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Efficient Synthesis of Rhodamine Conjugates Through the 2′-Position

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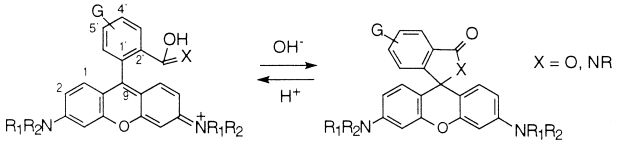
Abstract—Reaction of substrates containing primary amines with rhodamine 2′-esters cleanly produces fluorescent rhodamine 2′-amide conjugates at ambient temperature. Only primary amines react with the esters under these conditions. Chemoselectivity can thus be achieved in substrates containing different types of amines

Fluorescent probes have long been used to study complex biological systems. In particular, rhodamine conjugates have been utilized as fluorescent markers for a variety of biotechnological applications, including amidase activity measurement,[1] flow cytometry,[2] photoswitching technology,[3] antibody binding studies,[4] and chemodosimetry work.[5,6] Rhodamine derivatives are prized for their great photostability, pH insensitivity over a broad range (low to neutral pH), and the ability of their fluorescence characteristics to be tailored for a particular application, since ring and nitrogen substituents affect the fluorescence properties (absorption maximum, emission maximum, quantum yield, etc.).[7] In contrast to fluorescein, which exists in a non-fluorescent spiro form at acidic pH, rhodamines exist in a fluorescent quinone form at neutral and acidic pH, and a non-fluorescent spiro form at basic pH.[5]



While many rhodamine conjugates are synthesized from 4′- or 5′-activated rhodamine derivitives (G = acid chloride, active ester, etc.),[5,7–9] conjugates have also been prepared through an activated 2′-carboxyl group.[10] Utilization of bis-*N*-alkylated (R_1, R_2 = alkyl) rhodamines or protection, however, is required to prevent side reactions of any active group with unprotected amines (R_1, R_2 thus cannot = H). Chemoselectivity can be a problem in molecules with multiple amines, since such activated derivatives can react with different types of amines.

We theorized that nucleophilic amines could undergo reversible addition reactions at the 9-position of the quinone state of a rhodamine (vide supra). If this addition reaction could be followed by intramolecular trapping of the amine intermediate with a proximal 2′-functionality such as an ester, then a fluorescent rhodamine amide could be formed by ring opening of the spirolactam intermediate. Such a concept would allow for direct conjugation of rhodamine to amine containing substrates through the native 2′-carboxyl group. We report here a previously undescribed method for the preparation of 2′-rhodamine conjugates.

Initially, we tested our concept by examining the reaction of phenethylamine with a DMF solution of rhodamine ester 6G[11] at ambient temperature by analytical HPLC and electrospray mass spectrometry (ESMS). Immediately after amine addition, ESMS displayed an $(M+H)^+$ at 564.3, verifying the presence of the intermediate derived from 9-position phenethylamine addition prior to cleavage of the ethyl ester.[12] After stirring for 12 h, HPLC showed formation of one major new product, which ESMS confirmed as the amide product derived by loss of ethanol [$(M+H)^+$ at 518.3].

Subsequently, we tested the reactivity of several different amines (Table 1). In all cases, reaction of the amine containing substrate with the rhodamine ester resulted in formation of the rhodamine conjugate as shown by

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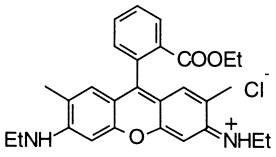
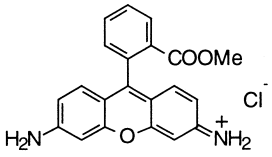
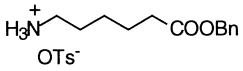
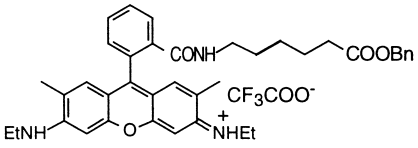
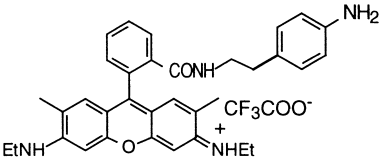
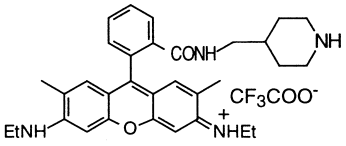
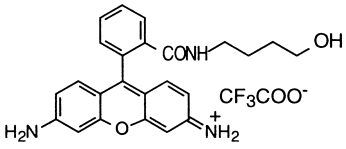
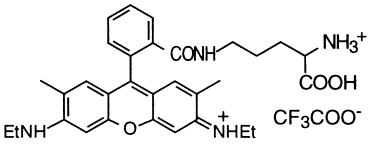
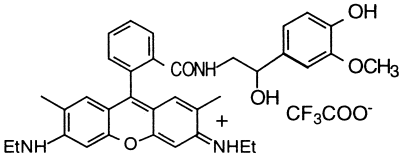
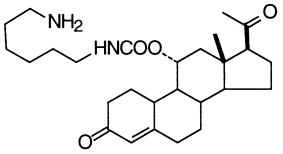
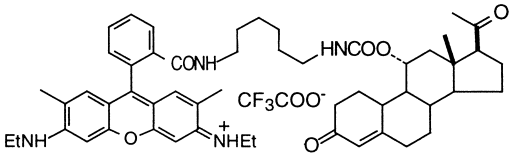
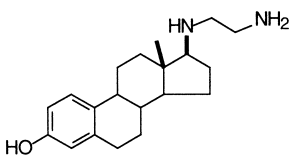
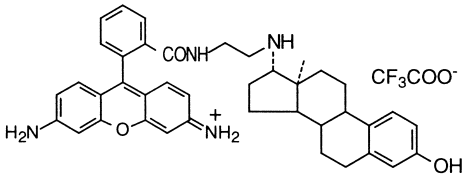
Table 1. Reaction of rhodamine esters[11,13] with amine containing substrates

ester "A"   ester "B"

Entry	Ester	Amine	Rhodamine conjugate	Isol. yield (%)
1	A			87[a,c]
2	B	1-(-4-Aminophenyl)-ethylamine		92[a]
3	A	4-Aminomethyl-piperidine		83[a]
4	B	4-Aminobutanol		82[a]
5	A	Lysine		61[b,c]
6	A	Normetanephrine		82[b,c]
7	A			54[b,c]
8	B			77[b,c]

[a]3 Equiv amine used.
[b]2 Equiv of rhodamine ester used.
[c]DIEA added for neutralization of amine salts.

HPLC together with ESMS. Purification by HPLC and lyophilization produced good yields of the desired conjugates as red solids. In Entries 1–4, 3 equiv of the amine per 1 equiv rhodamine ester was utilized, and the reaction was complete in 12 h. In Entries 5–8, 2 equiv of the ester was used per 1 equiv of the amine to illustrate the labeling of an amine containing substrate as the limiting reagent and employ a reaction time of 96 h.

In all cases, since trifluoroacetic acid was used as the aqueous modifier for the preparative reversed phase HPLC purifications, the rhodamine conjugates were isolated in their open quinone form (vide supra) as red colored mono- or bis-trifluoroacetate salts depending on whether an amine or diamine was used in the conjugation reaction. Rhodamine 6G conjugates showed $\lambda_{max\text{-}ex}$ at 525–526 nm and $\lambda_{max\text{-}em}$ at 550–553 nm, while rhodamine 123 conjugates showed $\lambda_{max\text{-}ex}$ at 502–503 nm and $\lambda_{max\text{-}em}$ at 532–533 nm, similar to the corresponding rhodamine acids.[7] The conjugates had extinction coefficients of 37,300–56,500. Analysis of the individual products by NMR spectroscopy, however, proved difficult, due to poor solubility of the salts in a variety of organic solvents. For ease of analysis, neutralization with sodium bicarbonate or sodium carbonate was thus routinely employed to fully convert each conjugate into its neutral non-fluorescent spirolactam form (typically an off-white solid), which showed satisfactory solubility in organic solvents and was easily analyzed by NMR.[13] The ^{13}C NMR contained a signal at 64–65 ppm, consistent for an sp^3-hybridized 9-position carbon attached to a nitrogen atom[14] and the closed spirolactam form.

As shown in Table 1, only primary amines on primary carbons reacted with the rhodamine esters. This chemoselectivity allowed for reaction in the presence of primary amines on secondary carbons, secondary amines, anilines, primary and secondary alcohols, and phenols. Such chemoselectivity also permits the use of multiple equivalents of the label for improving the yield of the conjugation, and allows for the use of rhodamines containing unprotected amines.

In conclusion, rhodamine 2′-esters readily react with primary amine containing substrates to provide a variety of fluorescent rhodamine conjugates under mild, chemoselective conditions. The reactions of the rhodamine esters are generally high yielding, and constitute an especially attractive method for conjugation to amine containing substrates at the 2′-position. Both non-*N*-alkylated and mono-*N*-alkylated rhodamines are excellent substrates for this reaction, allowing for considerable flexibility in the fine-tuning of the fluorescence properties of the resulting probes. Such probes would be particularly useful for multiplexing, i.e., detection of multiple analytes with a single bioassay. The rhodamine amides in their closed, non-fluorescent spirolactam form also represent masked fluorophores which are attractive as chemosensors.

References and Notes

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10. For an example of 2′-conjugates, see Arnost, M. J.; Meneghini, F. A.; Palumbo, P. S.; Stroud, S. G. US Patent 4,900,686, 1991.
11. The structures of rhodamine 6G and rhodamine 123 (Aldrich) are referred to as rhodamine esters A and B (bottom of page 3) to be totally unambiguous. Rhodamines are named according to several different conventions depending on the source.
12. We have demonstrated ESMS to be a useful tool for studying reaction intermediates: see Adamczyk, M.; Fishpaugh, J.; Gebler, J.; Mattingly, P. G.; Shreder, K. *Eur. Mass Spec*. **1998**, *4*, 121.
13. Representative example: Normetanephrine HCl (23 mg, 104 mmol) was dissolved in anhyd DMF (300 mL), and diisopropylethylamine (58 mL, 320 mol%) was added, followed by rhodamine 6G (100 mg, 200 mol%). After stirring for 96 h, the reaction mixture was purified directly by preparative reversed phase HPLC (CH_3CN/0.2% aq TFA mixt). Concentration on a rotovap, followed by lyophilization, provided 59 mg (82%) of a dark red solid: excitation/ emission λ_{max} 525/551 nm; ϵ_{525} = 41,400 (0.1 N HCl). Neutralization (satd $NaHCO_3/CH_2Cl_2$) and concentration provided an off-white solid: ^1H NMR (DMSO-d_6) δ 7.77 (m, 1H), 7.50 (m, 2H), 6.98 (m, 1H), 6.53 (d, 2H, J = 1.7 Hz), 6.49 (m, 2H), 6.30 (m, 4H), 6.06 (s, 1H), 5.88 (s, 1H), 5.05 (m, 3H), 4.03 (m, 1H), 3.65 (s, 3H), 3.11 (m, 3H), 2.96 (dd, 1H, J = 14.4 Hz, J = 5.2 Hz), 1.84 (s, 3H), 1.78 (s, 3H), 1.21 (m, 6H); ^{13}C NMR (DMSO-d_6) δ 167.6, 153.0, 151.1, 147.7, 147.6, 147.0, 145.4, 133.8, 132.7, 130.6, 128.2, 127.8, 127.7, 123.7, 122.3, 118.3, 118.1, 114.5, 109.6, 104.5, 104.4, 95.4, 71.0, 64.9, 55.2, 48.7, 17.0, 14.1; ESMS 580.4 $(M+H)^+$.
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