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

Cedrik Massif, a Sebastien Dautrey, a Alexandre Haefele, d Raymond Ziessel, d Pierre-Yves Renard a,b,c and Anthony Romieu a,b,c

a Université de Rouen, Laboratory COBRA UMR 6014 & FR 3038, IRCOF, 1 Rue Tesnière, 76821 Mont St Aignan Cedex - France
Fax: + 33 (0)2 35 52 29 71
Tel: + 33 (0)2 35 52 24 27
E-mail: anthony.romieu@univ-rouen.fr
Web: http://ircof.crihan.fr (Thématique Bioorganique)

b INSA de Rouen, Avenue de l’Université, 76800 St Etienne du Rouvray - France

c CNRS Délégation Normandie, 14 Rue Alfred Kastler, 14052 Caen Cedex - France

d Laboratoire de Chimie Moléculaire et Spectroscopies Avancées (LCOSA), Ecole Européenne de Chimie, Polymères et Matériaux, 25 rue Becquerel, 67087 Strasbourg Cedex 02, France
Fax: (+) 33 3-68-85-27-61
E-mail: ziesel@unistra.fr
Web: http://www-lmspc.u-strasbg.fr/lcosa

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Abbreviations
The following abbreviations are used throughout the text of the ESI file: AcOEt, ethyl acetate; β-Ala(SO3H)-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; DCM, N,N'-dicyclohexylurea; DCM, N,N'-diisopropylcarbodiimide; DMF, N,N-dimethylformamide; DMSO, dimethylsulfoxide; Fmoc, 9-fluorenylmethoxy carbonyl; 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. CH2Cl2 (stabilised with amylene) was dried by distillation over P2O5. 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(SO3H)-OH was prepared from β-Ala(SO3H)-OH using an improved synthetic procedure recently reported by us and is now commercially available from Iris Biotech GmbH (#HAA1915). 3-Azidopropylamine, tert-butyl isonipecotate and [Pd(Ph3)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. 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 (CH3CN) and methanol (CH3OH) 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

triethylammonium bicarbonate (TEAB, 1.0 M) buffers were prepared from distilled triethylamine and glacial acetic acid or CO₂ gas.

**Instruments and methods.**

¹H and ¹³C 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 CDCl₃ (δ_H = 7.26, δ_C = 77.16), D₂O (δ_H = 4.79) or DMSO-d₆ (δ_H = 2.54, δ_C = 40.45). J values are expressed in Hz. ¹³C 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 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:

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

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.

<table>
<thead>
<tr>
<th>Fluorophore (F)</th>
<th>Solvent</th>
<th>λ Ex. (nm)</th>
<th>Standard (std)</th>
<th>Φstd / solvent</th>
<th>Φx</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>PBS</td>
<td>270 nm</td>
<td>E-stilbene¹⁰</td>
<td>0.05 / hexane&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.56</td>
</tr>
<tr>
<td>17</td>
<td>PBS</td>
<td>270 nm</td>
<td>E-stilbene¹⁰</td>
<td>0.05 / hexane&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.02</td>
</tr>
<tr>
<td>9</td>
<td>PBS</td>
<td>360 nm</td>
<td>7-OH-coumarine¹¹</td>
<td>0.76 / PBS&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.78</td>
</tr>
</tbody>
</table>

<sup>a</sup> See http://omlc.ogi.edu/spectra/.

**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$_3$CN 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$_3$CN] 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$_3$CN 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$_3$CN] 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$_3$CN]. 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$_3$CN]. 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$_3$CN and aq. TFA 0.1% as eluents [100% TFA (5 min), then linear gradient from 0 to 100% (55 min) of CH$_3$CN] 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 450 nm.

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*refractive index = 1.375,*$^b$*refractive index = 1.337,*$^c$*refractive index = 1.333,*$^d$*refractive index = 1.362.

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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 C18 column, 10 µm, 21.2 × 250 mm) with CH3CN 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 CH3CN] 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 C18 column, 5 µm, 21.2 × 250 mm) with CH3CN and aq. TFA 0.1% as eluents [100% TFA (5 min), then linear gradient from 0 to 100% (55 min) of CH3CN] 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 CH3CN] 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 CH3CN] 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 CH2Cl2 (6 mL) for 15 min. After filtration, the resin was suspended in a mixture of CH2Cl2-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 CH2Cl2 (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 CH3OH and three times with CH2Cl2, 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 CH3OH and three times with CH2Cl2. For a complete deprotection, this step was performed twice.

**Coupling of Fmoc-β-Ala(SO3H)-OH**: A clear and limpid solution of Fmoc-β-Ala(SO3H)-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 CH3OH and three times with CH2Cl2, and dried.

**Fmoc removal**: vide supra.

**Coupling of propiolic acid**: The batch of β-Ala(SO3H)-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 CH2Cl2 (2.42 mL). A
solution of propionic 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 CH3OH and three times with CH2Cl2, and dried.

Cleavage of the resin: The resin cleavage was performed by adding a mixture of TFA-CH2Cl2 (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 CHCl3, and finally purified by RP-HPLC (system G, tR = 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%).

Azido-BODIPY (5). 8-(4-Iodophenyl)-1,3,5,7-tetramethyl-BODIPY 15 (50 mg, 0.10 mmol), was dissolved in dry CH2Cl2 (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 MgSO4 and evaporated to dryness. The resulting residue was purified by column chromatography on a silica gel column with petroleum ether/CH2Cl2 (gradient from 80:20 to 70:30) as eluents, to give azido derivative 5 (50 mg, yield 90%).

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 CH2Cl2. 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® Isola One), and using a linear gradient of CH3OH (0-10%) in CH2Cl2 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] cale
**C₃₀H₂₉NO₆ 499.20; HPLC (system C): tᵣ = 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 CH₂Cl₂ (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, tᵣ = 17.00-20.0 min). The product-containing fractions were lyophilised to give fluorescein carboxylic acid as an orange amorphous powder (66 mg, 0.14 mmol, yield 43%).

**δ(H(300 MHz, DMSO-d₆)) 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, CH₂), 3.65-3.58 (m, 1H, CH₂), 3.00-2.50 (m, 2H, CH₂), 2.45-2.20 (m, 1H, CH), 2.00-1.60 (m, 2H, CH₂), 1.40-1.00 (m, 2H, CH₂);

**δ(C(75.5 MHz, DMSO-d₆)) 175.3, 166.3, 156.6, 152.6, 135.8, 131.6, 130.6, 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): tᵣ = 20.1 min, purity > 90%; (ESI+): m/z 444.20 [M + H]+; (ESI-): m/z 442.27 [M - H] -, calcd C₂₆H₂₁NO₆ 443.14; λ max(PBS) nm 499 (ε/dm³ mol⁻¹ cm⁻¹ 48 000); λ max em (PBS) nm 520 (Φₑ 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, tᵣ = 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-d₆)) 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, CH₂), 3.70-3.60 (m, 1H, CH₂), 3.31 (t, 2H, J = 6.6 Hz, CH₂), 3.10-2.50 (m, 4H, 2 × CH₂), 2.50-2.10 (m, 1H, CH), 1.70-1.60 (m, 4H, 2 × CH₂), 1.40-1.00 (m, 2H,CH₂); δ(C(75.5 MHz, DMSO-d₆)) 173.6, 166.5, 136.6, 132.1, 130.6, 130.5, 129.9, 129.6, 127.3, 125.9, 115.5, 109.6, 84.5, 46.6, 42.5, 41.3, 36.6, 35.8, 28.5, 27.9, 25.2; HPLC (system D): tᵣ = 22.2 min, purity > 90%; (ESI+): m/z 526.27 [M + H]+ calcd C₂₉H₂₇N₅O₅ 525.19; λ max(PBS) nm 500 (ε/dm³ mol⁻¹ cm⁻¹ 23 400); λ max em (PBS) nm 520 (Φₑ 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 mixture was dissolved in 0.1% aq. TFA and CH₃CN and purified by semi-preparative RP-HPLC (system I, 1 injection, tᵣ = 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, CDCl₃)) 8.46 (dd, 1H, J = 2.4 Hz, J = 6.0 Hz,CH₃), 7.80-7.72 (m, 3H, CH₃), 6.78 (s, 2H, CH₃), 6.64 (s, 2H, 2 × CH₃), 6.60-6.58 (bs, 2H, 2 × NH), 4.51 (s, 2H, OCH₂), 3.50-3.40 (m, 4H, N–CH₂–CH₃), 3.27-3.19 (m, 4H,CH₃), 2.12 (s, 6H,2 × CH₃), 1.69 (qt, 2H, J = 6.6Hz, CH₂), 1.40-1.00 (t, 6H, J = 7.2Hz, N–CH₂–CH₃);
\( \delta_c (75.5 \text{ MHz, CDCl}_3) 167.0, 164.3, 157.7, 157.3, 156.1, 134.3, 133.3, 132.0, 130.6, 129.3, 128.9, 127.7, 113.8, 94.1, 63.8, 49.5, 38.8, 37.0, 28.7, 17.5, 13.9; \) 

HPLC (system C): \( t_R = 20.1 \text{ min, purity > 90\%; (ESI+): m/z 555.27 [M + H]^+ \text{ calcd C}_{31}\text{H}_{35}\text{N}_6\text{O}_4^+ 555.27; \) 
\( \lambda_{\text{max}} \text{(PBS)} \text{ nm 527 (} \varepsilon/\text{dm}^3 \text{ mol}^{-1} \text{ cm}^{-1}\ 70 000)\); \( \lambda_{\text{max em}} \text{(PBS) nm 552 (} \Phi_F 0.75). \)

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%). 

\( \delta_H (300 \text{ MHz, DMSO-}d_6) 6.70 (s, 1H), 6.78 (d, 1H, \ J = 6.4 \text{ Hz}), 7.49 (d, 1H, \ J = 8.5 \text{ Hz}), 8.63 (s, 1H); \delta_C (75.5 \text{ MHz, DMSO-}d_6) 80.3, 102.0, 113.0, 113.5, 129.0, 152.7, 155.5, 157.6, 161.8. \) 

(ESI-): \( m/z 287.07 [M - H]^- , \text{ calcd C}_{9}\text{H}_{5}\text{IO}_3 287.92. \)

HPLC (system A): \( t_R = 22.0 \text{ min, purity 99\%;} \)

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\(_2\)O (0.67 mg, 2.7 \(\mu\)mol, 0.05 equiv.) were mixed together in a mixture of degassed DMSO-H\(_2\)O (1 : 1, v/v, 1.5 mL) and the resulting reaction mixture was stirred at rt overnight. The reaction was checked for completion 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, \( t_R = 23.4-27.9 \text{ min})\). 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\%). \( \delta_H (300 \text{ MHz, DMSO-}d_6) 3.55 (t, 1H, \ J = 6.7 \text{ 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 \text{ Hz}), 8.57 (s, 1H), 9.41 (s, 1H); \delta_C (75.5 \text{ 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+): \( m/z 448.13 [M + H]^+ , \text{ (ESI-): m/z 446.00 [M - H]^- , calcd C}_{18}\text{H}_{17}\text{N}_5\text{O}_7\text{S 447.08; HPLC (system A): t_R = 19.8-20.4 \text{ min, purity 99\%;}} \)

Too hygroscopic compound for suitable IR and elemental analyses.

Monosulfonated triazole-based 7-hydroxycoumarine (9). Purification by RP-HPLC (system E, \( t_R = 17.2-17.9 \text{ min})\). 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\%). \( \delta_H (300 \text{ MHz, DMSO-}d_6) 3.57 (t, 1H, \ J = 6.4 \text{ Hz}), 3.68-3.90 (m, 4H), 6.85 (s, 1H), 6.91 (d, 1H, \ J = 8.6 \text{ Hz}), 7.76 (d, 1H, \ J = 8.6 \text{ Hz}), 8.18( t, 1H, \ J = 4.9 \text{ Hz, NH}), 8.43 ( t, 1H, \ J = 4.7 \text{ Hz, NH}), 8.65 (s, 1H), 8.90 s, 1H); \delta_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 + H2O]^+ (water cluster formed during the ionisation process), (ESI-): m/z 479.93 [M - H] -, calcd C_{17}H_{15}N_{5}O_{10}S 481.05; HPLC (system A): t_R = 15.0 min, purity 98.5%; \( \lambda_{max} \) (PBS)/nm 393 (\( \epsilon/dm^3\ mol^{-1}\ cm^{-1} \) 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%). \( \delta_t \) (300 MHz, DMSO-\( d_6 \)) 0.71 (t, 3H, J = 7.0 Hz, CH_2-CH_3), 1.28 (s, 3H, CH_3), 1.34 (s, 3H, CH_3), 2.33 (q, 4H, J = 7.3 Hz, 2 × CH_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 (s, 1H), 8.29 (t, 1H, J = 5.2 Hz, NH); \( \delta_c \) (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%). \( \delta_h \) (300 MHz, DMSO-\( d_6 \)) 1.13-1.23 (m, 2H, CH_2), 1.54-1.60 (m, 2H, CH_2), 1.94 (t, J = 6.6 Hz, CH_2-CH_2-CH_2), 2.21 (t, J = 9.7 Hz, CH), 2.71-3.20 (m, 4H, 2 × CH, CH_2-CH_2-CH_2), 3.49 (t, J = 6.6 Hz, CH), 3.63-3.96 (m, 6H), 4.36 (t, J = 6.6 Hz, CH_2-CH_2-CH_2), 7.06-7.14 (m, 4H), 7.43-7.46 (d, 1H, J = 7.5 Hz), 7.62 (t, 2H, J = 5.6 Hz, CH arom.), 7.68 (d, 2H, J = 5.6 Hz, CH arom.), 8.15 (s, 1H), 8.26 (t, 1H, J = 5.1 Hz, NH); \( \delta_c \) (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, 131.3, 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%; \( \lambda_{max} \) (PBS)/nm 497 (\( \epsilon/dm^3\ mol^{-1}\ cm^{-1} \) 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%). \( \delta_t \) (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.), 7.43-7.46 (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, NH), 8.30 (t, 1H, J = 5.1 Hz, NH), 8.37-8.40 (d, 1H, J = 7.5 Hz), 8.43 (s, 1H, CH); \( \delta_c \) (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%; \( \lambda_{max} \) (PBS)/nm 530 (\( \epsilon/dm^3\ mol^{-1}\ cm^{-1} \) 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, 1:1)
v/v/v). Then, Pd(PPh₃)₄ (7 mg, 6.0 µmol, 0.1 equiv.) and Cul (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 completion 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%). δH(300 MHz, DMSO-d₆) 3.54-4.01 (m, 5H, 2 × CH₂, 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.); δC(75.5 MHz, DMSO-d₆) 41.2, 63.8 (CH), 83.3 (C=CC), 84.2 (C=CC), 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 18H16N2O7S 404.07; HPLC (system A): tR = 20.4 min, purity 100%; λmax(PBS)/nm 244 (ε/dm³ 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, tR = 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%). δH(300 MHz, DMSO-d₆) 3.49-3.86 (m, 5H, 2 × CH₂, 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, NH), 8.36-8.38 (m, 2H, NH, H arom.); δC(75.5 MHz, DMSO-d₆) 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 + H₂O]+• (water cluster formed during the ionisation process), (ESI-): m/z 437.00 [M - H] -, calcd C 17H14N2O10S 438.03; HPLC (system A): tR = 14.7 min, purity 99%; λmax(PBS)/nm 422 (ε/dm³ 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 19 as an orange amorphous powder (11.0 mg, overall isolated yield 90%). δH(300 MHz, DMSO-d₆) 1.36 (s, 6H, 2 × CH₃), 2.45 (s, 6H, 2 × CH₃), 3.50-3.90 (m, partially masked by H₂O 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, NH), 8.43 (t, 1H, J = 4.2 Hz, NH); δC(75.5 MHz, DMSO-d₆) 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 27H27BF₂N₄O₇S 600.16; HPLC (system A): tR = 27.1 min, purity 99%; Too hygroscopic compound for suitable IR and elemental.

**Monosulfonated alkyne-based fluorescein (20).** Purification by RP-HPLC (system E, tR = 19.7-24.3 min). The product-containing fractions were lyophilised to give the water-soluble fluorescein 20 as a yellow amorphous powder (24.0 mg, overall isolated yield 70%). δH(300 MHz, DMSO-d₆) 3.56-3.90 (m, 5H, 2x CH₂, 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); δC(75.5 MHz, DMSO-d₆) 41.3, 63.9, 81.3, 85.3, 102.3, 109.1, 113.0, 121.9, 125.1,

S11
127.1, 128.6, 129.5, 138.9, 151.8, 152.1, 153.0, 160.0, 167.6, 171.2; (ESI+): m/z 609.00 [M + H]^+, (ESI-): m/z 607.07 [M - H]^-, calcd C_{28}H_{20}N_{2}O_{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.

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(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.