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# Fluorescent sodium ion indicators based on the 1,7-diaza-15-crown-5 system

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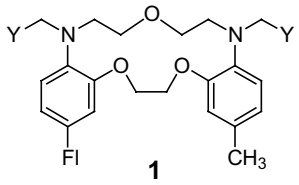
**Abstract**—A series of novel sodium ion-sensitive fluorescent reagents suitable for biological applications is described. The chelator nitrogen atom substituents affect the selectivity and affinity of cation binding, while the nature of the fluorophore determines the type of fluorescent response to metal ion chelation.


Metal ions play critical roles in a diverse array of biological systems. Cells utilize metal ions for a wide variety of functions, such as regulating enzyme activity and cellular signaling. Consequently, the accurate measurement of cation concentrations in both cellular compartments and in extracellular spaces is essential for molecular biological studies and for clinical diagnostics. Among numerous quantification methods, those based on fluorescence enjoy the benefits of high sensitivity and convenience of dynamic studies as well as the possibility of imaging applications.[1] The sensing element in these fluorescence methods is a fluoroionophore,[2] which is a combination of a chelator (common sodium chelators include crown ethers,[3] cyptands,[4] and calixarenes[5]) and a fluorescent moiety, capable of producing a fluorescence change upon interaction with the cation. Despite intensive research efforts[1,6] only a few sodium fluoroionophores have enjoyed research practice, including two commercially available reagents: the UV-excitable SBFI[7] and Sodium Green.[8] Sodium Green is excited by visible light, but exhibits only a modest fluorescence change in response to sodium binding. Because each of these probes contains two fluorophores, they are bulky, resulting in difficulties loading into live cells even as cell permeable AM ester forms.[9]

Here we report the synthesis and spectroscopic properties of a new family of sodium-specific fluoroionophores **1a**–**h**, based on the 1,7-diaza-4,10,13-trioxa-15-crown-5 ring system. Compounds **1** have a fluorophore attached to the aromatic ring annelated to a bis-aza crown ether moiety, in contrast to pendant substituents on the aza-crown nitrogen, which is the motif found in SBFI or Sodium Green. Similar benzannelation structures were used in previously reported $^{19}F$ NMR[10] probes and cyptand $K^+$ sensors.[11] Another key structural feature is the sidearm substituent (Y) on each anilino nitrogen atom, which can be changed to modulate metal ion selectivity.




**1**

Fl = fluorophore
Y = sidearm substituent

The synthesis of the compounds **1** began with diamine **2**[11] and included macrocyclization by bis-acylation, reduction of the amide groups, and introduction of the sidearm substituents by alkylation with methyl bromoacetate or bromoacetonitrile. Vilsmeier formylation of the resulting dibenzo-crowns **3a**,**b** yielded the aldehydes **4a**,**b** (Scheme 1). In **4b** the cyano groups were converted into amide sidearms to give **4c**.

Reactivity of the formyl group in the key intermediates **4** was used to build fluorophores to produce a series of

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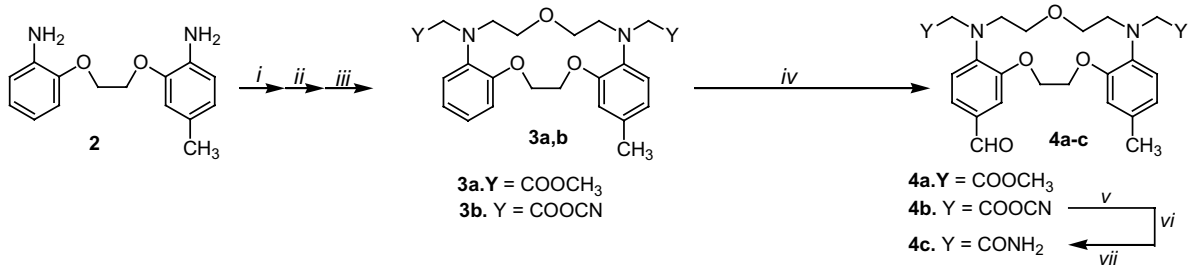
probes **5**–**9**.[12] Hydrolysis of the methoxycarbonyl groups in **5a** gave **5d** (Scheme 2).

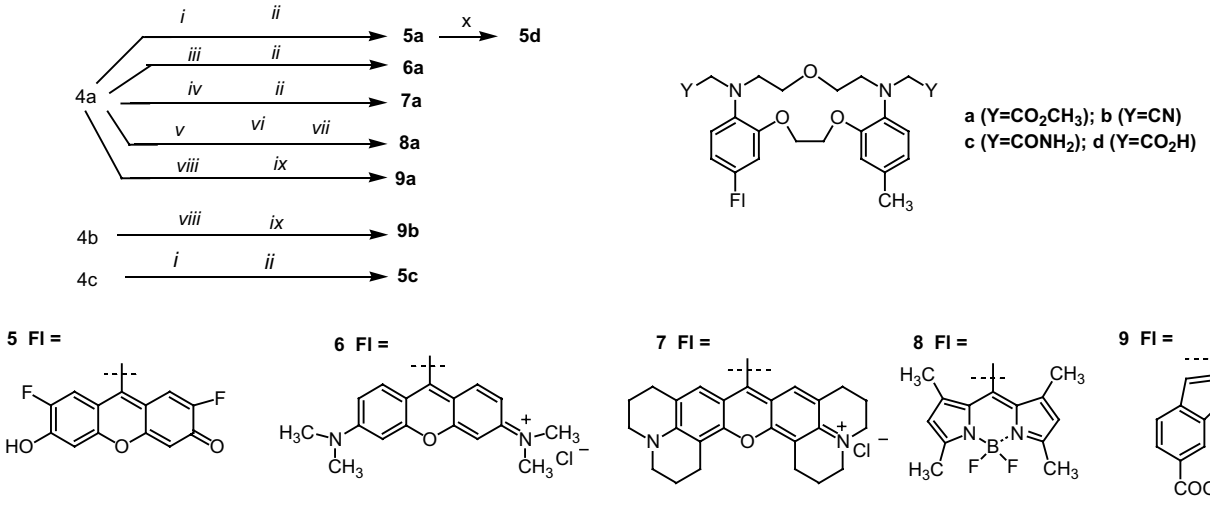
Compound **12** having a furan ring annelated to the crown ether system (Scheme 3) was synthesized from the key α-hydroxy aldehyde **11**, similar to preparation of the Fura-2 $Ca^{2+}$ indicator.[12] Aldehyde **11** was prepared from the benzyloxy diamine **10** similar to the synthesis of compounds **4a**,**b**. A protective benzyl group was cleaved by catalytic hydrogenation.

The study of sodium ion binding by fluorescence spectroscopy in a series of pH 7.0 buffer solutions (50 mM MOPS, indicator concentration 1–10 μM, containing sodium ion in the 0–1000 mM range) revealed that the response to the sodium ion depends in part upon the nature of the fluorophore. Compounds **5a**–**8a** behave like typical Photoinduced Electron Transfer (PET) sensors with large fluorescent increases and no significant wavelength shift (on–off switches).[13] On the other hand, compounds **9a**,**b** and **12** exhibit the features of Photoinduced Charge Transfer (PCT) sensors[14] with ratiometric responses to sodium ion binding in the excitation (compound **12**) or emission (compounds **9a**,**b**) spectra (Fig. 1).

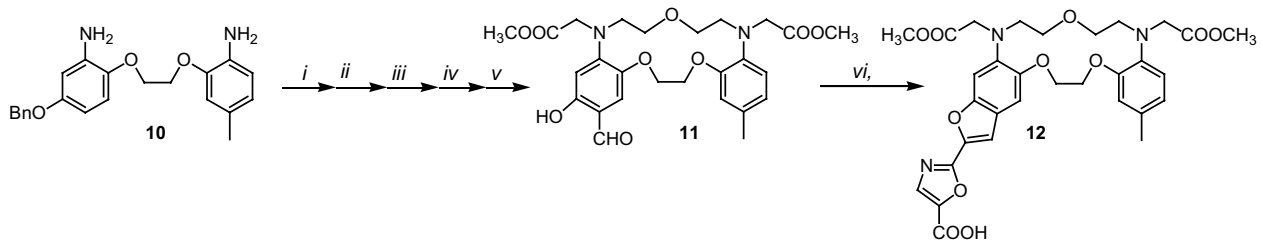
The data summarized in Table 1 demonstrate that when a donor fluorophore is attached to the ion binding crown ether, the $Na^+$ binding affinity is consistent (e.g., **5a**, **8a**, **9a**, **12a**). However, when the attached fluorophore is an acceptor (**6a**, **7a**), the $Na^+$ affinity drops considerably, a natural consequence of the reduced participation of the dye-attached aniline nitrogen lone pair in ion binding. The binding data also show that sidearm substituents affect both the affinity and selectivity of cation binding. Compounds having methoxycarbonyl groups in the sidearm are not sensitive toward divalent cations ($Ca^{2+}$, $Zn^{2+}$), while compound **5d** with *N*-acetic acid residues shows $Zn^{2+}$ sensitivity in addition to $Na^+$ sensitivity. However, the sensitivity of **5d** toward sodium ion and its relatively low affinity for $Zn^{2+}$ make it an unlikely candidate for physiological $Zn^{2+}$ measurements in light of existing fluorescent probes specifically designed for that purpose (see[15] and references cited therein). The sodium ion affinity drops by an order of magnitude (relative to **9a**) upon the introduction of the electron acceptor cyano group in the sidearms in compound **9b**. An even more significant decrease in affinity and selectivity is observed in bis-amide derivative **5c**.




**Scheme 1.** Reagents and conditions: (i) $(CH_2COCl)_2/Et_3N$, THF; (ii) $BH_3$/THF; (iii) $BrCH_2Y$/NaI, DIEA, $CH_3CN$; (iv) $POCl_3$/DMF; (v) $HOCH_2CH_2OH$, TsOH; (vi) $KOH/H_2O_2$; (vii) $HCl/H_2O$.




**Scheme 2.** Reagents and conditions: (i) 4-fluororesorcinol, $CH_3OSO_3H$; (ii) chloranil, MeOH/$CHCl_3$; (iii) 3-dimethylaminophenol, $CH_3CH_2COOH$/TsOH; (iv) hydroxujulolidine, $CH_3CH_2COOH$/TsOH; (v) 2,4-dimethyl pyrrole/TFA; (vi) DDQ; (vii) $(CH_2CH_2)_2OBF_3$; (viii) 4-methoxycarbonyl-2-nitro-phosphorane/$K_2CO_3$/DMF; (ix) $P(OEt)_3$; (x) $KOH/H_2O$, then HCl.




**Scheme 3.** Reagents and conditions: (i) $O(CH_2COCl)_2$/Et3N, THF; (ii) $BH_3$/THF; (iii) $BrCH_2Y$/NaI, DIEA, $CH_3CN$; (iv) $POCl_3$/DMF; (v) $H_2$, Pd/C, AcOH; (vi) 4-carboxy-2-chloromethyloxazole/$K_2CO_3$, DMF.

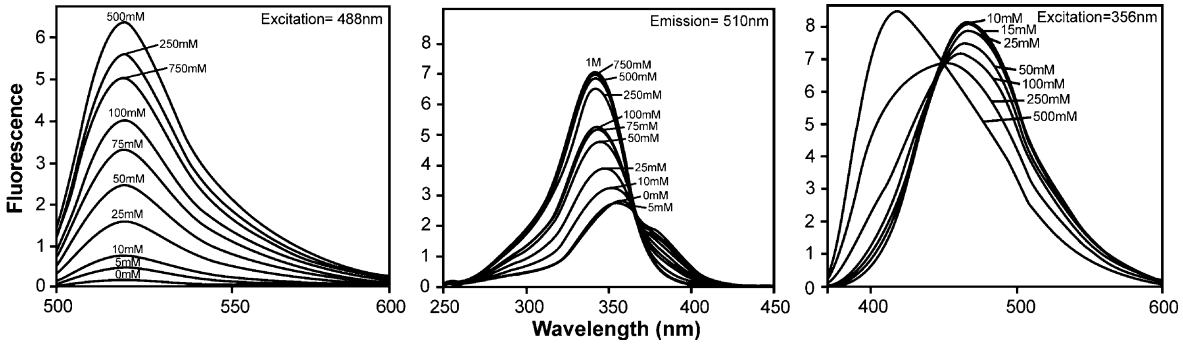



**Figure 1.** Fluorescence responses to sodium ion binding in 50 mM MOPS (pH 7.0 adjusted with tetramethylammonium hydroxide). (a) Emission spectra of **5a**; (b) excitation spectra of **12**; (c) emission spectra of **9b**.

**Table 1.** Cation binding data

| Compound | Fluorescence (nm) | | $Na^+$ binding $K_d$, (mM) | S[a] | Type[b] | Other $K_d$ |
|---|---|---|---|---|---|---|
| | Excit. | Emiss. | | | | |
| **5a** | 488 | 516 | 60[c] | 49 | Sw | $K^+$ (205 mM) |
| **6a** | 551 | 576 | 219 | 18 | Sw | $K^+$ (460 mM) |
| **7a** | 579 | 602 | 103[d] | 15 | Sw | $K^+$ (204 mM) |
| **8a** | 488 | 515 | 42[e] | 43 | Sw | — |
| **9a** | 340 | 495–441 | 32[e] | 7 | EmR | $K^+$ (115 mM) |
| **5d** | 488 | 516 | 36 | 4 | Sw | $Zn^{2+}$ (1 mM) |
| **9b** | 356 | 470–415 | 220 | 4 | EmR | |
| **5c** | 488 | 518 | 1500 | 5 | Sw | $Zn^{2+}$ (600 mM)[f] |
| **12** | 380–337 | 510 | 52 | 3 | ExR | $K^+$ (329 mM) |

[a] S = S[bound]/S[free] fluorescent response to a cation binding.
[b] Response type: SW—on–off switch, EmR—emission ratiometric, ExR—excitation ratiometric.
[c] $K_d$ ($Na^+$) = 87 mM in 100 mM [$K^+$].
[d] $K_d$ ($Na^+$) = 104 mM in 100 mM [$K^+$].
[e] In 50% DMSO.
[f] No detectable $Ca^{2+}$ sensitivity.

PET sensors **5a**–**8a** have the affinity and response slopes within the requirement range for extracellular $Na^+$ sensors.[3,7] Ratiometric indicators **9a** and **12** show a smaller intensity response; however, because of the measurement mode they have an advantage of easier calibration.[14] The binding data in Table 1 indicate that compounds **5a**–**9a**, **12** exhibit apparent selectivity of sodium over potassium in the range 2.0–6.3. This level of selectivity is not optimal, but is typical of crown ether chelators.[1,6] Nevertheless, the sodium ion $K_d$ for indicators **5a** and **7a** was little perturbed by performing the titrations in the presence of 100 mM potassium ion (Table 1 notes), indicating functional selectivity for sodium over potassium in physiologically relevant concentration ranges. Further increases in selectivity may be achievable by using cryptand as the chelating moiety.[4]

Loading studies in live NIH 3T3 cells were performed using compound **6a**. The net positive charge of **6a** permits passive loading using 1 μM solutions of probe in the extracellular medium. After incubation at 37 °C for up to 30 min, followed by washing with dye-free medium, fluorescence microscopy clearly showed intracellular loading with localization in the mitochondria. Cells loaded with **6a** were also studied by flow cytometry, using 488 nm excitation. Sorting experiments showed that in multiple trials the probe loaded into $\leqslant$30% of the cell population. The labeled cells responded to

$\leqslant$150 mM extracellular sodium ion, transported into cells through ligand-gated cation pores by addition of 20 μM monensin, by fluorescence increases of 2.0–4.0 fold.

In conclusion, a novel series of fluorescent sodium ion indicators has been synthesized and evaluated for ion binding in aqueous solutions. The synthetic methods allowed for the preparation of a variety of fluoroionophores to cover a wide spectral range and different types of fluorescent responses to ion binding.

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