The first "ready-to-use" benzene-based heterotrifunctional cross-linker for multiple bioconjugation

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Supporting Information

Abbreviations

The following abbreviations are used throughout the text of the ESI file: APCI, Atmospheric pressure chemical ionization; BSA, bovine serum albumin; Cy, cyanine; DIEA, *N*,*N*-diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; DMSO, dimethylsulfoxide; ESI, electrospray ionisation; ODN, oligodeoxynucleotide; NHS, *N*-hydroxysuccinimide; NMP, *N*-methylpyrrolidone; PBS, phosphate buffered saline; RP-HPLC, reversed-phase high performance liquid chromatography; R6G, rhodamine 6G; rt, room temperature; Sulfo-MBS, *m*-maleimidobenzoyl-*N*-hydroxysulfosuccinimide ester; Sulfo-SIAB, sodium sulfosuccinimidyl[4-iodoacetyl]aminobenzoate; TEAA, triethylammonium acetate; TEAB, triethylammonium bicarbonate; TFA, trifluoroacetic acid; TSTU, *O*-(*N*-succinimidyl)-1,1,3,3-tetramethyluronium tetrafluoroborate.

High-performance liquid chromatography separations

Several chromatographic systems were used for the analytical experiments and the purification steps: System S1: RP-HPLC (Thermo Hypersil GOLD C_{18} column, 5 µm, 2.1 × 100 mm) with CH_3CN and triethylammonium acetate buffer (TEAA 25 mM, pH 7.0) as eluents [100% TEAA (5 min), followed by linear gradient from 0 to 80% (40 min) of CH_3CN] at a flow rate of 0.25 mL min^{-1}. UV-vis detection with the "Max Plot" (*i.e.*, chromatogram at absorbance maximum for each compound) mode (220-750 nm). System S2: semi-preparative RP-HPLC (Thermo Hypersil GOLD C_{18} column, 5 µm, 10 × 250 mm) with CH_3CN and TEAB (50 mM, pH 7.5) as eluents [100% TEAB (5 min) followed by linear gradient from 0 to 80% (40 min) of CH_3CN] at a flow rate of 4.0 mL min^{-1}. Visible detection was achieved at 680 nm. System S3: semi-preparative RP-HPLC (Thermo Hypersil GOLD C_{18} column, 5 µm, 21.2 × 250 mm) with CH_3CN and 0.1% aq. trifluoroacetic acid (aq. TFA, 0.1%, v/v, pH 2.2) as eluents [100% TFA (5 min), followed by linear gradient from 0 to 20% (15 min) and 20 to 100% (80 min) of CH_3CN] at a flox rate of 15 mL min^{-1}. Dual UV detection was achieved at 224 and 275 nm. System S4: system S1 with CH_3CN and aq. TFA (0.1%, pH 2.2) as eluents. System S5: system S3 with the following gradient [100% TFA (5 min), followed by linear gradient from 0 to 20% (12.5 min) and 20 to 40% (60 min) of CH_3CN]. Dual UV detection was achieved at 225 and 280 nm. System S6: LC-MS under the following conditions: RP-HPLC (Thermo Hypersil GOLD C_{18} column, 5 µm, 2.1 × 150 mm) with CH_3CN and TEAB (25 mM, pH 7.5) as eluents [100% TEAB (5 min) followed by linear gradient from 0 to 80% (40 min) of CH_3CN] at a flow rate of 0.25 mL min^{-1}. Dual UV detection was achieved at 260 and 345 nm. ESI-MS detection in the negative mode (full scan, 150-1500 a.m.u., data type: centroid, sheat gas flow rate: 60 arb unit, aux/sweep gas flow rate: 20, spray voltage: 4.5 kV, capillary temp: 270 °C, capillary voltage: -10 V, tube lens offset: -50 V). System S7: semi-preparative RP-HPLC (Thermo Hypersil GOLD C_{18} column, 5 µm, 10 × 100 mm) with CH_3CN and TEAB (50 mM, pH 7.5) as eluents [100% TEAB (5 min) followed by linear gradient from 0 to 15% (10 min) and 15 to 65% (100 min) of CH_3CN] at a flow rate of 4.0 mL min^{-1}. Dual UV detection was achieved at 250 and 330 nm. System S8: system S4 with the following gradient [100% TFA (5 min), followed by linear gradient from 0 to 100% (35 min) of CH_3CN] at a flow rate of 0.25 mL min^{-1}. UV-vis detection with the "Max Plot" (*i.e.*, chromatogram at absorbance maximum for each compound) mode (220-750 nm). System S9: semi-preparative RP-HPLC (Thermo Hypersil GOLD C_{18} column, 5 µm, 10 × 100 mm) with CH_3CN and aq. TFA (0.1%, pH 2.2) as eluents [100% TFA (5 min), followed by linear gradient from 0 to 20% (15 min) and 20% to 40% (80 min) of CH_3CN]. Dual visible detection was achieved at 647 and 700 nm.

Table S1 Relative quantum yields of cyanine and Eu(III) chelate derivatives studied in this work.

Fluorophore (F)	Solvent	λ Ex. (nm)	Standard (std)	Φ_{std} / solvent	Φ_F
Cy 7.0 alkyne 12	PBS[a]	710	IR125[1]	0.11 / DMSO[b]	0.12
Cy 7.0 alkyne 12	PBS + 5% BSA[a]	710	IR125[1]	0.11 / DMSO[b]	0.17
Cy 5.0 labelled tripod 13	PBS + 5% BSA[a]	600	Cy 5.0[2]	0.29 / PBS + 5% BSA	0.40
FRET cascade 15	PBS	500	R6G[3]	0.76 / PBS	0.003[c, d]
Cy 3.0	PBS	500	R6G[3]	0.76 / PBS	0.065[c,d]
Eu(III) labelled peptide-tripod 20	H_2O	345	Eu(TMT)-AP_3[4]	0.169 / H_2O	0.082[e]
Eu(III) labelled peptide-ODN 21	TEAA (0.1 M, pH 7.0)	345	Eu(TMT)-AP_3[4]	0.182 / TEAA	0.095[e]

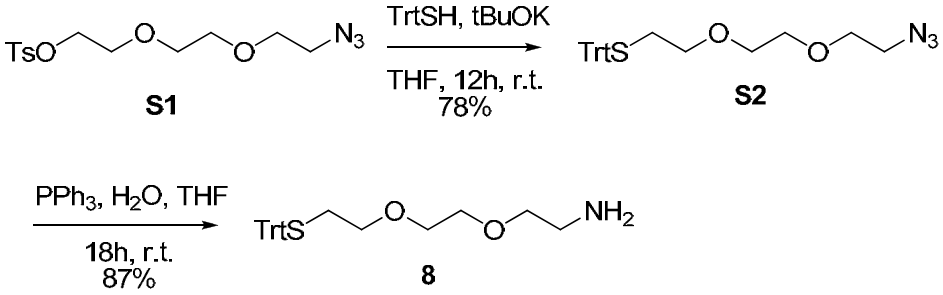
[a]refractive index = 1.337, [b]refractive index = 1.479. [c]determined for the emission range of donor Cy 3.0 (510 - 630 nm) because this is the only spectral region for which there is no residual emission of other cyanine units. [d]Energy transfer efficiency E.T.E (%) was calculated based on the equation: E.T.E = {100 × [1 - (Φ_F (donor Cy 3.0 in the FRET cascade))/(Φ_F (free donor Cy 3.0))]%} to be 95%.[5] [e]emission spectra were recorded in "phosphorescence mode" (500 - 800 nm) with the following parameters: delay time: 0.1 ms, gate time: 3 ms and total decay time: 10 ms, and after excitation at 345 nm.

Synthesised compounds

[1] S. Reindl, A. Penzkofer, S. H. Gong, M. Landthaler, R. M. Szeimies, C. Abels and W. Baeumler, *J. Photochem. Photobiol., A: Chem.*, 1997, **105**, 65.
[2] R. B. Mujumdar, L. A. Ernst, S. R. Mujumdar, C. J. Lewis and A. S. Waggoner, *Bioconjugate Chem.*, 1993, **4**, 105.
[3] J. Olmsted, III, *J. Phys. Chem.*, 1979, **83**, 2581.
[4] N. Maindron, S. Poupart, M. Hamon, J.-B. Langlois, N. Plé, L. Jean, A. Romieu and P.-Y. Renard, *Org. Biomol. Chem.*, 2011, **9**, 2357.
[5] G.-S. Jiao, A. Loudet, H. B. Lee, S. Kalinin, L. B. A. Johansson and K. Burgess, *Tetrahedron*, 2003, **59**, 3109.

Azido-PEG linkers **5** and **S1** have been already reported in the literature and synthesised according to published protocols.[6,7]

S-Trityl amino-PEG linker **8** was prepared from tosyl derivative **S1** by using the two-step synthetic procedure described in Scheme S1:



Scheme S1 Synthetic reactions used for the synthesis of **8**.

2-(2-(2-(Tritylthio)ethoxy)ethoxy)ethanamine (8).
(a) S_N2 reaction with TrtSH: To a stirred solution of triphenylmethanethiol (TrtSH, 0.67 g, 2.42 mmol) in dry THF (10 mL) at 0 °C was added *t*BuOK (0.29 g, 2.59 mmol). The resulting mixture was stirred 5 min at 0 °C before it was added a solution of product **S1** (0.57 g, 1.73 mmol) in dry THF (5 mL). The resulting reaction mixture was stirred at rt for 12 h before it was quenched with sat. aq. NH_4Cl (10 mL). The layers were separated and the aqueous layer was extracted with EtOAc (3 × 15 mL). The combined organic layers were washed with brine (20 mL), dried (over anhydrous Na_2SO_4) and concentrated in *vacuo*. Flash-column chromatography (silica gel, cyclohexane-EtOAc, 9 : 1, v/v) afforded azide **S2** (584 mg, yield 78%) as a colourless oil. R_f 0.30 (cyclohexane-EtOAc, 8 :2, v/v); ν_{max}(neat)/cm^{-1} 2863, 2096, 1586, 1491, 1443, 1286, 1111, 1032, 741, 697, 675, 628, 621, 616; δ_H(300 MHz; $CDCl_3$) 7.60-7.50 (m, 6 H), 7.46-7.35 (m, 9 H), 3.80-3.65 (m, 4 H), 3.62-3.55 (m, 2 H), 3.48-3.40 (m, 4 H), 2.57 (t, *J* 6.9, 2 H); δ_C(75 MHz; $CDCl_3$) 144.9, 129.7, 127.9, 126.9, 70.6, 70.3, 70.1, 69.8, 66.7, 50.7, 31.7; LRMS (APCI+): *m/z* 451.16 $[M + H_2O]^{+\bullet}$ (water cluster formed during the ionisation process), calcd for $C_{25}H_{27}N_3O_2S$ 433.18.
(b) Staudinger reduction: To a stirred solution of azide **S2** (292 mg, 0.67 mmol) in THF (5 mL) were added deionised water (0.1 mL) and PPh_3 (350 mg, 1.33 mmol). The resulting mixture was stirred at rt for 18 h before it was diluted with deionised water (15 mL) and extracted with EtOAc (3 × 15 mL). The combined organic layers were washed with brine (20 mL), dried (over anhydrous Na_2SO_4) and concentrated in *vacuo*. Flash-column chromatography (silica gel, CH_2Cl_2-MeOH, 9 : 1, v/v) afforded primary amine **8** (240 mg, yield 87%) as a colourless oil. R_f 0.30 (CH_2Cl_2-MeOH, 9 : 1, v/v); ν_{max}(neat)/cm^{-1} 2865, 1594, 1488, 1443, 1351, 1105, 1034, 742, 698, 616; δ_H(300 MHz; $CDCl_3$) 7.55-7.50 (m, 6 H), 7.40-7.20 (m, 9 H), 3.62-3.50 (m, 6 H), 3.40 (t, *J* 6.7, 2 H), 2.91 (t, *J* 4.9, 2 H), 2.53 (t, *J* 6.7, 2 H), 2.42 (bs, 2 H); δ_C(75 MHz; $CDCl_3$) 144.8, 129.6, 127.9, 126.7, 72.8, 70.1, 70.1, 69.6, 66.6, 41.5, 31.7; HRMS (ESI+): *m/z* 408.1993 $[M + H]^+$, calcd for $C_{25}H_{30}NO_2S^+$ 408.1997.

[6] (a) R. Appel, *Angew. Chem. Int. Ed.*, 1975, **14**, 801; (b) E. Klein, S. DeBonis, B. Thiede, D. A. Skoufias, F. Kozielski and L. Lebeau, *Bioorg. Med. Chem.*, 2007, **15**, 6474; (c) C. A. Hurley, J. B. Wong, J. Ho, M. Writer, S. A. Irvine, M. J. Lawrence, S. L. Hart, A. B. Tabor and H. C. Hailes, *Org. Biomol. Chem.*, 2008, **6**, 2554; (d) J. Xiao and T. J. Tolbert, *Org. Lett.*, 2009, **11**, 4144.
[7] D. J. Leaver, R. M. Dawson, J. M. White, A. Polyzos and A. B. Hughes, *Org. Biomol. Chem.*, 2011, **9**, 8465.

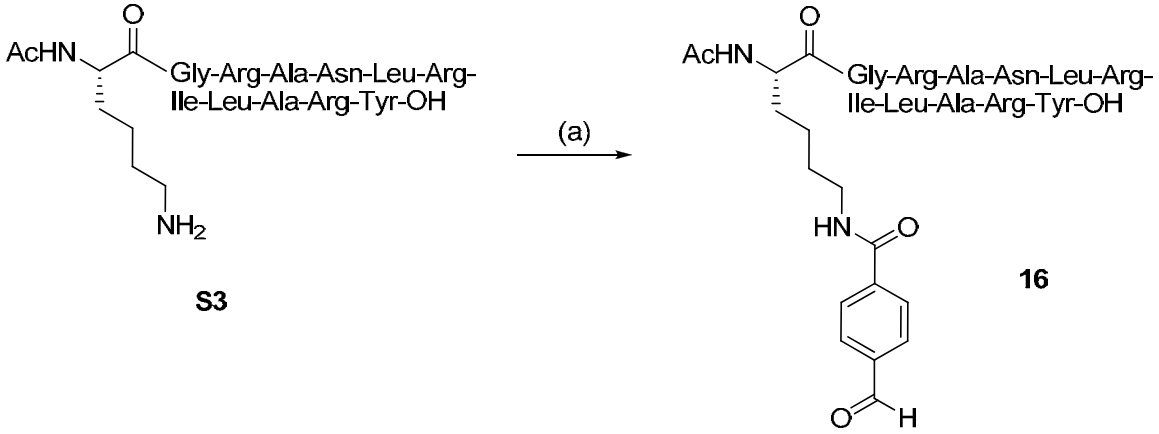
Fluorescent labelling and bioconjugation

Bioconjugatable cyanine dyes Cy 5.0-aldehyde **10** and Cy 3.0-IAB **11** have been already reported by us.[8]

Preparation of Cy 7.0-alkyne (12). Cy 7.0 carboxylic acid[2] (4.0 mg, 5.2 µmol) was introduced into a Reacti-Vial™ and dissolved in 0.3 mL of dry NMP. TSTU reagent (1.6 mg, 5.3 µmol) and DIEA (3.6 µL, 345 µmol) were added and the resulting reaction mixture was protected from light and stirred at rt for 1 h. The reaction was checked for completion by RP-HPLC (system S1) and the resulting solution was added to a solution of propargylamine (3.3 µL, 156 µmol) in 30 µL of dry NMP at 4 °C. The resulting reaction mixture was protected from light and stirred at 4 °C for 2 h. The reaction was checked for completion by RP-HPLC (system S1) and the mixture was evaporated to dryness and dissolved in aq. TEAB (5 mL, 50 mM, pH 7.5) and purified by semi-preparative RP-HPLC (system S2). The product containing fractions were lyophilised to give Cy 7.0-alkyne **12** as a green amorphous powder (0.26 mg, yield 6%). HPLC (system S1): t_R = 24.7 min (purity >95%); LRMS (ESI+): *m/z* 806.33 $[M + H]^+$, calcd for $C_{42}H_{51}N_3O_9S_2$: 805.31; λ_{max} (PBS) nm 750 (ε/dm^3 mol^{-1} cm^{-1} 200 000), λ_{max} em (PBS) nm 770 (Φ_F 0.12 in PBS).

Dodecapeptide (S3).[9] Purification by semi-preparative RP-HPLC (system S3). The product-containing fractions were lyophilised to give the TFA salt of dodecapeptide **S3** as a white amorphous powder (83 mg, yield 43%). HPLC (system S4): t_R = 23.7 min (purity >95%); LRMS (ESI+): *m/z* 737.07 $[M + 2H]^{2+}$, found 1472.14, calcd 1472.77.

Peptide-aldehyde (16).



Scheme S2 (a) 4-Formylbenzoic acid, CH_3CN, DMF, NHS, DCC, rt, 3 h, then dodecapeptide **S6**, 0.1 M aq. $NaHCO_3$ buffer, pH 8.5, , 4 °C, 2 h, 41% after RP-HPLC purification.

4-Formylbenzoic acid (10.0 mg, 66.6 µmol) was dissolved in a mixture of dry CH_3CN (0.6 mL) and DMF (0.2 mL). NHS (7.7 mg, 66.6 µmol) and DCC (13.7, 66.6 µmol) were sequentially added. The resulting reaction mixture was stirred at rt for 3 h. TFA salt of dodecapeptide **S3** (20 mg, 10 µmol) was dissolved in 0.1 M aq. $NaHCO_3$ buffer (0.5 mL) and cooled to 4 °C. Then, 200 µL of mixture containing the NHS ester of 4-formylbenzoic acid was added dropwise. The reaction mixture was stirred at 4 °C for 2 h. The reaction was checked for completion by RP-HPLC (system S4) and then quenched by addition of aq. TFA

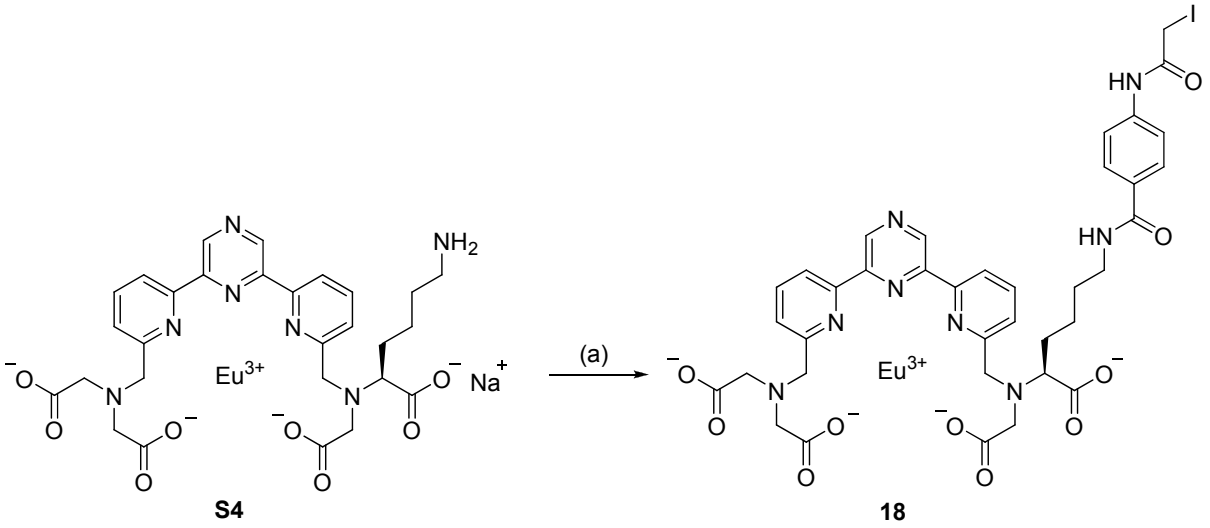
[8] G. Clavé, H. Volland, M. Flaender, D. Gasparutto, A. Romieu and P.-Y. Renard, *Org. Biomol. Chem.*, 2010, **8**, 4329.

[9] T. Priem, C. Bouteiller, D. Camporese, A. Romieu and P.-Y. Renard, *Org. Biomol. Chem.*, 2012, **10**, 1068.

0.1% (4 mL). The mixture was purified by semi-preparative RP-HPLC (system S5, 2 injections). The product-containing fractions were lyophilised to give peptide-aldehyde **16** as a white amorphous powder (8.7 mg, yield 41%). HPLC (system S4): t_R = 25.9 min (purity >95%); LRMS (ESI-): *m/z* 1602.67 $[M - H]^-$, calcd for $C_{73}H_{117}N_{23}O_{18}$: 1603.89.

16-mer ODN-alkyne (17).[8] HPLC (system S6): t_R = 16.9 min (purity >95%); LRMS (ESI-): *m/z* 1622.94 $[M - 3H]^{3-}$, found 4871.82, calcd 4872.20.

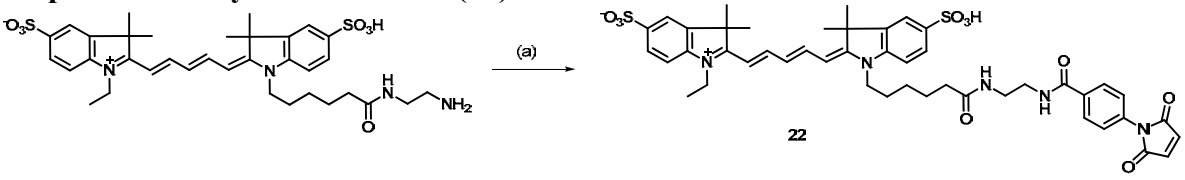
Thiol-reactive Eu(III) chelate (18).



Scheme S3 (a) Eu(III) chelate **S4**, 0.1 M aq. $NaHCO_3$ buffer, pH 8.5, Sulfo-SIAB reagent, H_2O, rt, 2 h, RP-HPLC purification.

Amine-containing Eu(III) chelate[4] **S4** (1.4 mg, 1.88 µmol) was introduced into a Reacti-Vial™ and dissolved in 0.1 M aq. $NaHCO_3$ buffer (0.4 mL, pH 8.5). 100 µL of a solution of Sulfo-SIAB reagent (1.4 mg, 2.78 µmol) in ultrapure water were added. The reaction mixture was protected from light and stirred at rt for 2 h. The reaction was checked for completion by RP-HPLC (system S1) then quenched by addition of 50 mM aq. TEAB (2 mL). The mixture was purified by semi-preparative RP-HPLC (system S7, 2 injections). The product-containing fractions were lyophilised to give the thiol-reactive Eu(III) chelate **16** as a white amorphous powder. HPLC (system S1): t_R = 20.1 min (purity >95%); LRMS (ESI-): *m/z* 1029.13 and 1031.07 $[M - H]^-$, calcd for $C_{37}H_{35}EuIN_8O_{10}$: 1031.07.

Preparation of Cy 5.0-maleimide (22).

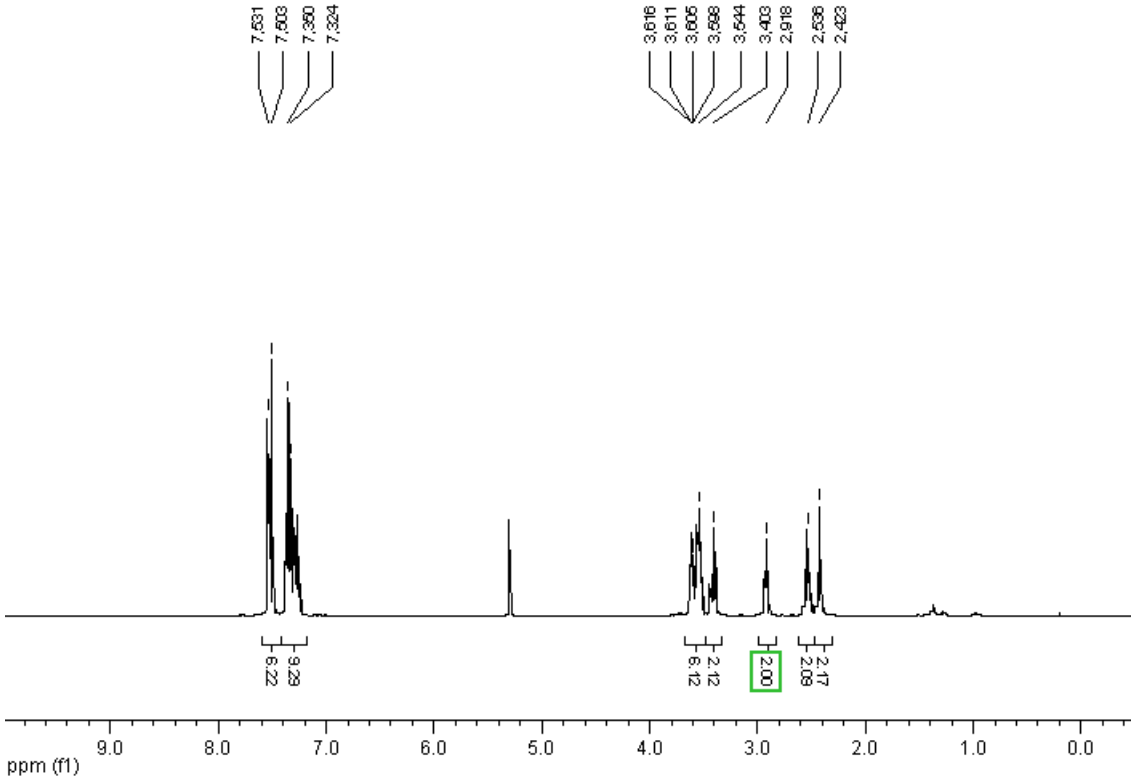


Scheme S4 (a) Cy 5.0 amine, 0.1 M aq. phosphate buffer, pH 8.0, Sulfo-MBS reagent, rt, 2 h, RP-HPLC purification.

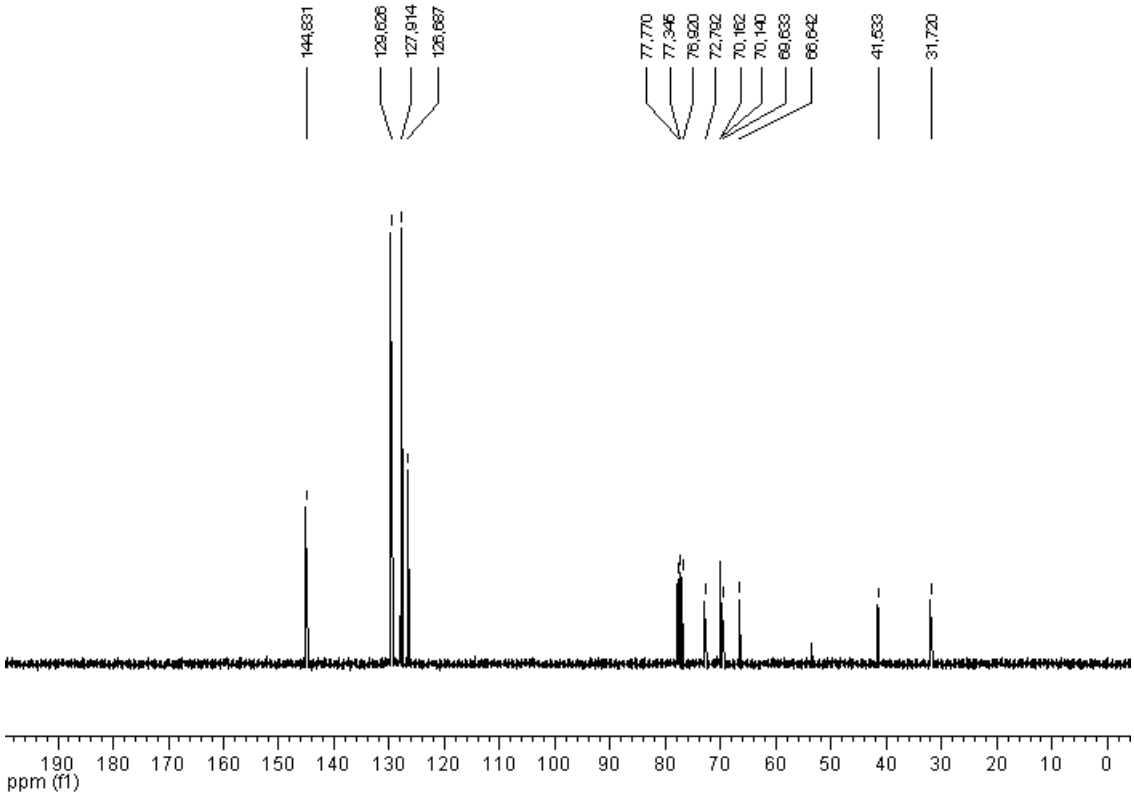
To a stirring solution of Cy 5.0 amine[8] (2.3 mg, 3.3 µmol) in 1.0 mL of 0.1 M aq. phosphate buffer (pH 8.0) was added Sulfo-MBS reagent (2.0 mg, 4.8 µmol) and the resulting reaction mixture was protected from light and stirred at rt for 2 h. The reaction was checked for

completion by RP-HPLC (system S8) and the resulting solution was dissolved in aq. TFA 0.1% (2 mL) and purified by semi-preparative RP-HPLC (system S9). The product containing fractions were lyophilised to give Cy 5.0-maleimide **22** as a blue amorphous powder (1.2 mg, yield 40%). HPLC (system S8): t_R = 22.1 min (purity >95%); LRMS (ESI+): *m/z* 898.27 [M + H]$^+$; LRMS (ESI-): *m/z* 896.27 [M - H]$^-$, calcd for $C_{46}H_{51}N_5O_{10}S_2$: 897.31.

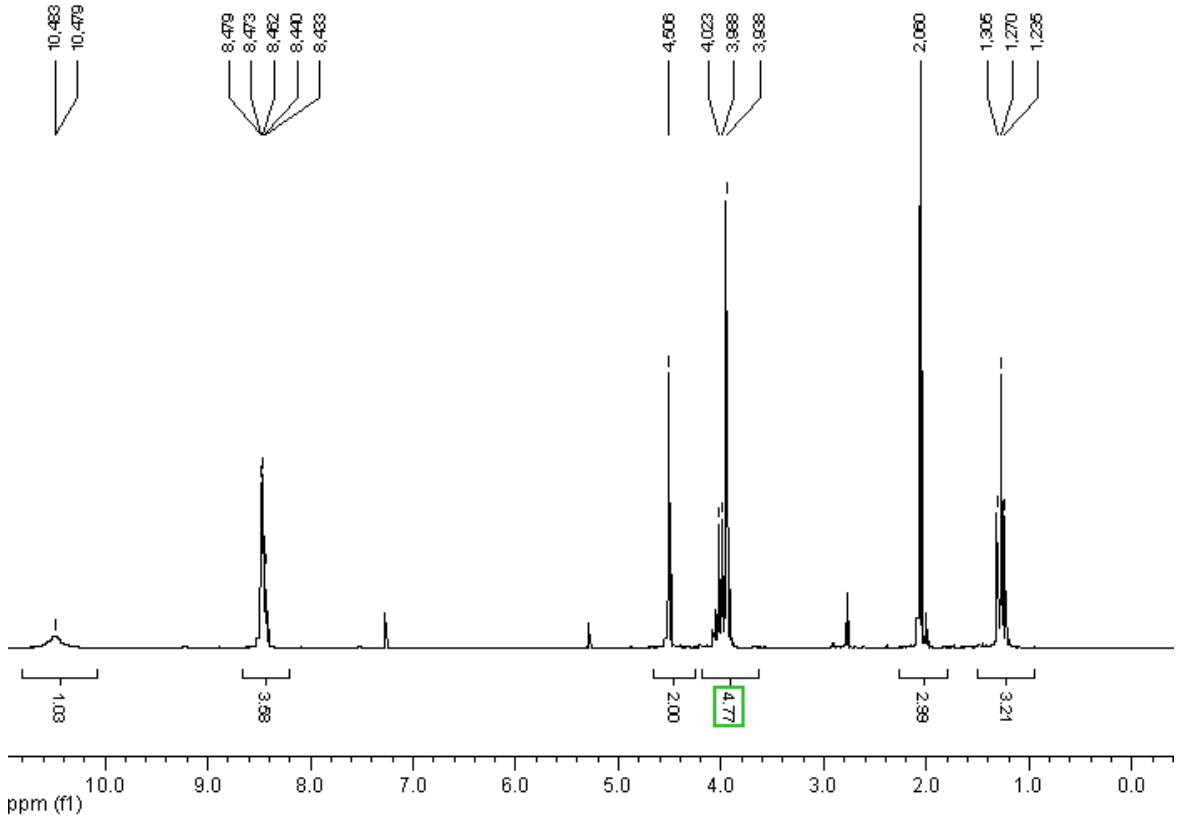
^{1}H NMR spectrum of compound 8 recorded in $CDCl_3$ at 300 MHz.



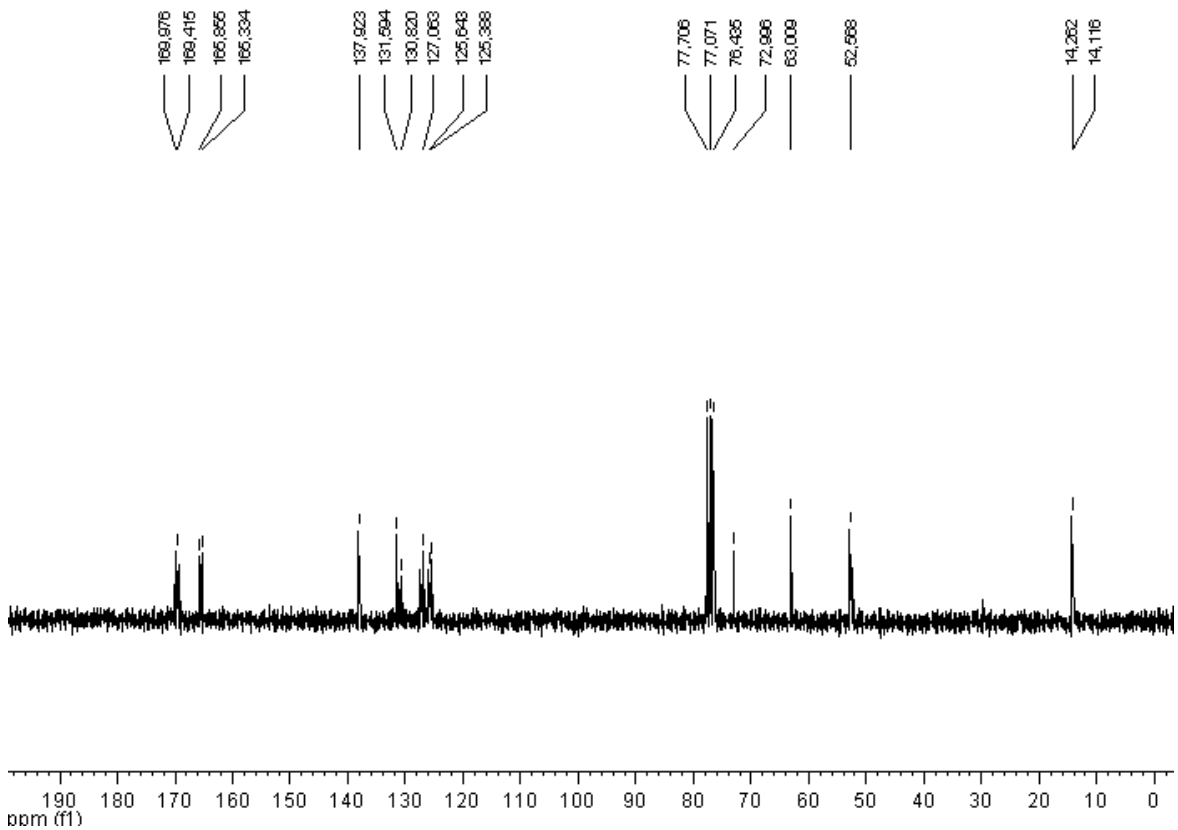
^{13}C NMR spectrum of compound 8 recorded in $CDCl_3$ at 75 MHz.



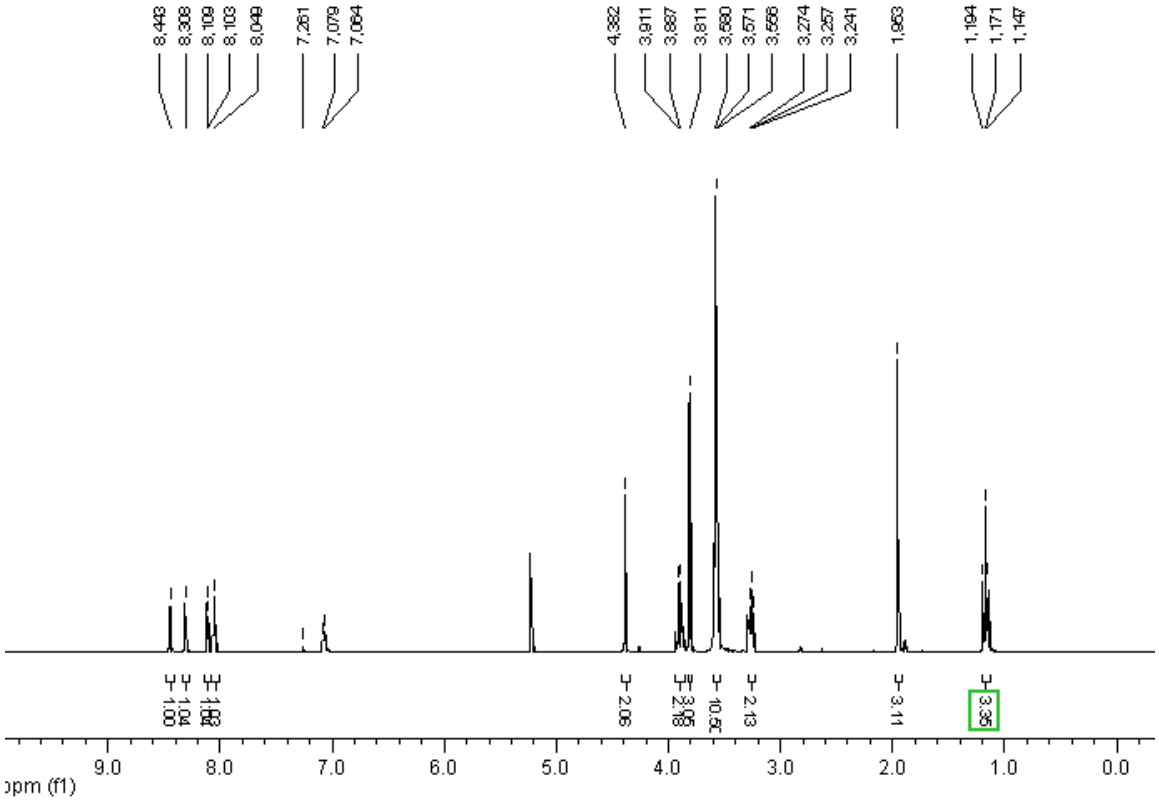
^{1}H NMR spectrum of compound 4 recorded in $CDCl_3$ at 300 MHz.



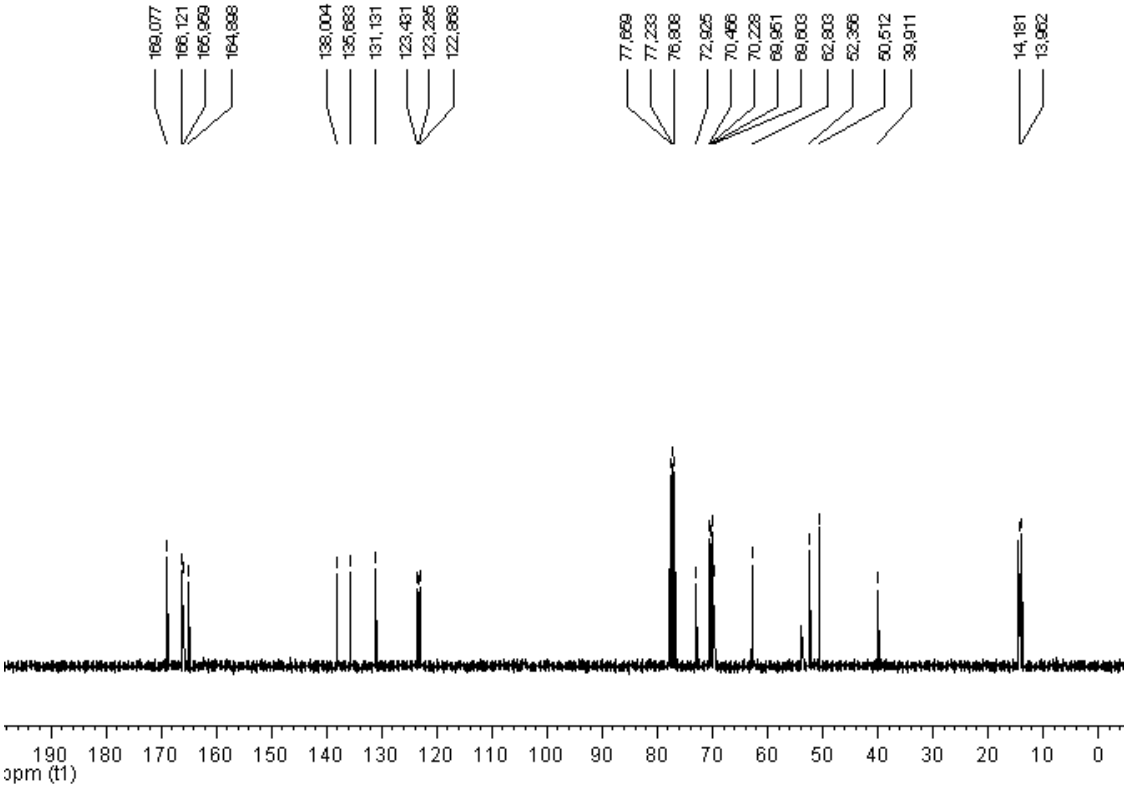
^{13}C NMR spectrum of compound 4 recorded in $CDCl_3$ at 75 MHz.



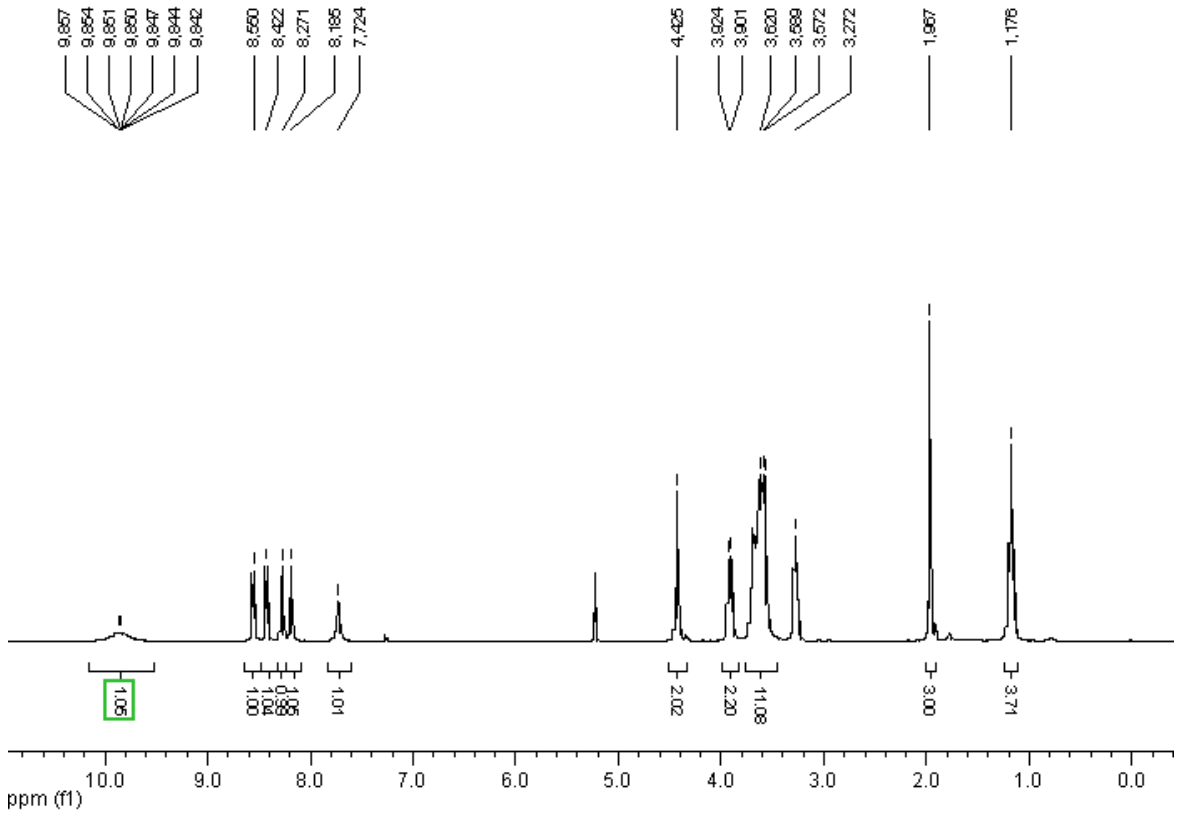
^{1}H NMR spectrum of compound 6 recorded in $CDCl_3$ at 300 MHz.



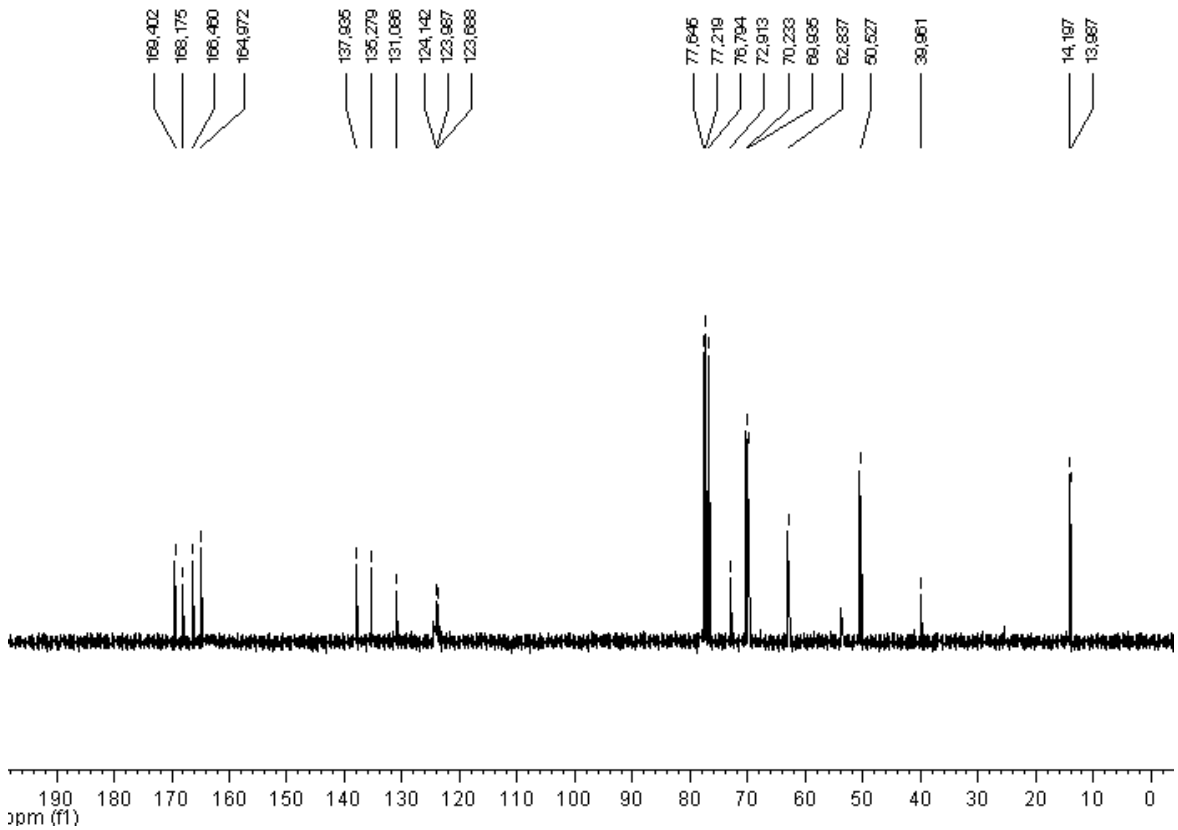
^{13}C NMR spectrum of compound 6 recorded in $CDCl_3$ at 75 MHz.



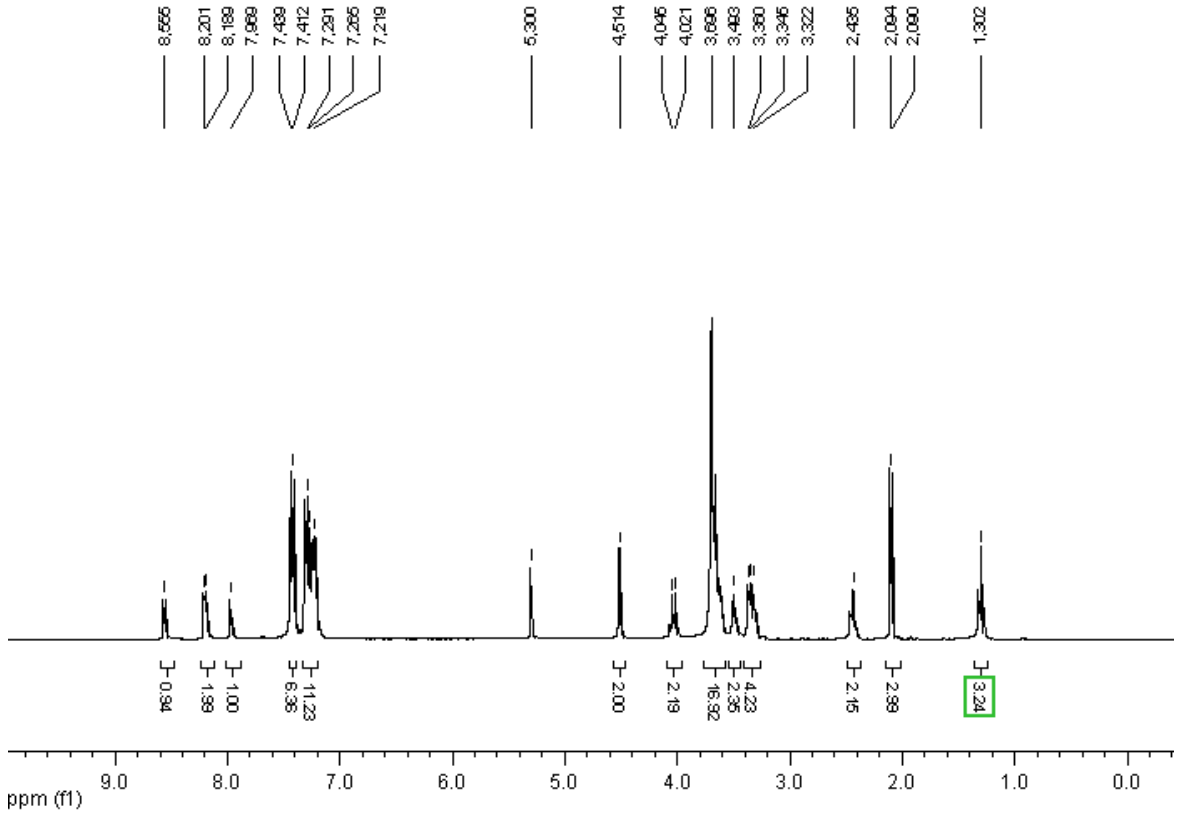
^{1}H NMR spectrum of compound 7 recorded in $CDCl_3$ at 300 MHz.



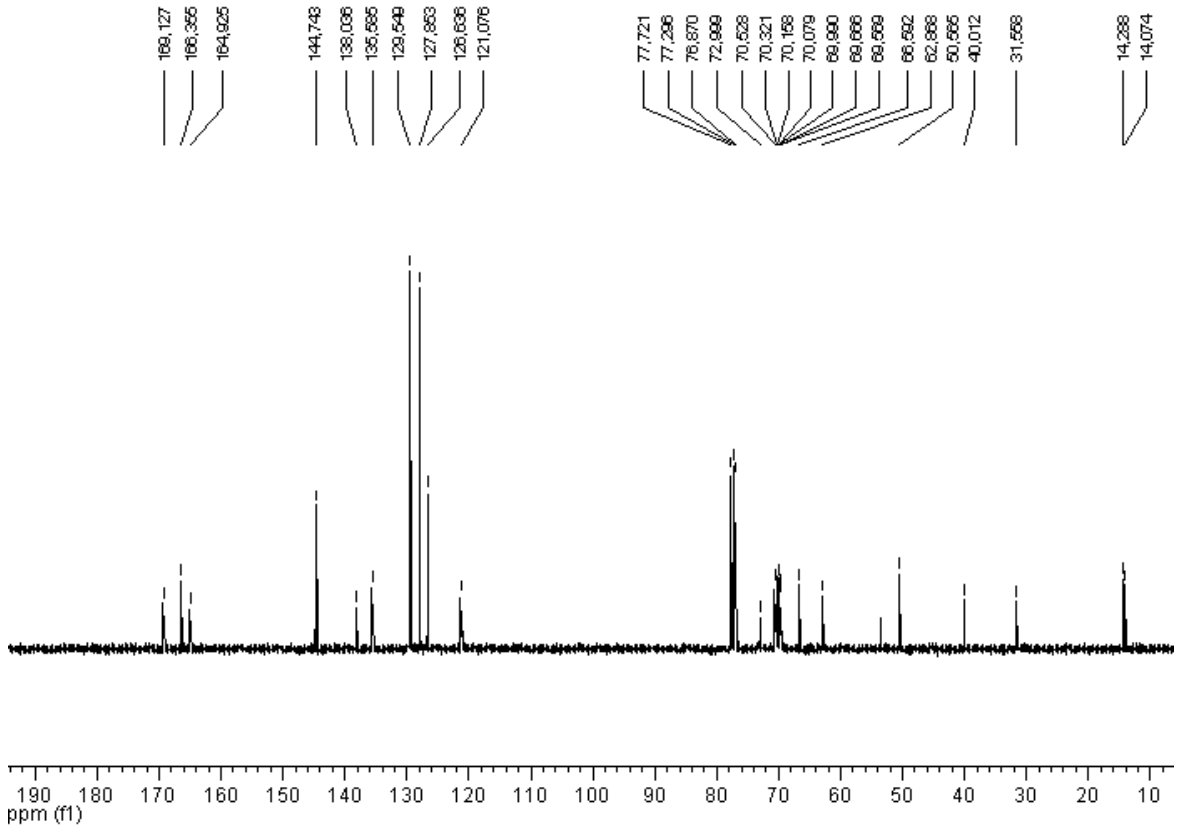
^{13}C NMR spectrum of compound 7 recorded in $CDCl_3$ at 75 MHz.



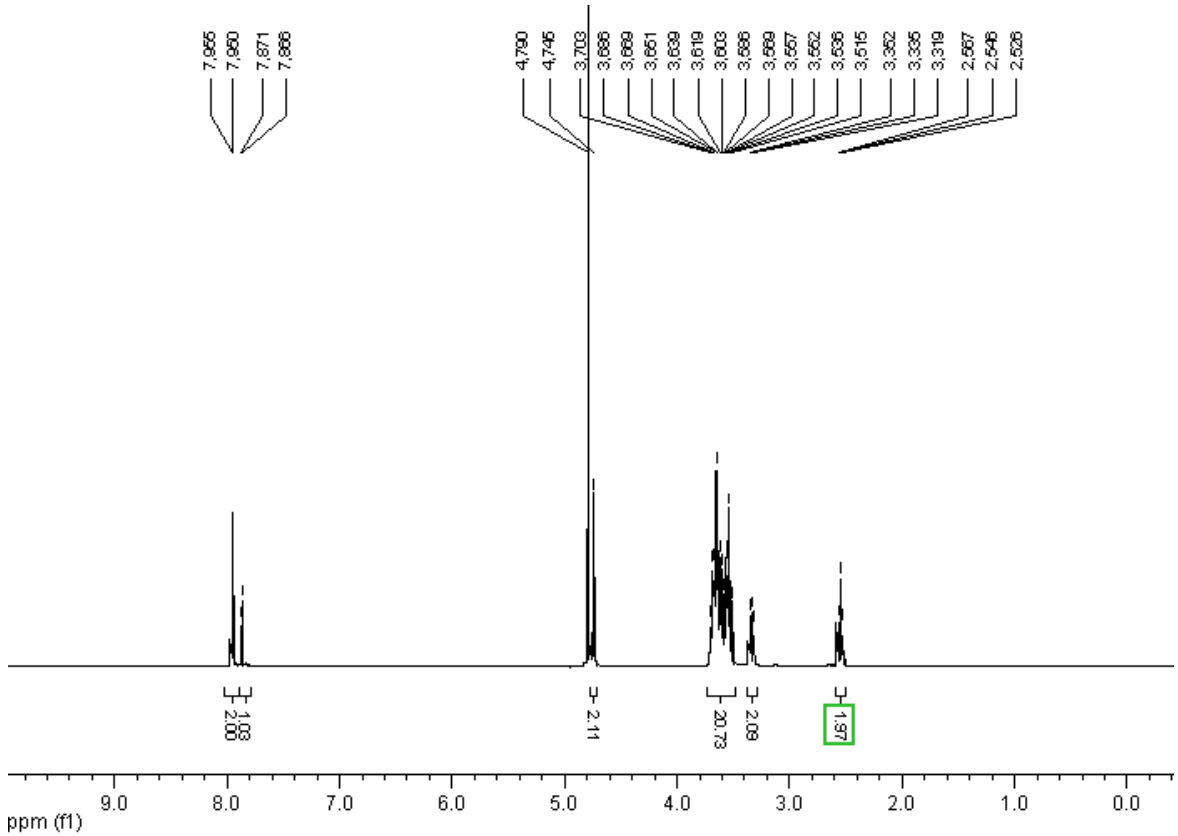
^{1}H NMR spectrum of compound 9 recorded in $CDCl_3$ at 300 MHz.



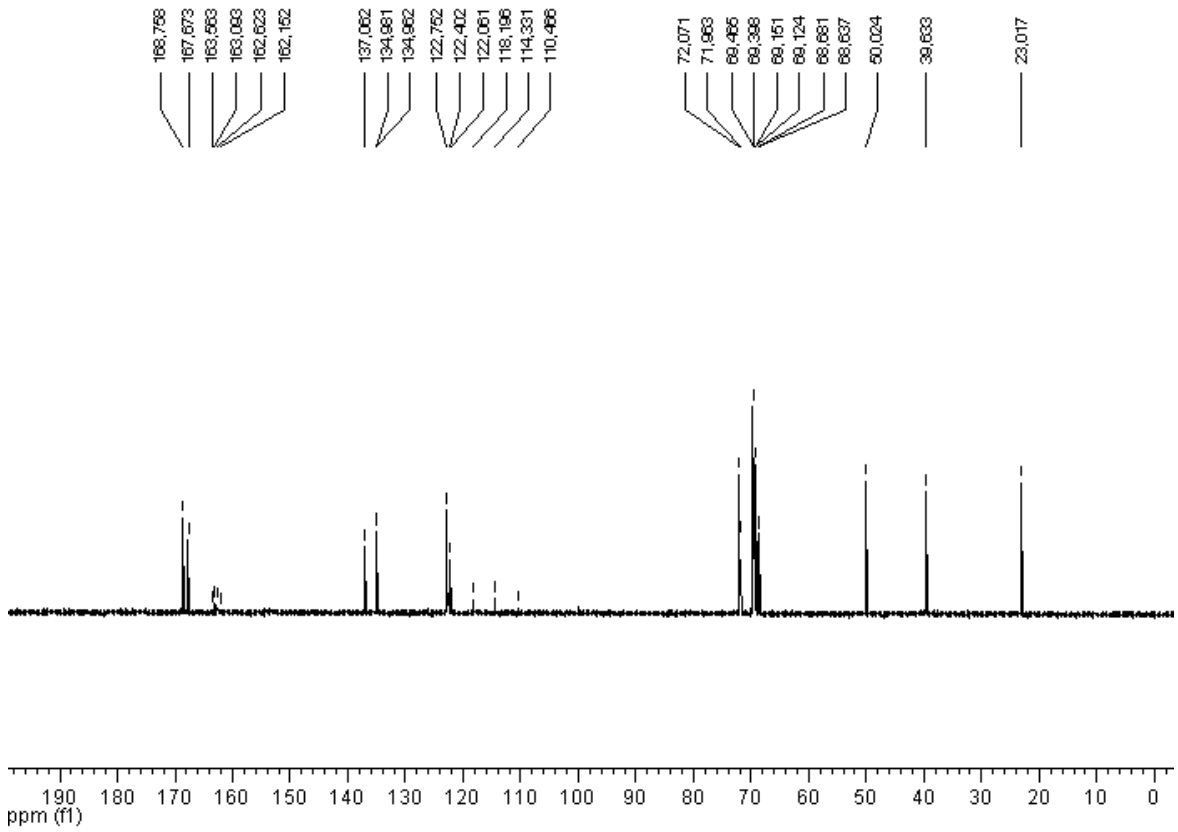
^{13}C NMR spectrum of compound 9 recorded in $CDCl_3$ at 75 MHz.



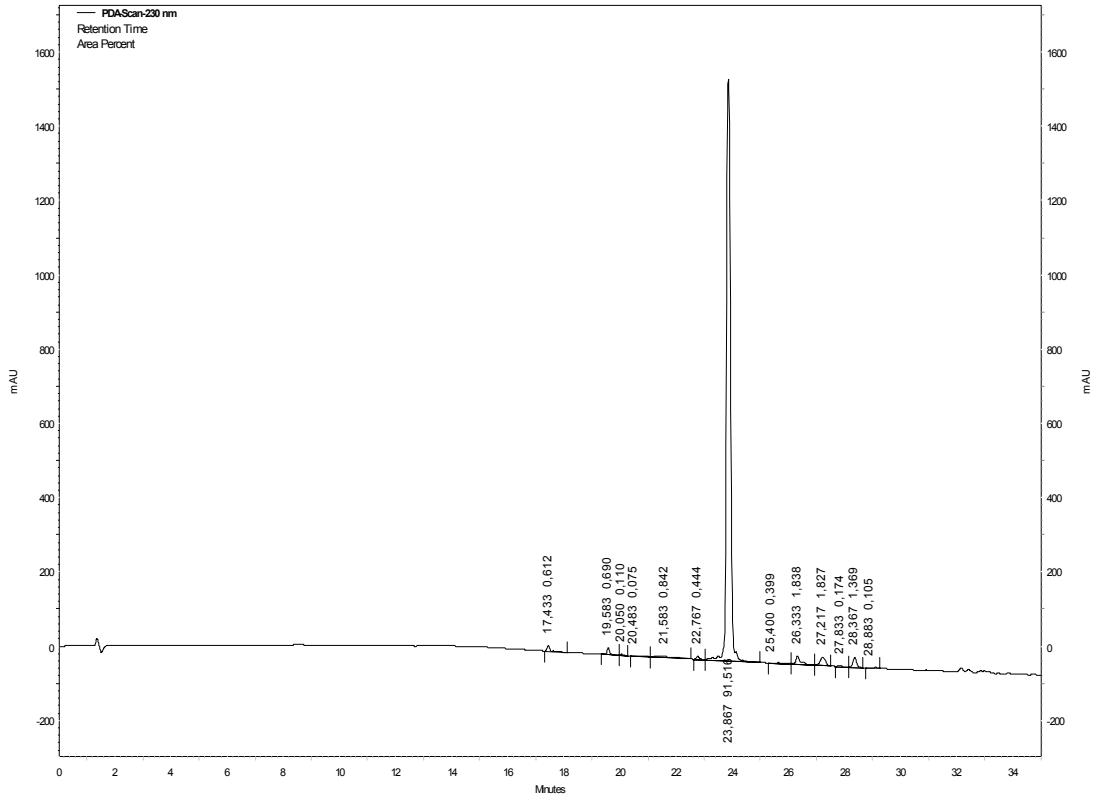
^{1}H NMR spectrum of benzenic "tripod" 1 recorded in D_2O at 300 MHz.



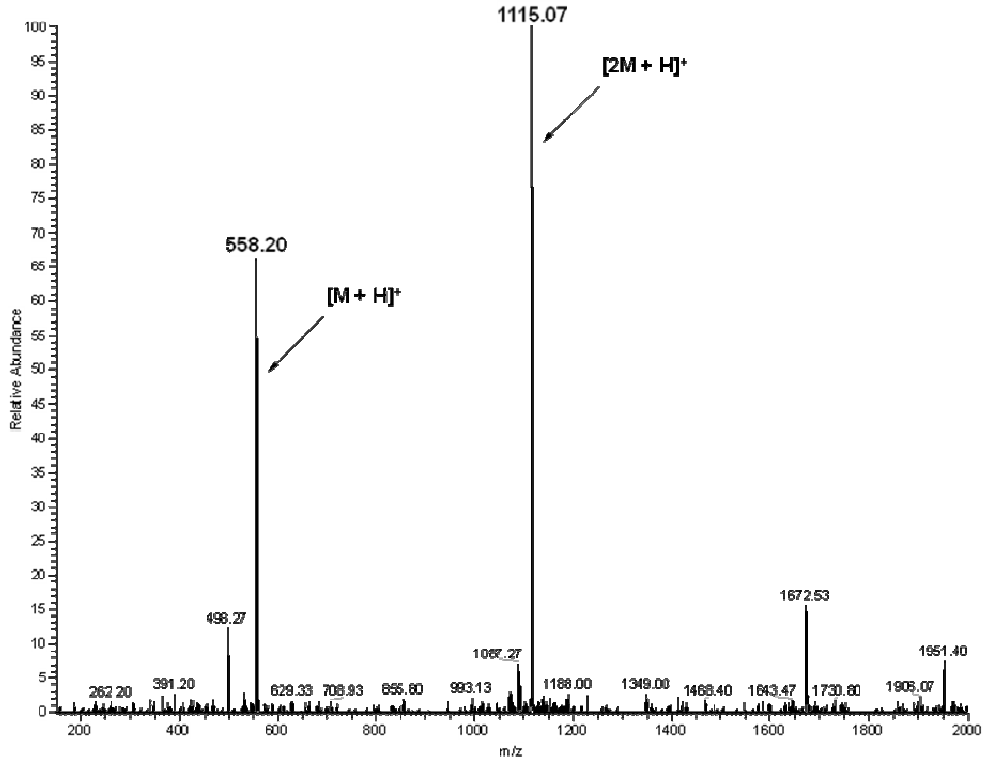
^{13}C NMR spectrum of benzenic "tripod" 1 recorded in D_2O at 75 MHz.



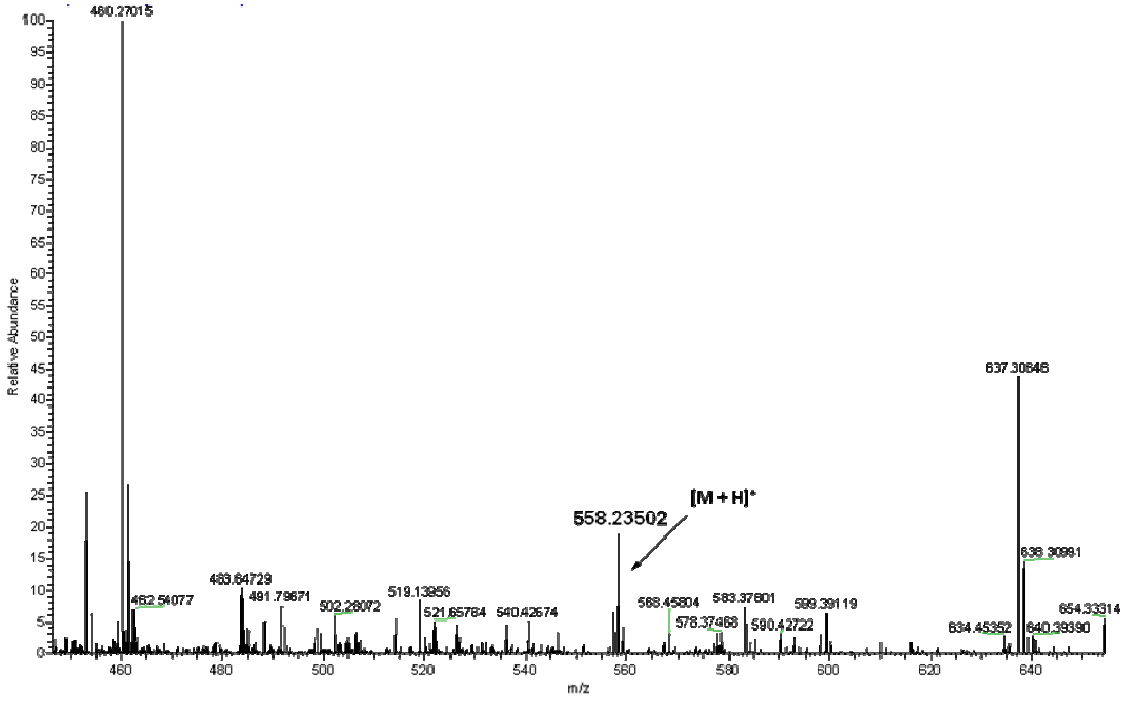
RP-HPLC elution profile (system A) of benzenic "tripod" 1.



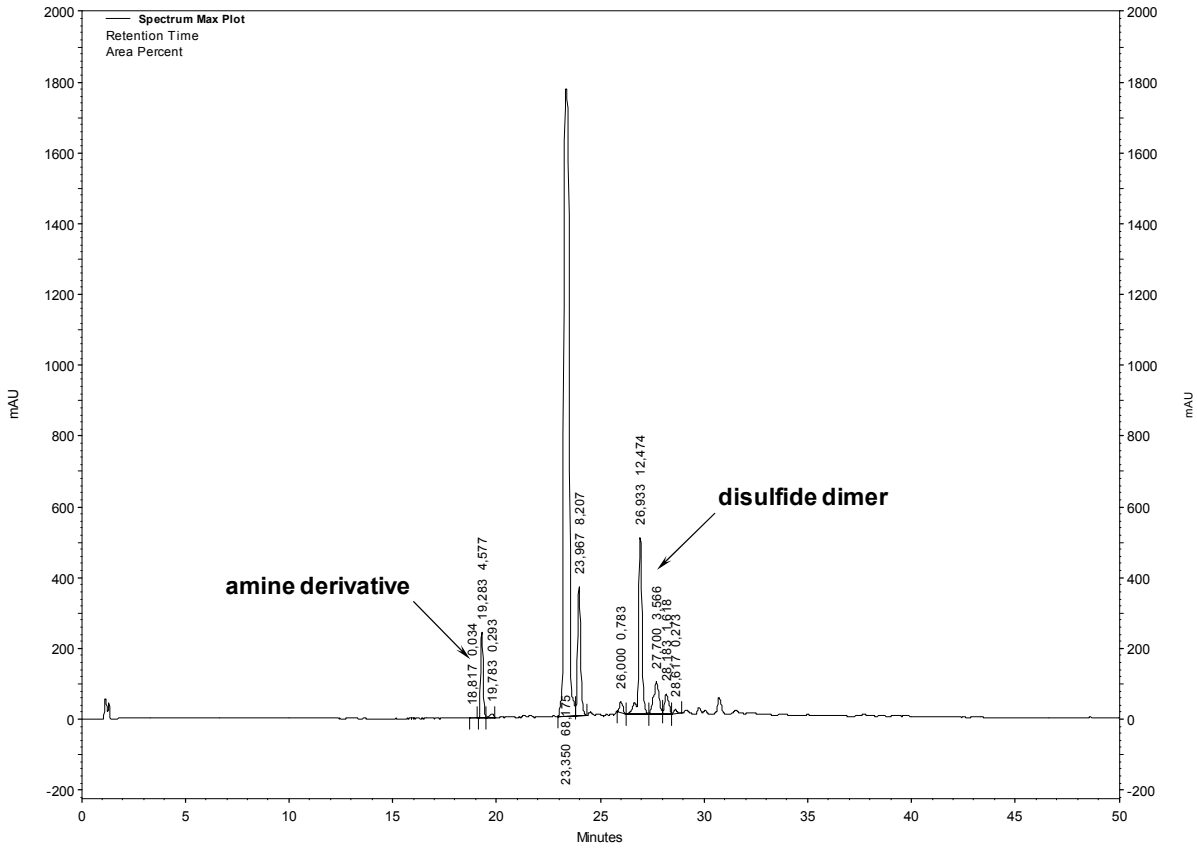
(ESI+) mass spectrum (low resolution) of benzenic "tripod" 1.



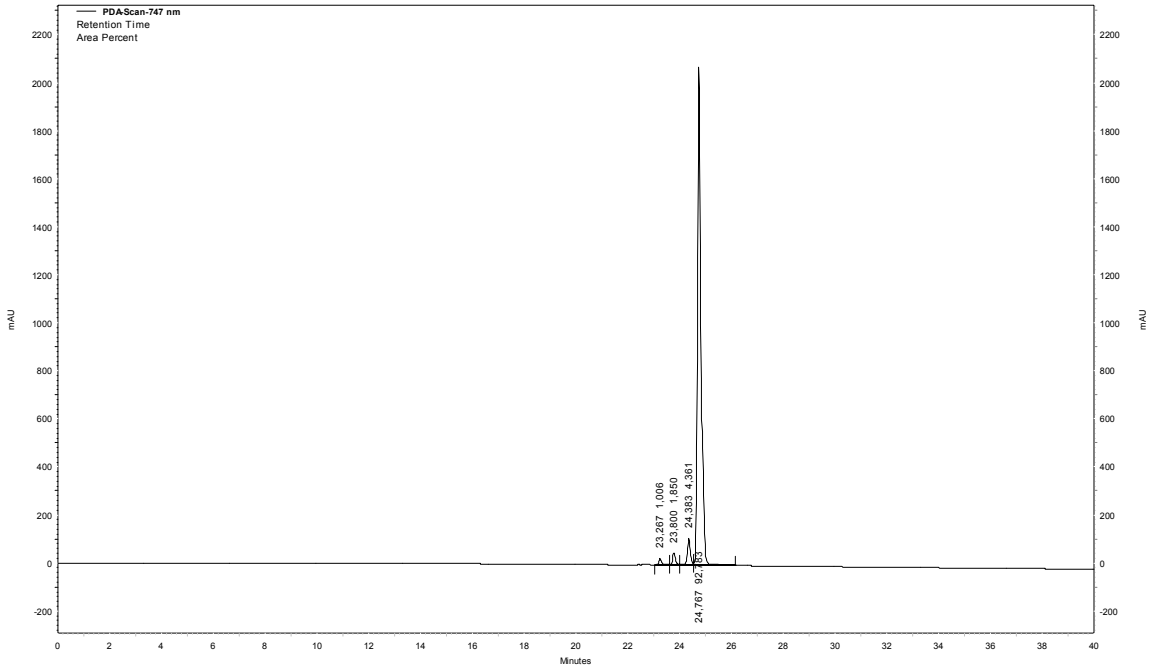
(ESI+) mass spectrum (high resolution) of "benzenic" tripod 1.



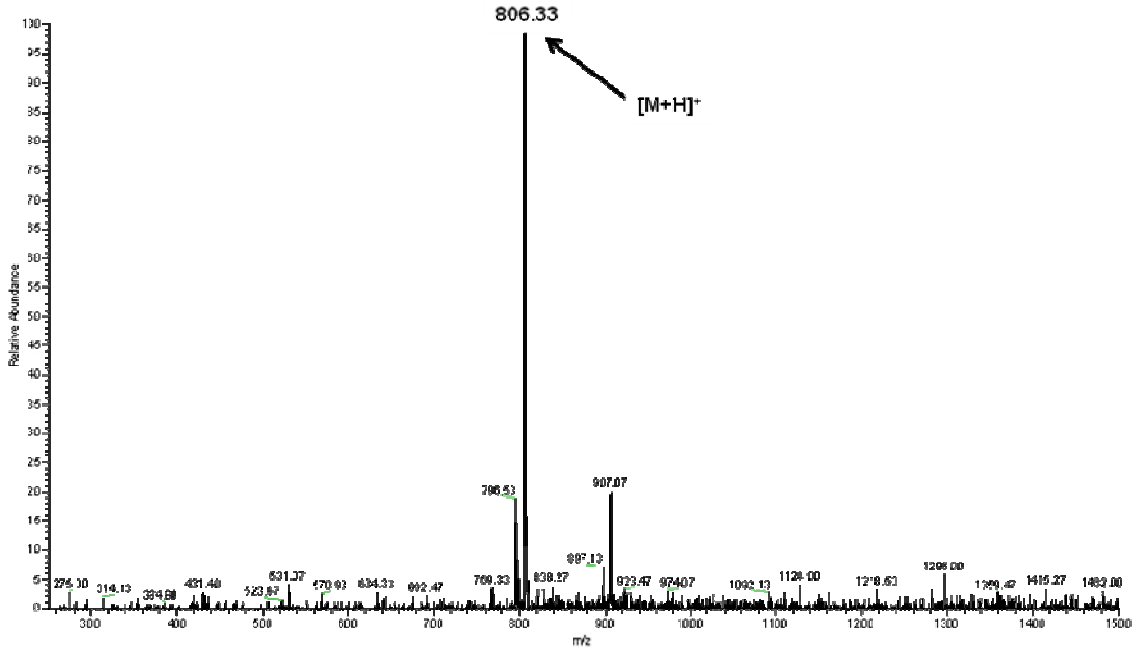
RP-HPLC elution profile (system A) of benzenic "tripod" 1 after two months of storage.



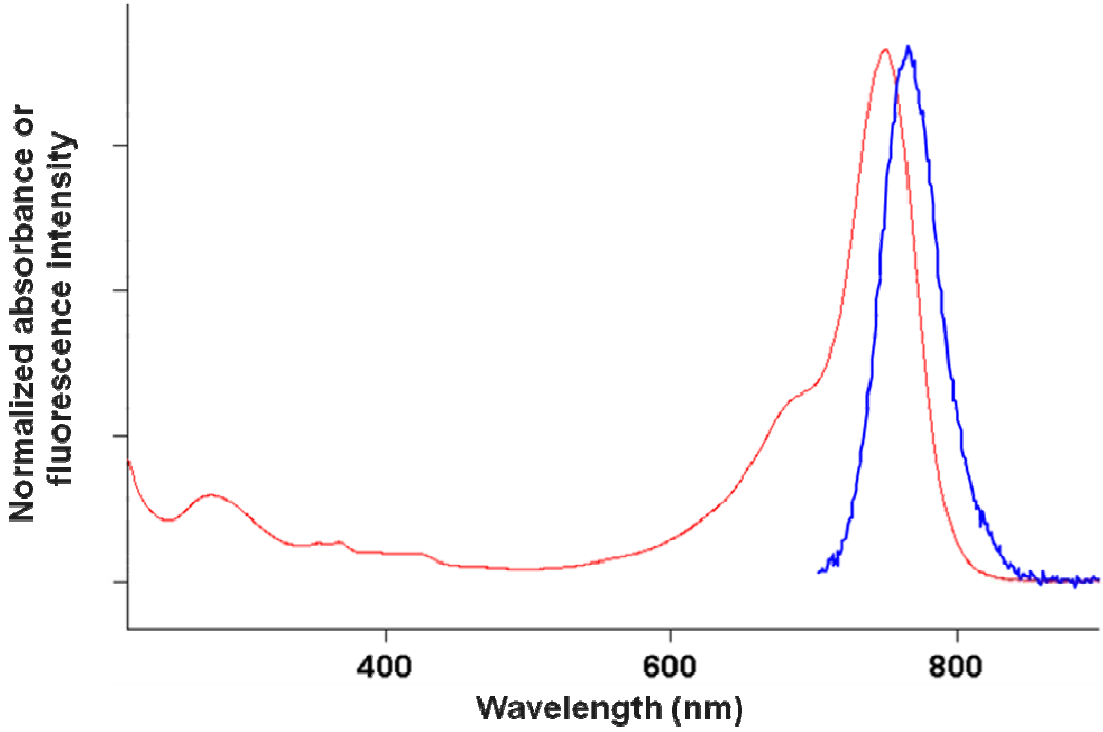
RP-HPLC elution profile (system S1) of Cy 7.0-alkyne 12.



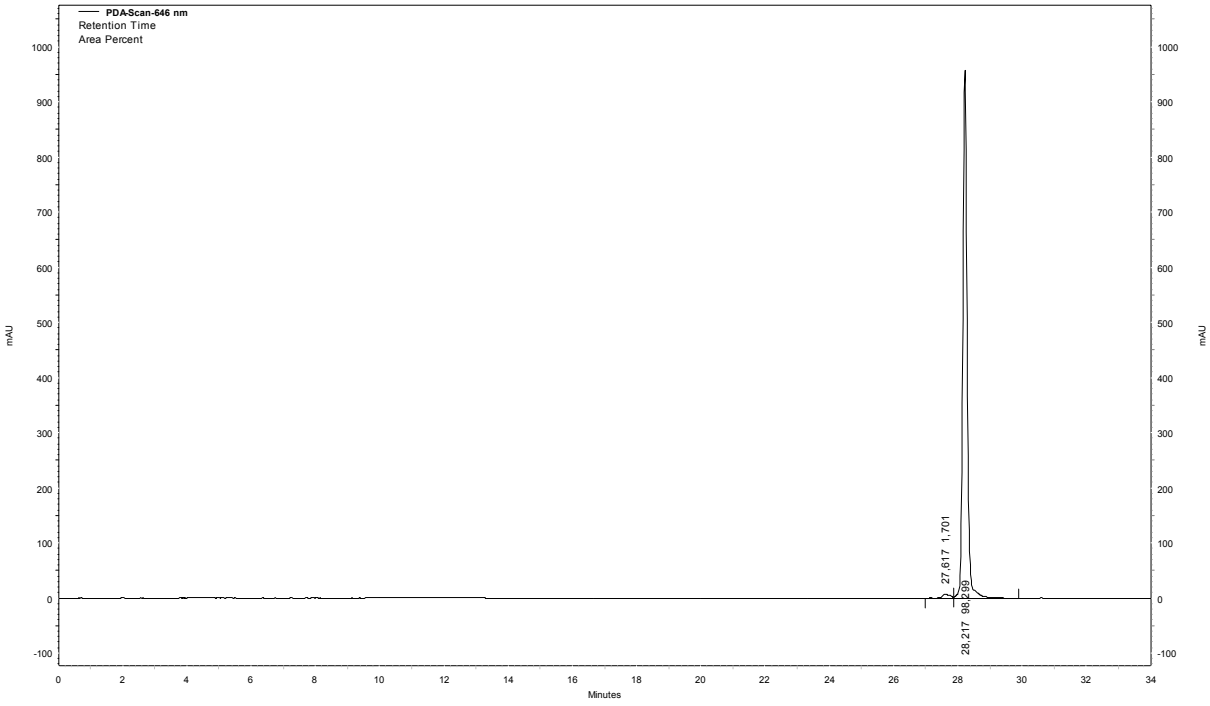
(ESI+) mass spectrum of Cy 7.0-alkyne 12.



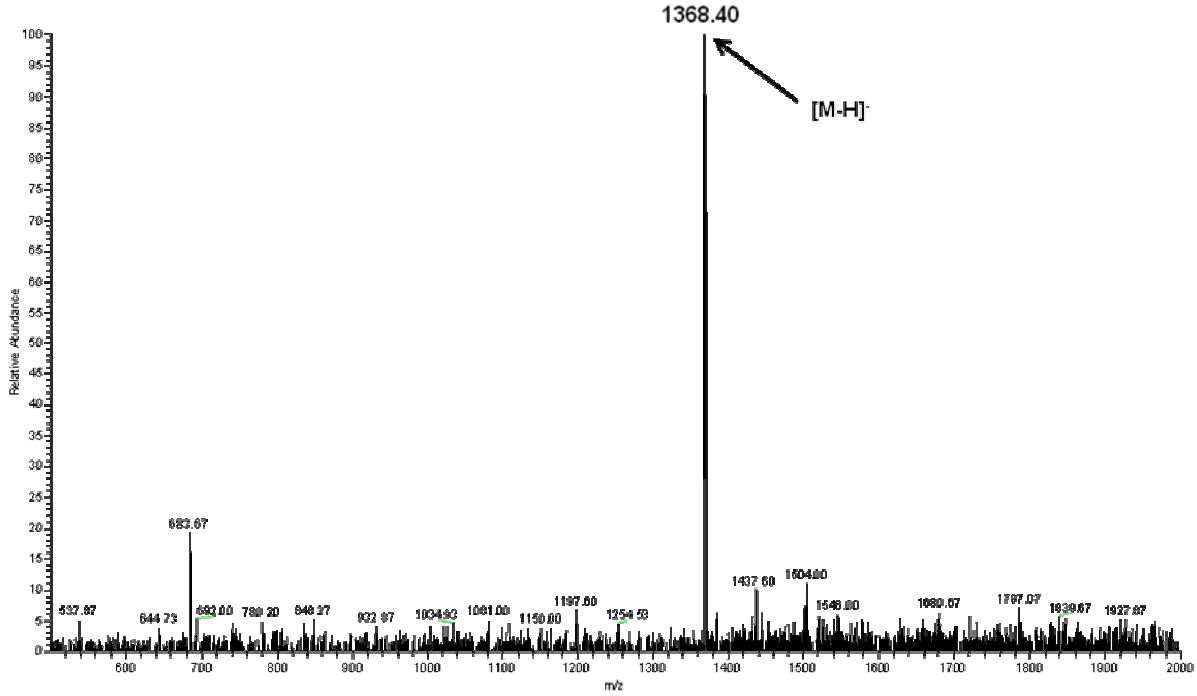
Normalised absorption (—) and fluorescence emission (—) (Ex. at 700 nm) spectra of Cy 7.0-alkyne 12 recorded in PBS at 25 °C.



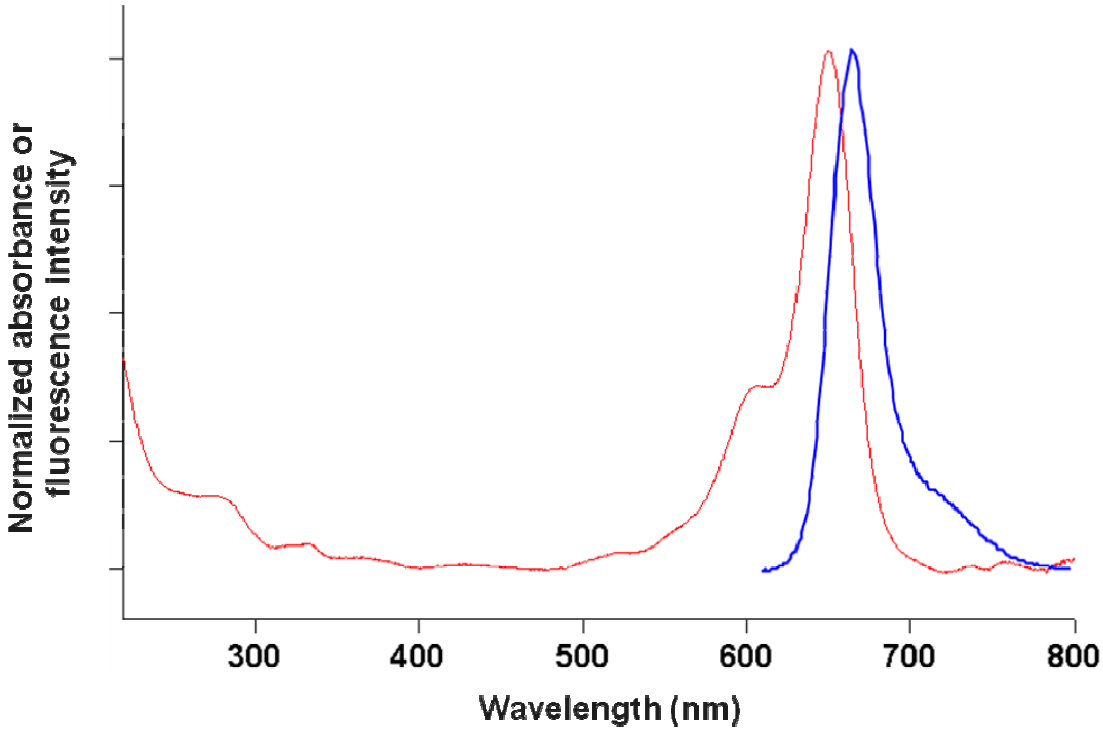
RP-HPLC elution profile (system D) of Cy 5.0-labelled tripod 13.



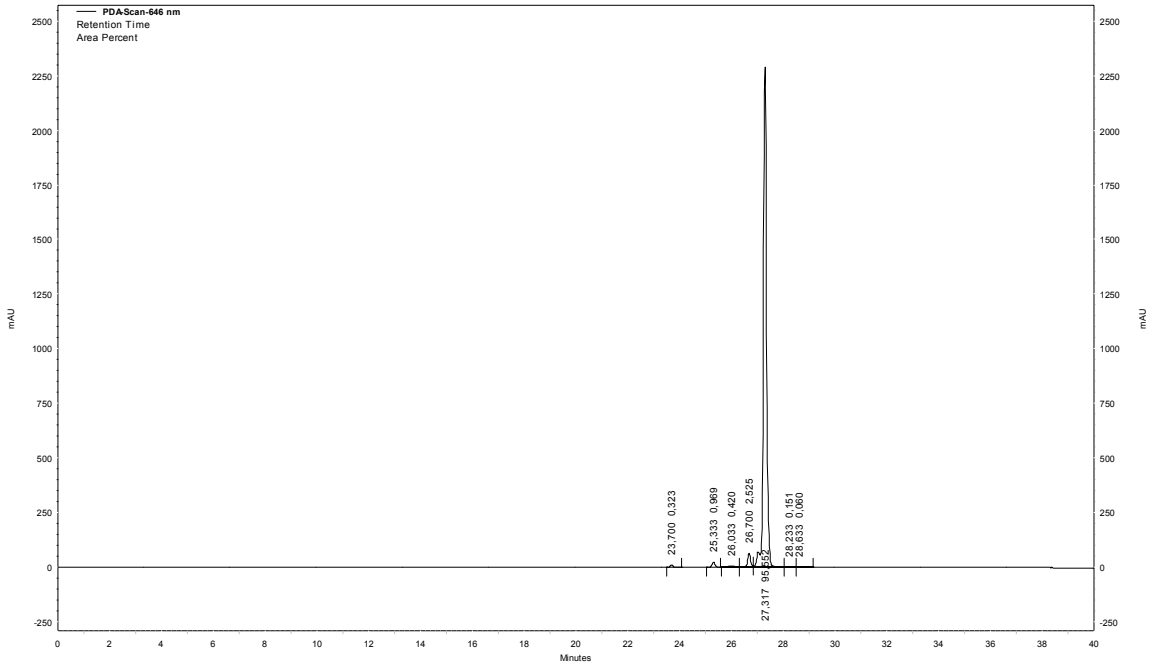
(ESI-) mass spectrum of Cy 5.0-labelled tripod 13.



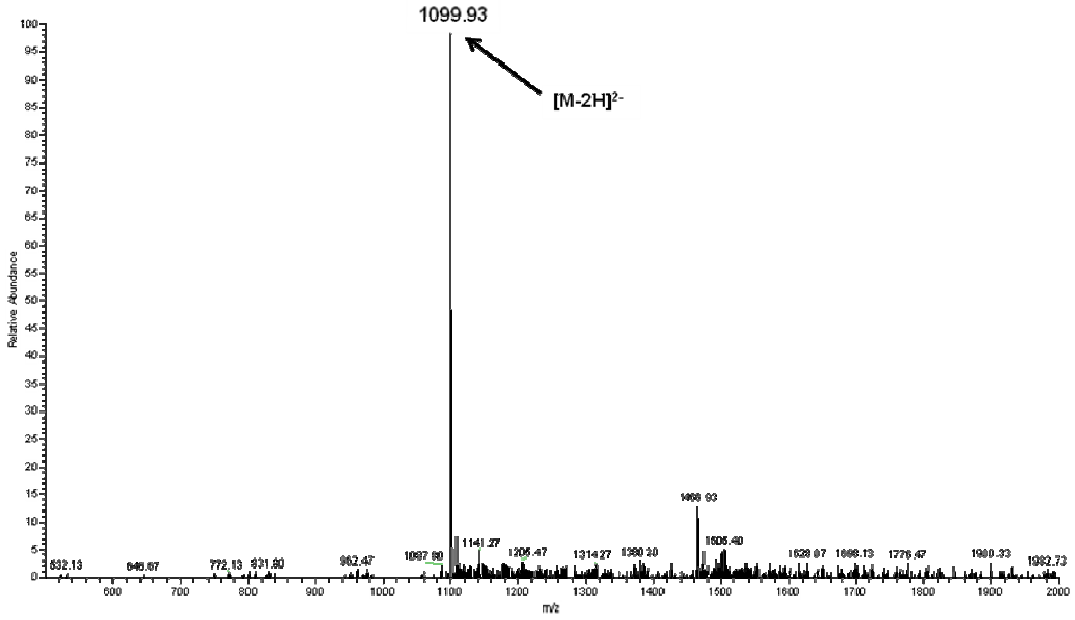
Normalised absorption (—) and fluorescence emission (—) (Ex. at 600 nm) spectra of Cy 5.0-labelled tripod 13 recorded in PBS at 25 °C.



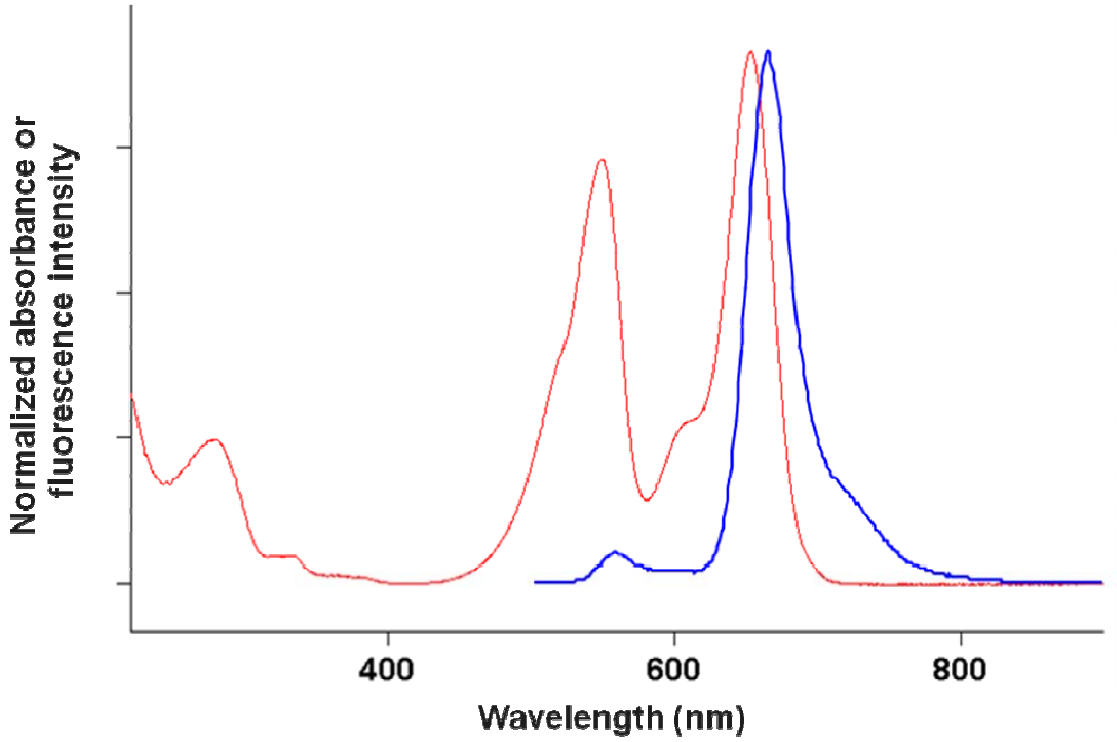
RP-HPLC elution profile (system D) of Cy 3.0/Cy 5.0-labelled tripod 14.



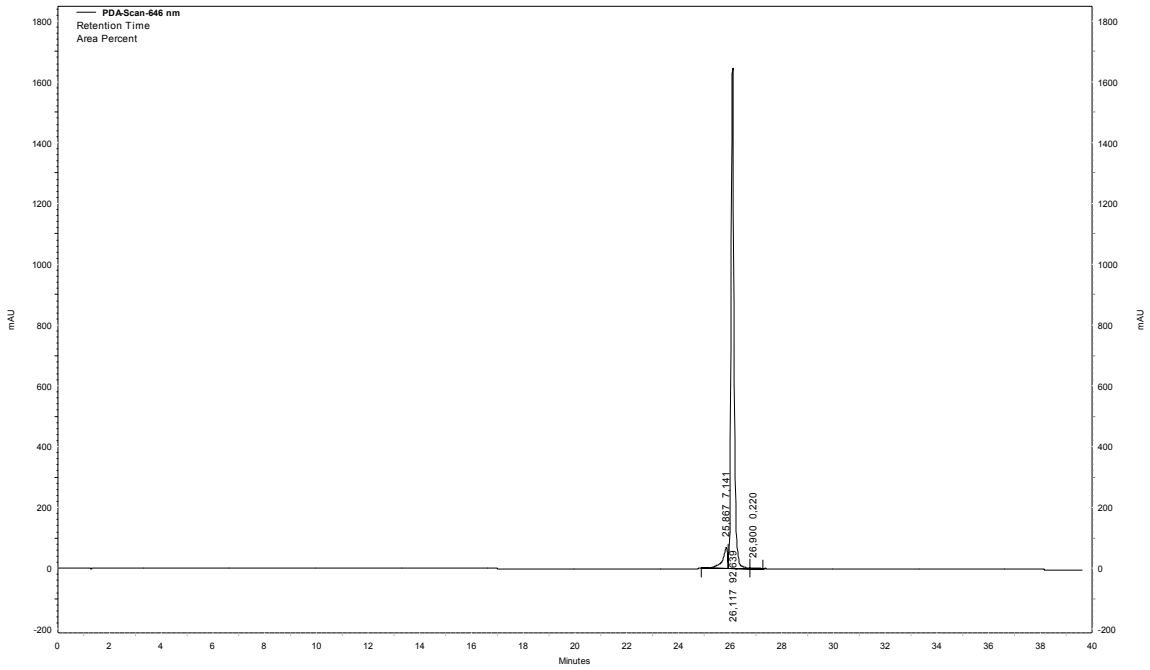
(ESI-) mass spectrum of Cy3.0/Cy 5.0-labelled tripod 14.



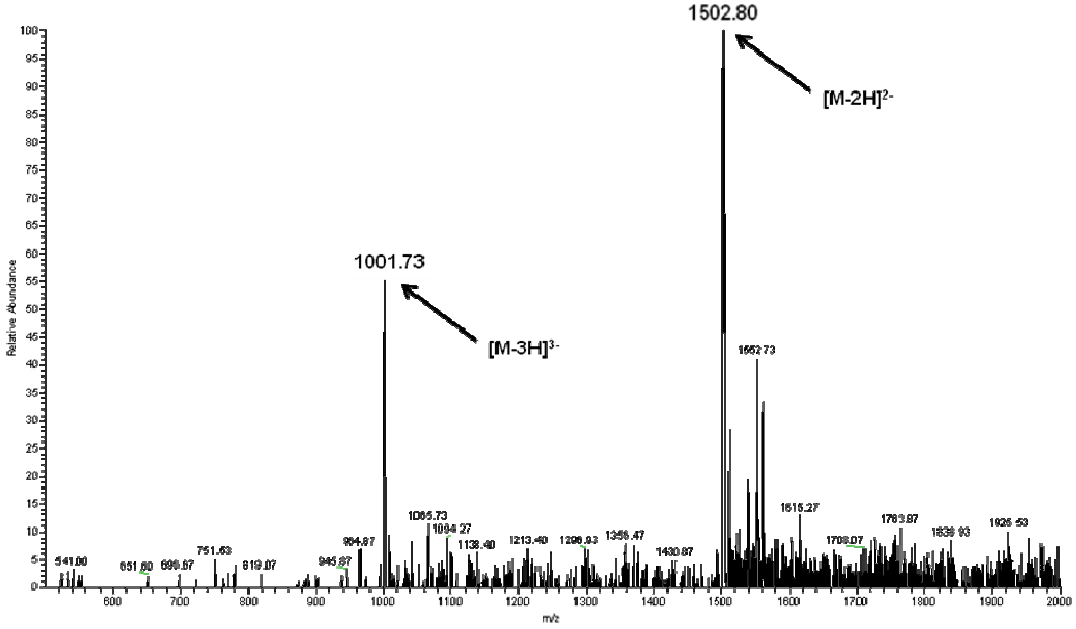
Normalised absorption (—) and fluorescence emission (—) (Ex. at 500 nm) spectra of Cy3.0/ 5.0-labelled tripod 14 recorded in PBS at 25 °C.



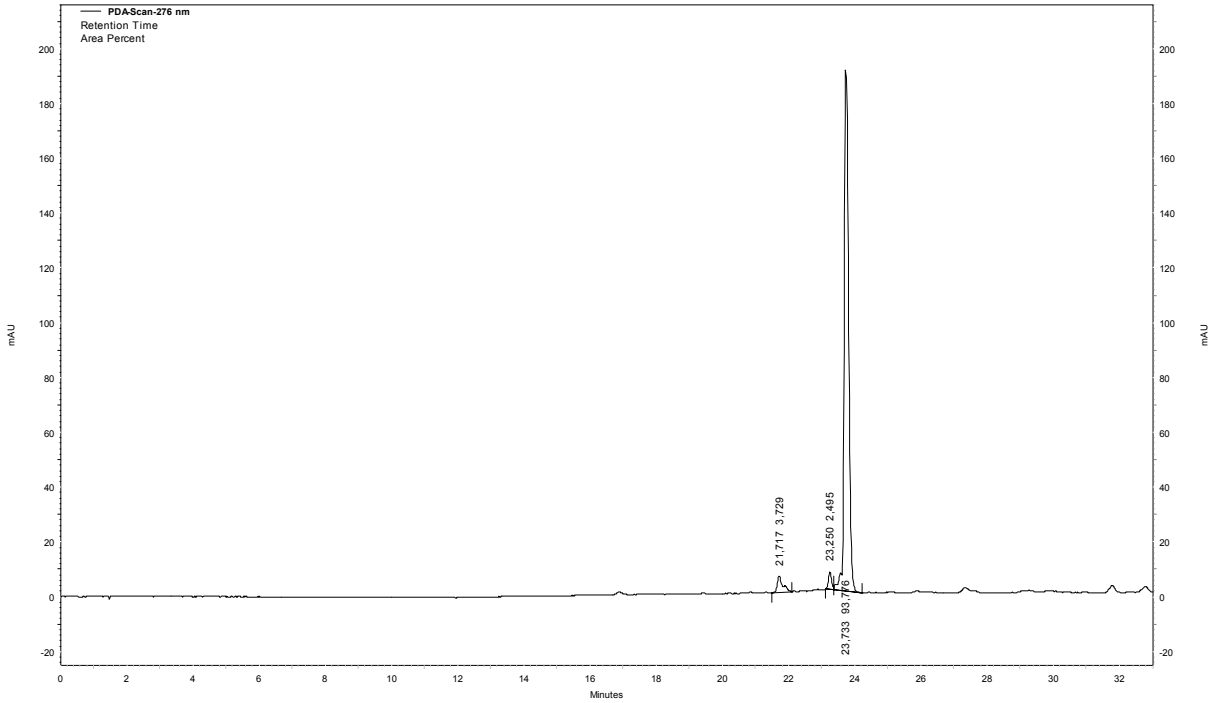
RP-HPLC elution profile (system D) of FRET cascade 15.



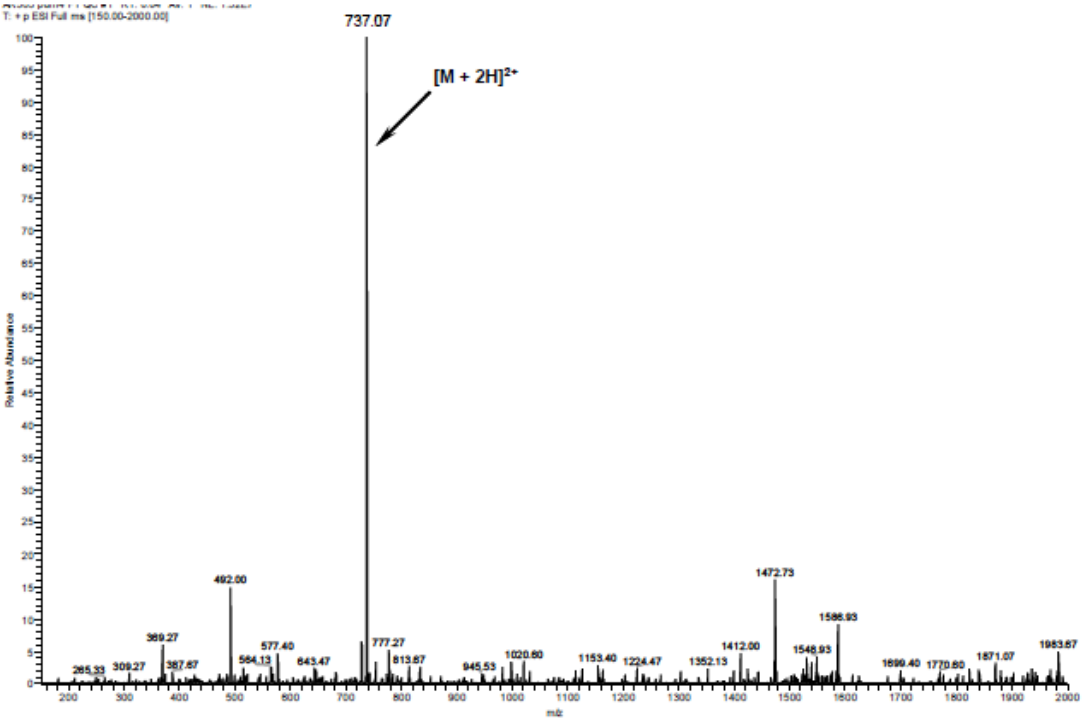
(ESI-) mass spectrum of FRET cascade 15.



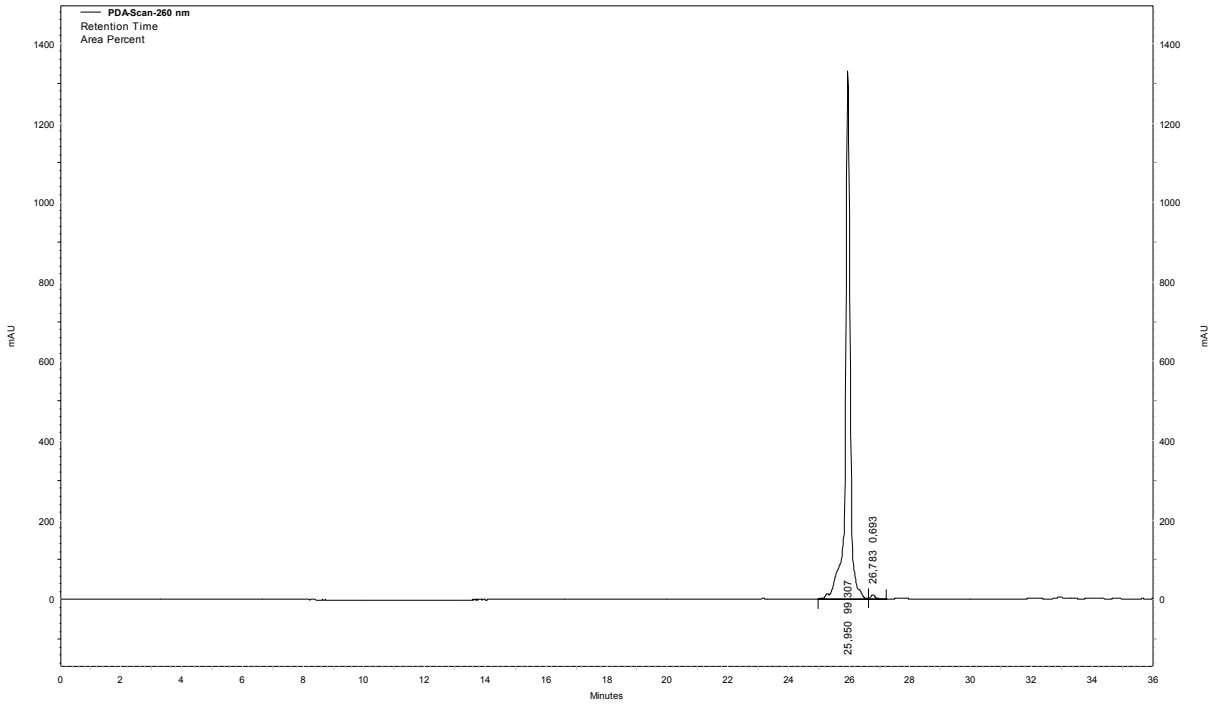
RP-HPLC elution profile (system S4) of dodecapeptide S3.



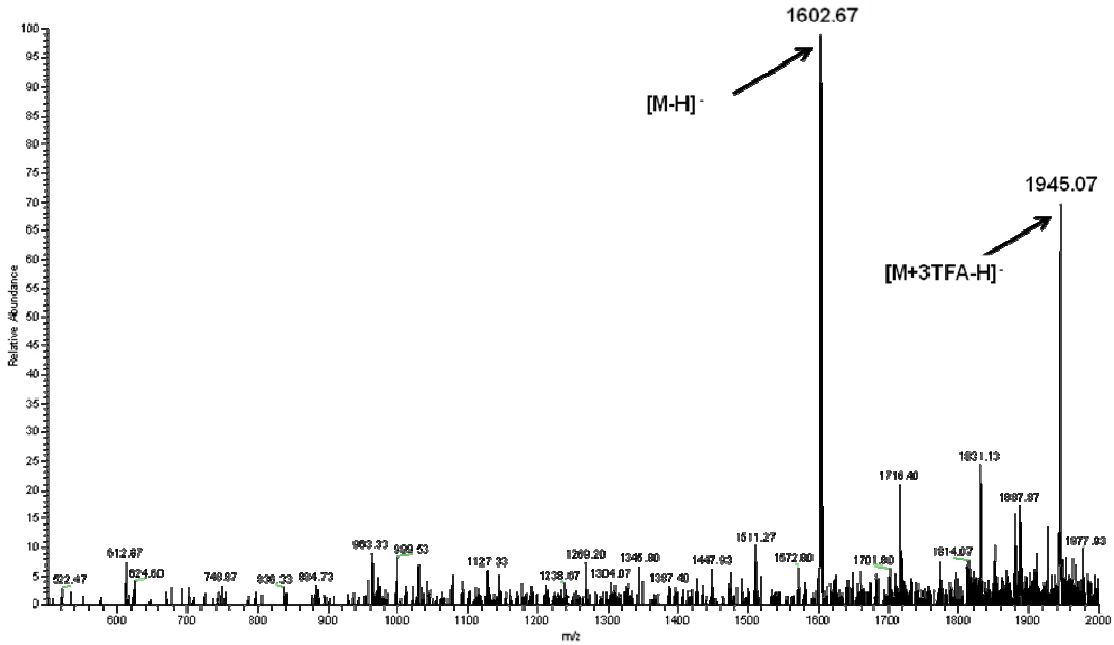
(ESI+) mass spectrum of dodecapeptide S3.



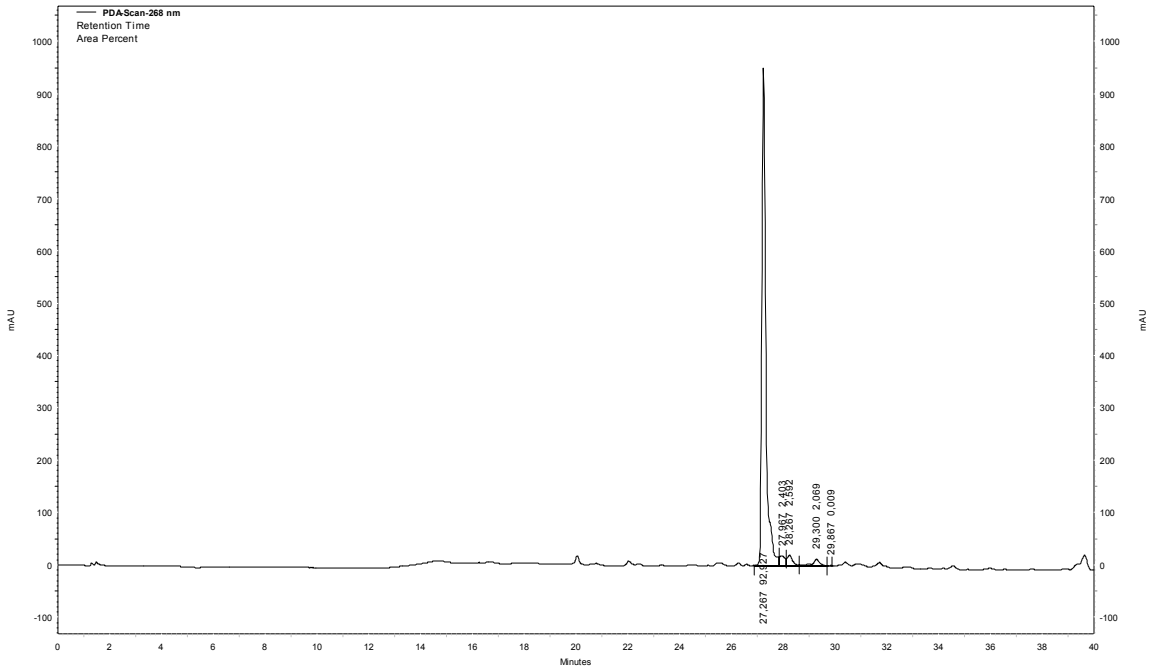
RP-HPLC elution profile (system S4) of peptide-aldehyde 16.



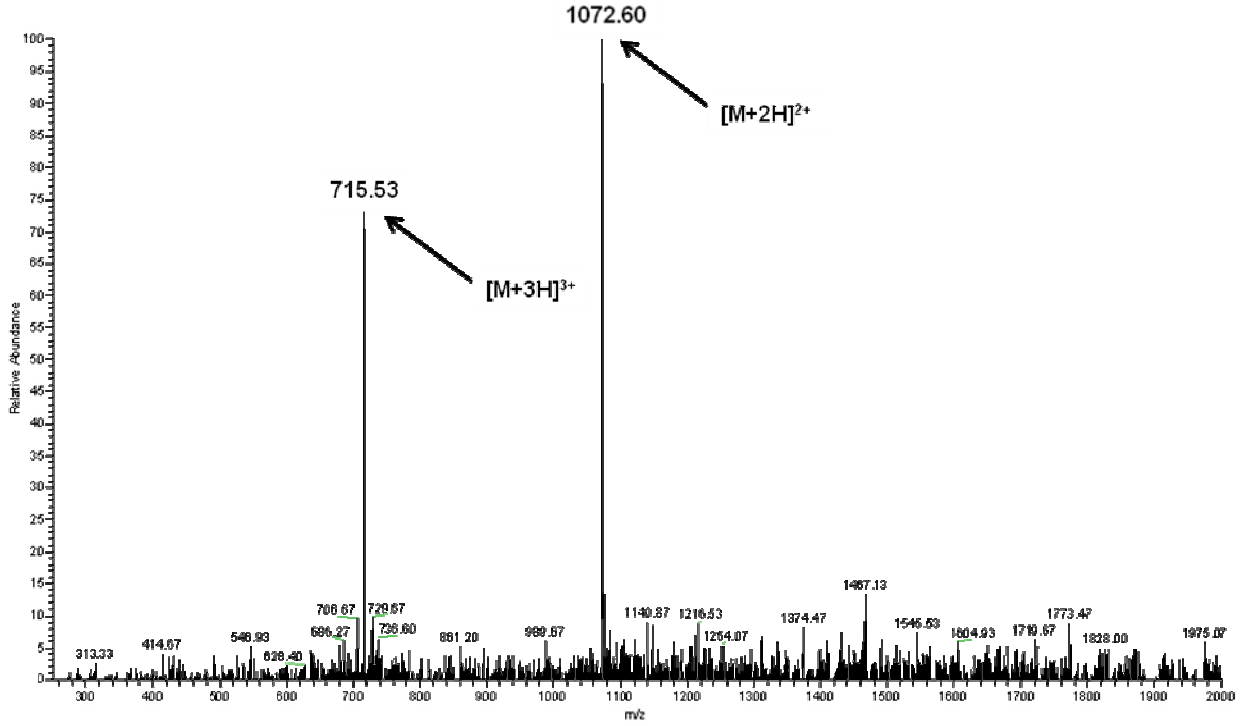
(ESI-) mass spectrum of peptide-aldehyde 16.



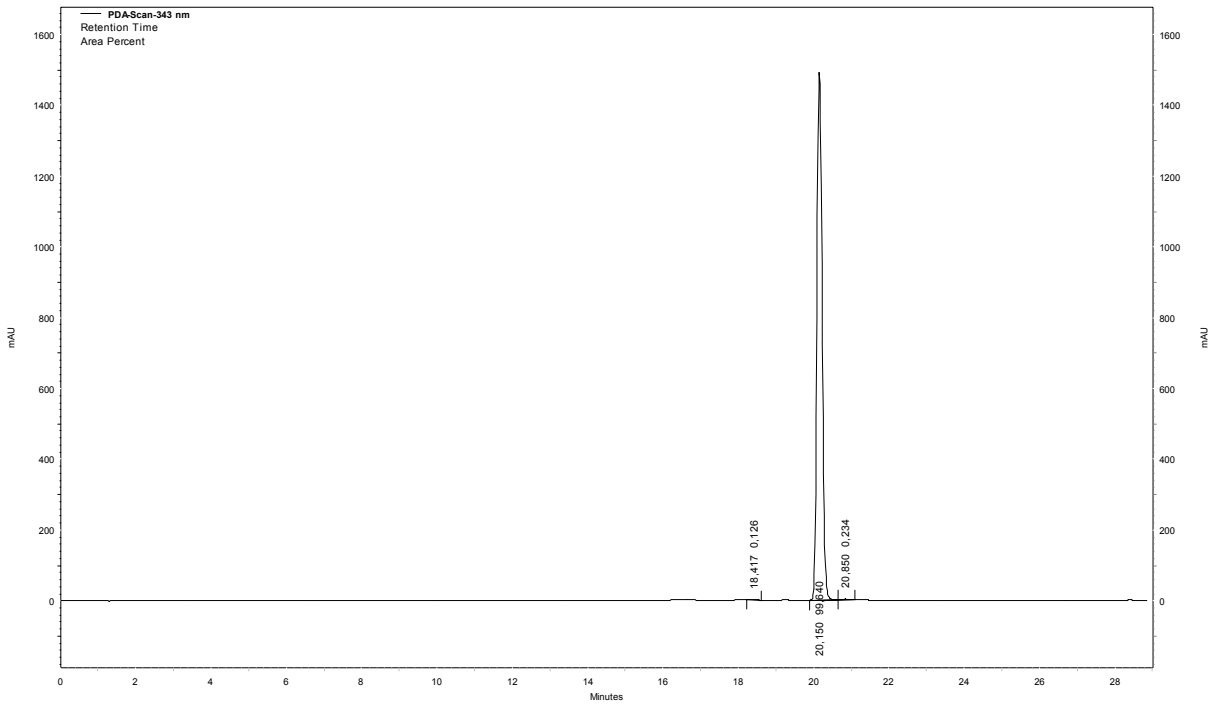
RP-HPLC elution profile (system D) of peptide-tripod conjugate 19.



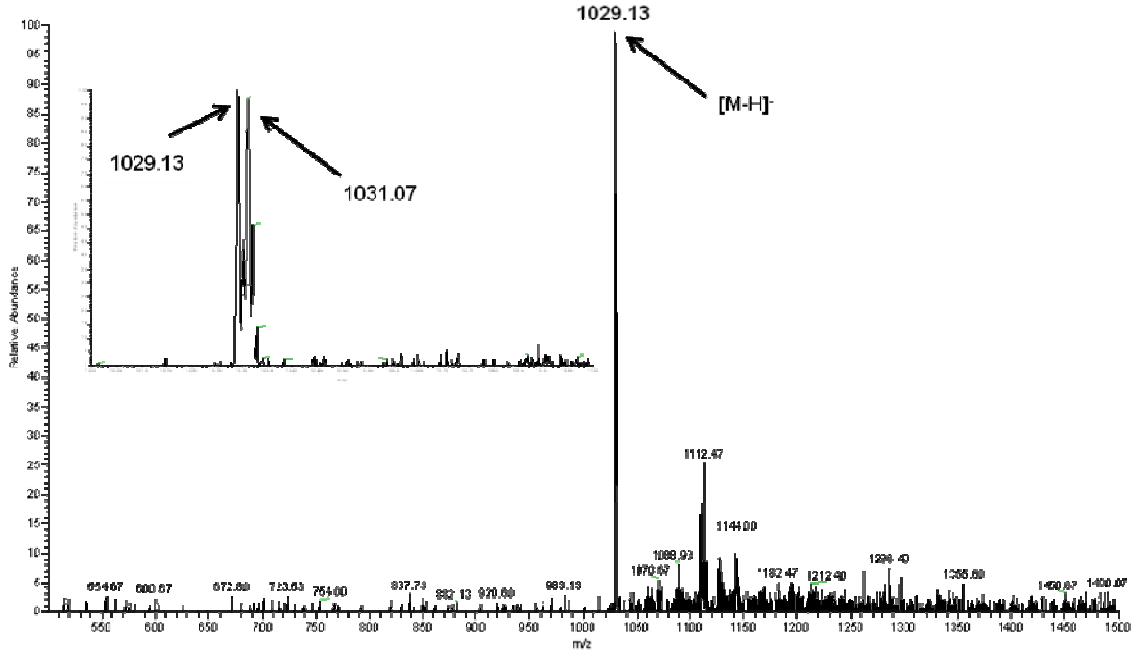
(ESI+) mass spectrum of peptide-tripod conjugate 19.



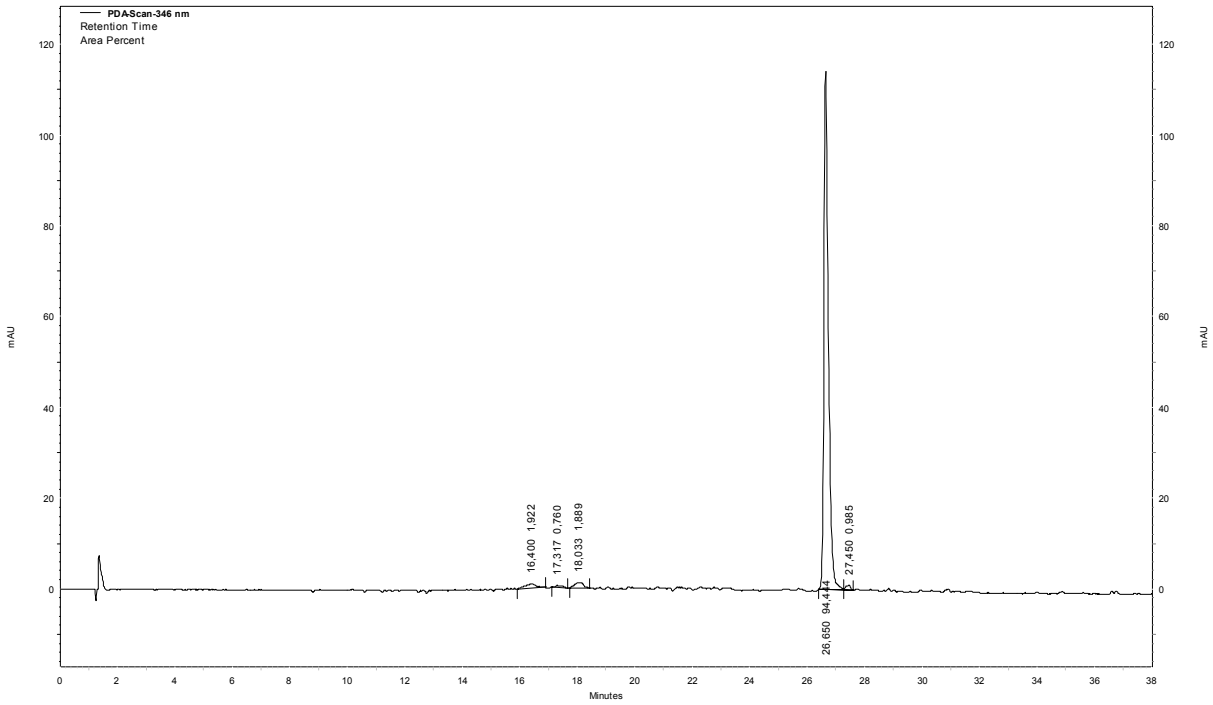
RP-HPLC elution profile (system S1) of thiol-reactive Eu(III) chelate 18.



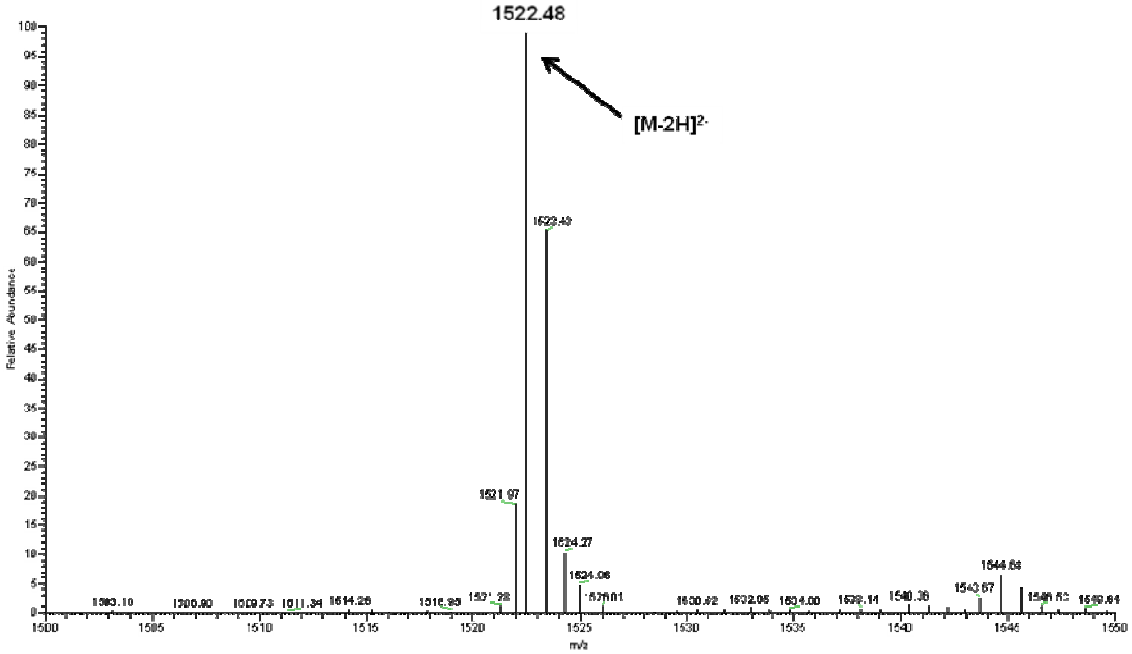
(ESI-) mass spectrum of thiol-reactive Eu(III) chelate 18.



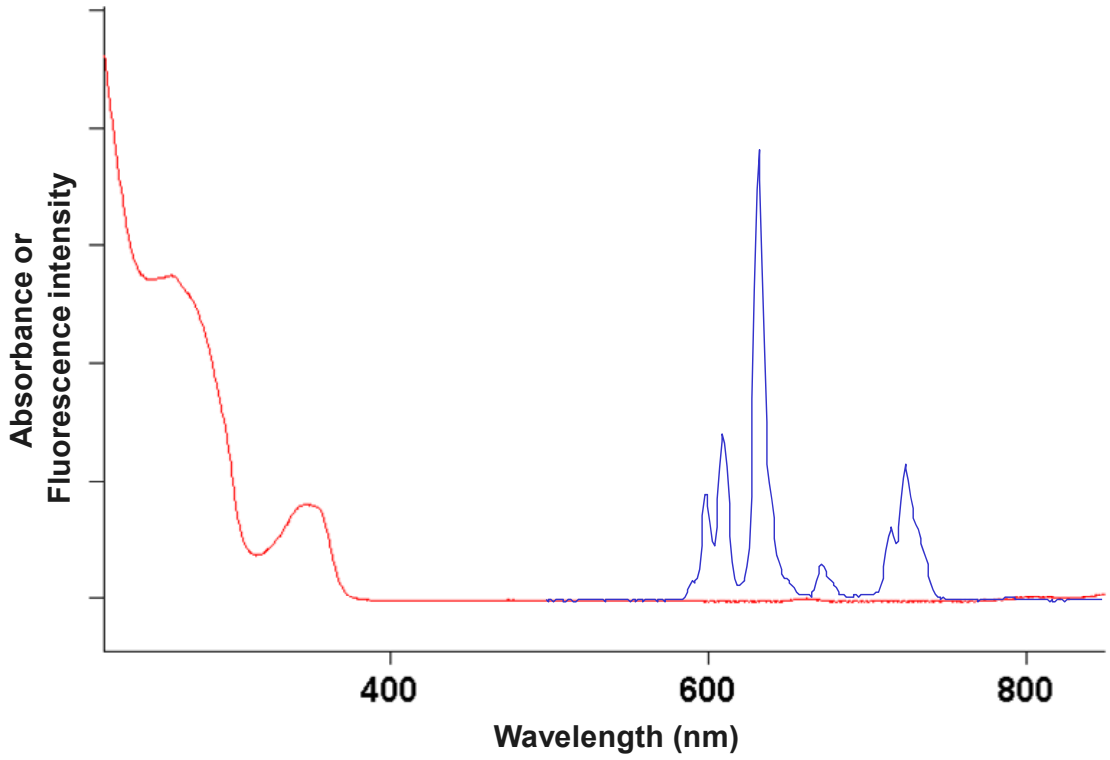
RP-HPLC elution profile (system D) of luminescent peptide-tripod conjugate 20.



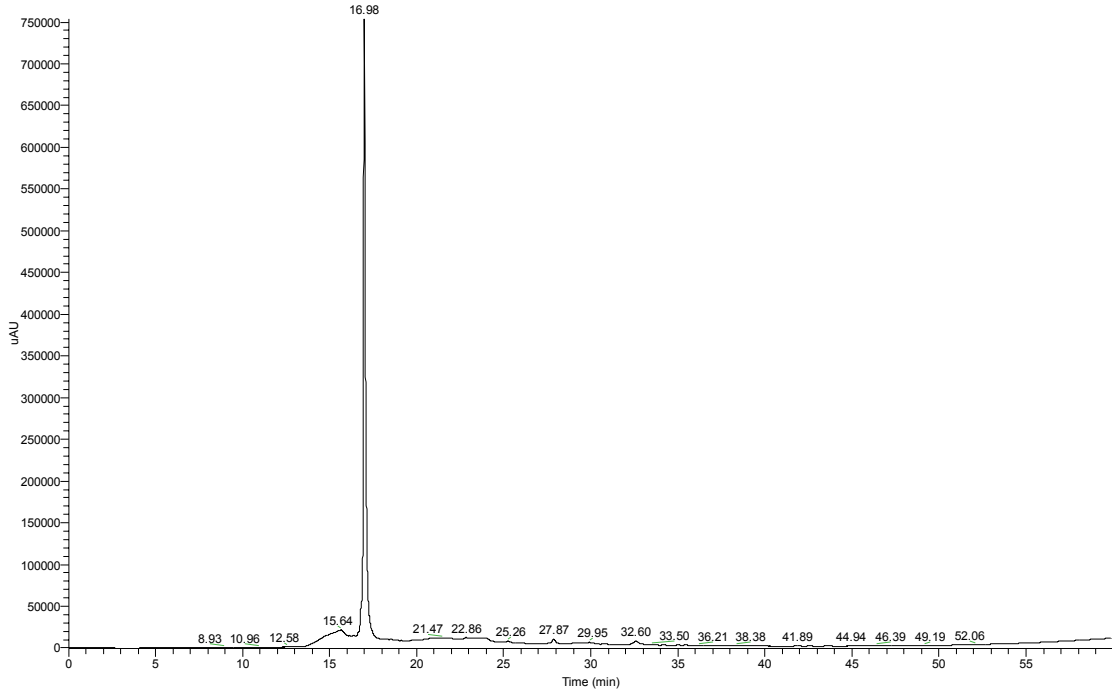
(ESI-) mass spectrum of luminescent peptide-tripod conjugate 20.



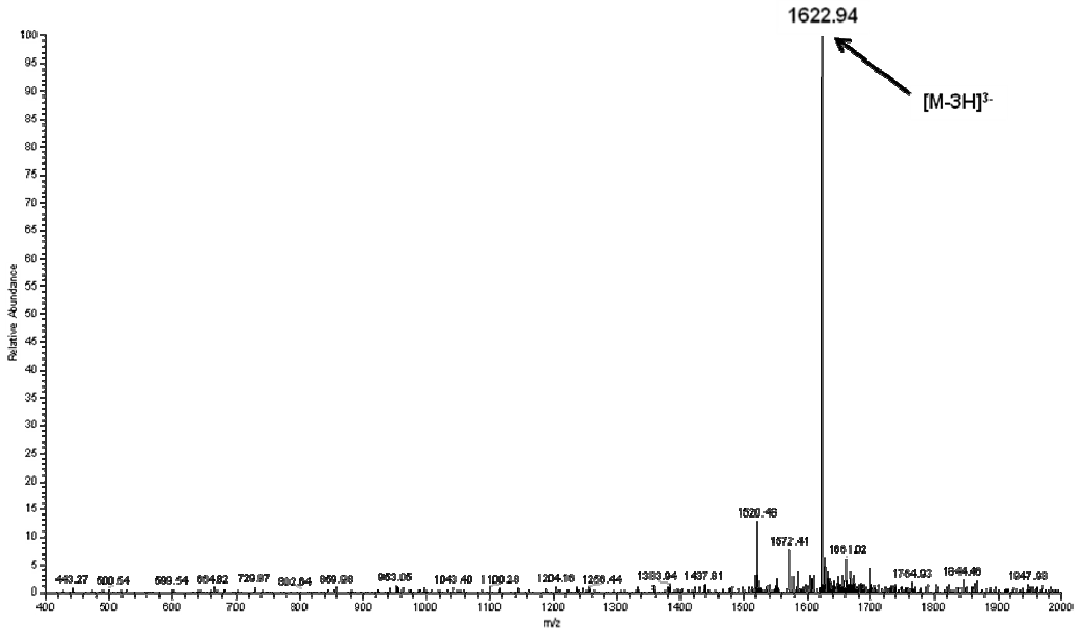
Normalised absorption (—) and luminescence (—) (Ex. at 345 nm) spectra of Eu(III) chelate-labelled peptide-tripod 20 recorded in water at 25 °C.



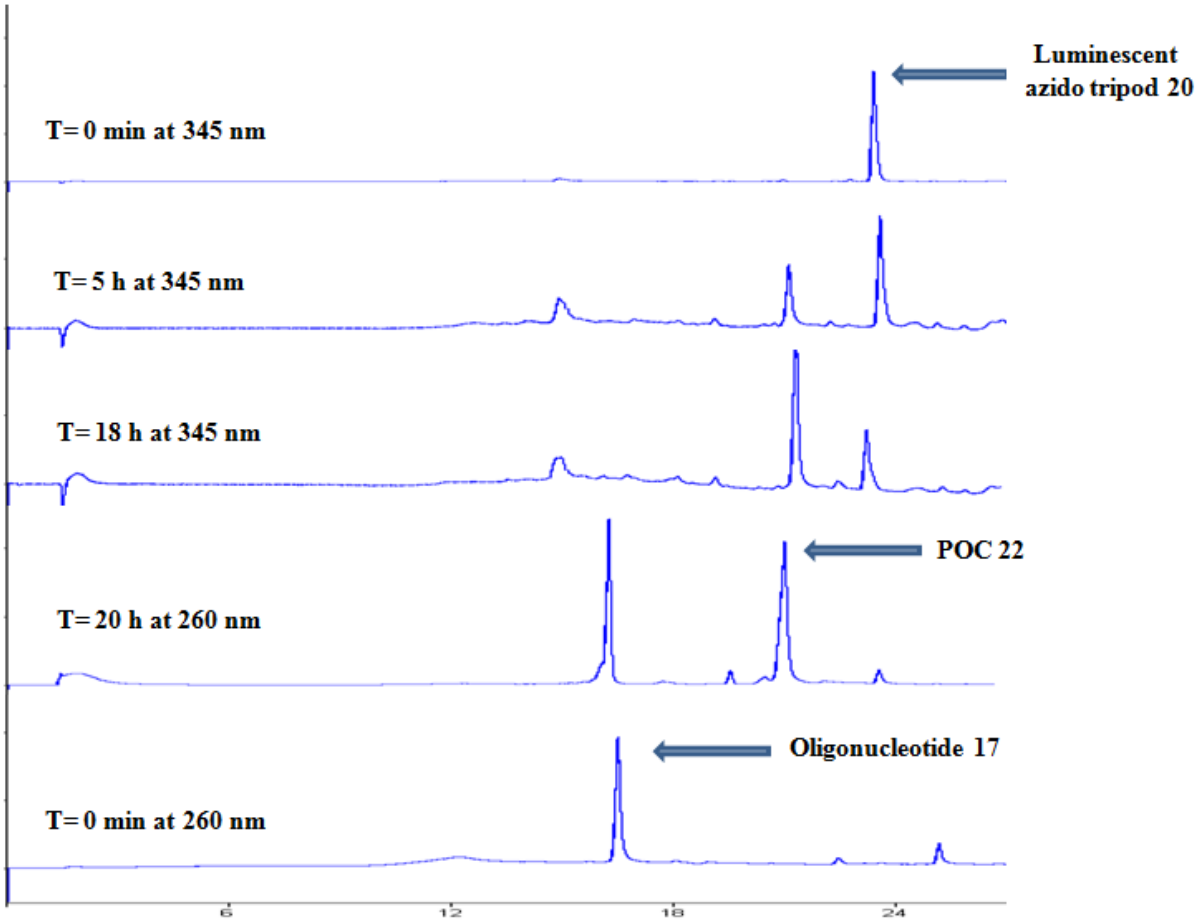
RP-HPLC elution profile (system S6) of ODN-alkyne 17.



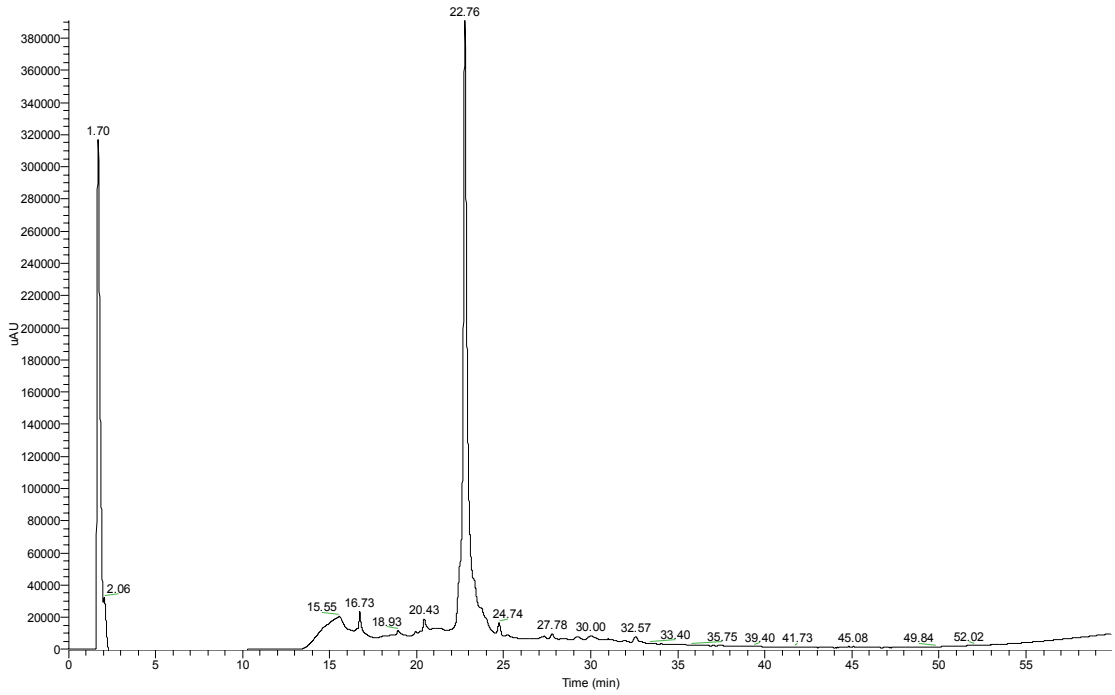
(ESI-) mass spectrum of ODN-alkyne 17.



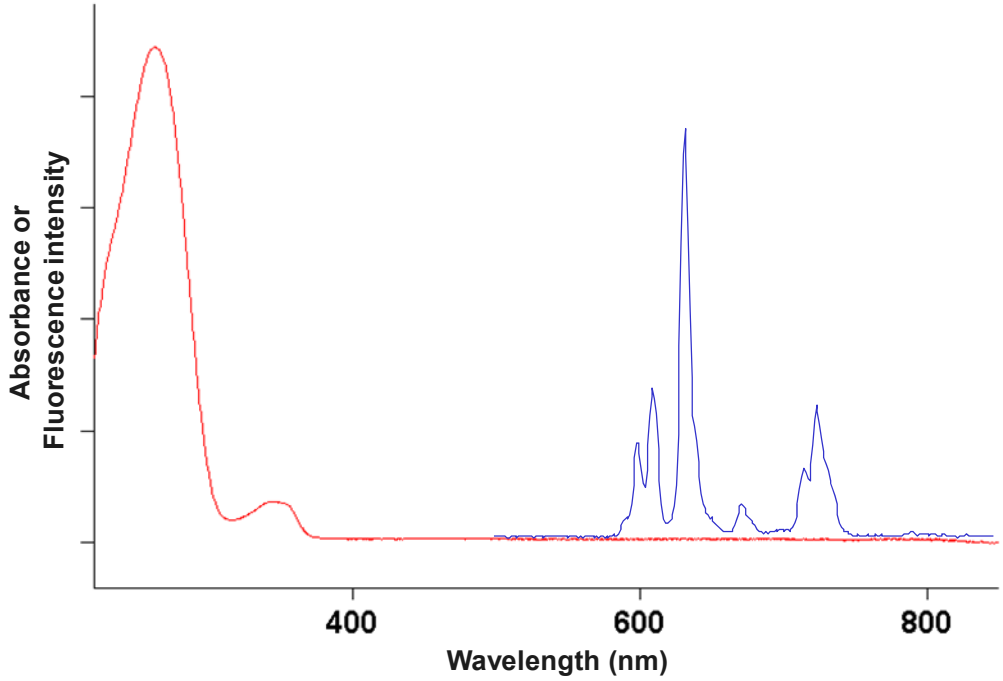
RP-HPLC elution profiles (system I) of the crude of the CuAAC reaction between 20 and ODN-alkyne 17.



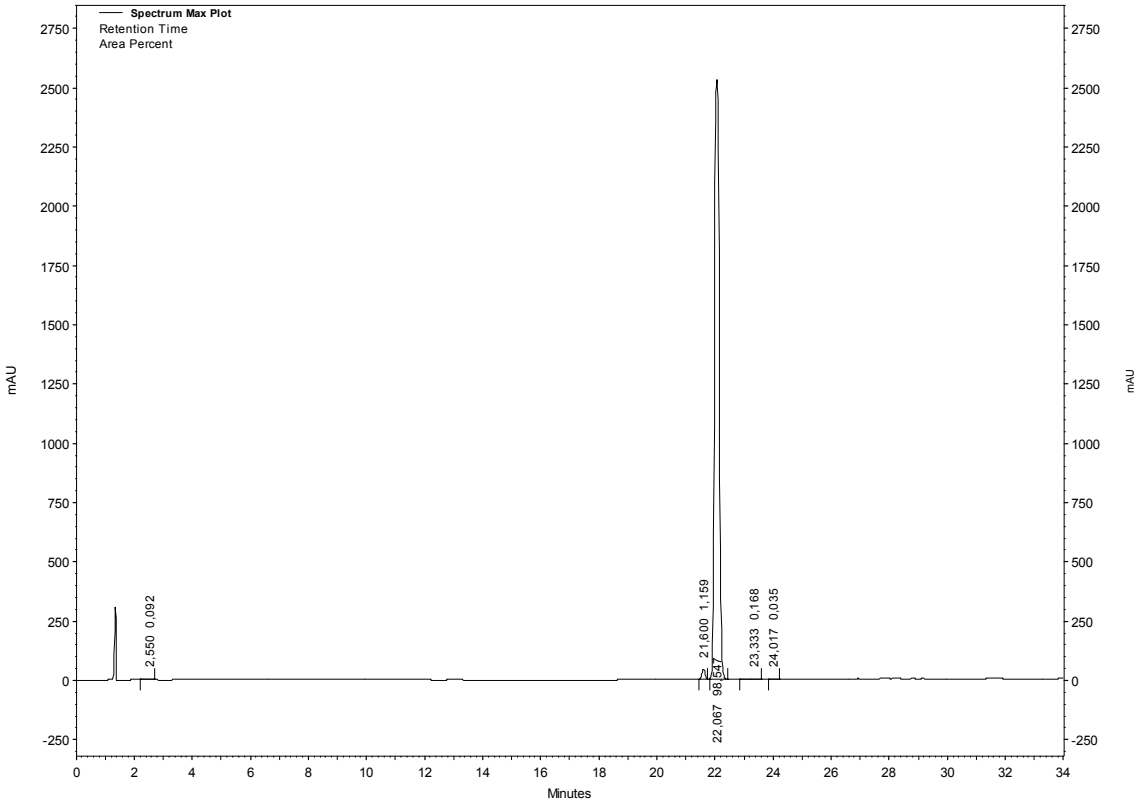
RP-HPLC elution profile (system J) of luminescent peptide-ODN conjugate 21.



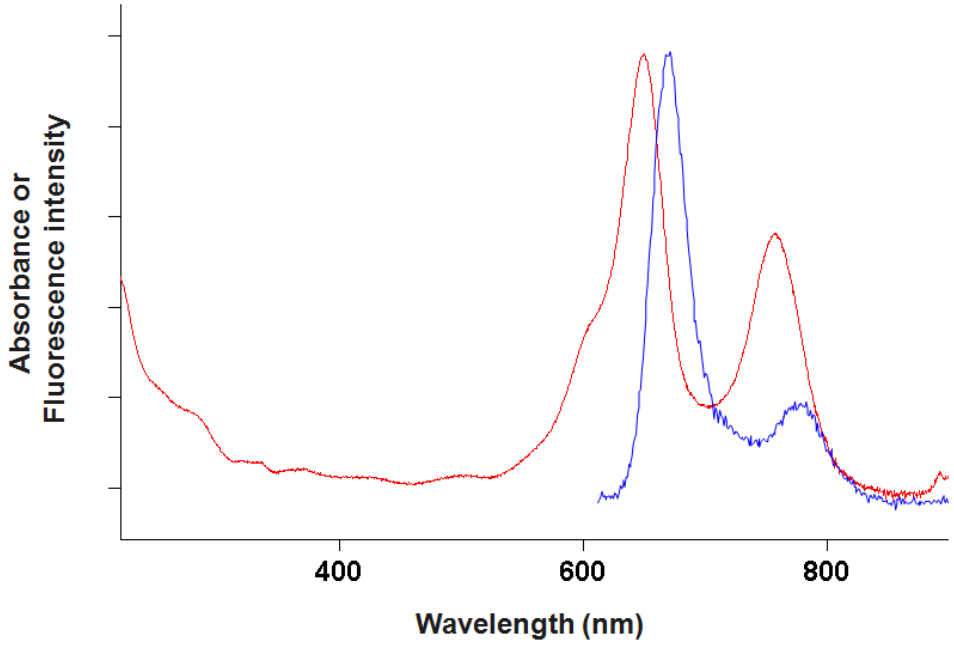
Normalised absorption (—) and luminescence (—) (Ex. at 345 nm) spectra of Eu(III) chelate-labelled peptide-ODN conjugate 21 recorded in TEEA (0.1 M, pH 7.0) at 25 °C.



RP-HPLC elution profile (system S8) of Cy 5.0-maleimide 22.



Normalised absorption (—) and fluorescence emission (—) (Ex. at 600 nm) spectra of Cy 5.0 / Cy 7.0-labelled peptide 23 recorded in deionised water at 25 °C.



The distance between the two cyanine dye molecules within fluorescent conjugate **23** was determined by applying a methodology previously described for a FRET fluorogenic caspase-3 probe[10] and was found to be 53 ±1 Å (for an energy transfer efficiency E = 0.71 and R_0 (Cy 5.0 - Cy 7.0) = 62 Å[11]).

[10] M. Lapeyre, J. Leprince, M. Massonneau, H. Oulyadi, P.-Y. Renard, A. Romieu, G. Turcatti and H. Vaudry, *Chem. Eur. J.*, 2006, **12**, 3655.
[11] S. Lee, J. Lee and S. Hohng, *PLoS ONE*, 2010, **5**, e12270.