke any signal in the influx assay up to 100 μM (data not shown). ML218 has a decidedly different structure than mibefradil and displays dissimilar selectivity profiles when tested in vivo.[102] This compound inhibiting CatSper currents provides a second control molecule for researchers studying CatSper currents.

Section 2.4 The Discovery of Steroidal CatSper Blockers

Section 2.4.1 The Inactive Steroids Successfully Block CatSper Activation

Of the 55 compounds tested for their ability to cause calcium influx in human MPA, LNG, and ALDO. Having identified these compounds, we sought to determine if they were capable of blocking the progesterone-, PGE_1-, sirenin-, and high K^+/pH-induced activation of the CatSper channel, or if they were simply inactive. Cholic acid showed no activation when previously tested and also did not block any method of eliciting calcium influx (not shown). Therefore, cholic acid was deemed inactive in the assay and was not pursued further. Cells treated with increasing concentrations of MPA (**2.16**), LNG (**2.28**) and ALDO (**2.35**) showed dose-dependent decreases in the signal elicited by treatment with an EC_{80} dose (30 nM) of progesterone (Figure 2.15, A). The selected steroids not only block the CatSper activation via progesterone, but also that of PGE_1 (Figure 2.15, B) and sirenin (Figure 2.15, C).

As shown in Figure 2.15 and Table 2.7, MPA (**2.16**) is the most potent compound of the three discovered steroidal blockers, with IC_{50} values of 9.7 ± 2.9, 9.2 ± 1.6 and 12 ± 3 μM for the respective activators as shown in Table 2.7. LNG (**2.28**) blocked each signal elicited with IC_{50} values of 32 ± 6, 20 ± 4 and 81 ± 9 μM, respectively, and ALDO (**2.35**) showed the weakest activity with IC_{50} values of 43 ± 9, 56 ± 11 and 78 ± 12 μM, respectively.

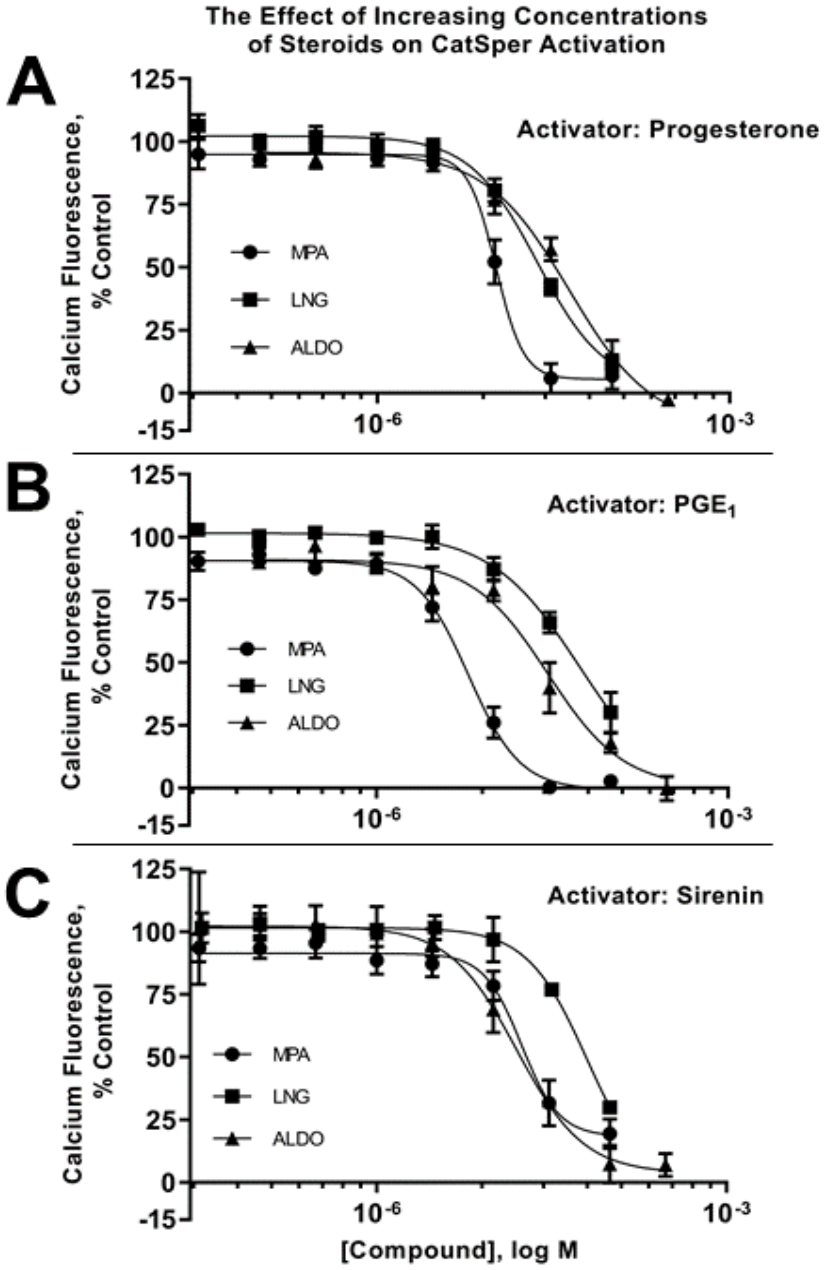
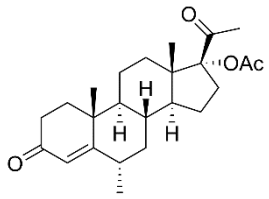
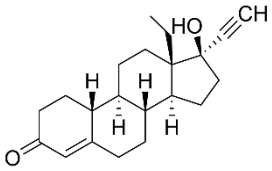
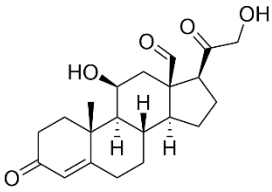


Figure 2.15. Dose-Response Inhibition Curves Showing Inhibition of CatSper Activation by Selected Steroids. The activator used in each graph is shown in the top right. In all experiments, the concentration of the opener used corresponded to an EC_{80} dose (see text). The data are plotted as the mean ± the standard error with the number of repeated experiments and IC_{50} values given in Table 2.7. Smooth curves represent the best fits of a nonlinear regression model to the data for each compound.

TABLE 2.7. Inhibition by selected steroids of various modes of CatSper activation

Compound	Structure	Activator	IC_{50}, µM[a]	Hill Slope[b]	*n*
2.16		*Progesterone*	9.7 ± 2.9	–1.3	12
		PGE_1	9.2 ± 1.6	–1.2	11
		l-Sirenin	13 ± 4	–1.2	9
2.28		*Progesterone*	32 ± 6	–2.2	12
		PGE_1	20 ± 4	–1.4	6
		l-Sirenin	81 ± 9	–2.8	6
2.35		*Progesterone*	43 ± 9	–2.3)	10
		PGE_1	56 ± 11	–2.2	10
		l-Sirenin	78 ± 12	–2.3	9

[a] IC50 values given as the mean ± standard error. [b] Hill slope for each compound is given. Each opener was dosed at a constant EC_{80} concentration corresponding to concentrations listed in the text.

Section 2.4.2 The Steroidal Blockers Cannot Inhibit the K^+/pH-Induced Calcium Influx

In general, including the studied t-type CCBs from the previous section, blockers most potently inhibit progesterone activation, followed by PGE_1 and *l*-sirenin activation. Also, the inhibition curves generated from all compounds except MPA frequently showed Hill slopes less than -1.5, whereas those from MPA are closer to -1.

Figure 2.16 shows the concentration-inhibition curves resulting from high K^+/pH-induced calcium influx in the presence of increasing concentrations of each steroidal blocker. From the figure it is clear that the studied steroidal compounds have minimal effect on this method of eliciting calcium influx, as the IC_{50} for each steroid is well above 100 μM. Conversely, as was seen in the last section, cells treated with increasing concentrations of mibefradil and ML218 fail to response to this method of evoking calcium influx, with IC_{50} values for each compound of 18 ± 2 and 11 ± 3 μM, respectively (Table 2.6, K^+/pH).

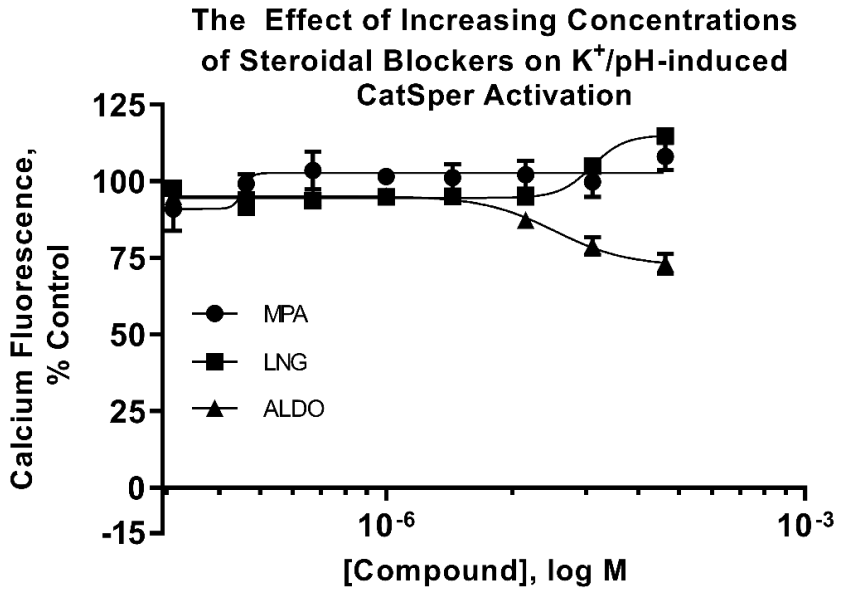


Figure 2.16. Dose-Response Inhibition Curves Showing Inability of Selected Steroids to Block K+-Induced Calcium Influx. The data are plotted as the mean ± the standard error. Experiments were repeated 4 times to ensure results. A smooth curve represents the best fits of a nonlinear regression model to the data for ALDO and LNG.

In order to ensure that the observed discrepancies, and indeed, all the observed inhibitory activity, was due to CatSper blockage and not indiscriminate interference of the compounds, all studied blockers were tested for their ability to inhibit the diffusion-based calcium ionophore, A23187.[103] By incubating sperm cells in the presence of 30 μM of each

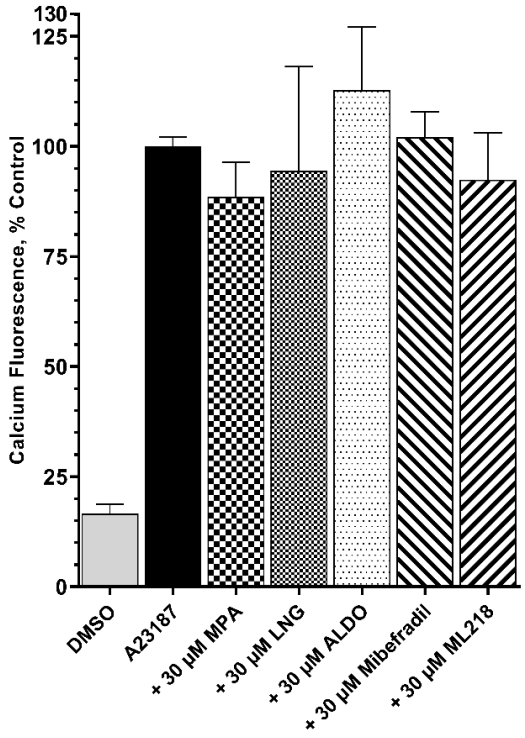


Figure 2.17. All Studied Blockers Do Not Block the Diffusion-Based Ionophore, A23187. The data are plotted as the mean ± the standard error. Experiments were repeated 3 times.

blocker, then treating with the ionophore and reading fluorescence, it was shown that the studied blockers do not indiscriminately reduce fluorescence or interfere with the dye used in the assay, Fluo-4-AM. As shown in Figure 2.17, all tested blockers were unable to significantly reduce the signal generated by this ionophore that facilitates ion diffusion across membranes, serving to validate the results shown previously in this section.

Section 2.5 Mode of Inhibition Studies of Steroids versus T-type CCBs

Section 2.5.1 T-type CCBs Display an Insurmountable Block of CatSper Activation

While screening the purchased and synthesized steroids for their ability to activate CatSper, 30 μM mibefradil was included in each assay to ensure that the observed influx

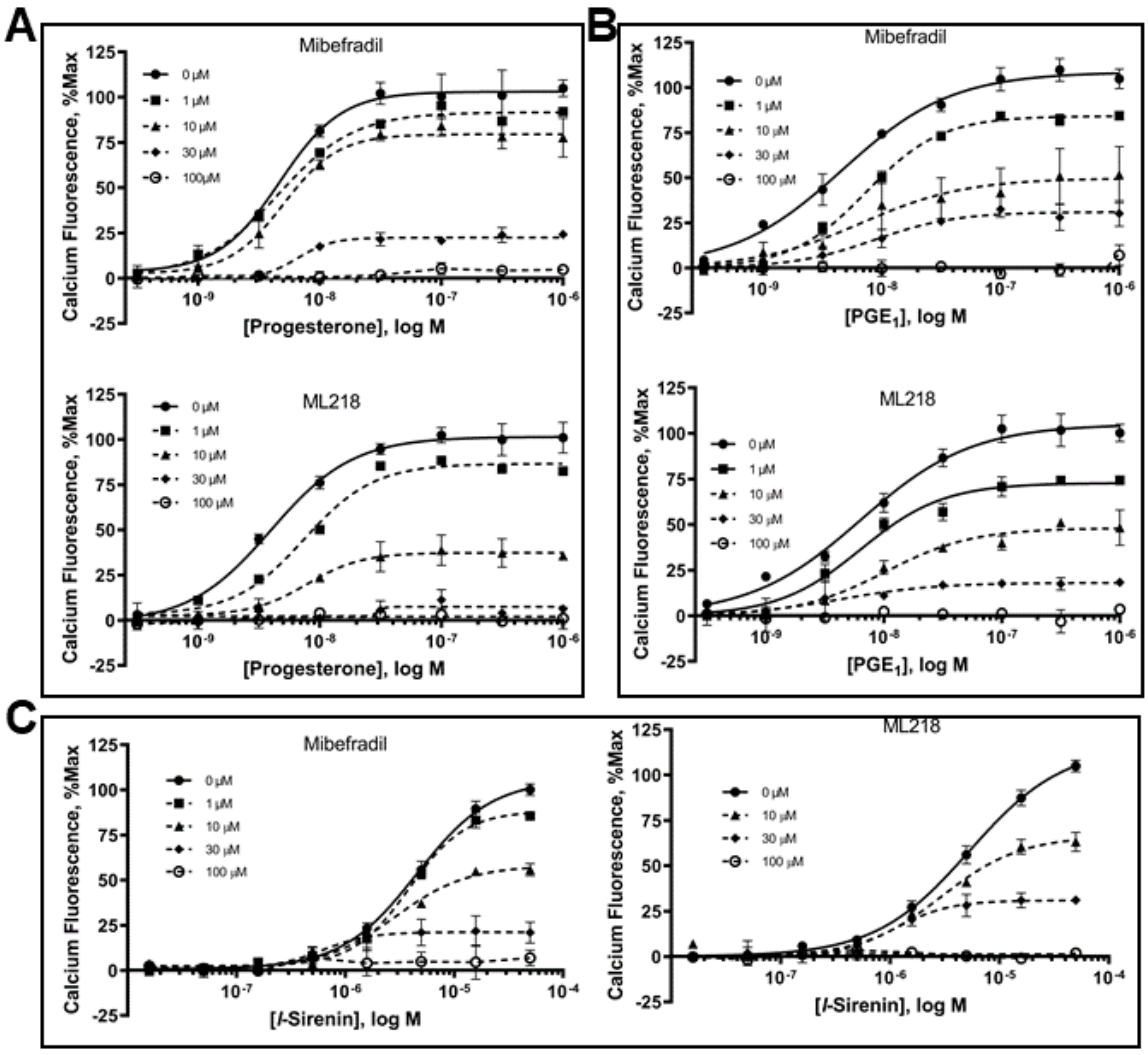


Figure 2.18. T-type CCBs Cause an Insurmountable Block of Ca2+-Influx in Human Sperm. Increasing concentrations of mibefradil and ML218 reduce E_{max} of progesterone (A), PGE_1 (B) and *l*-sirenin (C). The $EC_{50(app)}$ remains unchanged from control in each case. Calculated E_{max}, $EC_{50(app)}$, and the number of repeated experiments are given in Table 2.8. The data are plotted as the mean ± the standard error relative to the signal evoked by 3 μM progesterone. Smooth curves represent the best fits of a nonlinear regression model to the data for each compound. If $E_{max} < 10\%$, then a line connecting points is shown instead of best fit data.

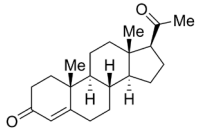
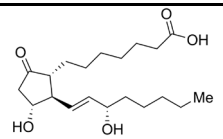
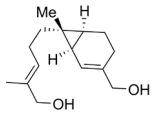
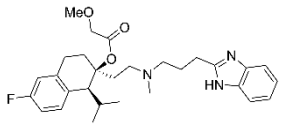
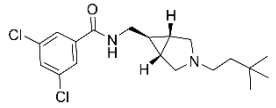
was due to CatSper-specific activation. It was noticed in each case that mibefradil reduced the E_{max} of every effective opener while leaving the EC_{50} unaffected (Tables 2.1 – 2.5, rightmost columns). These results are consistent with insurmountable or non-competitive inhibition. To further study and confirm these observations, the mechanism by which t-type CCBs inhibit the calcium influx caused by the studied openers was examined by measuring the effects of fixed concentrations of mibefradil and ML218 on the dose-response curves of progesterone, PGE_1 and sirenin.

As seen in Table 2.8, the presence of 1, 10, 30, and 100 μM mibefradil or ML218 reduced E_{max} while each respective EC_{50} was insignificantly altered, consistent with non-competitive inhibition. Furthermore, as seen previously in Tables 2.1–2.5, each studied steroid was blocked insurmountably by 30 μM mibefradil corroborating that t-type CCBs have binding sites distinct from those of steroids, PGE_1 and sirenin.

Section 2.5.2 The Block Produced by Steroidal Compounds is Surmountable

Contrary to the non-competitive block caused by the t-type CCBs, the effect of the studied steroidal blockers is completely surmountable as shown in Figures 2.19 – 2.21. With respect to MPA, increasing concentrations of the progestin caused parallel rightward shifts of the progesterone dose-response curves, indicating competitive inhibition (Figure 2.19, A).

Table 2.8. Effects of mibefradil and ML218 on progesterone-, PGE_1- and sirenin-induced increase in $[Ca^{2+}]_i$ in human sperm

		Opener									
	Blocker	Conc. (μM)	$EC_{50(app)}$[a]	E_{max}[b]	*n*	$EC_{50(app)}$[a]	E_{max}[b]	*n*	$EC_{50(app)}$[a]	E_{max}[b]	*n*
2.40		0	7.7 ± 1.4	100	9	3.1 ± 0.3	94	9	2.9 ± 0.7	99	9
		1	4.1 ± 1.5	92	3	7.4 ± 0.1	84	3	3.8 ± 1.1	88	3
		10	7.9 ± 1.7	80	5	6.7 ± 2.1	50	5	2.8 ± 2.1	58	4
		30	6.6 ± 2.4	22	5	8.6 ± 2.4	9	3	6.7 ± 3.4	21	4
		100	ND	5	4	ND	7	5	ND	9	4
2.41		0	7.7 ± 1.4	100	9	3.1 ± 0.3	94	9	2.9 ± 0.7	99	9
		1	7.8 ± 1.4	87	3	1.7 ± 0.2	73	3	ND	ND	–
		10	7.9 ± 2.4	37	3	10 ± 1	48	3	2.9 ± 1.9	66	3
		30	ND	7	3	4.3 ± 1.1	18	3	1.1 ±0.3	31	3
		100	ND	2	3	ND	5	3	ND	2	3

[a]EC50 values given in nM as the mean ± standard error. [b]Data presented as % of saturating dose of progesterone (3 μM). ND = not determined

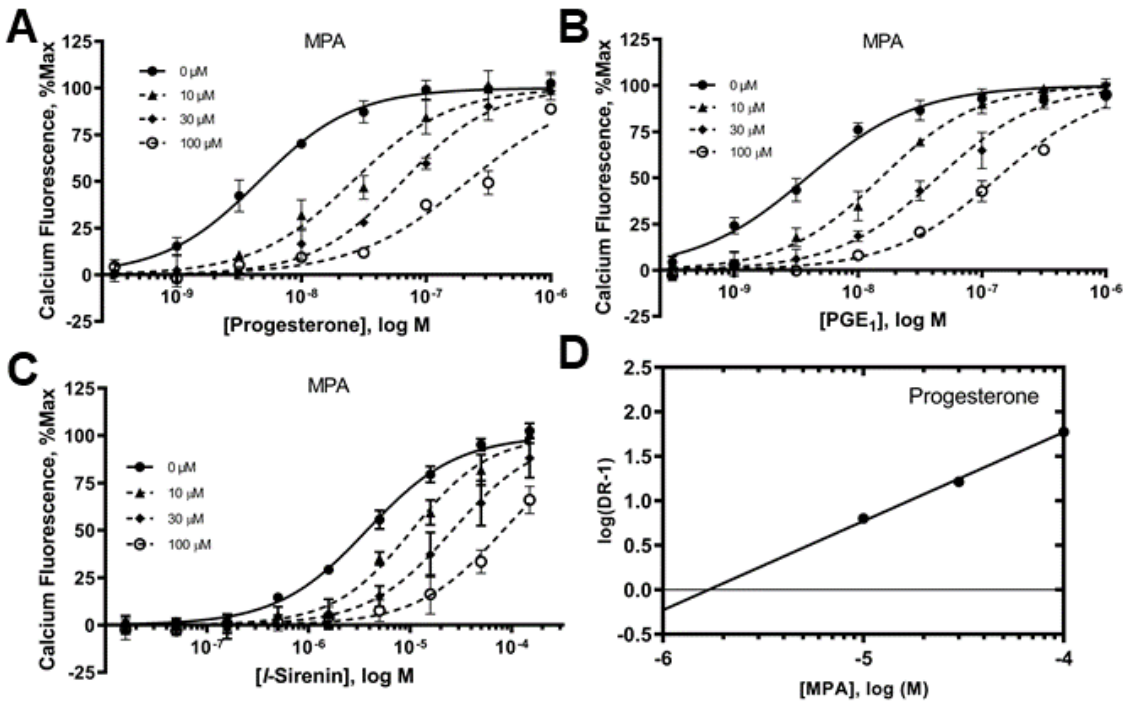


Figure 2.19. Effects of Increasing Concentrations of Medroxyprogesterone Acetate (MPA) on Increase of $[Ca^{2+}]_i$. in Human Sperm. (A-C) Increasing concentrations of MPA increase the $EC_{50(app)}$ value of progesterone (A), PGE_1 (B) and *l*-sirenin (C) while insignificantly affecting Emax, consistent with competitive inhibition. Calculated E_{max} and $EC_{50(app)}$ values, and the number of repeated experiments are given in Table 2.9. Data are plotted as the mean ± the standard error. (D) Representative Schild regression for the competitive block of progesterone by MPA. Dose ratios were calculated from EC_{50} values for each curve fitted independently to a non-linear regression model. The line was fitted by linear regression with its slope set to unity. K_B values for all interactions are given in Table 2.10.

Competitive inhibition, by definition, signifies that the blocker and opener at least partially share a binding site. Interesting, MPA also displays competitive inhibition of the signal evoked by PGE1 and l-sirenin (Figure 2.19, B & C, respectively). Pre-incubation of cells with 30 µM MPA caused approximately a 10-fold shift in the dose-response curve for progesterone giving an EC50(app) of 67 ± 14 nM vs the EC50 of 7.7 ± 1.4 without added

MPA. Table 2.9 summarizes the interactions of each steroidal blocker with each studied opener.

A similar pattern is observed when cells are treated with increasing concentrations of LNG, instead of MPA (Figure 2.20, A–C). LNG displayed a similar effect to those observed when cells were treated with MPA, though the effect on the influx evoked by *l*-sirenin was diminished at 10 μM, compared the inhibition by MPA. At this concentration, LNG was unable to alter the response of cells to *l*-sirenin.

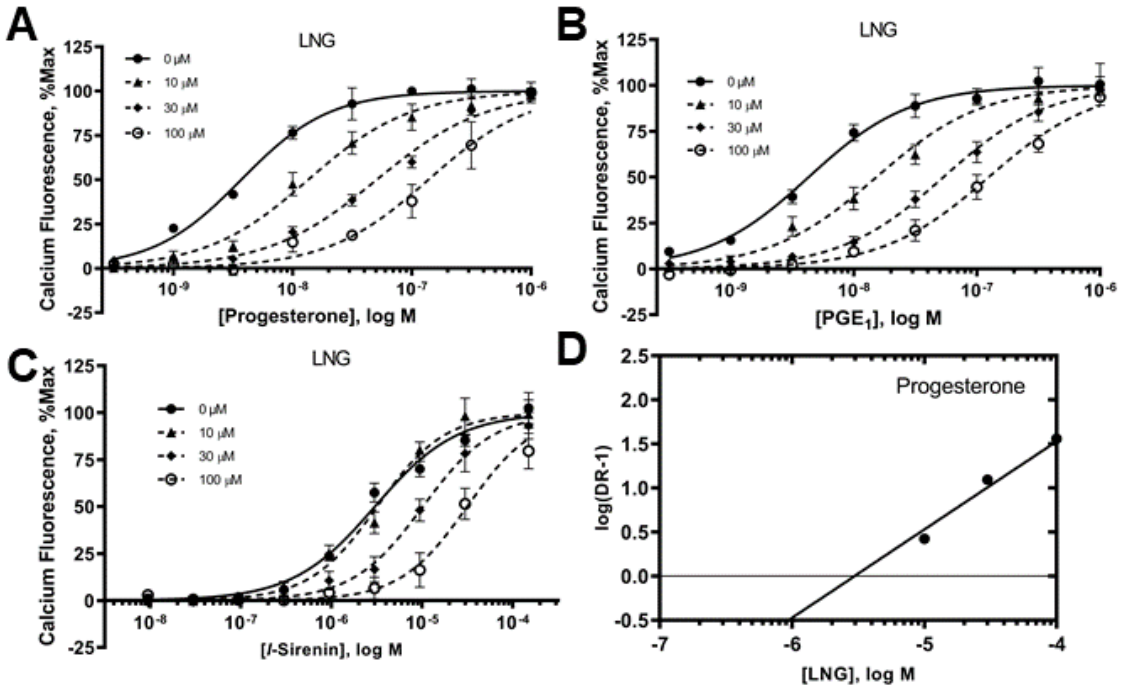
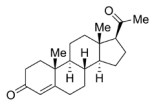
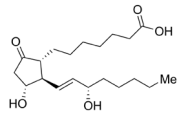
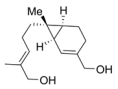
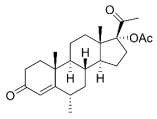
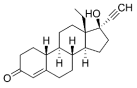
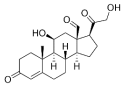


Figure 2.20. Effects of Increasing Concentrations of Levonorgestrel (LNG) on Increase of $[Ca^{2+}]_i$. in Human Sperm. (A-C) Increasing concentrations of LNG increase the $EC_{50(app)}$ value of progesterone (A), PGE_1 (B) and *l*-sirenin (C) while insignificantly affecting Emax, consistent with competitive inhibition. Calculated E_{max} and $EC_{50(app)}$ values, and the number of repeated experiments are given in Table 2.9. The data are plotted as the mean ± the standard error. (D) Representative Schild regression for the competitive block of progesterone by LNG. Dose ratios were calculated from EC_{50} values for each curve fitted independently to a non-linear regression model. The line was fitted by linear regression with its slope set to unity. K_B values for all interactions are given in Table 2.10.

Table 2.9. Effects of MPA, LNG and ALDO on progesterone-, PGE_1- and *l*-sirenin-induced increase in $[Ca^{2+}]_i$ in human sperm

		Opener									
	Blocker	Conc. (μM)	$EC_{50(app)}$, nM[a]	E_{max}[b]	*n*	$EC_{50(app)}$, nM[a]	E_{max}[b]	*n*	$EC_{50(app)}$, μM[a]	E_{max}[b]	*n*
2.16		0	7.7 ± 1.4	100	9	3.1 ± 0.3	94	9	2.9 ± 0.7	99	9
		10	28 ± 8	102	3	15 ± 2	101	3	10 ± 1	108	3
		30	67 ± 14	105	3	55 ± 5	108	3	26 ± 5	111	3
		100	234 ± 19	≥100	3	232 ± 14	≥100	3	83 ± 6	≥100	3
2.28		0	7.7 ± 1.4	100	9	3.1 ± 0.3	94	9	2.9 ± 0.7	99	9
		10	19 ± 6	95	8	15 ± 3	102	4	3.2 ± 0.6	104	4
		30	49 ± 12	102	4	54 ± 4	109	4	10 ± 5	100	4
		100	139 ± 27	≥100	4	125 ± 7	≥100	4	32 ± 6	85	4
2.35		0	7.7 ± 1.4	100	9	3.1 ± 0.3	94	9	2.9 ± 0.7	99	9
		10	28 ± 9	105	4	12 ± 6	105	4	11 ± 2	95	4
		30	84 ± 21	103	4	29 ± 2	104	4	28 ± 5	108	4
		100	253 ± 45	≥100	4	96 ± 8	96	4	85 ± 7	≥100	4

[a]EC50 values given as the mean ± standard error. [b]Data presented as % of saturating dose of progesterone (3 μM). A constraint of E_{max} = 100 was placed on certain curves displaying a significant rightward shift and is indicated by a "≥100" for E_{max}. ND = not determined

Finally, Figure 2.21 shows the effect of increasing aldosterone on the signals caused by each opener. Again, rightward shifts are observed with increasing concentrations of the

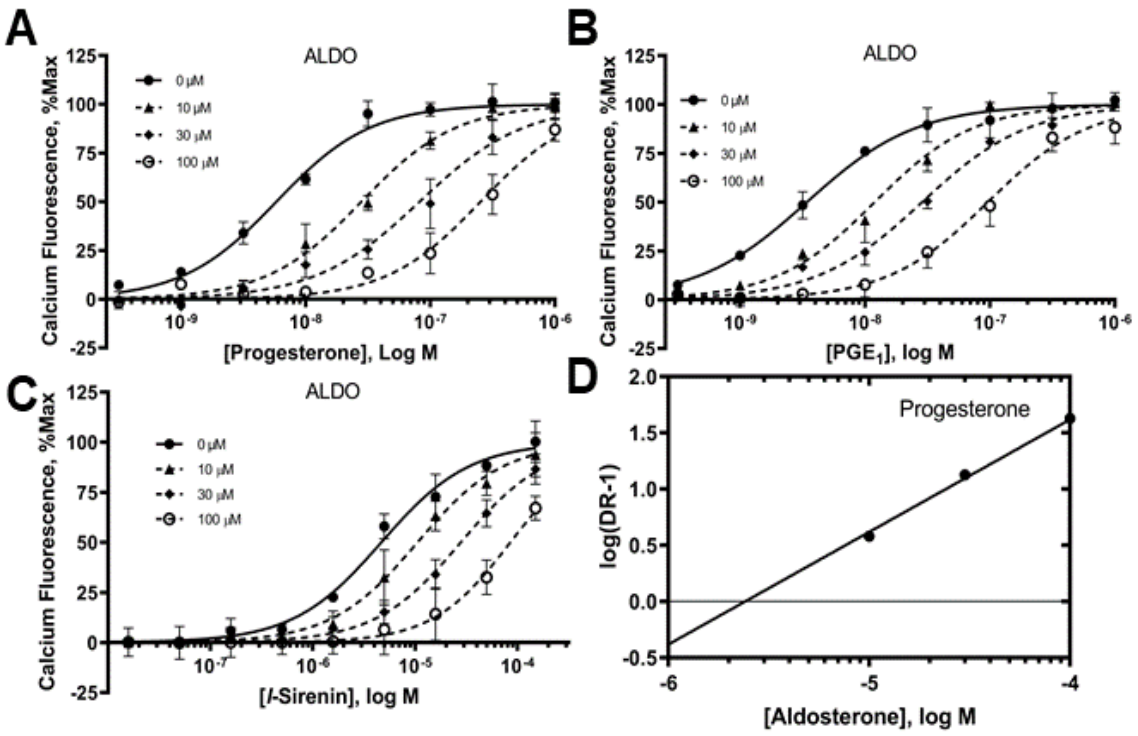


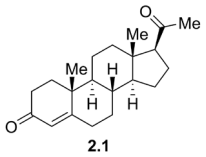
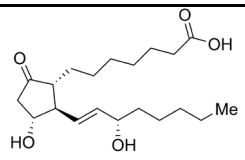
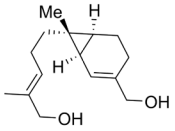
Figure 2.21. Effects of Increasing Concentrations of Aldosterone (ALDO) on Progesterone-Induced Increase of $[Ca^{2+}]_i$. in Human Sperm. (A-C) Increasing concentrations of ALDO increase the $EC_{50(app)}$ value of progesterone (A), PGE_1 (B) and *l*-sirenin (C) while insignificantly affecting Emax, consistent with competitive inhibition. Calculated E_{max} and $EC_{50(app)}$ values, and the number of repeated experiments are given in Table 2.9. The data are plotted as the mean ± the standard error. (D) Representative Schild regression for the competitive block of progesterone by ALDO. Dose ratios were calculated from EC_{50} values for each curve fitted independently to a non-linear regression model. The line was fitted by linear regression with its slope set to unity. K_B values for all interactions are given in Table 2.10.

mineralocorticoid while the E_{max} is unaffected. ALDO, unlike LNG reduced $EC_{50(app)}$ of all the studied openers, having the greatest effect at higher concentrations (30 and 100 μM).

Given this apparent competitive inhibition, we sought to verify the competitive nature of these interactions by performing Schild analysis (Figures 2.19–2.21, D).[104] K_B values (Table 2.10) were determined according to Schild regression experiments and the more generalized Leff-Dougall approach.[105] There was good agreement between the two

methods of analysis and Schild regression plots had slopes of unity within the 95% CI, helping to confirm a competitive interaction between the steroidal blockers and progesterone, PGE_1 and *l*-sirenin.

Table 2.10. Calculated dissociation constants for the studied steroidal blockers of calcium influx into human sperm

Opener	Blocker	pK_B	
		Schild[a]	*Leff-Dougall*[b]
 2.1	*MPA*	5.77	5.66
	LNG	5.53	5.14
	ALDO	5.62	5.02
 2.2	*MPA*	5.51	5.60
	LNG	5.48	5.26
	ALDO	5.34	4.82
 2.3	*MPA*	5.29	4.80
	LNG	ND	3.97
	ALDO	5.2	3.98

[a]For all Schild analyses, the slopes of the regressions used in determinations had slopes of unity within a 95% CI. [b]pK_B calculated from equation presented in ref. 102. ND = not determined

Section 2.6 Computer-Aided Sperm Analysis (CASA) as a Confirmatory Assay

Section 2.6.1 CASA as a Technique for Motility Parameter Acquisition

So far, the data presented, and any conclusions made from it, are derived from the results of a single type of assay. While great effort was put towards making this assay as reliable as possible (and this was achieved), a secondary assay confirming the observations of the first would greatly validate our results. To this end, we turned to computer-aided

sperm analysis (CASA). CASA has been utilized to analyze sperm motility since the late 80s, when it became readily available commercially. The technique has numerous successful studies to its credit in many different species.[106, 107]

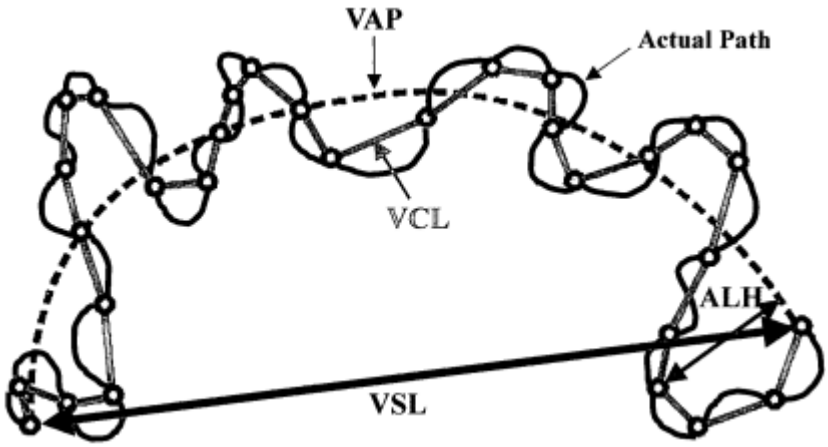


Figure 2.22. The Kinematic Parameters Measured by CASA Systems. VSL, straight line velocity; VCL, curvilinear velocity; VAP, average path velocity, ALH; amplitude of lateral head displacement; LIN; linearity: the linearity of the curvilinear path (ratio of VSL/VCL).

CASA detects the position of sperm heads and generates tracks relating to sperm movement over a short interval. Kinematic parameters (Figure 2.22) can be derived from these tracks describing velocities, path curvature, beat cross frequency, and amplitude of lateral head displacement (ALH). These parameters are monitored in real time from multiple (up to around 60) cells per field of view during short 1 second videos. Cells are then sorted into populations of cells including static, weakly motile (slow), motile, and progressively motile.[108]

Section 2.6.2 CASA as a Technique for Monitoring HAM: Early Studies

It should be noted here that CatSper function has little effect on these normal motility parameters and disruption of channel activity mainly leads to the inability of sperm

to achieve hyperactivated motility (HAM) with only a marginal effect on normal motility expected.[55] HAM is a complex mode of motility integrally intertwined with the even more complex process of capacitation.[109] As such, parameters for its detection in CASA instruments vary greatly from group to group and are dependent on every aspect of sperm cell preparation and isolation including the buffers used, the frame rate of instrument, and individual chamber depths.[110-113] With this in mind we first set out to ensure we could elicit HAM in cells from our various donors involved in the project, while also confirming that our core openers in the study had no effect themselves on normal motility parameters.

First, one of the steps in the procedure for isolating sperm cells can involve a short centrifugation step, utilized to ensure consistent concentrations of cells are used in a given treatment. This centrifugation step was shown to have no direct effect on observed motility of isolated cells and was not taken into any further considerations (data not shown). Next,

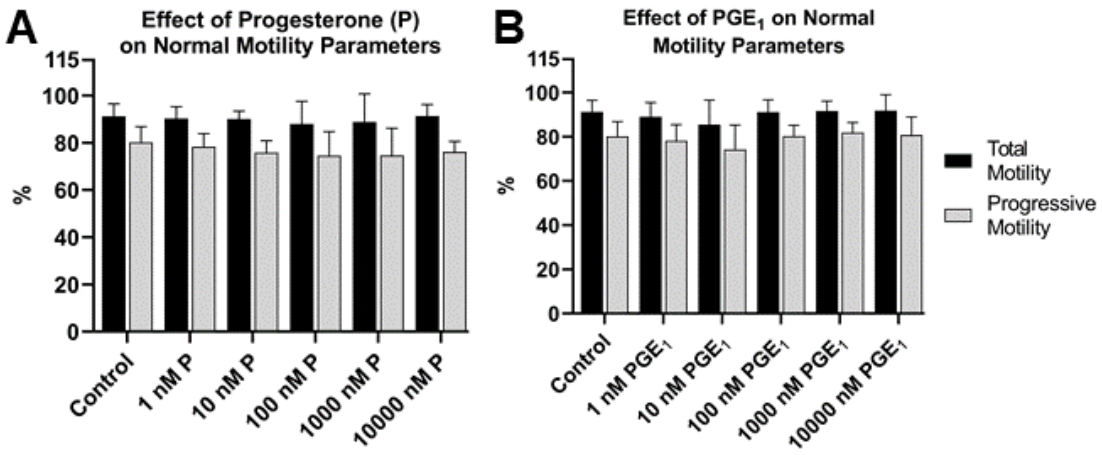


Figure 2.23. Progesterone and PGE_1 Show No Effect on Normal Motility Parameters. Data are represented as means ± SD and represent at least 6 independent experiments. "%" is percent of total cell count.

Figure 2.23 shows that up to 10 μM progesterone (A) and PGE_1 (B) had no significant effect on total or progressive motility in human sperm cells.

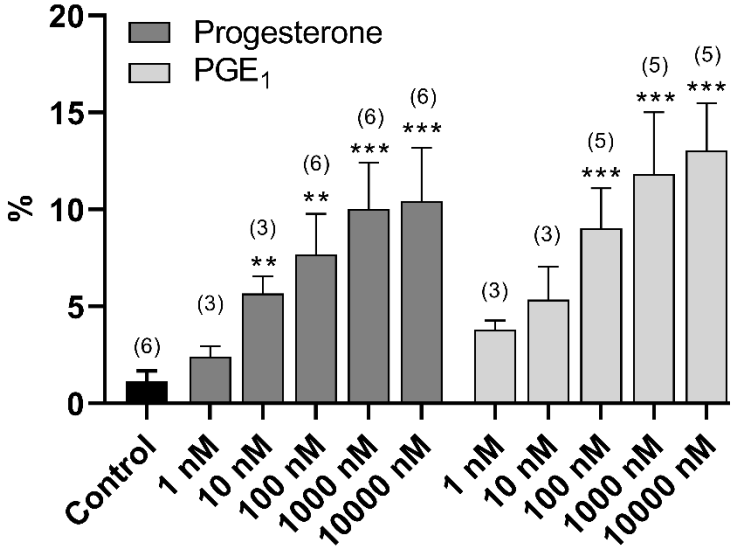


Figure 2.24. Progesterone and PGE_1 Effectively Increase the Population of Cells Displaying HAM in Human Sperm Cells. Data are represented as means ± SD; numbers in parentheses indicate the number of experiments; and $^{**}P < 0.005$, $^{***}P < 0.0005$.

Having shown that progesterone and PGE_1 do not affect normal motility parameters, we sought conditions to raise the population of cells displaying HAM. Many experiments were dedicated to this end and conditions were found in which a concentration-depended increase in cells displaying HAM was observed as displayed in Figure 2.24. Both progesterone and PGE_1, in the presence of 15 mM $NaHCO_3$ and 5% (*w/v*) BSA can successfully and significantly increase the population of cells displaying HAM at 100 nM. Furthermore, increases were seen with a concentration as low as 10 nM, but these increases were only significant for progesterone.

Section 2.6.3 Effect of Studied T-Type CCBs on Sperm Motility

Having established the effect of progesterone and PGE_1 on motility, the next experiments were carried out to observe the effects of all previously studied blockers on

sperm motility, both normal and hyperactivated. It has previously been shown that CatSper knockout mice lose motility over time, so blocking CatSper should show a slight drop in normal motility parameters with an increased, robust effect on HAM.[55] Figure 2.25 shows the effects of mibefradil and ML218 on normal motility parameters in sperm. Also included is the closely related t-type CCB NNC 55 0396 (NNC).

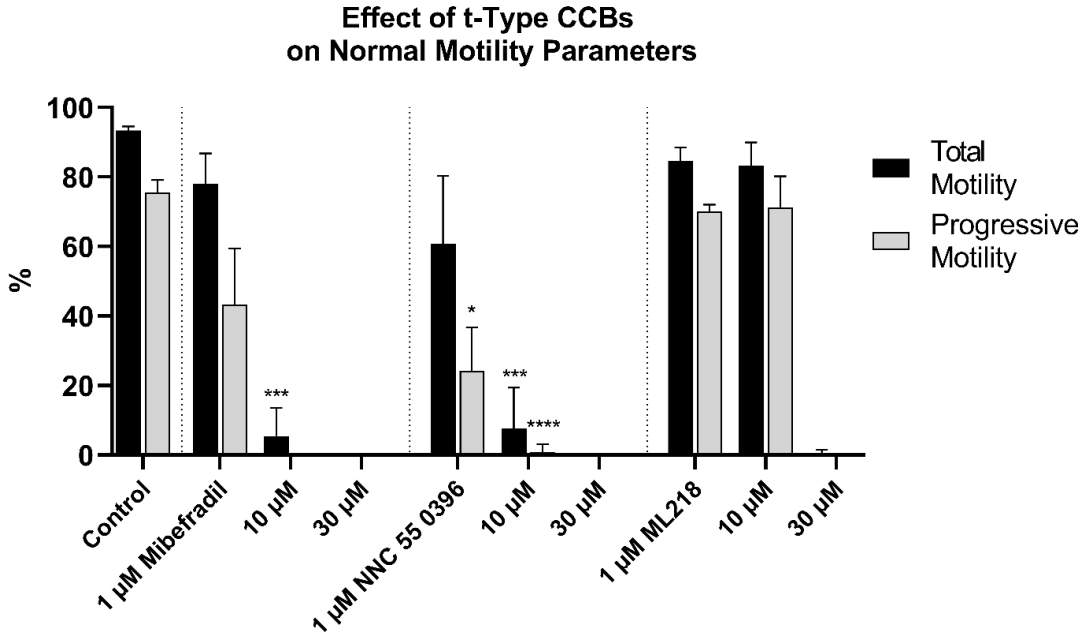


Figure 2.25. Effect of T-Type CCBs on Total and Progressive Motility. Data are represented as means ± SD; data represent the means of 6 individual experiments in all cases; and **P* < 0.05, ****P* <0.0005, *****P* <0.0001. Statistics not performed on data showing complete inhibition of motility.

As shown in Figure 2.25, the t-type CCBs mibefradil and NNC, have a significant effect on motility in human sperm at 10 µM and above, completely reducing total and progressive motility to 0% at 30 µM. Only slight motility reduction is observed at 1 µM though a pronounced effect can be seen, especially on progressive motility. ML218 shows a reduced effect on normal motility compared to mibefradil and NNC, though complete

ablation of motility is still seen at 30 µM. This reduced effect at 10 µM by ML218 is an interesting finding which will be discussed further later in this chapter.

Section 2.6.4 Steroidal Blockers Show Little Effect on Sperm Motility

Before determining the effects of the steroidal blockers on HAM, we first sought to determine their effects on normal motility parameters. We expected these compounds to show a minimal effect on normal motility and this was observed, as shown in Figure 2.26. When tested at 1, 10 and 30 µM, only MPA showed any significant effect on total and progressive motility. LNG and ALDO did not impact normal motility parameters even at the highest concentrations tested, likely reflecting their diminished potencies observed in the FLIPR assay.

Section 2.6.5 Both Classes of Blockers Effectively Reduce HAM

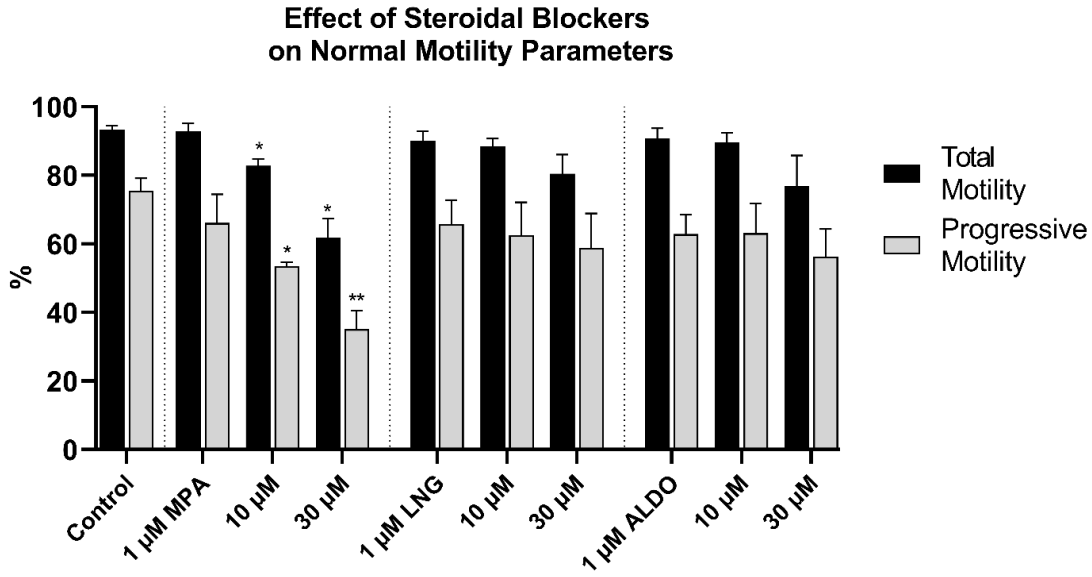


Figure 2.26. Effect of Steroids on Total and Progressive Motility. Data are represented as means ± SD; data represent the means of 8 individual experiments in all cases; and **P* < 0.05, ***P* <0.005.

With the effects and discrepancies between classes of blockers, the next step in the progression of this project was to show the effects of the blockers on HAM specifically. Starting with the t-type CCBs, and given their pronounced effect on normal motility, it was expected that their effect on HAM would be substantial. Figure 2.27 shows that this is the case, with no cells achieving HAM above a concentration of 1 μM. The only exception

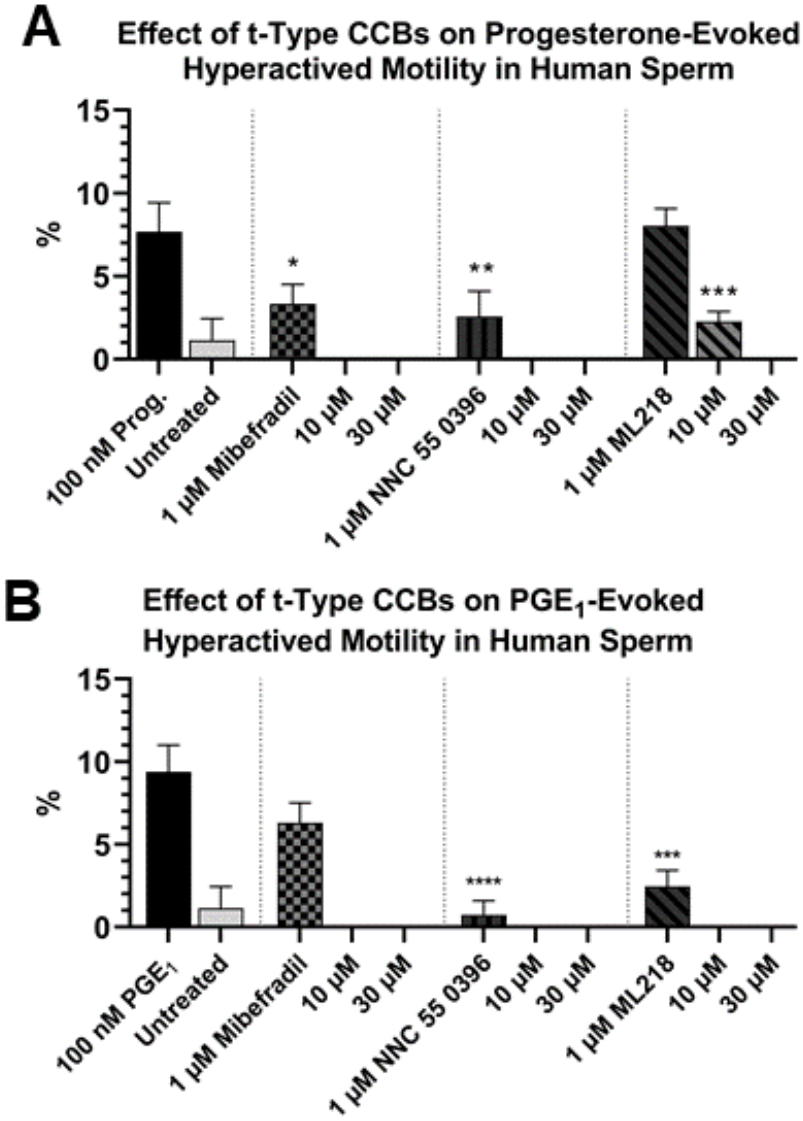


Figure 2.27. Effect of T-Type CCBs on Hyperactivated Motility (HAM). Data are represented as means ± SD; data represent the means of four individual experiments in all cases; and **P* < 0.05, ***P* <0.005, ****P* <0.0005, *****P* <0.0001.

was ML218 affecting progesterone-evoked HAM, in which a small fraction of cells was still able to hyperactivate. The effect of the compounds at 1 μM is pronounced in almost all cases. This class of compounds showed little effect on normal motility parameters at this concentration. Thus, the observed effect at 1 μM on HAM could point towards the efficacy of these compounds inhibiting CatSper specifically.

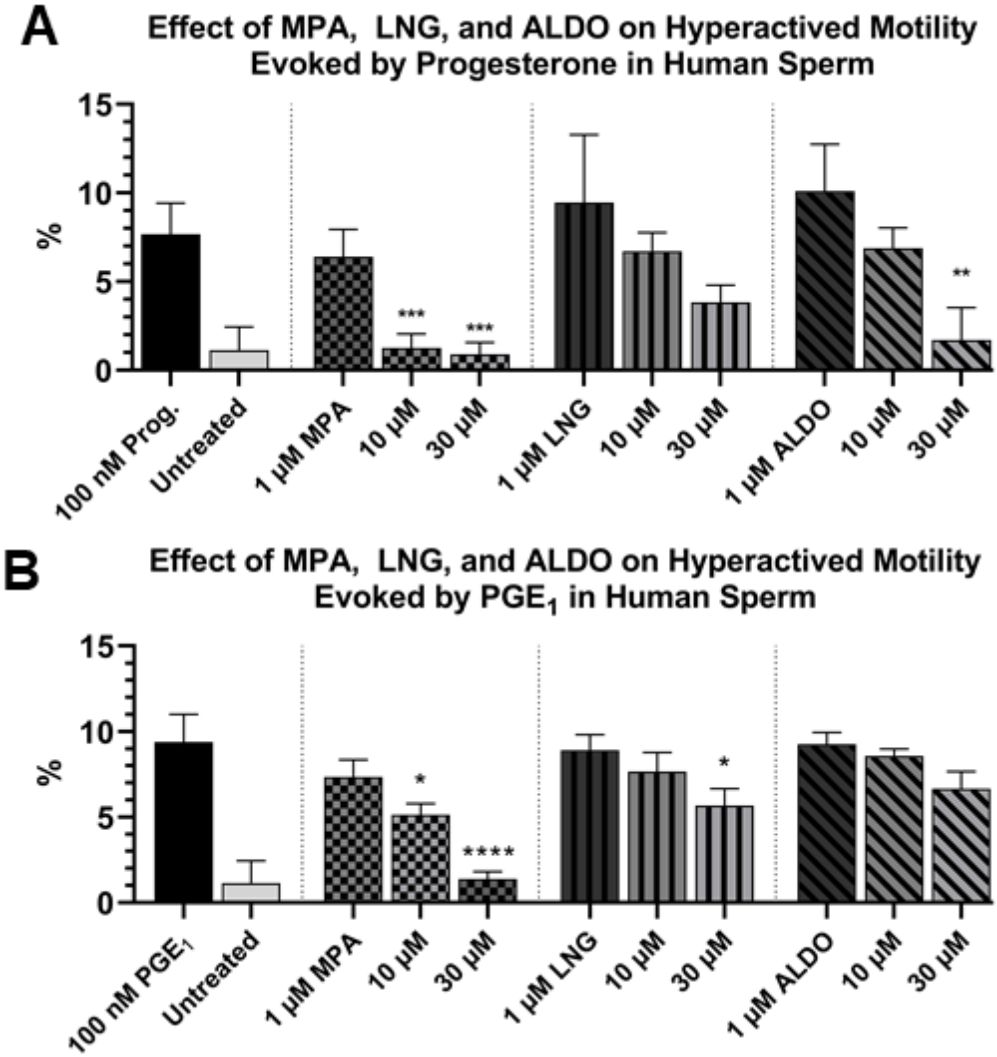


Figure 2.28. Effect of MPA, LNG and ALDO on Hyperactivated Motility (HAM). Data are represented as means ± SD; data represent the means of six individual experiments in all cases; and **P* < 0.05, ***P* <0.005, ****P* <0.0005, *****P* <0.0001.

With respect to the steroidal blockers, the effects on HAM are less pronounced and follow the trend observed with normal motility parameters, with MPA proving most effective in all cases and both LNG and ALDO providing only weak inhibition of HAM. Figure 2.28 shows the effects of the steroidal blockers on HAM.

MPA at 10 and 30 μM is able to significantly reduce the population of sperm cells displaying HAM when both progesterone and PGE_1 are used. Like the t-type CCBs, the effect from MPA is more potent with respect to HAM, compared to normal motility modes. Again, this can point towards CatSper-specific inhibition. Interestingly, LNG and ALDO show a reduced ability to inhibit HAM with respect to both studied openers. Both show efficacy at 30 μM individually, but neither is consistent across both openers, as either steroid shows increased efficacy for only one of the openers studied.

Section 2.6.6 Recovery of Motility is Only Possible with Steroidal Blockers

As exhibited in the FLIPR assay, there exists great discrepancies between the two types of blockers (Section 2.5) with respect to all methods of opening. To explore this observation further, making use of CASA, a simple experiment was envisioned in which the motility of cells could be recovered after treatment with a blocker in the presence an opener if that block was surmountable in nature. If, in the presence of opener, the motility was recovered despite the blocker being present this would indicate surmountable inhibition while, insurmountable inhibitors will have their effects unperturbed by the presence of an opener.

The detailed experiment was only carried out with MPA the steroidal blocker, since this compound was the only compound to reduce normal motility parameters. As expected, when cells were treated with 30 μM MPA a reduction in total motility was observed

consistent with previous results (Figure 2.29, A). When these cells were then challenged with 100 nM progesterone significant recovery was observed, even approaching untreated levels though falling short marginally.

Conversely, 100 µM progesterone was unable to increase motility when cells were previously dosed with 10 µM mibefradil, consistent with results from the FLIPR studies. Unexpectedly, when cells were dosed with 30 µM MPA and then dosed with 100 nM PGE_1, no recovery of motility was observed. Furthermore, recovery of motility was observed in cells pretreated with 10 µM mibefradil, which to this point had only shown insurmountable inhibition. The increase in the presence of 100 nM PGE_1 was statistically not significant, though the trend is undeniable. Further experiments are needed to explore this interesting, unexpected discrepancy between the t-type CCBs and the steroidal blockers.

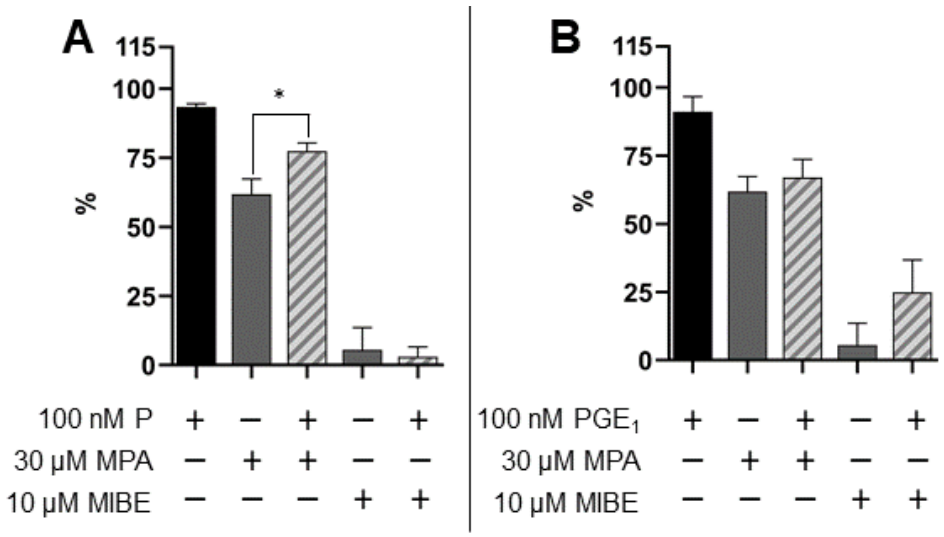


Figure 2.29. Modulation of Total Motility in the Presence of MPA or Mibefradil by Progesterone or PGE_1. (A) Progesterone, P, rescues motility when coapplied with MPA but not mibefradil. (B) PGE_1 is unable to recover motility degraded by steroidal blockers but can slightly increase motility in the presence of t-type CCB. Data are represented as means ± SD; data represent the means of three individual experiments in all cases; and $*P < 0.05$.

Section 2.7 Discussion of Results

Section 2.7.1 The CatSper channel is promiscuously activated

The recent observations that exogenous EDCs and odorants can activate CatSper gives credence to the idea that CatSper can be activated by a wide variety of small molecules, including several classes of steroids.[101] This study corroborates this notion by showing that over 30 steroid compounds activate CatSper. The systematic modification of the steroid skeleton has led to insights into the activity of steroids acting at CatSper which are summarized in Figure 2.30.

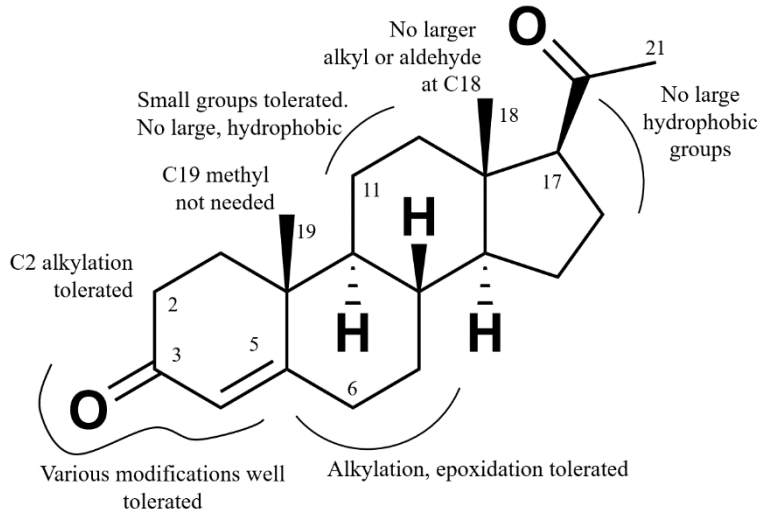


Figure 2.30. Summary of the SAR of Progesterone Activating CatSper.

Modifications to the A-ring appear to be well tolerated, with C2 alkylated **2.4** (Table 2.1), as well as reduction of both the C4,C5-olefin and C3 carbonyl groups appearing to have no significant effect on activity (**2.5**–**2.8**, Table 2.1). These observations correspond well with previous studies showing that an *O3*-linked BSA-progesterone conjugate was still able to activate CatSper.[83] The first reductions in potency were seen when the C21-hydroxyl bearing THDOCs **2.9** and **2.10** were tested, with EC_{50} values

falling above 1 μM for the first time (Table 2.1). This was the first indication that perhaps the D-ring of the steroid scaffold could be impactful, with respect to modulating channel activity.

Further exploration of the B ring revealed that alkylations of this area are only somewhat well tolerated. Considerably reduced potencies were observed when modification of the B-ring is combined with other detrimental modifications, such as C17 alkylations, like those seen in Table 2.2. An interesting trend was observed with the clinically used progestin medroxyprogesterone (**2.15**), which itself showed a modest potency of 480 nM. However, acetylation of the 17α-hydroxyl group to give **2.16** (MPA) completely ablated activity. Isolating these modifications, without the accompanying C6 alkylation gives two compounds of extremely different activities with 17α-acetoxyprogesterone **2.17** showing almost no ability to activate CatSper while 17α-hydroxyprogesterone **2.13** is one of the most potent compounds identified at 7.2 nM.

Whether or not the C6 modifications could also exert this sort of influence over activity remained to be seen so the synthesis of 6α-methylprogesterone (**2.18**) was undertaken, generating several additional analogs described in Table 2.3. Testing **2.18** in the influx assay revealed it to be a moderately potent activator of CatSper, further pointing away from the B-ring and instead towards the D-ring. Two interesting observations came from testing these synthesized steroids. First, 5α-hydroxy-6β-methylprogesterone **2.23** displayed a much lower potency than other, structurally similar, compounds. And second, every steroid bearing a C3,C20 ketal rather than the natural ketone was completely inactive in the influx assay (**2.19**–**2.22**).

Several C-ring modified steroids were also tested. Bulky alkylations, such as the 4-(dimethylamino)phenyl substituent found in the clinically relevant antiprogestins ulipristal (**2.31)**, ulipristal acetate (**2.32**) and mifepristone (**2.30**) markedly reduce the potency of these compounds (Table 2.4). While the potencies of all three were reduced from progesterone, **2.31**, bearing a 17α—hydroxyl group showed much great efficacy than its acetylated counterpart **2.32.** Small groups such as hydroxy and methyl substituents were tolerated at the C11 position, indicating that large substitutions at this position greatly reduce the ability of these compounds to activate CatSper. This agrees nicely with a previous study which showed a lack of CatSper activation by a progesterone analog bearing a BSA conjugated to the steroid via a C11 hydroxy group.[83]

It is worth noting that, while previous studies reported that mifepristone and ulipristal acetate were unable to block the progesterone-induced activation of CatSper, this is the first time these two compounds have been shown to actually activate the channel, albeit at elevated concentrations ($EC_{50} \approx 5$ μM).[114] The fact that these nuclear hormone receptor *antagonists* are able to instead *activate* CatSper serves as a fascinating example of the differences between CatSper and the progesterone nuclear hormone receptor.

Recently, a synthetic androgen, 11β-methyl-19-nortestosterone dodecylcarbonate has passed safety trials and is being investigated further as a potential male contraceptive.[28] The free hydroxyl analog of this prodrug was included as a generous gift of synthetic androgens from the National Institute of Child Health and Human Development (NICHD) and was tested in the influx assay (Table 2.5) 11β-Methyl-19-nortestosterone (**2.36**) activates the CatSper complex with nanomolar efficacy (EC_{50} = 280 nM). Two of the synthetic androgens provided by the NICHD (**2.36** and **2.37**) displayed submicromolar

efficacy and two (**2.38** and **2.39**) showed micromolar efficacy for activating CatSper. The contraceptive efficacy of these compounds has not yet been determined, however if these studies were performed, the data presented herein could provide information to be considered for clinical trial design.

In general, modifications to the D-ring of the scaffold produce much greater effects on the activity of the tested steroids, leading to the interpretation that this portion of the scaffold plays an important role in CatSper activation. This conclusion is strengthened by the significant difference in potency observed for 17α-hydroxyprogesterone (**2.13**) and 17α-acetoxyprogesterone (**2.17**) (Table 2.2). It was frequently observed that all of the least potent potential activators tested in the influx assay bear acetoxy groups or similar hydrophobic substitutions at the C17 position, e.g. ulipristal acetate (**2.32**), mifepristone (**2.30**), nestorone (**2.29**), medroxyprogesterone acetate (**2.16**), and levonorgestrel (**2.28**).

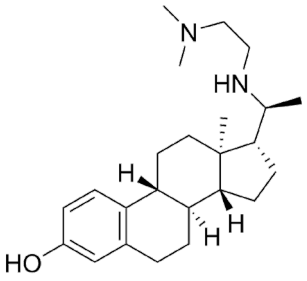


Figure 2.31 Structure of RU1968. A cross-species inhibitor of CatSper.

Furthermore, modification of the C18 position produced inactive compounds bearing either an ethyl or an aldehyde as seen in **2.28** (LNG) and **2.35** (ALDO), respectively, though LNG also bears a C17 modification. All these modifications are located in proximity to the D-ring of the scaffold and inhibitor development in the future should follow the lead of Strünker *et al*. in their development of RU1968, shown in Figure 2.31.[115]

Section 2.7.2 ML218 blocks CatSper

When studying CatSper physiology or pharmacology, investigators have few options beyond the published t-type CCBs mibefradil and the structurally similar NNC 55 0396 (NNC). The mentioned RU1968 serves as another option to investigators but a t-type calcium channel showing a distinct structure from mibefradil and NNC would be an advantage to the field. In this study, we show for the first time that ML218 (**2.41**) is able to block all methods of evoking calcium influx in sperm, be it progesterone, PGE_1, sirenin or elevated pH/K^+ with IC_{50} values ranging from 9 to 15 μM depending on mode of activation (Table 2.6). For each method, the potency of ML218 was similar to that of mibefradil (Table 2.6). Additionally, both these t-type CCBs exhibit Hill slope values well below -1.0, pointing to a non-competitive, cooperative inhibition. Additionally, at concentrations up to 100 μM, ML218 showed insignificant calcium influx in sperm cells, in contrast to previous studies using mibefradil.[82] Altogether, ML218 serves as another viable option for investigators interested in the pharmacology and physiology of CatSper and can be included as a tool compound in future CatSper studies.

Section 2.7.3 Steroidal blockers of CatSper

Of the 41 compounds tested for their ability to evoke calcium influx in sperm, four compounds showed no activity: cholic acid, MPA, LNG and ALDO. When tested for their ability to block the progesterone-induced activation of CatSper, all but cholic acid were active (Table 2.7). MPA, LNG and ALDO can now be added to the growing number of steroidal inhibitors of the CatSper complex. Furthermore, these compounds were able to

prevent the PGE_1- and sirenin-induced calcium influx, a first in the field, as no steroidal blockers of these modes of activation have been shown previously.

Of the steroidal blockers, MPA was the most potent across all modes of activation, while the IC_{50} values of ALDO and LNG were below 20 μM. This trend continued for PGE_1- and sirenin-induced calcium influx. When studied for its ability to effect conception, MPA concentrations in the endometrium have been shown to rise towards the IC_{50} values obtained herein.[116] This leaves the possibility that MPA could be having contraceptive effects by a unique, previously unknown, mechanism. The IC_{50} values of ALDO and LNG are sufficiently high so as to preclude their study of CatSper in vivo. Nevertheless, biochemical assays could still make use of these compounds to observe varying effects on human sperm function.

Interestingly, the studied steroids were unable to block the pH/K^+-induced calcium influx (Figure 2.16) indicating they prevent binding of activators rather than interfering with channel shape and function or directly blocking the pore of the channel. Also, given that these compounds do not prevent the signal evoked by ionophore A23187 (Figure 2.17), they are not non-specifically reducing the signal in the assay. This finding contributes further to the idea these compounds are binding and preventing activator binding.

As mentioned before, the concentrations required for a total block of CatSper within these trials is well above those found in individuals taking any of these medications with respect to LNG and ALDO.[117, 118] Even when taken as a higher dose for emergency contraception, LNG concentrations do not approach those corresponding to the IC_{50} value.[119] That is not to say, however, that the observed effects on motility associated with

MPA are not, in small part, due to CatSper activity. Given the intricacies involved in gamete transport, a minimal effect on CatSper could have important effects on fertility.

Section 2.7.4 Mode of inhibition: Steroids vs T-Type CCBs

Over the course of studying the SAR of steroids activating CatSper, each compound was concomitantly blocked with mibefradil to assure its activity was via CatSper (rightmost columns of Tables 2.1 – 2.5). In every instance, each tested compound was blocked by mibefradil in an apparent insurmountable manner, meaning there was no rightward shift in the dose-response curve but E_{max} was substantially reduced.

This observation was reinforced by treating cells with increasing doses of progesterone, PGE_1 and sirenin in the presence of 1, 10, 30 and 100 μM mibefradil or ML218 and observing a dose dependent reduction of E_{max} (Figure 2.18, and Table 2.8). These results suggest that mibefradil and ML218, and likely NNC given its structural similarity to mibefradil, bind CatSper at a site unique to that of the studied openers. The fact that these CCBs can block the ligand-free pH/K^+-induced calcium influx suggests they alter the conformation of the channel upon binding, closing the pore of CatSper.

In contrast, cells treated with the studied openers were consistently able to achieve maximum efficacy in the influx assay despite treatment with high concentrations (100 μM) of steroidal blocker (Figures 2.19 – 2.21). Only an apparent decrease in potency, not efficacy, was observed, consistent with competitive inhibition for each steroidal opener. (Table 2.9) For competitive inhibition to be observed, an overlap of binding sites is required. Given that MPA displays competitive inhibition with respect to both progesterone and PGE_1, it follows that the binding sites for progesterone and PGE_1 are either overlapping, the same, or in close proximity.

It is not unexpected that MPA and progesterone share a binding site given their structural similarity, however that they could also share a site with PGE_1, which is perhaps more interesting, especially given the previous studies reporting distinct sites for both molecules.[84] Stranger still is that the action of *l*-sirenin, a molecule from an entirely different species with no human counterpart observed, is also inhibited competitively by all three steroidal blockers. Taken together, these observations point to a shared or overlapping binding pocket for sirenin, progesterone and PGE_1.

With the identification of ABHD2 as a binding partner for progesterone in 2016, a potential explanation for the observed differences between the t-type and steroidal blockers of CatSper needs to be considered (Figure 2.32).[84] It is possible that the t-type blockers

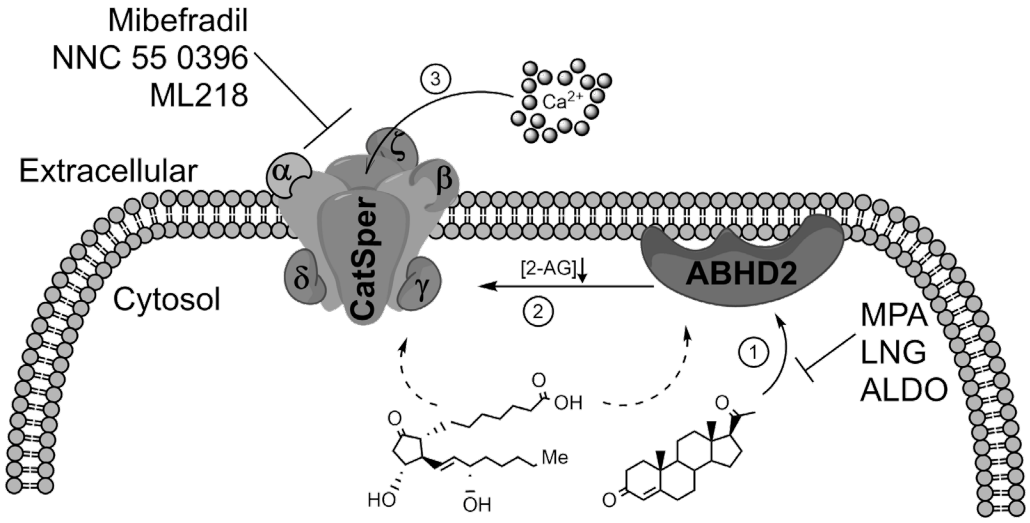


Figure 2.32. Graphical Summary of CatSper Activation and Pharmacological Interventions. (1) Progesterone binds to and activates the hydrolase ABHD2. (2) ABHD2 hydrolyses 2-arachodonyl glycerol (2-AG), decreasing the concentration of this lipid in the plasma membrane of sperm. (3) Decreasing 2-AG levels in the membrane drastically shift CatSper activation thresholds and allow for potentiation of current through the channel. T-type CCBs directly affect CatSper while steroidal blockers affect ABHD2. There is insufficient data to conclude with confidence where PGE_1 binds and exerts its influence.

bind to and inhibit the CatSper complex, while the steroidal blockers bind to membrane-associated ABHD2. Without further studies beyond those conducted here and previously, however, the promiscuity with which CatSper is activated will not be fully understood, especially given the complexity of CatSper as a whole.

Section 2.7.5 CASA Studies of Selected Blockers

To possibly give more credence to these observations, we employed computer-aided sperm analysis (CASA) to determine the effect of both t-type CCBs and steroidal blockers on sperm from healthy men. It was found that the t-type CCBs, mibefradil, ML218 and NNC 55 0396 have profound effects on normal motility parameters (total and progressive motility, Figure 2.25) while also abolishing hyperactivated motility (HAM, Figure 2.27). The effect on normal motility versus HAM was not differentiated with this class of compounds, meaning the effects on both modes were substantial.

Contrasting these results, the effects of the steroids on normal motility were minimal, with only MPA showing a slight effect on normal motility (Figure 2.26). The steroids also showed a diminished effect on HAM (Figure 2.28), with only MPA showing significant inhibition. LNG also significantly inhibited HAM at 30 μM, but MPA by far showed the most promising inhibition, consistent with its potency in the influx assay compared to LNG and ALDO.

Of note, during the course of these CASA studies, a small percent of the sperm population was shown to achieve HAM in the buffers used (Figure 2.27 and 2.28, "untreated"). The t-type CCBs completely abolished HAM, reducing the population to zero at high concentrations. Conversely, MPA never reduced the population of cells displaying HAM to zero, instead only reducing to levels consistent with cells receiving no treatment.

This observation shows again the differences between these two classes of blockers. Given that the steroids are likely blocking activation via ABHD2 inhibition, a basal level of $I_{CatSper}$ is still achievable, leading to these baseline HAM populations. The t-type CCBs block all CatSper activation and, as such, abolish all HAM.

Section 2.8 Summary and Prospects of the Data Presented

In summary, I have used a calcium influx assay to study the SAR of the progesterone activating CatSper through systematic modifications of the steroid scaffold. From these experiments, I conclude that the D-ring of the scaffold plays a prominent role in CatSper activation while the A, B, and C ring better tolerate modifications, confirming observations made from previous work.[83] Within these studies, three compounds showed negligible influx: medroxyprogesterone acetate (**2.16**), levonorgestrel (**2.28**) and aldosterone (**2.35**).

These compounds were subsequently shown to block not only the progesterone-induced calcium influx, but also that of PGE_1 and sirenin. Furthermore, I showed that these steroidal blockers act in a competitive nature with respect to progesterone, PGE_1 and sirenin, in contrast to the studied t-type CCBs, mibefradil and ML218, which display an insurmountable block. This is the first study showing ML218 blocks CatSper activation, giving researchers another tool compound beyond mibefradil and NNC55-0396 when studying CatSper physiology and pharmacology. Furthermore, the fascinating interplay of the steroidal blocks and t-type CCBs was studied in depth, with more questions being raised than answered.

Future work on this project should focus on further studying the potential inhibition (or lack thereof) of ABHD2 by relevant compounds. Binding assays, such as those

previously reported, should be repeated with UV-probes of PGE_1 and, potentially, steroidal blockers to gain better insight into this intricate, fascinating system that continues to confound the field with its complexity.[84]

Chapter 3: Hit-to-Lead Development of Two Scaffolds Discovered via a High Throughput Calcium Influx Assay

Section 3.1 Introduction

As presented in the previous chapter, birth control has been available to women since the early 1960s.[6] Since then research to discover and develop a male equivalent has focused on hormonal contraceptives.[120] However, no agent has reached the market to date due to side effects such as decrease of high-density lipoprotein (HDL), acne, low libido, and weight gain.[39] Therefore, efforts have centered in recent years on developing non-hormonal p\harmacological agents that specifically target the testis, the epididymis, or sperm. Nonhormonal targets relevant to male contraceptive development are briefly outlined in Chapter 1.

To date, little progress has been made in developing CatSper-specific blockers or antagonists. Along with the previously discussed NNC 55-0396 (**3.1**)[121] and mibefradil (**3.2**),[122, 123] limited screening of compounds identified HC-056456 (**3.3**) and its

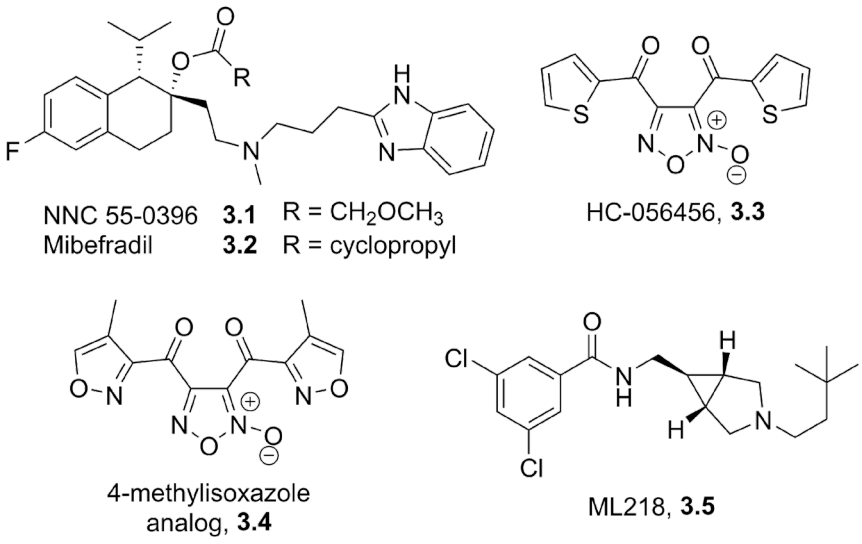


Figure 3.1. Structures of CatSper Blockers Discovered to Date.

methylisoxazole analog (**3.4**) as CatSper blockers (Figure 3.1). Compounds **3.1** and **3.2** are both antihypertensive agents showing minimal selectivity for t-type currents over l-type currents and have shown cardiotoxicity, leading to their withdrawal from clinical trials.[124]

Compounds **3.3** and **3.4** slow the rise of intracellular calcium in sperm and prevent hyperactivated motility in the low micromolar range, but contain structural liabilities precluding their further development. In the previous chapter we discussed the t-type CCB ML218 (**3.5**, Figure 3.1). This compound is exquisitely selective for t-type currents but its safety profile is under explored.[102]

As mentioned previously, heterologous expression of a functional CatSper channel has not yet been achieved and is likely due to a failure in proper assembly of the CatSper complex.[125] This issue has hampered traditional drug discovery efforts for this ion channel target and thus ex vivo experimentation using live sperm is currently the best method to discover modulators of CatSper in a high throughput manner. This chapter describes the results of a high throughput screen completed in the fall of 2012 which led to several hit compounds which were subsequently verified and elaborated upon. The full scope of this screen is beyond the scope of this chapter, but a brief description follows.

The HTS screen leading to the discovered compounds is found in Figure 3.2. To begin, over 36K compounds were screened at 10 μM using elevated potassium/pH conditions to elicit calcium influx into human sperm. This resulted in 220 compounds that exhibited > 60% inhibition. These 220 compounds were selected for dose response experiments in which 104 showed well-defined sigmoidal dose-response curves with IC_{50} values below 55 μM.

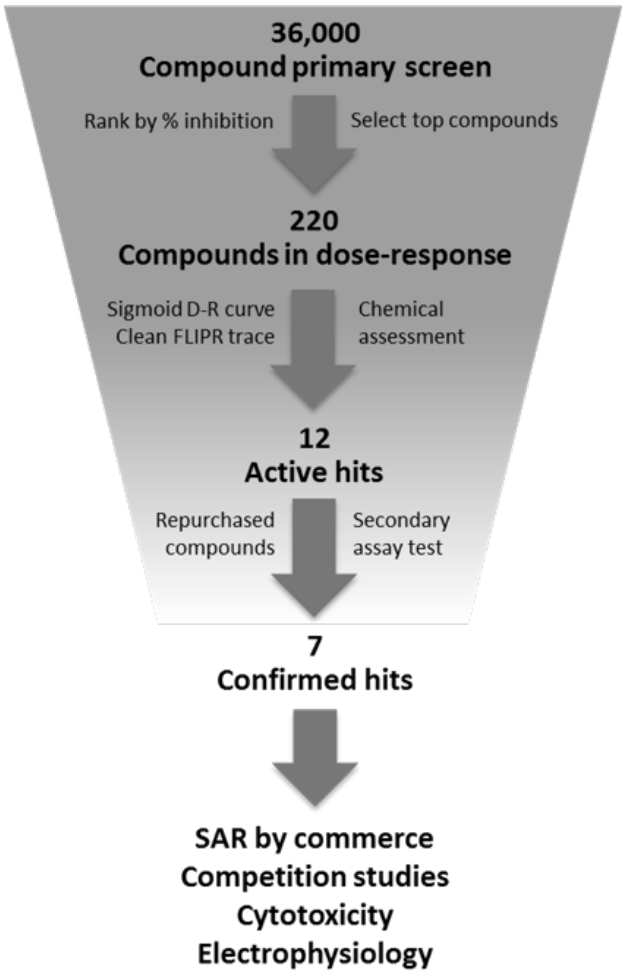


Figure 3.2. CatSper HTS Campaign Workflow.

Potency, physiochemical properties and lack of fluorescence interference were taken into account to reduce the number of compounds down to 12. These compounds were repurchased and verified to be pure by UPLC, then subjected again to the influx assay, only now the endogenous activator progesterone was used to elicit influx. Eight compounds reconfirmed in this assay and were chosen for further elaboration. One of these compounds was the known non-selective dopamine agonist apomorphine. This compound was removed from consideration considering its well-defined pharmacology, leaving seven scaffolds (**3.6** to **3.12**, Figure 3.3) for potential elaboration into lead molecules.

Several rounds of SAR studies were carried out with each confirmed hit using commercially available molecules, with data obtained in each round driving the selection

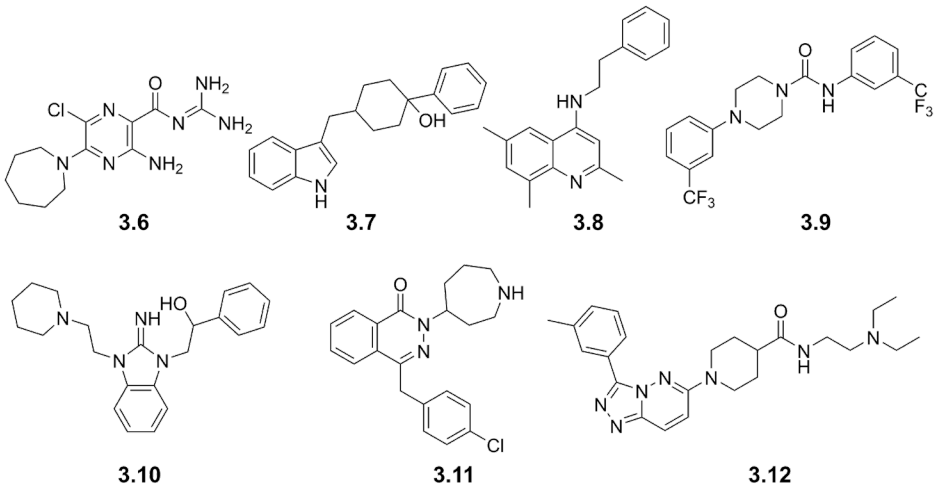


Figure 3.3. Structures of Screening Hits from HTS Campaign.

of successive analogs. Analogs were also selected from the GPHR-library within the ITDD, as only a small portion of the library was screened originally. A total of 67 analogs were selected for purchase from commercial sources based on structural diversity, high ligand efficiencies, and cost/availability. From these purchased compounds, an evaluation of the SAR of each scaffold was obtained, though in all cases the most potent compound in the FLIPR assay was the original hit compound.

In an attempt to narrow the number of scaffolds for SAR studies, cytotoxicity studies using the AlamarBlue assay were performed. The fibroblast cell line IMR-90 was selected as a non-transfected, non-germline control in addition to sperm viability assays. From these assays, **3.9** (GPHR-00032750) and **3.12** (GPHR-0036795) showed a complete lack of cytotoxicity in both sperm and control cell lines. This fact, coupled with promising

initial electrophysiology and CASA data, led to the selection of these two scaffolds for a comprehensive SAR study. The results of which are described herein.

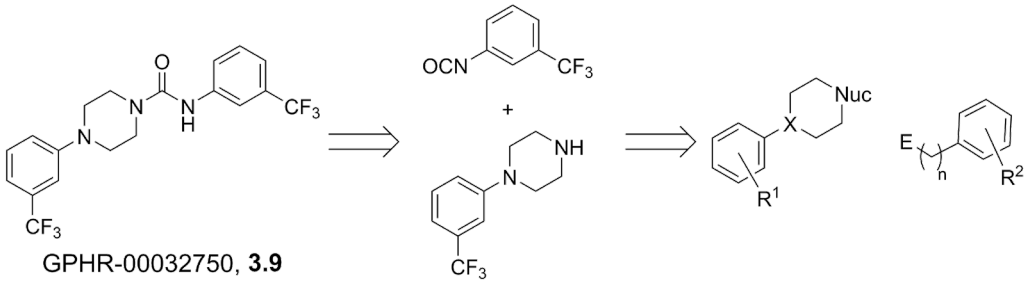
Section 3.2 Development of GPHR-00032750 via Matrix Chemistry

Section 3.2.1 Initial Observations and Retrosynthetic Strategy

Hit **3.9** was originally found from a library of ChemBridge compounds and obtained the designation GPHR-0032750 in the GPHR Library. In the original screen, this compound was one of the more potent hits found, with an IC_{50} value of 4.1 ± 1.2 μM for progesterone-induced influx and 9.2 ± 4.0 μM for potassium-mediated influx. Additionally, electrophysiology work performed in the lab of Dr. Polina Lishko at UC–Berkeley showed that the parent compound is a CatSper blocker in healthy human sperm (data not shown).

Based on these results, **3.9** was subjected to further SAR studies. To this end, a synthetic chemistry effort utilizing matrix chemistry was undertaken. Matrix chemistry allows for the generation of multiple compounds in rapid succession by forming all possible iterations of two different reaction partners. With respect to the retrosynthetic analysis of scaffold **3.9,** the combination of a nucleophile (piperazine) and an electrophile (isocyanate) will provide an array of analogs as shown in Scheme 3.1.

Scheme 3.1 Retrosynthetic approach to analog generation centered on **3.9**



The chemistry leading to analogs of **3.9** allows for the rapid generation of analogs by coupling various piperazines, alcohols, thiols and amines to isocyanates, carboxylic acids, sulfonyl and acyl chlorides and others. In this library, every compound was produced on a sub 20 mg scale, but sufficient quantities of every compound were purified by flash chromatography to properly characterize all synthesized compounds. All compounds were verified to be > 85% pure by qNMR before testing in the influx assay. This influx assay is the same as described in Chapter 2 and progesterone was utilized to activate CatSper in these assays, as it is the more physiologically relevant activator compared to elevated potassium/pH. Any compound that proved to be a rather potent analog was resynthesized, purified to >95% purity as determined by qNMR and, frequently, though not always, also by UPLC.

The generation of the focused library and its synthetic efforts follow.

Section 3.2.2 Initial Synthetic Efforts Towards GPHR-00032750 Analogs

Initial forays into the SAR of analogs of **3.9** focused on studying the effect of the *meta* trifluoromethyl groups while varying the internal piperazine moiety in an attempt to improve potency. The first compounds synthesized in the first library sought to hold the *m*-trifluoromethyl group of the western phenyl ring relatively constant while varying the eastern phenyl ring substituents. The general synthetic scheme for the development of these compounds is shown in Scheme 3.2. Mixing of the appropriate piperazine with an isocyanate of interest in the presence of a mild base at r.t. was sufficient to obtain target molecules in good yield, generating a set of 40 compounds that were assayed for their ability to block the progesterone-evoked calcium influx.

Scheme 3.2 General reaction scheme for the synthesis of analogs **3.10–3.48** in Table 3.1

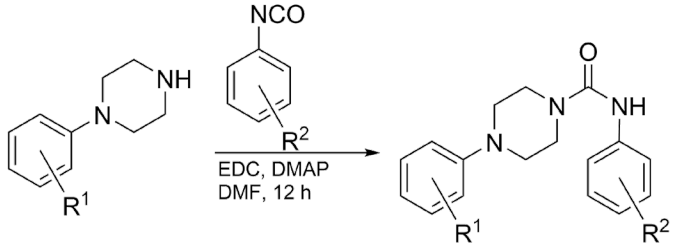


Table 3.1. Potency of Piperazinyl Analogs from Scheme 3.2

Compound	R^1	R^2	IC_{50}[a]
3.10	H	H	> 100
3.11	H	3-Me	>100
3.12	H	3-CF_3	>100
3.13	H	3-CN	25 ± 6
3.14	H	3-NO_2	>100
3.15	H	3-CO_2Me	>100
3.16	3-Me	H	69 ± 5
3.17	3-Me	3-Me	46 ± 11
3.18	3-Me	3-CF_3	9.2 ± 0.8
3.19	3-Me	3-CN	32 ± 4
3.20	3-Me	3-NO_2	25 ± 2
3.21	3-Me	3-CO_2Me	28 ± 3
3.22	3-Me	3,5-(CO_2Me)$_2$	28 ± 8
3.23	3-Me	4-Indole	>100
3.24	3-CF_3	H	26 ± 5
3.25	3-CF_3	3-Me	9.6 ± 0.4
3.9	3-CF_3	3-CF_3	5.2 ± 1.7
3.26	3-CF_3	3,5-(CF_3)$_2$	2.9 ± 0.8
3.27	3-CF_3	3-CN	>100
3.28	3-CF_3	3-NO_2	17 ± 6
3.29	3-CF_3	3-CO_2Me	24 ± 6
3.30	3-CF_3	3,5-(CO_2Me)$_2$	20 ± 7
3.31	3-CF_3	3-CO_2H	>100
3.32	3-CF_3	4-Indole	42 ± 9
3.33	3-CF_3	5-Indole	34 ± 3
3.34	3,5-(CF_3)$_2$	H	>100
3.35	3,5-(CF_3)$_2$	3-Me	13 ± 4
3.36	3,5-(CF_3)$_2$	3-CF_3	4.1 ± 1.2

3.37	3,5-$(CF_3)_2$	3,5-$(CF_3)_2$	>100
3.38	3,5-$(CF_3)_2$	3-CN	13 ± 1
3.39	3,5-$(CF_3)_2$	3-NO_2	7.0 ± 1.2
3.40	3,5-$(CF_3)_2$	3-CO_2Me	7.5 ± 0.9
3.41	3,5-$(CF_3)_2$	3,5-$(CO_2Me)_2$	>100
3.42	3,5-$(CF_3)_2$	3-CO_2H	>100
3.43	3,5-$(CF_3)_2$	5-Indole	92 ± 9
3.44	3-CN	3-CF_3	8.6 ± 3.3
3.45	3-CN	3,5-$(CF_3)_2$	5.0 ± 1.1
3.46	3-CN	3-CN	>100
3.47	3-CN	3-NO_2	>100
3.48	3-CN	3-CO_2Me	>100

From this initial set of compounds, a series of observations can be made regarding SAR patterns. First, the western phenyl group of the scaffold needs to be substituted. The type of substitution on this ring is not as important, as a great variety of substitutions retain some level of activity, but a phenyl group alone in this region is not sufficient to confer activity in the influx assay (compounds **3.10** – **3.15**). Building on this observation, the more electron withdrawing the substitution, the more likely the compound is to show good potency in the assay, in the following order: *m*-CF_3 ≈ *m*-NO_2 > CN > Me. In general, the eastern phenyl ring of the scaffold is more tolerant of modifications compared to the western ring. A wider variety of substitutions still show activity including indoles (**3.32**, **3.33**), though potencies are diminished greatly.

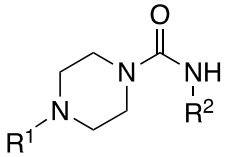
Importantly, included in this set of compounds was the original HTS hit (**3.9**). While this compound had been repurchased from vendors, its resynthesis, purification and assay all on site served to validate this series. From the SAR of this library of compounds it became apparent that the most potent modifications to the phenyl rings were going to remain the *m*-CF_3 groups, though the *m*-methyl analog **3.17** retained activity, indicating that sterics of this group may play a bigger role than electronics. Building on the *m*-CF_3

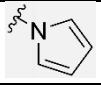
groups, several compounds were synthesized bearing 3,5-bis-CF_3 groups. These compounds proved rather potent in many cases (**3.26**, **3.35**, **3.36**), however 3,5-disubstitution with CF_3 (**3.37**) and a methyl ester moiety (**3.41**) on the eastern phenyl ring produced inactive compounds. The carboxylic acid-containing compounds **3.31** and **3.42** showed no activity compared to their ester counterparts **3.29** and **3.40**. Presumably, the carboxylic acid is preventing cellular penetration.

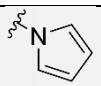
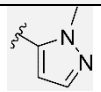
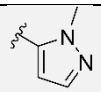
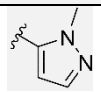
Section 3.2.3 Heterocyclic Analogs of Parent Compound Maintain Activity

Perhaps the most important observation from the SAR study (Table 3.1) is that none of the synthesized compounds showed an increase in potency compared to the original hit compound **3.9**. From Table 3.1 it is apparent that the eastern phenyl ring is more amenable to changes than the western phenyl ring. From this we sought to synthesize a small series of analogs in which a small heterocyclic group was appended to the eastern ring. In this series of analogs, we also investigated N-phenyl piperazines substituted with pyrrole (**3.49**–**3.51**) and *N*-methylpyrazole (**3.52–3.54**), pyridine (**3.55–3.59**) and pyrimidine (**3.60**) moieties. The results derived from these compounds are shown in Table 3.2. The synthesis of these compounds remains unchanged from Scheme 3.2.

Table 3.2. Potencies of Heterocycles-Containing Analogs **3.49–3.60**



Compound	R^1	R^2	IC_{50}[a]
3.49	3-CF_3C_6H4		19 ± 6
3.50	3,5-$(CF_3)_2C_6H_3$		5.5 ± 1.8

3.51	3-CNC_6H_4		21 ± 6
3.52	3-$CF_3C_6H_4$		11 ± 2
3.53	3,5-$(CF_3)_2C_6H_3$		4.3 ± 0.9
3.54	3-CNC_6H_4		25 ± 5
3.55	3-py.	3-Me	>100
3.56	3-py.	3-NO_2	>100
3.57	4-py.	3-Me	>100
3.58	4-py.	3-CF_3	10 ± 2
3.59	4-py.	3-NO_2	43 ± 22
3.60	2-pyr.	3-CF_3	>100

[a]Data presented in μM as mean ± SD and represent 3 individual experiments in all cases. py. = pyridine and pyr. = pyrimidine.

From the data for these compounds several observations were made. The eastern phenyl ring can accept heterocyclic substituents and retain activity, with potencies for **3.50** and **3.53** that are comparable to the parent compound **3.9**. For these compounds, the modifications to the western phenyl ring follow the same trend that was observed previously, that an increase in electron withdrawing modifications leads to improved potency (**3.50** > **3.49** > **3.51** and **3.53** > **3.52** > **3.54**). Compounds **3.55**–**3.58** continue the trend that the western phenyl ring is less accepting of substitutions, as compounds in which the phenyl ring is changed to a pyridine (py) or pyrimidine (pyr), all activity is lost, save compound **3.58** which retained activity.

Section 3.2.4 Analogs with Extended Aryl System Retain Activity

After analysis of the SAR data for these compounds it became clear that pursuing analogs modified at the eastern phenyl ring would be advantageous. Also, since the

heterocyclic substituents retained activity, we hypothesized that additional steric bulk could be added in this area. As such we expanded the aryl system by adding another ring via linkage through an amide bond, making use of the previously synthesized **3.31** and **3.42** as the starting material. The synthetic scheme leading to these analogs are shown in Scheme 3.3.

Scheme 3.3 Synthesis of extended aryl system analogs

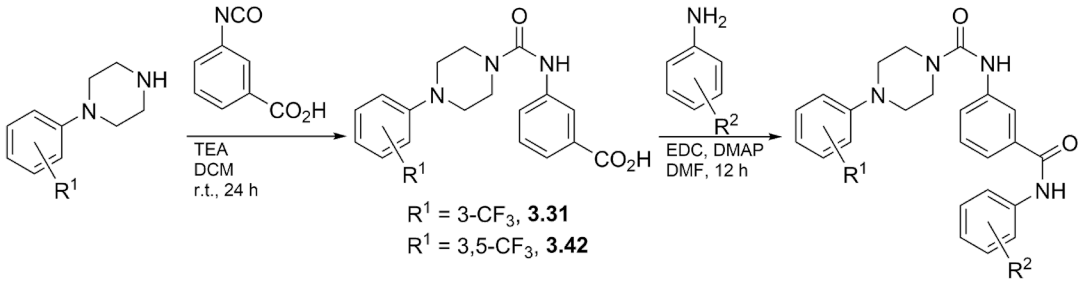
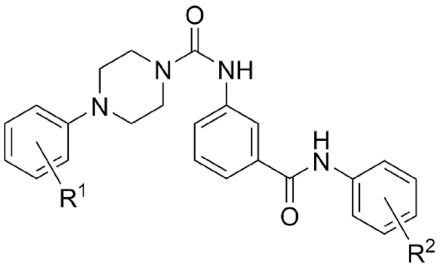


Table 3.3 shows that addition of the extra aryl system to the scaffold is tolerated, as evinced by compounds **3.61** and **3.68**. Large additions to this phenyl ring are not tolerated, however, as shown by compounds **3.66**, **3.67**, **3.74** and **3.75**, smaller groups, such as the chloro- groups found in **3.64**, **3.65** and **3.72** led to compounds displaying potencies slightly better than the parent compound. Conversely, heterocycles are not tolerated at this additional phenyl ring (**3.76** and **3.77**) and when the lipophilicity of the compound is too high all activity is lost, demonstrated by the inactive compounds **3.71** and **3.73** through **3.76**. In general, these molecules did not provide significant gains in potency over the parent compound and the molecular weights of these compounds were starting to be above 500. With further modifications needed to improve potency the molecular weight of additional analogs would above 550 molecular weight, and therefore a different direction was chosen by modifying the central core of the molecule.

Table 3.3. Potencies of extended aryl amide analogs



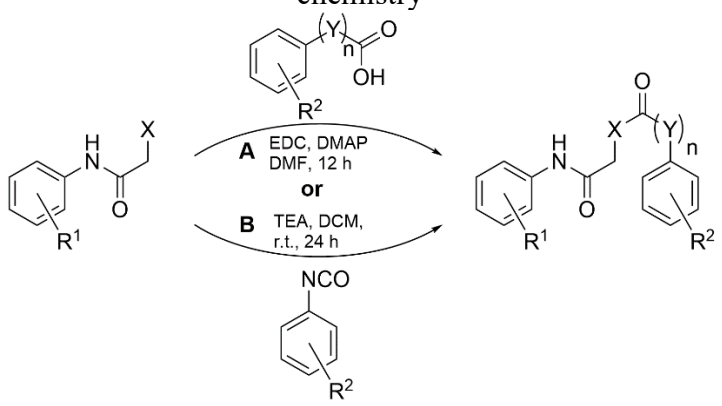
Compound	R^1	R^2	IC_{50}[a]
3.61	3-CF_3	H	13 ± 5
3.62	3-CF_3	4-Cl	13 ± 5
3.63	3-CF_3	4-OMe	>100
3.64	3-CF_3	3,4-$(Cl)_2$	2.4 ± 0.7
3.65	3-CF_3	3-Cl	3.2 ± 0.6
3.66	3-CF_3	4-$C(CH_3)_3$	>100
3.67	3-CF_3	3-CF_3, 4-Cl	>100
3.68	3,5-$(CF_3)_2$	H	6.5 ± 2.4
3.69	3,5-$(CF_3)_2$	4-Cl	>100
3.70	3,5-$(CF_3)_2$	4-OMe	>100
3.71	3,5-$(CF_3)_2$	3,4-Cl	>100
3.72	3,5-$(CF_3)_2$	3-Cl	3.8 ± .3
3.73	3,5-$(CF_3)_2$	4-NMe_2	>100
3.74	3,5-$(CF_3)_2$	4-$C(CH_3)_3$	>100
3.75	3,5-$(CF_3)_2$	3-CF_3, 4-Cl	>100
3.76	3,5-$(CF_3)_2$	4-py.	>100
3.77	3,5-$(CF_3)_2$	3-py.	>100
3.78	3,5-$(CF_3)_2$	4-CF_3	7.1 ± 3.1

[a]Data presented in μM as mean ± SD and represent 3 individual experiments in all cases. Py. = pyridine with numbering representing the position of endocyclic nitrogen.

Section 3.2.5 Modifications to the Piperazinyl Urea Core are not Well Tolerated

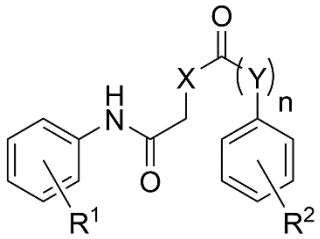
Having seen little to no improvement in potency when synthesizing targets similar in structure to the parent compound, analogs that displayed a greater variety in molecular structure were designed and synthesized as shown in Scheme 3.4.

Scheme 3.4 The synthesis of ring opened analogs of **3.9** via EDC coupling or isocyanate chemistry



Modifications to the scaffold included removal of the eastern nitrogen of the urea linker, replacing it with a methylene group or simply removing the spacer all together, resulting in several compounds with an aryl ketone rather than the usual aniline.

Table 3.4. Activities of Ring-Opened Analogs of **3.9**



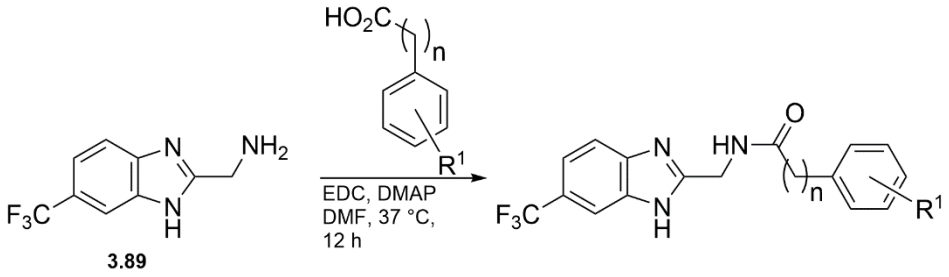
Compound	R^1	R^2	X	Y	n	IC_{50}[a]
3.79	3-CF_3, 4-Cl	3-CF_3	NH	CH_2	1	6.4 ± 1.4
3.80	3-CF_3, 4-Cl	3-CN	NH	–	0	26 ± 1
3.81	3-CF_3, 4-Cl	3,5-CF_3	NH	–	0	>100
3.82	3-CF_3, 6-Cl	3-CF_3	S	CH_2	1	29 ± 6
3.83	3-CF_3, 6-Cl	3-Me	S	NH	1	>100
3.84	3-CF_3	3-Me	S	NH	1	39 ± 11
3.85	3-CF_3	3-CN	S	NH	1	>100
3.86	3-CF_3	3-CO_2Me	S	NH	1	32 ± 2
3.87	3-CF_3	3,5-(CO_2Me)$_2$	S	NH	1	25 ± 8
3.88	3-CF_3	4-Indole	S	NH	1	15 ± 3

[a]Data presented in μM as mean ± SD and represent 3 individual experiments in all cases. "–" represents nonexistent site

In all synthesized compounds, the piperazine ring was opened to give either diamide linkers or carbamothioic esters in place of the normal urea. The activity of the resulting compounds is described in Table 3.4.

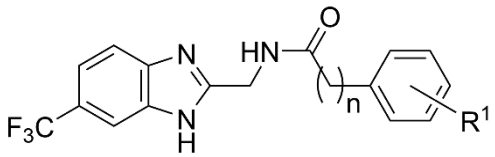
As observed in Table 3.4, opening the ring to a more flexible linking moiety resulted in compounds that retained activity in the assay, but displayed diminished activities. In almost all cases double digit micromolar inhibitors were observed except for compound **3.79** whose potency matched that of the parent compound. No discernable trends were observed with these compounds regarding thiol linkers over amide linkers or how ring opening affected phenyl substitution patterns, but the fact that these compounds retained activity prompted further exploration of ring-opened analogs. Scheme 3.5 shows the additional analogs synthesized, centered on a commercially available azaindole methylamine nucleophile **3.89**.

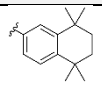
Scheme 3.5 The synthesis of additional ring opened analogs of **3.9**



When first tested, these compounds appeared to be submicromolar inhibitors in the FLIPR assay. Closer inspection of the data, however, revealed these compounds substantially interfered with the fluorescent readings of the assay (not shown), prompting their removal from consideration and truncating their testing at n =2.

Table 3.5. Potencies of Analogs Using **3.89** as Nucleophile



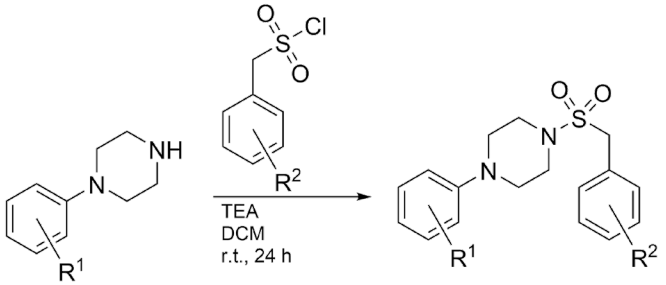
Compound	R^1	n	IC_{50}[a]
3.90	3-CF_3	0	>100
3.91	3,5-$(CF_3)_2$	0	>100
3.92	3-CN	0	>100
3.93	4-CN	1	>100
3.94	3-CF_3, 6-Cl	0	>100
3.95		0	>100
3.96			>100

[a]Data presented in μM as mean ± SD and represent 2 individual experiments in all cases. For R^1, when drawn, eastern phenyl group is replaced by entire drawing.

Section 3.2.6 Sulfonamide Analogs of **3.9** *Are Completely Inactive Save One Compound*

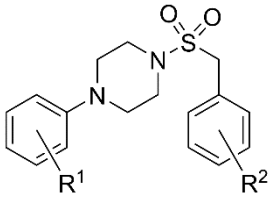
With the lackluster potencies seen in all ring-opened compounds the decision was made to stop drastically altering the piperazinyl core and instead, and change the urea linking group to improve potency. Sulfonamides retain many of the physicochemical properties of amides and ureas and, as such, were sought as potential analogs. Synthesis and testing of compound **3.97** showed that the sulfonamide analog corresponding to parent substitution pattern of **3.9** retained activity. With this knowledge, a series of sulfonamides were synthesized and assayed in the FLIPR assay. The synthesis of these compounds is shown in Scheme 3.6, though the chemistry is identical to the previously discussed isocyanate couplings.

Scheme 3.6 Synthesis of sulfonamide derivatives of **3.9**



As can be seen in Table 3.6, while analog **3.97** retained activity in the influx assay, almost every other sulfonamide synthesized showed no ability to block the progesterone-mediated calcium influx in human sperm.

Table 3.6. Potencies of Sulfonamide Derivatives



Compound	R^1	R^2	IC_{50}[a]
3.97	3-CF_3	3-CF_3	7.4 ± 0.9
3.98	H	H	>100
3.99	H	3-Me	>100
3.100	H	2-CF_3	>100
3.101	H	3-CF_3	>100
3.102	H	4-CF_3	>100
3.103	H	3,5-$(CF_3)_2$	>100
3.104	3-Me	H	>100
3.105	3-Me	2-CF_3	>100
3.106	3-Me	3-CF_3	>100
3.107	3-Me	4-CF_3	>100
3.108	3-Me	3,5-$(CF_3)_2$	>100
3.109	3-CF_3	H	>100
3.110	3-CF_3	3-Me	>100
3.111	3-CF_3	4-CF_3	52 ± 9

3.112	3-CF_3	3,5-$(CF_3)_2$	>100
3.113	3-CN	H	19 ± 4.4
3.114	3-CN	3-Me	>100
3.115	3-CN	3-CF_3	>100
3.116	3,5-$(CF_3)_2$	H	76 ± 32
3.117	3,5-$(CF_3)_2$	3-Me	>100
3.118	3,5-$(CF_3)_2$	2-CF_3	>100
3.119	3,5-$(CF_3)_2$	3-CF_3	59 ± 7
3.120	3,5-$(CF_3)_2$	4-CF_3	2.2 ± 0.4

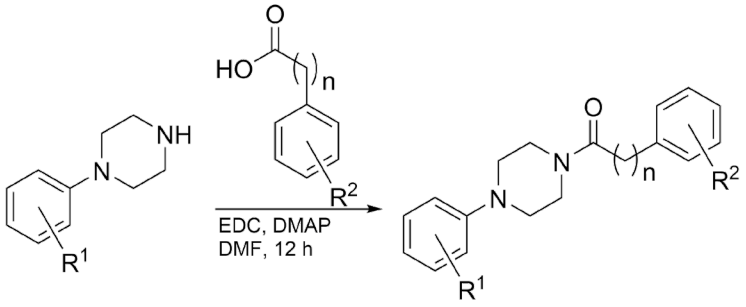
[a]Data presented in μM as mean ± SD and represent 2 individual experiments. In cases where an IC_{50} value < 100 μM is observed, 3 experiments were performed.

A disappointing result to be sure, given the initial success of **3.97**. This series of compounds was the first to introduce an *o*-CF_3 group into any analog, though in all cases this substitution was inactive and not pursued in additional analogs. Compound **3.120** bears a *p*-CF_3 group and is the most potent sulfonamide tested, though only moderately more potent than **3.97** and hit compound **3.9**.

Section 3.2.7 Exploration of the Nitrogen Spacer Reveals Promising Results

Having synthesized and tested over 100 compounds to this point, several observations were used to drive further analog synthesis. First, the electron deficiency of both phenyl rings is crucial for retention of activity, with very few analogs showing activity independent of –CF_3 groups. Second, the piperazine ring should be kept if possible, as most ring opened compounds showed reduced activity. Finally, the urea moiety is amenable to alteration only if the western nitrogen of this group is maintained, and it absolutely should not be changed to a sulfonamide.

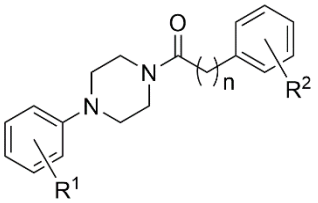
Scheme 3.7 EDC-mediated couplings leading to varied spacer analogs



Throughout the tested analogs there exist several examples in which the NH group of the urea linker is changed to a methylene linker (see compounds **3.79** and **3.97**). Also, compound **3.80** retains some potency with a truncated spacer, keeping just an aryl amide rather than a full urea. We next sought to explore the need for this spacer by synthesizing compounds according to Scheme 3.7, in which zero or one methylene groups or nitrogen atoms could modulate potency in the influx assay.

As seen in Table 3.7, most of the compounds in which n = 0, leaving an aryl amide, show little reduced potency in the influx assay, with several showing no activity whatsoever (**3.121**, **3.124** and **3.125**). These compounds closely resemble the parent compound, so their lack activity reflects the need for this spacer region to exist. Further validating the need for this spacer are compounds **3.126** and **3.127**, which retain activity and are the same as the parent compound except the urea NH group is a methylene linker. These two compounds are less potent then their urea counter parts (see **3.126** vs **3.9** and **3.127** vs **3.36**).

Table 3.7 Potency of truncated or methylene-containing compounds



Compound	R^1	R^2	n	IC_{50}[a]
3.121	3-CF_3	3-CF_3	0	>100
3.122	3-CF_3	3,5-$(CF_3)_2$	0	26 ± 3
3.123	3,5-$(CF_3)_2$	3-CF_3	0	11 ± 1
3.124	3,5-$(CF_3)_2$	3,5-$(CF_3)_2$	0	>100
3.125	3,5-$(CF_3)_2$	3-CN	0	>100
3.126	3-CF_3	3-CF_3	1	9.8 ± 0.2
3.127	3,5-$(CF_3)_2$	3-CF_3	1	7.7 ± 0.7
3.128	3-CN	3-CF_3	0	17 ± 2
3.129	3-CN	3,5-$(CF_3)_2$	0	26 ± 6
3.130	3-CN	3-CN	0	64 ± 1

[a]Data presented in μM as mean ± SD and represent 3 individual experiments in all cases.

Having explored truncated linkers with respect to the urea portion of the molecule, we next set out to increase the linker length. Having demonstrated with compounds **3.126** and **3.127** that a methylene is tolerated at this position, we sought to explore whether potency could be gained via alkylation of this methylene group. We also utilized a phenoxy linker in these compounds to explore the effect of this change on compound potency.

Scheme 3.8 Synthesis of phenoxy derivatives with alkylated linker regions

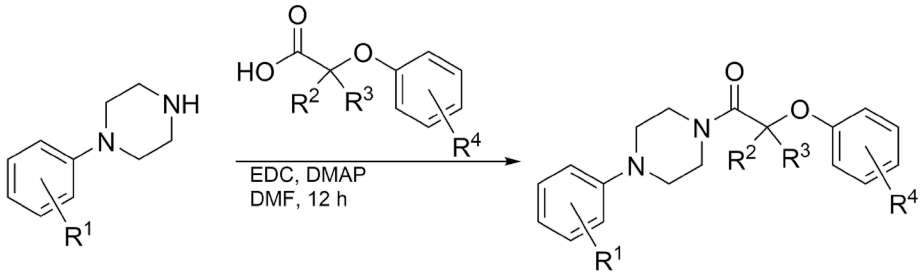
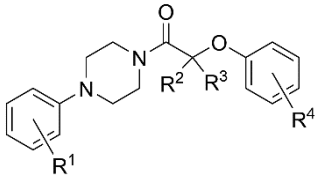


Table 3.8. Potency of phenoxymethylene derivatives with and without alkylated linker regions



Compound	R^1	R^2	R^3	R^4	IC_{50}[a]

3.131	3-CF_3	H	H	3-CF_3	6.9 ± 1.6
3.132	3,5-$(CF_3)_2$	H	H	3-CF_3	4.7 ± 0.9
3.133	3-CN	H	H	3-CF_3	15 ± 4
3.134	3-CF_3	H	H	3,4-F_2	8.6 ± 1.2
3.135	3,5-$(CF_3)_2$	H	H	3,4-F_2	7.5 ± 1.3
3.136	3-CN	H	H	3,4-F_2	66 ± 13
3.137	3-CF_3	H	Me	3-CF_3	9.6 ± 0.5
3.138	3,5-$(CF_3)_2$	H	Me	3-CF_3	10 ± 4
3.139	3-CN	H	Me	3-CF_3	20 ± 4
3.140	3-CF_3	Me	Me	3-CF_3	5.2 ± 0.9
3.141	3,5-$(CF_3)_2$	Me	Me	3-CF_3	43 ± 26
3.142	3-CN	Me	Me	3-CF_3	12 ± 4

[a]Data presented in μM as mean ± SD and represent 3 individual experiments in all cases.

From Table 3.8 there are several observations that can be made starting with the fact that, for the first time, all compounds in a synthesized set retained activity. This phenoxymethylene linker present in each of these compounds is an accepted change to the scaffold. Compounds **3.131**, **3.132**, **3.134**, **3.135**, and **3.140** display potencies equal to the parent compound. Though no significant increases in potency were observed, these results indicate a positive trend.

Also, the need for an electron withdrawing groups continues as previously observed trends continue to be seen, with 3,5-CF_3 > 3-CF_3 > 3-CN (see compounds **3.132** vs **3.131** vs **3.133**. The new substitution pattern of 3,4-difluorophenyl seen in compounds **3.134** – **3.136** retained activity, prompting the synthesis of future compounds that to explore monofluorinated phenyl ring systems. Finally, in general, it appears alkylation of this methylene linker is not beneficial, as methyl and *gem*-dimethyl variants did not show an increased potency in the influx assay as seen with compound **3.131** vs **3.137** vs **3.140**. While the alkylated derivatives did not lose activity, they did not gain any.

Section 3.2.8 Further Exploration of Spacer Region Results in Most Potent Compounds Discovered to Date

With the phenoxymethylene linker-containing compounds proving efficacious in the influx assay, we sought to further explore compounds of a similar nature by changing the phenoxy moiety to an aniline, more closely resembling the parent compound. Furthermore, the spacer was elongated in some cases to two methylene groups and monofluorinated phenyl substitution patterns were included given the success of compounds **3.134** – **3.136**. The synthesis of one of the final libraries of compounds is shown in Scheme 3.9 and follows the same EDC-mediated amide formations prevalent through earlier sections.

Scheme 3.9 Synthesis of anilino derivatives with methylene and ethyl linkers

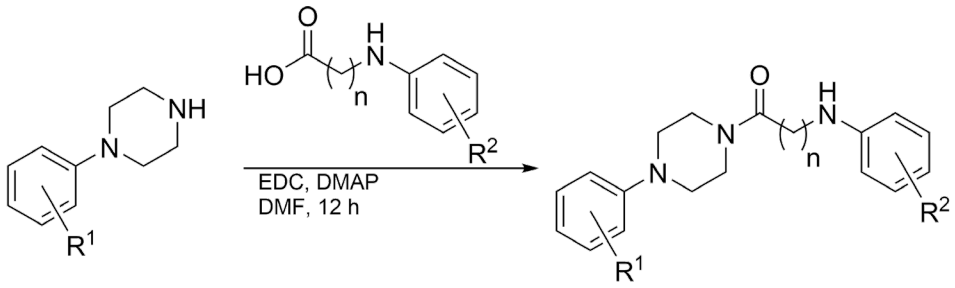
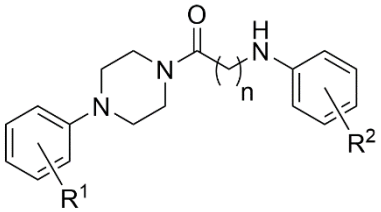


Table 3.9. Synthesis of anilino derivatives with methylene and ethyl linkers



Compound	R^1	R^2	n	IC_{50}[a]
3.143	3-CF_3	3-CF_3	1	5.9 ± 1.7
3.144	3,5-$(CF_3)_2$	3-CF_3	1	1.9 ± 0.5
3.145	3-CF_3	2,4-F_2	1	8.3 ± 1.2

3.146	3-CF_3	4-Cl	1	4.6 ± 0.6
3.147	3-CF_3	2-F	1	0.51 ± 0.12
3.148	3-CF_3	4-F	1	0.46 ± 0.03
3.149	3-Me	2,4-F_2	1	5.9 ± 1.0
3.150	3-Me	4-Cl	1	11 ± 1.7
3.151	3-Me	2-F	1	6.6 ± 1.4
3.152	3-Me	4-F	1	5.1 ± 0.2
3.153	H	H	2	>100
3.154	3-Me	H	2	>100
3.155	3-CF_3	H	2	>100
3.156	H	2-NO_2, 4-CF_3	2	>100
3.157	3-Me	2-NO_2, 4-CF_3	2	>100
3.158	3-CF_3	2-NO_2, 4-CF_3	2	>100
3.159	H	3-F	2	>100
3.160	3-Me	3-F	2	2.9 ± 0.3
3.161	3-CF_3	3-F	2	5.5 ± 2.1

[a]Data presented in μM as mean ± SD and represent 3 individual experiments in all cases.

The synthesized compounds were tested for their ability to inhibit progesterone-induced calcium influx as per our standard protocol. These compounds were expected to be rather potent based on the compounds found in Table 3.8, and this is indeed the case with the two most potent compounds to date being found within this library: **3.147** and **3.148**. These two compounds are fluorinated anilines with a methylene spacer between the piperazinylamide and the eastern aryl group.

It is perhaps not surprising that these compounds have a *m*-CF_3 group on the western phenyl ring. Nonetheless, these compounds are the first submicromolar inhibitors discovered in this campaign and consistently displayed a potent block in our FLIPR assay as shown in Figure 3.4. Other very potent compounds are seen in Table 3.9 including **3.144** and **3.160**. Of particular interest is **3.160**, since this compound was one of only two compounds with an ethyl linker to show activity in the assay, see compounds **3.153** – **3.159**.

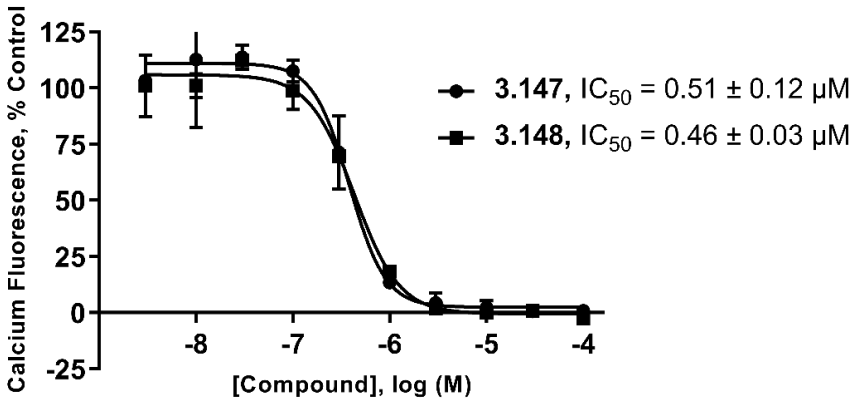


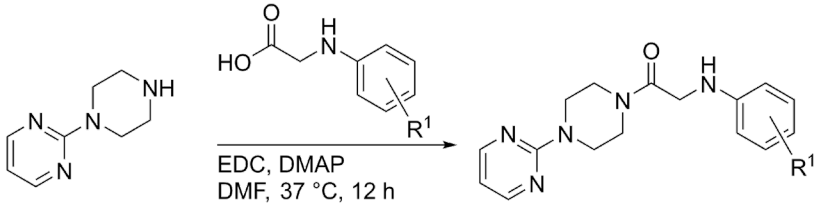
Figure 3.4. Dose-Response Curves of **3.147** and **3.148** from influx assay. Data are plotted as mean ± standard error, presented as a percent response relative to an EC_{50} dose of progesterone (30 nM). Smooth curves represent the best fits of a nonlinear regression model to the data for each compound. IC_{50} values determined using Prism v8.02.

Compound **3.161** was the other active ethyl linker analog. Interestingly, **3.160** is the more potent of the compounds, yet bears a *m*-methyl substitution, while **3.161** has the typically more potent *m*-CF_3 substitution. An interesting deviation from a consistent trend.

Section 3.2.9 Probing the Piperazinyl Nitrogen via Heterocyclic and CH Replacement

In an attempt to possibly find yet more potent compounds, an additional small collection of compounds was synthesized as shown in Scheme 3.10.

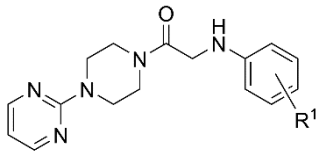
Scheme 3.10 Synthesis of pyrimidinylpiperazine analogs



Combining the potent carboxylic acids from Scheme 3.9 with the underutilized pyrimidinylpiperazine, it was hypothesized that this combination could potentially lead to

potent compounds. As seen in Table 3.10, this was not the case. Previously, compound **3.60** was the only compound with a pyrimidinylpiperazine moiety and was totally inactive in the assay. Optimism winning out, 5 compounds were synthesized and tested. One of the compounds did show activity in the assay though the potency left much to be desired.

Table 3.10. Potencies of synthesized pyrimidinylpiperazine analogs



Compound	R^1	IC_{50}[a]
3.162	3-CF_3	>100
3.163	2,4-$(F)_2$	>100
3.164	4-Cl	>100
3.165	2-F	22 ± 2
3.166	4-F	>100

[a]Data represented as mean ± SD and represent 2 individual experiments in all cases except where activity was observed, then 3 replicates were performed.

While synthesizing the various analogs of **3.9**, the obvious electronic contributions of the western phenyl ring were observed consistently, with electron withdrawing groups seemingly required. As a final experiment a piperidine analog was synthesized, in which the Western nitrogen of the piperazine ring is replaced by a carbon. *meta* Electron withdrawing groups will lower the electron density of the piperazinyl nitrogen, raising its pKa. Given that this nitrogen is less likely to be protonated at relevant pHs, the activity of the parent compound should be "protonated nitrogen" agnostic. This is indeed the case, Figure 3.5 shows that piperidine compound **3.167**, having a -CH- group in place of the anilinic nitrogen, is just as potent as **3.9**. While not conclusive, this result suggests future analogs of this scaffold need not contain this potential metabolic liability.

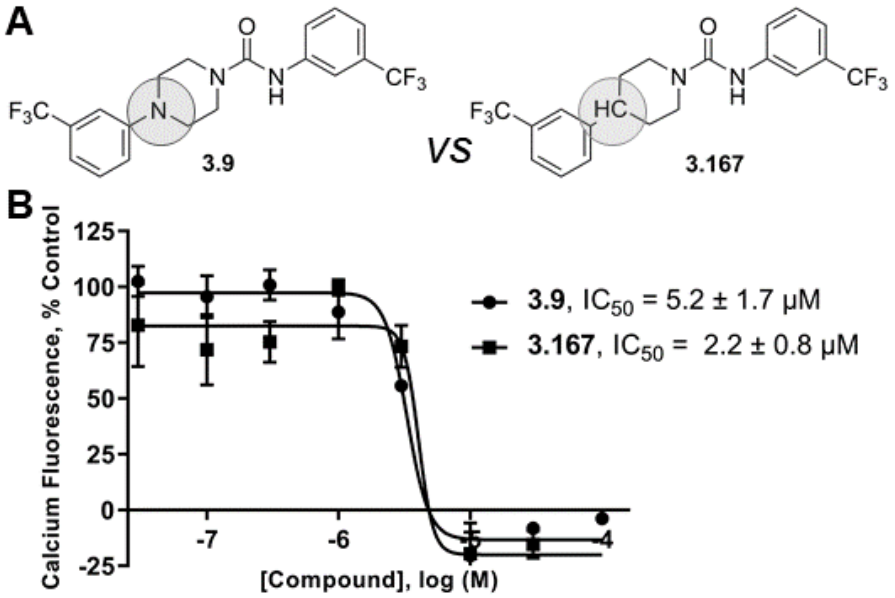


Figure 3.5. A Piperazinyl Nitrogen is not Needed for Activity of Analogs. (A) Structures of **3.9** and **3.167** with circle highlighting difference in structure. (B) Dose-response curves of piperidine-containing **3.167** and piperazine-containing **3.9**. Data are plotted as mean ± standard error, presented as a percent response relative to an EC_{50} dose of progesterone (30 nM). Smooth curves represent the best fits of a nonlinear regression model to the data for each compound. IC_{50} values determined using Prism v8.02.

Section 3.2.10 Verifying Synthesized Compounds Via CASA

As described before, CASA is computer-aided sperm analysis and measures the motility of live human sperm cells by tracking key kinematic parameters such as velocity and the amplitude of lateral head displacement. Having synthesized and tested over 160 compounds in the FLIPR assays, validation of the two submicromolar compounds in a secondary assay was sought. The secondary assay used was CASA. Figure 3.6 summarizes the effect of compounds **3.9** (initial hit), **3.147**, and **3.148**.

All the tested compounds affect normal motility at the concentrations tested to a significant degree. The parent compound **3.9** was tested at higher concentrations than the synthesized compounds due to the discrepancies in potency between the compounds. The

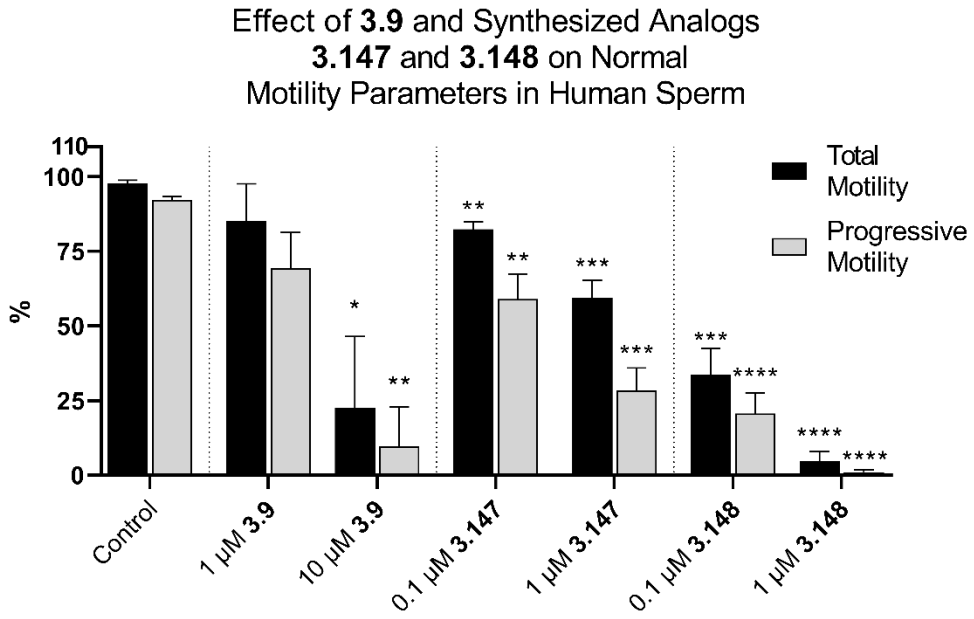


Figure 3.6. Compound **3.9** and Derivatives **3.147** and **3.148** Reduce both Total (black) and Progressive (gray) Motility in Human Sperm. Data are plotted as mean ± standard error, representing the mean of 4 replicate experiments, and $*P < 0.05$, $**P < 0.005$, $***P < 0.0005$, $****P < 0.0001$.

two synthesized compounds effectively reduce motility in human sperm at 0.1 and 1 μM. Compound **3.148** is especially potent, showing only 33% remaining motility at 0.1 μM.

It would be expected that a CatSper inhibitor would have minimal effect on normal motility parameters, however as observed in Chapter 2, the known t-type CCBs mibefradil and NNC 55 0396 have potent effects on normal motility in human sperm, indicating precedence for the observed effects these synthesized analogs are having on normal motility parameters.

With respect to HAM, the synthesized compounds drastically impact HAM evoked by both PGE_1 (Figure 3.7) and progesterone (Figure 3.8). At 100 nM **3.147** is able to effectively prevent cells from achieving HAM and at 1 μM both compounds are able to completely ablate the population of cells displaying HAM to zero or near zero.

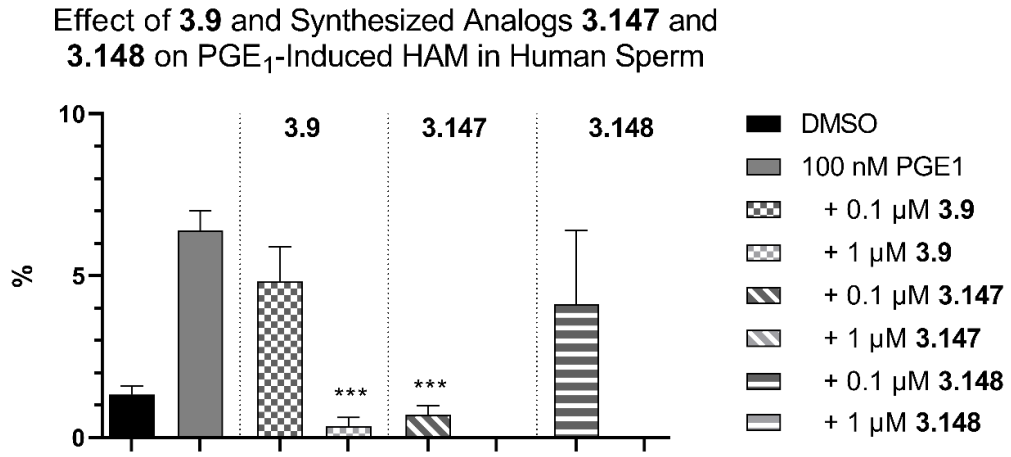


Figure 3.7. Effect of compound **3.9** and Derivatives **3.147** and **3.148** on HAM Induced by Treatment with 100 nM PGE_1. Data are represented as means ± SD; data represent the means of 4 individual experiments in all cases; and ***$P < 0.0005$.

The three compounds all show an improved efficacy with respect to inhibiting HAM over normal motility. For instance, at 1 µM parent compound **3.9** shows little effect on normal motility, but significantly impacts HAM. The same can be said for **3.147**, which has limited impact on normal motility at 100 nM, yet nearly completely knocks down HAM at this concentration. Compound **3.148** is a bit of a stranger case in that it shows a pronounced effect on normal motility even at 100 nM, but, at this concentration, has a negligible effect on HAM elicited by both PGE_1 and progesterone, though complete inhibition of HAM at 1 µM is seen. This oddity requires further investigation and the recruitment of additional progesterone-sensitive donors to fully elucidate this interaction.

Section 3.3 Fragment-Inspired Approach towards Development of GPHR-00213869

Section 3.3.1 Initial Observations and Retrosynthetic Strategy

While the development of hit compound **3.9** was ongoing, forays into the development of **3.12** were planned and begun. Hit compound **3.12** was found, perhaps unsurprisingly given its structure, from a kinase inhibitor library. Showing an initial IC_{50} value of 21 ± 2 μM and 10 ± 2 μM for progesterone- and K^+-induced calcium influx respectively, this hit compound was chosen for further development based on promising electrophysiology data in human sperm and a lack of cytotoxicity in both sperm and somatic cells up to 100 μM.

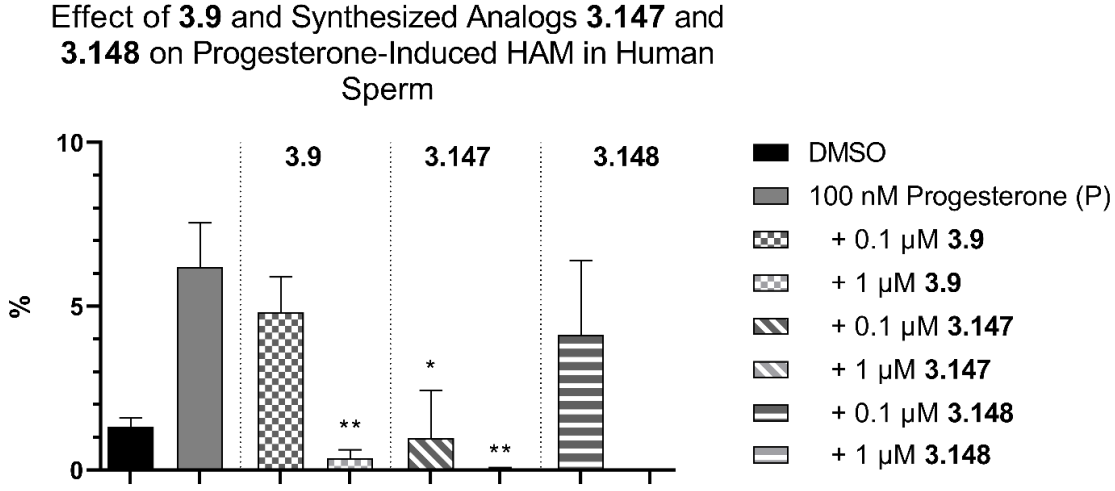
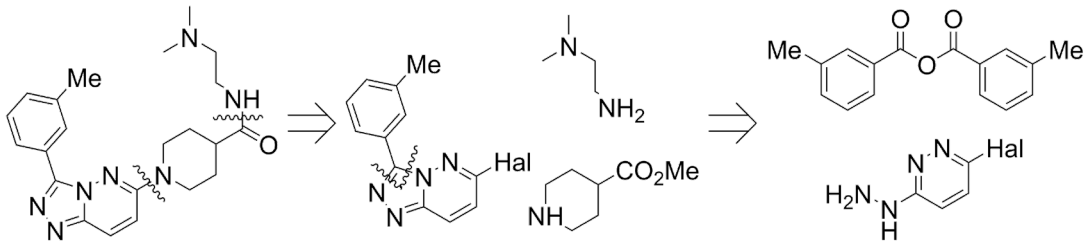


Figure 3.8. Effect of Compound **3.9** and its Synthesized Derivatives **3.147** and **3.148** on HAM Induced by Treatment with 100 nM Progesterone (P). Data are represented as means ± SD; data represent the means of 4 individual experiments in all cases; and **P* < 0.05, ***P* < 0.005.

Scheme 3.11 Retrosynthetic analysis of hit compound **3.12**



Hit compound **3.12** has a more complicated structure than **3.9**, containing a triazolopyridine core with a piperidine linker tying into an amide side chain. Scheme 3.11 shows the retrosynthetic analysis for synthesis of target molecule **3.12**. Disconnecting the side chain in the northeastern quadrant of the molecule and subsequent decoupling of the piperidine linker from the aromatic heterocycle gives a core heterocycle which can be disconnected to reveal 3-hydrazinylpyridazine and anhydrides as the starting materials.

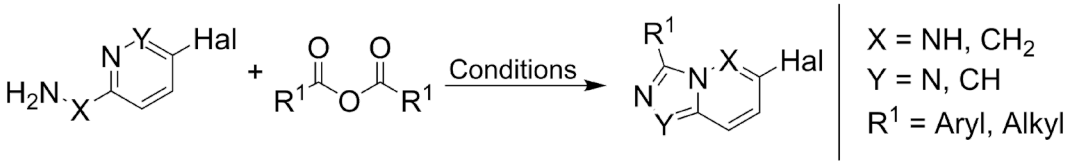
While compound **3.9** lent itself to the rapid generation of analogs via matrix chemistry, it would not be prudent to undertake the same strategy with **3.12**. Requiring four steps to complete the synthesis, though rather abridged, would still give an exceedingly high number of analogs if each modification were to be explored in full. As such, a fragment-inspired approach to the development of **3.12** was undertaken and the description of the preliminary results follow.

*Section 3.3.2 General Strategy Towards Potent Analogs of GPHR-00213869 (***3.12***)*

As can be seen clearly in Scheme 3.11, the first step of the synthesis towards **3.12** is a condensation cyclization reaction akin to those pioneered by Bischler and Napieralski. This robust chemistry allows for the electrophilic intramolecular formation of the triazolo or imidazole cores found in many natural products and drug molecules. For our efforts, this

chemistry allowed us to synthesize a series of compounds with varying nitrogen compositions. While varying the anhydride partner used in the reaction, a series a low molecular weight fragments could be envisioned as shown in Scheme 3.12.

Scheme 3.12 General synthesis of southwestern fragments



The starting material for this chemistry was affordable and the reactions were quite robust, as will be described. This chemistry was used to generate a focused library of fragments containing 64 compounds all of which were tested in the influx assay at two concentrations for their ability to inhibit calcium influx. The synthesis and testing of these analogs are described below.

Section 3.3.3 Synthesis and Evaluation of 1,2,4-triazolo[4,3-b]pyridazine Core-Containing Fragments

The fragment library containing all four nitrogen atoms present in parent compound **3.12** constitute 16 of the 64 compounds synthesized and bears a triazolopyridazine core. Interestingly, it was found that the phosphorous (IV) oxychloride usually required for these intramolecular cyclization chemistries to function was not needed, with the reaction proceeding smoothing in toluene under reflux as shown in Scheme 3.13 below.

Scheme 3.13 Synthesis of [1,2,4]-triazolo[4,3-b]pyridazine heterocycles

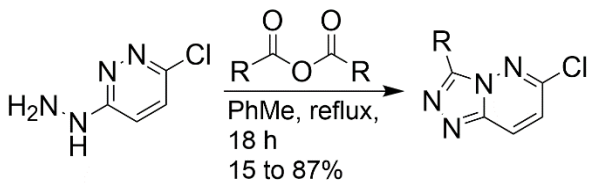
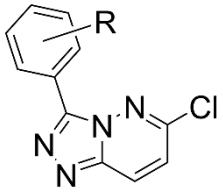
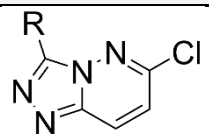


Table 3.11. Percent inhibition of triazolopyridazine core compounds



Compound	R	%Inhibition (30 µM)	%Inhibition (100 µM)
3.168	H	65 ± 12	65 ± 16
3.169	3-Me	54 ± 10	53 ± 19
3.170	3-CF_3	50 ± 12	42 ± 14
3.171	4-Me	55 ± 17	63 ± 20
3.172	4-CF_3	43 ± 21	22 ± 6
3.173	4-OMe	44 ± 10	37 ± 11
3.174	4-F	35 ± 19	49 ± 14
3.175	4-Cl	53 ± 17	50 ± 20
3.176	3-py.	22 ± 22	41 ± 23
3.177	3,4-Cl	15 ± 9	34 ± 21
3.178	4-$C(CH_3)_3$	61 ± 11	43 ± 13



3.179	Me	29 ± 17	61 ± 8
3.180	*n*-Butyl	51 ± 12	50 ± 20
3.181	$C(CH_3)_3$	35 ± 18	31 ± 15
3.182	CF_3	49 ± 17	48 ± 13
3.183	CCl_3	72 ± 7	58 ± 15

Data represented as mean ± SD and represent 3 individual experiments. In all cases, signal evoked by 30 nM progesterone. py. = Pyridine with numbering representing the position of endocyclic nitrogen.

The substitution pattern stemming from the variable group on the symmetric anhydride were selected with inspirations taken from the work of Topliss, featuring different hydrophobicity constants (π) and substituent constants (electronics, σ) among all analogs synthesized.[126] As mentioned, the synthesized compounds were tested for their ability to inhibit the progesterone-mediated calcium influx in our FLIPR assay. Given the low molecular weight of the compounds, and the desire to keep the number of assays to a minimum, the compounds were screened initially at two concentrations of 100 and 30 μM. The results of these assays are provided in Table 3.11.

From Table 3.11 it is apparent that a rather narrow SAR is observed, with all compounds showing some level of activity, but no compound displaying markedly increased potency over the rest. Since potencies were similar across the tested compounds, favor was given to those compounds which showed a good dose-dependent action. Many of the compounds displayed the same levels of inhibition at both concentrations, so the compounds showing good potency while also showing a reasonable improvement of potency at 100 μM over 30 μM (**3.171**, **3.174**, **3.179**) were marked as targets for resynthesis and retesting.

The *m*-Me substitution pattern found in compound **3.169** performed well, showing some of the better potency, though a dose-dependent effect was not observed. Interestingly, the unmodified phenyl substitute found in compound **3.168** proved to be quite a potent compound in the assay. Furthermore, bulky aliphatic groups, such as those seen in **3.178**

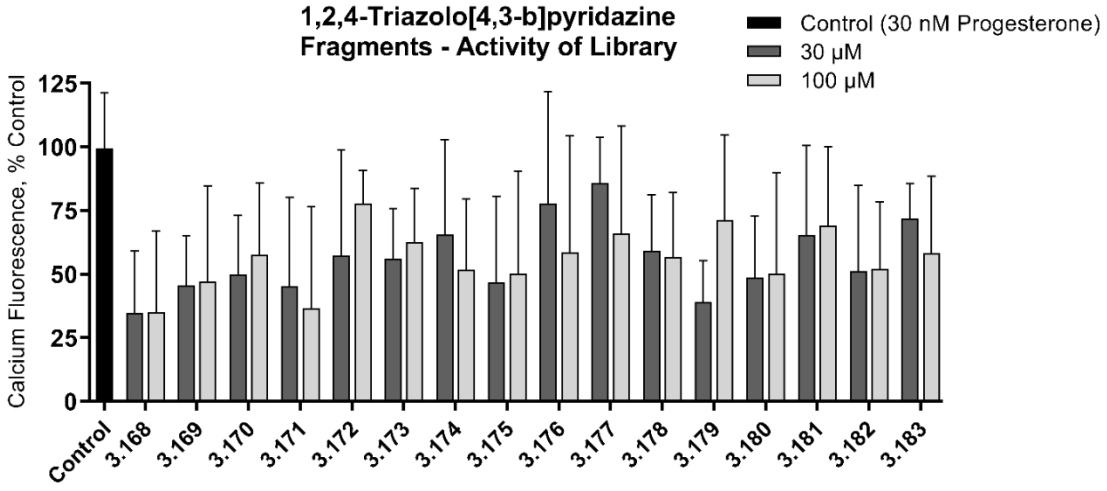
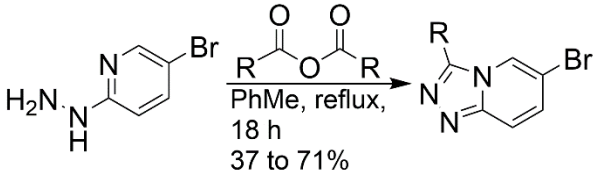


Figure 3.8. Activity of Triazolopyridazine Library in a Calcium Influx Assay. Data are plotted as mean ± standard deviation, representing the mean of 3 replicate experiments. Compounds tested at 30 µM (dark grey) and 100 µM (light grey) against 30 nM progesterone.

and **3.181** did not perform well, neither did heterocyclic replacements (**3.176**) nor trihalomethyl groups that were appended to phenyl rings (**3.182**, **3.183**). Figure 3.8 shows the graphs associated with the data from Table 3.11.

Section 3.3.4 Compounds containing a [1,2,4]triazolo[4,3-a]pyridine as Additional Fragments

Scheme 3.14 Synthesis of [1,2,4]-triazolo[4,3-a]pyridine heterocycles



As part of generating the fragment library, the systematic removal of the endocyclic nitrogen atoms of the southwestern ring system was planned. As such, the next series of compounds from which a library was synthesized removed one of the pyridazinyl nitrogen atoms, giving a triazolopyridine core. The chemistry used to synthesize this library is shown in Scheme 3.14 but is unchanged from Scheme 3.13. Again, the need for $POCl_3$ was not observed, with refluxing conditions in toluene sufficient to induce the intramolecular

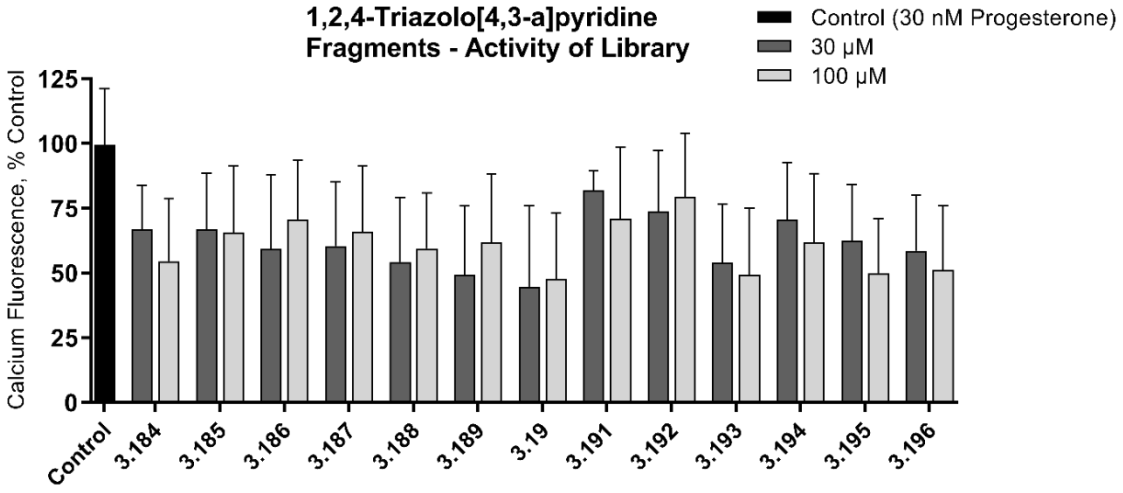


Figure 3.9. Activity of Triazolopyridine Library in a Calcium Influx Assay. Data are plotted as mean ± standard deviation, representing the mean of 3 replicate experiments. Compounds tested at 30 µM (dark grey) and 100 µM (light grey) against 30 nM progesterone.

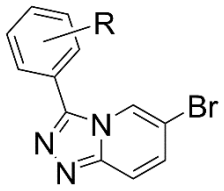
cyclization.

The synthesis of this class of fragment proceeded smoothly and 13 fragments were added to the library. The activity of these compounds is shown in Figure 3.9. Again, these compounds were tested at two concentrations to minimize the number of assays run and the data is presented as %remaining activity of a 30 nM dose of progesterone.

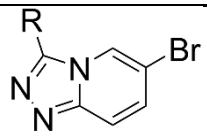
In general, removal of the superfluous nitrogen atoms (with respect to the chemistry) improved the activity of the compounds slightly, indicating that this extra

nitrogen atom is not needed for activity. Indeed, the fact that all the compounds showed activity aids this point. The percent remaining activity of each compound is given in Tables 3.12 for the phenyl and alkyl derivatives, respectively.

Table 3.12. Percent inhibition data for triazolopyridine core compounds



Compound	R	%Inhibition (30 µM)	%Inhibition (100 µM)
3.184	H	33 ± 8	45 ± 12
3.185	3-Me	34 ± 11	34 ± 13
3.186	3-CF_3	41 ± 12	29 ± 9
3.187	4-Me	40 ± 10	34 ± 10
3.188	4-CF_3	46 ± 10	40 ± 9
3.189	4-OMe	51 ± 11	38 ± 11
3.190	4-F	55 ± 16	52 ± 13
3.191	4-Cl	28 ± 4	29 ± 14
3.192	3,4-Cl	26 ± 10	21 ± 10



3.193	3-py.	46 ± 9	51 ± 10
3.194	*n*-Butyl	30 ± 9	39 ± 11
3.195	$C(CH_3)_3$	37 ± 9	50 ± 8
3.196	3-CF_3	42 ± 9	49 ± 11

Data represented as mean ± SD and represent 3 individual experiments. In all cases, signal evoked by 30 nM progesterone. py. = Pyridine with numbering representing the position of endocyclic nitrogen.

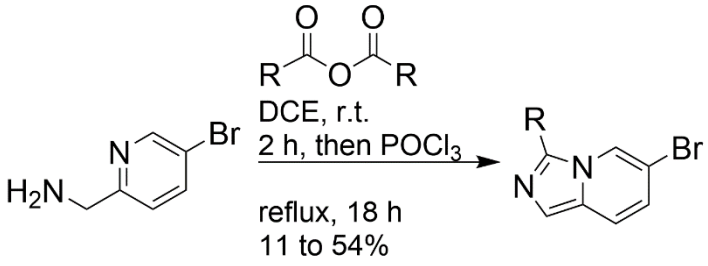
Similar trends were observed between the triazolopyridine and triazolopyridazine libraries. To start, the unsubstituted phenyl analog **3.184** shows similar activity to its triazolopyridazine counterpart **3.168** though its potency is reduced (68 vs 35% remaining activity). There is no significant trend between closely related analogs of the first two

libraries. That is to say not all triazolopyridazines are more/less potent than there triazolopyridine counterparts. That said, several compounds from this library showed good inhibition of the progesterone-induced influx, especially the lower molecular weight alkyl derivatives from Table 3.12 (**3.194 – 3.196**).

Section 3.3.5 Generation of a Fragment Library with Compounds Containing an Imidazo[1,5-a]pyridine Core

The last library systematically modifying the endocyclic nitrogen atoms of the southwestern fragment was a collection of compounds containing an imidazopyridine core. The synthesis of these compounds followed that of the previous libraries, though in this case the $POCl_3$ was needed for the intramolecular cyclization to proceed. DCE was needed to access temperatures high enough to breach the activation energy of the reaction; DCM proved insufficient.

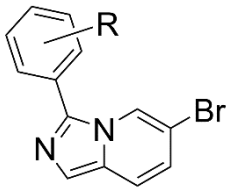
Scheme 3.15 Synthesis of imidazo[1,5-a]pyridine heterocycles



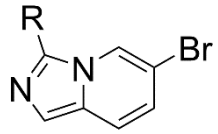
The synthesis of a small library of imidazopyridine core compounds was completed in short time and these compounds were assayed in the influx assay previously described. The results of these assays are shown in Table 3.13 and Figure 3.10. In general, the imidazopyridine library contained compounds showing the best potency of all compounds tested. In particular, compounds **3.198**–**3.200** are the most potent fragments with nearly

70% inhibition at 30 μM and near complete inhibition at 100 μM. Unfortunately, @ 100 μM **3.198** affected the signal of the assay to such a degree that a reliable IC_{50} value could not be obtained. Nonetheless its potency at 30 μM was quite promising.

Table 3.13. Percent inhibition data for triazolopyridine core compounds



Compound	R	%Inhibition (30 μM)	%Inhibition (100 μM)
3.197	H	65 ± 11	48 ± 14
3.198	3-Me	72 ± 6	ND
3.199	3-CF_3	61 ± 9	98 ± 2
3.200	4-Me	70 ± 6.8	91 ± 12
3.201	4-CF_3	37 ± 9	62 ± 5
3.202	4-F	53 ± 7	36 ± 8
3.203	4-Cl	62 ± 10	44 ± 5



3.204	3-py.	63 ± 9	61 ± 10
3.205	Me	45 ± 8	42 ± 15
3.206	*n*-Butyl	49 ± 11	57 ± 9

Data represented as mean ± SD and represent 3 individual experiments. In all cases, signal evoked by 30 nM progesterone. py. = Pyridine with numbering representing the position of endocyclic nitrogen. ND = not determined.

Yet again, as in all libraries synthesized so far, the phenyl derivative **3.197** shows good potency at 30 μM, it its activity at 100 μM is lower. The *p*-CF_3 group found in **3.201** confers good potency at 30 μM, like its *m*-CF_3 counterpart in compound **3.199**, however the activity at 100 μM was not increased compared to 30 μM, so this compound was not pursued further. Compound **3.204** is one of the first heterocyclic substitutions to show good activity at 30 μM, though at 100 μM no increase is observed.

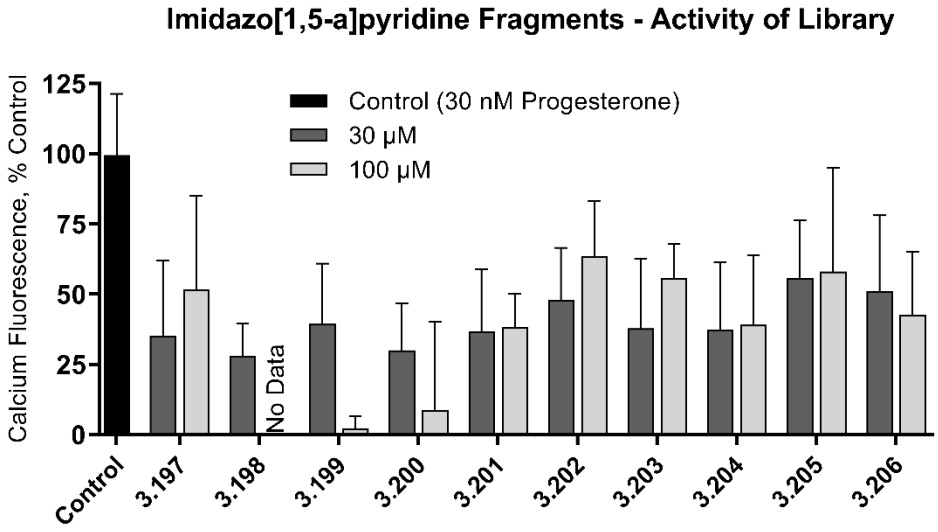


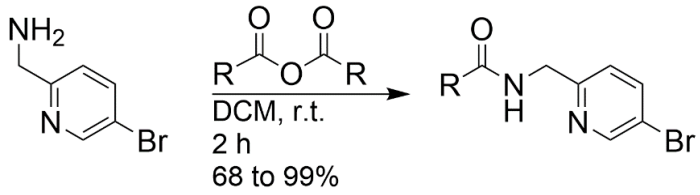
Figure 3.10. Activity of imidazopyridine library in a calcium influx assay. Data presented as percent remaining activity compared to signal evoked by 30 nM progesterone. Compounds tested at 30 μM (dark grey) and 100 μM (light grey). Data are plotted as mean ± standard deviation, representing the mean of 3 replicate experiments. No data for 100 μM **3.198** was obtained.

Section 3.3.6 Further Amide Fragments from Imidazo[1,5-a]pyridine Core Library

The final library to be discussed resulted from a mistake by an undergraduate research assist who was working in the lab at the time. As can be seen in Scheme 3.15, the solvent for the intramolecular cyclization needed to be DCE to access higher temperatures.

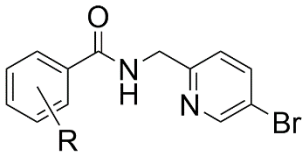
However, the researcher misread the lab notebook and used DCM in its place. In this solvent, only the initial addition product is observed as shown in Scheme 3.16.

Scheme 3.16 Synthesis of an amide library

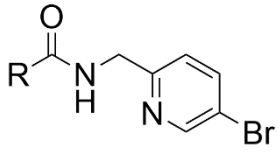


Several analogs corresponding to these ring-opened amides were isolated before this oversight could be remedied. However, given the nature of the project, these compounds were simply treated as additional, easily accessible compounds.

Table 3.14. Percent inhibition data for amide library



Compound	R	%Inhibition (30 μM)	%Inhibition (100 μM)
3.207	H	32 ± 18	63 ± 8
3.208	3-Me	37 ± 14	60 ± 12
3.209	4-Me	33 ± 19	66 ± 21
3.210	4-CF_3	49 ± 18	72 ± 19
3.211	4-OMe	58 ± 17	41 ± 4
3.212	4-F	53 ± 17	45 ± 8
3.213	4-Cl	57 ± 25	47 ± 11
3.214	3,5-Cl	30 ± 23	89 ± 25
3.215	4-$C(CH_3)_3$	50 ± 15	83 ± 22



3.216	3-py.	67 ± 19	52 ± 18
3.216	Me	25 ± 5	66 ± 17
3.217	*n*-Butyl	42 ± 5	60 ± 14

Data represented as mean ± SD and represent 3 individual experiments. In all cases, signal evoked by 30 nM progesterone. py. = Pyridine with numbering representing the position of endocyclic nitrogen.

A small library of amides resulted and these compounds were tested as previously described. Of note, while $POCl_3$ was added to the first few reactions, once it was decided to synthesize the remaining analogs, this step was obviously not performed.

As shown in Table 3.14, some of these serendipitous fragments are actually quite potent inhibitors of the progesterone-induced calcium influx, showing that methylamine derivatives can be used in the future development of this compound in addition to the cyclized products. In general, the same trends observed with the ring-closed analogs

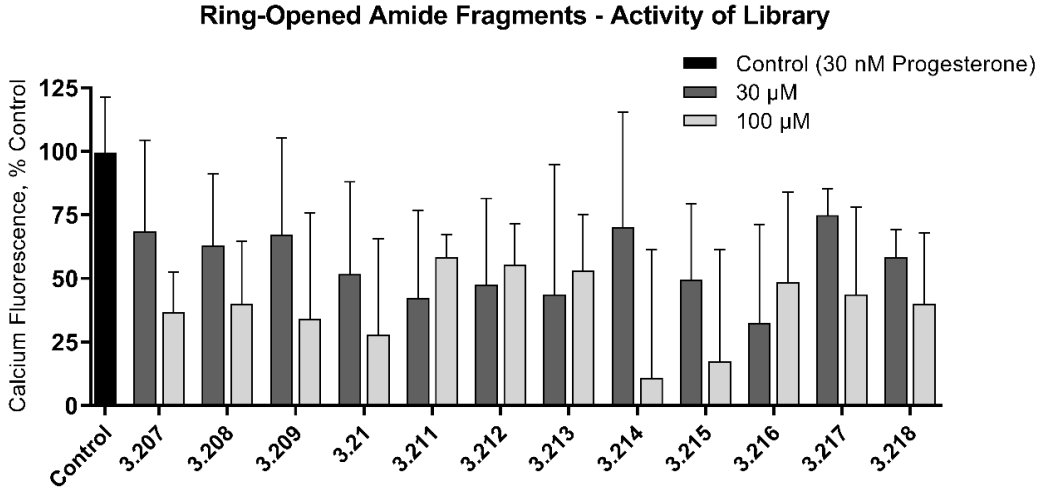


Figure 3.11. Activity of Ring-Opened Amide Fragment Library in the Calcium Influx Assay. Data presented as percent remaining activity of signal evoked by 30 nM progesterone. Compounds tested at 30 µM (dark grey) and 100 µM (light grey). Data are plotted as mean ± standard deviation, representing the mean of 3 replicate experiments.

continue with this library of amides, with phenyl (**3.207**), *m*-Me (**3.208**) and CF_3 groups **(3.210**) presenting the most active compounds. The 3,5-dichlorophenyl derivative **3.214**

showed a great dose response, however solubility issues prevented this compound from being pursued further.

Section 3.3.7 Validating the Fragment-Based Approach Via Synthesis and Evaluation of Test Compounds

During the course of generating and evaluating the discussed libraries, a valid question was raised: What percent of a particular compound's activity is derived from the omnipresent halogen? This halogen was included on each molecule because it serves as the handle for second step chemistries involving either S_NAr displacements or Buchwald-Hartwig couplings. This handle was kept on the tested analogs in an attempt to save costs and time, as not all compound was used in to assay activity and remaining powder could be taken into these next steps immediately.

That said, as a control, several dehalogenated analogs were synthesized and tested for their ability to inhibit calcium influx in human sperm. Comparing their activities to their halogenated counterparts would presumably show activity caused by the halogen. It

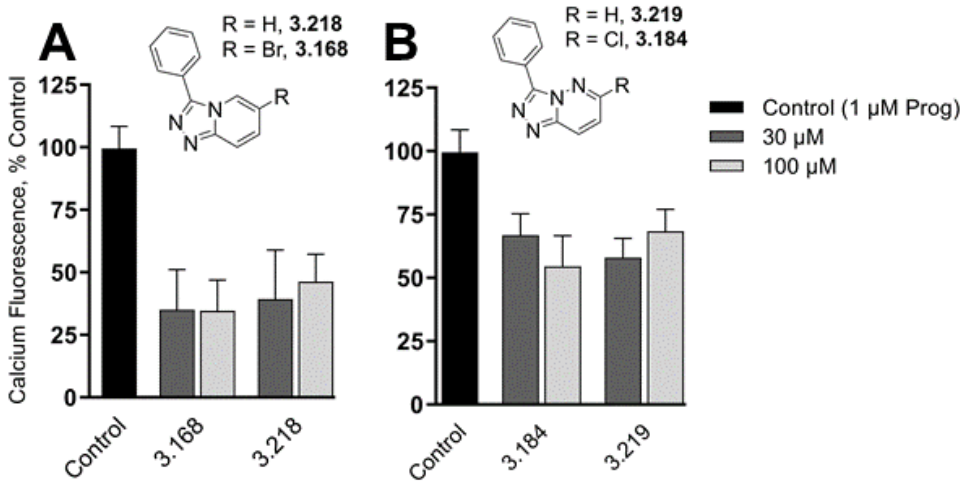


Figure 3.12 Comparison of Test Compounds with or without an Aryl Halide. Data presented as percent of signal evoked by 30 nM Progesterone. Compounds tested at 30 µM (dark grey) and 100 µM (light grey). Data are plotted as mean ± standard deviation, representing the mean of 3 replicate experiments.

was expected that the dehalogenated compounds would be slightly less potent than their halogenated partners, but that activity profiles would remain similar. If this were the case, then the strategy used so far would be legitimized.

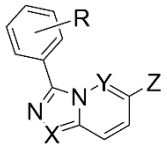
The synthesis of these control compounds is identical to that of their halogenated derivatives in the relevant schemes shown previously. Figure 3.12 shows the results of this test to be positive.

In both tested compounds, the dehalogenated derivative **3.218** and **3.219** show sufficiently similar activity to that of the originally tested compound (**3.168** and **3.184**) so as to not induce doubt in the previously reported data. While not conclusive, this experiment helps to show that the activity observed in the assay is almost certainly from the other molecular features present on the tested molecules and not that of the halogen.

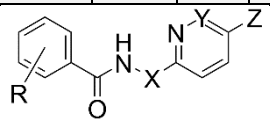
Section 3.3.8 Fragment Library Part Two: Confirming Activity of Most Potent Fragments in Influx Assay

In the synthesis and characterization of the four focused libraries, 51 compounds were generated and tested in the influx assay. Of these 51 compounds several showed a promising ability to inhibit the progesterone-mediated calcium influx in human sperm. Before continuing with the next step of the synthetic route (transition-metal couplings or S_NAr displacements), verification of a select few compounds and subsequent selection of a few key fragments was desired. To this end, as shown in Table 3.15, a small cohort of promising fragments, as determined by their initial results, were resynthesized, verified pure by qNMR and UPLC and assayed again in the influx assay. This time, given their already promising activity, the compounds were assayed at lower concentrations, being 10 and 30 μM, rather than 30 and 100 μM.

Table 3.15. Resynthesized compounds and their potencies at lower concentrations



Compound	R	X	Y	Z	%Inhibition (10 µM)	%Inhibition (30 µM)
3.220 (3.198)	3-Me	CH	CH	Br	31 ± 10	52 ± 5
3.221 (3.199)	3-CF_3	CH	CH	Br	30 ± 9	62 ± 5
3.222 (3.200)	4-Me	CH	CH	Br	15 ± 6	33 ± 8
3.223 (3.168)	H	N	N	Cl	9 ± 2	30 ± 5
3.224 (3.169)	3-Me	N	N	Cl	26 ± 6	15 ± 5
3.225 (3.171)	4-Me	N	N	Cl	8 ± 2	29 ± 3
3.226 (3.174)	4-F	N	N	Cl	16 ± 7	29 ± 3



3.227 (3.210)	4-CF_3	CH_2	CH	Br	17 ± 13	23 ± 2
3.228 (3.216)	4-$C(CH_3)_3$	CH_2	CH	Br	9 ± 4	26 ± 10

Data represented as mean ± SD and represent 3 individual experiments. Compound number in parenthesis corresponds to previously synthesized compound number. 30 nM Progesterone was used to evoke signal in all assays.

As can be seen from Table 3.15 and Figure 3.13, the resynthesized compounds retained activities close their original levels of inhibition, a promising observation. For the sake of comparison, the original compound number for the first iteration of synthesis if provided in parenthesis in the table. Compounds **3.223** – **3.228** show around 70% remaining activity when retested at these lower concentrations. These values are similar to those previously seen with these compounds. Moreover, a dose response is seen in all compounds except **3.224** which showed equal inhibition at both concentrations tested.

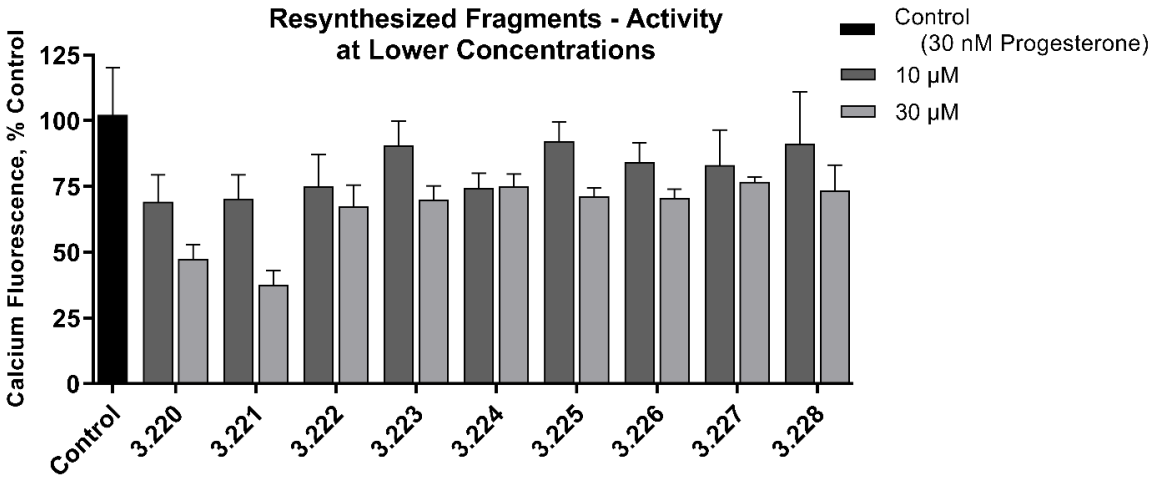
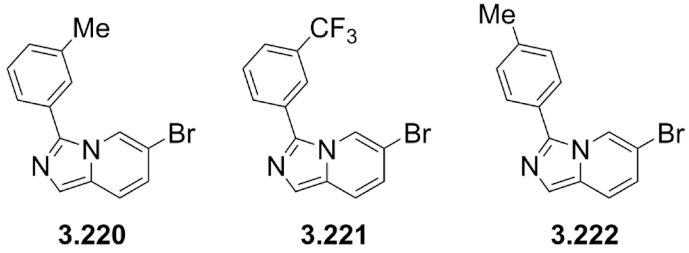


Figure 3.13. Activity of Resynthesized Compounds in a Calcium Influx Assay. Data presented as percent remaining activity compared to signal evoked by 30 nM Progesterone. Compounds tested at 10 µM (dark grey) and 30 µM (light grey). Data are plotted as mean ± standard deviation, representing the mean of 3 replicate experiments.



3.220 **3.221** **3.222**

Figure 3.14. Structures of Chosen Fragments from Synthesized Fragment Libraries.

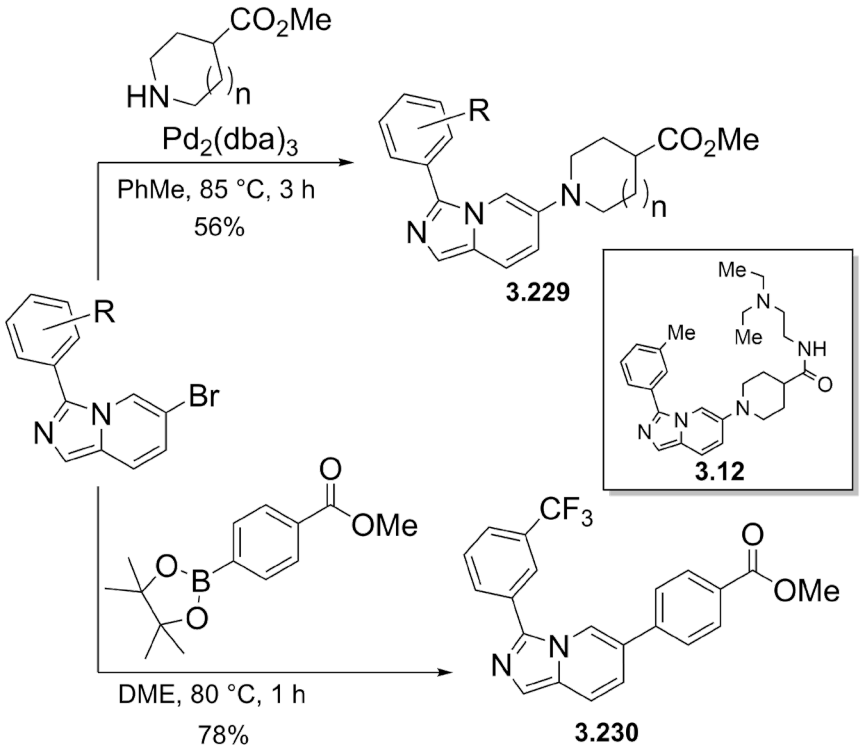
In general, the fact that the compounds inhibited the influx at these lower concentrations, given their low molecular weights, is a promising result. However only three compounds were chosen for further elaboration. The compounds **3.220**, **3.221** and **3.222** are shown in Figure 3.14 that showed the best potency of all the retested fragments.

Section 3.3.9 Ongoing Work: Towards Larger Molecular Weight Fragments of GPHR-00213869

The chosen fragments from Figure 3.14 are to be elaborated upon as shown in Scheme 3.17. It should be noted that, while not discussed, the 3-Me triazolopyridazine-containing fragment, bearing the same substitution pattern seen in **3.12,** will also be subjected to coupling reactions to synthesize a small number of compounds bearing this 3-Me triazolopyridazine southwestern fragment. This small subset of compounds can serve as a pseudo control set, since their substitution pattern will eventually result in the resynthesis of the parent compound. Increases in potency from the novel analogs would be desired over the parent substitution pattern.

The chemistries currently being utilized are meant to explore the linking of the piperidine ring found in **3.12**. Modifications to this ring include ring expansions and contractions to both the corresponding homo-piperidine and pyrrolidine (Scheme 3.17, top reaction). Further nitrogen-containing rings are being pursued including piperazine, homo-piperazine as well as pyrrole linkers. Fourteen commercially available nitrogen coupling partners have been purchased and will be coupled to each of the fragments by Buchwald-Hartwig chemistry. Additionally, Suzuki-Miyaura reactions can be utilized to introduce aryl-aryl linkages via the corresponding pinacol boronic esters (Scheme 3.17, bottom

Scheme 3.17 Current and future reactions exploring linker region of **3.12** (inset)



reaction). A series of 15 boronic esters and acids have been purchased and will be coupled to the chosen fragments to generate libraries of compounds for testing.

Each potential analog building off the southwestern heterocycle contains a methyl ester group, so as to standardize this group's reactivity, much like the halogens in the discussed libraries. By holding this group constant, increases/decreases in activity can be correlated to the other introduced molecular features. After completion of this linker region library of compounds, compounds can be chosen in a manner similar to that described in this section. Then this methyl ester can be hydrolyzed using $LiOH{\cdot}2H_2O$ (Scheme 3.18) and further analogs can be generated via EDC couplings to generate a large variety of analogs. Fischer esterification could also be utilized to explore the activity of esters at this position. Though, given their metabolic instabilities, it is perhaps merited to explore amides

to a greater degree. Preliminary SAR by commerce explored this modification and therefore it is known that amide formation is a viable approach to generate active analogs.

Section 3.4 Discussion and Overarching Observations from Hit-to-Lead Development Endeavors

After the preliminary work of Dr. Francis revealed narrow SAR patterns for all the HTS hit compounds, the best indicators for which of the hits should be developed further came from electrophysiology data provided by the Lishko lab at UC–Berkeley. This data showed the two compounds GPHR-00032750 (**3.9**) and GPHR-00213869 (**3.12**) were able to fully block the progesterone-mediated current in human sperm at 1μM. From this, the development of these two compounds was undertaken and some amount of success was achieved with respect to both of them. It should be said that the development of both these inhibitors is still ongoing and the work discussed in this chapter constitutes only a portion of an ongoing story.

Section 3.4.1 The Development of GPHR-00032750

The development of GPHP-00032750 (**3.9**) was begun in earnest in early 2015. The biphenylpiperazine scaffold lent itself to the rapid generation of analogs via facile chemistries. As such, the first library of compounds synthesized sought to explore the two phenyl ring substitutions present in the molecule via basic modifications probing the electronics of the two phenyl rings. In this library it was shown for the first, though not the last, that EWGs at the *meta* position are highly coveted and bring about the best activity, especially when comparing compounds **3.9** to **3.17**. From this library, and confirmed in subsequent libraries, the general trend of $–CF_3 = –NO_2 > Me >> Ph$ was found. With respect to the western phenyl ring, substitutions at the *meta* position were the only which retained activity.

As seen from the data for this library heterocyclic appendages to the eastern phenyl ring seen in compounds **3.32** and **3.33** retained activity, indicating a tolerance in this area for bulkier substitutions. So, a series of compounds in which the eastern phenyl ring was substituted with heteroaromatic groups was synthesized (Table 3.2, compounds **3.49** – **3.54**). These compounds further showed the tolerance of this eastern phenyl ring for bulky substitutions, so analogs in which an additional aryl system was appended were synthesized and assayed. As seen in Table 3.3, these extended analogs retained activity in many cases, but given the quickly rising molecular weights and lipophilicity of these compounds, a turn in strategy was taken.[127]

Given the lack of success in modifying the two phenyl rings, alterations of the core of the molecule, such as probing the piperazinylurea moiety was sought. The piperazine ring was opened to give diamide-containing analogs or thiocarbamoic esters and the urea

group was replaced with a sulfonamide in several analogs (Tables 3.4 – 3.6). This strategy failed to yield compounds with improved activity, ultimately leading to a great majority of inactive compounds.

Knowing now that modification of the piperazine, and furthermore the western phenyl group, was not likely to increase potency, the spacing group adjacent to the eastern phenyl group was explored. Analogs were synthesized in which the urea group was replaced with an aryl ketone (**3.121**–**3.125** and **3.128**–**3.130**). These compounds were largely inactive, leading to the conclusion that the aniline moiety of the phenyl urea was needed, or perhaps at least a methylene to serve as a spacer between the eastern phenyl ring and the urea since compounds **3.126** and **3.127** retained activity.

Extending this spacer group to phenoxymethylene linkers seen in Table 3.8 started to show promising results, with every compound showing potency equal to the parent compound. Also, now that this spacer was extend by a carbon, an additional point of modification (the methylene carbon) became possible. However, alkylation of this carbon did not significantly increase potency as demonstrated by compounds **3.136**–**3.142** and was abandoned.

With momentum turning in our favor we sought to further explore analogs of similar structure to those compounds in Table 3.8. It was promising that compounds showing submicromolar inhibition in our calcium influx assay were achieved by changing of the phenoxy group to an aniline while making use of less bulky, still electron withdrawing *o*- and *p*-fluoro substitutions. Compounds **3.147** and **3.148** show submicromolar IC_{50} values and constitute the most potent CatSper blockers observed to date (Figure 3.4).

These compounds were subjected to CASA to evaluate their abilities to reduce sperm motility (both normal and hyperactivated) characterized. Both compounds show an impressive ability to reduce both normal and hyperactivated motility (Figures 3.6 – 3.8). Compound **3.147** is able to significantly diminish the population of cells displaying HAM at 100 nM and at 1 μM completely ablates this population. Analog **3.148** had a diminished effect on HAM at 100 nM but also shows complete reduction of HAM at 1 μM. These compounds both significantly reduce normal motility parameters at both concentrations tested.

Going forward, further analogs of **3.147** and **3.148** should focus on removing the anilines from the compound while maintaining potency, since this group can be a metabolic liability. Further work should confirm that these compounds do indeed inhibit CatSper and not the recently characterized activity of the ubiquitously expressed ABHD2.[128] Confirmation of the activity of these compounds via electrophysiology is ongoing and will serve as the final test for these potent inhibitors of this intriguing channel.

Section 3.4.2 Fragment-Inspired Development of GPHR-00213869

As mentioned previously, **3.12** or GPHR-00213869 is a more drug-like hit compound compared to **3.9**. This compound, however, requires more synthetic steps to generate analogs. To avert this set back, a fragment-inspired approach was taken towards the development of this compound, with optimization of the southwestern heterocycle and adjacent phenyl system serving as a starting point.

Four libraries of compounds are described in Sections 3.3.3 through 3.3.6. These libraries focused on discerning the need for the individual nitrogen atoms of the original triazolopyridazine core. It was found that not all nitrogen atoms are necessary for the

Bischler-Napieralski chemistry to function and could be removed and fragments with good potency could still be obtained as exemplified by compounds **3.220** and **3.221**. Compounds containing more than the two necessary nitrogen atoms were still effective inhibitors, but the imidazopyridine fragments were the best performing compounds.

The phenyl ring adjacent to this heterocyclic core was also explored at great length, with substitution patterns being heavily influenced by Topliss considerations surrounding lipophilicity and substituent constants.[126] A great variety of both hydrophilic vs hydrophobic along with EWG vs EDG were synthesized. In general, as for **3.9**, EWGs were favored, with $–CF_3$ groups predominating the list of most active compounds as demonstrated with analog **3.199**. Several analogs were synthesized in which the entirety of the phenyl ring was removed, replaced by alkyl chains of varying length and configuration. These compounds retained a modicum of activity but were usually less active than their phenyl counterparts (see **3.198** vs **3.205**).

Given that each fragment tested thus far contained an aryl halogen, effort was put towards ensuring the activity of the synthesized compounds was derived from molecular features which would be present in the final molecules, rather than a halogen. To this end, test compounds **3.218** and **3.219** were synthesized and their activities compared to their halogenated counterparts. As seen in Figure 3.12, the phenyl and halogenated derivatives displayed similar levels of activity, helping to prove that the pertinent molecular features of the synthesized fragments were the cause of activity, though further testing would be needed for conclusive assertions.

In total, 51 fragments were synthesized and three were chosen after a series of compounds were resynthesized and retested at lower concentrations (Section 3.3.8). These

three compounds (**3.220**, **3.221** and **3.222**) will be taken forward into subsequent chemistries to explore the next fragment of the molecules, being the piperidine linker. Work is ongoing and several S_NAr reactions have been completed to generate an initial library with these three chosen fragments being coupled to a series of commercially available nitrogen nucleophiles. Generation of additional analogs via Suzuki and Buchwald chemistry is planned and following subsequent testing and confirmation of activity, a series of EDC reactions will generate a final library of compounds resembling very closely **3.12**, though hopefully optimized for CatSper inhibition. If all goes to plan, potent compounds resembling **3.12** should result from these synthetic efforts with only around 150 compounds being synthesized rather than the over 10K possible analogs.

Chapter 4: Structure-Activity Studies of Aminocyclopentitols: Discovery of Potent and Selective Inhibitors of GBA1 and GBA2

Section 4.1 Project Aim and Background

Found widespread in plants and bacteria, iminosugars are compounds in which the endocyclic oxygen of the sugar is replaced by a nitrogen atom.[129] While minimally changing the overall structure of the compound, this substitution results in different biological properties. Comparing the amine to the ether moiety, the differences in the electrostatic properties of these two functional groups become apparent (Figure 4.1). As such, the protonated amine **4.1** found in iminosugars make them excellent modulators of

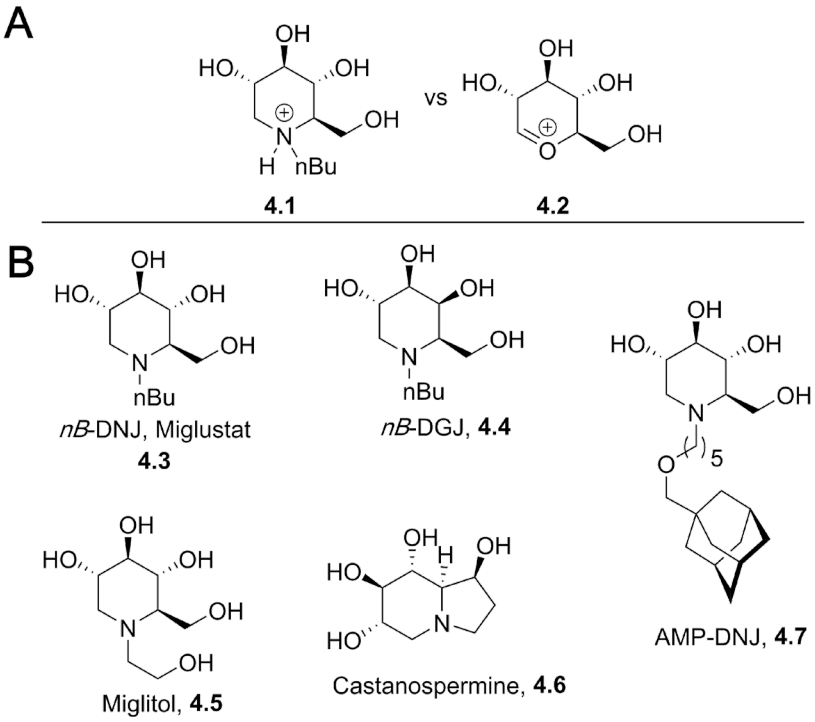


Figure 4.1. Iminosugars as a Molecular Class of Compounds. (A) Representation of protonated amine **4.1** versus oxocarbenium ion transition state **4.2** found in carbohydrate processing enzymes. (B) Structures of example iminosugars. Including approved drugs *N*B-DNJ **4.3** and miglitol (**4.5**).

carbohydrate processing enzymes, mimicking intermediate oxocarbenium ion **4.2** found in enzymatic catabolism of glycosidic linkages.[130, 131]

Iminosugars have been tested as potential therapeutics for numerous indications. Given their similarity to carbohydrates, saccharide processing deficiencies and lysosomal storage disorders were the first disease states that are treated with iminosugars.[132, 133] In 2002 and 2003, respectively, miglustat (*N*-butyl deoxy-nojirimycin, *N*B-DNJ, **4.3**) was approved as an orphan drug by the Federal Drug Administration and the European Medicines Evaluation Agency for the treatment of type 1 Gaucher disease.[134] Also, miglitol

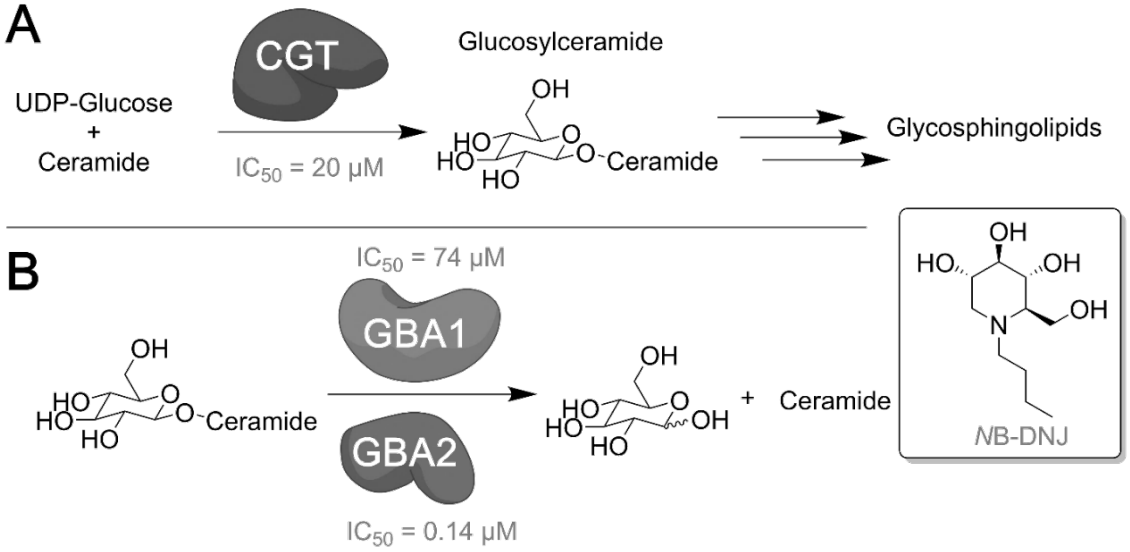


Figure 4.2. Inhibitory Effects on CGT, GBA1 and GBA2. These enzymes are responsible for the synthesis (A) and degradation (B) of glycosphingolipids, respectively. The IC_{50} values for *N*B-DNJ inhibition are shown in light grey. CGT = ceramide-specific glucosyltransferase. GBA1 = Glucocerebrosidase 1 (lysosomal) GBA2 = Glucosylceramidase 2 (non-lysosomal) Figure and IC_{50} values adapted from previous reports. [141]

(**4.5**) is an approved treatment for type 2 diabetes.[135] Moreover, iminosugars have shown several other properties including antiviral,[136] and, antibacterial[137] activity. More recently,

iminosugars have been reported as cystic fibrosis transmembrane conductance (CFTR) correctors.[138, 139] Several examples are shown at the bottom of Figure 4.1.

The biological activities of iminosugars are extensive but of relevance to this work is the ability of these compounds to modulate the glycosphingolipid metabolic pathway. Figure 4.2 illustrates this pathway containing several key enzymes that are inhibited by *N*B-DNJ and its diastereomer *N*-butyldeoxy-galactonojirimycin (**4.4**, *N*B-DGJ). These compounds can inhibit the enzymes responsible for both the formation and degradation of glucosylceramide: ceramide-specific glucosyltransferase (CGT) and β-glucosidase 1 (lysosomal) and 2 (non-lysosomal).[140-143]

In 2002, it was shown that inhibition of these two enzymes can cause reversible loss of fertility in C57BL/6 mice.[144] The study showed that these compounds affected formation of the acrosome, resulting in decreased motility and abnormal sperm morphology. However, these results could not be reproduced in humans, nor other species such as rats, rabbits and even different strains of mice.[49, 145, 146] The discrepancies in these results among species as well as strains of mice is an ongoing area of research.[147]

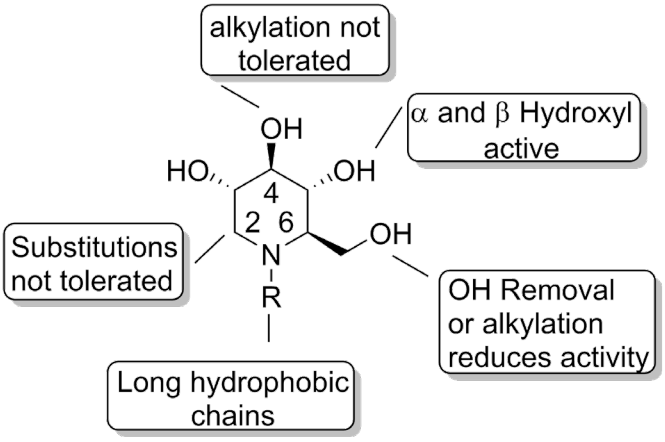


Figure 4.3. SAR of Six-Membered Iminosugars with Respect to CGT Inhibition. Figure adapted from reference 138.

The six-membered iminosugars *N*B-DNJ (**4.3**) and *N*B-DGJ **(4.4**) inhibit GBA1 and GBA2 more effectively than CGT. This observation served as evidence that inhibition of GBA2 rather than CGT is responsible for the male contraceptive effects.[148] Furthermore, it was shown later that GBA2 knockout mice were infertile.[149] The SAR of the six-membered iminosugars acting at these enzymes is well known with many studies seeking to improve both potency and selectivity of the scaffold for aforementioned reasons. The known SAR is shown in Figure 4.3. Any substitution to the C2 position of the ring, as well as alkylation of the C4 hydroxyl group completely ablates activity. The adjacent stereocenter at the C5 position can be either in alpha or beta orientation. Ring size of the glucose scaffold can vary greatly while retaining activity with analogs comprising 4- to 8-membered rings.[150]

Perhaps the most studied structural modification is the alkylation of the endocyclic nitrogen that generate N-alkyl iminosugar analogs.[143] Studies have shown the inhibitory activity of compounds bearing hydrophobic alkyl groups at this nitrogen directly correlate with hydrophobicity.[48] The most potent 6-membered iminosugar inhibitor of CGT, GBA1 and GBA2 bears a [(5-adamantan-1-yl-methoxy)pentyl] group off the nitrogen. This compound, AMP-DNJ (Figure 4.1, **4.7**) displays selective inhibitory activity for GBA2.[151]

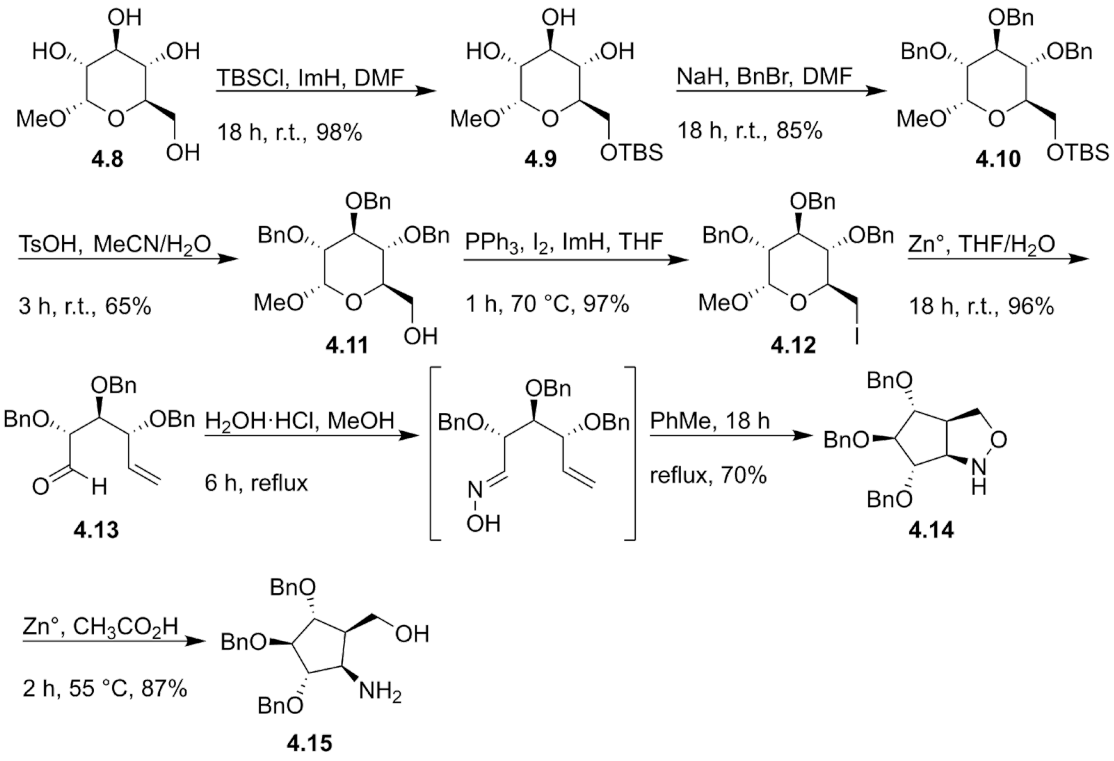
In an effort to further improve selectivity and potency for GBA2 we turned towards another class of iminosugars known as aminocyclopentitols.[152] These compounds are known inhibitors of glucosidases but had not yet been tested as inhibitors of CGT, GBA1 or GBA2.[153] Aminocyclopentitols bear an exocyclic amine that is protonated at relevant pH values, which mimics the oxocarbenium intermediate in the same manner as their 6-membered iminosugar counterparts. Previous work from the Georg group had shown that

monoalkylated aminocyclopentitols bearing butyl and nonyl alkyl chains were potent inhibitors of GBA1 and GBA2, with little to no effect on CGT.[143] Thus, the work discussed herein attempted to further improve the activity of this aminocyclopentitol scaffold at GBA1 and GBA2 by synthesizing dialkylated compounds similar to those previously synthesized, hypothesizing that the increased hydrophobicity of analogs would further increase activity. The synthesis and biological characterization of these compounds follows.

Section 4.2 Synthesis of Aminocyclopentitol Targets

The synthesis of aminocyclopentitol derivatives up to the late stage alkylation precursor **4.15** is shown in Scheme 4.1. Starting the synthetic route from methyl-alpha-D-glucopyranoside (**4.8**), the primary alcohol was protected as the tert-butyl silyl ether to form trihydroxy compound **4.9**. Subsequent protection of the remaining secondary alcohols was achieved with benzyl bromide and resulted in the fully protected pyranoside **4.10**.

Scheme 4.1 Synthesis of benzylated aminocyclopentitol alkylation precursor **4.15**



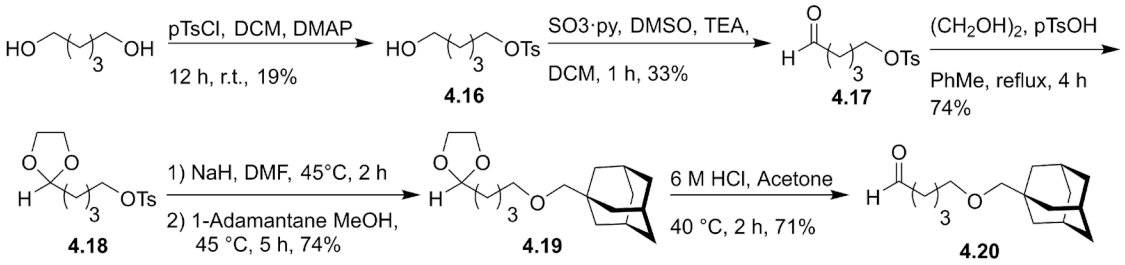
4.8 **4.9** **4.10** **4.11** **4.12** **4.13** **4.14** **4.15**

Deprotection of the primary alcohol under acidic conditions provided compound **4.11**, which was converted to the corresponding iodide compound using triphenylphosphine and molecular iodine to give Vasella fragmentation precursor **4.12**.[154] The aforementioned chemistry was used to open acetal **4.12** to the primary aldehyde while concomitantly converting the primary iodide to the olefin **4.13**. This aldehyde, after reaction with hydroxylamine hydrochloride, formed an intermediate oxime, which underwent 1,3-dipole cycloaddtion in toluene to furnish the [4:2] bicyclic system **4.14**. Cleavage of the *N-O* bond proceeded under reductive conditions to reveal the primary amine **4.15**, which was the precursor for the final steps of the reaction.

Before proceeding onto the final steps of target molecule synthesis, one of the side chains desired was an adamantyl ether side chain that required a five-step synthesis.[155] As described previously, this adamantyl sidechain was desired based on glucose mimic **4.7**

which bears this modification and displays great activity. Scheme 4.2 shows the synthesis of the adamantyl aldehyde **4.20**, that later served as the coupling partner with **4.15**, beginning from 1,5-pentanediol.

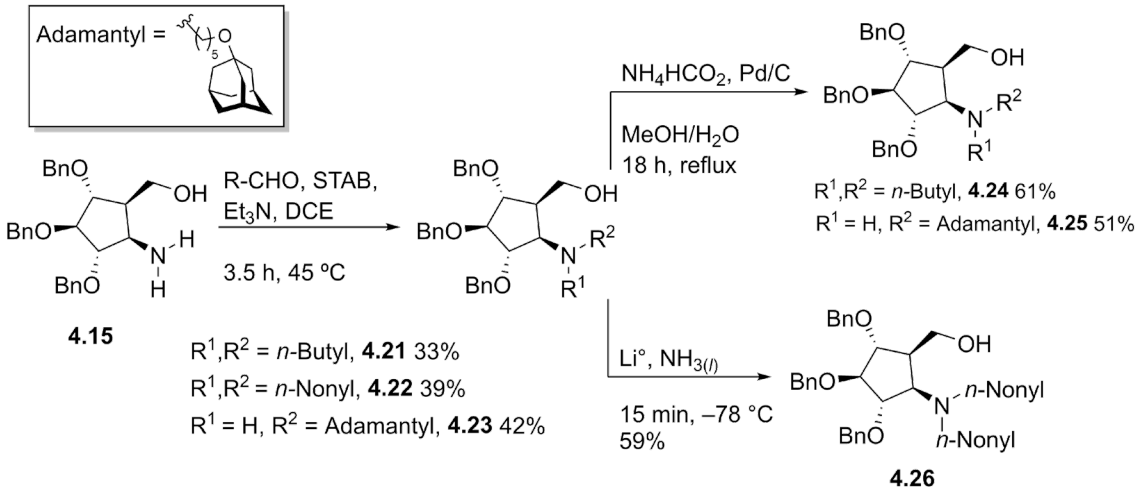
Scheme 4.2 Synthesis of target adamantyl aldehyde **4.20**



Monotosylation of 1,5-pentanediol gave tosylate **4.16**, which after Parikh-Doering oxidation of the alcohol was converted to aldehyde **4.17**. Subsequent protection of this aldehyde as the corresponding acetal **4.18**, followed by S_N2 displacement of the tosyl group by deprotonated 1-adamantylmethanol furnished compound **4.19**. This reaction required elevated temperatures and would not proceed under 40 °C. Finally, deprotection of the acetal under acidic conditions proceeded smoothly and gave the desired aldehyde **4.20** in a 2% overall yield due to a disappointing yield in the initial monotosylation. However, 1,5–pentanediol is inexpensive and therefore scale-up of the first step to accommodate this low yield is not cost prohibitive.

With the required synthesis of the adamantyl aldehyde accomplished, the synthetic route could be completed, and target molecules **4.24–4.26** were obtained (Scheme 4.3). Previous work on this project subjected the benzylated primary amine to reductive amination conditions after forming the HCl salt of the primary amine. Since dibenzylated derivatives were the targets, the reaction conditions leading to the alkylated debenzylation precursors were modified to the conditions shown in Scheme 4.3.

Scheme 4.3 Completion of synthetic route and acquisition of target aminocyclopentitols



Dialkylation of the primary amine was achieved using sodium triacetoxyborohydride (STAB) in dichloroethane (DCE) at elevated temperature and provided the benzylated compounds **4.21**–**4.23**. From here two different methods were employed to deprotect the three hydroxyl groups. *In situ* generation of H_2 via decomposition of ammonium formate in the presence of palladium on carbon was sufficient for the deprotection of **4.21** and **4.23**. However, this reaction was sluggish with respect to benzylated dinonyl compound **4.22**. As such, Birch conditions were utilized to deprotect this lipophilic molecule. In the end, target aminocyclopentitols **4.24**, **4.25**, and **4.26** were obtained in either 10 linear steps for **4.24** and **4.26** or 15 steps for **4.25**.

Section 4.3 Biological Evaluation of Synthesized Aminocyclopentitols

Until work performed by another student in the Georg group, no aminocyclopentitol derivatives had been tested for their ability to inhibit CGT. As such, novel analogs **4.24**–**4.26** were assayed for CGT, as well as GBA2 inhibitory activities.[48] The full details on the assays used to determine CGT, GBA1 and GBA2 activities have been reported.[48]

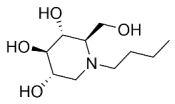
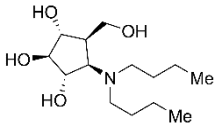
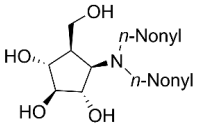
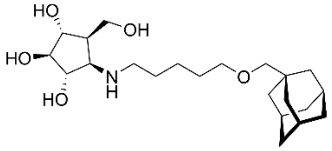
Briefly, for CGT, a HEPES-buffered solution containing UDP-glucose, target iminosugar, and microsomes isolated from either C57BL/6 mice or LE rats were incubated for 30 min in the presence of a BODIPY-tagged ceramide. Enzymatic activity transfers a glucose molecule to this fluorescent ceramide. The product resulting from CGT activity is determined by elution on a TLC plate and the fluorescent product is quantified in a transilluminator. For GBA1 and GBA2, a fluorescence-based assay utilizing 4-methylumbelliferyl β-D-glucoside (MUG) was used. Iminosugar dilutions are combined with testicular microsomes from the species of interest. GBA1 or GBA2 activity leads to the hydrolysis of the MUG probe, releasing the umbelliferone thereby increasing fluorescence.

Table 4.1 shows the testing results for these compounds as inhibitors of GBA2 and CGT. *N*B-DNJ (**4.3**) was used as a reference compound in these assays and showed an IC_{50} value of 48 and 7.4 μM for GBA2 and CGT, respectively. (Table 4.1) These results corroborate previous studies and highlight the lack of selectivity of this compound.[48, 156]

Dibutyl derivative **4.24** showed a modest potency of 95 μM (Entry 4.2) when assayed for GBA2 inhibition but displayed no inhibition of CGT up to 1 mM. Compound **4.24** is less potent than the monoalkylated derivative previously synthesized during the course of this project (3.3 μM, data not shown) with respect to GBA2 inhibition.[143] The monoalkylated butyl analog also showed no inhibition of CGT up to 1 mM (data not shown).[143] Continuing this trend, dinonyl aminocyclopentitol **4.26** was also less potent than its monoalkylated counterpart, which inhibited GBA2 with an IC_{50} value of 0.043 μM. However, compound **4.26** still shows a potent inhibition of GBA2 at 0.89 μM with no inhibition of CGT up to 1000 mM. Interestingly, shown in Entry 4.4, compound **4.25**

showed 100% inhibition in the assay, even at concentrations as low as 1 pM. No inhibition CGT was observed up to 1 mM with **4.25**.

Table 4.1. Inhibitory activity of synthesized Iminosugars on enzymes of interest[a]

Compound		K_i (μM)	
		GBA2	*CGT*
	**4.3**	48	7.4[b]
	**4.24**	95	>1000
	**4.26**	0.89	>1000
	**4.25**	<< 1 nM	> 1000

[a]The details and procedures of the enzyme inhibition assay for GBA and CGT are reported.[48] [b Value taken from previous reports.[48] GBA2, β-glucosidase 2; CGT, ceramide-specific glucosyltransferase. Data presented as mean of 3 experiments.

Effort was then undertaken to deduce the cause of this exquisitely potent compound. The assay was functioning properly, as the controls worked well, and **4.25** itself was not interfering with the fluorescence of the assay. It was eventually reasoned that the sample sent for testing was contaminated with chelated palladium from the final deprotection step. To remedy this setback, the altered synthetic route displayed in Scheme 4.4 was devised in which the debenzylation was moved up in the reaction sequence. It should be noted that this redefined scheme was needed because **4.25** proved unstable in the presence of a trimercaptotriazine resin meant to sequester palladium out of solutions.

Scheme 4.4 Altered synthetic route to avert palladium contamination

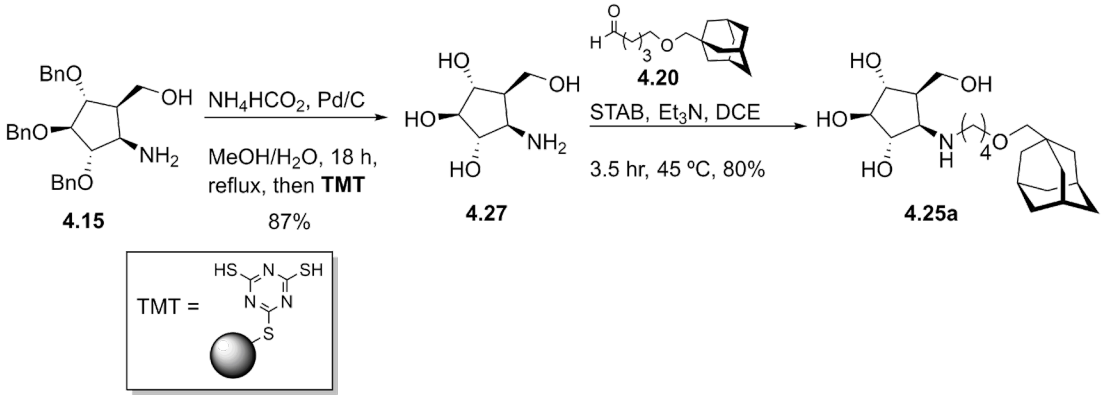
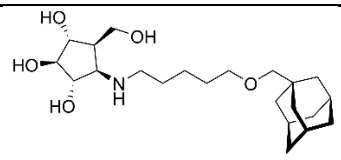


Table 4.2 Inhibitory activity of resynthesized adamantyl compound on enzymes of interest[a]

Compound		K_i (μM)		
		GBA1	*GBA2*	*CGT*
	**4.25a**	≤0.016[b]	0.014	≈1000

[a]The details and procedures of the enzyme inhibition assay for GBA and CGT are reported previously.[48] [b]Below limit of detection in assay. Data presented as mean of three independent trials.

Resynthesis of the target compound commenced and the alternative scheme was used for the final steps towards the synthesis of **4.25**. Deprotection of benzylated aminocyclopentitol core **4.15** via *in situ* generation of H_2 in the presence of palladium afforded the aminocyclopentitol **4.27**, which was stable in the presence of the TMT resin. Compound **4.27** could then be subjected to the previously optimized reductive alkylation conditions to afford **4.25** after purification. qICP analysis performed by the UMN core facilities revealed only trace amounts of palladium, as well as other heavy metals. This compound was then retested and performed similarly to expected efficacy for the compounds of this class.

As shown in Table 4.2, **4.25** displayed a much more realistic IC_{50} value of 14 nM in the GBA2 assay. This updated IC_{50} value aligns with expected values, given previous results with monoalkylated aminocyclopentitols. That said, the resynthesized iminosugar shows an excellent selectivity for GBA1 and GBA2 over CGT (> 10,000). This is promising since *N*B-DNJ inhibits all three enzymes with nearly equal potency, with IC_{50} values of 34, 38 and 7.4 μM for GBA1, GBA2 and CGT, respectively (Table 4.1, GBA1 data not shown).

The synthesized aminocyclopentitols described here inhibit GBA1 and GBA2 to a much greater extent than CGT. As expected, compounds bearing alkyl chains of increasing length showed concomitant increase in GBA inhibition, as demonstrated by the inhibitory trend observed in compounds **4.24** (butyl, 95 μM) versus **4.26** (nonyl, 0.89 μM). As mentioned, these dialkylated analogs are less potent than their previously synthesized mono-alkylated derivatives, indicating that there is a limit to the correlation of hydrophobicity with activity at these enzymes. Indeed, the cLogP of these dialkylated compounds is above 6 and solubility is likely an issue, especially at the higher concentrations.

Furthermore, it is possible that only secondary amines display full activity and that a hydrogen bond donor is a necessary molecular feature of the GBA2 pharmacophore. However, despite their reduced activity, the selectivity profile for these compounds is still exquisite and future studies would do well to utilize these compounds.

Section 4.4 Species-Dependent Action of Iminosugar Activity

As discussed previously, differences in the efficacy between species has been observed with respect to iminosugars and infertility.[144, 146, 157] Compound **4.3** (*N*B-DNJ)

inhibits CGT, GBA1 and GBA2 in vitro and in vivo with similar efficacies. Also, though **4.3** was shown to be an effective and reversible oral male contraceptive agent when tested in C57BL/6 mice, the same level of efficacy was not observed in several other species, including rabbit, human and other mouse strains. In C57BL/6 mice, however, reversible inhibition of fertility was observed three weeks after daily oral administration of 15 mg/kg *N*B-DNJ, with return to normal parameters observed after three weeks of cessation of treatment. [144] Separately, glucosylceramide levels were elevated in the testes, brain and spleen of mice treated with these iminosugars, indicating the enzymes responsible for the breakdown of glucosylceramide, i.e. GBA2, are more pertinent to contraceptive effects observed in vivo.[158]

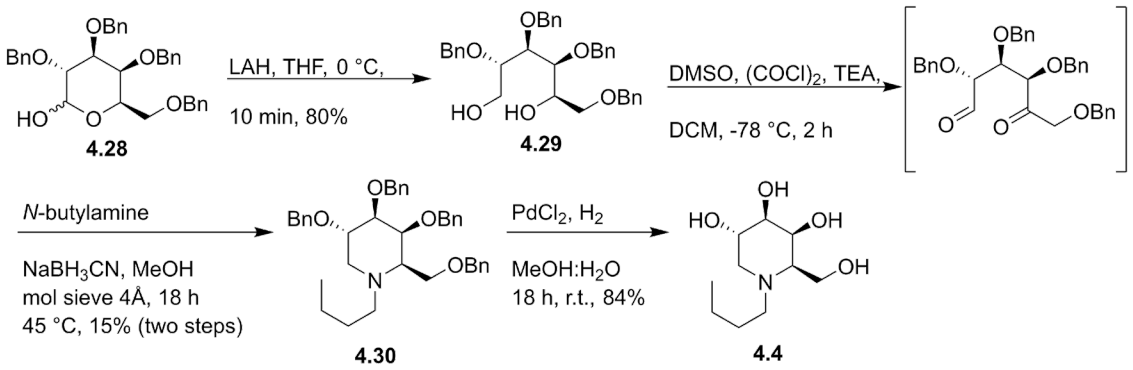
It has been shown that *N*B-DGJ (**4.4**) can disrupt spermiogenesis in C57BL/6 mice and does so at much lower concentrations than *N*B-DNJ (**4.3**) .[144] Furthermore, *N*B-DGJ has shown efficacy in species that remain fertile after treatment with *N*B-DNJ.[159] The observed discrepancies between the two diastereomers could be caused by differences in their ADMET properties. To address this, a pharmacokinetic study with the two diastereomers was planned in an attempt to determine if plasma levels of **4.3** and **4.4** were significantly different, and thus a possible cause for the observed differences.

Section 4.4.1 Synthesis of N*B-DGJ and* N*B-DNJ*

Since **4.4** is not commercially available, its synthesis was carried out according to known procedures from the literature (Scheme 4.5).[160] The four-step synthetic route begins with an LAH-mediated reductive ring opening of tetra-*O*-benzyl-D-glucopyranose **4.28** to reveal diol **4.29**. Swern oxidation of **4.29** formed the intermediate ketoaldehyde that after double reductive amination reformed the six-membered ring observed in **4.30**, now

containing the alkylated endocyclic nitrogen. Finally, debenzylation of this compound gave target molecule *N*B-DGJ (**4.4**) in 53% overall yield.

Scheme 4.5 Gram-scale synthesis of *N*B-DGJ (**4.4**)



While *N*B-DNJ is commercially available, purchasing the amount needed for even this small PK study was cost prohibitive. Therefore, we synthesized the amounts needed using the same route as shown in Scheme 4.3 but starting from the glucopyranoside. In short, we obtained sufficient amounts so as to perform the study with both compounds.

Section 4.4.2 Bioavailability Determinations of N*B-DNJ and* N*B-DGJ in Male CD Rats*

Per our original aim, we sought to correlate observed in vivo activity with the bioavailability of *N*B-DGJ, and perhaps discrepancies would be observed when compared to *N*B-DNJ. Previously, a study in collaboration between the Tash group at the University of Kansas Medical Center and the Georg group showed that *N*B-DGJ inhibits GBA2 from CD rats at 10 μM (unpublished results). This study also showed that *N*B-DJG was capable of causing a reversible contraceptive effect in vivo, while *N*B-DNJ showed no efficacy (unpublished results). To evaluate if bioavailability could be the cause of these discrepancies a small PK study was performed in which plasma levels following a single bolus oral dose of 150 mg/kg of either *N*B-DNJ or *N*B-DGJ were examined. Male CD rats

were orally dosed with the synthesized compounds by gavage (n=4) and at various timepoints following dosing, blood was taken. The resulting plasma was analyzed by LC/MS/MS.

As seen in Figure 4.4, oral dosing of *N*B-DGJ resulted in a $t_{1/2}$ of 6.5 hr and a C_{max} of 11,0167 ng/mL. These results agree with previously reported literature values for the

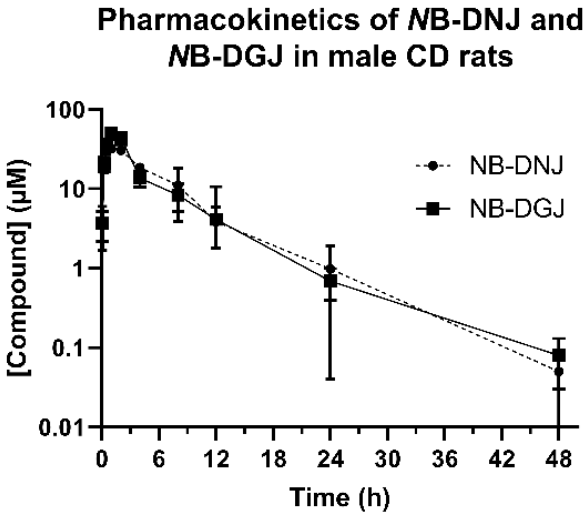


Figure 4.4 Pharmacokinetics of *N*B-DGJ and NB-DNJ in Male CD Rats. Rats with in-dwelling jugular vein cannulas were orally dosed with compound (150 mg/kg) and at various times (0.083, 0.25, 0.5, 1, 2, 4, 8, 12, 24 and 48 h) afterwards, approximately 0.1 ml whole blood was collected from each animal. Target compound was quantified in the resulting plasma using LC/MS/MS. Data represent the mean plasma levels ± SD and are the average of 4 independent experiments.

compound.[161] Unfortunately, these data show no significant difference between *N*B-DNJ and *N*B-DGJ, indicating some other cause of the species-specific differences causing infertility. Based on this study we are confident that the variation of efficacies for these compounds is not due to differences in the metabolism of the compounds in this strain of rat.

Section 4.5 Discussion and Summary of Aminocyclopentitol Work

Herein, the synthesis and biological evaluation of several novel iminosugars of a sub classed known as aminocyclopentitols are reported. Previously, monoalkylated derivatives of this compound had been synthesized and been tested for their inhibition of GBA1 and GBA2. Using known trends in the SAR of this class of molecules affecting the enzymes, the hydrophobicity of the amine substitution was increased by dialkylation with alkyl chains of increasing length.

Similar to their previously synthesized monoalkylated derivatives, the dialkylated aminocyclopentitols described here display increasing activity with alkyl chain length, though the dialkylated compounds proved less potent than their monoalkylated counterparts.

One compound, **4.25**, bearing an adamantyl chain was found to be a 14 nM inhibitor of GBA2, while showing only a slight inhibition of CGT (IC_{50} = 1000 μM). When first assayed this compound showed complete inhibition at 1 pM, but a redesigned synthesis allowed for us to obtain compound free of this metal contaminant and reliable assay results were achieved.

Compound **4.25** is the most selective iminosugar for GBA2 vs CGT published to date and could serve as a future tool molecule for studying glucosidase inhibition-derived infertility, a field that is plagued by species-dependent results. Previous studies used a tool compound, *N*B-DNJ (**4.3**), which displays poor selectivity amongst GBA1, GBA2 and CGT.[145] Therefore, molecules that selectively inhibit any one enzyme of the group would be preferred for future studies and these aminocyclopentitols fill a much-needed niche.

Finally, a portion of this project was dedicated to the synthesis and subsequent study of *N*B-DGJ (**4.4**), the galactose epimer of *N*B-DNJ (**4.3**). Compound **4.4** has shown efficacy in species outside the originally studied C57BL/6 mice, the only species in which **4.3** has shown efficacy.[159, 162, 163] We hypothesized that discrepancies in metabolism of these two compounds could be the cause for the differences in efficacy. Therefore, we synthesized both diastereomers of the compound of interest in gram quantity and dosed LE rats orally to observe any differences in the pharmacokinetic properties of **4.4** as compared to published values for **4.3**. Unfortunately, the clearance and $t_{1/2}$ of the two analogs were nearly identical, pointing to other causes for the observed species-dependent infertility results. Further pharmacological work is needed to understand the discrepancies in results between species and the synthesized aminocyclopentitols could serve as useful tool compounds in such studies.

Experimental Data and Procedures

General Synthetic Considerations

All chemicals and reagents were purchased from commercial sources and used directly without further purification. Solvents were dried using standard procedures. All non-aqueous reactions were performed under an atmosphere of nitrogen in flame-dried glassware. Reaction progress was monitored by thin layer chromatography using silica gel plates (silica gel 60 F_{254}) and eluted TLC plates were visualized with UV light (254 nm) and developing the plate with either $KMnO_4$ or $Ce(SO_4)_2$ stain. Reaction products were isolated by silica gel flash column chromatography. NMR experiments were performed on a 400/100 MHz Bruker instrument (unless noted otherwise). NMR spectra were processed through MestReNova 9.0 (Mestrelab Research). Chemical shifts are reported as ppm relative to $CDCl_3$ (7.26 ppm for ^1H, 77.0 ppm for ^{13}C) or CD_3OD (3.33 ppm for ^1H, 49.0 ppm for ^{13}C). ^1H NMR coupling constants (*J*) are expressed in Hz, and multiplicity is described as follows: s = singlet; d = doublet; t = triplet; q = quartet; quin = quintet; sext = sextet; br = broad resonance; m = multiplet. Mass spectrometry experiments were recorded with APCI unless noted otherwise. Melting points, when present, were determined with Electrothermal Digital Mel-Temp 3.0 melting point apparatus and are uncorrected. For compounds centered on the GPHR-00032750 and GPHR-00213869 scaffolds, yields are not reported, as only a small fraction of the compound was collected after chromatography to ensure purity of the to-be-tested compound.

Protocol for the Isolation of Motile Human Sperm

Semen is collected from healthy male donors (IRB: 1102M96152) and allowed to liquify at 37 °C for at least 40 min. Concomitantly, for each mL of semen sample, a canonical tube

containing 5 mL of HAMs-F10 is prepared and allowed to warm to 37 ˚C at a 45 degree angle. After liquefaction, 1 mL of sample is layered beneath the buffer in each tube. The tubes are then left to incubate at 37 °C in a 5% CO_2 environment for 1 h. After this 1 h, the top 2 mL of the buffer is siphoned off and combined among the different tubes. The concentration of this combined population is then determined through use of a hemocytometer and if a concentration of $10x10^6$ cell/mL is not achieved the sample is centrifuged at 400 x *g* for 7 min. Post centrifugation, the supernatant is discarded and the pelleted cells are resuspended in an appropriate buffer, using a volume sufficient to achieve $10x10^6$ cells/mL. Note: it is crucial to ensure no loss of motility is observed due to the centrifugation. It is recommended that additional motility data is obtained post centrifugation to ensure cell health. If an experiment requires capacitation, cells (post swim up and concentration) are suspended in HAM's-F10 containing 5% (*w/v*) BSA and 15 mM $NaHCO_3$ and left to incubate for 3.5 h at 37 ˚C in an atmosphere of 5% CO_2 either in the presence of compound or a DMSO vehicle.

Calcium Influx Assay and the Isolation of Sperm for this Assay

Semen from healthy human donors (IRB: 1102M96152) is collected and incubated in a shaker at 37 ˚C until complete liquefaction is observed (no longer than 1 hr post collection). The sample is diluted to 50 mL in low pH/low K^+ buffer (lo/lo buffer) containing (in mM): 101 NaCl, 4.69 KCl, 0.2 $MgSO_4$, 0.36 KH_2PO_4, 25 $NaHCO_3$, 0.32 sodium pyruvate, 2.78 glucose, 94 sodium lactate, 0.2 $CaCl_2$, pH 6.7 adjusted with 1 M HCl. The sample is then centrifuged at 800 x *g* for 10 min at RT followed by aspiration of the supernatant; this centrifugation is repeated an additional time. Following this second centrifugation, the pellet was resuspended in 10 mL lo/lo buffer containing 10 µg/mL Fluo-4-AM (Life

Technologies, Grand Island, NY) and 1 mM probenicid After, incubation for 30 min at 37 ˚C (caution: light sensitive!), the sample was again diluted to 50 mL with low pH/low K^+ buffer, centrifuged, and the supernatant aspirated to remove extracellular dye. The pellet was then resuspended in ca. 8 mL lo/lo buffer. Dye-loaded sperm were plated into black clear-bottom 384-well assay plates (Corning Inc, Tewksbury, MA) and transferred to the FLIPR® Tetra platform (Molecular Devices, Sunnyvale, CA). The calcium-induced fluorescence signal was continuously monitored for 7 min at 2 s intervals. When tested, blocking compound was added after a 10 s delay and allowed a 2 min binding period prior to opener addition. The signal was monitored for 5 min after opener addition. Opening of the channel was afforded by either 3 μM progesterone in lo/lo buffer or an activation buffer containing (in mM): 10 NaCl, 140 KCl, 0.198 $MgSO_4 \cdot 7\ H_2O$, 0.36 KH_2PO_4, 24.99 $NaHCO_3$, 0.32 sodium pyruvate, 2.78 glucose, 94.08 sodium lactate, $CaCl_2 \cdot 2\ H_2O$.

Sperm Motility Analysis

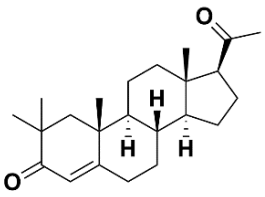
Viable human spermatozoa were selected by swim-up, and a subset of cells was allowed to capacitate at 37°C in normal capacitation media for 3.5 h. Sperm motility was analyzed by computer-assisted semen analysis (CASA, HTM-IVOS sperm analysis system, version 12.3, Hamilton Thorne Biosciences, Beverly, MA) system that measured average path velocity (VAP, μm/s), straight-line velocity (VSL, μm/s,) and curvilinear velocity (VCL, μm/s). From these measurements, we can determine linearity of progression [LIN = (VSL/VCL) × 100], and straightness [STR = (VSL/VAP) × 100]. Data were normalized to vehicle matched controls and presented as the average of (n) individual experiments ± S.E.M. For all experimental conditions, a minimum of 10 fields of view was analyzed containing at least 200 cells total, and all experiments were performed at 37°C.

Data Analysis

Data were analyzed in Prism 8.0.2 (GraphPad Software, Inc, San Diego, CA, USA). Statistical data were calculated and represented everywhere in the text as the mean ± S.E.M or S.D where applicable, and (*n*) indicates the number of repeated experiments. Throughout, statistical significance is indicated by $*P < 0.05$, $**P < 0.005$, $***P < 0.0005$ and $****P < 0.0001$.

Synthesized Steroids

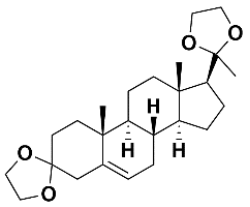
2,2-Dimethylprogesterone (2.4)



Progesterone (500 mg, 1.59 mmol) was dissolved in anhydrous diethylether (50 mL) and chilled to -78 °C followed by dropwise addition of n-butyllithium (1.74 mmol) as a 2.5 M solution in hexanes. The solution was stirred for 30 min followed by dropwise addition of methyliodide (4.77 mmol, 0.296 mL) after which the temperature of the reaction was allowed to raise to room temperature and stirred for 24 hr. A saturated solution of NH_4Cl (5 mL) was used to quench excess alkyllithium reagent. After quenching, the contents of the flask were transferred to a separatory funnel and the organics were extracted with EtOAc (2 x 100 mL). The combined organics were washed with a saturated solution of NaCl (2 x 100 mL) and dried over Na_2SO_4, after which the solvent was removed *in vacuo* to afford an off white, viscous oil as the crude product. Purification by silica gel flash column chromatography (0 to 50% EtOAc in hexanes, followed by a second column at 0 to 20% EtOAc) yielded target compound as a clear oil in 3.6% yield (26% BRSM). ^1H

NMR (400 MHz, $CDCl_3$) δ 5.56 (dd, J_1 = 2.45, J_2 = 5.14, 1H), 2.52 (m, 3H), 2.13 (s, 3H), 1.23 (s, 3H), 0.86 (s, 6H), 0.64 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 216.5, 209.4, 149.8, 119.7, 63.6, 56.9, 48.8, 48.7, 44.0, 38.8, 37.1, 33.7, 32.1, 31.6, 31.5, 31.2, 30.2, 27.3, 24.49, 22.9, 21.3, 19.3, 13.3; HRMS (APCI-TOF) m/z calcd for $C_{23}H_{38}NO_2^+$ $[M + NH_4]^+$ 360.2897, found 360.2903.

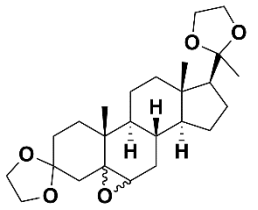
3,3:20,20-bis(Ethylenedioxy)-pregn-5-ene (2.19)



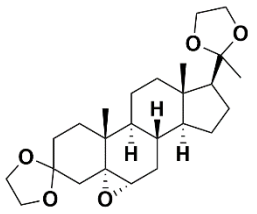
Progesterone (5 g, 15 mmol) was dissolved in toluene (250 mL) containing a catalytic amount of p-toluenesulfonic acid (172 mg, 0.9 mmol). To this mixture was added ethylene glycol (40 mL) followed by attachment of a Dean-Stark trap and heating of the reaction to 145 °C held for 24 h. At this time, the reaction was cooled to room temperature and concentrated under reduced pressure to ca 25% of its original volume and then was transferred to a separatory funnel. A saturated solution of sodium bicarbonate was added to the separatory funnel and the crude product was extracted with EtOAc (3 x 100 mL). The organic layers were then combined, dried with Na_2SO_4 and concentrated under reduced pressure to give crude product as an off-white solid which was then purified silica gel column chromatography (5 to 30% EtOAc in hexanes), affording 4.5 g (86% yield) of the desired bis-ketal as a white solid. mp 166-169 °C; ^1H NMR (400 MHz, $CDCl_3$) δ 5.35 (td, J_1 = 1.95, J_2 = 1.95, J_3 = 4.46, 1H), 4.02-3.83 (m, 8H), 2.56 (ddd, J_1 = 2.89, J_2 = 5.51, J_3 = 14.10, 1H), 2.11 (dd, J_1 = 2.86, J_2 = 14.19j, 1H), 2.06 (dt, J_1 = 3.90, J_2 = 3.90, J_3 = 12.85

1H), 1.96 (dddd, J_1 = 2.96, J_2 = 5.19, J_3 = 5.19, J_4 = 17.51, 1H), 1.30 (s, 3H), 1.03 (s, 3H), 0.78 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 140.2, 122.1, 112.0, 109.5, 65.2, 64.5, 64.24, 63.3, 58.2, 56.6, 49.7, 41.8, 39.4, 36.7, 36.4, 31.7, 31.5, 31.1, 24.6, 23.9, 23.0, 20.9, 18.9, 12.9; HRMS (APCI-TOF) m/z calcd for $C_{25}H_{39}O_4^+$ $[M + H]^+$ 403.2843, found 403.2860.

3,3:20,20-bis(Ethylenedioxy)-5,6-epoxypregnane

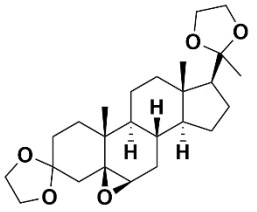


m-Chloroperoxybenzoic acid (*m*CPBA, 1.84 g, 10 mmol, accounting for 77% purity of *m*CPBA), was added at room temperature to a foiled flask containing 1.01 g of **2** (2.5 mmol) and 0.630 g $NaHCO_3$ (7 mmol) dissolved in 200 mL anhydrous DCM. The reaction was stirred for 2 hr after which time 100 mL of sat. $NaHCO_3$ solution was added. The contents were transferred to a separatory funnel where the layers were separated and the organics were washed twice more with 75 mL sat. $NaHCO_3$. The combined organics were washed with brine, dried over Na_2SO_4 and concentrated *in vacuo* to afford crude product as a white solid. Purification by silica gel flash column chromatography (5% to 30% EtOAc in hexanes) gave 837 mg (80% yield) of 5α,6α-epoxide as a white solid, the more polar diastereomer (R_f = 0.15 @ 30% EtOAc) compared to 167 mg (16% yield) of the 5β,6β-epoxide (R_f = 0.30 @ 30% EtOAc in hexanes) which was also a white solid.



3,3:20,20-bis(Ethylenedioxy)-5*α*,6*α*-epoxypregnane (2.20)

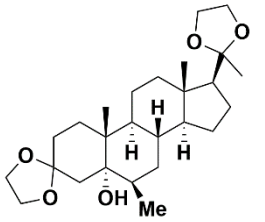
mp 175-178 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.93 (m, 8H), 2.81 (d, J_1 = 4.22, 1H) 2.37 (d, J_1 = 13.99, 1H), 1.99 (dd, J_1 = 3.14, J_2 = 11.62, 1H), 1.92 (ddd, J_1 = 4.59, J_2 = 6.61, J_3 = 15.15, 1H), 1.27 (s, 3H), 1.07 (s, 3H), 0.71 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 112.1, 109.1, 65.3, 64.9, 64.7, 64.2, 63.4, 58.03, 57.6, 56.9, 42.3, 42.0, 39.3, 39.2, 35.2, 31.5, 31.1, 29.5, 28.6, 24.7, 23.7, 23.0, 20.6, 15.8, 13.0; (APCI-TOF) m/z calcd for $C_{25}H_{39}O_5^+$ [M + H]$^+$ 419.2792, found 419.2803.



3,3:20,20-bis(Ethylenedioxy)-5β,6β-epoxypregnane (2.21):

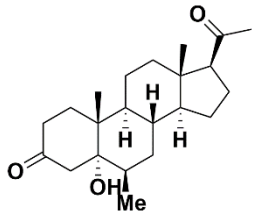
mp 132-134 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.92 (m, 8H), 3.06 (d, J_1 = 1.99, 1H), 2.33 (d, J_1 = 14.02, 1H), 2.04 (ddt, J_1 = 3.18, J_2 = 3.18, J_3 = 14.46, J_4 = 26.30, 2H), 1.28 (s, 3H), 1.00 (s, 3H), 0.74 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 111.9, 108.9, 65.1, 64.7, 64.6, 64.1, 63.2, 57.9, 57.4, 56.7, 42.2, 41.8, 39.1, 39.1, 35.1, 31.3, 31.0, 29.4, 28.4, 24.5, 23.6, 22.8, 20.4, 15.7, 12.9; HRMS (ESI-TOF) m/z calcd for $C_{50}H_{76}NaO_{10}$ [Dimer + Na]$^+$ 859.5331, found 859.5319.

3,3:20,20-bis(Ethylenedioxy)-5α-hydroxy-6β-methylpregnane (2.22)



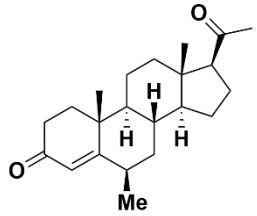
To a solution of 3,3:20,20-bis(ethylenedioxy)-5α,6α-epoxypregnane (71 mg, 0.17 mmol) dissolved in 5 mL THF was added MeMgBr (1.3 mL, 20 equiv, 3.4 mmol) as a 3 M solution in THF. The solution was then brought to reflux for 18 hr, after which it was cooled to room temperature and excess Grignard reagent was quenched with 3 mL of a saturated NH_4Cl solution. Organics were then extracted with EtOAc (3 X 25 mL) and the organics were combined, washed with a saturated NaCl solution and dried over Na_2SO_4, then concentrated *in vacuo* to afford crude product as a clear, viscous oil. Purification by chromatography (0 to 20% EtOAc in hexanes) yielded 33 mg (45%, 75% based on RSM) of **5** as a white powder. mp 187-189; ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.25 (s, 1H), 3.92 (m, 8H), 2.20 (d, J_1 = 14.04, 1H), 1.29 (s, 3H), 1.02 (s, 3H), 0.98 (d, J_1 = 7.8, 3H), 0.77 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 112.02, 110.18, 76.33, 65.19, 64.35, 63.85, 63.19, 58.36, 55.71, 45.63, 42.15, 40.43, 39.66, 39.51, 33.03, 32.56, 31.13, 30.14, 24.57, 23.82, 22.93, 20.90, 18.15, 17.18, 13.19; HRMS (APCI-TOF) m/z calcd for $C_{26}H_{43}O_5^+$ $[M + H]^+$ 435.3105, found 435.3127.

5α-Hydroxy-6β-methylpregnan-3,20-dione (2.23)



To a stirring solution of 3,3:20,20-bis(ethylenedioxy)-5α-hydroxy-6β-methylpregnane (96 mg, 0.221 mmol) in 2 mL THF was added 1 mL 3 M HCl. The reaction was stirred at room temperature for 3 hr, after which it was diluted with 50 mL of EtOAc and neutralized with a saturated solution of $NaHCO_3$. The layers were separated and the organic layer was washed with brine, dried over Na_2SO_4 and then concentrated *in vacuo*. Silica gel flash column chromatography (0 to 40% EtOAc in hexanes) afforded 39 mg (51% yield) of **6** as a white solid. mp 227-229; ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.01 (d, J_1 = 14.7, 1H), 2.55 (t, J_1 = 8.97, 1H), 2.36 (t, J_1 = 6.29, 1H), 2.34 (d, J_1 = 4.78, 1H) 2.13 (s, 3H), 1.25 (s, 3H), 1.09 (d, J_1 = 7.49, 3H), 0.66 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 211.4, 209.5, 80.0, 63.8, 56.0, 50.6, 46.4, 44.3, 42.0, 40.0, 39.0, 38.0, 35.7, 33.4, 31.6, 30.6, 29.7, 24.6, 22.8, 21.4, 17.9, 13.6; HRMS (ESI-TOF) m/z calcd for $C_{44}H_{68}NaO_6$ $[Dimer + Na]^+$ 715.4908, found 715.4861.

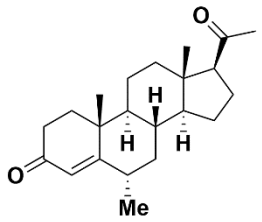
6β-Methylprogesterone (2.24)



Thionyl chloride (310 µL, 0.04 mmol) was added to a stirring solution of 3,3:20,20-bis(ethylenedioxy)-5,6-epoxypregnane (20 mg, 0.058 mmol) in pyridine (1 mL) at 0 °C.

After 5 minutes, 5 mL of ice water was added to the reaction and organics were extracted 2 X 10 mL EtOAc. The combined organics were then washed with brine (20 mL), dried over Na_2SO_4 and concentrated *in vacuo*. Purification by silica gel column chromatography (0 to 30 % EtOAc in hexanes) afforded 11 mg (60% yield) of **7** as a white solid. mp; 171-172; ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.76 (s, 1H), 2.65 (p, J_1 = 7.01, 1H), 2.50 (m, 2H), 2.36 (ddd, J_1 = 2.68, J_2 = 3.84, J_3 = 17.45, 1H), 2.12 (s, 3H), 1.26 (s, 3H), 1.22 (d, J_1 = 7.57, 3H), 0.69 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 209.3, 199.8, 175.3, 125.2, 63.6, 56.1, 53.2, 44.0, 38.7, 38.4, 38.3, 37.7, 37.4, 34.1, 31.5, 30.3, 24.5, 23.1, 22.8, 21.1, 20.4, 13.4; mp; 171-172; HRMS (APCI-TOF) m/z calcd for $C_{22}H_{33}O_2^+$ $[M + H]^+$ 329.2475, found 329.2483.

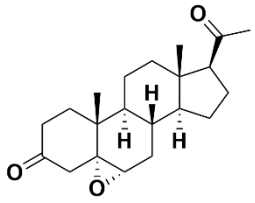
6α-Methylprogesterone (2.18)



After 6β-methylprogesterone (11 mg, 0.033 mmol) was dissolved in ethanol (95%, 1 mL) with sonication, 0.1 mL of 1 M HCl was added and the reaction was stirred under reflux for 2 hr. The reaction was cooled and concentrated under reduced pressure. The resulting slurry was diluted with sodium bicarbonate (10 mL) and extracted with EtOAc (2 X 10 mL). The combined organic layers were washed with brine (15 mL), dried over Na_2SO_4 and concentrated *in vacuo*. Purification by silica gel column chromatography (0 to 30% EtOAc in hexanes) and HPLC (50% to 95% MeCN in H_2O over 20 min) afforded 6α-methylprogesterone (10.5 mg, 95%) as a white powder. mp 116-119 °C; ^{1}H NMR (400

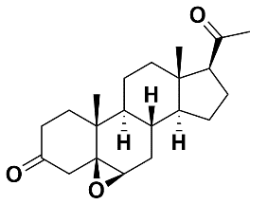
MHz, $CDCl_3$) δ 5.80 (s, 1H), 2.53 (t, J_1 = 9.02, 1H), 2.12 (s, 3H), 1.85 (td, J_1 = 3.95, J_2 = 12.70, 1H), 1.19 (s, 3H), 1.07 (d, J_1 = 6.40, 3H), 0.67 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 209.3, 199.8, 174.1, 121.3, 63.5, 55.9, 53.9, 44.0, 40.9, 38.9, 38.7, 36.0, 35.4, 33.9, 33.7, 31.5, 24.3, 22.9, 21.2, 18.4, 18.3, 13.4; HRMS (APCI-TOF) m/z calcd for $C_{22}H_{33}O_2^+$ $[M + H]^+$ 329.2475, found 329.2484.

5α,6α-Epoxypregnan-3,20-dione (2.25)



To a stirring solution of 3,3:20,20-bis(ethylenedioxy)-pregn-5-ene (alpha isomer, 27 mg, 0.065 mmol) in 1 mL THF was added 0.7 mL 3 M HCl. The reaction was stirred at room temperature for 3 hr, after which it was diluted with 40 mL of EtOAc and neutralized with a saturated solution of $NaHCO_3$. The layers were separated and the organic layer was washed with brine, dried over Na_2SO_4 and then concentrated *in vacuo*. Silica gel column chromatography (0 to 40% EtOAc in hexanes) afforded deprotected product as a white powder in 70% yield. mp 193-195 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.82 (m, 1H), 3.37 (d, J_1 = 15.49, 1H), 2.54 (t, J_1 = 8.94, 1H), 2.13 (s, 3H), 1.45 (s, 3H), 0.69 (s, 3H); ^{13}C NMR (100 MHz, CDCl3) δ 210.5, 209.3, 78.7, 63.6, 63.4, 55.4, 50.7, 45.6, 44.2, 39.2, 38.8, 37.8, 35.3, 34.8, 31.5, 30.1, 24.4, 22.8, 21.3, 17.8, 13.5; HRMS (APCI-TOF) m/z calcd for $C_{21}H_{34}NO_3^+$ $[M + NH_4]^+$ 348.2533, found 348.2534.

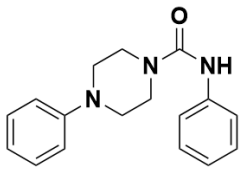
5β,6β-Epoxypregnan-3,20-dione (2.26)



To a stirring solution of 3,3:20,20-bis(ethylenedioxy)-pregn-5-ene (beta-isomer, 16.8 mg, 0.04 mmol) in 1 mL THF was added 0.7 mL 3 M HCl. The reaction was stirred at room temperature for 3 hr, after which it was diluted with 40 mL of EtOAc and neutralized with a saturated solution of $NaHCO_3$. The layers were separated and the organic layer was washed with brine, dried over Na_2SO_4 and then concentrated *in vacuo*. Silica gel column chromatography (0 to 40% EtOAc in hexanes) afforded 7.3 mg of deprotected product as a white powder in 58% yield. mp 154-152 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.18 (s, 3H), 0.96 (s, 3H), 0.65 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 211.08, 208.99, 208.52, 63.30, 57.49, 56.61, 53.30, 46.38, 44.34, 41.14, 38.38, 38.08, 37.79, 37.35, 36.96, 31.48, 24.18, 22.78, 21.62, 13.41, 12.60; HRMS (CI-TOF) m/z calcd for $C_{21}H_{34}NO_3^+$ $[M + NH_4]^+$ 348.2533, found 348.2538.

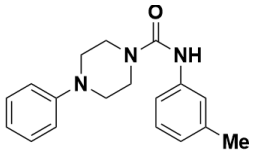
GPHR-00032750 Analogs

N,4-Diphenylpiperazine-1-carboxamide (3.10)



To a stirring solution of 1-phenylpiperazine (30 mg, 0.18 mmol, 1.0 equiv), triethylamine (1.9 µL, 0.018 mmol, 0.1 equiv) in DCM (5 mL) was added isocyanatobenzene (27 mg, 0.21 mmol, 1.2 equiv) and the solution was stirred for 30 min at RT. After confirming consumption of limiting reagent by TLC, 3 mL of distilled water was added to the reaction vial. The organic layer was separated and removed under reduced atmosphere. Column chromatography (0 to 50% EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for$C_{17}H_{20}N_3O^+$ [M+H$^+$], 282.3665 found 284. 1H NMR (400 MHz, CD_3OD) δ 7.37 (d, *J* = 8.6 Hz, 2H), 7.30 - 7.23 (m, 4H), 7.06 - 7.00 (m, 3H), 6.88 (t, *J* = 7.3 Hz, 1H), 3.74 - 3.66 (m, 4H), 3.25 - 3.18 (m, 4H).

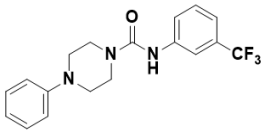
4-Phenyl-N-(m-tolyl)piperazine-1-carboxamide (3.11)



To a stirring solution of 1-phenylpiperazine (30 mg, 0.18 mmol, 1.0 equiv), triethylamine (1.9 µL, 0.018 mmol, 0.1 equiv) in DCM (5 mL) was added 1-isocyanato-3-methylbenzene (29 mg, 0.21 mmol, 1.2 equiv) and the solution was stirred for 30 min at RT. After confirming consumption of limiting reagent by TLC, 3 mL of distilled water was added to

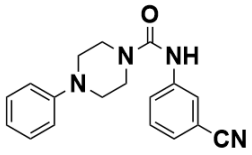
the reaction vial. The organic layer was separated and removed under reduced atmosphere. Column chromatography (0 to 50% EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd $C_{18}H_{22}N_3O^+$ [M+H$^+$], 296.3935 found 297. 1H NMR (400 MHz, CD_3OD) δ 7.26 (t, *J* = 7.4 Hz, 2H), 7.21 (s, 1H), 7.19 - 7.12 (m, 2H), 7.02 (d, *J* = 7.6 Hz, 2H), 6.92 - 6.84 (m, 2H), 3.72 - 3.65 (m, 4H), 3.23 - 3.17 (m, 4H), 2.32 (s, 3H).

4-Phenyl-N-(3-(trifluoromethyl)phenyl)piperazine-1-carboxamide (3.12)



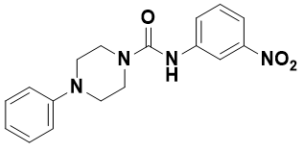
To a stirring solution of 1-phenylpiperazine (20 mg, 0.13 mmol, 1.0 equiv), triethylamine (1.8 µL, 0.013 mmol, 0.1 equiv) in DCM (5 mL) was added 1-isocyanato-3-(trifluoromethyl)benzene (27 mg, 0.16 mmol, 1.2 equiv) and the solution was stirred for 30 min at RT. After confirming consumption of limiting reagent by TLC, 3 mL of distilled water was added to the reaction vial. The organic layer was separated and removed under reduced atmosphere. Column chromatography (0 to 50% EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{18}H_{19}F_3N_3O^+$ [M+H$^+$], 350.36 found 360. 1H NMR (400 MHz, $CDCl_3$) δ 7.68 (s, 1H), 7.60 (d, *J* = 8.1 Hz, 1H), 7.41 (t, *J* = 7.9 Hz, 1H), 7.35 - 7.24 (m, 3H), 7.01 - 6.91 (m, 3H), 6.59 (br, 1H), 3.75 - 3.68 (m, 4H), 3.30 - 3.23 (m, 4H).

N-(3-Cyanophenyl)-4-phenylpiperazine-1-carboxamide (3.13)



To a stirring solution of 1-phenylpiperazine (30 mg, 0.18 mmol, 1.0 equiv), triethylamine (1.9 µL, 0.018 mmol, 0.1 equiv) in DCM (5 mL) was added 3-isocyanatobenzonitrile (32 mg, 0.21 mmol, 1.2 equiv) and the solution was stirred for 30 min at RT. After confirming consumption of limiting reagent by TLC, 3 mL of distilled water was added to the reaction vial. The organic layer was separated and removed under reduced atmosphere. Column chromatography (0 to 50% EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{18}H_{19}N_4O^+$ [M+H$^+$], 307.3765 found 307. 1H NMR (400 MHz, CD_3OD) δ 7.85 (s, 1H), 7.69 (d, *J* = 7.4 Hz, 1H), 7.45 (t, *J* = 7.9 Hz, 1H), 7.36 (d, *J* = 7.6 Hz, 1H), 7.27 (t, *J* = 7.5 Hz, 2H), 7.02 (d, *J* = 8.2 Hz, 1H), 6.68 (t, *J* = 7.3 Hz, 1H), 3.77 - 3.64 (m, 4H), 3.27 - 3.17 (m, 4H).

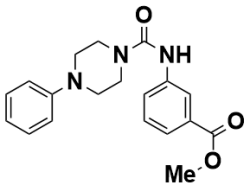
N-(3-Nitrophenyl)-4-phenylpiperazine-1-carboxamide (3.14)



To a stirring solution of 1-phenylpiperazine (20 mg, 0.12 mmol, 1.0 equiv), triethylamine (1.8 µL, 0.013 mmol, 0.1 equiv) in DCM (5 mL) was added 1-isocyanato-3-nitrobenzene (24 mg, 0.16 mmol, 1.2 equiv) and the solution was stirred for 30 min at RT. After confirming consumption of limiting reagent by TLC, 3 mL of distilled water was added to the reaction vial. The organic layer was separated and removed under reduced atmosphere.

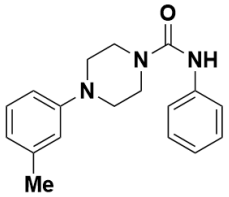
Column chromatography (0 to 50% EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{17}H_{19}N_4O_3^+$ [M+H$^+$], 327.36 found 328. 1H NMR (400 MHz, $CDCl_3$) δ 8.24 (t, J = 2.2 Hz, 1H), 7.89 (dd, J_1 = 2.1, J_2 = 8.1 Hz, 1H), 7.84 (dd, J_1 = 2.1, J_2 = 8.2 Hz, 1H), 7.45 (t, J = 8.2 Hz, 1H), 7.31 (t, J = 7.2 Hz, 2H), 7.01 - 6.91 (m, 3H), 6.77 (br, 1H), 3.77 - 3.70 (m, 4H), 3.31 - 3.25 (m, 4H).

Methyl 3-(4-phenylpiperazine-1-carboxamido)benzoate (3.15)



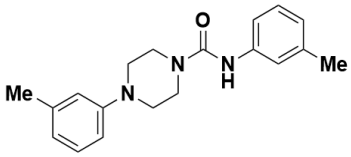
To a stirring solution of 1-phenylpiperazine (30 mg, 0.18 mmol, 1.0 equiv), triethylamine (1.9 µL, 0.018 mmol, 0.1 equiv) in DCM (5 mL) was added methyl 3-isocyanatobenzoate (39 mg, 0.21 mmol, 1.2 equiv) and the solution was stirred for 30 min at RT. After confirming consumption of limiting reagent by TLC, 3 mL of distilled water was added to the reaction vial. The organic layer was separated and removed under reduced atmosphere. Column chromatography (0 to 50% EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{19}H_{22}N_3O_3^+$ [M+H$^+$], 340.4025 found 340. 1H NMR (400 MHz, CD_3OD) δ 8.09 (s, 1H), 7.74 - 7.63 (m, 3H), 7.45 - 7.36 (m, 1H), 7.27 (t, J = 7.3 Hz, 1H), 7.02 (d, J = 7.2 Hz, 1H), 6.87 (t, J = 7.0 Hz, 1H), 3.93 - 3.87 (m, 5H), 3.74 - 3.68 (m, 4H), 3.24 - 3.17 (m, 4H).

N-Phenyl-4-(m-tolyl)piperazine-1-carboxamide (3.16)



To a stirring solution of 1-(m-tolyl)piperazine (30 mg, 0.14 mmol, 1.0 equiv), triethylamine (1.7 µL, 0.14mmol, 0.1 equiv) in DCM (5 mL) was added isocyanatobenzene (25 mg, 0.17 mmol, 1.2 equiv) and the solution was stirred for 30 min at RT. After confirming consumption of limiting reagent by TLC, 3 mL of distilled water was added to the reaction vial. The organic layer was separated and removed under reduced atmosphere. Column chromatography (0 to 50% EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd $C_{18}H_{22}N_3O^+$ [M+H$^+$], 296.3935 found 297. 1H NMR (400 MHz, CD_3OD) δ 7.37 (d, *J* = 8.6 Hz, 2H), 7.28 (t, *J* = 7.7 Hz, 2H), 7.14 (t, *J* = 7.8 Hz, 1H), 7.03 (t, *J* = 7.2 Hz, 1H), 6.86 - 6.79 (m, 2H), 6.72 (d, *J* = 7.4 Hz, 1H), 3.72 - 3.65 (m, 4H), 3.22 - 3.15 (m, 4H), 2.31 (s, 3H).

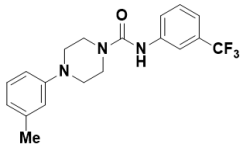
N,4-di-m-Tolylpiperazine-1-carboxamide (3.17)



To a stirring solution of 1-(m-tolyl)piperazine (30 mg, 0.15 mmol, 1.0 equiv), triethylamine (1.8 µL, 0.015 mmol, 0.1 equiv) in DCM (5 mL) was added 1-isocyanato-3-methylbenzene (27 mg, 0.18 mmol, 1.2 equiv) and the solution was stirred for 30 min at RT. After confirming consumption of limiting reagent by TLC, 3 mL of distilled water was added to

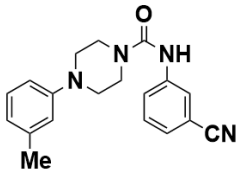
the reaction vial. The organic layer was separated and removed under reduced atmosphere. Column chromatography (0 to 50% EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd $C_{19}H_{24}N_3O^+$ [M+H$^+$], 310.4205 found 310. 1H NMR (400 MHz, CD_3OD) δ 7.21 (s, 1H), 7.18 - 7.11 (m, 3H), 6.88 - 6.83 (m, 2H), 6.80 (dd, J_1 = 2.4, J_2 = 8.1 Hz, 1H), 6.72 (d, J = 7.4 Hz, 1H), 3.72 - 3.64 (m, 4H), 3.21 - 3.13 (m, 4H), 2.31 (s, 6H).

4-(m-Tolyl)-N-(3-(trifluoromethyl)phenyl)piperazine-1-carboxamide (3.18)



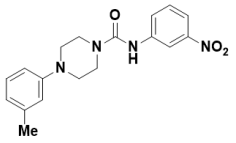
To a stirring solution of 1-(m-tolyl)piperazine (20 mg, 0.11 mmol, 1.0 equiv), triethylamine (1.8 µL, 0.011 mmol, 0.1 equiv) in DCM (5 mL) was added 1-isocyanato-3-(trifluoromethyl)benzene (25 mg, 0.14 mmol, 1.2 equiv) and the solution was stirred for 30 min at RT. After confirming consumption of limiting reagent by TLC, 3 mL of distilled water was added to the reaction vial. The organic layer was separated and removed under reduced atmosphere. Column chromatography (0 to 50% EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{19}H_{21}F_3N_3O^+$ [M+H$^+$], 364.39 found 365. 1H NMR (400 MHz, $CDCl_3$) δ 7.68 (s, 1H), 7.59 (d, J = 8.2 Hz, 1H), 7.41 (t, J = 7.9 Hz, 1H), 7.30 (d, J = 8.1 Hz, 1H), 7.20 (t, J = 7.8 Hz, 1H), 6.84 - 6.73 (m, 3H), 3.71 (br, 4H), 3.29 - 3.23 (m, 4H), 2.34 (s, 3H). 13C NMR (176 MHz, CDCl3) δ 154.6, 139.5, 139.2, 131.2 (q, J = 33 Hz), 129.4, 129.2, 123.9 (q, J = 272 Hz), 123.0, 119.8 (d, J = 4 Hz), 117.7, 116.6 (d, J = 4 Hz), 113.9, 49.6, 44.0, 21.8.

N-(3-Cyanophenyl)-4-(m-tolyl)piperazine-1-carboxamide (3.19)



To a stirring solution of 1-(m-tolyl)piperazine (30 mg, 0.17 mmol, 1.0 equiv), triethylamine (1.8 µL, 0.017 mmol, 0.1 equiv) in DCM (5 mL) was added 3-isocyanatobenzonitrile (29 mg, 0.20 mmol, 1.2 equiv) and the solution was stirred for 30 min at RT. After confirming consumption of limiting reagent by TLC, 3 mL of distilled water was added to the reaction vial. The organic layer was separated and removed under reduced atmosphere. Column chromatography (0 to 50% EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{19}H_{21}N_4O^+$ [M+H$^+$], 321.4035 found 322. 1H NMR (400 MHz, CD_3OD) δ 7.84 (s, 1H), 7.68 (d, *J* = 8.4 Hz, 1H), 7.45 (t, *J* = 7.8 Hz, 1H), 7.35 (d, *J* = 6.4 Hz, 1H), 7.14 (t, *J* = 7.8 Hz, 1H), 6.85 (s, 1H), 6.81 (d, *J* = 8.1 Hz, 1H), 6.72 (d, *J* = 7.5 Hz, 1H), 3.73 - 3.67 (m, 4H), 3.24 - 3.15 (m, 4H), 2.31 (s, 3H).

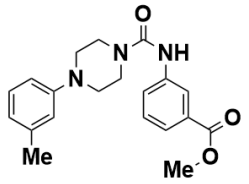
N-(3-Nitrophenyl)-4-(m-tolyl)piperazine-1-carboxamide (3.20)



To a stirring solution of 1-(m-tolyl)piperazine (20 mg, 0.11 mmol, 1.0 equiv), triethylamine (1.8 µL, 0.011 mmol, 0.1 equiv) in DCM (5 mL) was added 1-isocyanato-3-nitrobenzene (22 mg, 0.16 mmol, 1.2 equiv) and the solution was stirred for 30 min at RT. After

confirming consumption of limiting reagent by TLC, 3 mL of distilled water was added to the reaction vial. The organic layer was separated and removed under reduced atmosphere. Column chromatography (0 to 50% EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{18}H_{21}N_4O_3^+$ [M+H$^+$], 341.39 found 342. 1H NMR (400 MHz, $CDCl_3$) δ 8.24 (t, J = 2.2 Hz, 1H), 7.89 (dd, J_1 = 2.2, J_2 = 8.2 Hz, 1H), 7.84 (dd, J_1 = 2.2, J_2 = 8.2 Hz, 1H), 7.45 (t, J = 8.2 Hz, 1H), 7.20 (t, J = 7.7 Hz, 1H), 6.85 - 6.74 (m, 3H), 3.74 (m, 4H), 3.30 - 3.24 (m, 4H), 2.34 (s, 3H).

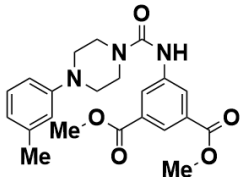
Methyl 3-(4-(m-tolyl)piperazine-1-carboxamido)benzoate (3.21)



To a stirring solution of 1-(m-tolyl)piperazine (30 mg, 0.17 mmol, 1.0 equiv), triethylamine (1.8 µL, 0.013 mmol, 0.1 equiv) in DCM (5 mL) was added methyl 3-isocyanatobenzoate (36 mg, 0.20 mmol, 1.2 equiv) and the solution was stirred for 30 min at RT. After confirming consumption of limiting reagent by TLC, 3 mL of distilled water was added to the reaction vial. The organic layer was separated and removed under reduced atmosphere. Column chromatography (0 to 50% EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{20}H_{24}N_3O_3^+$ [M+H$^+$], 354.4295 found 354. 1H NMR (400 MHz, CD_3OD) δ 8.09 (s, 1H), 7.75 - 7.62 (m, 3H), 7.45 - 7.35 (m, 1H), 7.14 (t, J = 7.8 Hz, 1H),

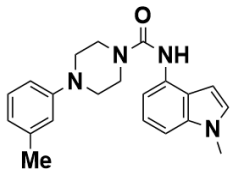
6.85 (s, 1H), 6.81 (d, *J* = 8.2 Hz, 1H), 6.72 (d, *J* = 7.4 Hz, 1H), 3.91 (s, 3H), 3.75 - 3.65 (m, 4H), 3.23 - 3.16 (m, 4H), 2.32 (s, 3H).

Dimethyl 5-(4-(m-tolyl)piperazine-1-carboxamido)isophthalate (3.22)



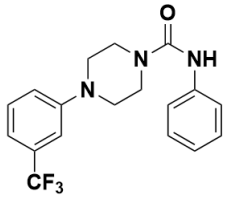
To a stirring solution of 1-(m-tolyl)piperazine (30 mg, 0.17 mmol, 1.0 equiv), triethylamine (1.8 µL, 0.017 mmol, 0.1 equiv) in DCM (5 mL) was added dimethyl 5-isocyanatoisophthalate (48 mg, 0.20 mmol, 1.2 equiv) and the solution was stirred for 30 min at RT. After confirming consumption of limiting reagent by TLC, 3 mL of distilled water was added to the reaction vial. The organic layer was separated and removed under reduced atmosphere. Column chromatography (0 to 50% EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{22}H_{26}N_3O_5^+$ [M+H$^+$], 412.4655 found 412. 1H NMR (400 MHz, CD_3OD) δ 8.34 (s, 2H), 8.28 (s, 1H), 7.14 (t, *J* = 7.8 Hz, 1H), 6.85 (s, 1H), 6.81 (d, *J* = 8.2 Hz, 1H), 6.72 (d, *J* = 7.4 Hz, 1H), 3.94 (s, 6H), 3.75 - 3.68 (m, 4H), 3.23 - 3.16 (m, 4H), 2.32 (s, 3H).

N-(1-Methyl-1H-indol-4-yl)-4-(m-tolyl)piperazine-1-carboxamide (3.23)



To a stirring solution of 1-(m-tolyl)piperazine (30 mg, 0.17 mmol, 1.0 equiv), triethylamine (1.8 µL, 0.013 mmol, 0.1 equiv) in DCM (5 mL) was added 4-isocyanato-1-methyl-1H-indole (35 mg, 0.20 mmol, 1.2 equiv) and the solution was stirred for 30 min at RT. After confirming consumption of limiting reagent by TLC, 3 mL of distilled water was added to the reaction vial. The organic layer was separated and removed under reduced atmosphere. Column chromatography (0 to 50% EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{18}H_{19}N_4O^+$ [M+H$^+$], 307.3765 found 307. 1H NMR (400 MHz, CD_3OD) δ 7.22 - 7.10 (m, 4H), 7.06 (d, *J* = 7.4 Hz, 1H), 6.86 (s, 1H), 6.83 (d, *J* = 8.0 Hz, 1H), 6.72 (d, *J* = 7.5 Hz, 1H), 6.47 (d, *J* = 3.1 Hz, 1H), 3.80 (s, 3H), 3.77 - 3.69 (m, 4H), 3.30 - 3.18 (m,4 H), 2.32 (s, 3H).

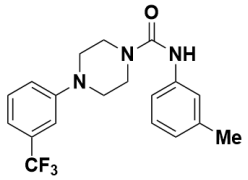
N-Phenyl-4-(3-(trifluoromethyl)phenyl)piperazine-1-carboxamide (3.24)



To a stirring solution of 1-(3-(trifluoromethyl)phenyl)piperazine (30 mg, 0.12 mmol, 1.0 equiv), triethylamine (1.8 µL, 0.012 mmol, 0.1 equiv) in DCM (5 mL) was added isocyanatobenzene (19 mg, 0.15 mmol, 1.2 equiv) and the solution was stirred for 30 min at RT. After confirming consumption of limiting reagent by TLC, 3 mL of distilled water was added to the reaction vial. The organic layer was separated and removed under reduced atmosphere. Column chromatography (0 to 50% EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization.

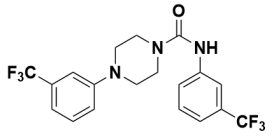
LRMS (APCI - quad) m/z cal'd $C_{18}H_{19}F_3N_3O^+$ [M+H$^+$], 350.3647 found 351. 1H NMR (400 MHz, CD_3OD) δ 7.42 (t, *J* = 7.8 Hz, 1H), 7.40 - 7.36 (m, 2H), 7.31 - 7.25 (m, 2H), 7.25 - 7.20 (m, 2H), 7.12 (d, *J* = 7.7 Hz, 1H), 7.03 (t, *J* = 6.4 Hz, 1H), 3.74 - 3.67 (m, 4H), 3.34 - 3.26 (m, 4H).

N-(m-Tolyl)-4-(3-(trifluoromethyl)phenyl)piperazine-1-carboxamide (3.25)



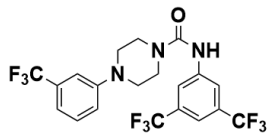
To a stirring solution of 1-(m-tolyl)piperazine (30 mg, 0.11 mmol, 1.0 equiv), triethylamine (1.6 µL, 0.011 mmol, 0.1 equiv) in DCM (5 mL) was added 1-isocyanato-3-methylbenzene (27 mg, 0.14 mmol, 1.2 equiv) and the solution was stirred for 30 min at RT. After confirming consumption of limiting reagent by TLC, 3 mL of distilled water was added to the reaction vial. The organic layer was separated and removed under reduced atmosphere. Column chromatography (0 to 50% EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd $C_{19}H_{21}F_3N_3O^+$ [M+H$^+$], 364.3917 found 365. 1H NMR (400 MHz, CD_3OD) δ 7.43 (t, *J* = 7.9 Hz, 1H), 7.25 - 7.09 (m, 6H), 6.87 (d, *J* = 6.2 Hz, 1H), 3.73 - 3.68 (m, 4H), 3.34 - 3.25 (m, 4H), 2.32 (s, 3H). 13C NMR (176 MHz, CDCl3) δ 155.0, 151.0, 138.9, 138.6, 131.6 (q, *J* = 31 Hz), 129.7, 128.7, 124.3, 124.0 (d, *J* = 272 Hz), 120.8, 119.1, 117.1, 116.5 (d, *J* = 4 Hz), 112.5 (d, *J* = 4 Hz), 48.6, 43.8, 21.5.

N,4-bis(3-(Trifluoromethyl)phenyl)piperazine-1-carboxamide (3.9).



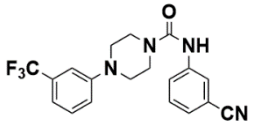
To a stirring solution of 1-(3-triflouromethylphenyl)piperazine (30 mg, 0.13 mmol, 1.0 equiv), triethylamine (1.8 µL, 0.013 mmol, 0.1 equiv) in DCM (5 mL) was added 3-trifluoromethylphenylisocyanate (29 mg, 0.15 mmol, 1.2 equiv) and the solution was stirred for 30 min at RT. After confirming consumption of limiting reagent by TLC, 3 mL of distilled water was added to the reaction vial. The organic layer was separated and removed under reduced atmosphere. Column chromatography (0 to 50% EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{19}H_{18}F_6N_3O^+$ [M+H$^+$] 418.36, found 418. 1H NMR (400 MHz, CDCl3) δ 7.66 (s, 1H), 7.58 (d, *J* = 8.2 Hz, 1H), 7.39 (q, *J* = 7.2 Hz, 2H), 7.30 (d, *J* = 7.8 Hz, 1H), 7.14 (d, *J* = 7.8 Hz, 1H), 7.12 (s, 1H), 7.07 (d, *J* = 8.4 Hz, 1H), 6.69 (s, 1H), 3.69 (t, *J* = 13.1 Hz, 4H), 3.29 (t, *J* = 5.2 Hz, 4H). 13C NMR (176 MHz, CDCl3) δ 160.4, 154.9 (d, *J* = 11 Hz), 154.8 (d, *J* = 11 Hz), 150.9, 139.5 (d, *J* = 9 Hz), 131.6 (d, *J* = 29 Hz), 131.2 (d, *J* = 36 Hz), 129.8, 129.4, 124.7 (d, *J* = 46 Hz), 123.5, 123.2 (d, *J* = 27 Hz), 119.8, 119.2, 117.0, 116.6, 112.6, 48.6, 43.8.

N-(3,5-Bis(trifluoromethyl)phenyl)-4-(3-(trifluoromethyl)phenyl)piperazine-1-carboxamide (3.26)



To a stirring solution of 1-(3-(trifluoromethyl)phenyl)piperazine (30 mg, 0.13 mmol, 1.0 equiv), triethylamine (1.8 µL, 0.013 mmol, 0.1 equiv) in DCM (5 mL) was added dimethyl 1-isocyanato-3,5-bis(trifluoromethyl)benzene (40 mg, 0.16 mmol, 1.2 equiv) and the solution was stirred for 30 min at RT. After confirming consumption of limiting reagent by TLC, 3 mL of distilled water was added to the reaction vial. The organic layer was separated and removed under reduced atmosphere. Column chromatography (0 to 50% EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{20}H_{17}F_9N_3O^+$ [$M+H^+$], 486.3611 found 486. 1H NMR (400 MHz, CD_3OD) δ 8.10 (s, 2H), 7.55 (s, 1H), 7.44 (t, *J* = 7.9 Hz, 1H), 7.26 - 7.22 (m, 2H), 7.13 (d, *J* = 7.6 Hz, 1H), 3.81 - 3.69 (m, 4H), 3.38 - 3.30 (m, 4H). 13C NMR (101 MHz, CDCl3) δ 153.9, 150.8, 140.3, 132.2 (q, *J* = 34 Hz), 131.7 (q, *J* = 32 Hz), 129.8, 123.9 (d, *J* = 272 Hz), 123.1 (d, *J* = 273 Hz), 119.4, 119.3, 116.9 (d, *J* = 4 Hz), 116.5, 112.8 (d, *J* = 4 Hz), 48.7, 43.9.

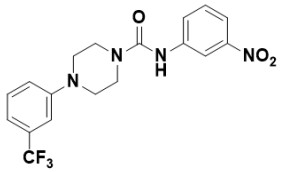
N-(3-Cyanophenyl)-4-(3-(trifluoromethyl)phenyl)piperazine-1-carboxamide (3.27)



To a stirring solution of 1-(3-(trifluoromethyl)phenyl)piperazine (30 mg, 0.13 mmol, 1.0 equiv), triethylamine (1.8 µL, 0.013 mmol, 0.1 equiv) in DCM (5 mL) was added 3-isocyanatobenzonitrile (22 mg, 0.16 mmol, 1.2 equiv) and the solution was stirred for 30 min at RT. After confirming consumption of limiting reagent by TLC, 3 mL of distilled water was added to the reaction vial. The organic layer was separated and removed under reduced atmosphere. Column chromatography (0 to 50% EtOAc/Hex) on silica gel was

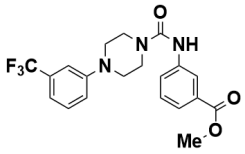
used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{19}H_{18}F_3N_4O^+$ [M+H$^+$], 375.3747 found 375. 1H NMR (400 MHz, CD_3OD) δ 7.85 (s, 1H), 7.69 (d, *J* = 9.6 Hz, 1H), 7.48 - 7.40 (m, 2H), 7.35 (d, *J* = 7.7 Hz, 1H), 7.26 - 7.20 (m, 2H), 7.12 (d, *J* = 7.7 Hz, 1H), 3.77 - 3.70 (m, 4H), 3.36 - 3.30 (m, 4H).

N-(3-Nitrophenyl)-4-(m-tolyl)piperazine-1-carboxamide (3.28)



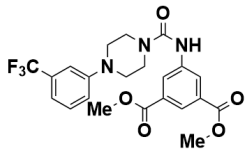
To a stirring solution of 1-(3-(trifluoromethyl)phenyl)piperazine (20 mg, 0.09 mmol, 1.0 equiv), triethylamine (1.8 µL, 0.009 mmol, 0.1 equiv) in DCM (5 mL) was added 1-isocyanato-3-nitrobenzene (16 mg, 0.12 mmol, 1.2 equiv) and the solution was stirred for 30 min at RT. After confirming consumption of limiting reagent by TLC, 3 mL of distilled water was added to the reaction vial. The organic layer was separated and removed under reduced atmosphere. Column chromatography (0 to 50% EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{18}H_{21}N_4O_3^+$ [M+H$^+$], 341.39 found 342. 1H NMR (400 MHz, $CDCl_3$) δ 8.23 (t, *J* = 2.2 Hz, 1H), 7.89 (dd, J_1 = 2.1, J_2 = 8.1 Hz, 1H), 7.84 (dd, J_1 = 2.1, J_2 = 8.1 Hz, 1H), 7.46 (t, *J* = 8.1 Hz, 1H), 7.40 (t, *J* = 8.0 Hz, 1H), 7.19 - 7.14 (m, 2H), 7.11 (d, *J* = 8.4 Hz, 1H), 6.79 (br, 1H), 3.79 - 3.70 (m, 4H), 3.37 - 3.30 (m, 4H).

Methyl 3-(4-(3-(trifluoromethyl)phenyl)piperazine-1-carboxamido)benzoate (3.29)



To a stirring solution of 1-(3-(trifluoromethyl)phenyl)piperazine (30 mg, 0. 13 mmol, 1.0 equiv), triethylamine (1.8 µL, 0.013 mmol, 0.1 equiv) in DCM (5 mL) was added methyl 3-isocyanatobenzoate (28 mg, 0.16 mmol, 1.2 equiv) and the solution was stirred for 30 min at RT. After confirming consumption of limiting reagent by TLC, 3 mL of distilled water was added to the reaction vial. The organic layer was separated and removed under reduced atmosphere. Column chromatography (0 to 50% EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{20}H_{21}F_3N_3O_3^+$ [M+H$^+$], 408.4007 found 408. 1H NMR (400 MHz, CD_3OD) δ 8.14 (s, 1H), 7.75 - 7.67 (m, 2H), 7.49 - 7.40 (m, 2H), 7.29 - 7.24 (m, 2H), 7.16 (d, *J* = 7.6 Hz, 1H), 3.94 (s, 3H), 3.81 - 3.73 (m, 4H), 3.39 - 3.30 (m, 4H).

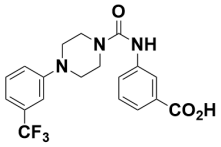
Dimethyl 5-(4-(3-(trifluoromethyl)phenyl)piperazine-1-carboxamido)isophthalate (3.30)



To a stirring solution of 1-(3-(trifluoromethyl)phenyl)piperazine (30 mg, 0.13 mmol, 1.0 equiv), triethylamine (1.8 µL, 0.013 mmol, 0.1 equiv) in DCM (5 mL) was added dimethyl 5-isocyanatoisophthalate (36 mg, 0.16 mmol, 1.2 equiv) and the solution was stirred for 30 min at RT. After confirming consumption of limiting reagent by TLC, 3 mL of distilled

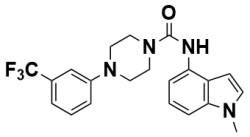
water was added to the reaction vial. The organic layer was separated and removed under reduced atmosphere. Column chromatography (0 to 50% EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{22}H_{23}F_3N_3O_5^+$ [M+H$^+$], 466.4367 found 466. 1H NMR (400 MHz, CD_3OD) δ 8.44 - 8.18 (m, 2H), 7.45 (s, 1H), 7.34 - 7.04 (m, 2H), 4.13 (br, 2H), 4.03 - 3.63 (m, 7H).

3-(4-(3-(Trifluoromethyl)phenyl)piperazine-1-carboxamido)benzoic Acid (3.31)



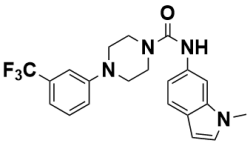
A solution of methyl 3-(4-(3-(trifluoromethyl)phenyl)piperazine-1-carboxamido)benzoate (EJC-3-136, 483 mg, 1.18 mmol, 1.0 equiv) was dissolved in a H_2O/THF cosolvent system (1:5, H_2O to THF) at rt. To the reaction was added LiOH·H_2O (442 mg, 5.88 mmol, 5 equiv) and the reaction was left to stir overnight. After confirming consumption of starting material by TLC, the THF was removed under reduced pressure and the resulting slurry was taken up in EtOAc and washed several times (2x25 mL) with 0.1 N HCl. The organic layer was separated and removed under reduced atmosphere. Column chromatography (0 to 100% EtOAc/Hex) resulted in target molecule as a white powder in moderate yield (202 mg, 47%). LRMS (APCI - quad) m/z cal'd $C_{19}H_{19}F_3N_3O_3^+$ [M+H$^+$], 394.3737 found 395. 1H NMR (400 MHz, $CDCl_3$) δ 7.97 (t, J = 2.0 Hz, 1H), 7.59 (td, J_1 = 1.3, J_2 = 7.7 Hz, 1H), 7.55 (ddd, J_1 = 1.0, 2.3, 8.2 Hz, 1H), 7.33 (t, J = 7.9 Hz, 1H), 7.28 (t, J = 7.9 Hz, 1H), 7.16 - 7.10 (m, 2H), 7.01 (d, J = 7.6 Hz, 1H), 3.66 - 3.59 (m, 4H), 3.27 - 3.17 (m, 4H).

N-(1-Methyl-1H-indol-4-yl)-4-(3-(trifluoromethyl)phenyl)piperazine-1-carboxamide (3.32)



To a stirring solution of 1-(3-(trifluoromethyl)phenyl)piperazine (30 mg, 0.13 mmol, 1.0 equiv), triethylamine (1.8 µL, 0.013 mmol, 0.1 equiv) in DCM (5 mL) was added 4-isocyanato-1-methyl-1H-indole (27 mg, 016 mmol, 1.2 equiv) and the solution was stirred for 30 min at RT. After confirming consumption of limiting reagent by TLC, 3 mL of distilled water was added to the reaction vial. The organic layer was separated and removed under reduced atmosphere. Column chromatography (0 to 50% EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{21}H_{22}F_3N_4O^+$ [M+H$^+$], 403.4287 found 404. 1H NMR (400 MHz, CD_3OD) δ 7.33 (t, *J* = 7.9 Hz, 1H), 7.18 - 7.11 (m, 2H), 7.10 - 7.04 (m, 2H), 7.03 - 7.00 (m, 2H), 6.96 (d, *J* = 7.3 Hz, 1H), 6.36 (d, *J* = 3.2 Hz, 1H), 3.69 (s, 3H), 3.68 - 3.63 (m, 4H).

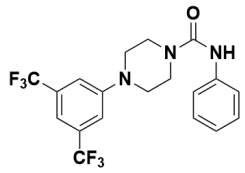
N-(1-Methyl-1H-indol-5-yl)-4-(3-(trifluoromethyl)phenyl)piperazine-1-carboxamide (3.33)



To a stirring solution of 1-(3-(trifluoromethyl)phenyl)piperazine (30 mg, 0.13 mmol, 1.0 equiv), triethylamine (1.8 µL, 0.013 mmol, 0.1 equiv) in DCM (5 mL) was added 5-isocyanato-1-methyl-1H-indole (27 mg, 0.16 mmol, 1.2 equiv) and the solution was stirred for 30 min at RT. After confirming consumption of limiting reagent by TLC, 3 mL of

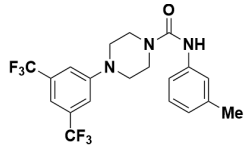
distilled water was added to the reaction vial. The organic layer was separated and removed under reduced atmosphere. Column chromatography (0 to 50% EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{21}H_{22}F_3N_4O^+$ [M+H$^+$], 403.4287 found 403. 1H NMR (400 MHz, CD_3OD) δ 8.12 (d, *J* = 11 Hz, 1H), 7.51 (d, *J* = 2.0 Hz, 1H), 7.44 (t, *J* = 7.9 Hz, 1H), 7.31 (d, *J* = 8.8 Hz, 1H), 7.28 - 7.21 (m, 1H), 7.16 - 7.10 (m, 3H), 6.38 (d, *J* = 3.1 Hz, 1H), 3.79 (s, 3H), 3.75 - 3.70 (m, 4H).

4-(3,5-bis(Trifluoromethyl)phenyl)-N-phenylpiperazine-1-carboxamide (3.34)



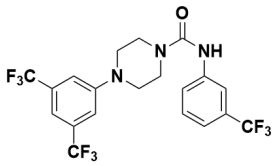
To a stirring solution of 1-(3,5-bis(trifluoromethyl)phenyl)piperazine (30 mg, 0.09 mmol, 1.0 equiv), triethylamine (1.5 μL, 0.009 mmol, 0.1 equiv) in DCM (5 mL) was added isocyanatobenzene (15 mg, 0.11 mmol, 1.2 equiv) and the solution was stirred for 30 min at RT. After confirming consumption of limiting reagent by TLC, 3 mL of distilled water was added to the reaction vial. The organic layer was separated and removed under reduced atmosphere. Column chromatography (0 to 50% EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd $C_{19}H_{18}F_6N_3O^+$ [M+H$^+$], 418.3629 found 419. 1H NMR (400 MHz, CD_3OD) δ 7.46 - 7.36 (m, 4H), 7.32 - 7.25 (m, 4H), 7.03 (q, *J* = 6.4 Hz, 2H), 3.76 - 3.70 (m, 4H), 3.43 - 3.39 (m, 4H).

4-(3,5-bis(Trifluoromethyl)phenyl)-N-(m-tolyl)piperazine-1-carboxamide (3.35)



To a stirring solution of 1-(3,5-bis(trifluoromethyl)phenyl)piperazine (30 mg, 0.09 mmol, 1.0 equiv), triethylamine (1.5 µL, 0.009 mmol, 0.1 equiv) in DCM (5 mL) was added 1-isocyanato-3-methylbenzene (16 mg, 0.11 mmol, 1.2 equiv) and the solution was stirred for 30 min at RT. After confirming consumption of limiting reagent by TLC, 3 mL of distilled water was added to the reaction vial. The organic layer was separated and removed under reduced atmosphere. Column chromatography (0 to 50% EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd $C_{20}H_{20}F_6N_3O^+$ [M+H$^+$], 432.3899 found 433. 1H NMR (400 MHz, CD_3OD) δ 7.44 (s, 2H), 7.31 (s, 1H), 7.26 - 7.12 (m, 4H), 6.89 - 6.82 (m, 1H), 3.75 - 3.68 (m, 4H), 3.44 - 3.35 (m, 4H), 2.31 (s, 3H).

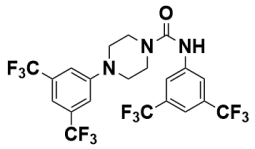
4-(3,5-bis(Trifluoromethyl)phenyl)-N-(3-(trifluoromethyl)phenyl)piperazine-1-carboxamide (3.36).



To a stirring solution of 1-(3,5-*bis*-triflouromethylphenyl)piperazine (30 mg, 0.10 mmol, 1.0 equiv), triethylamine (1.8 µL, 0.01 mmol, 0.1 equiv) in DCM (5 mL) was added 3-trifluoromethylphenylisocyanate (29 mg, 0.12 mmol, 1.2 equiv) and the solution was stirred for 30 min at RT. After confirming consumption of limiting reagent by TLC, 3 mL of distilled water was added to the reaction vial. The organic layer was separated and

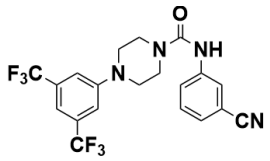
removed under reduced atmosphere. Column chromatography (0 to 50% EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{20}H_{17}F_9N_3O^+$ [M+H$^+$] 486.36, found 487. 1H NMR (400 MHz, CDCl3) δ 7.67 (s, 1H), 7.58 (t, *J* = 8.2 Hz, 1H), 7.42 (t, *J* = 8.0 Hz, 1H), 7.36 (s, 1H), 7.32 (d, *J* = 7.5 Hz, 1H), 6.57 (s, 1H), 3.74 (t, *J* = 5.1 Hz, 4H), 3.40 (t, *J* = 5.4 Hz, 4H). 13C NMR (176 MHz, CDCl3) δ 154.5, 151.1, 139.1, 132.6 (d, *J* = 33 Hz) 131.4 (d, *J* = 32 Hz), 129.5, 124.2, 123.2, 122.7, 120.1, 116.8, 115.0, 112.9, 100.0, 47.9, 43.6.

N,4-bis(3,5-bis(Trifluoromethyl)phenyl)piperazine-1-carboxamide (3.37)



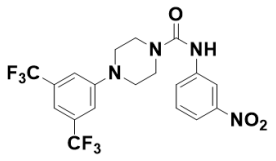
To a stirring solution of 1-(3,5-bis(trifluoromethyl)phenyl)piperazine (30 mg, 0.11 mmol, 1.0 equiv), triethylamine (1.7 µL, 0.011 mmol, 0.1 equiv) in DCM (5 mL) was added 1-isocyanato-3,5-bis(trifluoromethyl)benzene (30 mg, 0.13 mmol, 1.2 equiv) and the solution was stirred for 30 min at RT. After confirming consumption of limiting reagent by TLC, 3 mL of distilled water was added to the reaction vial. The organic layer was separated and removed under reduced atmosphere. Column chromatography (0 to 50% EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{23}H_{22}F_6N_3O_5^+$ [M+H$^+$], 534.4349 found 534. 1H NMR (400 MHz, CD_3OD) δ 8.10 (s, 2H), 7.54 (s, 1H), 7.46 (s, 2H), 7.31 (s, 1H), 3.38 - 3.69 (m, 4H), 3.50 - 3.38 (m, 4H).

4-(3,5-bis(Trifluoromethyl)phenyl)-N-(3-cyanophenyl)piperazine-1-carboxamide (3.38)



To a stirring solution of 1-(3,5-bis(trifluoromethyl)phenyl)piperazine (30 mg, 0.11 mmol, 1.0 equiv), triethylamine (1.7 µL, 0.011 mmol, 0.1 equiv) in DCM (5 mL) was added 3-isocyanatobenzonitrile (17 mg, 0.14 mmol, 1.2 equiv) and the solution was stirred for 30 min at RT. After confirming consumption of limiting reagent by TLC, 3 mL of distilled water was added to the reaction vial. The organic layer was separated and removed under reduced atmosphere. Column chromatography (0 to 50% EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{20}H_{17}F_6N_4O^+$ [M+H$^+$], 443.3729 found 443. 1H NMR (400 MHz, $CDCl_3$) δ 7.86 (s, 1H), 7.69 (d, *J* = 6.3 Hz, 1H), 7.48 - 7.42 (m, 3H), 7.36 (d, *J* = 7.7 Hz, 1H), 7.31 (s, 1H), 3.79 - 3.71 (m, 4H), 3.47 - 3.39 (m, 4H).

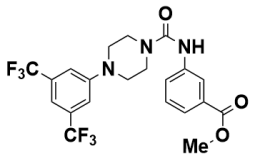
4-(3,5-bis(Trifluoromethyl)phenyl)-N-(3-nitrophenyl)piperazine-1-carboxamide (3.39)



To a stirring solution of 1-(3,5-bis(trifluoromethyl)phenyl)piperazine (30 mg, 0.09 mmol, 1.0 equiv), triethylamine (1.6 µL, 0.009 mmol, 0.1 equiv) in DCM (5 mL) was added 1-isocyanato-3-nitrobenzene (20 mg, 0.11 mmol, 1.2 equiv) and the solution was stirred for

30 min at RT. After confirming consumption of limiting reagent by TLC, 3 mL of distilled water was added to the reaction vial. The organic layer was separated and removed under reduced atmosphere. Column chromatography (0 to 50% EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd $C_{19}H_{17}F_6N_4O_3^+$ [M+H$^+$], 463.3599 found 464. 1H NMR (400 MHz, $CDCl_3$) δ 8.24 (t, J = 2.2 Hz, 1H), 7.91 (dd, J_1 = 2.3, J_2 = 8.3 Hz, 1H), 7.83 (dd, J_1 = 2.3, J_2 = 8.2 Hz, 1H), 7.47 (t, J = 8.2 Hz, 1H), 7.37 (s, 1H), 7.28 (s, 2H), 6.70 (s, 1H), 3.81 - 3.74 (m, 4H), 3.45 - 3.39 (m, 4H).

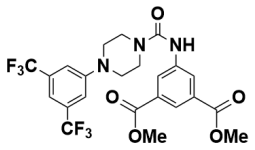
Methyl 3-(4-(3,5-bis(trifluoromethyl)phenyl)piperazine-1-carboxamido)benzoate (3.40)



To a stirring solution of 1-(3,5-bis(trifluoromethyl)phenyl)piperazine (30 mg, 0.11 mmol, 1.0 equiv), triethylamine (1.7 μL, 0.011 mmol, 0.1 equiv) in DCM (5 mL) was added methyl 3-isocyanatobenzoate (20 mg, 0.14 mmol, 1.2 equiv) and the solution was stirred for 30 min at RT. After confirming consumption of limiting reagent by TLC, 3 mL of distilled water was added to the reaction vial. The organic layer was separated and removed under reduced atmosphere. Column chromatography (0 to 50% EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{21}H_{20}F_6N_3O_3^+$ [M+H$^+$], 476.3989 found 477. 1H NMR (400 MHz, CD_3OD) δ 8.10 (s, 1H), 7.72 - 7.64 (m, 2H), 7.46 (s, 2H), 7.40 (t, J = 7.9 Hz, 1H), 7.31 (s, 1H), 3.91 (s, 3H), 3.79 - 3.72 (m, 4H), 3.46 - 3.40 (m, 4H).

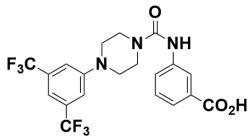
13C NMR (101 MHz, CDCl3) δ 166.8, 154.6, 151.2, 138.9, 132.5 (q, *J* = 33 Hz), 130.9, 129.1, 124.6, 124.5, 123.5 (q, *J* = 273 Hz), 120.8, 114.9, 112.7, 52.2, 47.9, 43.6.

Dimethyl 5-(4-(3,5-bis(trifluoromethyl)phenyl)piperazine-1-carboxamido)isophthalate (3.41)



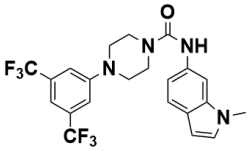
To a stirring solution of 1-(3,5-bis(trifluoromethyl)phenyl)piperazine (30 mg, 0.11 mmol, 1.0 equiv), triethylamine (1.7 μL, 0.011 mmol, 0.1 equiv) in DCM (5 mL) was added dimethyl 5-isocyanatoisophthalate (28 mg, 0.14 mmol, 1.2 equiv) and the solution was stirred for 30 min at RT. After confirming consumption of limiting reagent by TLC, 3 mL of distilled water was added to the reaction vial. The organic layer was separated and removed under reduced atmosphere. Column chromatography (0 to 50% EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{23}H_{22}F_6N_3O_5^+$ [M+H$^+$], 534.4349 found 534. 1H NMR (400 MHz, CD_3OD) δ 8.34 (s, 2H), 8.29 (s, 1H), 7.47 (s, 2H), 7.32 (s, 1H), 4.59 (br, 3H), 3.94 (s, 6H), 3.81 - 3.71 (m, 4H), 3.50 - 3.37 (m, 4H). 13C NMR (176 MHz, CDCl3) δ 165.9, 157.9, 150.9, 132.0 (q, *J* = 32 Hz), 131.5 (q, *J* = 32 Hz), 130.3, 129.8, 124.0 (dq, J_1 = 70, J_2 = 273 Hz), 119.4, 118.5 (d, *J* = 4 Hz), 117.8, 116.9 (d, *J* = 4 Hz), 112.8 (d, *J* = 5 Hz), 111.8 (d, *J* = 4 Hz), 67.5, 49.3, 48.8, 45.1, 41.2.

3-(4-(3,5-bis(Trifluoromethyl)phenyl)piperazine-1-carboxamido)benzoic Acid (3.42)



A solution of methyl 3-(4-(3,5-bis(trifluoromethyl)phenyl)piperazine-1-carboxamido)benzoate (EJC-3-137, 499 mg, 1.05 mmol, 1.0 equiv) was dissolved in a H_2O/THF cosolvent system (1:5, H_2O to THF) at rt. To the reaction was added $LiOH \cdot H_2O$ (210 mg, 5.25 mmol, 5 equiv) and the reaction was left to stir overnight. After confirming consumption of starting material by TLC, the THF was removed under reduced pressure and the resulting slurry was taken up in EtOAc and washed several times (2x25 mL) with 0.1 N HCl. The organic layer was separated and removed under reduced atmosphere. Column chromatography (0 to 100% EtOAc/Hex) resulted in target molecule as a white powder in moderate yield (327 mg, 68%). LRMS (APCI - quad) m/z cal'd $C_{20}H_{18}F_6N_3O_3^+$ [$M+H^+$], 462.3719 found 463. 1H NMR (400 MHz, $CDCl_3$) δ 7.97 (t, J = 1.9, 1H), 7.60 (td, J_1 = 1.3, J_2 = 7.8 Hz, 1H), 7.56 (ddd, J_1 = 1.0, J_2 = 2.2, J_3 = 8.2 Hz, 1H), 7.35 (s, 2H), 7.28 (t, J = 7.9 Hz, 1H), 7.20 (s, 1H), 3.68 - 3.59 (m, 4H), 3.36 - 3.28 (m, 4H).

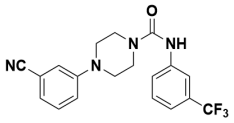
4-(3,5-bis(Trifluoromethyl)phenyl)-N-(1-methyl-1H-indol-6-yl)piperazine-1-carboxamide (3.43)



To a stirring solution of 1-(3,5-bis(trifluoromethyl)phenyl)piperazine (30 mg, 0.11 mmol, 1.0 equiv), triethylamine (1.7 µL, 0.011 mmol, 0.1 equiv) in DCM (5 mL) was added 4-isocyanato-1-methyl-1H-indole (21 mg, 0.14 mmol, 1.2 equiv) and the solution was stirred

for 30 min at RT. After confirming consumption of limiting reagent by TLC, 3 mL of distilled water was added to the reaction vial. The organic layer was separated and removed under reduced atmosphere. Column chromatography (0 to 50% EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{22}H_{21}F_6N_4O^+$ [M+H$^+$], 471.4269 found 472. 1H NMR (400 MHz, CD_3OD) δ 7.51 (d, *J* = 1.9 Hz, 1H), 7.46 (s, 2H), 7.33 - 7.28 (m, 2H), 7.17 - 7.10 (m, 2H), 6.38 (d, *J* = 3.1 Hz, 1H), 3.79 (s, 3H), 3.77 - 3.71 (m, 4H), 3.46 - 3.39 (m, 4H).

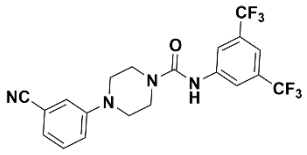
4-(3-Cyanophenyl)-N-(3-(trifluoromethyl)phenyl)piperazine-1-carboxamide (3.44).



To a stirring solution of 1-(3-cyanophenyl)piperazine (30 mg, 0.16 mmol, 1.0 equiv), triethylamine (1.8 μL, 0.016 mmol, 0.1 equiv) in DCM (5 mL) was added 3-trifluoromethylphenylisocyanate (29 mg, 0.18 mmol, 1.2 equiv) and the solution was stirred for 30 min at RT. After confirming consumption of limiting reagent by TLC, 3 mL of distilled water was added to the reaction vial. The organic layer was separated and removed under reduced atmosphere. Column chromatography (0 to 50% EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{19}H_{18}F_3N_4O^+$ [M+H$^+$] 375.37, found 375. 1H NMR (400 MHz, CDCl3) δ 7.67 (s, 1H), 7.59 (d, *J* = 8.1 Hz, 1H), 7.41 (t, *J* = 7.8 Hz, 1H), 7.36 (t, *J* = 8.7 Hz, 1H), 7.30 (d, *J* = 7.8 Hz, 1H), 7.16 (d, *J* = 7.0 Hz, 1H), 7.12 (s, 1H), 6.71 (s, 1H), 3.70 (t, *J* = 5.1 Hz, 4H), 3.29 (t, *J* = 5.2 Hz, 4H). 13C NMR

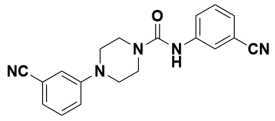
(176 MHz, CDCl3) δ 154.7, 150.8, 139.5, 131.2, 130.1, 129.4, 123.7 (q, *J* = 274 Hz), 123.3, 123.2, 120.3, 119.8, 119.8, 119.3, 118.7, 116.8, 113.0, 100.0, 48.2, 43.7.

N-(3,5-bis(Trifluoromethyl)phenyl)-4-(3-cyanophenyl)piperazine-1-carboxamide (3.45)



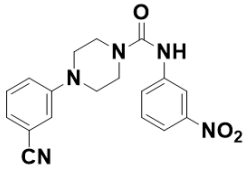
To a stirring solution of 3-(piperazin-1-yl)benzonitrile (30 mg, 0.16 mmol, 1.0 equiv), triethylamine (1.8 µL, 0.013 mmol, 0.1 equiv) in DCM (5 mL) was added 1-isocyanato-3,5-bis(trifluoromethyl)benzene (30 mg, 0.19 mmol, 1.2 equiv) and the solution was stirred for 30 min at RT. After confirming consumption of limiting reagent by TLC, 3 mL of distilled water was added to the reaction vial. The organic layer was separated and removed under reduced atmosphere. Column chromatography (0 to 50% EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{20}H_{17}F_6N_4O^+$ [M+H$^+$] 443.3729, found 443. 1H NMR (400 MHz, $CDCl_3$) δ 7.90 (s, 2H), 7.52 (s, 1H), 7.37 (t, *J* = 7.8 Hz, 1H), 7.19 - 7.10 (m, 3H), 6.97 (s, 1H), 3.83 - 3.61 (m, 4H), 3.36 - 3.22 (m, 4H). 13C NMR (101 MHz, CDCl3) δ 154.5, 150.8, 139.3, 131.2 (q, *J* = 33 Hz), 130.1, 129.5, 125.3, 123.3, 123.0, 122.6, 120.3, 119.9 (q, *J* = 4 Hz), 119.1, 118.7, 116.6 (q, *J* = 4 Hz), 113.2, 48.2, 43.7.

N,4-bis(3-Cyanophenyl)piperazine-1-carboxamide (3.46)



To a stirring solution of 3-(piperazin-1-yl)benzonitrile (30 mg, 0.16 mmol, 1.0 equiv), triethylamine (1.8 µL, 0.013 mmol, 0.1 equiv) in DCM (5 mL) was added 3-isocyanatobenzonitrile (28 mg, 0.19 mmol, 1.2 equiv) and the solution was stirred for 30 min at RT. After confirming consumption of limiting reagent by TLC, 3 mL of distilled water was added to the reaction vial. The organic layer was separated and removed under reduced atmosphere. Column chromatography (0 to 50% EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{19}H_{18}N_5O^+$ [M+H$^+$] 332.3865, found 332. 1H NMR (400 MHz, $CDCl_3$) δ 7.76 (s, 1H), 7.62 (d, *J* = 7.2 Hz, 1H), 7.43 - 7.32 (m, 3H), 7.18 - 7.11 (m, 3H), 6.62 (s, 1H), 3.74 - 3.66 (m, 4H), 3.35 - 3.29 (m, 4H).

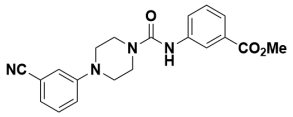
4-(3-Cyanophenyl)-N-(3-nitrophenyl)piperazine-1-carboxamide (3.47)



To a stirring solution of 3-(piperazin-1-yl)benzonitrile (30 mg, 0.16 mmol, 1.0 equiv), triethylamine (1.8 µL, 0.013 mmol, 0.1 equiv) in DCM (5 mL) was added 1-isocyanato-3-nitrobenzene (32 mg, 0.19 mmol, 1.2 equiv) and the solution was stirred for 30 min at RT. After confirming consumption of limiting reagent by TLC, 3 mL of distilled water was added to the reaction vial. The organic layer was separated and removed under reduced atmosphere. Column chromatography (0 to 50% EtOAc/Hex) on silica gel was used to

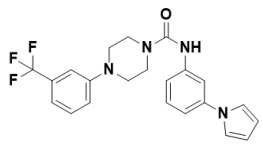
purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd $C_{18}H_{18}N_5O_3^+$ [M+H$^+$], 352.3735 found 354. 1H NMR (400 MHz, $CDCl_3$) δ 8.20 (s, 2H), 7.89 (d, *J* = 8.3, 1H), 7.82 (d, *J* = 8.1 Hz, 1H), 7.45 (t, *J* = 7.4 Hz, 1H), 6.93 (s, 1H), 3.67 (br, 4H), 3.07 (br, 4H).

Methyl 3-(4-(3-cyanophenyl)piperazine-1-carboxamido)benzoate (3.48)



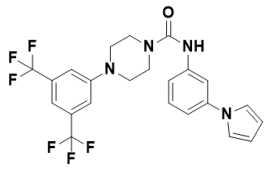
To a stirring solution of 3-(piperazin-1-yl)benzonitrile (30 mg, 0.16 mmol, 1.0 equiv), triethylamine (1.8 µL, 0.013 mmol, 0.1 equiv) in DCM (5 mL) was added methyl 3-isocyanatobenzoate (34 mg, 0.19 mmol, 1.2 equiv) and the solution was stirred for 30 min at RT. After confirming consumption of limiting reagent by TLC, 3 mL of distilled water was added to the reaction vial. The organic layer was separated and removed under reduced atmosphere. Column chromatography (0 to 50% EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{20}H_{21}N_4O_3^+$ [M+H$^+$] 365.4125, found 365. 1H NMR (400 MHz, $CDCl_3$) δ 7.94 (s, 1H), 7.74 - 7.67 (m, 2H), 7.40 - 7.30 (m, 2H), 7.16 - 7.08 (m, 3H), 6.86 (s, 1H), 3.88 (s, 3H), 3.70 - 3.64 (m, 4H), 3.28 - 3.22 (m, 4H).

N-(3-(1H-Pyrrol-1-yl)phenyl)-4-(3-(trifluoromethyl)phenyl)piperazine-1-carboxamide (3.49)



To a stirring solution of 1-(3-triflouromethylphenyl)piperazine (31 mg, 0.13 mmol, 1.0 equiv), triethylamine (1.8 µL, 0.013 mmol, 0.1 equiv) in DCM (5 mL) was added 1-(3-isocyanatophenyl)-1H-pyrrole (30 mg, 0.15 mmol, 1.2 equiv) and the solution was stirred for 30 min at RT. After confirming consumption of limiting reagent by TLC, 3 mL of distilled water was added to the reaction vial. The organic layer was separated and removed under reduced atmosphere. Column chromatography (0 to 50% EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{22}H_{22}F_3N_4O^+$ [M+H$^+$] 415.44, found 416. 1H NMR (400 MHz, CDCl3) δ 7.59 (t, *J* = 2.1 Hz, 1H), 7.38 (t, *J* = 8.0 Hz, 1H), 7.32 (t, *J* = 8.1 Hz, 1H), 7.19 - 7.11 (m, 3H), 7.11 - 7.06 (m, 3H), 6.61 (br, 1H), 6.32 (t, *J* = 2.1 Hz, 2H), 3.69 (t, *J* = 5.1 Hz, 4H), 3.28 (t, *J* = 5.2 Hz, 4H).

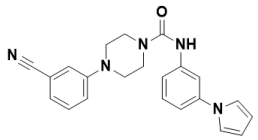
N-(3-(1H-Pyrrol-1-yl)phenyl)-4-(3,5-bis(trifluoromethyl)phenyl)piperazine-1-carboxamide (3.50)



To a stirring solution of 1-(3,5-*bis*-triflouromethylphenyl)piperazine (40 mg, 0.13 mmol, 1.0 equiv), triethylamine (1.8 µL, 0.013 mmol, 0.1 equiv) in DCM (5 mL) was added 3,5-bistrifluoromethylphenylisocyanate (30 mg, 0.015 mmol, 1.2 equiv) and the solution was stirred for 30 min at RT. After confirming consumption of limiting reagent by TLC, 3 mL of distilled water was added to the reaction vial. The organic layer was separated and removed under reduced atmosphere. Column chromatography (0 to 50% EtOAc/Hex) on

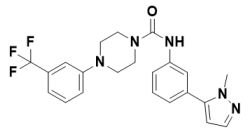
silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{23}H_{21}F_6N_4O^+$ [M+H$^+$] 483.44, found 484. 1H NMR (400 MHz, CDCl3) δ 7.57 (t, *J* = 2.0 Hz, 1H), 7.36 - 7.30 (m, 2H), 7.23 (s, 2H), 7.16 (d, *J* = 8.0 Hz, 1H), 7.12 - 7.05 (m, 3H), 3.70 (t, *J* = 4.9 Hz, 4H), 3.33 (t, *J* = 5.3 Hz, 4H)

N-(3-(1H-Pyrrol-1-yl)phenyl)-4-(3-cyanophenyl)piperazine-1-carboxamide (3.51)



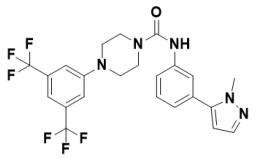
To a stirring solution of 1-(3-cyanophenyl)piperazine (30 mg, 0.16 mmol, 1.0 equiv), triethylamine (1.8 μL, 0.016 mmol, 0.1 equiv) in DCM (5 mL) was added 1-(3-isocyanatophenyl)-1H-pyrrole (29 mg, 0.19 mmol, 1.2 equiv) and the solution was stirred for 30 min at RT. After confirming consumption of limiting reagent by TLC, 3 mL of distilled water was added to the reaction vial. The organic layer was separated and removed under reduced atmosphere. Column chromatography (0 to 50% EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{22}H_{22}N_5O^+$ [M+H$^+$] 372.45, found 372. 1H NMR (400 MHz, CDCl3) δ 7.60 (t, *J* = 2.2 Hz, 1H), 7.38 (t, *J* = 8.6 Hz, 1H), 7.33 (t, *J* = 7.8 Hz, 1H), 7.21-7.14 (m, 4H), 7.10 (t, *J* = 2.4 Hz, 3H), 6.49 (br, 1H), 6.33 (t, *J* = 2.2 Hz, 2H), 3.74 (t, *J* = 5.2 Hz, 4H), 3.32 (t, *J* = 5.4 Hz, 4H).

N-(3-(1-Methyl-1H-pyrazol-5-yl)phenyl)-4-(3-(trifluoromethyl)phenyl)piperazine-1-carboxamide (3.52)



To a stirring solution of 1-(3-triflouromethylphenyl)piperazine (28 mg, 0.12 mmol, 1.0 equiv), triethylamine (1.8 µL, 0.012 mmol, 0.1 equiv) in DCM (5 mL) was added 5-(3-isocyanatophenyl)-1-methyl-1H-pyrazole (30 mg, 0.14 mmol, 1.2 equiv) and the solution was stirred for 30 min at RT. After confirming consumption of limiting reagent by TLC, 3 mL of distilled water was added to the reaction vial. The organic layer was separated and removed under reduced atmosphere. Column chromatography (0 to 50% EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{22}H_{23}F_3N_5O^+$ [M+H$^+$] 430.45, found 431. 1H NMR (400 MHz, CDCl3) δ 7.54 - 7.51 (m, 2H), 7.41 - 7.35 (m, 3H), 7.16 - 7.07 (m, 4H), 6.71 (br, 1H), 6.32 (d, *J* = 1.9 Hz, 1H), 3.92 (s, 3H), 3.72 (t, *J* = 5.3 Hz, 4), 3.31 (t, *J* = 5.3 Hz, 4H),

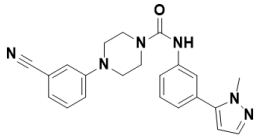
4-(3,5-bis(Trifluoromethyl)phenyl)-N-(3-(1-methyl-1H-pyrazol-5-yl)phenyl)piperazine-1-carboxamide (3.53)



To a stirring solution of 1-(3,5-*bis*-triflouromethylphenyl)piperazine (37 mg, 0.12 mmol, 1.0 equiv), triethylamine (1.8 µL, 0.012 mmol, 0.1 equiv) in DCM (5 mL) was added 5-(3-isocyanatophenyl)-1-methyl-1H-pyrazole (30 mg, 0.14 mmol, 1.2 equiv) and the solution was stirred for 30 min at RT. After confirming consumption of limiting reagent by TLC, 3

mL of distilled water was added to the reaction vial. The organic layer was separated and removed under reduced atmosphere. Column chromatography (0 to 50% EtOAc/Hex)) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{23}H_{22}F_6N_5O^+$ [M+H$^+$] 498.45, found 499. 1H NMR (400 MHz, CDCl3) δ 7.54 - 7.51 (m, 2H), 7.43 - 7.38 (m, 2H), 7.34 (br, 1H), 7.15 - 7.07 (m, 1H), 6.69 (br, 1H), 6.33 (d, *J* = 1.5 Hz, 1H), 3.93 (s, 3H), 3.75 (t, *J* = 5.0 Hz, 4H), 3.40 (t, *J* = 5.3 Hz, 4H). 13C NMR (176 MHz, CDCl3) δ 154.8, 151.2, 143.3, 139.0, 138.5, 132.7, 132.5, 131.4, 129.3, 124.2, 123.7, 122.7, 121.1, 120.4, 120.0, 115.0, 112.8, 106.1, 48.0, 43.6, 37.6.

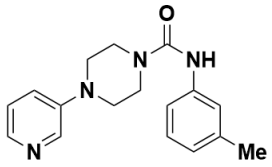
4-(3-Cyanophenyl)-N-(3-(1-methyl-1H-pyrazol-5-yl)phenyl)piperazine-1-carboxamide (3.54)



To a stirring solution of 1-(3-cyanophenyl)piperazine (30 mg, 0.16 mmol, 1.0 equiv), triethylamine (1.8 µL, 0.016 mmol, 0.1 equiv) in DCM (5 mL) was added 1-(3-isocyanatophenyl)-1H-pyrrole (29 mg, 0.19 mmol, 1.2 equiv) and the solution was stirred for 30 min at RT. After confirming consumption of limiting reagent by TLC, 3 mL of distilled water was added to the reaction vial. The organic layer was separated and removed under reduced atmosphere. Column chromatography (0 to 50% EtOAc/Hex)) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{22}H_{23}N_6O^+$ [M+H$^+$] 387.47, found 387. 1H NMR (400 MHz, CDCl3) δ 7.55 (br, 1H), 7.53 (d, *J* = 2.0 Hz, 1H), 7.41 - 7.33 (m,

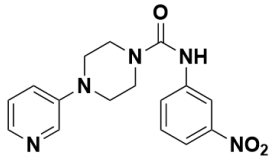
3H), 7.18 - 7.09 (m, 4H), 6.69 (1H), 6.33 (d, J = 2.0 Hz, 1H), 3.94 (s, 3H), 3.72 (t, J = 5.0 Hz, 4H), 3.31 (t, J = 5.3 Hz, 4H). 13C NMR (176 MHz, CDCl3) δ 154.7, 151.1, 141.4, 139.9, 132.8, 132.6, 132.4, 132.3, 129.9, 124.2, 122.7, 119.3, 116.9, 115.3, 115.0, 112.7, 112.2, 110.5, 47.9, 43.6.

4-(Pyridin-3-yl)-N-(m-tolyl)piperazine-1-carboxamide (3.55)



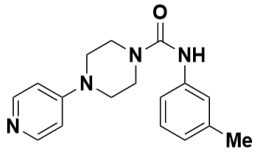
To a stirring solution of 1-(pyridin-3-yl)piperazine (30 mg, 0.18 mmol, 1.0 equiv), triethylamine (1.9 µL, 0.018 mmol, 0.1 equiv) in DCM (5 mL) was added 1-isocyanato-3-methylbenzene (29 mg, 0.21 mmol, 1.2 equiv) and the solution was stirred for 30 min at RT. After confirming consumption of limiting reagent by TLC, 3 mL of distilled water was added to the reaction vial. The organic layer was separated and removed under reduced atmosphere. Column chromatography (0 to 50% EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{17}H_{21}N_4O^+$ [M+H$^+$], 297.38 found 299. 1H NMR (400 MHz, $CDCl_3$) δ 8.31 (dd, J_1 = 1.6, J_2 = 5.0 Hz, 2H), 7.23 (s, 1H), 7.18 (t, J = 7.8 Hz, 1H), 7.14 - 7.10 (m, 1H), 6.88 (d, J = 8.8 Hz, 1H), 6.70 - 6.63 (m, 2H), 6.41 (br, 1H), 3.72 - 3.64 (m, 4H), 3.48 - 3.40 (m, 4H), 2.33 (s, 3H).

N-(3-Nitrophenyl)-4-(pyridin-3-yl)piperazine-1-carboxamide (3.56)



To a stirring solution of 1-(pyridin-3-yl)piperazine (30 mg, 0.18 mmol, 1.0 equiv), triethylamine (1.9 µL, 0.018 mmol, 0.1 equiv) in DCM (5 mL) was added 1-isocyanato-3-nitrobenzene (29 mg, 0.21 mmol, 1.2 equiv) and the solution was stirred for 30 min at RT. After confirming consumption of limiting reagent by TLC, 3 mL of distilled water was added to the reaction vial. The organic layer was separated and removed under reduced atmosphere. Column chromatography (0 to 50% EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{17}H_{21}N_4O^+$ [M+H$^+$], 297.38 found 299. 1H NMR (400 MHz, $CDCl_3$) δ 8.33 (dd, J_1 = 1.7, J_2 = 5.0 Hz, 2H), 8.23 (t, J = 2.2 Hz, 1H), 7.91 (ddd, J_1 = 1.0, J_2 = 2.2, J_3 = 8.2 Hz, 1H), 7.82 (ddd, J_1 = 0.8, J_2 = 2.2, J_3 = 8.2 Hz, 1H), 7.47 (t, J = 8.2 Hz, 1H), 6.71 - 6.65 (m, 3H), 3.77 - 3.70 (m, 4H), 3.52 - 3.44 (m, 4H).

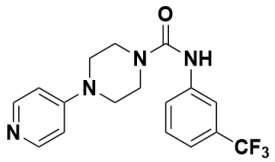
4-(Pyridin-4-yl)-N-(m-tolyl)piperazine-1-carboxamide (3.57)



To a stirring solution of 1-(pyridin-4-yl)piperazine (30 mg, 0.18 mmol, 1.0 equiv), triethylamine (1.9 µL, 0.018 mmol, 0.1 equiv) in DCM (5 mL) was added 1-isocyanato-3-methylbenzene (29 mg, 0.21 mmol, 1.2 equiv) and the solution was stirred for 30 min at RT. After confirming consumption of limiting reagent by TLC, 3 mL of distilled water was added to the reaction vial. The organic layer was separated and removed under reduced

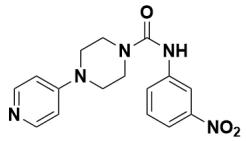
atmosphere. Column chromatography (0 to 50% EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{17}H_{21}N_4O^+$ [M+H$^+$], 297.38 found 298. 1H NMR (400 MHz, $CDCl_3$) δ 8.32 (t, *J* = 1.9 Hz, 1H), 8.15 (t, *J* = 2.9 Hz, 1H), 7.24 (s, 1H), 7.22 - 7.19 (m, 2H), 7.17 (d, *J* = 7.5 Hz, 1H), 7.15 - 7.11 (m, 1H), 6.88 (d, *J* = 7.3 Hz, 1H), 6.45 (br, 1H), 3.72 - 3.62 (m, 4H), 3.29 - 3.21 (m, 4H), 2.33 (s, 3H).

4-(Pyridin-4-yl)-N-(3-(trifluoromethyl)phenyl)piperazine-1-carboxamide (3.58)



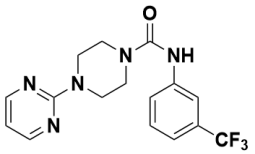
To a stirring solution of 1-(pyridin-4-yl)piperazine (30 mg, 0.18 mmol, 1.0 equiv), triethylamine (1.9 µL, 0.018 mmol, 0.1 equiv) in DCM (5 mL) was added 1-isocyanato-3-(trifluoromethyl)benzene (41 mg, 0.21 mmol, 1.2 equiv) and the solution was stirred for 30 min at RT. After confirming consumption of limiting reagent by TLC, 3 mL of distilled water was added to the reaction vial. The organic layer was separated and removed under reduced atmosphere. Column chromatography (0 to 50% EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{17}H_{18}F_3N_4O^+$ [M+H$^+$], 351.35 found 352. 1H NMR (400 MHz, $CDCl_3$) δ 8.31 (t, *J* = 2.0 Hz, 1H), 8.15 (t, *J* = 3.1 Hz, 1H), 7.66 (t, *J* = 2.0 Hz, 1H), 7.58 (dd, J_1 = 2.1, J_2 = 8.0 Hz, 1H), 7.39 (t, *J* = 7.9 Hz, 1H), 7.29 (d, *J* = 7.8 Hz, 1H), 7.22 - 7.19 (m, 2H), 6.89 (br, 1H), 3.75 - 3.64 (m, 4H), 3.30 - 3.22 (m, 4H).

N-(3-Nitrophenyl)-4-(pyridin-4-yl)piperazine-1-carboxamide (3.59)



To a stirring solution of 1-(pyridin-4-yl)piperazine (30 mg, 0.18 mmol, 1.0 equiv), triethylamine (1.9 µL, 0.018 mmol, 0.1 equiv) in DCM (5 mL) was added 1-isocyanato-3-nitrobenzene (29 mg, 0.21 mmol, 1.2 equiv) and the solution was stirred for 30 min at RT. After confirming consumption of limiting reagent by TLC, 3 mL of distilled water was added to the reaction vial. The organic layer was separated and removed under reduced atmosphere. Column chromatography (0 to 50% EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{17}H_{21}N_4O^+$ [M+H$^+$], 297.38 found 299. 1H NMR (400 MHz, $CDCl_3$) δ 8.32 (t, J = 2.0 Hz, 1H), 8.22 (t, J = 2.2 Hz, 1H), 8.16 (t, J = 2.6 Hz, 1H), 7.88 (ddd, J_1 = 1.0, J_2 = 3.2, J_3 = 8.2 Hz, 1H), 7.83 (ddd, J_1 = 1.0, J_2 = 3.2, J_3 = 8.2 Hz, 1H), 7.45 (t, J = 8.2, 1H), 7.23 - 7.20 (m, 2H), 6.97 (br, 1H), 3.77 - 3.68 (m, 4H), 3.34 - 3.24 (m, 4H).

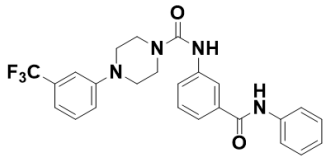
4-(Pyrimidin-2-yl)-N-(3-(trifluoromethyl)phenyl)piperazine-1-carboxamide (3.60)



To a stirring solution of 2-(piperazin-1-yl)pyrimidine (30 mg, 0.11 mmol, 1.0 equiv), triethylamine (1.8 µL, 0.011 mmol, 0.1 equiv) in DCM (5 mL) was added 1-isocyanato-3-(trifluoromethyl)benzene (41 mg, 0.14 mmol, 1.2 equiv) and the solution was stirred for 30 min at RT. After confirming consumption of limiting reagent by TLC, 3 mL of distilled

water was added to the reaction vial. The organic layer was separated and removed under reduced atmosphere. Column chromatography (0 to 50% EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{16}H_{17}F_3N_5O^+$ [M+H$^+$], 352.34 found 353. 1H NMR (400 MHz, $CDCl_3$) δ 8.33 (d, *J* = 4.8 Hz, 2H), 7.67 (s, 1H), 7.59 (d, *J* = 8.2 Hz, 1H), 7.40 (t, *J* = 7.9 Hz, 1H), 7.29 (d, *J* = 7.8 Hz, 1H), 6.63 (br, 1H), 6.56 (t, *J* = 4.7 Hz, 1H), 3.98 - 3.86 (m, 4H), 3.66 - 3.58 (m, 4H).

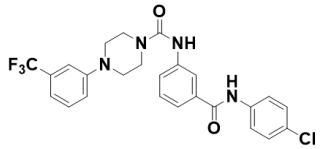
N-(3-(Phenylcarbamoyl)phenyl)-4-(3-(trifluoromethyl)phenyl)piperazine-1-carboxamide (3.61)



To a stirring solution of 3-(4-(3-(trifluoromethyl)phenyl)piperazine-1-carboxamido)benzoic acid (20 mg, 0.05 mmol, 1 equiv) in DCM (5 mL) was added EDC (21 mg, 0.1 mmol, 2.0 equiv) and DMAP (0.085 mg, 0.005 mmol, 0.01 equiv). A small amount of DMF was needed for complete dissolution of the acid. This solution was stirred for 10 min before addition of aniline(7 mg, 0.075 mmol, 1.5 equiv). The reaction slurry was brought to 37 ˚C for 12 h before quenching with 1 N HCl solution (3 mL). Organics were separated and the solvent was removed under reduced pressure. Column chromatography (0 to 100% (EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd $C_{25}H_{24}F_3N_4O_2^+$ [M+H$^+$], 469.4877 found 470. 1H NMR (400 MHz, $CDCl_3$) δ 8.01 (s, 1H), 7.87 (t, *J* = 2.0, 1H), 7.67 - 7.60 (m, 3H), 7.51 (d, *J* = 7.9 Hz, 1H),

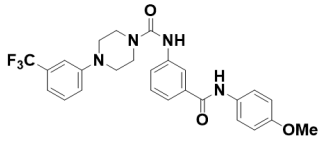
7.42 - 7.32 (m, 3H), 7.18 - 7.10 (m, 2H), 7.08 (d, *J* = 8.2 Hz, 1H), 6.81 (br, 1H), 3.72 - 3.64 (m, 4H), 3.32 - 3.22 (m, 4H).

N-(3-((4-Chlorophenyl)carbamoyl)phenyl)-4-(3-(trifluoromethyl)phenyl)piperazine-1-carboxamide (3.62)



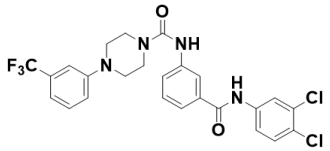
To a stirring solution of 3-(4-(3-(trifluoromethyl)phenyl)piperazine-1-carboxamido)benzoic acid (20 mg, 0.05 mmol, 1 equiv) in DCM (5 mL) was added EDC (21 mg, 0.1 mmol, 2.0 equiv) and DMAP (0.085 mg, 0.005 mmol0.01 equiv). A small amount of DMF was needed for complete dissolution of the acid. This solution was stirred for 10 min before addition of 4-chloroaniline (6 mg, 0.075 mmol, 1.5 equiv). The reaction slurry was brought to 37 ˚C for 12 h before quenching with 1 N HCl solution (3 mL). Organics were separated and the solvent was removed under reduced pressure. Column chromatography (0 to 100% (EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd $C_{25}H_{23}ClF_3N_4O_2^+$ [$M+H^+$], 503.9297 found 504. 1H NMR (400 MHz, $CDCl_3$) δ 8.12 (s, 1H), 7.87 (s, 1H), 7.62 - 7.54 (m, 3H), 7.50 (d, *J* = 7.8 Hz, 1H), 7.42 - 7.35 (m, 2H), 7.31 (d, *J* = 8.8 Hz, 2H), 7.15 (d, *J* = 7.8 Hz, 1H), 7.12 (s, 1H), 7.07 (d, *J* = 8.3 Hz, 1H), 6.75 (br, 1H), 3.74 - 3.62 (m, 4H), 3.35 - 3.21 (m, 4H).

N-(3-((4-Methoxyphenyl)carbamoyl)phenyl)-4-(3-(trifluoromethyl)phenyl)piperazine-1-carboxamide (3.63)



To a stirring solution of 3-(4-(3-(trifluoromethyl)phenyl)piperazine-1-carboxamido)benzoic acid (20 mg, 0.05 mmol, 1 equiv) in DCM (5 mL) was added EDC (21 mg, 0.1 mmol, 2.0 equiv) and DMAP (0.085 mg, 0.005 mmol, 0.01 equiv). A small amount of DMF was needed for complete dissolution of the acid. This solution was stirred for 10 min before addition of 4-methoxyaniline (7 mg, 0.075 mmol, 1.5 equiv). The reaction slurry was brought to 37 ˚C for 12 h before quenching with 1 N HCl solution (3 mL). Organics were separated and the solvent was removed under reduced pressure. Column chromatography (0 to 100% (EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd $C_{26}H_{26}F_3N_4O_3^+$ [M+H$^+$], 499.5137 found 500. 1H NMR (400 MHz, $CDCl_3$) δ 7.91 (t, *J* = 2.0 Hz, 1H), 7.77 - 7.71 (m, 2H), 7.43 - 7.36 (m, 2H), 7.18 - 7.09 (m, 2H), 6.52 (br, 1H), 3.91 (s, 3H), 3.75 - 3.68 (m, 4H), 3.36 - 3.29 (m, 4H).

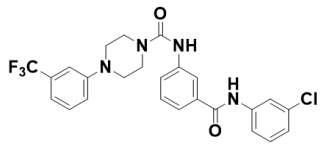
N-(3-((3,4-Dichlorophenyl)carbamoyl)phenyl)-4-(3-(trifluoromethyl)phenyl)piperazine-1-carboxamide (3.64)



To a stirring solution of 3-(4-(3-(trifluoromethyl)phenyl)piperazine-1-carboxamido)benzoic acid (20 mg, 0.05 mmol, 1 equiv) in DCM (5 mL) was added EDC

(21 mg, 0.1 mmmol, 2.0 equiv) and DMAP (0.085 mg, 0.005 mmol, 0.01 equiv). A small amount of DMF was needed for complete dissolution of the acid. This solution was stirred for 10 min before addition of 3,4-dichloroaniline (8 mg, 0.075 mmol, 1.5 equiv). The reaction slurry was brought to 37 ˚C for 12 h before quenching with 1 N HCl solution (3 mL). Organics were separated and the solvent was removed under reduced pressure. Column chromatography (0 to 100% (EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd $C_{25}H_{22}Cl_2F_3N_4O_2^+$ [M+H$^+$], 538.3717 found 540. 1H NMR (400 MHz, $CDCl_3$) δ 8.35 (br, 1H), 7.89 (d, *J* = 2.5 Hz, 1H), 7.83 (t, *J* = 2.0 Hz, 1H), 7.52 - 7.43 (m, 3H), 7.42 - 7.29 (m, 3H), 7.15 (d, *J* = 7.8 Hz, 1H), 7.13 (s, 1H), 7.09 (d, *J* = 8.3 Hz, 1H), 6.80 (br, 1H), 3.76 - 3.61 (m, 4H), 3.35 - 3.22 (m, 4H).

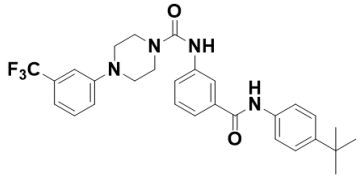
N-(3-((3-Chlorophenyl)carbamoyl)phenyl)-4-(3-(trifluoromethyl)phenyl)piperazine-1-carboxamide (3.65)



To a stirring solution of 3-(4-(3-(trifluoromethyl)phenyl)piperazine-1-carboxamido)benzoic acid (20 mg, 0.05 mmol, 1 equiv) in DCM (5 mL) was added EDC (21 mg, 0.10 mmol, 2.0 equiv) and DMAP (0.085 mg, 0.005 mmol, 0.01 equiv). A small amount of DMF was needed for complete dissolution of the acid. This solution was stirred for 10 min before addition of 3-chloroaniline (7 mg, 0.075 mmol, 1.5 equiv). The reaction slurry was brought to 37 ˚C for 12 h before quenching with 1 N HCl solution (3 mL). Organics were separated and the solvent was removed under reduced pressure. Column

chromatography (0 to 100% (EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd $C_{25}H_{23}ClF_3N_4O_2^+$ [M+H$^+$], 503.9297 found 505. 1H NMR (400 MHz, $CDCl_3$) δ 8.26 (s, 1H), 7.83 (t, *J* = 2.0 Hz, 1H), 7.77 (t, *J* = 2.1 Hz, 1H), 7.56 (d, *J* = 8.0, 1H), 7.50 - 7.44 (m, 2H), 7.42 - 7.31 (m, 2H), 7.26 (t, *J* = 7.0 Hz, 1H), 7.19 - 7.08 (m, 4H), 6.91 (s, 1H), 3.74 - 3.64 (m, 4H), 3.32 - 3.24 (m, 4H).

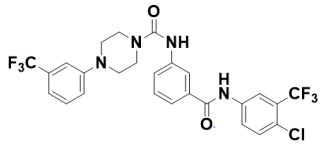
N-(3-((4-(tert-Butyl)phenyl)carbamoyl)phenyl)-4-(3-(trifluoromethyl)phenyl)piperazine-1-carboxamide (3.66)



To a stirring solution of 3-(4-(3-(trifluoromethyl)phenyl)piperazine-1-carboxamido)benzoic acid (20 mg, 0.05 mmol, 1 equiv) in DCM (5 mL) was added EDC (21 mg, 0.1 mmol, 2.0 equiv) and DMAP (0.085 mg, 0.005 mmol, 0.01 equiv). A small amount of DMF was needed for complete dissolution of the acid. This solution was stirred for 10 min before addition of 4-(tert-butyl)aniline (8 mg, 0.075 mmol, 1.5 equiv). The reaction slurry was brought to 37 ˚C for 12 h before quenching with 1 N HCl solution (3 mL). Organics were separated and the solvent was removed under reduced pressure. Column chromatography (0 to 100% (EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd $C_{29}H_{32}F_3N_4O_2^+$ [M+H$^+$], 525.5957 found 527. 1H NMR (400 MHz, $CDCl_3$) δ 7.92 (s, 1H), 7.87 (t, *J* = 1.9 Hz, 1H), 7.66 (d, *J* = 9.9 Hz, 1H), 7.58 - 7.49

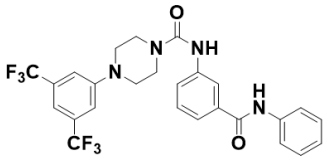
(m, 2H), 7.44 - 7.35 (m, 3H), 7.19 - 7.08 (m, 2H), 6.72 (br, 1H), 3.78 - 3.65 (m, 4H), 3.36 - 3.24 (m, 4H).

N-(3-((4-Chloro-3-(trifluoromethyl)phenyl)carbamoyl)phenyl)-4-(3-(trifluoromethyl)phenyl)piperazine-1-carboxamide (3.67)



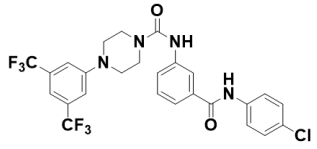
To a stirring solution of 3-(4-(3-(trifluoromethyl)phenyl)piperazine-1-carboxamido)benzoic acid (20 mg, 0.05 mmol, 1 equiv) in DCM (5 mL) was added EDC (21 mg, 0.10 mmol, 2.0 equiv) and DMAP (0.085 mg, 0.005 mmol, 0.01 equiv). A small amount of DMF was needed for complete dissolution of the acid. This solution was stirred for 10 min before addition of 4-chloro-3-(trifluoromethyl)aniline (10 mg, 0.075 mmol, 1.5 equiv). The reaction slurry was brought to 37 ˚C for 12 h before quenching with 1 N HCl solution (3 mL). Organics were separated and the solvent was removed under reduced pressure. Column chromatography (0 to 100% (EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd $C_{26}H_{22}ClF_6N_4O_2^+$ [M+H$^+$], 571.9279 found 572. 1H NMR (400 MHz, $CDCl_3$) δ 8.41 (s, 1H), 8.00 (d, J = 2.5 Hz, 1H), 7.93 (t, J = 1.9 Hz, 1H), 7.89 (dd, J_1 = 2.6, J_2 = 8.8 Hz, 1H), 7.54 (d, J = 7.6 Hz, 1H), 7.51 - 7.45 (m, 2H), 7.43 - 7.36 (m, 2H), 7.19 - 7.09 (m, 2H), 6.68 (br, 1H), 3.79 - 3.65 (m, 4H), 3.36 - 3.26 (m, 4H).

4-(3,5-bis(Trifluoromethyl)phenyl)-N-(3-(phenylcarbamoyl)phenyl)piperazine-1-carboxamide (3.68)



To a stirring solution of 3-(4-(3,5-bis(trifluoromethyl)phenyl)piperazine-1-carboxamido)benzoic acid (20 mg, 0.05 mmol, 1 equiv) in DCM (5 mL) was added EDC (13 mg, 0.1 mmol, 2.0 equiv) and DMAP (0.052 mg, 0.005 mmol, 0.01 equiv). A small amount of DMF was needed for complete dissolution of the acid. This solution was stirred for 10 min before addition of aniline (6 mg, 0.075 mol, 1.5 equiv). The reaction slurry was brought to 37 ˚C for 12 h before quenching with 1 N HCl solution (3 mL). Organics were separated and the solvent was removed under reduced pressure. Column chromatography (0 to 100% (EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd $C_{26}H_{23}F_6N_4O_2^+$ [M+H$^+$], 537.4859 found 537. 1H NMR (400 MHz, $CDCl_3$) δ 7.94 (s, 1H), 7.90 (t, *J* = 2.0 Hz, 1H), 7.69 - 7.60 (m, 3H), 7.54 (d, *J* = 7.7 Hz, 1H), 7.46 - 7.36 (m, 4H), 7.16 (t, *J* = 7.4 Hz, 1H), 6.69 (br, 1H), 3.79 - 3.68 (m, 4H), 3.43 - 3.35 (m, 4H).

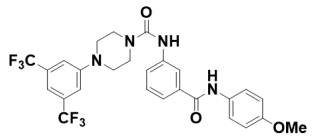
4-(3,5-bis(Trifluoromethyl)phenyl)-N-(3-((4-chlorophenyl)carbamoyl)phenyl)piperazine-1-carboxamide (3.69)



To a stirring solution of 3-(4-(3,5-bis(trifluoromethyl)phenyl)piperazine-1-carboxamido)benzoic acid (20 mg, 0.05 mmol, 1 equiv) in DCM (5 mL) was added EDC

(13 mg, 0.1 mmol, 2.0 equiv) and DMAP (0.052 mg, 0.005 mmol, 0.01 equiv). A small amount of DMF was needed for complete dissolution of the acid. This solution was stirred for 10 min before addition of 4-chloroaniline (8 mg, 0.075 mmol, 1.5 equiv). The reaction slurry was brought to 37 ˚C for 12 h before quenching with 1 N HCl solution (3 mL). Organics were separated and the solvent was removed under reduced pressure. Column chromatography (0 to 100% (EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd $C_{26}H_{22}ClF_6N_4O_2^+$ [M+H$^+$], 571.9279 found 572. 1H NMR (400 MHz, $CDCl_3$) δ 8.02 (s, 1H), 7.93 (s, 1H), 7.65 - 7.52 (m, 3H), 7.43 (t, *J* = 7.8 Hz, 1H), 7.39 - 7.30 (m, 2H), 6.63 (br, 1H), 3.82 - 3.67 (m, 4H), 3.47 - 3.33 (m, 4H).

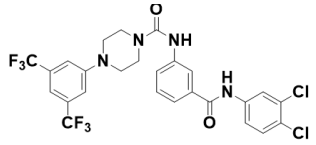
4-(3,5-bis(Trifluoromethyl)phenyl)-N-(3-((4-methoxyphenyl)carbamoyl)phenyl)piperazine-1-carboxamide (3.70)



To a stirring solution of 3-(4-(3,5-bis(trifluoromethyl)phenyl)piperazine-1-carboxamido)benzoic acid (20 mg, 0.05 mmol, 1 equiv) in DCM (5 mL) was added EDC (13 mg, 0.1 mmol, 2.0 equiv) and DMAP (0.052 mg, 0.005 mmol, 0.01 equiv). A small amount of DMF was needed for complete dissolution of the acid. This solution was stirred for 10 min before addition of 4-methoxyaniline (8 mg, 0.075 mmol, 1.5 equiv). The reaction slurry was brought to 37 ˚C for 12 h before quenching with 1 N HCl solution (3 mL). Organics were separated and the solvent was removed under reduced pressure. Column chromatography (0 to 100% (EtOAc/Hex) on silica gel was used to purify

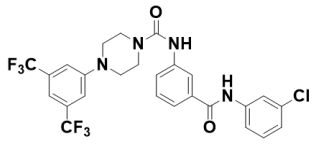
sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd $C_{27}H_{25}F_6N_4O_3^+$ [M+H$^+$], 567.5119 found 567. 1H NMR (400 MHz, $CDCl_3$) δ 7.88 (s, 1H), 7.81 (s, 1H), 7.64 (d, *J* = 8.0 Hz, 1H), 7.58 - 7.49 (m, 3H), 7.43 (t, *J* = 7.9 Hz, 1H), 7.35 (s, 1H), 6.91 (d, *J* = 8.8 Hz, 2H), 6.55 (br, 1H), 3.82 (s, 3H), 3.77 - 3.69 (m, 4H), 3.45 - 3.36 (m, 4H).

4-(3,5-bis(Trifluoromethyl)phenyl)-N-(3-((3,4-dichlorophenyl)carbamoyl)phenyl)piperazine-1-carboxamide (3.71)



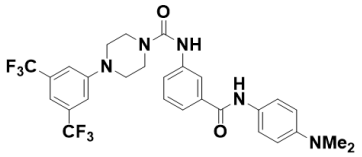
To a stirring solution of 3-(4-(3,5-bis(trifluoromethyl)phenyl)piperazine-1-carboxamido)benzoic acid (20 mg, 0.05 mmol, 1 equiv) in DCM (5 mL) was added EDC (13 mg, 0.1 mmol, 2.0 equiv) and DMAP (0.052 mg, 0.005 mmol, 0.01 equiv). A small amount of DMF was needed for complete dissolution of the acid. This solution was stirred for 10 min before addition of 3,4-dichloroaniline (11 mg, 0.075 mmol, 1.5 equiv). The reaction slurry was brought to 37 ˚C for 12 h before quenching with 1 N HCl solution (3 mL). Organics were separated and the solvent was removed under reduced pressure. Column chromatography (0 to 100% (EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd $C_{26}H_{21}Cl_2F_6N_4O_2^+$ [M+H$^+$], 606.3699 found 607. 1H NMR (400 MHz, $CDCl_3$) δ 8.83 (s, 1H), 7.89 (d, *J* = 2.4 Hz, 1H), 7.84 (s, 1H), 7.56 - 7.44 (m, 3H), 7.42 - 7.32 (m, 3H), 7.27 (s, 1H), 6.84 (br, 1H), 3.80 - 3.65 (m, 4H), 3.43 - 3.29 (m, 4H).

4-(3,5-bis(Trifluoromethyl)phenyl)-N-(3-((3-chlorophenyl)carbamoyl)phenyl)piperazine-1-carboxamide (3.72)



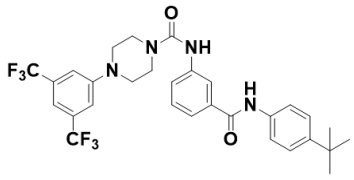
To a stirring solution of 3-(4-(3,5-bis(trifluoromethyl)phenyl)piperazine-1-carboxamido)benzoic acid (20 mg, 0.05 mmol, 1 equiv) in DCM (5 mL) was added EDC (13 mg, 0.1 mmol, 2.0 equiv) and DMAP (0.052 mg, 0.005 mmo, 0.01 equiv). A small amount of DMF was needed for complete dissolution of the acid. This solution was stirred for 10 min before addition of 3,4-dichloroaniline (8 mg, 0.075 mmol, 1.5 equiv). The reaction slurry was brought to 37 ˚C for 12 h before quenching with 1 N HCl solution (3 mL). Organics were separated and the solvent was removed under reduced pressure. Column chromatography (0 to 100% (EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd $C_{26}H_{22}ClF_6N_4O_2^+$ [M+H$^+$], 571.9279 found 572. 1H NMR (400 MHz, $CDCl_3$) δ 8.07 (s, 1H), 7.88 (t, J = 2.0 Hz, 1H), 7.78 (t, J = 2.1 Hz, 1H), 7.60 (d, J = 8.0 Hz, 1H), 7.54 - 7.46 (m, 2H), 7.41 (t J = 7.9 Hz, 1H), 7.36 (s, 1H), 7.29 (d, J = 8.1 Hz, 1H), 7.13 (ddd, J_1 = 1.0, J_2 = 3.0, J_3 = 8.0 Hz, 1H), 6.71 (br, 1H), 3.83 - 3.66 (m, 4H), 3.46 - 3.30 (m, 4H).

4-(3,5-bis(Trifluoromethyl)phenyl)-N-(3-((4-(dimethylamino)phenyl)-carbamoyl)phenyl)piperazine-1-carboxamide (3.73)



To a stirring solution of 3-(4-(3,5-bis(trifluoromethyl)phenyl)piperazine-1-carboxamido)benzoic acid (20 mg, 0.05 mmol, 1 equiv) in DCM (5 mL) was added EDC (13 mg, 0.1 mmol, 2.0 equiv) and DMAP (0.052 mg, 0.005 mmol, 0.01 equiv). A small amount of DMF was needed for complete dissolution of the acid. This solution was stirred for 10 min before addition of N1,N1-dimethylbenzene-1,4-diamine (9 mg, 0.075 mmol, 1.5 equiv). The reaction slurry was brought to 37 ˚C for 12 h before quenching with 1 N HCl solution (3 mL). Organics were separated and the solvent was removed under reduced pressure. Column chromatography (0 to 100% (EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd $C_{28}H_{28}F_6N_5O_2^+$ [M+H$^+$], 580.5549 found 580. 1H NMR (400 MHz, $CDCl_3$) δ 7.91 (s, 1H), 7.69 (d, *J* = 7.9 Hz, 1H), 7.58 - 7.50 (m, 2H), 7.40 (t, *J* = 8.0 Hz, 1H), 7.34 (s, 1H), 7.25 (s, 1H), 3.80 - 3.65 (m, 4H), 3.42 - 3.33 (m, 4H), 2.98 (s, 6H).

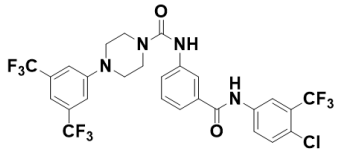
4-(3,5-bis(Trifluoromethyl)phenyl)-N-(3-((4-(tert-butyl)phenyl)carbamoyl)phenyl)piperazine-1-carboxamide (3.74)



To a stirring solution of 3-(4-(3,5-bis(trifluoromethyl)phenyl)piperazine-1-carboxamido)benzoic acid (20 mg, 0.05 mmol, 1 equiv) in DCM (5 mL) was added EDC (13 mg, 0.1 mmol, 2.0 equiv) and DMAP (0.052 mg, 0.005 mmol, 0.01 equiv). A small amount of DMF was needed for complete dissolution of the acid. This solution was stirred for 10 min before addition of 4-(tert-butyl)aniline (10 mg, 0.075 mmol, 1.5 equiv). The

reaction slurry was brought to 37 ˚C for 12 h before quenching with 1 N HCl solution (3 mL). Organics were separated and the solvent was removed under reduced pressure. Column chromatography (0 to 100% (EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd $C_{30}H_{31}F_6N_4O_2^+$ [M+H$^+$], 593.5939 found 594. 1H NMR (400 MHz, $CDCl_3$) δ 7.91 - 7.83 (m, 2H), 7.68 (d, *J* = 8.5 Hz, 1H), 7.57 - 7.51 (m, 3H), 7.47 - 7.33 (m, 4H), 6.61 (br, 1H), 3.78 - 3.69 (m, 4H), 3.45 - 3.35 (m, 4H), 1.32 (s, 9H).

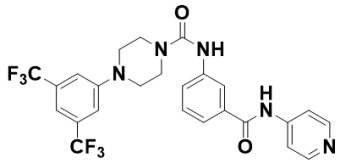
4-(3,5-bis(Trifluoromethyl)phenyl)-N-(3-((4-chloro-3-(trifluoromethyl)phenyl)carbamoyl)phenyl)piperazine-1-carboxamide (3.75)



To a stirring solution of 3-(4-(3,5-bis(trifluoromethyl)phenyl)piperazine-1-carboxamido)benzoic acid (20 mg, 0.05 mmol, 1 equiv) in DCM (5 mL) was added EDC (13 mg, 0.1 mmol, 2.0 equiv) and DMAP (0.052 mg, 0.005 mmol, 0.01 equiv). A small amount of DMF was needed for complete dissolution of the acid. This solution was stirred for 10 min before addition of 4-chloro-3-(trifluoromethyl)aniline (13 mg, 0.075 mmol, 1.5 equiv). The reaction slurry was brought to 37 ˚C for 12 h before quenching with 1 N HCl solution (3 mL). Organics were separated and the solvent was removed under reduced pressure. Column chromatography (0 to 100% (EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd $C_{27}H_{21}ClF_9N_4O_2^+$ [M+H$^+$], 639.9261 found 695. 1H NMR (400 MHz, $CDCl_3$) δ 8.34 (s, 1H), 7.99 (d, *J* = 2.5 Hz, 1H), 7.93 (s, 1H), 7.88 (dd, J_1 = 2.6,

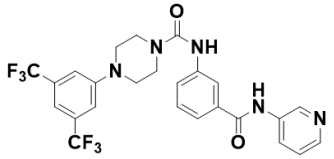
J_2 = 8.8 Hz, 1H), 7.57 - 7.45 (m, 3H) 7.41 (t, J = 7.8 Hz, 1H), 7.36 (s, 1H), 7.27 (s, 2H), 6.67 (br, 1H), 3.79 - 3.71 (m, 4H), 3.44 - 3.35 (m, 4H).

4-(3,5-bis(Trifluoromethyl)phenyl)-N-(3-(pyridin-4-ylcarbamoyl)phenyl)piperazine-1-carboxamide (3.76)



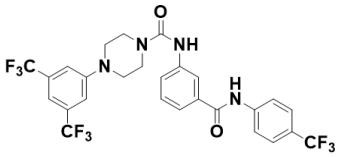
To a stirring solution of 3-(4-(3,5-bis(trifluoromethyl)phenyl)piperazine-1-carboxamido)benzoic acid (20 mg, 0.05 mmol, 1 equiv) in DCM (5 mL) was added EDC (13 mg, 0.1 mmol, 2.0 equiv) and DMAP (0.052 mg, 0.005mmol, 0.01 equiv). A small amount of DMF was needed for complete dissolution of the acid. This solution was stirred for 10 min before addition of pyridin-4-amine (6 mg, 0.075 mmol, 1.5 equiv). The reaction slurry was brought to 37 ˚C for 12 h before quenching with 1 N HCl solution (3 mL). Organics were separated and the solvent was removed under reduced pressure. Column chromatography (0 to 100% (EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd $C_{25}H_{22}F_6N_5O_2^+$ [M+H$^+$], 538.4739 found 539. 1H NMR (400 MHz, $CDCl_3$) δ 8.54 (d, J = 5.4 Hz, 1H) 8.03 - 7.89 (m, 2H), 7.64 (d, J = 6.5 Hz, 1H), 7.55 (t, J = 6.9 Hz, 1H), 7.46 - 7.32 (m, 3H), 6.70 (br, 1H), 3.80 - 3.66 (m, 4H), 3.45 - 3.32 (m, 4H).

4-(3,5-bis(Trifluoromethyl)phenyl)-N-(3-(pyridin-3-ylcarbamoyl)phenyl)piperazine-1-carboxamide (3.77)



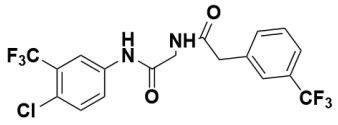
To a stirring solution of 3-(4-(3,5-bis(trifluoromethyl)phenyl)piperazine-1-carboxamido)benzoic acid (20 mg, 0.05 mmol, 1 equiv) in DCM (5 mL) was added EDC (13 mg, 0.1 mmol, 2.0 equiv) and DMAP (0.052 mg, 0.01 equiv). A small amount of DMF was needed for complete dissolution of the acid. This solution was stirred for 10 min before addition of pyridin-3-amine (6 mg, 0.075 mmol, 1.5 equiv). The reaction slurry was brought to 37 ˚C for 12 h before quenching with 1 N HCl solution (3 mL). Organics were separated and the solvent was removed under reduced pressure. Column chromatography (0 to 100% (EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd $C_{25}H_{22}F_6N_5O_2^+$ [M+H$^+$], 538.4739 found 539. 1H NMR (400 MHz, $CDCl_3$) δ 11.1 (s, 1H), 9.37 (s, 1H), 9.14 (s, 1H), 8.29 (br, 1H), 8.16 (d, *J* = 5.6 Hz, 1H), 8.11 (br, 1H), 7.70 (s, 1H), 7.63 (d, *J* = 8.2, 1H), 7.58 (d, *J* = 7.8 Hz, 1H), 7.32 (s, 1H), 7.21 (s, 2H), 7.10 (t, *J* = 7.8 Hz, 1H), 3.76 (br, 4H), 3.29 (br, 4H).

4-(3,5-bis(Trifluoromethyl)phenyl)-N-(3-((4-(trifluoromethyl)phenyl)carbamoyl)phenyl)piperazine-1-carboxamide (3.78)



To a stirring solution of 3-(4-(3,5-bis(trifluoromethyl)phenyl)piperazine-1-carboxamido)benzoic acid (20 mg, 0.05 mmol, 1 equiv) in DCM (5 mL) was added EDC (13 mg, 0.1 mmol, 2.0 equiv) and DMAP (0.052 mg, 0.005 mmol, 0.01 equiv). A small amount of DMF was needed for complete dissolution of the acid. This solution was stirred for 10 min before addition of 4-(trifluoromethyl)aniline (10 mg, 0.075 mmol, 1.5 equiv). The reaction slurry was brought to 37 ˚C for 12 h before quenching with 1 N HCl solution (3 mL). Organics were separated and the solvent was removed under reduced pressure. Column chromatography (0 to 100% (EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd $C_{27}H_{22}F_9N_4O_2^+$ [M+H$^+$], 605.4841 found 606. 1H NMR (400 MHz, $CDCl_3$) δ 8.18 (s, 1H), 7.96 (s, 1H), 7.79 (d, *J* = 8.3 Hz, 2H), 7.63 (d, *J* = 8.2 Hz, 2H), 7.57 (d, *J* = 7.5 Hz, 1H), 7.48 -7.37 (m, 2H), 7.19 - 7.06 (m, 2H), 6.58 (br, 1H), 3.73 (br, 4H), 3.33 (br, 4H).

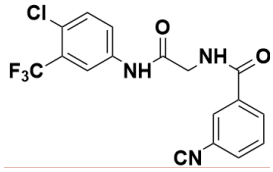
N-(4-Chloro-3-(trifluoromethyl)phenyl)-2-(2-(3-(trifluoromethyl)phenyl)acetamido)acetamide (3.79)



To a stirring solution of 2-amino-N-(4-chloro-3-(trifluoromethyl)phenyl)acetamide (38 mg, 0.14 mmol, 1.0 equiv), triethylamine (1.8 µL, 0.013 mmol, 0.1 equiv) in DCM (5 mL) was added 2-(3-(trifluoromethyl)phenyl)acetyl chloride (40 mg, 0.17 mmol,1.2 equiv) and the solution was stirred for 30 min at RT. After confirming consumption of limiting reagent by TLC, 3 mL of distilled water was added to the reaction vial. The organic layer was separated and removed under reduced atmosphere. Column chromatography (0 to 50%

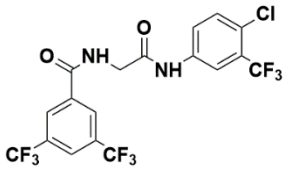
EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{18}H_{14}ClF_6N_2O_2^+$ [M+H$^+$] 439.7619, found 439. 1H NMR (400 MHz, CDCl3) δ 8.86 (s, 1H), 7.85 (d, *J* = 2.6 Hz, 1H), 7.61 - 7.53 (m, 3H), 7.51 - 7.46 (m, 2H), 7.39 (d, *J* = 8.8 Hz, 1H), 6.52 (s, 1H), 4.11 (d, *J* = 5.3 Hz, 2H), 3.72 (s, 4H).

N-(4-Chloro-3-(trifluoromethyl)phenyl)-2-(3-(3-cyanophenyl)ureido)acetamide (3.80)



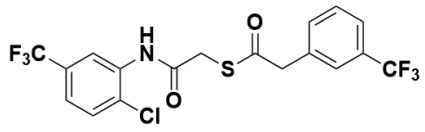
To a stirring solution of 3-cyanobenzoic acid (15 mg, 0.12 mmol, 1 equiv) in DCM (5 mL) was added EDC (32 mg, 0.24 mmol, 2.0 equiv) and DMAP (32 mg, 0.30 mmol, 2.5 equiv). This solution was stirred for 10 min before addition of 2-amino-N-(4-chloro-3-(trifluoromethyl)phenyl)acetamide (40 mg, 0.16 mmol,1.5 equiv). The reaction slurry was brought to 37 ˚C for 12 h before quenching with 1 N HCl solution (3 mL). Organics were separated and the solvent was removed under reduced pressure. Column chromatography (0 to 45% (EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{17}H_{13}ClF_3N_4O_2^+$ [M+H$^+$] 397.7617, found 398. 1H NMR (400 MHz, CD_3OD) δ 8.14 (t, *J* = 1.7 Hz, 1H), 8.08 (td, J_1 = 1.4, J_2 = 6.6 Hz, 1H), 8.0 (d, *J* = 2.6 Hz, 1H), 7.82 (td, J_1 = 1.4, J_2 = 7.8 Hz, 1H), 7.69 (dd, J_1 = 2.6, J_2 = 8.8 Hz, 1H), 7.58 (t, *J* = 7.8 Hz, 1H), 7.43 (d, *J* = 8.7 Hz, 1H), 4.12 (s, 2H).

2-(3-(3,5-bis(Trifluoromethyl)phenyl)ureido)-N-(4-chloro-3-(trifluoromethyl)phenyl)acetamide (3.81)



To a stirring solution of 3,5-bistrifluoromethylbenzoic acid (27 mg, 0.12 mmol, 1 equiv) in DCM (5 mL) was added EDC (32 mg, 0.24 mmol, 2.0 equiv) and DMAP (32 mg, 0.30 mmol, 2.5 equiv). This solution was stirred for 10 min before addition of 2-amino-N-(4-chloro-3-(trifluoromethyl)phenyl)acetamide (40 mg, 0.16 mmol,1.5 equiv). The reaction slurry was brought to 37 ˚C for 12 h before quenching with 1 N HCl solution (3 mL). Organics were separated and the solvent was removed under reduced pressure. Column chromatography (0 to 45% (EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{18}H_{12}ClF_9N_3O_2^+$ [M+H$^+$] 508.7481, found 509. 1H NMR (400 MHz, CD_3OD) δ 8.58 (s, 2H), 8.20 (s, 1H), 8.13 (d, J = 2.6 Hz, 1H), 7.82 (dd, J_1 = 2.6, J_2 = 8.8 Hz, 1H), 7.56 (d, J = 8.8 Hz, 1H), 4.27 (s, 3H).

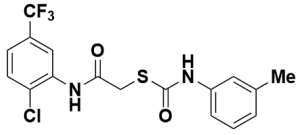
S-(2-((2-Chloro-5-(trifluoromethyl)phenyl)amino)-2-oxoethyl) 2-(3-(Trifluoromethyl)phenyl)ethanethioate (3.82)



To a stirring solution of N-(2-chloro-5-(trifluoromethyl)phenyl)-2-mercaptoacetamide (40 mg, 0.15 mmol, 1.0 equiv), triethylamine (1.8 µL, 0.013 mmol, 0.1 equiv) in DCM (5 mL) was added 2-(3-(trifluoromethyl)phenyl)acetyl chloride (40 mg, 0.18 mmol, 1.2 equiv) and

the solution was stirred for 30 min at RT. After confirming consumption of limiting reagent by TLC, 3 mL of distilled water was added to the reaction vial. The organic layer was separated and removed under reduced atmosphere. Column chromatography (0 to 50% EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{18}H_{14}ClF_6N_2O_2^+$ [M+H$^+$] 439.7619, found 440. 1H NMR (400 MHz, CDCl3) δ 8.67 (d, *J* = 2.0 Hz, 1H), 8.53 (br, 1H), 7.62 - 7.56 (m, 2H), 7.52 - 7.45 (m, 3H), 7.29 (dd, J_1 = 2.1, J_2 = 8.4 Hz, 1H), 4.01 (s, 2H), 3.75 (s, 2H).

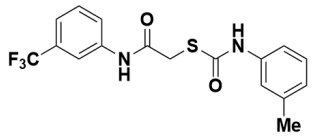
S-(2-((2-Chloro-5-(trifluoromethyl)phenyl)amino)-2-oxoethyl) m-tolylcarbamothioate (3.83)



To a stirring solution of N-(2-chloro-5-(trifluoromethyl)phenyl)-2-mercaptoacetamide (30 mg, 0.10 mmol, 1.0 equiv), triethylamine (1.7 µL, 0.01 mmol, 0.1 equiv) in DCM (5 mL) was added 1-isocyanato-3-methylbenzene (20 mg, 0.12 mmol, 1.2 equiv) and the solution was stirred for 30 min at RT. After confirming consumption of limiting reagent by TLC, 3 mL of distilled water was added to the reaction vial. The organic layer was separated and removed under reduced atmosphere. Column chromatography (0 to 50% EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd $C_{17}H_{15}ClF_3N_2O_2S^+$ [M+H$^+$], 403.8237 found 404. 1H NMR (400 MHz, CD_3OD) δ 8.49 (s, 1H), 7.64 (d, *J* = 8.4 Hz,

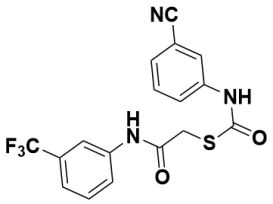
1H), 7.43 (dd, J_1 = 2.1, J_2 = 8.4 Hz, 1H), 7.36 - 7.29 (m, 2H), 7.18 (t, J = 7.7 Hz, 1H), 6.93 (d, J = 7.6 Hz, 1H), 3.89 (s, 2H), 2.32 (s, 3H).

S-(2-Oxo-2-((3-(trifluoromethyl)phenyl)amino)ethyl) m-tolylcarbamothioate (3.84)



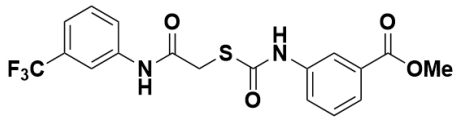
To a stirring solution of 2-mercapto-N-(3-(trifluoromethyl)phenyl)acetamide (30 mg, 0.10 mmol, 1.0 equiv), triethylamine (1.7 µL, 0.010 mmol, 0.1 equiv) in DCM (5 mL) was added 1-isocyanato-3-methylbenzene (20 mg, 0.12 mmol, 1.2 equiv) and the solution was stirred for 30 min at RT. After confirming consumption of limiting reagent by TLC, 3 mL of distilled water was added to the reaction vial. The organic layer was separated and removed under reduced atmosphere. Column chromatography (0 to 50% EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd $C_{17}H_{16}F_3N_2O_2S^+$ [M+H$^+$], 369.3817 found 370. 1H NMR (400 MHz, CD_3OD) δ 8.04 (s, 1H), 7.77 (br, 1H), 7.51 (d, J = 8.4 Hz, 1H), 7.40 (d, J = 7.6 Hz, 1H), 7.34 - 7.25 (m, 2H), 7.18 (t, J = 7.8 Hz, 1H), 6.92 (d, J = 7.4 Hz, 1H) 3.38 (s, 2H), 2.32 (s, 3H).

S-(2-Oxo-2-((3-(trifluoromethyl)phenyl)amino)ethyl) (3-cyanophenyl)carbamothioate (3.85)



To a stirring solution of 2-mercapto-N-(3-(trifluoromethyl)phenyl)acetamide (36 mg, 0.15 mmol, 1.0 equiv), triethylamine (1.8 µL, 0.013 mmol, 0.1 equiv) in DCM (5 mL) was added 3-cyanophenylisocyanate (40 mg, 0.18 mmol, 1.2 equiv) and the solution was stirred for 30 min at RT. After confirming consumption of limiting reagent by TLC, 3 mL of distilled water was added to the reaction vial. The organic layer was separated and removed under reduced atmosphere. Column chromatography (0 to 50% EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{17}H_{13}F_3N_3O_2S^+$ [M+H$^+$] 380.3647, found 381. 1H NMR (400 MHz, CD_3OD) δ 7.93 (br, 1H), 7.85 (t, *J* = 1.9 Hz, 1H), 7.68 - 7.62 (m, 2H), 7.44 - 7.27 (m, 4H), 3.80 (s, 2H).

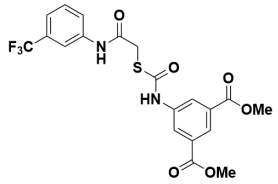
Methyl 3-((((2-oxo-2-((3-(trifluoromethyl)phenyl)amino)ethyl)thio)carbonyl)amino)benzoate (3.86)



To a stirring solution of 2-mercapto-N-(3-(trifluoromethyl)phenyl)acetamide (36 mg, 0.15 mmol, 1.0 equiv), triethylamine (1.8 µL, 0.013 mmol, 0.1 equiv) in DCM (5 mL) was added 4-isocyanato-1-methyl-1H-indole (40 mg, 0.19 mmol, 1.2 equiv) and the solution was stirred for 30 min at RT. After confirming consumption of limiting reagent by TLC, 3 mL of distilled water was added to the reaction vial. The organic layer was separated and removed under reduced atmosphere. Column chromatography (0 to 50% EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{18}H_{16}F_3N_2O_4S^+$ [M+H$^+$]

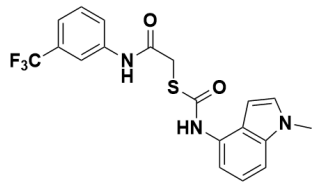
413.3907, found 414. 1H NMR (400 MHz, CD_3OD) δ 8.08 (s, 1H), 7.92 (s, 1H), 7.70 - 7.60 (m, 3H), 7.40 (t, *J* = 8.0 Hz, 1H), 7.33 - 7.25 (m, 2H), 3.79 (s, 3H), 3.78 (s, 2H).

Dimethyl 5-((((2-oxo-2-((3-(trifluoromethyl)phenyl)amino)ethyl)thio)carbonyl)amino)isophthalate (3.87)



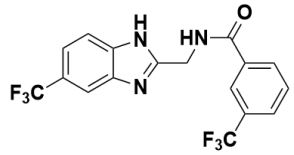
To a stirring solution of 2-mercapto-N-(3-(trifluoromethyl)phenyl)acetamide (36 mg, 0.15 mmol, 1.0 equiv), triethylamine (1.8 µL, 0.013 mmol, 0.1 equiv) in DCM (5 mL) was added 4-isocyanato-1-methyl-1H-indole (43 mg, 0.19 mmol, 1.2 equiv) and the solution was stirred for 30 min at RT. After confirming consumption of limiting reagent by TLC, 3 mL of distilled water was added to the reaction vial. The organic layer was separated and removed under reduced atmosphere. Column chromatography (0 to 50% EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{20}H_{18}F_3N_2O_6S^+$ [$M+H^+$] 471.4267, found 471. 1H NMR (400 MHz, CD_3OD) δ 8.30 (d, *J* = 1.4 Hz, 2H), 8.22 (s, 1H), 7.93 (s, 1H), 7.68 (d, *J* = 8.2 Hz, 1H), 7.41 (t, *J* = 8.0 Hz, 1H), 7.29 (d, *J* = 7.7 Hz, 1H), 3.83 (s, 6H), 3.81 (s, 2H).

S-(2-Oxo-2-((3-(trifluoromethyl)phenyl)amino)ethyl) (1-methyl-1H-indol-4-yl)carbamothioate (3.88)



To a stirring solution of 2-mercapto-N-(3-(trifluoromethyl)phenyl)acetamide (36 mg, 0.15 mmol, 1.0 equiv), triethylamine (1.8 µL, 0.013 mmol, 0.1 equiv) in DCM (5 mL) was added 4-isocyanato-1-methyl-1H-indole (42 mg, 0.18 mmol, 1.2 equiv) and the solution was stirred for 30 min at RT. After confirming consumption of limiting reagent by TLC, 3 mL of distilled water was added to the reaction vial. The organic layer was separated and removed under reduced atmosphere. Column chromatography (0 to 50% EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{19}H_{17}F_3N_3O_2S^+$ [M+H$^+$] 408.4187, found 408. 1H NMR (400 MHz, CD_3OD) δ 7.94 (s, 1H), 7.66 (d, *J* = 4.3 Hz, 1H), 7.39 (t, *J* = 7.96, 1H), 7.31 - 7.19 (m, 2H), 7.11 (d, *J* = 8.2 Hz, 1H), 7.05 - 6.99 (m, 2H), 6.46 (d, *J* = 3.2 Hz, 1H), 3.76 (s, 2H), 3.68 (s, 3H).

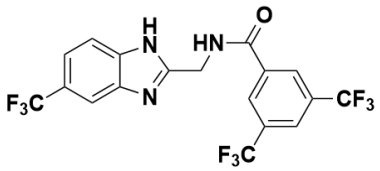
3-(Trifluoromethyl)-N-((5-(trifluoromethyl)-1H-benzo[d]imidazol-2-yl)methyl)benzamide (3.90)



To a stirring solution of 3-(trifluoromethyl)benzoic acid (20 mg, 0.11 mmol, 1 equiv) in DCM (5 mL) was added EDC (36 mg, 0.22 mmol, 2.0 equiv) and DMAP (35 mg, 0.26 mmol, 2.5 equiv). This solution was stirred for 10 min before addition of (5-

(trifluoromethyl)-1H-benzo[d]imidazol-2-yl)methanamine (30 mg, 0.16 mmol, 1.5 equiv). The reaction slurry was brought to 37 ˚C for 12 h before quenching with 1 N HCl solution (3 mL). Organics were separated and the solvent was removed under reduced pressure. Column chromatography (0 to 45% (EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{17}H_{12}F_6N_3O^+$ [M+H$^+$] 388.2929, found 388. 1H NMR (400 MHz, CD_3OD) δ 8.98 (s, 1H), 8.23 (s, 1H), 8.12 (d, *J* = 7.8 Hz, 1H), 7.83 (s, 1H), 7.77 (d, *J* = 7.8 Hz, 1H), 7.66 - 7.47 (m, 3H), 4.89 (d, *J* = 5.7 Hz, 2H).

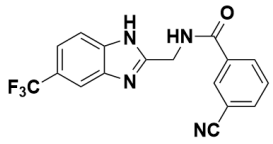
3,5-bis(Trifluoromethyl)-N-((5-(trifluoromethyl)-1H-benzo[d]imidazol-2-yl)methyl)benzamide (3.91)



To a stirring solution of 3,5-bis(trifluoromethyl)benzoic acid (20 mg, 0.11 mmol, 1 equiv) in DCM (5 mL) was added EDC (36 mg, 0.22 mmol, 2.0 equiv) and DMAP (35 mg, 0.26 mmol, 2.5 equiv). This solution was stirred for 10 min before addition of (5-(trifluoromethyl)-1H-benzo[d]imidazol-2-yl)methanamine (30 mg, 0.16 mmol, 1.5 equiv). The reaction slurry was brought to 37 ˚C for 12 h before quenching with 1 N HCl solution (3 mL). Organics were separated and the solvent was removed under reduced pressure. Column chromatography (0 to 45% (EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{18}H_{11}F_9N_3O^+$ [M+H$^+$] 456.2911, found 456. 1H NMR (400 MHz,

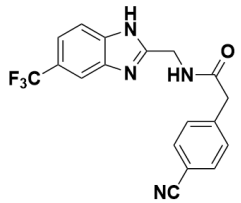
$CDCl_3$) δ 8.49 (br, 1H), 8.42 (s, 1H), 8.05 (s, 1H), 7.87 (s, 1H), 7.64 (d, *J* = 7.9 Hz, 1H), 7.54 (d, *J* = 7.8 Hz, 1H), 4.89 (d, *J* = 5.7 Hz, 2H).

3-Cyano-N-((5-(trifluoromethyl)-1H-benzo[d]imidazol-2-yl)methyl)benzamide (3.92)



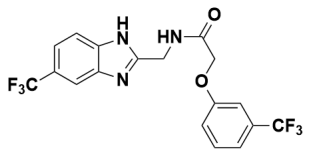
To a stirring solution of 3-cyanobenzoic acid (20 mg, 0.11 mmol, 1 equiv) in DCM (5 mL) was added EDC (36 mg, 0.22 mmol, 2.0 equiv) and DMAP (35 mg, 0.26 mmol, 2.5 equiv). This solution was stirred for 10 min before addition of (5-(trifluoromethyl)-1H-benzo[d]imidazol-2-yl)methanamine (30 mg, 0.16 mmol, 1.5 equiv). The reaction slurry was brought to 37 ˚C for 12 h before quenching with 1 N HCl solution (3 mL). Organics were separated and the solvent was removed under reduced pressure. Column chromatography (0 to 45% (EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{17}H_{12}F_3N_4O^+$ [M+H^+] 345.3047, found 346. 1H NMR (400 MHz, $CDCl_3$) δ 8.58 (t, *J* = 5.9 Hz, 1H), 8.25 (s, 1H), 8.16 (d, *J* = 7.9 Hz, 1H), 7.82 (d, *J* = 7.7 Hz, 1H), 7.59 (d, *J* = 7.9 Hz, 1H), 7.54 (t, *J* = 8.7 Hz, 1H), 4.87 (d, *J* = 5.7 Hz, 2H).

2-(4-Cyanophenyl)-N-((5-(trifluoromethyl)-1H-benzo[d]imidazol-2-yl)methyl)acetamide (3.93)



To a stirring solution of 2-(4-cyanophenyl)acetic acid (20 mg, 0.11 mmol, 1 equiv) in DCM (5 mL) was added EDC (36 mg, 0.22 mmol, 2.0 equiv) and DMAP (35 mg, 0.20 mmol, 2.5 equiv). This solution was stirred for 10 min before addition of (5-(trifluoromethyl)-1H-benzo[d]imidazol-2-yl)methanamine (30 mg, 0.16 mmol, 1.5 equiv). The reaction slurry was brought to 37 ˚C for 12 h before quenching with 1 N HCl solution (3 mL). Organics were separated and the solvent was removed under reduced pressure. Column chromatography (0 to 45% (EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{18}H_{14}F_3N_4O^+$ [M+H$^+$] 359.3317, found 359. 1H NMR (400 MHz, $CDCl_3$) δ 10.43 (br, 1H), 7.64 (d, *J* = 8.2 Hz, 2H), 7.52 (s, 1H), 7.38 (d, *J* = 8.1 Hz, 2H), 6.81 (br, 1H), 4.62 (d, *J* = 5.9 Hz, 2H), 3.69 (s, 2H).

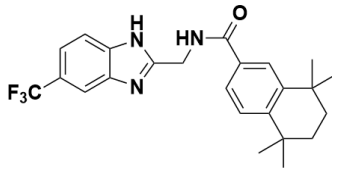
N-((5-(Trifluoromethyl)-1H-benzo[d]imidazol-2-yl)methyl)-2-(3-(trifluoromethyl)phenoxy)acetamide (3.94)



To a stirring solution of 2-(3-(trifluoromethyl)phenoxy)acetic acid (20 mg, 0.11 mmol, 1 equiv) in DCM (5 mL) was added EDC (36 mg, 0.22 mmol, 2.0 equiv) and DMAP (35 mg, 0.26 mmol, 2.5 equiv). This solution was stirred for 10 min before addition of (5-(trifluoromethyl)-1H-benzo[d]imidazol-2-yl)methanamine (30 mg, 0.16 mmol, 1.5 equiv). The reaction slurry was brought to 37 ˚C for 12 h before quenching with 1 N HCl solution (3 mL). Organics were separated and the solvent was removed under reduced pressure. Column chromatography (0 to 45% (EtOAc/Hex) on silica gel was used to purify sufficient

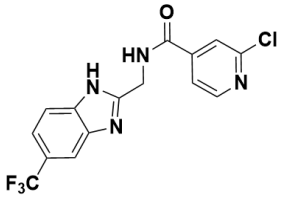
amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{18}H_{14}F_6N_3O_2^+$ [M+H$^+$] 418.3189, found 419. 1H NMR (400 MHz, $CDCl_3$) δ 10.94 (br, 1H), 8.09 (s, 1H), 7.95 (br, 1H), 7.74 (br, 1H), 7.51 (s, 1H), 7.39 (t, *J* = 8.0 Hz, 1H), 7.09 (s, 1H), 7.03 (d, *J* = 8.2, 1H), 4.77 (d, *J* = 6.1, 2H), 4.58 (s, 3H).

5,5,8,8-Tetramethyl-N-((5-(trifluoromethyl)-1H-benzo[d]imidazol-2-yl)methyl)-5,6,7,8-tetrahydronaphthalene-2-carboxamide (3.95)



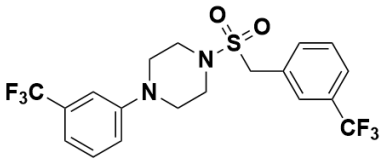
To a stirring solution of 5,5,8,8-tetramethyl-5,6,7,8-tetrahydronaphthalene-2-carboxylic acid (41 mg, 0.11 mmol, 1 equiv) in DCM (5 mL) was added EDC (36 mg, 0.22 mmol, 2.0 equiv) and DMAP (35 mg, 0.20 mmol, 2.5 equiv). This solution was stirred for 10 min before addition of (5-(trifluoromethyl)-1H-benzo[d]imidazol-2-yl)methanamine (30 mg, 0.16 mmol, 1.5 equiv). The reaction slurry was brought to 37 ˚C for 12 h before quenching with 1 N HCl solution (3 mL). Organics were separated and the solvent was removed under reduced pressure. Column chromatography (0 to 45% (EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{24}H_{27}F_3N_3O^+$ [M+H$^+$] 430.4947, found 431. 1H NMR (400 MHz, $CDCl_3$) δ 8.00 (t, *J* = 8.0 Hz, 1H), 7.86 (d, *J* = 1.9 Hz, 2H), 7.60 (dd, J_1 = 2.1, J_2 = 8.3 Hz, 2H), 7.50 (d, *J* = 8.6 Hz, 1H), 7.35 (d, *J* = 8.2 Hz, 1H), 4.87 (d, *J* = 5.9 Hz, 2H), 1.68 (s, 6H), 1.37 - 1.23 (m, 16H).

2-Chloro-N-((5-(trifluoromethyl)-1H-benzo[d]imidazol-2-yl)methyl)isonicotinamide (3.96)



To a stirring solution of 2-chloroisonicotinic acid (28 mg, 0.11 mmol, 1 equiv) in DCM (5 mL) was added EDC (36 mg, 0.22 mmol, 2.0 equiv) and DMAP (35 mg, 0.20 mmol, 2.5 equiv). This solution was stirred for 10 min before addition of (5-(trifluoromethyl)-1H-benzo[d]imidazol-2-yl)methanamine (30 mg, 0.16 mmol, 1.5 equiv). The reaction slurry was brought to 37 ˚C for 12 h before quenching with 1 N HCl solution (3 mL). Organics were separated and the solvent was removed under reduced pressure. Column chromatography (0 to 45% (EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{15}H_{11}ClF_3N_4O^+$ [M+H$^+$] 355.7247, found 356. 1H NMR (400 MHz, $CDCl_3$) δ 8.88 (br, 1H), 8.52 (dd, J_1 = 0.7, J_2, = 5.2 Hz, 1H), 7.86 (s, 2H), 7.71 (dd, J_1 = 1.5, J_2 = 5.2 Hz, 1H), 7.64 (d, J = 8.5 Hz, 1H), 7.55 (d, J = 8.5 Hz, 1H), 4.85 (d, J = 5.7 Hz, 2H).

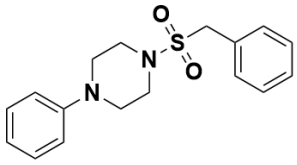
1-((3-(Trifluoromethyl)benzyl)sulfonyl)-4-(3-(trifluoromethyl)phenyl)piperazine (3.97)



A stirring solution of 1-(3-(trifluoromethyl)phenyl)piperazine (20 mg, 0.09 mmol, 1 equiv) in DCM (3 mL) was chilled to 0 °C and to this solution was added pyridine (21 mg, 0.27 mmol, 3 equiv) and (3-(trifluoromethyl)phenyl)methanesulfonyl chloride (22 mg, 0.11

mmol, 1.2 equiv). The ice bath was removed and after confirming consumption of limiting reagent by TLC, 2 mL of distilled water was added to the reaction vial. The organic layer was separated and removed under reduced atmosphere. Column chromatography (0 to 50% EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{19}H_{19}F_6N_2O_2S^+$ [M+H$^+$] 453.4229, found 453. 1H NMR (400 MHz, $CDCl_3$) δ 7.70 -7.61 (m, 3H), 7.54 (t, *J* = 7.6 Hz, 1H), 7.36 (t, *J* = 8.0 Hz, 1H), 7.14 (d, *J* = 7.6 Hz, 1H), 7.08 (s, 1H), 7.03 (d, *J* = 8.64, 1H), 4.29 (s, 2H), 3.36 - 3.28 (m, 4H), 3.23 - 3.14 (m, 4H). 13C NMR (176 MHz, CDCl3) δ 150.9, 134.1, 131.4 (ddd, J_1 = 32, J_2 = 48, J_3 = 64), 129.8, 129.7, 129.4, 127.5 (d, *J* = 4 Hz), 125.7 (d, *J* = 4 Hz), 123.9 (dq, J_1 = 72, J_2 = 273 Hz), 119.7, 117.2, 113.2, 56.4, 49.4, 45.9.

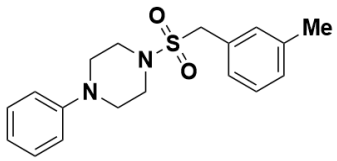
1-(Benzylsulfonyl)-4-phenylpiperazine (3.98)



A stirring solution of 1-phenylpiperazine (20 mg, 0.18 mmol, 1 equiv) in DCM (3 mL) was chilled to 0 °C and to this solution was added pyridine (21 mg, 0.56 mmol, 3 equiv) and phenylmethanesulfonyl chloride (28 mg, 0.21 mmol, 1.2 equiv). The ice bath was removed and after confirming consumption of limiting reagent by TLC, 2 mL of distilled water was added to the reaction vial. The organic layer was separated and removed under reduced atmosphere. Column chromatography (0 to 50% EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{17}H_{21}N_2O_2S^+$ [M+H$^+$], 317.4265 found 318. 1H NMR

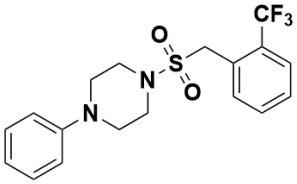
(400 MHz, $CDCl_3$) δ 7.38 - 7.28 (m, 4H), 7.23 - 7.15 (m, 4H), 6.86 - 6.77 (m, 2H), 4.19 (s, 2H), 3.25 - 3.15 (m, 4H), 3.08 - 3.00 (m, 4H). 13C NMR (176 MHz, CDCl3) δ 165.9, 157.8, 151.2, 132.5 (q, J = 33 Hz), 132.0 (q, J = 33 Hz), 130.3, 123.5 (dq, J_1 = 60, J_2 = 272 Hz), 118.5 (d, J = 3 Hz), 117.8, 115.3 (d, J = 4 Hz), 113.0, 111.8 (d, J = 4 Hz), 67.6, 48.6, 48.2, 44.8, 41.6.

1-((3-Methylbenzyl)sulfonyl)-4-phenylpiperazine (3.99)



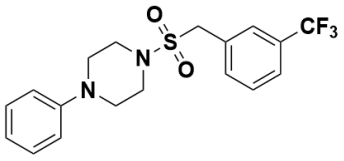
A stirring solution of 1-phenylpiperazine (20 mg, 0.12 mmol, 1 equiv) in DCM (3 mL) was chilled to 0 °C and to this solution was added pyridine (21 mg, 0.36 mmol, 3 equiv) and m-tolylmethanesulfonyl chloride (30 mg, 0.15 mmol, 1.2 equiv). The ice bath was removed and after confirming consumption of limiting reagent by TLC, 2 mL of distilled water was added to the reaction vial. The organic layer was separated and removed under reduced atmosphere. Column chromatography (0 to 50% EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{18}H_{23}N_2O_2S^+$ [$M+H^+$], 331.4535 found 331. 1H NMR (400 MHz, $CDCl_3$) δ 7.35 - 7.15 (m, 6H), 6.94 - 6.85 (m, 3H), 4.22 (s, 2H), 3.33 - 3.22 (m, 4H), 3.15 - 3.09 (m, 4H), 2.37 (s, 3H).

1-Phenyl-4-((2-(trifluoromethyl)benzyl)sulfonyl)piperazine (3.100)



A stirring solution of 1-phenylpiperazine (20 mg, 0.18 mmol, 1 equiv) in DCM (3 mL) was chilled to 0 °C and to this solution was added pyridine (21 mg, 0.56 mmol, 3 equiv) and (2-(trifluoromethyl)phenyl)methanesulfonyl chloride (38 mg, 0.21 mmol, 1.2 equiv). The ice bath was removed and after confirming consumption of limiting reagent by TLC, 2 mL of distilled water was added to the reaction vial. The organic layer was separated and removed under reduced atmosphere. Column chromatography (0 to 50% EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{18}H_{20}F_3N_2O_2S^+$ [M+H$^+$], 385.4247 found 386. 1H NMR (400 MHz, $CDCl_3$) δ 7.85 (d, J = 7.8 Hz, 1H), 7.73 (dd, J_1 = 1.3, J_2 = 8.0 Hz, 1H), 7.60 (t, J = 7.6 Hz, 1H), 7.49 (t, J = 7.6 Hz, 1H), 7.31 - 7.24 (m, 2H), 6.95 - 6.68 (m, 3H), 4.44 (s, 2H), 3.41 - 3.34 (m, 4H), 3.21 - 3.12 (m, 4H).

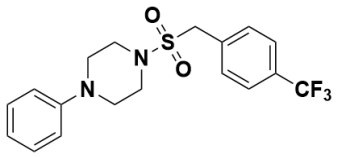
1-Phenyl-4-((3-(trifluoromethyl)benzyl)sulfonyl)piperazine (3.101)



A stirring solution of 1-phenylpiperazine (20 mg, 0.18 mmol, 1 equiv) in DCM (3 mL) was chilled to 0 °C and to this solution was added pyridine (21 mg, 056 mmol, 3 equiv) and (3-(trifluoromethyl)phenyl)methanesulfonyl chloride (38 mg, 0.21 mmol, 1.2 equiv). The ice bath was removed and after confirming consumption of limiting reagent by TLC, 2 mL of

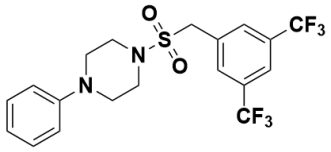
distilled water was added to the reaction vial. The organic layer was separated and removed under reduced atmosphere. Column chromatography (0 to 50% EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{18}H_{20}F_3N_2O_2S^+$ [M+H$^+$], 385.4247 found 386. 1H NMR (400 MHz, $CDCl_3$) δ 7.71 - 7.61 (m, 3H), 7.53 (t, *J* = 7.7 Hz, 1H), 7.31 - 7.26 (m, 2H), 6.95 - 6.86 (m, 3H), 4.27 (s, 2H), 3.36 - 3.29 (m, 4H), 3.18 - 3.11 (m, 4H).

1-Phenyl-4-((4-(trifluoromethyl)benzyl)sulfonyl)piperazine (3.102)



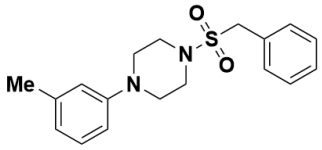
A stirring solution of 1-phenylpiperazine (20 mg, 0.18 mmol, 1 equiv) in DCM (3 mL) was chilled to 0 °C and to this solution was added pyridine (21 mg, 0.56 mmol, 3 equiv) and (4-(trifluoromethyl)phenyl)methanesulfonyl chloride (38 mg, 0.21 mmol, 1.2 equiv). The ice bath was removed and after confirming consumption of limiting reagent by TLC, 2 mL of distilled water was added to the reaction vial. The organic layer was separated and removed under reduced atmosphere. Column chromatography (0 to 50% EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{18}H_{20}F_3N_2O_2S^+$ [M+H$^+$], 385.4247 found 387. 1H NMR (400 MHz, $CDCl_3$) δ 7.66 (d, *J* = 8.1 Hz, 2H), 7.56 (d, *J* = 8.0 Hz, 2H), 7.31 - 7.26 (m, 2H), 6.94 - 6.87 (m, 2H), 4.26 (s, 2H), 3.35 - 3.30 (m, 4H), 3.19 - 3.14 (m, 4H).

1-((3,5-bis(Trifluoromethyl)benzyl)sulfonyl)-4-phenylpiperazine (3.103)



A stirring solution of 1-phenylpiperazine (20 mg, 0.18 mmol, 1 equiv) in DCM (3 mL) was chilled to 0 °C and to this solution was added pyridine (21 mg, 0.52 mmol, 3 equiv) and (3,5-bis(trifluoromethyl)phenyl)methanesulfonyl chloride (48 mg, 0.21 mmol, 1.2 equiv). The ice bath was removed and after confirming consumption of limiting reagent by TLC, 2 mL of distilled water was added to the reaction vial. The organic layer was separated and removed under reduced atmosphere. Column chromatography (0 to 50% EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{19}H_{19}F_6N_2O_2S^+$ [M+H$^+$], 453.4229 found 453. 1H NMR (400 MHz, $CDCl_3$) δ 7.31 - 7.26 (m, 2H), 7.02 - 6.95 (m, 2H), 6.95 - 6.88 (m, 3H), 6.85 (dddd, J_1 = 2.4, J_2 = 4.8, J_3 = 8.9, J_4 = 11.2 Hz, 1H), 4.19 (s, 2H), 3.40 - 3.31 (m, 4H), 3.22 - 3.11 (m, 4H).

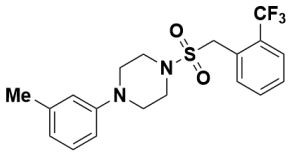
1-(Benzylsulfonyl)-4-(m-tolyl)piperazine (3.104)



A stirring solution of 1-(m-tolyl)piperazine (20 mg, 0.18 mmol, 1 equiv) in DCM (3 mL) was chilled to 0 °C and to this solution was added pyridine (21 mg, 0.56 mmol, 3 equiv) and phenylmethanesulfonyl chloride (26 mg, 0.2 mmol, 1.2 equiv). The ice bath was removed and after confirming consumption of limiting reagent by TLC, 2 mL of distilled

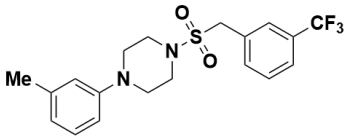
water was added to the reaction vial. The organic layer was separated and removed under reduced atmosphere. Column chromatography (0 to 50% EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{18}H_{23}N_2O_2S^+$ [M+H$^+$], 331.4535 found 331. 1H NMR (400 MHz, $CDCl_3$) δ 7.44 - 7.37 (m, 4H), 7.15 (t, *J* = 7.7 Hz, 1H), 6.76 - 6.66 (m, 3H), 4.26 (s, 2H), 3.30 - 3.22 (m, 4H), 3.14 - 3.08 (m, 4H), 2.31 (s, 3H).

1-(m-Tolyl)-4-((2-(trifluoromethyl)benzyl)sulfonyl)piperazine (3.105)



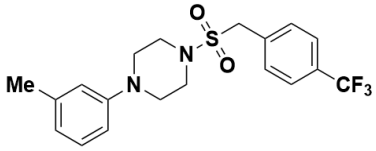
A stirring solution of 1-(m-tolyl)piperazine (20 mg, 0.18 mmol, 1 equiv) in DCM (3 mL) was chilled to 0 °C and to this solution was added pyridine (21 mg, 0.56 mmol, 3 equiv) and (2-(trifluoromethyl)phenyl)methanesulfonyl chloride (35 mg, 0.21 mmol, 1.2 equiv). The ice bath was removed and after confirming consumption of limiting reagent by TLC, 2 mL of distilled water was added to the reaction vial. The organic layer was separated and removed under reduced atmosphere. Column chromatography (0 to 50% EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{19}H_{22}F_3N_2O_2S^+$ [M+H$^+$], 399.4517 found 400. 1H NMR (400 MHz, $CDCl_3$) δ 7.85 (d, *J* = 7.8 Hz, 1H), 7.73 (d, *J* = 8.0 Hz, 1H), 7.61 (t, *J* = 7.7 Hz, 1H), 7.49 (t, *J* = 7.7 Hz, 1H), 7.16 (t, *J* = 7.7 Hz, 1H), 6.76 - 6.67 (m, 3H), 4.44 (s, 2H), 3.41 - 3.33 (m, 4H), 3.20 - 3.12 (m, 4H), 2.32 (s, 3H).

1-(m-Tolyl)-4-((3-(trifluoromethyl)benzyl)sulfonyl)piperazine (3.106)



A stirring solution of 1-(m-tolyl)piperazine (20 mg, 0.18 mmol, 1 equiv) in DCM (3 mL) was chilled to 0 °C and to this solution was added pyridine (21 mg, 0.27 mmol, 3 equiv) and (3-(trifluoromethyl)phenyl)methanesulfonyl chloride (35 mg, 0.21 mmol, 1.2 equiv). The ice bath was removed and after confirming consumption of limiting reagent by TLC, 2 mL of distilled water was added to the reaction vial. The organic layer was separated and removed under reduced atmosphere. Column chromatography (0 to 50% EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{19}H_{22}F_3N_2O_2S^+$ [M+H$^+$], 399.4517 found 399. 1H NMR (400 MHz, $CDCl_3$) δ 7.71 - 7.58 (m, 3H), 7.53 (t, *J* = 7.7 Hz, 1H), 7.16 (t, *J* = 7.7 Hz, 1H), 6.76 - 6.66 (m, 3H), 4.27 (s, 2H), 3.36 - 3.28 (m, 4H), 3.17 - 3.09 (m, 4H), 2.31 (s, 3H).

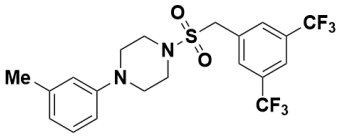
1-(m-Tolyl)-4-((4-(trifluoromethyl)benzyl)sulfonyl)piperazine (3.107)



A stirring solution of 1-(m-tolyl)piperazine (20 mg, 0.18 mmol, 1 equiv) in DCM (3 mL) was chilled to 0 °C and to this solution was added pyridine (21 mg, 0.56 mmol, 3 equiv) and (4-(trifluoromethyl)phenyl)methanesulfonyl chloride (35 mg, 0.21 mmol, 1.2 equiv). The ice bath was removed and after confirming consumption of limiting reagent by TLC, 2 mL of distilled water was added to the reaction vial. The organic layer was separated and

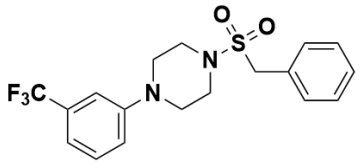
removed under reduced atmosphere. Column chromatography (0 to 50% EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{19}H_{22}F_3N_2O_2S^+$ [M+H$^+$], 399.4517 found 400. 1H NMR (400 MHz, $CDCl_3$) δ 7.67 (d, *J* = 8.1 Hz, 2H), 7.55 (d, *J* = 8.1 Hz, 2H), 7.16 (t, *J* = 7.6 Hz, 1H), 6.77 - 6.64 (m, 3H), 4.28 (s, 2H), 3.35 - 3.25 (m, 4H), 3.17 - 3.10 (m, 4H), 2.31 (s, 3H).

1-((3,5-bis(Trifluoromethyl)benzyl)sulfonyl)-4-(m-tolyl)piperazine (3.108)



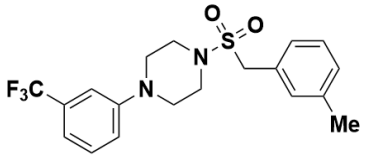
A stirring solution of 1-(m-tolyl)piperazine (20 mg, 0.18 mmol, 1 equiv) in DCM (3 mL) was chilled to 0 °C and to this solution was added pyridine (21 mg, 0.56 mmol, 3 equiv) and (3,5-bis(trifluoromethyl)phenyl)methanesulfonyl chloride (44 mg, 0.21 mmol, 1.2 equiv). The ice bath was removed and after confirming consumption of limiting reagent by TLC, 2 mL of distilled water was added to the reaction vial. The organic layer was separated and removed under reduced atmosphere. Column chromatography (0 to 50% EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{20}H_{21}F_6N_2O_2S^+$ [M+H$^+$], 467.4499 found 468. 1H NMR (400 MHz, $CDCl_3$) δ 7.18 (s, 1H), 7.09 (t, *J* = 7.7 Hz, 1H), 6.95 - 6.87 (m, 2H), 6.86 (tt, J_1 = 2.3, J_2 = 8.8 Hz, 1H), 6.70 - 6.61 (m, 2H), 4.11 (s, 2H), 3.30 - 3.21 (m, 4H), 3.13 - 3.04 (m, 4H), 2.25 (s, 3H).

1-(Benzylsulfonyl)-4-(3-(trifluoromethyl)phenyl)piperazine (3.109)



A stirring solution of 1-(3-(trifluoromethyl)phenyl)piperazine (20 mg, 0. 09 mmol, 1 equiv) in DCM (3 mL) was chilled to 0 °C and to this solution was added pyridine (21 mg, 0.27 mmol, 3 equiv) and phenylmethanesulfonyl chloride (19 mg, 0.11 mmol, 1.2 equiv). The ice bath was removed and after confirming consumption of limiting reagent by TLC, 2 mL of distilled water was added to the reaction vial. The organic layer was separated and removed under reduced atmosphere. Column chromatography (0 to 50% EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{18}H_{20}F_3N_2O_2S^+$ [M+H$^+$] 385.4247, found 386. 1H NMR (400 MHz, $CDCl_3$) δ 7.46 - 7.32 (m, 6H), 7.13 (d, *J* = 7.6 Hz, 1H), 7.06 (s, 1H), 7.01 (d, *J* = 8.3 Hz, 1H), 4.28 (s, 2H), 3.30 - 3.23 (m, 4H), 3.18 - 3.12 (m, 4H).

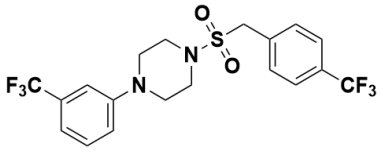
1-((3-Methylbenzyl)sulfonyl)-4-(3-(trifluoromethyl)phenyl)piperazine (3.110)



A stirring solution of 1-(3-(trifluoromethyl)phenyl)piperazine (20 mg, 0.09 mmol, 1 equiv) in DCM (3 mL) was chilled to 0 °C and to this solution was added pyridine (21 mg, 0.27 mmol, 3 equiv) and m-tolylmethanesulfonyl chloride (18 mg, 0.11 mmol, 1.2 equiv). The ice bath was removed and after confirming consumption of limiting reagent by TLC, 2 mL

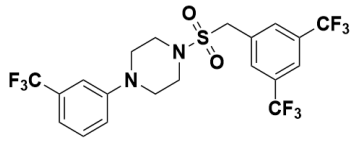
of distilled water was added to the reaction vial. The organic layer was separated and removed under reduced atmosphere. Column chromatography (0 to 50% EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{19}H_{22}F_3N_2O_2S^+$ [M+H$^+$] 399.4517, found 399. 1H NMR (400 MHz, $CDCl_3$) δ 7.35 (t, *J* = 8.0 Hz, 1H), 7.30 - 7.26 (m, 1H), 7.25 - 7.16 (m, 3H), 7.13 (d, *J* = 7.7 Hz, 1H), 7.06 (s, 1H), 7.02 (d, *J* = 8.8 Hz, 1H), 4.23 (s, 2H), 3.32 - 3.24 (m, 4H), 3.22 - 3.13 (m, 4H), 2.36 (s, 3H).

1-((4-Methylbenzyl)sulfonyl)-4-(3-(trifluoromethyl)phenyl)piperazine (3.111)



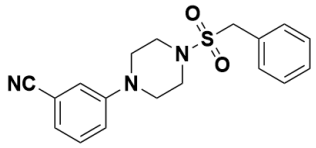
A stirring solution of 1-(3-(trifluoromethyl)phenyl)piperazine (20 mg, 0.09 mmol, 1 equiv) in DCM (3 mL) was chilled to 0 °C and to this solution was added pyridine (21 mg, 0.27 mmol, 3 equiv) and 4-methylphenylmethanesulfonyl chloride (18 mg, 0.11 mmol, 1.2 equiv). The ice bath was removed and after confirming consumption of limiting reagent by TLC, 2 mL of distilled water was added to the reaction vial. The organic layer was separated and removed under reduced atmosphere. Column chromatography (0 to 50% EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{19}H_{22}F_3N_2O_2S^+$ [M+H$^+$] 399.4517, found 399. 1H NMR (400 MHz, $CDCl_3$) δ 7.66 (d, *J* = 8.1 Hz, 2H), 7.55 (d, *J* = 8.1 Hz, 2H), 7.37 (t, *J* = 7.96 Hz, 1H), 7.15 (d, *J* = 7.7 Hz, 1H), 7.09 (s, 1H), 7.03 (d, *J* = 8.3 Hz, 1H), 4.29 (s, 2H), 3.38 - 3.29 (m, 4H), 3.25 - 3.17 (m, 4H).

1-((3,5-bis(Trifluoromethyl)benzyl)sulfonyl)-4-(3-(trifluoromethyl)phenyl)piperazine (3.112)



A stirring solution of 1-(3-(trifluoromethyl)phenyl)piperazine (20 mg, 0.8 mmol, 1 equiv) in DCM (3 mL) was chilled to 0 °C and to this solution was added pyridine (21 mg, 0.24 mmol, 3 equiv) and (3,5-bis(trifluoromethyl)phenyl)methanesulfonyl chloride (34 mg, 0.11 mmol, 1.2 equiv). The ice bath was removed and after confirming consumption of limiting reagent by TLC, 2 mL of distilled water was added to the reaction vial. The organic layer was separated and removed under reduced atmosphere. Column chromatography (0 to 50% EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{20}H_{18}F_9N_2O_2S^+$ [M+H$^+$], 521.4211 found 521. 1H NMR (400 MHz, $CDCl_3$) δ 7.37 (t, J = 8.0 Hz, 1H), 7.15 (d, J = 7.6 Hz, 1H), 7.09 (s, 1H), 7.04 (dd, J_1 = 2.5, J_2 = 8.3 Hz, 1H), 7.02 - 6.95 (m, 2H), 6.86 (tt, J_1 = 2.3, J_2 = 8.8 Hz, 1H), 4.20 (s, 2H), 3.39 - 3.32 (m, 4H), 3.25 - 3.18 (m, 4H).

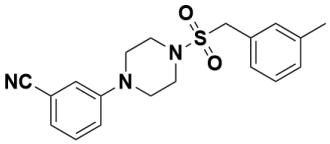
3-(4-(Benzylsulfonyl)piperazin-1-yl)benzonitrile (3.113)



A stirring solution of 3-(piperazin-1-yl)benzonitrile (30 mg, 0.09 mmol, 1 equiv) in DCM (3 mL) was chilled to 0 °C and to this solution was added pyridine (21 mg, 0.27 mmol, 3

equiv) and phenylmethanesulfonyl chloride (20 mg, 0.11 mmol, 1.2 equiv). The ice bath was removed and after confirming consumption of limiting reagent by TLC, 2 mL of distilled water was added to the reaction vial. The organic layer was separated and removed under reduced atmosphere. Column chromatography (0 to 50% EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{18}H_{20}N_3O_2S^+$ [M+H$^+$] 342.4365, found 342. 1H NMR (400 MHz, $CDCl_3$) δ 7.45 - 7.37 (m, 4H), 7.32 (t, *J* = 7.6 Hz, 1H), 7.22 (d, *J* = 7.6 Hz, 1H), 7.14 (d, *J* = 6.7 Hz, 1H), 7.09 - 7.04 (m, 2H), 4.28 (s, 2H), 3.30 - 3.19 (m, 4H), 3.18 - 3.08 (m, 4H).

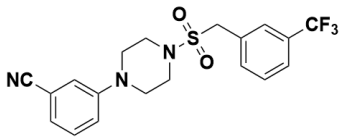
3-(4-((3-Methylbenzyl)sulfonyl)piperazin-1-yl)benzonitrile (3.114)



A stirring solution of 3-(piperazin-1-yl)benzonitrile (30 mg, 0.09 mmol, 1 equiv) in DCM (3 mL) was chilled to 0 °C and to this solution was added pyridine (21 mg, 0.11 mmol, 0.27 mmol, 3 equiv) and m-tolylmethanesulfonyl chloride (20 mg, 1.2 equiv). The ice bath was removed and after confirming consumption of limiting reagent by TLC, 2 mL of distilled water was added to the reaction vial. The organic layer was separated and removed under reduced atmosphere. Column chromatography (0 to 50% EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{19}H_{22}N_3O_2S^+$ [M+H$^+$] 356.4635, found 356. 1H NMR (400 MHz, $CDCl_3$) δ 7.33 (dt, J_1 = 2.0, J_2 = 7.4 Hz, 1H), 7.30 - 7.26

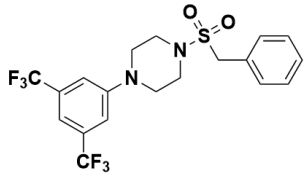
(m, 1H), 7.23 (s, 1H), 7.19 (d, *J* = 8.3 Hz, 2H), 7.15 (d, *J* = 8.0 Hz, 1H), 7.09 - 7.04 (m, 2H), 4.23 (s, 2H), 3.29 - 3.23 (m, 4H), 3.17 - 3.10 (m, 4H), 2.37 (s, 3H).

3-(4-((3-(Trifluoromethyl)benzyl)sulfonyl)piperazin-1-yl)benzonitrile (3.115)



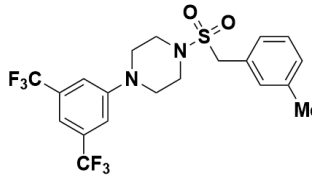
A stirring solution of 3-(piperazin-1-yl)benzonitrile (30 mg, 0.09 mmol, 1 equiv) in DCM (3 mL) was chilled to 0 °C and to this solution was added pyridine (21 mg, 0.27 mmol, 3 equiv) and (3-(trifluoromethyl)phenyl)methanesulfonyl chloride (20 mg, 0.11 mmol, 1.2 equiv). The ice bath was removed and after confirming consumption of limiting reagent by TLC, 2 mL of distilled water was added to the reaction vial. The organic layer was separated and removed under reduced atmosphere. Column chromatography (0 to 50% EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{19}H_{19}F_3N_3O_2S^+$ [M+H$^+$] 410.4347, found 411. 1H NMR (400 MHz, $CDCl_3$) δ 7.67 (d, *J* = 8.1 Hz, 2H), 7.56 (d, *J* = 8.0 Hz, 2H), 7.34 (t, *J* = 7.9 Hz, 1H), 7.17 (d, *J* = 6.4 Hz, 1H), 7.11 - 7.07 (m, 2H), 4.30 (s, 2H), 3.35 - 3.30 (m, 4H), 3.21 - 3.15 (m, 4H).

1-(Benzylsulfonyl)-4-(3,5-bis(trifluoromethyl)phenyl)piperazine (3.116)



A stirring solution of 1-(3,5-bis(trifluoromethyl)phenyl)piperazine (20 mg, 0.09 mmol, 1 equiv) in DCM (3 mL) was chilled to 0 °C and to this solution was added pyridine (21 mg, 0.27 mmol, 3 equiv) and phenylmethanesulfonyl chloride (15 mg, 0.11 mmol, 1.2 equiv). The ice bath was removed and after confirming consumption of limiting reagent by TLC, 2 mL of distilled water was added to the reaction vial. The organic layer was separated and removed under reduced atmosphere. Column chromatography (0 to 50% EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{19}H_{19}F_6N_2O_2S^+$ [M+H$^+$] 453.4229, found 455. 1H NMR (400 MHz, $CDCl_3$) δ 7.45 -7.37 (m, 5H), 7.33 (s, 1H), 7.19 (s, 1H), 4.29 (s, 2H), 3.29 - 3.23 (m, 4H), 3.23 - 3.18 (m, 4H).

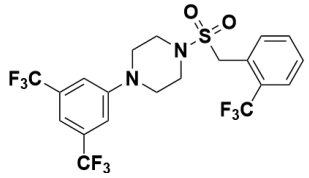
1-(3,5-bis(Trifluoromethyl)phenyl)-4-((3-methylbenzyl)sulfonyl)piperazine (3.117)



A stirring solution of 1-(3,5-bis(trifluoromethyl)phenyl)piperazine (20 mg, 0.09 mmol, 1 equiv) in DCM (3 mL) was chilled to 0 °C and to this solution was added pyridine (21 mg, 0.27 mmol, 3 equiv) and m-tolylmethanesulfonyl chloride (16 mg, 0.11 mmol, 1.2 equiv). The ice bath was removed and after confirming consumption of limiting reagent by TLC, 2 mL of distilled water was added to the reaction vial. The organic layer was separated and removed under reduced atmosphere. Column chromatography (0 to 50% EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{20}H_{21}F_6N_2O_2S^+$ [M+H$^+$] 467.45,

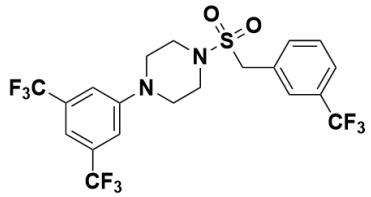
found 468. 1H NMR (400 MHz, $CDCl_3$) δ 7.33 (br, 1H), 7.30 - 7.23 (m, 2H), 7.22 - 7.18 (m, 4H), 4.26 (s, 2H), 3.30 - 3.20 (m, 8H), 2.36 (s, 3H).

1-(3,5-bis(Trifluoromethyl)phenyl)-4-((2-(trifluoromethyl)benzyl)sulfonyl)piperazine (3.118)



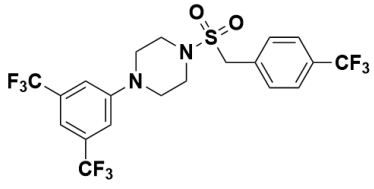
A stirring solution of 1-(3,5-bis(trifluoromethyl)phenyl)piperazine (30 mg, 0.11 mmol, 1 equiv) in DCM (3 mL) was chilled to 0 °C and to this solution was added pyridine (21 mg, 0.33 mmol, 3 equiv) and (2-(trifluoromethyl)phenyl)methanesulfonyl chloride (31 mg, 0.14 mmol, 1.2 equiv). The ice bath was removed and after confirming consumption of limiting reagent by TLC, 2 mL of distilled water was added to the reaction vial. The organic layer was separated and removed under reduced atmosphere. Column chromatography (0 to 50% EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{20}H_{18}F_9N_2O_2S^+$ [$M+H^+$], 521.4211 found 521. 1H NMR (400 MHz, $CDCl_3$) δ 7.86 (d, *J* = 7.7 Hz, 1H), 7.74 (d, *J* = 7.9 Hz, 1H), 7.62 (t, *J* = 7.5, 1H), 7.51 (t, *J* = 7.7 Hz, 1H), 7.34 (s, 1H), 7.21 (s, 2H), 4.17 (s, 2H), 3.40 - 3.34 (m, 4H), 3.29 - 3.25 (m, 4H).

1-(3,5-bis(Trifluoromethyl)phenyl)-4-((3-(trifluoromethyl)benzyl)sulfonyl)piperazine (3.119)



A stirring solution of 1-(3,5-bis(trifluoromethyl)phenyl)piperazine (20 mg, 0.9 mmol, 1 equiv) in DCM (3 mL) was chilled to 0 °C and to this solution was added pyridine (21 mg, 0.27 mmol, 3 equiv) and (3-(trifluoromethyl)phenyl)methanesulfonyl chloride (21 mg, 0.11 mmol, 1.2 equiv). The ice bath was removed and after confirming consumption of limiting reagent by TLC, 2 mL of distilled water was added to the reaction vial. The organic layer was separated and removed under reduced atmosphere. Column chromatography (0 to 50% EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{20}H_{18}F_9N_2O_2S^+$ [M+H$^+$] 521.4211, found 522. 1H NMR (400 MHz, $CDCl_3$) δ 7.69 - 7.62 (m, 3H), 7.55 (t, *J* = 8.0 Hz, 1H), 7.35 (s, 1H), 7.21 (s, 2H), 4.30 (s, 2H), 3.38 - 3.28 (m, 4H), 3.27 - 3.19 (m, 4H).

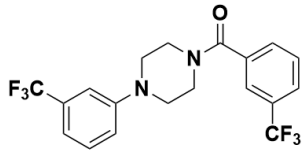
1-(3,5-bis(Trifluoromethyl)phenyl)-4-((4-(trifluoromethyl)benzyl)sulfonyl)piperazine (3.120)



A stirring solution of 1-(3,5-bis(trifluoromethyl)phenyl)piperazine (20 mg, 0.09 mmol, 1 equiv) in DCM (3 mL) was chilled to 0 °C and to this solution was added pyridine (21 mg,

0.27 mmol, 3 equiv) and (4-(trifluoromethyl)phenyl)methanesulfonyl chloride (21 mg, 0.11 mmol, 1.2 equiv). The ice bath was removed and after confirming consumption of limiting reagent by TLC, 2 mL of distilled water was added to the reaction vial. The organic layer was separated and removed under reduced atmosphere. Column chromatography (0 to 50% EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{20}H_{18}F_9N_2O_2S^+$ [M+H$^+$] 521.4211, found 521. 1H NMR (400 MHz, $CDCl_3$) δ 7.77 (d, *J* = 8.1 Hz, 2H), 7.56 (d, *J* = 8.0, 2H), 7.36 (s, 1H), 7.22 (s, 2H), 4.31 (s, 2H), 3.37 - 3.30 (m, 4H), 3.28 - 3.22 (m, 4H).

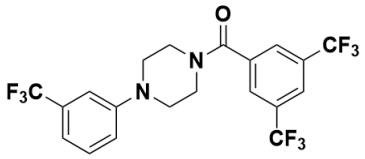
(3-(Trifluoromethyl)phenyl)(4-(3-(trifluoromethyl)phenyl)piperazin-1-yl)methanone (3.121)



To a stirring solution of 3-trifluoromethylbenzoic acid (22 mg, 0.12 mmol, 1 equiv) in DCM (5 mL) was added EDC (36 mg, 0.24 mmol, 2.0 equiv) and DMAP (35 mg, 0.30 mmol, 2.5 equiv). This solution was stirred for 10 min before addition of 1-(3-(trifluoromethyl)phenyl)piperazine (40 mg, 0.17 mmol, 1.5 equiv). The reaction slurry was brought to 37 ˚C for 12 h before quenching with 1 N HCl solution (3 mL). Organics were separated and the solvent was removed under reduced pressure. Column chromatography (0 to 45% (EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{19}H_{17}F_6N_2O^+$ [M+H$^+$] 403.3479, found 404. 1H NMR (400 MHz, CD_3OD) δ 7.74 - 7.68

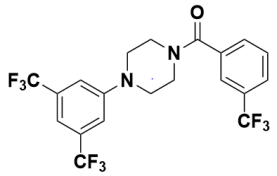
(m, 2H), 7.67 - 7.56 (m, 2H), 7.32 (t, *J* = 7.7 Hz, 1H), 7.16 - 7.09 (m, 2H), 7.01 (d, *J* = 7.6 Hz, 1H), 3.84 (br, 2H), 3.50 (br, 2H), 3.26 (br, 2H), 3.15 (br, 2H).

(3,5-bis(Trifluoromethyl)phenyl)(4-(3-(trifluoromethyl)phenyl)piperazin-1-yl)methanone (3.122)



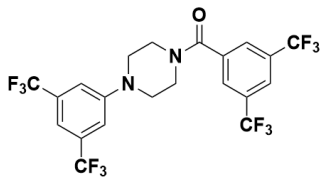
To a stirring solution of 3,5-bistrifluoromethylbenzoic acid (16 mg, 0.12 mmol, 1 equiv) in DCM (5 mL) was added EDC (36 mg, 0.24 mmol, 2.0 equiv) and DMAP (35 mg, 0.30 mmol, 2.5 equiv). This solution was stirred for 10 min before addition of 1-(3-(trifluoromethyl)phenyl)piperazine (40 mg, 0.17 mmol, 1.5 equiv). The reaction slurry was brought to 37 ˚C for 12 h before quenching with 1 N HCl solution (3 mL). Organics were separated and the solvent was removed under reduced pressure. Column chromatography (0 to 45% (EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{20}H_{16}F_9N_2O^+$ [M+H$^+$] 471.3461, found 471. 1H NMR (400 MHz, CD_3OD) δ 8.14 (s, 1H), 8.13 (s, 2H), 7.44 (t, *J* = 8.0 Hz, 1H), 7.27 - 7.22 (m, 2H), 7.14 (d, *J* = 7.6 Hz, 1H), 3.98 (br, 2H), 3.61 (br, 2H), 3.41 (br, 2H), 3.28 (br, 2H).

(4-(3,5-bis(Trifluoromethyl)phenyl)piperazin-1-yl)(3-(trifluoromethyl)phenyl)methanone (3.123)



To a stirring solution of 3-trifluoromethylbenzoic acid (16 mg, 0.12 mmol, 1 equiv) in DCM (5 mL) was added EDC (36 mg, 0.24 mmol, 2.0 equiv) and DMAP (35 mg, 0.30 mmol, 2.5 equiv). This solution was stirred for 10 min before addition of 1-(3,5-bis(trifluoromethyl)phenyl)piperazine (40 mg, 0.18 mmol, 1.5 equiv). The reaction slurry was brought to 37 ˚C for 12 h before quenching with 1 N HCl solution (3 mL). Organics were separated and the solvent was removed under reduced pressure. Column chromatography (0 to 45% (EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{20}H_{16}F_9N_2O^+$ [M+H$^+$] 471.3461, found 471. 1H NMR (400 MHz, CD_3OD) δ 7.85 - 7.79 (m, 2H), 7.76 - 7.70 (m, 2H), 7.46 (s, 2H), 7.32 (s, 1H), 3.96 (br, 2H), 3.63 (br, 2H), 3.48 (br, 2H), 3.37 (br, 2H).

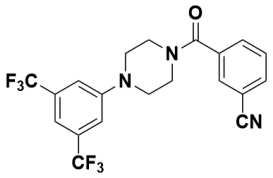
(3,5-bis(Trifluoromethyl)phenyl)(4-(3,5-bis(trifluoromethyl)phenyl)piperazin-1-yl)methanone (3.124)



To a stirring solution of 3,5-bis(trifluoromethyl)benzoic acid acid (22 mg, 0.08 mmol, 1 equiv) in DCM (5 mL) was added EDC (36 mg, 0.16 mmol, 2.0 equiv) and DMAP (35 mg, 0.20 mmol, 2.5 equiv). This solution was stirred for 10 min before addition of 1-(3,5-bis(trifluoromethyl)phenyl)piperazine (40 mg, 0.13 mmol, 1.5 equiv). The reaction slurry was brought to 37 ˚C for 12 h before quenching with 1 N HCl solution (3 mL). Organics were separated and the solvent was removed under reduced pressure. Column chromatography (0 to 45% (EtOAc/Hex) on silica gel was used to purify sufficient amounts

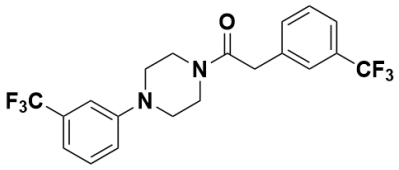
of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{21}H_{15}F_{12}N_2O^+$ [M+H$^+$] 539.3443, found 539. 1H NMR (400 MHz, CD_3OD) δ 8.15 - 8.12 (m, 3H), 7.46 (s, 2H), 7.32 (s, 1H), 3.98 (br, 2H), 3.62 (br, 2H), 3.51 (br, 2H), 3.39 (br, 2H).

3-(4-(3,5-bis(Trifluoromethyl)phenyl)piperazine-1-carbonyl)benzonitrile (3.125)



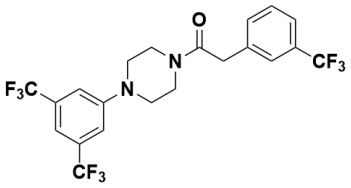
To a stirring solution of 3-cyanobenzoic acid acid (13 mg, 0.08 mmol, 1 equiv) in DCM (5 mL) was added EDC (36 mg, 0.16 mmol, 2.0 equiv) and DMAP (35 mg, 0.20 mmol, 2.5 equiv). This solution was stirred for 10 min before addition of 1-(3,5-bis(trifluoromethyl)phenyl)piperazine (40 mg, 0.13 mmol, 1.5 equiv). The reaction slurry was brought to 37 ˚C for 12 h before quenching with 1 N HCl solution (3 mL). Organics were separated and the solvent was removed under reduced pressure. Column chromatography (0 to 45% (EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{20}H_{16}F_6N_3O^+$ [M+H$^+$] 428.3579, found 429. 1H NMR (400 MHz, CD_3OD) δ 7.92 - 7.84 (m, 2H), 7.79 (td, J_1 = 1.5, J_2 = 7.8 Hz, 1H), 7.48 - 7.39 (m, 2H), 7.33 (s, 2H), 7.23 (d, J = 8.1 Hz, 1H), 3.95 (br, 2H), 3.62 (br, 2H), 3.49 (br, 2H), 3.38 (br, 2H).

2-(3-(Trifluoromethyl)phenyl)-1-(4-(3-(trifluoromethyl)phenyl)piperazin-1-yl)ethan-1-one (3.126)



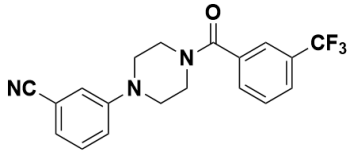
To a stirring solution of 1-(3-(trifluoromethyl)phenyl)piperazine (34 mg, 0.15 mmol, 1.0 equiv), triethylamine (1.8 μL, 0.013 mmol, 0.1 equiv) in DCM (5 mL) was added 2-(3-(trifluoromethyl)phenyl)acetyl chloride (40 mg, 0.18 mmol, 1.2 equiv) and the solution was stirred for 30 min at RT. After confirming consumption of limiting reagent by TLC, 3 mL of distilled water was added to the reaction vial. The organic layer was separated and removed under reduced atmosphere. Column chromatography (0 to 50% EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{20}H_{19}F_6N_2O^+$ [M+H$^+$] 417.3749, found 417. 1H NMR (400 MHz, CDCl3) δ 7.56 - 7.50 (m, 2H), 7.47 - 7.44 (m, 2H), 7.36 (t, J = 8.0 Hz, 1H), 7.13 (d, J = 7.7 Hz, 1H), 7.08 (s, 1H), 7.04 (dd, J_1 = 2.5, J_2 = 8.4 Hz, 1H), 3.85 - 3.81 (m, 4H), 3.67 - 3.61 (m, 2H), 3.22 - 3.19 (m, 2H), 3.13 - 3.10 (m, 2H). 13C NMR (176 MHz, CDCl3) δ 168.9, 150.9, 135.7, 132.3, 131.5 (q, J = 32 Hz), 131.0 (q, J = 28), 129.8, 129.2, 125.6 (d, J = 4 Hz), 124.1 (dq, J_1 = 29, J_2 = 273 Hz), 293.2 (d, J = 4 Hz), 119.3, 116.7 (d, J = 4 Hz), 112.9 (d, J = 4 Hz), 49.0, 48.7, 45.7, 41.6, 40.2.

1-(4-(3,5-bis(Trifluoromethyl)phenyl)piperazin-1-yl)-2-(3-(trifluoromethyl)phenyl)ethan-1-one (3.127)



To a stirring solution of 1-(3,5-bis(trifluoromethyl)phenyl)piperazine (44 mg, 0.15 mmol, 1.0 equiv), triethylamine (1.8 µL, 0.013 mmol, 0.1 equiv) in DCM (5 mL) was added 2-(3-(trifluoromethyl)phenyl)acetyl chloride (40 mg, 0.18 mmol, 1.2 equiv) and the solution was stirred for 30 min at RT. After confirming consumption of limiting reagent by TLC, 3 mL of distilled water was added to the reaction vial. The organic layer was separated and removed under reduced atmosphere. Column chromatography (0 to 50% EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{21}H_{18}F_9N_2O^+$ [M+H$^+$] 485.3731, found 386. 1H NMR (400 MHz, CDCl3) δ 7.57 - 7.50 (m, 2H), 7.48 - 7.45 (m, 2H), 7.33 (s, 1H), 7.22 (s, 2H), 3.88 - 3.81 (m, 4H), 3.69 - 3.63 (m, 2H), 3.32 - 3.25 (m, 2H), 3.23 - 3.15 (m, 2H). 13C NMR (176 MHz, CDCl3) δ 168.9, 151.2, 135.5, 132.5 (q, *J* = 33 Hz), 132.3, 131.1 (q, *J* = 32 Hz), 129.3, 125.6, 123.9 (q, *J* = 272 Hz), 124.0 (d, *J* = 4 Hz), 123.4 (q, *J* = 273 Hz), 115.2 (d, *J* = 4 Hz), 112.9, 48.3, 48.2, 45.5, 41.4, 40.3.

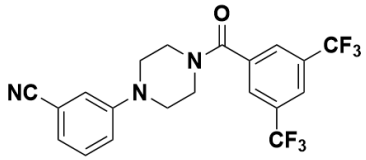
3-(4-(3-(Trifluoromethyl)benzoyl)piperazin-1-yl)benzonitrile (3.128)



To a stirring solution of 3-trifluoromethylbenzoic acid (20 mg, 0.12 mmol, 1 equiv) in DCM (5 mL) was added EDC (36 mg, 0.24 mmol, 2.0 equiv) and DMAP (35 mg, 0.30 mmol, 2.5 equiv). This solution was stirred for 10 min before addition of 3-(piperazin-1-yl)benzonitrile (30 mg, 0.16 mmol, 1.5 equiv). The reaction slurry was brought to 37 ˚C for 12 h before quenching with 1 N HCl solution (3 mL). Organics were separated and the solvent was removed under reduced pressure. Column chromatography (0 to 45%

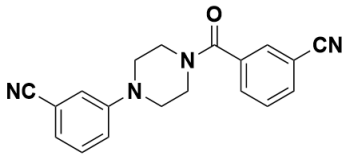
(EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{19}H_{17}F_3N_3O^+$ [M+H$^+$], 360.3597 found 362. 1H NMR (400 MHz, $CDCl_3$) δ 7.75 - 7.68 (m, 2H), 7.65 - 7.57 (m, 2H), 7.36 (dd, J_1 =7.3, J_2 = 7.6 Hz, 1H), 7.19 - 7.10 (m, 3H), 3.94 (br, 2H), 3.61 (br, 2H), 3.24 (br, 4H).

3-(4-(3,5-bis(Trifluoromethyl)benzoyl)piperazin-1-yl)benzonitrile (3.129)



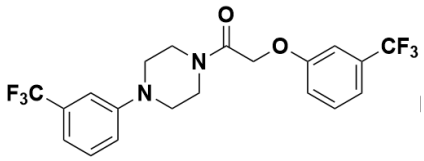
To a stirring solution of 3,5-bis(trifluoromethyl)benzoic acid (27 mg, 0.12 mmol, 1 equiv) in DCM (5 mL) was added EDC (36 mg, 0.24 mmol, 2.0 equiv) and DMAP (35 mg, 0.30 mmol, 2.5 equiv). This solution was stirred for 10 min before addition of 3-(piperazin-1-yl)benzonitrile (30 mg, 0.16 mmol, 1.5 equiv). The reaction slurry was brought to 37 ˚C for 12 h before quenching with 1 N HCl solution (3 mL). Organics were separated and the solvent was removed under reduced pressure. Column chromatography (0 to 45% (EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{20}H_{16}F_6N_3O^+$ [M+H$^+$], 428.3579 found 427. 1H NMR (400 MHz, $CDCl_3$) δ 7.97 (s, 1H), 7.90 (s, 2H), 7.37 (dd, J_1 = 4.6, J_2 = 7.6 Hz, 1H), 7.17 (d, J = 6.5 Hz, 1H), 7.15 - 7.10 (m, 2H), 3.96 (br, 2H), 3.60 (br, 2H), 3.28 (br, 4H).

3-(4-(3-Cyanobenzoyl)piperazin-1-yl)benzonitrile (3.130)



To a stirring solution of 3-cyanobenzoic acid (15 mg, 0.12 mmol, 1 equiv) in DCM (5 mL) was added EDC (36 mg, 0.24 mmol, 2.0 equiv) and DMAP (35 mg, 0.30 mmol, 2.5 equiv). This solution was stirred for 10 min before addition of 3-(piperazin-1-yl)benzonitrile (30 mg, 0.16 mmol, 1.5 equiv). The reaction slurry was brought to 37 ˚C for 12 h before quenching with 1 N HCl solution (3 mL). Organics were separated and the solvent was removed under reduced pressure. Column chromatography (0 to 45% (EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{19}H_{17}N_4O^+$ [M+H$^+$], 317.3715 found 317. 1H NMR (400 MHz, $CDCl_3$) δ 7.76 - 7.72 (m, 2H), 7.68 (d, *J* = 7.8 Hz, 1H), 7.58 (t, *J* = 7.9 Hz, 1H), 7.36 (dd, J_1 = 5.0, 7.7 Hz, 1H), 7.16 (d, *J* = 7.6 Hz, 1H), 7.14 - 7.10 (m, 2H), 3.93 (br, 2H), 3.59 (br, 2H), 3.25 (br, 4H).

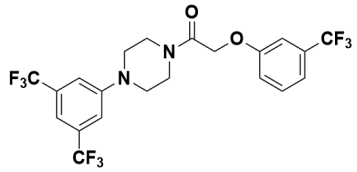
2-(3-(Trifluoromethyl)phenoxy)-1-(4-(3-(trifluoromethyl)phenyl)piperazin-1-yl)ethan-1-one (3.131)



To a stirring solution of 2-(3-(trifluoromethyl)phenoxy)acetic acid (19 mg, 0.12 mmol, 1 equiv) in DCM (5 mL) was added EDC (36 mg, 0.24 mmol, 2.0 equiv) and DMAP (35 mg, 0.30mmol, 2.5 equiv). This solution was stirred for 10 min before addition of 1-(3-(trifluoromethyl)phenyl)piperazine (40 mg, 0.17 mmol, 1.5 equiv). The reaction slurry was

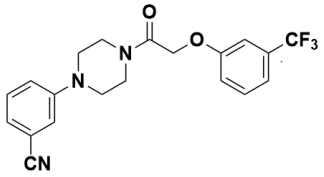
brought to 37 ˚C for 12 h before quenching with 1 N HCl solution (3 mL). Organics were separated and the solvent was removed under reduced pressure. Column chromatography (0 to 45% (EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{20}H_{19}F_6N_2O_2^+$ [M+H$^+$] 433.3739, found 433. 1H NMR (400 MHz, CD_3OD) δ 7.49 (t, *J* = 8.1 Hz, 1H), 7.43 (t, *J* = 7.8 Hz, 1H), 7.30 - 7.23 (m, 3H), 7.13 (d, *J* = 7.7 Hz, 1H), 4.97 (s, 2H), 3.83 - 3.72 (m, 4H), 3.30 - 3.23 (m, 4H).

1-(4-(3,5-bis(Trifluoromethyl)phenyl)piperazin-1-yl)-2-(3-(trifluoromethyl)phenoxy)ethan-1-one (3.132)



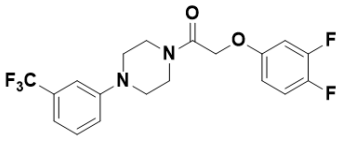
To a stirring solution of 2-(3-(trifluoromethyl)phenoxy)acetic acid (19 mg, 0.08 mmol, 1 equiv) in DCM (5 mL) was added EDC (36 mg, 0.16 mmol, 2.0 equiv) and DMAP (35 mg, 0.2 mmol, 2.5 equiv). This solution was stirred for 10 min before addition of 1-(3,5-bis(trifluoromethyl)phenyl)piperazine (31 mg, 0.13mmol, 1.5 equiv). The reaction slurry was brought to 37 ˚C for 12 h before quenching with 1 N HCl solution (3 mL). Organics were separated and the solvent was removed under reduced pressure. Column chromatography (0 to 45% (EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{21}H_{18}F_9N_2O_2^+$ [M+H$^+$] 501.3721, found 501. 1H NMR (400 MHz, CD_3OD) δ 7.50 (t, *J* = 8.0 Hz, 1H), 7.47 (s, 2H), 7.33 (s, 1H), 7.39 (s, 2H), 7.27 (d, *J* = 8.8 Hz, 1H), 4.99 (s, 2H), 3.85 - 3.76 (m, 4H), 3.48 - 3.43 (m, 2H), 3.34 - 3.36 (m, 2H).

3-(4-(2-(3-(Trifluoromethyl)phenoxy)acetyl)piperazin-1-yl)benzonitrile (3.133)



To a stirring solution of 2-(3-(trifluoromethyl)phenoxy)acetic acid (23 mg, 0.12 mmol, 1 equiv) in DCM (5 mL) was added EDC (36 mg, 0.24 mmol, 2.0 equiv) and DMAP (35 mg, 0.30 mmol, 2.5 equiv). This solution was stirred for 10 min before addition of 3-(piperazin-1-yl)benzonitrile (30 mg, 0.16 mmol, 1.5 equiv). The reaction slurry was brought to 37 ˚C for 12 h before quenching with 1 N HCl solution (3 mL). Organics were separated and the solvent was removed under reduced pressure. Column chromatography (0 to 45% (EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{20}H_{19}F_3N_3O_2^+$ [M+H$^+$], 390.3857 found 391. 1H NMR (400 MHz, $CDCl_3$) δ 7.41 (t, J = 8.0 Hz, 1H), 7.34 (dd, J_1 = 4.6, J_2 = 7.6 Hz, 1H), 7.28 - 7.24 (m, 1H), 7.19 (s, 1H), 7.14 (d, J = 7.9 Hz, 2H), 7.13 - 7.08 (m, 2H), 4.78 (s, 2H), 3.83 - 3.71 (m, 4H), 3.27 - 3.16 (m, 4H).

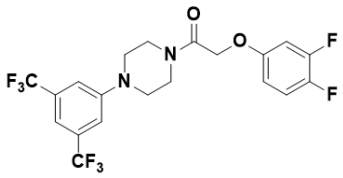
2-((3,4-Difluorophenyl)amino)-1-(4-(3-(trifluoromethyl)phenyl)piperazin-1-yl)ethan-1-one (3.134)



To a stirring solution of (3,4-difluorophenyl)glycine (30 mg, 0.13 mmol, 1 equiv) in DCM (5 mL) was added EDC (44 mg, 0.26 mmol, 2.0 equiv) and DMAP (1.9 mg, 0.013 mmol, 0.1 equiv). This solution was stirred for 10 min before addition of 1-(3-

trifluoromethylphenyl)-piperazine (55 mg, 0.19 mmol, 1.5 equiv). The reaction slurry was brought to 37 ˚C for 12 h before quenching with 1 N HCl solution (3 mL). Organics were separated and the solvent was removed under reduced pressure. Column chromatography (0 to 45% (EtOAc/Hex)) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{19}H_{18}F_5N_2O_2^+$ [M+H$^+$] 401.36, found 402. 1H NMR (400 MHz, CDCl3) δ 738 (t, J = 8.4 Hz, 1H), 7.15 (d, J = 8.0 Hz, 1H), 7.11 (br, 1H), 7.11 - 7.04 (m, 2H), 6.81 (ddd, J_1 = 3.44, J_2 = 6.5, J_3 = 9.4 Hz, 1H), 6.68 (dddd, J_1 = 1.8, J_2 = 3.2, J_3 = 6.4, J_4 = 9.1 Hz, 1H), 4.70 (s, 2H), 3.80 (t, J = 5.2, 2H), 3.75 (t, J = 5.1 Hz, 2H), 3.26 - 3.19 (m, 4H). 13C NMR (176 MHz, CDCl3) δ 165.9, 153.9 (d, J = 11 Hz), 151.2 (d, J = 14 Hz), 150.8, 149.8 (d, J = 13 Hz), 149.8 (d, J = 13 Hz), 144.9 (d, J = 13 Hz), 131.2 (q, J = Hz), 129.8, 124 (d, J = 272 Hz), 119.4, 117.5 (d, J = 18 Hz), 116.9 (d, J = 4 Hz), 112.9 (d, J = 4 Hz), 109.9 (dd, J_1 = 4 Hz, J_2 = 6 Hz), 104.7 (d, J = 20 Hz), 68.2, 49.3, 48.9, 45.1, 41.8.

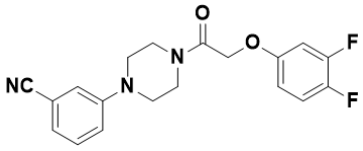
1-(4-(3,5-bis(Trifluoromethyl)phenyl)piperazin-1-yl)-2-((3,4-difluorophenyl)amino)ethan-1-one (3.135)



To a stirring solution of (3,4-difluorophenyl)glycine (30 mg, 0.13 mmol, 1 equiv) in DCM (5 mL) was added EDC (44 mg, 0.26 mmol, 2.0 equiv) and DMAP (1.9 mg, 0.013 mmol, 0.1 equiv). This solution was stirred for 10 min before addition of 1-(3,5-bistrifluoromethylphenyl)-piperazine (71 mg, 0. 20 mol, 1.5 equiv). The reaction slurry was brought to 37 ˚C for 12 h before quenching with 1 N HCl solution (3 mL). Organics

were separated and the solvent was removed under reduced pressure. Column chromatography (0 to 45% (EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{20}H_{17}F_8N_2O_2^+$ [M+H$^+$] 469.3547, found 470. 1H NMR (400 MHz, CDCl3) δ 7.36 (s, 1H), 7.25 (s, 2H), 7.08 (q, J = 9.4 Hz, 1H), 6.81 (ddd, J_1 = 3, J_2 = 6.4, J_3 = 9.4 Hz, 1H), 6.71 - 6.65 (m, 1H), 4.71 (s, 2H), 3.82 (t, J = 5.2 Hz, 2H), 3.78 (t, J = 5.1 Hz, 2H), 3.35 - 3.26 (m, 4H). 13C NMR (176 MHz, CDCl3) δ 166.0, 153.9 (d, J = 10 Hz), 151.2, 149.8 (d, J = 14 Hz), 146.4 (d, J = 13 Hz), 145.0 (d, J = 13 Hz), 132.5 (q, J = 33 Hz), 123.3 (q, J = 272 Hz), 117.6 (d, J = 19 Hz), 115.3, 113.1, 109.8 (dd, J_1 = 4, J_2 = 6 Hz), 104.6 (d, J = 21 Hz), 68.3, 48.7, 48.2, 44.6, 41.6.

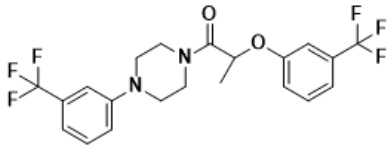
3-(4-((3,4-Difluorophenyl)glycyl)piperazin-1-yl)benzonitrile (3.136)



To a stirring solution of (3,4-difluorophenyl)glycine (30 mg, 0.16 mmol, 1 equiv) in DCM (5 mL) was added EDC (44 mg, 0.32 mmol, 2.0 equiv) and DMAP (1.9 mg, 0.016 mmol, 0.1 equiv). This solution was stirred for 10 min before addition of 3-(piperazin-1-yl)benzonitrile (44 mg, 0.24 mmol, 1.5 equiv). The reaction slurry was brought to 37 ˚C for 12 h before quenching with 1 N HCl solution (3 mL). Organics were separated and the solvent was removed under reduced pressure. Column chromatography (0 to 45% (EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{19}H_{18}F_2N_3O_2^+$ [M+H$^+$] 358.3683, found 358. 1H NMR (400 MHz, CDCl3) δ 7.36 (dd, J_1 = 7.6, J_2 = 9.24,

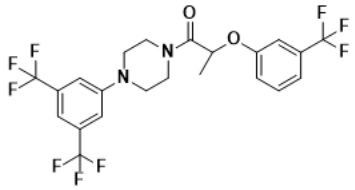
1H), 7.17 (d, J = 7.6, 1H), 7.13 - 7.05 (m, 3H), 6.81 (ddd, J_1 = 3, J_2 = 6.4, J_3 = 9.4 Hz, 1H), 6.68 (dddd, J_1 = 1.8, J_2 = 3.2, J_3 = 6.4, J_4 = 9.1 Hz, 1H), 4.70 (s, 2H), 3.80 (t, J = 5.2 Hz, 2H), 3.75 (t, J = 5.0 Hz, 2H), 3.25 - 3.16 (m, 4H).

2-(3-(Trifluoromethyl)phenoxy)-1-(4-(3-(trifluoromethyl)phenyl)piperazin-1-yl)propan-1-one (3.137)



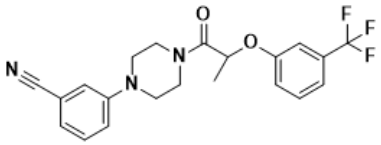
To a stirring solution of 2-(3-(trifluoromethyl)phenoxy)propanoic acid (30 mg, 0.13 mmol, 1 equiv) in DCM (5 mL) was added EDC (40 mg, 0.26 mmol, 2.0 equiv) and DMAP (1.2 mg, 0.1 equiv). This solution was stirred for 10 min before addition of 1-(3-trifluoromethylphenyl)-piperazine (44 mg, 0.19 mmol, 1.5 equiv). The reaction slurry was brought to 37 ˚C for 12 h before quenching with 1 N HCl solution (3 mL). Organics were separated and the solvent was removed under reduced pressure. Column chromatography (0 to 45% (EtOAc/Hex)) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{21}H_{21}F_6N_2O_2^+$ [M+H$^+$] 447.40, found 447. 1H NMR (400 MHz, CDCl3) δ 7.38 (app. qt, 2H), 7.24 (d, J = 7.7 Hz, 1H), 7.17 - 7.06 (m, 4H), 7.04 (d, J = 8.0 Hz, 1H), 5.04 (q, J = 6.9 Hz, 1H), 3.89 - 3.73 (m, 4H), 3.26 - 3.16 (m, 2H), 3.16 - 3.07 (m, 1H), 3.07 - 2.99 (m, 1H), 1.67 (d, J = 6.8 Hz, 3H). 13C NMR (176 MHz, CDCl3) δ 169.1, 157.2, 150.9, 132.3 (d, J = 36 Hz), 131.7, (d, J = 40 Hz) 130.4, 129.7, 123.0, 119.3, 118.4, 117.9, 116.9, 112.8, 112.1, 74.5, 49.3, 49.0, 44.8, 42.2, 17.9.

1-(4-(3,5-bis(Trifluoromethyl)phenyl)piperazin-1-yl)-2-(3-(trifluoromethyl)phenoxy)propan-1-one (3.138)



To a stirring solution of 2-(3-(trifluoromethyl)phenoxy)propanoic acid (30 mg, 0.12 mmol, 1 equiv) in DCM (5 mL) was added EDC (40 mg, 0.24 mmol, 2.0 equiv) and DMAP (1.59 mg, 0.1 mmol, 2.0 equiv). This solution was stirred for 10 min before addition of 1-(3,5-bistrifluoromethylphenyl)-piperazine (57 mg, 0.18 mmol, 1.5 equiv). The reaction slurry was brought to 37 ˚C for 12 h before quenching with 1 N HCl solution (3 mL). Organics were separated and the solvent was removed under reduced pressure. Column chromatography (0 to 45% (EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{22}H_{20}F_9N_2O_2^+$ [M+H$^+$] 515.40, found 515. 1H NMR (400 MHz, CDCl3) δ 7.42 (t, *J* = 8.4 Hz, 1H), 7.34 (s, 1H), 7.25 (obscured doublet, 1H), 7.21 (s, 1H), 7.16 (br, 1H), 7.11 (d, *J* = 8.4 Hz, 1H), 5.05 (q, *J* = 7.1 Hz, 1H), 3.93 - 3.77 (m, 4H), 3.33 - 3.25 (m, 2H), 3.22 - 3.14 (m, 1H), 3.13 - 3.04 (m, 1H), 1.68 (d, *J* = 6.8 Hz, 3H).

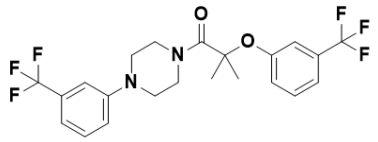
3-(4-(2-(3-(Trifluoromethyl)phenoxy)propanoyl)piperazin-1-yl)benzonitrile (3.139)



To a stirring solution of 2-(3-(trifluoromethyl)phenoxy)propanoic acid (30 mg, 0.16 mmol, 1 equiv) in DCM (5 mL) was added EDC (40 mg, 0.24 mmol, 1.5 equiv) and DMAP (31

mg, 0.24 mmol, 1.5 equiv). This solution was stirred for 10 min before addition of 1-(cyanophenyl)-piperazine (35 mg, 0.24 mmol, 1.5 equiv). The reaction slurry was brought to 37 ˚C for 12 h before quenching with 1 N HCl solution (3 mL). Organics were separated and the solvent was removed under reduced pressure. Column chromatography (0 to 45% (EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{21}H_{21}F_3N_3O_2^+$ [M+H$^+$] 404.41, found 404. 1H NMR (400 MHz, CDCl3) δ 7.41 (t, J = 8.2 Hz, 1H), 7.33 (dd, J_1 = 7.6, J_2, = 9.2 Hz, 1H), 7.25 (obscured doublet, 1H), 7.17 - 7.13 (m, 2H), 7.12 - 7.06 (m, 3H), 5.04 (q, J = 6.9 Hz, 1H), 3.91 - 3.75 (m, 4H), 3.24 - 3.16 (m, 2H), 3.16 - 3.05 (m, 1H), 3.05 - 2.79 (m, 1H), 1.67 (d, J = 6.8 Hz, 3H).

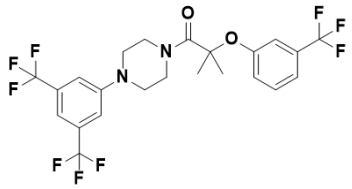
2-Methyl-2-(3-(trifluoromethyl)phenoxy)-1-(4-(3-(trifluoromethyl)phenyl)piperazin-1-yl)propan-1-one (3.140)



To a stirring solution of 2-methyl-2-(3-(trifluoromethyl)phenoxy)propanoic acid (30 mg, 0.12 mmol, 1 equiv) in DCM (5 mL) was added EDC (40 mg, 0.24 mmol, 2.0 equiv) and DMAP (31 mg, 0.24 mmol, 2.0 equiv). This solution was stirred for 10 min before addition of 1-(3-trifluoromethylphenyl)-piperazine (48 mg, 0.18 mmol, 1.5 equiv). The reaction slurry was brought to 37 ˚C for 12 h before quenching with 1 N HCl solution (3 mL). Organics were separated and the solvent was removed under reduced pressure. Column chromatography (0 to 45% (EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z

cal'd for $C_{22}H_{23}F_6N_2O_2^+$ [M+H$^+$] 461.63, found 462. 1H NMR (400 MHz, CDCl3) δ 7.46 - 7.39 (m, 2H), 7.35 (s, 1H), 7.30 (d, *J* = 7.8 Hz, 1H), 7.23 - 7.17 (m, 2H), 7.12 - 7.04 (m, 3H), 4.08 (br, 2H), 3.92 (br, 2H), 3.22 (br, 2H), 2.95 (br, 2H), 1.78 (s, 6H). 13C NMR (176 MHz, CDCl3) δ 171.2, 155.5, 150.8, 132.2 (d, *J* = 32 Hz), 131.6 (d, *J* = 33 Hz), 130.0, 129.7, 124.9, 123.3, 119.7, 119.1, 118.3, 116.7, 114.5, 112.6, 81.4, 49.0, 48.8, 45.6, 42.8, 26.0.

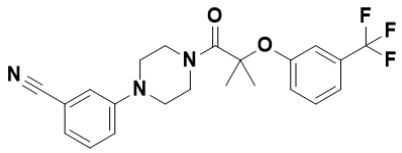
1-(4-(3,5-bis(Trifluoromethyl)phenyl)piperazin-1-yl)-2-methyl-2-(3-(trifluoromethyl)phenoxy)propan-1-one (3.141)



To a stirring solution of 2-methyl-2-(3-(trifluoromethyl)phenoxy)propanoic acid (30 mg, 0.12 mmol, 1 equiv) in DCM (5 mL) was added EDC (40 mg, 0.24 mmol, 2.0 equiv) and DMAP (31 mg, 0.24 mmol, 2.0 equiv). This solution was stirred for 10 min before addition of 1-(3,5-bistrifluoromethylphenyl)-piperazine (48 mg, 0.18 mmol, 1.5 equiv). The reaction slurry was brought to 37 ˚C for 12 h before quenching with 1 N HCl solution (3 mL). Organics were separated and the solvent was removed under reduced pressure. Column chromatography (0 to 45% (EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{23}H_{22}F_9N_2O_2^+$ [M+H$^+$] 529.43, found 529. 1H NMR (400 MHz, CDCl3) δ 7.35 (t, *J* = 7.8 Hz, 1H), 7.30 (s, 1H), 7.22 (td, J_1 = 0.7 Hz, J_2 = 7.0 Hz, 1H), 7.15

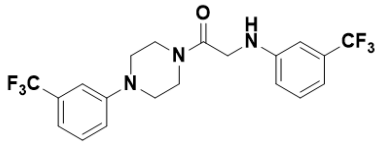
- 7.11 (m, 3H), 7.01 (dd, J_1 = 2.6, J_2 = 8.3 Hz, 1H), 4.01 (br, 2H), 3.84 (br, 2H), 3.20 (br, 2H), 2.92 (br, 2H), 1.70 (s, 6H).

3-(4-(2-Methyl-2-(3-(trifluoromethyl)phenoxy)propanoyl)piperazin-1-yl)benzonitrile (3.142)



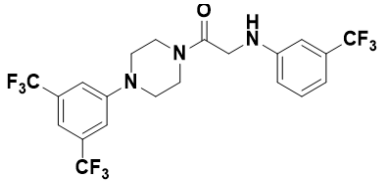
To a stirring solution of 2-methyl-2-(3-(trifluoromethyl)phenoxy)propanoic acid (30 mg, 0.12 mmol, 1 equiv) in DCM (5 mL) was added EDC (40 mg, 0.24 mmol, 2.0 equiv) and DMAP (31 mg, 0.24 mmol, 2.0 equiv). This solution was stirred for 10 min before addition of 1-(3-cyanophenyl)-piperazine (48 mg, 0.18 mmol, 1.5 equiv). The reaction slurry was brought to 37 ˚C for 12 h before quenching with 1 N HCl solution (3 mL). Organics were separated and the solvent was removed under reduced pressure. Column chromatography (0 to 45% (EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{22}H_{23}F_3N_3O_2^+$ [M+H$^+$] 418.44, found 419. 1H NMR (400 MHz, CDCl3) δ 7.36 (t, J = 7.8 Hz, 1H), 7.33 - 7.28 (m, 1H), 7.23 (J_1, = 0.7, J_2 = 7.0 Hz, 1H), 7.14 - 7.10 (m, 2H), 7.04 - 7.00 (m, 3H), 4.00 (br, 2H), 3.83 (br, 2H), 3.12 (br, 2H), 2.84 (br, 2H), 1.70 (s, 6H).

2-((3-(Trifluoromethyl)phenyl)amino)-1-(4-(3-(trifluoromethyl)phenyl)piperazin-1-yl)ethan-1-one (3.143)



To a stirring solution of (3-(trifluoromethyl)phenyl)glycine (40 mg, 0.17 mmol, 1 equiv) in DCM (5 mL) was added EDC (56 mg, 0.34 mmol, 2.0 equiv) and DMAP (2.2 mg, 0.17 mmol, 0.1 equiv). This solution was stirred for 10 min before addition of 1-(3-trifluoromethylphenyl)-piperazine (63 mg, 0.25 mmol, 1.5 equiv). The reaction slurry was brought to 37 ˚C for 12 h before quenching with 1 N HCl solution (3 mL). Organics were separated and the solvent was removed under reduced pressure. Column chromatography (0 to 45% (EtOAc/Hex)) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{20}H_{20}F_6N_3O^+$ [M+H$^+$] 432.39, found 434. 1H NMR (400 MHz, CDCl3) δ 7.41 (t, *J* = 7.9 Hz, 1H), 7.28 (t, *J* = 7.8 Hz, 1H), 7.21 - 7.12 (m, 3H), 6.68 (d, *J* = 7.6 Hz, 1H), 6.84 - 6.80 (m, 2H), 3.96 (s, 1H), 3.90 (t, *J* = 5.3 Hz, 2H), 3.69 (t, *J* = 5.2, 2H), 3.31 (t, *J* = 5.2 Hz, 2H), 3.28 (t, *J* = 5.2 Hz, 2H). 13C NMR (176 MHz, CDCl3) δ 167.0, 150.9, 147.3, 131.8, (d, *J* = 11), 131.6, (d, *J* = 11 Hz), 129.8, 129.7, 125.1, (d, *J* = 37 Hz), 123.6 (d, *J* = 37 Hz), 119.6, 117.1, 116.5, 114.1, 113.0, 108.5, 49.1, 49.0, 44.8, 44.1, 41.9.

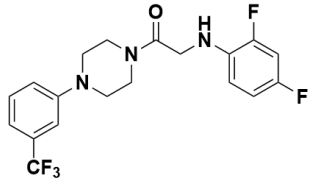
1-(4-(3,5-bis(Trifluoromethyl)phenyl)piperazin-1-yl)-2-((3-(trifluoromethyl)phenyl)amino)ethan-1-one (3.144)



To a stirring solution of (3-(trifluoromethyl)phenyl)glycine (40 mg, 0.13 mmol, 1 equiv) in DCM (5 mL) was added EDC (56 mg, 0.26 mmol, 2.0 equiv) and DMAP (2.2 mg, 0.013 mmol, 0.1 equiv). This solution was stirred for 10 min before addition of 1-(3,5-bistrifluoromethylphenyl)-piperazine (63 mg, 0.19 mmol, 1.5 equiv). The reaction slurry

was brought to 37 ˚C for 12 h before quenching with 1 N HCl solution (3 mL). Organics were separated and the solvent was removed under reduced pressure. Column chromatography (0 to 45% (EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{21}H_{19}F_9N_3O^+$ [M+H$^+$] 500.39, found 501. 1H NMR (400 MHz, CDCl3) δ 7.37 (s, 1H), 7.32 - 7.27 (m, 3H), 7.00 (d, *J* = 7.4 Hz, 1H), 6.87 - 6.81 (m, 2H), 3.98 (s, 2H), 3.89 (t, *J* = 5.3 Hz, 2H), 3.69 (t, *J* = 4.7 Hz, 2H), 3.38 (t, *J* = 5.4 Hz, 2H), 3.34 (t, *J* = 5.6 Hz, 2H). 13C NMR (176 MHz, CDCl3) δ 167.1, 151.7, 147.2, 132.6 (q, *J* = 40 Hz), 131.6 (d, *J* = 38 Hz), 121.7, 124.1 (d, *J* = 272 Hz), 123.4 (d, *J* = 272 Hz), 116.5, 115.4, 114.1 (d, *J* = 4 Hz), 113.2 (d, *J* = 3 Hz), 108.5 (d, *J* = 4 Hz), 48.3, 44.8, 43.8, 41.6.

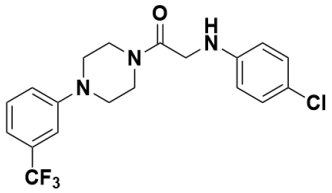
2-((2,4-Difluorophenyl)amino)-1-(4-(3-(trifluoromethyl)phenyl)piperazin-1-yl)ethan-1-one (3.145)



To a stirring solution of (2,4-difluorophenyl)glycine (16 mg, 0.07 mmol, 1 equiv) in DCM (5 mL) was added EDC (27 mg, 0.14 mmol, 2.0 equiv) and DMAP (0.10 mg, 0.007 mmol, 0.01 equiv). This solution was stirred for 10 min before addition of 1-(3-(trifluoromethyl)phenyl)piperazine (30 mg, 0.011 mmol, 1.5 equiv). The reaction slurry was brought to 37 ˚C for 12 h before quenching with 1 N HCl solution (3 mL). Organics were separated and the solvent was removed under reduced pressure. Column chromatography (0 to 100% (EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI -

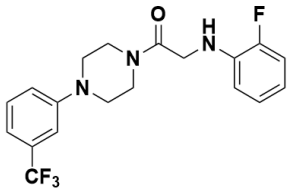
quad) m/z cal'd for $C_{19}H_{19}F_5N_3O^+$ [M+H$^+$], 400.37 found 401. 1H NMR (400 MHz, $CDCl_3$) δ 7.39 (t, J = 8.0 Hz, 1H), 7.20 - 7.07 (m, 3H), 6.85 - 6.71 (m, 2H), 6.55 (dd, J_1 = 5.3, J_2 = 9.3 Hz, 1H), 3.95 (s, 2H), 3.89 - 3.82 (m, 4H), 3.73 - 3.60 (m, 4H), 3.34 - 3.21 (m, 4H).

2-((4-Chlorophenyl)amino)-1-(4-(3-(trifluoromethyl)phenyl)piperazin-1-yl)ethan-1-one (3.146)



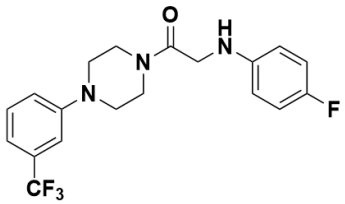
To a stirring solution of (4-chlorophenyl)glycine (16 mg, 0.07 mmol, 1 equiv) in DCM (5 mL) was added EDC (27 mg, 0.14mmol, 2.0 equiv) and DMAP (0.10 mg, 0.007 mmol, 0.01 equiv). This solution was stirred for 10 min before addition of 1-(3-(trifluoromethyl)phenyl)piperazine (30 mg, 0.10 mmol, 1.5 equiv). The reaction slurry was brought to 37 ˚C for 12 h before quenching with 1 N HCl solution (3 mL). Organics were separated and the solvent was removed under reduced pressure. Column chromatography (0 to 100% (EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{19}H_{20}ClF_3N_3O^+$ [M+H$^+$], 398.83 found 399.1H NMR (400 MHz, $CDCl_3$) δ 7.39 (t, J = 7.9 Hz, 1H), 7.19 - 7.11 (m, 4H), 7.09 (d, J = 8.3 Hz, 1H), 6.59 (d, J = 8.7 Hz, 2H), 3.91 (s, 2H), 3.88 - 3.84 (m, 2H), 3.66 - 3.61 (m, 2H), 3.31 - 3.22 (m, 4H). 13C NMR (101 MHz, CDCl3) δ 167.3, 150.9, 145.8, 131.6 (q, J = 32 Hz), 129.8, 129.2, 120.7 (q, J = 315 Hz), 117.1 (d, J = 4 Hz), 114.0, 113.0 (d, J = 4 Hz), 49.1, 49.0, 45.2, 44.1, 41.9.

2-((2-Fluorophenyl)amino)-1-(4-(3-(trifluoromethyl)phenyl)piperazin-1-yl)ethan-1-one (3.147)



To a stirring solution of (2-fluorophenyl)glycine (15 mg, 0.07 mmol, 1 equiv) in DCM (5 mL) was added EDC (27 mg, 0.14 mmol,2.0 equiv) and DMAP (0.10 mg, 0.007 mmol, 0.01 equiv). This solution was stirred for 10 min before addition of 1-(3-(trifluoromethyl)phenyl)piperazine (30 mg, 0.1 mmol, 1.5 equiv). The reaction slurry was brought to 37 ˚C for 12 h before quenching with 1 N HCl solution (3 mL). Organics were separated and the solvent was removed under reduced pressure. Column chromatography (0 to 100% (EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{19}H_{20}F_4N_3O^+$ [M+H$^+$], 382.38 found 383. 1H NMR (400 MHz, $CDCl_3$) δ 7.40 (t, *J* = 7.9 Hz, 1H), 7.20 - 7.09 (m, 3H), 7.03 - 6.96 (m, 2H), 6.72 - 6.59 (m, 2H), 3.98 (s, 2H), 3.92 - 3.85 (m, 2H), 3.70 - 3.64 (m, 2H), 3.31 - 3.24 (m, 4H).

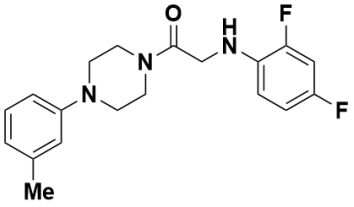
2-((4-Fluorophenyl)amino)-1-(4-(3-(trifluoromethyl)phenyl)piperazin-1-yl)ethan-1-one (3.148)



To a stirring solution of (4-fluorophenyl)glycine (15 mg, 0.07 mmol, 1 equiv) in DCM (5 mL) was added EDC (27 mg, 0.14 mmol, 2.0 equiv) and DMAP (0.10 mg, 0.007, 0.01

equiv). This solution was stirred for 10 min before addition of 1-(3-(trifluoromethyl)phenyl)piperazine (30 mg, 0.11 mmol, 1.5 equiv). The reaction slurry was brought to 37 ˚C for 12 h before quenching with 1 N HCl solution (3 mL). Organics were separated and the solvent was removed under reduced pressure. Column chromatography (0 to 100% (EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{19}H_{20}F_4N_3O^+$ [M+H$^+$], 382.38 found 382. 1H NMR (400 MHz, $CDCl_3$) δ 7.39 (t, *J* = 7.9 Hz, 1H), 7.14 (d, *J* = 7.7 Hz, 1H), 7.13 (s, 1H), 7.08 (d, *J* = 8.2 Hz, 1H), 6.92 (t, *J* = 8.7 Hz, 2H), 6.67 - 6.58 (m, 2H), 3.92 (s, 2H), 3.89 - 3.82 (m, 2H), 3.67 - 3.61 (m, 2H), 3.31 - 3.22 (m, 4H).

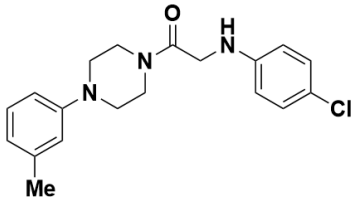
2-((2,4-Difluorophenyl)amino)-1-(4-(m-tolyl)piperazin-1-yl)ethan-1-one (3.149)



To a stirring solution of (4-fluorophenyl)glycine (21 mg, 0.12 mmol, 1 equiv) in DCM (5 mL) was added EDC (35 mg, 0.24 mmol, 2.0 equiv) and DMAP (0.13 mg, 0.012 mmol, 0.01 equiv). This solution was stirred for 10 min before addition of 1-(m-tolyl)piperazine (30 mg, 0.16 mmol, 1.5 equiv). The reaction slurry was brought to 37 ˚C for 12 h before quenching with 1 N HCl solution (3 mL). Organics were separated and the solvent was removed under reduced pressure. Column chromatography (0 to 100% (EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{19}H_{22}F_2N_3O^+$ [M+H$^+$], 346.40

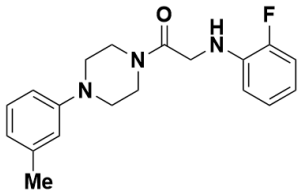
found 346. 1H NMR (400 MHz, $CDCl_3$) δ 7.19 (t, J = 7.7 Hz, 1H), 6.87 - 6.69 (m, 5H), 6.54 (dt, J_1 = 5.3, J_2 = 9.2 Hz, 1H), 4.95 (br, 1H), 3.94 (s, 2H), 3.89 - 3.83 (m, 2H), 3.67 - 3.60 (m, 2H), 3.26 - 3.16 (m, 4H), 2.34 (s, 3H).

2-((4-Chlorophenyl)amino)-1-(4-(m-tolyl)piperazin-1-yl)ethan-1-one (3.150)



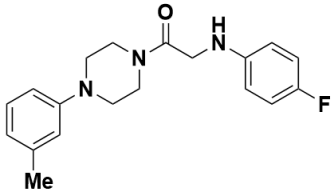
To a stirring solution of (4-chlorophenyl)glycine (21 mg, 0.12 mmol, 1 equiv) in DCM (5 mL) was added EDC (35 mg, 0.24 mmol, 2.0 equiv) and DMAP (0.13 mg, 0.012 mmol, 0.01 equiv). This solution was stirred for 10 min before addition of 1-(m-tolyl)piperazine (30 mg, 0.16 mmol, 1.5 equiv). The reaction slurry was brought to 37 ˚C for 12 h before quenching with 1 N HCl solution (3 mL). Organics were separated and the solvent was removed under reduced pressure. Column chromatography (0 to 100% (EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{19}H_{23}ClN_3O^+$ [$M+H^+$], 344.86 found 345. 1H NMR (400 MHz, $CDCl_3$) δ 7.21 (t, J = 7.7 Hz, 1H), 7.15 (d, J = 8.3 Hz, 2H), 6.89 - 6.80 (m, 3H), 6.56 (d, J = 8.4 Hz, 2H), 3.94 - 3.88 (m, 4H), 3.69 (br, 2H), 3.28 - 3.19 (m, 4H), 2.35 (s, 3H).

2-((2-Fluorophenyl)amino)-1-(4-(m-tolyl)piperazin-1-yl)ethan-1-one (3.151)



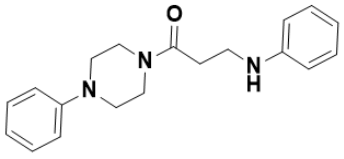
To a stirring solution of (2-fluorophenyl)glycine (19 mg, 0.12 mmol, 1 equiv) in DCM (5 mL) was added EDC (35 mg, 0. 24 mmol, 2.0 equiv) and DMAP (0.13 mg, 0.012 mmol, 0.01 equiv). This solution was stirred for 10 min before addition of 1-(m-tolyl)piperazine (30 mg, 0.16 mmol, 1.5 equiv). The reaction slurry was brought to 37 ˚C for 12 h before quenching with 1 N HCl solution (3 mL). Organics were separated and the solvent was removed under reduced pressure. Column chromatography (0 to 100% (EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{19}H_{23}FN_3O^+$ [M+H$^+$], 328.41 found 329. 1H NMR (400 MHz, $CDCl_3$) δ 7.19 (t, J = 7.6 Hz, 1H), 7.04 - 6.95 (m, 2H), 6.82 - 6.72 (m, 3H), 6.71 - 6.58 (m, 2H), 5.12 (br, 1H), 3.97 (s, 2H), 3.88 - 3.82 (m, 2H), 3.66 - 3.60 (m, 2H), 3.25 - 3.16 (m, 4H), 2.34 (s, 3H). 13C NMR (176 MHz, CDCl3) δ 167.3, 152.7 (dd, J_1 = 11, J_2 = 238 Hz), 151.0 (dd, J_1 = 11, J_2 = 238 Hz), 150.4, 132.6 (dd, J_1 = 3, J_2 = 12 Hz), 131.6 (q, J = 33 Hz), 129.8, 124.2 (q, J = 273 Hz), 119.5, 116.9 (d, J = 4 Hz), 112.9 (d, J = 4 Hz), 112.3 (dd, J_1 = 5, J_2 = 9 Hz), 110.5 (dd, J_1 = 4, J_2 = 22 Hz), 103.7 (dd, J_1 = 23, J_2 = 27 Hz), 49.0, 48.9, 45.4, 44.1, 41.8.

2-((4-Fluorophenyl)amino)-1-(4-(m-tolyl)piperazin-1-yl)ethan-1-one (3.152)



To a stirring solution of (4-fluorophenyl)glycine (19 mg, 0.11 mmol, 1 equiv) in DCM (5 mL) was added EDC (35 mg, 0.22 mmol, 2.0 equiv) and DMAP (0.13 mg, 0.011 mmol, 0.01 equiv). This solution was stirred for 10 min before addition of 1-(m-tolyl)piperazine (30 mg, 0.15 mmol, 1.5 equiv). The reaction slurry was brought to 37 ˚C for 12 h before quenching with 1 N HCl solution (3 mL). Organics were separated and the solvent was removed under reduced pressure. Column chromatography (0 to 100% (EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{19}H_{23}FN_3O^+$ [M+H$^+$], 328.41 found 330. 1H NMR (400 MHz, $CDCl_3$) δ 7.19 (t, *J* = 7.6 Hz, 1H), 6.91 (t, *J* = 8.5 Hz, 2H), 6.82 - 6.74 (m, 3H), 6.62 - 6.54 (m, 2H), 3.90 (s, 2H), 3.87 - 3.82 (m, 2H), 3.66 - 3.60 (m, 2H), 3.25 - 3.16 (m, 4H), 2.34 (s, 3H).

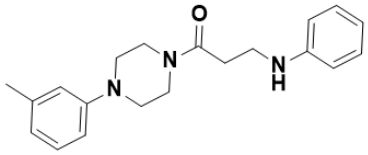
3-(Phenylamino)-1-(4-phenylpiperazin-1-yl)propan-1-one (3.153)



To a stirring solution of 3-(phenylamino)propanoic acid (50 mg, 0.30 mmol, 1 equiv) in DCM (5 mL) was added EDC (94 mg, 0.6 mmol, 2.0 equiv) and DMAP (92 mg, 0.75 mmol, 2.5 equiv). This solution was stirred for 10 min before addition of 1-phenylpiperazine (73 mg, 0.45 mmol, 1.5 equiv). The reaction slurry was brought to 37 ˚C

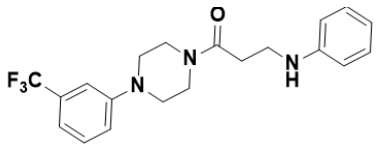
for 12 h before quenching with 1 N HCl solution (3 mL). Organics were separated and the solvent was removed under reduced pressure. Column chromatography (0 to 100% (EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{19}H_{24}N_3O^+$ [M+H$^+$], 310.42 found 310. 1H NMR (400 MHz, $CDCl_3$) δ 7.28 (t, *J* = 7.5 Hz, 2H), 7.18 (t, *J* = 7.3 Hz, 2H), 6.95 - 6.87 (m, 3H), 6.71 (t, *J* = 7.3 Hz, 1H), 6.65 (d, *J* = 8.0 Hz, 2H), 4.45 (br, 1H), 3.82 - 3.75 (m, 2H), 3.61 - 3.54 (m, 2H), 3.53 (t, *J* = 6.8 Hz, 2H), 3.19 - 3.09 (m, 4H), 2.66 (t, *J* = 6.0 Hz, 2H)

3-(Phenylamino)-1-(4-(m-tolyl)piperazin-1-yl)propan-1-one (3.154)



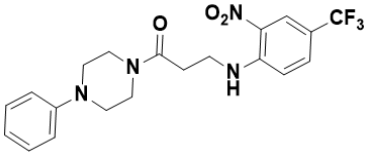
To a stirring solution of 3-(phenylamino)propanoic acid (50 mg, 0.29 mmol, 1 equiv) in DCM (5 mL) was added EDC (94 mg, 0.58 mmol, 2.0 equiv) and DMAP (92 mg, 0.75 mmol, 2.5 equiv). This solution was stirred for 10 min before addition of 1-(m-tolyl)piperazine (80 mg, 0.45 mmol, 1.5 equiv). The reaction slurry was brought to 37 ˚C for 12 h before quenching with 1 N HCl solution (3 mL). Organics were separated and the solvent was removed under reduced pressure. Column chromatography (0 to 100% (EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{20}H_{26}N_3O^+$ [M+H$^+$], 324.45 found 325. 1H NMR (400 MHz, $CDCl_3$) δ 7.22 - 7.13 (m, 3H), 6.77 - 6.67 (m, 4H), 6.64 (d, *J* = 8.4 Hz, 2H), 4.34 (br, 1H), 3.81 - 3.75 (m, 2H), 3.60 - 3.50 (m, 4H), 3.17 - 3.08 (m, 4H), 2.65 (t, *J* = 6.0 Hz, 2H), 2.33 (s, 3H).

3-(Phenylamino)-1-(4-(3-(trifluoromethyl)phenyl)piperazin-1-yl)propan-1-one (3.155)



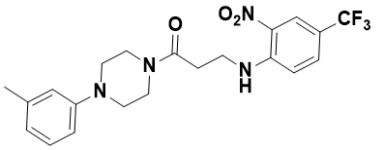
To a stirring solution of 3-(phenylamino)propanoic acid (50 mg, 0.22 mmol, 1 equiv) in DCM (5 mL) was added EDC (94 mg, 0.44 mmol, 2.0 equiv) and DMAP (92 mg, 0.51 mmol, 2.5 equiv). This solution was stirred for 10 min before addition of 1-(3-(trifluoromethyl)phenyl)piperazine (104 mg, 0.33 mmol, 1.5 equiv). The reaction slurry was brought to 37 ˚C for 12 h before quenching with 1 N HCl solution (3 mL). Organics were separated and the solvent was removed under reduced pressure. Column chromatography (0 to 100% (EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{20}H_{23}F_3N_3O^+$ [M+H$^+$], 378.42 found 379. 1H NMR (400 MHz, $CDCl_3$) δ 7.37 (t, *J* = 8.0 Hz, 1H), 7.18 (t, *J* = 7.3 Hz, 2H), 7.13 (d, *J* = 7.7 Hz, 1H), 7.10 (s, 1H), 7.05 (d, *J* = 8.3 Hz, 1H), 6.72 (t, *J* = 7.3 Hz, 1H), 6.65 (d, *J* = 8.6 Hz, 2H), 4.45 (br, 1H), 3.83 - 3.76 (m, 2H), 3.61 - 3.57 (m, 2H), 3.54 (t, *J* = 6.0 Hz, 2H), 3.24 - 3.13 (m, 4H), 2.66 (t, *J* = 6.0 Hz, 2H).

3-((2-Nitro-4-(trifluoromethyl)phenyl)amino)-1-(4-phenylpiperazin-1-yl)propan-1-one (3.156)



To a stirring solution of 3-((2-nitro-4-(trifluoromethyl)phenyl)amino)propanoic acid (50 mg, 0.6 mmol, 1 equiv) in DCM (5 mL) was added EDC (55 mg, 1.2 mmol, 2.0 equiv) and DMAP (54 mg, 1.5 mmol, 2.5 equiv). This solution was stirred for 10 min before addition of 1-phenylpiperazine (43 mg, 0.9 mmol, 1.5 equiv). The reaction slurry was brought to 37 ˚C for 12 h before quenching with 1 N HCl solution (3 mL). Organics were separated and the solvent was removed under reduced pressure. Column chromatography (0 to 100% (EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{20}H_{22}F_3N_4O_3^+$ [M+H$^+$], 423.42 found 424. 1H NMR (400 MHz, $CDCl_3$) δ 8.52 (t, J = 6.5 Hz, 1H), 8.47 (d, J = 2.1 Hz, 1H), 7.64 (dd, J_1 = 2.2, J_2 = 9.0 Hz, 1H), 7.29 (t, J = 7.1 Hz, 2H), 7.03 (d, J = 9.0 Hz, 1H), 6.96 - 6.90 (m, 2H), 3.85 - 3.73 (m, 4H), 3.67 - 3.60 (m, 2H), 3.22 - 3.14 (m, 4H), 2.77 (t, J = 6.6 Hz, 2H).

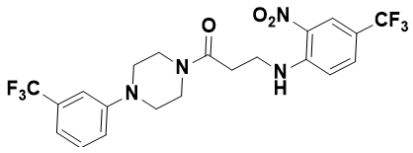
3-((2-Nitro-4-(trifluoromethyl)phenyl)amino)-1-(4-(m-tolyl)piperazin-1-yl)propan-1-one (3.157)



To a stirring solution of 3-((2-nitro-4-(trifluoromethyl)phenyl)amino)propanoic acid (50 mg, 0.29 mmol, 1 equiv) in DCM (5 mL) was added EDC (55 mg, 0.6 mmol, 2.0 equiv) and DMAP (54 mg, 0.75 mmol, 2.5 equiv). This solution was stirred for 10 min before addition of 1-(m-tolyl)piperazine (47 mg, 0.45 mmol, 1.5 equiv). The reaction slurry was brought to 37 ˚C for 12 h before quenching with 1 N HCl solution (3 mL). Organics were separated and the solvent was removed under reduced pressure. Column chromatography

(0 to 100% (EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{21}H_{24}F_3N_4O_3^+$ [M+H$^+$], 437.44 found 438. 1H NMR (400 MHz, $CDCl_3$) δ 8.51 (br, 1H), 8.48 (s, 1H), 7.64 (dd, J_1 = 2.2, J_2 = 9.1 Hz, 1H), 7.18 (t, J = 7.7 Hz, 1H), 7.03 (d, J = 9.0 Hz, 1H), 6.78 - 6.73 (m, 2H), 3.84 - 3.73 (m, 4H), 3.65 - 3.60 (m, 2H), 3.20 - 3.14 (m, 4H), 2.77 (t, J = 6.6 Hz, 2H), 2.33 (s, 3H).

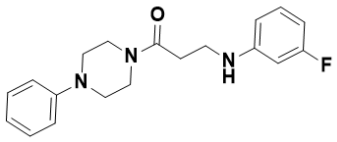
3-((2-Nitro-4-(trifluoromethyl)phenyl)amino)-1-(4-(3-(trifluoromethyl)phenyl)piperazin-1-yl)propan-1-one (3.158)



To a stirring solution of 3-((2-nitro-4-(trifluoromethyl)phenyl)amino)propanoic acid (50 mg, 0.17 mmol, 1 equiv) in DCM (5 mL) was added EDC (55 mg, 0.34 mmol, 2.0 equiv) and DMAP (54 mg, 0.42 mmol, 2.5 equiv). This solution was stirred for 10 min before addition of 1-(3-(trifluoromethyl)phenyl)piperazine (62 mg, 0.26 mmol, 1.5 equiv). The reaction slurry was brought to 37 ˚C for 12 h before quenching with 1 N HCl solution (3 mL). Organics were separated and the solvent was removed under reduced pressure. Column chromatography (0 to 100% (EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{21}H_{21}F_6N_4O_3^+$ [M+H$^+$], 491.41 found 492. 1H NMR (400 MHz, $CDCl_3$) δ 8.52 (t, J = 5.8 Hz, 1H), 8.47 (d, J = 2.1 Hz, 1H), 7.64 (dd, J_1 = 2.2, J_2 = 9.1 Hz, 1H), 7.38 (t, J = 7.9 Hz, 1H), 7.14 (d, J = 7.8 Hz, 1H), 7.11 (s, 1H), 7.70 (d, J = 8.3

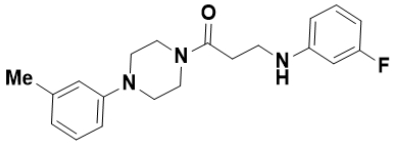
Hz, 1H), 7.03 (d, J = 9.0 Hz, 1H), 3.84 - 3.76 (m, 4H), 3.68 - 3.62 (m, 2H), 3.28 - 3.19 (m, 4H), 2.78 (t, J = 6.6 Hz, 2H).

3-((2-Fluorophenyl)amino)-1-(4-phenylpiperazin-1-yl)propan-1-one (3.159)



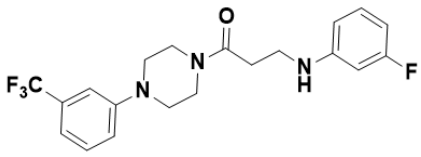
To a stirring solution of 3-((2-fluorophenyl)amino)propanoic acid (50 mg, 0.26 mmol, 1 equiv) in DCM (5 mL) was added EDC (85 mg, 0.52 mmol, 2.0 equiv) and DMAP (83 mg, 0.63 mmol, 2.5 equiv). This solution was stirred for 10 min before addition of 1-phenylpiperazine (66 mg, 0.40 mmol, 1.5 equiv). The reaction slurry was brought to 37 ˚C for 12 h before quenching with 1 N HCl solution (3 mL). Organics were separated and the solvent was removed under reduced pressure. Column chromatography (0 to 100% (EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{19}H_{23}FN_3O^+$ [M+H$^+$], 328.41 found 329. 1H NMR (400 MHz, $CDCl_3$) δ 7.32 - 7.26 (m, 2H), 7.08 (q, J = 6.9 Hz, 1H), 6.94 - 6.89 (m, 3H), 6.42 - 6.36 (m, 2H), 6.32 (td, J_1 = 2.4, J_2 = 11.6 Hz, 1H), 3.81 - 3.77 (m, 2H), 3.61 - 3.56 (m, 2H), 3.50 (t, J = 5.9 Hz, 2H), 3.18 - 3.11 (m, 4H), 2.64 (t, J = 5.9 Hz, 2H).

3-((2-Fluorophenyl)amino)-1-(4-(m-tolyl)piperazin-1-yl)propan-1-one (3.160)



To a stirring solution of 3-((2-fluorophenyl)amino)propanoic acid (50 mg, 0.28 mmol, 1 equiv) in DCM (5 mL) was added EDC (85 mg, 0.54 mmol, 2.0 equiv) and DMAP (83 mg, 0.69 mmol, 2.5 equiv). This solution was stirred for 10 min before addition of 1-(m-tolyl)piperazine (72 mg, 0.42 mmol, 1.5 equiv). The reaction slurry was brought to 37 ˚C for 12 h before quenching with 1 N HCl solution (3 mL). Organics were separated and the solvent was removed under reduced pressure. Column chromatography (0 to 100% (EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{20}H_{25}FN_3O^+$ [M+H$^+$], 342.44 found 343. 1H NMR (400 MHz, $CDCl_3$) δ 7.17 (t, *J* = 7.7 Hz, 1H), 7.08 (q, *J* = 7.9 Hz, 1H), 6.77 - 7.60 (m, 3H), 6.42 - 6.27 (m, 3H), 4.51 (br, 1H), 3.81 - 3.75 (m, 2H), 3.61 - 3.54 (m, 2H), 3.50 (t, *J* = 5.8 Hz, 2H), 3.18 - 3.09 (m, 4H), 2.64 (t, *J* = 5.9 Hz, 2H), 2.32 (s, 3H). 13C NMR (176 MHz, CDCl3) δ 169.9, 164.0 (d, *J* = 243 Hz), 150.9, 149.6 (d, *J* = 10 Hz), 139.0, 130.4 (d, *J* = 10 Hz), 129.1, 121.7, 117.6, 113.8, 109.0, 103.7 (d, *J* = 22 Hz), 99.4 (d, *J* = 25 Hz), 49.8, 49.5, 45.4, 41.6, 39.3, 31.9, 21.7.

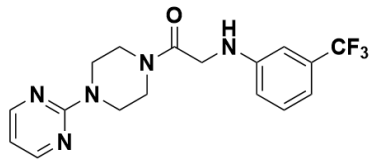
3-((2-Fluorophenyl)amino)-1-(4-(3-(trifluoromethyl)phenyl)piperazin-1-yl)propan-1-one (3.161)



To a stirring solution of 3-((2-fluorophenyl)amino)propanoic acid (50 mg, 0.26 mmol, 1 equiv) in DCM (5 mL) was added EDC (85 mg, 0.52 mmol, 2.0 equiv) and DMAP (83 mg, 0.69 mmol, 2.5 equiv). This solution was stirred for 10 min before addition of 1-(3-(trifluoromethyl)phenyl)piperazine (94 mg, 0.40 mmol, 1.5 equiv). The reaction slurry was

brought to 37 ˚C for 12 h before quenching with 1 N HCl solution (3 mL). Organics were separated and the solvent was removed under reduced pressure. Column chromatography (0 to 100% (EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{20}H_{22}F_4N_3O^+$ [M+H$^+$], 396.41 found 397. 1H NMR (400 MHz, $CDCl_3$) δ 7.37 (t, J = 7.9 Hz, 1H), 7.16 - 7.02 (m, 4H), 6.42 - 6.64 (m, 2H), 6.31 (td, J_1 = 2.4, J_2 = 11.6 Hz, 1H), 4.50 (br, 1H), 3.83 - 3.77 (m, 2H), 3.63 - 3.57 (m, 2H), 3.51 (t, J = 5.9 Hz, 2H), 3.24 - 3.16 (m, 4H), 2.65 (t, J = 5.9 Hz, 2H). 13C NMR (176 MHz, CDCl3) δ 170.0, 164.0 (d, J = 243), 150.9, 149.5 (d, J = 11 Hz), 131.5 (d, J = 32 Hz), 130.4 (d, J = 11 Hz), 129.8, 124.1 (d, J = 273 Hz), 119.4, 117.0, 112.8 (d, J = 4 Hz), 109.0 (d, J = 2 Hz), 103.9 (d, J = 21 Hz), 99.4 (d, J = 25 Hz), 49.0, 48.9, 45.1, 41.3, 39.3, 31.9.

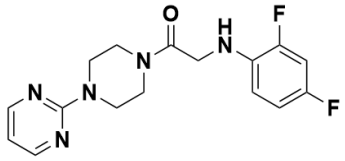
1-(4-(Pyrimidin-2-yl)piperazin-1-yl)-2-((3-(trifluoromethyl)phenyl)amino)ethan-1-one (3.162)



To a stirring solution of (3-(trifluoromethyl)phenyl)glycine (27 mg, 0.11 mmol, 1 equiv) in DCM (5 mL) was added EDC (37 mg, 0.22 mmol, 2.0 equiv) and DMAP (0.14mg, 0.01mmol, 0.01 equiv). This solution was stirred for 10 min before addition of 2-(piperazin-1-yl)pyrimidine (30 mg, 0.15 mmol, 1.5 equiv). The reaction slurry was brought to 37 ˚C for 12 h before quenching with 1 N HCl solution (3 mL). Organics were separated and the solvent was removed under reduced pressure. Column chromatography (0 to 100% (EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for

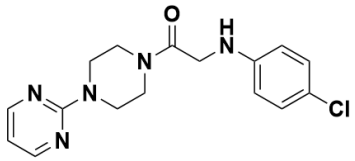
biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{17}H_{19}F_3N_5O^+$ [M+H$^+$], 366.37 found 367. 1H NMR (400 MHz, $CDCl_3$) δ 8.30 (d, *J* = 4.8 Hz, 2H), 7.25 - 7.19 (m, 1H), 6.91 (d, *J* = 7.6 Hz, 1H), 6.78 - 6.71 (m, 2H), 6.53 (t, *J* = 4.8 Hz, 1H), 3.93 - 3.87 (m, 4H), 3.86 - 3.80 (m, 2H), 3.77 - 3.70 (m, 2H), 3.53 - 3.46 (m, 2H).

2-((2,4-Difluorophenyl)amino)-1-(4-(pyrimidin-2-yl)piperazin-1-yl)ethan-1-one (3.163)



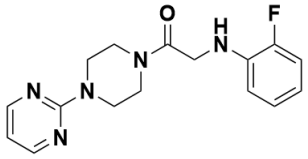
To a stirring solution of (2,4-difluorophenyl)glycine (23 mg, 0.11 mmol, 1 equiv) in DCM (5 mL) was added EDC (37 mg, 0.22 mmol, 2.0 equiv) and DMAP (0.14 mg, 0.01mmol, 0.01 equiv). This solution was stirred for 10 min before addition of 2-(piperazin-1-yl)pyrimidine (30 mg, 0.16 mmol, 1.5 equiv). The reaction slurry was brought to 37 ˚C for 12 h before quenching with 1 N HCl solution (3 mL). Organics were separated and the solvent was removed under reduced pressure. Column chromatography (0 to 100% (EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{16}H_{18}F_2N_5O^+$ [M+H$^+$], 334.35 found 335. 1H NMR (400 MHz, $CDCl_3$) δ 8.35 (d, *J* = 4.8 Hz, 2H), 6.85 - 6.71 (m, 2H), 6.61 - 6.52 (m, 2H), 3.97 - 3.86 (m, 6H), 3.81 - 3.74 (m, 2H), 3.58 - 3.50 (m, 2H).

2-((4-Chlorophenyl)amino)-1-(4-(pyrimidin-2-yl)piperazin-1-yl)ethan-1-one (3.164)



To a stirring solution of 4-chlorophenyl)glycine (22 mg, 0.11mmol, 1 equiv) in DCM (5 mL) was added EDC (37 mg, 0.22 mmol, 2.0 equiv) and DMAP (0.14 mg, 0.011 mmol, 0.01 equiv). This solution was stirred for 10 min before addition of 2-(piperazin-1-yl)pyrimidine (30 mg, 0.16 mmol, 1.5 equiv). The reaction slurry was brought to 37 ˚C for 12 h before quenching with 1 N HCl solution (3 mL). Organics were separated and the solvent was removed under reduced pressure. Column chromatography (0 to 100% (EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{16}H_{19}ClN_5O^+$ [M+H$^+$], 332.81 found 333. 1H NMR (400 MHz, $CDCl_3$) δ 8.47 (d, *J* = 5.0 Hz, 2H), 7.16 (d, *J* = 8.6 Hz, 2H), 6.74 (t, *J* = 5.0 Hz, 1H), 6.62 (d, *J* = 8.5 Hz, 2H), 4.16 - 4.08 (m, 2H), 4.04 - 3.98 (m, 2H), 3.39 (s, 2H), 3.85 - 3.78 (m, 2H), 3.67 - 3.60 (m, 2H).

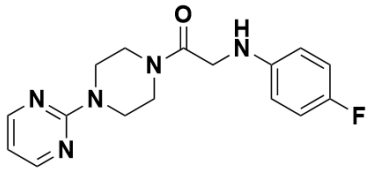
2-((2-Fluorophenyl)amino)-1-(4-(pyrimidin-2-yl)piperazin-1-yl)ethan-1-one (3.165)



To a stirring solution of (2-fluorophenyl)glycine (22 mg, 0.11 mmol, 1 equiv) in DCM (5 mL) was added EDC (37 mg, 0.22 mmol, 2.0 equiv) and DMAP (0.14 mg, 0.011 mmol, 0.01 equiv). This solution was stirred for 10 min before addition of 2-(piperazin-1-yl)pyrimidine (30 mg, 0.16 mmol, 1.5 equiv). The reaction slurry was brought to 37 ˚C for

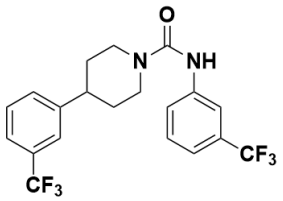
12 h before quenching with 1 N HCl solution (3 mL). Organics were separated and the solvent was removed under reduced pressure. Column chromatography (0 to 100% (EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{16}H_{19}FN_5O^+$ [M+H$^+$], 316.36 found 317. 1H NMR (400 MHz, $CDCl_3$) δ 8.36 (d, *J* = 4.8 Hz, 2H), 7.04 - 6.95 (m, 2H), 6.69 - 6.57 (m, 3H), 4.00 - 3.87 (m, 6H), 3.82 - 3.75 (m, 2H), 3.59 - 3.52 (m, 2H).

2-((4-Fluorophenyl)amino)-1-(4-(pyrimidin-2-yl)piperazin-1-yl)ethan-1-one (3.166)



To a stirring solution of (4-fluorophenyl)glycine (22 mg, 0.11 mmol, 1 equiv) in DCM (5 mL) was added EDC (37 mg, 0.22 mmol, 2.0 equiv) and DMAP (0.14 mg, 0.01 mmol, 0.01 equiv). This solution was stirred for 10 min before addition of 2-(piperazin-1-yl)pyrimidine (30 mg, 0. 16 mmol, 1.5 equiv). The reaction slurry was brought to 37 ˚C for 12 h before quenching with 1 N HCl solution (3 mL). Organics were separated and the solvent was removed under reduced pressure. Column chromatography (0 to 100% (EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{16}H_{19}FN_5O^+$ [M+H$^+$], 316.36 found 318. 1H NMR (400 MHz, $CDCl_3$) δ 8.36 (d, *J* = 4.8 Hz, 2H), 6.92 (t, *J* = 8.7 Hz, 2H), 6.65 - 6.56 (m, 3H), 3.98 - 3.86 (m, 6H), 3.80 - 3.73 (m, 2H), 3.61 - 3.50 (m, 2H).

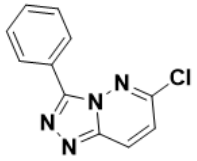
N,4-bis(3-(Trifluoromethyl)phenyl)piperidine-1-carboxamide (3.167)



To a stirring solution of 4-(3-(trifluoromethyl)phenyl)piperidine (30 mg, 0.13 mmol, 1.0 equiv), triethylamine (1.8 µL, 0.013 mmol, 0.1 equiv) in DCM (5 mL) was added 1-isocyanato-3-(trifluoromethyl)benzene (29 mg, 0.16 mmol, 1.2 equiv) and the solution was stirred for 30 min at RT. After confirming consumption of limiting reagent by TLC, 3 mL of distilled water was added to the reaction vial. The organic layer was separated and removed under reduced atmosphere. Column chromatography (0 to 50% EtOAc/Hex) on silica gel was used to purify sufficient amounts of the target molecule for biological testing and characterization. LRMS (APCI - quad) m/z cal'd for $C_{20}H_{19}F_6N_2O^+$ [M+H$^+$], 417.37 found 419. 1H NMR (400 MHz, $CDCl_3$) δ 7.67 (s, 1H), 7.58 (d, J = 8.2 Hz, 1H), 7.52 - 7.37 (m, 5H), 7.29 (d, J = 7.8 Hz, 1H), 6.59 (s, 1H), 4.25 (d, J = 13.4 Hz, 2H), 3.04 (t, J = 12.9 Hz, 2H), 2.82 (tt, J_1 = 3.3, J_2 = 6.9 Hz, 1H), 1.96 (d, J = 15.0 Hz, 2H), 1.75 (dq, J_1 = 4.1, J_2 = 12.7 Hz, 2H), 13C NMR (101 MHz, CDCl3) δ 154.4, 145.9, 139.7, 131.1 (quint, J = 32 Hz), 130.1, 129.4, 129.1, 124.1 (dq, J_1 = 16, J_2 = 272), 123.5 (sext, J = 4 Hz), 122.9, 119.6 (q, J = 4 Hz), 116.4 (q, J = 4 Hz), 44.9, 42.5, 32.9.

GPHR-00213869 Analogs

6-Chloro-3-phenyl-[1,2,4]triazolo[4,3-b]pyridazine (3.168)

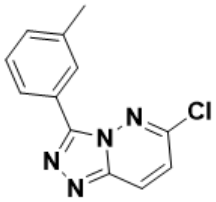


To a stirring suspension of 3-chloro-6-hydrazinylpyridazine (100 mg, 0.69 mmol, 1 equiv) in toluene (20 mL) was added DIPEA (0.2 mL, 0.069 mmol, 0.01 equiv) and benzoic anhydride (313 mg, 1.38 mmol, 2 equiv). The resulting suspension was heated to reflux and stirred for 12 h. After confirmation of consumption of starting material by TLC, the reaction was cooled to rt and the toluene was removed under reduced pressure. DCM (50 mL) was added and reaction was washed with a saturated $NaHCO_3$ solution (3 x 25 mL). The organic layer was then dried over Na_2SO_4, filtered and solvent removed under reduced pressure. Column chromatography (0 to 10% MeOH/DCM) on silica gel was used to purify sufficient amounts of target molecule for biological testing and characterization.

LRMS (CI-quad) m/z cal'd for $C_{11}H_8N_4Cl^+$ [M+H$^+$] 231.65, found 232.

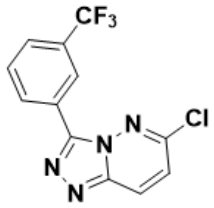
1H NMR (400 MHz, CDCl3) δ 10.29 (s, 1H), 8.19 (d, J = 9.2 Hz, 1H), 7.65 (d, J = 7.1 Hz, 2H), 7.56 (d, J = 7.7 Hz, 2H), 7.48 (d, J = 9.2 Hz, 1H), 7.42 (dd, J_1 = 7.6, J_2 = 15.0 Hz, 1H), 7.38 - 7.30 (m, 3H), 7.20 (t, J_1 = 7.7 Hz, 2H).

6-Chloro-3-(m-tolyl)-[1,2,4]triazolo[4,3-b]pyridazine (3.169)



To a stirring suspension of 3-chloro-6-hydrazinylpyridazine (100 mg, 0.69 mmol, 1 equiv) in toluene (20 mL) was added DIPEA (0.2 mL, 0.069 mmol, 0.01 equiv) and 3-methylbenzoic anhydride (261 mg, 1.38 mmol, 2 equiv). The resulting suspension was heated to reflux and stirred for 12 h. After confirmation of consumption of starting material by TLC, the reaction was cooled to rt and the toluene was removed under reduced pressure. DCM (50 mL) was added and reaction was washed with a saturated $NaHCO_3$ solution (3 x 25 mL). The organic layer was then dried over Na_2SO_4, filtered and solvent removed under reduced pressure. Column chromatography (0 to 10% MeOH/DCM) on silica gel was used to purify sufficient amounts of target molecule for biological testing and characterization. LRMS (CI-quad) m/z cal'd for $C_{12}H_{10}N_4Cl^+$ [M+H$^+$] 246.69, found 247. 1H NMR (400 MHz, CDCl3) δ 9.05 (s, 1H), 8.21 (d, *J* = 9.3 Hz, 1H), 7.54 (d, *J* = 9.2 Hz, 1H), 7.50 (d, *J* = 11.0 Hz, 1H), 7.44 (d, *J* = 7.5 Hz, 1H), 7.33 - 7.27 (m, 1H), 2.35 (s, 3H).

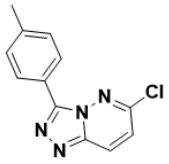
6-Chloro-3-(3-(trifluoromethyl)phenyl)-[1,2,4]triazolo[4,3-b]pyridazine (3.170)



To a stirring suspension of 3-chloro-6-hydrazinylpyridazine (100 mg, 0.69 mmol, 1 equiv) in toluene (20 mL) was added DIPEA (0.2 mL, 0.069 mmol, 0.01 equiv) and 3-(trifluoromethyl)benzoic anhydride (247 mg, 1.38 mmol, 2 equiv). The resulting suspension was heated to reflux and stirred for 12 h. After confirmation of consumption of starting material by TLC, the reaction was cooled to rt and the toluene was removed under reduced pressure. DCM (50 mL) was added and reaction was washed with a saturated

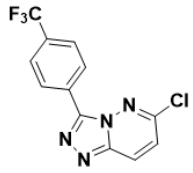
$NaHCO_3$ solution (3 x 25 mL). The organic layer was then dried over Na_2SO_4, filtered and solvent removed under reduced pressure. Column chromatography (0 to 10% MeOH/DCM) on silica gel was used to purify sufficient amounts of target molecule for biological testing and characterization. LRMS (CI-quad) m/z cal'd for $C_{12}H_7N_4F_3Cl^+$ [M+H$^+$] 300.66, found 300. 1H NMR (400 MHz, CDCl3) δ 8.79 (s, 1H) 8.70 (d, *J* = 7.9 Hz, 1H), 8.19 (d, *J* = 9.6 Hz, 1H), 7.79 (*J* = 7.8 Hz, 1H), 7.71 (t, *J* = 7.9 Hz, 1H), 7.21 (d, *J* = 9.6 Hz, 1H).

6-Chloro-3-(p-tolyl)-[1,2,4]triazolo[4,3-b]pyridazine (3.171)



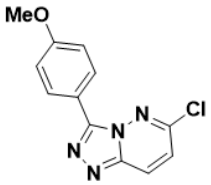
To a stirring suspension of 3-chloro-6-hydrazinylpyridazine (100 mg, 0.69 mmol, 1 equiv) in toluene (20 mL) was added DIPEA (0.2 mL, 0.069 mmol, 0.01 equiv) and 4-methylbenzoic anhydride (175 mg, 1.38 mmol, 2 equiv). The resulting suspension was heated to reflux and stirred for 12 h. After confirmation of consumption of starting material by TLC, the reaction was cooled to rt and the toluene was removed under reduced pressure. DCM (50 mL) was added and reaction was washed with a saturated $NaHCO_3$ solution (3 x 25 mL). The organic layer was then dried over Na_2SO_4, filtered and solvent removed under reduced pressure. Column chromatography (0 to 10% MeOH/DCM) on silica gel was used to purify sufficient amounts of target molecule for biological testing and characterization. LRMS (CI-quad) m/z cal'd for $C_{12}H_{10}N_4Cl^+$ [M+H$^+$] 246.69, found 247. 1H NMR (400 MHz, CDCl3) δ 8.35 (d, *J* = 8.3 Hz, 2H), 8.16 (d, *J* = 9.6 Hz, 1H), 7.38 (d, *J* = 8.0 Hz, 2H), 7.14 (d, *J* = 9.6, 2H), 2.45 (s, 3H).

6-Chloro-3-(4-(trifluoromethyl)phenyl)-[1,2,4]triazolo[4,3-b]pyridazine (3.172)



To a stirring suspension of 3-chloro-6-hydrazinylpyridazine (100 mg, 0.69 mmol, 1 equiv) in toluene (20 mL) was added DIPEA (0.2 mL, 0.069 mmol, 0.01 equiv) and 4-(trifluoromethyl)benzoic anhydride (249 mg, 1.38 mmol, 2 equiv). The resulting suspension was heated to reflux and stirred for 12 h. After confirmation of consumption of starting material by TLC, the reaction was cooled to rt and the toluene was removed under reduced pressure. DCM (50 mL) was added and reaction was washed with a saturated $NaHCO_3$ solution (3 x 25 mL). The organic layer was then dried over Na_2SO_4, filtered and solvent removed under reduced pressure. Column chromatography (0 to 10% MeOH/DCM) on silica gel was used to purify sufficient amounts of target molecule for biological testing and characterization. LRMS (CI-quad) m/z cal'd for $C_{12}H_7N_4F_3Cl^+$ [M+H$^+$] 300.66, found 300. 1H NMR (400 MHz, CDCl3) δ 8.62 (d, *J* = 7.8 Hz, 2H), 8.19 (d, *J* = 9.6 Hz, 1H), 7.83 (d, *J* = 8.0 Hz, 2H), 7.21 (d, *J* = 9.6 Hz, 1H).

6-Chloro-3-(4-methoxyphenyl)-[1,2,4]triazolo[4,3-b]pyridazine (3.173)



To a stirring suspension of 3-chloro-6-hydrazinylpyridazine (100 mg, 0.69 mmol, 1 equiv) in toluene (20 mL) was added DIPEA (0.2 mL, 0.069 mmol, 0.01 equiv) and 4-

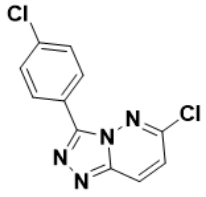
methoxybenzoic anhydride (294 mg, 1.38 mmol, 2 equiv). The resulting suspension was heated to reflux and stirred for 12 h. After confirmation of consumption of starting material by TLC, the reaction was cooled to rt and the toluene was removed under reduced pressure. DCM (50 mL) was added and reaction was washed with a saturated $NaHCO_3$ solution (3 x 25 mL). The organic layer was then dried over Na_2SO_4, filtered and solvent removed under reduced pressure. Column chromatography (0 to 10% MeOH/DCM) on silica gel was used to purify sufficient amounts of target molecule for biological testing and characterization. LRMS (CI-quad) m/z cal'd for $C_{12}H_{10}N_4OCl^+$ [M+H$^+$] 262.69, found 263. 1H NMR (400 MHz, CDCl3) δ 9.06 (s, 1H), 7.95 (d, *J* = 8.8 Hz, 1H), 7.83 (d, *J* = 8.9 Hz, 2H), 6.97 (d, *J* = 8.9 Hz, 2H), 3.87 (s, 3H).

6-Chloro-3-(4-fluorophenyl)-[1,2,4]triazolo[4,3-b]pyridazine (3.174)

To a stirring suspension of 3-chloro-6-hydrazinylpyridazine (100 mg, 0.69 mmol, 1 equiv) in toluene (20 mL) was added DIPEA (0.2 mL, 0.069 mmol, 0.01 equiv) and 4-fluorobenzoic anhydride (180 mg, 1.38 mmol, 2 equiv). The resulting suspension was heated to reflux and stirred for 12 h. After confirmation of consumption of starting material by TLC, the reaction was cooled to rt and the toluene was removed under reduced pressure. DCM (50 mL) was added and reaction was washed with a saturated $NaHCO_3$ solution (3 x 25 mL). The organic layer was then dried over Na_2SO_4, filtered and solvent removed under reduced pressure. Column chromatography (0 to 10% MeOH/DCM) on silica gel was used

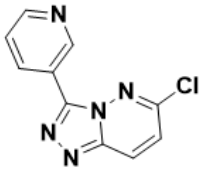
to purify sufficient amounts of target molecule for biological testing and characterization. LRMS (CI-quad) m/z cal'd for $C_{11}H_7N_4FCl^+$ [M+H$^+$] 250.65, found 251. 1H NMR (400 MHz, CDCl3) δ 8.49 (dddd, J_1 = 3.0, J_2 = 5.1, J_3 = 8.4, J_4 = 10.1 Hz, 2H), 8.16 (d, J = 9.6 Hz, 1H), 7.27 (dddd, J_1 = 3.0, J_2 = 5.1, J_3 = 10.1 Hz, 2H), 7.16 (d, J = 9.6 Hz, 1H).

6-Chloro-3-(4-chlorophenyl)-[1,2,4]triazolo[4,3-b]pyridazine (3.175)



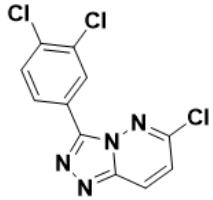
To a stirring suspension of 3-chloro-6-hydrazinylpyridazine (100 mg, 0.69 mmol, 1 equiv) in toluene (20 mL) was added DIPEA (0.2 mL, 0.069 mmol, 0.01 equiv) and 4-chlorobenzoic anhydride (203 mg, 1.38 mmol, 2 equiv). The resulting suspension was heated to reflux and stirred for 12 h. After confirmation of consumption of starting material by TLC, the reaction was cooled to rt and the toluene was removed under reduced pressure. DCM (50 mL) was added and reaction was washed with a saturated $NaHCO_3$ solution (3 x 25 mL). The organic layer was then dried over Na_2SO_4, filtered and solvent removed under reduced pressure. Column chromatography (0 to 10% MeOH/DCM) on silica gel was used to purify sufficient amounts of target molecule for biological testing and characterization. LRMS (CI-quad) m/z cal'd for $C_{11}H_7N_4Cl_2^+$ [M+H$^+$] 267.11, found 267. 1H NMR (400 MHz, CDCl3) δ 8.43 (d, J = 8.7 Hz, 2H), 8.16 (d, J = 9.6 Hz, 1H) 7.54 (d, J = 8.6 Hz, 2H), 7.17 (d, J = 9.6 Hz, 1H).

6-Chloro-3-(pyridin-3-yl)-[1,2,4]triazolo[4,3-b]pyridazine (3.176)



To a stirring suspension of 3-chloro-6-hydrazinylpyridazine (100 mg, 0.69 mmol, 1 equiv) in toluene (20 mL) was added DIPEA (0.2 mL, 0.069 mmol, 0.01 equiv) and nicotinic anhydride (157 mg, 1.38 mmol, 2 equiv). The resulting suspension was heated to reflux and stirred for 12 h. After confirmation of consumption of starting material by TLC, the reaction was cooled to rt and the toluene was removed under reduced pressure. DCM (50 mL) was added and reaction was washed with a saturated $NaHCO_3$ solution (3 x 25 mL). The organic layer was then dried over Na_2SO_4, filtered and solvent removed under reduced pressure. Column chromatography (0 to 10% MeOH/DCM) on silica gel was used to purify sufficient amounts of target molecule for biological testing and characterization. LRMS (CI-quad) m/z cal'd for $C_{10}H_7N_5Cl^+$ [M+H$^+$] 233.65, found 234. 1H NMR (400 MHz, CDCl3) δ 9.72 (d, J_1 = 2.0 Hz, 1H), 8.80 - 8.72 (m, 2H), 8.19 (d, J_1 = 9.6 Hz, 1H), 7.52 (dd, J_1 = 4.9, J_2 = 8.0 Hz, 1H), 7.21 (d, J_1 = 9.6 Hz, 1H).

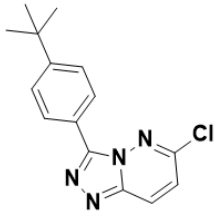
6-Chloro-3-(3,4-dichlorophenyl)-[1,2,4]triazolo[4,3-b]pyridazine (3.177)



To a stirring suspension of 3-chloro-6-hydrazinylpyridazine (100 mg, 0.69 mmol, 1 equiv) in toluene (20 mL) was added DIPEA (0.2 mL, 0.069 mmol, 0.01 equiv) and 3,4-dichlorobenzoic anhydride (251 mg, 1.38 mmol, 2 equiv). The resulting suspension was

heated to reflux and stirred for 12 h. After confirmation of consumption of starting material by TLC, the reaction was cooled to rt and the toluene was removed under reduced pressure. DCM (50 mL) was added and reaction was washed with a saturated $NaHCO_3$ solution (3 x 25 mL). The organic layer was then dried over Na_2SO_4, filtered and solvent removed under reduced pressure. Column chromatography (0 to 10% MeOH/DCM) on silica gel was used to purify sufficient amounts of target molecule for biological testing and characterization. LRMS (CI-quad) m/z cal'd for $C_{11}H_6N_4Cl_3^+$ [M+H$^+$] 301.55, found 302. 1H NMR (400 MHz, CDCl3) δ 8.64 (d, J_1 = 2.0 Hz, 1H), 8.38 (dd, J_1 = 2.0, J_2 = 8.5 Hz, 1H), 8.18 (d, J = 9.6 Hz, 1H), 7.65 (d, J = 8.5 Hz, 1H), 7.20 (d, J = 9.6 Hz, 1H).

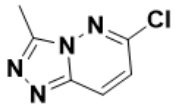
3-(4-(tert-Butyl)phenyl)-6-chloro-[1,2,4]triazolo[4,3-b]pyridazine (3.178)



To a stirring suspension of 3-chloro-6-hydrazinylpyridazine (100 mg, 0.69 mmol, 1 equiv) in toluene (20 mL) was added DIPEA (0.2 mL, 0.069 mmol, 0.01 equiv) and 4-(tert-butyl)benzoic anhydride (233 mg, 1.38 mmol, 2 equiv). The resulting suspension was heated to reflux and stirred for 12 h. After confirmation of consumption of starting material by TLC, the reaction was cooled to rt and the toluene was removed under reduced pressure. DCM (50 mL) was added and reaction was washed with a saturated $NaHCO_3$ solution (3 x 25 mL). The organic layer was then dried over Na_2SO_4, filtered and solvent removed under reduced pressure. Column chromatography (0 to 10% MeOH/DCM) on silica gel was used to purify sufficient amounts of target molecule for biological testing and characterization.

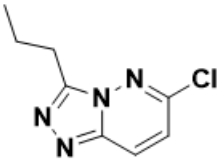
LRMS (CI-quad) m/z cal'd for $C_{15}H_{16}N_4Cl^+$ [M+H$^+$] 289.77, found 290. 1H NMR (400 MHz, CDCl3) δ 8.39 (dd, J_1 = 2.0, J_2 = 2.4 Hz, 1H), 8.37 (dd, J_1 = 1.5, J_2 = 1.9 Hz, 1H), 8.14 (d, J = 9.6 Hz, 1H), 7.61 (dd, J_1 = 2.1, J_2 = 2.6 Hz, 1H), 7.59 (dd, J_1 = 1.7, J_2 = 2.1 Hz, 1H), 7.13 (d, J = 9.6 Hz, 1H), 1.39 (s, 9H).

6-Chloro-3-methyl-[1,2,4]triazolo[4,3-b]pyridazine (3.179)



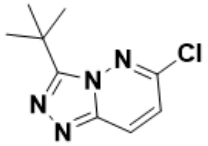
To a stirring suspension of 3-chloro-6-hydrazinylpyridazine (100 mg, 0.69 mmol, 1 equiv) in toluene (20 mL) was added DIPEA (0.2 mL, 0.069 mmol, 0.01 equiv) and acetic anhydride (70 mg, 1.38 mmol, 2 equiv). The resulting suspension was heated to reflux and stirred for 12 h. After confirmation of consumption of starting material by TLC, the reaction was cooled to rt and the toluene was removed under reduced pressure. DCM (50 mL) was added and reaction was washed with a saturated $NaHCO_3$ solution (3 x 25 mL). The organic layer was then dried over Na_2SO_4, filtered and solvent removed under reduced pressure. Column chromatography (0 to 10% MeOH/DCM) on silica gel was used to purify sufficient amounts of target molecule for biological testing and characterization. LRMS (CI-quad) m/z cal'd for $C_6H_6N_4Cl^+$ [M+H$^+$] 170.59, found 170. 1H NMR (400 MHz, CDCl3) δ 8.03 (d, J = 9.6 Hz, 1H), 7.08 (d, J = 9.6 Hz, 1H), 2.80 (s, 3H).

6-Chloro-3-propyl-[1,2,4]triazolo[4,3-b]pyridazine (3.180)



To a stirring suspension of 3-chloro-6-hydrazinylpyridazine (100 mg, 0.69 mmol, 1 equiv) in toluene (20 mL) was added DIPEA (0.2 mL, 0.069 mmol, 0.01 equiv) and butyric anhydride (107 mg, 1.38 mmol, 2 equiv). The resulting suspension was heated to reflux and stirred for 12 h. After confirmation of consumption of starting material by TLC, the reaction was cooled to rt and the toluene was removed under reduced pressure. DCM (50 mL) was added and reaction was washed with a saturated $NaHCO_3$ solution (3 x 25 mL). The organic layer was then dried over Na_2SO_4, filtered and solvent removed under reduced pressure. Column chromatography (0 to 10% MeOH/DCM) on silica gel was used to purify sufficient amounts of target molecule for biological testing and characterization. LRMS (CI-quad) m/z cal'd for $C_8H_{10}N_4Cl^+$ [M+H$^+$] 198.64, found 198. 1H NMR (400 MHz, CDCl3) δ 8.04 (d, *J* = 9.6 Hz, 1H), 7.07 (d, *J* = 9.6 Hz, 1H), 3.15 (t, *J* = 7.4 Hz, 2H), 1.95 (sext, *J* = 7.5 Hz, 2H), 1.05 (t, *J* = 7.4 Hz, 3H).

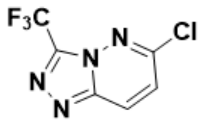
3-(tert-Butyl)-6-chloro-[1,2,4]triazolo[4,3-b]pyridazine (3.181)



To a stirring suspension of 3-chloro-6-hydrazinylpyridazine (100 mg, 0.69 mmol, 1 equiv) in toluene (20 mL) was added DIPEA (0.2 mL, 0.069 mmol, 0.01 equiv) and pivalic anhydride (128 mg, 1.38 mmol, 2 equiv). The resulting suspension was heated to reflux and stirred for 12 h. After confirmation of consumption of starting material by TLC, the reaction was cooled to rt and the toluene was removed under reduced pressure. DCM (50 mL) was added and reaction was washed with a saturated $NaHCO_3$ solution (3 x 25 mL). The organic layer was then dried over Na_2SO_4, filtered and solvent removed under reduced

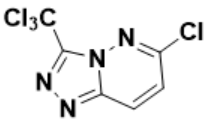
pressure. Column chromatography (0 to 10% MeOH/DCM) on silica gel was used to purify sufficient amounts of target molecule for biological testing and characterization. LRMS (CI-quad) m/z cal'd for $C_9H_{13}N_4Cl^+$ [M+H$^+$] 213.68, found 214. 1H NMR (400 MHz, CDCl3) δ 8.05 (d, *J* = 9.6 Hz, 1H), 7.06 (d, *J* = 9.7, 1H), 1.61 (s, 9H).

6-Chloro-3-(trifluoromethyl)-[1,2,4]triazolo[4,3-b]pyridazine (3.182)



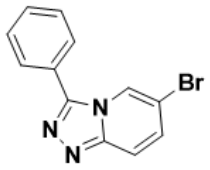
To a stirring suspension of 3-chloro-6-hydrazinylpyridazine (100 mg, 0.69 mmol, 1 equiv) in toluene (20 mL) was added DIPEA (0.2 mL, 0.069 mmol, 0.01 equiv) and 2,2,2-trifluoroacetic anhydride (144 mg, 1.38 mmol, 2 equiv). The resulting suspension was heated to reflux and stirred for 12 h. After confirmation of consumption of starting material by TLC, the reaction was cooled to rt and the toluene was removed under reduced pressure. DCM (50 mL) was added and reaction was washed with a saturated $NaHCO_3$ solution (3 x 25 mL). The organic layer was then dried over Na_2SO_4, filtered and solvent removed under reduced pressure. Column chromatography (0 to 10% MeOH/DCM) on silica gel was used to purify sufficient amounts of target molecule for biological testing and characterization. LRMS (CI-quad) m/z cal'd for $C_6H_3N_4F_3Cl^+$ [M+H$^+$], 224.56, found 224. 1H NMR (400 MHz, CDCl3) δ 8.22 (d, *J* = 10.0 Hz, 1H), 7.34 (d, *J* = 9.7, 1H).

6-Chloro-3-(trichloromethyl)-[1,2,4]triazolo[4,3-b]pyridazine (3.183)



To a stirring suspension of 3-chloro-6-hydrazinylpyridazine (100 mg, 0.69 mmol, 1 equiv) in toluene (20 mL) was added DIPEA (0.2 mL, 0.069 mmol, 0.01 equiv) and 2,2,2-trichloroacetic anhydride (213 mg, 1.38 mmol, 2 equiv). The resulting suspension was heated to reflux and stirred for 12 h. After confirmation of consumption of starting material by TLC, the reaction was cooled to rt and the toluene was removed under reduced pressure. DCM (50 mL) was added and reaction was washed with a saturated $NaHCO_3$ solution (3 x 25 mL). The organic layer was then dried over Na_2SO_4, filtered and solvent removed under reduced pressure. Column chromatography (0 to 10% MeOH/DCM) on silica gel was used to purify sufficient amounts of target molecule for biological testing and characterization. LRMS (CI-quad) m/z cal'd for $C_6H_3N_4Cl_4^+$ [M+H$^+$] 273.93, found 274. 1H NMR (400 MHz, CDCl3) δ 8.21 (d, *J* = 9.7 Hz, 1H), 7.33 (d, *J* = 9.5 Hz, 1H).

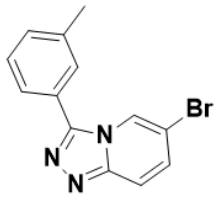
6-Bromo-3-phenyl-[1,2,4]triazolo[4,3-a]pyridine (3.184)



To a stirring suspension of 5-bromo-2-hydrazinylpyridine (100 mg, 0.69 mmol, 1 equiv) in toluene (20 mL) was added benzoic anhydride (54 mg, 0.69 mmol, 1 equiv). The resulting suspension was heated to reflux and stirred for 24 h. After confirmation of consumption of starting material by TLC, the reaction was cooled to rt and the toluene was removed under reduced pressure. DCM (50 mL) was added and reaction was washed with a saturated $NaHCO_3$ solution (3 x 25 mL). The organic layer was then dried over Na_2SO_4, filtered and solvent removed under reduced pressure. Column chromatography (0 to 10%

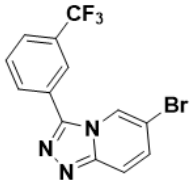
MeOH/DCM) on silica gel was used to purify sufficient amounts of target molecule for biological testing and characterization. LRMS (CI-quad) m/z cal'd for $C_{12}H_9N_3Br^+$ [M+H$^+$] 276.12, found 276. 1H NMR (400 MHz, CDCl3) δ 8.15 (d, *J* = 2.2 Hz, 1H), 7.89 (d, *J* = 7.5 Hz, 2H), 7.62 (dd, J_1 = 2.3, J_2 = 8.9 Hz, 1H), 7.56 (t, *J* = 7.2 Hz, 1H), 7.47 (d, *J* = 7.8 Hz, 2H), 6.74 (d, *J* = 8.9 Hz, 1H).

6-Bromo-3-(m-tolyl)-[1,2,4]triazolo[4,3-a]pyridine (3.185)



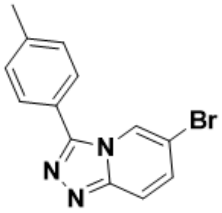
To a stirring suspension of 5-bromo-2-hydrazinylpyridine (100 mg, 0.69 mmol, 1 equiv) in toluene (20 mL) was added 3-methylbenzoic anhydride (134 mg, 0.69 mmol, 1 equiv). The resulting suspension was heated to reflux and stirred for 24 h. After confirmation of consumption of starting material by TLC, the reaction was cooled to rt and the toluene was removed under reduced pressure. DCM (50 mL) was added and reaction was washed with a saturated $NaHCO_3$ solution (3 x 25 mL). The organic layer was then dried over Na_2SO_4, filtered and solvent removed under reduced pressure. Column chromatography (0 to 10% MeOH/DCM) on silica gel was used to purify sufficient amounts of target molecule for biological testing and characterization. LRMS (CI-quad) m/z cal'd for $C_{13}H_{11}N_3Br^+$ [M+H$^+$] 290.15, found 290. 1H NMR (400 MHz, CDCl3) δ 8.18 (d, *J* = 2.1 Hz, 1H), 7.68 (s, 1H), 7.65 (d, *J* = 6.7 Hz, 1H), 7.59 (dd, J_1 = 2.3, J_2 = 8.8 Hz, 1H), 7.38 - 7.33 (m, 2H), 6.70 (d, *J* = 8.8 Hz, 1H), 2.40 (s, 3H).

6-Bromo-3-(3-(trifluoromethyl)phenyl)-[1,2,4]triazolo[4,3-a]pyridine (3.186)



To a stirring suspension of 5-bromo-2-hydrazinylpyridine (100 mg, 0.53 mmol, 1 equiv) in toluene (20 mL) was added 3-(trifluoromethyl)benzoic anhydride (191 mg, 0.53 mmol, 1 equiv). The resulting suspension was heated to reflux and stirred for 24 h. After confirmation of consumption of starting material by TLC, the reaction was cooled to rt and the toluene was removed under reduced pressure. DCM (50 mL) was added and reaction was washed with a saturated $NaHCO_3$ solution (3 x 25 mL). The organic layer was then dried over Na_2SO_4, filtered and solvent removed under reduced pressure. Column chromatography (0 to 10% MeOH/DCM) on silica gel was used to purify sufficient amounts of target molecule for biological testing and characterization. LRMS (CI-quad) m/z cal'd for $C_{13}H_8N_3F_3Br^+$ [M+H$^+$] 344.12, found 344. 1H NMR (400 MHz, CDCl3) δ 8.21 (d, *J* = 2.1 Hz, 1H), 8.14 (s, 1H), 8.05 (d, *J* = 7.8 Hz, 1H), 7.81 (d, *J* = 7.8 Hz, 1H), 7.60 (t, *J* = 6.7 Hz, 1H), 6.69 (d, *J* = 8.8 Hz, 1H).

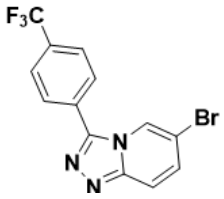
6-Bromo-3-(p-tolyl)-[1,2,4]triazolo[4,3-a]pyridine (3.187)



To a stirring suspension of 5-bromo-2-hydrazinylpyridine (100 mg, 0.53 mmol, 1 equiv) in toluene (20 mL) was added 4-methylbenzoic anhydride (134 mg, 0.53 mmol, equiv).

The resulting suspension was heated to reflux and stirred for 24 h. After confirmation of consumption of starting material by TLC, the reaction was cooled to rt and the toluene was removed under reduced pressure. DCM (50 mL) was added and reaction was washed with a saturated $NaHCO_3$ solution (3 x 25 mL). The organic layer was then dried over Na_2SO_4, filtered and solvent removed under reduced pressure. Column chromatography (0 to 10% MeOH/DCM) on silica gel was used to purify sufficient amounts of target molecule for biological testing and characterization. LRMS (CI-quad) m/z cal'd for $C_{13}H_{11}N_3Br^+$ [M+H$^+$] 290.15, found 290. 1H NMR (400 MHz, CDCl3) δ 8.16 (d, J = 2.3 Hz, 1H), 7.76 (d, J = 8.1 Hz, 2H), 7.58 (dd, J_1 = 2.4, J_2 = 8.8 Hz, 1H), 7.25 (d, J = 7.9 Hz, 2H), 6.70 (d, J = 9.6 Hz, 1H), 2.41 (s, 3H).

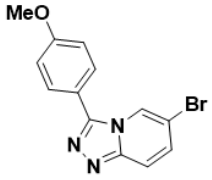
6-Bromo-3-(4-(trifluoromethyl)phenyl)-[1,2,4]triazolo[4,3-a]pyridine (3.188)



To a stirring suspension of 5-bromo-2-hydrazinylpyridine (100 mg, 0.53 mmol, 1 equiv) in toluene (20 mL) was added 4-(trifluoromethyl)benzoic anhydride (191 mg, 0.53 mmol, equiv). The resulting suspension was heated to reflux and stirred for 24 h. After confirmation of consumption of starting material by TLC, the reaction was cooled to rt and the toluene was removed under reduced pressure. DCM (50 mL) was added and reaction was washed with a saturated $NaHCO_3$ solution (3 x 25 mL). The organic layer was then dried over Na_2SO_4, filtered and solvent removed under reduced pressure. Column chromatography (0 to 10% MeOH/DCM) on silica gel was used to purify sufficient

amounts of target molecule for biological testing and characterization. LRMS (CI-quad) m/z cal'd for $C_{13}H_8N_3F_3Br^+$ [M+H$^+$] 344.12, found 344. 1H NMR (400 MHz, CDCl3) δ 8.23 (d, J = 2.0 Hz, 1H), 7.99 (d, J = 8.0 Hz, 2H), 7.75 (d, J = 8.0 Hz, 2H), 7.65 (dd, J_1 = 2.3, J_2 = 8.8 Hz, 1H), 6.71 (d, J = 8.8 Hz, 1H).

6-Bromo-3-(4-methoxyphenyl)-[1,2,4]triazolo[4,3-a]pyridine (3.189)

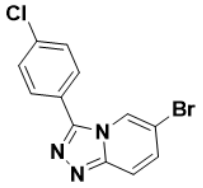


To a stirring suspension of 5-bromo-2-hydrazinylpyridine (100 mg, 0.53 mmol, 1 equiv) in toluene (20 mL) was added 4-methoxybenzoic anhydride (151 mg, 0.53 mmol, equiv). The resulting suspension was heated to reflux and stirred for 24 h. After confirmation of consumption of starting material by TLC, the reaction was cooled to rt and the toluene was removed under reduced pressure. DCM (50 mL) was added and reaction was washed with a saturated $NaHCO_3$ solution (3 x 25 mL). The organic layer was then dried over Na_2SO_4, filtered and solvent removed under reduced pressure. Column chromatography (0 to 10% MeOH/DCM) on silica gel was used to purify sufficient amounts of target molecule for biological testing and characterization. LRMS (CI-quad) m/z cal'd for $C_{13}H_{11}ON_3Br^+$ [M+H$^+$] 306.15, found 306. 1H NMR (400 MHz, CDCl3) δ 8.15 (d, J = 2.2 Hz, 1H), 7.83 (dt, J_1 = 2.8, J_2 = 4.8 Hz, 2H), 7.55 (dd, J_1 = 2.4, J_2, = 8.8 Hz, 1H), 6.92 (dt, J_1 = 2.8, J_2 = 4.7 Hz, 2H), 6.67 (d, J = 8.8, 1H), 3.85 (s, 3H).

6-Bromo-3-(4-fluorophenyl)-[1,2,4]triazolo[4,3-a]pyridine (3.190)

To a stirring suspension of 5-bromo-2-hydrazinylpyridine (100 mg, 0.53 mmol, 1 equiv) in toluene (20 mL) was added 4-fluorobenzoic anhydride (138 mg, 0.53 mmol, equiv). The resulting suspension was heated to reflux and stirred for 24 h. After confirmation of consumption of starting material by TLC, the reaction was cooled to rt and the toluene was removed under reduced pressure. DCM (50 mL) was added and reaction was washed with a saturated $NaHCO_3$ solution (3 x 25 mL). The organic layer was then dried over Na_2SO_4, filtered and solvent removed under reduced pressure. Column chromatography (0 to 10% MeOH/DCM) on silica gel was used to purify sufficient amounts of target molecule for biological testing and characterization. LRMS (CI-quad) m/z cal'd for $C_{12}H_8N_3FBr^+$ [M+H$^+$] 294.11, found 294. 1H NMR (400 MHz, CDCl3) δ 8.24 (d, J = 2.3 Hz, 1H), 7.89 (dd, J_1 = 5.3, J_2 = 8.4 Hz, 2H), 7.64 (dd, J_1 = 2.3, J_2 = 8.8 Hz, 1H), 7.17 (t, J = 8.5 Hz, 2H), 6.69 (d, J = 8.7 Hz, 1H).

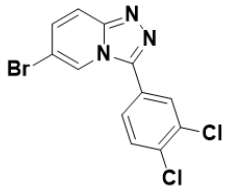
6-Bromo-3-(4-chlorophenyl)-[1,2,4]triazolo[4,3-a]pyridine (3.191)



To a stirring suspension of 5-bromo-2-hydrazinylpyridine (100 mg, 0.53 mmol, 1 equiv) in toluene (20 mL) was added 4-chlorobenzoic anhydride (156 mg, 0.53 mmol, equiv). The

resulting suspension was heated to reflux and stirred for 24 h. After confirmation of consumption of starting material by TLC, the reaction was cooled to rt and the toluene was removed under reduced pressure. DCM (50 mL) was added and reaction was washed with a saturated $NaHCO_3$ solution (3 x 25 mL). The organic layer was then dried over Na_2SO_4, filtered and solvent removed under reduced pressure. Column chromatography (0 to 10% MeOH/DCM) on silica gel was used to purify sufficient amounts of target molecule for biological testing and characterization. LRMS (CI-quad) m/z cal'd for $C_{12}H_8N_3ClBr^+$ [M+H$^+$] 310.57, found 310. 1H NMR (400 MHz, CDCl3) δ 8.08 (d, J = 2.3 Hz, 1H), 7.75 (d, J = 8.4 Hz, 2H), 7.54, (dd, J_1 = 2.4, J_2 = 8.8 Hz, 1H), 7.36 (d, J = 8.5, 2H), 6.63 (d, J = 8.8 Hz, 1H).

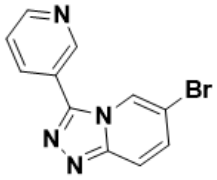
6-Bromo-3-(3,4-dichlorophenyl)-[1,2,4]triazolo[4,3-a]pyridine (3.192)



To a stirring suspension of 5-bromo-2-hydrazinylpyridine (100 mg, 0.53 mmol, 1 equiv) in toluene (20 mL) was added 3,4-dichlorobenzoic anhydride (192 mg, 0.53 mmol, equiv). The resulting suspension was heated to reflux and stirred for 24 h. After confirmation of consumption of starting material by TLC, the reaction was cooled to rt and the toluene was removed under reduced pressure. DCM (50 mL) was added and reaction was washed with a saturated $NaHCO_3$ solution (3 x 25 mL). The organic layer was then dried over Na_2SO_4, filtered and solvent removed under reduced pressure. Column chromatography (0 to 10% MeOH/DCM) on silica gel was used to purify sufficient amounts of target molecule for

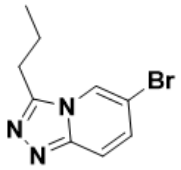
biological testing and characterization. LRMS (CI-quad) m/z cal'd for $C_{12}H_7N_3Cl_2Br^+$ [M+H$^+$] 345.01, found 345. 1H NMR (400 MHz, CDCl3) δ 8.23 (d, *J* = 2.3 Hz, 1H), 7.97 (d, 2.04 Hz, 1H), 7.69 (dd, J_1 = 1.8, J_2 = 8.3 Hz, 1H), 7.64 (dd, J_1 = 2.3, J_2 = 4.8 Hz, 1H), 7.57 (d, *J* = 8.3 Hz, 1H), 6.68 (d, *J* = 8.8 Hz, 1H).

6-Bromo-3-(pyridin-3-yl)-[1,2,4]triazolo[4,3-a]pyridine (3.193)



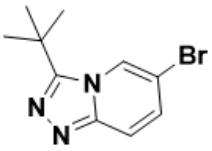
To a stirring suspension of 5-bromo-2-hydrazinylpyridine (100 mg, 0.53 mmol, 1 equiv) in toluene (20 mL) was added nicotinic anhydride (120 mg, 0.53 mmol, equiv). The resulting suspension was heated to reflux and stirred for 24 h. After confirmation of consumption of starting material by TLC, the reaction was cooled to rt and the toluene was removed under reduced pressure. DCM (50 mL) was added and reaction was washed with a saturated $NaHCO_3$ solution (3 x 25 mL). The organic layer was then dried over Na_2SO_4, filtered and solvent removed under reduced pressure. Column chromatography (0 to 10% MeOH/DCM) on silica gel was used to purify sufficient amounts of target molecule for biological testing and characterization. LRMS (CI-quad) m/z cal'd for $C_{11}H_8N_4Br^+$ [M+H$^+$] 277.11, found 277. 1H NMR (400 MHz, CDCl3) δ 9.08 (s, 1H), 8.70 (d, *J* = 4.7 Hz, 1H), 8.15 (d, *J* = 7.9 Hz, 1H), 8.11 (d, *J* = 2.1 Hz, 1H), 7.50 (dd, J_1 = 2.3, J_2 = 8.9 Hz, 1H), 7.33 (dd, J_1 = 4.9, J_2 = 8.1 Hz, 1H), 6.63 (d, *J* = 8.8 Hz, 1H).

6-Bromo-3-propyl-[1,2,4]triazolo[4,3-a]pyridine (3.194)



To a stirring suspension of 5-bromo-2-hydrazinylpyridine (100 mg, 0.53 mmol, 1 equiv) in toluene (20 mL) was added butyric anhydride (83 mg, 0.53 mmol, equiv). The resulting suspension was heated to reflux and stirred for 24 h. After confirmation of consumption of starting material by TLC, the reaction was cooled to rt and the toluene was removed under reduced pressure. DCM (50 mL) was added and reaction was washed with a saturated $NaHCO_3$ solution (3 x 25 mL). The organic layer was then dried over Na_2SO_4, filtered and solvent removed under reduced pressure. Column chromatography (0 to 10% MeOH/DCM) on silica gel was used to purify sufficient amounts of target molecule for biological testing and characterization. LRMS (CI-quad) m/z cal'd for $C_9H_{11}N_3Br^+$ [$M+H^+$] 242.11, found 242. 1H NMR (400 MHz, CDCl3) δ 8.17 (d, J = 2.3 Hz, 1H), 7.60 (dd, J_1 = 2.4, J_2 = 8.9 Hz, 1H), 6.61 (d, J = 8.8 Hz, 1H, 2.27 (t, J = 7.4 Hz, 2H), 1.73 (sext, J = 7.5 Hz, 2H), 0.99 (t, J = 7.4 Hz, 3H).

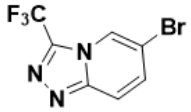
6-Bromo-3-(tert-butyl)-[1,2,4]triazolo[4,3-a]pyridine (3.195)



To a stirring suspension of 5-bromo-2-hydrazinylpyridine (100 mg, 0.53 mmol, 1 equiv) in toluene (20 mL) was added pivalic anhydride (98 mg, 0.53 mmol, equiv). The resulting suspension was heated to reflux and stirred for 24 h. After confirmation of consumption of

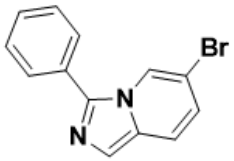
starting material by TLC, the reaction was cooled to rt and the toluene was removed under reduced pressure. DCM (50 mL) was added and reaction was washed with a saturated $NaHCO_3$ solution (3 x 25 mL). The organic layer was then dried over Na_2SO_4, filtered and solvent removed under reduced pressure. Column chromatography (0 to 10% MeOH/DCM) on silica gel was used to purify sufficient amounts of target molecule for biological testing and characterization. LRMS (CI-quad) m/z cal'd for $C_{10}H_{13}N_3Br^+$ [M+H$^+$] 256.13, found 257. 1H NMR (400 MHz, CDCl3) δ 8.17 (d, J = 2.1 Hz, 1H), 8.06 (s, 1H), 7.56 (dd, J_1 = 2.2 Hz, J_2 = 8.8, 1H), 6.54 (d, J = 8.8 Hz, 1H), 1.26 (s, 9H).

6-Bromo-3-(trifluoromethyl)-[1,2,4]triazolo[4,3-a]pyridine (3.196)



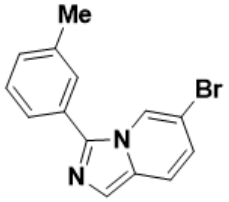
To a stirring suspension of 5-bromo-2-hydrazinylpyridine (100 mg, 0.53 mmol, 1 equiv) in toluene (20 mL) was added 2,2,2-trifluoroacetic anhydride (111 mg, 0.53 mmol, equiv). The resulting suspension was heated to reflux and stirred for 24 h. After confirmation of consumption of starting material by TLC, the reaction was cooled to rt and the toluene was removed under reduced pressure. DCM (50 mL) was added and reaction was washed with a saturated $NaHCO_3$ solution (3 x 25 mL). The organic layer was then dried over Na_2SO_4, filtered and solvent removed under reduced pressure. Column chromatography (0 to 10% MeOH/DCM) on silica gel was used to purify sufficient amounts of target molecule for biological testing and characterization. LRMS (CI-quad) m/z cal'd for $C_7H_4N_3F_3Br^+$ [M+H$^+$] 268.03, found 268. 1H NMR (400 MHz, CDCl3) δ 8.25 (d, J = 2.4 Hz, 1H), 7.69 (dd, J_1 = 2.4, J_2 = 8.8 Hz, 1H), 6.65 (d, J = 8.8 Hz, 1H).

6-Bromo-3-phenylimidazo[1,5-a]pyridine (3.197)



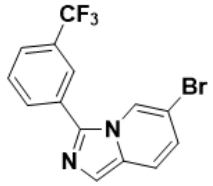
To a stirring suspension of (5-bromopyridin-2-yl)methanamine (100 mg, 0.69 mmol, 1 equiv) in dichloroethane (20 mL) was added benzoic anhydride (131 mg, 0.76 mmol, 1.1 equiv). The resulting suspension was stirred at rt for 30 min before dropwise addition of $POCl_3$ (88 mg, 0.76 mmol, 1.1 equiv) as a solution in *ca* 3 mL DCM. The reaction was then heated to reflux for 3 hr. After confirmation of consumption of starting material by TLC, the reaction was cooled to rt and the reaction was stirred with 5 mL of 0.1 N HCl (caution:exothermic reaction) for 15 mins to hydrolyze excess $POCl_3$. Post stirring, the reaction was washed with a saturated $NaHCO_3$ solution (3 x 25 mL, caution:gas evolution). The organic layer was then dried over Na_2SO_4, filtered and solvent removed under reduced pressure. Column chromatography (0 to 10% MeOH/DCM) on silica gel was used to purify sufficient amounts of target molecule for biological testing and characterization. LRMS (CI-quad) m/z cal'd for $C_{13}H_{10}N_2Br^+$ [M+H$^+$] 276.14, found 276. 1H NMR (400 MHz, CDCl3) δ 8.40 (s, 1H), 7.78 (d, *J* = 7.6 Hz, 2H), 7.60 (s, 1H), 7.56 (t, *J* = 7.5 Hz, 2H), 7.48 (t, *J* = 7.4 Hz, 1H), 7.41 (d, *J* = 9.6 Hz, 1H), 6.80 (d, *J* = 9.6 Hz, 1H).

6-Bromo-3-(m-tolyl)imidazo[1,5-a]pyridine (3.198)



To a stirring suspension of (5-bromopyridin-2-yl)methanamine (100 mg, 0.69 mmol, 1 equiv) in dichloroethane (20 mL) was added 3-methylbenzoic anhydride (141 mg, 1.58 mmol, 2.3 equiv). The resulting suspension was stirred at rt for 30 min before dropwise addition of $POCl_3$ (88 mg, 1.12 mmol, 1.7 equiv) as a solution in *ca* 3 mL DCM. The reaction was then heated to reflux for 3 hr. After confirmation of consumption of starting material by TLC, the reaction was cooled to rt and the reaction was stirred with 5 mL of 0.1 N HCl (caution:exothermic reaction) for 15 mins to hydrolyze excess $POCl_3$. Post stirring, the reaction was washed with a saturated $NaHCO_3$ solution (3 x 25 mL, caution:gas evolution). The organic layer was then dried over Na_2SO_4, filtered and solvent removed under reduced pressure. Column chromatography (0 to 10% MeOH/DCM) on silica gel was used to purify sufficient amounts of target molecule for biological testing and characterization. LRMS (CI-quad) m/z cal'd for $C_{11}H_8N_2Br^+$ [M+H$^+$] 249.10, found 249. 1H NMR (400 MHz, CDCl3) δ 8.38 (s, 1H), 7.60 (s, 1H), 7.57 (s, 1H), 7.55 (d, J = 7.7 Hz, 1H), 7.43 (t, J = 7.6 Hz, 1H), 7.38 (d, J = 9.5 Hz, 1H), 7.28 (d, J = 7.6, 1H), 6.77 (dd, J_1 = 1.5, J_2 = 10.9 Hz, 1H), 2.45 (s, 3H).

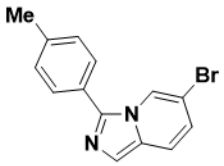
6-Bromo-3-(3-(trifluoromethyl)phenyl)imidazo[1,5-a]pyridine (3.199)



To a stirring suspension of (5-bromopyridin-2-yl)methanamine (100 mg, 0.69 mmol, 1 equiv) in dichloroethane (20 mL) was added 3-(trifluoromethyl)benzoic anhydride (210 mg, 0.76 mmol, 1.1 equiv). The resulting suspension was stirred at rt for 30 min before

dropwise addition of $POCl_3$ (105 mg, 0.89 mmol, 1.3 equiv) as a solution in *ca* 3 mL DCM. The reaction was then heated to reflux for 3 hr. After confirmation of consumption of starting material by TLC, the reaction was cooled to rt and the reaction was stirred with 5 mL of 0.1 N HCl (caution:exothermic reaction) for 15 mins to hydrolyze excess $POCl_3$. Post stirring, the reaction was washed with a saturated $NaHCO_3$ solution (3 x 25 mL, caution:gas evolution). The organic layer was then dried over Na_2SO_4, filtered and solvent removed under reduced pressure. Column chromatography (0 to 10% MeOH/DCM) on silica gel was used to purify sufficient amounts of target molecule for biological testing and characterization. LRMS (CI-quad) m/z cal'd for $C_{14}H_9N_2F_3Br^+$ [M+H$^+$] 343.13, found 343. 1H NMR (400 MHz, CDCl3) δ 8.35 (s, 1H), 8.08 (s, 1H), 7.96 (d, *J* = 7.5 Hz, 1H), 7.73 - 7.67 (m, 2H), 7.63 (s, 1H), 7.43 (d, *J* = 9.6 Hz, 1H), 6.85 (d, *J* = 9.6 Hz, 1H).

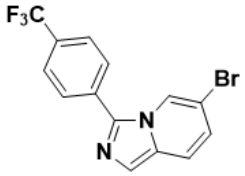
6-Bromo-3-(p-tolyl)imidazo[1,5-a]pyridine (3.200)



To a stirring suspension of (5-bromopyridin-2-yl)methanamine (100 mg, 0.69 mmol, 1 equiv) in dichloroethane (20 mL) was added nicotinic anhydride (147 mg, 0.76 mmol, 1.1 equiv). The resulting suspension was stirred at rt for 30 min before dropwise addition of $POCl_3$ (105 mg, 0.89 mmol, 1.3 equiv) as a solution in *ca* 3 mL DCM. The reaction was then heated to reflux for 3 hr. After confirmation of consumption of starting material by TLC, the reaction was cooled to rt and the reaction was stirred with 5 mL of 0.1 N HCl (caution:exothermic reaction) for 15 mins to hydrolyze excess $POCl_3$. Post stirring, the

reaction was washed with a saturated $NaHCO_3$ solution (3 x 25 mL, caution:gas evolution). The organic layer was then dried over Na_2SO_4, filtered and solvent removed under reduced pressure. Column chromatography (0 to 10% MeOH/DCM) on silica gel was used to purify sufficient amounts of target molecule for biological testing and characterization. LRMS (CI-quad) m/z cal'd for $C_{14}H_{12}N_2Br^+$ [M+H$^+$] 289.16, found 289. 1H NMR (400 MHz, CDCl3) δ 8.36 (s, 1H), 7.65 (d, *J* = 7.9 Hz, 2H), 7.56 (s, 1H), 7.39 - 7.32 (m, 3H), 6.76 (d, *J* = 9.5 Hz, 1H), 2.44 (s, 3H).

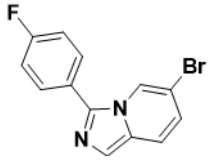
6-Bromo-3-(4-(trifluoromethyl)phenyl)imidazo[1,5-a]pyridine (3.201)



To a stirring suspension of (5-bromopyridin-2-yl)methanamine (100 mg, 0.69 mmol, 1 equiv) in dichloroethane (20 mL) was added 4-(trifluoromethyl)benzoic anhydride (210 mg, 0.76 mmol, 1.1 equiv). The resulting suspension was stirred at rt for 30 min before dropwise addition of $POCl_3$ (105 mg, 0.89 mmol, 1.3 equiv) as a solution in *ca* 3 mL DCM. The reaction was then heated to reflux for 3 hr. After confirmation of consumption of starting material by TLC, the reaction was cooled to rt and the reaction was stirred with 5 mL of 0.1 N HCl (caution:exothermic reaction) for 15 mins to hydrolyze excess $POCl_3$. Post stirring, the reaction was washed with a saturated $NaHCO_3$ solution (3 x 25 mL, caution:gas evolution). The organic layer was then dried over Na_2SO_4, filtered and solvent removed under reduced pressure. Column chromatography (0 to 10% MeOH/DCM) on silica gel was used to purify sufficient amounts of target molecule for biological testing

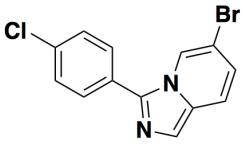
and characterization. LRMS (CI-quad) m/z cal'd for $C_{14}H_9N_2F_3Br^+$ [M+H$^+$] 343.13, found 343. 1H NMR (400 MHz, CDCl3) δ 8.40 (s, 1H), 7.93 (d, *J* = 8.1 Hz, 2H), 7.81 (d, *J* = 8.2 Hz, 2H), 7.64 (s, 1H), 7.44 (d, *J* = 9.5 Hz, 1H), 6.85 (d, *J* = 9.5 Hz, 1H).

6-Bromo-3-(4-fluorophenyl)imidazo[1,5-a]pyridine (3.202)



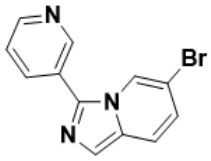
To a stirring suspension of (5-bromopyridin-2-yl)methanamine (100 mg, 0.69 mmol, 1 equiv) in dichloroethane (20 mL) was added 4-fluorobenzoic anhydride (322 mg, 1.58 mmol, 2.3 equiv). The resulting suspension was stirred at rt for 30 min before dropwise addition of $POCl_3$ (140 mg, 1.12 mmol, 1.7 equiv) as a solution in *ca* 3 mL DCM. The reaction was then heated to reflux for 3 hr. After confirmation of consumption of starting material by TLC, the reaction was cooled to rt and the reaction was stirred with 5 mL of 0.1 N HCl (caution:exothermic reaction) for 15 mins to hydrolyze excess $POCl_3$. Post stirring, the reaction was washed with a saturated $NaHCO_3$ solution (3 x 25 mL, caution:gas evolution). The organic layer was then dried over Na_2SO_4, filtered and solvent removed under reduced pressure. Column chromatography (0 to 10% MeOH/DCM) on silica gel was used to purify sufficient amounts of target molecule for biological testing and characterization. LRMS (CI-quad) m/z cal'd for $C_{13}H_9N_2FBr^+$ [M+H$^+$] 293.13, found 293. 1H NMR (400 MHz, CDCl3) δ 8.24 (s, 1H), 7.69 (dd, J_1 = 5.4, J_2 = 8.2 Hz, 2H), 7.51 (s, 1H), 7.33 (d, *J* = 9.5 Hz, 1H), 7.17 (d, *J* = 8.6 Hz, 1H), 6.73 (d, *J* = 9.6 Hz, 1H).

6-Bromo-3-(4-chlorophenyl)imidazo[1,5-a]pyridine (3.203)



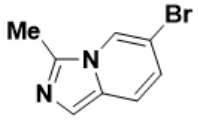
To a stirring suspension of (5-bromopyridin-2-yl)methanamine (100 mg, 0.69 mmol, 1 equiv) in dichloroethane (20 mL) was added 4-chlorobenzoic anhydride (361 mg, 1.58 mmol, 2.3 equiv). The resulting suspension was stirred at rt for 30 min before dropwise addition of $POCl_3$ (141 mg, 1.12 mmol, 1.7 equiv) as a solution in *ca* 3 mL DCM. The reaction was then heated to reflux for 3 hr. After confirmation of consumption of starting material by TLC, the reaction was cooled to rt and the reaction was stirred with 5 mL of 0.1 N HCl (caution:exothermic reaction) for 15 mins to hydrolyze excess $POCl_3$. Post stirring, the reaction was washed with a saturated $NaHCO_3$ solution (3 x 25 mL, caution:gas evolution). The organic layer was then dried over Na_2SO_4, filtered and solvent removed under reduced pressure. Column chromatography (0 to 10% MeOH/DCM) on silica gel was used to purify sufficient amounts of target molecule for biological testing and characterization. LRMS (CI-quad) m/z cal'd for $C_{13}H_8N_2ClBr^+$ [M+H$^+$] 293.13, found 293. 1H NMR (400 MHz, CDCl3) δ 8.27 (s, 1H), 7.66 (d, *J* = 8.3 Hz, 1H), 7.54 (s, 1H), 7.46 (d, *J* = 8.2 Hz, 1H), 7.35 (d, *J* = 9.7 Hz, 1H), 6.75 (d, *J* = 9.6 Hz, 1H).

6-Bromo-3-(pyridin-3-yl)imidazo[1,5-a]pyridine (3.204)



To a stirring suspension of (5-bromopyridin-2-yl)methanamine (100 mg, 0.69 mmol, 1 equiv) in dichloroethane (20 mL) was added nicotinic anhydride (132 mg, 0.76 mmol, 1.1 equiv). The resulting suspension was stirred at rt for 30 min before dropwise addition of $POCl_3$ (105 mg, 0.89 mmol, 1.3 equiv) as a solution in *ca* 3 mL DCM. The reaction was then heated to reflux for 3 hr. After confirmation of consumption of starting material by TLC, the reaction was cooled to rt and the reaction was stirred with 5 mL of 0.1 N HCl (caution:exothermic reaction) for 15 mins to hydrolyze excess $POCl_3$. Post stirring, the reaction was washed with a saturated $NaHCO_3$ solution (3 x 25 mL, caution:gas evolution). The organic layer was then dried over Na_2SO_4, filtered and solvent removed under reduced pressure. Column chromatography (0 to 10% MeOH/DCM) on silica gel was used to purify sufficient amounts of target molecule for biological testing and characterization. LRMS (CI-quad) m/z cal'd for $C_{12}H_9N_3Br^+$ [M+H$^+$] 276.12, found 276. 1H NMR (400 MHz, CDCl3) δ 9.07 (d, J = 2.3 Hz, 1H) 8.70 (d, J = 5.0 Hz, 1H), 8.38 (s, 1H), 8.13 (d, J = 8.1 Hz, 1H), 7.63 (s, 1H), 7.50 (dd, J_1 = 4.8, J_2 = 7.9 Hz,1H), 7.44 (d, J = 9.6 Hz, 1H), 6.85 (d, J = 9.5 Hz, 1H).

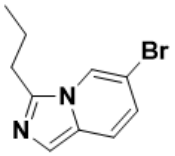
6-Bromo-3-methylimidazo[1,5-a]pyridine (3.205)



To a stirring suspension of (5-bromopyridin-2-yl)methanamine (100 mg, 0.69 mmol, 1 equiv) in dichloroethane (20 mL) was added acetic anhydride (59 mg, 0.76 mmol, 1.1 equiv). The resulting suspension was stirred at rt for 30 min before dropwise addition of $POCl_3$ (105 mg, 0.89 mmol, 1.3 equiv) as a solution in *ca* 3 mL DCM. The reaction was

then heated to reflux for 3 hr. After confirmation of consumption of starting material by TLC, the reaction was cooled to rt and the reaction was stirred with 5 mL of 0.1 N HCl (caution:exothermic reaction) for 15 mins to hydrolyze excess $POCl_3$. Post stirring, the reaction was washed with a saturated $NaHCO_3$ solution (3 x 25 mL, caution:gas evolution). The organic layer was then dried over Na_2SO_4, filtered and solvent removed under reduced pressure. Column chromatography (0 to 10% MeOH/DCM) on silica gel was used to purify sufficient amounts of target molecule for biological testing and characterization. LRMS (CI-quad) m/z cal'd for $C_8H_8N_2Br^+$ [M+H$^+$] 213.07, found 213. 1H NMR (400 MHz, CDCl3) δ 7.76 (s, 1H), 7.29 (s, 1H), 7.22 (t, *J* = 11.8 Hz, 1H), 6.63 (d, *J* = 10.1 Hz, 1H), 2.57 (s, 3H).

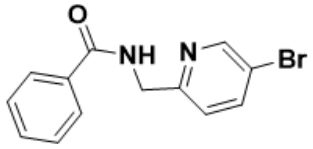
6-Bromo-3-propylimidazo[1,5-a]pyridine (3.206)



To a stirring suspension of (5-bromopyridin-2-yl)methanamine (100 mg, 0.69 mmol, 1 equiv) in dichloroethane (20 mL) was added butyric anhydride (91 mg, 0.76 mmol, 1.1 equiv). The resulting suspension was stirred at rt for 30 min before dropwise addition of $POCl_3$ (105 mg, 0.89 mmol, 1.3 equiv) as a solution in *ca* 3 mL DCM. The reaction was then heated to reflux for 3 hr. After confirmation of consumption of starting material by TLC, the reaction was cooled to rt and the reaction was stirred with 5 mL of 0.1 N HCl (caution:exothermic reaction) for 15 mins to hydrolyze excess $POCl_3$. Post stirring, the reaction was washed with a saturated $NaHCO_3$ solution (3 x 25 mL, caution:gas evolution). The organic layer was then dried over Na_2SO_4, filtered and solvent removed under reduced

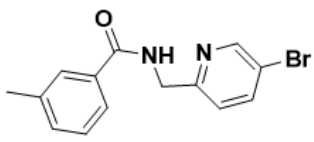
pressure. Column chromatography (0 to 10% MeOH/DCM) on silica gel was used to purify sufficient amounts of target molecule for biological testing and characterization. LRMS (CI-quad) m/z cal'd for $C_{10}H_{12}N_2Br^+$ [M+H$^+$] 242.12, found 242. 1H NMR (400 MHz, CDCl3) δ 7.88 (s, 1H), 7.39 (s, 1H), 7.30 (t, *J* = 9.6 Hz, 1H), 6.70 (d, *J* = 9.6 Hz, 1H), 2.93 (t, *J* = 7.6 Hz, 2H), 1.90 (sext, *J* = 7.4 Hz, 2H), 1.05 (t, *J* = 7.4 Hz, 3H).

N-((5-Bromopyridin-2-yl)methyl)benzamide (3.207)



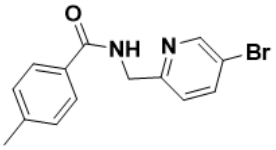
To a stirring suspension of (5-bromopyridin-2-yl)methanamine (20 mg, 0.106 mmol, 1 equiv) in dichloroethane (20 mL) was added benzoic anhydride (26 mg, 0.76 mmol, 1.1 equiv). The resulting suspension was stirred at rt for 30 min. After confirmation of consumption of starting material by TLC, reaction solvent was removed under reduced pressure and column chromatography (0 to 10% MeOH/DCM) on silica gel was used to purify sufficient amounts of target molecule for biological testing and characterization. LRMS (CI-quad) m/z cal'd for $C_{13}H_{12}ON_2Br^+$ [M+H$^+$] 294.15, found 294. 1H NMR (400 MHz, CDCl3) δ 8.62 (d, *J* = 1.9 Hz, 1H), 7.85 (d, *J* = 7.0 Hz, 2H), 7.81 (dd, J_1 = 2.3, J_2 = 8.3 Hz, 1H), 7.51 (t, *J* = 7.4 Hz, 1H), 7.45 (t, *J* = 7.6 Hz, 2H), 7.27 (t, *J* = 6.6 Hz, 1H), 4.72 (d, *J* = 5.0 Hz, 2H).

N-((5-Bromopyridin-2-yl)methyl)-3-methylbenzamide (3.208)



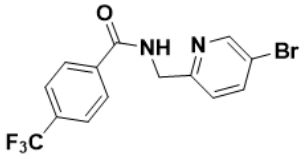
To a stirring suspension of (5-bromopyridin-2-yl)methanamine (20 mg, 0.106 mmol, 1 equiv) in dichloroethane (20 mL) was added 3-methylbenzoic anhydride (30 mg, 0.76 mmol, 1.1 equiv). The resulting suspension was stirred at rt for 30 min. After confirmation of consumption of starting material by TLC, reaction solvent was removed under reduced pressure and column chromatography (0 to 10% MeOH/DCM) on silica gel was used to purify sufficient amounts of target molecule for biological testing and characterization. LRMS (CI-quad) m/z cal'd for $C_{14}H_{14}ON_2Br^+$ [M+H$^+$] 307.18, found 307. 1H NMR (400 MHz, CDCl3) δ 8.63 (d, J = 2.0 Hz, 1H), 7.81 (dd, J_1 = 2.2, J_2 = 8.3 Hz, 1H), 7.67 (s, 1H), 7.66 - 7.59 (m, 1H), 7.37 - 7.29 (m, 2H), 7.26 (t, J = 4.8 Hz, 1H), 4.71 (d, J = 5.1 Hz, 2H), 2.40 (s, 3H).

N-((5-Bromopyridin-2-yl)methyl)-4-methylbenzamide (3.209)



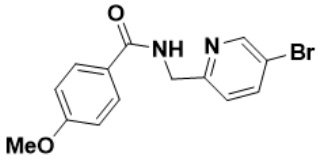
To a stirring suspension of (5-bromopyridin-2-yl)methanamine (20 mg, 0.106 mmol, 1 equiv) in dichloroethane (20 mL) was added 4-methylbenzoic anhydride (30 mg, 0.76 mmol, 1.1 equiv). The resulting suspension was stirred at rt for 30 min. After confirmation of consumption of starting material by TLC, reaction solvent was removed under reduced pressure and column chromatography (0 to 10% MeOH/DCM) on silica gel was used to purify sufficient amounts of target molecule for biological testing and characterization. LRMS (CI-quad) m/z cal'd for $C_{14}H_{14}ON_2Br^+$ [M+H$^+$] 307.18, found 308. 1H NMR (400 MHz, CDCl3) δ 8.62 (d, J = 2.1 Hz, 1H), 7.80 (dd, J_1 = 2.3, J_2 = 8.4 Hz, 1H), 7.75 (d, J = 8.2 Hz, 2H), 7.36 (br, 1H), 7.29 - 7.20 (m, 3H), 4.71 (d, J = 5.1 Hz, 2H), 2.40 (s, 3H).

N-((5-Bromopyridin-2-yl)methyl)-4-(trifluoromethyl)benzamide (3.210)



To a stirring suspension of (5-bromopyridin-2-yl)methanamine (20 mg, 0.106 mmol, 1 equiv) in dichloroethane (20 mL) was added 4-(trifluoromethyl)benzoic anhydride (42 mg, 0.76 mmol, 1.1 equiv). The resulting suspension was stirred at rt for 30 min. After confirmation of consumption of starting material by TLC, reaction solvent was removed under reduced pressure and column chromatography (0 to 10% MeOH/DCM) on silica gel was used to purify sufficient amounts of target molecule for biological testing and characterization. LRMS (CI-quad) m/z cal'd for $C_{14}H_{11}ON_3F_3Br^+$ [M+H$^+$] 375.16, found 375. 1H NMR (400 MHz, CDCl3) δ 8.65 (d, J = 2.2 Hz, 1H), 7.99 (d, J = 8.1 Hz, 2H), 7.87 (dd, J_1 = 2.3, J_2 = 8.4 Hz, 1H), 7.74 (d, J = 8.1 Hz, 2H), 7.57 (br, 1H), 7.29 (d, J = 8.3, 1H), 4.75 (d, J = 4.9 Hz, 2H).

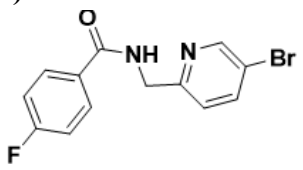
N-((5-Bromopyridin-2-yl)methyl)-4-methoxybenzamide (3.211)



To a stirring suspension of (5-bromopyridin-2-yl)methanamine (20 mg, 0.106 mmol, 1 equiv) in dichloroethane (20 mL) was added 4-methoxybenzoic anhydride (34 mg, 0.76 mmol, 1.1 equiv). The resulting suspension was stirred at rt for 30 min. After confirmation of consumption of starting material by TLC, reaction solvent was removed under reduced

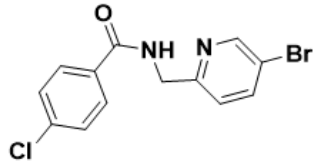
pressure and column chromatography (0 to 10% MeOH/DCM) on silica gel was used to purify sufficient amounts of target molecule for biological testing and characterization. LRMS (CI-quad) m/z cal'd for $C_{14}H_{14}O_2N_2Br^+$ [M+H$^+$] 323.18, found 323. 1H NMR (400 MHz, CDCl3) δ 8.63 (d, *J* = 2.2 Hz, 1H), 7.88 - 7.77 (m, 3H), 7.35 (br, 1H), 7.28 (d, *J* = 8.8 Hz, 2H), 4.72 (d, *J* = 5.1 Hz, 2H), 3.86 (s, 3H).

N-((5-Bromopyridin-2-yl)methyl)-4-fluorobenzamide (3.212)



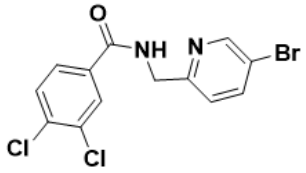
To a stirring suspension of (5-bromopyridin-2-yl)methanamine (20 mg, 0.106 mmol, 1 equiv) in dichloroethane (20 mL) was added 4-fluorobenzoic anhydride (31 mg, 0.76 mmol, 1.1 equiv). The resulting suspension was stirred at rt for 30 min. After confirmation of consumption of starting material by TLC, reaction solvent was removed under reduced pressure and column chromatography (0 to 10% MeOH/DCM) on silica gel was used to purify sufficient amounts of target molecule for biological testing and characterization. LRMS (CI-quad) m/z cal'd for $C_{13}H_{11}ON_2FBr^+$ [M+H$^+$] 311.14, found 311. 1H NMR (400 MHz, CDCl3) δ 8.65 (d, *J* = 2.3 Hz, 1H), 7.89 (ddd, J_1 = 2.8, J_2 = 3.3, J_3 = 5.4 Hz, 2H), 7.85 (dd, J_1 = 2.4, J_2 = 8.3 Hz, 1H), 7.41 (br, 1H), 7.28 (d, *J* = 8.4 Hz, 2H), 7.14 (ddd, J_1 = 3.0, J_2 = 4.8, J_3 = 11.5 Hz, 2H), 4.73 (d, *J* = 5.0 Hz, 2H).

N-((5-Bromopyridin-2-yl)methyl)-4-chlorobenzamide (3.213)



To a stirring suspension of (5-bromopyridin-2-yl)methanamine (20 mg, 0.106 mmol, 1 equiv) in dichloroethane (20 mL) was added 4-fluorobenzoic anhydride (34 mg, 0.76 mmol, 1.1 equiv). The resulting suspension was stirred at rt for 30 min. After confirmation of consumption of starting material by TLC, reaction solvent was removed under reduced pressure and column chromatography (0 to 10% MeOH/DCM) on silica gel was used to purify sufficient amounts of target molecule for biological testing and characterization. LRMS (CI-quad) m/z cal'd for $C_{13}H_{11}ON_2ClBr^+$ [M+H$^+$] 327.60, found 327. 1H NMR (400 MHz, CDCl3) δ 8.63 (d, J = 2.4 Hz, 1H), 7.85 (dd, J_1 = 2.6, J_2 = 8.6 Hz, 1H), 7.80 (d, J = 8.3 Hz, 2H), 7.43 (d, J = 8.7 Hz, 2H), 7.27 (d, J = 7.4 Hz, 1H), 4.71 (d, J = 5.0 Hz, 2H).

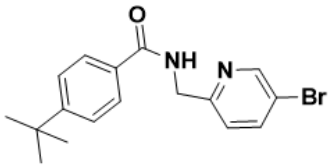
N-((5-Bromopyridin-2-yl)methyl)-3,4-dichlorobenzamide (3.214)



To a stirring suspension of (5-bromopyridin-2-yl)methanamine (20 mg, 0.106 mmol, 1 equiv) in dichloroethane (20 mL) was added 3,4-dichlorobenzoic anhydride (42 mg, 0.76 mmol, 1.1 equiv). The resulting suspension was stirred at rt for 30 min. After confirmation of consumption of starting material by TLC, reaction solvent was removed under reduced pressure and column chromatography (0 to 10% MeOH/DCM) on silica gel was used to purify sufficient amounts of target molecule for biological testing and characterization.

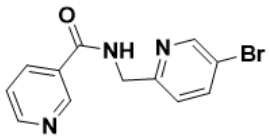
LRMS (CI-quad) m/z cal'd for $C_{13}H_{10}ON_2Cl_2Br^+$ [M+H$^+$] 362.04, found 362. H1 NMR (400 MHz, CDCl3) 8.64 (d, *J* = 2.2 Hz, 1H), 7.96 (d, *J* = 2.0 Hz, 1H), 7.85 (dd, J_1 = 2.3, J_2 = 8.4 Hz, 1H), 7.68 (dd, J_1 = 2.0, J_2 = 8.4 Hz, 1H), 7.53 (d, *J* = 8.4 Hz, 1H), 7.50 (br, 1H), 7.28 (s, 1H), 4.71 (d, *J* = 4.9 Hz, 2H).

N-((5-Bromopyridin-2-yl)methyl)-4-(tert-butyl)benzamide (3.215)



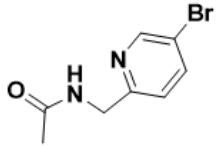
To a stirring suspension of (5-bromopyridin-2-yl)methanamine (20 mg, 0.106 mmol, 1 equiv) in dichloroethane (20 mL) was added 4-(tert-butyl)benzoic anhydride (39 mg, 0.76 mmol, 1.1 equiv). The resulting suspension was stirred at rt for 30 min. After confirmation of consumption of starting material by TLC, reaction solvent was removed under reduced pressure and column chromatography (0 to 10% MeOH/DCM) on silica gel was used to purify sufficient amounts of target molecule for biological testing and characterization. LRMS (CI-quad) m/z cal'd for $C_{17}H_{16}ON_2Br^+$ [M+H$^+$] 345.23, found 345. 1H NMR (400 MHz, CDCl3) δ 8.61 (d, *J* = 2.1 Hz, 1H), 7.83 - 7.77 (m, 3H), 7.46 (d, *J* = 8.6 Hz, 2H), 7.27 (d, *J* = 8.3 Hz, 1H), 4.71 (d, *J* = 5.1 Hz, 2H), 1.34 (s, 9H).

N-((5-Bromopyridin-2-yl)methyl)nicotinamide (3.216)



To a stirring suspension of (5-bromopyridin-2-yl)methanamine (20 mg, 0.106 mmol, 1 equiv) in dichloroethane (20 mL) was added nicotinic anhydride (27 mg, 0.76 mmol, 1.1 equiv). The resulting suspension was stirred at rt for 30 min. After confirmation of consumption of starting material by TLC, reaction solvent was removed under reduced pressure and column chromatography (0 to 10% MeOH/DCM) on silica gel was used to purify sufficient amounts of target molecule for biological testing and characterization. LRMS (CI-quad) m/z cal'd for $C_{12}H_{11}ON_3Br^+$ [M+H$^+$] 294.14, found 294. 1H NMR (400 MHz, CDCl3) δ 9.14 (d, J = 2.2 Hz, 1H), 8.76 (dd, J_1 = 1.6, J_2 = 5.0 Hz, 1H), 8.64 (d, J = 2.2 Hz, 1H), 8.23 (td, J_1 = 2.0, J_2 = 4.0 Hz, 1H), 7.84 (dd, J_1 = 2.5, J_2 = 8.4 Hz, 1H), 7.68 (br, 1H), 7.46 (dd, J_1 = 4.9, J_2 = 8.0 Hz, 1H), 7.28 (d, J = 8.4 Hz, 1H), 4.74 (d, J = 4.9 Hz, 2H).

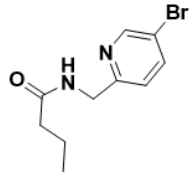
N-((5-Bromopyridin-2-yl)methyl)acetamide (3.217)



To a stirring suspension of (5-bromopyridin-2-yl)methanamine (20 mg, 0.106 mmol, 1 equiv) in dichloroethane (20 mL) was added 4-chlorobenzoic anhydride (12 mg, 0.76 mmol, 1.1 equiv). The resulting suspension was stirred at rt for 30 min. After confirmation of consumption of starting material by TLC, reaction solvent was removed under reduced pressure and column chromatography (0 to 10% MeOH/DCM) on silica gel was used to purify sufficient amounts of target molecule for biological testing and characterization. LRMS (CI-quad) m/z cal'd for $C_8H_{10}ON_2Br^+$ [M+H$^+$] 231.08, found 231. 1H NMR (400

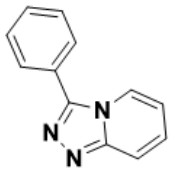
MHz, CDCl3) δ 8.60 (d, J = 2.1 Hz, 1H), 7.80 (dd, J_1 = 2.2, J_2 = 8.3 Hz, 1H), 7.20 (d, J = 7.9 Hz, 1H), 6.60 (s (b), 1H), 2.07 (s, 3H).

N-((5-Bromopyridin-2-yl)methyl)butyramide (3.218)



To a stirring suspension of (5-bromopyridin-2-yl)methanamine (20 mg, 0.106 mmol, 1 equiv) in dichloroethane (20 mL) was added butyric anhydride (18 mg, 0.76 mmol, 1.1 equiv). The resulting suspension was stirred at rt for 30 min. After confirmation of consumption of starting material by TLC, reaction solvent was removed under reduced pressure and column chromatography (0 to 10% MeOH/DCM) on silica gel was used to purify sufficient amounts of target molecule for biological testing and characterization. LRMS (CI-quad) m/z cal'd for $C_{10}H_{14}ON_2Br^+$ [M+H$^+$] 259.13, found 259. 1H NMR (400 MHz, CDCl3) δ 8.59 (d, J = 2.1 Hz, 1H), 7.79, (dd, J_1 = 2.3, J_2 = 8.3 Hz, 1H), 7.19 (d, J = 8.3 Hz, 1H), 6.58 (s, 1H), 4.51 (d, J = 5.2 Hz, 2H), 2.25 (t, J = 7.4 Hz, 2H). 1.69 (sext, 7.44 Hz, 2H), 0.96 (t, J = 7.4 Hz, 3H).

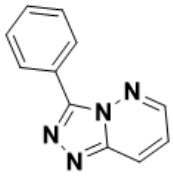
3-Phenyl-[1,2,4]triazolo[4,3-a]pyridine (3.218)



To a stirring suspension of 2-hydrazinylpyridine (100 mg, 0.91 mmol, 1 equiv) in toluene (20 mL) was added benzoic anhydride (208 mg, 0.91 mmol, 1 equiv). The resulting

suspension was heated to reflux and stirred for 24 h. After confirmation of consumption of starting material by TLC, the reaction was cooled to rt and the toluene was removed under reduced pressure. DCM (50 mL) was added and reaction was washed with a saturated $NaHCO_3$ solution (3 x 25 mL). The organic layer was then dried over Na_2SO_4, filtered and solvent removed under reduced pressure. Column chromatography (0 to 10% MeOH/DCM) on silica gel was used to purify sufficient amounts of target molecule for biological testing and characterization. LRMS (CI-quad) m/z cal'd for $C_{12}H_{10}N_3^+$ [M+H$^+$] 197.26, found 197. 1H NMR (400 MHz, CDCl3) δ 8.14 (dd, J_1 = 1.6, J_2 = 5.6 Hz, 1H), 7.90 (d, J = 7.3 Hz, 2H), 7.58 - 7.50 (m, 2H), 7.47 (t, J = 7.7 Hz, 2H), 6.80 (t, J = 4.2 Hz, 1H).

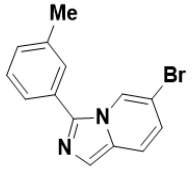
3-Phenyl-[1,2,4]triazolo[4,3-b]pyridazine (3.219)



To a stirring suspension of 3-hydrazinylpyridazine (100 mg, 0.69 mmol, 1 equiv) in toluene (20 mL) was added DIPEA (0.2 mL, 0.069 mmol, 0.01 equiv) and benzoic anhydride (205 mg, 1.38 mmol, 2 equiv). The resulting suspension was heated to reflux and stirred for 12 h. After confirmation of consumption of starting material by TLC, the reaction was cooled to rt and the toluene was removed under reduced pressure. DCM (50 mL) was added and reaction was washed with a saturated $NaHCO_3$ solution (3 x 25 mL). The organic layer was then dried over Na_2SO_4, filtered and solvent removed under reduced pressure. Column chromatography (0 to 10% MeOH/DCM) on silica gel was used to purify sufficient

amounts of target molecule for biological testing and characterization. LRMS (CI-quad) m/z cal'd for $C_{11}H_9N_4^+$ [M+H$^+$] 198.22, found 198. 1H NMR (400 MHz, CDCl3) δ 8.51 - 8.44 (m, 3H), 8.21 (dd, J_1 = 1.6, J_2 = 9.5 Hz, 1H), 7.59 - 7.52 (m, 3H), 7.16 (dd, J_1 = 4.1, J_2 = 9.5 Hz, 1H).

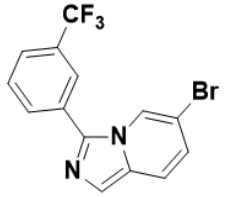
6-Bromo-3-(m-tolyl)imidazo[1,5-a]pyridine (3.220)



To a stirring suspension of (5-bromopyridin-2-yl)methanamine (100 mg, 0.69 mmol, 1 equiv) in dichloroethane (20 mL) was added 3-methylbenzoic anhydride (314 mg, 1.58 mmol, 2.3 equiv). The resulting suspension was stirred at rt for 30 min before dropwise addition of $POCl_3$ (141 mg, 1.12 mmol, 1.7 equiv) as a solution in *ca* 3 mL DCM. The reaction was then heated to reflux for 3 hr. After confirmation of consumption of starting material by TLC, the reaction was cooled to rt and the reaction was stirred with 5 mL of 0.1 N HCl (caution:exothermic reaction) for 15 mins to hydrolyze excess $POCl_3$. Post stirring, the reaction was washed with a saturated $NaHCO_3$ solution (3 x 25 mL, caution:gas evolution). The organic layer was then dried over Na_2SO_4, filtered and solvent removed under reduced pressure. Column chromatography (0 to 10% MeOH/DCM) on silica gel was used to purify sufficient amounts of target molecule for biological testing and characterization. LRMS (CI-quad) m/z cal'd for $C_{14}H_{12}N_2Br^+$ [M+H$^+$] 289.16, found 289. 1H NMR (400 MHz, CDCl3) δ 8.36 (s, 1H), 7.59 (s, 1H), 7.57 (s, 1H), 7.54 (d, J = 7.9 Hz,

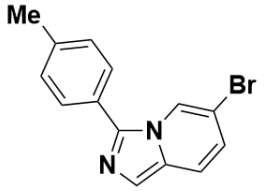
1H), 7.43 (d, *J* = 7.6 Hz, 1H), 7.38 (d, *J* = 9.5, 1H), 7.28 (d, *J* = 8.8 Hz, 1H), 6.77 (d, *J* = 9.5 Hz, 1H), 2.45 (s, 3H).

6-Bromo-3-(3-(trifluoromethyl)phenyl)imidazo[1,5-a]pyridine (3.221)



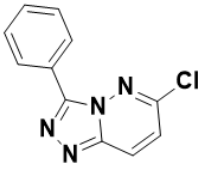
To a stirring suspension of (5-bromopyridin-2-yl)methanamine (100 mg, 0.69 mmol, 1 equiv) in dichloroethane (20 mL) was added 3-(trifluoromethyl)benzoic anhydride (448 mg, 1.58 mmol, 2.3 equiv). The resulting suspension was stirred at rt for 30 min before dropwise addition of $POCl_3$ (141 mg, 1.12 mmol, 1.7 equiv) as a solution in *ca* 3 mL DCM. The reaction was then heated to reflux for 3 hr. After confirmation of consumption of starting material by TLC, the reaction was cooled to rt and the reaction was stirred with 5 mL of 0.1 N HCl (caution:exothermic reaction) for 15 mins to hydrolyze excess $POCl_3$. Post stirring, the reaction was washed with a saturated $NaHCO_3$ solution (3 x 25 mL, caution:gas evolution). The organic layer was then dried over Na_2SO_4, filtered and solvent removed under reduced pressure. Column chromatography (0 to 10% MeOH/DCM) on silica gel was used to purify sufficient amounts of target molecule for biological testing and characterization. LRMS (CI-quad) m/z cal'd for $C_{14}H_7N_2F_3Br^+$ [M+H$^+$] 341.12, found 341. 1H NMR (400 MHz, CDCl3) δ 8.27 (s, 1H), 8.00 (s, 1H), 7.88 (d, *J* = 7.5 Hz, 1H), 7.65 - 7.57 (m, 2H), 7.54 (s, 1H), 7.35 (d, *J* = 9.5 Hz, 1H), 6.76 (d, *J* = 9.5 Hz, 1H).

6-Bromo-3-(p-tolyl)imidazo[1,5-a]pyridine (3.222)



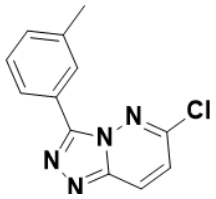
To a stirring suspension of (5-bromopyridin-2-yl)methanamine (100 mg, 0.69 mmol, 1 equiv) in dichloroethane (20 mL) was added 4-methylbenzoic anhydride (314 mg, 1.58 mmol, 2.3 equiv). The resulting suspension was stirred at rt for 30 min before dropwise addition of $POCl_3$ (141 mg, 1.12 mmol, 1.7 equiv) as a solution in *ca* 3 mL DCM. The reaction was then heated to reflux for 3 hr. After confirmation of consumption of starting material by TLC, the reaction was cooled to rt and the reaction was stirred with 5 mL of 0.1 N HCl (caution:exothermic reaction) for 15 mins to hydrolyze excess $POCl_3$. Post stirring, the reaction was washed with a saturated $NaHCO_3$ solution (3 x 25 mL, caution:gas evolution). The organic layer was then dried over Na_2SO_4, filtered and solvent removed under reduced pressure. Column chromatography (0 to 10% MeOH/DCM) on silica gel was used to purify sufficient amounts of target molecule for biological testing and characterization. LRMS (CI-quad) m/z cal'd for $C_{14}H_{12}N_2Br^+$ [$M+H^+$] 289.16, found 289. 1H NMR (400 MHz, CDCl3) δ 8.28 (s, 1H), 7.58 (d, *J* = 7.7 Hz, 2H), 7.49 (s, 1H), 7.33 - 7.25 (m, 3H), 6.68 (d, *J* = 9.5 Hz, 1H), 2.37 (s, 3H).

6-Chloro-3-phenyl-[1,2,4]triazolo[4,3-b]pyridazine (3.223)



To a stirring suspension of 3-chloro-6-hydrazinylpyridazine (100 mg, 0.69 mmol, 1 equiv) in toluene (20 mL) was added DIPEA (0.2 mL, 0.069 mmol, 0.01 equiv) and benzoic anhydride (78 mg, 0.69 mmol, 1 equiv). The resulting suspension was heated to reflux and stirred for 12 h. After confirmation of consumption of starting material by TLC, the reaction was cooled to rt and the toluene was removed under reduced pressure. DCM (50 mL) was added and reaction was washed with a saturated $NaHCO_3$ solution (3 x 25 mL). The organic layer was then dried over Na_2SO_4, filtered and solvent removed under reduced pressure. Column chromatography (0 to 10% MeOH/DCM) on silica gel was used to purify sufficient amounts of target molecule for biological testing and characterization. LRMS (CI-quad) m/z cal'd for $C_{11}H_7N_4Cl^+$ [M+H$^+$] 231.65, found 232. 1H NMR (400 MHz, CDCl3) δ 8.45 (d, *J* = 7.9 Hz, 2H), 8.15 (d, *J* = 9.6 Hz, 1H), 7.59 - 7.49 (m, 3H), 7.15 (d, *J* = 9.6 Hz, 1H).

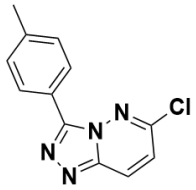
6-Chloro-3-(m-tolyl)-[1,2,4]triazolo[4,3-b]pyridazine (3.224)



To a stirring suspension of 3-chloro-6-hydrazinylpyridazine (100 mg, 0.69 mmol, 1 equiv) in toluene (20 mL) was added DIPEA (0.2 mL, 0.069 mmol, 0.01 equiv) and 3-methylbenzoic anhydride (87 mg, 0.69 mmool, 1 equiv). The resulting suspension was heated to reflux and stirred for 12 h. After confirmation of consumption of starting material by TLC, the reaction was cooled to rt and the toluene was removed under reduced pressure. DCM (50 mL) was added and reaction was washed with a saturated $NaHCO_3$ solution (3 x

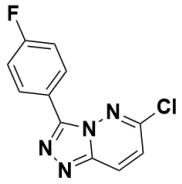
25 mL). The organic layer was then dried over Na_2SO_4, filtered and solvent removed under reduced pressure. Column chromatography (0 to 10% MeOH/DCM) on silica gel was used to purify sufficient amounts of target molecule for biological testing and characterization. LRMS (CI-quad) m/z cal'd for $C_{12}H_{10}N_4Cl^+$ [M+H$^+$] 246.69, found 247. 1H NMR (400 MHz, CDCl3) δ 8.29 - 8.20 (m, 2H), 8.17 (d, *J* = 9.6 Hz, 1H), 7.46 (t, *J* = 7.9 Hz, 1H), 7.36 (d, *J* = 7.7 Hz, 1H), 7.17 (d, *J* = 9.6 Hz, 1H), 2.48 (s, 3H).

6-Chloro-3-(p-tolyl)-[1,2,4]triazolo[4,3-b]pyridazine (3.225)



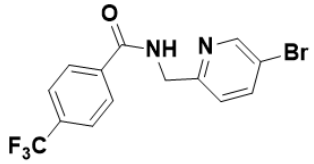
To a stirring suspension of 3-chloro-6-hydrazinylpyridazine (100 mg, 0.69 mmol, 1 equiv) in toluene (20 mL) was added DIPEA (0.2 mL, 0.069 mmol, 0.01 equiv) and 4-methylbenzoic anhydride (87 mg, 0.69 mmol, 1 equiv). The resulting suspension was heated to reflux and stirred for 12 h. After confirmation of consumption of starting material by TLC, the reaction was cooled to rt and the toluene was removed under reduced pressure. DCM (50 mL) was added and reaction was washed with a saturated $NaHCO_3$ solution (3 x 25 mL). The organic layer was then dried over Na_2SO_4, filtered and solvent removed under reduced pressure. Column chromatography (0 to 10% MeOH/DCM) on silica gel was used to purify sufficient amounts of target molecule for biological testing and characterization. LRMS (CI-quad) m/z cal'd for $C_{12}H_{10}N_4Cl^+$ [M+H$^+$] 246.69, found 247. 1H NMR (400 MHz, CDCl3) δ 8.35 (d, *J* = 8.0 Hz, 2H), 8.17 (d, *J* = 9.6 Hz, 1H), 7.39 (d, *J* = 8.0 Hz, 2H), 7.16 (d, *J* = 9.6 Hz, 1H), 2.45 (s, 3H).

6-Chloro-3-(4-fluorophenyl)-[1,2,4]triazolo[4,3-b]pyridazine (3.226)



To a stirring suspension of 3-chloro-6-hydrazinylpyridazine (100 mg, 0.69 mmol, 1 equiv) in toluene (20 mL) was added DIPEA (0.2 mL, 0.069 mmol, 0.01 equiv) and 4-fluorobenzoic anhydride (87 mg, 0.69 mmol, 1 equiv). The resulting suspension was heated to reflux and stirred for 12 h. After confirmation of consumption of starting material by TLC, the reaction was cooled to rt and the toluene was removed under reduced pressure. DCM (50 mL) was added and reaction was washed with a saturated $NaHCO_3$ solution (3 x 25 mL). The organic layer was then dried over Na_2SO_4, filtered and solvent removed under reduced pressure. Column chromatography (0 to 10% MeOH/DCM) on silica gel was used to purify sufficient amounts of target molecule for biological testing and characterization. LRMS (CI-quad) m/z cal'd for $C_{11}H_7N_4FCl^+$ [M+H$^+$] 250.65, found 251. 1H NMR (400 MHz, CDCl3) δ 8.49 (dd, J_1 = 5.4, J_2 = 8.6 Hz, 2H), 8.17 (d, J = 9.6 Hz, 1H), 7.26 (t, J = 8.5 Hz, 2H), 7.17 (d, J = 9.6 Hz, 1H).

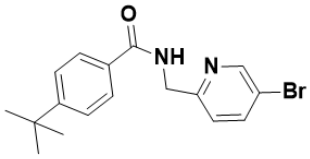
N-((5-Bromopyridin-2-yl)methyl)-4-(trifluoromethyl)benzamide (3.227)



To a stirring suspension of (5-bromopyridin-2-yl)methanamine (50 mg, 0.27 mmol, 1 equiv) in dichloroethane (20 mL) was added 4-(trifluoromethyl)benzoic anhydride (193

mg, 0.54 mmol, 2 equiv). The resulting suspension was stirred at rt for 30 min. After confirmation of consumption of starting material by TLC, reaction solvent was removed under reduced pressure and column chromatography (0 to 10% MeOH/DCM) on silica gel was used to purify sufficient amounts of target molecule for biological testing and characterization. LRMS (CI-quad) m/z cal'd for $C_{14}H_{11}ON_2F_3Br^+$ [M+H$^+$] 361.15, found 361. 1H NMR (400 MHz, CDCl3) δ 8.63 (d, J = 2.2 Hz, 1H), 7.97 (d, J = 8.0 Hz, 2H), 7.84 (dd, J_1 = 2.3, J_2 = 8.3 Hz, 1H), 7.72 (d, J = 8.1 Hz, 2H), 7.58 (br, 1H), 7.27 (d, J = 7.2 Hz, 1H), 4.73 (d, J = 4.9 Hz, 2H).

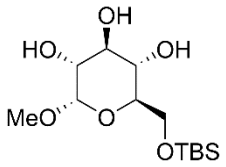
N-((5-Bromopyridin-2-yl)methyl)-4-(tert-butyl)benzamide (3.228)



To a stirring suspension of (5-bromopyridin-2-yl)methanamine (50 mg, 0.27 mmol, 1 equiv) in dichloroethane (20 mL) was added 4-(tert-butyl)benzoic anhydride (193 mg, 0.27 mmol, 2 equiv). The resulting suspension was stirred at rt for 30 min. After confirmation of consumption of starting material by TLC, reaction solvent was removed under reduced pressure and column chromatography (0 to 10% MeOH/DCM) on silica gel was used to purify sufficient amounts of target molecule for biological testing and characterization. LRMS (CI-quad) m/z cal'd for $C_{17}H_{20}ON_2Br^+$ [M+H$^+$] 349.26, found 349. 1H NMR (400 MHz, CDCl3) δ 8.61 (d, J = 2.2 Hz, 1H), 7.82 - 7.77 (m, 3H), 7.46 (d, J = 8.6 Hz, 2H), 7.39 (br, 1H), 7.25 (d, J = 8.4 Hz, 1H), 4.70 (d, J = 5.1 Hz, 2H), 1.33 (s, 9H).

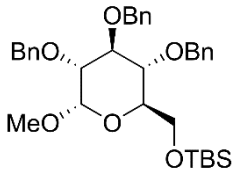
Aminocyclopentitol Synthesis

(2*R*,3*S*,4*S*,5*R*,6*S*)-2-(((tert-Butyldimethylsilyl)oxy)methyl)-6-methoxytetrahydro-2H-pyran-3,4,5-triol (4.9).



Methyl-α-D-glucopyranoside (5.10 g, 26.3 mmol) was dissolved in anhydrous DMF (60 mL) and cooled to 0 °C. Imidazole (4.40 g, 64.6 mmol) and *tert*-butyldimethylsilyl chloride (4.70 g, 31.0 mmol) were added to the solution in sequence. The mixture was allowed to stir at room temperature overnight. Upon the consumption of starting material, monitored by TLC, the solvent was removed under high vacuum and the residue was dissolved in EtOAc (400 mL). The solution was washed with water (2 x 200 mL) and brine (2 x 200 mL), dried over $MgSO_4$, and concentrated in vacuo. The residue was purified by flash column chromatography on silica gel, using 100% EtOAc as the eluent. After chromatographic purification, an amorphous solid was obtained (7.3 g, 24 mmol, 98%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 4.75 (d, *J* = 3.9 Hz, 1H), 3.89 (dd, *J* = 4.9, 10.6 Hz, 1H), 3.82 (dd, *J* = 5.2, 10.6 Hz, 1H), 3.74 (t, *J* = 9.1 Hz, 1H), 3.61 (dd, *J* = 5.0, 9.7 Hz, 1H), 3.53 (t, *J* = 9.0 Hz, 1H), 3.51 (m, 1H), 3.42 (s, 3H), 3.14 (s, 1H), 2.82 (s, 1H), 2.19 (d, *J* = 7.6 Hz, 1H), 0.91 (s, 9H), 0.10 (s, 6H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 100.0, 75.6, 73.3, 73.1, 71.2, 65.2, 56.2, 26.7, 19.1, -4.6; ESI-HRMS: calc'd m/e for [M+Na$^+$] $C_{13}H_{28}NaO_6Si$: 309.1733, found 309.2209; IR (neat, NaCl, cm^{-1}): 3474, 2954, 2929, 2858, 1655, 1472, 1464, 1361, 1253, 1194, 1152, 1112, 1045, 1002, 903, 854, 837, 777, 749; $[\alpha]_D^{25}$ +98 (*c* 1.0, $CHCl_3$).

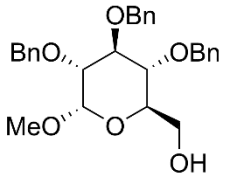
tert-Butyldimethyl(((2*R*,3*R*,4*S*,5*R*,6*S*)-3,4,5-tris(benzyloxy)-6-methoxytetrahydro-2H-pyran-2-yl)methoxy)silane (4.10).



The starting pyranoside (7.00 g, 22.7 mmol) was dissolved in anhydrous DMF (100 mL) and cooled to 0 °C. Sodium hydride (3.30 g, 82.5 mmol) was added to the solution. After the evolution of H_2 ceased, benzyl bromide (12.2 mL, 103 mmol) was added to the reaction. The mixture was stirred under an atmosphere of nitrogen overnight. Upon the consumption of the starting material, monitored by TLC, the reaction was quenched with MeOH (10 mL), then poured into water (500 mL) and extracted with EtOAc (5 x 100 mL). The combined organic layers were washed with water (2 x×200 mL) and brine (2 x 200 mL), dried over $MgSO_4$, and concentrated in vacuo. The residue was purified by flash column chromatography on silica gel, using 10% EtOAc:hexanes (v/v) as the eluent. After chromatographic purification, a colorless oil was obtained (11.2 g, 19.3 mmol, 85%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.32 - 7.21 (m, 15H), 4.93 (d, *J* = 10.8 Hz, 1H), 4.84 (d, *J* = 10.9 Hz, 1H), 4.78 (d, *J* = 10.9 Hz, 1H), 4.76 (d, *J* = 12.1 Hz, 1H), 4.64 (d, *J* = 12.1 Hz, 1H), 4.60 (d, *J* = 11.0 Hz, 1H), 4.57 (d, *J* = 3.6 Hz, 1H), 3.96 (t, *J* = 9.3 Hz, 1H), 3.75 (d, *J* = 3.2 Hz, 1H), 3.58 (dt, *J* = 3.7, 10.0 Hz, 1H), 3.50 (d, *J* = 9.1 Hz, 1H), 3.48 (d, *J* = 3.6 Hz, 1H), 3.46 (d, *J* = 3.6 Hz, 1H), 3.33 (s, 3H), 0.85 (s, 9H), 0.00 (d, *J* = 2.0 Hz, 6H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 139.2, 138.9, 138.6, 128.8, 128.7, 128.4, 128.39, 128.2, 128.0, 127.9, 98.2, 82.5, 80.6, 78.1, 76.2, 75.3, 73.7, 71.9, 62.6, 55.2, 26.3, 18.7, -4.8, -5.0; ESI-HRMS: calc'd m/e for [M+Na^+] $C_{34}H_{46}NaO_6Si$: 601.2961, found 601.2932; IR (neat, NaCl, cm^{-1}): 3065, 3031, 2928, 2856, 1606, 1497, 1455, 1361, 1252, 1201, 1193, 1160, 1136,

1092, 1072, 910, 836, 778, 735, 697; $[\alpha]_D^{25}$ +130 (*c* 0.89, $CHCl_3$).

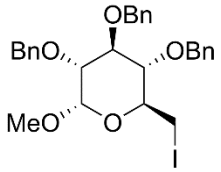
((2*R*,3*R*,4*S*,5*R*,6*S*)-3,4,5-tris(Benzyloxy)-6-methoxytetrahydro-2H-pyran-2-yl)methanol (4.11).



The starting pyranoside (11.5 g, 19.9 mmol) was dissolved in acetonitrile (120 mL). H_2O (25 mL) was added, and the pH of the solution adjusted to pH = 3 by the addition of *p*-toluenesulphonic acid. The reaction mixture was stirred at room temperature overnight. Upon the consumption of the starting material, monitored by TLC, EtOAc (300 mL) was added to the reaction mixture. The mixture was washed with saturated aqueous sodium bicarbonate (2 x 150 mL) and brine (150 mL). The organic layers were combined, dried over $MgSO_4$, and concentrated in vacuo. The residue was purified by flash column chromatography on silica gel, using 30% EtOAc:hexanes (v/v) as the eluent. After chromatographic purification, a colorless oil was obtained (6.00 g, 12.9 mmol, 65%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.37 - 7.28 (m, 15H), 4.99 (d, *J* = 10.9 Hz, 1H), 4.88 (d, *J* = 11.1 Hz, 1H), 4.84 (d, *J* = 10.9 Hz, 1H), 4.80 (d, *J* = 12.1 Hz, 1H), 4.65 (t, *J* = 12.2 Hz, 2H), 4.57 (d, *J* = 3.6 Hz, 1H), 4.00 (t, *J* = 9.3 Hz, 1H), 3.77 (m, 1H), 3.69 (m, 1H), 3.65 (m, 1H), 3.53 (d, *J* = 9.2 Hz, 1H), 3.50 (dd, *J* = 3.6, 6.1 Hz, 1H), 3.37 (s, 3H), 1.60 (dd, *J* = 5.3, 7.4 Hz, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 139.1, 138.5, 128.8, 128.8, 128.5, 128.4, 128.3, 128.3, 128.2, 128.0, 98.6, 82.3, 80.4, 76.1, 75.4, 73.8, 71.0, 62.3, 55.6.; ESI-HRMS: calc'd m/e for $[M+Na^+]$ $C_{28}H_{32}NaO_6$: 487.2097, found 487.2104; IR (neat, NaCl, cm^{-1}): 3486, 3063, 3034, 2921, 1732, 1606, 1497, 1454, 1360, 1260, 1192, 1076, 1072,

1068, 911, 802, 737, 697; $[\alpha]_D^{25}$ +23 (*c* 0.99, $CHCl_3$).

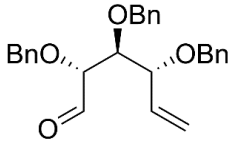
(2*S*,3*S*,4*S*,5*R*,6*S*)-3,4,5-Tris(benzyloxy)-2-(iodomethyl)-6-methoxytetrahydro-2H-pyran (4.12).



Triphenylphosphine (5.10 g, 19.4 mmol) and imidazole (1.80 g, 26.4 mmol) were successively dissolved in THF (150 mL). The resultant mixture was cooled to 0 °C, and iodine (4.90 g, 19.3 mmol) was added in portions over 30 min. A solution of starting pyranoside (6.00 g, 12.9 mmol) in THF (50 mL) was cannulated into the reaction flask. The reaction was then heated to 66 °C and stirred at that temperature for 1 h. The reaction was then cooled to room temperature and quenched with H_2O (25 mL); the aqueous layer was extracted with EtOAc (250 mL x 2). The organic layers were combined, washed with 10% sodium thiosulfate, dried over $MgSO_4$, and concentrated in vacuo. The crude material was purified by flash column chromatography on silica gel, using 10% EtOAc:hexanes (v/v) as the eluent. After chromatographic purification, a light yellow, oil was obtained (10.8 g, 18.8 mmol, 97%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.37 - 7.27 (m, 15H), 4.99 (d, *J* = 10.8 Hz, 1H), 4.94 (d, *J* = 11.0 Hz, 1H), 4.81 (d, *J* = 2.0 Hz, 1H), 4.79 (d, *J* = 3.3 Hz, 1H), 4.67 (t, *J* = 11.0 Hz, 2H), 4.61 (d, *J* = 3.6 Hz, 1H), 4.01 (t, *J* = 9.0 Hz, 1H), 3.54 (dd, *J* = 3.6 Hz, 1H), 3.46 (m, 2H), 3.42 (s, 3H), 3.34 (t, *J* = 9.0 Hz, 1H), 3.29 (dd, *J* = 6.7, 11.0 Hz, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 138.9, 138.4, 138.36, 128.9, 128.85, 128.8, 128.4, 128.35, 128.3, 128.1, 98.5, 81.9, 81.9, 80.4, 76.1, 75.7, 73.8, 69.7, 55.9, 8.0; ESI-HRMS:

calc'd m/e for [M+Na$^+$] $C_{28}H_{31}INaO_5$: 597.1114, found 597.1162; IR (neat, NaCl, cm^{-1}): 3062, 3030, 2909, 1496, 1454, 1359, 1260, 1196, 1120, 1102, 1088, 1048, 1028, 736, 697; $[\alpha]_D^{25}$ +32 (*c* 1.1, $CHCl_3$).

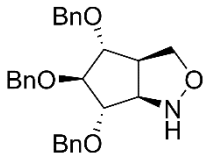
(2*R*,3*S*,4*R*)-2,3,4-Tris(benzyloxy)hex-5-enal (4.13).



The starting Iodo-compound (4.6 g, 8.0 mmol) was dissolved in a mixture of THF/H_2O (9:1, 200 mL) and then activated zinc (5.20 g, 79.5 mmol) was added. The flask was placed in an ultrasonic cleaner and sonicated overnight. The reaction progress was monitored by mass spectroscopy due to the fact that the R*f* value of the product is identical to that of the starting material on TLC. Upon the consumption of the starting material, the reaction was diluted with EtOAc (50 mL), and the aqueous layer was extracted with EtOAc (50 mL x 2). The organic layers were combined, washed with sodium bicarbonate (20 mL), brine (20 mL) and dried over $MgSO_4$. The solvent was removed in vacuo and the residue was purified by flash column chromatography on silica gel, using 10% EtOAc:hexanes (v/v) as the eluent. After chromatographic purification, a colorless oil was obtained (3.1 g, 7.5 mmol, 96%). ^{1}H NMR ($CDCl_3$, 400 MHz): 9.63 (dd, *J* = 1.6, 2.2 Hz, 0.6 H), 9.57 (d, *J* = 1.0 Hz, 0.4H), 7.29 - 7.21 (m, 15H), 5.84 - 5.76 (m, 1H), 5.29 (dt, *J* = 1.0, 4.3 Hz, 1H), 5.20 (dd, *J* = 1.0, 7.0 Hz, 1H), 4.64 (dd, *J* = 2.2, 10.0 Hz, 1H), 4.60 (t, *J* = 13.1 Hz, 1H), 4.54 (m, 1H), 4.50 (m, 1H), 4.44 (dd, *J* = 8.0, 19.8 Hz, 1H), 4.30 (dd, *J* = 3.3, 11.9 Hz, 1H), 4.04 (m, 1H), 3.91 (t, *J* = 6.8 Hz, 1H), 3.78 (m, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 201.6, 201.0, 138.1,

138.07, 137.8, 137.7, 137.2, 134.8, 134.4, 128.6, 128.5, 128.4, 128.4, 128.34, 128.31, 128.26, 128.2, 128.1, 128.0, 127.9, 127.8, 127.79, 127.7, 127.6, 127.0, 119.5, 119.4, 82.4, 81.8, 80.9, 80.0, 75.9, 74.5, 73.3, 73.1, 71.0 70.7, 45.2; ESI-HRMS: calc'd m/e for [M+Na^+] $C_{27}H_{28}NaO_4$: 439.1885, found 439.1954; IR (neat, NaCl, cm^{-1}): 3064, 3031, 2866, 1727, 1497, 1454, 1393, 1352, 1208, 1071, 1027, 932, 735, 697; $[\alpha]_D^{25}$ +4.5 (*c* 0.85, $CHCl_3$).

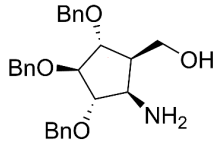
(3a*R*,4*R*,5*S*,6*S*,6a*R*)-4,5,6-Tris(benzyloxy)hexahydro-1H-cyclopenta[c]isoxazole (4.14).



The starting aldehyde (3.8 g, 9.1 mmol) was dissolved in MeOH (40 mL). To the stirring solution, hydroxylamine hydrochloride (2.50 g, 36.0 mmol) was added. The suspension was neutralized with sodium bicarbonate (4.30 g, 40.6 mmol). The solution was then stirred under refluxing conditions for 6 h. After the consumption of the starting material, the solvent was removed in vacuo. The residue was dissolved in EtOAc, washed with 10% HCl solution, saturated sodium bicarbonate, and brine in sequence, and dried over $MgSO_4$. The solvent was removed to yield an oily residue which was passed through a silica plug with 25% EtOAc:hexanes (v/v) as the eluent, giving a mixture of inseparable E/Z isomers as a light green oil. The resulting oxime (970 mg, 2.20 mmol) was then dissolved in dry toluene (35 mL) and heated at reflux for 15 h under nitrogen. Upon the consumption of the starting material, monitored by TLC, the reaction was cooled; and the solvent was removed in vacuo. The crude product was purified by flash column chromatography on silica gel,

using 40% EtOAc in hexanes as the eluent. After chromatographic purification, a yellow oil was obtained (2.7 g, 6.4 mmol, 70%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.38 - 7.28 (m, 15 H), 4.85 (d, *J* = 11.8 Hz, 1H), 4.79 (d, *J* = 11.9 Hz, 2H), 4.71 (d, *J* = 5.9 Hz, 2H), 4.58 (d, *J* = 11.8 Hz, 1H), 3.94 (t, *J* = 8.4 Hz, 1H), 3.87 (t, *J* = 5.8 Hz, 2H), 3.83 (t, *J* = 6.6 Hz, 1H), 3.68 (t, *J* = 7.4 Hz, 1H), 3.46 (t, *J* = 6.9 Hz, 1H), 2.91 (dd, *J* = 5.5, 7.0 Hz, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 138.5, 138.3, 138.1, 128.5, 128.4, 128.3, 127.9, 127.8, 127.75, 127.7, 127.6, 127.5, 86.0, 85.6, 84.7, 76.0, 72.7, 72.4, 72.3, 66.3, 49.6; ESI-HRMS: calc'd m/e for [M+Na^+] $C_{27}H_{29}NNaO_4$: 454.1994, found 454.1996; IR (neat, NaCl, cm^{-1}): 3435, 3031, 2922, 2863, 1742, 1724, 1497, 1454, 1364, 1208, 1094, 1072, 736, 697; $[\alpha]_D^{25}$ +25 (*c* 0.54, $CHCl_3$).

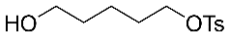
((1*R*,2*R*,3*S*,4*S*,5*R*)-2,3,4-Tris(benzyloxy)-5-amino-cyclopentyl)methanol (4.15).



To a stirring solution of starting isoxazole (675 mg, 1.60 mmol) in 85% acetic acid in H_2O (25 mL), active zinc dust (510 mg, 8.00 mmol) was added. The reaction was then stirred at 55 °C for 2 h. After the consumption of the starting material, monitored by TLC, the mixture was cooled to room temperature and the zinc dust was filtered off. The filtrate was diluted with H_2O (25 mL) and basified with 1M NaOH. The resultant solution was extracted with CH_2Cl_2 (3 x 25 mL); the organic layers were combined, dried over $MgSO_4$, and concentrated in vacuo. The crude product was purified by flash column chromatography on silica gel, using 10% MeOH/CH_2Cl_2 + 1% NH_4OH as the eluent. After chromatographic purification, a colorless oil was obtained (0.97 g, 2.2 mmol, 87%). ^{1}H

NMR ($CDCl_3$, 400 MHz): δ 7.30 - 7.28 (m, 15 H), 5.56 - 5.23 (br s, 3H), 4.68 (d, *J* = 12.0 Hz, 2H), 4.64 (d, *J* = 11.1 Hz, 2H), 4.54 (m, 2H), 4.53 (d, *J* = 11.6 Hz, 1H), 4.48 (d, *J* = 11.6 Hz, 1H), 4.02 (t, *J* = 5.7 Hz, 1H), 3.92 (t, *J* = 4.4 Hz, 1H), 3.87 (t, *J* = 5.0 Hz, 1H), 3.82 (m, 1H), 3.69 (t, *J* = 6.7 Hz, 1H), 2.02 (m, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 138.2, 138.18, 138.0, 128.8, 128.3, 128.2, 128.16, 87.6, 85.6, 83.0, 72.8, 72.4, 72.3, 61.1, 55.8, 45.3; ESI-HRMS: calc'd m/e for $[M+H^+]$ $C_{27}H_{32}NO_4$: 434.2326, found 434.2295; IR (neat, NaCl, cm^{-1}): 3308, 3063, 3030, 2927, 2871, 1496, 1454, 1363, 1207, 1091, 1071, 1028, 735, 697.

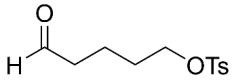
5-Hydroxypentyl 4-methylbenzenesulfonate (4.16).



To a stirring solution of 1,5-pentanediol (3 g, 28 mmol, 1 equiv) in DCM (50 mL) was added DMAP (342 mg, 2.8 mmol, 0.1 equiv) followed by p-toluenesulfonyl chloride (pTsCl, 5.49 g, 28 mmol, 1 equiv). The pTsCl was added as a solution in DCM over 10 mins. After the addition was complete the reaction was left to stir overnight at room temperature under nitrogen. After confirming consumption of starting materials, the reaction was transferred to a separatory funnel and was successively with water (2x100 mL) and brine (2X100 mL). The remaining organics were dried over Na_2SO_4, filtered and concentrated *en vacuo*. The crude product was purified by flash column chromatography on silica gel, using 10% to 30% EtOAc/Hexanes (v/v) as the eluent. After purification a colorless, viscous oil was obtained whose spectra matched reported literature. (1.41 g, 19%). ^1H NMR ($CDCl_3$, 400 MHz): δ 7.78 (d, *J* = 8.3 Hz, 2H), 7.34 (d, *J* = 8.0 Hz, 2H),

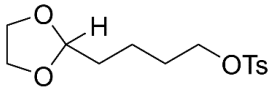
4.03 (t, J = 6.4 Hz, 2H), 3.60 (t, J = 6.3 Hz, 2H), 2.45 (s, 3H), 1.67 (q, J = 7.2 Hz, 2H), 1.55 – 1.48 (m, 2H), 1.44 – 1.35 (m, 2H).

5-Oxopentyl 4-methylbenzenesulfonate (4.17)



The starting tosyl compound (1.41 g, 5.45 mmol, 1 equiv) was dissoved in 50 mL of a 4:1 mix of DCM to DMSO. To this was incrementally added sulfur trioxide pyridine complex (3.4 g, 21.8 mmol, 4 equiv) over 10 min. The reaction was stirred at room temperature for 30 min and after confirming consumption of starting material, it was quenched with 50 mL water. The mixture was transferred to a separatory funnel and washed once with brine (50 mL) followed by a saturated solution of citric acid (50 mL). The organics were dried over Na_2SO_4, filtered and concentrated *en vacuo* to afford 1.28 g of crude product which was purified via flash chromatographing using 20 to 50% EtOAc/Hexanes (*v/v*) as the eluent. A viscous, colorless oil was obtained after purification. (464 mg, 1.81 mmol, 33%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 9.72 (s, 1H), 7.78 (d, J = 8.3 Hz, 2H), 7.35 (d, J = 8.1 Hz, 2H), 4.03 (t, J = 5.6 Hz, 2H), 2.48 – 2.37 (m, 5H), 1.74 – 1.61 (m, 4H).

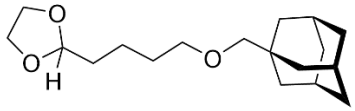
4-(1,3-Dioxolan-2-yl)butyl 4-methylbenzenesulfonate (4.18).



An excess of ethylene glycol (7 mL, 125 mmol, 100 equiv) and para-toluene sulfonic acid (31 mg, 0.18 mmol, 0.1 equiv) were dissolved in 20 mL of toluene. To this flask was attached a Dean-Stark apparatus and the flask was refluxed for 1 h, cooled, and to it was

added the starting aldehyde (464 mg, 1.8 mmol, 1 equiv) as a solution in anhydrous toluene (10 mL). The reaction was brought to reflux again for 4 hr after which time starting material was no longer observed by TLC. The reaction was cooled, transferred to a separatory funnel and washed with sat. $NaHCO_3$ until neutralized. The organics were dried over Na_2SO_4, filtered and collected *en vacuo*. The crude product was purified using flash column chromatography to afford target acetal as a colorless, very viscous oil. (398 mg, 1.33 mmol, 74%). ^1H NMR ($CDCl_3$, 400 MHz): δ 7.80 (d, J = 8.2 Hz, 2H), 7.35 (d, J = 7.9 Hz, 2H), 4.81 (t, J = 4.63 Hz, 1H), 4.03 (t, J = 6.4 Hz, 2H), 3.99 – 3.79 (m, 4H), 2.46 (s, 3H), 1.75 – 1.66 (m, 2H), 1.65 – 1.58 (m, 2H), 1.50 – 1.41 (m, 2H).

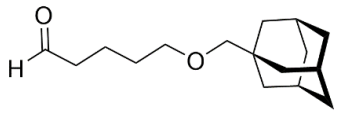
2-(4-((Adamantan-1-yl)methoxy)butyl)-1,3-dioxolane (4.19)



To a stirring solution of 1-adamantane methanol (615 mg, 3.87 mmol, 1.5 equiv) in DMF (15 mL) was added sodium hydride (300 mg, 7.5 mmol, 3 equiv) incrementally over 20 min after which the reaction was heated to 40 °C for 1 hr. At this time, the starting acetal (EJC-4-114, 775 mg, 2.5 mmol, 1 equiv) was added to the reaction as a solution in DMF (10 mL). The reaction was stirred for a further 3.5 hr until starting material was confirmed consumed. At which time the reaction was cooled to room temperature, diluted with 100 mL water and transferred to a separatory funnel. The organics were extracted 2X150 mL EtOAc, then the combined organics were washed 2X100 mL brine, dried over Na_2SO_4, filtered and concentrated *en vacuo*. The crude product was purified by flash colum chromatography using 15 to 45% EtOAc/Hex (v/v) as the eluent. Pure product was

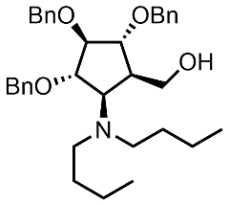
obtained as a colorless, viscous oil. (543 mg, 1.8 mmol, 74%) ^{1}H NMR ($CDCl_3$, 400 MHz): δ 4.86 (br, 1H), 3.98 – 3.82 (m, 4H), 3.38 (t, *J* = 6.1 Hz, 2H), 2.95 (s, 2H), 1.95 (s, 3H), 1.72 – 1.57 (m, 11H), 1.52 (br, 6H), 1.49 – 1.46 (m, 2H).

5-((Adamantan-1-yl)methoxy)pentanal (4.20)



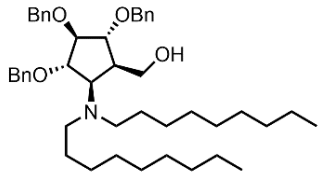
Starting acetal (540 mg, 1.8 mmol, 1 equiv) was dissolved in a 1:1 mixture of 6 M HCl and acetone. The reaction was heated to 40 °C for 2 hr. Upon confirmation of consumption of starting material, the reaction was neutralized with a 0 °C slurry of 6 mL of a 1:1 mix of 3 N NaOH and diethyl ether. Ensuring the pH of the reaction was raised above 7, the reaction was then transferred to a separatory funnel and the layers separated. The aqueous layer was extracted twice more with 30 mL diethyl ether. The combined organic layers were then washed with brine (2X50 mL), dried over Na_2SO_4, filtered and concentrated *en vacuo*. The crude product was purified by flash column chromatography using 3 to 10% EtOAc/Hexanes (v/v) as the eluent. The colorless, viscous oil recovered from the column was verified as target molecule and used within 3 days. (324 mg, 1.3 mmol, 71%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 9.77 (s, 1H), 3.39 (t, *J* = 6.4 Hz, 2H), 2.95 (s, 2H), 2.47 (t, *J* = 7.4 Hz, 2H), 1.95 (br, 3H), 1.74 – 1.58 (m, 12H), 1.52 (s, 6H).

((1R,2R,3S,4S,5R)-2,3,4-tris(Benzyloxy)-5-(dibutylamino)cyclopentyl)methanol (4.21)



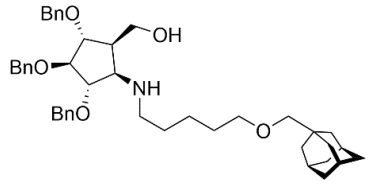
To a solution of the free amine (288 mg, 0.666 mmol, 1 equiv) in DCE (15 mL) was added butanal (191.8 mg, 2.66 mmol, 4 equiv). After 10 min, sodium triacetoxyborohydride (705 mg, 3.33 mmol, 5 equiv) was added to the mixture, and the reaction was stirred at room temperature for 15 h. After the consumption of the starting material, monitored by TLC, the reaction was quenched with a saturated sodium bicarbonate solution and extracted with CH_2Cl_2 (2 x 50 mL). The organic layers were combined, dried over $MgSO_4$, and concentrated in vacuo. The crude product was purified by flash column chromatography on silica gel, using 5% MeOH/CH_2Cl_2 + 1% NH_4OH as the eluent. Product was isolated as a colorless oil (119.2 mg, 33%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.38 – 7.25 (m, 15H), 4.68 (t, J = 11.9 Hz, 2H), 4.62 – 4.52 (m, 4H), 4.15 (dd, J_1 = 6.0 Hz, J_2 = 8.3 Hz, 1H), 3.99 (t, J = 5.4 Hz, 1H), 3.8 (m, 2H), 3.56 (t, J = 5.2 Hz, 1H), 3.51 (t, J = 8.6 Hz, 1H), 2.60 – 2.53 (m, 5H), 1.57 (b, 1H), 1.48 – 1.42 (m, 4H), 1.27 – 1.21 (m, 4H), 0.89 (t, J = 7.0 Hz, 6H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 138.0, 128.5, 128.4, 128.35, 128.30, 128.2, 127.9, 127.83, 127.77, 127.6, 89.3, 88.5, 87.6, 83.0, 82.8, 81.2, 77.2, 72.3, 71.8, 71.1, 68.1, 65.2, 63.4, 63.1, 52.5, 46.9, 44.7, 35.0, 33.4, 31.7, 29.7, 20.7, 19.2, 14.12, 14.05.; ESI-HRMS: calc'd m/e for [M^+] $C_{35}H_{47}NO_4$: 545.3505, found 545.3495. $[\alpha]_D^{25}$ +50 (*c* 0.100, MeOH).

(1R,2S,3S,4R,5R)-2,3,4-tris(Benzyloxy)-5-((benzyloxy)methyl)-N,N-dinonylcyclopentan-1-amine (4.22)



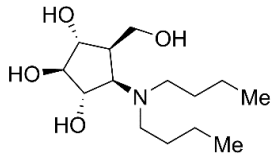
To a solution of the free amine (287 mg, 0.666 mmol, 1 equiv) in DCE (15 mL) was added nonanal (472.7 mg, 3.32 mmol, 5 equiv). After 10 min, sodium triacetoxyborohydride (844 mg, 3.98 mmol, 6 equiv) was added to the mixture, and the reaction was stirred at room temperature for 15 h. After the consumption of the starting material, monitored by TLC, the reaction was quenched with a saturated sodium bicarbonate solution and extracted with CH_2Cl_2 (2 x 50 mL). The organic layers were combined, dried over $MgSO_4$, and concentrated in vacuo. The crude product was purified by flash column chromatography on silica gel, using 5% MeOH/CH_2Cl_2 + 1% NH_4OH as the eluent. Product was isolated as a colorless oil (157 mg, 39%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.37 – 7.25 (m, 15H), 5.62 (b, 1H), 4.69 (d, *J* = 11.2 Hz, 1H), 4.64 (t, *J* = 11.9 Hz, 1H), 4.61 – 4.52 (m, 4H), 4.14 (t, *J* = 7.6 Hz, 1H), 3.99 (t, *J* = 5.4 Hz, 1H), 3.80 – 3.70 (m, 2H), 3.56 (t, *J* = 5.2 Hz, 1H), 3.50 (t, *J* = 8.6 Hz, 1H), 2.6 – 2.49 (m, 5H), 1.50 – 1.40 (m, 4H), 1.31 – 1.17 (m, 24H), 0.88 (t, *J* = 6.5 Hz, 6H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 138.27, 138.26, 138.1, 128.55, 128.54, 128.52, 128.51, 128.39, 128.38, 127.99, 127.98, 127.92, 127.89, 127.88, 127.87, 127.86, 127.85, 127.7, 89.4, 83.1, 83.06, 83.05, 72.4, 71.91, 71.88, 65.3, 63.4, 63.0, 52.80, 52.79, 44.8, 32.93, 32.92, 32.0, 29.69, 29.67, 29.56, 29.55, 29.38, 29.37, 27.6, 27.5, 25.9, 22.8, 14.2; ESI-HRMS: calc'd m/e for [M+H$^+$] $C_{45}H_{67}NO_4$:686.5143, found 686.5135. $[\alpha]_D^{25}$ +62 (*c* 0.81, MeOH).

((1R,2R,3S,4S,5R)-2-((6-(Adamantan-1-ylmethoxy)hexyl)amino)-3,4,5-tris(benzyloxy)cyclopentyl)methanol (4.23)



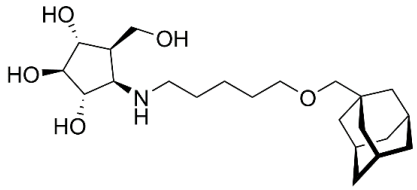
To a solution of the free amine (200 mg, 0.463 mmol, 1 equiv) in MeOH (15 mL) was added aldehyde **4.20** (130 mg, 0.522 mmol, 1.12 equiv). After 10 min, sodium triacetoxyborohydride (294 mg, 1.39 mmol, 3 equiv) was added to the mixture, and the reaction was stirred at room temperature for 15 h. After the consumption of the starting material, monitored by TLC, the reaction was quenched with a saturated sodium bicarbonate solution and extracted with CH_2Cl_2 (2 x 50 mL). The organic layers were combined, dried over $MgSO_4$, and concentrated in vacuo. The crude product was purified by flash column chromatography on silica gel, using 5% MeOH/CH_2Cl_2 + 1% NH_4OH as the eluent. Product was isolated as a colorless oil (119 mg, 33%). %). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.38 – 7.27 (m, 15H), 4.69 (d, J_1 = 12 Hz, 1H), 4.63 – 4.50 (m, 5H), 3.97 (t, J_1 = 4.5 Hz, 1H), 3.85 – 3.94 (m, 4H), 3.34 (t, J_1 = 6.5 Hz, 2H), 3.24 (t, J_1 = 7.0 Hz, 1H), 2.94 (s, 2H), 2.59 (t, J_1 = 6.9 Hz, 2H), 2.36 (dd, J_1 = 5.9 Hz, J_2 = 12 Hz, 1H), 1.95 (bs, 3H), 1.74 – 1.61 (m, 7H), 1.60 – 1.25 (m, 14H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 137.5, 128.6, 128.6, 127.8, 127.4, 86.5, 82.8, 81.8, 78.5, 73.3, 73, 71.3, 57.5, 48.3, 46.4, 40, 36.8, 34, 31.6, 30.8, 30.2, 28.4, 27, 25.6; ESI-HRMS: calc'd m/e for [M+H$^+$] $C_{44}H_{59}NO_5$: 681.4393, found 681.4400.

(1R,2S,3S,4R,5R)-4-(Dibutylamino)-5-(hydroxymethyl)cyclopentane-1,2,3-triol (4.24).



A mixture of the tri-benzyl precursor (119 mg, 0.22 mmol, 1 equiv), ammonium formate (138 mg, 1.94 mmol, 10 equiv) and 10% palladium on carbon (0.3 g per mmol of *O*-benzyl group) was refluxed in MeOH in a sealed reaction vessel for 5 h. Thereafter, the catalyst was filtered off by passing the reaction mixture through a Celite pad. The solvent was subsequently removed under vacuum. The neutral residue was purified by flash column chromatography on silica gel, using 10% MeOH/ CH_2Cl_2 + 1% NH_4OH (v/v) as the eluent. Compound was obtained as a colorless oil (39.5 mg, 51%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 5.05 (br, 4H), 4.01 (m, 1H), 3.85 (m, 1H), 3.69 (m, 3H), 3.32 (t, J = 9.25 Hz, 1H), 2.61 (m, 4H), 2.26 (b, 1H), 1.48 (m, 4H), 1.29 (m, 4H), 0.92 (t, J = 7.15 Hz, 6H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 81.5, 75.5, 75.4, 64.4, 62.4, 52.8, 45.2, 29.7, 20.6, 14.1; ESI-HRMS: calc'd m/e for [M+H$^+$] $C_{14}H_{30}NO_4$: 276.2169, found 276.2180. $[\alpha]_D^{25}$ +18 (*c* 0.36, MeOH).

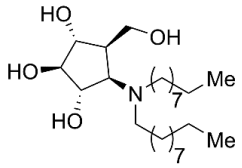
(1*R*,2*S*,3*S*,4*R*,5*R*)-4-((5-((Adamantan-1-yl)methoxy)pentyl)amino)-5-(hydroxymethyl)cyclopentane-1,2,3-triol (4.25).



A mixture of the tri-benzyl precursor (129.7 mg, 0.194 mmol, 1 equiv), ammonium formate (122 mg, 1.94 mmol, 10 equiv) and 10% palladium on carbon (0.3 g per mmol of *O*-benzyl

group) was refluxed in MeOH in a sealed reaction vessel for 5 h. Thereafter, the catalyst was filtered off by passing the reaction mixture through a Celite pad. The solvent was subsequently removed under vacuum. The neutral residue was purified by flash column chromatography on silica gel, using 10% MeOH/ CH_2Cl_2 + 1% NH_4OH (v/v) as the eluent. Compound was obtained as a colorless oil (39.5 mg, 51%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 3.97 (t, *J* = 7.3 Hz, 1H), 3.88 (d, *J* = 10.9 Hz, 1H), 3.73 (q, *J* = 8.2 Hz, 2H), 3.65 (t, *J* = 8.1 Hz, 1H), 3.37 (t, *J* = 6.5 Hz, 2H), 3.11 (t, *J* = 8.7 Hz, 1H), 2.95 (s, 2H), 2.66 (m, 2H), 2.05 (m, 1H), 1.96 (m, 3H), 1.68 (app. q, 6H), 1.54 (m, 10H), 1.36 (m, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 82.0, 81.0, 75.1, 71.5, 61.9, 49.8, 45.0, 39.8, 37.3, 34.1, 30.0, 29.5, 28.3, 23.9.; ESI-HRMS: calc'd m/e for [M+H$^+$] $C_{22}H_{40}NO_5$: 398.2901, found 398.2912;$[a]\frac{25}{D}$ +11 (*c* 0.46, MeOH).

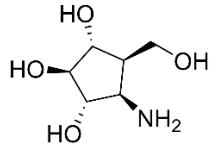
(1R,2S,3S,4R,5R)-4-(Dinonylamino)-5-(hydroxymethyl)cyclopentane-1,2,3-triol (4.26)



A fask, fitted with a dry ice condenser, was charged with anhydrous liquid ammonia (8.0 mL) and then an amount of lithium was added piecewise so as to cause to persist the blue color within the flask. A solutin of tri-benzyl compound (0.157 g, 0.229 mmol, 1 equiv) was dissolved in THF and added dropwise into the flask over 5 min. After an additional 10 min, ammonium chloride was added to the solution until the blue color faded. The dry ice condenser was removed and the ammonia was allowed to evaporate. The residue was extracted with EtOAc (2 X 20 mL). The organic layers were combined, dried over Mg_2SO_4

and concentrated en vacuo. The crude product was purified by flash column chromatography on silica gel with 10% MeOH/DCM + 1% NH_4OH (v/v) as the eluent. After chromatographic purification, a colorless, viscous oil was obtained (88 mg, 92%). ^1H NMR ($CDCl_3$, 400 MHz): δ 4.90 (b, 2H), 3.98 (t, J = 7.8 Hz, 1H), 3.85 (dd, J_1 = 4.8 Hz, J_2 = 11.7 Hz, 1H), 3.7 (p, J = 8.1 Hz, 2H), 3.64 (dd, J_1 = 6.3 Hz, J_2 = 12 Hz 1H), 3.28 (t, J = 10 Hz, 1H), 2.56 (t, J = 7.6 Hz, 4H), 2.22 (m, 1H), 1.48 (m, 4H), 1.27 (m, 26H), 0.88 (t, J = 6.3 Hz, 6H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 81.5, 75.7, 75.5, 64.3, 62.5, 53.0, 45.4, 31.9, 29.7, 29.4, 27.6, 22.7, 14.1; ESI-HRMS: calc'd m/e for $[M+H^+]$ $C_{24}H_{50}NO_4$: 416.3734, found 416.3783. $[\alpha]_D^{25}$ +23 (*c* 1.00, MeOH).

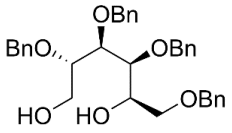
(1*R*,2*S*,3*S*,4*R*,5*R*)-4-Amino-5-(hydroxymethyl)cyclopentane-1,2,3-triol (4.27).



A mixture of the tribenzyl precursor (0.155 g, 0.358 mmol, 1 equiv), ammonium formate (225 mg, 3.58 mmol, 10 equiv) and 10% palladium on carbon (0.3 g per mmol of *O*-benzyl group, 430 mg) was refluxed in MeOH in a sealed reaction vessel for 3 h. Thereafter, the catalyst was filtered off by passing the reaction mixture through a Celite pad. The solvent was subsequently removed under vacuum, yielding the product as a tan solid. This solid was taken up in MeOH and shaken with MP-TMT resin (Biotage, cat#801469, 0.3 g per mmol of Pd used in reaction) for 24 hr. After this, the contents of the vial were filtered through a celite pad and the solvent was removed under vacuum to afford an off-white solid of suitable purity to carry on to the final step (50.6 mg, 0.31 mmol, 87%). ^1H NMR

(CD_3OD, 400 MHz) 3.83 – 3.77 (ddd, J_1 = 3.9 Hz, J_2 = 11 Hz, 2H), 3.76 – 3.71 (m, 2H), 3.62 (t, J = 7.8 Hz, 1H), 3.43 (t, J = 9.2 Hz, 1H), 2.18 – 2.11 (m, 1H); ^{13}C NMR (CD_3OD, 100 MHz) δ 81.8, 79.2, 75.8, 59.9, 56.3, 45.9; ESI-HRMS: calc'd m/e for [M^+] $C_6H_{13}NO_4$: 163.0845, found 163.0865; $[a]\frac{25}{D}$ +8 (c 0.61, MeOH).

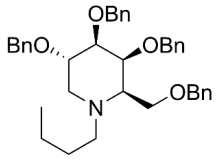
(2S,3R,4S,5R)-2,3,4,6-tetrakis(Benzyloxy)hexane-1,5-diol (4.29).



To a 0 °C solution of commercially available 2,3,4,6-tetra-*O*-benzyl-α-galactopyranose (5.25 g, 9.71 mmol, 1 equiv) in anhydrous THF (150 mL), $LiAlH_4$ (1.25 g, 33 mmol, 3.43 equiv) was added carefully in small portions. The mixture was stirred overnight at room temperature and then cooled to 0 °C. After the excess of $LiAlH_4$ was destroyed by the careful addition of ethyl acetate (20 mL), additional ethyl acetate (500 mL) was added. Then 2 N aq HCl (250 mL) was added and the reaction mixture was stirred for 10 minutes. The organic layer was separated, washed successively with sat. aq. $NaHCO_3$ (150 mL), dried with Na_2SO_4, and evaporated affording 4.22 g (80%) of **1.19** as a colorless viscous syrup.

^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.27 (m, 20H), 4.6 (m, 8H), 4.02 (m, 1H), 3.89 (dd, J = 3.6, 6.2 Hz, 1H), 3.75 (m, 3H), 3.63 (d, J = 3.8 Hz, 2H), 3.55 (dd, J = 4.5, 11.9 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 138.27, 138.01, 128.59, 128.28, 128.14, 128.06, 127.98, 127.09, 79.62, 79.22, 77.47, 74.66, 73.60, 73.41, 73.23, 71.27, 70.84, 61.95. $[\alpha]\frac{25}{D}$ +14.7 (c = 1.12, $CHCl_3$). ESI-HRMS: calc'd m/z for [M+H^+] $C_{22}H_{40}NO_5$: 543.2741, found 543.2771.

(2R,3S,4R,5S)-3,4,5-tris(Benzyloxy)-2-((benzyloxy)methyl)-1-butylpiperidine (4.30).

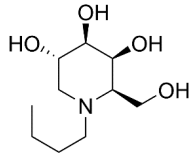


To a -78 °C mixture of dry CH_2Cl_2 (12 mL) and anhydrous DMSO (3.65 g, 3.32 mL, 46.9 mmol, 6 equiv) under an inert gas atmosphere was added dropwise a solution of oxalyl chloride (4.04 g, 2.73 mL, 31 mmol, 4 equiv) in CH_2Cl_2 (25 mL) over 15 min. After the mixture was stirred for 1.5 h at -78 °C, a solution of **1.19** (4.22 g, 7.78 mmol, 1 equiv) in CH_2Cl_2 (15 mL) was added dropwise while maintaining the temperature of the reaction mixture below -78 °C during the addition. The mixture was stirred for an additional 2 h at –78°C and then a solution of Et_3N (6.29 g, 8.68 mL, 8 equiv) in CH_2Cl_2 (10 mL) was added slowly dropwise at -78 °C. After that, the mixture was allowed to warm to room temperature and the solvents were removed under reduced pressure at 40 °C. The residue containing the crude ketoaldehyde **2** was used in the next step without purification.

The ketoaldehyde was dissolved in anhydrous methanol (150 mL) and then powdered 4 Å molecular sieves (0.3g per mmol limiting reagent) were added. A solution of n-butylamine (1.71 g, 2.31 mL, 23.3 mmol, 3 equiv) in anhydrous methanol (25 mL) was added, followed by sodium cyanoborohydride (1.46 g, 23.34 mmol, 3 equiv). *The pH should be maintained below 7 during this reaction. If needed, acetic acid is added to the mix*. After the mixture was stirred at 50 °C overnight, the mixture was made alkaline via addition of 1.0 M NaOH. The mixture was filtered through Celite and the filtrate was diluted with water (50 mL), extracted twice with CH_2Cl_2 (100 mL) and dried over Na_2SO_4. Silica gel column chromatography, employing hexanes/ethyl acetate (80:20) furnished

1.21 as a pale yellow solid (696 mg, 15% over two steps). ^{1}H NMR (400 MHz, $CDCl_3$) δ = 7.3 (m, 20H), 4.95 (d, *J* = 11.1 Hz, 1H), 4.87 (d, *J* = 10.9 Hz, 1H), 4.80 (d, *J* = 11.1 Hz, 1H), 4.68 (d, *J* = 11.6 Hz, 1H), 4.64 (d, *J* = 11.6 Hz, 1H), 4.48 (d, *J* = 12.3 Hz, 1H), 4.45 (d, *J* = 12.3 Hz, 1H), 4.42 (d, *J* = 10.9 Hz, 1H), 3.60 (m, 4H), 3.45 (t, *J* = 9.0 Hz, 1H), 3.10 (dd, *J* = 4.8, 11.1 Hz, 1H), 2.60 (m, 2H), 2.30 (d, *J* = 9.5Hz, 1H), 2.25 (t, *J* = 10.8 Hz, 1H), 1.35 (m, 2H), 1.19 (m, 2H), 0.86 (t, *J* = 7.2 Hz, 3H), ^{13}C NMR (100 MHz, $CDCl_3$): δ = 138.24, 138.79, 138.00, 128.59, 128.53, 128.48, 128.02, 127.78, 127.67, 127.57, 87.57, 78.81, 78.77, 75.46, 75.34, 73.64, 72.91, 63.95, 54.66, 52.29, 25.92, 20.84, 14.71. $[\alpha]\frac{25}{D}$ -14.0 (c = 1.23, $CHCl_3$). ESI-HRMS: calc'd m/z for $[M+H^+]$ $C_{38}H_{45}NO_4$: 580.3421, found 580.3341.

***N*-Butyl-1-deoxynojirimycin (4.31).**



To a solution containing **4.30** (696 mg, 1.2 mmol, 1 equiv) dissolved in methanol (50 mL) and H_2O (15 mL) was added palladium chloride (851 mg, 4.8 mmol, 1.1 equiv per benzyl group). The reaction was stirred under hydrogen gas until the uptake of hydrogen stopped. The reaction mixture was filtered through Celite and solvent was removed *en vacuo*. The crude product was purified using flash column chromatography (10% MeOH in DCM) to afford 248 mg (94%) of target compound. Mp = 129-130 °C Optical rotation $[\alpha]_D^{25}$ -15 (*c* = 1.23, H_2O). ^{1}H NMR (D_2O, 400 MHz): δ 3.91 (dd, *J* = 2.3, 12.8 Hz, 1H), 3.83 (dd, *J* = 2.7, 12.8 Hz, 1H), 3.54 (ddd, *J* = 4.9, 10.2, 14.3 Hz, 1H), 3.38 (d,d, *J* = 9.4 Hz, 1H), 3.25

(d,d, J = 9.3 Hz, 1H), 3.03 (dd, J = 5.0, 11.4 Hz, 1H), 2.74 (m, 1H), 2.60 (m, 1H), 2.30 (d, J = 11.1 Hz, 1H), 2.24 (dd, J = 2.7, 12.5 Hz, 1H), 1.46 (m, 2H), 1.28 (m, 2H), 0.90 (t, J = 7.3 Hz, 3H). ^{13}C NMR (100 MHz, D_2O): δ 74.7, 70.0, 66.7, 62.6, 60.2, 55.5, 52.1, 24.8, 20.0, 13.1; ESI-HRMS: calc'd m/z for $[M+H^+]$ $C_{38}H_{45}NO_4$: 219.1471, found 220.1521. HRMS calc'd for $C_{10}H_{22}NO_4$ $(M+1)^+$; found 220.1521.

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