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Dyes

Straightforward Access to Water-Soluble Unsymmetrical Sulfoxanthene Dyes: Application to the Preparation of Far-Red Fluorescent Dyes with Large Stokes' Shifts

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Abstract: An efficient synthesis of water-soluble unsymmetrical sulforhodamine/sulforhodol fluorophores containing a single julolidine fragment is presented. Owing to their valuable spectral properties in aqueous buffers, these dyes, especially those bearing a free aniline or phenol moiety, are valuable components of fluorogenic probes for a variety of biosensing applications. A further extension of this synthetic methodology to unusual phenols, namely 7-*N*,*N*-dialkylamino-4-hydroxy coumarins has enabled us to provide a new family water-soluble dyes of large Stokes' shift with far-red spectral features.

Fluorescence molecular imaging is now regarded as a powerful technique for visualizing biological processes and detecting biologically relevant species in tissues and in whole living organisms. This technique allows attractive opportunities to noninvasively diagnose disease stages at the molecular level, and thus, may help revolutionize medical diagnosis.[1] The key to effective implementation of this imaging technique relies on the use of fluorescent probes with appropriate photophysical properties in the form of excitation and emission in the far-red to near-infrared (NIR) (>650 nm) spectral region in order to minimize photodamage to biological samples, increase tissue penetration, minimize Rayleigh–Tyndall scattering of light, and minimize interference from background autofluorescence of cellular components.[2] Consequently, during the past decade, tremendous research efforts have been devoted to the development of far-red or NIR fluorophores that are both soluble and brightly fluorescent in aqueous environments.[3] The most common structures for such biocompatible emitters belong to the cyanine family (that is, Cy5 and Cy7 derivatives), even though their photo- and chemical stabilities are frequently limited, and their fluorescence quantum yields are often suboptimal.[4] Some improvements to these properties have been made with π-extended derivatives of the boron–dipyrromethene (BODIPY) scaffold, but most of them display complex structures and their preparation is not necessarily trivial.[5] Alternative large planar π-conjugated fluorescent organic dyes belonging to the xanthene family have recently received particular attention owing to their valuable properties: 1) excellent photophysical properties such as high molar extinction coefficients, excellent fluorescent quantum yields, and good photostability; 2) high chemical stability, especially under harsh conditions of pH and temperature; 3) easy modulation of their fluorescence properties through the spirocyclic/open-ring switching mechanism and/or through the reversible chemical modification (for example, amidification or esterification) of their aniline or phenol moieties.[6] Thus, the design of "smart" optical bioprobes that involve a xanthene dye as a fluorescent label and that are suitable for sensing/imaging of a specific bioanalyte (for example, an enzyme, a biomolecule, or a biologically relevant anion or cation) is currently the subject of intensive research, as illustrated by some recent reviews.[7,8] In this context, significant efforts have been recently devoted to the design of rhodamine-based far-red to NIR dyes,[9] either by replacing the oxygen-atom bridge of the xanthene core with a Group 14 element (that is, C, Si, or Ge)[10] or an oxidized tellurium atom,[11] or by merging a rhodamine analogue with a hemicyanine scaffold to give Changsha NIR fluorophores.[12] Furthermore, hybrid fluorophores of 7-*N*,*N*-diethylcoumarin and benzopyrilium moieties have recently been proposed as rhodamine-inspired dyes in the construction of NIR and ratiometric fluorescent probes for the selective detection of various analytes, including Hg^{2+} and biothiols.[13] Several synthetic approaches are now available to enhance water solubility of these fluorophores through the site-specific attachment (at either step of their synthesis) of polar residues, including hydroxyl, phosphate, and sulfonate groups to the xanthene core structure, and to convert them into bioconjugatable reagents for covalent labeling.[14] However, most of these far-red-emitting rhodamine dyes have two tertiary aniline moieties (mainly julolidine or *N*-alkyl 2,2-dimethyl-1,2-dihydroquinoline fragments) that prevent the design of fluorescence "turn-on" probes based on a selective X–N bond-cleavage reaction,

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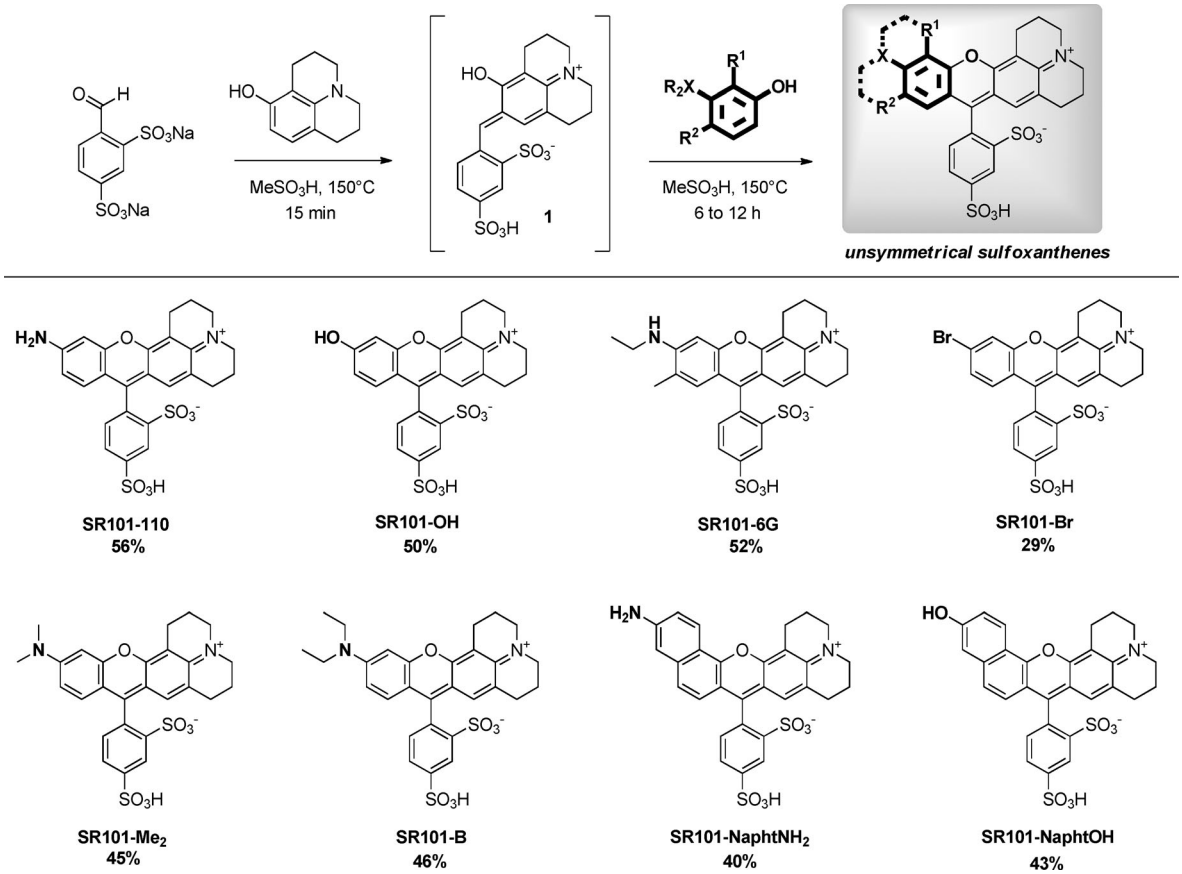
probes that are recognized as being key tools for imaging biologically important analytes and enzymes (for example, proteases).[15] To circumvent this issue, long-wavelength unsymmetrical rhodamines obtained from the condensation of two distinct *meta*-hydroxyaniline partners (ideally, a primary and a tertiary aromatic amine) with a phthalic acid derivative, could be used. To the best of our knowledge, no general and effective method for synthesizing such unusual xanthene dyes has been reported to date. Nonetheless, there are some reports focused on the preparation of primary-amine-functionalized rosamine dyes (that is, rhodamine dyes that do not bear the 2′-carboxylic acid group)[16] through effective organometallic addition to unsymmetrical 3,6-bis(amino)xanthone derivatives,[17] and their successful use in various live-cell-imaging applications.[18] Significant synthetic efforts have also been devoted to another class of unsymmetrical xanthene dyes, namely the rhodols, which are structural hybrids of fluorescein and rhodamine. However, few far-red-emitting derivatives (for example, the naphthorhodols) are currently available and such compounds are isolated in low yields through suboptimal synthetic routes.[19,20]

In this context, there is a serious need to develop an alternative synthetic route to unsymmetrical xanthene dyes, based on a condensation process involving either two different *meta*-aminophenol derivatives (for rhodamines) or a *meta*-aminophenol and a *meta*-hydroxyphenol (for rhodols), and an aryl-carbonyl-based electrophilic partner. Herein, we report the practical implementation of this new strategy, which was used primarily for the synthesis of unsymmetrical derivatives of the red fluorescent dye, sulforhodamine 101 (SR101, also known as Texas Red for its sulfonyl chloride derivative).[21] Indeed, this popular fluorophore exhibits structural features (that is, two sulfonate groups on the *meso* phenyl ring and positively charged julolidine units) that are valuable to retain within the core structure of targeted fluorescent compounds so as to convey water solubility and red-shifted emission. With this synthetic strategy in hand, further extension of this methodology to reach original pro-fluorescent phenols derived from 7-*N*,*N*-dialkylaminocoumarins was studied to provide analogues of rhodamine NIR derivatives with large Stokes' shift.

Unsymmetrical sulforhodamines were synthesized from 4-formylbenzene-1,3-disulfonic acid in two steps (Scheme 1). First, condensation between this disulfonated benzaldehyde and 8-hydroxyjulolidine was achieved at 150 °C, using methanesulfonic acid as a solvent. Under these conditions, quantitative conversion into Michael-type acceptor **1** was obtained by keeping the reaction time under 15 min (see the Supporting Information for the corresponding RP-HPLC elution profile of the crude reaction mixture) and without further addition of the 8-hydroxyjulolidine moiety. Purification by precipitation with Et_2O led to a compound with a high degree of purity (up to 95%), the structure of which was unambiguously confirmed



Scheme 1. Synthesis of unsymmetrical sulfoxanthene dyes. All compounds were recovered as TFA salts.

by detailed measurements that included ESI-HRMS and NMR spectroscopic analyses. Notable, the use of less acidic conditions, that is, 5% trifluoroacetic acid (TFA) in *N*,*N*-dimethylformamide (DMF), lead to a less efficient condensation reaction. Subsequent reaction of **1** with a *meta*-substituted phenol or a 6-substituted naphthol was next conducted under the same acidic and temperature conditions over a more extended period of time. Conducting the reaction at a higher temperature (165 °C) enables reduction of reaction times but leads to the formation of side products. Six different commercially available *meta*-substituted phenols, most of them bearing structural units found in popular symmetrical xanthene dyes (for example, fluorescein, rhodamine 110, rhodamine 6B, rhodamine B), were chosen so as to obtain an array of unsymmetrical sulforhodamines/sulforhodols covering a large part of the visible-light spectrum (Scheme 1). These compounds were readily purified by RP-HPLC and recovered with satisfying yields ranging from 29% to 56%. The additional conjugation of the π system of such unsymmetrical xanthene dyes was also targeted through the preparation of naphthyl derivatives **SR101-NaphtNH$_2$** and **SR101-NaphtOH** from commercially available 6-aminonaphthol and 1,6-dihydroxynaphthalene, respectively. Notably, using this sequential two-step condensation, **SR101-NaphtOH** was isolated in a yield that was four times higher than that reported by Hilderbrand and Weissleder, who employed a one-pot protocol involving the microwave irradiation (200 °C, 15 min) of a mixture of 1,6-dihydroxynaphthalene, 8-hydroxyjulolidine, and 4-formylbenzene-1,3-disulfonic acid disodium salt in 85% phosphoric acid at the same approximate scale (43 and 10% for 0.16 and 0.25 mmol of 1,6-dihydroxynaphthalene, respectively). The structures of these novel aniline- or phenol-based fluorophores were confirmed by detailed measurements including ESI-HRMS and NMR analyses. The purity of each compound (determined through RP-HPLC analyses) was found to be equal to or above 98%, and thus being suitable for an accurate and reliable determination of their photophysical properties. Furthermore, these sulfonated dyes were found to be

Table 1. Photophysical properties of unsymmetrical sulfoxanthene dyes at 25 °C. For structures, see Scheme 1.

Compound	Solvent	Abs. λ_{max} [nm][a]	Em. λ_{max} [nm]	Stokes' shift [nm]	Φ_F [%]
SR101–110	MeCN	542	567	25	87[b]
	DMSO	553	576	23	88[b]
	MeOH	548	570	22	76[b]
	PBS	554	576	22	55[b]
SR101-OH	MeCN (cation)[c]	500/536	570	70/34	24[b]
	MeCN (dianion)[d]	533	555	22	61[b]
	DMSO (cation)[c]	504/539	571	67/32	37[b]
	DMSO (dianion)[d]	538	562	24	65[b]
	MeOH (cation)[c]	502/536	568	66/32	26[b]
	MeOH (dianion)[d]	539	562	23	57[b]
	PBS	548	572	24	35[b]
	0.1 M NaOH (pH 13)	548	572	24	36[b]
SR101–6G	MeCN	552	575	23	99[e]
	DMSO	562	584	22	98[e]
	MeOH	558	579	21	84[e]
	PBS	565	586	21	68[e]
SR101-Br[f]	MeCN	504	–	–	–
	DMSO	507	–	–	–
	MeOH	507	–	–	–
	PBS	510	–	–	–
SR101-Me$_2$	MeCN	560	580	20	89[e]
	DMSO	570	591	21	96[e]
	MeOH	565	583	18	82[e]
	PBS	573	593	20	66[e]
SR101-B	MeCN	563	582	19	95[e]
	DMSO	572	592	20	98[e]
	MeOH	568	586	18	86[e]
	PBS	575	595	20	65[e]
SR101-NaphtNH$_2$	MeCN	581	611	30	63[g]
	DMSO	599	638	39	61[g]
	MeOH	598	629	31	48[g]
	PBS	546/589, 597[h]	625, 626[h]	79/36, 29[h]	19[g], 24[g,h]
SR101-NaphtOH	MeCN (cation)[c]	532/568	599	67/31	32[e]
	MeCN (dianion)[d]	633	675	42	35[i]
	DMSO (cation)[c]	535/573	602	67/29	36[e]
	DMSO (dianion)[d]	660	682	22	37[i]
	MeOH (cation)[c]	533/570	596	63/26	32[e]
	MeOH (dianion)[d]	603	652	49	35[g]
	PBS	537/580, 582[h]	619, 604[h]	82/39, 22[h]	15[g], 25[g,h]
	0.1 M NaOH (pH 13)	595	637	42	19[g]

[a] Most of the sulfoxanthene dyes were not obtained in sufficient amounts for highly accurate measurements of absorption coefficients; [b] Rhodamine B (RB, $\Phi_F = 72\%$ in MeOH, Ex. $\lambda = 510$ nm (**SR101–110**) or 500 nm (**SR101-OH**)) was used as a standard[37]; [c] organic solvent + 0.5% TFA; [d] organic solvent + 0.5% 0.1 M NaOH; [e] Sulforhodamine 101 (SR101, $\Phi_F = 95\%$ in EtOH, Ex. $\lambda = 515$ nm (**SR101–6G**), 520 nm (**SR101-Me$_2$** and **SR101-B**) or 530 nm (**SR101-NaphtOH** (cation))) was used as a standard[37]; [f] Non-fluorescent compound; [g] Cresyl violet (CV, $\Phi_F = 56\%$ in EtOH, Ex. $\lambda = 540$ nm (**SR101-NaphtNH$_2$** or 550 nm (**SR101-NaphtOH** (dianion))) was used as a standard[37]; [h] PBS + 5% BSA; [i] Sulfoindocyanine dye Cy 5.0 (CV, $\Phi_F = 20\%$ in PBS, Ex. $\lambda = 590$ nm (dianion in MeCN) or 600 nm (dianion in DMSO)) was used as standard[38].

soluble in water and related aqueous buffers ranging in concentration from 1 μM to 20 mM, thus emphasizing the relevance of our "SR101-hybrid" approach to rapidly obtain water-soluble xanthene-based fluorescent dyes, which could potentially be used in biological media. Conversion of these dyes (except those bearing a primary aniline or phenol group) into amine-reactive reagents for labeling biomolecules, may be readily achieved by derivatization of their "remote" sulfonic acid residue with an unusual amino acid, namely, isonipecotic acid, and through the corresponding sulfonyl chloride intermediate.[22] Indeed, this is the most common way to avoid undesired ring-chain tautomerism of sulforhodamine-amine conjugates, a transformation that leads to the formation of the colorless and nonfluorescent sultam.[23] Further conversion of the carboxylic acid into the corresponding *N*-hydroxysuccinimidyl (NHS) ester should enable stable bioconjugatable fluorophores to be obtained.

The spectroscopic properties of these sulforhodamines/sulforhodols were characterized both in polar aprotic solvents (MeCN and DMSO) and in protic solvents (MeOH and phosphate-buffered saline (PBS), pH 7.5). The results are summarized in Table 1 and representative examples of absorption/fluorescence spectra recorded for samples in PBS are shown in Figure 1. All sulforhodamines display a broad and intense absorption band with a maximum in the range 542–599 nm, depending on the bis(amino)xanthene substitution pattern and the solvent used, and assigned to the 0–0 band of the S0→S1 transition. A less pronounced shoulder peak at the higher energy side is also observed and is attributed to the vibronic relaxation (the 0–1 vibrational band). Furthermore, the bathochromic shift observed in both absorption and fluorescence spectra (see Table 1 and the Supporting Information) with increasing solvent polarity (compare spectra for solvents that do not donate hydrogen bonds, such as MeCN and DMSO, or compare spectra for different protic solvents, such as MeOH and aqueous buffer) suggests that the fluorescence involves the $\pi\rightarrow\pi^*$ transition. Such a solvent effect on the spectral properties of sulforhodamines has been recently reported for the commercial sulforhodamine B and meaningfully interpreted through the evaluation of ground- and excited-state dipole moments of this dye.[24] As revealed by fluorescence emission profiles shown in Figure 1 (and those available in the Supporting Information), all these unsymmetrical sulforhodamines exhibit an intense emission peak with a maximum in the range 567–638 nm. In all cases, the quantum yields are high, and to our great satisfaction, are especially high for compounds in simulated physiological environments (Table 1, entries 4, 16, 24 and 28). These results support the beneficial effects of introducing negatively charged ionizable groups (that is, sulfonic acids) onto the *meso*-phenyl ring of these xanthene dyes, both to impart water solubility and to promote dye–dye repulsion and resistance to aggregation-induced fluorescence quenching in aqueous media. Indeed, a perfect match between the absorption and excitation spectra recorded in PBS (see the Supporting Information) was observed, except for the more hydrophobic π-extended derivative **SR101-NaphtNH$_2$**, the two sulfonate groups of which are not sufficient to completely prevent dye–dye aggregation, which causes a subsequent decrease in the quantum yield (Table 1). However, the strong red fluorescence of **SR101-NaphtNH$_2$** is recovered by adding 5% (*w*/*v*) of bovine serum albumin (BSA), an additive often used in buffers in order to mimic body fluids ($\Phi_F = 25\%$). Indeed, this protein is known to enhance the emission of many fluorophores owing to a combination of rigidification, reduction in the polarity of the dye's microenvironment (binding in the hydrophobic BSA pocket), and deaggregation.[25] As a final matter, small Stokes' shifts, all within the range 20–30 nm, are observed and are comparable to those of traditional symmetrical rhodamine dyes. For the two sulforhodol derivatives, **SR101-OH** and **SR101-NaphtOH**, some interesting differences in their spectral behavior at physiological pH are noted (Figure 1 and Table 1). The fluorescein–SR101 hybrid was found to display an intense orange fluorescence assigned to its anionic form (that is, a phenolate species) that may be the predominant form of **SR101-OH** in PBS. The fluorescence quantum yield (35%) is of the same order of magnitude as that of a similar rhodol dye bearing a single carboxylic acid instead of the two sulfonic acid moieties on the *meso*-phenyl ring, known as Peroxy Orange 1 ($\Phi_F = 46\%$ in 20 mM HEPES, pH 7.0).[26] In contrast, absorption and emission spectra of the naphthol derivative appear as broad red-shifted bands that suggest the presence of several distinct absorbing/emissive species at pH 7.5, including mainly the protonated and deprotonated forms of **SR101-NapthOH**. The presence of two local maxima at 537 and 580 nm in the UV/Vis absorption spectrum also supports the presence of two predominant species in PBS even if the formation of H-type dimers (known to lead to a remarkable blue-shift of the absorption peak), which may partly explain the moderate fluorescence quantum yield of

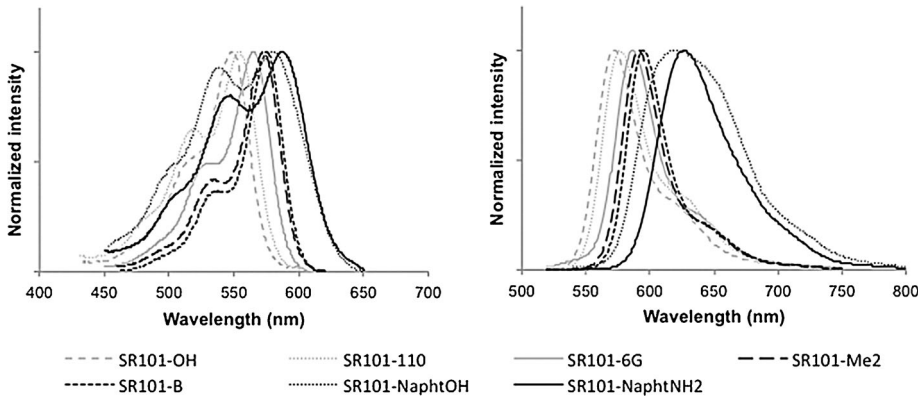


Figure 1. Normalized absorption (left) and emission (right) spectra of unsymmetrical sulforhodamine/sulforhodol dyes recorded in PBS (pH 7.5) at 25 °C.

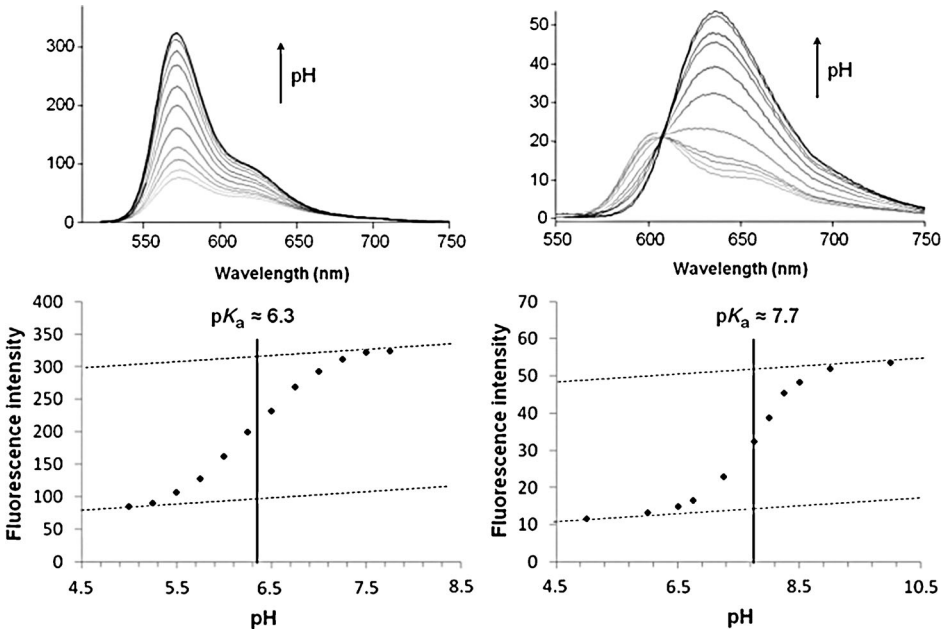
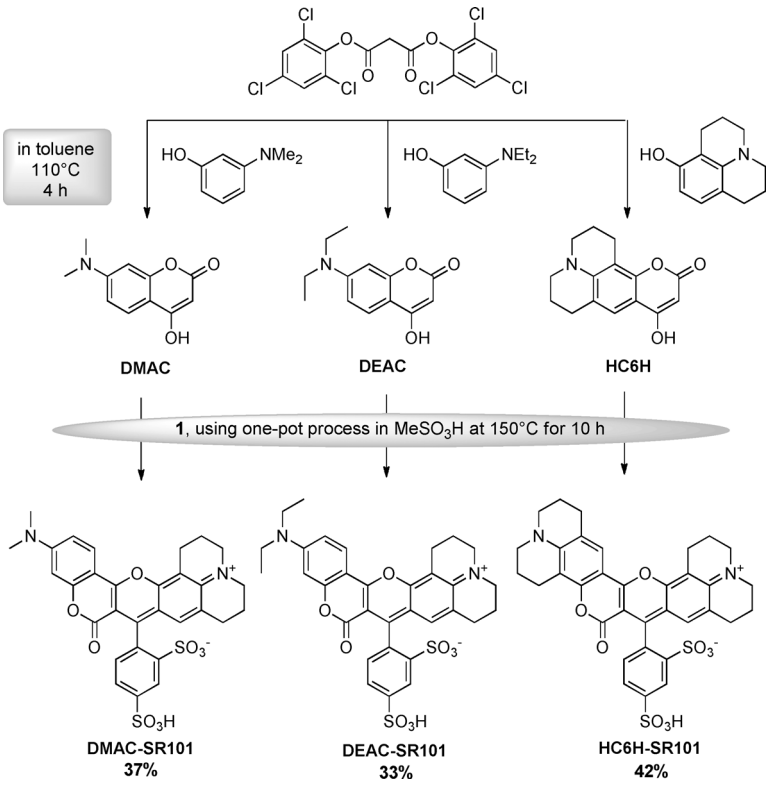


Figure 2. Determination of pK_a values for phenols of sulforhodols **SR101-OH** (left) and **SR101-NaphtOH** (right).

SR101-NaphtOH (15%), cannot be entirely excluded. To rationalize the differences in spectral features of these two rhodols, the pK_a of their phenol moiety was evaluated by monitoring their fluorescence emission as a function of the environmental pH (Figure 2). **SR101-OH** and **SR101-NapthOH** have pK_a values of 6.3 and 7.7, respectively. Thus, the relative low pK_a of **SR101-OH** confirms that, under physiological conditions, the phenolate form of this dye is predominant, whereas its naphthyl congener is fully deprotonated only in more basic aqueous media (namely, pH > 9.0). A fluorescence quantum yield of 19% was determined for **SR101-NapthOH** in aqueous 0.1 M NaOH, a value that is comparable to that reported for the seminaphthorodafluor, **C.SNARF-X**, a structural analogue of **SR101-NapthOH** bearing a dicarboxyphenyl ring instead of the aryl disulfonate moiety, as developed by Molecular Probes in the early 90s.[19] Conversely, this value is significantly different from that reported by Hilderbrand and Weissleder ($\Phi_F = 30\%$ in bicarbonate/carbonate buffer, pH 10). It is nevertheless difficult to make an accurate comparison between these two values because a different quantum yield standard was used for such determination (cresyl violet versus Cy5). A further examination of their spectral properties in organic solvents "buffered" with TFA (0.5% *v/v*) or aqueous 0.1 M NaOH (0.5% *v/v*) was also conducted and summarized in Table 1 (see the Supporting Information for the corresponding absorption/emission spectra). Different spectral behaviors for the phenol and phenolate forms of these rhodols were observed. Under acidic conditions, absorption spectra of **SR101-OH** and **SR101-NapthOH** display two distinct maxima at around 500 and 540 nm and 530 and 570 nm, respectively, which may be assigned to cationic species, namely xanthene carbocation and julolidinium salt. In contrast, absorption spectra of dianion species show relatively broad and featureless peaks, with a single maximum located in the range 533–539 nm and 603–660 nm, respectively. As expected, strong fluorescence emission in the orange or in the red/far-red region was observed for these basic phenolate forms of **SR101-OH** and **SR101-NapthOH**, as inferred by the high quantum yields obtained in basic media (ca. 60% for **SR101-OH** and in the range 25–35% for **SR101-NaphtOH**). An additional unusual monobromo xanthene dye, **SR101-Br** (Table 1) has also been synthesized with the goal in mind to use this derivative to further obtain unsymmetrical sulforhodamine derivatives, specifically functionalized at their aniline moiety, by using either a palladium-catalyzed Buchwald–Hartwig type amination reaction[27] or the Ullmann coupling.[28] As expected, the lack of a detectable fluorescence emission, whatever the solvent used, can be attributed to both the internal heavy-atom effect[29] and the removal of the electron-donating group (OH or NR_2) within its xanthene scaffold.

Because one of the main drawbacks of rhodamine dyes is their small Stokes' shifts (usually 20–40 nm), there is a strong interest in novel molecular design and synthetic approaches aimed at improving this spectral parameter without resorting to the Förster resonance energy transfer (FRET) or through-bond energy transfer (TBET) effect.[30] Interestingly, 7-*N*,*N*-dialkylaminocoumarins (DAAC) are known to exhibit large Stokes' shifts (> 100 nm) as the result of an excited-state intramolecular charge-transfer (ICT) effect;[31] we thought to take advantage of this unique property through the structural merging of DAAC and SR101 scaffolds within the same fluorescent architecture by using the two-step condensation process described above. Thus, three phenols derived from 7-*N*,*N*-dimethyl or 7-*N*,*N*-diethylaminocoumarin (DMAC or DEAC) or coumarin 6H[32] were independently reacted with **1** under acidic conditions to give, after RP-HPLC purification, the targeted 7-*N*,*N*-dialkylaminocoumarin-sulforhodamine hybrids in satisfying yields (33–42%) (Scheme 2). The structure of **DMAC-SR101**, **DEAC-SR101**, and **HC6H-SR101** were characterized by NMR spectroscopy and ESI-HRMS.

We next investigated the photophysical properties of these hybrids in the same solvents previously used for sulforhodamines/sulforhodols (see above). Table 2 provides the corresponding spectral features, and the absorption/emission profiles recorded under simulated physiological conditions are displayed in Figure 3. As a general trend, the absorption and fluorescence emission maxima are red-shifted in the **DMAC-SR101**→**DEAC-SR101**→**HC6H-SR101** series, albeit there is only a minute difference between the first two members. This trend follows the increasing donor ability of the dialkylamino



Scheme 2. Synthesis of 7-*N*,*N*-dialkylaminocoumarin-fused sulforhodamine analogues. All compounds were recovered as TFA salts.

Table 2. Photophysical properties of 7-*N*,*N*-dialkylaminocoumarin-fused sulforhodamines analogues at 25 °C. For structures, see Scheme 2.

Compound	Solvent	Abs. λ_{max} [nm][a]	Em. λ_{max} [nm]	Stokes' shift [nm]	Φ_F [%]
DMAC-SR101	MeCN	598	635	34	58[b]
	DMSO	608	644	36	56[b]
	MeOH	599	636	37	48[b]
	PBS	561/603, 605[c]	642, 637[c]	81/39, 32[c]	9[b], 14[b,c]
DEAC-SR101	MeCN	602	635	33	58[b]
	DMSO	611	644	33	57[b]
	MeOH	604	638	34	47[b]
	PBS	570/608, 610[c]	642, 644[c]	72/34, 34[c]	10[b], 15[b,c]
HC6H-SR101	MeCN	617	675	58	18[d]
	DMSO	628	684	56	19[d]
	MeOH	621	673	52	15[d]
	PBS	624, 626[c]	–[e], 654[c]	–[e], 28[c]	–[e], 15[c,d]

[a] These coumarin-sulforhodamine hybrids were not obtained in sufficient amounts for highly accurate measurements of absorption coefficients; [b] Cresyl violet (CV, $\Phi_F = 56\%$ in EtOH, Ex. $\lambda = 570$ nm) was used as a standard[37]; [c] PBS + 5% BSA; [d] Sulfoindocyanine dye Cy 5.0 ($\Phi_F = 20\%$ in PBS, Ex. $\lambda = 600$ nm) was used as a standard[38]; [e] very weakly fluorescent ($\Phi_F < 0.01$) without BSA.

group in the coumarin moiety. Thus, our SR101-hybrid approach is a rapid and convenient synthetic method toward long-wavelength sulforhodamine dyes with absorption/emission beyond 600 nm. A strong NIR emission centered at around 680 nm was obtained for **HC6H-SR101** in polar organic solvents. However, its fluorescence quantum yield in PBS is extremely low and consistent with the formation of dye–dye aggregates. Nevertheless, once more, the addition of BSA led to strong emission of this NIR dye under physiological conditions. Conversely, it is not essential to use this aggregate-disrupting agent to obtain significant far-red emission (Φ_F close to 10%) for **DMAC-SR101** and **DEAC-SR101** in PBS, thus further underlining the positive effect of the sulfonate groups to partly prevent dye aggregation. This is also evidenced by the recent work of Chen et al., who showed that 7-*N*,*N*-dialkylaminocoumarin-rhodamine B hybrids are not fluorescent in water.[33] In this latter case, the single carboxylic acid on the *meso*-phenyl ring is not effective for conveying water solubility. As expected, the three compounds display a larger Stokes' shift relative to that of the (sulfo)rhodamine dyes, such as the unsymmetrical derivatives described in this work. These values are comparable to those recently reported by Tian et al. for rhodamine dyes containing 1,4-diethyl-1,2,3,4-tetrahydroquinoxaline as an effective electron donor group (35–60 nm) and attributed to the ICT observed for the 7-*N*,*N*-dialkylaminocoumarin moiety.[34,35] Thus, all these valuable photophysical properties under simulated physiological conditions make these novel coumarin- and sulforhodamine-fused deep red fluorescent dyes promising candidates for designing biolabeling reagents or fluorescence "turn-on" probes with optimized properties, especially for applications in complex biological media.

In summary, we have prepared an array of novel water-soluble unsymmetrical sulforhodamine/sulforhodolfluorophores through a highly efficient and concise synthetic route based on the sequential condensation of two different *meta*-aminophenol units with a disulfonated benzaldehyde. To the best of our knowledge, this is the first synthesis of unsymmetrical xanthene dyes that does not require either 1) the post-functionalization of fluorescein derivatives through a palladium-catalyzed cross-coupling reaction, 2) the addition of organometallic species to unsymmetrical xanthones, or 3) time-consuming and yield-decreasing purifications. Furthermore, the presence of two sulfonate groups on the *meso*-phenyl ring is effective to make these SR101 analogs both readily soluble and highly fluorescent in polar organic

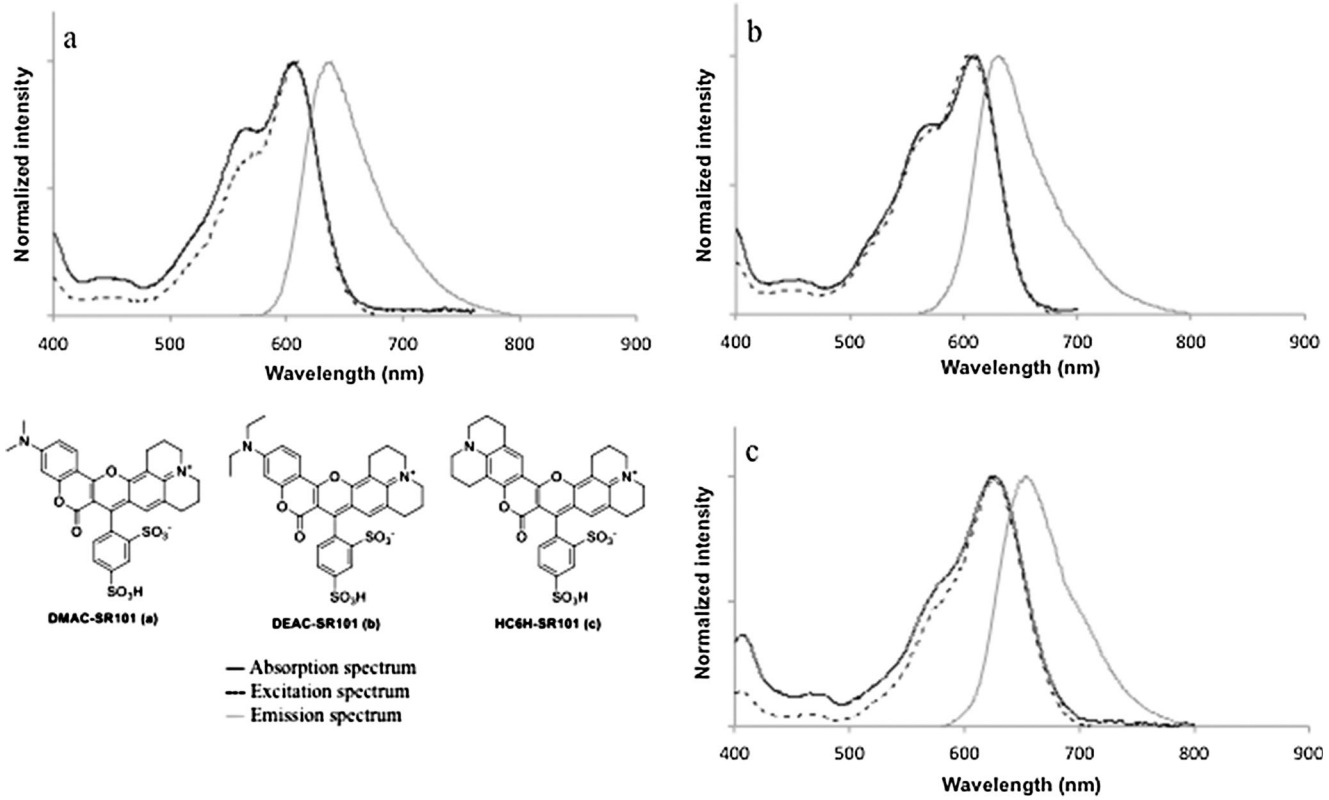


Figure 3. Normalized absorption, emission and excitation spectra 7-*N*,*N*-dialkylaminocoumarin-fused sulforhodamine analogues at 25 °C in PBS + 5% BSA: a) **DMAC-SR101** (Ex λ = 570 nm, Em λ = 710 nm); b) **DEAC-SR101** (Ex λ = 570 nm, Em λ = 710 nm); c) **HC6H-SR101** (Ex λ = 600 nm, Em λ = 730 nm).

solvents and in aqueous media. Further conversion of the free aniline or phenol derivatives (**SR101–110**, **SR101-OH**, **SR101-NaptNH$_2$** and **SR101-NaphtOH**) to latent fluorophores through the transformation of their NH_2/OH group into an enzyme-reactive moiety (for example, amide, ester, ether, or diazo bridge) is currently in progress in our laboratory, an investigation that should provide fluorescence "turn-on" probes suitable for sensing/imaging various enzyme activities including proteases, esterases, galactosidases,[8] and redox enzymes.[36] Versatility of the present synthetic methodology was also demonstrated with the rapid preparation of the first 7-*N*,*N*-dialkylaminocoumarin–sulforhodamine hybrids, a novel class of far-red fluorescent dyes with attractive structural and spectral features suitable for biolabeling and biosensing applications.

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Keywords: far-red dyes · fluorescent probes · rhodamines · rhodols · Stokes' shift

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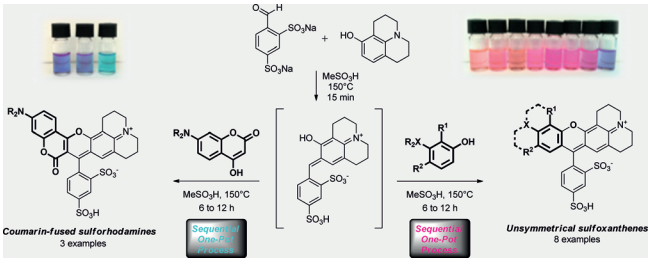

Unity is strength! A concise and efficient synthetic route toward unsymmetrical sulforhodamine/sulforhodol dyes has been developed (see scheme). Ten different fluorophores both soluble and fluorescent in polar organic solvents and in aqueous media were obtained. Those with a fused coumarin fragment are a novel class of far-red fluorescent dyes with attractive spectral features especially for use under physiological conditions.

Dyes

A. Chevalier, P.-Y. Renard, A. Romieu**

■■ – ■■

Straightforward Access to Water-Soluble Unsymmetrical Sulfoxanthene Dyes: Application to the Preparation of Far-Red Fluorescent Dyes with Large Stokes' Shifts