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Synthesis of Probes with Broad pH Range Fluorescence

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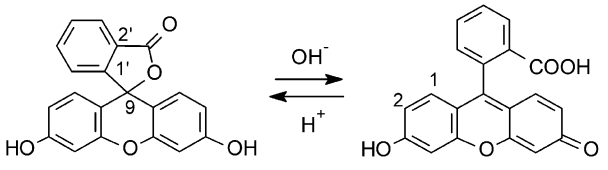
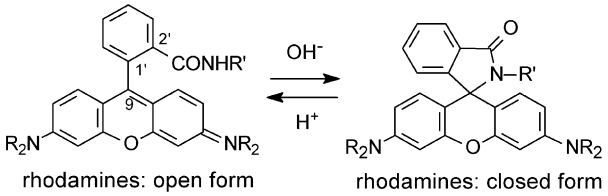
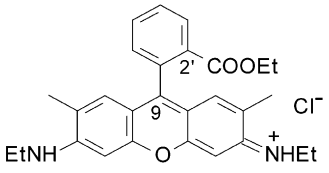
Abstract—Reaction of a rhodamine 2′-ester with an excess of alkyldiamines provides amino-functionalized rhodamine spirolactams, which when subsequently conjugated with carboxyfluorescein, provides probes which are fluorescent at acidic, neutral, and basic pH ranges.

Recent reports of fluorescent probes attest to their continued utility in the study of complex biological systems.[1–5] In particular, there is a need for new probes which display fluorescence over a broad pH range, since the lack of a signal resulting from fluorescence attenuation due to environmental factors such as pH can appear similar to the lack of accessibility of the probe to a particular environment.[6–8] Carboxyfluorescein, for example, suffers from the fact that in some relatively acidic intracellular environments its fluorescence intensity is reduced due to closure to the non-fluorescent spirolactone form.[8] In contrast to fluorescein, the recently disclosed rhodamine spirolactams[9] exist in a fluorescent quinone form at acidic or neutral pH, and a non-fluorescent spiro form at basic pH. The complementarity of the pH ranges of these two labels at which fluorescence is displayed suggests the intriguing possibility that the two labels could together serve as a pair of fluorophores with broad pH range utility. Furthermore, covalent linkage of the two labels would alleviate any relative migratory aptitude or permeability differences between the labels if individually used, and provide a single conjugate which would be fluorescent in almost any physiological pH environment. This report describes the synthesis and properties of such conjugates.







rhodamine 6G

For our study, rhodamine 6G was chosen due to its lipophilicity and permeability in cell membranes.[5] Initially, we tested the reactivity of rhodamine 6G with alkyldiamines (Table 1). In all cases, reaction of the rhodamine ester substrate with excess diamine did not produce detectable quantities of the bis-substituted product (ESMS), apparently since the unreacted alkylamine of the mono-substituted product cannot attain a suitably uncongested orientation to the 9-position for reaction with a second rhodamine ester.[9] This regioselectivity allowed the use of excess diamine, which eliminated the need for supplemental tertiary amine base for neutralization of the rhodamine salts. The reactions also showed rapid conversion (complete in <2 hr in all cases) to the amides (**1a**–**f**). Direct purification of the reaction mixture by HPLC using trifluoroacetic acid (TFA) as the aqueous modifier, followed by lyophilization, produced good yields of the desired amino functionalized amides as red solids in their open quinone forms. For NMR analysis, the individual products

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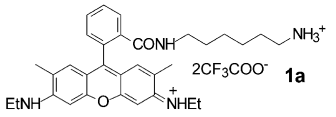
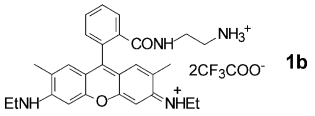
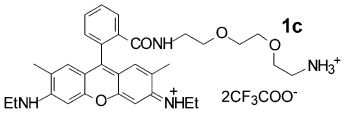
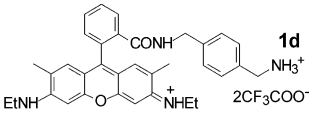
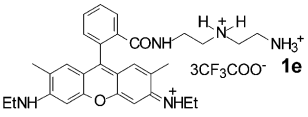
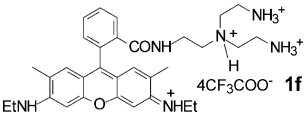
Table 1. Reaction of rhodamine 6G with diamines

Entry	Amine	Product	Isol. yield (%)
1	Hexanediamine	 **1a**	79
2	Ethylenediamine	 **1b**	83
3	Bis(oxyethylamino)ethane	 **1c**	89
4	*p*-Diaminoxylene	 **1d**	74
5	Bis(aminoethyl)amine	 **1e**	75
6	Tris(aminoethyl)amine	 **1f**	89

were neutralized with saturated aqueous sodium bicarbonate to fully convert each derivative into its symmetrical, off-white non-fluorescent spirolactam form, which could be isolated by extraction with dichloromethane. ^{13}C NMR analysis confirmed interconversion to the closed spirolactam form, by observation of a signal at 64–65 ppm, assignable to the sp^3 hybridized 9-position carbon attached to the nitrogen atom of the amide.[10]

As shown in Table 2, reaction of the amines **1a**–**f** with 6-carboxyfluorescein *N*-hydroxysuccinimide active ester (6CFAE, diisopropylethylamine, DMF) produced the target probes. In all cases, isolation of the product was achieved by preparative reversed phase HPLC with TFA as the aqueous modifier, producing the conjugates as red solids (rhodamine open, fluorescein closed). The structures of the probes were elucidated by ESMS, ^1H NMR, and ^{13}C NMR. The amine salts were neutralized for NMR analysis, resulting in degenerate aromatic resonances due to the interconversion of the rhodamine to its closed form.[11] Additionally, probe **2e** was synthesized by reaction of the carboxyfluorescein derivative of **1e** with succinic anhydride. Two probes (**2e** and **2f**) equipped with functionality for bioconjugation were thus prepared.

Each fluorescein conjugate and its amino functionalized rhodamine amide precursor was analyzed by UV spectroscopy at a fixed concentration and at three different pH values (pH 4, pH 6, and pH 8) in phosphate buffers. The spectra of each precursor (e.g., spirolactam **1d**, Fig. 1) and its corresponding conjugate (e.g., **2d**, Fig. 2) both demonstrated a strong pH dependence and a good correlation between the rhodamine portion of the two spectra, indicating that conjugation had little

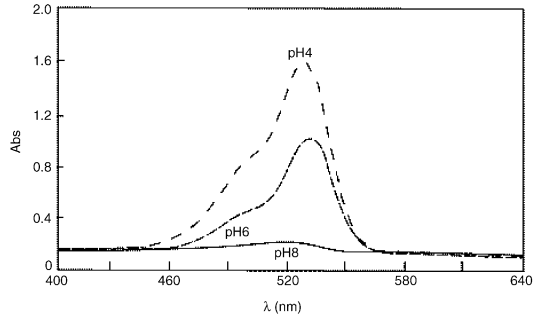


Figure 1. UV spectrum of spirolactam **1d**.

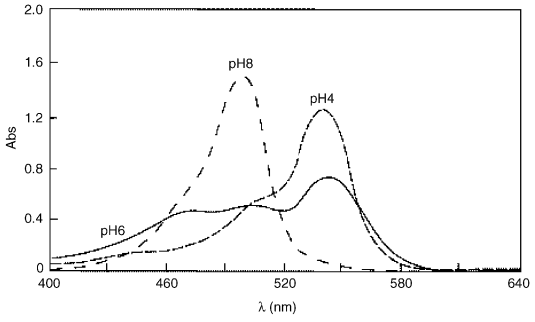
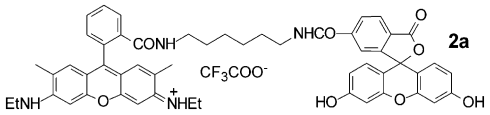
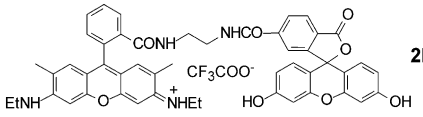
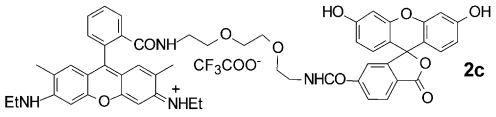
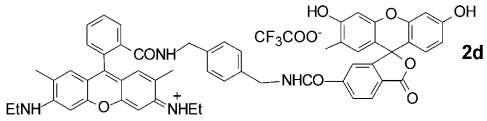
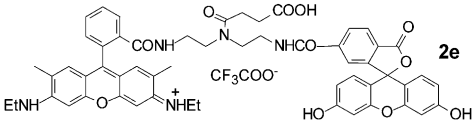
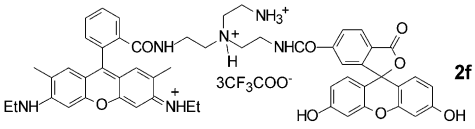


Figure 2. UV spectrum of conjugate **2d**.

Table 2. Conjugation of amino rhodamine amides with carboxyfluorescein N-hydroxysuccinimide active ester

Entry	Amine	Product	Isol. yield (%)
1	**1a**	 **2a**	42
2	**1b**	 **2b**	25
3	**1c**	 **2c**	59
4	**1d**	 **2d**	45
5	**1e**	 **2e**	30[a]
6	**1f**	 **2f**	32

[a]Two step yield: yield of conjugation 44%; yield of succinylation 68%.

effect on the interconversion of the rhodamine spirolactam. Examination of these spectra revealed that the conjugates all demonstrated one λ_{max} near 497 nm at pH 8 and above, consistent with the fluorescein label existing in its open form, and another λ_{max} near 540 nm at pH 4 and below, consistent with the rhodamine being in its open form.[9] The solutions were visibly pink at pH 4 (from the rhodamine chromophore) and yellow at pH 8 (from the fluorescein chromophore). Solutions at all pH values were visibly fluorescent.

The extinction coefficients of each precursor and its conjugate were determined at pH 4 and pH 8 by serial dilution in the corresponding buffers (Table 3). Interestingly, despite a change in the linker length (**1a** vs **1b**), hydrophilicity (vs **1c**), rigidity (vs **1d**), or the presence of charged groups (vs **1e**, **1f**), all precursors showed reasonably good agreement in their extinction coefficients at the two pH values tested. Similarly, the conjugates all showed approximately equivalent extinction coefficients at the two pH values tested.

The rhodamine values of the conjugates are attenuated when compared to their precursors, as are the fluorescein values of the conjugates, when compared to the extinction coefficient of 6-carboxyfluorescein at pH 8 (λ_{max} 492 nm, $\varepsilon = 80{,}000$).

The probes were also analyzed by fluorescence emission spectroscopy[12] at pH 4 and pH 8. At pH 4, the emission spectra of the probes displayed a $\lambda_{max\text{-}emis}$ of 561–562 nm, red shifted relative to rhodamine 6G ($\lambda_{max\text{-}emis}$ 554). The relative quantum yields varied from 0.35–0.82 (Table 4). The emission spectra at pH 8 demonstrated approximately the same $\lambda_{max\text{-}emis}$ as 6-carboxyfluoresceine (517 nm), and quantum yields of 0.19–0.64.

In conclusion, the conjugation of amino functionalized rhodamine spirolactams to carboxyfluorescein provides a variety of new fluorescent conjugates under mild conditions. All of the conjugates synthesized generate fluorescent signals across a broad pH range, making them useful as fluorescent probes at a variety of pH values, or conversely as indicators of pH, based on the quantity and wavelength of fluorescence observed.

Table 3. Extinction coefficients of rhodamine spirolactam precursors and conjugates at their λ_{max}

Precursor	pH 4[a] = ε	pH 8[a] = ε	Conjugate	pH 4[b] = ε	pH 8[c] = ε
1a	67,700	1500	**2a**	36,200	66,000
1b	60,900	500	**2b**	32,800	67,900
1c	68,800	750	**2c**	35,100	69,600
1d	60,200	1000	**2d**	27,500	54,900
1e	57,000	1000	**2e**	26,300	62,400
1f	63,500	400	**2f**	27,700	58,800

[a]530 nm.
[b]540 nm.
[c]497 nm.

Table 4. Quantum yields of new probes[a]

Probe	pH 4[b]	pH 8[c]
2a	0.41	0.24
2b	0.45	0.27
2c	0.35	0.19
2d	0.82	0.64
2e	0.52	0.35
2f	0.39	0.23

[a]Values are the average of duplicate determinations.
[b]Relative to rhodamine 6G.
[c]Relative to 6-carboxyfluoresceine.

Probes **2e** and **2f** also contain an amine or acid functionality, respectively, making them suitable for covalent attachment to generate a variety of bioprobes.[13] Since different rhodamine esters and fluoresceins would be serve as suitable substrates for this reaction, considerable flexibility in the designer's fine-tuning of the fluorescence properties of these broad range fluorophores can be achieved.

References and Notes

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10. Representative example: Rhodamine 6G hydrochloride (100 mg, 209 μmol) was dissolved in anhydrous DMF (500 μL), and hexanediamine (135 mg, 300 mol%) was added. After stirring for 12 h, the reaction mixture was purified directly by preparative reversed phase HPLC (CH_3CN/0.05% aqueous TFA mixtures). Concentration on a rotovap, followed by lyophilization, provided 122 mg (79%) of **1a**: excitation λ_{max} 530 nm; $\varepsilon_{530}=67{,}700$ (pH 4). Neutralization (saturated $NaHCO_3/CH_2Cl_2$) and concentration provided an off-white solid: ^{1}H NMR (DMSO-d_6) δ 7.74 (m, 1H), 7.48 (m, 2H), 6.97 (m, 1H), 6.24 (s, 2H), 6.03 (s, 2H), 5.05 (t, 2H, $J=5.2$ Hz), 3.26 (s, 4H), 3.11 (m, 3H), 2.92 (m, 2H), 2.41 (t, 2H, $J=6.8$ Hz), 1.84 (s, 6H), 1.19 (t, 6H, $J=6.7$ Hz), 0.95 (m, 6H); ^{13}C NMR (DMSO-d_6) δ 166.7, 153.3, 151.0, 147.6, 147.6, 132.5, 130.8, 128.2, 127.6, 123.6, 122.1, 118.1, 118.1, 104.9, 95.5, 64.2, 41.0, 37.5, 31.9, 27.6, 26.3, 25.8, 17.0, 14.1; ESMS 513.3 $(M+H)^+$.
11. To a solution of **1a** (50 mg, 68 μmol) and 6-carboxyfluorescein *N*-hydroxysuccinimide active ester (34 mg, 71 μmol) in anhyd DMF was added diisopropylethylamine (41 μL, 350 mol%). After stirring for 14 hr, the reaction mixture was purified directly by preparative reversed phase HPLC (CH_3CN/0.05% aqueous TFA mixtures). Concentration on a rotovap, followed by lyophilization, provided 28 mg (42%) of **2a**: excitation λ_{max} 540 nm, $\varepsilon_{540}=36{,}200$ (pH 4); excitation λ_{max} 497 nm, $\varepsilon_{497}=66{,}000$ (pH 8). Neutralization (pH 6 phosphate buffer) and concentration provided an off-white solid: ^{1}H NMR (d_6DMSO) δ 8.43 (m, 1H), 8.01 (d, 1H, $J=8.0$ Hz), 7.91 (d, 1H, $J=7.9$ Hz), 7.77 (m, 1H), 7.47 (m, 2H), 6.98 (m, 1H), 6.53 (s, 1H), 6.49 (s, 1H), 6.22 (s, 2H), 6.05 (m, 6H), 5.02 (m, 2H), 3.08 (m, 4H), 2.91 br, 2H), 1.82 (s, 6H), 1.17 (m, 6H), 0.95 (m, 6H); ^{13}C NMR (DMSO-d_6) δ 169.0, 166.7, 165.1, 153.3, 151.0, 147.6, 132.5, 130.9, 130.1, 128.2, 127.6, 123.6, 122.2, 118.2, 109.1, 104.9, 102.5, 95.6, 64.2, 37.5, 28.9, 26.1, 17.0, 14.1; ESMS 871.5 $(M+H)^+$.
12. Fluorescence emission spectra were obtained on an ISS PC1 spectrofluorometer (Champaign, IL).
13. In a trial study, probe **2e** was conjugated to an eledoisin related hexapeptide using TBTU as a coupling reagent. Purification by reversed-phase HPLC provided a conjugate that demonstrated 1:1 incorporation (ESMS), and displayed fluorescence properties similar to those of the probe described above (pH 4: $\lambda_{max\text{-}abs}$ 539, $\lambda_{max\text{-}emis}$ 561; pH 8: $\lambda_{max\text{-}abs}$ 499; $\lambda_{max\text{-}emis}$ 517).