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Brevisin: An Aberrant Polycyclic Ether Structure from the Dinoflagellate *Karenia brevis* and Its Implications for Polyether Assembly

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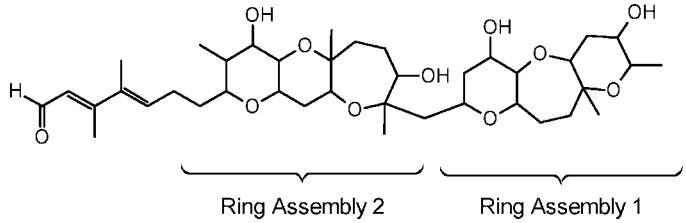

Brevisin is an unprecedented polycyclic ether isolated from the dinoflagellate *Karenia brevis*, an organism well-known to produce complex polycyclic ethers. The structure of brevisin was determined by detailed analyses of MS and 2D NMR spectra and is remarkable in that it consists of two separate fused polyether ring assemblies linked by a methylene group. One of the polycyclic moieties contains a conjugated aldehyde side chain similar to that recently observed in other *K. brevis* metabolites, though the "interrupted" polyether structure of brevisin is novel and provides further insight into the biogenesis of such fused-ring polyether systems. On the basis of the unusual structure of brevisin, principles underlying the initiation of polyether assemblies are proposed. Brevisin was found to inhibit the binding of [^{3}H]-PbTx-3 to its binding site on the voltage-sensitive sodium channels in rat brain synaptosomes.

Introduction

One of the most distinctive features of the chemistry of dinoflagellates has been the large diversity of polycyclic ether compounds that they produce.[1−3] Such compounds have been of great interest to many scientific groups because of their biological activity and unusual structures, the unique aspects of their biosynthesis, and the synthetic challenge that they present.[4−7] While such compounds are assembled from building blocks such as acetate, a particular area of interest has been to understand the principles underlying the conversion of a putative polyketide chain to the assemblies of fused ring (or ladder frame) polyethers seen in such compounds as PbTx-2 (**2**; also referred to as brevetoxin-B)[8] and PbTx-1 (**3**; also referred to as brevetoxin-A).[9] In the absence of enzymes or even gene sequences for such biosynthetic pathways, researchers have been restricted to developing biosynthetic principles based on labeling experiments and structural features within the suite of known polyethers. An early success using this approach came from Nakanishi, who observed that such assemblies could be produced by a cascade of successive endotet cyclizations from a polyepoxide precursor.[10] Such a process would account for the highly consistent patterns of ether bond occurrence within these compounds that are unaffected by ring size. A more recent advancement came from the observation by Gallimore and Spencer that, when restricted to the simpler fused ring polyethers similar to the brevetoxins,[11] there is a stereochemical regularity at the ether ring junctures that supports Nakanishi's hypothesis

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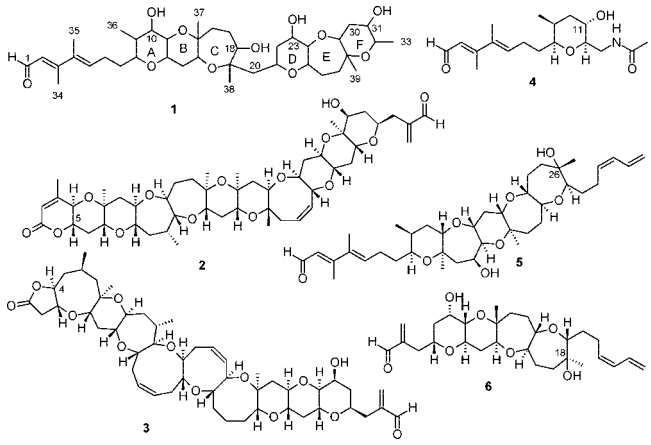
and further suggests a polyepoxide precursor produced by uniform oxidation at the same face of all *E* double bonds within an earlier polyene precursor.[12] This observation makes the polyepoxide cascade mechanism more attractive by reducing the degree of logistical complexity required for the process. Other aspects of this process that can be induced from the structures are that it is flexible in terms of both the number of rings within a system and in the sizes of the ether rings formed (five- to nine-membered rings have all been observed).



Two recent discoveries have shed light on the process of initiation of ladder frame formation, i.e., formation of the first ether ring and its importance to the whole cascade. The first came from chemical synthesis performed by Vilotijevic and Jamison which showed that ladder frame formation via endotet cyclization can only proceed spontaneously in aqueous media at neutral pH from a suitable polyepoxide if a template or initial β-hydroxy-tetrahydropyran ring is built into the precursor.[13] Otherwise, cyclization proceeds via the more kinetically favored exotet process.[14] This result suggests that after the first ether ring has been formed, the entropic cost of frame extension via endotet cyclization is much lower, perhaps enabling catalysis of ring formation by an enzyme providing minimal steric guidance of the process. Such a scheme could eliminate the need for active site cavities customized for each size of ring formed in a fused ring polyether. The second discovery is that of brevisamide (**4**), a monocyclic ether with apparent structural relatedness to the polycyclic ether brevenal (**5**), both isolated from *K. brevis*, the same organism that produces brevetoxins.[15] The single ring of brevisamide strongly resembles Jamison's template ring noted above, although in this case there are no further epoxides to propagate a ladder frame. Moreover, the occurrence of an apparent glycine starter unit in the putative polyketide precursor suggests that the direction of ether ring cascade propagation is opposite to the direction of polyketide chain synthesis. We report here the isolation of brevisin (**1**), a new polycyclic ether from *K. brevis* with an unprecedented structure, which contains two ring assemblies connected to one another by a methylene group. This phenomenon illuminates aspects of ladder frame initiation and propagation not apparent from the more regular fused ring polyethers.

Results and Discussion

The purification of brevisin (**1**) from the combined extract of cells and culture media was accomplished by a series of reversed-phase chromatography steps. The HR-FABMS and NMR data indicated a molecular formula of $C_{39}H_{62}O_{11}$ ([M + Na]$^+$ 729.4187, Δ −0.2 mmu) for **1**. The UV absorption maximum of **1** at 290 nm was identical to that of brevenal (**5**), suggesting that **1** contained a 3,4-dimethylhepta-2,4-dienal side chain similar to that in **5**. The ^{1}H NMR, ^{13}C NMR, DEPT, and HSQC data showed that **1** contained five singlet and two doublet methyls, 10 aliphatic methylenes, one aliphatic methine, 13 oxymethines, three quaternary oxycarbons, two olefinic methines, two quaternary olefinic carbons, and one aldehyde (Table 1). Based on the molecular formula and NMR data, brevisin contains nine double bond equivalents that can be accounted for by one aldehyde, two double bonds, and six ether rings. The ^{1}H NMR chemical shift data showed two of the singlet methyls were vinylic (δ_H 1.74 and 2.17 ppm), while the other three were attached to quaternary oxycarbons, a common feature at the ring junctions of polyethers. Detailed analysis of ^{1}H−^{1}H COSY and TOCSY spectra led to identification of five spin systems representing H-1 to H-2, H-5 to H-14, H$_2$-16 to H-18, H$_2$-20 to H$_2$-27, and H-29 to H$_3$-33 (Figure 1). The 3,4-dimethylhepta-2,4-dienal side chain, originally suggested by the UV data, was found to span C-1 to C-5 as confirmed by COSY correlations from H-1 to H-2 and from H-5 to H$_2$-6 and by long-range HMBC correlations from H$_3$-34 to C-2, C-3, and C-4 and from H$_3$-35 to C-3, C-4, and C-5. Similarly, the connectivities between the other spin systems were established by HMBC correlations. Thus, the observed cross-peaks from H$_3$-37 to C-14, C-15, and C-16; from H$_3$-38 to C-18, C-19, and C-20; and from H$_3$-39 to C-27, C-28, and C-29 made it possible to trace the carbon skeleton from C-1 to C-33. The observed ROESY correlations H-8/H-12, H-11/H$_3$-37, and H-14/H$_3$-38 identified the locations of the ether rings A, B, and C. Additional ROESY correlations H-24/H-29 and H$_3$-39/H-32 confirmed the positions of ether rings E and F and, hence, accounted for five of the six rings suggested by the molecular formula. Unexpectedly, there was no ROESY correlation from H-18 to H-25 (indicative of a nine-membered ring) or, indeed, between H-18 and any oxymethine proton. Instead a strong ROESY cross peak between H-25 and H-21 was observed, which identified ring D as the sixth and final ether ring in **1**. Taken together, the combined evidence indicate that the ether rings in **1** are not fused in a contiguous manner as has been the case for all polyethers from *K. brevis* up to now; instead, **1** is constructed from two fused polyether ring assemblies linked by a methylene group (6/6/7-CH$_2$-6/7/6).

This unprecedented architecture required further confirmation, which was provided by determination of the locations of the four hydroxyl groups in **1** by three independent methods: measurement of the effect of hydroxyl deuterium exchange on oxymethine carbon chemical shift, identification of COSY correlations between hydroxyl protons and oxymethines observed in pyridine-d_5, and the preparation and ^{1}H NMR characterization of a tetra-acetate derivative.[16] The deuterium shift experiment revealed that among all oxygenated carbons, only the four signals for C-10, C-18, C-23, and C-31 shifted significantly to lower field when measured in CD$_3$OH as

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TABLE 1. ^{1}H and ^{13}C NMR Data for **1** in Pyridine-d_5[a]

pos	^{1}H ($^{3}J_{HH}$ mult. Hz)	^{13}C	ROESY	HMBC
1	10.31 (d 8)	191.8		2
2	6.17 (d 8)	125.8	35	4, 34
3		157.8		
4		136.2		
5	6.14 (d,d 8,8)	135.5	6, 7a, 7b, 8, 34	3, 6, 7, 35
6	2.34 (m)	26.6	5, 7b, 35	5, 7
7a	1.78 (m)	32.1	5, 8ax, 36	
7b	1.43 (m)		5, 6, 8ax, 36	
8ax	4.25 (m)	74.5	5, 6, 7a, 7b, 9eq, 12ax	
9eq	2.14 (m)	41.1	8ax, 10eq, 36	
10eq	4.18 (br s)	72.2	9eq, 11ax, 36, 10-OH	
11ax	3.74 (d,d 10,3)	70.4	10eq, 13ax, 36, 37	
12ax	4.07 (d,d,d 10,10,3)	71.7	8ax, 13eq, 14	
13eq	2.36 (m)	35.6	12ax, 13ax, 14ax	14, 15
13ax	1.97 (m)		11ax, 13eq, 37	14
14ax	4.42 (d,d 5,9)	71.6	12ax, 13eq, 16a, 38	13, 15, 19
15		78.8		
16a	2.70 (d,d 11,11)	35.8	14ax, 16b, 17a, 17b	15
16b	1.75 (m)		16a, 17a, 17b	
17a	2.05 (m)	26.8	16a, 16b, 18	
17b	2.04 (m)		16a, 16b, 18	
18	4.45 (d,d 3,3)	73.5	20a, 20b, 37, 18-OH	
19		81.1		
20a	1.96 (m)	48.0	18, 21	18, 19, 21, 38
20b	1.72 (m)		18, 21	18, 19
21ax	4.40 (d,d 10,10)	68.9	20a, 20b, 22eq, 25ax	
22ax	2.03 (m)	40.8	22eq, 23eq	24
22eq	1.69 (m)		21ax, 22ax, 23eq	21
23eq	4.37 (br d 3)	67.9	22ax, 22eq, 24ax, 23-OH	
24ax	3.33 (d,d 10,3)	83.8	23eq, 26, 27a, 29ax	
25ax	4.03 (m)	74.8	21ax, 26, 27b, 39	27
26	2.02 (m)	30.1	24ax, 25ax	25, 27
27a	2.13 (m)	39.5	24ax, 27b, 29	25, 26, 28, 39
27b	1.88 (d,d,d 5,5,5)		25ax, 27a, 39	25, 26, 28, 39
28		77.0		
29ax	3.69 (d,d 10,4)	80.7	24ax, 27a, 30eq, 31ax	24, 27, 28, 30, 39
30eq	2.45 (d,d,d 10,3,3)	37.5	29ax, 30ax, 31ax	28, 29, 31, 32
30ax	2.03 (d,d,d 10,10,3)		30eq, 39	28, 29
31ax	3.59 (d,d,d 10,10,3)	72.4	29ax, 30ax, 33, 31-OH	32
32ax	3.74 (m)	71.7	33, 39	31
33	1.49 (d 6)	19.2	32	32
34	2.17 (s)	14.3	5	2, 3, 4
35	1.74 (s)	13.8	2, 6	3, 4, 5
36	1.05 (d 7)	11.6	7a, 9eq, 10eq, 11ax	8, 9, 10
37	1.54 (s)	16.7	11ax, 13ax, 18	14, 15, 16
38	1.66 (s)	21.8	14ax	18, 19, 20
39	1.31 (s)	18.3	25ax, 27b, 30ax, 32ax	27, 28, 29
10-OH	5.98 (s)		10eq	
18-OH	6.39 (d 4)		18	
23-OH	5.99 (s)		23eq	
31-OH	6.62 (s)		31ax	

[a] ^{1}H and ^{13}C NMR measured at 500 and 125 MHz, respectively, and referenced to residual solvent peaks at 7.58 and 135.5 ppm, respectively.

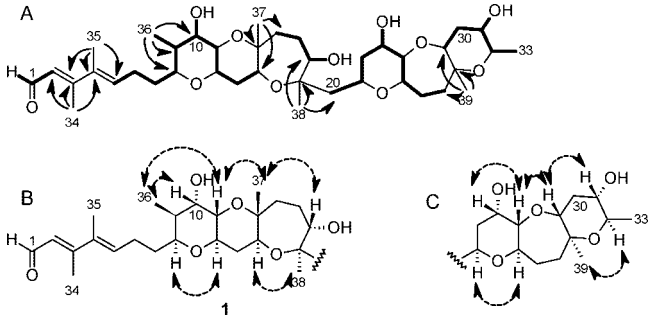

FIGURE 1. Structure of **1** deduced from NMR data: (A) $^{1}H-^{1}H$ COSY (bold lines) and HMBC (arrows) correlations. The relative configurations determined independently for rings A−C (B) and for rings D−F (C) are shown including key ROESY (dashed arrows) correlations.

compared to CD_3OD.[17] When the NMR spectra were measured in pyridine-d_5, four hydroxyl protons at δ_H 5.98, 6.39, 5.99, and 6.62 ppm, were found by COSY to be coupled with H-10, H-18, H-23, and H-31, respectively. Finally, acetylation of **1** with pyridine/acetic anhydride yielded a tetraacetate derivative ($C_{47}H_{70}O_{15}$; $[M + H]^+$ 875.4802, Δ +0.9 mmu). The ^{1}H NMR spectrum of this derivative revealed the characteristic downfield shift of four oxymethine protons assigned to H-10, H-18, H-23, and H-31 which now appeared at δ_H 4.95, 5.26, 5.26, and 4.38 ppm, respectively. Having definitively located the four hydroxyl groups at C-10, C-18, C-23, and C-31, we must infer that the other 12 oxycarbons in brevisin all participate in ether linkages.

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The ROESY correlations and proton coupling constants revealed that the ether rings in each three-ring assembly were fused in the usual *trans*−*cisoid* manner as in the brevetoxins. The ROESY correlation H-8/H-12 indicated that the dienal side chain in **1** is equatorial to ring A in contrast to the brevetoxins for which the side chains are located axially. Other observed ROESY correlations from H_3-36 to H-10 and H-11 indicated an axial location for H_3-36 on C-9. The presence of a strong ROESY cross peak of the appropriate phase for H-10/H-11 and a small coupling ($^3J_{HH}$ 5 Hz) between H-10 and H-11 pointed to an axial orientation for the hydroxyl group in ring A. Additional ROESY and *J* coupling data were used to establish the orientation of the remaining hydroxyl groups as follows: An α orientation for 18-OH on oxepane ring C was suggested by a weak ROESY correlation between H_3-37/H-18, and an axial orientation was deduced for 23-OH from the ROESY cross peak H-23/H-24, which had a high intensity and the expected phase for a ROESY interaction, and a coupling constant ($^3J_{HH}$ 5 Hz) between H-23 and H-24. Finally, a ROESY correlation H-31/H-29 and a large coupling constant ($^3J_{HH}$ 10 Hz) between H-31 and H-32 indicated an equatorial orientation for 31-OH.

In the side chain, *E* geometry of the $\Delta^{2,3}$ and $\Delta^{4,5}$ double bonds was based on NOE correlations from H-1 to H_3-34, from H_3-35 to H_2-6, and by the high-field ^{13}C chemical shifts of the olefinic methyls, C-34 and C-35 (Table 1). Absence of any $^1H-^1H$ coupling around the quaternary carbon C-19 hampered elucidation of the stereochemical orientation between C-19 and C-21. Consequently, the relative stereochemistry of the polyether ring assemblies A−C and D−F in **1** were assigned separately as shown in parts B and C of Figure 1. Attempts to prepare MTPA derivatives were unsuccessful, probably due to steric hindrance of the ring systems and the stereochemical orientation of the hydroxyl groups themselves.

The discovery and characterization of brevisin is important from a number of perspectives. First, it adds to the suite of polyether metabolites obtained from the dinoflagellate *K. brevis* which includes brevisamide (**4**),[15] hemibrevetoxin (**6**),[18] brevenal (**5**),[19,20] and the toxic brevetoxins (cf. **2** and **3**), causative agents of red tide episodes in the Gulf of Mexico and off the Florida Gulf coast.[8,9,21] These molecules represent a variety of polyether structures, ranging in molecular weight from 323 to 894 Da, and containing 1−10 fused ether rings. Brevisin inhibited the binding of PbTx-3 to the VSSC at nearly 10 μM (ED_{50}). This value is more than 1000-fold less potent than that of PbTx-2 (**2**) but similar to that of brevenal (**5**). Whether brevisin has antagonistic activity toward the brevetoxins will be tested and reported elsewhere along with detailed results of inhibition of the binding of PbTx-3 to VSSC.

The discovery of brevisin also has important biogenetic implications. As described in the Introduction, there is a growing belief that such polyether ring systems are generated by a cascade mechanism involving a putative polyepoxide biosynthetic intermediate derived from a preformed *E*-polyene. This hypothesis originally stemmed from isotopic labeling studies with the bacterial products lasalocid[22,23] and monensin,[24] which led to a unified proposal of polyene−polyepoxide intermediates in polyether production.[25] Such a mechanism has been further bolstered by recent findings regarding the biosynthesis of monensin and lasalocid and the role of a putative epoxy hydrolase in the formation of the ether rings.[26−28] However, the ether rings in monensin are not fused and hence are formed by the more kinetically favored exotet mechanism,[14] unlike fused polyethers which would be generated by the less favored endotet process.[12] It is important to note that in lasalocid the last ether ring is formed by an endotet mechanism, and a purified epoxide hydrolase from the lasalocid producer *Streptomyces lasaliencis* has been shown to convert a synthetically produced polyepoxide precursor to the energetically disfavored endotet natural product.[28] Although these collective results apply to bacterial metabolites that do not contain fused polyether systems, epoxide hydrolase catalyzed ring opening of aliphatic oxiranes and subsequent fused ether ring formation remains an attractive biogenetic scheme to explain the origin of such dinoflagellate polyethers, especially in light of the reported in vitro spontaneous formation of small fused ring polyethers from polyepoxide templates.[13] An important outcome of such a mechanism is that the last ether ring formed by the cascade should be identifiable by the presence of an oxygen substituent β to the ether ring oxygen, a common feature in most fused ring polyethers, albeit sometimes masked as part of a lactone or ketal functional group (e.g., C-18 and C-31 in **1**; C-5 in **2**; C-4 in **3**; C-11 in **4**; C-26 in **5**; C-18 in **6**).

Using the observation noted above as a guide to determining the polarity of a putative ether-forming cascade, further biosynthetic considerations suggest that the direction of propagation of the ether-forming cascade is opposite in sense to the direction of polyketide chain growth. In other words, the first ether ring to be generated in the assembly process arises from the last epoxide ring formed on the polyketide chain, assuming that the putative polyene is epoxidised in a stepwise fashion as the nascent polyketide chain grows. The evidence for this is limited in that the only ladder-frame polyethers for which the direction of polyketide chain assembly has been unambiguously determined are PbTx-1 (**3**)[29] and PbTx-2 (**2**).[30,31] Additional evidence is provided by brevisamide (**4**), where a compelling argument can be made for the incorporation of glycine as a starter unit based on precedence with other dinoflagellate metabolites.[15,32] In the case of the brevetoxins, brevisamide (**4**), and by extension the related compounds brevenal (**5**) and brevisin (**1**), the direction of the ring cascade appears to be opposite the direction

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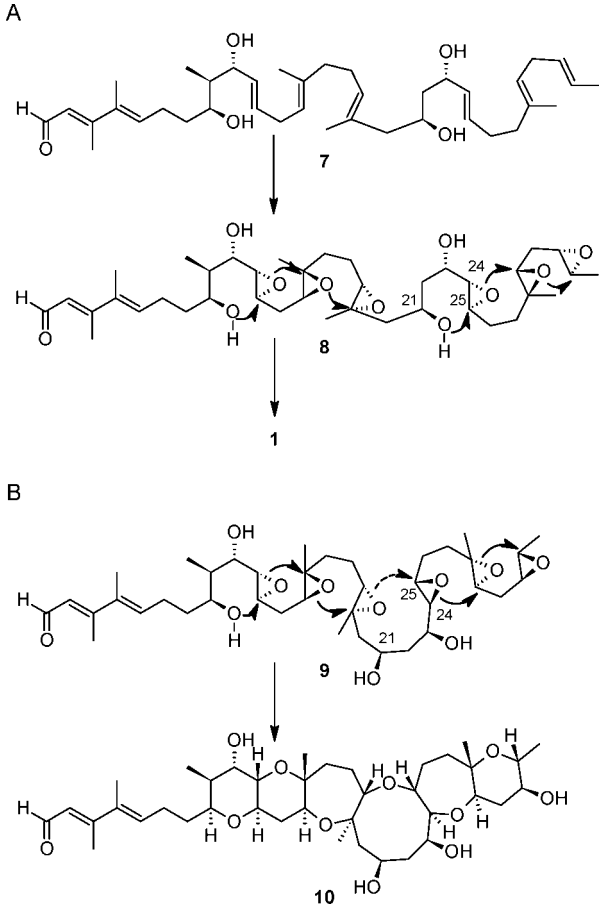

FIGURE 2. Two possible ring systems that can be formed from a putative polyepoxide precursor to brevisin. (A) Formation of the interrupted system with two initiators and two ring assemblies. (B) Formation of a contiguous ring assembly in which a putative initiator hydroxyl group is incorporated as a pendant group on a larger ring (shown with dashed arrow).

of polyketide chain growth. This is entirely consistent with the early labeling data obtained with lasalocid and monensin.[22−25]

The unique nature of the two methylene-bridged ladder frames found in brevisin probes this cascade process further. In brevisin (**1**), the putative polyepoxide cascade pathway is interrupted so that a structure containing a series of contiguous fused polyethers rings is not formed. Instead, one group of ladder frame ether rings are formed (D−F), followed by a second group (A−C). Figure 2A illustrates a process resulting from the occurrence of two cascade-initiating events within a putative polyepoxide precursor such as **8**, itself potentially derived from a polyene such as **7**. One cascade initiation starts at ring D, the other at ring A (Figure 2A). While it is impossible to determine whether the two ring assemblies are formed sequentially or simultaneously, it should be noted that an alternative continuous cyclization cascade derived from an intermediate similar to **9** could conceivably yield **10**, an uninterrupted fused ring product containing a 9-membered ring (Figure 2B). As noted earlier, 9-membered rings have already been found to occur in polyethers from *K. brevis*, so there appears to be no inherent mechanistic barrier to their formation by these cyclization cascades. To date, there is no evidence that **10** is formed, and its absence suggests that at the time of ring C formation (simultaneous to the formation of the alcohol at C-18), the epoxide at C-24/C-25 no longer exists, perhaps by virtue of having already condensed with the alcohol at C-21 to form ring D. This in turn would suggest that the logic underlying formation of the initiator ring in fused ring polyether cyclization cascades is a simple one based merely on the spacing in carbon atoms between an epoxide group and the nearest downstream (in the directional sense of polyketide chain biosynthesis) hydroxyl group.

In conclusion, the discovery of brevisin (**1**) highlights the remarkable diversity of polyether structures present in a single isolate of *K. brevis*. The structure of **1** bears an identical side chain to those found in **4** and **5**, yet each in the series contain distinct features in the architecture of their ether rings. In brevisamide (**4**), the cyclization process terminates after formation of the first ring due to the lack of an upstream epoxide. In brevisin (**1**), there are two suitable locations where initiator rings (A and D) can form, leading to an interrupted ladder structure. In brevenal (**5**) and the brevetoxin-like compounds, there is only one location suitable for initiator ring formation, and an appropriate arrangement of epoxides leads to a contiguous ladder-frame structure. While much remains to be understood, the discovery of brevisamide (**4**), brevisin (**1**), and brevenal (**5**) begins to shed some light on the biosynthetic assembly of these fascinating molecules.

Experimental Section

General Experimental Procedures. NMR spectra were acquired at 500 and 125 MHz for ^{1}H and ^{13}C, respectively. Spectra were acquired using pyridine-d_5 as a solvent and referenced to solvent signals at δ_H 7.58 and δ_C 135.5 ppm. Low-resolution mass spectra were acquired on an instrument using electrospray ionization and interfaced to an HPLC. High-resolution mass spectra were acquired using time-of-flight mass analysis and electrospray ionization. All solvents used for purification were of HPLC grade.

Culturing, Extraction, and Isolation. The dinoflagellate *K. brevis* (Wilson's 58 clone) was cultured in 10 L glass carboys, each containing 8 L of seawater media enriched with NH-15 nutrients, for 35 days at 24 °C. The combined cells and spent media from 200 L of culture were extracted with $CHCl_3$. Evaporation of the $CHCl_3$ extract yielded a thick oily residue that was partitioned between hexane and 90% MeOH in water. The aqueous MeOH phase was subjected to Si gel flash chromatography using a $CHCl_3$ to MeOH gradient, and selected fractions were further purified by a C_{18} flash column eluted with a linear gradient from 10% MeCN in water to 100% MeCN. Fractions containing **1** were combined and rechromatographed using a C_8 column (Waters Spherisorb C8, 5 μm, 1 × 25 cm) and an eluant gradient from 25% to 60% MeCN in water. Final purification was accomplished by HPLC on a C_{18} column (Waters μBondapak C_{18}, 10 μm, 3.9 × 150 mm) and elution with 28% MeCN in water. Throughout the purification, elution of **1** was monitored by UV absorption at 214 and 290 nm to yield **1** as a colorless amorphous solid (2.8 mg). Assays for the ability of **1** to competitively displace binding of tritiated PbTx-3 to rat brain synaptosomes were carried out as previously described for brevenal (**5**).[19]

Brevisin (1): UV (MeOH) λ_{max} 290 nm (log ε 4.18); $[\alpha]^{25}_D$ −21 (*c* 0.33, MeOH); IR (KBr) ν_{max} 3450, 2940, 1650, 1619, 1457, 1377, 1220, 1062 cm^{-1}; ^{1}H and ^{13}C NMR (Table 1); HRFABMS *m/z* 729.4187 (calcd for $C_{39}H_{62}O_{11}$ $[M + Na]^+$ 729.4189).

Peracetylation of 1. A 2.0 mg portion of **1** was dissolved in 250 μL dry pyridine to which 200 μL of acetic anhydride were added. The reaction was quenched with MeOH after 16 h at room temperature. The solvent was evaporated under a stream of N_2 and the sample characterized by 1D ^{1}H NMR and COSY.

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Supporting Information Available: One- and two-dimensional NMR data for brevisin. This material is available free of charge via the Internet at http://pubs.acs.org.

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