A New ^{19}F NMR Indicator for Intracellular Sodium

Gerry A. Smith,* Heide L. Kirschenlohr, James C. Metcalfe and Sonia D. Clarke
Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge, UK CB2 1QW

The effect of increased benzo substitution on the cation-binding strength of diaza-18-crown-6 has been investigated. The planarity constraint imposed by increased substitution reduces the effective cavity size thus giving rise to an increased selectivity for sodium over potassium, magnesium and calcium that is sufficient to provide a class of indicators suitable for measuring intracellular free sodium. The cation binding parameters and the associated spectral changes of a selection of compounds with varied aryl fluorination patterns have shown that there is very little electronic interaction between the aromatic rings and the bound cation through the heteroatoms of the crown ether. Only small adjustments to the cation affinity can therefore be achieved *via* alteration of the aryl substitution patterns. Aryl substitution adjacent to the hetero atoms gives a reduction in the cation affinity of the tribenzo-crown ether, similar to that found on fluorination of the calcium chelator BAPTA *ortho* to the oxyethylene bridge. These observations have led to the preparation of a new indicator for intracellular sodium that we have called FCrown-1. The new indicator has a dissociation constant for sodium of 11 mmol dm^{-3} and a selectivity for sodium over potassium of 173-fold. The sodium concentration is reported by the chemical shift (fast exchange) of one of the two types of fluorine present with a maximum sodium-induced shift of 4.6 ppm downfield. The resonance arising from the other fluorine substituents is insensitive to sodium and acts as an internal chemical shift reference at 8.8 to 13.4 ppm upfield from the reporting signal.

The cytosolic free Ca^{2+}, Na^+ and H^+ concentrations are important in regulating contraction in the heart. To quantify these directly we have designed and synthesised F-labelled NMR indicators for Ca^{2+}, Mg^{2+} and pH and incorporated them in Langendorff perfused hearts.[1-3] The polycarboxylate indicators are first converted into their acetoxymethyl (AM) esters which are hydrophobic and can diffuse through the plasma membrane of cells. The cytoplasm of eukaryotic cells contains esterases which hydrolyse esters of fatty acids and the acetoxy methyl esters are converted into hydroxymethyl esters which rapidly lose formaldehyde in the cytosol to give the free acids. The free acids are charged at normal intracellular pH and no longer permeate the plasma membrane.[4] As the p*K*s of carboxylic acids are sufficient to allow some protonation at normal intracellular pH values, it is necessary for an indicator to carry multiple carboxy groups to ensure retention in the cytosol.

The only spectroscopic reporter currently used for the measurement of intracellular $[Na^+]$ is the fluorescent indicator SBFI,[5,6] a 1,3-diaza-15-crown-5 linked to a pair of Fura-like fluorophores *via* the nitrogen atoms (Fig. 1), which has a measured dissociation constant (K_d) for Na^+ of 17–18 mmol dm^{-3} and a reported 18–22-fold selectivity for Na^+ over K^+.[6,7] However, fluorescent indicators are of limited use in solid tissues and an NMR reporter group is required. We have previously designed and synthesised the ^{19}F NMR indicator for sodium, FCryp-1 (Fig. 2).[8] Unfortunately the AM ester of FCryp-1 was not hydrolysed sufficiently in perfused heart to act as an indicator. This inability of cells to take up FCryp-1 *via* the AM ester also applies to the fluorescent cryptand analogue FCryp-2.[9] It seemed likely that the AM ester loading problem arose from the size and complexity of the indicator structures. New Na^+ indicators, with structures that are simpler than FCryp-1 and more closely related to 5-FBAPTA (Fig. 1),[1] have therefore been developed.

The crown ethers[10] and the cryptands[11] are structures that potentially offer specificity and selectivity for monocation chelation. The ether linkages confer specificity for alkali-metal ions and replacement of the oxygen with the soft base nitrogen, a much stronger ligand for the softer acid heavy metal ions, has an adverse effect on specificity. The binding selectivities between alkali-metal ions arise from steric and hydration effects. The

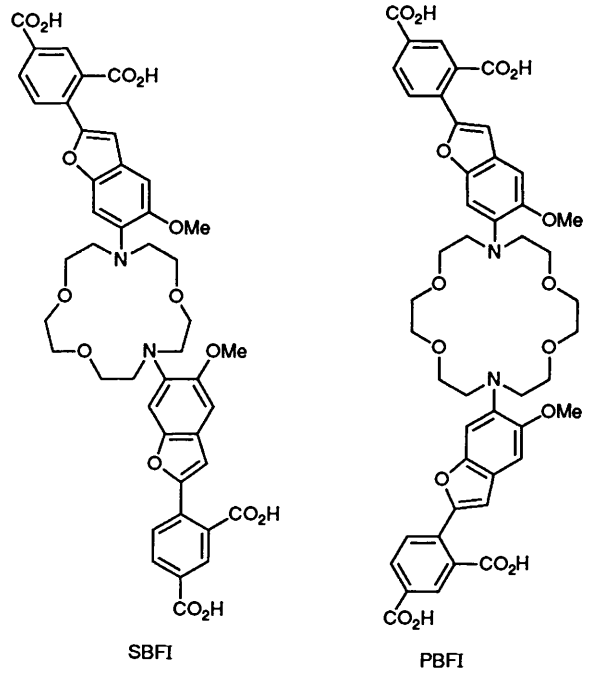

Fig. 1

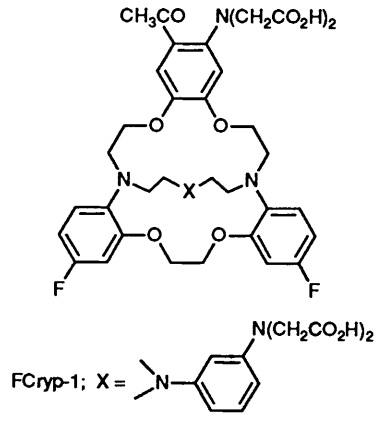

Fig. 2

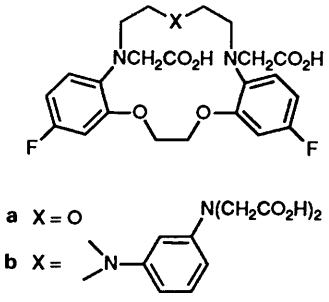

Fig. 3

variations in selectivity of binding between derivatives of these molecules are mostly steric in origin, as exemplified by the addition of a benzo or cycloalkyl substituent (ref. 12, p. 100). There is no selectivity for Na^+ over K^+ for the simple 15-crown-5 in aqueous solution although the cavity of 1.7–2.2 Å allows a good fit for the Na^+ ion (diameter 1.9 Å), and the lack of selectivity is attributed to differences in ion hydration.[12] The difluoro-dibenzo-1,3-diaza-15-crown-5 [Fig. 3(*a*)][8] showed a 16-fold selectivity for Na^+ over K^+ which must reflect favourable structural effects of aromatic rigidity in spite of the replacement of two oxygen donor atoms in the crown with two nitrogen atoms. This dibenzo-crown ether bound to Na^+ with a K_d of 10 mmol dm^{-3} (induced chemical shift of 2.05 ppm) but it was unsuitable as an indicator of cytosolic Na^+ as it leaked from cells. This presumably occurs because the cation-free compound possessed only two charges; complexes with calcium and magnesium, both of which would be formed at normal cytosolic concentrations of these ions, would be uncharged and relatively hydrophobic. The exchange of another crown oxygen atom with a third aza linkage coupled to a phenyl ring with carboxy-carrying substituents [Fig. 3(*b*)][8] increased the polarity but abolished the Na^+ and K^+ binding, consistent with the inability of an aryl trisubstituted nitrogen to chelate the alkali-metal ions. The addition of charged functionality to the fluorine carrying rings was attempted but it was fraught with the difficulties encountered in synthesising tetrasubstituted benzene rings.

An alternative way of altering the lipophilicity of the indicator is to add an extra benzo ring to carry the water-soluble substituents, which requires enlarging the crown structure. The simple 18-crown-6 has a cavity of 2.6–3.3 Å, the right size for preferential K^+ (diameter 2.66 Å) complexation (selectivity for K^+ over Na^+ is 16.9-fold).[12,13] The fluorescent potassium indicator, PBFI (Fig. 1),[6] a 1,4-diaza-18-crown-6 analogue of SBFI, has a selectivity for K^+ 2.6 times that for Na^+. Dibenzo-18-crown-6 is less selective for K^+ over Na^+ (3.24 times) compared with 18-crown-6. The introduction of linkages to form cryptands reduces the size of the cavity (*e.g.*, compare 18-crown-6 with its analogue cryptand [2.2.1] which has a cavity of 2.2 Å and a selectivity in water at 25 °C for Na^+ over K^+ of 28.2-fold).[12] FCryp-1,[8] incorporating a ^{19}F NMR reporter group into a tribenzo derivative of a [2.2.1] cryptand structure, had suitable properties for measuring cytosolic levels of Na^+ in solution but could only be loaded into lymphocytes as the AM ester with difficulty and not at all into perfused heart to act as an indicator.

FCryp-1 and its cryptand analogues showed very large selectivity for Na^+ over K^+ (400- to 5000-fold) which might not be completely abolished by removal of the steric hindrance of the substituted mono-aza bridge. We therefore decided to investigate the suitability of a tribenzo-18-crown-6 as a selective Na^+ binding site.

Results and Discussion

The tribenzo-diaza-18-crown-6 compounds (**7a** to **7e**) were prepared following an adaptation of the procedures described in Smith *et al.*,[8] 1986 (Fig. 4). The dissociation constants and ^{19}F NMR shifts associated with cation binding to the crown ethers in this study are summarised in Tables 1 and 2. The first step was to prepare the simplest mono-fluoro compounds (**7a** and **7b**). Convenient starting compounds were 1,2-bis(2-amino-4-fluorophenoxy)ethane (**2a**) and its 5-fluoro analogue (**2b**) as they were available from the preparation of FCryp-1, its fluorescent analogue FCryp-2,[9] and 4- and 5FBAPTA.[1]

The binding selectivity of **7a** for Na^+ over K^+ was 71-fold and the Na^+, K^+ and Ca^{2+} K_ds were 143 μmol dm^{-3}, 10 mmol dm^{-3} and 2 μmol dm^{-3}, respectively. Compound **7a** showed poor upfield chemical shifts induced by Na^+ (0.2 ppm) and K^+ (0.1 ppm). Ca^{2+} binding to **7a** produced a split resonance shifted downfield by 1.8 and 2.0 ppm. The two ^{19}F resonances from the calcium-bound form, which were equal in magnitude and in slow exchange condition with the free resonance, might arise either from the molecular asymmetry, or from two types of complex with the carboxylates *cis* and *trans* to the crown ring. Relocation of the fluorine substituents in **7a** gave compound **7b** which had similar binding properties; a selectivity of 94-fold for Na^+ over K^+ and dissociation constants for Na^+, K^+ and Ca^{2+} of 330 μmol dm^{-3}, 31 mmol dm^{-3} and 2.5 μmol dm^{-3}, respectively. At 30 °C the low-field ^{19}F NMR peak of the free form was broad (Tables 1 and 2, Fig. 4). Addition of saturating Na^+ concentrations elicited no chemical shift of the fluorine atoms *meta* to the oxyethylene bridge or change in the exchange which causes the line broadening, and hence no alteration to the appearance of the spectrum. Consistent with this observation were the very small changes in the ultraviolet spectrum associated with sodium binding to **7b**. Larger UV spectral changes were found only on cation binding to the benzo-crown ethers when a significant fluorine chemical shift change occurs. Calcium binding to **7b** resulted in only a single ^{19}F resonance with a slightly greater chemical shift change than for **7a**.

The double substitution of hydrogen atoms with fluorine atoms *meta* and *para* to the oxyethylene bridge gave compound **7c** (Fig. 4). The inclusion of two electronegative substituents had a very small effect on the affinity for alkali-metal cations although the calcium affinity was reduced by a factor of ten (Table 1). The latter difference possibly results from the aromatic tertiary amines having a much greater mesomeric interaction with the aromatic ring and stronger chelation of calcium than the aromatic ethers. The ^{19}F resonance signals of the free indicator (**7c**) appeared at much higher field and both showed increased cation-induced downfield shifts compared with the mono-fluoro substituted molecules **7a** and **7b** (Table 2). The resonance of **7c** at lower field was assigned to the fluorine *meta* to the oxygen bridge from both its position and its appearance as a singlet with greater downfield shift on binding

Table 1

Compound	Substituent position	K_d/mmol dm^{-3} Na	K	Ca	Mg	Selectivity ratio Na:K
7a	*p*	0.143	10.16	0.002		71
7b	*m*	0.33	31	0.0025		94
7c	*m,p*	0.387	32	0.026		83
7d	*m,p,m'*	0.153	20	0.151		131
7e	*o,m*	11	1900	0.135	150	173

Table 2

Compound	Substituent position see Fig. 4	δ_F(5FBAPTA)	Cation-induced $\Delta\delta$ Na	K	Ca	Mg
7a	*p*	−0.5	−0.2	−0.1	1.8, 2	
7b	*m*	3.9	0	0.75	2.7	
7c	*p*	−26	0.1	0.6	2.3, 2.4	
	m	−22.7	1.6	2.7	4.4	
7d	*p*	−47.7	0.3	0.2	2.5, 4.3	
	m,m'	−18.3	2.1	0	−1.2, 2.9	
		−16.3	2.3	0	2.3, 6.4	
7e	*o*	−32.6	0		1.4, 2.7	0.6*
	m	−23.8	4.6		5.7	0.9*

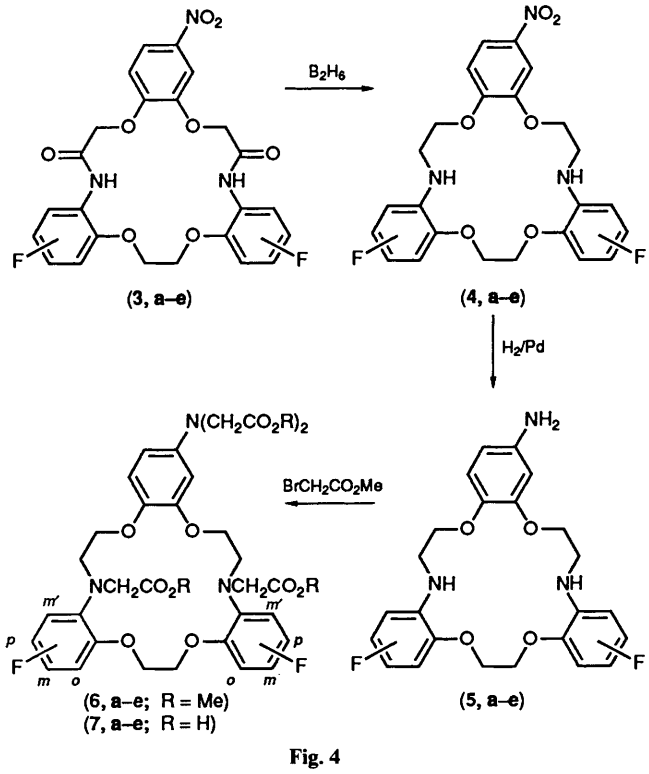

Fig. 4

calcium, more like the properties of the resonance arising from the *meta* fluorine in compound **7b** than the *para* fluorine in compound **7a**. The relative magnitudes of the fast-exchange, sodium-induced chemical shifts of the signals arising from the *meta* fluorine and the *para* fluorine were consistent with this assignment when compared with those induced by sodium in **7a** and **7b**. The lack of an appreciable effect of the increased substitution on alkali-metal ion binding was reflected in the retention of selectivity for Na^+ over K^+ (82-fold).

Introduction of a further (*meta'*) fluorine atom to give triple substitution (compound **7d**, Fig. 4) also had very little effect on the binding constants for sodium and potassium, causing a small increase in selectivity for sodium and the expected further decrease in affinity for calcium (Table 1). The resonance arising from the *para* fluorine was again shifted to much higher field by the mesomeric effect of the extra adjacent fluorine substituent. However the resonances of the fluorine atoms *meta* and *meta'* to the oxyethylene bridge were downfield to the *meta* fluorine resonance in compound **7c**, as expected from their mutual inductive effects (Table 2). The cation-induced shifts of all three resonances were further increased compared with those of **7c** such that the signals from both the *meta* and *meta'* fluorines appeared as doublets when calcium bound and could not be unambiguously assigned.

K_d^{Na} for a useful prototype of a cytosolic Na^+ indicator needs to be in the range 5–50 mmol dm^{-3}; hence further modifications of the parent structures, other than electronegative substitution, were required to reduce the Na^+ binding strength. The selectivity of Na^+ over K^+ needed to be retained or preferably enhanced. The effects of variation in the positions and number of fluorine substituents on the calcium dissociation constants of compounds **7b–d** followed the same pattern as seen for the nF$_m$BAPTA Ca^{2+} chelators (unpublished data). The inclusion of fluorine atoms *ortho* to the oxyethylene bridge in 6FBAPTA [1] greatly decreased the strength of cation binding owing to hindrance by the fluorine atom to the free rotation of the CH_2 groups on the oxyethylene bridge. This substitution also gave a ^{19}F resonance which did not shift on Ca^{2+} binding.

Introduction of fluorines *ortho* to the oxyethylene bridge of the diaza-18-crown-6 **7b** to give **7e** (FCrown-1) had an effect on all cation binding (Fig. 4, Table 1) similar to that found for calcium with 6FBAPTA (Fig. 1).[1] The dissociation constant of FCrown-1 for Na^+ was increased to 11 mmol dm^{-3}, suitable for an indicator of cytosolic Na^+, and the K_d of FCrown-1 for K^+ was 1.9 mol dm^{-3} giving a good selectivity for Na^+ over K^+ of 173-fold. The K_d of FCrown-1 for Ca^{2+} (0.135 mmol dm^{-3}) ensures negligible binding to calcium in the cytosol and virtually complete binding to calcium when in the external medium or in an intracellular calcium store. The highest pK_a of FCrown-1 was 4.8, which is well below the physiological range. Saturating concentrations of Na^+ induce a downfield chemical shift of 4.6 ppm for the lower field of the two signals, which is also assigned to the *meta* fluorine atom on the basis of its

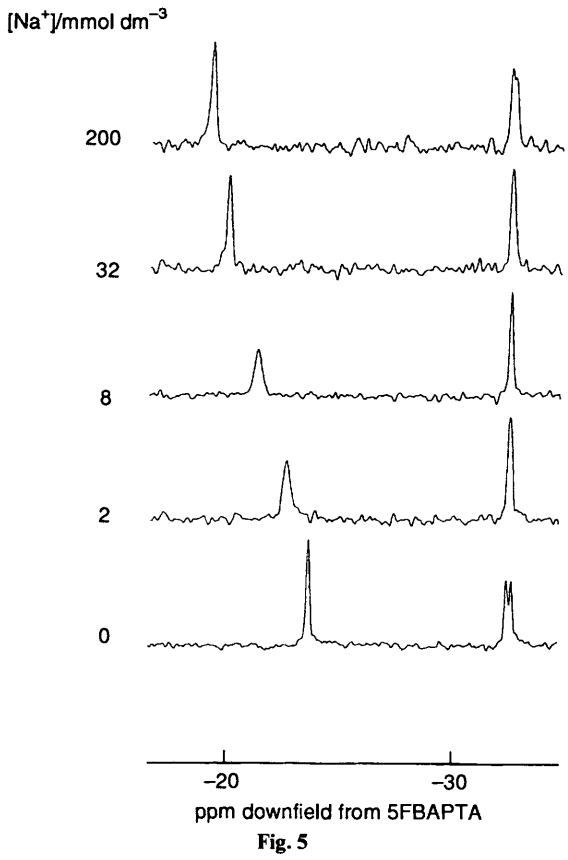

Fig. 5

chemical shift and its appearance as a singlet on calcium binding (Table 2). The extent of sodium binding to FCrown-1 is reported by the chemical shift (fast exchange) of the resonance arising from the fluorine *meta* in relation to the oxyethylene bridge, providing a direct read-out of the free sodium concentration. The resonance from the fluorine atoms *ortho* to the methylene bridge does not shift on sodium binding and hence provides a fixed internal standard of chemical shift upfield from the resonance of the fluorine signal which reports the Na^+ concentration (8.8 ppm in sodium-free medium, Fig. 5). The potassium-induced chemical shift could not be estimated as it was small and saturating concentrations could not be reached. Both the magnesium- and potassium-associated chemical shifts are very much less than that for sodium, whereas that for calcium is comparable to that for sodium. The K_d values are well above the maximal intracellular concentrations by a factor of approximately 20 for Mg^{2+}, K^+ and Ca^{2+}, and therefore under normal physiological conditions FCrown-1 will report $[Na^+]_i$ unaffected by perturbations in the other major intracellular cations.

The electronic effects on the affinity of the tribenzo-crown ethers for alkali-metal ions resulting from alteration of the substitution of the fluorinated rings (**7a–d**) were very small. The weak connection betwen the benzo-rings and the bound alkali-metal ion through the hetero-atoms was confirmed by altering the substitution of the catechol ring. Replacement of the amino-diacetic acid group in FCrown-1 with a nitro group (**8e**) resulted in only a 1.4-fold decrease in the affinity for Na^+ and no effect on the induced chemical shift. Therefore the Na^+ induced chemical shifts and UV spectral changes seen with the polyfluorinated compounds arise mostly from sterically induced alterations to the mesomeric interaction of the aza groups with the fluorinated rings and so are largest for the sterically perturbed *o,m*-difluorinated compounds (FCrown-1 and **8e**).

The AM ester of FCrown-1 is hydrolysed by porcine liver esterase *in vitro* but to date the indicator has not been loaded into the Langendorff perfused ferret heart and experiments to define a loading protocol are in progress.

Experimental

Cation-binding Constant Titrations.—The cation affinities were determined by difference UV spectrometric titrations of the indicators at 10 μmol dm^{-3}, over the range 250–350 nm, in 1 cm pathlength cells at 30 °C. The data were processed by Sips plots (log[bound/free] *vs.* log[M^{n+}]) which all showed single-site binding (slopes 1.00 ± 0.02). The solutions of indicator were prepared by hydrolysis of the esters with tetramethyl-ammonium hydroxide (see synthesis, **7** and **8**), dilution with a solution of tetramethyl ammonium chloride (100 mmol dm^{-3}), EGTA (0.1 mmol dm^{-3}) and HEPES (25 mmol dm^{-3}) and the pH adjusted to 7.2 with hydrochloric acid. The cation concentration was varied by the addition of the chloride salt as a concentrated stock in the indicator solution. The calcium titrations were in the absence of EGTA and for those indicators with affinities in the micromolar range were performed by either back titration of the sodium complex or by replacement of the 100 mmol dm^{-3} chloride solution with 40 mmol dm^{-3} citrate, and calculating the free calcium concentration using a dissociation constant of 316 μmol dm^{-3} for the calcium citrate complex. Titration of the single fluorine substituted isomer **7b** with sodium required a high concentration of probe and the signal-to-noise of the UV difference spectrum was poor; the value obtained was therefore confirmed by back titration of the calcium complex.

The ^{19}F chemical shifts were measured at 376 MHz, with 100–500 μmol dm^{-3} probe concentration in the same solutions as the titrations, with 50% D_2O lock signal at 30 °C and using 5FBAPTA as an internal standard, unless otherwise stated.

Synthesis.—The preparation of FCrown-1 (**7e**) is described in detail. All other analogues were obtained by similar procedures except for the nitro compound (**8e**) for which the hydrogenation step was omitted (Fig. 4). In general the methyl esters were preferred for synthesis as they give products that are more crystalline than the ethyl esters. This is of no consequence for eventual use in biological systems as the esters are removed prior to preparation of the AM esters. All reactions were followed and the purity of products ensured by thin layer chromatography on silica gel developed with ethyl acetate in toluene in proportions adjusted to give R_f values around 0.5.

1,2-*Bis*(2,3-*difluoro*-6-*nitrophenoxy*)*ethane* (**1e**).—A solution of 2,3-difluoro-6-nitrophenol (Aldrich) in 50% aqueous methanol was adjusted to pH 10 with 30% aqueous potassium hydroxide. The resulting solution was evaporated to dryness *in vacuo* and dried overnight under high vacuum. The potassium phenoxide (22 g, 103 mmol) was heated with 1,3-dibromoethane (12.5 g, 67 mmol) in dimethylformamide (50 cm^3) at 100 °C for 1.5 h. TLC on silica in 5% ethyl acetate–toluene indicated the presence of polar phenolic material along with the expected product. Addition of water produced a gum that was extracted into toluene, separated, washed with sodium carbonate solution (1 mol dm^{-3}), dried with sodium carbonate and evaporated to dryness *in vacuo*. The product was separated by chromatography on silica gel in 5% ethyl acetate in toluene and crystallised from chloroform–light petroleum (b.p. 40–60 °C) to give the dinitro compound (10 g, 51%), m.p. 64–67 °C; δ(60 MHz; $CDCl_3$) 4.71 (4 H, s), 7.08 (2 H, dt, *J* 9, 8) and 7.75 (2 H, ddd, *J* 2, 6, 9); *m/z* (EI) 376.

1,2-*Bis*(2,3-*difluoro*-6-*aminophenoxy*)*ethane* (**2e**).—The dinitro compound (10 g) in methanol was hydrogenated over

palladium-on-charcoal until the uptake of H_2 ceased, the catalyst was removed by filtration, and the solvent was removed *in vacuo* to give the diamine which was crystallised from methanol (6.0 g, 71%), m.p. 112–114 °C; δ(60 MHz; $CDCl_3$) 4.0 (4 H, br s), 4.39 (4 H, s), 6.4 (2 H, br dd, *J* 9, 4.5) and 6.78 (2 H, br q, *J* 9); *m/z* (EI) 316.

2,3,18,19-*Tetrafluoro*-11-*nitro*-22,23-*dihydrotribenzo*[b,h,n][1,4,10,13]*tetraoxa*[7,16]*diazacyclooctadecine*-6(5H),15(16H)-*dione* (**3e**).—The diamino compound (6 g, 19 mmol) was dissolved in dichloromethane (350 cm^3) and pyridine (2.8 g). 4-Nitrocatechol-*O*,*O*-diacetoyl chloride (5.8 g, 19 mmol) was dissolved in dichloromethane (350 cm^3) and the two solutions added concurrently to dichloromethane (50 cm^3) with stirring over 1 h. After a further 10 min the solution was washed with hydrochloric acid (2 × 100 cm^3; 2 mol dm^{-3}), passed through a column of silica gel (200 cm^3) and elution of the product was completed with chloroform. The eluate containing the product was evaporated to dryness and the cyclic diamide crystallised from a small volume of dichloromethane (6.1 g, 57%) m.p. 235–239 °C; δ_H(400 MHz; [$^2H_6$]DMSO; dissolution in 5 min at 80 °C; spectra consistent with mixed *syn–anti* of two amides) 3.29 (s), 3.94 (br d, *J* 15), 4.14 (br d, *J* 15), 4.23 (br d, *J* 10), 4.44 (br d, *J* 12), 4.48 (s), 4.55 (m), 4.67 (m), 4.92 (d, *J* 3.7), 5.0 (m), 6.9 (m), 7.05 (br q, *J* 9), 7.08 (q, *J* 9), 7.16 (q, *J* 9), 7.36 (d, *J* 9), 7.81 (br q, *J* 9), 7.98 (d, *J* 9), 8.0 (br s), 8.08 (br d, *J* 9), 8.18 (s), 9.23 (br d, *J* 7) and 9.46 (br s); δ_F(376 MHz; DMSO; no reference) 0.0 (tt, *J* 8, 28), 0.050 (dd, *J* 8, 21), 1.56 (d, *J* 21), 2.78 (br s), 11.94 (d, *J* 20), 12.95 (m) and 17.17 (d, *J* 20); *m/z* (EI) 551.

2,3,18,19-*Tetrafluoro*-11-*nitro*-6,7,14,15,22,23-*hexahydrotribenzo*[b,h,n][1,4,10,13]*tetraoxa*[7,16]*diazacyclooctadecine* (**4e**).—The cyclic diamide (6 g, 10.9 mmol) was dissolved in borane THF (1 mol dm^{-3}; 165 cm^3) under reflux whilst water* (4.2 cm^3) in THF (36 cm^3) was added with care over 30 min. When the frothing had ceased a further 50 cm^3 of water was added and the THF removed *in vacuo* to give a solid which was recovered by filtration, washed with water and dried under high vacuum to give the diazacrown ether (5.5 g, 97%) m.p. 205–210 °C; δ_H(400 MHz; $CDCl_3$–CD_3OD) 3.62 (4 H, ψt, *J* 4.4), 4.25 (2 H, ψt, *J* 4.4), 4.26 (2 H, ψt, *J* 4.4), 4.34 (4 H, s), 6.38 (2 H, dd, ψt, *J* 2.1, 9.2, 4.5), 6.86 (2 H, dd, ψt, *J* 3.8, 9.2, 8.5), 6.89 (1 H, d, *J* 8.8), 7.72 (1 H, d, *J* 2.6) and 7.89 (1 H, dd, *J* 2.2, 8.8); *m/z* (EI) 523.

2,3,18,19-*Tetrafluoro*-6,7,14,15,22,23-*hexahydrotribenzo*[b,h,n][1,4,10,13]*tetraoxa*[7,13]*diazacyclooctadecin*-11-*amine* (**5e**).—The nitro compound (5.5 g, 10.5 mmol) was hydrogenated in ethanol (400 cm^3) over 10% palladium-on-charcoal (600 mg); uptake of hydrogen was complete after 36 h. The catalyst was removed by filtration, the ethanol removed and the crystalline product dried under vacuum (5.2 g, 100%) m.p. 138–142 °C; δ_H(376 MHz; $CDCl_3$–CD_3OD) 3.49 (2 H, t, *J* 4.7), 3.54 (2 H, t, *J* 4.7), 4.10 (2 H, t, *J* 4.7), 4.11 (2 H, t, *J* 4.7), 4.34 (4 H, AB, *J* 6), 6.25 (1 H, dd, *J* 2.7, 8.5), 6.32 (1 H, d, *J* 2.7), 6.35 (2 H, ddd, *J* 4.5, 9, 14), 6.70 (1 H, d, *J* 8.5), 6.81 (1 H, t, *J* 9) and 6.83 (1 H, t, *J* 9); δ_F(no ref. 0.0) (dd, *J* 9, 21), 3.17 (ddd, *J* 4.5, 14, 21) and 3.21 (ddd, *J* 4.5, 14, 21); *m/z* (EI) 493.

5,16-*Bis*(*methoxycarbonylmethyl*)-11-[*bis*(*methoxycarbonylmethyl*)*amino*]-6,7,14,15,22,23-*hexahydrotribenzo*[b,h,n][1,4,10,13]*tetraoxa*[7,13]*diazacyclooctadecine* (**6e**).—The hydrogenation product (5.2 g, 10.5 mmol) was stirred under nitrogen with Proton Sponge (10 g, 47 mmol), methyl bromoacetate (10 g, 65 mmol) and acetonitrile (10 g) and heated under reflux for 18 h. The resulting suspension was mixed with toluene (100 cm^3) and the salts were removed by filtration and washed with more toluene. The combined filtrate was washed three times with ammonium phosphate (1 mol dm^{-3}; pH 4.0), dried with magnesium sulfate and evaporated to dryness *in vacuo* to give a gum (7.9 g). The gum was separated by chromatography on silica gel (1 dm^3) eluting with 30–50% ethyl acetate in toluene taking care not to collect the slightly less polar impurity. Evaporation of the eluate gave the tetramethyl ester of the indicator as a colourless glass which was a single component by TLC (5.3 g, 64%); δ_H(400 MHz; $CDCl_3$) 3.64 (6 H, s), 3.72 (6 H, s), 3.74 (2 H, t, *J* 4.7), 3.79 (2 H, t, *J* 4.7), 3.93 (2 H, t, *J* 4.7), 3.96 (2 H, t, *J* 4.7), 4.06 (4 H, s), 4.31 (4 H, AB), 4.37 (4 H, d, *J* 5), 6.07 (1 H, dd, *J* 2.8, 8.7), 6.17 (1 H, d, *J* 2.8), 6.67 (1 H, d, *J* 8.7), 6.85 (2 H, dt, *J* 4.5, 9.2) and 6.92 (2 H, dt, *J* 4.5, 9.2); λ_{max}/nm 252 (ε/dm^3 mol^{-1} cm^{-1} 33 000) and 292 infl. (10 600) (EtOH) [Found: *m/z* (EI) 781.2472, $C_{36}H_{39}F_4N_3O_{12}$ −0.3 ppm].

Hydrolysis of the Esters to the Acids **7a–e** *and* **8e**.—In general the indicator-free acids were prepared by dissolution in dichloromethane methanol (1:1), treatment with a twofold excess of potassium, or tetramethylammonium, hydroxide (2 mol dm^{-3}), followed by evaporation of the organic solvents at 50 °C under a stream of nitrogen and heating for a further 60 min until hydrolysis was complete and a single product obtained as shown by TLC on silica gel developed in chloroform–methanol–water–ammonia (880), 45:35:8:2. The solution was then either used for the titrations or diluted to the minimum convenient volume, the pH adjusted to 2.5 with dilute hydrochloric acid and the free acid recovered by centrifugation. The free acids showed considerable aqueous solubility. For **7e**, δ_H(400 MHz; Na_2CO_3, 1 mol dm^{-3}; D_2O) 4.50 (HOD), 3.11 (2 H, t, *J* 4.5), 3.12 (2 H, t, *J* 4.5), 3.19 (4 H, s), 3.49 (4 H, s), 3.64 (2 H, t, *J* 4.5), 3.68 (2 H, t, *J* 4.5), 4.25 (4 H, s), 5.67 (1 H, dd, *J* 2.7, 9), 5.75 (1 H, d, *J* 2.7), 6.45 (1 H, d, *J* 9), 6.63 (2 H, ddd, *J* 5, 9, 14) and 6.77 (2 H, dt, *J* 5, 9).

* The reduction of amides with borane is not normally complete after 24 h under reflux. We have found that the reaction proceeds rapidly to completion on the controlled addition of three equivalents of water at reflux temperature, Smith *et al.*,[4] 1986.

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