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Brevisamide: An Unprecedented Monocyclic Ether Alkaloid from the Dinoflagellate *Karenia brevis* That Provides a Potential Model for Ladder-Frame Initiation

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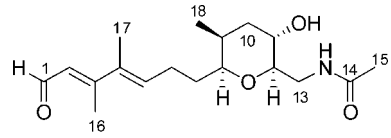
ABSTRACT



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The dinoflagellate *Karenia brevis* is known for the production of brevetoxins, a family of polycyclic ether toxins, as well as their antagonist brevenal. Further examination of organic extracts of *K. brevis* has uncovered yet another unprecedented cyclic ether alkaloid named brevisamide. This report describes the structure elucidation of brevisamide based on detailed MS and NMR spectral analysis, and the importance of this new compound in shedding light on the biogenesis of fused polyethers is discussed.

The toxins produced by dinoflagellates have attracted much interest both for their ecological and human health effects and for their structural complexity. The most characteristic toxins of the marine dinoflagellates are the cyclic polyethers which may take the form of ladder-frame fused rings or linear polycyclic ether rings.[1−4] For as long as the structures of the ladder-frame polyethers have been known, the biosynthetic processes underlying the assembly of these complex architectures have been the subject of much speculation and experimentation.[5−9]

One particularly appealing hypothesis on the formation of the ladder-frame was put forward by Nakanishi that involves formation of ether rings by a stepwise or cascading series of condensations starting from a putative polyepoxide intermediate.[5] Whatever the underlying process, the outstanding versatility in terms of the sizes of the polyether rings formed and the number of rings in a given system is readily apparent from the structures of the various known ladder-frame polyethers. An intriguing new development in the assembly of ladder-frame polyethers was heralded by the recent report that polyether ladders form spontaneously from a suitable polyexpoxide intermediate in polar solvent, but only if a template or nucleating hydroxytetrahydropyranyl

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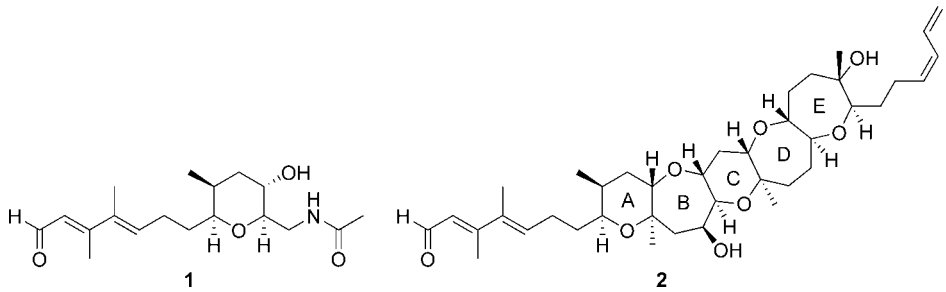

Figure 1. Brevisamide and brevenal from *Karenia brevis*.

functionality is built in to the intermediate.[10] This suggests the feasibility of a model in which only the first ether ring formed requires catalysis by an enzyme and that the remaining cyclizations proceed spontaneously, governed by the spacing and configuration of the epoxide functionalities.

The seminal studies on ladder-frame polyethers were carried out on the brevetoxins from the red tide dinoflagellate *Karenia brevis*.[11–13] Further studies with *K. brevis* have identified brevenal (**2**; Figure 1), a smaller ladder-frame polyether that exhibits antagonism of the sodium channel-blocking activity of the brevetoxins.[14,15] In addition to containing only five fused ether rings, the structure of brevenal is characterized in particular by a conjugated 3,4-dimethylhepta-2,4-dienal side chain, which displays characteristic UV absorption at 292 nm. During our continuing search for new metabolites of *K. brevis*, LC/UV/MS analysis of various chromatography fractions indicated the presence of other compounds displaying similar UV absorption at 292 nm. A very minor component displaying these UV spectral properties was successfully isolated following various column chromatography steps, and here we report the structure of brevisamide (**1**), a unique amide containing a single ether ring that matches the expected template ring possibly capable of initiating ladder-frame formation.

The dinoflagellate *K. brevis* (Wilson's 58 clone) was cultured in 10 L carboys each containing 10 L of seawater media enriched with NH-15 nutrients for 4−6 weeks at 25 °C. The purification of **1** was accomplished as follows: First, cultured cells and media were extracted with $CHCl_3$, and following evaporation in vacuo, the residue was partitioned between hexane and 80% CH_3OH in water. Flash chromatography of the aqueous CH_3OH phase on silica gel using an increasing gradient of CH_3OH in $CHCl_3$ was followed by a second flash chromatography step using a reversed-phase C18 column and elution with a gradient from 10% CH_3CN in water to 100% CH_3CN. All UV-active fractions exhibiting absorption at 290 nm were combined and further separated using a reversed-phase C8 column and eluting with a linear gradient elution from 25% to 60% CH_3CN in water. Final purification was accomplished by reversed-phase HPLC and elution with 22% CH_3CN in water. Throughout the purification scheme, elution of **1** was monitored with UV absorption at 290 and 214 nm. Finally, 0.2 mg of the major compound (**1**) was obtained as a colorless amorphous solid from approximately 400 L cultured cells.

Brevisamide (**1**): amorphous solid; $[\alpha]^{22}_D = -13$ (*c* 0.18, MeOH); UV_{max} (MeOH) 289 nm (ε 6700); IR ν_{max} (KBr) 3324, 2927, 2857, 1657, 1650, 1376, 1106, 1070 cm^{-1}. The molecular formula of $C_{18}H_{29}NO_4$ ($[M + Na]^+$ 346.1994, Δ −0.3 ppm), determined by HRMS and NMR data, indicated that **1** was a N-containing compound having five double-bond equivalents. Initially, the extremely limited amount of brevisamide hampered direct observation of the ^{13}C NMR spectra, and a combination of ^1H NMR, HSQC, and $^1H-^{13}C$ HMBC spectra were used to determine that **1** contained two olefinic methyls, one carbonyl methyl and one doublet methyl, three aliphatic methylenes, one aliphatic methine, one amino methylene, three oxymethines, two olefinic methines, two quaternary olefinic carbons, and one aldehyde. Later, when more material was accumulated, these assignments were confirmed by direct analysis of the ^{13}C NMR data (Table 1).

The ^1H NMR spectra of **1** indicated a skeletal structure distinctly different from those of the known brevetoxins, and while the rest of the spectral data was quite different, **1** shared a similar UV profile to brevenal.[15] Indeed, consistent with the UV data and analogous with brevenal, **1** possesses a 3,4-dimethylhepta-2,4-dienal side chain, which was assigned by $^1H-^1H$ COSY correlations H-1/H-2 and H-5/H_2-6, and long-range $^1H-^{13}C$ HMBC correlations from the vinylic methyl group (H_3-16, δ_H 2.33) to C-2 (δ_C 126.3), C-3 (δ_C 160.9), and C-4 (δ_C 137.2) and from the second vinylic methyl group (H_3-17, δ_H 1.86) to C-3 (δ_C 160.9), C-4 (δ_C 137.2), and C-5 (δ_C136.8; Table 1). This was also consistent with the ^{13}C NMR chemical shift data (Table 1), which closely matched the resonances for this portion of the molecule in brevenal.[15] Further analysis of the $^1H-^1H$ COSY and TOCSY data extended the proton connectivity from H_2-6 to H_2-13 and thus established the largest spin system in the molecule from H-5 through H_2-13 (Figure 2). In addition, the presence of an acetyl group was determined by an HMBC correlation

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Table 1. ^{1}H NMR and ^{13}C NMR data for **1** (CD_3OD)[a]

no.	δ_H (mult, J in Hz)	δ_C (mult)	HMBC
1	10.10 (d, 7.9)	194.4 (d)	C-2
2	6.04 (d, 7.9)	126.3 (d)	C-4, C-16
3		160.9 (s)	
4		137.2 (s)	
5	6.23 (t, 7.1)	136.8 (d)	C-3, C-6, C-7, C-17
6	2.34 m	26.9 (t)	C-5, C-7, C-8
7a	1.65 m	33.2 (t)	C-5, C-6, C-8
7b	1.44 m		
8ax	3.39 m	80.3 (d)	
9eq	1.85 m	34.3 (d)	C-11
10eq	1.90 m	40.9 (t)	C-11
10ax	1.65 m		
11ax	3.42 m	65.0 (d)	C-12
12ax	3.08 (ddd, 2.6, 7.0, 9.3)	83.1 (d)	
13a	3.53 (dd, 2.6, 14.0)	42.5 (t)	C-14
13b	3.32 (dd, 7.1, 14.0)		C-14
14		173.7 (s)	
15	1.95 s	22.5 (q)	C-14
16	2.33 s	14.6 (q)	C-2, C-3, C-4
17	1.86 s	14.0 (q)	C-3, C-4, C-5
18	0.95 (d, 6.9)	13.1 (q)	C-8, C-9, C-10

[a] Referenced to residual CD_3OD solvent signals at δ_H 3.30 ppm and δ_C 49.0 ppm. ^{1}H and ^{13}C NMR measurements were made at 500 and 125 MHz, respectively. Multiplicities for ^{13}C signals are from a DEPT experiment.

from the methyl group at H_3-15 to the carbonyl carbon C-14 resonating at 173.7 ppm. Collectively, the 3,4-dimethylhepta-2,4-dienal side chain and the acetyl function account for four of the five double-bond equivalents in the molecule, establishing that **1** contains a single ring.

A NOESY correlation between the oxymethines H-8 and H-12 indicated an ether link between C-8 and C-12 and, hence, that brevisamide possesses a tetrahydropyran (THP) ring system containing a methyl substituent at C-9 and a hydroxyl group at C-11. The ^{13}C chemical shift of the methylene carbon C-13 was observed at δ_C 42.5 by HSQC, representative of a carbon bearing nitrogen. In addition, the low-field ^{1}H chemical shifts of the H_2-13 protons at δ_H 3.32 and 3.53 were consistent with an *N*-acetyl moiety. In support of this, the HMBC correlations from the H_3-15 methyl group and H_2-13 protons to C-14 (δ_C 173.7) confirmed the partial structure around the amide group, and thus all the carbons in the molecule were assigned.

The large ^{1}H−^{1}H coupling constant ($J_3 = 9.3$ Hz) between

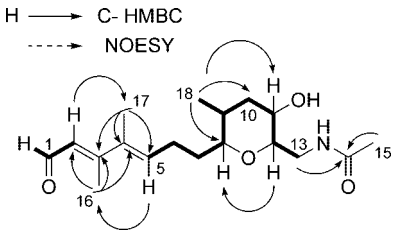

Figure 2. 2D NMR correlations establishing the structure of **1**. Bold bonds indicate spin systems observed by TOCSY.

H-11 and H-12 and the absence of an H-11/H-12 NOE correlation indicated a chair conformation of the ether ring. A NOE correlation from H_3-18 to H-11 indicated an axial location for C-18 and an equatorial orientation of the C-11 hydroxyl group. The geometry of both double bonds in the side chain was deduced as *E* based on the carbon chemical shifts of C-16 and C-17 (Table 1) and the NOE correlations H_3-16/H-5 and H_3-17/H-2. Therefore, the relative stereochemistry of brevisamide is as shown in **1**, though at present the absolute stereochemistry of brevisamide is unknown.

The discovery of **1** augments the suite of polycyclic ethers known to be produced by the dinoflagellate *K. brevis*[15] which we have now shown is composed of compounds ranging in size from 1 to 10 ether rings. The unprecedented structure of **1** represents the smallest ether-containing metabolite produced by a dinoflagellate and as such provides some interesting biogenetic clues as to the assembly of polycyclic ether systems in general. By comparison with other dinoflagellate metabolites where the biosynthetic pathway has been established, glycine can be considered to be a possible source of the nitrogen in brevisamide. In the two reported cases where glycine is incorporated in the biosynthesis of dinoflagellate metabolites, it is utilized as an extender unit in the biosynthesis of DTX-5 metabolites,[16] and as a starter unit in the biosynthesis of spirolides.[17] By analogy, glycine plausibly serves as a starter unit in a NRPS/PKS biosynthetic pathway leading to the formation of brevisamide, and the nascent chain is assembled from right to left as drawn (Figure 3), though the addition and modification of additional acetate

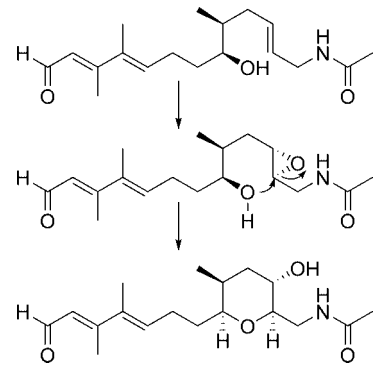

Figure 3. Possible biosynthetic mechanism for the formation of the ether ring of **1**.

units must remain speculative. Such a polarity of chain growth would be consistent with previous labeling results with the brevetoxins.[6–8]

Nevertheless, one point is clear: Brevisamide (**1**), with only a single ether ring, represents an ideal model to study the biogenesis of dinoflagellate polyether ring systems. An epoxide-based mechanism can be proposed for the formation

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of the ether ring in brevisamide (Figure 3). Following formation of the polyketide chain, the resulting hydroxy epoxide intermediate undergoes cyclization to form the ether ring following S_N2 attack of the β-hydroxyl group on the α-epoxide at C11−C12 in a flow from left to right—opposite the flow of polyketide chain assembly.[18] Interestingly, this leads to an ether ring containing an oxygen substituent anti to the ring oxygen, a structural characteristic found in the terminal ether ring of several fused polyethers such as ring B of brevetoxin A and brevetoxin B (though in both cases the oxygen is part of a lactone ring) and in many others including the gymnocins, gambierol, and the yessotoxins,[4] and supports a unified ring formation mechanism proposed for these other more complex polyethers. At the same time, this also identifies the direction of polyketide chain assembly, assuming that this is always opposite to the polyepoxide cascade mechanism. Having the direction of ether ring formation be the opposite of the direction of polyketide chain synthesis is appealing since it allows a single priming, perhaps enzymatic, step to be propagated in a self-sustaining cascade across the multiple epoxide functionalities installed during the process of chain elongation as seen in the work of Vilotijevic and Jamison with synthetic intermediates.[10]

However, the hydroxyl-THP structure present in brevisamide goes further in our understanding of the cascade process. The location of nitrogen in **1** precludes formation of 6/6 polyether systems via a cascade mechanism as described above, though it does suggest the tantalizing notion such a THP derivative could serve as a progenitor for other N-containing polyether systems in *K. brevis*. At the same time, the discovery of **1** also suggests that a brevisamide-like precursor lacking nitrogen could serve as the initiating unit in a cascade process leading to the formation of other polyethers in *K. brevis*. For example, since **1** is clearly a truncated analogue of brevenal (**2**) which contains six fused polyether rings, a polyepoxide cascade mechanism, initiated by a putative hydroxyl-THP derivative, could be proposed for the biogenesis of this multicyclic polyether.

(18) Other cyclization mechanisms can also be envisaged involving an imine intermediate, but in all cases the cyclization flow is always opposite the polyketide flow.

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Supporting Information Available: Details on instrumentation used; ^1H, ^{13}C, and DEPT 1D NMR spectra; TOCSY and NOESY 2D NMR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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