Supplementary material

Discovery of Novel Cyanamide-Based Inhibitors of Cathepsin C

Authors

Dramane Lainé, Michael Palovich, Brent McClelland, Emilie Petitjean, Isabelle Delhom, Haibo Xie, Jianghe Deng, Guoliang Lin, Roderick Davis, Anais Jolit, Neysa Nevins, Baoguang Zhao, Jim Villa, Jessica Schneck, Patrick McDevitt, Robert Midgett, Casey Kmetz, Sandra Umbrecht, Brian Peck, Alicia Bacon Davis and David Bettoun*

Corresponding Author: Dramane I. Lainé, GlaxoSmithKline, Respiratory CEDD, 709 Swedeland Road, P.O. Box 1539, King of Prussia, PA 19406-0939, Tel 1-610-270-7889, Fax 1-610-270-4451, dramane.i.laine@gsk.com

Contents

1. General experimental
2. Methods of preparation of compounds **1-17**
3. Recombinant cathepsins in vitro assays
 - 3.1. Cathepsin C
 - 3.2. Cathepsin K
 - 3.3. Cathepsin B
 - 3.4. Cathepsin L
 - 3.5. Cathepsin S
4. Plasma Stability Studies
 - 4.1. General Protocol
 - 4.2. Human plasma stability data
 - 4.3. Rat plasma stability studies of **8, 11 and 12**

- 4.4. Rat plasma stability studies of **8** in the presence of rat albumin, cysteine, glutathione or a broad spectrum esterase inhibitor
- 5. In vivo protocols
 - 5.1. Cathepsin C assay with (H-Gly-Arg)₂-R110 substrate
 - 5.2. Oral administration
 - 5.3. Intra-nasal administration
 - 5.4. Cigarette smoke
 - 5.5. Exposure levels

1. **General experimental**

All materials and reagents were used as is unless otherwise indicated. Air- or moisture-sensitive reactions were carried out under a nitrogen atmosphere. Flash chromatography was performed using silica gel (EM Science, 230–400 mesh) under standard techniques or using silica gel cartridges (RediSep normal phase disposable flash columns) on an Isco CombiFlash. Preparative and analytical HPLC were carried out on a Gilson 306 HPLC system. Unless otherwise stated, the conditions for the preparative reverse phase HPLC purifications used YMC Combiscreen ODS-A 75 mm × 30 mm (30 mL/min; gradient, (A) acetonitrile, (B) water; 10–80% A during 10 min) with UV detection at 214 nm. The ¹H NMR spectra were recorded at 400 MHz using a Bruker Avance 400 spectrometer. Chemical shifts are reported in parts per million (ppm) downfield of tetramethylsilane (δ scale). Multiplicity is indicated as follows: s (singlet), d (doublet), t (triplet), q (quartet), and m (multiplet). Coupling constants (*J*) are reported in hertz and refer to apparent multiplicities and not true coupling constants. Mass spectra were recorded on an Applied Biosystems MDS Sciex API 150EX single quadrupole mass spectrometer with an electrospray ionization (ESI) source. High-resolution mass spectral (HRMS) data were determined on a Bruker Daltonics 7T FTICR-MS, equipped with an Apollo ESI interface. The purity of the tested compounds not subjected to combustion analysis was determined by liquid chromatography (LC) on a Schimadzu LC system. Unless stated otherwise, the conditions for the LC used an Aquasil C18 40 mm x 1 mm column (flow rate: 300 μ L/min, 10% solution A, 90% solution B; A: water with 0.02 % TFA; B: acetonitrile with 0.018% TFA, duration 4.2 mins) with UV detection at 214 nm. All the reported test-compounds possess a purity of at least 95% as determined by LC.

2. Methods of preparation

3-(2-phenylethyl)-1-pyrrolidinecarbonitrile (1)

To a solution of 3-(2-phenylethyl)pyrrolidine (0.101 g, 0.576 mmol) in DCM (4 ml) was added DIEA (0.40 ml, 2.30 mmol). The reaction mixture was stirred at room temperature for 5 minutes. To this solution was added BrCN (0.112 g, 1.06 mmol). The reaction was stirred at room temperature overnight. The reaction was diluted with DCM (5 ml), washed with water (5 ml) and 1 N NaOH (0.5ml). The organic layer was dried, evaporated and then purified by preparatory HPLC (without TFA) to afford the title compound (0.0472 g). LC-MS: m/z, 201 (M+H), rt 2.12 min. ¹H NMR (400 MHz, CDCl₃) δ ppm 7.28 - 7.35 (2 H, m) 7.24 (1 H, d, *J*=7.28 Hz) 7.18 (2 H, d, *J*=7.03 Hz) 3.47 - 3.53 (2 H, m) 3.38 (1 H, m) 3.02 (1 H, t, *J*=8.78 Hz) 2.63 - 2.68 (2 H, m) 2.16 - 2.22 (1 H, m) 2.07 (1 H, m) 1.71 - 1.78 (2 H, m) 1.56 - 1.62 (1 H, m).

Cyano-pyrrolidine (2)

Compound 2 was prepared according to known experimental procedures (J. Org. Chem. (1953), 18 1003-13).

3-(2-phenylethyl)-1-piperidinecarbonitrile (3)

To a chilled (0°C) solution of triphenyl(phenylmethyl)phosphonium bromide (541 mg, 1.25 mmol) in THF (5 ml) was added *n*-BuLi solution in hexane (2.5 M, 0.5 ml, 1.25 mmol). The reaction mixture was stirred at room temperature for 15 min. followed by addition of a solution of 1,1-dimethylethyl 3-formyl-1-piperidinecarboxylate (213 mg, 1.0 mmol). The reaction mixture was stirred at room temperature for 2h. The reaction was quenched with water and diluted with EtOAc. The organic layer was washed with water (2x) then brine, dried MgSO₄, filtered and concentrated. The residue was purified by automated chromatography to give 1,1-dimethylethyl 3-[(*E*)-2-phenylethenyl]-1-piperidinecarboxylate, which was mixed with 10% Pd/C (250 mg) and hydrogenated via a balloon. The reaction was kept stirring for 3h followed by filtration. The filtrate was concentrated to give 1,1-dimethylethyl 3-(2-phenylethyl)-1-piperidinecarboxylate, which was mixed with 4M HCl in 1,4-dioxane (4ml). The reaction was stirred at room temperature for 1 h followed by evaporation. The dry material was then diluted with DCM (10 ml), and mixed with DIEA (0.57 mL, 3.2 mmol) and BrCN (3M in DCM, 0.80 mL, 2.4 mmol). The resultant mixture was stirred at room temperature overnight. The solvent was evaporated and the solid purified by preparatory HPLC (without TFA) to afford the title compound (0.10 g, 47%). LC-MS: *m/z*, 215 (*M*+*H*), *rt* 2.19 min. ¹H NMR δ ppm 7.26 - 7.35 (m, 2 H) 7.15 - 7.25 (m, 3 H) 3.33 - 3.45 (m, 2 H) 2.98 (td, *J*=11.94, 3.41 Hz, 1 H) 2.60 - 2.76 (m, 3 H) 1.87 - 1.98 (m, 1 H) 1.48 - 1.79 (m, 7 H) 1.04 - 1.16 (m, 1 H). HRMS calcd for (C₁₄H₁₉N₂) 215.1548, found 215.1538.

N-[(3*S*)-1-cyano-3-pyrrolidinyl]benzenesulfonamide (4)

To a chilled (0°C) solution of 1,1-dimethylethyl (3*S*)-3-amino-1-pyrrolidinecarboxylate (0.094 ml, 0.538 mmol) in DMF (1 ml) was added triethylamine (0.30 ml, 2.15 mmol) and benzenesulfonyl

chloride (0.137 ml, 1.07 mmol). The reaction mixture was stirred at room temperature overnight, diluted with water, extracted with EtOAc and concentrated. To the concentrate was added 4N HCl in 1,4-dioxane (5.0 ml). After stirring at room temperature overnight, the solvent was evaporated, the residue diluted with DCM (10 ml), and mixed with DIEA (0.2 mL, 1.15 mmol) and BrCN (0.40 mL, 1.2 mmol). The resultant mixture was stirred at room temperature overnight. The solvent was evaporated and purified by preparatory HPLC (without TFA) to afford the title compound (0.028 g). LC-MS: m/z, 252 (M+H), rt 1.47 min. ¹H NMR (400 MHz, CDCl₃) δ ppm 7.87 - 7.93 (2 H, m) 7.64 (1 H, t, J=7.40 Hz) 7.56 (2 H, t, J=7.53 Hz) 5.80 (1 H, br. s.) 3.88 (1 H, quin, J=5.33 Hz) 3.52 (1 H, dt, J=9.47, 7.43 Hz) 3.40 - 3.47 (2 H, m) 3.21 (1 H, dd, J=10.29, 4.27 Hz) 2.00 - 2.07 (1 H, m) 1.86 - 1.93 (1 H, m). HRMS calcd for (C₁₁H₁₄N₃ O₂ S) 252.0807, found 252.0797.

N-[(3R)-1-cyano-3-pyrrolidinyl]benzenesulfonamide (5)

To a chilled (0°C) solution of 1,1-dimethylethyl (3R)-3-amino-1-pyrrolidinecarboxylate (0.091 ml, 0.536 mmol) in DMF (1 ml) was added triethylamine (0.30 ml, 2.15 mmol) and benzenesulfonyl chloride (0.137 ml, 1.07 mmol). The reaction mixture was stirred at room temperature overnight, diluted with water, extracted with EtOAc and concentrated. To the concentrate was added 4N HCl in 1,4-dioxane (5.0 ml). After stirring at room temperature overnight, the solvent was evaporated, the residue diluted with DCM (10 ml), and mixed with DIEA (0.2 mL, 1.15 mmol) and BrCN (0.40 mL, 1.2 mmol). The resultant mixture was stirred at room temperature overnight. The solvent was evaporated and purified by preparatory HPLC (without TFA) to afford the title compound (0.063 g). LC-MS: m/z, 252 (M+H), rt 1.47 min. ¹H NMR (400 MHz, CDCl₃) δ ppm 7.87 - 7.92 (2 H, m) 7.60 - 7.65 (1 H, m) 7.52 - 7.59 (2 H, m) 5.95 (1 H, d, J=7.03 Hz) 3.83 - 3.90 (1 H, m) 3.48 - 3.55 (1 H, m) 3.39 - 3.46 (2 H, m) 3.21 (1 H, dd, J=10.16, 4.14 Hz) 1.99 - 2.05 (1 H, m) 1.85 - 1.92 (1 H, m). HRMS calcd for (C₁₁H₁₄N₃ O₂ S) 252.0807, found 252.0800.

N-[(3R)-1-cyano-3-pyrrolidiny]-4-fluorobenzamide (6)

To a cooled (0°C) solution of (*R*)-(+)-*N*-Boc-3-aminopyrrolidine (0.77 μl, 0.45 mmol) in DCM (3 ml) was added triethylamine (190 μl, 1.35 mmol, 3 eq) and 4-Chlorobenzoyl chloride (0.079 g, 0.5 mmol, 1.1 eq). The reaction mixture was stirred at room temperature for 18 hours. Excess acid chloride was removed by addition of PS-Trisamine (3.0 mmol/g, 3.5 eq) and stirring for 1 hour. The resin was filtered and the solvent was removed to provide the crude 1,1-dimethylethyl (3*R*)-3-[(4-fluorophenyl)carbonylamino]-1-pyrrolidinecarboxylate. This material was dissolved in 1,4 dioxane (1 ml) and a solution of 4N HCl in 1,4 dioxane (2 ml) was added and stirred at room temperature for 1 hour. The solvent was removed to provide crude 4-fluoro-*N*-[(3*R*)-3-pyrrolidiny]benzamide hydrochloride. This compound was dissolved in DCM (3 ml) and mixed with DIEA (0.175 ml, 1 mmol) followed by cyanogen bromide (0.333 ml, 1 mmol). The mixture was stirred for 18 hours at room temperature. The solvent was concentrated and the crude product was purified by reverse phase HPLC using a gradient of 10-60% Acetonitrile (with 0.1% TFA) / Water (with 0.1% TFA) to afford the title compound (0.031 g combined yield of 27% for the three steps). LC/MS *m/z* = 234 (*M*+*H*), *R*_t = 0.69 min. ¹H NMR (400 MHz, CD₃OD) δ 7.87 - 7.94 (m, 2H), 7.17 - 7.25 (m, 2H), 4.56 - 4.63 (m, 1H), 3.75 (dd, *J* = 6.40, 9.91 Hz, 1H), 3.64 (dt, *J* = 7.28, 9.29 Hz, 1H), 3.55 (td, *J* = 5.77, 8.66 Hz, 1H), 3.42 (dd, *J* = 4.52, 9.79 Hz, 1H), 2.24 - 2.32 (m, 1H), 2.05 - 2.12 (m, 1H). HRMS calcd for (C₁₂H₁₃N₃OF) 234.1043, found 234.1033.

N-[(3R)-1-cyano-3-pyrrolidiny]-*N'*-phenylurea (7)

To a solution of 1,1-dimethylethyl (3*R*)-3-amino-1-pyrrolidinecarboxylate (167.0 mg, 0.9 mmol) in DCM (3 ml) was added isocyanatobenzene (119.2 mg, 1.0 mmol). The reaction mixture was stirred at room temperature overnight. The reaction was diluted with EtOAc, washed with water (2x) then brine, dried MgSO₄, filtered and concentrated. Then 4N HCl in 1,4-dioxane (.15 ml) was added. After stirring at room temperature overnight, the solvent was evaporated. The dry material was then diluted with DCM (3 ml), and mixed with DIEA (0.2 mL, 1.15 mmol) and BrCN (0.40 mL, 1.2 mmol). The resultant mixture was stirred at room temperature overnight. The solvent was evaporated and the solid purified by preparatory HPLC (without TFA) to afford the title compound (17.95 mg). LC-MS: m/z, 231 (M+H), rt 1.18 min. ¹H NMR (500 MHz, DMSO-*d*₆) δ ppm 8.33 (s, 1H) 7.33 (d, *J*=1.8Hz, 2H) 7.17 (t, *J*=1.8 Hz, 1H) 6.86 – 6.91 (m, 1H) 6.48 – 6.53 (m, 1 H) 4.16 - 4.22 (m, 1H) 3.51 - 3.57 (dd, *J*=10.5, 3.4 Hz, 1 H, m, 1H) 3.38 (t, *J*=12.2Hz, 2 H) 3.14 (dd, *J*=10.8, 3.2 Hz, 1H) 2.00 - 2.10 (m, 1H) 1.75 - 1.82 (m, 1H). HRMS calcd for (C₁₂H₁₅N₄O₂) 231.1246, found 231.1238.

3-Bromo-N-[(3*R*)-1-cyano-3-pyrrolidinyl]benzenesulfonamide (8)

To a chilled (0°C) solution of 1,1-dimethylethyl (3*R*)-3-amino-1-pyrrolidinecarboxylate (0.095 ml, 0.0560 mmol) in DMF (1 ml) was added triethylamine (0.310 ml, 2.22 mmol) and 3-bromobenzenesulfonyl chloride (0.290 g, 1.14 mmol). The reaction mixture was stirred at room temperature overnight. The reaction was diluted with EtOAc, washed with water (2x) then brine, dried MgSO₄, filtered and concentrated. Then 4N HCl in 1,4-dioxane (5.0 ml) was added. After stirring at room temperature overnight, the solvent was evaporated. The dry material was then diluted with DCM (10 ml), and mixed with DIEA (0.2 mL, 1.15 mmol) and BrCN (0.40 mL, 1.2 mmol). The resultant mixture was stirred at room temperature overnight. The solvent was evaporated and the solid purified by preparatory HPLC (without TFA) to afford the title compound (0.0916 g). LC-MS: m/z, 330 (M+H), rt 1.70 min. ¹H NMR (400 MHz, CDCl₃) δ ppm 8.05 (t, *J*=1.76 Hz, 1 H) 7.81 - 7.86 (m, 1 H) 7.74 - 7.79 (m, 1 H) 7.45 (t, *J*=7.91 Hz, 1 H) 5.70 (d, *J*=7.28 Hz, 1 H) 3.88 - 3.96 (m, *J*=11.11, 5.77, 5.49, 5.49 Hz, 1 H) 3.43 - 3.59 (m, 3 H) 3.25

(dd, $J=10.29, 3.26$ Hz, 1 H) 2.04 - 2.14 (m, 1 H) 1.88 - 1.97 (m, 1 H). HRMS calcd for ($C_{11}H_{13}N_3O_2SBr$) 329.9912, found 329.9911.

N-[(3R)-1-cyano-3-pyrrolidinyl]-2,5-bis(methoxy)benzenesulfonamide (9)

To 2,5-bis(methoxy)benzenesulfonyl chloride (0.119 g, 0.50 mmol) and triethylamine (0.275 ml, 1.97 mmol) in DCM (2 mL) was added 1,1-dimethylethyl (3R)-3-amino-1-pyrrolidinecarboxylate (0.080 ml, 0.471 mmol). The reaction mixture was stirred at room temperature overnight. Water (1.5 ml) was added to the reaction mixture with stirring. The mixture was then diluted with DCM (2 ml) and water (1.5 ml) and put through a phase separator to dry it. Then 4N HCl in 1,4-dioxane (2.0 ml) was added. After 6 hours, the mixture was blown down to dryness. The dry material was then diluted with DCM (10 ml), and mixed with DIEA (0.45 mL, 2.58 mmol) and BrCN (0.40 mL, 1.2 mmol). The resultant mixture was stirred at room temperature overnight. The solvent was evaporated under vacuum and the solid purified by preparatory HPLC (without TFA) to afford the title compound (0.0757 g). LC-MS: m/z , 312 ($M+H$), rt 1.45 min. 1H NMR (400 MHz, $DMSO-d_6$) δ ppm 7.77 (d, $J=7.03$ Hz, 1 H) 7.27 (d, $J=2.51$ Hz, 1 H) 7.20 (d, $J=4.27$ Hz, 2 H) 3.87 (s, 3 H) 3.77 (s, 4 H) 3.39 - 3.47 (m, 1 H) 3.30 - 3.36 (m, 2 H) 3.10 (dd, $J=9.54, 4.77$ Hz, 1 H) 1.80 - 1.90 (m, 1 H) 1.67 - 1.76 (m, 1 H). HRMS calcd for ($C_{11}H_{18}N_3O_4S$) 312.1021, found 312.1018.

2,5-Dibromo-N-[(3R)-1-cyano-3-pyrrolidinyl]benzenesulfonamide (10)

To a chilled (0°C) solution of 1,1-dimethylethyl (3*R*)-3-amino-1-pyrrolidinecarboxylate (0.085 ml, 0.0501 mmol) in DMF (1 ml) was added triethylamine (0.275 ml, 1.97 mmol) and 2,5-dibromobenzenesulfonyl chloride (0.167 g, 0.50 mmol). The reaction mixture was stirred at room temperature overnight, diluted with water, extracted with DCM and concentrated. To the concentrate was added DCM (15 ml) and 4*N* HCl in 1,4-dioxane (5.0 ml). After stirring at room temperature overnight, the solvent was evaporated, the residue diluted with DCM (10 ml), and mixed with DIEA (0.2 mL, 1.15 mmol) and BrCN (0.20 mL, 0.6 mmol). The resultant mixture was stirred at room temperature overnight. The solvent was evaporated and purified by preparatory HPLC (without TFA) to afford the title compound (0.069 g). LC-MS: *m/z*, 408 (*M*+*H*), *rt* 1.72 min. ¹H NMR (400 MHz, CDCl₃) δ ppm 8.27 (d, *J*=2.26 Hz, 1H) 7.61 - 7.65 (m, 1H) 7.56 - 7.60 (m, 1H) 6.04 (d, *J*=7.28 Hz, 1H) 3.87 - 3.95 (m, 1H) 3.54 - 3.62 (m, 1H) 3.47 (ddd, *J*=12.61, 9.85, 5.65 Hz, 2H) 3.30 (dd, *J*=10.04, 4.27 Hz, 1H) 2.01 - 2.12 (m, 1H) 1.87 - 1.97 (m, 1H). HRMS calcd for (C₁₁H₁₂N₃O₂SBr₂) 407.9017, found 407.9006.

3-bromo-*N*-[(3*R*)-1-formyl-3-pyrrolidinyl]benzenesulfonamide (11)

To a solution of 1,1-dimethylethyl (3*R*)-3-[(3-bromophenyl)sulfonyl]amino-1-pyrrolidinecarboxylate (0.4696 g, 1.158 mmol) in DCM (4 ml) was added 4*N* HCl in 1,4-dioxane (4.0 ml). After stirring at room temperature overnight, the solvent was evaporated to afford crude 3-bromo-*N*-[(3*R*)-3-pyrrolidinyl]benzenesulfonamide (0.399 g). Formic acid (0.022 g, 0.333 mmol), 3-bromo-*N*-[(3*R*)-3-pyrrolidinyl]benzenesulfonamide (0.100 g, 0.328 mmol), 2-chloro-4,6-dimethoxy[1,3,5]triazine (0.0633 g, 0.360 mmol), 4-dimethylaminopyridine (0.002 g, 0.016 mmol) and *N*-methylmorpholine (0.04 ml, 0.363 mmol) were placed in a flask containing DCM (1.0 ml) and stirred overnight. The solvent was evaporated and purified by preparatory HPLC (without TFA) to afford the title compound (0.0335 g). LC-MS: *m/z*, 333 (*M*+*H*), *rt* 1.39 min. ¹H NMR (400 MHz, CDCl₃) δ ppm 8.15 (1 H, d, *J*=11.80 Hz) 8.02 - 8.09 (1 H, m) 7.84 (1 H, d, *J*=7.78 Hz) 7.73 (1 H, d, *J*=8.03 Hz) 7.42 (1 H, t, *J*=7.91 Hz) 6.56-6.79 (1 H, m) 3.80 - 3.95 (1 H, m) 3.63 - 3.70 (1 H, m) 3.55 - 3.59 (1 H, m) 3.48 - 3.54 (1 H, m) 3.30 - 3.44 (1 H, m) 1.86 - 2.11 (2 H, m). HRMS calcd for (C₁₁H₁₄N₂ O₃ SBr) 332.9909, found 332.9897.

1,1-dimethylethyl (3R)-3-[[[(3-bromophenyl)sulfonyl]amino]-1-pyrrolidinecarboxylate (12)

To a chilled (0°C) solution of 1,1-dimethylethyl (3R)-3-amino-1-pyrrolidinecarboxylate (1 g, 5.37 mmol) in DMF (10 ml) was added triethylamine (3.0 ml, 21.5 mmol) and 3-bromobenzenesulfonyl chloride (1.51 g, 5.91 mmol). The reaction mixture was stirred at room temperature overnight and concentrated. The residue was diluted in DCM and washed with water. The organic layer was dried and concentrated to afford the title compound (1.9 g). LC-MS: *m/z*, 405 (M+H), *rt* 2.09 min. ¹H NMR (400 MHz, DMSO-*d*₆) δ ppm 8.13 (1 H, br. s.) 7.97 (1 H, s) 7.89 (1 H, d, *J*=8.03 Hz) 7.83 (1 H, d, *J*=8.53 Hz) 7.59 (1 H, t, *J*=8.03 Hz) 3.70 (1 H, br. s.) 3.21 - 3.30 (3 H, m) 3.16 (1 H, m) 2.91 - 2.98 (1 H, m) 1.88-1.90 (1 H, m) 1.60-1.74 (1 H, m) 1.36 (9 H, d, *J*=7.53 Hz). HRMS calcd for (C₁₅H₂₂N₂O₄ SBr) 405.0484, found 405.0473.

3-bromo-N-[(3R,5R)-1-cyano-5-methyl-3-pyrrolidinyl]benzenesulfonamide (13) **1-(1,1-dimethylethyl) 2-methyl (2S,4S)-4-[[[(1,1-dimethylethyl)(dimethyl)silyl]oxy]-1,2-pyrrolidinedicarboxylate**

To a mixture of the commercially available 1-(1,1-dimethylethyl) 2-methyl (2S,4S)-4-hydroxy-1,2-pyrrolidinedicarboxylate (5 g, 20.4 mmol) in DMF (100 mL) was added imidazole (3.05 g, 44.9 mmol) and TBSCl (3.68 g, 24.4 mmol). The resultant mixture was stirred at room temperature for 16 hours. The solvent was evaporated under vacuum. The residue was partitioned between water and DCM. The DCM layer was separated, washed with water, dried over MgSO₄ and the solvent was removed to give a colorless oil (7.7 g). LC-MS: *m/z*, 360 (M+H). ¹H NMR (400 MHz, CDCl₃) δ 4.22 - 4.39 (m, 2H), 3.64 - 3.69 (m, 3H), 3.49 - 3.63 (m, 1

H), 3.25 - 3.32 (m, 1 H), 2.24 - 2.30 (m, 1 H), 2.01 - 2.10 (m, 1 H), 1.41 - 1.46 (m, 3 H), 1.34 - 1.41 (m, 6 H), 0.77 - 0.84 (m, 9 H), -0.04 - 0.03 (m, 6 H)

1,1-dimethylethyl (2S,4S)-4-[[[(1,1-dimethylethyl)(dimethyl)silyl]oxy]-2-(hydroxymethyl)-1-pyrrolidinecarboxylate

To a solution of 1-(1,1-dimethylethyl) 2-methyl (2S,4S)-4-[[[(1,1-dimethylethyl)(dimethyl)silyl]oxy]-1,2-pyrrolidinedicarboxylate (7.3 g, 20.3 mmol) in THF (100 mL) at 0 °C was added a solution of LiBH₄ (664 mgs, 30.5) in THF dropwise over one hour. The resulting mixture was stirred from 0 °C to room temperature over 16 hours. The reaction mixture was cooled down to 0 °C, neutralized with 1 N HCl, (ca 150 mL). The reaction mixture was then partitioned between water and ethyl acetate (300 mL) were added. The layers were separated and the aqueous layer was extracted with ethyl acetate (2 X 300 mL). The organic layers were combined, washed with a sat. aq. NaHCO₃ solution, dried on MgSO₄ and evaporated under vacuum to afford the title compound as a colorless oil (6.1 g). LC-MS: m/z, 332 (M+H).

1,1-dimethylethyl (2R,4S)-4-hydroxy-2-methyl-1-pyrrolidinecarboxylate

To a solution of 1,1-dimethylethyl (2S,4S)-4-[[[(1,1-dimethylethyl)(dimethyl)silyl]oxy]-2-(hydroxymethyl)-1-pyrrolidinecarboxylate (2 g, 6.0 mmol) in DCM (~20 mL) was added triethylamine (1.69 mL, 12.19 mmol), followed by methanesulfonyl chloride (0.70 mL, 9.0 mmol). The resulting mixture was stirred at room temperature for 16 hours. The reaction mixture was then diluted in DCM (300 mL) and washed with 1 N HCl (2 x 100 mL), sat. aq. NaHCO₃ (100 mL), dried on MgSO₄ and evaporated under vacuum to afford 1,1-dimethylethyl (2S,4S)-4-[[[(1,1-dimethylethyl)(dimethyl)silyl]oxy]-2-[[[(methanesulfonyl)oxy]methyl]-1-pyrrolidinecarboxylate as a colorless oil (2.54 g). The oil was diluted in THF (100 mL) and the mixture was cooled down to 0°C under argon. To the resultant mixture, a 1M solution of LiEt₃BH in THF, (24 mL, 24 mmol) was added dropwise. The mixture was stirred at room temperature for 2 hours then diluted with ethyl acetate (300 mL) and water (100 mL). The organic layer was separated, washed with 1 N HCl (100 mL) then sat. aq NaHCO₃ (100 mL), dried on MgSO₄ and evaporated to give a crude oily residue (2.05 g). Purification of the oil by flash chromatography (silica) eluting with a 0 to 20% gradient of ethyl acetate in hexanes afforded pure 1,1-dimethylethyl (2S,4S)-4-[[[(1,1-dimethylethyl)(dimethyl)silyl]oxy]-2-(hydroxymethyl)-1-pyrrolidinecarboxylate as a colorless oil (777 mgs), which was dissolved in THF (5 mL). Treatment of the solution with a 1M solution of TBAF in THF (4.9 mL, 4.9 mmol) gave a crude reaction mixture which was

evaporated onto silica gel and purified by flash chromatography to give 1,1-dimethylethyl-(2R,4S)-4-hydroxy-2-methyl-1-pyrrolidinecarboxylate a white solid (432 mg). LC-MS: m/z, 202 (M+H).

1,1-dimethylethyl (2R,4S)-2-methyl-4-[(methylsulfonyl)oxy]-1-pyrrolidinecarboxylate

To a solution of 1,1-dimethylethyl (2R,4S)-4-hydroxy-2-methyl-1-pyrrolidinecarboxylate (430 mg, 2.14 mmol) and triethylamine (6.41 mmol) in DCM (10 mL) was added methanesulfonyl chloride (331 μ L, 4.28 mmol). The resultant mixture was stirred for 16 hours, diluted with DCM (~100 mL), washed with 1 N HCl, sat. aq. NaHCO₃, dried on MgSO₄ and the solvent was evaporated under vacuum to afford 1,1-dimethylethyl (2R,4S)-2-methyl-4-[(methylsulfonyl)oxy]-1-pyrrolidinecarboxylate as a pale yellow oil (612.3 mg). LC-MS: m/z, 280 (M+H).

1,1-dimethylethyl (2R,4R)-4-amino-2-methyl-1-pyrrolidinecarboxylate

A mixture of 1,1-dimethylethyl (2R,4S)-2-methyl-4-[(methylsulfonyl)oxy]-1-pyrrolidinecarboxylate (612 mg, 2.19 mmol) and tertbutylammonium azide (685.4 mg, 2.41 mmol) in acetonitrile (10 mL) was allowed to stir at reflux for 2 hours then at room temperature for 2 days. TLC showed some starting material left so the mixture was heated at reflux for another 24 hours. More tertbutylammonium azide (470 mg, 1.65 mmol) was added and the mixture was allowed to heat at reflux for another 16 hours. The solvent was removed under vacuum and the resulting crude material was diluted in ethyl acetate, washed with water, dried over MgSO₄ then concentrated under vacuum to give a yellow oil (514 mg), which was redissolved in methanol (50 mL) and hydrogenated over 10% Pd/C in a H-CubeTM reactor (ThalesNano Nanotechnology). The solvent was evaporated under vacuum to afford the title compound as a yellow oil (377 mg). LC-MS: m/z, 201 (M+H).

1,1-dimethylethyl (2R,4R)-4-[(3-bromophenyl)sulfonyl]amino-2-methyl-1-pyrrolidinecarboxylate

To a solution of 1,1-dimethylethyl (2R,4R)-4-amino-2-methyl-1-pyrrolidinecarboxylate (117 mg, 0.58 mmol) in DCM (5 mL) were added DIPEA (0.20 mL, 1.16 mmol) and 3-bromobenzenesulfonyl chloride (84 μ L, 0.58 mmol). The resultant mixture was stirred at room temperature for 3.5 hours then loaded onto a 5 g silica SPE cartridge, eluting sequentially with dichloromethane and ethyl acetate. The relevant ethyl acetate fractions were combined and evaporated to afford the title compound (99 mg). LC-MS: m/z, 419 (M). ¹H NMR (400 MHz, CDCl₃) δ 7.95 (t, *J* = 1.76 Hz, 1H), 7.73 (d, *J* = 7.78 Hz, 1H), 7.66 (d, *J* = 8.03 Hz, 1H), 7.34 (t, *J* = 7.91 Hz, 1H), 4.65 (br. s., 1H), 3.77 - 3.91 (m, 1H), 3.38 (br. s., 1H), 3.07 (dd, *J* = 5.90, 11.42 Hz, 1H), 1.70 (br. s., 1H), 1.36 - 1.42 (m, 1H), 1.36 (s, 9H), 1.10 (d, *J* = 6.53 Hz, 3H).

3-bromo-N-[(3R,5R)-1-cyano-5-methyl-3-pyrrolidinyl]benzenesulfonamide

A mixture of 1,1-dimethylethyl (2R,4R)-4-[(3-bromophenyl)sulfonyl]amino-2-methyl-1-pyrrolidinecarboxylate (99 mg, 0.236 mmol, 1 eq.) in a 4 N HCl solution in dioxane (3 mL, 12 mmol) was stirred for over 2 hours and the dioxane was evaporated under vacuum to yield a pale yellow oil (99 mg) which was diluted in DCM (1 mL) and treated with triethylamine (0.161 mL, 1.18 mmol) and a 3M solution of BrCN in DCM (0.238 mL, 0.71 mmol). The reaction mixture was then diluted with DCM, washed with water, dried over MgSO₄ and evaporated to give a crude oil which was redissolved in DCM and then loaded onto a 2 g silica SPE cartridge, eluting sequentially with dichloromethane and ethyl acetate. The relevant ethyl acetate fractions were combined and evaporated to afford the title compound (62 mg). LC-MS: m/z, 345 (M+H). ¹H NMR (400 MHz, CDCl₃) δ 8.04 (d, J = 1.76 Hz, 1H), 7.82 (d, J = 7.78 Hz, 1H), 7.78 (d, J = 7.28 Hz, 1H), 7.43 - 7.49 (m, 1H), 4.95 (d, J = 6.78 Hz, 1H), 3.96 (br. s., 1H), 3.79 - 3.87 (m, 1H), 3.59 - 3.67 (m, 1H), 3.17 - 3.26 (m, 1H), 2.00 - 2.10 (m, 1H), 1.71 - 1.78 (m, 1H), 1.34 (dd, J = 2.01, 6.27 Hz, 3H). HRMS calcd for (C₁₂H₁₅N₃O₂SBr) 344.0068, found 344.0067.

N-[(3R,5R)-1-cyano-5-methyl-3-pyrrolidinyl]-2,5-bis(methoxy)benzenesulfonamide (14)

1,1-dimethylethyl (2R,4R)-4-([2,5-bis(methoxy)phenyl]sulfonyl)amino-2-methyl-1-pyrrolidinecarboxylate

To a solution of 1,1-dimethylethyl (2R, 4R)-4-amino-2-methyl-1-pyrrolidinecarboxylate (100 mg, 0.5 mmol) in DCM (5 mL) were added DIPEA (0.20 mL, 1.16 mmol) and 2,5-bis(methoxy)benzenesulfonyl chloride (119 mg, 0.5 mmol). The resultant mixture was stirred at room temperature overnight then loaded onto a 5 g silica SPE cartridge, eluting sequentially with hexane, dichloromethane and ethyl acetate. The relevant fractions were combined and evaporated to afford the title compound (79 mg). LC-MS: m/z, 400.8 (M⁺). ¹H NMR (400 MHz, CDCl₃) δ 7.47 (d, J = 3.26 Hz, 1H), 7.11 (dd, J = 3.26, 9.03 Hz, 1H), 7.00 (d, J = 9.03 Hz, 1H),

5.07 (d, $J = 7.78$ Hz, 1H), 3.96 (s, 3H), 3.86 - 3.93 (m, 2H), 3.84 (s, 3H), 3.34 (br. s., 1H), 3.12 (br. s., 1H), 1.45 (s, 9H), 1.15 (d, $J = 6.27$ Hz, 3H).

N-[*(3R,5R)*-1-cyano-5-methyl-3-pyrrolidinyl]-2,5-bis(methyloxy)benzenesulfonamide (14)

A mixture of 1,1-dimethylethyl (2*R*,4*R*)-4-([2,5-bis(methyloxy)phenyl]sulfonyl)amino)-2-methyl-1-pyrrolidinecarboxylate (79 mg) in a 4 N HCl solution in dioxane (3 mL, 12 mmol) was stirred for 16 hours and the dioxane was evaporated under vacuum to yield a crude product which was diluted in DCM (1 mL) and treated with triethylamine (0.22 mL) and a 3M solution of BrCN in DCM (0.3 mL). The reaction mixture was then treated with DCM and water. The organic phase was separated by passing onto a hydrophobic frit and then loaded onto a 2 g silica SPE cartridge, eluting sequentially with dichloromethane and ethyl acetate. The relevant ethyl acetate fractions were combined and evaporated to afford the title compound (49 mg). LC-MS: m/z , 328.9 (M^+). ^1H NMR (400 MHz, CDCl_3) δ 7.36 (d, $J = 3.01$ Hz, 1H), 7.04 (dd, $J = 3.26$, 9.03 Hz, 1H), 6.95 (d, $J = 9.03$ Hz, 1H), 5.26 (s, 1H), 3.91 (s, 3H), 3.77 - 3.85 (m, 1H), 3.76 (s, 3H), 3.71 - 3.76 (m, 1H), 3.40 (dd, $J = 5.52$, 10.54 Hz, 1H), 3.09 (dd, $J = 2.76$, 10.29 Hz, 1H), 1.91 - 1.99 (m, 1H), 1.52 - 1.63 (m, 2H), 1.22 (d, $J = 6.53$ Hz, 3H).

3-Bromo-*N*-[(3*R*,5*S*)-1-cyano-5-methyl-3-pyrrolidinyl]benzenesulfonamide (15)

methyl (4S)-4-hydroxy-*D*-prolinate

In a three necked flask under argon atmosphere equipped with a magnetic stir bar and a reflux condenser was placed MeOH (7 mL). To the system was added acetyl chloride (0.898 g, 11.4 mmol) in an ice bath followed by (4*S*)-4-hydroxy-*D*-proline (1 g, 7.6 mmol). The resultant mixture was heated at reflux for 12 hours and cooled down to room temperature. Ether was added to cause precipitation and the resulting brown powder was filtered off to afford the title compound. LC-MS: m/z , 146 ($M+H$).

1-(1,1-dimethylethyl) 2-methyl (2R,4S)-4-hydroxy-1,2-pyrrolidinedicarboxylate

To methyl (4S)-4-hydroxy-D-prolinate (1.1147 g, 7.6 mmol) in DCM (8 mL) was added triethylamine (3.19 mL, 22.9 mmol) while the bath cooled down to 0 °C. To the resultant mixture, di-tert-butyl dicarbonate (1.8318 g, 8.4 mmol) was added, stirred from 0 °C to room temperature over the weekend. The resultant mixture was washed with 1 N HCl and NaHCO₃ solution, dried over MgSO₄ and evaporated to afford the title compound as a yellow oil (1.4406 g). LC-MS: m/z, 246 (M+H).

1-(1,1-dimethylethyl) 2-methyl (2R,4S)-4-[[1,1-dimethylethyl)(dimethyl)silyl]oxy}-1,2-pyrrolidinedicarboxylate

To 1-(1,1-dimethylethyl) 2-methyl (2R,4S)-4-hydroxy-1,2-pyrrolidinedicarboxylate (1.4406 g, 5.9 mmol) in DMF (~4 mL) was added imidazole (0.4399 g, 6.46 mmol) and TBSCl (1.8512 g, 12.3 mmol). The resultant mixture was stirred at room temperature for 1.5 hours and diluted in ether and water. The organic layers were washed with 1 N HCl and NaHCO₃ solution, dried on MgSO₄ and the ether removed

1,1-dimethylethyl (2R,4S)-4-[[1,1-dimethylethyl)(dimethyl)silyl]oxy}-2-(hydroxymethyl)-1-pyrrolidinecarboxylate

1-(1,1-dimethylethyl) 2-methyl (2R,4S)-4-[[1,1-dimethylethyl)(dimethyl)silyl]oxy}-1,2-pyrrolidinedicarboxylate (2.3591 g, 6.56 mmol) was diluted in THF (30 mL) and the mixture was cooled down to 0 °C under argon. To the resultant mixture a solution of LiBH₄ in THF (15 mL) was added dropwise over 30 minutes, and stirred from 0 °C to room temperature overnight. The reaction mixture was cooled down to 0 °C, neutralized with 1 N HCl, and water (50 mL) and ethyl acetate (50 mL) were added. The layers were separated and the aqueous layer was extracted with ethyl acetate (~30 mL). The organic layers were combined and washed with NaHCO₃ solution (3 x 20 mL), dried on MgSO₄ and evaporated under vacuum to afford the title compound as a colorless liquid (2.02 g). LC-MS: m/z, 332 (M+H).

1,1-dimethylethyl (2R,4S)-4-[[1,1-dimethylethyl)(dimethyl)silyl]oxy}-2-[[1,1-dimethylethyl)(dimethyl)silyl]oxy}-2-(hydroxymethyl)-1-pyrrolidinecarboxylate

To 1,1-dimethylethyl (2R,4S)-4-[[1,1-dimethylethyl)(dimethyl)silyl]oxy}-2-(hydroxymethyl)-1-pyrrolidinecarboxylate (2.02 g, 6.09 mmol) in DCM (~12 mL) was added triethylamine (1.69 mL, 12.19 mmol). The resultant mixture was placed under argon and cooled down to -10 °C. To the resultant mixture methanesulfonyl chloride (0.71 mL, 9.14 mmol) was added and stirred from -10 °C to room temperature overnight. The reaction mixture was diluted in DCM (~50 mL) and

water (~50 mL) and the layers were separated. The organic layer was washed with 1 N HCl (2 x 20 mL), neutralized with NaHCO₃ solution, dried on MgSO₄ and the DCM was evaporated under vacuum to afford the title compound as a colorless liquid (1.9462 g). LC-MS: m/z, 410 (M+H).

1,1-dimethylethyl (2S,4S)-4-[[[(1,1-dimethylethyl)(dimethyl)silyl]oxy]-2-methyl-1-pyrrolidinecarboxylate

1,1-dimethylethyl (2R,4S)-4-[[[(1,1-dimethylethyl)(dimethyl)silyl]oxy]-2-[[[(methylsulfonyl)oxy]methyl]-1-pyrrolidinecarboxylate (0.1 g, 0.24 mmol) was diluted in THF (1 mL) and the mixture was cooled down to 0°C under argon. To the resultant mixture LiEt₃BH (superhydride), (2.44 mL, 2.44 mmol) was added dropwise and stirred at room temperature for 2 hours. The reaction mixture was diluted in water (40 mL) and ethyl acetate (60 mL), the layers were separated and the aqueous layer was extracted with ethyl acetate. The organic layers were combined, washed with 1 N HCl and NaHCO₃ solution, dried on MgSO₄ and the solvent was evaporated to afford the title compound as an orange oil (0.0824 g). LC-MS: m/z, 316 (M+H).

1,1-dimethylethyl-(2S,4S)-4-hydroxy-2-methyl-1-pyrrolidinecarboxylate

To 1,1-dimethylethyl (2S,4S)-4-[[[(1,1-dimethylethyl)(dimethyl)silyl]oxy]-2-methyl-1-pyrrolidinecarboxylate (0.19201 g, 6.08 mmol) in THF (~1 mL) was added TBAF (12 mL, 12.17 mmol) under argon. The resultant mixture was stirred at room temperature for 3 hours and purified by flash chromatography (silica gel) with gradient ethyl acetate:hexanes (1:2) to (2:1) to afford the title compound (1.0326 g). LC-MS: m/z, 202 (M+H).

1,1-dimethylethyl (2S,4S)-2-methyl-4-[(methylsulfonyl)oxy]-1-pyrrolidinecarboxylate

1,1-dimethylethyl (2S,4S)-4-hydroxy-2-methyl-1-pyrrolidinecarboxylate (1.0326 g, 5.13 mmol) in DCM (~2 mL) was placed under argon and cooled to 0 °C. To the reaction mixture was added triethylamine (1.2973 g, 12.8 mmol) and methanesulfonyl chloride (1.1753 g, 10.3 mmol). The resultant mixture was stirred for 16 hours and diluted in ether (~100 mL) and water (~60 mL). The aqueous layer was extracted with ethyl acetate (x2). The organic layers were combined, washed with 1 N HCl and NaHCO₃ solution, dried on MgSO₄ and the solvent evaporated under vacuum to afford the title compound as a yellow liquid (0.6395 g). LC-MS: m/z, 280 (M+H).

1,2-dimethylethyl-(2S,2R)-4-azido-2-methyl-1-pyrrolidinecarboxylate

To 1,1-dimethylethyl (2S,4S)-2-methyl-4-[(methylsulfonyl)oxy]-1-pyrrolidinecarboxylate (1.55 g, 5.55 mmol) in acetonitrile (15 mL) was added tertbutylammonium azide (1.77 g, 6.22 mmol) in

acetonitrile (10 ml). The resultant mixture was heated at reflux overnight. The solvent was removed under vacuum and the resulting crude material was diluted in ethyl acetate (~300 ml) and water (~100 ml). Both layers were separated and the aqueous layer was extracted again with ethyl acetate (x 2). The organic layers were combined, dried over MgSO₄ then concentrated under vacuum to give a crude material, which was purified by automated flash chromatography (40 g silica cartridge) to afford the title compound as a colorless liquid (0.966 g). LC-MS: m/z, 227 (M+H).

1,1-dimethylethyl (2S,4R)-4-amino-2-methyl-1-pyrrolidinecarboxylate

1,1-dimethylethyl (2S,4R)-4-azido-2-methyl-1-pyrrolidinecarboxylate (0.966 g, 4.26 mmol) in MeOH (~10 mL) was hydrogenated over 10% Pd/C in a H-Cube™ reactor (ThalesNano Nanotechnology Inc., H1031 Budapest, Zahony u. 7., Graphisoft Park Hungary, <http://www.thalesnano.com/products/h-cube-midi>). The solvent was evaporated under vacuum to afford the title compound as a colorless liquid (0.898 g). LC-MS: m/z, 201 (M+H).

1,1-dimethylethyl (2S,4R)-4-[(3-bromophenyl)sulfonyl]amino-2-methyl-1-pyrrolidinecarboxylate

To 1,1-dimethylethyl (2S,4R)-4-amino-2-methyl-1-pyrrolidinecarboxylate (0.100 g, 0.50 mmol) in DCM (~5 mL) was added DIPEA (0.176 mL, 1 mmol) and 3-bromobenzenesulfonyl chloride (0.128 g, 0.50 mmol). The resultant mixture was stirred at room temperature overnight, the solvent evaporated under vacuum and the resulting crude diluted in DCM. The DCM layer was isolated and loaded onto a silica SPE cartridge, eluting sequentially with DCM and ethyl acetate. The relevant fractions were combined and evaporated to afford the title compound (0.0615 g). LC-MS: m/z, 421 (M+H).

3-bromo-N-[(3R,5S)-1-cyano-5-methyl-3-pyrrolidinyl]benzenesulfonamide

1,1-dimethylethyl (2S,4R)-4-[(3-bromophenyl)sulfonyl]amino-2-methyl-1-pyrrolidinecarboxylate (0.1351 g, 0.32 mmol) was mixed with 4 N HCl (3 mL). The reaction mixture was stirred at room temperature for 12 hours, the solvent evaporated and the resulting solid was dissolved in DCM (~3 mL). To the resultant mixture was added DIEA (0.32 mL, 0.19 mmol) and BrCN (0.47 mL, 0.14 mmol). The resultant mixture was stirred for 4 hours at room temperature and then PS-trisamine (4 eq.) was added to quench the reaction. The reaction was stirred for another 2 hours at room temperature, filtered, concentrated and purified by preparatory HPLC (without TFA) to afford the title compound (0.0465 g). LC-MS: m/z, 326 (M+H). ¹H NMR (400 MHz, CDCl₃) δ ppm 8.02 (1 H, s) 7.81 (1 H, d, J=7.55 Hz) 7.75 (1 H, d, J=8.06 Hz) 7.44 (1 H, t, J=7.81 Hz) 5.88

(1 H, d, $J=6.80$ Hz) 3.80 - 3.88 (1 H, m) 3.58 - 3.68 (1 H, m) 3.52 (1 H, t, $J=8.56$ Hz) 3.23 (1 H, t, $J=8.44$ Hz) 2.25 (1 H, dt, $J=12.59, 6.29$ Hz) 1.46 (1 H, q) 1.34 (3 H, d, $J=6.04$ Hz).

N-[(3R,5S)-1-Cyano-5-methyl-3-pyrrolidinyl]-2,5-bis(methoxy)benzenesulfonamide (16)

1,1-Dimethylethyl (2S,4R)-4-([2,5-bis(methoxy)phenylsulfonyl]amino)-2-methyl-1-pyrrolidinecarboxylate (0.0615 g, 0.15 mmol, 1eq.) was mixed with 4 N HCl (3 mL). The reaction mixture was stirred at room temperature for 12 hours, the solvent evaporated and the resulting solid was dissolved in DCM (~3 mL). To the resultant mixture was added DIEA (0.32 mL, 0.19 mmol) and BrCN (0.47 mL, 0.14 mmol). The resultant mixture was stirred for 4 hours at room temperature and then PS-trisamine (4 eq.) was added to quench the reaction. The reaction was stirred for another 2 hours at room temperature, filtered, concentrated and purified by preparatory HPLC (without TFA) to afford the title compound (0.0252 g). LC-MS: m/z , 326 (M+H). ^1H NMR (400 MHz, CDCl_3) δ ppm 7.43 (1 H, d, $J=2.27$ Hz) 7.12 (1 H, dd, $J=8.81, 2.27$ Hz) 7.02 (1 H, d, $J=9.06$ Hz) 5.37 (1 H, d, $J=7.81$ Hz) 3.97 (3 H, s) 3.85 - 3.89 (1 H, m) 3.83 (3 H, s) 3.54 - 3.61 (1 H, m) 3.40 (1 H, t, $J=8.44$ Hz) 3.17 (1 H, t) 2.21 (1 H, ddd, $J=12.72, 6.55, 6.42$ Hz) 1.42 (1 H, q) 1.33 (3 H, d, $J=6.04$ Hz).

2,5-Dibromo-N-[(3R,5S)-1-cyano-5-methyl-3-pyrrolidinyl]benzenesulfonamide (17)

1,1-dimethylethyl (2S,4R)-4-[(2,5-dibromophenyl)sulfonyl]amino}-2-methyl-1-pyrrolidinecarboxylate

To 1,1-dimethylethyl (2S,4R)-4-amino-2-methyl-1-pyrrolidinecarboxylate (0.080 g, 0.40 mmol) in DCM (~3 mL) was added DIPEA (0.12 mL, 0.70 mmol) and 2,5-dibromobenzenesulfonyl chloride (0.117 g, 0.35 mmol). The resultant mixture was stirred at room temperature for 3 hours; the solvent was evaporated under vacuum and loaded onto a 5 g silica SPE cartridge, eluting sequentially with dichloromethane and ethyl acetate. The ethyl acetate fractions were combined and evaporated to afford the title compound (0.1627 g). LC-MS: m/z, 500 (M+H).

2,5-Dibromo-N-[(3R,5S)-1-cyano-5-methyl-3-pyrrolidinyl]benzenesulfonamide

1,1-Dimethylethyl (2S,4R)-4-[(2,5-dibromophenyl)sulfonyl]amino}-2-methyl-1-pyrrolidinecarboxylate (0.1627 g, 0.326 mmol, 1 eq.) in 4 N HCl (3 mL, 12 mmol) was mixed for 1 hour and the dioxane was evaporated under vacuum to yield a white solid which was diluted in DCM (~3 mL), mixed with DIEA (0.228 mL, 1.31 mmol) and BrCN (0.327 mL, 0.98 mmol). The resultant mixture was stirred at room temperature overnight; PS-trisamine (0.3275g, 4 eq.) was added and the mixture was stirred at room temperature for another 2 hours. The resin was filtered out, the solvent evaporated under vacuum and the residue purified by preparatory HPLC (without TFA) to afford the title compound (0.0805 g). LC-MS: m/z, 424 (M+H). ¹H NMR (400 MHz, CDCl₃) δ ppm 8.29 (1 H, d, J=2.26 Hz) 7.59 - 7.66 (1 H, m) 7.63 (1 H, q, J=8.45 Hz) 5.44 (1 H, d, J=7.78 Hz) 3.89 (1 H, dd, J=15.94, 7.91 Hz) 3.63 (1 H, dt, J=9.10, 6.37 Hz) 3.51 (1 H, dd, J=9.91, 7.40 Hz) 3.26 (1 H, dd, J=9.91, 7.15 Hz) 2.29 (1 H, dt, J=12.86, 6.49 Hz) 1.47 - 1.53 (1 H, m) 1.38 (3 H, d, J=6.27 Hz). HRMS calcd for (C₁₂H₁₄N₃O₂SBr₂) 421.9173, found 421.9158.

3. Recombinant Cathepsin in vitro assay

3.1 Cathepsin C

The activity of recombinant human cathepsin C is measured by the cleavage of a fluorogenic substrate, H-Ser-Tyr-AMC. Briefly, 24 pM cathepsin C is incubated with test compound (e.g. inhibitor) in a buffer consisting of 50 mM sodium acetate, 30 mM sodium chloride, 1 mM CHAPS, 1 mM dithiothreitol, 1 mM EDTA, pH 5.5, at room temperature for one hour. After one hour of incubating test compound with cathepsin C, the activity assay is initiated by the addition of an equal volume of 0.010 mM H-Ser-Tyr-AMC in the same buffer. After one hour, the activity assay is stopped by the addition of 1/10 volume of 100 μM E-64 or 10mM Aldrithiol. The reaction product is measured on a fluorescence reader set at an excitation wavelength of 360 nm and emission wavelength of 460 nm and equipped with a 400 nm dichroic mirror.

3.2 Cathepsin K

The activity of recombinant human cathepsin K is measured by the cleavage of a fluorogenic substrate, Z-Leu-Arg-AFC. Briefly, 80 pM cathepsin K is incubated with test compound (e.g. inhibitor) in a buffer consisting of 100 mM sodium acetate, 150 mM sodium chloride, 1 mM CHAPS, 1 mM dithiothreitol, 5 mM EDTA, pH 5.5, at room temperature for one hour. After one hour of incubating test compound with cathepsin K, the activity assay is initiated by the addition of an equal volume of 0.010 mM Z-Leu-Arg-AFC in the same buffer. After one hour, the activity assay is stopped by the addition of 1/10 volume of 100 μ M E-64. The reaction product is measured on a fluorescence reader set at an excitation wavelength of 405 nm and emission wavelength of 535 nm and equipped with a 505 nm dichroic mirror.

3.3 Cathepsin B

The activity of recombinant human cathepsin B is measured by the cleavage of a fluorogenic substrate, Z-Phe-Arg-AFC. Briefly, 500 pM cathepsin B is incubated with test compound (e.g. inhibitor) in a buffer consisting of 100 mM sodium acetate, 150 mM sodium chloride, 1 mM CHAPS, 50 μ M dithiothreitol, 5 mM EDTA, pH 5.5, at room temperature for 30 mins. After 30 mins of incubating test compound with cathepsin B, the activity assay is initiated by the addition of an equal volume of 0.050 mM Z-Phe-Arg-AFC in the same buffer. After one hour, the activity assay is stopped by the addition of 1/10 volume of 100 μ M E-64. The reaction product is measured on a fluorescence reader set at an excitation wavelength of 405 nm and emission wavelength of 535 nm and equipped with a 505 nm dichroic mirror.

3.4 Cathepsin L

The activity of recombinant human cathepsin L is measured by the cleavage of a fluorogenic substrate, Z-Leu-Arg-AMC. Briefly, 40 pM cathepsin L is incubated with test compound (e.g. inhibitor) in a buffer consisting of 100 mM sodium acetate, 1 mM CHAPS, 1 mM dithiothreitol, 5 mM EDTA, pH 5.5, at room temperature for one hour. After one hour of incubating test compound with cathepsin L, the activity assay is initiated by the addition of an equal volume of 0.004 mM Z-Leu-Arg-AFC in the same buffer. After one hour, the activity assay is stopped by the addition of 1/10 volume of 100 μ M E-64. The reaction product is measured on a fluorescence reader set at an excitation wavelength of 360 nm and emission wavelength of 460 nm and equipped with a 400 nm dichroic mirror.

3.5 Cathepsin S

The activity of recombinant human cathepsin S is measured by the cleavage of a fluorogenic substrate, KQKLR-AMC. Two nM cathepsin S is mixed with test compound (e.g. inhibitor) in a buffer consisting of 50 mM MES, 10 mM L-Cys, 5 mM EDTA, 0.5mM CHAPS pH 6.5, at room temperature. The activity assay is initiated by the addition of an equal volume of 0.060 mM KQKLR-AMC in the same buffer. After two hours, the activity assay is stopped by the addition of 1/10 volume of 100 μ M E-64. The reaction product is measured on a fluorescence reader set at an excitation wavelength of 360 nm and emission wavelength of 460 nm and equipped with a 400 nm dichroic mirror.

4. Plasma stability studies

4.1. General Protocol

Preparation of test compound

Prepare stock solution of the test compound at 1 mg/ml with DMSO or suitable solvent

Prepare an intermediate solution at 0.2 mg/ml by adding 20 μ L of the stock solution to 80 μ L of MeOH.

Preparation of plasma

Thaw plasma and keep at approx +4 °C until required.

Centrifuge thawed plasma to remove debris.

Adjust pH to approximately 7.4 with diluted HCl or NaOH.

Warm the plasma to 37 °C.

Preparation of 'time 0 sample'

Spike 2.5 μ L of the intermediate solution (0.2 mg/ml) into 997.5 ml of acetonitrile containing internal standard and vortex immediately. The resulting concentration of the test article is 500 ng/mL.

For the 0 min sample, add 50 mL diluted compound solution prepared from above step (in acetonitrile containing internal standard) to 150 mL of acetonitrile containing internal standard followed by addition of 50 mL plasma, vortex immediately

Initiation of incubation

Spike 2.5 μ L of intermediate solution (0.2 mg/ml) into 997.5 ml plasma and vortex immediately. Final incubation concentration is 500 ng/ml.

Sampling protocol

At 0.5, 30, 60, 120 min after initiation of test incubation, take a triplicate (3) of 50 μ L of the sample after vortex and add into 200 μ L of internal standard in acetonitrile to stop the reaction. Vortex and store in dry ice before transfer into a -80 $^{\circ}$ C freezer.

Measurement

The percentage of compound remaining is measured using specific HPLC-MS/MS methods as a ratio to the internal standard.

Data Analysis

The results are expressed as peak area ratio of test compound to internal standard. The remaining percentage of parent compound is calculated using the peak area ratio at specific time point divided by the peak area ratio at time 0 min.

The above protocol was adjusted as necessary to assess the stability of the compounds in rat and human plasma (see 4.2) as well as the rat plasma stability studies of **8** in the presence of rat albumin, cysteine, glutathione or a broad spectrum esterase inhibitor (see 4.3)

4.2. Human plasma stability data

Table 3: Stability of cpds **1-17** in human plasma

Cpd #	Human Plas. St. (% left @30 mins)
1	87
2	NT
3	NT
4	≥ 100
5	91
6	NT
7	NT

8	96
9	≥ 100
10	92
11	≥ 100 ^a
12	96 ^a
13	≥ 100
14	96
15	NT
16	NT
17	88

^a NT: Not tested; ^b Values are the mean of two or more independent assays.

4.3. Rat plasma stability studies with **8**, **11** and **12**

Figure 4. Stability of **8**, **11** and **12** in rat plasma

4.4. Rat plasma stability studies of **8** in the presence of rat albumin, cysteine, glutathione or a broad spectrum esterase inhibitor

Figure 5. Studies to determine the mechanism of action of the plasma instability of **8**

c

5. In vivo protocols

5.1. Cathepsin C assay with (H-Gly-Arg)₂-R110 substrate

The activity of cathepsin C in plasma or lung homogenate is measured by the cleavage of a fluorogenic substrate, (H-Gly-Arg)₂-R110. Briefly, the test sample (see below) is diluted in a buffer consisting of 1M sodium acetate, 1 M sodium chloride, 0.5 M EDTA, 1 M DTT, 1 mM EDTA in water at room temperature. The activity assay is initiated by the addition of a 10 mM (H-Gly-Arg)₂-R110 solution in the same buffer. The reaction product is measured on a fluorescence reader set at an excitation wavelength of 485 nm and emission wavelength of 530 nm and equipped with a 505 nm dichroic mirror.

5.2. Oral administration

Plasma time course:

Compound 17 was dissolved at 3 mg/ml in 10% dimethyl acetamide (DMA) and q.s.ed with sesame oil. Mice were treated p.o. with 30 mg/kg of compound or vehicle at a volume of 10 ml/kg. At either 30 min, 1.5 hr or 3 hr after p.o. administration, mice were euthanized with Fatal Plus i.p. (0.1 ml) and exsanguinated via cardiac puncture or jugular cutdown with a heparinized syringe. Blood was placed on ice, and then centrifuged at 15000g for 5 min to separate the plasma for the assay. The cathepsin C activity was assessed as described in 5.1

Lung homogenate time-course

C57BL/6J mice were orally dosed with the test compound (n=5). At the appropriate time (30 mins, 90 min or 180 min) mice were euthanized with 0.1 ml Fatal Plus i.p., then bronchoalveolar lavaged with 0.7 ml Dulbecco's phosphate-buffered saline with 1x complete (dPBS, in-house Media Prep lab) through a tracheal tube. Lungs were then removed en bloc and homogenized as indicated below. The cathepsin C activity was assessed as described in 5.1 with a 1/9 dilution factor in the sodium acetate buffer.

5.3. Intra-nasal administration

The protocols are similar as described in 5.2 with the exception that the compound was administered intra-nasal by instilling 50µL of compound in saline/5% DMSO intranasally to the mice under isoflurane anesthesia.

5.4. Cigarette smoke

The protocol uses 5 cigarettes/day for 3 days, with bronchoalveolar lavage on the 4th day.

Thirty minutes after compound administration, 10 mice are placed into a plexiglass box (Braintree Scientific, size 10" x 4" x 4") with an input for aerosol and another port for exhaust. Cigarette smoke is introduced through the aerosol port via a peristaltic pump (Masterflex L/S, Digital Economy Drive) set at 40 ml/min. Breathing air is also introduced through the same port via an elbow in the tubing at a rate of 0.4 L/min. With these settings, smoke concentration delivered to the box is approximately 10%. 3 cigarettes (research type 4A1, University of Kentucky Tobacco Institute) are given back-to-back with a 1 min period between each one, when the mice breathe fresh air for one minute. Two to 3 hours after the first group of cigarettes, 2 additional cigarettes are given, again allowing 1 min between each cigarette. After the last cigarette, the mice are returned to their normal housing. This procedure is repeated for 3 consecutive days.

On the 4th day, mice are euthanized with 0.1 ml Fatal Plus i.p., and then bronchoalveolar lavaged with 0.7 ml Dulbecco's phosphate-buffered saline with 1x complete (dPBS, in-house Media Prep lab) through a tracheal tube a total of 5 times.. Lungs are then removed en bloc and homogenized. The cathepsin C activity was assessed as described in 5.1 with a 1/9 dilution factor in the sodium acetate buffer.

5.5. Exposure levels

Plasma levels of 17 (level of detection 2 ng/ml) for Figure 2a

Animal	Concentration, timepoint	17
		Calculated Concentration (ng/mL)
1	Vehicle 1.5hr	NQ
2	Vehicle 1.5hr	NQ
3	Vehicle 30 min	NQ
4	Vehicle 3hr	NQ
5	Vehicle 3hr	NQ
6	30mg/kg 1.5hr	
7	30mg/kg 1.5hr	NQ
8	30mg/kg 1.5hr	NQ
9	30mg/kg 1.5hr	NQ
10	30mg/kg 1.5hr	NQ
11	30mg/kg 30min	NQ

12	30mg/kg 30min	NQ
13	30mg/kg 30min	NQ
14	30mg/kg 30min	NQ
15	30mg/kg 30min	NQ
16	30mg/kg 3 hr	NQ
17	30mg/kg 3 hr	NQ
18	30mg/kg 3 hr	NQ
19	30mg/kg 3 hr	NQ
20	30mg/kg 3 hr	NQ