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Water solubilization of xanthene dyes by post-synthetic sulfonation in organic media

Anthony Romieu [a,b,*], Delphine Tavernier-Lohr [c], Stéphane Pellet-Rostaing [d,*], Marc Lemaire [c], Pierre-Yves Renard [a,b,e]

[a] Equipe de Chimie Bio-Organique, COBRA-CNRS UMR 6014 & FR 3038, rue Lucien Tesnière, 76131 Mont-Saint-Aignan, France
[b] Université de Rouen, Place Emile Blondel, 76821 Mont-Saint-Aignan, France
[c] ICBMS, UMR 5246, Université Claude Bernard Lyon1, CPE, 43 Boulevard du 11 Novembre 1918, 69100 Villeurbanne, France
[d] ICSM, UMR 5257, Bâtiment 426, BP 17171, 30207 Bagnols Sur Ceze Cedex, France
[e] Institut Universitaire de France, 103 Boulevard Saint-Michel, 75005, Paris, France

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ABSTRACT

Highly water-soluble fluorescent fluorescein and rhodamine dyes were synthesized through amidification of their carboxylic acid functionality with original di- or tri-sulfonated amino linkers derived from taurine or α-sulfo-β-alanine. This post-synthetic derivatization was performed in organic media both to minimize the premature hydrolysis and to suppress the precipitation of the involved active ester of fluorophore, frequently encountered using standard Schotten–Baumann conditions.

1. Introduction

Fluorescent organic dyes are widely used as non-radioactive labels and as a key component of optical bio-probes for various biosensing and imaging applications.[1] Xanthene-based dyes such as rhodamines[2] and fluoresceins[3] (and more recently rhodols[4] and rosamines[5]) are among the most commonly used class of fluorescent detection reagents. Their spectral properties, especially in the near-infrared (NIR) range and/or in physiological conditions, are often less optimal than those exhibited by BODIPY and cyanine dyes. However, their use is preferred when the following valuable properties are required: (1) high photo- and chemical stability, (2) easy reversible formation of a colorless and non-fluorescent spirocyclic structure, and (3) modulation of fluorescence properties through reversible chemical modification (e.g., amidation or esterification) of aniline (for rhodamines) or phenol (for fluoresceins) moiety. Indeed, the latter two features are essential for the current design of spectroscopic off-on type probes (also named fluorogenic probes, latent or pro-fluorophores)[6] used as chemo- or biosensors for detecting various analytes (enzymes, heavy metal ions, etc.).[7]

For most biological applications, xanthene dyes must also exhibit both good water solubility and resistance to the formation of non-fluorescent dimers and higher aggregates, especially after conjugation to biological material.[8] Consequently, recent efforts have been devoted to the development of water-soluble analogues of rhodamines, through the introduction of some ionizable hydrophilic groups (carboxylic acid and/or sulfonic acid) within the xanthene core structure of these fluorescent dyes.[9] Most of them are commercialized by Molecular Probes (Invitrogen) and sold under the trade name Alexa Fluor®.[10] However, such chemical functionalization often requires: (1) the use of aggressive reagents such as oleum or concentrated sulfuric acid often not compatible with the stability of the fluorophore, or (2) restarting the synthesis of the fluorescent core from the beginning with building blocks bearing the water-solubilizing groups (de novo approach). Thus, these synthetic processes often lead to the desired fluorophore in a modest overall yield and are not amenable to large-scale preparation (i.e., gram scale). Recently, we have reported a straightforward method to enhance the water solubility of organic dyes (BODIPYs, cyanines and rhodamines) by a post-synthetic chemical derivatization of their carboxylic acid functionality with a poly-sulfonated peptide-based linker (i.e., α-sulfo-β-alanine di- or tripeptide) under standard Schotten–Baumann conditions.[11,12] This methodology has not yet been applied to the fluorescein derivatives and high hydrophobic extended conjugated rhodamines. However, the corresponding water-soluble analogues of these latter long-wavelength fluorophores could be useful as laser dyes and fluorescent

* Corresponding authors. Tel.: +33 2 35 52 24 15; fax: +33 2 35 52 29 71 (A.R.).
E-mail address: anthony.romieu@univ-rouen.fr (A. Romieu).

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markers in numerous bio-labeling, bio-imaging, and single-molecule-based spectroscopy applications.

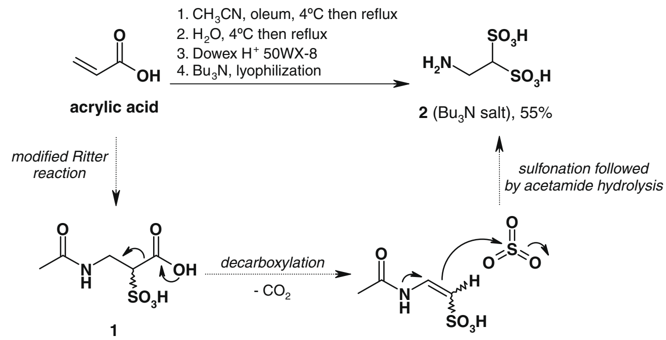
In this Letter, we report the water-solubilization of fluorescein, benzo[*b*]thiophene-, and indole-containing rhodamine dyes, through amidification of their carboxylic acid function with 2-aminoethane-1,1-disulfonic acid and tripeptide (α-sulfo-β-alanine)$_3$, respectively. The use of tributylammonium salts of these two poly-sulfonated linkers was considered in order to perform the derivatization reaction in organic media (instead of a mixture of DMF and aq buffer), especially to prevent both the premature hydrolysis and precipitation of the involved *N*-hydroxysuccinimide (NHS) active ester of hydrophobic rhodamine dyes, previously observed by us when the reaction was conducted under standard Shcotten–Baumann conditions. The spectral properties of the resulting water-soluble xanthene dyes were then evaluated under physiological conditions.

2. Results and discussion

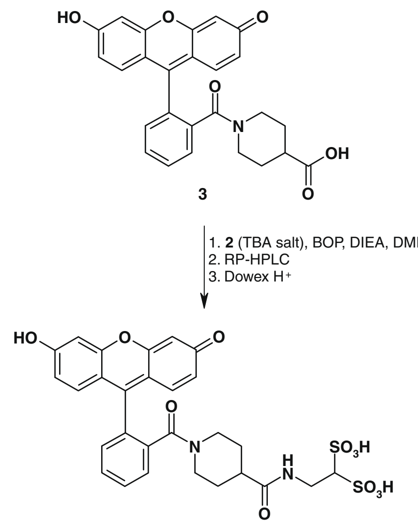
2.1. Synthesis of water-soluble fluorescein derivative 4

First, we focused on the water-solubilization of fluorescein. To our knowledge, only the di-sulfonated rhodamine derivative developed by Molecular Probes and named Alexa Fluor® 488 is currently used as an hydrophilic substitute of this xanthene dye. In order to offer an alternative to this expensive commercial fluorescent marker, we have explored the post-synthetic sulfonation of xanthamide derivative **3** with the original di-sulfonated amino linker **2** (Scheme 2). In the present case, it is not essential to use a water-solubilizing linker bearing a terminal reactive group for bioconjugation because the ultimate goal of this synthesis was to provide a new phenol-based fluorophore useful for the preparation of fluorogenic probes based on the pro-fluorescent concept (involving the reversible modification of its phenol moiety). The use of a fluorescein derivative bearing a tertiary amide of isonipecotic acid at the 2′-position instead of the current carboxyl group was preferred to provide steric protection and so to improve the photostability of fluorescein.[13] Furthermore, this is the most common way to avoid the undesired cyclization equilibrium leading to the formation of the colorless and non-fluorescent spirolactone (especially at pH <6). Synthesis of 2-aminoethane-1,1-disulfonic acid **2** was accomplished in one step from acrylic acid, oleum, and acetonitrile using the conditions reported by Wagner et al. for the preparation of α-sulfo-β-alanine through a modified Ritter reaction (Scheme 1).[14] Yet, in our hands, such experimental conditions did not provide this amino acid but the taurine-like di-sulfonated linker **2**. Since the heating temperature of the sulfonation reaction mixture was not indicated by Wagner et al. in their publication (dating from 1968), we chose to heat the reaction mixture under reflux and we suspect that under those harsh experimental conditions, once *N*-acetyl derivative of α-sulfo-β-alanine **1** was formed by the modified Ritter reaction, a spontaneous decarboxylation followed by the sulfonation reaction occurred, leading to **2** (Scheme 1). The structure of **2** was confirmed by detailed measurements, including ESI mass spectrometry and NMR analyses. This compound was converted into the corresponding tributylammonium salt (TBA salt) and dissolved in dry *N*-methylpyrrolidone (NMP) to yield a stock solution (0.5 M) suitable for post-synthetic derivatizations in organic media.

Thereafter, di-sulfonated amino linker **2** could be readily coupled to fluorescein derivative **3** by using BOP phosphonium salt[15] and DIEA in dry DMF to give the desired water-soluble analogue **4** in a satisfying yield (40%). Purification and desalting of **4** were sequentially achieved by semi-preparative reversed-phase HPLC (RP-HPLC) and ion-exchange chromatography (Dowex H$^+$), respectively. The introduction of the di-sulfonated linker onto the isonipecotic acid arm of fluorescein **4** was confirmed by ^{1}H NMR and ESI mass analyses.



Scheme 1. Synthesis of 2-aminoethane-1,1-disulfonic acid and proposed mechanism for its formation.



4 (acid form), 40%

Scheme 2. Synthesis of water-soluble fluorescein **4**.

2.2. Synthesis of water-soluble far-red emitting rhodamine derivatives 12 and 13

To demonstrate the full potential of this water-solubilizing procedure performed in organic media, the post-synthetic sulfonation of benzo[*b*]thiophene- and indole-containing rhodamine dyes **5** and **6** was also investigated. These two compounds belong to a novel class of highly fluorescent rhodamine derivatives with long-wavelength absorption and emission, reported in 2003 by Liu et al.[16] We have recently developed an alternative pathway to these valuable far-red emitting fluorophores, requiring an aryl–aryl coupling through C–H activation followed by a Pictet–Spingler reaction, to facilitate the synthetic access to these new rigid rhodamine derivatives in fewer steps.[17] Our preliminary experiments aiming at the post-synthetic derivatization of these fluorophores with poly-sulfonated amino linkers under previously reported standard Schotten–Baumann conditions failed. This was due to the complete precipitation of the corresponding NHS esters in the mixture of DMF and aq buffer. Furthermore, the small amounts of derivatized fluorophores that we obtained showed that their great hydrophobic character could not be countered by the introduction of only two sulfonate groups.