BIOSYNTHESIS OF GRISEOLIC ACIDS: INCORPORATION OF ^{13}C-LABELED COMPOUNDS INTO GRISEOLIC ACID A

SHUNICHI MIYAKOSHI, HIDEYUKI HARUYAMA†, TETSUYA SHIOIRI, SHUJI TAKAHASHI, AKIO TORIKATA and MITSUO YAMAZAKI

Fermentation Research Laboratories, Sankyo Co., Ltd.,
†Analytical and Metabolic Research Laboratories, Sankyo Co., Ltd.,
1-2-58 Hiromachi, Shinagawa-ku, Tokyo 140, Japan

(Received for publication July 1, 1991)

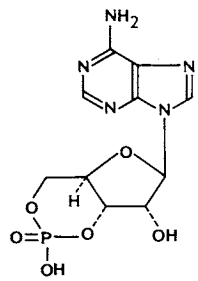
Biosynthesis of griseolic acids, competitive inhibitors of cyclic nucleotide phosphodiesterase, was investigated with the culture of a producing strain of *Streptomyces griseoaurantiacus*. ^{13}C-Labeled and ^{15}N-labeled compounds were added into the culture, and ^{13}C-enriched and ^{15}N-enriched griseolic acid A was isolated from the culture medium and analyzed by ^{13}C NMR and ^{15}N NMR spectroscopy. The compounds added to growth medium were [2-^{13}C]acetate, [1,2-^{13}C]acetate, [1,4-^{13}C]succinate, [1-^{13}C]glucose, [6-^{13}C]glucose, [2-^{13}C]ribose, and [1-^{13}C, ^{15}N]glycine.

The results suggest that adenosine, which is formed from amino acids and sugars contributes the adenine and ribose moieties to griseolic acid A. The data also suggest that a dicarboxylic acid from the Krebs tricarboxylic acid cycle contributes to the dicarboxylic part of the compound.

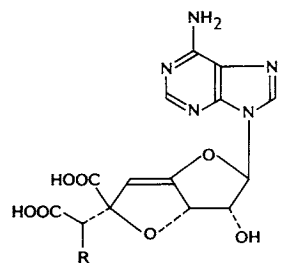
In the course of our extensive search for cyclic nucleotide phosphodiesterase (PDE) inhibitors from microorganisms, we discovered a series of cyclic AMP (**1**) analogues: griseolic acids A (**2**), B (**3**) and C (**4**) from the culture filtrate of *Streptomyces griseoaurantiacus*.[1~3] Griseolic acids have unique skeletons, that resemble cAMP (**1**) with an adenine group, a sugar moiety and a carboxylic function in the molecules. They inhibit PDE at low concentration in the homoginate of various tissues, and the inhibition is competitive with regard to cAMP, the substrate.[1,4]

Carbon-13 NMR spectroscopy is a useful technique for the identification of specific carbon atoms in complex organic molecules.[5] In this paper, we describe the determination of biochemical origines of the unique skeleton of these molecules by measuringthe incorporation of various ^{13}C-labeled and ^{15}N-labeled substrates into griseolic acid A using NMR spectroscopy.

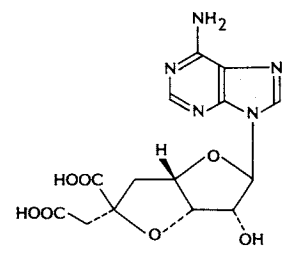
Fig. 1. Structures of cyclic AMP, griseolic acids A, B, and C.



Cyclic AMP (**1**)



Griseolic acid A (**2**) R=OH
Griseolic acid B (**3**) R=H



Griseolic acid C (**4**)

Materials and Methods

Microorganism

Streptomyces griseoaurantiacus SANK63479[1,3] was used for biosynthesis studies. The mutant strain 8-28 was induced from the wild strain SANK63479 by using UV-radiation, which shows low productivity for griseolic acids but no additional nutritional requirements.

Cultivation

Seed medium consisting of 5% maltose, 1% soybean meal, Polypepton (Wako), 0.4% meat extract (Kyokuto), 0.1% yeas extract (Bacto), 0.25% NaCl and 0.5% $CaCO_3$ (pH 7.1) was dispensed in 20-ml portions into 100 ml Erlenmeyer flasks, stopped with a cotton plug, and autoclaved at 121°C for 20 minutes. The flasks were inoculated with a spore suspension. Spores were harvested from the culture on YM slants consisting of 0.4% glucose, 1% Malt extract (Bacto), 0.4% Yeast extract (Bacto), and 2% agar (Wako) (pH 7.1), suspended in 20% glycerol, and stored at −80°C until the inoculation. After 3 days of incubation at 28°C on a rotary shaker (200 rpm), one ml of the seed was inoculated into sterilized 100 ml Erlenmeyer flasks containing following medium: 10% maltose, 3% soybean meal, 0.7% corn steep liquor, 0.5% Polypepton (Wako), 0.1% yeast extract (Bacto), 0.1% KH_2PO_4 (pH 7.1). The inoculated flasks were incubated on a rotary shaker (200 rpm) at 28°C for 12 days.

^{13}C-Incorporation Studies

^{13}C-Labeled compounds were added to the fermentation media 0 to 9 days after inoculation as described by WANG *et al.*[6] After 14 days cultivation, the culture broth was centrifuged at $3,000 \times g$ for 15 minutes. The supernatant was passed through a Diaion HP-20 column (4 ml) and the unbound fraction was collected. After the pH of the eluate was adjusted to 3.0 with HCl, it was centrifuged again. The supernatant was absorbed to Diaion HP-20 column (4 ml) and washed with water. Griseolic acid A was eluted with 20% methanol, and purified by reverse phase high performance liquid chromagography. The fraction of griseolic acid A was concentrated *in vacuo* and resulting powder was subjected to ^{13}C NMR spectroscopy.

Chemicals

Sodium [2-^{13}C]pyruvate and sodium [1,4-^{13}C]succinate were purchased from MSD Isotopes, Montreal, Canada, and other ^{13}C-labeled and ^{15}N-labeled compounds were from Aldrich, Milwaukee, U.S.A.

Results

Selection of Conditions for Isotopic Enrichment

It is known that the production of griseolic acids is markedly depressed by the supplementation of sodium salts of low-molecular-weight fatty acids in early stages of growth, although the incorporation of precursors increases in this stage. To minimize the inhibitory effect on griseolic acid production, 10 to 20 mg of the supplement was added on day 3, 6, and 9. With this feeding schedule, a total of 1.5 to 3.0 mg/ml of ^{13}C-labeled compounds supplied, which reduced the production of griseolic acids by less than 20% from that obtained in unsupplemented cultures.

Incorporation of ^{13}C-Labeled Sugars

The proton-noise-decoupled ^{13}C NMR spectrum of griseolic acid A derived from D-[2-^{13}C]-ribose showed enrichment of C-2′ carbon atom (Table 1). This highly-efficient incorporation (3%) suggests that D-ribose is a direct precursor of the sugar moiety of griseolic acid A.

The ^{13}C atom of D-[6-^{13}C]glucose was specifically incorporated into C-5′ position, but no specific incorporation from D-[1-^{13}C]glucose was observed (Table 1). Known sugar metabolisms support

Table 1. Incorporation of ^{13}C into griseolic acid A from ^{13}C-labeled compounds.

Carbon atom	Chemical shift (ppm)	Relative enrichment						
		[2-^{13}C]-Acetate	[1,2-^{13}C]-Acetate	[2-^{13}C]-Pyruvate	[1,4-^{13}C]-Succinate	[1-^{13}C]-Glucose	[6-^{13}C]-Glucose	[2-^{13}C]-Ribose
C-2	152.9	1.2	0.7	1.3	0.7	0.9	1.7	1.0
C-4	148.5	**2.8**	1.2	**2.6**	1.4	**2.3**	1.3	1.8
C-5	118.8	1.8	0.7	**2.5**	1.0	1.0	1.2	1.1
C-6	156.0	1.4	**4.1**	1.9	0.9	1.5	1.9	1.2
C-8	139.8	1.5	0.7	1.2	0.7	1.5	**2.0**	1.3
C-1′	98.9	1.1	1.1	1.4	1.0	1.6	**2.9**	1.3
C-2′	70.0	1.5	0.7	1.0	1.0	1.2	1.0	**3.9**
C-3′	82.3	1.0	1.0	1.0	1.0	1.0	1.0	1.0
C-4′	157.9	1.2	0.8	1.3	1.0	1.5	1.1	1.3
C-5′	96.4	1.3	0.9	0.5	1.0	1.7	**5.0**	1.3
C-6′	98.8	**3.2**	**2.2**	**2.4**	1.7	**2.5**	**4.5**	**2.0**
C-7′	72.8	**4.9**	**3.7**	1.5	1.1	**2.0**	**4.0**	1.4
C-8′	171.4	**2.9**	**6.0**	**2.5**	**2.3**	1.4	**2.0**	1.2
C-9′	171.5	**3.0**	**5.9**	1.8	**3.2**	1.6	1.9	1.6

decarboxylation at C-1 position of ^{13}C-labeled D-glucose and the formation of D-[5-^{13}C]ribose from D-[1,6-^{13}C]glucose.

Incorporation of ^{13}C, ^{15}N-Labeled Glycine

Strain 8-28 which shows low productivity for griseolic acids recovered their productivity on the additon of adenosine (0.05%～0.3%), but not by that of adenine. Therefore, adenosine may be one of the precursors of griseolic acids.

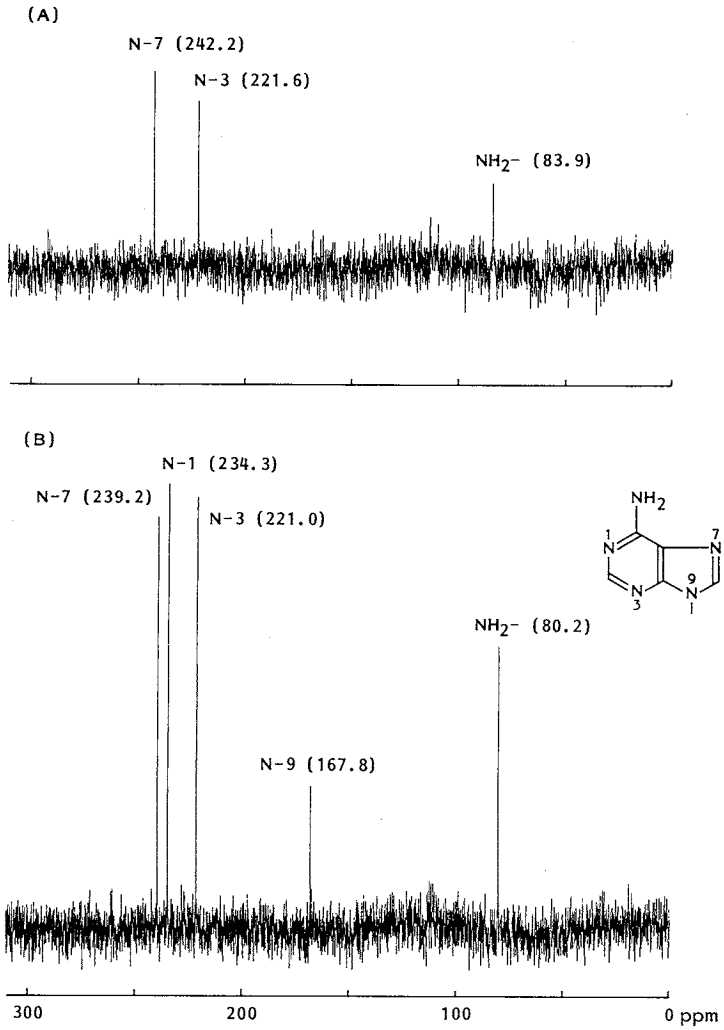
Incorporation of [1-^{13}C, ^{15}N]glycine into griseolic acid A was examined to determine the biosynthetic origin of the adenine moiety of the compound. ^{13}C-Labeled carbonyl carbon was incorporated into C-4 position of the adenine ring at a high efficiency of 3% (Table 1). The nitrogen atom at N-7 position of adenine moiety was most abundantly enriched by the addition of ^{15}N-labeled glycine (Fig. 2). These results suggest that glycine was incorporated into C-4, C-5 and N-7 positions of griseolic acids, which was consistent with known purine biosynthetic pathway (Fig. 3). Two other nitrogen atoms at N-3 position and amino group were also enriched. On ^{1}H-^{15}N spin echo difference NMR spectra, incorporation of ^{15}N atoms into N-7 and N-3 was supported by satelite peaks of 2-H and 8-H by ^{15}N-^{1}H couplings (data not shown). It is proposed that ^{15}N atoms of N-3 is derived from L-glutamine which is synthesized from L-glutamate and $^{15}NH_2$- of glycine.

Incorporation of ^{13}C-Labeled Organic Acids

^{13}C NMR spectra of griseolic acid A derived from sodium [2-^{13}C]acetate showed enrichment of four carbon atoms: C-6′ and C-7′ were the most efficiently enriched and two carbonyl carbons (C-8′ and C-9′) were also enriched. From sodium [1,2-^{13}C]acetate, the same four carbon atoms were enriched and ^{13}C-^{13}C couplings between C-6′ and C-9′ ($J=32.3$ Hz) and between C-7′ and C-8′ ($J=29.4$ Hz) were observed. These results suggest that acetate units were incorporated "head-to-head" into the carboxylic acid part of griseolic acid A (Fig. 3).

Both of the carbonyl carbon atoms (C-8′ and C-9′) were specifically enriched (1 to 2%) by the supplementation of [1,4-^{13}C]succinate. The specific incorporation of these carbon atoms from the same

Fig. 2. ^{15}N NMR spectra of griseolic acid A enriched by the supplementation of [^{15}N]glycine (A) and adenosine (B).



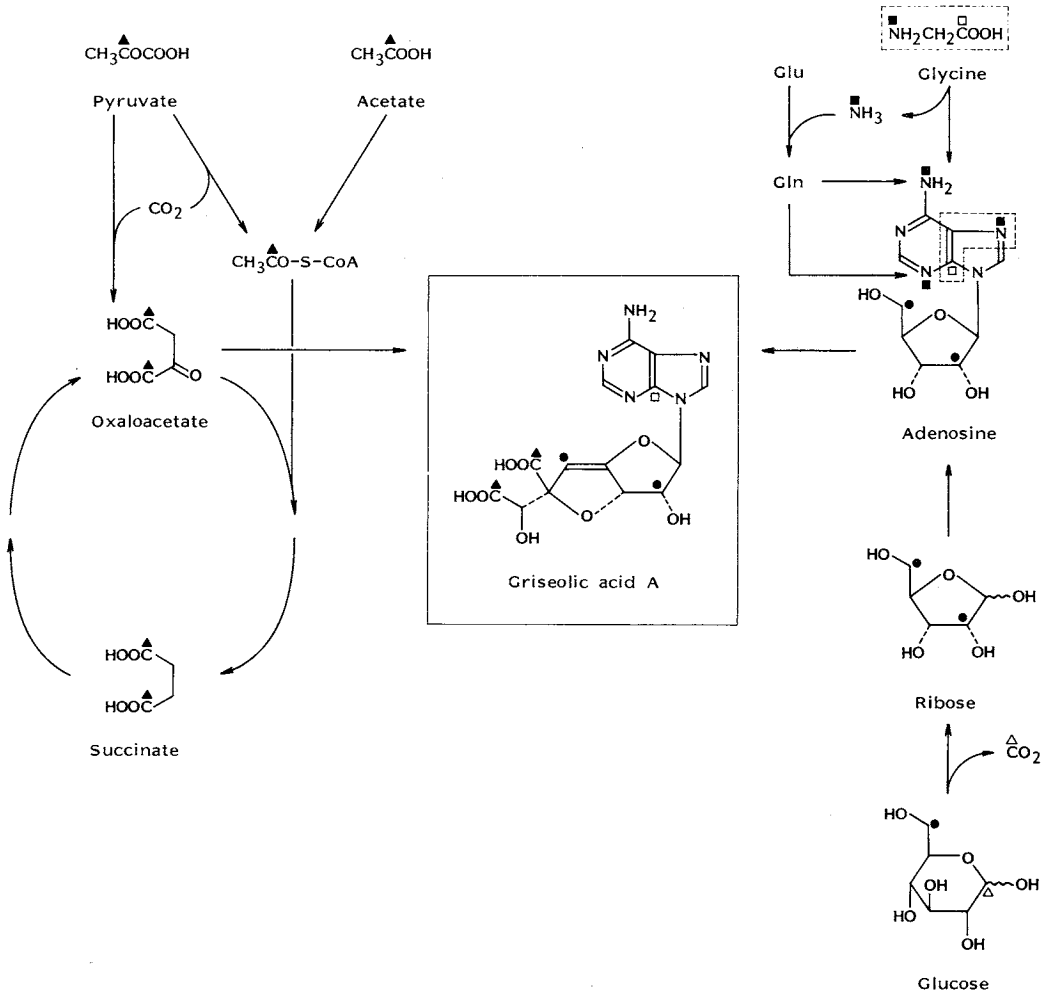
^{13}C-labeled succinate into griseolic acid B was also observed (data not shown). From [2-^{13}C]pyruvate, ^{13}C was most efficiently incorporated into the two carbons of griseolic acid A, and it was also incorporated into C-6′ and C-7′.

Discussion

Incorporation experiments with ^{13}C-labeled sugars and ^{13}C, ^{15}N-labeled glycine suggest that adenosine is one of the biosynthetic of griseolic acids. It is not known whether adenosine is the direct precursor or the adenine moiety is incorporated after the formation of the carboxylic parts. By the findings that the productivity was recovered by supplementing adenosine but not adenine in the culture of the low-productivity mutant 8-28, it is suggested that adenosine is incorporated without cleavage of the nucleoside bond. Similar results were reported by BERRY and ABBOTT for the biosynthesis of sinefungin[7]. This ambiguity will be clarified by the experiment using [*U*-^{14}C]adenosine.

Incorporation experiments with ^{13}C-labeled organic acids indicate that a four-carbon dicarboxylic

Fig. 3. Hypothetic pathway of griseolic acid A synthesis.



acid is another precursor of griseolic acids. The observations that not only C-6′ and C-7′ but also carbonyl carbons (C-8′ and C-9′) were enriched by the addition of [2-^{13}C]acetate suggest that griseolic acids are synthesized *via* the Krebs tricarboxylic acid cycle and not by dicarboxylic acid cycles. The incorporation pattern of pyruvate into griseolic acid A suggests that dicarboxylic acids in the Krebs cycle participate in griseolic acid biosynthesis and that oxaloacetate which is formed directly from pyruvate may also be a precursor.

Thus it is strongly suggested that griseolic acids are synthesized from adenosine and a four-carbon dicarboxylic acid. Similarly, ISONO *et al.* proposed the presence of an aldol condensation in the first step in polyoxin biosynthesis.[8] Studies on each biosynthetic step are under investigation using mutant strains and cell-free systems.

References

1) NAKAGAWA, F.; T. OKAZAKI, A. NAITO, Y. IIJIMA & M. YAMAZAKI: Griseolic acid, an inhibitor of cyclic adenosine 3′,5′-monophosphate phosphodiesterase. I. Taxonomy, isolation and characterization. J. Antibiotics 38: 823～829, 1985

2) TAKAHASHI, S.; F. NAKAGAWA, K. KAWAZOE, Y. FURUKAWA, S. SATO, C. TAMURA & A. NAITO: Griseolic acid an inhibitor of cyclic adenosine 3′,5′-monophosphate phosphodiesterase. II. The structure of griseolic acid. J. Antibiotics 38: 830～834, 1985

3) TAKAHASHI, S.; F. NAKAGAWA & S. SATO: Griseolic acids B and C, an inhibitor of cyclic adenosine 3′,5′-monophosphate phosphodiesterase. J. Antibiotics 41: 705～706, 1988
4) IIJIMA, Y.; F. NAKAGAWA, S. HANDA, T. ODA, A. NAITO & M. YAMAZAKI: Biological properties of griseolic acid, a cyclic AMP phosphodiestrase inhibitor with an adenine group. FEBS Lett. 192: 179～183, 1985
5) SETO, H.: Utilization of ^{13}C-^{13}C coupling in structural and biosynthetic studies of natural products. (in Japanese) Nippon Nogeikagaku Kaishi 50: R217～R223, 1976
6) WANG, I.-K.; L. C. VINING, J. A. WALTER & A. G. MCINNES: Use of carbon-13 in biosynthetic studies: Origin of the malonyl coenzyme A incorporated into tetracycline by *Streptomyces aureofaciens*. J. Antibiotics 39: 1281～1287, 1986
7) BERRY, D. R. & B. J. ABBOTT: Incorporation of ^{14}C-labeled compounds into sinefungin (A9145), a nucleoside antifungal antibiotic. J. Antibiotics 31: 185～191, 1978
8) ISONO, K.; T. SATO, K. HIRASAWA, S. FUNAYAMA & S. SUZUKI: Biosynthesis of the nucleoside skeleton of polyoxins. J. Am. Chem. Soc. 100: 3937～3939, 1978