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Brevisulcenal-F: A Polycyclic Ether Toxin Associated with Massive Fish-kills in New Zealand

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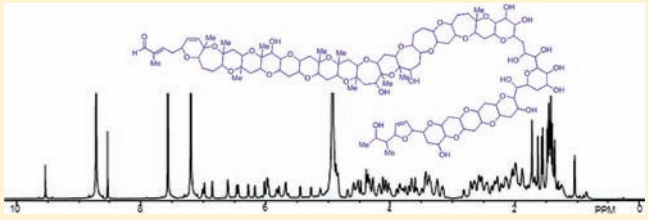
Supporting Information

ABSTRACT: A novel marine toxin, brevisulcenal-F (KBT-F, from karenia brevisulcata toxin) was isolated from the dinoflagellate *Karenia brevisulcata*. A red tide of *K. brevisulcata* in Wellington Harbour, New Zealand, in 1998 was extremely toxic to fish and marine invertebrates and also caused respiratory distress in harbor bystanders. An extract of *K. brevisulcata* showed potent mouse lethality and cytotoxicity, and laboratory cultures of *K. brevisulcata* produced a range of novel lipid-soluble toxins. A lipid soluble toxin, KBT-F, was isolated from bulk cultures by using various column chromatographies. Chemical investigations showed that KBT-F has the molecular formula $C_{107}H_{160}O_{38}$ and a complex polycyclic ether nature. NMR and MS/MS analyses revealed the complete structure for KBT-F, which is characterized by a ladder-frame polyether scaffold, a 2-methylbut-2-enal terminus, and an unusual substituted dihydrofuran at the other terminus. The main section of the molecule has 17 contiguous 6- and 7-membered ether rings. The LD_{50} (mouse i.p.) for KBT-F was 0.032 mg/kg.



INTRODUCTION

A widespread bloom of a red tide dinoflagellate was observed on the central and southern east coast of the North Island of New Zealand from January to March 1998.[1] The red tide resulted in massive kills of fish and invertebrates in Wellington Harbour. Over 500 cases of human respiratory distress were reported during the bloom with symptoms including a dry cough, severe sore throat, runny nose, skin and eye irritations, severe headaches, and facial sun-burn sensations. The causative dinoflagellate was identified as the new algal species *Karenia brevisulcata*.[2] This species is similar to *K. brevis* and *K. mikimotoi*, which produce brevetoxins[3] and gymnocins,[4] respectively. These toxins have structures characterized by a trans-fused polycyclic ether ring backbone with an unsaturated aldehyde terminus.

The cell extract from the *K. brevisulcata* exhibited strong mouse lethality and cytotoxicity,[5] and was partitioned between chloroform and aqueous methanol under neutral and acidic conditions.[1] Each fraction contained different toxins. Lipophilic toxins demonstrated strong mouse lethality and cytotoxicity, and they were named brevisulcenals (initially called karenia brevisulcata toxins (KBTs) in ref 1). Their ^{1}H NMR spectra contained large numbers of protons connected to oxygenated carbons, and their sodiated molecular cations in MALDI MS were observed at m/z over 2000. These observations indicated that KBTs were large polycyclic ethers analogous to maitotoxin (MTX)[6] and prymnesins.[7] However, their complex NMR spectra derived from the high molecular weights have hampered elucidation of their structures. In this paper, we report the structure of brevisulcenal-F (KBT-F, **1**) which is one of the most abundant toxins in *K. brevisulcata*.

RESULTS

Extraction and Isolation of Brevisulcenal-F (1). Brevisulcenals (KBTs) were extracted from mature cultures using resin. Cells were lysed by addition of acetone. After stirring for 1 h, the cultures were diluted with water and passed through HP20 resin. Brevisulcenals were recovered with acetone from the resin. The crude extract was dissolved in 55% MeOH with pH 7.2 phosphate buffer, and the lipophilic toxins were extracted with $CHCl_3$. The $CHCl_3$ extract was chromatographed on a diol−silica column with stepwise elution and guided by cytotoxicity assay against mouse leukemia P388 cells. Final purification of KBTs was by two stages of reversed phase chromatography using isocratic elution with 90% MeOH/H_2O followed by 85% MeOH/H_2O and monitored by UV absorbance at 230 nm. From 1450 L cultures, 3.1 mg of **1** was isolated. Similarly, 1.3 mg of ^{13}C enriched **1** was isolated from 250 L cultures with added ^{13}C-$NaHCO_3$ at about 35 mg/L.

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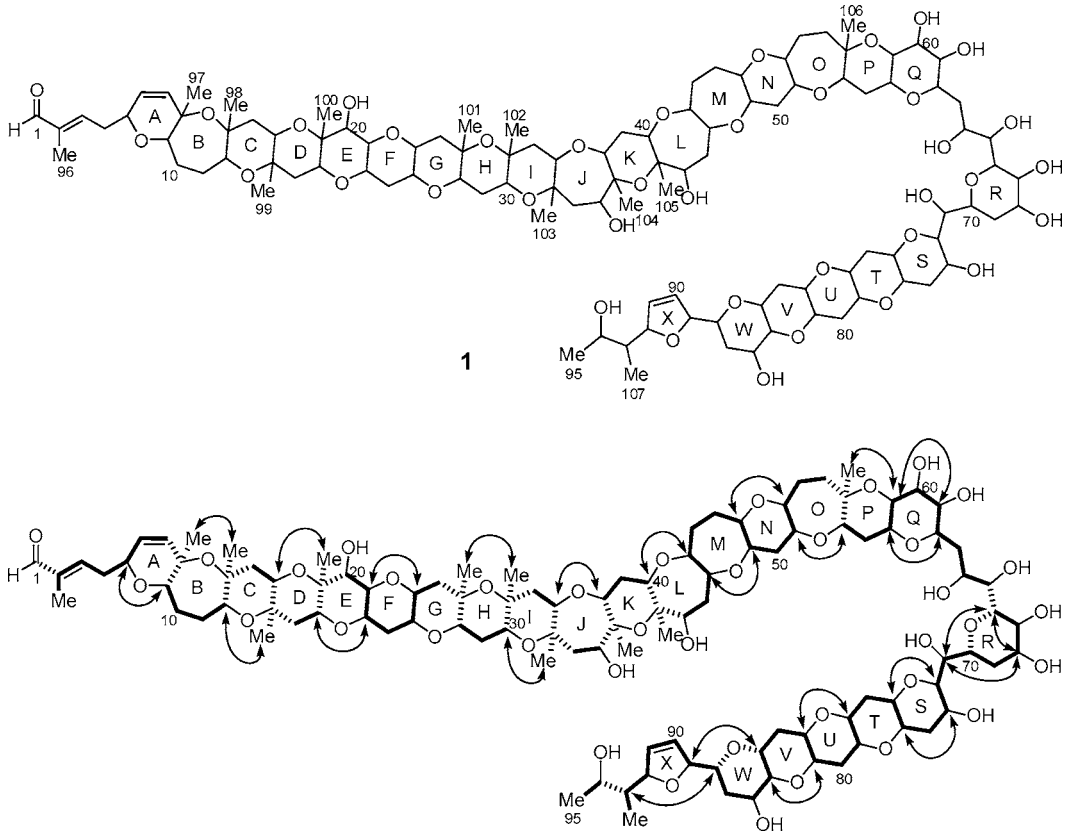

Figure 1. Structure and NMR interpretation of brevisulcenal-F (**1**). Bold lines indicated connectivities elucidated on the basis of the $^{1}H-^{1}H$ COSY and TOCSY data. Double-headed arrows show the important NOE correlations observed in the NOESY spectra of **1**. Broken lines depicted the key HMBC correlations of **1**.

Spectroscopic Measurements of Brevisulcenal-F. A UV absorption maximum at 227 nm indicated that KBT-F possessed a conjugated system in the molecule. A sodium adduct ion peak in the positive ion MALDI mass spectrum was observed at m/z 2076; the strong positive ion peak suggested KBT-F does not have sulfate groups. The MALDI-SpiralTOF accurate mass[8] and ^{1}H and ^{13}C NMR spectra indicated that the molecular formula of KBT-F was $C_{107}H_{160}O_{38}$ ($[M + Na]^{+}$ 2076.0476, calcd 2076.0480).

The ^{1}H NMR spectrum showed two methyl doublets, 11 methyl singlets, five olefinic protons, and an aldehyde in addition to aliphatic methylenes and methines bearing oxygen. The ^{1}H decoupled ^{13}C and DEPT, and HSQC spectra showed signals of 13 methyls, one methine and 25 methylene aliphatic carbons, 51 oxgenated methine and 10 oxygenated quaternary carbons, five methine and a quaternary olefinic carbons, and an aldehyde carbon. The number of methyl singlets was equal to the number of the quaternary carbons. This means that the quaternary carbons, except the olefinic carbon, bear a singlet methyl and an oxygen atom. On the other hand, the number of methyl doublets was not equal to the number of aliphatic methines. Therefore, the doublet methyl observed at δ_H 1.37 is attached to an oxymethine carbon and located at one terminus. These facts revealed that the scaffold of KBT-F consisted of a 95-carbon chain substituted with 12 branched methyls but lacking alicycles or longer carbon branches.

The structural elucidation of KBT-F was mainly carried out using both the intact and ^{13}C-enriched KBT-F molecules by analyses of extensive 2D NMR spectra measured with 400, 500, and 800 MHz instruments. Detailed analysis of $^{1}H-^{1}H$ COSY and TOCSY spectra led to identification of spin systems representing H-3 to H-7, H-9 to H-12, H-14 to H-15, H-17 to H-18, H-20 to H-26, H-28 to H-30, H-32 to H-33, H-35 to H-36, H-38 to H-40, H-42 to H-54, H-56 to H-65, H-66 to H-93, and H-94 to Me-95 (Figure 1). A 2-methylbut-2-enal side chain, originally suggested by the UV data, was confirmed by COSY correlations, including a long-range correlation from Me-96 to H-3 and H-3 to H-4, and by HMBC correlations from Me-96 to C-1, C-2, and C-3. Although interproton couplings between H-65 and H-66 and between H-93 and H-94 were not observed in the COSY spectra, the HMBC correlations from H-67 to C-65 and from Me-107 to C-94 confirmed the carbon connections C-65−C-66 and C-93−C-94, respectively. Other carbon connectivity was interrupted by the oxygenated quaternary carbons bearing the methyls, so all the partial structures were assembled by HMBC experiments. Long range correlations from a methyl to the vicinal quaternary carbon and neighboring carbons in the HMBC spectra made it possible to trace the carbon skeleton from C-1 to C-95. Interestingly, all the quaternary carbons are localized on the ring A−Q assembly. Isotope shift experiments measured in pyridine-d_5/CD_3OD (20:1) and pyridine-d_5/CD_3OH (20:1) showed that 13 oxycarbons (C-20, C-36, C-42, C-60, C-61, C-64, C-65, C-67, C-68, C-71, C-73, C-86, and C-94) were bearing hydroxyl groups. The rest of the oxycarbons were involved in ether linkages, so the number of ether rings in the molecule was deduced to be 24. These observations matched the r.+d.b. count of the molecular formula. The locations of ether linkages were determined by NOE and HMBC correlations (Figure 1) and the isotope shift experiments.

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Although the close chemical shifts H-24/H-28, Me-104/Me-105, and H-75/H-79 hampered confirmation of rings G, K, and T, the proton chemical shifts and signal shapes of methylenes CH_2-26, CH_2-39, and CH_2-77 were typical for methylenes residing in a six-membered ether ring. Judging from NOE correlations and interproton coupling constants shown in 2D spectra, the polycyclic ethers of rings A–Q and S–W were deduced to be trans–cisoid fused, as is the case with other polycyclic ether compounds such as the brevetoxins. The left-hand portion of the molecule consisted of the 2-methylbut-2-enal side chain and 17 contiguous ether rings A–Q including seven contiguous six-membered ether rings C–I. The right-hand portion has an unprecedented terminal structure consisting of dihydrofuran X and six-membered ether ring W that was deduced as follows. A strong HMBC correlation H-88/C-84 and NOE correlations between H-84/H-89 were observed. The equatorial proton H-88 gave rise to the strong HMBC correlation indicating a dihedral angle H–C-88–O–C-84 of nearly 180°. Therefore, ring W was a six-membered ether ring and C-89 resided on an axial orientation. Moreover, NOE correlations between H-88/H-93 and the ^{13}C chemical shifts of C-89 (δ 87.3) and C-92 (δ 91.2) observed in the lower field indicated that ring X was a dihydrofuran (Table 1). Therefore, an alternative regio-isomer, a fused 7/6 ether ring structure, was ruled out and the sequence for rings W-X was determined.

High energy collision induced dissociation MS/MS has been shown to be a practical method for structural verification of polycyclic ethers. Studies on yessotoxin,[9] maitotoxin,[6] and the gymnocins[4] revealed that fused polycyclic ethers give rise to characteristic ring cleavages with product ions that enable deduction of ether ring sizes and position and type of substituents directly. Therefore, MALDI-SpiralTOF-TOF was used for structural confirmation of KBT-F.[10]

In CID MS/MS analysis of YTX and MTX, the sulfate esters at the terminus of the molecules played an important role to simplify the product ion spectra because a negative charge is localized at that position, resulting in charge remote fragmentation. However, KBT-F does not contain a functional group for charge remote fragmentation. To introduce a charge site into the molecule, KBT-F was reacted with 3-(hydrazinecarbonyl)benzene sulfonate in 80% pyridine (Figure 2). Negative ion MALDI MS of a KBT-F benzene sulfonate derivative (**2**) revealed a molecular ion at m/z 2250 $[M - Na]^-$.

The MALDI-SpiralTOF-TOF produced prominent product ions generated by bond cleavage at the characteristic sites of ether rings (Figure 3). A mass difference of 56 Da between prominent ions indicated the presence of a six-membered ether ring. A mass difference of 70 Da could indicate either a seven-membered ether ring or a six-membered ether ring bearing a methyl; however, the NMR analyses enabled distinguishing the absence or presence of a methyl on an ether ring by the NOESY and HMBC experiments as mentioned above.

The prominent product ions showed the sequence of cyclic ethers for rings A–Q and S–W. The sequence of seven contiguous six-membered ether rings C–I was clearly confirmed by differences of 70/70/72/56/70/70/70 Da (Figure 3a). The differences of 70, 70, and 56 Da between m/z 727 and 797, 1037 and 1107, and 1868 and 1924 confirmed the positions of rings G, K, and T. The differences of 100 Da between m/z 937, 1037, and 86 Da between m/z 1107 and 1193 indicated the location of seven-membered ether rings (rings J and L) bearing a hydroxyl group. As depicted in Figure

Table 1. ^1H and ^{13}C NMR Assignments (δ) of Brevisulcenal-F (1) in Pyridine-d_5

no.	^1H	^{13}C	no.	^1H	^{13}C	no.	^1H	^{13}C
1	9.51	197.1	36	4.13	78.6	71	5.13	67.5
2		143.3	37		82.9	72	4.35	82.6
3	6.61	152.0	38	4.33	80.3	73	4.61	67.3
4	2.45	37.1	39	2.14	33.1	74	2.54	41.7
	2.55			2.29			2.83	
5	4.37	76.6	40	4.18	78.3	75	3.38	80.2
6	5.45	127.2	41		82.7	76	3.81	78.8
7	5.98	140.1	42	4.04	75.9	77	1.80	38.6
8		78.8	43	2.30	42.2		2.65	
9	3.86	80.5		2.68		78	3.51	79.6
10	1.98	26.7	44	3.84	84.4	79	3.36	80.2
	2.04		45	4.09	86.4	80	2.21	37.9
11	1.89	27.1	46	2.13	33.3		2.59	
	2.05			2.46		81	3.38	80.5
12	4.08	75.7	47	1.95	30.4	82	3.46	79.7
13		81.9		2.01		83	1.73	39.0
14	2.05	44.1	48	3.27	85.4		2.49	
	2.08		49	3.26	85.2	84	4.29	66.9
15	3.91	73.0	50	1.90	42.6	85	3.26	83.5
16		76.9		2.55		86	4.40	67.5
17	1.99	41.9	51	3.44	82.3	87	2.00	34.4
	2.24		52	3.19	82.7		2.60	
18	4.54	74.5	53	1.67	31.2	88	3.77	79.0
19		80.1		1.86		89	6.00	87.3
20	4.40	75.4	54	1.76	40.6	90	6.19	132.3
21	3.68	82.7		2.02		91	6.45	132.9
22	4.20	75.7	55		80.1	92	5.28	91.2
23	1.89	38.2	56	3.40	86.0	93	1.70	49.0
	2.73		57	2.17	35.9	94	4.37	69.0
24	3.43	82.2		2.33		95	1.37	23.8
25	3.59	80.9	58	4.02	73.2	96	1.74	11.7
26	1.75	46.7	59	3.72	75.9	97	1.47	24.6
	2.39		60	4.48	72.7	98	1.58	23.8
27		76.7	61	4.13	75.4	99	1.48	17.6
28	3.45	86.2	62	4.85	76.2	100	1.49	18.0
29	2.01	30.4	63	2.67	38.5	101	1.45	24.8
	2.06			2.73		102	1.64	23.8
30	3.67	76.5	64	4.98	70.8	103	1.56	23.3
31		77.5	65	4.60	77.5	104	1.41	21.5
32	1.89	45.2	66	4.30	77.0	105	1.44	24.6
	2.35		67	4.52	75.5	106	1.43	19.9
33	4.86	81.4	68	4.56	72.4	107	1.05	12.4
34		82.1	69	2.17	35.9			
35	2.31	48.7		3.17				
	2.59		70	4.70	74.3			

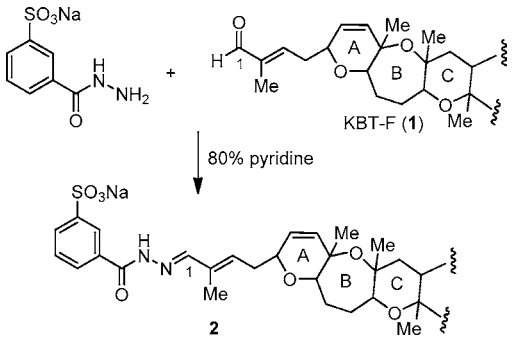

Figure 2. Preparation of KBT-F benzene sulfonate derivative (**2**).

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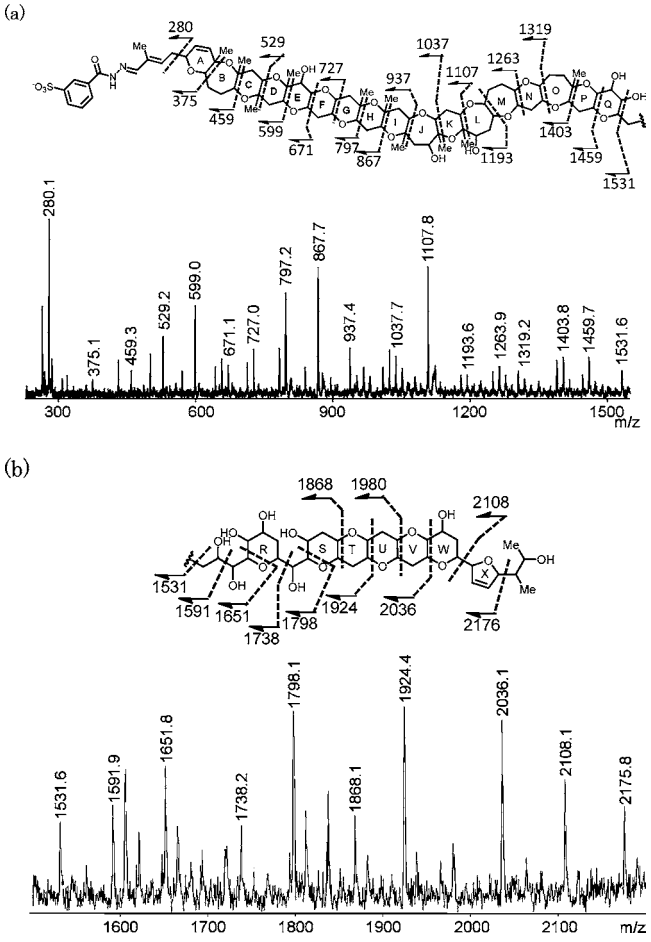

Figure 3. Product ion mass spectrum of **2**. The spectrum was taken for the precursor ion $[M - Na]^-$ at m/z 2250 and is shown in two parts: (a) m/z 250−1550 (C1−C63); (b) m/z 1500−2200 (C63−C95).

3b, the observed product ions at m/z 1798, 1738, 1651, 1592, and 1531 well matched the position of ring R, the hydroxyl groups at C-64 and C-65, and an acyclic structure. Rings W−X were also confirmed. The product ions at m/z 2176 and 2108 were generated by the bond cleavage between C-92 and C-93, and C-89 and C-88, respectively. The difference of 68 Da corresponded to the cleavage of the dihydrofuran X. The difference of 72 Da between m/z 2108 and 2036 was the characteristic cleavage of a six-membered ether ring bearing a hydroxyl group, confirming the size of ring W. Thus, all the prominent ions observed in the product ion spectra supported the planar structure deduced from the NMR data.

Stereochemistry of KBT-F. The stereochemistry of KBT-F was determined by combining NOE data and proton coupling constants (Figure 4). The NOE correlations from angular methyls to hydroxy bearing protons Me-103/H-36 and Me-105/H-42 were assigned the β orientation of C-36-OH and C-42-OH on rings J and L. The NOE due to Me-100/H-20 and the small coupling constant (2 Hz) H-20/H-21 indicated C-20-OH on ring D was orientated in an axial direction. The NOE correlations H-59/H-60 and H-59/H-61 and the small coupling constants H-59/H-60 and H-60/H-61 suggested that C-60-OH and C-61-OH on ring Q were orientated in axial and equatorial directions, respectively. The NOE correlation H-73/H-75 indicated an equatorial direction of C-73-OH on ring S. The proton coupling constants of H-66/H-67 and H-68/H-69 were 8.6 and 9.7 Hz, respectively. Additionally, NOESY cross peaks were observed between H-66/H-68, H-66/H-71, and H-68/H-71. Thus, ring R has a chair conformation, and the orientations of C-67-OH and C-68-OH on ring R both were determined to be equatorial. Configurations of other stereogenic centers (C-64, C-65, C-93, and C-94) on acyclic moieties and the diastereomeric relationships between rings Q−R, R−S, and W−X remain unknown because signal overlapping and small coupling constants prevented application of *J*-based conformational analysis.[11]

Biological Properties of KBT-F. The details of the biological properties of KBTs are reported in ref 1. The mouse lethality of KBT-F was estimated to be 0.032 mg/kg. KBT-F was also toxic against mouse leukemia P388 cells at 2.7 nM.

■ CONCLUSION

The NMR and MS/MS analyses led to elucidation of the structure of brevisulcenal-F (KBT-F) and its partial configurations. KBT-F has 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 A−Q, which is the longest of known polycyclic ethers. The long contiguous ether ring assembly with the 2-methylbut-2-enal terminus is reminiscent of the gymnocins. Unlike the brevetoxins, KBT-F does not have unsaturated

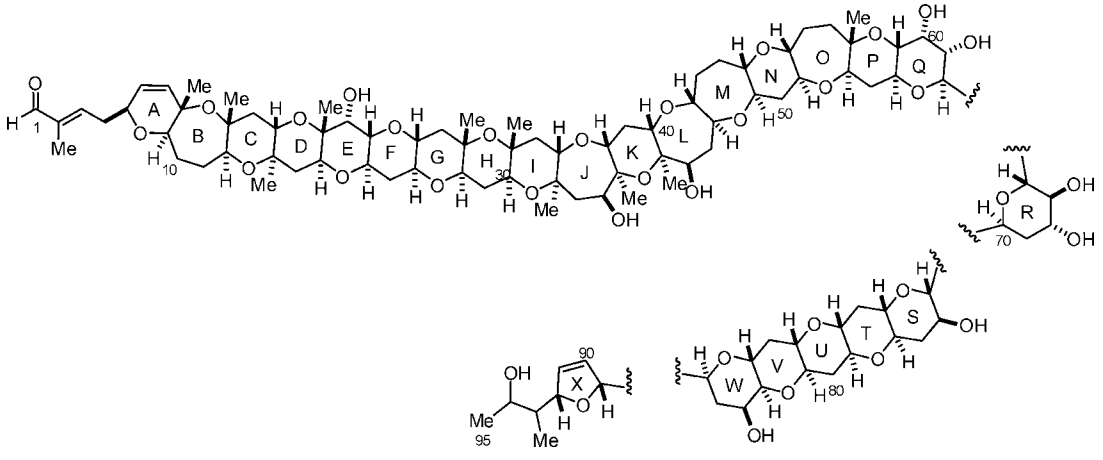

Figure 4. Relative configurations determined independently for rings A−G, ring R, rings S−W, and ring X.

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middle sized ether rings. The molecule consists of two parts: one is the rigid ether ring assembly, rings A−Q, and the other is a flexible acyclic part and the short ether ring assembly, rings S−X. The majority of the 13 hydroxyl groups reside in the flexible part of the molecule from C-60 to C-94, while all the angular methyls reside on the rigid ring assembly A−Q. Although the pharmacological mode of action of KBTs has not been elucidated, these structural features must be related to their potent biological activities, which are much higher than those of the gymnocins.

From a biosynthetic point of view, the terminal dihydrofuran X is also unusual among the marine polyethers. Formation of the fused ether rings in biosynthesis of marine ladder-frame polyethers is proposed to proceed by cascade *endo*-tet closure of a polyepoxide intermediate[12] whereas biosynthesis of dihydrofurans in terrestrial organisms is proposed to be by *exo* epoxide opening of an epoxide precursor.[13]

Structural elucidation of other analogues and configurational analyses of unelucidated stereostructures are now underway.

EXPERIMENTAL SECTION

General Methods. All solvents were purchased at highest commercial grade and used as supplied unless otherwise noted. Optical rotations were obtained on a JASCO DIP-350 polarimeter in $CHCl_3$. UV−visible absorption spectra were measured on a JASCO V-550 UV-spectrometer. NMR spectra were recorded on three NMR instruments with ^1H for 500 MHz (^{13}C for 125 MHz), ^1H for 400 MHz (^{13}C for 100 MHz), or ^1H for 800 MHz (^{13}C for 200 MHz). Chemical shift values are reported in ppm (δ) referenced to internal signals of residual protons [^1H NMR; C_5HD_4N (7.21); ^{13}C NMR, C_5D_5N (125.8)].

Culture Growth and Harvesting. *K. brevisulcata* (CAWD82) was collected from the Wellington Harbour in 1998 and is held at the Cawthron Institute Culture Collection of Microalgae (CICCM), Cawthron Institute, Nelson. Bulk cultures (150−250 L batches) were grown in 12 L carboys using 100% GP+Se media[14] under a 12/12 h day/night timed cool white fluorescent lighting regime and 25 min aeration every 30 min. Starter culture (14−21 days old) was added to 100% GP + Se media at a ratio of 1:10 to 1:15. Cultures were maintained for up to 21 days. Aliquots of culture were assessed for cells numbers with an inverted microscope. For ^{13}C enrichment, cultures were augmented at 0 and 7 days with $NaH^{13}CO_3$ (0.25 g per 12 L). Production of toxins was assessed by liquid chromatography−mass spectrometry (LC-MS) following SPE using a 50 mL aliquot of culture extracted with Strata-X (60 mg, Phenomenex Inc., CA), washed with Milli Q water and 20% methanol, and they were eluted with methanol or methanol followed by acetone (3 mL each).

Toxins were extracted from mature cultures using Diaion HP20 resin. The prewashed resin was packed in a polypropylene column. *K. brevisulcata* cultures were transferred to a 200 L barrel, and cells were lysed by addition of acetone to 7% v/v. The cultures were settled for one hour and diluted with reversed osmosis purified water (RO water) to 5% v/v acetone before pumping at 0.3 L/min through a filter system followed by the HP20 resin column. The column was then washed with water, and the HP20 resin was transferred to a 2 L flask. Toxins were recovered by soaking the resin with AR acetone (1 L) and decanting (3×). The combined acetone extract was rotary evaporated to produce a dried crude extract.

Isolation of Toxins. The crude HP20 extract was dissolved in methanol and diluted to 55% v/v with pH 7.2 phosphate buffer. The solution was partitioned with chloroform (2×), and the combined chloroform fraction containing neutral toxins was evaporated. Brevisulcenals were isolated from the neutral fractions of 1450 L of bulk cultures by column chromatography using a diol cartridge with stepwise elution (ethyl acetate to methanol) and guided by P388 cytoxicity assay. Final purification was by two stages of preparative HPLC (250 mm × 4.6 mm id Develosil C30-UG-5, Nomura Chemical Co., Japan) with isocratic elution (90% MeOH/H_2O followed by 85% MeOH/H_2O) and guided by UV absorbance at 230 nm.

Preparation of the Benzene Sulfonate Derivative (2). KBT-F (20 μg) was treated with an excessive amount of 3-(hydrazinecarbonyl)benzene sulfonate sodium salt for 2 h in 80% pyridine. After solvent elimination, the reaction mixture was partitioned between $CHCl_3$ and H_2O. The chloroform fraction was used for the MS/MS experiments (MALDI-SpiralTOF-TOF). The product ion spectra were analyzed using mMass software.[15]

MALDI-SpiralTOF MS and SpiralTOF-TOF Measurements. MALDI-SpiralTOF MS spectra were recorded on a JEOL-S3000 instrument using 2,5-dihydroxybenzoic acid (DHB, Wako) or α-cyano-*p*-hydroxycinnamic acid (CHCA, Wako) as a matrix. The KBT-F derivative was dissolved in MeOH/$CHCl_3$ and mixed with norharmane matrix and subjected to MALDI-SpiralTOF-TOF measurements in a negative mode. The product ion spectra were recorded with a laser irradiation at 349 nm, a laser frequency at 250 Hz, and −20 kV of acceleration voltage in the first TOF stage. The collision energy was 20 keV to induce high energy-collision induced dissociation. Product ions formed by collision induced dissociation were reaccelerated by 9 kV for the analysis in the second TOF stage.

Brevisulcenal-F (1). Isolated as a colorless amorphous solid: $[\alpha]_D^{19}$ 50.9 (*c* 0.05, $CHCl_3$); UV maxima (λ) 227 (ε 7900) nm. The high resolution MALDI-TOF MS of **1** gave $[M + Na]^+$ at *m/z* 2076.0476 (calcd 2076.0480 for $C_{107}H_{160}O_{38}Na$). ^1H and ^{13}C NMR data were described in Table 1.

ASSOCIATED CONTENT

Supporting Information

$^1H-^1H$ COSY, TOCSY, NOESY, HSQC, and HMBC spectra of KBT-F. 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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