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Visible Fluorescence Chemosensor for Saxitoxin

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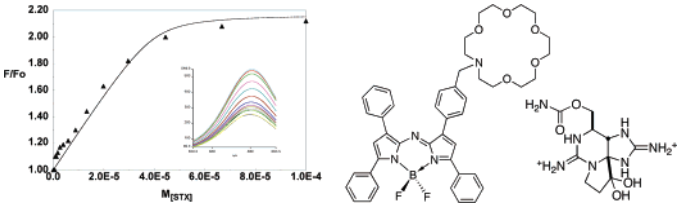

Absorption spectra of a number of shellfish extracts have been obtained and reveal prominent absorptions in all samples at 210 and 260 nm and at 325 nm in some of them. These absorptions preclude the use of chromophores with similar absorptions in testing of shellfish samples for paralytic shellfish toxins. Two crown ether chemosensors featuring a boron azadipyrrin chromophore have been synthesized; both have absorption maxima at 650 nm, where all the shellfish extracts are transparent. The synthetic sensors feature either 18- or 27-membered crown ether rings and have been evaluated as visible sensors for the paralytic shellfish toxin saxitoxin. The binding constant for one of them is in the range of $3-9 \times 10^5$ M^{-1} and exhibits a fluorescence enhancement of over 100% at 680 nm in the presence of 40 μM saxitoxin.

Introduction

Contamination of shellfish by paralytic shellfish toxins or poisons (PSTs, PSPs) continues to be a near-worldwide health risk.[1,2] Monitoring of shellfish beds for the presence of PSTs continues to use the mouse bioassay as the most common means of detection,[3,4] although recent advances in HPLC methods have been made and approved for use by monitoring agencies.[5] The mouse bioassay has obvious economic and ethical drawbacks,[6] and alternatives are being actively sought in a number of laboratories.[4,7−9]

Our work in this area has focused on optical methods, and we have developed and explored a number of chemosensor systems that are based on the concept of photoinduced electron transfer, or PET. In PET-based chemosensors, a host for a given analyte is designed in which a chromophore is separated from the host by a spacer (see Figure 1).[10−12] In our case, the host component of the sensor is a crown ether that is separated from the fluorophore by a methylene spacer. In the absence of a guest such as saxitoxin, PET from the crown to the fluorophore quenches the excited state after photon absorption, and fluorescence is turned off (or, more accurately, is minimized). When the host is complexed to the toxin, the relative energies of the molecular orbitals are perturbed so that PET is no longer

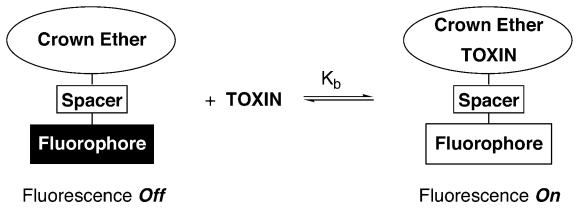

FIGURE 1. General design of a PET-based chemosensor for saxitoxin.

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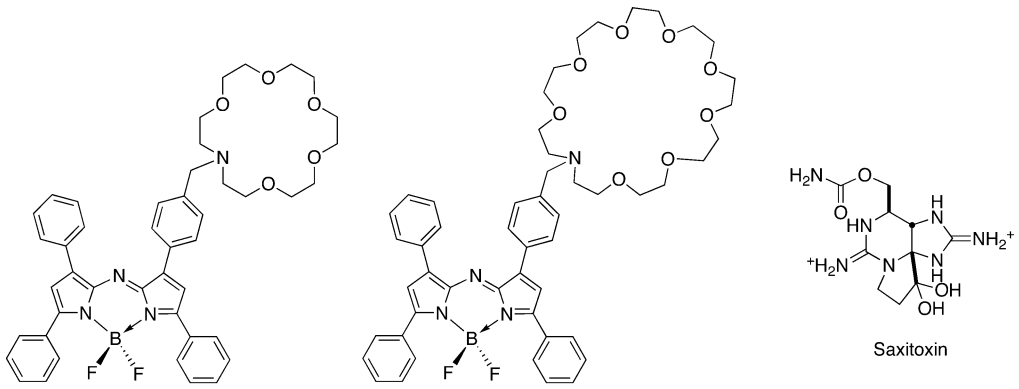
CHART 1



1 **2**

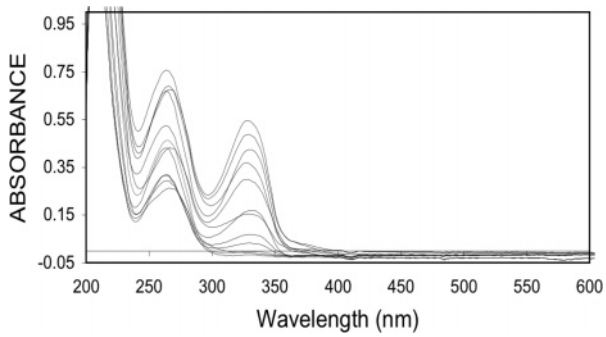


FIGURE 2. Absorption spectra of 12 extracts of shellfish from mouse bioassay.

favorable, and fluorescence is "turned on".[13,14] The sensitivity of a PET-based chemosensor is influenced by a number of factors, including the quantum yield of the fluorophore and the equilibrium, or binding constant, K_b, between the chemosensor and the fluorescent complex.

In past studies, we have evaluated anthracene,[13,14] coumarin,[15,16] and acridine[17] fluorophores in our chemosensors, all of which have absorption maxima in the ultraviolet. This class of chemosensors is selective for detection of saxitoxin over tetrodotoxin,[17] metal ions such as sodium and potassium,[15] and several other analytes.[13] They can also be incorporated into self-assembled monolayers for sensing on a surface.[16,18,19] Recently, we reported that incorporation of larger crown ether rings can significantly increase the binding constant.[14] We now report the synthesis and evaluation of boron azadipyrrins **1** and **2**, having absorption maxima in the visible region of the spectrum, and with both 18-crown-6 (**1**) and 27-crown-9 (**2**) ring hosts.

Results

The absorption spectra of extracts of 12 shellfish, collected in Washington in July and August of 2004, are shown in Figure 2. The data are from extracts of blue mussels (*Mytilus trossulus*), geoduck clams (*Panope generosa*), butter clams (*Saxidomus giganteus*), and littleneck clams (*Protothaca staminea*). The PSP number for these samples ranged from <38 to 2484 μg/100 g meat. Prominent absorption peaks for all extracts occur at 210 and 260 nm, with several of the extracts showing absorption bands at 330 nm, as well. There was no correlation between the intensity of this band and the toxicity of the sample as indicated by mouse bioassay data obtained from the Washington Department of Health (data not shown).

The synthesis of the boron azadipyrrin crowns **1** and **2** is shown in Scheme 1 and is patterned after the elegant work of O'Shea.[20,21] Monoaza 18-crown-6, **3a**, and monoaza 27-crown-9, **3b**,[14] were N-alkylated with *p*-bromomethylbenzaldehyde[22] to give crown-substituted aldehydes **4a**,**b** in 96 and 94% yield, respectively. Condensation with acetophenone afforded chalcones **5a**,**b** in 92 and 90%, respectively, and Henry addition of nitromethane gave **6a**,**b** in equally excellent yields of 96 and 95%. Nef reaction and condensation with ammonia afforded pyrroles **7a**,**b** in modest yields of 49%. Condensation of **7a**,**b** with nitrosopyrrole **8**, according to the method of O'Shea,[20] afforded azadipyrrins **9a**,**b** in good yields (69 and 65%, respectively), as dark blue oils. Treatment with boron trifluoride in the presence of base gave the target compounds, **1** and **2**, in yields of 77 and 78%, respectively, as dark green oils.

The absorption and emission spectra for **1** and **2** are shown in Figure 3. As expected, both are very similar. The binding isotherms were determined by titration with saxitoxin in methanol, buffered to pH 7.1 with tetrabutylammonium phosphate. The pH of a nonaqueous buffer is a complicated issue.[23−25] In this case, pH 7.1 refers to a meter reading of 7.1

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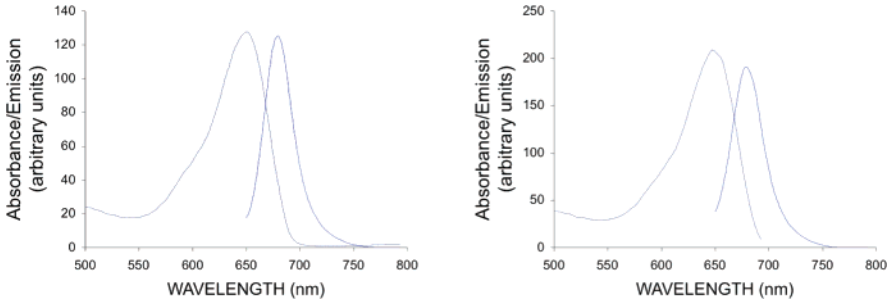


FIGURE 3. Excitation and emission spectra of boron azadipyrrins **1** (left) and **2**, in methanol; λ_{ex} = 652 and 681 nm; λ_{em} = 650 nm.

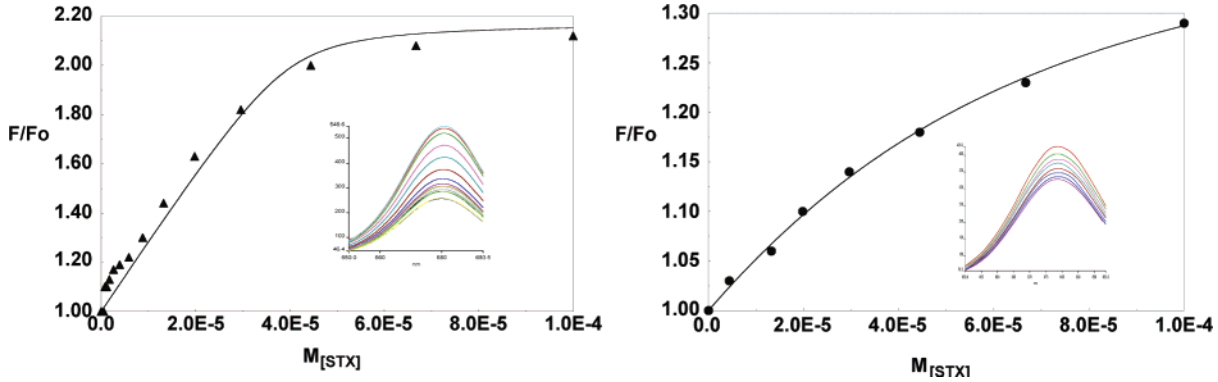
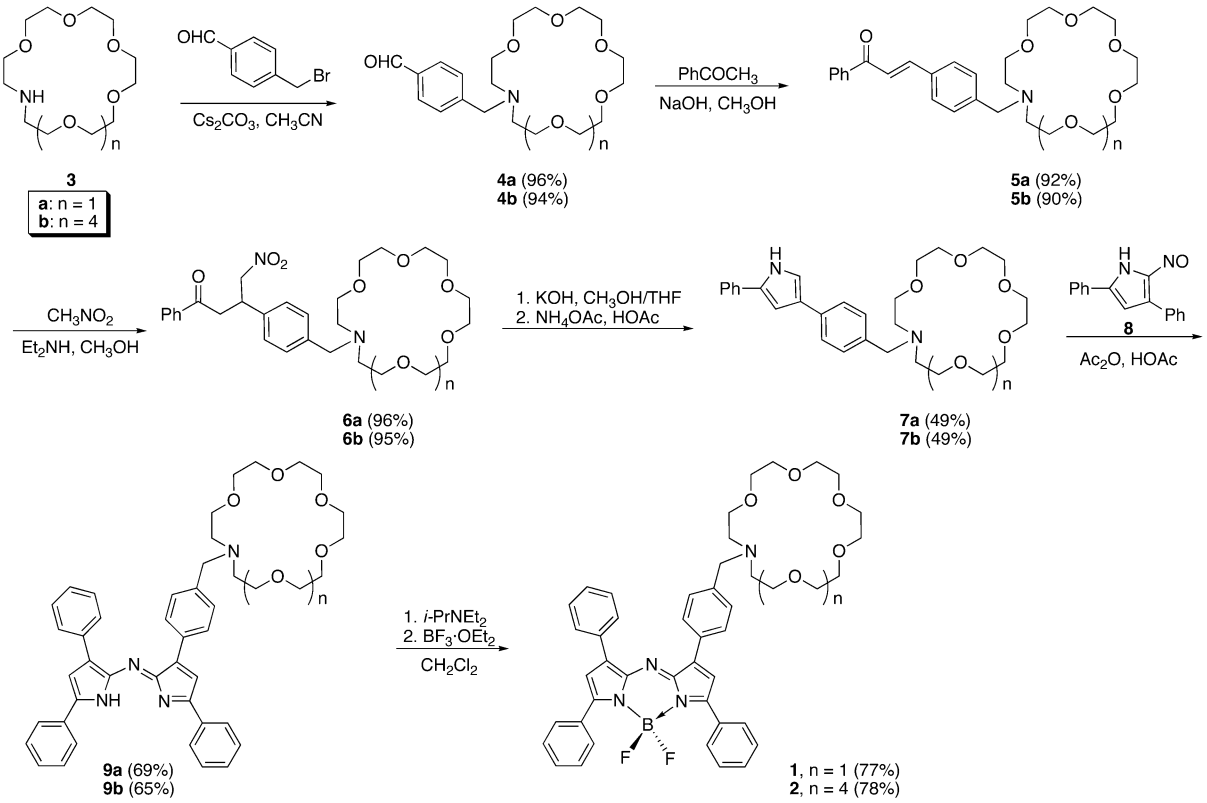


FIGURE 4. Binding isotherms for titration of saxitoxin against fluorescence response for **1** (left) and **2** (right), in methanol. The curve fit assumes a 1:1 binding stoichiometry. For **1**, $K_b = 8.9 \times 10^5$ M^{-1}; for **2**, $K_b = 1.4 \times 10^4$ M^{-1}.

SCHEME 1. Synthesis of Boron Azadipyrrin Chemosensors



in methanol, after calibration of the meter in aqueous buffer. In methanol, the pK_a for **1** and **2** is approximately 6.5. The concentration of the crown chemosensors was held constant at 40 μM; typical binding isotherms are shown in Figure 4. Nonlinear least-squares analysis,[26] assuming a 1:1 host/guest stoichiometry, reveals binding constants of 6.2 $\times$ 10^5 M^{-1} for

1 (average of three runs), and $K_b = 1.4 \times 10^4$ M^{-1} for **2** (best run; see Discussion).

Discussion

The chromophores we have incorporated into the sensor design in the past all absorb in the ultraviolet: coumarin at 328 nm, acridine at 350, and anthracene with three bands from 360 to 390 nm. The 330 nm UV absorption band of the shellfish extracts excludes coumarin as a viable chromophore for evaluating PSPs in shellfish. The tailing of this band to 350 nm in several samples also suggests that acridine is not ideal either. Anthracene has three prominent absorption bands, one of which is at 390 nm, and is in a region of little absorption by the shellfish matrix. Nevertheless, we decided to investigate a chromophore with absorption in the visible, far from any absorption bands due to the matrix. With this in mind, we were intrigued by the recent reports from the O'Shea group, in which boron azadipyrrins were incorporated into PET sensors (for pH sensing) having absorption bands in the region of 650 nm.[21,27] Particularly appealing was the recent report of a general synthesis that permits preparation of a variety of unsymmetrical azadipyrrins.[20] Chemosensors **1** and **2** incorporate the boron azadipyrrin as the chromophore, with crown ethers as the host moiety. Previously, we have found that larger crown rings produce larger binding constants; with anthracene as the fluorophore, the binding constant for the 18-crown-6 host was 5.3×10^4 M^{-1}; for the 27-crown-9 host, it was 1.69×10^5 M^{-1}.[14] We decided to prepare boron azadipyrrin chemosensors with both 18-crown-6 and 27-crown-9 rings.

The syntheses went smoothly in six steps, as illustrated in Scheme 1, with an overall yield of 22% for **1** and 20% for **2**. The lowest-yielding steps were the conversion of nitroketones **6a**,**b** to pyrroles **7a**,**b** and subsequently to dipyrrins **9a**,**b**. Our observed yields for these steps are somewhat lower than those O'Shea observed in similar reactions,[20] but since adequate amounts were obtained for evaluation, we did not attempt further optimization.

The optical properties of **1** and **2** were as expected (Figure 3). The fluorescence response of these chromophores is not as large as we have seen with coumarin and anthracene chromophores, possibly due to the instrumentation used. In order to see a good fluorescence response, it was necessary to employ 40 μM concentrations of sensors **1** and **2** in the titrations. At this concentration, **1** showed excellent sensitivity to very low concentrations of saxitoxin. At ~1:1 toxin/crown stoichiometry, the fluorescence enhancement, F/F_0, is well over 100%. This level of fluorescence enhancement is reproducible in methanol, buffered at pH 7.1. The legal limit of saxitoxin equivalents in shellfish extracts is 80 μg/100 g meat, but the detection limit in the mouse bioassay is about half that. This detection limit in the mouse bioassay corresponds to an approximately 1 μM solution of toxin.

The average binding constant for **1** to saxitoxin, 6.2×10^5 M^{-1}, is among the highest we have observed for any of our chemosensors. Since changing from an 18-crown-6 ring to a 27-crown-9 ring improved the binding with an anthracene fluorophore,[14] we anticipated a similar increase with **2**. As shown in Figure 4, a similar enhancement was not observed; to the contrary, the binding constant fell to a disappointing 1.4×10^4 M^{-1}. Moreover, **2** exhibited properties that we have not seen before. For example, fluorescence emission of 40 μM **2** in buffered methanol or in pure methanol (in the absence of toxin) decreased over a period of 3−10 min, suggesting an (unknown) equilibration. The fluorescence enhancement was minimal at best, and sometimes was negligible. The reasons for this difference in behavior are unknown.

We have previously suggested, based on molecular modeling studies, that the PET mechanism in these systems involves π-stacking of one of saxitoxin's guanidiniums to the fluorophore, and that this π-stacking perturbs the relative energies of the pertinent molecular orbitals to "turn off" photoinduced electron transfer. π-Stacking of guanidiniums to arenes is well-known in DNA-binding proteins,[28−30] in proteins engineered to bind guanidinium ion,[31] and even in binding of arginine to C_{60}, with the arginine sandwiched between the buckyball and a tryptophan.[32] Although the precise orientation of saxitoxin's guanidinium to the arene is not known, it should be similar among coumarin, acridine, and anthracene chromophores we have studied in the past. In the present case, the fluorophore is much larger, and features a zwitterionic boron−nitrogen dative bond. In such systems, although the negative charge is formally on the boron and the positive charge on the nitrogen, the negative charge density is mostly on the fluorines, while the positive charge is delocalized by resonance. One can easily imagine that having such a functionality proximal to the binding site could alter the geometry of the π-stacking of the toxin to the fluorophore, thereby affecting the ability of the toxin to inhibit the PET. We speculate that the larger crown, being able to offer more hydrogen-bonding acceptor heteroatoms, holds the toxin more firmly in an orientation that precludes the guanidinium π-stack that we believe is necessary to turn off PET.

In summary, we have shown that boron azadipyrrins, functionalized with an 18-crown-6 ring, make excellent visible chemosensors for the marine toxin saxitoxin, the most toxic component of paralytic shellfish poisons. Analysis of shellfish extracts for individual paralytic shellfish toxins (saxitoxin and its 20+ analogues) and the fluorescence response to each by our chemosensors is underway and will be reported in due course.

Experimental Section

Extracts of four species of shellfish used in routine mouse bioassays were obtained courtesy of Bob Lona, Department of Health, Seattle, Washington. Saxitoxin was obtained from Sherwood Hall, U.S. Food and Drug Administration. Details of the chemical syntheses of the boron azadipyrrin crowns, including characterization data and copies of the NMR spectra for all intermediates, are found in the Supporting Information. Titrations and binding constant calculations were done as described previously,[13] with the excitation and emission slits set at 10 and 15 nm, respectively. Methanol is buffered with tetrabutylammonium phosphate to pH 7.1 (phosphate concentration = 300 mM) by adding 600 μL of 5 M phosphoric

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acid to ~7 mL of methanol, and raising the pH to 7.1 (meter reading on a pH meter calibrated with aqueous buffer) with Bu_4NOH (55−60% in water). The crown sensor **1** or **2** is added such that the final concentration will be 40 μM (e.g., 140 μL of a 2.9 mM solution in methanol) and the solution diluted to 10 mL with methanol.

UV−Visible absorption spectra of shellfish extracts were obtained as follows: 100 μL of shellfish extract was diluted with 10 mL of pH 7.1 phosphate buffer (1:100); buffer solutions were then filtered using a 0.45 μM PTFE syringe filter and scanned on a UV-vis spectrophotometer.

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Supporting Information Available: Details of the synthesis procedures and characterization data; proton, carbon, and DEPT-135 NMR spectra of all new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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