SUPPLEMENTARY MATERIAL

Identification of Protein Palmitoylation Inhibitors from a Scaffold Ranking Library

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SUPPLEMENTAL MATERIALS AND METHODS

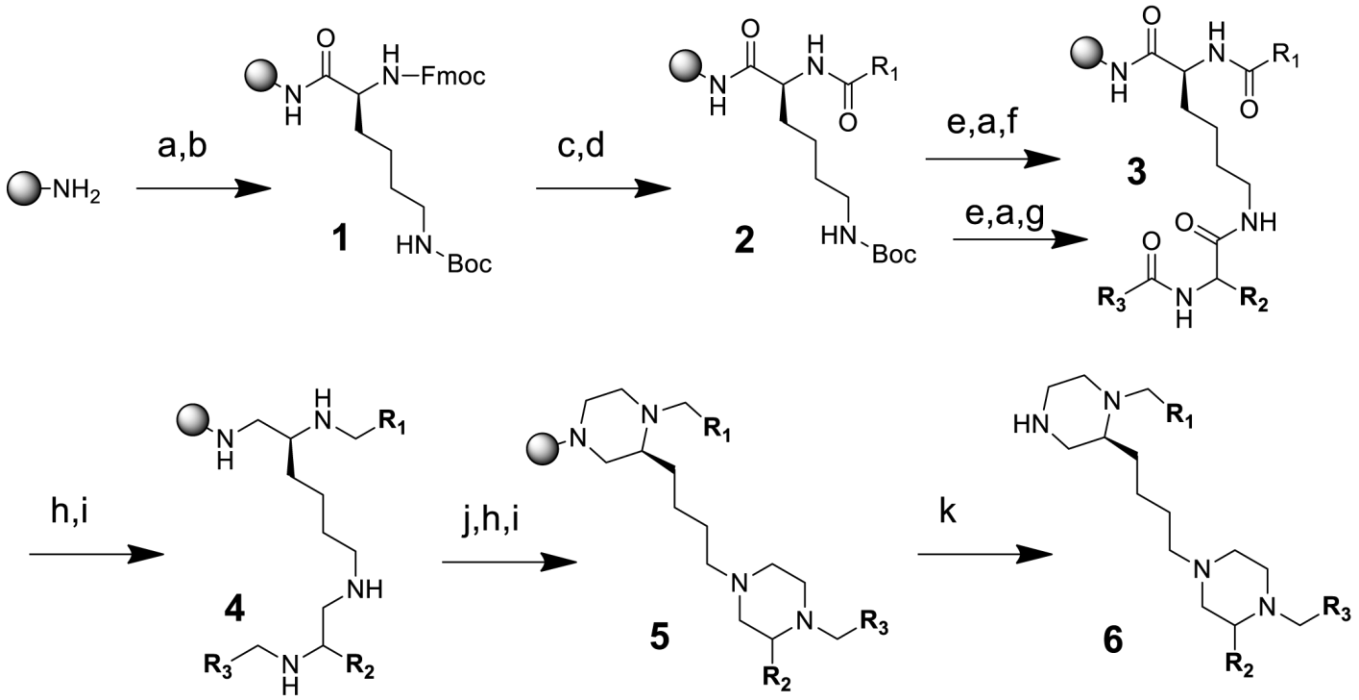
Synthesis of Library 2160 and Individual Compounds and Construction of Scaffold Ranking Plate

Library 2160 as well as the individual compounds reported herein (Compounds 1-54) were synthesized following the same synthetic scheme (Fig. **S1**) [1, 2]. Utilizing the "tea-bag" methodology [3], 100 mg of p-methylbenzhydrylamine (MBHA) resin (1.1 mmol/g, 100-200 mesh) was sealed in a mesh "tea-bag", neutralized with 5% diisopropylethylamine (DIEA) in dichloromethane (DCM) and subsequently swelled with additional DCM washes. Fmoc-L-Lys(Boc)-OH was coupled in Dimethylformamide (0.1M DMF) for 120 mins in the presence of Diisopropylcarbodiimide (DIC, 6 equiv.) and 1-Hydroxybenzotriazole hydrate (HOBt, 6 equiv.) (Step 1, Fig. **S1**). The Fmoc protecting group was removed with 20% piperidine in DMF for 20 mins and the R1 carboxylic acids was coupled (10 equiv.) in the presence of DIC (10 equiv.) and HOBt (10 equiv.) in DMF (0.1 M) for 120 mins (Step 2, Fig. **S1**). The Boc protecting group was then removed with Trifluoroacetic Acid (TFA) in DCM for 30 mins and subsequently neutralized with 5% DIEA/DCM (3x). Boc-Amino Acids (R2) were coupled utilizing standard coupling procedures (6 equiv.) with DIC (6 equiv.) and HOBt (6 equiv.) in DMF (0.1 M) for 120 mins. The Boc group was removed with 55% TFA/DCM for 30 mins and subsequently neutralized with 5% DIEA/DCM (3x). Carboxylic acids (R3) were coupled using (10 equiv.) in the presence of DIC (10 equiv.) and HOBt (10 equiv.) in DMF (0.1 M) for 120 mins (Step 3, Fig. **S1**). All coupling reactions were monitored for completion using Ninhydrin. The reduction was performed in a 4000 mL Wilmad LabGlass vessel under nitrogen. 1.0 M Tetrahydrofuran (THF) borane complex solution was used in 40-fold excess for each amide bond. The vessel was heated to 65°C and maintained at this temperature for 96 hrs. The solution was then removed and the bags were washed with THF and methanol (MeOH). Once completely dry, the bags were treated overnight with piperidine at 65°C and washed several times with DMF, DCM and methanol (Step 4, Fig. **S1**). As previously reported by our group and others, the reduction of polyamides with borane is free of racemization [4-6]. Before proceeding, completion of reduction was monitored by LCMS analysis of a control compound (Step 4, Fig. **S1**) that was cleaved from the solid support (HF, anisole, 0°C 7 hr). Cyclization was performed with a 5-fold excess (for each cyclization) of oxalyldiimidazole in a 0.1 M anhydrous DMF solution overnight. Following the cyclization, the bags were rinsed with DMF and DCM and the resulting diketopiperarzines were reduced down to their corresponding piperazines (Step 5, Fig. **S1**) using the same borane reduction procedure as above. The resin was cleaved with HF in the presence of anisole in an ice bath at 0°C for 7 hours (Step 6, Fig. **S1**). After removal of the HF by gaseous N_2, the products were then extracted from the vessels with 95% acetic acid in water, transferred to scintillation vials, frozen and lyophilized. The compounds were then reconstituted in 50% acetonitrile and water, frozen and lyophilized three more times. For initial screening the individual compounds were tested as crude material in case the activity is driven by some side reaction that was also present in the original positional scanning library. After this initial screening, compounds, 13, 14, 19, 22, 25, 27, 28, 30, 34, and 43 were selected for purification and NMR characterization.

LCMS Analysis

The purity and identity of all compounds was verified using a Shimadzu 2010 LCMS system, consisting of a LC-20AD binary solvent pump, a DGU-20A degasser unit, a CTO-20A column oven, and a SIL-20A HT autosampler. A Shimadzu SPD-M20A diode array detector was used for detection. A full spectra range of 190-600 nm was obtained during analysis. Chromatographic separations were obtained using a Phenomenex Luna C18 analytical column (5 µm, 50 x 4.6 mm i.d.) preceded by a Phenomenex C18 column guard (5 µm, 4 x 3.0 mm i.d.). All equipment was controlled and integrated by Shimadzu LCMS solutions software version 3. Mobile phases for LCMS analysis were HPLC grade or LCMS grade obtained from Sigma Aldrich and Fisher Scientific. The mobile phases consisted of a mixture LCMS grade Acetonitrile /water (both with 0.1%

formic acid for a pH of 2.7). The initial setting for analysis was set at 5% Acetonitrile (v/v), then was linearly increased to 95% Acetonitrile over 6 mins. The gradient was then held at 95% Acetonitrile for 2 mins, linearly decreased to 5% over 0.10 mins and held for an additional 1.90 mins. The total run time was equal to 12 mins. The total flow rate was set to 0.5 mL/minute. The column oven and flow cell temperature for the diode array detector was set at 30°C. The autosampler temperature was held at 15°C. 5 µl of compound was injected for analysis.



Supplemental Fig. (1). Synthetic Scheme of Bis-cyclic Piperazines. *a*, 5% DIEA/DCM; *b*, Fmoc-Lys(Boc)-OH, DIC, HOBt, DMF; *c*, 20% Piperidine/DMF; *d*, R_1COOH, DIC, HOBt, DMF;e*E*, 55% TFA/DCM; *f*, Boc-AA(R_2), DIC, HOBt, DMF; *g*, R_3COOH, DIC, HOBt, DMF; *h*, BH_3-THF, 65˚C, 96 hours; *i*, Piperidine, 65˚C, 24 hours; *j*, Oxalyldiimidazole, DMF; *k*, HF, anisole, 0˚C.

HPLC Purification (Compounds 13, 14, 19, 22, 25, 27, 28, 30, 34, and 43)

All purifications were performed on a Shimadzu Prominence preparative HPLC system, consisting of LC-8A binary solvent pump, a SCL-10A system controller, a SIL-10AP autosampler, and a FRC-10A fraction collector. A Shimadzu SPD-20A UV detector was used for detection. The wavelength was set at 214 nm during analysis. Chromatographic separations were obtained using a Phenomenex Luna C18 preparative column (5 µm, 150 x 21.5 mm i.d.) preceded by a Phenomenex C18 column guard (5 µm, 15 x 21.2 mm i.d.). Prominence prep software was used to set all detection and collection parameters. The mobile phases for HPLC purification were HPLC grade obtained from Sigma Aldrich and Fisher Scientific. The mobile phase consisted of a mixture of Acetonitrile/water (both with 0.1% formic acid). The initial setting for separation was set at 2% (v/v) Acetonitrile, which was held for 2 mins and the gradient was linearly increased to 20% (v/v) Acetonitrile over 4 mins. The gradient was then linearly increased to 55% (v/v) Acetonitrile over 36 mins. The HPLC system was set to automatically flush and re-equilibrate the column after each run for a total of 4 column volumes. The total flow rate was set to 12 mL/min and the total injection volume was set to 3900 µl. The fraction collector was set to collect from 6 to 40 mins. The corresponding fractions were then combined and lyophilized. The 1H spectra were obtained utilizing the Bruker 400 Ascend (400 MHz). NMR chemical shifts were reported in δ (ppm) using the δ 7.26 signal of CDCl3 (1H NMR).

Chemical Synthesis of Individual Compounds

4-(((2S)-1-(2-(4-isobutylphenyl)propyl)-4-(4-((2S)-1-(2-(4-isobutylphenyl)propyl)piperazin-2-yl)butyl)piperazin-2-yl)methyl) phenol (Compound 13)

Using the synthetic approach described in Fig. (**S1**) for the synthesis of compound 13 was synthesized using the following reagents: 4-Isobutyl-alpha-methylphenylacetic acid (R1), Boc-L-Tyrosine(BrZ) (R2), 4-Isobutyl-alpha-methylphenylacetic acid (R3). Final crude product was purified by HPLC as described above. 1H NMR (400 MHz, CHLOROFORM-d): δ ppm 7.28 (br. s., 1 H) 7.08 - 7.16 (m, 4 H) 7.05 (br. s., 4 H) 6.92 (br. s., 2 H) 6.80 (br. s., 3 H) 2.98 (br. s., 8 H) 2.64 - 2.84 (m, 5 H) 2.58 (br. s., 4 H) 2.45 (t, J=8.01 Hz, 6 H) 2.32 (d, J=16.26 Hz, 2 H) 2.01 (br. s., 1 H) 1.86 (d, J=6.60 Hz, 2 H) 1.52 (br. s., 1 H) 1.42 (br. s., 1 H) 1.36 (br. s., 1 H) 1.30 (br. s., 3 H) 1.12 - 1.26 (m, 4 H) 1.01 (br. s., 1 H) 0.91 (br. s., 12 H). LCMS (ESI+) Calcd for C45H68N4O: 681.54, found [M+H]+:681.25.

4-(((2S)-1-(3,5-bis(trifluoromethyl)phenethyl)-4-(4-((2S)-1-(2-(4-isobutylphenyl)propyl)piperazin-2-yl)butyl)piperazin-2-yl) methyl)phenol (Compound 14)

Using the synthetic approach described in Fig. (**S1**) for the synthesis of compound 14 was synthesized using the following reagents: 4-Isobutyl-alpha-methylphenylacetic acid (R1), Boc-L-Tyrosine(BrZ) (R2), 3,5-Bis(Trifluoromethyl)-Phenylacetic Acid (R3). Final crude product was purified by HPLC as described above. 1H NMR (400 MHz, CHLOROFORM-d): δ ppm 7.75 (br. s., 1 H) 7.65 (br. s., 3 H) 7.28 (s, 1 H) 7.00 - 7.08 (m, 4 H) 6.95 (br. s., 2 H) 6.81 (br. s., 3 H) 3.07 (br. s., 5 H) 2.93 (br. s., 5 H) 2.76 (br. s., 4 H) 2.65 (br. s., 4 H) 2.43 (d, J=6.36 Hz, 4 H) 2.36 (br. s., 2 H) 2.19 (br. s., 1 H) 2.01 (br. s., 1 H) 1.84 (br. s., 1 H) 1.47 (br. s., 2 H) 1.39 (br. s., 1 H) 1.27 (br. s., 1 H) 1.22 (br. s., 3 H) 1.05 (br. s., 1 H) 0.90 (d, J=5.50 Hz, 6 H). LCMS (ESI+) Calcd for C42H56F6N4O: 747.44, found [M+H]+:747.20.

(2S)-1-(2-(4-isobutylphenyl)propyl)-4-(4-((2S)-1-(2-(4-isobutylphenyl)propyl)piperazin-2-yl)butyl)-2-(naphthalen-2-ylmethyl) piperazine (Compound 19)

Using the synthetic approach described in Fig. (**S1**) for the synthesis of compound 19 was synthesized using the following reagents: 4-Isobutyl-alpha-methylphenylacetic acid (R1), Boc-3-(2-naphthyl)-L-alanine (R2), 4-Isobutyl-alpha-methylphenylacetic acid (R3). Final crude product was purified by HPLC as described above. 1H NMR (400 MHz, CHLOROFORM-d): δ ppm 7.78 (d, J=9.05 Hz, 3 H) 7.61 (br. s., 1 H) 7.44 (br. s., 2 H) 7.29 (br. s., 1 H) 6.99 - 7.20 (m, 7 H) 4.16 (br. s., 1 H) 3.09 - 3.34 (m, 1 H) 3.00 (d, J=12.59 Hz, 3 H) 2.91 (br. s., 2 H) 2.84 (br. s., 2 H) 2.78 (br.s., 2 H) 2.58 (d, J=12.84 Hz, 2 H) 2.45 (dd, J=15.89, 6.72 Hz, 7 H) 2.28 (br. s., 3 H) 2.20 (br. s., 2 H) 1.97 (br. s., 1 H) 1.86 (dd, J=13.08, 6.85Hz, 2 H) 1.60 (br. s., 1 H) 1.18 - 1.46 (m, 9 H) 1.15 (br. s., 2 H) 0.70 - 0.95 (m, 12 H). LCMS (ESI+) Calcd for C49H70N4: 715.56, found [M+H]+:715.30.

(2R)-1-(2-(4-isobutylphenyl)propyl)-4-(4-((2S)-1-(2-(4-isobutylphenyl)propyl)piperazin-2-yl)butyl)-2-(naphthalen-2-ylmethyl) piperazine (Compound 22)

Using the synthetic approach described in Fig. (**S1**) for the synthesis of compound 22 was synthesized using the following reagents: 4-Isobutyl-alpha-methylphenylacetic acid (R1), Boc-L-Tyrosine(BrZ) (R2), 4-Isobutyl-alpha-methylphenylacetic acid (R3). Final crude product was purified by HPLC as described above. 1H NMR (400 MHz, CHLOROFORM-d): δ ppm 7.71 - 7.85 (m, 3 H) 7.60 (br. s., 1 H) 7.44 (br. s., 2 H) 7.28 - 7.35 (m, 1 H) 7.01 - 7.26 (m, 8 H) 3.19 (d, J=10.15 Hz, 1 H) 2.94 - 3.07 (m, 3 H) 2.89 (d, J=9.66 Hz, 3 H) 2.82 (br. s., 3 H) 2.60 - 2.74 (m, 2 H) 2.55 (br. s., 1 H) 2.24 - 2.50 (m, 10 H) 2.19 (br. s., 2 H) 1.97 (br. s., 1 H) 1.74 - 1.92 (m, 2 H) 1.34 (d, J=8.68 Hz, 7 H) 1.22 (br. s., 3 H) 1.13 (br. s., 2 H) 0.91 (br. s., 13 H). LCMS (ESI+) Calcd for C49H70N4: 715.56, found [M+H]+:715.20.

4-(((2S)-4-(4-((S)-1-(3,5-bis(trifluoromethyl)phenethyl)piperazin-2-yl)butyl)-1-(2-(4-isobutylphenyl)propyl)piperazin-2-yl) methyl)phenol (Compound 25)

Using the synthetic approach described in Fig. (**S1**) for the synthesis of compound 25 was synthesized using the following reagents: 3,5-Bis(Trifluoromethyl)-Phenylacetic Acid (R1), Boc-L-Tyrosine(BrZ) (R2), 4-Isobutyl-alpha-methylphenylacetic acid (R3). Final crude product was purified by HPLC as described above. 1H NMR (400 MHz, CHLOROFORM-d): δ ppm 7.74 (br. s., 2 H) 7.62 (br. s.,1 H) 7.29 (s, 2 H) 7.07 - 7.26 (m, 4 H) 6.93 (s, 1 H) 6.96 (s, 1 H) 6.82 (d, J=6.97 Hz, 2 H) 3.19 (br. s., 2 H) 3.10 (br. s., 2 H) 3.00 (br. s., 5 H) 2.84 (br. s., 5 H) 2.70 (br. s., 4 H) 2.62 (br. s., 3 H) 2.46 (d, J=6.60 Hz, 3 H) 2.19 (br. s., 1 H) 2.02 (br. s., 1 H) 1.86 (br. s., 1 H) 1.56 (br. s., 1 H) 1.43 (br. s., 2H) 1.30 (br. s., 3 H) 1.17 (d, J=6.24 Hz, 1 H) 1.10 (br. s., 2 H) 0.91 (d, J=6.11 Hz, 6 H). LCMS (ESI+) Calcd for C42H56F6N4O: 747.44, found [M+H]+:747.10.

4-(((S)-1-(adamantan-1-ylmethyl)-4-(4-((S)-1-(3,5-bis(trifluoromethyl)phenethyl)piperazin-2-yl)butyl)piperazin-2-yl)methyl) phenol (Compound 27)

Using the synthetic approach described in Fig. (**S1**) for the synthesis of compound 27 was synthesized using the following reagents: 3,5-Bis(Trifluoromethyl)-Phenylacetic Acid (R1), Boc-L-Tyrosine(BrZ) (R2), 1-Adamantanecarboxylic Acid (R3). Final crude product was purified by HPLC as described above. 1H NMR (400 MHz, CHLOROFORM-d): δ ppm 8.44 (br. s., 1H) 8.05 (br. s., 1 H) 7.96 (br. s., 1 H) 7.83 (br. s., 1 H) 7.73 (br. s., 1 H) 7.63 (br. s., 2 H) 7.28 (s, 1 H) 7.00 (br. s., 2 H) 6.83 (d,J=6.48 Hz, 2 H) 3.17 (br. s., 1 H) 2.91 - 3.13 (m, 5 H) 2.84 (br. s., 5 H) 2.54 - 2.77 (m, 7 H) 2.40 (br. s., 2 H) 2.10 (br. s., 1 H) 1.99 (d, J=6.36 Hz, 4 H) 1.69 - 1.85 (m, 3 H) 1.64 (d, J=11.37 Hz, 4 H) 1.50 (br. s., 8 H) 1.28 (br. s., 1 H) 1.13 (br. s., 2 H). LCMS (ESI+) Calcd for C40H54F6N4O: 721.42, found [M+H]+:721.15.

(2S)-4-(4-((S)-1-(3,5-bis(trifluoromethyl)phenethyl)piperazin-2-yl)butyl)-1-(2-(4-isobutylphenyl)propyl)-2-phenylpiperazine (Compound 28)

Using the synthetic approach described in Fig. (**S1**) for the synthesis of compound 28 was synthesized using the following reagents: 3,5-Bis(Trifluoromethyl)-Phenylacetic Acid (R1), Boc-L-Phenylglycine (R2), 4-Isobutyl-alpha-methylphenylacetic acid (R3). Final crude product was purified by HPLC as described above. 1H NMR (400 MHz, CHLOROFORM-d): δ ppm 7.74 (br. s., 1 H) 7.65 (br. s., 2 H) 7.28 - 7.40 (m, 2 H) 7.12 - 7.26 (m, 2 H) 6.85 - 7.07 (m, 5 H) 3.43 - 3.66 (m, 1 H) 3.36 (br. s., 1 H) 3.30 (d, J=7.58 Hz, 1 H) 3.22 (d, J=11.37 Hz, 1 H) 3.15 (br. s., 1 H) 3.02 (br. s., 2 H) 2.83 - 2.96 (m, 6 H) 2.78 (d, J=11.37 Hz, 1 H) 2.53 - 2.72 (m, 2 H) 2.36 - 2.52 (m, 4 H) 2.26 - 2.36 (m, 3 H) 2.21 (br. s., 1 H) 1.98 - 2.16 (m, 2 H) 1.76 - 1.96 (m, 2

H) 1.48 (br. s., 3 H) 1.36 (br. s., 1 H) 1.27 (br. s., 1 H) 1.20 (d, J=6.24 Hz, 2 H) 1.08 (d, J=6.24 Hz, 2 H) 0.96 (d, J=5.99 Hz, 3 H) 0.88 (d, J=6.11 Hz, 3 H). LCMS (ESI+) Calcd for C41H54F6N4: 717.43, found [M+H]+:17.15.

(S)-1-(adamantan-1-ylmethyl)-4-(4-((S)-1-(3,5-bis(trifluoromethyl)phenethyl)piperazin-2-yl)butyl)-2-phenylpiperazine (Compound 30)

Using the synthetic approach described in Fig. (**S1**) for the synthesis of compound 30 was synthesized using the following reagents: 3,5-Bis(Trifluoromethyl)-Phenylacetic Acid (R1), Boc-L-Phenylglycine (R2), 1-Adamantanecarboxylic Acid (R3). Final crude product was purified by HPLC as described above. 1H NMR (400 MHz, CHLOROFORM-d): δ ppm 7.74 (br. s., 1 H) 7.66 (br. s., 2 H) 7.35 (br. s., 2 H) 7.28 - 7.32 (m, 2 H) 3.49 - 3.76 (m, 3 H) 3.34 - 3.49 (m, 1 H) 3.15 - 3.33 (m, 2 H) 3.03 (d, J=10.64 Hz, 2 H) 2.78 - 2.96 (m, 6 H) 2.59 - 2.73 (m, 2 H) 2.48 - 2.58 (m, 1 H) 2.45 (br. s., 1 H) 2.28 - 2.43 (m, 4 H) 2.20 (t, J=9.90 Hz, 1 H) 2.10 (d, J=13.57 Hz, 1 H) 2.01 (br. s., 1 H) 1.87 (br. s., 3 H) 1.71 (br. s., 1 H) 1.64 (d, J=11.98 Hz, 3 H) 1.55 (d, J=12.10 Hz, 4 H) 1.35 - 1.51 (m, 6 H) 1.25 (s, 3 H) 1.23 (s, 2 H). LCMS (ESI+) Calcd for C39H52F6N4: 691.41, found [M+H]+:691.10.

(2R)-4-(4-((S)-1-(3,5-bis(trifluoromethyl)phenethyl)piperazin-2-yl)butyl)-1-(2-(4-isobutylphenyl)propyl)-2-(naphthalen-2-ylmethyl)piperazine (Compound 34)

Using the synthetic approach described in Fig. (**S1**) for the synthesis of compound 34 was synthesized using the following reagents: 3,5-Bis(Trifluoromethyl)-Phenylacetic Acid (R1), Boc-3-(2-naphthyl)-D-alanine (R2), 4-Isobutyl-alpha-methylphenylacetic acid (R3). Final crude product was purified by HPLC as described above. 1H NMR (400 MHz, CHLOROFORM-d): δ ppm 7.71 - 7.82 (m, 4 H) 7.59 (s, 1 H) 7.63 (s, 2 H) 7.43 (br. s., 2 H) 7.28 (br. s., 1 H) 7.07 - 7.20 (m, 4 H) 3.54 (br. s., 2 H) 3.33 (br. s., 1 H) 3.19 (d, J=10.64 Hz, 1 H) 3.01 (br. s., 3 H) 2.89 (br. s., 4 H) 2.83 (br. s., 4 H) 2.61 (br. s., 2 H) 2.56 (br. s., 2 H) 2.40 - 2.52 (m, 5 H) 2.36 (br. s., 1 H) 2.26 (br. s., 1 H) 2.19 (br. s., 2 H) 1.88 (d, J=5.99 Hz, 1 H) 1.39 (br. s., 3 H) 1.27 - 1.37 (m, 4 H) 1.23 (br. s., 1 H) 1.16 (br. s., 1 H) 0.92 (br. s., 6 H). LCMS (ESI+) Calcd for C46H58F6N4: 781.46, found [M+H]+:781.15.

(2S)-4-(4-((S)-1-((4-(tert-butyl)cyclohexyl)methyl)piperazin-2-yl)butyl)-1-(2-(4-isobutylphenyl)propyl)-2-(naphthalen-2-ylmethyl)piperazine (Compound 43)

Using the synthetic approach described in Fig. (**S1**) for the synthesis of compound 43 was synthesized using the following reagents: 4-tert-Butyl-Cyclohexanecarboxylic Acid (R1), Boc-3-(2-naphthyl)-L-alanine (R2), 4-Isobutyl-alpha-methylphenylacetic acid (R3). Final crude product was purified by HPLC as described above. 1H NMR (400 MHz, CHLOROFORM-d): δ ppm 7.71 - 7.84 (m, 3 H) 7.60 (br. s., 1 H) 7.45 (br. s., 2 H) 7.29 (br. s., 1 H) 7.02 - 7.26 (m, 4 H) 3.18 (d, J=9.54 Hz, 1 H) 3.04 (br. s., 2 H) 2.97 (br. s., 2 H) 2.91 (br. s., 3 H) 2.83 (br. s., 1 H) 2.73 (d, J=9.54 Hz, 2 H) 2.52 - 2.67 (m, 2 H) 2.48 (br. s., 3 H) 2.41 (br. s., 2 H) 2.31 (br. s., 2 H) 2.22 (br. s., 2 H) 1.91 - 2.09 (m, 2 H) 1.88 (d, J=7.34 Hz, 1 H) 1.75 (d, J=13.94 Hz, 2 H) 1.60 (d, J=13.20 Hz, 1 H) 1.50 (br. s., 3 H) 1.43 (br. s., 3 H) 1.26 - 1.38 (m, 5 H) 1.21 (br. s., 2 H) 0.89 - 1.06 (m, 9 H) 0.65 - 0.89 (m, 10 H). LCMS (ESI+) Calcd for C47H72N4: 694.58, found [M+H]+:694.40.

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