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

Vladimir V. Martin, Anca Rothe and Kyle R. Gee*

Molecular Probes Invitrogen Detection Technologies, 29851 Willow Creek Road, Eugene, OR 97402, USA

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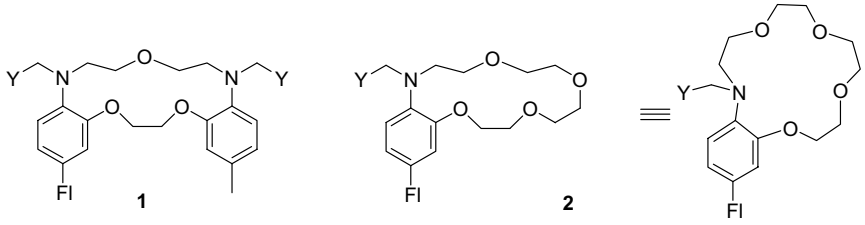
Abstract—The synthesis and metal binding properties of cation-sensitive fluorescent indicators intended for biological applications are described. The increase of the crown ether ring size enhances the affinity for larger cations, but weakens the fluorescent response and selectivity. A compound having a 15-crown-5 chelator directly attached to a 2,7-difluoroxanthenone fluorophore loads into live cells and responds to sodium ion concentration changes with large fluorescence increases in the visible wavelength range.

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 = $COOCH_3$; 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.
* Corresponding author. Tel.: +1 541 335 0366; fax: +1 541 344 6504; e-mail: kyle.gee@probes.com

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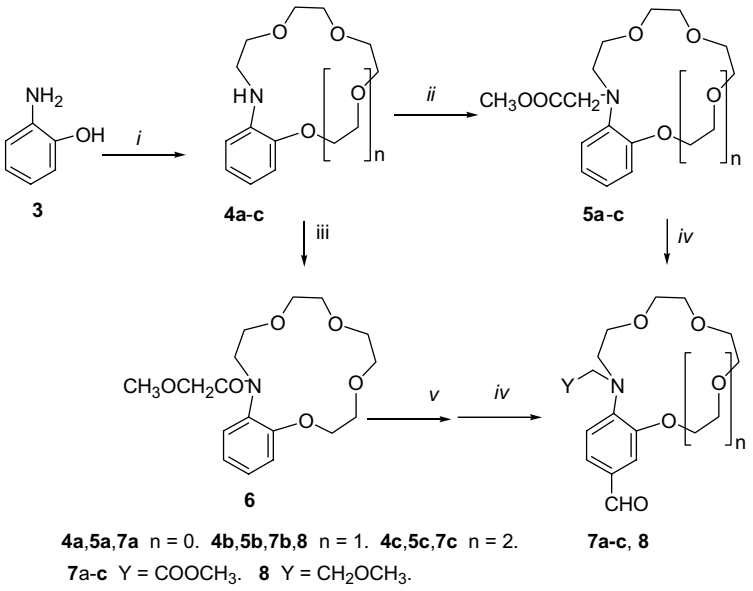


Scheme 1. Reagents and conditions: (i) $TsO(CH_2CH_2O)_n(CH_2CH_2O)_3Ts$, CsF, MeCN (25–62%); (ii) $BrCH_2COOCH_3$, DIEA, MeCN (52–65%); (iii) CH_3OCH_2COCl, DIEA, $CHCl_3$ (55%); (iv) $POCl_3$, DMF (55–78%); (v) BH_3, THF (77%).

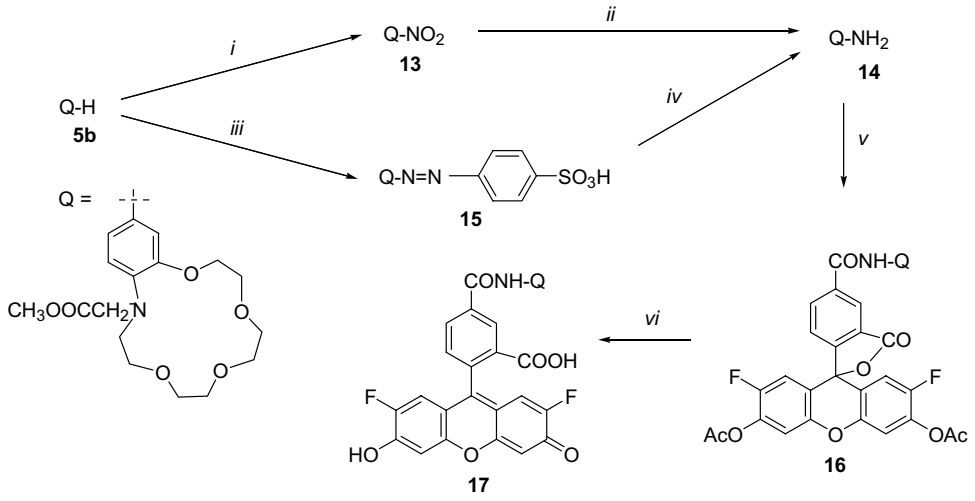
We also wanted to investigate the effect of the size of the crown ring on binding of different monovalent cations.

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 ($Y = CH_2OCH_3$), was prepared from compound **5b** in a three step sequence, which included 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+} indicators[15] 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-fluororesorcinol, CH_3SO_3H (75–90%); (ii) chloranil, MeOH/$CHCl_3$ (25–39%); (iii) KOH/H_2O (59%); (iv) 3-dimethylaminophenol, EtCOOH/TsOH (60%); (v) 4-methoxycarbonyl-2-nitrobenzylphosphorane/K_2CO_3, DMF (62–71%); (vi) $P(OEt)_3$ (64–75%); (vii) 2,4-dimethylpyrrole/TFA; (viii) DDQ; (ix) Et_2OBF_3 (30% in three steps).



Scheme 3. Reagents and condition: (i) HNO_3/Ac_2O (57%); (ii) H_2/Pd (62%); (iii) sulfanilic acid/$NaNO_2$, AcOH (8%); (iv) $Na_2S_2O_4$ (85%); (v) 3,6-diacetyl-5′-carboxy-2,7-difluorofluorescein/$(COCl)_2$, CH_2Cl_2/Et_3N (63%); (vi) Et_3N/H_2O (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. 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^+.

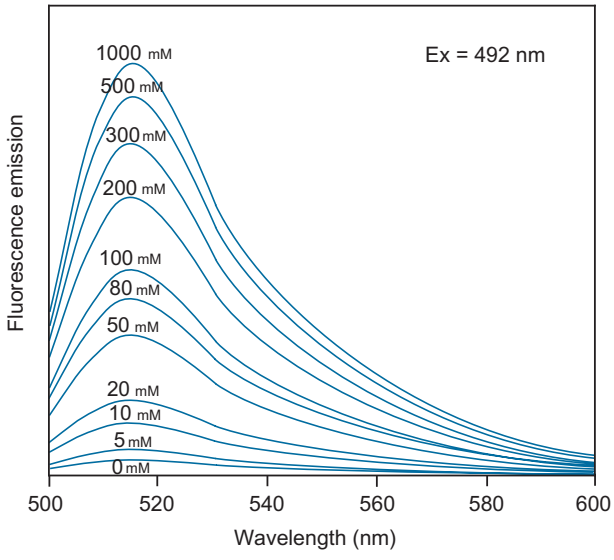


Figure 1. Fluorescence response of compound **9b** to increasing $[Na^+]$ in 50 mM MOPS (pH 7 adjusted with tetramethylammonium hydroxide).

(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.

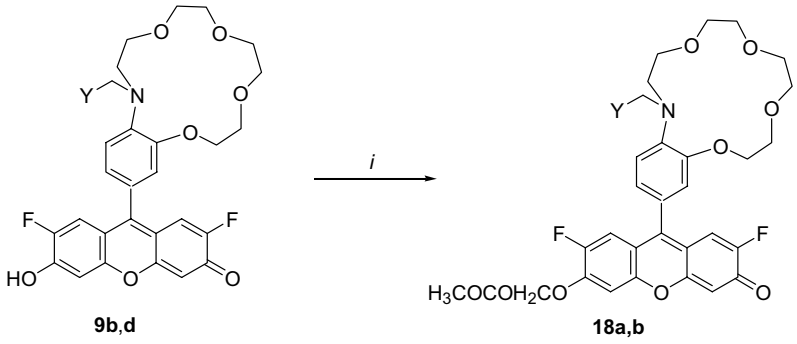
(d) Selectivity of Na^+ versus K^+ binding is about 4 for the CoroNa Green (**9b**), and decreases to less than 2 for tetramethylrhodamine (**10b**) or BODIPY (**12**) derivatives. This is in line with other crown ether indicators, including our previous series **1**. Compound **9b** displays a binding 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 $[Ca^{2+}]$ 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] $S = I$[bound]/I[free] fluorescent response to a cation binding (I = fluorescence intensity).
[b] Low sensitivity (outside the error margin).



9b, 18a Y = $COOCH_3$. **9d, 18b** Y = CH_2OCH_3.

Scheme 4. Reagents and condition: (i) $BrCH_2OCOH_3$/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, $\leqslant 4x$ 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 AM[16]) performed well as a fluorescent indicator for sodium ions in cyto.

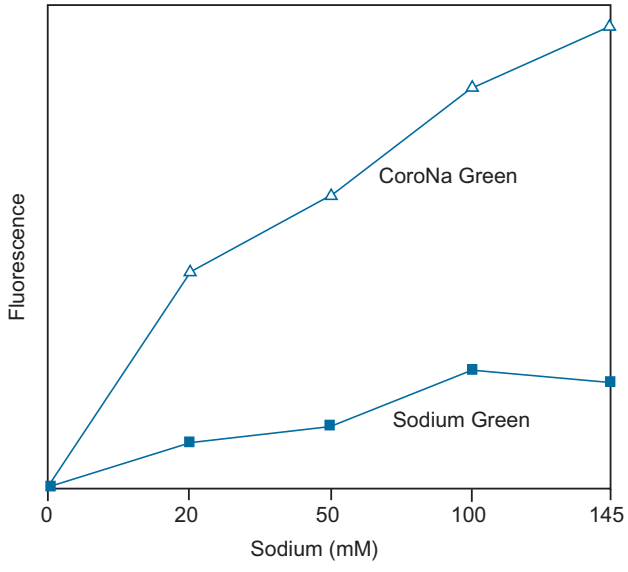


Figure 2. Fluorescence intensity of live Jurkat cells loaded with 10 μM **18a** (CoroNa Green AM) or 10 μM Sodium Green tetraacetate in the presence of 20 μM monensin. Fluorescence was measured by flow cytometry.

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