BIOSYNTHESIS OF BENASTATIN A

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The biosynthesis of benastatin A, produced by *Streptomyces* sp. MI384-DF12, has been studied by feeding experiments with ^{14}C- and ^{13}C-labeled compounds followed by measurement of radioactivity and ^{13}C NMR analysis. The results indicate that benastatin A is derived from two methionine units and fourteen acetate units, condensed in the "head-to-tail" fashion of typical polyketide biosynthesis.

Benastatin A, a new inhibitor of glutathione S-transferase, has been found in the culture broth of *Streptomyces* sp. MI384-DF12[1]. It has unique structure which consist of the chromophore of 13,13-dimethyl-8-oxo-benzo[*a*]naphthacene-2-carboxylic acid and a pentyl group bonded to C-3 of the chromophore[2] (Fig. 1). The closely related natural products possessing a benzo[*a*]naphthacene quinone skeleton, G-2N, G-2A[3] and KS-619-1[4], were reported previously, and the biosynthetic studies of a benzo[*a*]naphthacene quinone skeleton were also carried out concerning with benanomicins A and B[5] and pradimicin A[6], previously. The biosynthetic pathway of benastatin A was studied since it did not contain a quinone skeleton.

In this paper, we report the biosynthetic origin of the carbon atoms in benastatin A based on feeding experiments with ^{14}C- and ^{13}C-labeled compounds.

Materials and Methods

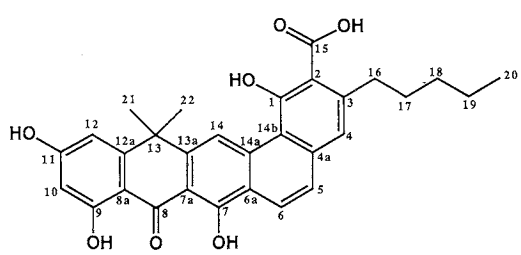
Labeled Compounds

Sodium [1-^{14}C]acetate, sodium [1-^{14}C]propionate and L-[*methyl*-^{14}C]methionine were purchased from New England Nuclear, Boston, U.S.A. The specific radioactivities are shown in Table 1. Sodium [1-^{13}C]acetate (99% ^{13}C enriched), sodium [2-^{13}C]acetate (99%), sodium [1,2-$^{13}C_2$]acetate (99%) and L-[*methyl*-^{13}C]methionine (99%) were obtained from Aldrich Chemical Co.

General Procedure

Radioactivities were measured in a 20-ml vial with a Beckman LS9800 liquid scintillation analyzer by using 10 ml of Aquasol-2 (NEN Research Products) as a scintillation cocktail. ^{13}C NMR spectra were obtained in MeOH-d_4 on a Jeol JNM-GX400 NMR spectrometer at 100 MHz. Chemical shifts were recorded in ppm down field from internal TMS. Preparative TLC was done on a Silica gel 60 F_{254} plate 0.25 mm thick (E. Merck, Art. No. 5715) using $CHCl_3$-MeOH (4:1) as the developing solvent. Benastatin A on the TLC

Fig. 1. Structure of benastatin A.



plates was detected either by its color or by use of UV light (254 nm).

Fermentation

A loopful of slant culture of *Streptomyces* sp. MI384-DF12 (FERM P-11270) was inoculated into 110 ml of a seed medium containing galactose 2.0%, dextrin 2.0%, Bacto Soytone 1.0%, corn steep liquor 0.5%, $(NH_4)_2SO_4$ 0.2% and $CaCO_3$ 0.2% (pH 7.4) in a 500-ml Erlenmeyer flask and cultured at 30°C for 72 hours on a rotary shaker (180 rpm). Two ml of this seed culture were transferred into 110 ml of the production medium consisting of glycerol 2.0%, soy bean meal (Ajinomoto Co., Inc.) 1.5%, K_2HPO_4 0.1% and $CoCl_2 \cdot 6H_2O$ 0.0005% (pH 6.2 adjusted with 1 M KH_2PO_4 before sterilization) in a 500-ml Erlenmeyer flask and cultured at 27°C on a rotary shaker (180 rpm). For the feeding experiments of ^{14}C-labeled compounds, the additions were made at 48 hours after inoculation and the fermentation was continued for 48 hours. In the case of ^{13}C-labeled compounds, singly labeled acetates and labeled methionine were dissolved in water at a concentration of 40 mg/ml, and doubly labeled acetate was dissolved in water at a concentration of 80 mg/ml and diluted with an equal volume of non-labeled acetate solution (80 mg/ml). An aqueous solution (0.25 ml) of each ^{13}C-labeled compound were added at 36, 42 and 48 hours after inoculation, and the cultures were then incubated further for 24 hours.

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

The culture broth was filtered and separated into the mycelial cake and the culture filtrate. The mycelial cake was extracted with 70 ml of MeOH; the extract was filtered and concentrated *in vacuo* to an aqueous solution. The solution was combined with the culture filtrate and extracted with an equal volume of EtOAc (pH 2). The extract was concentrated to dryness under reduced pressure and purified by preparative TLC. A yellowish zone containing radioactive benastatin A (Rf value: 0.46) was collected and suspended in the scintillation cocktail. The radioactivity of ^{14}C-labeled benastatin A was measured with a liquid scintillation analyzer and total incorporation ratios were calculated.

Preparation of ^{13}C-Labeled Benastatin A Sodium Salt

Sodium [1-^{13}C]acetate (30 mg), sodium [2-^{13}C]acetate (30 mg), sodium [1,2-$^{13}C_2$]acetate (30 mg) and L-[*methyl*-^{13}C]methionine (30 mg) were fed to each flask. The culture broth was filtered and separated into the mycelial cake and the culture filtrate. Extractions by MeOH and EtOAc were performed as described above. The crude powder was further purified by a reverse phase HPLC using a Capcell Pak C_{18} column (2.0 × 25 cm, flow rate 8 ml/minute, using GILSON's system) with a solvent mixture of CH_3CN-H_2O-AcOH (78:22:1). The eluate containing benastatin A was evaporated to dryness to obtain a yellow powder. In order to prepare benastatin A sodium salt, the yellow powder was suspended with 20 ml of water and extracted with 20 ml of EtOAc (pH 2). After the concentration of the EtOAc layer, three equivalents of $CH_3COONa \cdot 3H_2O$ was added to the solution of benastatin A in MeOH (10 mg/ml) and the mixture was stirred at room temperature for 10 minutes. The solution was concentrated to dryness and chromatographed on Sephadex LH-20 (1.3 × 110 cm) using MeOH as eluant. Benastatin A sodium salt was eluted and evaporated to give ^{13}C-labeled benastatin A sodium salt (1.6 ~ 3.3 mg).

Results and Discussion

As shown in Table 1, [1-^{14}C]acetate and L-[*methyl*-^{14}C]methionine were efficiently incorporated into benastatin A, but [1-^{14}C]propionate was negligibly incorporated. These results suggest that the benastatin skeleton is derived from a polyacetate intermediate and *C*-methylation occurs.

The feeding experiments using [1-^{13}C], [2-^{13}C] and [1,2-$^{13}C_2$]acetates and L-[*methyl*-^{13}C]-methionine to the cultures of *Streptomyces* sp. MI384-DF12 were carried out in order to clarify the biosynthetic origin of the chromophore and the pentyl group of benastatin A. The ^{13}C NMR spectra of benastatin A sodium salt derived from [1-^{13}C], [2-^{13}C] and [1,2-$^{13}C_2$]acetates are shown in Figs. 2 and 3.

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

Labeled compounds	Specific activity (mCi/mol)	Radioactivity (dpm)		Incorporation into benastatin A (%)
		Fed	Benastatin A	
Sodium [1-^{14}C]acetate	59.0	3.51×10^7	1.94×10^6	5.5
Sodium [1-^{14}C]propionate	57.0	4.26×10^7	3.82×10^4	0.09
L-[*Methyl*-^{14}C]methionine	55.0	4.43×10^7	8.99×10^6	20.3

Fig. 2. ^{13}C NMR spectra of benastatin A sodium salt derived from sodium [1-^{13}C] and [2-^{13}C]acetates and L-[*methyl*-^{13}C]methionine in CD_3OD.

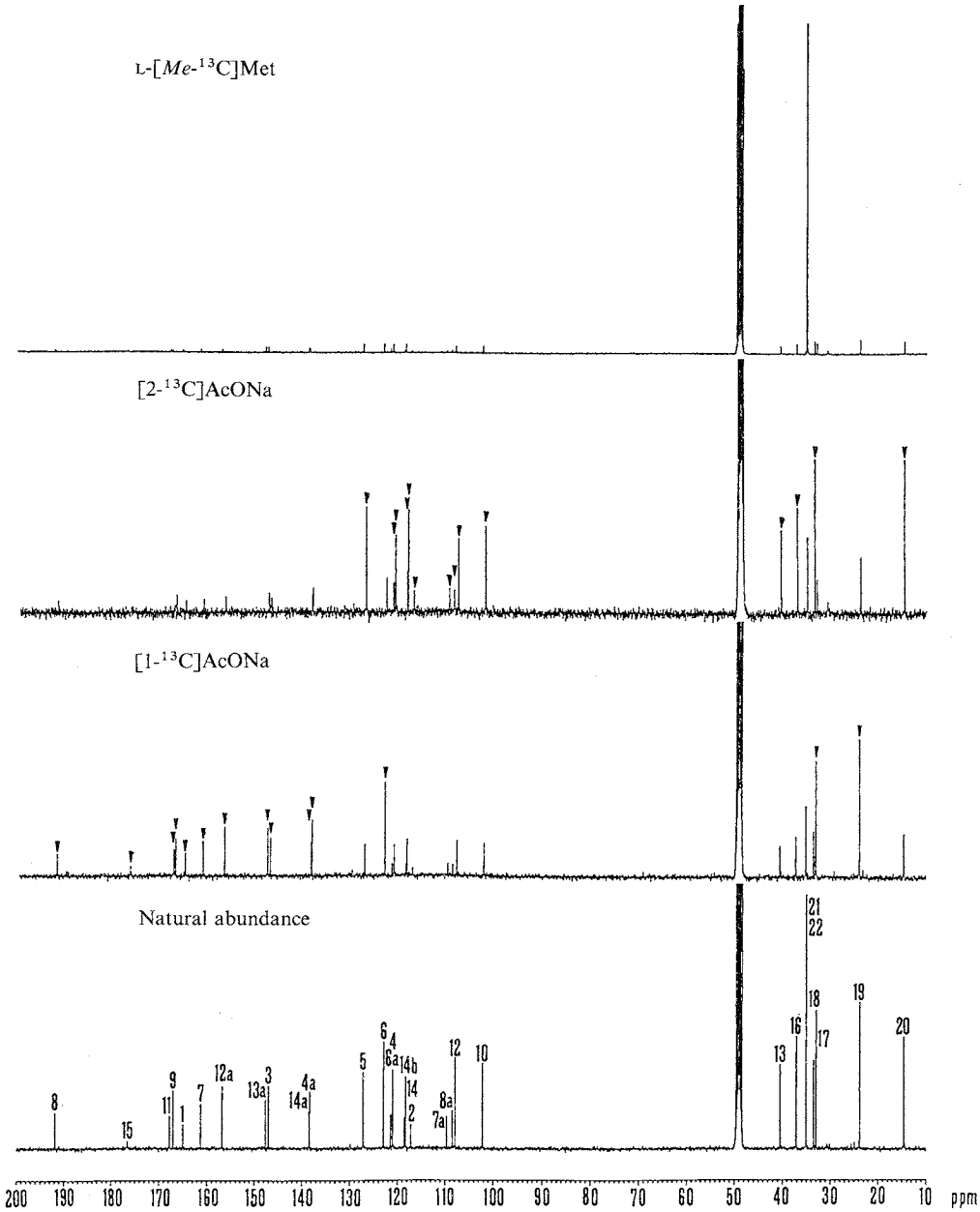
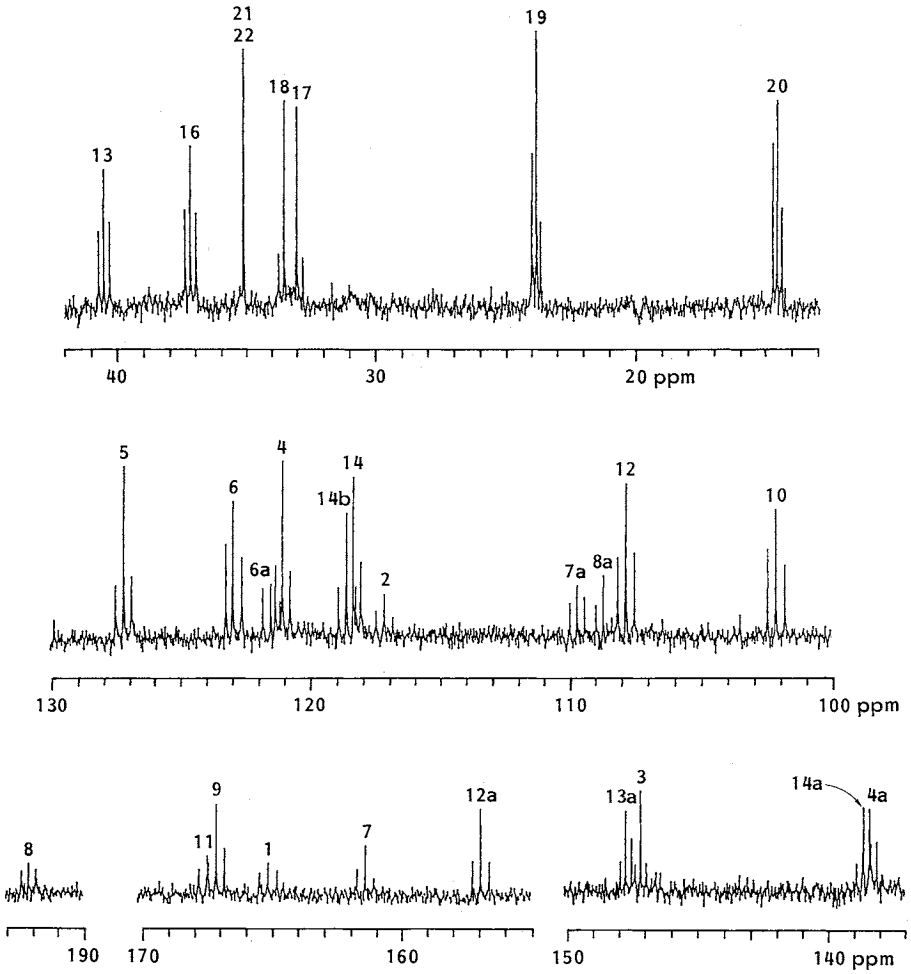


Fig. 3. ^{13}C NMR spectrum of benastatin A sodium salt derived from sodium [1,2-$^{13}C_2$]acetate in CD_3OD.



The chemical shift assignment of ^{13}C NMR signals of benastatin A sodium salt was determined by the aid of the HMBC (heteronuclear multiple bond connectivity) spectrum. Arrows indicate the enriched carbon signals in the ^{13}C NMR spectra of [1-^{13}C] and [2-^{13}C]acetate-labeled benastatin A sodium salt. Enrichment ratios and ^{13}C-^{13}C coupling constants of [1-^{13}C], [2-^{13}C] and [1,2-$^{13}C_2$]acetate-labeled benastatin A sodium salt are listed in Table 2. Enrichment ratios were calculated from the relative intensity of C-21, 22 as 1.0. In the ^{13}C NMR spectrum of [1-^{13}C]acetate-labeled benastatin A sodium salt, fourteen alternating carbon signals except the signal of *gem*-dimethyl carbons (C-21, 22) were enriched by ^{13}C incorporation. A similar experiment with sodium [2-^{13}C]acetate enhanced the ^{13}C NMR signals for fourteen carbons adjacent to the first set. These data suggest that acetate is incorporated into benastatin A by the way of a polyketide intermediate.

In regard to [1,2-$^{13}C_2$]acetate-labeled benastatin A sodium salt, twenty-seven carbon signals 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. 3. Acetate arrangements proved by J_{cc} are shown in Fig. 4.

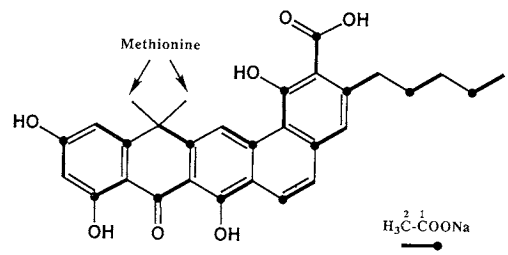
Table 2. Incorporation of sodium [1-^{13}C], [2-^{13}C] and [1,2-$^{13}C_2$]acetates and L-[*methyl*-^{13}C]methionine into benastatin A sodium salt.

Carbon	δ	Enrichment ratio			
		I	II	III (J_{cc}, Hz)	IV
1	165.1	3.6	2.0	4.4 (66.8)	0.9
2	117.2	1.4	3.3	4.3 (64.6)	0.8
3	147.1	2.2	0.9	2.6 (42.9)	0.8
4	121.1	1.5	3.3	4.0 (55.9)	1.1
4a	138.3	3.6	1.5	3.0 (55.9)	0.7
5	127.2	1.5	4.6	3.8 (62.3)	1.1
6	123.0	3.1	1.1	2.9 (62.3)	0.8
6a	121.5	1.4	3.2	4.4 (66.5)	1.2
7	161.3	2.9	1.2	2.7 (66.5)	0.7
7a	109.7	1.5	2.8	4.4 (55.9)	0.6
8	191.9	2.3	1.3	2.8 (55.9)	0.7
8a	108.6	1.2	2.2	3.3 (60.8)	0.7
9	167.1	2.4	ND	3.1 (60.8)	0.3
10	102.3	1.4	3.4	3.4 (66.0)	0.8
11	167.8	3.0	2.0	3.6 (66.0)	0.8
12	108.0	1.4	2.7	3.5 (64.0)	0.7
12a	156.8	2.8	1.0	3.2 (64.0)	0.5
13	40.5	1.3	3.3	3.6 (42.4)	0.9
13a	147.7	3.6	1.4	3.5 (42.4)	1.1
14	118.3	1.9	4.8	4.4 (55.3)	1.2
14a	138.5	3.4	1.8	4.4 (55.3)	1.2
14b	118.6	1.5	4.7	5.0 (66.8)	1.0
15	176.6	5.4	ND	ND	ND
16	37.2	1.3	3.1	3.2 (42.9)	0.9
17	33.0	3.0	0.8	2.0 (45.0)	0.8
18	33.5	1.8	5.7	3.3 (45.0)	1.5
19	23.8	3.3	1.3	3.4 (34.2)	1.0
20	14.6	1.4	4.5	4.1 (34.2)	1.1
21, 22	35.1	1.0	1.0	1.0 (—)	13.6

I: [1-^{13}C]acetate, II: [2-^{13}C]acetate, III: [1,2-$^{13}C_2$]acetate, IV: L-[*methyl*-^{13}C]methionine.
ND: Not detected.

On the other hand, the ^{13}C NMR spectrum of benastatin A sodium salt derived from L-[*methyl*-^{13}C]methionine indicated the enhancement of carbon signal only at *gem*-dimethyl carbons (C-21, 22) as shown in Fig. 2. In Table 2, enrichment ratio of the signal was calculated from the relative intensity of C-19 as 1.0.

Fig. 4. Building blocks for benastatin A.



From the above results, the 8-oxo-3-pentylbenzo[*a*]naphthacene-2-carboxylic acid of benastatin A is derived from a tetradecaketide intermediate due to head-to-tail condensation of fourteen intact acetate units from C-20 to C-15. Furthermore, the *gem*-dimethyl carbons (C-21, 22) are introduced from methionine-derived methyl groups. The building blocks of benastatin A are shown in Fig. 4. Among *Streptomyces* metabolites, the introduction of two C_1-units onto the same carbon has been reported in biosynthetic studies of resistomycin[7] and aplasmomycin[8].

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