Electronic Supplementary Material (ESI) for Organic & Biomolecular Chemistry

Supporting Information

Highly Specific Multi-branched Fluorescent Reporters for Analysis of Human Neutrophil Elastase

Nicolaos Avlonitis,[1,2] Manuelle Debunne,[2] Tashfeen Aslam,[1] Neil McDonald,[2] Chris Haslett,[2] Kevin Dhaliwal[2] & Mark Bradley[1*]

[1]EastCHEM, School of Chemistry, University of Edinburgh, West Mains Road, EH9 3JJ, Edinburgh (UK), [2]MRC Centre for Inflammation Research, University of Edinburgh, 47 Little France Crescent, EH16 4TJ, Edinburgh (UK)

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CHEMISTRY

General information

Commercially available reagents were used without further purification. NMR spectra were recorded using Bruker AC spectrometers operating at 250, 360 and 500MHz for ^{1}H. Chemical shifts are reported on the δ scale in ppm and are referenced to residual non-deuterated solvent resonances. Normal phase purifications by column chromatography were carried out on silica gel 60 (230-400 mesh).

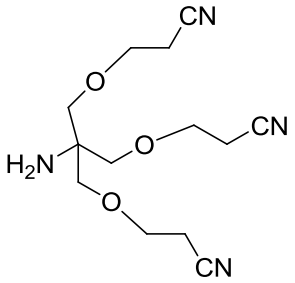
Analytical reverse-phase high-performance liquid chromatography (RP–HPLC) was performed on an HP1100 system equipped with a Discovery C18 reverse-phase column (5 cm x 4.6 mm, 5 µm) with a flow rate of 1 mL/min and eluting with H_2O/MeOH/HCOOH (95/5/0.05) to H_2O/MeOH/HCOOH (5/95/0.05), over 6 min, holding at 95% MeOH for 4 min, with detection at 254 and 495nm and by evaporative light scattering.

Semi-preparative RP–HPLC was performed on an HP1100 system equipped with a Phenomenex Prodigy C18 reverse-phase column (250 x 10 mm, 5 µm) with a flow rate 2.5 mL/min and eluting with 0.1% HCOOH in H_2O (A) and 0.1% HCOOH in CH_3CN (B), with a gradient of 5 to 95% B over 18 min and an initial isocratic period of 5 min.

Electrospray ionization mass spectrometry (ESI–MS) analyses were carried out on an Agilent Technologies LC/MSD Series 1100 quadrupole mass spectrometer (QMS) in an ESI mode. MALDI spectra were acquired on a Voyager-DE™ STR MALDI-TOF MS (Applied Biosystems) with a matrix solution of sinapinic acid (10 mg/mL) in 50% MeCN in water with 0.1% TFA.

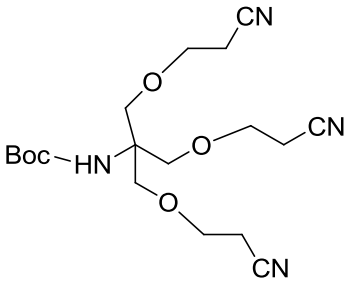
S1. Synthesis of monomer (6)

3-[2-Amino-3-(2-cyano-ethoxy)-2-(2-cyano-ethoxymethyl)-propoxy]propionitrile (1)



To a solution of tris(hydroxymethyl)aminomethane (6.0g, 49mmol) in THF (100mL), were added sequentially 40% KOH aqueous solution (2mL) and acrylonitrile (12.9mL, 200mmol) and the resulting solution was stirred overnight. The solvent was removed *in vacuo* and water (100mL) was added to the residue. The aqueous layer was extracted with dichloromethane (3x100mL), and the organic layer was dried with Na_2SO_4. The organic solvent was evaporated *in vacuo* and the product (10.7g of an oil, 73%) was used in the next step without further purification; **^{1}H-NMR** (500MHz, $CDCl_3$) δ: 5.31 (s, 2H, N*H*$_2$), 3.69 (t, *J* = 6 Hz, 6H, OC*H*$_2$), 3.44 (s, 6H, C*H*$_2$O), 2.62 (t, *J* = 6 Hz, 6H, C*H*$_2$CN); **MS** (ES) *m/z*: 281 [$(M+1)^+$, 100], 303 [$(M+Na)^+$, 20]. Data in good agreement with the literature[1].

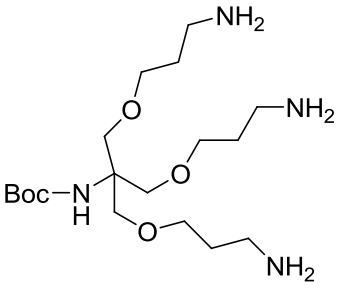
[2-(2-cyano-ethoxy)-1,1-bis-(2-cyano-ethoxymethyl)-ethyl]-carbamic acid tert-butyl ester (2)



To a stirred solution of amine (**1**) (10.7g, 38mmol) in THF (100mL) was added a solution of di-*tert*-butyl dicarbonate (12.4g, 57mmol) in THF (30mL) at 0 °C followed by the addition of DIEA (10.0mL, 57mmol). The reaction was allowed to warm to room temperature and was stirred overnight. The THF was evaporated *in vacuo* and the residue was dissolved in ethyl acetate (250mL). The organic layer was washed with 1N $KHSO_4$ (100mL), saturated $NaHCO_3$

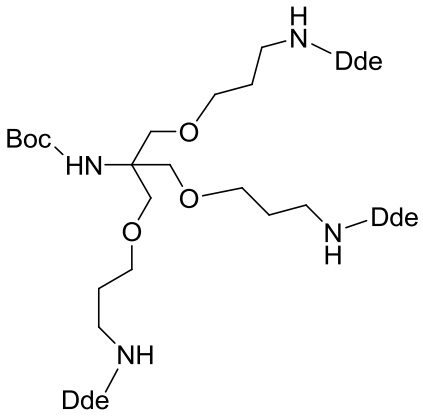
(100mL) and brine (100mL), dried over Na_2SO_4 and the solvent was evaporated to give the compound (2) as oil (14.4g, 100%); **^{1}H-NMR** (500MHz, $CDCl_3$) *δ*: 4.90 (s, 1H, N*H)*, 3.78 (t, *J* = 6 Hz, 6H, OCH_2*),* 3.70 (s, 6H, CH_2O*),* 2.62 (t, *J* = 6 Hz, 6H, CH_2CN*)*, 1.44 (s, 9H, CH_3); **MS** (ES) *m/z*: 403 [$(M+Na)^+$, 30], 281 [$(M\text{-Boc})^+$, 100]. Data in good agreement with the literature[1].

[2-(3-Amino-propoxy)-1,1-bis-(3-amino-propoxymethyl)-ethyl]-carbamic acid tert-butyl ester (3)



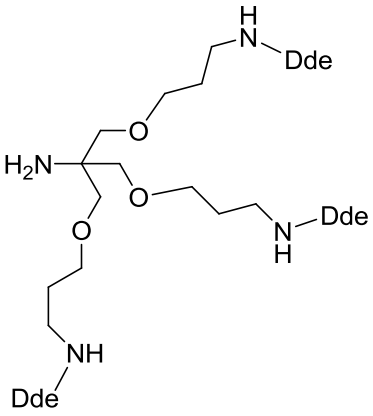
To a stirred solution of tris-nitrile (**2**) (4.5g, 12mmol) in dry THF (50mL) was added dropwise BH_3·THF complex (1M solution in THF, 72mmol, 72mL) and the resulting mixture was stirred at 55 °C for 5h. Following cooling, 2M HCl was added to give an apparent pH between 1-2. The mixture was neutralized with NaOH (aq 1M), and the solvent was removed *in vacuo*. The crude product was used without purification for the next step.

[2-{3-[1-(4, 4-Dimethyl-2,6-dioxocyclohexylidene)ethylamino]propoxy}-1,1-bis-{3-[1-(4,4-dimethyl-2,6 -dioxocyclohexylidene)ethylamino]propoxymethyl}-ethyl]-carbamic acid tert-butyl ester (4)



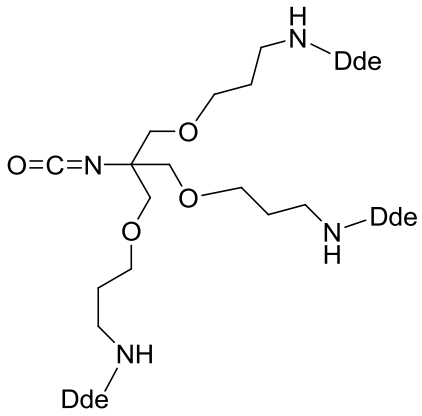
The crude product (3) (4.5g, 11.4mmol) was dissolved in methanol (75mL) and DIPEA (2.4mL, 13.68mmol) was added. A solution of 2-acetyl-dimedone[3] (DdeOH, 7.3g, 40.2mmol) in dichloromethane (40mL) was added and the resulting mixture was stirred overnight. The solvents were removed *in vacuo* and the residue was purified using column chromatography (eluting with dichloromethane/methanol 9/1) to afford the product as a colorless oil (2.4g, 23%); **^{1}H-NMR** (250Hz, $CDCl_3$) δ: 3.78 (s, 6H, CH_2O), 3.65-3.60 (m, 12H, CH_2), 2.68 (s, 9H, CH_3), 2.48 (s, 12H, CH_2), 2.12-2.01 (m, 6H, CH_2), 1.52 (s, 9H, CH_3), 1.14 (s, 18H, CH_3); **MS** (ES) *m/z*: 885 [M^+, 100]. Data were in good agreement with the literature[1].

[2-{3-[1-(4, 4-Dimethyl-2,6-dioxocyclohexylidene)ethylamino]propoxy}-1,1-bis-{3-[1-(4,4-dimethyl-2,6-dioxocyclohexylidene)ethylamino]propoxymethyl}-ethyl]amine (5)



The protected amine (4) (2.3g, 2.7mmol) was dissolved in 20% TFA in dichloromethane (40mL) and the resulting mixture was stirred for 2h. The solvent was removed *in vacuo* and the residue was dissolved in dichloromethane (150mL) and washed with saturated aqueous $NaHCO_3$ solution (75mL) and water (75mL). The organic layer was dried with Na_2SO_4 and the solvents removed *in vacuo*. The crude product (2.1g) was used directly in the next step without purification; **^{1}H-NMR** (360MHz, $CDCl_3$) δ: 13.15 (bs, 2H, NH_2), 3.63 (s, 6H, CH_2O), 3.58-3.47 (m, 12H, CH_2), 2.51 (s, 9H, CH_3), 2.32 (s, 12H, CH_2), 1.93-1.85 (m, 6H, CH_2), 0.98 (s, 18H, CH_3); **MS** (ES) *m/z*: 785 [M^+, 100], 786 [$(M+1)^+$, 45], 787 [$(M+2)^+$, 10]; **HPLC** t_R = 3.75 min. Data were in good agreement with the literature[1].

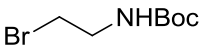
[2-{3-[1-(4, 4-Dimethyl-2,6-dioxocyclohexylidene)ethylamino]propoxy}-1,1-bis-{3-[1-(4,4-dimethyl-2,6 - dioxocyclohexylidene)ethylamino]propoxymethyl}-ethyl]isocyanate (6)



To a mixture of amine **5** (2.1g, 2.7mmol) and DMAP (0.36g, 2.97mmol) in dry DCM (20mL) was added, dropwise, a solution of Boc_2O (0.82g, 3.4mmol) in dry DCM (10mL) and the reaction mixture was stirred for 1h.[2] The solvent was removed *in vacuo* to give **6** (2.0g, 91%). The isocyanate **6** was used immediately. **^{1}H-NMR** (360MHz, $CDCl_3$) *δ*: 3.63-3.41 (m, 18H, C*H*$_2$), 2.49 (s, 9H, C*H*$_3$), 2.28 (s, 12H, C*H*$_2$), 1.89-1.86 (m, 6H, C*H*$_2$), 0.95 (s, 18H, C*H*$_3$); **MS** (ES) *m/z*: 811 [M^+, 100]; **IR** (neat) *v* (cm^{-1}): 2953, 2867, 2244, 1637, 1569, 1461, 1332, 1107, 806, 720. Data were in good agreement with the literature[1].

S2. Synthesis of monomer (12)

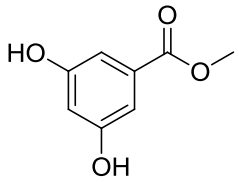
2-N-(-tert-Butoxycarbonylamino)ethyl bromide



A suspension of (2-bromoethyl)amine (5.0g, 25 mmol) and di-*tert*-butyl dicarbonate (5.4g, 25 mmol) in dichloromethane (12 mL) was cooled to 0 °C, and triethylamine (4mL, 3.9 mmol) was added dropwise. After stirring for 24 hours, dichloromethane (150mL) was added and the solution was washed with 1 M $KHSO_4$ (100mL), water (100mL) and brine (100mL), the mixture was dried (Na_2SO_4) and concentrated *in vacuo*. Product was isolated as clear yellow oil (5.22g, 90%); **^{1}H-NMR** (500 MHz, $CDCl_3$) δ: 4.96 (1H, bs, NH), 3.61-3.43 (4H, m, $BrCH_2$, CH_2NH), 1.48 (9H, s, $C(CH_3)_3$); **^{13}C-NMR** (125 MHz,

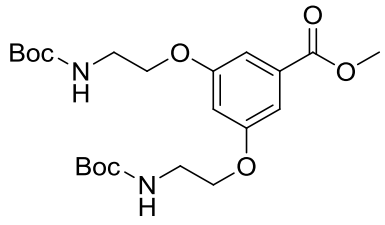
$CDCl_3$) δ: 155.4 (C=O), 79.6 (C), 42.2 (CH_2), 32.4 (CH_2), 28.1 (CH_3); Data were in good agreement with the literature[4].

Methyl 3,5-dihydroxybenzoate (8)



To a solution of 3,5-dihydroxybenzoic acid (5.0 g, 32 mmol) in methanol (170 mL) was added a catalytic amount of sulphuric acid (0.3 mL). After stirring at reflux overnight, the mixture was cooled and neutralized with 4M NaOH (aq. solution). After concentration, the residue was dissolved in ethyl acetate (100 mL) and washed with water (100mL) and brine (100mL). The organic layer was dried (Na_2SO_4) and concentrated *in vacuo*. Compound **2** was isolated as a white solid (5.13g, 95%); **m.p.** 164-165°C (*ethyl acetate*); **^{1}H-NMR** (500 MHz, d6-DMSO) δ: 9.65 (2H, s), 6.81 (2H, d, *J* 2.3 Hz, CH_{ar}), 6.43 (1H, d, *J* 2.3 Hz, CH_{ar}), 3.78 (3H, s, CH_3); **^{13}C-NMR** (125 MHz, d6-DMSO) δ: 166.2 (C=O), 158.4 (C x2), 131.2 (C), 107.1 (CH), 107.0 (CH x2), 51.9 (CH_3); **MS** $(ES)^-$ *m/z*: 167 $[M-H]^-$; **HPLC** t_R = 3.11; **IR** (neat) *v* (cm^{-1}): 3229, 1688, 1600, 1486, 1305, 1161, 1102, 995, 765. Data were in good agreement with the literature.[4]

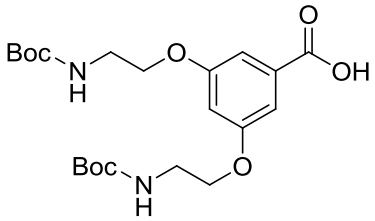
Methyl 3,5-[di-N-(-tert-butoxycarbonyl)ethoxy]-benzoate (9)



A mixture of 2-(Boc-amino)ethyl bromide (36g, 170 mmol), compound **8** (11.4g, 68 mmol), potassium carbonate (37.3g, 270 mmol) in anhydrous dimethylformamide (110mL) was stirred at 50°C for 16 hrs. The mixture was filtered through Celite® and the filtrate was reduced. The residue was dissolved in ethyl acetate (200mL) and washed with water (200mL) and brine (200mL), the organic layer was dried (Na_2SO_4) and concentrated *in vacuo*.

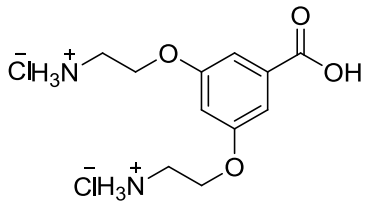
Crystallisation (EtOAc/hexane) afforded compound **3** as a white solid (11.9g). Remaining mother liquor was reduced *in vacuo* and the residual oil was purified via silica column chromatography using 20% EtOAc in hexane to give 13.7g of **9** (total yield 25.6g, 83%); **m.p.** 96-98°C (*EtOAc/hexane*); **^{1}H-NMR** (500 MHz, $CDCl_3$) δ: 7.18 (2H, d, *J* 2.2 Hz, CH_{ar}), 6.63 (1H, t, *J* 2.2 Hz, CH_{ar}), 4.98 (2H, bs, NH), 4.06 (4H, t, *J* 5.0 Hz, CH_2), 3.92 (3H, s, CH_3), 3.60-3.56 (4H, m, CH_2), 1.47 (18H, s, $C(CH_3)_3$); **^{13}C- NMR** (125 MHz, $CDCl_3$) δ: 166.5 (C=O), 159.6 (C x2), 155.8 (C=O), 132.1 (C), 108.1 (CH x2), 106.4 (CH), 79.5 (C), 67.5 (CH_2), 52.1 (CH_3), 40.0 (CH_2), 28.3 (CH_3); **MS** $(ES)^+$ *m/z*: 477 $[M+Na]^+$; **HPLC** t_R = 4.46 min; **IR** (neat) *v* (cm^{-1}): 3281, 1722, 1687, 1537, 1276, 1229, 1066, 841, 766. Data were in good agreement with the literature.[4]

***3,5-[Di-N-(-tert-butoxycarbonyl)ethoxy]-benzoic acid (10)*[4]**



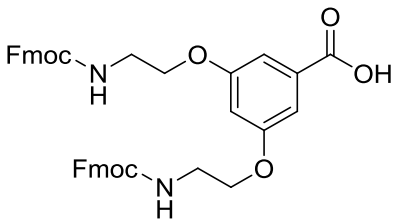
Compound **9** (1.06g, 2.3 mmol) was dissolved in dioxane/methanol/4M $NaOH_{(aq)}$ (14:5:2, 20 mL) and stirred for 5 hrs. The pH of the mixture was adjusted to 2 with 1M $KHSO_2$ and the mixture was concentrated *in vacuo*. The residue was dissolved in dichloromethane (50mL) and water (50mL). The organic layer was washed with water (50mL) and brine (50mL), and dried (Na_2SO_4). Compound **10** was isolated as a white solid (985mg, 96%); **m.p.** 132-134 °C (*dichloromethane*); **^{1}H-NMR** (500 MHz, $CDCl_3$) δ: 7.21 (2H, bs, CH_{ar}), 6.71 (1H, bs, CH_{ar}), 4.51 (2H, bs, NH), 4.16-4.21 (4H, m, Hz, CH_2), 3.58-3.52 (4H, m, CH_2), 1.52 (18H, s, $C(CH_3)_3$); **^{13}C- NMR** (125 MHz, $CDCl_3$) δ: 159.8 (C=O), 159.6 (C x2), 157.3 (C=O), 156.1 (C=O), 131.5 (C), 108.5 (CH x2), 107.6 (CH), 79.9 (C), 67.5 (CH_2), 67.4 (CH_2), 67.1 (CH_2 x2), 40.1 (CH_2), 28.4 (CH_3); **MS** $(ES)^+$ *m/z*: 463 $[M+Na]^+$; **HRMS** $(ESI)^+$ *m/z*: Calculated for $C_{21}H_{32}N_2O_8$ $[M+H]^+$ 441.2231, Found 441.2283; **HPLC** t_R = 4.26 min; **IR** (neat) *v* (cm^{-1}): 3389, 2980, 1715, 1598, 1518, 1174, 1070, 871. 786.

3,5-(Di-aminoethoxy)-benzoic acid, hydrochloride (11)



Benzoic acid **10** (2.28g, 5.2mmol) was dissolved in dichloromethane (30 mL) and diethyl ether (30 mL) saturated with hydrochloric acid was added. After stirring for 2 hrs, the mixture was concentrated *in vacuo*. The hydrochloride salt **11** was obtained as a white solid (1.64g, quantitative); **m.p.** <250 °C (*dichloromethane/ether*); **^{1}H-NMR** (500 MHz, D_2O) δ: 7.20 (2H, d, *J* 2.2 Hz, CH_{ar}), 6.81 (1H, t, *J* 2.2 Hz, CH_{ar}), 4.23 (4H, t, *J* 5.0 Hz, CH_2), 3.38 (4H, t, *J* 5.0 Hz, CH_2); **^{13}C-NMR** (125 MHz, D_2O) δ: 170.6 (C=O), 158.7 (C x2), 128.0 (C), 108.6 (CH x2), 106.5 (CH), 64.2 (CH_2), 38.8 (CH_2); **MS** $(ES)^+$ *m/z*: 241 $[M+Na]^+$; **HRMS** $(ES)^+$ *m/z*: Calculated for $C_{11}H_{16}N_2O_4$ $[M+H]^+$ 241.118, Found 241.120; **HPLC** t_R = 0.77 min.

3,5-[Di(2-fluorenylmethyloxycarbonylamino)ethoxy]-benzoic acid (12)



Hydrochloride salt (**11)** (717mg, 2.2 mmol) was dissolved in acetone:water (1:1, 100 mL) containing sodium carbonate (950mg, 9 mmol). To this solution was added Fmoc-OSu (1.57 g, 4.6 mmol) in acetone (25 mL) dropwise at room temperature. The solution was stirred at room temperature for 18 hrs. The reaction mixture was concentrated and the residue dissolved in water (50mL) and extracted with ether (2 x 50 mL). The aqueous layer was cooled in an ice bath and acidified with 2M HCl to pH3. The white solid (**12**) obtained was filtered, washed with water and dried under vacuum (1.15g, 77%); **m.p.** 192-195 °C (*water*); **^{1}H-NMR** (500 MHz, d6-DMSO) δ: 13.01 (1H, bs, OH), 7.87 (4H, d, *J* 7.5 Hz, CH_{ar}), 7.67 (4H, d, *J* 7.5 Hz, CH_{ar}), 7.51 (2H, t, *J* 5.5 Hz, NH), 7.39 (4H, t, *J* 7.5 Hz, CH_{ar}), 7.29 (4H, t, *J* 7.5 Hz, CH_{ar}), 7.06 (2H, d, *J* 2.0 Hz, CH_{ar}), 6.95 (1H, broad s, CH_{ar}), 4.31 (4H, d, *J* 6.9 Hz, 2xCH_2), 4.21

(2H, t, *J* 6.9 Hz, 2xCH), 4.01 (4H, t, *J* 5.5 Hz, CH_2), 3.38-3.35 (4H, m, CH_2); **^{13}C-NMR** (125 MHz, d6-DMSO) δ: 159.8 (C=O), 156.7 (C=O x2), 144.2 (C x4), 141.2 (C x4), 128.0, 128.9, 127.5 & 125.6 (CH), 120.5 (CH x2), 108.2 (CH), 67.0 (CH_2 x2), 65.8 (CH_2 x2), 55.1 (CH_2 x2), 47.2 (CH x2); **MS** $(ES)^+$ *m/z*: 707 $[M+Na]^+$; **HRMS** $(ES)^+$ *m/z*: Calculated for $C_{41}H_{36}N_2O_8$ $[M+H]^+$ 685.2544, Found 685.2616; **HPLC** t_R = 4.81 min; **IR** (neat) *v* (cm^{-1}): 3320, 1699, 1603, 1543, 1449, 1268, 1168, 916, 760.

S3. Synthesis of resin-bound dendrimeric scaffolds (14) and (15)

Resin-bound 3-branched dendrimeric scaffold (14)

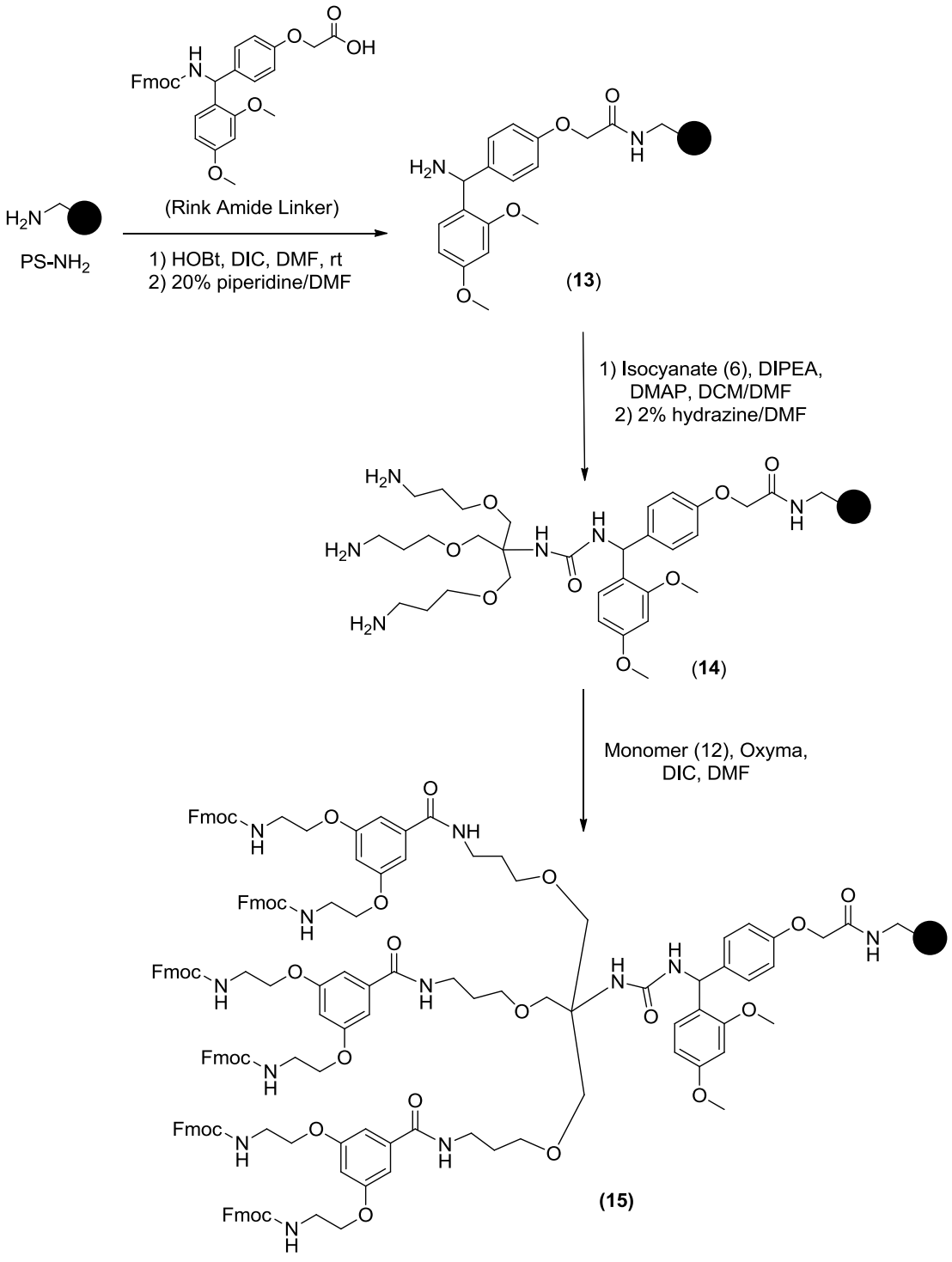
Resin (**13**) was synthesized (Scheme 3) using a 4-[(2,4-dimethoxyphenyl)-(Fmoc-amino)methyl]phenoxyacetic acid (Rink amide linker) attached to aminomethyl PS resin (1.6 mmol/g, 1% DVB, 100-200 mesh). The Fmoc-Rink-amide linker (2.6g, 4.8mmol) was dissolved in DMF (16mL) and HOBt (0.7g, 4.8mmol) was added and the mixture was stirred for 10min. DIC (0.7mL, 4.8mmol) was then added and the resulting mixture was stirred for a further 5 min. The solution was added to aminomethyl polystyrene resin (1g, 1.6mmol/g) and shaken for 2 hours. The resulting resin was washed with DMF (3×10mL), DCM (3×10mL) and MeOH (3×10mL).

General procedure for the Fmoc deprotection

To the resin (pre-swollen in DCM) was added 20% piperidine in DMF (5mL) and the reaction mixture was shaken for 10 min. The solution was drained and the resin was washed with DMF (3×10mL), DCM (3×10mL) and MeOH (3×10mL). This procedure was repeated twice.

Isocyanate coupling

To resin (**13**) (625mg, 1.0mmol), pre-swollen in DCM (10mL), was added a solution of isocyanate (**6**) (2.7g, 3.0mmol), DIPEA (0.5mL, 3.0mmol) and DMAP (7mg, 0.6mmol) in a mixture of DCM/DMF (1:1, 10mL) and the mixture was shaken overnight and the reaction monitored by a quantitative ninhydrin test. The solution was drained and the resin was washed with DMF (3×20mL), DCM (3×20mL) and MeOH (3×20mL) and ether (3×20mL). (3×20mL).



Scheme 3: Synthesis of 3 and 6-branched scaffolds (**14**) and (**15**)

Dde deprotection

To the resin (200mg, 0.32mmol), pre-swollen in DCM (5mL), was added 2% hydrazine in DMF (3mL) and the reaction mixture was shaken for 2h. The

solution was then drained and the resin (**14**) was washed with DMF (3×20mL), DCM (3×20mL) and MeOH (3×20mL).

Resin-bound 6-branched scaffold (15)

A solution of the monomer (**12**) (379mg, 0.55mmol, 4.5eq) and oxyma (79mg, 0.55mmol, 4.5eq) in DMF (0.5mL, 1M) was stirred for 10min. DIC (87µL, 0.55mmol, 4.5eq) was then added and the resulting solution was stirred for further 2 min. The solution was then added to the resin (**14**) (100mg, 0.12mmol, 1eq), pre-swollen in DCM (2mL), and the reaction mixture was shaken for 2 hours. The solution was drained and the resin (**9**) was washed DMF (3×2mL), DCM (3×2mL) and MeOH (3×2mL).

S4. Peptide synthesis

Peptide sequence

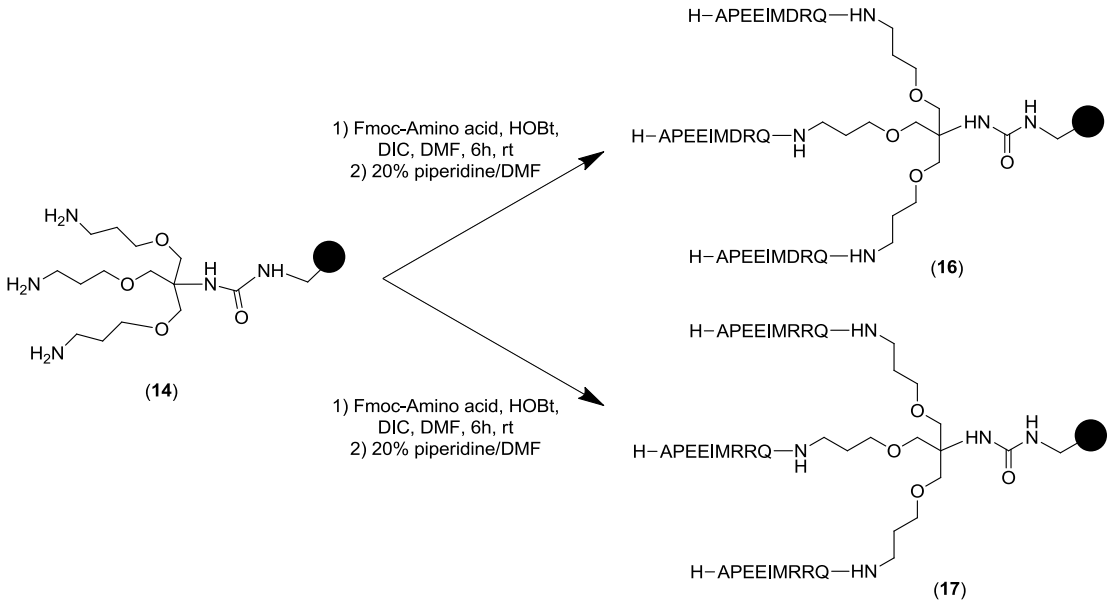
Two specific substrates for HNE were synthesised with the following sequences:

a) APEEIMDRQ (**S1**) and

b) APEEIMRRQ (**S2** and **S3**)

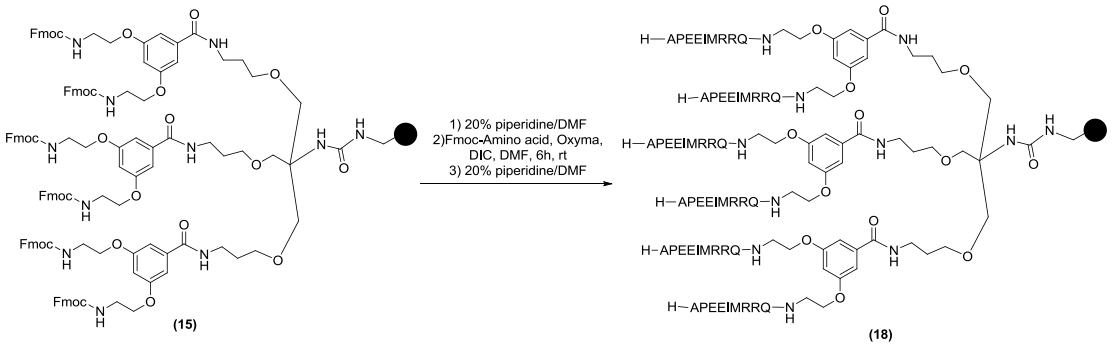
Peptide coupling (**16**), (**17**) and (**18**) (**Scheme 4 – 5)**

A solution of the appropriate Fmoc-amino acid (3.2mmol, 10eq) (Fmoc-Gln(Trt)-OH (1.95g), Fmoc-Arg(Pbf)-OH (2.1g), Fmoc-Met-OH (1.2g), Fmoc-Ile-OH (1.1g), Fmoc-Glu(tBu)-OH (1.4g), Fmoc-Pro-OH (1.1g), Fmoc-Ala-OH (1.0g), and HOBt (0.46g, 3.2mmol, 10eq) in DMF (3.2mL, 1M) was stirred for 10min. DIC (500µL, 3.2mmol, 10eq) was then added and the resulting solution was stirred for further 5min. The solution was then added to resin (**14**) (200mg, 0.32mmol, 1eq), pre-swollen in DCM (10mL), and the reaction mixture was shaken for 6 hours. The solution was drained and the resin (**16**) or (**17**) washed DMF (3×20mL), DCM (3×20mL) and MeOH (3×20mL). The coupling reactions were monitored by a quantitative ninhydrin test[5].



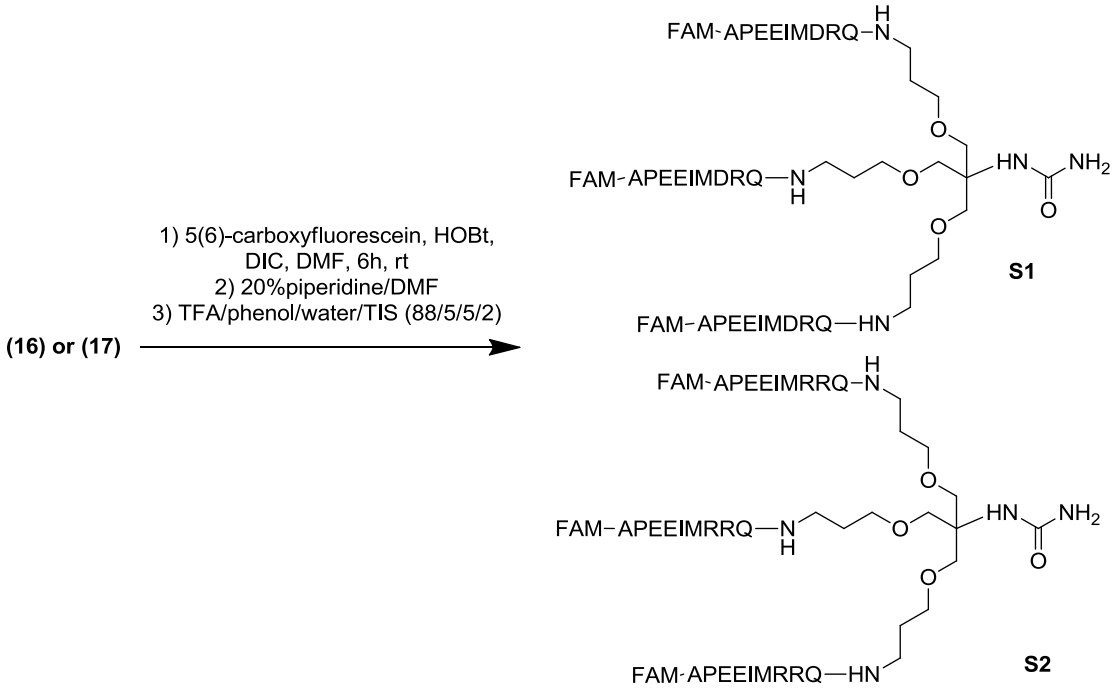
Scheme 4: Synthesis of peptide dendrimers (**16**) and (**17**)

To build the peptide on the 6-branched scaffold, above coupling procedure using DIC/oxyma was carried out on 58mg (0.025mmol) of resin (**15**). Solutions of the appropriate Fmoc-amino acids (0.45mmol, 18eq) [(Fmoc-Gln(Trt)-OH (275mg), Fmoc-Arg(Pbf)-OH (584mg), Fmoc-Met-OH (167mg), Fmoc-Ile-OH (159mg), Fmoc-Glu(tBu)-OH (382mg), Fmoc-Pro-OH (152mg), Fmoc-Ala-OH (140mg)] in DMF were used in conjunction with 18 equivalents of oxyma (65mg, 0.45mmol) and DIC (71µL, 0.45mmol). Fmoc-removal was carried out using 20% piperidine in DMF for 20 min and the reaction was monitored by quantitative ninhydrin test[5].



Scheme 5: Synthesis of peptide dendrimer (**18**)

S5. Synthesis of reporters S1, S2



Scheme 6: Synthesis of reporter S1 and S2

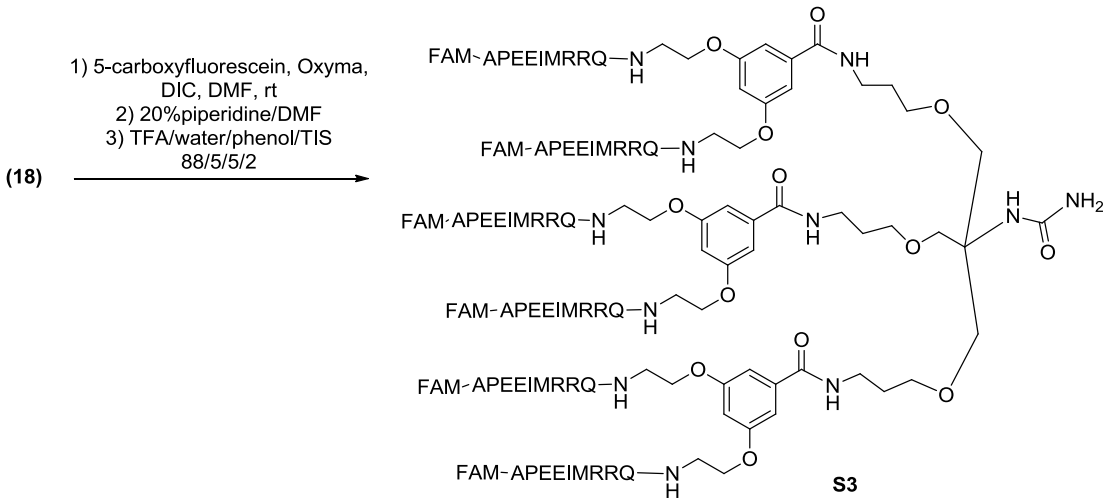
A solution of 5(6)-carboxyfluorescein (28mg, 0.075mmol, 10eq) and HOBt (10mg, 0.075mmol, 10eq) in DMF (700µL) was stirred for 10min. DIC (11µL, 0.075mmol, 10eq) was then added and the resulting solution was stirred for a further 5min. This solution was added to resin **16** or **17** (50mg, loading value = 0.15mmol/g, 1eq), pre-swollen in DCM, and the reaction mixture was shaken for 6h. The solution was drained and the resin washed with DMF (×3), DCM (×3) and MeOH (×3). The coupling reactions were monitored by a quantitative ninhydrin test[5]. Before cleavage, the resin was washed with 20% piperidine to remove any fluorescein phenol esters[6].

TFA cleavage and purification of reporter S1 and S2

The resin (50mg), pre-swollen in DCM, was treated with a cleavage cocktail of TFA/phenol/water/TIS (88/5/5/2, 1mL) for 2h. The solution was drained and the resin was washed with the cleavage cocktail and the solution was

removed *in vacuo*. The crude material was dissolved in a minimum amount of cleavage cocktail (500µL) and added to ice-cold ether (7.5mL). The precipitated solid was collected by centrifugation and the solvent removed by decantation and the precipitate was washed with cold ether (3x5mL). The precipitate was then purified by reverse phase preparative HPLC and the required fractions were pooled and lyophilized to afford S1 and S2. **S1**: **HPLC**: t_R = 8.4 min, purity>98% by ELSD; **MALDI**: $C_{209}H_{276}N_{44}O_{70}S_3$: [$M^+$] calcd: 4620.88, [$M^+$] found: 4620.56, **S2**: **HPLC** t_R = 8.2 min, purity>98% by ELSD; MALDI: $C_{215}H_{294}N_{50}O_{64}S_3$ [M^+] calcd: 4696.05, found [M+2$^+$]: 4698.79.

S6. Synthesis of reporter S3



Scheme 7: Synthesis of reporter S3

A solution of 5-carboxyfluorescein[7] (85mg, 0.23mmol, 9eq) and oxyma (32mg, 0.23mmol, 9eq) in DMF (0.5mL) was stirred for 10 min. DIC (24µL, 0.23mmol, 9eq) was then added and resulting solution was stirred for a further 2 min. This solution was added to resin (0.025mmol), pre-swollen in DCM, and the mixture was shaken for 3 hours. The solution was drained and the resin washed with DMF (×3), DCM (×3) and MeOH (×3). The coupling reactions were monitored by a quantitative ninhydrin test[5]. Before cleavage, the resin was washed with 20% piperidine to remove any fluorescein phenol esters[6].

TFA cleavage and purification of reporter S3

The resin (50mg), after Fmoc deprotection, was treated with a cleavage cocktail of TFA/phenol/water/TIS (88/5/5/2, 200µL) for 2.5h. The solution was drained and resin was washed with the cleavage cocktail (300µL). The filtrate was added to ice-cold ether (7.5mL). The precipitated solid was collected by centrifugation and the solvent removed by decantation and the precipitate was washed with cold ether (3x5mL). The yellow precipitate **S3** (31 mg) was dried under vacuum for 15h. **HPLC**: t_R = 5.17 min, purity>95% by ELSD.

S7. References

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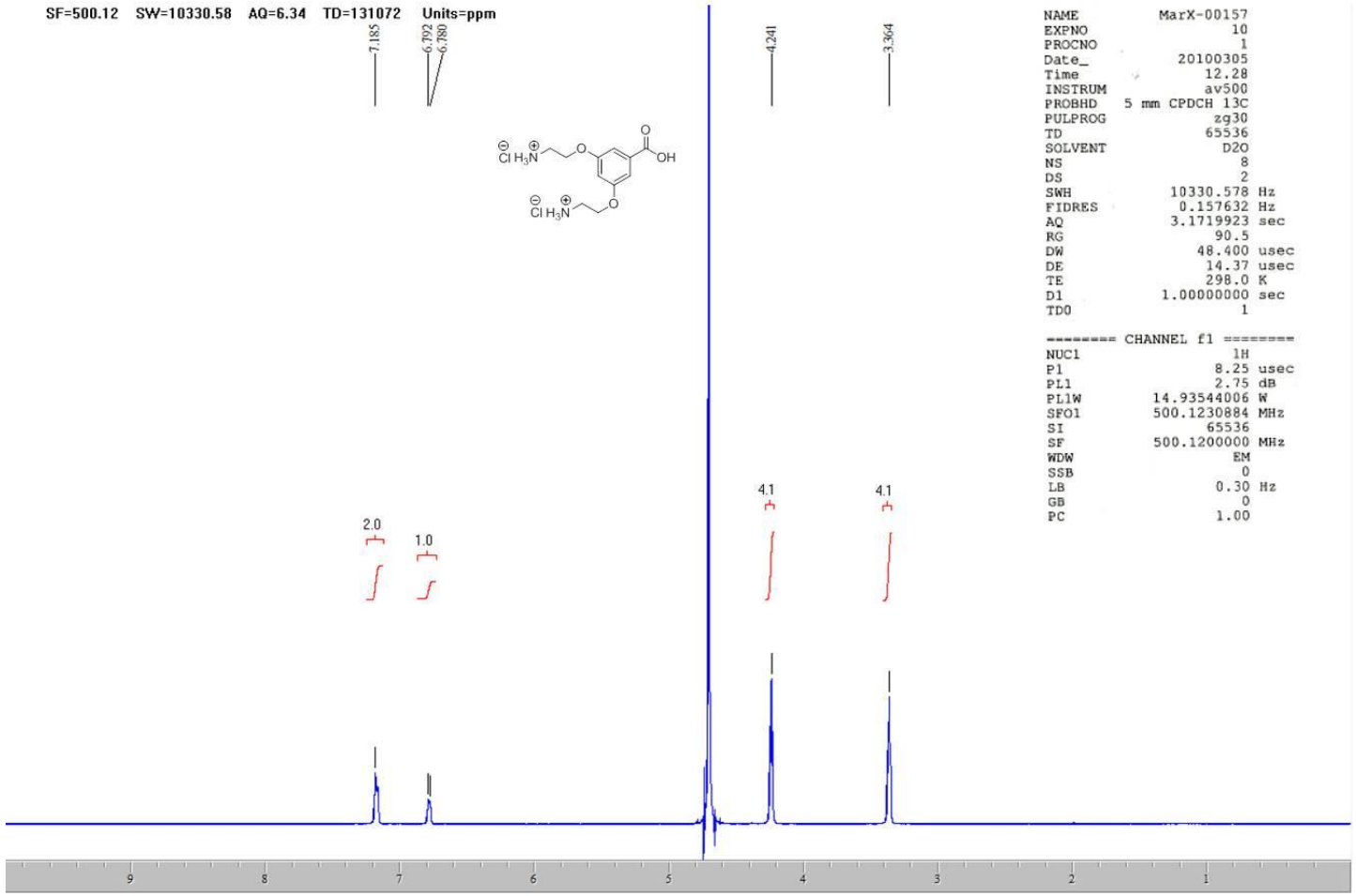
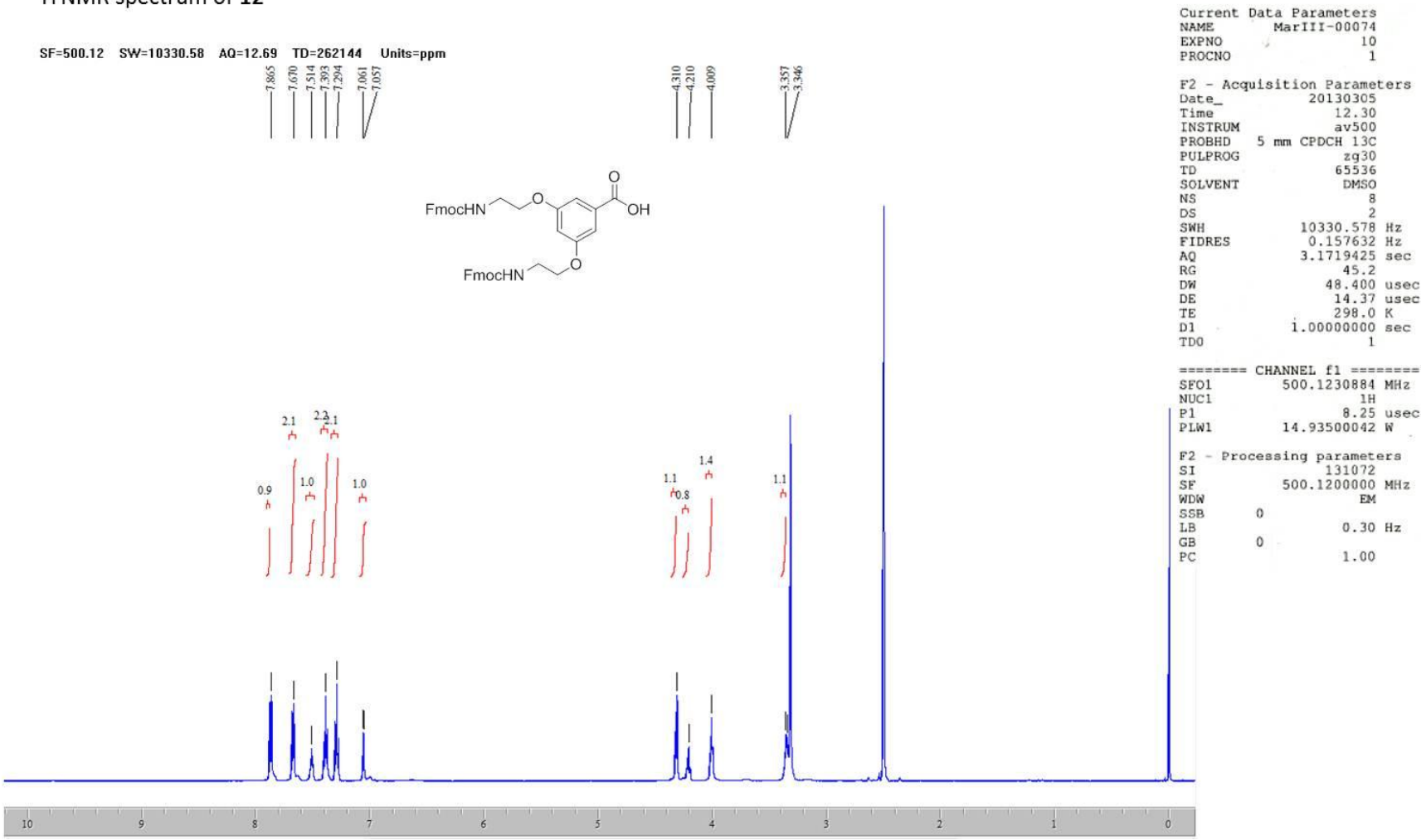
S8. NMR spectra

^{1}H HMR spectrum of **11**



^{1}H NMR spectrum of **12**

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D1            1.00000000 sec
TD0                    1

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P1                 8.25 usec
PLW1          14.93500042 W

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LB                 0.30 Hz
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PC                 1.00
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BIOLOGY

S9. In vitro cleavage of probes by recombinant proteinases

Recombinant enzymes (Human Neutrophil Elastase, Proteinase 3, Cathepsin G, Athens Research and Technology, Athens, GA), Silvelestat (Sigma, UK) and reporters (S1, S2 and S3) were diluted as previously described[1] in reaction buffer (50 mM HEPES buffer, pH 7.4, 0.75M NaCl, 0.05 % Igepal CA-630 v/v) to obtain the appropriate concentration. Enzyme and reporters were incubated with or without inhibitor and the cleavage of the S1, S2 or S3 was followed using a Biotek fluorescent microplate reader (excitation: 485nm, emission 530nm). Initial velocity was calculated by linear regression for each probe.

S10. Cell isolation and culture

Human Peripheral Blood leukocytes from healthy volunteers were prepared as previously described[2]. Briefly, citrated blood was centrifuged at room temperature for 20 minutes at 350g, and platelet-rich plasma was removed. Autologous serum was prepared by recalcification of platelet-rich plasma by addition of $CaCl_2$ to a final concentration of 2 mM. Leukocytes were separated from erythrocytes by dextran sedimentation using 0.6% dextran T500 (Pharmacia, Milton Keynes, UK), and the leukocyte-rich upper layer was then fractionated using isotonic Percoll (Pharmacia). Neutrophils and mononuclear leukocytes were harvested from the 68%/81% and 55/68% interfaces, respectively. In some experiments, neutrophils were labeled with DiD (Invitrogen, molecular probes) (2.5 µM) in D-PBS (w/o Ca/Mg) for 20 mins at room temperature.

S11 Evaluation of cellular and lysate HNE activity

PMNs and (equivalent lysate) were seeded in 96 well microplates (125000 cells per well). In some experiments inhibitors were added 5 minutes before probe addition (sivelestat, 10µM, SLPI at 10 µg/ml, alpha-1 antitrypsin at 2 µg/ml, and alpha-1 antichymotrypsin at 2 µg/ml). Cells were then incubated with 0.5µM probe and kinetics of probe cleavage measured (37°C; >30 mins)

using a Biotek fluorescent microplate reader (excitation: 485nm; emission 530nm).

S12 A549 Cell layer integrity

A549 cells (ATCC CCL-185) were maintained at 80-90 % confluence in culture medium which consisted of Dulbecco's modified Eagle's medium (4.5 $g.L^{-1}$ glucose) supplemented with 10 % (v/v) heat inactivated fetal bovine serum (FBS), L-glutamine (2 mM), streptomycin (50 $\mu g.ml^{-1}$) and penicillin (50 $units.ml^{-1}$) in a thermostatted (37 °C) and humidified atmosphere of 5 % CO_2/ 95 % air. Suspensions of exponentially growing cells (2×10^6 cells), detached following trypsin exposure were then seeded onto coverslips or 6-well microplates at 150×10^3 cells/ 30mm coverslip/well then grown to confluence 24-48 hours, cells were than incubated with 1µM of probe for 24h. The cells are then assessed visually by confocal microscopy.

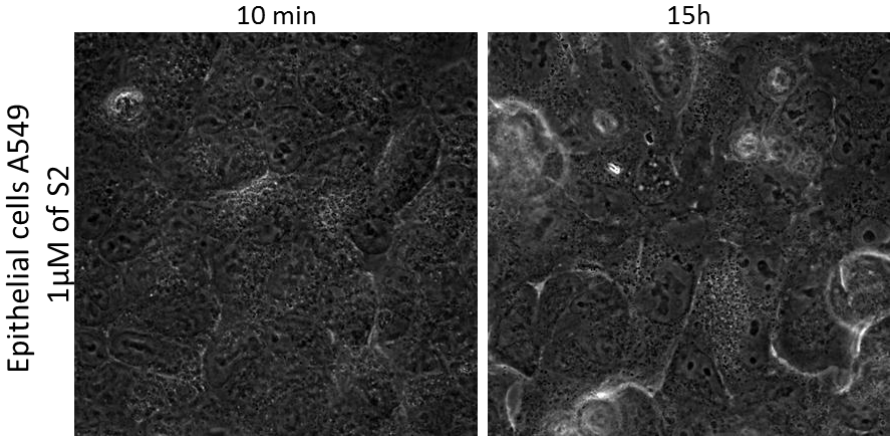

Fig 1: Confocal microscope images of epithelial cell A549 incubated with 1µM of S2for 15h. The cell layer integrity is not impaired by the incubation with the probe.

S13 Cells viability assay

96 well microplates were seeded with 20000 HeLa in DMEM medium supplemented with 10% of FBS, 1% Penicillin / streptomycin and 1% Glutamine. After of 6h incubation, medium was replaced with starving medium composed of DMEM supplemented with 2% FBS 1% Penicillin / streptomycin and 1% Glutamine. Cells were starved in serum O/N, then 100µM of each probe were added. As positive control for cell death, 1µM of staurosporine was added to the starving medium. After 24h of incubation with probes or 6h with Staurosporine, cell viability was assessed using cell titer glo kit

(Promega) according to manufacturer description and luminescence signal was read using Biotek luminescent microplate reader.

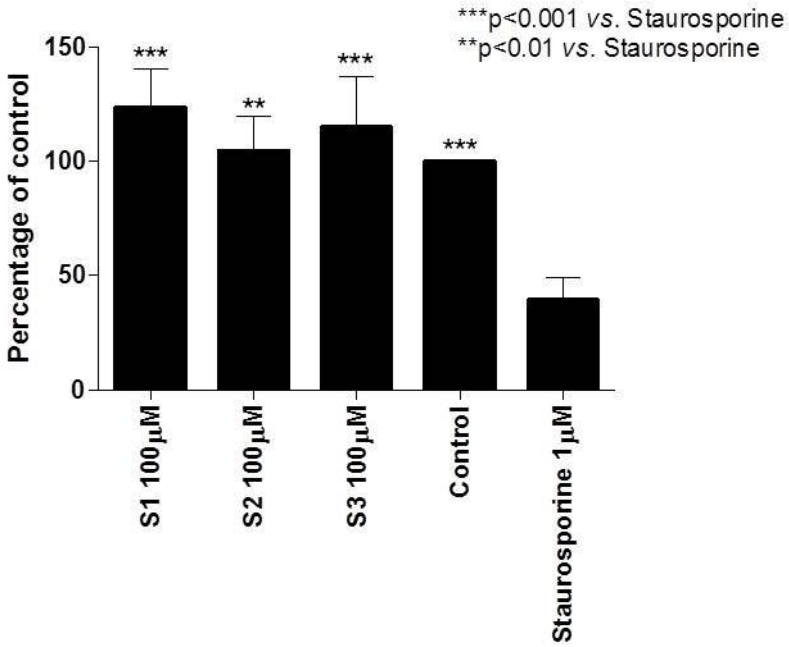

Fig2: Assessment of cellular toxicity of S1, S2 and S3 (100µM of probe incubated for 24 hours at 37 °C with HeLa cells; Cell viability assessed by Cell Titer Glo assay according to manufacturer's instructions (Promega)

S14. Biological References

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