Synthesis of stilbene carboxylic acids as scaffolds for calcium sensors†

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This communication describes the synthesis and characterization of calcium binding stilbene carboxylic acids.

The study of calcium as a cellular messenger has been greatly enhanced by the use of fluorescent calcium probes, first developed by Tsien and colleagues.[1,2] These probes have been used to study calcium concentration changes both inside and outside of cells.[3–5] One major limitation, however, has been the inability to target calcium probes to specific components of cells.[6] To accomplish this, some modifications of the original class of [1,2-bis-(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid] (BAPTA) based calcium probes developed by the Tsien laboratory have been created that involve the addition of an alkyl chain[7] or dextran[8,9] moiety to facilitate loading and help sequester the probes in different regions of the cell. However, the targeting of specific sites could be greatly improved by attaching peptides to calcium sensors. Other types of fluorophores with peptides have been reported for organelle tracking. These fluorophores were shown to localize in different, discrete areas within cells depending on the peptide sequence attached to them.[10,11]

We report here a new class of derivatizable, calcium-binding scaffolds that can be modified with peptides on solid phase to create targeted, small-molecule calcium sensors. Our laboratory has previously synthesized several phenylene vinylene–lysine conjugates that are noncytotoxic, and are endocytosed by different cell types,[12] and we describe here the modification of the aromatic scaffold for calcium binding with carboxylic acids as the binding ligands.

Computational modeling was performed to determine where the acids should be placed on the aromatic scaffold. Using an energy minimized structure derived from the modeling program Cerius2, it was found that placing the carboxylic acids *ortho* to the vinylene-linkage would create an average distance of 2.38 Å between each carboxylic acid with a Ca^{2+} ion between them. This distance is

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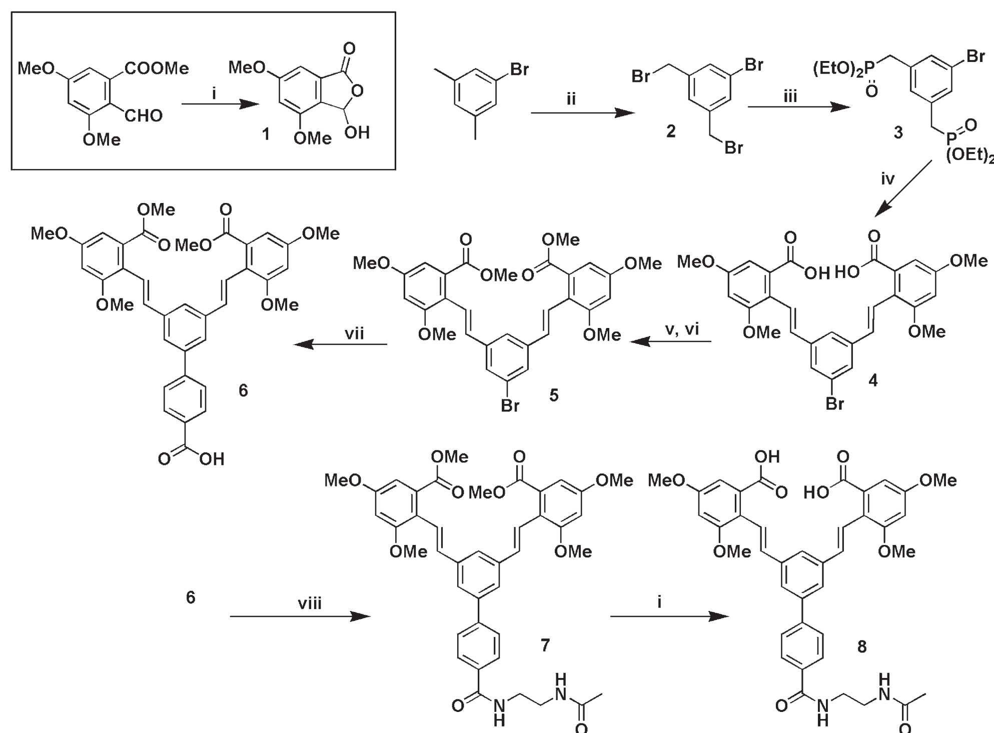
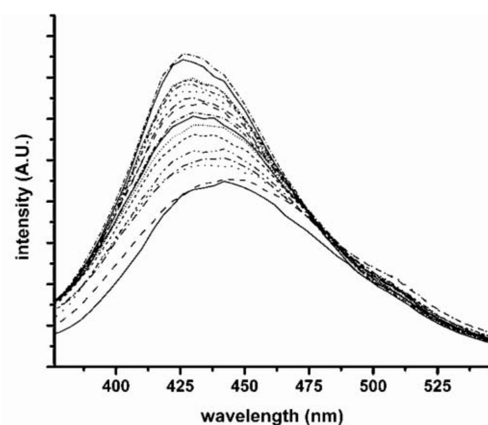
† Electronic supplementary information (ESI) available: Syntheses for compounds **1–8** and graph for determining K_d values. See http://dx.doi.org/10.1039/b506423f



Scheme 1 Synthesis of stilbene acid **8**. (i) LiOH, THF : H2O (5 : 1), rt, 3 h, 95%; (ii) NBS, BPO, CCl_4, reflux, 12 h, 30%; (iii) $P(OEt)_3$, 130 °C, 10 h, 68%; (iv) Potassium *tert*-butoxide, **1**, ether, 0 °C to rt, 16 h, 52%; (v) DMSO, KOH, 30 min, −78 °C; (vi) CH_3I, to rt, 3 h, 87%; (vii) 4-carboxyphenylboronic acid, K_2CO_3, $Pd(PPh_3)_4$, DME : H_2O (3 : 1), 80 °C, 12 h, 71%; (viii) HBTU, DIEA, *N*-acetylethylaminediamine, 8 h, 43%.

comparable to the distances observed in calcium binding proteins.[13,14] For the synthesis of the *ortho* acids, 2-formyl-3,5-dimethoxybenzoic acid methyl ester was treated with lithium hydroxide for three hours to obtain the deprotected *ortho* acid-aldehyde **1** for use in the Horner–Emmons reaction (see Scheme 1). Diphosphonate **3** was synthesized from dibromide **2** using standard Arbuzov conditions, as has previously been reported.[15–17] The novel Horner–Emmons product **4** was then methylated with methyl iodide[18] and methyl ester **5** was coupled to 4-carboxyphenyl boronic acid in a palladium catalyzed Suzuki coupling reaction.[19] The resulting acid was then ready for coupling in solid phase reactions. To test reactivity of the compound standard solution phase amination of acid **6** was performed, yielding the protected product **7**. Deprotection of the acids with lithium hydroxide affords **8** as a potential calcium binding compound. This synthesis was also applied to the *ortho* acid-aldehyde with no methoxyl groups.

A stock solution of **8** in DMSO was diluted into aqueous buffer (10 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS) buffer, pH 7.2) (1% v/v) for spectroscopic characterization. The molecule's absorption spectra reveal two maxima at 290 nm and 325 nm. Excitation at either wavelength gives an emission maximum at 442 nm (quantum yield (Φ) = 0.01, lifetime < 1 ns). Upon titration with calcium the wavelength maximum blue-shifts 20 nm, and a three-fold increase in intensity is observed (quantum yield (Φ) = 0.01, lifetime 11 ns) (Fig. 1). The analogue of **8** without methoxyl groups on the terminal phenyl rings did not display a shift upon binding, suggesting that electron density on the rings is beneficial to sensing applications. The dissociation constant, K_d, of 6.3 μM was calculated by plotting the logarithmic ratio of fluorescence intensity *versus* calcium concentration, as established in the literature.[1] This calcium affinity is consistent with other low affinity sensors used *in vitro* to monitor fast kinetic changes in calcium concentration.[20–22] The sensitivity of **8** towards other metal ions was also determined. Copper was the only metal ion tested that quenched the fluorescence of **8**, and the 20 nm shift observed was found to be selective for calcium (Fig. 1).



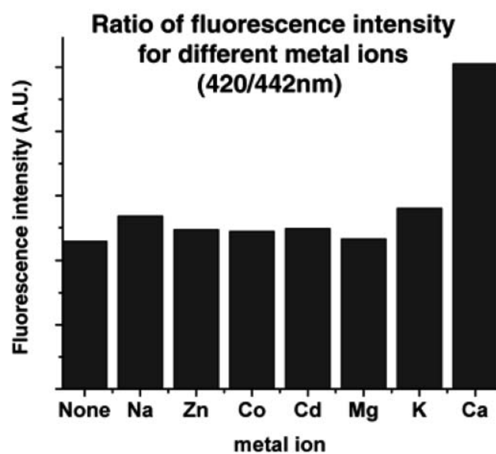


Fig. 1 Titration of **8** (100 mM MOPS buffer, pH 7.2) with calcium chloride (0.–1.1 μM in 0.1 μM steps, 1.3, 1.5, 2.0, 2.5, and 5 μM). The fluorescence intensity increases and shifts to the blue. The ratio of intensities for calcium and other ions is shown on the bottom.

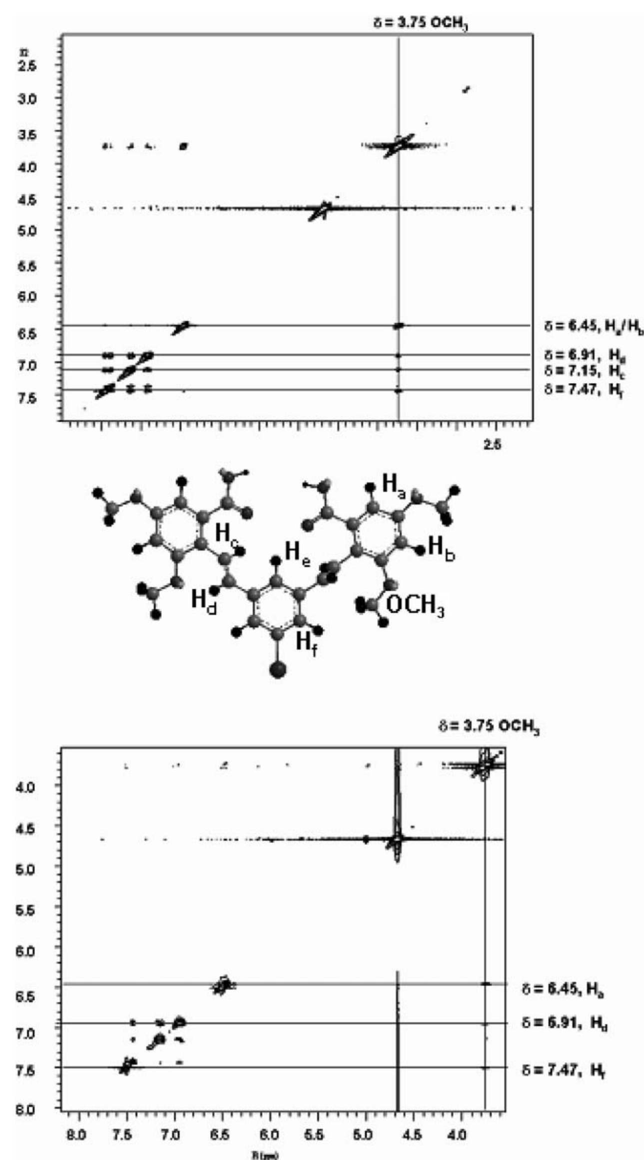


Fig. 2 NOE spectra of **4** both without (top) and with (bottom) calcium. Line crossing indicates close contacts between the methoxyl protons and the aromatic protons on the ring.

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In order to demonstrate compatibility with solid phase reactions, acid **6** was coupled to the peptide sequence NH2-FFKDEL-COOH (Phe-Phe-Lys-Asp-Glu-Leu) which has been shown to target the endoplasmic reticulum.[10] The peptide was synthesized on an acid sensitive Wang resin that is cleaved by treatment with 3% TFA in methylene chloride for 15 minutes, conditions that do not affect aromatic bonds in the fluorophore. The acidic amino acid residues were protected with groups cleaved under the same mild conditions. Free acid **6** was attached to the amino terminus of the peptide on solid phase with 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) as a coupling reagent. After the coupling was complete and the conjugate was cleaved from the resin, the lysine side chains were deprotected by anhydrous HCl in dioxanes. The material was added in DMSO to the media of 3T3 fibroblasts and tested for cell toxicity. Proliferation and growth was monitored compared to the control, and presence of the compound did not affect cell proliferation or growth as observed for precursor molecules.[12]

Diacid **8** has properties that could make it a good scaffold for calcium sensors, with the possibility of improvement by increasing conjugation of the ring systems to allow for excitation at lower wavelengths. This may also enhance the shift observed upon calcium binding. To determine the mechanism of calcium binding and aid in tailoring future structures for the sensors, a series of 2D NMR Nuclear Overhauser Effect (NOE) experiments were conducted on diacid **4** to elucidate the position of the acids in the presence and absence of calcium, as free rotation is possible around the stilbene double bonds. In the absence of calcium, the NOE close contacts (< 3Å) between the methoxyl protons and aromatic protons indicate that the molecule explores all conformations. However, upon the addition of calcium, the only specific NOE close contacts observed suggest a conformation of "both arms-in," with the calcium ion bound between them (Fig. 2). This structural insight can help direct the next designs of these stilbene-based calcium sensors.

In summary, we have synthesized a scaffold for calcium binding that shows a shift and increase in fluorescence intensity upon calcium binding. Most importantly, this molecular scaffold can be covalently linked to peptides on solid phase for purposes of cell targeting.

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