A novel heterotrifunctional peptide-based cross-linking reagent for facile access to bioconjugates. Applications to peptide fluorescent labelling and immobilisation

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Supporting Information

Experimental : Detailed synthetic procedures for compounds **4**, **7**, **8**, **9**, **10**, **11**, **12**, **13** and **A**

2-(2-(2-Azidoethoxy)ethoxy)ethanol (7)[1]. 2-(2-(2-Chloroethoxy)ethoxy)ethanol **6** (1.29 mL, 8.9 mmol) was added to a suspension of NaN_3 (0.70 g, 10.7 mmol) and NaI (0.14 g, 0.93 mmol) in dry EtOH. The resulting yellow mixture was refluxed for 5 days under an argon atmosphere. The reaction was checked for completion by TLC (CH_2Cl_2-MeOH, 9 : 1, v/v). The mixture was filtered over Celite® 545 to remove sodium salts and evaporated to dryness. The resulting oily residue was dissolved in CH_2Cl_2 (*ca.* 10 mL) and stored at 4 °C for 1 h. After filtration over a cotton bowl and concentration, 1.8 g (10.7 mmol) of compound **7** was obtained as colorless oil (quantitative yield). R_f (CH_2Cl_2-MeOH, 9 : 1, v/v) 0.69; IR (neat): ν_{max} 935, 1118, 1287, 1346, 1453, 2110, 2874, 2915, 3390 (broad) cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 2.63 (t, J = 6.0 Hz, 1H, OH), 3.43 (t, J = 4.9 Hz, 2H), 3.61-3.77 (m, 10H).

(2-(2-Azidoethoxy)ethoxy)acetic acid (8)[2]. 2-(2-(2-Azidoethoxy)ethoxy)ethanol **7** (1.56 g, 8.9 mmol) was dissolved in acetone (90 mL) and the resulting solution was cooled to 4 °C. Freshly prepared 3 M Jones' reagent (8.9 mL) was added dropwise (a green precipitate immediately formed) and the resulting reaction mixture was stirred at room temperature for 1 h. The reaction was checked for completion by TLC (CH_2Cl_2-MeOH, 9 : 1, v/v) and quenched by addition of propan-2-ol (*ca.* 4 mL). After 15 min, further amount of acetone (100 mL) was added and the green precipitate of Cr(III) salts was removed by filtration over Celite® 545. The filtrate was evaporated to dryness. The resulting oily residue was immediately purified by chromatography on a silica gel column with a step gradient of MeOH (0-5%) in CH_2Cl_2 as the mobile phase, giving 1.53 g (8.1 mmol) of carboxylic acid **8** as a yellow oil (yield 91%). R_f (CH_2Cl_2-MeOH, 9 : 1, v/v) 0.23; ^1H NMR (300 MHz, $CDCl_3$): δ 3.43 (t, J = 4.9 Hz, 2H), 3.66-3.80 (m, 6H), 4.19 (s, 2H); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 50.7, 68.6, 70.2, 70.6, 71.4, 174.2.

(2-(2-Aminoethoxy)ethoxy)acetic acid (9)[3]. (2-(2-Azidoethoxy)ethoxy)acetic acid **8** (1.53 g, 8.2 mmol) was dissolved in EtOH (150 mL) and the solution was cooled to 4 °C. Pd-C (0.32 g, 10% Pd) was added and the resulting reaction mixture was stirred at room temperature for 12 h under an H_2 atmosphere. The reaction was checked for completion by TLC (CH_2Cl_2-MeOH, 8 : 2, v/v) and the mixture was filtered over Celite® 545 to remove Pd-C. The filtrate was evaporated to dryness and the resulting oily residue was dried under vacuum to give 1.3 g (8.2 mmol) of amino acid **9** as a yellow oil (quantitative yield). R_f (CH_2Cl_2-MeOH, 8 : 2, v/v) 0.0; Spectroscopic data are identical to that reported in the literature.

(2-(2-(*tert*-Butyloxycarbonyl)aminoethoxy)ethoxy)acetic acid (10)[4]. (2-(2-Aminoethoxy)ethoxy)acetic acid **9** (0.7 g, 4.3 mmol) was dissolved in a mixture of THF-H_2O (2 : 1, v/v, 15 mL). Freshly prepared 2 M aq. NaOH solution (6.5 mL) was added and the solution was cooled to 4 °C. Boc_2O (1.4 g, 6.4 mmol) was added and the reaction mixture was stirred at room temperature for 1 h. The reaction was checked for completion by TLC (CH_2Cl_2-MeOH, 7 : 3, v/v) and acidified by adding 1 M aq. $KHSO_4$ solution (*ca.* 3.5 mL). The mixture was evaporated close to dryness. H_2O (30 mL) was added and the solution was extracted with CH_2Cl_2 (3 x 30 mL). The combined organic extracts were dried over Na_2SO_4, filtrated and evaporated to dryness. The resulting oily residue was purified by chromatography on a silica gel column with a step gradient of MeOH (0-3%) in CH_2Cl_2 as the mobile phase, giving 0.71 g (2.7 mmol, yield 63%) of protected amino acid **10** as a colorless oil. R_f (CH_2Cl_2-MeOH, 7 : 3, v/v) 0.21; ^1H NMR (300 MHz, $CDCl_3$): δ 1.44 (s, 9H), 3.34 (bm, 2H), 3.50-3.77 (m, 6H), 4.17 (s, 2H), 4.97, (bs, 1H, NH).

(2-(2-Aminoethoxy)ethoxy)acetic acid methyl ester (11). (2-(2-Aminoethoxy)ethoxy)acetic acid **9** (0.70 g, 3.35 mmol) was suspended in 2,2-dimethoxypropane (30.9 mL) and 37% HCl (3.35 mL) was added. The resulting reaction mixture was stirred at room temperature for 1 h. The reaction was checked for completion by TLC (CH_2Cl_2-MeOH-TEA, 80 : 20 : 2, v/v/v) and the mixture was evaporated to dryness. Deionised water (10 mL) was added and the resulting aq. solution was lyophilised thrice to give 714 mg (3.35 mmol) of methyl ester **11** as a yellow oil (quantitative yield). This compound was used in the next step without further purification. R_f (CH_2Cl_2-MeOH-TEA, 80 : 20 : 2, v/v/v) 0.46; ^{1}H NMR (300 MHz, CD_3CN + 5% D_2O): δ 3.08 (t, J = 5.3 Hz, 2H), 3.56-3.76 (m, 11H), 4.13 (s, 2H); MS (ESI+): *m/z* 178.20 $[M + H]^+$, calcd for $C_7H_{15}NO_4$ 177.20.

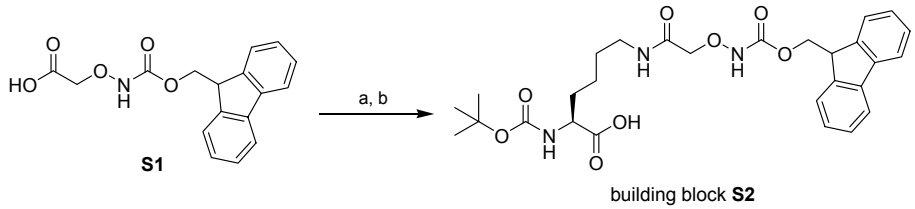
Boc-protected amino-PEG-acid spacer (A). A mixture of (2-(2-(*tert*-butyloxycarbonyl)aminoethoxy)ethoxy)acetic acid **10** (0.15 g, 0.83 mmol) and (2-(2-aminoethoxy)ethoxy)acetic acid methyl ester **11** (0.23 g, 0.87 mmol) was dissolved in dry CH_3CN. DIEA (0.44 mL, 2.5 mmol) and BOP reagent (0.37 g, 0.83 mmol) were sequentially added and the resulting reaction mixture was stirred at room temperature overnight under an argon atmosphere. The reaction was checked for completion by TLC (CH_2Cl_2-MeOH, 8 : 2, v/v) and the mixture was evaporated to dryness. Thereafter, the resulting residue was taken up in ethyl acetate, washed with 10% aq. citric acid, sat. aq. $NaHCO_3$, brine, dried over Na_2SO_4, filtrated and evaporated to dryness. The orange oily residue was dissolved in MeOH (5 mL) and the solution was cooled to 4 °C. 1 M aq. LiOH (0.83 mL) was added and the reaction mixture was stirred at room temperature for 30 min. The reaction was checked for completion by TLC (CH_2Cl_2-MeOH, 8 : 2, v/v) and acidified with 1 M aq. $KHSO_4$ solution (*ca.* 1 mL). The solution was evaporated to dryness without warming and the resulting residue was purified by chromatography on a silica gel column with a step gradient of MeOH (0-50%) in CH_2Cl_2 as the mobile phase. 0.17 g (0.42 mmol, overall yield for the two steps 51%) of *N*-Boc pseudo-PEG linker **A** was obtained as a colorless oil. R_f (CH_2Cl_2-MeOH, 8 : 2, v/v) 0.54; IR (neat): ν_{max} 559, 666, 771, 843, 1114 (broad), 1251, 1367, 1455, 1538, 1666 (broad), 2930, 3352 (broad) cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.43 (s, 9H), 3.30 (bm, 2H), 3.47-3.66 (m, 14H), 4.01 (s, 2H), 4.04 (s, 2H), 5.16 (bs, 1H, NH), 7.34 (bs, 1H, NH); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 28.5 (3C), 38.7, 40.3, 70.1 (2C), 70.4 (2C), 70.6 (2C), 70.9 (2C), 79.5, 156.3, 170.9 (2C); MS (ESI+): *m/z* 431.27 $[M + Na]^+$, 453.27 $[M + 2Na - H]^+$; MS (ESI-): *m/z* 407.47 $[M - H]^-$, calcd for $C_{17}H_{32}N_2O_9$ 408.45.

***N*-Phthaloyl protected aminooxyacetic acid (12)[5].**
(a) Synthesis of full-protected aminooxyacetic acid derivative: *N*-Hydroxyphthalimide (1.7 g, 10.3 mmol) was dissolved in dry NMP (24 mL). Anhydrous K_2CO_3 (2.1 g, 15.5 mmol) was then added and the resulting mixture was stirred at 40 °C under an argon atmosphere for 10 min. *tert*-Butyl bromoacetate (1.5 mL, 10.3 mmol) was then slowly added and the temperature increased to 50 °C for 3 h. The reaction was checked for completion by TLC (100% CH_2Cl_2). Precipitation of the desired product was achieved by adding cold deionised water. The solid was collected by filtration and washed with cold deionised water until the solid remains colorless. The resulting white solid was then dissolved in CH_2Cl_2, evaporated to dryness. Residual water was removed by lyophilisation to give *tert*-butyl phthalimidooxyacetate as a white powder (2.3 g, 8.3 mmol, yield 81%). R_f (100% CH_2Cl_2) 0.7; ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.49 (s, 9H, *t*Bu), 4.71 (s, 2H, CH_2), 7.74-7.86 (m, 4H, phthalimide); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 28.2, 73.6, 83.2, 123.8, 129.0, 134.8, 163.2, 166.5.
(b) Removal of *tert*-butyl ester: *tert*-Butyl phthalimimidooxyacetate (2.3 g, 8.3 mmol) was dissolved in dry CH_2Cl_2 (20 mL) and the mixture was cooled to 0 °C. TFA (6.16 mL, 83

mmol) was then added dropwise and the reaction mixture was stirred at room temperature for 1 h under an argon atmosphere. The reaction was checked for completion by TLC (100% CH_2Cl_2). Further amount of TFA was added (1.05 mL, 16.6 mmol) and the mixture was stirred again for 20 min. Thereafter, the mixture was concentred under reduced pressure and the resulting residue was co-evaporated thrice with chloroform (3 x 10 mL). Finally, 10 mL of deionised water was added and the aq. solution was lyophilised to give phthalimimidooxyacetic acid **12** as a white powder (1.8 g, 8.3 mmol, quantitative yield). R_f (100% CH_2Cl_2) 0.17; ^{1}H NMR (300 MHz, $CDCl_3$): δ 4.77 (s, 2H, CH_2), 7.83 (s, 4H, phthalimide); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 74.0, 124.3, 129.9, 135.9, 164.1, 168.3.

Synthesis of full-protected heterotrifunctional reagent (4).

The highly convergent synthetic strategy based on solution phase peptide couplings and developed for the preparation of *N*-Phthaloyl aminooxy heterotrifunctional cross-linking reagent **5** was used:

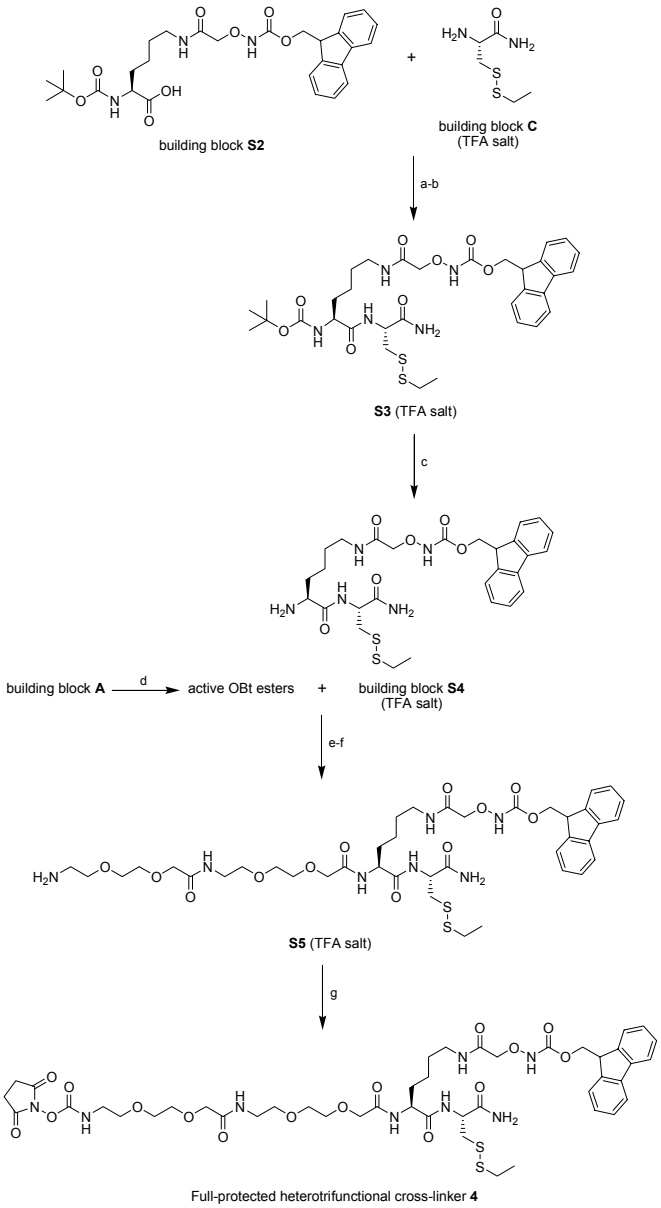


Scheme S1 *Reagents and conditions*: a) DCC (1.2 equiv), HOBt.H_2O (1.2 equiv), CH_3CN-DMF (1 : 1, v/v), rt, 2 h; b) Boc-Lys-OH (1 equiv), DMF, rt, 2 h, 50%. DCC = *N*,*N*'-dicyclohexylcarbodiimide, HOBt.H_2O = hydroxybenzotriazole monohydrate.

(9-Fluorenylmethoxycarbonyl)aminooxyacetic acid (S1)[6]. Carboxymethoxylamine hemihydrochloride (0.5 g, 4.6 mmol) was dissolved in an aq. solution of Na_2CO_3 (1.2 g in 20 mL) and the resulting solution was cooled to 4 °C. Then 9-fluorenylmethyl chloroformate (1.31 g, 5.0 mmol) in 1,4-dioxane (10 mL) was added dropwise and the reaction mixture was stirred at room temperature overnight. The reaction mixture was partially evaporated, acidified to pH 4-5 with 5% aq. HCl (*ca.* 5 mL); the crude product quickly precipitated. It was collected by filtration and washed with deionised water and pentane. Residual water was removed by lyophilisation to give 1.05 g of crude product. Further purification by chromatography on a silica gel column was undertaken with a step gradient of MeOH (0-50%) in CH_2Cl_2 as the mobile phase, giving 0.677 g (2.2 mmol, yield 47%) of Fmoc-Aoaa-OH **S1** as a white foam. ^{1}H NMR (300 MHz, CD_3OD): δ 4.22-4.27 (m, 3H), 4.44 (d, J = 6.8 Hz, 2H), 7.29-7.42 (m, 4H), 7.64 (d, J = 7.5 Hz, 2H), 7.80 (d, J = 7.5 Hz, 2H); HPLC (system B): t_R = 17.8 min, purity 94%.

N^{α}-(*tert*-Butyloxycarbonyl)-N^{ε}-[(9-fluorenylmethoxycarbonyl)aminooxyacetyl]-L-lysine (S2). Fmoc-Aoaa-OH **S1** (230 mg, 0.73 mmol) was dissolved in a mixture of dry CH_3CN-DMF (1:1, v/v, 9 mL). Hydroxybenzotriazole monohydrate (119 mg, 0.88 mmol) and DCC (182 mg, 0.88 mmol) were sequentially added and the resulting reaction mixture was stirred at room temperature for 2 h under an argon atmosphere. Thereafter, a solution of N^{α}-Boc-L-lysine (180 mg, 0.73 mmol) in dry DMF (2 mL) was added and the resulting reaction mixture was stirred at room temperature. The reaction was checked for completion by TLC (CH_2Cl_2-MeOH 8 : 2, v/v). After 2 h, the mixture was evaporated to dryness. The resulting residue was taken up with ethyl acetate, washed by 10% aq. citric acid, deionised water, dried over Na_2SO_4, filtrated, concentrated by rotator evaporation, then purified by chromatography on a silica gel column with a step gradient of ethyl acetate (0-80%) in CH_2Cl_2 as the mobile phase,

giving 296 mg (0.36 mmol, yield 50 %) of lysine building block **S2** as a white foam. R_f 0.59 (CH_2Cl_2-MeOH, 8 : 2, v/v); ^{1}H NMR (300 MHz, CD_3CN): δ 1.37-1.83 (m, 15H), 3.17-3.19 (d, J = 6.0 Hz, 2H), 4.00 (m, 1H), 4.04 (s, 2H), 4.26 (t, J = 6.8 Hz, 1H), 4.49 (d, J = 6.8 Hz, 2H), 5.61 (bd, J = 7.5 Hz, 1H, NH), 7.31-7.44 (m, 4H), 7.51 (bs, 1H, NH), 7.63 (d, J = 7.5 Hz, 2H), 7.83 (d, J = 7.5 Hz, 2H), 8.9 (bs, 1H, NH); ^{13}C NMR (75.5 MHz, CD_3CN): δ 23.5, 28.5 (3C), 29.5, 31.6, 38.9, 47.7, 54.2, 68.0, 76.3, 79.8, 120.9 (2C), 126.0 (2C), 128.1 (2C), 128.7 (2C), 142.1 (2C), 144.6 (2C), 156.7, 158.9, 169.3, 174.5; HPLC (system B): t_R = 21.2 min, purity 95%; UV-visible (recorded during the HPLC analysis) λ_{max} = 216, 262, 293 nm; MS (MALDI-TOF, positive mode): *m/z* 564.57 $[M + Na]^+$, 580.55 $[M + K]^+$, calcd for $C_{28}H_{35}N_3O_8$ 541.61.



Scheme S2 *Reagents and conditions*: a) DCC (1.2 equiv), HOBt.H_2O (1.2 equiv), CH_3CN-DMF (2 : 1, v/v), rt, 2 h; b) DIEA (2 equiv), DCC (2 equiv), rt, overnight, 88% (a+b); c) 15% TFA, CH_2Cl_2, 4 °C to rt, 90 min, quant. yield; d) DCC (1.2 equiv), HOBt.H_2O (1.2 equiv), CH_3CN, rt, 2 h; e) DIEA (2.5 equiv), DCC (0.3 equiv), rt, overnight then acetic acid (1.9 equiv), 61% after RP-HPLC purification; f) 11% TFA, CH_2Cl_2, 4 °C to rt, 1 h, quant. yield; g) DSC (2.5 equiv), TEA (1 equiv), DMF, rt, 90 min, 74% after RP-HPLC purification. DSC = *N*,*N*′-disuccinimidyl carbonate, TEA = triethylamine.

N^{α}-(*tert*-Butyloxycarbonyl)-N^{ε}-[(9-fluorenylmethoxycarbonyl)aminooxyacetyl]-L-lysinyl-*S*-(ethylthio)-L-cysteine carboxamide (S3). Lysine building block **S2** (0.196 g, 0.36 mmol) was dissolved in a mixture of dry CH_3CN-DMF (2 : 1, v/v, 3 mL). Hydroxybenzotriazole monohydrate (58.4 mg, 0.43 mmol) and DCC (89.1 mg, 0.43 mmol) were sequentially added and the resulting reaction mixture was stirred at room temprature for 2 h under an argon atmosphere. Thereafter, a solution of TFA salt of H-Cys(SEt)-OH **C** (106.0 mg, 0.36 mmol) in dry DMF (1 mL) was added and the resulting reaction mixture was stirred at room temperature under an argon atmosphere. The reaction was checked for completion by RP-HPLC (system B) and TLC (CH_2Cl_2-MeOH, 8 : 2, v/v). After 2, 4 and 6 h of stirring, DIEA (31 μL, 0.18 mmol) was added and a further amount of DCC (44.9 mg, 0.18 mmol) was added after 6 h. The round-bottom flask was stored at -20 °C overnight. Thereafter, further amounts of DIEA (31 μL, 0.18 mmol) and DCC (44.9 mg, 0.18 mmol,) were added. After 2 h, the reaction was checked to completion and the mixture was evaporated to dryness. The resulting residue was taken up with ethyl acetate, successively washed with 10% aq. citric acid, sat. $NaHCO_3$, deionised water, dried over Na_2SO_4 and evaporated to dryness. The resulting residue was purified by chromatography on a silica gel column with a step gradient of MeOH (0-10%) in CH_2Cl_2 as the mobile phase, giving 222 mg (0.31 mmol, yield 88%) of full-protected dipeptide **S3** as a white foam. R_f (CH_2Cl_2-MeOH, 8 : 2, v/v) 0.60; IR (KBr): ν_{max} 586 (broad), 621, 741, 760, 861, 1045, 1118, 1167, 1254, 1367, 1392, 1451, 1516 (broad), 1666 (broad), 2868, 2928, 3068, 3312 (broad) cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 1.26-1.31 (t, 3H, J = 7.1 Hz, CH_3(SEt) Cys), 1.42 (s, 9H, tBu) 1.51-1.92 (m, 6H, CH_2 β, δ, γ Lys), 2.65-2.72 (q, 2H, J = 7.5 Hz, CH_2(SEt) Cys), 3.08-3.10 (d, 2H, J = 6.0 Hz, CH_2 β Cys), 3.26-3.33 (m, 2H, CH_2 ε Lys), 4.04-4.06 (t, J = 6.0 Hz, 1H, CH α Lys), 4.20-4.25 (t, J = 7.2 Hz, 1H, CH Fmoc), 4.33 (s, 2H), 4.49-4.51 (d, 1H, J = 6.4 Hz, CH_2 Fmoc), 4.71-4.78 (q, J = 6.4 Hz, 1H, CH α Cys), 7.26-7.78 (m, 8H, CH Fmoc); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 14.2, 22.3, 28.6, 30.1, 32.4, 38.0, 39.3, 46.7, 52.3, 52.2, 68.0, 76.1, 80.8, 120.3, 125.1, 127.4, 128.1, 141.4, 143.3, 156.4, 158.6, 169.0,172.6, 172.8. MS (MALDI-TOF, positive mode): *m/z* 726.77 $[M + Na]^+$, 742.74 $[M + K]^+$, calcd for $C_{33}H_{45}N_5O_8S_2$ 703.88.

N^{ε}-[(9-Fluorenylmethoxycarbonyl)aminooxyacetyl]-L-lysinyl-*S*-(ethylthio)-L-cysteine carboxamide (S4). Dipeptide **S3** (191 mg, 0.27 mmol) was dissolved in dry CH_2Cl_2 (7 mL) and the solution was cooled to 4 °C. TFA (1.2 mL, 16.6 mmol) was added dropwise and the resulting reaction mixture was stirred at room temperature for 90 min. The reaction was checked for completion by TLC (CH_2Cl_2-MeOH, 9 : 1, v/v) and the mixture was evaporated to dryness. The resulting oily residue was dissolved in deionised water and lyophilised to give the dipeptide building block **S4** as a white amorphous powder (199 mg, 0.27 mmol, quantitative yield). This compound was used in the next coupling reaction step without further purification. R_f (CH_2Cl_2-MeOH, 9 : 1, v/v) 0.14; IR (KBr): ν_{max} 740, 1117, 1257, 1456, 1506, 1538, 1558, 1652, 1689, 1717, 1732, 2103, 2351, 2928, 3288 cm^{-1}; ^1H NMR (300 MHz, CD_3OD): δ 1.26-1.28 (t, 3H, J = 3.8 Hz, CH_3(SEt) Cys), 1.40-1.56 (m, 4H, CH_2 β, γ Lys), 1.83-1.90 (m, 2H, CH_2 δ Lys), 2.67-2.74 (q, 2H, J = 7.2 Hz, CH_2(SEt) Cys), 2.93-2.96 (d, 2H, J = 9.4 Hz, CH_2 β Cys), 3.20-3.28 (m, 2H, CH_2 ε Lys), 3.84-3.88 (t, J = 6.0 Hz, 1H, CH α Lys), 4.23 (s, 3H, CH Fmoc, CH_2), 4.46-4.48 (d, 2H, J = 6.4 Hz, CH_2 Fmoc), 4.63-4.68 (q, J = 4.9 Hz, 1H, CH α Cys), 7.26-7.78 (m, 8H, CH Fmoc), 8.32 (bs, 1H, NH); ^{13}C NMR (75.5 MHz, CD_3OD): δ 14.7, 22.3, 30.7, 32.2, 33.2, 39.5, 41.0, 48.2, 53.9, 54.2, 68.6, 76.5, 121.0, 126.0, 128.2, 129.0, 142.7, 144.8, 170.2, 171.2; MS (MALDI-TOF, positive mode): *m/z* 604.70 $[M + H]^+$, 626.68 $[M + Na]^+$, 642.66 $[M + K^+]$, calcd for $C_{28}H_{37}N_5O_6S_2$ 603.76.

PEG-peptide (S5).
(a) Coupling reaction: Boc-protected amino-PEG-acid spacer **A** (78 mg, 0.19 mmol) was dissolved in dry CH_3CN (2 mL). Hydroxybenzotriazole monohydrate (31 mg, 0.23 mmol) and DCC (47.3 mg, 0.23 mmol) were sequentially added and the resulting reaction mixture was stirred at room temperature for 2 h under an argon atmosphere. Thereafter, TFA salt of dipeptide **S4** (136 mg, 0.19 mmol) was added and the resulting reaction mixture was stirred at room temperature for 2 h. The round-bottom flask was stored at -20 °C overnight. Thereafter, DIEA (32 µL, 0.19 mmol) was added. The reaction was checked for competion by RP-HPLC (system B). After 3, 5 and 7 h of stirring, DIEA (16 µL, 95 µmol) was added a further amount of DCC (12 mg, 58 µmol) was added after 3 h. Finally, reaction was quenched by the addition of acetic acid (21 µL, 360 µmol) and the round-bottom flask was stored at -20 °C overnight. After dilution in a mixture of CH_3CN-H_2O (2 : 1, v/v, 2 mL), the crude coupling product was purified by RP-HPLC (system D, 3 injections, t_R = 35.8-27.2 min). The product-containing fractions were lyophilised to give the coupling product as a white amorphous powder (119 mg, 0.12 mmol, yield 61%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.25-1.30 (t, 3H, J = 7.1 Hz, CH_3(SEt) Cys), 1.43 (s, 9H, tBu), 1.52-1.90 (m, 4H, CH_2 γ, δ Lys), 2.32 (bs, 2H, CH_2 β Lys), 2.64-2.69 (q, 2H, J = 7.2 Hz, CH_2(SEt) Cys), 2.97-3.16 (m, 2H, CH_2 β Cys), 3.30 (s, 2H, CH_2 linker), 3.45-3.65 (m, 11H, CH_2 ε Lys + 4 x CH_2 linker), 4.0 (s, 3H, CH_2 linker + CH Fmoc), 4.21-4.25 (q, J = 6.8 Hz, 1H, CH α Lys), 4.34 (s, 2H, CH_2), 4.44-4.50 (d, 2H, J = 10.0 Hz, CH_2 Fmoc), 4.70-4.72 (q, 1H, J = 2.3 Hz CH α Cys), 5.23 (bs, 1H, NH), 6.08 (bs, 1H, NH), 6.9 (bs, 1H, NH), 7.26-7.77 (m, 8H, CH Fmoc); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 14.4, 22.6, 28.5, 28.7, 31.0, 31.6, 32.5, 38.7, 38.8, 39.5, 40.5, 52.5, 53.3, 53.6, 70.0, 70.2, 70.5, 70.5, 70.6, 71.0, 71.1, 77.8, 79.5, 124.1, 128.6, 135.2, 156.1, 163.8, 167.1, 170.4, 170.7, 171.9; HPLC (system B): t_R = 20.8 min, purity 86%; MS (MALDI-TOF, positive mode): *m/z* 1016.66 $[M + Na]^+$, calcd for $C_{45}H_{67}N_7O_{14}S_2$ 994.20.
(b) Removal of Boc group: Full-protected pseudo-peptide (62 mg, 62 µmol) was dissolved in dry CH_2Cl_2 (3 mL). The resulting solution was cooled to 4 °C and TFA (368 µL, 4.96 mmol) was added dropwise. The resulting reaction mixture was stirred at room temperature for 1 h. The reaction was checked for completion by RP-HPLC (system B) and the mixture was evaporated to dryness. Deionised water was added and the resulting solution was lyophilised to give the PEG-peptide **S5** as a white foam (83 mg, 82 µmol, yield 86%). This compound was used in the next step without further purification. HPLC (system B): t_R = 16.5 min, purity 88%. MS (MALDI-TOF, positive mode): *m/z* 894.64 $[M + H]^+$, calcd for $C_{40}H_{59}N_7O_{12}S_2$ 894.08.

Full-protected heterotrifunctional cross-linker (4). TFA salt of PEG-peptide **S5** (36 mg, 36.7 µmol) was dissolved in dry DMF (500 µL). TEA (5 µL, 36.7 µmol) and 50 µL of a solution of DSC reagent in dry DMF (24 mg, 91.8 µmol) were sequentially added and the resulting reaction mixture was stirred at room temperature for 90 min. The reaction was checked for completion by RP-HPLC (system B). Finally, the reaction mixture was quenched by dilution with aq. TFA 0.1% (pH 2, 4 mL) and purified by RP-HPLC (system E, 2 injections, t_R = 31.3-32.7 min). The product-containing fractions were lyophilised to give **4** as a white amorphous powder (25 mg, 24.2 µmol, yield 74%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.24-1.27 (t, 3H, J = 7.1 Hz, CH_3(SEt) Cys), 1.32-1.90 (m, 6H, CH_2 β, γ, δ Lys), 2.63-2.69 (q, 2H, J = 7.1 Hz, CH_2(SEt) Cys), 2.81 (s, 4H, 2 x CH_2 succinimide), 2.24-3.28 (m, 2H, CH_2 β Cys), 3.24-3.69 (m, 11H, 5 x CH_2 linker + CH_2 ε Lys), 4.22-4.27 (t, 1H, CH Fmoc), 4.32 (s, 2H, CH_2), 4.51-4.53 (d, 2H, J = 6.4 Hz, CH_2 Fmoc), 4.58-4.66 (q, J = 7.5 Hz, 1H, CH α Lys), 4.70-4.76 (q, 1H, J = 6.0 Hz CH α Cys), 6.17 (bs, 1H, NH), 6.83 (s, 1H, NH), 7.27-7.83 (m, 8H, CH Fmoc), 9.2 (bs, 1H, NH); HPLC (system B): t_R = 18.5 min, purity 97%; MS

(MALDI-TOF, positive mode): *m/z* 1035.69 $[M + H]^+$, 1057.71 $[M + Na]^+$, 1073.68 $[M + K]^+$, calcd for $C_{45}H_{62}N_8O_{16}S_2$ 1035.17.

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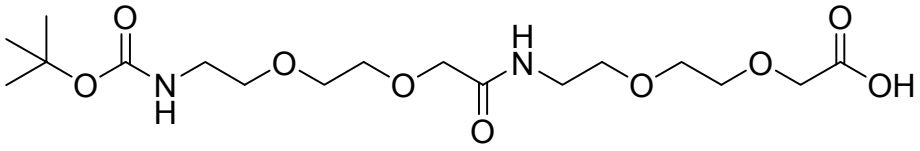
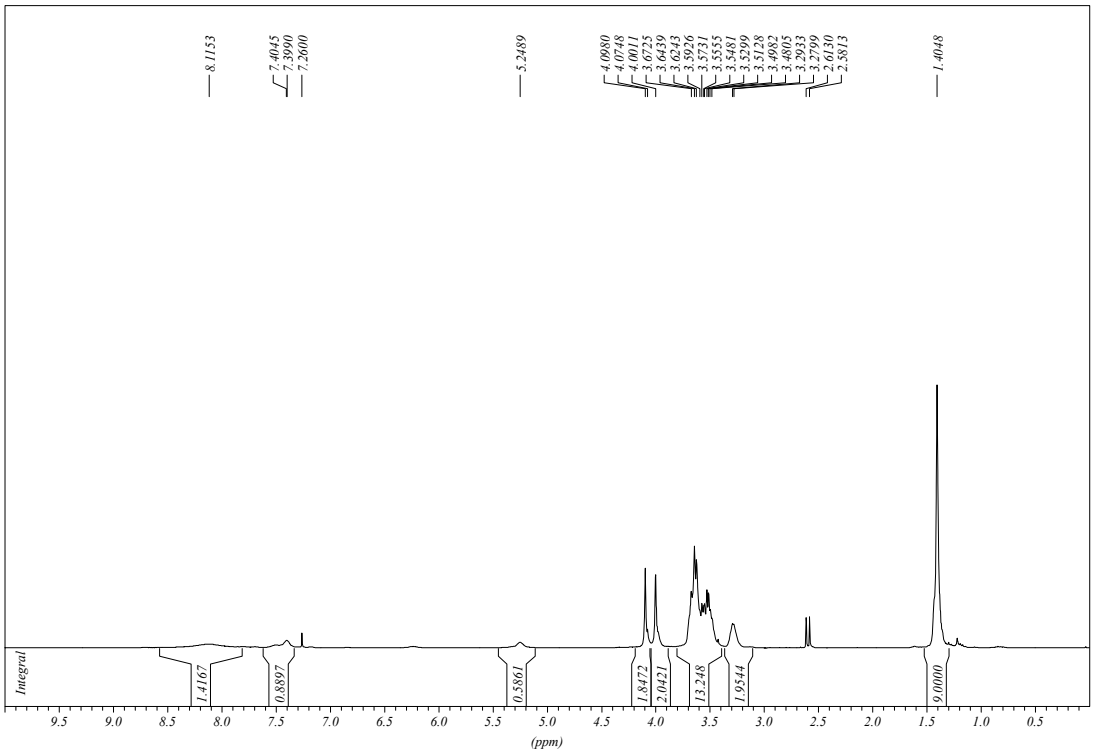
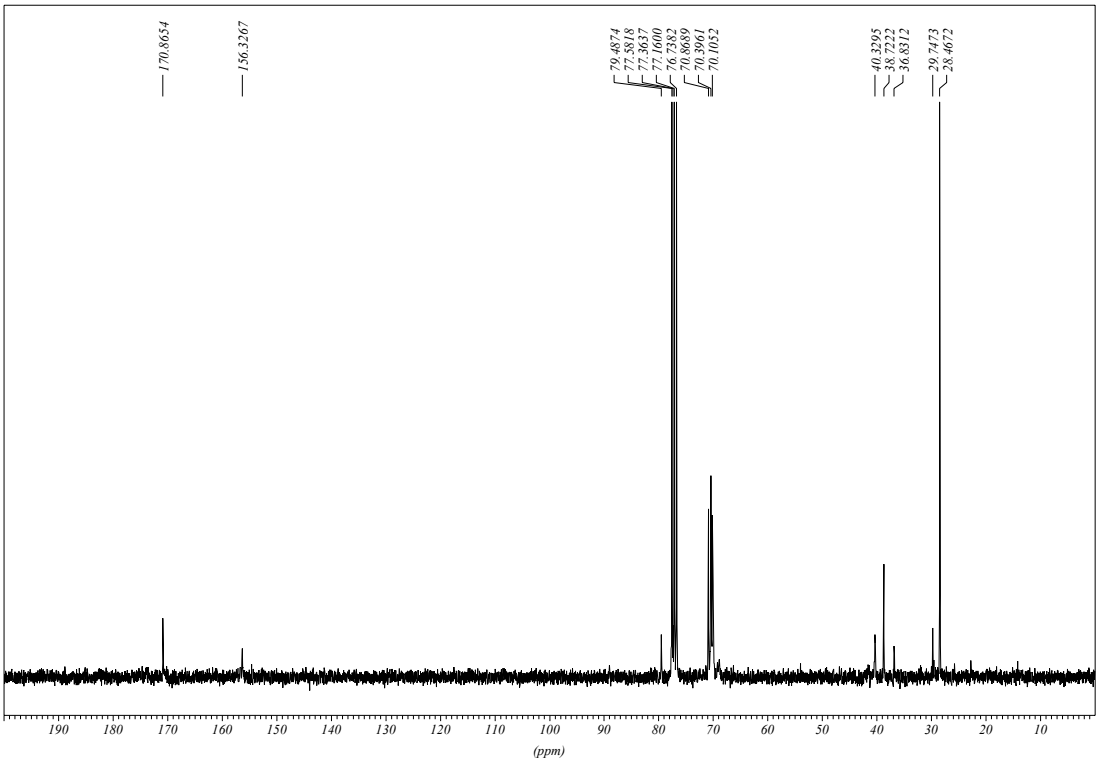
Boc-protected amino-PEG-acid spacer A



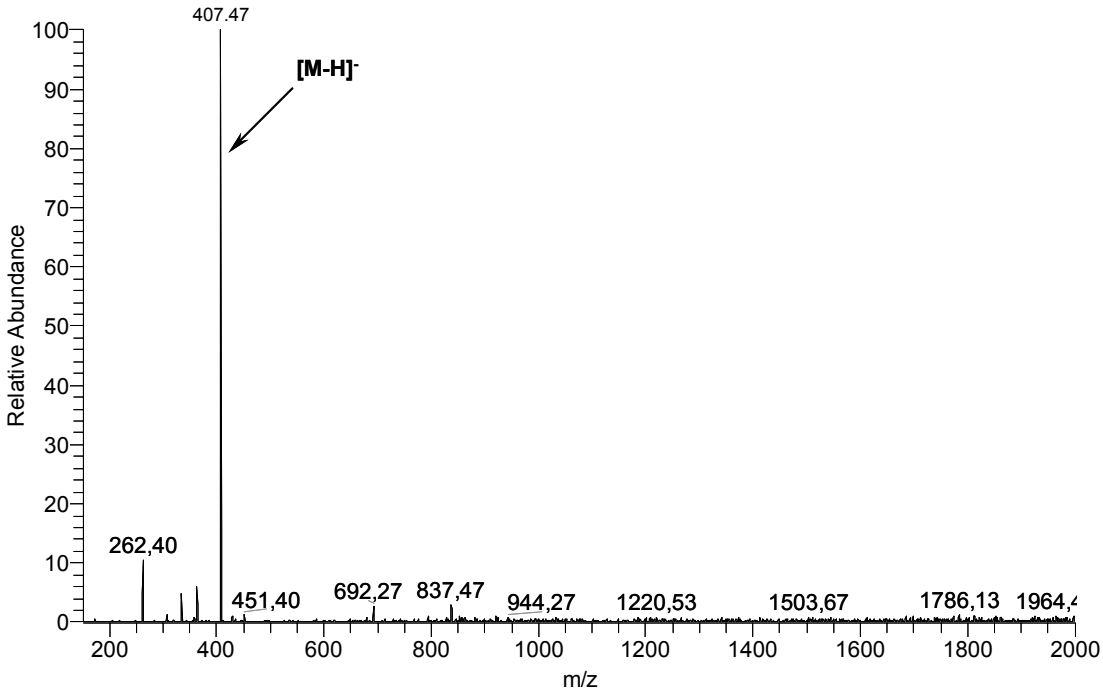
^{1}H NMR spectrum of **A** recorded in $CDCl_3$.



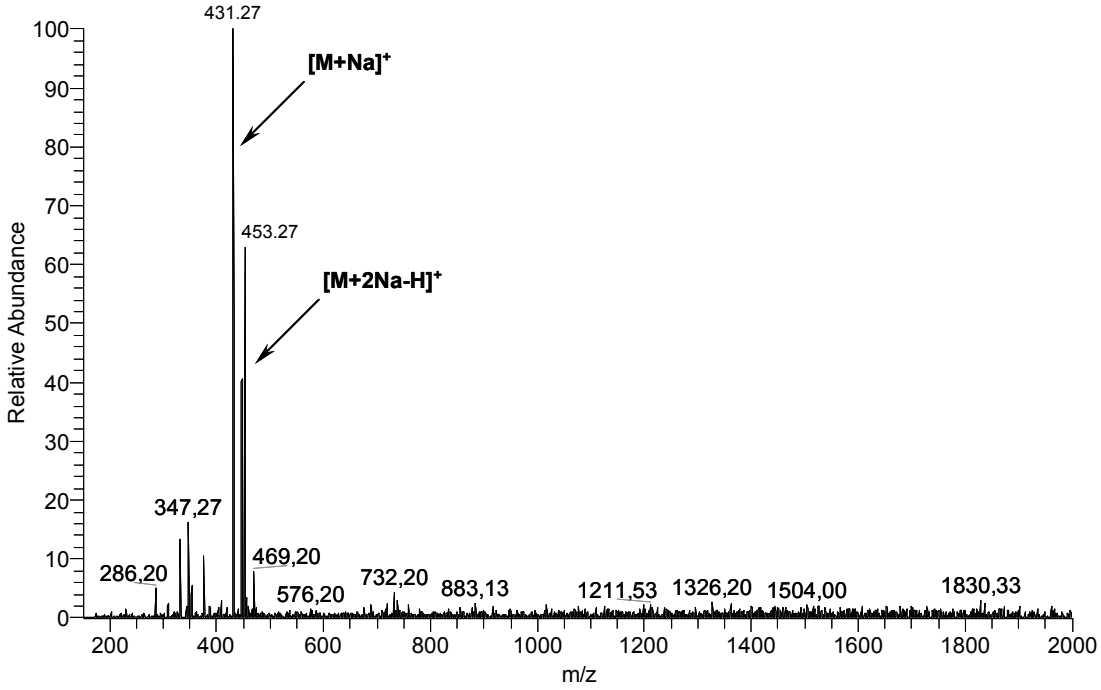
^{13}C NMR spectrum of **A** recorded in $CDCl_3$.



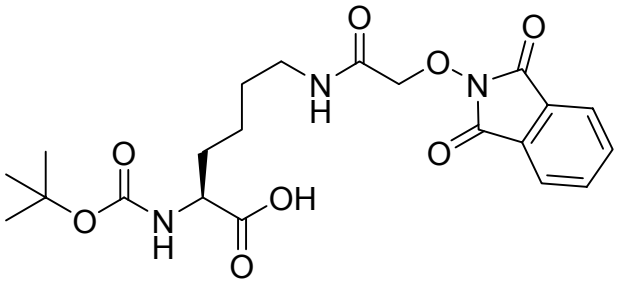
ESI-MS spectrum of **A** recorded in the negative mode.



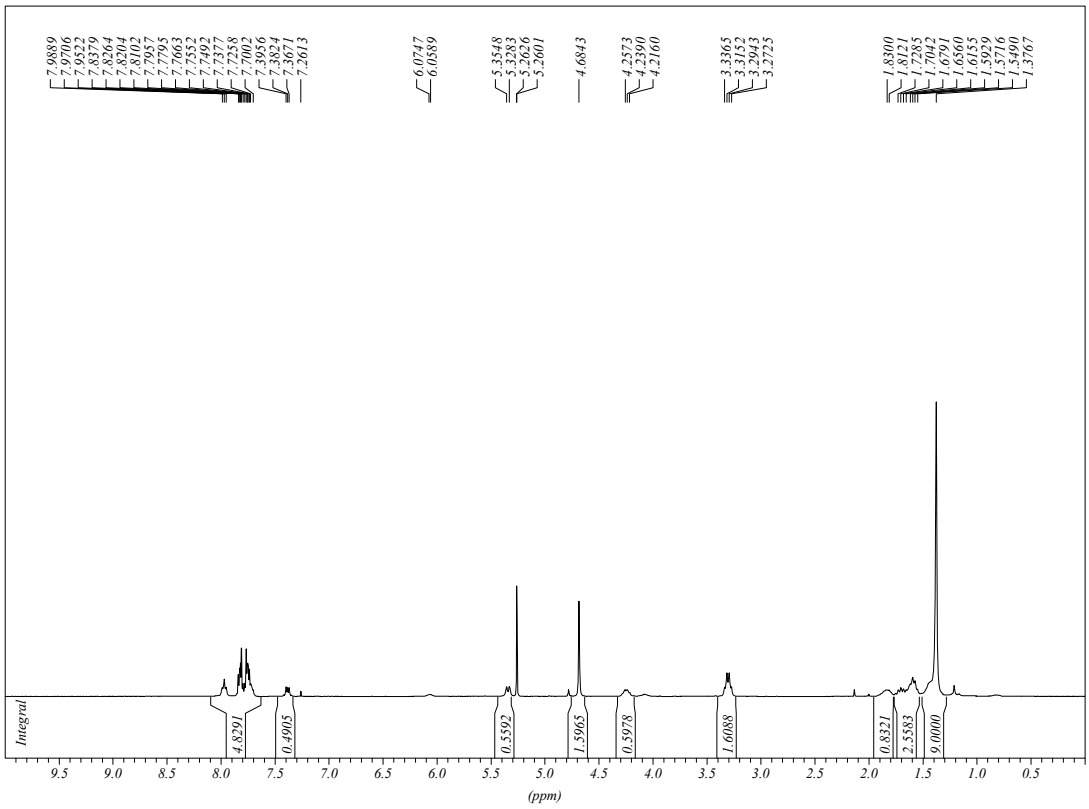
ESI-MS spectrum of **A** recorded in the positive mode.



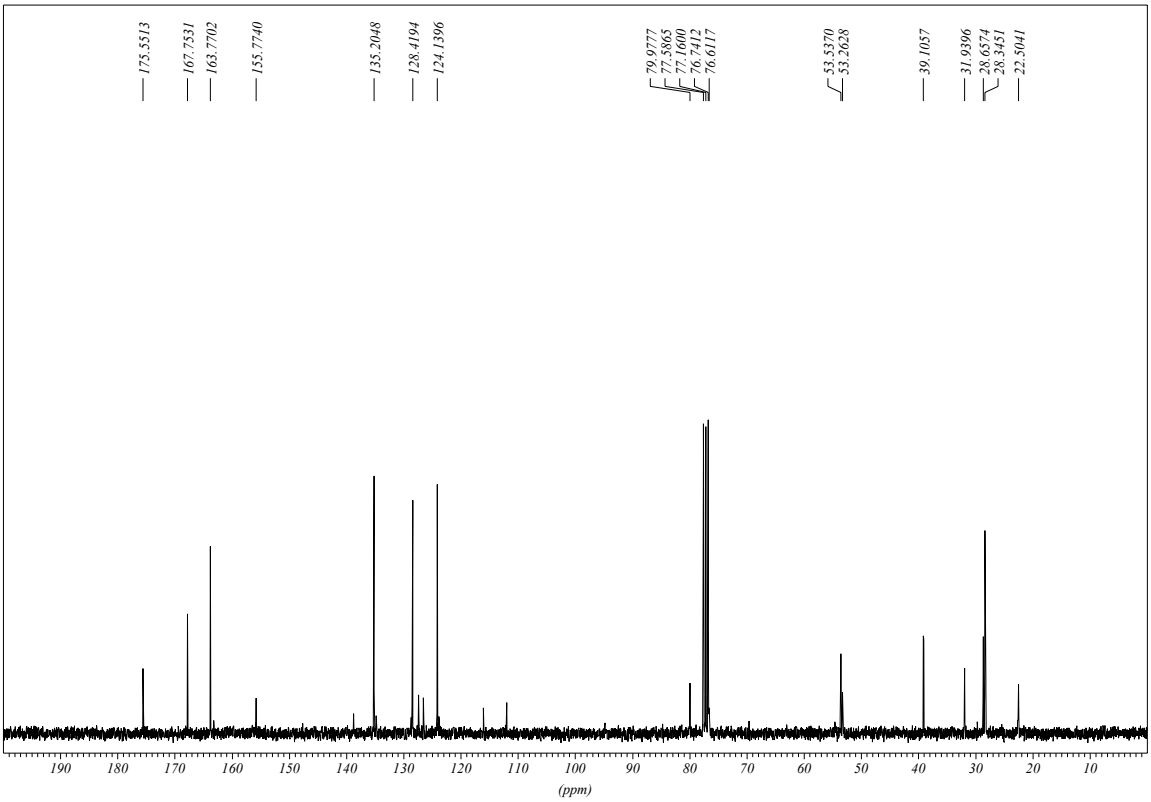
Aminooxy-containing lysine building block B



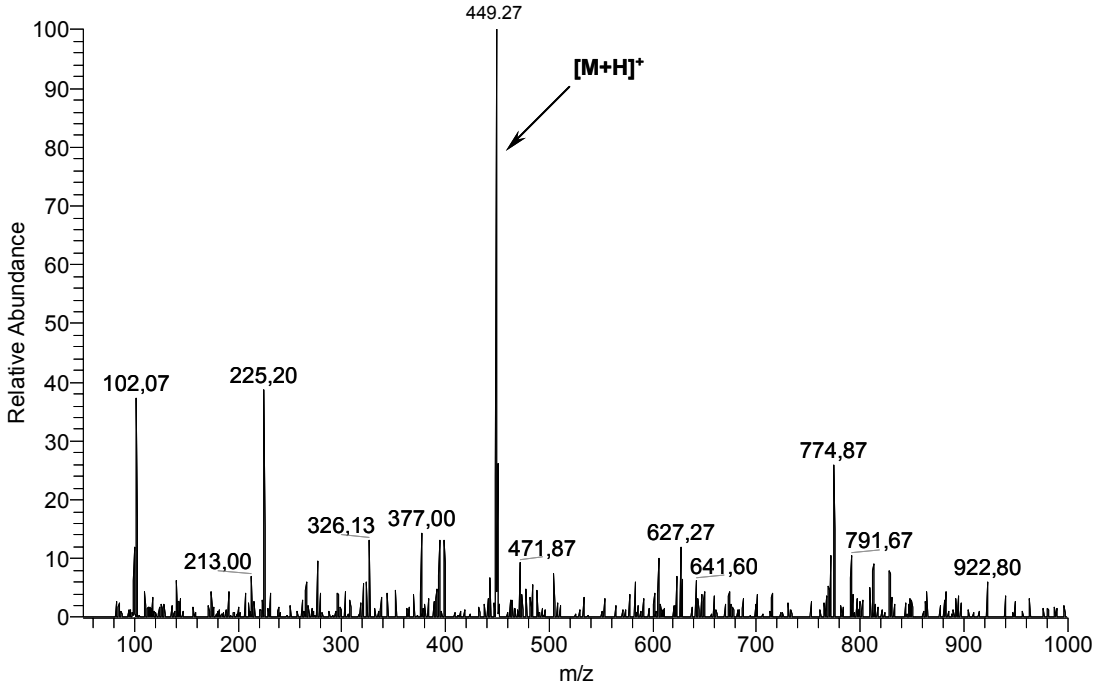
^{1}H NMR spectrum of **B** recorded in $CDCl_3$.



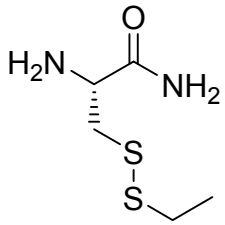
^{13}C NMR spectrum of **B** recorded in $CDCl_3$.



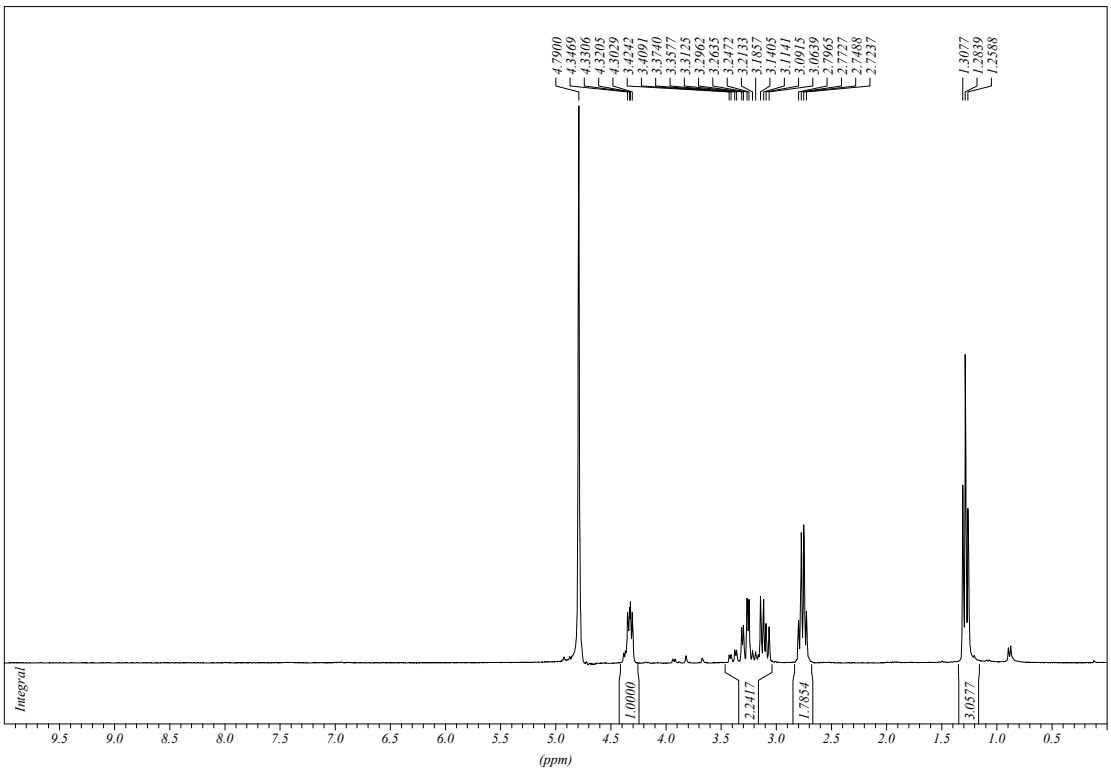
ESI-MS spectrum of **B** recorded in the positive mode.



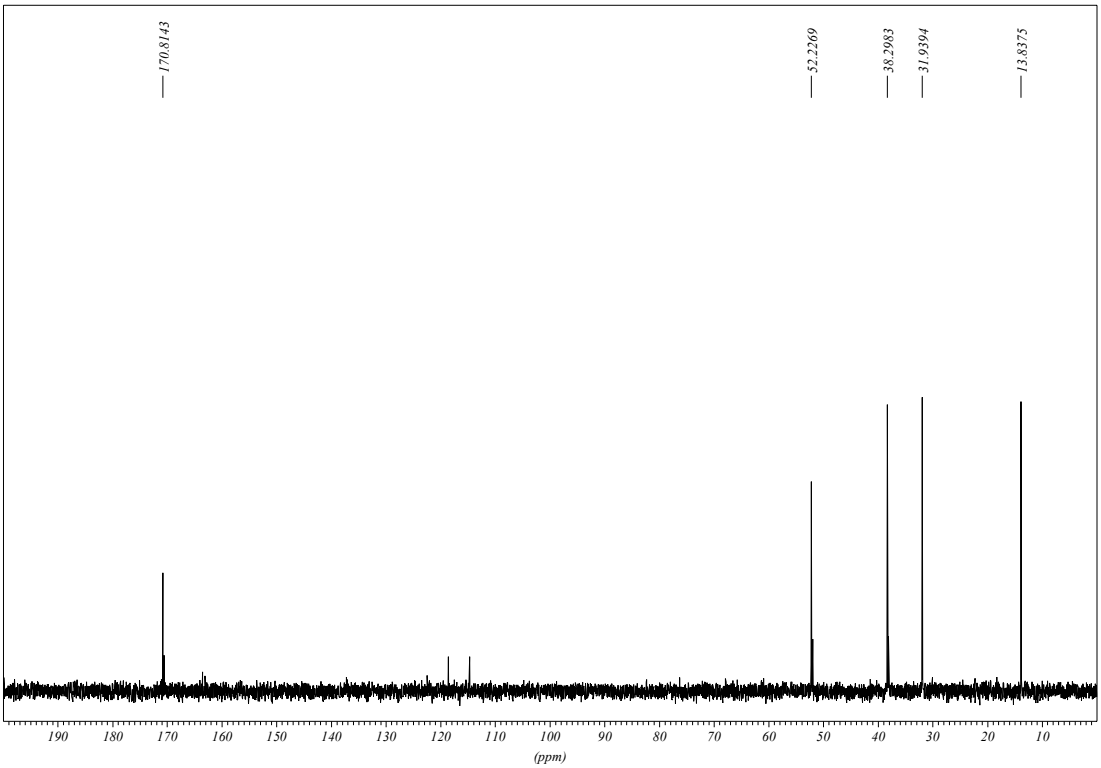
Cysteine building block C (TFA salt)



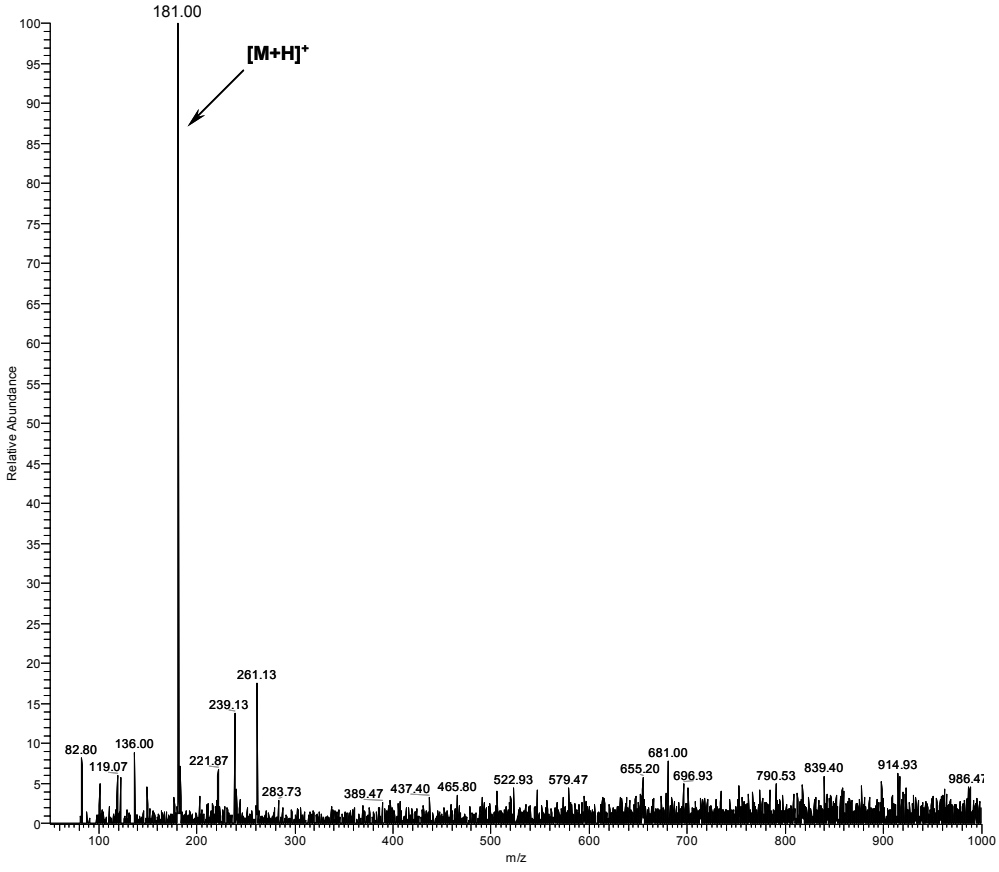
^{1}H NMR spectrum of **C** recorded in D_2O.



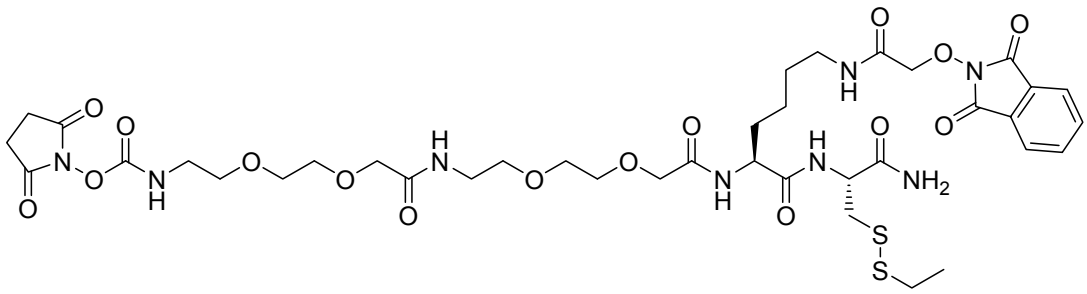
^{13}C NMR spectrum of **C** recorded in D_2O.



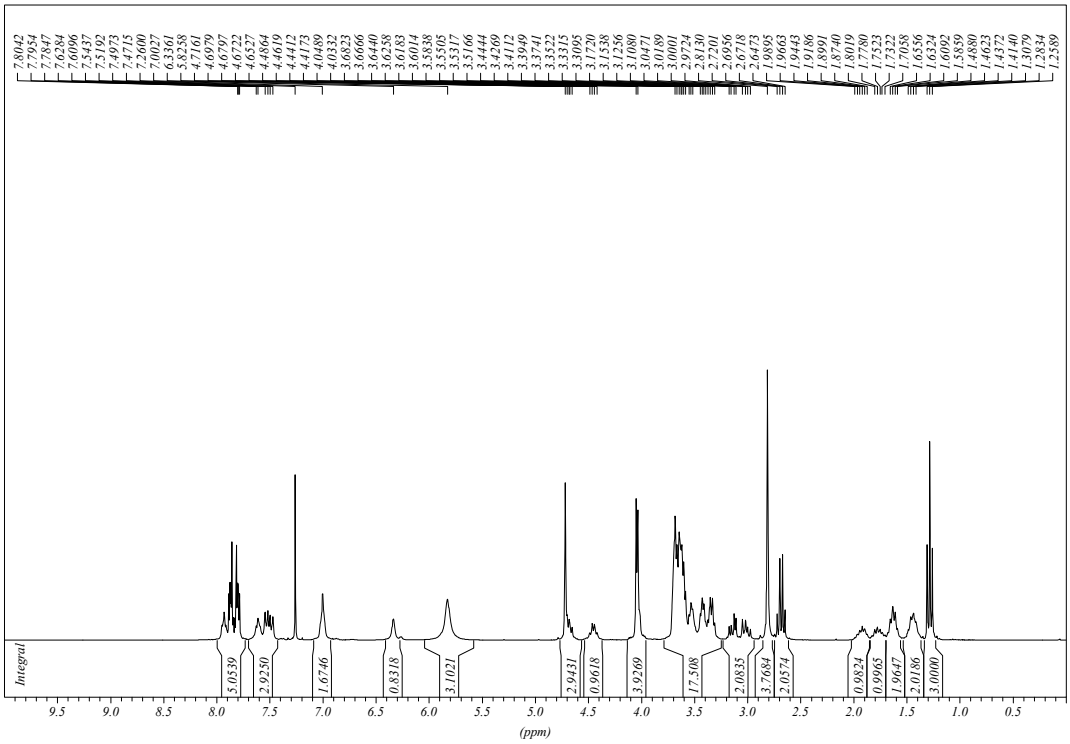
ESI-MS spectrum of **C** recorded in the positive mode.



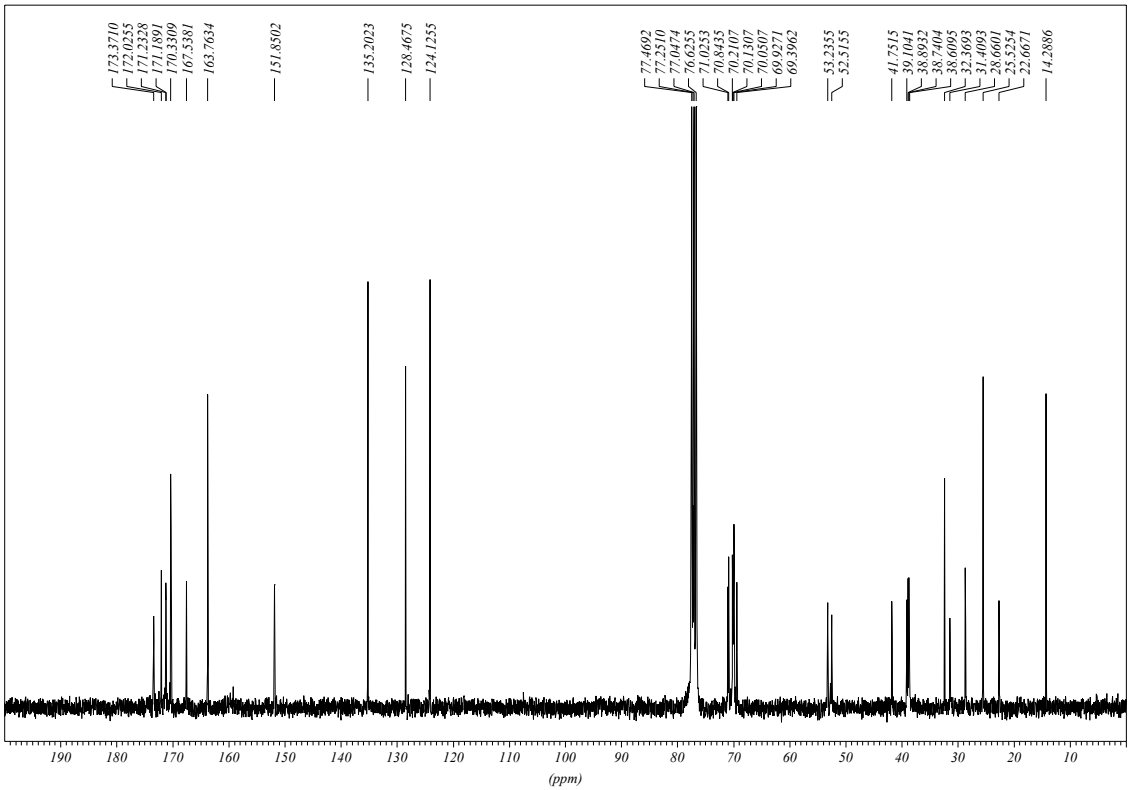
Heterotrifunctional cross-linking reagent 5



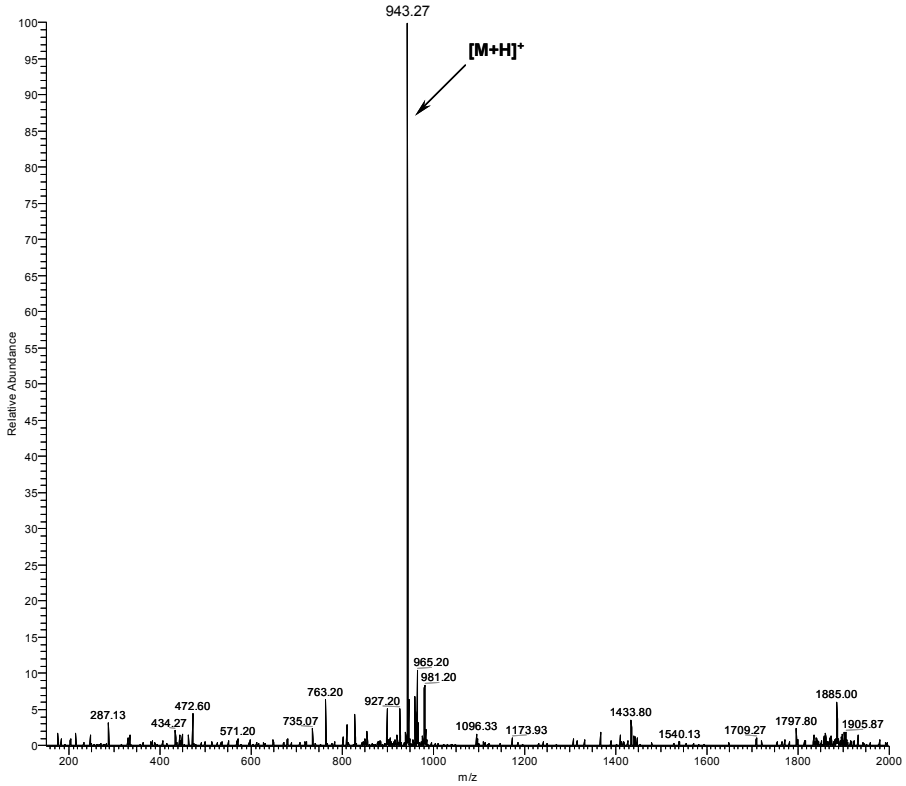
^{1}H NMR spectrum of **5** recorded in $CDCl_3$.



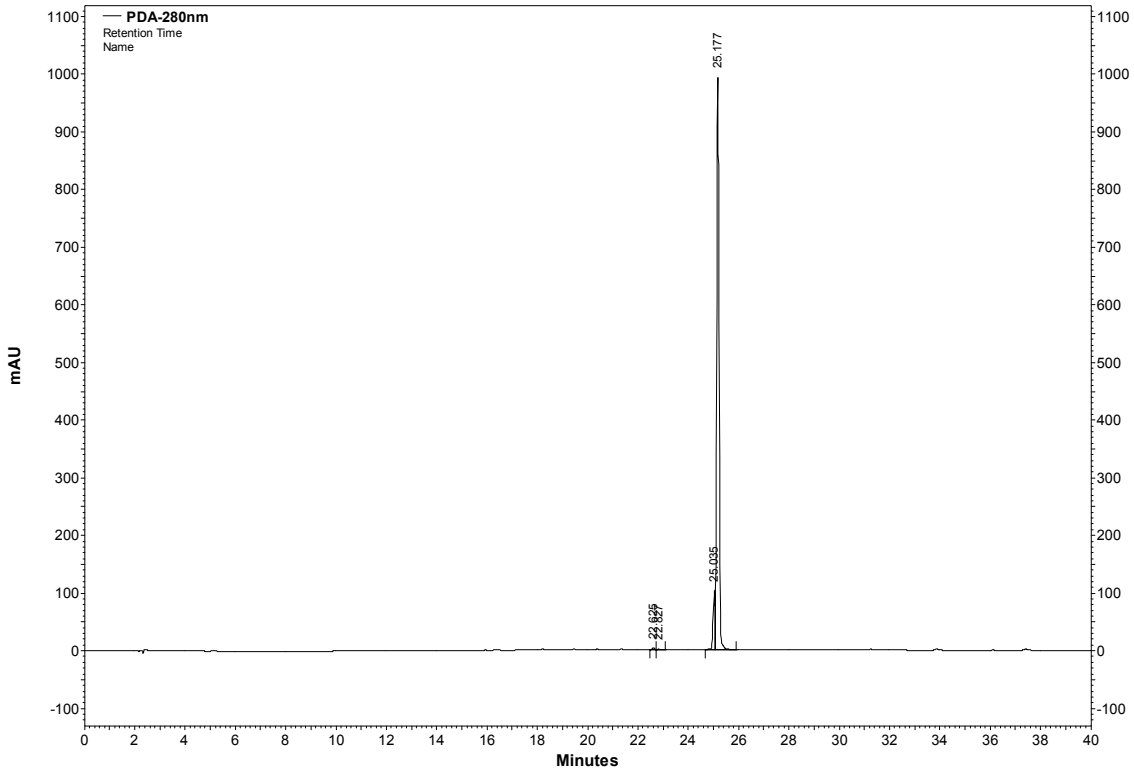
^{13}C NMR spectrum of **5** recorded in $CDCl_3$.



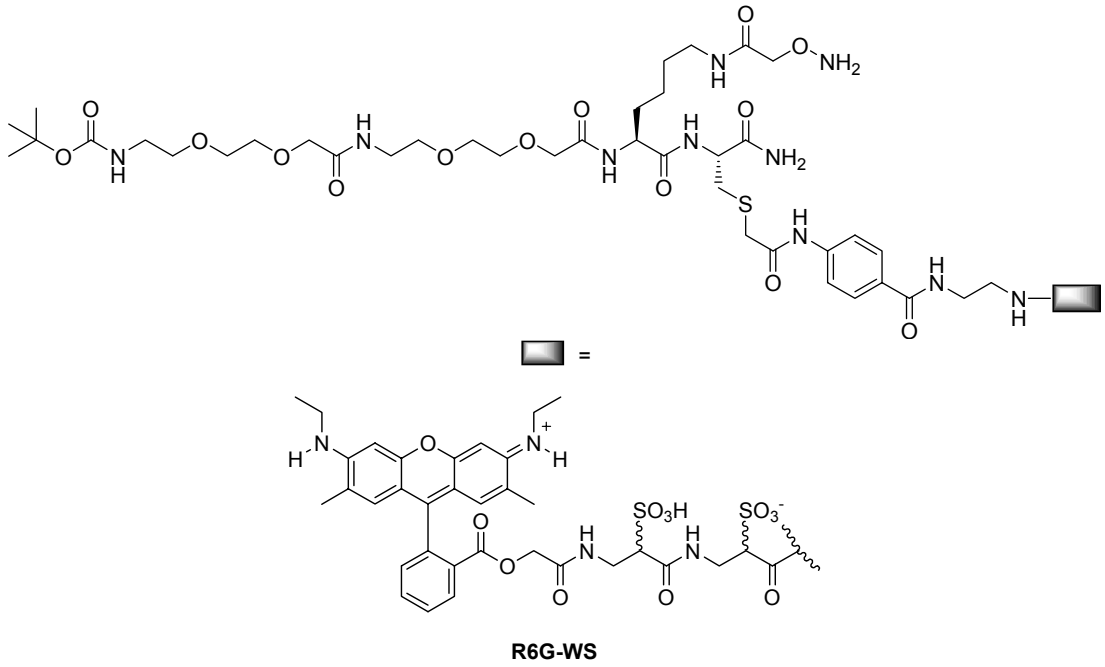
ESI-MS spectrum of **5** recorded in the positive mode.



RP-HPLC elution profile of **5** (system A).

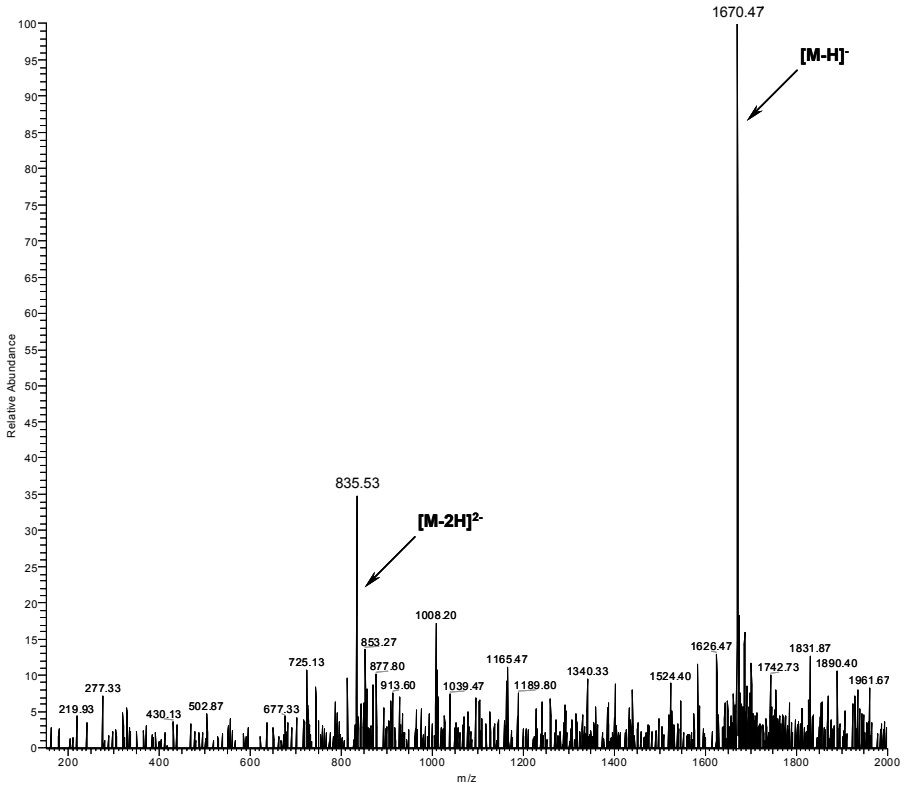


R6G-WS labeled aminooxy reagent 19



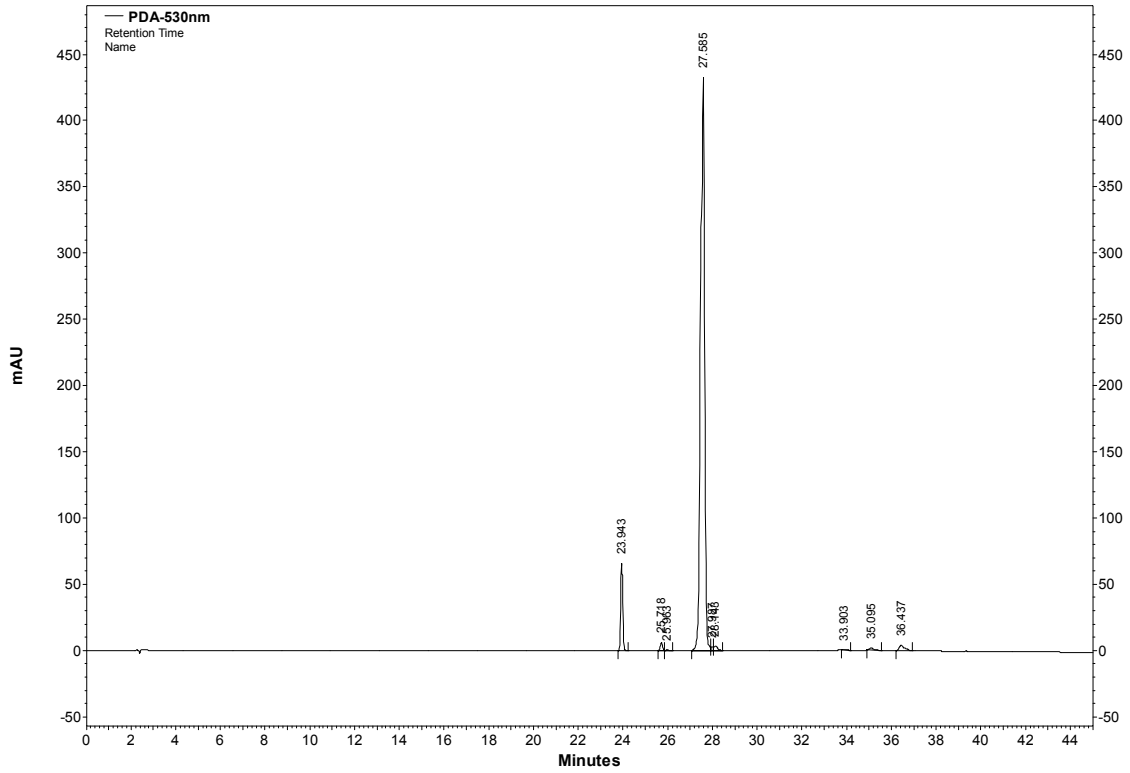
R6G-WS

ESI-MS spectrum of **19** recorded in the negative mode.[a]



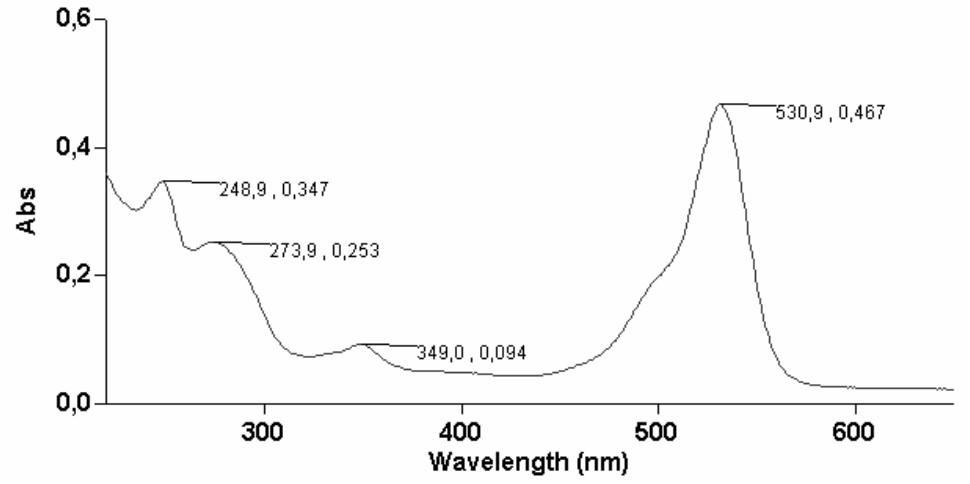
[a]Under our ionisation conditions, the aminooxy group of **19** was converted into the corresponding alcohol (ΔM of -15.0 Da). Such degradation reaction has been already reported by Bruné *et al.* (*Rapid. Commun. Mass Spectrom.* 2000, **14**, 2158) for the mass analysis of (aminooxy)acetyl peptides involved in oxime ligation reactions.

RP-HPLC elution profile of **19** (system B).[a]

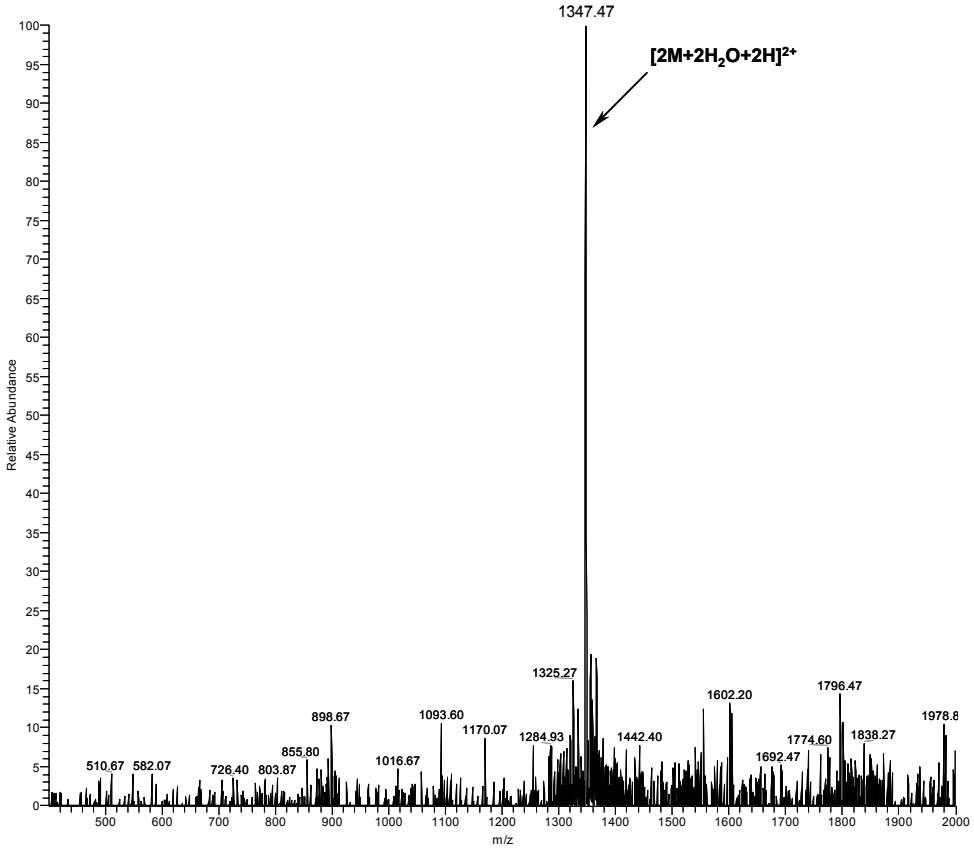


[a]Partial cleavage of Boc group was occurred during the HPLC analysis due to the acidity of aq. mobile phase.

UV-visible absorption of **19** in deionised water at 25°C (concentration = 4.2 μM).



ESI-MS spectrum of fluorescent substance P-tripod **23** recorded in the positive mode.[a]



[a]Only the doubly charged hydrated ion of **23** ($[M + 2H_2O + 2H]^{2+}$: *m/z* calcd mass 1347.10, found 1347.47) was observed under our ionisation conditions. Production of such solvated ions through ESI ionisation has been already reported especially for peptidyl biopolymers (see: Rodriguez-Cruz *et al. J. Am. Soc. Mass Spectrom.*, 1999, **10**, 958 and Wyttenbach *et al. Int. J. Mass Spectrom.*, 2005, **240**, 221).