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Synthesis and Characterization of a New Red-Emitting Ca^{2+} Indicator, Calcium Ruby

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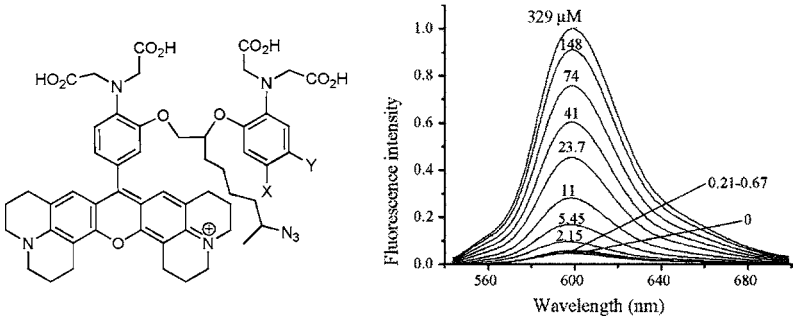
ABSTRACT



Calcium Ruby m-Cl (X = H, Y = Cl) is a visible-light excited red-emitting calcium concentration ($[Ca^{2+}]$) indicator dye (579/598 nm peak excitation/emission) with a side arm for conjugation via EDC or click chemistry. Its large molar extinction and high quantum yield rank it among the brightest long-wavelength Ca^{2+} indicators. Calcium Ruby is a promising alternative to existing dyes for imaging $[Ca^{2+}]$ in multicolor fluorescence applications or in the presence of yellow-green cellular autofluorescence.

Measuring the temporal and/or spatial variations in the intracellular free calcium concentration ($[Ca^{2+}]_i$) is a common theme in cytometry, cell biology, and neuroscience. Following the seminal work by Tsien and co-workers in the early 1980s,[1,2] a whole family of fluorescent indicators with different fluorescence properties and Ca^{2+}-binding affinities has been generated.[3,4] These indicators have in common the principle of covalently linking an organic fluorophore to a Ca^{2+}-chelating moiety, mostly to BAPTA. Ca^{2+} binding rotates the carbon−nitrogen bond which diverts the nitrogen free electron pairs out of conjugation, resulting in modulating the fluorescence intensity and/or in spectral shifts. Most of these Ca^{2+} indicators and certainly the most commonly used are fluorescein-derived fluorophores and hence emit green to yellow fluorescence upon blue-turquoise excitation.

However, for many cell and neurobiological applications, red-emitting Ca^{2+}-sensitive dyes would offer a distinctive advantage over green-emitting probes. Visible-light excited red emission would permit a better discrimination against cellular autofluorescence, allow the detection of Ca^{2+} release generated by photoreceptors,[5] and be compatible with the use of photoactivated "caged" Ca^{2+} compounds. Most

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Table 1. Comparison of the Spectral Properties of Calcium Ruby-Cl with Other Red-Emitting Ca^{2+}-Sensitive Dyes[a]

	$\lambda_{abs}^{max}/\lambda_{em}^{max}$ (nm)	ϵ (M^{-1} cm^{-1})	$\varphi_{Ca^{2+}-free}/\varphi_{Ca^{2+}-bound}$	F_{max}/F_{min}[b]
Calcium Orange	549/576	80000	*e*	~3
Rhod-1[18]	556/578	*e*	0.0014/0.021	*e*
Rhod-2	553/576	82000	0.03/0.102	14 in situ,[3] 100[c,10]
X-Rhod-1	574/594	92000	*e*	4−8 in situ,[19] 100[c,10]
ICPBC[20]	(558)485/(586)582[d]	*e*	*e*	0.25
Calcium Ruby	579/598	100000	0.026/0.42	32
Calcium Crimson	590/615	113,000	*e*	~2.55

[a] ϵ = molar extinction, φ = quantum yield, F = fluorescence at peak emission. [b] Defined as the relative change in fluorescence at peak emission wavelength. [c] Depending on the purification (no data available). [d] (Ca^{2+}-free) Ca^{2+}-bound. [e] No data available.

important, a red Ca^{2+} sensor would also permit the simultaneous detection of $[Ca^{2+}]_i$ and enhanced green fluorescent protein (EGFP) in a dual-emission detection scheme. The green-yellowish emission of EGFP results in an appreciable cross-talk that obscures the fluorescence emission of most current Ca^{2+} indicators.[6]

Among the long-wavelength Ca^{2+} indicators available, the X-Rhod family and Calcium Crimson result from an expansion of the rhodamine chromophore into a seven-ring system of a Texas Red-type fluorophore. Despite their red-shifted emission, the rhodamine derivatives Calcium Orange (549/575 nm peak absorbance/peak emission), Rhod-2 (553/576 nm), X-Rhod-1 (574/594 nm), and Calcium Crimson (590/615 nm), see Table 1, are not being widely used, either due to their intracellular compartmentalization, small absorption cross-section, inappropriate K_d value, or because they are simply no longer commercialized. Hence, the demand for red-emitting Ca^{2+} indicators displaying little spectral overlap with EGFP and cellular autofluorescence is still strong.

In this letter, we describe the synthesis of a visible-light excited red-emitting Ca^{2+} dye with a BAPTA moiety grafted on an extended rhodamine fluorophore (Figure 1). The novel

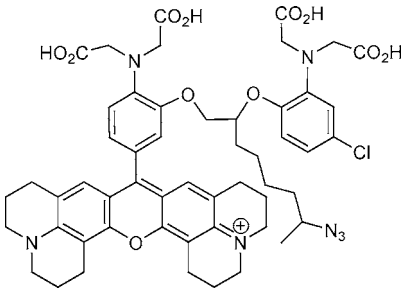

Figure 1. Calcium Ruby-Cl, compound (**11**) in Scheme 1.

Calcium Ruby indicator also includes a linker side chain that we attached to the ethylene glycol bridge. This bridge anchor was considered to be less interfering with the functional part of the indicator and more versatile than the aromatic ring anchoring used earlier by Gee and co-workers in Fluo-4 conjugates.[7] The linker offers a terminal azido group for conjugation, either via click chemistry using acetylenic-tagged molecules or through EDC-assisted coupling to a COOH bearing carrier after reduction to an amino group. These reactions permit the attachment of Calcium Ruby to a dextran, such as for retrograde labeling,[8] or its attachment to the surface of colloidal semiconductor nanocrystals in a hybrid bioassay combining inorganic and organic fluorophores.[9]

Advantageously, substitutions on the BAPTA moiety enable us to precisely control the indicator apparent Ca^{2+}-binding affinity, $K_d(Ca^{2+})$, denoted K_d in the sequel. By substituting the BAPTA in positions *X* and/or *Y*, we generate a whole family of red-emitting Ca^{2+} dyes with K_d values in the 0.5−300 μM range. In the present work, we specifically describe the chloride-substituted Calcium Ruby.

The plot of the Hammett constants σ of the substitutions on the aromatic ring of the BAPTA system of known BAPTA-based Ca^{2+} dyes against their $\log(K_d)$ (dissociation constant) displays a good correlation (Figure 2). From this relationship, we would anticipate a K_d on the order of 20−25 μM for the chloride-substituted Calcium Ruby molecule ($\sigma = 0.37$). Such a low-affinity Ca^{2+} indicator is of particular use when imaging Ca^{2+} microdomains, that is, highly localized and fast, transient micromolar excursions from resting $[Ca^{2+}]_i$ that are observed, for example, in response to voltage-gated Ca^{2+} channel opening or localized release from internal Ca^{2+} stores.[12,13]

Synthesis. The synthesis of the BAPTA part of **11**, depicted in Scheme 1, started from the commercially

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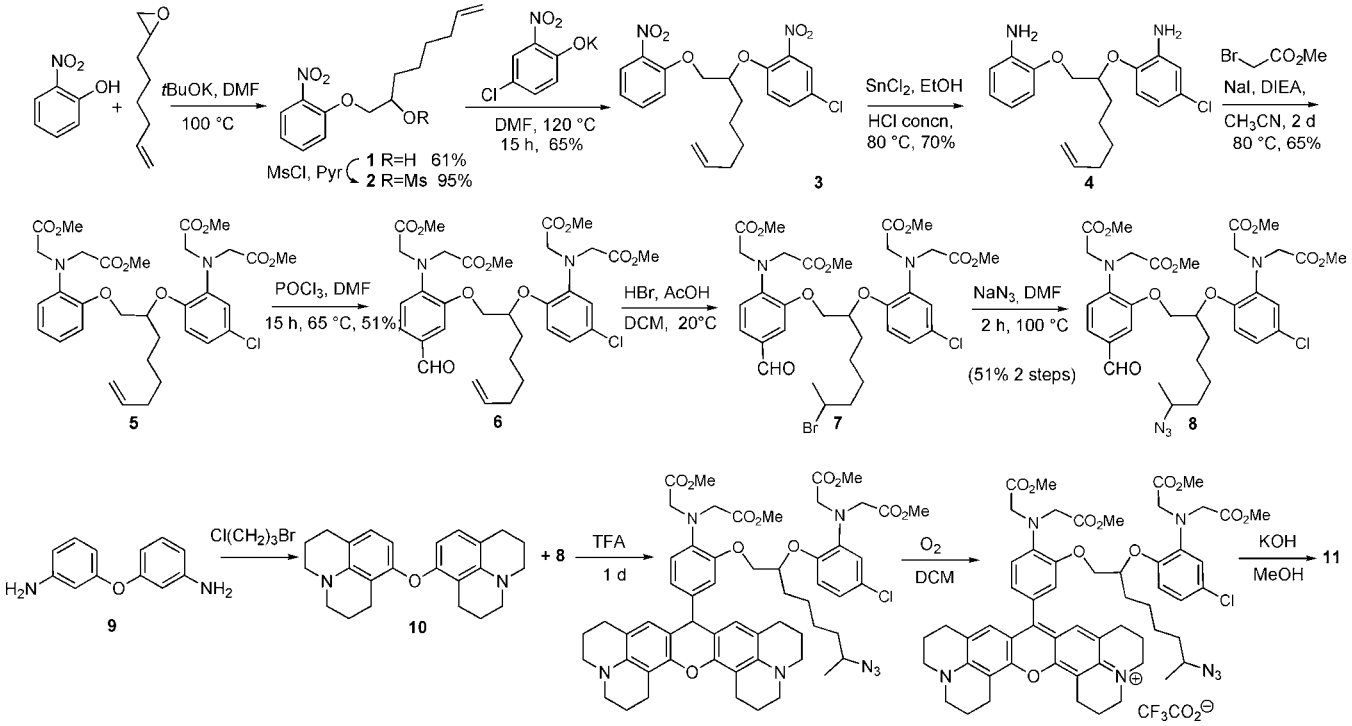
Scheme 1. Synthesis of **11**



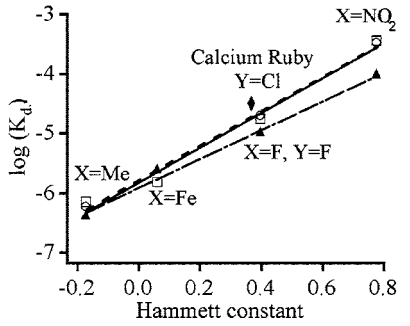


Figure 2. Correlation between log(K_d) (from ref 10) versus Hammett constants[11] for different aromatic substitutes of BAPTA at position *X* and *Y* (▲, Fluo; ○, Rhod; □, X-Rhod series of Ca^{2+}-sensitive dyes; and ◆, Calcium Ruby-Cl).

available 1,2-epoxy-7-octen. The epoxy ring was opened with potassium *o*-nitrophenate to give mainly the secondary alcohol **1**. This alcohol was mesylated, and the mesylate **2** reacted with potassium nitrochlorophenate to give **3**. Reduction of the two nitro groups and alkylation of the resulting amino groups with methyl bromoacetate in the presence of diisopropyl ethyl amine (DIEA) gave the key intermediate **5**. Vilsmeier formylation of **5** was followed by the double bond activation. This was achieved in a two-step procedure: ionic addition of HBr in dichloromethane (DCM) then substitution of the bromo to an azido group using NaN_3 in DMF.

In classical syntheses of X-Rhod dyes, the aromatic aldehyde (such as **8**) is opposed to the commercially available 8-hydroxy julolidine in hot and acid conditions. In our hands, the drastic conditions needed for this reaction were not successful but rather led to the formation of an intractable mixture of colored products. Thus, we preferred to preform the diphenyl ether part **10**. The known diamino diphenyl ether[14] is octa-alkylated[15] with 1-bromo 3-chloropropan to give **10** (Scheme 1).

The coupling reaction between **8** and **10** was conducted in neat trifluoroacetic acid (TFA) at room temperature.[16,17] The intermediate was oxidized by oxygen bubbling in dichloromethane, and the four ester functions were saponified using methanolic KOH. The final product was purified on TLC plate RP18 using MeOH/AcOH 70:3.

Absorption. As expected, the absorbance spectrum of Calcium Ruby-Cl (**11**) was similar to that of other dyes of the X-Rhod family, with a 2−3 nm shift to longer wavelengths. Absorbance was maximal at 578−580 nm ($n = 4$) and did not appreciably change upon Ca^{2+} binding (between nominally 0−1.2 mM [Ca^{2+}], data not shown).

Due to the hygroscopic nature of the product, we estimated the concentrations of **11** solutions using a extinction coefficient ϵ of ∼100 000 M^{-1} cm^{-1}, as reported for other X-Rhod dyes. We next determined the [Ca^{2+}] dependence of the fluorescence of **11** using the titration technique of Minta et al.[18]

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Fluorescence. 11 exhibits a fluorescence emission spectrum similar to that of other X-Rhod derivatives (see ref 4 for review) with a peak at 598−600 nm. Peak fluorescence increased ~32-fold upon Ca^{2+} binding, with no appreciable spectral shift (Figure 3). As with other rhodamine derivatives,

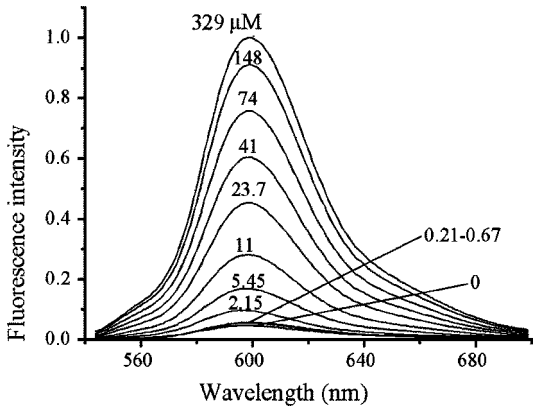

Figure 3. Fluorescence emission upon 535 nm excitation of **11** with [Ca^{2+}] ranging from 0.21 (bottom trace) to 329 μM (top). Titration buffer contained 100 mM KCl, 30 mM MOPS (pH 7.2), 10 mM NTA, and 0.01 mM Calcium Ruby-Cl.

fluorescence was very photostable, particularly when compared to that of fluorescein (not shown).

Quantum Yield φ. Comparison with Sulforhodamine 101 and Rhodamine B yielded an estimate of $\varphi = 0.42 \pm 0.03$ ($n = 4$) for the quantum yield of **11** in the presence of 329 μM free Ca^{2+}. Ca^{2+} binding increased the quantum yield ~17-fold compared to that of the Ca^{2+}-free form (0.026 ± 0.001, $n = 4$). In summary, **11** has a low basal fluorescence comparable to that of X-Rhod-1 and Rhod-2 fluorophores and a high dynamic range upon Ca^{2+} binding relative to other red-emitting fluorophores; see Table 1 for a quantitative comparison.

Ca^{2+}-Binding Affinity. Calcium Ruby-Cl with a chloride in the meta position (Figure 1) had an apparent dissociation constant (30.3 ± 2.6 μM, $n = 4$), measured at 20 °C and 0.1 M ionic strength. This value is in the range predicted for a chloride-substituted BAPTA (Figure 2).

In summary, our results indicate that we have successfully synthesized a visible-light red-emitting [Ca^{2+}] indicator dye (579/598 nm peak excitation/emission). Its large molar extinction and high quantum yield ($\epsilon\varphi \sim 42\,000$ M^{-1} cm^{-1}) ranks our novel Calcium Ruby Ca^{2+} indicator among the brightest red-fluorescent Ca^{2+}-sensitive dyes. Its high photostability and large red shift compared to existing Ca^{2+}-sensitive dyes should make the Calcium Ruby family a versatile alternative to conventional green-yellow and orange-emitting organic Ca^{2+} indicators. With its spectral properties, they respond to the demands of many biological multicolor fluorescence applications, specifically when imaging cells expressing enhanced green fluorescent protein (EGFP).

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Supporting Information Available: Additional experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

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