NEW ANTIBIOTICS, CARBAZOMYCINS A AND B

III. TAXONOMY AND BIOSYNTHESIS

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The carbazomycin-producing microorganism, strain H 1051-MY 10, was determined to a strain of *Streptoverticillium ehimense*.

Biosynthesis of carbazomycin B was studied using ^{14}C-labeled and ^{13}C-enriched precursors in combination with ^{13}C NMR spectroscopy. The C-2 carbon of [2-^{13}C]tryptophan was shown to be involved at the C-3 carbon in carbazomycin B and both carbons of [1,2-^{13}C]acetate at the C-1 and C-10 moiety of the antibiotic.

[CH_3-^{13}C]Methionine was involved at the methoxyl group but not at the methyl group on the C-2 carbon of the antibiotic. Neither of the labeled carbons, [1-^{14}C]tryptophan nor [2,3-^{13}C]propionic acid, was detected in the antibiotic, and a progenitor of the C-2 and C-11 moiety of the antibiotic has not been determined.

Carbazomycins A and B were produced by an unidentified microorganism, tentatively designated as strain H 1051-MY 10. Carbazomycins inhibited mainly the growth of phytopathogenic fungi and also showed weak antibacterial and antiyeast activities[1]. The structure of carbazomycin B (**I**) was determined to be 4-hydroxy-3-methoxy-1,2-dimethylcarbazole by ^1H and ^{13}C NMR spectra[2] and by X-ray crystallographic analysis[3]. Consequently, the structure of carbazomycin A (**II**) was postulated to be 3,4-dimethoxy-1,2-dimethylcarbazole[1,2]. Thus, carbazomycins are the first antibiotics shown to contain carbazole nucleus[1].

Taxonomy of the carbazomycin-producing microorganism and the biosynthesis of carbazomycin B are reported in this paper.

Taxonomy of Carbazomycin-Producing Microorganism

Strain H 1051-MY 10 was isolated from a soil sample collected in Ni-imi City, Okayama Prefecture, Japan. Strain H 1051-MY 10 grows well on the media recommended in the International Streptomyces Project (ISP)[4]. It produces good aerial mycelium on ISP-media 2, 3 and 4. The color of the aerial mycelium is pale brown with grayish pink tinge. Aerial mycelium is cottony, and, when examined microscopically, consists of long and branched hyphae bearing many whorls (Biverticulate forms) as shown in Fig. 1. The spore surface is smooth as shown in Fig. 2. Morphological characteristics of strain H 1051-MY 10, place it in the genus *Streptoverticillium*. For microscopic observation, an agar medium consisting of 1% soluble starch or maltose, 0.2% yeast extract and 1.5% agar (pH 7.0) is superior to ISP-media.

Table 1. Cultural characteristics of strain H 1051-MY 10.

	Growth	Aerial mycelium	Reverse side of colony	Soluble pigment
Yeast extract - malt extract agar (ISP medium 2)	Good	Good Pale brownish yellow [3 ba]	Brown [3 pi~4 pi]	Light brown
Oatmeal agar (ISP medium 3)	Good	Good Pale brown with pink tint [3 cb]	Brown [3 le]	Light brown
Inorganic salts - starch agar (ISP medium 4)	Good	Good Pale brown with pink tint [3 cb]	Brown [3 ie~3 lg]	Pale brown
Glycerol - asparagine agar (ISP medium 5)	Moderate	Poor White to pale brownish yellow [3 ba]	Pale pinkish yellow [3 ca]	None

Hue numbers in parentheses were described according to the Color Harmony Manual.[6]

The cultural and physiological characteristics of strain H 1051-MY 10 are shown in Table 1 and Table 2, respectively. The following species were selected from BERGEY's manual[5] on the basis of cultural and physiological properties, and were compared with strain H 1051-MY 10 according to ISP procedures: *Streptoverticillium cinnamoneum* (ISP 5005), *Streptoverticillium hachijoense* (ISP 5114), *Streptoverticillium kentuckense* (ISP 5259) and *Streptoverticillium ehimense* (ISP 5253). The results indicate that strain H 1051-MY 10 is similar to *S. ehimense*. Differences observed

Fig. 1. Photomicrograph of strain H 1051-MY 10. (on maltose - yeast extract agar, ×100)

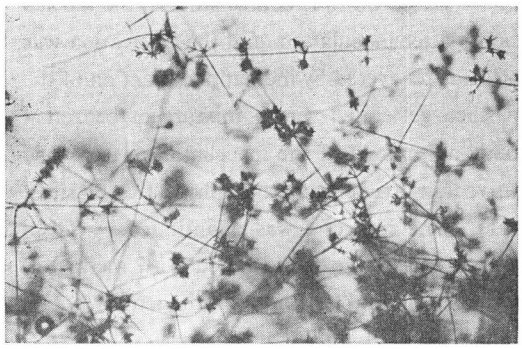


Fig. 2. Electron micrograph of spore chains of strain H 1051-MY 10.

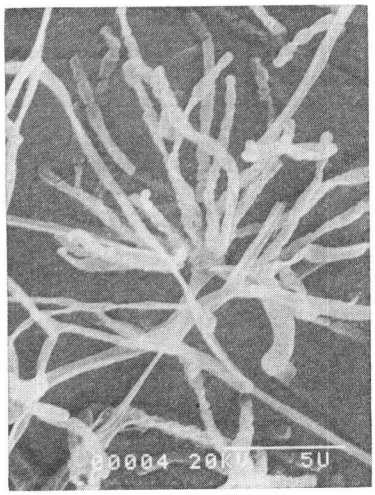


Table 2. Physiological characteristics of strain H 1051-MY 10.

Melanin production on Tyrosine agar (ISP medium 7)	Negative
Peptone - yeast extract iron agar (ISP medium 6)	Positive
Tryptone yeast extract broth (ISP medium 1)	Weakly positive
H_2S production	Positive
Starch hydrolysis	Positive
Gelatin liquefaction	Positive
Skim milk	
Coagulation	Negative
Peptonization	Positive
Carbon utilization	
D-Glucose	+
L-Arabinose	−
D-Xylose	−
D-Fructose	+
D-Mannitol	−
Sucrose	−
L-Rhamnose	−
Raffinose	−
Cellulose	−
i-Inositol	+
Salicin	−

between these two strains were as follows: the color of aerial mycelium of strain H 1051-MY 10 on ISP medium 4 was lighter (3 cb)[6] than that of *S. ehimense* (5 cb)[6]. *S. ehimense* utilized D-mannitol as a carbon source, but strain H 1051-MY 10 did not.

D-Mannitol utilization by *S. ehimense* is positive according to the ISP description[4]; but is described as trace utilization in BERGEY's manual. Hydrogen sulfide production by *S. ehimense* is negative according to BERGEY's manual, but was positive in our experiments.

Since the above differences between *S. ehimense* and strain H 1051-MY 10 are not enough to conclude that these two strains are distinct species, strain H 1051-MY 10 was assigned as a strain of *Streptoverticillium ehimense* H 1051-MY 10.

Biosynthesis of Carbazomycin B

Carbazole alkaloids have been isolated from higher plants (mostly Rutaceae) and have been classified into three groups, namely C_{13}-, C_{18}- and C_{23}-skeleton groups[7~9]. Carbazomycins are unique because of the C_{14}-skeleton and the 1,2,3,4-tetrasubstituted carbazole nucleus. Thus, biosynthesis of the antibiotics can be expected to be different from that of the alkaloids of higher plants[7~9] in which the contribution of mevalonic acid was proved. Determination of the progenitors of the substituted benzene nucleus is especially interesting from a viewpoint of biosynthesis.

The biosynthesis of **I** was studied in shake cultures of *S. ehimense* H 1051-MY 10, and the fermentation experiments were performed as described previously[1]. Based on time-course studies, the labeled precursors were added to the cultures aseptically at 48 hours after inoculation, and the cultivation was continued for additional 48 hours. About 50 mg/liter of **I** was recovered by methanol extraction of the mycelial cake followed by column chromatography on silica gel eluted with a mixture of benzene - acetone (30: 1). For radioactivity measurement, the methanol extract of the mycelial cake from one flask was evaporated to dryness and separated by preparative thin-layer chromatography (TLC) on silica gel developed with a mixture of benzene - acetone (10: 1). The spot of **I** was quantitated by TLC-chromatoscanner (Shimadzu CS-920) at 245 nm. The spot was scraped off, eluted with methanol, evaporated to dryness and dissolved in a toluene-based scintillation fluid to measure radioactivity. For ^{13}C NMR measurements, **I** was purified by silica gel column chromatography of the extract of the mycelial cake using a mixture of benzene - acetone (30: 1) as the eluent. Recrystallization from a benzene - *n*-hexane mixture yielded yellow prisms with a melting point of 160~162°C.

The structure of **I** strongly suggests that tryptophan and a C-1 unit would be logical precursors. In our first experiments with ^{14}C-labeled precursors, DL-[3-^{14}C]tryptophan, L-[1-^{14}C]tryptophan, L-[CH_3-^{14}C]methionine, sodium[1-^{14}C]-acetate and sodium[2-^{14}C]acetate were added to the culture medium composed of 1.5% soluble starch, 1.0% glucose, 2.0% soy bean meal, 0.5% Ebios (dried yeast, distributed by Tanabe Pharmaceutical Co., Ltd.), 0.25% NaCl and 0.3% $CaCO_3$ (pH 7.6 before sterilization).

As shown in Table 3, DL-[3-^{14}C]tryptophan and L-[CH_3-^{13}C]methionine gave good total in-

Table 3. Incorporation of ^{14}C-labelled precursors to carbazomycin B (**I**).

Labelled compounds	Radioactivity fed (μCi/flask)	Yield (μmole/flask)	Total incorporation (%)
DL-[3-^{14}C]-Tryptophan	2.5	22	14.7
L-[$^{14}CH_3$]-Methionine	2.5	15	1.9
Sodium[1-^{14}C]-acetate	2.5	17	0.12
Sodium[2-^{14}C]-acetate	2.5	14	0.24
L-[1-^{14}C]-Tryptophan	2.5	20	0.0

corporations, 14.7% and 1.9%, respectively, into **I**. While, [1-^{14}C] and [2-^{14}C]acetates gave lower incorporations of 0.12% and 0.24%, respectively, and L-[1-^{14}C]tryptophan was not incorporated at all. These high incorporation data prompted us to carry out feeding experiments with ^{13}C-enriched compounds to locate the labeled carbons. By feeding L-[CH_3-^{13}C]methionine, isolated **I** showed the ^{13}C NMR spectrum only enriched at the carbon signal due to the *O*-methyl group as seen in Fig. 3. DL-[2-^{13}C]Tryptophan mixed with DL-[3-^{14}C]tryptophan (already known to be involved) was fed to obtain radioactive **I** in 12.1% incorporation ratio. The ^{13}C NMR spectrum of the compound in acetone-d_6 showed the significant increment of the signal of C-3 (δ 139.3, singlet) as shown in Fig. 4. This fact proved the contribution of tryptophan to C-3 and C-4 of the hexa-substituted benzene ring besides the indole ring. Though the incorporation ratios of radioactive acetates were not very high, doubly labeled sodium[1,2-^{13}C]acetate was fed expecting the appearance of satellite signals due to ^{13}C-^{13}C coupling. As seen in Fig. 5, the ^{13}C NMR signals of C-1 and C-10 of **1** appeared along with satellite peaks of J=46 Hz, reasonable for sp^3-sp^2 coupling constant.

As for remaining two carbons, namely C-2 and C-11, suitable precursors are not yet found. Plau-

Fig. 3. ^{13}C NMR spectrum of carbazomycin B derived from [$^{13}CH_3$]methionine (in $CDCl_3$).

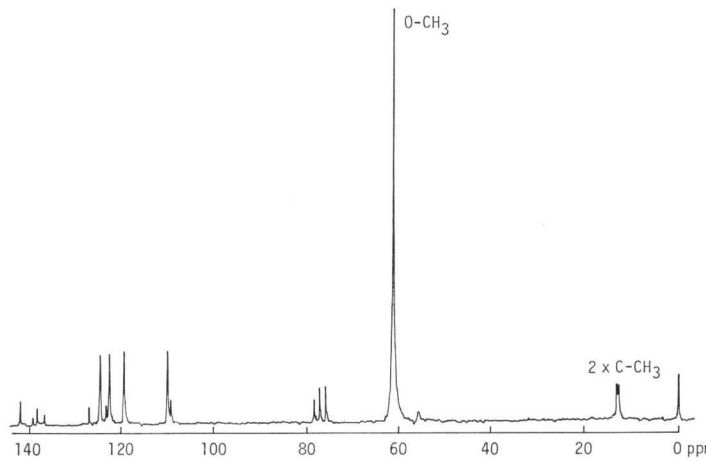


Fig. 4. ^{13}C NMR spectra of carbazomycin B; (A) derived from [2-^{13}C]tryptophan, (B) natural abundance (in acetone-d_6).

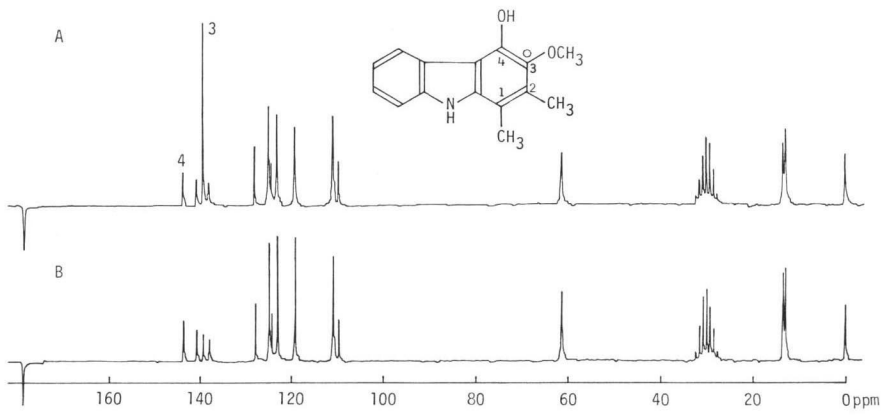


Fig. 5. ^{13}C NMR spectrum of carbazomycin B derived from sodium[1,2-^{13}C]acetate (in $CDCl_3$).

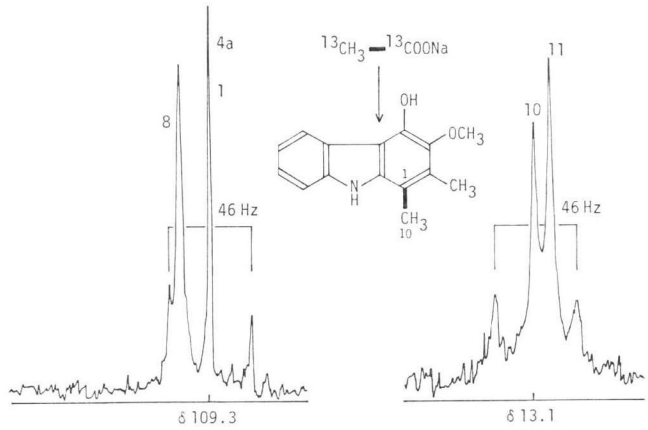
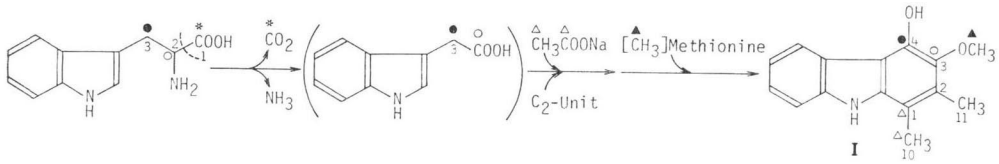


Fig. 6. Biosynthetic scheme for carbazomycin B.

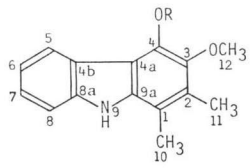


sible candidates, such as [1,2- or 3-^{14}C]propionate, [1-^{14}C]glycolate, [2-^{14}C]pyruvate and L-[U-^{14}C]alanine gave negative results.

Although the origin of the C_2-part (C-2 and C-11) of **I** has not been clarified, the present study has indicated a biosynthetic scheme for **I**, where tryptophan reacts with acetate and an unknown unit after decarboxylation and deamination, followed by *O*-methylation with methionine as shown in Fig. 6.

During our study of biosynthesis, it was concluded that the assignments of ^{13}C NMR of **I** and **II** previously reported[2,3] should be revised as indicated in Table 4. The ^1H NMR (270 MHz) assignments in acetone-d_6 for H-5, H-6, H-7 and H-8 in **I** are δ 8.25, 7.11, 7.27 and 7.41, respectively. Then, C-5, C-6, C-7 and C-8 of **I** were assigned by proton selective decoupling experiments. The C-1 signal previously assigned to δ 110.0 (overlapped with C-8) should be assigned to δ 109.3 (overlapped with C-4a), because previous assignments were incorrect due to misreading of multiplicity of off-resonance spectra. When the spectrum was measured in acetone-d_6, there appeared two singlet peaks at δ 110.6 (C-4a) and δ 109.7 (C-1), and a doublet at δ 111.0 (C-8). Two *C*-methyl signals at δ 12.7 and 13.1 had previously been assigned to C-10 and C-11, respectively. In the double labeled experiments, as already described, satellite signals of **I** were observed at the lower methyl signal (δ 13.1) and at the C-1 (δ 109.3) which was quite distinct from that of C-2 (δ 127.0). Further, the lower methyl signal in the ^1H NMR (δ 2.37, 10-CH_3) of **I** corresponded with the lower carbon signal (δ 13.3) in acetone-d_6 by proton selective decoupling experiments at 270 MHz (67.5 MHz for ^{13}C). Thus, previous assignments of two *C*-methyl signals should be reversed. The previous assignments of C-3 (δ 142.0) and C-4 (δ 138.5) should also be reversed as shown in Table 4. When [2-^{13}C]tryptophan was fed, the signal at δ 138.5 (δ 139.3 in acetone-d_6) of **I** was enlarged. This labeled compound of **I** was methylated with dimethyl sulfate to obtain labeled **II**. The ^{13}C NMR of labeled **II** showed an enlarged peak at δ 144.4 ($\Delta\delta$ +3.9). This substitution-induced

Table 4. Revised assignments of ^{13}C-chemical shifts (δ) of carbazomycins A and B.



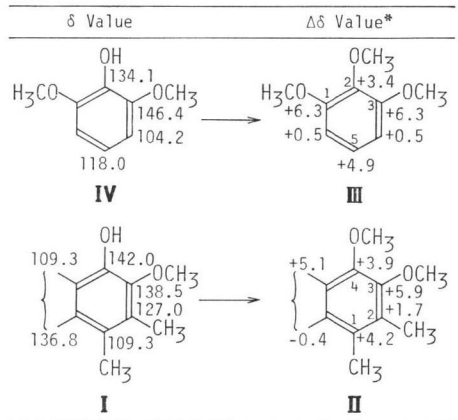
Carbazomycin B: R=H (I)
Carbazomycin A: R=CH_3 (II)

Carbon No.	Carbazomycin B	Carbazomycin A
C-1	109.3 (109.7)	113.5
C-2	127.0 (127.8)	128.7
C-3	138.5 (139.3)	144.4
C-4	142.0 (143.6)	145.9
C-4a	109.3 (110.6)	114.4
C-4b	123.3 (124.3)	122.8
C-5	122.7 (123.1)	122.5
C-6	119.5 (119.2)	119.4
C-7	124.7 (124.8)	125.0
C-8	110.0 (111.0)	110.3
C-8a	139.3 (140.7)	139.4
C-9a	136.8 (137.9)	136.4
C-10 (1-CH_3)	13.1 (13.3)	13.6
C-11 (2-CH_3)	12.7 (12.8)	12.6
C-12 (3-OCH_3)	61.4 (61.3)	61.1*
C-13 (4-OCH_3)	—	60.5*

Spectra were taken with JEOL PFT-100 spectrometer equipped with EC-6 computer at 25.15 MHz using tetramethylsilane as an internal standard and $CDCl_3$ as a solvent. Values in parentheses were taken in acetone-d_6. Parameters are as follows: spectral width 5 KHz, pulse width 16 μseconds (45°), repetition time 2 seconds, computer limited resolution $\pm$0.1 ppm.

* May be reversed.

Fig. 7. Substitution-induced shifts of aromatic carbons by *O*-methylation.



shift value ($\Delta\delta$) was compared with that of a model compound, 1,2,3-trimethoxybenzene (**III**)[10]. As shown in Fig. 7, on going from 1, 3-dimethyl-2-hydroxybenzene (**IV**), C-1 and C-3 carbons of **III** showed the largest $\Delta\delta$ value (+6.3), while the substituted position (C-2) showed smaller $\Delta\delta$ value (+3.4). Further, the *para*-carbon (C-5) of **III** showed fairly large $\Delta\delta$ value (+4.9). In the previous assignments of ^{13}C NMR of **I**, all signals except C-3 and C-4 were in good accordance with this shift trend. Thus, assignments of C-3 and C-4 can only be explained by being reversed.

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