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# New Chelating Agents for Colorimetric Determination of Calcium

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**Abstract.** Two new calcium chromoionophores **2** and **3** are described and their potential application to colorimetric determination of calcium is assessed. Chromogenic compound **2** bears two acetic acid chelating groups and a 4-(4′-nitrophenyl)azophenol chromophore. Its complexation of $Ca^{2+}$ at pH 9.0 is accompanied by a 109-nm bathochromic shift and increase in the absorptivity. Triaryl chromoionophore **3** bears two phosphonic acid chelating groups and the same azophenol chromophore. Compound **3** exhibits a 107-nm bathochromic shift of the absorption maximum, an increase in the absorptivity upon complexation with $Ca^{2+}$, and good selectivity for $Ca^{2+}$ over $Mg^{2+}$ at pH 7.0. Both chromoionophores exhibit linear responses from 0 to $4 \times 10^{-3}$ M $Ca^{2+}$.

**Key words:** Calcium complexation, chromogenic chelating agent, chromoionophore.

## 1. Introduction

The *o*-cresolphthaleine complexone method (CPC) [1] is probably the most commonly used spectrophotometric method for determining $Ca^{2+}$ in blood serum. Despite its widespread use, the method suffers from various deficiencies [2] and, therefore, development of an alternative calcium reagent with superior characteristics is highly desirable. Various chromogenic calcium chelating agents based on crown ether phenols [3], cryptands [4] and calixarenes [5], for use in extraction photometry and optical fiber sensors, have been reported. Recently, we have described chromogenic calcium chelating agents derived from aza-crown ethers [6]. We have also reported an acyclic chromogenic tetracarboxylic acid **1** [7]. This new chelating agent provided the basis for a method which has several advantages over the CPC method. In this paper we report two new acyclic chromoionophores **2** and **3** and their complexing abilities toward $Ca^{2+}$. We decided to synthesize compound **2** in order to probe whether a 'half-cut' version of the previously described chelating agent **1** could be utilized in the determination of calcium. Compound **3** was designed in hopes of achieving better selectivity for $Ca^{2+}$ over $Mg^{2+}$ at physiological pH.

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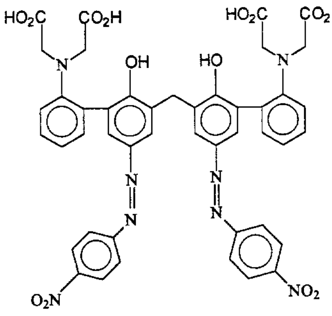
## 2. Experimental

Melting points are uncorrected. $^1$H-NMR spectra were recorded on a Varian Gemini spectrometer at 200 MHz. The UV-spectra were recorded on a Cary-3 spectrophotometer. Acetone was kept over anhydrous $K_2CO_3$ and distilled before use. Tetrahydrofuran was distilled from sodium benzophenone ketyl. 2-Methoxy-3-(methoxymethyl)-5-methylboronic acid and 2-(benzyloxy)-1,3-dibromobenzene were prepared according to literature procedures [8].



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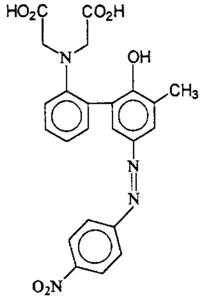

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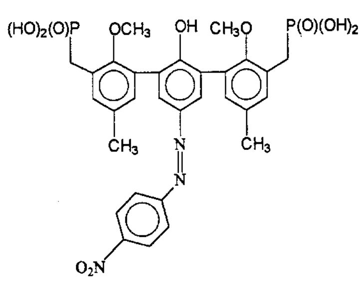

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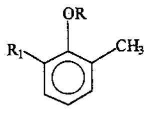
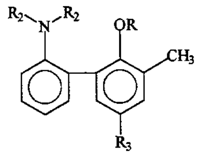
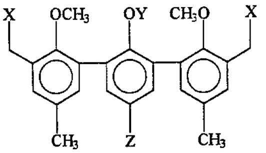


| | R | R1 |
|---|---|---|
| 4 | Bzl | Br |
| 5 | Bzl | B(OH)2 |



| | R | R2 | R3 |
|---|---|---|---|
| 6 | Bzl | H | H |
| 7 | Bzl | $CH_2CO_2Et$ | H |
| 8 | H | $CH_2CO_2Et$ | H |
| 9 | H | $CH_2CO_2Et$ | $N{=}N{-}C_6H_4{-}NO_2$ |



| | X | Y | Z |
|---|---|---|---|
| 10 | OMe | Bzl | H |
| 11 | Br | H | H |
| 12 | P(O)(OEt)2 | H | H |
| 13 | P(O)(OEt)2 | H | $N{=}N{-}C_6H_4{-}NO_2$ |

### 2.1. PREPARATION OF BENZYL ETHER 4

Benzyl bromide (32.4 g, 113.3 mmol) was added to a solution of 2-bromo-5-methylphenol (21.2 g, 113.3 mmol) in acetone (570 mL) containing anhydrous $K_2CO_3$ (28.5 g, 206.3 mmol) and the mixture was refluxed for 12 h. The solvent and excess benzyl bromide were removed in vacuo and the residue was chromatographed on silica gel with petroleum ether–EtOAc (50 : 1) to produce benzyl ether **4** (29.4 g, 94%) as a colorless liquid. $^1$H-NMR ($CDCl_3$): $\delta$ = 2.29 (s, 3H), 4.93 (s, 2H), 6.90 (t, 1H), 7.12 (d, 1H), 7.29–7.47 (m, 4H), 7.54 (d, 2H). *Anal. Calcd. for* $C_{14}H_{13}BrO$: C, 60.67; H, 4.73. *Found*: C, 60.81; H, 4.78.

### 2.2. PREPARATION OF BORONIC ACID 5

2.4 M *n*-Butyllithium (23.9 mL, 57.4 mmol) was added at −78 °C to a solution of benzyl ether **4** (15.0 g, 54.1 mmol) in THF (200 mL). After 10 min the solution was cannulated into a solution of trimethylborate (49.0 g, 472 mmol) in THF (130 mL), also cooled to −78 °C. The mixture was stirred at −78 °C for 30 min and allowed to warm up to 0 °C over 45 min. 2N HCl (400 mL) was added and the mixture was stirred at room temperature for 1 h. Ethyl ether (500 mL) was added and the mixture was stirred for 5 h. The organic layer was separated and extracted with 3 N NaOH. During extraction a white solid precipitated and was filtered. The solid was combined with the alkaline aqueous extract and carefully acidified with cold concentrated HCl. The aqueous layer was extracted with ethyl ether (4 × 50 mL) and the solvent from the combined extracts was removed in vacuo to afford boronic acid **5** (8.77 g, 66%) as a wet, white solid. The crude material was used in the next step without additional purification.

### 2.3. PREPARATION OF AMINE **6**

2M aqueous $Na_2CO_3$ (91 mL) and tetrakis(triphenylphosphine)palladium (1.83 g, 1.58 mmol) were added to a solution of 2-iodoaniline (6.60 g, 30.1 mmol) and boronic acid **5** in a mixture of toluene (182 mL) and ethanol (46 mL). The mixture was vigorously stirred and heated at 85 °C under argon for 24 h. The organic layer was separated, and the aqueous layer was extracted with $CH_2Cl_2$. The combined organic extracts were dried ($CaCO_3$), the solvent was removed in vacuo, and the residue was chromatographed on silica gel with petroleum ether–EtOAc (4 : 1) as eluent to give amine **6** (8.72 g, 100%) as a yellow viscous oil. $^1$H-NMR($CDCl_3$): $\delta = 2.32$ (s, 3H), 3.82 (br s, 2H), 4.49 (s, 2H), 6.81 (q, 2H), 6.99–7.30 (m, 10H). *Anal. Calcd.* for $C_{20}H_{19}NO$: C, 83.01; H, 6.62. *Found*: C, 82.94; H, 6.46.

### 2.4. PREPARATION OF DIETHYL ESTER **7**

A solution of amine **6** (4.10 g, 14.2 mmol), ethyl bromoacetate (11.9 g, 71.0 mmol), and lutidine (7.6 g, 71.0 mmol) in dry $CH_3CN$ (150 mL) was refluxed for 117 h. Additional amounts of lutidine (7.36g, 68.7 mmol) and ethyl bromoacetate (12.05 g, 72.1 mmol) were added after 45 h and 69 h. The solvent was removed in vacuo and the residue was partitioned between $CH_2Cl_2$ and water. The organic layer was dried ($MgSO_4$), the solvent was removed in vacuo, and the residue was chromatographed on silica gel with petroleum ether–EtOAc (5 : 1) as eluent to afford diethyl ester **7** (5.15 g, 79%) as a colorless oil. $^1$H-NMR($CDCl_3$): $\delta=1.11$ (t, 6H), 2.31 (s, 3H), 3.84 (br s, 4H), 3.99 (q, 4H), 4.43 (s, 2H), 6.96–7.48 (m, 12H). *Anal. Calcd.* for $C_{28}H_{31}NO_5$: C, 72.86; H, 6.77. *Found*: C, 72.71; H, 6.75.

### 2.5. PREPARATION OF DIETHYL ESTER PHENOL **8**

A mixture of diethyl ester **7** (5.05 g, 10.9 mmol), 10% Pd/C (1.0 g), acetic acid (2 mL), and ethanol (100 mL) was shaken under 50 psi of hydrogen over 24 h. The catalyst was filtered and the solvent was removed in vacuo to produce diethyl ester phenol **8** (4.02 g, 99%) as a light-brown, viscous oil which crystallized during storage. Column chromatography on silica gel with petroleum ether–EtOAc (5 : 1) as eluent provided an analytical sample of crystals with mp 87–89 °C. $^1$H-NMR($CDCl_3$): $\delta$=1.14 (t, 6H), 2.30 (s, 3H), 3.85 (br s, 4H), 4.06 (q, 4H), 6.87 (t, 1H), 6.99–7.16 (m, 4H), 7.25 (t, 2H), 7.95 (s, 1H). *Anal. Calcd.* for $C_{21}H_{25}NO_5$: C, 67.91; H, 6.78. *Found*: C, 68.21; H, 6.82.

## 2.6. PREPARATION OF CHROMOGENIC DICARBOXYLIC ACID 2

A cold suspension of 4-nitrobenzenediazonium tetrafluoroborate (3.70 g, 15.6 mmol) in a 1 : 1 mixture of THF-water (60 mL) was added dropwise to a solution of diethyl ester phenol **8** (1.83 g, 4.93 mmol) and $NaHCO_3$ (4.8 g) in a 1 : 1 mixture of THF–water. The mixture was stirred overnight at room temperature. The solvent was removed in vacuo and the residue was partitioned between $CH_2Cl_2$ and water. The organic layer was evaporated to dryness and the residue was chromatographed on silica gel with $CH_2Cl_2$–MeOH (99 : 1) to give slightly impure diester **9** (1.22 g, 47%) which was used in the next step without additional purification.

To a solution of **9** (0.29 g, 0.56 mmol) in a 5 : 1 mixture of dioxane–water was added LiOH (0.28 g) and the mixture was stirred overnight at room temperature. The solvent was removed in vacuo and the residue was partitioned between ethyl ether and water (pH 5). The organic layer was separated and the solvent was evaporated to give dicarboxylic acid **2** (0.23 g, 88%) as a brown foam. An analytical sample obtained by additional purification on a silica gel column with $CH_2Cl_2$–MeOH–AcOH (85 : 14 : 1) as eluent was a red glass. $^1$H-NMR ($CD_3OD$): $\delta$ = 2.36 (s, 3H), 3.96 (br s, 4H), 7.11–7.33 (m, 4H), 7.81 (s, 1H), 7.84 (s, 1H), 8.20 (ABq, 4H). *Anal. Calcd.* for $C_{23}H_{20}N_4O_7 \cdot 1.5H_2O$: C, 56.21; H, 4.72. *Found*: C, 56.00; H, 4.44.

## 2.7. PREPARATION OF TERPHENYL PENTAETHER 10

A mixture of 2-methoxy-3-(methoxymethyl)-5-methylphenylboronic acid [8] (9.97 g, 47.5 mmol), 2-(benzyloxy)-1,3-dibromobenzene [8] (6.76 g, 19.8 mmol), tetrakis (triphenylphosphine)palladium (1.20 g, 1.04 mmol), 2M aqueous $Na_2CO_3$ (60 mL), and toluene (120 mL) was refluxed under argon over 24 h. An additional amount of the catalyst (0.12 g) was added and reflux was continued for another 24 h. The mixture was cooled and diluted with toluene (50 mL) and 10% aqueous NaCl (150 mL). The organic layer was separated, dried ($MgSO_4$), and concentrated to 10 mL. Chromatography on a silica gel column with petroleum ether–EtOAc (95 : 5) as eluent gave **10** (8.5 g, 57%) as a viscous, pale-yellow oil. $^1$H-NMR($CDCl_3$): $\delta$=2.29 (s, $ArCH_3$, 6H), 3.43 (s, $CH_3O$, 6H), 3.45 (s, $CH_3O$, 6H), 4.32 (s, $ArCH_2OAr$, 2H), 4.52 (s, $ArCH_2OCH_3$, 4H), 6.60 (d, ArH, 2H), 6.95–7.40 (m, ArH, 10H). *Anal. Calcd.* for $C_{33}H_{36}O_5$: C, 77.32; H, 7.08. *Found*: C, 77.01; H, 6.99.

## 2.8. PREPARATION OF DIBROMIDE PHENOL **11**

HBr gas was bubbled for 10 min through a solution of **10** (7.50 g, 14.6 mmol) in chloroform (300 mL) cooled to 0 °C. The reaction mixture was washed with saturated aqueous $NaHCO_3$, dried ($Na_2SO_4$), and the solvent was removed in vacuo to give a syrup from which, after treatment with EtOAc and petroleum ether, pale-yellow crystals of dibromide phenol **11** were obtained (6.0 g, 79%); mp 121–122 °C. $^1$H-NMR($CDCl_3$): $\delta = 2.33$ (s, $ArCH_3$, 6H), 3.57 (s, $CH_3O$, 6H), 4.59 (s, $CH_2Br$, 4H), 7.00–7.39 (m, ArH+OH, 9H). *Anal. Calcd.* for $C_{24}H_{24}Br_2O_3$: C, 55.41; H, 4.65. *Found*: C, 55.33; H, 4.66.

## 2.9. PREPARATION OF TETRAETHYLDIPHOSPHONATE PHENOL **12**

A solution of dibromide phenol **11** (5.00 g, 9.61 mmol) in triethyl phosphite (50 mL) was heated for 1 h at 150 °C. The excess $(EtO)_3P$ was removed in vacuo at 50 °C (bath temperature) and the residue was chromatographed on silica gel with EtOAc and EtOAc-MeOH (8 : 2) as eluent to afford tetraethyldiphosphonate ester **12** (6.1 g, 100%) as a colorless, viscous oil. $^1$H-NMR($CDCl_3$): $\delta = 1.25$ (t, $CH_3CH_2O$, 12H), 2.30 (s, $ArCH_3$, 6H), 3.23 (d, $CH_2P$, 4H), 3.46 (s, $CH_3O$, 6H), 4.04 (pentet, $CH_2OP$, 8H), 6.98–7.08 (m, ArH+OH, 4H), 7.17–7.33 (m, ArH, 4H). *Anal. Calcd. for* $C_{32}H_{44}O_9P_2 \cdot 0.5H_2O$: C, 59.71; H, 7.01. *Found*: C, 59.37; H, 7.05.

## 2.10. PREPARATION OF CHROMOGENIC TETRAETHYLDIPHOSPHONATE **13**

A cold suspension of 4-nitrobenzenediazonium tetrafluoroborate (1.38 g, 5.82 mmol) in 22 mL of THF–$H_2O$ (1 : 1) was added dropwise at 0 °C to a solution of tetraethyldiphosphonate phenol **12** (1.13 g, 1.78 mmol) in 22 mL of THF–$H_2O$ (1 : 1) containing $NaHCO_3$ (1.80 g, 21.4 mmol). The mixture, which immediately turned violet, was stirred at room temperature for 2 h, the solvent was removed in vacuo and the residue was partitioned between $CH_2Cl_2$ and water. The organic phase was separated, the solvent was removed in vacuo and the residue was chromatographed on silica gel with $CH_2Cl_2$-MeOH (90 : 10) as eluent to produce **13** (0.70 g, 50%) as a red foam. $^1$H-NMR($CDCl_3$): $\delta = 1.26$ (t, $CH_3CH_2O$, 12H), 2.34 (s, $ArCH_3$, 6H), 3.25 (d, $CH_2OP$, 4H), 3.54 (s, $CH_3O$, 6H), 4.06 (pentet, $CH_2OP$, 8H), 7.14 (s, ArH,2H), 7.27 (s, ArH, 2H), 7.72 (br s, OH, 1H), 7.95 (s, ArH, 2H), 8.17 (ABq, ArH, 4H). *Anal. Calcd.* for $C_{38}H_{47}N_3O_{11}P_2$: C, 58.24; H, 6.04. *Found*: C, 58.04; H, 6.16.

## 2.11. PREPARATION OF CHROMOGENIC DIPHOSPHONIC ACID **3**

A solution of tetraethyldiphosphonate **13** (0.69 g, 0.88 mmol) in $CH_2Cl_2$ (15 mL) was cooled to −70 °C and trimethylsilyl bromide (1.4 mL, 10.8 mmol) was added in one portion and the mixture was allowed to warm-up to room temperature and was stirred for 36 h. The mixture was cooled to 0 °C and made basic with 5% aqueous LiOH. The solvent was removed in vacuo, the residue was dissolved in a small amount of water and passed through a Dowex 50X8 cation exchange resin to give diphosphonic acid **3** (0.50 g, 85%) as a red amorphous solid. $^1$H-NMR($CD_3OD$): $\delta$=2.33 (s, $ArCH_3$, 6H), 3.23 (d, $ArCH_2P$, 4H), 3.50 (s, $CH_3O$, 6H), 7.09 (s, ArH, 2H), 7.29 (s, ArH, 2H), 7.92 (s, ArH, 2H), 8.17 (ABq, ArH, 4H). *Anal. Calcd.* for $C_{30}H_{31}N_3O_{11}P_2 \cdot 2H_2O$: C, 50.93; H, 4.99. *Found*: C, 50.79; H, 4.63.

### 2.12. UV-VIS SPECTROSCOPIC PROPERTIES OF CHROMOGENIC COMPOUNDS 2 AND 3 AND THEIR RESPONSES TO CALCIUM AND MAGNESIUM

Chromogenic compounds **2** and **3** were dissolved in 1.0 M TMA(OH) to make a stock solution of $1.0 \times 10^{-2}$ M. For the nonionized form, a solution was made from 0.010 mL of the stock solution and 2.0 mL of 1 M HCl and scanned in a 1-cm cuvette from 700 to 300 nm with a Cary 3 spectrophotometer. Molar absorptivities ($\varepsilon$) at wavelength maxima ($\lambda_{max}$) were calculated according to Beer's law. A similar procedure was used for the ionized form where 1.0 M TMA(OH) was substituted for the 1.0 M HCl.

The reagent for obtaining calcium and magnesium responses consisted of $5.0 \times 10^{-5}$ M **2** or $1.0 \times 10^{-4}$ M **3**, and an appropriate buffer (Table I).

The reagent for demonstrating linear response of **2** to calcium (Figure 1) was formulated as follows: $2.0 \times 10^{-4}$M **2** in 0.1 M CHES buffer (pH 9.0) and 1.0% (w/v) Brij-35. To 1.0 mL of this solution 0.03 mL of aqueous calcium chloride solutions (0–4 mM) were added, and the change in absorbance at 550 nm was measured in a 1-cm cuvette. For compound **3** the linearity reagent consisted of 4.0 $\times\ 10^{-4}$ M **3** in 0.2 M imidazole hydrochloride buffer (pH 7.0) and 1.0% (w/v) Brij-35. To 2.0 mL of the reagent were added 0.04 mL aqueous solutions of calcium chloride, and the change in absorbance was measured at 505 nm.

## 3. Results and Discussion

### 3.1. SYNTHESES

In the synthesis of chromogenic compound **2**, commercially-available 2-bromo-5-methylphenol was benzylated with benzyl bromide–$K_2CO_3$ in acetone to give benzyl ether **4** (94%) which was metalated with BuLi in THF at −78 °C and the organometallic compound was treated with trimethylborate. Acid hydrolysis of the arylboronate ester provided arylboronic acid **5** which was treated with 2-iodoaniline in the Suzuki coupling [9] to produce amine **6** (100%). Reaction of **6** with excess ethyl bromoacetate and lutidine in $CH_3CN$ afforded diethyl ester **7** in 79% yield. The benzyl group of **7** was removed with $H_2$–10%Pd/C to give diethylester phenol **8** in an almost quantitative yield. Coupling of **8** with 4-nitro-benzenediazonium tetrafluoroborate provided crude chromogenic diethyl ester **9** which was subjected

TABLE I. Spectral characteristics and calcium and magnesium responses of chromogenic compounds **2** and **3** in the absence and presence of surfactant at optimum pH[a].

| Compound | pH | Form[b] | $\lambda_{max}$, nm | $\epsilon(\lambda_{max})$ |
|---|---|---|---|---|
| **2** | | HL | 381.0 | 14120 |
| | | $L^-$ | 536.0 | 28860 |
| | 9.0 | L | 390.0 | 17850 |
| | | CaL | 499.0 | 19630 |
| | | MgL | 468.0 | 15160 |
| | Brij-35 | L | 407.0 | 18050 |
| | (1.0% w/v) | CaL | 527.0 | 21350 |
| | | MgL | 498.0 | 15550 |
| **3** | | HL | 387.0 | 12200 |
| | | $L^-$ | 540.0 | 30000 |
| | 7.0 | L | 385.0 | 13640 |
| | | CaL | 465.0 | 10000 |
| | | MgL | 396.0 | 10500 |
| | Brij-35 | L | 398.0 | 18000 |
| | (1.0% w/v) | CaL | 505.0; 312.0 | 21000; 12890 |
| | | MgL | 406.0 | 13650 |

[a] Buffers: pH 7.0, 0.2 M imidazole acetate; pH 9.0, 0.1 M CHES ((cyclohexylamino)ethanesulfonic acid).

[b] $L^-$ is fully ionized ligand in 1.0 M TMA(OH); HL is nonionized ligand in 1.0 M HCl; CaL and MgL is the compound in the presence of a 100-fold excess of $Ca^{2+}$ and $Mg^{2+}$, respectively.

to basic hydrolysis with LiOH to give chromogenic diacid **2** in 88% yield, for the two-step process.

In the synthesis of diphosphonic acid **3**, the known 2-methoxy-3-(methoxymethyl)-5-methylphenylboronic acid [8] and 2-(benzyloxy)-1,3-dibromobenzene [8] were subjected to a 2-fold Suzuki aryl-aryl coupling reaction to give terphenyl pentaether **10** in 57% yield. Treatment of **10** with HBr in $CHCl_3$ caused bromination of the benzylic positions and cleavage of the benzyl group to provide dibromide phenol **11** in 79% yield. Heating **11** with excess triethyl phosphite at 150 °C gave tetraethyl ester phenol **12** (100%) which was coupled with 4-nitrobenzenediazonium tetrafluoroborate to afford chromogenic tetraethyl ester **13** in 50% yield. Treatment of **13** with trimethylsilyl bromide at −70 °C produced chromogenic diphosphonic acid **3** in 85% yield.

New compounds were characterized by $^1H$ NMR spectra and elemental analysis.

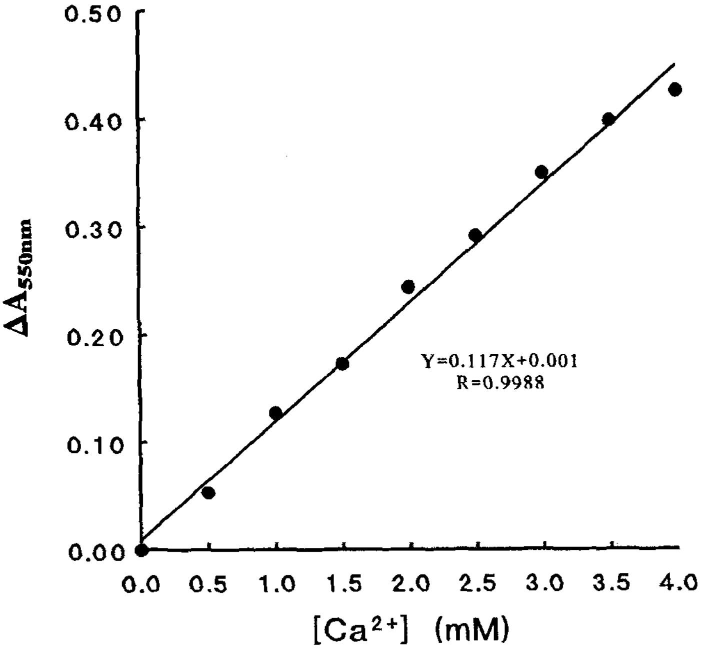


Fig. 1. Linear relationship between the absorbance of the calcium complex of **2** and the concentration of $Ca^{2+}$ at pH 9.0.

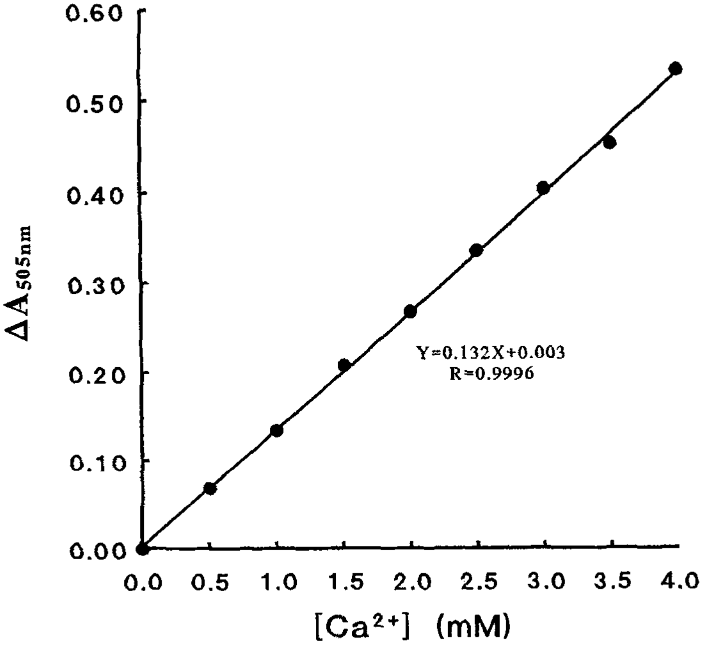


Fig. 2. Linear relationship between the absorbance of the calcium complex of **3** and the concentration of $Ca^{2+}$ at pH 7.0.

## 3.2. SPECTRAL CHARACTERISTICS AND CATION RESPONSES

Wavelength maxima ($\lambda_{max}$) and molar absorptivities ($\varepsilon$) of the acid (HL) and base ($L^-$) forms of chromogenic chelating agents **2** and **3** are recorded in Table I. To suppress ionization of the phenolic hydroxyl and thereby obtain the HL spectra, absorbances were determined in 1.0 M HCl. Likewise, 1.0 M TMA(OH) was used when obtaining the $L^-$ spectra to ensure ionization of the O—H bond. Compound **2** exhibited an absorption maximum for the acid form (HL) at 381 nm which upon ionization shifted to 536 nm with an approximately 2-fold increase in the molar absorptivity. At the optimum pH of 9.0, chromoionophore **2** exhibited its best selectivity for $Ca^{2+}$ over $Mg^{2+}$ (Table I). Complexation with $Ca^{2+}$ was accompanied by a large 109-nm bathochromic shift and a slight increase in absorptivity. Compound **2** also responded to magnesium but this interference could be completely eliminated by incorporating 8-hydroxyquinoline-5-sulfonic acid in the reagent. Addition of a nonionic surfactant, Brij-35, caused an even larger bathochromic shift to 527 nm and a further increase in absorptivity for the calcium complex (CaL), while the spectrum of the free form (L) of **2** was only slightly affected.

For triaryl chromogenic compound **3**, which incorporates two phosphonic acid groups, instead of carboxylic groups commonly used in calcium binders, there was a very large 153-nm wavelength maxima difference between the acid and base form. Compound **3** responded to $Ca^{2+}$ at pH 7.0. In the presence of Brij-35, complexation with calcium caused a 107-nm bathochromic shift and an increase in absorbance. At these conditions, magnesium response was marginal and could easily be eliminated.

As shown in Figures 1 and 2, the responses of **2** and **3** to $Ca^{2+}$ were linear over the entire clinical range from 0 to $4 \times 10^{-3}$ M $Ca^{2+}$. It is worth noting that pH optima for calcium response of both compounds differ by two pH units. Compound **2** has higher pH optimum than compound **3** due to possible contribution from hydrogen bonding between the phenolic group and the neighboring nitrogen atom.

In conclusion, despite its structural similarity to chromoionophore **1**, compound **2** exhibits only about half of the sensitivity found for **1** which can be related to the additive effect of chromogenic groups. This in turn limits its use to low sample dilutions at which interferences, e.g. from proteins, can become significant. Chromoionophore **3** is more promising because of the low pH required for the optimum calcium response, at which the azophenol chromophore is less susceptible to oxidation. However, similarly to compound **2**, dilution limitations apply due to inadequate sensitivity. Both new chromoionophores have potential for use in the colorimetric determination of calcium ions in physiological fluids.

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