PREPARATION OF ^{13}C- AND ^{3}H-LABELED CERULENIN AND BIOSYNTHESIS WITH ^{13}C-NMR

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Since the establishment of the mode of action of antifungal antibiotic cerulenin[1] by BLOCH, ŌMURA, VAGELOS, and OTHERS,[2~8] this antibiotic has become an important tool in the field of biochemistry as a specific inhibitor of lipid synthesis. In addition, cerulenin, [(2S)(3R)-2,3-epoxy-4-oxo-7,10-dodecadienoylamide],[9] is one of the derivatives of dodecanoic acid and thus presents several features of general interest from biosynthetic mechanisms viewpoint. The presence of two double bonds in a fatty acid and its derivatives is not uncommon, but the fact that both are in a *trans* configuration and that one is ω_2 is a novel structure. The *cis*-epoxide ring between two carbonyl groups, ketone and amide, also appears to be unique.

To resolve the problem of whether the cerulenin skeleton is synthesized from acetate or acetate plus another precursor such as succinate or glycerol, the biosynthesis of cerulenin was studied by using ^{13}C-NMR. As a preliminary experiment in order to know the most suitable condition to yield ^{13}C-labeled cerulenin, the incorporation of ^{3}H-acetate into cerulenin was examined.

Materials and Methods

Culture

Cephalosporium caerulens KF-140 was used for the production of cerulenin. Laboratory fermentations were run in 500-ml SAKAGUCHI flasks each containing 100 ml of the indicated medium at 27°C or on a reciprocal shaker (8-cm throw and 120 strokes/min). The seed culture was incubated for 48 hours in a seed medium containing 2% glucose, 0.5% peptone, 0.5% meat extract, 0.3% dry yeast, 0.5% NaCl and 0.3% $CaCO_3$. The resulting culture was used to inoculate flasks (1% volume transfer) of the second seed medium containing 3% glycerol, 1% glucose, 0.5% peptone, and 0.2% NaCl and further incubated for 24 hours.

Cultures for the preparation of labeled cerulenin were grown for 45 hours in the production medium (1 K medium) containing 1% glucose, 0.1% urea, 0.2% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, and 0.001% $FeSO_4 \cdot 7H_2O$.

Measurement of the amount of cerulenin

The amount of cerulenin was determined by a biological method used of *Candida albicans* KF-7 as described in our previous report.[10]

Assay of radioactivity

After the radioactive cerulenin was purified by the method described in Fig. 1, pre-blended scintillator (0.4% PPO and 0.005% POPOP in toluene) was added and the radioactivity of the sample was measured by Packard Tri-Carb Liquid Scintillation Spectrometer Model 3375.

Chemicals

Sodium acetate [U-^{3}H] was purchased from Daiichi Chemicals, Co., Tokyo and sodium acetate [1-^{13}C] from MSD (Japan) Co., Tokyo.

Results and Discussion

1. Effect of glucose concentration on the incorporation of ^{3}H-acetate into cerulenin

The effect of glucose concentration on the incorporation of ^{3}H-acetate into cerulenin was examined in the range of 1~4% of glucose as the productivity of cerulenin decreased suddenly when cultivated at the concentration of less than 1% of glucose in the production medium. As shown in Table 1, the highest incorporation of radioactive cerulenin was obtained when the fungus was cultivated in the medium containing 1~2% of glucose. In this experiment, the purification of cerulenin to measure the radioactivity was carried out by using the same method as described for the preparation of ^{13}C-labeled cerulenin. (Fig. 1).

2. Effect of the time of adding ^{3}H-acetate on the incorporation of the radioactivity into cerulenin

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Fig. 1. Preparation of ^{13}C-labeled cerulenin.

Seed culture I
↓ 27°C, 48 hours, shaking culture
Seed culture II
↓ 27°C, 24 hours, shaking culture
Main culture
↓ ^{13}C-acetate added after 14 hours harvested at 45 hours
Culture filtrate (cerulenin, 94 μg/ml)
↓ extracted with $CHCl_3$
$CHCl_3$ layer
↓ dried *in vacuo*
Crude powder
↓ Silica gel column chromatography developed with $CHCl_3$-EtOAc (50 : 0~2, v/v)
Active fraction
↓ dried *in vacuo*
Crude powder
↓ crystallized from benzene - petroleum ether
^{13}C-Labeled cerulenin (yield 11.6 mg, 15.4 %)

Modified production medium containing 2 % of glucose was used to test the effect of the time adding ^{3}H-acetate on the incorporation of the radioactivity into cerulenin. Then the time of adding ^{3}H-acetate was examined during 6~8 hours centering around 10 hours after the seeding. As shown in Table 2, the incorporation of labeled precursor reached maximum at 14 hours.

3. Preparation of ^{13}C-labeled cerulenin

On the bases of the above results, preparation of ^{13}C-labeled cerulenin was carried out as shown in Fig. 1. At the time when incubation of culture reached 14 hours after addition of the second seed culture of 10 % inoculum, 50 mg of ^{13}C-acetate was added to each 100 ml of the culture, and the fermentation continued as described in Materials and Methods. The purification of cerulenin was accomplished by chloroform extraction, silica gel chromatography and crystallization, and 11.6 mg of crystalline ^{13}C-labeled cerulenin was obtained from 800 ml of culture filtrate.

4. ^{13}C-NMR spectra of natural and ^{13}C-labeled cerulenin

^{13}C-NMR spectra of natural and ^{13}C-labeled cerulenin are shown in Figs. 2 and 3. Evidence for the biosynthetic labeling pattern comes from the ^{13}C-NMR spectrum of the antibiotic. Carbon signal assignment (see Table 3) for cerulenin has been based on the chemical shift rules. Differentiation of the four ethylenic and two epoxymethine carbons has been made by observing enhanced signals on the spectrum of the material from ^{13}C-acetate and by assuming an alternating labeling on the basis of the absence of any ^{13}C-^{13}C direct coupling (see Fig. 2). Among the three methylene carbons, C-5, being the neighbor of a ketone, should be in the lowest field (40.9 ppm) while the negative β-effect of the carbonyl group[11] brings C-6 to the high fileds (26.1 ppm) differentiating it from C-9 (35.5 ppm).

It is interesting to notice the triplet-like pattern for both C-5 and C-3 on the ^{13}C-NMR spectrum of cerulenin from the ^{13}C-acetate experiment. Although geminal C-C-C couplings are generally small,[11] this interaction is markedly increased if a carbonyl group is between two ^{13}C atoms.[11] DREESKAMP and his coworkers[12] have reported +16.1 Hz for this coupling constant in acetone. The central

Table 1. Effect of glucose concentration on the incorporation of ^{3}H-acetate into cerulenin

Glucose (%)	Radioactivity (cpm/μg of cerulenin)
4	2,950
3	3,610
2	4,950
1	4,945

^{3}H-Acetate (1 μCi/ml) was added at 8 hours after initiation of the cultivation. To assay the radioactivity, cerulenin was prepared from the culture filtrate after cultivation for 45 hours.

Table 2. Effect of the time adding ^{3}H-acetate on the incorporation of the radioactivity into cerulenin

Addition time (hours)	Radioactivity (cpm/μg of cerulenin)
6	2,178
8	2,908
10	4,010
12	4,502
14	4,510

^{3}H-Acetate (1 μCi/ml) was added at the time indicated.
Cultivation and assay of the radioactivity were carried out as described in the text.

Fig. 2. ^{13}C-NMR spectrum of natural cerulenin.

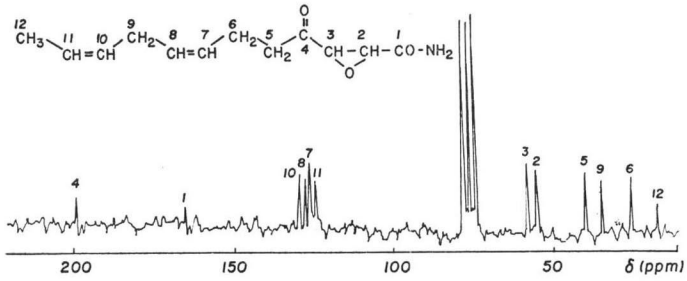

Fig. 3. ^{13}C-NMR spectrum of ^{13}C-acetate labeled cerulenin.
The ^{13}C incorporation yield based on the intensity ratio of the enhanced and natural abundance peaks may be estimated to be 12~15 %.

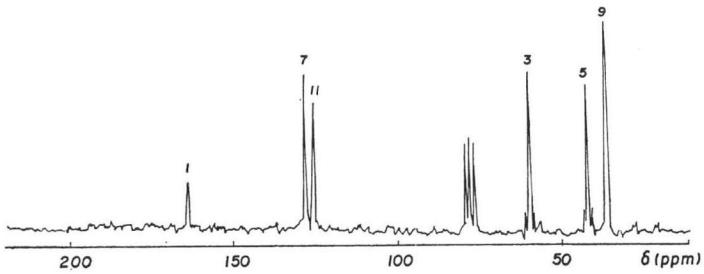

Table 3. ^{13}C-Chemical shifts for cerulenin

Carbon atoms No.	Chemical shifts (δ) from $(CH_3)_4Si$
C-1	167.2
C-2	55.4
C-3	58.4
C-4	202.0
C-5	40.9
C-6	26.1
C-7	127.8
C-8	129.3
C-9	35.5
C-10	130.8
C-11	125.8
C-12	17.9

line of the triplet at 58.4 ppm represents C-3 in those species where C-5 is a ^{12}C, while the two "outer lines" of the triplet at 58.8 and 58.0 ppm indicates C-3 in those species where C-5 is a ^{13}C. The observed coupling constant of 18.8 Hz excludes any direct ^{13}C-^{13}C coupling and is in agreement with a geminal coupling constant through a carbonyl group.

These results suggested that biosynthesis of cerulenin was closely related with fatty acid synthesis probably catalyzed by fatty acid synthetase.

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