BIOSYNTHESIS OF BENANOMICINS†

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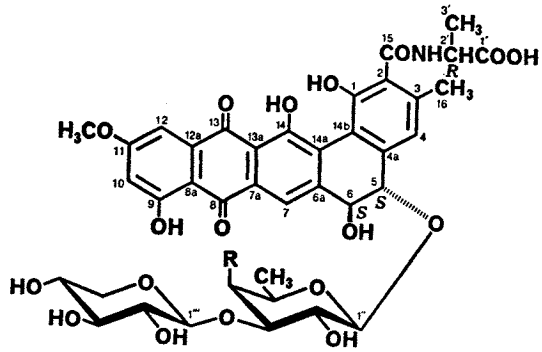
The biosynthesis of benanomicins A and B, produced by actinomycete strain MH193-16F4, was investigated by feeding experiments with ^{14}C- and ^{13}C-labeled compounds followed by measurement of radioactivity and ^{13}C NMR analysis. A 2D double quantum coherence NMR spectrum of benanomicin A derived from sodium [1,2-$^{13}C_2$]acetate provided the location of intact acetate units, and confirmed the ^{13}C assignments of benanomicins. The results indicate that the aglycone of benanomicins is derived from a dodecaketide, methionine and alanine.

New antifungal antibiotics, benanomicins A (**1**) and B (**2**) are produced by actinomycete strain MH193-16F4 related to *Actinomadura* or *Microtetraspora*, and contain a benzo[*a*]naphthacene quinone, D-alanine and disaccharide in their molecules[1]. The antibiotics exhibited excellent therapeutic effects against systemic fungal infections in mice[2], and inhibited infection of T-cell with human immunodeficiency virus and syncytium formation by the virus[3]. The absolute structures of benanomicins A and B were determined to be **1** and **2**, respectively, by NMR analysis†† and chemical degradation[4], and are shown in Fig. 1.

In this paper, we report the biosynthetic origin of the carbon atoms in the aglycone of benanomicins based on feeding experiments with ^{14}C- and ^{13}C-labeled compounds. The structures of benanomicins and ^{13}C NMR assignments were confirmed by the location of ^{13}C-enriched and ^{13}C-^{13}C connected carbons in benanomicins derived from singly- and doubly-labeled acetates.

Fig. 1. Structures of benanomicins.



Benanomicin A (**1**) R=OH
Benanomicin B (**2**) R=NH_2

† Most of this work was reported at the 243rd JARA Research Meeting held in Tokyo on Dec. 13, 1988.

†† As previously reported[1,4], the nuclear Overhauser effect (NOE) between 5-H and 1″-H in the ^1H-^1H NOE correlation spectra of benanomicins was obtained. Furthermore, in the sequential NMR analysis NOE's between 4-H and 6″-H, 16-H and 6″-H were clearly shown in the NOE difference spectra of benanomicins. From these results, the remaining absolute configurations at C-5 and C-6 positions were confirmed to be 5*S* and 6*S*, as in the structure of pradimicin A[5].

Materials and Methods

Labeled Compounds

Sodium [1-^{14}C]acetate, sodium [1-^{14}C]propionate, L-[*methyl*-^{14}C]methionine, L- and D-[1-^{14}C]alanines and D-[*U*-^{14}C]glucose were purchased from ICN Radiochemicals, Inc., CA, U.S.A. The specific radioactivities are shown in Table 1. Sodium [1-^{13}C]acetate (98% ^{13}C enriched), sodium [1,2-$^{13}C_2$]acetate (90%), L-[*methyl*-^{13}C]methionine (99%) and L-[1-^{13}C]alanine (99%) were obtained from SCETI Co., Ltd., France.

General Procedure

Radioactivities were measured in a 20-ml vial with a TRI-CARB Model 2000CA liquid scintillation analyzer by using 10 ml of AQUASOL-2 (NEN Research Products, Boston) as a scintillation cocktail. ^{13}C NMR spectra were obtained at 40°C in DMSO-d_6 on a Jeol JNM-GX400 spectrometer at 100 MHz. Chemical shifts were recorded in ppm using a center peak of DMSO-d_6 (39.5 ppm) as the internal reference. The 2D double quantum coherence (INADEQUATE) experiment on **1** (30 mg/0.5 ml, 5-mm tube) derived from sodium [1,2-$^{13}C_2$]acetate was carried out as follows: Spectral width, 21 KHz; data points, 2K (zero-filled to 4K); column points, 64 (zero-filled to 256); scans, 1,152; pulse delay time, 2.5 seconds; pulse interval ($1/4J_{CC}$), 4.167 mseconds. Preparative TLC was done on a Silica gel 60 F_{254} plate 0.5 mm thick (E. Merck, Art. No. 5744) using BuOH - AcOH - pyridine - water (6:1:4:3) as the developing solvent. Antibiotics on the TLC plates were detected either by their color or by use of UV light (254 nm). The antimicrobial activity was determined by the paper-disc method using *Candida albicans* M9001 as a test organism.

Fermentation

A slant culture of strain MH193-16F4 (FERM P-9529) was used to inoculate 80 ml of a medium containing starch 1.0%, soybean flour (Ajinomoto) 3.0%, silicone oil 0.01% and Adekanol (Adeka Co., Ltd.) 0.01% (pH 7.0) in a 500-ml Sakaguchi flask. The vegetative growth (2.5 ml) on a rotary shaker (135 rpm) at 28°C for 72 hours was transferred into a 500-ml baffled Erlenmeyer flask containing 80 ml of the same medium. The 48-hour culture (3.2 ml) was transferred into 80 ml of a production medium consisting of glycerol 2.0%, soluble vegetable protein (Sun-grain, Suntory Co.) 1.5%, silicone oil 0.03%, Adekanol 0.01%, K_2HPO_4 0.0025%, KH_2PO_4 0.1125% and $CoCl_2 \cdot 6H_2O$ 0.005% (pH 6.2) in a 500-ml baffled Erlenmeyer flask. Fermentation was carried out under the conditions described above. After 48 hours when benanomicins were being produced, an aqueous solution (0.5~1.0 ml) of each labeled compound was added to the medium and the fermentation was continued for 48 hours. In all feeding experiments, the productivity of benanomicins was about 500 μg/ml.

Determination of Incorporation Ratios of ^{14}C-Labeled Compounds

To the fermentation broth was added 80 ml of acetone. The mixture was stirred for 20 minutes and filtered. After evaporation of the filtrate, the residue was dissolved in 100 ml of water. ^{14}C-Labeled benanomicins in the solution (1 ml) were adsorbed on a column of Diaion HP-20. The column was washed with water and eluted with 50% aqueous acetone (20 ml). The eluate (1.6 ml) was concentrated and purified by preparative TLC. Two reddish purple zones containing radioactive **1** and **2** (Rf value: 0.57 and 0.45, respectively) were collected and suspended in the scintillation cocktail. The radioactivities of ^{14}C-labeled **1** and **2** were measured with a liquid scintillation analyzer and total incorporation ratios were calculated.

Preparation of ^{13}C-Labeled Benanomicins

Sodium [1-^{13}C]acetate (30 mg), sodium [1,2-$^{13}C_2$]acetate (35 mg), L-[*methyl*-^{13}C]methionine (20 mg) and L-[1-^{13}C]alanine (20 mg) were fed to each flask. To the fermentation broth, 80 ml of acetone was added. The mixture was stirred for 20 minutes, and filtered. The filtrate was concentrated and the residue was dissolved in water. Purification by Diaion HP-20 column chromatography described above followed by column chromatography of Sephadex LH-20 developed with methanol gave ^{13}C-labeled **1** (23~28 mg) and **2** (9~11 mg).

Results and Discussion

As shown in Table 1, [1-^{14}C]acetate, L-[*methyl*-^{14}C]methionine and D-[*U*-^{14}C]glucose were efficiently incorporated into benanomicins. Both D- and L-[1-^{14}C]alanines were moderately, but [1-^{14}C]propionate was negligibly incorporated. These results suggest that the benanomicin skeleton is derived from a polyacetate intermediate and that acylation of alanine and *O*-methylation occur.

In order to clarify the mode of incorporation of acetate, L-methionine and L-alanine into benanomicins, feeding experiments using ^{13}C-labeled compounds were carried out. The ^{13}C NMR spectra of **1** derived from [1-^{13}C] and [1,2-$^{13}C_2$]acetates are shown in Fig. 2. Arrows indicate the enriched carbon signals in the ^{13}C NMR spectrum of [1-^{13}C]acetate-labeled **1**. Enrichment ratios and ^{13}C-^{13}C coupling constants of [1-^{13}C] and [1,2-$^{13}C_2$]acetate-labeled benanomicins are listed in Table 2. Enrichment ratios were calculated from the relative intensity of C-1‴ as 1.0. In the ^{13}C NMR spectra of [1-^{13}C]acetate-labeled benanomicins, 12 alternate carbon signals of the aglycone except the methoxy

Table 1. Incorporation of ^{14}C-labeled compounds into benanomicins.

Labeled compounds	Specific activity (mCi/mol)	Radioactivity (dpm)			Incorporation into benanomicins (%)
		fed	**1**	**2**	
Sodium [1-^{14}C]acetate	9.6	3.32×10^7	3.15×10^6	1.44×10^6	13.8
Sodium [1-^{14}C]propionate	57.0	3.67×10^7	2.28×10^5	1.49×10^5	1.0
L-[*Methyl*-^{14}C]methionine	49.5	3.63×10^7	5.27×10^6	2.84×10^6	22.3
L-[1-^{14}C]Alanine	56.8	2.45×10^7	7.68×10^5	2.56×10^5	4.2
D-[1-^{14}C]Alanine	46.0	1.93×10^7	8.97×10^5	2.69×10^5	6.0
D-[*U*-^{14}C]Glucose	5.5	2.25×10^7	1.51×10^6	8.81×10^5	10.6

Fig. 2. ^{13}C NMR spectra of **1** derived from sodium [1-^{13}C] and [1,2-$^{13}C_2$]acetates.

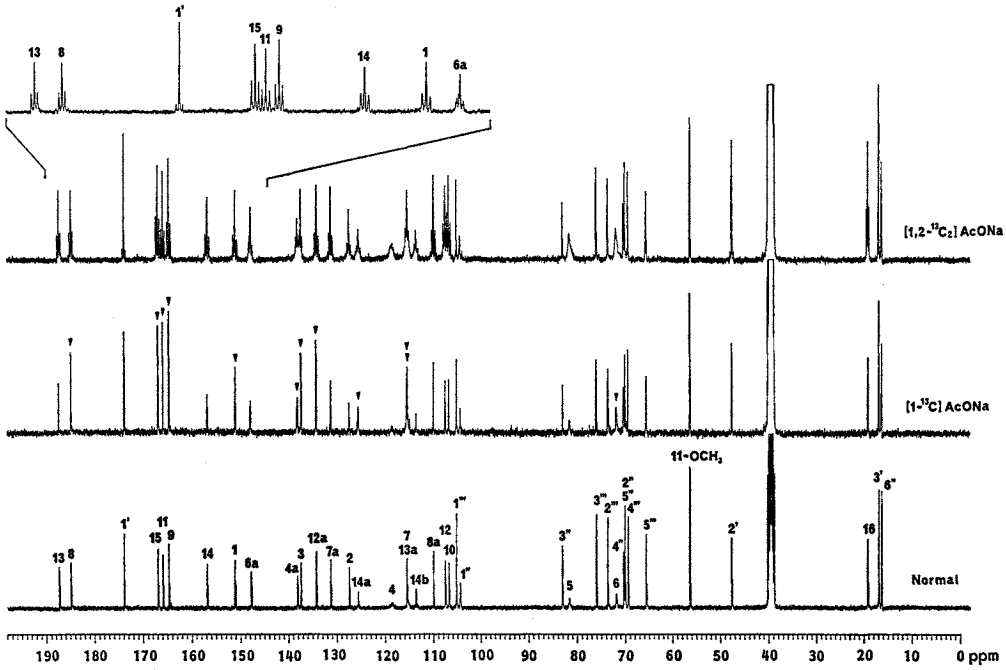


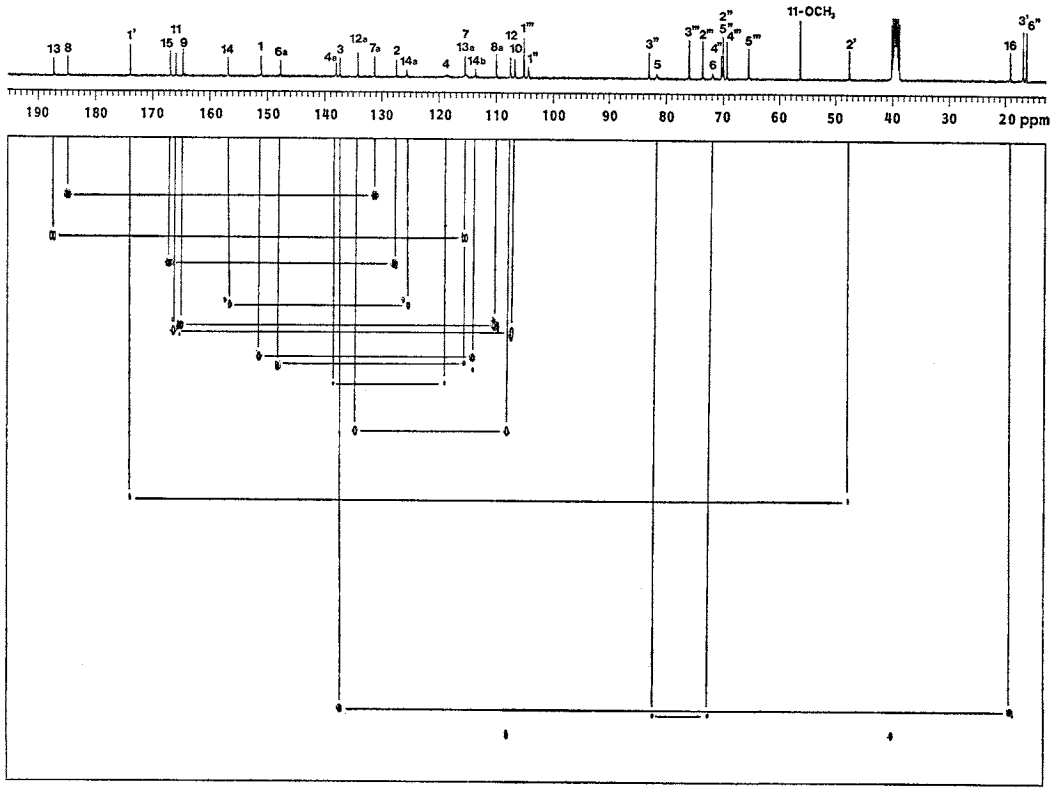
Table 2. Incorporation of sodium [1-^{13}C] and [1,2-$^{13}C_2$]acetates into benanomicins.

Carbon	1				2			
	δ	Enrichment ratio		J_{CC} (Hz)	δ	Enrichment ratio		J_{CC} (Hz)
		I	II			I	II	
C-1	151.1	2.2	3.5	70.5	151.0	2.7	2.8	ND
C-2	127.5	1.0	3.8	64.1	127.5	1.0	2.6	ND
C-3	137.4	2.1	3.8	43.6	137.3	2.4	2.8	43.6
C-4	118.6	0.8	3.3	ND	118.9	ND	3.0[a]	ND
C-4a	138.1	1.8	3.5	61.6	137.8	2.3	2.7	60.3
C-5	81.7	1.0	4.4	*ca.* 40	81.0	1.2	3.5	39.7
C-6	71.9	2.0	3.7	*ca.* 40	71.5	3.1	2.6	39.7
C-6a	147.7	0.9	3.2	60.3	148.0	1.0	2.6	61.6
C-7	115.4	2.4[a]	3.7[a]	ND	115.9	2.3[a]	3.0[a]	ND
C-7a	131.3	1.0	3.3	55.1	131.2	0.9	2.7	55.1
C-8	184.9	2.2	3.5	55.1	184.9	2.5	2.4	ND
C-8a	110.0	1.2	3.9	61.6	110.0	1.0	2.9	62.8
C-9	164.7	2.7	3.9	61.6	164.7	2.5	2.6	62.8
C-10	106.8	1.2	4.4	69.2	106.8	1.1	2.4	69.2
C-11	165.9	2.3	3.4	69.2	165.9	2.7	2.6	69.2
11-OCH_3	56.3	1.2	1.4	—	56.3	1.2	1.0	—
C-12	107.5	1.1	4.2	65.4	107.6	1.0	2.6	64.1
C-12a	134.2	2.3	3.3	65.4	134.2	2.3	2.6	64.1
C-13	187.3	1.1	3.8	56.4	187.4	1.0	2.7	55.1
C-13a	115.5	2.4[a]	3.7[a]	56.4	115.5	2.3[a]	3.0[a]	55.1
C-14	156.8	1.0	3.4	70.5	156.8	1.0	2.2	ND
C-14a	125.6	2.1	3.5	70.5	125.7	2.4	2.7	67.3
C-14b	113.7	1.1	3.5	70.5	113.7	1.0	2.6	ND
C-15	166.9	2.4	4.6	64.1	166.9	2.5	2.8	64.1
C-16	19.1	1.1	3.6	43.6	19.1	1.2	3.5	43.6
C-1′	173.9	1.4	2.0	57.7	173.9	1.4	1.5	59.0
C-2′	47.6	1.1	2.0	57.7	47.6	1.3	1.6	59.0
C-3′	16.9	1.0	1.8	—	16.9	1.2	1.4	—
C-1″	104.4	0.8	1.0	—	104.1	1.0	0.9	—
C-2″	70.1	0.8[a]	1.0[a]	—	69.8	1.1	1.0	—
C-3″	83.0	0.7	1.0	—	77.4	1.0	0.8	—
C-4″	70.3	0.8	1.0	—	54.2	1.0	0.9	—
C-5″	70.1	0.8[a]	1.0[a]	—	67.0	1.0	1.0	—
C-6″	16.3	0.8	1.0	—	16.3	1.2	1.0	—
C-1‴	105.2	1.0	1.0	—	104.4	1.0	1.0	—
C-2‴	73.6	0.7	1.0	—	73.3	1.1	1.0	—
C-3‴	76.0	0.7	1.0	—	75.9	1.0	0.9	—
C-4‴	69.4	0.9	1.0	—	69.4	1.2	0.9	—
C-5‴	65.6	0.8	1.0	—	65.7	1.1	1.0	—

I: [1-^{13}C]Acetate, II: [1,2-$^{13}C_2$]acetate.
ND: Not detected.
[a] Values are mean values for coincident signals.

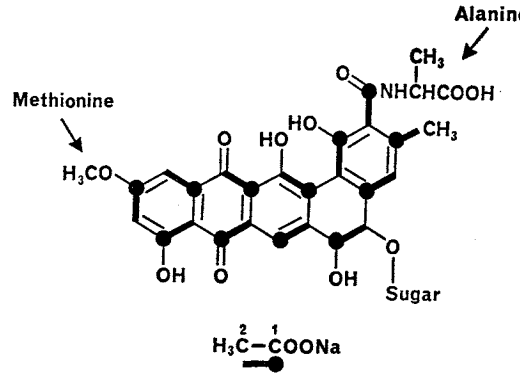
group and alanine side chain were enriched by ^{13}C incorporation. In regard to [1,2-$^{13}C_2$]acetate-labeled benanomicins, all 24 carbon signals of the chromophore were enriched and flanked by satellite signals owing to the ^{13}C-^{13}C coupling of intact doubly-labeled acetate units as shown in Fig. 2. The 2D INADEQUATE NMR spectrum (Fig. 3) of **1** derived from doubly-labeled acetate showed the location of 12 intact acetate units and clearly confirmed the ^{13}C NMR assignments of benanomicins. On

Fig. 3. 2D INADEQUATE NMR spectrum of **1** derived from sodium [1,2-$^{13}C_2$]acetate.



the other hand, the ^{13}C NMR spectra of labeled benanomicins derived from L-[*methyl*-^{13}C]methionine and L-[1-^{13}C]alanine indicated the enhancement of carbon signals only at the 11-methoxy group and C-1′, respectively. No incorporation of ^{13}C-labeled compounds into the disaccharide moieties of benanomicins was detectable in any experiment. These results show that the benzo[*a*]naphthacene quinone chromophore of benanomicins is derived from a dodecaketide intermediate due to head-to-tail condensation of 12 intact acetate units from C-16 to C-15. Furthermore, the 11-methoxy group is introduced from a methionine-derived methyl group and L-alanine is incorporated into the alanine side chain. The building blocks of benanomicins are shown in Fig. 4. The stereochemistry of the labeled benanomicins in respect to the incorporation of L- or D-alanine remains to be proved.

Fig. 4. Building blocks for benanomicins.



Compounds G-2N and G-2A[6], KS-619-1[7,8], SF2446 complex[9,10] and pradimicin A[5] were reported as microbial metabolites possessing a benzo[*a*]naphthacene quinone skeleton but there have been no biosynthetic studies. Except for G-2N and G-2A, these metabolites are structurally similar

to benanomicins, especially in regard to the orientation of substituents in both end-rings. Therefore, we suggest that the biosynthetic routes to these metabolites closely resemble that of benanomicins. Recently, RICKARDS[11] proposed the revised structures of G-2N and G-2A to be 8,13-dioxo-3-methyl-5,6,8,13-tetrahydro-1,7,9,11-tetrahydroxybenzo[*a*]naphthacene and its 2-carboxyl derivative, respectively, after comparing their ^{1}H NMR and electronic absorption spectra with those of KS-619-1 and others. The results of our biosynthetic studies on benanomicins support the revised structures.

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