Synthesis and characterization of a new red-emitting Ca²⁺ indicator, Calcium Ruby

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Supplementary materials

Synthesis

Compound (1)

A solution of 1,2-epoxyoctene (15.5 mL, 104 mmol) and nitrophenol (13.2 g, 95 mmol) in dry DMF was stirred two days at 100 °C under argon and concentrated. The residue was dissolved in dichloromethane and washed twice with water. The organic layer was separated, dried (MgSO₄) and concentrated and the residue was purified by flash chromatography on silica gel (AcOEt/Cyclohexane 1:7) to yield a mixture of two regio isomers : the required compound (80-70%, a secondary alcohol) and its regio isomer (20-30%, a primary alcohol). The latter was selectively tritylated to allow chromatographic separation: The mixture of regioisomers (9.44 g, 35.6 mmol) and trityl chloride (3.97g, 14 mmol) in dry pyridine was stirred overnight at 20 °C, concentrated and the residue was purified by flash chromatography on silica gel (AcOEt/Cyclohexane 1:7) to give **1** (61%). ¹H(CDCl₃, 250 MHz) d: 1.44(m, 4H), 1.53(m, 2H), 2.03(m, 2H), 3.9-4.2(m, 3H), 4.9-5.1(m, 2H), 5.75(m, 1H), 7.05(m, 2H), 7.55(dd, 1H), 7.82(d, 1H). ¹³C(CDCl₃, 250 MHz) d: 24.93, 29.11, 32.68, 33.60, 69.62, 74.00, 114.62, 115.08, 120.73, 125.74, 134.49, 138.73, 139.65, 152.31.

Compound (2)

1 (5.3 g, 20 mmol) and DMAP (0.24 g, 2 mmol) were dissolved in dry pyridine under argon at 0 °C. Mesyl chloride (6.4 ml, 80 mmol) was added dropwise to the solution and the mixture was stirred for 4 h at 20 °C, concentrated. A solution of the residue in dichloromethane was washed twice with water, dried and concentrated. The residue was purified by flash chromatography on silica gel (AcOEt/Cyclohexane 1:7) to yield **2** (95%). ¹H(CDCl₃, 250MHz) d: 1.45(m, 4H), 1.85(m, 2H), 2.10(m, 2H), 3.09(s, 3H), 4.25(m, 2H), 5.00(m, 3H), 5.80(m, 1H), 7.11(m, 2H), 7.59(t, *J*=8 Hz, 1H), 7.82(d, *J*=8Hz, 1H). ¹³C (CDCl₃, 250 MHz) d: 24.18, 28.42, 31.57, 33.33, 38.31, 70.54, 80.22, 114.78, 114.82, 121.38, 125.69, 134.36, 138.35, 139.98, 151.34.

Compound (3)

A solution of KOH (1.12 g, 20mmol) in water (20mL) was added to a solution of 4-chloro-2-nitrophenol (3.47 g, 20mmol) in hot ethanol. After complete dissolution, the solution was concentrated in vacuum and freeze-dried.

A solution of the potassium salt of 4-chloro-2-nitrophenol (4.23 g, 20 mmol) and **2** (6.83 g, 20 mmol) in dry DMF was stirred overnight at 120 °C under argon and concentrated. The solution of the residue in dichloromethane was washed twice with aq NaHCO₃, dried and concentrated. The residue was purified by flash chromatography on silica gel (AcOEt/Cyclohexane 1:7) to yield **3** (5.3 g, 62%). ¹H(CDCl₃, 250 MHz) d: 1.45(m, 4H), 1.90(m, 2H), 2.10(m, 2H), 4.20(dd, 1H), 4.35(m, 1H), 4.82(m, 1H), 5.00(m, 2H), 5.80(m, 1H), 7.10(m, 2H), 7.35(d, *J*=9Hz, 1H), 7.55(m, 2H), 7.79(d, *J*=2Hz, 1H), 7.85(d, *J*=8Hz, 1H). ¹³C(CDCl₃, 250 MHz) d: 24.35, 28.61, 31.46, 33.39, 71.86, 79.98, 114.77, 114.79, 118.73, 121.16, 125.09, 125.71, 126.06, 133.92, 134.28, 138.36, 140.00, 140.96, 150.54, 151.61. HRMS (CI, NH₃, *m/z*): calculated for C₂₀H₂₁ClN₂O₆ + NH₄: 438.1432. Found: 438.1429.

Compound (4)

A solution of **3** (5.00 g, 11.8 mmol), SnCl₂ (16 g, 70.8 mmol, 6 equiv), ethanol (100 ml), and concn aq HCl (14 ml) was refluxed for 10 min, cooled and brought to pH>11 with aqueous NaOH (20% w:w). The resulting suspension was extracted with ether (450ml). The pooled organic layers were dried, concentrated and the residue was purified by flash chromatography on silica gel (AcOEt/Cyclohexane 1:5) to yield **4** (3.0 g, 70%). ¹H(CDCl₃, 250 MHz) d: 1.50(m, 4H), 1.85(m, 2H), 2.12(m, 2H), 4.00(s broad, 4H), 4.15(m, 2H), 4.55(m, 1H), 5.00(m, 2H), 5.80(m, 1H), 6.6-6.9(m, 7H). ¹³C(CDCl₃, 250 MHz) d: 24.88, 28.89, 31.52, 33.58, 70.52, 78.52, 112.18, 114.77, 115.19, 115.57, 115.92, 117.67, 118.64, 121.89, 126.91, 136.29, 138.61, 139.05, 144.42, 146.31. HRMS (CI, NH₃, *m/z*): calculated for C₂₀H₂₅ClN₂O₂+H: 361.1683. Found: 361.1683.

Compound (5)

A solution of **4** (3 g, 8.3 mmol), methyl bromoacetate (3 ml) and diisopropylamine (DIPEA) (4 ml) in acetonitrile (30 ml) was stirred at reflux for 6 h. Methyl bromoacetate (3 ml) and DIEA (4 ml) were added to the solution and heating was continued for further 48 h. A solution of the residue in dichloromethane was washed twice with sat. aq. NaHCO₃, dried (MgSO₄), concentrated and the residue was purified by flash chromatography on silica gel (AcOEt/Cyclohexane 1:5) to yield **5** (2.3g, 65%). ¹H(CDCl₃, 250 MHz) d: 1.45(m, 4H), 1.75(m, 2H), 2.07(m, 2H), 3.65(m, 12H), 4.10(m, 10H), 4.62(m, 1H), 5.00(m, 2H), 5.80(m, 1H), 6.7-6.9(m, 7H). ¹³C(CDCl₃, 250 MHz) d: 24.70, 28.86, 31.15, 33.54, 51.49, 53.18, 70.05, 77.66, 113.82, 114.53, 116.52, 119.70,

120.09, 121.60, 121.79, 122.64, 126.37, 138.56, 139.15, 141.38, 148.27, 150.71, 171.39. HRMS (CI, NH₃, *m/z*): calculated for C₃₂H₄₁ClN₂O₁₀+H: 649.2528. Found: 649.2521.

Compound 6

5 (1 g, 1.54 mmol) in DMF (3 ml) was slowly added to a mixture of DMF (6 ml) and POCl₃ (0.29 ml, 3.08 mmol) at 20 °C. The solution was stirred at rt for 30 min then at 65 °C overnight. The solution was concentrated and a solution of the residue in dichloromethane was washed twice with an aqueous saturated solution of NaHCO₃, dried, concentrated and the residue was purified by flash chromatography on silica gel (AcOEt/Cyclohexane 1:4) to yield **6** (51% , 540 mg).

¹H (CDCl₃, 250 MHz) δ: 1.45(m, 4H), 1.75(m, 2H), 2.07(m, 2H), 3.65(m, 12H), 4.10(m, 10H), 4.58(m, 1H), 4.98(m, 2H), 5.78(m, 1H), 6.7-6.9(m, 4H), 7.38(m, 2H). ¹³C (CDCl₃, 250 MHz) δ: 24.66, 28.81, 30.95, 33.49, 51.68, 53.33, 70.20, 77.42, 111.58, 114.64, 116.74, 117.43, 119.88, 121.86, 126.40, 126.65, 130.24, 138.47, 141.53, 144.80, 148.06, 149.78, 170.87, 171.31, 190.48. HRMS (CI, NH₃, *m/z*): calculated for C₃₃H₄₁ClN₂O₁₀+H: 677.2477. Found: 677.2473.

Compound 8

HBr (33% in AcOH) (1 ml) was added to a solution of **6** (540 mg, 0.79 mmol) in dichloromethane (10 ml). After 10 min, the mixture was concentrated and dissolved in dichloromethane. The solution was washed with aq. sat. NaHCO₃ dried, concentrated. A solution of the residue **7** (539 mg, 0.71 mmol) and sodium azide (139 mg, 2.13 mmol) in dry DMF was heated 2h at 100 °C and concentrated. A solution of the residue in dichloromethane was washed with aq. sat. NaHCO₃. The aqueous layer was further extracted with ether (100 ml). The organic layers were pooled, dried, concentrated and the residue was purified by flash chromatography on silica gel (AcOEt/Cyclohexane 1:4) to yield **8** (51%, two steps). ¹H (CDCl₃, 250 MHz) δ: 1.26(d, 3H, J=7Hz), 1.50(m, 6H), 1.81(m, 2H), 3.46(m, 1H), 3.65(m, 12H), 4.12(s, 6H), 4.23(d, 4H, J=4Hz), 4.61(m, 1H), 6.8-6.92(m, 4H), 7.38(m, 2H), 9.81(s, 1H). ¹³C (CDCl₃, 250 MHz) δ: 19.45, 24.96, 26.11, 31.13, 36.02, 51.90, 53.35, 57.83, 68.12, 70.40, 111.60, 116.95, 117.52, 120.00, 121.99, 126.52, 126.87, 130.32, 141.57, 144.82, 148.07, 149.81, 170.91, 171.33, 190.54. HRMS (CI, NH₃, *m/z*): calculated for C₃₃H₄₁ClN₂O₁₀+H: 720.2648. Found: 720.2643.

Compound 9

A solution of 1-bromo-3-nitrobenzene (14.34 g, 71 mmol) in dry DMF (20 ml) was heated at 140 °C in the presence of Cu powder (0.3 g, 5.6 mmol). The potassium salt of the 3-nitrophenol (71 mmol) in dry DMF (20 ml) was added slowly to the hot solution. The mixture was stirred for 2 days at 140 °C. The mixture was cooled and poured into cold water (300 ml). The water layer was removed and the sticky solid was dissolved in acetone (300 ml) and mixed with activated carbon. The solution was filtered and concentrated to give a solid which was washed several times with cold methanol (TLC control AcOEt/cyclohexane 1:3) to yield bis-(3-nitro-phenyl)-ether (27%) as a white solid.

A mixture of bis-(3-nitro-phenyl)-ether (1 g, 3.8 mmol) palladium hydroxide 15% on carbon (0.53 g, 0.76 mmol) and ammonium formate (2.52 g, 40 mmol) in methanol (10 ml) was stirred overnight at 20 °C. The solution was filtered on a celite plug and the filtrate was concentrated. The bis-(3-amino-phenyl)-ether was used without further purification in the next step.

Compound 10

A mixture of bis-(3-amino-phenyl)-ether (2 g, 7.7 mmol) and 1-bromo-3-chloropropane (4.6 ml, 46.1 mmol) was heated 12h at 100 °C and then 24h at 140 °C. The mixture was diluted with dichloromethane (100 ml) and the solution was washed with a sat. aq. NaHCO₃ (100 ml). The organic layer was dried (MgSO₄) and concentrated. The residue was purified by flash chromatography on silica gel (AcOEt/Cyclohexane 1:7) to yield **10** (10%) as white solid. ¹H(CDCl₃, 250 MHz) δ: 2.00(sxt, 8H, j=6.5Hz), 2.75(t, 8H, j=6.5Hz), 3.18(dd, 8H, j=5.8Hz), 6.11(d, 2H, j=8.2Hz), 6.73(d, 2H, j=8.25Hz). ¹³C(CDCl₃, 250 MHz) δ: (21.40, 22.34, 27.33, 49.74, 50.16) CH₂, 105.56 PhCH, 112.37 PhCq, 116.12 PhCH, 126.58 PhCq, 144.01 PhCq, 153.30 PhCq. MS (CI, NH₃, *m/z*): m+H= 361.

Compound 11 : Calcium Ruby -Cl

A mixture of **10** (50 mg, 0.139 mmol) and **8** (100 mg, 0.139 mmol) in trifluoroacetic acid (1 ml) was stirred for 1 day, and concentrated in vacuum. A solution of the residue in dichloromethane (3 ml) was oxidized by oxygen bubbling for 1h and concentrated. The residue was purified on HPTLC plate RP18-WF (MeOH/dichloromethane 90:10) and dissolved in MeOH (1 ml) in the presence of an excess of KOH (1 M). The solution was stirred overnight at rt and concentrated. The residue is purified on TLC plate RP18 (MeOH/AcOH 70:3) to give **11** (20 mg). IR (KBr) 2112cm⁻¹

Hammett constants

Table 1' – Hammett constants (Σσ) ref. [1] of the YX substitute and K_d (μM), ref.[2], of BAPTA-based Ca²⁺ dyes

Fluoresceine	K _d	Rhodamine	K _d	X Rhodamine	K _d	Y	X	Σ σ
Fluo-4	0.39	Rhod-2	0.57	X-rhod-1	0.7	H	Me	-0.17
Fluo-5F	2.3		-	X-rhod-5f	1.5	H	F	0.062
Fluo FF	9.7	Rhod-FF	19	X-rhod-FF	17	F	F	0.399
Fluo-5N	90	Rhod-5N	320	X-rhod-5N	350	H	NO ₂	0.778
				Calcium ruby-CL		Cl	H	0.373

Photophysical properties of Calcium Ruby-Cl

List of abbreviations – BAPTA – O,O'-Bis(2-aminophenyl)ethyleneglycol-N,N,N',N'-tetraacetic acid, tetrapotassium salt, CFP – EtOH – ethanol (purum), GFP – green fluorescent protein, MOPS – 3-(N-morpholino)propane-sulfonic acid, NTA – Nitrilotriacetic acid.

Reagents – Ethanol (Merck N 100983); Methanol (Merck 106009); H₂O (Fluka 95305); MOPS (Sigma M1254); KCl – (Prolabo 215GC); 1M CaCl₂ (Fluka 21115); Rhodamine B (Sigma R-6626); SulfoRhodamine 101 (Sigma S-7635); NTA (Sigma N-9877)

Experimental procedures – 1 mM Calcium Ruby-Cl stock solutions were prepared in absolute methanol and kept at 20 °C for up to 3 months without showing signs of precipitation or degradation. We recorded room-temperature (RT, 20 °C) UV absorbance spectra on an Uvikon 933 double-beam UV/VIS spectrophotometer (Kontron Instruments AG, Zürich, Switzerland). From the same samples, fluorescence spectra in the 544-700 nm range were recorded using a QuantaMaster spectrofluorometer (model QM-4/2005; Photon Technology Int.), housing a 70-W Hg-arc lamp. Excitation and emission slits were set to 4-nm width. All measurements were carried out at RT in 0.5 ml quartz cuvettes with 10-mm optical path (QS-115 Helma, Germany). We used for reference a buffer containing (in mM: 30 MOPS; 100 KCl; 10 NTA; pH 7.2) (**figure 1'A**).

The fluorescence quantum yield φ of Calcium Ruby-Cl was evaluated relative to that of Sulforhodamine 101 (SR101, $\varphi = 1.0$) and Rhodamine B ($\varphi = 0.49$ in absolute ethanol at 20 °C), ref.[3], by integrating the spectral emission of Calcium Ruby over φ and using correction for solvents with different refractive index,

$$\varphi = \varphi_{ref} \frac{s}{s_{ref}} \cdot \frac{\eta^2}{\eta_{ref}^2} \quad (\text{eq.1})$$

where φ is the quantum yield, η is the refractive index ($\eta(\text{H}_2\text{O}) = 1.33$, $\eta(\text{EtOH}) = 1.36$) and s is the slope of a plot of the integrated fluorescence intensity vs. absorbance, measured at 535-nm excitation. The subscript *ref* refers to a reference fluorophore of known quantum yield, φ_{ref} , see ref.[4]. To avoid self-absorption, we worked with solutions of OD 0.01–0.1.

For titration, we modified the technique of Minta et al. ref.[5]. At low μM $[\text{Ca}^{2+}]$, we used 10 mM NTA/ Ca^{2+} buffer added to the 100 mM KCl, 30 mM MOPS (pH 7.2/KOH) solution so as to reduce the effect of contaminating Ca^{2+} . $[\text{Ca}^{2+}]$ was changed in the 0.2–350 μM range by adding CaCl₂. Total and free $[\text{Ca}^{2+}]$ in the presence of NTA and MOPS were calculated with GEOCHEM, ref.[6], employing the microscopic binding constants for Ca^{2+} and H^+ obtained from Martell & Smith ref.[7] and NIST ref.[8]. Free $[\text{Ca}^{2+}]$ was equally determined experimentally with a Ca^{2+} -sensitive electrode ref.[9]. (ISE25Ca, Radiometer Analytical, Villeurbanne, France). Potentiometrically determined $[\text{Ca}^{2+}]$ values fell within the interval defined by the two calculated estimates. Nominally 'zero' $[\text{Ca}^{2+}]$ solution contained 10 mM K₂H₂EGTA instead of 10 mM NTA. Points in the 400 to 1200 μM range of free $[\text{Ca}^{2+}]$ were measured in absence of NTA. The graph on **figure 1'** plots the evolution of the peak fluorescence at 598 nm vs. $[\text{Ca}^{2+}]$. The K_d was obtained from a fit of Hill's equation with the experimental data (**figure 1'B**).

Figure 1' *Spectral properties of CALCIUM RUBY.* **A** – Normalized absorption and fluorescence emission spectra of CALCIUM RUBY in a Ca²⁺-buffered solution containing 329 μM free [Ca²⁺]. **B** - Titration curve of fluorescence emission for CALCIUM RUBY, averaged from $n = 5$ data sets. Fitting a Hill function to the experimental data yields a K_d of 30.3 μM.

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Supplementary materials- part 2 (NMR spectrum)

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