Applying a Multitarget Rational Drug Design Strategy: a First Set of Modulators with Potent and Balanced Activity toward Dopamine D3 Receptor and Fatty Acid Amide Hydrolase

Alessio De Simone, Gian Filippo Ruda, Clara Albani, Glauco Tarozzo, Tiziano Bandiera, Daniele Piomelli, Andrea Cavalli, Giovanni Bottegoni.

Supporting Information

1. Ligand Docking

1.1 Preparation of Protein Structures

The coordinates of Fatty Acid Amide Hydrolase 1 from Rattus norvegicus (rFAAH-1) in complex with a noncovalent inhibitor (PDBid: 3QJ8) [1] and the coordinates of human dopamine receptor D3 (DRD3) in complex with eticlopride (PDBid: 3PBL) [2] were retrieved from the PDB and used in docking simulations. Hydrogen atoms were added. Polar hydrogen atoms and the positions of asparagine and glutamine side chain amidic groups were optimized and assigned the lowest energy conformation. After optimization, histidines were automatically assigned the tautomerization state that improved the hydrogen bonding pattern. Finally, the original ligands were deleted from the holo structures.

1.2 Binding Pocket Definition

Binding sites were identified by means of the IcmPocketFinder tool as implemented in ICM3.7.[3] The tolerance value was set equal to 4.6. The macro provides a mesh associated to every detected pocket. The graphical object closest to the co-crystallized ligand position was selected. All the residues with at least one side chain non- hydrogen atom in the range of 3.5 Å from the selected mesh were considered part of the pocket.

1.3 Preparation of the Ligands

Exploiting the overlap between the pharmacophoric features of the D3 selective modulators (**1-3**) and FAAH inhibitors (**4-6**), a prototype ligand was designed (Figure S1) and used for the generation of an in silico combinatorial library encompassing 280 compounds devised by combination of the chemical moieties reported in Figure S2.

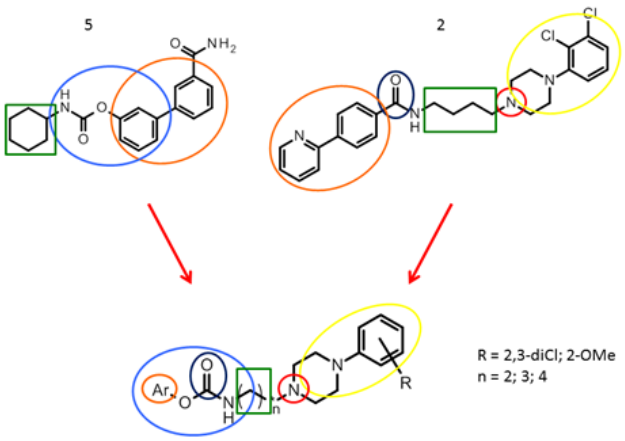


Figure S1. A dual-target pharmacophore encompassing the features of DRD3 selective partial agonists and FAAH inhibitors from the O-aryl carbamate series.

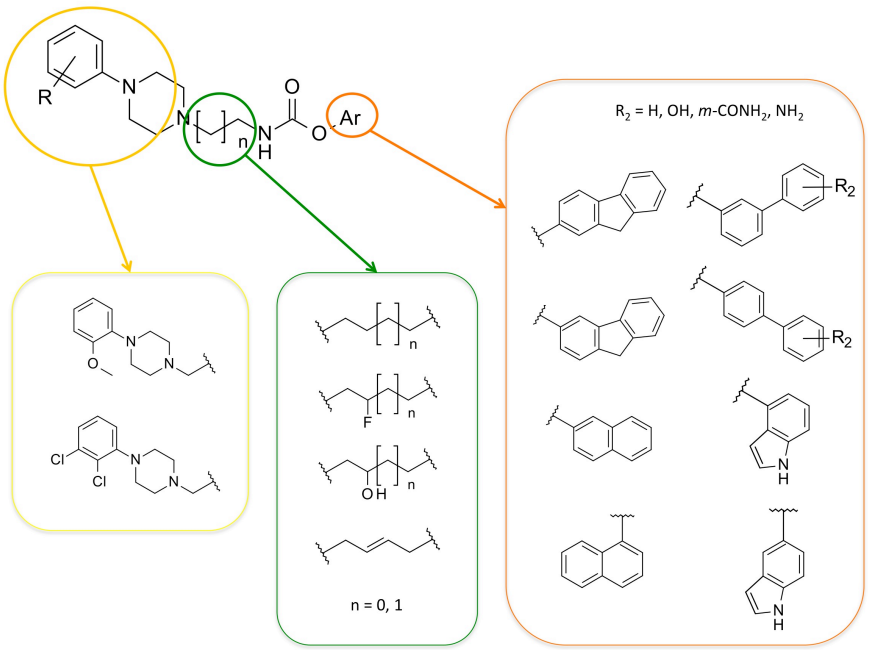


Figure S2. *In silico* combinatorial library. The molecules were assembled according to the general formula combining 2 differently substituted aryl-piperazines (yellow), linkers (green), and 20 different *O*-aryl moieties (orange).

In the resulting library, each molecule had a univocal arrangement of chemical features likely relevant for the activity on both FAAH and DRD3. Ligands were assigned the right bond orders, stereochemistry, hydrogen atoms, and protonation states at pH 7.4. Each ligand was assigned the MMFF force field atom types and charges.[4]

1.4 Docking Simulations

The docking engine employed was the Biased Probability Monte Carlo (BPMC) stochastic optimizer as implemented in ICM3.7 (Molsoft LLC, San Diego, CA – USA).[5] The ligand binding site at the receptor was represented by pre-calculated 0.5 Å spacing potential grid maps, representing van der Waals potentials for hydrogens and heavy probes, electrostatics, hydrophobicity, and hydrogen bonding. The van der Waals interactions were described by a smoother form of the 6-12 Lennard-Jones potential with the repulsive contribution capped at 4.0 kcal/mol. The electrostatic contribution was buffered, artificially increasing the distance between oppositely charged atoms in order to avoid their collapse when the electrostatic attractive energy prevailed on the softened van der Waals repulsion. The molecular conformation was described by means of internal coordinate variables. The adopted force field was a modified version of the ECEPP/3 force field with a distance-dependent dielectric constant.[6] Given the number of rotatable bonds in the ligand, the basic number of BPMC

steps to be carried out was calculated by an adaptive algorithm (thoroughness 3.0). In docking runs at rFAAH, the initial step of the catalytic Ser241 carbamylation reaction, which characterizes the mechanism of action of o-aryl carbamate inhibitors, was simulated by ICM covalent docking protocol according to the reaction described in Figure S3.[7]

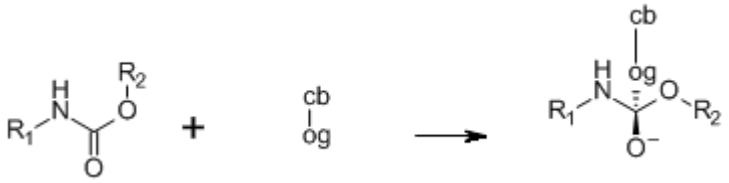


Figure S3. The carbamylation reaction that was used as an input in the covalent docking protocol performed at the binding site of rFAAH.

The binding energy was assessed by means of the standard ICM empirical scoring function.[8] For each ligand in the library, the best scoring pose was selected as representative of the ligand bound conformation.

1.5 Docking Results

The accuracy of the proposed docking protocol could be assessed attempting a re-docking exercise of eticlopride at its DRD3 cognate receptor structure. The native pose could be reproduced within a 0.41 Å RMSD, capturing the key interaction with Asp110. Docking at the same structure the D3 antagonist R-22, it was possible to generate a binding mode in qualitative agreement with the one reported by Chien and co-workers,[2] capturing the key interactions with Asp110 and Glu90. Similar results could be obtained testing the proposed protocol on FAAH. The native non-covalent ligand binding mode could be reproduced within 0.3 Å RMSD. Attempting to generate non-covalent and covalent binding modes for **5**, our results were in agreement with those already reported by Lodola and co-workers for **4**, and by Mor and coworkers for **5**, with the carboxyamide substituent interacting with the side chain of Gln273.[9]

In FAAH, our compounds bound according to the orientation suggested for *O*-aryl carbamate derivatives by the crystal structure of URB597-carbamoylated humanized rat FAAH [10] as well as by extensive computational studies performed on **4**.[11] The carbon linker mimicked the cyclohexyl group of **4** and **5** with the phenyl-piperazine moiety extending into the ACBP and pointing toward the membrane. The piperazine basic nitrogen likely interacted with the backbone carbonyl of Leu192. The *O*-aryl moieties were lodged in the cytoplasmic access channel. The *m*-biphenyl group of **7** (Figure 3a in the main text) and **8** (Figure S4a) established hydrophobic interactions with the side chains of Met191 and Ile238. The 3'-carbamoyl group made hydrogen bond interactions with the side chain of Gln273. The *p*-

biphenyl group of **15** (Figure S4c) established hydrophobic interactions with the side chains of Met191, Ile238, and Leu278. In **15**, the 3'-carbamoyl group made hydrogen bond interactions with the backbone of Val270. In derivative **16** (Figure S4e), the 2-naphtyl substituent established hydrophobic interactions with the side chains of Met191 and Ile238. Finally, in **17** (Figure S4g), the 1-naphtyl group was enclosed in a hydrophobic cage formed by the side chain of residues Met191, Ile238, Leu192, and Leu278.

The proposed binding poses for derivatives **7**,**8**,**15**-**17** were in fairly good agreement with the binding mode previously proposed by Chien and colleagues for DRD3 selective modulators.[2] Also in our case, the aryl-piperazine occupied the same region occupied by eticlopride in the crystal structure of DRD3, a conserved pocket defined by the side chains of residues from helices II, III, V, VI, and VII. Mimicking the interaction of the basic nitrogen of dopamine with conserved Asp110, the proposed binding mode was characterized by the direct interaction of the piperazine nitrogen with the same residue. The carbamate was involved in hydrogen bonding with the oxydryl group of Tyr365 side chain and the backbone of Cys181. The length of the linker did not seem to affect the ability of derivative **8** to establish these hydrogen bonds. The *O*-aryl substituents protruded toward the region of the binding pocket described by extracellular loop 1 and extracellular loop 2 together with helices I, II, and VII. The *m*-biphenyl moiety of **7** (Figure 3b in the main text) and **8** (Figure S4b) established hydrogen bond interactions with the backbone of Leu89 and Thr92. Derivative **15** (Figure S4d) bound in such a way that the proximal ring of the *p*-biphenyl moiety occupied the same region of derivatives **7** and **8**, thus establishing hydrogen bond interactions with the backbone of Leu89 and Thr92.

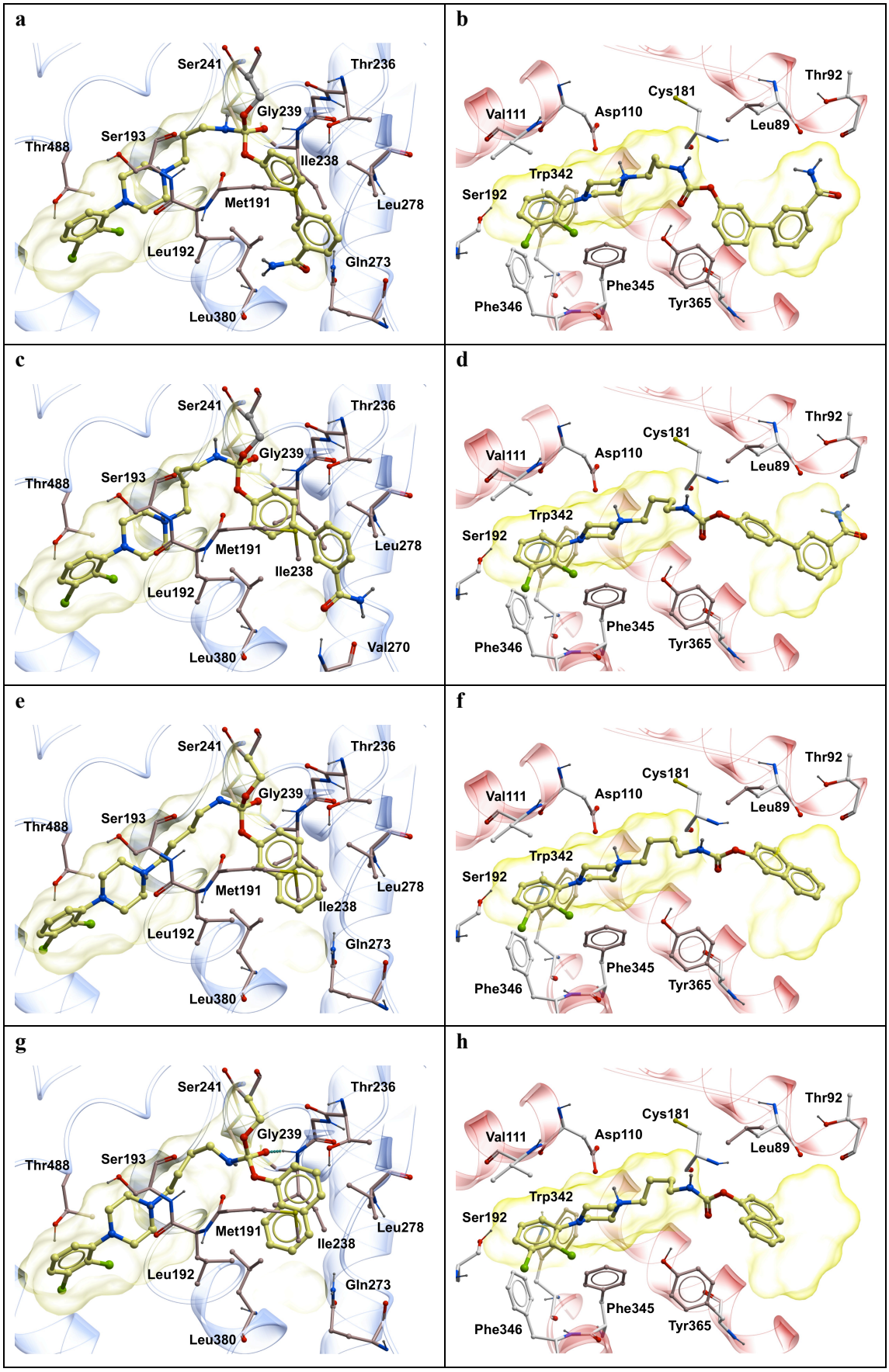


Figure S4. Bound poses of derivatives a) **8** at FAAH binding site; b) **8** at DRD3 binding site; c) **15** at FAAH binding site; d) **15** at DRD3 binding site; e) **16** at FAAH binding site; f) **16** at DRD3 binding

site; g) **17** at FAAH binding site; h) **17** at DRD3 binding site. Ligands are displayed in sticks representation with yellow carbon atoms. Binding site residues are displayed in sticks representation and labeled explicitly. The rest of the proteins is displayed as transparent cartoons. The boundaries of the binding pockets are highlighted by transparent meshes.

1.6 Physico-Chemical Properties

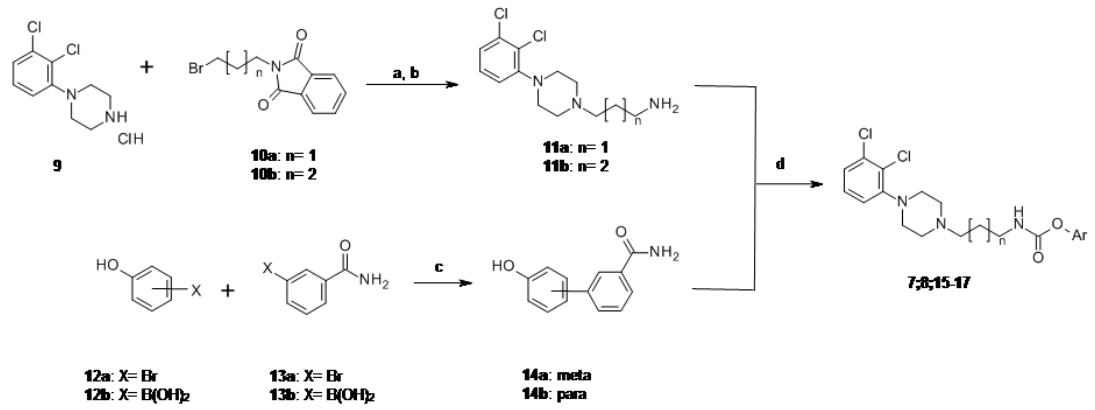
The therapeutic efficacy of our compounds will depend on their ability to cross the blood-brain barrier (BBB), engaging their targets in the central nervous system (CNS). In order to provide a preliminary assessment of the physico-chemical features that can modulate the propensity to cross the BBB, for each synthesized compound, we calculated: molecular weight (MW), LogP and the sp^3/sp^2 carbon ratio. Results are reported in Table S1. The biphenyl-bearing compounds (**7**, **8** and **15**) showed high MW (>500) and cLogP (>5). These values are in line with those calculated for known D3 modulators **1** and **2** (Table S1). Synthesizing the naphthilic derivatives **16** and **17**, we were able to reduce MW below the Lipinski's threshold of 500, maintaining, at the same time, cLogP values in line with those reported for **1**. Interestingly, the sp^3/sp^2 ratio was similar to the values reported for **1-3**. In light of these considerations, thank to their interesting physico-chemical profile, we believe that the proposed compounds are worth preceding to their next development step, testing *in vivo* their ability to exert their activity in the CNS.

Table S1. Physico-chemical properties of reported compounds

Compound	MW	cLogP	sp^3/sp^2 Carbon Ratio
1 (NGB2904)	494.46	6.44	0.47
2 (CJB090)	483.43	5.57	0.42
3 (BP897)	417.55	4.34	0.53
7	541.47	5.62	0.40
8	527.45	5.16	0.35
15	541.47	5.62	0.40
16	472.41	6.03	0.47
17	472.41	6.03	0.47

2. Organic Synthesis

2.1 Synthetic scheme



Scheme S1. a) CH_3CN, K_2CO_3, reflux, 6 h; b) Hydrazine monohydrate, Methanol, 80°C, 2 h, then HCl 2N, 1 h; c) $Pd(OAc)_2$, K_2CO_3, EGME/H_2O, RT, 5 min; d) $(Boc)_2O$, DMAP, CH_3CN, rt, 23 h.

Amines **11a,b** were obtained by alkylation of arylpiperazine derivative **9** with different commercially available bromoalkylphthalimides **10a,b**. The obtained phthalimides were deprotected by treatment with hydrazine. Biphenyl alcohols **14a,b** were prepared employing a modified Suzuki cross-coupling [12] between commercially available aryl halides **12a** and **13a** and boronic acid derivatives **12b** and **13b**, in the presence of a catalytic amount of palladium acetate in a medium of water and ethylene glycol monomethyl ether (EGME). This procedure provided the desired products in high yield and good reproducibility. In the final step, amines **11a,b** were activated as isocyanates, by reaction with slight excess of Boc anhydride and 4-dimethylamino pyridine (DMAP) in acetonitrile, and then reacted with the appropriate biphenyl alcohol 14a,b in order to get carbamates **7** and **8**.[13]

2.2 General reagents and materials

Automated column chromatography purifications were performed on a Teledyne ISCO apparatus (CombiFlashTM Rf) with pre-packed silica gel columns of different sizes (from 4 g to 120 g). Mixtures of increasing polarity of cyclohexane and ethyl acetate or dichloromethane and methanol were used as eluents. NMR experiments were run on a Bruker Avance III 400 system (400.13 MHz for 1H, and 100.62 MHz for 13C), equipped with a BBI probe and Z-gradients. Spectra were acquired at 300 K, using deuterated dimethylsulfoxyde (DMSO-d_6) or deuterated chloroform (Chloroform-*d*) as solvents. Chemical shifts for ^1H and ^{13}C spectra were recorded in parts per million using the residual non-deuterated solvent as the internal standard (for Chloroform-*d*: 7.26 ppm, 1H and 77.16 ppm, 13C; for DMSO-d_6: 2.50

ppm, 1H; 39.52 ppm, 13C). Signals were attributed based on DEPT 135, COSY, HSQC and HMBC experiments. UPLC/MS analyses were run on a Waters ACQUITY UPLC/MS system consisting of a SQD (Single Quadrupole Detector) Mass Spectrometer equipped with an Electrospray Ionization interface and a Photodiode Array Detector. PDA range was 210-400 nm. Electrospray ionization in positive and negative mode was applied. UPLC mobile phases were: (A) 10mM NH_4OAc in H_2O, pH 5; (B) 10mM NH_4OAc in MeCN/H_2O (95:5) pH 5. Analyses were performed either with method A, B or C below reported.

Method A (generic)

Gradient: 5 to 95% B over 3 min. Flow rate 0.5 mL/min. Temp. 40 °C

Pre column: Vanguard BEH C18 (1.7µm 2.1x5mm). Column: BEH C18 (1.7µm 2.1x50mm)

Method B (polar)

Gradient: 0 to 50% B over 3 min. Flow rate 0.5 mL/min. Temp. 40 °C

Pre column: VanGuard HSS T3 C18 (1.7µm 2.1x5 mm). Column HSS T3 (1.8µm 2.1x50mm)

Method C (apolar)

Gradient: 50 to 100% B over 3 min. Flow rate 0.5 mL/min. Temp. 40 °C

Pre column: Vanguard BEH C18 (1.7µm 2.1x5mm). Column: BEH C18 (1.7µm 2.1x50mm)

Accurate mass measurement was performed on a quadrupole time-of-flight instrument (Synapt G2 QTof, Waters, USA), equipped with an ESI ion source. Compounds were diluted to 20µM in water/acetonitrile and analyzed. Leucine Enkephalin (2ng/ml) was used as lockmass reference compound for spectra calibration.

2.3 Synthesis of 3-[4-(2,3-dichlorophenyl)piperazin-1-yl]propan-1-amine (11a)

A mixture of 1-(2,3-dichlorophenyl)piperazine hydrochloride (400.0 mg, 1.49 mmol), *N*-(3-bromopropyl)phthalimide (440.8 mg, 1.64 mmol) and K_2CO_3 (516.4 mg, 3.74 mmol) in 7 mL of acetonitrile was heated to reflux for 6 hours. The hot suspension was filtrated and the residue washed with acetone several times. The filtrates were concentrated under reduced pressure to give the phthalimide intermediate which was dissolved in methanol and heated to reflux in presence of hydrazine hydrate (0.07 mL, 1.53 mmol) for 2 hours. To the hot solution was added 2 mL of 2N HCl, and reflux was continued for one more hour. After cooling to ambient temperature, the mixture was filtered, the residue was washed with methanol, and the filtrate was evaporated to dryness. This dry residue was suspended in water and neutralized with 2N NaOH. Extraction with EtOAc afforded a colourless oily product, which was pure enough for the next step (233 mg, 54% over two steps). UPLC-MS (method A): Rt 1.81 min; m/z 288 $[M-H]^+$. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.18 – 7.09 (m, 2H), 6.95 (dd, J = 6.5, 3.1 Hz, 1H), 3.87 (s, 3H), 3.11 (s, 4H), 2.99 (t, J = 6.2 Hz, 2H), 2.72 (s, 4H), 2.59 (t, J = 6.5 Hz, 2H), 1.98 (s, 2H), 1.82 (p, J = 6.4 Hz, 2H).

2.4 Synthesis of 4-[4-(2,3-dichlorophenyl)piperazin-1-yl]butan-1-amine (11b)

A mixture of 1-(2,3-dichlorophenyl)piperazine hydrochloride (1.87 mmol, 500 mg), *N*-(4-bromobutyl)phthalimide (2.06 mmol, 579.9 mg) and K_2CO_3 (5.61 mmol, 774.7 mg) in 7 mL of acetonitrile was heated to reflux for 6 hours. The hot suspension was filtrated and the residue washed with acetone several times. The filtrates were concentrated under reduced pressure to give the phthalimide intermediate which was dissolved in methanol and heated to reflux in presence of hydrazine hydrate (0.10 mL, 2.14 mmol) for 2 hours. To the hot solution was added 2 mL of 2N HCl, and reflux was continued for one more hour. After cooling to ambient temperature, the mixture was filtered, the residue was washed with methanol, and the filtrate evaporated to dryness. This dry residue was suspended in water and neutralized with 2N NaOH. Extraction with EtOAc afforded a colourless oily product, which was pure enough for the next step (351 mg, 62% over two steps). UPLC-MS (method A): Rt 1.64 min; m/z 302 $[M\text{-}H]^+$. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.18 – 7.09 (m, 2H), 6.95 (dd, *J* = 6.5, 3.1 Hz, 1H), 3.16 – 2.98 (m, 4H), 2.72 (t, *J* = 6.8 Hz, 2H), 2.63 (d, *J* = 7.0 Hz, 4H), 2.48 – 2.36 (m, 2H), 1.62 – 1.42 (m, 4H), 1.37 (s, 2H).

2.5 Synthesis of 3-(3-hydroxyphenyl)benzamide (14a)

3-benzamideboronic acid (143.0 mg, 0.87 mmol) was added to a solution of 3-bromophenol (100.0 mg, 0.58 mmol) in EGME/water 3:1 (4 mL), followed by the addition of $Pd(OAc)_2$ (1.3 mg, 0.01 mmol) and K_2CO_3 (199.7 mg, 1.45 mmol). After few minutes the yellowish suspension turned dark-black and the reaction reached full conversion. The mixture was stirred for further 5 min., then diluted with water, acidified with 2N HCl and extracted with DCM. The organic phase was dried over Na_2SO_4 and concentrated to dryness. The crude residue was adsorbed on silica and purified by flash chromatography (Eluent: 5% MeOH in DCM), to provide the title compound as brown solid (119 mg, 97%). UPLC-MS (method A): Rt 1.59 min; m/z 214 $[M\text{-}H]^+$. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.57 (s, 1H), 8.11 (t, *J* = 1.86 Hz, 1H), 7.85 (dt, *J* = 1.37, 7.80 Hz, 1H), 7.75 (dt, *J* = 1.37, 7.75 Hz, 1H), 7.53 (t, *J* = 7.72 Hz, 1H), 7.40 (s, 2H), 7.29 (t, *J* = 7.84 Hz, 1H), 7.14 (dt, *J* = 1.20, 7.74 Hz, 1H), 7.10 (t, *J* = 2.10 Hz, 1H), 6.80 (dd, *J* = 2.38, 7.96 Hz, 1H).

2.6 Synthesis of 3-(4-hydroxyphenyl)benzamide (14b)

4-hydroxybenzeneboronic acid (450.0 mg, 3.26 mmol) was added to a solution of 3-bromobenzamide (783.2 mg, 3.92 mmol) in EGME/water 3:1 (4 mL), followed by the addition of $Pd(OAc)_2$ (7.3 mg, 0.03 mmol) and K_2CO_3 (1127.5 mg, 8.16 mmol). After few minutes the yellowish suspension turned dark-black and the reaction reached full conversion. The mixture was stirred for further 5 min., then diluted with water, acidified with 2N HCl and extracted with DCM. The organic phase was dried over Na_2SO_4 and concentrated to dryness.

The crude residue was adsorbed on silica and purified by flash chromatography (Eluent: 5% MeOH in DCM), to provide the pure compound as brown solid (625 mg, 90%). UPLC-MS (method A): Rt 1.53 min; m/z 214 $[M-H]^+$. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.58 (s, 1H), 8.12 – 8.02 (m, 2H), 7.75 (dddd, J = 20.2, 7.7, 1.8, 1.1 Hz, 2H), 7.61 – 7.52 (m, 2H), 7.48 (t, J = 7.7 Hz, 1H), 7.37 (q, J = 3.6, 3.0 Hz, 1H), 6.92 – 6.83 (m, 2H).

2.7 Synthesis of [3-(3-carbamoylphenyl)phenyl] N-[4-[4-(2,3-dichlorophenyl)piperazin-1-yl]butyl] carbamate (7)

To a solution of di-*tert*-butyldicarbonate (50.5 mg, 0.23 mmol) in acetonitrile (1 mL) were added in sequence: a solution of 4-dimethylaminopyridine (20.2 mg, 0.17 mmol) in acetonitrile (1 mL) and a solution of the appropriate amine **11b** (50.0 mg, 0.17 mmol) in acetonitrile (1 mL). After stirring for 10 minutes at room temperature, the phenol derivative **14a** (49.4 mg, 0.23 mmol) was added. The reaction mixture was stirred for 22 h at room temperature, after which the solvent was evaporated under reduced pressure. The crude residue was solubilized in ethyl acetate and washed with a saturated solution of $NaHCO_3$. The organic layer was dried (Na_2SO_4), concentrated and purified by flash chromatography (Eluent: 5% MeOH in DCM) to afford the desired compound as white solid (51 mg, 57%). UPLC-MS (method A): Rt 2.36 min; m/z 541 $[M-H]^+$. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.19 – 8.11 (m, 2H), 7.91 – 7.80 (m, 3H), 7.62 – 7.42 (m, 5H), 7.35 – 7.26 (m, 2H), 7.19 – 7.07 (m, 2H), 3.12 (d, J = 6.19 Hz, 2H), 2.99 (s, 4H), 2.55 (s, 4H), 2.38 (d, J = 6.39 Hz, 2H), 1.56 – 1.49 (m, 4H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 168.26, 158.58, 151.67, 141.45, 140.73, 135.31, 133.07, 130.42, 129.73, 129.30, 128.88, 126.98, 126.45, 126.07, 124.75, 123.81, 120.61, 119.96, 118.01, 115.18, 57.93, 53.25, 51.42, 28.46, 27.68, 24.13. ESI+ (m/z): $[M+H]^+$ calculated for C_{28} H_{31} Cl_2 N_4 O_3 541.1773; found 541.178 $[M+H]^+$. UPLC-MS Purity (UV @ 215 nm): 99%

2.8 Synthesis of [3-(3-carbamoylphenyl)phenyl] N-[4-[4-(2,3-dichlorophenyl)piperazin-1-yl]butyl] carbamate (8)

To a solution of di-*tert*-butyldicarbonate (95.4 mg, 0.44 mmol) in acetonitrile were added in sequence: a solution of 4-dimethylaminopyridine (38.1 mg, 0.31 mmol) in acetonitrile and a solution of the appropriate amine **11a** (90 mg, 0.31 mmol) in acetonitrile. After stirring for 10 minutes at room temperature, the phenol derivative **14a** (79.9 mg, 0.37 mmol) was added. The reaction mixture was stirred for 22 h at room temperature, after which the solvent was evaporated under reduced pressure. The crude residue was solubilized in ethyl acetate and washed with a saturated solution of $NaHCO_3$. The organic layer was dried (Na_2SO_4), concentrated and purified by flash chromatography (Eluent: 5% MeOH in DCM) to afford the desired compound as white solid (45 mg, 27%). UPLC-MS (method A): Rt 2.44 min; m/z 527

$[M-H]^+$. ^1H NMR (400 MHz, DMSO-d_6) δ 8.17 (m, 1H), 8.12 (m, 1H), 7.92 – 7.84 (m, 1H), 7.84 (m, 2H), 7.62 – 7.45 (m, 4H), 7.41 (m, 1H), 7.30 (q, *J* = 3.71, 4.39 Hz, 2H), 7.20 – 7.08 (m, 2H) 3.16 (q, *J* = 6.51 Hz, 2H), 3.00 (t, *J* = 4.84 Hz, 4H), 2.56 (m, 4H), 2.42 (t, *J* = 7.04 Hz, 2H), 1.69 (p, *J* = 6.95 Hz, 2H). ^{13}C NMR (101 MHz, DMSO) δ 167.7, 157.87, 151.17, 140.96, 140.25, 134.96, 132.58, 129.93, 129.24, 128.97, 128.81, 128.39, 126.50, 125.59, 124.27, 120.15, 119.52, 117.51, 114.69, 113.63, 55.45, 52.79, 50.91, 37.72, 27.22. ESI+ (m/z): $[M+H]^+$ calculated for C_{27} H_{29} Cl_2 N_4 O_3 527.1617; found 527.1622 $[M+H]^+$. UPLC-MS Purity (UV @ 215 nm): 94%

2.9 Synthesis of [4-(3-carbamoylphenyl)phenyl] N-[4-[4-(2,3-dichlorophenyl)piperazin-1-yl]butyl] carbamate (15)

To a solution of di-*tert*-butyldicarbonate (80.9 mg, 0.37 mmol) in acetonitrile were added in sequence: a solution of 4-dimethylaminopyridine (32.3 mg, 0.26 mmol) in acetonitrile and a solution of the appropriate amine **11b** (80.0 mg, 0.26 mmol) in acetonitrile. After stirring for 10 minutes at room temperature, the phenol derivative **14b** (79.0 mg, 0.37 mmol) was added. The reaction mixture was stirred for 22 h at room temperature, after which the solvent was evaporated under reduced pressure. The crude residue was solubilized in ethyl acetate and washed with a saturated solution of $NaHCO_3$. The organic layer was dried (Na_2SO_4), concentrated and purified by flash chromatography (Eluent: 5% MeOH in DCM) to afford the desired compound as white solid (59 mg, 41%). UPLC-MS (method A): Rt 2.32 min; m/z 541 $[M-H]^+$. ^1H NMR (400 MHz, DMSO-d_6) δ 8.15 (t, *J* = 1.8 Hz, 1H), 8.09 (s, 1H), 7.83 (dddd, *J* = 17.5, 7.8, 2.3, 1.2 Hz, 3H), 7.78 – 7.70 (m, 2H), 7.55 (t, *J* = 7.7 Hz, 1H), 7.41 (s, 1H), 7.34 – 7.27 (m, 2H), 7.26 – 7.19 (m, 2H), 7.15 (dd, *J* = 6.5, 3.1 Hz, 1H), 3.12 (q, *J* = 6.3 Hz, 2H), 3.00 (d, *J* = 5.0 Hz, 4H), 2.56 (s, 4H), 2.39 (d, *J* = 6.8 Hz, 2H), 1.60 – 1.47 (m, 4H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 168.22, 154.70, 151.68, 151.40, 139.93, 136.70, 135.42, 133.08, 129.74, 129.40, 128.89, 128.16, 126.95, 126.47, 126.03, 124.78, 122.68, 120.00, 57.87, 53.26, 51.42, 40.92, 27.67, 24.03. ESI+ (m/z): $[M+H]^+$ calculated for C_{28} H_{31} Cl_2 N_4 O_3 541.1773; found 541.1776 $[M+H]^+$. UPLC-MS Purity (UV @ 215 nm): 97%

2.10 Synthesis of 2-naphthyl N-[4-[4-(2,3-dichlorophenyl)piperazin-1-yl]butyl]carbamate (16)

To a solution of di-*tert*-butyldicarbonate (80.9 mg, 0.37 mmol) in acetonitrile were added in sequence: a solution of 4-dimethylaminopyridine (32.3 mg, 0.26 mmol) in acetonitrile and a solution of the appropriate amine **11b** (80.0 mg, 0.26 mmol) in acetonitrile. After stirring for 10 minutes at room temperature, commercially available 2-naphthol (53.4 mg, 0.37 mmol) was added. The reaction mixture was stirred for 22 h at room temperature, after which the solvent was evaporated under reduced pressure. The crude residue was solubilized in EtOAc

and washed with a saturated solution of $NaHCO_3$. The organic layer was dried (Na_2SO_4), concentrated and purified by flash chromatography (Eluent: 5% MeOH in DCM) to afford the desired compound as white solid (37 mg, 30%). UPLC-MS (method A): Rt 2.88 min; m/z 472 $[M-H]^+$. ^1H NMR (400 MHz, DMSO-d_6) δ 7.96 – 7.85 (m, 4H), 7.64 (d, *J* = 2.4 Hz, 1H), 7.56 – 7.45 (m, 2H), 7.34 – 7.26 (m, 3H), 7.15 (dd, *J* = 6.0, 3.6 Hz, 1H), 3.13 (q, *J* = 6.2 Hz, 2H), 3.07 – 2.93 (m, 4H), 2.56 (s, 4H), 2.45 – 2.34 (m, 2H), 1.54 (t, *J* = 3.9 Hz, 4H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 156.09, 151.47, 149.17, 133.44, 132.79, 132.25, 130.36, 128.83, 128.56, 128.22, 128.03, 127.70 (d, *J* = 2.9 Hz), 125.65, 120.69, 118.11, 112.26, 56.77, 52.29, 50.51, 40.39, 27.46, 25.54. ESI+ (m/z): $[M+H]^+$ calculated for $C_{25}\ H_{28}\ Cl_2\ N_3\ O_2$ 472.1559; found 472.1555 $[M+H]^+$. UPLC-MS Purity (UV @ 215 nm): 99%

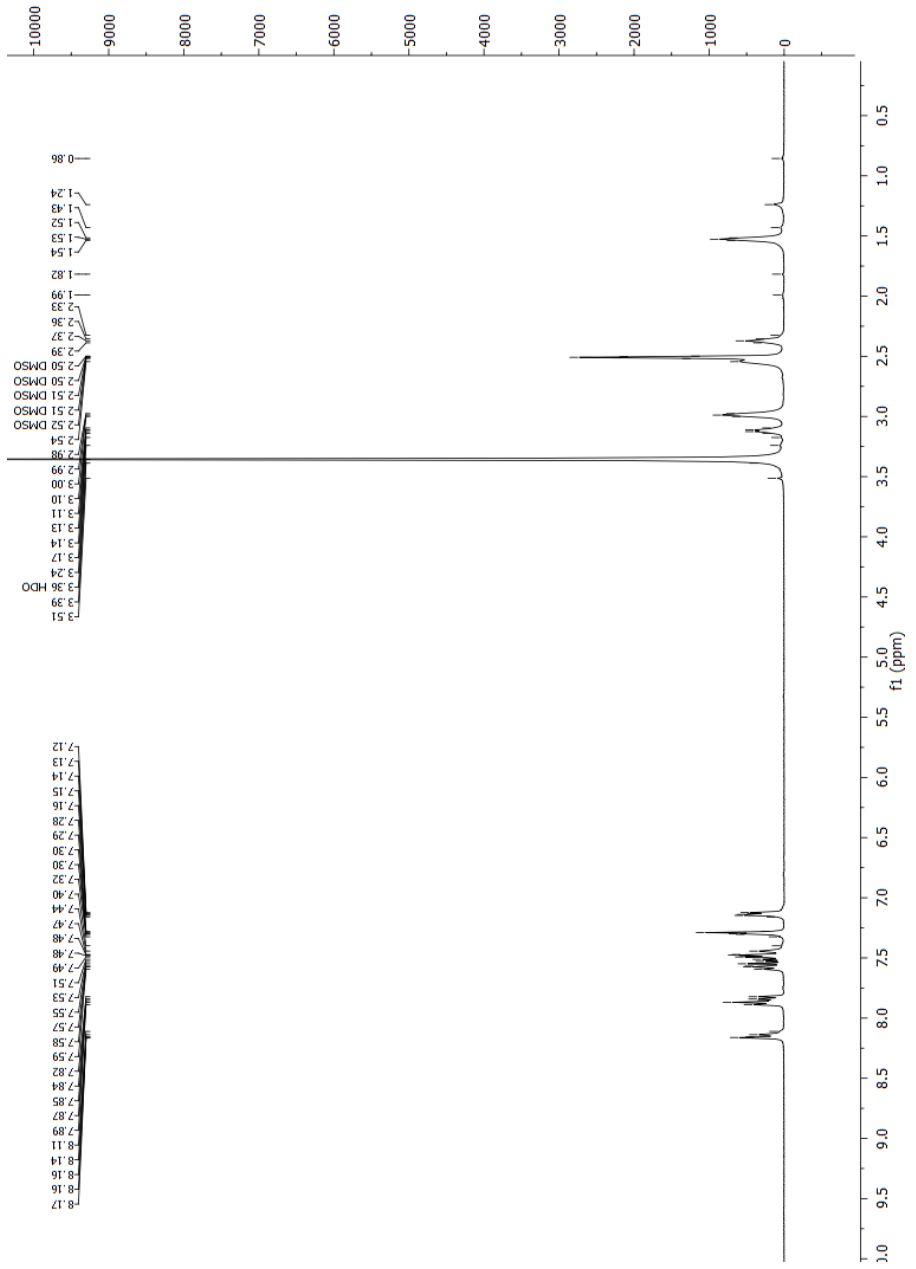
2.11 Synthesis of 1-naphthyl N-[4-[4-(2,3-dichlorophenyl)piperazin-1-yl]butyl]carbamate hydrochloride (17)

To a solution of di-*tert*-butyldicarbonate (101.1 mg, 0.46 mmol) in acetonitrile were added in sequence: a solution of 4-dimethylaminopyridine (40.4 mg, 0.33 mmol) in acetonitrile and a solution of the appropriate amine **11b** (100.0 mg, 0.33 mmol) in acetonitrile. After stirring for 10 minutes at room temperature, commercially available 1-naphthol (49.1 mg, 0.43 mmol) was added. The reaction mixture was stirred for 22 h at room temperature, after which the solvent was evaporated under reduced pressure. The crude residue was solubilized in ethyl acetate and washed with a saturated solution of $NaHCO_3$. The organic layer was dried (Na_2SO_4), concentrated and purified by flash chromatography (Eluent: 5% MeOH in DCM) to afford the desired compound pure which was dissolved in a little amount of 1,4-dioxane and a 4M solution of HCl in 1,4-dioxane was added. The solvent was removed under vacuum and the product was recovered as white solid (52 mg, 31%). UPLC-MS (method A): Rt 2.84 min; m/z 472 $[M-H]^+$. ^1H NMR (400 MHz, DMSO-d6) δ 11.09 (s, 1H), 8.13 (t, J = 5.7 Hz, 1H), 7.98 (dd, J = 7.4, 1.9 Hz, 1H), 7.92 (dd, J = 7.9, 1.7 Hz, 1H), 7.81 (d, J = 8.3 Hz, 1H), 7.58 (pd, J = 6.8, 1.5 Hz, 2H), 7.52 (t, J = 7.9 Hz, 1H), 7.41 – 7.35 (m, 2H), 7.35 – 7.28 (m, 1H), 7.21 (dd, J = 7.1, 2.5 Hz, 1H), 3.57 (m, 4H), 3.31 – 3.07 (m, 8H), 1.86 (pd, J = 9.4, 8.1, 3.0 Hz, 2H), 1.60 (p, J = 7.1 Hz, 2H). ^{13}C NMR (101 MHz, DMSO-d6) δ 155.06 , 150.03 , 147.17 , 134.57 , 133.21 , 129.13 , 128.37 , 127.70 , 126.91 (d, J = 4.4 Hz), 126.53 , 126.19 , 125.76 , 125.58 , 121.55 , 120.27 , 118.94 , 66.82 , 55.60 , 51.54 , 48.19 , 26.95 , 20.99. ESI+ (m/z): $[M+H]^+$ calculated for $C_{25}\ H_{28}\ Cl_2\ N_3\ O_2$ 472.1559; found 472.1562 $[M+H]^+$. UPLC-MS Purity (UV @ 215 nm): 98%

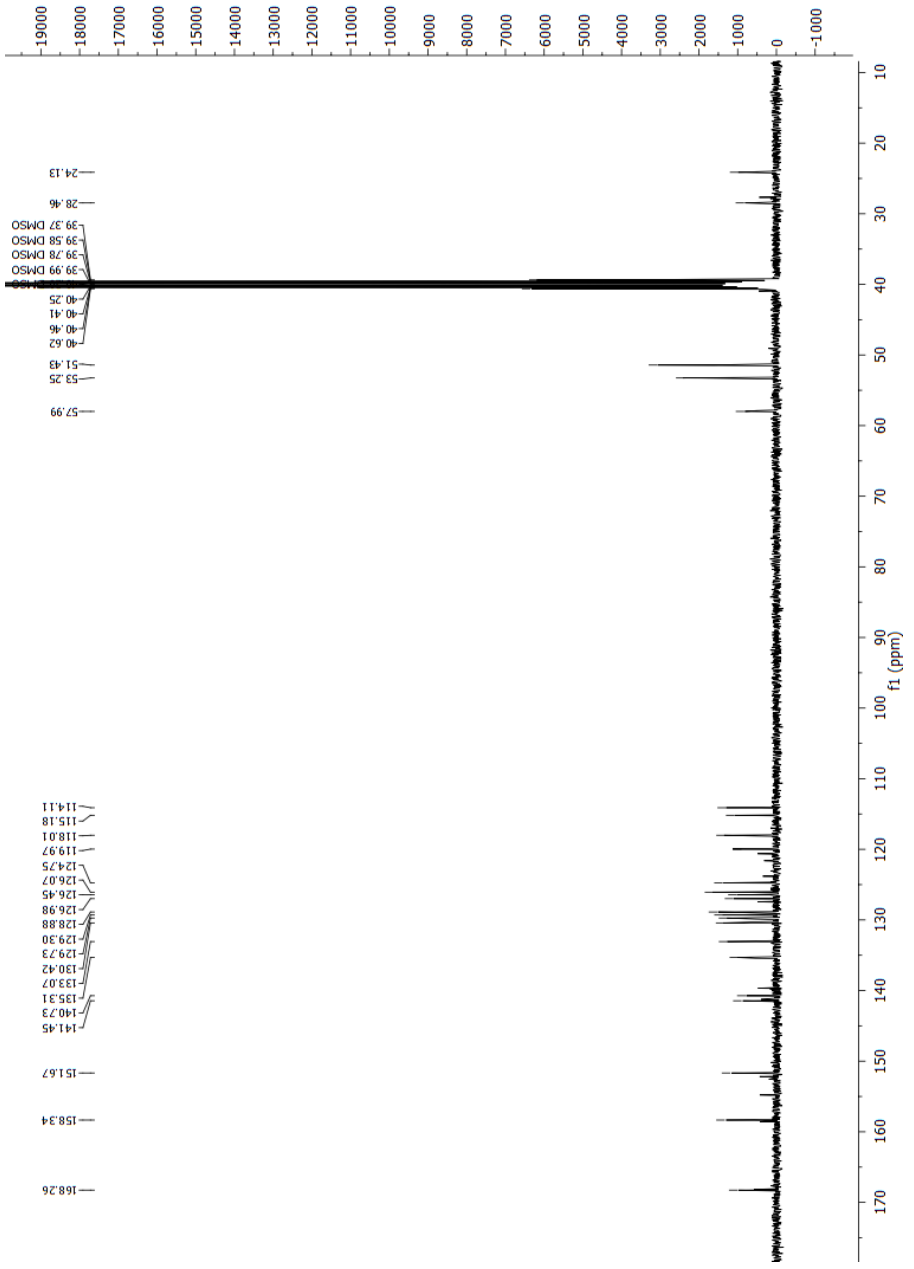
3. ^{1}H and ^{13}C NMR spectra

3.1 Compound 7

^{1}H NMR (DMSO-*d6*):

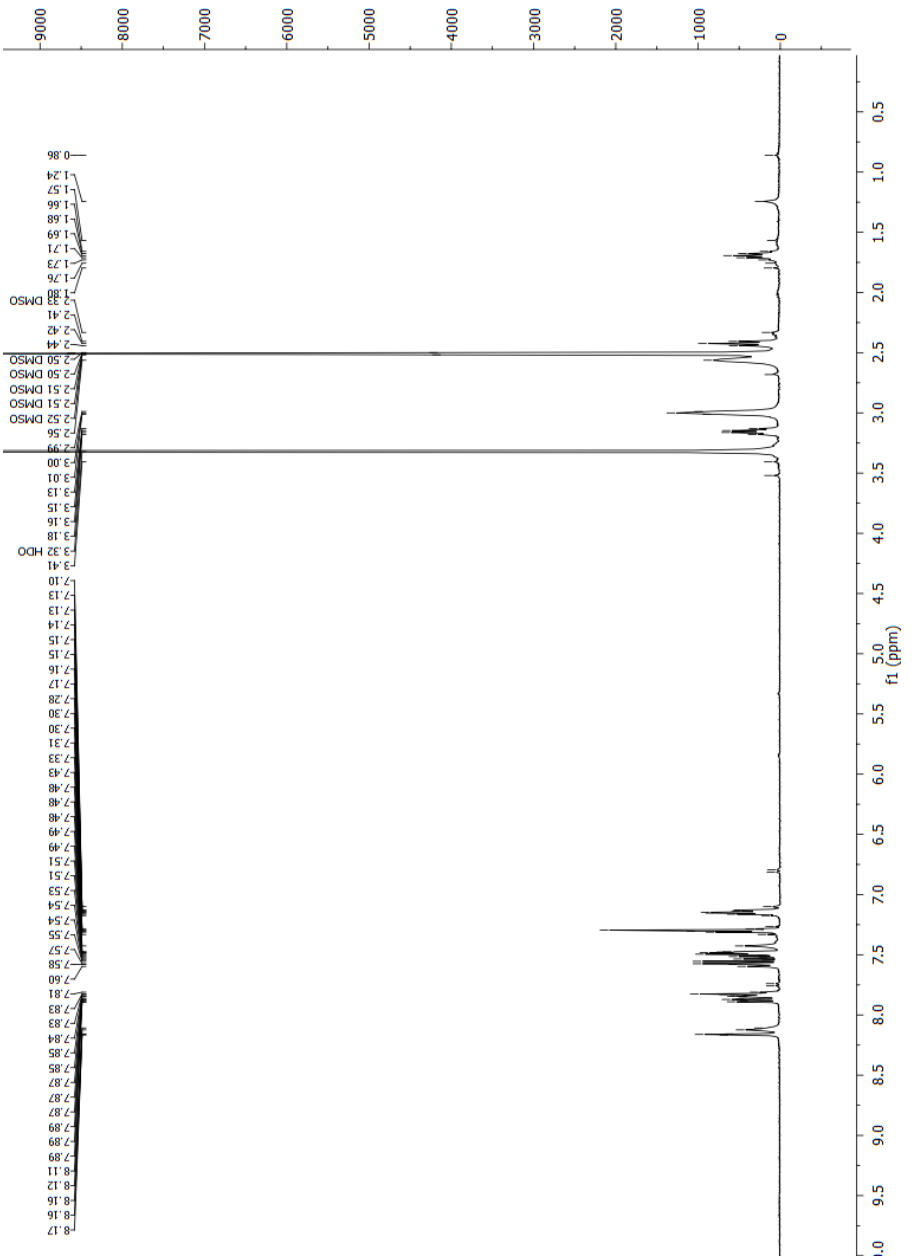


^{13}C NMR (DMSO-*d6*):

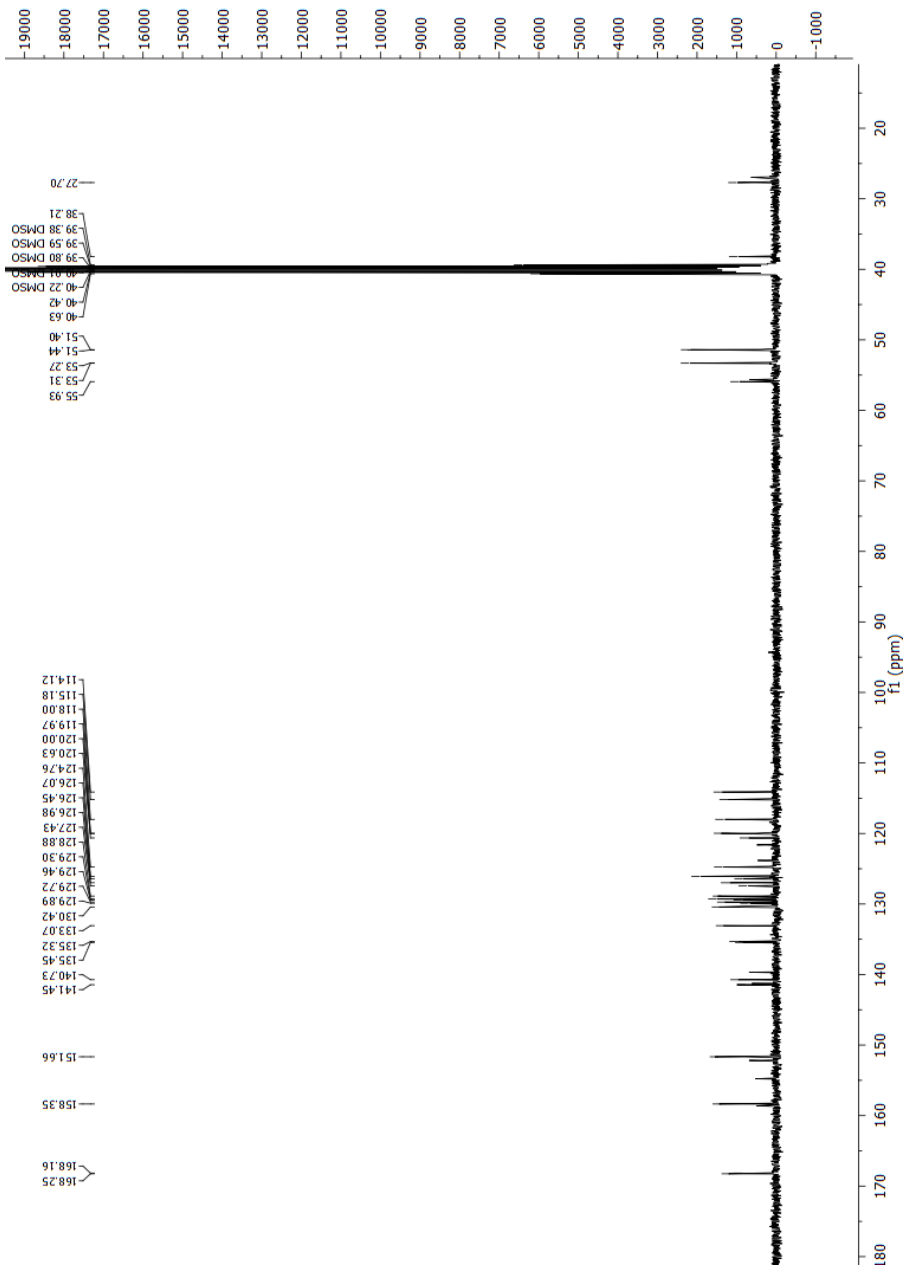


3.2 Compound 8

^{1}H NMR (DMSO-*d6*):

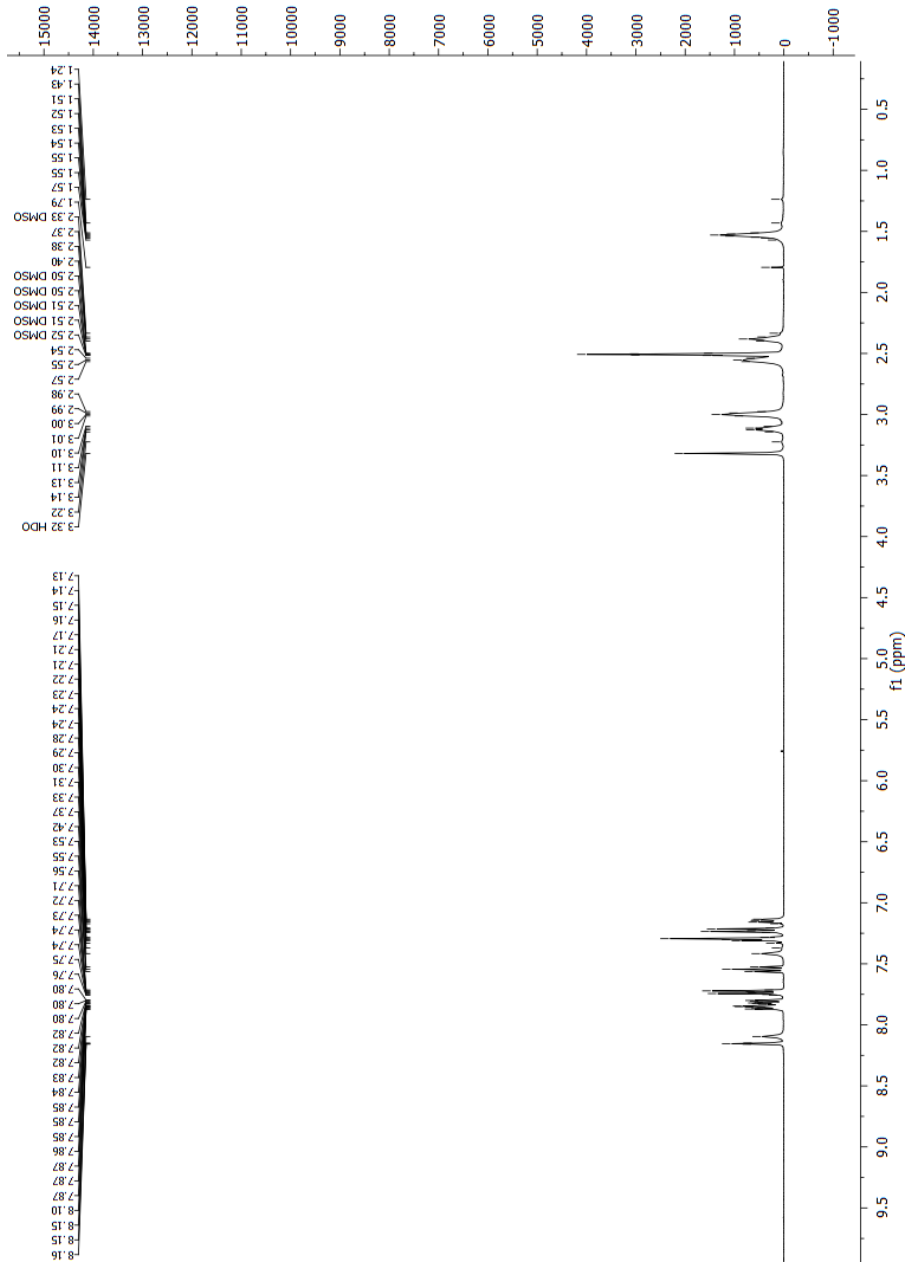


^{13}C NMR (DMSO-*d6*):

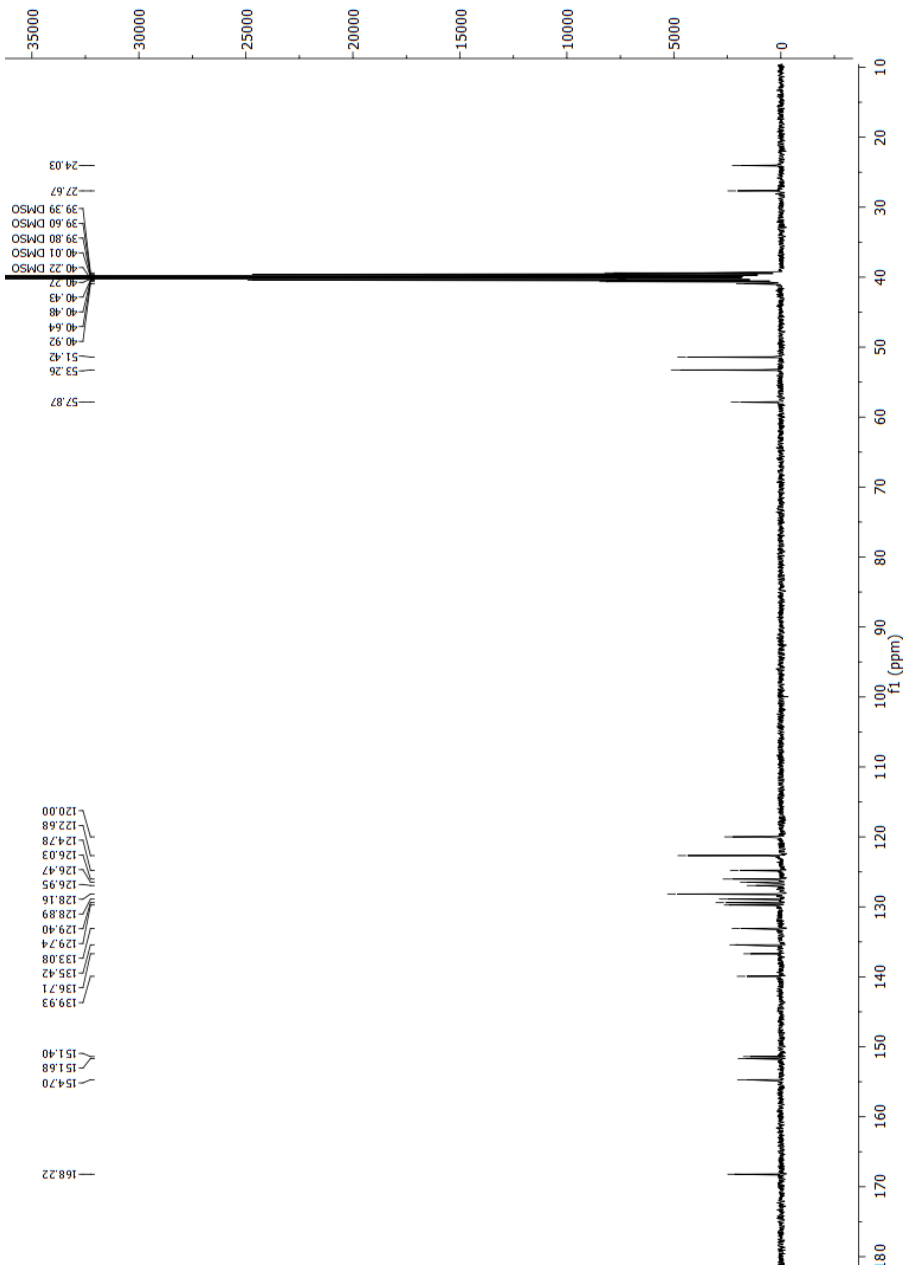


3.3 Compound 15

^{1}H NMR (DMSO-*d6*):

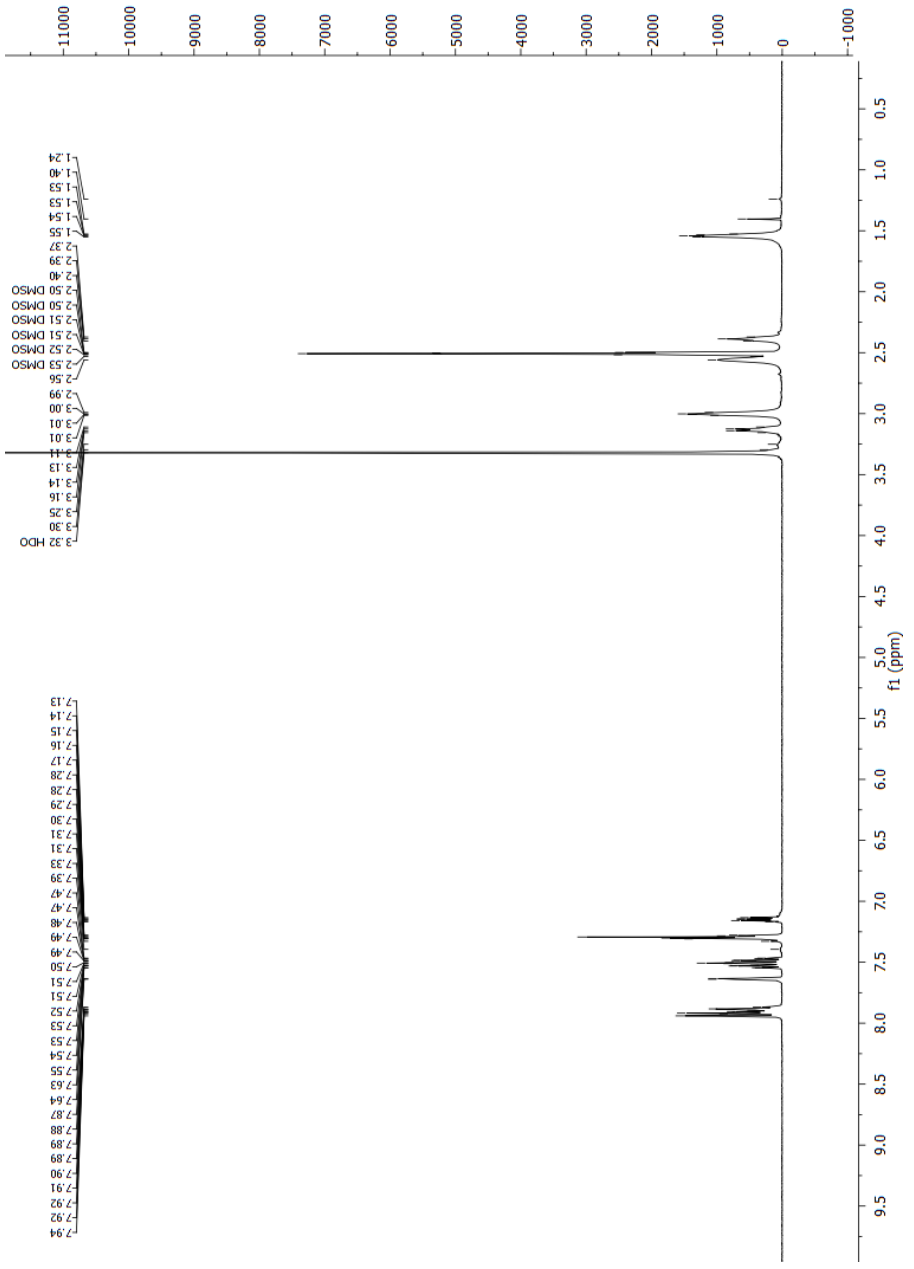


^{13}C NMR (DMSO-*d6*):

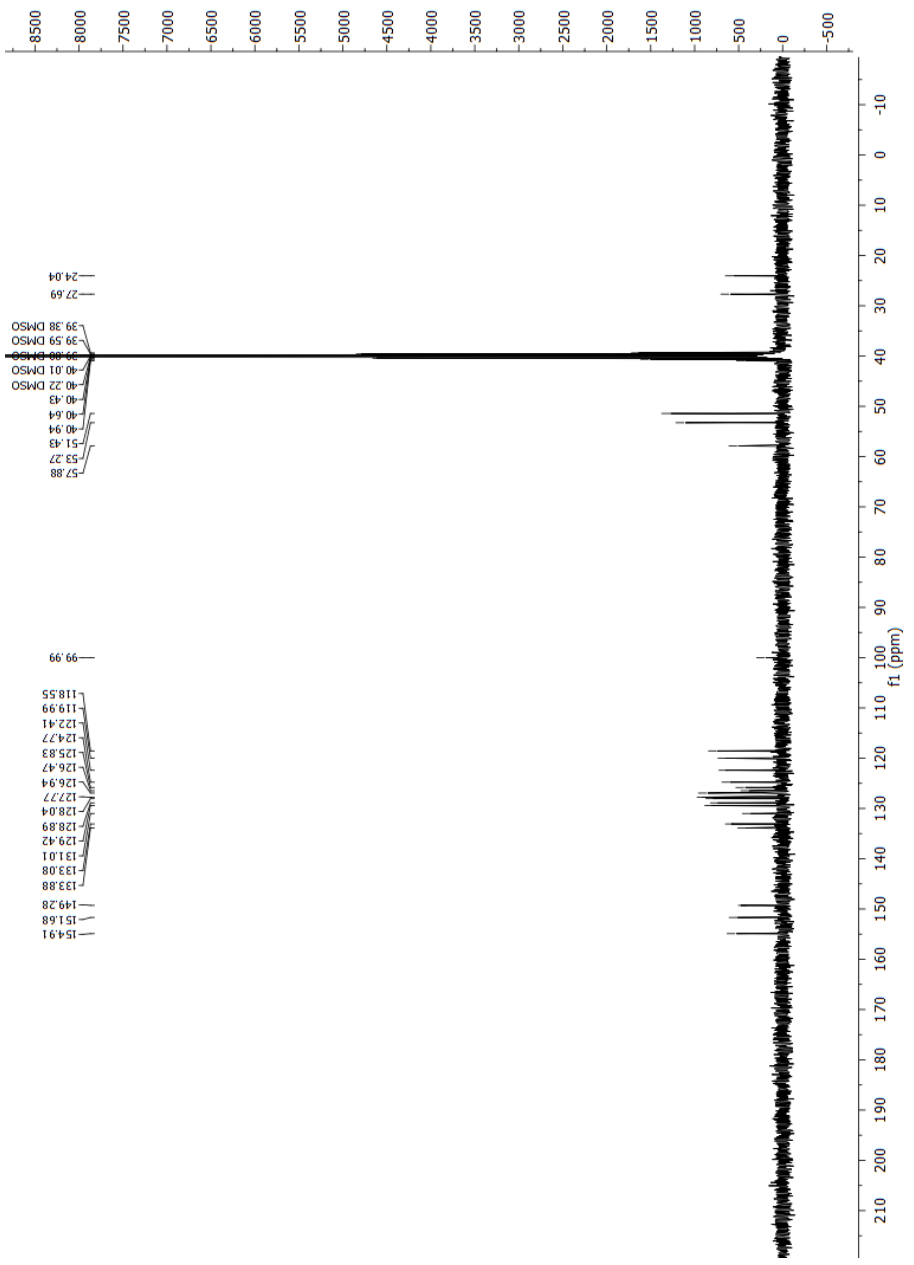


3.4 Compound 16

^{1}H NMR (DMSO-*d6*):

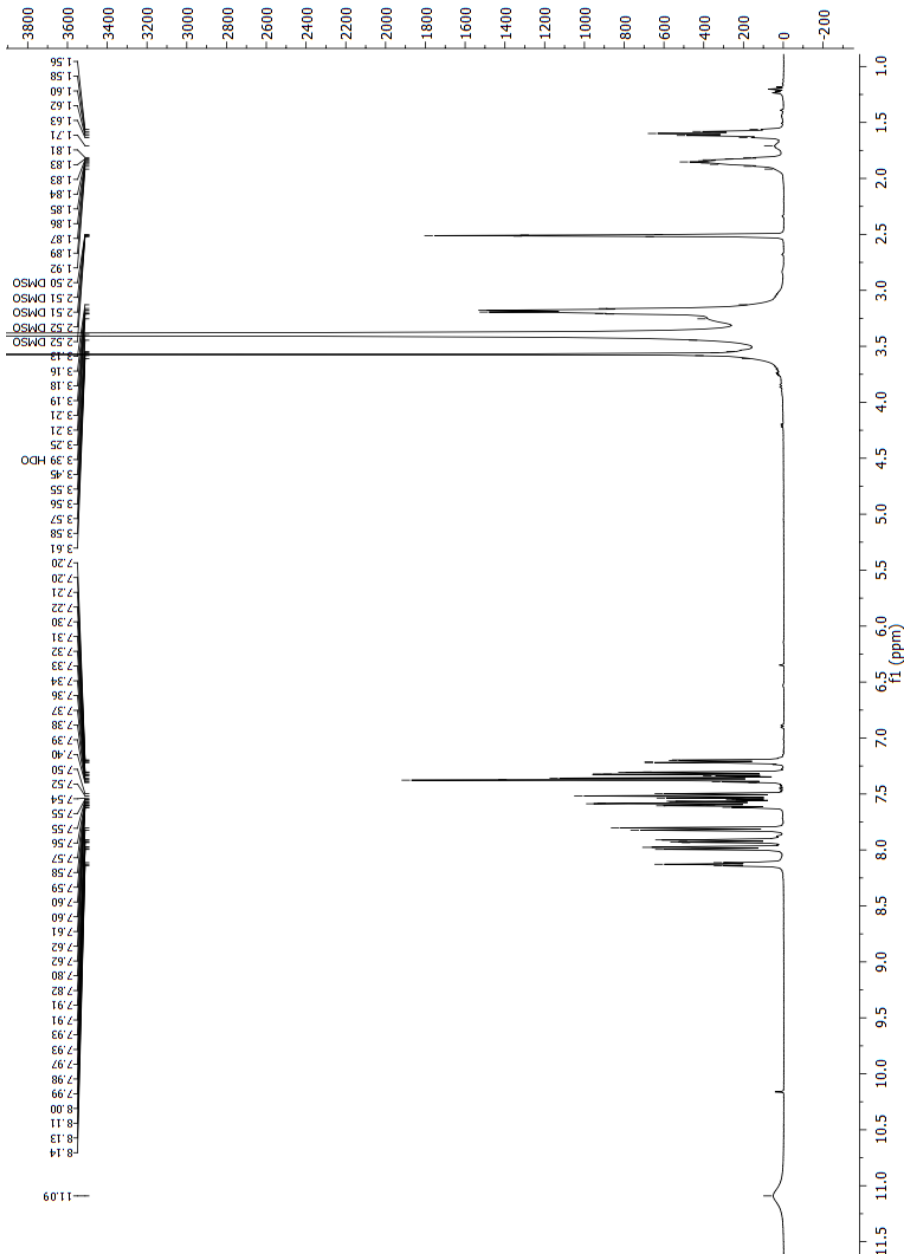


^{13}C NMR (DMSO-*d6*):

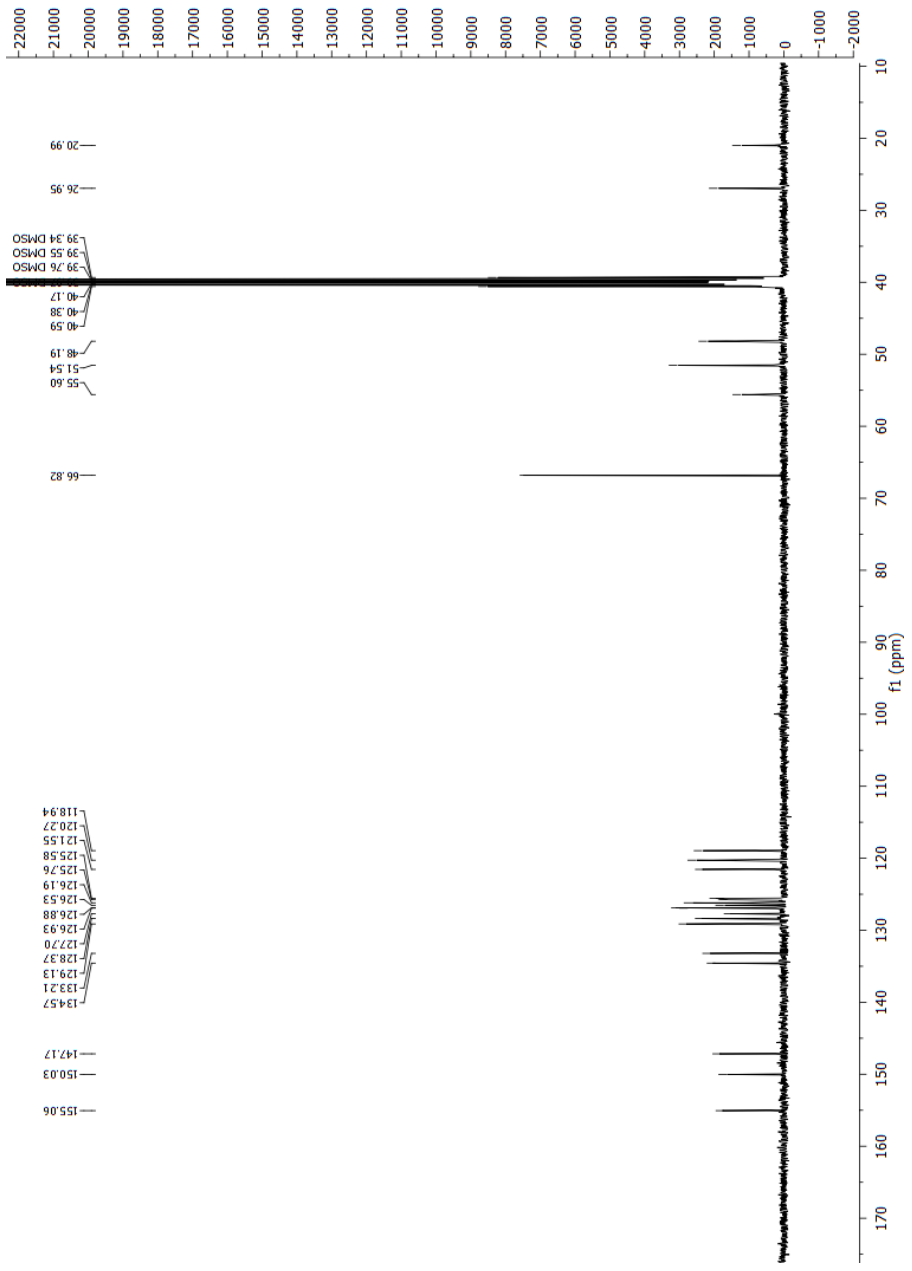


3.5 Compound 17

^{1}H NMR (DMSO-*d6*):



^{13}C NMR (DMSO-*d6*):



4. Biological Assays

4.1 Rat Fatty acid amide hydrolase (FAAH) assay

Male Sprague Dawley rats brains were homogenized in a potter in 20mM Tris-HCl pH 7.4, 0.32M sucrose, protein concentration was measured and samples aliquot stored at -80°C until use. FAAH activity was measured using 50 µg of total rat brain homogenate pre-incubated for 10 minutes at 37°C in 445.5 µL of assay buffer (50mM Tris-HCl pH7.4, 0.05% fatty acid-free BSA) with 4.5 µL of inhibitor at appropriate concentration in DMSO, or DMSO alone to measure FAAH total activity. The blank (no activity sample) was prepared with 445.5 µL of assay buffer and 4.5 µL of DMSO without the 50 µg of total rat brain homogenate.

After 10 minutes of pre-incubation, the reaction was started by the addition of 50 µL of substrate for 30 minutes at 37°C. The substrate is prepared in assay buffer in order to achieve the final concentration of 1µM Arachidonoyl-ethanolamide (N.90050, Cayman Chemical) and 0.6nM Anandamide [ethanolamine-1-3H] (American Radiolabeled Chemicals Inc., ART.0626, 1mCi/ml, specific activity 60Ci/mmol).

The reaction was stopped by with the addition of cold 1:1 $CHCl_3$/Methanol. After 10 minutes of centrifugation (845xg at 4°C) 600 µL of the aqueous phase were transferred into scintillation vials previously filled with 3 mL of scintillation fluid (ULTIMA GOLD, Cat.6013329, Perkin Elmer Inc.).

Radioactivity was measured by liquid scintillation counting (Microbeta2 Lumijet, Perkin Elmer Inc.).

4.2 Human recombinant Fatty acid amide hydrolase (FAAH) fluorescent assay

Hek293 cells stably transfected with human FAAH were maintained in DMEM containing 10% FBS, 1% pen/strep and 500µg/mL G418 to maintain selective pressure. Cells were scraped off with cold PBS 1x pH7.4 from plates and collected by centrifugation (500xg, 10 minutes, 4°C); cells pellets were re-suspended in homogenizing buffer (20mM Tris-HCl pH7.4, 0.32M sucrose), disrupted by sonication (10 pulses, 5 times) and centrifuged at 800xg for 15 minutes at 4°C; the resultant supernatants collected were then centrifuged at 105,000xg for 1hour at 4°C and membranes pellets were re-suspended in PBS 1x pH7.4.

Protein concentration was measured and samples aliquot stored at -80°C until use.

The fluorescent assay used to measure FAAH activity was performed in 96 wells plates (OptiPlate-96 Black, cat.6005279, Perkin Elmer Inc.) in a volume of 180 µL/well of assay buffer (50mM Tris-HCl pH7.4, 0.05% Fatty acid-free BSA) containing 2.5 µg/well of Hek293-hFAAH-1 membrane preparation with 10 µL of inhibitor at appropriate concentration in DMSO, or 10 µL of DMSO alone to measure FAAH total activity. The blank (no activity) was prepared using 180 µL of assay buffer without Hek293-hFAAH membrane

preparation and 10 µL of DMSO. After 50 minutes of pre-incubation of compounds with the enzyme at 37°C, the reaction was started by the addition of 10 µL of substrate (AMC Arachidonyl Amide, N.10005098, Cayman Chemical) for 30 minutes at 37°C. The substrate is prepared in Ethanol in order to achieve the final concentration of 2 µM. Fluorescence was detected with Tecan Infinite M200 nanoquant (Tecan Group Ltd.) at an excitation wavelength 350 nm and an emission wavelength 460nm.

4.3 DRD3, DRD2-short Dopamine receptors and CB-1 cellular assay

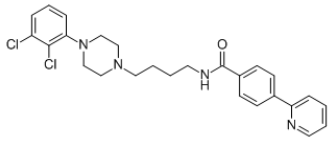
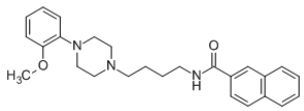
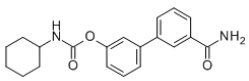
DRD3, DRD2 and CB-1 assays were run by CEREP (Le Bois l'Evêque, FR). Briefly, activities on DRD3 and CB-1 were tested with an HTRF-cAMP functional assay on stably transfected human-DRD3 and human CB-1 expressing CHO cells. The cells are suspended in HBSS buffer (Invitrogen) complemented with 20 mM Hepes/NaOH (pH 7.4), 70 mM NaCl, 5.33 mM KCl, 1.25 mM CaCl2, 0.5 mM MgCl2, 0.41 mM MgSO4, 0.441 mM KH2PO4, 0.3 mM Na2HPO4, 0.1% glucose, 0.1% BSA and 500 µM IBMX (DRD3) or simply 20 mM HEPES (pH 7.4) (CB-1). Cells are then seeded in 96 multiwell microplates at a density of 104 (DRD3) or 5x103 (CB-1) cells/well in the presence of either of the HBSS (basal control), the reference agonist (stimulated control) or various concentrations of the test compounds. Thereafter, the adenylyl cyclase activator NKH 477 is added at a final concentration of 3 µM. Following 10 (DRD3) or 20 (CB-1) minutes incubation at 37°C, the cells are lysed and the fluorescence acceptor (D2-labeled cAMP) and fluorescence donor (anti-cAMP antibody labeled with europium cryptate) are added. After 60 min at room temperature, the fluorescence transfer is measured at λex=337 nm and λem=620 and 665 nm using a microplate reader (Rubystar, BMG). The cAMP concentration is determined by dividing the signal measured at 665 nm by that measured at 620 nm (ratio). Activities on DRD2 were assayed on a DRD2short-stably transfected Hek-293 cell line using a label-free cellular dielectric spectroscopy technology (CellKey, Molecular Devices LLC, USA). Cells are seeded onto 96-well plate coated with fibronectin at 2x105 cells/well in HBSS buffer + 20 mM HEPES (Invitrogen) with 0.1% BSA and are allowed to equilibrate for 45 min at 28°C before the start of the experiment. Plates are placed onto the system and measurements are made at a temperature of 28°C. Solutions are added simultaneously to all 96 wells using an integrated fluidics system: HBSS (basal control), reference agonist (stimulated control), or the test compounds. Impedance measurements are monitored for 10 minutes after ligand addition. In all assays the compounds were tested at 8 different concentrations ranging from 0.1nM up to 10 µM in duplicates. The results are expressed as a percent of the control response to either 300 nM dopamine (DRD3) 3 µM dopamine (DRD2) or 30 nM CP 55940 (CB-1), while antagonstic effects were tested using dopamine at the concentrations of 10nM or 30nM for DRD3 and DRD2 respectively .

4.4 Analysis of the Biological Data

EC50 or IC50 values (concentrations causing half-maximal response or inhibition of control agonist response) were determined by non-linear regression analysis of the Log [concentration]/response curves generated with mean replicate values using a four parameter Hill equation curve fitting with GraphPad Prism 5 (GraphPad Software Inc., CA – USA).

4.5 Biological Data of known compounds

Table S1. Biological data of activities of known compounds.

Compound	Structure	rat FAAH IC50 (nM)	human FAAH IC50 (nM)	DRD3 EC50 (nM)	DRD3% Efficacy *	DRD2 EC50 (nM)	DRD2% Efficacy **	Ratio D2/D3	CB1 EC50 (nM)	Ratio CB1/D3
2 (CJB090)		17% @ 100μM	0% @ 100μM	0.6 [14]	29.7 [14]	57.9 [14]	A [14]	97 [14]	840.0	1400
3 (BP897)		0% @ 100μM	0% @ 100μM	0.9 [15]	55 [15]	61.0 [15]	A [15]	66	N.C.	
5 (URB597)		1.7	3.3	N.C.		N.C.			N.C.	

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