EurJOC European Journal of Organic Chemistry

FULL PAPER

DOI: 10.1002/ejoc.201403215

A Synthetic Route to 3-(Heteroaryl)-7-hydroxycoumarins Designed for Biosensing Applications

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Keywords: Fluorescent probes / Fluorescence / Biosensors / Oxygen heterocycles

A straightforward method to synthesize 3-(2-benzimidazolyl)-7-hydroxycoumarins, based on the condensation reaction of 7-acetoxy-3-formylcoumarin with various *C*- and/or *N*-substituted *ortho*-phenylenediamine derivatives is presented. This unusual approach proved particularly effective for introducing different hydrophilic groups (carboxylic or sulfonic acids or trimethylalkylammonium moieties) onto the heteroaryl scaffold, leading to cyan-green emitting coumarins that were both water-soluble and strongly fluorescent under physiological conditions. The further extension of this condensation reaction to bis(2-aminophenyl)diselenide enabled the first synthesis of 3-(2-benzoselenazolyl)-7-hydroxycoumarin. The potential utility of these new 7-hydroxycoumarins was demonstrated through the synthesis and spectroscopic and analyte-responsive behavior of fluorogenic probes suitable for sensing biologically relevant thiols and urokinase, a protease that plays a key role in cancer invasion and metastasis.

Introduction

7-Hydroxycoumarin derivatives are popular fluorescent organic dyes. They are currently used as reporters in various biological and/or bioanalytical applications that involve biosensing or biolabeling operations.[1,2] This widespread use is particularly due to their valuable and unique photophysical properties (especially their broad Stokes' shift and good-to-high quantum yields under physiological conditions), mainly in the blue or violet spectral regions, which are often associated with a good (photo)chemical stability. Thus, a huge number of "smart" fluorescent (bio)probes (also known as fluorescent chemodosimeters or chemosensors, or profluorophores) based on a 7-hydroxycoumarin scaffold have been developed and described in the literature for the detection of various (bio)analytes including enzymes, (bio)thiols, polluting chemicals, cations, and anions.[3,4] Interestingly, 7-*O*-substituted coumarin compounds have also been extensively investigated in medicinal chemistry to obtain therapeutic agents effective against various diseases (e.g., neurodegenerative diseases).[5]

In the early 1980s, the Wolfbeis group carried out detailed work aimed at redshifting the absorption/emission maxima of the 7-hydroxycoumarin scaffold by the introduction of a heteroaryl substituent (mainly 2-benzimidazolyl, 2-benzothiazolyl, 2-benzoxazolyl, and 2-pyridyl) at the C-3 position, and possibly a cyano group at the C-4 position of this coumarin moiety.[6] These structural modifications induce a 20–150 nm bathochromic displacement of the emission band, depending on the substitution pattern, and this is particularly relevant when considering these responsive fluorogenic reporters for use in various biosensing and bioimaging applications.[7] However, such π-extended coumarins are often more hydrophobic than the parent umbelliferone, they are more prone to self-aggregation, and they show lower fluorescence quantum yields, especially in aqueous media. Furthermore, the lack of a bioconjugatable handle (typically a carboxylic acid) within their core structure prevents their use as fluorescent labels for biomolecules. There is thus a real need to design bioconjugatable long-wavelength 7-hydroxycoumarins that are both soluble and strongly fluorescent in water. Unfortunately, most current strategies for enhancing the aqueous solubility of umbelliferone, based on the site-specific introduction of some hydrophilic groups (carboxylic or sulfonic acids, pyridine moieties, and PEG-type chains) at the C-3 or C-4 position of the oxabenzopyran scaffold,[8,9] cannot be applied to 3-heteroaryl derivatives. Two independent publications have described the synthesis of some heterocycle-substituted 7-hydroxycoumarin dyes equipped with a conjugatable carboxylic acid, and possibly with an anionic solubilizing moiety (Scheme 1).[10] However, the synthetic strategies used for their preparation rely on acid- or base-catalyzed condensation reactions that are difficult to generalize to a wide range of 3-(heteroaryl)umbelliferone derivatives (Scheme 1). Alternatively, more sophisticated water-solubilizing ap-

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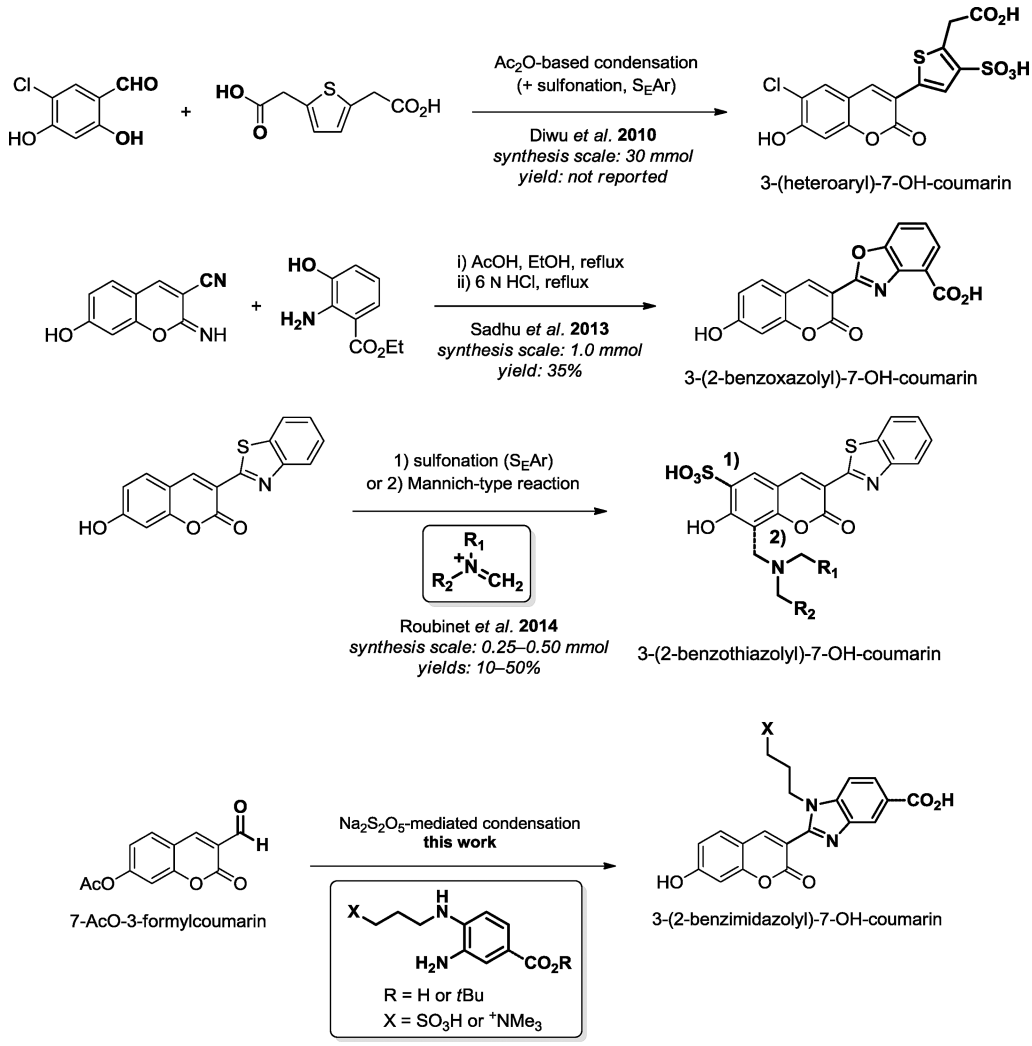
Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/ejoc.201403215.



Scheme 1. Background information about the synthesis of water-soluble and bioconjugatable 3-heteroaryl-7-hydroxycoumarin dyes, and the synthetic strategy explored in this work.

proaches have recently been explored, especially by our group. Electrophilic aromatic sulfonation of the C-6 position of 3-(2-benzothiazolyl)-7-hydroxycoumarin, and Mannich-type reactions to functionalize the C-8 position with an aminomethyl arm derived from a hydrophilic secondary amine (i.e., iminodiacetic acid, sarcosine, or isonipecotic acid) have led to water-soluble 7-hydroxycoumarins with unprecedentedly high fluorescence quantum yields under physiological conditions (Scheme 1).[11,12] However, the presence of these polar substituents in close proximity to the 7-OH group makes the synthesis of reaction-based fluorescent probes through chemical modification of the phenol moiety difficult.[12] Furthermore, these water-solubilizing groups negatively influence both the stability and the fluorogenic reactivity of the resulting profluorophores in aqueous buffers. To overcome the difficulties associated with these synthetic methods, a substantially different approach based on the facile functionalization of the 3-heteroaryl moiety of such long-wavelength coumarins with anionic or cationic hydrophilic groups (trimethylalkylammonium, carboxylate, or sulfonate), and possibly with a bioconjugatable arm (typically a carboxylic acid), should be considered. The goal would be to impart to 3-(heteroaryl)-umbelliferone derivatives both water solubility and the possibility for bioconjugation without compromising the (fluorogenic) reactivity of their phenol moiety. Inspired by recent work by Yoon et al.[24] on the efficient synthesis of 1,2-disubstituted benzimidazoles through a condensation reaction between an *ortho*-phenylenediamine derivative and an aromatic aldehyde, we have devised a concise synthetic route to functionalized 3-(heteroaryl)-7-hydroxycoumarins using 7-acetoxy-3-formylcoumarin as the common starting material (Scheme 1).

In this paper, we report the practical implementation of this new synthetic strategy for the preparation of original water-soluble and/or bioconjugatable derivatives of 3-(2-benzimidazolyl)-7-hydroxycoumarin.[13–15] A further extension of this method to the direct synthesis of the unknown 3-(2-benzoselenazolyl)-7-hydroxycoumarin was also explored. To demonstrate the beneficial effects of the remoteness of water-solubilizing groups from the phenol moiety on the reactivity of these new fluorogenic dyes, some thiol-

sensitive profluorophores were synthesized, and their fluorescence behavior was studied in detail. Finally, the bioconjugation ability of this new family of heterocycle-substituted coumarins was also confirmed through the preparation of Förster resonance energy transfer (FRET)-based probes suitable either for biothiol sensing or in vitro/in cellulo detection of urokinase-type plasminogen activator (uPA), a serine protease known to play an important role in tumor-associated proteolysis.[16]

Results and Discussion

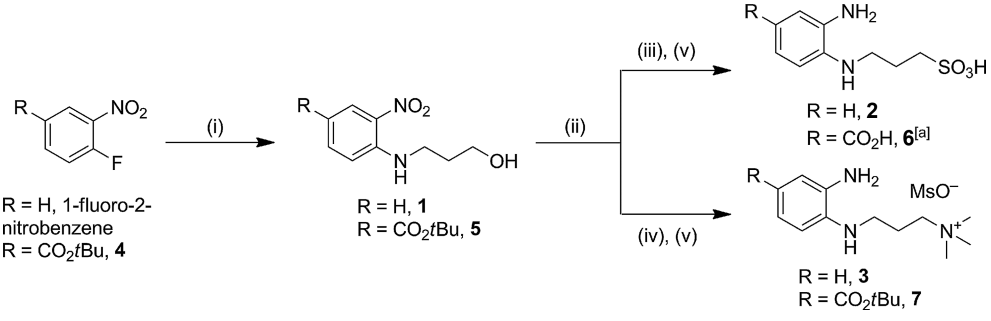
Numerous synthetic methods are currently available for the preparation of 3-(heteroaryl)coumarins [especially 3-(2-benzimidazolyl) derivatives]. These include Pechmann, Perkin, or Knoevenagel reactions,[14,17] Pd-catalyzed cross-coupling reactions,[18] condensation reactions of 2-chloro-2-arylacetaldehyde with salicylaldehyde catalyzed by *N*-heterocyclic carbenes (NHC),[19] BBr_3-induced domino reactions starting from homophthalic anhydride and a 2-methoxybenzaldehyde derivative,[20] and acid-catalyzed condensation reactions between an *ortho*-phenylenediamine derivative and a 3-(ethoxycarbonyl)coumarin.[21] However, most of these methods are not really suitable for the introduction of charged functional groups into the heteroaryl moiety, and they are not easily transferable to more sensitive 7-hydroxycoumarin derivatives. Since the key step of our synthetic strategy is a condensation reaction between an *N*-monosubstituted aryl diamine and the known 7-acetoxy-3-formylcoumarin (Scheme 1), our initial efforts were devoted to the development of an efficient and versatile synthetic route to hydrophilic *ortho*-phenylenediamine derivatives bearing a hydrophilic substituent.

Synthesis of Hydrophilic *ortho*-Phenylenediamine Derivatives

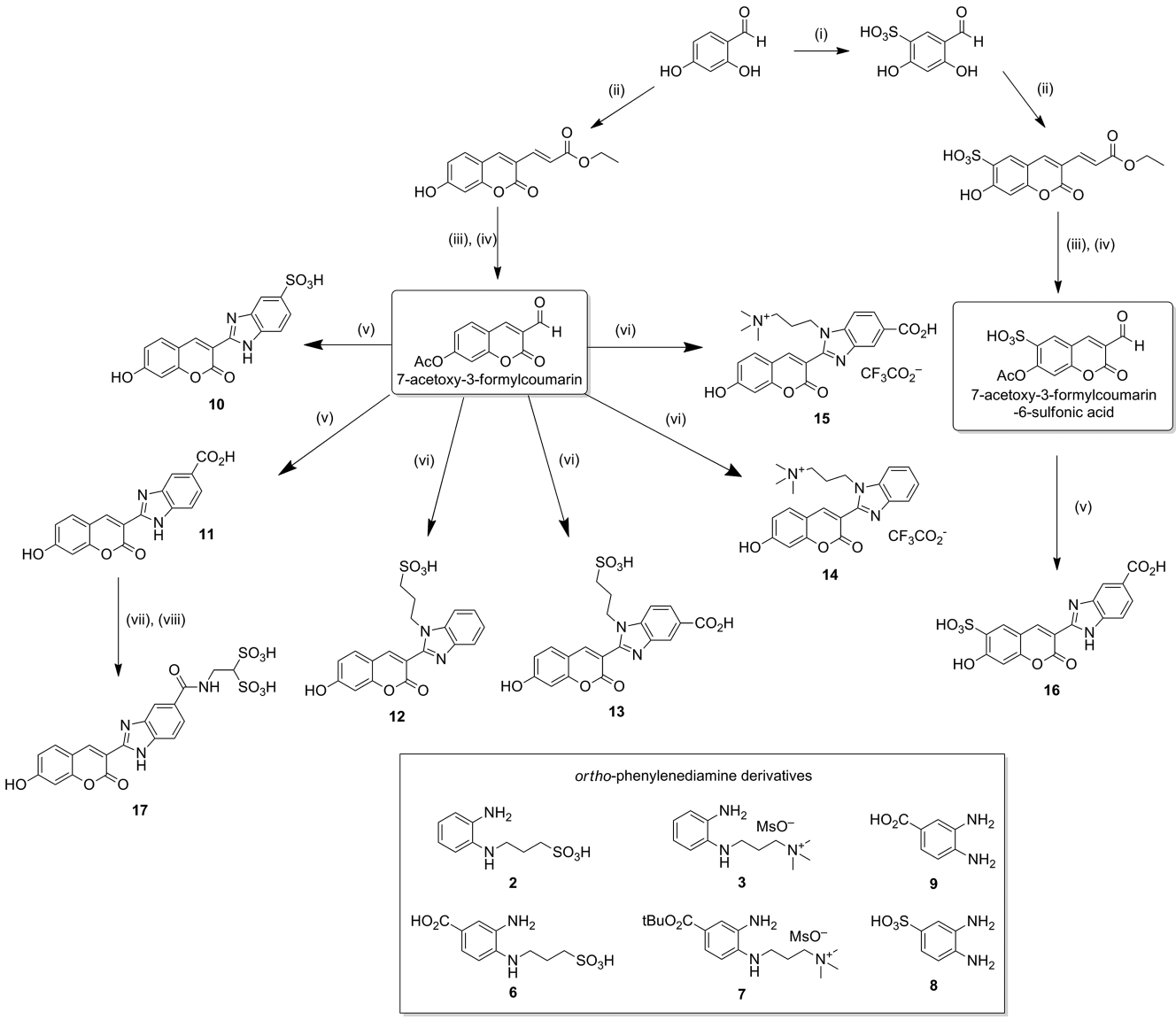
The synthesis of *ortho*-phenylenediamine derivatives **2**, **3**, **6**, and **7** is shown in Scheme 2. The introduction of a single trimethylammonium- or sulfonate-terminated alkyl chain substituent onto one of the two anilines was achieved through an S_N2 reaction of trimethylamine (TMA) or sodium sulfite with a mesylate derived from the corresponding primary alcohol (i.e., **1** or **5**). To access these alcohols, an S_NAr reaction between *ortho*-nitro-fluorobenzene derivatives and 3-aminopropan-1-ol was carried out. This reaction worked well under mild conditions. The final step of this synthetic route was the reduction of the nitro group by catalytic hydrogenation over Pd/C, to install the primary aniline of the target *ortho*-phenylenediamines **2**, **3**, **6**, and **7** (overall yields: 52, 55, 20, and 27%, respectively). Concerning the preparation of derivative **6**, which bears a free carboxylic acid, a further TFA (trifluoroacetic acid)-mediated deprotection step was required for the complete removal of the *tert*-butyl ester from its nitro precursor. The premature partial cleavage of this protecting group occurred during the S_N2 reaction with sodium sulfite, and thus we decided to isolate the free carboxylic acid form (for the synthesis of the coumarin, vide infra) by chromatography over reverse-phase silica gel (for synthetic details and analytical data, see the Supporting Information).

Synthesis of Water-Soluble 3-(2-Benzimidazolyl)-7-hydroxycoumarins − Extension to Unknown 3-(2-Benzoselenazolyl)-7-hydroxycoumarin

Condensation of *ortho*-phenylenediamine derivatives, including the *N*-monosubstituted derivatives described above and bis-primary anilines such as 3,4-diaminobenzenesulfonic acid **8**[22] and 3,4-diaminobenzoic acid **9**, with 7-acetoxy-3-formylcoumarin[23] was achieved in DMSO at 120 °C for 3 h to give six new 3-(2-benzimidazolyl)-7-hydroxycoumarins **10**–**15** (Scheme 3). The successful synthesis of the 6-sulfonated derivative of 7-acetoxy-3-formylcoumarin according to the three-step procedure reported by Lim et al.[23] (see Scheme 3 and the Supporting Information for synthetic details) enabled us to achieve the preparation of a further water-soluble derivative (compound **16**). Except for compounds **10**, **11**, and **16**, this reaction required the



Scheme 2. Synthesis of *ortho*-phenylenediamine derivatives. Reagents and conditions: i) 3-aminopropan-1-ol (1.6 equiv.), DIEA (diisopropylethylamine; 1.2 equiv.), CH_2Cl_2, room temp., overnight, 80% (for **1**) and 58% (for **5**); ii) MsCl (1.1 equiv.), TEA (triethylamine; 1.2 equiv.), dry CH_2Cl_2 (0 °C), 1 h, 94–96%; iii) Na_2SO_3 (5 equiv.), EtOH/H_2O (2:1, v/v), reflux, 12 h, 72% (for nitro intermediate leading to **2**) and 37% (for nitro intermediate leading to **6**; premature cleavage of the *t*Bu ester occurred during this reaction step); iv) TMA, THF (1.0 M solution; 5 equiv.), reflux, 12 h, 76% (for nitro intermediate leading to **3**) and 51% (for nitro intermediate leading to **7**); v) H_2, Pd/C (10%), MeOH, room temp., 3 h, 97% (for **2**, **3**, **6**, and **7**); [a]A further treatment with CH_2Cl_2/TFA (5:2, v/v), 0 °C to room temp., 1 h, between steps (iii) and (v) was used to completely remove the *t*Bu ester.



Scheme 3. Synthesis of water-soluble 3-(2-benzimidazolyl)-7-hydroxycoumarin dyes. Reagents and conditions: i) oleum (6 equiv.), 0 °C, 30 min, then room temp., 2 h, 54%; ii) diethyl glutaconate (1 equiv.), piperidine (1.2 equiv.), EtOH, reflux, 24 h, 67% (for intermediate leading to 7-acetoxy-3-formylcoumarin) and 42% (for intermediate leading to 7-acetoxy-3-formylcoumarin-6-sulfonic acid); iii) Ac_2O (60 equiv.), pyridine, room temp., 1 h, 88% (for intermediate leading to 7-acetoxy-3-formylcoumarin) and 97% (for intermediate leading to 7-acetoxy-3-formylcoumarin-6-sulfonic acid); iv) OsO_4 (4% in water), $NaIO_4$ (2.9 equiv.), THF, room temp., 4 d, 79% (for 7-acetoxy-3-formylcoumarin) and 65% (for 7-acetoxy-3-formylcoumarin-6-sulfonic acid); v) *ortho*-phenylenediamine **8** or **9** (1 equiv.), DMSO, 120 °C, 3 h, 19% (**10**), 20% (**11**), and 25% (**16**); vi) *ortho*-phenylenediamine **2**, **3**, **6**, or **7** (1 equiv.), $Na_2S_2O_5$ (1 equiv.), DMSO, 120 °C, 3 h, 23% (**12**), 21% (**13**), 24% (**14**) and 23% (**15**); vii) DCC (5.3 equiv.), NHS (5.3 equiv.), NMP (*N*-methyl-2-pyrrolidone), room temp., 2 h; viii) **11**-NHS (1 equiv.), TBA^+ salt of 2-aminoethane-1,1-disulfonic acid (13 equiv.), DIEA (5 equiv.), NMP, 0 °C then room temp., overnight, 28%; 3-(2-benzimidazolyl)-7-hydroxycoumarin derivatives were isolated as TEA salts (**10**–**13** and **16**) or TFA salts (**14** and **15**), except for **17** (acid form, after Dowex H^+ desalting).

addition of sodium metabisulfite ($Na_2S_2O_5$) to take place. As suggested by Yoon et al.,[24] this unusual additive may favor the in situ formation of the more reactive bisulfite adduct of the aromatic aldehyde derived from umbelliferone. Furthermore, it is important to point out that the temporary protection of the 7-OH group of 3-formyl-umbelliferone as an acetate ester was also found to be beneficial to enhancing the electrophilic reactivity of the aldehyde, even if its premature cleavage occurred during the reaction due to the severe conditions of heating. For coumarin synthesis involving *ortho*-phenylenediamine **7**, these reaction conditions also led to the removal of the *tert*-butyl protecting group to reveal the carboxylic acid. Due to the high polarity of the resulting 3-heteroaryl-coumarins, all reaction mixtures were purified by semipreparative RP (reverse-phase) HPLC or using an automated flash-purification system equipped with an RP-C_{18} cartridge, with aqueous triethylammonium hydrogen carbonate buffer (TEAB; 50 mM, pH 7.5) and CH_3CN as eluents to recover compounds **10**–**13** and **16** in a pure form. Indeed, our first attempts at puri-

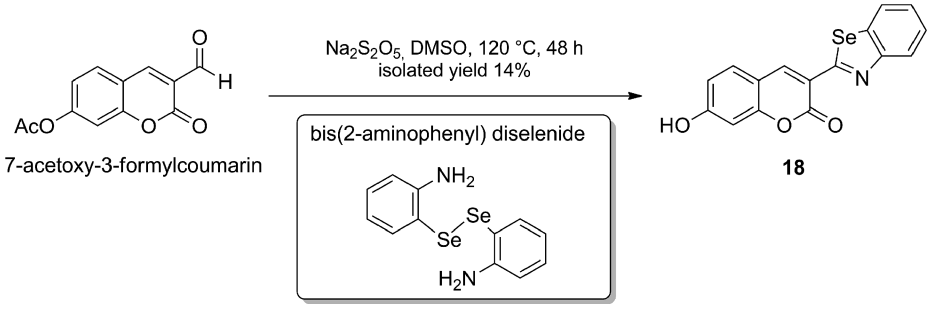
fication by reverse-phase chromatography using a more conventional aqueous mobile phase [TFA (0.1% aq.), pH 2.0] partly failed due to nonspecific adsorption of these polar compounds over the stationary phase under the acidic conditions. The use of this TEAB buffer was found to be the only way to achieve acceptable yields of the desired water-soluble 3-(heteroaryl)-7-hydroxycoumarins. On the other hand, compounds **14** and **15** could be recovered using aq. TFA and CH_3CN as eluents. However, all the isolated yields were modest (in the range 20–25%), which is in contrast to the good conversions (in the range 70–80%), as determined by RP-HPLC analysis of the crude reaction mixtures (data not shown). This is related to the loss of material during the RP chromatographic purification and lyophilization steps.

To dramatically improve the solubility of 3-(2-benzimidazolyl)-7-hydroxycoumarin **11** in aqueous media, and to check the reactivity of its benzoic acid moiety, we next investigated a "post-synthetic" sulfonation process through amidification of its carboxylic acid with 2-aminoethane-1,1-disulfonic acid.[25] This synthesis involves the initial conversion of the carboxylic acid of **11** into the corresponding *N*-hydroxysuccinimidyl (NHS) active ester by treatment with an excess of DCC (*N*,*N*′-dicyclohexylcarbodiimide) and *N*-hydroxysuccinimide. Despite its lower reactivity, this reagent was preferred over more effective uronium- or phosphonium-based coupling agents [i.e., TSTU (2-succinimido-1,1,3,3-tetramethyluronium tetrafluoroborate), PyBOP (benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate), or PyBrOP (bromo-tris-pyrrolidinophosphonium hexafluorophosphate)] because these latter reagents are known to readily react with the phenol moiety of umbelliferone derivatives (7-hydroxycoumarins, vide infra, Scheme 8). After this activation step, subsequent reaction with 2-aminoethane-1,1-disulfonic acid (tributylammonium, TBA^+ salt) led to the desired disulfonated 7-hydroxycoumarin (i.e., **17**), which was purified by flash chromatography over reverse-phase silica gel using aqueous TEAB and CH_3CN as eluents, followed by desalting with Dowex H^+ resin. The structures of all of the hydrophilic 3-(2-benzimidazolyl)-7-hydroxycoumarins were confirmed by detailed measurements, including ESI-HRMS and NMR spectroscopic analysis (see the Supporting Information for the corresponding spectra). Furthermore, the purity of each compound (determined by RP-HPLC analysis) was found to be equal to or above 95%, and thus suitable for an accurate and reliable determination of their photophysical properties.

Next, we wanted to extend this promising strategy to the preparation of 3-(2-benzoselenazolyl)-7-hydroxycoumarin (Scheme 4), especially to assess the effects of the heteroatom on the spectral properties of the 3-(heteroaryl)-7-hydroxycoumarin scaffold. By analogy with the recent work of Conley et al. on the synthesis and bioluminescence properties of a selenium analog of firefly D-luciferin,[26] we hypothesized that this atom replacement [Se instead of N/S of already known 3-(heteroaryl)-7-hydroxycoumarins] would result in a redshift of the absorption/emission maxima of the fluorescent heterocyclic scaffold. Although there is growing interest in the synthesis of organoselenium-based molecular dyes and their applications, particularly in photodynamic therapy (PDT) or as fluorescent probes for the detection of various endogenous (bio)analytes in physiological systems,[27] this selenium-containing coumarin dye has never been reported in the literature. Commercially available bis(2-aminophenyl)diselenide was treated with 7-acetoxy-3-formylcoumarin under the same conditions as described above, to give the target 3-(2-benzoselenazolyl)-7-hydroxycoumarin (i.e., **18**), which was isolated by conventional silica gel column chromatography in a modest 14% yield. In this case, $Na_2S_2O_5$ promoted the Se–Se bond cleavage, probably through the radical anion $SO_2^{\cdot-}$ generated from $S_2O_5^{2-}$ by heating.[28]

Photophysical Properties of Water-Soluble 3-(2-Benzimidazolyl)-7-hydroxycoumarin Dyes

All the synthesized coumarin dyes (except **18**) have excellent water solubility (greater than 10 mM). Thus, their photophysical properties were evaluated in phosphate-buffered saline (PBS; pH 7.5), which mimics physiological conditions, and the results are compiled in Table 1 (see the Supporting Information for the corresponding spectra). In order to compare their spectral behavior in aqueous and organic media, further measurements in DMSO were also carried out. As previously reported by Deligeorgiev et al.,[15] the introduction of a 2-benzimidazolyl substituent onto the C-3 position of umbelliferone led to a long-wavelength 7-hydroxycoumarin with absorption/emission max-



Scheme 4. Synthesis of 3-(2-benzoselenazolyl)-7-hydroxycoumarin.

Table 1. Spectral properties of water-soluble 3-(2-benzimidazolyl)-7-hydroxycoumarin derivatives at 25 °C.

Dye	Solvent	Absorption λ_{max} [nm][a]	Emission λ_{max} [nm]	Φ_F [%][b]
3-(2-BZM)-7-OH[c]	pH 7.0	420	480	22
	pH 8.0			61
3-(2-BZM)-7-OH	PBS	417	477	57
3-(2-BZM)-7-OH	DMSO	390	493	78
10	PBS	420	476	66
10	DMSO	389	484	60
11	PBS	421	478	59
11	Tris·HCl pH 8.0	424	478	69
11	DMSO	389	474	70
12	PBS	392	467	78
12	DMSO	350	478	79
13	PBS	394	466	78
13	DMSO	350	471	84
14	PBS	395	467	77
14	DMSO	350	480	78
15	PBS	396	468	73
15	DMSO	351	459	83
16	PBS	426	481	57
16	DMSO	389	474	76
17	PBS	426	477	67
17	DMSO	388	474	72

[a] Most of the dyes were not obtained in sufficient amounts for highly accurate measurements of molar absorption coefficients. [b] Determined at 25 °C by using 7-hydroxycoumarin [Φ_F = 76% in phosphate buffer (PB), pH 7.4, λ_{ex} = 390 nm] as standard.[50] [c] BZM = benzimidazolyl, spectral properties reported by Deligeorgiev et al.[15]

ima close to 420/480 nm in PBS, and 390/495 nm in DMSO. Further functionalization of the heteroaryl scaffold with a carboxylic or sulfonic acid group has no significant effect on the position of the absorption/emission bands (see compounds **10** and **11**), and good fluorescence quantum yields in the range 60–70% (both in PBS and DMSO) are retained. Contrary to our previous observations with 3-(2-benzothiazolyl)-7-hydroxycoumarin,[12] *ortho*-sulfonation of the phenol moiety does not lead to a significant increase of the quantum yield (see compound **16**). The presence of the more hydrophilic benzimidazole heterocycle (compared to a benzothiazolyl ring) is probably a major factor in the resistance to aggregation-induced fluorescence quenching of this 3-heteroaryl-7-hydroxycoumarin scaffold. The post-synthetic derivatization of the carboxylic acid of **11** with 2-aminoethane-1,1-disulfonic acid leads to compound **17**, which also shows a good fluorescence quantum yield under physiological conditions, close to 70%. Interestingly, *N*-alkylation of the benzimidazolyl moiety with a propyl chain terminated by a trimethylammonium group or a sulfonic acid induces hypsochromic shifts of ca. 30 nm for the absorption maximum and ca. 10 nm for the emission maximum of the 3-heteroaryl-7-hydroxycoumarin (see compounds **12**–**15**). Furthermore, both of these polar *N*-substituents have a positive impact on the fluorescence efficiency of the resulting hydrophilic coumarinic scaffold. Indeed, a 33% increase in quantum yield compared to parent 3-(2-benzimidazolyl)-7-hydroxycoumarin was observed, which can partly explained by the fact that such ionized/ionizable groups prevent the aggregation of the dye molecules and/or the formation of nonemitting dimers in aqueous solutions. Similar trends were observed in DMSO, although larger Stokes' shifts were obtained. This set of results clearly shows that a water-solubilizing approach based on the functionalization of the benzimidazolyl moiety with hydrophilic groups is effective for obtaining long-wavelength 7-hydroxycoumarins with strong cyan-green emission in physiological media.

Some photophysical properties of the parent 3-(2-benzimidazolyl)-7-hydroxycoumarin and its thio and seleno analogs [i.e., 3-(2-benzothiazolyl)-7-hydroxycoumarin and **18**, respectively] were also determined both in phosphate buffer (PB, pH 7.4) and DMSO, and the results are given in Table 2. In DMSO, a 14 nm bathochromic shift for the benzothiazole derivative, and a 19 nm bathochromic shift for **18** at the wavelength of the absorption maximum can be observed as the heteroatom becomes larger. The Stokes' shift is of the same order of magnitude (75–80 nm) for **18** and 3-(2-benzothiazolyl)-7-hydroxycoumarin, but that for the benzimidazolyl derivative is higher. As the chalcogen atom becomes larger, spin-orbit (heavy-atom) effects promote triplet formation over fluorescence, and Φ_F dramatically decreases from 81 to 13%.[29] Despite a low solubility in water (limited to the micromolar concentration range), the spectral characteristics of **18** were next measured in PB. A very broad absorption spectrum that does not match with the corresponding excitation curve was obtained (see the Supporting Information). This trend may suggest the presence of aggregates rather than the prevalence of the weakly emitting phenol form of **18**. Indeed, the pK_a of the phenol moiety of **18** was evaluated by monitoring the fluorescence emission of this fluorophore as a function of the environmental pH, and it was found to be slightly different from

Table 2. Spectral properties of 3-(heteroaryl)-7-hydroxycoumarin dyes at 25 °C.

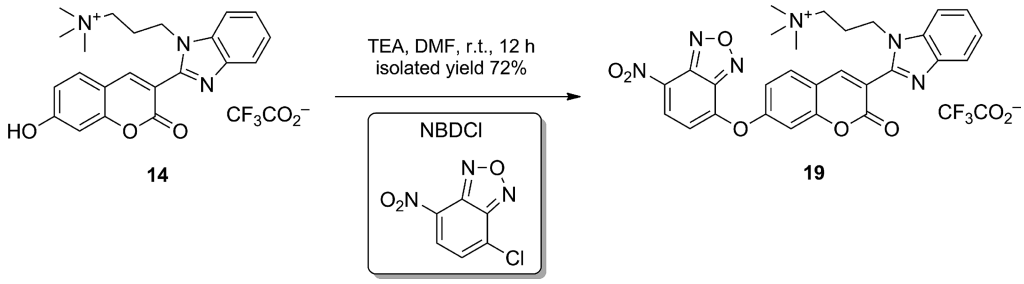
Dye	Solvent	Absorption λ_{max} [nm][a]	Emission λ_{max} [nm]	Φ_F [%][b]
3-(2-BZM)-7-OH	PBS	417	477	57
3-(2-BT)-7-OH[b]	PBS	430	486	43
18	PB	439	492	–[c]
18	PB + 5% BSA	473	495	60
18	H_2O + 2.5% SDS[d]	400	475	5
3-(2-BZM)-7-OH	DMSO	383	478	78
3-(2-BT)-7-OH[b]	DMSO	397	472	81
18	DMSO	402[e]	477	13
18	EtOH	399[e]	473	11

[a] Determined at 25 °C by using 7-hydroxycoumarin (Φ_F = 76% in PB, pH 7.4, λ_{ex} = 390 nm) as standard,[50] except for measurement in PB + 5% BSA [standard: fluorescein in NaOH (0.1 N), Φ_F = 95%, λ_{ex} = 440 nm].[51] [b] BT = benzothiazolyl. [c] A nonlinear relationship between fluorescence emission and absorption at 390 nm was obtained, and this prevented an accurate determination of the relative quantum yield in PB. [d] pH = 6.9, measurements with this additive were performed in water due to its poor solubility in PB. [e] Molar absorption coefficients: 36880 $M^{-1}cm^{-1}$ in DMSO, and 35125 $M^{-1}cm^{-1}$ in EtOH; SDS = sodium dodecyl sulfate buffer.

that of its benzimidazolyl and benzothiazolyl analogs (6.7 compared with 6.9 and 7.0, see the Supporting Information). This hypothesis was further supported by the fact that a nonlinear relationship between the fluorescence emission and the absorption value (at the wavelength used for excitation) was observed, which prevented the determination of the fluorescence quantum yield. However, the strong cyan-green fluorescence of **18** was recovered by adding 5% (w/v) of bovine serum albumin (BSA), an additive often used in buffers to mimic body fluids (Φ_F = 60%). Indeed, this protein is known to enhance the emission of many fluorophores owing to a combination of rigidification, a reduction in the polarity of the dye's microenvironment (binding in the hydrophobic BSA pocket), and deaggregation.[30] BSA also had a marked effect on both the shape and the position (redshifted) of the absorption band of this fluorescent phenol. A more comprehensive study of spectral properties of 3-(2-benzoselenazolyl)-7-hydroxycoumarin aimed at assessing its potential as a fluorogenic dye will be carried out, and the results will be reported in due course.

Water-Soluble Profluorophore for Thiol Sensing

In order to both demonstrate the potential utility of the new hydrophilic 3-(2-benzimidazolyl)-7-hydroxycoumarin derivatives and assess the possible benefits of the larger distance between the phenol and water-solubilizing moieties on their fluorogenic reactivity, we explored the synthesis and sensing ability of thiol-sensitive profluorophores. These probes are based on the reversible alkylation of the 7-OH group with quenching moieties that may incur photo-induced electron transfer (PeT) or strongly influence the internal charge transfer (ICT) state of the fluorophore.[31,32] As an illustrative example, phenol-based fluorophore **14** was converted into a thiol-sensitive fluorogenic probe using 7-nitro-2-oxa-1,3-diazole (NBD) as the quenching moiety (Scheme 5). NBD ethers have recently emerged as electrophilic triggers for reaction-based probes; they allow the dual colorimetric and fluorogenic detection of the gasotransmitter hydrogen sulfide (H_2S).[32,33,34] The preparation of phenolic ether **19** was achieved by reaction of **14** with NBD chloride (NBDCl) in the presence of TEA in dry DMF at room temperature (Scheme 5). This NBD probe was recovered in a pure form and in good yield (73%) by semipreparative RP-HPLC. This NBD ether is essentially nonfluorescent in both PBS and DMSO (see the Supporting Information), and its sensing response to thiols [Cys, thiophosphate (ThioPi), and HS^-, the predominant form of H_2S at pH 7.5] was also studied through time-dependent UV/Vis absorption and fluorescence analysis (Figure 1). Surprisingly, thiophosphate anion appeared to be the most reactive sulfhydryl analyte towards the NBD electrophilic site, since a plateau for fluorescence intensity at 480 nm was reached within 30 min, compared to 60 min for Cys (Figure 1, B). The fluorescence enhancement estimated by the emission intensity ratio of **19** at 480 nm in the presence and absence of added thiols, I/I_0, was 100 for ThioPi and 65.4



Scheme 5. Synthesis of 7-*O*-NBD fluorogenic ether **19**.

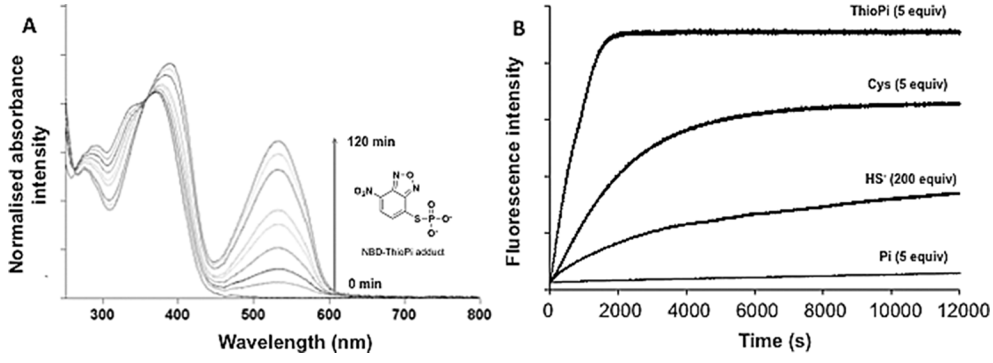


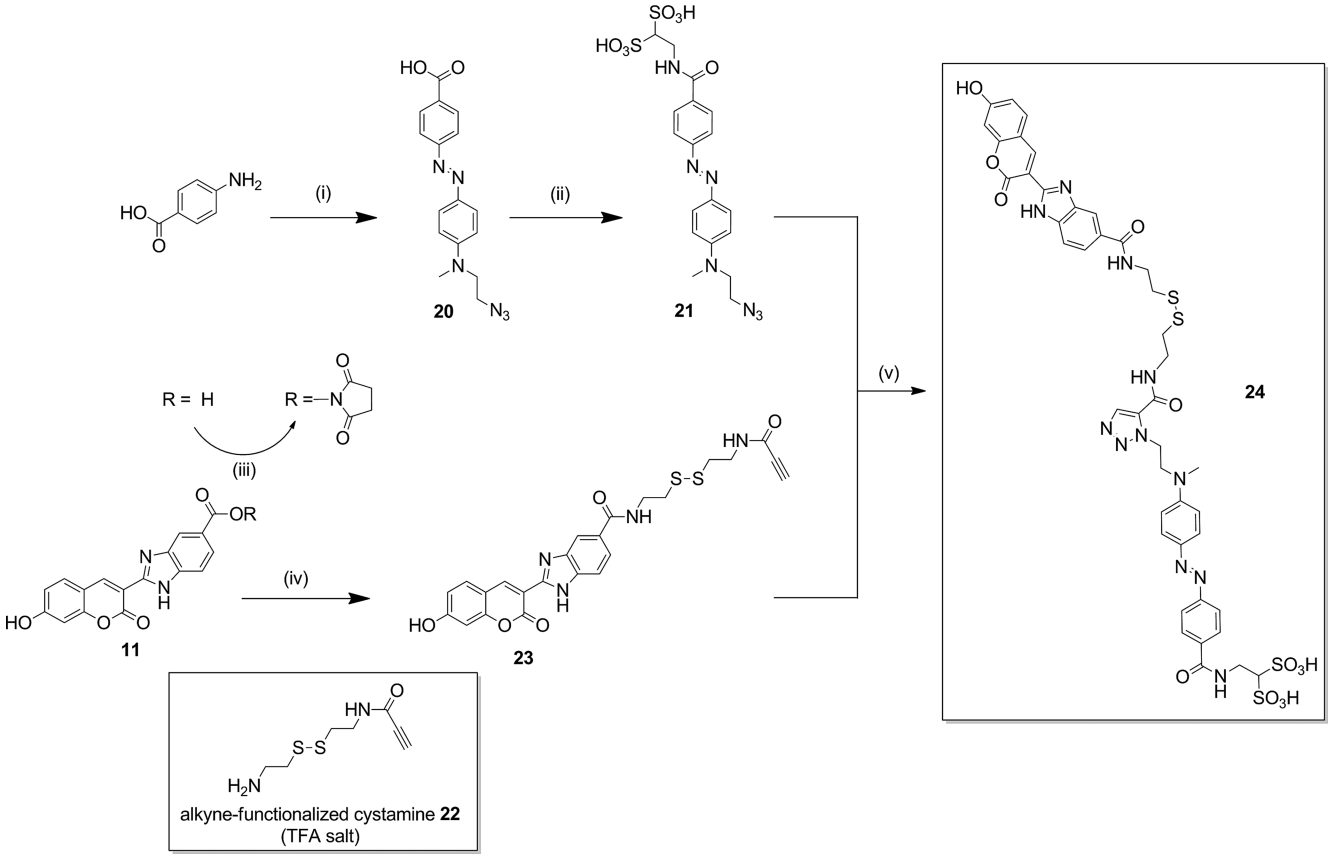
Figure 1. (A) Time-dependent changes in the UV/Vis spectrum of NBD probe **19** (8.6 μM) in PBS (100 mM phosphate + 150 mM NaCl, pH 7.5), in the presence of ThioPi (Na salt, 5 equiv.) at 25 °C. (B) Time-dependent fluorescence intensity of NBD probe **19** (1.1 μM) at 480 nm (λ_{ex} = 390 nm) in the presence of sulfhydryl analytes: Cys (5 equiv.), ThioPi (Na salt, 5 equiv.) and HS^- (Na salt, 200 equiv.), recorded in PBS at 25 °C; a further kinetics curve for Pi (K salt, 5 equiv.) was recorded under the same conditions.

for Cys. The thiolysis of NBD probes such as **19** proceeds by an S_NAr mechanism (addition-elimination process). In this case, this was supported by changes observed in the UV/Vis spectrum of **19** upon addition of ThioPi (Figure 1, A). The appearance of two strong absorption bands at 395 and 550 nm was observed, which were assigned to free 7-hydroxycoumarin **14** and the NBD-ThioPi adduct, respectively. Kinetic fluorescence measurements achieved with NaHS (200 equiv.) and nonnucleophilic phosphate anion (Pi; 50 equiv., used as a negative control) revealed that NBD probe **19** shows a moderate reactivity toward H_2S (I/I_0 = 52, but only after a prolonged incubation time and at much higher concentrations), and is not prone to hydrolysis at physiological pH. This preliminary biosensing application has enabled us to demonstrate that water-soluble 3-(2-benzimidazolyl)-7-hydroxycoumarins unsubstituted at their C-6 and/or C-8 positions can easily be converted into reaction-based fluorescent probes through chemical modification of their 7-OH group with various chemical moieties that can act as both the quencher and the fluorescence trigger.

Water-Soluble FRET Probe for Thiol Sensing

Since our synthetic method also gave access to water-soluble 3-(2-benzimidazolyl)-7-hydroxycoumarins bearing a bioconjugatable handle (i.e., a carboxylic acid), we also explored the preparation of thiol-sensitive fluorogenic probes based on the covalent association of a fluorophore of this type with a dark quencher chromophore (i.e., a nonfluorescent acceptor), together making up a FRET pair,[35] through a thiol-reactive linker (namely a disulfide bridge). In this way, we demonstrate the utility and reactivity of this additional functional group.

We focused on the preparation of FRET probe **24**. In this compound, coumarin-carboxylic acid **11** is linked to an original water-soluble analog of azo-based quencher DABCYL {4-([4-(dimethylamino)phenyl]azo)benzoic acid}, i.e., **21** ($\Delta\lambda_{1/2\ max}$ value of 112 nm, corresponding to a quenching range 394–506 nm, and fully compatible with the emission of **11**, centered at 478 nm), through a cleavable disulfide spacer (Scheme 6). The synthesis of azo-based quencher **21** was achieved as follows: in situ formation of



Scheme 6. Synthesis of thiol-sensitive FRET-based probe **24**. Reagents and conditions: i) a) $NOBF_4$ (1.1 equiv.), CH_3CN, 0 °C, 15 min; b) *N*-(2-azidoethyl)-*N*-methylaniline (1.2 equiv.), CH_3CN, 0 °C, 30 min, 86% (for the two steps a and b); ii) a) TSTU (1.3 equiv.), DIEA (3 equiv.), NMP, room temp., 30 min; b) TBA^+ salt of 2-aminoethane-1,1-disulfonic acid (10 equiv.), DIEA (3 equiv.), 0 °C then room temp., 2 h, 61% (for the two steps a and b); iii) DCC (5.3 equiv.), NHS (5.3 equiv.), NMP, room temp., 2 h; iv) alkyne-functionalized cystamine **22** (8.5 equiv.), NMP, DIEA (5 equiv.), overnight, 25% (for the two steps iii and iv); v) **21** (1.1 equiv.), **23** (1 equiv.), Cu^0 (0.5 equiv.), $CuSO_4$ (0.1 equiv.), DMSO/H_2O (2:1, v/v), 50 °C, 2 h, 48%; compounds were isolated as TEA salt (**24**), TFA salt (**23**) or in acid form after Dowex H^+ desalting (**21**).

the diazonium salt of *para*-aminobenzoic acid was readily achieved using the bench-stable, commercially available nitrosating agent nitrosonium tetrafluoroborate ($NOBF_4$) in dry CH_3CN.[36] Subsequent reaction with an electron-rich tertiary aniline, namely *N*-(2-azidoethyl)-*N*-methylaniline, led to azido-acid azo dye **20**. "Post-synthetic" sulfonation through amidification of its carboxylic acid with 2-aminoethane-1,1-sulfonic acid was achieved under conditions similar to those used for the synthesis of disulfonated 7-hydroxycoumarin **17**, except that the activation step involved the use of TSTU/DIEA in NMP, to give water-soluble "clickable" DABCYL analog **21** in a good overall yield (53%). On the other hand, a coupling reaction between 3-(2-benzimidazolyl)-7-hydroxycoumarin **11** and alkyne-functionalized cystamine **22**[37] was carried out under conditions previously optimized for the synthesis of **17** (vide supra), to give, after semipreprative RP-HPLC, the second molecular partner (i.e., **23**) for the copper-catalyzed azide–alkyne 1,3-dipolar cycloaddition (CuAAC).[38] This latter reaction was achieved using microsized Cu^0 in a mixture of DMSO/H_2O (2:1, v/v) at 50 °C.[39] The resulting disulfide-based FRET probe **24** was isolated in pure form by semipreparative RP-HPLC (yield 48%, purity 98%). Its structure was unambiguously confirmed by ESI-HRMS and NMR spectroscopic analysis (see the Supporting Information). The quenching efficiency (QE) of DABCYL analog **21** was measured as the difference in fluorescence (area under the emission curve) of FRET probe **24** before and after reductive cleavage of its disulfide bridge with a large excess of DTT (100 equiv.) in phosphate buffer (PB, pH 7.5), and expressed as a percentage (QE = 100 × [1 – I_0(em)/I(em)]). An excellent value of 98% was found. The fluorogenic reactivity of FRET probe **24** was next examined at pH 8.0 (Tris·HCl buffer) because the reductive disulfide bond cleavage mediated by Cys is known to be favored under slightly alkaline conditions (Figure 2, see also the Supporting Information for details of the same experiment performed in PB, demonstrating the poor fluorogenic reactivity of **24** towards Cys in this buffer). As expected, the optical response of **24** towards Cys is very fast, and a plateau for the fluorescence emission at 480 nm

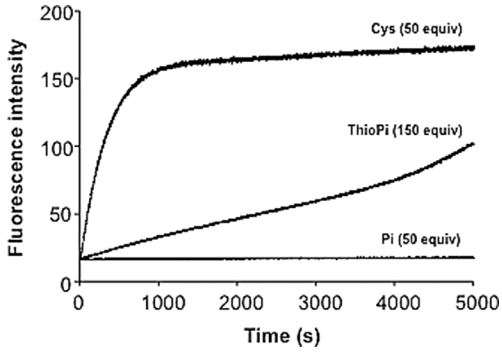
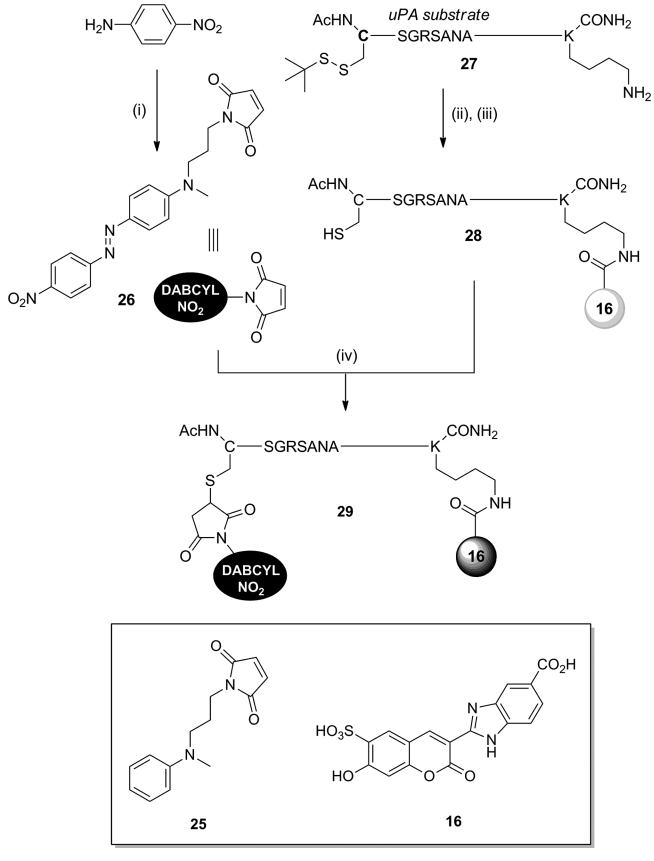


Figure 2. Time-dependent fluorescence intensity of disulfide-based FRET probe **24** (2.15 μM) at 480 nm (λ_{ex} = 390 nm) in the presence of sulfhydryl analytes: Cys (50 equiv.), and ThioPi (Na salt, 150 equiv.), recorded in Tris·HCl buffer (100 mM, pH 8.0) at 25 °C; a further kinetics curve for Pi (K salt, 50 equiv.) was recorded under the same conditions.

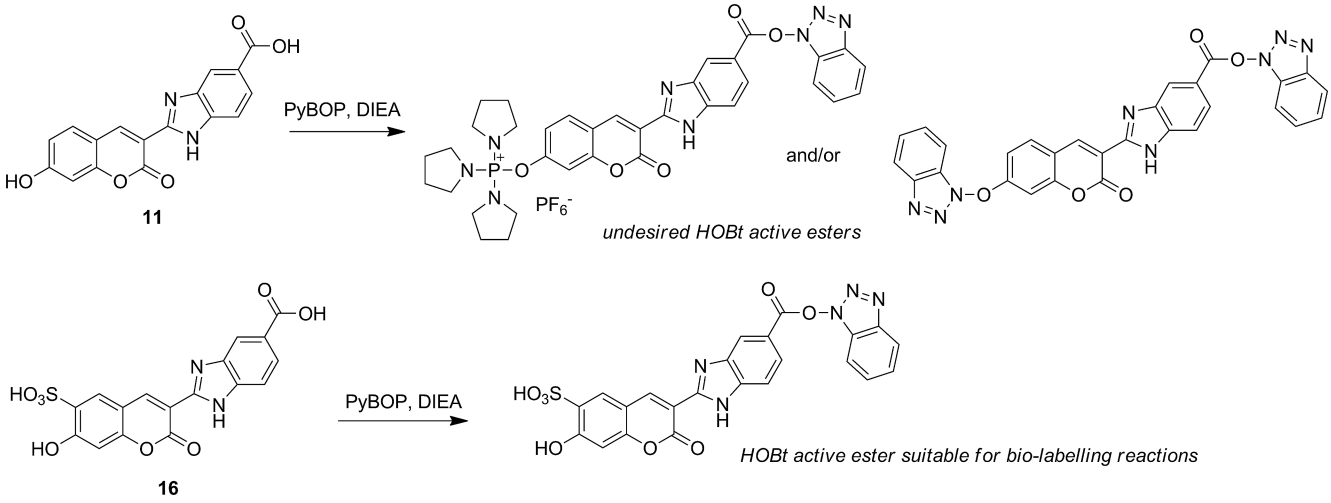
was obtained within 15 min of incubation. Conversely, 90 min was not enough time to obtain complete reaction when the ThioPi anion was used as reductant in a three-times larger amount. This is consistent with the lower oxidation–reduction potential of ThioPi (E'_0 = 0 V at pH 7.0) compared to that of Cys (E'_0 = –0.32 V at pH 7.0).[40] Once again, no increase in fluorescence was observed for the reaction involving the Pi anion, which proves the stability of this FRET probe at pH 8.0.

uPA-Sensitive FRET-Based Probe

As illustrated above, 3-(2-benzimidazolyl)-7-hydroxycoumarins functionalized with a carboxylic acid on their heteroaryl moiety are suitable reagents for covalent labeling.[2] Furthermore, their cyan-green fluorescence can be effectively switched off through the design of FRET probes involving a DABCYL derivative as the quencher. To expand the scope of these new bioconjugatable umbelliferone derivatives to more biologically relevant fluorescent probes, we next explored the preparation and in vitro validation of a FRET substrate for the detection of a serine protease,



Scheme 7. Synthesis of uPA-sensitive FRET-based probe **29**. Reagents and conditions: i) $NOBF_4$ (1.1 equiv.), CH_3CN, 0 °C, 15 min, then maleimide-terminated aniline **25**, 0 °C, 30 min, then NaOAc buffer (0.1 M, pH 4.0), 74%; ii) **16** (1.1 equiv.), PyBOP (1.1 equiv.), DIEA (5 equiv.), NMP, room temp., 2 h; iii) DTT (10 equiv.), $NaHCO_3$ (0.1 M aq.; pH 8.5), room temp., 1 h, 31% (for the two steps ii and iii); iv) NMP/$NaHCO_3$ (0.1 M aq.; pH 8.15), 2:1, v/v, room temp., 1 h, 62%.



Scheme 8. Comparative reactivity of bioconjugatable 3-(2-benzimidazolyl)-7-hydroxycoumarin **11** and **16** towards a phosphonium-based coupling reagent, namely PyBOP (generalized to all peptide coupling agents involving a basic catalysis: phosphonium and uronium salts). For clarity, only one of the three structures known for HOBt (hydroxybenzotriazole) ester[52] is shown.

namely urokinase-type plasminogen activator (uPA). This protease plays a critical role in malignancies, and its overexpression has been linked to poor clinical prognosis in breast cancer.[41] The ability to serially map uPA expression as a biomarker and through fluorogenic assays would thus have significant potential to improve new cancer therapies. Heptapeptide H-Ser-Gly-Arg-Ser-Ala-Asn-Ala-OH (also SGRSANA) has been reported to be a highly potent substrate of uPA (cleavage site between Arg and Ser residues);[42] therefore, we decided to label this peptide with a FRET pair consisting of 3-(2-benzimidazolyl)-7-hydroxycoumarin **16** as the fluorophore, and nitro-DABCYL derivative **26** as the quencher (Scheme 7). To achieve chemoselective labeling reactions, cysteine and lysine residues were incorporated at the *N*- and *C*-termini, respectively, as handles for the introduction of maleimide-DABCYL **26** and the amine-reactive fluorophore. Thus, peptide Ac-Cys(*St*Bu)-Ser-Gly-Arg-Ser-Ala-Asn-Ala-Lys-NH_2 **27** was readily obtained by standard solid-phase peptide synthesis (SPPS) techniques, and was doubly labeled according to a three-step procedure previously reported by us.[36] Interestingly, the use of a 6-sulfonated 7-hydroxycoumarin **16** whose phenol group is weakly reactive,[12] enabled us to carry out the acylation of the ε-amino group of **27** with this fluorescent carboxylic acid by using a phosphonium-based coupling reagent (PyBOP) and DIEA, which is in contrast to the amidification reactions involving 7-hydroxycoumarin **11** (vide supra and Scheme 8). The resulting uPA fluorogenic substrate (i.e., **29**) was isolated in pure form by semipreparative RP-HPLC (overall yield 20%, purity 100%). Its structure was unambiguously confirmed by ESI-MS analysis. The results from the fluorogenic cleavage assay with commercial uPA (from human urine) are summarized in Figure 3. Almost complete quenching (QE = 98%) was found for **29** until it was cleaved by uPA, which caused a 32-fold increase in fluorescence at λ_{em} = 480 nm over time (a plateau was reached within 120 min). As expected, no significant changes in the fluorescence signal were observed in the absence of protease.

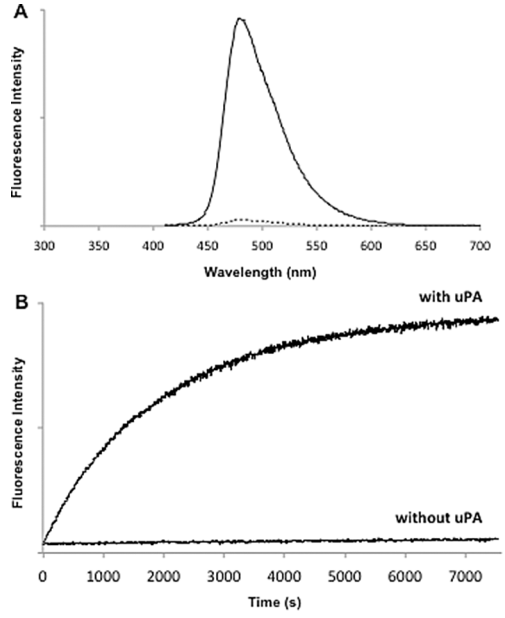


Figure 3. In vitro fluorescence-based assay of uPA-sensitive FRET-based probe **29**: (A) Fluorescence emission spectrum (λ_{ex} = 400 nm) of probe **29** in PBS before (dashed line) and after (solid line) incubation with uPA; (B) Time-dependent fluorescence intensity at 480 nm (λ_{ex} = 400 nm) of probe **29** (1.0 μM) with and without uPA from human urine (0.6 U, incubation time 25 min) recorded in PBS at 37.5 °C.

Conclusions

In this paper, we have reported the design, synthesis, and some biolabeling applications of a new family of water-soluble cyan-green emitting fluorophores based on the 3-(2-benzimidazolyl)-7-hydroxycoumarin scaffold. An original

synthetic pathway whose key step is a condensation reaction between 7-acetoxy-3-formylcoumarin and prefunctionalized *ortho*-phenylenediamine derivatives has, for the first time, enabled the introduction of both water-solubilizing moieties and a bioconjugatable handle onto the 3-heteroaryl substituent of these 7-hydroxycoumarin derivatives. Gratifyingly, high fluorescence quantum yields under physiological conditions were obtained. The versatility of this method was also demonstrated through the preparation of the unknown 3-(2-benzoselenazolyl)-7-hydroxycoumarin **18**, whose spectroscopic and biological properties are currently under investigation. In contrast to more conventional long-wavelength 7-hydroxycoumarins bearing a hydrophilic substituent at their C-6 or C-8 position, the lack of polar groups close to the phenol facilitates the preparation of thiol-sensitive profluorophores in good yields through etherification of 7-OH moiety with NBDCl. The high "fluorogenic" reactivity of "smart" fluorescent probe **19** toward Cys, ThioPi, and H_2S was proved by fluorescence-based in vitro assays. The reported water-solubilization strategy can be easily extended to other fluorescent phenols prefunctionalized with a formyl group, and should be particularly useful for developing biocompatible fluorogenic probes targeting nucleophilic (bio)analytes.[3,43] Furthermore, the introduction of a carboxylic acid within the benzimidazolyl substituent of these 7-hydroxycoumarins has allowed us to develop new coumarin-based reagents for fluorescent labeling of biomolecules, as demonstrated by the preparation of FRET probes reactive towards biothiols or proteases.

Experimental Section

General Remarks: Flash column chromatography was carried out on Geduran® Si 60 silica gel (63–200 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 (λ = 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. Bis(2-aminophenyl)diselenide was purchased from Fluorochem. All solvents were dried by standard procedures [CH_2Cl_2: distillation from P_2O_5; CH_3CN: distillation from CaH_2; absolute EtOH: storage over anhydrous Na_2SO_4, THF: distillation from sodium benzophenone diketyl; and triethylamine (TEA): distillation from KOH and storage over BaO]. Peptide synthesis grade NMP and anhydrous DMF were purchased from Carlo Erba, and stored over 4 Å molecular sieves. Peptide synthesis grade DIEA was provided by Iris Biotech GmbH. HPLC gradient grade acetonitrile (CH_3CN) was obtained from VWR. Aqueous buffers [PB, PBS, and aqueous 0.1 M acetate and borate buffers used for the pK_a determination of 3-(heteroaryl)-7-hydroxycoumarins] and mobile phases for HPLC were prepared with water purified using a MilliQ system (purified to 18.2 MΩ cm). Triethylammonium acetate (TEAA; 2.0 M) and triethylammonium hydrogen carbonate (TEAB; 1.0 M) buffers were prepared from distilled TEA and glacial acetic acid or CO_2 gas, respectively. The following starting materials were synthesized according to literature procedures: 3-(2-benzimidazolyl)-7-hydroxycoumarin,[15] 7-acetoxy-3-formylcoumarin,[23] 3-[(2-nitrophenyl)amino]-1-propanol,[44] 4-fluoro-3-nitrobenzoic acid *tert*-butyl ester,[45] 2-aminoethane-1,1-disulfonic acid (tributylammonium salt, TBA^+ salt),[25] alkyne-functionalized cystamine **22**,[37] TFA salt of 3,4-diaminobenzenesulfonic acid,[22] *N*-(2-azidoethyl)-*N*-methylaniline,[36] maleimide-terminated aniline **25**,[46] sodium thiophosphate (NaThioPi),[47] and peptide Ac-Cys(*St*Bu)-Ser-Gly-Arg-Ser-Ala-Asn-Ala-Lys-NH_2 **27**.[36]

Instruments and Methods: The synthesis of peptide **27** was carried out with an Applied Biosystems 433A synthesizer using standard Fmoc/*t*Bu chemistry.[48] ^1H and ^{13}C NMR 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), and the residual solvent peak was used for calibration.[49] *J* values are expressed in Hz. Infrared (IR) spectra were recorded with a universal ATR sampling accessory with a Perkin–Elmer FTIR Spectrum 100 spectrometer. Bond vibration frequencies are expressed in reciprocal centimeters (cm^{-1}). Analytical HPLC was carried out with a Thermo Scientific Surveyor Plus instrument equipped with a PDA detector. Semipreparative HPLC was carried out with a Thermo Scientific SPECTRASYSTEM liquid chromatography system (P4000) equipped with a UV/Vis 2000 detector. Automated flash purifications on RP-C_{18} cartridges were carried out with a Biotage Isolera™ One (ISO-1EW) system. Ion-exchange chromatography (for desalting disulfonated 7-hydroxycoumarin **17**) was carried out with an Econo-Pac® disposable chromatography column (Bio-Rad, #732–1010) filled with an aq. suspension of Dowex® 50WX8–400 (Alfa Aesar, ca. 5 g for 15 mg of dye, 15 × 50 mm bed), which was regenerated using HCl (10% aq.), and equilibrated with deionized water. Low-resolution mass spectra (LRMS) were obtained with a Finnigan LCQ Advantage MAX (ion trap) apparatus equipped with an electrospray ionisation (ESI) source. High-resolution mass spectra (HRMS) were recorded either with a Thermo LTQ Orbitrap XL apparatus equipped with an ESI source or with an LCT Premier XE benchtop orthogonal acceleration time-of-flight (oa-TOF) mass spectrometer (Waters Micromass) equipped with an ESI source. UV/Vis absorption spectra were obtained with a Varian Cary 50 scan spectrophotometer using a rectangular quartz cell (Varian, standard cell, Open Top, light path 10 × 10 mm, chamber volume: 3.5 mL). Fluorescence spectroscopic studies (emission/excitation spectra) were carried out with a Varian Cary Eclipse spectrophotometer with a semi-micro quartz fluorescence cell (Hellma, 104F-QS, light path: 10 × 4 mm, chamber volume: 1400 μL) or an ultra-micro quartz fluorescence cell (Hellma, 105.251-QS, light path: 3 × 3 mm, chamber volume: 45 μL) for uPA assays. All absorption spectra (of 3-benzimidazolyl-7-hydroxycoumarin derivatives, fluorogenic probes, and DABCYL-based quenchers) were recorded (220–700 nm or 300–700 nm) at 25 °C in the selected solvents (mainly PB, PBS, and DMSO). Excitation/emission spectra were recorded under the same conditions after emission/excitation at 390/510 (or 600 or 650) nm (excitation filter: auto and emission filter: open, excitation and emission slit: 5 nm). Fluorescence quantum yields were measured at 25 °C by a relative method using 7-hydroxycoumarin (Φ_F = 76% in PB, pH 7.4) or fluorescein (Φ_F = 95% in 0.1 N NaOH) as a standard.[50,51] 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 AU), *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 the standard and the unknown, respectively. The following refractive index values were used: 1.362 for EtOH, 1.479 for DMSO, and 1.337 for PB, PBS, and H_2O + 2.5% SDS.

General Procedure for in-vitro Thiolysis of Fluorogenic Probes – Fluorescence-Based Assays: Stock solutions (1.0 mg mL^{-1}) of water-soluble fluorophores **10**–**17** and azido-DABCYL **21** were prepared in ultrapure water, and a stock solution of profluorophore **19** was prepared in H_2O/DMSO (9:1, v/v). A stock solution of 3-(2-benzoselenazolyl)-7-hydroxycoumarin **18** was prepared in DMSO. Stock solutions (10 mg mL^{-1}) of analytes (L-cysteine, NaThioPi, and K_3PO_4) used for thiolysis of water-soluble profluorophores were prepared in ultrapure water. A micromolar solution (for each profluorophore) was obtained by dilution of the stock solution with PB + 45% DMSO (pH 8.3) for **24**, with PBS [phosphate (100 mM) + NaCl (150 mM), pH 7.5] for **19**, and with Tris·HCl (100 mM, pH 8.0) for **24**. Depending on the reactivity of the probe (i.e., thiolysis kinetics) and the fluorescence efficiency of the released fluorophore, a concentration between 1.1 and 8.6 μM was used. Stock solutions (100 μM) of the analytes (Cys, NaThioPi, K_3PO_4, and NaHS) used for the fluorescence-based assay of **19** were prepared in PBS buffer. A portion (3 mL) of this solution was transferred into a quartz fluorescence cell (Varian, fluorescence cell, open top, light path: 10 × 10 mm, chamber volume: 3.5 mL), and thermostatted at 25 °C. The required number of equivalents of analyte (5, 50, or 150 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 λ = 480 nm (emission slit = 5 nm) upon excitation at λ = 390 nm (excitation slit = 5 nm; excitation/emission filters: auto) over time, with measurements recorded every 1 s.

General Procedure for in-vitro Peptide Cleavage by uPA: A solution (1.0 μM) of fluorogenic peptide **29** was prepared in PBS (45 μL), and transferred into the ultra-micro quartz fluorescence cell. uPA solution [5 μL, 0.6 U; prepared from uPA (25 μg) in buffer (100 μL): Tris·HCl (500 mM), NaCl (1.0 M), PEG 6000 (1%), mannitol (2.0 M)] was added, and the resulting mixture was incubated at 37.5 °C. After excitation at λ = 400 nm, the fluorescence emission of the released fluorophore at 480 nm was monitored over time, with measurements recorded every 5 s.

HPLC Separations: Several chromatographic systems were used for the analytical experiments and purification steps (by semipreparative 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 trifluoroacetic acid (0.1% aq.; pH 2.0) as eluents [100% aq. TFA (5 min), then linear gradient from 0 to 100% (45 min) CH_3CN] at a flow rate of 0.25 mL min^{-1}. 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*: as for system A but with TEAA buffer (25 mM, pH 7.0) as aq. mobile phase [100% TEAA buffer (5 min), then linear gradient from 0 to 100% (45 min) CH_3CN]. *System C*: automated flash purification (Biotage® SNAP cartridge KP-C18-HS, 120 g) with aq. TEAB (50 mM, pH 7.5) as aq. mobile phase [100% TEAB (5 min), then linear gradient from 0 to 40% (50 min) CH_3CN] at a flow rate of 35.0 mL min^{-1}. Dual UV-detection was achieved at 220 and 365 nm. *System D*: semipreparative RP-HPLC (Varian Kromasil C_{18} column, 10 μm, 21.2 × 250 mm) with the following gradient [100% TEAB (5 min), then linear gradient from 0 to 40% (70 min) CH_3CN] at a flow rate of 20.0 mL min^{-1}. UV detection was achieved at 365 nm. *System E*: as for system D, but with TFA (0.1% aq.). *System F*: as for system D, but with the following gradient: [100% TEAB (5 min), then linear gradient from 0 to 30% (75 min) CH_3CN]. *System G*: as for system C, but with CH_3CN and TFA (0.1% aq.) as eluents [100% aq. TFA (5 min), then linear gradient from 0 to 50% (100 min) CH_3CN]. *System H*: as for system C, but with CH_3CN and TFA (0.1% aq.) as eluents [100% aq. TFA (5 min), then linear gradient from 0 to 40% (60 min) CH_3CN]. *System I*: as for system D, but with the following gradient: [100% TEAB (5 min), then linear gradient from 0 to 100% (100 min) CH_3CN]. *System J*: as for system E, but with the following gradient: [100% aq. TFA (5 min), then linear gradient from 0 to 100% (105 min) CH_3CN]. Visible detection was achieved at 430 nm. *System K*: semipreparative RP-HPLC (Thermo Hypersil GOLD C_{18} column, 5 μm, 10.0 × 250 mm) with CH_3CN and TFA (0.1% aq.) as eluents [100% aq. TFA (5 min), followed by linear gradient from 0 to 100% (60 min) CH_3CN] at a flow rate of 4.0 mL min^{-1}. Visible detection was achieved at 430 nm.

Synthesis: For the detailed synthetic procedures for *ortho*-phenylenediamine derivatives **2**, **3**, **5**–**7**, and 7-acetoxy-3-formylcoumarin-6-sulfonic acid, see the Supporting Information.

Most of 3-(2-benzimidazolyl)-7-hydroxycoumarins and related fluorogenic probes were found to be soluble in D_2O, but poor quality spectra were obtained (i.e., broad and poorly resolved peaks). Thus, all NMR spectra of water-soluble derivatives were recorded in [D_6]DMSO.

General Procedure A (GP A) for the Preparation of 3-(2-Benzimidazolyl)-7-hydroxycoumarin Derivatives: A mixture of 7-acetoxy-3-formylcoumarin (1 equiv.) and *ortho*-phenylenediamine derivative (1 equiv.) in DMSO was stirred for 3 h at 120 °C. Thereafter, the mixture was diluted with aq. TEAB buffer (pH 7.5), and purified on an RP-C_{18} cartridge using an automated flash purification system. The product-containing fractions were lyophilized three times to give the TEA salt of the 3-(2-benzimidazolyl)-7-hydroxycoumarin derivative as a yellow amorphous powder.

General Procedure B (GP B) for the Preparation of 3-(2-Benzimidazolyl)-7-hydroxycoumarin Derivatives: A mixture of 7-acetoxy-3-formylcoumarin derivative (1 equiv.), $Na_2S_2O_5$ (1 equiv.), and *ortho*-phenylenediamine derivative (1 equiv.) in DMSO was stirred for 3 h at 120 °C. Thereafter, the mixture was diluted with aq. TEAB buffer (pH 7.5), and purified on an RP-C_{18} cartridge using an automated flash purification system. The product-containing fractions were lyophilized three times to give the TEA salt of the 3-(2-benzimidazolyl)-7-hydroxycoumarin derivative as a yellow amorphous powder.

General Procedure C (GP C) for the Activation of the Carboxylic Acid of Bioconjugatable 3-(2-Benzimidazolyl)-7-hydroxycoumarin Derivatives: The 3-(2-benzimidazolyl)-7-hydroxycoumarin derivative (1 equiv.) was dissolved in NMP (0.1 mL), and a solution of *N*,*N*′-dicyclohexylcarbodiimide (DCC) in NMP (5.3 equiv. in 50 mL) and a solution of *N*-hydroxysuccinimide (NHS) in NMP (5.3 equiv. in 50 mL) were added sequentially. The resulting reaction mixture was stirred at room temp. for 2 h. Thereafter, the crude mixture was centrifuged to remove the DCU precipitate, and the supernatant (containing the corresponding NHS ester) used in the next coupling step without further purification.

Sulfonated 3-(2-Benzimidazolyl)-7-hydroxycoumarin (10): Compound **10** was synthesized from 7-acetoxy-3-formylcoumarin and *ortho*-phenylenediamine **8** according to GP A, and purified with an automated flash purification (system C). Isolated yield, 19%. IR (ATR): $\tilde{\nu}$ = 3330, 2972, 1688, 1596, 1611, 1453, 1235, 1175, 1082, 1028 cm^{-1}. ^{1}H NMR (300 MHz, [D_6]DMSO) [spectrum recorded with the first batch of **10**, purified under nonoptimized conditions (aq. TFA 0.1% as the aq. mobile phase)]: δ = 11.38 (s, 1 H, O*H*), 9.01 (s, 1 H), 8.21 (s, 1 H), 7.82 (d, $^3J_{H,H}$ = 8.6 Hz, 1 H), 7.76 (m, 2 H), 6.97 (dd, $^3J_{H,H}$ = 2.0 Hz, 8.6 Hz, 1 H), 6.91 (d, $^3J_{H,H}$ = 2.0 Hz, 1 H) ppm. ^{13}C NMR (75 MHz, [D_6]DMSO) [spectrum re-

corded with the second batch of **10** (TEA salt), purified under optimized conditions (TEAB buffer as the aq. mobile phase)]: δ = 163.0, 161.3, 160.5, 159.5, 147.1, 144.5, 143.6, 143.4, 131.4, 129.7, 121.0, 114.3, 113.2, 111.6, 111.0, 102.1, 45.8 (*N*-*C*H$_2$-CH$_3$, TEA), 8.6 (*N*-CH$_2$-*C*H$_3$, TEA) ppm. HRMS (ESI$^-$): calcd. for $C_{16}H_9N_2O_6S^-$ [M – H]$^-$ 357.01758; found 357.01817. HPLC (system A): t_R = 17.4 min, purity = 96%.

3-(2-Benzimidazolyl)-7-hydroxycoumarincarboxylic Acid (11): Compound **11** was synthesized from 7-acetoxy-3-formylcoumarin and *ortho*-phenylenediamine **9** according to GP A, and purified with an automated flash purification (system C). Isolated yield, 20%. IR (ATR): $\tilde{\nu}$ = 3329, 2981, 1678, 1613, 1562, 1305, 1266, 1231, 1198, 1130 cm^{-1}. ^{1}H NMR (300 MHz, [D$_6$]DMSO) [spectrum recorded with the first batch of **11**, purified under nonoptimized conditions (aq. TFA 0.1% as the aq. mobile phase)]: δ = 11.10 (s, 1 H, O*H*), 9.09 (s, 1 H), 8.28 (s, 1 H), 7.85 (m, 2 H), 7.73 (d, $^3J_{H,H}$ = 8.7 Hz, 1 H), 6.92 (dd, $^3J_{H,H}$ = 2.0 Hz, 8.7 Hz, 1 H), 6.86 (d, $^3J_{H,H}$ = 2.1 Hz, 1 H) ppm. ^{13}C NMR (75 MHz, [D$_6$]DMSO) [spectrum recorded with the second batch of **11** (TEA salt), purified under optimized conditions (TEAB buffer as the aq. mobile phase)]: δ = 167.7, 163.5, 159.4, 155.9, 148.5, 144.6, 140.9, 137.1, 131.6, 125.2, 124.1, 117.1, 115.2, 114.5, 111.5, 110.3, 102.1 ppm. HRMS (ESI$^-$): calcd. for $C_{17}H_9N_2O_5^-$ [M – H]$^-$ 321.05060; found 321.05040. HPLC (system A): t_R = 20.4 min, purity = 96%.

3-(2-Benzimidazolyl)-7-hydroxycoumarin-Substituted *N*-Propanesulfonic Acid (12): Compound **12** was synthesized from 7-acetoxy-3-formylcoumarin and *ortho*-phenylenediamine **2** according to GP B, and purified by semipreparative RP-HPLC (system D). Isolated yield, 23%. IR (ATR): $\tilde{\nu}$ = 3332, 2980, 1680, 1615, 1564, 1315, 1276, 1232, 1199, 1134 cm^{-1}. ^{1}H NMR (300 MHz, [D$_6$]DMSO): δ = 10.91 (s, 1 H, O*H*), 8.40 (s, 1 H), 7.72 (m, 2 H), 7.68 (d, $^3J_{H,H}$ = 10.0 Hz, 1 H), 7.31 (t, $^3J_{H,H}$ = 10.0 Hz, 1 H), 7.25 (t, $^3J_{H,H}$ = 10.0 Hz, 1 H), 6.87 (dd, $^3J_{H,H}$ = 5.0 Hz, 10.0 Hz, 1 H), 6.84 (d, $^3J_{H,H}$ = 5.0 Hz, 1 H), 4.33 (t, $^3J_{H,H}$ = 5.0 Hz, 2 H), 3.09 (q, $^3J_{H,H}$ = 6.0 Hz, 6 H, *N*-C*H*$_2$-CH$_3$, TEA), 2.35 (t, $^3J_{H,H}$ = 4.0 Hz, 2 H), 1.98 (qt, $^3J_{H,H}$ = 5.0 Hz, 2 H), 1.16 (t, $^3J_{H,H}$ = 6.0 Hz, 9 H, *N*-CH$_2$-C*H*$_3$, TEA) ppm. ^{13}C NMR (75 MHz, [D$_6$]DMSO): δ = 162.6, 159.2, 156.1, 148.5, 147.2, 142.5, 135.3, 130.9, 122.7, 122.0, 119.1, 113.8, 113.8, 111.2, 111.1, 102.1, 48.2, 45.7 (*N*-*C*H$_2$-CH$_3$, TEA), 43.4, 25.8, 8.6 (*N*-CH$_2$-*C*H$_3$, TEA) ppm. HRMS (ESI$^-$): calcd. for $C_{19}H_{15}N_2O_6S^-$ [M – H]$^-$ 399.07453; found 399.06508. HPLC (system B): t_R = 17.5 min, purity = 99%.

***N*-Propylsulfonated 3-(2-Benzimidazolyl)-7-hydroxycoumarincarboxylic Acid (13):** Compound **13** was synthesized from 7-acetoxy-3-formylcoumarin and *ortho*-phenylenediamine **6** according to GP B, and purified by semipreparative RP-HPLC (system D). Isolated yield, 21%. IR (ATR): $\tilde{\nu}$ = 3325, 2961, 1675, 1623, 1574, 1316, 1269, 1233, 1189, 1124 cm^{-1}. ^{1}H NMR (500 MHz, [D$_6$]DMSO): δ = 8.44 (s, 1 H), 8.24 (s, 1 H), 7.92 (d, $^3J_{H,H}$ = 10.0 Hz, 1 H), 7.78 (d, $^3J_{H,H}$ = 10.0 Hz, 1 H), 7.72 (d, $^3J_{H,H}$ = 10.0 Hz, 1 H), 6.88 (dd, $^3J_{H,H}$ = 5.0 Hz, 10.0 Hz, 1 H), 6.83 (d, $^3J_{H,H}$ = 5.0 Hz, 1 H), 4.37 (t, $^3J_{H,H}$ = 5.0 Hz, 2 H), 2.90 (q, $^3J_{H,H}$ = 6.0 Hz, 8 H, *N*-C*H*$_2$-CH$_3$, 1.33 TEA), 2.33 (t, $^3J_{H,H}$ = 4.0 Hz, 2 H), 1.98 (br. t, $^3J_{H,H}$ = 5.0 Hz, 2 H), 1.10 (t, $^3J_{H,H}$ = 6.0 Hz, 12 H, *N*-CH$_2$-C*H*$_3$, 1.33 TEA) ppm. ^{13}C NMR (126 MHz, [D$_6$]DMSO): δ = 168.1, 162.9, 159.2, 156.2, 150.4, 147.5, 142.2, 138.3, 131.0, 125.4, 123.9, 120.9, 114.0, 113.4, 111.1, 110.9, 102.2, 48.1, 45.7 (*N*-*C*H$_2$-CH$_3$, TEA), 43.6, 25.8, 9.4 (*N*-CH$_2$-*C*H$_3$, TEA) ppm. HRMS (ESI$^-$): calcd. for $C_{20}H_{15}N_2O_8S^-$ [M – H]$^-$ 443.05436; found 443.05331. HPLC (system B): t_R = 15.6 min, purity = 99%.

3-(2-Benzimidazolyl)-7-hydroxycoumarin *N*-(*N*′,*N*′,*N*′-Trimethyl)-propylammonium (14): Compound **14** was synthesized from 7-acetoxy-3-formylcoumarin and *ortho*-phenylenediamine **3** according to GP B, and purified by semipreparative RP-HPLC (system E). Isolated yield, 24% (TFA salt). IR (ATR): $\tilde{\nu}$ = 3315, 2991, 1672, 1597, 1461, 1373, 1322, 1233, 1180, 1123, 1020 cm^{-1}. ^{1}H NMR (300 MHz, [D$_6$]DMSO): δ = 8.50 (s, 1 H), 7.85 (d, $^3J_{H,H}$ = 8.7 Hz, 1 H), 7.75 (m, 2 H), 7.40 (m, 2 H), 6.93 (dd, $^3J_{H,H}$ = 2.1 Hz, 8.7 Hz, 1 H), 6.89 (d, $^3J_{H,H}$ = 2.1 Hz, 1 H), 4.31 (t, $^3J_{H,H}$ = 6.0 Hz, 2 H), 3.33 (t, $^3J_{H,H}$ = 3.0 Hz, 2 H), 2.51 (s, 9 H), 2.26 (qt, $^3J_{H,H}$ = 6.0 Hz, 2 H) ppm. ^{13}C NMR (75 MHz, [D$_6$]DMSO): δ = 163.4, 159.2, 156.3, 148.6, 147.7, 139.9, 134.4, 131.2, 123.8, 123.3, 118.3, 114.2, 111.6, 111.6, 111.0, 102.2, 62.6, 52.4, 52.3, 52.3, 41.9, 22.7 ppm. HRMS (ESI$^+$): calcd. for $C_{22}H_{24}N_3O_3^+$ [M]$^{+\cdot}$ 378.18122; found 378.18059. HPLC (system A): t_R = 16.4 min, purity = 98%.

3-(2-Benzimidazolyl)-7-hydroxycoumarin *N*-(*N*′,*N*′,*N*′-Trimethyl)-propylammonium Carboxylic Acid (15): Compound **15** was synthesized from 7-acetoxy-3-formylcoumarin and *ortho*-phenylenediamine **7** according to GP B, and purified by semipreparative RP-HPLC (system E). Isolated yield, 23% (TFA salt). IR (ATR): $\tilde{\nu}$ = 3340, 2974, 1675, 1599, 1485, 1442, 1305, 1231, 1177, 1123, 1009 cm^{-1}. ^{1}H NMR (300 MHz, [D$_6$]DMSO): δ = 8.48 (s, 1 H), 8.28 (s, 1 H), 7.97 (d, $^3J_{H,H}$ = 8.6 Hz, 1 H), 7.85 (d, $^3J_{H,H}$ = 8.6 Hz, 1 H), 7.73 (d, $^3J_{H,H}$ = 8.6 Hz, 1 H), 6.90 (m, 2 H), 4.29 (t, $^3J_{H,H}$ = 6.0 Hz, 2 H), 3.32 (t, $^3J_{H,H}$ = 3.0 Hz, 2 H), 3.00 (s, 9 H), 2.26 (qt, $^3J_{H,H}$ = 6.0 Hz, 2 H) ppm. ^{13}C NMR (75 MHz, [D$_6$]DMSO): δ = 167.7, 163.2, 159.5, 156.3, 150.4, 148.3, 142.0, 138.2, 131.1, 125.2, 124.2, 121.0, 114.2, 112.8, 111.1, 111.0, 102.2, 62.7, 52.3, 52.3, 52.2, 41.8, 22.9 ppm. HRMS (ESI$^+$): calcd. for $C_{23}H_{24}N_3O_5^+$ [M]$^{+\cdot}$ 422.17105; found 422.17056; HPLC (system A): t_R = 17.4 min, purity = 98%.

Sulfonated 3-(2-Benzimidazolyl)-7-hydroxycoumarincarboxylic Acid (16): Compound **16** was synthesized from 7-acetoxy-3-formylcoumarin-6-sulfonic acid and *ortho*-phenylenediamine **9** according to GP A, and purified with an automated flash purification system (system C). Isolated yield, 25%. IR (ATR): $\tilde{\nu}$ = 3299, 2987, 2712, 1711, 1610, 1520, 1389, 1216, 1160, 1119, 1076, 1010 cm^{-1}. ^{1}H NMR (300 MHz, [D$_6$]DMSO): δ = 12.70 (s, 1 H, O*H*), 9.14 (s, 1 H), 8.27 (s, 1 H), 8.18 (s, 1 H), 7.83 (d, $^3J_{H,H}$ = 8.3 Hz, 1 H), 7.71 (d, $^3J_{H,H}$ = 8.3 Hz, 1 H), 6.93 (s, 1 H), 2.98 (q, $^3J_{H,H}$ = 3.0 Hz, 10 H, *N*-C*H*$_2$-CH$_3$, 1.66 TEA), 1.12 (t, $^3J_{H,H}$ = 3.0 Hz, 14 H, *N*-CH$_2$-C*H*$_3$, 1.66 TEA) ppm. ^{13}C NMR (75 MHz, [D$_6$]DMSO): δ = 168.0, 159.4, 158.6, 155.6, 143.8, 129.8, 129.0, 125.1, 125.0, 123.7, 123.5, 120.2, 117.9, 114.8, 112.6, 111.5, 103.0, 45.7 (*N*-*C*H$_2$-CH$_3$, TEA), 9.1 (*N*-CH$_2$-*C*H$_3$, TEA) ppm. HRMS (ESI$^-$): calcd. for $C_{17}H_9N_2O_8S^-$ [M – H]$^-$ 401.00741; found 40100841. HPLC (system B): t_R = 16.8 min, purity = 95%.

Disulfonated 3-(2-Benzimidazolyl)-7-hydroxycoumarin (17): Compound **11**-NHS (12 mg, 0.0284 mmol, 1 equiv.) was synthesized according to GP C. Then, the NMP solution containing this active ester was added dropwise to a precooled (0 °C) solution (0.5 M) of 2-aminoethane-1,1-disulfonic acid (TBA$^+$ salt) in NMP (748 μL, 0.374 mmol, 13 equiv.) containing DIEA (2.0 M solution in NMP; 200 μL, 5 equiv.), over a period of 15 min. The resulting mixture was stirred at room temp. overnight. This amidification reaction was checked for completion by RP-HPLC (system B). The reaction was quenched by the addition of glacial acetic acid (100 μL), and then the mixture was diluted with aq. TEAB (ca. 8.0 mL), and purified by RP-HPLC (system F). The product-containing fractions were lyophilized three times to give the TEA salt of compound **17** as a mixture with TBA$^+$ salts. Desalting by ion-exchange chromatography (followed by lyophilisation) gave compound **17** (4.0 mg, 28%) as a yellow amorphous powder. IR (ATR): $\tilde{\nu}$ = 3321, 3053, 1703, 1601, 1590, 1462, 1171, 1060, 1015 cm^{-1}. ^{1}H NMR

(300 MHz, [D$_6$]DMSO): δ = 9.53 (s, 1 H), 8.66 (s, 1 H, N*H*), 8.08 (s, 1 H), 8.02 (d, $^3J_{H,H}$ = 9.0 Hz, 1 H), 7.85 (d, $^3J_{H,H}$ = 9.0 Hz, 1 H), 7.77 (d, $^3J_{H,H}$ = 9.0 Hz, 1 H), 6.94 (dd, $^3J_{H,H}$ = 9.0 Hz, 3.0 Hz, 1 H), 6.88 (d, $^3J_{H,H}$ = 3.0 Hz, 1 H), 4.01 (m, 1 H), 3.92 (m, 2 H) ppm. ^{13}C NMR (126 MHz, [D$_6$]DMSO): δ = 165.3, 164.2, 158.1, 156.6, 149.5, 145.6, 133.5, 132.9, 132.4, 131.2, 124.0, 114.9, 114.2, 113.1, 111.2, 104.5, 102.1, 73.4, 39.5 (masked by DMSO signal) ppm. HRMS (ESI$^-$): calcd. for $C_{19}H_{14}N_3O_{10}S_2^-$ [M – H]$^-$ 508.01151; found 508.01263. HPLC (system B): t_R = 18.5 min, purity = 95%.

3-(2-Benzoselenazolyl)-7-hydroxycoumarin (18): 7-Acetoxy-3-formylcoumarin (200 mg, 0.86 mmol, 1 equiv.) was dissolved in DMSO (20 mL), and $Na_2S_2O_5$ (163 mg, 0.86 mmol, 1 equiv.) and bis(2-aminophenyl)diselenide (235 mg, 0.69 mmol, 0.8 equiv.) were sequentially added. The resulting reaction mixture was stirred at 120 °C under an Ar atmosphere for 48 h. Thereafter, the mixture was diluted with satd. aq. NH_4Cl, and extracted with EtOAc. The combined organic phases were dried with anhydrous $MgSO_4$, and concentrated under reduced pressure. The resulting residue was purified by flash column chromatography on silica gel (CH_2Cl_2/EtOAc with a step gradient from 100:0 to 95:5) to give seleno-dye **18** (53 mg, 14%) as an orange solid. IR (ATR): $\tilde{\nu}$ = 1709, 1596, 1586, 1562, 1446, 1255, 1233, 1193, 1063 cm^{-1}. ^{1}H NMR (300 MHz, [D$_6$]DMSO): δ = 11.11 (s, 1 H, O*H*), 9.12 (s, 1 H), 8.18 (dd, $^3J_{H,H}$ = 7.9 Hz, 0.7 Hz, 1 H), 8.07 (d, $^3J_{H,H}$ = 7.6 Hz, 1 H), 7.93 (d, $^3J_{H,H}$ = 8.6 Hz, 1 H), 7.53 (t, $^3J_{H,H}$ = 7.2 Hz, 1 H), 7.35 (t, $^3J_{H,H}$ = 8.0 Hz, 1 H), 6.94 (dd, $^3J_{H,H}$ = 8.6 Hz, 2.2 Hz, 1 H), 6.86 (d, $^3J_{H,H}$ = 2.1 Hz, 1 H) ppm. ^{13}C NMR (75 MHz, [D$_6$]DMSO): δ = 163.5, 163.4, 160.6, 155.7, 153.9, 141.4, 139.0, 132.2, 126.4, 125.3, 124.8, 123.8, 116.6, 114.6, 111.5, 102.1 ppm. HRMS (ESI$^+$): calcd. for $C_{16}H_{10}NO_3Se^+$ [M + H]$^+$ 343.9805; found 343.9821. HPLC (system A): t_R = 28.3 min, purity = 99%.

***N*-(*N*′,*N*′,*N*′-Trimethyl)propylammonium NBD Ether (19):** Phenol **14** (10 mg, 0.020 mmol, 1 equiv.) was dissolved in dry DMF, and NBDCl (6 mg, 0.023 mmol, 1.5 equiv.) and TEA (15 μL, 0.115 mmol, 5.8 equiv.) were sequentially added. The resulting reaction mixture was stirred at room temp. overnight. Thereafter, the crude mixture was directly purified by semipreparative RP-HPLC (system G). The product-containing fractions were lyophilized to give the TFA salt of NBD ether **19** (11.0 mg, 72%) as a yellow amorphous solid. IR (ATR): $\tilde{\nu}$ = 3430, 3050, 1726, 1675, 1614, 1538 (NO_2), 1457, 1332 (NO_2), 1261, 1167, 1116, 997 cm^{-1}. ^{1}H NMR (300 MHz, [D$_6$]DMSO): δ = 8.74 (d, $^3J_{H,H}$ = 8.3 Hz, 1 H), 8.65 (s, 1 H), 8.10 (d, $^3J_{H,H}$ = 8.6 Hz, 1 H), 7.78 (m, 3 H), 7.53 (dd, $^3J_{H,H}$ = 2.3 Hz, 8.6 Hz, 1 H), 7.35 (m, 2 H), 7.11 (d, $^3J_{H,H}$ = 8.3 Hz, 1 H), 4.30 (t, J = 7.4 Hz, 2 H), 3.35 (q, J = 2.1 Hz, 7.4 Hz, 2 H), 3.03 (s, 9 H), 2.28 (br. t, $^3J_{H,H}$ = 2.1 Hz, 2 H) ppm. ^{13}C NMR (75 MHz, [D$_6$]DMSO): δ = 158.8, 158.2, 157.8, 157.1, 155.3, 151.2, 147.1, 145.5, 144.6, 142.2, 135.1, 131.7, 131.6, 123.4, 122.6, 119.3, 117.9, 117.4, 116.9, 112.9, 111.2, 108.4, 62.7, 52.4, 52.3, 52.3, 41.7, 22.9 ppm. HRMS (ESI$^+$): calcd. for $C_{28}H_{25}N_6O_6^+$ [M]$^{+\cdot}$ 541.18301; found 541.18150. HPLC (system A): t_R = 21.4 min, purity = 100%.

Alkyne-Functionalized 3-(2-Benzimidazolyl)-7-hydroxycoumarin (23): The NHS ester of carboxylic acid **11** (14 mg, 0.033 mmol, 1 equiv.) was synthesized according to GP C. Then, the NMP solution of this active ester was added dropwise to a precooled (0 °C) solution (0.57 M) of alkyne-functionalized cystamine **24** (TFA salt; 90 mg, 0.284 mmol, 8.5 equiv.) in NMP (0.5 mL) containing DIEA (2.0 M solution in NMP; 83 μL, 5 equiv.), over a period of 15 min. The resulting reaction mixture was stirred at room temp. overnight. This amidification reaction was checked for completion by RP-HPLC (system A). The reaction was quenched by the addition of glacial acetic acid (50 μL), and the mixture was purified by semipreparative RP-HPLC (system H). The product-containing fractions were lyophilized to give the TFA salt of compound **23** (5 mg, 25%) as a yellow amorphous powder. IR (ATR): $\tilde{\nu}$ = 3240, 2920, 2854, 2105 (-C≡C-), 1712, 1613, 1570, 1504, 1439, 1301, 1232, 1189, 1127, 1024 cm^{-1}. ^{1}H NMR (300 MHz, [D$_6$]DMSO): δ = 11.08 (s, 1 H, O*H*), 9.07 (s, 1 H), 8.90 [t, $^3J_{H,H}$ = 5.3 Hz, 1 H, C(O)N*H*], 8.67 [m, $^3J_{H,H}$ = 5.3 Hz, 1 H, C(O)N*H*], 8.17 (s, 1 H), 7.84 (d, $^3J_{H,H}$ = 8.6 Hz, 1 H), 7.71 (m, 2 H), 6.91 (dd, J = 2.2 Hz, 8.5 Hz, 1 H), 6.86 (d, $^3J_{H,H}$ = 2.2 Hz, 1 H), 4.14 (s, 1 H), 3.40 (m, 4 H, masked by water signal), 2.95 (t, $^3J_{H,H}$ = 6.6 Hz, 2 H), 2.83 (t, $^3J_{H,H}$ = 6.6 Hz, 2 H) ppm. ^{13}C NMR (75 MHz, [D$_6$]DMSO): δ = 166.8, 163.3, 159.5, 155.8, 151.8, 148.1, 144.2, 131.6, 128.9, 122.0, 114.4, 111.5, 110.8, 102.1, 78.1, 75.9, 39.5 (masked by DMSO signal), 38.7, 37.2, 36.6 ppm. Not enough of this compound was obtained for it to be well-characterized by ^{13}C spectroscopy (four carbons are missing). LRMS (ESI$^-$): calcd. for $C_{24}H_{19}N_4O_5S_2$ 507.07 [M – H]$^-$; found 507.07 and 620.60 [M + TFA – H]$^-$. HPLC (system A): t_R = 21.9 min, purity = 99%.

Azido-DABCYL Dye 20: Solid $NOBF_4$ (187 mg, 1.6 mmol, 1.1 equiv.) was added to a precooled solution of *para*-aminobenzoic acid (200 mg, 1.46 mmol, 1 equiv.) in dry CH_3CN (10 mL). The resulting mixture was stirred at 0 °C for 15 min. Then, a solution of *N*-(2-azidoethyl)-*N*-methylaniline (306 mg, 1.74 mmol, 1.2 equiv.) in CH_3CN (1 mL) was added dropwise, and the resulting mixture was stirred at 0 °C for 30 min. Thereafter, the product was precipitated by the addition of NaOAc buffer (0.1 M; pH 4.0). The orange solid was recovered by filtration, washed with a mixture of CH_3CN/H_2O (1:1, v/v), and finally lyophilized to give azido-DABCYL **20** (404 mg, 86%) as an orange amorphous powder. IR (ATR): $\tilde{\nu}$ = 2531, 2095, 1672, 1596, 1515, 1419, 1377, 1286, 1137, 945, 862, 814 cm^{-1}. ^{1}H NMR (300 MHz, [D$_6$]DMSO): δ = 8.08 (d, $^3J_{H,H}$ = 8.6 Hz, 2 H), 7.83 (dd, $^3J_{H,H}$ = 2.3 Hz, 8.6 Hz, 4 H), 6.91 (d, $^3J_{H,H}$ = 9.2 Hz, 2 H), 3.69 (t, $^3J_{H,H}$ = 5.9 Hz, 2 H), 3.55 (t, $^3J_{H,H}$ = 5.9 Hz, 2 H), 3.08 (s, 3 H) ppm. ^{13}C NMR (75 MHz, [D$_6$]-DMSO): δ = 167.0, 155.0, 151.8, 143.0, 131.2, 130.5, 125.3, 121.8, 111.7, 50.6, 48.4, 38.4 ppm. HRMS (ESI$^-$): calcd. for $C_{16}H_{15}N_6O_2^-$ [M – H]$^-$ 323.1262; found 323.1259. UV/Vis (DMSO, 25 °C): λ_{max} = 446 nm; ε (446 nm) = 21000 M^{-1} cm^{-1}; $\Delta\lambda_{1/2\ max}$ = 370–500 (130 nm).

Water-Soluble Azido-DABCYL Dye 21:

Azido-DABCYL **20** (40 mg, 0.123 mmol, 1 equiv.) and TSTU (48 mg, 0.16 mmol, 1.3 equiv.) were dissolved in NMP (0.5 mL), and the solution was stirred under an Ar atmosphere. Then, DIEA (2.0 M solution in NMP; 123 mL, 0.246 mmol, 3 equiv.) was added, and the reaction mixture was stirred at room temp. for 30 min. The resulting NHS active ester was used in the next step without purification.

2-Aminoethane-1,1-disulfonic acid (TBA$^+$ salt; 0.5 M solution in NMP; 2.5 mL, 1.25 mmol, 10 equiv.) and DIEA (2.0 M solution in NMP; 0.184 mL, 184 μL, 3 equiv.) were mixed, and the solution was cooled to 0 °C. Then, the solution of the crude NHS active ester was added dropwise, and the resulting reaction mixture was stirred at room temp. for 2 h. The reaction was checked for completion by RP-HPLC (system B), and the mixture was purified by semipreparative RP-HPLC (system I). The product-containing fractions were lyophilized to give the TEA salt of compound **21** as a mixture with TBA$^+$ salts. Desalting by ion-exchange chromatography (followed by lyophilisation) gave water-soluble azido-DABCYL dye **21** (38 mg, 61%) as a brown amorphous powder. IR (ATR): $\tilde{\nu}$ = 3359, 2107, 1616, 1597, 1538, 1388, 1265, 1219, 1183, 1141, 1016, 826 cm^{-1}. ^{1}H NMR (300 MHz, D_2O): δ = 7.37 (d, $^3J_{H,H}$

= 8.6 Hz, 2 H), 7.23 (d, $^3J_{H,H}$ = 9.5 Hz, 2 H), 7.05 (d, $^3J_{H,H}$ = 8.6 Hz, 2 H), 6.80 (d, $^3J_{H,H}$ = 9.5 Hz, 2 H), 4.18 (t, $^3J_{H,H}$ = 5.7 Hz, 1 H), 3.90 (d, $^3J_{H,H}$ = 5.7 Hz, 2 H), 3.71 (br. s, 2 H), 3.61 (br. s, 2 H), 3.17 (s, 3 H) ppm. ^{13}C NMR (75 MHz, D_2O): δ = 166.7, 159.1, 143.4, 136.6, 132.7, 130.9, 128.4, 117.3, 117.1, 73.3, 52.8, 48.4, 40.4, 39.0 ppm. HRMS (ESI$^-$): calcd. for $C_{18}H_{20}N_7O_7S_2^-$ [M – H]$^-$ 510.0871; found 510.0873. UV/Vis (DMSO, 25 °C): λ_{max} = 437 nm; ε (437 nm) = 27000 M^{-1} cm^{-1}; $\Delta\lambda_{1/2\ max}$ = 394–489 (95 nm). UV/Vis (PBS, 25 °C): λ_{max} = 461 nm; ε (461 nm) = 27000 M^{-1} cm^{-1}; $\Delta\lambda_{1/2\ max}$ = 394–506 (112 nm). HPLC (system B): t_R = 21.5 min, purity = 98%.

Thiol-Sensitive FRET Probe 24: Alkyne-functionalized 7-hydroxycoumarin **23** (5.0 mg, 9.8 μmol, 1 equiv.), water-soluble azido-DABCYL **21** (5.0 mg, 9.8 μmol, 1 equiv.), and Cu^0 microsized powder (0.3 mg, 4.9 μmol, 0.5 equiv.) were suspensed in a mixture of DMSO/H_2O (2:1, v/v). Then, $CuSO_4{\cdot}5H_2O$ (40 mM solution in water; 0.24 mg, 0.98 μmol, 0.1 equiv.) was added, and the resulting mixture was stirred at 50 °C for 2 h. The reaction was checked for completion by RP-HPLC (system B), and then the mixture was purified by semipreparative RP-HPLC (system J). The product-containing fractions were lyophilized three times to give FRET probe **24** (4.8 mg, 48%) as a yellow amorphous powder. ^{1}H NMR (500 MHz, [D$_6$]DMSO): δ = 12.60 (d, $^3J_{H,H}$ = 28.6 Hz, 1 H), 10.96 (s, 1 H, 1 O*H*), 9.07 (s, 1 H), 8.82 [m, 2 H, 2 C(O)N*H*], 8.60 (m, 4 H), 8.17 (s, 1 H), 7.79 (m, 8 H), 6.90 (dd, $^3J_{H,H}$ = 5.0, 10.0 Hz, 1 H), 6.86 (d, $^3J_{H,H}$ = 5.0 Hz, 1 H), 6.81 (d, $^3J_{H,H}$ = 10.0 Hz, 2 H), 4.65 (t, $^3J_{H,H}$ = 10.0 Hz, 2 H), 3.98 (t, $^3J_{H,H}$ = 10.0 Hz, 2 H), 3.84 (t, $^3J_{H,H}$ = 10.0 Hz, 2 H), 3.52 (m, 5 H), 3.10 (q, $^3J_{H,H}$ = 5.0 Hz, 10 H, *N*-C*H*$_2$-CH$_3$, 1.90 TEA), 2.93 (m, 4 H), 2.85 (s, 3 H), 1.20 (t, $^3J_{H,H}$ = 5.0 Hz, 16 H, *N*-CH$_2$-C*H*$_3$, 1.90 TEA) ppm. ^{13}C NMR (126 MHz, [D$_6$]DMSO): δ = 164.3, 159.7, 155.7, 153.8, 151.3, 143.0, 142.7, 135.2, 131.3, 128.7, 128.4, 128.3, 127.7, 127.1, 125.0, 121.7, 117.5, 114.2, 112.5, 112.0, 111.6, 101.9, 74.0, 57.8, 51.4, 45.7, 40.0 (*N*-*C*H$_2$-CH$_3$,TEA), 38.1, 37.9, 37.3, 37.1, 10.9, 8.6 (*N*-CH$_2$-*C*H$_3$,TEA), 8.5, 7.6 ppm. Not enough of this compound was obtained for it to be well-characterized by ^{13}C spectroscopy (nine carbons are missing), despite an extended acquisition time (more than 80000 scans) on a 500 MHz spectrometer (equipped with a 5 mm "BBFO" broadband ATMA gradient z probe four times more sensitive than a standard 300 MHz spectrometer). HRMS (ESI$^-$): calcd. for $C_{42}H_{40}N_{11}O_{12}S_4^-$ [M – H]$^-$ 1018.17352; found 1018.17566 and 508.58352 [M – 2 H]$^{2-}$. HPLC (system B): t_R = 21.5 min, purity = 98%.

Maleimide-nitro-DABCYL Dye (26): Solid $NOBF_4$ (18.6 mg, 0.16 mmol, 1.1 equiv.) was added to a precooled (0 °C) solution of *para*-nitroaniline (20 mg, 0.14 mmol, 1 equiv.) in dry CH_3CN (1.5 mL). The resulting reaction mixture was stirred at 0 °C for 15 min. Then, a solution of maleimide-terminated aniline **25** (42 mg, 0.17 mmol, 1.2 equiv.) in CH_3CN (0.2 mL) was added dropwise, and the resulting mixture was stirred at 0 °C for 30 min. Thereafter, the product was precipitated by the addition of NaOAc buffer (0.1 M; pH 4.0). The orange solid was recovered by filtration, washed with a mixture of CH_3CN/H_2O (1:1, v/v), and finally lyophilized to give maleimide quencher **26** (42 mg, 74%) as an orange amorphous powder. IR (ATR): $\tilde{\nu}$ = 1707, 1601, 1509, 1382, 1334, 1140, 858, 831, 694 cm^{-1}. ^{1}H NMR (300 MHz, [D$_6$]DMSO): δ = 8.32 (d, $^3J_{H,H}$ = 8.9 Hz, 2 H), 7.92 (d, $^3J_{H,H}$ = 7.3 Hz, 2 H), 7.89 (d, $^3J_{H,H}$ = 7.3 Hz, 2 H), 7.24 (m, 4 H), 3.60 (t, $^3J_{H,H}$ = 7.1 Hz, 2 H), 3.49 (t, $^3J_{H,H}$ = 7.1 Hz, 2 H), 3.10 (s, 3 H), 1.97 (qt, $^3J_{H,H}$ = 7.1 Hz, 2 H) ppm. ^{13}C NMR (75 MHz, [D$_6$]DMSO): δ = 170.8, 156.9, 152.4, 147.6, 144.0, 134.4, 126.3, 124.8, 122.8, 111.7, 50.1, 38.7, 35.8, 26.3. HRMS (ESI$^+$): calcd. for $C_{20}H_{20}N_5O_4^+$ [M + H]$^+$ 394.1510; found 394.1509. UV/Vis (DMSO, 25 °C): λ_{max} = 502 nm; ε (502 nm) = 39000 M^{-1} cm^{-1}; $\Delta\lambda_{1/2\ max}$(nm) = 438–553 (115 nm).

Urokinase-Sensitive FRET Probe (29):

The TFA salt of Ac-C(S*t*Bu)SGRSANAK-NH$_2$ **27** (9 mg, 8.8 μmol, 1 equiv.), coumarin **16** (3.9 mg, 9.7 μmol, 1.1 equiv.), and PyBOP (5.0 mg, 9.7 μmol, 1.1 equiv.) were dissolved in NMP (100 μL). DIEA (2.0 M solution in NMP; 22 mL, 44 μmol, 5 equiv.) was added, and the resulting reaction mixture was stirred at room temp. for 2 h. The reaction was checked for completion by RP-HPLC (system A). Thereafter, the mixture was diluted with TFA (0.1% aq.), and purified by semipreparative RP-HPLC (system J). The product-containing fractions were lyophilized to give the fluorescently-labeled peptide as a yellow amorphous powder. LRMS (ESI$^-$; CH_3CN/H_2O, 2:1, v/v): calcd. for $C_{56}H_{78}N_{17}O_{20}S_3^-$ [M – H]$^-$ 1404.48; found 1404.27. UV/Vis (PBS, 25 °C): λ_{max} = 430 nm. Fluorescence (PBS, 25 °C): λ_{em} = 481 nm; Φ_F = 74%. HPLC (system A): t_R = 20.7 min, purity = 97%.

The fluorescently-labeled peptide was dissolved in $NaHCO_3$ (0.1 M aq.; pH 8.5; 200 μL), and a solution of DTT (13.5 mg, 88 μmol, 10 equiv.) in $NaHCO_3$ (0.1 M aq.; 100 μL) was added. The resulting reaction mixture was stirred at room temp. for 1 h. The reaction was checked for completion by RP-HPLC (system A). Then, the mixture was diluted with TFA (0.1% aqueous; 700 μL), and purified by semipreparative RP-HPLC (system J). The product-containing fractions were lyophilized to give target free-sulfhydryl fluorescent peptide **28** (4.9 mg, 31% over two steps) as a yellow amorphous powder. LRMS (ESI$^-$; CH_3CN/H_2O, 2:1, v/v): calcd. for $C_{52}H_{70}N_{17}O_{20}S_2^-$ [M – H]$^-$ 1316.44; found 1316.53. LRMS (ESI$^+$; in CH_3CN/H_2O, 2:1, v/v): calcd. for $C_{52}H_{72}N_{17}O_{20}S_2^+$ [M + H]$^+$ 1318.46; found 1318.27. UV/Vis (PBS, 25 °C): λ_{max} = 433 nm. Fluorescence (PBS, 25 °C): λ_{em} = 481 nm; Φ_F = 26% (cyan-green emission of grafted 7-hydroxycoumarin partially quenched par free thiol). HPLC (system A): t_R = 17.9 min, purity = 99%.

Free-sulfhydryl fluorescent peptide **28** (1.0 mg, 0.7 μmol, 1 equiv.) and a solution of maleimide-nitro-DABCYL **26** (0.43 mg, 0.77 μmol, 1.1 equiv.) in CH_3CN (20 mM) were dissolved in a mixture of NMP and $NaHCO_3$ (0.1 M aq.; pH 8.5) (2:1, v/v; 100 μL). The resulting reaction mixture was protected from light (with aluminum foil), and stirred at room temp. for 1 h. The reaction was checked for completion by RP-HPLC (system A). Then, the mixture was diluted with TFA (0.1% aq.; 4 mL), and purified by semipreparative RP-HPLC (system K). The product-containing fractions were lyophilized to give uPA-sensitive FRET probe **29** (0.8 mg, 62%) as a yellow amorphous powder. LRMS (ESI$^+$; CH_3CN/H_2O, 2:1, v/v): calcd. for $C_{72}H_{91}N_{22}O_{24}S_2^+$ [M + H]$^+$ 1711.60; found 1712.20 and 856.27 [M + 2 H]$^{2+}$. HPLC (system A): t_R = 24.4 min, purity = 100%.

Supporting Information (see footnote on the first page of this article): Details of the synthesis of *ortho*-phenylenediamine derivatives **2**, **3**, **5**–**7**, and 7-acetoxy-3-formylcoumarin-6-sulfonic acid. Analytical data for all compounds/probes synthesized in this work.

Acknowledgments

This work was partially supported by INSA Rouen, Rouen University, Centre National de la Recherche Scientifique (CNRS), Labex SynOrg (ANR-11-LABX-0029), and Région Haute-Normandie (CRUNCh network). Financial support from the Région Haute-Normandie and Fonds Européen de Développement Régional (FEDER) (TRIPODE, grant number 33883) for Ph. D. grants to B. R. and A. C., respectively, is greatly acknowledged. A. R. thanks

the Institut Universitaire de France (IUF) for financial support, and the "Plateforme d'Analyse Chimique et de Synthèse Moléculaire de l'Université de Bourgogne (PACSMUB, http://www.wpcm.fr) for access to the Bruker Avance III 500 spectrometer and the Thermo LTQ Orbitrap XL mass spectrometer. The authors thank Marie-José Penouilh and Dr. Fanny Picquet (University of Burgundy, ICMUB, UMR CNRS 6302) and A. Marcual (laboratory COBRA, UMR CNRS 6014) for recording the HRMS spectra, and Patricia Martel (University of Rouen, laboratory COBRA, UMR CNRS 6014) for recording the IR spectra.

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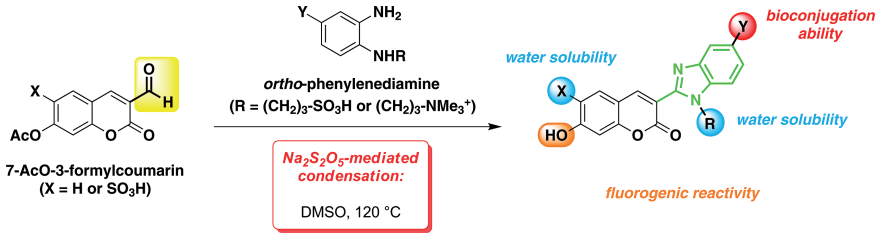
Received: September 15, 2014
Published Online: ■

Fluorescent Probes



A condensation reaction between 7-acetoxy-3-formylcoumarin and various *C*- and/or *N*-monosubstituted *ortho*-phenylendiamine derivatives has led to a new class of 3-(heteroaryl)-7-hydroxycoumarins meeting the requirements for biosensing applications (bioconjugation ability, water solubility, and high "fluorogenic" reactivity).

Keywords: Fluorescent probes / Fluorescence / Biosensors / Oxygen heterocycles

