Full Papers

A New Polyether Ladder Compound Produced by the Dinoflagellate *Karenia brevis*

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A new ladder-frame polyether compound containing five fused ether rings was isolated from laboratory cultures of the marine dinoflagellate *Karenia brevis*. This compound, named brevenal, and its dimethyl acetal derivative both competitively displace brevetoxin from its binding site in rat brain synaptosomes. Significantly, these compounds are also nontoxic to fish and antagonize the toxic effects of brevetoxins in fish. The structure and biological activity of brevenal, as well as the dimethyl acetal derivative, are described in this paper.

A number of bioactive polyether compounds have been isolated from the marine dinoflagellate *Karenia brevis*, the organism responsible for toxic red tides along Florida's Gulf Coast. The most well-known bioactive compounds isolated from *K. brevis* are a family of neurotoxins called the brevetoxins (Figure 1), which consist of nine different toxins with two different structural backbones: brevetoxin-A (containing 10 fused cyclic ether rings, 5,8,6,7,9,8,8,6,6,6)[1−3] and brevetoxin-B (containing 11 fused cyclic ether rings, 6,6,6,7,7,6,6,8,6,6,6).[3−8]

Brevetoxins bind with high affinity to site 5 of voltage sensitive sodium channels (VSSC) in neurons.[9,10] Binding of brevetoxins to tissues containing VSSC results in membrane depolarization, repetitive firing, and increased sodium currents.[11−14] Investigation of the effect of brevetoxins on excitable membranes using voltage clamp experiments indicates that brevetoxins activate VSSC by prolonging mean open time, inhibiting channel inactivation, and shifting the channel activation potential to more negative values.[12−14] During *K. brevis* red tides humans are most commonly affected by brevetoxins that have been aerosolized in sea spray or bioaccumulated in shellfish. Inhaled brevetoxins cause respiratory irritation and breathing difficulties in sensitive populations.[15−17] At sufficiently high concentrations ingested brevetoxins lead to a collection of symptoms commonly referred to as neurotoxic shellfish poisoning (NSP).[18,19] NSP in humans is characterized by sensory abnormalities, cranial nerve dysfunction, gastrointestinal symptoms, and sometimes respiratory failure.[19]

In 1989 Prasad and Shimizu[20] isolated and described another polyether ladder compound from *K. brevis* cultures that contained a different structural backbone and named it hemibrevetoxin-B. Hemibrevetoxin-B contains structural features similar to brevetoxin but is about half of the size and contains only four fused cyclic ether rings (6,6,7,7). Hemibrevetoxin-B (Figure 2) showed cytotoxicity in mouse neuroblastoma cells at concentrations of 5 μM, but no fish or mouse bioassays were reported.[20] Brevetoxin-specific receptor binding assays were not yet available at that time.

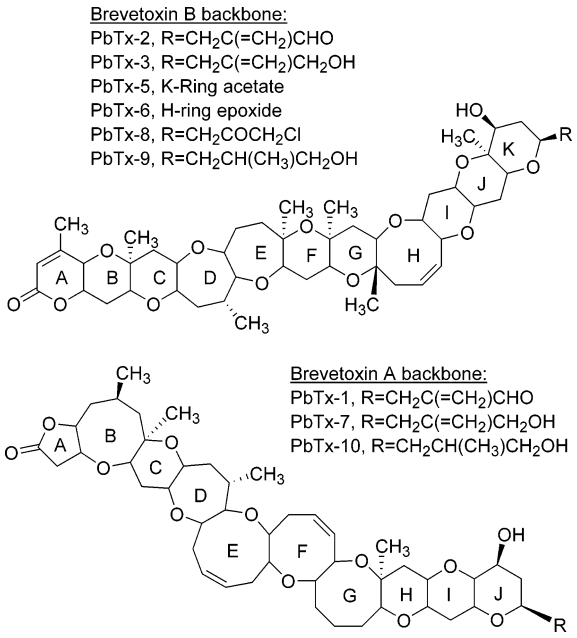

Figure 1. Structure of the nine known brevetoxins.

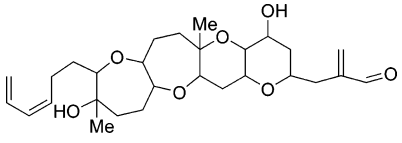

Figure 2. Hemibrevetoxin-B.

In this report we describe the isolation, structural characterization, and biological activity of a new ladder-frame polyether aldehyde named brevenal (Figure 3a), isolated from cultures of *K. brevis*. Brevenal as well as its dimethyl acetal derivative (Figure 3b) contain five fused cyclic ether rings, have low toxicity to fish, and have a

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Table 1. NMR Data for Brevenal and Brevenal Acetal in d_6-Benzene

	brevenal			brevenal acetal		
no.	δ_H	δ_C	HMBC	δ_H	δ_C	HMBC
1	10.1d	191.4 CH	C2	5.26d	101.5 CH	C40 C41
2	6.15d	126.4 CH	C4 C34	5.85d	124.0 CH	C3 C40 C41
3		157.1 C			136.8 C	
4		136.2 C			141.8 C	
5	5.82t	135.0 CH	C3 C6 C7 C35	5.73t	128.7 CH	C4 C35
6	2.02/2.14m	26.8 CH_2	C5 C7 C8	2.12/2.24m	26.3 CH_2	C5
7	1.10/1.48m	33.1 CH_2	C5 C6 C8	1.19/1.56m	33.5 CH_2	
8	3.29dt	71.3 CH	C6 C7 C9 C36	3.37m	71.1 CH	
9	1.47m	33.8 CH	C8	1.48m	33.8 CH	
10	1.76dd	35.7 CH_2	C11	1.49/1.74m	35.7 CH_2	
11	4.01dd	76.9 CH	C10 C12 C13 C37	4.01dd	77.0 CH	
12		77.6 C			77.5 C	
13	2.24/2.33m	48.4 CH_2	C12 C14 C37	2.26/2.36m	48.3 CH_2	C12 C37
14	4.09m	70.3 CH	C15	4.07m	70.2 CH	
15	3.46dd	75.4 CH	C16 C17	3.45dd	75.4 CH	
16	3.72m	74.1 CH		3.71m	74.0 CH	
17	1.86/2.25dd	34.9 CH_2	C16 C18	1.83/2.25m	34.9 CH_2	
18	2.95dd	82.4 CH	C20 C38	2.92dd	82.3 CH	
19		77.1 C			77.5 C	
20	1.69/1.78m	38.2 CH_2	C19 C21	1.69m	38.0 CH_2	C19
21	1.78/2.17m	30.2 CH_2	C19 C20 C22	2.15m	30.2 CH_2	C20 C22
22	3.36q	86.7 CH	C21 C23	3.33m	86.5 CH	C23
23	3.18m	85.2 CH	C22 C25	3.17m	85.0 CH	C22
24	1.78/1.91m	29.8 CH_2	C23 C26	1.78/1.19m	29.7 CH_2	
25	1.53/1.55m	39.2 CH_2	C24 C26 C27	1.51/1.53m	38.9 CH_2	C23 C26
26		74.3 C			74.4 C	
27	3.17m	87.8 CH_2	C25 C26 C28 C29 C39	3.16m	87.6 CH	C26
28	1.45/1.58m	31.0 CH_2	C27	1.43/1.60m	31.0 CH_2	C29
29	2.36m	25.7 CH_2	C27 C28 C30 C31	2.33m	25.5 CH_2	C28 C30
30	5.46q	133.4 CH	C28 C29 C31 C32	5.39q	133.3 CH	C32
31	6.08t	130.5 CH	C29 C30 C32 C33	6.09t	130.5 CH	
32	6.78dt	133.1 CH	C30 C31	6.78dt	133.0 CH	C31
33	5.06/5.17dd	117.4 CH_2	C31 C32	5.06/5.17dd	117.7 CH_2	C30 C31
34	1.81s	14.4 CH_3	C2 C3 C4	1.92s	15.2 CH_3	C2 C3 C4
35	1.56s	14.1 CH_3	C3 C4 C5	1.80s	14.5 CH_3	C3 C4 C5
36	0.96d	13.3 CH_3	C8 C9 C10	0.97d	13.2 CH_3	C8 C9 C10
37	1.19s	19.9 CH_3	C12 C13	1.2s	19.6 CH_3	C12 C13
38	1.14s	16.6 CH_3	C18 C19 C20	1.12s	16.5 CH_3	C18 C19 C20
39	0.99s	23.9 CH_3	C25 C26 C27	0.96s	23.9 CH_3	C25 C26 C27
C40, C41				3.23s	52.0 CH_3	C1

structural backbone different from both brevetoxins and hemibrevetoxins.

Results and Discussion

Brevenal (Figure 3a) was obtained as a lipid-soluble, colorless, noncrystalline compound. The UV spectrum showed two maxima at 227 and 290 nm, suggesting a conjugated carbonyl system. High-resolution positive ion FAB mass spectroscopy gave a protonated molecular ion at *m/z* 657.4043, corresponding to a molecular formula of $C_{39}H_{60}O_8$. This was confirmed by the ^{13}C and DEPT NMR spectra (Table 1), which revealed 39 carbons comprised of six methyls, 12 methylenes, 15 methines, five quaternary carbons, and one aldehyde carbon. The molecular formula corresponded to 10 double-bond equivalents, and by consideration of the DEPT and HSQC data, it was determined that brevenal contained five rings. The TOCSY and COSY spectra indicated five different spin systems (A−E) (Figure 4a). Spin system A, containing the aldehyde function, could be connected with system B through a series of HMBC correlations (Figure 4a) involving the olefinic methyl groups C-34 (δ_C 14.4; δ_H 1.81) and C-35 (δ_C 14.1; δ_H 1.56). Strong correlations were observed between the methyl protons H-34 and C-2, C-3, and C-4 and between H-35 and C-3, C-4, and C-5. An HMBC correlation between the H-37 methyl protons (δ_H 1.19) and C-12 and C-13, another from H-11 (δ_H 4.01) to C-12 and C-37, and from the methylene protons H-13 (δ_H 2.33/2.44) to H-37 established the relative location of spin systems B and C. Spin systems C and D were linked through a series of correlations between H-18 (δ_H 2.95) and C-20, H-20 (δ_H 1.69/1.78) and C-19, and a series of correlations between the C-38 methyl group (δ_C 1.14; δ_H 1.14) and C-18, C-19, and C-20. Finally, spin systems D and E were linked by a series of correlations between the methylene protons H-25 (δ_H 1.53/1.55) and C-26 and between the H-39 methyl protons (δ_H 0.99) and

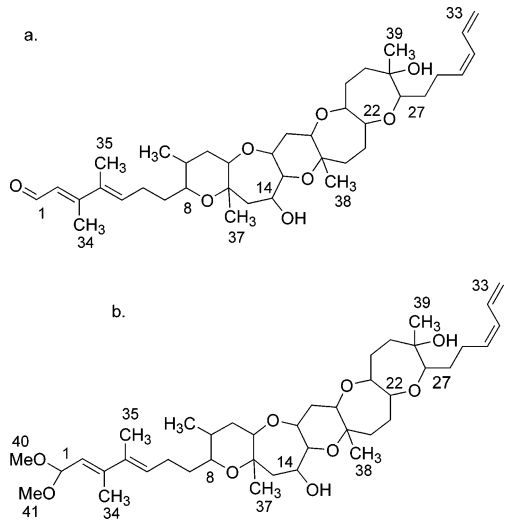

Figure 3. Structure of (a) brevenal and (b) brevenal acetal.

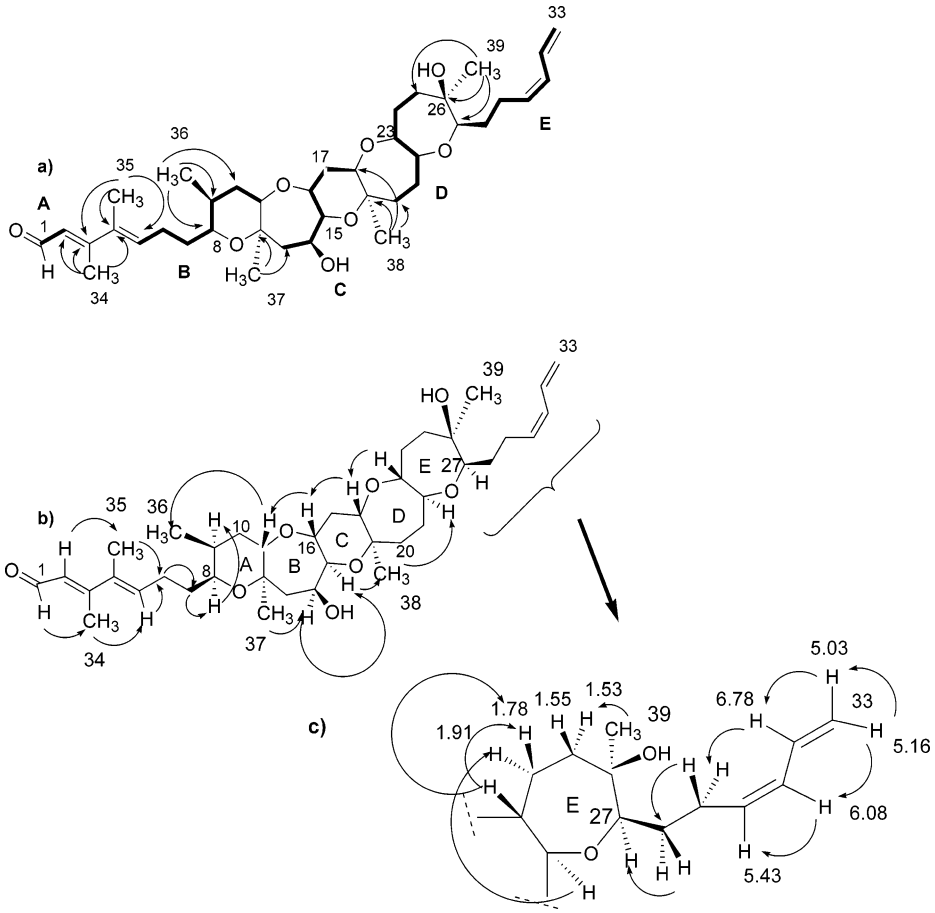

Figure 4. Structure of brevenal determined from NMR data in d_6-benzene: (a) ^{1}H−^{1}H-COSY (bold lines) and HMBC (arrows) correlations, (b) ^{1}H−^{1}H-NOESY correlations, (c) detailed view of ring E and side chain from part b.

C-25, C-26, and C-27. The remaining two oxygens in the molecular formula could be accounted for as two hydroxyl groups. The first was identified as a tertiary alcohol located at C26 on the basis of the chemical shift data (δ_C 74.3), while the second was located at C14 (δ_C 70.3) in spin system C, on the basis of the chemical shift data (δ_C 74.34) and HMBC correlations between the H-14 methine (δ_H 4.09) and C-13 and C-15. This was further supported by a series of NOE correlations between H-14 and the methyl protons H-37 and H-15.

The *E,E* configuration of the conjugated double bonds in the aldehyde side chain was deduced from the NOESY data (Figure 4b). Key correlations were observed between the aldehyde proton and the C-3 methyl group, which in turn showed a strong correlation with the olefinic proton H-5. As further support, the C-4 methyl group showed a strong NOE correlation with H-2 and the allylic methylene protons H-6. Similarly, NOE correlations between H-32 and the allylic methylenes H-29 established the *Z* configuration of protons on C-30 and C-31 (Figure 4c). Other NOE correlations observed for brevenal (Figure 4b) were consistent with a series of *trans*-fused polyether rings, typical of ladder-frame dinoflagellate polyethers.[21] Finally, the relative stereochemistry of the ring 5 substituents was determined by a series of NOE correlations. Significantly, a correlation was observed between the C-26 methyl group and C-25 proton at δ 1.53, but not the proton resonating at δ 1.55, corresponding to the α-proton at H-25 (Figure 4c).

During the course of these studies another compound was isolated from the toxin extract as a colorless noncrystalline solid named brevenal acetal (Figure 3b). The molecular formula of this second compound was determined to be $C_{41}H_{66}O_9$, $[M + Na]^+ = 725.4$ amu, from NMR and MS data, 46 amu (C_2H_6O) larger than brevenal, which was also consistent with the ^{13}C NMR data (Table 1). The UV data (λ_{max} 227 nm) suggested a loss of the conjugated carbonyl moiety, supported by the absence of a carbonyl (aldehyde) resonance in the NMR spectra. Apart from this obvious difference, the NMR spectra of the two compounds were remarkably similar, suggesting both compounds possessed the same carbon skeleton, except that the ^{13}C resonance of the aldehyde carbon in brevenal was replaced by a new signal at δ_C 101.5 ppm, consistent with an acetal carbon. In addition, the ^{1}H NMR spectrum of the second compound contained a sharp 6H singlet at δ_H 3.23 ppm, corresponding to a dimethyl acetal group. The remainder of the 1D and 2D NMR data for the second compound was essentially identical to that for brevenal and established the structure as the dimethyl acetal derivative of brevenal. We have evidence (data not shown) that the brevenal acetal is a product of the isolation procedure and is easily converted back to brevenal in mildly acidic solutions to give a sodiated molecular ion $[M + Na] = 679.6$ in LC/MS experiments.

Brevenal and brevenal acetal interactions with VSSC were evaluated using a rat brain synaptosome receptor-binding assay.[9] Both brevenal and brevenal acetal inhibited [^{3}H]-PbTx-3 binding in a concentration-dependent manner (Figure 5) with a K_i for brevenal of 1.85 μM and a K_i for brevenal acetal of 0.68 μM. These results provide direct evidence that brevenal and brevenal acetal bind to site 5 of the VSSC on rat brain synaptosomes.

The antagonistic effects of brevenal and brevenal acetal on brevetoxin toxicity were tested using a fish bioassay

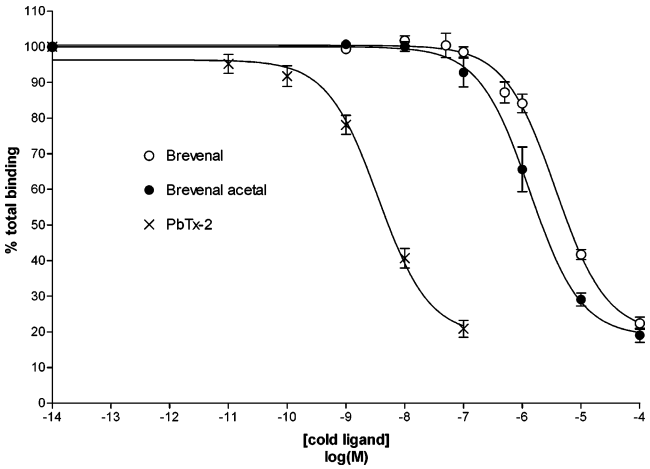

Figure 5. Displacement of tritium-labeled PbTx-3 ([^{3}H]-PbTx-3) from rat brain synaptosomes. All values are the mean of at least three experiments, with duplicate determinations at each concentration of competitor in each experiment. Radioactivity associated with the pellet (bound) and the supernatant (free) was determined. A displacement curve for PbTx-2 is provided for reference. Linear regression analysis of each double reciprocal plot yielded r^2 values > 0.98 (data not shown). Convergence of the plots near the ordinate indicates that the interaction between the ligands and [^{3}H]-PbTx-3 is competitive.

Table 2. Fish Bioassay

treatment	no. of subjects	time to death (min ± SEM)
control 200 μL ETOH	25	no deaths after 24 h
brevenal 1 μg/mL	10	no deaths after 24 h
brevenal acetal 1 μg/mL	5	no deaths after 24 h
PbTx-2 1 μg/mL	10	7.5 + 1.06 min
brevenal 1 μg/mL + PbTx-2 1 μg/mL	10	17.00 + 2.84 *p < 0.01
brevenal acetal 1 μg/mL + PbTx-2 1 μg/mL	5	9.36 + 0.72

with *Gambusia affinis*. In a 24 h toxicity test, brevenal and brevenal acetal were found to be nontoxic to *G. affinis* at concentrations of 1 μg/mL, with 100% of the fish surviving (Table 2). When the fish were exposed to 1 μg/mL PbTx-2, 100% mortality was observed within 7.5 ± 1.06 min. When the fish were exposed to both brevenal and PbTx-2 (1 μg/mL PbTx-2 and 1 μg/mL brevenal), mortality was delayed significantly. These results are consistent with the proposed antagonist role of brevenal and suggest that brevenal can inhibit or delay brevetoxin-induced mortality in fish. Fish exposed to both brevenal acetal and PbTx-2 did survive longer that those in PbTx-2 alone, but the delay did not achieve statistical significance ($p \geq 0.01$).

Brevenal, together with its dimethyl acetal derivative, represents a new fused-ring polyether compound isolated from large-scale laboratory cultures of the toxic dinoflagellate *K. brevis*. These compounds have also been found in extracts of natural red tide blooms from both the east and west coast of Florida and have been found to vary with the phase of the bloom as well as the toxicity of the blooms.[22,23] Both compounds contain five fused polyether rings, and while the terminal seven-membered ring and attached side chain are similar to the hemibrevetoxins,[20] the arrangement of 6,7,6,7,7 ether rings in brevenal is unique among dinoflagellate metabolites.

Like gambieric acid[24] both brevenal and the acetal displace natural brevetoxin from site 5 of the VSSC in rat brain synaptosomes, and brevenal also acts as a brevetoxin antagonist with *in vivo* bioassays. Unlike gambieric acid however, brevenal and the acetal are nontoxic in a fish bioassay. Interestingly, using aerosilized compound, brevenal pretreatment of sheep with airway hypersensitivity to *Ascaris suum* antigen significantly decreased bronchoconstriction produced by pure brevetoxins (PbTx-2 and -3) and crude *K. brevis* extract.[25] Brevenal and related derivatives have the potential to serve as therapeutic agents for the treatment of brevetoxin poisoning because of their low toxicity and ability to block the effects of brevetoxins *in vivo*.

Experimental Section

Isolation of Brevenal and Brevenal Acetal. Cultures of *K. brevis* (200 L; Wilson's 58 clone) were extracted with $CHCl_3$ after 30 days growth. The $CHCl_3$ extract was concentrated *in vacuo* and resuspended in petroleum ether (PE) 60/40 MeOH/H_2O (90:10:1; v/v/v). The MeOH layer was separated and concentrated in vacuo, resuspended in chloroform/MeOH/H_2O (90:10:1, v/v/v), and applied to a silica gel low-pressure column (Analtech, ICN, Silica, TSC, 60 Å). Elution with 90:10:1 $CHCl_3$/MeOH/acetic acid (v/v/v) yielded a dark brown band containing brevenal, brevenal acetal, and the usual suite of brevetoxins. This dried fraction was resuspended in a small volume of acetone and applied to a silica gel TLC plate (20 × 20 cm; Analtech) and eluted with $CHCl_3$/MeOH/H_2O, (90:10:1, v/v/v). Bands were visualized with UV light (254 nm), the band containing brevenal and the acetal was removed from the plate, and the organic compounds were eluted with acetone. The acetone extract was applied to a second TLC plate (20 × 20 cm; Analtech) and eluted with PE 60/40/acetone (70:30 v/v). The band containing brevenal and some brevetoxins was removed and eluted from the silica gel using acetone. This sample was purified as follows in a series of HPLC steps: the pigments and most of the brevetoxins were separated using a C-18 column (Varian; 8 mm × 250 mm; eluant 90% MeOH and 10% water, v/v, 3.4 mL/min, $\lambda = 215$ nm). Brevenal eluted at about 8 min as a broad complex peak containing PbTx-3, while the acetal eluted at around 12 min and contained some PbTx-2. The peaks were collected separately and further purified by reversed-phase HPLC (Phenomenex reverse-phase phenyl-hexyl; 8 mm × 250 mm; 99% MeOH and 1% water, flow rate 3.4 mL/min, $\lambda = 215$ nm) to remove any traces of brevetoxins. A third and final cleanup was performed (Phenomenex reverse-phase phenyl-hexyl; 4 mm × 250 mm; 90% MeOH and 10% water, flow rate 1.4 mL/min, $\lambda = 215$ nm) to yield brevenal (t_R 6 min; 20 mg) and brevenal acetal (t_R 7 min; 2.2 mg) with purity greater than 98% as measured by HPLC-UV.

Spectroscopy. The UV spectra were recorded on HP1100 HPLC (90% MeOH and 10% water, Agilent C-18, 4 × 250 mm column, flow rate 1.4 mL/min) equipped with a Waters UV diode array detector. NMR spectra were obtained with Bruker 400 and 500 MHz spectrometers in d_6-benzene, d_6-acetone, d_3-methanol, and $CDCl_3$. The following NMR experiments were run on 10 mg of brevenal or 2.2 mg of brevenal acetal: ^{1}H, ^{13}C, DEPT-135, COSY, HSQC, HMBC, and NOESY. High-resolution FAB$^+$MS for brevenal was performed at the UC Riverside Mass Spectrometry facility. Low-resolution mass spectroscopy for brevenal dimethyl acetal and acid hydrolysis of the acetal back to brevenal were performed at aaiPharma Inc. Low-resolution mass spectra were obtained on a ThermoFinnigan LCQ Deca ion trap mass spectrometer equipped with an electrospray ionization source. The ion spray voltage was set at 4.5 kV, and the capillary temperature was 250 °C. All other instrumental parameters were automatically tuned by the instrument's software to maximize the signal for the *m/z* of interest. The instrument was scanned from 200 to 1500 *m/z* with the spectra shown consisting of the sum of 20 scans. Samples for MS analysis were dissolved in either 50:50 water/acetonitrile or 49:49:2 water/acetonitrile/acetic acid before being directly injected at a flow rate of 10 μL/min.

Fish Bioassay. Male mosquito fish (*Gambusia affinis*) were placed individually in 50 mL beakers containing 20 mL of water. The test compounds (PbTx-2, brevenal, and brevenal

acetal) were dissolved in ethanol at a concentration 0.1 mg/mL and delivered to the beaker in 200 μL of ethanol solution. The control fish received 200 μL of ethanol ($n = 20$). Fish were exposed to toxin alone (1 μg/mL water, $n = 10$), brevenal alone (1 μg/mL water, $n = 10$), brevenal acetal alone (1 μg/mL water, $n = 5$), or both brevenal (1.0 μg/mL water) and PbTx-2 (1.0 μg/mL water, $n = 5$) or brevenal acetal (1.0 μg/mL water) and PbTx-2 (1.0 μg/mL water, $n = 5$). In competition experiments brevenal and brevenal acetal were added 3 min before the toxin. After addition of the different compounds the fish were monitored for 24 h or until the time of death. Significant differences were determined using a two-way Student's *t*-test.

Synaptosome Binding Assay. Briefly, as described by Poli et al. 1986,[9] synaptosomes were prepared from frozen whole rat brains (male, Sprague−Dawley) by homogenization in 0.32 M sucrose (containing protease inhibitors: 1 mM iodoacetamine, 1 mM 1,10-phenanthroline, 0.1 mM phenylmethylsulfonyl fluoride, 1 μM pepstatin A) followed by three centrifugation steps. The P3 fraction was diluted to 1 mg/mL protein (determined by a modified Lowry technique) with HEPES binding medium containing protease inhibitors. Binding was determined at 4 °C in 1 mL total volume in the presence of 0.1 mg of bovine serum albumin. Serial dilutions of competing ligands were prepared in ethanol and added (10 μL) to 790 μL of binding medium in 1.5 mL Eppendorf centrifuge tubes (duplicates at each concentration of competitor). A solution of [^{3}H]-PbTx-3 in binding medium (100 μL) was added to yield a final concentration of approximately 5 nM, near the reported K_D value for rat brain synaptosomes (2.2 nM). After addition of 100 μL of synaptosome suspension, the contents of each tube were vortexed and allowed to equilibrate for >1 h on ice. Tubes were then centrifuged, the supernatant was quickly aspirated, the pellet was washed with immediate aspiration, and the bottom of the tube (containing the pellet) was removed. The radioactivity associated with the pellet was quantified by liquid scintillation spectroscopy. Samples were run in triplicate. Nonlinear regression analysis was performed on the resulting competition binding data using GraphPad Prism version 3.02 for Windows (GraphPad Software, San Diego CA, www.graphpad.com).

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