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Brevisulcatic Acids, Marine Ladder-Frame Polyethers from the Red Tide Dinoflagellate *Karenia brevisulcata* in New Zealand

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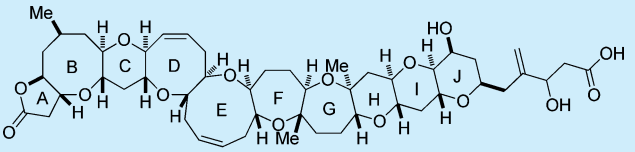
Supporting Information

ABSTRACT: The isolation and structural determination of new marine ladder-frame polyethers, brevisulcatic acids-1 (**1**) and -4 (**2**) are reported. Brevisulcatic acids were isolated from the dinoflagellate *Karenia brevisulcata*, which was identified as the causative species of a major red tide event in New Zealand in 1998. The ether ring composition and a β-hydroxy, γ-methylene valeric acid side chain of **1** and **2** are common, but **2** has a γ-lactone as the 5-membered A-ring while **1** is the seco acid analogue. Compound **2** has structural and bioactivity similarities to brevetoxin A.



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Red tides can cause serious problems such as human intoxications and massive kills of marine life. In 1998, a major red tide incident occurred in Wellington Harbour, New Zealand, that led to widespread ecological damage with mortality of many species from diatoms to fish. More than 500 human bystanders in the surrounding harbor suffered from respiratory distress with a dry cough, severe sore throat, runny nose, skin and eye irritations, severe headaches, and facial sunburn sensations. A new dinoflagellate, *Karenia brevisulcata*, was identified as the causative species of the incident.[1] Cell culture extracts of the dinoflagellate showed mouse lethality and cytotoxicity. From these extracts two new classes of marine biotoxins were isolated.[2] Brevisulcenals (initially called *Karenia brevisulcata* toxins (KBTs) in ref 2) were isolated from the neutral lipophilic fraction. The molecular weights of these toxins are more than *m*/*z* 2000,[2] and the structure of a major component, brevisulcenal-F (KBT-F, $C_{107}H_{160}O_{38}$), has been elucidated and reported.[3] Structural features of KBT-F are 24 ether rings including an unusual dihydrofuran, 13 hydroxyl groups, 13 methyl groups, and a 2-methylbut-2-enal terminus. KBT-F contains 17 contiguous ether rings, which is the longest of known polycyclic ethers. In addition to the KBTs, more water-soluble polycyclic ether toxins were obtained from an acidic fraction. These were named brevisulcatic acids (BSXs)[2] and had molecular weights of around *m*/*z* 900. BSXs were extracted with dichloromethane after adjustment of the crude extract to acidic conditions. Preliminary mass spectrometric analysis (ESI^+), NMR evidence, and toxicity data suggested the presence of polycyclic ethers containing terminal carboxylic acid groups with structural and bioactivity similarities to brevetoxins. However, conformer effects confounded the full interpretation of the NMR spectra.[2] In this paper, we report the structural elucidation of brevisulcatic acids-1 (BSX-1, **1**) and -4 (BSX-4, **2**) by detailed analysis of low-temperature NMR spectra and high energy MALDI TOF MS/MS spectra (Figure 1).

Brevisulcenals (KBTs) and brevisulcatic acids (BSXs) were extracted from mass cell cultures in stationary phase. After lysis of cells with acetone and stirring for 1 h, the cultures were diluted with water and passed through HP20 resin. Both classes of polyether toxins were extracted from the resin using acetone. The crude extract was dissolved in 55% MeOH with pH 7.2 phosphate buffer, and the KBTs were extracted with $CHCl_3$. The aqueous fraction was adjusted to pH 4, and then the BSXs were extracted with dichloromethane. The dichloromethane extract was subjected to solid-phase extraction (Strata-X) with stepwise elution using MeOH/water (1:1, 7:3, 8:2, 9:1, 1:0). The 100% MeOH fraction was chromatographed on an ODS reversed-phase column with a gradient elution of MeOH/water. From 150 L culture media, 0.17 mg of **1** and 0.62 mg of **2** were obtained by repeated chromatography on a reversed-phase column. To facilitate ^{13}C-related NMR measurements, ^{13}C labeled **1** (1.2 mg) and **2** (1.1 mg) were isolated from cultures grown in media enriched with ^{13}C $NaHCO_3$. The ^{13}C-enrichment of the BSXs was estimated to be about 10%.[3]

No UV maximum above 210 nm indicated that **1** and **2** did not contain conjugated bond systems. The HR ESI MS gave

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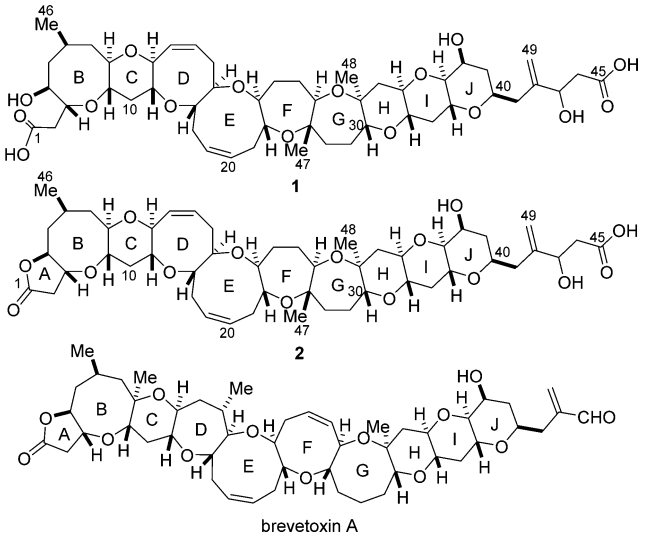

Figure 1. Structures of brevisulcatic acids-1 (**1**) and -4 (**2**) and brevetoxin A.

the molecular formula of **1** as $C_{49}H_{72}O_{16}$ ($[M + H]^+$ m/z 917.4827 calcd 917.4899) and that of **2** as $C_{49}H_{70}O_{15}$ ($[M + H]^+$ m/z 899.4747, calcd 899.4793) with a difference of H_2O. These molecular formulas are similar to that of brevetoxin A ($C_{49}H_{70}O_{13}$).[4] Structural interconversion under acidic and basic conditions suggested that **2** was a lactone form of **1**. The similarity of NMR spectra of **1** and **2** also suggested that the structures of **1** and **2** were identical except for this A ring. By analyses of COSY and HSQC measured at 25 °C, proton connectivities from H_2-2 to H_2-15, from H-32 to H_2-41, and from H-43 to H_2-44 were assigned, and observed HMBC cross peaks from H_2-2 to C1 (174.0 ppm), H_2-49 to C41, C42 (151.1 ppm) and C43, and H_2-44 to C45 (177.0 ppm) elucidated the structures around two carbonyls and an exomethylene (Figure 2). Those connectives belonged to rings A, B, C, H, I, and J and

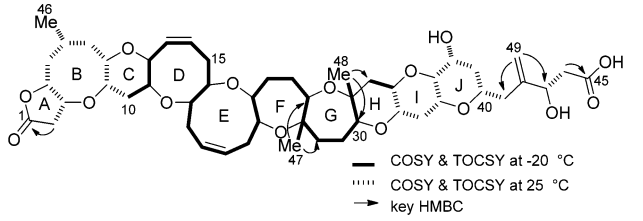

Figure 2. COSY and TOCSY at low temperature and room temperature and key HMBC correlations for structural elucidation of **2**.

a β-hydroxy, γ-methylene valeric acid side chain. The rings B, H, I, and J were identical in **1**, **2**, and brevetoxin A. However, considering the molecular formula, 18 carbons and 26 protons (C-16 to C-31) were not assignable because of extremely broad or missing NMR signals, from H-16 to Me-48, on the rings E to G in the middle portions of **1** and **2**. This was presumed to be caused by slow conformational perturbation of a 9-membered ether ring including a double bond as observed in brevetoxin A[4] and ciguatoxins.[5] NMR spectra of **1** and **2** were remeasured at low temperature (−20 °C), which was used to solve this problem for ciguatoxins.[5] This resulted in the appearance of new signals and sharpening of broad peaks. However, a new problem arose, with almost 70 ^{13}C signals, including some weak peaks, being observed in the ^{13}C NMR spectra. This was deduced to arise from **1** and **2** having more than one stable conformer at low temperatures, unlike the 9-membered ether ring in the ciguatoxins. The conformational perturbation at −20 °C affected the NMR signals for C10 to C33 (Figure 2), with two sets of ^{1}H and ^{13}C NMR peaks being observed (Table 1).

Table 1. NMR Data for BSX-4 (2) in Pyridine-d_5 at −20 °C

no.[a]	δ_H	δ_C	no.[a]	δ_H	δ_C	no.[a]	δ_H	δ_C
1		174.0	18′	2.24	37.1	30	3.33	83.5
2	2.95	38.1		2.91		30′	3.32	84.6
	3.20		19	5.76	127.9	31		76.3
3	4.38	81.9	19′	5.99	128.4	31′		76.4
4	4.28	85.2	20	5.76	129.3	32	1.86	45.1
5	1.41	43.2	20′	6.06	129.9		2.38	
	2.02		21	2.24	31.9	32′	1.86	45.3
6	1.67	29.4		2.96			2.38	
7	1.84	44.0	21′	2.02	32.8	33	3.49	77.7
	1.94			2.87		33′	3.49	77.6
8	3.03	82.9	22	3.65	71.2	34	3.19	78.4
9	3.33	82.6	22′	3.90	74.6	35	1.84	36.6
10	1.69	40.3	23	3.77	89.3		2.61	
	2.24		23′	3.33	84.6	36	4.51	62.9
10′	1.69	40.1	24	1.86	32.6	37	3.26	81.5
	2.24			2.22		38	4.45	65.7
11	3.25	79.4	24′	1.73	32.1	39	2.02	35.4
11′	3.25	78.3		2.24			2.10	
12	3.97	79.8	25	1.75	28.3	40	4.48	71.4
12′	3.97	79.9		2.10		41	2.94	36.7
13	5.91	134.1	25′	1.75	28.7		3.83	
13′	5.98	134.5		2.10		42		151.1
14	5.76	127.4	26	3.82	76.9	43	5.43	71.7
14′	5.86	127.5	26′	3.77	76.1	44	3.09	43.6
15	2.29	32.9	27		80.2		3.22	
	2.75		27′		79.3	45		177.0
15′	2.38	32.2	28	1.95	41.0	46	0.88	27.6
	2.69			2.10		47	1.41	20.8
16	3.81	91.5	28′	1.91	41.4	47′	1.31	19.3
16′	3.57	85.6		2.12		48	1.41	16.8
17	3.49	78.3	29	1.86	25.8	48′	1.47	16.9
17′	3.83	79.0		2.02		49	5.34	111.5
18	2.31	36.1	29′	1.86	25.8		5.72	
	2.93			2.02				

[a]Numbers with prime mean that they are assigned as positions in the minor conformer.

For example, in ^{13}C and HSQC spectra measured at −20 °C, four methyl singlet signals appeared, but the peak heights of these methyl signals were half of the methyl doublet (Me-46). They were deduced to arise from two quaternary methyl groups (Me-47 and Me-48) each present in two conformrs with similar abundances forming doublet ^{13}C signals. The signals derived from each conformer were assigned independently on the basis of the ^{13}C peak heights. The unobserved signals at 25 °C were assigned to six oxymethines, two olefinic methines, six methylenes, two methyl singlets, and two quaternary carbons. The NMR experiments at low temperature enabled the elucidation of the structure around the 9-membered ether ring E (Figure 2). The proton connectivity from H_2-15 to H-26 and H_2-28 to H-30 was assigned by the COSY and TOCSY measured at −20 °C. The partial structures in the skeletal

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structure were interupted by the quaternary oxycarbons bearing the methyl singlets, Me-47 and Me-48.

The structure around the quaternary carbons was elucidated by HMBC and DEPT experiments. HMBC correlations from Me-47 to C24, C25, and C26 and from Me-48 to C30, C31, and C32 were observed, even though the methyl signals were split. Thus, all the carbons were assembled into the structure. The positions of ether linkages were determined by the NOE correlations and proton coupling constants. The observed NOE correlations H-3/H-9, H-8/H-12, H-11/H-17, H-16/H-23, H-22/Me-47, H-26/Me-48, H-30/H-34, H-33/H-37, and H-36/H_2-41 and ^{1}H chemical shifts of angular protons confirmed the positions of the ether rings and the manner of fusing. The sizes of the fused ether rings D, E, F, and G are 8, 9, 7, and 7, respectively, for **1** and **2** versus 7, 9, 8, and 8 for brevetoxin A. The existence of the γ-lactone in **2** was deduced by the change in proton chemical shift of H-4 from δ 4.28 ppm for **2** in pyridine-d_5 (Table 1) to δ 3.25 ppm for **1** in CD_3OD (Supporting Information). Thus, the position of the lactone bond was determined at C4 and the structure of **2** was confirmed as the lactone form of **1**, as for ring A in brevetoxin A. LC−MS experiments showed that **1** was detected in the crude HP-20 extract from acetone elution of the resin. This supports **1** being a component of the algal culture and not an artifact produced by acid hydrolysis of **2** during partitioning. Similar ring-opened metabolites of brevetoxins have been reported.[6]

The stereochemistry of the methyl and the hydroxyl group substituted on the ether rings was clarified by NOE correlations and the proton coupling constants. The small coupling constant (3 Hz) between H-37 and H-38 indicated the hydroxyl group at C38 to be axial. Observed NOE signals H-4/H-5α and H-6, H-8/H-6 and H-7α, H-3/H-5β, and H-7β/H-9 indicated that the 8-membered ether ring B was in a crown conformation and the methyl at C6 was a β-methyl. Thus, the stereochemistry of the methyl and the hydroxy group on the rings B and J were also similar to that of brevetoxin A.[4]

In order to confirm the structure of **2**, a MALDI-spiralTOF−TOF experiment was conducted (Figure 3).[3,7] MALDI-spiralTOF−TOF is a useful technique for structural confirmation of ladder-frame polyether compounds because of the cleavages induced at high collision energies. Compound **2** did not give rise to an intense negative ion that was suitable as a precursor ion for MS/MS experiments because the carboxylic acid is a weak charge site for MALDI ionization. Therefore, **2** was conjugated with taurine to provide a sulfonic acid as a strong charge site (Scheme 1). *N*,*N*′-Dicyclohexylcarbodiimide (DCC) was added to a solution of **2** and *N*-hydroxy-5-norbornene-2,3-dicarboximide (HONB) in THF/dioxane at 0 °C, followed by mixing with the sodium salt of taurine in dioxane/H_2O at rt. The water layer was washed with ethyl acetate and purified on a reversed-phase column.[8] Only 0.01 mg of taurine amide **3** was obtained from 0.1 mg of **2** (Scheme1).

Negative-mode MALDI MS of **3** gave an intense precursor ion at *m*/*z* 1004.5 $[\mathbf{3} - Na]^-$ using 9-aminoacridine as a matrix. The MALDI-spiralTOF−TOF produced prominent product ions generated by bond cleavages at the characteristic sites for polycyclic ethers (Figure 3 A). The mass differences 58, 98, and 56 between products ions at *m*/*z* 946, 848, and 792 confirmed the sequence of the γ-lactone, an 8-membered ether ring bearing a methyl, and a 6-membered ether ring. Product ions at *m*/*z* 792, 710, 614, 544, and 460 confirmed the sequence of

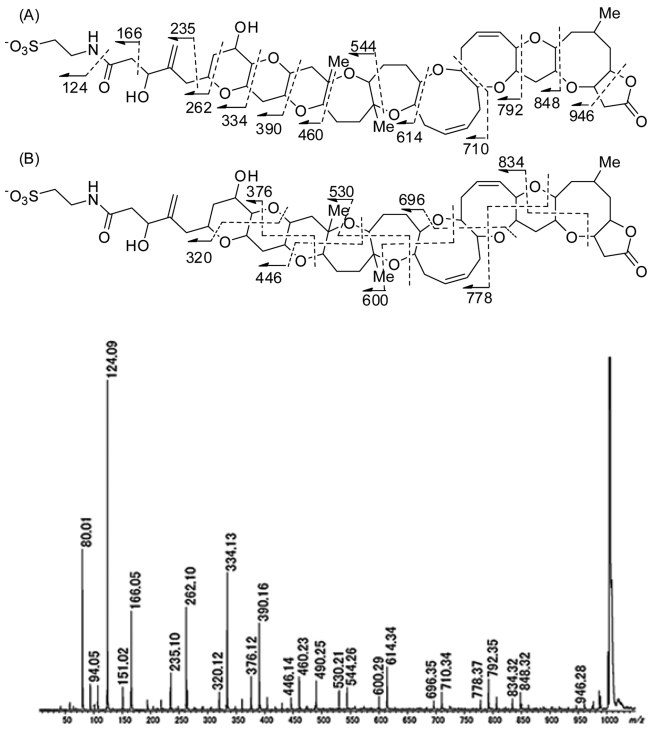
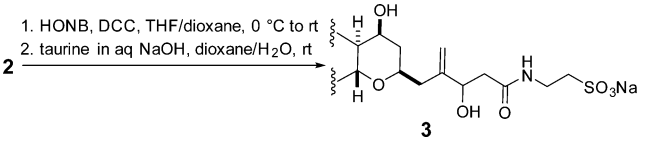


Figure 3. Cleavage sites: (A) A-type and (B) B-type fragmentation and product ion mass spectrum of **3** by MALDI-spiralTOF-TOF. The specrum was generated for the precursor ion $[M - Na]^-$ at *m*/*z* 1004.5.

Scheme 1. Derivatization of Taurine Amide (3)



ether rings around the 9-membered ether ring E. The mass differences 82, 96, 70, and 84 verified the ring size of 8-, 9-, 7-, and 7-membered ether rings and the sequence of the ether rings D, E, F, and G. The mass differences 70, 56, and 72 between *m*/*z* 460, 390, 334, and 262 also confirmed the three contiguous 6-membered ether rings H, I, and J with the angular methyl, nonsubstitution and the hydroxyl group, respectively. The lower intensity ions at *m*/*z* 834, 778, 696, 606, 530, 446, 376, and 320 are proposed to be generated from a type-B fragmentation (Figure 3B).[4b,7] These results supported the proposed structures of **1** and **2** deduced from the NMR spectral analysis. Therefore, the structures of BSX-1 and BSX-4 were determined as shown in **1** and **2**. Limited amount of purified materials has hampered determination of the absolute configuration of the ether ring portion and C43. Once more material has been generated, and the absolute configuration will be reported elsewhere.

The toxic dinoflagellate *K. brevisulcata* produces two kinds of ladder-frame polyethers which have different structures. Interestingly, the long contiguous ether ring assembly with the 2-methylbut-2-enal terminus of KBT-F is reminiscent of gymnocins[9] from *Karenia mikimotoi*, while the structure of **2** resembles that of brevetoxin A from *Karenia brevis*. The ring arrangement of both termini in **2** is identical with that in brevetoxin A, and there is a slow conformational change that occurs around the 9-membered ether ring. The 3-hydrox-

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ypropionic acid structure in the side chain is proposed to be generated by an aldol condensation of acetyl-CoA with a terminal enal such as that in brevetoxin A. In the neuroblastoma cell assay for voltage gated sodium channel activity (neuro-2a cells in the presence of veratridine and ouabain), **2** gave a cytotoxicity EC_{50} of 20 ng/mL, while that for **1** was 300 ng/mL.[2] The 15-fold higher cytotoxicity of **2** than that for **1** indicates that the γ-lactone in **2** plays a significant role in the cytotoxicity of **2**.[10] Without addition of veratridine and ouabain, **2** did not show cytotoxicity against neuro-2a, and therefore, the mode of action is activation of sodium channels, analogous with the brevetoxins and the ciguatoxins.[11] Since the brevetoxins have potent ichthyotoxicity, **2** likely contribute, along with other brevisulcatic acids and brevesulcenals, to the harmful effects of the *K. brevisulcata* red tide observed in New Zealand during 1998.[2] Therefore, the structures of **1** and **2** provide useful information to marine toxin scientists and lead to development of a trace detection method to monitor future incidents.[12]

■ ASSOCIATED CONTENT

Supporting Information

Details of the isolation procedures, derivatization of taurine amide (**3**), ESIMS, ^{1}H NMR, ^{13}C NMR, HSQC, DQF-COSY, HMBC, NOESY, and TOCSY spectra of **2**, ESIMS, ^{1}H NMR, and ^{13}C NMR spectra of **1**, and MALDIMS of **3**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) (a) Chang, F. H. *Harmful Algae News* **1999**, *19*, 3−4. (b) Chang, F. H. *Phycologia* **1999**, *38*, 377−384.
(2) Holland, P. T.; Shi, F.; Satake, M.; Hamamoto, Y.; Ito, E.; Beuzenberg, V.; McNabb, O.; Munday, R.; Briggs, L.; Truman, P.; Gooneratne, R.; Edwards, P.; Pascal, S. *Harmful Algae* **2012**, *13*, 47−57.
(3) Hamamoto, Y.; Tachibana, K.; Holland, P. T.; Shi, F.; Beuzenberg, V.; Itoh, Y.; Satake, M. *J. Am. Chem. Soc.* **2012**, *134*, 4963−4968.
(4) (a) Shimizu, Y.; Chou, H.-N.; Bando, H.; Duyne, G. V.; Clardy, J. *J. Am. Chem. Soc.* **1986**, *108*, 514−515. (b) Pawlik, J.; Tempesta, M. S.; Golik, J.; Zagorski, M. G.; Lee, M. S.; Nakanishi, K.; Iwashita, T.; Gross, M. L.; Tomer, K. B. *J. Am. Chem. Soc.* **1987**, *109*, 1144−1150. (c) Zagorski, M. G.; Nakanishi, K.; Qin, G-w.; Lee, M. S. *J. Org. Chem.* **1988**, *17*, 4156−4158.
(5) (a) Murata, M.; Legrand, A.-M.; Ishibashi, Y.; Fukui, M.; Yasumoto, T. *J. Am. Chem. Soc.* **1990**, *112*, 4380−4386. (b) Murata, M.; Legrand, A.-M.; Scheuer, P. J.; Yasumoto, T. *Tetrahedron Lett.* **1992**, *33*, 525−526. (c) Satake, M.; Murata, M.; Yasumoto, T. *Tetrahedron Lett.* **1993**, *34*, 1975−1978.
(6) Abraham, A.; Plakas, S. M.; Wang, Z.; Jester, E. L. E.; El Said, K. R.; Grande, H. R.; Henry, M. S.; Blum, P. C.; Pierce, R. H.; Dicky, R. W. *Toxicon* **2006**, *48*, 104−115.
(7) Naoki, H.; Murata, M.; Yasumoto, T. *Rapid Commun. Mass Spectrom.* **1993**, *7*, 179−182.
(8) Ienaga, K.; Higashiura, K.; Toyomaki, Y.; Matsuura, H.; Kimura, H. *Chem. Pharm. Bull.* **1988**, *36*, 70−77.
(9) (a) Satake, M.; Shoji, M.; Oshima, Y.; Naoki, H.; Fujita, T.; Yasumoto, T. *Tetrahedron Lett.* **2002**, *43*, 5829−5832. (b) Satake, M.; Tanaka, Y.; Ishikura, Y.; Oshima, Y.; Naoki, H.; Yasumoto, T. *Tetrahedron Lett.* **2005**, *46*, 3537−3540. (c) Tanaka, Y.; Satake, M.; Yotsu-Yamashita, M.; Oshima, Y. *Heterocycles* **2013**, *87*, 2037−2046.
(10) Gawley, R. E.; Rein, K. S.; Jeglitsch, G.; Adams, D. J.; Theodoraskis, E. A.; Tiebes, J.; Nicolaou, K. C.; Baden, D. G. *Chem. Biol.* **1995**, *2*, 533−541.
(11) Yasumoto, T.; Fukui, M.; Sasaki, K.; Sugiyama, K. *J. AOAC Int.* **1995**, *78*, 574−582.
(12) Harwood, D. T.; Shi, F.; Satake, M.; Holland, P. *Toxicon* **2014**, *84*, 19−27.

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