Supporting information

Fragment-Based Discovery of Mexiletine Derivatives as Orally Bioavailable Inhibitors of Urokinase-Type Plasminogen Activator

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Preparative details and analytical data for compounds **6-16**

uPA Assay protocols

X-Ray crystallographic details for uPA co-complexed with (*R*)-**5**, **6**, **9**, **11**, **14** and **15**

General synthetic details :

Reagents and solvents obtained from commercial suppliers were used without further purification. ^1H nuclear magnetic resonance spectra (^1H NMR) were recorded in deuterated solvents [either $\text{MeOH-}d_4$ or $\text{DMSO-}d_6$ as indicated] on a Bruker Avance 400 spectrometer operating at 400 MHz. Chemical shifts are reported in parts per million (δ) downfield from the tetramethylsilane resonance in the indicated solvent (δ TMS = 0.0 ppm). Data are reported as follows: chemical shift, multiplicity (br = broad, s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet), coupling constants (measured in Hertz), integration. Mass spectral data were determined using a Waters Fractionlynx/Micromass ZQ LC/MS platform using the positive electrospray ionization technique (+ES) using a mobile phase of acetonitrile/water with 0.1% formic acid. For compounds containing chlorine atoms the masses reported correspond to those for the higher abundance ^{35}Cl isotope.

($\pm$)-Mexiletine (**5**) was obtained from Sigma (as the hydrochloride salt) and was used without further purification.

Synthesis of acid intermediates : A, B & C

4-[2-(*tert*-Butyloxycarbonylamino)ethoxy]-3,5-dichlorobenzoic acid (A)

A stirred mixture of ethyl 3,5-dichloro-4-hydroxybenzoate (15.0 g, 63.75 mmol) and anhydrous potassium carbonate (10.5 g, 76.5 mmol) in *N,N*-dimethylformamide (100 ml) was heated to 120°C and was treated with *N*-(2-bromoethyl)phthalimide (24.3 g, 95.62 mmol) in small portions over a period of 1 hour. The resulting mixture was stirred at 120°C overnight and then allowed to cool to room temperature. Water was added and the mixture was extracted with dichloromethane. The organic layer was separated, dried over anhydrous magnesium sulphate, filtered and the solvent removed *in vacuo* to afford ethyl 3,5-dichloro-4-[2-(1,3-dioxo-1,3-dihydro-isoindol-2-yl)ethoxy]benzoate (26.0 g, 100%) as a colourless solid

that was used without further purification. ^1H NMR (400 MHz, $\text{MeOH-}d_4$): δ 1.35 (t, $J = 7.5$, 3H), 4.15 (q, $J = 7.5$, 2H), 4.35 (m, 4H), 7.85 (m, 2H), 7.90 (m, 2H), 7.95 (s, 2H).

Hydrazine monohydrate (3.09 ml, 63.75 mmol) was added dropwise to a stirred solution of ethyl 3,5-dichloro-4-[2-(1,3-dioxo-1,3-dihydro-isoindol-2-yl)ethoxy]benzoate (26.0 g, 63.75 mmol) in ethanol (100 ml) at room temperature. The mixture was stirred at 100°C for 3 hours and allowed to cool to room temperature. The resulting precipitate was removed by filtration and the filtrate was evaporated to dryness *in vacuo*. The residue was triturated with dichloromethane to afford ethyl 4-[(2-amino)ethoxy]-3,5-dichlorobenzoate (12.3 g, 70%) as a pale yellow solid that was used without further purification. ^1H NMR (400 MHz, $\text{MeOH-}d_4$): δ 1.25 (t, $J = 7.5$, 3H), 2.95 (t, $J = 7$, 2H), 4.05 (t, $J = 7$, 2H), 4.25 (q, $J = 7.5$, 2H), 7.90 (s, 2H).

Di-*tert*-butyl dicarbonate (9.69 g, 44.4 mmol) was added portionwise to a stirred solution of ethyl 4-[(2-amino)ethoxy]-3,5-dichlorobenzoate (12.3 g, 44.4 mmol) in dichloromethane (100 ml) at room temperature. The mixture was stirred for 2 hours and water was then added. The organic layer was separated, dried over anhydrous magnesium sulphate, filtered, the solvent removed *in vacuo* and the residue subjected to column chromatography on silica. Elution with 30% ethyl acetate in petroleum ether afforded ethyl 4-[2-(*tert*-butyloxycarbonylamino)ethoxy]-3,5-dichlorobenzoate (13.5 g, 81%) as a colourless solid. ^1H NMR (400 MHz, $\text{MeOH-}d_4$): δ 1.25 (t, $J = 7.5$, 3H), 1.40 (s, 9H), 3.40 (t, $J = 7$, 2H), 4.00 (t, $J = 7$, 2H), 4.25 (q, $J = 7.5$, 2H), 7.85 (s, 2H).

Lithium hydroxide (1.7 g, 71.6 mmol) and water (30 ml) were added to ethyl 4-[2-(*tert*-butyloxycarbonylamino)ethoxy]-3,5-dichlorobenzoate (13.5 g, 35.8 mmol) in tetrahydrofuran (100 ml) and the mixture was stirred at room temperature for 3 hours. The organic solvent was removed *in vacuo* and the resulting basic aqueous solution was acidified by the addition of 1 M hydrochloric acid. The precipitate was collected by suction filtration, rinsed with water, sucked dry under reduced pressure and dried in a vacuum oven at 50°C overnight to afford 4-[2-(*tert*-butyloxycarbonylamino)ethoxy]-3,5-dichlorobenzoic acid **A** (11.04 g, 88%) as a colourless solid. ^1H NMR (400 MHz, $\text{MeOH-}d_4$): δ 1.45 (s, 9H), 3.50 (t, $J = 7$, 2H), 4.15 (t, $J = 7$, 2H), 8.00 (s, 2H). MS (+ES): m/e 348 (M-H).

4-[2-(*tert*-Butyloxycarbonylamino)ethoxy]-3,5-dimethylbenzoic acid (B)

4-Hydroxy-3,5-dimethylbenzoic acid (10.94 g, 65.9 mmol) was dissolved in a solution of 2% (w/v) hydrogen chloride gas in methanol (100 ml) and the mixture was stirred and held at reflux for 3 hours. Upon cooling to room temperature the solvent was removed *in vacuo* to

afford methyl 4-hydroxy-3,5-dimethylbenzoate (11.8 g, 99%) as a colourless solid. ^1H NMR (400 MHz, $\text{DMSO}-d_6$): δ 2.19 (s, 6H), 3.78 (s, 3H), 7.58 (s, 2H), 9.13 (s, 1H). MS (+ES): m/e 181 (MH^+).

A stirred solution of methyl 4-hydroxy-3,5-dimethylbenzoate (8.65 g, 48.06 mmol) and caesium carbonate (23.5 g, 72.08 mmol) in *N,N*-dimethylformamide (50 ml) at 50°C was treated with [2-(*tert*-butoxycarbonylamino)ethyl] toluene-4-sulphonate (22.71 g, 72.09 mmol) [prepared as per WO 2002/083690] in small portions over 4 hours. The mixture was stirred at 50°C overnight and the solvent then removed *in vacuo*. The residue was partitioned between 2 M sodium hydroxide and dichloromethane, the organic layer separated and evaporated to dryness *in vacuo* and the residue was subjected to column chromatography on silica. Elution with 5-20% ethyl acetate in petroleum ether afforded methyl 4-[2-(*tert*-butoxycarbonylamino)ethoxy]-3,5-dimethylbenzoate (13.4 g, 86%) as a colourless solid. ^1H NMR (400 MHz, $\text{DMSO}-d_6$): δ 1.40 (s, 9H), 2.26 (s, 6H), 3.30 (m, 2H), 3.78 (m, 2H), 3.82 (s, 3H), 7.09 (t, $J = 6$, 1H), 7.65 (s, 2H). MS (+ES): m/e 324 (MH^+).

A stirred solution of methyl 4-[2-(*tert*-butoxycarbonylamino)ethoxy]-3,5-dimethylbenzoate (9.69 g, 30.0 mmol) in methanol (120 ml) at 50°C was treated with a solution of potassium hydroxide (5.6 g, 100.0 mmol) in water (40 ml) and the mixture was stirred at 50°C for 3 hours. The methanol was removed *in vacuo* and the resulting basic aqueous solution was acidified by the addition of 2 M hydrochloric acid. The resulting precipitate was collected by suction filtration, rinsed with water, sucked dry under reduced pressure and dried overnight in a vacuum oven at 50°C to afford 4-[2-(*tert*-butoxycarbonylamino)ethoxy]-3,5-dimethylbenzoic acid **B** (9.0 g, 97%) as a colourless solid. ^1H NMR (400 MHz, $\text{DMSO}-d_6$): δ 1.38 (s, 9H), 2.26 (s, 6H), 3.30 (m, 2H), 3.79 (m, 2H), 3.82 (s, 3H), 7.08 (t, $J = 6$, 1H), 7.62 (s, 2H), 12.70 (br s, 1H). MS (+ES): m/e 308 ($\text{M}-\text{H}^-$).

(*R*)-4-[2-(*tert*-Butoxycarbonylamino)propoxy]-3,5-dimethylbenzoic acid (C)

A stirred solution of (*R*)-2-(*tert*-butoxycarbonylamino)-1-propanol (7.0 g, 40.0 mmol) in pyridine (30 ml) at 0°C was treated with toluene-4-sulphonyl chloride (7.62 g, 40.0 mol) and the mixture stirred and allowed to warm to room temperature overnight. Pyridine was then removed *in vacuo* at room temperature and the residue partitioned between 2 M hydrochloric acid and dichloromethane. The organic layer was separated and the solvent removed *in vacuo* to afford (*R*)-2-[(*tert*-butoxycarbonyl)amino]propyl toluene-4-sulphonate (12.4 g, 94%) as a pale yellow oil that was used without purification. ^1H NMR (400 MHz, $\text{DMSO}-d_6$): δ 0.97 (d, $J = 7$, 3H), 1.34 (s, 9H), 2.42 (s, 3H), 3.12 (m, 1H), 3.85 (m, 2H), 6.86 (d, $J = 7$, 1H), 7.48 (d, $J = 7$, 2H), 7.78 (d, $J = 7$, 2H).

A stirred solution of methyl 4-hydroxy-3,5-dimethylbenzoate (5.4 g, 30.0 mmol) and caesium carbonate (14.67 g, 45.0 mmol) in *N,N*-dimethylformamide (30 ml) at 50°C was treated with (*R*)-2-[(*tert*-butoxycarbonyl)amino]propyl toluene-4-sulphonate (12.4 g, 37.6 mmol) in small portions over 8 hours. The mixture was stirred at 50°C overnight and the solvent removed *in vacuo*. The residue was partitioned between 2 M sodium hydroxide and dichloromethane, the organic layer separated and evaporated to dryness *in vacuo* and the residue was subjected to column chromatography on silica. Elution with 5-20% ethyl acetate in petroleum ether afforded methyl (*R*)-4-[2-(*tert*-butoxycarbonylamino)propyloxy]-3,5-dimethylbenzoate (4.2 g, 42%) as a colourless solid. ¹H NMR (400 MHz, DMSO-*d*₆): δ 1.20 (d, *J* = 7, 3H), 1.38 (s, 9H), 2.24 (s, 6H), 3.30 (m, 1H), 3.68 (m, 1H), 3.81 (m, 4H), 6.92 (d, *J* = 6, 1H), 7.62 (s, 2H). MS (+ES): *m/e* 338 (MH⁺).

A stirred solution of methyl 4-[2-(*tert*-butoxycarbonylamino)propyloxy]-3,5-dimethylbenzoate (4.2 g, 12.46 mmol) in methanol (60 ml) at 50°C was treated with a solution of potassium hydroxide (2.8 g, 50.0 mmol) in water (20 ml) and the mixture stirred at 50°C for 3 hours. The methanol was then removed *in vacuo* and the resulting basic aqueous solution was acidified to pH 2 or below by the addition of 2 M hydrochloric acid. The resulting precipitate was collected by suction filtration and was rinsed with water, sucked dry under reduced pressure and dried overnight in a vacuum oven at 50°C to afford (*R*)-4-[2-(*tert*-butoxycarbonylamino)propyloxy]-3,5-dimethylbenzoic acid **C** (3.6 g, 90%) as a colourless solid. ¹H NMR (400 MHz, DMSO-*d*₆): δ 1.18 (d, *J* = 7, 3H), 1.38 (s, 9H), 2.24 (s, 6H), 3.68 (m, 1H), 3.79 (m, 1H), 3.82 (m, 1H), 6.93 (d, *J* = 6, 1H), 7.60 (s, 2H), 12.70 (br s, 1H). MS (+ES): *m/e* 324 (MH⁺).

Synthesis of amine intermediates : **D**, **E**, **F** & **G**

3,5-Di-(isopropoxy)aniline hydrochloride (**D**)

A mixture of methyl 3,5-dihydroxybenzoate (3.36 g, 20.0 mmol), anhydrous potassium carbonate (8.28 g, 60.0 mmol) and isopropyl iodide (8 ml) in *N,N*-dimethylformamide (25 ml) was stirred at 80°C for 16 hours. The solvent was then removed *in vacuo* and the residue partitioned between dichloromethane and water. The organic layer was separated and the

solvent removed *in vacuo* to afford a pale yellow liquid. Methanol (60 ml), potassium hydroxide (3.0 g, 53.57 mmol) and water (20 ml) were added and the mixture stirred and held at reflux for 1 hour. The methanol was removed *in vacuo* and the aqueous solution acidified with 2 M hydrochloric acid. The resulting precipitate was collected by suction filtration, rinsed with water, sucked dry under reduced pressure and dried overnight in a vacuum oven at 50°C to afford 3,5-di-(isopropoxy)benzoic acid as a colourless solid (3.8 g, 80%). ¹H NMR (400 MHz, DMSO-*d*₆): δ 1.27 (d, *J* = 7.5, 12H), 4.63 (m, 2H), 6.68 (s, 1H), 6.99 (s, 2H), 12.92 (br s, 1H).

A stirred solution of 3,5-di-(isopropoxy)benzoic acid (1.19 g, 5.0 mmol) in toluene (30 ml) was treated with diphenylphosphoryl azide (1.08 ml, 5.0 mmol) and triethylamine (0.84 ml, 6.0 mmol) and the mixture was stirred at room temperature for 2 hours. The solvent was then removed *in vacuo* at room temperature and the residue partitioned between 1 M hydrochloric acid and dichloromethane. The organic layer was separated and the solvent removed *in vacuo* at room temperature to afford the crude acyl azide as a pale yellow oil. Toluene (20 ml) and *tert*-butanol (1.0 g, 13.51 mmol) were added and the mixture was stirred and held at reflux for 16 hours. Upon cooling to room temperature the solvent was removed *in vacuo* and the residue subjected to column chromatography on silica. Elution with 5% ethyl acetate in petroleum ether afforded *N*-(*tert*-butyloxycarbonyl)-3,5-di-(isopropoxy)aniline as a colourless solid. ¹H NMR (400 MHz, DMSO-*d*₆): δ 1.24 (d, *J* = 7.5, 12H) 1.47 (s, 9H), 4.48 (m, 2H), 6.04 (s, 1H), 6.64 (s, 2H), 9.17 (s, 1H). MS (+ES): *m/e* 310 (MH⁺). This material was dissolved in 4 M hydrogen chloride in 1,4-dioxane (8 ml) and the mixture was stirred at room temperature for 3 hours. Removal of the solvent *in vacuo* afforded the crude product which was triturated with 20% diethyl ether in petroleum ether. The solid was collected by suction filtration and dried overnight in a vacuum oven at 50°C to afford 3,5-di-(isopropoxy)aniline hydrochloride **D** (460 mg, 44%) as a pale tan solid. ¹H NMR (400 MHz, DMSO-*d*₆): δ 1.27 (d, *J* = 7.5, 12H), 4.56 (m, 2H), 6.41 (s, 3H), 9.90 (br s, 3H). MS (+ES): *m/e* 210 (MH⁺).

3-Fluoro-5-(4-morpholinyl)aniline dihydrochloride (E)

A stirred solution of 3-fluoro-5-(4-morpholinyl)benzoic acid (450 mg, 2.0 mmol) [prepared as per WO 2003/087087] in toluene (10 ml) was treated with diphenylphosphoryl azide (0.43 ml, 2.0 mmol) and triethylamine (0.34 ml, 2.4 mmol) and the mixture stirred at room temperature for 3 hours. The solvent was then removed *in vacuo* at room temperature and the residue partitioned between 1 M hydrochloric acid and dichloromethane. The organic layer was separated and the solvent removed *in vacuo* at room temperature to afford the crude acyl azide as a pale yellow oil. Toluene (10 ml) and *tert*-butanol (0.5 g, 6.76 mmol) were added and the mixture was stirred and held at reflux for 16 hours. The solvent was then removed *in*

vacuo and the residue was subjected to column chromatography on silica. Elution with 25-50% ethyl acetate in petroleum ether afforded *N*-(*tert*-butoxycarbonyl)-3-fluoro-5-(4-morpholinyl)aniline as a colourless solid. ¹H NMR (400 MHz, DMSO-*d*₆): δ 1.46 (s, 9H), 3.04 (m, 4H), 3.71 (m, 4H), 6.39 (d, *J* = 12.5, 1H), 6.77 (d, *J* = 11, 1H), 6.88 (br s, 1H), 9.37 (br s, 1H). This material was dissolved in 4 M hydrogen chloride in 1,4-dioxane (8 ml) and methanol (5 ml) and the mixture was stirred at room temperature for 2 hours. Removal of the solvent *in vacuo* afforded 3-fluoro-5-(4-morpholinyl)aniline dihydrochloride **E** (230 mg, 43%) as a pale tan solid. ¹H NMR (400 MHz, DMSO-*d*₆): δ 3.13 (m, 4H), 3.72 (m, 4H), 6.50 (d, *J* = 9, 1H), 6.64 (s, 1H), 6.72 (d, *J* = 12.5, 1H).

3-Chloro-5-(1-piperidiny)aniline (F)

Piperidine (1 ml, 10.12 mmol) was added to a mixture of 3,5-dichloro-nitrobenzene (1.27 g, 6.61 mmol) and anhydrous potassium carbonate (1.1 g, 7.97 mmol) in dimethylsulphoxide (3ml) and the mixture was stirred and heated to 120°C for 3 hours. The mixture was cooled to room temperature, diluted with ethyl acetate and extracted with 5% aqueous citric acid solution. The organic layer was separated, dried over anhydrous magnesium sulphate and filtered. The solvent was then removed *in vacuo* and the residue was subjected to column chromatography on silica. Elution with 5-10% ethyl acetate in petroleum ether afforded 3-chloro-5-(1-piperidiny)nitrobenzene (368 mg, 23%) as an orange oil. ¹H NMR (400 MHz, DMSO-*d*₆): δ 1.60 (m, 6H), 3.33 (m, 4H), 7.58 (t, *J* = 2, 1H), 7.47 (t, *J* = 2, 1H), 7.58 (t, *J* = 2, 1H). MS (+ES): *m/e* 241 (MH⁺).

Tin (II) chloride dihydrate (1.2 g, 5.31 mmol) was added to a solution of 3-chloro-5-(1-piperidiny)nitrobenzene (280 mg, 1.16 mmol) in ethanol (20 ml) and the mixture was stirred and held at reflux under a nitrogen atmosphere for 2 hours. The mixture was then allowed to cool to room temperature and the solvent was removed *in vacuo*. The mixture was dissolved in ethyl acetate and was washed with 2M sodium hydroxide, water and brine. The organic layer was separated, dried over anhydrous magnesium sulphate, filtered and the solvent removed *in vacuo* to afford 3-chloro-5-(1-piperidiny)aniline **F** (235 mg, 96%) as an orange oil. ¹H NMR (400 MHz, DMSO-*d*₆): δ 1.54 (m, 6H), 3.05 (m, 4H), 5.15 (br s, 2H), 6.02 (t, *J* = 2, 1H), 6.07 (m, 2H). MS (+ES): *m/e* 211 (MH⁺).

3-Chloro-2-ethoxy-5-(1-piperidiny)aniline (G)

A mixture of 2-chloro-4-fluoro-6-nitrophenol (7.30 g, 38.12 mmol) [prepared as per WO 2000/013508], anhydrous potassium carbonate (7.89 g, 57.18 mmol) and ethyl iodide (10 ml) in *N,N*-dimethylformamide (60 ml) was stirred at 60°C for 60 hours. Upon cooling to room temperature the solvent was removed *in vacuo* and the residue partitioned between

dichloromethane and water. The organic layer was separated and the solvent removed *in vacuo* to afford 1-chloro-2-ethoxy-5-fluoro-3-nitrobenzene (8.0 g, 96%) as a dark yellow liquid. ^1H NMR (400 MHz, DMSO- d_6): δ 1.33 (t, $J = 7.5$, 3H), 4.13 (q, $J = 7.5$, 2H), 7.98 (d, $J = 9$, 2H).

A stirred solution of 1-chloro-2-ethoxy-5-fluoro-3-nitrobenzene (5.1 g, 23.29 mmol), anhydrous potassium carbonate (7.87 g, 58.22 mmol) and piperidine (4.96 g, 58.22 mmol) in dimethylsulphoxide (15 ml) was stirred at 120°C for 3 hours. Upon cooling to room temperature the mixture was diluted with diethyl ether and the mixture extracted with water and then twice with 5% aqueous citric acid solution. The organic layer was separated and the solvent removed *in vacuo* to afford 1-chloro-2-ethoxy-3-nitro-5-(1-piperidinyl)benzene (3.58 g, 54%) as an orange oil that was used without further purification. ^1H NMR (400 MHz, DMSO- d_6): δ 1.30 (t, $J = 7$, 3H), 1.56 (m, 6H), 3.41 (m, 4H), 4.04 (q, $J = 7$, 2H), 7.32 (s, 2H). MS (+ES): m/e 285 (MH^+).

A stirred solution of 1-chloro-2-ethoxy-3-nitro-5-(1-piperidinyl)benzene (3.58 g, 12.58 mmol) and tin (II) chloride dihydrate (23.45 g, 104.0 mmol) in ethanol (100 ml) was stirred and held at reflux for 16 hours. Upon cooling to room temperature the solvent was removed *in vacuo* and the residue partitioned between dichloromethane and 2 M sodium hydroxide. The organic layer was separated, the solvent removed *in vacuo* and the residue was subjected to column chromatography on silica. Elution with 40% ethyl acetate in petroleum ether afforded 3-chloro-2-ethoxy-5-(1-piperidinyl)aniline **G** (1.6 g, 50%) as an orange oil. ^1H NMR (400 MHz, DMSO- d_6): δ 1.31 (t, $J = 7$, 3H), 1.50 (m, 2H), 1.55 (m, 4H), 3.00 (m, 4H), 3.82 (q, $J = 7$, 2H), 4.92 (br s, 2H), 6.12 (d, $J = 3$, 1H), 6.22 (d, $J = 3$, 1H). MS (+ES): m/e 255 (MH^+).

Synthetic details : Compounds 6-16

4-[(2-amino)ethoxy]-3,5-dichlorobenzoic acid (6). Lithium hydroxide monohydrate (16 mg, 0.38 mmol) was added to a solution of ethyl 4-[(2-amino)ethoxy]-3,5-dichlorobenzoate (70 mg, 0.25 mmol) in a mixture of tetrahydrofuran (1 ml), methanol (1 ml) and water (2.5 ml) and the mixture was stirred at room temperature for 3 hours. The mixture was then neutralized by the addition of 2 M hydrochloric acid and was evaporated to dryness *in vacuo*. The residue was purified by preparative HPLC-MS to afford 4-[(2-amino)ethoxy]-3,5-

dichlorobenzoic acid **6** (16 mg, 26%) as an off-white solid. ^1H (400 MHz, $\text{MeOH-}d_4$): δ 4.45 (t, $J = 7.5$, 2H), 4.30 (t, $J = 7.5$, 2H, t), 8.00 (s, 2H). MS (+ES): m/e 250 (M^+).

A mixture of the acid intermediate (**A**, **B** or **C**) (1.0 equivalent), the appropriately substituted aniline (**D**, **E**, **F**, **G** or a commercially available aniline) (1.0 equivalent), *N*-ethyl-*N'*-(3-dimethylaminopropyl)carbodiimide hydrochloride (1.1 - 2.0 equivalents), 1-hydroxy-7-azabenzotriazole or 1-hydroxybenzotriazole (1.0 equivalent) in DMF (5 mL per mmol of acid substrate) was stirred at room temperature or at 50-65°C for 16 hours. In cases where aniline hydrochloride salts were used, triethylamine (1.1 equivalent per hydrochloride equivalent) was also added. The mixture was then diluted with 2 M hydrochloric acid and either ethyl acetate or dichloromethane and the organic layer was separated. The organic layer was washed with dilute sodium hydroxide and water and was then dried over anhydrous magnesium sulphate, filtered and the solvent removed *in vacuo*. The residue was subjected to column chromatography on silica [eluting with mixtures of ethyl acetate in petroleum ether] or was purified by preparative HPLC/MS to yield the pure *N*-(butyloxycarbonyl) derivatives of **7-16** (60-90% yield).

The *N*-(butyloxycarbonyl) derivative was dissolved in either a saturated solution of hydrogen chloride in diethyl ether (5-10 ml per mmol of substrate) or in 4 M hydrogen chloride in 1,4-dioxane (5-10 ml per mmol of substrate) and the resulting solution was stirred at room temperature for 4-16 hours until the reaction was shown to be complete as evidenced by HPLC/MS analysis. The solvent was then removed *in vacuo* to yield amines **7-16** (80-100%

yield). If necessary the final products were further purified by HPLC/MS. Compounds were isolated as mono- or di-hydrochloride salts, or if purified by HPLC/MS as a formate salt as indicated below.

***N*-Phenyl 4-[(2-amino)ethoxy]-3,5-dichlorobenzamide formate (7).** From intermediate **A** and aniline. Colourless solid. ^1H NMR (400 MHz, $\text{MeOH-}d_4$): δ 3.45 (t, $J = 7$, 2H), 4.35 (t, $J = 7$, 2H), 7.15 (t, $J = 8$, 1H), 7.35 (t, $J = 8$, 2H), 7.70 (d, $J = 7$, 2H), 8.05 (s, 2H), 8.35 (s, 1H). MS (+ES): m/e 325 (MH^+).

***N*-(2-Methoxyphenyl) 4-[(2-amino)ethoxy]-3,5-dichlorobenzamide formate (8).** From intermediate **A** and 2-methoxyaniline. Colourless solid. ^1H NMR (400 MHz, $\text{MeOH-}d_4$): δ 3.45 (t, $J = 7$, 2H), 3.90 (s, 3H), 4.35 (t, $J = 7$, 2H), 7.00 (t, $J = 7.5$, 1H), 7.12 (t, $J = 7.5$, 1H), 7.21 (t, $J = 7.5$, 1H), 7.85 (d, $J = 7.5$, 1H), 8.00 (s, 2H), 8.45 (s, 1H). MS (+ES): m/e 355 (MH^+).

***N*-[3-(isopropoxy)phenyl] 4-[(2-amino)ethoxy]-3,5-dichlorobenzamide formate (9).** From intermediate **A** and 3-(isopropoxy)aniline. Tan foam. ^1H NMR (400 MHz, $\text{MeOH-}d_4$): δ 1.35 (d, $J = 7$, 6H), 3.45 (t, $J = 7$, 2H), 4.35 (t, $J = 7$, 2H), 4.60 (m, 1H), 6.75 (dd, $J = 7$, 2.5, 1H), 7.25 (m, 2H), 7.40 (s, 1H), 8.05 (s, 2H), 8.42 (s, 1H). MS (+ES): m/e 383 (MH^+).

***N*-[3-(isopropoxy)phenyl] 4-[(2-amino)ethoxy]-3,5-dimethylbenzamide hydrochloride (10).** From intermediate **B** and 3-(isopropoxy)aniline. Colourless solid. ^1H NMR (400 MHz, $\text{DMSO-}d_6$): δ 1.33 (d, $J = 7$, 6H), 2.35 (s, 6H), 3.31 (m, 2H), 4.00 (m, 2H), 4.55 (m, 1H), 6.65 (d, $J = 7.5$, 1H), 7.22 (t, $J = 7.5$, 1H), 7.37 (d, $J = 7.5$, 1H), 7.45 (s, 1H), 7.65 (s, 2H), 8.23 (br s, 3H), 10.05 (s, 1H). MS (+ES): m/e 343 (MH^+).

***N*-[(2,5-di-ethoxy)phenyl] 4-[(2-amino)ethoxy]-3,5-dimethylbenzamide hydrochloride (11).** From intermediate **B** and 2,5-diethoxyaniline. Off-white solid. ^1H NMR (400 MHz, $\text{MeOH-}d_4$): δ 1.42 (m, 6H), 2.41 (s, 6H), 3.43 (m, 2H), 4.02 (m, 2H), 4.11 (m, 4H), 6.70 (dd, $J = 9$, 3, 1H), 6.97 (d, $J = 9$, 1H), 7.64 (s, 2H), 7.75 (d, $J = 3$, 1H). MS (+ES): m/e 373 (MH^+).

***N*-[(3,5-di-isopropoxy)phenyl] 4-[(2-amino)ethoxy]-3,5-dimethylbenzamide hydrochloride (12).** From intermediates **B** and **D**. Colourless solid. ^1H NMR (400 MHz, $\text{DMSO-}d_6$): δ 1.27 (d, $J = 7.5$, 12H), 2.33 (s, 6H), 3.25 (m, 2H), 3.99 (t, $J = 7$, 2H), 4.52 (m, 2H), 6.18 (s, 1H), 7.02 (s, 2H), 7.65 (s, 2H), 8.30 (br s, 3H), 10.02 (s, 1H). MS (+ES): m/e 401 (MH^+).

***N*-[(3-fluoro-5-(4-morpholinyl)phenyl] 4-[(2-amino)ethoxy]-3,5-dimethylbenzamide dihydrochloride (13).** From intermediates **B** and **E**. Colourless solid. ^1H NMR (400 MHz,

DMSO-*d*₆): δ 2.32 (s, 6H), 3.13 (m, 4H), 3.25 (m, 2H), 3.73 (m, 4H), 4.00 (m, 2H), 6.53 (d, J = 12, 1H), 7.19 (s, 1H), 7.23 (d, J = 11, 1H), 7.68 (s, 2H), 8.31 (br s, 3H), 10.16 (s, 1H). MS (+ES): m/e 388 (MH⁺).

***N*-[(3-chloro-5-(1-piperidinyl)phenyl] 4-[(2-amino)ethoxy]-3,5-dimethylbenzamide dihydrochloride (14).** From intermediates **B** and **F**. Pale yellow solid. ¹H NMR (400 MHz, MeOH-*d*₄): δ 1.85 (m, 2H), 2.10 (t, J = 7.5, 4H), 2.40 (s, 6H), 3.45 (t, J = 7, 2H), 3.65 (t, J = 7.5, 2H), 4.10 (t, J = 7, 2H), 7.55 (s, 1H), 7.75 (s, 2H), 7.85 (s, 1H), 8.25 (s, 1H). MS (+ES): m/e 402 (MH⁺).

***N*-[(3-chloro-2-ethoxy 5-(1-piperidinyl)phenyl] 4-[(2-amino)ethoxy]-3,5-dimethylbenzamide dihydrochloride (15).** From intermediates **B** and **G**. Colourless solid. ¹H NMR (400 MHz, MeOH-*d*₄): δ 1.47 (t, J = 7, 3H), 1.82 (m, 2H), 2.06 (m, 4H), 2.42 (s, 6H), 3.44 (t, J = 7, 2H), 3.68 (t, J = 7.5, 4H), 4.12 (t, J = 7, 2H), 4.20 (q, J = 7, 2H), 7.29 (d, J = 3, 1H), 7.72 (s, 2H), 8.43 (d, J = 3, 1H). MS (+ES): m/e 446 (MH⁺).

(*R*)-*N*-[(3-chloro-2-ethoxy 5-(1-piperidinyl)phenyl] 4-[(2-amino)propyloxy]-3,5-dimethylbenzamide dihydrochloride (16). From intermediates **C** and **G**. Colourless solid. ¹H NMR (400 MHz, DMSO-*d*₆): δ 1.27 (t, J = 7.5, 3H), 1.34 (d, J = 7, 3H), 1.58 (m, 2H), 1.78 (m, 4H), 2.34 (s, 6H), 3.30 (m, 4H), 3.62 (m, 1H), 3.90 (m, 2H), 4.00 (q, J = 7, 2H), 7.70 (s, 2H), 8.33 (br s, 2H), 9.72 (br s, 1H). MS (+ES): m/e 460 (MH⁺).

HPLC Purity determination : Compounds 6-16

Analytical reversed phase high performance liquid chromatography – mass spectrometry (LC-MS) was performed on a Waters HPLC Separation Module 2795 system equipped with an autosampler, a Micromass Platform LC mass spectrometer and a Waters 2996 photodiode array detector. Purity of the final compounds was determined by LC-MS using two different chromatographic methods.

First method : (Basic method) - Column: Phenomenex Gemini (5 μ m, 2.0 mm x 50 mm); Mobile phase A: 10 mM ammonium bicarbonate in water adjusted to pH 9.2 with ammonium hydroxide; Mobile phase B: acetonitrile; Flow rate: 0.8 mL/min; Elution profile: gradient elution from 5% to 95% B over 3.5 minutes followed by isocratic elution at 95% B for 1 minute.

Second method : (Polar method) - Column: Phenomenex Synergi MAX-RP 80A (4 μ m, 2.0 mm x 50 mm); Mobile phase A: 0.1% formic acid in water; Mobile phase B: 0.1% formic acid in acetonitrile; Flow rate: 0.8 mL/min; Elution profile: gradient elution from 0% to 50% B over 3 minutes followed by isocratic elution at 95% B for 1.5 minutes.

Purity of key compounds as determined by LC-MS (two methods) :

Compound	LC-MS : Basic method		LC-MS : Polar method	
	Retention time (min)	Area under peak (%)	Retention time (min)	Area under peak (%)
6	0.38 ^a	100.0	1.90	98.4
7	2.74	100.0	2.68	95.4
8	2.80	100.0	2.71	93.8
9	3.07	100.0	2.84	98.1
10	2.88	100.0	2.74	96.3
11	3.02	100.0	3.01	100.0
12	3.19	95.6	3.17	96.7
13	2.66	100.0	2.55	100.0
14	3.33	98.0	2.95	95.9
15	3.60	100.0	2.87	95.4
16	3.72	98.8	2.96	96.3

a. Compound **6** runs at the solvent front under this HPLC method.

uPA Assay protocols :

Method 1 : Activity of uPA was measured using the peptide Z-Gly-Gly-Arg-AMC (peptide substrate 1) from Bachem. Compounds were incubated with uPA and 25 μ M peptide substrate 1 in 50 mM Tris, pH 7.5, 0.1 % PEG, in 2% DMSO in 96-well black, flat bottomed Cliniplates in a final assay volume of 50 μ l. The reaction rate was monitored at room temperature on a Gemini XS (Molecular Devices) plate reader with excitation and emission wavelengths of 365 nm and 460 nm respectively. Initial reaction rates were measured and IC₅₀'s were calculated from replicate curves using GraphPad Prism software.

Method 2 : Compounds that fluoresced under the conditions described above were assayed using the peptide Z-Val-Gly-Arg-pNA (peptide substrate 2) from Calbiochem. Compounds were incubated with uPA and 70 μ M peptide substrate 2 in 50 mM Tris, pH 7.5, 2% DMSO in 96-well clear, flat bottomed ½ area Costar plates in a final assay volume of 50 μ l. The reaction rate was monitored at room temperature on a SpectraMax plate reader (Molecular Devices) with absorbance at 405 nm. Initial reaction rates were measured and used to calculate IC₅₀'s as described above.

X-Ray crystallographic details for uPA co-complexed with (R)-5, 6, 9, 11, 14 and 15 :

	(R)-5	6	9	11	14	15
PDB Accession code	0000	0000	0000	0000	0000	0000
Crystal data						
Space group (#)	$P2_12_12_1$ (19)					
Z	4					
α, β, γ /°	90, 90, 90					
a /Å	52.470	52.404	52.488	52.176	52.338	52.336
b /Å	54.277	54.184	54.382	53.949	54.001	54.118
c /Å	80.923	81.483	81.029	81.756	81.938	80.526
Data collection						
λ /Å	1.5418					
Resolution range /Å	37.7-1.90	27.7-1.80	27.6-1.72	27.6-2.00	34.2-1.72	27.5-2.05
Overall R_{merge} /%	5.7	8.7	5.6	13.0	5.9	10.4
HRS ^a R_{merge} /%	31.4	40.0	30.4	42.1	29.1	40.7
HRS ^a $I/\sigma I$	2.3	1.5	2.1	2.1	2.0	2.0
Completeness /%	95.7	88.5	79.6	97.1	82.3	97.5
Refinement statistics						
Unique reflections	18010	19595	20118	15709	21002	14568
R_{work} /%	17.0	18.9	16.2	18.0	18.0	17.8
R_{free} /%	23.5	25.0	23.8	26.4	23.8	26.0
Number of atoms						
protein	1955	1957	1963	1961	1962	1952
ligand	13	15	25	27	28	31
ions ^b	9	4	9	4	4	4
waters	349	271	387	285	224	204
R.m.s. deviations						
bond lengths /Å	0.008	0.008	0.010	0.007	0.009	0.010
bond angles /°	1.0	1.1	1.0	1.1	1.2	1.0

a. Highest resolution shell

b. Acetate and / or sulphate

X-Ray crystallographic methods :

Crystallization : Protein samples used for crystallization were from uPA preparations [containing ~ 6 mg ml⁻¹ of uPA in 10 mM citrate buffer pH 4.0] that had either been stored at 4°C for less than 2 weeks or stored at -80°C. Protease inhibitor [ethyl 4-(6-

aminohexanoyloxy)benzoate hydrochloride] was added to the protein sample to a final concentration of 3 mM. Reservoirs of a Greiner low profile 96-well sitting drop plate were loaded with solutions composed of 22-24% PEG 4000, 0.17 M $(\text{NH}_4)_2\text{SO}_4$, 15% glycerol and 0.1 M NaOAc pH 4.0. Sitting drops containing 0.5 μl of protein and 0.5 μl of reservoir solution were dispensed using a PixSys robot from Genomic Solutions. The plate was incubated at 20°C. Crystals appeared within 24 hours and were harvested within 1 week.

Soaking and freezing of uPA crystals : Soaking solutions were composed of 28% PEG 4000, 5% glycerol, 0.29 M BisTris pH 6.6, 1 mM $(\text{NH}_4)_2\text{SO}_4$, 1 mM NH_4OAc and 1-40 mM of the test compound. The pH of the soaking solution was adjusted using NaOH or HCl if the pH was <6.0 or >7.0. Sitting drops containing at least one uPA crystal of 0.1 mm size were selected for soaking. uPA Crystals were washed with 4 μl of the soaking solution and were left in 6 μl of fresh soaking solution at 20°C for between 3 hours and 3 days. Crystals were removed from the crystallization plate and immediately transferred into liquid nitrogen.

Data collection and refinement : Data was collected on a Rigaku RU200/Jupiter CCD system and crystals manipulated automatically with an Actor robot. The structural models were refined and water and ligand molecules added using AutoSolve software. Model rebuilding was performed with AstexViewer. Autobuster controlled by AutoSolve was used for final rounds of structure refinement.