BIOSYNTHETIC PREPARATION OF LABELED CERULENIN WITH HIGH SPECIFIC RADIOACTIVITY

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Cerulenin, (2*R*)(3*S*)-2,3-epoxy-4-oxo-*E*,*E*-7,10-dodecadienoylamide,[1] is a potent inhibitor of fatty acid synthase systems isolated from various microorganisms and animal tissues.[1] This antibiotic specifically blocks the activity of 2-oxoacyl thioester synthase (condensing enzyme).[1] We have proposed that cerulenin covalently binds to the cysteine-SH at the active center of the condensing enzyme.[2,3] Cerulenin has also been found to inhibit the biosynthesis of various polyketide-derived secondary metabolites such as macrolides[4] and nanaomycin.[5] Because of its unique mode of action and usefulness as a biological reagent, attempts were made to prepare [^{3}H]- and [^{14}C]cerulenin by the present authors[6,7] and other research groups.[8] Labeled cerulenin has been used for the binding and active-site studies on fatty acid synthase.[6~8] However, the specific radioactivities of these preparations were not very high (0.4[6] and 0.016[7] mCi/mmol of [^{3}H]cerulenin) although TSUKAMOTO *et al.*[8] reported on [^{14}C]cerulenin with fairly high specific radioactivity (2.21 mCi/mmol). On the other hand, ROBERT and LEADLAY[9,10] prepared [^{3}H]tetrahydrocerulenin (45 Ci/mmol) by chemical reduction of cerulenin with tritium gas over PtO_2.

The purpose of this paper is to present a method of biosynthetic preparation of labeled cerulenin with high specific radioactivity. This method yielded 13.2 mCi/mmol of [^{3}H]-cerulenin.

Materials and Methods

Microorganism

Cephalosporium caerulens KF-140 was used for the production of cerulenin.

Cultivation

Spores and mycelia of *C. caerulens* KF-140 were inoculated into a test tube (20×2 cm) containing 10 ml of seed medium (glucose 2%, meat extract 0.5%, peptone 0.5%, dried yeast cells 0.3% and NaCl 0.5%, pH 7.0). The tube was incubated at 27°C for 2 days with reciprocal shaking (280 strokes/minute). The seed culture (1.0 ml) was transferred into a 500-ml Sakaguchi flask containing 100 ml of production medium for the preparation of labeled cerulenin (glycerol 3%, glucose 0.5%, peptone 0.1%, NaCl 0.2% and zeolite 1.0%). Zeolite, an ammonium ion-trapping agent, enhances cerulenin production as we reported previously.[11] A natural zeolite (Fusseki Kako Co.) and a synthetic mordenite powder (Nippon Chemical Industry Co.) allowed the best cerulenin production among zeolites commercially available. Labeled acetate was fed at the 14th and/or 24th hour of cultivation.

Measurement of the Production and Radioactivity of Labeled Cerulenin

One-ml of cultured broth withdrawn from flasks was extracted with 1 ml of EtOAc. After centrifugation, 0.5 ml of the EtOAc layer was concd *in vacuo*. The extracts were dissolved in 100 μl of MeOH and a 20-μl aliquot was used to determine the amount of cerulenin by HPLC. The conditions of HPLC were as follows: Apparatus; Jasco Tri Rotar V system, column; YMC-Pack A-302 ODS (4.6×150 mm), elutant; 40% CH_3CN in 10 mM KH_2PO_4 (pH 3.0), flow rate; 1.0 ml/minute, detection; UV at 210 nm. The retention times of cerulenin are 12.5 and 15.0 minutes for the acyclic and hydroxylactam forms.[3]

The fractions of the two forms of cerulenin purified by HPLC were poured directly into scintillation vials. After addition of 4 ml of a scintillator (ACS II, Amersham Corp.), the radioactivity of cerulenin was measured by a scintillation spectrometer (Aloka LSC-751 system).

Chemicals

Sodium [*U*-^{3}H]acetate (3.2 Ci/mmol, NEN

Research Products) and sodium [1-^{14}C]acetate (2.5 mCi/mmol, NEN Research Products) were purchased from Daiichi Chemical Co.

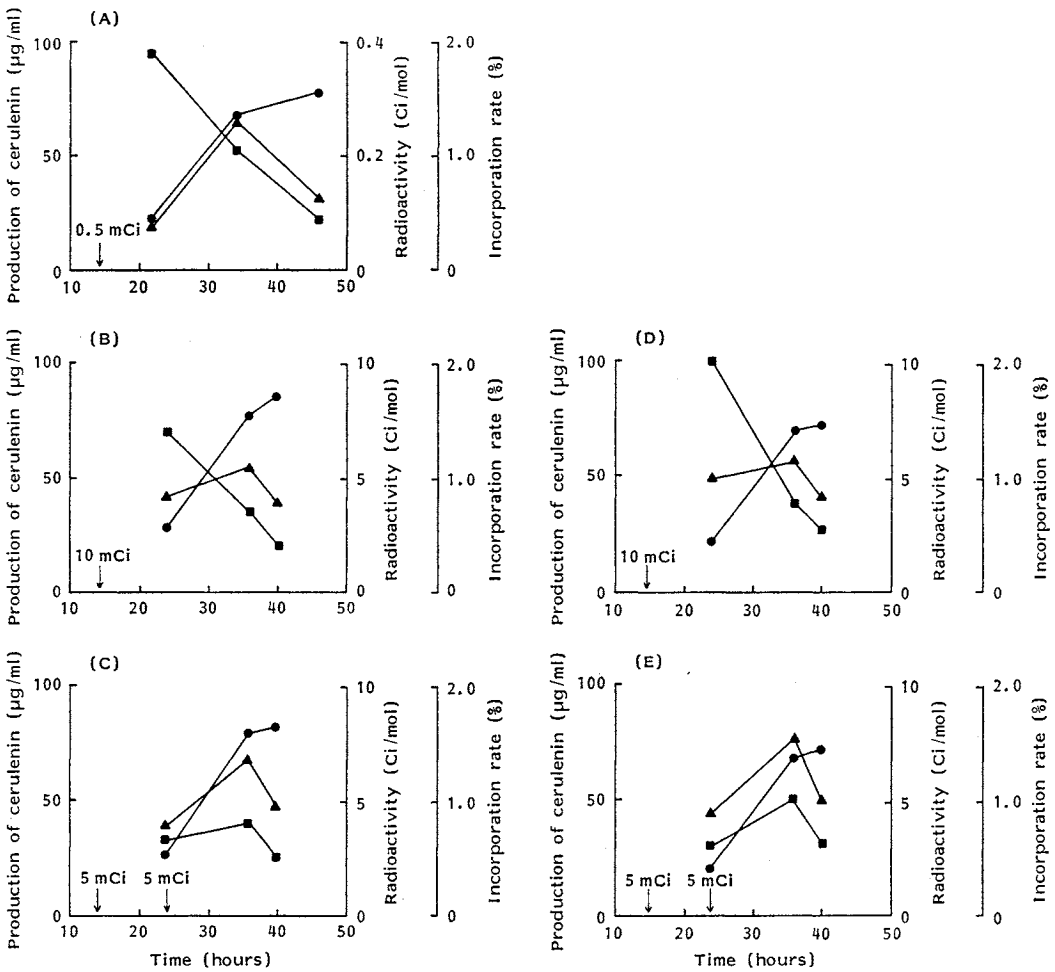
Results and Discussion

Several experiments were carried out to obtain labeled cerulenin with high specific radioactivity (Figs. 1 (A)~(E)). At the various times indicated, the amounts of labeled cerulenin, the specific radioactivity and the rate of [^{3}H]acetate incorporation into cerulenin were measured. In the previous paper,[12] 1.0% glucose was used in the production medium. Further studies revealed that the cerulenin production was almost the same when the fungus was cultivated in the medium containing 0.5% glucose. Then the concentrations of carbon sources, glycerol and glucose, in the production medium were fixed at 3.0 and 0.5%, respectively.

First, 0.5 mCi (A) or 10 mCi (B) of [^{3}H]-acetate was added at the 14th-hour of cultivation when cerulenin production begins as reported previously.[12] Increased amount of [^{3}H]-acetate added exogenously caused a corresponding elevation in specific radioactivity, while the

Fig. 1. Time course of [^{3}H]cerulenin production (●), specific radioactivity (■) and incorporation rate of [^{3}H]acetate into cerulenin (▲).

A: 0.5 mCi of [^{3}H]acetate was added at 14 hours, B and D: 10 mCi of [^{3}H]acetate was added at 14 hours, C and E: 5 mCi each of [^{3}H]acetate was added at 14 and 24 hours. Production medium: glycerol 3%, peptone 0.25%, glucose 0.5%, NaCl 0.2% and zeolite 1.0% for A, B and C and glycerol 3% ,peptone 0.1%, glucose 0.5%, NaCl 0.2% and zeolite 1.0% for D and E.



patterns of cerulenin production and incorporation rates of [^{3}H]acetate were almost the same.

When the content of peptone in the production medium was reduced from 0.25% (B and C) to 0.1% (D and E), cerulenin production decreased slightly, whereas the specific radioactivity of [^{3}H]cerulenin increased. A single addition of [^{3}H]acetate was associated with a steep decline in specific activity (A, B and D). This was overcome by intermittent additions of 5 mCi each at the 14th and 24th-hour (C and E). Thus, the specific radioactivity could be retained constant or even raised until 36 hours.

From the results described above, we adopted finally the conditions of experiment E except that the amount of [^{3}H]acetate to be added was increased further. At the 14th and 24th-hour, 14.4 mCi each of [^{3}H]acetate was added (a total of 28.8 mCi/flask). At the 39th-hour the cultured broth from two flasks was centrifuged to obtain 200 ml of a supernatant fluid containing 15 mg of [^{3}H]cerulenin (13.2 mCi/mmol). The fluid was extracted with 200 ml of EtOAc. The extracts were concd to dryness *in vacuo* to yield a yellowish paste (40 mg). The paste was purified by HPLC (Jasco Tri Rotar V, column; YMC-Pack A-343 ODS 20×250 mm, solvent; 50% aq CH_3CN, flow rate; 8.0 ml/minute, detection; UV at 220 nm). The fractions of cerulenin were combined and concd *in vacuo* to give a pure white powder of [^{3}H]cerulenin (13.2 mCi/mmol, 13.8 mg). [^{3}H]Acetate incorporation into the cerulenin molecule was 1.5%. The high radioactivity of the [^{3}H]cerulenin obtained is sufficient to use for binding or active-site studies on fatty acid synthase and related enzymes.

The same method was applied to prepare [^{14}C]cerulenin. It was considered that the rate of [^{14}C]acetate incorporation would be lower than that of [^{3}H]acetate incorporation because the specific radioactivity of [^{14}C]acetate (2.5 mCi/mmol) now available is about one thousandth of that of [^{3}H]acetate (3.2 Ci/mmol). Contrary to our expectation, 5 mg of [^{14}C]-cerulenin (0.55 mCi/mmol, incorporation rate of 1.2%) was obtained from 100 ml of a culture filtrate when 0.5 mCi [^{14}C]acetate was added at the 14th and 24th-hour (1 mCi in total) to production medium. Further experiments on [^{14}C]-cerulenin were not carried out, since [^{14}C]acetate is expensive. However, it is worthwhile to note that the final incorporation rates were almost the same and that the specific radioactivity of labeled cerulenin appeared to be proportional to the radioactive acetate added under the conditions employed. Therefore, it should be possible to prepare [^{14}C]cerulenin with a higher specific radioactivity if an increased amount of [^{14}C]acetate is added to the medium.

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