PNEUMOCANDINS FROM *Zalerion arboricola* V. GLUTAMIC ACID- AND LEUCINE-DERIVED AMINO ACIDS IN PNEUMOCANDIN A_0 (L-671,329) AND DISTINCT ORIGINS OF THE SUBSTITUTED PROLINE RESIDUES IN PNEUMOCANDINS A_0 AND B_0

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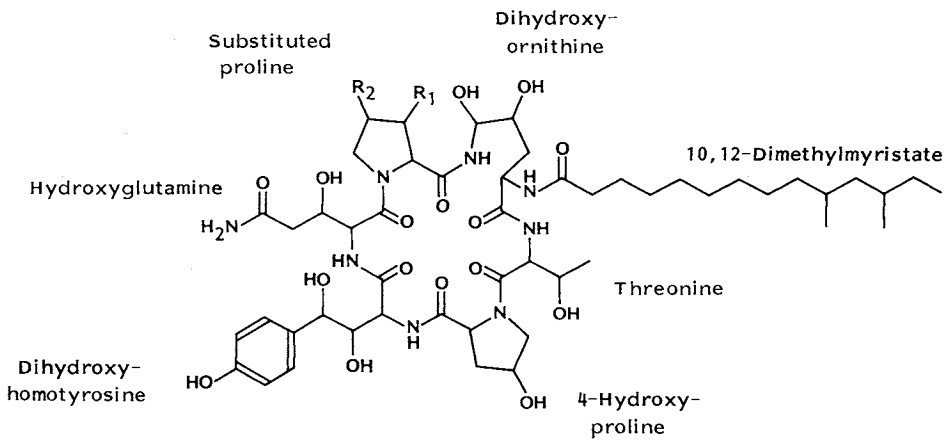
The accompanying papers[1~3] describe a family of pneumocandins produced by the helicosporous fungus, *Zalerion arboricola*. The pneumocandins are acylated cyclic hexapeptides belonging to the echinocandin group of antifungal antibiotics.[4] We have initiated a study of the biosynthesis of pneumocandin A_0 (formerly L-671,329) which is the most prominent pneumocandin in the fermentation. It contains threonine together with five unusual, hydroxylated, amino acid residues and a 10,12-dimethylmyristoyl side chain[5] (Fig. 1). Feeding experiments with ^{13}C-labeled precursors established that (i) tyrosine is condensed with acetate to form the homotyrosine unit of the antibiotic, (ii) proline is the precursor of the 4-hydroxyproline residue but not the 3-hydroxy-4-methylproline residue, (iii) leucine is cyclized to produce the 3-hydroxy-4-methylproline residue, and (iv) acetate units comprise the backbone of the dimethylmyristoyl group with methionine providing the methyl moieties.[6] The work described in this note explores several additional aspects of pneumocandin formation, namely, the biogenesis of the 3-hydroxyglutamine, 4,5-dihydroxyornithine, and 4-hydroxyproline residues, the mechanism of leucine cyclization, and the origin of the 3-hydroxyproline residue in pneumocandin B_0 (formerly L-688,786), the next most prominent product of the fermentation. This last point is relevant to the biosynthetic relationship of pneumocandins A_0 and B_0 which differ in only one respect (Fig. 1): pneumocandin B_0 has a 3-hydroxyproline residue at the position occupied by 3-hydroxy-4-methylproline in pneumocandin A_0. Because methylation of proline is not the mechanism by which the methylproline of pneumocandin A_0 arises,[6] pneumocandin B_0 cannot be the precursor of pneumocandin A_0. The formal possibility that pneumocandin B_0 is generated by demethylation of pneumocandin A_0 is addressed in the experiments presented here.

Fig. 1. Structures of pneumocandins A_0 and B_0.



	R_1	R_2	Substituted proline
Pneumocandin A_0	OH	CH_3	3-Hydroxy-4-methylproline
Pneumocandin B_0	OH	H	3-Hydroxyproline

Table 1. NMR determination of the incorporation of ^{13}C from L-[1,2-^{13}C]glutamic acid into pneumocandin A_0.

Positions labeled		δ (ppm)	^{13}C Enrichment factor	Satellites J_{CC} ($\pm$0.5 Hz)
3-Hydroxyglutamine	C-1	169.0	1.6	54.2
3-Hydroxyglutamine	C-2	55.6	1.6	54.2
4,5-Dihydroxyornithine	C-1	174.6	1.5	52.6
4,5-Dihydroxyornithine	C-2	51.4	1.5	52.7
4-Hydroxyproline	C-1	173.4	1.2	56.5
4-Hydroxyproline	C-2	62.5	1.2	56.5

3-Hydroxyglutamine, 4,5-Dihydroxyornithine and 4-Hydroxyproline Residues

L-[1,2-^{13}C]Glutamic acid was fed to *Z. arboricola* ATCC 20868 (MF5171; identical to MF5402) according to the protocol described elsewhere.[6] NMR analysis of the pneumocandin A_0 produced showed ^{13}C enrichment at both C-1 and C-2 of the hydroxyglutamine, dihydroxyornithine, and 4-hydroxyproline residues (Table 1). Although the magnitude of enrichment in these residues was relatively low, satellite peaks were observed in each case providing clear evidence that the coupled ^{13}C atoms in the precursor were incorporated into the antibiotic without randomization. These results suggest that glutamine synthetase and the standard enzymes of mitochondrial glutamate metabolism (glutamate acetyltransferase, acetylglutamate kinase, acetyl-γ-glutamylphosphate reductase, acetylornithine aminotransferase, acetylornithine-glutamate acetyltransferase) are involved in the generation of pneumocandin precursors in this fungus. The incorporation of ^{13}C into the hydroxyproline residue was somewhat lower than that into the other two residues; this may indicate that hydroxyproline is produced from a cytoplasmic pool of glutamic acid that can be rapidly utilized in several metabolic pathways, whereas, hydroxyglutamine and dihydroxyornithine are produced from a glutamic acid pool that is sequestered in the mitochondrial compartment and unavailable for general metabolism.

Leucine Cyclization and Hydroxylation

3-Hydroxy-4-methylproline is a unique component of echinocandin-type antibiotics, though 4-methylproline is found in a family of fungal products known as the leucinostatins[7,8] and in the streptomycete antibiotics, griselimycin[9] and monamycin.[10] An unsaturated form of 4-methylproline comprises the nucleus of glycerinopyrin which is produced by *Streptomyces violaceus*.[11] We have demonstrated that leucine is used by *Z. arboricola* to produce the 3-hydroxy-4-methylproline residue of pneumocandin A_0. Similarly, the substituted proline residues of monamycin and glycerinopyrin arise from leucine.[10,12] In the latter two examples, the mechanism of leucine cyclization has not been explored. As a step toward understanding the process in *Z. arboricola*, we sought (i) to determine if the amino nitrogen is retained during cyclization and (ii) to identify the hydrogen atoms that are displaced.

Table 2. Retention of deuterium atoms during the conversion of leucine to 3-hydroxy-4-methylproline.

Precursor fed	Number of deuterium atoms retained	Incorporation efficiency
DL-[2,3,3-d_3]Leucine	1	8%
DL-[2,3,3,4-d_4]Leucine	2	10%
DL-[4,5,5,5,6,6,6-d_7]Leucine	5	7%

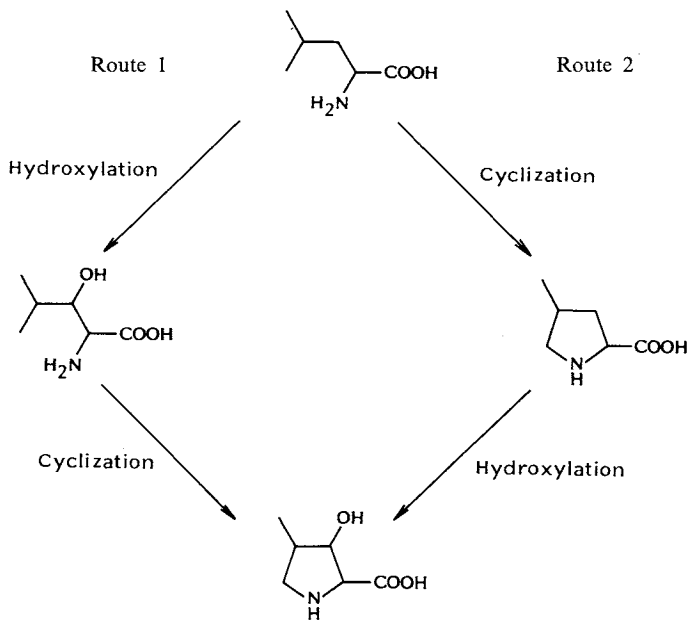
L-[^{15}N]Leucine and three forms of deuterium-labeled leucine were fed individually to *Z. arboricola* ATCC 20868 for incorporation into pneumocandin A_0. GC-coupled mass spectrometry was performed with the trimethylsilyl derivatives of the acid hydrolysis products from the antibiotic[5] to determine the presence and number of heavy atoms retained in the 3-hydroxy-4-methylproline residue. When L-[^{15}N]leucine was the precursor, ^{15}N was incorporated into the 3-hydroxy-4-methylproline showing that cyclization does not require the conversion of leucine to the 2-oxo-acid. However, the incorporation efficiency for ^{15}N (3%) was low relative to the values obtained with the deuterated substrates (7~10%) suggesting a significant isotope effect. The results obtained with the deuterated leucines are presented in the Table 2. With DL-[2,3,3-d_3]leucine as the precursor, a single deuterium atom was retained in the product whereas

two deuterium atoms were retained from DL-[2,3,3,4-d_4]leucine. This pair of results shows that the deuterium at C-4 of DL-[2,3,3,4-d_4]leucine is not displaced. (Insufficient fragmentation of the TMS-derivatives precluded assignment of the locations of deuterium atoms directly from the mass spectrometry data.) In the experiment with DL-[4,5,5,5,6,6,6-d_7]leucine, two deuterium atoms were lost. It is reasonable to assume that only one of the methyl groups of leucine is functionalized during cyclization to proline. Thus, three deuterium atoms on the non-functionalized methyl group along with a fourth at C-4 must be retained. This places the fifth atom at C-5. Loss of two deuterium atoms from C-5 during cyclization is consistent with an oxidative mechanism giving an aldehyde that can spontaneously cyclize with the amino nitrogen atom in a manner analogous to the conversion of glutamate-5-semialdehyde to 1-pyrroline-5-carboxylic acid in proline biosynthesis. Enzymatic reduction of the resulting SCHIFF's base would yield 4-methylproline. Precedent for the oxidation of a γ-methyl function of leucine in a biological system is provided by the honeylocust, *Gleditsia triacanthos*, which produces 4-methylglutamic acid from leucine.[13] Involvement of cytochrome P-450 monooxygenase systems in the direct oxidation of methyl groups is known, for example, in the 15-hydroxylation of ipomeamarone,[14] the detoxification of pisatin,[15] and the C-14 demethylation of lanosterol.[16] The retention of deuterium at C-4 eliminates the possibility of epoxide formation between C-4 and C-5 as a way of oxidizing C-5, a mechanism employed in the enzymatic closure of the D ring of agroclavine to generate chanoclavine-I during the biosynthesis of ergot alkaloids.[17,18]

Loss of one deuterium atom from DL-[2,3,3-d_3]leucine during the formation of the 3-hydroxy-4-methylproline would accompany the introduction of the hydroxyl function at C-3. Our data do not permit more than speculation regarding the loss of the second deuterium from this precursor. Tautomerization before reduction of the SCHIFF's base that results from cyclization could account for the loss. Alternatively, the electron withdrawing effects of the nitrogen and carboxyl function of the SCHIFF's base may facilitate hydrogen exchange at C-2. A third possibility is the formation of a double bond between C-2 and C-3 of leucine or 4-methylproline, resulting in the loss of one deuterium atom from each carbon; addition of water to this double bond could account for hydroxylation at C-3. In support of the last alternative, two natural products with C-2,3 unsaturations in their 4-methyl-2-pyrrole-carboxylic acid rings may be cited: The trail marker pheromone of the Texan leaf-cutting ant, *Atta texana*,[19] and glycerinopyrin, a metabolite of *Streptomyces violaceus*.[12]

Fig. 2. Potential pathways for the conversion of leucine to 3-hydroxy-4-methylproline.

Route 1 *via* 3-hydroxyleucine and route 2 *via* 4-methylproline.



An additional question regarding the pathway from leucine to 3-hydroxy-4-methylproline concerns the order in which hydroxylation and cyclization occur (Fig. 2). 3-Hydroxyleucine would be a pathway intermediate if hydroxylation precedes cyclization (route 1), whereas 4-methylproline is the expected intermediate if hydroxylation follows cyclization (route 2). We explored this issue with an isotope dilution experiment in which replicate suspensions of respiring cells were fed L-[*U*-^{14}C]leucine alone or in combination with unlabeled 3-hydroxyleucine or 4-methylproline. The specific activity of the antibiotic formed under each condition is presented in Table 3. 3-Hydroxyleucine did not alter the specific activity of the product significantly ($P>0.05$ by DUNNETT's *t*-test) whereas the 70% reduction caused by 4-methylproline was a highly significant change ($P<0.05$). This study provides evidence for route 2 with cyclization preceding hydroxylation.

Origins of the 3-Hydroxyproline Residue of Pneumocandin B_0

Although the wild-type strain of *Z. arboricola* makes pneumocandin A_0 as its primary antibiotic product, several other members of the pneumocandin family including pneumocandin B_0 are minor components of the fermentation. The isolation of a *Z. arboricola* mutant ATCC 74030 (MF5533) in which production is shifted in favor of pneumocandin B_0[2] made it feasible to determine the origin of the 3-hydroxyproline residue of pneumocandin B_0. We fed L-[1-^{13}C]proline and L-[1-^{13}C]leucine to the mutant under the same conditions used for the wild-type organism. NMR analysis of the antibiotic produced in each case gave results which contrast strikingly with those obtained earlier for pneumocandin A_0 (Table 4); they clearly establish that L-[1-^{13}C]proline is a precursor of not just one but both proline residues in pneumocandin B_0 and that L-[1-^{13}C]leucine is not involved in the formation of the 3-hydroxyproline residue of this pneumocandin. Thus, demethylation of pneumocandin A_0 cannot be the route of pneumocandin B_0 formation. In sum, our data indicate that pneumocandin B_0 can be considered neither a biosynthetic progenitor nor a degradation product of pneumocandin A_0. A defect in the ability to cyclize leucine to methylproline could account for the shift in pneumocandin formation from pneumocandin A_0 to pneumocandin B_0 in mutant ATCC 74030.

Table 3. Dilution of the radioactivity incorporated from L-[*U*-^{14}C]leucine into pneumocandin A_0 by potential intermediates in the leucine cyclization/hydroxylation pathway.

Addition	Pneumocandin A_0 specific activity (cpm/mg)	Standard deviation
None	4.03×10^4	0.07
3-Hydroxy-DL-leucine	3.97×10^4	0.16
4-Methyl-L-proline	1.29×10^4	0.01

Experimental

The NMR results presented in Tables 1 and 4 were obtained in CD_3OD at ambient temperature and at 125 MHz and 100 MHz, respectively. L-[1,2-^{13}C]Glutamic acid and L-[1-^{13}C]leucine were obtained from Cambridge Isotopes; L-[1-^{13}C]-proline was from MSD Isotopes. Enrichment of ^{13}C was 99% in the glutamic acid and leucine and 90% in the proline. The ^{13}C assignments listed in the Tables are taken from HENSENS *et al.*[3]

All deuterated and ^{15}N-labeled leucine precursors used for the experiment in Table 2 were obtained from MSD Isotopes and had >98% heavy atom enrichment. The pneumocandin A_0 made in the presence of each precursor was isolated and hydrolyzed with acid to release the constituent amino acids which were converted to trimethylsilyl derivatives and analyzed by GC-coupled mass spectrometry.[5] The number of heavy atoms retained in the 3-hydroxy-4-methylproline residue was indicated by the isotope cluster for the *m/z* 244 base peak. With each of the deuterated precursors there was no evidence of incorporation other than that listed.

Table 4. NMR determination of the incorporation of ^{13}C-labeled precursors into pneumocandin B_0.

Precursor fed	Possible position labeled		δ (ppm)	^{13}C Enrichment factor
L-[1-^{13}C]Proline	C-1	4-Hydroxyproline	173.4	45
L-[1-^{13}C]Proline	C-1	3-Hydroxyproline	172.9	45
L-[1-^{13}C]Leucine	C-1	3-Hydroxyproline	172.9	None

For the experiment that generated the data in Table 3, six 10-ml suspensions of respiring cells[6] were divided into three pairs. All received 4 μCi (12.9 nmol) of L-[*U*-^{14}C]leucine (310 mCi/mmol; Amersham), and one pair was incubated without further additions. One of the two remaining pairs was supplemented with 3-hydroxy-DL-leucine and the other received 4-methyl-L-proline. The amount of unlabeled amino acid used in each supplemented suspension (6.5 μmol) represented a 500-fold molar excess with respect to the labeled precursor. The mixtures were incubated for 10.5 hours before the antibiotic was extracted. HPLC[6] was used to quantify the pneumocandin A_0 in the extracts, and the radioactivity that eluted along with the antibiotic peak was measured by scintillation counting. Each specific activity shown is the mean for each pair. 3-Hydroxy-DL-leucine and 4-methyl-L-proline were obtained from United States Biochemical Corp. and the Merck chemical collection, respectively.

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