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# Biosynthetic Studies of Microbial Alkaloid Pyrindicin using C-13 Labelled Precursors

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The biosynthetic origins of pyrindicin, a metabolite of *Streptomyces griseoflavus* var. *pyrindicus*, have been investigated by the use of $^{13}C$ nuclear magnetic resonance spectroscopy. It has been established that pyrindicin is derived from five acetates and one propionate.

**Keywords**——biosynthesis; *Streptomyces*; alkaloid; $^{13}C$–NMR; acetate[$^{13}C$], propionate[$^{13}C$]

In the course of screening for microbial alkaloids, a new alkaloid, pyrindicin[2] was isolated from the culture filtrate of *Streptomyces griseoflavus* var. *pyrindicus*. In the subsequent studies on this alkaloid, 5-(*trans*-1′-butenyl)-3-methyl-3,4,6,7-tetrahydro-2H-1-pyrindine has been proposed as its structure.[3]

In a series of the detailed biosynthetic studies on hemlock and its related piperidine alkaloids, the piperidine ring is classified according to the biosynthetic pathway into three subgroups,[4] that is, lysine,[5] acetic acid[6] and isoprenoid[7] groups. For example, nigrifactin, a simple piperidine alkaloid produced by *Streptomyces nigrifaciens* var. FFD–101, is derived from six acetate units through "polyketide" as a hypothetical intermediate.[8]

Since the structure of pyrindicin resembled to that of nigrifactin, it was suggested that pyrindicin might be synthesized from acetate units through "polyketide". However, it is not clear whether the methyl carbon of C-10 is derived from $C_1$ unit or propionate. Thus we have carried out detailed biosynthetic studies on the origin of C-10 which has been one of the most intriguing subjects in this field.

This paper therefore deals with the biosynthetic origins of pyrindicin as determined by $^{13}C$-nuclear magnetic resonance ($^{13}C$–NMR) spectroscopy using $^{13}C$-labelled precursors.

We first investigated to determine whether or not acetate is incorporated into pyrindicin molecule using [1-$^{14}C$]acetate. The result indicated that acetate is a biosynthetic precursor of pyrindicin. The effect of addition at different concentrations of acetate and propionate on the production of pyrindicin was then studied as shown in Table I. Acetate and propionate did not show inhibition of pyrindicin production at a concentration of 1.0 mg/ml, but a small extent of inhibition was observed in the presence of 2.0 mg/ml of acetate and propionate.

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Table I. Effect of Addition of Acetate and Propionate on Pyrindicin Production

| Conc. (mg/ml) | Pyrindicin production (unit/ml) | |
|---|---|---|
| | Acetate | Propionate |
| 0 | 256 | 128 |
| 0.25 | 256 | 128 |
| 0.5 | 256 | 128 |
| 1.0 | 256 | 128 |
| 2.0 | 128 | 64 |

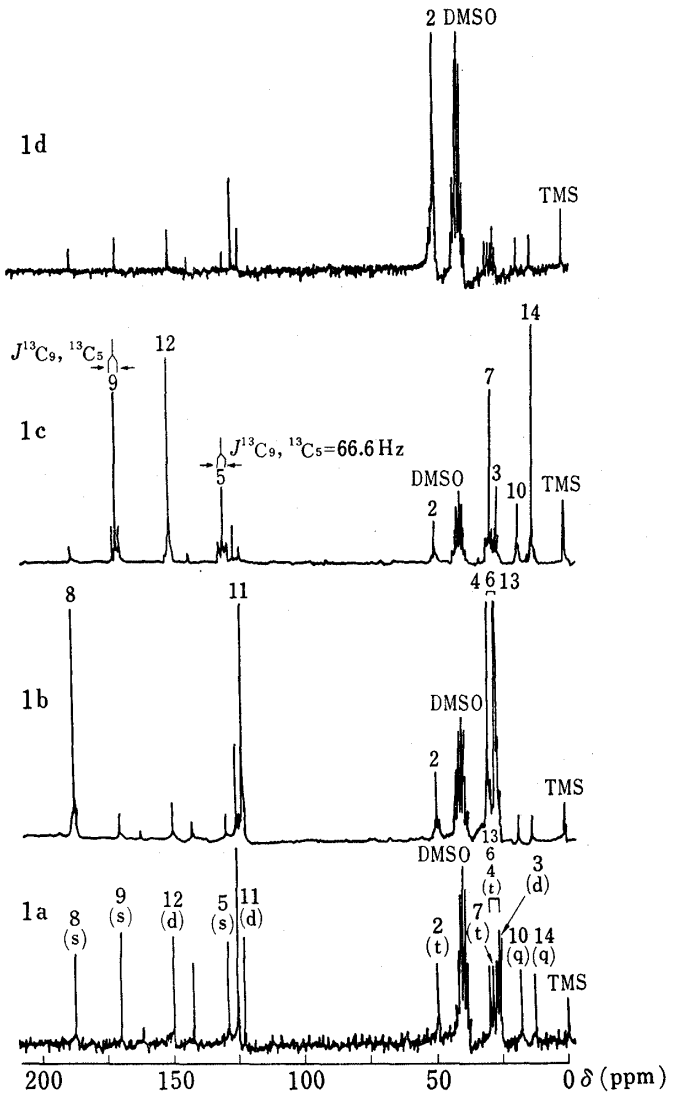


Fig. 1. $^{13}$C-NMR Spectra of Pyrindicin Picrate

Ia: natural abundance.
Ib: [1—$^{13}$C]acetate labelled.
Ic: [2—$^{13}$C]acetate labelled.
Id: [1—$^{13}$C]propionate labelled.
The abbreviations used are as follow: s, singlet, d, doublet; t, triplet; q. quartet.

Acetate[1-$^{13}$C] (1.0 mg/ml) was fed to the culture medium of *Streptomyces griseoflavus* var. *pyrindicus* and $^{13}$C-labelled pyrindicin picrate was isolated from the culture broth. The $^{13}$C–NMR spectra of natural and $^{13}$C-enriched pyrindicin picrate were shown in Fig. 1a, 1b, 1c and 1d. The spectrum of natural pyrindicin picrate indicated five $sp^2$-carbon signals in the 122—187 ppm region and eight $sp^3$-carbon signals in the 12—49 ppm region. The assignments of these carbons were based on their chemical shifts,[9] off-resonance decoupling and the labeling pattern expected from the incorporation experiments using $^{13}$C-labelled precursors. Comparison of the spectrum of the natural product (Fig. 1a) with that derived from [1-$^{13}$C]acetate (Fig. 1b) revealed that the six carbon signals at 26.2, 26.8, 29.4, 48.8, 122.5 and 186.1 ppm were enhanced by the addition of [1-$^{13}$C]acetate into the medium. It should be noted that the rate of increase of the signal height of the peak at 48.8 ppm was smaller than that of other enriched five carbons.

To obtain more conclusive evidence for the participation of acetate and propionate in the biosynthetic route, the incorporation of [2-$^{13}$C]-acetate and [1-$^{13}$C]propionate into pyrindicin was investigated in a

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similar manner as described above. When [2-$^{13}$C]acetate was added to the culture, enrichment of the signals at 12.4, 17.4, 25.4, 28.2, 48.8, 128.7, 149.0 and 168.8 ppm was observed (Fig. 1c). Upon addition of [1-$^{13}$C]propionate, only the signal at 48.8 ppm was enriched (Fig. 1d). These results led us to propose a working model for the biosynthesis of pyrindicin whose building unit consists of five acetates and propionate as illustrated in Fig. 2. This model satisfactorily explains the $^{13}$C-enrichment patterns with [1-$^{13}$C]acetate, [2-$^{13}$C]acetate and [1-$^{13}$C]propionate.

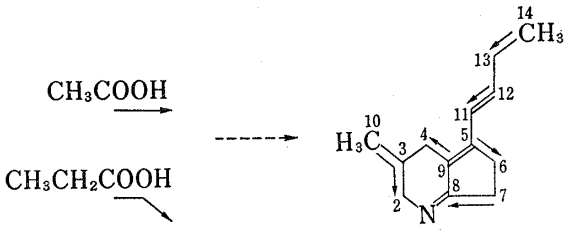


Fig. 2. Biosynthetic Pattern of Pyrindicin

Pyrindicin possesses five methylenic carbons C-4, C-6, C-7, C-13 and C-2. Four of these except C-2 are bonded to olefinic carbons and the last one is connected to nitrogen. The signal at 28.2 ppm enriched by the addition of [2-$^{13}$C]acetate could be assigned to C-7 in accord with the working model. On the other hand, extreme proximity in the chemical shifts of three other carbons, C-4, C-6 and C-13, which were enriched by [1-$^{13}$C]acetate, precluded their assignments. Since the signal at 48.8 ppm, the lowest field peak among the five methylenic carbons, was significantly enriched by [1-$^{13}$C]propionate, it was assigned to C-2. Among the two methyl signals at 12.4 and 17.4 ppm, the former was assigned to C-14 because of strong incorporation of [2-$^{13}$C]acetate and the latter assigned to C-10. Each of the two signals of olefinic carbons at 122.5 and 149.0 ppm was observed as a doublet by off-resonance decoupling. Since the carbons at 122.5 and 149.0 ppm were enriched by the addition of [1-$^{13}$C]-acetate and [2-$^{13}$C]acetate, respectively, these were assigned to C-11 and C-12, respectively. Of the two signals at 128.7 and 168.8 ppm which show $^{13}$C-$^{13}$C coupling ($J$=66.6 Hz) in the spectrum of pyrindicin labelled with [2-$^{13}$C]acetate, the lower field signal should correspond to C-9 and the other signal at 128.7 ppm was therefore assigned to C-5. The remaining olefinic carbon at 186.1 ppm was assigned to C-8.

Since the assignment of each carbon is in accord with the labeling pattern of a working model as mentioned above, it is reasonable to conclude that pyrindicin is derived from five acetates and one propionate as shown in Fig. 2.

It is unambiguously established that propionate is the precursor of C-2, C-3 and C-10 in the pyrindicin molecule. However, it should be noted that [1-$^{13}$C]acetate and [2-$^{13}$C]acetate were also incorporated into these three carbons to a certain extent. In the metabolic pathway from acetate to propionate in the pyrindicin biosynthesis, two routes, that is, glyoxylate and TCA cycles are conceivable. As shown in Fig. 1, only methylenic carbon C-2 corresponding to the carboxyl carbon of propionate was enriched by [1-$^{13}$C]acetate and each carbon of C-2, C-3 and C-10 was enriched by [2-$^{13}$C]acetate. If acetate is metabolized into propionate *via* either glyoxylate cycle or TCA cycle, we can expect such labeling pattern from each metabolic cycle as shown in Fig. 3. The results described above are difficult to explain with either one of the metabolic cycles. Therefore, it is suggested that the metabolism of acetate into propionate in pyrindicin biosynthesis may involve both glyoxylate and TCA cycles.

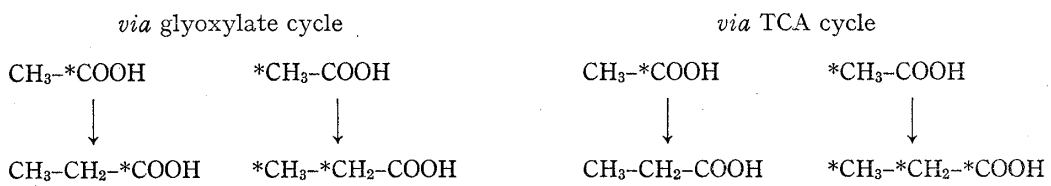


Fig. 3. Labeling Patterns of the Metabolism of Acetate into Propionate *via* Glyoxylate Cycle and *via* TCA Cycle

## Experimental

Carbon spectra were obtained on a JNM PFT-100 spectrometer operating at 25.15 MHz. Chemical shifts are reported in parts per million relative to internal tetramethylsilane. The CMR spectra of $^{13}C$-labelled compounds (27 to 66 mg) were measured in DMSO-$d_6$ (0.8 ml).

**Incorporation of [1-$^{14}C$]acetate into Pyrindicin**——Sodium[1—$^{14}C$]acetate (specific activity: 2.2 mCi/mmol) was obtained from New England Nuclear. $^{13}C$-labelled precursors (90 atom%) were purchased from Merck Sharp and Dohme of Canada. Sodium[1—$^{14}C$]acetate (5 μCi) and cold sodium acetate (100 mg) were added to 100 ml of the culture. Pyrindicin was isolated from the culture filtrate by solvent extraction and the extract was then evaporated *in vacuo* to dryness. The residue was purified by a preparative silica gel TLC developing with cyclohexane–diethylamine (9: 1, v/v). The resulting pyrindicin was dissolved in toluene-based scintillator [4 g of 2,5-diphenyl-oxazole, 50 mg of 1,4-bis-(2-(5-phenyl-oxazoyl))benzene in 1 l of toluene] and the radioactivities were counted by liquid scintillation counter (Aloka LSC-651).

**Preparation of $^{13}C$-Labelled Pyrindicin**——*Streptomyces griseoflavus*, a strain producing pyrindicin, was culture in modified Waksman medium (glucose 2.0%, peptone 0.5%, meat extract 0.5%, dried yeast 0.3%, $CaCO_3$ 0.3%, NaCl 0.5% (pH 7.0)). Each $^{13}C$-precursors (0.1% w/v), 90% enriched [1—$^{13}C$]acetate, [2—$^{13}C$]-acetate, or [1—$^{13}C$]propionate was added to the medium after 20 hr of culture and the cultivation was continued at 27° for 48 hr. The broth filtrates (1—2 l) were extracted with *n*-butyl acetate at pH 10, and alkaloid was transferred into 0.1 N aqueous HCl from the organic layer. The water layer was adjusted to pH 10 and extracted with ethyl ether. The ether extract was dried over anhydrous sodium sulfate and concentrated to a small volume, to which a saturated ether solution of picric acid was added until no more precipitates formed to obtain $^{13}C$-labelled pyrindicin picrate (27—66 mg) as yellow needles.

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