THE ENZYMIC CONVERSION OF SQUALENE, 2(3),22(23)-DIEPOXIDE TO α-ONOCERIN BY A CELL-FREE EXTRACT OF *ONONIS SPINOSA*

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1. Introduction

Squalene-2(3)-epoxide is the immediate non-cyclic precursor in sterol biosynthesis in animals [1, 2] and plants [3]. Corey and Ortiz de Montellano have demonstrated that the triterpene β-amyrin is biosynthesised from squalene-2(3)-epoxide by *Pisum sativum* seedlings [4]. Squalene on the other hand is cyclised directly in tetrahymanol biosynthesis by *Tetrahymena pyriformis* [5].

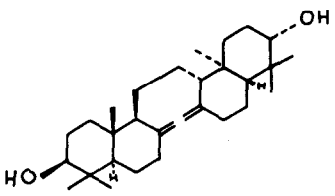

Fig. 1. The structure of α-onocerin.

α-Onocerin is a triterpene found in some species of *Ononis* and in *Lycopodium clavatum.* Its structure (fig. 1) suggests that squalene-2(3),22(23)-diepoxide, squalene-2(3)-epoxide or squalene itself may cyclise by analogous mechanisms to those referred to above. In this paper we provide evidence that squalene-2(3), 22(23)-diepoxide is the precursor of α-onocerin.

2. Materials and methods

2.1. α- *Onocerin extraction*

α- Onocerin was extracted from roots of *Ononis spinosa* by the method of Barton and Overton [6]. The crude α-onocerin was recrystallised from chloroform; m.p. = 204–205° (uncorr.) lit 202–203° [6].

A portion of this material was acetylated with acetic anhydride and pyridine and the product recrystallised from chloroform–acetone; m.p. = 222–224° (uncorr.) (lit 222–224° [6]).

2.2. *Substrates*

1-^{14}C-Squalene-2(3)-epoxide (specific activity = 2.3 μCi per mg) was a gift from Professor E.J. Corey. 3-^{3}H-Squalene (specific activity = 1.3 μCi per mg) and 3-^{3}H-squalene-2(3),22(23)-diepoxide (specific activity = 6.4 μCi per mg) were prepared by Dr. J.B. Greig by a modification of the method described by Nadeau and Hanzlik [7].

2.3. *Preparation of enzyme*

Seedlings of *Ononis spinosa* were grown by the University of Liverpool Botanic Gardens. Both roots and leaves of 2 month old seedlings were used as a source of enzyme. About 10 g of tissue were ground with a pestle and mortar under liquid nitrogen. The frozen powder was stirred into 0.1 M potassium phosphate pH 7.2 (30 ml), containing 2% (w/v) soluble polyvinyl pyrollidone, 0.05% (w/v) cysteine, ascorbate (0.05 M) and sucrose (0.2 M). The homogenate was strained through gauze and the filtrate centrifuged at 20,000 *g* for 20 min. The pellet was discarded. All manipulations were carried out at 0–4°.

2.4. *Assay for cyclase activity*

The enzyme preparation (0.8 ml) and an emulsion of the substrate (squalene-2(3),22(23)-diepoxide, 20,000 cpm in 0.2 ml) prepared as described by Dean

et al. [8] were incubated with shaking, under nitrogen at 25° for 15 hr. Control incubations were carried out with boiled preparation. The reaction was stopped by adding an equal volume of 2 M KOH in methanol. The solution was extracted with ether (5 times). Carrier α-onocerin (1 mg) was added to the ether extract and the products separated by thin layer chromatography (TLC) on silica gel G (solvent: 2% v/v methanol in chloroform).

Incubations were also carried out with squalene and squalene-2(3)-epoxide as substrates. The products of the reaction with squalene were separated by TLC on silica gel G impregnated with 20% (w/w) $AgNO_3$ (solvent: 50% v/v benzene in hexane). The extracts from the incubation with squalene-2(3)-epoxide were separated on silica gel G plates irrigated with 9% (v/v) ethyl acetate in benzene.

2.5. *Identification of ^{3}H-α-onocerin*

A large scale incubation was performed using 20 ml of root enzyme preparation and about 10^6 dpm of 3-^{3}H-squalene-2(3),22(23)-diepoxide as substrate.

After 18 hr, a suspension of 49 mg of authentic α-onocerin in 10 ml of water was added to the assay, and the reaction immediately stopped. The mixture was extracted five times with diethyl ether. The combined extracts were washed with water, dried ($MgSO_4$), and the solvent removed under reduced pressure. The product was washed several times with light petroleum (b.p. 40–60°) to remove unchanged squalene-2(3),22(23)-diepoxide. The residue was acetylated with pyridine and acetic anhydride and the resulting onocerin diacetate recrystallised from chloroform–acetone to constant specific radioactivity. An aliquot of the crystals was removed at each stage, weighed and the radioactivity determined by liquid scintillation.

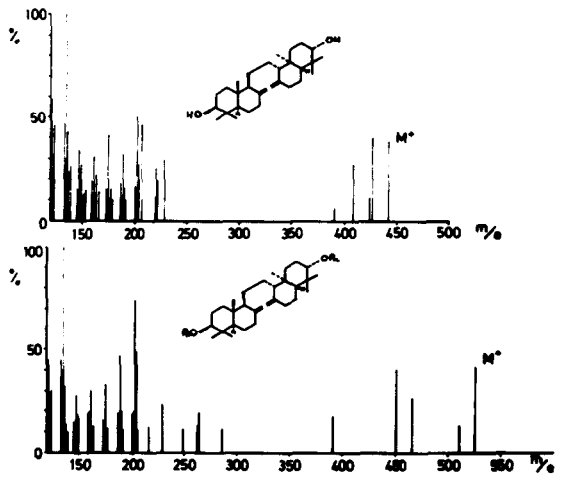

Fig. 2. Mass spectra of α-onocerin (top) and α-onocerin diacetate.

3. Results

3.1. *Mass spectra of α-onocerin and α-onocerin diacetate*

The mass spectra of α-onocerin and α-onocerin diacetate are shown in fig. 2. They were obtained with an AEI MS 12 mass spectrometer using a vacuum lock direct insertion probe. All the spectra were recorded at an ionising voltage of 70 eV and temperature of 180°.

3.2. *Biosynthesis of α-onocerin*

Two radioactive peaks were observed in anaerobic incubations in which squalene-2(3),22(23)-diepoxide was used as substrate. These correspond to unreacted squalene-2(3),22(23)-diepoxide (R_f 0.8) and α-onocerin (R_f 0.4). Enzyme preparations from 2 month old *O. spinosa* roots gave maximum yields of 11% of α-onocerin. Leaf preparations from the same plants gave conversions of about 5%.

No products were detected when squalene was used as substrate for the root enzyme preparations. When squalene-2(3)-epoxide was used as substrate a minor peak (1% yield) with chromatographic properties similar to cycloartenol was produced by both root and leaf enzyme preparations; the compound was not further characterized.

3.3. *Recrystallisation of ^{3}H-α-onocerin diacetate*

The results of a large scale incubation are summarised in table 1.

4. Discussion

Squalene-2(3),22(23)-diepoxide is the only substrate which is converted to α-onocerin under

Table 1
Recrystallisation of ^{3}H-α-onocerin diacetate.

Radioactivity	dpm
of substrate added	10^6
recovered after incubation in petrol soluble fraction (unchanged substrate)	954,500
	921,000
in residue	33,500

Recrystallisation of acetylated residue:

Recrystallisation number	Weight of crystals taken for counting (mg)	Radioactivity (dpm)	Specific radioactivity (dpm/mg)
1	4.87	808	166
2	5.70	855	150
3	3.45	535	155
4	3.69	557	151

anaerobic conditions. If it is assumed that only the (3 S, 22 S) isomer of squalene-2(3),22(23)-diepoxide is utilised by this enzyme the maximum conversion of 11% total squalene-2(3),22(23)-diepoxide to onocerin is equivalent to the cyclisation of 44% of the active isomer.

Under anaerobic conditions the other potential precursors, squalene and squalene-2(3)-epoxide, were not measurably converted to products which might be considered as intermediates in onocerin biosynthesis. Squalene was not converted to a bicyclic or tetracyclic triterpene by a reaction analogous to the squalene cyclisation in tetrahymanol biosynthesis [5]. Squalene-2(3)-epoxide was rather inefficiently (1%) converted to a compound similar to cycloartenol. Although the possibility that this compound has the structure shown in fig. 3 cannot yet be discounted, the cyclisation of squalene-2(3)-epoxide is neither as fast nor as reproducible as the cyclisation of squalene-2(3),22(23)-diepoxide.

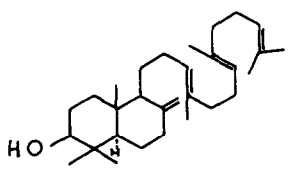

Fig. 3. A possible product of squalene-2(3)-epoxide cyclisation.

Under some conditions (pH 7.4 in the absence of ascorbate) it is possible to accumulate small quantities (1%) of a compound similar to cycloartanol-24(25)-epoxide using squalene-2(3),22(23)-diepoxide as substrate. Squalene-2(3),22(23)-diepoxide has been cyclised to lanosterol-24(25)-epoxide by an enzyme preparation from pig liver [9]. Similarly, bramble microsomes convert squalene-2(3),22(23)-diepoxide to cycloartanol-24(25)-epoxide [10]. These reactions are probably catalysed by enzymes for which the diepoxide is not the natural substrate. The present paper appears to be the first report of its cyclisation to a naturally occurring compound.

Acknowledgements

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