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Biosynthesis of Brevianamides A and B: In Search of the Biosynthetic Diels-Alder Construction

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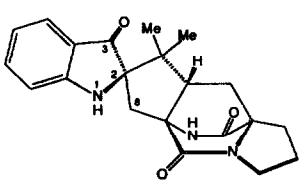
Abstract: A [^{13}C]labeled hypothetical intermediate metabolite that was invoked by Birch in the biogenesis of brevianamides A and B has been synthesized and used in a biosynthetic feeding experiment. No incorporation of this substance was evident nor could it be detected in culture extracts of the producing organism *Penicillium brevicompactum*. In contrast with this, [^{3}H]labeled deoxybrevianamide E was shown to be efficiently incorporated in brevianamides A, B, and E, while [^{3}H]labeled brevianamide E was not incorporated at all in these substances. Moreover, brevianamide E has been found not to be an artifact, in contrast with an earlier suggestion found in the literature. These results are discussed in terms of an alternative biosynthetic pathway for the brevianamides.

INTRODUCTION

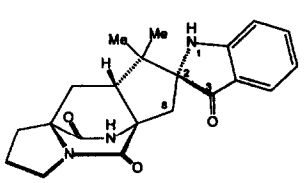
The Diels-Alder cyclization reaction is one of the most important ring-forming reactions in synthetic organic chemistry.[2] This versatile reaction and all of its heteroatom variants have been extensively utilized in the chemical syntheses of a large variety of natural products. It is therefore surprising that no documented cases of the enzyme-catalyzed Diels-Alder reaction in biosynthetic pathways are in the literature, despite their likelihood.[3]

In 1969, Birch and Wright [4a] reported the isolation of several neutral metabolites from culture extracts of the fungus *Penicillium brevicompactum*. These compounds, present in very small amounts in the extracts, were named brevianamides A-E. Based primarily on spectroscopic evidence, chemical degradation, and biogenetic considerations, the structure **1** was proposed [4b] for brevianamide A. This structure was later shown to be correct by single crystal X-ray analysis of its 5-bromo derivative. [5] The X-ray structure also established the relative and absolute configuration of **1**. In addition to being produced by *P. brevicompactum* ,[4] **1** is also found in both *P. viridicatum* [6] and *P. ochraceum* [7] cultures. Later research led to the discovery of a new member of this class, brevianamide F (**3**). Moreover, brevianamides C and D were shown to arise from irradiation of brevianamide A (**1**).[4c] Neither brevianamide C nor D is present in cultures of *P. brevicompactum* grown and worked-up in the dark, and are therefore considered artifacts.[4c]

The brevianamides belong to a class of mycotoxins that has been joined by the paraherquamides [8] (**1**, paraherquamide A) and the marcfortines [9] (**8**, marcfortine A). While **1** has been shown to possess antifeedant and insecticidal effects [10], several members of the paraherquamide family have very potent antiparasitic properties.[11] The most distinctive feature in the structures of **1**, **2**, **7**, and **8** is the presence of a core bicyclo[2.2.2] ring system which has been invoked to formally arise from a [4+2] cycloaddition.



1, *(+)-BREVIANAMIDE A*



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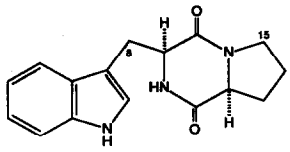

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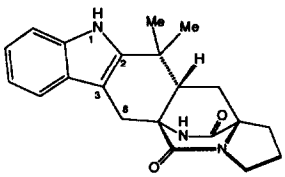

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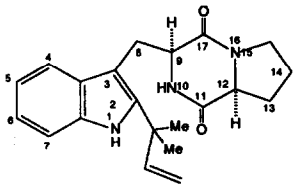
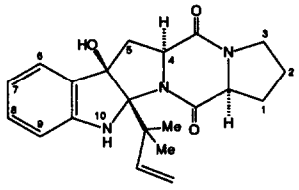
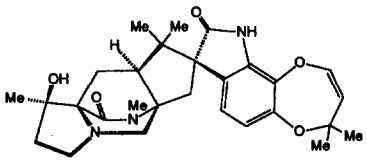


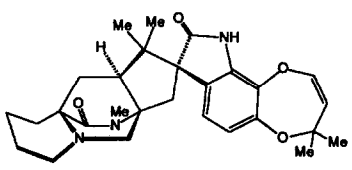
5, *DEOXYBREVIANAMIDE E*



6, *BREVIANAMIDE E*



7, *PARAHERQUAMIDE A*



8, *MARCFORTINE*

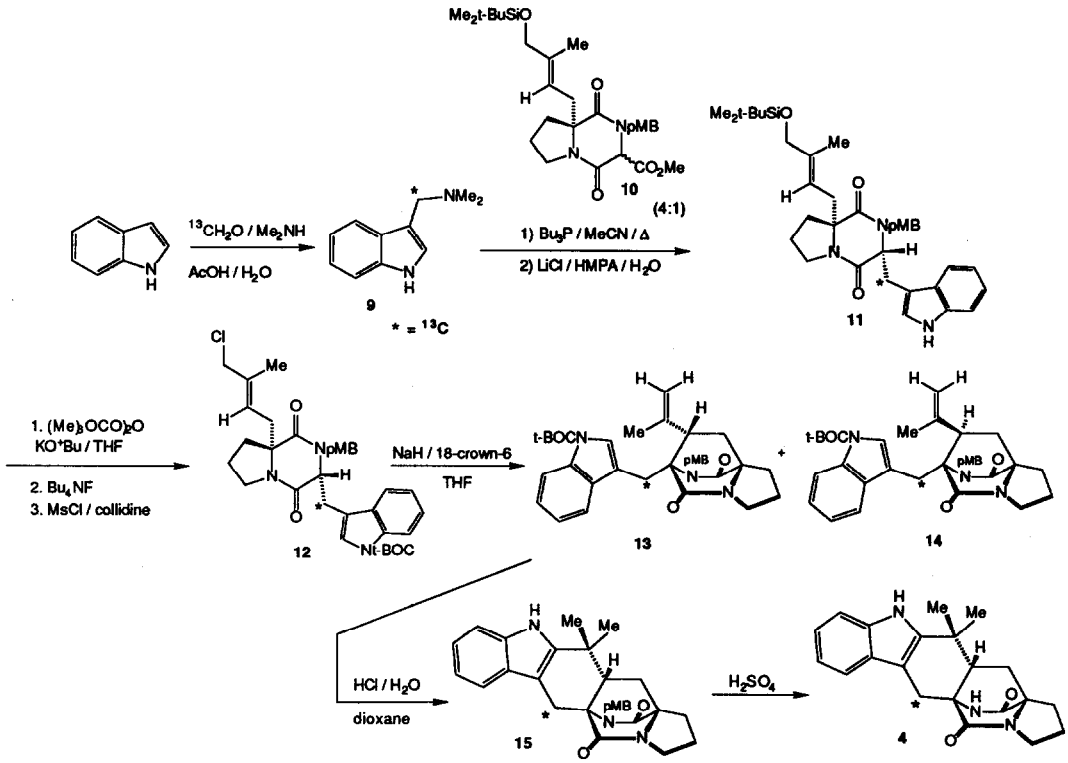
After Porter and Sammes published a suggestion on how the formation of the polycyclic system in these compounds takes place,[12a] Birch and associates carried out some preliminary studies on the biosynthesis of these compounds.[12b] They found that [15-^{3}H,8-^{14}C]brevianamide F ([15-^{3}H, 8-^{14}C]-**3**), [^{3}H]tryptophan, and [^{3}H]proline are biosynthetically incorporated into brevianamide A (**1**) in cultures of *P. brevicompactum*. From these observations, they proposed [12b] a biosynthetic pathway that involved prenylation of **3** to yield the indole **5** (deoxybrevianamide E) [13] as a first step. Formation of the bicyclo [2.2.2] dioxopiperazine nucleus would arise *via* oxidation of the tryptophanyl moiety, followed by a unique intramolecular [4+2] cycloaddition reaction to furnish the hexacyclic indole **4**. Well-precedented [14] oxidative spiro-rearrangement of **4** would afford the spiro-indoxyl system to give **1** and **2**.

During the course of our studies on the synthesis of the brevianamides [15] we observed that the structure of **2** is not the one proposed by Birch , but that of its *enantiomer*. Birch's original proposal was modified by us [16] to accommodate the fact that totally synthetic and semi-synthetic brevianamide B (**2**, obtained from brevianamide A (**1**) by a redox protocol) is, in fact, the enantiomer of the natural brevianamide B (**2**). Since the hypothetical biogenetic intermediate **4** was a key substance in our revised proposal on the biogenesis of the brevianamides, we decided to synthesize this substance in labeled, *racemic* form for biosynthetic feeding experiments and examine its incorporation in brevianamides A (**1**) and B (**2**). This would also allow us to ascertain the possible presence of this substance in cultures of *P. brevicompactum*, using the synthetic material as a reference.

Although deoxybrevianamide E (**5**) has been included in all proposals for the biosynthesis of the brevianamides thus far, no previous experiments have validated this hypothesis. We have undertaken the synthesis of a radio-labeled derivative of this substance, thus allowing us to perform feeding experiments in order to validate its intermediacy in this biosynthetic process. Herein we report our latest results on our work on the biosynthesis of the brevianamides; part of these studies have recently been published as a communication.[17]

RESULTS AND DISCUSSION

The synthesis of [^{13}C]-d,l-**4** was accomplished by the preparation of d,l-**10** as described previously.[15] In addition, >90% [^{13}C]labeled gramine (**9**) was prepared by the reaction of [^{13}C]formaldehyde with indole and dimethylamine in a Mannich condensation reaction (Scheme 1). The [^{13}C]gramine (**9**) was used in the Somei coupling[18] with the dioxopiperazine derivative **10**. A 4:1 diastereomeric mixture of racemic **10** [15] was



Scheme 1

directly treated with **9** in the presence of tri-n-butylphosphine in acetonitrile to furnish a single diastereoisomer. Hydrolysis of the carbomethoxy group of the resulting condensation product to the corresponding carboxylic acid with concomitant thermal decarboxylation was realized with LiCl in wet HMPA at 100°C to yield **11**. Protection of the indole nitrogen as its t-BOC derivative and removal of the silyl group furnished an alcohol, which was directly transformed to the allylic chloride (**12**) by standard methods. The treatment of **12** with NaH in THF in the presence of 18-crown-6 resulted in the two pentacyclic olefins **13** and **14** (3~5:1 ratio). After separation of this mixture using chromatography, **13** underwent clean, regiospecific cyclization and t-BOC deprotection to yield **15**. This compound was finally deprotected using conc. H_2SO_4, to yield d,l-[^{13}C]-**4**. Using an authentic sample of this key, proposed metabolite as a reference for comparison, we exhaustively screened fermentation extracts of *P. brevicompactum* for the production of **4**. Despite extensive extractive procedures on the mycelia and culture broth, ^{1}H NMR, TLC and HPLC analysis of the concentrated extracts provided no evidence that even a trace of this substance was being produced by the cells.

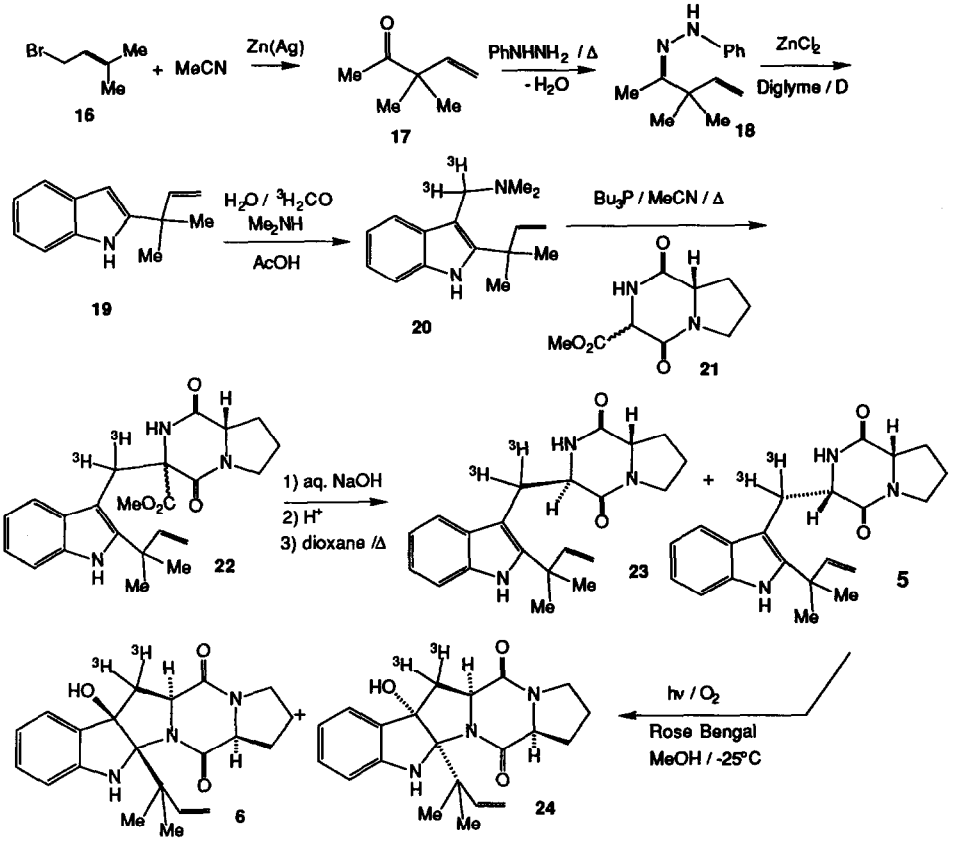
The biosynthetic feeding of [^{13}C]-d,l-**4** proved to be difficult due to its poor solubility in water. Eventually, we found that [^{13}C]-d,l-**4** could be dissolved in warm DMSO and diluted into the fermentation broth without precipitating. After growing *P. brevicompactum* on media spiked with [^{13}C]-d,l-**4** in DMSO, brevianamides A (**1**) and B (**2**) were isolated in the usual way. Brevianamide A (**1**) was subjected to ^{13}C NMR analysis. Within experimental error of NMR integration, we did not detect significant enhancement of C-8 in **1**. The sample of brevianamide B isolated from this feeding experiment, however, was too small for ^{13}C NMR experiments. Instead, the incorporation of d,l-[^{13}C]-**4** was determined by mass spectrometry. The $(M+1)^+/M^+$ intensity ratios in the EIMS spectra of **1** and **2** obtained in the feeding experiments were compared to the ones obtained from control cultures. Again, the difference was not significant for either **1** or **2**, indicating that there had been no incorporation of the labeled substance. The lack of incorporation of [^{13}C]-d,l-**4** into either **1** or **2**, and the fact that **4** was not detected in the fermentation extracts led us to pursue some alternative possibilities.

To ascertain the intermediacy of deoxybrevianamide E (**5**) we synthesized this substance labeled with tritium (Scheme 2). Our choice for tritium was based on the fact that it is much easier to detect and measure accurately than deuterium. This is an important consideration because the brevianamides are produced in very small amounts. Although we did not expect to be able to crystallize to constant activity the compounds resulting from the feeding experiments, HPLC would provide the necessary purity. Another possible benefit in using tritium was that any minor, intermediate metabolites would be very easy to identify and isolate from the culture extracts. Thus, [8-^{3}H]labeled deoxybrevianamide E ([8-^{3}H]-**5**)[13] was obtained following the sequence described by Kametani and collaborators.[19] One of the starting materials in this synthetic protocol is the 2-(1,1-dimethylprop-2-enyl)indole (**19**). Although several syntheses have been published for this compound,[20] none of them are really convenient, either due to lengthy sequences or difficulties in preparing useful quantities. We found that a simple reaction sequence yields this compound quite efficiently. Our method, based on the classic Fischer indole synthesis,[21] consists of the preparation of the phenylhydrazone **18** from phenylhydrazine and 3,3-dimethylpent-4-en-2-one (**16**). Although the latter can be synthesized by a variety of methods described in the literature,[22] we found it more convenient to adapt Rousseau and Conia's method for the synthesis of ketones.[23] Thus, acetonitrile reacts with 4-bromo-2-methylbut-2-ene (**16**) and Zn(Ag) to give **16** in 63% yield. The synthesis of its phenylhydrazone (**18**) was straightforward and quantitative. Finally, treatment of **18** with anhydrous $ZnCl_2$ in diglyme at reflux for 9 hours gave the desired 2-(1,1-dimethylprop-2-enyl)indole (**19**) in 45% yield.

The label was introduced in the reaction of **19** with [^{3}H]labeled formaldehyde and dimethylamine to give the gramine derivative [^{3}H]-**20**. In a departure from the originally published synthesis,[19] Somei coupling [18] of [^{3}H]-**20** with the dioxopiperazine **21** in presence of Bu_3P in acetonitrile under reflux gave the condensation product [^{3}H]-**22** as a mixture of diastereomers. This mixture was hydrolyzed with aqueous NaOH, and the resulting carboxylic acids were decarboxylated by heating in dioxane at 75-80°C to give a mixture of [8-^{3}H]deoxybrevianamide E ([8-^{3}H]-**5**) and its epimer, [8-^{3}H]-9-*epi*-deoxybrevianamide E ([8-^{3}H]-**23**). Chromatography yielded pure [8-^{3}H]-**5**.

Feeding experiments performed with [8-^{3}H]deoxybrevianamide E ([8-^{3}H]-**5**) (16.5mg with an activity of 1.605 μCi, and specific activity of 37.3 μCi/mmol) gave significant incorporation of the radioactivity into both brevianamide A (**1**) (7.8% total incorporation,[24] 12.1% specific incorporation, 0.125 μCi, 6.12 μCi/mmol) and brevianamide B (**2**) (0.9% total incorporation,[24] 1.4% specific incorporation, 0.015 μCi, 10.8 μCi/mmol). The specific activities of both **1** and **2** are comparable, thus confirming their common biosynthetic origin. As expected, the incorporation of [8-^{3}H]-**5** in the brevianamide E (**6**) was also high (24.9% total incorporation,[24] 38.5% specific incorporation, 0.40 μCi, 32.0 μCi/mmol). The high values for these incorporations confirm that deoxybrevianamide E (**5**) is a biosynthetic precursor of brevianamides A (**1**), B (**2**), and E (**6**). Careful inspection of the extracts obtained from these cultures showed no evidence of any further compounds with significant incorporation of radioactivity.

Since brevianamide E (**6**) could also be proposed as a reasonable intermediate *via* the indoxyl **26**, we decided to check this possibility. We obtained [5-^{3}H]-**6** from [8-^{3}H]-**5** by photooxidation and reduction of the resulting hydroperoxide with dimethylsulfide[19] (Scheme 2). In this case, however, the feeding experiment with [5-^{3}H]-**6** (17mg with an activity of 1.60 μCi, and a specific activity of 37.3 μCi/mmol), gave **1** and **2** with no significant incorporation. Solubility experiments showed that [5-^{3}H]-**6** was totally soluble in the broth under the feeding experiment conditions. It thus seems that **6** is a metabolite that does not lead to **1** or **2**.

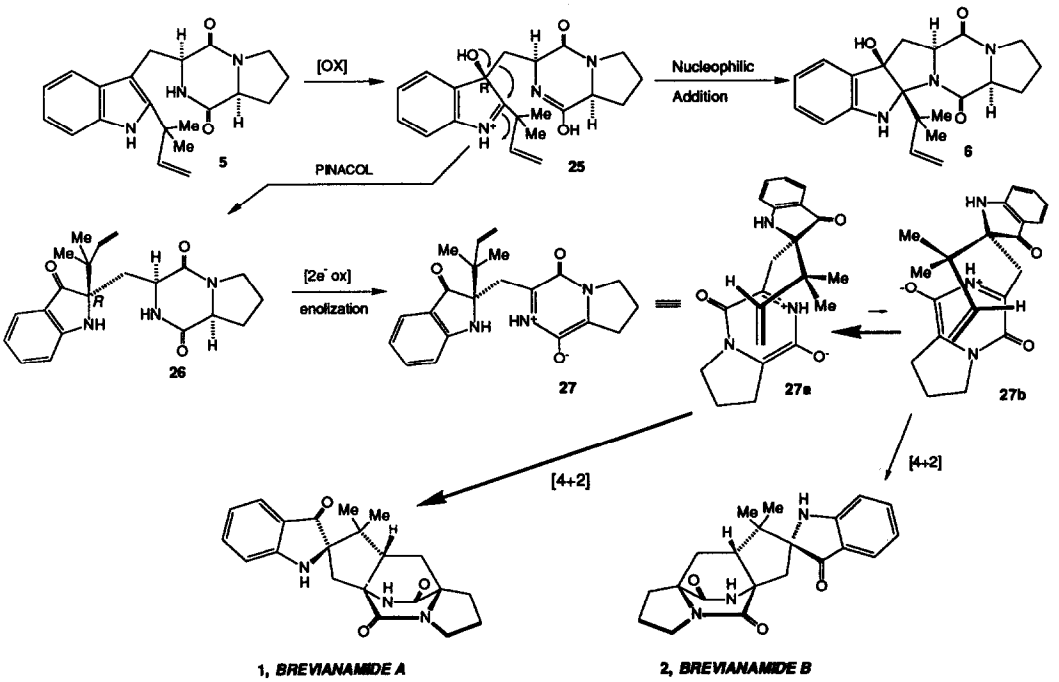


Scheme 2

In the biosynthetic pathways proposed thus far for the brevianamides it has been assumed that oxidation of the indole nucleus to the indoxyls occurs at the very end of the sequence. The results of our feeding experiments suggest, however, that a different biosynthetic pathway must be proposed. As mentioned in the introduction, deoxybrevianamide E (**5**) was included by Birch in his original biosynthetic proposal;[4,12b] the involvement of brevianamide E (**6**) in the biosynthetic pathway of this class of substances, however, has not been implicated thus far. Since autoxidation of deoxybrevianamide E (**5**) leads to the production of brevianamide E (**6**), it has been speculated that **6** may just be an artifact.[25] In our feeding experiments, however, **5** was quite stable under the culture conditions, being recovered from the experiment, in fact, in 35% of the initial amount. Moreover, in our cultures, brevianamide E (**6**) was always present in a fairly constant proportion relative to the brevianamide A (**1**) formed. In contrast with this, deoxybrevianamide E (**5**) has been isolated from cultures of *Aspergillus ustus*, but no trace of **6** was found.[13] We therefore think that, while some **6** may be formed by direct

autoxidation of **5**, compound **6** is not an artifact of the work-up or culture conditions, but rather a metabolite that represents a dead end in this biosynthetic pathway. The proposed pathway must therefore account for this fact.

An alternative biosynthetic scheme that explains the stereochemical outcome in the biosynthesis of the brevianamides A (**1**) and B (**2**) is depicted in Scheme 3. We now wish to propose that, after the conversion of brevianamide F (**3**) into deoxybrevianamide E (**5**) by prenylation, an R-selective hydroxylation reaction occurs at the 3-position of the indole **5**, furnishing the 3-hydroxyindolenine **25**.[17] Nucleophilic addition of the dioxopiperazine secondary amide N to the C=N bond of **25** gives brevianamide E (**6**). On the other hand, catalyzed pinacol-type rearrangement of the 3-hydroxyindolenine **25** [14] sets the R-absolute stereochemistry at the indoxyl quaternary center to give **26**. Oxidation of the dioxopiperazine subunit [26] in **26** forms the azadiene **27**. Finally, it is possible that a *catalyzed* intramolecular Diels-Alder cyclization [27] from a major rotamer (**27a**) directly leads to **1**, and a minor rotamer (**27b**) cyclizes to **2**. The preponderance of **1** over **2** would be due either to the relative activities of two different enzymes or the affinity of a single enzyme active site for the individual conformers. Thus, this proposal accommodates the existence of the two enantiomorphic bicyclo[2.2.2] ring systems.



Scheme 3

The question as to whether the hypothetical cycloaddition reaction is enzyme-catalyzed or not remains to be solved. There is little precedent for Diels-Alder reactions occurring in dioxopiperazines,[12a,27] and in these cases, highly electron-deficient dienophiles were involved in the cycloaddition. To the best of our knowledge, no examples of related intermolecular or intramolecular Diels-Alder reactions with neutral or electron-rich dienophiles in the dioxopiperazine manifold have been reported. FMO theory would predict [2a] that for the energy levels of a relatively electron-rich diene (such as that present in the dioxopiperazine) to effectively interact with a dienophile, powerful electron-withdrawing groups should be present on the dienophile. In the present case, the dienophile is an isolated and electron-neutral vinyl group. Thus, although experimental precedent does

exist for [4+2] cycloadditions in dioxopiperazines, one would not expect such reactions to be spontaneous without catalysis.

In summary, the stereocontrolled total synthesis of the hypothetical biosynthetic precursor **4** proposed by Birch has been achieved; the intermediacy of this compound in the biosynthesis of the brevianamides A (**1**) and B (**2**), however, is uncertain and, based on both the examination of culture extracts and feeding experiments, seems at present rather unlikely. Our feeding experiments also show that while deoxybrevianamide E (**5**) is a biosynthetic intermediate of both brevianamides A (**1**) and B (**2**), brevianamide E (**6**) is a metabolite which does not lead to these compounds. Although the original Sammes Diels-Alder proposal (leading up to **4**) now seems unlikely, the intermediacy of a pericyclic [4+2] cycloaddition reaction in the construction of the core bicyclo[2.2.2] nucleus of the brevianamide / paraherquamide / marcfortine class remains the most attractive conceptual framework from which to base future biosynthetic explorations. Efforts are presently underway to identify, isolate and clone the last enzyme in the biosynthesis of the brevianamides.

Acknowledgments: This work was supported by the National Institutes of Health (Grant CA 43969) and the Colorado State University Agricultural Experiment Station as part of USDA SAES Western Project W-122. R. M. W. also acknowledges additional support from Hoffman-La Roche, inc.; Merck, Sharp & Dohme Research Laboratories; and Symphony Pharmaceutical Co., inc. J.F.S.-C. thanks the DGICYT of Spain for a fellowship. We also thank Renee Gallegos for technical assistance and Prof. H. C. J. Ottenheijm for sharing with us unpublished information on the preparation of 2-(1,1-dimethylprop-2-enyl)indole.

EXPERIMENTAL

Growth of the cultures and isolation of the brevianamides. In a typical experiment, two Czapek Dox (glucose) slants (2 x 10 cm, 10mL of medium in each one) were inoculated with *P. brevicompactum* (ATCC 9056) spores, which had been stored in glycerol at -80°C. The slants were incubated in the dark at 25° for 7 days, to obtain a lush bluish-green lawn of growth. They were then used to inoculate the fermentation media by shaking them in the mouth of the flask, allowing the spores to settle on the surface. The fermentation media consisted of 40g of glucose and 22g of corn steep liquor (Sigma) per 1L of distilled water. The media were autoclaved prior to use. After the inoculation, the fermentation flasks were incubated in the dark at 25°C until a thick surface growth was obtained (10 days). In our experience, if a suspension of the spores in water is used for the inoculation of fermentation media, the resulting white mycelia will produce a much smaller amount of brevianamides or no brevianamides at all. The broth was separated by decantation, filtered, and extracted with EtOAc (4 x 100 mL). The mycelia were extracted at room temperature in the dark, under magnetic stirring, with MeOH (2 x 250 mL, 2h each time). After removal of the solvent *in vacuo*, these extracts were combined with the broth extract, washed with aq. satd $NaHCO_3$ soln (2 x 50 mL), dried over anh. Na_2SO_4, and the solvent was removed *in vacuo* . The individual products were purified by column chromatography on silica gel (CH_2Cl_2:MeOH 10:1), followed by HPLC. HPLC was performed with a 8 mm x 10 cm Bondapak reverse phase C18 column with pre-column, using the mixture MeOH-5 % aq. $(NH_4)_2CO_3$ (1:1) as eluent. Since **1** and **2** are photolabile, the whole process was carried out avoiding exposure to the light of the extracts and fractions containing these compounds. The purity of all compounds isolated from our feeding experiments was assessed by using HPLC.

Synthesis of [^{13}C]-4:

3-(Dimethylamino-[^{13}C]methyl)indole ([^{13}C]-9). To a cooled flask containing glacial acetic acid (0.85mL) 0.29g of dimethylamine were added. To this solution, cooled to 2°C, 0.20g of commercial [^{13}C]formaldehyde (6.45mmol, label >95%) were added, as a 20% aq. solution. This mixture was added in one portion to 0.756g of indole (6.45mmol) at room temperature, and the resulting solution was stirred overnight at room temperature under N_2. Then, the reaction mixture was basified with 2N aq. NaOH, which caused the gramine to precipitate. This solid was filtered *in vacuo*, washed with water, and dried to give 1.05g of the crude gramine as a white solid. This compound was used in the following step without further purification. As with the rest of the [^{13}C]labeled compounds in this work, the spectra for this substance were the same as for gramine, except for the splitting of the signals for the $^{13}CH_2$ hydrogens in the ^{1}H-NMR spectrum, due to the ^{1}H-^{13}C coupling (J_{HC}=130Hz).

(d,l)-6-(R)-[4-((tert-Butyldimethylsilyl)oxy)-3-methyl-butene-2E-yl]-2,5-dioxo-3-(R)-((3-indolyl)-[^{13}C]methyl)-4-(4-methoxybenzyl)-1.4-diazabicyclo-[4.3.0]-nonane ([^{13}C]-11). A solution of **10** (mixture of epimers, 1.692g, 3.20mmol, 1.0eq, obtained as previously described[15], but starting with d,l-proline), [^{13}C]-**9** (0.505g, 2.87mmol, 0.9eq) and tri-n-butylphosphine (0.32mL, 1.28mmol, 0.4eq) in acetonitrile (35mL) was gently refluxed under N_2 for 18h. The solvent was evaporated under reduced pressure and from the oily residue the condensation product was isolated on silica gel by column chromatography (eluted with EtOAc/hexanes 1:1) as a colorless glass (1.14g, 54%)[15]. A solution of this product (1.14g, 1.73mmol, 1.0 eq), lithium chloride (0.4g) and water (60μL) in HMPA (10mL) was heated at 85-90°C under N_2 for 9h. The reaction mixture was diluted with EtOAc/hexane (1:1) and thoroughly washed with water. The organic layer was dried over Na_2SO_4 and concentrated under reduced pressure to furnish an oily residue. Separation by silica gel column chromatography (EtOAc/hexane 1:1) yielded 0.947g (91%) of a mixture of the isomeric decarbomethoxylated products *syn*-[^{13}C]-**11** and *anti*-[^{13}C]-**11**.

The ^{1}H NMR spectrum for this substance was the same as for the unlabeled compound[15], except for the splitting of the signals for the $^{13}CH_2$ hydrogens in the ^{1}H NMR spectrum, due to the ^{1}H-^{13}C coupling. Its ^{13}C NMR spectrum (75.47MHz, $CDCl_3$) showed a single signal at δ29.7 ($^{13}\underline{C}H_2$), the rest of the peaks being buried under the noise.

(d,l)-3-(R)-[(N-tert-Butyloxycarbonyl-3-indolyl)-[^{13}C]methyl]-6-(R)-(4-chloro-3-methyl-buten-2E-yl)-2.5-dioxo-4-(4-methoxybenzyl)-1.4-diazabicyclo-[4.3.0]-nonane (d,l)-[^{13}C]-(12). To a solution of [^{13}C]-**11** (0.947g; 1.57mmol, 1.0eq) in THF (25mL) cooled to 0°C, a solution of potassium t-butoxide (0.194g, 1.73mmol, 1.1eq) in THF (2mL) was added. After 5 min. of stirring a solution of di-t-butylcarbonate (0.360g, 1.65mmol, 1.05eq) in THF (2mL) was added, the cooling bath was removed and the mixture was allowed to warm up to room temperature. The reaction was stirred until TLC showed no more starting material (2h). 5mL of MeOH were then added to quench the excess of di-t-butylcarbonate, and the reaction was stirred for 2h. more. A solution of n-Bu_4NF trihydrate (0.2M, 11.8mL, 2.36mmol, 1.5eq) in THF was added and the reaction mixture was stirred for 3 days at room temperature. The reaction mixture was diluted with water, extracted with EtOAc and dried over anhydrous Na_2SO_4. The crude reaction product was purified by radial chromatography with hexane-EtOAc 1:1 to give 0.800g of the corresponding protected allylic alcohol. To a cold (0°C) solution of this compound (0.800g; 1.36mmol, 1.0eq), collidine (0.52mL, 3.93mmol, 2.5eq) and LiCl (0.166g, 3.93mmol, 2.5eq) in DMF (15mL), methanesulfonyl chloride (0.385mL, 3.93mmol, 2.5eq) was added in one portion. The reaction mixture was allowed to warm to 25°C and then stirred for 4h. The mixture was poured on water, acidified with 3N HCl (2.0mL) and extracted with CH_2Cl_2. The organic extract was dried over anhydrous Na_2SO_4. The crude reaction mixture was kept under oil-pump vacuum for 2 days in order to remove the excess of methanesulfonyl chloride. This gave 0.89g of a crude reaction, containing the desired chloride [^{13}C]-**15**[15]. Its ^{13}C NMR spectrum showed a single peak at δ26.1. This compound was used in the following reaction without further purification.

(d,l)-1-(R)-[(N-tert-Butoxycarbonyl-3-indolyl)-[^{13}C]methyl]-2.8-dioxo-10-(R)-(1-methylethenyl)-9-(4-methoxybenzyl)-3,9-diazatricyclo-[5.2.2.0^{3,7}]-undecane ((d,l)-[^{13}C]-13) and (d,l)-1-(R)-[(N-tert-Butoxycarbonyl-3-indolyl)-[^{13}C]methyl]-2,8-dioxo-10-(S)-1-(1-methylethenyl)-9-(4-methoxybenzyl)-3,0-diazatricyclo-[5.2.2.0^{3,7}] undecane ((d,l)-[^{13}C]-14) from (d,l)-[^{13}C]-12. To a flask containing 0.64g of NaH (13.3mmol, 10.0eq) a solution of 1.75g of 18-crown-6 (6.65mmol, 5 eq) in 15mL of abs. THF was added. To this solution the crude allylic chloride [^{13}C]-**12** (0.139g, 0.23mmol, 1.0eq) and 0.145g of di-t-butylcarbonate (0.67mmol, 0.5eq) dissolved in 50mL of abs. THF were added. The resulting mixture was stirred and refluxed under N_2 for 2h. The reaction mixture was poured on water, neutralized with an equimolar amount of dilute HCl and extracted with ethyl acetate/hexane. The organic layer was dried over Na_2SO_4 concentrated under reduced pressure and the residue was separated on a silica gel column (EtOAc/hexane 2:1) to furnish a mixture of [^{13}C]-**13** and [^{13}C]-**14** (0.44g, 58%, ca. 3:1 ratio). A small portion of this crude reaction was purified using PTLC to give pure [^{13}C]-**13** and [^{13}C]-**14**.[15] For [^{13}C]-**13**, the ^{13}C NMR spectrum (75.47MHz, $CDCl_3$) showed a single signal at δ23.7 ($^{13}\underline{C}H_2$), while for [^{13}C]-**14** this signal appeared at δ24.0. In both cases, the rest of the peaks were buried under the noise.

Cyclization of (d,l)-[^{13}C]-13/(d,l)-[^{13}C]-14 (formation of (d,l)-[^{13}C]-15). A solution of the mixture of [^{13}C]-**13** and [^{13}C]-**14** (*ca.* 3:1 ratio) (0.44g, 0.77mmol) in 5mL of dioxane was cooled to -10°C and then a mixture of 45mL of conc. aq. HCl in 50mL of dioxane was added to it. The reaction was kept at 0°C for 3h, and then at 12°C for 24h. The reaction mixture was diluted with water and extracted with methylene chloride. The organic layer was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The oily residue was

separated by PTLC on silica gel (CH_2Cl_2-MeOH 50:1) to afford [^{13}C]-**15** (0.160g, 44%) and its C-19-epimer (0.053g, 18%)[15]. The compound [^{13}C]-**15** was purified by crystallization from MeOH to give 0.128g (35%) of the pure cyclized indole.[15] Its ^{13}C NMR spectrum (75.47MHz, $CDCl_3$) showed a single signal at δ21.9 ($^{13}\underline{C}H_2$). The rest of the peaks were buried under the noise.

Deprotection of (d,l)-[^{13}C]-15 (preparation of (d,l)-[^{13}C]-4). To a flask containing 1mL of conc. H_2SO_4 cooled to 10°C, solid [^{13}C]-**15** (31.3mg, 0.066mmol) was added in one portion with vigorous stirring. The dissolution of the compound was accompanied by pale yellow coloration of the solution, which changed to a purple color after a few minutes. The reaction was stirred at 10°C for 15 min. and it was then quenched by adding ice, diluting with water, and neutralizing with 10M aq. NaOH. The mixture was thoroughly extracted with CH_2Cl_2 and the insoluble material was filtered off. The deprotected amide [^{13}C]-**4** was isolated from the CH_2Cl_2 extract. It was purified by PTLC (silica gel plates, CH_2Cl_2-MeOH 30:1) to give 9mg of the pure compound as a colorless solid (38%) [15]. A comparison with an authentic sample of this compound obtained using a different deprotection method [15], showed that both compounds were identical, with the exception of the the splitting of the signals for the $^{13}CH_2$ hydrogens in the ^{1}H-NMR spectrum, due to the ^{1}H-^{13}C coupling.

d,l-[^{13}C]-**4**: ^{1}H NMR (270MHz, $CDCl_3$) δ TMS: 1.23 (3H, s); 1.28 (3H, s); 1.80-1.92 (1H, m); 2.0-2.15 (4H, m); 2.33-2.39 (1H, m); 2.75-2.87 (1H, m); 2.83 (1H, dd, J=18.0Hz, 130.0Hz); 3.54 (2H, t, J=6.8Hz); 3.92 (1H, dd, J=18.0Hz, 130.0Hz); 5.74 (1H, br. s); 7.08-7.19 (2H, m); 7.31 (1H, d, J=7.7Hz); 7.51 (1H, d, J=7.7Hz); 7.82 (1H, br. s). ^{13}C NMR (unlabeled **4**) (75MHz, DMSO-d_6) δ TMS: 22.5; 23.9; 24.3; 27.8; 28.4; 31.6; 34.1; 43.6; 45.5; 60.5; 66.4; 102.6; 110.7; 117.6; 118.1; 120.6; 126.9; 136.4; 140.8; 169.1; 172.4. Compound d,l-[^{13}C]-**4** showed only one peak in its ^{13}C NMR spectrum (75MHz, DMSO-d_6, TMS) at δ23.9 ($^{13}\underline{C}H_2$). The rest of the peaks were not discernible from the baseline.

Feeding experiments with d,l-[^{13}C]-4:

A fermentation culture of *P. brevicompactum* on glucose/corn steep liquor in which the medium (200mL) had been spiked with a solution of 8mg of d,l-[^{13}C]-**4** in 1mL of DMSO was inoculated, grown for 10 days and the brevianamides A and B were isolated and purified as described above. A ^{13}C NMR of the isolated brevianamide A (**1**) (*ca.* 1mg) did not show any significant enhancement of the methylene peak in which the incorporation of the ^{13}C label would have happened. This methylene signal was easily identified in the ^{13}C NMR spectrum of **1** using a HETCOR 2D experiment, in which the carbon at δ 35.3 (75MHz, DMSO-d_6) was clearly correlated to the easily discernable AB system in its ^{1}H NMR spectrum.[4b,c]

The sample of brevianamide B (**2**) isolated from this experiment was too small for ^{13}C NMR experiments. Instead, the incorporation of the [^{13}C]labeled substrate was determined by comparing the $(M+1)^+/M^+$ intensity ratios in the EIMS spectra of the brevianamides obtained in the feeding experiments and the ones obtained from control cultures. Again, the difference was not significant for either **1** or **2**.

Synthesis of [8-^{3}H]deoxybrevianamide E [8-^{3}H]-5[19] and [5-^{3}H]brevianamide E [8-^{3}H]-6[19].

3,3-Dimethylpent-4-en-2-one (16). A 250 mL 3-neck round bottom flask was charged with 13.1mL of abs. acetonitrile (10.28g, 0.25mol), 60mL of abs. THF, and 21.91g of Zn(Ag) alloy.[23] While stirring vigorously at room temperature under N_2, 38.7mL of 4-bromo-2-methylbut-2-ene (**16**) (50g, 0.335mol) were added dropwise over 12h. The reaction was stirred for 24h more, and then it was cooled to 0°C. To this solution 100mL of ice-cold, satd. aq. solution of NH_4Cl were added while sirring. The mixture was stirred for 10 min. more, and then it was transferred to a separatory funnel containing 150mL more of the NH_4Cl aq. satd. solution. The crude ketone was extracted with ether (4 x 75mL), and the organic extracts were combined and dried over anh. Na_2SO_4. Most of the solvent was removed by distillation at atmospheric pressure using a Vigreux column, and the residue was transferred to a smaller distillation set. The ketone distilled at atmospheric pressure at 110-128°C, to give 21g of the product with a purity of ca. 85% (by NMR). This ketone was carefully redistilled at 126-128°C to give pure 3,3-dimethylpent-4-en-2-one (**16**) (17.6g, 63% yield). This compound had the same physical and spectroscopic properties as previously described in the literature.[22]

2-(1,1-Dimethylprop-2-enyl)indole (19). A 250mL round bottom flask fitted with a Dean-Stark distillation head was charged with 17.58g of **16** (0.157mol), 100mL of toluene and 16.93g of phenylhydrazine (0.157mol, 1 eq.). The reaction was refluxed until no more water was formed (30 min.). The solvent was removed *in vacuo*, and the crude hydrazone **18** was kept under vacuum over P_2O_5 overnight, in order to remove any trace of water. The crude **18** was dissolved in 100mL of abs. diglyme, and to this solution, while stirring and under N_2, 42.79g of anh.

$ZnCl_2$ (0.33mol, 2eq.) were added in one portion. The mixture was refluxed for 9h. Then 100mL of toluene were added to the mixture, and the latter was stirred with a glass rod while cooling it to room temperature. The crystallized $ZnCl_2$ was filtered off and washed with several portions of hot toluene. The solvent in the filtrate was removed *in vacuo* and the residue was separated in column chromatography (silica gel, toluene). The 2-(1,1-dimethyprop-2-enyl)indole (**19**) was eluted in the first fractions, yielding 13.1g (45%) of the compound as a colorless oil. This compound rapidly darkens when exposed to the air. Its physical and spectroscopic properties were identical with those described in the literature.[20]

2-(1,1-Dimethylprop-2-enyl)-3-(dimethylamino-[^{3}H]methyl)indole ([^{3}H]-20). To a flask containing 2.8mL of 40% aq. solution of Me_2NH (22.16mmol, 1.1eq.) cooled to 2°C, 2.88mL of glacial acetic acid (3.02g, 50.36mmol, 2.5eq., pre-cooled to 2°C) were added. When the temperature of the mixture reached 2°C, 1.49mL of formalin (38% aq. soln. of formaldehyde) and 1mCi of [$^3H_2$]formaldehyde (0.0125mmol, 80mCi/mmol) were added. This mixture was stirred for 10 minutes, and then it was added in one portion to 4.10g of **19** (22.16mmol, 1.1eq.) at room temperature. Enough MeOH was added to this mixture to make it homogeneous (2mL), and the resulting solution was stirred for 18h at room temperature. The reaction mixture was then poured into 140mL of water containing 4mL of conc. aq. HCl, and extracted with 50mL of ether, in order to remove the excess of the 2-(dimethylallyl)indole. This extract was discarded, and the aq. layer was basified with a solution of 8g of NaOH in 50mL of water. This caused the crude gramine derivative to separate as an oil, which was extracted with ether (3 x 50mL). The organic extracts were combined, washed with water, and dried over anh. Na_2SO_4, to give 4.086g of the crude product (83% crude yield) as a yellowish oil. This compound was used in the following step without further purification.

Condensation of the dioxopiperazine 21 and 2-(1,1-dimethylprop-2-enyl)-3-(dimethylamino-[^{3}H]methyl)indole ([^{3}H]-20). A solution of 4.086g of **20** (16.02mmol, 1eq.), 4.27g of the dioxopiperazine **24**[19] (16.99mmol, 1.06eq), and 1.62g of n-Bu_3P (2.0mL, 0.5eq.) in 75mL of abs. CH_3CN was gently refluxed under N_2 for 5h. The solvent was removed *in vacuo* and the oily residue was dissolved in 100mL of 0.5M aq. HCl. This mixture was extracted with CH_2Cl_2 (3 x 50mL). The extracts were combined, washed with brine (50mL), and dried over anh. Na_2SO_4. Removal of the solvent *in vacuo* yielded 8.426g of crude condensation product [^{3}H]-**22**, which was used in the following step without further purification.

[8-^{3}H]Deoxybrevianamide E ([8-^{3}H]-5) and [8-^{3}H]-9-epi-deoxybrevianamide E ([8-^{3}H]-23). A solution of 1g of NaOH in 5mL of water was added to a solution of the crude [^{3}H]-**22** obtained in the previous reaction (8.426g) in 50mL of MeOH. The reaction was stirred overnight under inert atmosphere at room temperature. The MeOH was removed *in vacuo*, and a solution of 3mL of conc. aq. HCl in 140mL of water was added to the oily residue. This caused the yellow oil to turn to a white solid, which was extracted with CH_2Cl_2 (3 x 50mL). The organic extracts were combined and washed with brine (50mL). After drying over anh. Na_2SO_4, removal of the solvent *in vacuo* gave 6.989g of crude mixture. The crude reaction product was dissolved in 125mL of abs. dioxane, and the solution was kept at 75-80°C for 6h. The solvent was removed *in vacuo* to give a crude product, which was separated using column chromatography (silica gel, CH_2Cl_2-MeOH 50:1 to 20:1). A first fraction (2.29g) contained a mixture of both epimers. A second fraction (3.564g) consisted of pure [8-^{3}H]-9-epi-deoxybrevianamide E (**23**). The first fraction was submitted again to column chromatography separation (silica gel, CH_2Cl_2-MeOH 100:1 to 25:1). This gave 297mg of pure [8-^{3}H]deoxybrevianamide E [8-^{3}H]-**5** (5%yield, 31.5μCi, 37.3μCi/mmol), as well as 1.69g more of its epimer [8-^{3}H]-**23**. The total amount of [8-^{3}H]-**23** obtained was 3.98g (67%yield, 422μCi, 37.3μCi/mmol). Both substances were found to be identical with the corresponding unlabeled compounds, obtained by repeating a published synthesis.[19] The identity was assessed by IR, NMR, and TLC.

[5-^{3}H]Brevianamide E ([5-^{3}H]-6). To a solution of 90mg (0.256mmol) of [8-^{3}H]-**5** in 125mL of abs. MeOH 160mg (0.157mmol) of Rose Bengal were added. The solution was cooled to -25°C and it was irradiated for 4.5h with white light, using a 250W spot Hg lamp, while a stream of O_2 was bubbled through the reaction mixture. 4mL of Me_2S were then added, and the mixture was kept overnight at -20°C. After evaporation of the solvent *in vacuo* (using a bath at room temperature) the Rose Bengal was removed by filtration through a neutral alumina column (grade I, 40g), using the mixture CH_2Cl_2-MeOH 10:1 (250mL) as eluent. Thus, a mixture of two diastereomeric compounds was obtained. This mixture was separated by HPLC (Waters μ-Bondapak C18 column, MeOH-5% aq. $(NH_4)_2CO_3$ 2:3) to give 29mg of [5-^{3}H]brevianamide E ([5-^{3}H]-**6**) (32% yield, 2.95μCi, 37.3μCi/mmol) and 17.5mg of [5-^{3}H]-5a,10a-diepi-brevianamide E ([5-^{3}H]-**24**) (19% yield, 1.78μCi, 37.3μCi/mmol). Both substances were found to be identical with the corresponding unlabeled compounds.[19]

Feeding experiment with [8-^{3}H]deoxybrevianamide E ([8-^{3}H]-5).

A glucose-corn steep liquor culture of *P. brevicompactum* (200mL in a 1L Erlenmeyer flask), inoculated as described above, was incubated at 25°C in the dark until the mycelia started to to turn greenish (4 days). At that point, 3/4 of the fermentation broth was removed, and replaced with 175mL of sterilyzed H_2O. A solution of 16.5mg of [8-^{3}H]-**5** (1.605 μCi) in 2mL of DMSO was then added to the fermentation media while stirring carefully as not to disturb the mycelia. After 7 more days of incubation under the same conditions as above, mycelia and broth were separated by decantation, and the brevianamides were separated and purified as described above. The isolated compounds were homogeneous in HPLC. 35.3% of the compound used in this experiment was recovered unchanged.

Liquid scintillation counting of these compounds showed that there was incorporation of the radioactive label into **1**, **2**, and **7** (see text).

Feeding experiments with [5-^{3}H]brevianamide E ([8-^{3}H]-6).

The feeding experiment with [5-^{3}H]-**6** was performed as described above for [8-^{3}H]-**5**. In this case, 17mg of the labeled compound, (1.60 μCi, 37.3 μCi/mmol) were used. The brevianamides A (**1**) and B (**2**) were isolated from the culture, and purified as above to give an activity of 0.42 nCi (0.026% total incorporation, 0.14% specific incorporation, and 0.08 μCi/mmol of specific activity) for **1**, while for **2**, the activity was 0.53 nCi, (0.033% total incorporation, 0.18% of specific incorporation, and 1.01 μCi/mmol of specific activity). These compounds were homogeneous in HPLC. 81.6% of the compound used for this experiment was recovered. This feeding experiment was carried out twice, giving similar results each time.

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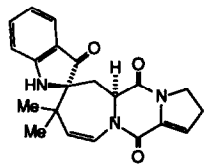

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