Dyes and Pigments xxx (2014) 1–15

Contents lists available at ScienceDirect

Dyes and Pigments

journal homepage: www.elsevier.com/locate/dyepig

New insights into the water-solubilization of thiol-sensitive fluorogenic probes based on long-wavelength 7-hydroxycoumarin scaffolds

Benoît Roubinet [a], Pierre-Yves Renard [a], Anthony Romieu [b,c,*]

[a] Normandie Université, COBRA UMR 6014 & FR 3038; UNIV Rouen; INSA Rouen; CNRS, IRCOF, 1, Rue Tesnières, 76821 Mont-Saint-Aignan Cedex, France
[b] Institut de Chimie Moléculaire de l'Université de Bourgogne, UMR CNRS 6302, Université de Bourgogne, 9, Avenue Alain Savary, 21078 Dijon, France
[c] Institut Universitaire de France, 103 Boulevard Saint-Michel, 75005 Paris, France

ARTICLE INFO

Article history:
Received 26 December 2013
Received in revised form
1 February 2014
Accepted 3 February 2014
Available online xxx

Keywords:
7-Hydroxycoumarins
Fluorogenic probe
Pro-fluorescence
Red-shift
Thiols
Water-solubility

ABSTRACT

The synthesis and photophysical properties of novel water-soluble phenol-based fluorophores derived from 3-benzothiazolyl-7-hydroxycoumarin and emitting in the range 485–631 nm are described. Further conversion into thiol-sensitive fluorogenic probes through the chemical modification of their hydroxyl group was next investigated. Depending on the type of thiol-reactive quenching moiety used (2,4-dinitrobenzenesulfonyl ester, 2,4-dinitrophenyl ether or benzoquinone-type Michael acceptors) and the water-solubilizing group(s) pre-introduced into the coumarin core, dramatic differences in the thiol-induced fluorescence activation of these pro-fluorophores under physiological conditions were observed. Results for this comparative study provide valuable informations for the selection of the most suitable structural features for designing 7-hydroxycoumarin-based long-wavelength fluorescent probes for thiol bioimaging.

1. Introduction

Thiols are important molecules in the environment and in biological processes. Cysteine (Cys), homocysteine (HCys), glutathione (GSH) and the gasotransmitter hydrogen sulfide (H_2S) play crucial roles in a wide range of physiological and pathological processes arising from their biological redox chemistry [1]. In contrast, aromatic thiols are versatile chemical intermediates currently used to produce pesticides, polymers and pharmaceuticals [2], identified as polluting compounds and highly toxic for human health causing serious damages to the central nervous system and related injuries [3]. Therefore, the design of reaction-based probes or related chemosensors for selective and quantitative detection of thiols by simple spectroanalysis in complex environmental matrices or biological samples has been the focus of increasing attention. Owing to their unique advantages, such as high sensitivity and operational simplicity, thiol-sensitive fluorogenic probes are valuable (bio) analytical tools for some applications in environmental pollution monitoring and disease diagnostic assays. Consequently, during the past decade, tremendous research efforts have been devoted to the development of "smart" reaction-based strategies for fluorescence sensing and bioimaging of thiols [4–7]. Most of them are based either on reductive cleavage reactions or nucleophilic reactions (Michael addition and S_NAr) and tandem processes, often implemented on an aniline- or a phenol-based fluorophore whose reversible chemical modification of amino/hydroxyl group causes dramatic changes in its spectral properties [8–20]. Current improvements to these pro-fluorophores aim at developing pro-fluorescent probes that can either (1) discriminate between benzenethiols and aliphatic thiols or differentiate between physiological thiols (*e.g.*, effective discrimination of cysteine from homocysteine) [8,10,14,21] and/or (2) improve and facilitate thiol detection in complex biological contexts (*i.e.*, *in cellulo* or *in vivo*) by red-shifting the spectral features of the released fluorescent aniline or phenol [22,23]. Surprisingly, to the best of our knowledge, no studies specifically focused on the optimization of physicochemical properties of thiol-sensitive fluorogenic probes have been reported to date. Since factors such as water-solubility and net electric charge are known to strongly influence their cell permeability and emission efficiency under physiological conditions of their unmasked fluorescent label (directly related to their resistance to

* Corresponding author. Institut de Chimie Moléculaire de l'Université de Bourgogne, UMR CNRS 6302, Université de Bourgogne, 9, Avenue Alain Savary, 21078 Dijon, France. Tel.: +33 3 80 39 36 24; fax: +33 3 80 39 61 17.
E-mail address: anthony.romieu@u-bourgogne.fr (A. Romieu).

http://dx.doi.org/10.1016/j.dyepig.2014.02.004
0143-7208/

aggregation in aq. media), these are key parameters to be considered in the rational design of thiol-imaging agents [24]. Furthermore, structure and specific position of water-solubilizing moieties onto the pro-fluorophore may complicate its synthesis (especially the introduction of the selected thiol-reactive quenching moiety) and affect its reactivity towards thiols under physiological conditions, mainly due to adverse electrostatic and/or steric effects. In this context, we have decided to make a comparative evaluation of different water-solubilizing methodologies, implemented to a family of thiol-sensitive pro-fluorophores derived from 3-benzothiazolyl-7-hydroxycoumarin and exhibiting distinct red-shifted emission in the range 485–631 nm, aimed at assessing their effects on the thiol-mediated probes' activation and on the fluorescence efficiency of the released phenols. The ultimate goal of the present work is to identify the best pair of molecular candidates acting as water-solubilizing and thiol-reactive quenching moieties respectively, to convert a specific hydrophobic long-wavelength 7-hydroxycoumarin derivative into a thiol-sensitive fluorogenic probe fulfilling all requirements for the targeted biosensing application.

2. Experimental

2.1. Chemicals and instruments

Flash column chromatography purifications were performed on Geduran® Si 60 silica gel (40–63 μm) from Merck. TLC were carried out on Merck DC Kieselgel 60 F-254 aluminium sheets. The spots were visualized by illumination with a UV lamp ($\lambda = 254/365$ nm) and/or staining with $KMnO_4$ solution. Unless otherwise noted, all chemicals were used as received from commercial sources without further purification. All solvents were dried by standard procedures (CH_2Cl_2: distillation over P_2O_5; pyridine: distillation over CaH_2 and stored over BaO; CH_3CN: distillation over CaH_2; absolute EtOH: storage over anhydrous Na_2SO_4 and triethylamine (TEA): distillation over KOH and storage over BaO). Peptide synthesis-grade NMP and anhydrous DMF were purchased from Carlo Erba and stored over 4Å molecular sieves. Peptide synthesis-grade *N*,*N*-diisopropylethylamine (DIEA) was provided by Iris Biotech GmbH. HPLC gradient-grade acetonitrile (CH_3CN) was obtained from VWR. Phosphate buffer (PB, 100 mM, pH 7.5) and aq. mobile phases for HPLC were prepared with water purified by means of a MilliQ 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 TEA and glacial acetic acid or CO_2 gas, respectively. 3-Benzothiazolyl-7-hydroxycoumarin (hydrochloride salt) and its 4-cyano derivative, 3-benzothiazolyl-7-hydroxycoumarin-6-sulfonic acid, di-*tert*-butyl iminodiacetate **A**, 2-aminoethane-1,1-disulfonic acid (tetrabutylammonium salt, TBA^+ salt) **B**, *N*-sulfopropyl-2-methylbenzothiazole **C**, *N*-sulfopropyl-4-methylpyridine **D**, 1-chloromethyl-2,5-dimethoxy-3,4,6-trimethylbenzene **E**, amino-trimethyl lock linker-functionalized quinone **F** and sodium thiophosphate (NaThioPi) were prepared according to literature procedures [25–36].

^1H and ^{13}C spectra were recorded with either a Bruker DPX 300 or a Bruker Avance III 500 spectrometer (Bruker, Wissembourg, France). Chemical shifts are expressed in parts per million (ppm) using the residual solvent peak for calibration [37]. *J* values are expressed in Hz. Infrared (IR) spectra were recorded with a universal ATR sampling accessory on a Perkin Elmer FT-IR Spectrum 100 spectrometer. The bond vibration frequencies are expressed in reciprocal centimetres (cm^{-1}). 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–Vis 2000 detector. Automated flash purifications on RP-C_{18} cartridges were performed with a Biotage Isolera™ One (ISO-1EW) system. Ion-exchange chromatography (for desalting fluorophores purified with TEAB as aq. mobile phase) was performed with an Econo-Pac® disposable chromatography column (Bio-Rad, #732–1010) filled with an aq. solution of Dowex® 50WX8-400 (Alfa Aesar, ~5 g for 15 mg of dye, 15 × 50 mm bed), regenerated using aq. 10% HCl solution and equilibrated with deionized water. Low-resolution 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 7-hydroxycoumarin derivatives were recorded (220–800 nm) in PB (100 mM, pH 7.5) at 25 °C. Excitation/emission spectra were recorded under the same conditions after emission/excitation at the corresponding wavelength (390/470/510/550 nm, excitation and emission filters: auto, excitation and emission slit: 5 nm). Fluorescence emission spectra of far-red emitting 7-hydroxycoumarin-hemicyanine dyes (**10**, **11** and **13**) were corrected. Fluorescence quantum yields were measured at 25 °C by a relative method using 7-hydroxycoumarin (Φ_F = 76% in PB, pH = 7.4) [38], sulforhodamine 101 (SR101, Φ_F = 95% in EtOH), fluorescein (Fluo, Φ_F = 91% in 0.1 N NaOH) or cresyl violet (CV, Φ_F = 56% in EtOH) as a standard [39]. 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 of 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. The following refractive index values were used: 1.479 for DMSO, 1.362 for EtOH and 1.337 for PB and PB + 5% BSA.

Several chromatographic systems were used for the analytical experiments and purification steps (by semi-preparative HPLC or automated flash purification system): System A: RP-HPLC (Thermo Hypersil GOLD C_{18} column, 5 μm, 2.1 × 100 mm) with CH_3CN and 0.1% trifluoroacetic acid (aq. TFA 0.1%, pH 2.0) as eluents [100% TFA (5 min) then linear gradient from 0% to 100% (45 min) of CH_3CN] at a flow rate of 0.25 mL/min. Triple UV–vis detection was achieved at 220, 260, and 380 nm and with the "Max Plot" (*i.e.*, chromatogram at absorbance maximum for each compound) mode (220–650 nm). System B: system A with the following gradient [80% TFA (5 min) then linear gradient from 20% to 100% (45 min) of CH_3CN]. System C: system A with TEAA buffer (25 mM, pH 7.0) as aq. mobile phase [100% TEAA (5 min) then linear gradient from 0% to 100% (45 min) of CH_3CN]. System D: 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 50% (100 min) of CH_3CN] at a flow rate of 20.0 mL/min. Visible detection was achieved at 420 nm. System E: automated flash purification (Biotage® SNAP cartridge KP-C18-HS, 60 g) with CH_3CN and ultrapure water as eluents [100% H_2O (5 min) then linear gradient from 0% to 100% (40 min) of CH_3CN] at a flow rate of 35.0 mL/min. Dual UV detection was achieved at 220 and 360 nm; System F: system E with CH_3CN and aq. TFA 0.1% as eluents [100% TFA (5 min) then linear gradient from 0% to 10% (10 min) and 10%–60% (60 min) of CH_3CN] at a flow rate of 35.0 mL/min. System G: system D with the following gradient [100% TFA 0.1% (5 min) then linear gradient from

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0% to 15% (10 min) and 15%–55% (65 min) of CH_3CN]. UV detection was achieved at 360 nm. System H: system G with aq. TEAB (50 mM, pH 7.5) as aq. mobile phase. System I: system G with the following gradient [100% TEAB (5 min) then linear gradient from 0% to 10% (10 min) and 10%–50% (45 min) of CH_3CN]. System J: system F with the following cartridge (Biotage® SNAP cartridge KP-C18-HS, 60 g) and gradient [100% TFA (5 min) then linear gradient from 0% to 20% (10 min) and 20%–50% (45 min) of CH_3CN] at a flow rate of 35.0 mL/min. System K: system D with the following gradient [100% TFA (5 min) then linear gradient from 0% to 30% (10 min) and 30%–60% (55 min) of CH_3CN]. UV detection was achieved at 360 nm. System L: system K with the following gradient [100% TFA (5 min) then linear gradient from 0% to 30% (10 min) and 30%–70% (65 min) of CH_3CN]. System M: system K with the following gradient [100% TFA (5 min) then linear gradient from 0% to 25% (15 min) and 25%–70% (70 min) of CH_3CN]. System N: system K with the following gradient [100% TFA (5 min) then linear gradient from 0% to 25% (10 min) and 25%–60% (55 min) of CH_3CN]. System O: system K with the following gradient [100% TFA 0.1% (5 min) then linear gradient from 0% to 20% (10 min) and 20%–70% (65 min) of CH_3CN]. System P: system H with the following gradient [100% TEAB (5 min) then linear gradient from 0% to 15% (10 min) and 15%–45% (40 min) of CH_3CN]. Visible detection at 400 nm. System Q: system G with the following gradient [100% TFA (5 min) then linear gradient from 0% to 20% (10 min) and 20%–60% (65 min) of CH_3CN].

For the detailed synthetic procedures of compounds **4a**, **4b**, **6**, **8**, **17a** and **17b**, see supplementary material.

Most of 7-hydroxycoumarins and related fluorogenic probes were found to be soluble in D_2O but bad quality spectra were obtained (*i.e.*, broad and poor-resolved peaks). Thus, all NMR spectra of water-soluble derivatives were recorded in DMSO-d_6.

2.2. Synthesis of water-soluble 3-benzothiazolyl-7-hydroxycoumarin derivatives

2.2.1. 4-Cyano 6-sulfonated derivative (**2**)

To a solution of 3-benzothiazolyl-7-hydroxycoumarin-6-sulfonic acid **1** (0.1 g, 0.24 mmol, 1 equiv) in DMF (2 mL), an aqueous solution of KCN (250 μL, 35 mg, 0.54 mmol, 2.3 equiv) was added and the resulting reaction mixture was stirred at 50 °C for 3 h. Thereafter, the mixture was cooled to 0 °C, DDQ (61 mg, 0.27 mmol, 1.1 equiv) was added and the mixture stirred at room temperature for a further 2 h. The crude solution was concentrated and the resulting residue was purified by semi-preparative RP-HPLC (System D). The product-containing fractions were lyophilized to give the TFA salt of compound **2** as an amorphous red solid (56 mg, yield 45%). IR: ν 2198 (CN), 1725, 1606, 1538, 1351, 1228, 1172, 1084, 1020. ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.23 (d, 1H, $J = 9.0$ Hz), 8.15 (d, 1H, $J = 6.0$ Hz), 8.11 (s, 1H), 7.63 (t, 1H, $J = 9.0$ Hz), 7.56 (t, 1H, $J = 9.0$ Hz), 7.04 (s,1H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 159.3, 158.7, 157.5, 154.3, 151.4, 136.5, 130.4, 127.1, 126.5, 125.9, 123.3, 122.2, 121.4, 120.8, 113.9, 109.8, 103.8. MS (ESI, negative mode): $m/z = 399.07$ $[M - H]^-$, calcd for $C_{17}H_8N_2O_6S_2$ 399.98. HPLC (system A): $t_R = 26.3$ min, purity = 95%.

2.2.2. Di-tert-butyl ester of 8-aminomethyl derivative (**3a**)

Paraformaldehyde (132 mg, 4.1 mmol, 6.8 equiv) was dissolved in dry EtOH (2 mL) and solid KOH (17 mg, 0.30 mmol, 0.5 equiv) was added at 0 °C and the resulting solution was stirred for 5 min. Then, di-*tert*-butyl iminodiacetate **A** (530 mg, 2.0 mmol, 3.4 equiv) was added and the mixture was stirred at rt for 1 h. The iminium salt formed *in situ* was slowly added to a solution of 3-benzothiazolyl-7-hydroxycoumarin (200 mg, 0.6 mmol, 1 equiv) in dry EtOH (5 mL) and the resulting reaction mixture was stirred under reflux for 24 h. Volatiles were removed under reduced pressure and the resulting residue was subjected to an automated flash purification on a RP-C_{18} cartridge (system E). The product-containing fractions were lyophilized to give compound **3a** as an amorphous yellow solid (80 mg, yield 24%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.95 (s, 1H), 8.03 (d,1H, $J = 6.0$ Hz), 7.93 (d, 1H, $J = 9.0$ Hz), 7.51 (m, 2H), 7.36 (t, 1H, $J = 9.0$ Hz), 6.91 (d, 1H, $J = 6.0$ Hz), 4.26 (s, 2H), 3.44 (s, 4H), 1.50 (s, 18H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 169.9, 164.0, 160.8, 160.0, 153.8, 152.6, 142.5, 136.6, 130.3, 126.4, 125.0, 122.6, 121.7, 115.6, 155.5, 111.7, 108.5, 82.6, 55.5, 48.1, 28.2. MS (ESI, positive mode): $m/z = 552.93$ $[M + H]^+$, calcd for $C_{29}H_{32}N_2O_7S$ 552.19.

2.2.3. Iminodiacetic acid derivative (**3b**)

Di-*tert*-butyl ester **3a** (59 mg, 0.11 mmol, 1 equiv) was dissolved in a mixture of TFA/CH_2Cl_2 (1:1, v/v, 20 mL) and the resulting reaction mixture was stirred at reflux for 2 h. Thereafter, the deprotection mixture was concentrated to dryness and triturated with Et_2O. The solid was recovered and dried under vacuum to give the TFA salt of compound **3b** as a yellow amorphous solid (40 mg, yield 66%). IR: ν 1720, 1606, 1573, 1431, 1384, 1298, 1196, 1087, 1002. ^{1}H NMR (300 MHz, DMSO-d_6): δ 12.24 (s, 2H, 2 × $CO_2\underline{H}$), 9.16 (s, 1H), 8.16 (d, 1H, $J = 6.0$ Hz), 8.05 (d, 1H, $J = 9.0$ Hz), 7.89 (d, 1H, $J = 9.0$ Hz), 7.57 (t, 1H, $J = 6.0$ Hz), 7.52 (t, 1H, $J = 6.0$ Hz), 6.93 (d, 1H, $J = 9.0$ Hz), 4.17 (s, 2H), 3.53 (s, 4H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 172.4, 163.1, 160.4, 159.6, 153.8, 152.0, 143.0, 135.6, 130.9, 126.5, 125.0, 122.2, 122.2, 114.50, 114.3, 111.4, 109.4, 53.8, 46.7. MS (ESI, negative mode): $m/z = 439.00$ $[M - H]^-$, calcd for $C_{21}H_{16}N_2O_7S$ 440.07. HPLC (system A): $t_R = 24.8$ min, purity = 97%.

2.2.4. Sarcosine derivative (**5**)

Paraformaldehyde (65 mg, 2.18 mmol, 7.2 equiv) was dissolved in dry EtOH (2 mL) and solid KOH (122 mg, 2.18 mmol, 7.2 equiv) was added at rt and the resulting solution was stirred for 5 min. Then, sarcosine *tert*-butyl ester hydrochloride (184 mg, 1.09 mmol, 3.6 equiv) was added and the mixture was stirred at reflux for 3 h. The iminium salt formed *in situ* was slowly added to a solution of 3-benzothiazolyl-7-hydroxycoumarin (100 mg, 0.3 mmol, 1 equiv) in dry EtOH (5 mL) and the resulting reaction mixture was stirred under reflux for 4 h. *Premature cleavage of tert-butyl ester occurr*ed during this Mannich-type reaction and the free sarcosine–coumarin conjugate was isolated as the major product as follows: after removal of volatiles under reduced pressure, the crude residue was subjected to an automated flash purification on a RP-C_{18} cartridge (system F). The product-containing fractions were lyophilized to give the TFA salt of compound **5** as an amorphous yellow solid (73 mg, yield 48%). IR: ν 1696, 1635, 1570, 1407, 1291, 1255, 1191, 1130, 1027. ^{1}H NMR (300 MHz, DMSO-d_6): δ 9.21 (s, 1H), 8.18 (d, 1H, $J = 6.0$ Hz), 8.03 (m, 2H), 7.56 (t, 1H, $J = 9.0$ Hz), 7.46 (t, 1H, $J = 9.0$ Hz), 7.05 (d, 1H, $J = 6.0$ Hz), 4.46 (s, 2H), 3.98 (s, 2H), 2.72 (s, 3H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 168.2, 163.3, 160.2, 159.2, 154.8, 152.0, 142.8, 135.6, 132.9, 126.6, 125.1, 122.2, 122.2, 114.7, 113.9, 11.5, 104.1, 55.5, 47.8, 41.1. MS (ESI, positive mode): $m/z = 396.87$ $[M + H]^+$, calcd for $C_{20}H_{16}N_2O_5S$ 396.08. HPLC (system A): $t_R = 24.5$ min, purity = 95%.

2.2.5. Di-sulfonated sarcosine derivative (**7**)

To a solution of 7-hydroxycoumarin carboxylic acid **5** (20 mg, 39.3 μmol, 1 equiv) in NMP (200 μL), a 0.38 M solution of PyBrOP in NMP (200 μL, 35 mg, 75.6 μmol, 1.5 equiv) and a 2.0 M solution of DIEA in NMP (56 μL, 111 μmol, 2.2 equiv) were sequentially added and the resulting reaction mixture was stirred at rt for 30 min. Then, this crude acyl bromide coumarin derivative was added dropwise to a pre-cooled (0 °C) 0.5 M solution of 2-aminoethane-1,1-disulfonic acid **B** (TBA^+ salt) in NMP (0.3 mL, 150 μmol, 3 equiv) containing 5 equiv of DIEA (125 μL of a 2.0 M solution in NMP), over a period of 15 min. The resulting reaction mixture was stirred at rt

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for 2 h. Then, a further amount of PyBrOP (23 mg, 50.4 μmol, in 75 μL of NMP) was added and the mixture was stirred for a further 3 h. This amidification reaction was checked for completion by RP-HPLC (system A), quenched by adding acetic acid (50 μL) and dilution with aq. TFA-CH_3CN (4:1, v:v, 5 mL) and finally purified by RP-HPLC (system G). The product-containing fractions were lyophilized to give the TFA salt of compound **7** in mixture with TBA^+ salts. Desalting by ion-exchange chromatography (followed by lyophilization) afforded compound **7** as a yellow amorphous powder (7 mg, yield 31%). IR: ν 1730, 1680, 1607, 1570, 1498, 1250, 1193, 1063, 1016. ^{1}H NMR (300 MHz, DMSO-d_6): δ 11.99 (s, 1H), 9.82 (s, 1H), 9.17 (s, 1H), 8.16 (d, 1H, J = 6.0 Hz), 8.09 (s, 1H), 8.03 (m, 2H), 7.54 (t, 1H, J = 9.0 Hz), 7.44 (t, 1H, J = 9.0 Hz), 7.06 (d, 1H, J = 6.0 Hz), 4.53 (s, 2H), 4.10 (s, 2H), 3.75 (s, 2H), 3.58 (t, 1H, J = 6.0 Hz), 2.81 (s, 3H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 163.4, 162.8, 160.2, 159.1, 155.0, 152.0, 142.6, 135.7, 133.3, 126.6, 125.1, 122.3, 115.0, 113.8, 111.6, 103.2, 74.2, 56.3, 47.4, 41.2, 38.6. MS (ESI, negative mode): m/z = 581.67 $[M - H]^-$, calcd for $C_{22}H_{21}N_3O_{10}S_3$ 583.04. HPLC (system A): t_R = 22.6 min, purity = 98%.

2.2.6. 3-Benzothiazolyl-8-formyl-7-hydroxycoumarin (**9**)

To a solution of 3-benzothiazolyl-7-hydroxycoumarin (500 g, 1.5 mmol, 1 equiv) in TFA (15 mL), hexamine (474 mg, 3.40 mmol, 2.3 equiv) was added and the resulting reaction mixture was stirred at reflux for 24 h. Then, aq. 1.0 M HCl (30 mL) was added and the mixture was stirred for a further 3 h. Thereafter, this aq. mixture was extracted with ethyl acetate (EtOAc) and the combined organic phases were washed with deionized water, dried over anhydrous Na_2SO_4 and finally evaporated to dryness. The resulting residue was purified by flash column chromatography on silica gel (petroleum ether/EtOAc with a step gradient from 1:0 to 3:2) in order to give aldehyde **9** as a yellow solid (280 mg, yield 58%). IR: ν 1722, 1658, 1586, 1473, 1313, 1290, 1188, 1166, 1069, 1008. ^{1}H NMR (300 MHz, DMSO-d_6): δ 10.48 (s, 1H), 9.19 (s, 1H), 8.18 (m, 2H, J = 9.0 Hz), 8.04 (d, 1H, J = 9.0 Hz), 7.56 (t, 1H, J = 9.0 Hz), 7.47 (t, 1H, J = 9.0 Hz), 7.07 (d, 1H, J = 9.0 Hz). ^{13}C NMR (75 MHz, DMSO-d_6): δ 190.0, 165.6, 159.8, 158.8, 155.5, 152.0, 142.4, 137.7, 135.7, 126.7, 125.3, 122.3, 122.2, 115.8, 115.2, 111.4, 109.4. MS (ESI, positive mode): m/z = 321.93 $[M + H]^+$, calcd for $C_{23}H_9NO_4S$ 323.03.

2.2.7. 7-Hydroxycoumarin-Hemicyanine dye (**10**)

To a solution of aldehyde **9** (50 mg, 0.16 mmol, 1 equiv) in dry EtOH (2 mL), *N*-sulfopropyl-2-methylbenzothiazole **C** (84 mg, 0.31 mmol, 2 equiv) and pyrrolidine (30 μL, 0.31 mmol, 2 equiv) were sequentially added and the resulting reaction mixture was stirred at rt under an Ar atmosphere for 2 h. The reaction was checked for completion by RP-HPLC (system C) and purified by semi-preparative RP-HPLC (System I). The product-containing fractions were lyophilized to give the TEA salt of compound **10** in mixture with TEAB salt. Desalting by ion-exchange chromatography (followed by lyophilization) afforded compound **10** as a brown amorphous powder (46 mg, yield 50%). IR: ν 1727, 1588, 1557, 1495, 1330, 1190, 1149, 1102, 1033. ^{1}H NMR (300 MHz, DMSO-d_6): δ 9.23 (s, 1H), 8.45 (m, 3H), 8.33 (s, 1H, O$\underline{H}$), 8.18 (d, 1H, J = 9.0 Hz), 8.11 (d, 1H, J = 9.0 Hz), 8.10 (m, 2H), 7.91 (t, 1H, J = 9.0 Hz), 7.82 (t, 1H, J = 9.0 Hz), 7.57 (t, 1H, J = 9.0 Hz), 7.47 (t, 1H, J = 9.0 Hz), 7.16 (d, 1H, J = 9.0 Hz), 5.02 (m, 2H), 2.68 (m, 2H), 2.50 (m, 2H, masked by DMSO signal). *Twisting and bending molecular motions of such hemicyanine dye in solution prevent the recording of a good quality ^{13}C NMR spectrum even for a reasonable sample concentration of 20 mg/mL.* MS (ESI, positive mode): m/z = 577.20 $[M + H]^+$, calcd for $C_{17}H_9NO_4S$ 576.05. HPLC (system C): t_R = 26.4 min, 99%.

2.2.8. 7-Hydroxycoumarin-hemicyanine dye (**11**)

To a solution of aldehyde **9** (30 mg, 93 μmol, 1 equiv) in dry EtOH (3 mL), *N*-sulfopropyl-4-methylpyridine **D** (60 mg, 279 μmol, 3 equiv) and pyrrolidine (24 μL, 279 μmol, 3 equiv) were sequentially added and the resulting reaction mixture was stirred at rt under an Ar atmosphere for 2 h. The reaction was checked for completion by RP-HPLC (system C), the mixture was concentrated and the resulting residue was purified by semi-preparative RP-HPLC (system P). The product-containing fractions were thrice lyophilized to give the TEA salt of **11** as a red amorphous solid (25 mg, yield 44%). IR: ν 1720, 1641, 1603, 1565, 1499, 1328, 1185, 1037. ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.98 (s, 1H), 8.84 (d, 2H, J = 9.0 Hz), 8.13 (m, 3H), 8.05 (d, 2H, J = 9.0 Hz), 7.98 (d, 1H, J = 9.0 Hz), 7.74 (d, 1H, J = 9.0 Hz), 7.51 (t, 1H, J = 9.0 Hz), 7.40 (t, 1H, J = 9.0 Hz), 6.76 (d, 1H, J = 9.0 Hz), 4.62 (t, 2H, J = 9.0 Hz), 3.10 (q, 2H, 1 × N–C$\underline{H}_2$–CH_3, 0.33 × TEA), 2.50 (m, 2H, masked by DMSO signal), 2.23 (m, 2H) 1.19 (t, 3H, 1 × N–CH_2–C$\underline{H}_3$, 0.33 × TEA). ^{13}C NMR (126 MHz, DMSO-d_6): δ 162.0, 160.3, 157.5, 155.2, 152.4, 143.6, 142.3, 135.1, 133.2, 132.1, 126.1, 124.0, 122.5, 121.9, 121.3, 109.8, 57.9, 47.1, 45.8 (N–C$\underline{H}_2$–CH_3, TEA), 27.3, 8.73 (N–CH_2–C$\underline{H}_3$, TEA), five carbons are missing despite long acquisition time on a 500 MHz spectrometer. MS (ESI, negative mode): m/z = 519.00 $[M - H]^-$, calcd for $C_{26}H_{20}N_2O_6S_2$ 520.08. HPLC (system B): t_R = 25.7 min, purity = 98%.

2.2.9. 3-Benzothiazolyl-8-formyl-7-hydroxycoumarin 6-sulfonic acid (**12**)

To a suspension of 3-benzothiazolyl-7-hydroxycoumarin 6-sulfonic acid **1** (100 mg, 0.24 mmol, 1 equiv) in CH_2Cl_2 (50 mL), 5 mL of an aq. solution of tetrabutylammonium hydroxide (40%, 1.5 M) was added and the resulting mixture was stirred at rt for 5 min. TBA^+ salt of **1** was extracted with CH_2Cl_2 and the resulting organic phase was dried over anhydrous Na_2SO_4 and evaporated to dryness. The TBA^+ salt of **1** was next dissolved in TFA (2 mL), hexamine (74 mg, 0.54 mmol, 2.2 equiv) was added and the resulting reaction mixture was stirred at reflux for 48 h. Thereafter, aq. 1.0 M HCl (30 mL) was added and the mixture was stirred for a further 3 h. Then, the mixture was concentrated to dryness and the crude product was subjected to an automated flash purification on a RP-C_{18} cartridge (system J). The product-containing fractions were lyophilized to give the TFA salt of compound **12** (in mixture with TBA^+ salts) as a yellow amorphous solid (32 mg). For spectroscopic characterizations, a sample (17 mg) was desalted by ion-exchange chromatography (followed by lyophilization) to afford aldehyde **12** as a yellow amorphous powder (4 mg, yield 7%). IR: ν 1736, 1647, 1596, 1484, 1383, 1192, 1138, 1045. ^{1}H NMR (300 MHz, DMSO-d_6): δ 10.47 (s, 1H), 9.25 (s, 1H), 8.50 (s, 1H), 8.16 (d, 1H, J = 9.0 Hz), 8.08 (d, 1H, J = 9.0 Hz), 7.56 (t, 1H, J = 9.0 Hz), 7.47 (t, 1H, J = 9.0 Hz). ^{13}C NMR (75 MHz, DMSO-d_6): δ 186.9, 160.9, 159.8, 158.9, 154.8, 151.0, 142.4, 135.8, 134.3, 130.0, 126.7, 125.3, 122.5, 122.2, 116.5, 111.1, 110.6. MS (ESI, negative mode): m/z = 402.07 $[M - H]^-$, calcd for $C_{17}H_9NO_7S_2$ 402.98. HPLC (system A): t_R = 24.5 min, purity = 95%.

2.2.10. 7-Hydroxycoumarin-hemicyanine dye (**13**)

To a solution of sulfonated aldehyde **12** (15 mg, 29.1 μmol, 1 equiv, contaminated with TBA^+ salts, *vide supra*) in dry EtOH (1 mL), *N*-sulfopropyl-2-methylbenzothiazole **C** (21 mg, 74.4 μmol, 2.6 equiv) and pyrrolidine (6 μL, 74.4 μmol, 2.6 equiv) were sequentially added and the resulting reaction mixture was stirred at rt under an Ar atmosphere for 2 h. The reaction was checked for completion by RP-HPLC (system C), the mixture was concentrated and the resulting residue was purified by semi-preparative RP-HPLC (system I). The product-containing fractions were twice lyophilized to give the TEA salt of compound **13** as a black

Please cite this article in press as: Roubinet B, et al., New insights into the water-solubilization of thiol-sensitive fluorogenic probes based on long-wavelength 7-hydroxycoumarin scaffolds, Dyes and Pigments (2014), http://dx.doi.org/10.1016/j.dyepig.2014.02.004

amorphous solid (20 mg, overall yield for the two steps 23%). IR: ν 1704, 1599, 1555, 1511, 1482, 1421, 1323, 1270, 1159, 1119, 1016. ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.85 (s, 1H), 8.74 (d, 1H, J = 15.0 Hz), 8.38 (d, 1H, J = 15.0 Hz), 8.27 (m, 2H), 8.10 (d, 1H, J = 9.0 Hz), 7.99 (m, 2H), 7.76 (t, 1H, J = 9.0 Hz), 7.65(t, 1H, J = 9.0 Hz), 7.50 (t, 1H, J = 9.0 Hz), 7.38 (t, 1H, J = 9.0 Hz), 4.82 (m, 2H), 3.10 (q, 12H, 6 × N–C$\underline{H}_2$–CH$_3$, 2 × TEA), 2.72 (m, 2H), 2.22 (m, 2H), 1.19 (t, 18H, 6 × N–CH$_2$–C$\underline{H}_3$, 2 × TEA). *Twisting and bending molecular motions of such hemicyanine dye in solution prevent the recording of a good quality ^{13}C NMR spectrum even for a reasonable sample concentration of 20 mg/mL*. MS (ESI, negative mode): m/z = 654.73 [M − H]$^-$ calcd for $C_{17}H_9NO_4S$ 656.01. HPLC (system C): t_R = 24.8 min, purity = 96%.

2.3. Synthesis of water-soluble thiol-sensitive fluorogenic probes

2.3.1. 2,4-Dinitrobenzenesulfonyl ester (**14**)

To a solution of 3-benzothiazolyl-7-hydroxycoumarin (120 mg, 0.39 mmol, 1 equiv) in dry DMF (20 mL), 2,6-lutidine (140 μL, 1.22 mmol, 3.1 equiv) and 2,4-dinitrobenzenesulfonyl chloride (DNBS-Cl, 163 mg, 6.10 mmol, 1.6 equiv) were sequentially added and was the resulting reaction mixture was stirred at 120 °C for 18 h. Thereafter, the mixture was evaporated to dryness. The resulting residue was dissolved in EtOAc, washed with deionized water (50 mL), dried over anhydrous Na_2SO_4 and concentrated. The crude product was purified by flash column chromatography on silica gel using CH_2Cl_2 as the eluent to afford the desired DNBS ester **14** as a yellow solid (23.5 mg, yield 12%). IR: ν 1722, 1609, 1534 (NO_2), 1405, 1388, 1347 (NO_2), 1119, 1111. ^{1}H NMR (300 MHz, DMSO-d_6): δ 9.28 (s, 1H), 9.14 (s, 1H), 8.63 (d, 1H, J = 9.0 Hz), 8.35 (d, 1H, J = 9.0 Hz), 8.18 (m, 2H), 8.10 (d, 1H, J = 9.0 Hz), 7.60 (t, 1H, J = 9.0 Hz), 7.53 (m, 2H), 7.31 (d, 1H, J = 9.0 Hz). ^{13}C NMR (75 MHz, DMSO-d_6): δ 159.4, 158.9, 153.9, 151.9, 151.7, 150.9, 148.1, 140.9, 136.0, 133.6, 132.1, 130.7, 127.7, 126.8, 125.7, 122.6, 122.3, 121.3, 120.2, 119.1, 118.8, 110.5. MS (ESI, positive mode): m/z = 526.00 [M + H]$^+$, calcd for $C_{22}H_{11}N_3O_9S_2$ 524.99. HPLC (system A): t_R = 36.5 min, purity = 99%.

2.3.2. 6-Sulfonated 2,4-dinitrobenzenesulfonyl ester (**15**)

To a solution of 3-benzothiazolyl-7-hydroxycoumarin 6-sulfonic acid **1** (50 mg, 0.12 mmol, 1 equiv) in dry pyridine (2 mL), DMAP (10 mg, 0.066 mmol, 0.55 equiv) and DNBS-Cl (107 mg, 0.40 mmol, 3.3 equiv) were sequentially added and the resulting reaction mixture was stirred at 50 °C for 24 h. Thereafter, the crude mixture was concentrated and purified by semi-preparative RP-HPLC (system K). The product-containing fractions were lyophilized to give the TFA salt of sulfonated DNBS ester **15** as a yellow amorphous solid (13 mg, yield 15%). IR: ν 1725, 1599, 1538 (NO_2), 1478, 1346 (NO_2), 1272, 1245, 1191, 1150, 1065. ^{1}H NMR (300 MHz, DMSO-d_6): δ 9.33 (s, 1H), 8.87 (s, 1H), 8.52 (s, 1H), 8.43 (d, 1H, J = 9.0 Hz), 8.20 (d, 1H, J = 9.0 Hz), 8.13 (d, 1H, J = 9.0 Hz), 7.60 (t, 1H, J = 9.0 Hz), 7.53 (m, 2H), 7.08 (d, 1H, J = 9.0 Hz). ^{13}C NMR (75 MHz, DMSO-d_6): δ 159.6, 159.1, 155.6, 154.5, 153.7, 152.0, 141.7, 141.2, 139.1, 137.9, 136.0, 130.8, 129.1, 126.8, 125.5, 122.7, 122.3, 121.5, 120.3, 119.3, 116.6, 110.45. MS (ESI, negative mode): m/z = 540.07 [M − H]$^-$ and 1080.53 [2M − H]$^-$, calcd for $C_{22}H_{11}N_3O_{12}S_3$: 604.95, *a non-identified side-reaction occurred within the ESI probe and led to a loss of 64 Da*. HPLC (system A): t_R = 28.7 min, purity = 98%.

2.3.3. Iminodiacetic acid 2,4-dinitrobenzenesulfonyl ester (**16b**)

*2.3.3.1. Di-tert-butyl iminodiacetate 2,4-dinitrobenzenesulfonyl ester (**16a**).* To a solution of 7-hydroxycoumarin derivative **3a** (46 mg, 83 μmol, 1 equiv) in dry CH_2Cl_2 (10 mL), 2,6-lutidine (100 μL, 830 μmol, 10 equiv) and DNBS-Cl (77 mg, 290 μmol, 3.5 equiv) were sequentially added and the resulting reaction mixture was stirred at rt for 18 h. Thereafter, the crude mixture was evaporated to dryness. The resulting residue was dissolved in EtOAc, washed with deionized water (50 mL), dried over anhydrous Na_2SO_4 and concentrated. The crude product was subjected to an automated flash purification on a Biotage® SNAP KP-SIL (10 g) cartridge and using a mixture of cyclohexane/EtOAc as eluents (step gradient from 1:0 to 3:2) to afford DNBS ester **16a** as a yellow solid (30 mg, yield 46%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 9.01(s, 1H), 8.71 (s, 1H), 8.62 (s, 1H), 8.08 (d, 1H, J = 9.0 Hz), 7.98 (d, 1H, J = 9.0 Hz), 7.69 (d, 1H, J = 9.0 Hz), 7.54 (t, 1H, J = 9.0 Hz), 7.42 (m, 2H), 7.26 (s, 1H), 4.12 (s, 2H), 3.46 (s, 4H), 1.38 (s, 18H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 170.4, 159.2, 158.8, 153.7, 152.6, 151.2, 151.1, 149.1, 140.5, 137.0, 134.3, 134.2, 129.6, 127.2, 126.8, 125. 8, 123.2, 121.9, 120.7, 118.9, 118.3, 81.3, 55.9, 47.2, 28.2. MS (ESI, positive mode): m/z = 805.07 [M + Na]$^+$, calcd for $C_{35}H_{34}N_4O_{13}S_2$: 782.16.

2.3.3.2. Acid removal of tert-butyl esters. Di-*tert*-butyl ester **16a** (46 mg, 58.8 μmol, 1 equiv) was dissolved in a mixture of TFA/CH_2Cl_2 (1:1, v/v, 20 mL) and the resulting reaction mixture was stirred at reflux for 2 h. Thereafter, the deprotection mixture was concentrated to dryness to give the TFA salt of compound **16b** as a brown amorphous solid (39 mg, yield 85%). IR: ν 1733, 1602, 1542 (NO_2), 1477, 1348 (NO_2), 1185, 1067, 1000. ^{1}H NMR (300 MHz, acetone-d_6): δ 9.50 (s, 2H, 2 × $CO_2\underline{H}$), 9.16 (s, 1H), 9.01(s, 1H), 8.80 (d, 1H, J = 9.0 Hz), 8.66 (d, 1H, J = 9.0 Hz), 8.10 (m, 3H), 7.58 (t, 1H, J = 9.0 Hz), 7.47 (m, 2H), 4.33 (s, 2H), 3.73 (s, 4H). ^{13}C NMR (75 MHz, acetone-d_6): δ 172.3, 160.2, 159.4, 154.6, 153.5, 152.8, 152.0, 149.7, 141.8, 137.6, 134.9, 133.6, 131.8, 128.7, 127.5, 126.5, 123.8, 122.0, 121.2, 120.1, 119.6, 119.6, 114.3, 55.9, 47.9. MS (ESI, negative mode): m/z = 668.67 [M − H]$^-$ and 782.53 [M + TFA − H]$^-$, calcd for $C_{27}H_{18}N_4O_{13}S_2$: 670.03. HPLC (system B): t_R = 20.0 min, purity = 97%.

2.3.4. 2,4-Dinitrophenyl ether (**18**)

To a solution of 3-benzothiazolyl-7-hydroxycoumarin (30 mg, 0.09 mmol, 1 equiv) in dry DMF (1 mL), anhydrous K_2CO_3 (35 mg, 0.254 mmol, 2.8 equiv) was added and the mixture was stirred at rt under an Ar atmosphere for 5 min. Then, 1-fluoro-2,4-dinitrobenzene (DNBF, 51 μL, 0.407 mmol, 4.5 equiv) was added and the reaction mixture was stirred at rt overnight. Thereafter, the crude mixture was evaporated to dryness. The resulting residue was dissolved in EtOAc and washed with deionized water, dried over anhydrous Na_2SO_4 and evaporated to dryness. The resulting crude product was purified by flash column chromatography on silica gel using a mixture of cyclohexane and CH_2Cl_2 as eluents (step gradient from 1:0 to 0:1) to give DNP ether **18** as a yellow solid (25 mg, yield 48%). IR: ν 1724, 1594, 1524 (NO_2), 1474, 1342 (NO_2), 1276, 1197, 1119. ^{1}H NMR (300 MHz, DMSO-d_6): δ 9.31 (s, 1H), 8.96 (d, 1H, J = 9.0 Hz), 8.55 (dd, 1H, J = 9.0 Hz, J = 3.0 Hz), 8.22 (m, 2H), 8.10 (d, 1H, J = 9.0 Hz), 7.60 (t, 1H, J = 9.0 Hz), 7.51 (m, 3H), 7.37 (dd, 1H, J = 9.0 Hz, J = 3.0 Hz). ^{13}C NMR (75 MHz, DMSO-d_6): δ 159.7, 159.2, 158.4, 154.8, 153.0, 151.9, 142.9, 141.5, 140.4, 135.9, 132.5, 130.0, 126.8, 125.5, 122.5, 122.3, 122.1, 122.0, 118.5, 116.6, 116.4, 107.0. MS (ESI, positive mode): m/z = 462.13 [M + H]$^+$, calcd for $C_{22}H_{11}N_3O_7S$ 461.03. HPLC (system A): t_R = 34.5 min, purity = 96%.

2.3.5. 6-Sulfonated 2,4-dinitrophenyl ether (**19**)

To a solution of 3-benzothiazolyl-7-hydroxycoumarin 6-sulfonic acid **1** (30 mg, 73 μmol, 1 equiv) in dry DMF (1 mL), anhydrous K_2CO_3 (30 mg, 200 μmol, 2.8 equiv) was added and the mixture was stirred at rt under an Ar atmosphere for 5 min. Then, DNBF (40 μL, 320 μmol, 4.5 equiv) was added and the reaction mixture was stirred at 50 °C for 2 h. Thereafter, the crude mixture was concentrated and the resulting residue was directly purified by semi-preparative RP-HPLC (system O). The product-containing fractions were lyophilized to give the TFA salt of sulfonated DNP ether **19** as a yellow amorphous solid (14 mg, yield 30%). IR: ν 1732,

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1600, 1536 (NO_2), 1470, 1409, 1345 (NO_2), 1253, 1120, 1076. ^{1}H NMR (300 MHz, DMSO-d_6): δ 9.31(s, 1H), 8.86 (d, 1H, J = 3.0 Hz), 8.51 (s, 1H), 8.42 (dd, 1H, J = 9.0 Hz, J = 3.0 Hz), 8.19 (d, 1H, J = 9.0 Hz), 8.11 (t, 1H, J = 9.0 Hz), 7.54 (m, 3H), 7.10 (d, 1H, J = 9.0 Hz). ^{13}C NMR (75 MHz, DMSO-d_6): δ 159.6, 159.2, 155.6, 154.6, 153.7, 152.0, 141.7, 141.3, 139.1, 137.8, 136.0, 130.9, 129.2, 126.8, 125.6, 122.7, 122.3, 121.6, 120.4, 119.4, 116.6, 110.5. MS (ESI, negative mode): m/z = 540.00 $[M - H]^-$ and 1080.53 $[2M-H]^-$, calcd for $C_{22}H_{11}N_3O_{10}S_2$ 540.99. HPLC (system A): t_R = 28.1 min, purity = 98%.

*2.3.6. 6-Sulfonated 4-cyano 2,4-dinitrophenyl ether (**20**)*

To a solution of 3-benzothiazolyl-4-cyano-7-hydroxycoumarin-6-sulfonic acid **2** (30 mg, 59 μmol, 1 equiv) in dry DMF (1 mL), anhydrous K_2CO_3 (31 mg, 225 μmol, 3.8 equiv) was added and the mixture was stirred at rt under an Ar atmosphere for 5 min. DNBF (37 μL, 300 μmol, 5.1 equiv) was added and the reaction mixture was stirred at 50 °C for 5 h. Thereafter, the crude mixture was concentrated and directly purified by semi-preparative RP-HPLC (system Q). The product-containing fractions were lyophilized to give the TFA salt of DNP ether **20** as a yellow amorphous solid (7 mg, yield 18%). IR: ν 2118 (CN), 1738, 1596, 1538 (NO_2), 1476, 1348 (NO_2), 1267, 1202, 1090, 1021.^{1}H NMR (300 MHz, DMSO-d_6): δ 8.89 (d, 1H, J = 3.0 Hz), 8.48 (m, 2H), 8.28 (d, 1H, J = 9.0 Hz), 8.21(d, 1H, J = 9.0 Hz), 7.63 (m, 3H), 7.11 (d, 1H, J = 9.0 Hz). ^{13}C NMR (75 MHz, DMSO-d_6): δ 158.2, 157.0, 155.1, 154.4, 153.3, 151.4, 141.7, 139.4, 138.1, 136.8, 129.4, 127.6, 127.3, 126.9, 125.1, 123.6, 122.4, 121.7, 120.6, 120.0, 114.6, 113.7, 110.8. MS (ESI, negative mode): m/z = 565.00 $[M - H]^-$ and 1130.73 $[2M - H]^-$, calcd for $C_{23}H_{10}N_4O_{10}S_2$ 565.98. HPLC (system A): t_R = 28.8 min, purity = 99%.

*2.3.7. N-Sulfopropylpyridinium 2,4-dinitrophenyl ether (**21**)*

To a solution of TEA salt of 7-hydroxycoumarin-hemicyanine dye **11** (20 mg, 32 μmol, 1 equiv) in dry DMF (1 mL), anhydrous K_2CO_3 (15 mg, 113 μmol, 3.5 equiv) was added and the mixture was stirred at rt under an Ar atmosphere for 5 min. DNBF (24 μL, 129 μmol, 4.0 equiv) was added and the reaction mixture was stirred at rt for 5 h. Thereafter, the crude mixture was concentrated and directly purified by semi-preparative RP-HPLC (system Q). The product-containing fractions were lyophilized to give the TFA salt of DNP ether **21** as a yellow amorphous solid (10 mg, yield 39%). IR: ν 1735, 1612, 1586, 1536 (NO_2) 1474, 1347 (NO_2), 1258, 1197, 1035. ^{1}H NMR (300 MHz, DMSO-d_6): δ 9.35 (s, 1H), 9.00 (m, 3H), 8.57 (d, 1H, J = 9.0 Hz), 8.33 (d, 2H, J = 9.0 Hz), 8.24 (m, 2H), 8.12 (d, 1H, J = 9.0 Hz), 8.05 (d, 1H, J = 16.0 Hz), 7.86 (d, 1H, J = 16.0 Hz), 7.63 (t, 1H, J = 9.0 Hz), 7.49 (m, 2H), 7.35 (d, 1H, J = 9.0 Hz), 4.68 (m, 2H), 2.50 (m, 2H, masked by DMSO signal), 2.22 (m, 2H). *Twisting and bending molecular motions of such hemicyanine dye in solution prevent the recording of a good quality ^{13}C NMR spectrum even for a reasonable sample concentration of 10 mg/mL.* MS (ESI, positive mode): m/z = 687.20 $[M + H]^+$, calcd for $C_{32}H_{22}N_4O_{10}S_2$ 686.08. HPLC (system A): t_R = 29.4 min, purity = 94%.

*2.3.8. 6-Sulfonated quinone methyl ether (**22b**)*

*2.3.8.1. 6-Sulfonated 2,5-dimethoxy-3,4,6-trimethylbenzyl ether (**22a**).* To a solution of 3-benzothiazolyl-7-hydroxycoumarin-6-sulfonic acid **1** (50 mg, 0.12 mmol, 1 equiv) in dry DMF (3 mL), anhydrous K_2CO_3 (55 mg, 0.40 mmol, 3.3 equiv) was added and the mixture was stirred at rt for 20 min. Thereafter, 1-chloromethyl-2,5-dimethoxy-3,4,6-trimethylbenzene **E** (36 mg, 0.32 mmol, 2.7 equiv) and KI (33 mg, 0.20 mmol, 1.7 equiv) were sequentially added and the resulting reaction mixture was stirred at rt for 24 h. Thereafter, the crude mixture was evaporated to dryness and directly purified by semi-preparative RP-HPLC (system L). The product-containing fractions were lyophilized to give the TFA salt of benzyl ether **22a** as a yellow amorphous solid (19 mg, yield 23%). ^{1}H NMR (300 MHz, DMSO-d_6): δ 9.16 (s, 1H), 8.31 (s, 1H), 8.16 (d, 1H, J = 9.0 Hz), 8.07 (d, 1H, J = 9.0 Hz), 7.56 (t, 1H, J = 9.0 Hz), 7.45 (m, 2H), 5.20 (s, 2H), 3.68 (s, 3H), 3.59 (s, 3H), 2.27 (s, 3H), 2.17 (s, 6H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 160.8, 160.3, 159.8, 156.0, 153.4, 152.6, 152.0, 142.8, 135.7, 134.5, 131.4, 130.3, 130.0, 127.4, 126.6, 125.2, 125.4, 122.1, 115.7, 110.7, 100.1, 64.0, 62.1, 59.9, 12.8, 12.4, 11.7. MS (ESI, positive mode): m/z = 567.87 $[M + H]^+$, calcd for $C_{28}H_{25}NO_8S_2$: 567.10. HPLC (system A): t_R = 29.7 min, purity = 98%.

2.3.8.2. CAN-mediated removal of methoxy groups. To a solution of dimethoxy-benzene derivative **22a** (25 mg, 37 μmol, 1 equiv) in a mixture of CH_2Cl_2/CH_3CN (2:1, v/v, 0.75 mL), cerium ammonium nitrate (CAN, 50 mg, 85 μmol, 2.3 equiv) was added and the resulting reaction mixture was stirred at rt for 2 h. Further amounts of CAN (2 × 50 mg) was added after 2 h and 4 h of stirring. The reaction was checked for completion by RP-HPC (system A) and volatiles were evaporated to dryness. The crude product was purified by semi-preparative RP-HPLC (system M). The product-containing fractions were lyophilized to give the TFA salt of **22b** as a yellow amorphous solid (7 mg, yield 30%). IR: ν 1720, 1607, 1555 (NO_2), 1365 (NO_2), 1292, 1253, 1226, 1173. ^{1}H NMR (300 MHz, DMSO-d_6): δ 9.17 (s, 1H), 8.30 (s, 1H), 8.17 (d, 1H, J = 9.0 Hz), 8.08 (d, 1H, J = 9.0 Hz), 7.57 (t, 2H, J = 9.0 Hz), 7.46 (t, 2H, J = 9.0 Hz), 5.13 (s, 2H), 2.15 (s, 3H), 2.02 (s, 3H), 2.00 (s, 3H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 187.0, 185.4, 160.2, 160.2, 159.8, 155.7, 152.0, 146.1, 142.7, 140.7, 139.9, 136.0, 135.7, 134.7, 130.0, 126.6, 125.2, 122.4, 122.1, 116.1, 111.1, 100.3, 61.6, 12.3, 12.2, 12.2. MS (ESI, negative mode): m/z = 535.80 $[M - H]^-$ and 649.33 $[M + TFA - H]^-$, calcd for $C_{26}H_{19}NO_8S_2$:537.06. HPLC (system A): t_R = 28.4 min, purity = 97%.

*2.3.9. 6-Sulfonated quinone "trimethyl lock" carbamate (**23**)*

To a pre-cooled mixture (0 °C) of phosgene (~20% w/v solution in toluene, 150 μL, 280 μmol, 3.3 equiv) and TEA (40 μL, 280 μmol, 3.3 equiv), a solution of *N*-methyl secondary amine **F** (90 mg, 280 μmol, 3.3 equiv) in dry toluene (1 mL) was added dropwise at 0 °C for 30 min and the resulting reaction mixture was stirred at rt for 24 h. Thereafter, this chlorocarbonylation mixture was concentrated and the residue was re-dissolved in dry pyridine (2 mL). Then, a solution of 3-benzothiazolyl-7-hydroxycoumarin-6-sulfonic acid **1** (35 mg, 85 μmol, 1 equiv) in dry pyridine (1 mL) was added dropwise at rt. The resulting reaction mixture was stirred for 24 h. After evaporation to dryness, the crude product was purified by semi-preparative RP-HPLC (system N). The product-containing fractions were lyophilized to give the TFA salt of carbamate **23** as a yellow amorphous solid (7 mg, yield 10%). IR: ν 1721, 1644, 1611, 1555 (NO_2), 1405, 1238 (NO_2), 1162, 1081, 1022. ^{1}H NMR (300 MHz, DMSO-d_6): δ 9.25 (s, 1H), 8.37 (s, 1H), 8.20 (d, 1H, J = 9.0 Hz), 8.12 (d, 1H, J = 9.0 Hz), 7.59 (t, 2H, J = 9.0 Hz), 7.49 (t, 2H, J = 9.0 Hz), 7.35 (s,1H), 3.70 (s, 2H), 3.57 (s, 2H), 3.42 (m, 1H), 3.32 (m, 1H), 3.18 (s, 1H), 3.02 (m, 3H), 2.87 (s, 2H), 2.79 (s, 1H), 2.73 (s, 1H), 2.05 (m, 3H), 1.86 (m, 6H), 1.33 (m, 6H). *Not enough product to record a good quality ^{13}C NMR spectrum.* MS (ESI, negative mode): m/z = 720.13 $[M - H]^-$ and 1440.47 $[2M - H]^-$, calcd for $C_{35}H_{35}N_3O_{10}S_2$: 721.18. HPLC (system A): t_R = 30.8 min, purity = 92%.

2.4. General procedure for in vitro thiolysis of fluorogenic probes – fluorescence assay

Stock solutions (1.0 mg/mL) of water-soluble pro-fluorophores **15**, **16b**, **17b** and **19-23** were prepared in H_2O/DMSO (9:1, v/v) whereas reference thiol probes **14** and **18** were dissolved in DMSO (1.0 mg/mL). Stock solutions (10 mg/mL) of analytes (Cys, NaThioPi, K_3PO_4 and 4-chlorothiophenol) were prepared in ultrapure water except for 4-chlorothiophenol (in CH_3CN). A micromolar solution

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(for each thiol probe) was obtained by dilution of stock solution with PB or PB/DMSO. Depending on the reactivity of the probe (*i.e.*, thiolysis kinetics) and the fluorescence efficiency of the released fluorophore, a concentration in the range from 2.2 μM to 8.6 μM was used. 3 mL of this solution was transferred into a quartz fluorescence cell (Varian, fluorescence cell, Open Top, 10 × 10 mm, 3.5 mL) and thermostated at 25 °C. The required number of equivalents of analyte (2, 5, 50 or 250 equiv) was added and the resulting mixture was homogenized through magnetic stirring for 2 min. The fluorescence emission of the released fluorophore was monitored at the suitable wavelength (λ = 490, 600 or 655 nm, emission slit = 5 nm, upon excitation at λ = 390, 510 or 500, excitation slit = 5 nm; excitation/emission filters: auto) over time with measurements recorded every 1 s.

3. Results and discussion

3.1. Synthesis of water-soluble 3-benzothiazolyl-7-hydroxycoumarin derivatives

In the early 1980s, the Wolfbeis group was interested in the synthesis and photophysical characterization of a series of 3-substituted 7-hydroxycoumarin derivatives with the aim of shifting the excitation and emission maxima of umbelliferone to longer wavelengths and simultaneously lowering the pKa value of its phenol moiety [25]. Of all the electron-withdrawing substituents explored, 2-benzothiazolyl group was identified as a promising molecular unit to achieve these ambitious goals (as inferred from the spectral features under simulated physiological conditions: Abs/Em max. 431/488 nm, Φ_F = 43% [40], pKa = 7.0 *vs.* Abs/Em max. 326/452 nm, Φ_F = 76%, pKa = 7.7 for umbelliferone [38]). Consequently, 3-benzothiazolyl-7-hydroxycoumarin has emerged as a valuable alternative to 7-hydroxycoumarin, and is frequently used as a fluorogenic dye in various (bio)sensing applications [29,30,41–44]. Although the pKa value of this phenolic fluorophore allows a high solubility in various alkaline aq. buffers (pH 8–10), it is surprising that little attention has been paid to the synthesis of hydrophilic analogues which exhibit a much higher water-solubility even if their 7-OH group is masked. This is particularly interesting to facilitate the design of phenol-based fluorogenic probes (*e.g.*, thiol-imaging agents derived from 7-hydroxycoumarins) readily soluble in biological media whatever the pH and without using an organic co-solvent. Contrary to 7-*N*,*N*-dialkylaminocoumarin derivatives, few synthetic methods aimed at introducing various polar or ionizable groups at specific positions of a 7-hydroxycoumarin scaffold have been reported in the literature. Particularly noteworthy, some umbelliferones whose 3-position is functionalized with a carboxyl moiety or the 4-position is substituted by an acetic acid arm or a α-sulfo-β-alanyl linker have been synthesized but these strategies cannot be applied to 3-heteroaryl-7-hydroxycoumarins [45–48]. There is clearly a need to explore alternative synthetic accesses to decorate the 3-substituted coumarin ring systems with one or several hydrophilic substituents. Thus, we decided to explore three different approaches to achieve this: (1) sulfonation of the 6-position through electrophilic aromatic substitution [33], (2) Mannich-type reaction to functionalize the 8-position with an aminomethyl arm derived from a hydrophilic secondary amine (*i.e.*, iminodiacetic acid, sarcosine, and isonipecotic acid) [49,50] optionally followed by a post-amidification reaction of the carboxylic acid function with 2-aminoethane-1,1-disulfonic acid **B** [32,35], and (3) Knoevenagel-type reaction between an aldehyde functionality pre-introduced in the 8-position and a sulfobetain derivative of 2- or 4-methyl-azaheterocyle (*N*-sulfopropyl-2-methylbenzothiazole **C** or -4-methylpyridine **D**) [51]. In addition to impart water-solubility of 3-benzothiazolyl-7-hydroxycoumarin, this latter approach will lead to a dramatic red-shift of its spectral features due to the extension of the aromatic π-system. For the two first water-solubilizing methodologies, a further chemical modification of the coumarin core, namely cyanation of 4-position, was also considered in order to obtain a further 100 nm red-shift in emission maximum of 3-benzothiazolyl-7-hydroxycoumarin. The practical implementation of these functionalization methods has led to eleven different water-soluble 3-benzothiazolyl-7-hydroxycoumarin derivatives, and is summarized in Scheme 1 (for aromatic sulfonation and Mannich approach) and Scheme 2 (for Knoevenagel approach). First, 6-sulfonated derivative **1** was readily synthesized from 5-formyl-2,4-dihydroxybenzenesulfonic acid and benzothiazole-2-acetonitrile according to a two-step protocol previously reported in the literature [27,28,33]. Compound **1** was next used for the preparation of water-soluble 4-cyano derivative **2** through Michael-type addition of cyanide anion on C3–C4 double bond and subsequent re-aromatization with DDQ [52]. The 6-sulfonated coumarin **2** was readily purified by semi-preparative RP-HPLC and recovered in a pure form with a satisfying 45% yield. Concerning the synthesis of 8-substituted derivatives **3b** and **4b**, aminomethylation reactions were carried out according to a protocol initially developed for the grafting of the bis(carboxymethyl)aminomethyl moiety onto 3-unsubstituted 7-hydroxycoumarins [50]. Thus, 3-benzothiazolyl-7-hydroxycoumarin and its 4-cyano derivative were treated with an excess of freshly prepared Mannich reagent (*i.e.*, iminium salt resulting from the reaction between paraformaldehyde and di-*tert*-butyl iminodiacetate with a catalytic amount of KOH) in refluxing EtOH. The reactions were found not to be complete even after a prolonged time of heating and all attempts to isolate the resulting 8-*N*,*N*-dialkylamino coumarins **3a** and **4a** by silica or alumina column chromatography failed. Alternative purification over reversed-phase silica gel provided **3a** and **4a** in a pure form but with modest yields (24% and 18% respectively), partly explained by losses of material through non-specific adsorption phenomena over the chromatographic stationary phase. Subsequent treatment of **3a** and **4a** with a 50% solution of TFA in CH_2Cl_2 to remove *tert*-butyl groups, afforded the targeted 8-iminodiacetic acid derivatives **3b** and **4b**. Much better yields were obtained for the Mannich reactions involving iminium salts derived from sarcosine *tert*-butyl ester and ethyl isonipecotate. Furthermore, the alkaline reaction conditions led to the premature cleavage of alkyl ester moieties of these unusual amino acids and has enabled us to directly recover the free carboxylic acid derivatives **5** and **6** in 48% and 38% yield respectively. In order to further increase the water-solubility of these latter hydrophilic coumarins, we next considered the amidification of their added carboxylic acid functionality with a di-sulfonated amino linker derived from taurine [32,35]. Our first attempts involving the aminolysis of *N*-hydroxysuccinimidyl (NHS) esters of **5** and **6** with an excess of 2-aminoethane-1,1-disulfonic acid failed. Thus, we have chosen to convert the carboxylic acid of **5** and **6** into the more reactive acyl bromide derivatives by using PyBrOP/DIEA. Upon this acid pre-activation step, subsequent reaction with 2-aminoethane-1,1-disulfonic acid (tetrabutylammonium, TBA^+ salt) led to the desired di-sulfonated coumarins **7** and **8** which were purified by semi-preparative RP-HPLC (31% and 20% yields respectively).

The synthetic access to the last three targeted water-soluble 3-benzothiazolyl-7-hydroxycoumarin derivatives **10**, **11** and **13** has required the prior preparation of the 8-formyl derivatives **9** and **12** using the Duff reaction [51] (Scheme 2). This aromatic formylation was carried out under standard conditions which are not really effective for the 6-sulfonated derivative **1** (isolated yield 7% *vs.* 58% for 3-benzothiazolyl-7-hydroxycoumarin) even after improving its solubility in TFA/hexamine mixture by a counter-ion exchange process (TBA^+ instead of H^+). However, this latter sulfonated benzaldehyde

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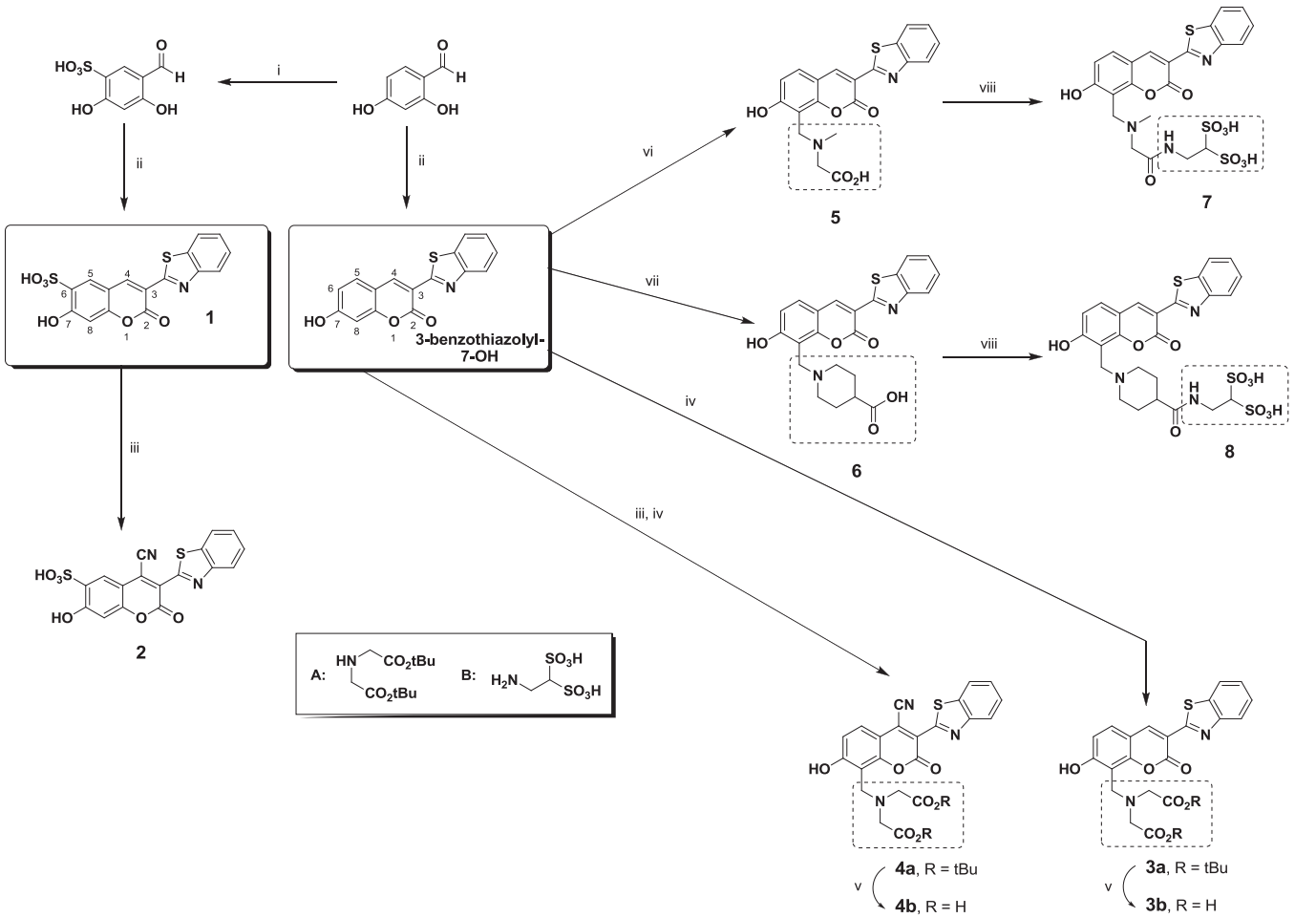

Scheme 1. Synthesis of water-soluble 3-benzothiazolyl-7-hydroxycoumarin derivatives through aromatic sulfonation and Mannich approach. *Reagents and conditions*: (i) oleum (6 equiv), 2,4-dihydroxybenzaldehyde (1 equiv), 0 °C, 30 min, then rt, 2 h, 54%; (ii) (a) benzothiazole-2-acetonitrile (1 equiv), piperidine (10 equiv), rt, 18 h, (b) HCl (6 M), reflux, 24 h, 71% (for the two steps); (iii) KCN (2.3 equiv), H_2O, DMF, 50 °C, 3 h, then DDQ (1.1 equiv), rt, 2 h, 48%; (iv) paraformaldehyde (6.8 equiv), **A** (3.4 equiv), KOH (0.5 equiv), EtOH, rt, 1 h, then reflux, 24 h, 24% (**3a**) and 18% (**4b**); (v) TFA, CH_2Cl_2, reflux, 2 h, 66% (**3b**) and 60% (**4b**); (vi) paraformaldehyde (7.2 equiv), sarcosine *tert*-butyl ester HCl salt (3.6 equiv), KOH (7.2 equiv), EtOH, reflux, 4 h, 48%; (vii) paraformaldehyde (6.8 equiv), ethyl isonipecotate (3.4 equiv), KOH (6.8 equiv), EtOH, reflux, 4 h, 43%; (viii) PyBrOP (3.2 equiv), DIEA (2.8 equiv), NMP, rt, 30 min, then TBA^+ salt of disulfonated amine **B** (3.8 equiv), DIEA (6.3 equiv), NMP, 0 °C then rt, 5 h, 31% (**7**) and 20% (**8**). *7-Hydroxycoumarin derivatives were isolated as HCl (3-benzothiazolyl-7-hydroxycoumarin and **1**) or TFA (**2**, **3b**, **4b**, **5** and **6**) salts, except for **7** and **8** (acid form, after Dowex H^+ desalting).*

was obtained in a sufficient amount to achieve the Konevenagel-type condensation reaction with *N*-sulfopropyl-2-methylbenzothiazole **C**. Experimental conditions currently used for the preparation of 7-*N*,*N*-dialkylamino- or 7-hydroxycoumarin-hemicyanine dyes (pyrrolidine in dry EtOH at rt) [53,54,51] were employed and led to water-soluble extended conjugated coumarins **10** and **13** readily purified by semi-preprative RP-HPLC (50% and 23% yields respectively). The less hydrophobic sulfobetain *N*-sulfopropyl-4-methylpyridine **D** was also subjected to the same base-catalysed condensation reaction with the 8-formyl derivative **9** to give a further water-soluble derivative of 3-benzothiazolyl-7-hydroxycoumarin (compound **11**, yield 44%).

Structures of these novel hydrophilic phenol-based fluorophores were confirmed by detailed measurements including ESI mass spectrometry and NMR analyses. Furthermore, the purity of each compound (determined through RP-HPLC analyses) was found to be equal or above to 95%, suitable for an accurate and reliable determination of their photophysical properties.

3.2. Photophysical properties of water-soluble 3-benzothiazolyl-7-hydroxycoumarin derivatives

Due to their excellent water-solubility (greater than 10 mM), the optical properties of these novel fluorophores were evaluated in phosphate buffer (pH 7.5) and compiled in Table 1. For some compounds, further measurements were achieved in DMSO, especially for assessing the differences in fluorescence quantum yield compared to an aq. medium, when the use of this organic co-solvent is required for fluorescence-based thiol detection assays involving the release of a coumarinic dye (*vide infra*). The first thing to note is that the introduction of the water-solubilizing moieties namely sulfonic acid and *N*,*N*-disubstituted aminomethyl onto the 6- or 8-position of the 3-benzothiazolyl-7-hydroxycoumarin scaffold has nearly no influence on absorption and (fluorescence) emission band positions (see entries 3, 7 and 9–12 compared to entry 1, and Fig. 1). Conversely, and as already reported for other 7-hydroxycoumarin derivatives [25,51], cyanation of the 4-position or grafting of a *N*-sulfopropyl azaheterocycle to the 8-position and through a dimethine chain, gave rise to dramatic red-shifts in absorption (63–109 nm) and emission (105–143 nm) maxima in aq. media (see entries 5, 8, 13, 15, 17 compared to entry 1, and Fig. 1). Moreover, and as foreseen, 6-sulfonic acid substituent and the *N*,*N*-disubstituted aminomethyl arms improve significantly the fluorescence efficiency of 3-benzothiazolyl-7-hydroxycoumarin scaffold in aq. media. Indeed, a 63% increase in fluorescence quantum yield was obtained for the 6-sulfonated fluorophore **1** whereas for the Mannich derivatives **3b** and **5**–**8**, increases ranged

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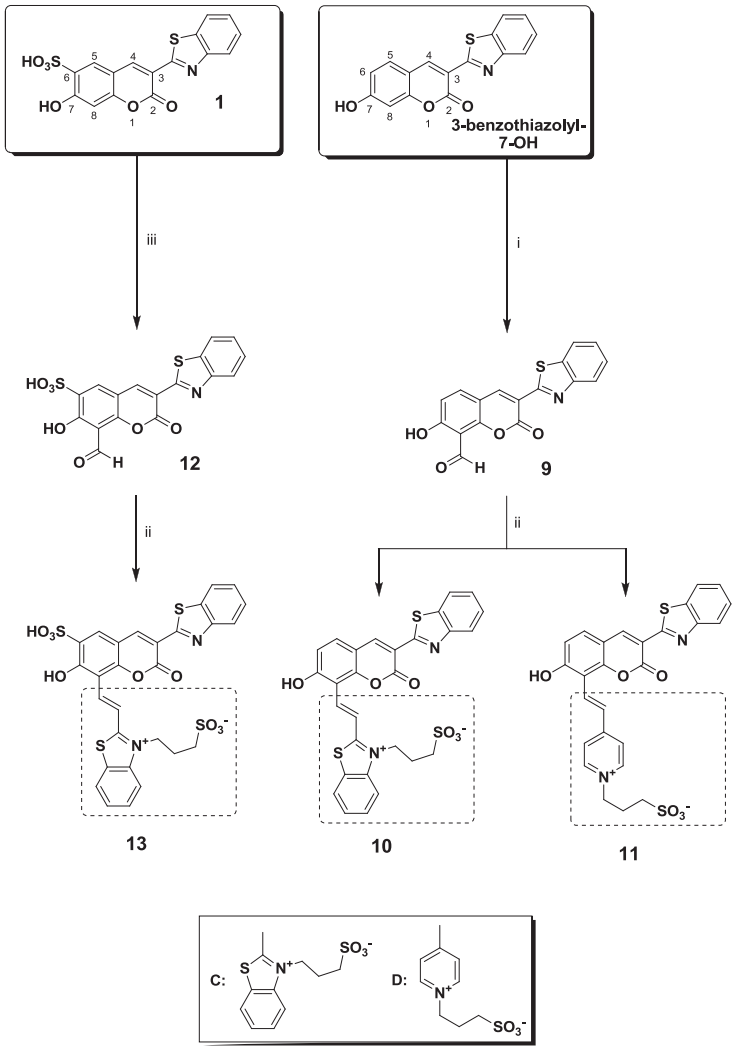

Scheme 2. Synthesis of water-soluble 3-benzothiazolyl-7-hydroxycoumarin derivatives through Knoevenagel approach. *Reagents and conditions*: (i) (a) hexamine (2.3 equiv), TFA, reflux, 24 h, (b) HCl (1 M), 3 h, rt, 58%; (ii) **C** or **D** (2–3 equiv), pyrrolidine (2–3 equiv), EtOH, rt, 2 h, 50% (**10**), 44% (**11**) and 23% (**13**, for the two steps ii–iii); (iii) (a) Bu_4NOH (1 equiv), H_2O, CH_2Cl_2, (b) hexamine (2.2 equiv), TFA, reflux, 48 h, (c) HCl (1 M), rt, 3 h. *7-Hydroxycoumarin derivatives were isolated as TEA salts (**11** and **13**) except for **12** (acid form, after Dowex H^+ desalting).*

from 44% to 151% were observed. For these latter 7-hydroxycoumarins, lowering the pKa of their phenol moiety through the formation of a strong hydrogen bond with the adjacent tertiary amino group that leads to an increase in the molar fraction of the emitting phenolate form, is generally accepted to assess their higher fluorescence intensity compared to the 8-unsubstituted parent compounds [49]. Thus, the highest quantum yield is obtained with the 7-hydroxycoumarin-isonipecotic acid conjugate **6** that bears the most basic *N*,*N*-dialkylaminomethyl moiety, as inferred from the comparison between the pKa values for the secondary amine of isonipecotic acid (10.85 ± 0.1), sarcosine (10.20 ± 0.1) and iminodiacetic acid (9.30 ± 0.5) [55]. A similar assumption could be claimed to partially explain the good quantum yield of **1** (compared to 3-benzothiazolyl-7-hydroxycoumarin) because *ortho*-sulfonation of a phenol moiety is known to decrease its pKa by *ca.* 0.5 units [56]. Furthermore, the presence of two negative charges at physiological pH within the coumarin scaffold (*i.e.*, sulfonate and phenolate) promotes dye–dye repulsion and resistance to aggregation-induced fluorescence quenching, as confirmed by the perfect match between the absorption and excitation spectra of **1** recorded in aq. buffer (Fig S2.1.1). For the hemicyanine dyes **10** and **11**, sulfobetain moiety of the grafted aza-heterocycle is not sufficient to prevent dye–dye aggregation in phosphate buffer and the free fluorescent monomers of **10** and **11** with satisfactory quantum yields (entries 13 and 15) are obtained by adding 5% (w/v) of bovine serum albumin (BSA), an additive often used in buffers for mimicking body fluids. Indeed, this protein is known to enhance the emission of many fluorophores due to a combination of rigidization, reduction in the polarity of the dye's microenvironment (binding in the hydrophobic BSA pocket), and deaggregation [57]. Surprisingly, the introduction of a further sulfonate group on the 6-position of coumarin scaffold of bis-benzothiazolyl derivative **13** has no beneficial effects to disrupt aggregates in phosphate buffer. Again, there is a need to add BSA to determine a significant value of fluorescence quantum yield for **13** under simulated physiological conditions (entry 17). For the 4-cyano derivatives **2** and **4b** that emit in the orange spectral region (maximal emission peaks at 599 and 593 nm respectively), no evidence of dye–dye aggregation is observed (see ESI for the corresponding absorption/excitation spectra, Fig S2.1.2 and S2.1.4) and quantum yields close to 20% are obtained (see entries 5 and 8). All these results clearly show that the chosen water-solubilizing

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Table 1
Photophysical properties of water-soluble 3-benzothiazolyl-7-hydroxycoumarins at 25 °C.

Dye	Solvent	$\lambda_{max,\ abs}$ (nm)[a]	$\lambda_{max,\ em}$ (nm)	Stokes shift (cm^{-1})	Φ_F (%)	Std[b]/$\lambda_{exc\ (nm)}$
3-benzothiazolyl-7-OH	PB	431	488	2710	43	7OH/390
3-benzothiazolyl-7-OH	DMSO	397	485	4570	81	7OH/390
1	PB	441	485	2057	70	7-OH/390
1	DMSO	395	475	4264	70	7-OH/390
2	PB	502	599	3226	19	SR101/510
2	DMSO	416	590	7089	29	Fluo/470
3b	PB	434	485	2423	68	7-OH/390
4b	PB	494	593	3379	24	SR101/510
5	PB	436	485	2317	72	7-OH/390
6	PB	436	485	2317	≈100[c]	7-OH/390
	–	–	–	–	≈100[c]	Fluo/440
7	PB	436	485	2317	62	7-OH/390
8	PB	436	485	2317	90	7-OH/390
10	PB + 5% BSA[d]	535	631	2844	24	CV/550
10	DMSO	598	704	2518	4	CV/550
11	PB + 5% BSA[d]	502	615	3660	26	SR101/510
11	DMSO	558	705	3737	4	CV/550
13	PB + 5% BSA[d]	540	626	2544	18	CV/550
13	DMSO	550	669	3234	7	CV/550

[a] Most of the water-soluble 3-benzothiazolyl-7-hydroxycoumarin derivatives were not obtained in sufficient amounts for highly accurate measurements of absorption coefficients. Molar absorptivity of 3-benzothiazolyl-7-hydroxycoumarin and 6-sulfonated derivative **1** in EtOH have been already reported in the literature: 30 050 M^{-1} cm^{-1} (at 398 nm) [28] and 42 400 M^{-1} cm^{-1} (at 400 nm) [33] respectively.

[b] 7-OH = 7-hydroxycoumarin (Φ_F = 76% in PB, λ_{ex} = 390 nm), SR101 = sulforhodamine 101 (Φ_F = 95% in EtOH, λ_{ex} = 510 nm), Fluo = fluorescein (Φ_F = 91% in 0.1 N NaOH, λ_{ex} = 470 nm), CV = cresyl violet (Φ_F = 56% in EtOH, λ_{ex} = 550 nm) [39].

[c] Relative quantum yield of **6** is slightly greater than 100% (107–108%) because this compound was found to be more fluorescent than the standards 7-hydroxycoumarin and fluorescein; no suitable standard is available to determine a quantum yield lower than 100%.

[d] BSA was added to the phosphate buffer to disrupt aggregates that prevent the determination of relative quantum yield (a non-linear relationship between fluorescence emission and absorbance at the excitation wavelength was obtained in PB).

strategies are particularly effective to get 7-hydroxycoumarin-based fluorophores with strong long-wavelength emission in aq. media.

3.3. Synthesis of water-soluble fluorogenic probes and optical responses to thiols

Among the numerous quenching moieties commonly found in fluorescent turn-on thiol probes, 2,4-dinitrobenzenesulfonyl (DNBS) group is probably one of the most heavily used, through either the esterification of a phenol or sulfonylation of an aniline moiety of the selected fluorescent organic dye [1]. Due to the high level of electron-deficiency on its phenyl ring, DNBS moiety can act as an electron sink when attached to a fluorophore and may incur photoinduced electron transfer (PeT) or strongly impacts the internal charge transfer (ICT) state of the molecule, both leading to the quenching of its native fluorescence. In the presence of thiols, DNBS arylesters or arylamides can easily undergo a S_NAr desulfonylation reaction (addition-elimination mechanism) whose driving force is the release of SO_2 gas, and thus resulting in a dramatic fluorescence increase. We have decided to implement this strategy to the water-soluble 3-benzothiazolyl-7-hydroxycoumarin derivatives previously synthesized (Scheme 3). Positive effects on the water-solubility of the resulting thiol probes are expected because polar substituents such as sulfonate, sulfobetain or *N*,*N*-dialkylaminomethyl should readily offset the hydrophobic character of DNBS moiety. This *O*-sulfonylation reaction was first conducted with the 6-sulfonated derivative **1** and was found not to work properly, probably due to the steric hindrance and electronic effects of the sulfonic acid substituent located close to the 7-OH group. A wide range of experimental conditions including the use of different bases (pyridine, DMAP, 2,6-lutidine, *t*BuOK and K_2CO_3), solvents (pyridine and DMF), temperatures (rt, 50 °C, 80 °C or reflux) and variable amounts of DNBS chloride (DNBS-Cl) has been evaluated aimed at optimizing the synthesis of fluorogenic 2,4-dinitrobenzenesulfonyl ester **15** [58]. Thus, the treatment of phenol **1** with 3.3 equiv of DNBS-Cl and 0.5 equiv of DMAP in dry pyridine at 50 °C has enabled us to obtain **15** which was recovered by semi-preparative RP-HPLC (yield 15%). The synthesis of 7-*O*-DNBS ester of 3-benzothiazolyl-7-hydroxycoumarin (compound **14**) was also achieved in order to provide a reference thiol probe. By contrast, all attempts to obtain the 7-*O*-DNBS ester derived from the 4-cyano derivative **2** in a significant amount have failed, highlighting the lower nucleophilicity of its phenol group. Not

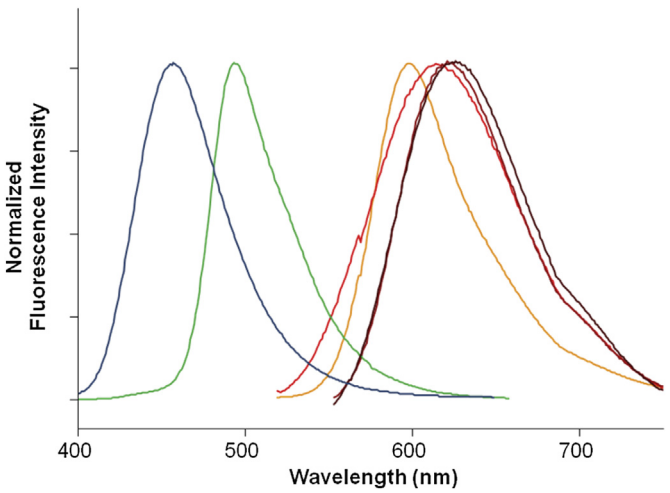

Fig. 1. Normalized fluorescence emission spectra of 7-hydroxycoumarin derivatives in PB (or in PB + 5% BSA) buffer (blue: 7-hydroxycoumarin, λ_{ex} = 390 nm; green: 3-benzothiazolyl-7-hydroxycoumarin-6-sulfonic acid, λ_{ex} = 390 nm; orange: 3-benzothiazolyl-4-cyano-7-hydroxycoumarin-6-sulfonic acid **2**, λ_{ex} = 510 nm; red: 7-hydroxycoumarin-hemicyanine **11**, λ_{ex} = 510 nm; dark red: 7-hydroxycoumarin-hemicyanine **13**, λ_{ex} = 550 nm and purple: 7-hydroxycoumarin-hemicyanine **10**, λ_{ex} = 550 nm). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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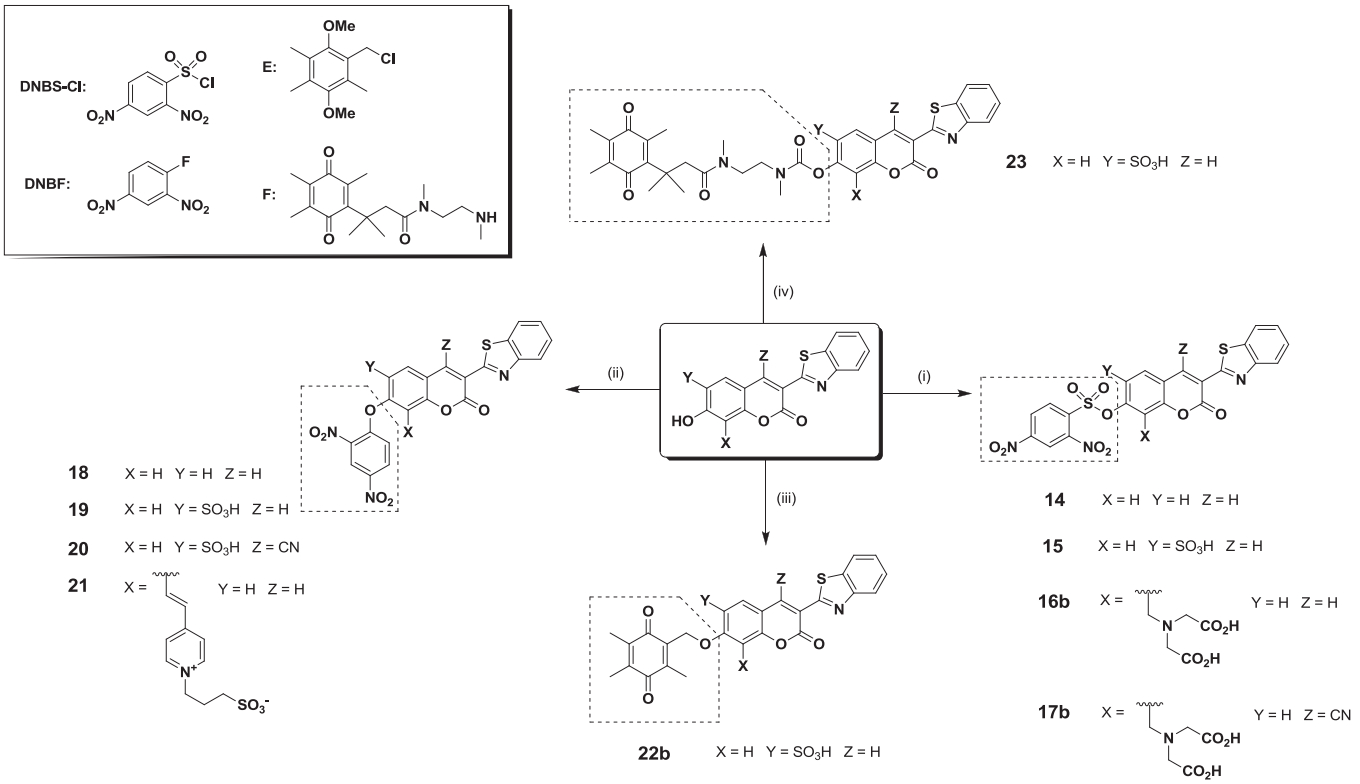

Scheme 3. Synthesis of water-soluble thiol-sensitive fluorogenic probes. *Reagents and conditions*: (i) DNBS-Cl (1.6 equiv), 2,6 lutidine (3.1 equiv), DMF, 120 °C, 18 h, 12% (**14**) or DNBS-Cl (3.3 equiv), DMAP (0.55 equiv), pyridine, reflux, 24 h, 15% (**15**) or (a) DNBS-Cl (3.5 equiv), 2,6 lutidine (10 equiv), CH_2Cl_2, rt, 18 h, (b) TFA, CH_2Cl_2, reflux, 2 h, 39% (**16b**, for the two steps) and 10% (**17b**, for the two steps); (ii) DNBF (4.5 equiv), K_2CO_3 (2.8 equiv), DMF, rt, overnight, 48% (**18**) and 39% (**21**) or DNBF (4.5 or 5.1 equiv), K_2CO_3 (2.8 or 3.8 equiv), DMF, 50 °C, 2 h or 5 h, 30% (**19**) and 18% (**20**); (iii) (a) K_2CO_3 (3.3 equiv), DMF, rt, 20 min, (b) **E** (2.7 equiv), KI (1.7 equiv), rt, 24 h, 23% (**22a**), (c) CAN (6.9 equiv), CH_2Cl_2/CH_3CN (2 : 1, v/v), rt, 6 h, 30%; (iv) (a) **F** (3.3 equiv), phosgene (3.3 equiv), TEA (3.3 equiv), toluene, rt, 24 h, quant. yield (b) **F** carbamoyl chloride (3.3 equiv), pyridine, rt, 24 h, 10%. *All fluorogenic probes were isolated as TFA salts except for **14** and **18**.*

surprisingly, sulfonylation conditions are not compatible with the moderate stability of hemicyanine dyes such as **10**, **11** and **13** and our initial attempts have led to the complete degradation of their dimethine chain. Finally, we have explored the *O*-sulfonylation of 7-hydroxycoumarins bearing an *N*,*N*-disubstitued aminomethyl arm on the 8-position. All reactions carried out with the free (di)carboxylic or di-sulfonic acid derivatives **5**–**8** failed to afford the targeted DNBS esters. Degradation of their 8-substituent in particular through decarboxylation and/or substitution (by pyridine at the benzylic position) reactions was obtained. To circumvent this issue, we have considered the sulfonylation of 8-substituted 7-hydroxycoumarins whose two carboxylic acids are protected as *tert*-butyl esters (compounds **3a** and **4a**). This then allows (1) to avoid the premature decarboxylation of the hydrophilic arm and (2) to use a less polar and unreactive solvent (*i.e.*, CH_2Cl_2). Compounds **3a** and **4a** were thus reacted with DNBS-Cl in the presence of excess 2,6-lutidine and in dry CH_2Cl_2. After purification by conventional column chromatography or preparative TLC, a further TFA treatment provided the DNBS esters **16b** and **17b** in 39% and 10% yield respectively. All spectroscopic data were in agreement with the structures assigned for **14**, **15**, **16b** and **17b**. Furthermore, the lack of free 7-OH parent fluorophore (as a minor impurity) was undoubtedly confirmed by RP-HPLC analyses and purity above 97% was found for all DNBS arylesters except for the 4-cyano derivative **17b** (purity = 78%). This latter compound is easily prone to hydrolysis in aq. solution, since electron-withdrawing properties of its cyano group may enhance the electrophilicity of sulfonate ester. The ability of DNBS group to effectively quench the blue-green fluorescence of water-soluble 7-hydroxycoumarins **14**, **15** and **16b** was confirmed through the determination of fluorescence quantum yields of these fluorogenic probes that do not exceed 5% in phosphate buffer (ESI, Table S2.3.1).

The sensing response of the three stable fluorogenic DNBS probes toward two different thiols namely Cys and thiophosphate anion (ThioPi) and the unreactive phosphate anion (Pi) was next examined. The time-dependant fluorescence intensity changes of probes **14**, **15** and **16b** with these analytes were studied and the results are shown in Fig. 2. Upon addition of Cys (5 equiv), the solution of non-sulfonated probe **14** showed an initial fast fluorescence increase followed by a gradual further increase in fluorescence intensity at 490 nm to reach a plateau after 200 min (Fig. 2a). A slower fluorescence response was obtained for the less nucleophilic ThioPi whereas the addition of Pi anion did not induce obvious variations in fluorescence intensity, suggesting that probe **14** was stable in the assay conditions. In sharp contrast, a much slower but steady fluorescence increase was observed for the 6-sulfonated derivative **15**, yet only in the presence of 250 equiv. of Cys (Fig. 2C1). No plateau has been reached after 16 h of incubation and a fluorescence level three times lower than that noted for **14** was obtained. This different behaviour can be explained by the steric effects of sulfonic acid substituent leading to a reduction in the accessibility of the thiol-reactive electrophilic centre of the probe, and by the fact that the fluorogenic S_NAr reaction involves a build-up of negative charge in the transition state (Meisenheimer complex) which can be destabilized by the negative electrostatic field induced by this charged water-solubilizing group [10]. Although the DNBS probe **15** is perfectly soluble in phosphate buffer alone, we found that the use of DMSO as a co-solvent

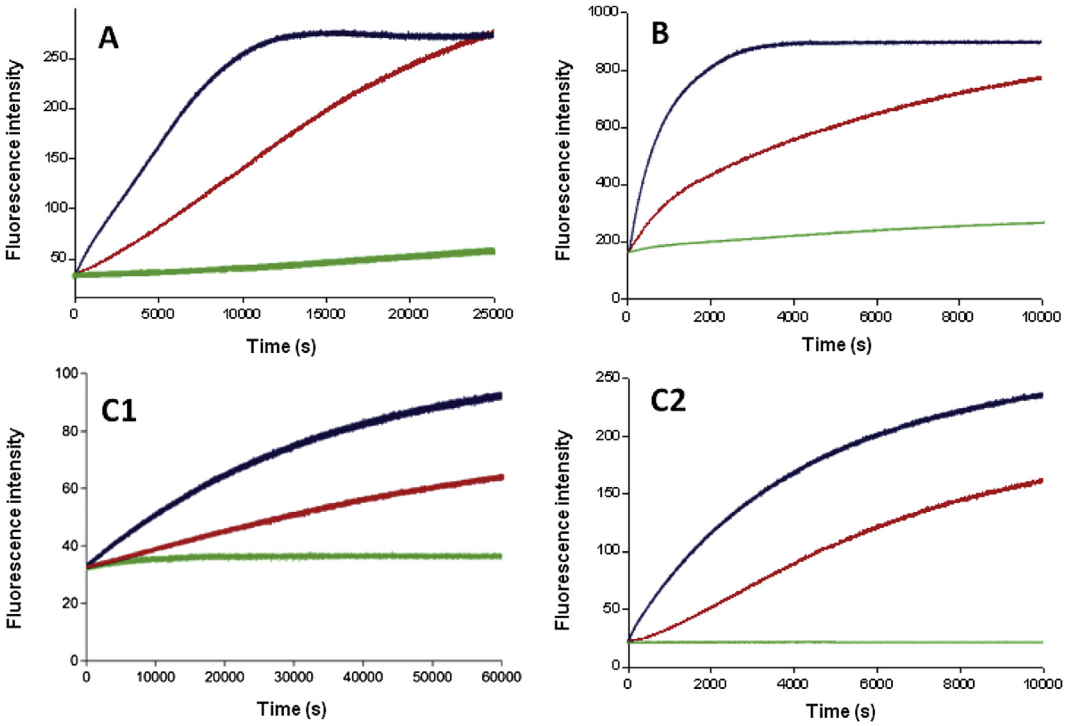

Fig. 2. Time-dependant fluorescence intensity of DNBS probes at 490 nm ($\lambda_{ex} = 390$ nm) in the presence of sulfhydryl analytes (Cys or ThioPi) and Pi anion in PB buffer (100 mM, pH 7.5) at 25 °C. (A) probe **14** (6.3 μM) with Cys (5 equiv) in blue, ThioPi (5 equiv) in red, and Pi (5 equiv) in green; (B) probe **16b** (3.4 μM) with Cys (5 equiv) in blue, ThioPi (5 equiv) in red, and Pi (5 equiv) in green; (C1) probe **15** (8.6 μM) with Cys (250 equiv) in blue, ThioPi (250 equiv) in red, and Pi (250 equiv) in green; (C2) probe **15** (8.6 μM) with Cys (50 equiv) in blue, ThioPi (250 equiv) in red, and Pi (250 equiv) in green recorded in PB (100 mM) + 45% DMSO (pH 8.3). *A lower concentration of probe **16b** was used to avoid exceeding the upper limit of fluorescence detection (1000 units).* (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

promoted the kinetics of this thiolysis process with lower amounts of Cys (50 equiv) and with ThioPi (250 equiv) (Fig. 2C2). However, to avoid the use of such additives, that are scarcely or not at all compatible for *in cellulo* or *in vivo* applications, we next assessed the fluorogenic reactivity of 8-substituted DNBS probe **16b**. As depicted in Fig. 2b, in the presence of non-nucleophilic Pi anion, this probe displayed no significant changes in fluorescence intensity at 490 nm. Conversely, when being treated with 5 equiv of Cys, the solution exhibited a dramatic increase in the emission intensity which reached a maximum in less than 50 min. Again, a slower fluorescence response was observed for ThioPi anion. However, for both sulfhydryl analytes, a large rate acceleration of the fluorogenic S_NAr process was observed, compared to thiolysis of DNBS probes **14** and **15**. Thus, 8-functionalization of 3-benzothiazolyl-7-hydroxycoumarin by Mannich reaction involving the use of a hydrophilic secondary amine, should be preferred in order to obtain blue-green emitting thiol-reactive DNBS probes soluble in aq. media.

In order to find alternative 7-*O*-quenching moieties whose thiol-reactivity is less or not affected by the presence of 6-SO_3H substituent onto the 7-hydroxycoumarin scaffold, we next explored the synthesis of water-soluble fluorogenic probes whose DNBS arylester is replaced either by the 2,4-dinitrophenyl ether (DNP) or a benzoquinone-type Michael acceptor grafted to the 7-OH group through a benzyl ether or a self-immolative carbamate linkage.

For 3-benzothiazolyl-7-hydroxycoumarin and its three water-soluble derivatives **1**, **2** and **11**, the *O*-etherification reaction was performed with Sanger reagent (2,4-dinitrofluorobenzene, DNBF) according to the procedure reported by Lin et al. [41]. The 6-sulfonated derivatives **1** and **2** were reacted with an excess of DNBF and K_2CO_3 in dry DMF at 50 °C. The resulting 7-*O*-DNP ethers **19** and **20** were isolated in a pure form by semi-preparative RP-HPLC (30% and 18% yields respectively). A similar protocol was applied to 3-benzothiazolyl-7-hydroxycoumarin and hemicyanine dye **11** and the lack of 6-SO_3H substituent allowed the reaction to work at rt. These milder conditions prevented the degradation of the dimethine chain of **11** previously observed during the synthesis of DNBS arylesters (*vide supra*). Thus, the DNP probes **18** and **21** were readily recovered by conventional column chromatography and semi-preparative RP-HPLC respectively. As expected, these four DNP ethers are essentially non-fluorescent in both phosphate buffer and DMSO (Table S2.3.1) and their sensing response to thiols were also studied through time-dependant analyses (Fig. 3). No significant changes in emission intensity of these DNP probes were observed in phosphate buffer upon addition of thiols (Cys and ThioPi) whatever the number of equivalents used (Fig. 3A1–C1). These results can be related to the study of Lin et al. which highlights the excellent selectivity of DNP probe **18** for benzenethiols over aliphatic thiols (such as Cys) at neutral pH (*i.e.*, phosphate buffer, pH 7.0) [41]. This property was attributed to the distinct pKa values of benzenethiols (pKa = 6.5) and aliphatic thiols (pKa = 8.5), and to the thiolysis of dinitrophenyl ethers proceeding *via* S_NAr by the nucleophilic thiolate. In our case, the addition of DMSO to phosphate buffer (45% v/v) seriously affects the pH of the medium (8.3 against 7.5 for the buffer alone) and a fluorescence turn-on response was then obtained for all DNP probes except for the 4-cyano derivative **20** (Fig. 3A2–C2). For this latter fluorogenic probe, thiolysis of DNP moiety and subsequent fluorescence spectral changes were observed only in DMSO and with 250 equiv. of thiol analytes (data not shown). The fastest response time was obtained with the non-sulfonated DNP ether **18** (Fig. 3A2), thereby demonstrating once again that *ortho*-sulfonation should not be the preferred approach to make water-soluble fluorogenic phenolic dyes. The small far-red fluorescence enhancement observed for the

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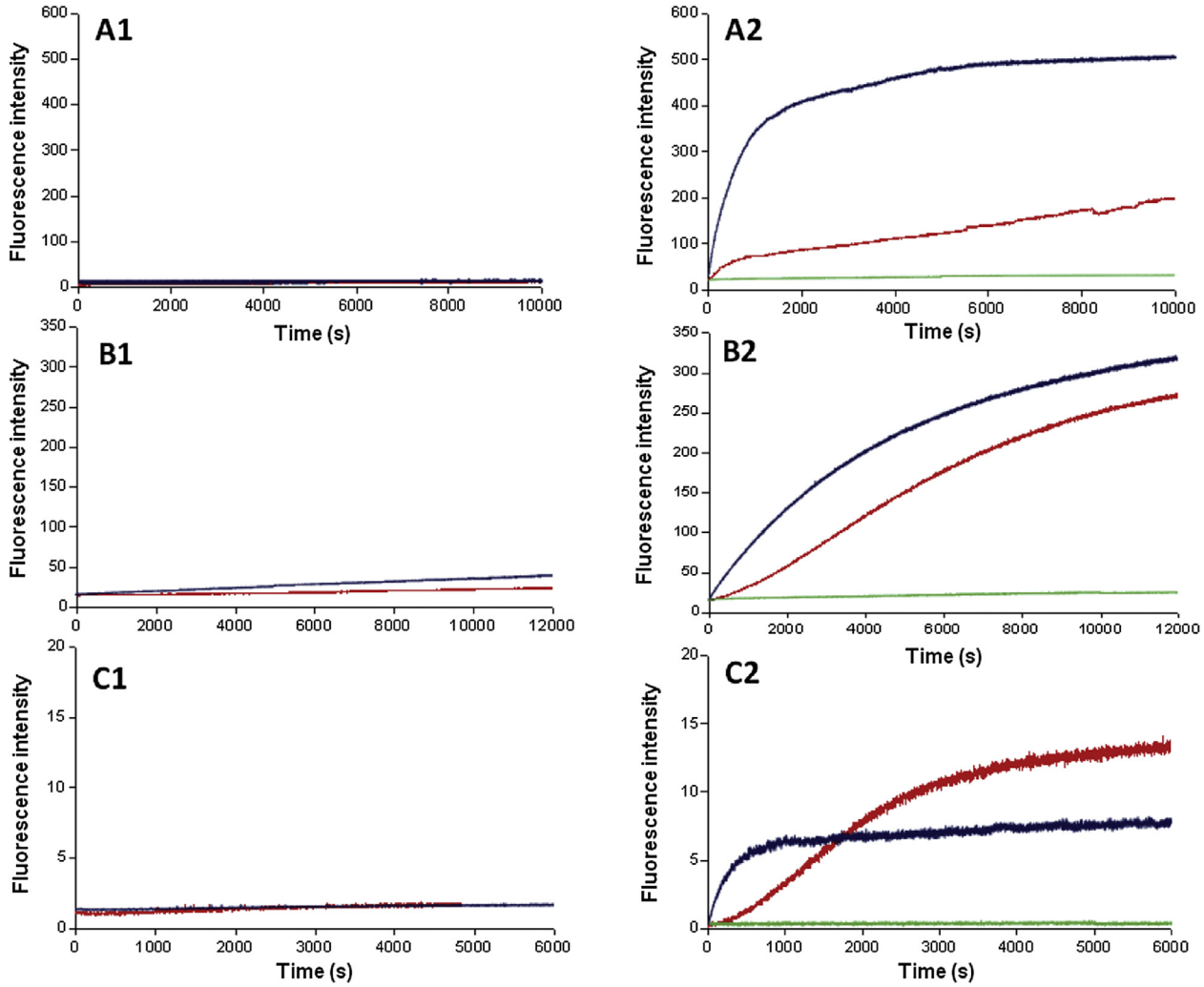

Fig. 3. Time-dependant fluorescence intensity of DNP probes in the presence of sulfhydryl analytes (Cys or ThioPi) and Pi anion in PB buffer (100 mM, pH 7.5) or in PB + 45% DMSO (pH 8.3) at 25 °C. (A1) probe **18** (8.6 μM) with Cys (50 equiv) in blue, ThioPi (250 equiv) in, $\lambda_{ex} = 390$ nm, $\lambda_{em} = 490$ nm; (A2) *ibid*. in PB + 45% DMSO, a further kinetic curve for Pi (50 equiv) in green; (B1) probe **19** (8.6 μM) with Cys (50 equiv) in blue, ThioPi (250 equiv) in, $\lambda_{ex} = 390$ nm, $\lambda_{em} = 490$ nm; (B2) *ibid*. in PB + 45% DMSO, a further kinetic curve for Pi (50 equiv) in green; (C1) probe **21** (8.6 μM) with Cys (50 equiv) in blue, ThioPi (250 equiv) in red, $\lambda_{ex} = 390$ nm, $\lambda_{em} = 490$ nm; (C2) *ibid*. in PB + 45% DMSO except for ThioPi (50 equiv.), a further kinetic curve for Pi (50 equiv) in green, $\lambda_{ex} = 500$ nm, $\lambda_{em} = 655$ nm. *Thiolysis of **21** leads to the release of 7-hydroxycoumarin-hemicyanine dye **11*** whose fluorescence emission maximum is highly dependent on the solvent used: 490 nm in PB, 615 nm in PB + 5% BSA, 655 nm in PB + 45% DMSO and 705 nm in DMSO. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

fluorogenic thiolysis of **21** can be linked to the poor quantum yield (less than 5%) of the released hemicyanine dye **11** in the mixture PB + 45% DMSO (Fig. 3C2). More generally, it is very obvious that the combined use of our water-solubilizing methodologies and thiol-reactive quenching moiety DNP is not a relevant approach to convert 7-hydroxycoumarin scaffolds into water-soluble fluorescent probes for detection of biological thiols (such as Cys) in aq. media but a very promising way to obtain water-soluble "OFF–ON" chemosensors for benzenethiol pollutants whose thiol pKa value is lower than physiological pH. Indeed, as shown in Fig. 4, a remarkable enhancement of the rate of DNP ether **18** thiolysis was obtained with 4-chlorothiophenol (pKa = 6.12) [55] compared to cysteine.

Alternative thiol sensing mechanisms based on quinone-methide-type rearrangement or tandem cyclization reactions ("trimethyl lock" cyclization and intramolecular cyclic urea formation) both induced by thiol-Michael addition on a benzoquinone moiety, were finally implemented through the preparation of fluorogenic probes **22b** and **23** from the 6-sulfonated fluorophore **1** (Scheme 3). We thus seeked to take advantage of the substantial distance between the thiol-reactive centre and the bulky fluorescent dye to minimize the steric interference and electronic effects of the water-solubilizing substituent, in order to improve the thiolysis kinetics. First, alkylation of phenol **1** with 1-chloromethyl-2,5-dimethoxy-3,4,6-trimethylbenzene **E** and subsequent removal of the two methoxy protecting groups with CAN, were achieved using literature protocols [30]. Due to the presence of 6-SO_3H substituent, purification of quinone-based probe **22b** was carried out by semi-preprative RP-HPLC but complicated by its limited stability in all aq. mobile phases tested (*i.e.*, TFA 0.1%, ammonium formate 50 mM, pH 6.4, ultrapure water and TEAB 50 mM, pH 7.5). A small amount of **22b** was recovered, sufficient for NMR analyses, but its gradual decomposition giving back the parent fluorescent phenol **1** prevented us to perform reliable measurements of quenching efficiency and fluorescence response to thiols. To increase the overall stability of such fluorogenic Michael addition-based probe, a "cloak trimethyl-lock benzoquinone" unit [59] was chosen as an alternative to previous benzyl-like group and grafted

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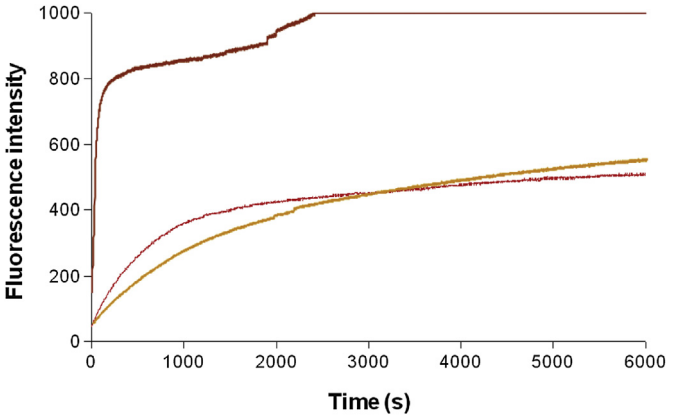

Fig. 4. Time-dependant fluorescence intensity of DNP probe **18** (8.6 μM) in the presence of sulfhydryl analytes (Cys or 4-chlorothiophenol) in PB + 45% DMSO (pH 8.3) at 25 °C (λ_{ex} = 390 nm, λ_{em} = 490 nm). (red) Cys (50 equiv); (brown) 4-chlorothiophenol (50 equiv) and (orange) 4-chlorothiophenol (5 equiv). *Thiolysis of probe* **18** *with* 50 *equiv of 4-chlorothiophenol leads to fluorescence signal saturation* (*>1000 units*). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

to the phenol moiety of **1** through a diamine spacer involving *N*-methylcarbamate linkages. The *O*-acylation of **1** with carbamoyl chloride derivative of **F** (pre-formed by reaction of *N*-methyl secondary amine **F** and phosgene) was achieved under conditions previously reported by us for the synthesis of protease-sensitive fluorogenic probes using 7-hydroxycoumarin as reporter group [60]. The resulting quinone-based probe **23** was purified by semi-preparative RP-HPLC but again, the loss of quenching moiety leading to the premature release of fluorophore **1** partially occurred in aq. mobile phase. Full characterization of this water-soluble fluorogenic probe by mass spectrometry and NMR spectroscopy has however been made but its limited stability in aq. buffers prevented us to reliably assess its thiol sensing ability through fluorescence measurements in phosphate buffer. It seems therefore that the *ortho*-substituent SO_3H favours the hydrolysis of *O*-aryl benzyl ether and carbamate linkages and prevents the design of latent fluorophores that possess a unique combination of chemical stability and thiol reactivity. This may be related to a better leaving group ability of 6-sulfonated coumarin **1** as a result of lower pKa of its phenol moiety compared to parent 3-benzothiazolyl-7-hydroxycoumarin (*vide supra*).

4. Conclusion

In summary, a series of water-soluble fluorophores based on the 3-benzothiazolyl-7-hydroxycoumarin scaffold were successfully synthesized by means of electrophilic aromatic sulfonation or Mannich- and Knoevenagel-type reactions with hydrophilic building blocks. Unprecedented high fluorescence quantum yields in physiological conditions were obtained for the majority of compounds, especially those emitting in the blue-green spectral range. To the best of our knowledge, the present work is the first study devoted to the water-solubilization of long-wavelength 7-hydroxycoumarins, now regarded as valuable tools in the field of fluorogenic probes. The masking of 7-OH group of these fluorescent organic dyes with various thiol-sensitive protecting groups acting as quenching moieties, was also investigated and ten novel water-soluble pro-fluorophores potentially usable for thiol detection in the visible region (485–631 nm), were synthesized. A comparative study focused on their ease of synthesis, chemical stability and thiol sensing ability under physiological conditions has led us to make the following main conclusions: (1) DNBS ester is a valuable thiol-responsive trigger group for long wavelength 7-hydroxycoumarins bearing a water-solubilizing moiety (other than $-SO_3H$) onto the 8-position and provide fluorescent probes with favourable features (*i.e.*, fast response time, significant fluorescence enhancement, solubility and stability at physiological pH) for biological thiol detection, (2) DNP ether is a more versatile thiol-reactive quenching moiety than DNBS ester, due to its easier introduction onto the phenol moiety of a wide range of 6- or 8-substituted 7-hydroxycoumarins. However, the sensing ability of the water-soluble DNP pro-fluorophores is limited to thiols completely converted into their thiolate forms in slightly alkaline media (*i.e.*, benzenethiols in the pH range 7.5–8.5), and (3) a sulfonic acid moiety located *ortho* to the phenol functionality of long-wavelength 7-hydroxycoumarins hampers the construction of water-soluble fluorogenic probes that possess both high hydrolytic stability and reactivity toward biological thiols. Thus, the results of this study provides the rationale for the design of 7-hydroxycoumarin-based pro-fluorophores with improved photophysical (*i.e.*, red-shifted absorption/emission and enhanced quantum yield in water) and physicochemical (*i.e.*, water-solubility) properties suitable for the sensing and bioimaging of thiol species. This could be also useful for developing fluorescent probes targeting other nucleophilic (bio)analytes [61–63].

Acknowledgements

This work was supported by the “ Région Haute-Normandie” (especially for the Ph. D. grant of Benoît Roubinet), the CRUNCh program (CPER 2007–2013) and “ Institut Universitaire de France” (IUF). We thank Dr. Michel Picquet (University of Burgundy, ICMUB, UMR CNRS 6302) for its helpful assistance for achieving NMR analyses on Bruker Avance III 500 spectrometer and Patricia Martel (University of Rouen, laboratory COBRA, UMR CNRS 6014) for the recording of IR spectra. Dr. Nicolas Maindron is also greatly acknowledged for the synthesis of di-*tert*-butyl-iminodiacetate.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.dyepig.2014.02.004.

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Please cite this article in press as: Roubinet B, et al., New insights into the water-solubilization of thiol-sensitive fluorogenic probes based on long-wavelength 7-hydroxycoumarin scaffolds, Dyes and Pigments (2014), http://dx.doi.org/10.1016/j.dyepig.2014.02.004