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Synthesis and characterization of a novel calcium-selective chelator

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Abstract—A calcium-selective photochromic chelator to study the effects of physiological [Ca^{2+}] oscillations on proteins in vitro or in vivo has been designed. The novel 2,2′-spirobi[2*H*-1-benzopyran] derivative **1** has been synthesized and the ion complexation properties of **1** with Mg^{2+}, Ca^{2+}, and Sr^{2+} in aqueous buffered solution have been investigated. The chelator **1** shows modest selectivity for calcium ion ($K_{11}=6720\ M^{-1}$) over magnesium ion ($K_{11}=564\ M^{-1}$) by ^1H NMR titration.

Calcium is an important second messenger in signal transduction systems that translate extracellular signals into cellular responses.[1] Intracellular calcium concentration oscillations, both rapid and slow, occur in many cell types. Both the frequency and amplitude of these oscillations influence cellular events.[2] Extensive work has been carried out to understand how such oscillations arise, but much less is known about the effects of calcium oscillations on a molecular level. In order to examine the effects of calcium oscillations on a single protein in vitro, or to isolate the effects of oscillations in a cell from those of the signal transduction cascade, a method to impose oscillations of defined frequency is needed. Photoreversible calcium-specific chelators appear to be an effective approach.

Photochromic compounds are well suited for controlled ion binding, because they undergo reversible structural changes upon irradiation that may be used to form or disrupt a binding cavity.[3] In order to mimic physiological calcium oscillations using light that will not damage proteins, the photochromic molecule must selectively bind calcium over magnesium and must have appropriate photophysical, kinetic, and thermodynamic properties.

Many photochromic chromophores have been incorporated into macrocyclic ethers designed for alkali, alkaline earth, and heavy metals.[4] These systems lack specificity for calcium over other univalent and divalent cations, and are poorly soluble in water. While the use of spiropyrans to reversibly bind metal ions has been extremely successful in both organic and aqueous systems, no water-soluble, calcium-selective binders have been synthesized.[5]

Investigators have used other approaches to generate calcium oscillations independent of agonist application. These include reversible electroporation of the cell membrane or rapid superfusion of an immobilized protein with alternating solutions of high and low calcium concentrations.[6] These approaches may introduce significant perterbations to the cell or proteins

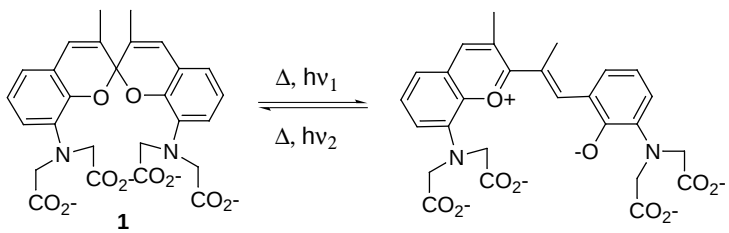

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Figure 1. Compound **1** can be interconverted between closed and open forms with light and/or heat.

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being examined. Photolabile calcium-selective chelators that react irreversibly upon irradiation have also been adapted to this problem.[7] These approaches are limited by the diminishment of unphotolyzed chelator with each light flash.

The photochromic chelator (**1**), whose synthesis and study are discussed here, is shown in Fig. 1 in equilibrium with its low-affinity form. The binding site of **1** is designed to have calcium selectivity over magnesium. Steric constraints should not allow the cavity to collapse to a smaller size, preventing adequate stabilization for the smaller magnesium cation.[8] The photochromic scaffold is based on a 2,2′-spirobi[2*H*-1-benzopyran] derivative, is well established to undergo heterolytic cleavage from closed to open forms. Representative physical data for the related compound 3,3′-spirobi[3*H*-naphtho[2,1-*b*]pyran] includes K_{eq} (closed to open form, 20°C, benzyl alcohol) $=4\times10^{-3}$. Colorization quantum yield (dioxane, 20°C, 8,8′-dinitro-3,3′-spirobi[3*H*-naphtho[2,1-*b*]pyran]) $=0.63$.[9] The photophysical properties of **1** can be readily tuned by altering substituents to change ground-state polarity. Photochemical ring opening will disrupt the binding site in the low-affinity form, so the focus here is whether calcium selectivity can be achieved.

The synthesis of compound **1**, shown in Scheme 1, proceeds from commercially available salicylaldehyde (R = H) and 3-pentanone. Depending on the substitution at R, different substituents may be incorporated into the final structure. This procedure results in an overall yield of 55% of the racemic product. Experimental data are provided for all new compounds.[10]

Compound **1** is soluble in aqueous solutions buffered at pH 8–10, with a critical aggregation concentration greater than 3 mM, as measured by ^{1}H NMR chemical shifts. The highest pK_a's are measured as 6.7, 6.1, 5.0, and 3.7.[11] To ensure >99% deprotonation of all acidic sites, binding studies were conducted at pH >8.7.

^{1}H NMR spectroscopy was used to determine affinities for metal ions.[12] The protons that shifted most significantly were the two aryl protons *ortho* and *para* to the dialkylamino substituents. Each proton had a maximal downfield shift of approximately 0.2 ppm upon binding. This shifting is consistent with metal ion binding diminishing the electron density of the dialkylamino substituent. The program NMRspec was used to analyze the chemical shift data and produce binding constants, maximal downfield shift values, and error analysis. The use of a 1:1 binding model resulted in a poor fit to the observed data. The following simultaneous binding equilibria were considered (Fig. 2), resulting in a marked improvement in fit and no pattern in the residuals. Incorporation of additional equilibria did not improve the fit. Note that K_{12} and K_{21} are overall stability constants, or betas (β_{12},β_{12}).

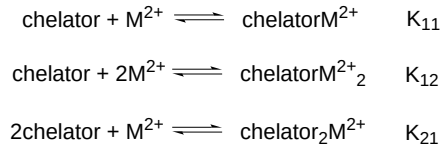


Figure 2. Simultaneous binding equilibria used to model ^{1}H NMR titration data.

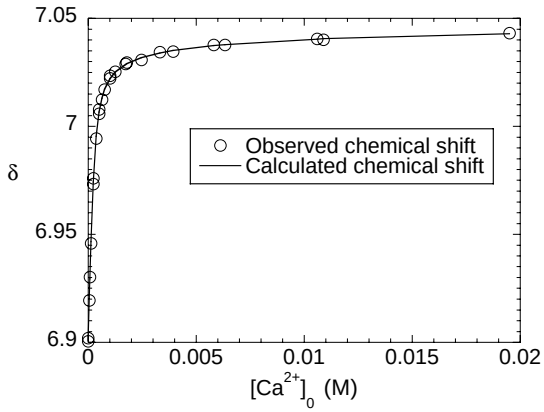
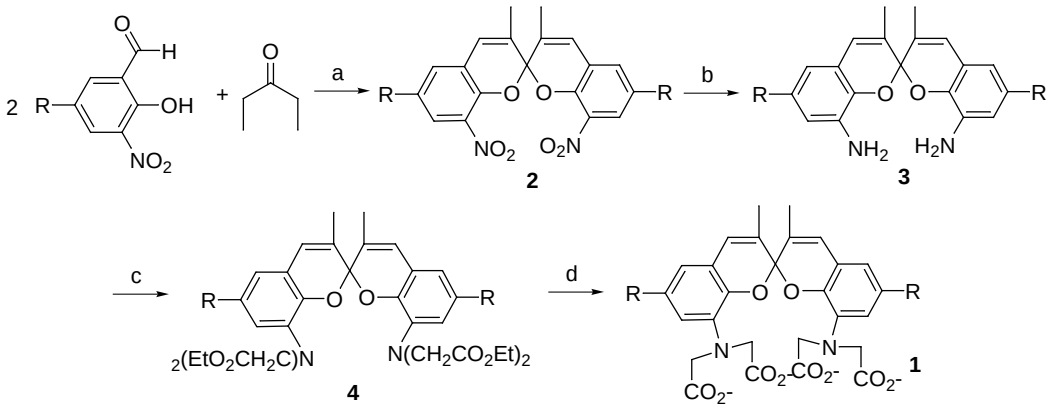


Figure 3. Observed chemical shift (○) and calculated chemical shift (—) for one proton of **1** during a titration with $CaCl_2$(aq).



Scheme 1. The synthesis of **1**. (a) HCl(g), EtOH; (b) $SnCl_2{\cdot}2H_2O$, EtOH, 80°C; (c) $BrCH_2COOEt$, CH_3CN, proton sponge, NaI, reflux; (d) NaOH H_2O/THF.

Table 1. Binding constants[a] of chelator $\mathbf{1}+M^{2+}$ in D_2O, 10 mM HEPES-d_{18} pH 9.8

	Mg^{2+}	Ca^{2+}	Sr^{2+}
K_{11} (M^{-1})	5.64×10^{2}	6.72×10^{3}	8.10×10^{3}
K_{12} (M^{-2})	2.87×10^{1}	5.10×10^{5}	4.77×10^{5}
K' (M^{-1})	5.09×10^{-3}	7.59×10^{1}	5.89×10^{1}
K_{21} (M^{-2})	2.11×10^{6}	4.41×10^{7}	1.39×10^{8}
K'' (M^{-1})	3.74×10^{3}	6.56×10^{3}	1.72×10^{4}

[a] Determined by ^{1}H NMR titration.

A sample plot and fit to the multiple equilibria model are shown in Fig. 3.

Comparing K_{11} for compound **1** and each of the three divalent metal cations reveals that calcium ions and strontium ions are more strongly bound than the magnesium ion. One may consider the overall stability constants K_{12} and K_{21} to be products of stepwise stability constants, such that

$$K_{12}=K_{11}^{*}K'$$

$$K_{21}=K_{11}^{*}K''$$

It was found that K' is quite small compared to K_{11}, so that the 1:1 complex is only weakly bound to a second metal ion for all three metal ions. K'' is comparable in magnitude to K_{11} for each of the metals, suggesting that the metal ion is not encapsulated entirely by the ligands of one chelator and that a second chelator may bind the metal ion equally well (Table 1).

In summary, a photochromic system that shows good selectivity for calcium ion over magnesium ion has been designed and synthesized. Enhancement of this selectivity may be afforded by lengthening the chelator arms. For example, using methyl 3-bromoproprionate instead of ethyl bromoacetate in the synthetic route would result in a chelator with a larger cavity. This cavity should still bind calcium ion well, but should be less able to collapse to stabilize the smaller magnesium ion. Additional future work is directed toward photophysical studies of **1**. The photochemical and binding properties may be optimized by varying substituents on the chelator. The modular synthetic scheme readily allows symmetric and asymmetric substitution on the product chelator.

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10. General methods. ^{1}H NMR spectra were recorded on a Bruker 400 MHz spectrometer. High resolution mass spectra were obtained at the University of California at Riverside Mass Spectroscopy Facility.
Compound ***2***. The synthetic procedure reported in the literature for related compounds (Appriou, P.; Trebaul, C.; Brelivet, J.; Garnier, F.; Guglielmetti, R. *Bull. Soc. Chim. Fr*. **1976**, 2039–2045.) was modified as follows. A solution of 3-nitrosalicylaldehyde (2 g, 12.3 mmol) and 3-pentanone (0.65 mL, 6.16 mmol) in 100 mL of anhydrous ethanol was stirred and cooled in an ice bath. Hydrogen chloride gas (Caution) was bubbled through the cooled reaction mixture for 2 h. The mixture was allowed to warm to room temperature, and after 24 h, the reaction was again chilled to 0°C and the procedure repeated for 2 h and left overnight. The crude product was filtered off and purified by recrystallization (80% isolated yield). ^{1}H NMR ($CDCl_3$) δ 7.78 (dd, $J=1.6$, 8.3 Hz, 2H), 7.36 (dd, $J=1.6$, 7.6 Hz, 2H), 7.07 (t, $J=8.16$

Hz, 2H), 6.72 (d, $J=1.5$ Hz, 2H), 2.04 (d, $J=1.4$ Hz, 6H); ^{13}C NMR ($CDCl_3$) δ 131.0, 130.3, 126.4, 125.8, 125.3, 123.9, 122.8, 121.7, 103.0, 18.9; HRMS (DEI) calculated M^+ 366.3300, found 366.0856.

Compound ***3***. A mixture of compound **2** (0.365 g, 1 mmol), tin(II) chloride dihydrate (2.256 g, 10 mmol), and 10 mL of ethanol were stirred under nitrogen and warmed to 70°C. (Bellamy, F. D.; Ou, K. *Tetrahedron Lett*. **1984**, *25*, 839–842.) Reaction progress was monitored by thin layer chromatography (TLC), and after 40 min, the reaction was poured over ice. A 5% bicarbonate solution was added to neutralize the reaction. The product was extracted (ethyl acetate) and dried (sodium sulfate). The extract was concentrated in vacuo and the crude product was not purified further. ^1H NMR ($CDCl_3$) δ 6.80 (t, $J=7.7$ Hz, 2H), 6.62 (dd, $J=1.3$, 7.9 Hz, 2H), 6.57 (dd, $J=1.2$, 7.5 Hz, 2H), 6.55 (d, $J=1.4$ Hz, 2H), 3.66 (br, 4H), 1.97 (s, 6H); ^{13}C NMR ($CDCl_3$) δ 137.9, 134.9, 129.2, 123.9, 121.9, 11.9, 116.2, 115.7, 102.7, 19.1; HRMS (DEI) calculated M^+ 306.3641, found 366.1363.

Compound ***4***. To a stirred solution of compound **3** (0.199 g, 0.650 mmol) in 5 mL anhydrous acetonitrile, sodium iodide (0.0336 g, 0.224 mmol), Proton Sponge™ (0.856 g, 3.99 mmol), and ethyl bromoacetate (1.1 mL, 4.38 mmol) were added. The system was kept under nitrogen and covered in foil. (Tsien, R. Y. *Biochemistry* **1980**, *19*, 2396–2404.) The reaction refluxed for 48 h while being monitored by TLC. The reaction cooled and was diluted with toluene. The precipitate was filtered and washed with toluene. The toluene layer was extracted with several portions of phosphate buffer at pH 2, then washed with water. The organic layer was dried and the resulting oil was purified by flash chromatography (6% ethyl acetate in methylene chloride). (70% isolated yield) ^1H NMR ($CDCl_3$) δ 6.81 (t, $J=8$ Hz, 2H), 6.66 (d, $J=7.6$ Hz, 4H), 6.45 (d, $J=1.2$ Hz, 2H) 4.03 (m, 4H), 3.98 (s, 8H), 3.88 (m, 4H), 1.87 (s, 6H); 1.14 (t, $J=7.1$ Hz, 12H); ^{13}C NMR ($CDCl_3$) δ 171.8, 141.2, 137.8, 128.6, 123.5, 121.6, 120.6, 119.4, 118.7, 103.1, 60.9, 54.1, 18.7, 14.5; HRMS (DEI) calculated M^+ 650.7259, found 650.2827.

Compound ***1***. To a stirred solution of compound **4** (0.030 g, 4.61×10^{-5} mol) in about 1 mL of tetrahydrofuran (THF) in a 5 mL point bottom flask was added 8 equivalents of NaOH (140 microliters of a 2.5 M NaOH (aq)). The reaction was left to stir for several days, with occasional addition of water, and monitored using reverse-phase TLC (10% CH_3CN in H_2O). The sample was lyophilized and acidified with 70 microliters of 6 M HCl. A minimum of acetonitrile was added to the sample to dissolve it, and it was then applied to a 6 mL SPE tube (Supelco Supelclean™ LC-18). Stepwise gradient elution using 10, 20, and 30% acetonitrile in water was used to isolate the pure product. (90% isolated yield) ^1H NMR (CD_3CN) δ 6.91 (t, $J=6.9$ Hz, 2H), 6.84 (m, 4H), 6.63 (d, $J=1.4$ Hz, 2H), 3.88 (AB, 8H), 1.90 (d, $J=1.5$ Hz, 6H); ^{13}C NMR (CD_3CN) δ 173.5, 141.3, 136.3, 128.8, 124.1, 122.1, 121.5, 120.8, 119.1, 117.7, 102.9, 55.7, 17.8; HRMS (FAB) calculated MNa^+ 561.5006, found 561.1485.

11. pK_a's of the chelator were determined by performing several titrations monitoring pH with an Orion Ag/AgCl microelectrode. (In a water bath, a gently stirred 3.00 mL solution of 8.89×10^{-4} M chelator, 0.100 M NaCl, and 7.11×10^{-3} M NaOH was titrated using a dilution of a volumetric standard 0.197N HCl solution). The potentiometric data were analyzed using the program Hyperquad2000 written by Dr. Peter Gans, University of Leeds. The first two ionizations of compound **1** could not be determined accurately since the compound is not soluble at low pH. Critical aggregation concentration was determined by examining ^1H NMR chemical shifts while increasing the concentration of chelator from approximately 1×10^{-4} to 3×10^{-3} M.
12. Binding constants were determined by performing a ^1H NMR titration of metal chloride added to chelator using the chemical shifts of three protons of the chelator referenced to internal standard of 3-(trimethylsilyl)-1-propanesulfonic acid, sodium salt (DSS) at 12–17 different guest to host concentration ratios. (Stock solutions were made up using 1.00 mL volumetric flasks; NMR tubes for the binding studies were made up such that [chelator] $=6.6\times10^{-4}$ M, [metal] $=0$–1.0×10^{-2} M in 10 mM HEPES-d_{18}, pH 9.8). These data were collected and three independent binding studies for each metal chloride were fit to 1:1, 1:2, and 2:1 binding models in an iterative least-squares refinement procedure found in the Pascal program NMRspec. This program, written by Richard E. Barrans, Jr. (PG Research Foundation), generates association constants, the chemical shifts of the bound chelator for the binding model specified, and confidence limits. Reduced χ^2 for the fits for Mg^{2+} (0.53), Ca^{2+} (0.10), and Sr^{2+} (0.42) were calculated using the uncertainty in chemical shifts to be 0.004 ppm.