A Novel 12-Membered Triazaoxamacrocycle-*N,N',N''*-triacetic Acid Indicator for Colorimetric Determination of Calcium

Richard A. Bartsch,*,† Eddy Chapoteau,‡ Bronislaw P. Czech,‡ Jan Krzykawski,† Anand Kumar,*,‡ and Thomas W. Robison†

Department of Chemistry and Biochemistry, Texas Tech University, Lubbock, Texas 79409, and Diagnostics Division, Miles Incorporated, Tarrytown, New York 10591

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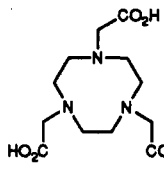
The synthesis of a novel chromogenic triazaoxamacrocycle bearing three acetic acid groups and a 2,4,6-trinitroanilino chromophore, for generation of a spectrophotometric response, is described. As determined by potentiometric titration conducted in water, tetraionizable chromoionophore **4** forms a rather weak 1:1 complex with calcium ($K_s = 9.5 \times 10^2$ M^{-1}) but exhibits very high selectivity for Ca^{2+} over Mg^{2+} at neutral pH. Cation responses at pH 7.0, 8.0, 9.0, and 10.0 are determined. The response of **4** to calcium is linear from 2×10^{-5} to 1×10^{-4} M Ca^{2+}.

Introduction

Chelating agents based on azacrown ethers bearing acetic acid moieties have attracted considerable interest due to their enhanced ligating abilities toward di- and trivalent cations. Acetic acid derivatives of tri- and tetraaza macrocycles, such as 9-membered NOTA (**1**), 12-membered DOTA (**2**), 14-membered TETA (**3**), and their derivatives,



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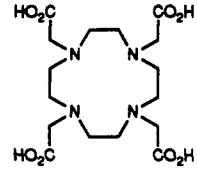

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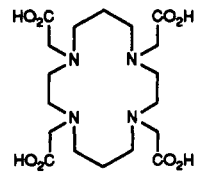

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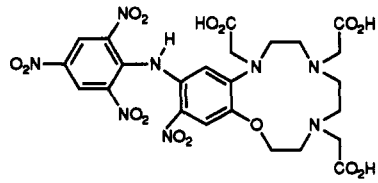


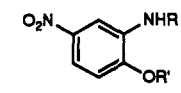
4

have received the most attention.[1] Some of these chelating agents have potential practical applications as contrast agents for magnetic resonance imaging and in cancer diagnosis and therapy.[2] Tetraacetic acid derivative **2** has been reported to complex Ca^{2+} with exceptional strength and selectivity.[3] Several currently available methods for the colorimetric determination of calcium in serum are based on acyclic chromogenic compounds, such as *o*-cresolphthalein complexone (CPC) and Arsenazo III. The most commonly used CPC method[4] suffers from a relatively short reagent stability and sensitivity to carbon dioxide. Both problems are related to the highly alkaline conditions (pH 10–12) required for the reagent to function. Since the compound binds Mg^{2+} as well, a masking agent must be incorporated into the reagent to eliminate magnesium interference.

Due to our continuing interest in the colorimetric determination of physiologically important ions, we have examined the possibility of designing an improved colorimetric calcium indicator based on a macrocyclic chelate. We envisioned a chromogenic compound **4** based on a benzotriazaoxamacrocycle-*N,N',N''*-triacetic acid which bears a 2,4,6-trinitroanilino chromophore. The chromophore was to be attached para to the sensing oxygen atom of the ring. Also, to decrease the pK_a of the NH proton to allow response at physiological pH, attachment of an additional nitro group ortho to the 2,4,6-trinitroanilino group was envisioned. In this paper we report the synthesis of this new cyclic chromoionophore and its applicability for the determination of calcium.

Results and Discussion

Synthesis. Commercially-available 2-amino-4-nitrophenol (**5**) was treated with K_2CO_3 and $TsOCH_2CH_2NHTs$ (**15**)[5] in DMF to give a 36% yield of nitro compound **6**,



	R	R'
5	H	H
6	H	CH_2CH_2NHTs
7	Ts	CH_2CH_2NHTs
8	Ts	H

which was also prepared in 43% yield by reaction of **5**

† Texas Tech University.

‡ Miles Incorporated.

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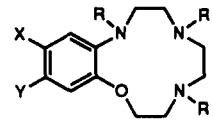
with *N*-tosylaziridine (16).[6] The slightly higher yield which was obtained with tosyl aziridine as the alkylating agent was more than offset by the additional step required to prepare 16 from tosyl sulfonamide 15 and by its tendency to undergo self-polymerization. Reaction of 6 with tosyl chloride and pyridine in CH_2Cl_2 afforded ditosylamide 7 in 69% yield.



	R	Y	X
9	Ts	H	NO_2
10	H	H	NO_2
11	CH_2CO_2Et	H	NO_2
12	CH_2CO_2Et	H	NH_2
13	CH_2CO_2Et	H	NH-picryl
14	CH_2CO_2Et	NO_2	NH-picryl

15 16 17

An alternative route to 7 involved selective tosylation (TsCl, pyridine, CH_2Cl_2) of the aniline nitrogen in 5 to produce sulfonamide 8 in 77% yield. Subsequent reaction of 8 with 15 (NaH, DMF) or 16 (K_2CO_3, DMF) gave 26 and 30% yields of 7, respectively.

For both routes the alkylation reactions were complicated by the presence of both nitrogen and oxygen nucleophilic centers. Also, the nucleophilicity of the phenolate anion is considerably reduced by the electron-withdrawing nitro group in the para position. These factors are probably the major contributors to the low yields in the oxygen-alkylation reactions.

The macrocyclic ring formation which involved high dilution reaction of 7, 3-(tosyl)-1,5-bis(tosyloxy)-3-azapentane (17),[7] and $CsCO_3$ in DMF produced cyclic tritosylamide 9 in 57% yield. It was found that Cs_2CO_3 typically gave a 15–20% higher cyclization yield than did K_2CO_3. Presumably this is due to the "cesium effect" which results from enhanced solubility of the cesium salt in the organic solvent.[8]

The tosyl groups of 9 were removed by heating in concentrated H_2SO_4 to provide cyclic triamine 10 in 94% yield.

Complete alkylation of the amine nitrogens in 10 with ethyl bromoacetate proved to be more difficult than expected. Reaction of 10 with excess ethyl bromoacetate and K_2CO_3 in refluxing acetonitrile for 36 h gave primarily a dialkylated product in which the two dialkylamine nitrogens had been alkylated. Presumably, addition of the third ester group was slow and incomplete due to steric problems and reduced nucleophilicity of the alkyl aryl amine nitrogen. The completely alkylated product 11 was obtained in 88% yield when alkylation was conducted in DMF at 100 °C for 48 h.

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Table I. Spectral Characteristics and Calcium and Magnesium Responses of Chromogenic Compound 4 at Different pH Values

pH[a]	form[b]	λ_{max}, nm	ϵ (λ_{max})
	HL	383.0	14 790
	L^-	449.0	21 970
7.0	L	400.0	18 900
(MES)	CaL	404.5	12 800
	MgL	400.0	18 800
8.0	L	402.3	19 200
(HEPES)	CaL	412.3	13 200
	MgL	401.1	17 300
9.0	L	407.0	19 800
(HEPES)	CaL	443.0	15 700
	MgL	443.0	16 450
10.0	L	427.0	18 560
(CHES)	CaL	444.0	13 210
	MgL	442.0	13 550

[a] Buffers: 0.1 M MES-2-(*N'*-morpholino)ethanesulfonic acid; 0.1 M HEPES-*N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; 0.1 M CHES-2-(cyclohexylamino)ethanesulfonic acid. [b] HL is nonionized 4 in 0.1 M HCl; L^- in fully ionized 4 in 0.1 M TMA(OH); L is the uncomplexed 4; CaL and MgL are 4 in the presence of a 100-fold excess of calcium and magnesium ions, respectively.

The nitro group of 11 was catalytically reduced (H_2, 10% Pd/C) to the amino group of 12 which, due to its tendency to decompose, was reacted immediately with picryl chloride and K_2CO_3 in CH_3OH to give 13 in an overall 60% yield for these two steps.

To lower the pK_a value for the chromophore unit, a nitro group was added to the benzocrown moiety. Nitration of 13 with fuming HNO_3 in $CHCl_3$–CH_3CO_2H afforded a 94% yield of nitro compound 14 in which the nitro group is located ortho to the picrylamine group. Hydrolysis of the ester groups of 14 was accomplished by treatment with $LiOH \cdot H_2O$ in dioxane–water to produce triacid 4 in 58% yield. Under more vigorous conditions for the basic hydrolysis, concomitant cleavage of the picryl group was experienced.

Spectral Characteristics and Cation Responses of Chromoionophore 4. Wavelength maxima (λ_{max}) and molar absorptivities (ϵ) of the acid and base form of the chromogenic compound 4 are recorded in Table I. To suppress ionization of the N–H bond and thereby obtain the HL spectrum, the absorbance was determined in 0.1 M HCl. Likewise, full ionization of the N–H bond was ensured by use of 0.1 M tetramethylammonium hydroxide, TMA(OH), to obtain the L^- form. The chromophore system of compound 4 exhibited an absorption maximum for the acid form at 383 nm which upon full ionization shifted bathochromically to 449 nm with an increase in the molar absorptivity.

It was found that at pH 7.0 compound 4 shows very high selectivity for Ca^{2+} over Mg^{2+} (Table I). However the complexation of calcium is accompanied by only a 4-nm bathochromic shift. Unlike other ionizable chromogenic ionophores, for which complexation is usually accompanied by both a bathochromic shift and increase in absorptivity, compound 4 exhibits about a 35% decrease in the absorptivity upon calcium complexation at pH 7.0 (Figure 1).

Stability constants (K_s) for complexes of 4 with calcium at pH 6.0, 7.0, 8.0, and 9.5 in buffered aqueous solutions at 25 °C were measured potentiometrically with an ion-selective electrode and are summarized in Table II. As

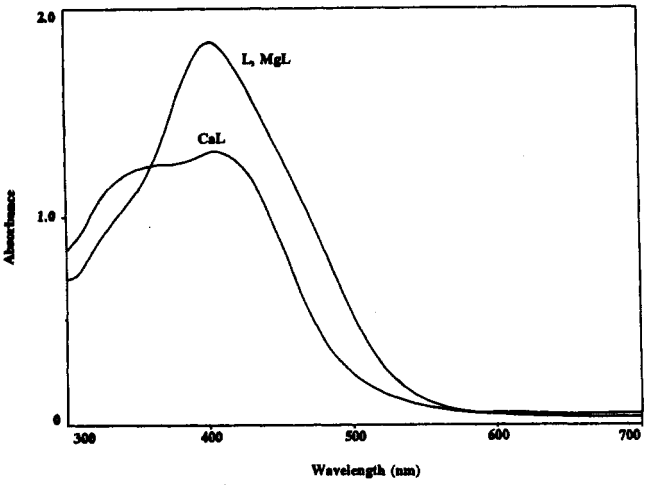

Figure 1. UV–vis spectra for chromogenic compound 4 (L) and for calcium (CaL) and magnesium (MgL) responses in water at pH 7.0.

Table II. Stability Constants[a] for Complexation of Ca^{2+} by Compound 4 in Buffered Aqueous Solutions at 25 °C

pH	6.0	7.0	8.0	9.5
buffer	MES	HEPES	HEPES	CHES
K_s (M^{-1})	100	9.5×10^2	3.1×10^3	1.4×10^4

[a] Determined by potentiometry using an ion-selective electrode.

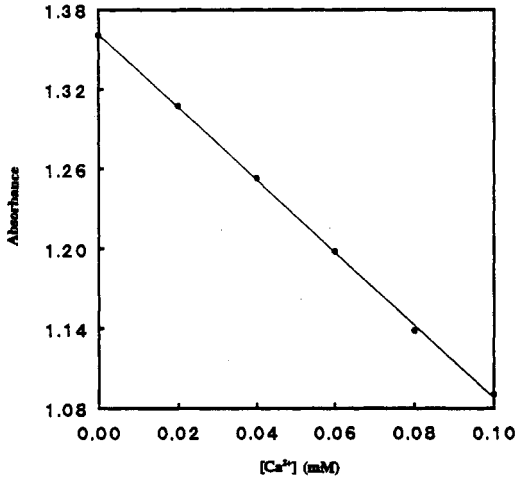

Figure 2. Linear relationship between the absorbance of the calcium complex of 4 and the concentration of calcium ions at pH 7.0.

the pH increases the formation constants (K_s) also increase, but the selectivity for calcium over magnesium diminishes (Table I), and the chromogenic compound was found to be less stable than at neutral pH. The response of 4 to calcium at neutral pH is linear from 0 to 1×10^{-4} M Ca^{2+} (Figure 2). It was found that the reagent maintained adequate sensitivity after 1 month at room temperature and neutral pH which is a definite improvement over the CPC method. Thus, chromogenic compound 4 possesses potential for use in the colorimetric determination of calcium ion.

Experimental Section

General Methods. Melting points were determined on a Fisher Johns melting point apparatus and are uncorrected. IR spectra were obtained with a Nicolet MX-S FT-IR spectrophotometer. ^1H and ^{13}C NMR spectra were recorded with a Bruker 200-MHz spectrometer, and chemical shifts are reported in parts per million (δ) downfield from TMS. The UV–visible spectra were recorded on a Beckman DU-8 spectrophotometer. An Orion 601-A digital ion analyzer was used in the pH measurements. Combustion analysis was performed by Galbraith Laboratories (Knoxville, TN) and Desert Analytics (Tucson, AZ).

Materials. Unless specified otherwise, reagent-grade reactants and solvents were used as received from chemical suppliers. DMF was distilled under vacuum and stored over molecular sieves. THF was purified by distillation from sodium metal. 2-(Tosylamino)ethyl *p*-toluenesulfonate (15),[5] tosylaziridine (16),[6] and *N*-(*p*-tolylsulfonyl)diethanolamine bis(*p*-toluenesulfonate) (17)[7] were prepared according to literature procedures.

Preparation of Tosylamide 6. Method A. Anhydrous potassium carbonate (13.9 g, 99.9 mmol) and 2-amino-4-nitrophenol (5) (7.00 g, 45.4 mmol) in DMF (30 mL) was stirred and heated at 100 °C for 30 min to form a dark red solution. A solution of 2-(tosylamino)ethyl *p*-toluenesulfonate[5] (15, 16.8 g, 45.4 mmol) in DMF (30 mL) was added during a 10-h period with a syringe pump. After an additional 2 h, the solvent was removed in vacuo. The residue was dissolved in water and acidified with 6 N HCl. Extraction with several portions of EtOAc, drying of combined extracts with $MgSO_4$, and evaporation of the solvent in vacuo gave a residue which was passed through a short alumina column with EtOAc as eluent. Evaporation of the solvent in vacuo gave a residue to which 100 mL of CH_2Cl_2 was added resulting in the precipitation of a yellow solid. The solid was collected by filtration and washed with several portions of cold CH_2Cl_2 and dried in vacuo to give 6 (5.75 g, 36%) as a yellow powder with mp 181–182 °C. IR (KBr): 3414, 3333 (NH), 1521 (NO_2), 1342, 1150 (SO_2) cm^{-1}. ^1H NMR ($CDCl_3$-$CD_3S(O)CD_3$): δ 2.35 (s, 3H), 3.35 (t, 2H), 3.97 (t, 2H), 5.00 (br s, 2H), 6.59 (d, 1H), 7.24 (d, 2H), 7.47 (dd, 1H), 7.53 (d, 1H), 7.55 (d, 2H), 7.85 (t, 1H). Anal. Calcd for $C_{15}H_{17}N_3O_5S$: C, 51.27; H, 4.88. Found: C, 51.39; H, 4.93.

Method B. Anhydrous K_2CO_3 (0.45 g, 3.35 mmol) and 5 (1.00 g, 6.49 mmol) in DMF (10 mL) were heated to 75 °C and *N*-tosylaziridine (16) (1.28 g, 6.49 g) in DMF (4 mL) was added over a 10-h period with a syringe pump. The reaction mixture was stirred overnight, the solvent was removed in vacuo, and the residue was partitioned between EtOAc and water. The organic layer was separated and dried ($MgSO_4$). Evaporation of the solvent in vacuo left an oily residue which was filtered through a short layer of alumina with EtOAc as eluent. Chromatography on silica gel with $CHCl_3$ as eluent gave 6 (0.98 g, 43%).

Preparation of Tosylamide 8. Solid tosyl chloride (2.47 g, 12.98 mmol) was added in one portion to a solution of 5 (2.00 g, 12.98 mmol) and pyridine (1.13 g, 14.28 mmol) in CH_2Cl_2 (20 mL) at −3 °C. The mixture was allowed to gradually warm to room temperature. After being stirred for 5 h the mixture was washed with 6 N HCl which produced a precipitate. The precipitate was collected and washed with water and CH_2Cl_2 to give 8 (3.09 g, 77%) as a light brown solid with mp 206.5–208 °C. IR (KBr): 3395 (OH), 1529, 1345 (NO_2), 1159 (SO_2) cm^{-1}. ^1H NMR ($CDCl_3$-$CD_3S(O)CD_3$): δ 2.36 (s, 3H), 6.86 (d, 1H), 7.23 (d, 2H), 7.71 (d, 2H), 7.78–7.84 (m, 2H), 8.28 (d, 1H), 10.66 (brs, 1H). Anal. Calcd for $C_{13}H_{12}N_2O_5S$: C, 50.65; H, 3.92. Found: C, 50.34; H, 3.89.

Preparation of Ditosylamide 7. Method A. Monotosylamide 6 (5.15 g, 14.7 mmol) was suspended in CH_2Cl_2 (250 mL), and pyridine (14 mL), was added. The mixture was cooled to 0 °C, and tosyl chloride (4.19 g, 22.0 mmol) was added. The mixture was allowed to warm to room temperature and was stirred for 2 h. After washing with 5% aqueous HCl followed by water, the organic layer was dried over $MgSO_4$. Evaporation of the solvent in vacuo gave a yellow solid which was washed with several portions of cold CH_2Cl_2 to give 7 (5.14 g, 69%) as a white solid with mp 188.5–189.5 °C. IR (KBr): 3327, 3223 (NH), 1528 (NO_2), 1342, 1162 (SO_2) cm^{-1}. ^1H NMR ($CDCl_3$-$CD_3S(O)CD_3$): δ 2.32 (s, 3H), 2.35 (s, 3H), 3.14 (t, 2H), 3.73 (t, 2H), 6.15 (t, 1H), 6.60–8.16 (m, 11H), 8.40 (d, 1H). Anal. Calcd for $C_{22}H_{23}N_3O_7S_2$: C, 52.77; H, 4.59. Found: C, 52.29; H, 4.52.

Method B. A mixture of 8 (2.00 g, 6.49 mmol) and K_2CO_3 (0.90 g, 6.49 mmol) in DMF (10 mL) was stirred at 60 °C for 5 min. A solution of 16 (0.90 g, 6.49 mmol) in DMF (10 mL) was added during a 30-min period, and the mixture was stirred for 24 h at 60 °C. The solvent was removed in vacuo, and the residue was

partitioned between CH_2Cl_2 and water. The organic layer was dried (Na_2SO_4), the solvent was removed in vacuo, and the residue was triturated with a small amount of cold CH_2Cl_2. The precipitate was filtered and washed with small portions of CH_2Cl_2 to afford 7 (0.98 g, 30%) as a light yellow solid.

Method C. Sodium hydride (0.29 g of 60% dispersion in mineral oil, 7.14 mmol) was added to a solution of 8 (2.00 g, 6.49 mmol) in DMF (10 mL), and the mixture was stirred at 80 °C for 5 min. A solution of tosylate tosylamide 15 (2.40 g, 6.49 mmol) in DMF (10 mL) was added during a 15-min period. After the mixture was stirred for 15 h, the solvent was removed in vacuo and the residue was partitioned between CH_2Cl_2 and water. The organic layer was dried (Na_2SO_4) and evaporated in vacuo. Addition of a small amount of cold CH_2Cl_2 to the residue gave a solid which was collected and washed several times with cold CH_2Cl_2 to give 7 (0.85 g, 26%).

Preparation of Cyclic Tritosylamide 9. A solution of 7 (7.00 g, 13.8 mmol) and ditosylate tosylamide 17 (7.57 g, 18.8 mmol) in DMF (20 mL) was added during 14 h to a suspension of anhydrous Cs_2CO_3 (9.00 g, 27.7 mmol) in DMF (30 mL), at 100 °C. Following completion of the addition, the mixture was stirred for 10 h, cooled, and filtered. The solvent was removed in vacuo, and the residue was partitioned between CH_2Cl_2 and water. The dichloromethane layer was concentrated in vacuo and chromatographed on alumina with CH_2Cl_2–EtOAc (49:1) as eluent to afford 9 (5.71 g, 57%) as a fluffy, white solid with mp 228–229 °C. IR (deposit on NaCl plate from $CDCl_3$ solution): 1520 (NO_2), 1344, 1167 (SO_2) cm^{-1}. ^1H NMR ($CDCl_3$): δ 2.41–2.47 (m, 9H), 2.55–4.50 (m, 12H), 6.99 (d, 1H), 7.20–7.85 (m, 12H), 8.22 (dd, 1H). Anal. Calcd for $C_{33}H_{36}N_4O_9S_3$: C, 54.39; H, 4.98. Found: C, 54.26; H, 5.08.

Preparation of Cyclic Triamine 10. Cyclic tritosylamide 9 (5.60 g, 7.68 mmol) was dissolved in concentrated H_2SO_4 (20 mL) and stirred at 100 °C for 12 h. The reaction mixture was cooled in an ice bath, and anhydrous Et_2O (200 mL) was slowly added resulting in a white precipitate. The solid was collected by vacuum filtration and dissolved in a minimum amount of water. Potassium hydroxide was added until pH >10 was obtained. The yellow solid precipitate was collected by filtration, and the aqueous layer was extracted with CH_2Cl_2. The organic extracts and the solid were combined, dried over Na_2SO_4, and passed through a short column of alumina with CH_2Cl_2–MeOH (19:1) as eluent to produce 10 (1.91 g, 94%) as a dark-red viscous oil: IR (deposit on NaCl plate from $CDCl_3$ solution): 3344 (NH), 1518 (NO_2) cm^{-1}. ^1H NMR ($CDCl_3$): δ 1.91 (br s, 2H), 2.50 (t, 2H), 2.60–2.80 (m, 4H), 2.89 (t, 2H), 3.21 (t, 2H), 4.25 (t, 1H), 5.89 (br s, 1H), 6.98 (d, 1H), 7.50–7.65 (m, 2H). Anal. Calcd for $C_{12}H_{18}N_4O_3$: C, 54.12; H, 6.81. Found: C, 53.80; H, 6.51.

Preparation of Nitro Triester 11. Cyclic crown triamine 10 (1.91 g, 7.17 mmol), ethyl bromoacetate (7.19 g, 43.0 mmol), and anhydrous K_2CO_3 (5.95 g, 43.0 mmol) were stirred in dry DMF (30 mL) at 90 °C for 48 h. After being cooled to room temperature, the mixture was filtered and the solvent was removed in vacuo. The residue was partitioned between CH_2Cl_2 and water. The organic layer was dried ($MgSO_4$) and evaporated in vacuo. The residue was chromatographed on alumina with CH_2Cl_2–EtOAc (19:1) as eluent to give 11 (3.31 g, 88%) as a viscous, orange oil. IR (deposit on NaCl plate from $CDCl_3$ solution): 1741 (C=O), 1516 (NO_2) cm^{-1}. ^1H NMR ($CDCl_3$): δ 1.22–1.33 (m, 9H), 2.75 (t, 2H), 2.92 (t, 2H), 3.00 (t, 2H), 3.18 (t, 2H), 3.36 (s, 2H), 3.43 (s, 2H), 3.55 (t, 2H), 4.00 (s, 2H), 4.10–4.27 (m, 8H), 6.83 (d, 1H), 7.66 (dd, 1H), 7.79 (dd, 1H). Anal. Calcd for $C_{24}H_{36}N_4O_9$: C, 54.95; H, 6.92. Found: C, 54.83; H, 6.93.

Preparation of Amino Triester 12. Triester 11 (0.78 g, 1.48 mmol) was dissolved in 95% EtOH (60 mL), and 10% Pd/C (0.12 g) and *p*-toluenesulfonic acid (0.10 g) were added. The mixture was shaken at room temperature under hydrogen at 50 psi for 12 h and filtered, and the filtrate was evaporated in vacuo. The residue was chromatographed on alumina with CH_2Cl_2–MeOH (19:1) as eluent to give slightly impure 12 (0.67 g) which was used immediately in the next step. IR (neat): 3439, 3366 (NH), 1734 (C=O) cm^{-1}. ^1H NMR ($CDCl_3$): δ 1.17–1.28 (m, 9H), 2.73 (t, 2H), 2.85–2.93 (m, 4H), 3.04 (t, 2H), 3.99–4.21 (m, 10H), 6.20 (dd, 1H), 6.27 (d, 1H), 6.63 (d, 1H).

Preparation of Chromogenic Triester 13. The slightly impure amino triester 12 (0.67 g, 1.37 mmol) was dissolved in dry MeOH (10 mL). Picryl chloride (0.43 g, 1.80 mmol) and anhydrous K_2CO_3 (0.24 g, 1.80 mmol) were added, and the mixture was stirred at room temperature for 4 h. The solvent was removed in vacuo at 30 °C, and the residue was chromatographed on deactivated silica gel with CH_2Cl_2–MeOH (10:1) as eluent to afford 13 (0.58 g, 60% overall from 11) as a dark-red semisolid. IR (deposit on NaCl plate from $CDCl_3$ solution): 1739 (C=O) cm^{-1}. ^1H NMR ($CDCl_3$): δ 1.20–1.31 (m, 9H), 2.77 (m, 2H), 2.89–3.00 (m, 4H), 3.12 (t, 2H), 3.38–3.51 (m, 6H), 3.98–4.22 (m, 10H), 6.54–6.59 (m, 2H), 6.74 (dd, 1H), 9.03 (s, 2H), 10.24 (s, 1H). Anal. Calcd for $C_{30}H_{39}N_7O_{13}\cdot 0.3\ CH_2Cl_2$: C, 49.77; H, 5.46. Found: C, 49.77; H, 5.40.

Preparation of Nitrated Chromogenic Triester 14. Triester 13 (0.22 g, 0.31 mmol) was dissolved in $CHCl_3$ and cooled to 0 °C with an ice bath. A solution of fresh fuming HNO_3 (0.5 mL), acetic acid (0.5 mL), and $CHCl_3$ (0.5 mL) was added dropwise, and the reaction mixture was stirred for 10 min during which time a dark-red precipitate formed. The solvent layer was decanted, and the residue was dissolved in a minimum amount of MeOH. The solution was passed through a short column of alumina with MeOH as eluent. Evaporation of the solvent followed by chromatography of the residue on silica gel with CH_2Cl_2–MeOH (19:1) as eluent gave 14 (0.22 g, 94%) as a dark-red, glassy solid. IR (deposit on NaCl plate from $CDCl_3$ solution): 1739 (C=O), 1521 (NO_2) cm^{-1}. ^1H NMR ($CDCl_3$): δ 1.13 (t, 3H), 1.27 (t, 6H), 2.80 (t, 2H), 2.89 (t, 2H), 3.18 (br t, 4H), 3.39–3.40 (m, 4H), 3.66 (t, 2H), 3.87 (s, 2H), 4.03–4.22 (m, 8H), 5.94 (s, 1H), 7.61 (s, 1H), 9.08 (s, 2H), 11.54 (br s, 1H). Anal. Calcd for $C_{30}H_{38}N_8O_{15}$: C, 48.00; H, 5.10. Found: C, 48.02; H, 4.94.

Preparation of Nitrated Chromogenic Tricarboxylic Acid 4. Nitro crown triester 14 (0.50 g, 0.66 mmol) was dissolved in 3.0 mL of dioxane–water (5:1). Lithium hydroxide monohydrate (0.28 g, 6.60 mmol) was added, and the solution was stirred for 4 h at room temperature. The solution was acidified with 5 drops of 6 N HCl, and the solvent was removed in vacuo. Benzene was added, and water was removed by azeotropic distillation. Ethanol (10 mL) was added to the residue, and the solution was filtered. Anhydrous Et_2O was added to the filtrate resulting in the precipitation of a solid which was collected by filtration and dried under vacuum to afford the dihydrochloride salt of 4 (0.28 g, 58%) as a red-brown solid with mp >300 °C. ^1H NMR ($CDCl_3$): δ 3.13–4.24 (m, 20H), 6.26 (s, 1H), 7.66 (s, 1H), 9.04 (s, 2H), 10.82 (s, 1H). Anal. Calcd for $C_{24}H_{28}N_8O_{15}Cl_2$: C, 38.98; H, 3.82. Found: C, 38.70; H, 4.10.

UV–Vis Spectroscopic Properties of Chromogenic Compound 4 and Its Responses to Calcium and Magnesium. Chromogenic compound 4 was dissolved in 0.1 M TMA(OH) to make a stock solution of 1.0×10^{-2} M. For the nonionized form, a solution was made from 0.02 mL of the stock solution and 0.2 mL of 0.1 M HCl and scanned in a 1-cm path length cuvette from 700 to 300 nm with a Beckman DU-8 spectrophotometer. Molar absorptivities (ε) at wavelength maxima (λ_{max}) were calculated according to Beer's law. A similar procedure was applied for the ionized form where 0.1 M HCl was replaced by 0.1 M TMA(OH).

The reagents for obtaining calcium and magnesium responses consisted of 1.0×10^{-4} M 4 and an appropriate buffer (Table I). The final concentration of calcium and magnesium ions in each cuvette was 1.0×10^{-2} M.

The reagent for demonstrating linear response to calcium (Figure 2) was formulated as follows: 3.0×10^{-4} M 4 in 0.2 M HEPES buffer (pH 7.0).

Determination of Stability Constants. The stability constants were determined for complexation of calcium ions by compound 4 in buffered aqueous solutions at 25 ± 0.5 °C by the Frensdorff method[9] using a calcium ion-selective electrode prepared according to the literature method.[10]

(9) Frensdorff, H. K. *J. Am. Chem. Soc.* **1971**, *93*, 600.
(10) Moody, G. J.; Nassory, N. S.; Thomas, J. D. R. *Analyst* **1978**, *103*, 68.