Conformational Analysis of the Sodium Channel Modulator, Brevetoxin A, Comparison with Brevetoxin B Conformations, and a Hypothesis about the Common Pharmacophore of the "Site 5" Toxins

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The marine neurotoxins brevetoxin A, brevetoxin B, and ciguatoxin bind to the same site (site 5) on the voltage-gated sodium channel. This work, and the following paper in this issue, describe efforts to identify the common pharmacophore and to develop a ligand–receptor model for the binding of these neurotoxins to site 5. Conformational analysis of brevetoxin A has been completed using an internal coordinate Monte Carlo search protocol. Within 6 kcal/mol of the global minimum (*in vacuo*), there are 48 conformations of brevetoxin A. In chloroform or water solvent, the calculated relative energies change, but no new minima appear. Like brevetoxin B, brevetoxin A has both straight and bent conformers available. Elimination of several G-ring crown conformers from consideration and comparison of the two brevetoxin backbones indicates that those that match most closely in overall shape and location of functional groups are straight. We postulate that the common pharmacophore is a roughly cigar-shaped molecule (~30 Å long) bound to its receptor primarily by hydrophobic and nonpolar solvation forces, possibly aided by strategically placed hydrogen bonds near the site of the lactone carbonyl in the receptor.

The natural marine toxins, brevetoxin A, brevetoxin, B and ciguatoxin (Figure 1) are xenobiotics that may contaminate seafood.[1] The intoxication signs in animals and symptoms in humans have been quite well characterized.[2] These three molecules exert their activity by association with a specific receptor site, in excitable membranes, which has been described with respect to binding characteristics.[3] This receptor site is known as site 5, and its location is on domain IV of the α-subunit of voltage-gated sodium channels.[4] Since no endogenous biological molecule that naturally binds to this site has been identified and no endogenous function for the receptor is known, site 5 can be classified as an "orphan receptor".[5]

Other avenues of research point to domain IV of the α-subunit of the sodium channel to be involved in many of the regulatory properties and voltage sensitivity of the channel. Two different human muscle diseases, *hyperkalemic periodic paralysis* and *paramytonia congenita*, have been shown to be due to point missense mutations associated with domain IV of the α-subunit of the voltage-gated sodium channel. These mutations result in persistent depolarization and abnormal noninactivating sodium conductances along with elevated serum potassium.[6] Since brevetoxins and ciguatoxin interact with excitable membranes in such a way as to produce nearly these identical effects,[7] one may postulate that polyether toxins interact with the activating and inactivating portions of the α-subunit at sites proximal to, or identical to, those modified by missense mutation. Consequently, the site specificity of these toxins may not only present an opportunity to employ a natural ligand to further probe activation and gating phenomena but also presents the potential for unmasking those regions of the channel which allosterically regulate sodium channel activity.

Our ultimate goal is to provide a molecular view of the site 5 orphan receptor and to identify the common pharmacophore of the three polyether ladder toxins that bind to site 5 of the voltage-gated sodium channel. Initial efforts toward this goal, reported in this and the accompanying paper, correlate the results of bioassays of native and synthetically modified toxins to conformational modeling data.

When studying receptor ligand interactions, the ideal situation would be to obtain a crystal structure of the binding site of the protein, or even better, a crystal structure of the protein with a ligand bound to it. While the site 1 neurotoxins tetrodotoxin and saxitoxin bind to purified sodium channel[8] and a recent report describes high affinity binding of brevetoxins to purified, *reconstituted* sodium channel,[9] we have been unable to demonstrate binding of brevetoxins to solubilized, purified sodium channel (i.e., the receptor protein must be incorporated into a membrane to be in it's "active" conformation). We therefore must use an indirect approach to the study of receptor–ligand interactions at site 5. This approach assumes that the receptor is topographically

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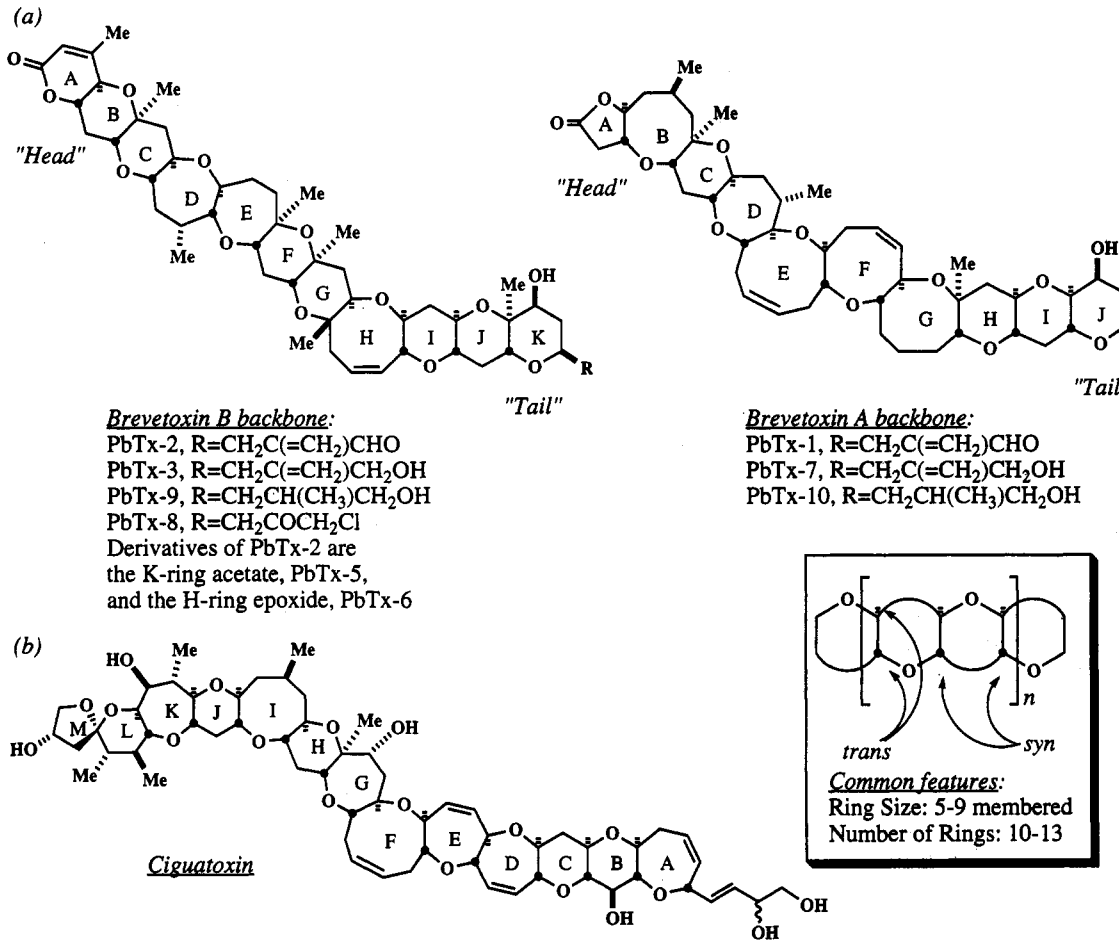

Figure 1. Toxins that bind to site 5 of the voltage-gated sodium channel.

complementary to the active molecules.[10] In order to understand binding on a molecular level we must first have a complete conformational picture of the active and inactive ligands. In comparing the number of potentially rotatable bonds in the medium sized rings of the three backbones, it is evident that the complexity of the conformational analysis increases from brevetoxin B (16 rotatable bonds) to brevetoxin A (31 rotatable bonds) to ciguatoxin (38 rotatable bonds), as shown by the dark bonds in Figure 2. Using Still's internal coordinate Monte Carlo search protocol,[11] we have completed the conformational analysis of the brevetoxin B backbone.[12] Herein, we describe the conformational analysis of the brevetoxin A backbone, an effort that further underscores the utility of the internal coordinate Monte Carlo method in conformational analysis of fused-ring polycyclics. We also compare the conformations of the brevetoxin A and B backbones and offer a hypothesis regarding their common pharmacophore.

Computational Methods

Internal coordinate Monte Carlo sampling, minimization using the MM2* force field, and structure comparisons were performed

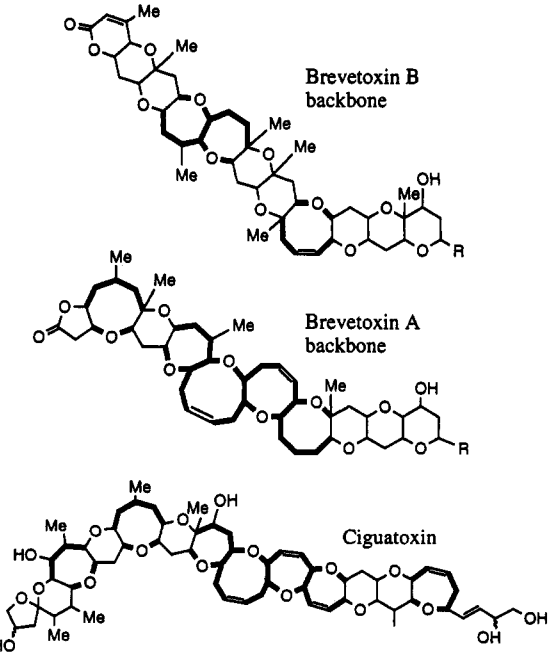

Figure 2. Rotatable bonds in the backbones of the site 5 toxins (in bold).

using MacroModel (v. 3.1×) and Batchmin (v. 3.1).[13] To simplify the structures and speed computation, the J-ring side chain and

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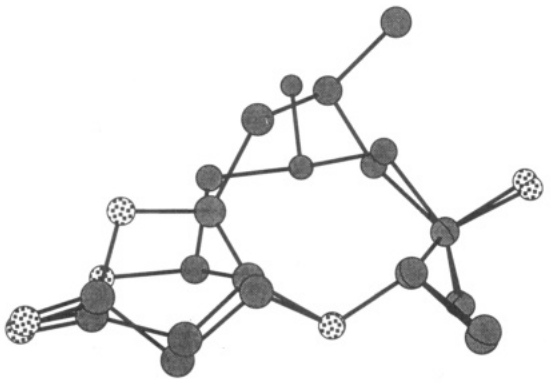

Figure 3. Superimposition of the two conformers of the B-ring of brevetoxin A.

hydroxyl were removed. Dihedral angles were varied randomly within a range of 0 to ±180°. A ring closure distance range of 0–2.0 Å was used. Torsional constraints of 0–5° were placed around double bonds. Using our experience gained with the modeling of the brevetoxin B backbone,[12] the truncated Newton Raphson algorithm with a maximum of 1500 iterations and a large (50 kJ/mol) or no energetic window provided optimal results. The user-directed structure selection technique was invoked in which the least-used conformation became the starting geometry for a new Monte Carlo cycle, provided it was within 25 kJ/mol of the current global minimum.

Efficient methods for searching conformational space of molecules with a large (>10) number of torsions have only recently been developed.[11] In an effort to simplify the problem, we found it helpful to model fragments of the molecule first and use the information gained to direct the whole-molecule search. The largest number of torsions varied in one of these "partial searches" was 21. All Monte Carlo searches were continued until each minimum had been found many times. The number of Monte Carlo steps varied from several hundred for the smaller fragments to about 5000 for searches that included 18–21 torsions and four ring closures (see supplementary material for details). Derivative convergence to 0.1 kJ/(Å·mol) proved to be sufficient for the smaller fragments (up to four rings) but was insufficient for the whole molecule searches (*i.e.*, duplicate conformations were not always eliminated). However, derivative convergence of the whole molecule to 0.01 kJ/(Å·mol) was too computationally time consuming. Thus, a convergence criteria of 0.1 kJ/(Å·mol) with a large (50 kJ/mol) or no energetic window (DEMX) was used for the initial search, and a final multiconformer minimization with 0.01 kJ/(Å·mol) convergence criteria eliminated duplicates. An energetic window of 25 kJ/mol was used in the final multiconformer run. The 24 minimized structures were then reminimized with chloroform and water "solvents," where the solvent is treated as a statistical continuum.[14] For discussion purposes, the final energies are converted to kcal/mol.

Results and Discussion

Modeling of the ABC-ring fragment revealed that the B-ring may occupy two essentially isoenergetic ($\Delta E = 0.014$ kcal/mol) conformations: a crown and a boat–chair. The two conformations are shown superimposed in Figure 3 and separately in Figure 4. In the superimposed conformers, note that the location of the A-ring lactone carbonyl oxygen is virtually unchanged, an observation that may have implications regarding the complementary region of the sodium channel binding site (*vide infra*).

While the six-membered HIJ-rings are rigid by virtue of their *trans*-decalin-like geometries, we were uncertain about the six-membered C-ring, since it is flanked by the eight-membered B-ring and the seven-membered D-ring. Conformational analysis of a BCD-ring fragment indicated that the C ring is indeed fixed in a single chair conformation. Since the C-ring is rigid and the B-ring conformational change does not alter the overall shape of the molecule, the B-ring was arbitrarily fixed in the crown conformation and torsional angles in the A–C rings were omitted in subsequent searches.

The fused "medium-sized" D–G rings present an interesting conformational problem, since each medium-sized ring might be expected to have a number of accessible conformations, and we were unsure of the effect their fusion might have on the potential energies of various (reasonable) conformations. We were particularly interested in the conformations of the nine-membered E-ring. Analysis of a C through H-ring fragment revealed only two low-energy conformations of the E-ring, E1 and E2 (Figure 4). A separate Monte Carlo search that varied only the torsions in the E ring, but which included the entire toxin backbone in the minimization, identified another E-ring conformation, E3 (Figure 4). The lower energy conformation is E1 (*cf.* Table 1, conformer pairs *a*/*b*, *c*/*q*, *d*/*h*, and *g*/*n*), which has the double bond rotated toward the β-face (as drawn in Figure 1), while the higher energy conformations E2 and E3 have the double bond rotated toward the α-face. Interestingly, the four bonds to the neighboring D and F rings are virtually superimposable in the E1 and E2 conformations, meaning that these two E-ring conformations have no influence on the overall shape of the toxin backbone. Indeed, both of these E-ring conformations were found in the unit cell of crystalline brevetoxin A.[15] For each conformation having the E-ring in conformation E1, a corresponding E2 conformer was not necessarily found. Since the energy difference for these two conformers could be rather small (*cf.* Table 1, conformer pairs *a*/*b* and *d*/*h*), this was a source of concern and prompted further investigation. Manual E1 to E2 inversion of either conformers *a* or *e* followed by minimization affords only conformer *b*. Similarly, conformations *d* and *f* both reduce to conformation *h*, and *c* and *l* both go to *q* upon manual E1 to E2 ring reversal. Thus, the D3 and D4 conformations are only available when the E-ring is in conformation E1. This is underscored by manual E-ring inversion of *k* and *m*, which produced conformers *j* and *r*, respectively, in which the D-ring also inverted. (Manual E1/E2 inversion of conformers *j*, *p*, *s*, *t*, *u*, and *x* produces conformers that are outside the energetic window being considered.) As expected, several conformations of the seven-membered D-ring were found (Figure 4), but only three conformations of the eight-membered F-ring, torsionally constrained by a double bond, were found.

We found five conformations for the saturated 8-membered G-ring, but there is a large preference of the G-ring for a boat–chair conformation. Conformer pairs *a*/*g*, *b*/*n*, *d*/*p*, *e*/*t*, and *f*/*x*, which differ only in the G-ring, have an average (gas phase) preference of 3.04 kcal/mol for boat–chair G1 over crown G2. Only in conformer pair *c*/*i* is the G-ring crown preferred (by 1.14 kcal/mol). Previous studies[12] indicated that the H-ring of brevetoxin B, which corresponds to the G-ring of brevetoxin A, has two conformations, which differ by almost 5 kcal/mol. This is in contrast to the nearly isoenergetic boat–chair and crown conformations of the other brevetoxin A eight-

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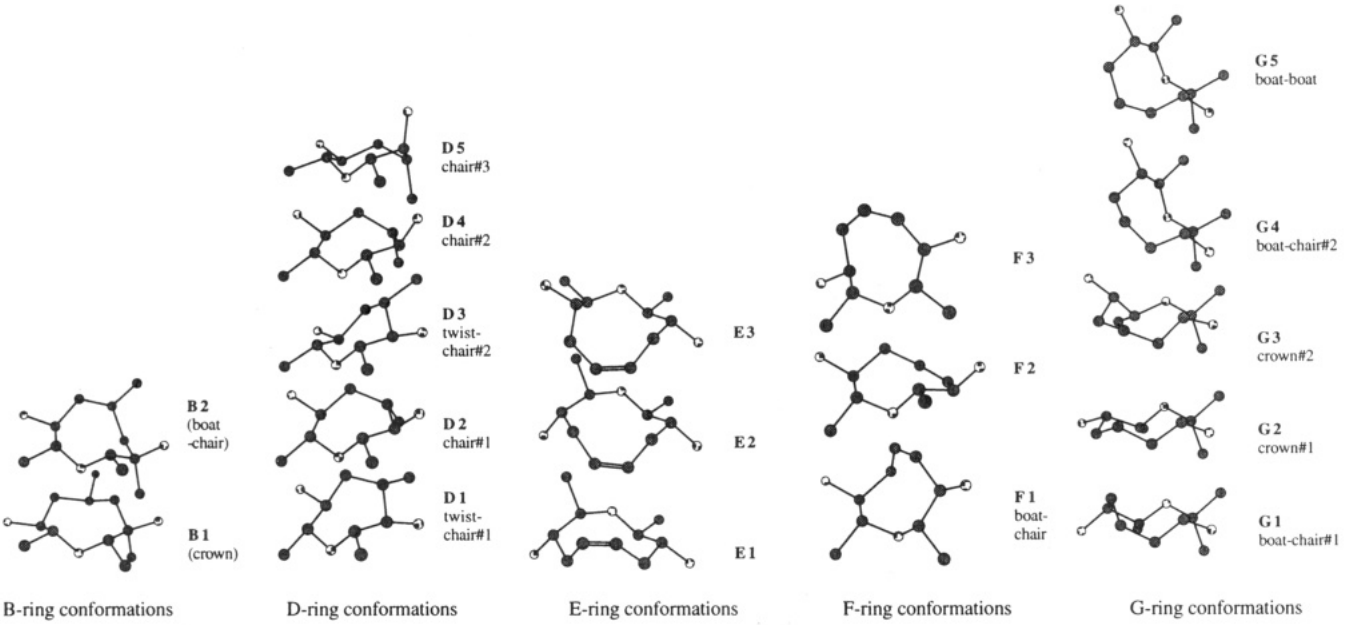

Figure 4. Low-energy conformations of the flexible rings of the brevetoxin A backbone.

Table 1. Low-Energy Conformations of Brevetoxin A. The Conformations of the Individual Rings Correspond to Those in Figure 4

entry	D-ring	E-ring	F-ring	G-ring	rank (gas)	ΔE(gas) (kcal/mol)	rank ($CHCl_3$)	ΔE($CHCl_3$) (kcal/mol)	rank (H_2O)	ΔE(H_2O) (kcal/mol)
a	D1	E1	F1	G1	1	0	1	0	1	0
b	D1	E2	F1	G1	2	2.03	11	3.04	12	3.17
c	D1	E1	F2	G2	3	2.37	4	1.72	6	2.16
d	D2	E1	F1	G1	4	2.44	8	2.13	7	2.28
e	D3	E1	F1	G1	5	2.68	3	1.45	4	1.46
f	D4	E1	F1	G1	6	2.82	9	2.48	10	2.84
g	D1	E1	F1	G2	7	2.99	5	1.91	5	2.00
h	D2	E2	F1	G1	8	3.23	2	1.45	2	1.12
i	D1	E1	F2	G1	9	3.51	7	2.10	9	2.53
j	D2	E2	F3	G1	10	4.40	13	3.56	11	2.93
k	D4	E1	F3	G1	11	4.54	12	3.44	13	3.44
l	D3	E1	F2	G2	12	5.04	15	4.28	16	4.80
m	D4	E1	F2	G3	13	5.07	16	4.42	17	5.09
n	D1	E2	F1	G2	14	5.11	10	2.99	8	2.51
o	D5	E3	F1	G1	15	5.28	14	3.92	14	4.23
p	D2	E1	F1	G2	16	5.47	24	8.27	24	9.15
q	D1	E2	F2	G2	17	5.47	18	4.99	18	5.13
r	D2	E2	F2	G3	18	5.56	21	5.56	21	6.02
s	D2	E1	F2	G2	19	5.64	19	5.19	20	5.76
t	D3	E1	F1	G2	20	5.66	23	6.21	23	6.64
u	D1	E1	F2	G4	21	5.69	17	4.54	15	4.52
v	D1	E1	F2	G5	22	5.71	20	5.21	19	5.14
w	D1	E2	F2	G1	23	5.87	22	5.78	22	6.31
x	D4	E1	F1	G2	24	5.95	6	2.03	3	1.46

membered ring (B, with a slight preference for the crown). Other researchers have also made note of this preference[15] which appears to have an interesting effect on binding to site 5. In the following paper in this issue,[16] we show that this eight-membered ring must be in the boat–chair conformation for binding, which corresponds very closely to conformation G1.

In summary, a total of two B-ring conformers, five D-ring conformers, three E-ring conformers, three F-ring conformers, and five G-ring conformers (see Figure 4) combine to give a total of 48 conformations within 6 kcal/mol of the global minimum. Table 1 lists the calculated relative energies of half of these conformations *in vacuo* and in chloroform and water solvents. Each of these conformations has a corresponding (approximately isoenergetic) conformer with the B-ring in a boat–chair. Conformations *d* and *h* correspond to the conformations found in the X-ray crystal structure (B-ring crown).[15]

Note that on changing solvents, the relative energies of a number of the conformations change dramatically. The many "anomeric arrangements" (OCCO linkages) in these molecules may not be adequately paramaterized by the MM2* (or MM2 or MM3) force fields,[17] and so we have conservatively included a number of relatively high-energy conformations in this list, but our conclusions are not predicated on relative energies. Rather, we have used a large enough energetic window that all reasonable conformations have been included, and we believe that one

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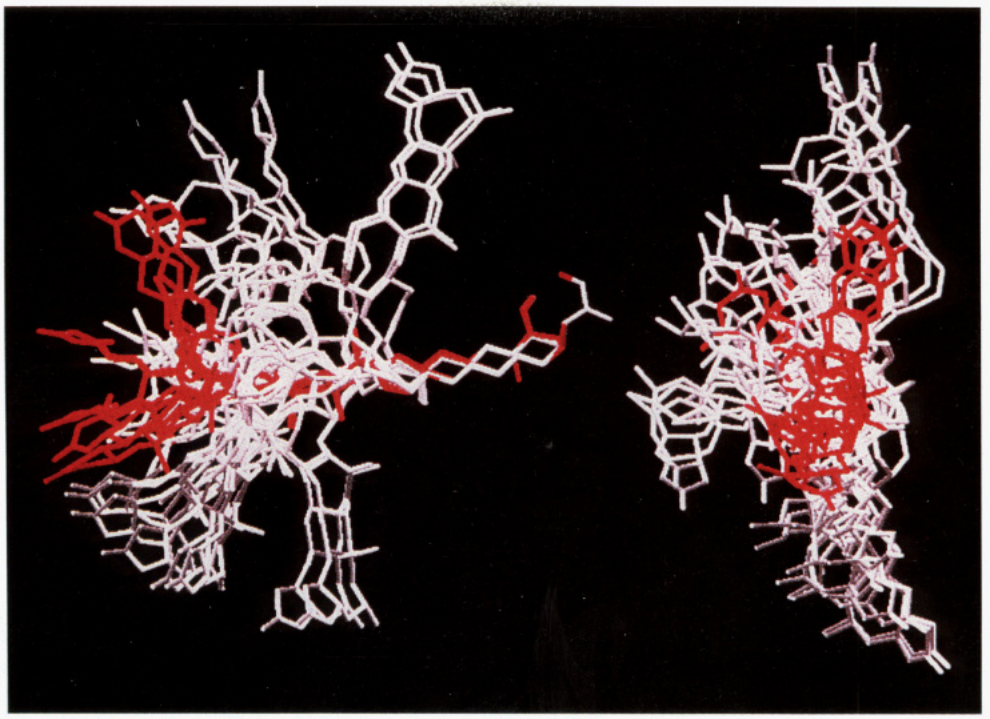

Figure 5. Superimposition of 24 conformations of the brevetoxin A backbone (white) and 7 conformations of the brevetoxin B backbone (red), with the hydrogens deleted for clarity. The tails of the two toxins are superimposed strictly, but the side chain is illustrated in an arbitrary conformation. Left: View showing the superimposed tails on the right, with the heads fanning out to the left. Right: Same superimposition, rotated 90° along the *y*-axis such that the heads are in the foreground.

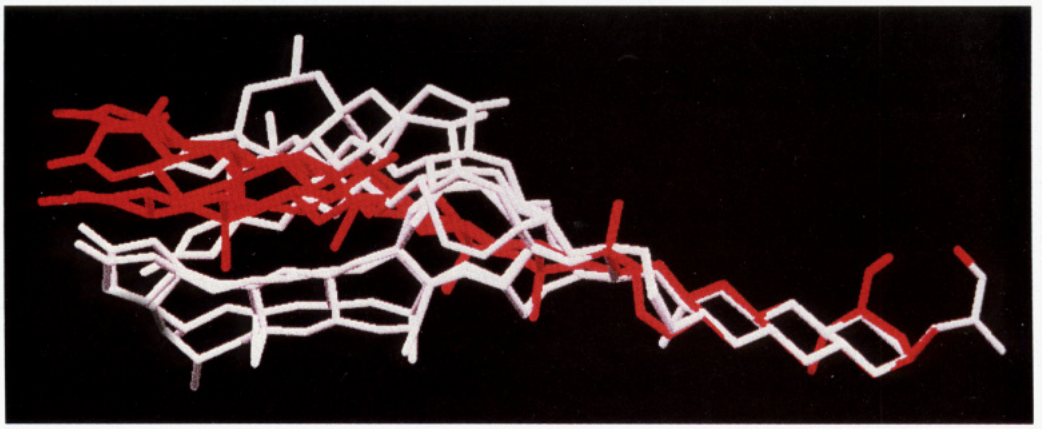

Figure 6. Superimposition of brevetoxin A conformers *d*, *f*, *i*, and *w* and brevetoxin B conformers 1, 2, and 5, with the hydrogens deleted for clarity.

(or more) of these approximates the geometry of the toxin when it is bound to site 5.

Detailed comparison of the brevetoxin A and B[12] backbones reveals a number of similarities in gross shape. Both toxins have straight and bent conformations available to them, and both are approximately the same length from end to end (~30 Å). Most noteworthy is the fact that the G-ring of brevetoxin A and the H-ring of brevetoxin B both exhibit a preference for the boat–chair conformation, which is apparently necessary for binding.[16] Because the GHIJ-rings of brevetoxin A and the HIJK-rings of brevetoxin B (including the side chains) have the same carbon skeleton, differing only in a double bond and the location of two angular methyl groups, and because the side chains of both toxin skeletons are structurally similar, we assume that these portions of the toxins occupy the same region of the binding site. The only other regions of the toxins which are similar are the A-ring lactones. One would reasonably expect these two similar regions, at opposite ends of the molecules, to coincide when their binding geometries are superimposed. Recall (Figure 2) that the two B-ring conformers of brevetoxin A have their carbonyl oxygens in almost exactly the same place.

Figure 5 illustrates the superimposition of all 24 brevetoxin A conformers (white) with seven brevetoxin B conformers having the required[16] H-ring boat–chair conformation (red). The "tails" of both are superimposed strictly. Clearly, a wider range of shapes are available to the brevetoxin A backbone, but since both backbones bind competitively to the same site, a similar geometry must be available to both backbones. Elimination of brevetoxin A conformers lacking the G1 conformation and any toxin (of either color) whose lactone carbonyl oxygen is >5 Å away from any carbonyl oxygen of the other color reduced the number of possibilities to those illustrated in Figure 6: brevetoxin A conformers *d*, *i*, *f*, and *w* and brevetoxin B conformers[12b] 1, 2, and 5. Figure 7 shows CPK models

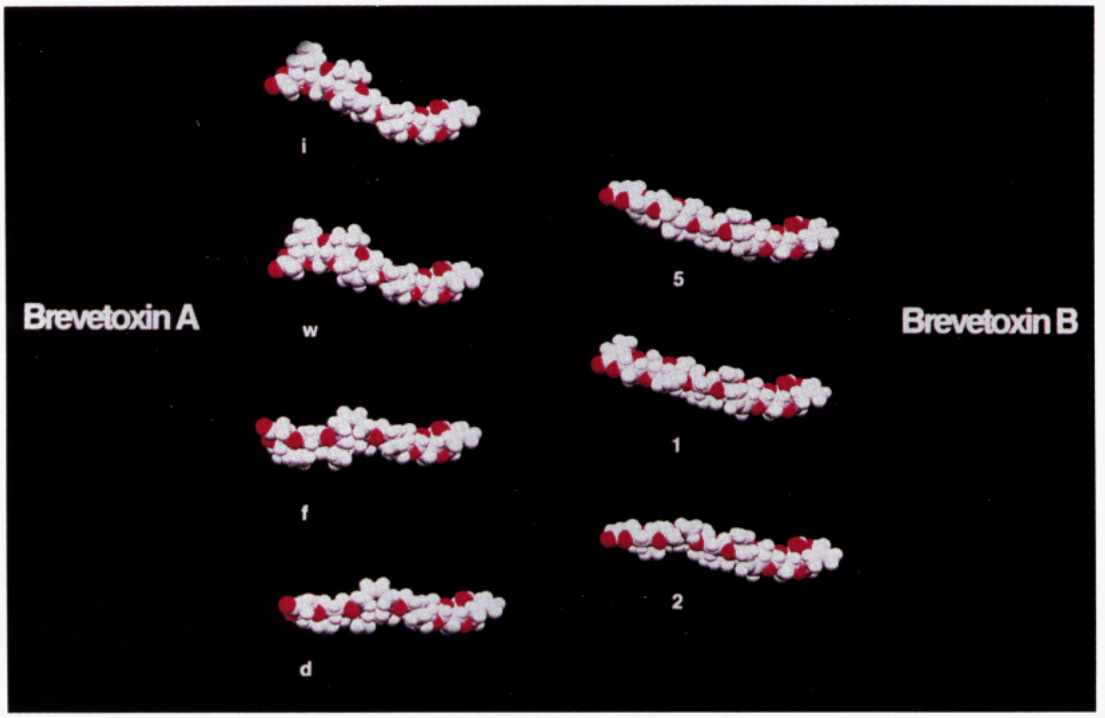

Figure 7. Same conformers as Figure 6, now separated and illustrated in the same orientation as Figure 6, as CPK models with hydrogens added.

of these seven structures, whose coordinates are also listed in the supplementary material.

In conclusion, we have completed a conformational analysis of brevetoxin A, the first natural product to have five-, six-, seven-, eight-, and nine-membered rings in the same molecule and which has a very flexible sequence of fused seven-, six-, eight-, and eight-membered rings. Both brevetoxins A and B have A-ring lactones at the "head" and also have very similar "tails". Since these two backbones bind competitively to the same receptor, we presume that the heads and tails of the two backbones occupy the same regions when they are bound. Since the B-ring flexibility of brevetoxin A has virtually no effect on the position of the A-ring carbonyl oxygen in space, and since several brevetoxin A and brevetoxin B conformers, when superimposed in the tail region, have the A-ring carbonyl oxygens of the two backbones in close proximity, we suggest that there is a functional group in the receptor region complementary to this carbonyl, probably a hydrogen bond donor such as a serine hydroxyl. We have compared the low-energy conformations of the brevetoxin A and B backbones and found that the best fit is for the straight conformations. These studies, along with the structure–activity studies of modified brevetoxin B reported in the following paper in this issue[16] prompt us to suggest that *the common pharmacophore for the toxins that bind to site 5 is a roughly cigar-shaped molecule, 30 Å long, bound to its receptor primarily with hydrophobic and nonpolar solvation forces, possibly aided by strategically placed hydrogen bond donors near the site of the A-ring carbonyls.*[18] Further identification and characterization of the receptor site for these potent channel modulators should help to define structure function relationships of the voltage-gated sodium channel.

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Supplementary Material Available: Atomic coordinates for *d*, *i*, *f*, and *w* and brevetoxin B conformers 1, 2, and 5 and details of the search methodology (24 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm edition of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

(18) Preliminary data on the conformational analysis of ciguatoxin have revealed a number of "straight" conformations but have also revealed some surprisingly bent "hair-pin" shapes. Additionally, there is no carbonyl in ciguatoxin, so if the hydrogen bond hypothesis for the brevetoxins is true, then the hydrogen bond receptor on ciguatoxin must be an ether oxygen or else a latent carbonyl must be unmasked *in vivo*.