THE BIOSYNTHESIS OF HAZIMICINS: POSSIBLE ORIGIN OF ISONITRILE CARBON

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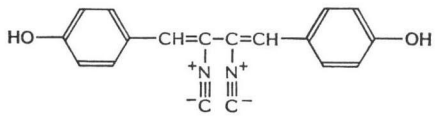
(Received for publication July 31, 1984)

Hazimicin factors 5 (*R,R*+*S,S*) and 6 (*R,S*), having comparable physico-chemical properties, are members of a new class of broad-spectrum antibacterial and antifungal antibiotics recently isolated from *Micromonospora echinospora* var. *challisensis* SCC 1411. The antibiotics are active against various Gram-positive, Gram-negative bacteria and yeasts, *e.g.*, *Staphylococcus aureus*, *Micrococcus luteus, Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa* and *Candida albicans*. The details of taxonomy, biological properties[1] and the structures[2] have been reported from our laboratories. The presence of an isonitrile ($-N^+\equiv C^-$) function was quite unusual and its biogenetic origin has been intriguing eversince the isolation of xanthocillin (**1**), the *Pencillium* metabolite[3]. Carbon-14 labeling experiments indicated the incorporation of tyrosine, but not of methionine or formate into **1**[4]. More recently the biosyntheses of isonitrile containing antibiotics, **2** and **3**, isolated from *Trichoderma hamatum* (Bon.) Bain. aggr. were reported to originate from tyrosine, however, the origin of the isonitrile carbon was not addressed[5,6]. The lack of data on the origin of an isonitrile carbon prompts us to report on the biosynthesis of hazimicins.

The fermentation was carried out either in flasks or 14-liter tanks according to the published procedure[1]. First the data on the incorporation of ^{14}C-labeled precursors (conc 0.20 μCi/ml), Na-[1-^{14}C]acetate, Na-[2-^{14}C]acetate, Na-[3-^{14}C]-pyruvate, L-[*methyl*-^{14}C]methionine, L-[1-^{14}C]-tyrosine, DL-[3-^{14}C]tyrosine, [1-^{14}C]formaldehyde were obtained. Then the ^{13}C-labeled DL-[3-^{13}C]-tyrosine (25 mg/liter), L-[*methyl*-^{13}C]methionine (200 mg/liter) and DL-[*N-methyl*-^{13}C]tyrosine* (**4**, conc 25 mg/liter) were added individually to the culture at the optimal time; additions were done at either day two or three.

The fermentation broth was extracted twice with double volumes of EtOAc and the combined extract was further concentrated. For ^{14}C-labeled studies, this extract was chromatographed in chloroform - methanol (9:1) on LK6DF Whatman silica plates and scanned for radioactivity. Duplicate plates were tested by bioautography on *Micrococcus luteus* plates. For ^{13}C NMR, the EtOAc extract was purified on Sephadex LH-20 column using ethanol as a solvent. Since the incorporations of ^{14}C-labeled precursors in components 5 and 6 were comparable, it was not necessary to separate them for the present study. Fully decoupled ^{13}C NMR spectra of the natural abundance ^{13}C and the enriched antibiotic samples were recorded in DMSO-d_6 on a Varian XL-100-15 NMR Spectrometer operating at 25.2 MHz. Percentage enrichments obtained from both components of hazimicin are presented in Table 1.



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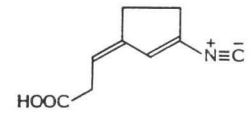

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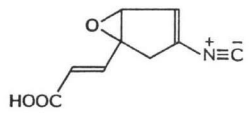

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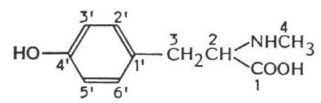

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* DL-[*N-Methyl*-^{13}C]tyrosine was prepared by *N*-methylation using $^{13}CH_3I$ of *N*-trifluoroacetyl benzyloxy tyrosine and subsequent removal of the protecting groups.

Table 1. Incorporation of ^{14}C- and ^{13}C-labeled percursors.[a]

C[b]	Hazimicin (ppm) Factors 5 & 6	[3-^{13}C]-Tyrosine		L-[*Methyl*-^{13}C]-methionine	
		I/Io	[% ^{14}C]	I/Io	[% ^{14}C]
1	167.0	1.00		1.00	
2	58.5	1.27		1.11	
3	37.7	8.11	14~16	0.97	
1′	126.0	1.12		1.05	
2′	129.0	1.14		0.92	
3′	125.7	1.32		1.13	
4′	153.5	1.00		1.00	
5′	115.6	1.13		1.09	
6′	132.1	0.80		0.78	
4	158.1	—		1.45	1.5

[a] Ratio of normalized intensities of the same carbon for enriched (I) and natural abundance (Io) samples. The spectra were obtained under essentially identical conditions with s/n of 37:1 for the methionine experiment.

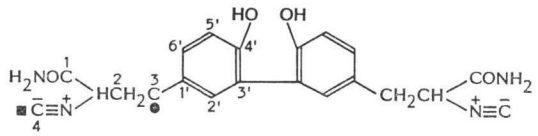
[b] The chemical shifts of the monomer are: δ (ppm) C-1=167.0, C-2=58.7, C-3=37.6, C-1′=125.9, C-2′,6′=130.2, C-3′,6′=115.0, C-4′=156.4 and C-4=158.0.

The ^{14}C-labeling studies indicated that only DL-tyrosine (16%), [2-^{14}C]acetate (0.04%) and methionine were incorporated into the antibiotic complex. In the case of methionine, however, 1.5% of radioactivity was found in components 5 and 6, the rest (3.5%) ended up in the bioinactive components. The addition of DL-[3-^{13}C]tyrosine (25 mg/liter) highly enriched C-3 with percentage comparable to the enrichment of 14~16% obtained from ^{14}C-labeled tyrosine. The incorporation of [^{13}C]methionine at the above concentration was considerably lower; the intensity of C-4 enhanced by 15~20%. When 8 times the initial concentration was used a significant enrichment of C-4 as well as a 3~4 fold increase in the production of hazimicins 5 and 6 was observed. It appears, therefore, that isonitrile carbon originated from L-[*methyl*-^{13}C]methionine, and it is probable that $-NHCH_3$ is converted to $-NC$ through the intermediacy of $-NHCHO$ based upon chemical analogy[2,7].

$$-HNCH_3 \rightarrow [-NH-CH_2OH] \rightarrow -HNCHO$$
$$\rightarrow -N=C=O \rightarrow -N^+\equiv C^-$$

The above sequence was further supported by the formation of the *N*-formyl product in some fermentations ($-CHNH-CHO$, $\delta_{CH}=4.9$ became a triplet on D_2O exchange of $\delta_{NH}=8.82$, $\delta_{CHO}=8.02$s)†. However attempts to incorporate ^{14}C-labeled formaldehyde into hazimicins failed as with the *Penicillium* metabolite[4]. Similarly the addition of DL-[*N-methyl*-^{13}C]tyrosine failed to show any incorporation of C-4 at δ 158.1 indicating either lack of incorporation or demethylation in the initial stages of the fermentation. Thus hazimicins (Fig. 1) are biosynthesized from tyrosine and methionine; the latter established an origin of isonitrile carbon.

Fig. 1. Biosynthesis of hazimicins.



● DL-[3-^{13}C]Tyrosine
■ L-[*Methyl*-^{13}C]methionine

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† After the completion of our work, Prof. SCHEUER, *et al.*, reported that in the sponge *Hymeniacidon* sp. an isolated isocyanoterpene coexisted with a formamide and an isothiocyanate and the -NC function is transformed to -NHCHO and -NCS (J. Am. Chem. Soc. 106: 2447~2448, 1984). It is conceivable, therefore, that the isocyano antibiotic was microbially converted to the formamide.

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