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BIOSYNTHETIC STUDIES WITH ^{13}C

THE ANTIFUNGAL ANTIBIOTIC ILICICOLIN H.

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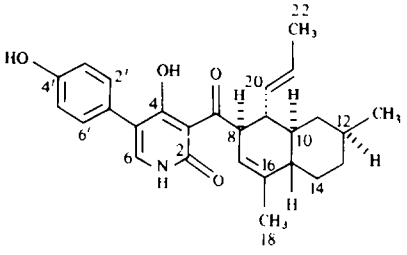
Abstract—The results of feeding experiments conducted with ^{13}C-labeled acetates, ^{15}N-labeled phenylalanine, and ^{14}C-labeled phenylalanine in fermentations of *Cylindrocladium ilicicola* MFC 870 are consistent with the polyketide pathway to ilicicolin H, the principal metabolite. The origin of the 5-(4-hydroxyphenyl-α-pyridone) unit in ilicicolin H is via an intermediate tetramic acid derivative, analogous to the biosynthesis of the same unit in tenellin. The two reduced carbocyclic rings of ilicicolin H are derived through cyclization of a linear polyketide precursor.

Investigation of the fungal metabolite ilicicolin H (**1**) is interesting because this compound has two distinctive structural features, whose origins we have been examining. The first feature is the 5-(4-hydroxyphenyl)-α-pyridone. The identical chromophore occurs in the yellow metabolite tenellin (**2**), which is produced by the insect-pathogenic fungus *Beauveria bassiana*.



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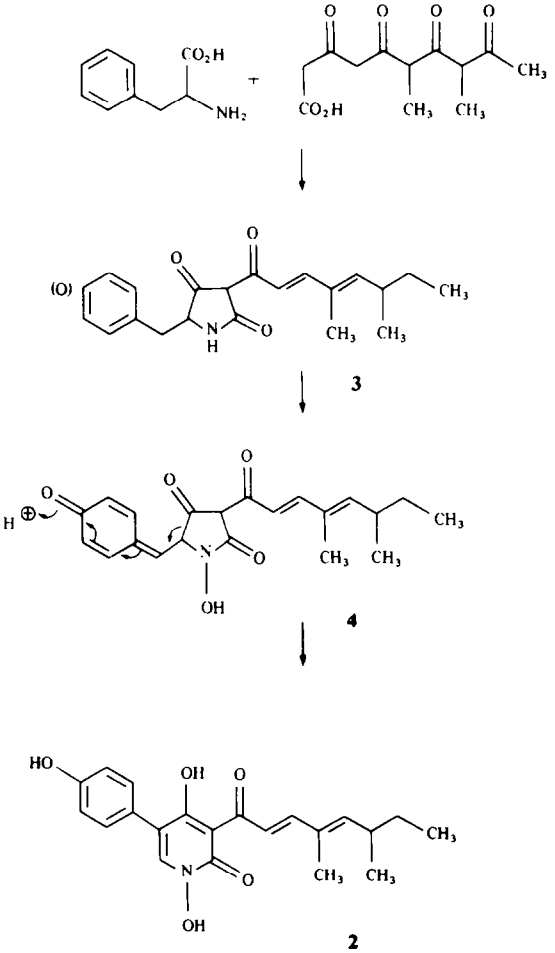
Extensive and detailed biosynthetic studies of tenellin have shown that it is derived from a polyketide precursor that incorporates phenylalanine,[1] as shown in Scheme 1. These investigators propose that the polyketide unit cyclizes to an intermediate tetramic acid derivative (**3**) and that a unique ring-expansion reaction of **3** accounts for the formation of the α-pyridone system. They described a plausible rearrangement route, proceeding from an intermediate *p*-quinomethide (**4**), which is formed by specific P_{450}-catalyzed *para*-oxygenation of the phenylalanine ring. This intermediate subsequently rearranges, by ring expansion, to the α-pyridone system found in tenellin.

In an additional biosynthetic study of tenellin, conducted with a doubly ^{13}C-labeled precursor, 1,3-^{13}C-phenylalanine, Leete[2] elegantly defined the detailed course of this rearrangement process that affords tenellin. The labeled tenellin isolated in this feeding study displayed homonuclear ^{13}C–^{13}C coupling signals for the C_4 and C_6 carbon signals in the ^{13}C-NMR spectrum, indicating that the rearrangement of the tetramic acid to the α-pyridone system proceeds by specific intramolecular migration of C_4 to C_6. These two carbons were originally



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Scheme 1. Biosynthesis of tenellin.

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derived from the C_1 and C_3 carbons of phenylalanine respectively.

The second structural feature of interest in ilicicolin H is the bicyclic decalin system. The presence of this structural element in a fungal metabolite formally derived from a linear polyketide precursor is relatively novel. The facile enzymic cyclization of such linear polyketide chains to afford a wide array of metabolites with aromatic rings is well established. The widespread occurrence of reduced carbocycles in terpenes and sterol metabolites and their elaboration from linear terpene precursors derived from mevalonic acid are also well documented.

To better understand the details of the formation of these two structural elements, the 5-(4-hydroxyphenyl-α-pyridone) ring and the decalin ring in ilicicolin H, we examined the biosynthesis of this metabolite by feeding studies conducted with stable isotope-labeled precursors and ^{13}C-NMR spectroscopy.

EXPERIMENTAL

Microbiological production of ilicicolin H. A two-stage fermentation was used for microbiological production of ilicicolin H.[3] Cultures of the producing organism, *Cyclindrocladium ilicicola* (MFC 870), were inoculated into 100 ml of a medium consisting of glucose (3%), peptone (2%), and NaCl (0.5%) at pH 6.8. The mixture was incubated for 7 days on a rotary shaker. The secondary stage was then incubated in a similar manner for an additional 7 days by inoculating 50 ml of the primary-stage culture into a 2-l. Erlenmeyer flask containing 800 ml of medium. Sterile aqueous solns of isotopically labeled compounds were added between the two stages of fermentation to achieve final concentrations of about 10 mM.

Ilicicolin H was isolated from the fermentation broth by thoroughly mixing the culture medium with 150 g Celite, for each flask, and stirring for 30 min. The suspension was centrifuged and the residue was extracted with MeOH (3 l./flask) by stirring the suspension for 2–3 hr at room temp. The suspension was allowed to stand overnight and then filtered. The extract was evaporated under reduced pressure at 40–50°, leaving an oily residue that was extracted with ether and washed with 5% HCl, 5% Na_2CO_3, and water. After evaporation of the ether soln, the residue was dissolved in benzene and chromatographed on silica gel. The less polar co-metabolites ilicicolin A to G were eluted first. Finally, ilicicolin H was eluted and isolated, in a yield of *ca* 1.25 g/l. of fermentation broth, and characterized as described.[3,4]

^{13}C-NMR Assignments for ilicicolin H. The assignment of the ^{13}C-NMR shifts for ilicicolin H shown in Table 1 were facilitated by comparison with the published ^{13}C-shift assignments for the 5-(4′-hydroxyphenyl)-4-hydroxy-α-pyridone moiety in tenellin.[1] The comparison for the 11 carbons that constitute this unit are shown in Table 2. The observed shift coincidences for the 4-hydroxyphenyl carbons are not unexpected, and the minor differences between the α-pyridine units can be attributed to the presence of the hydroxamic acid functionality in tenellin.

For the remaining 16 carbons, the lowest field signal at 210.1 ppm is assigned to the C-7 CO group. Of the four sp^2

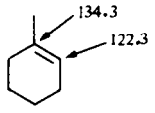
Table 1. ^{13}C-NMR Assignments for ilicicolin H and relative ^{13}C-enrichments from ^{13}C-precursors

Carbon No.	Chemical Shift (ppm)	$^{13}CH_3COONa$	$CH_3{}^{13}COONa$	$^{13}CH_3$-Methionine	$^{13}CH_3{}^{13}COONa$
2	163.7 s	–	+ 2.2	–	74.8 Hz
3	107.2 s	+ 3.0		–	74.2
4	176.6 s	–	–	–	–
5	115.5 s	–	–	–	–
6	138.66 d	–	–	–	–
7	210.1 s	–	+ 2.2	–	41.8
8	53.7 d	+ 3.3	–	–	41.8
9	45.1 d	–	+ 2.6	–	44.0
10	43.4 d	+ 3.3	–	–	33.8
11	39.5 t	–	+ 3.0	–	33.9
12	32.8 d	+ 3.4	–	–	33.8
13	35.6 d	–	+ 2.9	–	33.8
14	29.8 t	+ 3.8	–	–	33.1
15	44.5 d	–	+ 2.6	–	33.2
16	139.0 s	+ 2.5	–	–	72.8
17	119.0 d	–	+ 2.7	–	72.7
18	21.0 q	–	–	+ 3.7	–
19	22.7 q	–	–	+ 3.7	–
20	133.8 d	+ 2.5	–	–	44.1
21	126.5 d	–	+ 2.8	–	13.3
22	17.9 q	+ 2.9	–	–	42.9
1′	124.9 s	–	–	–	–
2′	130.5 d	–	–	–	–
3′	115.6 d	–	–	–	–
4′	155.6 s	–	–	–	–
5′	115.6 d	–	–	–	–
6′	130.5 d	–	–	–	–

Table 2. ^{13}C-NMR Comparisons of α-pyridone unit

	Ilicicolin H (ppm)	Tenellin (ppm)
2	163.7	157.5
	107.2	105.9
4	176.6	173.0
5	115.5	110.9
6	138.6	140.0
7	210.1	193.8
1'	124.9	122.8
2',6'	130.5	130.2
3',5'	115.6	115.0
4'	155.9	156.9

carbons in the carbocyclic system, C-16, C-17, C-20 and C-21, the signal at 139.0 ppm is unambiguously assigned to C-16 because of its lack of multiplicity in the off-resonance decoupled spectrum. The other three unsaturated carbons were assigned by comparison with model compounds **5** and **6**.



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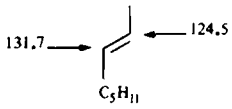

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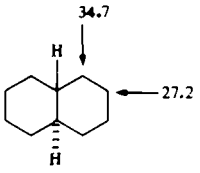
The calculated shifts for the side chain (E)-olefin, using Robert's procedure,[5] were remarkably close to the observed values.

	Observed	Calculated
C-20	133.8	134.4
C-21	126.5	128.4

The three methylene C signals, C-11, C-13 and C-14, were conveniently assigned by recourse to the known chemical shift assignments of *trans*-decalin (**7**), in which the β-carbons resonate at higher field (27.2 ppm) compared with the α-carbons (34.7 ppm).



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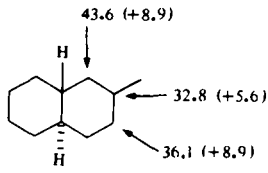

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The established effect of Me substituents on C chemical shifts applied in the decalin system (**8**) results in deshielding of the α-position by +5.6 ppm and of the β-position by +8.9 ppm.[6]

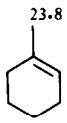
Furthermore, the carbons at C-14 and C-18 in ilicicolin H are in a γ-gauche relationship, resulting in a shielding of −5.5 ppm. Similarly, C-11 is also shielded by a γ-gauche interaction with C-20, resulting in a −3.4 ppm shift.[6,7] The combined effects of the Me substituent and the γ-gauche interactions lead to reasonable agreement for observed and calculated chemical shifts for C-11, C-13 and C-14, as shown.

	Observed	Calculated
C-11	39.5 ppm	40.2 ppm
C-13	35.6	36.1
C-14	29.8	29.2

The five methine C signals at C-8, C-9, C-10, C-12 and C-15 were assigned by considering known and established trends in C chemical shift assignments. The highest field signal at 32.8 ppm within the methine C group was assigned to C-12 because it is influenced only by interaction with the equatorial C-19 Me group. The lowest field signal in this group at 53.7 ppm was assigned to C-8 because it bears the ketonic C-7 carbon as well as two alkyl substituents, one of which is unsaturated, thus making C-8 an allylic C.

C-9, also an allylic C, is shielded by alkyl substituents at C-8 and C-10 and is therefore assigned the resonance at 45.1 ppm. The two ring-juncture carbons at C-10 and C-15 can interact with the alkyl substituents at C-9 and C-16. However, C-15 is also an allylic C and is more deshielded at 44.5 ppm than C-10 is at 43.4 ppm.

The assignments for the three Me signals at δ 17.9, 21.0 and 22.7 ppm were made for C-22, C-18 and C-19, respectively, by comparison with the three model compounds shown below. The γ-gauche interaction of C-14 and C-18 was also taken into consideration in assigning C-18 upfield of the C-19 signal.



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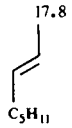

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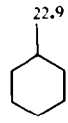

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Incorporation of sodium [1-^{13}C]-, [2-^{13}C]-, *and* [1,2-^{13}C]-*acetates.* From cultures of *Cylindrocladium ilicicola* supplemented with 90% sodium [2-^{13}C]-acetate to a final concentration of 10 mM, ilicicolin H was isolated as described. The distribution of label in the ^{13}C-enriched metabolite was determined by comparison of the ^{13}C-NMR spectra of the enriched and natural abundance material recorded under identical experimental conditions, as shown in Fig. 1. The signals of enhanced intensity were normalized to a reference peak at 22.7 ppm for C-19 because this signal is known not to be enhanced by this precursor.

Ilicicolin H biosynthesized from 90% enriched sodium [2-^{13}C]-acetate showed enhanced signal intensities for C-3 (3%), C-8 (3.3%), C.10(3.3%), C-12 (3.4%), C-14 (3.8%), C-16 (2.5%), C-20 (2.5%) and C-22 (2.9%).

Similarly, ilicicolin H isolated from cultures fortified with 90% sodium [1-^{13}C]-acetate had enhanced signal intensities for C-2 (2.2%), C-7 (2.2%), C-9 (2.6%), C-11 (3.0%), C-13 (2.9%), C-15 (2.6%), C-17 (2.7%) and C-21 (2.8%).

A complementary feeding study with the doubly labeled precursor [1,2-^{13}C]-acetate was also conducted. This precursor was 90% enriched at each carbon atom. It afforded a labeled ilicicolin H that exhibited the characteristic ^{13}C-^{13}C coupling signals flanking the center unenriched C signals in the ^{13}C-NMR spectrum because of the incorporation of intact acetic acid units, as shown in Fig. 1(c). The pairs of chemically bonded carbons associated with incorporation of intact AcOH units could be identified by matching the respective coupling constants. The couplings found in ilicicolin H were C_2, C_3, ^1Jcc 74.8 Hz; C_7, C_8, ^1Jcc 41.8 Hz; C_9, C_{20}, ^1Jcc 44.0 Hz; C_{10}, C_{11}, ^1Jcc 33.8 Hz; C_{12}, C_{13}, ^1Jcc 33.8 Hz; C_{14}, C_{15}, ^1Jcc 33.1 Hz; C_{16}, C_{17}, ^1Jcc 72.7 Hz; C_{21}, C_{22}, ^1Jcc 43.1 Hz.

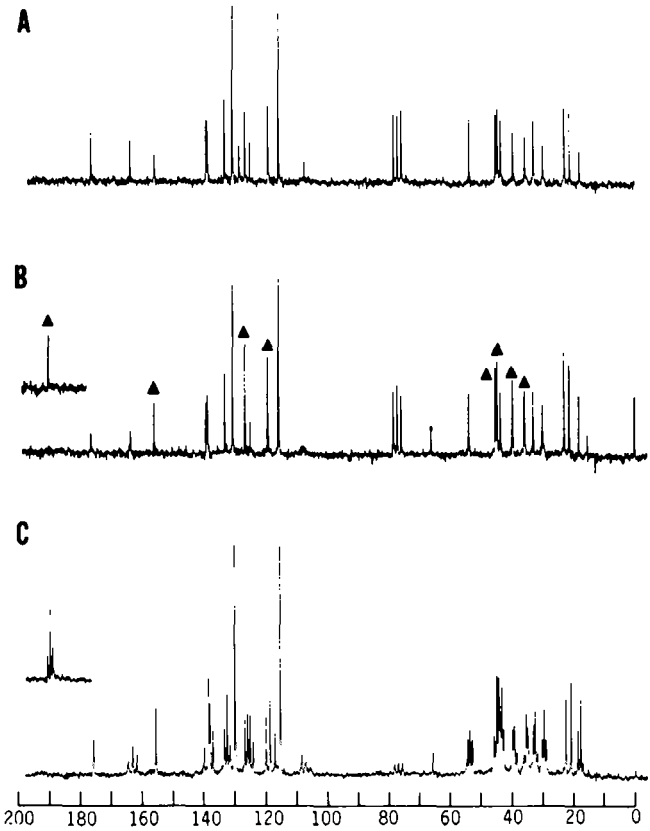


Fig. 1. ^{13}C ^{1}H-NMR spectra of ilicicolin H obtained under the following conditions: Varian XL-100, solvent $CDCl_3$ 25.1 MHz; spectral width 6016 Hz, 16K points, acquisition time 1.0 sec; 50,000 transients; (A) natural abundance (90 at.% C-13), (B) derived from [1-^{13}C]-sodium acetate, (C) derived from [1,2-^{13}C]-sodium acetate (90 at.% C-13).

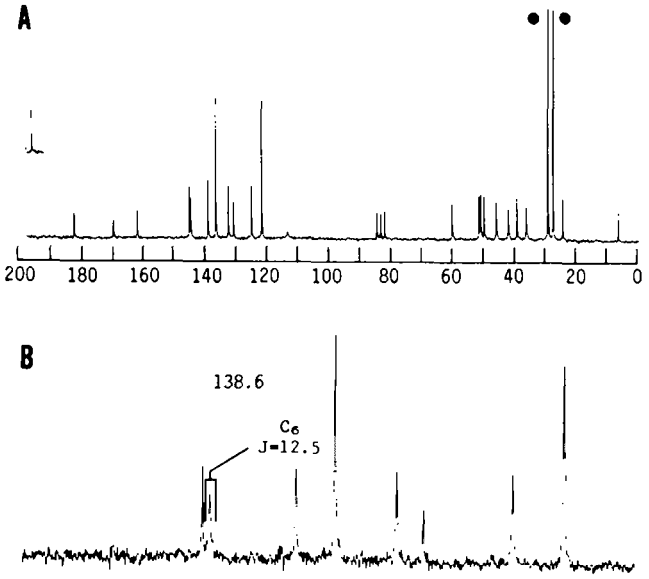


Fig. 2. ^{13}C ^{1}H-NMR spectra of ilicicolin H obtained under the following conditions: Varian XL-100, solvent $CDCl_3$, 25.1 MHz; 16K points, acquisition time 1.0 sec; 50,000 transients (A) derived from [^{13}C]-methionine (90 at.% C-13) (spectral width 6016 Hz), (B) derived from [^{15}N]-phenylalanine (96.6 at.% N-15) (spectral width 1000 Hz).

Incorporation of L-[$-CH_3-^{13}C$]-*methionine*. Ilicicolin H isolated from culture broths supplemented with 90% L [$-CH_3-^{13}C$]-methionine to a final concentration of 10 mM showed two very strong signal enhancements, as shown in Fig. 2(a). These signals, found at 21.0 ppm (3.7%) and 22.7 ppm (3.7%), were assigned to C-18 and C-19, respectively, as shown in Fig. 2.

Incorporation of L-U-(^{14}C)-*phenylalanine*. When the radioactive precursor L-U-(^{14}C-) phenylalanine (2.6 mCi/mmole) was added to the culture broth, it produced radiochemically labeled ilicicolin H with a total activity of 0.13 mCi and a specific activity of 13.0 μCi/mmole, giving an incorporation yield of 0.5%.

Incorporation of DL-[^{15}N]-*phenylalanine*. Additional confirmation for incorporation of phenylalanine into ilicicolin H was obtained by feeding studies conducted with DL-[^{15}N]-phenylalanine. The mass spectral analysis of ilicicolin H obtained from this study showed a ^{15}N-enrichment of 3% from a fragment ion with an *m/e* 231 ($C_{12}H_8O_4N$) produced by fragmentation of the C-7–C-8 bond. Furthermore, heteronuclear ^{13}C–^{15}N spin–spin coupling was detected in this labeled specimen in the ^{13}C-NMR spectrum. As indicated in Fig. 2(b), the observed coupling constant ^1J–^{13}C–^{15}N is 12.5 Hz. The magnitude of this one-bond coupling constant appears to be consistent with an sp^2 C (C-6) directly attached to the ^{15}N.[8] Thus, this labeling result is consistent with the view that an intact phenylalanine unit is incorporated and eliminates considerations of prior metabolism to phenylpyruvic acid before incorporation into ilicicolin H.

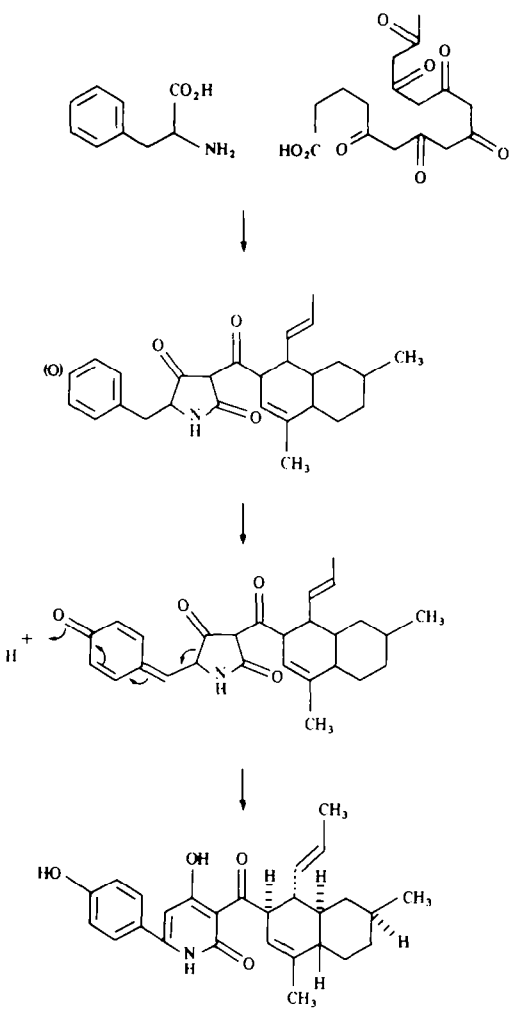
DISCUSSION

The ^{13}C-labeling results summarized in Table 1 and the results of the phenylalanine feeding studies are consistent for a biosynthetic pathway to ilicicolin H that utilizes a putative polyketide precursor derived from eight intact acetic acid units. Formation of the octaketide unit proceeds through the usual head-to-tail assembly via acetyl-Co-A and seven malonyl-Co-A units. Carbon alkylation at C-12 and C-16 of this octaketide unit by S-adenosylmethionine to introduce the C-18 and C-19 Me substituents is also a well-established biosynthetic process and is consistent with the observed labeling result.

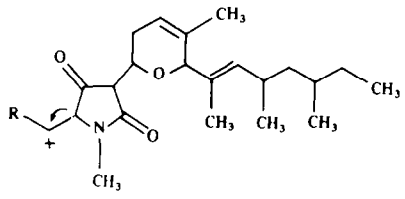
The octaketide unit can then cyclize by standard aldol processes between C-8 and C-9 and between C-10 and C-15 to produce an intermediate bicyclic-carbocycle. This carbocycle can then interact with phenylalanine to afford a tetramic acid intermediate. The precise order of events in the formation of this key tetramic acid intermediate is uncertain because the octaketide unit itself can interact with phenylalanine prior to its cyclization to the bicyclic carbocycle to produce a tetramic acid with a linear side chain that can subsequently cyclize.

The proposed intermediate role of the tetramic acid is to eventually provide an electron deficient center α to the tetramic acid ring to facilitate the rearrangement. Analogous to this role of the tetramic acid as the precursor for the α-pyridone unit in tennellin biosynthesis, our labeling results are compatible with the identical rearrangement pathway to produce the α-pyridone ring in ilicicolin H as shown in Scheme 2.

With regard to the biosynthesis of α-pyridone units, it is interesting to speculate about the nature of the process that generates the electron-deficient intermediate (**10**) involved in the biosynthesis of funicu-



Scheme 2. Biosynthesis of ilicicolin.



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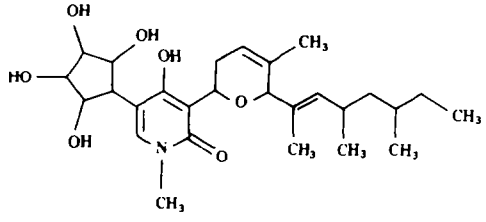

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losin (**11**), another fungal metabolite possessing an α-pyridone unit.[9] We presume that this metabolite is biosynthesized from an intermediate tetramic acid derived from an unusual, unknown amino acid and a heptaketide unit.

Reduced carbocyclic structures, as found in ilicicolin H, are prominent features of many new fungal metabolites. These new carbocyclics appear to be derived from acyclic polyketide precursors, as exemplified in the hypotholesteremic agents compactin and monoacolin K,[10] the antibiotics avermectin,[11] milbemycin α_1,[12] kijanimicin,[13] and tetrocarcin,[14] and the macrolide derivative nodusmicin.[15] Investigation of the details of the cyclization processes involved in their biosynthesis should be interesting.

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