Synthesis and Spectral Properties of New Fluorescent Probes for Potassium

Roger Crossley,[b] Zia Goolamali,[a] Jeffrey J. Gosper[a] and Peter G. Sammes*[,a]
[a] *Molecular Probes Unit, Department of Chemistry, Brunel University, Uxbridge, Middlesex, UK UB8 3PH*
[b] *Wyeth Research (UK) Ltd., Huntercombe Lane South, Taplow, Berks, UK SL6 0PH*

Studies on the preparation and properties of two new, selective fluorescent probes CD18, **1** and C18, **2** for potassium are described. The probes incorporate the 1,10-diaza-18-crown-6 chelating group for the ion and the coumarin group as the fluorophore. The probes are compared with the known reagent PBFI. CD18 shows considerably greater selectivity for potassium over sodium than PBFI.

Potassium (K^+) is a major cationic constituent of living cells, existing at an intracellular concentration of approximately 150 mmol dm^{-3}, whilst extracellular levels are typically around 4 mmol dm^{-3}. This concentration gradient is primarily maintained in conjugation with sodium (Na^+) by the so-called 'sodium pump', which uses up about a third of the cell's available energy.[1] The pump allows the cell to power many physiological processes such as nutrient-uptake and signalling. Consequently, methods for the detection and monitoring of K^+ levels provide the clinical biologist with a powerful means to study, for instance, the effects of drugs or disorders, *e.g.* hypertension, on the ionic equilibrium. The potassium–sodium ionic exchange often involves pulses (firing mechanisms) in which transient changes in the local concentrations of the ions occur and means for monitoring these rapid changes would also be of value. Of various analytical techniques available for the determination of potassium in aqueous media few either do not result in the destruction of the cells, or are suitable for real time monitoring studies. An analytical method for K^+ based on optical measurements which allow such studies is, therefore, an attractive concept that has become a possibility since Pederson's discovery that it is possible to make macrocyclic crown ethers that selectively complex alkali and alkaline earth cations.[2] Although several examples of chromogenic K^+-chelators have been reported in the last couple of decades,[3] these require extractions into organic media before measurement or operate in non-aqueous media only. Very few operate in aqueous fluids although Czech *et al.*[4] developed a chromogenic hemispherand that exhibited a change in the absorption spectrum upon K^+ binding. Minta and Tsien[5] have described the use of 'PBFI' (*vide infra*) and Golchini *et al.*[6] have employed fluorescence as a means of detection which offers greater sensitivity and excellent signal:noise ratios. Although these fluorescent probes are useful they suffer from two limitations, in that they work only at the higher range of biological potassium levels, and their response to potassium ion involves only an enhancement of fluorescent quantum yield without any concomitant measurable shift in the emission or excitation maxima, a feature of an ideal potassium probe that would allow direct measurement of K^+ concentration.[5]

Other desirable properties of a K^+ probe include the following. (1) High selectivity for K^+ over Na^+ and other biological ions so that any interference from these is inconsequential. (2) Water solubility, which is essential for use in biological applications. Polar carboxylic acid groups incorporated into the structure of the probe greatly aid water solubility. (3) If the probe is to be used intracellularly it must be capable of being noninvasively loaded into the interior of the cell without diffusing out again *i.e.* be irreversibly cell permeant. This has been made possible by using acetoxymethyl esters that are hydrolysed by the cytoplasmic hydrolases leaving a charged species inside the cell.[7] (4) Possession of a dissociation constant that falls into the concentration range of the potassium to be measured. (5) Fast response time, preferably in the millisecond or faster range. (6) Operation under cellular conditions (taking into account pH, toxicity, and stability). (7) Showing a sufficiently large Stoke's shift to avoid spectral overlap. (8) Exhibiting reasonably efficient fluorescence changes to allow measurement on an intracellular basis. (9) Possession of an excitation maximum over 340 nm and emission maximum over 500 nm to avoid use of expensive quartz apparatus and tissue autofluorescence.

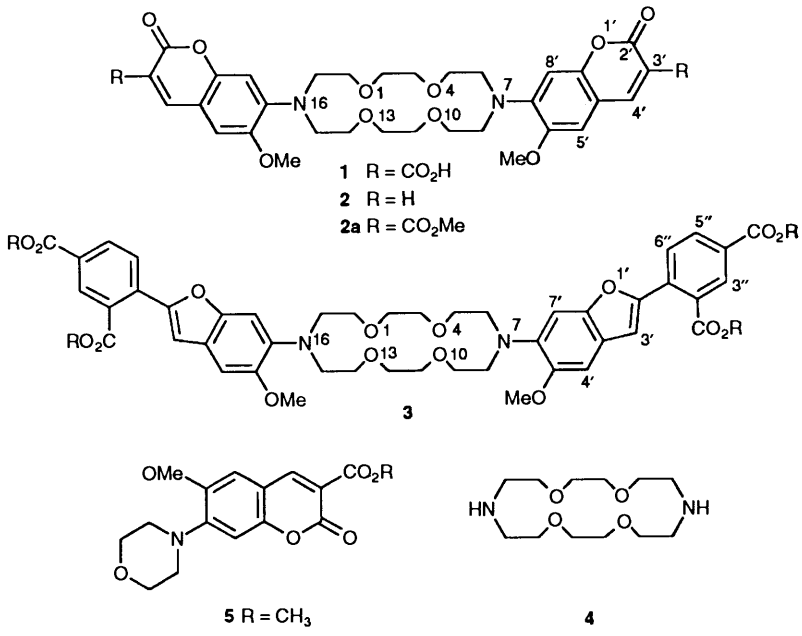
In reality the possibility of achieving all of the above in a single molecule poses an extremely difficult task that to date has not been achieved.

Herein, we describe the full synthesis of two new potassium selective fluorescent probes **1** (abbreviated to the acronym CD18) and **2** (abbreviated to the acronym C18) capable of measuring K^+ in an aqueous, physiological environment. Spectral properties are compared with those of the commercially available and structurally similar PBFI ('potassium-binding benzofuran isophthalate', **3**).[5]

Results and Discussion

Literature precedent in the development of fluorescent ionic probes has demonstrated that interactions between the ion recognition site and the associated fluorophore are complex and, therefore, it is difficult to predict the photophysical properties of any particular ligand–fluorophore combination. One general consequence is that upon binding, the ligand will generally become less mobile so that the vibrational modes of decay (internal conversion) of an excited state become less important; as a consequence specific binding of an ion to a ligand generally leads to increased fluorescence of the associated fluorophore. Direct electronic interactions can also affect the photophysical processes including alterations to the energy gap between the ground and excited states, so that shifts in the emission wavelength can be observed. Such ligand–fluorophore effects are therefore best studied experimentally.

The approach chosen was to utilise a known selective crown ether for potassium. The diaza-18-crown-6 ligand, **4**, is selective for K^+ ions[8] and hence was chosen as the core ligand. Since the potassium ion can accommodate up to ten chelating groups, further chelating atoms were desirable in the probe to assist the six atoms provided by the crown group. After some preliminary studies we chose the *o*-methoxy strategy adopted by Minta and Tsien.[5] Various alternative fluorophores were then considered before selecting the coumarin group, derivatives of which have the advantage of relatively good fluorescence quantum yields and photostability to light of $\lambda > 300$ nm.[9] The targets chosen were the coumarin derivatives **1** and **2**.



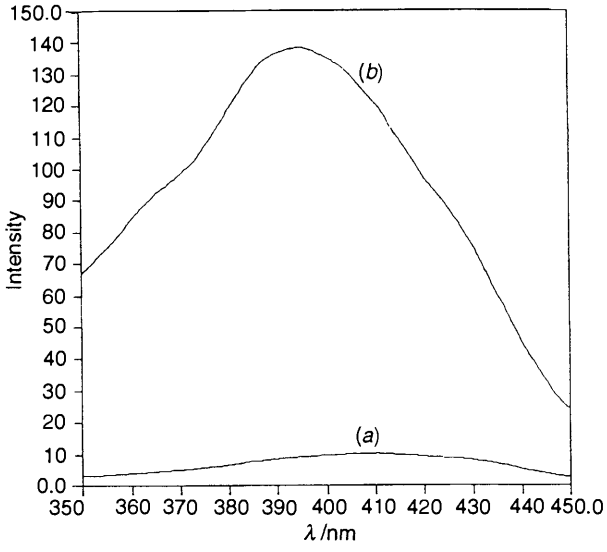
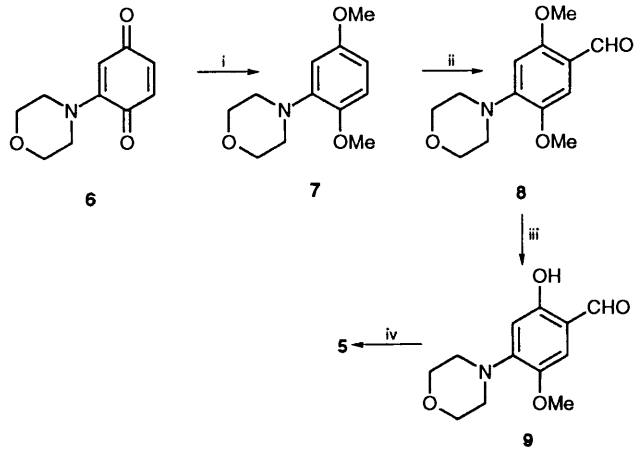


Fig. 1 Fluorescence excitation spectra of (a) 8×10^{-6} mol dm^{-3} morpholino-coumarin **5** and (b) 8×10^{-6} mol dm^{-3} CD18 emitted at 475 nm at pH 7

In order to gauge the effectiveness of the target compounds **1** and **2**, two reference compounds were also prepared, the commercial standard used in this area, PBFI **3**, and the non-chelating analogue of the diaza-18-crown-6 system **4**, the morpholine analogue **5**.

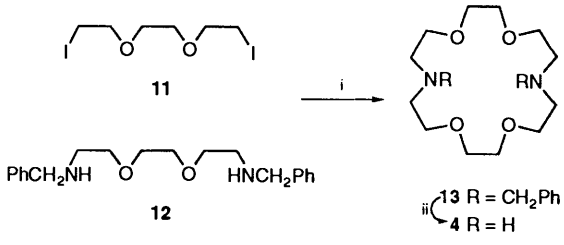
The morpholino derivative **5** was prepared as outlined in Scheme 1. Addition of morpholine to 1,4-benzoquinone proceeded smoothly to give the quinone adduct **6**. Reduction and alkylation with dimethyl sulfate gave the ether **7**. Formylation, using Vilsmeier–Haack conditions, gave the aldehyde **8** which could be selectively demethylated with boron trichloride to give the salicylaldehyde **9**. Condensation of the aldehyde with dimethyl malonate[10] gave the target reference coumarin **5**; the overall yield for this five-stage synthesis was 38%. The product coumarin showed only modest fluorescence in aqueous solvents (Fig. 1) but this was totally unaffected by the addition of cations such as Na^+, K^+, Ca^+ and Mg^{2+}.

Synthesis of the known PBFI probe and the target fluorophores required the initial synthesis of diaza-18-crown-6, **4**. Of



Scheme 1 i, H_2–Pd, Me_2SO_4–Triton B; ii, $POCl_3$–DMF; iii, BCl_3; iv, $(MeO_2C)_2CH_2$–piperidine–HOAc

several reported routes we chose that of Gokel *et al.*[11] This route utilised benzyl protecting groups and the reported method for debenzylation required catalytic hydrogenation. In our hands this step proved difficult and high pressure hydrogenation (at pressures > 60 psi) were required. In order to avoid this step an alternative route was developed, Scheme 2,

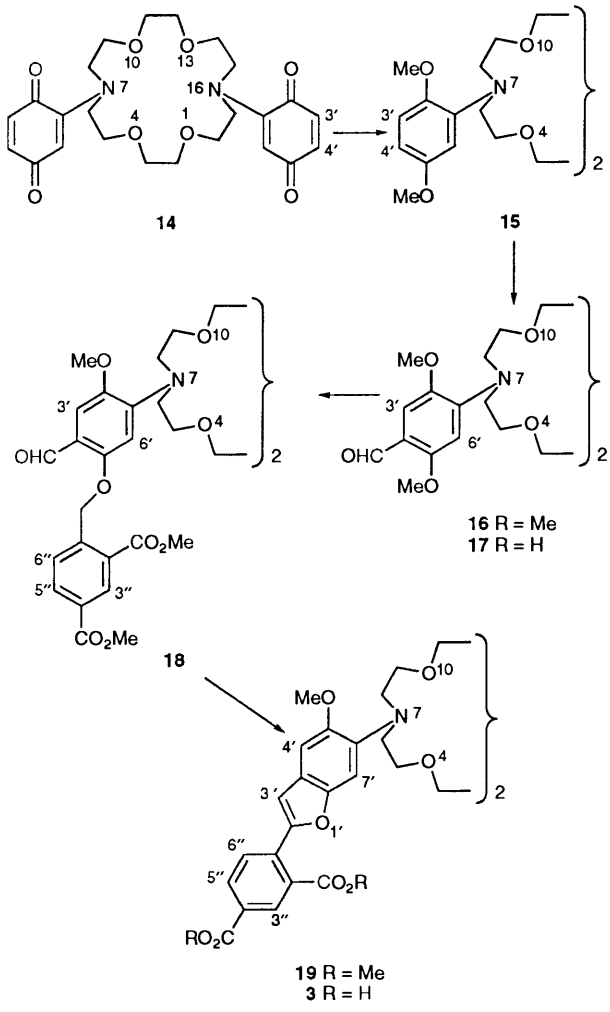


Scheme 2 i, high dilution; ii, Na–NH_3–THF

using sodium in liquid ammonia as the deprotection agent. Thus 1,2-bis(2-chloroethoxy)ethane **10** was separately converted into the diiodo compound **11** and the diamine **12**. Reaction of compound **11** with **12**, under high dilution conditions, afforded the protected crown **13**. Reductive cleavage of the *N*-benzyl groups, using sodium in liquid ammonia,[12]

initially failed and this was eventually traced to the high insolubility of the protected crown **13** in the ammonia. By using 2:1 liquid ammonia–tetrahydrofuran (THF) as the solvent and by slow addition of the amine, in solution in THF, to the sodium solution in the mixed solvent, a high yield (>90%) of the free crown **4** could be obtained.

The synthesis of PBFI followed a modified literature method,[5] as detailed in Scheme 3 (see Experimental). Because



Scheme 3

of the chelating properties of the incorporated crown unit, separations and purification of the intermediates **14** to **19** tended to be more difficult and the yields of each step much lower than for the morpholine model **5**, the overall yield of the tetra-acid **3** being only a few percent.

The bis-salicylaldehyde **17**, prepared en route to PBFI **3**, can be used to prepare a variety of heterocyclic systems, of which the coumarin group, the 1-benzopyran-2-one derivative, was of particular interest, since substituted coumarins are well-known fluorophores.[9] The salicylaldehyde **17** was condensed with dimethyl malonate under similar conditions to those used to prepare the morpholine analogue **5**; by using only trace quantities of piperidine and acetic acid as catalyst, the corresponding crown bis-coumarin ester **2a** was isolated in a clean reaction (78%).

Since eventual water solubility of the coumarin derivative is needed, selective removal of the ester groups to release the free carboxy groups was required; these carboxy groups also make possible the preparation of simple, transportable esters, such as acetoxymethyl derivatives, which can be loaded into cells followed by intracellular hydrolysis to prevent their egress.

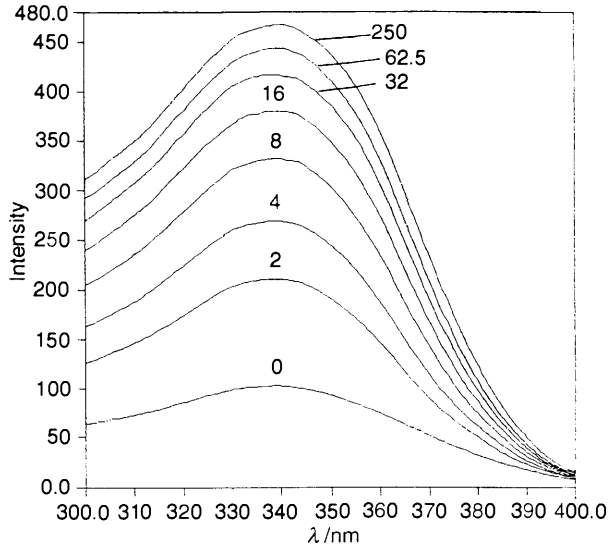


Fig. 2 Fluorescence excitation spectra of 8 × 10^{-6} mol dm^{-3} PBFI (**3**) as a function of 0–250 mmol dm^{-3} K^+ emitted at 505 nm at pH 7

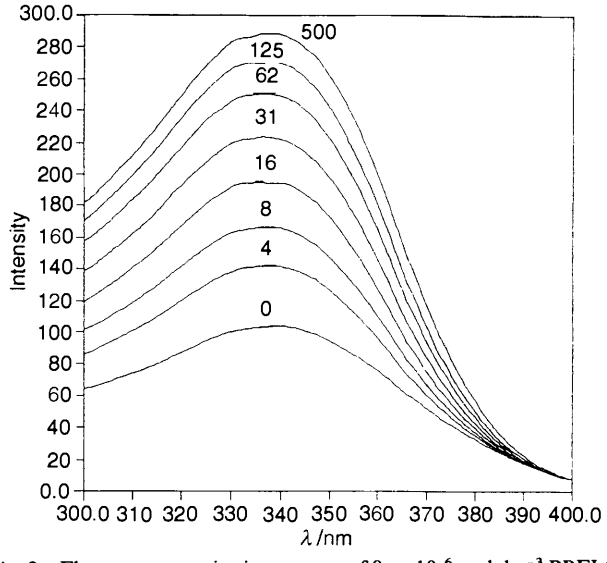


Fig. 3 Fluorescence excitation spectra of 8 × 10^{-6} mol dm^{-3} PBFI (**3**) as a function of 0–500 mmol dm^{-3} Na^+ emitted at 505 nm at pH 7

Direct hydrolysis by stirring with hot water or dilute aqueous acid failed to produce the desired acid. Use of aqueous methanolic potassium hydroxide for the hydrolysis appeared to be too harsh a condition, since degradation of the coumarin system accompanied ester hydrolysis. Consequently, milder base hydrolysis was applied, using lithium hydroxide,[13] which successfully produced a pure specimen of the diacid CD18 **1**.

The decarboxylated material C18, **2** was of interest as a possible membrane binding probe as well as a further reference compound to study the effect of the absence of the substituent carboxy groups. Preparation of **2**, was achieved by heating the diester **2a** in 9 mol dm^{-3} hydrochloric acid at reflux for 3 days; a yield of 70% was obtained for the decarboxylation.

The fluorescent properties of the synthesised probes were examined under standard conditions following those developed by Minta and Tsien.[5] The diester crown-bis-coumarin **2a** was too insoluble in aqueous media under neutral pH, but both the diacid **1** (CD18) and the decarboxylated derivative **2** (C18) were sufficiently soluble in water to perform the experiments. The spectroscopic and binding properties of these new probes, with the reference compound **3** (PBFI), are given in Table 1.

The properties of the PBFI tetra-acid compared well with the

Table 1 Spectral and binding properties of fluorescent probes PBFI, CD18 and C18[a]

Probe	M^+ ([M^+]/mmol dm^{-3})	Absorbance λ_{max}/nm (ε/10^3 dm^3 mol^{-1} cm^{-1})	Fluorescence λ_{max}/nm		Φ_F	K_D/mmol dm^{-3}
			Excitation	Emission		
PBFI 3	—	337 (37)	340	523	0.012	—
	K^+ (250)	336 (44)	340	486	0.038	6
	Na^+ (500)	335 (43)	340	510	0.022	17
	Mg^{2+} (500)	340 (35)	342	541	0.006	40
	Ca^{2+} (500)	327 (40)	340	523	0.038	15
	K^+/Na^+ (135)[b]	—	—	—	—	140
CD18 1	—	389 (25)	394	481	0.01	—
	K^+ (250)	370 (23)	382	473	0.021	22
	Na^+ (500)	372 (20)	384	480	0.004	200
	Mg^{2+} (500)	418 (26)	406	489	0.004	8
	Ca^{2+} (500)	408 (21)	401	486	0.006	6
	K^+/Na^+ (135)[b]	—	—	—	—	500
C18 2	—	366 (18)	385	475	0.008	—
	K^+ (135)	360 (16)	358	469	0.015	21
	Na^+ (135)	361 (16)	385	474	0.009	333

[a] Probe concentration 8 × 10^{-6} mol dm^{-3} in water maintained at pH 7: see Experimental. [b] Effective K_D for K^+ in the presence of Na^+, where [Na^+ + K^+] = 135 mmol dm^{-3}.

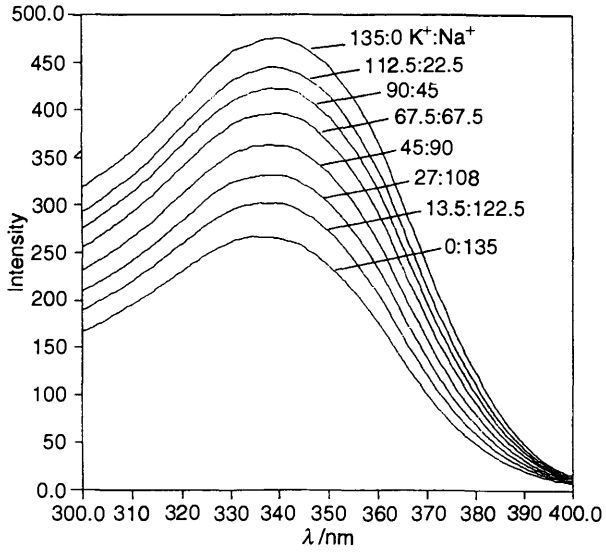


Fig. 4 Fluorescence excitation spectra of 8 × 10^{-6} mol dm^{-3} PBFI (3) as a function of increasing [K^+] and decreasing [Na^+], such that [K^+ + Na^+] = 135 mmol dm^{-3} emitted at 505 nm at pH 7

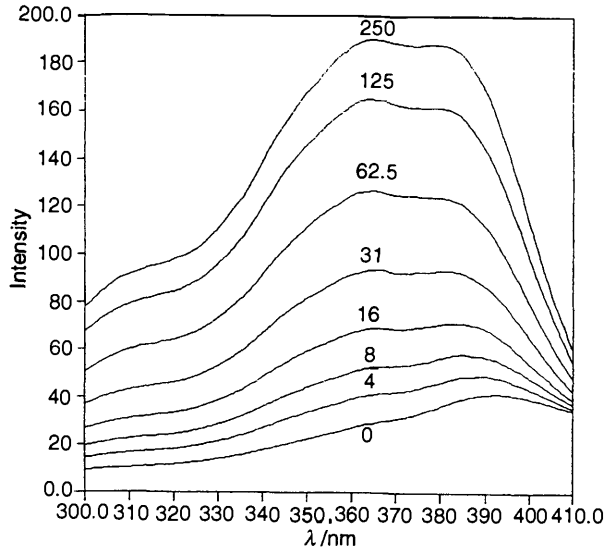


Fig. 5 Fluorescence excitation spectra of 8 × 10^{-6} mol dm^{-3} CD18 (1) as a function of 0–250 mmol dm^{-3} K^+ emitted at 450 nm at pH 7

literature values, as shown in Table 1. The molar extinction coefficient of the order of 40 000 dm^3 mol^{-1} cm^{-1} was as expected; the quantum efficiency for fluorescence increased threefold upon addition of 250 mmol dm^{-3} K^+ to the PBFI (maintained at a constant concentration of 8 × 10^{-6} mol dm^{-3} throughout the measurements) consistent with the available data. The addition of Na^+ and Ca^+ also increased the fluorescence intensity of the probe but to a lesser extent, whereas the effect of Mg^{2+} was to suppress the fluorescence of the systems Figs. 2 and 3 show the excitation spectra of PBFI in the presence of varying concentrations of K^+ and Na^+ respectively, at pH 7, and clearly indicate the increase in fluorescence just mentioned. From the corresponding emission spectra the dissociation constants (K_D) were measured according to the procedure of Bourson and Valeur[14] and gave values of 6 and 17 mmol dm^{-3} for K^+ and Na^+ respectively as compared to the reported literature values of 8 and 21 mmol dm^{-3}.[5] This indicates a relatively low potassium selectivity over sodium of approximately threefold.

In order to mimic biological conditions a mixture of K^+–Na^+ was used,[5] keeping the combined ionic concentrations of these two constant at 135 mmol dm^3 at neutral pH; the fluorescence excitation spectra of PBFI are shown in Fig. 4.

An increase in [K^+] and corresponding decrease in [Na^+] results in an overall two-fold enhancement in fluorescence intensity at the wavelength maximum with no substantial shift in wavelength. Under these competitive, high ionic strength conditions the measured K_D for K^+ increased to 140 mmol dm^{-3}.

The spectral and binding data for CD18 are shown in Table 1. Its molar extinction coefficient of 25 000 dm^3 mol^{-1} cm^{-1} is almost half that of the PBFI and the quantum efficiency is also slightly lower. The fluorescence excitation λ_{max} for the free probe (8 × 10^{-6} mol dm^{-3}) at pH 7 is 394 nm, longer than for PBFI, and emission λ_{max} is 481 nm, although still a useful Stoke's shift, this is less than the difference observed for PBFI (*ca.* 150 nm). Figs. 5 and 6 show the effect of addition of K^+ and Na^+ respectively. As predicted K^+ causes the fluorescence intensity to increase when emitting at 450 nm, from which the K_D of 22 mmol dm^{-3} can be derived. The addition of Na^+ induces a much smaller enhancement in the fluorescence excitation, reflecting the selectivity of the molecule and the corresponding K_D for sodium was calculated to be 200 mmol dm^{-3}. The latter effect is relatively minor compared to the response of PBFI to sodium. The K^+/Na^+ selectivity for CD18 is, therefore, of the order of nine, proving to be three times more selective than PBFI.

Fig. 7 shows the fluorescence excitation spectra for solutions

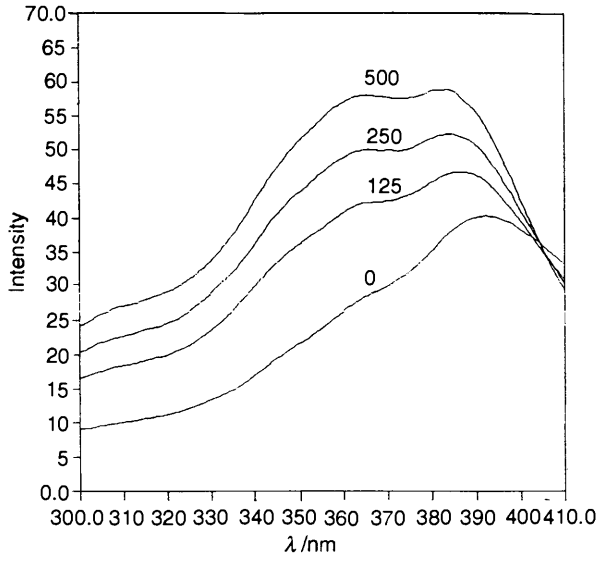


Fig. 6 Fluorescence excitation spectra of 8 × 10^{-6} mol dm^{-3} CD18 (**1**) as a function of 0–500 nmol dm^{-3} Na^+ emitted at 450 nm at pH 7

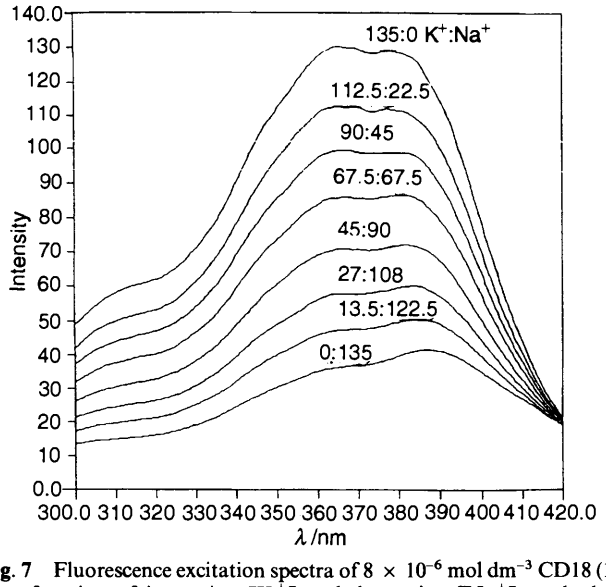


Fig. 7 Fluorescence excitation spectra of 8 × 10^{-6} mol dm^{-3} CD18 (**1**) as a function of increasing [K^+] and decreasing [Na^+], such that [$K^+ + Na^+$] = 135 mmol dm^{-3} emitted at 450 nm at pH 7

of 8 × 10^{-6} mol dm^{-3} CD18 with [$K^+ + Na^+$] = 135 mmol dm^{-3} emitting at 450 nm at pH 7. Again, as expected, a concomitant increase in fluorescence is observed as [K^+] rises. On going from the sodium-rich solution to the potassium-rich one there is an overall fourfold enhancement in the intensity, which is twice the effect observed with PBFI (see Fig. 4). In addition the shoulder in the sodium rich solution (λ_{max} of 387 nm) becomes more prominent in the potassium rich solution (λ_{max} of 365 nm) although this relatively small 'shift' is not really substantial enough to allow accurate dual-wavelength monitoring.[15]

A pH profile on CD18/135 mmol dm^{-3} K^+ (see Fig. 8) showed that over the range pH 6–8 there exists a plateau region, implying stability of the system in terms of fluorescence over this range. This is required for a system operable under physiological conditions. At pH 5 and below the fluorescence decreases rapidly and is attributable to the protonation of the nitrogens in the crown, thus reducing the chelating affinity of the probe for K^+. Under strong basic conditions the coumarin ring slowly cleaves eventually resulting in the loss of fluorescence.

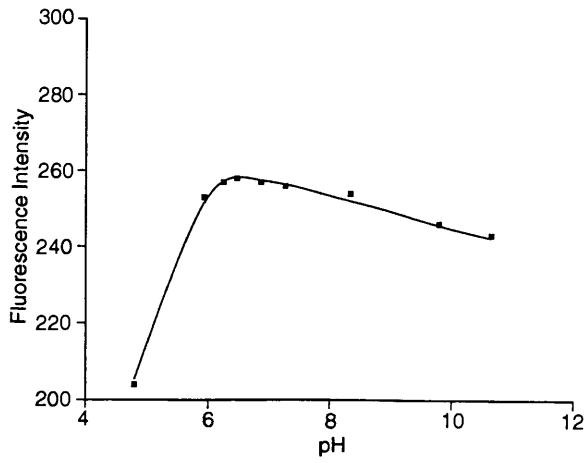


Fig. 8 Fluorescence excitation intensity of a solution of 8 × 10^{-6} mol dm^{-3} CD18 (**1**)/135 mmol dm^{-3} K^+ as a function of pH, emitted at 450 nm

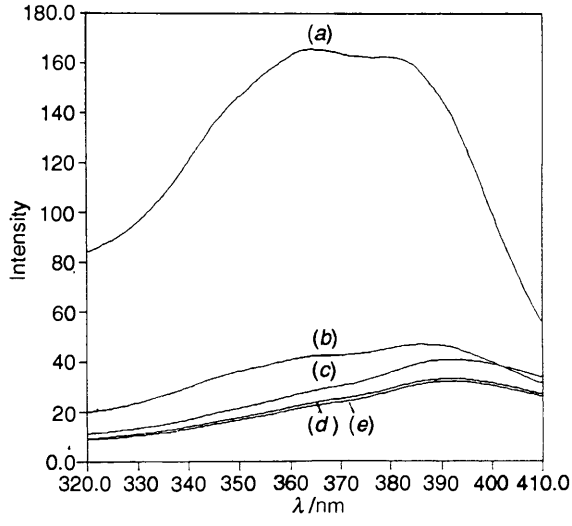


Fig. 9 Fluorescence excitation spectra of 8 × 10^{-6} mol dm^{-3} CD18 (**1**) in the presence of: (a) 125 mmol dm^{-3} K^+, (b) 125 mmol dm^{-3} Na^+, (c) no salt, (d) 1 mmol dm^{-3} Ca^{2+}, and (e) 1 mmol dm^{-3} Mg^{2+}. Emission was at 450 pH 7

Studies on the influence of other biological cations, such as calcium and magnesium show that, at physiological levels, no adverse competitive binding effects occur (see Fig. 9, see also Table 1) and similar fluorescence responses were obtained for K^+ either in the presence or absence of these ions; this suggests that the primary binding site for the divalent ions is not the cryptand group.

The decarboxylated probe, C18 exhibited different excitation characteristics. Fig. 10 shows the fluorescence excitation with increasing [K^+] and decreasing [Na^+]. The expected enhancement in fluorescence is observed upon K^+ binding but there is a clear hypsochromic shift in λ_{max} of approximately 40 nm on going from 0 to 135 mmol K^+. This clear spectral shift is the first that has been reported for K^+, although similar shifts have been seen for Ca^{2+}.[15] A similar shift was not seen in the emission spectra. The enhancement in fluorescence intensity is comparatively lower (about 80%) compared to PBFI and CD18 which indicates a slightly lower sensitivity threshold to potassium.

The probe **2** (C18) was also subjected to a simple ^1H NMR study to further illustrate the K^+ *versus* Na^+ binding. The results are seen in Fig. 11, which shows the effect of saturating levels of K^+ and Na^+ on the NMR signals of C18 in CD_3OD; solubility was too limited to carry out the studies in D_2O. In methanol, as compared with water, ion binding is increased and,

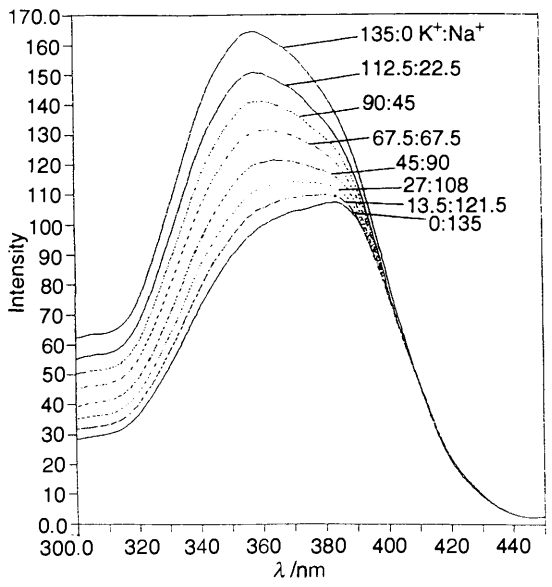


Fig. 10 Fluorescence excitation spectra of 8×10^{-6} mol dm^{-3} C18 (**2**) as a function of increasing [K^+] and decreasing [Na^+], such that [$K^+ + Na^+$] = 135 mmol dm^{-3} emitted at 475 nm at pH 7

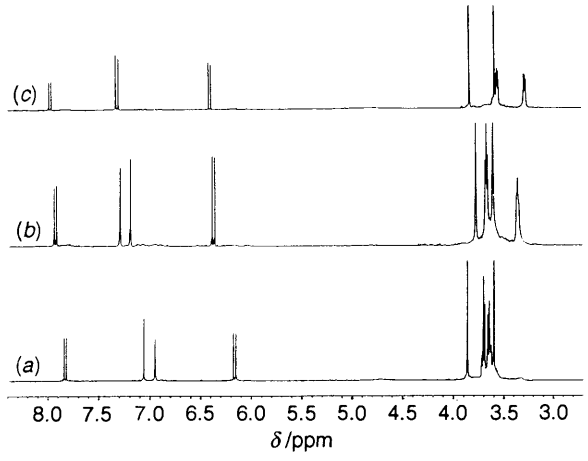


Fig. 11 ^{1}H NMR spectra of 'free' C18 in CD_3OD and with saturating levels of Na^+ and K^+: (a) C18 alone; (b) with saturating NaCl; (c) with saturating KCl

for potassium-selective probes, selectivity is enhanced,[16] thereby magnifying any effects of chelation. The effect of Na^+ and K^+ on the probe causes a deshielding influence and the aromatic signals move downfield, the largest shifts being observed in the presence of K^+. This can be explained in terms of the coumarin moieties adopting a conformation residing over the top and bottom of the K^+ ion, with the crown structure forming a cavity for the cation to sit in as suggested by Minta and Tsien.[5] The triplet signals for the protons next to the nitrogens are shifted upfield, the effect again being larger in the case for K^+. The results are consistent with a greater selectivity of the probe for K^+ rather than Na^+.

In conclusion, the new probes show different properties from those of PBFI, the diacid CD18 **1** showing a greater selectivity for potassium over sodium and the decarboxylated coumarin C18 **2** showing a relatively large hypsochromic shift in its excitation spectrum on binding to potassium.

Experimental

Optical Measurements.—Fluorescence spectra were recorded on a Perkin-Elmer LS50B luminescence spectrophotometer at room temperature; excitation and emission bandwidths were 10 nm. Probe concentration was maintained throughout at 8×10^{-6} mol dm^{-3} and, unless otherwise stated, solutions were buffered with 10^{-2} mol dm^{-3} MOPS [3-(morpholino)propanesulfonic acid] to pH 7 with Triton B (benzyltrimethylammonium hydroxide in methanol, 40%). For pH measurements, the pH of a stock solution (10 cm^3) was increased by small additions of the base Triton B. The pH of the solutions was monitored using a Corning pH meter 240. UV spectra were recorded on a Perkin-Elmer Lambda 5 UV spectrophotometer. Quantum efficiencies 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 the quantum efficiency of 0.59. Dissociation constants (K_D) were calculated according to the procedure of Bourson and Valeur,[14] using the fluorescence intensity of the free probe, I_F^0, and that of the solution, I_F, at the concentration of metal ion, ; plotting $I_F^0/(I_F - I_F^0)$ versus $[M]^{-1}$, gives the K_D by the gradient/intercept ratio.

Synthesis.—All purification by chromatography was performed on silica gel (Sorbasil). 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}. Unless otherwise stated, ^{1}H NMR spectra were recorded on either a Varian CFT-20 (80 MHz) or Jeol FX200 (200 MHz) spectrometer. Resonances are reported as (frequency; solvent); δ = shift in ppm from tetramethylsilane at 0 ppm and the multiplicity of signals as s, singlet; d, doublet; dd, double doublet; t, triplet; m, multiplet; br, broad; J values are in Hz. The numbering system used is shown in the Schemes. The NMR spectrum of Fig. 11 was recorded on a Bruker AMX 400 (400 MHz) instrument. IR spectra were measured on a Perkin-Elmer 1420 Ratio Recording spectrophotometer as KBr discs. Melting points were measured on a Reichert–Jung Thermovar melting point apparatus. Mass spectra were recorded either on an AEI MS902 spectrometer or at the SERC facility, Department of Chemistry, Swansea College, University of Wales. Microanalyses were performed by MEDAC Ltd., Brunel University. Ether refers to diethyl ether; light petroleum to the fraction of boiling range 60–80 °C.

1,2-*Bis*(2-*iodoethoxy*)*ethane* **11**.[17] A mixture of 1,2-bis(2-chloroethoxy)ethane (10 g, 53 mmol), sodium iodide (17.4 g, 116 mmol) in acetone (30 cm^3) was heated under reflux with stirring for 24 h. The reaction mixture was cooled, filtered, and the solvent removed *in vacuo*. The residue was dissolved in ether (100 cm^3) and washed with aqueous sodium thiosulfate (2 × 20 cm^3) to remove iodine. The ether was dried (Na_2SO_4) and removed *in vacuo* to leave the crude product which was vacuum distilled (120 °C, 0.6 mmHg) to give the pale yellow oil, **11** (17.7 g, 90%); δ_H(80 MHz; $CDCl_3$) 3.23 (4 H, t, J 7, CH_2I), 3.64 (4 H, s, CH_2CH_2I) and 3.74 (4 H, t, J 7, OCH_2); m/z 370 (M^+, 2%), 199 ($M^+ - OCH_2CH_2I$, 83) and 155 [$M^+ - (OCH_2CH_2)_2I$, 100].

1,10-*Bisbenzyl*-3,7-*dioxa*-1,10-*diazadecane* **12**.[18] To a refluxing solution of benzylamine (45.53 g, 430 mmol) and Na_2CO_3 (25 g, 240 mmol) in toluene (100 cm^3) in a Dean–Stark apparatus was added 1,2-bis(2-chloroethoxy)ethane (20 g, 110 mmol) over a period of 5 h. The reaction mixture was filtered to remove the inorganic materials and the filtrate evaporated. The crude product was vacuum distilled (200 °C, 0.4–0.5 mmHg) to give the slightly yellow oil **12** (30 g, 86%); δ_H(80 MHz; $CDCl_3$) 1.81 (2 H, s, D_2O exchangeable, NH), 2.76 (4 H, t, J 5, NCH_2), 3.56 (4 H, s, OCH_2), 3.57 (4 H, t, J 5, NCH_2CH_2), 3.76 (4 H, s, $ArCH_2$) and 7.24 (10 H, m, Ph); m/z 328 (M^+, 2%), 237 ($M^+ - CH_2Ph$, 15), 194 ($M^+ - CH_2CH_2NHCHPh$, 13), 178 ($M^+ - OCH_2CH_2NHCHPh$, 11) and 91 ($PhCH_2^+$, 100).

7,16-*Bisbenzyl*-1,4,10,13-*tetraoxa*-7,16-*diazacyclooctadecane* **13**.[11] A solution of **12** (6.6 g, 20 mmol), **11** (9.2 g, 25 mmol), Na_2CO_3 (10.6 g, 100 mmol) and NaI (1.5 g, 10 mmol) in MeCN

(400 cm^3) was heated under reflux overnight. The reaction mixture was cooled, filtered, and the solvent removed *in vacuo*. The residue was dissolved in a refluxing solution of acetone–dioxane (1 : 1, 80 cm^3) and left to crystallise in the freezer. The precipitate was dissolved in water (30 cm^3) and extracted with $CHCl_3$ (3 × 30 cm^3) and the solvent dried (Na_2SO_4) and removed *in vacuo* to leave white crystals of the crude product, **13** (5.6 g, 63%), m.p. 79–80 °C (lit.,[11] 80–81 °C); δ_H(80 MHz; $CDCl_3$) 2.79 (8 H, t, *J* 5, NCH_2), 3.57 (8 H, s, OCH_2), 3.61 (8 H, t, *J* 5, NCH_2CH_2), 3.67 (4 H, s, CH_2Ar) and 7.23 (10 H, m, Ph); *m/z* 442 (M^+, 8%), 381 (24), 351 ($M^+ - CH_2Ph$, 32), 208 (50), 176 (42), 91 ($PhCH_2^+$, 100).

1,4,10,13-*Tetraoxa*-7,16-*diazacyclooctadecane* **4**. To a stirred blue solution of liquid ammonia (150 cm^3), THF (100 cm^3) and sodium (6.25 g, 540 mmol) was added dropwise a solution of **13** (5 g, 11 mmol) in THF (50 cm^3). Stirring was continued for a further 2 h, after which the reaction was quenched with absolute EtOH (20 cm^3). The reaction mixture was left overnight at room temperature (to allow evaporation of the ammonia) and filtered to remove the inorganic materials. The solvent was removed *in vacuo* to a leave a residue which was dissolved in water (30 cm^3) and extracted with $CHCl_3$ (3 × 40 cm^3). The combined organic extracts were dried (Na_2SO_4) and the solvent removed *in vacuo* to yield crude pale yellow crystals of **4** (2.8 g, 95%). Recrystallisation from hexane afforded white crystals of the diaza-crown, m.p. 114–115 °C (from hexane) (lit.,[8] 114–115 °C) (Calcd. for $C_{12}H_{26}N_2O_4$: C, 54.9; H, 10.0; N, 10.7. Found: C, 54.9; H, 10.3; N, 10.5%); δ_H(80 MHz; $CDCl_3$) 2.31 (2 H, br s, D_2O exchangeable, NH), 2.77 (8 H, t, *J* 5, NCH_2), 3.57 (8 H, s, OCH_2) and 3.58 (8 H, t, *J* 5, NCH_2CH_2); *m/z* 262 (M^+, 4%), 132 (100), 118 (94) and 100 (77).

7,16-*Bis*-(2,5-*dioxocyclohexa*-1,4-*dienyl*)-1,4,10,13-*tetraoxa*-7,16-*diazacyclooctadecane* **14**.[5] To a refluxing solution of freshly sublimed *p*-benzoquinone (5.61 g, 0.05 mole) in dry THF (15 cm^3) was added dropwise a solution of **4** (1.36 g, 0.005 mol) in THF (5 cm^3) and the mixture was heated further under reflux overnight. The solvent was removed *in vacuo* and the residue dissolved in $CHCl_3$ (150 cm^3) and filtered. The filtrate was passed through a silica column with ethyl acetate as the eluent to remove the excess *p*-benzoquinone and the product **14** (1.46 g, 60%) was obtained as dark red crystals by eluting with MeOH–ethyl acetate (1 : 19), m.p. 151–153 °C (lit.,[5] 153–155 °C); δ_H(200 MHz; $CDCl_3$) 3.61 (8 H, s, OCH_2CH_2O), 3.74 (16 H, m, NCH_2CH_2O), 5.65 (2 H, d, *J* 2, 6′-H) and 6.48 (2 H, d, *J* 9, 3′-H) and 6.58 (2 H, dd, *J* 3 and 9, 4′-H); *m/z* (FAB) 475 (M + 1).

7,16-*Bis*-(2,5-*dimethoxyphenyl*)-1,4,10,13-*tetraoxa*-7,16-*diazacyclooctadecane* **15**.[5] This was prepared by the literature method. The product **15** (0.39 g, 35%) was isolated as a red solid; δ_H(200 MHz; $CDCl_3$) 3.50–3.80 (24 H, m, CH_2), 3.78 (12 H, s, Me), 6.45 (2 H, dd, *J* 3 and 9, 4′-H), 6.72 (2 H, d, *J* 6′-H) and 6.75 (2 H, d, *J* 9, 3′-H); *m/z* 534 (M^+, 100%), 473 (54), 354 (20), 267 (40), 254 (61), 237 (72), 222 (52) and 179 (82).

7,16-*Bis*-(2,5-*dimethoxy*-4-*formylphenyl*)-1,4,10,13-*tetraoxa*-7,16-*diazacyclooctadecane* **16**.[5] The bis-dimethoxy compound **15** (0.20 g, 0.38 mmol) was dissolved in dimethylformamide (DMF) (1 cm^3) and stirred at 0 °C, during which a mixture of $POCl_3$–DMF (1 : 4 v/v; 3 cm^3) was added. Stirring was continued for a further 1 h. Water (5 cm^3) was added to quench the reaction mixture which was then carefully basified with saturated K_2CO_3. This aqueous layer was extracted with $CHCl_3$ (3 × 20 cm^3) and the combined organic layers were backwashed with water, dried (Na_2SO_4) and evaporated *in vacuo*. The residue was column chromatographed with $CHCl_3$ and gradient eluting with MeOH to afford white crystals of **16** (0.15 g, 68%), m.p. 131–132 °C (lit.,[5] 131–133 °C); δ_H(200 MHz; $CDCl_3$) 3.61 (8 H, s, OCH_2CH_2O), 3.70 (16 H, m, NCH_2CH_2O), 3.80 (6 H, s, Me), 3.87 (6 H, s, Me), 6.52 (2 H, s, 6′-H), 7.26 (2 H, s, 3′-H) and 10.23 (2 H, s, CHO); *m/z* 590 (M^+, 7%), 561 ($M^+ - CHO$, 64), 534 (100), 501 (20), 487 (23), 473 (83), 459 (38), 165 (Ar, 23) and 135 (52).

7,16-*Bis*-(4-*formyl*-5-*hydroxy*-2-*methoxyphenyl*)-1,4,10,13-*tetraoxa*-7,16-*diazacyclooctadecane* **17**.[5] The bis-dimethoxy aldehyde **16** (50 mg, 0.085 mmol) was dissolved in nitromethane (3 cm^3) and a saturated solution of $ZnCl_2$ in nitromethane (3 cm^3) added and stirred at room temperature for 15 min. A solution of BCl_3 in dichloromethane (1 mol dm^{-3}, 6 cm^3) was added and stirred further for 1.5 h, when a mixture of water–MeOH (1 : 1, 6 cm^3) was added. After 0.5 h an aqueous solution of K_2CO_3–EDTA* (almost saturated, 12 cm^3) was added to leach out the $ZnCl_2$ and stirring continued for 0.5 h. The reaction mixture was extracted with $CHCl_3$ (3 × 10 cm^3), followed by ethyl acetate (1 × 10 cm^3). The combined organic extracts were backwashed with water (25 cm^3), dried (Na_2SO_4) and the solvent removed *in vacuo* to leave the crude product. Column chromatography using ethyl acetate isolated the bis-salicaldehyde **17** as an orange solid (20 mg, 45%), m.p. 134–135 °C (lit.,[4] 134–136 °C); R_F 0.36 (ethyl acetate); (ethyl acetate); δ_H(80 MHz; $CDCl_3$) 3.57 (8 H, s, OCH_2CH_2O), 3.68 (16 H, m, NCH_2CH_2O), 3.76 (6 H, s, Me), 6.30 (2 H, s, 6′-H), 6.76 (2 H, s, 3′-H), 9.50 (2 H, s, CHO) and 11.18 (2 H, s, OH); *m/z* 562 (M^+, 37%), 501 ($M^+ - C_2H_5O_2$, 27), 268 (68), 251 (36), 236 (40), 208 (41), 194 (78) and 28 (CO^+, 100).

7,16-*Bis*-[5-(2,4-*dimethoxycarbonylbenzyloxy*)-4-*formyl*-2-*methoxyphenyl*]-1,4,10,13-*tetraoxa*-7,16-*diazacyclooctadecane* **18**.[5] This was prepared by the literature method condensing the aldehyde **17** (15 mg, 0.027 mmol), with 4-bromomethylisophthalate[19] (60 mg, 0.21 mmol). The product ester (15 mg, 58%) showed R_F 0.25 (ethyl acetate); δ_H(80 MHz; $CDCl_3$) 3.53 (8 H, s, OCH_2CH_2O), 3.60 (16 H, br s, NCH_2CH_2O), 3.76 (6 H, s, OMe), 3.87 (6 H, s, ester Me), 3.92 (6 H, s, ester Me), 5.55 (4 H, s, Ar″CH_2O), 6.45 (2 H, s, 6′-H), 7.23 (2 H, s, 3′-H), 7.80 (2 H, d, *J* 8, 6″-H), 8.15 (2 H, dd, *J* 8 and 2, 5″-H), 8.61 (2 H, d, *J* 2, 3″-H) and 10.28 (2 H, s, CHO); *m/z* (FAB) 998 (M + Na^+) and 1013 (M + K^+).

7,16-*Bis*-[2-(2,4-*dicarboxyphenyl*)-5-*methoxy*-1-*benzofuran*-6-*yl*]-1,4,10,13-*tetraoxa*-7,16-*diazacyclooctadecane* PBFI (**3**).[5] The bis-ether **19** (15 mg, 0.015 mmol) was converted into PBFI, **3** *via* the ester **19** using the literature method. PBFI was isolated as a dark yellow solid (8 mg, 59%); δ_H(80 MHz; CD_3OD) 3.50–3.70 (24 H, m, CH_2), 4.03 (6 H, s, OMe), 7.20 (2 H, s, 7′-H), 7.46 (2 H, s, 4′-H), 7.82 (2 H, d, *J* 8, 6″-H), 7.91 (2 H, s, 3′-H), 8.15 (2 H, dd, *J* 2 and 8, 5″-H) and 8.33 (2 H, d, *J* 2, 3″-H); *m/z* (FAB) 884 (M + H) and 906 (M + Na).

7,16-*Bis*-(3-*methoxycarbonyl*-6-*methoxy*-2-*oxo*-2H-1-*benzopyran*-7-*yl*)-1,4,10,13-*tetraoxa*-7,16-*diazacyclooctadecane* **2a**. The bis-salicylaldehyde **17** (60 mg, 0.1 mmol), dimethyl malonate (280 mg, 2 mmol), piperidine (1 drop) and glacial acetic acid (1 drop) in MeOH (1 cm^3) were heated under reflux for 6 h during which a yellow precipitate appeared. The reaction mixture was cooled and filtered. The solid was recrystallised from MeOH to give pure **2a** (60 mg, 78%); m.p. 198–200 °C; R_F 0.56 [MeOH–$CHCl_3$ (1 : 9)] (Found: C, 59.4; H, 5.7; N, 3.75. $C_{36}H_{42}N_2O_{14}$ requires C, 59.5; H, 5.8; N, 3.9%); δ_H(200 MHz; $CDCl_3$) 3.61 (8 H, s, OCH_2CH_2O), 3.73 (16 H, m, NCH_2CH_2O), 3.84 (6 H, s, OMe), 3.89 (6 H, s, ester Me), 6.71 (2 H, s, 5′-H), 6.77 (2 H, s, 8′-H) and 8.36 (2 H, s, 4′-H); *m/z* (FAB) 727 (M + H) and 749 (M + Na).

7,16-*Bis*-(3-*carboxy*-6-*methoxy*-2-*oxo*-2H-1-*benzopyran*-7-*yl*)-1,4,10,13-*tetraoxa*-7,16-*diazacyclooctadecane* CD18 (**1**). A suspension of **2a** (20 mg, 0.028 mmol), LiOH·H_2O (7 mg, 0.17 mmol) and MeOH (2 drops) in water (3 cm^3) was stirred at room temperature for 6 days during which a yellow solution formed. The solution was neutralised with aqueous HCl (2 mol dm^{-3}) which precipitated the yellow diacid CD18 (17 mg, 88%). m.p. 218–220 °C (Found: M + H^+, 699.2401. $C_{34}H_{39}N_2O_{14}$

* Ethylenediaminetetra acetic acid.

requires $M + H^+$, 699.2435); δ_H(400 MHz; $CDCl_3$) 3.63 (8 H, s, OCH_2CH_2O), 3.70–3.80 (16 H, m, NCH_2CH_2O), 3.88 (6 H, s, OMe), 6.83 (2 H, s, 5'-H), 6.88 (2 H, s, 8'-H), 8.70 (2 H, s, 4'-H); *m/z* (FAB) 699 ($M + H^+$), 721 ($M + Na^+$) and 737 ($M + K^+$).

7,16-*Bis*-(6-*methoxy*-2-*oxo*-2H-1-*benzopyran*-7-*yl*)-1,4,10,13-*tetraoxa*-7,16-*diazacyclooctadecane* C18 (**2**). A solution of **2a** (40 mg, 0.05 mmol) in aqueous HCl (9 mol dm^{-3}; 3 cm^3) was heated under reflux for 72 h. The solution was cooled, neutralised with aqueous NaOH (3 mol dm^{-3}), and extracted with CH_2Cl_2 (3 × 15 cm^3). The organic layers were combined, dried (Na_2SO_4) and the solvent removed *in vacuo*; preparative TLC on silica using MeOH–$CHCl_3$ (1:10) as solvent afforded the title compound as a beige solid (23 mg, 70%); m.p. range 140–145 °C; R_F 0.3 [MeOH–$CHCl_3$ (1:10)] (Found: $M + H^+$, 611.2605. $C_{32}H_{39}N_2O_{10}$ requires $M + H^+$, 611.2635); δ_H(400 MHz; $CDCl_3$) 3.61 (8 H, s, OCH_2CH_2O), 3.65–3.80 (16 H, m, NCH_2CH_2O), 3.86 (6 H, s, OMe), 6.20 (2 H, d, *J* 9, 3'-H), 6.78 (2 H, s, 8'-H), 6.85 (2 H, s, 5'-H) and 7.56 (2 H, d, *J* 9, 4'-H); *m/z* (FAB) 611 ($M + H^+$) and 633 ($M + Na^+$).

N-(2,5-*Dioxocyclohexa*-1,4-*dienyl*)*morpholine* **6**. To a refluxing solution of freshly sublimed *p*-benzoquinone (0.62 g, 5.75 mmol) in dry THF (2 cm^3) was added morpholine (0.10 g, 1.15 mmol) and refluxing continued for a further 15 min. The solvent was removed *in vacuo* and the residue dissolved in $CHCl_3$ (10 cm^3) and filtered. The filtrate was passed through a silica column with ethyl acetate–light petroleum (1:1) as eluent and product **6** was obtained as red crystals (0.20 g, 91%), m.p. 130–131 °C; R_F 0.24 [ethyl acetate–light petroleum (1:1)] (Found: C, 61.95; H, 5.7; N, 7.15. $C_{10}H_{11}NO_3$ requires C, 62.2; H, 5.7; N, 7.25%); δ_H(80 MHz; $CDCl_3$) 3.37 (4 H, t, *J* 5, NCH_2), 3.80 (4 H, t, *J* 5, OCH_2), 5.72 (1 H, br s, 6-H) and 6.54–6.56 (2 H, m, 3-H, 4-H); *m/z* 193 (M^+, 100%), 136 (59), 137 ($M^+ - 2CO$) and 108 (62); ν_{max}/cm^{-1} 1670, 1640 (C=O); 1620 (C=C).

N-(2,5-*Dimethoxyphenyl*)*morpholine* **7**. The benzoquinone derivative **6** (0.10 g, 0.52 mmol) was dissolved in MeOH (2 cm^3) and hydrogenated with 10% palladium-on-charcoal (0.01 g) under atmospheric pressure and at room temperature. After reduction was complete (no red colour was present), Triton B (4.33 g, 10 mmol) was added to the reaction mixture *via* a syringe, followed by dimethyl sulfate (1.30 g, 10 mmol) under a hydrogen atmosphere and the mixture was left overnight. The reaction mixture was filtered and the MeOH removed *in vacuo*. The residue was dissolved in water (4 cm^3) and made acidic with acetic acid (2 drops). Extraction with $CHCl_3$ (2 × 20 cm^3) and removal of solvent *in vacuo* followed by column chromatography using ethyl acetate–light petroleum (1:1) afforded title ether **7** as a colourless oil (0.1 g, 87%); R_F 0.6 [ethyl acetate–light petroleum (1:1)] (Found: C, 64.6; H, 7.6; N, 6.0. $C_{12}H_{17}NO_3$ requires C, 64.55; H, 7.7; N, 6.3%); δ_H(80 MHz; $CDCl_3$) 3.03 (4 H, t, *J* 5, NCH_2), 3.73 (3 H, s, OMe), 3.78 (3 H, s, OMe), 3.84 (4 H, t, *J* 5, OCH_2) 6.42 (1 H, dd, *J* 3 and 9, 4-H), 6.47 (1 H, d, *J* 3, 6-H) and 6.74 (1 H, d, *J* 9, 3-H); *m/z* 223 (M^+, 100%), 208 ($M^+ - Me$, 92), 165 (26), 150 (61); ν_{max}/cm^{-1} 1180, 1060 (C–O).

N-(2,5-*Dimethoxy*-4-*formylphenyl*)*morpholine* **8**. The dimethoxy compound **7** (0.25 g, 1.12 mmol) was dissolved in DMF (1.5 cm^3) at 0 °C. A mixture of $POCl_3$–DMF (1:4 v/v; 5 cm^3) was added and stirred at room temperature for 4 h. Water (5 cm^3) was added and the mixture was neutralised with aqueous Na_2CO_3 upon which appeared a pale yellow precipitate of the title aldehyde **8** (0.25 g, 91%), m.p. 128–129 °C; R_F 0.64 (ethyl acetate) (Found: C, 58.3; H, 7.1; N, 5.1. $C_{13}H_{17}NO_4 \cdot H_2O$ requires C, 58.0; H, 7.1; N, 5.2%); δ_H(80 MHz; $CDCl_3$) 3.20 (4 H, t, *J* 5, NCH_2), 3.83 (3 H, s, OMe), 3.85 (4 H, t, *J* 5, OCH_2), 3.87 (3 H, s, OMe), 6.39 (1 H, s, 6-H), 7.26 (1 H, s, 3-H) and 10.24 (1 H, s, CHO); *m/z* 251 (M^+, 59%), 236 ($M^+ - Me$, 36), 223 ($M^+ - CO$, 20), 208 (21), 149 (100) and 86 (C_4H_8NO, 28); ν_{max}/cm^{-1} 1660 (C=O).

N-(4-*Formyl*-5-*hydroxy*-2-*methoxyphenyl*)*morpholine* **9**. To a solution of the dimethoxy aldehyde **8** (0.1 g, 0.4 mmol) in dichloromethane (2 cm^3) was added BCl_3 (1 mol dm^{-3} in dichloromethane; 3 cm^3) at room temp. Stirring was continued for 30 min then water (5 cm^3) was added carefully and the reaction mixture extracted with dichloromethane (3 × 20 cm^3). The solvent was dried (Na_2SO_4) and removed *in vacuo* to leave pale yellow crystals of the phenol (0.46 g, 98%), m.p. 112–113 °C; R_F 0.64 (ethyl acetate) (Found: C, 60.8; H, 6.4; N, 5.8. $C_{12}H_{15}NO_4$ requires C, 60.75; H, 6.4; N, 5.9%); δ_H(80 MHz; $CDCl_3$) 3.22 (4 H, t, *J* 5, NCH_2), 3.82 (3 H, s, OMe), 3.82 (4 H, t, *J* 5, OCH_2), 6.35 (1 H, s, 6-H), 6.83 (1 H, s, 3-H), 9.62 (1 H, s, CHO) and 11.08 (1 H, s, D_2O exchangeable, OH); *m/z* 237 (M^+, 26%), 222 ($M^+ - Me$, 14), 165 (34), 149 (100); ν_{max}/cm^{-1} 3500 (H-bonded OH), 1650 (C=O).

N-(3-*Carboxymethyl*-7-*methoxy*-2-*oxo*-2H-1-*benzopyran*-7-*yl*)*morpholine* **5**. A solution of **9** (230 mg, 1 mmol), dimethyl malonate (140 mg, 1.1 mmol), piperidine (4 drops) and glacial acetic acid (1 drop) in MeOH (3 cm^3) was heated under reflux for 3 h. A few drops of water were added to precipitate the coumarin **5** (50 mg, 16%). Column chromatography of the residues isolated from the filtrate, using ethyl acetate gave a further 118 mg (total 168 mg, 54%); m.p. 195 °C; R_F 0.47 (ethyl acetate) (Found: C, 60.4; H, 5.6; N, 4.4. $C_{16}H_{17}NO_6$ requires C, 60.2; H, 5.4; N, 4.4%); δ_H(400 MHz; $CDCl_3$) 3.26 (4 H, s, t, *J* 5, NCH_2), 3.88 (4 H, t, *J* 5, OCH_2), 3.91 (3 H, s, OMe), 3.94 (3 H, s, OMe), 6.78 (1 H, s, 5-H), 6.86 (1 H, s, 8-H) and 8.52 (1 H, s, 4-H); *m/z* 319 (M^+, 100%), 304 ($M^+ - Me$, 34), 288 ($M^+ - OMe$, 15), 261 (45), 233 ($M^+ - C_4H_8NO$, 23), 230 (34); ν_{max}/cm^{-1} 1735 (C=O), 1620 (C=O).

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