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Synthesis and Evaluation of Fluorinated Calcium Chelators with Enhanced Relaxation Characteristics

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The fluorinated calcium ion chelator 5F BAPTA [1,2-bis(2-amino-5-fluorophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetra- acetic acid] was used for the determination of cytosolic Ca^{2+} ion levels in cells and tissues by ^{19}F NMR. The possibility of enhancing the sensitivity of these measurements by modifying the relaxation behavior of the chelator was evaluated. These strategies involved the design of a chelator with reduced T_1 value viz. 5F RBAPTA {1,2-bis[2-amino-4-(1-carboxy-2-methyl-2-propyl)-5-fluorophenoxy]ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid}, and the evaluation of a chelator with slower exchange characteristics, viz. 5F BenzEGTA [1-(2-amino-5-fluorophenoxy)-2-(2-aminoethoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid]. Both analogs exhibit chemical shift differences on calcium complexation which are similar to those of the parent compound, 5F BAPTA. In the case of BenzEGTA, a considerably greater pH dependence of the apparent calcium dissociation constant, K_D, also results from the presence of a tertiary amine with a p*K* value above the physiological range. Additionally, the synthetic approach used for RBAPTA can lead to a wide variety of derivatives with variable chemical, biochemical and NMR properties.

KEY WORDS ^{19}F NMR Fluorinated calcium chelators Enhanced relaxation characteristics

INTRODUCTION

Considerable progress toward understanding the role of cytosolic calcium ions in the regulation of cellular metabolism and the mediation of cell injury has been made as a consequence of the development of intracellular calcium indicators. Some of the more useful of these are modifications of the calcium selective chelator EGTA, which contain fluorescent[1,2] or NMR-sensitive[3,4] groups such as ^{19}F. The use of NMR-active fluorinated indicators offers several advantages, including the ability to carry out studies in cells with significant fluorescent backgrounds such as erthrocytes,[5–7] the ablity to study perfused organs[8–10] and greater selectivity of NMR observations against other divalent and potentially interfering ions such as zinc or lead.[11–13] However, the NMR technique is considerably less sensitive, with the consequent limitation on time and spatial resolution. One approach to increasing the sensitivity of fluorinated NMR indicators involves the synthesis of chelators containing a larger number of magnetically equivalent fluorine nuclei. Such approaches are limited, however, by significant synthetic difficulties reflecting the high electronegativity of fluorine, and by the difficulty of obtaining sufficient chemical shift sensitivity for the potentially useful trifluoromethyl reporter group.

An alternative approach to the sensitivity problem is suggested by an analysis of the relaxation behavior of these chelators. Relaxation data obtained *in vitro*[3–5] and to a more limited extent *in vivo*[5] indicate that for the fluorinated chelators studied to date, the ^{19}F $T_1 \gg T_2$. Such relaxation behavior arises as a consequence of the large chemical exchange contribution to the transverse relaxation rate. Consequently, it becomes feasible to consider structural modifications specifically designed to alter relaxation behavior. In general, modifications which lead to an increased affinity for the ion will decrease the dissociation rate constant and hence reduce the exchange broadening contribution in the slow exchange limit which characterizes several of the chelators.[4] Of course, such modifications will also alter the affinity of the chelator for the target ion. Alternatively, the chelator structure can be modified with the goal of reducing the spin–lattice relaxation time T_1. Successful realization of these strategies would result, in the first case, in sharper and more easily observed peaks, whereas in the second case, the shorter T_1 makes possible greater accumulation of signal in a given time period. Two chelators, 1-(2-amino-5-fluorophenoxy)-2-(2-aminoethoxy) ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (5F BenzEGTA) and 1,2-bis[2-amino-4-(1-carboxy-2-methyl-2-propyl)-5-fluorophenoxy]ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid (5F RBAPTA), have been designed and synthesized to evaluate the feasibility of these strategies.

EXPERIMENTAL

Synthesis

Creation of the backbone of the new chelator 5F RBAPTA, a 1,2-diphenoxyethane derivative, was

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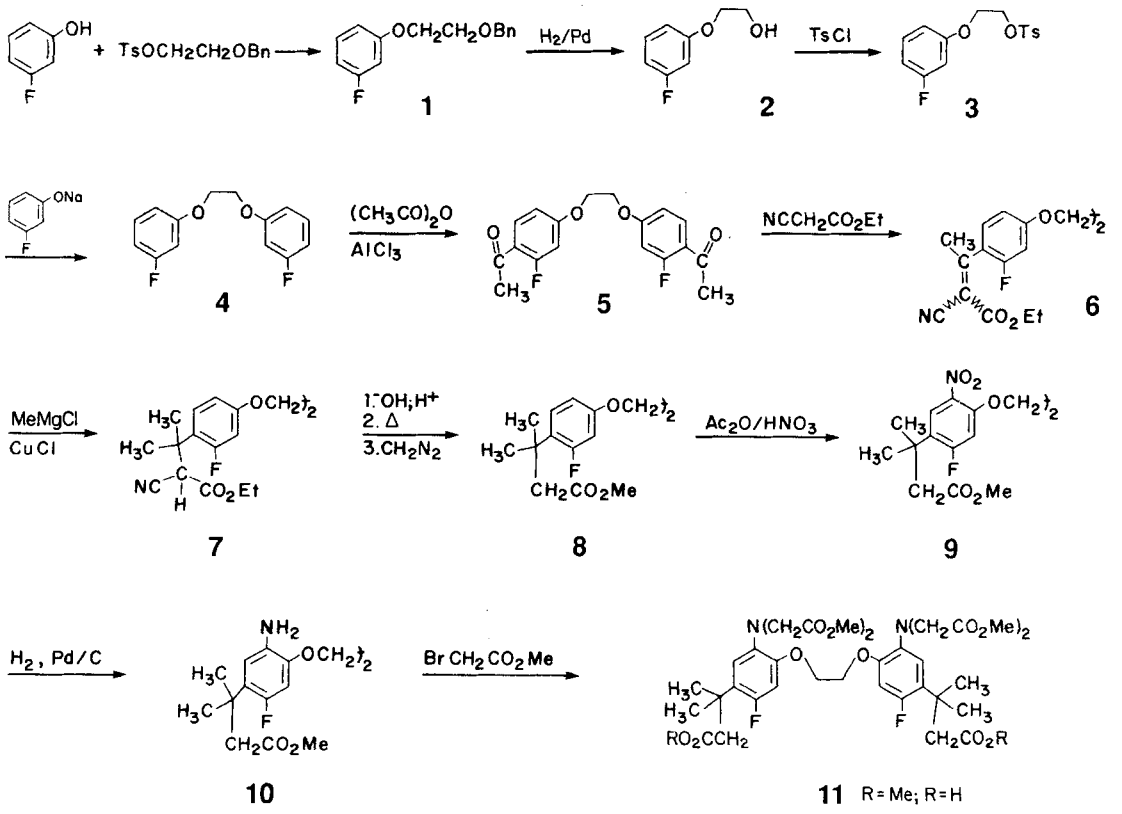

Figure 1. Synthetic scheme and structure of RBAPTA.

carried out as shown in Figure 1. Introduction of the two fluorophenoxy groups in one step, as had been effected in the original synthesis of BAPTA,[1] by reaction of a substituted *o*-nitrophenol with 1,2-dibromoethane, was not successful. Friedel–Crafts acylation of **4** following the reported procedure for the acylation of *m*-fluoroanisole[14] gave **5** accompanied by a small amount of monoacylated derivative. This impurity could be easily removed by simple extraction, as **5** is insoluble in most solvents. Development of the substituted *tert*-butyl group proceeded with the condensation of **5** and ethyl cyanoacetate to yield **6** as a mixture of geometric isomers. The presence of the cyano function was necessary to activate the conjugated system to copper-catalyzed methyl Grignard addition.[15] Although the isolated product **7** appeared to be homogeneous, it was probably a mixture of diastereoisomers. Hydrolysis of the two cyano functions and decarboxylation of the resulting bismalonic acid derivative to yield **8** removes this ambiguity. Nitration with acetyl nitrate occurs in the desired position to yield **9** and catalytic reduction of the nitro groups results in amine **10**. Alkylation with methyl bromoacetate as previously described yields the tetraacetic acid derivative **11**.[1]

Synthesis of the chelator of 5F BenzEGTA proceeded as shown in Fig. 2. Alkylation of 5-fluoro-2-nitrophenol was readily accomplished by tosylate displacement on the protected amine. Simultaneous deprotection and reduction of the aromatic nitro group was effected by catalytic hydrogenation and hydrogenolysis to yield intermediate **13**. Alkylation by the usual method yielded the ester derivative **14** of the chelator 5F BenzEGTA. Saponification of the esters **11** and **14** (hydrogenolysis of this benzyl ester was very slow) provided the chelators themselves.

1-(3-Fluorophenoxy)-2-benzyloxyethane (1). To a suspension of sodium hydride (55% dispersion in oil, 0.41 mol, which was rinsed twice with hexane) in 400 ml of dimethylformamide was added dropwise and with stirring a solution of *m*-fluorophenol (0.4 mol) in 100 ml of the same solvent. After hydrogen evolution had ceased, a solution of 1-benzyloxy-2-tosyloxyethane (0.4 mol) in 150 ml of dimethylformamide was added dropwise. The resulting mixture was heated to 65 °C and maintained at this temperature for 3 h, then cooled and stirred overnight at room temperature. The mixture was poured into water and extracted three times with diethyl ether. The ether solution was washed with water, dried and the solvent removed to yield 62.3 g (63%) of clear oil, homogeneous by thin-layer chromatography (TLC) (R_F = 0.6, 9 : 1 hexane–ethyl acetate).

NMR ($CDCl_3$): 3.82 (t, J = 5 Hz, 2H, $ArOCH_2CH_2OBn$), 4.13 (t, J = 5 Hz, 2H, $ArOCH_2CH_2OBn$), 4.63 (s, 2H, $PhCH_2O$), 6.7 (m, 3H, ArH), 7.2–7.4 (m, 6H, ArH).

2-(3-Fluorophenoxy)ethanol (2). A solution of 20 g (81 mmol) of **1** in 300 ml of ethanol was hydrogenolyzed using 4 g of 10% Pd–C at 55 °C and 3 atm H_2 in a Parr apparatus. The reaction, as followed by TLC, took 24 h to go to completion. The reaction mixture was then filtered through a Celite pad and the solvent removed to yield 11.8 g (93.7%) of homogeneous product (R_F = 0.3, 7 : 3 hexane–ethyl acetate).

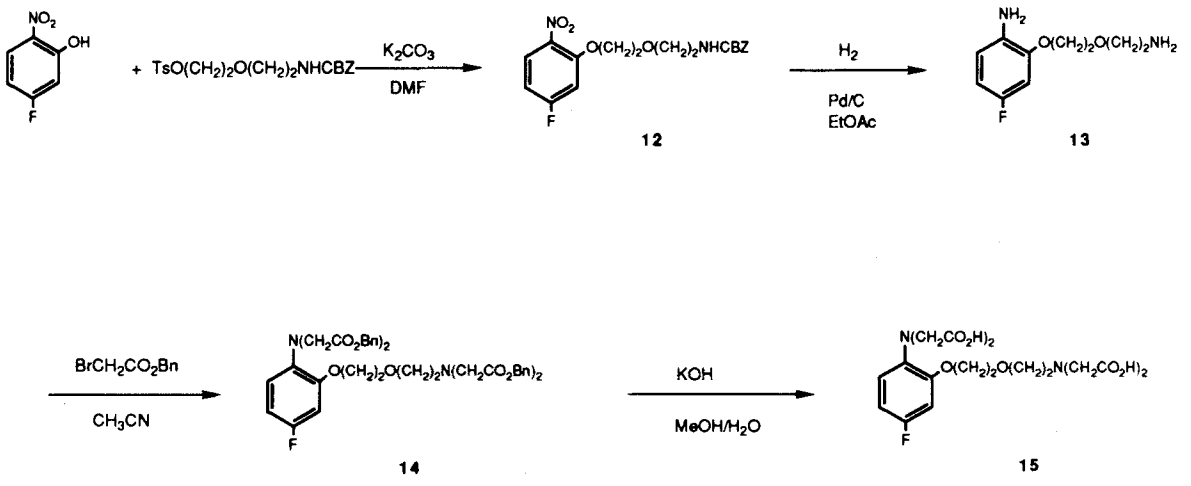

Figure 2. Synthetic scheme and structure of 5F BenzEGTA.

NMR ($CDCl_3$): 3.96 (t, $J = 4.2$ Hz, 2H, $ArCH_2CH_2OH$), 4.07 (t, $J = 4.2$ Hz, 2H, $ArCH_2CH_2OH$), 6.7 (m, 3H, ArH), 7.23 (ddd, $J = 8$, 8 and 8 Hz, 1H, ArH).

1-(3-Fluorophenoxy)-2-tosyloxyethane (3). To an ice-cooled and stirred solution of 37.6 g (0.24 mol) **2** in 320 ml of pyridine were added, portionwise, 47.5 g (0.25 mol) of tosyl chloride. The resulting solution was stirred at this temperature for an additional 2 h. The reaction mixture was poured into ice–water and the resultant crystalline product was filtered, washed with water and dried under high vacuum. The yield of white crystalline product ($R_F = 0.5$, 7:3 hexane–ethyl acetate) was 54.3 g (75%), m.p. 47–48 °C.

NMR ($CDCl_3$): 2.45 (s, 3H, $ArCH_3$), 4.13 (t, 2H, $ArOCH_2CH_2OTs$), 4.37 (t, 2H, $ArOCH_2CH_2OTs$), 6.46 (m, 1H, ArH), 6.57 (dd, $J = 2.1$ and 8.3 Hz, 1H, ArH), 6.66 (m, 1H, ArH), 7.19 (ddd, $J = 8$, 6 and 8 Hz, 1H, ArH), 7.3H d, $J = 8$ Hz, 1H, ArH), 7.81 (d $J = 8.2$ Hz, 2H, ArH).

1,2-Bis(3-fluorophenoxy)ethane (4). A stirred solution of 53.9 g (0.17 mol) of **3** and 38.7 g (0.17 mol) of sodium 3-fluorophenoxide in 300 ml of DMF was heated at 60 °C for 3 h. The cooled solution was poured into ice–water and the light-tan product filtered and dried to yield 42.5 g (98%) product. A sample recrystallized from ethanol had m.p. 73–74 °C.

NMR ($CDCl_3$): 4.30 (s, 4H, $ArOCH_2CH_2OAr$), 6.7 (m, 6H, ArH), 7.24 (ddd, $J = 8$, 8 and 8 Hz, 2H, ArH).

1,2-Bis(3-fluoro-4-acetylphenoxy)ethane (5). A 1 l flask equipped with a mechanical stirrer was charged with 20 g of **4** and 250 ml of carbon disulfide. Aluminum chloride (67 g) was added and then, dropwise, 20 g of acetic anhydride, which resulted in slight warming and a marked change in appearance of the reaction mixture. The mixture was brought to room temperature and then refluxed on a steam-bath until gas evolution ceased. The solvent layer was decanted from the cooled mixture and the residue poured on to a mixture of ice and HCl (75 ml). The precipitated product was filtered, dried and repeatedly extracted with acetone to remove any unreacted starting material. The yield of slightly off-white product was 14.1 g (53%), m.p. 177–179 °C.

NMR (CD_2Cl_2): 2.57 (d, $J = 5$ Hz, 6H, CH_3COAr), 4.39 (s, 4H, $ArOCH_2CH_2OAr$), 6.70 (dd, $J = 2.2$ and 13 Hz, 2H, ArH), 6.82 (dd, $J = 2.3$ and 8.8 Hz, 2H, ArH), 7.87 (dd, $J = 8.8$ and 8.8 Hz, 2H, ArH).

1,2-Bis[3-fluoro-4-(1-cyano-1-carboethoxyprop-2-en-2-yl)phenoxy]ethane (6). A mixture of 9.91 g of **5** (29.6 mmol), 9.7 g (86 mmol) of ethyl cyanoacetate, 6 g of acetic acid, 600 mg of ammonium acetate and 100 ml of benzene was refluxed for 2 days, water being removed by the use of a Dean–Stark trap. Additional ammonium acetate (1 g) was added at intervals for a total of 3 g. The cooled solution was washed with water, dried and the solvent removed to yield crude product, which was rapidly chromatographed to remove polar impurities. The mixture of product isomers, obtained as a clear, dark yellow oil in 78% yield, was not extensively resolved. The NMR spectrum of this mixture showed it to consist of geometrical isomers, presumably *EE*, *ZZ* and *EZ*, although the spectrum of the *EZ* isomer probably appears as a superposition of the *EE* and *ZZ* spectra.

NMR (CDl_3): 1.19 (t, $J = 7$ Hz, 6H, $CO_2CH_2CH_3$), 1.34 [t, $J = 7$ Hz, 6H, $CO_2CH_2CH_3$] 2.49 [s, 6H, $ArC(CH_3)=C(CN)(CO_2Et)$], 2.61 [s, 6H, $ArC(CH_3)=C(CN)(CO_2)Et)$], 4.08 (q, $J = 7$ Hz, 4, CO_2CH_2Me), 4.13 (q, $J = 7$ Hz, 4H, CO_2CH_2Me), 4.28 (s, 4H, OCH_2CH_2O) 4.30 (s, 4H, OCH_2CH_2O), 6.6–6.8 (m, 4H, ArH), 7.04 (dd, $J = 8.5$ and 8.5 Hz, 1H, ArH), 7.26 (dd, $J = 8$ and 8 Hz, 1H, ArH).

1,2-Bis[3-fluoro-4-(1-cyano-1-carboethoxy-2-methyl-2-propyl) phenoxy]ethane (7). In an ice-cooled flask equipped with a magnetic stirrer bar and an addition funnel and maintained under an argon atmosphere were placed 58 ml of 3.0 M methylmagnesium chloride in THF and 4.3 g of CuCl. The mixture was stirred for 10 min and then a solution of 11.5 g of **6** in 75 ml of diethyl ether was added dropwise over a period of 1.25 h. The solution was brought to room temperature and

stirring was continued overnight. The mixture was then poured on to a mixture of ice and HCl (20 ml) and extracted three times with diethyl ether. The extract was washed with water and $NaHCO_3$ solution, dried and the solvent removed to yield 11.9 g of crude product. Chromatographic purification of this material (7:3 hexane–ethyl acetate) yielded 5.21 g (43%) of pure product as a clear, colorless oil.

NMR ($CDCl_3$): 1.03 (t, J = 7 Hz, 6H, $CO_2CH_2CH_3$), 1.55 [d, J = 3.3 Hz, 12H, $ArC(CH_3)_2$], 3.99 (q, J = 7 Hz, 8H, CO_2CH_2Me), 4.20 [d, J = 3.3 Hz, 2H, $ArC(CH_3)_2CHCNCO_2Et$], 4.21 (s, 4H, OCH_2CH_2), 6.58–6.63 (m, 4H, ArH), 7.13 (dd, J = 9 and 9 Hz, 2H, ArG).

1,2-Bis[3-fluoro-4-(1-carbomethoxy-2-methyl-2-propyl)-phenoxy]ethane (8). A mixture of 4.9 g of 7, 2 g of KOH, 20 ml of ethylene glycol and 2 ml of water was refluxed for 36 h, at which time no ammonia could be detected either by odor or with moist pH paper. The solution was diluted with water, extracted with diethyl ether, acidified (HCl) and extracted three times with diethyl ether. After washing, drying and removal of the solvent from this extract the resulting product was heated at 180 °C (bath) for 6 h. The cooled residue was dissolved in diethyl ether and treated with diazomethane. The crude ester was chromatographed (7:3 hexane–ethyl acetate) to yield 975 mg (22%) of pure **8**, m.p. 53–54 °C.

NMR ($CDCl_3$): 1.45 [s, 12H, $ArC(CH_3)_2$], 2.76 (s, 4H, CH_2CO_2Me), 3.51 (s, 6H, CO_2CH_3), 4.27 (s, 4H, OCH_2CH_2O), 6.66 and 6.63 (two AB quartets as part of an ABX system, J = 2.5, 2.6 and 11 Hz, 4H, ArH), 7.18 (dd, J = 9 and 9Hz, 2H, ArH).

1,2-Bis[2-nitro-4-(1-carbomethoxy-2-methyl-2-propyl)-5-fluorophenoxy]ethane (9). A solution of acetyl nitrate was prepared by dropwise addition of 10 ml of 70% HNO_3 to 50 ml of ice-chilled acetic anhydride. This solution was added in one portion to a stirred solution, cooled to −25 °C, of 953 mg (1.99 mmol) of **8** in 25 ml of CH_2Cl_2. The reaction was maintained at −15 °C for 20 min and then poured into aqueous $NaHCO_3$. The mixture was extracted three times with diethyl ether, the ether layer was separated and dried and the solvent removed to yield 1.05 g of crude product as a yellow oil. Chromatography of this material (7:3 hexane–ethyl acetate) yielded 738 mg of product (65%) which, after crystallization from benzene–hexane had, m.p. 90–91 °C.

NMR ($CDCl_3$): 1.48 [s, 6H, $ArC(CH_3)_2$], 2.79 (s, 4H, CH_2CO_2Me), 3.54 (s, 6H, CO_2Me), 4.50 (s, 4H, OCH_2CH_2O), 6.90 (d, J = 13.3 Hz, 2H, ArH), 7.92 (d, J = 8.4 Hz, 2H, ArH).

1,2-[2-amino-4-(1-carbomethoxy-2-methyl-2-propyl)-5-fluorophenoxy]ethane (10). The nitro compound **9**, 681 mg (1.20 mmol) in 30 ml of ethyl acetate, was hydrogenated at atmospheric pressure using 100 mg of 10% Pd–C catalyst. Filtration of the catalyst and removal of the solvent yielded 541 mg (89%) if slightly off-white product, homogeneous on TLC (1:1 hexane–ethyl acetate), m.p. 149–159 °C.

NMR [$(CD_3)_2CO$]: 1.39 [s, 12H, $ArC(CH_3)_2$], 2.68 (s, 4H, CH_2CO_2Me), 3.47 (s, 6H, CO_2CH_2O), 4.28 (s, 4H, OCH_2CH_2O), 6.66 (d, J = 2.4 Hz, 2H, ArH), 6.69 (d, J = 7.9 Hz, 2H, ArH).

1,2-Bis[2-amino-4-(1-carbomethoxy-2-methyl-2-propyl)-5-fluoro phenoxy]ethane-*N,N,N′,N′*-tetraacetic acid, tetramethyl ester (11). Using a procedure described previously,[1] 520 mg of amine **10** were converted into the tetraalkylated ester in 83% yield, after chromatography (6:4 hexane–ethyl acetate). Originally obtained as an oil, the product slowly crystallized and had m.p. 67–68 °C.

NMR ($CDCl_3$): 1.39 [s, 12H, $ArC(CH_3)_2$], 2.70 (s, 4H, CH_2CO_2Me), 3.49 (s, 6H, CO_2Me), 3.56 (s, 12H, CO_2Me), 4.06 [s, 8H, $N(CH_2CO_2Me)_2$], 4.21 (s, 4H, OCH_2CH_2O), 6.54 (d, J = 3.5 Hz, 2H, ArH), 6.7 (d, J = 8.4 Hz, 2H, ArH).

1-(2-Nitro-5-fluorophenoxy)-2-(CBZ-2-aminoethoxy)ethane (12). A mixture of 3.99 g of 1-tosyloxy-2- (2-CBZ-aminoethoxy)ethane (CBZ = carbobenzoxy), 1.58 g of 5-fluoro-s-nitrophenol and 1.45 g of potassium carbonate in 22 ml of dry dimethylformamide was heated, with stirring and under argon, at 55 °C for 17 h. At this time TLC indicated considerable product. The cooled reaction mixture was poured into water and extracted with diethyl ether. The ether extract was washed with water, then brine and dried ($MgSO_4$). Removal of the ether yielded 3.05 g of crude product. Flash chromatography with 6:4 hexane–ethyl acetate yielded 1.03 g of pure product and 1.66 g of less pure product (contaminated with starting tosylate).

NMR ($CDCl_3$): 3.35 (dt, J = 5 and 5 Hz, 2H, OCH_2CH_2NHCBZ), 3.59 (t, J = 5 Hz, 2H, OCH_2X), 3.80 (t, J = 4.4 Hz, 2H, OCH_2CH_2Y), 4.15 (t, J = 4.5 Hz, 2H, OCH_2CH_2Z), 5.07 (s, 2H, CO_2CH_2Ph), 5.12 (s, 1H, NH), 6.65 (m, 1H, ArH), 6.75 (m, 1H, ArH), 7.3 (m, 5H, ArH), 7.86 (m, 1H, ArH).

1-(2-Amino-5-fluorophenoxy)-2-(2-aminoethoxy)ethane (13). A solution of 1.01 g of the corresponding nitro-CBZ-protected amine **12** in 45 ml of ethyl acetate was hydrogenolyzed and reduced at the same time using 110 mg of 10% Pd–C under 1 atm of H_2. The resulting solution was filtered through a short column of Celite and the solvent removed to yield 532 mg of slightly pink product that was homogeneous by TLC (R_F = 0.2, MeOH). It was used as such in the next step.

NMR ($CDCl_3$): 2.87 (t, J = 5 Hz, 2H, CH_2CH_2O), 3.55 (t, J = 5 Hz, 2H, OCH_2CH_2), 3.81 (t, J = 5 Hz, 2H, OCH_2CH_2N), 4.11 (m, 2H, $OCH_2CH_2NH_2$), 6.55 (m, 3H, ArH).

1-(2-Amino-5-fluorophenoxy)-2-(2-aminoethoxy)ethane-*N,N,N′,N′*-tetraacetic acid, tetrabenzyl ester (14). A solution of 527 mg of amine **13**, 2.64 g of benzyl bromoacetate and 2.56 g of proton sponge in 15 ml of dry acetonitrile was heated at gentle reflux for 12 h. Diethyl ether was added to the cooled reaction mixture and the precipitated salts were filtered. The resulting ether solution was washed with pH 2 buffer, water and brine and then dried ($MgSO_4$). The crude product was purified by flash chromatography (7:3 hexane–ethyl acetate) to yield 762 mg of pure product as a clear, very light yellow oil.

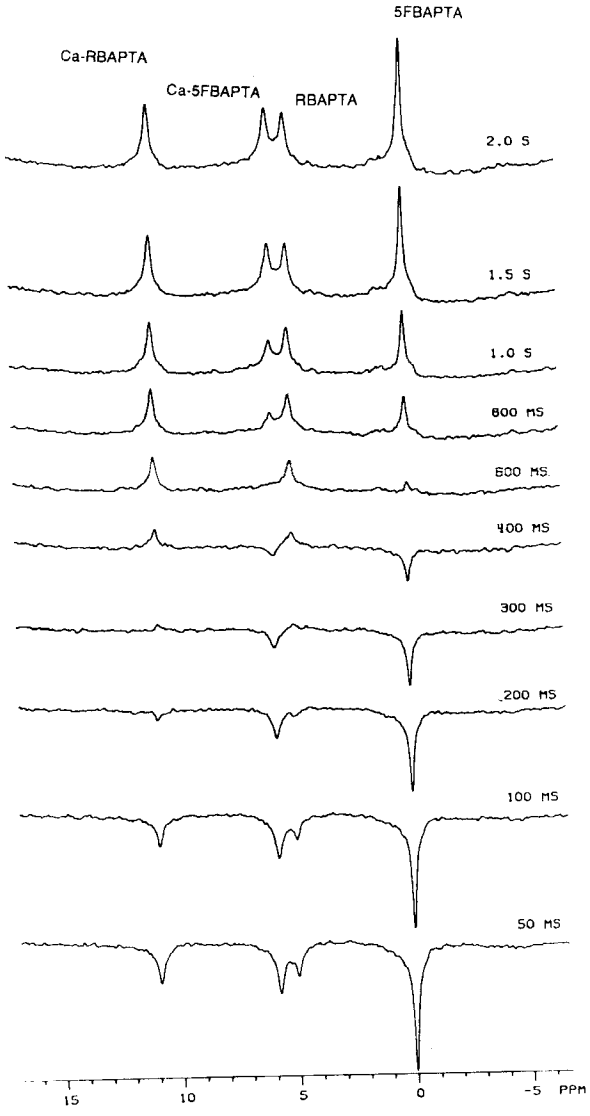

Figure 3. Fluorine-19 inversion–recovery study of the relaxation of 5F RBAPTA and 5F BAPTA. The solution contained 5 mM 5F RBAPTA, 4 mM 5F BAPTA and sufficient $CaCl_2$ to allow the observation of free and bound resonances for both chelators, in the standard buffer [120 mM KCl–20 mM NaCl–10mM Tris–HEPES (pH 7.3)]. Delays are indicated in milliseconds.

NMR ($CDCl_3$): 2.91 (t, $J = 5$ Hz, 2H, XCH_2CH_2Y), 3.54 (t, $J = 5$ Hz, 2H, XCH_2CH_2Y), 3.60 (t, $J = 5$ Hz, 2H, XCH_2CH_2Y), 3.61 [s, 4H, $N(CH_2CO_2Bn)_2$], 3.93 (t, $J = 5$ Hz, 2H, XCH_2CH_2Y), 4.12 (s, 4H, $NCH_2CO_2BN_2$), 5.07 (s, 4H, CO_2CH_2Ph), 5.08 s, 4H, CO_2CH_2Ph), 6.5 (m, 2H, ArH), 6.8 m, 1H, ArH), 7.2 m, 20H, ArH).

Hydrolysis of esters 11 and 14

A solution of 0.5 mmol of tetra- (**14**) or hexa- (**11**) ester in 10 ml of 0.8 M KOH in water–methanol (10:90) was kept at room temperature overnight. The solvents were removed and the product was used as such to determine K_D values.

Determination of K_D values

The calcium dissociation constants, K_D, for all chelators were determined in a solution made up to resemble the intracellular milieu. The solution contained 120 mM KCl, 20 mM NaCl and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) with the pH adjusted to 7.2 with Tris base. The K_D value of 5F RBAPTA was determined based on NMR studies of solutions containing both 5F BAPTA and 5F RBAPTA. At equilibrium, the free calcium level, Ca_i, will be given by

$$Ca_i = K_D^{5F\ BAPTA}\left(\frac{[Ca\text{–}5F\ BAPTA]}{[5F\ BAPTA]}\right) = K_D^{RBAPTA}\left(\frac{[Ca\text{–}5F\ RBAPTA]}{[5F\ RBAPTA]}\right) \quad (1)$$

where the concentrations of the free and bound species of both chelators can be determined directly from the ^{19}F NMR spectrum. The ratio of the dissociation constants is then given by

$$\frac{K_D^{RBAPTA}}{K_D^{5F\ BAPTA}} = \frac{[Ca\text{–}5F\ BAPTA]}{[5F\ BAPTA]} \times \frac{[5F\ RBAPTA]}{Ca\text{–}5F\ RBAPTA]} \quad (2)$$

The K_D value determined for 5F RBAPTA was based on a K_D value of 700 nM for 5F BAPTA.[3]

The K_D values for 5F BenzEGTA at different pH values were determined in solutions containing 5F BenzEGTA, the fluorescent indicator fura-2 and calcium–EGTA buffers.[16] Ionized calcium levels were determined based on fura-2 fluorescence, and K_D values were determined from the slope of a plot of [Ca–5F BenzEGTA]/[5F BenzEGTA] *vs.* ionized calcium.

NMR measurements

Fluorine-19 NMR measurements were carried out on a Nicolet NT-360 wide-bore multi-nuclear NMR spectrometer using a 5 mm proton probe retuned to the fluorine frequency of 339.7 MHz. Spin–lattice relaxation times were determined using an inversion–recovery sequence. Kinetic rate constants were carried out essentially as described by Perrin and Engler,[17] by carrying out two series of measurements in which the resonance of either the complexed or uncomplexed chelator was selectively inverted using the method of Robinson *et al.*[18] In general, only experimental data obtained using delay times $\tau \leqslant 3/k_{-1}$ provided sufficient accuracy to be useful for the determination of the rate constants. Data were fitted using a program written in Mathematica.

RESULTS AND DISCUSSION

The relaxation rates of ^{19}F nuclei in small molecules are generally dominated by dipolar and/or chemical shift anisotropy (CSA) mechanisms.[19] Since it is difficult to

Table 1. K_D values of calcium chelators determined in 0.115 M KCl–0.02 mM HEPES buffer

Chelator	pH	K_D (μM)
5F BAPTA	7.2	0.7
5F RBAPTA	7.2	0.38
5F BenzEGTA	6.9[a]	1.5
5F BenzEGTA	7.2[a]	1.2
5F BenzEGTA (pH 7.4)[b]	7.4[b]	0.82

[a] 37 °C.
[b] 22 °C.

alter predictably the strength of the latter mechanism, the most straightforward approach for lowering T_1 involves the construction of analogs with additional protons and/or shorter proton–fluorine internuclear distances. Using this approach, the analog '5F RBAPTA' (indicating 'relaxed BAPTA') shown in Fig. 1 was prepared. A ^{19}F NMR spectrum of a solution containing 5F RBAPTA, 5F BAPTA and sufficient Ca^{2+} to allow observation of bound and free resonances for both chelators is shown in Fig. 3. As is apparent from this spectrum, the exchange kinetics of calcium with both 5F BAPTA and 5F RBAPTA fall into the slow exchange limit, so that separate ^{19}F resonances are observed for the uncomplexed and calcium complexed indicators. The chemical shift difference between uncomplexed and calcium complexed ^{19}F resonances is very similar for the two chelators: 5.76 ppm for 5F BAPTA and 5.85 ppm for RBAPTA. Surprisingly, both ^{19}F resonances of the RBAPTA are shifted downfield relative to those of 5F BAPTA (Fig. 3). This is opposite to the upfield shift which the 4-methyl substituent produces in the calcium chelator 4-methyl-5-fluoro-BAPTA[4] and in the magnesium ion chelator 4-methyl-5-fluoro-APTRA[20] relative to the nonmethylated analogs. The basis for this difference is unknown at present, but probably involves the close steric interactions between the fluorine and the 3,3-dimethylpropionate substituent and/or an electrostatic interaction arising from the proximity of the fluorine to the carboxyl group.

As in our previous studies on 4-methyl-5-fluoro-BAPTA, introduction of the additional aklyl substituent at position 4 was anticipated to reduce the calcium dissociation constant K_D, reflecting primarily the increased basicity of the amino nitrogen.[1,4] That this is the case is readily apparent from inspection of the spectrum in Fig. 3, since the [Ca–5F RBAPTA]/[5F RBAPTA] intensity ratio is considerably greater than the observed [Ca–5F BAPTA]/[5F BAPTA] intensity ratio. A comparison of the two intensity ratios noted above yields a K_D ratio of 1.8 for the two indicators (Table 1). This results in a K_D value of 0.38 μM for RBAPTA, based on a 5F BAPTA K_D of 0.7 μM.

Time points in an inversion–recovery measurement of spin–lattice relaxation are shown in Fig. 3. It is clear from these data that as in previous studies, no significant difference is observable between the T_1 measurement of the uncomplexed and the calcium complexed indicator, and that 5F RBAPTA exhibits a significantly shorter T_1 value. T_1 values of 0.40 and 0.43 s for free and calcium-complexed RBATPA and of 0.78 and 0.75 s for free and calcium-complexed 5F BAPTA were obtained at 25 °C. As discussed, for example, by Ernst,[21] given a fixed total experiment time, the maximum signal-to-noise ratio in a pulsed NMR experiment is obtained for very rapid pulsing with delay times $T \ll T_1$. However, once the pulse repetition interval T becomes less than T_1, no significant improvement can be obtained by further shortening the interpulse delay. Hence, for a fixed experimental time, the optimum sensitivity is obtained by pulsing with a delay equal to T_1. As a consequence of the shorter RBAPTA T_1 value, a signal-to-noise ratio equivalent to that obtained using

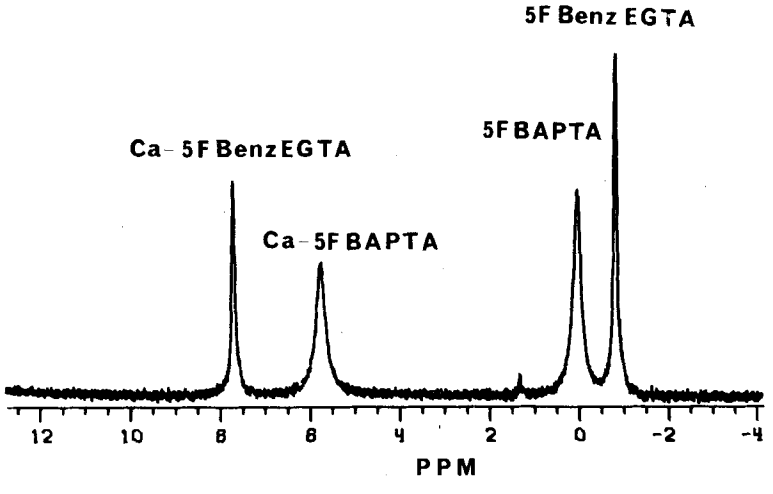

Figure 4. Fluorine-19 NMR spectrum of a solution containing 5 mM 5F BAPTA, 7.3 mM 5F BenzEGTA and sufficient $CaCl_2$ to allow the observation of free and bound resonances for both chelators, in a buffer containing 120 mM KCl, 20 mM NaCl and 10 mM Tris–HEPES (pH 7.4), measured at 22 °C

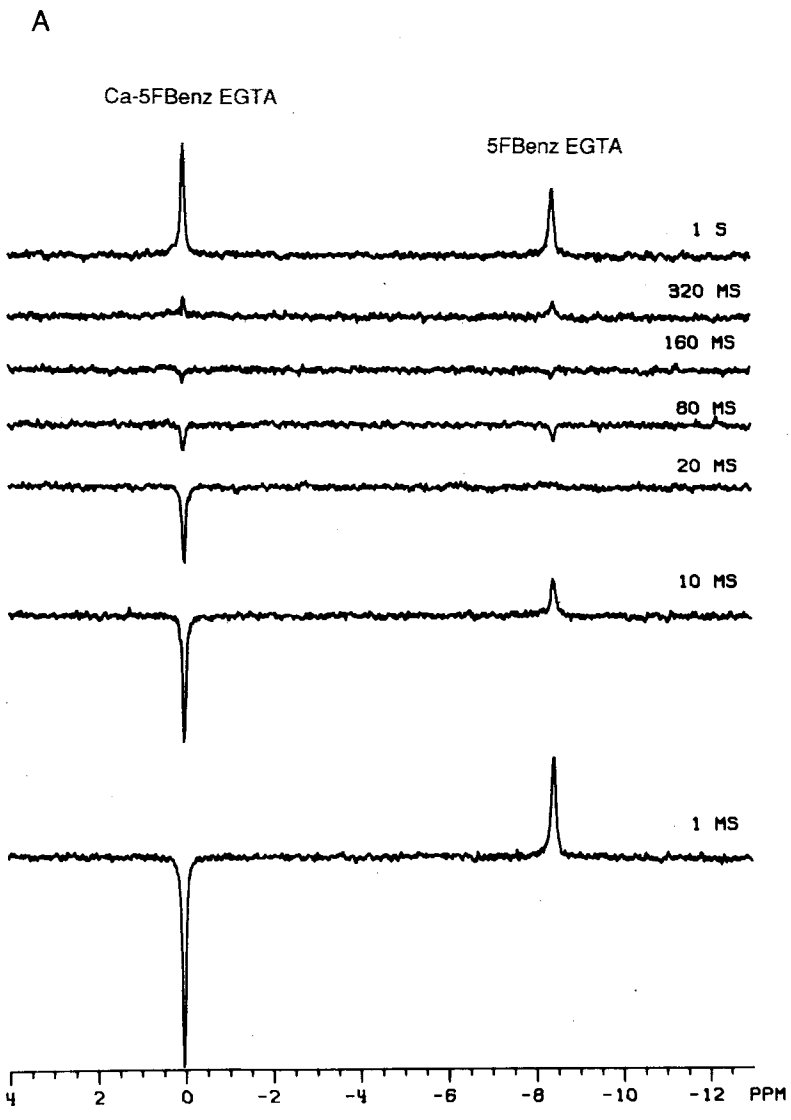

Figure 5

5F BAPTA can be obtained in approximately half the time. Alternatively, an improvement in signal-to-noise ratio of 1.4 would be obtained if equivalent experimental times were used in the comparison. This result is readily verified in studies carried out by overpulsing the above solution containing the two indicators.

The effects of calcium binding kinetics on line widths were further investigated by constructing the chelator 5-fluoro-BenzEGTA (5F BenzEGTA). In this system, the presence of an aliphatic amine with a much greater basicity than the aromatic amine leads to a more stable complex with a reduced dissociation rate constant, as is reflected in the narrower fluorine line width of the calcium–5F BenzEGTA complex (Fig. 4). In the slow exchange limit, the exchange contribution to the line width of the calcium chelator complex will be given by

$$\nu^{\text{exch}}_{\text{Ca–5F BenzEGTA}} = \frac{1}{\pi\tau} = \frac{k_{-1}}{\pi} \tag{3}$$

where k_{-1} is the dissociation rate constant for the complex. The line width corresponding to the uncomplexed chelator will, of course, be related to that of the complexed chelator by

$$\frac{\nu_{\text{5F BenzEGTA}}}{\nu_{\text{Ca–5F BenzEGTA}}} = \frac{[\text{Ca–5F BenzEGTA}]}{[\text{5F BenzEGTA}]} \tag{4}$$

where the system is assumed to be in equilibrium, and where the line width contributions on the left-hand side of the above equation refer only to the contributions due to chemical exchange of the chelator between free and calcium complexed forms. Hence, the decreased line widths are also expected to improve the sensitivity of the measurement, particularly in systems in which the line width is not dominated by magnetic field inhomogeneity or chemical shift dispersion. A direct measurement of the line widths does not, however, provide an accurate measure of the calcium exchange rates since other factors, particularly the exchange of H^+ and Mg^{2+} ions with the 'uncomplexed' chelator, can also contribute. A more accurate determination of the dissociation rate constant was carried out using a selective inversion experiment as discussed under Experimental.

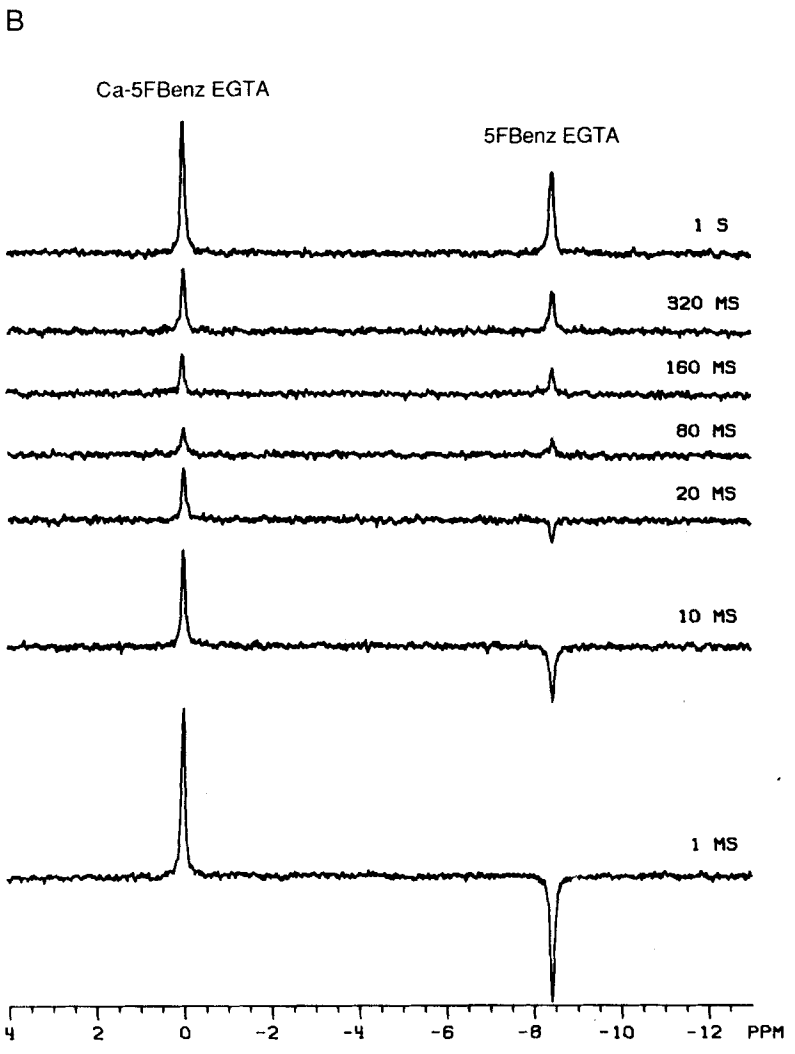

Figure 5. Magnetization transfer study of the Ca–5F BenzEGTA complex. The fluorine resonance of (A) the Ca–5F BenzEGTA complex or (B) the uncomplexed 5F BenzEGTA was selectively inverted using the method of Robinson *et al.*[18]

The ^{19}F resonance corresponding to either the Ca–5F BenzEGTA or to free 5F BenzEGTA was selectively inverted (Fig. 5). Analysis of the data using a Mathematica program based on the method of Perrin and Engler[17] yielded dissociation and association rate constants of $k_{-1} = 29.5\ s^{-1}$ and $k_1' = 45\ s^{-1}$ at 25 °C, where the pseudo-first-order association rate constant k_1' is related to the true association rate constant by $k_1' = k_1[Ca^{2+}]$. Based on the equilibrium dissociation constant determined above, this corresponds to $k_1 = 7.8 \times 10^7\ mol^{-1}\ s^{-1}$. The dissociation rate constant for Ca–5F BenzEGTA is approximately a factor of 3 slower than the reported value of $104\ s^{-1}$ reported for Ca–5F BAPTA, determined at 20 °C.[22]

As is apparent from the spectrum in Fig. 4, the calcium dissociation constant of the Ca–5F BenzEGTA complex measured at pH 7.4 is similar to that of Ca–5F BAPTA. Since at equilibrium $K_D = k_{-1}/k_1$, this result indicates that, in parallel with the reduced dissociation rate constant, a reduced association rate constant also results from the presence of the aliphatic amino group. The association rate constant of Ca^{2+} with 5F BenzEGTA is an order of magnitude slower than the association rate constant for calcium ions with 5F BAPTA of $8.1 \times 10^8\ lmol^{-1}\ s^{-1}$ determined at 37 °C.[3] In addition to the expected effect of temperature, this difference arises since the chelator is now predominantly protonated at the aliphatic amino group at neutral pH so that calcium complexation occurs only with the (rare) deprotonated form, and/or in a slower process involving a concerted deprotonation. The presence of the aliphatic amino group is also expected to lead to more pH-sensitive binding behavior. Dissociation constants measured at several different pH values are summarized in Table 1. The fluorine resonance of 5F BenzEGTA exhibits sensitivity to both the aniline ($pK \approx 5.5$) and the aliphatic amine ($pK \approx 9$) titration steps (Fig. 6). The pH dependence of the apparent calcium dissociation constant will depend on the affinity of the protonated forms for Ca^{2+}. For example, in the limit that 5F BenzEGTA protonated on the aliphatic amino group does not bind Ca^{2+} at all, the apparent $K_D(Ca^{2+})$ of the chelator would increase by approximately an order of magnitude for each pH unit below the amino pK. As a consequence of the pH-dependent chemical shift difference between the free and bound

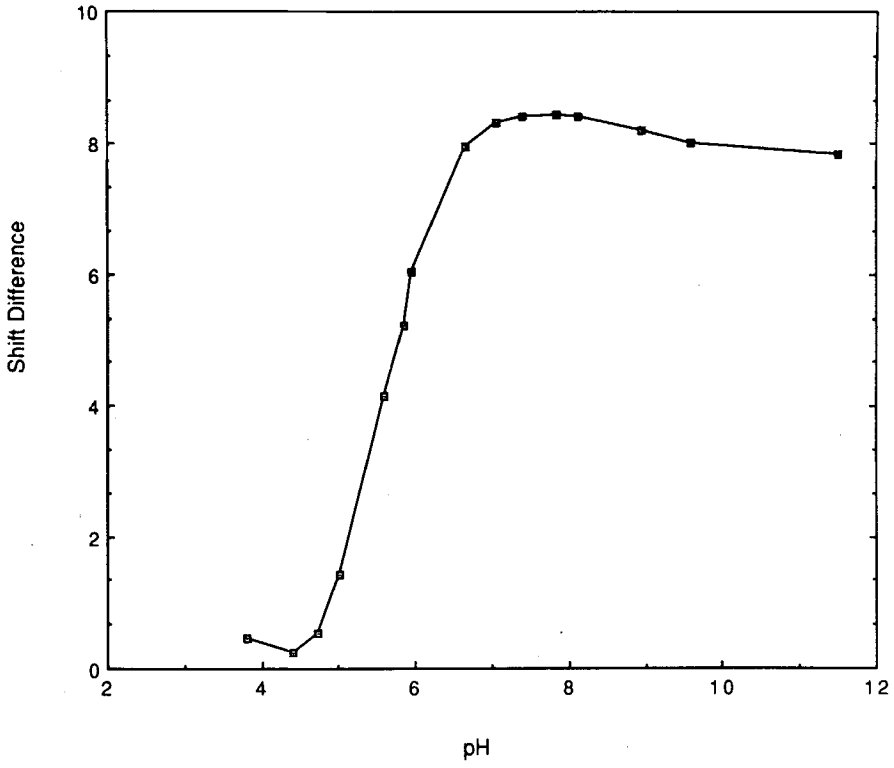

Figure 6. The ^{19}F chemical shift difference of Ca–5F BenzEGTA relative to uncomplexed 5F BenzEGTA as a function of pH. Other buffer components as in Fig. 3.

resonances (Fig. 6), it should be possible to determine the intracellular pH and hence to correct for the pH-dependent changes in K_D.

CONCLUSIONS

Modification of the structure of fluorinated chelators can be carried out to alter relaxation characteristics and thereby improve the sensitivity of the measurement. The introduction of a substituted *tert*-butyl group at position 4 results in a reduction of the ^{19}F T_1 by a factor of *ca.* 2, and in a decrease of the apparent calcium dissociation constant, K_D, by a factor of 1.8. The latter can also improve the signal-to-noise ratio by reducing the exchange contribution to the line width for chelators such as 5F RBAPTA which are in the slow exchange limit. Alternatively, chelators containing aliphatic amino groups such as 5F BenzEGTA yield significantly narrower resonances as a consequence of the longer lifetimes of the calcium complexed species. The calcium ion dissociation constant exhibits a significantly greater pH dependence compared with chelators such as 5F BAPTA which have only aryl amino groups with pK values below the physiological value; however, it is possible to assess independently the pH based on the resonance shifts, and this can be used in conjunction with the calcium binding data if the dependence of K_D on pH has been determined previously. From a synthetic standpoint, the approach used in the synthesis of RBAPTA can be modified to yield a broad variety of BAPTA analogs substituted at C-4. Such modifications can alter the chemical, physical or magnetic properties of the chelator, and could provide a range of useful analogs.

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