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

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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

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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