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Synthesis of 5- and 6-Carboxy-X-rhodamines

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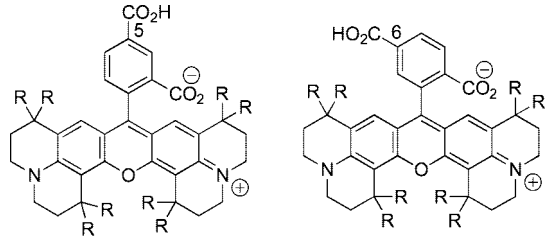
ABSTRACT



1a: R = H
1b: R = Me

2a: R = H
2b: R = Me

An efficient route is reported to 5- and 6-carboxy-X-rhodamines (compounds 1 and 2) that contain multiple *n*-propylene or γ,γ-dimethylpropylene groups bridging terminal nitrogen atoms and the central xanthene core. Gram quantities of these dyes are synthesized from inexpensive starting materials. The isolated products are activated by selective transformation of the carboxylic acid group into *N*-hydroxysuccinimidyl esters in situ and then conjugated with an amino group of a molecule of interest.

Rhodamine dyes are important because of their favorable photochemical and photophysical properties.[1] They have long wavelength absorption maxima, high molar absorptivities, and high quantum yields.[2] Compared to the fluorescein dyes, rhodamines are relatively resistant to photobleaching and their fluorescence spectra are pH independent over a range from 4 to 10.[3] For these reasons, they have broad application, not only in biotechnology for fluorescent labeling or single molecule detection but also in medicine for imaging living cells or live animals in preclinical research.[6,7] In recent years, a wide variety of rhodamine dyes have been commercialized for conjugation with biomolecules.[2] Of these, 5- and 6-carboxy-X-rhodamines (1 or 2) are of special interest because of their highly intense absorption and emission. This is possibly due to the presence of the added structural rigidity introduced by multiple *n*-propylene bridges, which prevents fluorescence deactivation by nonradiative processes. The high cost of these dyes from commercial sources limits their use in biomedical research. Although different methods are available in the literature for the synthesis of diverse rhodamine derivatives,[8−11] no report describes the synthesis of 5- and 6-carboxy-X-rhodamines. Therefore, as a part of our ongoing program to develop fluorescent ligands, we report the synthesis and structure of 5- and 6-carboxy-X-rhodamine dyes that contain repetitive *n*-propylene or γ,γ-

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dimethylpropylene bridging moieties. In addition, we describe an efficient route to activate and conjugate these dyes with the amino group of a molecule of interest to provide easy excess to fluorescent ligands that are useful for materials and biomedical and clinical research.

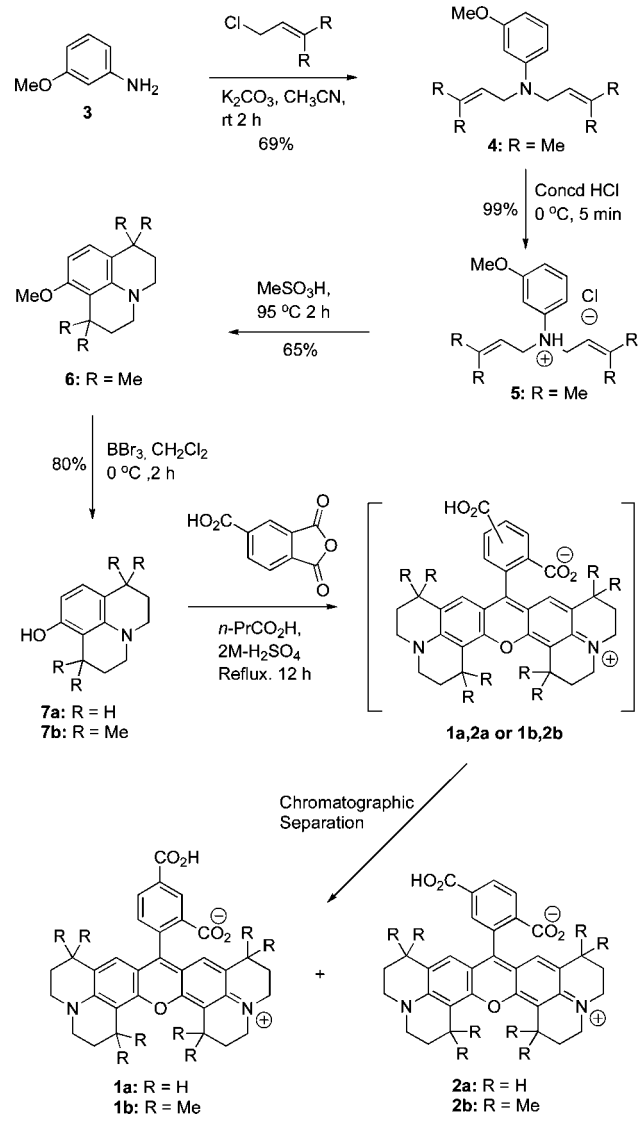
The alkylation of *m*-anisidine (**3**) using 1-chloro-3-methylbut-2-ene in the presence of K_2CO_3 gave 3-methoxy-*N*,*N*-bis(3-methylbut-2-enyl)aniline (**4**) in 69% yield. The treatment of compound **4** with concd HCl at 0 °C afforded 3-methoxy-*N*,*N*-bis(3-methylbut-2-enyl)aniline hydrochloride (**5**) in 99% yield. Intramolecular cyclization of compound **5** using neat $MeSO_3H$ at 95 °C gave 1,1,7,7-tetramethyl-8-methoxyjulolidine (**6**) in 65% yield, and *O*-desmethylation of compound **6** using BBr_3 gave the corresponding 1,1,7,7-tetramethyl-8-hydroxyjulolidine (**7b**) in 80% yield. The 8-hydroxyjulolidine (**7a**) was synthesized using a reported protocol.[12] The synthesis of 5- or 6-carboxy-X-rhodamine dyes (**1** or **2**) requires a symmetrical condensation of 2 equiv of compound **7** with 1 equiv of 4-carboxyphthalic anhydride. The overall process requires two sequential Friedel−Crafts-type electrophilic aromatic substitution reactions for the formation of the xanthene skeleton. We first tried a traditional condensation protocol involving fusion of compound **7** (R = H or Me) with 4-carboxyphthalic anhydride at high temperature (180 °C). This reaction gave minimal yield, possibly due to considerable sublimation of the starting materials arround the vessel wall. Next, we used a catalytic amount of Lewis acid ($ZnCl_2$), or a protic acid (H_2SO_4) that gave a poor yield of the desired products. However, the yield was dramatically increased by using a high-boiling weakly acidic solvent (*n*-$PrCO_2H$, pK_a 4.82) with a trace of 2 M H_2SO_4 under reflux (Scheme 1).

The separation of the two isomers from the regioisomeric mixture [**1a**, **2a** (R = H) or **1b**, **2b** (R = Me)] was achieved by flash chromatography. Extensive optimization was performed to develop conditions for the separation of significant quantities of **1** and **2**. Silica gel 60 (230−450 mesh) was slurry packed into a 10 in. column (1 × 15 in.) and eluted with methanol/chloroform gradients at a flow rate of 5 mL/min. The progress of separation was monitored with a Spectroline ENF-280C UV detector (365 nm window). The best yield was obtained using 0.5 g of crude sample with relatively shallow gradients. In a typical experiment, we obtained 34% and 32% isolated yields for compounds **1a** and **2a** or 42% and 15% isolated yield for compounds **1b** and **2b**, respectively.

The isomeric purity of each of the isolated compounds was determined by HPLC analysis to be greater than 99%.

A complete structural analysis of compounds **1** and **2** was conducted using 1D and 2D NMR spectroscopy. There are three aromatic protons in each of these compounds that are characteristic for their differentiation by ^{1}H NMR. For compound **1a**,

Scheme 1. Synthesis of Rhodamine-Based Fluorescent Dyes **1a**,**b** and **2a**,**b**



signals for two neighboring aryl protons at position-6 and position-7 (H_{arom}-6 and H_{arom}-7) were assigned as two separate doublets at 8.18 and 7.05 ppm, respectively, with a large vicinal coupling constant (J = 9.0 Hz). The assignment was confirmed by a ^{1}H−^{1}H COSY experiment, where H_{arom}-6 showed a strong correlation with H_{arom}-7 (Supporting Information). The proton located at position-4 (H_{arom}-4) was assigned at 8.27 ppm and showed a weak correlation with H_{arom}-6 in the COSY spectrum. This weak correlation indicates that H_{arom}-4 is located at least four bonds from H_{arom}-6 and supports the location of H_{arom}-4 within two carboxyl groups of the aryl ring. In compound **2a**, the resonances for the two neigboring aryl protons (H_{arom}-4 and H_{arom}-5) appeared as two doublets at 7.77 ppm and 8.04 ppm (J = 8.9 Hz), respectively. This assignment was confirmed by a ^{1}H−^{1}H COSY spectrum, in which H_{arom}-4 showed a strong correlation with H_{arom}-5. The sharp singlet located at 7.43 ppm was assigned as H_{arom}-7, that has a weak correlation with H_{arom}-5 in the COSY spectrum. This indicates that H_{arom}-7 is located

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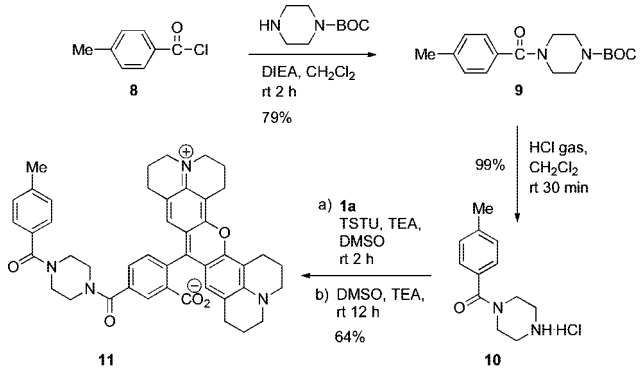
Scheme 2. Conjugate Chemistry



between the carboxyl-group and rhodamine scaffold. The chemical shifts for the two rhodaminyl/vinylic CH protons were assigned at 6.0 ppm. These two protons (H-8 and H-9) are equivalent. The CO_2H proton on the aryl ring of compound **1a** or **2a** appeared at 13.22 ppm as a singlet. The chemical shift resonances and coupling patterns for the aliphatic protons of compounds **1a** and **2a** are almost identical. Details are described in the Supporting Information section.

We synthesized 4-methylbenzoylpiperazine hydrochloride (**10**) using a protocol described in the Scheme 2. Briefly, acylation of *N*-BOC piperazine by 4-methylbenzoyl chloride (**8**) in the presence of *N,N*-diisoproylethylamine gave 4-(4-methylphenylcarbonyl)piperazine-1-carboxylate (**9**) in 79% yield. The 4-(4-methylphenylcarbonyl)piperazine-1-carboxylate (**9**) was then deprotected to compound **10** using hydrogen chloride gas. This reaction proceeded in quantitative yield. The fluorescent compound **1a** or **1b** was activated using *N,N,N,N*-tetramethyl-*O*-(*N*-succinimidyl)uronium tetrafluoroborate (TSTU). The reaction was performed in the presence of ≥2 equiv of base, such as triethylamine, to give the corresponding *N*-hydroxysuccinimidyl ester which was generated in situ. The ester was then reacted with the amino group of compound **10**. This coupling reaction proceeded efficiently with only a slight excess of TSTU. Air-free techniques were used for both activation and coupling reactions. The succinimidyl esterification was performed in an activator vessel under an inert atmosphere, and the activated dye was transferred via a cannula to the coupling vessel that contained compound **10** in dimethyl sulfoxide. The crude product was obtained by removal of solvent, and purification was conducted by a silica gel flash column chromatography using an isocratic elution of 35:7:1 $CHCl_3$/MeOH/concd NH_4OH. This procedure afforded 64% yield for compound **11**. The structures were confirmed by ^{1}H NMR and mass spectrometry. Compound **11** is a model for targeted small molecule−carboxy-X-rhodamine conjugates.

The rigid rhodamine dyes **1a**,**b** and **2a**,**b** exhibited strong fluorescence properties in aqueous buffer. Compounds **1a**,**2a** [**1a**: $\lambda_{exit} = 580$ nm ($\varepsilon = 3.6 \times 10^4$ M^{-1} cm^{-1}), $\lambda_{emit} = 604$ nm ($\phi = 0.94$); **2a**: $\lambda_{exit} = 581$ nm ($\varepsilon = 3.6 \times 10^4$ M^{-1} cm^{-1}), $\lambda_{emit} = 605$ nm ($\phi = 0.96$)] and **1b**,**2b** [**1b**: $\lambda_{exit} = 582$ nm ($\varepsilon = 4.1 \times 10^4$ M^{-1} cm^{-1}), $\lambda_{emit} = 605$ nm ($\phi = 0.91$); **2b**: $\lambda_{exit} = 583$ nm ($\varepsilon = 4.1 \times 10^4$ M^{-1} cm^{-1}), $\lambda_{emit} = 606$ nm ($\phi = 0.92$)] demonstrated similar photophysical properties. Also, their absorption and emission maxima are essentially identical with those of their conjugates **11** [$\lambda_{exit} = 581$ nm ($\varepsilon = 3.6 \times 10^4$ M^{-1} cm^{-1}), $\lambda_{emit} = 604$ nm ($\phi = 0.92$)]. Compared with a rhodamine dye [rhodamine B: $\lambda_{exit} = 554$ nm (9.7×10^4 M^{-1} s^{-1}), $\lambda_{emit} = 573$ nm ($\phi = 0.36$)][13] that lacks such rigidity, compounds **1**, **2**, or **11** exhibited red-shifted (30−40 nm) absorption and emission maxima and higher fluorescence quantum yields. Octamethyl substitution (**1a**,**2a** → **1b**,**2b**) modestly increases the molar absorptivity and quantum yields.

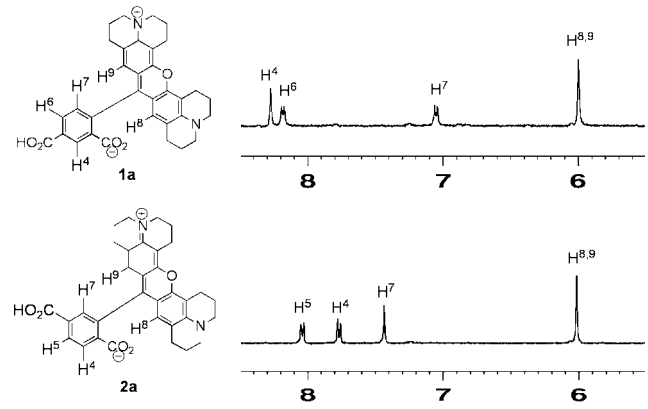


Figure 1. ^{1}H NMR spectra of compounds **1a** and **2a** dissolved in DMSO-d_6 at 500 MHz.

In summary, we report the first synthesis of 5- and 6-carboxy-X-rhodamines having multiple *n*-propylene or γ,γ-dimethylpropylene bridging moieties that provide rigidity for preventing nonradiative fluorescence deactivation processes. Synthesis of gram quantities of these fluorescent dyes is conducted from inexpensive starting materials. We describe the structural analysis and methodology for activation followed by conjugation of the dye with a molecule of interest. These dyes and their conjugates exhibit red-shifted absorption and emission maxima and high quantum yields for fluorescence. The availability of a facile and inexpensive route to these fluorophores should enable their adoption for a broad range of applications in material sciences, biotechnology, and biomedical imaging.

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Supporting Information Available: Complete description of the experimental details and product characterization. This material is available free of charge via the Internet at http://pubs.acs.org.

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