Reverse Prenyl Transferases Exhibit Poor Facial Discrimination in the Biosynthesis of Paraherquamide A, Brevianamide A, and Austamide

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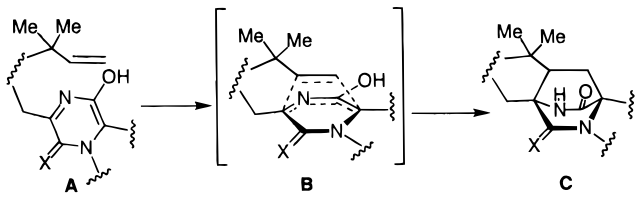
Abstract: The mode of attachment of dimethylallyl pyrophosphate (DMAPP) in the biosynthesis of the indole alkaloids paraherquamide A, austamide, and brevianamide A has been studied. Feeding experiments on *Penicillium fellutanum*, *Penicillium brevicompactum*, and *Aspergillus ustus* using [$^{13}C_2$]-acetate showed isotopic scrambling of the geminal methyl groups originating from C-2 of the indole ring precursors in paraherquamide A, brevianamide A, and austamide biosynthesis. The labeling patterns suggest that the methyl groups of dimethylallyl pyrophosphate become equivalent during the biosyntheses; a non-face-selective S_N' mechanism has been invoked to account for these observations.

Introduction

The indole alkaloids are a large, structurally and biologically diverse group of compounds.[1] Among the classes of indole alkaloids, the paraherquamides (**1**−**10**, Figure 1)[2] comprise a family of fungal metabolites that, together with sclerotamide (**11**),[3] marcfortine A (**12**),[4] asperparaline A (**14**, also named aspergillimide, and **15**, SB202327),[5] and the brevianamides (**16** and **17**),[6] have recently attracted much attention due to their anthelmintic, paralytic, and insecticidal activities.[2−6] Also fascinating in this family are the pathways which lead to the biosynthesis of these complex and structurally unique substances. These secondary metabolites are the consequence of mixed biogenetic origins, being derived from the oxidative polycyclization of amino acids and isoprene units. Especially interesting in this regard is the emerging body of evidence that supports the notion that the bicyclo [2.2.2] core structural motif common to **1**−**17** is formed by a biosynthetic intramolecular [4+2] cycloaddition of the isoprene-derived olefin across an azadiene moiety derived from a preformed, oxidized piperazinedione (**A** → **B** → **C**), as shown in Scheme 1.[7,8] Such pericyclic reactions are quite rare in nature, and in only a few cases has experimental evidence been obtained to support the intermediacy of a Diels−Alder-type of cycloaddition.[8,9] Our research efforts have thus focused on the biogenesis of these compounds, with particular emphasis on the key cycloaddition step.

Scheme 1



In the case of these metabolites, the intriguing possibility of a Diels−Alder cycloaddition necessitates the formation of a "reverse" prenylated intermediate in which dimethylallylpyrophosphate (DMAPP) suffers electrophilic attack not at C-1, the phosphorylated end, but at C-3, which bears the geminal methyl groups; this is formally an S_N2' process. The existence of this type of intermediate was first alluded to in 1971 when deoxybrevianamide E (**20**) was isolated from the austamide-producing (**21**) cultures of *Aspergillus ustus* by Steyn.[10] Further

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(1) Williams, R. M.; Stocking, E. M.; Sanz-Cervera, J. F. Biosynthesis of Prenylated Alkaloids Derived from Tryptophan. In *Topics in Current Chemistry*; Vol. 209, Biosynthesis-Terpenes and Alkaloids; Leeper, F., Vederas, J. C., Eds.; Springer-Verlag: New York, 2000; pp 97−173.

(2) (a) Yamazaki, M.; Okuyama, E.; Kobayashi, M.; Inoue, H. *Tetrahedron Lett.* **1981**, *22*, 135−136. (b) Ondeyka, J. G.; Goegelman, R. T.; Schaeffer, J. M.; Kelemen, L.; Zitano, L. *J. Antibiot.* **1990**, *43*, 1375−1376. (c) Liesch, J. M.; Wichmann, C. F. *J. Antibiot.* **1990**, *43*, 1380−1386. (d) Blanchflower, S. E.; Banks, R. M.; Everett, J. R.; Manger, B. R.; Reading, C. *J. Antibiot.* **1991**, *44*, 492−497. (e) Blanchflower, S. E.; Banks, R. M.; Everett, J. R.; Reading, C. *J. Antibiot.* **1993**, *46*, 1355−1363.

(3) Whyte, A. C.; Gloer, J. B. *J. Nat. Prod.* **1996**, *59*, 1093−1095.

(4) (a) Polonsky, J.; Merrien, M.-A.; Prange, T.; Pascard, C.; Moreau, S. *J. Chem. Soc., Chem. Commun.* **1980**, 601−602. (b) Prange, T.; Buillion, M.-A.; Vuilhorgne, M.; Pascard, C.; Polonsky, J. *Tetrahedron Lett.* **1980**, *22*, 1977−1980.

(5) (a) Hayashi, H.; Nishimoto, Y.; Nozaki, H. *Tetrahedron Lett.* **1997**, *38*, 5655−5658. (b) Banks, R. M.; Blanchflower, S. E.; Everett, J. R.; Manger, B. R.; Reading, C. *J. Antibiot.* **1997**, *50*, 840−846. (c) Hayashi, H.; Yukifumi, N.; Kohki, A.; Nozaki, H. *Biosci. Biotechnol. Biochem.* **2000**, *64*(*1*), 111−115.

(6) (a) Birch, A. J.; Wright, J. J. *J. Chem. Soc. D* **1969**, 644−645. (b) Birch, A. J.; Wright, J. J. *Tetrahedron* **1970**, *26*, 2329−2344. (c) Birch, A. J.; Russell, R. A. *Tetrahedron* **1972**, *28*, 2999−3008. (d) Bird, B. A.; Remaley, A. T.; Campbell, I. M. *Appl. Environ. Microbiol.* **1981**, *42*, 521−525. (e) Bird, B. A.; Campbell, I. M. *Appl. Environ. Microbiol.* **1982**, *43*, 345. (f) Robbers, J. E.; Straus, J. W. *Lloydia* **1975**, *38*, 355. (g) Paterson, R. R. M.; Hawksworth, D. L. *Trans. Br. Mycol. Soc.* **1985**, *85*, 95−100. (h) Wilson, B. J.; Yang, D. T. C.; Harris, T. M. *Appl. Microbiol.* **1973**, *26*, 633−635. (I) Coetzer, J. *Acta Crystallogr.* **1974**, *B30*, 2254−2256.

(7) Porter, A. E. A.; Sammes, P. G. *J. Chem. Soc., Chem. Commun.* **1970**, 1103.

(8) (a) Williams, R. M.; Kwast, E.; Coffman, H.; Glinka, T. *J. Am. Chem. Soc.* **1989**, *111*, 3064−3065. (b) Williams, R. M.; Glinka, T.; Kwast, E.; Coffman, H.; Stille, J. K. *J. Am. Chem. Soc.* **1990**, *112*, 808−821. (c) Domingo, L. R.; Sanz-Cervera, J. F.; Williams, R. M.; Picher, M. T.; Marco, J. A. *J. Org. Chem.* **1997**, *62*, 1662−1667. (d) Sanz-Cervera, J. F.; Glinka, T.; Williams, R. M. *J. Am. Chem. Soc.* **1993**, *115*, 347−348. (e) Sanz-Cervera, J. F.; Glinka, T.; Williams, R. M. *Tetrahedron* **1993**, *49*, 8471−8472. (f) Williams, R. M.; Sanz-Cervera, J. F.; Sancenon, F.; Marco, J. A.; Halligan, K. *J. Am. Chem. Soc.* **1997**, *119*, 1090−1091. (g) Williams, R. M.; Sanz-Cervera, J. F.; Sancenon, F.; Marco, J. A.; Halligan, K. *Bioorg. Med. Chem.* **1998**, *6*, 1233−1241.

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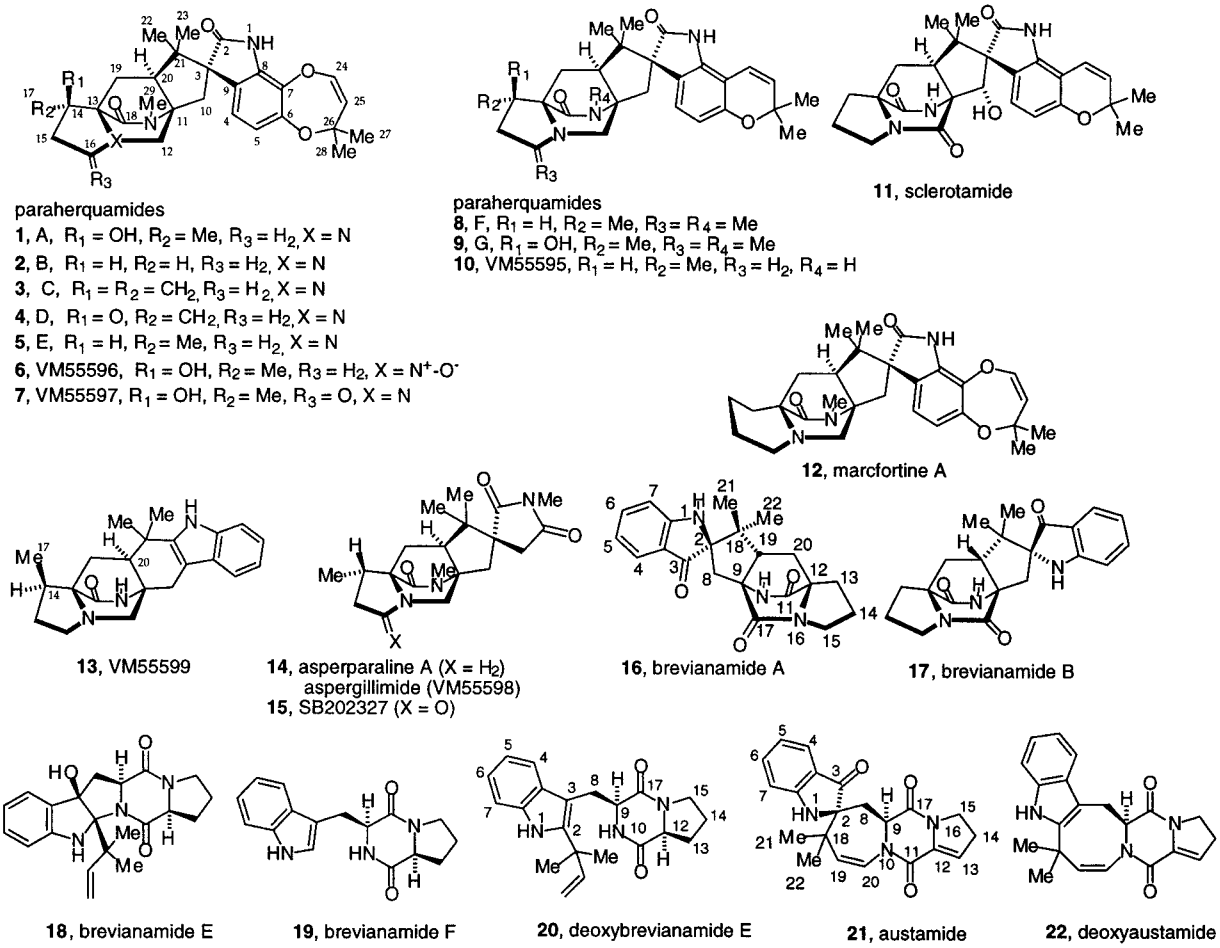


Figure 1.

evidence for a "reverse-prenylated" intermediate was reported by Williams and co-workers, who were able to experimentally demonstrate significant incorporation of synthetic tritiated deoxybrevianamide E (**20**) into brevianamide A (**16**), brevianamide B (**17**), and brevianamide E (**18**) in cultures of *Penicillium brevicompactum*.[8d,e]

It is interesting to note that deoxybrevianamide E, as a potential simple progenitor of the family of alkaloids displayed in Figure 1, is not the only "reverse" prenylated natural indole alkaloid to have been described in the literature. Several other natural substances such as roquefortine (**23**, Figure 2), isolated from *Penicillium roqueforti*, and some members of the echinulin family (**24**), isolated from *Aspergillus amstelodami*, as well as oxaline (**25**) and aszonalenin (**26**) and numerous other alkaloids have been described that also contain the "reverse" prenyl group.[11] The irregular structure of the "reverse" isoprene group in these and other metabolites has led to considerable speculation as to the mechanism of reverse prenylation in the biosynthesis of these substances.[1]

One possible mode of reverse prenylation that has been advanced for the biosynthesis of both roquefortine and the echinulins involves an aza-Claisen-type rearrangement from an *N*-prenylated indole (**27** → **28** via **29**), as shown in Scheme 2. This was first proposed by Barrow et al. in 1979 for the biosynthesis of roquefortine.[12] Thus, aza-Claisen rearrangement of **27** would yield the reverse prenylated 3-indolenine **29**, which could subsequently suffer 1,2-migration of the dimethylvinyl carbon substituent followed by loss of the C-2 hydrogen atom of the indole nucleus to yield **28**. Separate experimental observations to probe this mode of reverse prenylation have cast some doubt on this type of mechanism for both roquefortine and the echinulins.[1,13−15]

In the case of roquefortine, Bhat et al. showed retention of deuterium from C-2 of L-[2,4,5,6,7-$^2H_5$]tryptophan (**30**) at C-6 of roquefortine, thus questioning the intermediacy of **28** in the biosynthesis of this metabolite.[13]

It is of further significance that Gorst-Allman et al. observed partial scrambling (∼2:1 ratio) of the ^{13}C-label derived from

(9) Natural products implicated as being constructed by a biosynthetic Diels−Alder reaction: (a) Laschat, S. *Angew. Chem., Int. Ed. Engl.* **1996**, *35*, 289−291. (b) *Curr. Org. Chem.* **1998**, *2*, 365−394. (c) Oikawa, H.; Katayama, K.; Suzuki, Y.; Ichihara, A. *J. Chem. Soc., Chem. Commun.* **1995**, 1321−1322. (d) Cane, D. E.; Tan, W.; Ott, W. R. *J. Am. Chem. Soc.* **1993**, *115*, 527−535. (e) Bazan, A. C.; Edwards, J. M. *Tetrahedron* **1978**, *34*, 3005−3015. (f) Roush, W. R.; Peseckis, S. M.; Walts, A. E. *J. Org. Chem.* **1984**, *49*, 3429−3432. (g) Ito, S.; Hirata, Y. *Tetrahedron Lett.* **1972**, *25*, 2557−2560. (h) Bandaranayake, W. M.; Banfield, J. E. *J. Chem. Soc., Chem. Commun.* **1980**, 902−903. (i) Hofheinz, W.; Schonholzer, P. *Helv. Chim. Acta* **1977**, *60*, 1367−1370. (j) Hano, Y.; Nomura, T.; Ueda, S. *J. Chem. Soc., Chem. Commun.* **1990**, 610−613. (k) Witter, D. J.; Vederas, J. C. *J. Org. Chem.* **1996**, *61*, 2613−2623. (l) Kennedy, J.; Auclair, K.; Kendrew, S. G.; Park, C.; Vederas, J. C.; Hutchinson, C. R. *Science* **1999**, *284*, 1368−1372. (m) Binder, M.; Tamm, C. *Angew. Chem., Int. Ed.* **1973**, *12*, 370−380. (n) Baldwin, J. E.; Whitehead, R. C. *Tetrahedron Lett.* **1992**, *33*, 2059−2062. (o) Baldwin, J. E.; Claridge, T. D. W.; Culshaw, A. J.; Heupel, F. A.; Lee, V.; Spring, D. R.; Whitehead, R. C.; Boughtflower, R. J.; Mutton, I. M.; Upton, R. J. *Angew. Chem., Int. Ed. Engl.* **1998**, *37*, 2661−2663. (p) Shindo, K.; Sakakibara, M.; Kawai, H.; Seto, H. *J. Antibiot.* **1996**, *49*, 249−252. (q) Scott, A. I. *Acc. Chem. Res.* **1970**, *3*, 151−157. (r) Trfinov, L. S.; Dreiding, A. S.; Hoesch, L.; Rast, D. M. *Helv. Chim. Acta* **1981**, *64*, 1843−1846. (s) Nicolaou, K. C.; Simonsen, K. B.; Vassilikogiannakis, G.; Baran, P. S.; Vidali, V. P.; Pitsinos, E. N.; Couladouros, E. A. *Angew. Chem., Int. Ed. Engl.* **1999**, *38*, 3555−3559. (t) Lee, J. C.; Strobel, G. A.; Lobkovsky, E.; Clardy, J. *J. Org. Chem.* **1996**, *61*, 3232−3233. (u) Schreiber, S. L.; Kiessling, L. L. *J. Am. Chem. Soc.* **1988**, *110*, 631−633.

(10) Steyn, P. S. *Tetrahedron Lett.* **1971**, *36*, 3331−3334.

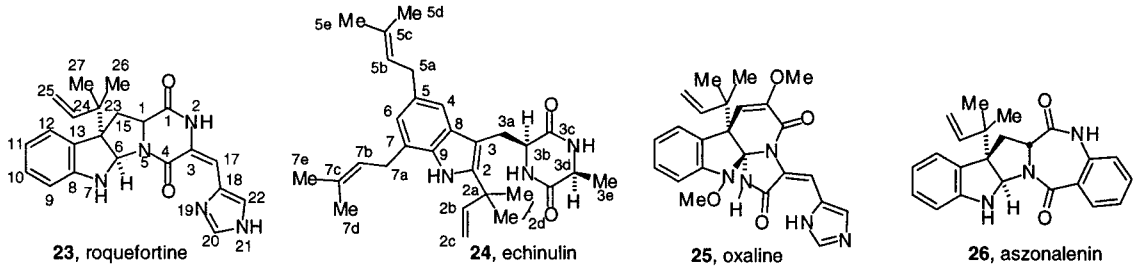


Figure 2.

[$^{13}C_2$]-acetate in the geminal carbons of roquefortine. To the best of our knowledge, this was the first reported experimental data that revealed a nonface-selective reverse prenylation. The same group also inferred the aza-Claisen pathway as a possible mechanism to accommodate these observations.[15]

In the instance of the echinulins, Grundon et al. concluded that the aza-Claisen type mechanism may not be operative in this system due to the lack of incorporation of the tritiated *N*-prenyl precursors 1-([1-^{3}H]-3,3-dimethylallyl)-L-tryptophan and *cyclo*-L-alanyl-1-([1-^{3}H]-3,3-dimethylallyl)-L-tryptophan (corresponding to **27**).[14]

Another possibility for the attachment of the "reverse" prenyl group, and perhaps the simplest explanation, is a direct formal S_N2' mechanism. This was first proposed by Bhat et al. for attachment of the dimethylallyl moiety at C-3 of the indole in roquefortine;[13] the direct S_N' mechanism was also postulated by our group with regard to the biosynthesis of the reverse prenyl unit in paraherquamide A (**1**).[16]

During the course of our investigations into the biosynthesis of paraherquamide A (**1**), we initially set out to determine if the isoprene units of paraherquamide were derived via the well-known mevalonic acid pathway,[17] or via the more recently discovered deoxyxylulose pathway.[18] Through feeding experiments with U-^{13}C-glucose and [$^{13}C_2$]-acetate we found that the isoprene units of paraherquamide A (**1**) arise from the classical mevalonic acid pathway, but we also found an unexpected stereochemical distribution of the isotopically enriched geminal methyl groups derived from DMAPP.[16] Through an analysis of the coupling of intact C_2 units by ^{13}C NMR spectroscopy, we determined that the stereochemical integrity of DMAPP is maintained in the assembly of the dioxepin moiety. On the other hand, we observed a scrambling of the ^{13}C labels for the geminal methyl groups at C-22 and C-23, which indicates a loss of stereochemical integrity during the biosynthetic construction of this quaternary center. This unanticipated result indicates that the reverse prenylation of the indole ring occurs via a nonface-selective mechanism. We have subsequently explored the stereochemical integrity of the reverse prenyl groups in the related yet simpler natural products, brevianamide A (**16**) and austamide (**21**), and report these results here along with full details of the previously communicated paraherquamide A (**1**) studies.[16]

Results and Discussion

The biosynthesis of both austamide (**21**), a metabolite of *Aspergillus ustus*, and brevianamide A (**16**), a metabolite of *Penicillium brevicompactum* and several related *Penicillium* sp., is thought to proceed through the intermediacy of deoxybrevianamide E (**20**), as shown in Scheme 3.[1,8,10] Thus, "reverse" prenylation of *cyclo*-L-Trp-L-Pro (**19**, brevianamide F) produces deoxybrevianamide E, which is converted by distinct modes of oxidative cyclization into brevianamides A and B in *Penicillium* sp., or into austamide (**21**) in *Aspergillus ustus*. The intermediacy of deoxybrevianamide E in the biosynthesis of brevianamides A and B has been confirmed through incorporation of tritium-

(11) **Roqufortine:** (a) Scott, P. M.: Merrien, M.; Polonsky, J. *Experientia* **1976**, *32*, 140−142. **Echinulin:** (b) Quilico, A.; Panizzi, L. *Chem. Ber.* **1943**, *76*, 348−358. (c) Quilico, A. *Res. Prog. Org. Biol. Med. Chem.* **1964**, *1*, 1964. **Flustramines:** (d) Carle, J. S.; Christophersen, C. *J. Am. Chem. Soc.* **1979**, *101*, 4012−4013. (e) Carle, J. S.; Christophersen, C. *J. Org. Chem* **1980**, *45*, 1586−1589. (f) Wulff, P.; Carle, J. S.; Christophersen, C. *J. Chem. Soc., Perkin Trans. 1* **1981**, 2895−2898. (g) Carle, J. S.; Christophersen, C. *J. Org. Chem.* **1981**, *46*, 3440−3443. (h) Wulff, P.; Carle, J. S.; Christophersen, C. *Comp. Biochem. Physiol. B* **1982**, *71B*, 523−524. (i) Sjoeblom, T.; Lars, B.; Carsten, C. *Acta Pharm. Suec.* **1983**, *20*(*6*), 415−418. (j) Wright, J. L. C. *J. Nat. Prod.* **1984**, *47*, 893−895. (k) Christophersen, C. *Acta Chem. Scand., Ser. B* **1985**, *B39*, 517−529. (l) Laycock, M. V.; Wright, J. L. C.; Findlay, J. A.; Patil, A. D. *Can. J. Chem.* **1986**, *64*, 1312−1316. **Ardeemins:** (m) Karnowski, J. P, Jackson, M.; Rasmussen, R. R.; Humphrey, P. E.; Poddig, J. B.; Kohl, W. L.; Scherr, M. H.; Kadam, S.; McAlpine, J. B. *J. Antibiot.* **1993**, *116*, 11143−11144. (n) Chou, T.-C.; Depew, K. M.; Zheng, Y.-H.; Safer, M. L.; Chan, D.; Helfrich, B.; Zatorska, D.; Zatorski, A.; Bornmann, W.; Danishefsky, S. *J. Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 8369−8374. **Amauromine:** (o) Takase, S.; Kawai, T.; Uchida, I.; Tanaka, H.; Aoki, H. *Tetrahedron* **1985**, *41*, 3307−3048. (p) Takase, S.; Kawai, T.; Uchida, I.; Tanaka, H.; Aoki, H. *Tetrahedron Lett.* **1984**, *25*, 4673−4676. (q) Takase, S.; Kawai, T.; Uchida, I.; Tanaka, H.; Aoki, H. *J. Antibiot.* **1984**, *37*, 1320−1323. (r) Takase, S.; Kawai, T.; Uchida, I.; Tanaka, H.; Aoki, H. *Tennen Yuki Kagobutsu Toronkai Koen Yoshishu* **1983**, *26th*, 94−101. **Verrucofortine:** (s) Hodge, R. P.; Harris, C. M.; Harris T. M. *J. Nat. Prod.* **1988**, *51*, 66−73. **Gypsetin:** (t) Shinohara, C.; Hasumi, K.; Takei, Y.; Endo, A. *J. Antibiot.* **1994**, *47*, 163−167. (u) Nuber, B.; Hansske, F. *J. Antibiot.* **1994**, *47*, 168−172. **Okaramines:** (v) Hayashi, H.; Takiuchi, K.; Murao, S.; Arai, M. *Agric. Biol. Chem.* **1989**, *53*, 461−469. (w) Hayashi, H.; Fujiwara, S.; Murao, S.; Arai, M. *Agric. Biol. Chem.* **1991**, *55*, 3143−3145. (x) Hayashi, H.; Asabu, Y.; Murao, S.; Arai, M. *Biosci. Biotechnol. Biochem.* **1995**, *59*, 246−250. (y) Hayashi, H.; Sakaguchi, A. *Biosci. Biotechnol. Biochem.* **1998**, *62*, 804−806. (z) Hayashi, H.; Furutsuka, K.; Shiono, Y. *J. Nat. Prod.* **1999**, *62*, 315−317. (aa) Shiono, Y.; Akiyama, K.; Hayashi, H. *Biosci. Biotechnol. Biochem.* **1999**, *63*, 1910−1920. (bb) Shiono, Y.; Akiyama, K.; Hayashi, H. *Biosci. Biotechnol. Biochem.* **2000**, *64*, 103−110. **Oxaline:** (cc) Nagel, D. W.; Pachler, K. G. R.; Steyn, P. S.; Wessels, P. L.; Gafner, G.; Kruger, G. J. *J. Chem. Soc., Chem. Commun.* **1974**, 1021−1022. (dd) Nagel, D. W.; Pachler, K. G. R.; Steyn, P. S.; Vleggaar, R.; Wessels, P. L. *Tetrahedron* **1976**, *32*, 2625−2631. **Aszonalenin:** (ee) Kimura, Y.; Hamasaki, T.; Nakajima, H.; Isogai, A. *Tetrahedron Lett.* **1982**, *23*, 225−228. (ff) Bhat, B.; Harrison, D. M.; Lamont, H. M. *Tetrahedron* **1993**, *49*, 10655−10662.

(12) Barrow, K. D.; Colley, P. W.; Tribe, D. E. *J. Chem. Soc., Chem. Commun.* **1979**, 225−226.

(13) (a) Bhat, B.; Harrison, D. M.; Lamont, H. M. *J. Chem. Soc., Chem. Commun*. **1990**, 1518−1519. (b) Bhat, B.; Harrison, D. M.; Lamont, H. M. *Tetrahedron* **1993**, *49*, 10663−10668.

(14) Grundon, M. F.; Hamblin, M. R.; Harrison, D. M.; Derry Logue, J. N.; McGuire, M.; McGrath, J. A. *J. Chem. Soc., Perkin Trans. 1* **1980**, 1294−1298.

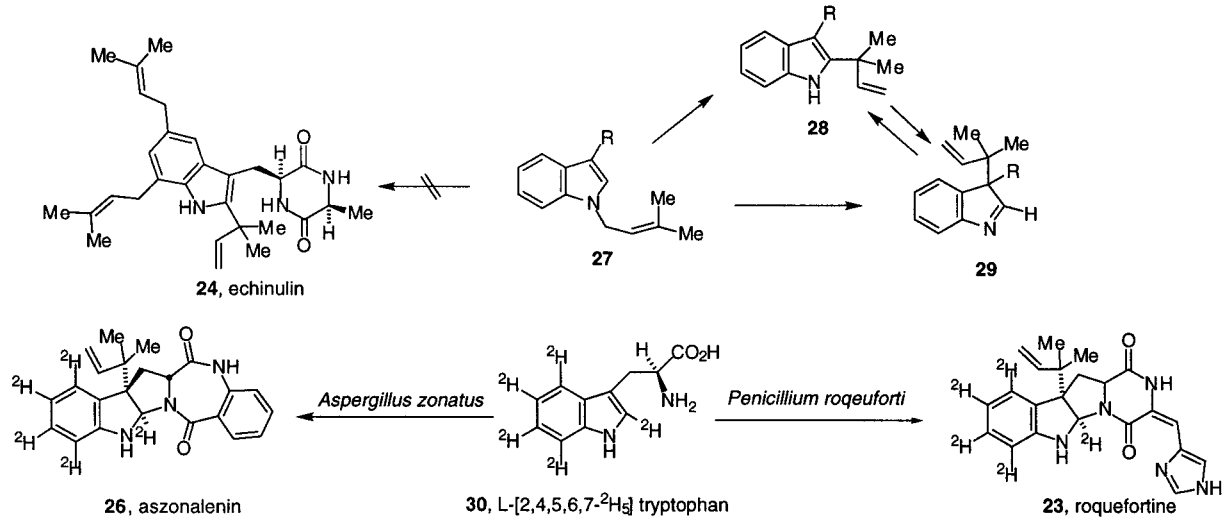
(15) Gorst-Allman, C. P.; Steyn, P. S.; Vleggaar, R. *J. Chem. Soc., Chem. Commun.* **1982**, 652−653.

(16) Stocking, E. M.; Sanz-Cervera, J. F.; Williams R. M. *Angew. Chem., Int. Ed.* **1999**, *38*, 786−789.

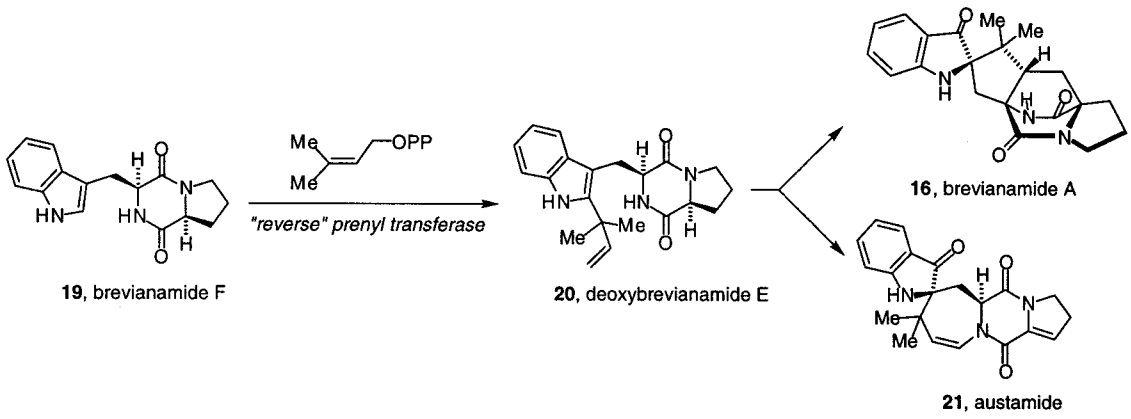
(17) (a) Cane, D. E. *Tetrahedron* **1980**, *36*, 1109−1159. (b) Poulter, C. D.; Rilling, H. C. In *Biosynthesis of Isoprenoid Compounds*; Porter, J. W., Spurgeon, S. L., Eds.: Wiley-Interscience, New York, 1981; p 161. (c) Herbert, R. B. In *The Biosynthesis of Secondary Metabolites*; Chapman and Hall: London, 1981. (d) Mann, J. In *Secondary Metabolism*; Oxford University Press: Oxford, 1987.

(18) Eisenrich, W.; Schwarz, M.; Cartayrade, A.; Arigoni, D.; Zenk, M. H.; Bacher, A. *Chem. Biol.* **1998**, *5*, R221−R233.

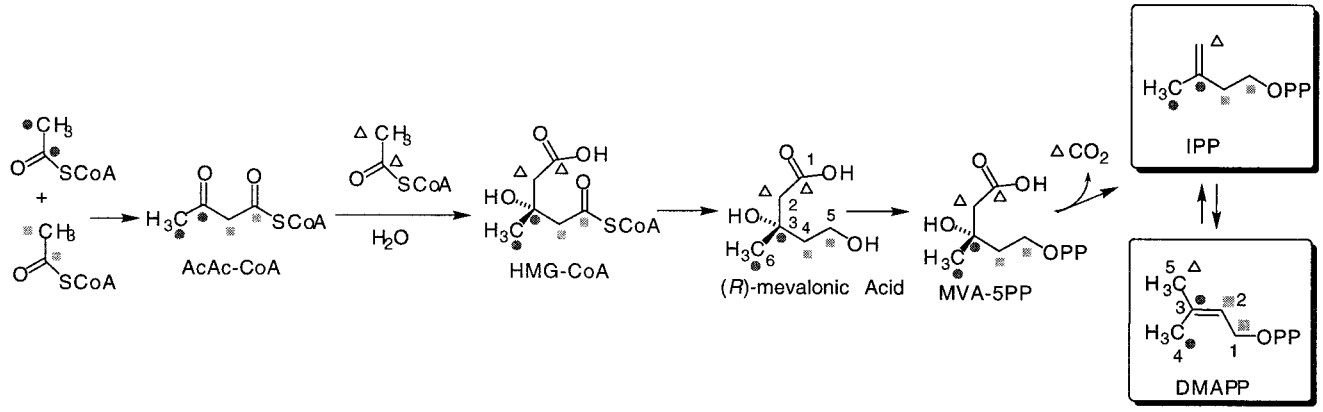
Scheme 2



Scheme 3



Scheme 4. The Classical Mevalonic Acid Pathway Showing the Labeling Pattern via 1,2-Doubly Labeled Acetate

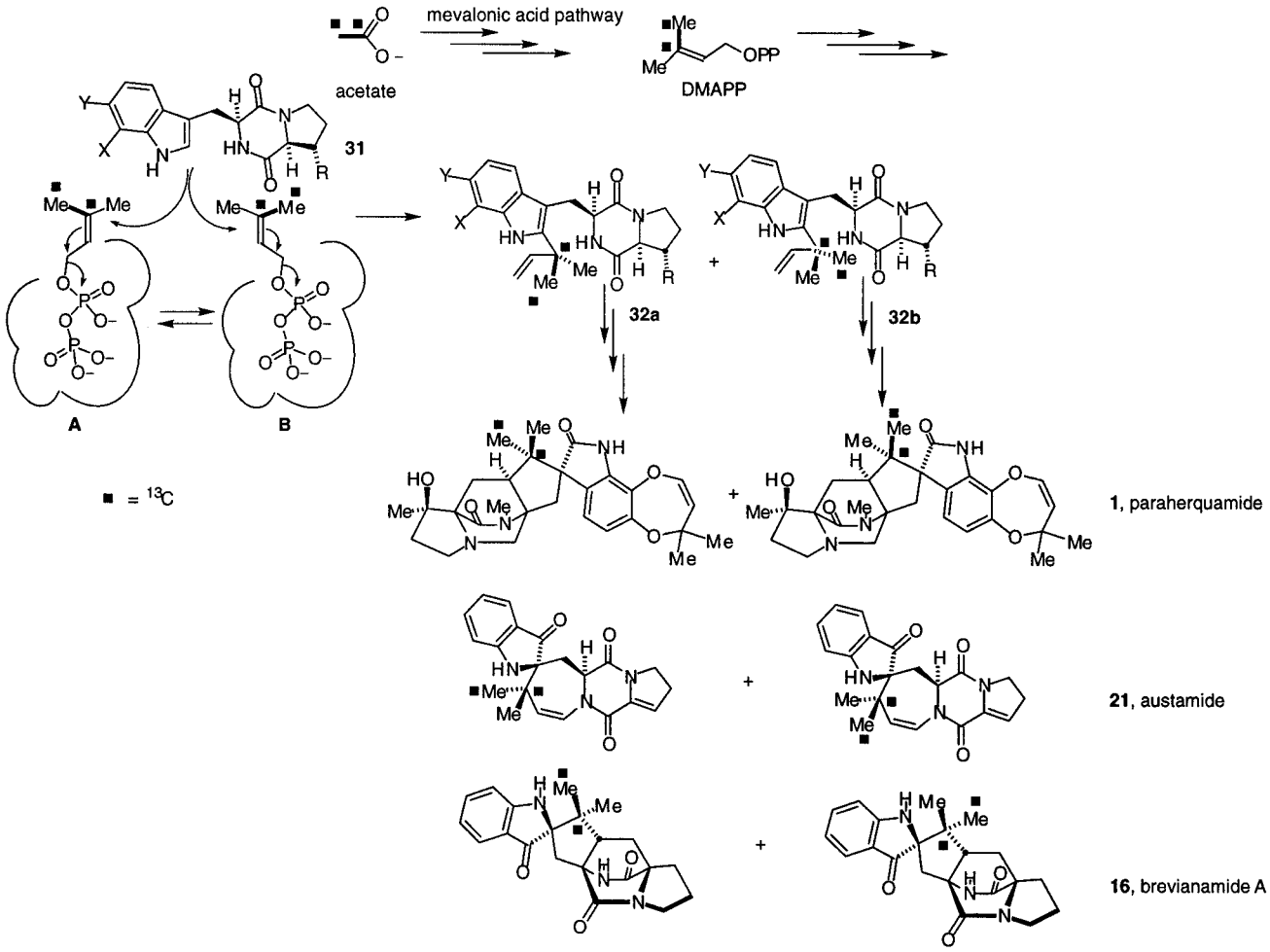


labeled deoxybrevianamide E in cultures of *Penicillium brevicompactum* as reported from this laboratory.[8d,e] In the case of austamide (**21**), deoxybrevianamide E is a co-metabolite that has been isolated along with deoxyaustamide (**22**) from cultures of *Aspergillus ustus*. The involvement of this substance in this pathway is presently based only on this circumstantial evidence.[10]

DMAPP arising from the mevalonic acid pathway is derived from three C-2 units followed by loss of a C-1 unit, as shown in Scheme 4. Thus, incorporation of [$^{13}C_2$]-acetate into isoprenylated metabolites should exhibit coupling between C-1 and C-2 and between C-3 and C-4 of DMAPP; however, no coupling should be observed between C-3 and C-5 since it has been demonstrated that the *E*-methyl group of DMAPP is derived from C-2 of mevalonic acid (i.e., the methyl group of mevalonic acid becomes C-4 of DMAPP; see Scheme 5).[17]

Paraherquamide A (**1**) contains two isoprene units, one comprising C-24 to C-28 in the dioxepin ring, and one comprising C-19 to C-23, which constitutes the bicyclo[2.2.2] ring system. Since [$^{13}C_2$]-acetate fed to *Penicillium fellutanum* was incorporated into both of these isoprene units as intact C-2 units, this unambiguously confirms that the paraherquamide A-producing fungi constructs the primary isoprene units via the classical mevalonic acid pathway (Tables 1 and 2). With respect

Scheme 5. A Possible Biosynthetic Sequence that May Explain How the Geminal Methyl Groups Are Rendered Equivalent in the Biosynthesis of Paraherquamide A (**1**), Austamide (**21**), and Brevianamide A (**16**)[a]



[a] The two black squares represent one intact C_2 unit from acetate, incorporated in C-3/C-5 of individual DMAPP molecules, and in C-21, C-22, and C-23 of **1** and C-18, C-21, and C-22 for both **16** and **21**. For the sake of clarity and simplicity, the labels that would appear in other positions are not represented.

Table 1. Specific Incorporations, Chemical Shifts, and Coupling Constants for the C_5 Carbon Atoms of Paraherquamide A (**1**) in the Feeding Experiment with [$^{13}C_2$]-Acetate

paraherquamide C no.	δ	J_{C-C} (Hz)	% ^{13}C at each C[a]	% ^{13}C specifically incorporated at each position from intact C_2 units
19	22.2	35	4.6	32
20	51.4	34	4.2	32
21	46.4	36	4.4	42
22	20.5	36	4.4	31
23	23.7	36	3.3	41
24	138.9	79	5.1	37
25	115.1	79	3.2	34
26	79.8	40	3.6	31
27	29.9	40	2.9	39
28	29.8		3.9	0

[a] % ^{13}C inclusive of natural abundance ^{13}C.

Table 2. Specific Incorporations, Chemical Shifts, and Coupling Constants for the C_5 Carbon Atoms of Paraherquamide A (**1**) in the Feeding Experiment with [U-$^{13}C_6$]-Glucose[19]

C no.	chemical shift (ppm)	$J_{^{13}C-^{13}C}$ (Hz)	% ^{13}C in each position[a]	% ^{13}C specifically incorporated at each position from intact C_2 units
19	22.2	34	2.1	41
20	51.4	34	3.1	32
21	46.4	36	2.6	36
22	20.5	36	3.2	14
23	23.7	36	2.9	21
24	138.9	81	4.0	35
25	115.1	79	3.5	37
26	79.8	40	3.5	37
27	29.9	40	2.1	40
28	29.8		2.8	0

[a] Percent ^{13}C is inclusive of natural abundance ^{13}C.

to the carbons that form the two C_5 units, C-19 to C-23, and C-24 to C-28, the results of the feeding experiment with [$^{13}C_2$]-acetate were essentially the same as those with [U-^{13}C]-glucose.[16] In both feeding experiments, the signal for C-28 at δ 29.80 showed enhancement with respect to the control spectrum, but no splitting. In the first C_5 fragment (C-19−C-23), the observed couplings mean that C19 is coupled to C-20, while C-21 is coupled to C-22 and C-23, but not to both simultaneously. For the second C_5 unit (C-24−C-28), the coupling constants show that C-24 and C-25 are coupled, while C-26 is coupled to C-27. In this case, C-28 shows no coupling.

It is significant that in the C_5 fragment comprised of carbons C-24 to C-28, carbon C-26 is coupled to C-27, but C-28 shows no coupling (Figure 3). This clearly establishes that the methyl groups in DMAPP are not equivalent in the biosynthesis of the dioxepin ring of this metabolite. In contrast, in the other C_5 fragment, comprised of carbons C-19 to C-23, both methyl groups show coupling with C-21, although not simultaneously.

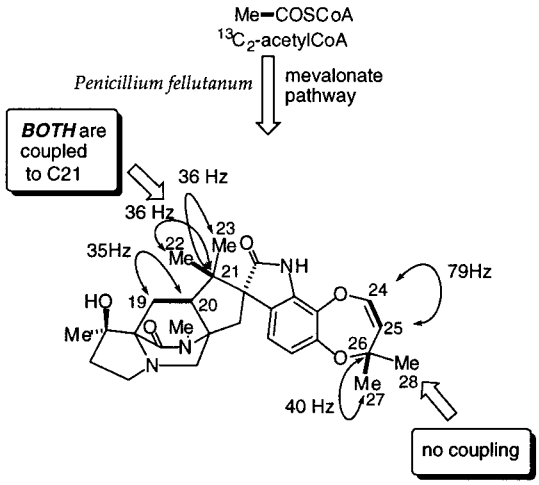


Figure 3.

Table 3. Specific Incorporations, Chemical Shifts, and Coupling Constants for the C_5 Carbon Atoms of Austamide (**21**) in the Feeding Experiment with [$^{13}C_2$]-Acetate

austamide C no.	δ	J_{C-C} (Hz)	% ^{13}C at each C[a]	%^{13}C specifically incorporated at each position from intact C_2 units
18	42.1	35	4.9	42
19	128.7	76	5.0	39
20	125.5	76	5.5	34
21	26.1	35	5.5	24
22	23.5	35	5.3	32

[a] % ^{13}C inclusive of natural abundance ^{13}C.

Table 4. Specific Incorporations, Chemical Shifts, and Coupling Constants for the C_5 Carbons of Brevianamide A (**16**) in the Feeding Experiment with [$^{13}C_2$]-Acetate

brevianamide A (**16**) C no.	δ	J_{C-C} (Hz)	% ^{13}C at each C[a]	% ^{13}C specifically incorporated at each position from intact C_2 units
18	48.9	37	3.9	30
19	55.9	37	3.5	28
20	29.2	37	4.1	29
21	19.8	37	4.0	13
22	23.9	37	3.9	22

[a] Percent of ^{13}C is inclusive of natural abundance ^{13}C.

These data clearly demonstrate that the stereochemical integrity of DMAPP is maintained in the formation of the quaternary C−O bond of the dioxepin ring, whereas the stereochemical integrity of the DMAPP that forms the bicyclo[2.2.2] nucleus is sacrificed at some stage in the biosynthesis.

To further examine the mode of attachment of reverse prenyl groups in the simpler monoprenylated metabolites brevianamide A (**16**) and austamide (**21**), feeding experiments were performed with [$^{13}C_2$]-acetate in cultures of *Penicillium brevicompactum* (ATCC: 9056), which produces brevianamide A (**16**), and with *Aspergillus ustus* (ATCC: 36063), which produces austamide (**21**). In brevianamide A (**16**), the reverse isoprene unit, analogous to C-19−C-23 in paraherquamide A (**1**), undergoes a net oxidative cyclization across the two amino acid α-carbons culminating in the formation of the bicyclo[2.2.2] ring system.[8] In austamide (**21**), the reverse isoprene unit suffers an alternative mode of oxidative cyclization to the tryptophyl amide nitrogen atom and thus becomes the unsaturated seven-membered ring.

Incorporation of intact C-2 units was observed for both austamide (**21**) and brevianamide A (**16**), as shown in Tables 3 and 4, respectively. Thus, as with paraherquamide A (**1**), the isoprene units are derived via the mevalonic acid pathway due to the significant levels of incorporation of the labeled acetate units into the isoprene moieties. Significantly, the two geminal methyl groups derived from the reverse isoprene units in both austamide (**21**) and brevianamide A (**16**) exhibit coupling to the quaternary carbon to which they are bonded (Figure 4).

In austamide (**21**), as expected, C-19 shows coupling to C-20. C-21 and C-22 both show coupling to C-18 with coupling constants of ∼35 Hz (Table 3, Figure 4). The coupling between both methyl groups, C-21 and C-22, and the adjacent quaternary carbon, C-18, can be clearly seen in the INADEQUATE spectrum shown in Figure 5.

In an INADEQUATE experiment, cross-peaks represent connectivity between adjacent carbons.[20] This type of experiment is not used frequently because of the extremely low sensitivity inherent to this technique. In this instance, however, since the austamide (**21**) sample has been enriched with $^{13}C_2$ units, the INADEQUATE is an excellent way to show which carbons arise from intact C_2 units. Besides the coupling observed between C-18 to C-21 and C-22, C-14 and C-15 give strongly coupled signals in the INADEQUATE spectrum, as shown in Figure 5. The high level of incorporation of [$^{13}C_2$]-acetate in the proline ring can be traced to the fact that L-proline is derived from L-glutamate, which in turn arises from α-ketoglutarate, a product of the condensation of acetyl-CoA in the citric acid cycle.[21]

As seen visually by the intensity of coupled ^{13}C-signals in the ^{13}C-spectrum (Figure 5) and from the calculated values shown in Table 3, the percentage of ^{13}C incorporated at C-21 and C-22 as C_2 units is approximately the same. There is thus essentially no facial bias in the mode of reverse prenylation in the biosynthesis of austamide (**21**).

An initial feeding experiment with [$^{13}C_2$]-acetate in *Penicillium brevicompactum* gave very high levels of specific incorporation of [$^{13}C_2$]-acetate into brevianamide A (**16**) with concomitant couplings generated between different intact C_2 units in the carbons arising from DMAPP in the same molecule. As a result, C-18 and C-19 showed coupling not only to the carbons that come from intact C_2 units but also to contiguous labeled carbons that most likely do not arise from intact $^{13}C_2$ units. From the splitting pattern seen in the ^{13}C spectrum, it was apparent that C-19 was coupled to C-20 (d, J = 37 Hz), but that C-19 was also coupled to C-20 and C-18 concurrently (dd, J = 37, 37 Hz). The resonance for C-18 also showed multiple couplings.

To avoid the complications that these simultaneous incorporations of two or more C_2 units introduced in the ^{13}C spectrum of the resulting brevianamide A (**16**), a second feeding experiment was performed in which 200 mg of [$^{13}C_2$]-acetate were used together with 1000 mg of unlabeled acetate. This lowered the probability of simultaneous incorporation of two labeled acetate units in the same molecule of brevianamide A (**16**), thereby simplifying the ^{13}C spectrum of this metabolite resulting from a feeding experiment. In effect, the resulting brevianamide A (**16**) showed a much simpler ^{13}C spectrum (Figure 6), with lower incorporation of labeled acetate (Table 4), and only doublets for the carbon couplings. As expected, C-18 (d, J = 37 Hz) exhibits couplings to C-21 (d, J = 37 Hz) and C-22 (d,

(19) It should be noted that the values published in ref 17 for the glucose feeding experiments were calculated incorrectly and the corrected values now appear in Table 2.

(20) (a) Bax, A.; Freeman, R.; Kempsell, S. P. *J. Am. Chem. Soc.* **1980**, *102*, 4849−4851. (b) Lambert, J. B.; Shurvell, H. F.; Lightner, D. A.; Cooks, G. R. *Organic Structural Spectroscopy*; Prentice Hall: Englewood Cliffs, NJ, 1998; pp 138−139.

(21) Stryer, L. *Biochemistry*; W. H. Freeman and Company: New York, 1988; p 579.

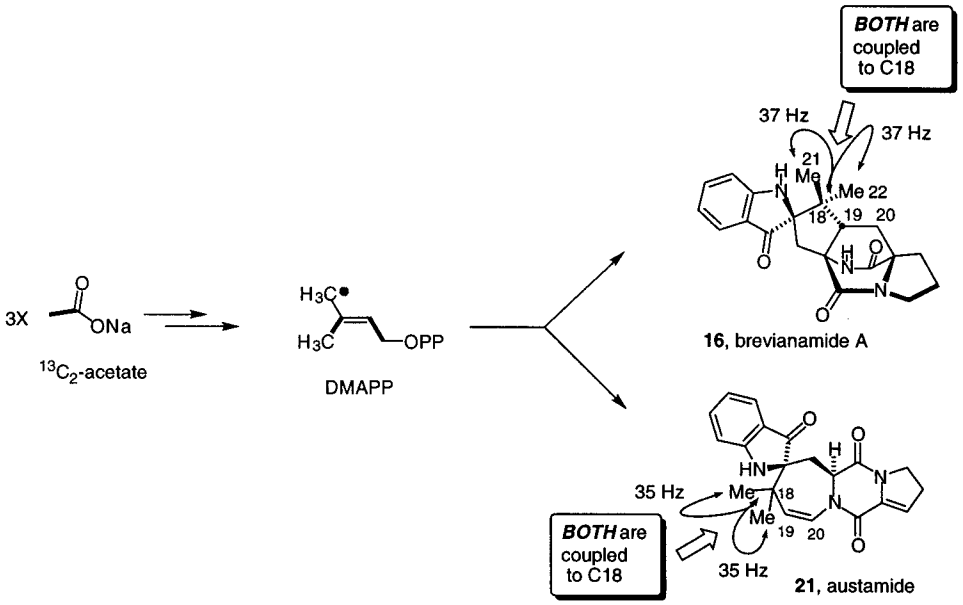


Figure 4.

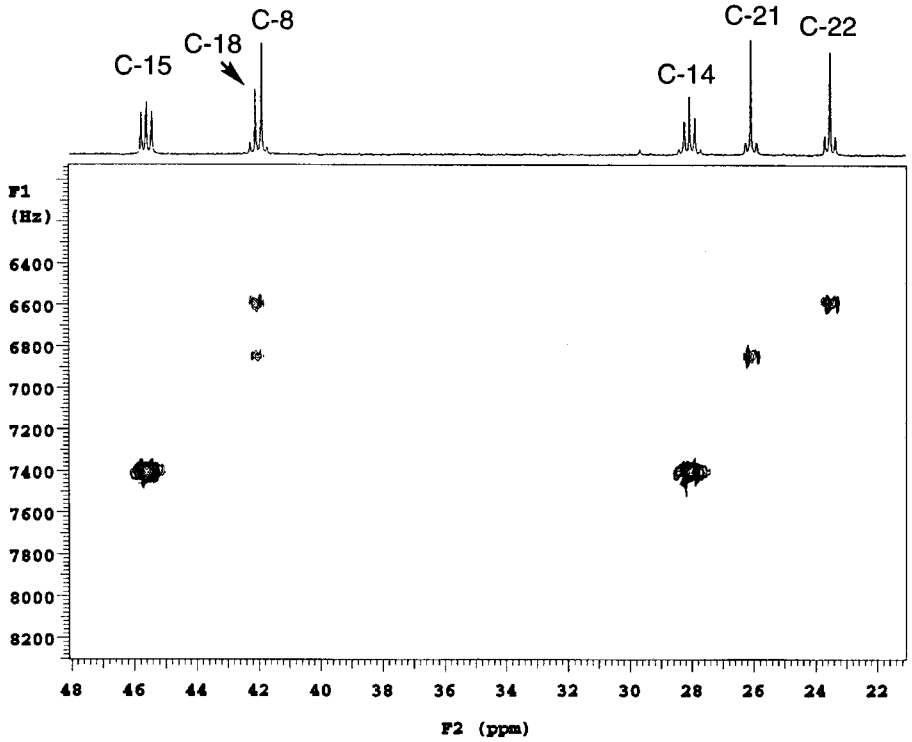


Figure 5. Portion of the INADEQUATE spectrum of austamide (**21**) derived from a [$^{13}C_2$]-acetate feeding experiment exhibiting the coupling between the geminal methyl groups and C-18. The corresponding portion of the ^{13}C-spectrum is shown at the top.

$J = 37$ Hz) (Figure 6), while C-19 (d, 37 Hz) is coupled only to C-20 (d, $J = 37$ Hz).

Another noteworthy feature of the ^{13}C NMR spectrum of brevianamide A (**16**) is that C-22, which we have assigned by ^{1}H NMR nOe experiments,[22] shows a higher percentage of specifically incorporated ^{13}C from intact C_2 units (22%) than C-21 (13%). Within experimental error, those values agree with the measured value for C-18 (30% of ^{13}C atoms specifically incorporated from intact C_2 units; see Table 4): C-18 is coupled to either C-21 or C-22, but not to both of them simultaneously. This indicates that, although there is loss of stereochemical integrity of the methyl groups derived from DMAPP in the biosynthesis of brevianamide A (**16**), there is *some degree* of stereofacial bias in the attachment of the reverse prenyl group to *cyclo*-L-Trp-L-Pro (brevianamide F) in the biosynthetic formation of deoxybrevianamide E, the key reverse-prenylated precursor.

The unusual lack of stereospecificity observed in the construction of the quaternary center at the indole 2-position in all of these metabolites can be interpreted to mean that the isoprene unit destined for C-2 of the indole ring in all of these metabolites is attached via a "reverse" prenyl transferase, which presents the olefinic π-system of DMAPP so that both faces of the π-system are susceptible to attack by the 2-position of the indole (Scheme 5).

(22) We have determined through ^{1}H NMR spectroscopy that both H-21 and H-19 in brevianamide A (**16**) show mutual nOe effects. This result unequivocally establishes the stereochemistry shown.

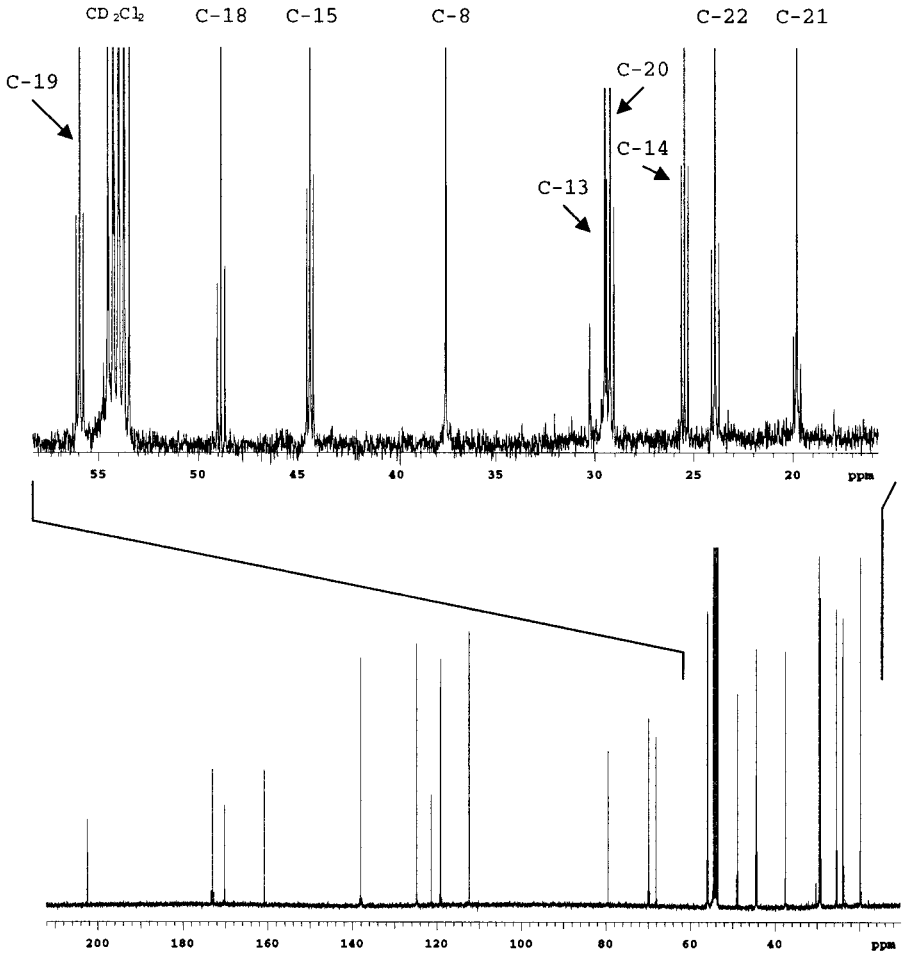


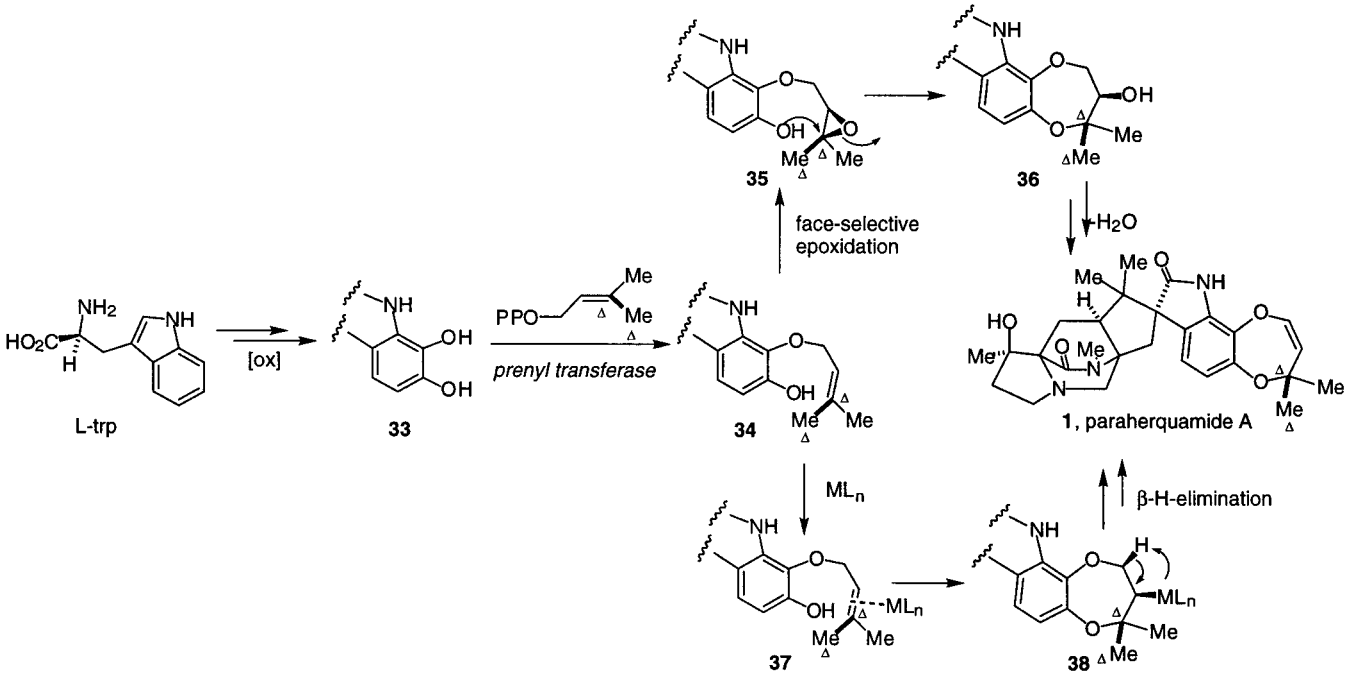
Figure 6. 100 MHz ^{13}C NMR spectrum of brevianamide A (**16**) in CD_2Cl_2 from a feeding experiment with [$^{13}C_2$]-acetate diluted with unlabeled acetate in a 1:5 proportion.

If the hydrophilic diphosphate portion of DMAPP is buried in an enzyme active site, "upside down" in relation to "normal" prenyl transferases, the hydrophobic isoprenyl moiety would be susceptible to a facially indiscriminate S_N' attack as shown in Scheme 5. In this instance, the isoprene group would be presented to the hypothetical generic indole substrate **31** in a conformationally flexible (**A** ⇌ **B**) disposition with respect to the tryptophan-derived substrate. This would result in loss of stereochemical integrity of the geminal methyl groups in the prenylated products **32a** and **32b**. The subsequent oxidative ring-closure reactions to each family of structural types would not be expected to affect the stereochemical integrity of the relevant quaternary centers once they have been set in the key C−C bond-forming reverse prenylation reactions.

In contrast, the methyl groups in the other C_5 unit constituting the quaternary carbon of the dioxepin moiety for paraherquamide A (**1**) are clearly differentiated. Thus, it is quite likely that this C_5 group (carbons 24 to 28) is introduced in the molecule via direct alkylation with DMAPP by a normal prenyl transferase (to give **34**, Scheme 6), followed by a net stereospecific oxidative addition to the olefinic π-system. Of several possibilities, a plausible mechanism for the formation of this ring system is via face-selective epoxidation of the olefin (to **35**, Scheme 6) followed by *a completely stereospecific ring-opening of the epoxide* and dehydration (**35** → **36** → **1**). Alternatively, face-selective complexation of a transition metallo-protein to the olefinic π-system (**37**) followed by stereospecific intramolecular nucleophilic addition (to give **38**) and reductive elimination to the enol-ether would yield the dioxepin moiety of paraherquamide A (**1**). We are aware of no biosynthetic precedent for the latter possibility and the former (via **35**), therefore, appears to be the most likely. Whatever the mechanism for the construction of this interesting ring system, the C−O bond-forming reaction in the construction of the dioxepin is fashioned without loss of stereochemical integrity.

Another possible explanation for the observed retention and loss of stereochemical integrity of the respective geminal methyl groups in paraherquamide A (**1**) is that the methyl groups of the DMAPP in C-19−C-23 are scrambled via a dimethyl vinyl carbinol-type intermediate derived from DMAPP. However, this would necessarily provide stereochemically scrambled isotopomers of DMAPP to the cells' cytosolic pool and scrambling would also be expected in the isoprene unit constituting C-24−C-28 unless there are two pools of *completely compartmentalized* DMAPP in the biosynthesis of paraherquamide A (**1**). In one of these pools the stereochemical integrity of DMAPP would be sacrificed through a dimethyl vinyl carbinol-type of intermediate that is then used *exclusively* for the assembly of the C-19−C-23 unit. In the other pool, DMAPP would retain the normal stereochemical integrity of the mevalonate pathway and is then used *exclusively* for fashioning the dioxepin moiety C-24−C-28. This possibility seems to be highly unlikely. Paraherquamide A (**1**) is therefore unique in that the mode of construction of each quaternary center derived from isoprene building blocks is distinct: one center is formed in a completely stereospecific manner and the other is formed in an entirely nonstereospecific manner.

Scheme 6



Conclusion

Isotopic enrichment experiments with [$^{13}C_2$]-acetate have shown that the isoprene units in the secondary metabolites paraherquamide A (**1**), brevianamide A (**16**), and austamide (**21**) all arise via the classical mevalonate pathway. In all three systems, we have observed a loss of stereochemical integrity at the isoprene-derived quaternary center attached to the 2-position of the indole ring. In the biosynthesis of paraherquamide A (**1**), the geminal methyl groups from the isoprene unit which antecedes the bicyclo[2.2.2] ring system shows approximately equal incorporation of ^{13}C from intact C-2 units, which indicates that these methyl groups become essentially equivalent at some point in the biosynthesis. The isoprene unit that constitutes the dioxepin ring displays retention of stereochemical integrity and infers a completely net face-selective biosynthetic addition reaction to the olefinic π-system derived from DMAPP via the tryptophyl ring hydroxyl group. In the biosynthesis of austamide (**21**), there are also approximately equal levels of specific ^{13}C enrichment from intact C_2 units at the isoprene derived geminal methyl groups. Brevianamide A (**16**), on the other hand, exhibits significant but incomplete loss of stereochemical integrity in the construction of the reverse prenyl unit. In this regard, it is most interesting to note that *both* brevianamide A (**16**) and austamide (**21**) are apparently fashioned from the *same* reverse-prenylated precursor, namely, deoxybrevianamide E (**20**). While *Penicillium* sp. and *Aspergillus* sp. are very similar genera of fungi genetically, it would appear that the reverse prenylases in each organism that construct deoxybrevianamide E display distinct levels of facial discrimination in the transfer of DMAPP to the 2-position of the indole. Studies to clarify these subtleties and to further elucidate the generality of the poor facial discrimination in reverse prenylations are in progress in these laboratories.

Experimental Section

Materials and Methods. [$^{13}C_2$]-Acetic acid, sodium salt, 99% atom % ^{13}C, and [U-$^{13}C_6$]-D-glucose 99% atom % ^{13}C were obtained from Aldrich Chemical Co. The ^{13}C spectra of labeled and unlabeled samples obtained for each of the fungal metabolites were obtained under comparable conditions (i.e. sample concentration, number of scans, etc.). ^{13}C NMR spectra of paraherquamide A (**1**) were obtained on a Bruker AMX 500 MHz NMR at Los Alamos National Laboratory and taken in $CDCl_3$ (^1H, 7.24 ppm; ^{13}C 77 ppm). The pulse sequence included a 4.0 s relaxation delay, an acquisition time of 0.79 s, and a spectral window of 41667 Hz. ^{13}C data were obtained with continuous WALTZ 16 composite pulse decoupling. The ^{13}C data were processed using 2 Hz exponential line broadening with a digital resolution of 2.54 Hz/pt. ^{13}C NMR spectra for both austamide (**21**), taken in $CDCl_3$ (^1H, 7.24 ppm; ^{13}C 77 ppm), and brevianamide A (**16**), taken in CD_2Cl_2 (^1H, 5.32 ppm; ^{13}C 54 ppm), were obtained on an INOVA 400 Varian NMR with a dual full band console at the Chemistry Central Instrument Facility at Colorado State University. The pulse sequence consisted of a 1.3 s relaxation delay, a pulse angle of 45°, an acquisition time of 0.64 s, and a spectral window of 25157 Hz. ^{13}C data were obtained with continuous WALTZ 16 composite pulse decoupling. The ^{13}C data were processed using a −0.634 Hz square sine bell correction and a −0.634 Hz sine bell shift with a digital resolution of 1.54 Hz/pt. The ^{13}C spectra were acquired until a satisfactory signal-to-noise ratio was obtained (ca. 16 h). The resulting ^{13}C spectra were thoroughly phased and the baseline carefully corrected to obtain a satisfactory, reproducible integral. The INADEQUATE spectrum for austamide was obtained on an INOVA 400 Varian NMR using an inadqt pulse sequence at the Chemistry Central Instrument Facility at Colorado State University. All mass spectra were obtained on a Fisons VG Quattro SQ at the Chemistry Central Instrument Facility at Colorado State University. Samples were dissolved in 1:1 water/acetonitrile without pH adjustment and measured with positive ion electrospray (20 scans, 8 s/scan) with a cone voltage of 25 V.

General Procedure for Isotopic Enrichment Experiments. Spores from the respective fungi suspended in a 15% aqueous glycerol solution were spread onto fungus specific sterile agar slants; 50 μL of the suspension was used per slant. The slants were placed in an incubator at 25 °C for 10−12 days. The spores from eight slants were shaken into four 6-L flasks containing 600 mL of sterile glucose corn steep liquor (40 g of glucose and 22 g of corn steep liquor per 1 L of distilled deionized water). The inoculated flasks were placed in an incubator at 25 °C for 6 days. The glucose corn steep liquor was removed leaving a disk of the fungus. The undersides of the disks, the mycelia cells, were rinsed with 100 mL of sterile water.

One hundred milliliters of sterile trace element solution (35 mM $NaNO_3$, 5.7 mM K_2HPO_4, 4.2 mM $MgSO_4$, 1.3 mM KCl, 36 μM $FeSO_4 \cdot 7H_2O$, 25 μM $MnSO_4 \cdot H_2O$, 7 μM $ZnSO_4 \cdot 7H_2O$, 1.5 μM $CuCl_2 \cdot$

$2H_2O$) containing 250 mg of [$^{13}C_2$]-sodium acetate (except for brevianamide A, where 200 mg of [$^{13}C_2$]-sodium acetate plus 1 g of unlabeled acetate were used) was placed into each of two flasks (four flasks in the case of brevianamide A) containing the fungus. Two control flasks were also set up, each containing 100 mL of sterile trace element solution. The flasks were put into the incubator at 25 °C for 10 days and swirled daily to ensure even distribution of the [$^{13}C_2$]-sodium acetate.

The aqueous media was decanted off and stored at 4 °C with 1−2 mL of chloroform. The mycelia cells from each flask were harvested, combined with the cells from the duplicate experiment, and pulverized with 500 mL of methanol in an Oster blender. The methanol suspensions of mycelia cells were placed in a shaker at room temperature for 24 h. Ten grams of Celite was added to each suspension before filtering through Whatman No. 2 paper. The filtrate was stored at 4 °C. The residual mycelia and Celite were re-suspended in methanol, placed in the shaker for an additional 42 h, and re-filtered.

The methanol solutions from both filtrations were combined and evaporated in vacuo. For paraherquamide A, the aqueous solution from each feeding experiment was added to the corresponding methanolic residue and the mixture was acidified to pH 4 with 12 mL of glacial acetic acid. The acidic solution was extracted 4 times with 150 mL portions of ethyl acetate. The organic layer was discarded. The aqueous layer was brought to pH 9−10 by the addition of 50 mL of 5 M NaOH. The aqueous layer was then extracted 4 times with ethyl acetate. The combined organic layers were washed with brine, dried over anhydrous sodium carbonate, and evaporated to dryness. For brevianamide A and austamide, the aqueous solution from each feeding experiment was added to the corresponding methanolic residue, taken at a pH of 7−8, and extracted 4 times with ethyl acetate. The combined organic layers were washed successively with 10% aqueous Na_2CO_3 solution and brine, then dried over anhydrous sodium sulfate and evaporated to dryness. The fungal metabolites were purified via radial chromatography and thin-layer chromatography on silica gel using a gradient elution of 4−10% methanol in methylene chloride. This was followed by preparative TLC on silica gel precoated plates with methylene chloride−methanol (10:1 for paraherquamide A (**1**); 25:1 for austamide (**21**)) or methylene chloride−acetone mixtures (2:1 for brevianamide A (**16**)).

Paraherquamide A (1): *Penicillium fellutanum* (ATTC: 20841) was initially grown on sterile malt extract agar slants (20 g of glucose, 1 g of peptone, and 20 g of agar per liter of distilled, deionized water). Yield of paraherquamide A (**1**): 18 mg, 0.036 mmol, from the control experiment, 15 mg, 0.030 mmol, from the [$^{13}C_2$]-acetate experiment, and 34 mg, 0.070 mmol from the U-$^{13}C_6$-glucose feeding experiment. The incorporation levels were as follows: 0.35% total incorporation and 0.07% specific total incorporation (intact C-2 units) of [$^{13}C_2$]-acetate into paraherquamide A (**1**) was observed.

Austamide (21): *Aspergillus ustus* (ATCC: 36063) was initially grown on sterile malt extract agar slants. Yield of austamide (**21**): 15 mg, 0.041 mmol, from the control experiment, and 14 mg, 0.039 mmol, from the [$^{13}C_2$]-acetate experiment. The incorporation levels were as follows: 0.57% total incorporation and 0.19% specific total incorporation (intact C-2 units) of [$^{13}C_2$]-acetate into austamide (**21**) was observed.

Brevianamide A (16): *Penicillium brevicompactum* (ATCC: 9056) was grown on sterile Czapek Dox slants (3.0 g of $NaNO_3$, 1.0 g of K_2HPO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.5 g of KCl, 0.01 g of $FeSO_4 \cdot 7H_2O$, 30 g of D-glucose, 15.0 g of agar per liter of distilled deionized water). [$^{13}C_2$]-Acetate (200 mg) plus 1 g of unlabeled acetate were used in the feeding experiment. Yield of brevianamide A (**16**): 17 mg, 0.047 mmol, from the control experiment, and 15 mg, 0.041 mmol, from the [$^{13}C_2$]-acetate experiment. The incorporation levels were as follows: 2.19% total incorporation and 0.32% specific total incorporation (intact C-2 units) of [$^{13}C_2$]-acetate into brevianamide A (**16**) was observed.

Determination of Isotopic Enrichment. Gated-decoupled experiments were precluded by the small amounts of these metabolites and the intrinsic lower sensitivity of this kind of experiment; for that reason, standard ^{13}C spectra were measured for the metabolite from both control experiments and from feeding experiments. The relative abundance of ^{13}C in each carbon of paraherquamide A (**1**), brevianamide A (**16**), and austamide (**21**) resulting from feeding experiments was determined through comparison of the integration for that peak with the total integration in the standard ^{13}C spectrum. When this relative integration was compared to the relative integration for each carbon signal in compounds from control experiments, it was possible to calculate the relative abundance of ^{13}C for each position with respect to the rest of carbon signals. The total abundance of ^{13}C in each metabolite was then determined by calculating the increase in intensity for the peaks corresponding to the isotopomers with one, two, three, and four ^{13}C atoms in the mass spectra for each of the metabolites resulting from feeding experiments, relative to the intensity of the peak that corresponds to molecules with ^{12}C atoms only. The ^{13}C abundance for each position was finally calculated taking into account the relative abundance of ^{13}C (from the ^{13}C NMR spectrum) and the average ^{13}C abundance for the metabolite (from the MS) isolated from the feeding experiment.[23]

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Supporting Information Available: Complete details of the calculations of ^{13}C incorporation into paraherquamide A (**1**), austamide (**21**), brevianamide A (**16**), and a control sample (Trp-OMe) are provided (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(23) The method used for the incorporation calculations was applied to three known dilutions of L-[10-^{13}C]-L-tryptophan methyl ester (99% enriched; carboxyl group is labeled). Comparison of the percentage of ^{13}C enrichment obtained from these calculations with the actual percentage of enrichment gave an average error of 20% (most likely arising from signal integration of the ^{13}C and mass spectra peaks; see Supporting Information).