Electronic Supplementary Material (ESI) for Organic and Biomolecular Chemistry

N-Fmoc-α-sulfo-β-alanine: a versatile building block for the water solubilisation of chromophores and fluorophores by solid-phase strategy

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Supporting Information

Abbreviations

The following abbreviations are used throughout the text of the ESI file: ATR, attenuated total reflectance; BOP, benzotriazol-1-yloxytris(dimethylamino)-phosphonium hexafluorophosphate; BSA, bovine serum albumin; DIC, *N*,*N'*-diisopropylcarbodiimide; DIEA, *N*,*N*-diisopropylethylamine; DMAP, *N*,*N*,-dimethylaminopyridine; DMF, *N*,*N*-dimethylformamide; DMSO, dimethylsulfoxide; Fmoc, 9-fluorenylmethyloxycarbonyl; Gly, glycine; JMOD, J-modulated spin-echo; NHS, *N*-hydroxysuccinimide; NMP, *N*-methylpyrrolidone; PBS, phosphate buffered saline; Rink amide MBHA resin, 4-(2',4'-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxyacetamido-norleucyl-4-methylbenzhydryl-amine resin ; RP-HPLC, reversed-phase high performance liquid chromatography; R6G, rhodamine 6G; rt, room temperature; TEA, triethylamine; TFA, trifluoroacetic acid; TSTU, *O*-(*N*-succinimidyl)-1,1,3,3-tetramethyluronium tetrafluoroborate.

Experimental Section

Chemicals and reagents.

Anhydrous 1,4-dioxane was purchased from Acros Organics. CH_2Cl_2 was dried by distillation over P_2O_5. Fmoc-Gly-OH, DMF (peptide synthesis grade), NMP (peptide synthesis grade) and polystyrene PHB Wang resin (1% DVB, 100-200 mesh, loading: 0.9 mmol g^{-1}) were provided by Iris Biotech GmbH. Rink Amide MBHA resin (100-200 mesh, loading: 0.5 mmol g^{-1}) was from Novabiochem. α-Sulfo-β-alanine was obtained through oleum sulfonation of β-alanine and is now commercially available from Iris Biotech GmbH (#HAA1860).[1,2] Rhodamine 6G carboxylic acid **5** was prepared from rhodamine 6G (R6G, Aldrich, dye content *ca* 95%) by using the three-step synthetic procedure developed by Afonso *et al.* (*i.e.*, pyrolysis, alkylation with benzyl bromoacetate, and hydrogenolysis of benzyl ester).[3] Cyanine dye **8** was prepared in four steps from 1,1,2-trimethyl-1*H*-benz[*e*]indole by using the convergent synthetic scheme reported by us.[4] Sulfobenzindocyanine dye Cy 5.5 (also named Cy5.205) was prepared by using a literature procedure.[5] Spectroscopy grade solvents : cyclohexane (C_6H_{12}) and ethanol (EtOH) were obtained from Aldrich ($\geq$ 99% A.C.S., #154741-1L) and Merck (Uvasol®, #1.00980.0500) respectively. The HPLC-gradient grade acetonitrile (CH_3CN) and methanol (CH_3OH) were obtained from VWR. Borate buffer (200 mM H_3BO_3/KCl + 200 mM NaOH, 50 : 5.9, v/v, pH 8.2), 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).

Instruments and methods.

Automated solid-phase peptide synthesis was performed with an Applied Biosystems 433A synthesizer. The ultrasound-activated fluorophore-resin coupling reactions were performed in an Elmasonics S120H ultrasonic cleaner. Size-exclusion chromatography (for purification of fluorescently labelled BSA protein) was performed with an Econo-Pac® Disposable chromatography column (Bio-Rad, #732-1010) filled with an aq. solution of Sephadex® G-25 Fine (Amersham Biosciences AB, 15 × 40 mm bed), equilibrated with PBS (0.01 M

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[1] D. Wagner, D. Gertner and A. Zilkha, *Tetrahedron Lett.*, 1968, 4479-4480.
[2] Now commercially available from Iris Biotech GmbH (#HAA1860).
[3] C. A. M. Afonso, V. Santhakumar, A. Lough and R. A. Batey, *Synthesis*, 2003, 2647-2654.
[4] B. Chipon, G. Clavé, C. Bouteiller, M. Massonneau, P.-Y. Renard and A. Romieu, *Tetrahedron Lett.*, 2006, **47**, 8279-8284. Corrigendum, 2007, **48**, 501.
[5] S. R. Mujumdar, R. B. Mujumdar, C. M. Grant and A. S. Waggoner, *Bioconjugate Chem.*, 1996, **7**, 356-362.

phosphate, 0.015 M NaCl, pH 7.5). ^{1}H and ^{13}C spectra were recorded on a Bruker DPX 300 spectrometer (Bruker, Wissembourg, France). Chemical shifts are expressed in parts per million (ppm) from D_2O (δ_H = 4.79) or DMSO-d_6 (δ_H = 2.54, δ_C = 40.45).[6] *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" (-).[7] 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. Fluorescence spectroscopic studies were performed with a Varian Cary Eclipse spectrophotometer. The absorption spectra of water-soluble chromophores/fluorophores were recorded (220-850 nm) in PBS (concentration: 1.0-10.0 μM) at 25 °C. Emission spectra were recorded under the same conditions after excitation at the corresponding wavelength (see Table S1, excitation and emission filters : auto, excitation and emission slit = 5 nm) in PBS. 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
10	PBS	488	R6G[8]	0.76 / water[a]	0.70
11	PBS	270	napthalene[9]	0.23 / C_6H_{12}[b]	0.23
12	PBS	254.5	anthracene[9]	0.27 / EtOH[c]	0.12
13	PBS	600	Cy 5.5 sym.[5]	0.23 / PBS[d]	0.05
13	PBS + 5% BSA	600	Cy 5.5 sym.[5]	0.23 / PBS	0.21
14	PBS	338	7-OH-coumarin[10]	0.76 / PBS	0.77

[6] (a) G. R. Fulmer, A. J. M. Miller, N. H. Sherden, H. E. Gottlieb, A. Nudelman, B. M. Stoltz, J. E. Bercaw and K. I. Goldberg, *Organometallics*, 2010, **29**, 2176-2179, (b) H. E. Gottlieb, V. Kotlyar and A. Nudelman, *J. Org. Chem.*, 1997, **62**, 7512-7515.
[7] C. Le Cocq and J. Y. Lallemand, *J. Chem. Soc., Chem. Commun.*, 1981, 150-152.
[8] J. Olmsted, III, *J. Phys. Chem.*, 1979, **83**, 2581-2584.
[9] See http://omlc.ogi.edu/spectra/.

[a]refractive index = 1.333, [b]refractive index = 1.426, [c]refractive index = 1.362, [d]refractive index = 1.337.

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

- System A: RP-HPLC (Thermo Hypersil GOLD C_{18}, 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 80% (40 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}, 5 µm, 21.2 × 250 mm) with CH_3CN and aq. TFA as eluents [90% TFA (5 min), then linear gradient from 0 to 40% (15 min) and 40 to 80% (80 min) of CH_3CN] at a flow rate of 15.0 mL min^{-1}. Dual UV detection was achieved at 260 and 300 nm.
- System C: RP-HPLC (Varian Kromasil C_{18} column, 10 µm, 21.2 × 250 mm) with CH_3CN and aq. TFA as eluents [90% TFA (5 min), linear gradient from 10 to 40% (15 min) and 40 to 80% (80 min) of CH_3CN] at a flow rate of 20.0 mL min^{-1}. UV detection was achieved at 290 nm.
- System D: system C with UV detection at 260 nm.
- System E: system C with the following gradient [100% TFA (5 min), linear gradient from 0 to 20% (15 min) and 40 to 80% (80 min) of CH_3CN]. Dual UV detection was achieved at 235 and 280 nm.
- System F: system C with the following gradient [90% TFA (5 min), linear gradient from 10 to 30% (15 min) and 30 to 70% (80 min) of CH_3CN]. Dual UV detection was achieved at 255 and 365 nm.
- System G: system C with the following gradient [85% TFA (5 min), linear gradient from 15 to 50% (12 min) and 50 to 100% (100 min) of CH_3CN]. Dual visible detection was achieved at 600 and 675 nm.
- System H: system C with the following gradient [100% TFA (5 min), linear gradient from 0 to 20% (15 min) and 20 to 60% (80 min) of CH_3CN]. Dual UV detection was achieved at 260 and 325 nm.

Fmoc-*β*-Ala(SO_3H)-OH (1)[11]. Racemic *α*-sulfo-*β*-alanine (5.0 g) was suspended in deionised water (70 mL) and Na_2CO_3 (4.83 g, 46.6 mmol) was added. Complete solubilisation of amino acid and carbon dioxide release were observed. Then, the homogeneous mixture was cooled to 4 °C and a solution of Fmoc-Cl (4.84 g, 18.7 mmol) in dry 1,4-dioxane (50 mL) was added dropwise over a period of 15 min. The resulting reaction mixture was stirred at rt for further 90 min. The reaction was checked for completion by HPLC (system A) and the mixture was acidified to pH ~ 2 with 10% aq. HCl, and evaporated to dryness. The solid residue was triturated with CH_3CN (2 × 100 mL) and finally washed with deionised water (50 mL) to remove NaCl salt. After isolation by filtration with a Büchner funnel, residual amount of water was removed by lyophilisation. The targeted *N*-Fmoc amino acid **1** was obtained as a white amorphous powder (7.25 g, quantitative yield) and in a pure form without futher purification. Spectroscopic data were identical to those previously reported by us. HPLC (system A): t_R = 23.6 min, purity 98%.

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[10] K.-I. Setsukinai, Y. Urano, K. Kikuchi, T. Higuchi and T. Nagano, *J. Chem. Soc., Perkin Trans. 2*, 2000, 2453-2457.
[11] Now commercially available from Iris Biotech GmbH (#FAA1915).

Model peptide coupling reaction between Fmoc-*β*-Ala(SO_3H)-OH and 4-methoxybenzyl alcohol. A mixture of Fmoc-*β*-Ala(SO_3H)-OH **1** (100 mg, 0.26 mmol) and 4-methoxybenzyl alcohol (37.5 mg, 0.27 mmol) were dissolved in dry DMF (1.1 mL). DIEA (181 μL, 1.04 mmol, 4 equiv.) and BOP reagent (115 mg, 0.26 mmol), were sequentially added and the resulting reaction mixture was stirred at rt overnight. The reaction was checked for completion (complete consumption of starting amino acid) by HPLC (system A). Thereafter, acetic acid (*ca* 100 μL) was added and the reaction mixture was evaporated to dryness. Purification was performed by RP-HPLC (system B, 2 injections) and the main isolated product (t_R = 29.5-31.5 min) was identified as the corresponding benzotriazole ester **2**. (ESI-): *m/z* 491.13 [M - H]$^-$, calcd for $C_{24}H_{20}N_4O_6S$ 492.51; HPLC (system A): t_R = 28.9 min, purity 95%.

Preparation of peptidyl resin *β*-Ala(SO_3H)-Wang using benzyloxybenzyl chloride resin.
(a) Chloration of Wang resin: The Wang resin (278 mg, 0.25 mmol), was suspended in dry CH_2Cl_2 (2.6 mL) and cooled to 4 °C. Then, $SOCl_2$ (90.6 μL, 1.25 mmol, 5 equiv.) was added and the resulting reaction mixture was stirred at 4 °C for 45 min. Then, the mixture was filtered, rinsed six times with CH_2Cl_2, and dried. The reaction was checked for completion (complete conversion of benzyl alcohol moiety into the chloride derivative) by IR measurement. ν_{max}/cm^{-1} 697, 757, 822, 1015, 1173, 1239 (broad), 1452, 1493, 1511, 1604, 2918 (broad), 3024.
(b) Nucleophilic substitution with Fmoc-β-Ala(SO_3H)-OH: The resin was suspended in DMF (2 mL) containing Fmoc-*β*-Ala(SO_3H)-OH **1** (293 mg, 0.75 mmol, 3 equiv.) and anhydrous KI (124 mg, 0.75 mmol, 3 equiv.). Thereafter, 6 equiv. of DIEA (2.0 M solution in NMP, 0.75 mL) were added and the reaction mixture was stirred at rt for 24 h. Then, the mixture was filtered, rinsed three times with NMP, three times with aq. NMP (NMP-H_2O, 9 : 1, v/v), three times with CH_3OH and three times with CH_2Cl_2, and dried. The loading of the Fmoc-amino acid on the resin was determined by spectrophotometry (UV quantification of fulvene-piperidine adduct at λ_{max}/nm 301 (ε/dm^3 mol^{-1} cm^{-1} 7 100)) and was found to be less 1.25 10^{-2} mmol g^{-1} (overall yield 5%).

Preparation of peptidyl resins *β*-Ala(SO_3H)-Gly-Wang and [*β*-Ala(SO_3H)]$_2$-Gly-Wang.
(a) Wang resin loading with Fmoc-Gly-OH: the symmetrical anhydride method was employed. Firstly, the Wang resin (278 mg, 0.25 mmol) was swelled in dry CH_2Cl_2 (2 mL) for 15 min. After filtration, the resin was suspended in a mixture of CH_2Cl_2-NMP (7 : 3, v/v, 2.6 mL) containing Fmoc-Gly-OH (297 mg, 1 mmol, 4 equiv.) and DIC (0.5 mL of a 1.0 M solution in NMP, 0.5 mmol, 2 equiv.). Thereafter, 0.36 mL of a 0.1 M solution of DMAP in NMP (0.036 mmol, 0.15 equiv.) and further amount of dry CH_2Cl_2 (0.6 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. This loaded resin was placed inside the 0.25/0.50 mmol reaction vessel (41 mL) of the ABI433A Peptide Synthesizer to perform the next deprotection-coupling steps automatically.
(b) Fmoc removal: This deprotection was performed under standard conditions (*i.e.*, treatment with a fresh solution of 20% piperidine in NMP) by using a preprogrammed and optimised "B" module of "large scale" (0.25 mmol) FastMoc Chemistry (SynthAssist software).
(c) Coupling of Fmoc-β-Ala(SO_3H)-OH: A clear and limpid solution of Fmoc-*β*-Ala(SO_3H)-OH (391 mg, 1 mmol, 4 equiv.) and BOP reagent (442 mg, 1 mmol, 4 equiv.) in a mixture of DMF-NMP (45 : 55, v/v, 4.06 mL) was prepared and transferred into an amino acid cartridge for ABI433A. This coupling procedure (vortexing and washings) was performed using preprogrammed and optimised modules of "large scale" FastMoc Chemistry, expect for the "E" module (add DIEA and transfer to reaction vessel) which was slightly modified to deliver

12 equiv. (or 16 equiv. for the coupling of the second Fmoc-β-Ala(SO_3H)-OH unit) of DIEA into the reaction mixture.

General procedure for the solid-phase derivatisation of chromophores/fluorophores. All solid-phase derivatisation reactions were performed manually in a single-neck round bottom flask (25 mL). Typically, 100 mg (ca. 0.09 mmol) of peptidyl resin β-Ala(SO_3H)-Gly-Wang (for 4-benzoylbenzoic acid **4** and R6G carboxylic acid **5**) and [β-Ala(SO_3H)]$_2$-Gly-Wang (for 2-naphthoic acid **6**, anthracene-9-carboxylic acid **7**, and cyanine dye **8**) were swelled in dry dry CH_2Cl_2 (1 mL) for 15 min. After filtration, the resin was suspended in a solution of NMP (2 mL) containing 5 equiv. of chromophore/fluorophore carboxylic acid (except cyanine dye **8**, only 3 equiv.) and 5 equiv. of BOP reagent (except R6G carboxylic acid **5**, previously activated with TSTU reagent and DIEA). Thereafter, 20 equiv. of DIEA (2.0 M solution in NMP) were added and the reaction mixture was allowed to stir at rt overnight. Then, the mixture was filtered, rinsed three times with NMP, three times with CH_3OH (except R6G carboxylic acid **5**, six washings were found to be necessary to completely remove any unreactive dye) and three times with CH_2Cl_2, and dried. After the coupling, a negative ninhydrin test was obtained and for cyanine and rhodamine dyes **8** and **5**, the efficiency of the derivatisation reaction was directly visualised through the colour change of resin beads (beige to blue and red respectively). The resin clevage was performed by adding a mixture of TFA-CH_2Cl_2 (1 : 1, v/v, 2 mL) and stirring for 1 h (except cyanine dye **8**, only 30 min), 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 dissolved in deionised water and lyophilised.

Water-soluble benzophenone (9). Purification by RP-HPLC (system C, 1 injection, t_R = 11.0-16.0 min). The product-containing fractions were lyophilised to give the water-soluble benzophenone **9** as a beige glassy solid (19.5 mg, overall isolated yield 50%). δ_H(300 MHz; D_2O) 7.70-7.57 (7H, m, Ph-benzophenone), 7.44-7.39 (2H, m, Ph-benzophenone), 4.15 (1H, q, *J* 9.0, *J* 5.3, C<u>*H*</u>-β-Ala(SO_3H)), 4.07-3.82 (4H, m, C<u>*H*</u>$_2$-β-Ala(SO_3H) & C<u>*H*</u>$_2$-Gly); δ_C(75.4 MHz; D_2O) 38.5 (CH_2), 41.3 (CH_2), 63.9 (CH), 127.1 (2 × CH), 128.4 (2 × CH), 130.2 (4 × CH), 133.7 (CH), 135.9 (Cq), 137.0 (Cq), 139.4 (Cq), 168.1 (C=O), 169.6 (C=O), 172.6 (C=O), 199.5 (C=O); (ESI+): *m/z* 435.00 $[M + H]^+$, 868.73 $[2M + H]^+$, (ESI-): *m/z* 433.20 $[M - H]^-$, 866.80 $[2M - H]^-$, calcd for $C_{19}H_{18}N_2O_8S$ 434.43; HPLC (system A): t_R = 18.6 min, purity 97%; λ_{max}(PBS)/nm 263 (ε/dm^3 mol^{-1} cm^{-1} 23 250).

Water-soluble rhodamine 6G (10). Purification by RP-HPLC (system D, 1 injection, t_R = 26.5-28.5 min). The product-containing fractions were lyophilised to give the water-soluble R6G **10** as a red amorphous powder (15.5 mg, overall yield 25%). δ_H(400 MHz; DMSO-d_6) 8.46 (1H, dd, *J* 7.9, *J* 0.8, Ph-R6G), 8.09 (1H, t, *J* 5.4, N<u>*H*</u>), 7.94-7.82 (3H, m, 2 × Ph-R6G & N<u>*H*</u>) 7.66 (2H, t, *J* 5.6, 2 × N<u>*H*</u>), 7.45 (1H, dd, *J* 7.6, *J* 1.0, Ph-R6G), 6.92 (2H, s, 2 × Ph-R6G), 6.79 (2H, s, Ph-R6G), 4.41 (2H, s, O-C<u>*H*</u>$_2$-C(O)), 3.84-3.29 (9H, m, C<u>*H*</u>-β-Ala(SO_3H), C<u>*H*</u>$_2$-β-Ala(SO_3H), C<u>*H*</u>$_2$-Gly & 2 × C<u>*H*</u>$_2$-Et-R6G), 2.09 (6H, s, 2 × CH_3-R6G), 1.26 (6H, t, *J* 7.1, 2 × CH_3-R6G); δ_C(75.4 MHz, DMSO-d_6) 13.7 (2 × CH_3), 17.5 (2 × CH_3), 37.7 (CH_2), 38.0 (2 × CH_2), 41.2 (CH_2), 63.2 (CH_2), 63.7 (CH), 93.6 (2 × CH), 112.8 (2 × Cq), 125.4 (2 × Cq), 128.4 (Cq), 128.5 (2 × CH), 130.4 (2 × CH), 131.5 (CH), 133.6 (CH), 134.2 (Cq), 157.8 (2 × Cq), 156.7 (2 × Cq), 157.2 (Cq), 163.6 (C=O), 165.5 (C=O), 167.1 (C=O), 171.0 (C=O); (ESI+): *m/z* 681.27 $[M + H]^+$, (ESI-): *m/z* 679.33 $[M - H]^-$, 792.67 $[M - H + TFA]^-$, 1472.80

$[2M - H + TFA]^-$, calcd for $C_{33}H_{36}N_4O_{10}S$ 680.74; HPLC (system A): t_R = 25.8 min, purity 99%; λ_{max}(PBS)/nm 528 (ε/dm^3 mol^{-1} cm^{-1} 65 000).

Water-soluble naphthalene (11). Purification by RP-HPLC (system E, 1 injection, t_R = 19.0-23.0 min). The product-containing fractions were lyophilised to give the water-soluble naphthalene **11** as a gray-beige glassy solid (21.6 mg, overall isolated yield 45%, mixture of two racemic diastereomers). δ_H(300 MHz; D_2O) 8.1 (1H, d, *J* 5.5, Ph-naphthalene), 7.91-7.48 (6H, m, Ph-naphthalene), 4.06-3.00 (8H, m, 2 × C*H*-*β*-Ala(SO_3H), 2 × C*H*$_2$-*β*-Ala(SO_3H) & C*H*$_2$-Gly); δ_H(75.4 MHz; D_2O) 38.0 (CH_2, 1 diastereomer), 38.3 (CH_2, 1 diastereomer), 38.4 (CH_2, 1 diastereomer), 38.5 (CH_2, 1 diastereomer), 40.7 (CH_2, 1 diastereomer), 40.9 (CH_2, 1 diastereomer), 63.5 (CH, 1 diastereomer), 63.9 (CH, 1 diastereomer), 64.2 (CH, 1 diastereomer), 64.3 (CH, 1 diastereomer), 123.0 & 123.2 (1 × CH, 2 diastereomers), 126.9 & 127.0 (1 × CH, 2 diastereomers), 127.6 (1 × CH, 2 diastereomers), 127.7 & 127.8 (1 × CH, 2 diastereomers), 128.1 & 128.2 (1 × CH, 2 diastereomers), 128.4 (1 × CH, 2 diastereomers), 128.9 (1 × CH, 2 diastereomers), 129.8 (Cq, 1 diastereomer), 130.1 (Cq, 1 diastereomer), 131.9 (Cq, 2 diastereomers), 134.5 (Cq, 2 diastereomers), 167.5 (C=O, 1 diastereomer), 167.6 (C=O, 1 diastereomer), 167.7 (C=O, 1 diastereomer), 167.9 (C=O, 1 diastereomer), 170.0 (C=O, 1 diastereomer), 170.4 (C=O, 1 diastereomer), 171.6 (C=O, 1 diastereomer), 172.3 (C=O, 1 diastereomer); (ESI-): *m/z* 530.13 $[M - H]^-$, calcd $C_{19}H_{21}N_3O_{11}S_2$ 531.52; HPLC (system A): t_R = 14.6 & 14.8 min, purity 94%; λ_{max}(PBS)/nm 235.5 (ε/dm^3 mol^{-1} cm^{-1} 39 900), 281.5 (ε/dm^3 mol^{-1} cm^{-1} 5 500).

Water-soluble anthracene (12). Purification by RP-HPLC (system F, 1 injection, t_R = 14.3-17.5 min). The product-containing fractions were lyophilised to give the water-soluble anthracene **12** as a gray-beige glassy solid (22.5 mg, overall yield 43%, mixture of two racemic diastereomers). δ_H(300 MHz; D_2O) 8.61 (1H, bs, Ph-anthracene), 8.08 (2H, bm, Ph-anthracene), 7.96-7.89 (2H, bm, Ph-anthracene), 7.55 (4H, bm, Ph-anthracene), 4.37-2.82 (8H, m, 2 × C*H*-*β*-Ala(SO_3H), 2 × C*H*$_2$-*β*-Ala(SO_3H) & C*H*$_2$-Gly); δ_H(75.4 MHz; D_2O) 38.1 (CH_2, 1 diastereomer), 38.2 (CH_2, 1 diastereomer), 38.4 (CH_2, 1 diastereomer), 38.5 (CH_2, 1 diastereomer), 40.6 (CH_2, 1 diastereomer), 41.1 (CH_2, 1 diastereomer), 63.5 (CH, 1 diastereomer), 64.0 (CH, 1 diastereomer), 64.3 (CH, 1 diastereomer), 64.7 (CH, 1 diastereomer), 124.0 (2 × CH, 2 diastereomers), 125.8 & 126.0 (4 × CH, 2 diastereomers), 127.1 & 127.2 (2 × Cq, 2 diastereomers), 128.5 & 128.6 (2 × CH, 2 diastereomers), 128.9 & 129.0 (2 × CH, 2 diastereomers), 129.6, 130.5, 130.6 & 130.8 (3 × CH, 2 diastereomers), 167.2 (C=O, 1 diastereomer), 167.5 (C=O, 1 diastereomer), 167.6 (C=O, 2 diastereomers), 171.5 (C=O, 1 diastereomer), 172.1 (C=O, 2 diastereomers), 172.4 (C=O, 1 diastereomer); (ESI+): *m/z* 582.00 $[M + H]^+$, 598.93 $[M + H_2O]^{+\bullet}$ (water cluster formed during the ionisation process), (ESI-): *m/z* 580.13 $[M - H]^-$, calcd $C_{23}H_{23}N_3O_{11}S_2$ 581.58; HPLC (system A): t_R = 16.0 & 16.5 min, purity 97%; λ_{max}(PBS)/nm 254 (ε/dm^3 mol^{-1} cm^{-1} 124 400), 346 (ε/dm^3 mol^{-1} cm^{-1} 5 135), 364.5 (ε/dm^3 mol^{-1} cm^{-1} 6 850), 383.5 (ε/dm^3 mol^{-1} cm^{-1} 5 700).

Water-soluble cyanine dye (13). The BOP-mediated coupling reaction mixture involving sterically hindered cyanine dye **8** and peptidyl resin [*β*-Ala(SO_3H)]$_2$-Gly-Wang was periodically sonicated for 15 min (bath temperature: 35-40 °C) in order to improve the yield of this reaction.[12] Purification by RP-HPLC (system G, 1 injection, t_R = 26.0-30.0 min). The product-containing fractions were lyophilised to give the water-soluble cyanine dye **13** as a blue amorphous powder (10 mg, overall isolated yield 10%, mixture of two racemic

[12] M. V. Anuradha and B. Ravindranath, *Tetrahedron*, 1997, **53**, 1123-1130.

diastereomers). δ_H(300 MHz; DMSO-d_6) 8.46-7.40 (21H, m, Ph-benzoindole, Ph-phthalimide, 2 × N*H* & 2 × CH=C*H*-CH=C), 6.70 (1H, t, *J* 13.5, C*H*=CH-CH=C), 6.56-6.43 (2H, m, CH=CH-C*H*=C), 4.26-3.35 (14H, m, 2 × (benzoindole)-N-C*H*$_2$-, (phthalimide)-N-C*H*$_2$-, 2 × C*H*-*β*-Ala(SO_3H), 2 × C*H*$_2$-*β*-Ala(SO_3H) & C*H*$_2$-Gly), 1.96 (6H, s, 2 × C*H*$_3$-benzoindole), 1.94 (6H, s, 2 × C*H*$_3$-benzoindole), 2.08-1.15 (12H, m, (benzoindole)-N-CH_2-C*H*$_2$-C*H*$_2$-CH_2-phthalimide, (benzoindole)-N-CH_2-C*H*$_2$-C*H*$_2$-C*H*$_2$-CH_2-C(O)); (ESI+): *m/z* 1129.20 $[M + H]^+$, (ESI-): *m/z* 1127.40 $[M - H]^-$, calcd $C_{59}H_{64}N_6O_{13}S_2$ 1129.33; HPLC (system A): t_R = 34.8 min, purity 97%; λ_{max}(PBS)/nm 632 (ε/dm^3 mol^{-1} cm^{-1} 65 860), 680 (ε/dm^3 mol^{-1} cm^{-1} 89 700), λ_{max}(PBS + 5% BSA)/nm 698 (ε/dm^3 mol^{-1} cm^{-1} 157 540).

Preparation of peptidyl resin *β*-Ala(SO_3H)-Rink amide MBHA.

(a) Fmoc removal: The commercial Rink amide MBHA resin (200 mg, 0.1 mmol) was deprotected under standard conditions (i.e., treatment with a fresh solution of 22% piperidine in NMP) by using a preprogrammed and optimised "B" module of "small scale" (0.1 mmol) FastMoc Chemistry (SynthAssist software).

(b) Coupling of Fmoc-β-Ala(SO_3H)-OH: A clear and limpid solution of Fmoc-*β*-Ala(SO_3H)-OH (391 mg, 1 mmol, 10 equiv.) and BOP reagent (442 mg, 1 mmol, 10 equiv.) in a mixture of DMF-NMP (45 : 55, v/v, 4.06 mL) was prepared and transferred into an amino acid cartridge for ABI433A. This coupling procedure (vortexing and washings) was performed using preprogrammed and optimised modules of "small scale" FastMoc Chemistry, expect for the "E" module (Add DIEA and transfer to RV) which was slightly modified to deliver 3 equiv. of DIEA into the reaction mixture.

Solid-phase derivatisation of 7-hydroxycoumarin-4-acetic acid.

This solid-phase derivatisation reaction was performed manually in a single-neck round bottom flask (25 mL). 207 mg (*ca.* 0.1 mmol) of peptidyl resin *β*-Ala(SO_3H)-Rink amide MBHA were suspended in a solution of NMP (1 mL) containing 5 equiv. of 7-hydroxycoumarin-4-acetic acid (110 mg, 0.5 mmol). 5 equiv of BOP reagent (221 mg, 0.5 mmol) and 20 equiv. of DIEA (2.0 M solution in NMP, 2 mL) were sequentially added and the resulting reaction 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. After the coupling, a negative ninhydrin test was obtained. The resin clevage was performed by adding a mixture of TFA-CH_2Cl_2 (1 : 1, v/v, 3 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 dissolved in deionised water and lyophilised. Purification was performed by RP-HPLC (system H, 1 injection, t_R = 15.5-20.0 min). The product-containing fractions were lyophilised to give the water-soluble umbelliferone **14** as a white amorphous powder (15.3 mg, overall isolated yield 41%). δ_H(300 MHz; D_2O) 7.33 (1H, d, *J* 8.9, H-coumarin), 6.72 (1H, dd, *J* 8.8, *J* 2.0, H-coumarin), 6.55 (1H, s, H-coumarin), 6.09 (1H, s, H-coumarin), 3.92 (1H, m, C*H*-*β*-Ala(SO_3H)), 3.76-3.59 (4H, m, C*H*$_2$-*β*-Ala(SO_3H) & C*H*$_2$-CO_2H); δ_C(75.4 MHz; D_2O) 38.0 (CH_2), 38.7 (CH_2), 63.8 (CH), 102.7 (CH), 111.8 (Cq), 111.9 (CH), 113.5 (CH), 126.1 (CH), 151.4 (Cq), 154.3 (Cq), 160.0 (Cq), 163.8 (Cq), 169.9 (Cq), 171.1 (Cq); (ESI-): *m/z* 369.13 $[M - H]^-$, calcd for $C_{14}H_{14}N_2O_8S$ 370.34; HPLC (system A): t_R = 12.1 min, purity 99%; λ_{max}(PBS)/nm 338 (ε/dm^3 mol^{-1} cm^{-1} 7 800), 364 (sh) (ε/dm^3 mol^{-1} cm^{-1} 6 800).

Fluorescent labelling of BSA protein.

*(a) Conversion of water-soluble rhodamine 6G **10** into amine-reactive derivative*: A 7.7 mM solution of water-soluble R6G dye carboxylic acid (concentration determined by UV-vis measurements at λ_{max}(H_2O)/nm 528, ε/dm^3 mol^{-1} cm^{-1} 65 000) was prepared in NMP and 20

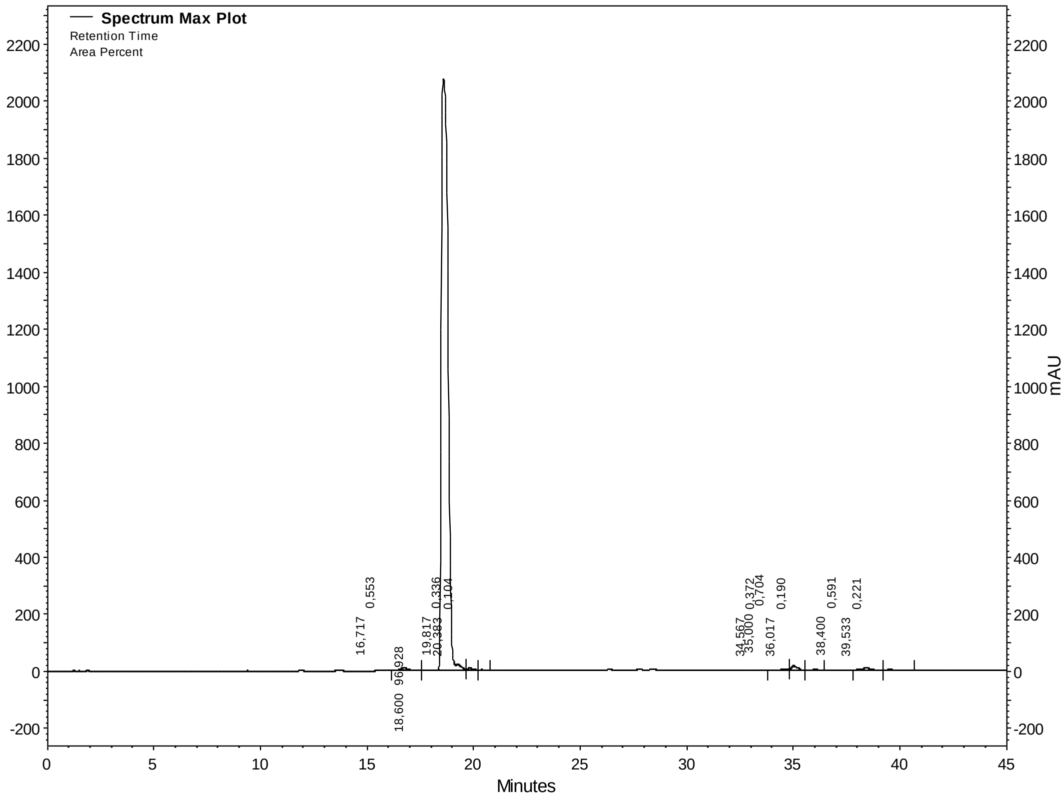
μL (153 nmol, 1 equiv.) was transferred in a 0.7 mL Eppendorf type microtube. 23 μL of a 10 mM solution of TSTU in NMP (230 nmol, 1.5 equiv.) and 0.3 μL of 2.0 M solution of DIEA in NMP were sequentially added. The resulting reaction mixture was protected from light and periodically vortexed for 8 h. The reaction was checked for completion by ESI-MS. The resulting NHS ester was used in the next labelling step without purification. (ESI+): *m/z* 778.13 $[M + H]^+$, (ESI-): *m/z* 776.40 $[M - H]^-$, calcd $C_{37}H_{39}N_5O_{12}S$ 777.81.
(b) Labelling of BSA: The solution of NHS ester (*vide supra*, 153 nmol, 12-fold excess) was added to a 500 μL solution of BSA (1.8 mg/mL, 13 nmol) in borate buffer (pH 8.2). The resulting mixture was protected from the light and periodically vortexed. The reaction was left at 4 °C overnight. Thereafter, R6G-BSA conjugate was purified by size-exclusion chromatography. λ_{max}(PBS)/nm 277, 502, 535. Φ_F(PBS) 0.24.

The R6G per protein ratio (*F* / *P*) was determined spectrophotometrically by measuring their absorbance at 280 and 528 nm and inserting the measured values into the following equation :

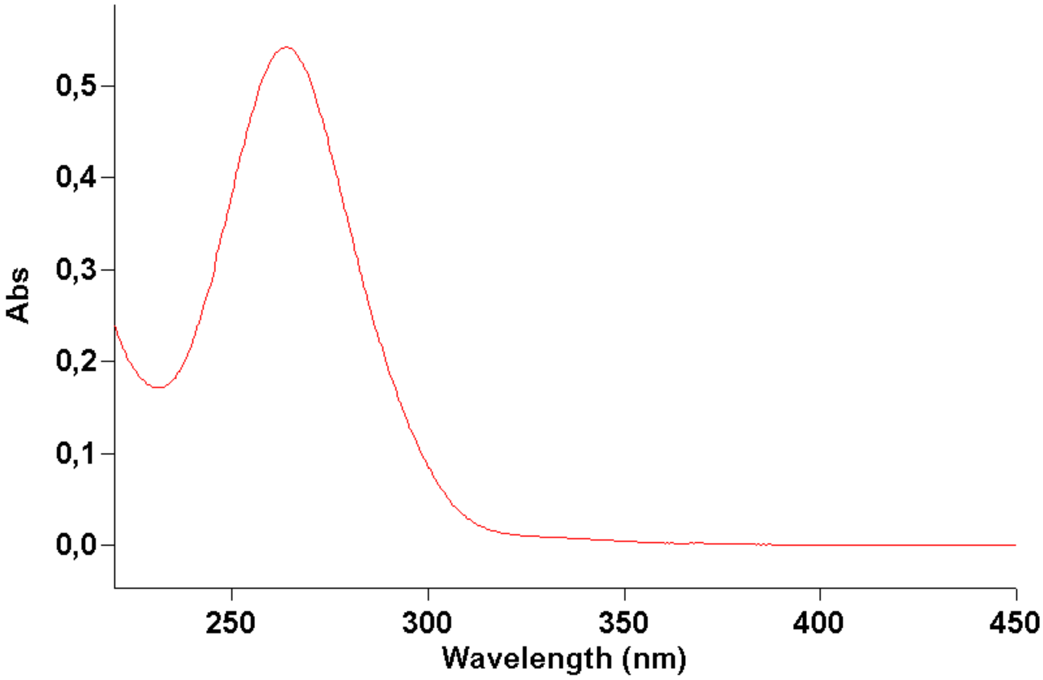
$$F / P = A_{max}\,{}^{P}\varepsilon_{280} / (A_{280}\,{}^{F}\varepsilon_{max} + A_{max}\,{}^{F}\varepsilon_{280})$$

Where A_{280} is the absorbance of the protein at 280 nm, ${}^{P}\varepsilon_{280}$ is the extinction coefficient of the protein at 280 nm, A_{max} is the absorbance of the R6G label as its absoprtion maximum, ${}^{F}\varepsilon_{max}$ is the extinction coefficient of the fluorophore at the absorption maximum, and ${}^{F}\varepsilon_{280}$ is the extinction coefficient of the fluorophore at 280 nm. BSA protein has an extinction coefficient at 280 nm of 43 824 $dm^3\ mol^{-1}\ cm^{-1}$. A value of 0.5 was found.

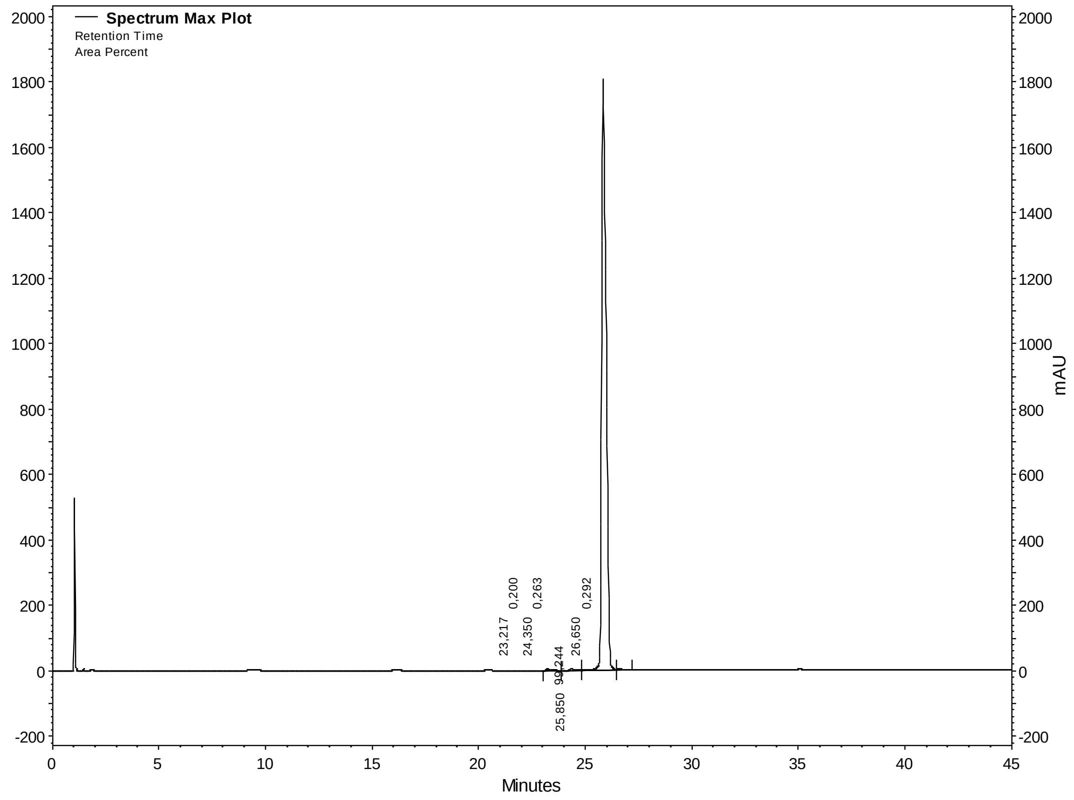
RP-HPLC elution profile (system A) of water-soluble benzophenone 9 in PBS at 25 °C.



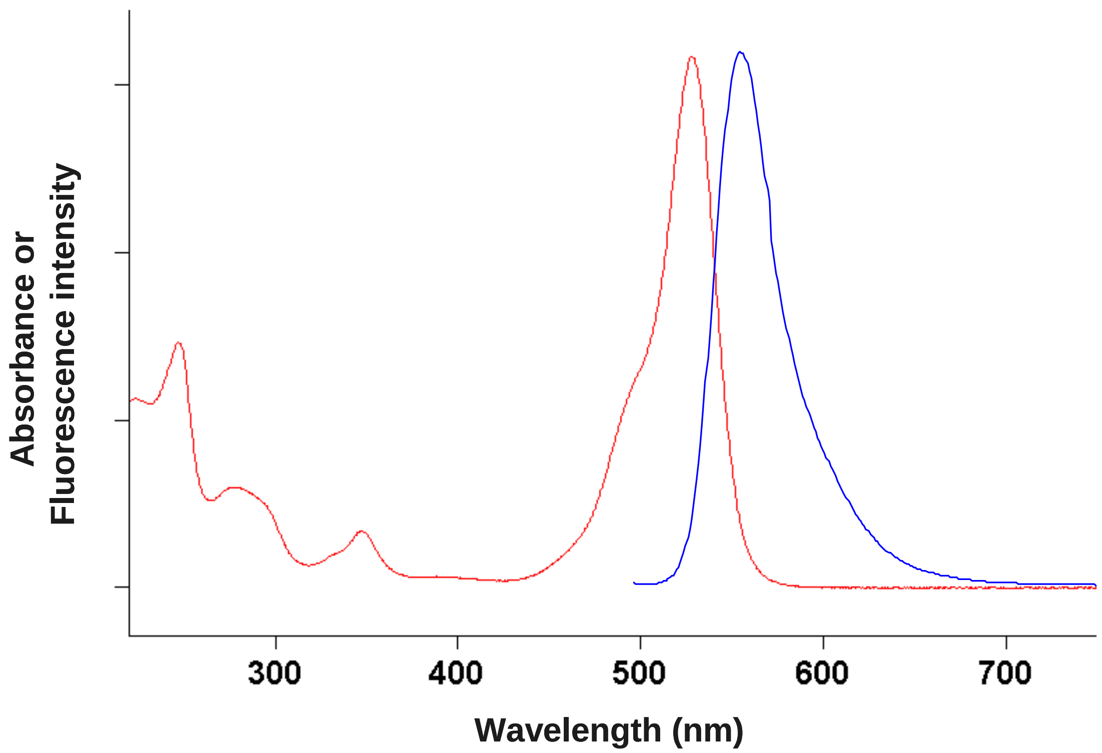
UV absorption spectrum of water-soluble benzophenone 9 in PBS (concentration : 23 μM) at 25 °C.



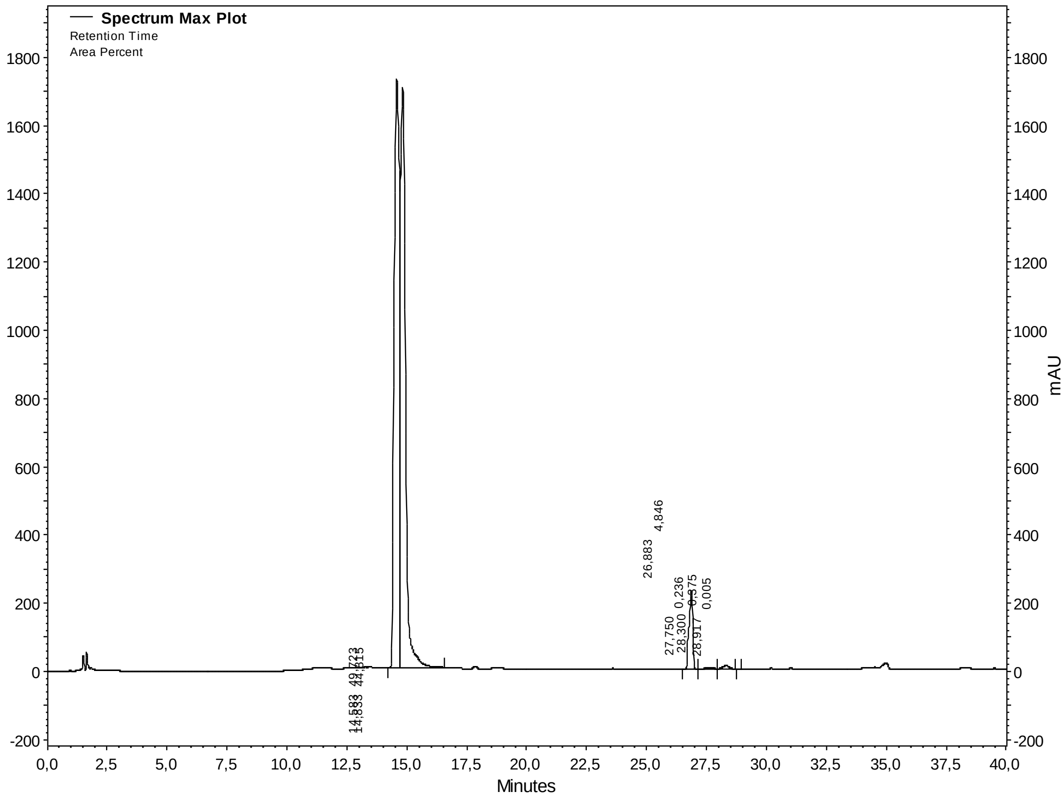
RP-HPLC elution profile (system A) of water-soluble R6G 10 in PBS at 25 °C.



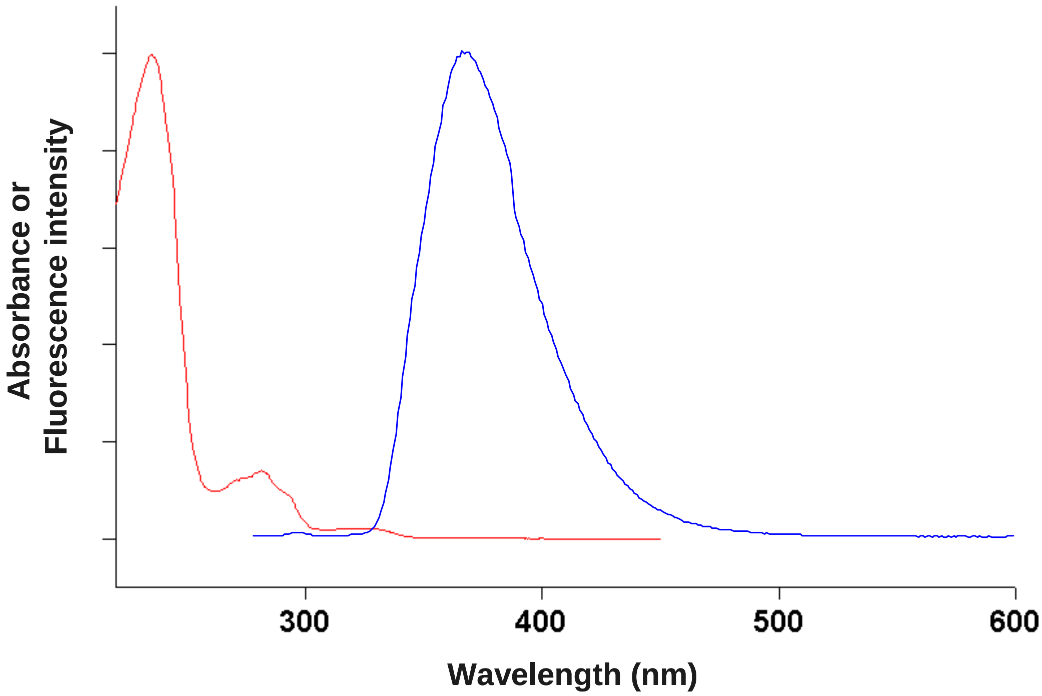
Normalised absorption (—) and emission (—) spectra of water-soluble R6G 10 in PBS at 25 °C.



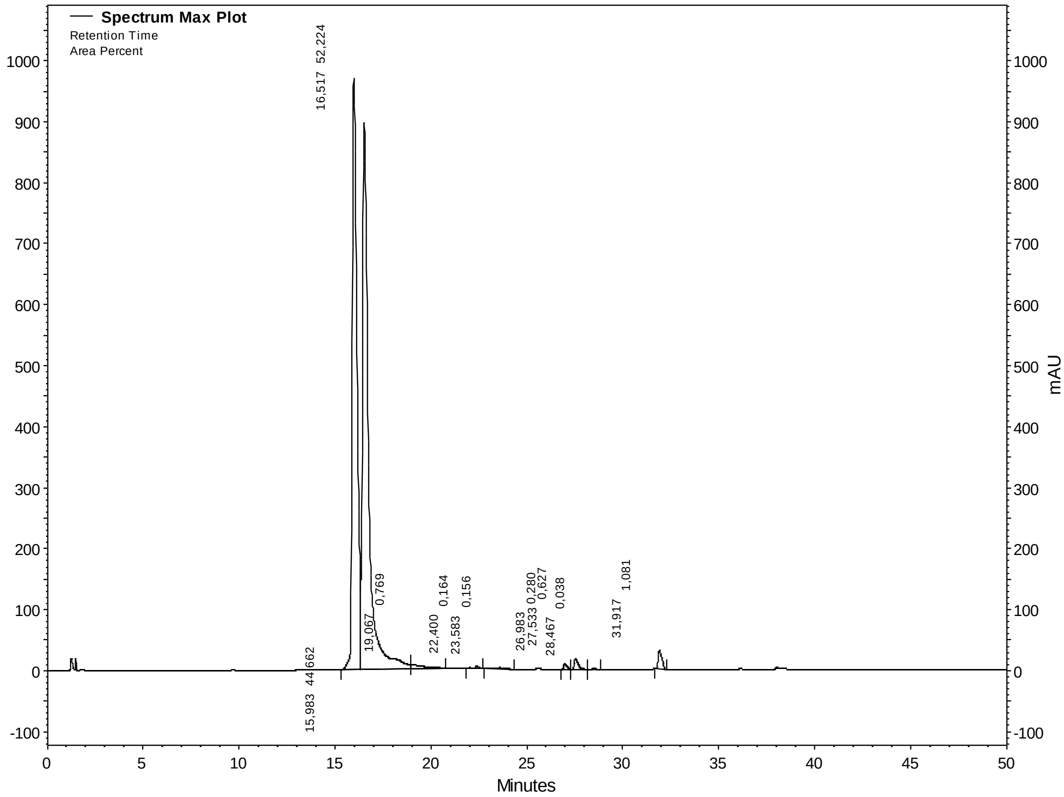
RP-HPLC elution profile (system A) of water-soluble naphthalene 11 in PBS at 25 °C.



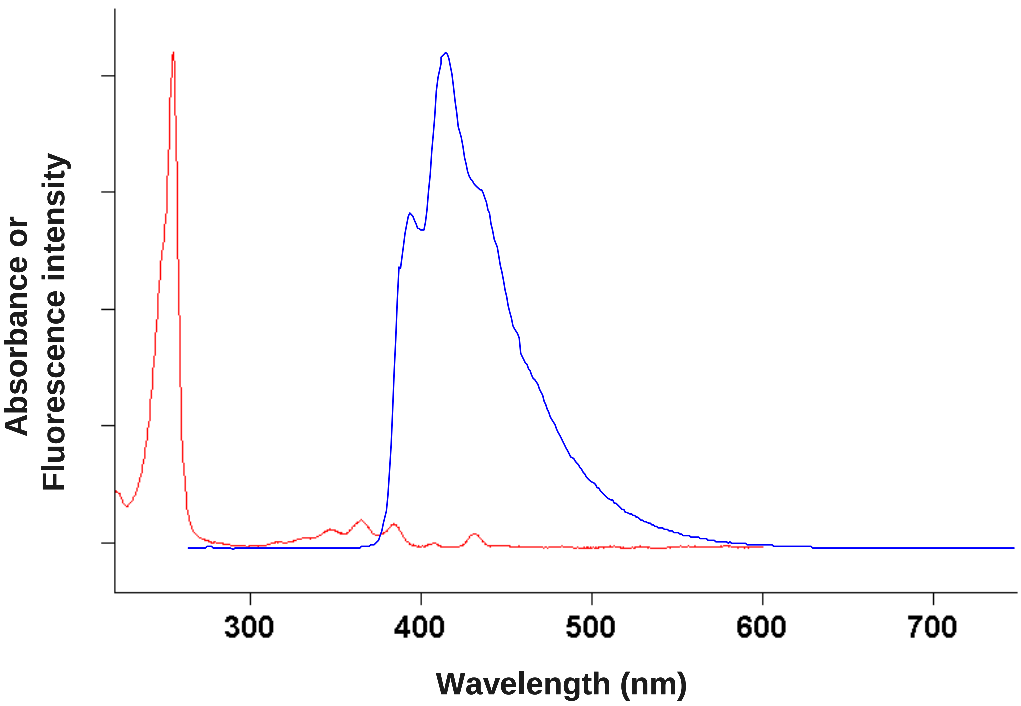
Normalised absorption (—) and emission (—) spectra of water-soluble naphthalene 11 in PBS at 25 °C.



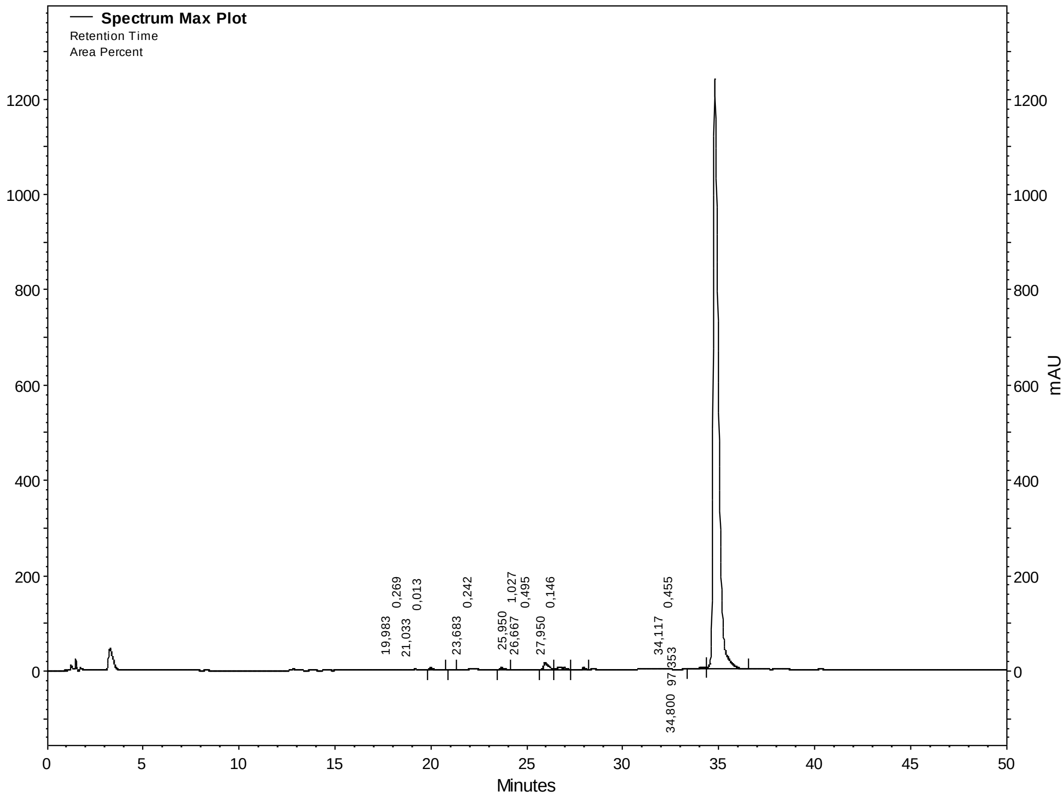
RP-HPLC elution profile (system A) of water-soluble anthracene 12 in PBS at 25 °C.



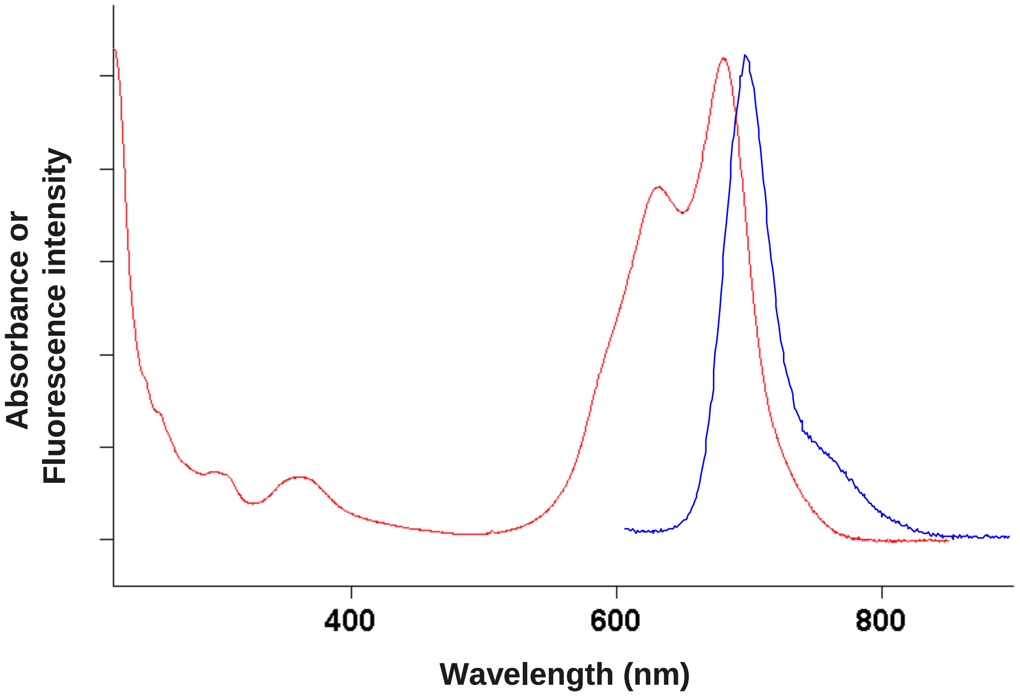
Normalised absorption (—) and emission (—) spectra of water-soluble anthracene 12 in PBS at 25 °C.



RP-HPLC elution profile (system A) of water-soluble cyanine dye 13 in PBS at 25 °C.

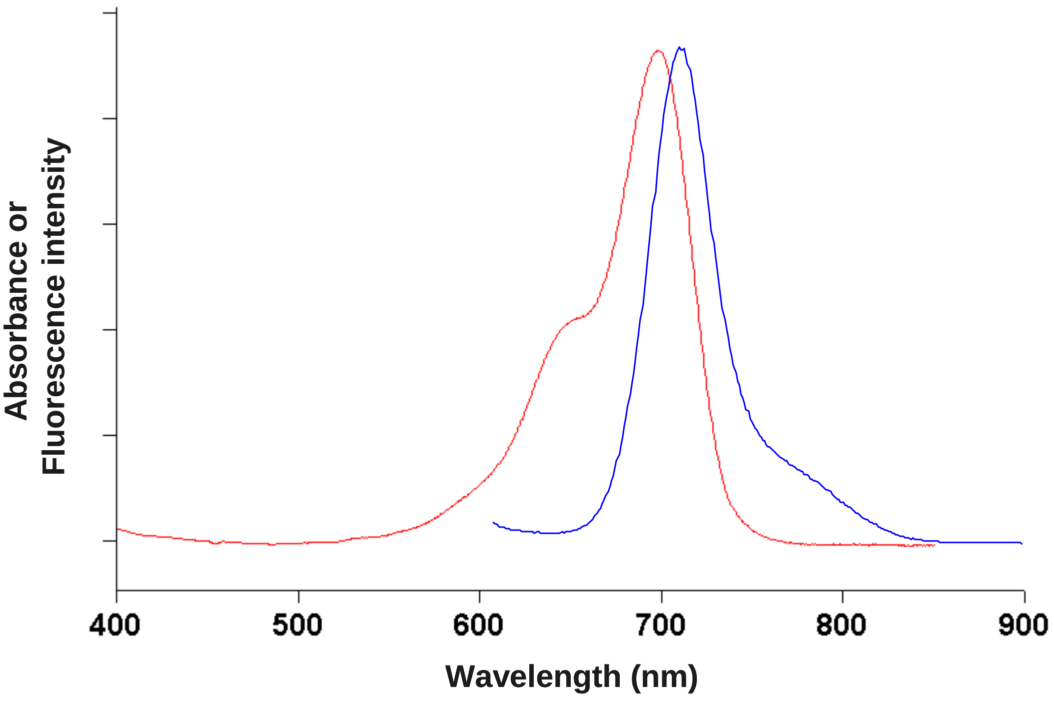


Normalised absorption (—) and emission (—) spectra[a] of water-soluble cyanine dye 13 in PBS at 25 °C.

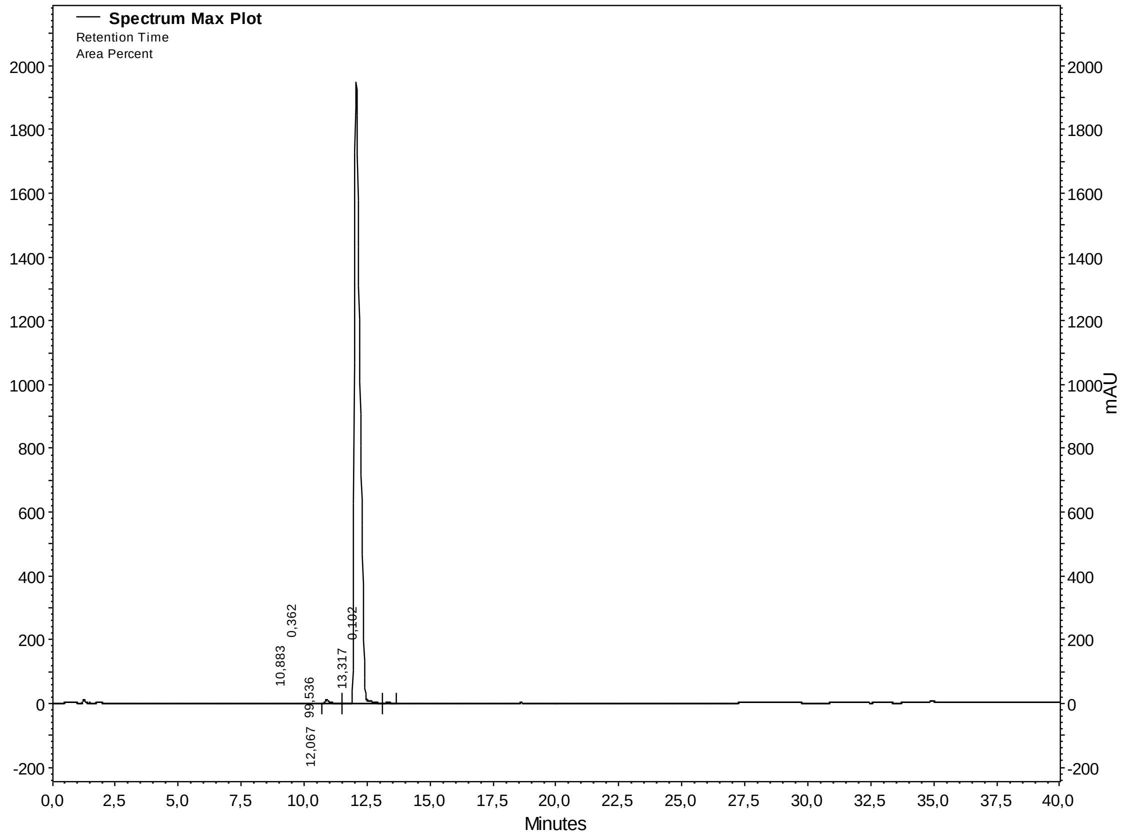


[a]Fluorescence emission intensity ratio @ 700 nm (between PBS and PBS + 5% BSA) = 0.24

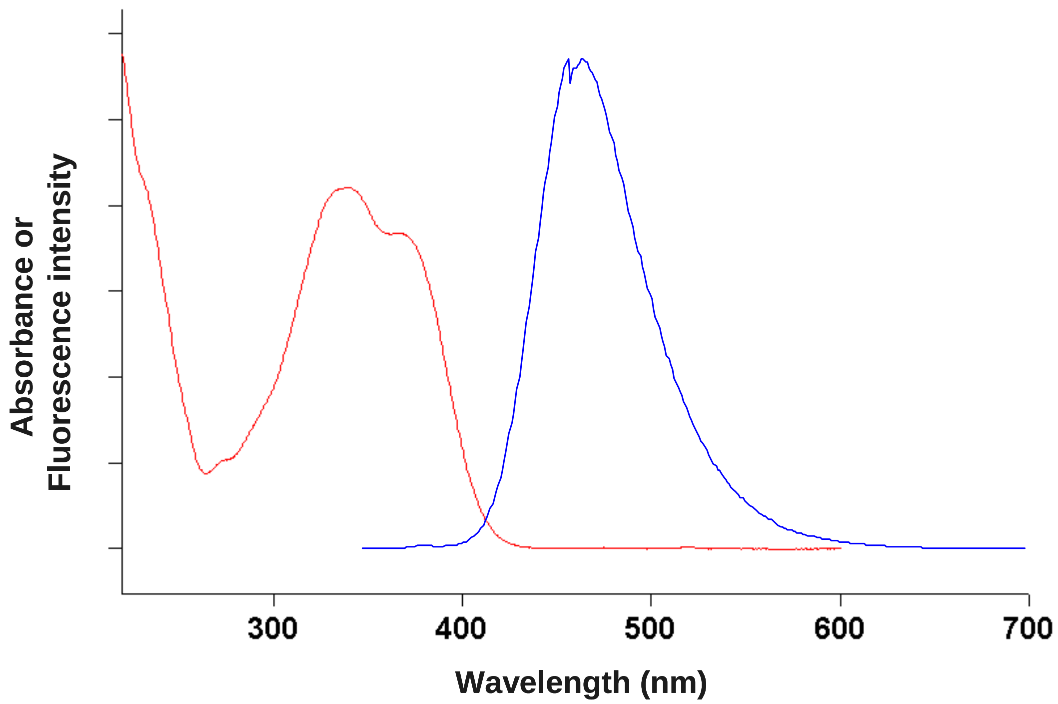
Normalised absorption (—) and emission (—) spectra of water-soluble cyanine dye 13 in PBS + 5% BSA at 25 °C.



RP-HPLC elution profile (system A) of water-soluble 7-hydroxycoumarin 14 in PBS at 25 °C.



Normalised absorption (—) and emission (—) spectra of water-soluble 7-hydroxycoumarin 14 in PBS at 25 °C.



Normalised absorption (—) and emission (—) spectra of 10-BSA conjugate in PBS at 25 °C.