Electronic Supplementary Material (ESI) for Organic & Biomolecular Chemistry

New insights into the water-solubilisation of fluorophores by post-synthetic "click" and Sonogashira reactions

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Supporting Information

Abbreviations

The following abbreviations are used throughout the text of the ESI file: AcOEt, ethyl acetate; β-Ala(SO_3H)-OH, α-sulfo-β-alanine; ATR, attenuated total reflectance; BODIPY, 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene ; BOP, benzotriazol-1-yloxytris(dimethylamino)-phosphonium hexafluorophosphate; DCC, *N*,*N'*-dicyclohexylcarbodiimide; DCU, *N*,*N'*-dicyclohexylurea; DIC, *N*,*N'*-diisopropylcarbodiimide; DIEA, *N*,*N*-diisopropylethylamine; DMAP, *N*,*N*,-dimethylaminopyridine; DMF, *N*,*N*-dimethylformamide; DMSO, dimethylsulfoxide; Fmoc, 9-fluorenylmethyloxycarbonyl; Gly, glycine; HOBt, 1-hydroxybenzotriazole; JMOD, J-modulated spin-echo; NBS, *N*-bromosuccinimide; NHS, *N*-hydroxysuccinimide; NMP, *N*-methylpyrrolidone; PBS, phosphate buffered saline; RP-HPLC, reversed-phase high performance liquid chromatography; R6G, rhodamine 6G; rt, room temperature; TEA, triethylamine; TEAA, triethylammonium acetate ; TEAB, triethylammonium bicarbonate; TFA, trifluoroacetic acid; TSTU, *O*-(*N*-succinimidyl)-1,1,3,3-tetramethyluronium tetrafluoroborate.

Experimental Section

Chemicals and reagents.

All chemicals were used as received from commercial sources without further purification unless otherwise stated. CH_2Cl_2 (stabilised with amylene) was dried by distillation over P_2O_5. Fmoc-Gly-OH, DMF (peptide synthesis grade), NMP (peptide synthesis grade), piperidine and polystyrene PHB Wang resin (1% DVB, 100-200 mesh, loading: 0.9 mmol g^{-1}) were provided by Iris Biotech GmbH. Fmoc-β-Ala(SO_3H)-OH was prepared from β-Ala(SO_3H)-OH using an improved synthetic procedure recently reported by us and is now commercially available from Iris Biotech GmbH (#HAA1915).[1] 3-Azidopropylamine, *tert*-butyl isonipecotate and [$Pd(Ph_3)_4$] catalyst were prepared according to literature procedures.[2,3,4] 2-Azidonaphthalene **3** and 3-azido-7-hydroxycoumarine **4** were readily synthesised according to published protocols.[5,6] Rhodamine carboxylic acid was prepared from rhodamine 6G (Aldrich, dye content ~ 95%) by using the 3-steps synthetic procedure developed by Afonso *et al.* (*i.e.*, pyrolysis, alkylation with benzyl bromoacetate and hydrogenolysis of benzyl ester).[7] 2-Iodonaphthalene **13** is commercially available and 8-(4-iodophenyl)-1,3,5,7-tetramethyl-BODIPY **15** and 5-iodofluorescein **16** were synthesised according to published procedures.[8,9] The HPLC-gradient grade acetonitrile (CH_3CN) and methanol (CH_3OH) were obtained from VWR. Phosphate buffered saline (PBS, 100 mM phosphate + 150 mM NaCl, pH 7.5) and aq. mobile-phases for HPLC were prepared using water purified with a Milli-Q system (purified to 18.2 MΩ.cm). Triethylammonium acetate (TEAA, 2.0 M) and

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triethylammonium bicarbonate (TEAB, 1.0 M) buffers were prepared from distilled triethylamine and glacial acetic acid or CO_2 gas.

Instruments and methods.
^1H and ^{13}C spectra were recorded either with a Bruker DPX 300 spectrometer (Bruker, Wissembourg, France) or with a Bruker AVANCE I 400 spectrometer (probe: BBFO, 5 mm). Chemical shifts are expressed in parts per million (ppm) from $CDCl_3$ (δ_H = 7.26, δ_C = 77.16), D_2O (δ_H = 4.79) or DMSO-d_6 (δ_H = 2.54, δ_C = 40.45). *J* values are expressed in Hz. ^{13}C substitutions were determined with JMOD experiments, differentiating signals of methyl and methine carbons pointing "up" (+) from methylene and quaternary carbons pointing "down" (-). Infrared (IR) spectra were recorded with an universal ATR sampling accessory on a Perkin Elmer FT-IR Spectrum 100 spectrometer. Analytical HPLC was performed on a Thermo Scientific Surveyor Plus instrument equipped with a PDA detector. Semi-preparative HPLC was performed on a Thermo Scientific SPECTRASYSTEM liquid chromatography system (P4000) equipped with a UV-visible 2000 detector. Mass spectra were obtained with a Finnigan LCQ Advantage MAX (ion trap) apparatus equipped with an electrospray source. UV-visible absorption spectra were obtained on a Varian Cary 50 scan spectrophotometer by using a rectangular quartz cell (Varian, standard cell, Open Top, 10 × 10 mm, 3.5 mL). Fluorescence spectroscopic studies (emission/excitation spectra) were performed on a Varian Cary Eclipse spectrophotometer with a semi-micro quartz fluorescence cell (Hellma, 104F-QS, 10 × 4 mm, 1400 µL). The absorption spectra of water-soluble fluorophores were recorded (220-650 nm) in PBS (concentration: 1.0-10.0 µM) at 25 °C. Excitation/emission spectra were recorded under the same conditions after emission/excitation at the corresponding wavelength (see Table S1, excitation and emission filters: auto, excitation and emission slit = 5 nm) in PBS. All fluorescence spectra were corrected. Relative quantum yields were measured in PBS at 25 °C by a relative method using a suitable standard (see Table). The following equation was used to determine the relative fluorescence quantum yield:

$$\Phi_F(x) = (A_S/A_X)(F_X/F_S)(n_X/n_S)^2 \Phi_F(s)$$

Where A is the absorbance (in the range 0.01-0.1 A.U.), F is the area under the emission curve, n is the refractive index of the solvents (at 25 °C) used in measurements, and the subscripts s and x represent standard and unknown, respectively.

Fluorophore (F)	Solvent	λ Ex. (nm)	Standard (std)	Φ_{std} / solvent	Φ_F
8	PBS	270 nm	E-stilbene[10]	0.05 / hexane[a]	0.56
17	PBS	270 nm	E-stilbene[10]	0.05 / hexane[a]	0.02
9	PBS	360 nm	7-OH-coumarine[11]	0.76 / PBS[b]	0.78

[10] See http://omlc.ogi.edu/spectra/.
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18	PBS	400 nm	7-OH-coumarine[11]	0.76 / PBS[b]	0.37
11	PBS	480 nm	fluorescein[12]	0.88 / NaOH 0.1N[c]	0.81
20	PBS	480 nm	fluorescein[12]	0.88 / NaOH 0.1N[c]	0.60
10	PBS	500 nm	R6G[12]	0.95 / EtOH[d]	0.25
19	PBS	480 nm	R6G[12]	0.95 / EtOH[d]	0.31
12	PBS	500 nm	R6G[12]	0.95 / EtOH[d]	0.86

[a]refractive index = 1.375, [b]refractive index = 1.337, [c]refractive index = 1.333, [d]refractive index = 1.362.

HPLC separations.
Several chromatographic systems were used for the analytical experiments and the purification steps:

- System A: RP-HPLC (Thermo Hypersil GOLD C_{18} column, 5 µm, 4.6 × 100 mm) with CH_3CN and aq. 0.1% trifluoroacetic acid (aq. TFA 0.1%, pH 2.0) as eluents [100% TFA (5 min), then linear gradient from 0 to 100% (50 min) of CH_3CN] at a flow rate of 1.0 mL min^{-1}. UV-vis detection with the "Max Plot" (*i.e.*, chromatogram at absorbance maximum for each compound) mode (220-750 nm).

- System B: RP-HPLC (Thermo Hypersil GOLD C_{18} column, 5 µm, 4.6 × 100 mm) with CH_3CN and aq. triethylammonium acetate (TEAA, 100 mM, pH 7.0) as eluents. [100% TEAA (5 min), then linear gradient from 0 to 100% (50 min) of CH_3CN] at a flow rate of 1.0 mL min^{-1}. UV-vis detection with the "Max Plot" mode (220-750 nm).

- System C: system A with the following gradient [80% TFA (5 min), then linear gradient from 20 to 100% (40 min) of CH_3CN]. UV detection with the "Max Plot" (*i.e.*, chromatogram at absorbance maximum for each compound) mode (220-400 nm).

- System D: system C with the following gradient [100% TFA (5 min), then linear gradient from 0 to 80% (40 min) of CH_3CN]. UV-vis detection with the "Max Plot" (*i.e.*, chromatogram at absorbance maximum for each compound) mode (220-650 nm).

- System E: semi-preparative RP-HPLC (Varian Kromasil C_{18} column, 10 µm, 21.2 × 250 mm) with CH_3CN and aq. TFA 0.1% as eluents [100% TFA (5 min), then linear gradient from 0 to 100% (55 min) of CH_3CN] at a flow rate of 18.0 mL min^{-1}. Dual visible detection was achieved at 254 and 290 nm for **8**, 300 and 344 nm for **9,** 500 and 544 nm for **10,** 410 and

[12] J. Olmsted, III, *J. Phys. Chem.*, 1979, **83**, 2581-2584.

444 nm for **11**, 500 and 528 nm for 12, 260 and 310 nm for **17**, 320 and 360 nm **18**, 220 and 365 nm for **19**, 228 and 275 nm for **20**.

- System F: semi-preparative RP-HPLC (Varian Kromasil C_{18} column, 10 µm, 21.2 × 250 mm) with CH_3CN and aq. triethylammonium bicarbonate (TEAB, 50 mM, pH 7.5) as eluents [100% TEAB (5 min), then linear gradient from 0 to 100% (55 min) of CH_3CN] at a flow rate of 18.0 mL min^{-1}. Dual visible detection was achieved at 254 and 290 nm for **8.**

- System G: semi-preparative RP-HPLC (Thermo Hypersil GOLD C_{18} column, 5 µm, 21.2 × 250 mm) with CH_3CN and aq. TFA 0.1% as eluents [100% TFA (5 min), then linear gradient from 0 to 100% (55 min) of CH_3CN] at a flow rate of 15.0 mL min^{-1}. Dual visible detection was achieved at 366 and 635 nm.

- System H: system E with the following gradient [90% TFA (5 min), then linear gradient from 10 to 90% (40 min) of CH_3CN] at a flow rate of 16.0 mL min^{-1}. Dual visible detection was achieved at 415 and 440 nm.

- System I: system H with the following gradient [90% TFA (5 min), then linear gradient from 10 to 85% (50 min) of CH_3CN] at a flow rate of 16.0 mL min^{-1}. Dual UV-vis detection was achieved at 415 and 440 nm for fluorescein, 270 and 350 nm for R6G.

Solid-phase synthesis of sulfonated terminal alkyne (1):

Wang resin loading with Fmoc-Gly-OH: the symmetrical anhydride method was employed. Firstly, the Wang resin (835 mg, 0.75 mmol) was swelled in dry CH_2Cl_2 (6 mL) for 15 min. After filtration, the resin was suspended in a mixture of CH_2Cl_2-NMP (7 : 3, v/v, 7.5 mL) containing Fmoc-Gly-OH (891 mg, 3 mmol, 4 equiv.) and DIC (1.5 mL of a 1.0 M solution in NMP, 1.5 mmol, 2 equiv.). Thereafter, 1.08 mL of a 0.1 M solution of DMAP in NMP (0.108 mmol, 0.15 equiv.) and a further amount of dry CH_2Cl_2 (1.8 mL) were added and the resulting mixture was stirred at rt overnight. Then, the mixture was filtered, rinsed three times with NMP, three times with CH_3OH and three times with CH_2Cl_2, and dried.

Fmoc removal: This deprotection was performed using a fresh solution of 20% piperidine in NMP (10 mL). The mixture was filtered, rinsed three times with NMP, three times with CH_3OH and three times with CH_2Cl_2. For a complete deprotection, this step was performed twice.

Coupling of Fmoc-β-Ala(SO_3H)-OH: A clear and limpid solution of Fmoc-β-Ala(SO_3H)-OH (1.56 g, 3 mmol, 4 equiv.) in NMP (12.0 mL) was added in a single-neck round bottom flask (25 mL) containing the Gly-Wang resin. Thereafter, 4.5 mL of a 2.0 M solution of DIEA in NMP (9 mmol, 12 equiv.) followed by BOP reagent (1.32 g, 3 mmol, 4 equiv.) were added and the resulting mixture was stirred at rt overnight. Then, the mixture was filtered, rinsed three times with NMP, three times with CH_3OH and three times with CH_2Cl_2, and dried.

Fmoc removal: *vide supra*.

Coupling of propiolic acid: *The batch of β-Ala(SO_3H)-Gly-Wang resin is divided into three equal parts (ca. 0.25 mmol) and the coupling reaction was conducted into three different flasks, each having a magnetic stirrer.* The resin was suspended in dry CH_2Cl_2 (2.42 mL). A

solution of propiolic acid (77 µL, 1.25 mmol, 5 equiv.) and DIC (1.25 mL of a 1.0 M solution in NMP, 1.25 mmol, 5 equiv.) were sequentially added. Thereafter, 0.75 mL of a 0.1 M solution of DMAP in NMP (0.075 mmol, 0.3 equiv.) was added and the mixture turned rapidly to a dark color. The resulting mixture was stirred at rt overnight. Then, the mixture was filtered, rinsed three times with NMP, three times with CH_3OH and three times with CH_2Cl_2, and dried.

Clevage of the resin: The resin clevage was performed by adding a mixture of TFA-CH_2Cl_2 (1 : 1, v/v, 15 mL) and stirring for 1 h, then filtered-off and washed with TFA (*ca.* 3 mL). The resulting filtrate was evaporated to dryness and the residue was co-evaporated three times with $CHCl_3$, and finally purified by RP-HPLC (system G, t_R = 1.3-1.5 min). The product-containing fractions were lyophilised to give the water-soluble terminal alkene **1** as an hygroscopic beige solid (95.5 mg, overall yield 45%). δ_H(300 MHz, D_2O) 3.36 (s, 1H, HC≡C), 3.70-4.06 (m, 5H); δ_C(75.5 MHz, D_2O) 37.4 (CH_2), 41.4 (CH_2), 64.0 (CH), 75.5 (Cq), 76.8 (Cq), 154.4 (C=O), 168.0 (C=O), 172.8 (C=O); (ESI-): *m/z* 277.13 $[M - H]^-$, calcd $C_8H_{10}N_2O_7S$ 278.02; HPLC (system A): t_R = 1.4 min, purity 95%; Too hygroscopic compound for suitable IR and elemental analyses.

Azido-BODIPY (5). 8-(4-Iodophenyl)-1,3,5,7-tetramethyl-BODIPY **15** (50 mg, 0.10 mmol), was dissolved in dry CH_2Cl_2 (2 mL) and NBS (18 mg, 0.10 mmol) was added. The resulting reaction mixture was stirred at rt in the dark for 30 min. Then, DMF (2 mL) and sodium azide (50 mg, 0.77 mmol) were added and the solution was stirred for a further 1 h. At this stage, the course of the reaction was followed by TLC. The solution was extracted with AcOEt, washed with deionised water (5 × 25 mL), NaCl (2 × 20 mL), dried over anhydrous $MgSO_4$ and evaporated to dryness. The resulting residue was purified by column chromatography on a silica gel column with petroleum ether/CH_2Cl_2 (gradient from 80:20 to 70:30) as eluents, to give azido derivative **5** (50 mg, yield 90%). δ_H(300 MHz, $CDCl_3$) 0.99 (t, 3H, ^3J = 7.5 Hz), 1.04 (t, 3H, ^3J = 7.7 Hz), 1.35 (s, 3H), 1.36 (s, 3H), 2.32 (q, 2H, ^3J = 7.5 Hz), 2.38 (q, 2H, ^3J = 7.7 Hz), 2.57 (s, 3H), 4.58 (s, 2H), 7.47 (AB sys, 4H, J_{AB} = 8.3 Hz, $\nu_o\delta$ = 240.5 Hz); δ_C(75.4 MHz, $CDCl_3$) 12.0, 12.4, 13.2, 14.5, 15.1, 17.2, 17.3, 45.3, 94.9, 130.2, 132.5, 133.2, 135.1, 135.3, 137.4, 138.6, 140.3, 141.1, 145.3, 159.6; δ_B(128.4 MHz, $CDCl_3$) 0.66 (t, $J_{B\text{-}F}$ = 32.7 Hz); (EI+): *m/z* (%) 547.1 (30) $[M]^{+\bullet}$, 506.1 (100) $[M\text{-}N_3]^{+\bullet}$, calcd $C_{23}H_{25}BF_2IN_5$ 547.12; HPLC (system A): t_R = 45.6 min, purity 97%; elemental analysis (%) calcd: C, 50.48; H, 4.61; N, 12.80; found: C, 50.22 ; H, 4.40 ; N, 12.59; $\lambda_{max}(CH_2Cl_2)$/nm 526 (ε/dm^3 mol^{-1} cm^{-1} 54 200), 502 (sh, ε/dm^3 mol^{-1} cm^{-1} 26 600), 486 (ε/dm^3 mol^{-1} cm^{-1} 7 400).

Azido-fluorescein (6).

DCC-HOBt mediated coupling: To a solution of fluorescein (115 mg, 0.35 mmol) and *tert*-butyl isonipecotate (115 mg, 0.63 mmol, 1.8 equiv.) in dry DMF (4 mL) were sequentially added HOBt monohydrate (55 mg, 0.42 mmol, 1.2 equiv.), DIEA (60 µL, 0.63 mmol,1 equiv.) and DCC (85 mg, 0.42 mmol, 1.2 equiv.). The resulting reaction mixture was heated at 75 °C overnight. Thereafter, DMF was evaporated under reduced pressure. The resulting residue was dissolved in CH_2Cl_2. DCU precipitate was filtered-off and the filtrate was purified on a SNAP flash-chromatography cartridge (100 g, Biotage® KP-Sil) by means of an automated flash purification system (Biotage® Isolera One), and by using a linear gradient of CH_3OH (0-10%) in CH_2Cl_2 as the mobile phase (flow rate 50 mL min^{-1} and UV detection at 260 nm) to finally obtain the expected product as an orange oil. Its structure was confirmed by mass analyse and purity checked by RP-HPLC. (ESI+): *m/z* 500.27 $[M + H]^+$ calcd

$C_{30}H_{29}NO_6$ 499.20; HPLC (system C): t_R = 16.5 min, purity > 85%. This *tert*-butyl ester was used in the next step without further purification.

Removal of the tert-butyl ester: The oily residue was dissolved in CH_2Cl_2 (5 mL) and TFA (2 mL) was added at 0 °C. After 3 h of stirring at rt, volatiles were removed, and the purification was performed by semi-preparative RP-HPLC (system H, 2 injections, t_R =17.00-20.0 min). The product-containing fractions were lyophilised to give fluorescein carboxylic acid as a an orange amorphous powder (66 mg, 0.14 mmol, yield 43%) δ_H(300 MHz, DMSO-d_6) 7.80-7.40 (m, 4H, 4 × CH_{ar}), 7.15-7.05 (m, 2H, 2 × CH_{ar}), 6.80-6.70 (m, 4H, 4 × CH_{ar}), 3.95-3.90 (m, 1H, CH_2), 3.65-3.58 (m, 1H, CH_2), 3.00-2.50 (m, 2H, CH_2), 2.45-2.20 (m, 1H, CH), 2.00-1.60 (m, 2H, CH_2), 1.40-1.00 (m, 2H, CH_2); δ_C(75.5 MHz, DMSO-d_6) 175.3, 166.3, 156.6, 152.6, 135.8, 131.6, 130.6, 130.3, 129.6, 127.3, 124.5 119.1, 115.2, 109.6, 102.9, 46.3, 42.4, 37.6, 34.3, 27.9, 27.4, 24.6; NMR spectra are complicated by the conformational equilibrium of the piperidine ring; HPLC (system D): t_R = 20.1 min, purity > 90%; (ESI+): *m/z* 444.20 $[M + H]^+$; (ESI-): *m/z* 442.27 $[M - H]^-$, calcd $C_{26}H_{21}NO_6$ 443.14; λ_{max}(PBS) nm 499 (ε/dm^3 mol^{-1} cm^{-1} 48 000); λ_{max} em (PBS) nm 520 (Φ_F 0.79). Too hygroscopic compound for suitable elemental analysis.

Coupling with 3-azidopropylamine: To a solution of fluorescein-isonipecotic acid derivative (66 mg,0.15 mmol) and 3-azidopropylamine (15 mg , 0.15 mmol) in dry DMF (5 mL) were added BOP salt (80 mg, 0.18 mmol, 1.2 equiv.) and DIEA (27 µL,0.3 mmol, 2 equiv.). After 5 h of stirring at rt, solvent was removed, and the purification was performed by semi-preparative RP-HPLC (system I, 2 injections, t_R = 22.00-24.0 min). The product-containing fractions were lyophilised to give azido-fluorescein **6** as an orange amorphous powder (39 mg, 0.07 mmol, yield 50%). ν_{max}/cm^{-1} 3278, 2920, 2097, 1593,1449; δ_H(300 MHz, DMSO-d_6) 7.82 (t, 1H, *J* = 5.4 Hz, CH_{ar}), 7.73-7.61 (m, 3H, 2 × CH_{ar} & NH), 7.53-7.49 (m, 1H, CH_{ar}), 7.20 (d, 2H, *J* = 9.0 Hz, 2 × CH_{ar}), 6.90-6.81 (m, 4H, 4 × CH_{ar}), 4.10-3.90 (m, 1H, CH_2), 3.70-3.60 (m, 1H, CH_2), 3.31 (t, 2H, *J* = 6.6 Hz, CH_2), 3.10-2.50 (m, 4H, 2 × CH_2), 2.50-2.10 (m, 1H, CH), 1.70-1.60 (m, 4H, 2 × CH_2), 1.40-1.00 (m, 2H,CH_2); δ_C(75.5 MHz, DMSO-d_6) 173.6, 166.5, 136.6, 132.1, 130.6, 130.5, 129.9, 129.6, 127.3, 115.5, 102.9, 48.45, 46.6, 42.5, 41.3, 36.6, 35.8, 28.5, 27.9, 25.2; HPLC (system D): t_R = 22.2 min, purity > 90%; (ESI+): *m/z* 526.27 $[M + H]^+$ calcd $C_{29}H_{27}N_5O_5$ 525.19; λ_{max}(PBS) nm 500 (ε/dm^3 mol^{-1} cm^{-1} 23 400) λ_{max} em (PBS) nm 520 (Φ_F 0.71). Too hygroscopic compound for suitable elemental analysis.

Azido-R6G (7). R6G carboxylic acid (50 mg, 0.1 mmol) synthesised according to Afonso *et al.* protocol was dissolved in dry DMF (1.5 mL). DIEA (18.5 µL, 0.2 mmol, 2 equiv.) and TSTU reagent (33 mg, 0.11 mmol, 1.1 equiv.) were sequentially added. The resulting mixture was stirred at rt for 2 h. The complete conversion into the corresponding NHS ester was confirmed by ESI mass analyse and this active ester was used directly in the next step. The 3-Azidopropylamine (10 mg, 0.1 mmol) and DIEA (18.5 µL, 0.2 mmol, 2 equiv.) were dissolved in dry DMF (5 mL), the solution of NHS ester of R6G was then added and the resulting reaction mixture was stirred at rt overnight. The reaction was checked for completion by RP-HPLC and the mixture was evaporated to dryness. The resulting residue was dissolved in 0.1% aq. TFA and CH_3CN and purified by semi-preparative RP-HPLC (system I, 1 injection, t_R = 29.4-36.2 min). The product-containing fractions were lyophilised to give azido-R6G **7** as a pink amorphous powder (33 mg, 0.06 mmol, yield 56%). ν_{max}/cm^{-1} 3050, 2095, 1598, 1434, 1241; δ_H(300 MHz, $CDCl_3$) 8.46 (dd, 1H, *J* = 2.4 Hz, *J* = 6.0 Hz,CH_{ar}), 7.80-7.72 (m, 3H, CH_{ar}), 6.78 (s, 2H, CH_{ar}), 6.64 (s, 2H, 2 × CH_{ar}), 6.60-6.58 (bs, 2H, 2 × NH), 4.51 (s, 2H, OCH_2), 3.50-3.40 (m, 4H, $NC\underline{H_2}CH_3$), 3.27-3.19 (m, 4H,CH_2), 2.12 (s, 6H,2 × CH_3), 1.69 (qt, 2H, *J* = 6.6Hz, CH_2), 1.40-1.00 (t, 6H, *J* = 7.2Hz, $NCH_2C\underline{H_3}$);

δ_C(75.5 MHz, $CDCl_3$) 167.0, 164.3, 157.7, 157.3, 156.1, 134.4, 133.3, 132.0, 130.6, 130.1, 129.3, 128.9, 127.7, 113.8, 94.1, 63.8, 49.5, 38.8, 37.0, 28.7, 17.5, 13.9; HPLC (system C): t_R = 20.1 min, purity > 90%; (ESI+): *m/z* 555.27 $[M + H]^+$ calcd $C_{31}H_{35}N_6O_4^+$ 555.27; λ_{max}(PBS) nm 527 (ε/dm^3 $mol^{-1}cm^{-1}$ 70 000); λ_{max} em (PBS) nm 552 (Φ_F 0.75). Too hygroscopic compound for suitable elemental analysis.

3-Iodo-7-hydroxycoumarine (14). 3-Acetamido-7-acetoxy-coumarine[6] (500 mg , 2.0 mmol) was refluxed in a solution of conc. HCl and ethanol (2 : 1, v/v, 5mL) for 90 min. Then, ice-water (8 mL) was added to the reaction mixture. The resulting dilute solution was then cooled to 4 °C with an ice bath and $NaNO_2$ (280 mg, 4.0 mmol, 2 equiv.) was added in portions. The mixture was stirred for 30 min and KI (1.2 g, 7.2 mmol, 7.2 equiv.) was added slowly. After stirring for a further 1 h, the mixture was extracted with AcOEt and washed with deionised water and brine. The organic layer was dried over anhydrous $MgSO_4$, filtrated, and the solvent was removed under reduced pressure. The resulting dark oil was purified by flash-chromatography on a silica gel column with a mixture of cyclohexane-AcOEt (4 : 6, v/v) as the mobile phase to afford 3-iodo-7-hydroxycoumarine **14** as a pinkish solid. (85 mg, yield 15%). R_f (cyclohexane-AcOEt, 4 : 6, v/v) 0.7; δ_H(300 MHz, DMSO-d_6) 6.70 (s, 1H), 6.78 (d, 1H, *J* = 6.4 Hz), 7.49 (d, 1H, *J* = 8.5 Hz), 8.63 (s, 1H); δ_C(75.5 MHz, DMSO-d_6) 80.3, 102.0, 113.0, 113.5, 129.0, 152.70, 155.5, 157.6, 161.8. (ESI-): *m/z* 287.07 $[M - H]^-$, calcd $C_9H_5IO_3$ 287.92. HPLC (system A): t_R = 22.0 min, purity 99%.

General procedure for sulfonation of fluorophores through the CuAAC reaction:
A mixture of azido-fluorophore (0.054 mmol, 1 equiv.), sulfonated terminal alkyne **1** (18 mg, 0.064 mmol, 1.2 equiv.), sodium ascorbate (2.13 mg, 0.0108 mmol, 0.2 equiv.) and $CuSO_4$,$5H_2O$ (0.67 mg, 2.7 µmol, 0.05 equiv.) were mixed together in a mixture of degassed DMSO-H_2O (1 : 1, v/v, 1.5 mL) and the resulting reaction mixture was stirred at rt overnight. The reaction was checked for completetion by RP-HPLC (system A) and purified by semi-preparative RP-HPLC. The product-containing fractions were lyophilised to give the targeted monosulfonated fluorophore.

Monosulfonated triazole-based naphthalene (8). Purification by RP-HPLC (system F followed by system G, t_R = 23.4-27.9 min). The product-containing fractions were lyophilised to give the water-soluble naphthalene **8** as a light brown amorphous powder (13 mg, overall isolated yield 54%). δ_H(300 MHz, DMSO-d_6) 3.55 (t, 1H, *J* = 6.7 Hz), 3.75-3.92 (m, 4H), 7.60-7.69 (m, 2H), 8.07-8.22 (m, 5H), 8.43(t, 1H, *J* = 5.3 Hz), 8.57 (s, 1H), 9.41 (s, 1H); δ_C(75.5 MHz, DMSO-d_6) 37.9 (CH_2), 41.2 (CH_2), 63.8 (CH), 118.4, 118.8, 124.8, 127.2, 127.5, 127.9, 128.4, 130.0, 132.5, 132.8, 133.8, 143.6, 158.9 (Cq), 167.4 (C=O), 170.9 (C=O); (ESI+): *m/z* 448.13 $[M + H]^+$, (ESI-): *m/z* 446.00 $[M - H]^-$, calcd $C_{18}H_{17}N_5O_7S$ 447.08; HPLC (system A): t_R = 19.8-20.4 min, purity 99%; λ_{max}(PBS)/nm 230 (ε/dm^3 mol^{-1} cm^{-1} 28 100); Too hygroscopic compound for suitable IR and elemental analyses.

Monosulfonated triazole-based 7-hydroxycoumarine (9). Purification by RP-HPLC (system E, t_R = 17.2-17.9 min). The product-containing fractions were lyophilised to give the water-soluble coumarine **9** as a white amorphous powder (8.0 mg, overall isolated yield 32%). δ_H(300 MHz, DMSO-d_6) 3.57 (t, 1H, *J* = 6.4 Hz), 3.68-3.90 (m, 4H), 6.85 (s, 1H), 6.91 (d, 1H, *J* = 8.6 Hz), 7.76 (d, 1H, *J* = 8.6 Hz), 8.18(t, 1H, *J* = 4.9 Hz, NH), 8.43 (t, 1H, *J* = 4.7 Hz, NH), 8,65 (s, 1H), 8.90 s, 1H), δ_C(75.5 MHz, DMSO-d_6) 38.0, 45.8, 63.9, 102.3, 110.3, 114.50, 119.1, 127.3, 131.3, 137.3, 142.6, 154.9, 156.4, 158.8, 162.8, 167.4, 171.1; (ESI+):

m/z 482.01 $[M + H]^+$, 498.71 $[M + H_2O]^{+\bullet}$ (water cluster formed during the ionisation process), (ESI-): *m/z* 479.93 $[M - H]^-$, calcd $C_{17}H_{15}N_5O_{10}S$ 481.05; HPLC (system A): t_R = 15.0 min, purity 98.5%; λ_{max}(PBS)/nm 393 (ε/dm^3 mol^{-1} cm^{-1} 17 650); Too hygroscopic compound for suitable IR and elemental.

Monosulfonated triazole-based BODIPY (10). Purification by RP-HPLC (system E, t_R = 29.4-35.7 min). The product-containing fractions were lyophilised to give the water-soluble BODIPY **10** as a red amorphous powder (7.0 mg, overall isolated yield 31%). δ_H(300 MHz, DMSO-d_6) 0.71 (t, 3H, *J* = 7.0 Hz, CH_2-C<u>*H*</u>$_3$), 0.95 (t, 3H, *J* = 7.0 Hz, CH_2-C<u>*H*</u>$_3$), 1.22 (s, 3H, CH_3), 1.28 (s, 3H, CH_3), 1.34 (s, 3H, CH_3), 2.33 (q, 4H, *J* = 7.3 Hz, 2 × C<u>*H*</u>$_2$-CH_3), 3.60-3.81 (m, 5H, 2 × CH_2, CH partially masked by H_2O peak), 5.80 (s, 2H), 7.25 (d, 2H, *J* = 8.1 Hz, CH *arom.*), 7.94 (d, 2H, *J* = 8.1 Hz, CH *arom.*), 8.15 (t, 1H, *J* = 5.1 Hz, N<u>*H*</u>), 8.29 (s, 1H), 8.31 (t, 1H, *J* = 5.2 Hz, N<u>*H*</u>); δ_C(75.5 MHz, DMSO-d_6) 11.3, 11.9, 14.1, 14.3, 16.2, 37.7, 43.1, 63.7, 96.0, 129.3, 131.9, 132.7, 133.6, 135.5, 136.7, 138.1, 141.0, 141.7, 142.5, 158.9, 160.4, 167.2, 170.9; (ESI-): *m/z* 823.94 $[M - H]^-$, calcd $C_{31}H_{35}BF_2IN_7O_7S$ 825.14; HPLC (system A): t_R = 14.7 min, purity 99%; Too hygroscopic compound for suitable IR and elemental.

Monosulfonated triazole-based fluorescein (11). Purification by RP-HPLC (system E, t_R = 20.5-22.5 min). The product-containing fractions were lyophilised to give the water-soluble fluorescein **11** as a yellow amorphous powder (11.0 mg, overall isolated yield 52%). δ_H(300 MHz, DMSO-d_6) 1.13-1.23 (m, 2H,CH_2), 1.54-1.60 (m, 2H, CH_2), 1.94 (t, 2H, *J* = 6.6 Hz, CH_2-CH_2-CH_2), 2.21 (t, 1H, *J* = 9.7 Hz, CH), 2.71-3.20 (m, 4H, 2 × CH, CH_2-CH_2-CH_2), 3.49 (t, 1H, *J* = 6.6 Hz, CH), 3.63-3.96 (m, 6H), 4.36 (t, 2H, *J* = 6.6 Hz, CH_2-CH_2-CH_2), 7.06-7.14 (m, 4H), 7.43 (d, 2H, *J* = 8.6 Hz), 7.54 (d, 1H, *J* = 8.6 Hz), 7.67-7.5 (m, 4H), 8.17 (t, 1H, *J* = 5.1 Hz, N<u>*H*</u>), 8.26 (t, 1H, *J* = 5.1 Hz, N<u>*H*</u>), 8.56 (s, 1H); δ_C(75.5 MHz, DMSO-d_6) 29.7, 35.5, 37.8, 41.2 47.5, 63.8, 102.5, 116.0, 120.5, 126.5, 127.4, 129.6, 130.2, 130.3, 133.1, 135.8, 142.5, 157.9, 159.2, 166.2, 167.3, 170.9, 173.7; (ESI+): *m/z* 804.13 $[M + H]^+$, (ESI-): *m/z* 802.13 $[M - H]^-$, calcd $C_{37}H_{37}N_7O_{12}S$ 803.22; HPLC (system A): t_R = 18.6 min, purity 86%; λ_{max}(PBS)/nm 497 (ε/dm^3 mol^{-1} cm^{-1} 48 350); Too hygroscopic compound for suitable IR and elemental.

Monosulfonated triazole-based R6G (12). Purification by RP-HPLC (system E, t_R = 27.5-29.7 min). The product-containing fractions were lyophilised to give the water-soluble R6G **12** as a pink amorphous powder (8.0 mg, overall isolated yield 40%). δ_H(300 MHz, DMSO-d_6) 1.24 (t, 6H, *J* = 7 Hz, CH_3), 1.83 (qt, 2H, *J* = 6.6 Hz, CH_2), 2.06 (s, 6H, CH_3), 2.95 (q, 2H, *J* = 6.0 Hz, CH_2), 3.5-3.9 (m, 6H, partially masked by H_2O peak) 4.19 (t, 2H, *J* = 6.7 Hz, CH_2), 4.44 (s, 2H, OCH_2), 6.78 (s, 2H, CH *arom.*), 6.86 (s, 2H, CH *arom.*), 73.43-7.46 (d, 1H, *J* = 7.5 Hz), 7.62 (t, 2H, *J* = 5.6 Hz), 7.83-7.95 (m, 2H), 8.05 (t, 1H, *J* = 5.6 Hz, N<u>*H*</u>), 8.30 (t, 1H, *J* = 5.1 Hz, N<u>*H*</u>), 8.37-8.40 (d, 1H, *J* = 7.5 Hz), 8.43 (s, 1H, CH); δ_C(75.5 MHz, DMSO-d_6) 13.6, 17.4, 29.8, 35.3, 37.9, 41.2, 47.2, 63.4, 63.9, 93.6, 112.8, 125.3, 126.3, 128.5, 128.7, 130.3, 130.5, 133.6, 134.1, 142.5, 155.7, 156.7, 157.0, 159.2, 164.2, 166.3, 167.4, 171.0; (ESI+): *m/z* 833.20 $[M + H]^+$, (ESI-): *m/z* 831.07 $[M - H]^-$, calcd $C_{18}H_{17}N_5O_7S$ 832.28; HPLC (system A): t_R = 25.4 min, purity 99%; λ_{max}(PBS)/nm 530 (ε/dm^3 mol^{-1} cm^{-1} 80 200); Too hygroscopic compound for suitable IR and elemental.

General procedure for sulfonation of fluorophores through the Sonogashira reaction:
Iodo-fluorophore (0.06 mmol, 1 equiv.) and sulfonated terminal alkyne **1** (20 mg, 0.072 mmol, 1.2 equiv.) were dissolved in 1 mL of a degassed solution of DMF-H_2O-Et_3N (2 : 1 : 1,

v/v/v). Then, $Pd(PPh_3)_4$ (7 mg, 6.0 μmol, 0.1 equiv.) and CuI (3.0 mg, 12 μmol, 0.2 equiv.) were sequentially added under an argon atmosphere and the resulting reaction mixture was stirred at rt for 2-3 h. The reaction was checked for completetion by RP-HPLC (system A). The crude was neutralised to pH ~ 7 by adding 37% HCl and was centrifugated to remove insoluble materials. The mixture was diluted with aq. TFA 0.1% (6 mL) and purified by semi-preparative RP-HPLC. The product-containing fractions were lyophilised to give the targeted monosulfonated fluorophore.

Monosulfonated alkyne-based naphthalene (17). Purification by RP-HPLC (system G). The product-containing fractions were lyophilised to give the water-soluble naphthalene **17** as a white amorphous powder (16.0 mg, overall isolated yield 82%). δ_H(300 MHz, DMSO-d_6) 3.54-4.01 (m, 5H, 2 × CH_2, CH), 7.56-7.61 (m, 2H), 7.96-8.12 (m, 4H, H *arom.*), 8.23 (s, 1H, -NH), 8.32 (s, 1H, H *arom.*), 8.41 (s, 1H, N*H*); δ_C(75.5 MHz, DMSO-d_6) 41.2, 63.8 (CH), 83.3 (C≡C), 84.2 (C≡C), 117.1, 127.1, 127.3, 127.6, 127.8, 127.9, 128.0, 128.2, 128.5, 128.7, 129.7, 130.6, 132.1, 132.3, 132.8, 133.0, 133.3, 152.0, 167.3, 171.1; (ESI+): *m/z* 403.00 $[M + H]^+$, (ESI-): *m/z* 405.07 $[M - H]^-$, calcd $C_{18}H_{16}N_2O_7S$ 404.07; HPLC (system A): t_R = 20.4 min, purity 100%; λ_{max}(PBS)/nm 244 (ε/dm^3 mol^{-1} cm^{-1} 33 190); Too hygroscopic compound for suitable IR and elemental analyses.

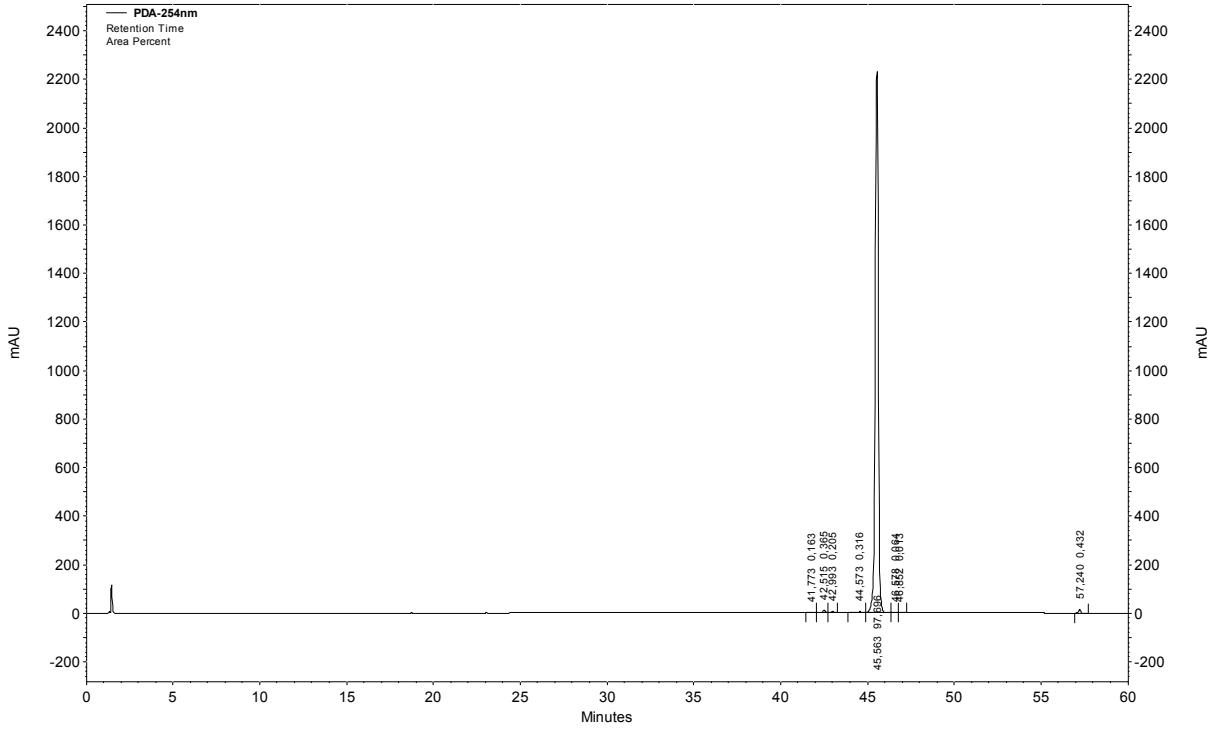
Monosulfonated alkyne-based 7-hydroxycoumarine (18). Purification by RP-HPLC (system E, t_R = 18.1-20.2 min). The product-containing fractions were lyophilised to give the water-soluble coumarine **18** as a white amorphous powder (9.0 mg, overall isolated yield 36%). δ_H(300 MHz, DMSO-d_6) 3.49-3.86 (m, 5H, 2 × CH_2, CH), 6.73 (s, 1H, H *arom.*), 6.83 (d, 1H, *J* = 8.5Hz, H *arom.), 7.57 (d, 1H, J* = 8.5Hz, H *arom.*), 8.10 (t, 1H, *J* = 4.9 Hz, N*H*), 8.36-8.38 (m, 2H, NH, H *arom.)*; δ_C(75.5 MHz, DMSO-d_6) 41.3, 63.6, 78.5, 87.2, 102.3, 104.1, 111.1, 114.1, 130.7, 149.8, 151.7, 155.6, 158.9, 163.1, 167.2, 171.2; (ESI+): *m/z* 439.07 $[M + H]^+$, 456.00 $[M + H_2O]^{+\bullet}$ (water cluster formed during the ionisation process), (ESI-): *m/z* 437.00 $[M - H]^-$, calcd $C_{17}H_{14}N_2O_{10}S$ 438.03; HPLC (system A): t_R = 14.7 min, purity 99%; λ_{max}(PBS)/nm 422 (ε/dm^3 mol^{-1} cm^{-1} 28 3000); Too hygroscopic compound for suitable IR and elemental.

Monosulfonated alkyne-based BODIPY (19). Purification by RP-HPLC (system E). The product-containing fractions were lyophilised to give the water-soluble BODIPY **17** as an orange amorphous powder (11.0 mg, overall isolated yield 90%). δ_H(300 MHz, DMSO-d_6) 1.36 (s, 6H, 2 × CH_3), 2.45(s, 6H, 2 × CH_3), 3.50-3.90 (m, 5H, partially masked by H_2O peak), 6.20 (s, 2H, H *arom.*), 7.48 (d, 2H, *J* = 6.2 Hz, H *arom.*), 7.79 (d, 2H, *J* = 6.2 Hz, H *arom.*), 8.09 (t, 1H, *J* = 4.2 Hz, N*H*), 8.43 (t, 1H, *J* = 4.2 Hz, N*H*); δ_C(75.5 MHz, DMSO-d_6) 114.1, 114.2, 38.7, 41.2, 63.7, 82.2, 84.9, 120.8, 121.6, 128.6; 130.3, 132.7, 132.9, 135.7, 140.6, 142.6, 151.8, 155.2, 167.2, 171.1; (ESI-): *m/z* 599.07 $[M - H]^-$, calcd $C_{27}H_{27}BF_2N_4O_7S$ 600.16; HPLC (system A): t_R = 27.1 min, purity 99%; Too hygroscopic compound for suitable IR and elemental.

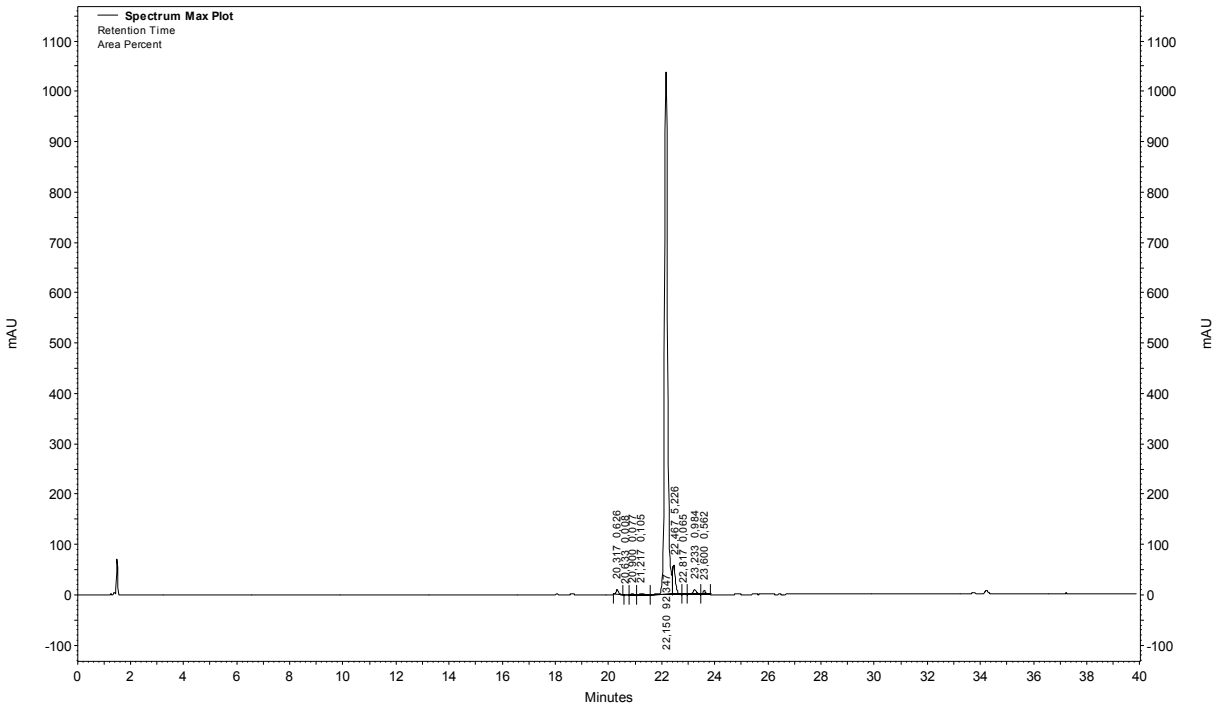
Monosulfonated alkyne-based fluorescein (20). Purification by RP-HPLC (system E, t_R = 19.7-24.3 min). The product-containing fractions were lyophilised to give the water-soluble naphthalene **20** as a yellow amorphous powder (24.0 mg, overall isolated yield 70%). δ_H(300 MHz, DMSO-d_6) 3.56-3.90 (m, 5H, 2x CH_2, CH), 6.55-6.70 (m, 6H, H *arom.*), 7.36 (d, 1H, *J* = 7.9 Hz, H *arom.*) 7.93 (d, 1H, *J* = 7.8 Hz, H *arom.*), 8.13 (m, 2H, CH, NH), 8.56 (s, 1H, NH); δ_C(75.5 MHz, DMSO-d_6); 41.3, 63.9, 81.3, 85.3, 102.3, 109.1, 113.0, 121.9, 125.1,

127.1, 128.6, 129.5, 138.9, 151.8, 152.1, 153.0, 160.0, 167.3, 167.6, 171.2; (ESI+): *m/z* 609.00 $[M + H]^+$, (ESI-): *m/z* 607.07 $[M - H]^-$, calcd $C_{28}H_{20}N_2O_{12}S$ 608.07; HPLC (system A): t_R = 18.5 min, purity 99%; λ_{max}(PBS)/nm 497 (ε/dm^3 mol^{-1} cm^{-1} 66 700); Too hygroscopic compound for suitable IR and elemental.

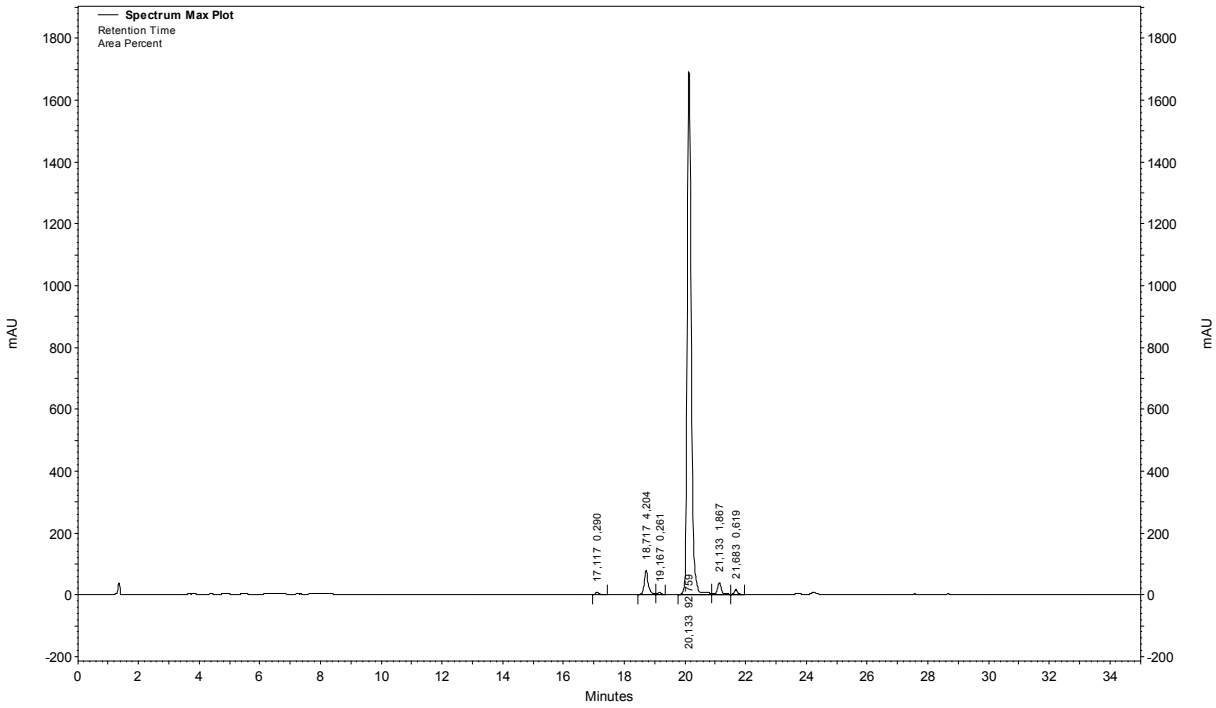
RP-HPLC elution profile (system A) of azido-BODIPY 5.



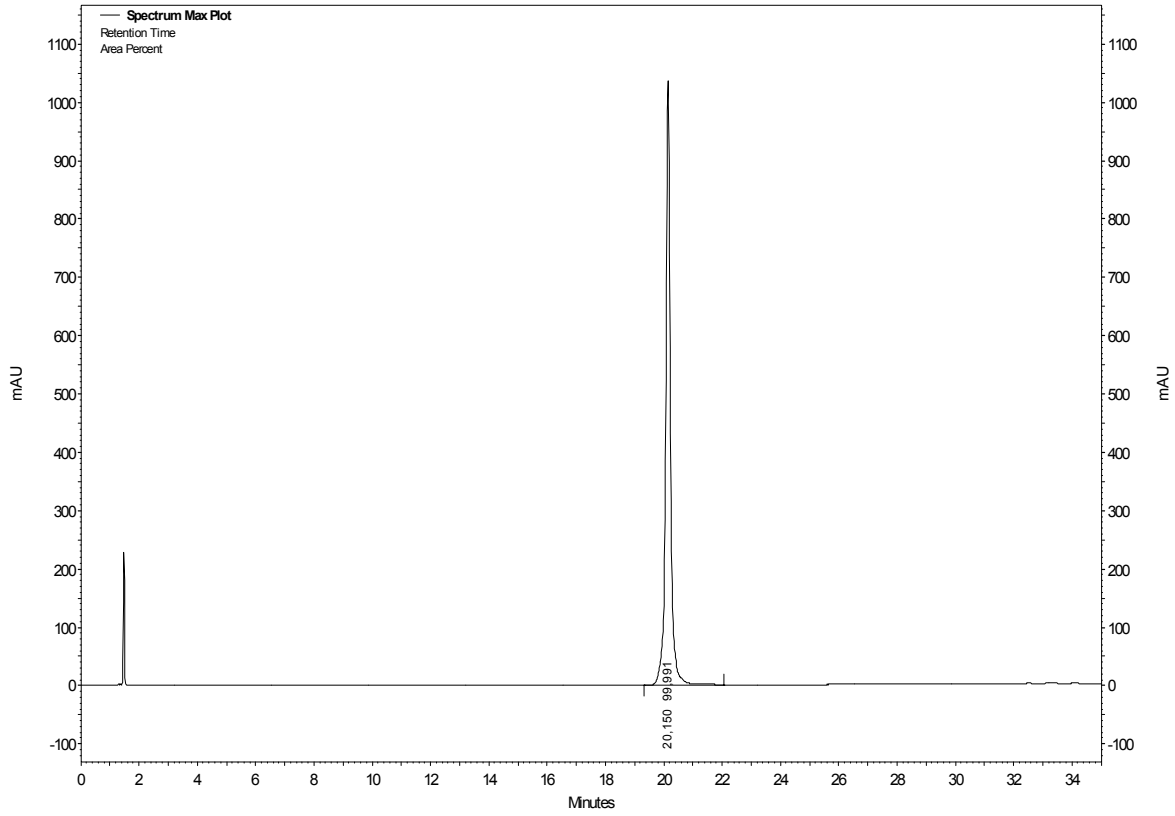
RP-HPLC elution profile (system D) of azido-fluorescein 6.



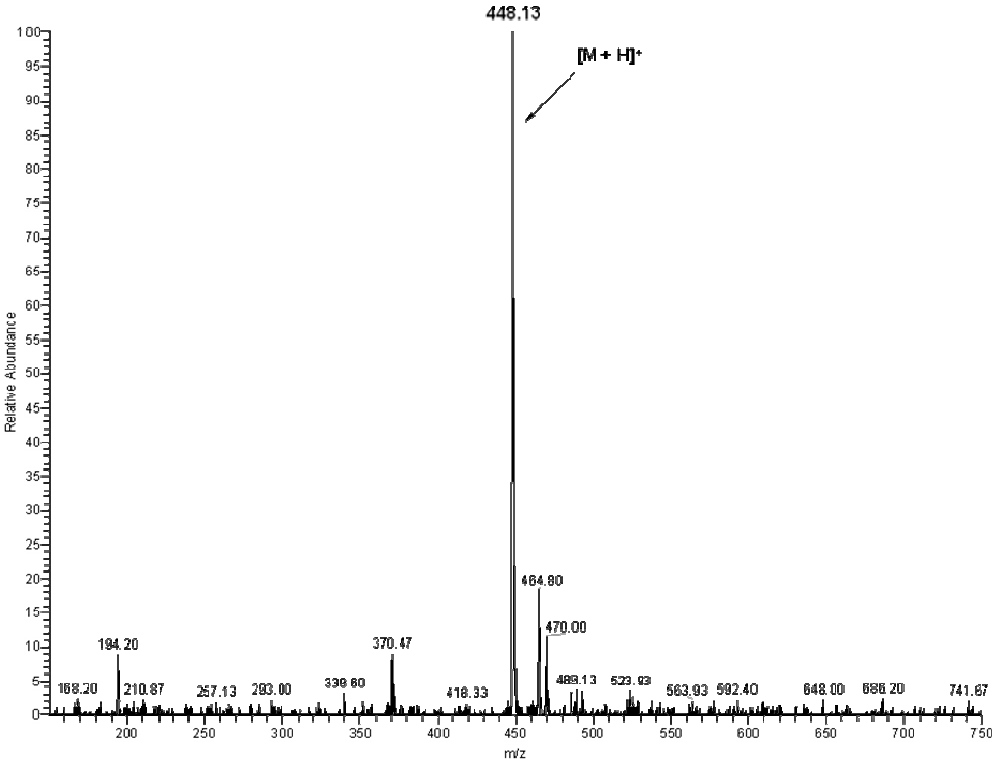
RP-HPLC elution profile (system C) of azido-R6G 7.



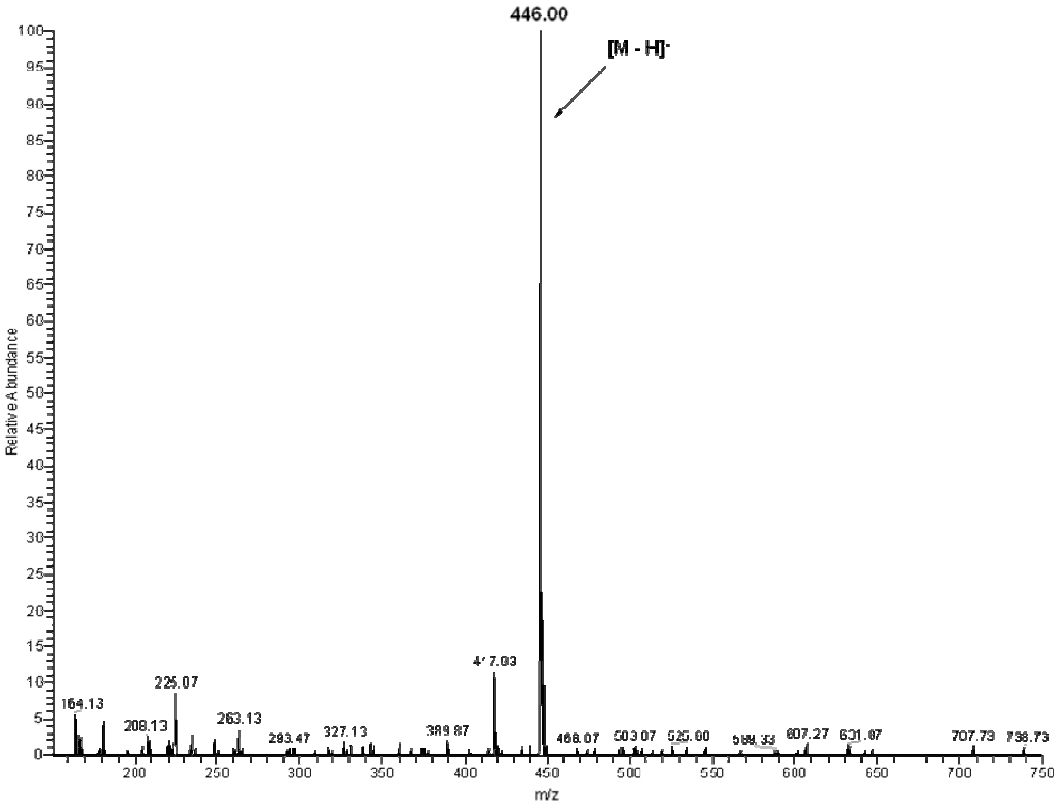
RP-HPLC elution profile (system A) of monosulfonated triazole-based naphthalene 8.



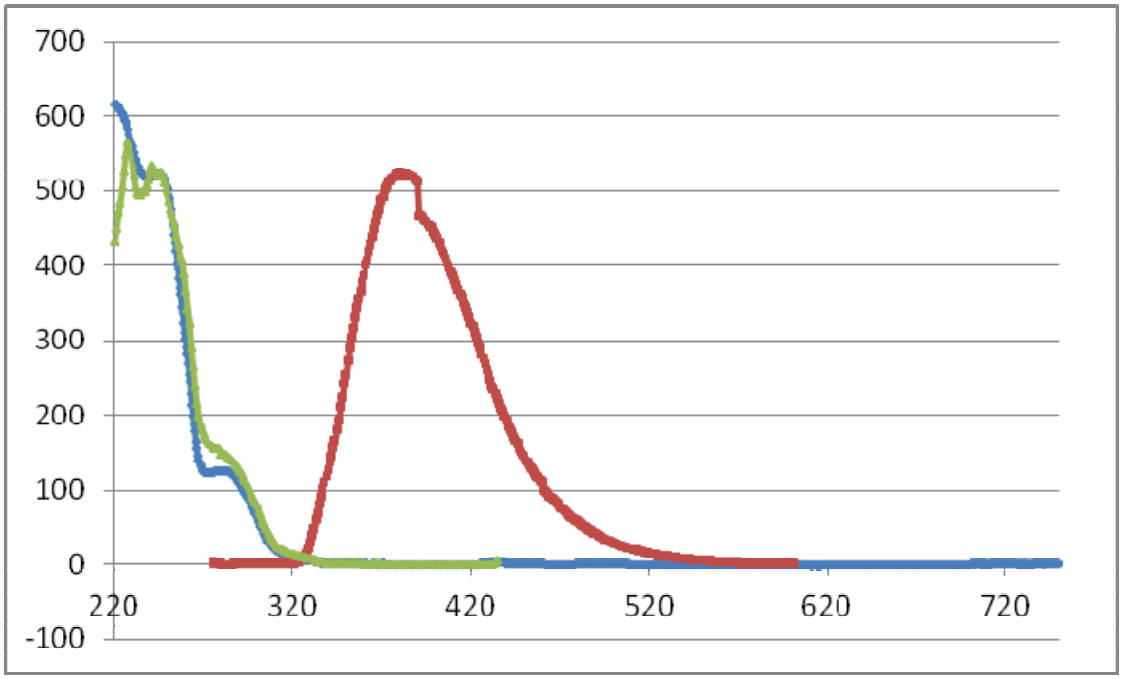
(ESI+) mass spectrum of monosulfonated triazole-based naphthalene 8.



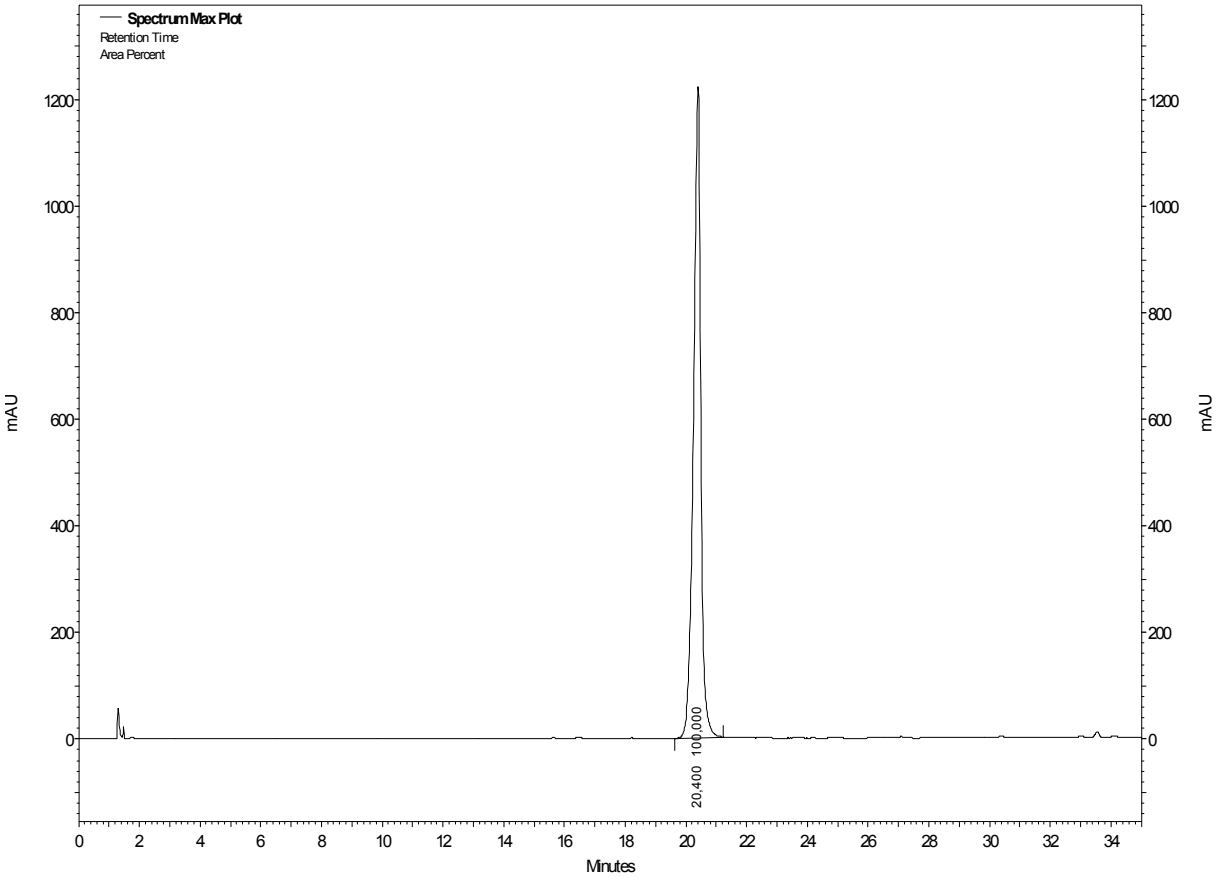
(ESI-) mass spectrum of monosulfonated triazole-based naphthalene 8.



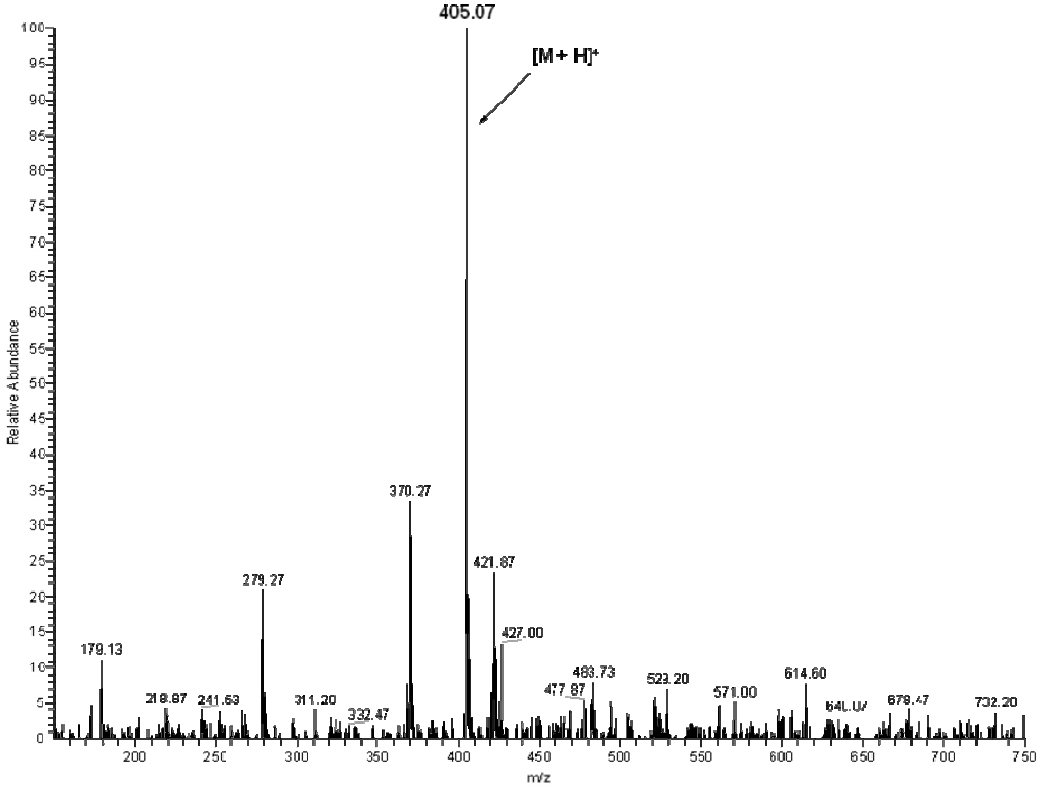
Normalised absorption (—), emission (—) and excitation (—) spectra of monosulfonated triazole-based naphthalene 8 in PBS at 25 °C.



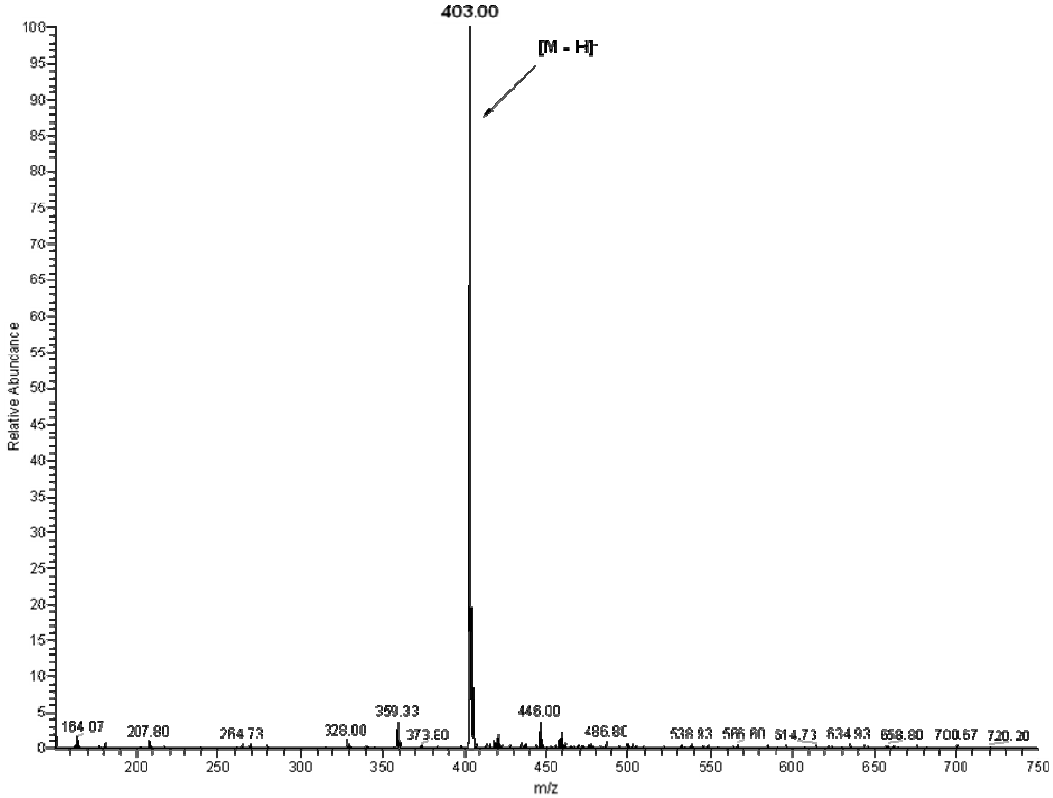
RP-HPLC elution profile (system A) of monosulfonated alkyne-based naphthalene 17.



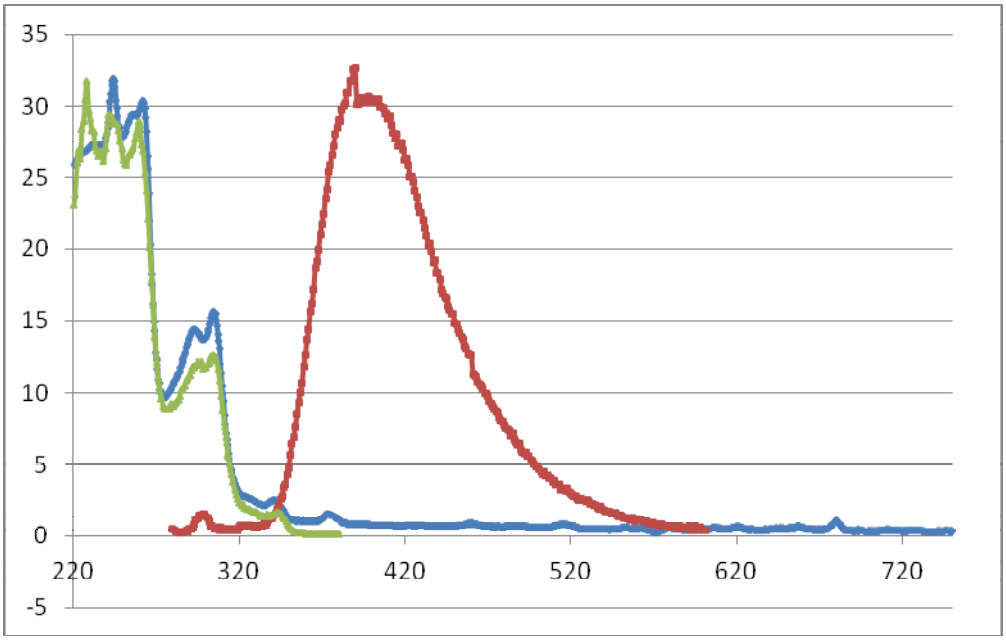
(ESI+) mass spectrum of monosulfonated alkyne-based naphthalene 17.



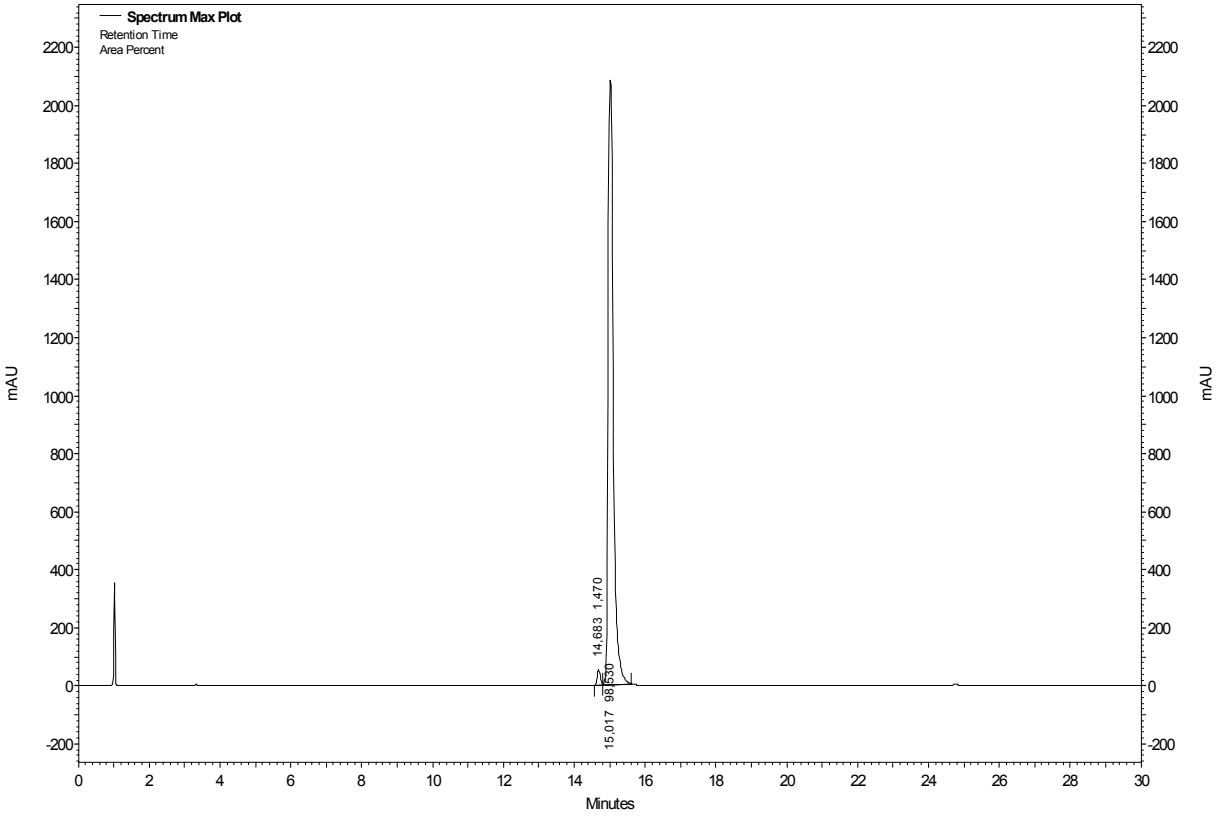
(ESI-) mass spectrum of monosulfonated alkyne-based naphthalene 17.



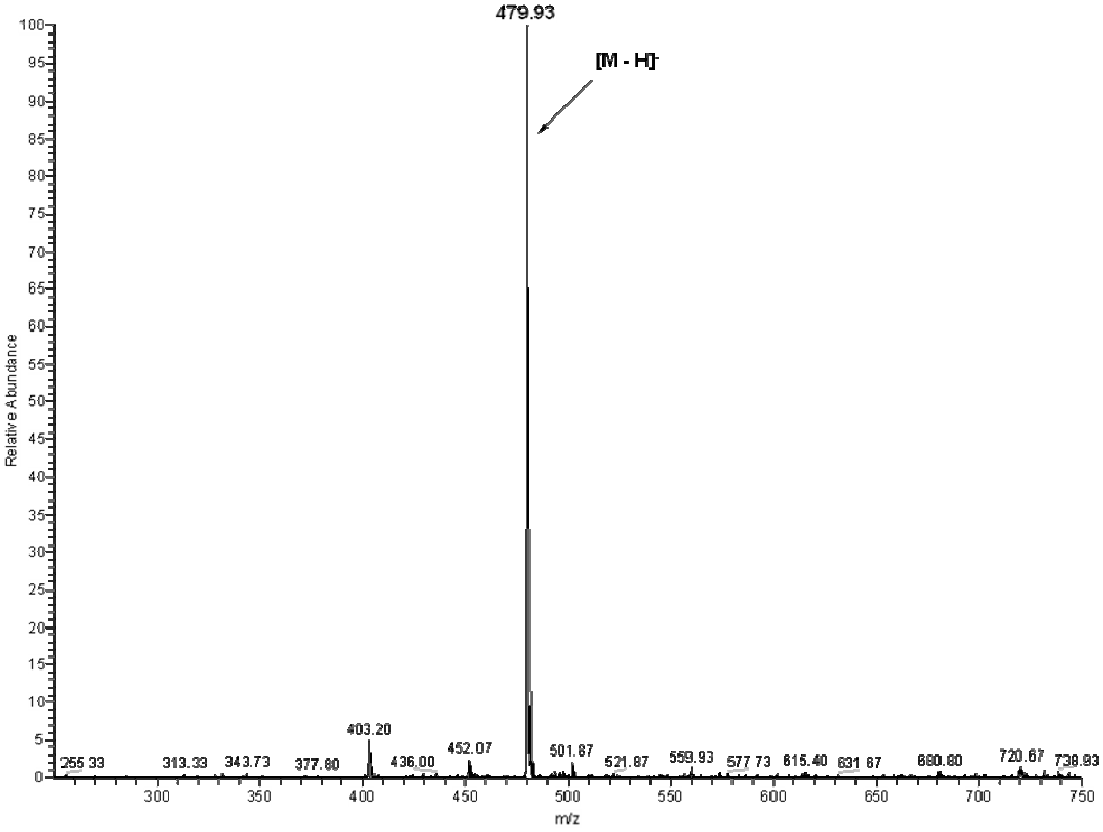
Normalised absorption (—), emission (—) and excitation (—) spectra of monosulfonated alkyne-based naphthalene 17 in PBS at 25 °C.



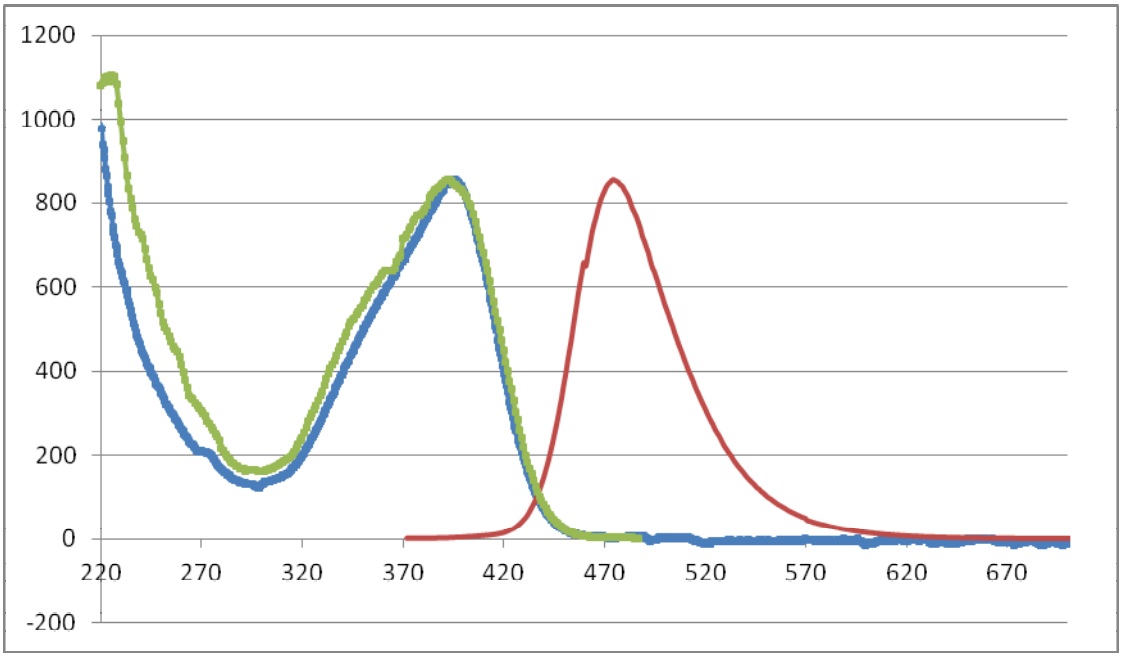
RP-HPLC elution profile (system A) of monosulfonated triazole-based coumarine 9.



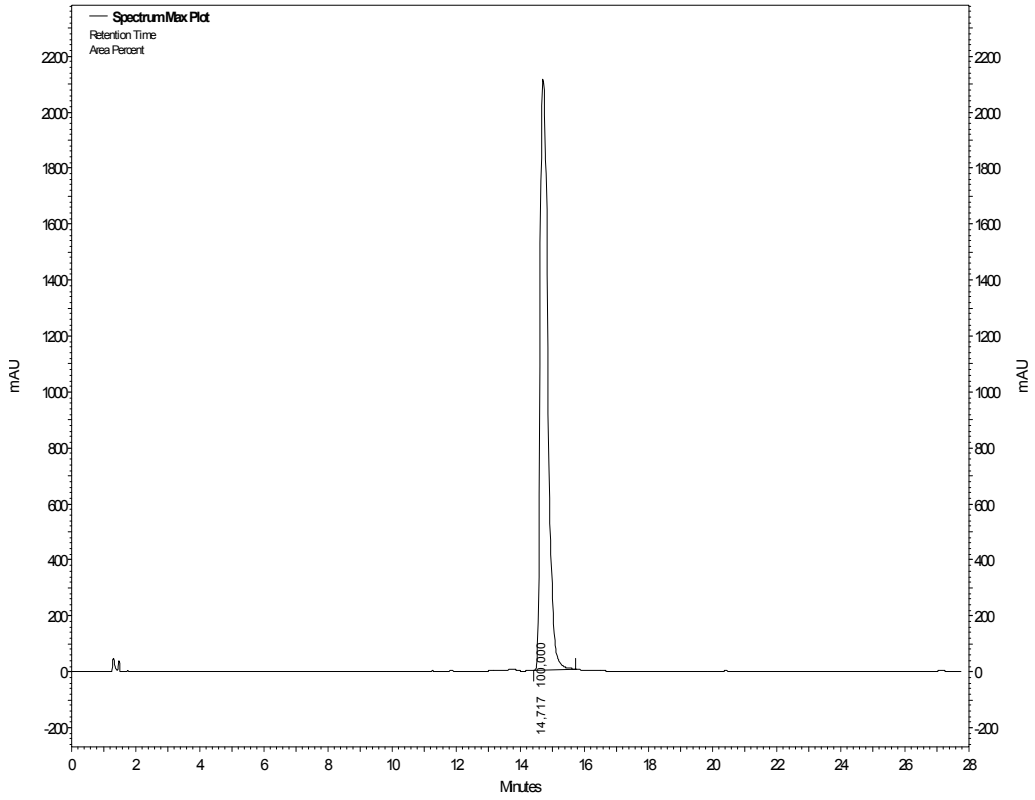
(ESI-) mass spectrum of monosulfonated triazole-based coumarine 9.



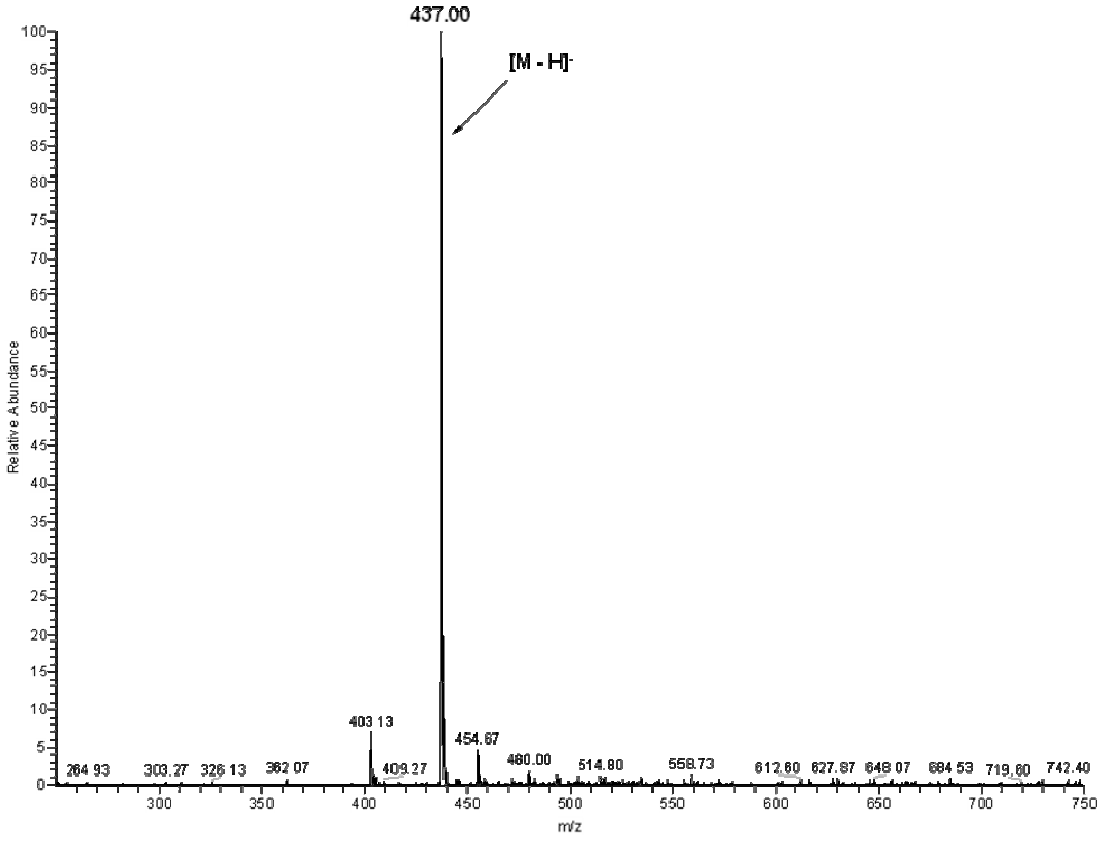
Normalised absorption (—), emission (—) and excitation (—) spectra of monosulfonated triazole-based coumarine 9 in PBS at 25 °C.



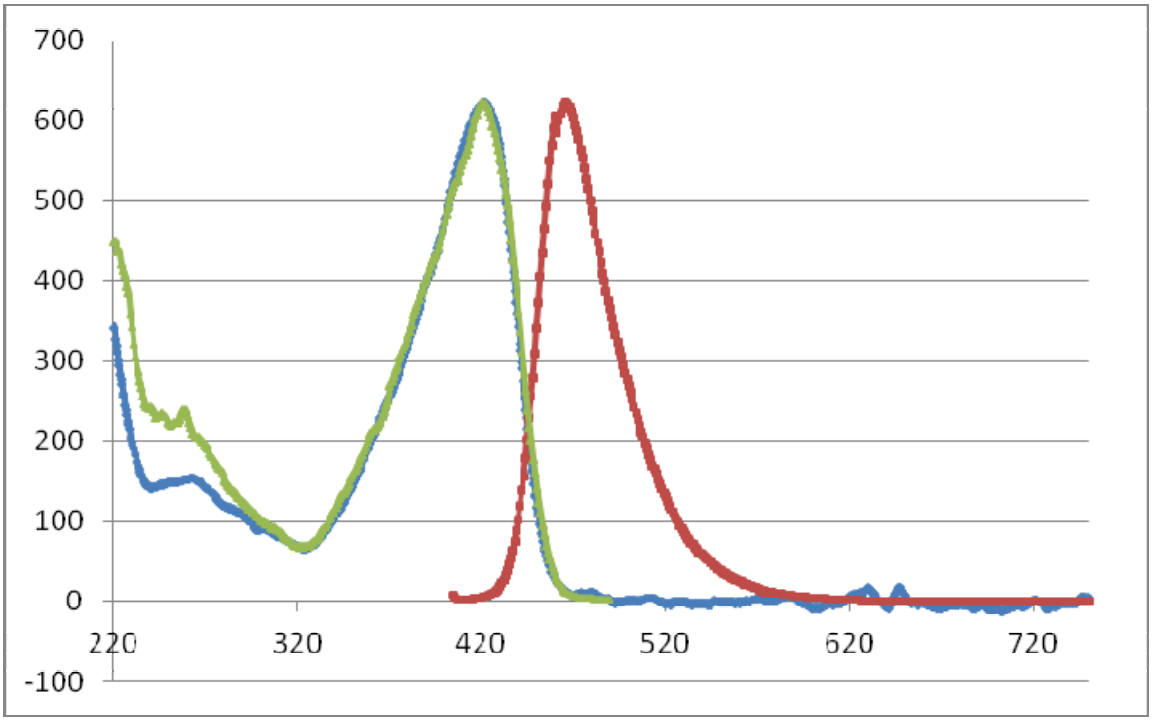
RP-HPLC elution profile (system A) of monosulfonated alkyne-based coumarine 18.



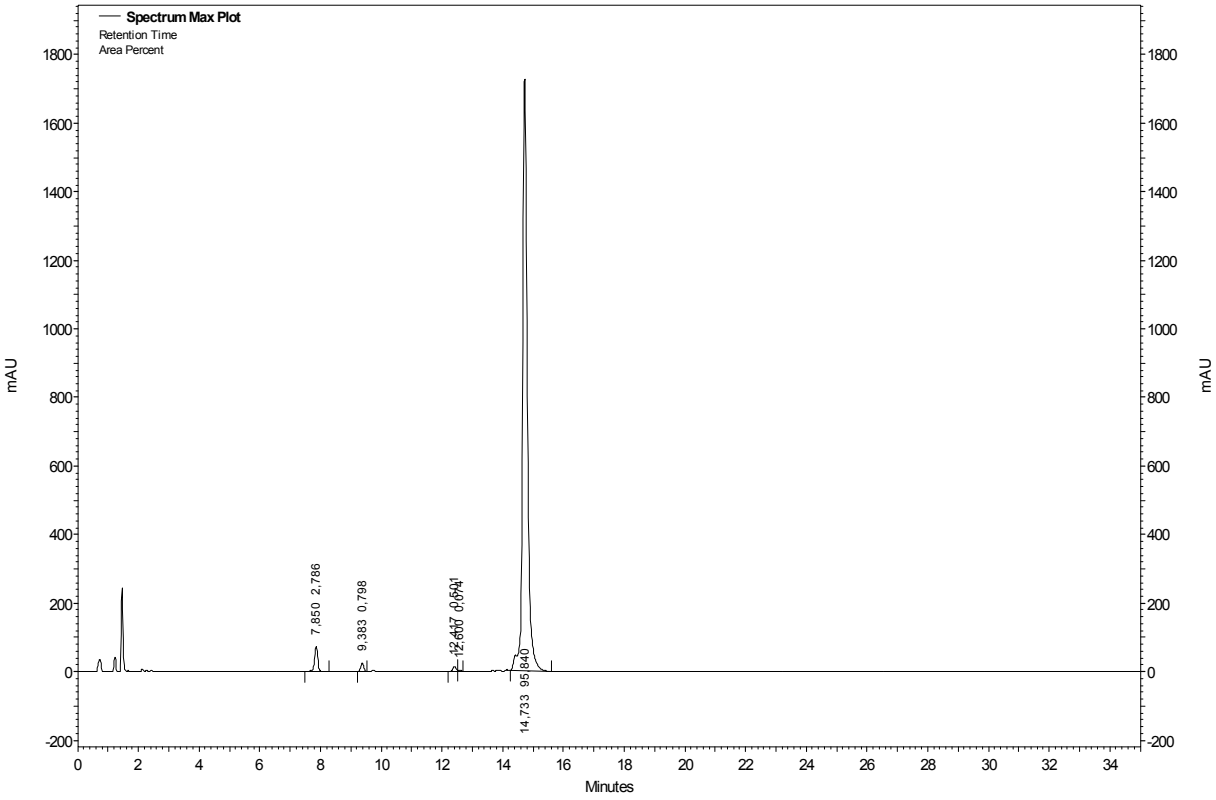
(ESI-) mass spectrum of monosulfonated alkyne-based coumarine 18.



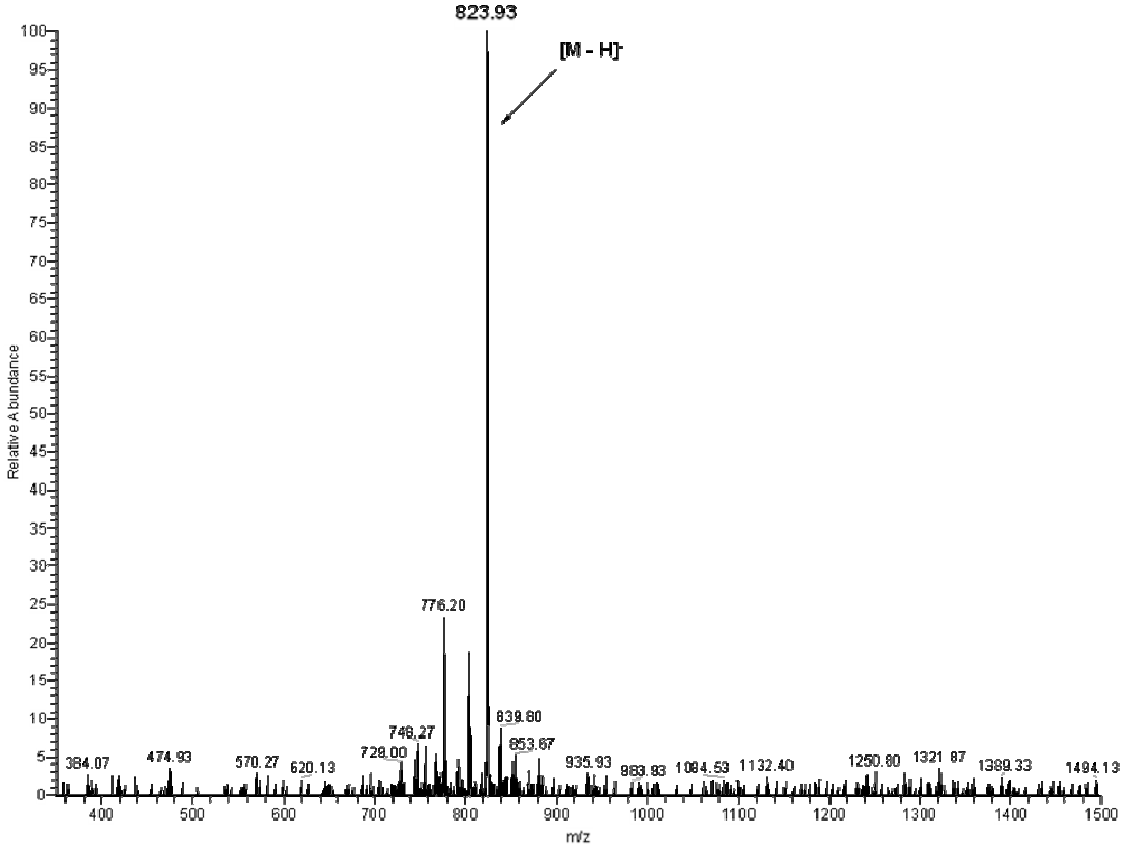
Normalised absorption (—), emission (—) and excitation (—) spectra of monosulfonated alkyne-based coumarine 18 in PBS at 25 °C.



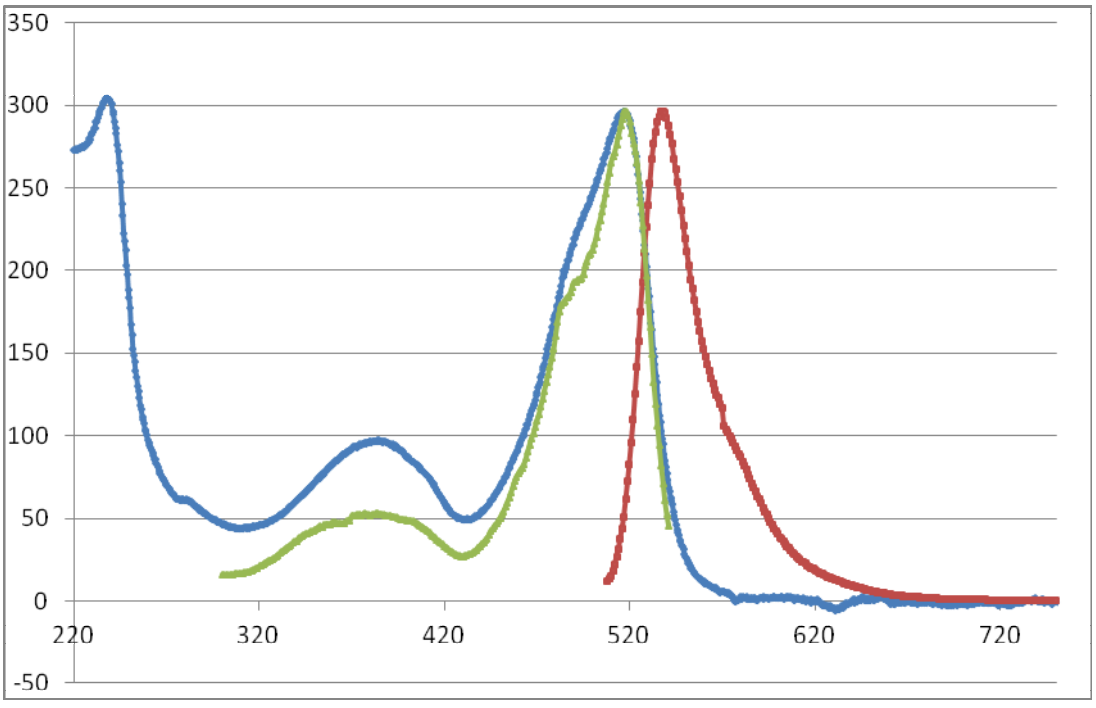
RP-HPLC elution profile (system A) of monosulfonated triazole-based BODIPY 10.



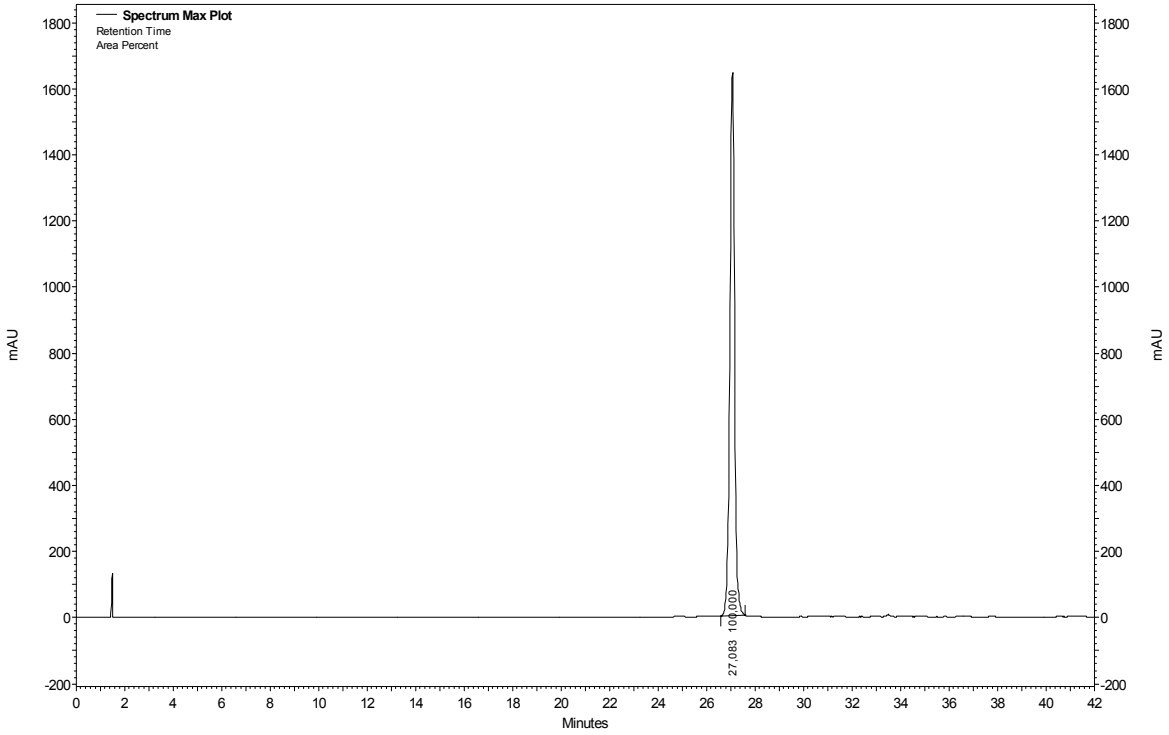
(ESI-) mass spectrum of monosulfonated triazole-based BODIPY 10.



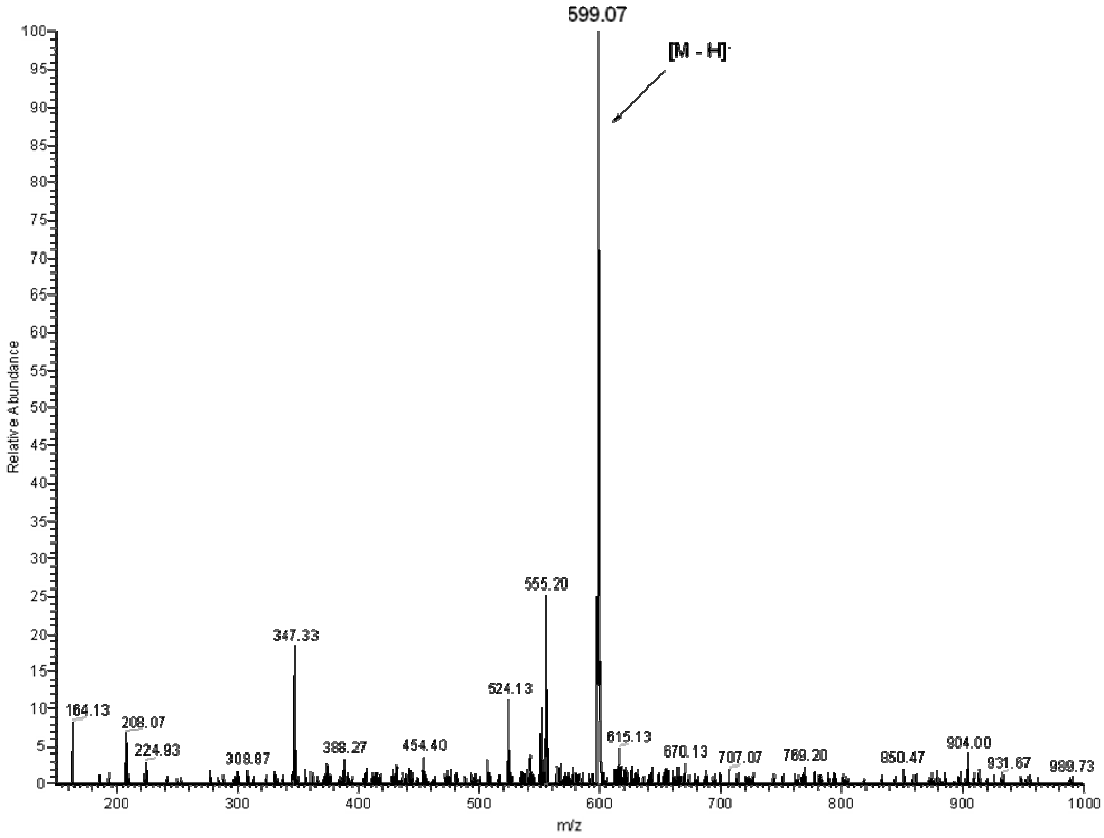
Normalised absorption (—), emission (—) and excitation (—) spectra of monosulfonated triazole-based BODIPY 10 in PBS at 25 °C.



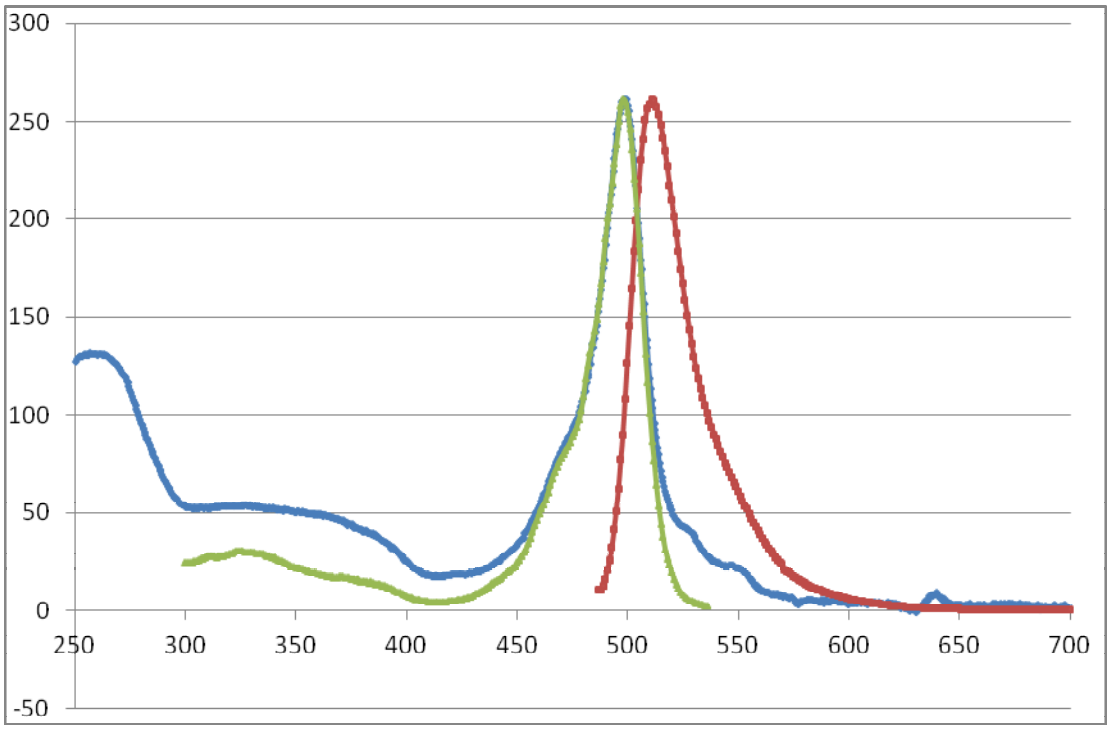
RP-HPLC elution profile (system A) of monosulfonated alkyne-based BODIPY 19.



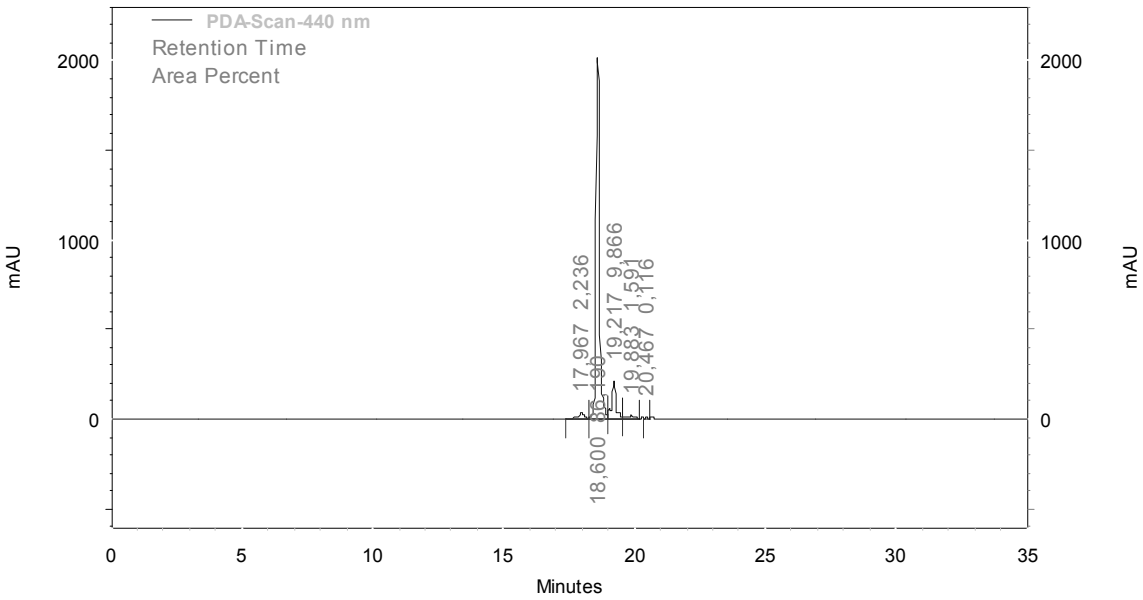
(ESI-) mass spectrum of monosulfonated alkyne-based BODIPY 19.



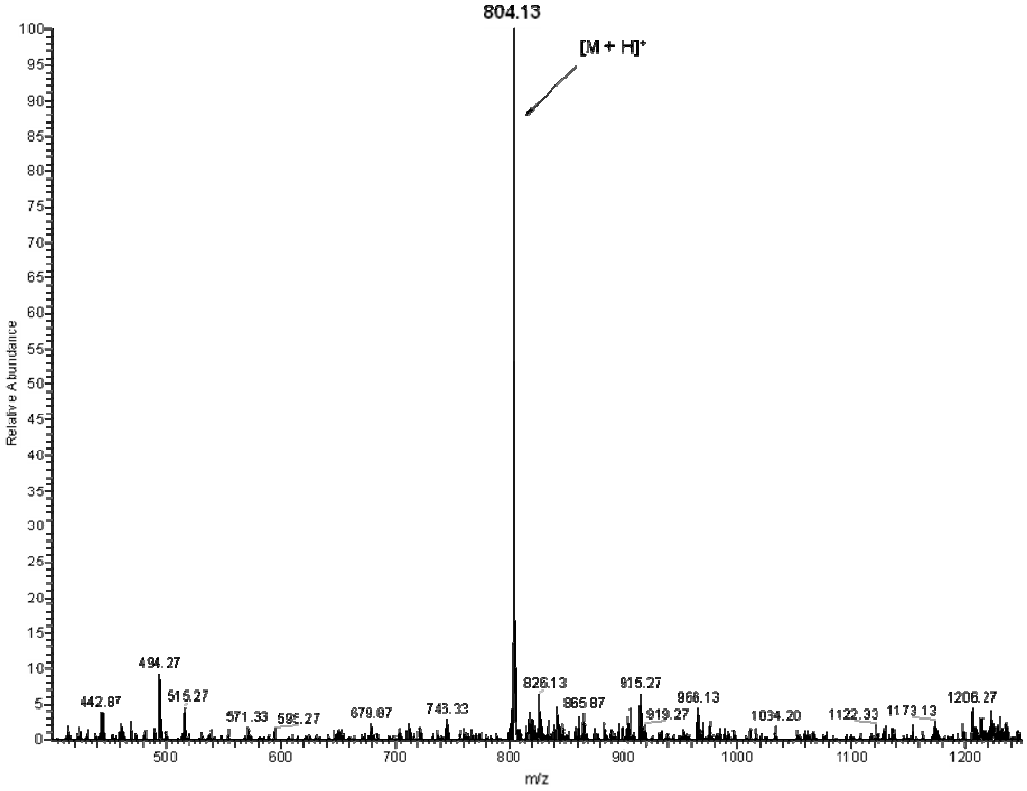
Normalised absorption (—), emission (—) and excitation (—) spectra of monosulfonated alkyne-based BODIPY 19 in PBS at 25 °C.



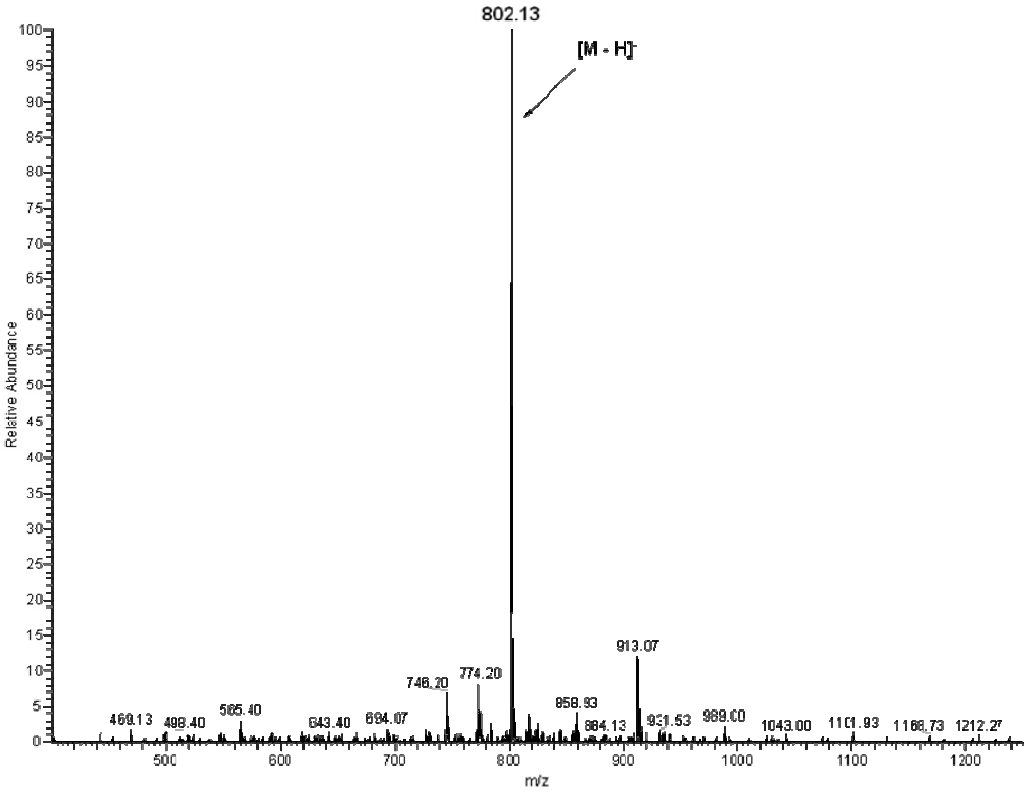
RP-HPLC elution profile (system A) of monosulfonated triazole-based fluorescein 11.



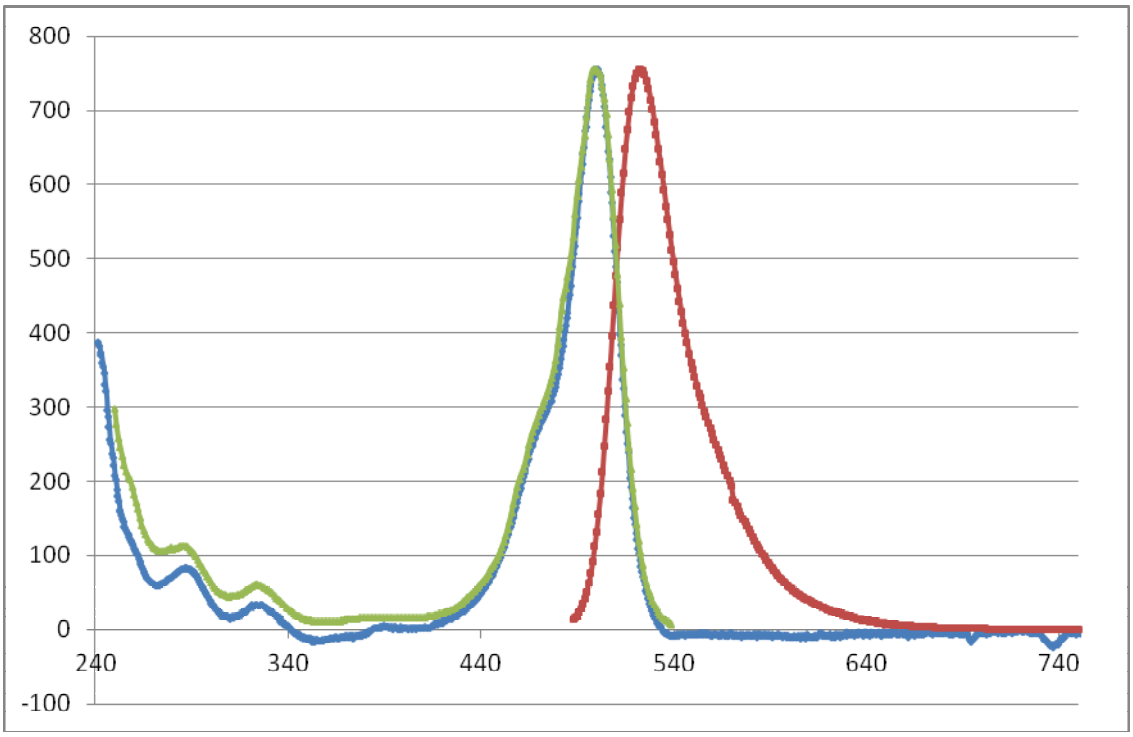
(ESI+) mass spectrum of monosulfonated triazole-based fluorescein 11.



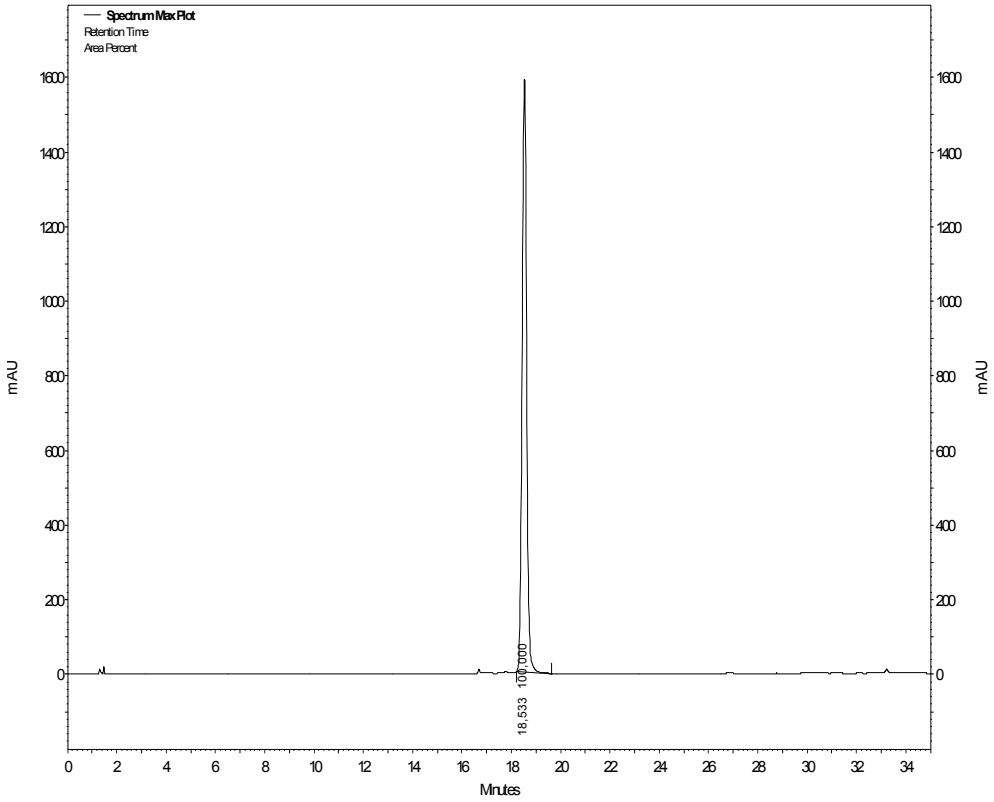
(ESI-) mass spectrum of monosulfonated triazole-based fluorescein 11.



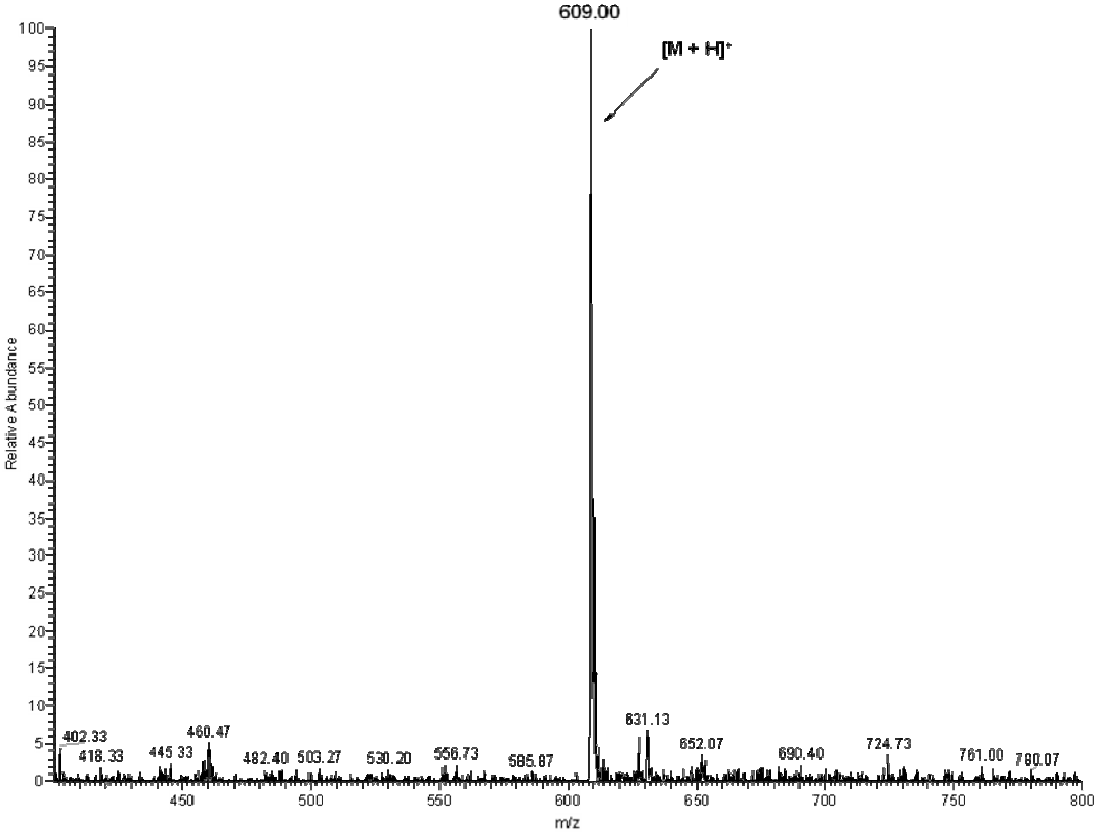
Normalised absorption (—), emission (—) and excitation (—) spectra of monosulfonated triazole-based fluorescein 11 in PBS at 25 °C.



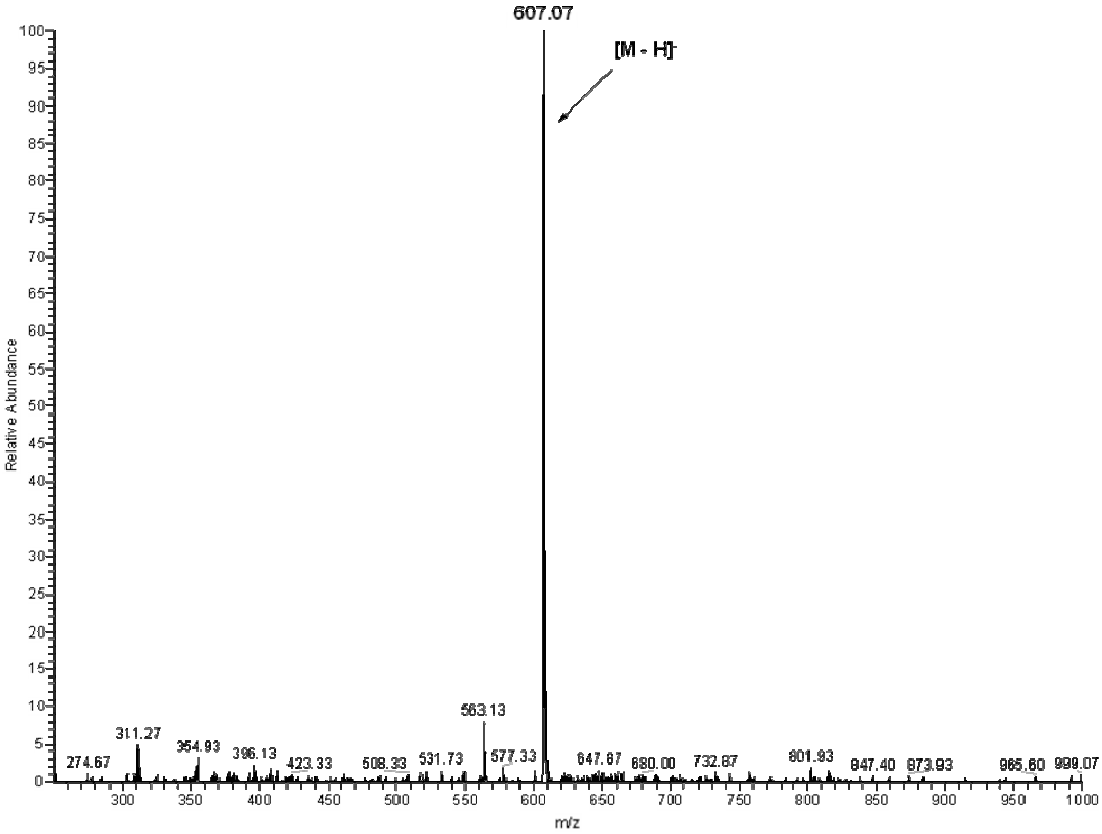
RP-HPLC elution profile (system A) of monosulfonated alkyne-based fluorescein 20.



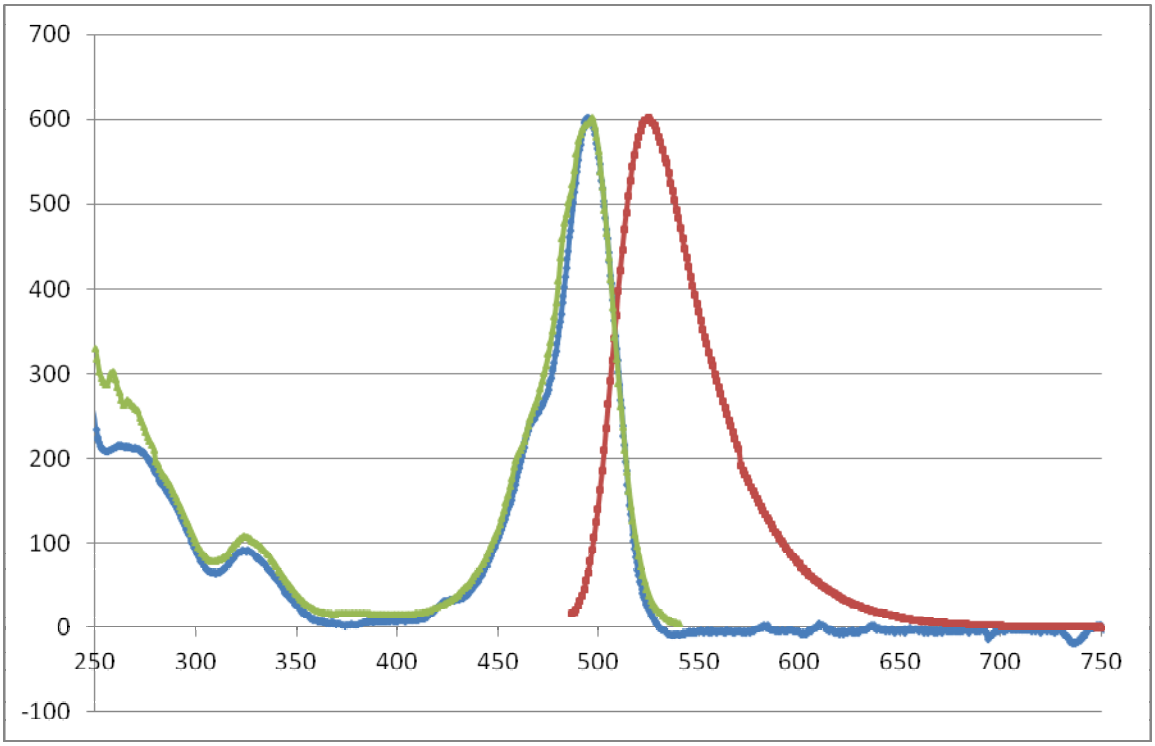
(ESI+) mass spectrum of monosulfonated alkyne-based fluorescein 20.



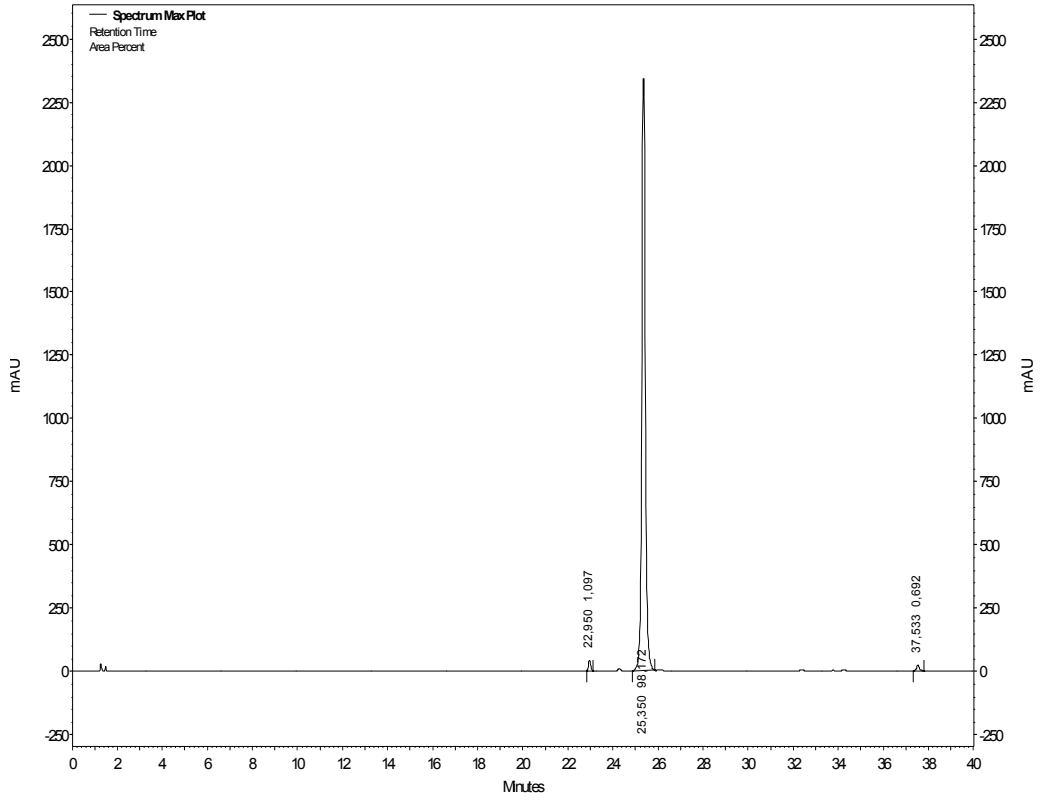
(ESI-) mass spectrum of monosulfonated alkyne-based fluorescein 20.



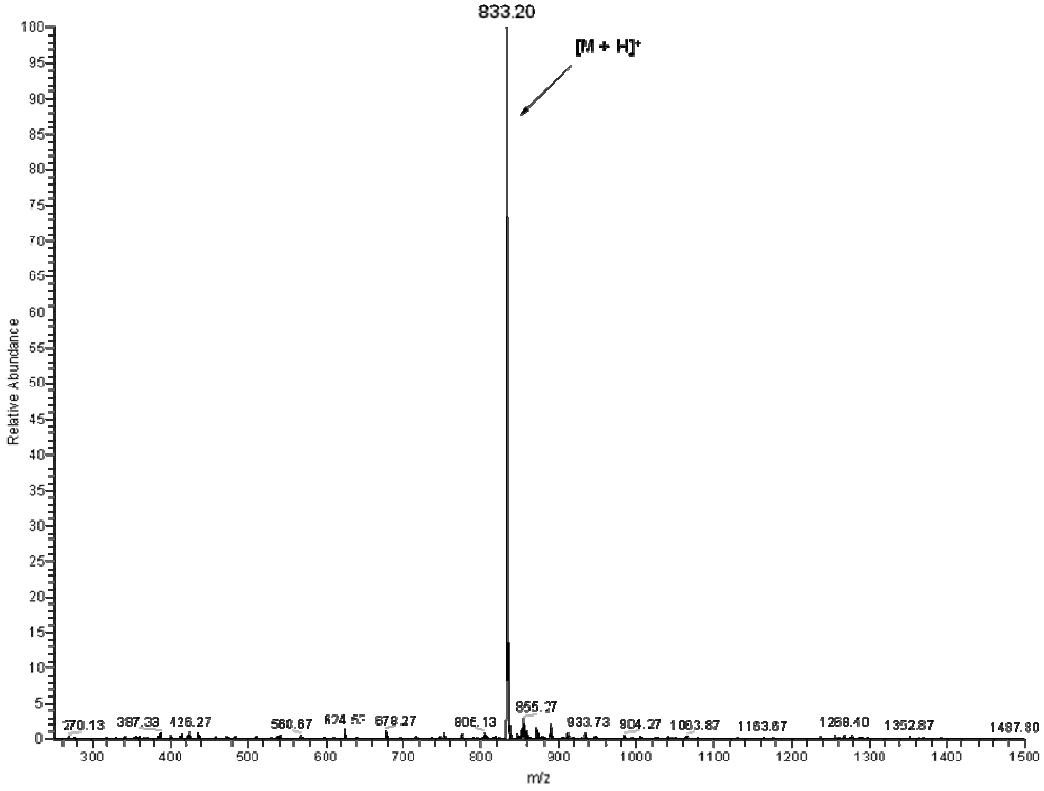
Normalised absorption (—), emission (—) and excitation (—) spectra of monosulfonated alkyne-based fluorescein 20 in PBS at 25 °C.



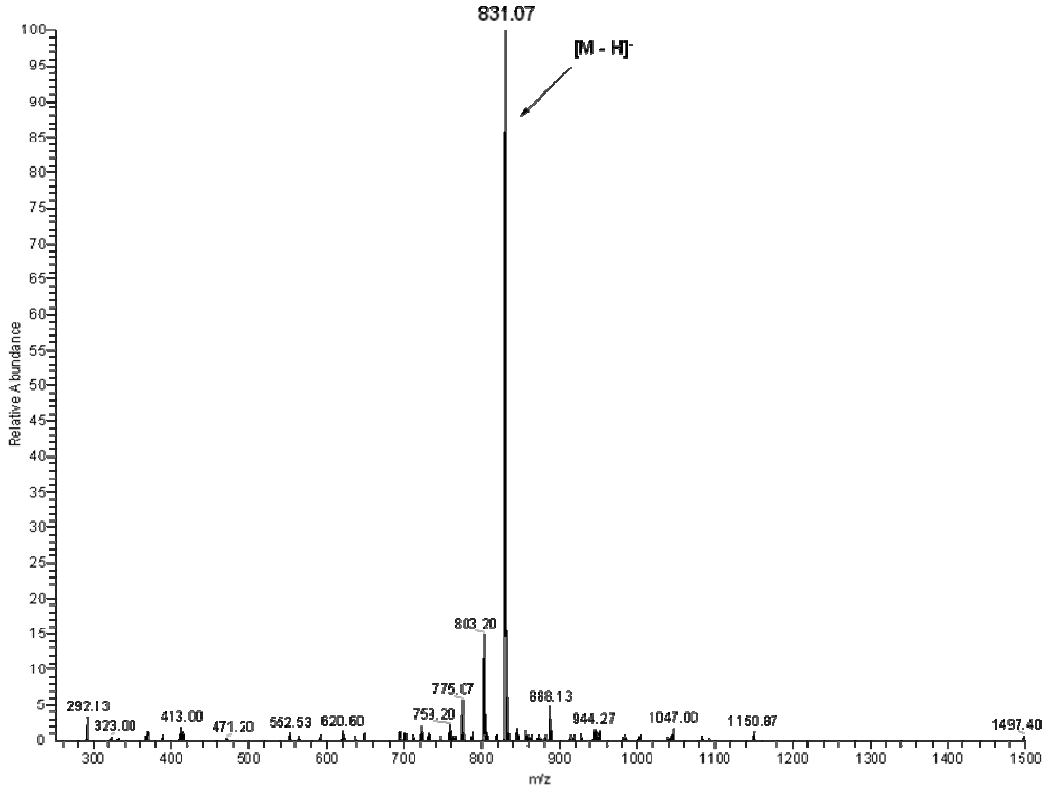
RP-HPLC elution profile (system A) of monosulfonated triazole-based R6G 12.



(ESI+) mass spectrum of monosulfonated triazole-based R6G 12.



(ESI-) mass spectrum of monosulfonated triazole-based R6G 12.



Normalised absorption (—), emission (—) and excitation (—) spectra of monosulfonated triazole-based R6G 12 in PBS at 25 °C.