[^{3}H]Tetrahydrocerulenin, a specific reagent for radio-labelling fatty acid synthases and related enzymes

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We have synthesised [^{3}H]tetrahydrocerulenin, a radio-labelled derivative of cerulenin, an antibiotic from *Cephalosporium caerulens* which specifically inhibits the condensing enzyme of fatty acid synthases and polyketide synthases from various sources. [^{3}H]Tetrahydrocerulenin binds to fatty acid synthase from pig liver with the same specificity as cerulenin. We have also used [^{3}H]tetrahydrocerulenin to monitor condensing enzyme activity in cell-free extracts of the erythromycin-producing organism *Streptomyces erythreus*.

Fatty acid synthase *Cerulenin* *Affinity labelling* *Macrolide antibiotic synthase* *Erythromycin*

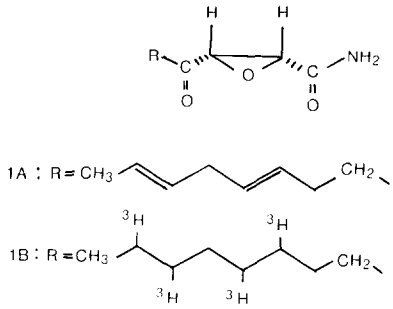
1. INTRODUCTION

Cerulenin [(2*R*)(3*S*)-2,3-epoxy-4-oxo-7,10-dodecadienoylamide] (structure 1A), an antibiotic isolated from *Cephalosporium caerulens* [1], is a potent inhibitor of fatty acid synthase systems from various micro-organisms and animal tissues [2–4]. The antibiotic specifically and irreversibly blocks the activity of β-ketoacyl thioester synthase (condensing enzyme) [3,4], and it has been proposed that cerulenin becomes covalently bound to the 'peripheral' cysteine residue at the active site of the condensing enzyme [5]. Cerulenin has also been found to inhibit the biosynthesis of various polyketide-derived natural products such as 6-methylsalicylic acid [6] and the macrolide antibiotics leucomycin [7] and candicidin [8], suggesting that the condensing enzyme in polyketide biosynthesis resembles the analogous component of fatty acid synthase.

This report describes the synthesis of [^{3}H]tetrahydrocerulenin (1b) and its use for specific labelling of fatty acid synthase. We also report the use of the reagent for detection of fatty acid synthase and related enzymes in bacterial extracts.

Structure 1

Structure of cerulenin (1A) and of [^{3}H]tetrahydrocerulenin (1B) showing the anticipated distribution of tritium label



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2. MATERIALS AND METHODS

CoA esters were purchased from Sigma Chemicals (St Louis MO). Cerulenin was supplied by C.P. Labs. (Bishop's Stortford, Herts). Tritiated tetrahydrocerulenin (about 45 Ci/mmol) was prepared by catalytic reduction of cerulenin

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with carrier-free tritium gas over PtO_2, essentially as in [9]. This was performed by the tritium labelling service of Amersham International (Amersham Bucks). Portions of the product were purified before use by preparative TLC on silica plates (Merck) eluted with chloroform–methanol (25:1, v/v). In this system [^{3}H]tetrahydrocerulenin migrated with an R_F of 0.35. The presumed labelling pattern is shown in structure 1B.

2.1. *Purification of fatty acid synthase*

Fatty acid synthase was purified from pig liver by a published procedure [10], except that gel-filtration on Bio-gel A1.5m (Bio-Rad) replaced fractionation on a sucrose density gradient. The final specific activity (overall complex) was 34 nkat/mg.

2.2. *Preparation of extracts of* Streptomyces erythreus

Streptomyces erythreus CA340 (Abbott), a mutant strain that overproduces erythromycin, was grown vegetatively as in [11]. For production of antibiotic, mycelium was transferred to a sucrose-containing medium [12]. Cells were washed, resuspended (2 ml/g wet wt) in 50 mM Tris–HCl (pH 7.4), containing 2 mM EDTA, 1 mM dithiothreitol, and glycerol (10%, v/v), and disrupted by two passes through a French pressure cell operated at maximum pressure. Cell debris was removed by centrifugation (12000 × *g*, 10 min) and streptomycin sulphate (final conc. 1%, w/v) was added to precipitate nucleic acids. The mixture was re-centrifuged (27000 × *g*, 20 min) and the supernatant was used for the labelling experiments described below.

2.3. *[^{3}H]Tetrahydrocerulenin labelling*

To 50 μg purified fatty acid synthase (or 200 μg protein from *S. erythreus* extracts) in 0.2 ml 50 mM Tris–HCl (pH 7.4) at 20°C, [^{3}H]tetrahydrocerulenin (final conc. 30 μM) was added, followed 10 min later by excess unlabelled cerulenin (0.2 mM). The cerulenin 'chase' was found to be essential to minimise non-specific labelling.

2.4. *Gel electrophoresis*

SDS–polyacrylamide gel electrophoresis was performed in slab gels [13]. Protein samples were precipitated with 5 vol. acetone. The pellets were washed twice with acetone and then dissolved in gel sample buffer [13] and heated at 100°C for 2 min before applying to the gel. After electrophoresis, overnight at 50 V, gels were stained with methanol–acetic acid–water (5:1:5, by vol.) containing 0.1% Coomassie brilliant blue for 30 min at 60°C, and subsequently destained in the same solvent mixture at room temperature.

2.5. *Fluorography*

The destained gels were photographed, and prepared for fluorography as in [14]. The dried gels were exposed at −70°C to pre-flashed X-ray film (Fuji RX) for 7 days.

3. RESULTS AND DISCUSSION

Incubation of purified fatty acid synthase from pig liver with [^{3}H]tetrahydrocerulenin resulted in apparently covalent attachment of the tritiated ligand to the protein. This labelling was

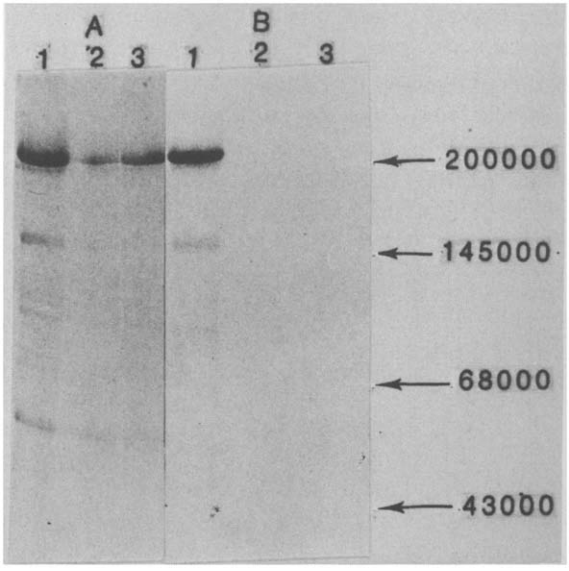

Fig.1. 12.5% SDS–polyacrylamide gel electrophoresis of pig liver fatty acid synthase treated with [^{3}H]tetrahydrocerulenin. Proteins were detected either (A) with Coomassie brilliant blue or (B) by fluorography: (1) fatty acid synthase (40 μg); (2) fatty acid synthase (10 μg) pre-incubated with acetyl-CoA (1 mM); (3) fatty acid synthase (20 μg) pre-incubated with cerulenin (0.5 mM).

demonstrated in fluorograms of gels containing fatty acid synthase, after electrophoresis in the presence of 0.1% SDS (fig.1). The labelling of fatty acid synthase was abolished either in the presence of cerulenin (which binds very much more tightly to fatty acid synthase than does tetrahydrocerulenin [3]) or in the presence of acetyl-CoA, which is known to compete with cerulenin for the cysteine sidechain in the condensing enzyme [5]. NADPH and malonyl-CoA gave very little protection against labelling by [^{3}H]tetrahydrocerulenin (not shown) which is also consistent with the idea that the tritiated ligand and cerulenin bind to the same unique site in fatty acid synthase [5].

Fatty acid synthase from various animal sources has been shown to consist of a dimer of identical subunits, each of M_r 220000–250000 [15–17]. The 7 individual enzyme activities of the multienzyme complex are therefore properties of a single type of polypeptide chain. The availability of [^{3}H]tetrahydrocerulenin, a radio-labelled active site-directed inhibitor of the condensing enzyme, should allow us to undertake structural studies on this enzyme component of the multifunctional polypeptide.

We have also used [^{3}H]tetrahydrocerulenin in attempts to detect fatty acid synthase-like enzymes in cell-free extracts of the erythromycin-producing organism *Streptomyces erythreus*. The core aglycone of erythromycin is constructed from 1 molecule of propionyl-CoA and 6 molecules of methylmalonyl-CoA, in a process analogous to fatty acid biosynthesis [18]. Unfortunately, very little is known about the enzyme responsible, erythronolide synthase. The analogy between this enzyme and fatty acid synthase is strengthened by our finding (unpublished) that cerulenin, when added to resting cells of *S. erythreus* in the antibiotic-producing phase at the end of exponential growth, totally inhibits production of erythromycin.

Only one polypeptide (M_r ~70000) is significantly labelled by [^{3}H]tetrahydrocerulenin in cell-free extracts of exponentially-growing *S. erythreus* (see fig.2). When extracts were examined from cells early in the antibiotic-producing phase, additional bands were detected after fluorography (fig.2). Interestingly, the major new band of radioactivity (M_r 35000) has the same mobility as the β-ketoacyl

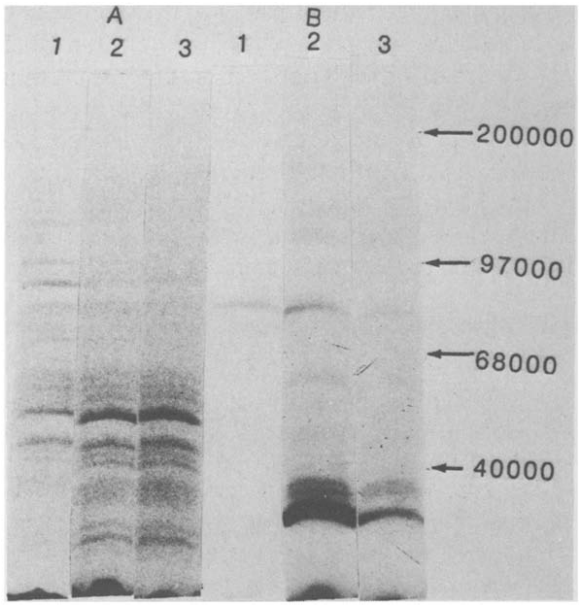

Fig.2. 12.5% SDS–polyacrylamide gel electrophoresis of *S. erythreus* extracts treated with [^{3}H]tetrahydrocerulenin. Proteins were detected either (A) with Coomassie brilliant blue or (B) by fluorography: (1) extract from exponentially-growing cells; (2) extract from differentiated cells (antibiotic-producing phase); (3) extract from differentiated cells, pre-incubated with propionyl-CoA (1 mM).

ACP synthase I (condensing enzyme) from *E. coli* [19,20]. Labelling of the M_r 35000 polypeptide was greatly reduced in the presence of propionyl-CoA. Cerulenin and acetyl-CoA were also effective in preventing labelling of the polypeptide (not shown). Further experiments will be required to clarify the relationship between this apparent condensing enzyme activity present in differentiated *S. erythreus*, and macrolide antibiotic synthesis which occurs specifically at this phase of growth.

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