Synthesis and spectroscopic characterisation of fluorescent indicators for Na^+ and K^+ based on (di)pyridino crown ethers

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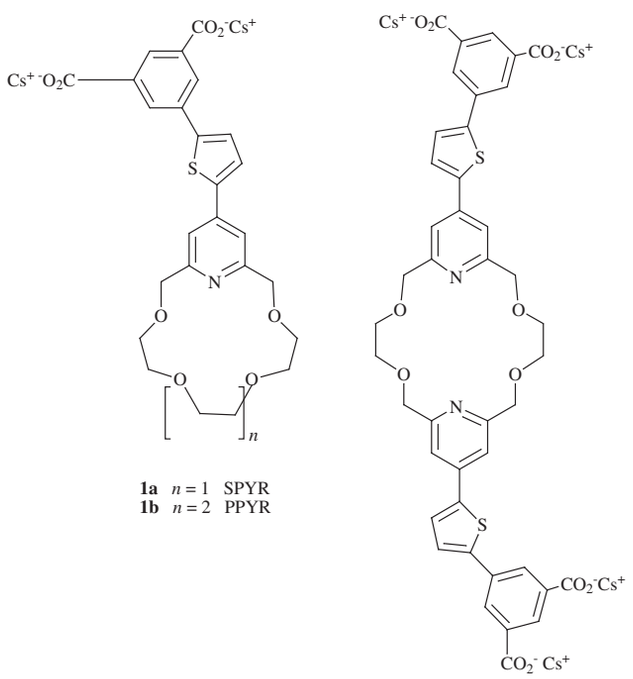
The synthesis and the spectral and cation binding properties of a fluorescent indicator for Na^+, caesium 5-{5-[3,6,9,12-tetraoxa-18-azabicyclo[12.3.1]octadeca-1(18),14,16-trien-16-yl]-2-thienyl}isophthalate (SPYR), and of two fluorescent indicators for K^+, caesium 5-{5-[3,6,9,12,15-pentaoxa-21-azabicyclo[15.3.1]henicosa-1(21),17,19-trien-19-yl]-2-thienyl}isophthalate (PPYR) and dicaesium 5-{5-[21-(5-{1-[3,5-di(caesiooxycarbonyl)phenyl]}-2-thienyl)-3,6,14,17-tetraoxa-23,24-diazatricyclo[17.3.1.1^{8,12}]tetracosa-1(23),8(24),9,11,19,21-hexaene-10,21-diyl]-2-thienyl}isophthalate (BIPY) are reported. To investigate the effect of an sp^2-nitrogen in the aza-crown ether cavity on the complex formation behaviour, a (bi)pyridino crown compound with an appropriate cavity for the sodium and the potassium cation, respectively, is linked to an aryl thiophene fluorophore. New, versatile routes for the synthesis of 4-substituted pyridino crown ethers and 4,4′-substituted *sym*-dipyridino crown ethers are described. The chelating abilities of the indicators with monovalent cations have been studied in aqueous solution by their fluorescence properties. Both the free and complexed forms of the indicators have an absorption maximum around 345 nm and an emission maximum at 410 nm, displaying only a decrease in the fluorescence intensity upon cation binding. While SPYR cannot discriminate between Na^+ and other monovalent cations, PPYR and BIPY show a peak selectivity for K^+ with a dissociation constant of 1.2 ± 0.3 mM and 4.4 ± 0.7 mM, respectively.

Introduction

The accurate measurement of free concentrations of biologically important ions and messengers, such as K^+, Na^+, Mg^{2+}, Ca^{2+} and H^+, inside living tissues, is of tremendous importance in the fields of cell physiology and clinical medicine.[1] Fluctuations in these concentrations are often fast and highly localised, and are an essential link in biological signal transduction. Optical probes are among the best tools for sensing such concentrations, because they have an excellent signal-to-noise ratio and are virtually instantaneous in their response time.[2] The development of fluorescent ion indicators has become feasible, given the availability of ionophores that selectively bind the desired ion in aqueous solutions with an affinity that matches normal cytosolic levels (for K^+ 50 mM < K_d < 150 mM; for Na^+ 0 mM < K_d < 50 mM, where K_d is the ground-state dissociation constant) or extracellular ion levels (for K^+ 0 mM < K_d < 20 mM; for Na^+ 120 mM < K_d < 450 mM).[3,4] Fluorescent derivatives of such ionophores exhibit a change, preferably a shift in the excitation and/or emission spectrum, upon ion binding.[5]

For the sodium and the potassium cations, the most popular indicators for intracellular cation determination are sodium binding benzofuran isophthalate (SBFI) and potassium binding benzofuran isophthalate (PBFI), respectively,[6] consisting of a diaza-crown ether with an appropriate cavity, linked to a benzofuran fluorophore. Their spectral responses upon ion binding permit excitation ratio measurements. In a previous article, the synthesis and the photophysical studies of two new fluorescent indicators for Na^+ and K^+ based on benzocrown ethers were reported.[7] Both indicators only showed a change in intensity upon ion binding and no corresponding spectral shifts. To investigate the effect of the presence of an sp^2 nitrogen in the aza-crown cavity on the spectral response to ion binding, we have developed a series of sodium and potassium indicators, based on (di)pyridino containing crown derivatives (Fig. 1).



1a $n = 1$ SPYR
1b $n = 2$ PPYR

2 BIPY

Fig. 1 Structures of the compounds SPYR, PPYR and BIPY synthesised in this study. SPYR = sodium binding pyridine crown ether, PPYR = potassium binding pyridine crown ether, BIPY = bispyridine crown ether.

Taking into account the principles of molecular recognition, a 15-crown-5 type crown ether was used as the chelating group for the sodium ion, and an 18-crown-6 type for the potassium ion. The *sym*-dipyridine crown unit in compound **2** (Fig. 1)

allows the attachment of two fluorophores; hence, an increase in the absorption and the fluorescence intensity can be expected. Macrocyclic ligands containing pyridino nitrogen groups have not been studied extensively, because the incorporation of pyridino groups into unsubstituted crown rings (*i.e.*, macrocyclic ligands bearing no ester functionalities) is difficult synthetically.[8] As it is known that introduction of a pyridino unit into the 18-crown-6 ring produces only a minor decrease in the stability of the complexes with Na^+ and K^+ compared to 18-crown-6, with selectivity among the cations remaining essentially unaltered,[9] it seemed worthwhile to use a pyridino crown compound as the chelating moiety in a fluorescent indicator. A thiophene derivative was chosen as the fluorophore, which was linked to the 4-position of the pyridino moiety, resulting in a 4-(2-thienyl)pyridine skeleton. Thienylpyridine compounds have found wide applications in designing fluorescent labeling reagents for HPLC and electrochromic materials.[10] Furthermore, the combination of the electron rich nature of the thienyl ring and the electron deficient pyridine centre results in nonlinear optical activity.[11] For the indicators described here, the thiophene group is linked further to a phenyl ring, substituted with two carboxylate functionalities to ensure water solubility. In this article, the syntheses of two indicators with a mono-pyridino crown, SPYR **1a** (caesium 5-{5-[3,6,9,12-tetraoxa-18-azabicyclo[12.3.1]octadeca-1(18),14,16-trien-16-yl]-2-thienyl}isophthalate) for Na^+ and PPYR **1b** (caesium 5-{5-[3,6,9,12,15-pentaoxa-21-azabicyclo[15.3.1]henicosa-1(21),17,19-trien-19-yl]-2-thienyl}isophthalate) for K^+, and one *sym*-dipyridino crown indicator, BIPY **2** (dicaesium 5-{5-[21-(5-{1-[3,5-di(caesiooxycarbonyl)phenyl]}-2-thienyl)-3,6,14,17-tetraoxa-23,24-diazatricyclo[17.3.1^{8,12}]tetracosa-1(23),8(24),9,11,19,21-hexaene-10,21-diyl]-2-thienyl}isophthalate) are presented.

Besides the syntheses of the indicators for Na^+ and K^+, the spectral and cation binding properties of the indicators are reported. From absorption and steady-state fluorescence measurements, the position of the spectra, the molar extinction coefficient (ε), the quantum yield of fluorescence (φ_f) of the free and saturated forms of the indicators, as well as the dissociation constant, K_d, in the ground state of the formed host–guest complexes, and the selectivity *vs.* other cations were determined.

Theory

Determination of K_d from fluorimetric titration

The expression of the fluorescence signal as a function of the ion concentration has been derived by Kowalczyk *et al.*[12] for the case of a 1:1 complex between a fluorescent indicator and a cation. For a consecutive multiple binding model (Scheme 1),

$$\boldsymbol{0} + X \rightleftharpoons \boldsymbol{1} \qquad K_{d1} = \frac{[\boldsymbol{0}][X]}{[\boldsymbol{1}]} \qquad (1.1)$$

$$\boldsymbol{1} + X \rightleftharpoons \boldsymbol{2} \qquad K_{d2} = \frac{[\boldsymbol{1}][X]}{[\boldsymbol{2}]} \qquad (1.2)$$

$$\vdots \quad \vdots \quad \vdots$$

$$(\boldsymbol{n}-\boldsymbol{1}) + X \rightleftharpoons \boldsymbol{n} \qquad K_{dn} = \frac{[(\boldsymbol{n}-\boldsymbol{1})][X]}{[\boldsymbol{n}]} \qquad (1.n)$$

$$\boldsymbol{0} + nX \rightleftharpoons \boldsymbol{n} \qquad K_d = \frac{[\boldsymbol{0}]\,[X]^n}{[\boldsymbol{n}]} = \prod_{i=1}^{n} K_{di} \qquad (2)$$

Scheme 1

this expression can be expanded by taking into account the formation of an n:1 ion–indicator complex. Consider a system consisting of species ***0*** that can undergo a reversible reaction with ion X to form species ***1*** (determined by the dissociation constant K_{d1}), which in turn can react with another equivalent of X, to yield species ***2*** (determined by the dissociation constant K_{d2}), *etc.* (eqn. (1.1)–(1.n)). Species ***0*** represents the free form of a fluorescent indicator, species ***1*** the 1:1 complex with a cation X and species ***n*** the n:1 ion–indicator complex. The global reaction is determined by the composite dissociation constant K_d (eqn. (2)).

If one assumes that the intermediate complexes (species ***1***–(***n*** − ***1***)) are present in concentrations low enough to give only a negligible contribution to the fluorescence intensity, and that the rate of ion binding in the excited state is negligible, the total fluorescence signal due to excitation at λ_{ex} and observed at λ_{em} can be expressed by eqn. (3), where F_{min} corresponds to the

$$F = \frac{[X]^n F_{max} + K_d F_{min}}{K_d + [X]^n} \qquad (3)$$

fluorescence signal of the free form of the indicator, F_{max} denotes the fluorescence signal of the saturated form of the indicator and $K_d = \prod_{i=1}^{n} K_{di}$. Fitting eqn. (3) to the fluorescence data F as a function of [X] yields values for K_d, n, F_{min}, and F_{max}.

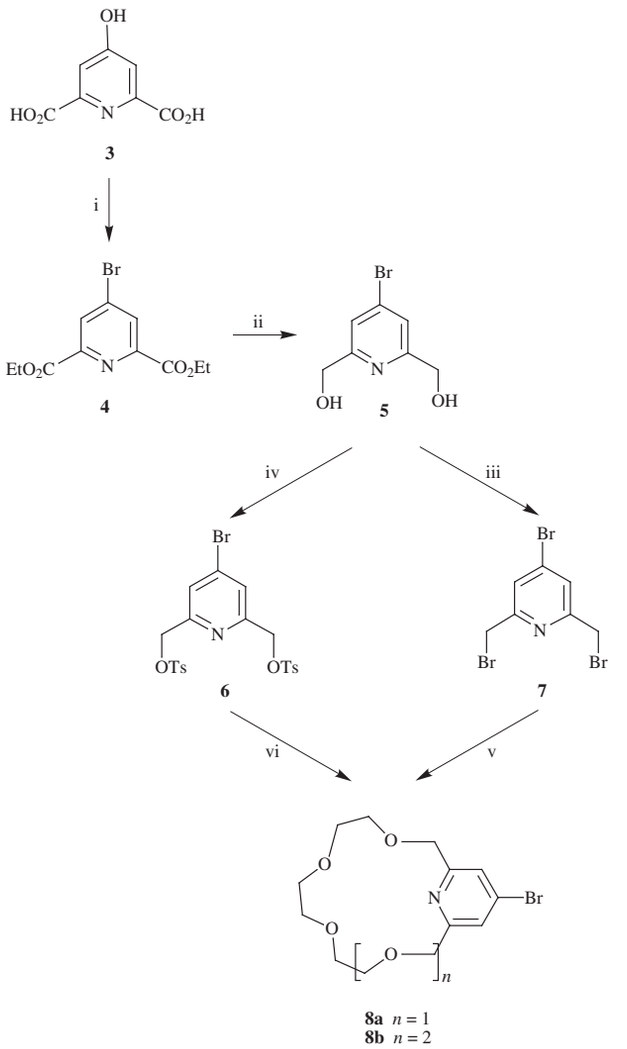
Results

Synthesis

The syntheses of SPYR **1a**, PPYR **1b** and BIPY **2** are approached in a convergent way to allow the attachment of the thiophene-based fluorophore, which the three indicators have in common, to the respective ionophores. In all cases, the coupling between the fluorophore and the ionophore is achieved *via* a Stille[13] reaction between the halogenated chelator and the stannylated fluorophore. Therefore, the availability of a halide-substituted ionophore is required. Although the synthesis of unsubstituted (di)pyridino crown compounds has long been known,[8] no (di)pyridino crown ethers substituted by a halide in the 4-position have, to our knowledge, been synthesised yet. The conversion of the acyclic starting material to the appropriately substituted pyridine analogue before cyclisation is recommended, for Bradshaw and co-workers have found that substitution of a 4-substituent on the pyridine ring after cyclisation is unsuccessful.[8]

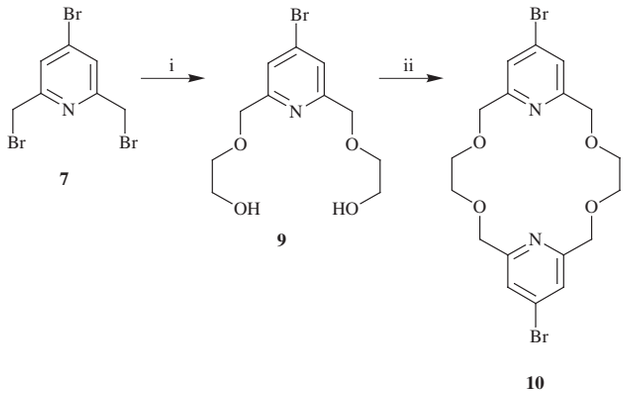
Synthesis of the monopyridine ionophores 10a,b. The chelating groups of SPYR **1a** and PPYR **1b** were synthesised from chelidamic acid (1,4-dihydro-4-oxopyridine-2,6-dicarboxylic acid) **3**, which was brominated and then esterified to yield diethyl 4-bromopyridine-2,6-dicarboxylate **4**, according to the procedure described by Takalo and Kankare[14] (Scheme 2). Upon reduction with sodium borohydride,[15,16] 4-bromopyridine-2,6-dimethanol **5** was obtained, which was converted either into the corresponding ditosylate **6**,[17] or into dibromide **7** by addition of phosphorus tribromide.[15] When toluene-*p*-sulfonyl chloride was used as the tosylating agent, the mass spectrum of compound **6** revealed a partial substitution of the bromide in the 4-position by a chloride group, originating from toluene-*p*-sulfonyl chloride. Therefore, toluene-*p*-sulfonyl bromide was used, freshly prepared by oxidative bromination of toluene-*p*-sulfonyl hydrazide.[18] For the synthesis of the 4-bromo-2,6-pyridino crown ethers **8a**, two synthesis pathways were investigated. In both cases, a solution of triethylene glycol in THF was converted into the corresponding dialcoholate using sodium hydride as a base, after which the dialcoholate reacted with the pyridino precursor **6** or **7**. When 4-bromo-2,6-bis(bromomethyl)pyridine **7** was used under high dilution conditions, much better yields (35%) were obtained than with 4-bromo-2,6-bis[(*p*-tolylsulfonyl)methyl]pyridine **6** under normal reaction conditions (15%). Therefore, compound **8b** was synthesised uniquely from the tribromide and could be isolated in 32%.

Synthesis of the *sym*-dipyridyl ionophore 10. Few reports on the synthesis of *sym*-dipyridyl-18-crown-6 compounds are



Scheme 2 *Reagents and conditions*: i: a) Br_2, PBr_3, hexane, 3 h at 90 °C, b) $CHCl_3$, EtOH; ii: $NaBH_4$, EtOH, 2 h at rt, 15 h reflux; iii: PBr_3, ether, 30 min at rt, overnight reflux; iv: a) **5**, THF, 2.2 equiv. KOH, 15 min at 0 °C, b) 2.1 equiv. toluene-*p*-sulfonyl bromide, THF, 5 h at 0 °C, 12 h at rt; v: a) 1.1 equiv. $HO(CH_2CH_2O)_3H$, 1.2 equiv. NaH, THF, 1 h reflux, b) −78 °C, **6**, THF, 2 days rt; vi: a) 1.1 equiv. NaH, THF, 0 °C, $HO(CH_2CH_2O)_nH$ ($n = 3$ or 4), b) 200 cm^3 THF, **7**, 2 h at 0 °C, rt overnight.

known in the literature.[17,19] They are all based on a one-pot reaction of two ethylene glycol units with two 2,6-disubstituted pyridino building blocks and are characterised by yields not exceeding 20%. Therefore, we built up the dipyridino containing ligand 4,4′-dibromo-*sym*-dipyridyl-18-crown-6 **10** in a two-step reaction. This allows a fast and high yield formation of the diol **9**, without polymerisation as a side reaction. Thus, 4,4′-dibromo-*sym*-dipyridyl-18-crown-6 **10** was synthesised from 4-bromo-2,6-bis(bromomethyl)pyridine **7** (Scheme 3), which was prepared as described above. By drop-wise addition of this tribromide at 0 °C to a solution of excess ethylene glycol in anhydrous THF in the presence of sodium hydride, compound **9** was formed. Yet, upon removal of the ethylene glycol, reaction at the 4-substituent of **9** occurred, due to the high temperatures required. Therefore, compound **9** was isolated by column chromatography in 95% yield. The acyclic precursor **9** was then cyclised to yield compound **10** by adding a second 4-bromo-2,6-bis(bromomethyl)pyridino unit **7** with potassium hydride acting as a template. In a first attempt, the diol **9** was added under high dilution conditions to a solution of 2 equiv. potassium hydride in THF at room temperature, followed by drop-wise addition of the tribromide **7** in THF. Due to a side-reaction with the 4-bromo substituent of **7**, the desired dipyridino crown **10** could be isolated only in 14% yield.



Scheme 3 *Reagents and conditions*: i: a) 3.3 equiv. NaH, excess ethylene glycol, 15 min at rt, 30 min at 60 °C, b) 0 °C, **7**, THF, 10 h at rt; ii: a) 1.1 equiv. KH, THF, 0 °C, **11**, b) THF, **7**, 0 °C, rt overnight.

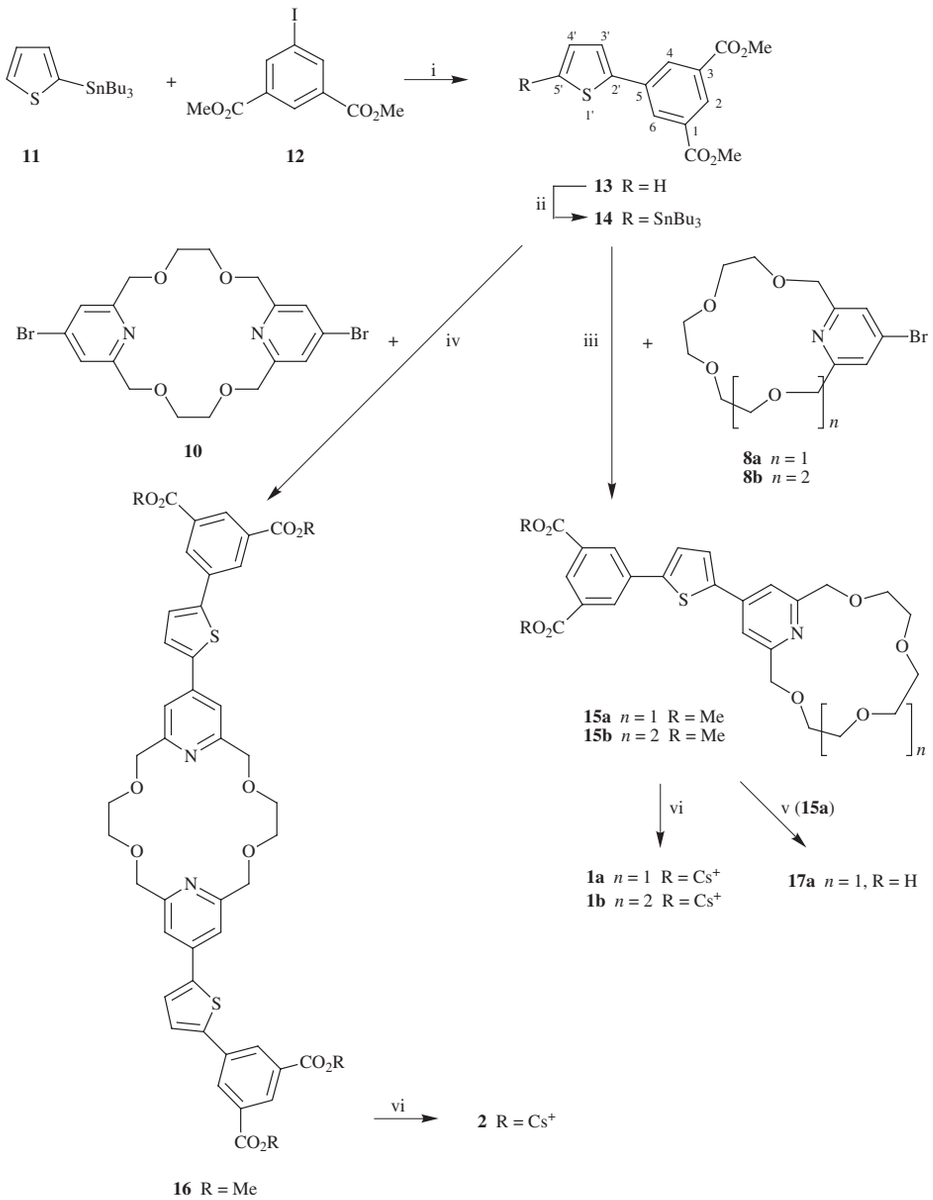
Reversal of the order of addition, *i.e.* addition of a solution of potassium hydride in THF to a solution of the diol **9** in THF, followed by diluted addition of the tribromide **7** to this mixture, all caried out at 0 °C, improved the yield to 61%.

Synthesis of the indicators SPYR 1a, PPYR 1b and BIPY 2. The next step comprises the coupling between both types of crown compounds **8a**,**b** and **10** and the fluorophore building block. This synthon, dimethyl 5-(5-tributylstannyl-2-thienyl)-isophthalate **14**, was synthesised by a Stille coupling between 2-(tributylstannyl)thiophene **11** and dimethyl 5-iodoisophthalate **12**,[7] followed by stannylation in the 5-position of the thiophene nucleus of **13** with lithium 2,2,6,6-tetramethylpiperidine–tributyltin chloride, as was described previously[7] (Scheme 4). The methyl esters of the fluorescent indicators **15a**,**b** and **16** were obtained in good yields by refluxing a mixture of 1.2 equiv. of the fluorophore building block **14** (for **15a**,**b**) or 2.4 equiv. of **14** (for **16**) and 1 equiv. of the appropriate crown compound **8a**, **8b** or **10** in anhydrous toluene under nitrogen atmosphere in the presence of 1 mol% tetrakis(triphenylphosphine)palladium. In the final reaction step, the esters **15a,b** and **16** were converted into the corresponding water soluble forms. The free acid **17a** was obtained by refluxing compound **15a** overnight in dry THF with 2.5 equiv. potassium trimethylsilanolate.[20] Since the free acid **17a** showed a limited solubility in aqueous solution at physiological pH (7.05), we preferred to prepare directly the caesium salt,[21] analogously to Minta and Tsien, who used Cs^+ as a truly inert background cation.[6] Hence, the caesium salts **1a**,**b** and **2** were obtained from the corresponding methyl esters **15a**,**b** and **16** by reflux in methanol with a large excess of caesium hydroxide and were used as such for the fluorescence measurements. Comparison of the UV spectra of the esters in methanol and the caesium salts in water indicates that the fluorophore structure remains unaltered.

Steady-state fluorescence

Steady-state fluorescence and absorption measurements were performed on the caesium salts of the indicators in aqueous solution at 20 °C and pH 7.05. The indicator concentrations were of the order of $(5–6) \times 10^{-6}$ M, yielding an absorbance per cm path length of approximately 0.1 at the absorption maximum. A physiological pH of 7.05 was obtained by buffering the solutions with 10^{-2} M MOPS (3-morpholinopropanesulfonic acid), adjusted with tetramethylammonium hydroxide.

SPYR 1a and PPYR 1b. The absorption spectra of SPYR and PPYR show a small decrease in the absorbance with increasing cation concentration, while the absorption maxima remain located at 345 nm for SPYR and 342 nm for PPYR (not shown). These decreases are reflected by small changes in



Scheme 4 *Reagents and conditions*: i: 2 mol% $(MeCN)_2PdCl_2$, DMF, air, rt; ii: a) 2,2,6,6-tetramethylpiperidine, BuLi, THF, 0 °C, b) **13**, tributyltin chloride, THF, 2 h at −78 °C, rt overnight; iii: 1.2 equiv. **14**, anhydrous toluene, **8a** or **8b**, 1 mol% $Pd(PPh_3)_4$, 15 h reflux; iv: 2.4 equiv. **14**, anhydrous toluene, **10**, 1 mol% $Pd(PPh_3)_2$, 15 h reflux; v: a) 2.5 equiv. $KOSiMe_3$, THF, overnight reflux, b) HCl; vi: excess CsOH, MeOH, overnight reflux.

the molar extinction coefficients, which for SPYR are $(23.6 \pm 0.1) \times 10^3$ M^{-1} cm^{-1} for the free form of the indicator and $(20.8 \pm 0.2) \times 10^3$ M^{-1} cm^{-1} for the Na^+ saturated form. The molar extinction coefficients of PPYR are somewhat lower: $(18.0 \pm 0.1) \times 10^3$ M^{-1} cm^{-1} and $(17.2 \pm 0.3) \times 10^3$ M^{-1} cm^{-1} for the free and the K^+-bound form, respectively.

The fluorescence excitation and emission spectra of SPYR with Na^+ and PPYR with K^+ are shown in Fig. 2 and Fig. 3, respectively. Both indicators show an emission maximum at 410 nm; the excitation maxima are identical to those of absorption. The relative decrease in the fluorescence intensity with increasing concentrations of all cations is much more pronounced for SPYR than for PPYR. The fluorescence intensity of SPYR with Na^+ reaches a first plateau at 7 mM Na^+, followed by a further decrease, which can be attributed to an excited-state reaction.[12] The fluorescence quantum yields for the free and bound form of SPYR are 0.73 and 0.63, respectively. PPYR is saturated with K^+ at 5 mM K^+, from whereon the intensity reaches a constant value. The fluorescence quantum yields of PPYR are even higher than those measured for SPYR ($\varphi_f = 0.84$ for the free and 0.80 for the K^+ bound form). The spectral properties of SPYR and PPYR are summarised in Table 1.

The ground-state dissociation constants, K_d, of the ion–indicator complexes were determined by fitting eqn. (3) to the fluorescence data F at $\lambda_{em} = 410$ nm and $\lambda_{ex} = 340$ nm, yielding additional values for n, F_{min}, and F_{max}. The non-linear fit for the binding of Na^+ by SPYR, which is given in Fig. 4, yields a value of 1.2 ± 0.2 mM for K_d. n equals 1.05 ± 0.04, indicating a 1 : 1 stoichiometry for the Na^+–indicator complex. The K_d values for the other monovalent cations with SPYR determined in this way are summarised in Table 2.

Analysis of the data for PPYR in a similar way (Fig. 5) yields a dissociation constant of 1.2 ± 0.3 mM for K^+–PPYR and a stoichiometry consistent with a 1 : 1 complex. The binding properties of PPYR with the other monovalent cations are given in Table 3.

BIPY 2. The absorbance of the *sym*-dipyridino crown indicator is rather insensitive to increasing cation concentration: the extinction coefficients are $(37.2 \pm 0.3) \times 10^3$ M^{-1} cm^{-1} for the free form and $(36.4 \pm 0.2) \times 10^3$ M^{-1} cm^{-1} for the K^+ complex. The changes in the emission and excitation spectra that accompany K^+ binding are shown in Fig. 6. The excitation spectrum (with $\lambda_{max} = 344$ nm) and the emission spectrum (with

Table 1 Spectral properties of SPYR, PPYR and BIBY at pH 7.05 and 20 °C. For SPYR, the Na^+ complex is taken as the bound form, while for PPYR and BIPY the K^+ complex is considered

	λ_{max}/nm		$\varepsilon/10^3$ M^{-1} cm^{-1}		φ_f	
Indicator	excitation	emission	free	bound	free	bound
SPYR	345	410	23.6 ± 0.1	20.8 ± 0.2	0.73	0.63
PPYR	342	410	18.0 ± 0.1	17.2 ± 0.3	0.84	0.80
BIPY	344	410	37.2 ± 0.3	36.4 ± 0.2	0.35	0.13

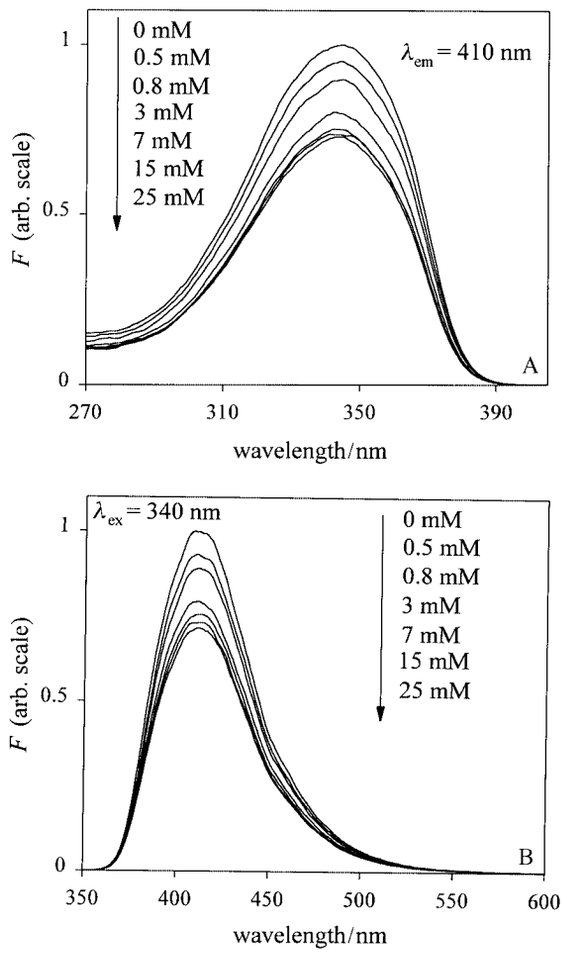


Fig. 2 Fluorescence excitation (A) and emission (B) spectra of SPYR as a function of $[Na^+]$ at 20 °C and pH 7.05.

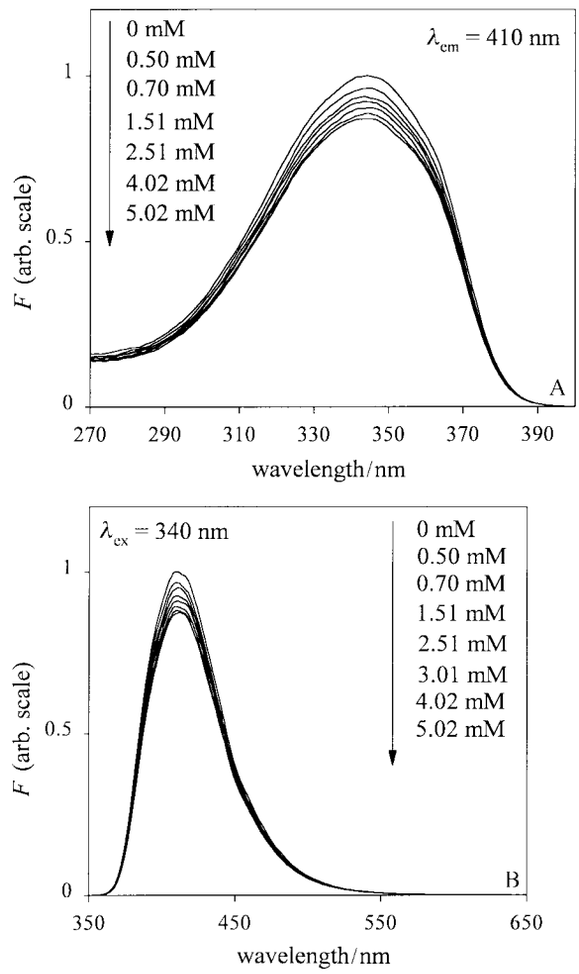


Fig. 3 Fluorescence excitation (A) and emission (B) spectra of PPYR as a function of $[K^+]$. The experimental conditions were as in Fig. 2.

λ_{max} = 410 nm) undergo a similar intensity decrease upon ion complexation, but the effect is more pronounced for this indicator than with the monopyridine derivatives. At elevated ion concentrations, a second band at longer wavelengths shows up in the emission spectrum, probably due to aggregate formation. In order to obtain reproducible results, freshly prepared and well stirred samples had to be used. Although enhanced fluorescence was expected for BIPY compared to SPYR and PPYR, given the presence of two fluorophore moieties, much lower quantum yields were observed: 0.35 for the free form, and 0.13 for BIPY saturated with K^+. The spectral properties of BIPY are summarised in Table 1.

For the K^+–BIPY complex, a dissociation constant of 4.4 ± 0.7 mM was determined by fitting eqn. (3) to the fluorescence data at 410 nm (Fig. 7). The selectivity of BIPY *vs.* other cations, like K^+, Li^+, Cs^+, Mg^{2+}, and Ca^{2+}, can be calculated from the ratio of their respective K_d values (Table 4). Comparison of the data for monovalent cations shows a peak selectivity for K^+ (K^+ binds more strongly to BIPY than Li^+ by a factor of 1.6, 3.6 times more strongly than Na^+ and 4.1 times more strongly than Cs^+). BIPY also shows a high affinity for the divalent cations Ca^{2+} and Mg^{2+}.

Discussion

Synthesis

The synthesis of the indicators SPYR, PPYR and BIPY involves the development of a synthetic route towards new 4-(4′-) (di)halogenated (di)pyridino crown ethers as key intermediates. In the 2,6-disubstituted pyridino building blocks a 4-bromo substituent is introduced prior to cyclisation which takes place under high-dilution conditions. By using a two-step reaction instead of the known one-pot synthesis,[17,19] the yield of *sym*-dipyridino crown ethers could be raised to 60%. These macrocyclic ionophores can be coupled in a Stille reaction to the stannylated fluorophore to yield the desired fluorescent indicators in good yields.

Table 2 Binding properties of SPYR with the respective cations determined by fitting eqn. (3) to the fluorescence data, with F_{max}, F_{min}, K_d, and n as adjustable parameters. Since eqn. (3) allows direct fitting for K_d and n, the errors on these values are reported. The dissociation constants were measured with 6.5 μM of the indicator and 10^{-2} M MOPS at pH 7.05 buffered by (5–6) × 10^{-3} M tetramethylammonium hydroxide. The emission intensity, originating from excitation at 340 nm, was recorded at 410 nm. No correction was made for the change in ionic strength of the solutions

Ion	K_d/mM	n stoichiometry
Li^+	1.1 ± 0.5	0.97 ± 0.05
Na^+	1.2 ± 0.2	1.05 ± 0.04
K^+	1.2 ± 0.7	0.95 ± 0.07
Cs^+	1.7 ± 0.4	1.00 ± 0.02

Table 3 Binding properties of PPYR with the respective cations determined by fitting eqn. (3) to the fluorescence data, with F_{max}, F_{min}, K_d, and n as adjustable parameters. For K^+, the excitation intensity, originating from emission at 410 nm, was recorded at 344 nm. For the other cations, the experimental conditions were as in Table 2

Ion	K_d/mM	n stoichiometry
Li^+	1.2 ± 0.1	0.96 ± 0.04
Na^+	2.4 ± 0.9	1.1 ± 0.3
K^+	1.2 ± 0.3	1.05 ± 0.04
Cs^+	2.8 ± 0.4	1.00 ± 0.02

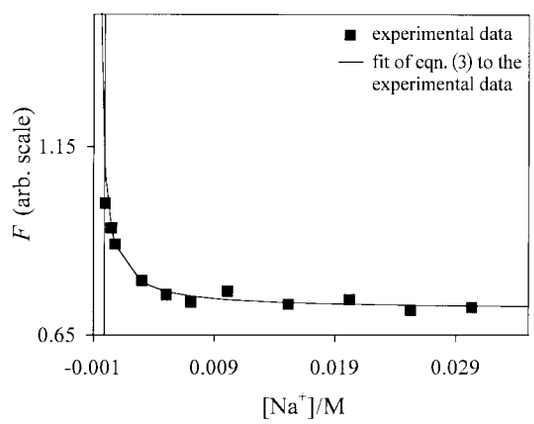


Fig. 4 Non-linear fit of eqn. (3) to the emission intensities of SPYR at λ_{em} = 410 nm as a function of the Na^+ concentration. The excitation wavelength was 340 nm and the other experimental conditions were as in Fig. 2.

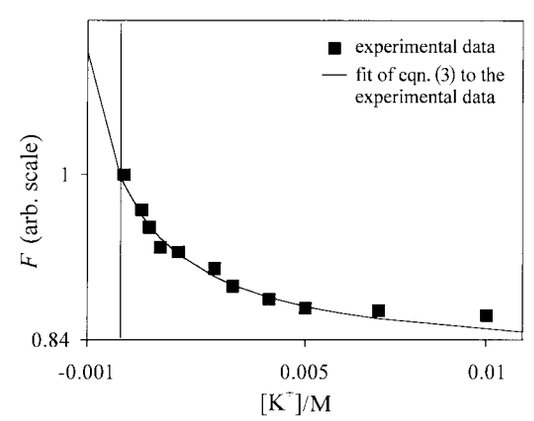


Fig. 5 Non-linear fit of eqn. (3) to the excitation intensities of PPYR at λ_{ex} = 344 nm as a function of the K^+ concentration. The emission wavelength was 410 nm and the other experimental conditions were as in Fig. 2.

Table 4 Binding properties of BIPY with the respective cations determined by fitting eqn. (3) to the fluorescence data, with F_{max}, F_{min}, K_d, and n as adjustable parameters. The experimental conditions were as in Table 2

Ion	K_d/mM	n stoichiometry
Li^+	7 ± 2	1.0 ± 0.1
Na^+	16 ± 3	1.0 ± 0.1
K^+	4.4 ± 0.7	0.98 ± 0.04
Cs^+	18 ± 5	1.1 ± 0.1
Ca^{2+}	4 ± 1	1.1 ± 0.1
Mg^{2+}	1.7 ± 0.4	1.0 ± 0.1

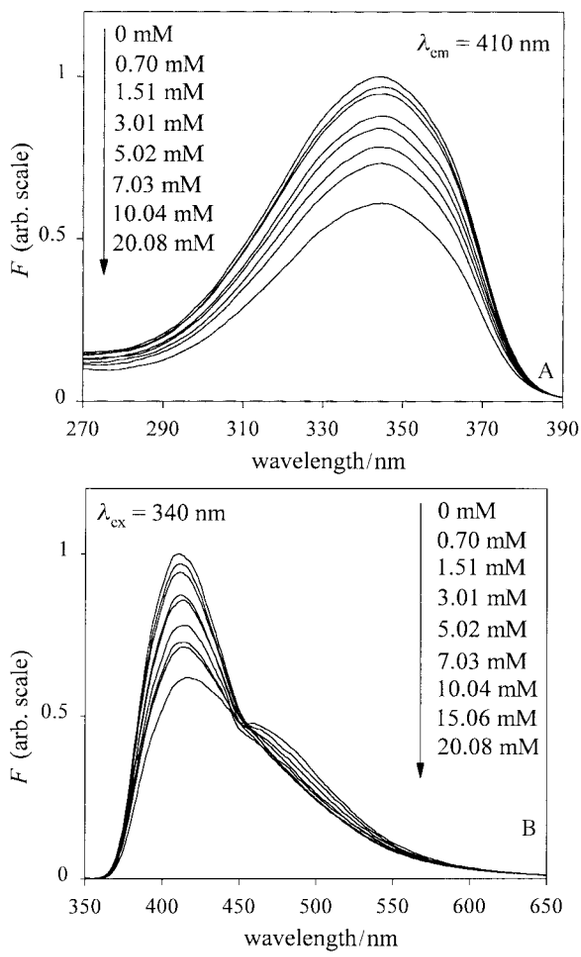


Fig. 6 Fluorescence excitation (A) and emission (B) spectra of BIPY as a function of $[K^+]$. The experimental conditions were as in Fig. 2.

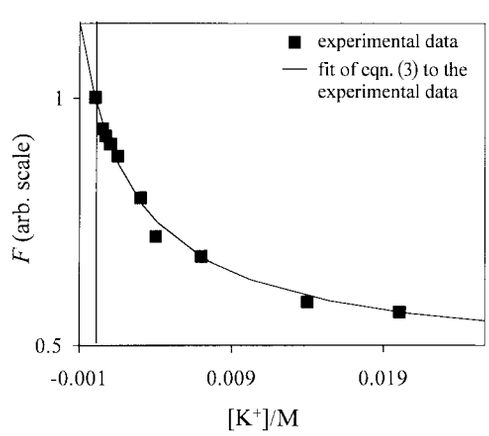


Fig. 7 Non-linear fit of eqn. (3) to the emission intensities of BIPY at λ_{em} = 410 nm as a function of the K^+ concentration. The excitation wavelength was 340 nm and the other experimental conditions were as in Fig. 2.

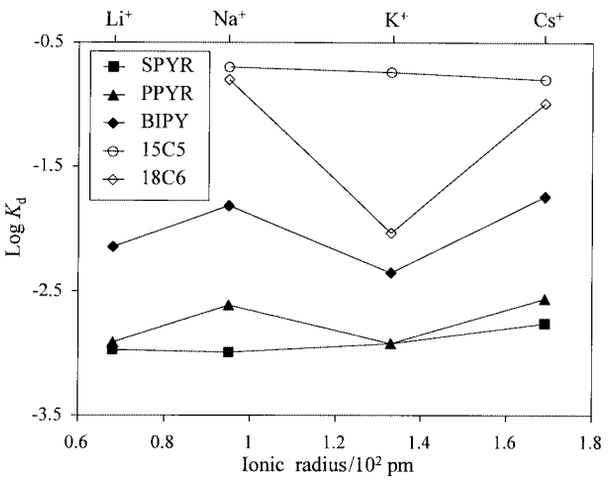


Fig. 8 Comparison of the cation binding properties of the indicators under study with 15C5 and 18C6 in water.

Basis for cation affinity and selectivity

From comparison of the binding behaviour of SPYR, PPYR and BIPY, a peak selectivity[22] is seen for the 18-crown-6 compounds PPYR and BIPY, while the 15-crown-5 derivative SPYR can hardly discriminate between the monovalent cations. As Tables 3 and 4 point out, the K_d values for PPYR and BIPY increase in the order $K^+ < Li^+ < Na^+ < Cs^+$. For an optimal complexation, the size of the cavity should be equal to the size of the cation to be recognised, as is the case for K^+ with 18-crown-6.[23,24] Both Li^+ and Na^+ have too small a radius to be effectively accomodated within the 18-crown-6 cavity of PPYR and BIPY. The unexpectedly strong complexation of Li^+ may be explained by the high degree of solvatation of the lithium ion compared to the other monovalent cations. This theory was proposed by Valeur *et al.*, who observed a similar strong complexation of the aza crown indicator BOZ-crown (BOZ = benzoxazinone) with Li^+.[24] By taking part in the complex formation under its solvated form, Li^+ would fit better into the 18-crown-6 cavity. Cs^+, with an ion radius exceeding the crown cavity radius, gives rise to a complex strongly destabilised by cation–ligand repulsions as well as ligand deformations.[25] Given the affinity of the respective indicators for Cs^+, competition between the other monovalent cations and Cs^+, which was used in preparing the caesium salts, can be expected. However, for an indicator to have optimal sensitivity for a given cation X, it is required that $0.1\ K_d \leq [X] \leq 10\ K_d$. In all samples $[Cs^+] = 5–6 \times 10^{-5}$ M, a concentration which is well below $0.1\ K_d$. Therefore, interference with Cs^+ is of minor importance. Although the unsolvated sodium ion fits rather well into the SPYR-cavity, an efficient complexation is prohibited by a high solvatation energy.[22] The high selectivity of BIPY for Ca^{2+} and Mg^{2+} may be due to an enhanced affinity between the soft pyridine nitrogen donors and more charge dense ions.[26]

Influence of the presence of a pyridino ring

In Fig. 8, the log K_d values of the ion complexes of SPYR, PPYR and BIPY are compared with those of the corresponding parent compounds 18C6 (18-crown-6) and 15C5 (15-crown-5). Although smaller fluctuations are observed for PPYR and BIPY compared to 18C6, the three 18-membered derivatives all follow the same tendency: $K_d(Cs^+) \geq K_d(Na^+) > K_d(K^+)$. The 15-membered compounds show hardly any selectivity among monovalent cations. The increased stability of the complexes of PPYR and SPYR compared to 18C6 and 15C5 is thought to be due to the stabilising presence of the electron rich thiophene fluorophore, which may exert an electron donating effect. Additionally, since the pyridine group introduces organisation and rigidity both in the free and complexed form,[8] the equilibrium conformation does not change much upon complexation, thus causing little ligand deformation. The weaker complex formation with the *sym*-dipyridyl compound BIPY compared to PPYR might be attributed to a higher degree of ligand deformation upon ion binding.[27] This hypothesis is supported by the more pronounced difference in the shape of the spectra and the fluorescence quantum yields for BIPY compared to PPYR and SPYR.

Spectral and cation binding properties

The most striking property of the mono-pyridino indicators SPYR and PPYR is their excellent fluorescence quantum yield, which in both cases lowers after ion binding. Although for BIPY, a fluorescence enhancement was expected compared to SPYR and PPYR, due to the presence of two fluorophores, quantum yields are much smaller. *Via* time-resolved fluorescence measurements and global compartmental analysis, the rate constants of the radiative and the non-radiative processes can be determined to gain more insight into the mechanism of the changes in the fluorescence intensity upon cation binding.[28]

Since the indicators display only a change in intensity upon cation binding and no corresponding spectral shifts, they are not suitable for ratiometric measurements at dual wavelengths. While SPYR has an equal chelating power *vs.* all monovalent cations, PPYR shows a two-fold selectivity for K^+ over Na^+, whereas BIPY displays a similar 3.5-fold selectivity. This compares favourably with PBFI, which shows a 2.6-fold selectivity for K^+ over Na^+. Furthermore, BIPY gives rise to a more pronounced response upon ion binding: a 40% decrease in the fluorescence intensity between the free and the K^+ saturated form is observed, compared to a 15% decrease for PPYR with K^+. The K_d value estimated for K^+–BIPY (4.4 ± 0.7 mM) is comparable to that for K^+–PBFI (5.1 mM), while K_d for K^+–PPYR (1.2 ± 0.3 mM) matches extracellular potassium levels (0–20 mM). The lower fluorescence quantum yields of BIPY compared to the monopyridine compounds still largely exceed those of PBFI (0.024 for the free form and 0.072 for the K^+-bound form, respectively).

Experimental

Materials and methods

When required, solvents and reagents were dried prior to use. Tetrahydrofuran (THF) and toluene were distilled over sodium. ^{1}H NMR spectra were recorded on a Bruker WM 250 or a Bruker AMX 400 instrument. The spectra were measured using deuteriochloroform ($CDCl_3$) or deuterated DMSO as solvent. The ^{1}H and ^{13}C chemical shifts are reported in ppm relative to tetramethylsilane or the deuterated solvent as an internal reference. The coupling constants J are given in Hz. Mass spectra were run using a Kratos MS50TC instrument and a DS90 data system. IR spectra were recorded on a Perkin-Elmer 1720 Fourier transform spectrometer. Melting points were determined with a Reichert Thermovar apparatus and are uncorrected.

The absorption measurements were performed on a Perkin-Elmer Lambda 6 UV/VIS spectrophotometer. Corrected steady-state excitation and emission spectra were recorded on a SPEX Fluorolog 212. Fluorescence quantum yields of the free and saturated forms of the indicators were determined using quinine sulfate in 0.05 M sulfuric acid as reference. The quantum yield of the reference was taken to be 0.54.[29] The samples were measured in Milli-Q water. No correction for the refractive index was necessary.

Synthesis

Diethyl 4-bromopyridine-2,6-dicarboxylate 4. Compound **4** was prepared according to the procedure described by Takalo

and Kankare.[14] Mp = 94–95 °C (lit:[14] 95–96 °C); ν_{max}(KBr)/cm^{-1} 3072–2980 (CH_3, CH_2), 1710 (CO), 1560 (pyridine), δ_H($CDCl_3$) 1.43 (6H, t, *J* 7.2, CH_3), 4.48 (4H, q, *J* 7.2, CH_2), 8.42 (2H, s, Pyr-H); δ_C($CDCl_3$) 14.18 (CH_3), 62.7 (CH_2), 131 (CH), 134.8 (CBr), 149.52 (C=N), 163.53 (CO); *m*/*z* 302 ($M^{+\bullet}$ + 1, 9%), 256 ($M^{+\bullet}$ − EtO, 12), 229 ($M^{+\bullet}$ − $C_3H_5O_2$, 100), 201 ($C_6H_2BrNO_2^+$, 22), 76 ($C_5H_2N^+$, 31); Found: 300.9948, Calc. for $C_{11}H_{12}BrNO_4$: 300.9949.

[4-Bromo-6-(hydroxymethyl)-2-pyridyl]methanol 5. Compound **5** was prepared according to the procedure described by Takalo *et al.*[15] and Lüning *et al.*[16]; mp = 163–164 °C (lit:[15] 162–164 °C); ν_{max}(KBr)/cm^{-1} 3350 (OH), 3090 (CH_2), 1580 (pyridine); δ_H(DMSO-d_6) 4.51 (4H, d, *J* 5.6, CH_2), 5.49 (2H, t, *J* 5.6, OH), 7.5 (2H, s, Pyr-H); δ_C(DMSO-d_6) 63.62 (CH_2), 121.03 (CH), 133.15 (CBr), 163.14 (C=N); *m*/*z* 217 ($M^{+\bullet}$, 22%), 199 ($M^{+\bullet}$ − H_2O, 39), 170 ($M^{+\bullet}$ − CH_2O − OH, 27), 90 ($C_6H_4N^+$, 63), 76 ($C_5H_2N^+$, 22), 63 (C_4HN^+, 63), 51 (C_3HN^+, 100); Found: 216.9736, Calc. for $C_7H_8BrNO_2$: 216.9738.

4-Bromo-2,6-bis[(*p*-tolylsulfonyl)oxymethyl]pyridine 6. To a suspension of the diol **5** (2.18 g, 10 mmol) in dry THF (80 cm^3), finely ground potassium hydroxide (1.45 g, 22 mmol, 2.2 equiv.) was added. After stirring for 15 min at 0 °C under an argon stream, toluene-*p*-sulfonyl bromide (4.94 g, 21 mmol, 2.1 equiv.) dissolved in THF (20 cm^3) was added drop-wise. The reaction mixture was then stirred for 5 h at 0 °C followed by 12 h at room temperature. The mixture was filtered on a glass filter and then evaporated. The obtained residue was purified by column chromatography on silica (CH_2Cl_2–hexane, 60 : 40) yielding a white powder (4.2 g, 77%); mp = 110–111 °C (CH_2Cl_2–hexane); ν_{max}(KBr)/cm^{-1} 3060 (CH_2), 1570 (pyridine), 1370–1170 (-O-SO_2), 670 (CBr); δ_H($CDCl_3$) 2.45 (6H, s, CH_3), 5.01 (4H, s, CH_2), 7.34 (4H, d, *J* 7.6, *m*-Ar-H), 7.4 (2H, s, Pyr-H), 7.8 (4H, d, *J* 7.6, *o*-Ar-H); δ_C($CDCl_3$) 21.67 (CH_3), 70.44 (CH_2), 124.48 (*C*H(Pyr)), 128.03 (C_o), 130 (C_m), 132.57 (C_p), 134.45 (C-Br), 145.42 (C-S), 154.89 (C=N); *m*/*z* 525 ($M^{+\bullet}$, 4%), 370 ($M^{+\bullet}$ − $C_7H_7SO_2$, 5), 355 ($M^{+\bullet}$ − $C_7H_7SO_3$, 8), 155 ($C_7H_7SO_2^+$, 15), 91 ($C_7H_7^+$, 100); Found: 524.9910, Calc. for $C_{21}H_{20}BrNO_6S_2$: 524.9915.

4-Bromo-2,6-bis(bromomethyl)pyridine 7. The tribromo derivative **7** was prepared from compound **5** as was described by Takalo *et al.*[15] To a suspension of the diol **5** (2.18 g, 10 mmol) in ether (100 cm^3) at 0 °C, phosphorus tribromide (4.07 g, 15 mmol) was added drop-wise, after which the mixture was stirred at room temperature for 30 min and then refluxed overnight. After cooling to room temperature, an aqueous solution of 5% $NaHCO_3$ was added gradually until neutral pH was reached. The solution obtained was extracted with ether (4 × 30 cm^3) and the combined organic layers were concentrated. After filtering the residue on silica (CH_2Cl_2), white crystals were obtained (2.63 g, 76%); mp = 131–132 °C (lit:[15] 128–129 °C) (CH_2Cl_2–hexane); ν_{max}(KBr)/cm^{-1} 3060 (CH_2), 1560 (pyridine); δ_H($CDCl_3$) 4.64 (4H, s, CH_2), 7.78 (2H, s, Pyr-H); δ_C($CDCl_3$) 33.2 (CH_2), 125.97 (CH), 133.11 (CBr), 158.34 (C=N); *m*/*z* 341 ($M^{+\bullet}$, 10%), 262 ($M^{+\bullet}$ − Br, 60), 183 ($M^{+\bullet}$ − 2 × Br, 12), 104 ($C_6H_4N^+$, 63); Found: 340.8055, Calc. for $C_7H_6Br_3N$: 340.8050.

16-Bromo-3,6,9,12-tetraoxa-18-azabicyclo[12.3.1]octadeca-1(18),14,16-triene 8a. *Method A.* In a three necked flask equipped with a septum and a reflux condensor triethylene glycol (165 mg, 1.1 mmol) in anhydrous THF (5 cm^3) was added to a solution of sodium hydride (36 mg, 1.2 mmol) in THF (10 cm^3) under argon. After refluxing the mixture for 1 h, the solution was cooled to −78 °C and a solution of the ditosylate **6** (526 mg, 1 mmol) in THF (10 cm^3) was added drop-wise. After stirring at room temperature for 2 days, the precipitate was filtered off and the filtrate was concentrated. The obtained residue was diluted with chloroform, washed with brine and dried over $MgSO_4$. After evaporation, the product was purified on alumina ($CHCl_3$–MeOH, 99 : 1). A viscous oil was obtained (50 mg, 15%).

Method B. In a 500 cm^3 three necked flask equipped with two septa sodium hydride (198 mg, 3.3 mmol, 1.1 equiv.) was dissolved in anhydrous THF (50 cm^3) under argon. After cooling to 0 °C, triethylene glycol (0.4 cm^3, 3 mmol) was added drop-wise, followed by stirring for 1 h while the temperature was kept constant. Then, THF (200 cm^3) was added, whereupon compound **7** (1.032 g, 3 mmol) dissolved in THF (50 cm^3) was added drop-wise at 0 °C over a period of 2 h. After stirring overnight at room temperature, the formed precipitate was filtered off and the filtrate was condensed by evaporation. The obtained residue was dissolved in chloroform (100 cm^3) and water (200 cm^3). The aqueous layer was extracted with chloroform (4 × 30 cm^3) and the combined organic layers were washed with brine and then dried over $MgSO_4$. A viscous oil (348 mg, 35%) was obtained after column chromatography on alumina ($CHCl_3$–MeOH, 98 : 2); ν_{max}($CHCl_3$)/cm^{-1} 3020–2920 (CH_2, CH), 1570–1520 (pyridine), 1100 (C-O); δ_H($CDCl_3$) 3.48 (4H, s, CH_2), 3.47–3.6 (4H, m, CH_2), 3.7–3.79 (4H, m, CH_2), 4.66 (4H, s, CH_2) 7.42 (2H, s, Pyr-H); δ_C($CDCl_3$) 70.34, 70.49, 70.77, 73.75 (CH_2), 124.66 (CH), 133.2 (CBr), 159.8 (C=N); *m*/*z* 332 (M^+, 72%), 288 [$M^{+\bullet}$ − (CH_2CH_2O), 43], 244 [$M^{+\bullet}$ − (CH_2CH_2O)$_2$, 66], 200 [$M^{+\bullet}$ − (CH_2CH_2O)$_3$, 100], 184 [$M^{+\bullet}$ − (CH_2CH_2O)$_3$ − O, 34]; Found: 331.0422, Calc. for $C_{13}H_{18}BrNO_4$: 331.0419.

19-Bromo-3,6,9,12,15-pentaoxa-21-azabicyclo[15.3.1]-henicosa-1(21),17,19-triene 8b. Compound **8b** was prepared as decribed in *Method B* for compound **8a** from the tribromide **7** (344 mg, 1 mmol) and tetraethylene glycol (172 µl, 1 mmol, 1 equiv.). A very viscous oil (120 mg, 32%) was obtained after column chromatography on alumina ($CHCl_3$–MeOH, 98 : 2); ν_{max}($CHCl_3$)/cm^{-1} 3015–2920 (CH_2, CH), 1570–1520 (pyridine), 1100 (C-O); δ_H($CDCl_3$) 3.59 (8H, s, CH_2), 3.66–3.69 (4H, m, CH_2), 3.73–3.76 (4H, m, CH_2), 4.74 (s, 4H, CH_2) 7.43 (2H, s, Pyr-H); δ_C($CDCl_3$) 69.8, 70.4, 70.8, 71.2, 73.1 (CH_2), 124.6 (CH), 133.5 (CBr), 159.7 (C=N); *m*/*z* 367 ($M^{+\bullet}$, 100%), 332 [$M^{+\bullet}$ − (CH_2CH_2O), 13], 297 ($M^{+\bullet}$ − Br, 12), 288 [$M^{+\bullet}$ − (CH_2CH_2O)$_2$, 3]; Found: 375.0683, Calc. for $C_{15}H_{22}BrNO_5$: 375.0681.

4-Bromo-2,6-bis[(2-hydroxyethoxy)methyl]pyridine 9. To a suspension of sodium hydride (304 mg, 12.6 mmol) ethylene glycol (40 cm^3) (freshly distilled) was added. The mixture was stirred at room temperature for 15 min, followed by heating at 60 °C for 30 min. After cooling to 0 °C, a solution of the tribromide **7** (1.45 g, 4.2 mmol) in THF (5 cm^3) was added drop-wise. After stirring at room temperature for 10 h, the mixture was poured into glacial brine (50 cm^3), whereupon ethyl acetate (20 cm^3) was added. Subsequently, the product was extracted with ethyl acetate (10 × 15 cm^3) and dried over $MgSO_4$. The yellow oil obtained after evaporation was filtered over alumina (CH_2Cl_2–MeOH, 90 : 10) yielding a white powder (1.17 g, 91%); mp 62–63 °C (CH_2Cl_2–hexane); ν_{max}(KBr)/cm^{-1} 3058 (CH_2), 1561 (pyridine); δ_H($CDCl_3$) 3.7–3.82 (8H, m, CH_2(O)), 4.28 (2H, t, *J* 5.87, OH), 4.64 (4H, s, CH_2C=N), 7.42 (2H, s, Pyr-H); δ_C($CDCl_3$) 61.47, 72.47, 73.13 (CH_2), 123.45 (CH), 134.2 (CBr), 159.19 (C=N); *m*/*z* 306 ($M^{+\bullet}$, 18%), 245 [$M^{+\bullet}$ − (O(CH_2)$_2$OH), 100], 184 [$M^{+\bullet}$ − (O(CH_2)$_2$OH)$_2$, 19], 104 ($C_7H_6N^+$, 65); Found: 305.0258, Calc. for $C_{11}H_{16}BrNO_4$: 305.0262.

10,21-Dibromo-3,6,14,17-tetraoxa-23,24-diazatricyclo-[17.3.1.1^{8,12}]tetracosa-1(23),8(24),9,11,19,21-hexaene 10. In a three necked flask equipped with a septum and a stopcock, potassium hydride (88 mg, 2.2 mmol, 1.1 equiv.) was dissolved in anhydrous THF (20 cm^3). After cooling to 0 °C, a solution of

compound **9** (306 mg, 1 mmol) in THF (10 cm^3) was added drop-wise, followed by stirring at 0 °C for 30 min. Then, THF (40 cm^3) was added, whereupon a solution of the tribromide **7** (344 mg, 1 mmol) in THF (30 cm^3) was added drop-wise at 0 °C over a period of 2 h. The reaction mixture was stirred overnight at room temperature and subsequently poured into water (50 cm^3) and ether (30 cm^3). The aqueous phase was extracted with ether (4 × 10 cm^3), and the combined organic layers were washed with brine and then dried over $MgSO_4$. After column chromatography on alumina (CH_2Cl_2–MeOH, 98:2) white crystals were obtained (300 mg, 61%); mp 163–164 °C (CH_2Cl_2–hexane); ν_{max}(KBr)/cm^{-1} 2920 (CH_2, CH), 1565–1440 (pyridine), 1100 (C-O), 680 (CBr); δ_H($CDCl_3$) 3.76 (8H, s, CH_2(O)), 4.55 (8H, s, CH_2C=N), 7.43 (4H, s, Pyr-H); δ_C($CDCl_3$) 70.22, 72.95 (CH_2), 133.53 (CH), 133.64 (CBr), 159.49 (C=N); *m/z* 489 ($M^{+\bullet}$, 15%), 459 ($M^{+\bullet}$ − CH_2O, 45), 445 [$M^{+\bullet}$ − ($O(CH_2)_2$), 100], 409 ($M^{+\bullet}$ − Br, 6), 199 ($C_7H_6BrNO^+$, 85), 104 ($C_6H_4N^+$, 81), 77 ($C_5H_3N^+$, 76); Found: 485.9803, Calc. for $C_{18}H_{20}BrN_2O_4$: 485.9793.

Dimethyl 5-(2-thienyl)isophthalate 13. Compound **13** was prepared from compound **12** according to the procedure described by Cielen *et al.*[7]

Dimethyl 5-(5-tributylstannyl-2-thienyl)isophthalate 14. Compound **14** was prepared from compound **13** according to the procedure described by Cielen *et al.*[7]

Dimethyl 5-{5-[3,6,9,12-tetraoxa-18-azabicyclo[12.3.1]octadeca-1(18),14,16-trien-16-yl]-2-thienyl}isophthalate 15a. To a solution of compound **14** (950 mg, 1.68 mmol, 1.2 equiv.) in anhydrous toluene (20 cm^3), compound **8a** (465 mg, 1.4 mmol) and $Pd(PPh_3)_4$ (16 mg, 1 mol%) were added. The reaction mixture was refluxed during 15 h under an argon stream. After cooling to room temperature, the solvent was evaporated and the yellow residue was purified on alumina ($CHCl_3$–hexane, 70:30 then $CHCl_3$–MeOH, 99:1). The obtained yellow oil (1.1 g) was crystallised from hot CH_2Cl_2 followed by addition of cold hexane. A white powder was obtained (443 mg, 60%); mp 115–117 °C (CH_2Cl_2–hexane); ν_{max}(KBr)/cm^{-1} 2860 (CH_3, CH_2, CH), 1720 (CO), 1600–1560 (pyridine), 1125 (C-O); δ_H($CDCl_3$) 3.5 (4H, s, CH_2), 3.61–3.64 (4H, m, CH_2), 3.79–3.83 (4H, m, CH_2), 3.99 (6H, s, CH_3), 4.73 (4H, s, CH_2), 7.44 (2H, s, Pyr-H), 7.48 (1H, d, *J* 3.6, 3′-H), 7.53 (1H, d, *J* 3.6, 4′-H), 8.45 (2H, s, 4-, 6-H), 8.59 (1H, s, 2-H); δ_C($CDCl_3$) 52.57 (CH_3), 70.18, 70.54, 70.87 (CH_2), 73.97 (CH_2), 117.61 (Pyr-CH), 125.68, 126.48 (3′-, 4′-C), 129.69 (2-C), 130.51 (4-, 6-C), 131.48 (1-, 3-C), 134.57 (2′-C), 141.62, 141.72 (Pyr-C), 143.35 (5′-C), 159.13 (C=N), 165.6 (C(O)), *m/z*: 527 ($M^{+\bullet}$, 10%), 496 ($M^{+\bullet}$ − CH_2OH, 16), 484 [$M^{+\bullet}$ − ($(CH_2)_2O$), 29], 438 [$M^{+\bullet}$ − $(CH_2)_2O)_2$, 29], 410 [$M^{+\bullet}$ − $(CH_2)_2O)(CH_2)_2$), 31], 396 [$M^{+\bullet}$ − $(CH_2)_2O)_3$, 100], 381 [$M^{+\bullet}$ − $(CH_2)_2O)_3CH_2$, 99] Found: 527.1612, Calc. for $C_{27}H_{29}NO_8S$: 527.1613.

Dimethyl 5-{5-[3,6,9,12,15-pentaoxa-21-azabicyclo[15.3.1]henicosa-1(21),17,19-trien-19-yl]-2-thienyl}isophthalate 15b. Compound **15b** was prepared as described for compound **15a**, starting from compounds **14** (216 mg, 0.38 mmol, 1.2 equiv.) and **8b** (120 mg, 0.32 mmol, 1 equiv.). A yellow powder was obtained (115 mg, 63%); mp 123–124 °C; ν_{max}(KBr)/cm^{-1} 2860 (CH_3, CH_2, CH), 1720 (CO), 1600–1560 (pyridine), 1125 (C-O); δ_H($CDCl_3$) 3.56–3.61 (8H, m, CH_2), 3.62–3.7 (4H, m, CH_2), 3.71–3.82 (4H, m, CH_2), 3.98 (6H, s, CH_3), 4.81 (4H, s, CH_2), 7.47 (1H, d, *J* 3.9, 3′-H), 7.48 (2H, s, Pyr-H), 7.54 (1H, d, *J* 3.9, 4′-H), 8.45 (2H, d, *J* 1.5, 4-, 6-H), 8.6 (1H, t, *J* 1.5, 2-H); δ_C($CDCl_3$) 52.5 (CH_3), 69.8, 70.5, 70.67, 71.15 (CH_2), 73.69 (CH_2), 116.5 (Pyr-CH), 125.5, 126.4 (3′-, 4′-C), 129.66 (2-C), 130.53 (4-, 6-C), 131.48 (1-, 3-C), 134.64 (2′-C), 141.7, 141.95 (Pyr-C, 5-C), 143.25 (5′-C), 159.11 (C=N), 165.8 (CO), *m/z*: 571 ($M^{+\bullet}$, 23%), 528 ($M^{+\bullet}$ − CH_2CHO, 45), 512 [$M^{+\bullet}$ − (OCH_2-CHO, 29], 484 [$M^{+\bullet}$ − (($(CH_2)_2OCH_2CHO$), 22], 396 [$M^{+\bullet}$ − $[(CH_2)_2O]_4H$, 100], 381 [$M^{+\bullet}$ − $CH_2[(CH_2)_2O)]_4H$, 76]; Found: 571.1873, Calc. for $C_{29}H_{33}NO_9S$: 571.1876.

Dimethyl 5-[5-(21-{5-[3,5-di(methoxycarbonyl)phenyl]-2-thienyl}-3,6,14,17-tetraoxa-23,24-diazatricyclo[17.3.1.1^{8,12}]-tetracosa-1(23),8(24),9,11,19,21-hexaen-10-yl)-2-thienyl]isophthalate 16. To a solution of compound **14** (334 mg, 0.57 mmol, 1.2 equiv.) in anhydrous toluene (10 cm^3) compound **10** (120 mg, 0.24 mmol) and $Pd(PPh_3)_4$ (6 mg, 1 mol%) were added. The reaction mixture was refluxed during 15 h under an argon stream. After cooling to room temperature, the solvent was evaporated and the yellow residue was purified on alumina ($CHCl_3$–hexane, 70:30 then $CHCl_3$–MeOH, 99.5:0.5). A yellow powder was obtained, which was recrystallised from hot CH_2Cl_2 followed by addition of cold hexane (443 mg, 60%); mp 269–270 °C (CH_2Cl_2–hexane); ν_{max}(KBr)/cm^{-1} 2950 (CH_3, CH_2, CH), 1720 (CO), 1600–1540 (pyridine), 1100 (C-O); δ_H($CDCl_3$) 3.92 (8H, s, CH_2), 3.99 (12H, s, CH_3), 4.56 (8H, s, CH_2C=N), 7.27 (2H, d, *J* 3.6, 3′-H), 7.34 (4H, s, Pyr-H), 7.40 (2H, d, *J* 3.6, 4′-H), 8.20 (4H, d, *J* 1.6, 4-, 6-H), 8.40 (2H, t, *J* 1.6, 2-H); δ_C($CDCl_3$) 52.52 (CH_3), 70.06 (CH_2O), 73.49 (CH_2C=N), 116.07 (Pyr-CH), 125.19, 126.31 (3′-, 4′-C), 129.26 (2-C), 128.89 (4-, 6-C), 131.13 (1-, 3-C), 134.28 (2′-C), 141.41, 141.78 (Pyr-C, 5-C), 142.89 (5′-C), 156.7 (C=N), 165.6 (CO), *m/z*: 878 ($M^{+\bullet}$, 2%), 849 ($M^{+\bullet}$ − CHO, 7), 835 [$M^{+\bullet}$ − (CH_2-CHO), 23], 396 ($M^{+\bullet}$ − $C_{25}H_{25}NO_4S$, 100), 381 ($M^{+\bullet}$ − $C_{26}H_{27}$-NO_4S, 40); Found: 878.2184, Calc. for $C_{46}H_{42}N_2O_{12}S_2$: 878.2179.

5-{5-[3,6,9,12-Tetraoxa-18-azabicyclo[12.3.1]octadeca-1(18),14,16-trien-16-yl]-2-thienyl}isophthalic acid 17a. To a suspension of the diester **15a** (105 mg, 0.2 mmol) in anhydrous THF (15 cm^3), $KOSiMe_3$ (64.2 mg, 0.5 mmol, 2.5 equiv.) was added. The mixture was refluxed overnight, then filtered on a glass filter, washed with ether, dissolved in water (10 cm^3) and acidified with an aqueous solution of hydrogen chloride (1 M) until pH 4 was reacted. The obtained yellow precipitate was filtered on a glass filter and washed with water. After drying in a dessicator in the presence of P_2O_5, a yellow powder was obtained (70 mg, 70%); mp 157–160 °C ; ν_{max}(KBr)/cm^{-1} 3000 (OH), 2900 (CH_2, CH), 1711 (CO), 1600–1440 (pyridine), 1100 (C-O); δ_H(DMSO-d_6) 3.37 (4H, s, CH_2), 3.46–3.56 (4H, m, CH_2), 3.67–3.93 (4H, m, CH_2), 4.73 (4H, s, CH_2), 7.66 (2H, s, 7′-, 11′-H), 7.86 (1H, d, *J* 3.8, 3′-H), 7.9 (1H, d, *J* 3.8, 4′-H), 8.41 (1H, s, 2-H), 8.42 (2H, s, 4-, 6-H); δ_C(DMSO-d_6) 69.5, 69.7, 69.8 (CH_2), 72.91 (CH_2), 117.22 (Pyr-CH), 126.6, 127.93 (3′-, 4′-C), 129.1 (2-C), 129.4 (4-, 6-C), 132.57 (1-, 3-C), 133.85 (2′-C), 140.67, 140.7 (Pyr-C), 142.56 (5′-C), 158.87 (C=N), 166.2 (CO), *m/z*: 499 ($M^{+\bullet}$, 5%), 470 ($M^{+\bullet}$ − CHO, 15), 456 [$M^{+\bullet}$ − $(CH_2)_2O$, 24], 410 [$M^{+\bullet}$ − $[(CH_2)_2O]_2H$, 28], 396 [$M^{+\bullet}$ − $O[(CH_2)_2O]_2$, 20], 382 [$M^{+\bullet}$ − $[(CH_2)_2O]_2CHO$, 50], 368 [$M^{+\bullet}$ − $[(CH_2)_2O]_2CH_2CHO$, 100], 353 [$M^{+\bullet}$ − $(CH_2CH_2O)_3$-CH_2), 96]; Found: 499.1288, Calc. for $C_{25}H_{25}NO_8S$: 499.1300.

Caesium salts. 1a,**b** and **2** were prepared from compounds **15a,b** and **16** according to the procedure described by Minta and Tsien.[21] A solution of ester (2.3 × 10^{-5} mol) and anhydrous caesium hydroxide (2.3 × 10^{-4} mol, 10 equiv.) in methanol (3 cm^3) was refluxed overnight. After evaporation of the methanol, the product was dissolved in water (100 cm^3) and used as such for the fluorescence measurements.

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