

Supporting Information

Synthesis of 5- and 6-Carboxy-X-rhodamine Dyes

Md. Jashim Uddin and Lawrence J. Marnett*

A. B. Hancock Jr. Memorial Laboratory for Cancer Research, Department of Chemistry, Biochemistry and Pharmacology, Vanderbilt Institute of Chemical Biology, Vanderbilt University School of Medicine, Nashville, Tennessee 37232-0146

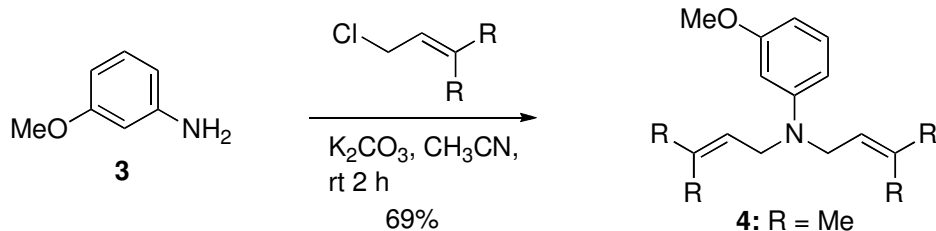
*Address correspondence to: E-mail: Larry.marnett@Vanderbilt.edu

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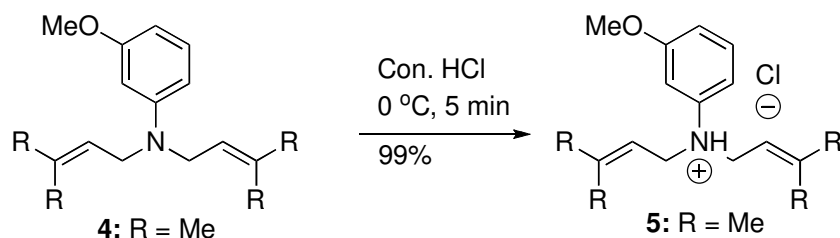
Experimental: Melting points were determined using Gallenkamp 7936G capillary melting point apparatus and uncorrected. Silica gel column chromatography was performed using Sorbent silica gel standard grade, porosity 60Å, particle size 32-63 µm (230 x 450 mesh), surface area 500 – 600 m²/g, bulk density 0.4 g/mL, pH range 6.5 – 7.5. All other reagents, purchased from the Aldrich Chemical Company (Milwaukee, WI), were used without further purification. ¹H NMR was taken on a Bruker AV-I console operating at 500.13 MHz. ¹H COSY experiments were acquired using a 9.4 T Oxford magnet equipped with a Bruker AV-I console operating at 500.13 MHz. Experimental conditions included 2048 x 512 data matrix, 13 ppm sweep width, recycle delay of 1.5 seconds and 4 scans per increment. The data was processed using squared sinebell window function, symmetrized, and displayed in magnitude mode. The steady state fluorescence excitation and emission were determined with Spex 1681 Fluorolog spectrofluorometer, equipped with a 450 W xenon arc lamp. The excitation and emission monochromator slit widths were 1-2 mm.

Figure 1. Synthesis of compound **4**.



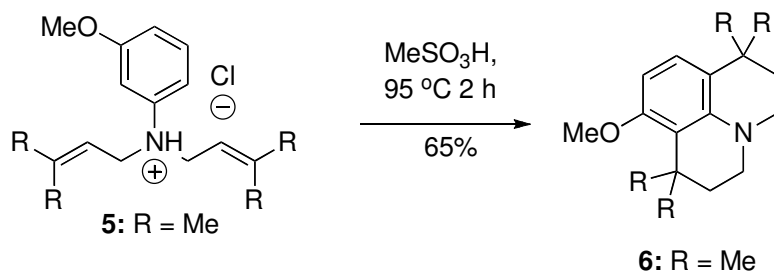
3-Methoxy-*N,N*-bis(3-methylbut-2-enyl)aniline (**4**). To a stirred solution of *m*-Anisidine (**3**) (1.23 g, 10 mmol) in MeCN (100 mL) K_2CO_3 (2.0 g), molecular sieves (MS 4Å) (2 g) and 1-chloro-3-methylbut-2-ene (2.08 g, 20 mmol) were added respectively. The reaction mixture was stirred for 2 h at room temperature. The reaction mixture was filtered and the filtrate was concentrated *in vacuo* and the crude product was purified by a silica gel column chromatography ($CHCl_3$: MeOH : NH_4OH , 35 : 7 : 1) to give the title compound 3-methoxy-*N,N*-bis(3-methylbut-2-enyl)aniline (**4**) (1.78 g, 69%) as brown oil. 1H NMR (500 MHz, $DMSO-d_6$) δ 1.50 (s, 6H, 2 X CH_3), 1.56 (s, 6H, 2 X CH_3), 3.77 (s, 3H), 4.08 (d, 4H, $J = 8.5$ Hz), 5.22 (t, 2H, $J = 7.2$ Hz), 6.53 (d, 1H, $J = 2.4$ Hz), 6.81 (dd, 1H, $J = 9.2, 2.4$ Hz), 7.12 (dd, 1H, $J = 9.2, 2.4$ Hz), 7.25 (t, 1H, $J = 8.9$ Hz). Mass (ESI) m/z M^+ calcd 260.19; found 260.22.

Figure 2. Synthesis of compound **5**.



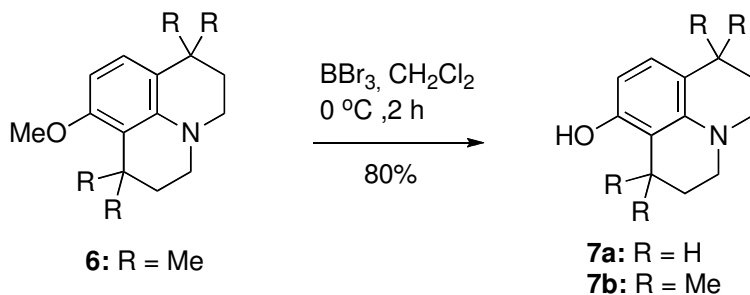
3-Methoxy-*N,N*-bis(3-methylbut-2-enyl)aniline hydrochloride (**5**). To a ice-cold (0 °C) solution of 3-methoxy-*N,N*-bis(3-methylbut-2-enyl)aniline (**4**) (2.6 g, 10 mmol) in *N,N*-dimethylformamide (50 mL) conc. HCl (10 mL) was added drop-wise. After stirring for 5 min at 0 °C $CHCl_3$ (1 mL) was added. The mixture was stirred for 1 h at room temperature. The solid was filtered and washed with THF that afforded the target compound **5** (2.9 g, 99 %) as a white solid, m.p. 262 °C dec. 1H NMR (500 MHz, $DMSO-d_6$) δ 1.52 (s, 6H), 1.55 (s, 6H), 3.79 (s, 3H), 4.52 (d, 4H, $J = 8.5$ Hz), 5.25 (t, 2H, $J = 7.2$ Hz), 6.55 (d, 1H, $J = 2.4$ Hz), 6.83 (dd, 1H, $J = 9.2, 2.4$ Hz), 7.15 (dd, 1H, $J = 9.2, 2.4$ Hz), 7.27 (t, 1H, $J = 8.9$ Hz) 8.51-8.55 (m, 1H, NH). Mass (ESI) m/z $(M-Cl)^+$ calcd 260.72; found 260.68.

Figure 3. Synthesis of compound **6**.



1,1,7,7-Tetramethyl-8-methoxyjulolidine (6). A solution of 3-methoxy-*N,N*-bis(3-methylbut-2-enyl)aniline hydrochloride (**5**) (1 g, 3.3 mmol) in MeSO_3H (10 mL) was heated ($95\text{ }^\circ\text{C}$) with stirring for 2 h. After cooling to $0\text{ }^\circ\text{C}$, NH_4OH (30%) (10 mL) was added drop-wise and adjusted the pH at 7.5. The solvent was removed *in vacuo* and residue was purified by a silica gel column chromatography (CH_2Cl_2 : MeOH , 5 : 1) to afford the compound **6** (0.56 g, 65%) as brown syrup. ^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ 1.22 (s, 6H), 1.43 (s, 6H), 1.69-1.75 (m, 4H), 3.20-3.36 (m, 4H, 2 X CH_2), 3.75 (s, 3H), 6.02 (d, 1H, 8.4 Hz), 6.64 (d, 1H, 8.4 Hz). Mass (ESI) m/z M^+ calcd 260.19; found 260.23.

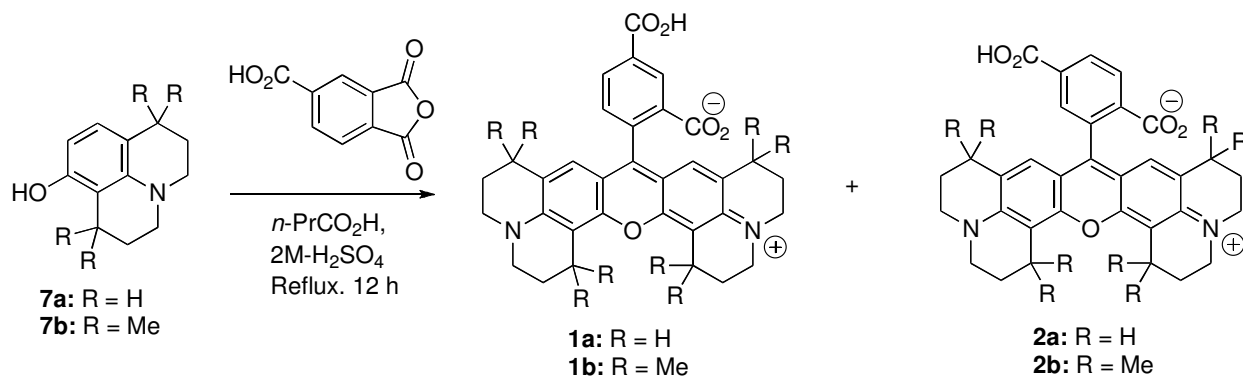
Figure 4. Synthesis of compound **7**.



8-Hydroxyjulolidine (7a). Compound **7a** was synthesized using a published protocol.¹

1,1,7,7-Tetramethyl-8-hydroxyjulolidine (7b). To a cold ($0\text{ }^\circ\text{C}$) solution of 1,1,7,7-tetramethyl-8-methoxyjulolidine (**6**) (0.78 g, 3 mmol) in CH_2Cl_2 (10 mL) was added BBr_3 (0.75 g, 3 mmol) and stirred for 1 h at $0\text{ }^\circ\text{C}$ to room temperature. The solvent was removed *in vacuo* to give a gummy mass that was purified by silica gel column chromatography (CH_2Cl_2 : MeOH , 5 : 1) to afford 1,1,7,7-tetramethyl-8-hydroxyjulolidine (**7b**) as a pale yellow solid (0.58 g, 80%), m.p. $125\text{-}127\text{ }^\circ\text{C}$. ^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ 1.23 (s, 6H), 1.45 (s, 6H), 1.70-1.76 (m, 4H), 3.22-3.38 (m, 4H), 6.12 (d, 1H, 8.4 Hz), 6.85 (d, 1H, 8.4 Hz). Mass (ESI) m/z M^+ calcd for 246.18; found 246.20.

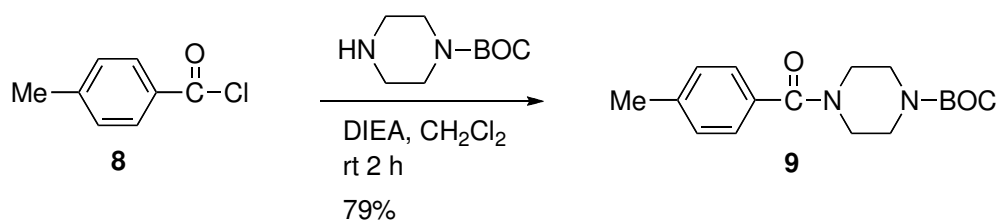
Figure 5. Synthesis of compounds **1a-b** and **2a-b**.



5- & 6-Carboxy-X-rhodamines (1a,2a): Method-1. To a stirred solution of 8-hydroxyjulolidine (**7a**) (0.95 g, 5 mmol) in *n*-PrCO₂H (40 mL) was added 2M-H₂SO₄ catalyst (2-drops) followed by 4-carboxyphthalic anhydride (0.48 g, 2.5 mmol). The reaction mixture was refluxed for 12 h. After cooling to room temperature, the solvent was removed *in vacuo* and the crude mass was chromatographed on a flash column to afford 5-carboxy-X-rhodamine (**1a**, 34%) (fraction-2) and 6-carboxy-X-rhodamine (**2a**, 32%) (fraction-1) respectively as dark purple solids. **Method-2.** To a stirred solution of 8-hydroxyjulolidine (**7a**) (0.95 g, 5 mmol) in EtCO₂H (40 mL) was added *p*-TsOH:H₂O catalyst (20 mg, 0.1 mmol) followed by 4-carboxyphthalic anhydride (0.48 g, 2.5 mmol). The reaction mixture was refluxed for 24 h and then cooled to room temperature. The solvent (EtCO₂H) was removed *in vacuo* and the crude mass was chromatographed on a flash column (CHCl₃ : MeOH, 5 : 1 → 1 : 1) to afford compound **1a** (30%) and **2a** (25%) respectively. **5-Carboxy-X-rhodamine (1a):** M.p. 224 °C dec. ¹H NMR (500 MHz, DMSO-*d*₆) δ 1.73-1.84 (m, 4H), 1.89-2.01 (m, 4H), 2.39-2.46 (m, 4H), 2.81-2.89 (m, 4H), 3.04-3.10 (m, 4H), 3.11-3.19 (m, 4H), 6.00 (s, 2H), 7.05 (d, *J* = 9.0 Hz, 1H), 8.18 (d, *J* = 9.0 Hz, 1H), 8.27 (s, 1H). Mass (ESI) *m/z* M⁺ calcd 535.22; found 535.23. **6-Carboxy-X-rhodamine (2a):** M.p. 226 °C dec. ¹H NMR (500 MHz, DMSO-*d*₆) δ 1.75-1.85 (m, 4H), 1.90-2.00 (m, 4H), 2.40-2.47 (m, 4H), 2.80-2.90 (m, 4H), 3.05-3.11 (m, 4H), 3.12-3.20 (m, 4H), 6.02 (s, 2H), 7.43 (s, 1H), 7.77 (d, *J* = 8.9 Hz, 1H), 8.04 (d, *J* = 8.9 Hz, 1H). Mass (ESI) *m/z* M⁺ calcd 535.22; found 535.13.

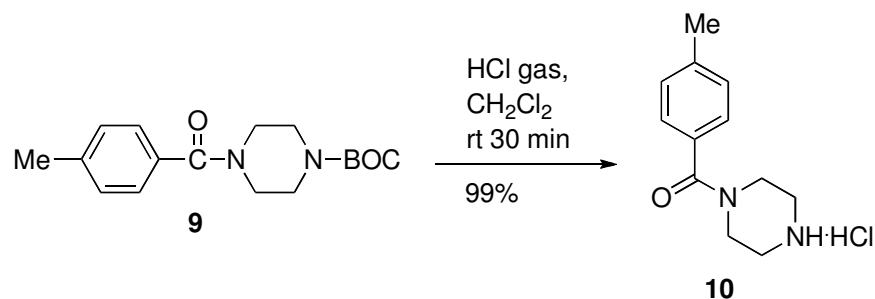
Octamethyl-5- & 6-carboxy-X-rhodamines (1b,2b): Synthesis of compounds **1b,2b** was conducted using a similar method described for compounds **1a,2a**, where 1,1,7,7-tetramethyl-8-hydroxyjulolidine (**7b**) was used in place of 8-hydroxyjulolidine (**7a**). *Octamethyl-5-carboxy-X-rhodamine (1b)*: Yield 42%, m.p. 217 °C dec. ¹H NMR (500 MHz, DMSO-*d*₆) δ 1.35 (s, 12H), 1.46 (s, 12H), 1.72-1.80 (m, 4H), 1.90-2.03 (m, 4H), 3.03-3.10 (m, 4H), 3.12-3.19 (m, 4H), 6.01 (s, 2H), 7.06 (d, *J* = 9.0 Hz, 1H), 8.20 (d, *J* = 9.0 Hz, 1H), 8.28 (s, 1H). Mass (ESI) (*M*+1) calcd for 647.34; found 647.32. *Octamethyl-6-carboxy-X-rhodamine (2b)*: Yield 15%, m.p. 216 °C dec. ¹H NMR (500 MHz, DMSO-*d*₆) δ 1.36 (s, 12H), 1.47 (s, 12H), 1.74-1.79 (m, 4H), 1.89-2.05 (m, 4H), 3.07-3.1 (m, 4H), 3.15-3.20 (m, 4H), 6.02 (s, 2H), 7.44 (s, 1H), 7.75 (d, *J* = 8.9 Hz, 1H), 8.01 (d, *J* = 8.9 Hz, 1H). Mass (ESI) *m/z* *M*⁺ calcd 647.34; found 647.36.

Figure 6. Synthesis of compound **9**.



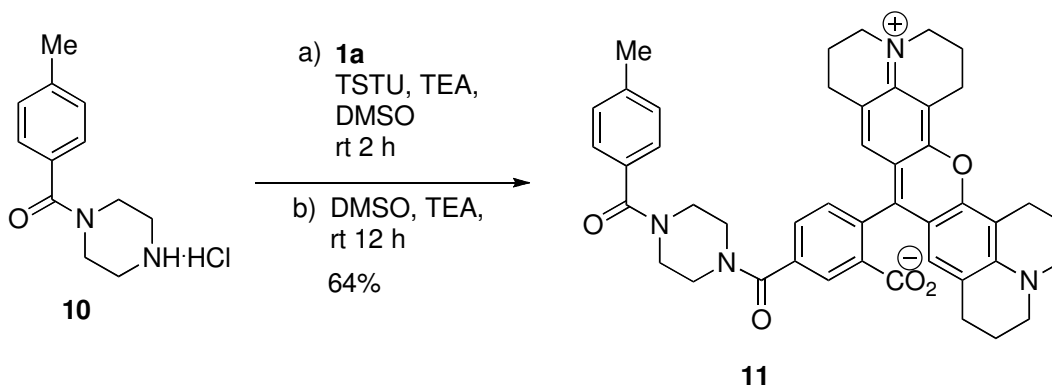
tert-Butyl 4-(4-methylphenylcarbonyl)piperazine-1-carboxylate (9): To a stirred solution of *N*-BOC piperazine (1.86 g, 10 mmol) in CH₂Cl₂ (10 mL) was added *N,N*-diisopropylethylamine (DIEA) (1.29 g, 10 mmol). After stirring for 5 min at 0 °C, 4-methylbenzoyl chloride (**8**) (1.54 g, 10 mmol) was added drop-wise keeping the temperature below 10 °C. The reaction mixture was stirred for 2 h at room temperature. A crude product was obtained after removal of solvent *in vacuo*, which was purified by a silica gel column chromatography (*n*-Hexane : EtOAc, 3 : 1) to give the title compound **9** (2.40 g, 79%) as a pale yellow gummy mass. ¹H NMR (500 MHz, DMSO-*d*₆) δ 1.35 (s, 9H), 2.26 (s, 3H), 3.35-3.41 (m, 4H), 3.62-3.68 (m, 4H), 7.34 (d, 2H), 7.86 (d, 2H, *J* = 8.5 Hz). Mass (ESI) *m/z* *M*⁺ calcd 305.18; found 305.23.

Figure 7. Synthesis of compound **10**.



N-(4-Methylbenzoyl)piperazine hydrochloride (**10**): HCl (gas) was bubbled through a solution of *tert*-butyl 4-(4-methylphenylcarbonyl)piperazine-1-carboxylate (**9**) (1.0 g, 3 mmol) in CH₂Cl₂ (10 mL) for 30 min at 0 °C. Removal of solvent *in vacuo* afforded a yellow residue, where *n*-hexane was added (20 mL) and stirred for 30 min to make good slurry. The slurry was filtered and washed with *n*-hexane to give the title compound **10** (0.8 g, 99%) as a brown solid, m.p. 241 °C dec. ¹H NMR (500 MHz, DMSO-*d*₆) δ 2.28 (s, 3H), 3.74-3.81 (m, 4H), 4.02-4.09 (m, 4H), 7.51 (d, 2H), 7.96 (d, 2H), 8.24-8.29 (m, 2H). Mass (ESI) *m/z* (M-Cl)⁺ calcd 205.13 found 205.19.

Figure 8. Synthesis of compound **11**.



1-(5-carboxy-*X*-rhodaminy)-4-(4-methylbenzoyl)piperazine (**11**). To a stirred solution of compound **1a** (5.3 mg) in dimethyl sulfoxide (DMSO) (5 mL) was added *N,N,N,N*-tetramethyl-*O*-(*N*-succinimidyl)uranium tetrafluoroborate (TSTU) (2.5 mg) and triethylamine (20 μL) (Vessel-1). The reaction mixture was stirred for 2 h at room temperature. In a separate flask (Vessel-2) *N*-(4-methylbenzoyl)piperazine hydrochloride (**10**) (2.4 mg) in DMSO (10 mL) was treated with a drop of triethylamine and stirred for 5 min at room temperature. The succinimidyl ester of compound **1a** generated in the Vessel-1 was added slowly to the Vessel-2 through a cannula. The reaction mixture was stirred for 24 h at room temperature. The solvent was

removed to give the crude product, which was purified by a silica gel gravity column chromatography (4.6 mg, 64%) as a deep blue solid, m.p. 232 °C dec. ¹H NMR (500 MHz, DMSO-*d*₆) δ 1.74-1.84 (m, 4H), 1.88-2.02 (m, 4H), 2.25 (s, 3H, CH₃), 2.40-2.46 (m, 4H), 2.82-2.88 (m, 4H), 3.05-3.11 (m, 4H), 3.12-3.19 (m, 4H), 3.36-3.40 (m, 4H), 3.60-3.66 (m, 4H), 6.02 (s, 2H), 7.06 (d, *J* = 9.0 Hz, 1H), 7.35 (d, 2H, *J* = 8.5 Hz), 7.85 (d, 2H, *J* = 8.5 Hz), 8.20 (d, *J* = 9.0 Hz, 1H), 8.28 (s, 1H). Mass (ESI) *m/z* M⁺ calcd 721.33; found 721.39.

Figure 9. $^1\text{H-NMR}$ spectra of compounds **1a** and **2a**.

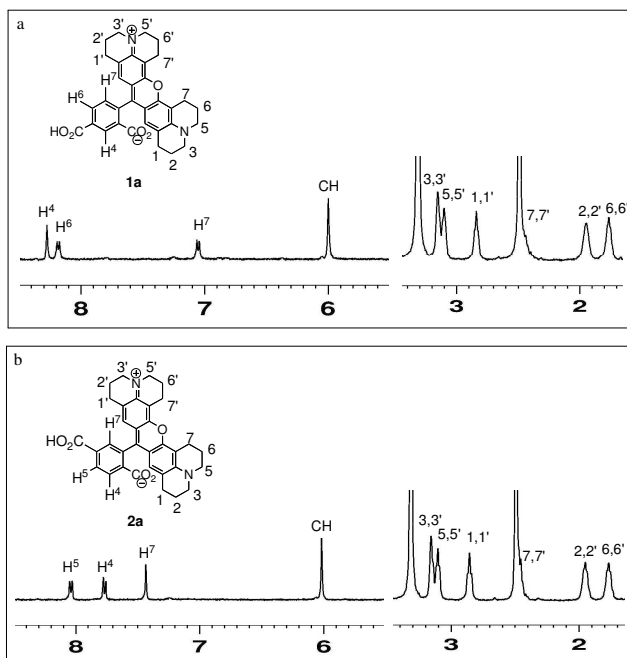
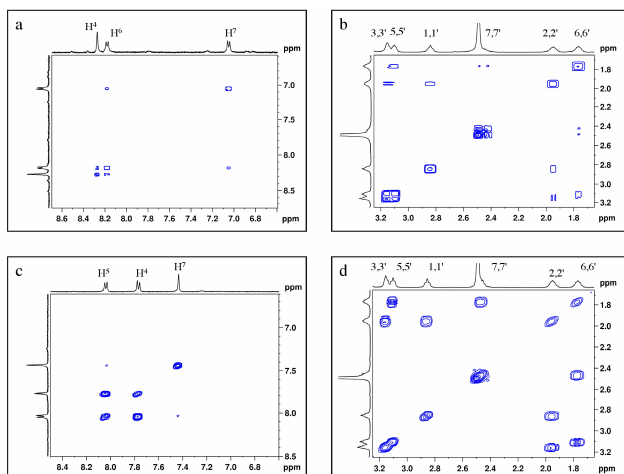


Figure 10. $^1\text{H-}^1\text{H}$ COSY spectra of compounds for **1a** and **2a**.^a



^a(a) Correlations of aromatic protons of compound **1a** ($\text{H}_{\text{arom-6}}$ and $\text{H}_{\text{arom-7}}$; $\text{H}_{\text{arom-4}}$ and $\text{H}_{\text{arom-6}}$), (c) correlations of aliphatic protons of compound **1a** (H-1,1' and H-2,2' and H-3,3' ; H-5,5' and H-6,6' and H-7,7') (c) correlations of aromatic protons of compound **2a** ($\text{H}_{\text{arom-4}}$ and $\text{H}_{\text{arom-5}}$; $\text{H}_{\text{arom-5}}$ and $\text{H}_{\text{arom-7}}$), and (d) correlations of aliphatic protons of compound **2a** (H-1,1' and H-2,2' and H-3,3' ; H-5,5' and H-6,6' and H-7,7').

Analysis of 1D and 2D NMR spectra of 1a and 2a: There are three aromatic protons in each of these dyes that are characteristic for their differentiation in ^1H NMR spectra. For compound **1a**, signals for two neighboring aryl protons at position-6 and position-7 ($\text{H}_{\text{arom-6}}$ and $\text{H}_{\text{arom-7}}$), were assigned as two separate doublets at 8.18 and 7.05 ppm respectively. They exhibited large vicinal coupling constants ($J_{6,7} = 9.0$ Hz and $J_{7,6} = 9.0$ Hz). The assignment was confirmed by a ^1H - ^1H COSY experiment, where $\text{H}_{\text{arom-6}}$ showed a strong correlation with $\text{H}_{\text{arom-7}}$. The proton located at position-4 ($\text{H}_{\text{arom-4}}$) was assigned at 8.27 ppm chemical shift position, which showed a weak correlation with $\text{H}_{\text{arom-6}}$ in COSY spectra. This weak correlation indicates that $\text{H}_{\text{arom-4}}$ is located at least four bonds apart from $\text{H}_{\text{arom-6}}$. This supports that $\text{H}_{\text{arom-4}}$ is located within two carboxyl-groups of the aryl ring. On the other hand, in compound **2a**, the chemical shift resonances for two neighboring aryl protons at position-4 and position-5 ($\text{H}_{\text{arom-4}}$ and $\text{H}_{\text{arom-5}}$) appeared as two separate strong doublets at 7.77 ppm ($J_{4,5} = 8.9$ Hz) and 8.04 ppm ($J_{5,4} = 8.9$ Hz) positions respectively. This was confirmed by a ^1H - ^1H COSY spectra, in which $\text{H}_{\text{arom-4}}$ showed a strong correlation with $\text{H}_{\text{arom-5}}$. The sharp singlet located at 7.43 ppm was assigned as $\text{H}_{\text{arom-7}}$, that has a weak correlation with $\text{H}_{\text{arom-5}}$ in the COSY spectra, which indicates that $\text{H}_{\text{arom-7}}$ is located between the carboxyl-group and rhodamine scaffold. The chemical shifts for two rhodaminyl vinylic CH resonance were assigned at about 6.0 ppm (**1a**, $\delta = 6.00$ ppm and **2a**, $\delta = 6.02$ ppm). These two protons became equivalent and appeared at the same chemical shift position. The proton of CO_2H on the aryl ring of compound **1a** or **2a** appeared at about 13.22 ppm as a singlet, which might be due to the rapid exchange of CO_2H proton with D_2O present in $\text{DMSO } d_6$. The chemical shift resonances and coupling patterns for the aliphatic protons of the compound **1a** and **2a** are almost identical in their ^1H -NMR. In compound **1a**, the chemical shifts for H-1,1', 2,2', 3,3' resonance assigned as multiplets at 2.81-2.89, 1.89-2.01, 3.04-3.10 ppm, which is confirmed by the COSY spectra, where these three groups of protons were correlated with each other. Similarly, H-5,5', 6-6', 7,7' appeared as multiplets in three different locations (3.11-3.19, 1.73-1.84, 2.39-2.46 ppm) and correlated strongly with each other in their H-H COSY spectra. On the other hand, the methylene protons (H-1,1', 2,2', 3,3', 5,5', 6-6', 7,7') of compound **2a** assigned as multiplets at 2.80-2.90, 1.90-2.00, 3.12-3.20, 3.05-3.11, 1.75-1.85, 2.40-2.47 ppm respectively, were confirmed by a similar COSY experiment, where H-1,1', 2,2', 3,3' were correlated with each other. A similar type of correlation was observed for H-5,5', 6-6', 7,7' in the COSY spectra.

Figure 11. Photophysical characteristics (figure edited).

Comp No.	λ_{\max} (nm) ^a		ϵ_{\max} (M ⁻¹ cm ⁻¹) ^b	Φ_f ^c
	Excitation	Emission		
1a	580	604	36,200	0.94
1b	582	605	40,540	0.91
2a	581	605	35,990	0.96
2b	583	606	40,190	0.91
11	581	604	36,350	0.92

^a The steady state fluorescence excitation and emission were determined with Spex 1681 Fluorolog spectrofluorometer, equipped with a 450 W xenon arc lamp using aqueous buffer pH 7.4. The excitation and emission monochromator slit widths were 1-2 mm.

^b Molar extinction coefficients were determined in HBSS buffer at 10 μ M, pH 7.5. Measurement uncertainty is $\leq 6\%$.

^c Quantum yields were determined according to the method described by Karstens et al.² Compounds to be evaluated were weighed on a microbalance and dissolved in DMSO at 1 mM. Measurements were conducted after dilution to 1 μ M in HBSS buffer, pH 7.5. Rhodamine101 was used as a reference standard ($\Phi_f = 1.00$ in ethanol). Emission spectra were integrated using SpectraAcq software. Measurement uncertainty is $\leq 5\%$.

Reference

- (1) Gompel, J. V.; Schuster, G. B. *J. Org. Chem.* **1987**, *52*, 1465.
- (2) Karstens, T.; Kobs, K. *J. Phys. Chem.* **1980**, *84*, 1871.