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7-Hydroxycoumarin−Hemicyanine Hybrids: A New Class of Far-Red Emitting Fluorogenic Dyes

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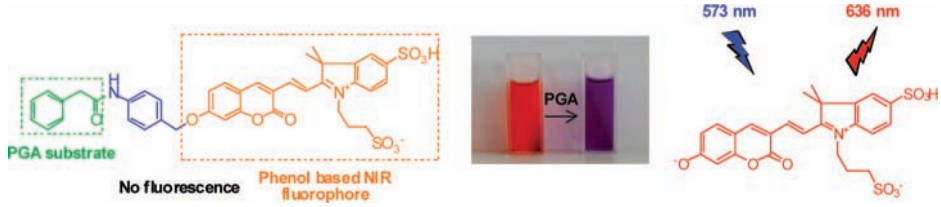
ABSTRACT



The design and synthesis of novel water-soluble far-red emitting phenol-based fluorophores derived from 7-hydroxycoumarin are described. These hemicyanine−coumarin hybrids display promising spectroscopic features such as large apparent Stokes shift (ranging from 60 to 140 nm) and fluorescence emission maxima between 620 and 720 nm in physiological conditions. Their utility was then illustrated by the preparation of an original fluorogenic probe of penicillin G acylase (PGA) whose fluorescence is unveiled through an enzyme-initiated domino reaction.

During the past two decades, intensive research efforts have been devoted to the development of numerous enzyme assays for various bioanalytical and biological applications including high-throughput screening or biomolecular imaging.[1] The use of spectroscopic probes whose fluorescence properties dramatically change upon reaction, covalent interaction, or reversible binding with target analytes is often required. Indeed, molecular fluorescence spectroscopy is one of the most powerful and simple methods to visualize *in vitro* and *in vivo* biochemical and cell biological processes in a real-time manner, with a high degree of accuracy, and by using simple instruments and facilities.[2] Thus, numerous profluorescent probes that unmask their intense fluorescence only by a user-designated chemical reaction have been elaborated for the visualization of biologically relevant molecules (metal cations, reactive oxygen species, thiols)[3] and the detection of various enzymes like esterases, phosphatases, proteases, or β-galactosidase.[4]

Recently, the research of optical bioprobes absorbing and emitting in the near-infrared (NIR) region has gained a huge interest.[5] Indeed, light in the region 650−900 nm is poorly

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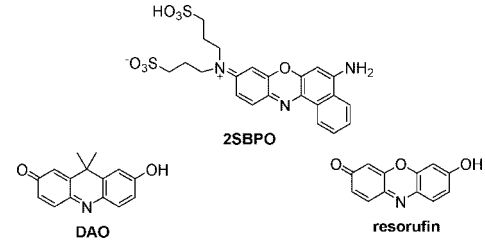
absorbed by biomolecules and thus can penetrate more deeply into tissues (up to several centimeters).[6] Autofluorescence is also minimized in this region as well as Rayleigh−Tyndall light scatttering artifacts.[7] Thus, much higher signal-to-noise ratios can be obtained, and the corresponding NIR fluorescent probes are expected to be suitable for emerging and challenging biomedical applications such as *in vivo* diagnostic imaging.

The most popular synthetic strategy to obtain NIR fluorophores consists of the extension of the $\pi-\pi$ conjugation system of conventional dyes (i.e., cyanine dyes, fluorescein, rhodamines, and BODIPYs) but such chemical modification often leads to molecules poorly or not soluble in aqueous media, sometimes chemically unstable (especially for the long polymethine chain cyanine dyes such as Cy 7.0 and its analogues) and/or highly sensitive to photobleaching.[8] Furthermore, few water-soluble NIR dyes having a reactive group (i.e., an aniline, a phenol, or a thiophenol moiety) whose reversible chemical modification (e.g., acylation, alkylation) significantly affects their fluorescence properties have been reported to date. The chemistry of such fluorogenic dyes was recently investigated by the group of Weissleder through the development of original Nile Blue analogues (i.e., benzo[*a*]phenoxazine dyes) and new symmetric and asymmetric xanthene dyes (i.e., naphthofluorescein and rhodanaphthofluor derivatives).[9] The potential utility of their disulfonated benzo[*a*]phenoxazine scaffold (2SBPO) was illustrated by the preparation of fluorogenic probes suitable for the *in vivo* imaging of two different hydrolytic enzymes: β-galactosidase and dipeptidyl peptidase IV.[10] Given their critical role in numerous diseases, the detection of specific protease activities *in vivo* is of great importance, especially for diagnostic and therapeutic purposes. In that context, we recently reported original profluorescent substrates of penicillin G acylase (PGA) and caspase-3, whose strong fluorescent 7-hydroxycoumarin dye is released through enzyme-initiated domino reactions.[11] Such a strategy requires the introduction of a self-immolative spacer (e.g, *p*-aminobenzyl alcohol, PABA) between the peptidyl substrate and the fluorophore that results in many beneficial effects such as higher stability in physiological media as well as better enzymatic recognition and cleavage kinetics. In order to extend this approach to the NIR range, we have applied the same synthetic strategy to the red-emitting acridinone and oxazinone phenol dyes already reported in the literature (i.e., DAO and resorufin). However, we found that thiols have a deleterious quenching effect on the fluorescence of these fluorophores thus preventing their use in imaging applications of living systems.[11] To circumvent this issue, we decided to explore a novel class of NIR dyes derived from 7-hydroxycoumarin that do not contain a thiol-sensitive quinoimine ring. Herein, we report the synthesis of these water-soluble far-red emitting phenol-based fluorophores, their promising spectral properties in physiological conditions (large Stokes shift and fluorescence emission maxima between 620 and 720 nm) and their application for the preparation of latent fluorescent probes aimed at the detection of the model protease, PGA.



The targeted fluorogenic phenol dyes were designed as follows: (1) the pro-fluorescence properties should be obtained taking into account that 7-alkoxycoumarins emit weakly compared to the phenol free 7-hydroxycoumarins;[12] (2) a push−pull device was designed between the phenol functionality of the 7-hydroxycoumarin and an indolium moiety; (3) the red-shift of the wavelength emission should be obtained by the introduction of one or two double bonds in the 3-position of the coumarin, thus extending the π-conjugation of the resulting dye; (4) water solubility of the fluorophore should be obtained through to the introduction of up to three sulfonate groups onto the indolium moiety (Figure 1).

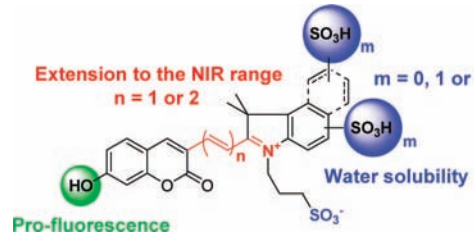


Figure 1. General structure of the targeted water-soluble far-red emitting phenol-based fluorophore.

Fluorophores **4a**−**e** were synthesized *via* a base-mediated electrophilic substitution of indolium derivatives **3a**−**c**[13] as depicted in Scheme 1. First, the reaction with 3-formylcoumarin **1** in ethanol in the presence of pyrrolidine gave the water-soluble hemicyanine−coumarin hybrids **4a**, **4c**, and **4e** in good yields. Interestingly, the removal of the acetyl

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Scheme 1. Synthesis of Water-Soluble Far-Red Emitting Phenol-Based Fluorophores **4a**−**e**

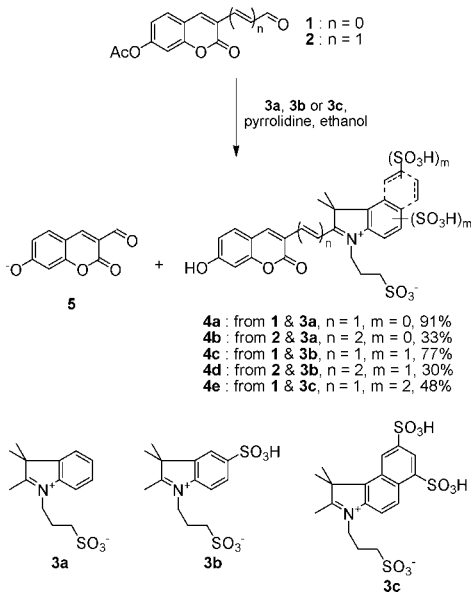

Table 1. Absorption and Fluorescence Data for Hemicyanine-Coumarin Hybrids

dye	λ_{max}(abs) (nm)	λ_{max}(em) (nm)	Stokes shift (nm)	ε^a ($\times 10^4$)	$\Phi_F{}^b$ (%)	solvent
4a	555	620	65	2.28	0.7	PBS[c]
	630	649	19	2.22	2.3	DMSO
	623	642	19	2.20	4.9	ethanol
4b	564	720	139	2.70	1.3	PBS
	491	653	162	2.45	1.3	DMSO
4c	573	636	63	7.04	1.3	PBS
	485	604	119	3.55	1.2	DMSO
	630	648	18	7.80	3.1	ethanol
4d	592	722	130	2.45	1.1	PBS
	494	655	161	2.35	1.7	DMSO
	508	654	146	2.50	1.0	ethanol
4e	578	643	65	6.55	2.7	PBS
	495	625	130	3.37	2.5	DMSO
	643	659	16	4.18	1.4	ethanol

[a] Molar extinction coefficient are in M^{-1} cm^{-1}. [b] The fluorescence quantum yields were determined at 25 °C by using rhodamine 6G (Φ_F = 90% in water at 25 °C) or sulfocyanine dye Cy 5.0 (Φ_F = 20% in PBS at 25 °C) as standards.[15] [c] PBS = 100 mM phosphate buffer + 150 mM NaCl, pH 7.4.

group of phenol moiety occurred simultaneously during this base-mediated condensation reaction. In order to shift the fluorescence emission to longer red wavelengths, an additional double bond was added to the coumarin core of **1** by means of a Wittig reaction and afforded 3-cinnamyl coumarin **2** in 60% yield (see the Supporting Information). This latter aldehyde was then condensed with indolium derivatives **3a**,**b** under the same conditions as used for **1**. However, the corresponding fluorophores **4b** and **4d** were obtained in lower yields (30−35%) than the dimethine dyes. These modest yields were explained by a competitive pyrrolidine-catalyzed retroaldol reaction of **2** transitorily leading to the 3-formylcoumarin **1** which subsequently reacts with indolium to give **4a** or **4c**. All spectroscopic data (see the Supporting Information), especially NMR and mass spectrometry, were in agreement with the structures assigned. As expected, fluorophores **4a**−**e** were found to be perfectly soluble in water and related aqueous buffers in the range of concentrations suitable for biomolecular labeling applications (1.0 μM to 5 mM). The inertness of these fluorescent phenols toward thiols was also demonstrated through incubation with 1,4-dithioerythritol (DTE) and subsequent LC−MS analyses (see the Supporting Information).

The photophysical properties of the five novel water-soluble phenol-based fluorophores available were evaluated in various solvents (i.e., PBS and polar organic solvents) and collected in Table 1 (for their normalized absorption and emission spectra, see the Supporting Information). As expected, the extension of the aromatic system conjugation of parent 7-hydroxycoumarin resulted in a dramatic red-shift of the emission maxima beyond 600 nm. Interestingly, compounds **4a**−**e** exhibited a large Stokes shift (ranging from 60 to 140 nm according to the solvent and the length of polymethine chain) which is beneficial for a better detection sensitivity thanks to the decrease of the reabsorption of photons and a diminution of the fluorophore self-quenching. This might be attributed to an excited-state intramolecular charge transfer (ICT) between the phenolate group (donor) and the indolium moiety (acceptor) within the same dye molecule. Furthermore, a marked negative solvatochromism in the absorption spectrum of dimethine hemicyanine−coumarin hybrids **4a**, **4c**, and **4e** (~60 nm shift from PBS to ethanol) was observed with increasing polarity of the solvents, but no apparent solvatochromism in the emission spectra was detected. These properties are due to hydrogen-bonding interaction between the solvents and the dye molecule and proved that an excited-state ICT should take place in the present case.[14] A slight improvement of the quantum yields was observed along with the increase in the number of negatively charged sulfonate groups directly attached to the (benzo)indole ring. Indeed, this hydrosolubilization strategy enabled reducing dye−dye interactions in water because of Coulombic repulsion and partially prevents static quenching through association. Brightness ($\Phi_F\varepsilon$) values in physiological conditions (e.g., 1770 for **4e** in PBS) are comparable to those determined for the commercially available red-emitting acridinone or oxazinone phenol dyes (e.g., 1930 for DAO in PBS).[11]

Several possible hypotheses to explain the relatively modest fluorescence efficiency of phenol dyes **4a**−**e** can be found: dynamic quenching of excited dye molecules through collision with dye in the ground-state and excited-state twisting (rotation/vibration) of the central tetramethine bridge separating the two (hetero)cyclic heads ("loose belt effect")[16] or static quenching through association (other than H-aggregation) of ground-state

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dye molecules. Thus, current work is ongoing in our laboratory to rigidify these pseudocyanines in order to improve the quantum yields in physiological conditions.

Prior to the use of these novel phenol-based fluorophores in the context of fluorogenic probes based on the pro-fluorescent concept, it was essential to check that the chemical modification of their hydroxyl group (i.e., modification of the phenol either to an ether or an ester) effectively quenched their red fluorescence emission. For this purpose, we synthesized a fluorogenic probe suitable for the detection of model amidase PGA. We chose to use hemicyanine−coumarin hybrid **4c** as the fluorescent phenol because it represented a good compromise between high molar extinction coefficient, acceptable quantum yield and ease of synthesis. Thus, our synthetic strategy to the PGA sensitive profluorescent probe **7** involved the introduction of the self-immolative PABA linker between the phenylacetyl moiety (i.e., PGA-sensitive substrate) and the phenol-based fluorophore (Figure 2A). However, to avoid the laborious and time-consuming handling and purification of highly polar synthetic intermediates, the disulfonated indole **3b** was introduced at the final stage of the synthesis through a K_2CO_3-mediated condensation reaction with aldehyde **6**. Spectroscopic measurements performed in PBS at pH 7.5 revealed that the etherification of the 7-OH group of **4c** effectively quenched its fluorescence at 636 nm (Figure 2B).[17] This particular trend confirmed that hemicyanine−coumarin hybrids **4a**−**e** can be used in the design and synthesis of fluorogenic probes sensitive to various analytes. Finally, profluorophore **7** was tested against PGA. Figure 2C showed the time-course for the amidase-catalyzed hydrolysis of **7**. After PGA was added to the substrate solution, a strong fluorescence signal generated at 636 nm indicated the catalytic cleavage of the carboxamide bond and the release of the free fluorescent phenol **4c**. Furthermore, no nonspecific cleavage of the probe was detected in a control reaction where **7** was incubated only in phosphate buffer. Overall, these results confirm that the latent red fluorophore **7** is a suitable substrate for penicillin amidase. Thus, in addition to the profluorescent character of our phenol-based fluorophores, the full stability of fluorogenic probe **7** as well as its suitability for the detection of a model protease have been shown.

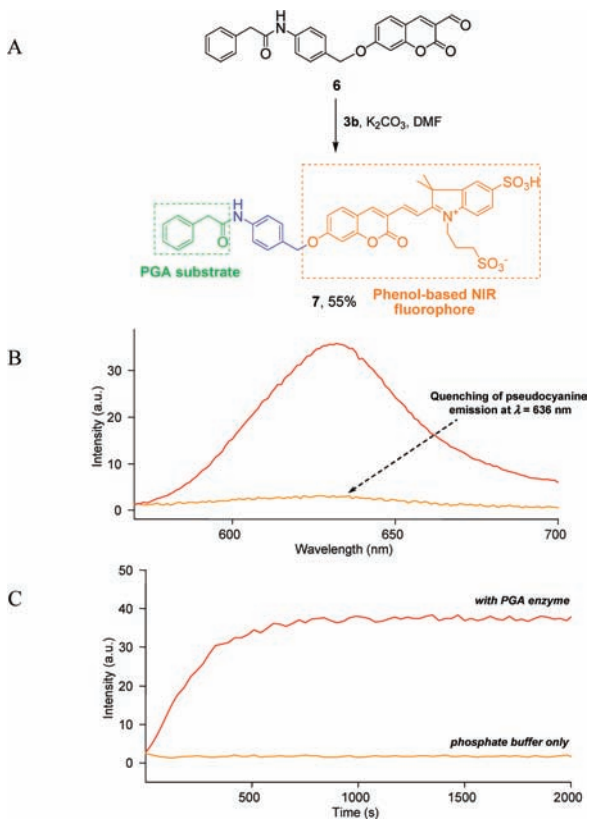

Figure 2. (A) Synthesis of the PGA sensitive fluorogenic probe **7**. (B) Emission spectra (ex λ = 560 nm) of pseudocyanine dye **4c** (red line) and fluorogenic probe **7** (orange line) at 37 °C in phosphate buffer (concentration 3.9 μM). (C) Fluorescence emission time-course of probe **7** (concentration 3.9 μM) with PGA (0.0105 U) in phosphate buffer (37 °C) at 636 nm (ex 560 nm).

In summary, we have designed a novel class of water-soluble far-red emitting phenol-based fluorophores by coupling a polysulfonated (benzo)indolium unit to the 3-formyl (or the 3-cinnamyl) derivative of umbelliferone. Contrary to the commercially available red-emitting acridinone and oxazinone phenol dyes, these fluorescent labels are not reactive toward biological thiols. They exhibit a large Stokes shift, and the emission maximum wavelength can be easily tuned between 620 and 720 nm thanks to the extension of the electron density to the indole moiety *via* a push−pull device. Furthermore, we confirmed the profluorescent features of our pseudocyanine dyes through the synthesis and *in vitro* validation of the PGA sensitive fluorogenic probe **7**. In order to expand the scope of these promising latent far-red fluorophores to the visualization of various biologically relevant molecules and activities inside living cells, further works are in progress to improve both the brightness of these fluorescent phenols and their cell permeability by using bioactivatable masking groups for the negative charges of sulfonate moieties.[18]

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Supporting Information Available: Procedures and additional data for syntheses and analyses (HPLC, MS, NMR) reported herein. This material is available free of charge via the Internet at http://pubs.acs.org.

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(17) Hypsochromic shift of absorption maximum (λ_{max} = 470 nm) was also observed.

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