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BREVISULCATIC ACIDS FROM A MARINE MICROALGAL SPECIES IMPLICATED IN A TOXIC EVENT IN NEW ZEALAND

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Abstract – Ladder-frame polyethers, known as brevisulcatic acids (BSXs) -1 (**1**), -2 (**2**), -4 (**3**), -5 (**4**) and -7 (**5**), were isolated from the dinoflagellate *Karenia brevisulcata* as potential causative toxins of a harmful algal event in New Zealand in 1998. The structures of **2**, **4**, and **5** were elucidated in this study. Brevisulcatic acids possessed a common fused nine ether-ring backbone, with a size and sequence determined to be 8/6/8/9/7/7/6/6/6. Rings B, C, H, I and J of the brevisulcatic acids were similar to those of brevetoxin A, a well-known polyether toxin. BSX-5 (**4**) had a γ-lactone as the 5-membered A-ring, analogous to brevetoxin A, while in BSX-2 (**2**) and BSX-7 (**5**) the lactone was present in its seco-acid form. In addition to this backbone variation, there were also some structural difference in side-chain substituents, with **2** and **4** having a 2-methylenepropionic acid unit, and **5** a 2-methylenepropanol unit. Cytotoxicity of **4** against neuroblastoma cells indicated that not only the structures but also activity of brevisulcatic acids are similar to those of brevetoxin A. This indicates that brevisulcatic acids potentially played a significant role in the toxicity observed during the only documented *Karenia brevisulcata* bloom to date.

INTRODUCTION

In 1998, a wide-spread dinoflagellate bloom occurred in Wellington Harbour, New Zealand that caused

mortality of many species, from diatoms to fish.[1] It also caused human illness, with more than 500 harbour bystanders reporting respiratory distress (dry cough, severe sore throat), skin and eye irritations, severe headaches, and facial sun-burn sensations. A new dinoflagellate, *Karenia brevisulcata*, was identified as the causative species.[2] Dinoflagellates within the *Karenia* genus are well known to cause red-tide events and have been shown to produce marine ladder-frame polyethers with unsaturated aldehyde side-chains such as brevetoxins,[3] and gymnocins.[4] Cell culture extracts of *K. brevisulcata* showed mouse lethality, cytotoxicity, and ichthyotoxicity. Two new classes of marine ladder-frame polyethers were isolated from the dinoflagellate extracts,[5] with one of these, the brevisulcenals (initially called karenia brevisulcata toxins (KBTs)), being contained in a neutral lipophilic fraction. The sodium adduct ions of KBTs in MALDI MS were observed as being greater than *m/z* 2000 and the structure of a major component, brevisulcenal-F (KBT-F, $C_{107}H_{160}O_{38}$), has been elucidated and reported.[5,6] KBT-F shows potent mouse lethality and cytotoxicity against mouse lymphoid P388 cells. Its structure consists of 24 ether rings (including an unusual dihydrofuran), 13 hydroxyl groups, 13 methyl groups, and a 2-methylbut-2-enal terminus. The sequence of 17 contiguous ether rings A–Q is the longest of any known polycyclic ether. In addition to the KBTs, smaller polycyclic ethers were isolated from an acidic fraction and the neutral lipophilic fraction. These were named brevisulcatic acids (BSXs) and had molecular weights around 900 g/mol.[5] Most of the BSXs were extracted with dichloromethane from an acidic methanolic fraction (55% MeOH). The molecular structures of brevisulcatic acids-1 (BSX-1, **1**) and -4 (BSX-4, **3**) were determined by detailed analysis of NMR spectra measured at both room and low temperature, and high energy MALDI-TOF MS/MS spectra (Figure 1).[7] The structures of **1** and **3** have similarity to brevetoxin A, which is a well-known marine ladder-frame polyether with a γ-lactone and a conjugated aldehyde side chain.[3] The fact that the size and sequence of fused ether rings of **1** and **3** was 8/6/8/9/7/7/6/6/6 suggested that the B, C, H, I and J ring-structures of these two compounds were similar to those of brevetoxin A. In addition to the fused ether-ring backbone, **3** had a γ-lactone in the A-ring that was structurally analogous to that in brevetoxin A. The difference of the molecular formula between **1** and **3** was H_2O, and the NMR analysis determined that **1** was a seco acid analogue of **3**. The NMR signals of the E ring were extremely broadened at room temperature due to slow conformational change around the 9-membered ether ring, as is observed with the brevetoxins and ciguatoxins.[8] For this reason, the molecular structure around the ring E was elucidated by low-temperature NMR. Under these conditions, the structure of rings D–G converged to two dominant conformers, generating two sets of complex NMR signals. Structural confirmation of **3** was determined by negative mode TOF-TOF experiments using a taurine derivative of **3**.

Further investigation of extracts generated from *K. brevisulcata* cultures has led to the isolation of brevisulcatic acids-2 (**2**), -5 (**4**), and -7 (**5**), in addition to **1** and **3**. In this paper we report the structures of brevisulcatic acid analogues **2**, **4** and **5**.

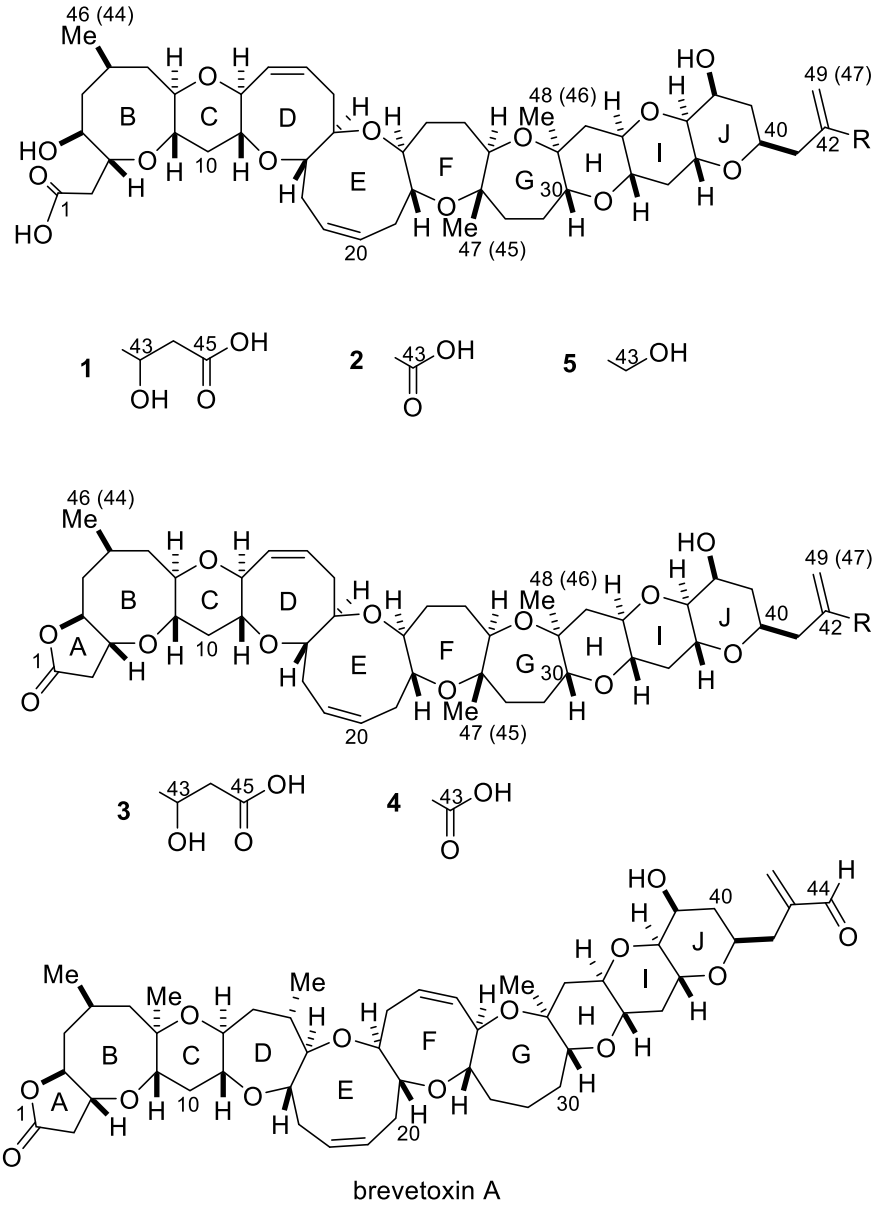

Figure 1. Structures of brevisulcatic acids-1 (BSX-1, **1**), -2 (BSX-2, **2**), -4 (BSX-4, **3**), -5 (BSX-5, **4**), and -7 (BSX-7, **5**) and brevetoxin A. The numbers in parentheses are positions of **2**, **4**, and **5**.

RESULTS AND DISCUSSION

Cultured *K. brevisulcata* cells were lysed with acetone and then stirred for one hour. The extract was diluted with water and passed through HP20 resin. Both the brevisulcenals and the brevisulcatic acids were retained and selectively extracted from the resin using acetone. The crude extract was dissolved in 55% MeOH with pH 7.2 phosphate buffer and the KBTs and BSX-7 (**5**) were extracted into $CHCl_3$. The aqueous fraction containing BSXs-1 (**1**), -2 (**2**), -4 (**3**), and -5 (**4**) was adjusted to pH 4, and then the BSXs in the acidic aqueous fraction were extracted into CH_2Cl_2. The CH_2Cl_2 extract was subjected to solid-phase extraction using a Strata-X cartridge with stepwise elution using MeOH–water (1:1, 6:4, 7:3, 1:0). The fractions

containing BSXs were combined and then chromatographed on a C_{30} reverse phase column with a linear gradient elution from MeOH–water containing 0.1% acetic acid. From 596 L culture media enriched with ^{13}C-$NaHCO_3$, 0.24 mg of **2** and 0.32 mg of **4** were obtained, in addition to 1.2 mg of **1** and 1.1 mg of **3**, by repeated chromatography on the reverse-phase column. Only **5** was isolated from the neutral chloroform fraction of ^{13}C-enriched extracts. The $CHCl_3$ fraction was subjected to chromatographic separation using a diol solid-phase extraction cartridge, and stepwise elution by ethyl acetate and MeOH. Fractions containing **5** were dissolved in $CHCl_3$, and subjected to alumina-column separation: the column was washed with $CHCl_3$, $CHCl_3$–MeOH (1:1) and then MeOH, and **5** was finally eluted with 1% NH_4OH–MeOH (1:1). Further purification of **5** was also performed using reverse-phase column chromatography using a MeOH–water gradient. From 200 L culture media, 0.067 mg of ^{13}C-enriched **5** was isolated.

The structural elucidation of **2**, **4**, and **5** was accomplished by a comparison of NMR and MS spectra to **1** and **3**. Spectral resemblance of **4** to **3** demonstrated that **4** contained a γ-lactone, while the resemblance of **2** and **5** to **1** indicated that **2** and **5** contained carboxylic acids at their C1 positions (Table 1).

The molecular formula of **4** was deduced to be $C_{47}H_{66}O_{14}$ ($[M+H]^+$ *m/z* 855.4593, calcd. 855.4531) by high resolution +ESI-MS. The difference of the molecular formula of **3** ($C_{49}H_{70}O_{15}$) and **4** was C_2H_4O, which corresponded to a methine possessing a hydroxyl group and an aliphatic methylene. It suggested that **4** lacked a –CHOH–CH_2– portion in the side chain of **3**. Oxymethine (δ_H 4.42, H-43 in **3**) and methylene (δ_H 2.26 and 2.44, H_2-44 in **3**) signals belonging to a side chain in **3** were not observed in the NMR spectra of **4**. In the ^{1}H NMR spectra, the exomethylene protons (H_2-47) in **4** were observed at 5.36 and 5.93 ppm, while those (H_2-49) in **3** were observed at 4.93 and 5.16 ppm. The lower-field shift of the chemical shifts of these signals suggested that the exomethylene in **4** was involved in a conjugated system. HMBC correlations from the exomethylene protons to an allylic methylene carbon (C-41) at 38.4 ppm and a carboxylic acid (C-43) at 176.2 ppm were observed. Moreover, HMBC correlations from an allylic methylene (H_2-41) at 2.82 and 2.99 ppm to an oxymethine (C-40) at 73.6 ppm, the exomethylene (C-47) at 123.7 ppm, a quaternary olefin (C-42) at 146.4 ppm, and the carboxylic acid (C-43) at 176.2 ppm were also observed. These HMBC correlations revealed that the side chain structure of **4** was 2-methylenepropionic acid. The structure of the ether-ring core of **4** was also assigned by analyses of ^{1}H–^{1}H COSY, HSQC, and HMBC at room temperature and low temperature. The proton connectivity from H_2-2 to H_2-16, and H_2-32 to H_2-41 was assigned by ^{1}H–^{1}H COSY and TOCSY measured at room temperature. However, analogous with **1** and **3**, NMR signals on rings D–G were observed as broad signals at 25 °C due to the slow conformational change of the E ring. Thus, the structure from CH-14 to CH-32 was assigned using NMR spectra measured at -15 °C. ^{1}H and ^{13}C chemical shifts from CH_2-2 to CH-40 of **4** in the HSQC agreed well with those of **3**. Overall, these spectral data demonstrated that the structure of the ether ring core of **4** was

identical to that of **3**. The proton chemical shift of H-4 at 4.21 ppm supported that **4** had a γ-lactone at the ring A. Therefore, the structure of **4** was elucidated as shown in Figure 1.

The molecular formula of **2** was determined to be $C_{47}H_{68}O_{15}$ ($[M+H]^+$ *m/z* 873.4578, calcd. 873.4617), and its mass being 18 amu greater than that of **4** suggesting it had a carboxylic acid at C1 (similar to **1**). The proton connectivity from H_2-2 to H_2-15 of **2** assigned by ^1H–^1H COSY measured at 25 °C was identical to that of **4**. However, the proton chemical shift (δ_H 3.27) of H-4 in **2** was nearly 0.94 ppm higher-field than that (δ_H 4.21) of **4**, and this H-4 chemical shift of **2** was identical with that of H-4 in **1**. These data indicated that **2** had not a γ-lactone at the A ring but a carboxylic acid at the C1 terminus. A 2-methylenepropionic acid side-chain was also assigned using HMBC correlations from an exomethylene (H_2-47) at 5.34 and 5.91 ppm to a methylene (C-41) at 38.4 ppm and to a carboxylic acid (C-43) at 176.5 ppm. The limited amount of **2** prevented ^{13}C NMR spectra being obtained, even when ^{13}C-enriched **2** was used, so ^{13}C chemical shifts were assigned by analysis of HSQC and HMBC spectra. The NMR data of ether rings C–J agreed with those of **4**. Thus **2** was determined to be a seco-acid analogue of **4**.

Isolation of **5** from ^{13}C-enriched extracts hampered determination of the molecular formula of **5** by high-resolution MS. The dominant $[M-H]^-$ ion observed for ^{13}C-enriched **5** was at *m/z* 858.2 in -ESI MS. It was 14 amu less than that of ^{13}C-enriched **2**. From this information, the molecular formula of **5** was determined to be $C_{47}H_{70}O_{14}$. Therefore, **5** was determined to be a reduced analogue of **2**. The chromatographic behavior and ESI-MS characteristics of **5** suggested it contained a primary alcohol in the side-chain instead of a carboxylic acid, as in **2**. Proton chemical shifts of an exomethylene in the side-chain of **5** observed at 4.92 ppm and 5.08 ppm indicated that the exomethylene in **5** was not involved in a conjugated system. In the HSQC spectra, an oxymethylene was observed at 4.04 ppm for ^1H and 66.0 ppm for ^{13}C. These chemical shifts were characteristic for an allylic primary alcohol. The HMBC correlations from the oxymethylene protons to a methylene (δ_C 38.0), the exomethylene (δ_C 112.4), and a quaternary olefin (δ_C 148.8) were also observed. Therefore, the side-chain in **5** was assigned to be 2-methylenepropanol, identical to the side-chain of 42-dihydrobrevetoxin B (brevetoxin-3).[9] **5** was obtained from the neutral chloroform fraction, in contrast to the other BSXs isolated from the acidic fraction. Although the limited amount of **5** hampered observation of HMBC correlations from H_2-2 to C-1, elution of **5** from the alumina column with 1% NH_4OH–MeOH (1:1) suggested that **5** also had an acidic functionality in the molecule. In addition, the similar proton chemical shifts of H_2-2, H-3, and H-4 in **5** to those in **2** suggested the presence of a carboxylic acid at C-1 in **5** (Table 1). The COSY and HSQC cross-peaks indicated that the ether-ring core structure of **5** was identical to that of **1** and **2**. Thus the structure of **5** was determined as shown in Figure 1.

Table 1. ^{1}H and ^{13}C NMR data[a] for BSX-2 (**2**), BSX-5 (**4**) and BSX-7 (**5**) in CD_3OD at -15 °C

	2			**4**			**5**		
no.	^{1}H	^{1}H	^{13}C	^{1}H	^{1}H	^{13}C	^{1}H	^{1}H	^{13}C
1[b]			181.2			176.3			
2	2.14	2.76	45.4	2.64	2.86	38.7	2.14	2.76	45.3
3	3.80		88.6	4.34		83.1	3.80		88.6
4	3.27		75.7	4.21		87.1	3.27		75.7
5	1.65	1.85	49.4	1.53	2.10	44.5	1.64	1.85	49.4
6	1.85		29.7	1.82		31.0	1.85		29.7
7	1.59	1.79	46.7	1.68	1.83	48.8	1.58	1.79	46.7
8	3.00		82.4	3.02		84.3	2.99		82.4
9	3.34		84.5	3.30		83.8	3.34		84.5
10	1.33	2.50	41.6	1.45	2.26	41.2	1.33	2.49	41.6
11	3.20		81.0	3.24		80.4	3.20		81.0
12	3.77		81.0	3.81		81.1	3.77		81.0
13	5.61		135.8	5.62		135.6	5.61		135.8
14	5.61		127.8	5.62		127.8	5.61		127.7
14'	5.62		127.8	5.64		127.8	5.62		127.7
15	2.25	2.64	33.8	2.28	2.65	33.8	2.25	2.65	33.8
15'	2.32	2.54	33.0	2.35	2.55	33.0	2.31	2.54	33.0
16	3.69		93.1	3.71		93.0	3.69		93.1
16'	3.45		87.0	3.47		86.9	3.45		87.0
17	3.22		80.2	3.26		79.5	3.23		80.3
17'	3.69		80.2	3.71		80.2	3.69		80.2
18	2.13	2.68	36.6	2.08	2.72	36.7	2.13	2.67	36.5
18'	2.03	2.73	32.2	2.00	2.77	32.4	2.03	2.73	32.3
19	5.50		129.0	5.52		128.7	5.49		129.0
19'	5.66		129.2	5.70		128.9	5.66		129.1
20	5.53		129.8	5.52		130.1	5.52		129.8
20'	5.75		130.6	5.76		130.7	5.75		130.7
21	1.95	2.68	37.9	1.95	2.68	37.7	1.95	2.68	37.9
21'	1.83	2.71	33.6	1.84	2.71	33.6	1.83	2.71	33.7
22	3.40		72.2	3.40		72.2	3.39		72.2
22'	3.84		75.9	3.85		75.9	3.84		76.1
23	3.64		91.2	3.64		91.1	3.64		91.2
23'	3.25		86.3	3.26		86.2	3.25		86.2
24	1.76	2.08	33.4	1.77	2.09	33.4	1.76	2.08	33.4
24'	1.66	2.26	33.1	1.67	2.27	33.1	1.66	2.25	33.1
25	1.50	1.91	29.0	1.50	1.91	29.0	1.50	1.91	29.0
25'	1.54	1.96	29.3	1.54	1.95	29.3	1.53	1.95	29.3
26	3.58		78.3	3.58		78.3	3.58		78.3
26'	3.56		77.7	3.56		77.7	3.56		77.6
27			81.6			81.6			81.4

27'			80.4			80.5			80.4
28	1.84		41.8	1.84		41.9	1.84		41.8
28'	1.84	1.93	42.3	1.84	1.92	42.3	1.84	1.93	42.2
29	1.71	1.84	26.6	1.71	1.84	26.6	1.71	1.84	26.6
29'	1.77	1.77	26.6	1.77	1.77	26.6	1.77	1.77	26.6
30	3.33		84.5	3.33		84.4	3.33		84.5
30'	3.28		85.7	3.28		85.8	3.28		85.8
31			77.3			77.5			77.2
31'			77.5			77.5			77.4
32	1.57	1.98	45.9	1.57	1.98	46.1	1.56	1.99	46.0
32'	1.57	1.98	46.1	1.57	1.98	46.2	1.56	1.99	46.1
33	3.25		79.0	3.26		78.9	3.27		78.9
34	3.10		79.4	3.10		79.4	3.09		79.4
35	1.37	2.17	37.1	1.37	2.17	37.0	1.39	2.13	37.0
36	3.94		64.0	3.95		64.0	3.90		63.8
37	3.08		82.0	3.08		82.0	3.09		81.8
38	4.13		67.1	4.13		67.1	4.13		67.1
39	1.92		35.0	1.93		35.1	1.85	1.99	35.9
40	4.01		73.6	4.00		73.6	4.00		72.8
41	2.84	2.96	38.4	2.82	2.99	38.4	2.36	3.02	38.0
42			146.1			146.4			148.8
43			176.5			176.2	4.04	4.04	66.0
44	1.04		28.9	1.11		28.4	1.04		28.9
45	1.29		21.1	1.29		21.1	1.29		21.1
45'	1.23		19.2	1.23		19.2	1.23		19.2
46	1.27		17.2	1.27		17.2	1.27		17.2
46'	1.30		17.2	1.30		17.3	1.30		17.2
47	5.34	5.91	123.5	5.36	5.93	123.7	4.92	5.08	112.4

[a] The ^{13}C chemical shifts of **2**, **4** and **5** were assigned based on HSQC and HMBC experiments.

[b] The ^{13}C chemical shift at C1 of **5** could not be assigned due to a small amount of **5**.

In this study the molecular structures of **2**, **4**, and **5** were determined. Structural changes of the BSXs occur in the side chain moieties. Compounds **2** and **4** have the 2-methylenepropionic acid side chains and **5** has the 2-methylenepropanol side chain. Detection of **1**, **2**, **3** and **4** in neutral extracts analyzed by LC–ESI MS indicated that **2** was not a hydrolyzed artifact of **4** formed during acid extractions.[5,10]

In the neuroblastoma cell assay for voltage-sensitive sodium channel activity (neuro-2a cells in the presence of veratridine and ouabain), **4** had a cytotoxicity EC_{50} of 26.9 nM, while that for **2** was 370 nM.[5] The 13-fold higher cytotoxicity of **4** than that for **2** indicates that the γ-lactone in BSX-5 plays a significant role in its neurotoxicity.[10] However, the cytotoxicity of **3** and **4** are very close, suggesting that slight structural modifications of the side-chains have less influence on the activity of these brevisulcatic acids. Without addition of veratridine and ouabain, **3** and **4** did not show cytotoxicity against the neuroblastoma cells. This

shows the activity of brevisulcatic acids are due to activation of voltage-sensitive sodium channels, analogous to the mode of action of brevetoxins and ciguatoxins.[11] Thus, brevisulcatic acids resemble brevetoxins in not only their molecular structures but also in their profile of cellular activities. Brevetoxins are also toxic to fish and humans, raising the possibility that brevisulcatic acids **3** and **4** might have contributed, together with brevisulcenals, to the harmful effects observed during the *K. brevisulcata* bloom in New Zealand.[2]

EXPERIMENTAL

General

All solvents and reagents were commercially available and HPLC grade solvents were used for all column chromatography. Optical rotations were recorded on a JASCO P2200 polarimeter. ESI-MS spectra were recorded on a Waters-Micromass Quattro Ultima mass spectrometer and a JEOL JMS-T100LC spectrometer. ^{1}H and ^{13}C NMR spectra were recorded on a JEOL JNM-ECA500 spectrometer (500 MHz), a Bruker AVANCE III HD600 spectrometer (600 MHz) equipped with a cryoQCI probe, and an Agilent INOVA 900 spectrometer (900 MHz). The NMR signals of CD_3OD were used as internal references, at 3.31 ppm for ^{1}H and at 49.8 ppm for ^{13}C.

Culture growth and harvesting of *Karenia brevisulcata*

Karenia brevisulcata (CAWD82) was collected at the Wellington Harbour in 1998 and has been kept in live culture at the Cawthron Institute, Nelson, New Zealand. The dinoflagellate was cultured in 12 L culture flasks for 21 days using 100% GP+Se media under a 12/12 hour day/night timed cool white fluorescent lighting regime and 25 min aeration every 30 min. For ^{13}C enrichment, $NaH^{13}CO_3$ (0.25 g/12L) was added to cultures at 0 and 7 days. Production of BSXs was monitored by liquid chromatography–mass spectrometry (LC–MS). Dinoflagellate cells were lysed by addition of acetone to 7% v/v for one hour and diluted with water to 5% v/v acetone. An extract was passed through HP20 resin which was packed in a polypropylene column at the flow rate 0.3 L/min. After the column was washed with water, the HP20 resin was transferred to a 2 L flask. Brevisulcatic acids were extracted by soaking the resin with acetone and decanting. The acetone extracts were combined and dried by evaporation.

Isolation of brevisulcatic acids

The crude HP20 extract was dissolved in 55% MeOH with pH 7.2 phosphate buffer. The solution was partitioned with $CHCl_3$. The aqueous MeOH fraction containing brevisulcatic acids (BSXs) -1 (**1**), -2 (**2**), -4 (**3**), and -5 (**4**) was adjusted to pH 4.0, and BSXs were then extracted with CH_2Cl_2. The extract was subjected to solid-phase extraction on Strata-X resin using stepwise elution with MeOH–water (1:1, 7:3,

8:2, 9:1, 1:0). The 70%, 80%, 90% and 100% MeOH fractions were combined and the combined fractions were subjected to purification on a C_{30} column with a gradient elution of 70–100% MeOH containing 0.1% acetic acid.

The neutral $CHCl_3$ fraction of ^{13}C-enriched extract was subjected to solid-phase extraction on a Diol cartridge column with EtOAc–MeOH (1:0, 9:1, 6:4, 0:1). The 90% and 100% EtOAc fractions were combined and further chromatographed on an alumina (Act III) column, eluting with $CHCl_3$, $CHCl_3$–MeOH (1:1), MeOH and 1% NH_4OH–MeOH (1:1). A final purification of an acid fraction was conducted on an ODS column with a linear gradient elution of 70–100% MeOH. Elution of brevisulcatic acid-7 (**5**) was monitored using LC–MS.

Brevisulcatic acid-2 (**2**): A white amorphous solid; $[\alpha]_D^{25}$ +95.7 (*c* 0.01, MeOH); UV (MeOH) λ_{max} (log ε) 210 (3.53); HR ESI MS $[M+H]^+$ *m/z* 873.4578; ^1H and ^{13}C NMR data (CD_3OD), see Table 1.

Brevisulcatic acid-5 (**4**): A white amorphous solid; $[\alpha]_D^{25}$ +45.1 (*c* 0.02, MeOH); UV (MeOH) λ_{max} (log ε) 210 (3.41); HR ESI MS $[M+H]^+$ *m/z* 855.4593; ^1H and ^{13}C NMR data (CD_3OD), see Table 1.

Brevisulcatic acid-7 (**5**): A white amorphous solid; ESI MS *m/z* 858.2 $[M-H]^-$ ($C_{47}H_{70}O_{14}$); ^1H and ^{13}C NMR data (CD_3OD), see Table 1.

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