The Design and Properties of a Series of Calcium Indicators which Shift from Rhodamine-like to Fluorescein-like Fluorescence on Binding Calcium†

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The prototype for a new series of ratio-mode fluorescence indicators of cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) has been developed. The fluorophore, termed FluoRhod,‡ is a hybrid containing elements of the closely related fluorescein and rhodamine structures. The novel feature of the prototype indicator, FluoRhod-2, which incorporates a tetracarboxylate chelating element similar to that of 1,2-bis[2-aminophenoxy]ethane-*N,N,N',N'*-tetraacetic acid (BAPTA), is that it shifts from rhodamine- to fluorescein-like fluorescence on binding Ca^{2+}. The synthesis and properties of a series of tetracarboxylate derivatives of the FluoRhod fluorophore are described which led to the FluoRhod-2 structure with an apparent dissociation constant for Ca^{2+} and pK_a adjusted to meet the requirements for a $[Ca^{2+}]_i$ indicator in the ratio mode. The excitation and emission maxima of FluoRhod-2 are 537 nm and 566 nm in the absence of Ca^{2+} and 480 nm and 537 nm in the presence of Ca^{2+}. The indicator can be used in either the dual excitation or dual emission mode or the ratio of these two ratios can be used by making dual excitation and dual emission measurements. The brightness of FluoRhod-2 is comparable to that of fura-2 with the advantage of excitation in the visible range. FluoRhod-2 is insensitive to pH from 6.5 to 7.5 when used in the ratio mode, irrespective of the extent to which the indicator is complexed to Ca^{2+}.

The uses and limitations of the current generation of fluorescent indicators of cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) are well established. Fura-2 has been most used; it is about 30 times brighter than the prototype indicator, quin2.[1] The calibration problem is overcome because the excitation spectrum of fura-2 shifts when Ca^{2+} is bound and therefore allows $[Ca^{2+}]_i$ to be measured directly from the ratio of fluorescent emission intensities at two excitation wavelengths (usually 340 and 380 nm).[2] The limitations of fura-2 are mainly the short wavelengths of the excitation maxima and the insolubility of the acetoxymethyl ester (AME) derivative[3,4] used to achieve cell permeation. To overcome this the indicator is frequently dispersed in a surfactant, pluronic F-127.[4,5] Fura-2 AME is hydrolysed more slowly than quin2 AME and under some conditions bright spots of particulate fluorescence are observed in cells.[6,7] Both the slow hydrolysis and the association of the indicator with intracellular structures may be due to the hydrophobicity of fura-2 AME.

The most recent versions of fluorescent indicators for $[Ca^{2+}]_i$ are based on the fluorescein or rhodamine chromophores incorporated into BAPTA derivatives to give fluo-3 and rhod-2 with excitation wavelengths at 499 nm and 521 nm respectively.[8] Calcium binding causes an increase in fluorescence emission but as the unchelated indicators are not significantly fluorescent the indicators cannot be used for ratio measurements of $[Ca^{2+}]_i$.

There are, however, major potential advantages of using the fluorescein or rhodamine fluorophores for $[Ca^{2+}]_i$ indicators designed to be used in the ratio mode. They are the most commonly used stable fluorophores operating at long excitation and emission wavelengths (480 nm to 580 nm), well away from the most endogenous fluorophores in cells. The brightness of the fluorescein and rhodamine fluorophores, defined as $Q \cdot E_{max}$, where Q is the quantum yield and E_{max} is the maximum extinction coefficient, is more than ten times that for fura-2, offering a potential increase in sensitivity in addition to the advantages of working at longer wavelengths. We have therefore aimed to incorporate into the parent BAPTA structure a fluorophore which would shift from rhodamine-like to fluorescein-like fluorescence when Ca^{2+} is bound to the tetracarboxylate chelator, while retaining the brightness of the parent fluorophores and allowing ratio measurements in both the excitation and emission modes. The starting point for this development was a rhodol[9] derivative incorporating an aminodiacetic acid group shown in Fig. 1(*a*) which we have termed FluoRhod. There are two possible mesomeric forms of the FluoRhod fluorophore, A' and A'' in Fig. 1(*a*), which would be expected to have fluorescein- and rhodamine-like fluorescence spectra, respectively.

Several structural prototypes were considered for the development of ratio mode indicators based on the FluoRhod fluorophore. The possibility of incorporating the 9-*o*-benzoic acid ring of the xanthine system as part of a BAPTA-like chelator was considered first. The 9-*o*-benzoic acid ring is held out of the plane of the fluorophore by the *o*-carboxylic acid group and therefore the rings cannot interact mesomerically. Removal of the carboxy group is required to allow mesomeric interaction and to obtain fluorescence changes, as in the structure of fluo-3[8] [Fig. 1(*b*)]. This structural modification is unsuitable because it results in changes in fluorescence intensity but not wavelength on binding Ca^{2+}, as for fluo-3, and because planar molecules of this class (*e.g.*, acriflavines and pyronines) are much more toxic than their fluorescein and rhodamine counterparts.[10]

A more promising way of exploiting the potential change in conjugation on binding Ca^{2+} to FluoRhod derivatives is to use the aminodiacetic acid group as part of the BAPTA-like chelator structure. The main problems to be tackled, beyond

† *Abbreviations used:* AME, acetoxymethyl ester; BAPTA, 1,2-bis[2-aminophenoxy]ethane-*N,N,N',N'*-tetraacetic acid; ($[Ca^{2+}]_i$), cytosolic free Ca^{2+} concentration; DCM, dichloromethane; DIPEA, diisopropylethylamine; DMF dimethylformamide; EBA, ethyl bromoacetate; EBP, ethyl 2-bromopropionate; $K_d(M^{n+})$, equilibrium dissociation constant of a cation, where M is the atomic symbol and n is the value of the charge.

‡ A preliminary account of the properties of FluoRhod-1, a precurser of FluoRhod-2, was presented at the XII International Meeting of the Society for Analytical Cytology, in Cambridge, UK, on 14th August, 1987.

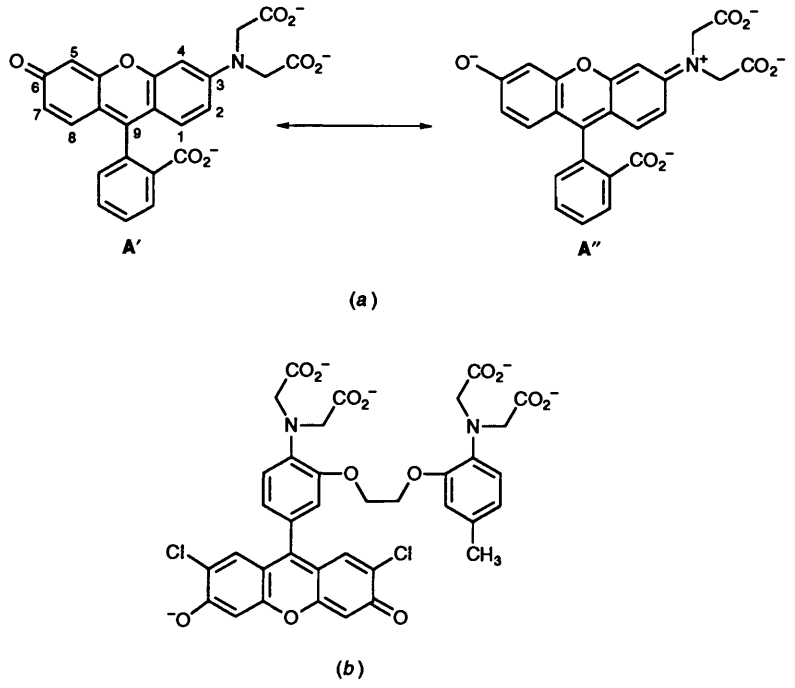
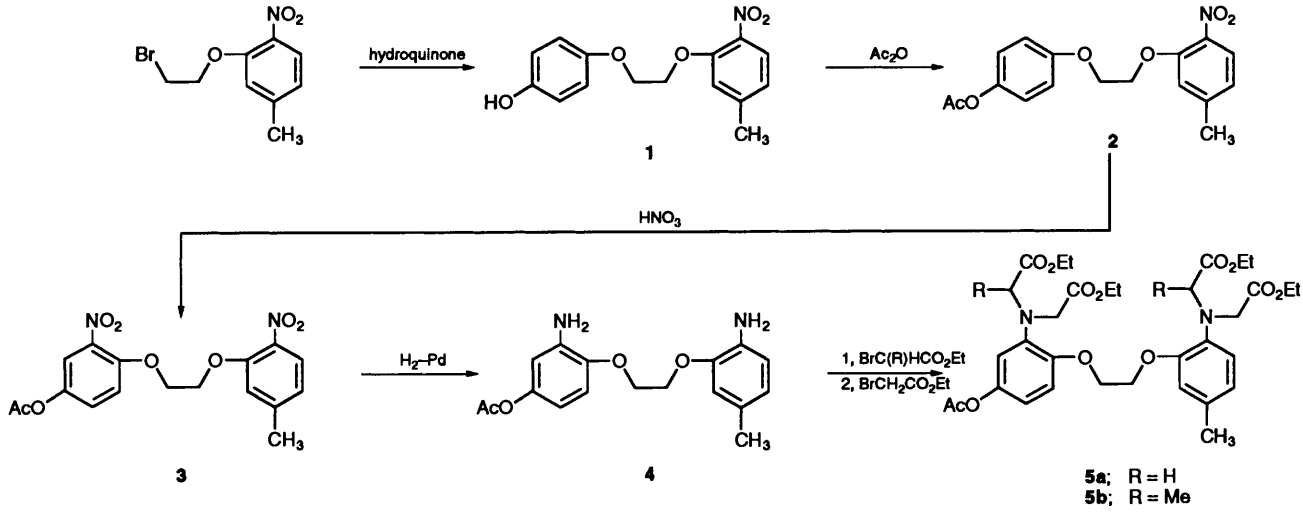


Fig. 1 Structures of fluo-3 and the mesomeric forms A′ and A″ of the FluoRhod fluorophore: (*a*) FluoRhod mesomers showing the aminoquinone (A′) and iminophenoxy (A″) forms; (*b*) fluo-3



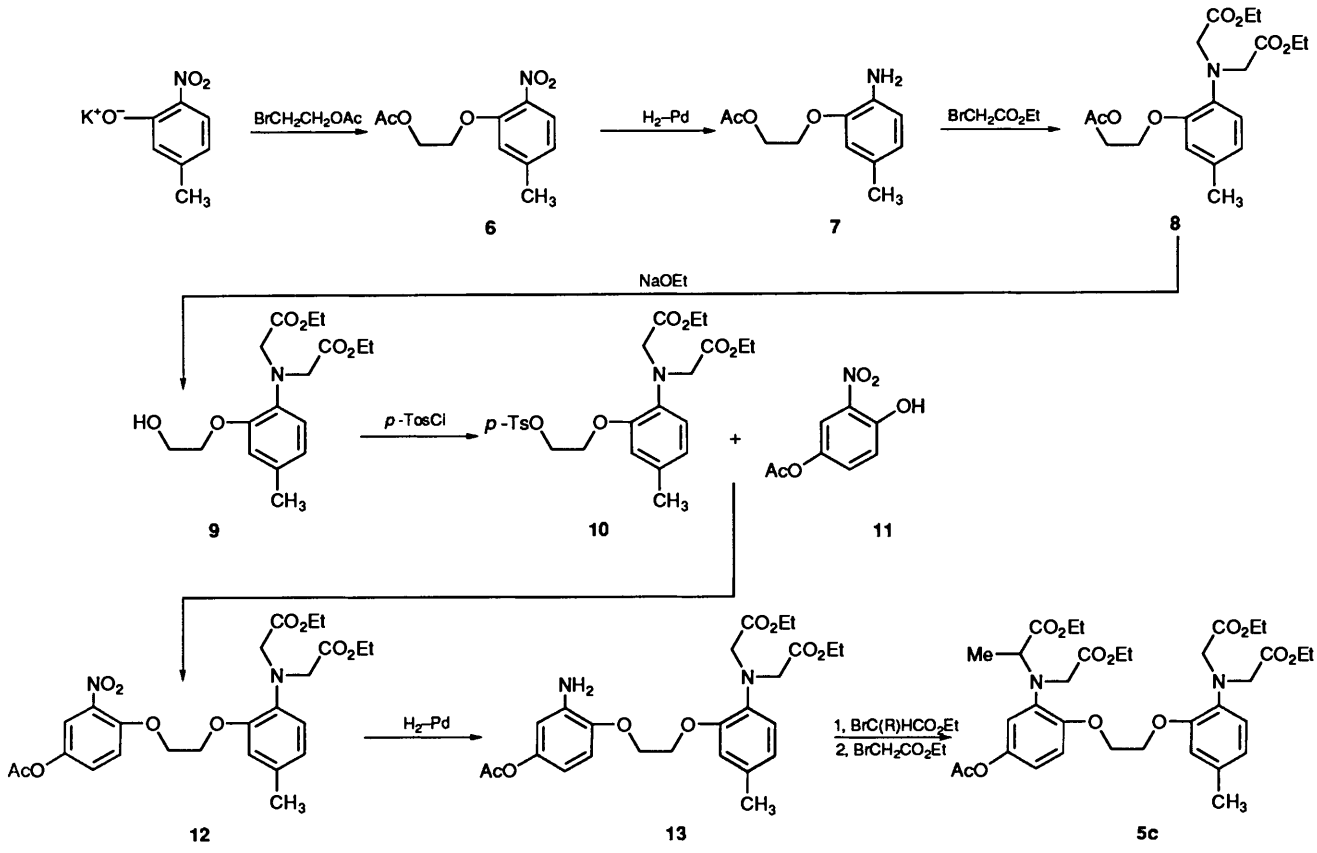
Scheme 1 Synthetic pathway to **5a** and **5b**

the inherent difficulty of the unpredictable effects of small structural modifications on fluorescence properties, were: (*i*) the incorporation of the aminodiacetic acid group of the FluoRhod fluorophore into BAPTA derivatives with appropriate fluorescence spectral shifts on binding Ca^{2+} and (*ii*) retention of an apparent K_d for Ca^{2+} in the normal $[Ca^{2+}]_i$ range.

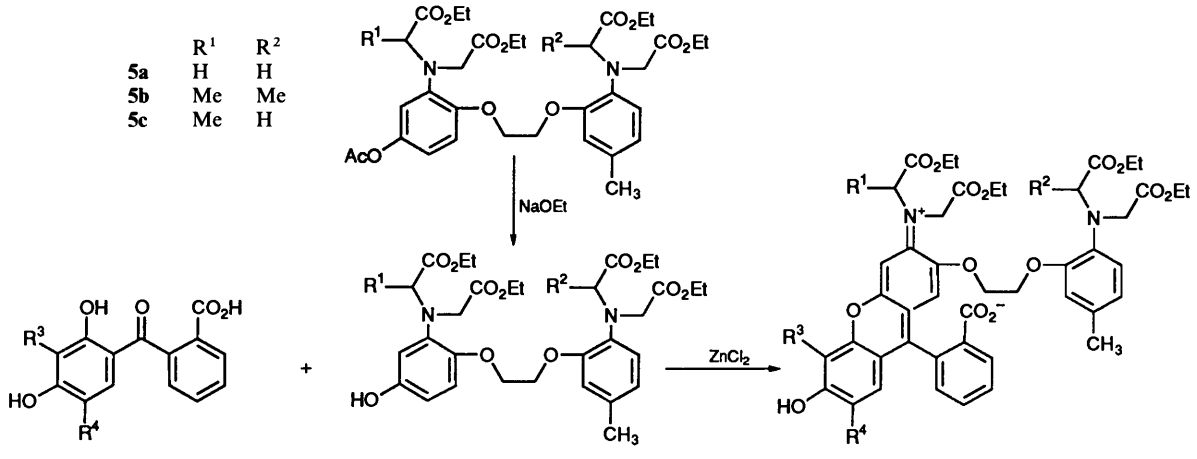
For a significant shift in the fluorescence spectrum on binding Ca^{2+} there must be a shift in the fractional contributions of the mesomeric forms of the fluorophore corresponding to A′ and A″ in Fig. 1(*a*). Ca^{2+} would be expected to bind preferentially to the mesomeric form equivalent to A′ which has the electron lone pair localised on the aminodiacetic acid nitrogen rather than to the A″ form which has a positive charge on the nitrogen. However, if the required shift in contributions from the mesomeric forms is to occur on binding Ca^{2+}, there must be a significant contribution to the structure of the uncomplexed, free indicator from the A″ form which has a low predicted affinity for Ca^{2+}. Tetracarboxylate derivatives of FluoRhod with useful fluorescence spectral shifts are therefore predicted to have substantially lower affinity for Ca^{2+} than BAPTA. For the prototype of the new series of indicators we aimed at an apparent K_d for Ca^{2+} of approximately 1 μmol dm^{-3}, which should be about optimal for measuring transient $[Ca^{2+}]_i$ spikes in single cells, where $[Ca^{2+}]_i$ rises into the micromolar concentration range.

(*iii*) The third problem was the inherent pH sensitivity of fluorescein-like structures which has been exploited in pH_i indicators (*e.g.*, BCECF).[11]

These design problems were analysed separately in a series of model compounds before attempting to assemble the optimal features for Ca^{2+} affinity and pH_i insensitivity in a single structure. We describe here the synthesis (Schemes 1–3) and main properties of a series of tetracarboxylate chelators incorporating the FluoRhod fluorophore, including FluoRhod-2, which is the structure which most closely meets the above design criteria.



Scheme 2 Synthetic pathway to **5c**



	R^1	R^2
5a	H	H
5b	Me	Me
5c	Me	H

	R^3	R^4
15a	H	H
15b	H	$CH_2CH_2CO_2Et$
15c	Me	$CH_2CH_2CO_2Et$
15d	Br	Br

	R^1	R^2
14a	H	H
14b	Me	Me
14c	Me	H

	R^1	R^2	R^3	R^4
16a	H	H	H	H
16b	H	H	H	$CH_2CH_2CO_2Et$
16c	H	H	Me	$CH_2CH_2CO_2Et$
16d	H	H	Br	Br
16e	Me	Me	H	H
16f	Me	Me	H	$CH_2CH_2CO_2Et$
16g	Me	Me	Me	$CH_2CH_2CO_2Et$
16h	Me	H	H	$CH_2CH_2CO_2Et$

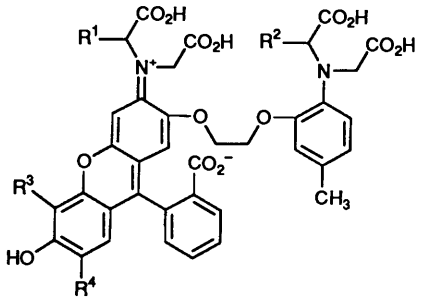
17a–h as for **16a–h** but with Et = H

Scheme 3 Synthetic pathway from **5a–c** to **17a–h**

Results and Discussion

Properties of the FluoRhod Fluorophore.—FluoRhod has fluorescence excitation and emission maxima at wavelengths intermediate between those of fluorescein and rhodamine (λ_{exc} = 520 nm, λ_{em} = 540 nm) and of similar brightness to the parent fluorophores. The fluoroescence intensity of FluoRhod

Table 1



Indicator	Substituents				log K^{Ca}	Fluorescence maxima				pK_a^1
	R^1	R^2	R^3	R^4		Ca^{2+} satd.		Ca^{2+} free		
17a	H	H	H	H	5.01	480	537	537	560	6.54
17b (FluoRhod-1)	H	H	H	$CH_2CH_2CO_2H$	5.12	480	537	537	566	6.71
17c	H	H	Me	$CH_2CH_2CO_2H$	5.20	543	570	543	570	6.60
17d	H	H	Br	Br	4.73	513	540	513	570	6.14
17e	Me	Me	H	H	6.02	480	537	537	563	7.25
17f	Me	Me	H	$CH_2CH_2CO_2H$	6.41	480	540	540	566	7.38
17g	Me	Me	Me	$CH_2CH_2CO_2H$	6.49	543	570	543	570	7.37
17h (FluoRhod-2)	Me	H	H	$CH_2CH_2CO_2H$	5.97	480	537	537	566	6.87

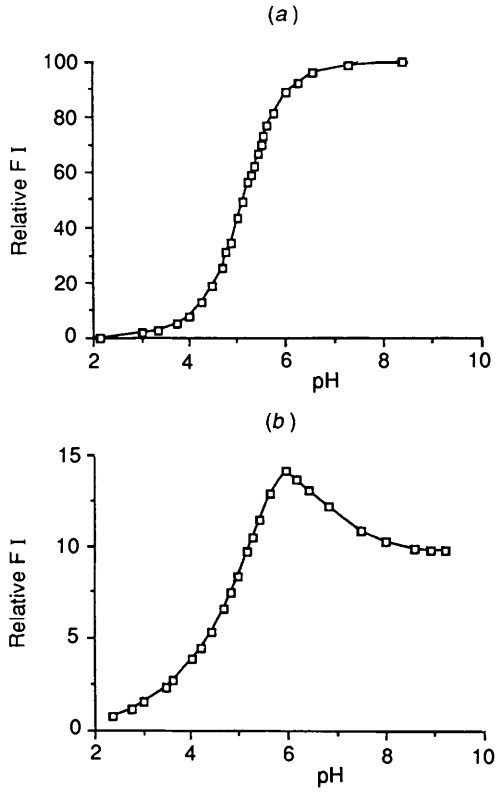


Fig. 2 pH titrations of the fluorescence of FluoRhod and **17a**. The free acids (2 μmol dm^{-3}) in 100 mmol dm^{-3} KCl, 25 mmol dm^{-3} Tris or orthophosphoric acid were mixed to give stepwise changes in pH at 37 °C. Fluorescence intensity as a function of pH for (*a*) FluoRhod with excitation at 520 nm and emission at 540 nm and (*b*) **17a** with excitation at 537 nm and emission at 560 nm.

was completely quenched on protonation with a single apparent pK_a of 5.15 [Fig. 2(*a*)]. The addition of molar concentrations of Ca^{2+} had no effect on fluorescence intensity above pH 8 but caused a progressive decrease in fluorescence as the pH was lowered below 8. This implies that FluoRhod is protonated between pH 6 and 8 without effect on the fluorescence and this protonation is attributed to the negatively charged phenoxide oxygen in the A″ mesomeric form (see below). Ca^{2+} binding to this protonated form, or a second protonation with a pK_a at 5.15, is attributed to lactonisation of the *o*-benzoic acid group with the consequent quenching of fluorescence.[12]

Incorporation of the FluoRhod Fluorophore into Tetracarboxylate Derivatives.—Two ring positions adjacent to the aminodiacetic acid group of FluoRhod are available for attachment of the 2-(2-aminophenoxy)ethoxy-*N*,*N*-diacetic acid auxochrome needed to construct BAPTA derivatives. Substitution at the xanthene 4 position was found to be inaccessible chemically and all subsequent FluoRhod derivatives were substituted at the xanthene 2 position [Fig. 1(*a*)]. The most easily synthesised compound, **17a** (R^1, R^2, R^3, R^4 = H), was prepared (see Table 1). The substitution of the auxochrome in FluoRhod at the xanthene 2 position in **17a** increased the excitation and emission maxima to 537 nm and 560 nm in the absence of Ca^{2+} (*i.e.*, towards those of rhodamine). Addition of Ca^{2+} produced a large shift in fluorescence excitation and emission maxima from rhodamine-like to fluorescein-like fluorescence at 480 nm and 537 nm (see Fig. 3). The shift in fluorescence wavelengths and the low affinity of **17a** for Ca^{2+} are both consistent with the uncomplexed, free chelator at pH 7.2 being predominantly present in the A″ mesomeric form with a positive charge on the aminodiacetic acid nitrogen. On binding Ca^{2+} the equilibrium shifts towards the A′ mesomeric form which accounts for the shift in wavelength from rhodamine-like to fluorescein-like fluorescence. The brightness of **17a** was comparable to that of fura-2 and was markedly reduced compared with that of FluoRhod. There was also a large reduction in the Ca^{2+} affinity of **17a** to $10^{5.01}$ dm^3 mol^{-1} compared with $10^{6.97}$ dm^3 mol^{-1} for BAPTA.

The effects of changing pH on the relative fluorescence intensities of FluoRhod and **17a** are illustrated in Fig. 2. Since the predominant form of the uncomplexed, free chelator is A″, the negatively charged phenoxide oxygen was assumed to be the site of the first protonation; this does not affect the fluorescence intensity of the FluoRhod fluorophore as noted earlier [see Fig. 2(*a*)] but in the presence of the substituent at the xanthene 2 position, which restricts the planarity of the xanthene 3-aminodiacetate, there is a significant contribution from the A′ which lacks rhodamine-like fluorescence. Protonation of the

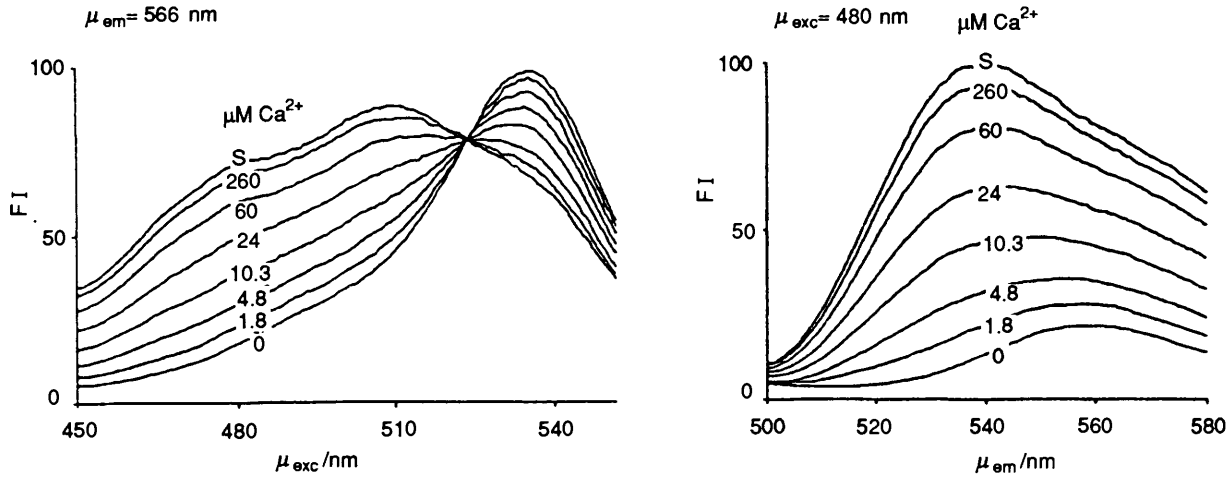


Fig. 3 Fluorescence spectra of **17a**: (*a*) fluorescence excitation and (*b*) emission spectra for **17a** (2 μmol dm^{-3}) in 40 mmol dm^{-3} citrate, 5 mmol dm^{-3} Hepes (pH 7.2) at the indicated $[Ca^{2+}]_{free}$ (s denotes saturating concentration) at 37 °C

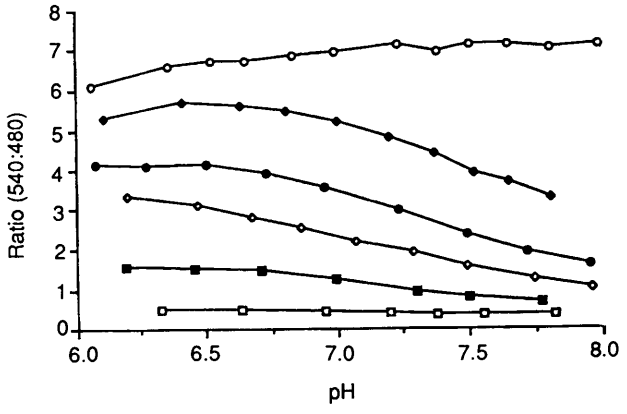


Fig. 4 Effect of pH on the ratio of fluorescence intensities of **17f** at 540 nm to 480 nm (with emission at 566 nm) as a function of $[Ca^{2+}]_{free}$. The $[Ca^{2+}]_{free}$ values are: (○) zero; (◆) 304 nmol dm^{-3}; (●) 807 nmol dm^{-3}; (◇) 1.71 μmol dm^{-3}; (■) 3.71 μmol dm^{-3}; (□) 24.3 μmol dm^{-3}.

phenolate fixes the fluorophore in the phenoxy-imino form (A″) which is reflected in an increase in rhodamine-like fluorescence with decreasing pH below 7.5; further decrease in pH causes a reduction in observed fluorescence intensity for both A′ and A″ due to lactonisation of the *o*-benzoic acid group [Fig. 2(*b*)].

These observations showed that the FluoRhod fluorophore could be incorporated into a BAPTA derivative to meet the first design requirement for a significant shift in wavelength on bonding Ca^{2+}. The problem was therefore to make substitutions in the structure of **17a** which increased the affinity for Ca^{2+} into the physiological range while retaining the substantial shift in fluorescence excitation and emission maxima on binding Ca^{2+}.

Modifications to the Structure of **17a**.—*R^3 and R^4 substituents*. It has been shown previously that the inductive effect of a carboxyethyl donor group *ortho* to the hydroxy group increases the pK_a of the fluorescein derivative, BCECF.[11] The addition of the same carboxyethyl group at R^4 (xanthene position 7) gave a modest improvement in the affinity for Ca^{2+} of **17b** with retention of the Ca^{2+}-induced rhodamine to fluorescein spectral changes and the brightness (see Table 1). The fluorescence intensity ratios at the rhodamine-like and fluorescein-like emission maxima for **17b**, termed FluoRhod-1, were independent of pH (over the physiological range) at all Ca^{2+} concentrations (data not shown). The introduction of the negatively charged carboxyethyl group also removed the small concentration dependence of the fluorescence yield noted for **17a**; this is presumably due to decreased intermolecular association and energy transfer from the bound to the free forms at higher indicator concentrations.

The improvement in affinity suggested that the addition of a second electron-donating group *ortho* to the phenol might further increase the affinity. However, substitution of a methyl group at R^3 (**17c**) gave only a small further increase in affinity for Ca^{2+} together with the unwanted effect of restricting the fluorophore to rhodamine-like fluorescence spectra in the presence and absence of Ca^{2+}. Loss of the wavelength shift also resulted from methylation at R^3 when R^1 and R^2 were methyl groups (**17g**, *cf.* **17f**).

The effect of substituting a strong inductive withdrawing group was examined. The compound with $R^3 = R^4 = Br$ showed a small decrease in affinity for Ca^{2+} (**17d**, *cf.* **17a**) as expected for an electron-withdrawing group, but substitution at R^3 almost completely abolished the fluorescein-like fluorescence of the Ca^{2+}-bound form in a similar way to **17c** (see Table 1).

R^1 and R^2 substituents. Substitution of the hydrogen atoms at R^1 and R^2 with alkyl groups was investigated. We have shown in the preceding paper that single alkyl substitution at each of the aminodiacetic acid groups in BAPTA analogues leads to increases in the Ca^{2+} affinity of the chelator with minimal effect on the spectral properties of the free and bound forms of the chelators. The increase in affinity for Ca^{2+} by methylation of the aminodiacetic acid groups was directly dependent on the extent of delocalisation of the amino groups. For ease of synthesis, a compound methylated at both R^1 and R^2 was prepared first, which resulted in a large increase in the affinity (Δ1.01 log units; *cf.* **17a** and **17e** in Table 1) which was maintained when the carboxyethyl group was added at R^4 (Δ1.29 log units; *cf.* **17b** and **17f**). Thus **17f** has high Ca^{2+} affinity ($K = 10^{6.4}$ dm^3 mol^{-1}), only four times lower than that of BAPTA, and the compound retained the Ca^{2+}-induced spectral and ratio changes of **17b** (see Fig. 4). However, the pK_a of **17f** was too high for it to be of value as an indicator: the pH dependence of the intensity ratios at the rhodamine-like and fluorescein-like emission wavelengths at Ca^{2+} concentrations which partially saturate the indicator (Fig. 4) show this clearly, from which the pK_a of ~ 7.5 can be estimated. The fluorescence ratios of the completely free and bound forms of the indicator are pH-insensitive and hence the pH sensitivity must result from the Ca^{2+}-chelating moiety that does not contribute to the fluorescence.

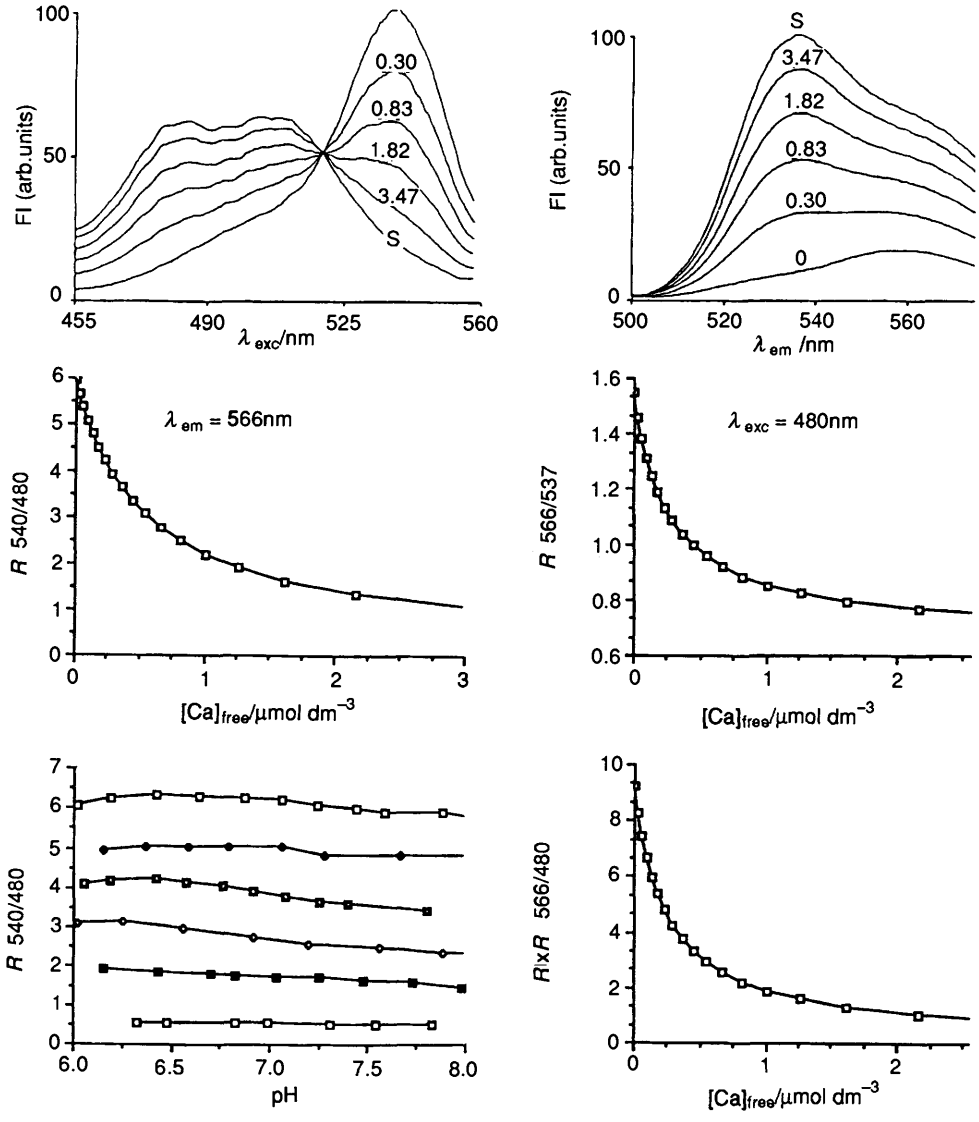
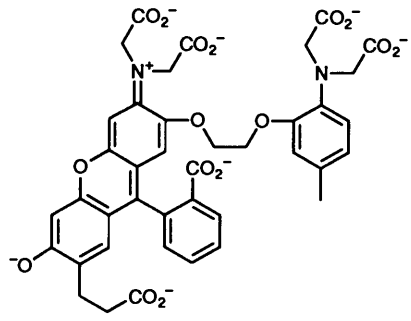


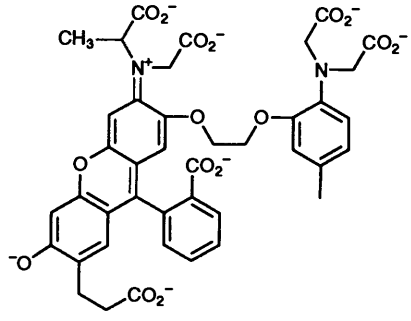
Fig. 5 Fluorescence properties of **17h**: (*a*) fluorescence excitation (emission at 566 nm) and (*b*) emission (excitation at 480 nm) spectra of **17h** (2 μmol dm^{-3}) in 40 mmol dm^{-3} citrate, 5 mmol dm^{-3} Hepes (pH 7.2) at the indicated $[Ca^{2+}]_{free}$ (s denotes saturating concentration) at 37 °C. (*c*) Ratio of fluorescence intensities at 540 nm to 480 nm (with emission at 566 nm) as a function of $[Ca^{2+}]_{free}$. (*d*) Ratio of fluorescence intensities at 566 nm to 537 nm (with excitation at 480 nm) as a function of $[Ca^{2+}]_{free}$. (*e*) Effect of pH on the ratio of fluorescence intensities shown in (*c*) at the indicated $[Ca^{2+}]_{free}$. The $[Ca^{2+}]_{free}$ are: (□) zero; (◆) 114 nmol dm^{-3}; (□) 338 nmol dm^{-3}; (◇) 723 nmol dm^{-3}; (■) 1.72 μmol dm^{-3}; (□) 10.3 μmol dm^{-3}. (*f*) The calculated product of the ratios shown in (*c*) and (*d*) [540:480 ratio (emission at 566 nm) × 566:537 ratio (excitation at 480 nm)] is approximately equivalent to the ratio of 566:480. It is expressed as a function of $[Ca^{2+}]_{free}$.

The highest group pK_a in compound **17f**, estimated from data in the preceding paper (compound **3**) as 7.6, is that of the non-fluorescent part of the chelator. Removal of the methyl group at R^2 to give **17h** reduced the highest group pK_a to 6.8. This is similar to the pK_a of FluoRhod-1, quin2 and fura-2 and would therefore be expected to give ratio measurements of $[Ca^{2+}]_i$ independent of pH in the physiological pH_i range. The Ca^{2+}-induced excitation and emission spectral changes for compound **17h** at pH 7.2 are shown in Figs. 5(*a*) and 5(*b*), with a rhodamine-like emission maximum at 566 nm and the fluorescein-like excitation maximum at 480 nm. Figs. 5(*c*) and 5(*d*) show the plots of ratio changes against $[Ca^{2+}]_{free}$ at pH 7.2 determined from Figs. 5(*a*) and 5(*b*) for the wavelengths indicated. These plots show that the 540/480 excitation ratio change is larger than that observed for the 566/537 emission ratio change. The reduction of the highest pK_a of **17h** to 6.8 was sufficient to render the fluorescence ratios essentially independent of pH over the physiological range at all Ca^{2+} concentrations [Fig. 5(*e*)]. The product of the ratios in Figs. 5(*c*) and 5(*d*) is shown in Fig. 5(*f*) indicating that it should be possible to use dual excitation and dual emission measurements to determine $[Ca^{2+}]_i$ in a single experiment. This property may be particularly useful in fluorescence microscopy. The selectivity for Ca^{2+} against Mg^{2+}[K_d (Mg^{2+}) > 1 mol dm^{-3}] of the parent indicator, **17b**, was retained on introduction of the methyl group to the chelating site at R^1, but the greatly reduced affinity of **17h** for the quenching ion Mn^{2+}[$K_d(Mn^{2+})$ = 0.1 μmol dm^{-3}] compared with BAPTA, quin2 and fura-2 was unexpected. The brightness, cation selectivity, Ca^{2+} affinity and pH insensitivity of **17h** in the ratio mode therefore meet the design requirements set out in the introduction and this indicator is termed FluoRhod-2 (Fig. 6).

FluoRhod-2 is being evaluated as a ratio-mode indicator by microinjection into a variety of cells in culture. Development of suitable acetoxymethyl ester derivatives for loading the indicator into cells is in progress. The peracetoxymethyl ester,



(a)



(b)

Fig. 6 Structures of (*a*) FluoRhod-1 and (*b*) FluoRhod-2

lacking the free benzoate carboxygroup, is non-zwitterionic and unable to lactonise. This derivative is thus prone to addition of nucleophiles (*e.g.*, water) in contrast with the stable ethyl ester (**16h**) and free acid (**17h**).

Experimental

Measurement of Cation Affinities.—Fluorescence spectra were recorded on a Perkin-Elmer 44E fluorescence spectrophotometer at 37 °C in the ratio mode to minimise distortion from the lamp output with both excitation and emission bandwidths at 3 nm. The time constant was adjusted in relation to the scan speed to give less than 3% distortion of the (uncorrected) spectra. Changes in fluorescence spectra of a solution containing 2 μmol dm^{-3} indicator, 40 mmol dm^{-3} citrate, 5 mmol dm^{-3} Hepes,* pH 7.2 were measured at specified wavelengths as a function of Ca^{2+} concentration. The Ca^{2+} concentrations were set by addition of known quantities of stock solutions of $CaCl_2$, 40 mmol dm^{-3} citrate, 5 mmol dm^{-3} Hepes, pH 7.2, containing 2 μmol dm^{-3} indicator, and the free Ca^{2+} concentrations were calculated with a computer program[13] using stability constants for citrate from Martell and Smith.[14] The values for K_d (Ca^{2+}) were obtained from plots of log [bound indicator]/[free indicator] against the log of the calculated $[Ca^{2+}]_{free}$. The computer program corrected for the effects of the presence of residual Ca^{2+} on the fluorescence intensities of the 'Ca^{2+}-free' chelators (measured by back-titration of the Ca^{2+} by addition of EGTA) and the effects of increased ionic strength on the fluorescence intensities of the 'Ca^{2+}-saturated' chelators. Slopes of the plots were 1 ± 0.02 which indicated a 1:1 indicator:Ca^{2+} binding and gave the corrected values for the $K_d(Ca^{2+})$. The program also calculated the ratio of fluorescence intensities from data at two wavelengths (for the Ca^{2+}-free and Ca^{2+}-bound species) as a function of free Ca^{2+} concentrations for the indicators for which λ_{max} shifted on addition of Ca^{2+}. The affinities for Mg^{2+} were determined by the same computer program. (The mathematical equations used in the program are described in ref. 2.) The highest pK_a values for the indicators were determined as described in references 12 and 15.

Chemical Synthesis.—The synthetic pathways to the new indicators are summarised in Schemes 1 to 3. The esters **16a–h** were hydrolysed to the acids **17a–h** by standard procedures.[1] All compounds were >99% purity as shown by thin layer chromatography on silica gel plates developed in either methanol–chloroform, ethyl acetate–toluene or chloroform–methanol–ammonia (or acetic acid)–water mixtures of varying compositions depending on the compound polarity. Proton NMR spectra were recorded on Hitachi Perkin-Elmer 60 and Bruker AM 400 spectrometers. Resonances are reported referenced to tetramethylsilane. The UV spectra are not reported as criteria of purity as they are extremely dependent on the conditions of measurement; the changes with ionic conditions, which are of no value for intracellular measurements, are reflected in the associated fluorescence spectra.

Preparation of Compounds in Scheme 1

1-(4-*Hydroxyphenoxy*)-2-(5-*methyl*-2-*nitrophenoxy*)*ethane* **1**.—1-Bromo-2-(5-methyl-2-nitrophenoxy)ethane[2] (13.5 g, 51.9 mmol) was added to a refluxing mixture of hydroquinone (108 g, 0.98 mmol) and anhydrous sodium carbonate (26 g, 0.24 mol) in dimethylformamide (DMF, 500 cm^3) under N_2 (1 h). The cooled mixture was poured into acetic acid solution (1.5 dm^3, 0.98 mol) and stirred (1 h). The crystalline product was filtered off, washed with water and dried (7.81 g, 45%), m.p. 114–116 °C; *m*/*z* (EI) 289.

1-(4-*Acetoxyphenoxy*)-2-(5-*methyl*-2-*nitrophenoxy*)*ethane* **2**.—**1** (7.8 g, 23.3 mmol) was stirred in 100 cm^3 pyridine and treated with 67 cm^3 acetic anhydride (1 h). The reaction mixture was cooled and cold water was carefully added until no increase in temperature was observed (< 50 °C) and then water (500 cm^3) was added to precipitate out the product (4 °C). The solid was filtered off, dissolved in 420 cm^3 hot ethanol with a little activated charcoal and filtered. As the solution cooled, light-brown crystals were formed (6.42 g, 82.2%), m.p. 93–95 °C; *m*/*z* (EI) 331.

1-(4-*Acetoxy*-2-*nitrophenoxy*)-2-(5-*methyl*-2-*nitrophenoxy*)-*ethane* **3**.—**2** (6.25 g, 18.8 mmol) was added to a nitrating mixture (comprising 45 cm^3 acetic acid, 45 cm^3 acetic anhydride and 9 cm^3 nitric acid cooled to below 10 °C) and stirred for 1 h. Ice–water (66 cm^3) was added to precipitate the product which was filtered off, dried, dissolved in the minimum of dichloromethane (DCM) and recrystallised from methanol to give white crystals (6.2 g, 87%), m.p. 136–138 °C, δ($CDCl_3$–CD_3OD; 60 MHz) 2.28 (t, 7 Hz, 12 H), 2.40 (s, 3 H), 4.48 (s, 4 H), 6.82 (d, 8 Hz, 1 H), 6.98 (s, 1 H), 7.28 (d, 2 Hz, 1 H), 7.29 (s, 2 H), 7.60 (d, 2 Hz, 1 H) and 7.73 (d, 8 Hz, 1 H); *m*/*z* (EI) 376.

1-(4-*Acetoxy*-2-*aminophenoxy*)-2-(2-*amino*-5-*methyl*-*phenoxy*)*ethane* **4**.—**3** (1.8 g, 4.8 mmol) in 150 cm^3 ethanol was hydrogenated with 500 mg 5% palladium-on-carbon (30 min; 750 cm^3 H_2 used). The mixture was filtered through Hyflo Supercel, washed with DCM and the mixed solvent was removed by evaporation. The residue was dispersed in 20 cm^3 ethanol, precipitated by addition of water and collected by filtration (1.48 g, 98%); *m*/*z* (EI) 316.

1-[4-*Acetoxy*-2-*bis*(*ethoxycarbonylmethyl*)*aminophenoxy*]-2-

* 4-(2-Hydroxyethyl)piperizin-1-ylethanesulfonic acid.

[2-*bis*(*ethoxycarbonylmethyl*)*amino*-5-*methylphenoxy*]*ethane* **5a**.—**4** (1.45 g, 6.9 mmol), diisopropylethylamine (DIPEA; 4.39 g, 34.1 mmol) and ethyl bromoacetate (EBA; 6.18 g, 34.1 mmol) were heated at 140 °C overnight. The mixture was cooled, diluted with toluene and washed with 1 mol dm^{-3} ammonium phosphate pH 4.0 (3 ×), 1 mol dm^{-3} phosphate buffer pH 2.0 (3 ×), water (1 ×) and saturated potassium hydrogencarbonate solution (2 ×). The solvent was dried and removed at low pressure and the excess reagent was transferred to a trap at high vacuum pressure (50 °C). The product was isolated on a silica gel column (150 g, pre-equilibrated in 5% ethyl acetate in toluene, elution with 5–20% ethyl acetate in toluene) to yield a light-yellow solid (recrystallised from ethanol) (2.13 g, 70%), m.p. 92–94 °C, $\delta(CDCl_3$; 60 MHz) 1.15 (t, *J* 7 Hz, 12 H), 2.25 (s, 3 H), 4.05 (q, *J* 7 Hz, 8 H), 4.13 (s, 8 H), 4.35 (s, 4 H) and 6.60 (m, 6 H); *m*/*z* (EI) 660.

1-{4-*Acetoxy*-2-[1-(*ethoxycarbonyl*)*ethyl*(*ethoxycarbonylmethyl*)*amino*]*phenoxy*}-2-{2-[1-*ethoxycarbonylethyl*(*ethoxycarbonylmethyl*)*amino*]-5-*methylphenoxy*}*ethane* **5b**.—**4** (640 mg, 2.03 mmol), acetonitrile (3 cm^3), proton sponge (1,8-bis(dimethylamino)naphthalene; 1.74 g, 8.1 mmol) and ethyl 2-bromopropionate (EBP; 1.47 g, 8.1 mmol) were stirred at 115 °C under N_2 (22 h). The rate of reaction of alkylation was followed by TLC. When **4** had been alkylated in two steps, giving a single product, a further 1.74 g Proton Sponge (8.1 mmol) and 4.06 g EBA (24.3 mmol) were added and the reaction was left at 115 °C (under N_2) until the other two positions were alkylated (24 h). The mixture was extracted into toluene and washed (see the preparation of **5a** for details). The product was isolated on 180 g silica gel (pre-equilibrated in 10% ethyl acetate in toluene, elution with 15–30% ethyl acetate in toluene), yellow oil (1.25 g, 90%), $\delta(CDCl_3$; 60 MHz) 1.12 (t, *J* 7 Hz, 6 H), 1.20 (t, *J* 7 Hz, 6 H), 1.62 (d, *J* 7 Hz, 3 H), 1.65 (d, *J* 7 Hz, 3 H), 2.43 (s, 3 H), 2.46 (s, 3 H), 4.25 (q, *J* 7 Hz, 8 H), 4.31 (s, 6 H), 4.50 (s, 4 H), 6.95 (s, 2 H) and 7.02 (m, 4 H); *m*/*z* (EI) 688.

Preparation of Compounds in Scheme 2

1-*Acetoxy*-2-(5-*methyl*-2-*nitrophenoxy*)*ethane* **6**.—Potassium 5-methyl-2-nitrophenoxide[2] (10 g, 52.3 mmol) and 2-bromoethyl acetate (7.5 cm^3) in 25 cm^3 DMF were heated at 120 °C (1 h). Anhydrous potassium carbonate (500 mg) was added and the mixture was maintained at 120 °C for a further hour during which time it slowly solidified. When cool, the reaction mixture was stirred with water. Potassium carbonate (2.0 g) was added with a few seeds of **6** and the slurry was diluted to 100 cm^3 with water (0 °C). The yellow solid was filtered off, washed with water, ice-cold ethanol–water (1:1 v/v) and then dried, m.p. 38–40 °C (10.8 g, 86%).

1-*Acetoxy*-2-(2-*amino*-5-*methylphenoxy*)*ethane* **7** *and* 1-*Acetoxy*-2-[2-*bis*(*ethoxycarbonylmethyl*)*amino*-5-*methylphenoxy*]-*ethane* **8**.—**6** (7.8 g, 32.6 mmol) in 500 cm^3 ethanol was hydrogenated with 700 mg 10% palladium-on-carbon (30 min; 2.2 dm^3 H_2 used). The solvent was removed by evaporation and the residue was dissolved in toluene and evaporated (4 ×) and dried (**7**). DIPEA (20 cm^3) and EBA (20 cm^3) in acetonitrile (40 cm^3) were heated with the **7** at 100 °C overnight. The mixture was cooled, diluted with toluene and washed with 1 mol dm^{-3} ammonium phosphate pH 4.0 (3 ×), 1 mol dm^{-3} phosphate buffer pH 2 (3 ×), water and saturated potassium hydrogencarbonate solution (2 ×). The solvent was dried over potassium carbonate, filtered and removed at low pressure and the excess of reagent was transferred to a trap at high vacuum pressure (50 °C). The product was separated on a silica gel column [250 g, pre-equilibrated in ethyl acetate–toluene (1:20 v/v), elution with a gradient of ethyl acetate–toluene 1:20 v/v to 1:10 v/v] to give a yellow gum (12 g, 96%); *m*/*z* (EI) 381.

2-[2-*Bis*(*ethoxycarbonylmethyl*)*amino*-5-*methylphenoxy*]-*ethanol* **9**.—A trace of 300 mmol dm^{-3} sodium ethoxide solution was added to a stirred solution of **8** (12 g) in absolute ethanol. The rate of the deacylation was monitored by TLC [ethyl acetate–toluene (1:10, v/v)]. When the reaction was complete (15 min) one drop of glacial acetic acid was added and the product was evaporated to dryness from toluene. The residue was dispersed in toluene and filtered to remove any sodium acetate and then dried. Pale-yellow crystalline solid (10.6 g, 99%).

1-*p*-*tosyloxy*-2-[2-*bis*(*ethoxycarbonylmethyl*)*amino*-5-*methylphenoxy*]*ethane* **10**.—Toluenesulfonyl chloride (15 g, 79 mmol) was stirred with **9** (10.5 g) in 50 cm^3 pyridine at 5–10 °C for 4 h. Water was added while the reaction temperature was maintained at less than 20 °C (stir 30 min), and the product was extracted into toluene, washed with dilute HCl (3 ×), water and saturated sodium carbonate solution. The organic layer was dried over anhydrous sodium carbonate, filtered and dried. The product was dissolved in a minimum of toluene and crystallised from light petroleum (b.p. 40–60 °C) (15 g, 98%), yellow solid, $\delta(CDCl_3$; 60 MHz) 1.20 (t, *J* 7 Hz, 6 H), 2.25 (s, 3 H), 2.42 (s, 3 H), 4.10 (s, 4 H), 3.9–4.3 (m, 8 H), 6.63 (s, 1 H), 6.83 (s, 2 H), 7.40 (d, *J* 9 Hz, 2 H) and 7.90 (d, 9 Hz, 2 H); *m*/*z* (EI) 493.

4-*Acetoxy*-2-*nitrophenol* **11**.—Acetic anhydride (102 g, 1 mol) was added to hydroquinone (110 g, 1 mol) dissolved in 380 cm^3 pyridine (60 °C) and the reaction was allowed to go to completion (1 h). The solution was cooled on ice and water (550 cm^3) was added to precipitate the diacetate compound which was filtered off (1,4-diacetoxybenzene, m.p. 121–122 °C). HCl (350 cm^3; 10 mol dm^{-3}) was added slowly to the ice-cooled, stirred filtrate (< 20 °C) to bring it to pH 7.0. Any precipitated solid was filtered off and the filtrate was acidified to pH 3.0, extracted into 1 dm^3 ethyl acetate (batch extraction) and the filtrate was back-extracted into 0.5 dm^3 ethyl acetate. The extracts were washed with water (2 ×) to remove the pyridine and dried over anhydrous magnesium sulfate. The solvent was removed to yield a pale yellow mobile oil which crystallised very slowly at room temperature and solidified at 4 °C. The white, low-melting 4-acetoxyphenol was stored at −30 °C.

A mixture of nitric acid (1.27 g, 20 mmol) and acetic acid (1.27 g) was added dropwise to 4-acetoxyphenol (2.05 g, 13 mmol) dissolved in 30 cm^3 acetic acid and stirred over ice (*n.b.* the mixture freezes below 4 °C). When the reaction was complete (10 min) the mixture was stirred rapidly and ice and water were added. The solid was filtered off and washed with cold water; it was crystallised from methanol (1.1 g, 43%), $\delta(CDCl_3$; 60 MHz) 2.3 (s, 3 H), 7.13 (d, *J* 9 Hz, 1 H), 7.47 (dd, *J* 9, 3 Hz, 1 H) and 7.83 (d, *J* 3 Hz, 1 H); *m*/*z* (EI) 197.

1-(4-*Acetoxy*-2-*nitrophenoxy*)-2-[2-*bis*(*ethoxycarbonylmethyl*)*amino*-5-*methylphenoxy*]*ethane* **12**.—**11** (320 mg, 1.5 mmol) in ethanol was treated with potassium carbonate (69 mg) in the minimum of water. The solvent was evaporated off and the potassium salt was dried and added to **10** (493 mg, 1 mmol) in DMF (4 cm^3) and heated at 100 °C (1 h). The mixture was cooled and partitioned between water and toluene. The product was extracted into toluene, washed with aqueous sodium carbonate and dried over magnesium sulfate. The product was isolated on a silica gel column [20 g, pre-equilibrated in the ethyl acetate–toluene (1:20 v/v), elution with ethyl acetate–toluene (gradient from 1:20 v/v to 3:20 v/v)] and it was crystallised from toluene and light petroleum (b.p. 40–60 °C) (373 mg, 72%, $\delta(CDCl_3$; 60 MHz) 2.30 (s, 3 H), 7.13 (d, *J* 9 Hz, 1 H), 7.47 (dd, *J* 9, 3 Hz, 1 H) and 7.83 (d, *J* 3 Hz, 1 H); *m*/*z* (EI) 518.

1-(4-*Acetoxy*-2-*aminophenoxy*)-2-[2-*bis*(*ethoxycarbonylmethyl*)*amino*-5-*methylphenoxy*]*ethane* **13** *and* 1-{4-*Acetoxy*-2-[1-*ethoxycarbonylethyl*(*ethoxycarbonylmethyl*)*amino*]-*phenoxy*}-2-[2-*bis*(*ethoxycarbonylmethyl*)*amino*-5-*methylphenoxy*]*ethane* **5c**.—**12** (350 mg, 675 mmol) was hydrogenated in ethanol with a palladium–carbon catalyst (see the preparation of **7**). The reduced compound **13**, dissolved in 500 cm^3 acetonitrile, was treated with EBP (400 mg, 2.21 mmol) and proton sponge (400 mg, 1.86 mmol) (6 h) [see the preparation of **8**]. Product isolation was by column chromatography [40.0 g silica gel pre-equilibrated in ethyl acetate–toluene (2:13 v/v), elution with ethyl acetate–toluene (2:13 v/v)]. The gum (300 mg, 76%, 511 mmol) was alkylated in 500 cm^3 acetonitrile with 850 mg EBA (5.10 mmol) and Proton Sponge (4.0 mmol) following the procedure for the preparation of **8** (10 h) to give a gum (230 mg, 67%), $\delta(CDCl_3$; 60 MHz) 0.86 (t, *J* 7 Hz, 3 H), 1.12 (t, *J* 7 Hz, 3 H), 1.18 (t, *J* 7 Hz, 6 H), 1.22 (t, *J* 7 Hz, 3 H), 1.40 (m, 4 H), 2.27 (s, 6 H), 3.9–4.4 (m, 9 H), 4.20 (s, 6 H), 4.35 (s, 4 H) and 6.6–7.1 (m, 6 H); *m*/*z* (EI) 674.

Preparation of Compounds in Scheme 3

1-[2-*Bis*(*ethoxycarbonylmethyl*)*amino*-4-*hydroxyphenoxy*]-2-[2-*bis*(*ethoxycarbonylmethyl*)*amino*-5-*methylphenoxy*]*ethane* **14a**.—**5a** (1.0 g, 1.5 mmol) was dissolved in 50 cm^3 absolute ethanol and sodium ethoxide (1.6 mmol) was added. The reaction was stirred in a moisture-tight conical flask (10 min) and then poured into acetic acid (6.4 mmol dm^{-3}; 250 cm^3), extracted into DCM and washed with dilute HCl. The aqueous layer was re-extracted with DCM (2 ×); the organic layer was dried over sodium sulfate, filtered, evaporated and dried to yield a gum; this was crystallised from DCM and light petroleum (b.p. 40–60 °C) (860 mg, 86%), m.p. 83–84 °C. (Minta *et al.*,[8] reported a melting point of 82–84 °C for their compound **I**, identical with **14a**), $\delta(CDCl_3$; 60 MHz) 1.10 (t, *J* 7 Hz, 12 H), 2.20 (s, 3 H), 4.02 (q, *J* 7 Hz, 8 H), 4.07 (s, 8 H), 4.12 (s, 4 H), 6.31 (s, 2 H) and 6.54 (m, 4 H).

1-{2-[1-*Ethoxycarbonylethyl*(*ethoxycarbonylmethyl*)*amino*]-4-*hydroxyphenoxy*}-2-{2-[1-*ethoxycarbonylethyl*(*ethoxycarbonylmethyl*)*amino*]-5-*methylphenoxy*}*ethane* **14b**.—**5b** was deacetylated to **14b** following the method for **14a**; $\delta(CDCl_3$; 60 MHz) 1.15 (t, *J* 7 Hz, 12 H), 1.20 (t, *J* 7 Hz, 12 H), 1.45 (d, *J* 7 Hz, 6 H), 2.27 (s, 3 H), 4.11 (q, 7 Hz, 8 H), 4.15 (s, 6 H), 4.30 (s, 4 H) and 6.62 (m, 6 H).

1-{2-[2-*Ethoxycarbonylethyl*(*ethoxycarbonylmethyl*)*amino*]-4-*hydroxyphenoxy*}-2-[2-*bis*(*ethoxycarbonylmethyl*)*amino*-5-*methylphenoxy*]*ethane* **14c**.—**5c** was deacetylated to **14c** following the method for **14a**: $\delta(CDCl_3$; 60 MHz) 1.20 (t, *J* 7 Hz, 12 H), 1.43 (d, *J* 7 Hz, 3 H), 2.26 (s, 3 H), 4.00 (q, *J* 7 Hz, 6 H), 4.14 (s, 7 H), 4.28 (s, 4 H), 6.56 (m, 2 H), 6.72 (m, 2 H) and 6.76 (m, 2 H).

2′-*Carboxy*-2,4-*dihydroxybenzophenone* **15a**.—Resorcinol (6.5 g, 59.1 mmol) was added to a solution of phthalic anhydride (9.0 g, 60.8 mmol) and aluminium chloride (18.3 g, 137.0 mmol) dissolved in 150 cm^3 nitrobenzene, flushed with N_2 and stirred at room temperature overnight. The mixture was poured into an emulsion of HCl (0.5 mol dm^{-3}; 700 cm^3) and light petroleum (b.p. 40–60 °C) and stirred rapidly (2 h) to precipitate the product. The solutions were decanted off and the product was filtered off and washed with 0.1 mol dm^{-3} HCl and light petroleum (b.p. 40–60 °C). The product was crystallised from 50% methanol in water and recrystallised from methanol (13.6 g, 89%), m.p. 196–198 °C, $\delta(CDCl_3$; 60 MHz) 6.30 (dd, *J* 3, 8 Hz, 1 H), 6.40 (s, 1 H), 6.95 (d, *J* 8 Hz, 1 H), 7.45 (m, 1 H), 7.65 (m, 2 H) and 8.05 (m, 1 H).

2′-*Carboxy*-2,4-*dihydroxy*-5-(2-*ethoxycarbonylethyl*)*benzophenone* **15b**.—Ethyl 3-(2,4-dihydroxyphenyl)propionate[11] (5.0 g, 23.8 mmol) was added to phthalic anhydride (3.76 g, 25.4 mmol) and aluminium chloride (7.6 g, 56.9 mmol) dissolved in 150 cm^3 nitrobenzene. The reaction procedure for **15a** was followed, but no solid precipitated while the mixture was being washed. The liquids were therefore decanted and the oily product was extracted into methanol–chloroform (1:20), adsorbed onto a column containing silica gel pre-equilibrated with the same solvent and eluted with methanol–chloroform (1:5). The gummy solid was triturated with diethyl ether (3 h) to yield a pale yellow crystalline solid (3.2 g, 37%), m.p. 140–145 °C, $\delta(CDCl_3$; 60 MHz) 1.03 (t, *J* 7 Hz, 3 H), 2.42 (m, 4 H), 3.89 (q, *J* 7 Hz, 2 H), 6.39 (s, 1 H), 6.78 (s, 1 H), 7.39 (m, 1 H), 7.66 (m, 2 H) and 7.96 (m, 1 H).

The analogue 2′-carboxy-2,4-dihydroxy-5-ethoxycarbonylethyl-3-methylbenzophenone **15c** was prepared from 7-hydroxy-8-methylcoumarin by the method for **15b** and 3,5-dibromo-2′-carboxy-2,4-dihydroxybenzophenone **15d** was the doubly brominated derivative of **15a**.

2-(2-{2-[2-*Bis*(*ethoxycarbonylmethyl*)*amino*-5-*methylphenoxy*]*ethoxy*}-3-[*bis*(*ethoxycarbonylmethyl*)*iminio*]-6-*hydroxy*-3H-*xanthen*-9-*yl*)*benzoate* **16a**.—A solution of fused zinc chloride (14 mg, 100 μmol) in 500 cm^3 tetrahydrofuran was added to **14a** (25.8 mg, 100 μmol) and **15a** (61.8 mg, 100 μmol) in 0.5 cm^3 tetrahydrofuran and heated at 140 °C (30 min). The red oil was dissolved in ethanol and partitioned between DCM and aqueous sodium acetate (0.5 mol dm^{-3}). The organic layer was dried and the product was isolated by column chromatography [10 g, pre-equilibrated in methanol–chloroform (1:10), elution with methanol–chloroform (1:10 v/v to 3:20 v/v)]. The product was further purified by preparative TLC [methanol–chloroform (1:10)]. Red solid (32 mg, 38%), $\delta(CDCl_3$; 60 MHz) 1.15 (t, 7 Hz, 12 H), 2.25 (s, 3 H), 3.97 (q, *J* 7 Hz, 8 H), 4.03 (s, 8 H), 4.08 (s, 4 H), 6.22 (s, 1 H), 6.55 (m, 7 H), 7.12 (m, 1 H), 7.60 (m, 2 H) and 8.02 (m, 1 H); λ_{max}/nm (ε/dm^3 mol^{-1} cm^{-1}) 495 (2300), 522 (3500) (EtOH); *m*/*z* (+FAB, NOBA, M + 1) 841.32080, $C_{45}H_{49}N_2O_{14}$ −2.9 ppm.

The other analogues were formed by cyclocondensation reactions of **14x** (**x** is **a**–**c**) and **15y** (**y** is **a**–**d**) similar to the preparation of **16a**.

2-(2-{2-[2-*Bis*(*ethoxycarbonylmethyl*)*amino*-5-*methylphenoxy*]*ethoxy*}-3-[*bis*(*ethoxycarbonylmethyl*)*iminio*]-6-*hydroxy*-7-(2-*ethoxycarbonylethyl*)-3H-*xanthen*-9-*yl*)*benzoate* **16b** (**14a** + **15b**). $\delta(CDCl_3$; 60 MHz) 1.12 (t, *J* 7 Hz, 6 H), 1.13 (t, *J* 7 Hz, 6 H), 1.21 (t, *J* 7 Hz, 3 H), 2.23 (s, 3 H), 2.63 (t, *J* 7 Hz, 2 H), 2.65–2.85 (m, 2 H), 3.9–4.2 (m, 8 H), 4.03 (s, 4 H), 4.18 (s, 4 H), 6.10 (s, 1 H), 6.43 (s, 1 H), 6.57 (d, *J* 1 Hz, 1 H), 6.64 (s, 1 H), 6.65 (dd, *J* 8, 1 Hz, 1 H), 6.71 (d, *J* 8 Hz, 1 H), 6.75 (s, 1 H), 7.10 (dd, *J* 6, 1 Hz, 1 H), (7.14, m, 1 H), 7.60 (m, 2 H) and 8.01 (dd, *J* 6, 1 Hz, 1 H); λ_{max}/nm (ε/dm^3 mol^{-1} cm^{-1}) 495 (24 000) and 522 (30 000) (EtOH); *m*/*z* (+FAB, NOBA, M + 1) 941.372 80, $C_{50}H_{57}N_2O_{16}$ −2.2 ppm.

2-(2-{2-[2-*Bis*(*ethoxycarbonylmethyl*)*amino*-5-*methylphenoxy*]*ethoxy*}-3-[*bis*(*ethoxycarbonylmethyl*)*iminio*]-5-*methyl*-6-*hydroxy*-7-(2-*ethoxycarbonylethyl*)-3H-*xanthen*-9-*yl*)*benzoate* **16c** (**14a** + **15c**).—$\delta(CDCl_3$; 60 MHz) 1.10 (t, *J* 7 Hz, 6 H), 1.13 (t, *J* 7 Hz, 9 H), 2.20 (s, 3 H), 2.29 (s, 3 H), 2.4–2.8 (m, 4 H), 3.8–4.3 (m, 8 H), 4.0 (s, 8 H), 4.2 (br s, 4 H), 6.25 (s, 1 H), 6.4 (s, 1 H), 6.5–6.8 (m, 4 H), 7.2 (m, 1 H), 7.6 (m, 2 H) and 8.1 (m, 1 H); λ_{max}/nm (ε/dm^3 mol^{-1} cm^{-1}) 502 (20 000) and 536 (25 000) (EtOH); *m*/*z* (+FAB, NOBA, M + 23) 978.372 70, $C_{51}H_{59}N_2NaO_{16}$ +3.6 ppm.

2-[2-(2-{2-[1-*Ethoxycarbonylethyl*(*ethoxycarbonylmethyl*)-

amino]-5-*methylphenoxy*}*ethoxy*)-3-[1-*ethoxycarbonylethyl*-(*ethoxycarbonylmethyl*)*iminio*]-6-*hydroxy*-7-(2-*ethoxycarbonylethyl*)-3H-*xanthen*-9-*yl*)*benzoate* **16f** (**14b** + **15b**).—δ($CDCl_3$–MeOD; 400 MHz) 1.14 (br t, *J* 7 Hz, 3 H), 1.166 (t, *J* 7 Hz, 6 H), 1.183 (t, *J* 7 Hz, 3 H), 1.24 (t, *J* 7 Hz, 3 H), 1.33 (br d, *J* 7 Hz, 3 H), 1.475 (d, *J* 7 Hz, 3 H), 2.25 (s, 3 H), 2.49 (t, *J* 7 Hz, 2 H), 2.6–2.8 (m, 2 H), 3.50 (q, *J* 7 Hz, 1 H), 3.66 (q, *J* 7 Hz, 1 H), 3.843 (m, 12 H), 4.47 (br q, *J* 7 Hz, 12 H), 6.24 (s, 0.5 H), 6.25 (s, 0.5 H), 6.49 (s, 1 H), 6.64 (d, *J* 8 Hz, 1 H), 6.65 (s, 1 H), 6.69 (d, *J* 8 Hz, 1 H), 6.85 (s, 0.5 H), 6.86 (s, 0.5 H), 6.88 (s, 0.5 H), 7.15 (m, 1 H), 7.63 (m, 2 H), 8.04 (m, 1 H); λ_{max}/nm (ε/dm^3 mol^{-1} cm^{-1}) 492 (66 000) and 524 (78 000) (EtOH); *m*/*z* (+FAB, NOBA, M + 1) 969.406 40, $C_{52}H_{61}N_2O_{16}$ −4.5 ppm.

2-[2-(2-{2-[*Bis*(*ethoxycarbonylmethyl*)*amino*]-5-*methylphenoxy*}*ethoxy*)-3-[1-*ethoxycarbonylethyl*(*ethoxycarbonylmethyl*)]*iminio*-6-*hydroxy*-7-(2-*ethoxycarbonylethyl*)-3H-*xanthene*-9-*yl*)*benzoate* **16h** (**14c** + **15b**).—δ($CDCl_3$–MeOD; 400 MHz) 1.115 (t, *J* 7 Hz, 1.5 H), 1.12 (t, *J* 7 Hz, 1.5 H), 1.15 (t, *J* 7 Hz, 6 H), 1.17 (t, *J* 7 Hz, 3 H), 1.24 (t, *J* 7 Hz, 3 H), 1.465 (d, *J* 7 Hz, 3 H), 2.24 (s, 3 H), 2.49 (t, *J* 7 Hz, 2 H), 2.6–2.8 (m, 2 H), 3.67 (q, 7 Hz, 2 H), 3.8–3.43 (m, 9 H), 4.01 (s, 4 H), 4.45 (q, *J* 7 Hz, 2 H), 6.16 (s, 1 H), 6.43 (s, 1 H), 6.60 (s, 0.5 H), 6.61 (s, 0.5 H), 6.66 (d, *J* 8 Hz, 1 H), 6.68 (s, 1 H), 6.72 (d, *J* 8 Hz, 1 H), 6.82 (s, 0.5 H), 6.83 (s, 0.5 H), 7.14 (m, 1 H), 7.65 (m, 2 H) and 8.02 (m, 1 H); λ_{max}/nm (ε/dm^3 mol^{-1} cm^{-1}) 492 (29 000) and 524 (35 000) (EtOH); *m*/*z* (+FAB, NOBA, M + 1) 955.390 50, $C_{51}H_{59}N_2O_{16}$ −4.3 ppm.

Compounds **16d**, **16e** and **16g** were prepared by identical procedures from **14a** + **15d**, **14b** + **15a** and **14b** + **15c**, respectively.

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