Design and Properties of New ^{19}F NMR Ca^{2+} Indicators: Modulation of the Affinities of BAPTA Derivatives *via* Alkylation†

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The effects of alkyl substitution in the aminodiacetic groups of 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid (BAPTA) have been examined systematically with the aim of increasing the affinity for Ca^{2+} of novel ^{19}F NMR and fluorescent Ca^{2+} indicators based on the BAPTA structure into the range required to measure the cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$). Single methylation on the β-carbon of each aminodiacetic acid group of BAPTA derivatives gave increases in the association constant for Ca^{2+} [$K(Ca^{2+})$] of up to 22-fold, depending on the structure of the parent BAPTA derivative. This was shown to be the most effective alkylation for BAPTA derivatives to raise $K(Ca^{2+})$ with a minimal increase in pK_a, and no loss of selectivity for Ca^{2+}, so that the insensitivity of Ca^{2+} binding to pH and $[Mg^{2+}]$ over the physiological ranges was maintained.

(1) Single methylation on the β-carbon of each aminodiacetic acid group of BAPTA derivatives symmetrically substituted with single fluorines at the 4-position (4FBAPTA) or at the 5-position (5FBAPTA) raised log $K(Ca^{2+})$ from 5.61 to 6.81 and 6.12 to 7.47, respectively. The corresponding pK_{av} values (average of the two highest pK_a values in the molecule) were increased from 4.50 to 6.02 and 5.85 to 6.59. The ^{19}F NMR spectroscopic properties of the methylated derivatives were unchanged compared with the parent compounds except for slower exchange rates. These new methylated derivatives of 4FBAPTA and 5FBAPTA therefore extend the range of $K(Ca^{2+})$ available for ^{19}F NMR indicators of $[Ca^{2+}]_i$.

(2) Single methylation on the β-carbon of each aminodiacetic acid group of BAPTA derivatives symmetrically substituted at the 5-position on the aromatic rings with trifluoromethyl groups increased log $K(Ca^{2+})$ from 4.83 to 6.14 and the pK_{av} from 5.39 to 5.90, respectively. There is a Ca^{2+}-induced chemical shift of the ^{19}F NMR resonance of 0.81 ppm upfield with slow exchange at 376 MHz and 30 °C. This compound was selected as a prototype for ^{19}F NMR indicators with enhanced sensitivity through the incorporation of two trifluoromethyl groups to replace the two single fluorine substituents in the current ^{19}F NMR indicators.

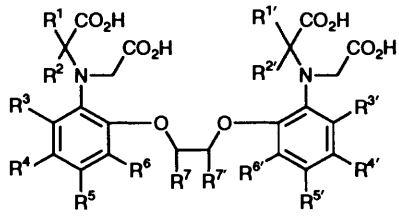
The ^{19}F NMR and fluorescent Ca^{2+} indicators in use are based on the EGTA analogue, BAPTA; 1,2-bis(2-aminophenoxy)-ethane-*N,N,N',N'*-tetracetic acid.[1] The indicators chelate Ca^{2+} selectively over Mg^{2+} through the two aminodiacetic acid groups with 1 : 1 stoichiometry and binding is quantitated by the changes induced in the spectra of substituted aromatic reporter groups. The ^{19}F NMR and fluorescence changes that occur on binding Ca^{2+} result from changes in the electronic interactions between the spectroscopic reporter group and the aminodiacetic acid chelating moieties. The spectral changes in intensity and/or frequency on Ca^{2+} binding to a BAPTA derivative are therefore normally enhanced by the incorporation of structural features which increase the electronic interaction between the chelator element and the spectroscopic reporter in the unchelated state. The amino groups are involved in the coordinate binding of Ca^{2+} and a decrease in their electron density will result in a reduction in the affinity of the chelator for Ca^{2+}. This implies that there are conflicting structural requirements for enhancing changes in the spectroscopic properties of the indicator on binding Ca^{2+} and maintaining the high Ca^{2+} affinity required for an indicator of cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$).

Our initial attempts to increase the sensitivity of both ^{19}F NMR and fluorescent Ca^{2+} indicators based on BAPTA gave substantially improved spectroscopic properties but the affinities for Ca^{2+} were too low for useful $[Ca^{2+}]_i$ indicators for the reasons outlined above. For example, the 5,5'-bis(trifluoromethyl) derivative (TFM) of BAPTA [5TFMBAPTA; Fig. 1(*a*)] has greater potential sensitivity than 5FBAPTA[2] and retains a chemical shift on binding Ca^{2+} of *ca.* 1 ppm. However, the electron-withdrawing power of the TFM group on each ring increases the dissociation constant (K_d) for Ca^{2+} from 0.76 μmol dm^{-3} to 14.8 μmol dm^{-3}.[3] We have also reported that a rhodol fluorophore, FluoRhod [Fig. 1(b)], incorporated into a Ca^{2+} chelator shifts from rhodamine-like to fluorescein-like fluorescence on binding Ca^{2+} with a K_d of approximately 10 μmol dm^{-3}.[3]

From these studies it was clear that the BAPTA derivatives with enhanced spectroscopic properties required structural modifications to increase their affinity for Ca^{2+} by more than 10-fold to bring the K_d into the useful affinity range for $[Ca^{2+}]_i$

† *Abbreviations used:* BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid [other derivatives of BAPTA are symmetrically substituted at positions indicated by number (see Fig. 2) with (F) fluorine, (M) methyl or (TFM) trifluoromethyl substituents]; benz-4, 1-(2-amino-5-methylphenoxy)-2-(2-aminophenoxy)ethane-*N,N,N',N'*-tetracetic acid; $[Ca^{2+}]_i$, cytosolic free Ca^{2+} concentration; $[Ca^{2+}]_{free}$, free Ca^{2+} concentration; *cis*-4, *erythro*-2-(2-amino-5-methylphenoxy)-3-(2-aminophenoxy)butane-*N,N,N',N'*-tetraacetic acid; *cis*-5, *cis*-1-(2-amino-5-methylphenoxy)-2-(2-aminophenoxy)cyclopentane-*N,N,N',N'*-tetraacetic acid; *cis*O-5, *cis*-3-(2-amino-5-methylphenoxy)-4-(2-aminophenoxy)tetrahydrofuran-*N,N,N',N'*-tetraacetic acid; DIPEA, diisopropylethylamine; EBA, ethyl bromoacetete; EBB, ethyl 2-bromobutyrate; EBIB, ethyl 2-bromoisobutyrate; EBP, ethyl 2-bromopropionate; HEDTA, *N*-(2-hydroxyethyl)ethylenediaminetriacetic acid; $K(M^{n+})$, equilibrium association constant of a cation, where M is the atomic symbol and *n* is the value of the charge; K_d, equilibrium dissociation constant; MFBAPTA, 1,2-bis(2-amino-5-fluoro-4-methylphenoxy)ethane-*N,N,N',N'*-tetraacetic acid; NTA, nitrilotriacetic acid; OBS, oxybis(succinic acid); pH_i, cytosolic pH; pK_{av}, average of the two highest pK_a values; Proton Sponge, 1,8-bis(dimethylamino)-naphthalene; *trans*-4, *threo*-2-(2-amino-5-methylphenoxy)-3-(2-aminophenoxy)butane-*N,N,N',N'*-tetraacetic acid.

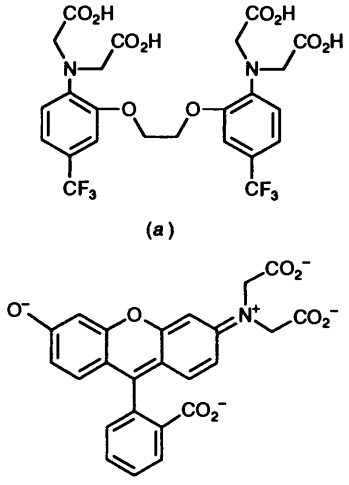
Table 1



$R^1 = R^{1'} = R^2 = R^{2'} = H$

Chelator	R^3	$R^{3'}$	R^4	$R^{4'}$	R^5	$R^{5'}$	R^6	$R^{6'}$	R^7	$R^{7'}$	$K_d(Ca^{2+})$/ nmol dm^{-3}	δ	Exchange (376 MHz)	pK_{av}	$K_d(Mg^{2+})$/ mmol dm^{-3}	Ref.
BAPTA	H	H	H	H	H	H	H	H	H	H	107	n.a.	n.a.	6.42[a]	17	1
5MBAPTA	H	H	H	H	Me	Me	H	H	H	H	38	n.a.	n.a.	7.10[a]	n.d.	1
3FBAPTA	F	F	H	H	H	H	H	H	H	H	646	5.0	slow	5.80[b]	n.d.	2
4FBAPTA	H	H	F	F	H	H	H	H	H	H	2 455	1.5	fast	<4.5[b]	>1 000	2
5FBAPTA	H	H	H	H	F	F	H	H	H	H	537	5.5	slow	5.85[b]	>65	2,11
6FBAPTA	H	H	H	H	H	H	F	F	H	H	25 119	0	fast	n.d.	n.d.	2
MFBAPTA	H	H	Me	Me	F	F	H	H	H	H	269	5.7	slow	n.d.	n.d.	5
benz4	H	H	H	H	Me	H	H	H	H	H	79	n.a.	n.a.	n.d.	8.10	6
trans-4	H	H	H	H	Me	H	H	H	Me	Me	102	n.a.	n.a.	n.d.	8.91	6
cis-4	H	H	H	H	Me	H	H	H	Me	Me	1 000	n.a.	n.a.	n.d.	7.76	6
cis-5	H	H	H	H	Me	H	H	H	$(CH_2)_3$		20	n.a.	n.a.	n.d.	4.17	6
*cis*O-5	H	H	H	H	Me	H	H	H	CH_2OCH_2		50	n.a.	n.a.	n.d.	7.20	6

[a] Highest. [b] Average of two highest.



(*a*)

(*b*)

Fig. 1

indicators. A further requirement was that the increase in affinity should be selective for Ca^{2+} compared with H^+ and Mg^{2+}. The highest pK_a for BAPTA is 6.4 (see Table 1) and this is expected to increase with the affinity for Ca^{2+}. If it is raised into the normal range for cytosolic pH (pH_i), then Ca^{2+} binding to the indicator will be sensitive to changes in pH_i.

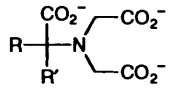
The chemical options to increase the Ca^{2+} affinity of BAPTA-based indicators by alkylation are shown in Table 1 and have been partially examined in previous studies. A small increase in affinity can be achieved by suitable substituents on the aromatic ring and examples of methylation at the 4 and 5 positions have been reported. Methylation of BAPTA at R^5 and $R^{5'}$, *para* to the amino groups (5MBAPTA) increase the Ca^{2+} affinity by a factor of 2.8 and although this increases the pK_a to just below 7 this modification has been used in the structures of quin2 and fura-2.[1,4] Similarly, introduction of a methyl group at R^4 and $R^{4'}$ in 5FBAPTA caused a 2.8-fold increase in Ca^{2+} affinity.[7] Fluorine substitution of BAPTA was used to develop indicators to measure $[Ca^{2+}]_i$ by Ca^{2+}-induced chemical shifts but the effects of the electron-withdrawing fluorine substituents were to decrease the Ca^{2+} affinity of the analogues.[2] Introduction of fluorine atoms at R^6 and $R^{6'}$ in BAPTA gave anomalously low affinity for Ca^{2+} compared with that for 4FBAPTA and hence we assumed that alkylation at this position would not be effective at increasing the affinity for Ca^{2+}. However, comparison of the pK_a and Ca^{2+} affinities of 3FBAPTA with 5FBAPTA indicated that substitution at the 3- and 5-positions had very similar effects. We found in preliminary experiments that methyl substitution at the *o*-position (R^3) in one aromatic ring of 5MBAPTA showed the expected increase in pK_a arising from the inductive effect of the methyl group, but the strength of Ca^{2+} chelation was considerably reduced (see compound **1** in Table 3). This results presumably from a strong steric interaction between the methyl group and one of the methylene groups of the aminodiacetic acid group and was not therefore useful for further development.

The only other positions for which the effect of substituents on Ca^{2+} affinity have been reported are in the bridging ethylenedioxy linkage at R^7 and $R^{7'}$. The addition of two methyl groups [either *erythro* or *threo*] to the ethylenedioxy linkage of 1-(2-amino-5-methylphenoxy)-2-(2-aminophenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (benz4), to give *cis*-4 and *trans*-4, decreased the Ca^{2+} affinity and the only useful increase in Ca^{2+} affinity (a four fold decrease in K_d) for sterically unhindered benz4 derivatives was obtained by replacing the linkage with a 1,2-*cis*-cyclopentane group, giving *cis*-5.[6] It is clear that none of the alkylations described would be sufficient by itself to bring the Ca^{2+} affinity of 5TFMBAPTA into the required range.

The introduction of additional chelating carboxylates to increase affinity was rejected as this is generally found greatly to reduce the selectivity for Ca^{2+} over Mg^{2+}.[1] The only other sites available for structural modification are on the chelating diaminoacetic acid groups (R^2 and $R^{2'}$) but no studies of the effect of alkylation of these groups in BAPTA or EGTA have been reported. However we noted that data were available for analogous derivatives of nitrilotriacetic acid [NTA, Table 2(*a*)] and EDTA [Table 2(*b*)].[7,8] Methylation at one of the chelating arms of NTA causes large increases in affinities for both Ca^{2+} and H^+ with an increase of up to 100-fold in $K(Ca^{2+})$ for two methyl groups. Increasing the size of the alkyl substituent was less effective at increasing the affinity of NTA for Ca^{2+}. However, symmetrical methyl substitution at the same

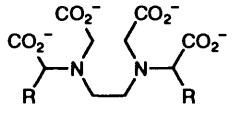
Table 2

(*a*)



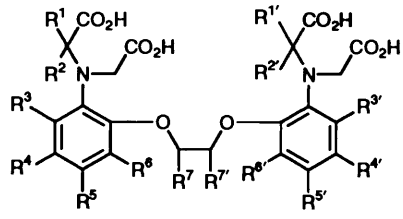
M^{n+}	Equil.	log $K(M^{n+})$ for NTA analogues: R = H, R′ = H	R = H, R′ = Me	R = Me, R′ = Me	R = H, R′ = Et	R = H, R′ = Pr^n
H^+	HL/H.L	9.71	10.47	11.86	9.88	9.94
H^+	H_2L/HL.H	2.48	2.46	2.52	2.48	2.46
Ca^{2+}	ML/M.L	6.41	6.97	8.32	6.46	6.40

(*b*)



M^{n+}	Equil.	log $K(M^{n+})$ for EDTA analogues: R = H	R = Me	R = Et	R = Pr^n
H^+	HL/H.L	10.24	10.42	10.42	10.47
H^+	H_2L/HL.H	6.16	6.65	6.09	6.15
Ca^{2+}	ML/M.L	10.69	10.01	9.02	9.24

Table 3



$R^4 = R^{4'} = R^6 = R^{6'} = R^7 = R^{7'} = H$

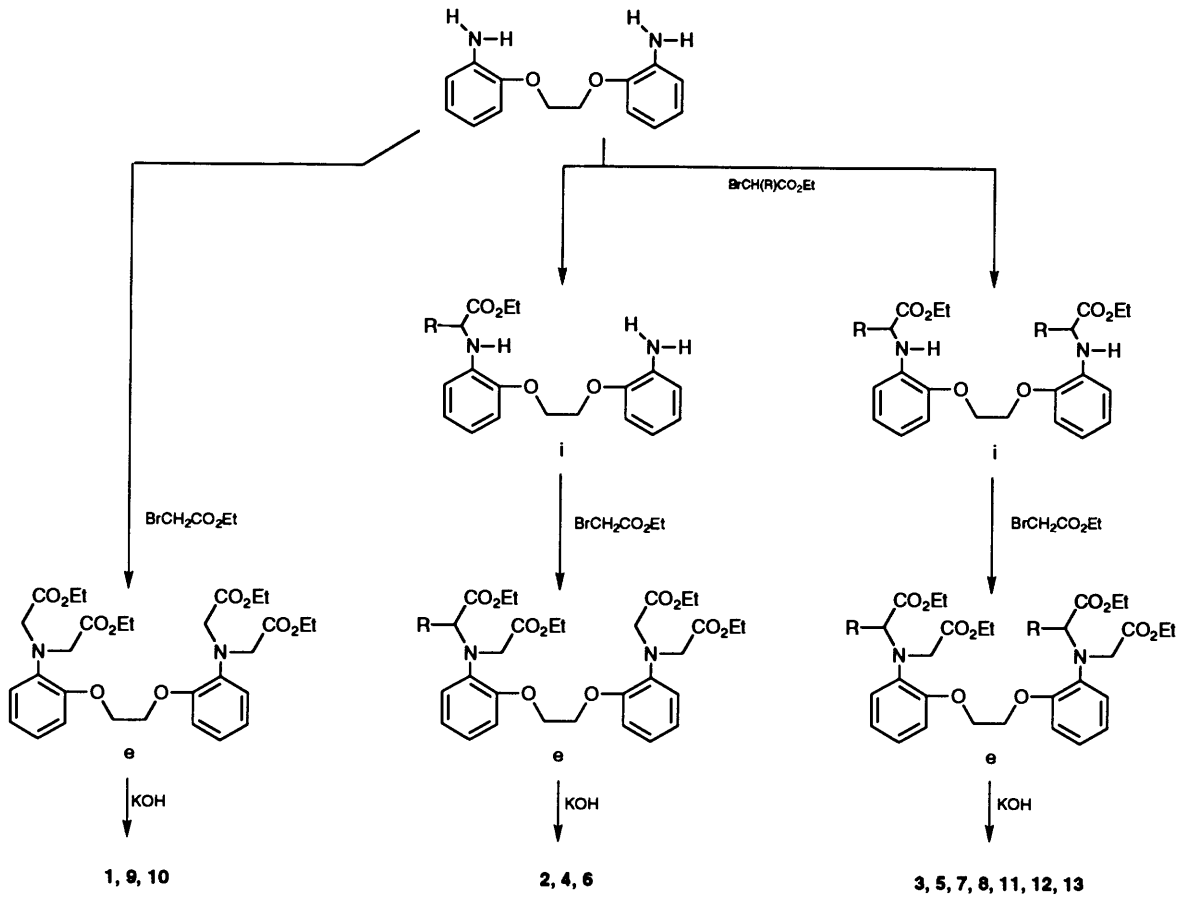
Chelator	R^1	$R^{1'}$	R^2	$R^{2'}$	R^3	$R^{3'}$	R^5	$R^{5'}$	K_d(Ca)/nmol dm^{-3}	pK_α	pK_β
1	H	H	H	H	Me	H	Me	Me	204	7.80	6.26
2	H	H	Me	H	H	H	Me	Me	32	7.33	6.00
3	H	H	Me	Me	H	H	Me	Me	14	7.60	7.00
4	H	H	Et	H	H	H	Me	Me	46	7.30	5.81
5	H	H	Et	Et	H	H	Me	Me	22	7.61	7.01
6	Me	H	Me	H	H	H	Me	Me	8	10.00	6.41
7	Me	Me	Me	Me	H	H	Me	Me	1	10.17	9.65
8	H	H	Me	Me	H	H	H	H	18	7.09	6.83

positions on two of the chelating arms of EDTA has the opposite effect on the affinity of the EDTA analogues for Ca^{2+} and increasing the size of the substituent caused a further decrease in affinity. The reason for this difference between NTA and EDTA presumably lies in different steric interactions or solvation effects of the substituent alkyl groups in the complexes of EDTA and NTA with Ca^{2+}.

On the basis of these data it was not possible to predict the effect on the affinity for Ca^{2+} of methyl substitution in the aminodiacetic acid groups of BAPTA derivatives. Here we have examined systematically the effects of alkyl substitution at this position in BAPTA on the affinity for Ca^{2+}, Mg^{2+} and H^+. From the study of these model chelators it was possible to design derivatives of 5FBAPTA, 4FBAPTA and 5TFMBAPTA with increased affinity for use in NMR experiments described elsewhere.[9] The data for the model BAPTA derivatives were also used to adjust the affinity of a novel fluorescent indicator into the required range for $[Ca^{2+}]_i$ measurements as described in the following paper.

Results and Discussion

Effects of Alkyl Substitutions in the Aminodiacetic Acid Groups on the Affinity for Ca^{2+} *of BAPTA and 5MBAPTA.*— Preliminary experiments were performed to determine whether a single methyl substituent in each aminodiacetic acid group in BAPTA caused an increase in affinity for Ca^{2+} as in NTA or a decrease as in EDTA. A methyl substituent at R^2 of the acetic acid moiety of an aminodiacetic acid group introduces a chiral centre and substitution at both aminodiacetic acid groups in BAPTA and its derivatives ($R^2 = R^{2'}$ = methyl) generates diastereoisomers. The (±) and *meso* forms of **8e** were separated by crystallisation and were shown to have different polarities by HPLC (see the Experimental section). They had Ca^{2+}



Scheme 1

and proton affinities that were indistinguishable within experimental error (Table 3). All subsequent model chelators described in Tables 3 and 4 with $R^2 = R^{2'}$ = methyl were therefore analysed as mixtures and the affinities of the unresolved diastereoisomers were assumed to be the same provided their binding data met the criteria of linearity and slope described in the Experimental section. The large increase of approximately six fold in Ca^{2+} affinity of **8** compared with BAPTA suggested that the steric interactions which reduce the affinity of EDTA for Ca^{2+} on alkylation of the acetic acid moiety are much less significant for BAPTA.

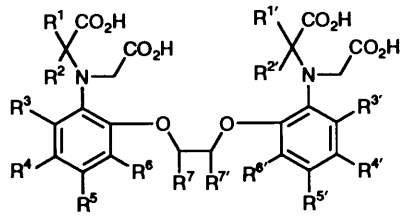
For subsequent studies, 5MBAPTA (Table 1) was used as the parent structure because it had several technical advantages for defining the properties of these model compounds (**1**–**7**). The most important of these was that accurate pK_a values were easier to determine by spectrophotometric measurement because ring methylation raised the highest pK_a values (*i.e.*, *N* protonation of the aminodiacetic acid groups) compared with BAPTA and separated them from the subsequent pK_a values. It was therefore possible to define more precisely the effects of structural modifications in 5MBAPTA on the highest pK_a values of these model compounds.

It was found that *N*-alkylation of the *bis*-amino precursor in Scheme 1 with esters of the higher α-bromo acids proceeded more slowly than with α-bromoacetate ester and could be controlled to produce readily separable mixtures of mono- and di-substituted intermediates. No product was obtained with more than one *N*-alkyl substituent on each amino group except for *N*-alkylation with α-bromoacetate ester. However, it was possible to obtain bis-methylation on one or both amino groups by using α-bromoisobutyrate ester. The additional carboxy groups required to complete the tetracarboxylate derivative were introduced into the mono- and di-*N*-alkylated intermediates with α-bromoacetate ester. Table 3 therefore contains examples of all of the types of *N*-alkylated compounds which were prepared.

Two methyl substituents (**3**) are more effective in increasing Ca^{2+} affinity than a single methyl group (**2**) and bis-methylation on the same aminodiacetic acid group (**6**) is more effective than a single methyl substituent on each amino-diacetic acid (**3**). The highest Ca^{2+} affinity was achieved with the tetramethylated derivative (**7**). However it is clear that all methyl substitutions also increase the pK_a values and although **6** and **7** are strong Ca^{2+} chelators, the higher degree of alkylation produced pK_a values that were too high for **6** and **7** to be useful for $[Ca^{2+}]_i$ measurements at normal intracellular pH values. Ethyl substitution gave smaller increases in the affinity for Ca^{2+} but similar increases in pK_a compared with the corresponding methyl substitutions. It is noted that this pattern of substituent effects on Ca^{2+} and H^+ affinities is similar to that for NTA rather than EDTA [Tables 2(a) and 2(b)].

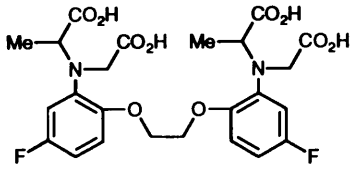
In summary, modifications of the BAPTA derivatives shown here and previously to raise Ca^{2+} affinity are: (*i*) methylation of the aromatic ring at R^5 and $R^{5'}$ [a 2.8-fold increase in $K(Ca^{2+})$];[1] (*ii*) alkylation of the ethylene bridge at R^7 and $R^{7'}$ to form a *cis*-cyclopentane ring [increase in $K(Ca^{2+})$ of approximately four fold];[6] (*iii*) single methylation on each aminodiacetic acid group at R^2 and $R^{2'}$ gives increases in $K(Ca^{2+})$ up to 22-fold depending on the parent BAPTA derivative. For example, the substitutions cause increases in $K(Ca^{2+})$ of 5.9-fold in **8** compared with BAPTA, of 2.7-fold in **3** compared with 5MBAPTA, of 22-fold in 2M5FBAPTA compared with 5FBAPTA and of 20-fold in 2M5TFMBAPTA compared with 5TFMBAPTA. These data therefore show that the greater the reduction in Ca^{2+} affinity caused by the substituents on the aromatic rings of BAPTA, the greater the

Table 4



$R^1 = R^{1'} = R^3 = R^{3'} = R^6 = R^{6'} = R^7 = R^{7'} = H$

Chelator		R^2	$R^{2'}$	R^4	$R^{4'}$	R^5	$R^{5'}$	K_d(Ca)/ nmol dm^{-3}	δ	Exchange (376 MHz)	pK_a	pK_β
9	4TFMBAPTA	H	H	TFM	TFM	H	H	4 677	−0.2	fast	5.96	5.10
10	5TFMBAPTA	H	H	H	H	TFM	TFM	14 790	−1.1	fast-int	5.69	5.09
11	2M4FBAPTA	Me	Me	F	F	H	H	155	2.07	slow	pK_{av} 6.02	
12	2M5FBAPTA	Me	Me	H	H	F	F	34	3.96	slow	pK_{av} 6.59	
13	2M5TFMBAPTA	Me	Me	H	H	TFM	TFM	724	−0.8	slow-int	6.08	5.72



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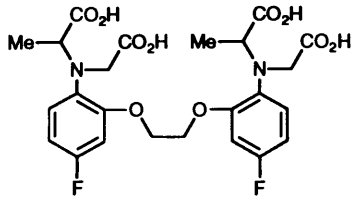

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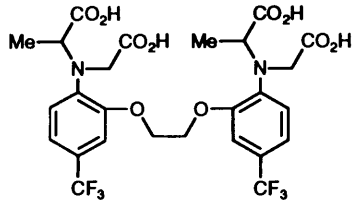

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Fig. 2

effect of the methylation on each aminodiacetic acid group in increasing the Ca^{2+} affinity of the derivative.

Of the structural modifications to BAPTA summarised in (*i*), (*ii*) and (*iii*) it is concluded that the most effective substituent to raise Ca^{2+} affinity with the least increase in pK_a is a single methyl group on the β-carbon of the aminodiacetate sidechain of the aromatic ring of a BAPTA derivative. The selectivity for Ca^{2+} over Mg^{2+} is maintained in these methyl derivatives as demonstrated for the ^{19}F NMR indicators (see compounds **11** and **12** below).

^{19}F *BAPTA Derivatives for NMR Indicators.*—The ^{19}F labelled chelators (Fig. 2 and Table 4) were synthesised with two objectives: (*i*) to explore the effects of methyl substitution of the aminodiacetic acid groups on the affinities and spectroscopic properties of 4FBAPTA and 5FBAPTA with the aim of obtaining a series of ^{19}F NMR indicators with a range of affinities for Ca^{2+} for use in perfused heart experiments and (*ii*) to examine the corresponding effects of similar substitutions on the properties of the novel TFM analogues of 4FBAPTA and 5FBAPTA (4TFMBAPTA and 5TFMBAPTA, respectively).

Compound **11** (*2M4FBAPTA*).—The methylated analogue (**11**) of 4FBAPTA had a log $K(Ca^{2+})$ of 6.8 compared with 5.6 for 4FBAPTA. Compound **11** complexed with Ca^{2+} exhibits a slow-exchange NMR spectrum at 376 MHz and 30 °C with a chemical shift of 1.1 ppm downfield. The pK_a is 6.02 and the chemical shifts of the free and bound forms are both insensitive to pH in the physiological range. These properties may be compared with those of 4FBAPTA which shows intermediate exchange under the same conditions with a 1.5 ppm shift downfield on Ca^{2+} binding.[2]

Compound **12** (*2M5FBAPTA*).—Addition of one methyl group into each of the aminodiacetic acid chelating groups of 5FBAPTA increased the log $K(Ca^{2+})$ from 6.2 to 7.5 at 30 °C. There was no significant change in the chemical shift of the resonance from either the free or the Ca^{2+}-bound forms compared with 5FBAPTA (5.5 ppm downfield on Ca^{2+} binding) at pH > 9 and the indicator was in the slow-exchange condition for Ca^{2+} binding at 376 MHz at 30 °C, consistent with its higher affinity compared with 5FBAPTA. The highest pK_a of compound **12** was 6.59 at 30 °C which is sufficiently low to ensure minimal changes in the apparent affinity for Ca^{2+} in the physiological pH_i range. However, the very large chemical shift (11 ppm downfield) caused by protonation of the free form of the indicator results in substantial pH-induced chemical shift changes for the resonance from the free form in the normal physiological pH range, whereas the resonance from the bound form does not shift. Hence in the absence of other effects on the resonances, the compound will act as an indicator of both pH (fast exchange) and $[Ca^{2+}]_{free}$ (slow exchange) simultaneously.

For compounds **11** and **12** the affinity for Mg^{2+} was < 0.2 dm^3 mol^{-1} measured by UV spectroscopy and no Mg^{2+}-induced chemical shift was observed at Mg^{2+} concentrations < 100 mmol dm^{-3}. It was therefore assumed that methylation of the aminodiacetic acid groups does not adversely affect the selectivity for Ca^{2+} over Mg^{2+}.

Compound **9** (*4TFMBAPTA*) *and Compound* **10** (*5TFMBAPTA*).—The properties of 6FBAPTA (Table 1) and 1-(2-amino-3,5-dimethylphenoxy)-2-(2-amino-5-methylphenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (**1** in Table 2) showed that incorporating the TFM reporter group at the 6 or 3 substitution positions was very unlikely to give useful indicators and

therefore only the 4TFM and 5TFM derivatives of BAPTA (**9** and **10**, respectively) were synthesised. It was found that the large inductive effect and the absence of any mesomeric effect resulted in affinities for Ca^{2+} of both **9** and **10** (log K of 5.33 and 4.83) that were considerably weaker and in reverse order to those of 4FBAPTA and 5FBAPTA (Table 1). The ^{19}F NMR chemical shifts induced by Ca^{2+} binding were much smaller (0.2 ppm for 4TFMBAPTA and 1.1 ppm for 5TFMBAPTA) and were in the reverse direction (i.e., upfield on Ca^{2+} binding) than for 4FBAPTA and 5FBAPTA. Both chelators were in fast exchange with Ca^{2+} at 376 MHz at 30 °C. These compounds are not useful, therefore, as ^{19}F NMR indicators of $[Ca^{2+}]_i$.

Compound **13** *(2M5TFMBAPTA).*—Although the Ca^{2+} affinity of 5TFMBAPTA (**10**) was approximately three times weaker than that of 4TFMBAPTA, the larger chemical shift of **10** suggested that it was a more promising compound for modification by alkylation and compound **13** was therefore prepared. This compound showed an affinity for Ca^{2+} (log K = 6.14) close to that of 5FBAPTA and a Ca^{2+}-induced chemical shift of 0.81 ppm upfield with slow to intermediate exchange at 376 MHz and 30 °C. This compound was therefore selected as a prototype for ^{19}F NMR indicators of enhanced sensitivity through the incoporation of two TFM groups into BAPTA.

Experimental

Spectroscopic Determination of Cation Affinity Constants.—The pK_a values for the new BAPTA analogues (see Tables 3 and 4) were measured by UV spectroscopy from 270 nm to 320 nm using 5 cm^3 cuvettes with a path length of 4 cm. Spectra were recorded of samples (10 μmol dm^{-3}) in 100 mmol dm^{-3} KCl, 1 mmol dm^{-3} EGTA, 50 mmol dm^{-3} Tris/acetate at 37 °C for **1–8** or at 30 °C for **9**, **10**, or in 40 mmol dm^{-3} trisodium citrate, 10 mmol dm^{-3} EGTA at 30 °C for **11–13**, titrated between pH 9.8 and 2.4 by varying the proportions of Tris and acetate in the solutions. The data obtained were analysed by using a computer curve-fitting program (based on least-squares minimisation) which calculated the three highest pK_a values (pK_α, pK_β and pK_γ) for each compound.[3]

Titrations to determine the Ca^{2+} affinities of the chelators with various pK_a values required suitable buffer systems at appropriate pH values. The samples were dissolved in 40 mmol dm^{-3} citrate, 5 mmol dm^{-3} Hepes at pH 7.6 (**1**, **2**) or pH 7.2 (**8**, **11**, **12**, **13**); or 40 mmol dm^{-3} oxybis(succinate) (OBS), 5 mmol dm^{-3} Hepes, pH 8.10 (**3**, **5**) or 40 mmol dm^{-3} OBS, 50 mmol dm^{-3} Tris, pH 11.10 (**6**); or 10 mmol dm^{-3} *N*-(2-hydroxyethyl)-ethylenediaminetriacetic acid (HEDTA), 50 mmol dm^{-3} tripotassium borate, pH 11.30 (**7**) or 100 mmol dm^{-3} KCl, 5 mmol dm^{-3} Tris/acetate (**9**, **10**). The UV absorbances were measured as a function of $[Ca^{2+}]_{free}$ at 37 °C for **1–8** or at 30 °C for **9–13**. Plots of log [Ca^{2+}-bound indicator/free indicator] *versus* log $[Ca^{2+}]_{free}$ were used with iterative computation to determine the affinities of the compounds for Ca^{2+}.[3,4] The linearity and slope (1.00 ± 0.05) provided sensitive test criteria for homogeneous and stoichiometric binding of a single Ca^{2+} ion by the chelators. log Equilibrium constants ML/M.L(Ca^{2+}), HL/H.L and H_2L/HL.H used for citrate were 3.50, 5.69 and 4.34, respectively[7] and for HEDTA were 8.20, 9.81 and 5.37.[8] log K(Ca^{2+}) is 5.26 for OBS (the pK_a values were not determined but are likely to be similar to citrate).[10]

Chemical Synthesis.—The tetraacetic acid analogues of BAPTA were prepared from the appropriately substituted nitrophenols following the general synthetic route described previously.[1] Deviations from this route occur at the alkylation of the diamino analogues and are shown in Scheme 1. The reaction of symmetrical diamines with 1.5 molar equivalents of ethyl 2-bromopropionate (EBP), ethyl 2-bromobutyrate (EBB) or ethyl 2-bromoisobutyrate (EBIB) gave approximately equal proportions of the monoalkylated and dialkylated intermediates (i) which, after separation and characterisation, were further alkylated with ethyl bromoacetate (EBA) to give tetraesters (e). All alkylation products were isolated as for **3i**. Hydrolysis of the tetraesters (**1e–13e**) by standard procedures[1] using potassium hydroxide in aqueous ethanol yielded the chelators (**1–13**) as white solids. Alkylation of a diamine with 2 or more molar equivalents of EBP yielded a symmetrically disubstituted intermediate which was subsequently alkylated with EBA and hydrolysed to yield the tetracid (see Scheme 1).

Proton NMR spectra were recorded on Hitachi Perkin-Elmer 60 and Bruker AM 400 spectrometers. Resonances are reported as NMR (solvent, frequency); δ = shift in ppm from tetramethylsilane at 0 ppm and the multiplicity of signals as s = singlet; d = doublet; dd = double doublet; t = triplet; q = quartet; m = multiplet; br = broad.

1-[2-*Di(ethoxycarbonylmethyl)amino*-3,5-*dimethylphenoxy*]-2-[2-*di(ethoxycarbonylmethyl)amino*-5-*methylphenoxy*]*ethane* **1e**.—Alkylation of 1-(2-amino-3,5-dimethylphenoxy)-2-(2-amino-5-methylphenoxy)ethane with EBA gave a yellow oil which was dissolved in a minimum of light petroleum (b.p. 40–60 °C) and crystallised from ethanol to yield white crystals (46% yield), m.p. 82 °C, δ(C^2HCl_3; 60 MHz) 1.16 (t, J 7 Hz, 6 H), 1.19 (t, J 7 Hz, 6 H), 2.24 (s, 6 H), 2.39 (s, 3 H), 3.78 (s, 4 H), 4.03 (q, J 7 Hz, 8 H), 4.04 (s, 4 H), 4.25 (m, 4 H), 6.53 (m, 3 H) and 6.65 (s, 2 H); λ_{max}/nm (ε/dm^3 mol^{-1} cm^{-1}) 256 (12 400) and 290 (3400) (EtOH); m/z (+EI) 630.3142. $C_{33}H_{46}N_2O_{10}$ +1.6 ppm.

Other tetraacetate analogues were **9e** which crystallised from light petroleum (b.p. 40–60 °C) (74% yield), m.p. 105–107 °C, δ(C^2HCl_3; 60 MHz) 1.17 (t, J 7 Hz, 12 H), 4.06 (q, J 7 Hz, 8 H), 4.17 (s, 8 H), 4.38 (s, 4 H), 6.95 (dd, J 9, 2 Hz, 2 H), 7.14 (d, 2 Hz, 2 H) and 7.25 (dd, J 9, 2 Hz, 2 H); λ_{max}/nm (ε/dm^3 mol^{-1} cm^{-1}) 264 (19 500) and 294 (5600) (EtOH); m/z (+EI) 724.2431. $C_{32}H_{38}F_6N_2O_{10}$ 0 ppm, and **10e** which was also crystallised from light petroleum (b.p. 40–60 °C) to give 77% yield, m.p. 128–130 °C, δ(C^2HCl_3; 60 MHz) 1.20 (t, J 7 Hz, 12 H), 4.10 (q, J 7 Hz, 8 H), 4.20 (s, 8 H), 4.33 (s, 4 H), 6.86 (d, 9 Hz, 2 H), 7.11 (d, 2 Hz, 2 H) and 7.35 (dd, J 9, 2 Hz, 2 H); λ_{max}/nm (ε/dm^3 mol^{-1} cm^{-1}) 265 (13 800) and 300infl (6400) (EtOH); m/z (+EI) 724.2391, $C_{32}H_{38}F_6N_2O_{10}$ +5.5 ppm, (M + 1) 725.2505, $C_{32}H_{39}F_6N_2O_{10}$ +0.5 ppm.

1-{2-[1-(*Ethoxycarbonyl)ethyl*]*amino*-5-*methylphenoxy*}-2-(2-*amino*-5-*methylphenoxy)ethane* **2i** *and* 1,2-*bis*{2-[1-(*ethoxycarbonyl)ethyl*(*ethoxycarbonylmethyl*)]*amino*-5-*methylphenoxy*}*ethane* **3i**.—1,2-Bis(2-amino-5-methylphenoxy)ethane[1] (2.32 g, 8.5 mmol), EBP (2.68 g, 12.8 mmol) and 1.8-bis(dimethylamino)naphthalene [(Proton Sponge); 2.92 g 14 mmol] in 10 cm^3 acetonitrile were refluxed at 110 °C under N_2 (30 min). The mixture was diluted with toluene and washed with 1 mol dm^{-3} ammonium phosphate pH 4.0 (4 ×), water (1 ×) and saturated potassium hydrogen carbonate solution (2 ×). The solvent was dried and removed at low pressure and then the excess of EBP was removed into a trap under high vacuum. The mixture was separated on a silica gel column [200 g, pre-equilibrated ethyl acetate–toluene (1 : 10 v/v), eluted with ethyl acetate–toluene (1 : 10 v/v to 1 : 5 v/v)]. The first eluted fraction (**3i**) was crystallised from ethanol (1.53 g, 38% yield), m.p. 68 °C, δ(C^2HCl_3; 400 MHz) 1.22 (t, J 7 Hz, 6 H), 1.46 (dd, J 8, 1 Hz, 6 H), 2.25 (s, 6 H), 4.01 (q, J 7 Hz, 2 H), 4.15 (q, J 7 Hz, 4 H), 4.35 (m, 4 H), 4.70 (m, 2 H), 6.44 (d, J 8 Hz, 2 H), 6.67 (dd, J 8, 1 Hz, 2 H) and 6.70 (d, J 1 Hz, 2 H). The light-brown crystals of the second fraction (**2i**) were dissolved in a minimum volume of diethyl ether and light petroleum (b.p. 40–60 °C) was added until the solution became opaque. White crystals formed on

scratching (27% yield), m.p. 72–74 °C, $\delta(C^2HCl_3$; 400 MHz) 1.23 (t, *J* 7 Hz, 3 H), 1.45 (d, *J* 8 Hz, 3 H), 2.25 (s, 3 H), 2.26 (s, 3 H), 4.09 (q, *J* 7 Hz, 1 H), 4.16 (q, *J* 7 Hz, 2 H), 4.36 (s, 4 H), 6.45 (d, *J* 8 Hz, 1 H) and 6.65 (m, 5 H).

Similarly, alkylation of 1,2-bis(2-amino-5-methylphenoxy)-ethane with 1.5 mol equiv. of EBB gave **4i**, a brown oil (25% yield), $\delta(C^2HCl_3$; 60 MHz) 0.98 (t, *J* 7 Hz, 3 H), 1.20 (t, *J* 7 Hz, 3 H), 1.79 (q, *J* 7 Hz, 1 H), 2.25 (s, 6 H), 4.17 (q, *J* 7 Hz, 2 H), 4.35 (s, 4 H), 6.61 (s, 2 H) and 6.65 (m, 4 H) and **5i**, a yellow oil which was crystallised from ethanol to give white crystals (28% yield), m.p. 73–75 °C, $\delta(C^2HCl_3$; 60 MHz) 0.98 (t, *J* 7 Hz, 6 H), 1.21 (t, *J* 7 Hz, 6 H), 1.85 (q, *J* 7 Hz, 4 H), 2.25 (s, 6 H), 4.15 (q, *J* 7 Hz, 4 H), 4.30 (m, 4 H), 4.40 (s, 4 H) and 6.60 (m, 6 H).

Alkylation of 1,2-bis(2-amino-5-methylphenoxy)ethane with 1.5 mol equiv. of EBIB gave the mono-substituted product **6i**, which was crystallised with ethanol to yield fine white needles (38%), m.p. 95 °C, $\delta(C^2HCl_3$; 60 MHz) 1.19 (t, *J* 7 Hz, 3 H), 1.51 (s, 6 H), 2.26 (s, 6 H), 4.10 (m, 3 H), 4.15 (q, *J* 7 Hz, 2 H) and 4.35 (s, 6 H) and the disubstituted product **7i** which was crystallised from ethanol (29% yield), m.p. 96–98 °C, $\delta(C^2HCl_3$; 60 MHz) 1.15 (t, *J* 7 Hz, 6 H), 1.50 (s, 12 H), 2.24 (s, 6 H), 4.10 (m, 2 H), 4.20 (q, *J* 7 Hz, 4 H), 4.35 (s, 4 H), 6.55 (m, 4 H) and 6.62 (s, 2 H).

1-{2-[1-(*Ethoxycarbonyl*)*ethyl*(*ethoxycarbonylmethyl*)]-*amino*-5-*methylphenoxy*}-2-[2-*bis*(*ethoxycarbonylmethyl*)-*amino*-5-*methylphenoxy*]*ethane* **2e**.—**2i** (660 mg, 1.8 mmol), Proton Sponge (1.5 g, 7 mmol) and EBA (2.0 g, 12 mmol) in acetonitrile (2.5 cm^3) was heated at 110 °C under N_2 (10 h). The product was isolated as above and it was crystallised from light petroleum (b.p. 40–60 °C) to yield large pale pink crystals (61%), m.p. 75–76 °C, $\delta(C^2HCl_3$; 60 MHz) 1.13 (t, *J* 7 Hz, 3 H), 1.17 (t, *J* 7 Hz, 6 H), 1.20 (t, *J* 7 Hz, 3 H), 1.42 (d, *J* 9 Hz, 3 H), 2.25 (s, 6 H), 4.10 (m, 15 H), 4.30 (s, 4 H) and 6.80 (m, 6 H); λ_{max}/nm (ε/dm^3 mol^{-1} cm^{-1}) 258 (15 300) and 298 (5400) (EtOH); *m*/*z* (+EI) 630.3162. $C_{33}H_{46}N_2O_{10}$ −1.6 ppm.

Other analogues were **4e**, an oil (62% yield), NMR $\delta(C^2CHl_3$; 60 MHz) 0.97 (t, *J* 7 Hz, 3 H), 1.13 (t, *J* 7 Hz, 3 H), 1.18 (t, *J* 7 Hz, 6 H), 1.75 (q, *J* 7 Hz, 2 H), 2.25 (s, 6 H), 4.10 (m, 15 H), 4.30 (s, 4 H) and 6.80 (m, 6 H); λ_{max}/nm (ε/dm^3 mol^{-1} cm^{-1}) 254 (14 600) and 290 (6300) (EtOH); *m*/*z* (+EI) 644.3274. $C_{34}H_{48}N_2O_{10}$ +5 ppm, and **6e** which was crystallised from ethanol to give a white powder (36%), m.p. 69–71 °C, $\delta(C^2HCl_3$; 400 MHz) 1.15 (t, *J* 7 Hz, 3 H), 1.18 (t, *J* 7 Hz, 6 H), 1.25 (t, *J* 7 Hz, 3 H), 1.36 (s, 6 H), 2.27 (s, 3 H), 2.30 (s, 3 H), 4.01 (q, *J* 7 Hz, 2 H), 4.02 (s, 2 H), 4.10 (q, *J* 7 Hz, 4 H), 4.12 (s, 4 H), 4.14 (q, *J* 7 Hz, 2 H), 4.26 (t, *J* 6 Hz, 2 H), 4.32 (t, *J* 6 Hz, 2 H), 6.66 (d, *J* 8 Hz, 1 H), 6.68 (s, 2 H), 6.71 (d, *J* 8 Hz, 1 H), 6.78 (d, *J* 8 Hz, 1 H) and 7.17 (d, *J* 8 Hz, 1 H); λ_{max}/nm (ε/dm^3 mol^{-1} cm^{-1}) 254 (12 500) and 288 (4800) (EtOH); *m*/*z* (+EI) 644.3268. $C_{34}H_{48}N_2O_{10}$ +6.3 ppm.

1,2-*Bis*{2-[1-(*ethoxycarbonyl*)*ethyl*(*ethoxycarbonylmethyl*)]-*amino*-5-*methylphenoxy*}*ethane* **3e**.—**3i** (600 mg, 1.27 mmol) was added to proton sponge (1.5 g, 7 mmol) and EBA (2.0 g, 10.2 mmol) in acetonitrile (2.5 cm^3). The reaction was heated at 110 °C under N_2 for 8 h. The isolated product was crystallised from light petroleum to yield white crystals (81% yield), m.p. 70–71 °C, $\delta(C^2HCl_3$; 60 MHz) 1.15 (t, *J* 7 Hz, 6 H), 1.20 (t, *J* 7 Hz, 6 H), 1.43 (d, *J* 8 Hz, 6 H), 2.27 (s, 6 H), 4.10 (m, 14 H), 4.34 (s, 4 H), 6.70 (d, *J* 8 Hz, 2 H), 6.77 (s, 2 H) and 6.95 (dd, *J* 8, 1 Hz, 2 H); λ_{max}/nm (ε/dm^3 mol^{-1} cm^{-1}) 256 (15 600) and 296 (5400) (EtOH); *m*/*z* (+EI) 644.3310. $C_{34}H_{48}N_2O_{10}$ −0.2 ppm.

Other analogues were **5e**, a brown oil (62%), $\delta(C^2HCl_3$; 60 MHz) 0.98 (t, *J* 7 Hz, 6 H), 1.15 (t, *J* 7 Hz, 6 H), 1.23 (t, *J* 7 Hz, 6 H), 1.78 (m, *J* 7 Hz, 4 H), 2.28 (s, 6 H), 4.12 (m, 10 H), 4.11 (s, 4 H), 4.33 (s, 4 H), 6.71 (d, *J* 8 Hz, 2 H), 6.77 (s, 2 H) and 7.17 (d, *J* 8 Hz, 2 H); λ_{max}/nm (ε/dm^3 mol^{-1} cm^{-1}) 256 (12 300) and 292 (4900) (EtOH); *m*/*z* (+EI) 672.3590, $C_{36}H_{52}N_2O_{10}$ +4.6 ppm, and **7e**, which was crystallised from ethanol, (44%), m.p. 65 °C, $\delta(C^2HCl_3$; 400 MHz) 1.17 (t, *J* 7 Hz, 6 H), 1.26 (t, *J* 7 Hz, 6 H), 1.35 (s, 12 H), 2.31 (s, 6 H), 4.02 (s, 4 H), 4.04 (q, *J* 7 Hz, 4 H), 4.15 (q, *J* 7 Hz, 4 H), 4.31 (s, 4 H), 6.68 (dd, *J* 8, 2 Hz, 2 H), 6.73 (d, 2 H) and 7.19 (d, 8 H); λ_{max}/nm (ε/dm^3 mol^{-1} cm^{-1}) 260 (7900) and 288 (5600) (EtOH); *m*/*z* (+EI) 672.3639. $C_{36}H_{52}N_2O_{10}$ −2.6 ppm.

1,2-*Bis*{2-[1-(*ethoxycarbonyl*)*ethyl*]*aminophenoxy*}*ethane* **8i**.—1,2-Bis(2-aminophenoxy)ethane (8.35 g, 32.4 mmol), *N*,*N*-diisopropylethylamine (DIPEA; 20.6 g, 160 mmol) and EBP (29.0 g, 160 mmol) were heated at 140 °C (4 h). Isolation and purification gave an oil (71% yield), $\delta(C^2HCl_3$; 60 MHz) 1.20 (t, *J* 7 Hz, 6 H), 1.47 (d, *J* 7 Hz, 6 H), 4.15 (q, *J* 7 Hz, 4 H), 4.17 (q, *J* 7 Hz, 2 H), 4.37 (s, 4 H), 4.75 (m, 4 H) and 6.50–7.00 (m, 6 H).

1,2-*Bis*{2-[1-(*ethoxycarbonyl*)*ethyl*(*ethoxycarbonylmethyl*)]-*aminophenoxy*}*ethane* **8e**.—**8i** (10 g, 22.5 mmol), DIPEA (20.6 g, 160 mmol) and EBA (26.7 g, 160 mmol) were heated without solvent at 140 °C for 4 h. The isolated product was crystallised from diisopropyl ether (IPE)/light petroleum (b.p. 40–60 °C) (1 : 1 v/v; slow) to give a white solid (36% yield), m.p. 62–64 °C, $\delta(C^2HCl_3$; 60 MHz) 1.12 (t, *J* 7 Hz, 6 H), 1.19 (t, *J* 7 Hz, 6 H), 1.42 (d, *J* 7 Hz, 6 H), 3.90–4.50 (m, 4 H), 4.17 (br s, 8 H), 4.35 (s, 4 H) and 6.98 (s, 6 H); λ_{max}/nm (ε/dm^3 mol^{-1} cm^{-1}) 258 (18 200) and 292 (7000) (EtOH); *m*/*z* (+EI) 616.2998. $C_{32}H_{44}N_2O_{10}$ −0.4 ppm. The mother-liquors contained the other stereoisomer which did not crystallise. $\delta(C^2HCl_3$; 60 MHz) 1.13 (t, *J* 7 Hz, 6 H), 1.26 (t, *J* 7 Hz, 6 H), 1.45 (d, *J* 7 Hz, 6 H), 3.90–4.40 (m, 4 H), 4.19 (br s, 8 H), 4.38 (s, 4 H) and 6.98 (s, 6 H); λ_{max}/nm (ε/dm^3 mol^{-1} cm^{-1}) 258 (18 000) and 292 (6800) (EtOH); *m*/*z* (+EI) 616.3010. $C_{32}H_{44}N_2O_{10}$ −2.3 ppm. The separated diasterisomers were loaded onto an HPLC column packed with Spherisorb silica and eluted with ethyl acetate–trimethylpentane (2:25) at 5 °C. Each product showed a single peak and a mixed sample of both showed a double peak indicating a small but distinct difference in the migration of the stereoisomers. Other analogues were **11e**, $\delta(C^2HCl_3$; 60 MHz) 1.15 (t, *J* 7 Hz, 6 H), 1.22 (t, 7 Hz, 6 H), 1.47 (d, *J* 7 Hz, 6 H), 4.16 (t, *J* 7 Hz, 2 H), 4.17 (q, *J* 7 Hz, 8 H), 4.19 (s, 4 H), 4.29 (s, 4 H) and 6.72 (m, 6 H); λ_{max}/nm (ε/dm^3 mol^{-1} cm^{-1}) 254 (12 000) and 300 (6800) (EtOH); *m*/*z* (+EI) 652.2819. $C_{32}H_{42}F_2N_2O_{10}$ −1.8 ppm; **12e** (oil), $\delta(C^2HCl_3$; 60 MHz) 1.11 (t, *J* 7 Hz, 6 H), 1.17 (t, *J* 7 Hz, 6 H), 1.36 (d, *J* 7 Hz, 3 H), 1.37 (d, *J* 7 Hz, 3 H), 4.06 (m, 12 H), 4.18 (q, *J* 7 Hz, 1 H), 4.19 (q, *J* 7 Hz, 1 H), 4.31 (s, 4 H), 6.52 (t, *J* 7, 3 Hz, 2 H), 6.65 (dd, *J* 10, 2 Hz, 2 H), 7.00 (dd, *J* 6, 2 Hz, 1 H) and 7.01 (dd, *J* 6, 2 Hz, 1 H); λ_{max}/nm (ε/cm^3 mol^{-1} cm^{-1}) 256 (6600) and 296 (3000) (EtOH); *m*/*z* (+EI) 652.2820. $C_{32}H_{42}F_2N_2O_{10}$ −1.9 ppm, and **13e** which was crystallised from light petroleum (28% yield), m.p. 95–96 °C, $\delta(C^2HCl_3$; 60 MHz) 1.07 (t, *J* 7 Hz, 6 H), 1.15 (t, *J* 7 Hz, 6 H), 1.40 (d, *J* 7 Hz, 6 H), 4.00 (q, *J* 7 Hz, 8 H), 4.13 (s, 4 H), 4.30 (t, *J* 7 Hz, 2 H), 4.31 (s, 4 H) and 7.10 (m, 6 H); λ_{max}/nm (ε/dm^3 mol^{-1} cm^{-1}) 265 (20 000) and 296infl (11 200) (EtOH); *m*/*z* (+EI) 752.2741. $C_{34}H_{42}F_6N_2O_{10}$ +0.2 ppm.

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References

1 R. Y. Tsien, *Biochemistry*, 1980, **19**, 2396.
2 G. A. Smith, T. R. Hesketh, J. C. Metcalfe, J. Feeney and P. G. Morris, *Proc. Natl. Acad. Sci. USA*, 1983, **80**, 7178.
3 S. D. Clarke, Ph.D Thesis, University of Cambridge, 1990.

4 G. Grynkiewicz, M. Poenie and R. Y. Tsien, *J. Biol. Chem.*, 1985, **260**, 3440.
5 L. A. Levy, E. Murphy and R. E. London, *Am. J. Physiol.*, 1987, **252**, C441.
6 S. R. Adams, J. P. Y. Kao, G. Grynkiewicz, A. Minta and R. Y. Tsien, *J. Am. Chem. Soc.*, 1988, **110**, 3212.
7 A. E. Martell and R. M. Smith, *Critical Stability Constants*, Plenum Press, New York, London, 1977, Vol. 3.
8 A. E. Martell and R. M. Smith, *Critical Stability Constants*, Plenum Press, New York, London, 1974, vol. 1.
9 H. L. Kirschenlohr, A. A. Grace, S. D. Clarke, Y. Shackar-Hill, J. C. Metcalfe, P. G. Morris and G. A. Smith, *Biochem. J.*, 1993, **110**, in press.
10 M. S. Nieuwenhuizen, A. P. G. Kieboom and H. van Bekkum, *Tenside Detergents*, 1985, **22**, 247.
11 J. C. Metcalfe, T. R. Hesketh and G. A. Smith, *Cell Calcium*, 1985, **6**, 183.

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