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Synthesis and evaluation of xanomeline analogs—Probing the wash-resistant phenomenon at the M_1 muscarinic acetylcholine receptor

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Abstract—A series of xanomeline analogs were synthesized and evaluated for binding at the M_1 muscarinic acetylcholine receptor (M_1 receptor). Specifically, compounds that substitute the *O*-hexyl chain of xanomeline with polar, ionizable, or conformationally restricted moieties were assessed for their ability to bind to the M_1 receptor in a wash-resistant manner (persistent binding). From our screen, several novel ligands that persistently bind to the M_1 receptor with greater affinity than xanomeline were discovered. Results indicate that persistent binding may arise not only from hydrophobic interactions but also from ionic interactions with a secondary M_1 receptor binding site. Herein, a qualitative model that accounts for both binding scenarios is proposed and applied to understand the structural basis to wash-resistant binding and long-acting effects of xanomeline-based compounds.

1. Introduction

Alzheimer's disease (AD) is the most common cause of dementia in the elderly, afflicting approximately 10% of those over the age of 65, and 47% of those over age 84.[1] With an aging population, the number of AD diagnoses is expected to triple from 4.5 million Americans diagnosed in 2000 to approximately 13 million by the year 2050.[2] Considering these staggering statistics, there is a tremendous need for therapeutics that can delay the onset of AD or ameliorate its symptoms.

One of the many proposed therapeutic options to treat AD is through the use of muscarinic receptor agonists.[3] Based upon the observation that M_1 receptors involved in learning and memory are largely conserved in AD, it has been speculated that pharmacologically selective M_1 receptor agonists would be viable therapeutics.[3] As such, several M_1 receptor agonists have been taken to the clinic. One of these compounds, xanomeline (Fig. 1), increased cognitive function of patients affected with AD (as measured by an assessment of memory, language, and motor skills).[4] Despite these promising results, xanomeline was ultimately discontinued in Phase III trials due to side effects including salivation, nausea, and diarrhea.[5]

At approximately the same time that xanomeline was discontinued, it was serendipitously discovered that it binds to the M_1 receptor in a wash-resistant manner.[6] Specifically, preincubation of Chinese hamster ovary (CHO) cells overexpressing the M_1 receptor with xanomeline followed by extensive washing resulted in persistent receptor activation.[6,7] This wash-resistant activation, as measured by elevated basal levels of both nNOS and phosphoinositide hydrolysis, can be silenced by the addition of the non-selective muscarinic antago-

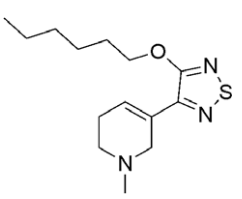

Figure 1. Structure of xanomeline.

Keywords: Xanomeline; Alzheimer's disease; Muscarinic; Plasma-lemma diffusion microkinetic model; Long-acting; Drug design; M_1 receptor; Wash-resistant binding.
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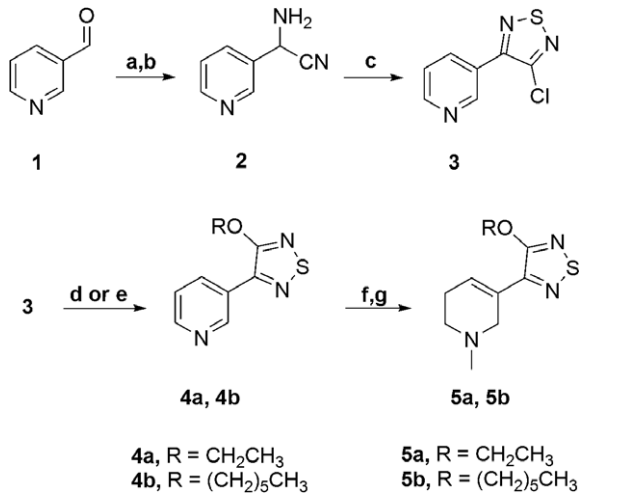
nist atropine.[6,7] If atropine is washed off, reactivation occurs. In order to further examine these unique properties, several other studies have been conducted.[7–12] Saturation binding experiments in cells preincubated with xanomeline followed by washing off free ligand exhibited reduced affinity for [^{3}H] *N*-methyl scopolamine (NMS) and a reduced receptor number.[7] As the concentration of xanomeline was increased, the observed effects on both the K_d and B_{max} of [^{3}H]NMS reached a limit.[7] Collectively, these data have led to the assertion that xanomeline wash-resistant binding occurs at an allosteric 'exosite' in a manner that is not mutually exclusive with atropine.[12]

More recent studies have examined how modification of the hydrophobic tail of xanomeline affects wash-resistant binding.[11] These studies modified the hexyl chain to shorter alkyl groups in order to assess if non-specific hydrophobic interactions are the impetus for wash-resistant binding. In summary, it was found that decreasing the length of the hydrophobic chain to a propyl or methyl group abolishes the wash-resistant binding.[11] Although these results point toward a hydrophobic site of interaction, it is not clear if the interaction is in the receptor, on the receptor surface, or in or on the membrane. Moreover, the data is far from exhaustive and does not rule out the existence of a pharmacophore specific exosite.

To date, there have been no studies that examine where this potential binding site (specific or non-specific) may be located. Studies involving reconstituted purified M_1 receptors implicate both the receptor and the membrane lipids in xanomeline wash-resistant binding.[11] In an effort to further define the structural requirements and potential location of this binding site, we have synthesized a series of xanomeline derivatives that replace the hydrophobic *O*-hexyl chain with polar and ionizable functional groups. In addition, we have synthesized several molecules that have been shown to have functional activity at the M_1 receptor.[13] The ligands were subsequently tested for their ability to bind reversibly and in a wash-resistant manner to the M_1 receptor. Experiments were also performed by varying the pH to evaluate the effect ionization has on both binding affinity and wash-resistant binding. The results are somewhat surprising and suggest a correlation exists between ligand structure and function that goes beyond simple hydrophobic effects in mediating wash-resistant binding. The data are further applied to develop a preliminary model that accounts for these unique phenomena through the existence of a secondary receptor exosite.

2. Chemistry

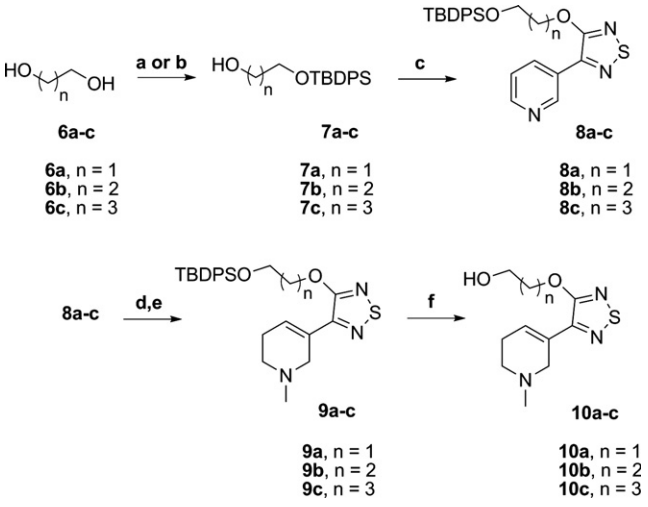
Xanomeline and its analogs were synthesized largely according to previously published procedures (Scheme 1).[13–15] Sequential addition of potassium cyanide and acetic acid to the commercially available 3-pyridine carboxyaldehyde (**1**) afforded 2-hydroxy-2-(3-pyridyl)acetonitrile. Further reaction with ammonium chloride in ammonium hydroxide led to 2-amino-2-(3-pyridyl)acetonitrile (**2**). Reaction of **2** with sulfur monochloride in DMF afforded 3-chloro-4-(3-pyridyl)-1,2,5-thiadiazole (**3**), a key intermediate for the synthesis of the majority of our derivatives.



Scheme 1. Synthesis of alkyl derivatives.[14] Reagents and conditions: (a) KCN, H_2O, AcOH, rt, 7.5 h; (b) NH_4Cl, H_2O/NH_4OH, rt, 18 h; (c) S_2Cl_2, DMF, 0 °C, 2h; (d) Na/ROH, 50 °C, 3 h; (e) ROH, NaH, THF, reflux, 18 h; (f) CH_3I, acetone, rt, 18 h; (g) $NaBH_4$, EtOH, 0 °C, 1 h.

The ethyl derivative of xanomeline was synthesized by stirring **3** in a solution of sodium ethoxide.[13] The resulting pyridine derivative (**4a**) was subsequently converted to its corresponding pyridinium salt via iodomethane in acetone. Treatment with $NaBH_4$ in ethanol (0 °C for 1 h) afforded 1,2,5,6-tetrahydropyridine **5a**. Xanomeline (**5b**), the hexyl derivative, was synthesized utilizing NaH and 1-hexanol in THF.[15] Subsequent addition of intermediate **3**, followed by refluxing for 18 h, led to **4b** in 83% yield. Similar to **4a**, this pyridine derivative was methylated and reduced to afford the 1,2,5,6-tetrahydropyridine product (**5b**).

A series of hydroxyl derivatives were synthesized starting from monoprotected diols (Scheme 2). Compound



Scheme 2. Synthesis of hydroxyl derivatives. Reagents and conditions: (a) TBDPSCl, DMF/DIEA, rt, 12 h; (b) TBDPSCl, DIEA, CH_2Cl_2, rt, 2 h; (c) NaH, **3**, THF, reflux, 18 h; (d) CH_3I, acetone, rt, 18 h; (e) $NaBH_4$, EtOH, reflux, 12 h; (f) 1.0 M TBAF/THF, 0 °C to rt, 3 h.

6a was monoprotected via dropwise addition of 1 equiv of *tert*-butylchlorodiphenylsilane (TBDPSCl) into a solution of ethylene glycol in DMF/DIEA.[16] The other two diols, 1,3-propanediol and 1,4-butanediol, were monoprotected according to a procedure reported by Freeman et al.[17] In short, 2 equiv of the diol, 1 equiv of DIEA, and 1 equiv of TBDPSCl were stirred in CH_2Cl_2. In all cases, silica gel column chromatography afforded the monoprotected product. Similar to the reaction that yielded **4b** (NaH in THF), the monoprotected alcohols (**7a**–**c**) were reacted with intermediate **3** to afford **8a**–**c**. Again, the methyl pyridinium salt was obtained by treatment with iodomethane in acetone. Reduction with $NaBH_4$ in EtOH at 0 °C for 1 h did not result in sufficient reduction of the pyridinium salt. Therefore, reaction temperature and time were increased until considerable amounts of **9a**–**c** could be obtained. Finally, deprotection of the TBDPS group with 1.0 M tetrabutylammonium fluoride (TBAF) in THF afforded compounds **10a**–**c**.

Important to note in this series of reactions is that the purity of **8a**–**c** can be increased via a work-up procedure consisting of an acid wash. Specifically, addition of a citric acid (pH = 4) solution to the CH_2Cl_2 layer results in intermediate **3** partitioning into the aqueous layer, leaving **8a**–**c** and the unreacted alcohol **7a**–**c** largely in the organic layer. Although this work-up procedure results in slightly lower yields (due to minor amounts of **8a**–**c** lost in the acidic aqueous layer), it is deemed worthwhile because it often eliminated the need for column chromatography.

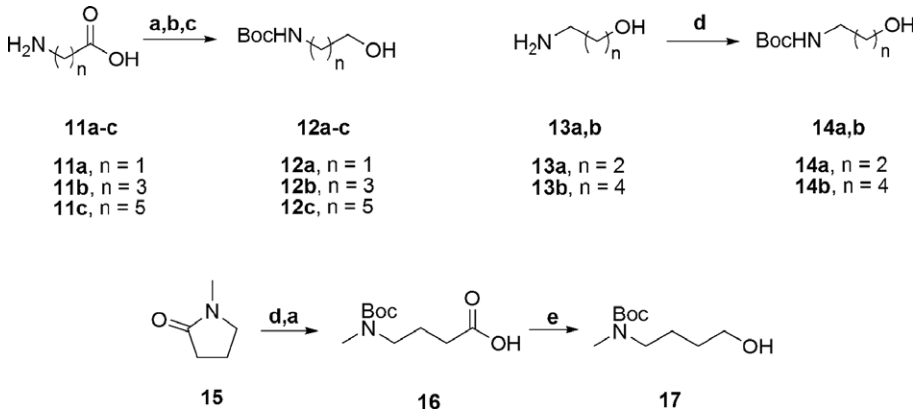
Amino derivatives were also synthesized in order to determine what affect, if any, a second positive charge has in the wash-resistant binding of xanomeline. These derivatives were synthesized from *N*-*tert*-butoxycarbonyl-protected amino alcohols, derived from commercially available amino acids or amino alcohols (Scheme 3). Using a published method, glycine (**11a**), 4-aminobutyric acid (GABA, **11b**), and caproic acid (**11c**) were Boc-protected.[18] Subsequent reaction with isopropylchloroformate or ethylchloroformate afforded a mixed carboxylic-carbonic anhydride, which was subsequently reduced with $NaBH_4$ to yield the desired Boc-amino alcohols (**12a**–**c**).[19,20] The other Boc-protected amino alcohols (**14a**,**14b**) were synthesized from 3-aminopropan-1-ol and 5-amino-pentan-1-ol (**13a** and **13b**). Treatment with di-*tert*-butyl dicarbonate and Et_3N in CH_2Cl_2 afforded compounds **14a** and **14b** in 90% and 99% yield, respectively.[21,22] In addition to these primary amines, a secondary amine, derived from *N*-methyl pyrrolidone (NMP), was synthesized. Acid mediated ring opening,[23] followed by Boc-protection,[18] yielded compound **17**. The carboxylic acid moiety was reduced to the alcohol using BH_3·THF.[24]

The Boc-protected amino alcohols were reacted with intermediate **3** as described earlier. Specifically, deprotonation of the alcohol with 2 equiv of NaH in THF followed by refluxing with intermediate **3** for 18 h afforded compounds **18a**–**e** and **21** (Scheme 4). Methylation and reduction led to the 1,2,5,6-tetrahydropyridine products (**19a**–**e**, **22**). Deprotection of the Boc group using 4.0 M HCl in dioxane or trifluoroacetic acid in CH_2Cl_2 afforded the HCl or TFA salt, respectively.

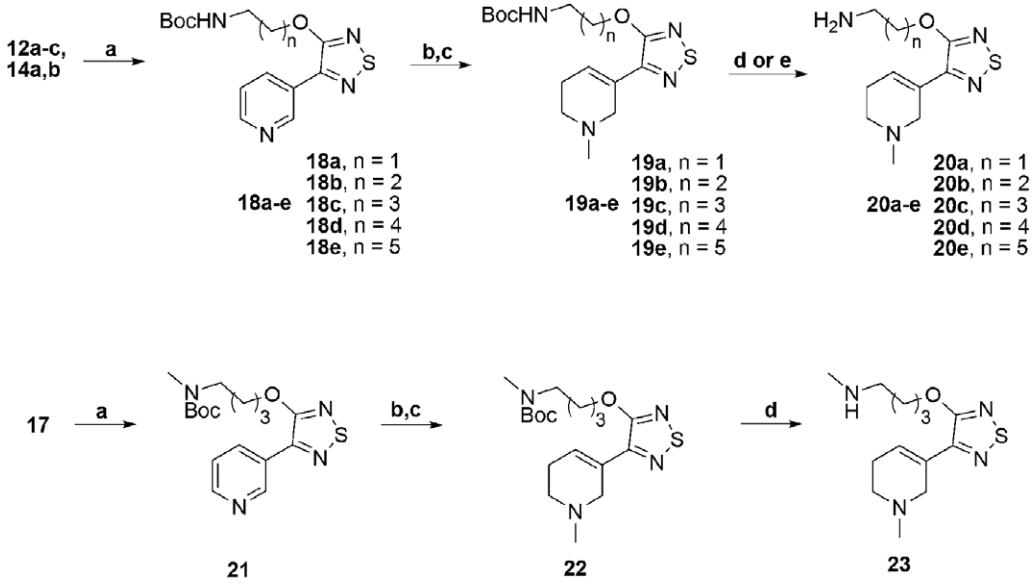
In the above sequence, it is noteworthy the reaction of **3** with 3-(*tert*-butoxycarbonylamino)-1-propanol (**14b**) led to significant amounts of undesired cyclic urethane.[22,25] In order to avoid this and increase the yield of **18b**, it was determined that the reaction mixture should be stirred at room temperature.

In addition to the above compounds, several analogs that incorporate aromatic rings into the side chain were synthesized (Scheme 5). Two such analogs, a 3-phenyl-2-propyn-1-ol and a 4-phenyl-1-butanol derivative, have been shown to possess significant M_1 receptor activity.[13] Starting from the commercially available alcohols, deprotonation with NaH in THF followed by addition of intermediate **3** yielded products **24** and **25**. Subsequent treatment with iodomethane and $NaBH_4$ afforded compounds **26** and **27**.

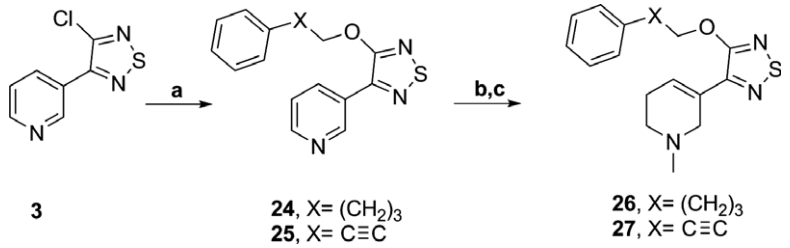
In order to assess the SAR of the phenyl ring of compound **27**, an imidazole and a pyrrole analog were synthesized. Reaction of **10c** with $SOCl_2$ afforded the



Scheme 3. Synthesis of Boc-amino alcohol derivatives. Reagents and conditions: (a) NaOH, dioxane/H_2O, Boc_2O; (b) alkyl chloroformate, THF, 0 °C, 30 min; (c) $NaBH_4$, MeOH; 0 °C to rt, 30 min; (d) $Et_3N/CH_2Cl_2/Boc_2O$, 0 °C to rt, 12 h; (e) concd HCl, reflux, 12 h; (f) BH_3·THF, 0 °C to rt, 12 h.



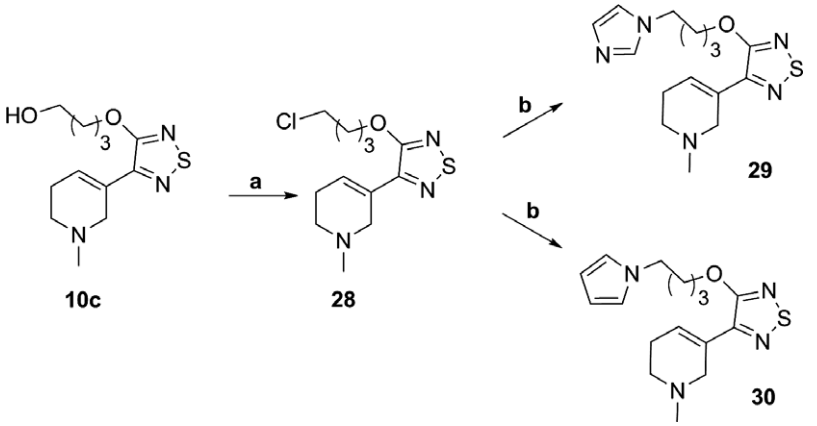
Scheme 4. Synthesis of amino derivatives. Reagents and conditions: (a) NaH, **3**, THF, reflux, 18 h; (b) CH_3I, acetone, rt, 18 h; (c) $NaBH_4$, EtOH, reflux, 12 h; (d) TFA/CH_2Cl_2, rt, 4 h; (e) 4.0 N HCl/dioxane, rt, 4 h.



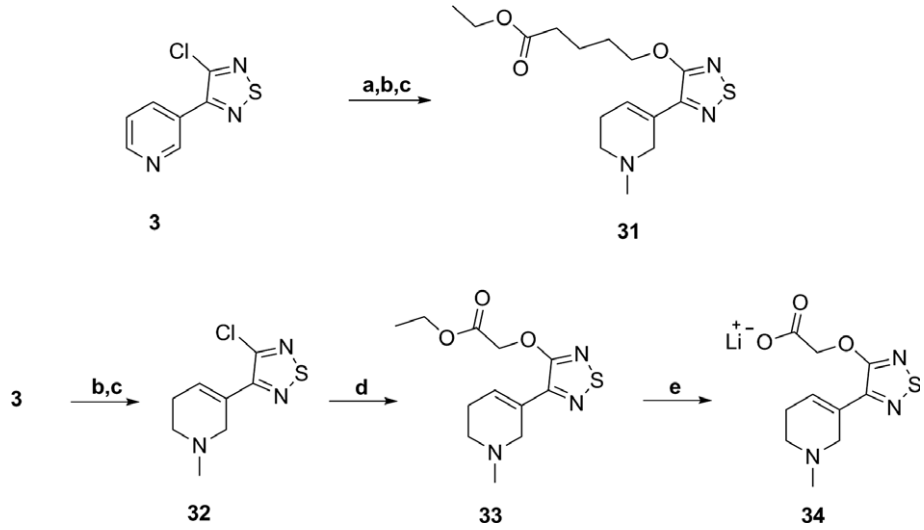
Scheme 5. Synthesis of aromatic derivatives. Reagents and conditions: (a) ROH, NaH, THF, reflux, 18 h; (b) CH_3I, acetone, rt, 18 h; (c) $NaBH_4$, EtOH, reflux, 3 h.

chloro-substituted derivative **28**, which was reacted with NaH and pyrrole or NaH and imidazole in DMF to afford compounds **29** and **30**, respectively (Scheme 6).

The synthesis of several carboxylic acid derivatives was pursued. Reaction of intermediate **3** with the sodium salt of 5-hydroxypentanoic acid followed by treatment with iodomethane and $NaBH_4$ afforded ethyl ester derivative **31**. Glycolic acid derivatives were also synthesized. Using similar reaction conditions as that for the hydroxyl and amino derivatives (NaH in THF), the glycolic ethyl ester was formed. However, in the following reduction step, $NaBH_4$ reduced the ester to primary alcohol **10a**. Attempts with a milder reducing agent, $NaCNBH_3$,



Scheme 6. Synthesis of imidazole and pyrrole derivatives. Reagents and conditions: (a) $SOCl_2$, CH_2Cl_2, 0 °C to rt, 4 h; (b) pyrrole or imidazole, NaH, DMF, reflux, 18 h.



Scheme 7. Synthesis of carboxylic acid derivatives. Reagents and conditions: (a) 5-hydroxypentanoic acid sodium salt, NaH, THF, reflux, 18 h; (b) CH_3I, acetone, rt, 18 h; (c) $NaBH_4$, EtOH, reflux, 12 h; (d) glycolic acid ethyl ester, NaH, THF, reflux, 18 h; (e) LiOH 1:1 H_2O/ACN.

also resulted in the formation of **10a**. Because of this, an alternative synthetic route which involves initially forming 1,2,5,6-tetrahydropyridine **32** was employed. Specifically, **3** treated with iodomethane in acetone yielded the corresponding pyridinium salt, which was subsequently reacted with $NaBH_4$ in ethanol. The resulting 1,2,5,6-tetrahydropyridine compound (**32**) was reacted with glycolic acid ethyl ester and NaH in THF to yield ethyl ester **33**. Finally, saponification with LiOH in 1:1 water/acetonitrile afforded compound **34** (Scheme 7).

3. Results and discussion

Two receptor binding protocols were applied to assess the physical behavior of the *O*-substituted xanomeline analogs. The first protocol, termed 'continuous presence,' is a traditional radioligand displacement assay wherein cells are simultaneously incubated with a fixed concentration of radioligand and various concentrations of the unlabeled analog. After 1 h, the cells are filtered and the amount of radioligand that remains bound is measured. The other protocol, termed the 'wash-resistant paradigm,' also measures how an unlabeled ligand dose-dependently affects the binding of a radioligand. However, in these studies the radioligand is not added until after the cells have been preincubated with the test compound and extensively washed. This washing procedure consists of diluting the incubation mixture with ~50 vol of buffer followed by centrifugation. The resulting pellet is subjected to this procedure (dilution and centrifugation) an additional two times in order to ensure that only irreversible (or pseudo-irreversible) binding remains.[7] The cells are resuspended in buffer and the radioligand [^{3}H] *N*-methylscopolamine ([^{3}H]NMS) is added. Lastly, the incubation mixture is filtered and the residual bound radioactivity is measured. In all cases non-specific binding was assessed in the presence of 10 μM atropine.

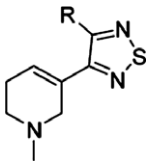
A comparison of the results from these two different binding assays provides valuable information about how the structure of an analog is linked to its differential ability to displace [^{3}H]NMS in the two assays. Since a K_i value for the wash-resistant binding studies cannot be determined (due to non-competitive pseudo-irreversible conditions), it is most fitting to compare pIC_{50} values ($-\log IC_{50}$). In most cases (for the *O*-alkyl analogs tested previously[11] as well as the novel compounds described herein), the difference between these two values is approximately 1.75–2.05 orders of magnitude. Results are summarized in Table 1.

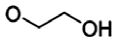
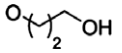
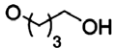
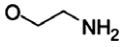
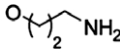
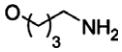
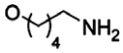
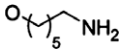
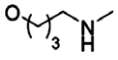
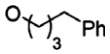
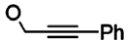
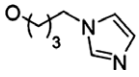
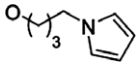
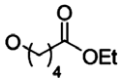
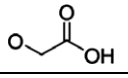
Starting with the *O*-alkyl derivatives of xanomeline, pIC_{50} values are in agreement with similar results obtained in cell membranes.[11] The ethyl derivative (**5a**), which was not evaluated in our previous studies on the *O*-alkyl derivatives, possesses a relatively high pIC_{50} value of 6.85 ± 0.06. Despite this high pIC_{50} value, **5a** did not possess wash-resistant binding. This result is consistent with our previous finding that a long alkyl chain is necessary for such binding.[11]

In addition to the *O*-alkyl derivatives, numerous polar analogs were synthesized. These compounds were designed in order to assess whether moieties that contain the ability to hydrogen bond or form ionic interactions would provide further insight into the mechanism responsible for the wash-resistant binding. The hydroxyl derivatives (**10a**–**c**) did not show a correlation between chain length and pIC_{50} values (pIC_{50} values in the continuous presence binding studies were in the order of hydroxybutyl > hydroxyethyl > hydroxypropyl; 6.51 ± 0.04, 5.97 ± 0.06, and 5.42 ± 0.08, respectively). Additionally, none of these derivatives possess wash-resistant binding.

The amino derivatives also fail to show a correlation between the position of the polar group (the amine) and the pIC_{50} values in the continuous presence experiments. The pIC_{50} values of **20a**–**e** (in order of increasing chain length) were found to be 6.00 ± 0.01, 6.79 ± 0.10, 4.11 ± 0.09, 5.72 ± 0.13, and 6.19 ± 0.09, respectively. In contrast to their hydroxyl counterparts, several

Table 1. Binding data for xanomeline analogs in continuous presence and wash-resistant binding studies



Compound	R	pIC_{50} continuous presence	pIC_{50} = washout conditions
5a		6.85 ± 0.06 (3)	N.B.
LY1		7.28 ± 0.06 (4)	N.B.
LY2		7.55 ± 0.05 (4)	4.11 ± 0.2
LY3		7.48 ± 0.16 (3)	5.07 ± 0.2
5b		7.09 ± 0.1 (3)	5.31 ± 0.04
10a		5.97 ± 0.06 (2)	N.B.
10b		5.42 ± 0.08 (2)	N.B.
10c		6.51 ± 0.04 (2)	N.B.
20a		6.00 ± 0.01 (4)	3.96 ± 0.24
20b		6.79 ± 0.10 (4)	4.83 ± 0.10
20c		4.11 ± 0.09 (4)	N.B.
20d		5.72 ± 0.13 (3)	4.35 ± 0.19
20e		6.19 ± 0.09 (4)	5.40 ± 0.07
23		5.15 ± 0.06 (5)	N.B.
26		6.84 ± 0.08 (3)	5.04 ± 0.05
27		7.43 ± 0.15 (4)	5.67 ± 0.34
29		6.06 ± 0.05 (7)	4.28 ± 0.15
30		6.56 ± 0.07 (3)	4.54 ± 0.26
31		7.23 ± 0.06 (3)	N.B.
34		6.21 ± 0.16 (2)	N.B.

The pIC_{50} values were determined in whole cell binding assays as outlined in Section 5. The number of individual determination is indicated in parentheses (*n*). Since the wash-resistant studies were done in parallel (*n*) is the same for the wash-resistant binding studies. N.B., no binding.

amino derivatives were observed to possess wash-resistant binding. The 6-aminohexyl derivative **20e** possessed excellent wash-resistance, displaying a pIC_{50} value of 5.40 ± 0.07. In a descending order of their pIC_{50} values, compounds **20b**, **20d**, and **20a** possess wash-resistant binding with pIC_{50} = 4.83 ± 0.10, 4.35 ± 0.19, and 3.96 ± ce:hsp sp="0.25"/>0.24, respectively. Interestingly, neither the 4-aminobutyl derivative (**20d**) nor its *N*-methyl analog (**23**) was observed to bind in a wash-resistant manner.

It was also of interest to determine if previously reported xanomeline analogs possess wash-resistant binding. The literature reveals that substitution of the hexyl chain with alkylphenyl or alkynylphenyl moieties affords compounds that maintain potent M_1 receptor activity.[13] Since we had previously reported that alkyl derivatives of butyl or larger possess wash-resistant binding,[11] we chose analogs that contained side chains of similar length for synthesis and evaluation (**26** and **27**). Consistent with previous reports, both **26** and **27** possess high affinity for the M_1 receptor (pIC_{50} = 6.84 ± 0.08 and 7.43 ± 0.15 in the continuous binding assay, respectively). Additionally, both ligands possess significant wash-resistant binding (pIC_{50} = 5.04 ± 0.05 and 5.67 ± 0.34, respectively). This is the first reported result of a xanomeline analog containing a phenyl ring that binds in a wash-resistant manner. Presumably, the exosite does not discriminate against analogs that possess moderate steric bulk.

In addition to xanomeline derivatives containing a phenyl ring, substitution of the hexyl chain with moieties containing heteroaromatic rings has been reported.[26,27] Given our results that several derivatives with dual cationic character maintain wash-resistant binding, we synthesized a potentially ionized imidazole derivative. In the continuous binding assay, imidazole analog **29** possessed moderate affinity for the M_1 receptor (pIC_{50} = 6.06 ± 0.05). The affinity of **29** was further diminished in the wash-resistant binding assay (pIC_{50} = 4.28 ± 0.15). A comparison to the non-ionizable pyrrole derivative **30** reveals similar activity in both the continuous binding assay (pIC_{50} = 6.56 ± 0.07) and the wash-resistant assay (pIC_{50} = 4.54 ± 0.26).

Ester derivative **31** was also tested. In the continuous binding assay, it inhibited the binding of [^{3}H]NMS to a moderate extent (pIC_{50} = 7.23 ± 0.06). In the wash-resistant binding assay, it did not possess a residual inhibitory effect on [^{3}H]NMS binding. Similar results were also observed with carboxylic acid derivative **34**. In the continuous binding assay, a pIC_{50} value of 6.21 ± 0.16 was obtained, and again, no residual wash-resistant binding was observed.

Upon further inspection of Table 1, amino derivatives **20c** and **23** stand out because unlike the other alkylamino derivatives, neither compound binds in a wash-resistant manner. Since the positive charge of both of these molecules is positioned at the same distance from the thiadiazole ring, we hypothesized that the exosite does not interact favorably with a positive charge at

the distance found for the butyl analogs. In order to further explore this hypothesis, pH-dependent studies with imidazole derivative **29** were conducted. It was envisioned that the wash-resistant binding of **29** could be abolished if the imidazole possessed a positive charge. In order to protonate the imidazole, the buffer in the binding studies was acidified to pH = 6.0. In the continuous binding assay, **29** possessed a pIC_{50} value slightly lower than the value obtained at pH = 7.4 ($pIC_{50} = 5.64 \pm 0.10$ at pH = 6.0 vs $pIC_{50} = 6.06 \pm 0.05$ at pH = 7.4, Table 2). Meanwhile, at pH = 8.0, **29** possessed the same pIC_{50} value as that observed under normal binding conditions ($pIC_{50} = 6.06 \pm 0.05$). This suggests that the imidazole moiety is positively charged at pH = 6.0 and neutral at pH = 7.4 and pH = 8.0. At pH = 8.0, **29** maintained a similar pIC_{50} value as that observed in Table 1 (4.27 ± 0.41 vs 4.28 ± 0.15). Remarkably, at pH = 6.0, **29** lost the ability to bind in a wash-resistant manner.

In order to determine if the observed data were due to pH-dependent changes in receptor structure, several control experiments were conducted. Specifically, hexyl derivative **5b** (xanomeline) and pyrrole derivative **30** were tested at both pH = 6.0 and pH = 8.0. In both cases, the binding profiles were consistent with previously determined values at pH = 7.4. Collectively, the data suggest that topological changes in the receptor are not responsible for the lack of wash-resistant binding of **29** (at pH = 6.0). Instead, it appears that the difference in wash-resistant binding is due to alterations of the ionization state of the xanomeline analog.

Also interesting to note from the data in Table 1 is that some analogs display significant variation in the difference between the pIC_{50} values determined in the continuous presence assay versus the pIC_{50} values determined in the wash-resistant binding assay (Table 3). While most of the compounds have a difference ranging from 1.75 to 2.05 log units, amino derivatives **20d** and **20e** fall well below this range (1.37 and 0.79, respectively). The relatively small difference between pIC_{50} values in the two assays found for **20e** is due to both a decreased pIC_{50} value for the M_1 receptor in the continuous binding protocol (compared to xanomeline) and an increased pIC_{50} value (again compared to xanomeline) in the wash-resistant binding studies. This is significant because it suggests that ligand structure can modulate at the site where the ligand interacts. If the chemical moieties responsible for obtaining high wash-resistant binding can be delineated from those that do not, it may be possible to rationally design ligands that bind wash-resistantly.

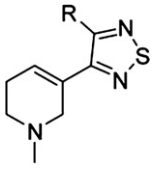
Table 3. Difference between the pIC_{50} values in the two different binding protocols

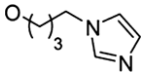
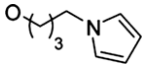
Compound	pIC_{50} (CP) − pIC_{50} (WO)	Compound	pIC_{50} (CP) − pIC_{50} (WO)
LY2	3.44	**20e**	0.79
LY3	2.41	**26**	1.8
5b	1.78	**27**	1.76
20a	2.04	**29**	1.78
20b	1.96	**30**	2.02
20d	1.37		

CP, continuous presence; WO, washout.

Toward this end, it is of great importance to determine how xanomeline elicits its wash-resistant binding. One possible explanation is that it interacts at an allosteric exosite found entirely within the M_1 receptor. Such allosteric sites have been proposed for several other M_1 receptor agonists.[28,29] According to Espinoza-Fonseca and Trujillo-Ferrara,[30] two of these allosteric sites are located in the extracellular loops and the other is located in the intracellular loops. Without chimeric or site-directed mutagenesis data, however, it is difficult to assess whether this hypothesis is well-founded. Wash-resistant phenomena have also been rationalized by the suggestion that some ligands preferentially partition non-specifically into the lipid bilayer. This type of interaction, termed the plasmalemma diffusion microkinetic model, does not fully explain the low affinity of xanomeline for several other neuronal receptors,[31,32] nor does it explain the absence of wash-resistant xanomeline in cells not transfected with the M_1 receptor. A third hypothesis that combines portions of the previous two is that xanomeline binds at an interface of the receptor and the cellular membrane. This hypothesis is supported

Table 2. pH dependence on wash-resistant binding



Compound	R	pIC_{50} ± SEM continuous presence	pIC_{50} ± SEM washout conditions
5b		pH = 6: 7.74 ± 0.13 (2) pH = 8: 7.54 ± 0.51 (2)	pH = 6: 5.62 ± 0.43 pH = 8: 6.02 ± 0.18
29		pH = 6: 5.64 ± 0.10 (5) pH = 8: 6.06 ± 0.05 (5)	pH = 6: N.B. pH = 8: 4.27 ± 0.41
30		pH = 6: 6.39 ± 0.08 (3) pH = 8: 6.66 ± 0.36 (3)	pH = 6: 4.40 ± 0.33 pH = 8: 4.92 ± 0.47

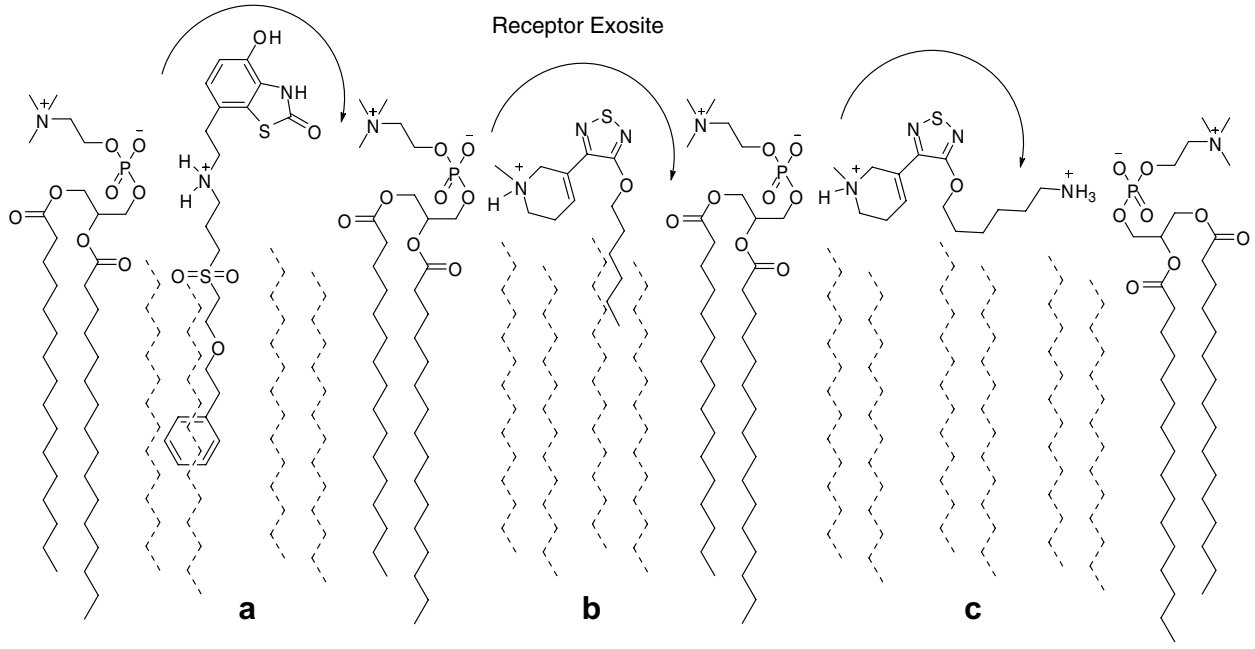


Figure 2. Schematic diagram of (a) benzthiazolone analog of salmeterol, (b) xanomeline (**5b**), and (c) **20e** binding within the cellular membrane. In all three models, the primary pharmacophore is also available for binding to the receptor.

by previous experiments that show that purified soluble M_1 receptors do not exhibit wash-resistant binding, while purified receptors reconstituted into a liposome do.[11] Such a dual interaction has also been speculated for the binding of salmeterol (a wash-resistant D_2/β-adrenergic receptor agonist, Fig. 2a).[33] Austin et al. suggest that salmeterol partitions into the cellular membrane in such a manner that the positive charge interacts with the negatively charged polar head groups while the hydrophobic region of the ligand interacts with the hydrophobic chains contained within the membrane (Fig. 2a).[34,35] Although they do not propose direct interactions of salmeterol with the D_2 or the β-adrenergic receptor, it is reasonable to suggest that the exposed benzthiazolone interacts with structural domains of the receptor at the lipid–protein interface. It is reasonable to propose an analogous mechanism for xanomeline (Fig. 2b). Similar to the benzthiazolone, the thiadiazole acts as a hydrogen bond acceptor for the positively charged head group. Additional interactions are possible between the positively charged 1,2,5,6-tetrahydropyridine ring and the negatively charged phosphate moiety. The results also indicate that the addition of charged groups to the lipophilic tail of xanomeline, such as an amine (as demonstrated by **20e**, Fig. 2c), increases the wash-resistant binding affinity. This lends considerable support to the general hypothesis that wash-resistant binding is conferred through specific interactions, perhaps at single or multiple exosites on the receptor.

4. Conclusions

In this paper, a series of xanomeline analogs were synthesized and tested for their ability to bind in two different binding paradigms. These compounds were specifically designed in order to complement a previous study in which we reported that the hydrophobicity of the *O*-alkyl group of xanomeline was strongly correlated to its wash-resistant binding.[11] The outcome of the current study suggests that wash-resistant binding is not simply related to hydrophobicity but may be a complex phenomena involving one or more exosites on or around the receptor. The data from Table 1 clearly show that wash-resistant binding occurs for charged amino alkyl substituted xanomeline analogs, while no continuous binding is noted for the carboxyl derivative (**34**). It is also significant to note that a couple of the amino derivatives possess pIC_{50} values in the wash-resistant binding assay that are comparable to those of the pIC_{50} value in the continuous presence assays. This lends strong support to the general hypothesis that wash-resistant binding is conferred through specific (as opposed to non-specific) interactions with the receptor, or at the lipid–receptor interface. While the results are provocative, it is important to note that wash-resistant binding is also significant for the *O*-arylalkyls reported here. It is therefore difficult to draw any strong conclusions at this point in time as to the precise nature of this unique physical property or to the structural architecture of the secondary binding site.

In terms of therapeutics, the development of long-acting drugs for the treatment of AD (and anti-psychotics) would be highly advantageous, especially when considering patient compliance in treating mental health. The finding that charged alkyl amino derivatives are active is not trivial. This may be of particular importance since past research has shown that the poor bioavailability and a short plasma half-life (major detriments of the development of xanomeline) are due to the *O*-butyl carboxylic acid metabolite.[36] Although the metabolic pathways of these new compounds have yet to be studied, it is conceivable that this *O*-butyl carboxylic acid

derivative can be avoided. It may therefore be of interest to further evaluate the amino alkyls, as well as the phenyl alkyls reported here for metabolic stability and toxicity, as these derivative may offer new hope in the design of long-acting AD therapeutics.

5. Experimental

5.1. Chemistry

^{1}H NMR spectra were recorded on a Varian Mercury 300 (300 MHz) spectrometer or a Varian 600 (600 MHz) spectrometer. Column chromatography was conducted using silica gel 60 (40–63 μM). Yield determinations were generally based on one experiment and have not been optimized. Several of the pyridine derivatives were not purified to >95% because in the following steps, the impurities are easily removed. In these cases, the experimental yield is determined by calculating the fractional masses from the NMR spectrum of the crude reaction mixture. High-resolution mass spectra (HRMS) were collected from a TOF–ESI Agilent LC–MS and analyzed using the Analyst QS software. Reversed-phase high-performance liquid chromatography (RP-HPLC) was performed on a Beckman Coulter 125S System Gold using an Agilent Zorbax Eclipse XDB-C18 5.0 μM 4.6 × 150 mm column monitoring UV at 210 nm on a 166 detector. The compounds tested in the biological assays were all determined to be of >95% purity based upon two separate chromatography methods. Method A was a 20 min gradient method with a flow rate of 1.0 mL/min. Over the first 15 min, the percentage of ACN/water was increased from 60% to 80%. Over the final 5 min, the gradient was increased to 100% ACN. Method B was a gradient method that started from a 65% MeOH/water with a flow rate of 1.0 mL/min. Here, the percentage of MeOH was increased to 100% over 20 min. Results were analyzed using the 32 Karat software package. Elemental analysis was not performed.

5.1.1. 2-Amino-2-(3-pyridyl)acetonitrile (2). Commercially available 3-pyridinecarboxaldehyde (22.09 g, 0.206 mol) was added dropwise (over a 60 min period) to a stirred solution of potassium cyanide (14.77 g, 0.277 mol) in H_2O (62 mL) at 5 °C. Once addition was complete, 12.8 mL of glacial acetic acid was added over a 30 min period (5 °C). The reaction was stirred at room temperature for 6 h and then cooled to 5 °C, in order to promote precipitation. However, since the product failed to precipitate, the aqueous solution was extracted with EtOAc (4× 200 mL). The combined organic extracts were washed with H_2O (200 mL) and brine (200 mL) and dried over $MgSO_4$. The solvent was concentrated to yield 18.01 g (66%) of the crude cyanohydrin product as a brown oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.58 (d, J = 2.4 Hz, 1H), 8.51 (dd, J = 1.5, 4.8 Hz, 1 H), 7.93 (dt, J = 1.8, 7.8 Hz, 1H), 7.40 (dd, J = 4.5, 7.8 Hz, 1 H). The crude cyanohydrin (16.42 g, 122 mmol) was added to a solution of NH_4Cl (40.10 g, 750 mmol) in H_2O (100 mL) and 29% NH_4OH (10 mL). The reaction mixture was stirred at room temperature for 18 h and extracted with CH_2Cl_2 (5× 100 mL). The combined organics were washed with brine, dried over $MgSO_4$, and concentrated to give 8.12 g (50% yield) of the title compound as a brown oil. This brown oil was carried forward without purification. An analytical sample (>95% purity) was obtained from silica gel column chromatography (4:1 EtOAc/MeOH). Overall yield from **1**: 33%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.82 (d, J = 2.4 Hz, 1 H), 8.67 (dd, J = 1.5, 4.8 Hz, 1H), 7.90–7.94 (m, 1H), 7.39 (dd, J = 4.8, 7.9 Hz, 1H), 5.00 (t, J = 7.5 Hz, 1 H). HRMS (ESI+) m/z: Calcd for $C_7H_8N_3$: 134.0713. Found: 134.0726.

5.1.2. 3-(3-Chloro-1,2,5-thiadiazol-4-yl)pyridine (3). Commercially available (Aldrich) sulfur monochloride (15.01 g, 111 mmol) was added to DMF (15 mL) at 0 °C. To this, a solution of **2** (7.40 g, 55.6 mmol) in DMF (7.5 mL) was added over an hour. After the addition was complete, the reaction mixture was stirred at 0 °C for an additional 45 min, whereupon 30 mL of ice water was added. The resulting precipitate was filtered and the filtrate was chilled to 0 °C. Over the next 5 min, 25 mL of a 9 M NaOH solution was added, resulting in a crystalline product (2.81 g). After filtration, the mother liquor was concentrated to yield a brown oil. This oil was dissolved in CH_2Cl_2, washed with 5% NaOH, brine, and concentrated. Silica gel column chromatography (3:1 hexane/EtOAc) afforded an additional 3.51 g. In total, 6.58 g of a white crystalline solid was obtained. Yield: 58%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.22 (dd, J = 0.9, 2.3 Hz, 1H), 8.73 (dd, J = 1.6, 4.8 Hz, 1H), 8.28 (ddd, J = 1.7, 2.3, 8.0 Hz, 1H), 7.45 (ddd, J = 0.9, 4.9, 8.0 Hz, 1H). HRMS (ESI+) m/z: Calcd for $C_7H_5N_3SCl$: 197.9887. Found: 197.9896.

5.1.3. 3-(3-(Ethoxy)-1,2,5-thiadiazol-4-yl)pyridine (4a). Elemental sodium (0.20 g, 8.7 mol) was slowly added to ethanol (10 mL). After the sodium had completely dissolved, compound **3** (294 mg, 1.49 mmol) in ethanol (5 mL) was added. The reaction was stirred for 3 h at 50 °C and then evaporated. The resulting residue was dissolved in H_2O and extracted with CH_2Cl_2 (3× 50 mL). The combined organics were washed with brine, dried over $MgSO_4$, and concentrated to yield 254 mg of a white solid. Yield: 82%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.42 (d, J = 0.8 Hz, 1H), 8.66 (dd, J = 1.8, 4.7 Hz, 1H), 8.45 (ddd, J = 1.7, 2.3, 8.0 Hz, 1H), 7.40 (ddd, J = 0.8, 4.8, 7.8 Hz, 1H), 4.51 (q, J = 6.2 Hz, 2H), 1.46 (t, J = 6.6 Hz, 3H). HRMS (ESI+) m/z: Calcd for $C_9H_{10}N_3SO$: 208.0539. Found: 208.0536.

5.1.4. 3-(3-(Hexyloxy)-1,2,5-thiadiazol-4-yl)pyridine (4b). A suspension of sodium hydride (447 mg, 11.2 mmol, 60% dispersion in mineral oil) was added to a solution of 1-hexanol (570 mg, 5.58 mmol) in THF (20 mL) at 0 °C. This reaction mixture was refluxed for 1 h and then cooled to 0 °C. After cooling, intermediate **3** (440 mg, 2.23 mmol) in THF (5 mL) was added. The reaction mixture was refluxed for 8 h, cooled, and then concentrated. The residue was taken up in CH_2Cl_2, washed with 5% NaOH, brine, and dried over $MgSO_4$. The or-

ganic layer was concentrated to yield crude **4b** as a white solid (490 mg). Yield: 83%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.41 (d, J = 1.5 Hz, 1H), 8.66 (dd, J = 1.7, 4.8 Hz, 1H), 8.44 (dt, J = 1.8, 7.8 Hz, 1H), 7.40 (ddd, J = 0.7, 4.8, 8.0 Hz, 1H), 4.53 (t, J = 6.6 Hz, 2H), 1.85–1.95 (m, 2H) 1.45–1.62 (m, 2H), 1.32–1.55 (m, 4H), 0.87–0.91 (m, 3H). HRMS (ESI+) *m*/*z*: Calcd for $C_{13}H_{18}N_3SO$: 264.1165. Found: 264.1187.

5.1.5. General procedure for the synthesis of 1,2,5-thiadiazol-4-yl 1,2,5,6-tetrahydro-1-methylpyridines: method A

5.1.5.1. 3-(3-(Ethoxy)-1,2,5-thiadiazol-4-yl)-1,2,5,6-tetrahydro-1-methylpyridine (5a). To a stirred solution of **4a** (252 mg, 1.22 mmol) in acetone (~5 mL) was added iodomethane (346 mg, 2.44 mmol). The reaction mixture was stirred at room temperature for 18 h. The pyridinium salt precipitated from solution and was filtered and washed with cold acetone. To a stirred solution of the pyridinium salt in absolute ethanol (296 mg, 0.85 mmol) at 0 °C was added $NaBH_4$ (136 mg, 3.4 mmol). The mixture was refluxed for 3 h, concentrated, and partitioned between CH_2Cl_2 and water. The aqueous layer was separated and further extracted with CH_2Cl_2 (2× 50 mL). The combined organics were washed with brine, dried over Na_2SO_4, and concentrated to furnish a brown oil. Silica gel column chromatography eluting with EtOAc/MeOH (3:1) afforded **5a** as a brown oil (92 mg). Yield: 33%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.04 (m, 1H), 4.46 (q, J = 7.1 Hz, 2H), 3.42 (d, J = 1.7 Hz, 2H), 2.54 (t, J = 5.6 Hz, 2H), 2.32–2.49 (m, 5H), 1.43 (t, J = 7.1 Hz, 3H). HRMS (ESI+) *m*/*z*: Calcd for $C_{10}H_{16}N_3SO$: 226.1009. Found: 226.1019. It is important to note that in many of the methylation reactions there was little or no precipitation. In these cases, the reaction mixture was concentrated, Et_2O was added, and then concentrated in vacuo. Although this sometimes provided a solid (which was subsequently washed with cold acetone), much of the time an oil resulted. In all cases, the mixture containing the pyridinium salt was carried forward without further purification.

5.1.5.2. 3-(3-(Hexyloxy)-1,2,5-thiadiazol-4-yl)-1,2,5,6-tetrahydro-1-methylpyridine (5b) HCl salt. The title compound was synthesized from **4b** according to method A. The freebase was converted to its hydrochloride salt by bubbling HCl (g) through an ethereal solution. Yield: 40%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.05–7.09 (m, 1H), 4.44 (t, J = 6.6 Hz, 2H), 3.52–3.55 (m, 2H), 2.67 (t, J = 6.0 Hz, 2H), 2.44–2.49 (m, 5H), 1.77–1.87 (m, 2H), 1.30–1.46 (m, 6H), 0.91 (t, J = 6.0 Hz, 3H). HRMS (ESI+) *m*/*z*: Calcd for $C_{14}H_{24}N_3SO$: 282.1635. Found: 282.1651.

5.1.5.3. 2-*tert*-Butyldiphenylsilyloxy-1-ethanol (7a). To a mixture containing ethylene glycol (1.00 g, 16.1 mmol), DMF (48 mL), and DIEA (21 mL) was added TBDPSCl (4.65 g, 16.92 mmol) dropwise. The reaction mixture was stirred for 12 h and was then quenched with cold water (~200 mL), and extracted with Et_2O. The organic layer was separated, successively washed with 1 M HCl, saturated $NaHCO_3$, and brine, and concentrated in vacuo. The residue was purified by silica gel column chromatography eluting with hexane/acetone (3:1) to furnish **7a** (368 mg) as a white solid. Yield: 76%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.70–7.74 (m, 4H), 7.39–7.49 (m, 6H), 3.68–3.82 (m, 4H), 2.34 (t, J = 6.2 Hz, 1H) 1.05 (s, 9H). HRMS (APCI+) *m*/*z*: Calcd for $C_{19}H_{27}O_2Si$: 301.1618. Found: 301.1602.

5.1.5.4. 3-*tert*-Butyldiphenylsilyloxy-1-propanol (7b). To a solution of 1,3-propanediol (4.19 g, 55.0 mmol) and DIEA (10 mL) in CH_2Cl_2 (20 mL) was added TBDPSCl (1.62 g, 5.92 mmol) in CH_2Cl_2 (10 mL) dropwise. The solution was stirred at room temperature for 12 h and then concentrated. Column chromatography (3:1 hexane/EtOAc) of the crude residue afforded a white solid (5.01 g). Yield 88%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.70–7.74 (m, 4H), 7.39–7.49 (m, 6H), 3.68–3.82 (m, 4H), 2.34 (t, J = 6.2 Hz, 1H) 1.05 (s, 9H). HRMS (APCI+) *m*/*z*: Calcd for $C_{20}H_{29}O_2Si$: 315.1775. Found: 315.1506.

5.1.5.5. 4-*tert*-Butyldiphenylsilyloxy-1-butanol (7c). The title compound was obtained using the same method as described for **7b**. The resulting residue was purified by column chromatography eluting with hexane/EtOAc (3:1). Yield 81%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.66–7.69 (m, 4H), 7.36–7.46 (m, 6H), 3.82–3.87 (m, 4H), 2.40 (t, J = 5.5 Hz, 1H) 1.80 (p, 4H) 1.05 (s, 9H). HRMS (APCI+) *m*/*z*: Calcd for $C_{20}H_{29}O_2Si$: 329.1931. Found: 329.1896.

5.1.6. General procedure for the synthesis of derivatives 8a–8c: method B

5.1.6.1. 3-(3-((2-(*tert*-Butyldiphenylsilyloxy)ethyl)oxy)-1,2,5-thiadiazol-4-yl)pyridine (8a). Compound **7a** (540 mg, 1.59 mmol) was dissolved in THF (~15 mL) and chilled to 0 °C. Sodium hydride (127 mg, 3.18 mmol, 60% dispersion in mineral oil) was added, and the reaction mixture was refluxed for 1 h. The solution was chilled to 0 °C, and intermediate **3** (470 mg, 2.38 mmol) in THF (10 mL/mmol) was added. The reaction mixture was refluxed for 18 h, cooled, and then concentrated. The residue was dissolved in CH_2Cl_2 (50 mL), washed with aqueous citric acid, pH = 4 (3× 50 mL), 5% NaOH, brine, dried over $MgSO_4$, and concentrated to afford crude **8a** (609 mg) as a colorless oil. Yield: 83%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.38 (d, J = 1.9 Hz, 1H), 8.59 (dd, J = 1.7, 4.8 Hz), 8.49 (dt, J = 1.8, 7.2 Hz, 1H), 7.64–7.79 (m, 5H), 7.29–7.43 (m, 6H), 4.67 (t, J = 4.9 Hz, 2H), 4.08 (t, J = 4.9 Hz, 2H), 1.12 (s, 9H). HRMS (ESI+) *m*/*z*: Calcd for $C_{25}H_{28}N_3O_2SSi$: 462.1666. Found: 462.1653.

5.1.6.2. 3-(3-((3-(*tert*-Butyldiphenylsilyloxy)propyl)oxy)-1,2,5-thiadiazol-4-yl)pyridine (8b). The title compound was synthesized according to method B. Yield: 75%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.29 (dd, J = 0.9, 2.3 Hz, 1H), 8.60 (dd, J = 1.7, 4.8 Hz, 1H), 8.30 (ddd, J = 1.7, 2.3, 8.0 Hz, 1H), 7.58–7.78 (m, 5H), 7.24–7.40 (m, 6H), 4.66 (t, J = 6.0 Hz, 2H), 3.87 (t, J = 6.0 Hz, 2H), 2.11 (p, J = 6.0 Hz, 2H), 1.12 (s, 9H). HRMS (ESI+) *m*/*z*: Calcd for $C_{26}H_{30}N_3O_2SSi$: 476.1823. Found: 476.1887.

5.1.6.3. 3-(3-((4-(*tert*-Butyldiphenylsilyloxy)butyl)oxy)-1,2,5-thiadiazol-4-yl)pyridine (8c). The title compound was synthesized from according to method B. Yield: 80%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.38, (dd, J = 0.8, 2.2 Hz, 1 H), 8.64 (dd, J = 1.7, 4.8 Hz, 1H), 8.41, (dt, J = 1.8, 8.0 Hz, 1H), 7.63–7.68 (m, 5H), 7.32–7.42 (m, 6H), 4.54 (t, J = 6.5 Hz, 2H), 3.74 (t, J = 6.1 Hz, 2H), 1.95–2.05 (m, 2H), 1.72–1.81 (m, 2H), 1.05 (s, 9H). HRMS (ESI+) *m*/*z*: Calcd for $C_{27}H_{32}N_3O_2SSi$: 490.1979. Found: 490.2024.

5.1.6.4. 3-(3-((2-(*tert*-Butyldiphenylsilyloxy)ethyl)oxy)-1,2,5-thiadiazol-4-yl)-1,2,5,6-tetrahydro-1-methylpyridine (9a). The title compound was synthesized from **8a** according to method A. Yield: 42%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.60–7.74 (m, 4H), 7.28–7.42 (m, 6H), 7.13–7.19 (m, 1H), 4.56–4.60 (m, 2H), 3.99–4.04 (m, 2H) 3.48–3.54 (m, 2H), 2.58–2.64 (m, 2H), 2.45–2.50 (m, 5H), 1.06 (s, 9H). HRMS (ESI+) *m*/*z*: Calcd for $C_{26}H_{34}N_3O_2SSi$: 480.2136. Found: 480.2158.

5.1.6.5. 3-(3-((3-(*tert*-Butyldiphenylsilyloxy)propyl)oxy)-1,2,5-thiadiazol-4-yl)-1,2,5,6-tetrahydro-1-methylpyridine (9b). The title compound was synthesized from **8b** according to method A. Yield: 30%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.62–7.73 (m, 4H), 7.30–7.43 (m, 6H), 7.03–7.09 (m, 1H), 4.59 (t, J = 6.0 Hz, 2H), 3.70–3.77 (m, 2H), 3.44–3.50 (m, 2H), 2.58 (t, J = 5.1 Hz, 2H), 2.48–2.54 (m, 5H), 1.93–2.04 (m, 2H), 1.06 (s, 9H). HRMS (ESI+) *m*/*z*: Calcd for $C_{27}H_{36}N_3O_2SSi$: 494.2292. Found: 494.2318.

5.1.6.6. 3-(3-((4-(*tert*-Butyldiphenylsilyloxy)butyl)oxy)-1,2,5-thiadiazol-4-yl)-1,2,5,6-tetrahydro-1-methylpyridine (9c). The title compound was synthesized from **8c** according to method A. Yield: 63%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.64–7.69 (m, 4H), 7.33–7.43 (m, 6H), 7.02–7.07 (m, 1H), 4.45 (t, J = 6.5 Hz, 2H) 3.67–3.75 (m, 2H), 3.42–3.46 (m, 2H), 2.56 (t, J = 5.3 Hz, 2H), 2.40–2.46 (m, 5H), 1.89–1.99 (m, 2H), 1.67–1.77 (m, 2H), 1.06 (s, 9H). HRMS (ESI+) *m*/*z*: Calcd for $C_{28}H_{38}N_3O_2SSi$: 508.2449. Found: 508.2477.

5.1.7. General procedure for TBDPS deprotection (10a–10c): method C

5.1.7.1. 3-(3-((4-Hydroxybutyl)oxy)-1,2,5-thiadiazol-4-yl)-1,2,5,6-tetrahydro-1-methylpyridine (10c). To a stirring solution of **9c** (690 mg, 1.36 mmol) in THF (5 mL) at 0 °C was added TBAF in THF (1.77 mL of a 1.0 M solution, 1.77 mmol). The reaction mixture was stirred at room temperature for 3 h and then concentrated. The resulting residue was purified by silica gel column chromatography (9:1:0.2 CH_2Cl_2/MeOH/Et_3N) to furnish **10c** as a yellow solid (169 mg). Yield: 46%. ^{1}H (300 MHz, $CDCl_3$) δ 7.05 (m, 1H), 4.47 (t, J = 6.5 Hz, 2H), 3.70 (t, J = 6.4 Hz, 2H), 3.45 (dd, J = 2.5, 4.4 Hz, 2H), 2.58 (t, J = 5.8 Hz, 2H), 2.44–2.47 (m, 5H), 1.88–1.98 (m, 2H), 1.66–1.77 (m, 2H). HRMS (ESI+) *m*/*z*: Calcd for $C_{12}H_{20}N_3SO_2$: 270.1271. Found: 270.1223.

5.1.7.2. 3-(3-((2-Hydroxyethyl)oxy)-1,2,5-thiadiazol-4-yl)-1,2,5,6-tetrahydro-1-methylpyridine (10a). The title compound was synthesized from **9a** according to method C. However, after the THF solution was concentrated, the resultant residue was partitioned between Et_2O and 5% NaOH. The organic layer was separated, washed with brine, dried over Na_2SO_4, and concentrated. Column chromatography eluting with CH_2Cl_2/MeOH/Et_3N (15:1:0.2) afforded alcohol **10a**. Yield: 20%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.04 (m, 1H), 4.57 (t, J = 4.5 Hz, 2H), 3.99 (t, J = 4.5 Hz, 2H), 3.45–3.49 (m, 2H), 2.61 (t, J = 5.7 Hz, 2H), 2.44–2.50 (m, 5H). HRMS (ESI+) *m*/*z*: Calcd for $C_{10}H_{16}N_3SO_2$: 242.0958. Found: 242.0967.

5.1.7.3. 3-(3-((3-Hydroxypropyl)oxy)-1,2,5-thiadiazol-4-yl)-1,2,5,6-tetrahydro-1-methylpyridine (10b). The title compound was synthesized from **9b** according to method C, with a similar work-up as that described for **10a**. Yield: 79%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.01 (m, 1H), 4.59 (t, J = 6.1 Hz, 2H), 3.78 (t, J = 6.1 Hz, 2H), 3.44 (dd, J = 2.4, 4.2 Hz, 2H), 2.57 (t, J = 5.7 Hz, 2H), 2.40–2.48 (m, 5H), 2.33 (br, 1H), 2.07 (p, J = 6.1 Hz, 2H). HRMS (ESI+) *m*/*z*: Calcd for $C_{11}H_{18}N_3SO_2$: 256.1114. Found: 256.1109.

5.1.8. General procedure for the synthesis of *N*-*tert*-butoxycarbonyl-protected amino acids: method D

5.1.8.1. *N*-*tert*-Butoxycarbonyl glycine.[18] Glycine (1.10 g, 14.8 mmol) and NaOH (590 mg, 14.8 mmol) were stirred in dioxane/H_2O (~10 mL each) until complete dissolution (~10 min). This mixture was cooled to 0 °C at which time Boc_2O (3.53 g, 16.2 mmol) was added in three equal portions. Stirring continued for 30 min at 0 °C and then for an additional 12 h at room temperature. The reaction mixture was concentrated and the resulting residue was redissolved in H_2O (50 mL). The aqueous layer was washed with diethyl ether (3× 75 mL), and then acidified with citric acid to pH = 4. The aqueous layer was extracted with EtOAc (3× 30 mL). The combined EtOAc layers were washed with brine, dried over $MgSO_4$, and concentrated to yield *N*-*tert*-butoxycarbonyl glycine (2.13 g) as a white solid. Yield: 82%. The ^{1}H NMR matched previously reported values.[18]

5.1.8.2. 2-(*tert*-Butoxycarbonylamino)-1-ethanol (12a).[37,38] *N*-*tert*-Butoxycarbonyl glycine (760 mg, 4.33 mmol) was dissolved in THF (20 mL) and cooled to 0 °C. Sequential addition of Et_3N (0.61 mL, 4.3 mmol) and isobutyl chloroformate (0.56 mL, 4.3 mmol) turned the reaction mixture from clear, to bright yellow, to rose. The reaction mixture was stirred for an additional 20 min (0 °C) and then $NaBH_4$ (491 mg, 13 mmol) was added. Over the next 10 min, MeOH (30 mL) was added dropwise. The reaction mixture was warmed to room temperature and stirred for an additional 30 min. The reaction mixture was concentrated and extracted with EtOAc (50 mL). The organic layer was washed with brine, dried over $MgSO_4$ and concentrated. Column chromatography eluting with a gradient of CH_2Cl_2/MeOH (9:1–6:1) afforded title compound **12a**. Yield: 45%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.94 (br, 1H), 3.72 (t, J = 4.5 Hz, 2H), 3.24–3.30 (m, 2H), 2.71 (br, 1H), 1.45 (s, 9H). HRMS (APCI+) *m*/*z*: Calcd for $C_7H_{16}NO_3$: 162.1125. Found: 162.1164.

5.1.8.3. 4-(*tert*-Butoxycarbonylamino)-1-butanol (12b). Starting from 4-aminobutanoic acid (GABA), *N*-*tert*-butoxycarbonyl-GABA was synthesized according to method D (88% yield).[18] The acid was reduced using a similar mixed anhydride reduction as **12a**, except that ethyl chloroformate was utilized instead of isobutyl chloroformate. Additionally, the $NaBH_4$ (3 equiv) was added as a solution in MeOH and the resulting mixture was allowed to stir at room temperature for 2 h.[20] The reaction mixture was concentrated and partitioned between EtOAc and H_2O. The organic layer was separated, washed with brine, dried over $MgSO_4$, and concentrated to afford **12b**. Yield: 65%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.72 (br, 1H), 3.60–3.69 (m, 2H), 3.08–3.19 (m, 2H), 2.12–2.20 (m, 1H), 1.51–1.61 (m, 4H), 1.44 (s, 9H). HRMS (APCI+) *m*/*z*: Calcd for $C_9H_{20}NO_3$: 190.1438. Found: 190.1452.

5.1.8.4. 6-(*tert*-Butoxycarbonylamino)-1-hexanol (12c). Starting from 6-aminohexanoic acid, the amino group was Boc-protected utilizing method D (89% yield).[18] The acid was reduced using the procedure detailed for **12b**.[20] Yield: 41%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.52 (br, 1H), 3.64 (t, J = 6.5 Hz, 2H), 3.06–3.16 (m, 2H), 1.28–1.60 (m, 17H). HRMS (APCI+) *m*/*z*: Calcd for $C_{11}H_{24}NO_3$: 218.1756. Found: 218.1747.

5.1.8.5. 3-(*tert*-Butoxycarbonylamino)-1-propanol (14a).[21] To a solution of 3-aminopropan-1-ol (628 mg, 8.50 mmol) and Et_3N (1.17 mL, 8.50 mmol) in CH_2Cl_2 (10 mL) at 0 °C, Boc_2O (1.69 g, 7.72 mmol) was added. The reaction mixture was stirred at 0 °C for 30 min and then stirred for an additional 12 h at room temperature. The reaction mixture was concentrated and the residue was dissolved in Et_2O (50 mL). The organic phase was washed with citric acid (pH ~ 4.0), saturated $NaHCO_3$, and brine. The ethereal layer was dried over $MgSO_4$ and concentrated to yield **14a** (1.22 g). Yield: 90%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.98 (br, 1H), 3.66 (q, J = 6.0 Hz, 2H), 3.39 (t, J = 6.0 Hz, 1H), 3.28 (q, J = 6.0 Hz, 2H), 1.67 (p, J = 6.0 Hz, 2H), 1.45 (s, 9H). HRMS (APCI+) *m*/*z*: Calcd for $C_8H_{18}NO_3$: 176.1281. Found: 176.1043.

5.1.8.6. 5-(*tert*-Butoxycarbonylamino)-1-pentanol (14b).[39] The title compound was synthesized in an analogous manner to afford propanol derivative **14a**. Yield: 99%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.60 (br, 1H), 3.65 (q, J = 5.8 Hz, 2H), 3.11–3.16 (m, 2H), 1.69 (t, J = 5.1 Hz, 1H), 1.50–1.59 (m, 6H), 1.44 (s, 9H). HRMS (APCI+) *m*/*z*: Calcd for $C_{10}H_{22}NO_3$: 204.1594. Found: 204.1590.

5.1.8.7. 4-(*tert*-Butoxycarbonyl(methyl)amino)butyric acid (16).[23] A solution of concentrated HCl (20 mL) and 1-methyl-2-pyrrolidinone (10 mL) was refluxed for 18 h. The reaction mixture was cooled and concentrated. The resulting white solid matched previously reported NMR data for 4-(methylamino)butanoic acid hydrochloride salt and was used without further purification.[40] The Boc group was installed using method D with an additional equivalent of NaOH to neutralize the HCl salt of the precursor. Yield from **15**: 52%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.28 (t, J = 6.0 Hz, 2H), 2.84 (s, 3H), 2.35 (t, J = 7.0 Hz, 2H), 1.80–1.87 (m, 2H), 1.45 (s, 9H). HRMS (ESI−) *m*/*z*: Calcd for $C_{10}H_{18}NO_4$: 216.1241. Found: 216.1252.

5.1.8.8. 4-(*tert*-Butoxycarbonyl(methyl)amino)-1-butanol (17).[24] To a solution of **16** (502 mg, 2.47 mmol) in THF (10 mL) at 0 °C was added BH_3·THF (2.6 mL of a 1.0 M solution). The reaction was allowed to warm to room temperature and was stirred for an additional 18 h. The reaction mixture was then chilled to 0 °C and ~3 mL of H_2O was added. The reaction mixture was concentrated, extracted with EtOAc, and washed with a citric acid solution (pH = 3.5). The organic layer was dried over $MgSO_4$ and concentrated. Column chromatography eluting with a gradient of CH_2Cl_2/MeOH (9:1–4:1) afforded compound **17** as a colorless oil (390 mg). Yield: 83%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.66 (t, J = 6.0 Hz, 2H), 3.23 (t, J = 6.6 Hz, 2H), 2.83 (s, 3H), 1.78 (br, 1H), 1.51–1.60 (m, 4H), 1.44 (s, 9H). HRMS (APCI+) *m*/*z*: Calcd for $C_{10}H_{22}NO_3$: 204.1594. Found: 204.1582.

5.1.9. General procedure for preparation of compounds 18a–e, 21, 24, and 25: method E

5.1.9.1. 3-(3-((4-(*tert*-Butoxycarbonyl)aminobutyl)oxy)-1,2,5-thiadiazol-4-yl) pyridine (18c). To a stirring solution of **12b** (250 mg, 1.32 mmol) in THF (~15 mL) at 0 °C was added NaH (105 mg, 2.63 mmol). The reaction mixture was refluxed for 1 h and then cooled to 0 °C. Intermediate **3** (260 mg, 1.32 mmol) in THF (10 mL) was added. The reaction mixture was refluxed for 18 h, cooled, and then concentrated. The residue was taken up in CH_2Cl_2 (50 mL/mmol), washed with 5% NaOH, brine, dried over Na_2SO_4, and concentrated. The resulting residue was purified using silica gel column chromatography eluting with CH_2Cl_2/MeOH (9:1). Compound **18c** was isolated as a brown oil (205 mg). Yield: 44%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.38 (d, J = 0.8 Hz, 1H), 8.64 (dd, J = 1.5, 4.8 Hz, 1H), 8.43–8.50 (m, 1H), 7.45 (dd, J = 5.0, 7.6 Hz, 1H), 4.70 (br, 1H), 4.53 (t, J = 6.4 Hz, 2H), 3.15–3.23 (m, 2H), 1.86–1.96 (m, 2H), 1.62–1.72 (m, 2H), 1.41 (s, 9H). HRMS (ESI+) *m*/*z*: Calcd for $C_{16}H_{23}N_4O_3S$: 351.1485. Found: 351.1531.

5.1.9.2. 3-(3-((2-(*tert*-Butoxycarbonyl)aminoethyl)oxy)-1,2,5-thiadiazol-4-yl)pyridine (18a). The title compound was synthesized according to method E. Instead of refluxing for 18 h, however, the reaction mixture was stirred at room temperature (18 h). Column chromatography eluting with CH_2Cl_2/MeOH (9:1) afforded compound **18a**. Yield: 30%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.37 (d, J = 1.6 Hz, 1H), 8.67 (dd, J = 1.7, 4.8 Hz, 1H), 8.44 (dt, J = 1.6, 8.1 Hz, 1H), 7.42 (ddd, J = 0.9, 4.8, 8.0 Hz, 1H), 5.04 (br, 1H), 4.62, (t, J = 5.3 Hz, 2H), 3.61–3.69 (m, 2H), 1.43 (s, 9H). HRMS (ESI+) *m*/*z*: Calcd for $C_{14}H_{19}N_4O_3S$: 323.1172. Found: 323.1177.

5.1.9.3. 3-(3-((3-(*tert*-Butoxycarbonyl)aminopropyl)oxy)-1,2,5-thiadiazol-4-yl)pyridine (18b). The title compound was synthesized according to method E with the same modification as that which was denoted in **18a**. Yield: 50%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.38 (dd, J = 0.8,

2.4 Hz, 1H), 8.66 (dd, J = 1.7, 4.8 Hz, 1H), 8.44 (dt, J = 1.8, 7.2 Hz, 1H), 7.42 (ddd, J = 0.9, 4.8, 8.0 Hz, 1H), 4.64 (t, J = 6.0 Hz, 2H), 5.73 (br, 1H), 3.38–3.45 (m, 2H), 2.12–2.22 (m, 2H), 1.45 (s, 9H). HRMS (ESI+) *m*/*z*: Calcd for $C_{15}H_{21}N_4O_3S$: 337.1329. Found: 337.1242.

5.1.9.4. 3-(3-((5-(*tert*-Butoxycarbonyl)aminopentyl)oxy)-1,2,5-thiadiazol-4-yl)pyridine (18d). The title compound was synthesized according to method E. Yield: 72%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.37–9.41 (m, 1H), 8.65 (dd, J = 1.7, 4.8 Hz, 1H), 8.39–8.44 (m, 1H), 7.37–7.43 (m, 1H), 4.90 (br, 1H), 4.50–4.54 (m, 2H), 3.12–3.26 (m, 2H), 1.86–1.97 (m, 2H), 1.50–1.64 (m, 4H), 1.43 (s, 9H). HRMS (ESI+) *m*/*z*: Calcd for $C_{17}H_{25}N_4O_3S$: 365.1642. Found: 365.1673.

5.1.9.5. 3-(3-((6-(*tert*-Butoxycarbonyl)aminohexyl)oxy)-1,2,5-thiadiazol-4-yl)pyridine (18e). The title compound was synthesized according to method E. Yield: 85%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.38 (d, J = 0.8 Hz, 1 H), 8.64 (dd, J = 1.2, 4.7 Hz, 1H), 8.44 (ddd, J = 1.9, 2.1, 8.1 Hz), 7.43 (ddd, J = 0.8, 4.9, 8.0 Hz), 4.62, (br, 1H), 4.50 (t, J = 6.6 Hz, 2H), 3.06–3.16 (m, 2H), 1.83–1.93 (m, 2H), 1.45–1.55 (m, 4H), 1.42 (s, 9H). HRMS (ESI+) *m*/*z*: Calcd for $C_{18}H_{27}N_4O_3S$: 379.1798. Found: 379.1813.

5.1.9.6. 3-(3-((2-(*tert*-Butoxycarbonyl)aminoethyl)oxy)-1,2,5-thiadiazol-4-yl)-1,2,5,6-tetrahydro-1-methylpyridine (19a). The title compound was synthesized from **18a** according to method A. Column chromatography eluting with CH_2Cl_2/MeOH/Et_3N (9:1:0.2) afforded **19a** as a brown oil. Yield: 46%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.00–7.05 (m, 1H), 4.89 (br, 1H), 4.52 (t, J = 5.4 Hz, 2H), 3.57–3.63 (m, 2H), 3.47–3.50 (m, 2H), 2.62 (t, J = 5.7 Hz), 2.45–2.50 (m, 5H), 1.42 (s, 9H). HRMS (ESI+) *m*/*z*: Calcd for $C_{15}H_{25}N_4O_3S$ 341.1642. Found: 341.1686.

5.1.9.7. 3-(3-((3-(*tert*-Butoxycarbonyl)aminopropyl)oxy)-1,2,5-thiadiazol-4-yl)-1,2,5,6-tetrahydro-1-methylpyridine (19b). The title compound was synthesized from **18b** according to method A. Column chromatography eluting with CH_2Cl_2/MeOH/Et_3N (9:1:0.2) afforded **19b** as a brown oil. Yield: 16%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.00–7.05 (m, 1H), 4.76 (br, 1H), 4.52 (t, J = 6.2 Hz, 2H), 3.44–3.48 (m, 2H), 3.26–3.34 (m, 2H), 2.59 (t, J = 5.3 Hz, 2H), 2.44–2.48 (m, 5H), 2.00–2.10 (m, 2H), 1.44 (s, 9H). HRMS (ESI+) *m*/*z*: Calcd for $C_{16}H_{27}N_4O_3S$: 355.1798. Found: 355.1814.

5.1.9.8. 3-(3-((4-(*tert*-Butoxycarbonyl)aminobutyl)oxy)-1,2,5-thiadiazol-4-yl)-1,2,5,6-tetrahydro-1-methylpyridine (19c). The title compound was synthesized from **18c** according to method A. Column chromatography eluting with CH_2Cl_2/MeOH/Et_3N (9:1:0.2) afforded **19c** as a brown oil. Yield: 16%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.03–7.08 (m, 1H), 4.62 (br, 1H), 4.47 (t, J = 6.5 Hz, 2H), 3.51–3.55 (m, 2H), 3.15–3.24 (m, 2H), 2.67 (t, J = 5.6 Hz, 2H), 2.49–2.54 (m, 5H), 1.83–1.94 (m, 2H), 1.60–1.71 (m, 2H), 1.45 (s, 9H). HRMS (ESI+) *m*/*z*: Calcd for $C_{17}H_{29}N_4O_3S$: 369.1955. Found: 369.1956.

5.1.9.9. 3-(3-((5-(*tert*-Butoxycarbonyl)aminopentyl)oxy)-1,2,5-thiadiazol-4-yl)-1,2,5,6-tetrahydro-1-methylpyridine (19d). The title compound was synthesized from **18d** according to method A. Yield: 33%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.00–7.07 (m, 1H), 4.77 (br, 1H), 4.53 (t, J = 6.0 Hz, 2H), 3.40–3.46 (m, 2H), 3.11–3.20 (m, 2H), 2.57 (t, J = 5.7 Hz, 2H), 2.42–2.47 (m, 5H), 1.82–1.94 (m, 2H), 1.47–1.65 (m, 4H), 1.44 (s, 9H). HRMS (ESI+) *m*/*z*: Calcd for $C_{18}H_{31}N_4O_3S$: 383.2111. Found: 383.2159.

5.1.9.10. 3-(3-((6-(*tert*-Butoxycarbonyl)aminohexyl)oxy)-1,2,5-thiadiazol-4-yl)-1,2,5,6-tetrahydro-1-methylpyridine (19e). The title compound was synthesized from **18a** according to method A. Column chromatography eluting with CH_2Cl_2/MeOH/Et_3N (9:1:0.2) afforded **19e** as a brown oil. Yield: 37%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.00–7.05 (m, 1H), 4.65 (br, 1H), 4.40 (t, J = 6.6 Hz, 2H), 3.43–3.47 (m, 2H), 3.06–3.10 (m, 2H), 2.58 (t, J = 5.9 Hz, 2H), 2.39–2.45 (m, 5H), 1.75–1.86 (m, 2H), 1.36–1.50 (m, 15H). HRMS (ESI+) *m*/*z*: Calcd for $C_{19}H_{33}N_4O_3S$: 397.2268. Found: 397.2293.

5.1.10. Boc-deprotection reactions. The Boc group was removed via one of two different methods. One method involved stirring the starting material in a 50/50 mixture of TFA and CH_2Cl_2.[41] After stirring for 4 h the reaction mixture was concentrated. Alternatively, the Boc group was removed by stirring in a solution of 4.0 M HCl in dioxane. Similarly, the reaction mixture was stirred for 4 h and was then concentrated.

5.1.11. 3-(3-((2-Aminoethyl)oxy)-1,2,5-thiadiazol-4-yl)-1,2,5,6-tetrahydro-1-methylpyridine (20a) 2·TFA salt. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.00–7.05 (m, 1H), 4.30–4.40 (m, 2H), 3.81–3.92 (m, 2H), 3.33–3.52 (m, 2H), 3.03–3.17 (m, 2H), 2.90–2.97 (m, 2H), 2.84–2.89 (m, 5H). HRMS (ESI+) *m*/*z*: Calcd for $C_{10}H_{17}N_4OS$: 241.1118. Found: 241.1112.

5.1.12. 3-(3-((3-Aminopropyl)oxy)-1,2,5-thiadiazol-4-yl)-1,2,5,6-tetrahydro-1-methylpyridine (20b). This was originally made as the TFA salt, but since it did not easily recrystallize, it was dissolved in 1 M K_2CO_3, extracted, concentrated, and stored as its freebase. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.00–7.06 (m, 1H), 4.54 (t, J = 5.1 Hz, 2H), 3.62–3.76 (m, 2H), 3.41–3.46 (m, 2H), 2.86–2.94, (m, 2H), 2.53–2.60 (m, 2H) 2.40–2.48 (m, 5H), 1.94–2.04 (m, 2H). HRMS (ESI+) *m*/*z*: Calcd for $C_{11}H_{19}N_4OS$: 255.1274. Found: 255.1234.

5.1.13. 3-(3-((4-Aminobutyl)oxy)-1,2,5-thiadiazol-4-yl)-1,2,5,6-tetrahydro-1-methylpyridine (20c) 2·HCl salt. Boc-deprotection was done using 4.0 M dioxane in HCl solution. ^{1}H NMR (300 MHz, D_2O) δ 6.88–6.94 (m, 1H), 4.24–4.29 (m, 2H), 3.68–3.78 (m, 2H), 3.27–3.37 (m, 2H), 3.00–3.05 (m, 2H), 2.70–2.77 (m, 7H), 1.49–1.68 (m, 4H). HRMS (ESI+) *m*/*z*: Calcd for $C_{12}H_{21}N_4OS$: 269.1431. Found: 269.1444.

5.1.14. 3-(3-((5-Aminopentyl)oxy)-1,2,5-thiadiazol-4-yl)-1,2,5,6-tetrahydro-1-methylpyridine (20d) 2·HCl salt. Boc-deprotection was done using 4.0 M dioxane in

HCl solution. ^{1}H NMR (300 MHz, D_2O) δ 7.06–7.11 (m, 1H), 4.42 (t, J = 4.5 Hz, 2H), 3.68–3.75 (m, 2H), 3.57–3.65 (m, 2H), 2.80–2.90 (m, 2H), 2.59–2.67 (m, 5H), 2.48–2.56 (m, 2H), 1.77–1.88 (m, 2H), 1.42–1.64 (m, 4H) HRMS (ESI+) *m*/*z*: Calcd for $C_{13}H_{23}N_4OS$: 283.1587. Found: 283.1604.

5.1.15. 3-(3-((6-Aminohexyl)oxy)-1,2,5-thiadiazol-4-yl)-1,2,5,6-tetrahydro-1-methylpyridine (20e). Boc-deprotection was done using 4.0 M dioxane in HCl solution. ^{1}H NMR (300 MHz, D_2O) δ 7.03–7.08 (m,1H), 4.28–4.34 (m, 2H), 3.82–3.91 (m, 2H), 3.05–3.14 (m, 2H), 2.85–2.90, (m, 5H), 2.81 (t, J = 5.0 Hz, 2H), 2.49–2.59 (m, 2H), 1.65–1.75 (m, 2H), 1.24–1.55 (m, 6H). HRMS (ESI+) *m*/*z*: Calcd for $C_{14}H_{25}N_4OS$: 297.1744. Found: 297.1779.

5.1.16. 3-(3-((4-(*tert*-Butoxycarbonyl(methyl)amino)butyl)-oxy)-1,2,5-thiadiazol-4-yl)pyridine (21). The title compound was synthesized according to method E. Yield: 82%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.37 (dd, J = 0.8, 2.3 Hz, 1H), 8.64 (dd, J = 1.7, 4.8 Hz, 1H), 8.40 (dt, J = 1.9, 7.2 Hz, 1H), 7.38 (ddd, J = 0.8, 4.8, 7.2 Hz, 1H), 4.55 (t, J = 6.4 Hz, 2H), 3.29 (t, J = 6.8 Hz, 2H), 2.84 (s, 3H), 1.83–1.92 (m, 2H), 1.66–1.75 (m, 2H), 1.43 (s, 9H). HRMS (ESI+) *m*/*z*: Calcd for $C_{17}H_{25}N_4O_3S$: 365.1642. Found: 365.1826.

5.1.17. 3-(3-((4-(*tert*-Butoxycarbonyl(methyl)amino)butyl)-oxy)-1,2,5-thiadiazol-4-yl)-1,2,5,6-tetrahydro-1-methylpyridine (22). The title compound was synthesized from **21** according to method A. Yield: 30%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.02–7.06 (m, 1H), 4.46 (t, J = 6.3 Hz, 2H), 3.42–3.46 (m, 2H), 3.22–3.33 (m, 2H), 2.84 (s, 3H), 2.58 (t, J = 5.4 Hz, 2H), 2.41–2.47 (m, 5H), 1.79–1.89 (m, 2H), 1.63–1.73 (m, 2H), 1.45 (s, 9H). HRMS (ESI+) *m*/*z*: Calcd for $C_{18}H_{31}N_4O_3S$: 383.2111. Found: 383.2042.

5.1.18. 3-(3-((4-Methylaminobutyl)oxy)-1,2,5-thiadiazol-4-yl)-1,2,5,6-tetrahydro-1-methylpyridine (23). Compound **22** was Boc-deprotected with TFA in CH_2Cl_2. Yield: 34%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.00–7.06 (m, 1H), 4.46 (t, J = 6.5 Hz, 2H), 3.41–3.47 (m, 5H), 2.79 (t, J = 4.5 Hz, 2H), 2.58 (t, J = 5.4 Hz, 2H), 2.42–2.46 (m, 5H), 1.87–1.94 (m, 2H), 1.70–1.78 (m, 2H). HRMS (ESI+) *m*/*z*: Calcd for $C_{13}H_{23}N_4OS$: 283.1587. Found: 283.1575.

5.1.19. 3-(3-((3-Phenyl-2-propyn)oxy)-1,2,5-thiadiazol-4-yl)pyridine (24). The title compound was synthesized according to method E. Yield: 75%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.43 (dd, J = 0.9, 2.4 Hz, 1H), 8.67 (dd, J = 1.8, 4.8 Hz, 1H), 8.46 (ddd, J = 1.8, 2.4, 7.8 Hz, 1H), 7.45–7.51 (m, 2H), 7.41 (ddd, J = 0.9, 5.1, 8.1 Hz, 1H), 7.30–7.37 (m, 3H), 5.39 (s, 2H). HRMS (ESI+) *m*/*z*: Calcd for $C_{16}H_{12}N_3OS$: 294.0696. Found: 294.0721.

5.1.20. 3-(3-((4-Phenylbutyl)oxy)-1,2,5-thiadiazol-4-yl)pyridine (25). The title compound was synthesized according to method E. Yield: 82%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.39 (dd, J = 0.9, 2.4 Hz, 1H), 8.64 (dd, J = 1.8, 4.8 Hz, 1H), 8.40 (ddd, J = 1.8, 2.4, 8.1 Hz, 1H), 7.38 (ddd, J = 0.9, 5.1, 8.1 Hz, 1H), 7.13–7.30 (m, 5 H), 4.53 (t, J = 6.3 Hz, 2H), 2.71 (t, J = 6.9 Hz, 2H), 1.80–1.99 (m, 4H). HRMS (ESI+) *m*/*z*: Calcd for $C_{17}H_{18}N_3OS$: 312.1165. Found: 312.1187.

5.1.21. 3-(3-((3-Phenyl-2-propyn)oxy)-1,2,5-thiadiazol-4-yl)-1,2,5,6-tetrahydro-1-methylpyridine (26).[13] The title compound was synthesized from **24** according to method A. Column chromatography eluting with CH_2Cl_2/MeOH/Et_3N (9:1:0.2) afforded **26**. Yield: 11%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.45–7.50 (m, 2H), 7.31–7.37 (m, 3H), 7.10–7.16 (m, 1H), 5.30 (s, 2H), 3.44–3.47 (m, 2H), 2.59 (t, J = 5.1 Hz, 2H), 2.47–2.51 (m, 5H). HRMS (ESI+) *m*/*z*: Calcd for $C_{17}H_{18}N_3OS$: 312.1165. Found: 312.1252.

5.1.22. 3-(3-((4-Phenylbutyl)oxy)-1,2,5-thiadiazol-4-yl)-1,2,5,6-tetrahydro-1-methylpyridine (27) HCl salt.[13] The title compound was synthesized from **24** according to method A. Column chromatography eluting with CH_2Cl_2/MeOH/Et_3N (9:1:0.2) afforded a mixture of **27** and 4-phenylbutan-1-ol. This mixture was dissolved in HCl/EtOH, stirred for 30 min, and concentrated. The resulting oil was repeatedly washed with Et_2O, which eventually led to the precipitation of the HCl salt of **27**. Yield: 19%. Freebase NMR: ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.26–7.32 (m, 2H), 7.16–7.23 (m, 3H), 7.02–7.07 (m, 1H), 4.46 (t, J = 6.3 Hz, 2H), 3.44–3.48 (m, 2H), 2.70 (t, J = 7.8 Hz, 2H), 2.57 (t, J = 5.1 Hz. 2H), 2.45–2.48 (m, 5H), 1.76–1.94 (m, 4H). HRMS (ESI+) *m*/*z*: Calcd for $C_{18}H_{24}N_3OS$: 330.1625. Found: 330.1681.

5.1.23. 3-(3-((4-Chlorobutyl)oxy)-1,2,5-thiadiazol-4-yl)-1,2,5,6-tetrahydro-1-methylpyridine (28). To a stirring solution of **10c** (29 mg, 0.11 mmol) in CH_2Cl_2 (5 mL) at 0 °C was added $SOCl_2$ (0.20 mL, 2.8 mmol). The reaction was warmed to room temperature and stirred for 12 h. After cooling to 0 °C, H_2O (~5 mL) was added, followed by K_2CO_3, until the pH of the aqueous layer reached ~12. To this mixture was added CH_2Cl_2 (~10 mL). The separated aqueous layer was further extracted with CH_2Cl_2 (3× 10 mL). The combined organics were washed with brine, dried over Na_2SO_4, and concentrated to yield the title compound (32 mg). Yield: 100%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.04 (m, 1H), 4.49 (t, J = 6.1 Hz, 2H), 3.63 (t, J = 6.2 Hz, 2H), 3.49 (dd, J = 2.4, 4.3 Hz, 2H), 2.62 (t, J = 5.8 Hz, 2H), 2.47–2.53 (m, 5 H), 1.91–2.08 (m, 4H). HRMS (ESI+) *m*/*z*: Calcd for $C_{12}H_{19}N_3SOCl$: 288.0932. Found: 288.0897.

5.1.24. 3-(3-(4-(1*H*-imidazol-1-yl)butoxy)-1,2,5-thiadiazol-4-yl)-1,2,5,6-tetrahydro-1-methylpyridine (29). To a stirring solution of imidazole (10.4 mg, 0.153 mmol) in DMF (5 mL) at 0 °C was added NaH (6.1 mg, 0.15 mmol). The reaction mixture was refluxed for 1 h and then chilled to 0 °C. Compound **28** (22 mg, 0.076 mmol) in DMF (~3 mL) was added and the reaction was refluxed for 18 h. The reaction mixture was cooled and concentrated. The resulting residue was dissolved in CH_2Cl_2 (30 mL) and subsequently washed

with 5% NaOH and brine. The organic layer was dried over Na_2SO_4 and concentrated to yield crude product. Column chromatography on silica gel eluting with CH_2Cl_2/MeOH/Et_3N (9:1:0.2) afforded **29** (3.2 mg). Yield: 13%. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.48 (s, 1H), 7.06 (s, 1H), 6.96–7.01 (m, 1H), 6.91 (s, 1H), 4.45 (t, J = 6.2 Hz, 2H), 4.02 (t, J = 6.8 Hz, 2H), 3.43–3.45 (m, 2H), 2.57 (t, J = 6.0 Hz, 2H), 2.45–2.49 (m, 5H), 1.91–1.99 (m, 2H), 1.80–1.88 (m, 2H). HRMS (ESI+) *m*/*z*: Calcd for $C_{15}H_{22}N_5SO$: 320.1540. Found: 320.1496.

5.1.25. 3-(3-(4-(1*H*-pyrrol-yl)butoxy)-1,2,5-thiadiazol-4-yl)-1,2,5,6-tetrahydro-1-methylpyridine (30). To a stirring solution of pyrrole (15.0 mg, 0.224 mmol) in DMF (5 mL) at 0 °C was added NaH (9.0 mg, 0.23 mmol). The reaction mixture was refluxed for 1 h and then chilled to 0 °C. Compound **28** (20.1 mg, 0.070 mmol) in DMF (~3 mL) was added and the reaction was refluxed for 18 h. The reaction mixture was cooled and concentrated. The resulting residue was dissolved in CH_2Cl_2 (30 mL) and subsequently washed with 5% NaOH and brine. The organic layer was dried over Na_2SO_4 and concentrated to yield crude product. Column chromatography on silica gel eluting with CH_2Cl_2/MeOH/Et_3N (9:1:0.2) afforded **30** (4.3 mg). Yield: 19%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.99–7.05 (m, 1H), 6.63–6.68 (m, 2H), 6.12–6.16 (m, 2H), 4.44 (t, J = 6.0 Hz, 2H), 3.96 (t, J = 7.0 Hz, 2H), 3.41–3.46 (m, 2H), 2.57 (t, J = 5.5 Hz, 2H), 2.41–2.47 (m, 5H), 1.88–2.06 (m, 4H). HRMS (ESI+) *m*/*z*: Calcd for $C_{16}H_{23}N_4SO$: 319.1587. Found: 319.1539.

5.1.26. Ethyl 5-(4-(1-methyl-1,2,5,6-tetrahydropyridin-3-yl)-1,2,5-thiadiazol-3-yloxy)pentanoate (31). To a stirring solution of the sodium salt of 5-hydroxypentanoic acid (185 mg, 1.32 mmol) in THF (20 mL) at 0 °C was added NaH (106 mg, 2.64 mmol). The reaction mixture was refluxed for 1 h and then cooled to 0 °C at which time intermediate **3** (261 mg, 1.32 mmol) in THF (5 mL) was added. The reaction was refluxed for an additional 18 h and then concentrated in vacuo. The resulting residue was partitioned between Et_2O and H_2O. The separated aqueous layer was acidified to pH = 2, washed with Et_2O (3× 30 mL), and concentrated. The resulting residue was suspended in DMF (10 mL) and filtered. To the filtrate, K_2CO_3 (365 mg, 2.64 mmol) and iodomethane (0.165 mL, 2.64 mmol) were added. This mixture was stirred at room temperature for 18 h and then concentrated. The resulting residue was dissolved in CH_2Cl_2 (30 mL), washed with water (30 mL) and brine. The aqueous layer was further extracted with CH_2Cl_2 (2× 30 mL). The combined organics were concentrated, dissolved in EtOH (20 mL), and chilled to 0 °C. To this solution was added $NaBH_4$ (110 mg, 2.9 mmol). The reaction mixture was refluxed for 4 h and then concentrated. The residue was taken up in CH_2Cl_2, washed with water, brine, dried over Na_2SO_4, and concentrated. Column chromatography eluting with CH_2Cl_2/MeOH/Et_3N (15:1:0.2) afforded **31** as a yellow solid (16 mg). Yield: 4%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.05 (m, 1H), 4.46 (t, J = 6.1 Hz, 2H), 4.13 (q, J = 7.1 Hz, 2H), 3.44–3.48 (m, 2H), 2,59 (t, J = 5.4 Hz, 2H), 2.47 (m, 5H), 2.38 (t, J = 7.1 Hz, 2H), 1.75–1.92 (m, 4H), 1.25 (t, J = 7.2 Hz, 3H). HRMS (ESI+) *m*/*z*: Calcd for $C_{15}H_{24}N_3SO_3$: 326.1533. Found: 326.1572.

5.1.27. 3-(3-Chloro-1,2,5-thiadiazol-4-yl)-1,2,5,6-tetrahydro-1-methylpyridine (32). The title compound was synthesized from **3** according to method A. Column chromatography eluting with CH_2Cl_2/MeOH/Et_3N (9:1:0.2) afforded **32** as a brown oil. Yield: 31%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.91–6.96 (m, 1H), 3.33–3.37 (m, 2H), 2.53 (t, J = 5.7 Hz, 2H), 2.38–2.45 (m, 5H). HRMS (ESI+) *m*/*z*: Calcd for $C_8H_{11}N_3SCl$: 216.0357. Found: 216.0356.

5.1.28. Ethyl 2-(4-(1-methyl-1,2,5,6-tetrahydropyridin-3-yl)-1,2,5-thiadiazol-3-yloxy)acetate (33). The title compound was synthesized according to method E, with modification to the amounts of glycolic acid ethyl ester (10 equiv) and NaH (10 equiv) used. Column chromatography on alumina (basic, Brockman Activity I) eluting with hexane/EtOAc (3:1) afforded the title compound. Yield: 20%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.11–7.17 (m, 1H), 5.00 (s, 2H), 4.27 (q, J = 7.2 Hz, 2H), 3.44–3.47 (m, 2H), 2.57 (t, J = 4.8 Hz, 2H), 2.43–2.47 (m, 5H), 1.29 (t, J = 7.2 Hz, 3H). HRMS (ESI+) *m*/*z*: Calcd for $C_{12}H_{18}N_3SO_3$: 284.1063. Found: 284.1041.

5.1.29. 2-(4-(1-Methyl-1,2,5,6-tetrahydropyridin-3-yl)-1,2,5-thiadiazol-3-yloxy)acetic acid (34) lithium salt. To a stirring solution of **33** (23.0 mg, 0.082 mmol) in CH_3CN/H_2O (5 mL each) was added $LiOH{\cdot}H_2O$ (3.40 mg, 0.082 mmol). The reaction mixture was heated at 50 °C for 2 h and then concentrated to afford a yellow solid (21.2 mg). Yield: 99%. ^{1}H NMR (600 MHz, D_2O) δ 7.00–7.06 (m, 1H), 4.68 (s, 2H), 3.29–3.34 (m, 2H), 2.52–2.58 (m, 2H), 2.28–2.36 (m, 5H). HRMS (ESI+) *m*/*z*: Calcd for $C_{10}H_{14}N_3SO_3$: 256.0750. Found: 256.0686. HRMS (ESI−) *m*/*z*: Calcd for $C_{10}H_{12}N_3SO_3$: 254.0605. Found: 254.0554.

5.2. Biological assays

[^{3}H] *N*-Methyl scopolamine ([^{3}H]NMS) was purchased from Perkin-Elmer Life Sciences (Boston, MA). Atropine (racemic mixture) was purchased from Sigma–Aldrich (St. Louis, MO). Xanomeline analogs (**LY1–LY3**) were a kind gift from Eli Lilly & Co. (Indianapolis, IN). The NNC series of ligands were obtained from the El-Fakahany laboratory.[42] The components of the buffered solutions were purchased from multiple commercial sources. Experiments were performed on Chinese hamster ovary (CHO) cells stably transfected with the human gene for the muscarinic M_1 receptor (kindly provided by Dr. M. Brann, Acadia Pharmaceuticals, Inc., San Diego, CA). Cells were grown in plastic dishes in Dulbecco's modified Eagle's medium with 10% bovine calf serum and 50 μg/mL geneticin. They were harvested by mild trypsinization 4 days after subculturing and washed twice by centrifugation (3 min at 300*g*) and resuspension in a Hepes medium (110 mM NaCl, 5.3 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgSO_4$, 25 mM glucose, 20 mM Hepes, 58 mM

sucrose, with pH adjusted to 7.4 and osmolarity to 340 mOsM).

5.2.1. Continuous binding experiments. CHO hm1 cells were incubated with a fixed concentration of [^{3}H]NMS (ranging from 0.14 to 0.20 nM) in the presence of increasing amounts of the analogs (1–0.1 mM). Non-specific binding was defined as residual binding in the presence of 10 μM atropine. Incubation proceeded for 1 h at 37 °C before termination by rapid filtration through Whatman GF/B filters (Whatman, Clifton, NJ), positioned on a Brandell cell harvester (Gaithersburg, MD). Filters were washed three times with 4 mL of ice-cold saline and dried. Radioactivity was measured via liquid scintillation counting. Assuming simple competition, the data were refitted according to both one- and two-site mass-action binding models, and the better model was determined by an extra-sum-of-squares test using Prism 3.0 (Prism 3.0, GraphPad, San Diego, CA).

5.2.2. Wash-resistant binding experiments. CHO hm1 cells were incubated with xanomeline or its analogs in the absence of the radioligand (1 h). The incubation mixture was diluted with ~50 vol of buffer and centrifuged. The resulting pellet was subjected to this procedure two additional times. The cells were resuspended in buffer and radioligand ([^{3}H]NMS) was added. Incubation proceeded for 1 h at 37 °C before termination via methods similar to those described in the continuous binding experiments.

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