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BIOSYNTHETIC STUDY USING ^{13}C-ENRICHED PRECURSORS

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3-Morpholinopropylamino-bleomycin (MOP-BLM) was produced by the culture of *Streptomyces verticillus* ATCC 15003 grown in the presence of a ^{13}C-enriched compound and 3-morpholinopropylamine in the medium, and the resulting MOP-BLM was analyzed by ^{13}C-NMR spectrometry. In the spectrum of MOP-BLM produced by addition of ^{13}C-methyl-L-methionine, the intensities of two signals assigned to the methyl carbon of the pyrimidine chromophore and of the 2-position of the 4-amino-3-hydroxy-2-methylpentanoic acid moiety were remarkably enhanced. When DL-alanine-3-^{13}C was added, the signals emanated from the 5-methyl and 2-methine carbons of the 4-amino-3-hydroxy-2-methylpentanoic acid moiety were markedly enhanced. These results definitely indicate that the methyl group of the pyrimidine moiety originates from methionine-methyl group and the carbon skeleton of the pentanoic acid moiety is formed from alanine, acetate and methionine-methyl group. This study also revealed that the former assignment of the two signals from the two methyl carbons of the 4-amino-3-hydroxy-2-methylpentanoic acid moiety must be reversed.

As reported briefly[1], we have previously studied the biosynthesis of bleomycin (BLM) by isolating of the biosynthetic intermediates and by testing the incorporation of ^{14}C-labeled compounds. These studies showed that the methionine-methyl group was incorporated into the methyl group of the pyrimidine chromophore after the synthesis of the pyrimidine-ring, and suggested that the 4-amino-3-hydroxy-2-methylpentanoic acid moiety was formed from alanine, acetate and methionine-methyl group. To confirm these findings and to clarify the ambiguity of the assignment of the two methyl-carbon signals of the 4-amino-3-hydroxy-2-methylpentanoic acid moiety, we studied the biosynthesis of bleomycin by ^{13}C-NMR spectrometry of the bleomycin produced by fermentation with specific ^{13}C-enriched substances.

Experimentals

L-Methionine-methyl-^{13}C (90 atom %) and DL-alanine-3-^{13}C (90 atom %) were purchased from Commissariat a L'Energie Atomique and Merck Sharp and Dohme Canada Limited, respectively.

^{1}H-Noise decoupled ^{13}C FT NMR spectra were recorded on a Varian XL-100-DISK FT spectrometer at 25.16 MHz and ambient probe temperature (30°C) using 5-mm tube. About 40 mg of the sample was dissolved in 0.3 ml of deuterium oxide and the pHm (pH meter reading uncorrected for deuterium isotope effects) of the solution was adjusted to 6.6~6.7. FT NMR measurement conditions were as follows: spectrum width, 5120 Hz; pulse flipping angle, 35°; acquisition time, 0.8 sec.; number of data points, 8,191; number of transients, 14,682. Dioxane was used as the internal reference of ^{13}C-chemical shift, which was adjusted to δ 67.40.

Fermentation

Streptomyces verticillus ATCC 15003 was cultured in seven 500-ml flasks each containing 100 ml of

the following medium: 6.4% millet jelly, 0.5% glucose, 3.5% soy bean meal, 0.75% corn steep liquor, 0.3% NaCl, 0.2% $NaNO_3$, 0.1% K_2HPO_4, 0.05% $ZnSO_4 \cdot 7H_2O$, 0.01% $CuSO_4 \cdot 5H_2O$, 0.01% 3-morpholinopropylamine. The inoculated flasks were aerated rotationally at 28°C for 7 days. The ^{13}C-enriched compound was added 7 times during the third to fifth day of the fermentation. The total amount of the ^{13}C-enriched methionine added to the 7 flasks was 34.3 mg. In another culture experiment 242 mg of the ^{13}C-enriched alanine was added to the seven flasks in the same way.

Extraction and Purification

The culture filtrate was passed through a column of Amberlite XAD-2 (90 ml). The adsorbed material was eluted with 1: 4 mixture of 0.01 N HCl and methanol. The eluate was dried under diminished pressure, the dried material was extracted with 80% methanol, and the methanol soluble fraction was passed through a column of alumina (20 ml). The effluent was dried under diminished pressure and the dried material was chromatographed on CM-Sephadex C-25 column (30 ml). Elution with a linear gradient of NaCl solution (0.05 M→1.0 M) gave blue-colored 3-morpholinopropylamino-bleomycin (MOP-BLM), a bleomycin containing 3-morpholinopropylamine as the terminal amine[2]. An appreciable amount of any other congener of bleomycin was not detected by this chromatography. The colorless copper-free MOP-BLM was obtained by treatment of the blue MOP-BLM with hydrogen sulfide in methanol. Thus, from the 700 ml cultures, 48 mg of the metal-free ^{13}C-methionine-incorporated MOP-BLM (^{13}C-Met-MOP-BLM), 58 mg of the ^{13}C-alanine-incorporated MOP-BLM (^{13}C-Ala-MOP-BLM), and 48 mg of MOP-BLM (without addition of a ^{13}C-enriched substance for the control) were obtained.

Results and Discussion

The ^{13}C-NMR spectra of ^{13}C-Ala-MOP-BLM, ^{13}C-Met-MOP-BLM and natural abundance MOP-BLM are shown in Fig. 1-a, -b, and -c. All of the signals have been already assigned by us except for

Fig. 1. ^{13}C-NMR spectra of ^{13}C-Ala-MOP-BLM, ^{13}C-Met-MOP-BLM and natural abundance MOP-BLM.

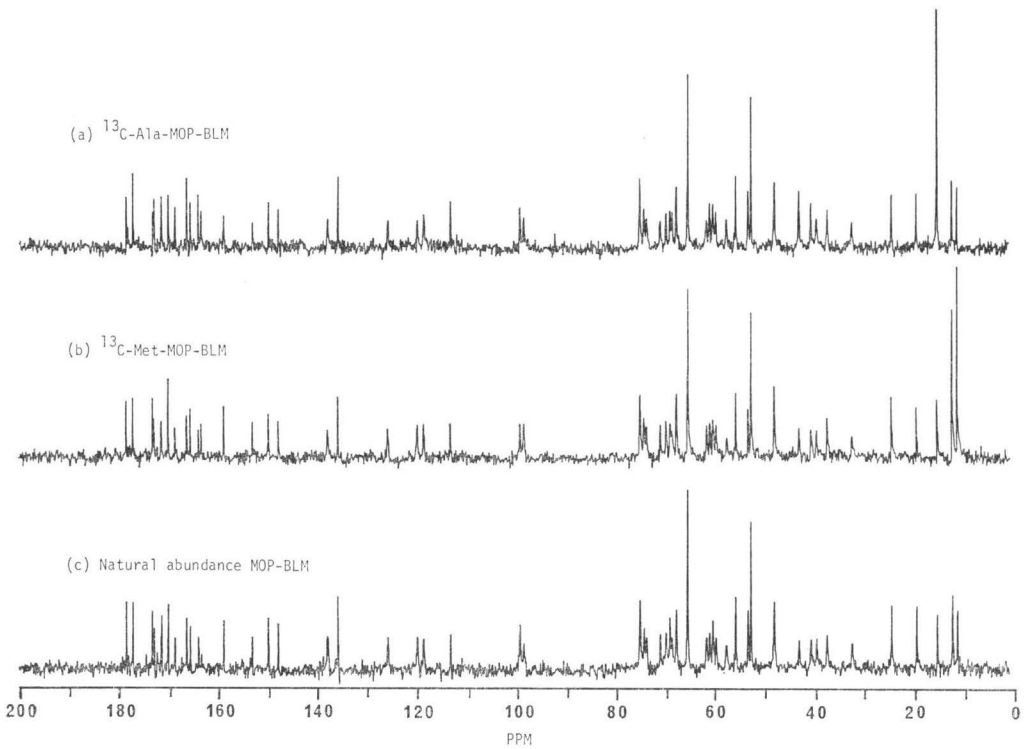


Table 1. The ^{13}C-chemical shifts and ^{13}C-enrichment ratios of ^{13}C-Ala-MOP-BLM and ^{13}C-Met-MOP-BLM.

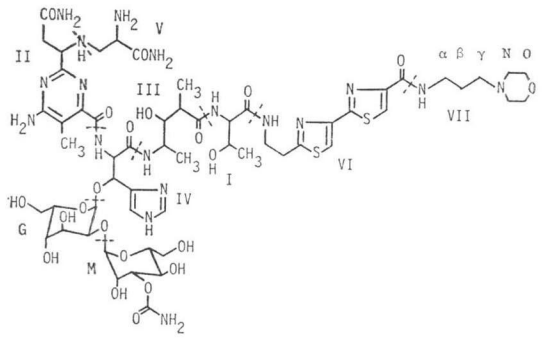
Carbon*	^{13}C-Ala-MOP-BLM		^{13}C-Met-MOP-BLM		Carbon*	^{13}C-Ala-MOP-BLM		^{13}C-Met-MOP-BLM	
	Chemical shift	Enrich ratio	Chemical shift	Enrich ratio		Chemical shift	Enrich ratio	Chemical shift	Enrich ratio
III-CO	178.3	1.04	178.3	0.93	G-4	69.9	1.06	69.9	1.21
II-S-CO	176.9	0.93	176.9	0.85	M-2	69.1	0.75	69.1	0.84
V-CO	172.9	0.72	173.0	1.04	IV-β-CH	68.7	1.12	68.8	0.98
I-CO	172.7	0.88	172.7	0.80	I-β-CH	67.9	0.99	67.9	1.13
VI-CO	171.2	0.99	171.2	0.73	G-5	67.9		67.9	
IV-CO	169.8	0.78	169.8	1.11	M-4	65.4	0.93	65.4	0.96
II-R-CO	168.4	0.92	168.5	0.77	VII-O-CH_2	65.4×2		65.4×2	
II-2	166.1	1.38	166.1	1.02	M-6	61.7	0.81	61.7	1.11
II-4	165.4	1.02	165.4	1.14	G-6	61.1	1.12	61.1	1.04
VI-2	163.8	1.44	163.8	0.90	V-CH	60.5	0.88	60.5	0.88
VI-2′	163.2	0.98	163.2	1.03	I-α-CH	59.9	1.13	59.9	1.08
M-CO	158.7	0.77	158.7	1.17	IV-α-CH	57.8	1.15	57.8	0.82
II-6	152.9	0.73	152.9	1.11	VII-γ-CH_2	55.9	0.94	55.9	0.95
VI-4	149.6	0.89	149.6	1.00	II-CH	53.4	1.02	53.5	1.03
VI-4′	147.6	0.84	147.7	0.90	VII-N-CH_2	52.9×2	1.00	52.9×2	1.00
IV-2	137.7	0.86	137.8	0.91	III-γ-CH	48.2	0.93	48.3	1.11
IV-4	135.6	0.96	135.7	0.97	V-CH_2	48.2		48.3	
VI-5′	125.7	0.88	125.7	0.98	III-α-CH	43.4	1.98	43.4	1.19
VI-5	119.8	0.90	119.8	1.05	II-CH_2	41.0	1.42	41.0	1.17
IV-5	118.5	1.16	118.5	1.25	VI-β-CH_2	40.0	0.91	39.9	1.03
II-5	113.0	1.01	113.1	0.95	VII-α-CH_2	37.7	1.03	37.7	1.16
M-1	99.0	1.16	99.0	1.04	VI-α-CH_2	32.8	0.84	32.8	0.91
G-1	98.3	0.75	98.3	0.90	VII-β-CH_2	24.8	0.84	24.9	0.97
III-β-CH	75.1	0.98	75.2	1.11	I-CH_3	19.7	0.78	19.7	0.89
M-3	75.1		75.2		III-γ-CH_3	15.7	4.01	15.7	1.14
G-2	74.3	1.02	74.3	1.09	III-α-CH_3	12.7	0.90	12.7	2.30
M-5	73.9	0.99	73.9	0.99	II-CH_3	11.7	1.02	11.6	2.90
G-3	71.1	0.70	71.1	0.95					

* For the numbering, see Fig. 2 and reference 3.

the signals from the terminal amine of MOP-BLM[3]. The ^{13}C-chemical shifts and ^{13}C-enrichment ratios of ^{13}C-Ala-MOP-BLM and ^{13}C-Met-MOP-BLM are shown in Table 1. The ^{13}C-enrichment is represented by the signal height ratio to the corresponding individual signal of the natural abundance ^{13}C-NMR spectrum, and the signal of VII-N-CH_2 (for the numbering, see Fig. 2 and reference 3) at δ 52.9 (×2) was taken as the reference (the enrichment ratio: 1.00), because the incorporation of the added ^{13}C-enriched material into the unnatural morpholine is impossible. The chemical shift difference between the two spectra was within 0.1 ppm. The slight difference in the chemical shift values from the table published earlier[3] is due to corrected processing of the data and the difference of the measurement pH*. The assignment of the methyl carbon at the 2-position (III-α-CH_3) and the 5 methyl carbon (III-γ-CH_3) of the 4-amino-3-hydroxy-2-methylpentanoic acid moiety in Table 1 is reversed from the former table[3], the reason for which is discussed below.

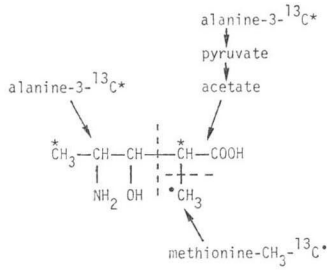
* H. NAGANAWA, T. TAKITA and H. UMEZAWA: Unpublished.

Fig. 2. Structure of MOP-BLM.



Boundaries between the labeled residues are indicated with broken lines.

Fig. 3. The biosynthetic origin of 4-amino-3-hydroxy-2-methylpentanoic acid (III) of bleomycin.



In the ^{13}C-NMR spectrum of ^{13}C-Met-MOP-BLM the signals at δ 11.6 and 12.7 were remarkably enhanced, while in the spectrum of ^{13}C-Ala-MOP-BLM the signal at δ 15.7 was greatly enhanced. The assignment of the signal at δ 11.6 to II-CH_3 has been conclusively confirmed by a single frequency ^{1}H-decoupling experiment[3]. However, the assignment of the signals at δ 12.7 and 15.7 was not definite, although the former was tentatively assigned to III-γ-CH_3 and the latter to III-α-CH_3 from the spectra of the peptide components[3]. The ambiguity arose from the fact that (1) both signals always appeared as a pair in the spectra, (2) the single frequency irradiation of these methyl protons for selective decoupling was impossible due to the signal overlapping of these methyl protons and (3) the selective long-range ^{1}H-decoupling did not give a conclusive result. The definitive assignment of these signals is only possible by this ^{13}C-enrichment technique. The previous biosynthetic study[1] suggested that the 4-amino-3-hydroxy-2-methylpentanoic acid moiety is formed from alanine, acetate and methionine-methyl group[1]. Therefore, the signal at δ 15.7 enhanced by addition of alanine-3-^{13}C must be assigned to the 5-methyl carbon (III-γ-CH_3) of the pentanoic acid moiety, and the signal at δ 12.7 enhanced by ^{13}C-methionine must be assigned to the methyl carbon at the 2-position (III-α-CH_3). This assignment is the reverse of the former assignment[3]. Thus, the incorporations of alanine into the C3 to C5 part and methionine-methyl into the side methyl of the 4-amino-3-hydroxy-2-methylpentanoic acid moiety of bleomycin were conclusively confirmed (Fig. 3).

In the spectrum of ^{13}C-Ala-MOP-BLM, the signal at δ 43.4 (III-α-CH) was also significantly enhanced (the ^{13}C-enrichment ratio: 1.98). It was confirmed by the repeated measurement of the spectrum (the ^{13}C-enrichment ratio: 1.93). Incidentally, in the same spectrum (Fig. 1a) the ^{13}C-enrichment ratios of other slightly enhanced signals at δ 166.1 (the ^{13}C-enrichment ratio 1.38), 163.8 (1.44) and 41.0 (1.42) were 1.25, 1.15 and 1.19 in the remeasured spectrum, respectively. The signal at δ 43.4 has previously been assigned to emanate from the α-methine carbon of the 4-amino-3-hydroxy-2-methylpentanoic acid moiety[3]. The previous biosynthetic study[1] suggested that this carbon atom originates from the methyl group of acetate. It is well-known that alanine is readily converted into acetyl-CoA *via* pyruvate. Therefore, the ^{13}C-enrichment at the α-methine carbon of the pentanoic acid moiety by addition of alanine-3-^{13}C is confirmatory evidence for the acetate-incorporation into the C1 and C2 part of the pentanoic acid moiety (Fig. 3).

On the basis of the fact that the carbon chain of the 4-amino-3-hydroxy-2-methylpentanoic acid moiety of bleomycin is biosynthesized from alanine and acetate, the peptide chain of bleomycin is sug-

gested to be elongated by a multienzyme system in a similar manner to gramicidin S[4].

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