**Supporting Information**

# Synthesis and characterization of the first inhibitor of *N*-acylphosphatidylethanolamine phospholipase D (NAPE-PLD)

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## 1. General Information

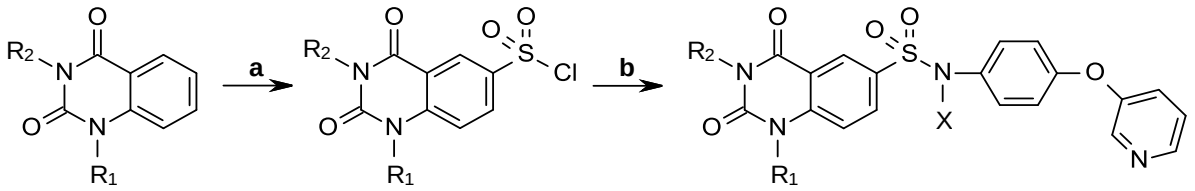
**Abbreviations.** Acetonitrile (MeCN), ammonium chloride ($NH_4Cl$), acetic acid (AcOH), formic acid (FA), ammonium acetate ($NH_4OAc$), ammonium formate ($NH_4HCO_2$), isopropyl alcohol (IPA), dichloromethane (DCM), dimethylformamide (DMF), dimethylsulfoxide (DMSO), ethanol (EtOH), ethyl acetate (EtOAc), methanol (MeOH), DIPEA (*N*,*N*-diisopropylethylamine), triethylamine (TEA), overnight (on), palladium on activated charcoal moistened with water (10% Pd/C), sodium sulfate ($Na_2SO_4$), tetrahydrofuran (THF), tetrakis(triphenylphosphine) palladium ($Pd(PPh_3)_4$), triethylamine ($Et_3N$), nitrogen ($N_2$). Other abbreviations used are: aqueous (aq.), hour (h), minute (min), second (s), room temperature (rt), maltose binding protein (MBP), Luria Broth (LB), deoxyribonuclease (DNase), standard deviation (SD), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), ethylenediaminetetraacetic acid (EDTA), liquid chromatography tandem-mass spectrometry (LC-MS/MS), ultra-performance liquid chromatography (UPLC), internal standard (IS), polymerase chain reaction (PCR), forward (fw), reverse (rev), phosphate-buffered saline (PBS),1,4-dithiothreitol (DTT), bovine serum albumin (BSA), fetal bovine serum (FBS), volume (vol), multiple reaction monitoring (MRM), *N*-acylphosphatidylethanolamine (NAPE), *N*-acylphosphatidylethanolamine phospholipase D (NAPE-PLD), oleoylethanolamide (OEA), palmitoylethanolamide (PEA), arachidonoylethanolamide (AEA), stearoylethanolamide (SEA), fatty acylethanolamide (FAE), volt (V), kiloDalton (kDa), phospholipase D (PLD), 4-fluoro-*N*-(2-(4-(5-fluoro-1H-indol-1-yl)piperidin-1-yl)ethyl)benzamide (FIPI), size-exclusion chromatography (SEC), sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), half-maximal inhibitory concentration ($IC_{50}$), relative fluorescent units (RFU).

**Chemicals, Materials and Methods.** All commercially available reagents and solvents were used as purchased from vendors without further purification. Dry solvents (e.g., THF, DMF) were obtained from Sigma-Aldrich (Italy). Automated column chromatography purifications were performed using a Teledyne ISCO apparatus (CombiFlash® Rf) with pre-packed silica gel columns of various sizes (from 4 - 40 g). Mixtures of cyclohexane/EtOAc and DCM/MeOH were used as eluents. Microwave heating was performed using the instrument Explorer®-48 positions (CEM). NMR experiments were run on a Bruker Avance III 400 system (400.13 MHz for $^1H$ and 100.62 MHz for $^{13}C$) equipped with a BBI probe and Z-gradients. Spectra were acquired at 300 K using deuterated dimethylsulfoxide (DMSO-$d_6$) as solvent. Chemical shifts for $^1H$ and $^{13}C$ spectra were recorded in parts per million using the residual non-deuterated solvent as the IS (for DMSO-$d_6$: 2.50 ppm, $^1H$; 39.52 ppm, $^{13}C$). LC-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. Analyses were performed on an ACQUITY UPLC HSS T3 C18 column (50 × 2.1 mm ID, particle size 1.8 µm) with a VanGuard HSS T3 C18 pre-column (5 × 2.1 mm ID, particle size 1.8 µm). Mobile phase was 10 mM $NH_4OAc$ in $H_2O$ at pH 5 adjusted with AcOH (A) and 10 mM $NH_4OAc$ in MeCN/$H_2O$ (95:5) at pH 5 (B). Electrospray ionization in positive and negative mode was applied. All intermediates and final compounds **10a-c, 25a-c, 3-9,11-25, 28a-b, 29a-c, 30-31a-e, 32** showed ≥ 95%

purity by NMR ($^{1}H$, $^{13}C$, $^{1}H$-$^{1}H$ COSY, $^{1}H$-$^{13}C$ HSQC) and UPLC-MS (UV). DMSO stock solutions of final compounds (30 mM) used for biological assays were evaluated prior to tests (NMR; LC-MS) and concentrations were assessed by quantitative $^{1}H$-NMR.

## 2. Synthetic Schemes

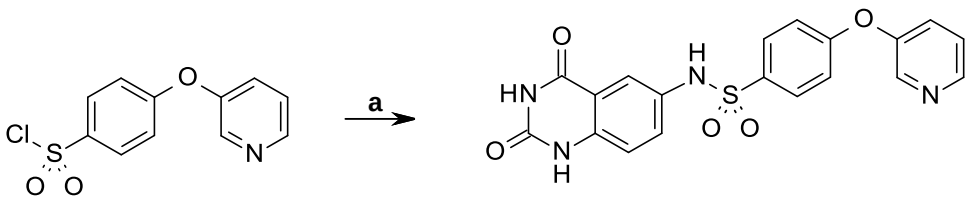
**Scheme 1**. General procedure for the synthesis of compounds **3-4** and **6-9**.




**10a-c** **25a-c** **3-4**, **6-9**

Reagents and conditions: a) $ClSO_3H$, -10°C to 50°C, 8 h (95%); b) $NR_1R_2$, DIPEA, THF, rt, on (10-72%).

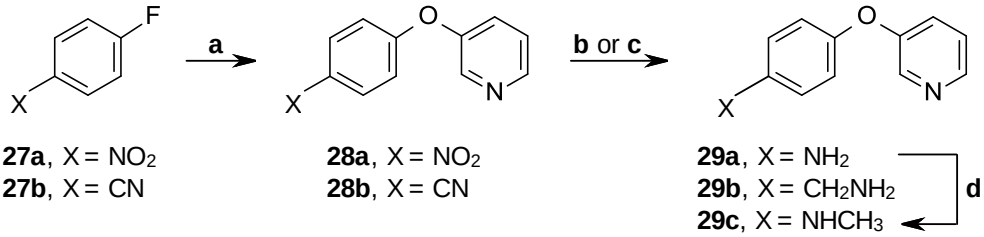
**Scheme 2**. Synthesis of compound **5**.




**26** **5**

Reagents and conditions: a) 6-amino-1H-quinazoline-2,4-dione, DIPEA, THF, rt, on (32%).

**Scheme 3**. Synthesis of amines **29a-c**.



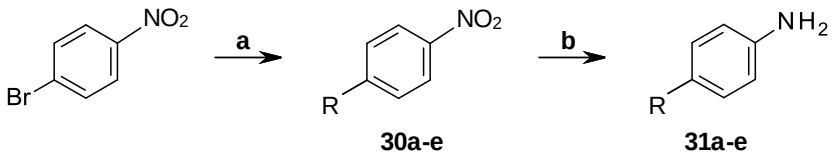

**27a**, X = $NO_2$
**27b**, X = CN

**28a**, X = $NO_2$
**28b**, X = CN

**29a**, X = $NH_2$
**29b**, X = $CH_2NH_2$
**29c**, X = $NHCH_3$

Reagents and conditions: a) 3-hydroxypyridine, $Cs_2CO_3$, DMF, 90°C, on (**28a**, 46%; **28b**, 84%); b) 10% Pd/C, $NH_4HCO_2$, EtOH, 80°C, 2 h (**29a**, 47%); c) 0.75 M $LiAlH_4$ at 0°C, THF, rt, 90 min (**29b**, 60%); d) *i:* $Ac_2O$, TEA, DCM, rt, 2 h (95%); *ii*: NaH, $CH_3I$, THF, rt, 2 h (quant.); *iii*: conc. HCl, reflux, 20 h (**29c**, quant.).

**Scheme 4**. Synthesis of amines **31a-e**.

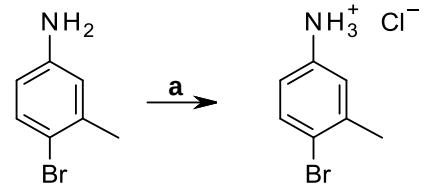



**30a-e** **31a-e**

**a**: R = 4-pyridyl
**b**: R = pyrimidin-5-yl
**c**: R = 2-fluoro-4-pyridinyl
**d**: R = 2-chloro-4-pyridinyl
**e**: R = 2-methyl-4-pyridinyl

Reagents and conditions: a) 4-pyridylboronic acid (**30a**), pyrimidine-5-boronic acid (**30b**), 2-fluoropyridine-4-boronic acid (**30c**), 2-chloropyridine-4-boronic acid (**30d**), 2-methylpyridine-4-boronic acid (**30e**), $Pd(PPh_3)_4$, $K_2CO_3$, dioxane/$H_2O$ (3:1, vol/vol) (54-90%); b) 10% Pd/C, $NH_4HCO_2$, EtOH, 80°C, 2 h (76%-quant).

**Scheme 5.** Synthesis of amine **32**.




**32**

Reagents and conditions: a) *i*: $Ac_2O$, TEA, DCM, rt, on (95%); *ii*: 4-pyridylboronic acid, $Pd(PPh_3)_4$, $K_2CO_3$, in dioxane/$H_2O$ (3:1, vol/vol) (50%); *ii*: conc. HCl, reflux, on (quant.).

## 3. General Procedures

### General procedure 1 (GP1): chlorosulfonation

Quinazoline-2,4-dione (1.0 eq.) was added portionwise to chlorosulfonic acid (5.0 eq.) by stirring at 0°C. The reaction was then stirred at 60°C on. Afterwards, the reaction mixture was carefully poured into crushed ice. A precipitate was obtained, filtered and washed with cold water to afford the desired product as a white solid (95%).

### General procedure 2 (GP2): nucleophilic aromatic substitution

Aryl fluoride (1.0 eq.), phenol (1.0 eq.) and $Cs_2CO_3$ (1.5 eq.) were suspended in DMF (3.6 M). The reaction mixture was stirred at 90°C on. The suspension was cooled to rt, washed with water and extracted with EtOAc (3 x 10 mL). The organic layers were combined, dried over $Na_2SO_4$, filtered, concentrated *in vacuo* and purified by flash chromatography.

### General procedure 3 (GP3): nitro reduction

To a stirring solution of nitro aryl derivatives (1.0 eq.) in EtOH (0.1 M), $NH_4HCO_2$ (5.0 eq.) and 10% Pd/C (0.1 eq.) were added at rt and the mixture was stirred at 80°C until the starting material was consumed (usually 1 h, otherwise stated) as indicated by TLC and UPLC-MS analyses. Once cooled, the reaction solution was passed through a bed of Celite® and washed with copious amounts of EtOH. The solvent was then removed under reduced pressure. The residue was taken up in EtOAc, passed through a silica plug and the solvent was evaporated under vacuum.

**General procedure 4 (GP4): Suzuki coupling**

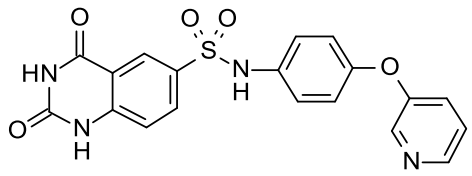
An oven-dried microwave tube was charged with aryl nitro bromide (1.0 eq.), phenylboronic acid (1.0 eq.), $Pd(PPh_3)_4$ (0.1 eq.) and $K_2CO_3$ (3.0 eq.). The reaction vessel was evacuated and backfilled with argon (3 times). Then, a mixture of dioxane/$H_2O$ (3:1, vol/vol, 0.2 M) was added *via* a syringe and the test tube was stirred under microwave irradiation at 120°C for 20 min until consumption of the starting material as indicated by UPLC-MS analysis. Brine was added and the mixture was extracted with EtOAc (3 x 10 mL). The organic layers were combined, dried over $Na_2SO_4$, filtered, concentrated *in vacuo* and purified by flash chromatography.

**General procedure 5 (GP5): sulfonamide synthesis**

To a stirring solution of amine (1.0 eq.) in THF (0.1 M) at 0°C, DIPEA (2.1 eq.) and sulfonyl chloride (1.2 eq.) were sequentially added. The reaction mixture was allowed to reach rt and stirred on. The resulting suspension was quenched with $NH_4Cl$ and extracted with EtOAc (3 x 10 mL). The combined organic layers were dried over $Na_2SO_4$, filtered, concentrated *in vacuo* and purified by flash chromatography.

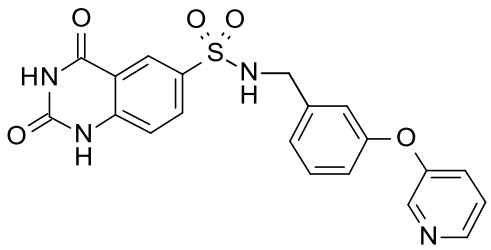
## 4. Synthesis and Characterization of Compounds 10a-c, 25a-c, 3-9,11-25, 28a-b, 29a-c, 30-31a-e, 32.

**2,4-dioxo-*N*-[4-(3-pyridyloxy)phenyl]-1H-quinazoline-6-sulfonamide (3)**



According to the GP5, **10** (0.18 g, 0.69 mmol), DIPEA (0.21 mL, 1.21 mmol) and **29a** (0.107 g, 0.57 mmol) in THF (5.7 mL) were used to obtain **3** as a light yellow solid (0.02 g, 10%) after purification by column chromatography (DCM/MeOH, 90:10, vol/vol). **$^1$H NMR** (400 MHz, DMSO-$d_6$) $\delta$ 11.57 (s, 1H), 11.55 (s, 1H), 10.27 (s, 1H), 8.38 – 8.34 (m, 1H), 8.14 (d, *J* = 2.2, 1H), 7.90 (dd, *J* = 8.6, 2.2, 1H), 7.46 (dd, *J* = 8.5, 4.7, 1H), 7.38 (ddd, *J* = 8.3, 2.6, 1.2, 1H), 7.28 (d, *J* = 8.6, 1H), 7.16 – 7.07 (m, 2H), 7.04 – 6.95 (m, 2H). **$^{13}$C NMR** (101 MHz, DMSO-$d_6$) $\delta$ 114.28, 116.28, 119.75, 123.22, 124.71, 125.41, 126.35, 132.57, 132.75, 133.47, 140.21, 143.84, 143.98, 149.97, 152.62, 153.51, 161.83. **MS** (ESI) *m/z*: 215 $[M + H]^+$, 237 $[M + Na]^+$.

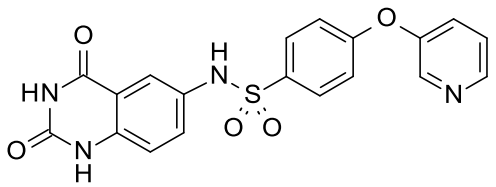
**2,4-dioxo-*N*-(4-(pyridin-3-yloxy)benzyl)-1,2,3,4-tetrahydroquinazoline-6-sulfonamide (4)**



According to the GP5, **29b** (0.05 g, 0.25 mmol), DIPEA (0.09 mL, 0.52 mmol) and **10** (0.08 g, 0.3 mmol) in THF (2.5 mL) were used to obtain **4** as a light yellow solid (0.04 g, 36%) after purification by column chromatography (DCM/MeOH, 98:2, vol/vol). **$^1$H NMR** (400 MHz, DMSO-$d_6$) $\delta$ 11.47 (br. s, 1H), 8.37 (dd, *J* = 4.6, 1.4 Hz, 0H), 8.33 (d, *J* = 2.8 Hz, 1H), 8.24 (br. s, 1H), 8.19 (d, *J* = 2.1 Hz, 1H), 7.94 (dd, *J* = 8.6, 2.2 Hz, 1H), 7.42 (ddd, *J*

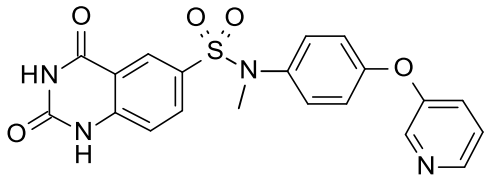
= 8.4, 4.7, 0.7 Hz, 1H), 7.32 (ddd, *J* = 8.4, 2.9, 1.4 Hz, 0H), 7.28 – 7.20 (m, 1H), 6.97 – 6.88 (m, 1H), 4.01 (d, *J* = 2.6 Hz, 1H).**$^{13}$C NMR** (101 MHz, DMSO-$d_6$) $\delta$ 162.40, 155.53, 153.66, 150.57, 144.97, 143.89, 141.34, 135.03, 133.50, 133.07, 130.04, 126.67, 125.82, 125.08, 118.81, 116.63, 114.68, 46.01. **MS** (ESI) *m/z*: 425 $[M + H]^+$, 424 $[M - H]^-$.

***N*-(2,4-dioxo-1H-quinazolin-6-yl)-4-(3-pyridyloxy)benzenesulfonamide (5)**



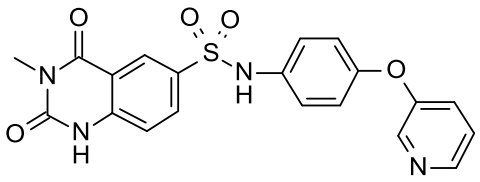
According to the GP5, 6-amino-1H-quinazoline-2,4-dione[1] (0.01 g, 0.06 mmol), DIPEA (0.02 mL, 0.13 mmol) and 4-(3-pyridyloxy)benzenesulfonyl chloride **26** (0.02 g, 0.07 mmol) in THF (0.6 mL) were used to give **5** as a light brown solid (0.008 g, 32%) after purification by preparative HPLC ($CH_3CN/H_2O$). **$^1$H NMR** (400 MHz, DMSO-$d_6$) $\delta$ 11.28 (s, 1H), 11.09 (s, 1H), 10.25 (s, 1H), 8.47 (dd, *J* = 4.6, 1.4 Hz, 1H), 8.45 (d, *J* = 2.9 Hz, 1H), 7.75 – 7.69 (m, 2H), 7.57 (ddd, *J* = 8.4, 2.9, 1.4 Hz, 1H), 7.54 (d, *J* = 2.5 Hz, 1H), 7.49 (dd, *J* = 8.4, 4.6 Hz, 1H), 7.40 (dd, *J* = 8.7, 2.6 Hz, 1H), 7.17 – 7.11 (m, 2H), 7.08 (d, *J* = 8.7 Hz, 1H).**$^{13}$C NMR** (101 MHz, DMSO-$d_6$) $\delta$ 162.81, 160.61, 151.95, 150.45, 146.46, 142.61, 138.33, 134.22, 132.55, 129.80, 129.39, 128.01, 125.43, 119.38, 118.30, 116.88, 115.17. **MS** (ESI) *m/z*: 411 $[M + H]^+$, 409 $[M - H]^-$.

***N*-methyl-2,4-dioxo-*N*-[4-(3-pyridyloxy)phenyl]-1H-quinazoline-6-sulfonamide (6)**



According to the GP5, **10** (0.09 g, 0.36 mmol), DIPEA (0.13 mL, 0.76 mmol) and **29c** (0.12 g, 0.43 mmol) in THF (3.6 mL) were used to give **6** as a brown solid (0.07 g, 44%) after purification by column chromatography (DCM/MeOH, 90:10, vol/vol). **$^1$H NMR** (400 MHz, DMSO-$d_6$) $\delta$ 11.60 (s, 2H), 8.43 (dd, *J* = 2.5, 1.1, 1H), 8.40 (dd, *J* = 4.1, 1.9, 1H), 7.83 (d, *J* = 2.2, 1H), 7.73 (dd, *J* = 8.6, 2.2, 1H), 7.49 – 7.45 (m, 2H), 7.29 (d, *J* = 8.6, 1H), 7.16 – 7.11 (m, 2H), 7.07 – 7.00 (m, 2H), 3.11 (s, 3H).**$^{13}$C NMR** (101 MHz, DMSO-$d_6$) $\delta$ 37.93, 114.30, 115.0, 119.61, 119.87, 126.19, 126.64, 126.2, 128.39, 128.57, 128.78, 133.38, 137.0, 137.8, 140.72, 144.44, 150.0, 154.04, 154.21, 162.0. **MS** (ESI) *m/z*: 425$[M + H]^+$, 447 $[M + Na]^+$.

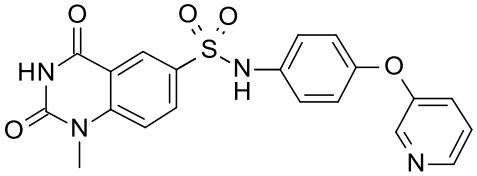
**3-methyl-2,4-dioxo-*N*-[4-(3-pyridyloxy)phenyl]-1H-quinazoline-6-sulfonamide (7)**



According to the GP5, **29a** (0.07 g, 0.36 mmol), DIPEA (0.13 mL, 0.76 mmol) and **25b** (0.12 g, 0.43 mmol) in THF (3.6 mL) were used to afford **7** as a light brown solid (0.11 g, 27%) after purification by column chromatography (DCM/MeOH, 90:10, vol/vol). **$^1$H NMR** (400 MHz, DMSO-$d_6$) $\delta$ 11.81 (s, 1H), 10.24 (s, 1H), 8.33 (d, *J* = 4.5 Hz, 1H), 8.30 (d, *J* = 2.8 Hz, 1H), 8.23 (d, *J* = 2.2 Hz, 1H), 7.91 (dd, *J* = 8.6, 2.2 Hz, 1H), 7.38 (dd, *J* = 8.4, 4.5 Hz, 1H), 7.32 (dd, *J* = 8.4, 2.8 Hz, 1H), 7.28 (d, *J* = 8.6 Hz, 1H), 7.16 – 7.06 (m, 2H), 7.03 – 6.95 (m, 2H), 3.24 (s, 3H). **$^{13}$C NMR**

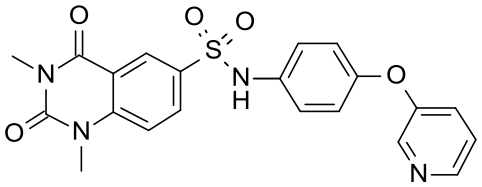
(101 MHz, DMSO-$d_6$) $\delta$ 161.80, 153.91, 153.18, 150.57, 144.83, 142.78, 141.00, 133.93, 133.33, 132.96, 127.20, 125.60, 125.06, 123.66, 120.24, 116.56, 114.15, 27.63. **MS** (ESI) *m/z*: 425 $[M + H]^+$, 423 $[M - H]^-$.

**1-methyl-2,4-dioxo-*N*-[4-(3-pyridyloxy)phenyl]quinazoline-6-sulfonamide (8)**



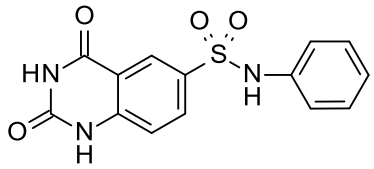
According to the GP5, **29a** (0.07 g, 0.37 mmol), DIPEA (0.14 g, 0.79 mmol) and **25a** (0.12 g,0.44 mmol) in THF (3.7 mL) were used to obtain **8** as a light yellow solid (0.85 g, 72%) after purification by column chromatography (DCM/MeOH, 90:10, vol/vol). **$^1$H NMR** (400 MHz, DMSO-$d_6$) $\delta$ 11.92 – 11.72 (m, 1H), 10.28 (s, 1H), 8.33 (dd, *J* = 4.6, 1.4, 1H), 8.30 (d, *J* = 2.8, 1H), 8.24 (d, *J* = 2.3, 1H), 7.98 (dd, *J* = 8.9, 2.3, 1H), 7.56 (d, *J* = 8.9, 1H), 7.39 (ddd, *J* = 8.4, 4.6, 0.7, 1H), 7.31 (ddd, *J* = 8.4, 2.9, 1.4, 1H), 7.15 – 7.07 (m, 2H), 7.03 – 6.93 (m, 2H), 3.43 (s, 3H). **$^{13}$C NMR** (101 MHz, DMSO-$d_6$) $\delta$ 160.94, 153.42, 152.74, 150.06, 144.64, 144.38, 140.57, 133.40, 132.81, 132.64, 126.35, 125.14, 124.63, 123.11, 119.79, 115.97, 115.64, 29.90. **MS** (ESI) *m/z*: 425 $[M + H]^+$.

**1,3-dimethyl-2,4-dioxo-*N*-[4-(3-pyridyloxy)phenyl]quinazoline-6-sulfonamide (9)**



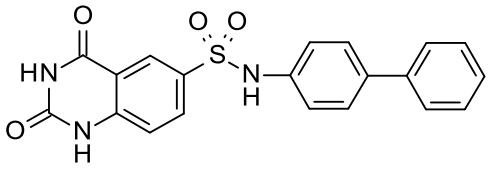
According to the GP5, **29a** (0.06 g, 0.34 mmol), DIPEA (0.12 mL, 0.72 mmol) and **25c** (0.12 g, 0.41 mmol) in THF (3.4 mL) were used to afford **9** as a light yellow powder (0.07 g, 48%) after purification by column chromatography (DCM/MeOH, 90:10, vol/vol). **$^1$H NMR** (400 MHz, DMSO-$d_6$) $\delta$ 10.50 (s, 1H), 8.60 – 8.51 (m, 2H), 8.31 (d, *J* = 2.3, 1H), 8.03 (dd, *J* = 8.9, 2.4, 1H), 7.86 – 7.72 (m, 2H), 7.61 (d, *J* = 8.9, 1H), 7.22 – 7.13 (m, 2H), 7.13 – 7.05 (m, 2H), 3.51 (s, 3H), 3.29 (s, 3H). **$^{13}$C NMR** (101 MHz, DMSO-$d_6$) $\delta$ 160.54, 153.44, 152.70, 150.39, 144.41, 143.20, 140.58, 133.48, 133.04, 132.54, 126.73, 125.18, 124.63, 123.05, 119.81, 115.80, 114.81, 31.03, 28.32. **MS** (ESI) *m/z*: 439 $[M + H]^+$, 461 $[M + Na]^+$.

**2,4-dioxo-*N*-phenyl-1H-quinazoline-6-sulfonamide (11)**



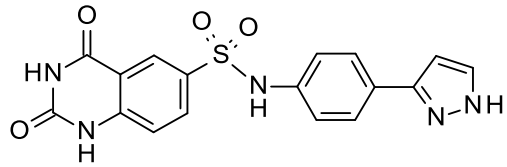
According to the GP5, aniline (0.03 mL, 0.31 mmol), DIPEA (0.11 mL, 0.64 mmol) and **10** (0.09 g, 0.37 mmol) in THF (3.1 mL) were used to afford **11** as a white solid (0.05 g, 54%) after purification by column chromatography (EtOAc/cyclohexane, 60:40, vol/vol). **$^1$H NMR** (400 MHz, DMSO-$d_6$) $\delta$ 11.52 (d, *J* = 1.8, 1H), 11.49 (s, 1H), 10.29 (s, 1H), 8.23 (d, *J* = 2.2, 1H), 7.91 (dd, *J* = 8.6, 2.2, 1H), 7.28 – 7.19 (m, 3H), 7.11 – 7.06 (m, 2H), 7.03 (td, *J* = 7.3, 1.2, 1H). **$^{13}$C NMR** (101 MHz, DMSO-$d_6$) $\delta$ 114.84, 116.79, 120.71, 124.74, 126.84, 129.69, 133.09, 133.45, 137.90, 144.34, 150.47, 162.33. **MS** (ESI) *m/z*: 318 $[M + H]^+$, 316 $[M - H]^-$.

**2,4-dioxo-*N*-(4-phenylphenyl)-1H-quinazoline-6-sulfonamide (12)**



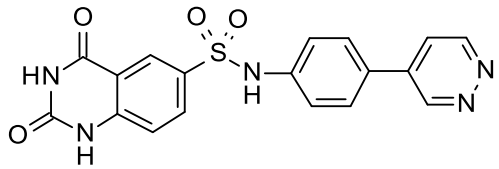
According to the GP5, 4-aminobiphenyl (0.064 g, 0.38 mmol), DIPEA (0.14 mL, 0.8 mmol) and **10** (0.12 g, 0.45 mmol) in THF (3.8 mL) were used to give **12** as a white solid (0.04 g, 31%) after purification by column chromatography (DCM/MeOH, 90:10, vol/vol). **$^{1}$H NMR** (400 MHz, DMSO-$d_6$) $\delta$ 11.53 (s, 1H), 11.51 (s, 1H), 10.44 (s, 1H), 8.28 (d, $J$ = 2.2, 1H), 7.97 (dd, $J$ = 8.6, 2.2, 1H), 7.61 – 7.50 (m, 4H), 7.41 (t, $J$ = 7.7, 2H), 7.31 (tt, $J$ = 7.4, 1.3, 1H), 7.26 (d, $J$ = 8.7, 1H), 7.20 – 7.14 (m, 2H).**$^{13}$C NMR** (101 MHz, DMSO-$d_6$) $\delta$ 114.43, 116.41, 120.35, 126.25, 126.37, 127.21, 127.43, 128.85, 132.61, 132.99, 135.85, 136.84, 139.18, 143.94, 149.99, 161.86.

***N*-(4-(1H-pyrazol-3-yl)phenyl)-2,4-dioxo-1,2,3,4-tetrahydroquinazoline-6-sulfonamide (13)**



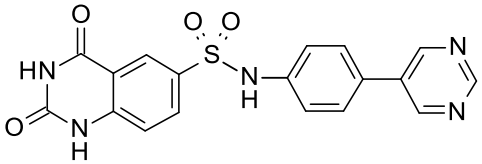
According to the GP5, 3-(4-aminophenyl)pyrazole (0.059 g, 0.37 mmol), DIPEA (0.14 mL, 0.78 mmol) and **10** (0.12 g, 0.41 mmol) in THF (4.0 mL) were used to afford **13** as a white solid (0.02 g, 14%) after purification by column chromatography (DCM/MeOH, 90:10, vol/vol). **$^{1}$H NMR** (400 MHz, DMSO-$d_6$) $\delta$ 12.79 (s, 1H), 11.61 – 11.42 (m, 2H), 10.34 (s, 1H), 8.27 (d, $J$ = 2.2Hz, 1H), 7.94 (dd, $J$ = 8.7, 2.2 Hz, 1H), 7.74 – 7.58 (m, 3H), 7.25 (d, $J$ = 8.6 Hz, 1H), 7.17 – 7.09 (m, 2H), 6.59 (d, 1H).**$^{13}$C NMR** (101 MHz, DMSO-$d_6$) $\delta$ 162.34, 150.46, 148.1, 144.38, 133.47, 133.11, 132.02, 127.21, 126.87, 126.45, 120.96, 116.80, 114.87, 102.42. **MS** (ESI) *m/z*: 384 $[M + H]^+$, 382 $[M - H]^-$.

**2,4-dioxo-*N*-(4-pyridazin-4-ylphenyl)-1H-quinazoline-6-sulfonamide (14)**



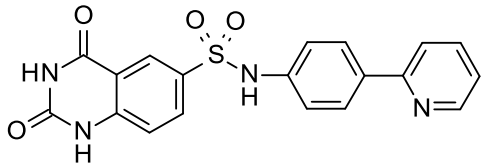
According to the GP5, 4-(pyridazin-4-yl)aniline (0.12 g, 0.70 mmol), DIPEA (0.25 mL, 1.5 mmol) and **10** (0.22 g, 0.84 mmol) in THF (7.0 mL) were used to obtain **14** as a light brown solid (0.15 g, 56%) after purification by column chromatography (DCM/MeOH, 90:10, vol/vol). **$^{1}$H NMR** (400 MHz, DMSO-$d_6$) $\delta$ 11.59 (s, 1H), 11.53 (s, 1H), 10.96 (s, 1H), 9.75 (d, $J$ = 2.3, 1H), 9.40 (d, $J$ = 5.7, 1H), 8.36 – 8.26 (m, 2H), 8.04 (dd, $J$ = 8.7, 2.3, 1H), 8.01 – 7.93 (m, 2H), 7.36 – 7.29 (m, 3H). **$^{13}$C NMR** (101 MHz, DMSO-$d_6$) $\delta$ 161.81, 149.94, 149.81, 149.15, 144.18, 140.68, 140.47, 132.74, 132.60, 129.09, 127.40, 126.39, 125.35, 119.51, 116.64, 114.46. **MS** (ESI) *m/z*: 396 $[M + H]^+$, 394 $[M - H]^-$.

**2,4-dioxo-*N*-(4-pyrimidin-5-ylphenyl)-1H-quinazoline-6-sulfonamide (15)**



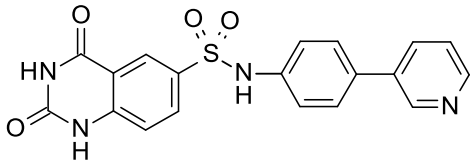
According to the GP5, **31b** (0.16 g, 0.93 mmol), DIPEA (0.34 mL, 1.95 mmol) and **10** (0.29 g, 1.11 mmol) in THF (9.3 mL) were used to afford **15** as a light yellow solid (0.07 g, 20%) after purification by preparative HPLC ($CH_3CN/H_2O$). **$^1$H NMR** (400 MHz, DMSO-$d_6$) $\delta$ 11.54 (s, 1H), 11.51 (s, 1H), 10.60 (s, 1H), 9.12 (s, 1H), 9.05 (s, 2H), 8.29 (d, $J$ = 2.2, 1H), 7.99 (dd, $J$ = 8.7, 2.3, 1H), 7.74 – 7.68 (m, 2H), 7.29 – 7.21 (m, 3H). **$^{13}$C NMR** (101 MHz, DMSO-$d_6$) $\delta$ 161.83, 156.94, 154.24, 149.97, 144.01, 138.28, 132.91, 132.59, 132.33, 129.20, 127.80, 126.38, 120.15, 116.49, 114.46. **MS** (ESI) *m/z*: 396 $[M + H]^+$.

**2,4-dioxo-*N*-[4-(2-pyridyl)phenyl]-1H-quinazoline-6-sulfonamide (16)**



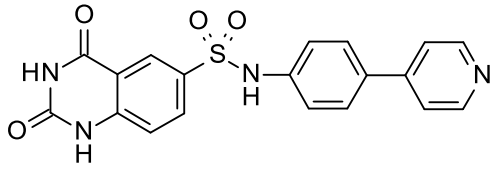
According to the GP5, 4-(2-pyridyl)aniline (0.05 g, 0.26 mmol), DIPEA (0.09 mL, 0.55 mmol) and **10** (0.08 g, 0.31 mmol) in THF (2.6 mL) were used to afford **16** as a light yellow solid (0.012 g, 11%) after purification by column chromatography (DCM/MeOH, 90:10, vol/vol). **$^1$H NMR** (400 MHz, DMSO-$d_6$) $\delta$ 11.54 (s, 2H), 10.76 (s, 1H), 8.68 (dd, $J$ = 5.3, 1.6, 1H), 8.29 (d, $J$ = 2.2, 1H), 8.14 (t, $J$ = 7.8, 1H), 8.07 – 7.99 (m, 2H), 7.99 – 7.93 (m, 2H), 7.56 (dd, $J$ = 7.4, 5.3, 1H), 7.31 – 7.23 (m, 3H).**$^{13}$C NMR** (101 MHz, DMSO-$d_6$) $\delta$ 114.93, 116.38, 118.85, 121.33, 123.15, 126.0, 126.78, 128.09, 131.30, 132.69, 133.21, 133.24, 140.59, 144.57, 146.0, 153.78, 162.29. **MS** (ESI) *m/z*: 393 $[M - H]^-$.

**2,4-dioxo-*N*-[4-(3-pyridyl)phenyl]-1H-quinazoline-6-sulfonamide (17)**



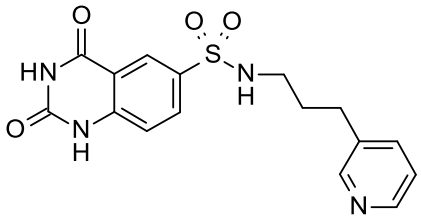
According to the GP5, 4-(3-pyridyl)aniline (0.06 g, 0.38 mmol), DIPEA (0.14 mL, 0.80 mmol) and **10** (0.12 g, 0.46 mmol) in THF (3.8 mL) were used to afford **17** as a light yellow solid (0.04 g, 28%) after purification by column chromatography (DCM/MeOH, 90:10, vol/vol). **$^1$H NMR** (400 MHz, DMSO-$d_6$) $\delta$ 11.54 (s, 1H), 11.52 (s, 1H), 10.54 (s, 1H), 8.81 (d, $J$ = 2.0, 1H), 8.52 (dd, $J$ = 4.8, 1.5, 1H), 8.28 (d, $J$ = 2.2, 1H), 8.03 – 7.94 (m, 2H), 7.63 (d, $J$ = 8.6, 2H), 7.43 (ddd, $J$ = 8.0, 4.8, 0.8, 1H), 7.26 (d, $J$ = 8.7, 1H), 7.22 (d, $J$ = 8.7, 2H).**$^{13}$C NMR** (101 MHz, DMSO-$d_6$) $\delta$ 114.44, 116.49, 120.15, 123.80, 126.27, 127.75, 132.55, 132.58, 132.94, 133.74, 134.65, 137.56, 143.97, 147.17, 148.11, 149.97, 161.84. **MS** (ESI) *m/z*: 395 $[M + H]^+$, 393 $[M - H]^-$.

**2,4-dioxo-*N*-[4-(4-pyridyl)phenyl]-1H-quinazoline-6-sulfonamide (18)**



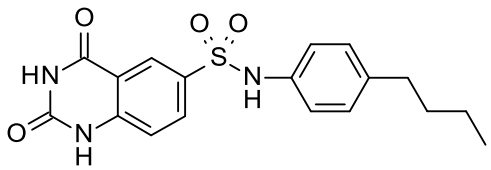
According to the GP5, **31a** (0.10 g, 0.58 mmol), DIPEA (0.21 mL, 1.22 mmol) and **10** (0.18 g, 0.69 mmol) in THF (5.8 mL) were used to afford **18** as a light yellow solid (0.070 g, 31%) after purification by column chromatography (DCM/MeOH, 90:10, vol/vol). **$^{1}$H NMR** (400 MHz, DMSO-$d_6$) $\delta$ 11.55 (s, 1H), 11.52 (s, 1H), 10.63 (s, 1H), 8.61 – 8.57 (m, 2H), 8.29 (d, $J$ = 2.2 Hz, 1H), 8.00 (dd, $J$ = 8.7, 2.2 Hz, 1H), 7.76 – 7.70 (m, 2H), 7.66 – 7.62 (m, 2H), 7.29 – 7.22 (m, 3H).**$^{13}$C NMR** (101 MHz, DMSO-$d_6$) $\delta$ 162.32, 150.46, 150.41, 144.51, 139.13, 133.35, 133.09, 132.80, 128.30, 126.86, 121.19, 120.40, 116.98, 114.94. **MS** (ESI) *m/z*: 395 $[M + H]^+$, 393 $[M - H]^-$.

**2,4-dioxo-*N*-[3-(3-pyridyl)propyl]-1H-quinazoline-6-sulfonamide (19)**



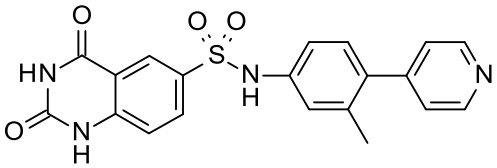
According to the GP5, 3-(3-pyridyl)propan-1-amine (0.03 g, 0.26 mmol), DIPEA (0.1 mL, 0.55 mmol) and **10** (0.08 g, 0.3 mmol) in THF (2.6 mL) were used to obtain **19** as a light yellow solid (0.06 g, 69%) after purification by column chromatography (DCM/MeOH, 90:10, vol/vol). **$^{1}$H NMR** (400 MHz, DMSO-$d_6$) $\delta$ 11.52 (s, 2H), 8.40 – 8.31 (m, 2H), 8.25 (d, $J$ = 2.2, 1H), 7.96 (dd, $J$ = 8.6, 2.2, 1H), 7.72 (t, $J$ = 5.8, 1H), 7.54 (dt, $J$ = 7.9, 2.0, 1H), 7.30 (d, $J$ = 8.6, 1H), 7.25 (dd, $J$ = 7.8, 4.8, 1H), 2.74 (q, $J$ = 6.5, 2H), 2.56 (t, $J$ = 7.7, 2H), 1.76 – 1.59 (m, 2H). **$^{13}$C NMR** (101 MHz, DMSO-$d_6$) $\delta$ 162.00, 150.07, 149.49, 147.13, 143.53, 136.63, 135.68, 134.03, 132.57, 126.04, 123.33, 116.33, 114.36, 41.78, 30.30, 28.98. **MS** (ESI) *m/z*: 361 $[M + H]^+$, 359 $[M - H]^-$.

***N*-(4-butylphenyl)-2,4-dioxo-1*H*-quinazoline-6-sulfonamide (20)**



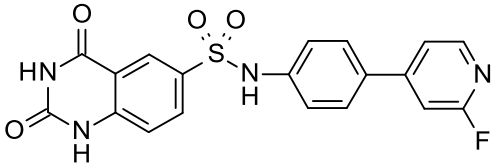
According to the GP5, 4-butylaniline (0.06 mL, 0.38 mmol), DIPEA (0.14 mL, 0.8 mmol) and **10** (0.12 g, 0.46 mmol) in THF (3.8 mL) were used to obtain **20** as a white solid (0.09 g, 64%) after purification by column chromatography (DCM/MeOH, 90:10, vol/vol). **$^{1}$H NMR** (400 MHz, DMSO-$d_6$) $\delta$ 11.51 (s, 1H), 11.48 (s, 1H), 10.12 (s, 1H), 8.21 (d, $J$ = 2.2, 1H), 7.89 (dd, $J$ = 8.7, 2.2, 1H), 7.23 (d, $J$ = 8.6, 1H), 7.03 (d, $J$ = 8.4, 2H), 6.97 (d, $J$ = 8.5, 2H), 2.44 (t, $J$ = 7.7, 2H), 1.53 – 1.37 (m, 2H), 1.29 – 1.13 (m, 2H), 0.83 (t, $J$ = 7.3, 3H). **$^{13}$C NMR** (101 MHz, DMSO-$d_6$) $\delta$ 161.86, 149.99, 143.79, 138.50, 134.93, 133.14, 132.61, 128.92, 126.37, 120.77, 116.25, 114.32, 34.04, 32.93, 21.62, 13.68. **MS** (ESI) *m/z*: 372 $[M - H]^-$.

***N*-[3-methyl-4-(4-pyridyl)phenyl]-2,4-dioxo-1H-quinazoline-6-sulfonamide (21)**



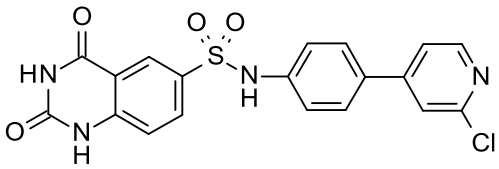
According to the GP5, **32** (0.21 g, 1.01 mmol), DIPEA (0.44 mL, 2.54 mmol) and **10** (0.34 g, 1.32 mmol) in THF (10 mL) were used to obtain **21** as a white solid (0.19 g, 48%) after purification by column chromatography (DCM/MeOH, 90:10, vol/vol). **$^{1}$H NMR** (400 MHz, DMSO-$d_6$) $\delta$ 11.57 (s, 1H), 11.54 (s, 1H), 10.60 (s, 1H), 8.71 – 8.64 (m, 2H), 8.30 (d, *J* = 2.2, 1H), 8.03 (dd, *J* = 8.6, 2.2, 1H), 7.58 – 7.50 (m, 2H), 7.31 (dd, *J* = 8.6, 1.8, 1H), 7.23 – 7.17 (m, 1H), 7.08 (d, *J* = 6.6, 2H), 2.20 (s, 3H). **$^{13}$C NMR** (101 MHz, DMSO-$d_6$) $\delta$ 161.86, 151.10, 149.98, 146.70, 144.02, 138.20, 136.22, 133.29, 133.07, 132.63, 130.53, 126.36, 125.03, 121.39, 117.15, 116.52, 114.46, 20.15. **MS** (ESI) *m/z*: 409 $[M + H]^+$, 407 $[M - H]^-$.

***N*-[4-(2-fluoro-4-pyridyl)phenyl]-2,4-dioxo-1H-quinazoline-6-sulfonamide (22)**



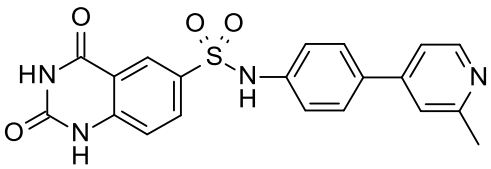
According to the GP5, **31c** (0.22 g, 1.18 mmol), DIPEA (0.43 mL, 2.48 mmol) and **10** (0.37 g, 1.41 mmol) in THF (11.8 mL) were used to obtain **22** as a light yellow solid (0.10 g, 20%) after purification by column chromatography (DCM/MeOH, 90:10, vol/vol). **$^{1}$H NMR** (400 MHz, DMSO-$d_6$) $\delta$ 11.54 (s, 1H), 11.51 (s, 1H), 10.68 (s, 1H), 8.28 (d, *J* = 2.2, 1H), 8.24 (d, *J* = 5.3, 1H), 7.99 (dd, *J* = 8.7, 2.2, 1H), 7.82 – 7.73 (m, 2H), 7.61 (dt, *J* = 5.5, 1.8, 1H), 7.43 (s, 1H), 7.31 – 7.20 (m, 3H). **$^{13}$C NMR** (101 MHz, DMSO-$d_6$) $\delta$ 164.10 (d, *J* = 234.1), 161.85, 152.20, 152.09, 149.99, 148.02 (d, *J* = 15.9), 144.06, 139.26, 132.73 (d, *J* = 25.0), 131.12, 128.15, 126.39, 119.67, 119.01 (d, *J* = 3.4), 116.53, 114.49, 105.85 (d, *J* = 38.7). **MS** (ESI) *m/z*: 413 $[M + H]^+$, 411 $[M - H]^-$.

***N*-[4-(2-chloro-4-pyridyl)phenyl]-2,4-dioxo-1H-quinazoline-6-sulfonamide (23)**



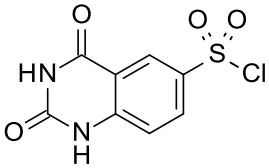
According to the GP5, **31d** (0.08 g, 0.36 mmol), DIPEA (0.13 mL, 0.76 mmol) and **10** (0.11 g, 0.43 mmol) in THF (3.6 mL) were used to afford **23** as a light yellow solid (0.05 g, 30%) after purification by column chromatography (DCM/MeOH, 90:10, vol/vol). **$^{1}$H NMR** (400 MHz, DMSO-$d_6$) $\delta$ 11.53 (s, 2H), 10.67 (s, 1H), 8.39 (d, *J* = 5.3, 1H), 8.29 (d, *J* = 2.2, 1H), 7.99 (dd, *J* = 8.7, 2.3, 1H), 7.80 – 7.71 (m, 3H), 7.64 (dd, *J* = 5.3, 1.6, 1H), 7.30 – 7.20 (m, 3H). **$^{13}$C NMR** (101 MHz, DMSO-$d_6$) $\delta$ 161.81, 151.24, 150.24, 149.96, 149.71, 144.04, 139.24, 132.87, 132.58, 130.86, 128.15, 126.38, 120.67, 120.05, 119.71, 116.51, 114.47. **MS** (ESI) *m/z*: 429 $[M + H]^+$, 427 $[M - H]^-$.

***N*-[4-(2-methyl-4-pyridyl)phenyl]-2,4-dioxo-1H-quinazoline-6-sulfonamide (24)**



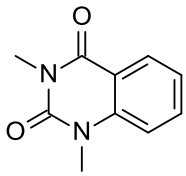
According to the GP5, **31e** (0.15 g, 0.8 mmol), DIPEA (0.3 mL, 1.71 mmol) and **10** (0.25 g, 0.96 mmol) in THF (8 mL) were used to give **24** as a white solid (0.05 g, 14%) after purification by column chromatography (DCM/MeOH, 90:10, vol/vol). **$^1$H NMR** (400 MHz, DMSO-$d_6$) $\delta$ 11.54 (s, 1H), 11.52 (s, 1H), 10.65 (s, 1H), 8.48 (d, *J* = 5.4 Hz, 1H), 8.29 (d, *J* = 4.8 Hz, 1H), 8.00 (dd, *J* = 8.7, 2.0 Hz, 1H), 7.77 – 7.70 (m, 2H), 7.62-7.58 (m, 1H), 7.51 (d, *J* = 5.5 Hz, 1H), 7.32 – 7.21 (m, 2H), 2.51 (s, 3H). **$^{13}$C NMR** (101 MHz, DMSO-$d_6$) $\delta$ 162.31, 158.09, 150.46, 148.50, 148.10, 144.52, 139.33, 133.36, 133.08, 132.62, 128.45, 126.87, 120.98, 120.37, 118.76, 116.98, 116.54, 114.93, 23.75. **MS** (ESI) *m/z*: 409 $[M + H]^+$, 407 $[M - H]^-$.

**2,4-dioxo-1*H*-quinazoline-6-sulfonyl chloride (25)**



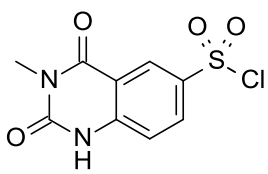
According to the GP1, 1H-quinazoline-2,4-dione **10** (2.0 g, 12.33 mmol) and chlorosulfonic acid (4.1 mL) were employed to afford **25** as a white solid (95%). **$^1$H NMR** (400 MHz, DMSO-$d_6$) δ 11.27 (s, 1H), 11.21 (s, 1H), 8.10 (d, *J* = 1.8, 1H), 7.82 (dd, *J* = 8.4, 1.9, 1H), 7.12 (d, *J* = 8.4, 1H). **MS** (ESI) *m/z*: 259 $[M - H]^-$.

**1,3-dimethylquinazoline-2,4-dione (10c)**



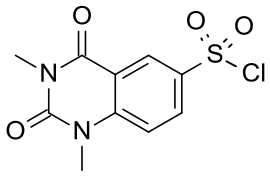
To a solution of 1-methylquinazoline-2,4-dione **10a**[2] (0.16 g, 0.91 mmol) in dioxane (2.75 mL, 0.33 M), 0.1 M $Na_2CO_3$ (13.6 mL, 1.36 mmol) and $CH_3I$ (0.08 mL, 1.36 mmol) were added and the reaction mixture was stirred at rt for 24 h. The mixture was diluted with DCM, washed with brine and extracted with DCM (3 x 10 mL). The combined organic layers were dried over $Na_2SO_4$, filtered, concentrated *in vacuo* and purified by flash chromatography (cyclohexane/EtOAc, 60:40, vol/vol) to afford **10c** as a white solid (0.12 g, 70%). **$^1$H NMR** (400 MHz, DMSO-$d_6$) $\delta$ 8.05 (dd, *J* = 7.8, 1.6, 1H), 7.77 (ddd, *J* = 8.7, 7.2, 1.7, 1H), 7.45 (d, *J* = 8.4, 1H), 7.30 (ddd, *J* = 8.0, 7.3, 0.9, 1H), 3.52 (s, 3H), 3.31 (s, 3H). **MS** (ESI) *m/z*: 191 $[M + H]^+$, 213 $[M + Na]^+$.

**3-methyl-2,4-dioxo-1H-quinazoline-6-sulfonyl chloride (25b)**



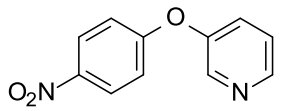
According to the GP1, 3-methyl-1H-quinazoline-2,4-dione **10b**[3] (0.15 g, 0.85 mmol) and chlorosulfonic acid (0.30 mL, 4.26 mmol) were employed to afford **25b** as a white solid (0.20 g, 86%). **$^1$H NMR** (400 MHz, DMSO-$d_6$) $\delta$ 11.49 (s, 1H), 8.16 (d, *J* = 1.9 Hz, 1H), 7.84 (dd, *J* = 8.4, 1.9 Hz, 1H), 7.12 (d, *J* = 8.4 Hz, 1H) 3.27 (s, 3H). **MS** (ESI) *m/z*: 273 $[M - H]^-$.

**1,3-dimethyl-2,4-dioxo-quinazoline-6-sulfonyl chloride (25c)**



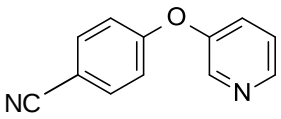
According to the GP1, **10c** (0.12 g, 0.63 mmol) and chlorosulfonic acid (0.21 mL, 3.15 mmol) were used to afford **25c** as a brown solid (0.17 g, 63%). **$^1$H NMR** (400 MHz, DMSO-$d_6$) $\delta$ 8.25 (d, $J$ = 2.1, 1H), 7.92 (dd, $J$ = 8.7, 2.1, 1H), 7.41 (d, $J$ = 8.7, 1H), 3.52 (s, 3H), 3.31 (s, 3H). **MS** (ESI) *m/z*: 289 [M + H]$^+$.

**3-(4-nitrophenoxy)pyridine (28a)**



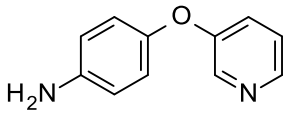
According to the GP2, 1-fluoro-4-nitrobenzene **27a** (1.5 g, 10.52 mmol), 3-hydroxypyridine (1.0 g, 10.52 mmol) and $Cs_2CO_3$ (5.14 g, 15.78 mmol) were suspended in DMF (2.92 mL) and stirred at 90°C on. The reaction mixture was cooled to rt, washed with $H_2O$ and extracted with EtOAc (3 × 10 mL). The combined organic layers were dried over $Na_2SO_4$, filtered, concentrated *in vacuo* and purified by column chromatography (cyclohexane/EtOAc, 60:40, vol/vol) to afford **28a** as a brown solid (1.05 g, 46%). **$^1$H NMR** (400 MHz, DMSO-$d_6$) $\delta$ 8.56 – 8.49 (m, 2H), 8.32 – 8.23 (m, 2H), 7.69 (ddd, $J$ = 8.4, 2.9, 1.4, 1H), 7.54 (ddd, $J$ = 8.4, 4.7, 0.7, 1H), 7.26 – 7.15 (m, 2H). **MS** (ESI) *m/z*: 217 [M + H]$^+$.

**4-(3-pyridyloxy)benzonitrile (28b)**



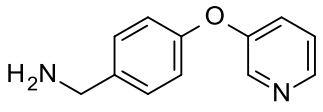
According to the GP2, 3-hydroxypyridine (0.19 g, 1.98 mmol), 4-fluorobenzonitrile **27b** (0.20 g, 1.65 mmol) and $Cs_2CO_3$ (0.64 g, 1.98 mmol) were suspended in DMF (0.55 mL) to afford **28b** as a light yellow solid (0.27 g, 84%) after purification by column chromatography (cyclohexane/EtOAc, 30:70, vol/vol). **$^1$H NMR** (400 MHz, DMSO-$d_6$) $\delta$ 8.53 – 8.47 (m, 2H), 7.92 – 7.85 (m, 2H), 7.64 (ddd, $J$ = 8.4, 2.9, 1.4 Hz, 1H), 7.52 (dd, $J$ = 8.4, 4.7 Hz, 1H), 7.22 – 7.15 (m, 2H). **MS** (ESI) *m/z*: 197 [M + H]$^+$, 195 [M - H]$^-$.

**4-(3-pyridyloxy)aniline (29a)**



According to the GP3, **28a** (1.05 g, 4.85 mmol), $NH_4HCO_2$ (1.53 g, 24.25 mmol) and 10% Pd/C (0.51g, 0.48 mmol) in EtOH (48.5 mL) were used to afford **29a** as an orange solid (0.41 g, 47%). **$^1$H NMR** (400 MHz, DMSO-$d_6$) $\delta$ 8.27 – 8.20 (m, 2H), 7.32 (ddd, $J$ = 8.4, 4.6, 0.7, 1H), 7.21 (ddd, $J$ = 8.4, 2.9, 1.4, 1H), 6.85 – 6.77 (m, 2H), 6.64 – 6.56 (m, 2H), 5.04 (s, 2H). **MS** (ESI) *m/z*: 187 [M + H]$^+$.

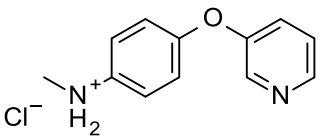
**[4-(3-pyridyloxy)phenyl]methanamine (29b)**



A solution of 0.75 M $LiAlH_4$ in THF (1 mL, 0.75 mmol) was added dropwise to a solution of **28b** (0.10 g, 0.50 mmol) in THF (1 mL) at 0°C. The reaction mixture was stirred at rt for 90 min. NaOH (1 mL, 2 M) was then added and the mixture was stirred at rt for 1 h. The resulting emulsion was filtered through Celite® and the phases were separated. The aqueous layer was extracted with EtOAc (3 x 10 mL) and the combined organic layers were dried over $Na_2SO_4$, filtered, concentrated *in vacuo* and purified by flash

chromatography (DCM/1% ammonia in MeOH, 90:10, vol/vol) to afford **29b** as a light yellow oil (0.06 g ,60%). **$^1$H NMR** (400 MHz, DMSO-$d_6$) $\delta$ 8.42 – 8.30 (m, 2H), 7.49 – 7.32 (m, 4H), 7.09 – 6.94 (m, 2H), 3.72 (s, 2H), 3.31 (br. s, 2H). **MS** (ESI) *m/z*: 201 $[M + H]^+$, 199 $[M - H]^-$.

**methyl-[4-(3-pyridyloxy)phenyl]ammonium chloride (29c)**

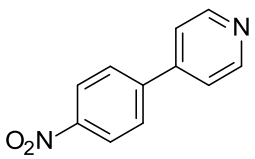


**Step1:** to a solution of **29a** (0.248 g, 1.33 mmol) and TEA (0.27 mL, 1.99 mmol) in DCM (13.3 mL, 0.1M), acetic anhydride (0.188 mL, 1.99 mmol) was added dropwise. The reaction mixture was stirred at rt for 2 h and the solvent was removed under reduced pressure afterwards. The resulting residue was diluted with EtOAc and washed with $NH_4Cl$. The phases were separated and the aqueous layer was extracted with EtOAc (3 x 10 mL). The combined organic phases were dried over $Na_2SO_4$, filtered, and concentrated *in vacuo* to afford *N*-[4-(3-pyridyloxy)phenyl]acetamide as a pink oil (0.28 g, 95%). **$^1$H NMR** (400 MHz, DMSO-$d_6$) $\delta$ 9.98 (s, 1H), 8.39 – 8.29 (m, 2H), 7.66 – 7.57 (m, 2H), 7.39 (ddd, *J* = 8.4, 4.4, 0.8, 1H), 7.35 (ddd, *J* = 8.4, 2.8, 1.6, 1H), 7.10 – 6.99 (m, 2H), 2.04 (s, 3H). **MS** (ESI) *m/z*: 229 $[M + H]^+$.

**Step 2:** to a solution of the acetamide (0.28 g, 1.25 mmol) in dry THF (12.5 mL, 0.1 M), NaH (dry, 95%, w/w) (0.36 g, 1.5 mmol) and $CH_3I$ (0.09 mL, 1.5 mmol) were added at 0°C. The reaction mixture was then warmed to rt and stirred for 2 h. Upon completion of the reaction, the suspension was diluted with EtOAc and washed with $NH_4Cl$. The phases were separated and the aqueous layer was extracted with EtOAc (3 x 10 mL). The combined organic phases were dried over $Na_2SO_4$, filtered, and concentrated *in vacuo* to afford *N*-methyl-*N*-[4-(3-pyridyloxy)phenyl]acetamide as a brown solid (0.34 g, quant.). **$^1$H NMR** (400 MHz, DMSO-$d_6$) $\delta$ 8.45 – 8.35 (m, 2H), 7.45 (dd, *J* = 8.3, 4.5, 2H), 7.37 (d, *J* = 8.3, 2H), 7.17 – 7.04 (m, 2H), 3.13 (s, 3H), 1.78 (s, 3H). **MS** (ESI) *m/z*: 243$[M + H]^+$, 265 $[M + Na]^+$, 281 $[M + K]^+$.

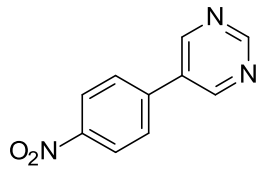
**Step 3:** concentrated HCl (1.4 mL) was added to a stirring solution of *N*-methylacetamide (0.34 g, 1.4 mmol). The reaction mixture was stirred at reflux on. When the reaction was completed, the mixture was portioned between water and EtOAc. The organic layer was separated and the aqueous phase was extracted with EtOAc (3 x 10 mL). The combined organic phases were dried over $Na_2SO_4$, filtered, and concentrated *in vacuo* to afford **29c** as a brown solid (0.46 g, quant.). **$^1$H NMR** (400 MHz, DMSO-$d_6$) $\delta$ 8.69 (d, *J* = 2.7, 1H), 8.62 (dd, *J* = 5.3, 1.1, 1H), 8.00 (dd, *J* = 8.7, 2.7, 1H), 7.87 (dd, *J* = 8.6, 5.2, 1H), 7.63 – 7.52 (m, 2H), 7.38 – 7.25 (m, 2H), 2.88 (s, 3H). **MS** (ESI) *m/z*: 201 $[M + H]^+$.

**4-(4-nitrophenyl)pyridine (30a)**



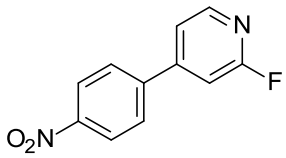
According to the GP4, 1-bromo-4-nitro-benzene (0.49 g, 2.44 mmol), 4-pyridylboronic acid (0.30 g, 2.44 mmol), $Pd(PPh_3)_4$ (0.28 g, 0.24 mmol) and $K_2CO_3$ (1.01 g, 7.32 mmol) in dioxane/$H_2O$ (10.8/5.4 mL) were used to give **30a** as a white solid (0.42 g, 89%) after purification by column chromatography (EtOAc/cyclohexane, 70:30, vol/vol). **$^1$H NMR** (400 MHz, DMSO-$d_6$) $\delta$ 8.76 – 8.69 (m, 2H), 8.42 – 8.31 (m, 2H), 8.14 – 8.04 (m, 2H), 7.83 – 7.78 (m, 2H). **MS** (ESI) *m/z*: 201 $[M + H]^+$.

**5-(4-nitrophenyl)pyrimidine (30b)**



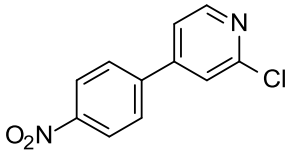
According to the GP4, 1-bromo-4-nitro-benzene (0.33 g, 1.62 mmol), pyrimidine-5-boronic acid (0.20 g, 1.62 mmol), $Pd(PPh_3)_4$ (0.18 g, 0.16 mmol) and $K_2CO_3$ (0.67 g, 4.84 mmol) in dioxane/$H_2O$ (7.2/3.6 mL) were used to obtain **30b** as a white solid (0.20 g, 62%) after purification by column chromatography (EtOAc/cyclohexane, 70:30, vol/vol). **$^1$H NMR** (400 MHz, DMSO-$d_6$) $\delta$ 9.27 (m, 3H), 8.45 – 8.30 (m, 2H), 8.18 – 8.06 (m, 2H). **MS** (ESI) *m/z*: 202 $[M + H]^+$.

**2-fluoro-4-(4-nitrophenyl)pyridine (30c)**



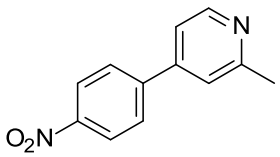
According to the GP4, 1-bromo-4-nitro-benzene (0.28 g, 1.42 mmol), 2-fluoropyridine-4-boronic acid (0.20 g, 1.42 mmol), $Pd(PPh_3)_4$ (0.16 g, 0.14 mmol) and $K_2CO_3$ (0.59 g, 4.26 mmol) in dioxane/$H_2O$ (6.3/3.1 mL) were used to afford **30c** as a white solid (0.29 g, 95%) after purification by column chromatography (EtOAc/cyclohexane, 80:20, vol/vol). **$^1$H NMR** (400 MHz, DMSO-$d_6$) $\delta$ 8.40 (d, *J* = 5.3, 1H), 8.39 – 8.33 (m, 2H), 8.18 – 8.11 (m, 2H), 7.81 (dt, *J* = 5.3, 1.8, 1H), 7.68 (d, *J* = 1.5, 1H). **MS** (ESI) *m/z*: 217 $[M + H]^-$.

**2-chloro-4-(4-nitrophenyl)pyridine (30d)**



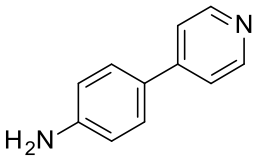
According to the GP4, 1-bromo-4-nitro-benzene (0.26 g, 1.27 mmol), 2-chloropyridine-4-boronic acid (0.20 g, 1.27 mmol), $Pd(PPh_3)_4$ (0.15 g, 0.13 mmol) and $K_2CO_3$ (0.53 g, 3.81 mmol) in dioxane/$H_2O$ (8.45/4.2 mL) were used to give **30d** as a light brown solid (0.26 g, 90%) after purification by column chromatography (EtOAc/cyclohexane, 30:70, vol/vol). **$^1$H NMR** (400 MHz, DMSO-$d_6$) $\delta$ 8.56 (dd, *J* = 5.2, 0.7, 1H), 8.38 – 8.33 (m, 2H), 8.18 – 8.12 (m, 2H), 8.00 (dd, *J* = 1.6, 0.7, 1H), 7.86 (dd, *J* = 5.2, 1.7, 1H). **MS** (ESI) *m/z*: 235 $[M + H]^+$.

**2-methyl-4-(4-nitrophenyl)pyridine (30e)**



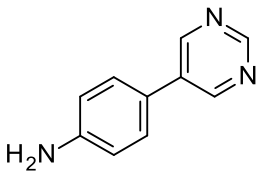
According to the GP4, 1-bromo-4-nitro-benzene (0.29 g, 1.46 mmol), 2-methylpyridine-4-boronic acid (0.20 g, 1.46 mmol), $Pd(PPh_3)_4$ (0.17 g, 0.15 mmol) and $K_2CO_3$ (0.60 g, 4.38 mmol) in dioxane/$H_2O$ (9.6/4.8 mL) were used to obtain **30e** as a white solid (0.17 g, 54%) after purification by column chromatography (EtOAc/cyclohexane, 70:30, vol/vol). **$^1$H NMR** (400 MHz, DMSO-$d_6$) $\delta$ 8.58 (dd, *J* = 5.2, 0.8, 1H), 8.40 – 8.30 (m, 2H), 8.11 – 8.03 (m, 2H), 7.72 – 7.66 (m, 1H), 7.59 (dd, *J* = 5.2, 1.8, 1H), 2.56 (s, 3H). **MS** (ESI) *m/z*: 215 $[M + H]^+$.

**4-(4-pyridyl)aniline (31a)**



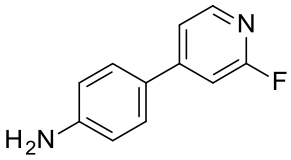
According to the GP3, **30a** (0.42 g, 2.1 mmol), $NH_4HCO_2$ (0.66 g, 10.48 mmol) and 10% Pd/C (0.23 g, 0.21 mmol) in EtOH (21 mL) were used to afford **31a** as a white solid (0.34 g, 98%). **$^1$H NMR** (400 MHz, DMSO-$d_6$) $\delta$ 8.54 – 8.41 (m, 2H), 7.57 – 7.53 (m, 4H), 6.76 – 6.60 (m, 2H), 5.49 (s, 2H). **MS** (ESI) *m/z*: 171 $[M + H]^+$.

**4-pyrimidin-5-yl-aniline (31b)**



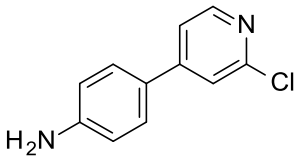
According to the GP3, **30b** (0.20 g, 0.99 mmol), $NH_4HCO_2$ (0.31 g, 4.95 mmol) and 10% Pd/C (0.10 g, 0.099 mmol) in EtOH (9.9 mL) were employed to afford **31b** as a light brown solid (0.16 g, 97%). **$^1$H NMR** (400 MHz, DMSO-$d_6$) $\delta$ 9.03 – 8.97 (m, 3H), 7.52 – 7.47 (m, 2H), 6.76 – 6.59 (m, 2H), 5.45 (s, 2H). **MS** (ESI) *m/z*: 172 $[M + H]^+$.

**4-(2-fluoro-4-pyridyl)aniline (31c)**



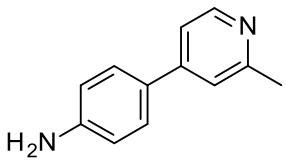
According to the GP3, **30c** (0.29 g, 1.33 mmol), $NH_4HCO_2$ (0.42 g, 6.65 mmol) and 10% Pd/C (0.14 g, 0.13 mmol) in EtOH (13.3 mL), were reacted together to afford **31c** as a light yellow solid (0.22 g, 89%). **$^1$H NMR** (400 MHz, DMSO-$d_6$) $\delta$ 8.13 (d, *J* = 5.4, 1H), 7.68 – 7.48 (m, 3H), 7.32 (d, *J* = 1.5, 1H), 6.71 – 6.60 (m, 2H), 5.62 (s, 2H). **MS** (ESI) *m/z*: 189 $[M + H]^+$.

**4-(2-chloro-4-pyridyl)aniline (31d)**



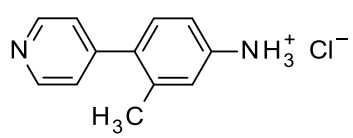
To a solution of **30d** (0.10 g, 0.43 mmol) in THF (2 mL), $NH_4Cl$ (0.23 g, 4.3 mmol) in water (1 mL) was added, followed by portionwise addition of zinc powder (0.22 g, 3.4 mmol). The reaction mixture was stirred at rt on. Upon completion, the mixture was filtered through Celite®, diluted with water and extracted with EtOAc (3 x 10 mL). The organic layers were pooled, dried over $Na_2SO_4$ and concentrated to afford **31d** as a light yellow solid (0.08 g, 76%). **$^1$H NMR** (400 MHz, DMSO-$d_6$) $\delta$ 8.28 (d, *J* = 5.5, 1H), 7.65 (d, *J* = 1.7, 1H), 7.58 (dd, *J* = 7.9, 2.2, 3H), 6.71 – 6.62 (m, 2H), 5.62 (s, 2H). **MS** (ESI) *m/z*: 205 $[M + H]^+$.

**4-(2-methyl-4-pyridyl)aniline (31e)**



According to the GP3, **30e** (0.17 g, 0.79 mmol), $NH_4HCO_2$ (0.25 g, 3.96 mmol) and 10% Pd/C (0.08 g, 0.08 mmol) in EtOH (7.9 mL) were used to afford **31e** as a light brown solid (0.15 g, quant.). **$^1$H NMR** (400 MHz, DMSO-$d_6$) $\delta$ 8.34 (dd, *J* = 5.4, 0.7, 1H), 7.56 – 7.46 (m, 2H), 7.43 (d, *J* = 1.8, 1H), 7.34 (dd, *J* = 5.4, 1.8, 1H), 6.77 – 6.57 (m, 2H), 5.45 (s, 2H), 2.46 (s, 3H). **MS** (ESI) *m/z*: 185 $[M + H]^+$.

**[3-methyl-4-(4-pyridyl)phenyl]ammonium chloride (32)**



**Step1:** to a solution of 4-bromo-3-methyl-aniline (0.50 g, 2.7 mmol) and TEA (0.56 mL, 4.03 mmol) in DCM (13.5 mL, 0.2 M), acetic anhydride (0.38 mL, 4.03 mmol) was added dropwise. The reaction mixture was stirred at rt on and the solvent was removed under reduced pressure afterwards. The resulting residue was diluted with EtOAc and washed with $NH_4Cl$. The phases were separated and the aqueous layer was extracted with EtOAc (3 x 10 mL). The combined organic phases were dried over $Na_2SO_4$, filtered, and concentrated *in vacuo* to afford *N*-(4-bromo-3-methyl-phenyl)acetamide as a light yellow solid (0.47 g, 95%). **$^1$H NMR** (400 MHz, DMSO-$d_6$) $\delta$ 9.95 (s, 1H), 7.56 (d, *J* = 2.8, 1H), 7.45 (d, *J* = 8.7, 1H), 7.36 (dd, *J* = 8.7, 2.6, 1H), 2.29 (s, 3H), 2.03 (s, 3H). **MS** (ESI) *m/z*: 228 $[M + H]^+$, 227 $[M - H]^-$.

**Step 2:** according to the GP4, *N*-(4-bromo-3-methyl-phenyl)acetamide (0.47 g, 2.06 mmol), 4-pyridylboronic acid (0.25 g, 2.06 mmol), $Pd(PPh_3)_4$ (0.24 g, 0.21 mmol) and $K_2CO_3$ (0.85 g, 6.18 mmol) in dioxane/$H_2O$ (10.0/5.0 mL) were used to afford *N*-[3-methyl-4-(4-pyridyl)phenyl]acetamide as a light yellow solid (0.23 g, 50%) after purification by column chromatography (EtOAc/cyclohexane, 98:2, vol/vol). **$^1$H NMR** (400 MHz, DMSO-$d_6$) $\delta$ 9.98 (s, 1H), 8.69 – 8.53 (m, 2H), 7.53 (d, *J* = 6.9, 2H), 7.40 – 7.29 (m, 2H), 7.22 – 7.14 (m, 1H), 2.24 (s, 3H), 2.06 (s, 3H). **MS** (ESI) *m/z*: 227 $[M + H]^+$, 225 $[M - H]^-$.

**Step 3:** concentrated HCl (0.03 mL) was added to a stirring solution of *N*-[3-methyl-4-(4-pyridyl)phenyl]acetamide (0.23 g, 1.01 mmol). The reaction mixture was stirred at reflux on. When the reaction was completed, the mixture was portioned between water and EtOAc. The organic layer was separated and the aqueous phase was extracted with EtOAc (3 x 10 mL). The combined organic phases were dried over $Na_2SO_4$, filtered and concentrated *in vacuo* to afford **32** as a light yellow solid (0.21 g, quant.). **$^1$H NMR** (400 MHz, DMSO-$d_6$) $\delta$ 9.03 – 8.86 (m, 2H), 8.13 – 7.99 (m, 2H), 7.42 (d, *J* = 8.0, 1H), 7.19 (d, *J* = 8.5, 2H), 2.33 (s, 3H). **MS** (ESI) *m/z*: 185 $[M + H]^+$.

## 5. Computational procedures

**Model building.** Chain A of the crystal structure of human NAPE-PLD (PDB 4QN9)[4] was used for docking studies. The protein was refined using the Protein Preparation Wizard of the Schrödinger suite 2015–4;[5] hydrogen atoms were added to the structure and bond orders were assigned and checked. Zero-order bonds connecting the two zinc ions to their coordinating atoms were added to obtain an octahedral geometry. The protonation state of ionizable amino acids was selected to be consistent with physiological pH. The overall hydrogen bonding network was optimized by sampling the orientation of hydroxyl and thiol groups, as well as the orientation of asparagine, glutamine and histidine side chains. Sulfate ions and deoxycholic acid molecules co-crystallized with the enzyme were removed. The system was subjected to a first energy minimization where hydrogen atoms were free to move. A second minimization run was then performed on all atoms, restraining the positions of protein heavy atoms until a root-mean-square deviation (RMSD) value of 0.3 Å was reached. All crystallized water molecules were

removed before docking studies. The Q320S NAPE-PLD mutant was modeled replacing glutamine 320 with serine.

**Docking calculations.** Docking studies were carried out using Glide version 6.9[6,7] and the SP scoring function. Glide grids were centered on the two zinc ions and the dimensions of bounding and enclosing boxes were set to 20 and 40 Å, respectively. No van der Waals scaling was applied to protein atoms. Inhibitor **18** was built in Maestro 10.4[8] and prepared with LigPrep 3.6[9] using default settings. The neutral species of the inhibitor was subjected to docking studies. 100 poses were collected for each docking run and subsequently ranked according to their G-score values. The top-ranked pose was retained and analyzed.

**6. Biology – Materials and Methods**

**Animals.** Wild-type C57BL/6J (20-30 g) male mice were housed at room temperature on a 12 h light/dark cycle. Water and standard chow pellets were freely available. Mice were sacrificed at 8 weeks of age. 5-7 cm segments of small intestine were removed and used for microsome preparation.[10] All procedures were performed in accordance with European Union (O.J. of E.C. L 358/1 12/18/1986) and Italian (D.M. 116192) regulations on the protection of animals used for experimental and other scientific purposes**.**

**Cell cultures.** HEK-293 cells (ATCC, VA, USA) were cultured at 37°C and 5% $CO_2$ in Dulbecco's Modified Eagle's Medium (DMEM) (Euroclone, Italy) supplemented with 10% FBS (Gibco™, Thermo Fisher Scientific, MA, USA), 2 mM L-glutamine and 1% penicillin/streptomycin (Euroclone) in 100 mm x 20 mm dishes (Sigma-Aldrich, Italy). Cells (1-2 x $10^6$ cell/dish) were incubated with vehicle (0.5% DMSO, vol/vol, Sigma–Aldrich) or **18** at 50 µM for 0 - 4 h. Cells were scraped in cold PBS (Euroclone) after treatment and harvested by centrifugation at 500 x *g* for 10 min at 4°C. Cells were then subjected to lipid extraction.[11] Data are reported in pmol as the mean ± SD of NAPEs or FAEs per mg of protein. Protein concentration was determined using the bicinchoninic acid assay (Thermo Fisher Scientific, MA, USA).

**Expression and purification of human recombinant NAPE-PLD.** A truncated form of wild-type or mutant human NAPE-PLD containing amino acid D47 (GAT, G is nucleotide 467) to F393 (TTT, T is nucleotide 1505) with an N-terminus MBP tag and a C-terminus hexahistidine tag was produced in *E. coli* Rosetta-gami B(DE3)pLysS cells (EMD Chemicals Inc., CA, USA) as previously reported.[4] Cells expressing the recombinant protein were grown in a 0.5 L of culture medium (LB mixed with 100 µg/mL ampicillin, 34 µg/mL chloramphenicol, 0.2% glucose) (Sigma-Aldrich) at 37°C to an absorbance of 0.7-0.8 and at 600 nm. Expression was induced with 0.5 mM isopropyl-β-D-thiogalactoside (Sigma-Aldrich) at 28°C for 20 h. Cells were harvested by centrifugation (3,000 x *g*, 30 min, 4°C) and stored at -80°C. Cell pellets were thawed on ice and suspended in cold lysis buffer (20 mM HEPES pH 7.8, 200 mM NaCl) mixed with EDTA-free protease inhibitor cocktail (Sigma-Aldrich). Cells were lysed by sonication

on ice for 7 min. Cell lysates were incubated for 60 min at 4°C in the presence of 1% Triton X-100 (Sigma-Aldrich), DNase (5 µg/mL, Sigma-Aldrich) and 2 mM $MgCl_2$ (Sigma-Aldrich). Cell lysates were centrifuged at 30,000 x *g* for 40 min at 4°C to remove cell debris. Supernatants were mixed with 3 mL amylose resin (New England BioLabs, MA, USA) and gently stirred for 2 h at 4°C. The recombinant protein was eluted at 4°C with 10 - 15 resin volumes of buffer (20 mM HEPES pH 7.8, 200 mM NaCl), followed by further 5 resin volumes of the same buffer containing 10 mM maltose (Sigma-Aldrich). The recombinant protein was concentrated to 1 mL and loaded in cold buffer (20 mM HEPES pH 7.8, 200 mM NaCl) onto a Superdex 200 Increase 10/300 GL (GE Healthcare, IL, USA) column for SEC. Fractions of highest purity were pooled, snap frozen and stored at -80°C to be used in activity assays.

**Site-directed mutagenesis.** Primers (Q320A fw: 5' CGA GGT GGT TTA TGA AAT ACG CGC ATG TAG ACC CAG AAG AAG C 3' and Q320A rev: 5' GCT TCT TCT GGG TCT ACA TGC GCG TAT TTC ATA AAC CAC CTC G 3'; Q320S fw: 5' CGA GGT GGT TTA TGA AAT ACT CGC ATG TAG ACC CAG AAG AAG C 3' and Q320S rev: 5' GCT TCT TCT GGG TCT ACA TGC GAG TAT TTC ATA AAC CAC CTC G 3') were purchased from Metabion (Germany). Q320A and Q320S mutations were introduced in the vector pMAL™- c5X (New England Biolabs, MA, USA) by PCR as indicated in the QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies, CA, USA). Mutated plasmids were used for the transformation of XL1-Blue competent *E. coli* cells (Agilent Technologies) through a standard heat-shock procedure. Cells were plated on agar plates containing 100 µg/mL ampicillin (Sigma-Aldrich). Colonies were amplified on in LB medium (4 mL, Sigma-Aldrich) containing 100 µg/mL ampicillin (Sigma-Aldrich) and plasmid DNA isolation was carried out using the NucleoSpin® Plasmid kit (Macherey-Nagel GmbH, Germany) from a 3-mL culture. Plasmids were verified by sequencing. Those harboring correct point mutations were transformed into Rosetta-gami B(DE3)pLysS competent cells (EMD Chemicals Inc., CA, USA) to express the corresponding recombinant protein mutants with an N-terminus MBP tag and a C-terminus hexahistidine tag, as previously reported.[4] Mutant Q158S/Y159S was produced as described.[4]

**Standard NAPE-PLD assay.** Purified recombinant wild-type human MBP-Δ47 hexahistidine-tagged NAPE-PLD[4] (592.7 ng) was preincubated with inhibitors (0.3 – 300 µM or 0.3 – 500 µM) or vehicle (1% DMSO, vol/vol) for 15 min at 37°C in 0.3 mL of assay buffer[12] (50 mM Tris-HCl pH 7.4, 0.1% Triton X-100). 100-fold concentrated scalar dilutions of inhibitors were prepared in DMSO (Sigma-Aldrich). Following preincubation, 2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-arachidonoyl (5 µM, Avanti Polar Lipids) was added and samples were incubated for additional 15 min at 37°C. The reactions were terminated by dilution (1:6) in ice-cold MeCN containing AEA-$d_4$ (0.5 µM, Cayman Chemical, MI, USA) as IS. Samples were centrifuged at 3,000 x *g* at 4°C for 20 min. The organic layers were collected for AEA analysis. AEA was measured by LC-MS/MS using an Acquity UPLC system coupled with a Xevo TQ-MS triple quadrupole mass spectrometer. Chromatographic separation was achieved using a BEH C18 column (2.1 x 50 mm, particle size 1.7 µm) eluted at a flow rate of 0.5 mL/min and set at 45°C. Instrument and columns were from Waters Inc. Milford, MA, USA. The mobile phase consisted of 0.1%

FA in water as eluent A and 0.1% FA in MeCN as eluent B. A gradient was developed for the best separation of AEA from Triton X-100 (0.1%, vol/vol, Sigma-Aldrich) used in the assay buffer, in order to avoid matrix effects.[13] The step gradient was: 0-0.5 min 60% B, 0.5-2.5 min 60 to 100% B, 2.5-3 min 100% B. The column was then reconditioned to 60% B for 0.5 min. The total run time for analysis was 3.5 min and the injection volume was 3 µL. Under these conditions AEA and AEA-$d_4$ eluted from the column at 1.6 min. The mass spectrometer was operated in the positive ESI mode. AEA (*m/z* = 348) and AEA-$d_4$ (*m/z* = 352) were quantified in multiple reaction monitoring (MRM) mode by selecting fragments with *m/z* = 62 and *m/z* = 66, respectively. The capillary voltage was set at 3 kV and the cone voltage at 25 V. The source temperature was set to 125°C. Desolvation gas and cone gas ($N_2$) flows were set to 800 and 80 L/h, respectively. Desolvation temperature was set to 400°C. Data were acquired by MassLynx software and quantified by TargetLynx software. Calibration curves were constructed by plotting the analyte to IS peak areas ratio versus the corresponding analyte concentration using weighted (1/*x*) least square regression analysis. $IC_{50}$ values were computed by nonlinear four-parameter dose-response analysis with GraphPad Prism version 5.03 (GraphPad Software, CA, USA). The bottom parameter of each curve was constrained to equal zero. Data were reported as the mean ± SD. Experiments were carried out in biological triplicates.

**Single concentration assay on NAPE-PLD.** Purified recombinant wild-type human MBP-Δ47 hexahistidine-tagged NAPE-PLD[4] (592.7 ng) was preincubated with test compounds at 75 µM or vehicle (1% DMSO, vol/vol, Sigma-Aldrich) for 15 min at 37°C in 0.3 mL of assay buffer[12] (50 mM Tris-HCl pH 7.4, 0.1% Triton X-100). 100-fold concentrated stock solutions of inhibitors were prepared in DMSO (Sigma-Aldrich). Following preincubation, 2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-arachidonoyl substrate (5 µM, Avanti Polar Lipids) was added and samples were incubated for additional 60 min at 37°C. The reactions were terminated by dilution (1:6) in ice-cold MeCN containing AEA-$d_4$ (0.5 µM, Cayman Chemical) as IS. Samples were centrifuged at 3,000 x *g* at 4°C for 20 min. The organic layers were collected for LC-MS/MS analysis. AEA was analyzed as described above. Percent of inhibition relative to control samples (1% DMSO, vol/vol) was calculated using GraphPad Prism version 5.03 (GraphPad Software). The experiments were carried out in biological triplicates.

**Concentration-response curves of compound 18.** Purified recombinant human wild-type MBP-Δ47 hexahistidine-tagged NAPE-PLD[4] and the corresponding Q158S/Y159S, Q320A or Q320S mutants (592.7 ng) were preincubated with **18** or vehicle (1% DMSO, vol/vol, Sigma-Aldrich) for 15 min at 37°C in 0.3 mL of assay buffer[12] (50 mM Tris-HCl pH 7.4, 0.1% Triton X-100). 100-fold concentrated scalar dilutions of **18** (0.3 – 300 µM) were prepared in DMSO (Sigma-Aldrich). Following preincubation, 2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-arachidonoyl (3 µM, Avanti Polar Lipids) was added to initiate reactions and samples were incubated for additional 10 min at 37°C. Reactions were terminated by dilution (1:6) in ice-cold MeCN containing AEA-$d_4$ (0.5 µM, Cayman Chemical) as IS. Samples were centrifuged at 3,000 x *g* at 4°C for 20 min. The organic layers were collected for LC-MS/MS analysis. AEA was analyzed as described above. $IC_{50}$ values were calculated using GraphPad Prism version 5.03

(GraphPad Software) by nonlinear four-parameter dose-response analysis. Data are reported as percent activity respect to total activity samples (1% DMSO, vol/vol). Experiments were carried out in biological triplicates.

**Kinetics of NAPE-PLD with compound 18.**[14] Reaction progress curves were carried out in 0.3 mL of assay buffer[12] (50 mM Tris-HCl pH 7.4, 0.1% Triton X-100). Purified recombinant wild-type human MBP-Δ47 hexahistidine-tagged NAPE-PLD[4] (592.7 ng) was preincubated for 15 min at 37°C with vehicle (DMSO, 1% vol/vol, Sigma-Aldrich) or **18** at 50 µM or 100 µM. Samples were incubated for 1 h at 37°C with varying concentrations of 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-arachidonoyl as substrate (0.5 – 10 µM, Avanti Polar Lipids) to initiate reactions. 100-fold concentrated scalar dilutions of substrate were prepared in DMSO (Sigma-Aldrich). Reactions were stopped at 0, 2, 6, 10, 12, 15, 30 and 60 min by dilution (1:6) in ice-cold MeCN containing AEA-$d_4$ (0.5 µM, Cayman Chemical) as IS. Samples were subjected to centrifugation at 3,000 x *g* at 4°C for 20 min. The organic layers were collected for LC-MS/MS analysis. AEA was analyzed as described above. The rates of the reaction progress curves were calculated within the linear range (0 - 15 min) and plotted against concentration of substrate. Kinetic parameters (Km and Vmax) were calculated by Michaelis-Menten nonlinear regression analysis using GraphPad Prism version 5.03 (GraphPad Software). Experiments were carried out in biological duplicates and values are reported as the mean ± SD of the initial velocity ($V_0$) expressed as concentration of product (AEA, µM) per min over concentration of substrate (µM).

**Kinetic analysis of wild-type and mutant NAPE-PLD.**[14] Reaction progress curves of wild-type and mutant (Q158S/Y159S, Q320A and Q320S) human MBP-Δ47 hexahistidine-tagged NAPE-PLD[4] were carried out in 0.3 mL of assay buffer[12] (50 mM Tris-HCl pH 7.4, 0.1% Triton X-100). Proteins (592.7 ng) were preincubated for 15 min at 37°C with vehicle (1% DMSO, vol/vol, Sigma-Aldrich). Samples were then incubated for 60 min at 37°C with varying concentrations of 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-arachidonoyl (0.5 – 10 µM, Avanti Polar Lipids) to initiate reactions. 100-fold concentrated scalar dilutions of the substrate were previously prepared in DMSO (Sigma-Aldrich). Reactions were stopped at 0, 2, 6, 10, 15, 20, 30 and 60 min after dilution (1:6) in ice-cold MeCN containing AEA-$d_4$ (0.5 µM, Cayman Chemical) as IS. Samples were centrifuged at 3,000 x *g* at 4°C for 20 min. The organic layers were collected for LC-MS/MS analysis. AEA was analyzed as described above. The rates of the reaction progress curves were calculated within different linear ranges depending on the activity of the protein under investigation and then plotted against concentration of substrate to afford the Michaelis-Menten plot. The kinetic parameters (Km and Vmax) of wild-type protein, Q158S/Y159S mutant and Q320A or Q320S mutants were computed in 10, 15 and 60 min, respectively. Kinetic parameters were determined using GraphPad Prism version 5.03 (GraphPad Software) by Michaelis-Menten nonlinear regression analysis. Experiments were carried out in biological duplicates and reported as the mean ± SD of the initial velocity ($V_0$) expressed as concentration of product (AEA, µM) per min over concentration of substrate (µM).

**Rapid dilution assay.**[15] Samples of purified recombinant wild-type human MBP-Δ47 hexahistidine-tagged NAPE-PLD[4] (7.5 µg) were preincubated for 15 min at 37°C with vehicle (1% DMSO, vol/vol, Sigma-Aldrich) or **18** (100 µM) in 0.020 mL assay buffer[12] (50 mM Tris-HCl, 0.1% Triton X-100). Samples were then diluted 100-fold (up to 2 mL) with assay buffer containing 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-arachidonoyl substrate (5 µM, Avanti Polar Lipids) to initiate reactions. Reactions were stopped at 0, 3, 5, 10, 15 min after dilution (1:6) in ice-cold MeCN spiked with AEA-$d_4$ (0.5 µM, Cayman Chemical) as IS. Samples were centrifuged at 3,000 x *g* at 4°C for 20 min. The organic layers were collected for LC-MS/MS analysis. AEA was analyzed as described above. The experiments were carried out in biological triplicates and values are expressed as the mean ± SD of pmol of product (AEA) per µg of protein over time (min). Data are reported using GraphPad Prism version 5.03 (GraphPad Software).

**Preincubation assay on human recombinant NAPE-PLD.** Samples of purified recombinant wild-type human MBP-Δ47 hexahistidine-tagged NAPE-PLD[4] (296 ng) were preincubated for 0 - 30 min at 37°C with vehicle (1% DMSO, vol/vol, Sigma-Aldrich) or **18** (50 µM or 100 µM). Substrate (1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-arachidonoyl substrate, 4.7 µM, Avanti Polar Lipids) was added at different time points during preincubation (0, 3, 5, 10, 15, 20, 25 and 30 min). Reactions were terminated after 10 min from each time point, by dilution (1:6) in ice-cold MeCN spiked with AEA-$d_4$ (0.5 µM, Cayman Chemical) as IS. Samples were centrifuged at 3,000 x *g* at 4°C for 20 min. The organic layers were collected for LC-MS/MS analysis. AEA was analyzed as described above. Data are reported using GraphPad Prism version 5.03 (GraphPad Software) as the mean ± SD of concentration of AEA (nM) released over time (min). The experiments were carried out in biological duplicates.

**Time-course experiments on human recombinant NAPE-PLD.** Samples (592 ng) of purified recombinant human wild-type and mutant (Q158S/Y159S or Q320A or Q320S) protein were incubated with substrate (1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-arachidonoyl, 3 µM, Avanti Polar Lipids) to initiate reactions in 0.3 mL of assay buffer[12] (50 mM Tris-HCl pH 7.4, 0.1% Triton X-100). Reactions were conducted at 37°C and stopped at 0, 5, 8, 15, 30 and 60 min by dilution (1:6) in ice-cold MeCN spiked with AEA-$d_4$ (0.5 µM, Cayman Chemical) as IS. Samples were centrifuged at 3,000 x *g* at 4°C for 20 min. The organic layers were collected for LC-MS/MS analysis. AEA was analyzed as described above. Results are reported as the mean ± SD of percent of product released relative to wild-type protein. Experiments were carried out in biological duplicates.

**Lipid extraction of HEK-293 cells.**[11] HEK-293 cells were suspended in cold PBS (1 mL, Euroclone, Italy), sonicated on ice (10 pulses, 10 s) and then mixed with MeOH (1 mL, Sigma-Aldrich) spiked with IS (50 nM C18:0–C22:6–*N*17:0 NAPE[16]; 100 nM OEA-$d_4$, PEA-$d_4$, AEA-$d_4$ and SEA-$d_3$, Cayman Chemical). After mixing for 30 s with a Vortex®, chloroform (2 mL, Sigma-Aldrich) was added. After a further mix of 30 s, samples were centrifuged for 20 min at 3,500 x *g* at 4°C. The layers were separated and the organic phases were collected, transferred into glass vials and evaporated under $N_2$. The

resulting lipid residues were dissolved in chloroform (2 mL, Sigma-Aldrich) and loaded onto silica (Sigma-Aldrich) columns (1 mL, chloroform/silica, 1:1, vol/vol). FAEs were eluted with a mixture of chloroform/MeOH (2 mL, 9:1, vol/vol). NAPEs were next eluted in the same glass vials with the addition of chloroform/MeOH (2 mL, 1:1, vol/vol). The organic mixtures were evaporated under $N_2$ and suspended in chloroform/MeOH (0.1 mL, 1:9, vol/vol) for LC-MS/MS analysis.

**NAPE and FAE analyses[17] in HEK-293 cells.**

NAPE and FAE analyses were carried out by LC-MS/MS using an Acquity UPLC system coupled with a Xevo TQ-MS triple quadrupole mass spectrometer. NAPEs and FAEs were separated on a HSS T3 C18 column (2.1 x 50 mm, particle size 1.8 µm) at a flow rate of 0.4 mL/min and set at 55°C. Instrument and columns were from Waters. The mobile phase consisted of 10 mM $NH_4HCO_2$ (pH 5) in MeCN/water (60:40, vol/vol) as solvent A and 10 mM $NH_4HCO_2$ (pH 5) in MeCN/IPA (10:90, vol/vol) as solvent B. A gradient program was used for the separation of all metabolites: 0-1 min 5% B, 1-7 min 5% to 100% B, 7-7.9 min 100% B, 7.9-8 min 5% B and 8-9 min maintained at 5% B. The total run time for analysis was 9 min, and the injection volume was set to 5 µL. The mass spectrometer was operated in the positive ESI mode and analytes were analyzed in MRM. The capillary voltage was set at 3 kV and the cone voltage at 25 V. The source temperature was set to 125°C. Desolvation gas and cone gas ($N_2$) flows were set to 800 and 50 L/h, respectively. Desolvation temperature was set to 450°C. Data were acquired by MassLynx software and quantified by TargetLynx software. Calibration curves were constructed by plotting the analyte to IS peak areas ratio versus the corresponding analyte concentration using weighted (1/*x*) least square regression analysis.

| **FAEs** | **Parent ion** (m/z) | **Daughter ion** (m/z) | **Acquisition time** (min) | **Retention time** (min) | **Collision** (V) |
|---|---|---|---|---|---|
| PEA | 300 | 62 | 0-4 | 2.82 | 20 |
| PEA-$d_4$ | 304 | 62 or 66 | 0-4 | 2.82 | 20 |
| OEA | 326 | 62 | 0-4 | 3.13 | 20 |
| OEA-$d_4$ | 330 | 66 | 0-4 | 3.13 | 20 |
| SEA | 328 | 62 | 0-4.50 | 3.87 | 20 |
| SEA-$d_3$ | 331 | 62 | 0-4.50 | 3.87 | 20 |
| AEA | 348 | 62 | 0-4 | 2.45 | 20 |
| AEA-$d_4$ | 352 | 66 | 0-4 | 2.45 | 20 |

MRM transitions of FAEs at a specific collision energy and the corresponding acquisition and retention times.

| NAPEs | Parent ion (m/z) | Daughter ion (m/z) | Acquisition time (min) | Retention time (min) | Collision (V) |
|---|---|---|---|---|---|
| 18:0-22:6-N18:0 | 1058.81 | 310.31 | 4-9 | 6.57 | 25 |
| 18:0-20:4-N18:0 | 1034.82 | 310.31 | 4-9 | 6.61 | 25 |
| P16:0-22:6-N18:0 | 1014.78 | 310.31 | 4-9 | 6.52 | 25 |
| 16:0-22:6-N18:0 | 1030.80 | 310.31 | 4-9 | 6.44 | 25 |
| P18:0-22:6-N18:0 | 1042.82 | 310.31 | 4-9 | 6.64 | 25 |
| 18:0-22:6-N16:0 | 1030.80 | 282.27 | 4-9 | 6.45 | 25 |
| P18:0-22:6-N16:0 | 1014.78 | 282.27 | 4-9 | 6.53 | 25 |
| 18:0-22:6-N17:0 | 1044.54 | 293.36 | 4-9 | 6.51 | 25 |

MRM transitions of NAPEs at a specific collision energy and the corresponding acquisition and retention times.

## 7. Off-Target Pharmacology

**Preparation of human Fatty Acid Amide Hydrolase (FAAH-1).**[18] Membrane-enriched fractions of human recombinant wild-type FAAH-1 were obtained from a stable cell line of HEK-293 cells overexpressing the FAAH-1 variant. Cells were grown in 75 $cm^2$ flasks (Corning, Sigma-Aldrich) in Dulbecco's modified Eagle medium (DMEM, Sigma-Aldrich) containing 10% FBS (Gibco™, Thermo Fisher Scientific), 1% glutamine and 500 µg/mL G418 (Euroclone). Once reached 80% confluence cells were then scraped off in PBS (Euroclone) and collected by centrifugation at 300 x *g* for 7 min at 4°C. Cell pellets were suspended in 20 mM Tris-HCl pH 7.4, 320 mM sucrose buffer and disrupted by sonication on ice (10 pulses, 10 s, 2 times) and then centrifuged at 1,000 x *g* for 10 min at 4°C. The resulting surpernatant was then centrifuged at 12,000 x *g* for 10 min at 4°C. Supernatants were ultracentrifuged at 105,000 x *g* for 1 h at 4°C. Membrane pellets were suspended in PBS (Euroclone) to afford the FAAH-1-enriched preparation which was quantified by Bradford Protein Assay (Bio-Rad Laboratories, Italy) and aliquoted for storage at -80°C.

**FAAH activity assay.**[18] Activity of human FAAH-1 was measured using a fluorimetric assay carried out in 96-well microplates (Black OptiPlate™-96 F, PerkinElmer, MA, USA). Human FAAH-1 membrane-enriched fractions (2.5 µg) were preincubated for 50 min at 37°C in 0.18 mL assay buffer (50 mM Tris-HCl pH 7.4, 0.05% fatty acid-free BSA) with 10 µL vehicle (5% DMSO, vol/vol, Sigma-Aldrich) or inhibitor. **18** or assay reference compound were tested in triplicate at 8 concentrations ranging from 3 pM to 30 µM. Background samples were prepared with 0.18 mL assay buffer without human FAAH-1 and with 10 µL DMSO (5%, vol/vol, Sigma-Aldrich). Reactions were started after substrate addition (2 µM, 7-amino-4-methyl coumarin-arachidonamide, Cayman Chemical) dissolved in ethanol previously. Reactions were run for 45 min at 37°C in the dark. Fluorescence was detected with a Tecan Infinite M200 NanoQuant plate reader with excitation and emission filters set at 350 nm and 460 nm, respectively. $IC_{50}$ were computed by nonlinear four-parameter dose-response analysis with GraphPad

Prism version 5.03 (GraphPad Software, CA, USA). Data were reported as the mean ± SD of percent activity respect to total activity (5% DMSO, vol/vol). Experiments were carried out in biological triplicates.

**Preparation of human *N*-acylethanolamine acid amide hydrolase (NAAA).**[19] NAAA-enriched fractions were prepared as described.[19] Briefly, HEK-293 cells stably transfected with the human NAAA coding sequence cloned from a human spleen cDNA library (Clontech Laboratories Inc., CA, USA) were used as enzyme source. Once reached 80% confluence, cells were scraped off in PBS (Euroclone) and collected by centrifugation at 300 x *g* for 7 min at 4°C. Cell pellets were suspended in 20 mM Tris-HCl pH 7.4, 320 mM sucrose buffer, sonicated on ice and centrifuged at 800 x *g* for 15 min at 4°C. Supernatants were ultracentrifuged at 12,000 x *g* for 30 min at 4°C. The resulting pellets were suspended in PBS (Euroclone) and subjected to three freeze-thaw cycles at -80°C. The suspension was finally ultracentrifuged at 105,000 x *g* for 1 h at 4°C, supernatants were collected, protein concentration was measured and samples aliquoted for storage at -80°C.

**NAAA activity assay.**[19] Activity of human NAAA was measured using a fluorimetric assay carried out in 96-well microplates (Black OptiPlate™-96 F, PerkinElmer) in 0.2 mL reaction buffer (100 mM citrate/phosphate buffer pH 4.5, 3 mM DTT, 0.1% NP-40, 0.05% BSA, 150 mM NaCl). Human NAAA-enriched preparations (4 µg) were preincubated for 10 min with **18** or reference compound at 10 concentrations ranging from 476.6 pM to 125 µM or vehicle (5% DMSO, vol/vol, Sigma-Aldrich). Reactions were started after addition of 5 µM *N*-(4-methyl-2-oxo-chromen-7-yl)-hexadecanamide (PAMCA) as substrate.[20,21] Reactions were run for 50 min at 37°C. Fluorescence was detected with an EnVision 2014 Multilabel Reader (PerkinElmer) using 340 nm and 450 nm as excitation and emission wavelengths, respectively. $IC_{50}$ values were calculated by non-linear regression analysis with a standard slope curve fitting using GraphPad Prism version 5.03 (GraphPad Software). Data were reported as the mean ± SD of percent activity relative to total activity (5% DMSO, vol/vol). Experiments were carried out in biological triplicates.

**Preparation of brain cytosolic fractions.**[22] Wild type C57BL/6J mouse brains were homogenized on ice with 4 volumes of 20 mM Tris-HCl pH 7.4, 320 mM sucrose buffer[18] in a glass homogenizer endowed with a hand-operated Teflon® pestle. 3 additional buffer volumes were added and the resulting homogenate was spun at 20,000 x *g* for 20 min at 4°C. The supernatant was kept at 4°C and the pellet was suspended in 4 volumes of buffer (20 mM Tris-HCl pH 7.4, 320 mM sucrose) and centrifuged at 20,000 x *g* for 20 min at 4°C. Supernatants were combined and centrifuged at 55,000 x *g* for 90 min at 4°C. The resulting supernatant was snap frozen in liquid $N_2$ and stored at -80°C until use. Protein concentration was determined by bicinchoninic acid quantification (Pierce BCA Protein Assay Kit, Thermo Fisher Scientific).

**Preparation of small intestinal microsomes.**[10] Segments of small intestine (5-7 cm) were removed from wild-type male C57BL/6J mice (20-30 g), rinsed with cold PBS (Euroclone) containing 1 mM DTT

(Sigma-Aldrich) and protease inhibitor cocktail (Sigma-Aldrich). The small intestine was everted[23] and the mucosal layer was carefully scraped off with a spatula onto a Petri dish on ice. The scraped cells were collected in cold PBS (Euroclone) and pelleted by spinning at 2,000 x *g* for 10 min at 4°C. Cells were suspended in 20 mM Tris-HCl pH 7.4, 320 mM sucrose buffer. The cell suspension was sonicated on ice (10 pulses, 10 s). The cell lysate was centrifuged at 1,000 x *g* for 10 min to remove cell debris. The supernatant was centrifuged at 12,000 x *g* for 10 min.[18] The resultant supernatant was collected and centrifuged at 100,000 x *g* for 60 min. The pellet was suspended in a minimum volume of PBS (Euroclone) and stored at -80°C until use. Protein concentration was determined by bicinchoninic acid quantification (Pierce BCA Protein Assay Kit).

**PLD activity assay.** Mouse brain cytosolic fractions (9.12 µg) (Figure S5a) or intestinal mouse microsomes (32.8 µg) (Figure S5b) were preincubated in PBS (0.02 mL) for 60 min at 37°C in the presence of vehicle (10% DMSO or MilliQ® water) or FIPI[24,25], sodium oleate[26] or **18** at 3 mM. Corresponding negative controls were prepared in PBS (Euroclone) for the detection of basal fluorescent signals. Samples (0.01 mL) were then assayed for PLD activity using the Phospholipase D Assay Kit (Sigma-Aldrich). Samples were diluted 1:10 in the reaction mixture (0.09 mL) that was added according to manufacturer's instruction. Fluorescence was measured for 60 min with a Tecan Infinite M200 NanoQuant plate reader with excitation and emission filters set at 530 nm and 585 nm, respectively. The instrument gain was set to 97 and 98 for the detection of the cytosolic and microsomal activities, respectively. RFU values of control samples were subtracted from the corresponding RFU values of enzyme samples, thus affording ΔRFU. Data were reported as the mean ± SD of ΔRFU calculated from 4 replicates in 35 min of incubation (Figure S5a) and as the mean ± SD of slopes (ΔRFU/min) calculated from 6 replicates in 20 min of incubation (Figure S5b). Statistical analyses were carried out with GraphPad Prism version 5.03 (GraphPad Software).

**Statistical analyses.** Statistical analyses were carried out using GraphPad Prism version 5.03 for Windows, (GraphPad Software, CA, USA). Data were evaluated by one-way ANOVA followed by Dunnett's test for multiple comparisons or by the unpaired Student's *t*-Test. Results were considered statistically significant at values of $p < 0.05$. Data were expressed as mean ± SD.

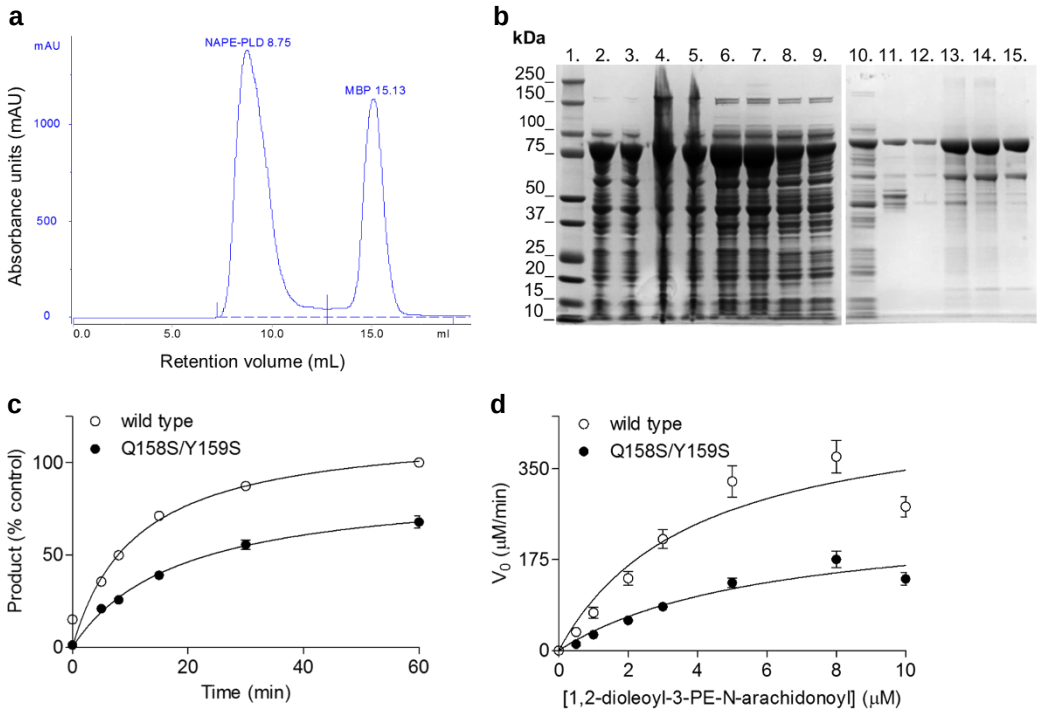
## 8. Supplementary Figures and Tables

**Table S1.** Kinetic parameters of purified human recombinant NAPE-PLD in the absence or presence of compound **18**.

| | Vehicle[a] | **18**, 50 µM | **18**, 100 µM |
|---|---|---|---|
| Km (µM) | 4.8 ± 2.1 | 10.1 ± 3.7 | 12.1 ± 8.9 |
| Vmax µM/min) | 619.1 ± 123.8 | 314.5 ± 69.0 | 232.7 ± 109.2 |

[a]DMSO (1% in assay buffer, vol/vol).

**Figure S1**



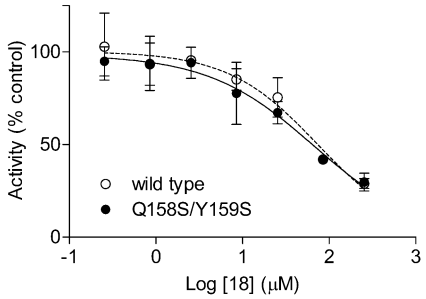

Purification and kinetics of human recombinant Q158S/Y159S NAPE-PLD mutant. a) SEC tracing showing elution of Q158S/Y159S NAPE-PLD mutant and MBP at 8.75 mL and 15.13 mL retention volumes, respectively. b) SDS-PAGE of protein aliquots collected during purification: molecular weight ladder (lane 1); cell lysate after 4 min sonication on ice (lane 2 - 3); cell lysate after 6 min sonication on ice (lane 4 - 5); cell lysate after DNase I treatment (lane 6 - 7); supernatant after centrifugation at 30,000 x *g* (lane 8 - 9); flow-through from amylose resin (lane 10); SEC fractions (lane 11-15). c) Time-course of product formation by wild-type and Q158S/Y159S NAPE-PLD under standard assay conditions. d) Michaelis-Menten kinetics analysis of wild-type and Q158S/Y159S NAPE-PLD.

**Table S2.** Kinetic parameters of wild-type and Q158S/Y159S NAPE-PLD.

| **NAPE-PLD** | **Km** (μM)[a] | **Vmax** (μM/min)[a] | **Kcat** ($s^{-1}$) |
|---|---|---|---|
| Wild type | 3.9 ± 2.2 | 276.7 ± 19.2 | 197 |
| Q158S/Y159S | 6.1 ± 2.8 | 138.1 ± 11.9 | 98 |

[a]Values are the mean ± SD of at least two independent experiments.

**Figure S2**



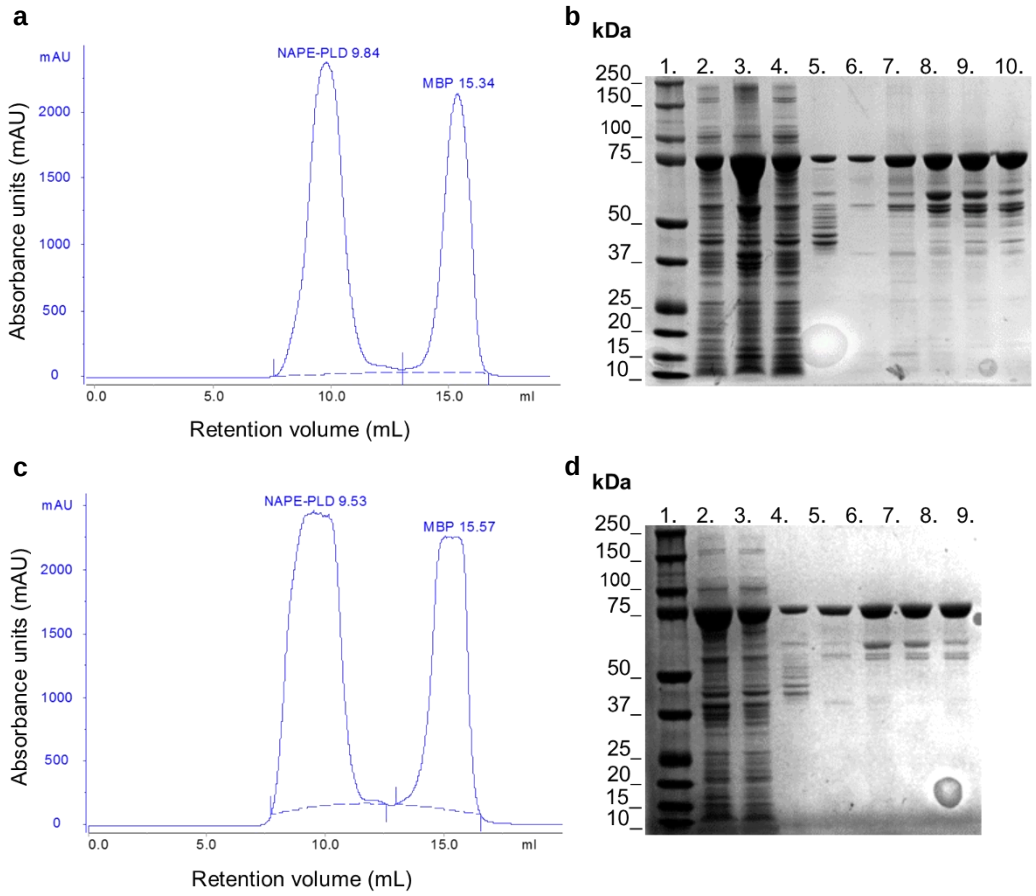

Inhibitory effects of **18** on purified wild-type or Q158S/Y159S human recombinant NAPE-PLD. The results are the mean ± SD of three independent experiments.

**Table S3.** Inhibitory potency ($IC_{50}$) of **18** on wild-type or Q158S/Y159S human recombinant NAPE-PLD.

| **NAPE-PLD** | **$IC_{50}$** (μM)[a] | **Residual activity** (% control) |
|---|---|---|
| Wild type | 46.20 ± 1.56 | 18.45 |
| Q158S/Y159S | 37.78 ± 1.83 | 18.69 |

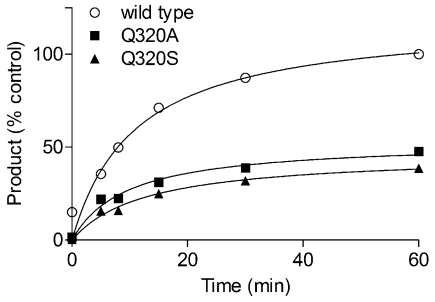
[a]Values are the mean ± SD of at least three independent experiments.

**Figure S3**




Purification of Q320A or Q320S human recombinant NAPE-PLD mutants. a) SEC tracing showing elution of Q320S NAPE-PLD mutant and MBP at 9.84 mL and 15.34 mL retention volumes, respectively. b) SDS-PAGE of aliquots collected during purification of Q320S NAPE-PLD mutant: molecular weight ladder (lane 1); cell lysate after 7 min sonication on ice (lane 2); supernatant after centrifugation at 30,000 x *g* (lane 3); flow-through from amylose resin (lane 4); final eluate in maltose buffer (before SEC) (lane 5); SEC fractions (lane 6 -10). c) SEC tracing showing elution of Q320A NAPE-PLD mutant and MBP at 9.53 mL and 15.57 mL retention volumes, respectively. d) SDS-PAGE of aliquots collected during purification of Q320A NAPE-PLD mutant: molecular weight ladder (lane 1); cell lysate after 7 min sonication on ice (lane 2); flow-through from amylose resin (lane 3); final eluate in maltose buffer (before SEC) (lane 4); SEC fractions (lane 5 - 8).

**Figure S4**




Time-course experiment with wild-type, Q320A and Q320S NAPE-PLD. The results are expressed as the mean ± SD of two independent experiments.

**Table S4.** Michaelis-Menten parameters of wild-type, Q320A or Q320S NAPE-PLD.

| **NAPE-PLD** | **Km** (μM)[a] | **Vmax** (μM/min)[a] | **Kcat** ($s^{-1}$) |
|---|---|---|---|
| Wild type | 3.9 ± 2.2 | 276.7 ± 19.2 | ≈ 196 |
| Q320A | 9.8 ± 2.9 | 24.9 ± 3.9 | ≈ 18 |
| Q320S | 14.7 ± 1.6 | 35.8 ± 1.6 | ≈ 25 |

[a]Values are expressed as the mean ± SD of two independent experiments.

**Table S5.** Inhibitory potency of compound **18** at 50 μM on three functionally distinct metallo-enzymes.[a]

| **Enzyme tested** | **Classification** | **Source** | **Inhibition**[a] (% control) |
|---|---|---|---|
| Carbonic Anhydrase II[27,28] | Hydro-Lyase | Human erythrocytes | 12 |
| Neutral Endopeptidase[29,30] | Metalloendopeptidase | Human Raji cells | -5.2 |
| Angiotensin-converting enzyme[31] | Peptidyl-Dipeptidase | Human recombinant | -15.6 |

[a]The screening was performed by Eurofins Cerep Panlabs using compound **18** at 50 μM. Results are expressed as mean of percent inhibition of control enzyme activity in three independent experiments. Moderate negative values are indicative of the variability of the signal around the control level.

**Figure S5**

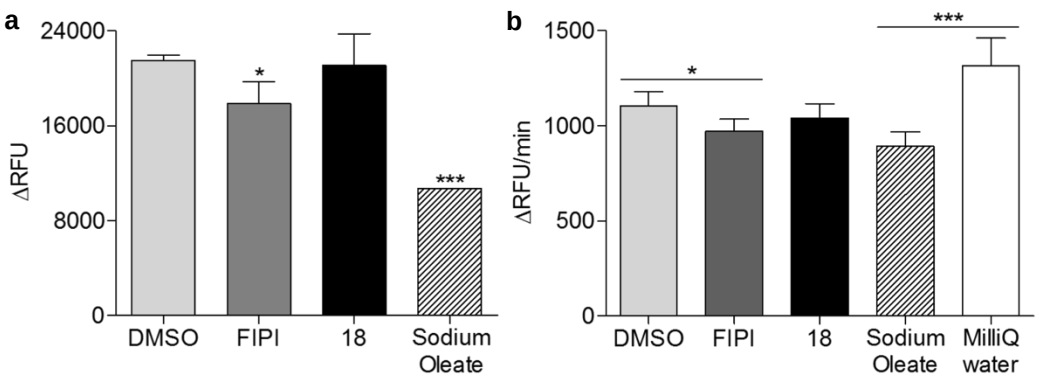
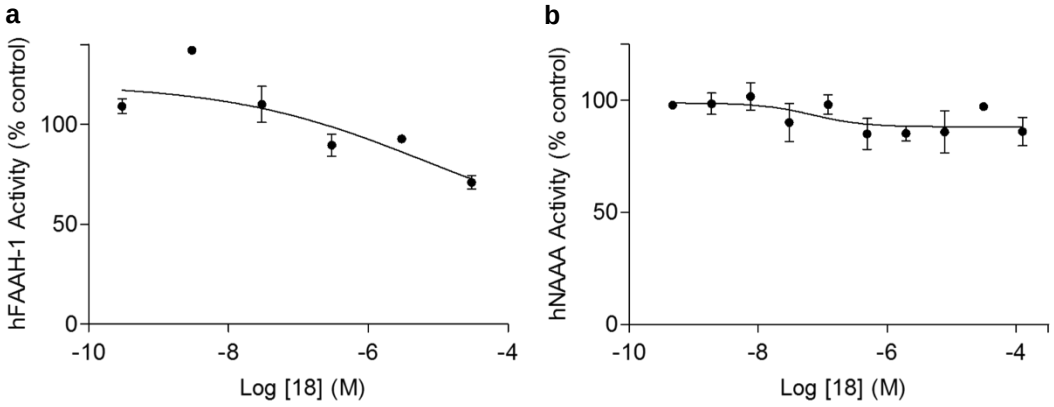


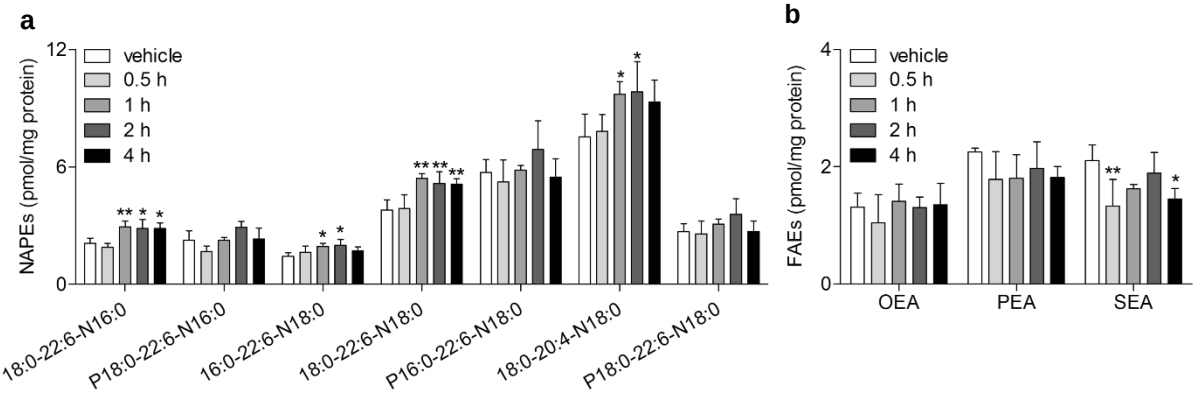
**Figure S5.** Off-target pharmacology of **18** on mouse PLD activities. a) Effects of vehicle (1% DMSO), 300 µM FIPI, **18** or sodium oleate on PLD activity in mouse brain cytosolic fractions. Student's unpaired *t*-Test (* $p < 0.05$, 1% DMSO vs FIPI, n=4/group) or Student's unpaired *t*-Test run on reciprocal values, in an attempt to equalize variances (*** $p < 0.0001$, 1% DMSO vs sodium oleate, n=4/group). b) Effects of vehicle (1% DMSO or MilliQ® water), 300 µM FIPI, **18** or sodium oleate on PLD activity in intestinal mouse microsomes. One-way ANOVA followed by Dunnett's post hoc test (* $p < 0.5$, 1% DMSO vs FIPI, n=6/group) or Student's unpaired *t*-Test (*** $p < 0.0001$, MilliQ® water vs sodium oleate, n=6/group). Results are expressed as the mean ± SD.

**Figure S6**




Off-target pharmacology of compound **18** by fluorimetric protein assays. The compound was tested on two FAE-hydrolyzing enzymes: a) human fatty acid amide hydrolase (FAAH)-1 and b) human *N*-acylethanolamine acid amidase (NAAA). The results are the mean ± SD of three independent experiments.

**Figure S7**




Effects of compound **18** (50 µM, 0 - 4 h) or its vehicle (0.5% DMSO) on NAPE and FAE levels in human HEK-293 cells. a) NAPE species differing in *sn*-1, *sn*-2 and *N*-acyl chains. b) FAE species, oleoylethanolamide (OEA), palmitoylethanolamide (PEA) and stearoylethanolamide (SEA). Results are expressed as the mean ± SD. One-way ANOVA followed by Dunnett's test for multiple comparison (*, **, $p < 0.05$, vehicle vs treatment, n=4/group).

## 9. References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