The enzyme activity was determined[17] by measuring incorporation of ^{32}P from [γ-^{32}P]ATP into H1 histone (Sigma). The reaction mixture contained 20 mM Tris-HCl (pH 7.5), 15 μM ATP (1 μCi [γ-^{32}P]ATP), 10 mM magnesium acetate, 20 μg/mL phosphatidylserine, 0.5 mM EGTA, 100 nM of the lyngbyatoxin A analogue, and 2 μg of the enzyme preparation in a final volume of 200 μL. The reaction was stopped with 1 mL of 25% trichloroacetic acid after incubation at 28 °C for 10 min. The precipitated protein was washed four times and counted in a liquid scintillation counter.

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Biosynthetic Studies of Brevetoxins, Potent Neurotoxins Produced by the Dinoflagellate *Gymnodinium breve*

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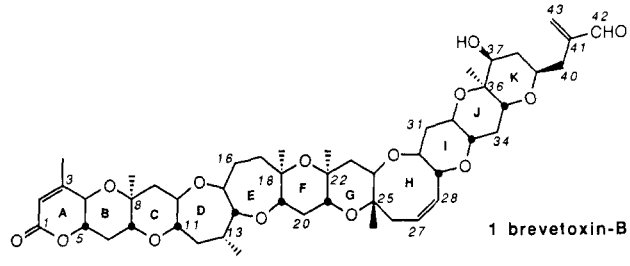
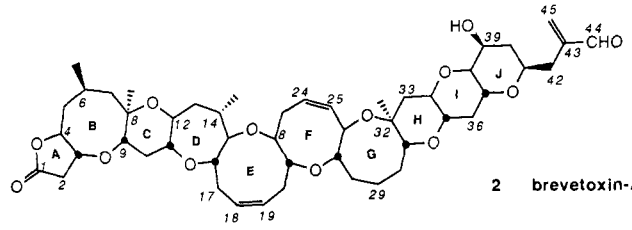
Abstract: Blooms of the dinoflagellate *Gymnodinium breve* (*Ptychodiscus brevis*) commonly known as "red tide" have led to massive fish kills, mollusk contamination, and human food intoxications along the Florida coast and the Gulf of Mexico. The toxins from *G. breve* responsible for these phenomena are the brevetoxins (BTX's), a group of potent neurotoxins with polycyclic trans-fused ether rings which presumably depolarize the sodium channels of the excitable membranes. BTX-B, $C_{50}H_{70}O_{14}$, the first of these neurotoxins whose structure was elucidated, has an unprecedented structure consisting of 6/6/6/7/7/6/6/8/6/6/6 ether rings trans-fused in a ladder-like manner. Another member of these toxins, BTX-A, $C_{49}H_{70}O_{13}$, has another remarkable structure consisting of trans-fused 5/8/6/7/9/6/6/6 ether rings. Although the carbon skeletons of BTX-B and BTX-A are different, both consist of a single carbon chain that is polyoxygenated with methyl substituent groups. This is consistent with polyketide biosynthesis, i.e., condensation of acetate units with the methyl groups originating from either *S*-adenosylmethionine or propionate. Labeling experiments using sodium [1-^{13}C]- and [2-^{13}C]acetate and [*methyl*-^{13}C]methionine demonstrate that the labeling patterns of BTX-B and BTX-A are similar and that the biosynthesis of brevetoxins is not of simple polyketide origin. These labeling studies suggest that the citric acid cycle is involved in the biosynthetis of BTX-B and BTX-A, the degree of its involvement being unusually high. Furthermore, CO_2 participates in a unique manner in the biosynthesis of C-1 of BTX-B and BTX-A.

Blooms of dinoflagellates commonly known as "red tide" have received much attention due to their toxic effects on the environment. One of the most toxic species, *Gymnodinium breve* (*Ptychodiscus brevis*), occurring along the Gulf of Mexico and Florida coast, has received much attention due to its devastating effects on fishing and tourist industries as well as its effects on the ecosystem.[1] Since 1968 numerous attempts had been made to isolate the lipid-soluble neurotoxins of *G. breve*[2] that depolarize the sodium channels of the excitable membranes.[3]

The structure of the first of these neurotoxins, brevetoxin-B (BTX-B) (**1**) $C_{50}H_{70}O_{14}$, was of unprecedented nature consisting of 11 ethereal 6/6/6/7/7/6/6/8/6/6/6 rings trans-fused in a ladder-like manner.[4] The structure of the major toxin BTX-B was determined by X-ray crystallography and the absolute configuration by the dibenzoate chirality method. The structures of five additional toxins in this series have been elucidated, i.e., BTX-C,[5] GB-3,[6] GB-5,[7] and GB-6.[7] This was followed by structure elucidation of BTX-A (**2**), $C_{49}H_{70}O_{13}$, the most potent and challenging of the brevetoxins which also had a remarkable structure consisting of trans-fused 5/8/6/7/9/8/8/6/6/6 ether rings. The structure of BTX-A (**2**) was elucidated by X-ray crystallography[8] and independently by extensive MS and NMR;[9] the spectroscopically derived structure was correct except for the C-6 configuration, which was assigned as 6α instead of 6β. The difficulty associated with NMR studies of BTX-A was due to the medium-sized rings which makes the molecule flexible, thus leading to broadening of signals; in contrast, BTX-B is quite stiff except around rings D/E where it can fold.



1 brevetoxin-B



2 brevetoxin-A

Recently, another class of toxins responsible for diarrheic shellfish poisoning (DSP) has received wide interest. Okadaic

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(1) Shimizu, Y. In *Marine Natural Products*; Scheuer, P. J., Ed.; Academic: New York, 1978; Vol. 1, Chapter 1.
(2) (a) Spikes, J. J.; Ray, S. M.; Aldrich, D. V.; Nash, J. B. *Toxicon* **1968**, *5*, 171. (b) Padilla, G. M.; Kim, Y. S.; Rauckmen, E. J.; Rosen, G. M. In *Toxic Dinoflagellate Blooms*; Taylor, D. L., Seliger, H. H., Eds.; Elsevier North-Holland: New York, 1979; pp 351-354. (c) Risk, M.; Lin, Y. Y.; MacFarlan, R. D.; Sadagopa Ramunujam, V. M.; Smith, L. L.; Trieff, N. M. In *Toxic Dinoflagellate Blooms*; Taylor, D. L., Seliger, H. H., Eds.; Elsevier North-Holland: New York, 1979; pp 335-344. (d) Baden, D. G.; Mende, T. J.; Block, R. E. In *Toxic Dinoflagellate Blooms*; Taylor, D. L., Seliger, H. H., Eds.; Elsevier North-Holland: New York, 1979; pp 327-334.

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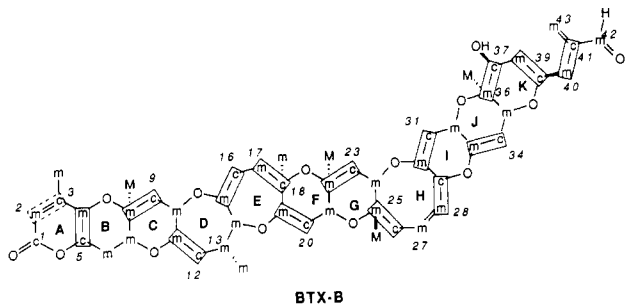
acid, a toxin responsible for DSP and its episulfide derivative, were first isolated from the sponges *Pandaros acanthifolium*,[10] *Halichondria okadai*,[11] and *Halichondria melanodocia*.[11] Subsequently it was found that dinoflagellates *Prorocentrum lima*, *Dinophysis fortii*, and *Dinophysis accuminata* were the progenitor of okadaic acid and its derivative known as dinophysistoxin.[12] Also isolation and structural determination of toxins known as pectenotoxins's (PTX), polyether macrocyclic lactones, have been carried out.[12] Another group of polyether macrolides from a marine sponge *Halichondria okadai*, known as halichondrins, has also been isolated.[13] The structure of yessotoxin (YTX) with remarkable structural similarities to brevetoxins has been elucidated.[14] This toxin was isolated from digestive glands of the scallop *Pantinopecten yessoensis* that has been implicated in DSP. During this isolation, pectenotoxin as well as okadaic acid derivatives were also isolated. It will be of interest to determine the origin of this toxin and that of halicondrins since they both yield okadaic acid derivatives but belong to different species. Thus, it is likely that the progenitors of these toxins are from organisms other than sponges or scallops and that toxins like okadaic acid are produced by dinoflagellates.

The structures of these bioactive polyether marine toxins somewhat resemble the polyether ionophores from terrestrial microorganisms. Extensive biosynthetic studies have been carried out on polyether antibiotics produced by terrestrial microorganisms, exemplified by monensin-A,[15] lasalocid, narasin, salinomycin, lysocellin, lonomycin-A, and dianemycin.[16] These polyether antibiotics represent a group of more than 60 naturally occurring ionophores, the vast majority of which are produced by the genus *Streptomyces*. Unlike the polyether antibiotics produced by terrestrial microorganisms, biosynthetic studies of polyether toxins produced by dinoflagellates are in their infancy. Biosynthetic investigation of one of the toxins produced by *G. breve*, BTX-B, was reported recently.[17,18] Here a fuller account of biosynthetic studies of BTX-A and BTX-B is presented.

BTX-B and BTX-A consist of a single polyoxygenated carbon chain with methyl substituents. The prevailing thought about this trait is that the skeleton is derived from a single polyketide chain with the methyl groups originating from either *S*-adenosylmethionine or propionate. An example of this is monensin-A



BTX-B

Figure 1. Biosynthetic carbon origins of BTX-B. Positions of the 16 carbons enriched by [1-^{13}C]acetate are represented as "c", the 30 carbons enriched by [2-^{13}C]acetate as "m", and the four methyls enriched by [*methyl*-^{13}C]methionine as "M". The result of 2D INADEQUATE measurements of [1,2-$^{13}C_2$]acetate-labeled BTX-B identified the 14 intact acetate units, enclosed in solid rectangles. Although the intensity of the dotted rectangular box is weaker relative to the other intact acetate units, the INADEQUATE experiment indicates that this is also an intact acetate unit.

where the carbon chain is assembled from five acetates, seven propionates, and one butyrate, while the *O*-methyl is derived from methionine.[15]

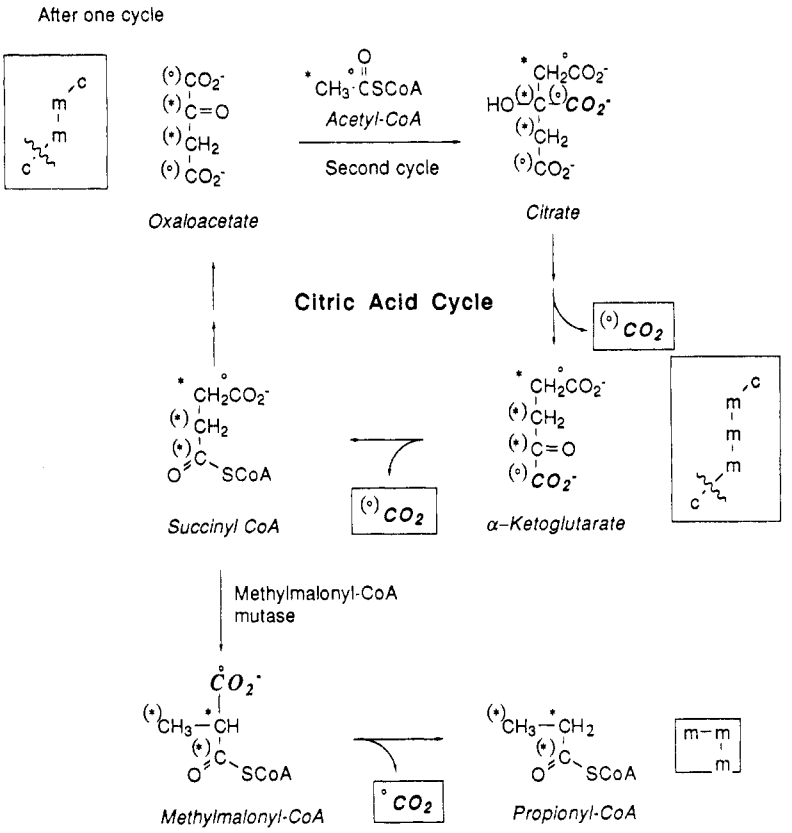
Biosynthesis of BTX-B. The first labeled experiment was incubation of the *G. breve* culture[19] with sodium [1-^{13}C]acetate.[17] The proton decoupled ^{13}C NMR of purified BTX-B showed that only 16 of the 50 carbons were enriched by [1-^{13}C]acetate. This immediately poses a problem and strongly suggested that BTX-B biosynthesis does not follow a straightforward polyketide path.[20]

With the carbon assignments already determined for BTX-B,[17,18] the positions of ^{13}C-enriched carbons by [1-^{13}C]acetate can be readily located (Figure 1). In this figure as well as the following figures the notation "c" represents positions in BTX-B that are labeled by the acetate [1-^{13}C] (1, 3, 5, 9, 12, 16, 18, 20, 23, 26, 29, 31, 34, 37, 39, and 40). Although the acetate carbonyl is oxygenated in some cases, unlike normal polyketide biosynthesis, there is no obvious pattern for this oxygenation. A further interesting observation made during this [1-^{13}C]acetate feeding was that prolonged growth led to the appearance of ^{13}C–^{13}C couplings; a possible rationalization of this is given later. The toxin resulting from feeding with sodium [2-^{13}C]acetate showed enhancement of 30 carbon peaks noted as "m" in Figure 1 (2, 3-Me, 4, 6, 7, 8, 10, 11, 13, 13-Me, 14, 15, 17, 18-Me, 19, 21, 22, 24, 25, 27, 28, 30, 32, 33, 35, 36, 38, 40, 42, and 43). The ^{13}C NMR of BTX-B enriched with [2-^{13}C]acetates showed ^{13}C–^{13}C couplings clearly more pronounced than the case of [1-^{13}C]acetate (discussed below).

The C_2 units which originate from the same acetate unit was determined by incubation with sodium [1, 2-$^{13}C_2$]acetate. Identification of these C_2 units, which could not be performed from ^{13}C–^{13}C *J* constants due to similarities in values, was solved by 2D INADEQUATE measurements.[17] This experiment showed that 14 C_2 acetate units (boxed), 4/5, 8/9, 11/12, 15/16, 17/18, 19/20, 22/23, 25/26, 28/29, 30/31, 33/34, 36/37, 38/39, and 40/41, are derived from the same acetate unit (all "m–c" respectively) (Figure 1). Furthermore, observation of the weaker intensity of ^{13}C–^{13}C coupled cross peaks for 2/3 (dotted box) suggests that this is also an intact acetate unit. The application of the 2D INADEQUATE technique[21] was instrumental for determining the presence of intact acetate units in BTX-B (Figure 1). The advantage of this technique is obvious since the intensity of natural abundance carbon signals is 0.01% as compared to the 2% value for each coupled pair of carbon atoms, thus representing a 200-fold increase in sensitivity.

(3) Baden, D. G.; Bikhazi, G.; Decker, S. J.; Foldes, F. F.; Leung, I. *Toxicon* **1984**, *22*, 75–84, and references cited therein.
(4) Lin, Y. Y.; Risk, M.; Ray, S. M.; Van Engen, D.; Clardy, J.; Golik, J.; James, J. C.; Nakanishi, K. *J. Am. Chem. Soc.* **1981**, *103*, 6773–6775. BTX represents another neurotoxin batrachotoxin. Although GBTX has been considered for brevetoxin, BTX is used here until a suitable abbreviation has been reached by consensus; an alternative abbreviation could be BrTX.
(5) Golik, J.; James, J. C.; Nakanishi, K.; Lin, Y. Y. *Tetrahedron Lett.* **1982**, *23*, 2535–2538.
(6) Chou, H. N.; Shimizu, Y. *Tetrahedron Lett.* **1982**, *23*, 5521–5524.
(7) Chou, H. N.; Shimizu, Y.; Van Duyne, G.; Clardy, J. *Tetrahedron Lett.* **1985**, *26*, 2865–2868.
(8) Shimizu, Y.; Chou, H. N.; Bando, H.; Van Duyne, G.; Clardy, J. C. *J. Am. Chem. Soc.* **1986**, *108*, 514–515.
(9) Pawlak, J.; Tempesta, M. S.; Golik, J.; Zagorski, M. G.; Lee, M. S.; Nakanishi, K.; Iwashita, T.; Gross, M. L.; Tomer, K. B. *J. Am. Chem. Soc.* **1987**, *109*, 1144–1150.
(10) Schmitz, F. J.; Prasad, R. S.; Gopichand, Y.; Hossain, M. B.; van der Helm, D.; Schmidt, P. *J. Am. Chem. Soc.* **1981**, *103*, 2467–2469.
(11) Tachibana, K.; Scheuer, P. J.; Kikuchi, H.; Tsukitani, Y.; Van Engen, D.; Clardy, J.; Gopichand, Y.; Schmitz, F. J. *J. Am. Chem. Soc.* **1981**, *103*, 2469–2471.
(12) Yasumoto, T.; Murata, M.; Oshima, Y.; Sano, M.; Matsumoto, G. K.; Clardy, J. *Tetrahedron* **1985**, *41*, 1019–1025.
(13) (a) Uemura, D.; Takahashi, K.; Yamamoto, T.; Katayama, C.; Tanaka, J.; Okumura, Y.; Hirata, Y. *J. Am. Chem. Soc.* **1985**, *107*, 4796–4798. (b) Hirata, Y.; Uemura, D. *Pure Appl. Chem.* **1986**, *58*, 701–710.
(14) Murata, M.; Kumagai, M.; Lee, J. S.; Yasumoto, T. *Tetrahedron Lett.* **1987**, *28*, 5869–5872.
(15) (a) Cane, D. E.; Liang, T.-c.; Hasler, H. *J. Am. Chem. Soc.* **1981**, *103*, 5962. (b) Cane, D. E.; Liang, T.-c.; Hasler, H. *J. Am. Chem. Soc.* **1982**, *104*, 7274–7281, and references cited therein.
(16) (a) Cane, D. E.; Celmer, W. D.; Westly, J. W. *J. Am. Chem. Soc.* **1983**, *105*, 3594–3600, and references cited therein. (b) Westly, J. W. *J. Natural Products* **1986**, *49*, 35–47.
(17) (a) Lee, M. S.; Repeta, D. J.; Nakanishi, K.; Zagorski, M. G. *J. Am. Chem. Soc.* **1986**, *108*, 7855–7856.
(18) Chou, H.-N.; Shimizu, Y. *J. Am. Chem. Soc.* **1987**, *109*, 2184–2185.
(19) Gates, E. J.; Wilson, W. B. *Limnol. Oceanogr.* **1960**, *5*, 171–174.
(20) Simpson, T. J. *Natural Product Rep.* **1985**, *4*, 321–347.
(21) (a) Chan, J. K.; Moore, R. N.; Nakashima, T. T.; Vederas, J. C. *J. Am. Chem. Soc.* **1983**, *105*, 3334–3336. (b) Carter, G. T.; Fantini, A. A.; James, J. C.; Borders, D. B.; White, R. J. *Tetrahedron Lett.* **1984**, *25*, 255–258. (c) Moore, R. N.; Bigam, G.; Chan, J. K.; Hogg, A. M.; Nakashima, T. T.; Vederas, J. C. *J. Am. Chem. Soc.* **1985**, *107*, 3694–3701.

Scheme I. Citric Acid Cycle[a]



[a] Parenthesized asterisks and circles denote labeling patterns for the first cycle using $*CH_3{}^{\circ}CO_2Na$(m–c). Moieties that are lost are shown in bold italics.

The combination of two labeling experiments, [1-^{13}C]- and [2-^{13}C]acetates, accounted for the origin of 46 of the 50 carbons in BTX-B. The four remaining carbons whose origin as yet not determined were methyl groups. This suggests that they may be derived from *S*-adenosylmethionine. Indeed when a labeling experiment was carried out with [*methyl*-^{13}C]methionine, enrichment of the four unaccounted methyls in BTX-B was observed (Figure 1, "M"). The feeding experiments described thus far have accounted for the origin of all 50 carbons in BTX-B. However, the position of [1-^{13}C] (c) and [2-^{13}C] (m) shows no logical pattern. Indeed, the single carbon chain which constitutes the backbone of the ladder-like oxacyclic skeleton is not a simple polyketide. More puzzling is the presence of consecutive methyl acetates (m) prevalent in the molecule. Indeed there are six "m–m" moieties (10/11, 21/22, 24/25, 27/28, 32/33, and 35/36), one "m–m–m" moiety (6/7/8), and even one "m–m–m–m" moiety (13-Me/13/14/15).

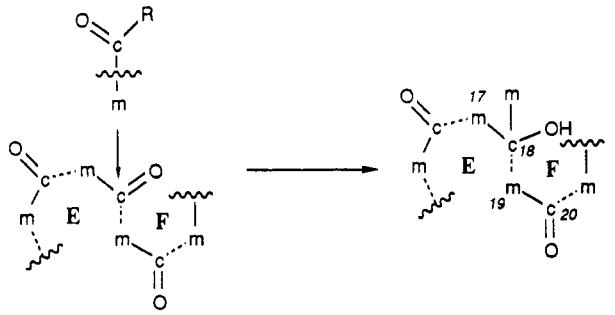
In polyketide biosynthesis, the acetate can be converted directly into the metabolite, or the acetate can be turned through the citric acid cycle into an advanced precursor before it is used in polyketide biosynthesis.[22] In the case of *G. breve*, it is very likely that the citric acid cycle is involved in the biosynthesis of this secondary metabolite BTX-B as proposed by Chou and Shimizu from [^{13}C]acetate feeding experiments.[18] The ^{13}C NMR data of BTX-B formed by feeding experiments with [1-^{13}C]- and [2-^{13}C]acetates show ^{13}C–^{13}C coupling patterns compatible with participation of the citric acid cycle in its biosynthesis.

The citric acid cycle[23] (Scheme I) begins with the condensation of acetyl-CoA with oxaloacetate. After one cycle, the original acetate added in the first step is distributed throughout the regenerated oxaloacetate. This is because free succinate is a symmetrical molecule, and the enzyme that acts upon it does not have the ability to distinguish the two ends. Thus, the two methyl carbons are equivalent as well as the two carboxyl carbons. For example, [1-^{13}C]acetate after one cycle will label oxaloacetate at the 1- and 4-positions with both positions having half the original ^{13}C content. Since only one end is labeled by ^{13}C, the enzyme has a 50% probability of being attached to the labeled end or the other nonlabeled end. In the second cycle, after condensation of acetyl-CoA, the lost CO_2 is ^{13}C-labeled. In the case of [1-^{13}C]acetate, regardless of the number of cycles, only the carboxyl becomes labeled, subsequent decarboxylations always resulting in the loss of $^{13}CO_2$ (Scheme I). Since the dinoflagellate *G. breve* is photosynthetic, the presence of ^{13}C–^{13}C coupled peaks in [1-^{13}C]acetate-labeled BTX-B is probably due to uptake of the $^{13}CO_2$ (produced in Scheme I) during the photosynthesis. In the case of [2-^{13}C]acetate, after one cycle the oxaloacetate will be labeled at positions 2 and 3, with subsequent loss of unlabeled CO_2. Consequently, condensation of [2-^{13}C]acetate to [2,3-$^{13}C_2$]- (actually it is 2-^{13}C and 3-^{13}C) oxaloacetate leads to a ^{13}C–^{13}C coupling pattern due to [2-^{13}C]acetate and [2-^{13}C]oxaloacetate condensations (Scheme I). Indeed, distinct "triplet" peaks characteristic of ^{13}C–^{13}C coupling are observed throughout the ^{13}C NMR spectrum of BTX-B enriched with [2-^{13}C]acetate (Supplementary Material).

The labeled pattern of acetates strongly reflects the citric acid cycle pathway in which the C_4 diacids participate in the biosynthesis of BTX-B. The presence of m–m–c in BTX-B (Figure 1) indicates that this C_3 unit is derived from an intermediate in the citric acid cycle, namely by decarboxylation of C_4 diacids from the cycle. There are six such "m–m–c" C_3 units in BTX-B as shown by 10/11/12, 21/22/23, 24/25/26, 27/28/29, 32/33/34, and 35/36/37. All "–m–c's" in this "m–m–c" are intact acetate units. The addition of a C_3 unit (m–m–c) derived from an intermediate in the citric acid cycle has also been suggested to occur in marticin, a metabolite of *Fusarium martii*.,[24] and in glauconic acid, a fungal metabolite of *Penicillium purgurogenm* (IMI

(22) Herbert, R. B. In *The Biosynthesis of Secondary Metabolites*; Chapman and Hall: New York, 1981; Chapter 3.
(23) Zubay, G. *Biochemistry*; Addison-Wesley: MS, 1983; Chapter 9.
(24) Holenstein, J. E.; Kern, H.; Stoessl, A.; Atothers, J. B. *Tetrahedron Lett.* **1983**, *24*, 4059–4062.

Scheme II. Proposed Pathway of Attachment of the Methyl Group to C-3 and 18 of BTX-B and C-8 of BTX-A[a]

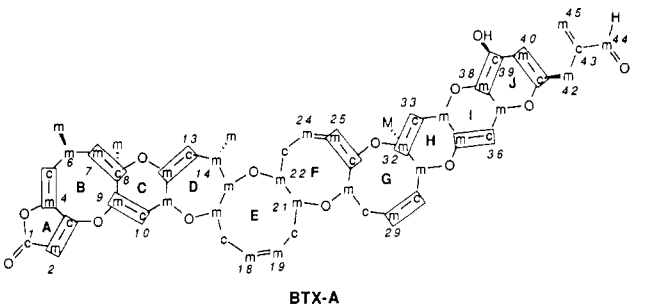


[a] The methylation at these carbons is unusual in the sense that the carboxyl "c" of acetate is methylated by a methyl "m" of acetate with subsequent loss of one C_1 unit.

90178);[25] what is characteristic of BTX-B is the high extent in which this C_3 unit is involved.

The occurrence of "m–m–m–c" at 6/7/8/9 can be explained by passage of acetate through the citric acid cycle.[18] After the first cycle using [2-^{13}C]acetate, the labeled oxaloacetate condenses with a second [2-^{13}C]acetate; subsequent isomerization and decarboxylation yields the α-ketoglutarate C_5 unit (Scheme I), the decarboxylation and condensation of which leads to the biosynthetic pattern observed for 6/7/8/9 (m–m–m–c) of BTX-B. The "m–m–m" unit comprising 13-Me/13/14 could be derived from propionate. More correctly the indirect incorporation of [2-^{13}C]acetate via the citric acid cycle is responsible for this formation. After completing the citric acid cycle using [2-^{13}C]acetate, the propionate derived from succinyl-CoA will be labeled at C-1 and C-3 propionate (Scheme I). However, after the second cycle, the C-1, C-2, and C-3 of propionate derived from succinate will be labeled with an enrichment ratio of 0.5:1.0:0.5, respectively. In this case the succinyl-CoA labeled by [2-^{13}C]acetate is isomarized by methylmalonyl-CoA by methylmalonyl-CoA mutase (Scheme I).[26] Subsequently, after decarboxylation, this C_3 unit is incorporated into BTX-B as a propionate "m–m–m" (13-Me/13/14). Such examples have been encountered in cationomycin, a polyether ionophore antibiotic produced by *Actinomadura azurea*.[27] A feeding experiment using ethyl [2, 3-$^{13}C_2$]succinate has shown enrichment at the C-2 and C-3 positions of propionates in the macrolides, erythromycin A and B.[28] Similar pathways for propionate formation has been suggested for the polyketide antibiotic IC13963[29] and macrolide geldanamycin.[30] The labeling pattern of the side chain in BTX-B suggests that it is derived from isopentenyl pyrophosphate with loss of the carbon attached to the phosphate group.[31]

Three of the seven methyl groups originate from [2-^{13}C]acetate and the remaining four from [*methyl*-^{13}C]methionine. Methylation by *S*-adenosylmethionine at the "m" position of acetates in BTX-B (8, 22, 25, and 36) represents normal methylation in a biological system.[32] Of the remaining three methyls, those at C-3 and C-18 have not been accounted for. Methylation here



BTX-A

Figure 2. Biosynthetic carbon origins of BTX-A. Positions of the 17 carbons enriched by [1-^{13}C]acetate are represented as "c", the 31 carbons enriched by [2-^{13}C]acetate as "m", and the single methyl stemming from [*methyl*-^{13}C]methionine as "M". The result of 2D INADEQUATE measurements of [1,2-$^{13}C_2$]acetate-labeled BTX-B clarified the 11 intact acetate units, enclosed in solid rectangles. The other possible intact acetate units, 16/17, 19/20, 22/23, 27/28, and 42/43, do not exhibit ^{13}C–^{13}C coupling from 2D INADEQUATE. This is due to sample size, but more importantly it is due to the line width of these peaks caused by the slow molecular motion; this has resulted in the difficulty in assigning the carbons.[35]

is unusual in the sense that an acetate of carboxy (c) is methylated by an acetate of methyl (m). The methyls could be the result of participation of 3-hydroxy-3-methylglutarate and isoprenoid unit, 1/2/[3-Me]/4/5 and 17/18[18-Me]/19/20, respectively, as proposed by Chou et al.[18] However, we believe that the methylation at C-3 and C-18 is by "m" of an acetate unit with subsequent loss of one C_1 unit (Scheme II). A similar methylation of a carboxyl group has been suggested to occur in the antibiotic virginiamycin M from *Streptomyces virginiae*[33] and the macrolide antibiotic myxovirescin A_1 from *myxococus virescens*.[34] In Figure 1, the 2D INADEQUATE shows a weak intensity ^{13}C–^{13}C coupling between C-2/C-3 (2/3) but not between 1/2 and 3/3-Me. We believe that the 2/3 is an intact acetate unit with methylation at C-3 by an acetate followed by decarboxylation (Scheme II). This excludes any possibility of mevalonic acid participating in the formation of ring A in BTX-B. This leaves an intriguing question as to the origin of C-1 of BTX-B.

BTX-A Biosynthesis. Although BTX-B and BTX-A are trans-fused polycyclic ethers, they have different carbon skeletons. The question remains whether the two toxins have similar or different biosynthetic patterns. Feeding experiments with BTX-A using [1-^{13}C]acetate (c) resulted in the enrichment of 17 out of 49 carbons in BTX-A (1, 3, 5, 8, 10, 13, 17, 20, 23, 26, 28, 30, 33, 36, 39, 41, and 43) (Figure 2). Feeding of [2-^{13}C]acetate (m) showed enrichment of 31 carbons (2, 4, 6, 6-Me, 7, 8-Me, 9, 11, 12, 14-Me, 14, 15, 16, 18, 19, 21, 22, 24, 25, 27, 29, 31, 32, 34, 35, 37, 38, 40, 42, 44, and 45) (Figure 2). These two feeding experiments permitted determination of the origin of 48 carbons in BTX-A. The remaining one methyl carbon was labeled by [*methyl*-^{13}C]methionine (Figure 2, "M").

The 2D INADEQUATE of BTX-A labeled with [1,2-$^{13}C_2$]-acetates showed the presence of 11 C_2 intact acetate units. This experiment shows that 2/3, 4/5, 7/8, 9/10, 12/13, 25/26, 29/30, 32/33, 35/36, 38/39, and 40/41 are derived from same acetate units as boxed in Figure 2. The other possible C_2 acetate units, 16/17, 19/20, 22/23, 27/28, and 42/43, do not show ^{13}C–^{13}C coupling. As noted before when assigning the carbons for BTX-A,[35] this is probably due to the broad line width of these peaks resulting from slow molecular motion. The labeling pattern is indeed similar to BTX-B. There are seven "m–m" moieties (11/12, 18/19, 21/22, 24/25, 31/32, 34/35, and 37/38), one "m–m–m" moiety (6-Me/6/7), and one "m–m–m–m" moiety (14-Me/14/15/16). As before the presence of "m–m–c" C_3 units are derived from acetates participating in the citric acid cycle. The

(25) Cox, R. E.; Holker, J. S. E. *J. Chem. Soc., Chem. Commun.* **1976**, 583–584.

(26) (a) Zubay, G. *Biochemistry*; Addison-Wesley: MS, 1983; Chapter 13. (b) Lehninger, A. L. *Biochemistry*; Worth: New York, 1975; p 555.

(27) Ubukata, M.; Uzawa, J.; Isono, K. *J. Am. Chem. Soc.* **1984**, *106*, 2213–2214.

(28) Cane, D. E.; Hasler, H.; Taylor, P. B.; Liang, T. *Tetrahedron* **1983**, *39*, 3449–3455.

(29) Bulsing, J. M.; Laue, E. D.; Leeper, F. J.; Staunton, J.; Davies, D. H.; Ritchie, G. A. F.; Davies, A.; Davies, A. B.; Mabelis, R. P. *J. Chem. Soc., Chem. Commun.* **1984**, 1301–1302.

(30) Haber, A.; Johnson, R. D.; Rinehart, K. L. *J. Am. Chem. Soc.* **1977**, *99*, 3541–3544.

(31) (a) Herbert, R. B. In *The Biosynthesis of Secondary Metabolites*; Chapman and Hall: New York, 1981; Chapter 4. (b) Torssell, K. B. G. In *Natural Product Chemistry*; John Wiley and Sons: New York, 1983; Chapter 5. (c) Mann, J. In *Secondary Metabolism*; Clarendon: Oxford, 1978; Chapter 3.

(32) Herbert, R. B. In *The Biosynthesis of Secondary Metabolites*; Chapman and Hall: New York, 1981; Chapters 1 and 3.

(33) (a) Kingston, D. G. I.; Kolpak, M. X. *J. Am. Chem. Soc.* **1980**, *102*, 5964–5966. (b) Kingston, D. G. I.; Kolpak, M. X.; Lefevre, J. W.; Borup-Grochtmann, I. *J. Am. Chem. Soc.* **1983**, *105*, 5105–5110.

(34) Trowitzsch, W.; Gerth, K. K.; Wray, V.; Höfle, G. *J. Chem. Soc., Chem. Commun.* **1983**, 1174–1175.

C_4 diacids from the citric acid cycle condense with decarboxylation thus resulting in chain elongation by a C_3 unit. There are seven such "m–m–c" units in BTX-A as represented by 11/12/13, 18/19/20, 21/22/23, 24/25/26, 31/32/33, 34/35/36, and 37/38/39. The "m–m–m" moiety of 14-Me/14/15 is similar to the 13-Me/13/14 (m–m–m) of BTX-B and most likely derives from propionate (Scheme I). The "m–m–m–c" moiety of 6-Me/6/7/8 can originate from a C_5 unit in the citric acid cycle which decarboxylates and subsequently condenses as a C_4 unit. The unusual methylation at the carboxyl center (c) 8 by "m" was encountered previously in BTX-B (Scheme II) and the antibiotics virginiamycin[33] and myxovirescin A_1.[34] The side chain of ring J is most likely isoprenoid as in BTX-B.

The origin of all carbons and the possible biosynthetic scheme for BTX-A have been explained except for the origin of C-1, which represents a novel case in polyketide biosynthesis in that it is probably derived from CO_2. This may not be so unusual if one looks at the conversion of acetyl-CoA to malonyl-CoA. However, one major difference between the two is that malonyl-CoA decarboxylates, whereas in the case of C-1 in BTX-A the carboxyl group is retained and plays a vital role in the biosynthetic process. The involvement of the citric acid cycle is strengthened by C-1 being labeled by [1-^{13}C]acetate. As shown in Scheme I, as [1-^{13}C]acetate goes through the citric acid cycle, only the carboxyl group in C_4 diacids becomes labeled. The lost CO_2 during the second cycle is always labeled $^{13}CO_2$. Also, decarboxylation of the C_4 unit to give a C_3 unit always results in the loss of $^{13}CO_2$. This labeled CO_2 is used in the construction of C-1, thereby resulting in apparent enrichment at this position by [1-^{13}C]acetate. The feeding experiment involving $NaH^{13}CO_3$ resulted in scrambling of the labels since *G. breve* is photosynthetic. However, in this case, the relative intensities of C-1 and C-43, two quaternary sp^2 carbon centers apparently labeled by [1-^{13}C]acetate, can be used to explain the participation of CO_2. The relative intensity of C-43 is higher than C-1 in both nonlabeled and [1-^{13}C]-acetate-labeled BTX-A; however, the relative intensities of the two signals are reversed in BTX-A labeled by $NaH^{13}CO_3$. The fact that the relative intensity of C-1 is higher than C-43 indicates that although the labeled bicarbonate is getting scrambled, there is systematic use of labeled CO_2 in the construction of C-1. Thus, comparison of the relative intensities of the two quaternary centers of nonlabeled, [1-^{13}C]acetate, and $NaH^{13}CO_3$-labeled BTX-A under similar experimental conditions suggests that C-1 is derived from CO_2. The origin of all 49 carbons in BTX-A has been clarified. Likewise, we believe that C-1 of BTX-B is also derived from CO_2.

Succinate Feeding Experiment. In order to substantiate the involvement of the citric acid cycle, radioactive as well as stable isotopic labeling experiments were carried out with labeled succinate. The feeding experiment with the use of [2,3-$^{14}C_2$]succinate clearly indicated that succinate was incorporated into both BTX-B and BTX-A (Figure 3b). To prove and show the actual location of the C_3 unit of decarboxylated succinate, ^{13}C-labeled sodium succinate was incubated with *G. breve* culture. However, the HPLC-purified BTX-B showed no discernable ^{13}C-enriched peaks due to the low enrichment level.

Propionate Feeding Experiment. To substantiate the participation of propionates in BTX-B and BTX-A, radioactive and stable isotope experiments were carried out. The HPLC and radioactivity profile (Figure 3c) of feeding experiment with [1-^{14}C]propionate clearly indicated that propionate was incorporated into both BTX-B and BTX-A. However, a labeled feeding experiment with [1-^{13}C]propionate showed no discernable enriched NMR peaks for BTX-B due to low incorporation.

In an attempt to circumvent this problem, the *N*-acetylcysteamine (NAC) thio ester of propionate was prepared. Thio esters have been used widely for in vitro studies of fatty acids as donors or acceptors of enzyme substrates or products via transesterification.[36] Thio esters have also been used successfully in

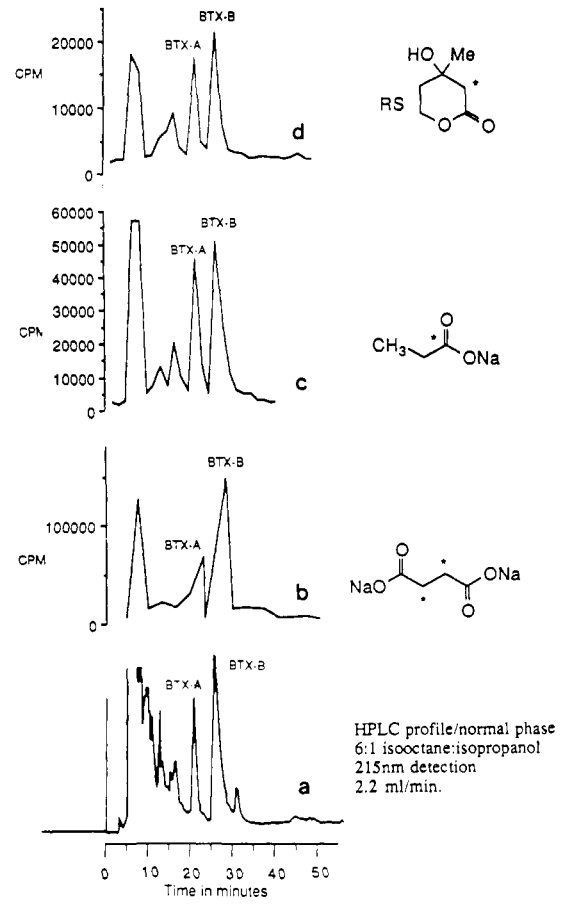


Figure 3. (a) HPLC profile of crude extract of *Gymnodinium breve* culture using 5 μ normal phase column: solvent, 6:1 isooctane/isopropyl alcohol; flow rate, 2.2 mL/min; detection, λ 215 nm. (b) Radioactivity profile of HPLC fractions of *G. breve* culture grown with disodium [2,3-$^{14}C_2$]succinate. (c) Radioactivity profile of HPLC fractions of *G. breve* culture grown with sodium [1-^{14}C]propionate. (d) Radioactivity profile of HPLC fractions of *G. breve* culture grown with (*RS*)-[2-^{14}C]mevalonolactone. The three radioactive trace experiments clearly show that succinate, propionate, and mevalonolactone are incorporated into both BTX-B and BTX-A.

biosynthetic studies of the macrolide antibiotic tylosin from *Streptomyces fradiae* where C_6 and C_9 units were incorporated into the macrolide without catabolism of precursors to propionates.[37] A similar experiment has been carried out using the thio ester of a C_6 unit for the macrolide antibiotic erythromycin B biosynthesized from *Streptomyces erythreus*.[38]

The incubation of *S*-[1-^{13}C]propionyl-*N*-acetylcysteamine with *G. breve* culture could be performed for only 4 days due to the extreme weakness of the culture. Presence of the propionyl thio ester hindered the culture growth and interfered with the isolation of BTX's, but sufficient BTX-B was isolated to measure the ^{13}C NMR which again showed no apparent enrichment. The BTX-A sample also showed no enrichment. The apparent lack of incorporation is probably due to the culture not being incubated with the precursor sufficiently long and also to the sensitivity of ^{13}C NMR.

Mevalonolactone Feeding Experiment. Feeding experiment involving radioactive and stable isotopes have been carried out for mevalonolactone to check for the participation of isoprenoid units in BTX-B and BTX-A. The HPLC and radioactive profile of feedcing experiments with (*RS*)-[2-^{14}C]mevalonolactone clearly indicated that mevalonolactone was incorporated into both BTX-B

(35) Zagorski, M. G.; Nakanishi, K.; Qin, G.-W.; Lee, M. S. *J. Org. Chem.* **1988**, *53*, 4156–4158.

(36) Rainwater, D. L.; Kolattukudy, P. E. *J. Biol. Chem.* **1985**, *260*, 616–623.

(37) Yue, D.; Duncan, J. S.; Yamamoto, Y.; Hutchinson, C. R. *J. Am. Chem. Soc.* **1987**, *109*, 1253–1255.

(38) Cane, D. E.; Simpson, T. J.; Scott, A. I.; Shishido, K. *J. Am. Chem. Soc.* **1987**, *109*, 1255–1257.

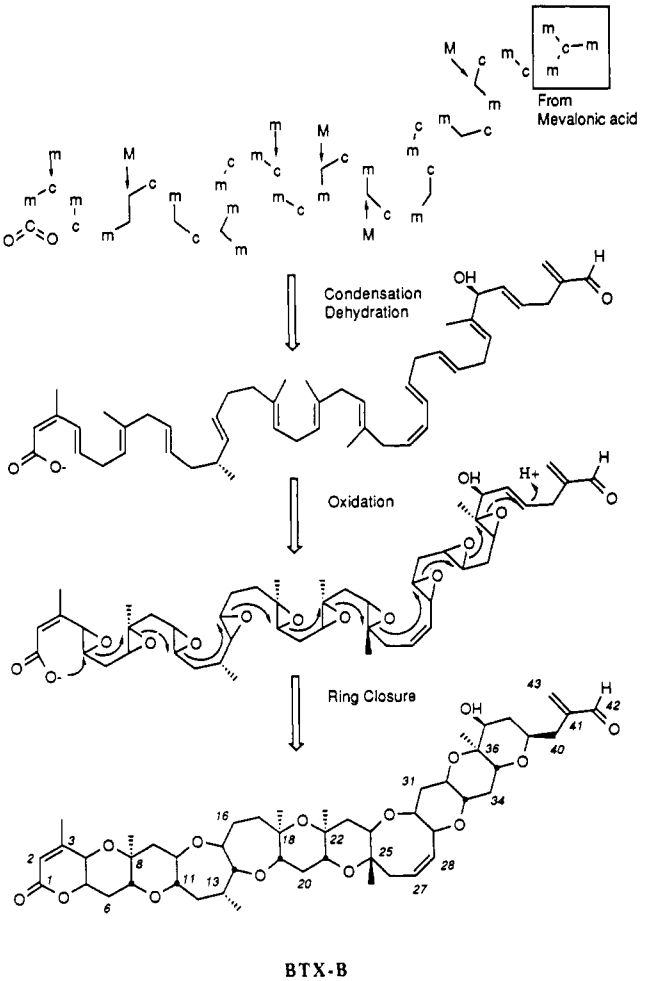


Figure 4. Biogenesis of BTX-B.

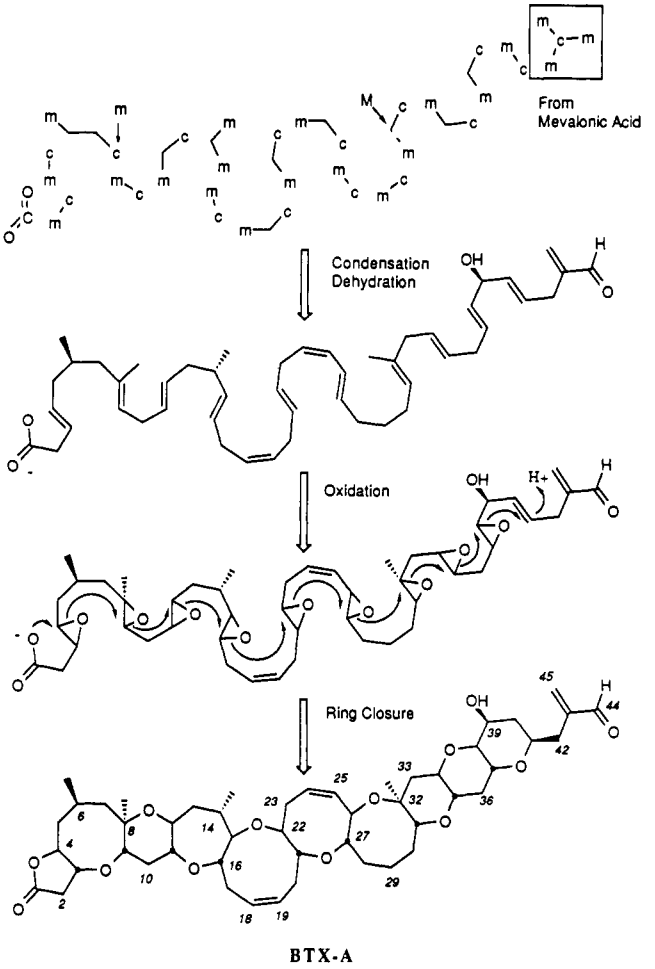


Figure 5. Biogenesis of BTX-A.

and BTX-A (Figure 3d). (*RS*)-[2-^{13}C]mevalonolactone was prepared and utilized in a feeding experiment in order to locate the isoprenoid unit(s) in BTX's; however, the ^{13}C NMR of BTX-B showed no apparent enrichment.

Discussion

Thus far attempts to show the location of the succinic, propionic, and mevalonic precursors involved in the biosynthesis of BTX-B and BTX-A have failed due to the low incorporation level. However, the corresponding ^{14}C-labeled precursors have been incorporated, thus implicating a mixed polyketide origin in the biosynthesis of BTX-B and BTX-A. The C_1, C_2, C_3, and C_4 units comprising both BTX-B and BTX-A are identical. Schematic biosynthetic pathways for BTX-B and BTX-A are shown in Figures 4 and 5. BTX-B is composed of one CO_2 (C_1), seven acetates (C_2), one "m–m–m" unit (C_3, propionate), six "m–m–c" units (C_3), one "m–m–m–c" (C_4) unit, four methionines (C_1), two methylations by [2-^{13}C]acetate (C_1), and one decarboxylated isoprenoid unit (C_4). BTX-A is composed of one CO_2 (C_1), seven acetates (C_2), one "m–m–m" unit (C_3, propionate), seven "m–m–c" units (C_3), one "m–m–m–c" (C_4) unit, one methionine (C_1), one methylation by [2-^{13}C]acetate (C_1), and one decarboxylated isoprenoid unit (C_4). The cyclization cascade of the polyepoxides, included in these figures, was proposed earlier[39] as an intriguing biogenetic scheme and should not be taken seriously; moreover, the origins of the oxygen atoms are unknown. However, it is interesting that 27,28-epoxy-BTX-B (GB-6) has been isolated from *G. breve* culture.[7] In the biosynthesis of the polyether antibiotic monensin A, isotopically shifted ^{13}C NMR peaks resulting from incorporation of ^{18}O was instrumental in proposing a polyepoxide cyclization cascade to account for a stereocontrolled formation.[15] Subsequently, a similar stereocontrolled cyclization of epoxides by chemical means, i.e., under acidic conditions, has been demonstrated.[40] Also enantiomerically pure diepoxide has been converted to the stereospecifically cyclized product where the cyclization cascade was initiated upon treatment with pig live esterase.[41]

Conclusion

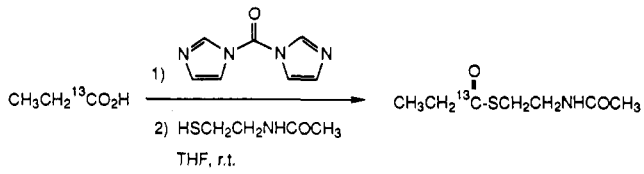
The involvement of the citric acid cycle in the biosynthesis of BTX's as described by Chou and Shimizu[18] and in this paper is not novel, but the degree of its involvement is unusual. The participation of C_3 units in the biosynthesis of BTX's differs from monensin. The C_3 units in monensin is derived directly from propionates, whereas in BTX's the C_3 units are derived indirectly through the citric acid cycle; C_4 diacids from the cycle are used in the biosynthesis with decarboxylation. Perhaps this constitutes one of the most important distinctions in the metabolites that are produced by terrestrial and marine microorganisms. During evolution, these marine microorganisms followed different paths such that for these organisms, the citric acid cycle became intimately involved in the production of some of their secondary metabolites. This may represent a more general difference between terrestrial and marine microorganisms. Thus, it would be interesting to see the outcome of a biosynthetic study of compounds exemplified by okadaic acid (marine)[10,11] in comparison to the well-established studies on compounds like monensin (terrestrial).[15,16] Similarly, biosynthetic studies on macrolide molecules

(39) Nakanishi, K. *Toxicon* **1985**, *23*, 473–479. K.N. is indebted to Professor Robert Thomas who suggested this cascade scheme, October 19, 1983.

(40) (a) Still, W. C.; Romero, A. G. *J. Am. Chem. Soc.* **1986**, *108*, 2105–2106. (b) Schreiber, S. L.; Sammakia, T.; Hulin, B.; Schulte, G. *J. Am. Chem. Soc.* **1987**, *109*, 2106–2108.

(41) Russell, S. T.; Robinson, J. A.; Williams, D. *J. Chem. Soc., Chem. Commun.* **1987**, 351–352.

Scheme III



such as pectenotoxin (marine)[12] and on yessotoxin,[14] remarkably similar to BTX-B, would be most interesting.

Experimental Section

General Methods. ^{13}C NMR measurements were carried out with Bruker WM-250 (62.9 MHz for ^{13}C) and Bruker AM-500 (125.1 MHz for ^{13}C) in benzene-d_6. Chemical shifts are referenced to internal benzene-d_6 at δ 128 ppm for carbon. For the 2D INADEQUATE NMR measurements, delays were optimized for $^1J_{CC}$ = 40 Hz. Typically, 128 increments, each consisting of 384 scans separated by a 1.2-s repetition delay were time-averaged over a 128-step phase cycle. Also, the protons were decoupled by using a Waltz-16 composite decoupling sequence for all ^{13}C measurements. Spectral width in F_1 and F_2, respectively, were 5952 and 23810 Hz, with a data block of 128 × 1 K, which after transformation yielded a 512 × 512 matrix. In F_2 6 Hz Lorentzian line broadening, while in F_1 a sine bell shifted ($\pi/2$) window function was used. Total accumulation times were between 11 and 13 h. All 2D spectra are displayed in the absolute mode.

Commercially available stable isotopes were purchased from Cambridge Isotope Laboratories or Merck Sharp and Dome. The commercially available radioactive compounds and the scintillation cocktail "Aquasol" were purchased from New England Nuclear (NEN). Radioactivity counts were performed with a Beckman LS 6800 liquid scintillation counter, channel setting 400–670.

Incubation of Sodium [1-^{13}C]- and [2-^{13}C]Acetate. A typical feeding experiment involved inoculation of a 12-L media with 6 L of *G. breve* culture (smaller scale; 6 L of media inoculated with 3 L of culture). This 18-L culture was allowed to grow under constant illumination with standard cool white fluorescent light at 20 °C for 10 days, at which time penicillin-G (40 units/mL) and streptomycin sulfate (200 units/mL) were added. The antibiotics were dissolved in 100 mL of media and poured into the culture followed by a 100-mL rinse. On day 11, the labeled acetate (0.67 mM) was added. The *G. breve* culture was harvested 7 days after addition of the labeled precursor by ether extraction. The dark green extract was applied to prepatory TLC and was developed with 5:1 hexane/isopropyl alcohol. The resulting crude toxins were further purified by using 5 μ normal phase HPLC, detection 215 nm, 6:1 isooctane/isopropyl alcohol, flow rate 3 mL/min. The elution time is as follows: BTX-A, 15 min; BTX-B, 20 min; GB-3, 30 min.

Incubation of Sodium [^{13}C]Bicarbonate. Incubation conditions were exactly the same as those for acetate incorporation.

Incubation of [*methyl*-^{13}C]Methionine. The same procedure was used as above except that the concentration of [*methyl*-^{13}C]methionine in a 9-L culture was 95.7 μM.

Incubation of Disodium [1,4-$^{13}C_2$]Succinate. Six liters of media were inoculated with 3 L of *G. breve* culture. On day 10, penicillin-G (42 units/mL) and streptomycin sulfate (168 units/mL) and, on day 11, disodium [1,4-$^{13}C_2$]succinate (0.47 mM) prepared from commercially available [1,4-$^{13}C_2$]succinic acid were added to the 9-L culture. The disodium salt was dissolved in 125 mL of culture and added to the *G. breve* culture followed by a 100-mL rinse. Succinic acid was converted into disodium salt by dissolving the diacid in hot ethanol, and subsequently NaOH/EtOH was added until a pH of 9 was reached. The crude salt precipitation was recrystallized in MeOH/H_2O to afford a white crystalline solid. Eight days after the addition of labeled precursor the culture was extracted with ether. The isolation procedure was carried out by preparative TLC followed by HPLC as before.

Incubation of Sodium [1-^{13}C]Propionate. The same procedure was used as above except that 12 L of culture with propionate concentration of 0.68 mM was employed for the labeling experiment. The culture was harvested after 7 days of incubation with labeled precursor.

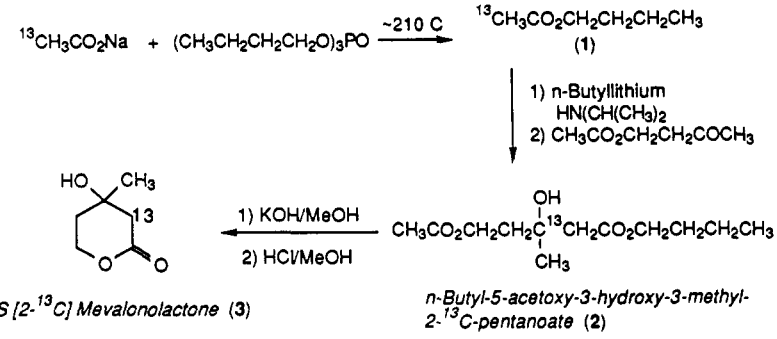
Incubation of *S*-[1-^{13}C]Propionyl-*N*-acetylcysteamine. Prior to feeding of this labeled material to the culture, a compatibility test was carried out. Various concentrations of *S*-propionyl-*N*-acetylcysteamine were administered to the culture. The most obvious outcome of this experiment was that the cell density was a function of the concentration of the added precursor, *S*-propionyl-*N*-acetylcysteamine. The presence of this precursor interfered with the growth of the culture. Thus, higher concentrations of precursor resulted in a weaker culture. This prompted us to test the effect of *N,S*-diacetylcysteamine because propionate had the effect of weakening the culture; thus, the propionyl thio ester may have had the same effect. However, the same weakening of culture was observed upon administration of *N,S*-diacetylcysteamine. After inoculation, the 9 L of culture were grown 12 days before addition of antibiotics. On day 13, *S*-[1-^{13}C]propionyl-*N*-acetylcysteamine (0.78 mM) was added by dissolving the labeled precursor in 150 mL of media and subsequently pouring into the culture followed by a 150-mL rinse. The culture was harvested 4 days after the addition of the labeled precursor due to the weak condition of the culture. The same purification scheme as above was used.

Incubation of (*RS*)-[2-^{13}C]Mevalonolactone. After inoculation, 18 L of culture was grown for 10 days before the addition of antibiotics. On day 11, 969 mg of (*RS*)-[2-^{13}C]mevalonolactone was added by dissolving the labeled precursor in 75 mL of culture followed by 2 × 75 mL rinse. On day 13, further addition of (*RS*)-[2-^{13}C]mevalonolactone (1.18 g) was carried out as before. The culture was harvested on day 18.

Incubation of Disodium [2,3-$^{14}C_2$]Succinate. The medium (200 mL) was inoculated with 200 mL of culture which was then allowed to grow for 10 days before addition of penicillin-G (50 units/mL) and streptomycin sulfate (188 units/mL). The antibiotics were dissolved in 10 mL of medium and pipetted into the culture. On day 11, radiolabeled disodium succinate was added. Commercially available [2,3-$^{14}C_2$]succinic acid in ethanol (specific activity 56.0 mCi/mmol, total radioactivity 0.05 mCi) was converted to disodium salt by addition of 2 mol equiv of 0.1 N NaOH. Ethanol was removed by a stream of argon. The remaining solid residue was dissolved in 2 mL of media and was pipetted into the culture followed by 2 mL of media rinse. Seven days after addition of the precursor, 100 mL of the above culture was extracted with ether, and the ether extract was passed through a 4-cm silica/pipet to remove insoluble material. This crude extract was then injected onto HPLC, 2.2 mL/min, 215-nm detection, and 6:1 isooctane/isopropyl alcohol as solvent. Fractions were collected directly in scintillation vials from the time of injection to the end of the HPLC run. The solvent was then allowed to evaporate and subsequently 10 mL of scintillation cocktail (Aquasol) were added to the vials containing dry residue. The radioactivity count was carried out on all of the collected fractions by using the liquid scintillation counter with channel settings at 400 to 670. The remaining 300-mL of culture were harvested 11, 14, and 21 days after the addition of precursor (each containing 100 mL of culture).

Incubation of Sodium [1-^{14}C]Propionate. The same procedure as described above was followed. The antibiotics were added 11 days after inoculation. Sodium [1-^{14}C]propionate in ethanol with a specific activity of 59 mCi/mmol and a total radioactivity of 0.05 mCi was used. Ethanol was removed by a stream of argon, and the propionate was dissolved in 0.5 mL of culture and transferred into the 400-mL culture followed by a 2 × 0.5 mL rinse which was also added to the culture. The culture was harvested on days 8, 10, and 14 after addition of the radiolabeled precursor (100, 100, and 200 mL, respectively). The isolation procedure and

Scheme IV



radioactivity count followed the same procedure as described above.

Incubation of (*RS*)-[2-^{14}C]Mevalonolactone. The same procedure as described above was followed. Antibiotics were added 11 days after inoculation of 200 mL of medium with 200-mL culture. On day 13, (*RS*)-[2-^{14}C]mevalonolactone in ethanol with a specific activity of 50.1 mCi/mmol and a total activity of 0.05 mCi was added. Ethanol was removed by a stream of argon, and the radioactive mevalonolactone was dissolved in 2 mL of culture and pipetted into the 400-mL culture, followed by a 2 × 2 mL rinse. Subsequently, the culture was harvested on days 8, 10, and 14 after addition of the precursor. The isolation and radioactivity count were performed in the same manner as previously described.

Preparation of *S*-[1-^{13}C]Propionyl-*N*-acetylcysteamine[37] (Scheme III). Preparation of *N*,*S*-Diacetylcysteamine.[42] A three-necked, 500-mL, round-bottomed flask equipped with a pH electrode and two addition funnels were charged with 2-aminoethanethiol hydrochloride (21.21 g, 0.187 mol) in 170 mL of water. This was then chilled to 0 °C with an ice/water bath. The pH was adjusted to pH 8 by addition of 8 N KOH. One addition funnel was charged with acetic anhydride, while the other was charged with 8 N KOH. The acetic anhydride (57.2 g, 0.56 mol) was added dropwise while keeping the pH of the reaction at pH 8 by addition of 8 N KOH. After all the acetic anhydride had been added, 2 N HCl was used to adjust the pH to pH 7, and the reaction mixture was then allowed to stir at 0 °C for 1.5 h. The workup was carried out by addition of NaCl until saturation, and the mixture was extracted with CH_2Cl_2. The organic layer was dried over Na_2SO_4, and the solvent was removed in vacuo to give a white solid (31.32 g, 96%): ^{1}H NMR ($CDCl_3$) δ 3.35 (q, 2 H, N—CH_2), 2.95 (t, 2 H, S—CH_2), 2.28 (s, 3 H, CH_3—C(O)S), and 1.90 ppm (s, 3 H, CH_3—C(O)N).

Preparation of *N*-Acetylcysteamine.[42] To a solution of *N*,*S*-diacetylcysteamine (25.2 g, 0.156 mol) in H_2O at 0 °C was added solid KOH (32 g, 0.50 mol). This mixture was warmed to room temperature and stirred for 50 min. This reaction mixture was then cooled to 0 °C, and the pH was adjusted to pH 7 with 2 N HCl. NaCl was added until saturation, and the mixture was extracted with CH_2Cl_2 and dried over Na_2SO_4 to yield a clear viscous liquid (15.5 g, 78%). ^{1}H NMR ($CDCl_3$) δ 3.35 (q, 2 H, N—CH_2), 2.59 (dt, 2 H, S—CH_2), 2.92 (s, 3 H, CH_3—C(O)N), and 1.35 (t, 1 H, HS).

Preparation of *S*-[1-^{13}C]Propionyl-*N*-acetylcysteamine. [1-^{13}C]-Propionic acid (1.0 g, 13 mmol) in 20 mL of THF was treated with carbonyldiimidazole (3.57 g, 22 mmol) at room temperature. After stirring for 2 h, *N*-acetylcysteamine (3.85 g, 32 mmol) was added, and the reaction mixture was stirred for 2 h at room temperature. The workup was carried out by diluting the reaction mixture with ethyl acetate and washing with H_2O. The aqueous wash was back extracted with ethyl acetate. The organic layers were dried, and the solvent was removed in vacuo. The resulting viscous liquid was flash chromatographed in ether to yield *S*-[1-^{13}C]propionyl-*N*-acetylcysteamine (1.96 g, 83.9%, pale yellowish viscous liquid): ^{1}H NMR ($CDCl_3$) δ 3.41 (q, 2 H, N—CH_2), 3.00 (m, 2 H, S—CH_2), 2.58 (dq, 2 H, —$CH_2{}^{13}$CO), 1.94 (s, 3 H, CH_3—CO), and 1.16 ppm (dt, 3 H, CH_3—).

Preparation of (*RS*)-[2-^{13}C]Mevalonolactone[44] (Scheme IV). Preparation of *n*-Butyl [2-^{13}C]Acetate.[43] A suspension of sodium [2-^{13}C]acetate (3.32 g, 39.5 mmol) in tributyl phosphate (17.6 g, 18 mL, 66 mmol) was refluxed at ~210 °C for 6 h. The reaction mixture was cooled to room temperature, and the product was distilled into a cold trap (liquid N_2) by heating to ~120 °C for 1.5 h at ~0.1 mm with the reflux condenser running, to yield *n*-butyl [2-^{13}C]acetate, clear liquid (4.14 g, 88%).

Preparation of *n*-Butyl 5-Acetoxy-3-hydroxy-3-methyl[2-^{13}C]pentanoate. To a solution of dry ether and diisopropylamine (4.3 g, 6 mL, 42.8 mmol) at −20 °C, *n*-butyllithium (16.5 mL, 2.6 M in hexane) was added dropwise. After 1 h of stirring the mixture was cooled to −70 °C, treated with *n*-butyl [2-^{13}C]acetate, stirred for 30 min, treated with 4-acetoxy-2-butanone, and stirred for 30 more min. The reaction was terminated by addition of 2 N HCl (40 mL), and the reaction mixture was warmed to room temperature. This mixture was diluted with H_2O and extracted with ether and dried over $MgSO_4$. This crude was used directly for the next reaction.

Preparation of (*RS*)-[2-^{13}C]Mevalonolactone.[44] *n*-Butyl 5-acetoxy-3-hydroxy-3-methyl[2-^{13}C]pentanoate was dissolved in KOH/MeOH at 0 °C, allowed to warm to room temperature, and stirred for 12 h. The pH was adjusted to pH 2 by addition of MeOH/HCl. The KCl salt was filtered, MeOH was removed in vacuo, and the dried reaction mixture was flash chromatographed in ether to yield (*RS*)-[2-^{13}C]mevalonolactone (4.1 g, 88.0%).

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Supplementary Material Available: Tables of proton and carbon-13 chemical shifts and the origin of each carbon of BTX-A and BTX-B, ^{13}NMR spectra of BTX-A and BTX-B derived from [1-^{13}C]acetate, [2-^{13}C]acetate, and [*methyl*-^{13}C]methionine, 2D INADEQUATE spectra of BTX-A and BTX-B enriched with [1,2-$^{13}C_2$]acetate, and proton NMR spectra of (*RS*)-[2-^{13}C]-mevalonolactone and *S*-[1-^{13}C]propionyl-*N*-acetylcysteamine (17 pages). Ordering information is given on any current masthead page.

(42) Schwab, J. M.; Klassen, J. B. *J. Am. Chem. Soc.* **1984**, *106*, 7217–7227.

(43) Bardshiri, E.; Simpson, T. J.; Scott, A. I.; Shishido, K. *J. Chem. Soc., Perkin Trans. I* **1984**, 1765–1767.

(44) Ellison, R. A.; Bhatnagar, P. K. *Synthesis* **1974**, *10*, 719.