

Fluorescent metal ion indicators based on benzoannelated crown systems: a green fluorescent indicator for intracellular sodium ions

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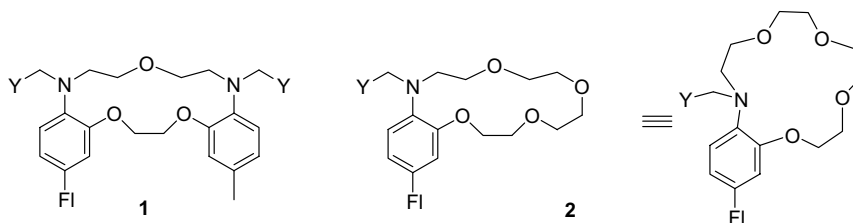
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Abstract—The synthesis and metal binding properties of cation-sensitive fluorescent indicators intended for biological applications are described. The increase of the crown ether ring size enhances the affinity for larger cations, but weakens the fluorescent response and selectivity. A compound having a 15-crown-5 chelator directly attached to a 2,7-difluoroxanthone fluorophore loads into live cells and responds to sodium ion concentration changes with large fluorescence increases in the visible wavelength range.
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Ion channels controlling the flow of monovalent sodium and potassium cations form the signal transduction networks of intra- and intercellular communication systems.¹ The study of ion concentrations, gradients, and currents is integral to most areas of cellular research,² drug screening,³ and clinical diagnostics.⁴ Fluorescence-based reporting enjoys a centerpiece role in biological detection techniques by providing quantitative, temporal, and spatial information.⁵ Several fluorescent reagents have been developed for the quantification and imaging of biologically important metal ions, such as magnesium,⁶ zinc,⁷ and especially calcium.⁸ However, only a few fluorescent reagents for the detection of sodium and potassium ions inside live cells have been reported and only two of them, SBFI and Sodium Green, are available commercially.^{9,10} The disadvantage of SBFI is that the excitation wavelength is in the UV

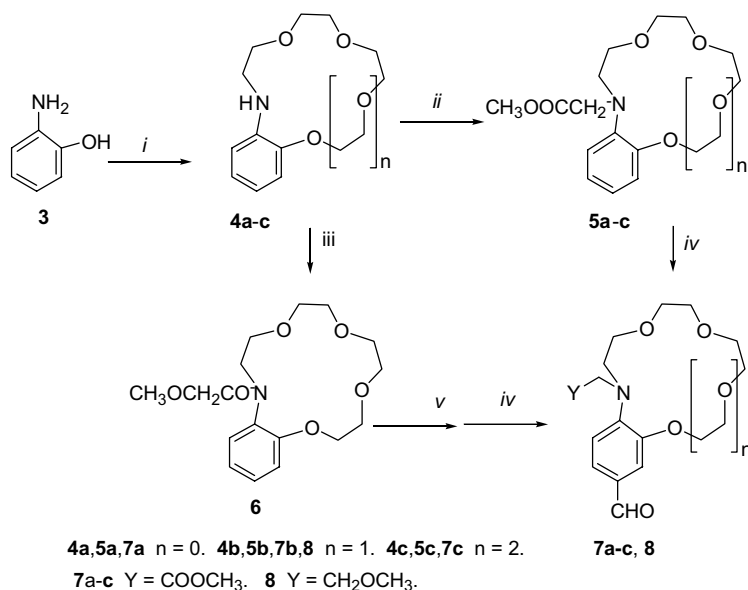
range, while visible wavelength-excited Sodium Green displays only a modest fluorescence increase in response to Na⁺ binding. Also, because both molecules include two fluorophores, they are bulky and exhibit difficulties loading into live cells. Recently we reported the synthesis of new fluorescent Na⁺ indicators of the general formula **1** based on a benzoannelated crown ether system.¹¹ Sodium indicators of this series respond well to Na⁺ binding in vitro; however only the tetramethylrhodamine derivative **1** (Y = COOCH₃; Fl = TMR), aka CoroNa Red is capable of loading into live cells where it compartmentalizes in mitochondria.^{11,12} We hypothesized that substitution of the second aniline fragment, which does not participate in fluorescent reporting, by an ethyleneoxy bridge (i.e., transforming compounds **1** into a new series **2**) could decrease the molecules' bulk and improve loading properties and binding affinities.



Fl = fluorophore
Y = sidearm substituent

Keywords: Fluorescence; Sodium indicator; Fluoroionophore.

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Scheme 1. Reagents and conditions: (i) $\text{TsO}(\text{CH}_2\text{CH}_2\text{O})_n(\text{CH}_2\text{CH}_2\text{O})_n\text{Ts}$, CsF, MeCN (25–62%); (ii) $\text{BrCH}_2\text{COOCH}_3$, DIEA, MeCN (52–65%); (iii) $\text{CH}_3\text{OCH}_2\text{COCl}$, DIEA, CHCl_3 (55%); (iv) POCl_3 , DMF (55–78%); (v) BH_3 , THF (77%).

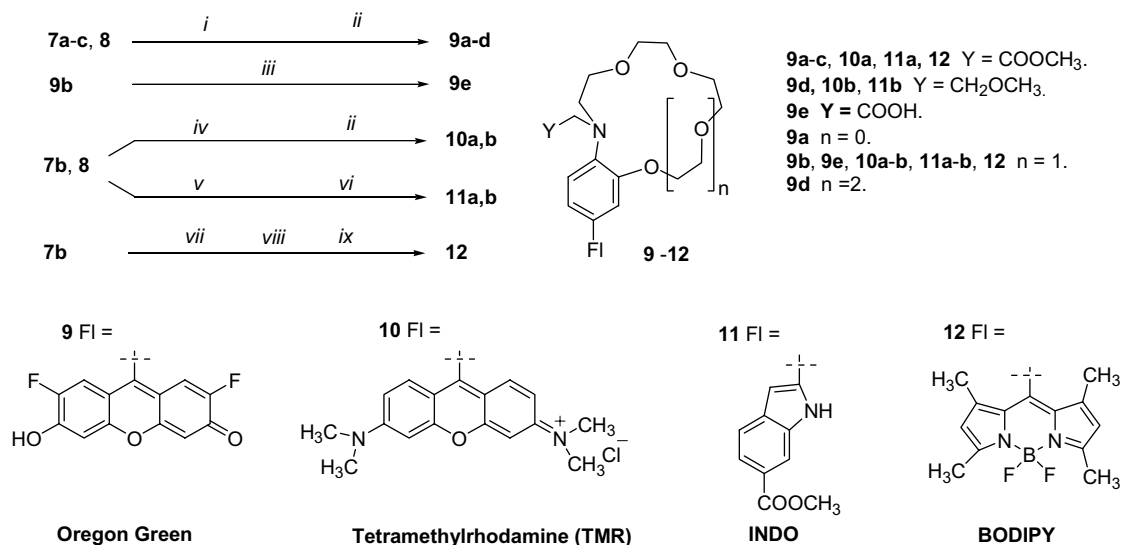
We also wanted to investigate the effect of the size of the crown ring on binding of different monovalent cations.

The synthesis of compounds **2** started with N,O-dialkylation of 2-aminophenol **3** with polyethyleneglycol ditosylates (**Scheme 1**) in the presence of CsF.^{13,14} Compounds **4a–c** were converted into aldehydes **7a–c** by alkylation with methyl bromoacetate, followed by Vilsmeier formylation of the intermediates **5a–c**. As a result, we synthesized a series of key benzaldehydes containing the 12-crown-4 (**7a**), 15-crown-5 (**7b**), or 18-crown-6 (**7c**) chelators. Aldehyde **8**, having a 15-crown-5 moiety and methoxyethyl sidearm ($Y = \text{CH}_2\text{OCH}_3$), was prepared from compound **5b** in a three step sequence, which in-

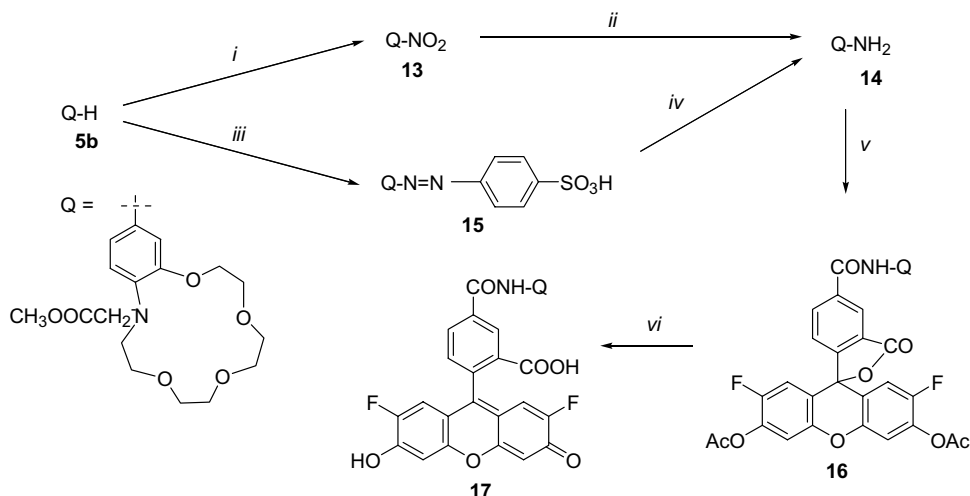
cluded acylation with methoxyacetyl chloride, followed by borane reduction of the resulting amide **6** and introduction of the formyl group.

The aldehyde group in compounds **7, 8** was transformed into several fluorophores by the procedures developed for the synthesis of fluorescent Ca^{2+} indicators¹⁵ and also used earlier in preparation of Na^+ indicators **1**¹¹ (**Scheme 2**).

Another 15-crown-5 compound **17** having an amide linkage between the sensor and crown moiety, similar to that of the calcium indicator Oregon Green 488 BAPTA 488,¹⁵ was prepared starting with acylation of amino



Scheme 2. Reagents and conditions: (i) 4-fluoresoresorcinol, $\text{CH}_3\text{SO}_3\text{H}$ (75–90%); (ii) chloranil, MeOH/ CHCl_3 (25–39%); (iii) $\text{KOH}/\text{H}_2\text{O}$ (59%); (iv) 3-dimethylaminophenol, EtCOOH/TsOH (60%); (v) 4-methoxycarbonyl-2-nitrobenzylphosphorane/ K_2CO_3 , DMF (62–71%); (vi) $\text{P}(\text{OEt})_3$ (64–75%); (vii) 2,4-dimethylpyrrole/TFA; (viii) DDQ; (ix) Et_2OBF_3 (30% in three steps).



Scheme 3. Reagents and condition: (i) $\text{HNO}_3/\text{Ac}_2\text{O}$ (57%); (ii) H_2/Pd (62%); (iii) sulfanilic acid/ NaNO_2 , AcOH (8%); (iv) $\text{Na}_2\text{S}_2\text{O}_4$ (85%); (v) 3,6-diacetyl-5'-carboxy-2,7-difluorofluorescein/ $(\text{COCl})_2$, $\text{CH}_2\text{Cl}_2/\text{Et}_3\text{N}$ (63%); (vi) $\text{Et}_3\text{N}/\text{H}_2\text{O}$ (48%).

crown **14** with a protected Oregon Green acid chloride, followed by hydrolytic deprotection with aqueous Et_3N . The key aminocrown ether **14** was prepared by the reduction of either nitro derivative **13** or azo dye **15**; the former reaction produced a mixture which was easier to purify (Scheme 3).

The in vitro study of ion binding was conducted by fluorescence spectroscopy using a series of pH 7.0 buffer solutions containing 0–1000 mM concentration of monovalent Li^+ , Na^+ , K^+ , and Rb^+ cations in 50 mM MOPS at 20 °C (Fig. 1). Several conclusions can be drawn from the results, listed in Table 1.

- (a) Increase of the crown ring size from 15 to 18 (compound **9c** vs **9b**) increases binding affinity of the larger K^+ and Rb^+ cations; however the magnitude of the fluorescent response to binding drops significantly.

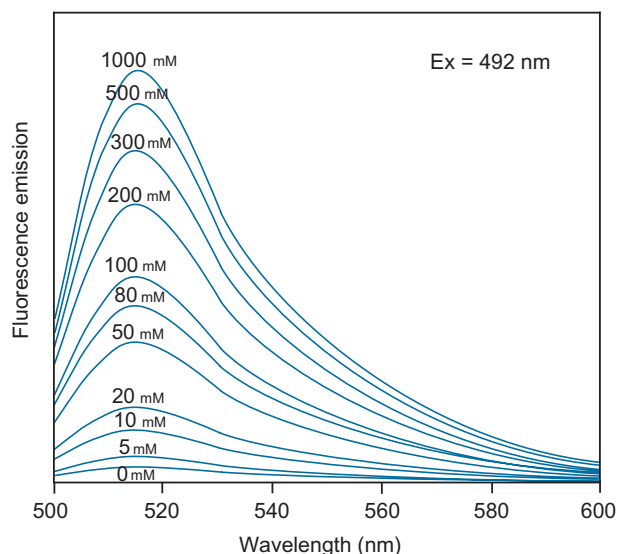


Figure 1. Fluorescence response of compound **9b** to increasing $[\text{Na}^+]$ in 50 mM MOPS (pH 7 adjusted with tetramethylammonium hydroxide).

cantly. Surprisingly, reduction of the crown size from 15 to 12 (compound **9a** vs **9b**) reduces both binding and fluorescence response to every tested cation, including the smaller Li^+ .

- (b) Sodium binding affinities of the 15-crown-5 compounds are similar for the compounds having neutral fluorophores (**9b**, **11a**, and **12**), while decreasing for the positively charged tetramethylrhodamine derivative **10a**. This effect can be attributed to the reduced electron density on the aniline nitrogen of the indicator group. The large fluorescence increase as a function of Na^+ binding to **9b** (aka CoroNa Green) is shown in Figure 1. Compounds **11a–b** having Indo-type fluorophores do not display a pronounced ratiometric response, as in the earlier series **1**,¹¹ but behave as on-off switches.
- (c) The change in sidearm substituent Y from COOCH_3 to CH_2OCH_3 (compounds **9b**, **10a**, and **11a** vs **9d**, **10b**, and **11b**) results in a small increase in binding affinity, possibly because of the replacement of an electron-withdrawing carbonyl group by methylene group; however the magnitude of the fluorescent response to ion binding decreases. A similar effect is observed for compound **9e**, which should be negatively charged at pH 7.
- (d) Selectivity of Na^+ versus K^+ binding is about 4 for the CoroNa Green (**9b**), and decreases to less than 2 for tetramethylrhodamine (**10b**) or BODIPY (**12**) derivatives. This is in line with other crown ether indicators, including our previous series **1**. Compound **9b** displays a binding K_d of about 100 mM when titrated in the presence of 100 mM KCl making it possible to use in physiological conditions. As for selectivity over Ca^{2+} binding, **9b** is impervious to this important divalent cation at physiologically relevant levels. A very small fluorescence increase ($S = 1.3$) is observed upon addition of Ca^{2+} , but not until the $[\text{Ca}^{2+}]$ reaches several hundred micromolar (K_d 470 μM).

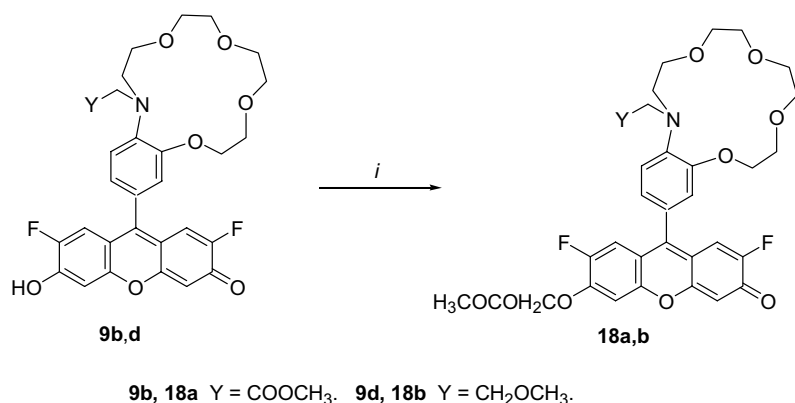
For in cyto studies Jurkat cells were loaded with 10 μM dye for 1 h at 37 °C. Cells were washed and resuspended

Table 1. Cation binding data

Compound	Fluorescence (nm)		Cation binding, K_d (mM)							
	Excit.	Emiss.	Li^+		Na^+		K^+		Rb^+	
			K_d	S^a	K_d	S	K_d	S	K_d	S
9a	493	517	438	2.8	226	19.7	978	9.7	376	2.9
9b	492	516	142	6.8	82	28.5	291	6.9	319	2.7
9c	492	516		^b	15	1.5	11	2.2	25	1.5
9d	494	516			78.8	5.9	269	2.8		
9e	491	518			20	3.6				
10a	546	576			163	41	254	10.6		
10b	546	576			150	12				
11a	351	444			89	25		^b		
11b	351	444			89	2				
12	498	510			100	7.3	170	2.9		
17	494	522	65	3.1	59	3.9	144	2.7	157	1.9

^a $S = I[\text{bound}]/I[\text{free}]$ fluorescent response to a cation binding (I = fluorescence intensity).

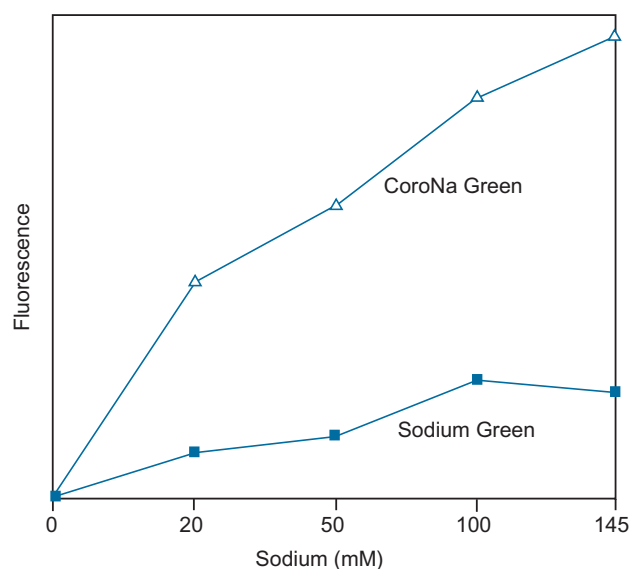
^b Low sensitivity (outside the error margin).

**Scheme 4.** Reagents and condition: (i) BrCH₂COCH₃/DIEA, DMF (72–86%).

in sodium gluconate in the presence of 20 μM monensin for 30 min. Fluorescence was measured by flow cytometry. The initial results showed only poor loading with every 15-crown-5 derivative **9**–**12**. The cell loading increased dramatically when compound **9b** was converted into acetoxymethyl ether **18a** (Scheme 4).

In side-by-side experiments compound **18a** loads into 20% of a cell population compared to 6% for **9b** (Fig. 2) and 3% for Sodium Green tetraacetate. Also, **18a** displayed a larger fluorescence response to sodium influx, compared with Sodium Green tetraacetate (Fig. 2), that is, $\leq 4\times$ fluorescence increases were regularly observed.¹⁶ Surprisingly, the structurally similar compound **18b** loaded into live cells poorly. A report appeared recently describing a somewhat similar green fluorescent sodium ion indicator.¹⁷ However, the binding affinity and fluorescence increases observed upon Na^+ binding were lower than those obtained for **9b**; additionally no live cell data were presented.

In conclusion, a novel series of fluorescent sodium ion indicators has been synthesized and evaluated for ion binding in aqueous solutions and in live cells. The 15-crown-5 derivative **18a** (aka CoroNa Green AM¹⁶) performed well as a fluorescent indicator for sodium ions in cyto.

**Figure 2.** Fluorescence intensity of live Jurkat cells loaded with 10 μM **18a** (CoroNa Green AM) or 10 μM Sodium Green tetraacetate in the presence of 20 μM monensin. Fluorescence was measured by flow cytometry.

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