Absolute Configuration of Brevisamide and Brevisin: Confirmation of a Universal Biosynthetic Process for *Karenia brevis* Polyethers

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The discovery of brevisin, the first example of an "interrupted" polycyclic ether, obtained from the dinoflagellate *Karenia brevis*, posed some important questions regarding the mechanism of the cyclization process. Consequently, we have established absolute configurations of brevisin and its related metabolite brevisamide using a modified Mosher's esterification method. For brevisin, analysis was carried out on both the 31-monokis- and the 10,31-bis-MTPA esters. The results suggest that both metabolites, like other polyethers from *K. brevis*, result from polyepoxide precursors with uniform (*S*, *S*) configurations for all epoxides and provide further support for a universal stereochemical model for dinoflagellate polyether formation.

More than 20 years after their initial discovery, the fused-ring polyethers of dinoflagellates remain subjects of intense study. Although many recent studies have identified PKS gene fragments in toxin-producing dinoflagellates, the inherent complexity of dinoflagellate genome structure and posttranscriptional RNA processing have proven formidable obstacles toward the goal of identifying a complete biosynthetic pathway for a known toxin.[1−5] In spite of this, the ongoing discovery of new polyether metabolites and the development of new and innovative techniques for their synthesis have continued to expand both the information available for and the interest in the processes leading to their production. In particular, many researchers have focused on the potential formation of these compounds via epoxide hydrolase-mediated cascades from polyepoxide precursors, by analogy to bacterial polyether compounds.[6−13] The recent discovery of brevisamide (**1**)[12] from a brevetoxin-producing strain of the dinoflagellate *Karenia brevis* has shed light on the process of initiation of these cascades. The subsequent discovery of brevisin (**2**)[13] as an "interrupted" polyether was surprising and posed some questions as to the universality of the polyether cyclization process. It was reasoned that knowledge of the absolute configuration of these compounds would provide crucial details in this regard since stereochemical observations first led to the polyepoxide hypothesis and subsequent extensions of it.[6−9,14] However, initial limitations in the amount of these metabolites prevented the determination of the absolute configuration of both products, but with the accumulation of additional material we now report the determination of the absolute configuration of brevisamide (**1**) and brevisin (**2**).

The *R*- and *S*-MTPA monoesters of brevisamide (**1**) were readily formed using DMAP and triethylamine as bases. Assignments of δ_H were made for all protons in the two derivatives using a combination of ^{1}H NMR, TOCSY, MQ-COSY, HSQC, and HMBC experiments. The resulting $\Delta\delta_{S-R}$ values for each position are shown in Figure 1. Using a traditional modified Mosher's method analysis of the results indicates an 11-*S* configuration, which, combined with the relative configuration originally reported[12] and subsequently confirmed by synthesis,[15] indicates the absolute configuration shown in Figure 1. This configuration is identical to that of synthetic brevisamide (**1**), confirming the earlier observation of the synthetic compound having a specific rotation similar in sign to that of the natural product, though differing in magnitude.[15]

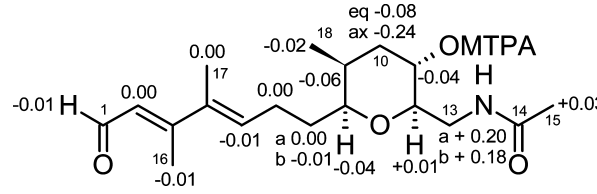
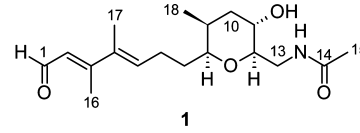
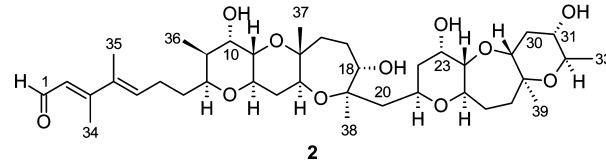


Figure 1. Observed $\Delta\delta_{S-R}$ values for the MTPA ester of brevisamide (**1**).



1



2

The esterification of brevisin (**2**) with MTPA was carried out at 4 °C using stoichiometric control of reagents. This was found to be necessary after preliminary experiments yielded instead the tetrakis-MTPA ester of brevisin, leading to interactions in $\Delta\delta_{S-R}$ effects among the different modification sites. Complete NMR assignments of all hydrogen atoms were made for the 31-monokis- and the 10,31-bis-MTPA esters based on ^{1}H NMR, TOCSY, MQ-COSY, ROESY, HSQC, and HMBC experiments. The esterification sites were identified on the basis of downfield shifts in δ_C for the esterified alcohol carbon atoms relative to brevisin (**2**) and by the disappearance of the corresponding hydroxy proton signals. Comparison of the derived $\Delta\delta_{S-R}$ values for the monokis-ester to those of the bis-ester showed that the two corresponded within ±0.02 ppm for positions 29−33 and 39, whereas for all other positions the monokis-ester had $\Delta\delta_{S-R}$ values of 0.00 ± 0.03 ppm. This indicates that the effects of the two esterification sites do not interfere with one another in any way. Figure 2 shows the observed $\Delta\delta_{S-R}$ values for 10,31-bis-MTPA-brevisin. Each of the two sites shows a clear pattern of $\Delta\delta_{S-R}$ values with negative values on one side and positive values on the other, following the expected pattern for the Mosher method[16] and allowing the unambiguous assignment of an *S* configuration for both C-10 and C-31. Thus, by using the relative stereochemical relationships noted previously for brevisin

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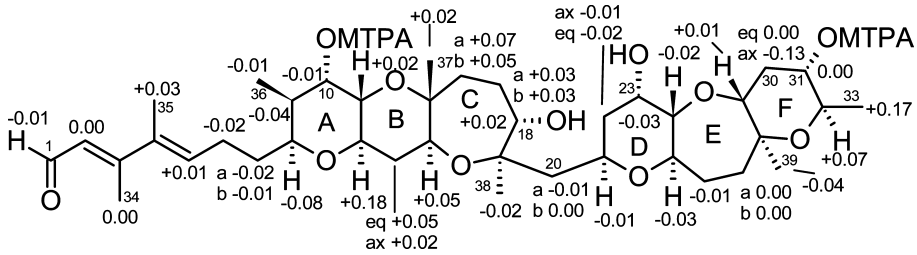

Figure 2. Observed $\Delta\delta_{S-R}$ values for the 10,31-bis-MTPA-ester of brevisin (**2**).

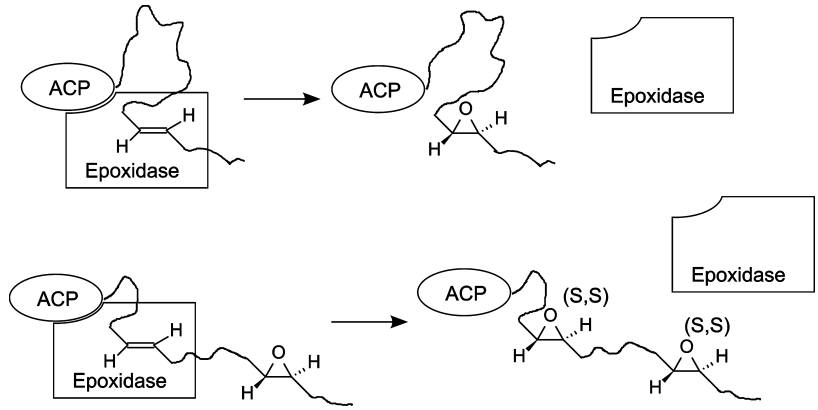

Figure 3. Illustration of how uniform configuration in epoxides might be achieved by a promiscuous epoxidase that forms contacts with an acyl carrier protein (ACP).

(**2**),[13] it is possible to assign the absolute configuration of brevisin as shown in Figure 2.

The absolute configurations for brevisamide and brevisin fit well within the framework of polyether production in *K. brevis*. For both compounds, as for brevenal, PbTx-1, and PbTx-2, the carbon atom bearing the alcohol group (or ester in the case of the brevetoxins) β to the ether oxygen on the terminal ring has an *S* configuration. Indeed, for brevisin both such carbinol moieties have the same configuration. In a ring-closing cascade based on a polyepoxide, this position must retain the configuration of the precursor epoxide because it occurs on the carbon atom that is not subject to nucleophilic attack. This suggests that the stereochemical uniformity originally noted for the brevetoxins and related compounds extends to brevisamide (**1**) and brevisin (**2**),[9] which appear to result from polyepoxide precursors where every epoxide group has an (*S*, *S*) configuration for its respective carbon atoms.[9] Crucially, this result rules out the "interrupted" frame of brevisin as resulting from a stereochemical incompatibility of an isolated epoxide group with continuous ring frame formation. Before the absolute configuration of brevisin (**2**) was known, it was possible (however unlikely) to conjecture that the epoxides from which rings A−C are formed might have an absolute configuration opposite that of the epoxides from which rings D−F are formed. Thus, this stereochemical incompatibility might cause the epoxide ring cascade to falter, favoring the interrupted frame over the continuous frame. However, our results show that a putative polyepoxide precursor to brevisin is completely uniform with respect to the configuration of all epoxides, just as such precursors are for the other *K. brevis* polyethers. This indicates that the formation of an interrupted frame results from either some other property of the polyepoxide precursor (such as spacing between hydroxy group nucleophiles and upstream epoxides) or some property of the enzyme(s) catalyzing the cascade.

Previously we suggested that the ring-forming cascade from a polyepoxide precursor flows in the opposite direction of nascent polyketide chain biosynthesis.[12] Such a polyepoxide precursor might be derived from an all *E*-polyene.[9] Although the degree of substitution of the olefinic groups to be modified varies from unit to unit within a metabolite, without exception the same olefin face is oxidized if one views the nascent chain with the side of attachment to the PKS as an orienting landmark (e.g., always on the left side; Figure 3). If our proposal for the direction of ring formation with respect to the direction of chain formation is correct, this in turn suggests that the formation of epoxides occurs at a time when the polarity of direction of the nascent chain can be distinguished, i.e., before the nascent chain has been cleaved from the PKS. Similarly, the oxidative cyclization of precursors to bacterial polyethers has been proposed to take place on a polyketide chain tethered to a dedicated ACP in the monensin pathway.[10] This also suggests that the logic underlying epoxide formation is simple, requiring only an *E* olefin of variable substitution with stereochemical orientation being provided by attachment of one end of the nascent chain to the PKS. As such, it is possible that a very small set of oxygenases (possibly a single enzyme) is required for this aspect of producing all polyethers in *K. brevis*.[9]

A second (logically, not necessarily temporally)[9,14] set of events crucial for polyether assembly is the formation of the initial ring, after which frame extension by *endo-tet* ring openings could in principle proceed spontaneously in water[11,17] or under the control of a single epoxide hydrolase.[14,18,19] Here again, there is a remarkable consistency among the polyethers of *K. brevis*. We have previously argued that the occurrence of ring frame initiation can be reliably predicted from a putative polyepoxide precursor structure based solely on the spacing between candidate nucleophile hydroxy groups and their nearest upstream epoxides.[13] Our results indicate that the two initial epoxide groups in the putative precursor to brevisin (at C-11/C-12 and C-24/C-25, respectively) would have identical absolute configurations, a property known to be crucial for bacterial enzymes in favoring the otherwise disfavored 6-*endo-tet* cyclization.[20]

In conclusion, the absolute configuration of the anomalous metabolites brevisamide (**1**) and brevisin (**2**) highlights commonalities with the brevetoxins in the enzymatic reactions occurring en route to polyether formation. Epoxidation occurs with universal stereochemical regularity.[9] Initial ring formation occurs invariably and exclusively when spacing requirements are met between alcohol and epoxide functionalities.[13] As more information regarding the

various polyether metabolites of *K. brevis* becomes available, more inferences can be drawn on the principles underlying their assembly.

Experimental Section

General Experimental Procedures. NMR spectra were acquired on a 500 MHz Bruker Avance spectrometer with a 1.7 mm TXI probe. NMR data were analyzed using Topspin 2.0 (Bruker Biospin, Inc.). Preparative HPLC was accomplished using a system with two Waters 515 HPLC pumps, a gradient controller, and a Waters 2487 dual-wavelength UV detector. All solvents used were HPLC grade.

Formation of MTPA Esters of Brevisamide (1). A solution of 4.4 mg of DMAP and 3.4 μL of triethylamine in 50 μL of dry CH_2Cl_2 was prepared. Two 500 μg portions of brevisamide (**1**) were dried in vacuo; then each was dissolved in 20 μL of the aforementioned solution, to which was immediately added 1.7 μL of *S*-(+)- or *R*-(−)-MTPA chloride (Fluka, Inc.).[21] The solutions were left at room temperature for 1 h and quenched by addition of 30 μL of CH_2Cl_2 and 50 μL of H_2O. The solutions were separately agitated by vortex and the organic layers extracted by syringe. Each reaction product was purified by HPLC using a Gemini-NX 150 × 4.6 mm, 3 μ C18 column (Phenomenex, Inc.) with a binary mobile phase system consisting of 0.1% formic acid (A) and MeCN (B). Each sample was injected onto the column under isocratic elution at 20% B (0.8 mL/min) followed by a linear gradient to 100% B over 80 min. The MTPA esters eluted at 45 min and were detected by absorbance at 290 nm.

Formation of 31-Monokis- and 10,31-Bis-MTPA Esters of Brevisin (2). For each reaction, 1.4 mg of brevisin (**2**) was dried in vacuo, then equilibrated at 4 °C. Each sample was dissolved in 120 μL of a solution prepared by dissolving 5.8 mg of DMAP in 290 μL of dry CH_2Cl_2. To this solution was added 6 μL of a solution prepared by mixing 1 μL of *R*-(−)- or *S*-(+)-MTPA chloride with 12 μL of dry CH_2Cl_2. The reactions were maintained at 4 °C for 3 h and quenched by addition of 100 μL of chilled H_2O to each vessel. The reactions were agitated by vortex and the organic layers removed by syringe. Purification of each was achieved using a Gemini-NX 150 × 4.6 mm, 3 μ C18 column (Phenomenex, Inc.) with a binary mobile phase system consisting of H_2O (A) and MeCN (B). The reactions yielded a mixture of esterified products consisting approximately of 50% 31-monokis-MTPA ester and 25% 10,31-bis-MTPA ester. Smaller amounts (15% and 10%, respectively) were obtained of a second bis- and a tris-MTPA ester, but these minor products were not characterized. The product mixture was fractionated using a stepped gradient at 0.8 mL/min of 67% B from 0−10 min, 85% B from 10−20 min, and 94% B from 20−30 min, yielding the four esters eluting at 12, 21, 22, and 25 min, respectively.

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Supporting Information Available: ^{1}H 1D NMR spectra and TOCSY, HSQC, HMBC, DQF-COSY, and ROESY 2D NMR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) Plumley, F. G. *Limnol. Oceanogr.* **1997**, *42*, 1252–1264.
(2) Snyder, R. V.; Gibbs, P. D.; Palacios, A.; Abiy, L.; Dickey, R.; Lopez, J. V.; Rein, K. S. *Mar. Biotechnol.* **2003**, *5*, 1–12.
(3) Snyder, R. V.; Guerrero, M. A.; Sinigalliano, C. D.; Winshell, J.; Perez, R.; Lopez, J. V.; Rein, K. S. *Phytochemistry* **2005**, *66*, 1767–1780.
(4) Kubota, T.; Iinuma, Y.; Kobayashi, J. I. *Biol. Pharm. Bull.* **2006**, *29*, 1314–1318.
(5) Monroe, E. A.; Van Dolah, F. M. *Protist* **2008**, *159*, 471–482.
(6) Cane, D. E.; Celmer, W. D.; Westley, J. W. *J. Am. Chem. Soc.* **1983**, *105*, 3594–3600.
(7) Nakanishi, K. *Toxicon* **1985**, *23*, 473–479.
(8) Shimizu, Y. In *Natural Toxins: Animal, Plant, and Microbial*; Harris, J. B., Ed.; Oxford University Press: New York, 1986; pp 115−125.
(9) Gallimore, A. R.; Spencer, J. B. *Angew. Chem., Int. Ed.* **2006**, *45*, 4406–4413.
(10) Harvey, B. M.; Mironenko, T.; Sun, Y.; Hong, H.; Deng, Z.; Leadlay, P. F.; Weissman, K. J.; Haydock, S. F. *Chem. Biol.* **2007**, *14*, 703–714.
(11) Vilotijevic, I.; Jamison, T. F. *Science* **2007**, *317*, 1189–1192.
(12) Satake, M.; Bourdelais, A. J.; Van Wagoner, R. M.; Baden, D. G.; Wright, J. L. C. *Org. Lett.* **2008**, *10*, 3465–3468.
(13) Satake, M.; Campbell, A.; Van Wagoner, R. M.; Bourdelais, A. J.; Jacocks, H.; Baden, D. G.; Wright, J. L. C. *J. Org. Chem.* **2009**, *74*, 989–994.
(14) Gallimore, A. R.; Stark, C. B. W.; Bhatt, A.; Harvey, B. M.; Demydchuk, Y.; Bolanos-Garcia, V.; Fowler, D. J.; Staunton, J.; Leadlay, P. F.; Spencer, J. B. *Chem. Biol.* **2006**, *13*, 453–460.
(15) Kuranaga, T.; Shirai, T.; Baden, D. G.; Wright, J. L. C.; Satake, M.; Tachibana, K. *Org. Lett.* **2009**, *11*, 217–220.
(16) Seco, J. M.; Quinoa, E.; Riguera, R. *Chem. Rev.* **2004**, *104*, 17–117.
(17) Morten, C. J.; Jamison, T. F. *J. Am. Chem. Soc.* **2009**, *131*, 6678–6679.
(18) Gallimore, A. R. *Nat. Prod. Rep.* **2009**, *26*, 266–280.
(19) Liu, T.; Cane, D. E.; Deng, Z. *Methods Enzymol.* **2009**, *459*, 187–214.
(20) Matsuura, Y.; Shichijo, Y.; Minami, A.; Migita, A.; Oguri, H.; Watanabe, M.; Tokiwano, T.; Watanabe, K.; Oikawa, H. *Org. Lett.* **2010**, *12*, 2226–2229.
(21) Ohtani, I.; Kusumi, T.; Kashman, Y.; Kakisawa, H. *J. Am. Chem. Soc.* **1991**, *113*, 4092–4096.

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