Journal of Medicinal Chemistry

Article

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J. Med. Chem., **Just Accepted Manuscript** • DOI: 10.1021/acs.jmedchem.6b01033 • Publication Date (Web): 01 Sep 2016

ACS Publications

Journal of Medicinal Chemistry is published by the American Chemical Society. 1155 Sixteenth Street N.W., Washington, DC 20036

Discovery of a Potent, Selective and Cell-active Dual Inhibitor of Protein Arginine Methyltransferase 4 and Protein Arginine Methyltransferase 6

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ABSTRACT: Well-characterized selective inhibitors of protein arginine methyltransferases (PRMTs) are invaluable chemical tools for testing biological and therapeutic hypotheses. Based on **4**, a fragment-like inhibitor of type I PRMTs, we conducted structure–activity relationship (SAR) studies and explored three regions of this scaffold. The studies led to the discovery of a potent, selective and cell-active dual inhibitor of PRMT4 and PRMT6, **17** (MS049). As compared to **4, 17** displayed much improved potency for PRMT4 and PRMT6 in both biochemical and cellular assays. It was selective for PRMT4 and PRMT6 over other PRMTs and a broad range of other epigenetic modifiers and non-epigenetic targets. We also developed **46** (MS049N), which was inactive in biochemical and cellular assays, as a negative control for chemical biology studies. Considering possible overlapping substrate specificity of PRMTs,

17 and **46** are valuable chemical tools for dissecting specific biological functions and dysregulation of PRMT4 and PRMT6 in health and disease.

INTRODUCTION

Arginine residues contain a characteristic guanidinium moiety, which is protonated and positively charged at physiological conditions.[1] The arginine guanidinium group can interact with other polar groups through its five potential hydrogen bond donors. Consequently, through stable bidentate salt bridges and hydrogen bonds, arginine is frequently involved in protein/protein and protein/nucleic acid complex formations.[1-3] Arginine methylation, a major post-translational modification of arginine residues, does not change its cationic charge character.[4] Instead, this modification increases the bulkiness of the guanidinium moiety and alters the charge distribution, hydrophobicity and hydrogen bond formation potential.[5] As a result, arginine methylation can have dramatic effects on the interactions of the modified protein with other proteins and nucleic acids, thus its physiological function,[6] including RNA processing, DNA repair, transcriptional activation/repression, signal transduction, cell differentiation, and embryonic development.[7]

As a common post-translational modification in eukaryotic cells,[8-10] arginine methylation is catalyzed by S-5′-adenosyl-*L*-methionine (SAM)-dependent PRMTs.[7] Currently, nine PRMTs have been identified and grouped into three categories based on their product specificity.[9, 11, 12] Type I PRMTs, including PRMT1-4, PRMT6 and PRMT8, catalyze arginine mono- and asymmetric dimethylation. Type II PRMTs, including PRMT5 and PRMT9, catalyze arginine mono- and symmetric dimethylation. PRMT7, which catalyzes arginine monomethylation only, is the sole type III PRMT.[11] PRMTs have a vital role in the regulation of the arginine methylation pattern and level of a plethora of different substrates, including both histones and non-histone proteins.[7] Thus, it is not surprising that dysregulation of PRMTs has been linked to a variety of human diseases.[7, 11, 12] For example, PRMT4 (also known as coactivator-associated arginine methyltransferase 1, CARM1) has a broad range of substrates including histones (e.g., H3R17, H3R26),[13] transcriptional factors (e.g., SOX2, SOX9, PAX7, RUNX1),[14-17] chromatin remodeling factor (e.g., BAF155),[18] transcriptional coactivators (e.g., CBP, p300, SRC3),[19-21] RNA binding proteins (e.g., PABP1, HuR, HuD),[22-24] splicing factors (e.g., CA150,

SAP49, SmB, U1C),[25] T cell-specific factor (e.g., TARPP),[26] RNA polymerase II,[27] and mediator of RNA polymerase II transcription (e.g., Med12).[28] Overexpression of PRMT4 has been associated with AML,[29] breast,[29] prostate,[30] lung[31] and colorectal[31] cancers. The primary methylation targets of PRMT6 are histone H3R2[32] and DNA polymerase β.[33] PRMT6 can also methylate HIV Tat[34] and HMGA1a.[35] PRMT6 is overexpressed in melanoma[36], bladder,[37] lung[37] and prostate[38] cancers. Thus, growing evidence suggests PRMT4 and PRMT6 as potential therapeutic targets.[11, 12] In addition, both PRMT4 and PRMT6 dimethylated H3R42 *in vitro* and *in vivo*.[39] Furthermore, PRMT4 and PRMT6 showed synergic effect in stimulating estrogen receptor alpha (ERα)-dependent transcription.[40] Therefore, targeting PRMT4 and PRMT6 simultaneously may provide a beneficial therapeutic strategy.

Potent, selective and cell-active small-molecule inhibitors of PRMTs[41, 42] would be invaluable tools for investigating the biology of these protein targets and testing the disease and therapeutic hypotheses associated with them. Over the last decade, a number of small-molecule PRMT inhibitors have been reported,[43-60] which include a series of PRMT4 inhibitors represented by compound **1**[50] and a PRMT6 selective inhibitor, **2**[57] (Figure 1A). Based on the PRMT4 and PRMT6 inhibitors,[50, 57] we previously discovered **3** (MS023), a potent, selective and cell-active inhibitor of type I PRMTs (Figure 1A).[47] Recently, benzylpiperidinylethanamine **4 (**Figure 1B) was identified as a fragment-like inhibitor of type I PRMTs (IC_{50} = 0.3–19 μM).[48] The crystal structure of PRMT6 in complex with **4** revealed that the ethylenediamino moiety was anchored in the substrate binding channel and serves as an arginine mimetic (Figure 1B).[48] Here, we report structure–activity relationship (SAR) studies that focus on exploring three regions of the scaffold represented by compound **4**. We describe the design, synthesis, and biological evaluation of novel compounds, which resulted in the discovery of a potent, selective and cell-active dual inhibitor of PRMT4 and PRMT6, **17** (MS049), and a negative control, **46** (MS049N).

RESULTS

Design and Synthesis. To improve potency and selectivity of **4**, we investigated the following three regions: (1) the right-hand side (RHS) piperidinylethanamine moiety, (2) the middle linker moiety, and (3) the left-hand side (LHS) aromatic moiety (Figure 1B).

The piperidinylethanamine portion of **4** contains an ethylenediamino group, which is also present in the PRMT4 inhibitor **1**,[50] PRMT6 selective inhibitor **2**,[57] and type I PRMT selective inhibitor **3**.[47] The cocrystal structures[47, 48, 50, 57] of these inhibitors in complex with PRMT4 or PRMT6 clearly indicate that the ethylenediamino group forms direct and water-mediated hydrogen bonds (H-bonds) with the proteins in the arginine binding pockets, thus serving as an arginine mimetic. Previous SAR studies suggested that the ethylenediamino group is a key contributor to the PRMT4 and PRMT6 inhibitory activities.[47, 48, 50, 57] We therefore designed a relatively small set of compounds (Scheme 1A and Table 1) to explore the SAR of the piperidinylethanamine region. We focused on investigating substituents on the terminal amino group, the number of carbons between two nitrogen atoms, the substitution position at the piperidinyl ring, and the ring size. Synthetic routes for preparing compounds **5**−**10** are outlined in Scheme 1A. Compounds **5**−**7** and **9**−**10** were synthesized via a standard sodium triacetoxyborohydride mediated reductive amination reaction of commercially available benzylpiperidines or benzylpyrrolidines with corresponding *tert*-butyloxycarbonyl (Boc) protected α- or β-amino aldehydes, followed by the removal of the Boc protecting group under acidic conditions. Compound **8** was synthesized using a nucleophilic substitution reaction between benzylpiperidine and Boc protected aminomethylepoxide, followed by a deprotection reaction.

The X-ray crystal structure of PRMT6−compound **4**−S-5′-adenosyl-*L*-homocysteine (SAH, the cofactor product) complex showed that the middle linker and LHS phenyl moiety of compound **4** make hydrophobic interactions with PRMT6.[48] However, it was not clear what was required for these hydrophobic interactions and whether additional interactions could be made. We therefore explored these two regions to potentially increase H-bond formations and/or polar interactions. For the middle linker, we explored different linker lengths (from no linker to a three-atom linker) as well as different

linker types including various heteroatoms such as O, S, and NH (Scheme 1B and Table 2). In addition, we investigated linkers containing a hydroxyl methylene, alkyl ether, alkyl amine, ester, or amide functional group (Scheme 1B and Table 2). Synthetic routes for preparing **11**−**23** are outlined in Scheme 1B. Compounds **11**−**18** and **20**−**23** were synthesized utilizing reductive amination reactions of commercially available substituted piperidines with *N*-Boc (methylamino)acetaldehyde followed by the removal of the protecting group. Compound **19** was prepared by using a 6-step synthetic sequence. First, reductive amination of aniline with Boc protected piperidine-4-carboxaldehyde provided Boc protected piperidinylmethylaniline, the aniline nitrogen of which was subsequently protected with a trifluoroactyl group. Selective removal of the Boc protecting group produced intermediate **I1**, which underwent another reductive amination reaction. Sequential removal of the protecting groups afforded the desired compound **19**.

To investigate SAR of the LHS phenyl moiety, we selected compound **17** as the starting point because compound **17** was identified as the most potent dual inhibitor of PRMT4 and PRMT6 from our SAR studies on the middle linker region (see Table 2 and discussion below). We designed compounds **24**−**45** to explore a number of electron donating and withdrawing groups with different steric bulkiness at the 2-, 3- or 4-postion at the phenyl ring (Scheme 1C and Table 3). These compounds were synthesized using a 4-step parallel synthetic sequence. Nucleophilic substitution reactions of Boc protected 4-hydroxypiperidine with various substituted benzyl chlorides or bromides and the subsequent deprotection afforded 4-benzyloxypiperidine intermediates **I2**, which were then converted to the desired compounds **24**−**45** via reduction amination with *N*-Boc (methylamino)acetaldehyde, followed by the removal of the Boc protecting group.

All of the synthesized compounds were evaluated in human PRMT4 and PRMT6 biochemical assays, which measure the potency of test compounds at inhibiting the transfer of the methyl group from ^{3}H-SAM to the peptide substrates. IC_{50} values of the compounds are summarized in Tables 1−3.

SAR of the RHS Piperidinylethanamine Moiety. The X-ray crystal structure of **4** in the complex with PRMT6 and SAH revealed that two nitrogen atoms of the piperidinylethanamine moiety formed direct and water mediated H-bonds with Glu155, Glu164, and His317 (Figure 1B).[48] Consistent with this observation, installation of a small substituent on the terminal primary amino group of **4**, which does not disturb these H-bond interactions, was well tolerated, while changing the distance between these two nitrogen atoms, which interrupts these interactions, greatly reduced the potency for both PRMT4 and PRMT6. Specifically, switching the terminal primary amino group (**4**) to a secondary methylamino group provided **5**, which displayed similar potency (PRMT4, IC_{50} = 890 ± 140 nM; PRMT6, IC_{50} = 170 ± 39 nM) as compound **4** (Table 1). On the other hand, a bulkier ethylamino group (**6**) decreased the potency for PRMT4 and PRMT6 by 3- to 4-fold, compared with **4**. Increasing the distance between those two nitrogen atoms using 1,3-diaminopropyl group (**7**) totally abolished the inhibitory activity against both enzymes. Interestingly, adding a free hydroxyl group to the 1,3-diamino-propyl group (**8**) partially recovered the potency for PRMT4 (IC_{50} = 1,220 ± 140 nM) and PRMT6 (IC_{50} = 3,040 ± 270 nM), presumably due to extra H-bond interaction(s). These results confirm that the ethylenediamino group is a preferred moiety for interacting with the arginine binding site. We also explored the piperidinyl region. Switching the 4-benzyl group at the piperidinyl ring to 3-position (**9**) increased potency for PRMT4 (IC_{50} = 120 ± 28 nM) while maintaining potency for PRMT6 (IC_{50} = 290 ± 30 nM). Replacement of the 6-membered piperidinyl ring with the 5-membered pyrrolidinyl ring (**10**) resulted in high potency for both PRMT4 (IC_{50} = 80 ± 28 nM) and PRMT6 (IC_{50} = 70 ± 6 nM). Compound **10** could be a valuable lead for future SAR studies. Because compounds **9** and **10** contain a chiral center and would require enantiomeric resolution to obtain pure enantiomers, we chose **5**, which is achiral, as the starting point to study SAR of the middle linker for the simplicity reason.

SAR of the Middle Linker. We next investigated the middle linker region by exploring various linker lengths and introducing various heteroatoms and functional groups. We postulated that changing the linker length could direct the phenyl group to different locations within the binding site, thus

affecting inhibitor−protein interactions. Furthermore, heteroatoms such as O and NH and various functional groups could increase polar interactions or H-bond formations, and in turn improve potency for PRMT4 and PRMT6. Without the methylene linker, **11** displayed 7-fold and 55-fold potency loss against PRMT4 and PRMT6, respectively, compared with **5** (Table 2). Among analogs with a one-atom linker, thioether (**13**) and hydroxymethine (**15**) linkers slightly impaired the potency, while amino linker (**14**) showed similar potency for PRMT4 (IC_{50} = 616 ± 70 nM) and PRMT6 (IC_{50} = 186 ± 23 nM) as compound **5**. Interestingly, with an ether linker, **12** displayed higher potency (~6-fold) for PRMT4 (IC_{50} = 149 ± 24 nM) and similar potency for PRMT6 (IC_{50} = 90 ± 16 nM) as compared to **5**. Except an amide-linker containing compound (**22)**, the synthesized compounds with a two-atom linker, such as ethylene (**16**), ethers (**17** and **18**), methyleneamine (**19**), ester (**20**) and amide (**21**), displayed higher potency for PRMT4, resulting in inhibitors with almost equal potency for PRMT4 and PRMT6. Among all derivatives, **17** was the most potent dual inhibitor of PRMT4 (IC_{50} = 34 ± 10 nM) and PRMT6 (IC_{50} = 43 ± 7 nM). Compared with **5**, **17** was significantly more potent (~26-fold) for PRMT4 and had moderately improved potency (~4-fold) for PRMT6. The compound with a three-atom ester-linker (**23**), however, was less potent than compound **18**, which has a two-atom ether-linker, and compound **20**, which has a two-atom ester-linker. Overall, a linker with two atoms between the phenyl and piperidinyl groups is preferred and the methyleneoxy linker in compound **17** yields the highest potency for PRMT4 and PRMT6. Thus, compound **17** was used as the starting scaffold to investigate the LHS phenyl moiety.

SAR of the LHS Phenyl Moiety. To examine the effect of substitution at the phenyl moiety of **17**, a number of electron-withdrawing groups, such as F, Cl, Br and CF_3, and electron donating groups, such as phenyl, were installed at the 2-, 3- or 4-position of the phenyl ring. SAR results of these substituted phenyl compounds (**24**−**38**) showed that both electron-deficient and electron-rich groups were well tolerated (Table 3). With the exception of **33**, 4-substituted phenyl groups (**26**, **29**, **32** and **38**) slightly improved (by about 2-fold) potency for PRMT4, compared with **17**. We therefore explored a

few additional substituents (**39**−**45**) at this position. Similar to the substituents in **24**−**38**, these additional substituents (in **39**−**45**) were also well tolerated. In general, substitution at the phenyl ring did not affect PRMT4 and PRMT6 potency drastically. Several compounds (e.g., **30**, **38**, **45**) exhibited about a 2-fold potency improvement over **17** and could serve as leads for future SAR studies. Because compound **17** has the simplest structure, we selected it for full characterization in the following studies.

Characterization of 17 in Selectivity, Biophysical and Mechanism of Action Studies. We evaluated selectivity of compound **17** and its two close analogs (**25** and **37**) against other PRMTs (Table 4). We were pleased to find none of these compounds showed inhibition against type II (PRMT5) and type III (PRMT7) PRMTs. In addition, all three compounds were more potent against PRMT4 and PRMT6 than other type I PRMTs. For example, **17** and **25** had good selectivity over PRMT8 (>30-fold) and excellent selectivity over PRMT1 and PRMT3 (>300-fold). To further assess selectivity of **17**, we tested it against 25 protein lysine methyltransferases (PKMTs), DNA and RNA methyltransferases (DNMTs/RNMTs), 3 histone lysine demethylases (KDMs), and 9 methyllysine and methylarginine reader proteins. We found that **17** did not inhibit any of the methyltransferases at 50 μM (Figure 2) or demethylases (Supporting Figure S1) at 10 μM, and did not display appreciable binding to any of the methyllysine/methylarginine reader proteins using the DSF assay at 200 μM (Supporting Table S1). Moreover, we also evaluated the selectivity of **17** over a broad range of non-epigenetic targets in two selectivity profiling panels: CEREP and PDSP[61]. The CEREP platforms cover 100 GPCRs, ion channels, transporters, and kinases. Compound **17** showed no appreciable inhibition (no more than 50% inhibition at 10,000 nM) in this panel (Supporting Table S2). The PDSP selectivity panel consists of 44 GPCRs. Compound **17** showed no more than 50% inhibition at 10,000 nM against 41 targets and >50% inhibition at 10,000 nM against 3 targets in the panel. K_i determinations in the radioligand binding assay for each of the 3 interacting targets was subsequently performed. Compound **17** had K_i values of 64 nM, 87 nM, and 574 nM for sigma1, histamine H3, and sigma2, respectively (Supporting Table S3). However, it is not clear whether **17** has activity in sigma1, histamine H3 and sigma2 functional assays.

Overall, **17** demonstrated excellent selectivity over a broad range of epigenetic and non-epigenetic targets.

We next confirmed binding of **17** to PRMT4 using isothermal titration calorimetry (ITC) in the presence of SAH with a K_d value of 100 nM (n = 2) (Figure 3A). In the case of PRMT6 binding was confirmed by ITC in the presence of SAM with a K_d value of 87 ± 35 nM (Figure 3B). Binding of **17** to PRMT4 and PRMT6 was confirmed by another orthogonal method, differential scanning fluorimetry (DSF).[62] In DSF experiments, an increase in melting temperature (ΔT_m) of PRMT4 (3.3 °C) and PRMT6 (11.3 °C) was observed upon binding of **17** at 200 μM (Figure 3C and 3D).

To assess mechanism of action (MOA) of **17**, we evaluated the effect of SAM and peptide concentrations on IC_{50} values of **17** against PRMT4 and PRMT6. As illustrated in Figures 3E and 3F, increasing the cofactor SAM or peptide substrate concentrations had no effects on IC_{50} values of **17** for either PRMT4 or PRMT6, suggesting that this inhibitor is noncompetitive with both the cofactor SAM and peptide substrate. This is not surprising as similar noncompetitive inhibition phenomena were observed for arginine binding-site occupying PRMT inhibitors, such as compounds **2**, **3** and **4**.[47, 48, 57] Considering its similar chemical structure to **4**, compound **17** most likely occupies the substrate (arginine) binding site of PRMT4 and PRMT6. It is likely that the binding affinity of the peptide substrate mainly derived from the interactions outside of the arginine-binding site. Thus **17** can't compete off the peptide substrate.[47, 48, 63, 64] Another plausible interpretation is that the binding of **17** induces major protein conformational changes and traditional enzymatic kinetic may not apply.[47]

Cell-based Studies. We first assessed the effect of **17** on inhibition of ectopically expressed PRMT6 in HEK293 cells. Overexpression of the wild type (WT) PRMT6 but not catalytic mutant (V86/D88A) robustly increased the asymmetric dimethylation of histone H3 arginine 2 (H3R2me2a) level (Figure 4A, first two columns).[32] Compound **17** (20 h exposure) reduced the H3R2me2a mark in HEK293 cells in a concentration dependent manner (IC_{50} = 0.97 ± 0.05 μM (n = 3)) (Figure 4A). The effect of the 8 μM **17** treatment matched with that of the catalytically inactive mutant (Figure 4A).

Recently, Med12 was identified as a non-histone substrate of PRMT4.[28] Compound **17** treatment (72 h exposure) inhibited endogenous PRMT4 methyltransferase activity in a concentration dependent manner resulting in reduced levels of cellular asymmetric arginine dimethylation of Med12 (Med12-Rme2a, IC_{50} = 1.4 ± 0.1 μM (n = 3)) in HEK293 cells (Figure 4B). To further confirm the cellular assay results, we designed and synthesized a negative control compound, **46**, by replacing the methyl amino group with the methyl ether group on the RHS of **17** (Figure 5 and Supporting Scheme 1). This single atom switch (from nitrogen to oxygen) completely abolished inhibitory activity against PRMT4 and PRMT6 *in vitro* (Figure 5). As expected, **46** did not decrease cellular levels of H3R2me2a or Med12me2a at concentrations up to 30 μM (Supporting Figure S2A and S2B). It is worth noting that in our experiments further characterizing compound **17**, we observed that **17** unexpectedly reduced asymmetric dimethylation of histone H4 arginine 3 (H4R3me2a) in HEK293 cells while compound **46** did not (Supporting Figure S2C). It was shown previously that PRMT1 is the major isoform responsible for methylation of H4R3, however PRMT6 is also able to methylate H4R3 *in vitro*[65] and in cells when overexpressed.[66] It is unclear what contributes to the inconsistency between the biochemical and cellular assay results, which will be a subject of future investigations. Lastly, to evaluate cell toxicity of **17** and **46,** we investigated effects of 17 and 46 on growth inhibition of HEK293 cells over 4 days (Supporting Figure S3). Both compounds did not display significant cell toxicity at up to 50 μM within the time of the treatment.

CONCLUSIONS

We conducted SAR studies and explored three regions of the fragment-like inhibitor of PRMTs, **4**. By optimizing the three regions of this hit, we discovered compound **17** as a potent, selective and cell-active dual inhibitor of PRMT4 and PRMT6. In biochemical assays, **17** was highly potent and selective for PRMT4 and PRMT6 over a broad range of epigenetic modifiers, including other PRMTs, PKMTs, DNMTs, KDMs, and methyllysine/methylarginine reader proteins, and non-epigenetic targets, including GPCRs, ion channels, transporters, and kinases. In cellular assays, **17** potently inhibited the

methyltransferase activity of PRMT4 and PRMT6 and reduced levels of Med12me2a and H3R2me2a in HEK293 cells. We also discovered **46**, a close analog of **17**, which was inactive in biochemical and cellular assays, as a negative control for chemical biology studies. Both **17** and **46** were not toxic and did not affect the growth of HEK293 cells. Therefore, **17** and **46** are valuable chemical tools for the biomedical community to test biological and therapeutic hypotheses concerning PRMT4 and PRMT6 in cellular systems.

EXPERIMENTAL SECTION

Chemistry General Procedures. HPLC spectra for all compounds were acquired using an Agilent 1200 Series system with DAD detector. Chromatography was performed on a 2.1×150 mm Zorbax 300SB-C_{18} 5 μm column with water containing 0.1% formic acid as solvent A and acetonitrile containing 0.1% formic acid as solvent B at a flow rate of 0.4 mL/min. The gradient program was as follows: 1% B (0−1 min), 1−99% B (1−4 min), and 99% B (4−8 min). High-resolution mass spectra (HRMS) data were acquired in positive ion mode using an Agilent G1969A API-TOF with an electrospray ionization (ESI) source. Nuclear Magnetic Resonance (NMR) spectra were acquired on eith a Bruker DRX-600 spectrometer (600 MHz ^{1}H, 150 MHz ^{13}C) or a Bruker Avance-III 500 MHz spectrometer (500 MHz ^{1}H, 125 MHz ^{13}C). Chemical shifts are reported in ppm (δ). Preparative HPLC was performed on Agilent Prep 1200 series with UV detector set to 254 nm. Samples were injected into a Phenomenex Luna 75 x 30 mm, 5 μm, C_{18} column at room temperature. The flow rate was 40 mL/min. A linear gradient was used with 10% (or 50%) of MeOH (A) in H_2O (with 0.1 % TFA) (B) to 100% of MeOH (A). HPLC was used to establish the purity of target compounds. All final compounds had > 95% purity using the HPLC methods described above. Because compound **20** was not very stable when treated with HCl, the free base form was used in biological assays. All of the rest of compounds were tested in biological assays using their HCl salt forms.

General procedure A for reductive amination (step a). To a solution of the amine (1 equiv.) in CH_2Cl_2 (0.13 M solution) was added aldehyde (1.1 equiv.). The resulting solution was stirred at room

temperature for 15 mins prior to the addition of sodium triacetoxyborohydride (1.4 equiv.), after which the solution was stirred for an additional 16 h. The reaction was then quenched with aqueous sodium bicarbonate solution and extracted with EtOAc (3 x). The combined organic extracts were dried with anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure. The residue was then purified by silica gel column chromatography to afford the Boc protected amine. (Note: When the amine starting material is in the HCl salt form, Et_3N (1.1 equiv.) was added to the reaction before aldehyde addition.)

General procedure B for Boc deprotection (step b). To a solution of the Boc protected amine (1 equiv.) in CH_2Cl_2 (0.20 M solution) was added HCl/dioxane (4 M solution, 10 equiv.). The resulting solution was stirred at room temperature for 2 h prior to removal of all solvents under reduced pressure, and dissolution of the residue in water. The aqueous solution was frozen, and lyophilized for 16 h to afford the hydrochloride salt. Alternatively, HCl/MeOH (3 M solution, 10 equiv.) was used to remove the Boc protecting group at rt for 12 h.

General procedure C for benzyl ether formation (step g). To a solution of *tert*-butyl 4-hydroxypiperidine-1-carboxylate (1 equiv.) in DMF (0.13 M solution) was added NaH (60% dispersion in mineral oil, 2 equiv.) in portions at 0 °C. After 15 min, a solution of benzyl bromide or chloride (2 equiv.) in DMF (1 M solution) was added dropwise. The resulting mixture was slowly warmed to room temperature and stirred for overnight, before being quenched with water. The reaction was extracted with CH_2Cl_2 (3 x). The combined organic extracts were dried with anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure. The resulting residue was purified by silica gel column chromatography to afford the desired benzyl ether.

General procedure D for converting compound 17 and 24−46 to free base form. The ^1H NMR of the HCl salt contains different sets of signals, presumably due to different salt forms. A small portion of the HCl salt was converted to free base by washing with aqueous saturated $NaHCO_3$ and extraction with EtOAc. The combined organic layers were dried over Na_2SO_4 and concentrated. The resulting residue was used directly to obtain NMR spectra. Note: The HPLC-MS spectra were identical for the HCl salt

form and free base form. In addition, the HCl salt form and free-base form of compound **17** showed identical results in the biochemical assay.

2-(4-Benzylpiperidin-1-yl)-*N*-methylethan-1-amine (5). The title compound was prepared according to the general procedure A and B (HCl/dioxane) (68% over two steps). ^{1}H NMR (500 MHz, DMSO-d_6): δ 10.90 (br s, 1H), 10.18 (br s, 2H), 7.30 – 7.24 (m, 2H), 7.20 – 7.13 (m, 3H), 3.74 – 3.65 (m, 1H), 3.53 – 3.45 (m, 1H), 2.81 – 2.76 (m, 2H), 2.53 – 2.47 (m, 2H), 2.30 (t, J = 6.5 Hz, 2H), 2.26 (s, 3H), 1.85 – 1.75 (m, 2H), 1.54 – 1.48 (m, 2H), 1.48 – 1.41 (m, 1H), 1.22 – 1.10 (m, 2H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 139.9, 129.4 (2), 128.8 (2), 126.5, 52.7, 52.2 (2), 42.7, 41.9, 35.3, 32.7, 29.1 (2). HRMS (ESI-TOF) *m/z*: $[M+H]^+$ calcd for $C_{15}H_{25}N_2$, 233.2018; found: 233.2019.

2-(4-Benzylpiperidin-1-yl)-*N*-ethylethan-1-amine (6). The title compound was prepared according to the general procedure A and B (HCl/MeOH) (64% over two steps). ^{1}H NMR (600 MHz, CD_3OD): δ 7.29 (t, J = 7.5 Hz, 2H), 7.23 – 7.13 (m, 3H), 3.66 (d, J = 12.2 Hz, 2H), 3.55 – 3.48 (m, 2H), 3.46 (t, J = 6.6 Hz, 2H), 3.15 (q, J = 7.2 Hz, 2H), 3.07 – 2.96 (m, 2H), 2.62 (d, J = 6.8 Hz, 2H), 1.96 – 1.84 (m, 3H), 1.74 – 1.59 (m, 2H), 1.36 (t, J = 7.3 Hz, 3H). HRMS (ESI-TOF) *m/z*: $[M+H]^+$ calcd for $C_{16}H_{27}N_2$, 247.2174; found: 247.2176.

3-(4-Benzylpiperidin-1-yl)-*N*-methylpropan-1-amine (7). The title compound was prepared according to the general procedure A and B (HCl/MeOH) (68% over two steps). ^{1}H NMR (600MHz, CD_3OD): δ 7.29 (t, J = 7.6 Hz, 2H), 7.23 – 7.14 (m, 3H), 3.59 (d, J = 12.2 Hz, 2H), 3.20 (t, J = 8.1 Hz, 2H), 3.11 (t, J = 7.8 Hz, 2H), 2.97 (t, J = 12.8 Hz, 2H), 2.74 (s, 3H), 2.62 (d, J = 6.6 Hz, 2H), 2.24 – 2.11 (m, 2H), 1.98 – 1.82 (m, 3H), 1.66 – 1.51 (m, 2H). HRMS (ESI-TOF) *m/z*: $[M+H]^+$ calcd for $C_{16}H_{27}N_2$, 247.2174; found: 247.2165.

1-(4-Benzylpiperidin-1-yl)-3-(methylamino)propan-2-ol (8). To a solution of 4-benzylpiperidine (0.053 g, 0.3 mmol) in *i*-PrOH (5 mL) was added *tert*-butyl methyl(oxiran-2-ylmethyl)carbamate (0.067 g, 0.36 mmol). The reaction solution was heated at 80 °C for 16 h before being concentrated and passed

through a short silica gel plug. After concentration under reduced pressure, the Boc protecting group was remove using the general procedure B (HCl/MeOH) (53% over two steps). ^{1}H NMR (600 MHz, CD_3OD): δ 7.29 (t, *J* = 7.6 Hz, 2H), 7.23 – 7.17 (m, 3H), 4.47 – 4.39 (m, 1H), 3.72 – 3.65 (m, 1H), 3.64 – 3.59 (m, 1H), 3.24 – 3.13 (m, 3H), 3.10 – 2.93 (m, 3H), 2.75 (s, 3H), 2.62 (d, *J* = 6.8 Hz, 2H), 1.96 – 1.84 (m, 3H), 1.70 – 1.53 (m, 2H). HRMS (ESI-TOF) *m/z*: $[M+H]^+$ calcd for $C_{16}H_{27}N_2O$, 263.2123; found: 263.2122.

2-(3-Benzylpiperidin-1-yl)-*N*-methylethan-1-amine ((±)-9). The title compound was prepared according to the general procedure A and B (HCl/dioxane) (77% over two steps). ^{1}H NMR (500 MHz, DMSO-d_6): δ 10.90 (br s, 1H), 9.53 (br s, 2H), 7.35 – 7.29 (m, 2H), 7.26 – 7.20 (m, 1H), 7.20 – 7.16 (m, 2H), 3.74 – 3.65 (dm, 1H), 3.57 – 3.40 (m, 6H), 2.90 – 2.80 (m, 1H), 2.80 – 2.70 (m, 1H), 2.65 – 2.45 (dm, 1H), 2.57 (s, 3H), 2.25 – 2.15 (m, 1H), 1.85 – 1.75 (m, 2H), 1.70 – 1.61 (m, 1H), 1.20 – 1.06 (m, 1H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 138.9, 129.5 (2), 128.9 (2), 126.8, 57.2, 53.6, 52.4, 42.6, 39.1 (hidden by solvent), 35.6, 32.7, 27.8, 22.5. HRMS (ESI-TOF) *m/z*: $[M+H]^+$ calcd for $C_{15}H_{25}N_2$, 233.2018; found: 233.2017.

2-(3-Benzylpyrrolidin-1-yl)-*N*-methylethan-1-amine ((±)-10). The title compound was prepared according to the general procedure A and B (HCl/dioxane) (80% over two steps). ^{1}H NMR (500 MHz, DMSO-d_6): δ 11.40 (br s, 1H), 9.42 (br s, 2H), 7.35 – 7.29 (m, 2H), 7.28 – 7.20 (m, 3H), 3.75 – 3.65 (m, 1H), 3.65-3.55 (m, 1H), 3.55-3.45 (m, 3H), 3.35 – 3.10 (m, 3H), 2.90 – 2.66 (m, 3H), 2.58 (s, 3H), 2.20 – 1.95 (m, 1H), 1.85 – 1.55 (m, 1H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 140.1, 129.1 (2), 129.0 (2), 126.8, 60.7, 58.1, 52.9, 44.1, 44.0, 39.1, 38.4, 32.7. HRMS (ESI-TOF) *m/z*: $[M+H]^+$ calcd for $C_{14}H_{23}N_2$, 219.1861; found: 219.1862.

***N*-Methyl-2-(4-phenylpiperidin-1-yl)ethan-1-amine (11).** The title compound was prepared according to the general procedure A and B (HCl/dioxane) (72% over two steps). ^{1}H NMR (500 MHz, DMSO-d_6): δ 10.99 (br s, 1H), 9.45 (br s, 2H), 7.38 – 7.32 (m, 2H), 7.28 – 7.22 (m, 3H), 3.73 – 3.65 (m, 2H), 3.50 – 3.52 (m, 4H), 3.20 – 3.09 (m, 2H), 2.90 – 2.81 (m, 1H), 2.62 (s, 3H), 2.16 – 2.06 (m, 2H), 2.04 – 1.96

(m, 2H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 144.6, 129.1 (2), 127.1, 127.0 (2), 53.0, 52.2 (2), 42.8, 38.9, 32.8, 30.2 (2). HRMS (ESI-TOF) *m/z*: $[M+H]^+$ calcd for $C_{14}H_{23}N_2$, 219.1861; found: 219.1859.

***N*-Methyl-2-(4-phenoxypiperidin-1-yl)ethan-1-amine (12).** The title compound was prepared according to the general procedure A and B (HCl/dioxane) (60% over two steps). ^{1}H NMR (500 MHz, DMSO-d_6): δ 11.15 (br s, 1H), 9.52 (s, 2H), 7.35 – 7.28 (m, 2H), 7.08 – 6.93 (m, 3H), 3.70 – 3.63 (m, 2H), 3.54 – 3.43 (m, 5H), 3.27 – 3.11 (m, 2H), 2.60 (s, 3H), 2.30 – 2.15 (m, 2H), 2.11 – 1.92 (m, 2H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 157.1, 130.1 (2), 121.8, 116.8 (2), 70.5, 51.1, 48.0 (2), 42.7, 32.7, 28.6, 26.7. HRMS (ESI-TOF) *m/z*: $[M+H]^+$ calcd for $C_{14}H_{23}N_2O$, 235.1810; found: 235.1811.

***N*-Methyl-2-(4-(phenylthio)piperidin-1-yl)ethan-1-amine (13).** The title compound was prepared according to the general procedure A and B (HCl/dioxane) (81% over two steps). ^{1}H NMR (500 MHz, DMSO-d_6): δ 11.26 – 10.94 (br s, 1H), 9.49 (s, 2H), 7.46 – 7.42 (m, 2H), 7.40 – 7.35 (m, 2H), 7.32 – 7.28 (m, 1H), 3.64 – 3.55 (m, 2H), 3.55 – 3.40 (m, 5H), 3.15 – 3.00 (m, 2H), 2.58 (s, 3H), 2.14 – 2.04 (m, 2H), 1.98 – 1.82 (m, 2H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 133.4, 131.6 (2), 129.7 (2), 127.6, 52.4 (2), 51.9, 42.6, 40.0 (hidden by solvent), 32.6, 29.7 (2). HRMS (ESI-TOF) *m/z*: $[M+H]^+$ calcd for $C_{14}H_{23}N_2S$, 251.1582; found: 251.1577.

1-(2-(Methylamino)ethyl)-*N*-phenylpiperidin-4-amine (14). The title compound was prepared according to the general procedure A and B (HCl/dioxane) (78% over two steps). ^{1}H NMR (500 MHz, DMSO-d_6): δ 11.00 – 10.80 (br s, 1H), 9.52 (s, 2H), 7.44 – 7.27 (m, 2H), 7.26 – 7.03 (m, 3H), 4.95 (br s, 1H), 3.70 – 3.63 (m, 2H), 3.52 – 3.45 (m, 1H), 3.45 – 3.35 (m, 4H), 3.15 – 3.04 (m, 2H), 2.59 (t, J = 5.0 Hz, 3H), 2.22 – 2.10 (m, 2H), 2.08 – 1.94 (m, 2H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 137.7, 130.2 (2), 126.5, 121.6 (2), 66.8, 51.9, 51.3 (2), 42.7, 32.8, 26.8 (2). HRMS (ESI-TOF) *m/z*: $[M+H]^+$ calcd for $C_{14}H_{24}N_3$, 234.1970; found: 234.1968.

(1-(2-(Methylamino)ethyl)piperidin-4-yl)(phenyl)methanol (15). The title compound was prepared according to the general procedure A and B (HCl/dioxane) (85% over two steps). ^{1}H NMR (500 MHz,

DMSO-d_6): δ 10.46 (br s, 1H), 9.39 (br s, 2H), 7.36 – 7.29 (m, 4H), 7.29 – 7.24 (m, 1H), 5.45 (br s, 1H), 4.30 (br s, 1H), 3.74 – 3.65 (dm, 1H), 3.62 – 3.54 (m, 2H), 3.54 – 3.44 (m, 2H), 2.95 – 2.80 (m, 2H), 2.58 (s, 3H), 1.98 – 1.90 (m, 1H), 1.80 – 1.70 (m, 1H), 1.70 – 1.57 (m, 2H), 1.46 – 1.37 (m, 1H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 144.4, 128.5 (2), 127.5, 126.9 (2), 66.8, 52.8, 52.6, 52.2, 42.8, 41.0, 32.8, 26.2, 25.3. HRMS (ESI-TOF) *m/z*: $[M+H]^+$ calcd for $C_{15}H_{25}N_2O$, 249.1967; found: 249.1966.

***N*-Methyl-2-(4-phenethylpiperidin-1-yl)ethan-1-amine (16).** The title compound was prepared according to the general procedure A and B (HCl/dioxane) (69% over two steps). ^{1}H NMR (500 MHz, DMSO-d_6): δ 10.77 (br s, 1H), 9.56 (br s, 2H), 7.30 – 7.25 (m, 2H), 7.24 – 7.14 (m, 3H), 3.75 – 3.63 (m, 2H), 3.63 – 3.43 (m, 7H), 3.00 – 2.90 (m, 2H), 2.51 (s, 3H), 1.93 – 1.86 (m, 2H), 1.60 – 1.45 (m, 4H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 142.4, 128.8 (2), 128.7 (2), 126.2, 52.8 (2), 52.1, 42.7, 37.6, 32.8, 32.7, 32.5, 29.2 (2). HRMS (ESI-TOF) *m/z*: $[M+H]^+$ calcd for $C_{16}H_{27}N_2$, 247.2174; found: 247.2172.

2-(4-(Benzyloxy)piperidin-1-yl)-*N*-methylethan-1-amine (17). The title compound was prepared according to the general procedure A (with Et_3N) and B (HCl/MeOH) (64% over two steps). A small portion of the HCl salt was converted to the free base form using the general procedure D to obtain the NMR sample. ^{1}H NMR (600 MHz, CD_3OD): δ 7.39 – 7.30 (m, 4H), 7.29 – 7.22 (m, 1H), 4.55 (s, 2H), 3.54 – 3.44 (m, 1H), 2.84 – 2.74 (m, 2H), 2.71 (t, J = 6.8 Hz, 2H), 2.50 (t, J = 6.8 Hz, 2H), 2.41 (s, 3H), 2.28 – 2.16 (m, 2H), 2.00 – 1.89 (m, 2H), 1.71 – 1.59 (m, 2H); ^{13}C NMR (150 MHz, CD_3OD): δ 140.2, 129.3, 128.8, 128.6, 75.4, 70.9, 58.2, 52.4, 49.3, 36.1, 32.0. HRMS (ESI-TOF) *m/z*: $[M+H]^+$ calcd for $C_{15}H_{25}N_2O$, 249.1967; found: 249.1970.

***N*-Methyl-2-(4-(phenoxymethyl)piperidin-1-yl)ethan-1-amine (18).** The title compound was prepared according to the general procedure A and B (HCl/MeOH) (67% over two steps). ^{1}H NMR (600 MHz, CD_3OD): δ 7.26 (t, J = 7.8 Hz, 2H), 6.96 – 6.88 (m, 3H), 3.91 (d, J = 5.6 Hz, 2H), 3.75 (d, J = 12.0 Hz, 2H), 3.59 – 3.47 (m, 4H), 3.14 (t, J = 12.5 Hz, 2H), 2.81 (s, 3H), 2.21 – 2.10 (m, 3H), 1.90 – 1.77 (m, 2H). HRMS (ESI-TOF) *m/z*: $[M+H]^+$ calcd for $C_{15}H_{25}N_2O$, 249.1967; found: 249.1969.

***N*-((1-(2-(Methylamino)ethyl)piperidin-4-yl)methyl)aniline (19).** To a solution of aniline (0.027 mL, 0.3 mmol) in CH_2Cl_2 (5 mL) was added *tert*-butyl 4-formylpiperidine-1-carboxylate (0.64 g, 0.3 mmol). The resulting solution was stirred for 30 min before being treated with sodium triacetoxyborohydride (0.095 g, 0.45 mmol) for 16 h. The reaction was quenched with aqueous saturated $NaHCO_3$ and the product was extracted with EtOAc (3 x). The combined organic layers were dried over Na_2SO_4 and concentrated. The resulting residue was passed through a short silica gel plug to get the crude desired product. The resulting intermediate (0.087 g, 0.3 mmol) was dissolved in CH_2Cl_2 (5 mL). To the solution was added triethylamine (0.084 mL, 0.6 mmol) followed by the addition of trifluoroacetic anhydride (0.054 mL, 0.36 mmol) at 0 °C. The reaction was allowed to warm to rt and stirred for 16 h at rt. The reaction was quenched with aqueous saturated $NaHCO_3$ and the product was extracted with EtOAc (3 x). The combined organic layers were dried over Na_2SO_4 and concentrated. The resulting residue was purified by silica gel column to provide *tert*-butyl 4-((2,2,2-trifluoro-*N*-phenylacetamido)methyl)piperidine-1-carboxylate (0.098 g, 85% over 2 steps). ^{1}H NMR (600 MHz, CD_3OD): δ 7.54 – 7.42 (m, 3H), 7.36 (d, J = 7.4 Hz, 2H), 4.05 (d, J = 13.4 Hz, 2H), 3.71 (d, J = 7.3 Hz, 2H), 2.86 – 2.58 (m, 2H), 1.83 – 1.72 (m, 1H), 1.68 (d, J = 13.3 Hz, 2H), 1.45 (s, 9H), 1.22 – 1.10 (m, 2H). To a solution of *tert*-butyl 4-((2,2,2-trifluoro-*N*-phenylacetamido)methyl)piperidine-1-carboxylate (0.077g, 0.2 mmol) in CH_2Cl_2 (2 mL) was added trifluoroacetic acid (1 mL). The reaction was stirred for 2 h at rt before being concentrated under reduce pressure. After the resulting residue was dissolved in CH_2Cl_2 (5 mL), *tert*-butyl methyl(2-oxoethyl)carbamate (0.035 g, 0.2 mmol) was added. The solution was stirred for 30 mins, before triethylamine (0.041 mL, 0.3 mmol) and sodium triacetoxyborohydride (0.064 g, 0.3 mmol) were added. The reaction was stirred for 16 h at rt. The reaction was quenched with aqueous saturated $NaHCO_3$ and the product was extracted with EtOAc (3 x). The combined organic layers were dried over Na_2SO_4 and concentrated. The resulting residue was passed through a short silica gel plug. After evaporation of solvents, the resulting residue was dissolved in methanol (2.5 mL) and aqueous NaOH (5 N, 0.5 mL). The reaction mixture was heated at 50 °C for 2 h before being diluted with EtOAc and water. The mixture was extracted with EtOAc (3 x). The combined organic layers were

dried over Na_2SO_4 and concentrated. The residue was purified by silica gel column to provide *tert*-butyl methyl(2-(4-((phenylamino)methyl)piperidin-1-yl)ethyl)carbamate. A solution of *tert*-butyl methyl(2-(4-((phenylamino)methyl)piperidin-1-yl)ethyl)carbamate in 3 N HCl (2 mL) was stirred for 12 h. After concentration of the reaction mixture under reduced pressure, the resulting residue was dissolved in MeOH (0.5 mL). The resulting solution was triturated with ethyl ether to precipitate the desired product as white solid (0.047 g, 66% over 4 steps). ^{1}H NMR (600 MHz, D_2O): δ 7.43 (t, J = 7.7 Hz, 2H), 7.37 (t, J = 7.4 Hz, 1H), 7.30 (d, J = 7.9 Hz, 2H), 3.65 – 3.47 (br s, 2H), 3.40 (s, 4H), 3.31 (d, J = 6.6 Hz, 2H), 3.09 – 2.88 (m, 2H), 2.67 (s, 3H), 2.09 – 1.93 (m, 3H), 1.62 – 1.44 (m, 2H). HRMS (ESI-TOF) *m/z*: $[M+H]^+$ calcd for $C_{15}H_{26}N_3$, 248.2127; found: 248.2130.

1-(2-(Methylamino)ethyl)piperidin-4-yl benzoate (20). The title compound was prepared according to the general procedure A and B (HCl/MeOH). The product was further purified by prep-HPLC. After evaporation of solvents, the residue was basified with aqueous saturated $NaHCO_3$ and extracted with CH_2Cl_2 (3x). The combined organic layers were dried over Na_2SO_4 and concentrated to provide desired product as a free base (65% over two steps). Note: Conversion the free base to its HCl salt was attempted but led to slow decomposition. ^{1}H NMR (600 MHz, CD_3OD): δ 8.06 – 7.99 (m, 2H), 7.66 – 7.57 (m, 1H), 7.48 (t, J = 7.8 Hz, 2H), 5.10 – 4.99 (m, 1H), 2.93 (t, J = 6.3 Hz, 2H), 2.84 – 2.74 (m, 2H), 2.61 (t, J = 6.3 Hz, 2H), 2.57 (s, 3H), 2.50 – 2.41 (m, 2H), 2.09 – 2.00 (m, 2H), 1.93 – 1.82 (m, 2H). HRMS (ESI-TOF) *m/z*: $[M+H]^+$ calcd for $C_{15}H_{23}N_2O_2$, 263.1760; found: 263.1761.

***N*-(1-(2-(Methylamino)ethyl)piperidin-4-yl)benzamide (21).** The title compound was prepared according to the general procedure A and B (HCl/MeOH). The product was further purified by prep-HPLC. After evaporation of solvents, the residue was basified with aqueous saturated $NaHCO_3$ and extracted with CH_2Cl_2 (3x). The combined organic layers were dried over Na_2SO_4 and concentrated. The resulting residue was dissolved in 3 mL of MeOH and was treated with 3 N HCl in MeOH (0.5 mL) for 1 h. After concentration, the title compound was obtained as white solid (59% over two steps). ^{1}H NMR (600 MHz, CD_3OD): δ 7.83 (d, J = 7.7 Hz, 2H), 7.59 – 7.50 (m, 1H), 7.47 (t, J = 7.6 Hz, 2H),

4.20 (t, *J* = 12.2 Hz, 1H), 3.79 (d, *J* = 12.3 Hz, 2H), 3.59 – 3.52 (m, 4H), 3.25 (t, *J* = 12.8 Hz, 2H), 2.81 (s, 3H), 2.30 – 2.24 (m, 2H), 2.17 – 2.02 (m, 2H). HRMS (ESI-TOF) *m/z*: $[M+H]^+$ calcd for $C_{15}H_{24}N_3O$, 262.1919; found: 262.1920.

1-(2-(Methylamino)ethyl)-*N*-phenylpiperidine-4-carboxamide (22). The title compound was prepared according to the general procedure A and B (HCl/dioxane) (65% over two steps). ^{1}H NMR (500 MHz, DMSO-d_6): δ 10.94 (br s, 1H), 10.67 (br s, 1H), 10.22 (br s, 2H), 7.57 (d, *J* = 8.0 Hz, 2H), 7.25 – 7.20 (m, 2H), 6.99 – 6.94 (m, 1H), 3.63 – 3.55 (m, 2H), 3.44 – 3.29 (m, 6H), 3.04 – 2.91 (m, 2H), 2.68 – 2.58 (m, 1H), 2.45 – 2.42 (m, 1H), 2.10 – 1.89 (m, 4H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 172.2, 139.6, 129.1 (2), 123.7, 119.7 (2), 52.3, 52.0 (2), 42.8, 38.7 (hidden by solvent), 32.8, 26.1 (2). HRMS (ESI-TOF) *m/z*: $[M+H]^+$ calcd for $C_{15}H_{24}N_3O$, 262.1919; found: 262.1921.

(1-(2-(Methylamino)ethyl)piperidin-4-yl)methyl benzoate (23). The title compound was prepared according to the general procedure A and B (HCl/MeOH). The product was further purified by prep-HPLC. After evaporation of solvents, the residue was basified with aqueous saturated $NaHCO_3$ and extracted with CH_2Cl_2 (3x). The combined organic layers were dried over Na_2SO_4 and concentrated. The resulting residue was dissolved in 3 mL of MeOH and was treated with 3 N HCl in MeOH (0.5 mL) for 1 h. After concentration, the title compound was obtained as white solid (62% over two steps). ^{1}H NMR (600 MHz, CD_3OD): δ 8.10 – 8.02 (m, 2H), 7.65 – 7.60 (m, 1H), 7.49 (t, *J* = 7.8 Hz, 1H), 4.28 (d, *J* = 5.8 Hz, 2H), 3.75 (d, *J* = 12.1 Hz, 2H), 3.58 – 3.48 (m, 4H), 3.19 – 3.09 (m, 2H), 2.81 (s, 3H), 2.24 – 2.15 (m, 1H), 2.15 – 2.07 (m, 2H), 1.97 – 1.84 (m, 2H).

2-(4-((2-Fluorobenzyl)oxy)piperidin-1-yl)-*N*-methylethan-1-amine (24). The title compound was prepared in four steps according to the general procedure C, B (HCl/MeOH), A (with Et_3N) and B (HCl/MeOH) (35% over four steps). A small portion of the HCl salt was converted to the free base form using the general procedure D to obtain the NMR sample. ^{1}H NMR (600 MHz, CD_3OD): δ 7.44 (t, *J* = 7.1 Hz, 1H), 7.36 – 7.24 (m, 1H), 7.15 (t, *J* = 7.4 Hz, 1H), 7.06 (t, 1H), 4.60 (s, 2H), 3.58 – 3.39 (m,

1H), 2.86 – 2.73 (m, 2H), 2.71 (t, *J* = 6.8 Hz, 2H), 2.50 (t, *J* = 6.8 Hz, 2H), 2.41 (s, 3H), 2.30 – 2.16 (m, 2H), 2.06 – 1.84 (m, 2H), 1.77 – 1.58 (m, 2H). MS (ESI) *m/z* 267.2 $[M+H]^+$.

2-(4-((3-Fluorobenzyl)oxy)piperidin-1-yl)-*N*-methylethan-1-amine (25). The title compound was prepared in four steps according to the general procedure C, B (HCl/MeOH), A (with Et_3N) and B (HCl/MeOH) (38% over four steps). A small portion of the HCl salt was converted to the free base form using the general procedure D to obtain the NMR sample. ^{1}H NMR (600 MHz, CD_3OD): δ 7.39 – 7.24 (m, 1H), 7.14 (d, *J* = 7.6 Hz, 1H), 7.08 (d, *J* = 9.8 Hz, 1H), 6.98 (t, *J* = 8.5 Hz, 1H), 4.55 (s, 2H), 3.55 – 3.39 (m, 1H), 2.89 – 2.66 (m, 4H), 2.52 (t, *J* = 6.7 Hz, 2H), 2.46 (s, 3H), 2.33 – 2.07 (m, 2H), 2.00 – 1.90 (m, 2H), 1.77 – 1.58 (m, 2H). ^{13}C NMR (150 MHz, CD_3OD): δ 163.7, 162.1, 141.9, 129.7, 122.7, 113.7, 74.2, 68.6, 55.9, 50.8, 47.4, 34.1, 30.6. MS (ESI) *m/z* 267.2 $[M+H]^+$. HRMS (ESI-TOF) *m/z*: $[M+H]^+$ calcd for $C_{15}H_{24}FN_2O$, 267.1867; found 267.1874.

2-(4-((4-Fluorobenzyl)oxy)piperidin-1-yl)-*N*-methylethan-1-amine (26). The title compound was prepared in four steps according to the general procedure C, B (HCl/MeOH), A (with Et_3N) and B (HCl/MeOH) (44% over four steps). A small portion of the HCl salt was converted to the free base form using the general procedure D to obtain the NMR sample. ^{1}H NMR (600 MHz, CD_3OD): δ 7.58 – 7.24 (m, 2H), 7.23 – 6.72 (m, 2H), 4.51 (s, 2H), 3.64 – 3.40 (m, 1H), 2.89 – 2.73 (m, 4H), 2.54 (t, *J* = 6.6 Hz, 2H), 2.50 (s, 3H), 2.33 – 2.17 (m, 2H), 2.03 – 1.86 (m, 2H), 1.72 – 1.58 (m, 2H). MS (ESI) *m/z* 267.2 $[M+H]^+$.

2-(4-((2-Chlorobenzyl)oxy)piperidin-1-yl)-*N*-methylethan-1-amine (27). The title compound was prepared in four steps according to the general procedure C, B (HCl/MeOH), A (with Et_3N) and B (HCl/MeOH) (30% over four steps). A small portion of the HCl salt was converted to the free base form using the general procedure D to obtain the NMR sample. ^{1}H NMR (600 MHz, CD_3OD): δ 7.51 (d, *J* = 7.1 Hz, 1H), 7.36 (d, *J* = 8.4 Hz, 1H), 7.32 – 7.23 (m, 2H), 4.62 (s, 2H), 3.68 – 3.45 (m, 1H), 2.93 – 2.71 (m, 4H), 2.54 (t, *J* = 6.5 Hz, 2H), 2.49 (s, 3H), 2.33 – 2.21 (m, 2H), 2.04 – 1.91 (m, 2H), 1.77 – 1.61 (m, 2H). MS (ESI) *m/z* 283.1 $[M+H]^+$.

2-(4-((3-Chlorobenzyl)oxy)piperidin-1-yl)-*N*-methylethan-1-amine (28). The title compound was prepared in four steps according to the general procedure C, B (HCl/MeOH), A (with Et_3N) and B (HCl/MeOH) (35% over four steps). A small portion of the HCl salt was converted to the free base form using the general procedure D to obtain the NMR sample. ^{1}H NMR (600 MHz, CD_3OD): δ 7.36 (s, 1H), 7.33 – 7.28 (m, 1H), 7.28 – 7.23 (m, 2H), 4.53 (s, 2H), 3.53 – 3.43 (m, 1H), 2.85 (t, *J* = 6.5 Hz, 2H), 2.82 – 2.74 (m, 2H), 2.54 (t, *J* = 6.5 Hz, 2H), 2.52 (s, 3H), 2.35 – 2.18 (m, 2H), 2.01 – 1.87 (m, 2H), 1.74 – 1.58 (m, 2H). MS (ESI) *m/z* 283.1 $[M+H]^+$.

2-(4-((4-Chlorobenzyl)oxy)piperidin-1-yl)-*N*-methylethan-1-amine (29). The title compound was prepared in four steps according to the general procedure C, B (HCl/MeOH), A (with Et_3N) and B (HCl/MeOH) (44% over four steps). A small portion of the HCl salt was converted to the free base form using the general procedure D to obtain the NMR sample. ^{1}H NMR (600 MHz, CD_3OD): δ 7.32 (s, 4H), 4.52 (s, 2H), 3.52 – 3.44 (m, 1H), 2.77 (t, *J* = 6.5 Hz, 4H), 2.51 (t, *J* = 6.7 Hz, 2H), 2.46 (s, 3H), 2.30 – 2.16 (m, 2H), 1.99 – 1.89 (m, 2H), 1.72 – 1.59 (m, 2H). MS (ESI) *m/z* 283.1 $[M+H]^+$.

2-(4-((2-Bromobenzyl)oxy)piperidin-1-yl)-*N*-methylethan-1-amine (30). The title compound was prepared in four steps according to the general procedure C, B (HCl/MeOH), A (with Et_3N) and B (HCl/MeOH) (27% over four steps). A small portion of the HCl salt was converted to the free base form using the general procedure D to obtain the NMR sample. ^{1}H NMR (600 MHz, CD_3OD): δ 7.55 (d, *J* = 7.9 Hz, 1H), 7.50 (d, *J* = 7.3 Hz, 1H), 7.34 (t, *J* = 7.3 Hz, 1H), 7.18 (t, *J* = 8.2 Hz, 1H), 4.58 (s, 2H), 3.59 – 3.48 (m, 1H), 2.84 – 2.69 (m, 4H), 2.52 (t, *J* = 6.7 Hz, 2H), 2.46 (s, 3H), 2.32 – 2.18 (m, 2H), 2.04 – 1.88 (m, 2H), 1.78 – 1.60 (m, 2H). MS (ESI) *m/z* 327.1 $[M+H]^+$.

2-(4-((3-Bromobenzyl)oxy)piperidin-1-yl)-*N*-methylethan-1-amine (31). The title compound was prepared in four steps according to the general procedure C, B (HCl/MeOH), A (with Et_3N) and B (HCl/MeOH) (38% over four steps). A small portion of the HCl salt was converted to the free base form using the general procedure D to obtain the NMR sample. ^{1}H NMR (600 MHz, CD_3OD): δ 7.51 (s, 1H), 7.41 (d, *J* = 7.8 Hz, 1H), 7.30 (d, *J* = 7.6 Hz, 1H), 7.24 (t, *J* = 7.8 Hz, 1H), 4.52 (s, 2H), 3.52 – 3.43 (m,

1H), 2.78 (t, *J* = 6.6 Hz, 4H), 2.52 (t, *J* = 6.6 Hz, 2H), 2.47 (s, 3H), 2.30 – 2.18 (m, 2H), 1.99 – 1.90 (m, 2H), 1.72 – 1.60 (m, 2H). MS (ESI) *m/z* 327.0 $[M+H]^+$.

2-(4-((4-Bromobenzyl)oxy)piperidin-1-yl)-*N*-methylethan-1-amine (32). The title compound was prepared in four steps according to the general procedure C, B (HCl/MeOH), A (with Et_3N) and B (HCl/MeOH) (30% over four steps). A small portion of the HCl salt was converted to the free base form using the general procedure D to obtain the NMR sample. ^{1}H NMR (600 MHz, CD_3OD): δ 7.48 (d, *J* = 8.3 Hz, 2H), 7.27 (d, *J* = 8.2 Hz, 2H), 4.51 (s, 2H), 3.53 – 3.43 (m, 1H), 2.81 (t, *J* = 6.5 Hz, 4H), 2.53 (t, *J* = 6.6 Hz, 2H), 2.48 (s, 3H), 2.30 – 2.17 (m, 2H), 2.04 – 1.88 (m, 2H), 1.72 – 1.60 (m, 2H). MS (ESI) *m/z* 327.0 $[M+H]^+$.

***N*-Methyl-2-(4-((2-(trifluoromethyl)benzyl)oxy)piperidin-1-yl)ethan-1-amine (33).** The title compound was prepared in four steps according to the general procedure C, B (HCl/MeOH), A (with Et_3N) and B (HCl/MeOH) (45% over four steps). A small portion of the HCl salt was converted to the free base form using the general procedure D to obtain the NMR sample. ^{1}H NMR (600 MHz, CD_3OD): δ 7.72 (d, *J* = 7.7 Hz, 1H), 7.66 (d, *J* = 7.8 Hz, 1H), 7.61 (t, *J* = 7.5 Hz, 1H), 7.44 (t, *J* = 7.6 Hz, 1H), 4.70 (s, 2H), 3.58 – 3.45 (m, 1H), 2.85 – 2.77 (m, 2H), 2.74 (t, *J* = 6.7 Hz, 2H), 2.51 (t, *J* = 6.7 Hz, 2H), 2.44 (s, 3H), 2.34 – 2.15 (m, 2H), 2.03 – 1.88 (m, 2H), 1.77 – 1.55 (m, 2H). HRMS (ESI-TOF) *m/z*: $[M+H]^+$ calcd for $C_{16}H_{24}F_3N_2O$, 317.1835; found 317.1832.

***N*-Methyl-2-(4-((3-(trifluoromethyl)benzyl)oxy)piperidin-1-yl)ethan-1-amine (34).** The title compound was prepared in four steps according to the general procedure C, B (HCl/MeOH), A (with Et_3N) and B (HCl/MeOH) (50% over four steps). A small portion of the HCl salt was converted to the free base form using the general procedure D to obtain the NMR sample. ^{1}H NMR (600 MHz, CD_3OD): δ 7.65 (s, 1H), 7.60 (d, *J* = 7.6 Hz, 1H), 7.56 (d, *J* = 7.8 Hz, 1H), 7.52 (t, *J* = 7.7 Hz, 1H), 4.62 (s, 2H), 3.55 – 3.48 (m, 1H), 2.85 – 2.75 (m, 2H), 2.72 (t, *J* = 6.8 Hz, 2H), 2.50 (t, *J* = 6.8 Hz, 2H), 2.42 (s, 3H), 2.31 – 2.16 (m, 2H), 2.06 – 1.89 (m, 2H), 1.80 – 1.62 (m, 2H). HRMS (ESI-TOF) *m/z*: $[M+H]^+$ calcd for $C_{16}H_{24}F_3N_2O$, 317.1835; found 317.1834.

***N*-Methyl-2-(4-((4-(trifluoromethyl)benzyl)oxy)piperidin-1-yl)ethan-1-amine (35).** The title compound was prepared in four steps according to the general procedure C, B (HCl/MeOH), A (with Et_3N) and B (HCl/MeOH) (44% over four steps). A small portion of the HCl salt was converted to the free base form using the general procedure D to obtain the NMR sample. ^{1}H NMR (600 MHz, CD_3OD): δ 7.62 (d, *J* = 8.0 Hz, 2H), 7.53 (d, *J* = 7.9 Hz, 2H), 4.63 (s, 2H), 3.56 – 3.46 (m, 1H), 2.88 – 2.73 (m, 4H), 2.54 (t, *J* = 6.6 Hz, 2H), 2.49 (s, 3H), 2.34 – 2.17 (m, 2H), 2.07 – 1.88 (m, 2H), 1.80 – 1.60 (m, 2H). HRMS (ESI-TOF) *m/z*: $[M+H]^+$ calcd for $C_{16}H_{24}F_3N_2O$, 317.1835; found 317.1841.

2-(4-([1,1'-Biphenyl]-2-ylmethoxy)piperidin-1-yl)-*N*-methylethan-1-amine (36). The title compound was prepared in four steps according to the general procedure C, B (HCl/MeOH), A (with Et_3N) and B (HCl/MeOH) (45% over four steps). A small portion of the HCl salt was converted to the free base form using the general procedure D to obtain the NMR sample. ^{1}H NMR (600 MHz, CD_3OD): δ 7.53 – 7.43 (m, 1H), 7.50 – 7.37 (m, 2H), 7.37 – 7.29 (m, 5H), 7.27 – 7.17 (m, 1H), 4.39 (s, 2H), 3.35 – 3.24 (m, 1H), 2.75 – 2.60 (m, 4H), 2.46 (t, *J* = 6.7 Hz, 2H), 2.41 (s, 3H), 2.21 – 2.03 (m, 2H), 1.84 – 1.71 (m, 2H), 1.59 – 1.45 (m, 2H). MS (ESI) *m/z* 325.2 $[M+H]^+$.

2-(4-([1,1'-Biphenyl]-3-ylmethoxy)piperidin-1-yl)-*N*-methylethan-1-amine (37). The title compound was prepared in four steps according to the general procedure C, B (HCl/MeOH), A (with Et_3N) and B (HCl/MeOH) (41% over four steps). A small portion of the HCl salt was converted to the free base form using the general procedure D to obtain the NMR sample. ^{1}H NMR (600 MHz, CD_3OD): δ 7.66 – 7.54 (m, 3H), 7.52 (d, *J* = 7.7 Hz, 1H), 7.49 – 7.36 (m, 3H), 7.36 – 7.26 (m, 2H), 4.59 (s, 2H), 3.54 – 3.46 (m, 1H), 2.85 – 2.73 (m, 4H), 2.51 (t, *J* = 6.6 Hz, 2H), 2.48 (s, 3H), 2.26 – 2.15 (m, 2H), 2.01 – 1.91 (m, 2H), 1.73 – 1.60 (m, 2H). MS (ESI) *m/z* 325.2 $[M+H]^+$.

2-(4-([1,1'-Biphenyl]-4-ylmethoxy)piperidin-1-yl)-*N*-methylethan-1-amine (38). The title compound was prepared in four steps according to the general procedure C, B (HCl/MeOH), A (with Et_3N) and B (HCl/MeOH) (40% over four steps). A small portion of the HCl salt was converted to the free base form using the general procedure D to obtain the NMR sample. ^{1}H NMR (600 MHz, CD_3OD): δ 7.63 – 7.56

(m, 4H), 7.45 – 7.39 (m, 4H), 7.32 (t, *J* = 7.3 Hz, 1H), 4.59 (s, 2H), 3.57 – 3.47 (m, 1H), 2.87 – 2.70 (m, 4H), 2.52 (t, *J* = 6.7 Hz, 2H), 2.46 (s, 3H), 2.35 – 2.15 (m, 2H), 2.02 – 1.89 (m, 2H), 1.76 – 1.62 (m, 2H). HRMS (ESI-TOF) *m/z*: $[M+H]^+$ calcd for $C_{21}H_{29}N_2O$, 325.2274; found 325.2269.

***N*-Methyl-2-(4-((4-(trifluoromethoxy)benzyl)oxy)piperidin-1-yl)ethan-1-amine (39).** The title compound was prepared in four steps according to the general procedure C, B (HCl/MeOH), A (with Et_3N) and B (HCl/MeOH) (34% over four steps). A small portion of the HCl salt was converted to the free base form using the general procedure D to obtain the NMR sample. ^1H NMR (600 MHz, CD_3OD): δ 7.44 (d, *J* = 8.3 Hz, 2H), 7.24 (d, *J* = 8.2 Hz, 2H), 4.56 (s, 2H), 3.55 – 3.45 (m, 1H), 2.86 – 2.73 (m, 2H), 2.70 (t, *J* = 6.8 Hz, 2H), 2.50 (t, *J* = 6.8 Hz, 2H), 2.41 (s, 3H), 2.31 – 2.17 (m, 2H), 2.06 – 1.90 (m, 2H), 1.75 – 1.61 (m, 2H). HRMS (ESI-TOF) *m/z*: $[M+H]^+$ calcd for $C_{16}H_{24}F_3N_2O_2$, 333.1784; found 333.1776.

***N*-Methyl-2-(4-((4-(2,2,2-trifluoroethoxy)benzyl)oxy)piperidin-1-yl)ethan-1-amine (40).** The title compound was prepared in four steps according to the general procedure C, B (HCl/MeOH), A (with Et_3N) and B (HCl/MeOH) (21% over four steps). A small portion of the HCl salt was converted to the free base form using the general procedure D to obtain the NMR sample. ^1H NMR (600 MHz, CD_3OD): δ 7.31 (d, *J* = 8.2 Hz, 2H), 6.98 (d, *J* = 8.5 Hz, 2H), 4.56 – 4.44 (m, 4H), 3.49 – 3.43 (m, 1H), 2.84 – 2.72 (m, 4H), 2.52 (t, *J* = 6.6 Hz, 2H), 2.47 (s, 3H), 2.28 – 2.15 (m, 2H), 1.98 – 1.89 (m, 2H), 1.70 – 1.59 (m, 2H). HRMS (ESI-TOF) *m/z*: $[M+H]^+$ calcd for $C_{17}H_{26}F_3N_2O_2$, 347.1941; found 347.1938.

2-(4-((4-isobutylbenzyl)oxy)piperidin-1-yl)-*N*-methylethan-1-amine (41). The title compound was prepared in four steps according to the general procedure C, B (HCl/MeOH), A (with Et_3N) and B (HCl/MeOH) (20% over four steps). A small portion of the HCl salt was converted to the free base form using the general procedure D to obtain the NMR sample. ^1H NMR (600 MHz, CD_3OD): δ 7.24 (d, *J* = 7.6 Hz, 2H), 7.12 (d, *J* = 7.6 Hz, 2H), 4.50 (s, 2H), 3.55 – 3.40 (m, 1H), 2.82 – 2.75 (m, 2H), 2.73 (t, *J* = 6.7 Hz, 2H), 2.50 (t, *J* = 6.7 Hz, 2H), 2.46 (d, *J* = 7.3 Hz, 2H), 2.43 (s, 3H), 2.25 – 2.17 (m, 2H), 1.97 –

1.91 (m, 2H), 1.85-1.82 (m, 1H), 1.68 – 1.59 (m, 2H), 0.89 (d, *J* = 6.6 Hz, 6H). HRMS (ESI-TOF) *m/z*: $[M+H]^+$ calcd for $C_{19}H_{33}N_2O$, 305.2587; found 305.2580.

2-(4-((4-Cyclohexylbenzyl)oxy)piperidin-1-yl)-*N*-methylethan-1-amine (42). The title compound was prepared in four steps according to the general procedure C, B (HCl/MeOH), A (with Et_3N) and B (HCl/MeOH) (10% over four steps). A small portion of the HCl salt was converted to the free base form using the general procedure D to obtain the NMR sample. ^{1}H NMR (600 MHz, CD_3OD): δ 7.24 (d, *J* = 7.9 Hz, 2H), 7.17 (d, *J* = 7.8 Hz, 2H), 4.49 (s, 2H), 3.50 – 3.43 (m, 1H), 2.85 – 2.69 (m, 4H), 2.50 (t, *J* = 6.8 Hz, 2H), 2.44 (s, 3H), 2.29 – 2.12 (m, 2H), 1.97 – 1.89 (m, 2H), 1.89 – 1.78 (m, 4H), 1.78 – 1.71 (m, 1H), 1.69 – 1.58 (m, 2H), 1.49 – 1.35 (m, 4H), 1.36 – 1.21 (m, 2H). HRMS (ESI-TOF) *m/z*: $[M+H]^+$ calcd for $C_{21}H_{35}N_2O$, 331.2744; found 331.2745.

***N*-methyl-2-(4-((4-(*tert*-pentyl)benzyl)oxy)piperidin-1-yl)ethan-1-amine (43).** The title compound was prepared in four steps according to the general procedure C, B (HCl/MeOH), A (with Et_3N) and B (HCl/MeOH) (11% over four steps). A small portion of the HCl salt was converted to the free base form using the general procedure D to obtain the NMR sample. ^{1}H NMR (600 MHz, CD_3OD): δ 7.31 (d, *J* = 8.0 Hz, 2H), 7.26 (d, *J* = 8.1 Hz, 2H), 4.51 (s, 2H), 3.51 – 3.44 (m, 1H), 2.86 – 2.73 (m, 2H), 2.69 (t, *J* = 6.9 Hz, 2H), 2.49 (t, *J* = 6.8 Hz, 2H), 2.40 (s, 3H), 2.27 – 2.16 (m, 2H), 1.99 – 1.89 (m, 2H), 1.71 – 1.59 (m, 4H), 1.27 (s, 6H), 0.65 (t, *J* = 7.5 Hz, 3H). HRMS (ESI-TOF) *m/z*: $[M+H]^+$ calcd for $C_{20}H_{35}Cl_2N_2O$, 319.2744; found 319.2744.

2-(4-((4-(2,3-dichlorophenoxy)benzyl)oxy)piperidin-1-yl)-*N*-methylethan-1-amine (44). The title compound was prepared in four steps according to the general procedure C, B (HCl/MeOH), A (with Et_3N) and B (HCl/MeOH) (25% over four steps). A small portion of the HCl salt was converted to the free base form using the general procedure D to obtain the NMR sample. ^{1}H NMR (600 MHz, CD_3OD): δ 7.40 – 7.30 (m, 3H), 7.26 (t, *J* = 8.2 Hz, 1H), 6.97 – 6.85 (m, 3H), 4.53 (s, 2H), 3.55 – 3.45 (m, 1H), 2.85 – 2.76 (m, 2H), 2.72 (t, *J* = 6.8 Hz, 2H), 2.50 (t, *J* = 6.8 Hz, 2H), 2.42 (s, 3H), 2.29 – 2.19 (m, 2H),

2.01 – 1.89 (m, 2H), 1.73 – 1.60 (m, 2H). HRMS (ESI-TOF) *m/z*: $[M+H]^+$ calcd for $C_{21}H_{27}Cl_2N_2O_2$, 409.1444; found 409.1439.

2-(4-((4-(3,5-dimethyl-1*H*-pyrazol-1-yl)benzyl)oxy)piperidin-1-yl)-*N*-methylethan-1-amine (45). The title compound was prepared in four steps according to the general procedure C, B (HCl/MeOH), A (with Et_3N) and B (HCl/MeOH) (25% over four steps). A small portion of the HCl salt was converted to the free base form using the general procedure D to obtain the NMR sample. ^{1}H NMR (600 MHz, CD_3OD): δ 7.50 (d, *J* = 8.1 Hz, 2H), 7.39 (d, *J* = 8.0 Hz, 2H), 6.06 (s, 1H), 4.62 (s, 2H), 3.57 – 3.47 (m, 1H), 2.85 – 2.76 (m, 2H), 2.74 (t, *J* = 6.8 Hz, 2H), 2.51 (t, *J* = 6.8 Hz, 2H), 2.43 (s, 2H), 2.30 – 2.18 (m, 8H), 2.02 – 1.91 (m, 2H), 1.75 – 1.62 (m, 2H). HRMS (ESI-TOF) *m/z*: $[M+H]^+$ calcd for $C_{19}H_{30}N_4O$, 343.2492; found 343.2490.

4-(Benzyloxy)-1-(2-methoxyethyl)piperidine (46). To a mixture of 4-(benzyloxy)piperidine hydrochloride (0.23 g, 1.0 mmol), potassium carbonate (0.28 g, 2.0 mmol), potassium iodide (0.016 g, 0.1 mmol) in acetonitrile (10 mL) was added 1-bromo-2-methoxyethane (0.15 g, 1.1 mmol). After being refluxed for overnight, the reaction mixture was cooled down to rt and quenched with water. The mixture was extracted with EtOAc (3 x). Combined organic layers were dried over Na_2SO_4 and concentrated under reduced pressure. The resulting residue was purified by flash chromatography on silica gel column to provide desired product as yellow oil (20%). ^{1}H NMR (600 MHz, CD_3OD): δ 7.69 – 7.28 (m, 4H), 7.28 – 7.22 (m, 1H), 4.53 (s, 2H), 3.51 (t, *J* = 5.6 Hz, 2H), 3.49 – 3.42 (m, 1H), 3.32 (s, 3H), 2.88 – 2.74 (m, 2H), 2.55 (t, *J* = 5.6 Hz, 2H), 2.32 – 2.18 (m, 2H), 1.98 – 1.87 (m, 2H), 1.73 – 1.59 (m, 2H). ^{13}C NMR (150 MHz, CD_3OD): δ 138.7, 127.9, 127.3, 127.1, 73.8, 69.7, 69.4, 57.5, 57.1, 51.0, 30.3. MS (ESI) *m/z* 250.2 $[M+H]^+$. HRMS (ESI-TOF) *m/z*: $[M+H]^+$ calcd for $C_{15}H_{24}NO_2$, 250.1802; found 250.1799.

PRMT biochemical assays. A radioactivity based scintillation proximity assay (SPA) was used to assess the effect of test compounds on inhibiting the methyl transfer reaction catalyzed by PRMTs as described previously.[45] The full length human PRMT4 (residues 1-608) and PRMT6 (residues 1-375)

were expressed and purified as described previously[48, 49] and were used in all of the biochemical and biophysical assays. The tritiated S-adenosyl-*L*-methionine (^{3}H-SAM) obtained from PerkinElmer Life Sciences (cat#NET155V001MC; specific activity range 12−18 Ci/mmol) served as the methyl donor. The (^{3}H) methylated biotin labelled peptide would be captured in streptavidin/scintillant-coated scintillation proximity FlashPlates Plus (PerkinElmer Life Sciences) which brings the incorporated ^{3}H-methyl and the scintillant to close proximity resulting in light emission that is quantified by tracing the radioactivity signal (counts per minute) as measured by a TopCount NXT™ Microplate Scintillation and Luminescence Counter (PerkinElmer Life Sciences). When necessary, non-tritiated SAM (AK Scientific, Union City, CA) was used to supplement the reactions. The reaction mixture for the PRMT6 assay (10 μL) contained 20 mM bis-tris-propane (pH 7.5) containing 0.01% Tween-20, 10 mM dithiothreitol (DTT), 25 nM PRMT6, 0.6 μM H4 1-24 peptide (SGRGKGGKGLGKGGAKRHRKVLRDK-Biotin) and 2.3 μM SAM. For PRMT4 assay, the reaction mixture contained 20 mM bicine (pH 8.5), 0.01% Triton X-100, 25 nM PRMT4, 0.7 μM H3 1-25 peptide (ARTKQTARKSTGGKAPRKQLATKAAGK-Biotin) and 1.9 μM SAM. For the IC_{50} determination the compounds were serially diluted in the reaction buffer and were added to the reaction mixture before starting the reactions by addition of the substrate. The reactions were quenched by addition of equal volume of 7.5 M guanidine hydrochloride and the volume finalized to 120 μL by the addition of 20 mM Tris-HCl, pH 8.0. After the transfer to the SPA plates (FlashPlate® PLUS; PerkinElmer Life Sciences), the plates were incubated for at least 3 h before measuring the signals using a TopCount NXT™ Microplate Scintillation and Luminescence Counter (PerkinElmer Life Sciences). The IC_{50} values were determined under balanced conditions at K_m concentrations of both substrate and cofactor by titration of test compounds in the reaction mixture. The ^{3}H-SAM can be purchased only at low concentration (40-50 μM) in acidic solution which need to be diluted at least in 1:10 ratio to prepare the final reaction mixture (maximum of 5 μM). All IC_{50} values were determined at K_m of substrates. For enzymes that have K_m values higher than 5 μM for SAM (for example PRMT3) we needed to increase the concentration of SAM by adding unlabeled SAM. Same limitation existed when assessing the

competition with SAM which saturation concentration of SAM was required. In case of the enzymes with apparent SAM K_m below 5 μM, due to high cost of ^{3}H-SAM, we still diluted ^{3}H-SAM with unlabeled SAM as long as the signal-to-noise was reliable for assays.

Selectivity assays. The methyltransferase selectivity of **17** was assessed at two compound concentrations of 10 and 50 μM as described previously.[67-70] The inhibitory effect of **17** against three lysine demethylases, KDM1A (LSD1), KDM3A (JMJD1A), and KDM4A (JMJD2A) was assessed using the method described by Yu et. al.[71] The selectivity also was tested against 9 reader proteins (UHRF1, BRPF1, WDR5, HGDF2, TDRD3, RBBP1, FXR1, EED, and SND1) using DSF and/or DSLS based assays as described elsewhere.[72] Selectivity assays of 100 non-epigenetic targets (kinases, GPCRs, ion channels, and transporters) were conducted by CEREP. The 5HT2B radiolabel binding assay was performed by the NIMH Psychoactive Drug Screening Program.

Mechanism of action (MOA) studies. To determine the MOA of the **17**, the competition of the compound with SAM and peptide substrate was determined individually. In brief, the peptide concentration was kept at saturation and compound potency was monitored at different SAM concentrations (0.5, 1, 4, 8, 12, 16, 20, and 25×K_m). To test the competition with peptide, the SAM concentration was kept at saturation and IC_{50} values were determined at different peptide concentrations (0.5, 1, 2, 4, 8, 12, 16, and 20×K_m).

Biophysical assays. Isothermal titration calorimetry (ITC) was performed in a Nano ITC instrument (TA Instruments, USA) following the procedure described previously.[47] The ITC titrations were performed at 25 °C by using 2 μL injections with a total of 25 injections. For PRMT6, the cell was loaded with 50 μM PRMT6, 100 μM SAM with final DMSO concentration of 2.5% DMSO in 50 mM Tris-HCl (pH 8.5) containing 150 mM NaCl. The syringe was loaded with 200 μM of **17**, 100 μM SAM and 2.5% DMSO prepared in the same buffer. For PRMT4, the cell was loaded with 35 μM PRMT4, 120 μM SAH and 3% DMSO in phosphate buffered saline. The syringe was loaded with 300 μM **17**,

125 μM SAH and 3% DMSO prepared in the same buffer. The data were fitted with a one-binding-site model using "Nano Analyze" software supplied by the instrument manufacturer. Differential scanning fluorimetry (DSF) was performed using a Light Cycler 480 II instrument from Roche Applied Science as described previously.[47, 72] The experiments were done with 100 mM HEPES buffer (pH 7.4) containing 150 mM NaCl, 0.1 mg/mL PRMT6, and 5X Sypro Orange (Invitrogen). DSF was carried out by increasing the temperature by 4 °C/min from 20 to 95 °C, and data points were collected at 1 °C intervals. The temperature scan curves were fitted to a Boltzmann sigmoid function, and the T_m values were obtained from the midpoint of the transition as described previously.[73] Differential static light scattering (DSLS) was performed as previously described.[73]

Cellular PRMT4 assay. HEK293 cells were grown in 12-well plates in DMEM supplemented with 10% FBS, penicillin (100 units/mL) and streptomycin (100 μg/mL). 30% confluent cells were treated with different concentrations of compounds in triplicates or DMSO control for 72 h. Cells were lysed in 100 μL of total lysis buffer (20 mM Tris-HCl pH 8, 150 mM NaCl, 1 mM EDTA, 10 mM $MgCl_2$, 0.5% TritonX-100, 12.5 U/mL benzonase (Sigma), complete EDTA-free protease inhibitor cocktail (Roche)). After 3 min incubation at RT, SDS was added to final 1% concentration. Lysates were run on SDS-PAGE and immunoblotting was done as outlined below to determine Med12-Rme2a levels in Western blot.

Cellular PRMT6 assay. HEK293 cells were grown in 12-well plates in DMEM supplemented with 10% FBS, penicillin (100 U/mL) and streptomycin (100 μg/mL). 50 % confluent cells were transfected with FLAG-tagged PRMT6 or mutant V86K/D88A PRMT6 (1 μg of DNA per well) using jetPRIME® transfection reagent (Polyplus-Transfection), following manufacturer instructions. After 4 h media were removed and cells were treated with compounds at indicated concentrations or DMSO control. After 20 h, media was removed and cells were lysed in 100 μL of total lysis buffer. Lysates were run on SDS-PAGE and immunoblotting was done as outlined below to determine H3R2me2a levels in Western blot.

Western blot. Total cell lysates were resolved in 4-12% Bis-Tris Protein Gels (Invitrogen) with MOPS buffer (Invitrogen) and transferred in for 1.5h (80 V) onto PVDF membrane (Millipore) in Tris-Glycine transfer buffer containing 20% MeOH and 0.05% SDS. Blots were blocked for 1 h in blocking buffer (5% milk in 0.1% Tween 20 PBS) and incubated with primary antibodies: mouse anti-H4 (1:1000, Abcam #174628), rabbit anti-H4R3me2a (1:1000, Active Motif #39705), mouse anti-H3 (1:1000, Abcam #ab10799), rabbit anti-H3R2me2a (1:1000, Millipore #04-808), rabbit anti-Rme2a (1:1000, Cell Signaling Technology #13522), mouse anti-Med12 (1:1000, Abnova # H00009968-A01), rabbit anti-Med12-Rme2a (1:1000, gift from Dr. Mark Bedford) in blocking buffer overnight at 4 ºC. After five washes with 0.1% Tween 20 PBS the blots were incubated with goat-anti rabbit (IR800 conjugated, LiCor #926-32211) and donkey anti-mouse (IR 680, LiCor #926-68072) antibodies (1:5000) in Odyssey Blocking Buffer (LiCor) for 1 h at RT and washed five times with 0.1% Tween 20 PBS. The signal was read on an Odyssey scanner (LiCor) at 800 nm and 700 nm.

Cell growth assay. HEK293 cells grown in DMEM supplemented with 10% FBS, penicillin (100 U/mL) and streptomycin (100 µg/mL) were seeded on 96-well plates at density 3000/well and treated with **17** or **46** at 0, 1, 10 and 50 µM concentrations for 96 h. The confluency was measured using IncuCyte™ ZOOM live cell imaging device (Essen Bioscence) and analyzed with IncuCyte™ ZOOM (2015A) software based on phase contrast images.

ASSOCIATED CONTENT

Supporting information

This material is available free of charge via the internet at http://pubs.acs,org.

^{1}H and ^{13}C NMR spectra of compound **17**; Selectivity of **17** against KDMs, methyllysine and methylarginine reader proteins, CEREP and PDS panels; Synthetic scheme of **46**; Effect of cellular assay results of **46**; Effects of **17** and **46** on cell growth; Molecular formula strings.

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Notes

The authors declare no competing financial interests.

ACKNOWLEDGEMENTS

We thank Albina Bolotokova for compound management and Taraneh Hajian for protein purification. The research described here was supported by the grant R01GM103893 (to J.J.) from the U.S. National Institutes of Health. The SGC is a registered charity (number 1097737) that receives funds from AbbVie, Bayer Pharma AG, Boehringer Ingelheim, Canada Foundation for Innovation, Eshelman Institute for Innovation, Genome Canada, Innovative Medicines Initiative (EU/EFPIA) [ULTRA-DD grant no. 115766], Janssen, Merck & Co., Novartis Pharma AG, Ontario Ministry of Economic Development and Innovation, Pfizer, São Paulo Research Foundation-FAPESP, Takeda, and the Wellcome Trust. 7TM, kinase, and ion channel off-target selectivity screening was kindly supplied by Eurofins-Cerep. Further Ki determinations and receptor binding profiles were generously provided by the National Institute of Mental Health's Psychoactive Drug Screening Program, Contract # HHSN-271-2013-00017-C (NIMH PDSP). The NIMH PDSP is Directed by Bryan L. Roth MD, PhD at the University of North Carolina at Chapel Hill and Project Officer Jamie Driscoll at NIMH, Bethesda MD, USA. Y.S. was supported by a postdoctoral fellowship from the SGC.

ABBREVIATIONS USED

PMT, protein methyltransferase; PRMT, protein arginine methyltransferase; CARM1, coactivator associated arginine methyltransferase 1; SAM, S-5′-adenosyl-*L*-methionine; SAH, S-5′-adenosyl-*L*-

homocysteine; DSF, differential scanning fluorimetry; DSLS, differential static light scattering; SAR, structure–activity relationship; Boc, *tert*-butyloxycarbonyl; PKMT, protein lysine methyltransferase, DNMT, DNA methyltransferase; RNMT, RNA methyltransferases; KDM, histone lysine demethylase; MOA, mechanism of action; Rme2a, asymmetric arginine dimethylation; H3R2me2a , asymmetric dimethylation of histone H3 arginine 2; H4R3me2a, asymmetric dimethylation of histone H4 arginine 3; H4R3me2a, asymmetric dimethylation of histone H4 arginine 3.

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FIGURES, TABLES AND SCHEMES.

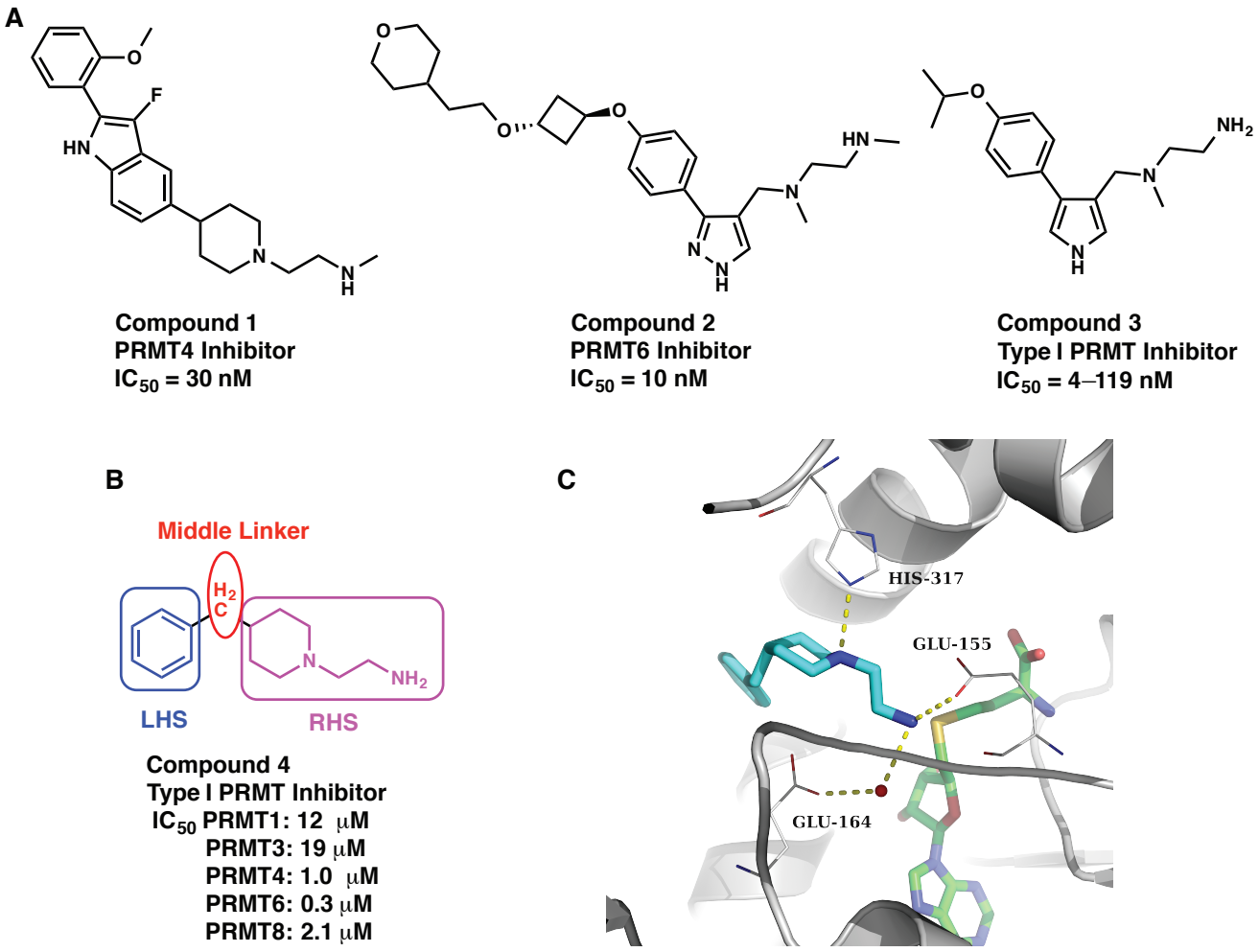
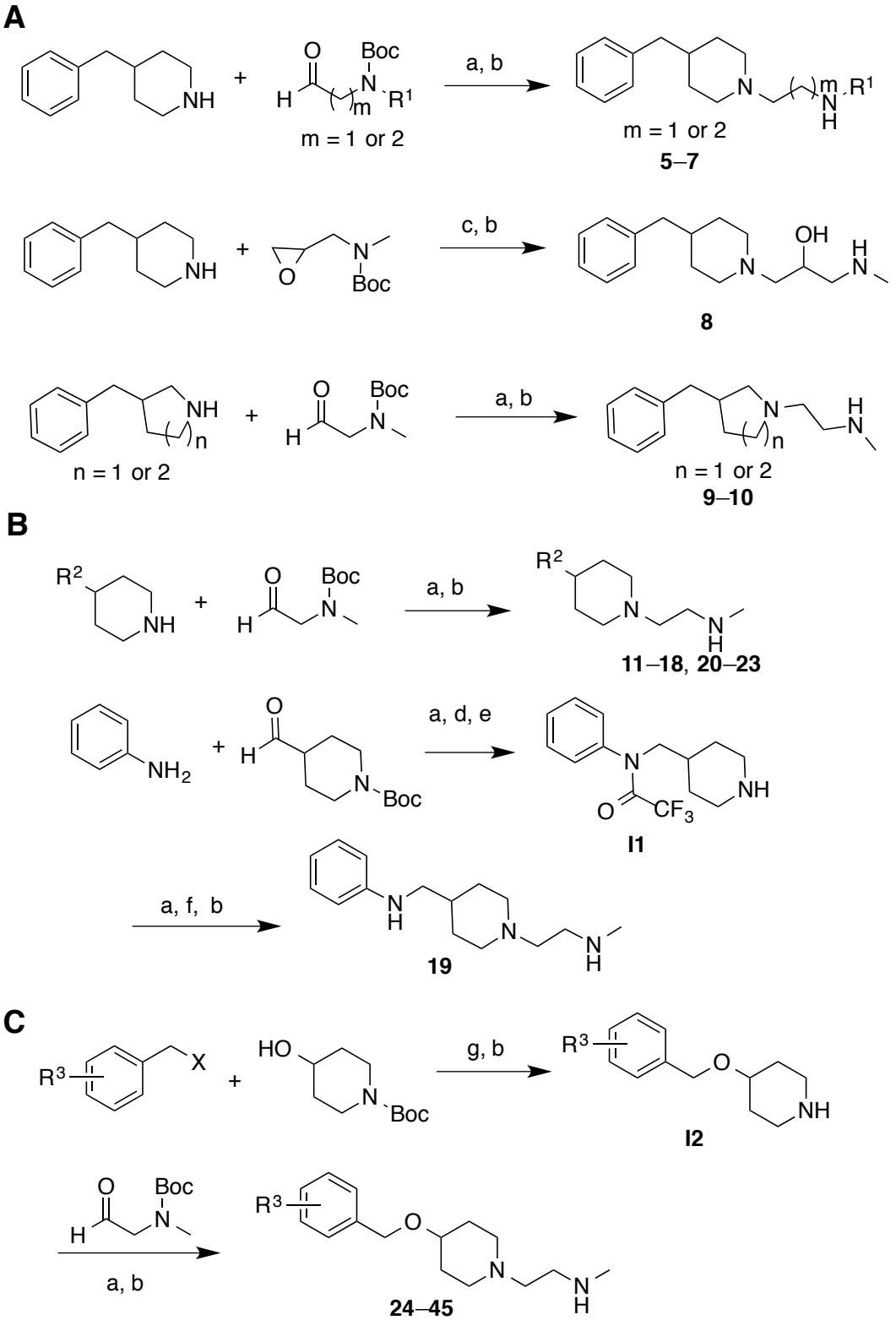


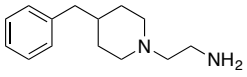
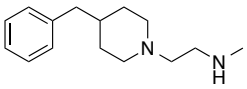
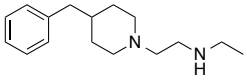
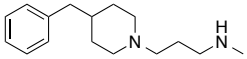
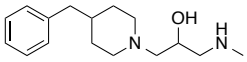
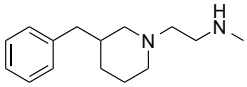
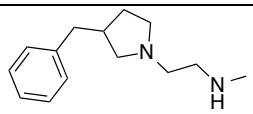
Figure 1. (A) Structure and potency of compounds 1 (PRMT4 inhibitor), 2 (PRMT6 selective inhibitor), and 3 (selective type I PRMT inhibitor). (B) Structure and potency of compound 4, a fragment-like inhibitor of type I PRMTs. (C) X-ray cocrystal structure of compound 4 (cyan) in the complex with PRMT6 (gray) and SAH (green) (PDB: 5EGS). The key H-bond interactions are shown in yellow dotted lines.

Scheme 1. Synthetic routes for preparing designed compounds. Synthesis of compounds in Table 1 (A), Table 2 (B), and Table 3 (C).



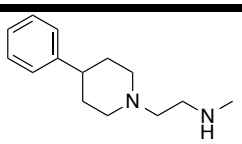
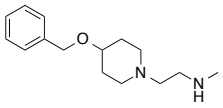
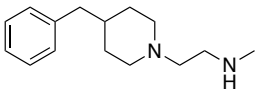
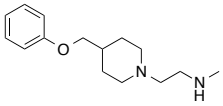
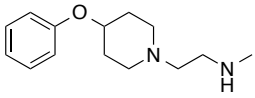
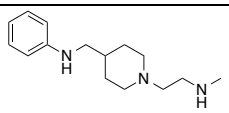
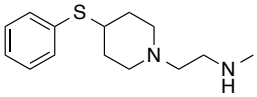
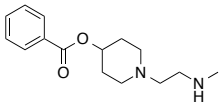
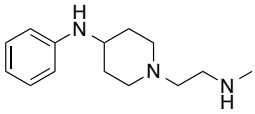
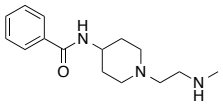
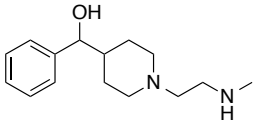
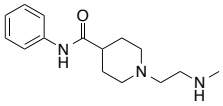
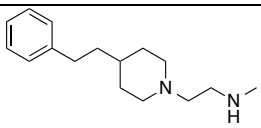
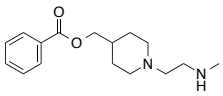
Reagents and conditions: (a) $NaBH(OAc)_3$, DCM; (b) HCl in MeOH or dioxane, rt, 10-85% over 2-4 steps; (c) *i*-PrOH, 50 °C (d) TFAA, DCM, 0 °C to rt, 85% over two steps; (e) TFA, DCM, rt; (f) NaOH, MeOH, H_2O, 50 °C; (g) NaH, DMF, 0 °C to rt.

Table 1. SAR of the RHS moiety.

Compound	Structure	IC_{50} (nM)	
		PRMT4	PRMT6
4		1,000 ± 40[a]	400 ± 100[a]
5		890 ± 140	170 ± 39
6		>4,700	830 ± 54
7		>10,000	>10,000
(±)-**8**		1,220 ± 140	3,040 ± 270
(±)-**9**		120 ± 28	290 ± 30
(±)-**10**		80 ± 28	70 ± 6

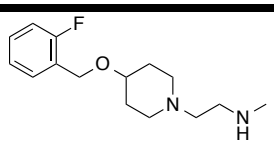
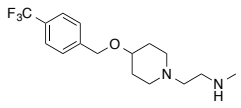
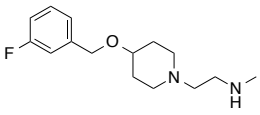
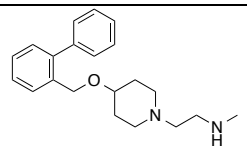
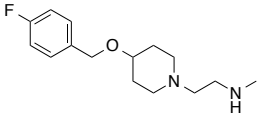
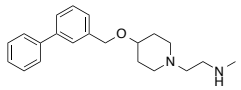
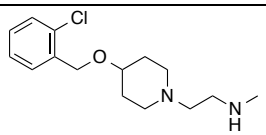
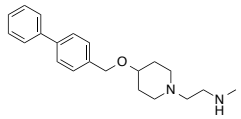
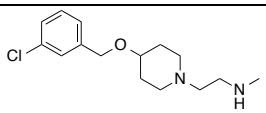
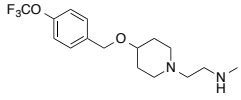
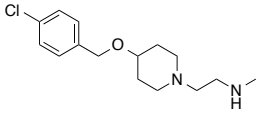
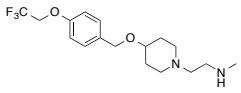
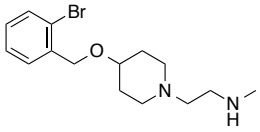
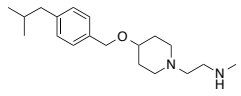
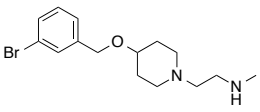
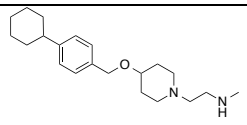
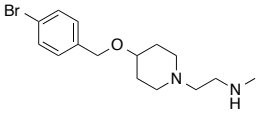
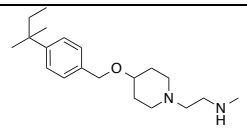
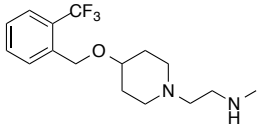
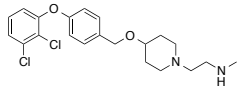
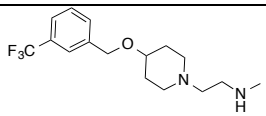
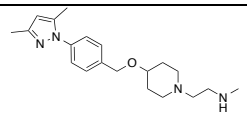
IC_{50} determination experiments were performed at substrate and cofactor concentrations equal to the respective K_m values for each enzyme. IC_{50} determination experiments were performed in triplicate and the values are presented as Mean ± SD. *a.* IC_{50} values were from reference 48.

Table 2. SAR of the middle linker.

Com-pound	Structure	IC_{50} (nM)		Com-pound	Structure	IC_{50} (nM)	
		PRMT4	PRMT6			PRMT4	PRMT6
11		6,250 ± 280	9,340 ± 1,820	**MS049 (17)**		34 ± 10	43 ± 7
5		890 ± 140	170 ± 39	**18**		98 ± 13	92 ± 11
12		149 ± 24	90 ± 16	**19**		103 ± 7	116 ± 24
13		3,680 ± 640	488 ± 75	**20**		177 ± 19	107 ± 25
14		616 ± 70	186 ± 23	**21**		231 ± 18	129 ± 17
15		1,637 ± 68	469 ± 56	**22**		1,540 ± 190	5,360 ± 390
16		108 ± 21	56 ± 7	**23**		1,070 ± 160	515 ± 91

IC_{50} determination experiments were performed at substrate and cofactor concentrations equal to the respective K_m values for each enzyme. IC_{50} determination experiments were performed in triplicate and the values are presented as Mean ± SD.

Table 3. SAR of the LHS phenyl moiety.

Compound	Structure	IC_{50} (nM)		Compound	Structure	IC_{50} (nM)	
		PRMT4	PRMT6			PRMT4	PRMT6
24		35 ± 7	47 ± 5	**35**		62 ± 11	144 ± 15
25		41 ± 6	56 ± 7	**36**		41 ± 11	48 ± 8
26		19 ± 4	38 ± 8	**37**		27 ± 9	30 ± 5
27		18 ± 3	30 ± 3	**38**		17 ± 4	27 ± 1
28		33 ± 5	78 ± 8	**39**		28 ± 5	46 ± 4
29		18 ± 5	40 ± 4	**40**		53 ± 5	48 ± 2
30		14 ± 4	25 ± 4	**41**		85 ± 32	72 ± 7
31		17 ± 3	53 ± 10	**42**		42 ± 6	28 ± 1
32		15 ± 2	44 ± 8	**43**		16 ± 2	67 ± 9
33		23 ± 2	64 ± 6	**44**		22 ± 4	26 ± 2
34		28 ± 6	117 ± 20	**45**		15 ± 2	25 ± 2

IC_{50} determination experiments were performed at substrate and cofactor concentrations equal to the respective K_m values for each enzyme. IC_{50} determination experiments were performed in triplicate and the values are presented as Mean ± SD.

Table 4. PRMT selectivity of compounds 17, 25 and 37.

Compound	IC_{50} (nM)						
	PRMT1	**PRMT3**	**PRMT4**	**PRMT5**	**PRMT6**	**PRMT7**	**PRMT8**
17	>13,000	>22,000	34 ± 10	NI	43 ± 7	NI	1,600 ± 81
25	>16,000	>28,000	41 ± 6	NI	56 ± 7	NI	1,850 ± 160
37	>5,800	1,970 ± 170	27 ± 9	NI	30 ± 5	NI	573 ± 61

IC_{50} determination experiments were performed at substrate and cofactor concentrations equal to the respective K_m values for each enzyme. IC_{50} determination experiments were performed in triplicate and the values are presented as Mean ± SD. NI: no inhibition.

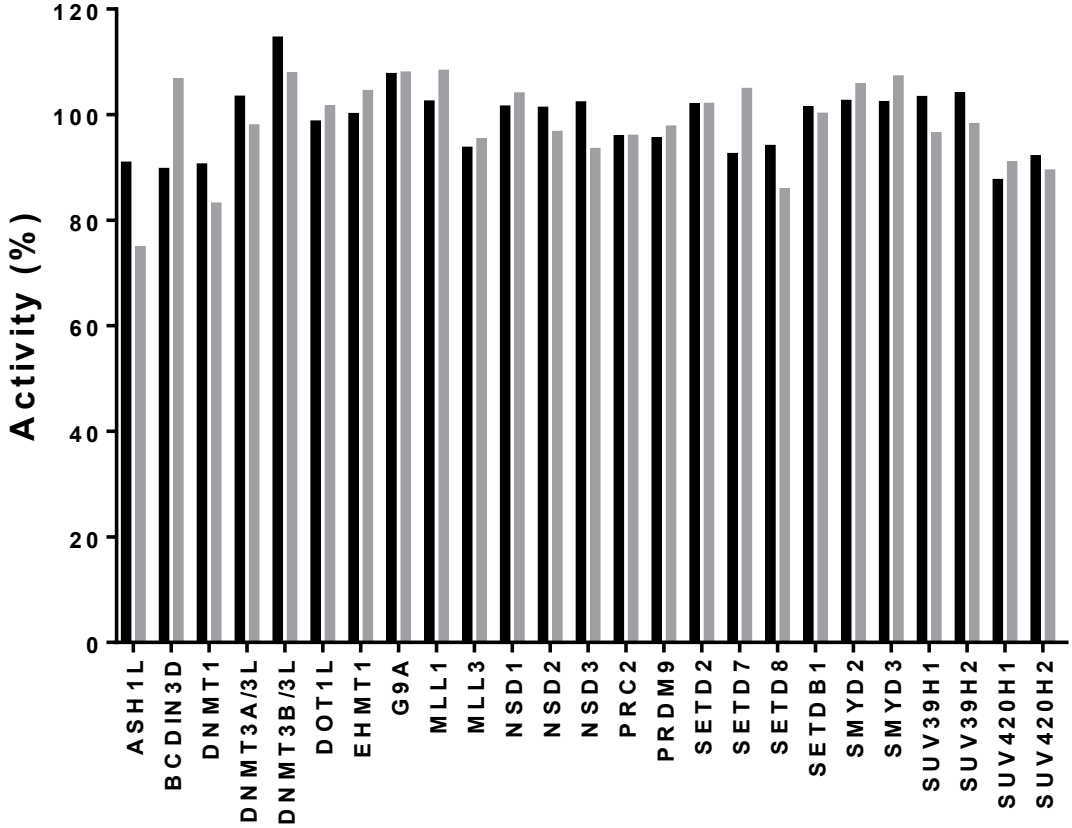


Figure 2. Selectivity of 17 against methyltransferases. Selectivity of **17** against a panel of 25 PKMTs and DNMTs was determined at two compound concentrations of 10 μM (■) and 50 μM (■).

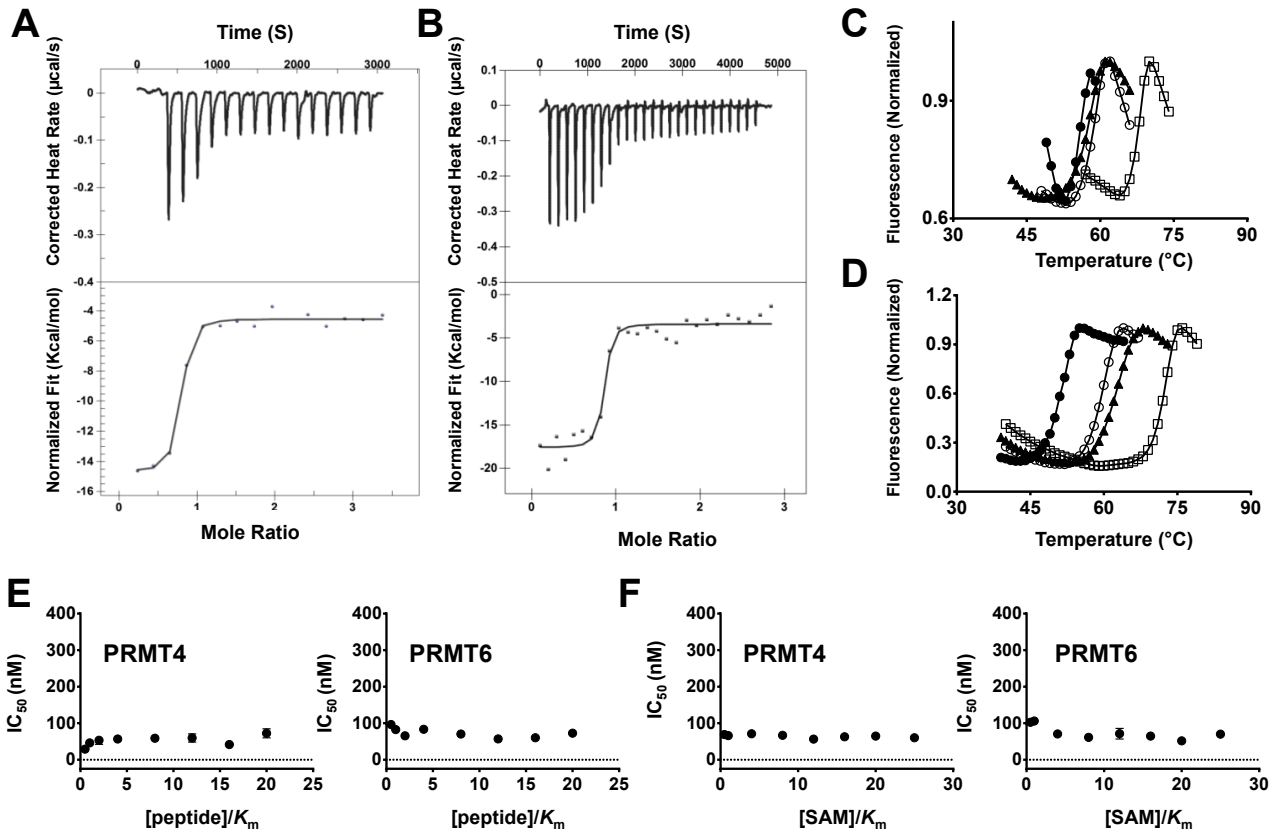


Figure 3. Binding confirmation and MOA of 17. (A) Isothermal titration calorimetry (ITC) was used to confirm binding of **17** to PRMT4 with a K_d of 100 nM (B) and to PRMT6 with a K_d of 87 ± 35 nM. ITC experiments for PRMT4 and PRMT6 were performed in duplicate and triplicate, respectively. Differential scanning fluorimetry (DSF) was also used to confirm the binding of **17** to PRMT4 (C) and PRMT6 (D). Experiments were performed (●) in the absence of any compound as a negative control and in the presence of (○) 100 µM SAH as a positive control with PRMT4 (ΔT_m of 4 °C) and PRMT6 (ΔT_m of 8.4 °C). Presence of (▲) 200 µM **17** resulted in stabilization of both proteins with ΔT_m of 3.3 and 11 °C for PRMT4 and PRMT6, respectively. Presence of (□) 100 µM SAM plus 200 µM **17** had the highest effect with ΔT_m of 13 and 21 °C for PRMT4 and PRMT6, respectively. The inflection point of each transition curve is considered melting temperature (T_m) and the increase in T_m is an indication of binding. The IC_{50} values for **17** with PRMT4 and PRMT6 was unchanged when determined under various peptide (E) and SAM (F) concentrations suggesting an apparent noncompetitive pattern of inhibition

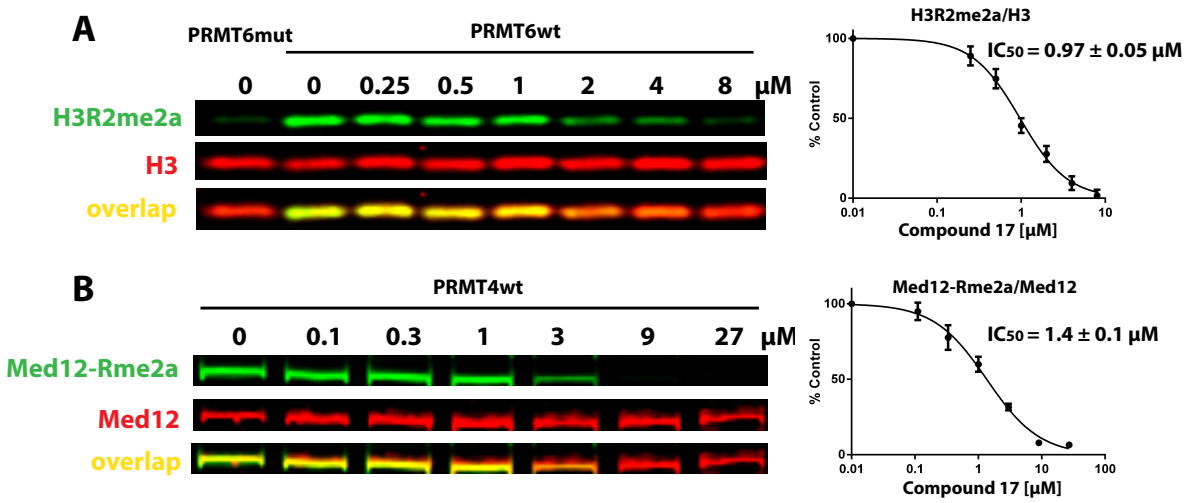
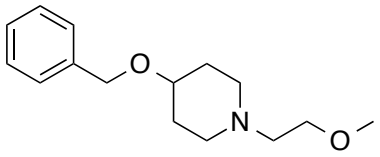


Figure 4. **The effect of 17 on inhibiting PRMT6 and PRMT4 in cells.** (A) Compound **17** inhibits PRMT6 methyltransferase activity in HEK293 cells. HEK293 cells were transfected with FLAG-tagged PRMT6 (wt) or its catalytically inactive mutant V86K/D88A (mut) and treated with **17** at indicated concentrations for 20 h. H3R2me2a levels were determined by Western blot. The graphs represent nonlinear fits of H3R2me2a signal intensities normalized to total histone H3. The results are MEAN ± SEM of 3 replicates. (B) Compound **17** inhibits endogenous PRMT4 methyltransferase activity in HEK293 cells. HEK293 cells were treated with **17** at indicated concentrations for 72 h and Med12-Rme2a levels were determined by Western blot. The graphs represent nonlinear fits of Med12-Rme2a signal intensities normalized to total Med12. The results are MEAN ± SEM of 3 replicates.



MS049N (46)
IC_{50} PRMT4: NI
PRMT6: NI

Figure 5. Compound 46 a close analog of compound 17, has no inhibitory activity against PRMT4 and PRMT6. NI: no inhibition.

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