Synthesis and Properties of a Potential Extracellular Fluorescent Probe for Potassium

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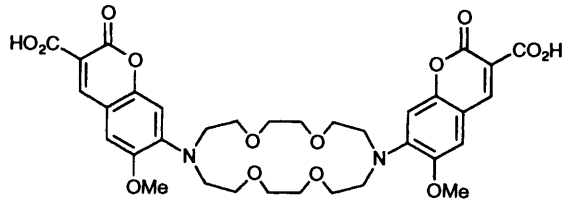
Development of a new, selective, high affinity, fluorescent probe CD222, **2**, for potassium is described. Coincidentally two related probes, CDF18, **3**, and CTF18, **4**, were prepared and showed selectivity for the cations sodium and calcium, respectively, and possible reasons for these differences are discussed. The bicyclic probe CD222, **2**, with observed K_D values for K^+ ranging from 1 to 10 mmol dm^{-3}, has potential applications as an extracellular probe for potassium.

The intracellular concentration of potassium ions in living cells is around 150 mmol dm^{-3}, well above that generally found in the surrounding media, typically about 4 mmol dm^{-3}.[1] This concentration gradient is mainly maintained by a sophisticated ionic pump, the 'sodium pump', which involves exchange of potassium ions with sodium ions.[2] The concentration gradient allows cells to power many physiological processes such as nutrient uptake and, in particular, various aspects of cell signalling. The potassium–sodium exchange is often carried out in pulses (firings) in which transient changes in the local concentrations of these ions occur, for example, by the discharge of potassium ions into the surrounding medium.

In a recent publication[3] we have described the requirements for an intracellular potassium probe and have developed reagents such as CD18, **1**; in the present paper the development of a potential extracellular probe for potassium, the compound CD222 ('coumarin diacid cryptand [2.2.2]'), **2** is detailed. Its fluorescent properties are compared with those of the related derivatives CDF18 ('coumarin diacid fused 18-crown-6'), **3** and CTF18 ('coumarin tetraacid fused 18-crown-6'), **4**.

During the firing of cells the percentage change in intracellular K^+ concentrations change only by a few percent (< 5%) so that any intracellular potassium probe has to be able to signal these small changes against the relatively large background of K^+ ions present. As a consequence, as for the reagent CD18, **1**, corresponding changes of fluorescence of only a few percent can be expected. One way to overcome this problem is to concentrate on the subsequent, relatively larger changes in intracellular sodium ion concentration that follow firing, although these are generally much slower. This approach has been explored by Smith *et al.*[4] and Minta and Tsien.[5] One non-fluorescent example of the latter approach is the use of a variety of fluorinated probes for the chelation of sodium, designed to be studied by ^{19}F NMR spectroscopy,[6] a method for monitoring changes in bulk samples of cells only. It should be noted, however, that fluorescent probes have certain advantages and applications not met by non-fluorescent approaches, such as the use of ion-selective electrode probes.[7]

We have explored an alternative approach, a study of the extracellular changes in potassium concentration. Such an approach would require the cell(s) to be suspended in a minimum volume of medium as well as the following properties: (1) high selectivity for K^+ over Na^+ and other common physiological cations in water; (2) water solubility; (3) a dissociation constant in the concentration range of the potassium to be measured (1–10 mmol dm^{-3}) in order to observe maximum sensitivity to changes in the K^+ concentration in this range; (4) a change in fluorescence upon chelation with potassium; (5) an excitation maximum longer than 340 nm with an emission maximum over 500 nm to avoid self-quenching and autofluorescence signals; (6) rapid equilibration with changing potassium fluxes in order to give meaningful changes in fluorescent signals against time.



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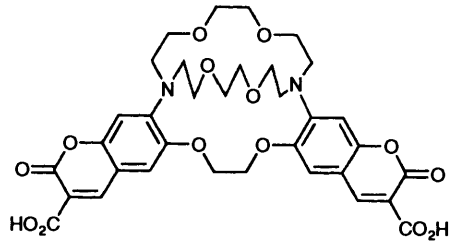

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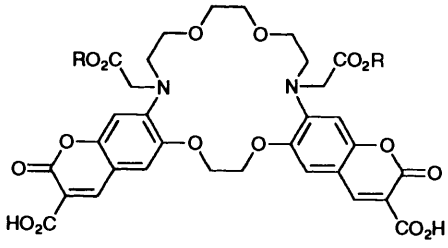


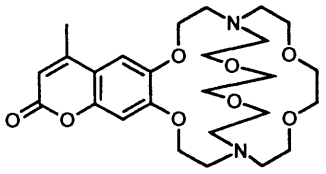
3 R = CH_3
4 R = H

The main difference between the extracellular potassium probe as against an intracellular one is the need for a higher binding constant, *i.e.*, a smaller dissociation constant, K_D, ideally in the range 1–10 mmol dm^{-3}. As in our earlier work,[3] essential requirements are for agents that work in water and that show no strong hydrophobic properties that could lead to binding to cell walls, *etc.*

Results and Discussion

In order to attain the high, yet selective binding of a potassium ion chelator some further rigidity in the system was considered necessary as against the selective binder CD18, **1**. The properties

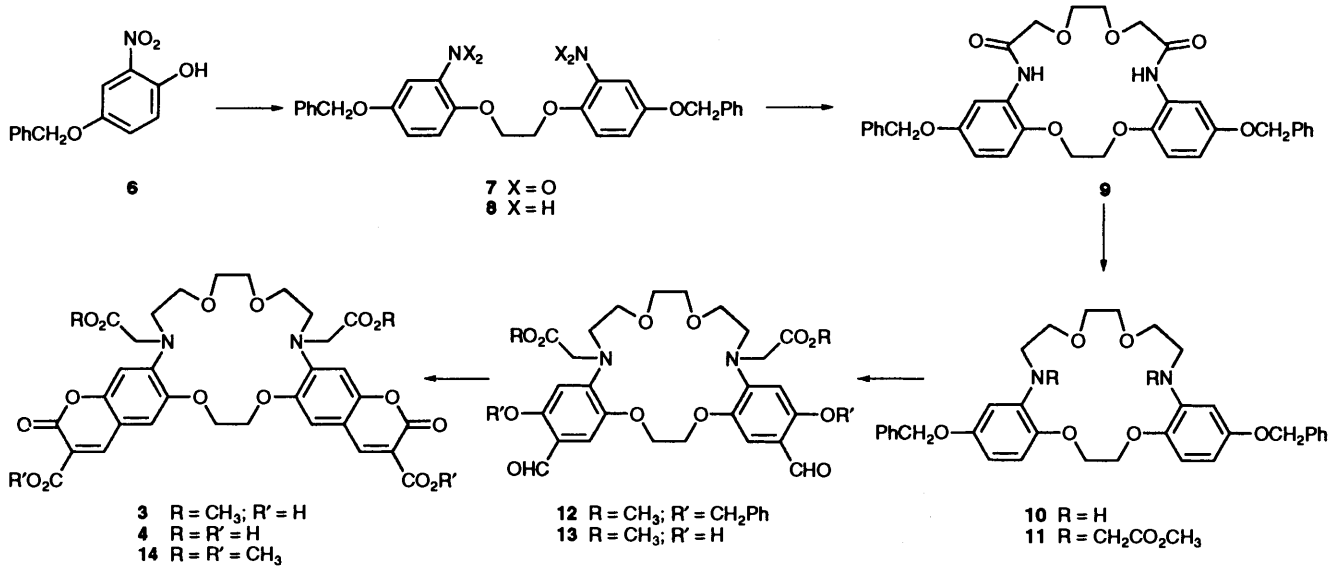
of a range of coronands and cryptands, including the effects of the incorporation of benzo-rings to decrease flexibility have been covered in full reviews by Izatt *et al.*[8] One example of this approach is that of Golchini *et al.*[9] who developed the coumarin derivative **5**. Whilst this showed a high selectivity for K^+ over



5

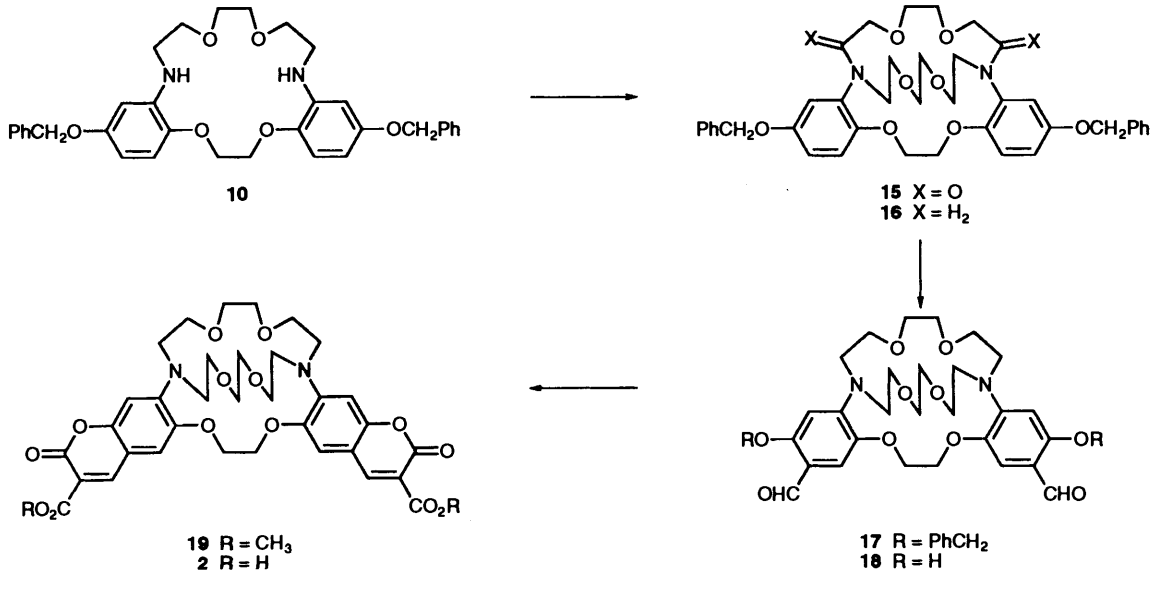
other physiological cations, certain concerns arose: the lack of polar groups suggested that it might be sufficiently hydrophobic preferentially to bind to cell walls during biological studies. Another concern was that, because of the relative rigidity of the bicyclic cryptand group, the rate of exchange of K^+ ions may not be fast enough to be able to monitor physiological changes in K^+ concentrations. However, Golchini *et al.* reported that, for their probe **5**, a relatively fast response to changing potassium ion fluxes occurred.[9]

We initially adopted an approach in which the increased rigidity of the potassium chelator was achieved by introducing aromatic rings into the ligand structure, to produce **4**. Structure **4** incorporates a central dibenzo-diazacryptand system similar to that prepared by de Silva *et al.*,[10] although they did not report on the cation selectivity of their compound and the product was too hydrophobic for studies in water. Our targets incorporate a more oxygenated aromatic precursor and the syntheses of the ligands **2**–**4** are oulined in Schemes 1 and 2. The nitrophenol **6**[11] was alkylated with 1,2-dibromoethane in dimethylformamide to give the bis-ether **7**. Selective catalytic reduction of the nitro groups afforded the diamine **8**. Ring cyclisation was effected with 1,2-ethylene-*O*,*O*-diglycolic acid chloride[12] and the diamine **8** under high dilution conditions in tetrahydrofuran to give the lactam **9** in 60% yield. The lactam was smoothly reduced to the key diamine intermediate **10** with lithium aluminium hydride. Subsequent alkylation of the amine groups, using an excess of methyl bromoacetate and proton sponge [1,8-bis(dimethylamino)naphthalene] gave a high yield



6 **7** X = O, **8** X = H **9** **10** R = H, **11** R = $CH_2CO_2CH_3$ **12** R = CH_3; R′ = CH_2Ph **13** R = CH_3; R′ = H **3** R = CH_3; R′ = H **4** R = R′ = H **14** R = R′ = CH_3

Scheme 1



10 **15** X = O, **16** X = H_2 **17** R = $PhCH_2$ **18** R = H **19** R = CH_3 **2** R = H

Scheme 2

Table 1 Spectral and binding properties of fluorescent probes CDF18, SBF1 and FCryp-2

Probe	M^+ ($[M^+]$/mmol dm^{-3})	Absorbance λ_{max}/nm (ε/10^3 dm^3 mol^{-1} cm^{-1})	Fluorescence λ_{ex}/nm	Fluorescence λ_{em}/nm	Φ_b	K_D/mmol dm^{-3}
CDF18 (**3**)		390 (23)	393	470	0.07	—
	K^+ (135)	375 (18)	386	466	0.08	666
	Na^+ (135)	367 (19)	374	463	0.12	40
	Mg^{2+} (1)	390 (20)	393	470	0.06	8
	Ca^{2+} (1)	390 (23)	393	470	0.06	11
	Na^+/K^+ (135)[b]	—	—	—	—	166
SBF1 (**21**)		346 (42)	350[d]	551	0.045	—
	Na^+	334 (47)	341[d]	525	0.083	7.4
	Na^+/K^+ (135)[b]	—	—	—	—	17–18
FCryp-2 (**22**)		340 (5)	325	450	0.03[e]	
	Na^+(K^+ 120)	—	325	395	0.7	6.0

[a] Probe concentration 10^{-6} mol dm^{-3} in water maintained at pH 7: see the Experimental section. [b] Effective K_D for Na^+ in the presence of K^+, where $[Na^+ + K^+]$ = 135 mmol dm^{-3}. [c] Ref. 5. [d] Data extracted from Fig. 2, p. 412 in ref. 15. [e] Data estimated from ref. 4.

Table 2 Spectral and binding properties of fluorescent probes CTF18, and fura-2

Probe	M^+ ($[M^+]$/mmol dm^{-3})	Absorbance λ_{max}/nm (ε/10^3 dm^3 mol^{-1} cm^{-1})	Fluorescence λ_{ex}/nm	Fluorescence λ_{em}/nm	Φ_b	K_D/mmol dm^{-3}
CTF18 (**4**)		394 (29)	391	477	0.06	—
	K^+ (135)	383 (31)	391	477	0.04	35
	Na^+ (135)	376 (31)	389	475	0.03	4.4
	Mg^{2+} (1)	393 (30)	391	477	0.05	833
	Ca^{2+} (1)	358 (30)	357	458	0.11	0.009
fura-2[b] (**23**)		362 (27)	368[c]	512	0.23	—
	Ca^{2+}	335 (33)	341[c]	505	0.49	0.000 14[d]

[a] Probe concentration 10^{-6} mol dm^{-3} in water maintained at pH 7: see the Experimental section. [b] Ref. 8. [c] Data extracted from Fig. 20.1, p. 114 in ref. 15. [d] Effective K_D in the presence of 100 mmol dm^{-3} K^+.

of the diester **11**. Vilsmeier–Haack formylation of the diester **11** afforded the dialdehyde **12**, which was debenzylated under hydrogen, using 10% palladium-on-charcoal as the catalyst, to produce the bis-salicylaldehyde **13**. The debenzylation needed to be carefully monitored since, upon prolonged exposure to hydrogen, the aldehyde groups were further reduced to produce unstable benzylic alcohols. Condensation of the salicylaldehyde with malonic acid[13] gave the probe CDF18, **3**, whilst condensation with dimethyl malonate initially gave the tetraester **14**; this could be hydrolysed to the tetraacid, CTF18, **4**, by use of lithium hydroxide in aqueous methanol over 7 days at room temperature.[14] The overall yield from the phenol **6** to CDF18, **3**, was 14% whilst that to CTF18, **4**, was 8%.

The diamine intermediate **10** could be reacylated with triethylene glycolic acid chloride, under high dilution conditions (Scheme 2), using pyridine as the base, to give the cyclic lactam **15** in 43% yield and subsequent reduction with diborane in tetrahydrofuran[15] afforded the cryptand **16**. Vilsmeier–Haack formylation, in the manner described above, gave the bis-aldehyde **17**. Debenzylation by catalytic hydrogenolysis was hampered by limitations of solubility and was best carried out portionwise with frequent TLC monitoring to prevent over-reduction as observed during the reduction of **12** (see above). The product bis-salicylaldehyde **18** was readily condensed with dimethyl malonate to produce the corresponding coumarin ester **19** and this could be hydrolysed to the target probe CD222, **2**, with lithium hydroxide; the yield of the latter probe from the intermediate **10** was 14%. All the probes **2**–**4** were fully characterised by mass spectrometry and ^{1}H NMR spectroscopy. For example, in the ester **19** at room temperature the coumarin groups were characterised by the presence of only three aromatic proton signals, at δ 6.68, 6.93 and 8.43, and a doubling up of the signals for the chain hydrogens, indicating, on the ^{1}H NMR timescale, the presence of symmetry in the structure (see the Experimental section). Presumably, under ambient conditions, the cryptand is undergoing rapid fluxional interconversions of various conformations.[16]

The fluorescent properties of the new probes were examined under the conditions used in our earlier studies[3] and followed the procedure described by Minta and Tsien.[5] All three probes readily gave 10^{-6} mol dm^{-3} solutions in water maintained at pH 7. The spectroscopic and binding properties of these probes are presented in Tables 1–3. The dissociation constants, K_D against various cations were measured according to the method described by Bourson and Valeur.[17]

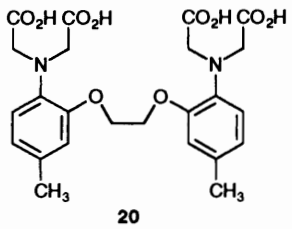
Our initial expectation was that the presence of the 18-membered diaza-crown systems would endow all three compounds with a reasonably high and selective affinity for potassium, although the structure of the tetraacid **4** does show some resemblance to BAPTA, **20**, a known selective calcium binding system[18] and interference from this cation might be observed. In the event all three compounds showed completely different chelation behaviour.

The diester diacid CDF18, **3**, may be compared with the probe CD18, **1**, the two structures sharing a similar diaza-18-crown-6 system except that, in the former, the aromatic rings are attached in an endocyclic manner whilst in the latter they are exocyclic; in addition, the two *ortho*-methoxy groups in CD18

Table 3 Spectral and binding properties of fluorescent probes CD222 and **5**

Probe	M^+ ($[M^+]$/mmol dm^{-3})	Absorbance λ_{max}/nm ($\varepsilon/10^3$ dm^3 mol^{-1} cm^{-1})	Fluorescence λ_{ex}/nm	Fluorescence λ_{em}/nm	Φ_b	K_D/mmol dm^{-3}
CD222 (**2**)		390 (31)	384	476	0.03	—
	K^+ (135)	360 (31)	364	465	0.08	1.0
	Na^+ (135)	360 (29)	364	476	0.02	0.7
	Mg^{2+} (1)	380 (17)	384	476	0.04	**b**
	Ca^{2+} (1)	380 (17)	384	476	0.04	**b**
	$K^+(Na^+$ 135)[c]	—	—	476	—	13.5
				—		
(**5**)[d]		—	340	420	0.045	—
	K^+	—	340	420	—	1.9
	$K^+(Na^+$ 140)[c]	—	—	—	—	10.7

[a] Probe concentration 10^{-6} mol dm^{-3} in water maintained at pH 7: see the Experimental section. [b] No measurable binding. [c] Effective K_D for K^+ in the presence of a background of $[Na^+]$ = 135 mmol dm^{-3}. [d] Ref. 7. [e] Effective K_D for K^+ in the presence of a background of $[Na^+]$ = 140 mmol dm^{-3}.



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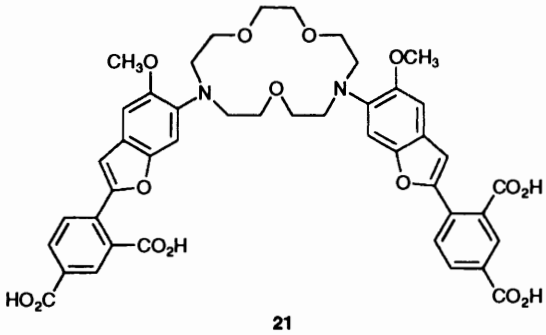

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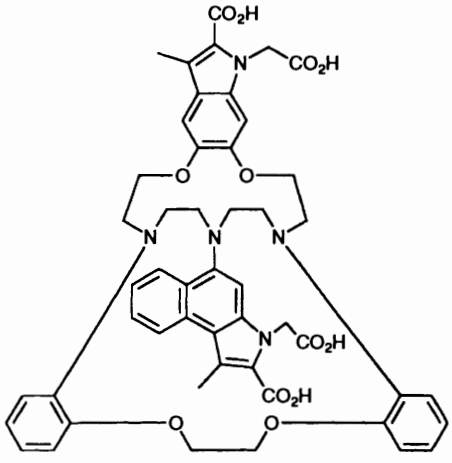

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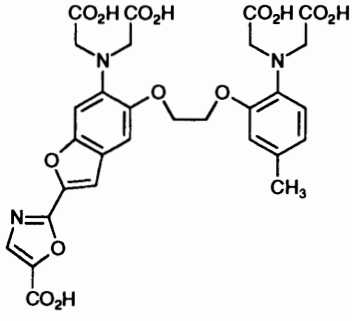

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are replaced by the two ligating ester groups in the probe CDF18. Molecular modelling indicated that the two systems were capable of adopting similar spatial conformations around potassium.

The extinction coefficient of CDF18 was 22 000 dm^3 mol^{-1} cm^{-1} with λ_{max} at 393 and 470 nm for the excitation and emission, respectively. Its behaviour in the presence of biologically important cations was unexpected since it proved to be much more selective for the smaller sodium cation than for K^+ ions, with a K_D of 40 as against the weak value of 666 for potassium (Table 1), a Na^+/K^+ selectivity of *ca.* 17. The quantum yield for fluorescence (Fig. 1) approximately doubles on going from 0 to 135 mmol dm^{-3} Na^+, in contrast with the very small change caused by potassium. The effect of competing potassium ions at up to 135 mmol was minimal and, under these conditions, the effective $K_D(Na^+)$ is 166. Although the dissociation constants, K_D, were smaller for Mg^{2+} and Ca^{2+} (*i.e.*, higher affinities) at physiological levels these cations had little effect on the fluorescence characteristics of the probe and did not seem to interfere with the effect caused by sodium ions. Similar behaviour was recorded for the probe **1**,[3] and was explained by the fact that a different mode of binding is possibly involved with the latter cations, one that does not involve the diaza-crown group. The unexpected selectivity of the probe **3** for Na^+ rather than K^+ is presumed to be caused by the two endocyclic aromatic functions, probably making the diaza-crown ring adopt a conformation with a smaller pocket suitable only for the sodium ion. The probe CDF18, **3**, gave a similar fluorescence response throughout the physiological pH range of 6–8 and only fell away slightly at the extremes of the range 5 to 10. The properties of this probe compare favourably with those reported for the commercially available Na^+ probe SBFI, **21** [5,19] (see Table 1) developed for studying intracellular sodium ion concentrations. There is a hypsochromic shift of 14 nm in the excitation maximum, λ_{ex}, twice that observed with SBFI and the latter operates at an excitation wavelength some 40 nm

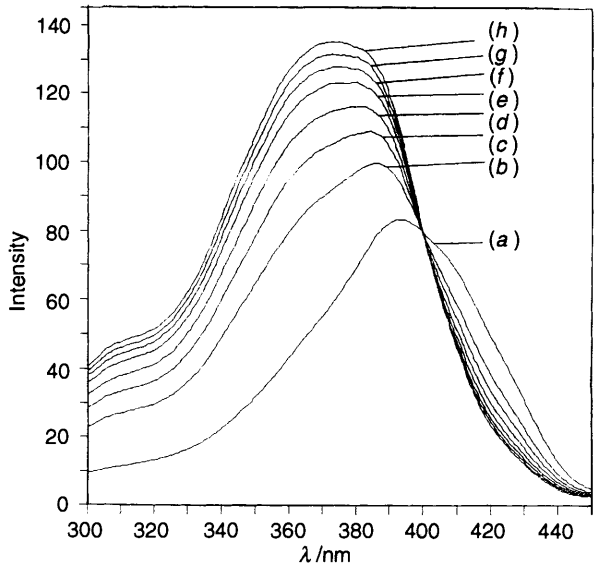

Fig. 1 Fluorescence excitation spectra of 10^{-6} mol dm^{-3} CDF18 as a function of 0–200 mmol dm^{-3} Na^+ emitted at 475 nm at pH 7: [Na^+]/mmol dm^{-3} = 0 (*a*); 20 (*b*); 40 (*c*); 67 (*d*); 100 (*e*); 133 (*f*); 167 (*g*); 200 (*h*)

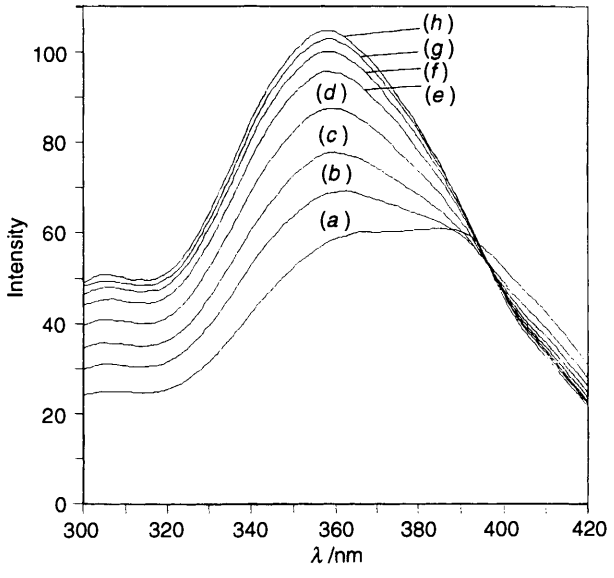

Fig. 2 Fluorescence excitation spectra of 10^{-6} mol dm^{-3} CTF18 as a function of 0–1 mmol dm^{-3} Ca^{2+} emitted at 460 nm at pH 7: [Ca^{2+}]/mmol dm^{-3} = 0 (*a*); 0.005 (*b*); 0.01 (*c*), 0.02 (*d*); 0.04 (*e*); 0.08 (*f*); 0.5 (*g*); 1 (*h*)

shorter than that required for CDF18. The working range for sodium ions with CDF18 is between 0 and 135 mmol dm^{-3} whilst for SBFI it is 0–54 mmol dm^{-3}. These values may also be compared with those reported by Smith *et al.*[4] for the sodium probe FCryp-2, **22**, which has a working range of 0–20 mmol dm^{-3}; the latter probe also has the advantage of showing a large hypsochromic shift in the *emission* spectrum on complexing sodium (*ca.* 55 nm) but none in the excitation spectrum (see Table 1).

Removal of the two methyl ester groups from CDF18, **3**, to give the tetraacid CTF18, **4**, completely changes the binding behaviour. Perversely, for CTF18 both the presence of either Na^+ or K^+ ions *decreased* the intensity of fluorescence. The system behaved as a selective, relatively strong, calcium ion chelator and also showed a strong increase in fluorescence as the Ca^{2+} concentration increased to a saturating level of *ca.* 0.1

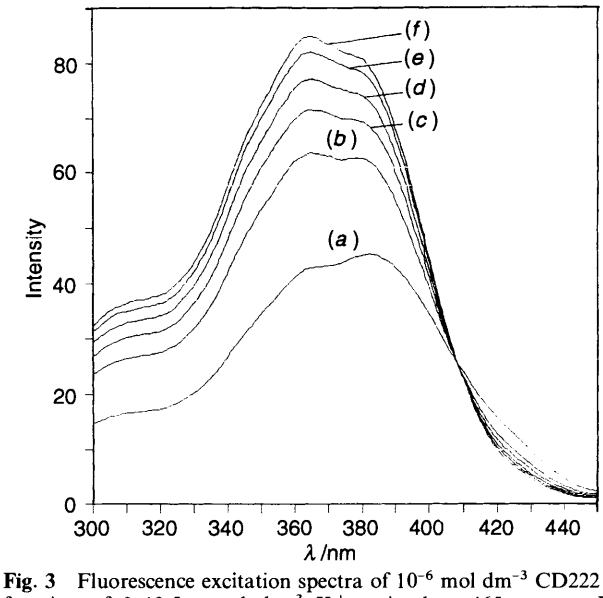

Fig. 3 Fluorescence excitation spectra of 10^{-6} mol dm^{-3} CD222 as a function of 0–13.5 mmol dm^{-3} K^+ emitted at 465 nm at pH 7: [K^+]/mmol dm^{-3} = 0 (*a*); 0.8 (*b*); 1.5 (*c*); 3.5 (*d*); 7 (*e*); 13.5 (f)

mmol dm^{-3}. An accompanying shift in the λ_{ex} of *ca.* 35 nm was also observed, see Fig. 2, allowing the possibility of dual wavelength monitoring.[18] The probe was highly selective for calcium, magnesium showing a very large dissociation constant (K_D 833, Table 2); thus a concentration study against Ca^{2+} in the presence of 100 mmol dm^{-3} K^+ and 1 mmol dm^{-3} Mg^{2+} gave results very similar to those illustrated in Fig. 2. Included in Table 2 are comparative data for the commercially popular calcium ion probe Fura-2 (**23**),[11] whose structure is based on BAPTA. Whilst the Φ_f values for Fura-2 are higher in comparison to those for CTF18, Φ_f for the latter doubles upon Ca^{2+} complexation. In addition, the shift of λ_{ex} is slightly greater for CTF18 and the latter exhibits an approximately 60-fold weaker binding affinity for Ca^{2+}. A possible explanation is that the 'open chain' Fura-2 is not as conformationally restrained during complexation as in CTF18. To our knowledge, CTF18 is the first reported fluorescent Ca^{2+} probe constructed from a macrocyclic ring.

The calcium-specific behaviour of CTF18, **4**, can be explained by a combination of two factors: (*a*) the two acid groups attract the higher charge density of the divalent calcium ion to form an octacoordinate binding site as observed by Grynkiewicz *et al.*,[11] and (*b*) for the structurally similar diester, CDF18, **3**, the system favours the size of the sodium ion (0.098 nm radius)[20] and, in the case of CTF18, the calcium ion has the closest size amongst the divalent ions (0.106 nm radius).[20]

In contrast with the systems **3** and **4**, the probe CD222, **2**, did exhibit the predicted selective binding to potassium ions, as illustrated by the observed excitation spectra (Fig. 3). The fluorescence of CD222 increased by over a factor of two up to the saturating concentration of K^+ of 13.5 mmol dm^{-3}. The dissociation constant, K_D, was found to be 1 mmol dm^{-3} in the absence of background sodium; this value is slightly lower than the value of 1.9 mmol dm^{-3} reported by Golchini *et al.*[9] for the monocoumarin cryptand **5** (Table 3). In the presence of sodium ions, at a concentration of 135 mmol dm^{-3}, the K_D for potassium rose to 13.5 mmol dm^{-3}. This change is considered to be a general salt effect since the fluorescence response of the probe to potassium in the presence of Na^+ was similar, *except* that sodium ions *decreased* the initial fluorescence yield of the cryptand (Table 3); in the presence of sodium ions, the overall fluorescence intensity showed the same relative increase on proceeding from no K^+ to saturating concentrations. The behaviour of the probe was unaffected by the presence of Ca^{2+}

or Mg^{2+} ions. Fluorescence was also unaffected by pH changes over the range 6–8.

One final check on the behaviour of CD222, **2**, was carried out. We were initially concerned that the cage structure of this probe would slow down the rate of exchange of potassium ions so that equilibration times would preclude its use as a potassium ion monitor (*vide supra*). Fluorescence against time studies showed that exchange was quite rapid (< a second) upon addition of K^+ and that equilibration was complete within the mixing times. Studies on the possible use of this probe to measure extracellular changes in K^+ concentrations are in hand and will be reported elsewhere.

Experimental

M.p.s were determined on a hot-stage apparatus and are uncorrected. All chromatographic purifications were carried out using Sorbasil silica gel and redistilled solvents. Thin layer chromatography (TLC) was performed on Whatman 2.5 × 7.5 cm glass-backed plates with a 0.25 mm layer of silica gel 60 F_{254}.

Solvents were redistilled and, where necessary, dried before use.

^1H NMR spectra were obtained on either a Varian CFT-20 (80 MHz) instrument or a Bruker AMX200 (200 MHz) or 400 (400 MHz) spectrometers and, unless otherwise stated, for solutions in deuteriochloroform, using tetramethylsilance as an internal reference. Mass spectra were recorded on an AEI MS902 spectrometer; the high resolution and FAB spectra were obtained on the SERC facilities, Department of Chemistry, University College, Swansea. Infrared spectra were obtained on a Perkin-Elmer 1420 Ratio recording spectrophotometer, using KBr discs unless otherwise stated. UV spectra were obtained on a Perkin-Elmer Lambda 5 spectrophotometer and fluorescence data were recorded using a Perkin-Elmer LS50B luminescence spectrometer at room temperature with the excitation slitwidth set at 10 nm and the emission bandwidth at 10 nm, generally with a scan speed of 500 nm min^{-1}. In making solutions for the fluorescence measurements water purified (18 MΩ cm) on a MILLI-Q Plus water system (Millipore) was employed. AnalaR grade chloride salts of Na^+, K^+, Mg^+, and Ca^+ were used. Probe solutions were prepared by initially dissolving the probe in a few drops of dimethyl sulfoxide before diluting to volume with water and using immediately. The probe concentrations were kept at 1×10^{-6} mol dm^{-3} and the solutions were buffered with 10^{-2} mol dm^{-3} MOPS [3-(morpholino)propanesulfonic acid] brought to pH 7 with Triton B (benzyltrimethylammonium hydroxide in methanol, 40%). Dilutions were obtained by mixing samples from stock solutions of the components to give a final volume of 2 cm^3; pH measurements were monitored using a Corning pH meter 240, standardised against two buffers at pH 4 and 7. Quantum yields (Φ_f) were calculated by comparison of the integral of the emission spectrum with the corresponding integral for quinine bisulfate in 1 mol dm^{-3} perchloric acid of equal absorbance, assuming a quantum yield of 0.59. Dissociation constants (K_D) were calculated according to the procedure of Bourson and Valeur[17] using the fluorescence intensity of the free probe, $I_F°$, and that of the solution, I_F, at the concentration of the metal ion, M^+; plotting $I_F°/(I_F - I_F°)$ *versus* $[M^+]^{-1}$, gives the K_D by the ratio gradient/intercept.

Timed fluorescence changes were carried out using the Timedrive facility of the LS50B instrument, using an interval response of 0.5 s.

4-Benzyloxy-2-nitrophenol **6**.—This was prepared according to the method of Grynkiewicz *et al.*[11] as yellow crystals (16 g, 62%), m.p. 68–70 °C (from methanol–water), (lit.,[11] 67–70 °C); R_F 0.66 diisopropyl ether (Found: C, 63.7; H, 4.5; N, 5.7. Calc. for $C_{13}H_{11}NO_4$: C, 63.7; H, 4.5; N, 5.7%).

1,2-Bis(4-benzyloxy-2-nitrophenoxy)ethane **7**.—A mixture of **6** (15 g, 60 mmol), 1,2-dibromoethane (5.76 g, 30 mmol) and K_2CO_3 (9.29 g, 67 mmol) in dimethylformamide (DMF) (25 cm^3) was heated at 120 °C overnight. The reaction was cooled, after which water (30 cm^3) was added and stirred for 30 min at room temp. The resulting brown precipitate was collected, washed with water and dried *in vacuo* at 80 °C. Recrystallisation from glacial acetic acid (AcOH) afforded yellow/beige crystals of the *bis-nitro* compound **7** (12.41 g, 80%), m.p. 184–185 °C (from AcOH) (Found: C, 65.2; H, 4.6; N, 5.35. $C_{28}H_{24}N_2O_8$ requires C, 65.1; H, 4.7; 6, 5.4%); δ(200 MHz; [$^2H_6$]DMSO) 4.42 (4 H, s, OCH_2CH_2O), 5.10 (4 H, s, OCH_2Ar) and 7.30–7.60 (16 H, m, 3-H, 5-H, 6-H, Ph); *m/z* 516 (M^+, 26) 486 ($M^+ - NO$, 19), 181 ($C_8H_7NO_4$, 6) and 91 (CH_2Ph, 100); ν_{max}/cm^{-1} 1520, 1350 (conjugated NO_2) and 1220 C–O).

1,2-Bis(4'-benzyloxy-2-aminophenoxy)ethane **8**.—A suspension of **7** (5 g, 100 mmol), 5% platinum-on-charcoal (0.1 g) in tetrahydroduran (THF) (200 cm^3) and MeOH (100 cm^3) was hydrogenated for 3 h at atmospheric pressure at room temp. The reaction mixture was filtered and the solvent removed *in vacuo* leaving a light-grey solid, which was recrystallised from acetone. The *bis-amine* **8** was obtained as light-brown crystals (91%), m.p. 160–161 °C (decomp.) (from acetone) (Found: C, 73.9; H, 6.15; N, 6.1. $C_{28}H_{28}N_2O_4$ requires C, 73.7; H, 6.15; N, 6.1%); δ(200 MHz; [$^2H_6$]DMSO) 4.18 (4 H, s, OCH_2CH_2O), 4.48 (4 H, br s, D_2O exchangeable, NH_2), 4.92 (4 H, s, OCH_2Ar), 6.15 (2 H, dd, *J* 3 and 9, 5'-H), 6.35 (2 H, d, *J* 3, 3'-H), 6.69 (2 H, d, *J* 9, 6'-H) and 7.31 (10 H, m, Ph); *m/z* 456 (M^+, 66), 366 (14), 241 (19) and 91 (CH_2Ph, 100); ν_{max}/cm^{-1} 3410 and 3425 (NH_2).

1⁴,6⁴-Dibenzyloxy-2,5,10,13-tetraoxa-7,16-diaza-1(1,2),6-(1,2)-benzenacyclohexadecaphane-8,15-dione **9**.—The reaction between 1,2-ethylene-*O,O*-diglycolic acid chloride[12] and **8** was performed under high dilution and dry conditions at 0 °C. To a 3 dm^3 three-necked flask equipped with a mechanical stirrer and two 250 cm^3 pressure equalising funnels, was added a solution of triethylamine (1.33 g, 13 mmol) in THF (1 cm^3) and maintained at 0 °C. The bis-amine **8** (3 g, 6.6 mmol) in THF (500 cm^3), and the diacid chloride (1.42 g, 6.6 mmol) in THF (500 cm^3) were added dropwise, simultaneously through the dropping funnels. Addition was complete after 10 h, maintaining the reaction temperature at 0 °C. Stirring was continued for a further 24 h and the reaction mixture was filtered to remove the salts. Removal of the solvent *in vacuo* left a beige residue, which was crystallised from cold ethyl acetate to afford white crystals of the *diamide*, **9** (2.36 g, 60%), m.p. 158–159 °C (from ethyl acetate); R_F 0.49 ethyl acetate (Found: C, 68.1; H, 5.7; N, 4.6. $C_{34}H_{34}N_2O_8$ requires C, 68.2; H, 5.7; N, 4.7%); δ(200 MHz) 3.81 (4 H, s, 11-CH_2, 12-CH_2), 4.10 (4 H, s, 3-CH_2, 4-CH_2), 4.32 (4 H, s, 9-CH_2, 14-CH_2), 5.03 (4 H, s, OCH_2Ph), 6.63 (2 H, dd, *J* 3 and 9, 1^5-H, 6^5-H), 6.75 (2 H, d, *J* 9, 1^6-H, 6^6-H), 7.28–7.45 (10 H, m, Ph), 8.19 (2 H, d, *J* 3, 1^3-H, 6^3-H) and 9.08 (2 H, br s, D_2O exchangeable, NH); *m/z* 598 (M^+, 10%), 91 (CH_2Ph, 67) and 28 (CO, 100); ν_{max}/cm^{-1} 3400, 3120 and 1690 (secondary amide).

1⁴,6⁴-Dibenzyloxy-2,5,10,13-tetraoxa-7,16-diaza-1(1,2),6-(1,2)-dibenzenacyclohexadecaphane **10**.—To a suspension of $LiAlH_4$ (1.5 g, 40 mmol) in dry THF (200 cm^3) at 0 °C was added dropwise a solution of the amide **9** (1.8 g, 3 mmol) in THF (50 cm^3). When the addition was complete the reaction mixture was heated under reflux overnight. The excess of $LiAlH_4$ was destroyed by the dropwise addition of aqueous NaOH (3 mol

dm^{-3}), the reaction mixture filtered and the filtrate was evaporated to dryness to leave a brown residue. Column chromatography was performed with $CHCl_3$ and gradient elution MeOH (up to 1%) to give the *title diazacrown* as white crystals (1.32 g, 77%), m.p. 151–152 °C (from ethyl acetate); R_F 0.18 MeOH–$CHCl_3$ (1:9) (Found: C, 71.45; H, 6.7; N, 4.7. $C_{34}H_{38}N_2O_6$ requires C, 71.6; H, 6.7; N, 4.9%); δ(200 MHz) 3.32 (4 H, t, *J* 5, NCH_2CH_2O) 3.59 (4 H, s, 11-CH_2, 12-CH_2), 3.68 (4 H, t, *J* 5, OCH_2CH_2N), 4.28 (4 H, s, 3-CH_2, 4-CH_2), 5.00 (4 H, s, OCH_2Ar), 6.23 (2 H, dd, *J* 3 and 9, 1^5-H, 6^5-H), 6.28 (2 H, d, *J* 3, 1^1-H, 6^3-H), 6.67 (2 H, d, *J* 9, 1^6-H, 6^6-H) and 7.20–7.50 (10 H, m, Ph); *m/z* 570 (M^+, 8%), 479 ($M^+ - CH_2Ph$, 6), 268 (68), 254 (24), 165 (40), 136 (44) and 91 (CH_2Ph, 100); ν_{max}/cm^{-1} 3420 (NH).

$1^4,6^4$-*Dibenzyloxy*-7,16-*di*(*methoxycarbonylmethyl*)-2,5,10,13-*tetraoxa*-7,16-*diaza*-1(1,2),6(1,2)-*dibenzenacyclohexadecaphane* **11**.—A solution of **10** (1.10 g, 1.9 mmol), methyl bromoacetate (1.0 g, 6.54 mmol), 1,8-bis(dimethylamino)-naphthalene ('proton-sponge', 0.83 g, 3.8 mmol) in acetonitrile (10 cm^3) was heated under reflux overnight. After being cooled, the reaction mixture was filtered, the solvent was removed *in vacuo* and the residue chromatographed with $CHCl_3$ and gradient elution with MeOH (up to 1%). Following re-crystallisation from MeOH the *title ester* was isolated as white needles (1.28 g, 91%), m.p. 131–132 °C (from MeOH), R_F 0.1 MeOH–$CHCl_3$ (1:9) (Found: C, 67.2; H, 6.45; N, 3.8. $C_{40}H_{46}N_2O_{10}$ requires C, 67.2; H, 6.5; N, 3.9%); δ(400 MHz) 3.50 (4 H, t, *J* 5, NCH_2CH_2O), 3.51 (4 H, s, 11-CH_2, 12-CH_2, 3.59 (4 H, t, *J* 5, OCH_2CH_2N), 3.60 (6 H, s, Me), 4.27 (4 H, s, 3-CH_2, 4-CH_2), 4.29 (4 H, s, CH_2CO_2), 5.00 (4 H, s, OCH_2Ar), 6.50 (2 H, dd, *J* 3 and 9, 1^5-H, 6^5-H), 6.70 (2 H, d, *J* 3, 1^3-H, 6^3-H), 6.81 (2 H, d, *J* 9, 1^6-H, 6^5-H) and 7.30–7.44 (10 H, m, Ph); *m/z* 714 (M^+, 5%), 655 ($M^+ - CO_2Me$, 3), 623 ($M^+ - CH_2Ph$, 4) and 91 (CH_2Ph, 100); ν_{max}/cm^{-1} 2875–2950 (Ar), 1740 (C=O), 1610 (Ar), 1500 (Ar), 1200–1000 (C–O), 720 and 740 (Ar).

$1^4,6^4$-*Dibenzyloxy*-7,16-*di*(*methoxycarbonylmethyl*)-2,5,10,13-*tetraoxa*-7,16-*diaza*-1(1,2),6(1,2)-*dibenzenacyclohexadecaphane*-$1^5,6^5$-*dicarbaldehyde* **12**.—To an ice-cooled solution of the bis-ester **11** (0.5 g, 0.84 mmol) in DMF (5 cm^3) at 0 °C was added a mixture of $POCl_3$–DMF (1:4 v/v, 10 cm^3). The reaction was allowed to reach room temp. and stirring was continued for a further 3 h. Upon careful quenching with water (10 cm^3) a light green precipitate of crude product appeared and the suspension was neutralised using saturated aqueous K_2CO_3. Column chromatography, using ethyl acetate as the eluent, gave the *bis-formyl compound* (0.5 g, 77%), m.p. 158–159 °C; R_F 0.62 ethyl acetate (Found: $M^+ + H$, 771.3325. $C_{42}H_{47}N_2O_{12}$ requires *M*, 771.3339; δ(400 MHz) 3.47 (4 H, s, 11-CH_2, 12-CH_2), 3.50–3.56 (8 H, m, OCH_2CH_2N), 3.55 (6 H, s, Me), 4.28 (4 H, s, CH_2CO_2), 4.42 (4 H, s, 3-CH_2, 4-CH_2), 6.39 (2 H, s, 1^3-H, 6^3-H), 7.28 (2 H, s, 1^6-H, 6^6-H), 7.30–7.40 (10 H, m, Ph) and 10.31 (2 H, s, CHO); *m/z* 771 (M^+, 2%) and 91 (CH_2Ph, 100); ν_{max}/cm^{-1} 2875–2950 (Ar), 1740 (ester C=O), 1640 (aryl C=O), 1600, 1510 (Ar) and 1210–1000 (C–O).

$1^4,6^4$-*Dihydroxy*-7,16-*di*(*methoxycarbonylmethyl*)-2,5,10,13-*tetraoxa*-7,16-*diaza*-1(1,2),6(1,2)-*dibenzenacyclohexadecaphane*-$1^5,6^5$-*di-carbaldehyde* **13**.—The bis-aldehyde **12** (0.4 g, 0.52 mmol) in THF (10 cm^3) and MeOH (6 cm^3) was hydrogenated with 10% palladium-on-charcoal (40 mg) for 2 h. The reaction mixture was filtered and the solvent removed *in vacuo* to leave a *pale yellow solid* (0.29 g, 97%). A small portion was column chromatographed, using $CHCl_3$–MeOH gradient elution (up to 2% MeOH) and the product was recrystallised from MeOH to give the *salicylaldehyde* **13**, m.p. 155–156 °C (from MeOH); R_F 0.39 MeOH–$CHCl_3$ (1:9) (Found: C, 56.7; H, 5.8; N, 4.6. $C_{28}H_{34}N_2O_{12}$ requires C, 56.95; H, 5.8; N, 4.7%); δ(200 MHz) 3.56 (4 H, s, 11-CH_2, 12-CH_2), 3.58 (6 H, s, Me), 3.59 (4 H, t, *J* 5, NCH_2CH_2O), 3.73 (4 H, t, *J* 5, OCH_2CH_2N), 4.27 (4 H, s, CH_2CO_2), 4.55 (4 H, s, 3-CH_2, 4-CH_2), 6.31 (2 H, s, 1^3-H, 6^3-H), 6.84 (2 H, s, 1^6-H, 6^6-H), 9.59 (2 H, s, CHO) and 11.25 (2 H, s, OH); *m/z* 590 (M^+, 100), 559 ($M^+ - OMe$, 33), 531 ($M^+ - CO_2Me$, 97), 561 ($M^+ - CHO$, 4), 44 (25) and 28 (CO, 84); ν_{max}/cm^{-1} 1750 (ester C=O), 1730 (aryl C=O) and 1510 (Ar).

7,6-*Di*(*methoxycarbonylmethyl*)-$1^2,6^2$-*dioxo*-2,5,10,13-*tetraoxa*-7,16-*diaza*-1(7,6),6(6,7)-*di*(2H-2-*benzopyrana*)*cyclohexadecaphane*-$1^3,6^3$-*dicarboxylic Acid* CDF18 (**3**).—The bis-salicylaldehyde **13** (300 mg, 0.5 mmol), malonic acid (160 mg, 1.5 mmol) and pyrrolidine (3 drops) in DMF (4 cm^3) were heated at 60 °C for 2 h. Water (1 cm^3) was added followed by aqueous HCl (2 mol dm^{-3}, 6 cm^3) which precipitated the *diacid* CDF18 as a yellow solid (130 mg, 35%). A sample was recrystallised from MeOH, m.p. 155–156 °C (decomp.) (Found: $M^+ + H$, 727.2022. $C_{34}H_{35}N_2O_{16}$ requires *M*, 727.1987); δ(200 MHz) 3.58 (6 H, s, Me), 3.60 (4 H, s, 11-CH_2, 12-CH_2), 3.27 (4 H, t, *J* 5, NCH_2CH_2O), 3.80 (4 H, t, *J* 5, OCH_2CH_2N), 4.40 (4 H, s, CH_2CO_2), 4.60 (4 H, s, 3-CH_2, 4-CH_2), 6.88 (2 H, s, 1^5-H, 6^5-H), 6.97 (2 H, s, 1^8-H, 6^8-H) and 8.74 (2 H, s, 1^4-H, 6^4-H); *m/z* (FAB) 727 (M + H) and 749 (M + Na).

Dimethyl 7,16-*Di*(*methoxycarbonylmethyl*)-$1^2,6^2$-*dioxo*-2,5,10,13-*tetraoxa*-7,16-*diaza*-1(7,6),6(6,7)-*di*(2H-2-*benzopyrana*)-*cyclohexadecaphane*-$1^3,6^3$-*dicarboxylate* **14**.—A solution of the salicylaldehyde **13** (200 mg, 0.34 mmol), dimethyl malonate (200 mg, 1.5 mmol), piperidine (2 drops) and glacial AcOH (1 drop) in MeOH (5 cm^3) were heated under reflux overnight. A yellow precipitate of the crude coumarin appeared, which was chromatographed, using chloroform and MeOH (up to 1%), to give the *tetra-ester* 200 mg, 88%), m.p. 250–252 °C (decomp.) from MeOH; R_F 0.23 ethyl acetate (Found: C, 57.1; H, 5.05; N, 3.7. $C_{36}H_{38}N_2O_{16}$ requires C, 57.3; H, 5.1; N, 3.7%); δ(200 MHz) 3.59 (4 H, s, 11-CH_2, 12-CH_2), 3.57 (6 H, s, 1^3-,6^3-CO_2Me), 3.63 (4 H, t, *J* 5, NCH_2CH_2O), 3.74 (4 H, t, *J* 5, OCH_2CH_2N), 3.94 (6 H, s, 7-, 16-CO_2Me), 4.36 (4 H, s, CH_2CO_2), 4.54 (4 H, s, 3-CH_2, 4-CH_2), 6.72 (2 H, s, 1^5-H, 6^5-H), 6.88 (2 H, s, 1^8-H, 6^8-H) and 8.49 (2 H, s, 1^4-H, 6^4-H); *m/z* 754 (M^+, 8), 723 ($M^+ - OMe$, 3), 695 ($M^+ - CO_2Me$), 44 (65), 28 (CO, 100); ν_{max}/cm^{-1} 1770–1700 (C=O), 1620 and 1510 (Ar).

7,16-*Di*(*carboxymethyl*)-$1^2,6^2$-*dioxo*-2,5,10,13-*tetraoxa*-7,16-*diaza*-1(7,6),6(6,7)-*di*-(2H-2-*benzopyrana*)*cyclohexadecaphane*-$1^3,6^3$-*dicarboxylic Acid* CTF18 (**4**).—*Method A*. A solution of the diacid diester CDF18 (10 mg, 0.014 mmol), $LiOH{\cdot}H_2O$ (4 mg, 0.08 mmol) in water (2 cm^3) was stirred at room temp. overnight and neutralised with aqueous HCl (3 mol dm^{-3}) upon which a yellow precipitate of product CTF18 appeared (5 mg, 54%, purity 90% by NMR).

Method B. A suspension of the tetra-ester **14** (20 mg, 0.03 mmol) in water (2 cm^3) and MeOH (1 cm^3) containing $LiOH{\cdot}H_2O$ (8 mg, 0.18 mmol) was stirred at room temperature for 7 days until a fluorescent yellow/brown solution was observed. Aqueous HCl (3 mol dm^{-3}; 1 cm^3) was added which precipitated the desired tetra-acid, CTF18 of greater purity by NMR spectroscopy than by method A (13 mg, 72%), m.p. 218–220 °C (decomp.) (Found: $M + H^+$, 699.1694. $C_{32}H_{31}N_2O_{16}$ requires $M + H^+$, 699.1674); δ(200 MHz; [$^2H_6$]DMSO) 3.48 (4 H, s, 11-CH_2, 12-CH_2), 3.64 (8 H, br s, NCH_2CH_2O), 4.37 (4 H, s, CH_2CO_2), 4.44 (4 H, s, 3-CH_2, 4-CH_2), 6.78 (2 H, s, 1^5-H, 6^6-H), 7.40 (2 H, s, 1^8-H, 6^8-H), 8.62 (2 H, s, 1^4-H, 6^4-H) and 12.60 (2 H, br s, D_2O exchangeable, CO_2H); *m/z* (FAB) 699 (*M* + H) and 721 (*M* + Na).

$19^4,24^4$-*Dibenzyloxy*-4,7,13,16,20,23-*hexaoxa*-1,10-*diaza*-19-(1,2),24(1,2)-*dibenzenabicyclo*[8.8.6]*tetracosaphane*-2,9-*dione* **15**.—This reaction was performed under high dilution and dry conditions between 0–20 °C. To a 3 dm^3 three-necked flask equipped with a mechanical stirrer and two 250 cm^3 pressure-equalising funnels was placed a solution of pyridine (0.83 g, 11 mmol) in THF (1.5 dm^3) maintained at 0 °C. The diaza-18-crown-6 **12** (3 g, 5.3 mmol) in THF (250 cm^3) and 1,2-ethylene-*O*,*O*-diglycolic acid chloride[12] (1.13 g, 5.3 mmol) in THF (250 cm^3) were simultaneously added dropwise to the solution *via* the funnels over a period of 8 h. Stirring was continued for a further 48 h and the solvent removed *in vacuo* to leave a brown gummy paste which was dissolved in dichloromethane (200 cm^3) and washed with aqueous HCl (3 mol dm^{-3}; 2 × 100 cm^3). The organic layer was dried (Na_2SO_4) and the solvent removed *in vacuo*. The resulting brown foam was column chromatographed with $CHCl_3$ and gradient eluted with MeOH (up to 5%) to afford the *bis-amide* **15** as a white solid (1.6 g, 43%), m.p. 217–218 °C (from MeOH); R_F 0.35 MeOH–$CHCl_3$ (1:9) (Found: $M + H^+$, 713.3112. $C_{40}H_{45}N_2O_{10}$ requires $M + H^+$, 713.3074); δ(90 MHz) 3.40–4.00 (20 H, m, OCH_2, NCH_2), 4.33 (4 H, br s, 21-CH_2, 22-CH_2), 4.96 (4 H, s, OCH_2Ar), 6.68 (2 H, br s, 19^5-H, 24^5-H), 6.87 (4 H, br s, 19^3-, 19^6-H, 24^3-, 24^6-H) and 7.32 (10 H, m, Ph); *m*/*z* 712 (M^+, 54%), 621 (M^+ − CH_2Ph, 27) and 91 (CH_2Ph); ν_{max}/cm^{-1} 1690 (tertiary amide) and 1525 (Ar).

$19^4,24^4$-*Dibenzyloxy*-4,7,13,16,20,23-*hexaoxa*-1,10-*diaza*-19-(1,2),24(1,2)-*dibenzenabicyclo*[8.8.6]*tetracosaphane* **16**.—The general method of Pettit *et al.*[15] was employed with a modified work-up procedure. To an ice-cooled solution of borane in THF (1 mol dm^{-3}; 8.2 cm^3; 8.2 mmol) was added dropwise the bis-amide **15** (1.3 g, 1.8 mmol) in THF (15 cm^3). The solution was heated under reflux for 1.5 h, cooled and water (2 cm^3) carefully added followed by aqueous HCl (6 mol dm^{-3}; 16 cm^3). The solvent was removed *in vacuo* at 50 °C and the resulting white suspension/emulsion was brought to pH 8 with aqueous LiOH. The aqueous layer was extracted with chloroform (3 × 75 cm^3) and dried (Na_2SO_4). Removal of the solvent *in vacuo* left a yellow/brown gum which was triturated with hot ethyl acetate to give white crystals of the *bis-amine* (1.1 g, 88%), m.p. 173–174 °C; R_F 0.1 MeOH–$CHCl_3$ (1:9) (Found: $M^+ + H$, 685.3522. $C_{40}H_{49}N_2O_8$ requires $M + H^+$, 685.3489); δ(90 MHz) 3.00–3.90 (24 H, m, OCH_2, NCH_2), 4.26 (4 H, s, 21-CH_2, 22-CH_2), 4.96 (4 H, s, OCH_2Ar), 6.52 (2 H, dd, *J* 3 and 19^5-H, 24^5-H), 6.56 (2 H, d, *J* 3, 19^3-H, 24^3-H), 6.85 (2 H, d, *J* 9, 19^6-H, 24^6-H) and 7.32 (10 H, m, Ph); *m*/*z* 684 (M^+, 92%), 623 (34), 609 (45), 593 (M^+ − CH_2Ph, 78), 188 (23) and 91 (CH_2Ph, 19); ν_{max}/cm^{-1} 2680–2920 (Ar), 2800 (NCH_2), 1610 (Ar) and 1505 (Ar).

$19^5,24^5$-*Dibenzyloxy*-4,7,13,16,20,23-*hexaoxa*-1,10-*diaza*-19-(1,2),24(1,2)-*dibenzenabicyclo*[8.8.6]*tetracosaphane*-$19^4,24^4$-*dicarbaldehyde* **17**.—To an ice-cooled suspension of the bis-amine **16** (1.0 g, 1.5 mmol) in DMF (10 cm^3) was added a mixture of $POCl_3$–DMF (1:4 v/v, 30 cm^3). The reaction was allowed to reach room temp. and stirring was continued for a further 3 h. The dark yellow/brown solution was carefully quenched with water (10 cm^3) and neutralised with saturated Li_2CO_3 upon which a beige precipitate of the *title bis-aldehyde* appeared (0.95 g, 88%), m.p. 190–200 °C (decomp.) (from MeOH) (Found: $M^+ + H$, 741.3423. $C_{42}H_{49}N_2O_{10}$ requires $M + H^+$, 741.3387); δ(90 MHz) 3.00–3.70 (24 H, m, OCH_2, NCH_2), 4.28 (4 H, s, 21-CH_2), 22-CH_2), 5.12 (4 H, s, OCH_2Ar), 6.26 (2 H, s, 19^6-H, 24^6-H), 7.21 (2 H, s, 19^3-H, 24^3-H), 7.33 (10 H, m, Ph) and 10.28 (2 H, s, CHO); *m*/*z* 740 (M^+), 711 (M^+ − CHO, 51), 649 (M^+ − CH_2Ph, 26), 622 (20) and 91 (CH_2Ph, 100); ν_{max}/cm^{-1} 2870–2950 (Ar), 1665 (aryl C=O), 1610 and 1510 (Ar).

$19^5,24^5$-*Dihydroxy*-4,7,13,16,20,23-*hexaoxa*-1,5-*diaza*-19(1,2),24(1,2)-*dibenzenabicyclo*[8.8.6]*tetracosaphane*-$19^4,24^4$-*dicarbaldehyde* **18**.—A suspension of the bis-aldehyde cryptand **17** (0.4 g, 0.54 mmol), 10% palladium-on-charcoal (40 mg) in THF (200 cm^3) and MeOH (20 cm^3) was stirred under a hydrogen atmosphere for 45 min. The reaction mixture was filtered and the solvent removed *in vacuo* to leave the title phenol as a gum, which was used directly in the next step of the reaction sequence (0.25 g, 83%); δ(90 MHz) 3.00–3.70 (24 H, m, OCH_2, NCH_2), 4.29 (4 H, s, 21-CH_2, 22-CH_2), 6.29 (2 H, s, 19^6-H, 24^6-H), 6.93 (2 H, s, 19^3-H, 24^3-H), 9.56 (2 H, s, CHO) and 11.18 (2 H, s, D_2O exchangeable, OH); *m*/*z* 560 (M^+, 100%), 532 (M^+ − CO, 6), 499 (17), 485 (49), 460 (23), 182 (23), 167 (35) and 149 (28).

Dimethyl $19^2,24^2$-*Dioxo*-4,7,13,16,20,23-*hexaoxa*-1,10-*diaza*-19(7,6),24(6,7)-*di*(2H-2-*benzopyrana*)*bicyclo*[8.8.6]*tetracosaphane*-$19^3,24^3$-*dicarboxylate* **19**.—The crude bis-salicylaldehyde **18** (200 mg, 0.36 mmol), dimethyl malonate (240 mg, 1.8 mmol), piperidine (2 drops), and glacial acetic acid (1 drop) in MeOH (10 cm^3) were heated under reflux for 6 h during which time an orange/yellow precipitate appeared of the desired *bis-coumarin* (165 mg, 64%), m.p. 240 °C (decomp.) (from MeOH); R_F 0.26 MeOH–$CHCl_3$ (1:9) (Found: $M^+ + H$, 725.2055. $C_{36}H_{41}N_2O_{14}$ requires $M + H^+$, 725.2020); δ(90 MHz) 3.30–4.00 (24 H, m, OCH_2, NCH_2), 3.91 (6 H, s, CH_3) 4.37 (4 H, s, 21-CH_2, 22-CH_2), 6.68 (2 H, s, 19^5-H, 24^5-H), 6.93 (2 H, s, 19^8-H, 24^8-H) and 8.43 (2 H, s, 19^4-H, 24^4-H); *m*/*z* (FAB) 725 (*M* + H); ν_{max}/cm^{-1} 1780 (C=O), 1625 and 1515 (Ar).

$19^2,24^2$-*Dioxo*-4,7,13,16,20,23-*hexaoxa*-1,10-*diaza*-19(7,6),24(6,7)-*di*(2H-2-*benzopyrana*)*bicyclo*[8.8.6]*tetracosaphane*-$19^3,24^3$-*dicarboxylic Acid* CD222 (**2**).—A suspension of the diester **19** (120 mg, 0.14 mmol) in water (10 cm^3) and MeOH (2 cm^3) containing LiOH·H_2O (150 mg, 0.83 mmol) was stirred at room temperature for 5 h. The resulting solution was acidified with aqueous HCl (3 mol dm^{-3}) which precipitated the desired *diacid* CD222 as a yellow solid that was collected, washed with water and dried *in vacuo* over P_2O_5 (90 mg, 78%) (Found: $M^+ + H$, 697.2299). $C_{34}H_{37}N_2O_{14}$ requires $M^+ + H$, 697.2245); δ(90 MHz; [$^2H_6$]DMSO) 3.30–3.90 (24 H, m, OCH_2NCH_2), (4 H, s, 21-CH_2, 22-CH_2), 6.87 (2 H, s, 19^5-H, 24^5-H), 7.45 (2 H, s, 19^8-H, 24^8-H) and 8.58 (2 H, s, 19^4-H, 24^4-H); *m*/*z* (FAB) 697 (*M* + H) and 719 (*M* + Na).

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