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Chemosensors for the Marine Toxin Saxitoxin

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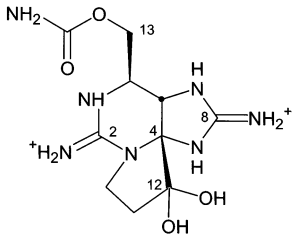
Abstract: Eleven anthracylmethyl crown ethers have been synthesized and evaluated as fluorescence sensors for the marine toxin saxitoxin. Fluorescence enhancement data are consistent with a 1:1 binding complex for all crowns. The binding constants are in the range of 10^4 M^{-1} in ammonium phosphate buffer (pH 7.1) in 80% ethanol solvent. Selectivity for sensing saxitoxin versus several organic analytes has been demonstrated for the first time. Possible modes of binding are presented, and relevance to saxitoxin monitoring programs are discussed.

Introduction

Harmful algal blooms (red tides) produce a wide variety of secondary metabolites,[1] but saxitoxin is virtually alone among them in being capable of causing human mortality by consumption of tainted shellfish.[2] The most severe symptom of saxitoxin consumption, also known as paralytic shellfish poisoning,[3] is respiratory paralysis.[4,5] Saxitoxin and its congeners are known as paralytic shellfish poisons (PSPs).[6] The tragedy of human mortality events due to PSP consumption is that recovery is virtually guaranteed if the disease is properly diagnosed and treated by mechanical ventilation for 24 h.[7] Currently, governments of many countries monitor shellfish beds for the presence of saxitoxin by using one of several tests, the most reliable of which is mouse bioassay.[8] Unfortunately, not all countries can afford such monitoring facilities, and as recently as 1987, a harmful algal bloom (HAB) on the Pacific coast of Guatemala resulted in numerous deaths, including 50% of the children who were reported ill.[9] Despite massive monitoring efforts by the respective governments, disease outbreaks in the United States and Portugal have been documented as recently as the past decade.[5] In the spring of 2002, the lay press reported a number of saxitoxin poisonings from puffer fish caught near Cape Canaveral, Florida. This is the first time this toxin has been reported in the Atlantic, south of New England!



saxitoxin, STX

Saxitoxin detection programs may use a number of techniques,[10] but mouse bioassay is by far the most common, and is the current benchmark technique.[8] New approaches include insect bioassay,[11] tissue biosensors,[12] molecular pharmacology,[13]

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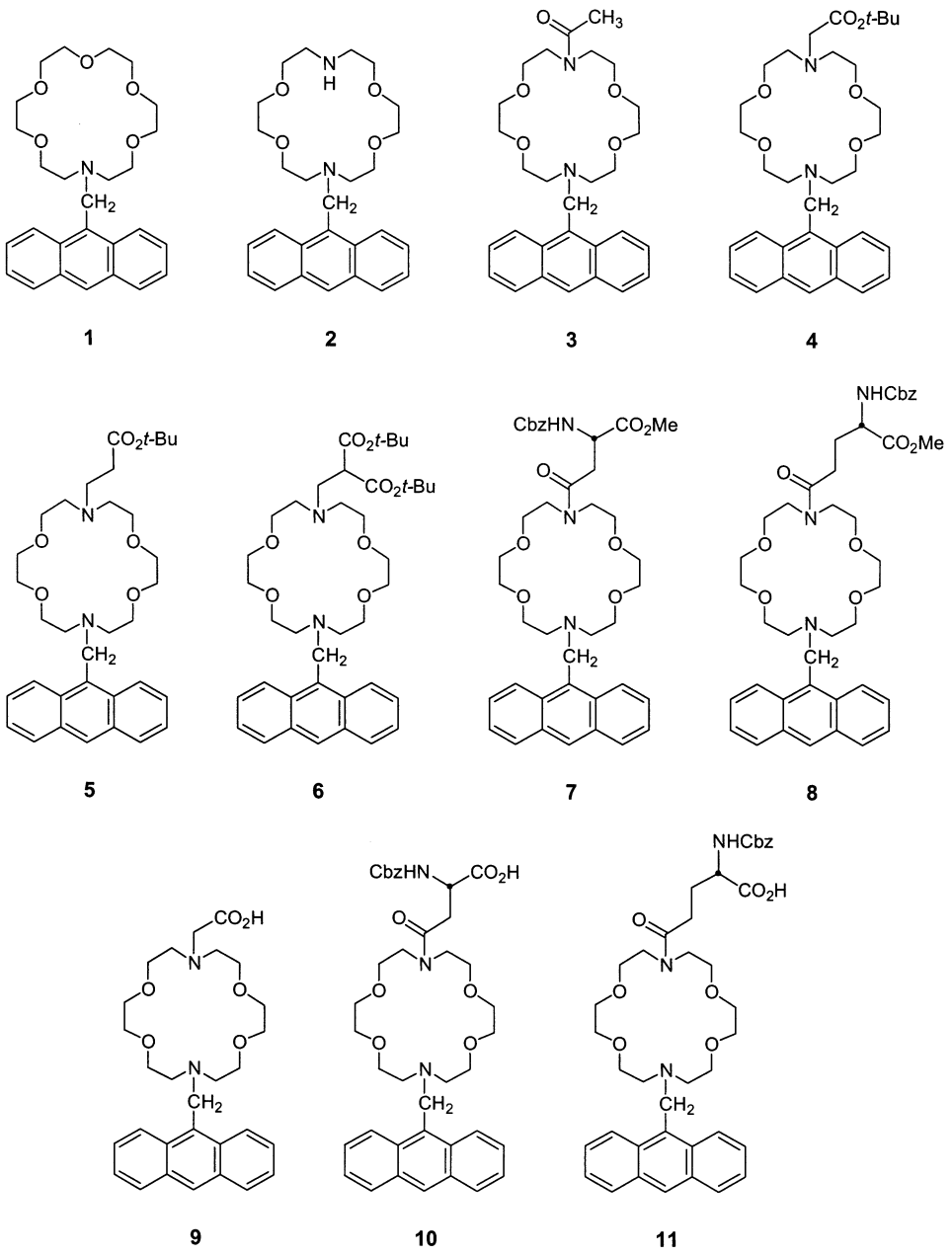

Figure 1. Crown sensors used in this study.

neurophysiology,[14] whole-cell bioassay,[15] HPLC with postcolumn oxidation of the C4−C12 bond and aromatization,[16] and HPLC-linked mass spectroscopy.[17] For both economic and ethical reasons, an alternative to mouse bioassay is desired.[18] Herein, we report our efforts to date in the development of a chemosensor for saxitoxin using fluorescence sensing,[19] with the ultimate aim of incorporating the sensor into an optical fiber.[20]

Synthesis. The crown ether sensors used in this study are shown in Figure 1. Crown **1** was reported in 1985[21] and was the better of two STX sensors evaluated in preliminary studies.[19]

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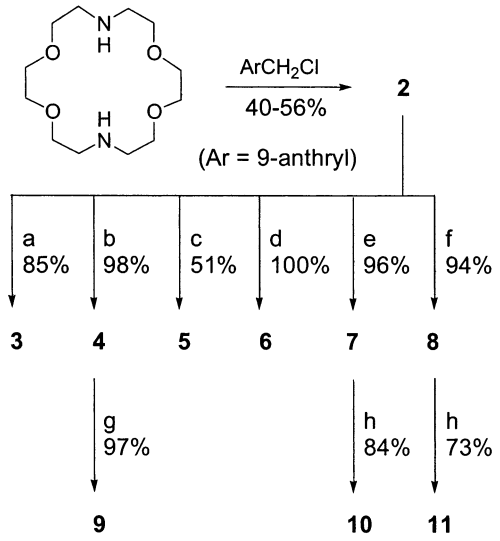
Scheme 1 [a]



[a] Reagents and conditions: (a) HOAc, DCC, CH_2Cl_2, RT; (b) Et_3N, $BrCH_2CO_2t$-Bu, toluene, reflux; (c) $CH_2{=}CHCO_2t$-Bu, *i*-PrOH, 85°; (d) $CH_2{=}C(CO_2t\text{-Bu})_2$, *i*-PrOH, rt; (e) Cbz-Asp-αOMe, EDCI, CH_2Cl_2, rt; (f) Cbz-Glu-αOMe, EDCI, CH_2Cl_2, RT; (g) 96% HCO_2H, 75°; (h) K_2CO_3, $MeOH/H_2O$, rt.

Crowns **2**−**11** were prepared to compare structural effects on sensitivity and, in some cases, as derivatives suitable for incorporation into peptidic libraries. Crown **1** was prepared by the method of de Silva;[21] the others were prepared by routine transformations as outlined in Scheme 1.

Fluorescence Titrations. Our initial work[19] began with crown ether **1**.[21] This sensor responds to alkali cations in (strictly anhydrous!) methanol solution. Aminomethylanthracenes fluoresce poorly as free bases because the nitrogen lone pair quenches the excited state by photoinduced electron transfer (PET).[22] The sensing mechanism invoked for sensors such as **1** is protonation, hydrogen bonding, or coordination to the nitrogen, thereby inhibiting electron-transfer such that the anthracene fluorophore emits normally. Because the C-8 guanidinium in STX has a pK_a of 8.24,[23] and because we wanted to eliminate the possibility of simple proton-transfer enhancing fluorescence, we did our binding studies in ethanol/water solvent mixtures, buffered to pH 7.1 with 6×10^{-3} M ammonium phosphate. Control experiments showed that this buffer had no effect on the fluorescence intensity of **1** in 80% ethanol. In water, this type of crown sensor is insensitive to metal ions such as Na^+, K^+, and Ca^{2+}.[21,24]

To determine the binding constant, fluorescence spectra were recorded at concentrations of [STX] ranging from 1.0×10^{-7} to 1.0×10^{-4} M, at concentrations of 1.0×10^{-7}, 2.5×10^{-7}, 5.0×10^{-7}, and 7.5×10^{-7} M, and so forth, over this concentration range. The concentration of crown ether was held constant at 1.0×10^{-6} M and the buffer at 6×10^{-3} M, with irradiation at $\lambda = 366$ nm.

The raw data and the normalized binding isotherm for **1** are shown in Figure 2a, along with the emission spectra corresponding to the first and last datapoints (Figure 2b), at [STX] = 0 and 1.0×10^{-4} M. It was not possible to determine the stoichiometry of binding using either a continuous variation or mole ratio plot,[25] for the following reasons: (i) at high concentrations, the fluorescence intensity was not linear; (ii) at lower concentrations, F/F_0 was too small. Therefore, as a working hypothesis, we assume that the binding is 1:1.

The binding constant was determined from the experimental data using a nonlinear least-squares fit to eq 1,[26] where F and F_0 are the observed fluorescence intensities in the presence and absence of STX, respectively, integrated from 380 to 600 nm; k_{crown} and k_{11} are constants related to fluorescence intensities of the crown and the presumed 1:1 crown·STX complex, respectively; K_{11} is the binding constant for the 1:1 complex; and [STX] is the equilibrium concentration of unbound saxitoxin.

$$\frac{F}{F_0} = \frac{1 + \left(\frac{k_{11}}{k_{crown}}\right)K_{11}[\mathrm{STX}]}{1 + K_{11}[\mathrm{STX}]} \qquad (1)$$

A good fit was obtained ($R = 0.991$), and revealed a binding constant of $(1.38 \pm 0.46) \times 10^4$ M^{-1}. Figure 2a also shows data obtained from similar experiments with arginine, adenine, guanidinium hydrochloride, and *o*-bromophenol as controls. None produced any fluorescence enhancement in crown **1**. Arginine and guanidinium hydrochloride were tested because guanidinium ions are known to bind to crowns, and STX has two guanidinium moieties. Adenine was chosen because it is a biomolecule and has a purine ring system, as does STX. *o*-Bromophenol was chosen because it has a pK_a of 8.45,[27] similar to that of saxitoxin. Clearly, none shows any evidence of binding, indicating that crown **1** binds saxitoxin selectively over all of these analytes and that the observed fluorescence response *is not* due to simple proton transfer to the benzylic nitrogen of the crown.

With these preliminary results in hand, we examined a series of 10 more diazacrowns, **2**−**11** for binding. There were two objectives in evaluating these compounds: probing the effect of structural variation on binding and designing derivatives that are suitable for incorporation into combinatorial libraries. The results of the binding studies are listed in Table 1, along with the data from **1** for comparison. Least-squares fit of all the titrations resulted in excellent correlations ($r \geq 0.96$). Individual binding isotherms and emission spectra are reported in the Supporting Information.

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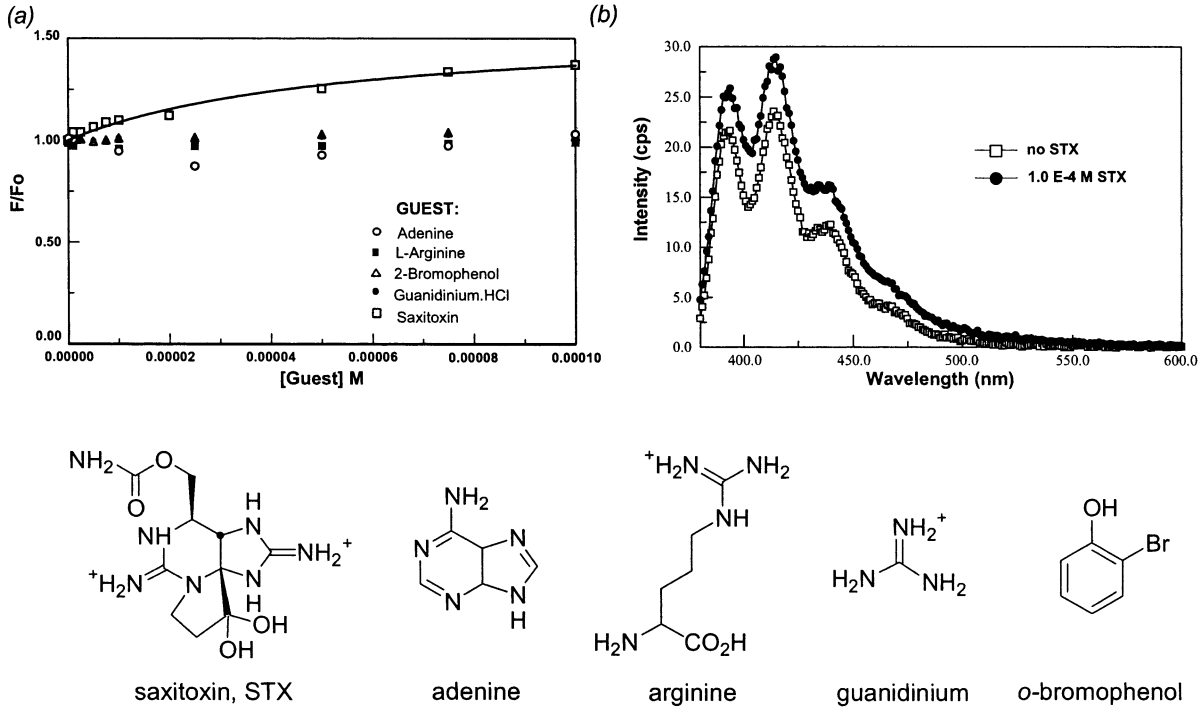

Figure 2. (a) Binding isotherm for crown 1 with STX, and comparison data for adenine, arginine, guanidinium hydrochloride and o-bromophenol. Crown concentration is 1.0×10^{-6} M, and the guest concentration is varied from 1.0×10^{-7} to 1.0×10^{-4} M. The solvent is 80:20 ethanol/water for STX, guanidinium and *o*-bromophenol and 98:2 ethanol/water for adenine and arginine. Both solvent mixtures were buffered to pH 7.1 with ammonium phosphate: 6×10^{-3} M in 80/20 and 2×10^{-4} M in 98:2 EtOH/H_2O. (b) Emission spectra for crown 1 (1.0×10^{-6} M) in the absence and presence of 1.0×10^{-4} M STX, corresponding to the first and last datapoints for the binding isotherm in Figure 2a.

Table 1. Binding Constants Determined by Fluorescence Titration and Assuming a 1:1 STX·Crown Complex (Buffered 80:20 Ethanol/Water)

cmpd	$K_{11} \times 10^4$ (M^{-1})	*r*	cmpd	$K_{11} \times 10^4$ (M^{-1})	*r*
1	1.38 ± 0.46	.990	**7**	1.51 ± 0.87	.974
2	3.58 ± 1.11	.982	**8**	$.50 \pm 0.21$	.995
3	3.26 ± 1.32^a	.974	**9**	1.80 ± 0.52^a	.993
4	2.25 ± 0.73^a	.986	**10**	1.63 ± 0.97	.972
5	0.88 ± 0.24	.996	**11**	1.13 ± 0.56	.988
6	1.63 ± 0.50	.991			

[a] Average of two runs.

Discussion

Comparison of monoazacrown **1** with the diazacrown analogue **2** shows a slight increase in binding, and acylation of the basic nitrogen, as in **3**, gives no further change. The three *tert*-butyl esters, **4**−**6**, have similar binding constants, as does *N*- and *O*-protected aspartate derivative **7**. Curiously, the homologue **8**, a similarly protected glutamate derivative, has a somewhat lower binding constant. Also interesting is the fact that incorporation of a carboxylic acid moiety, which ought to be ionized at pH 7.1, as in **9**−**11**, has no measurable increase on binding. The excellent fits of the experimental isotherms to the theoretical 1:1 binding equation strongly suggests that a 1:1 complex is indeed formed in all cases.

It is of interest to determine the nature of the binding. The mechanism by which these types of sensors are believed to work is through photoinduced electron transfer, PET.[28] In PET, the fluorophore fails to fluoresce because the excited state is quenched by electron transfer, unless the relative energies of the fluorophore are perturbed by a binding event. For example, in aminomethylanthracenes, PET can be inhibited by protonation or hydrogen bonding.[22] Figure 3 shows the X-ray crystal

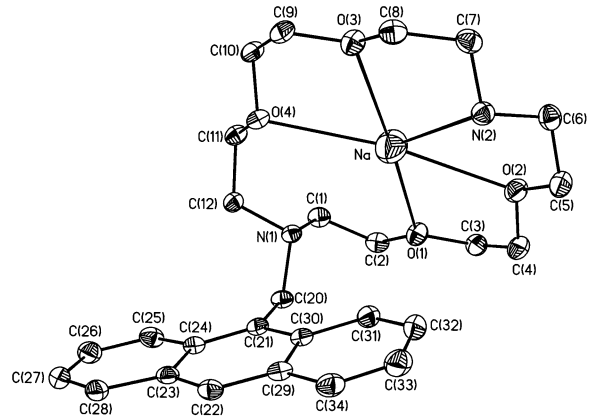

Figure 3. ORTEP drawing of **2**·NaCl at the 20% probability level. Two waters of hydration and the chloride anion are deleted for clarity.

structure of crown **2**, with sodium complexed to the crown heteroatoms in this way. In the solid state, the sodium is in close contact with all the heteroatoms in the crown except the benzylic nitrogen.

Our initial thinking was that saxitoxin, molecular formula $C_{10}H_{19}N_7O_4$, would be an excellent candidate for fluorescence sensing by quenching of PET, because of the large number (11) of potential hydrogen-bond donors. Moreover, STX is a bis-guanidinium dication, and it has long been known that guanidinium ions bind to crown ethers.[29] This hypothesis is supported by the fact that we observe a fluorescence response.

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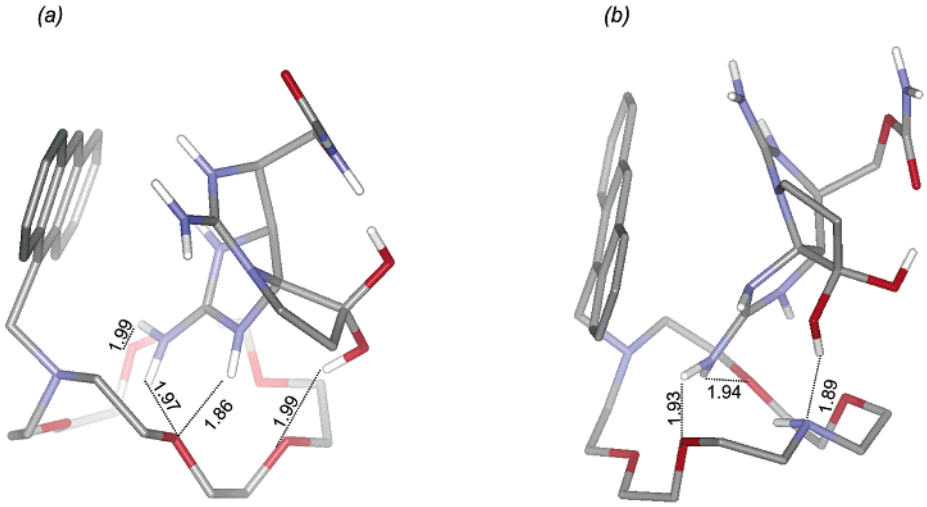

Figure 4. Global minima for STX docked into crowns **1** and **2**, with distances (in Å) between STX hydrogen bond donors and crown heteroatoms indicated. (a) Crown **1**·STX; (b) Crown **2**·STX.

Several crystal structures of 18-crown-6 and its aza analogues with urea, uronium ions, amides, and amidines show hydrogen bonding with crown heteroatoms,[30] but several of the analytes tested (Figure 1) against crown **1** have hydrogen bond donors but provide no detectable fluorescence enhancement. Attempts to grow crystals of a crown·STX complex have, thus far, failed. Although NMR titrations can be used to evaluate structural features in a complex, such studies are not possible at the concentrations we are studying (10^{-6} M). As an alternative, we have used computational techniques to probe possible modes of binding.

Monte Carlo docking searches using the explicit hydrogen atom AMBER* force field were performed using the "low-mode docking" search procedure, which performs atomic movement on the crown·STX complex in a manner such that atoms are moved along a trajectory that is consistent with the low-frequency vibrational modes of the complex.[31] Further, STX was subjected to explicit translation and rotation in the crown ether binding site, and explicit torsional variation was performed for the STX and crown ether side chains that can undergo free rotation. The Supporting Information contains details of these calculations and the software employed. The global minima for the docking of STX with crowns **1** and **2**, each of which were found multiple times in the search, are shown in Figure 4.

The lack of apparent participation by the benzylic nitrogen in the binding of sodium in the solid state (Figure 3) and of STX in these computational models (Figure 4) is interesting. If the structures in Figures 3 and 4 bear any resemblance to the structure of the crown complexes in solution, it would seem that coordination or hydrogen bonding to the benzylic nitrogen is too simple an explanation to account for the PET. Molecular orbital calculations and time-resolved fluorescence measurements are in progress to clarify this point.

The parameters utilized by AMBER* for this study contained no low-quality torsional parameters, which could have adversely affected the accuracy of computed conformational energies. The point charges used for the calculations were derived from charges fitted to the electrostatic potential derived from the 6-31G** wave function. Moreover, since AMBER itself was parametrized using electrostatic potential fitted charges, we believe that the energies calculated are reliable within the molecular mechanics paradigm. Nonetheless, we performed a second Monte Carlo search using the OPLS all-atom force field (OPLS-AA) for **1**·STX. The lowest-energy structures (i.e., the global minimum and all structures within 1 kcal/mol of it) possessed hydrogen bonds between the C-8 guanidinium of STX and the crown ether oxygens, while some of the higher-energy structures possessed hydrogen bonds between the C-2 guanidinium of STX and the crown oxygens.

Relevance to Saxitoxin Monitoring Programs. The benchmark method for detecting saxitoxin and its congeners in shellfish is mouse bioassay. The current legal limit for STX in shellfish is 80 μg/100 g of shellfish, and the mouse bioassay can reliably detect down to 40 μg of STX/100 g of shellfish. In the AOAC technique, extraction of shellfish that contains 40 μg of STX/100 g of shellfish produces an aqueous solution that is $\sim 10^{-6}$ M in STX, and this is what is injected into the mouse. As it stands now, our crown sensors show excellent fluorescence enhancement at STX concentrations of 10^{-4} M. Many show significant (10−20%) enhancement at concentrations of 5×10^{-6} M, which is very encouraging for development of better sensors. A more *sensitive* STX sensor, having a larger binding constant, would have a steep binding isotherm in the low-concentration range, and this is what we will be seeking as this work proceeds. Nevertheless, we are already very close to the limit of detection by the mouse bioassay!

Selectivity of binding of STX to all our crowns has already been demonstrated relative to ammonium ion, and crown **1** is selective for STX relative to arginine, adenine, guanidinium ion, and *o*-bromophenol. Very recently, we have found that coumaryl crown ethers have binding constants to STX in water that are an order of magnitude higher than those in aqueous ethanol, and which are selective for STX in the presence of sodium and

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potassium ions in aqueous solution.[24] Thus, it appears that crown ether sensors such as those reported herein will prove useful in testing for STX in environmental samples. Such experiments are being planned, and will be reported in due course.

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Supporting Information Available: Experimental details for the preparation of crowns **2**−**11**, proton and carbon NMR spectra, mass spectra, crystal data for crowns **2** and **9**, plots of the binding isotherms for crowns **2**−**11**, details of the molecular modeling calculations (PDF) and an X-ray crystallographic file (CIF). This material is available free of charge via the Internet at http://www.pubs.acs.org.

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