PLANT STEROL METABOLISM

EVIDENCE FOR THE PRESENCE OF AN ENZYME CAPABLE OF OPENING THE CYCLOPROPANE RING OF CYCLOEUCALENOL.

R. HEINTZ, P. BENVENISTE and T. BIMPSON

Laboratoire des Applications Biologiques du Groupe de Laboratoires de Strasbourg-Cronenbourg - 23, rue du Loess 67 - Strasbourg-Cronenbourg (France).

Received November 23, 1971

Summary

For the first time an enzyme-mediated opening of the 9β-19β cyclopropane ring of cycloeucalenol (I)* is reported. A cell-free system from bramble (Rubus Fruticosus) tissues grown in vitro converts cycloeucalenol into obtusifoliol (II). The implications of these results in terms of the biosynthetic pathway of phytosterols are discussed.

Some limitations on the use of cell-free systems in the study of plant-sterol biosynthesis have become evident in our laboratory. For instance, only squalene is synthesised in vitro starting from mevalonate (1). No product of the biosynthetic pathway after squalene could be isolated. It has been suggested that the mixed-fonction oxidases required for sterol formation are inhibited in cell-free systems derived

---

* Trivial Name

(I) = 4α, 14α-Dimethyl-24 methylene-9β, 19β cyclocholestan-3β-c

(II) = 4α, 14α-Dimethyl-24 methylene-cholest 8 en-3β-ol

(III) = 9β, 19β Cyclolanost-24 en-3β-ol

(IV) = 24 methylene-9β, 19β Cyclolanostan-3β-ol

Lanosterol = Lanosta-8, 24 dien-3β-ol.

Copyright © 1972, by Academic Press, Inc.

from plant tissues (1). In particular the transformation of squalene into squalene-2,3-oxide does not occur (1). However, some of the non-oxidative steps in the biosynthetic sequence are not inhibited. The in vitro cyclisation of squalene-2,3-oxide into cycloartenol (III) has been observed (2,3). Similarly, the in vitro methylation of both squalene-2,3-oxide and cycloartenol, in the presence of S-adenosyl methionine, has been demonstrated (4,5).

One of the later steps of particular interest is the opening of the cyclopropane ring. This enzymatic step is a unique one distinguishing biosynthesis in plants from that in animals, since the involment of 9β, 19β cyclopropane ring in sterol biosynthesis is restricted to plants. Furthermore, the absence of lanosterol in detectable amounts in higher plants implies that this enzymatic step does not fonction at the level of cycloartenol, but only with later intermediates.

We therefore investigated the ability of a cell-free system from tissue cultures of bramble to transform cycloeucalenol into obtusifoliol. This communication reports the results of these investigations.

Experimental

3-α-T cycloeucalenol was prepared by the reduction of cycloeucalenone with $NaBT_4$ (50 mC/μM, Commissariat à l'Energie Atomique, 91 - Gif-sur-Yvette). After addition of unlabelled cycloeucalenol, the product was purified by silica gel T.L.C. and recrystallised from methanol. The final product had a purity greater than 98 % on the base of g.l.c. analysis, and a specific radioactivity of 100 μC/μM.

A 1000 g fraction was prepared from bramble tissues as described previously (1). Incubations of the resuspended 1000 g pellet were carried out in a medium (70 mM Tris/HCl buffer) containing : 20 μmoles nicotinamide, 20 μmoles mercapto-ethanol, 32 μmoles $MgCl_2$, 16 μmoles ATP, 5 μmoles NADPH, 5 μmoles NADH, 32 μmoles KOH. The total incubation vo-

lume was 6 ml (5 mg protein/ml). Incorporations were carried out at 30°C for 5 hrs, after the addition of 20 nanomoles labelled cycloeucalenol, plus 200-300 µg unlabelled obtusifoliol as a trap.

The incubation was terminated by addition of 10 % KOH in ethanol ; the non-saponifiable lipid was extracted with petroleum ether and the 4α-methyl sterols were purified by silica gel T.L.C., acetylated and epoxidised with p-nitroperbenzoïc acid (6) after the addition of the unlabelled acetates of cycloeucalenol and obtusifoliol as carriers. The monoepoxide of cycloeucalenol acetate and the diepoxide of obtusifoliol acetate were separated by silica gel T.L.C. and the diepoxide recrystallised after addition of unlabelled carrier. All experiments were carried out with a control consisting of an identical enzyme preparation inactivated by heating at 100°C for 30 min.

Results and Discussion

After silica gel T.L.C. of the non-saponifiable lipid, radioscanning showed the presence of only one peak, corresponding to a product of R_f equivalent to that of cycloeucalenol. This gave, after acetylation and epoxidation, two peaks corresponding to the monoepoxide of cycloeucalenol acetate and the diepoxide of obtusifoliol acetate. It is known that sterols can be separated on the basis of number and position of double bonds by silica gel T.L.C. of their acetate epoxides (6). This method has limitations in that, during epoxidation of the steryl acetate, a loss of approximately 40 % occurs because of the rather drastic conditions used. This makes it difficult to put the results on a quantitative basis since the yields of recovery of the monoepoxide of cycloeucalenol acetate and the diepoxide of obtusifoliol acetate may be different.

However, the figures obtained from one experiment, shown in table I, clearly demonstrate that a significant

TABLE I

Activity in the 4α-methyl sterol fractions from the control and experimental preparations

	experimental (d.p.m)	control (d.p.m.)
Added before incubation	10×10^6	11×10^6
Alcohol recovered after incubation	9.4×10^6	10.2×10^6
Monoepoxides	2.3×10^6	2.08×10^6
Diepoxides	1.7×10^6	0.08×10^6

transformation of cycloeucalenol into obtusifoliol has taken place with the active enzyme preparation, the diepoxide of obtusifoliol acetate containing 42 % of the total activity. Compared to this the control experiment with an inactivated enzyme preparation contained only 2.7 % of the total activity at the R_f of diepoxide of obtusifoliol acetate. Furthermore, after a second chromatography on T.L.C. less than 1,5 ‰ of this radioactivity remained, making the recrystallisation of obtusifoliol acetate diepoxide from the control unnecessary.

The diepoxide of obtusifoliol acetate from the active enzyme preparation was further purified by recrystallisation from methanol after addition of unlabelled carrier ; the results are given in table II. The product retained 65 % of its initial total activity after reaching constant specific radioactivity.

These results demonstrate that the cell-free fraction used contains an enzymatic system capable of opening the 9β, 19β cyclopropane ring of cycloeucalenol to transform it

TABLE II

Recrystallisation of the diepoxide of obtusifoliol acetate.

		Specific radioactivity of the diepoxide (d.p.m./mg)	Specific radioactivity the material remaining in the mother-liquor (d.p.m./mg)
after addition of carrier		72,000 ± 4000	
recrystallisation	1)	66,000 ± 4000	
	2)	63,000 ± 4000	75,000 ± 4500
	3)	60,000 ± 3500	64,000 ± 4000
	4)	61,000 ± 3500	61,500 ± 3500

The diepoxide (8.5×10^5 d.p.m.) was recrystallised from methanol after the addition of 11.75 mg of unlabelled diepoxide of obtusifoliol acetate.

into obtusifoliol. The presence of such an enzyme has been postulated on biosynthetic grounds (7,8), but this is the first clear evidence of its existence. Previously, the conversion of cycloartenol into lanosterol by the latex of Euphorbia latyris had been reported (9). However it must be noted that this case is unique for several reasons. This tissue is the only one reported in the plant kingdom where lanosterol is present in appreciable quantities. Furthermore the conversion of cycloartenol into lanosterol does not occur in other species of Euphorbia examinated. Finally the tissue of Euphorbia does not appear to synthetise sterols to an appreciable extent (10).

But in our case, with the microsomal fraction derived from bramble tissues cultures ; under similar conditions

of incubation no transformation products of cycloartenol (III) or 24-methylene (IV) cycloartanol, which would indicate an opening of the 9β, 19β-cyclopropane ring, have been detected (11). Furthermore, incubation of such cell-free systems with squalene-2,3-oxide leads only to cycloartenol (3).

It therefore seems that the enzymatic systems of bramble tissues are able to open the 9β, 19β-cyclopropane ring in the presence of a 4α-methyl group, but not (or very slowly) in the presence of the 4,4-dimethyl group. This was one of the key hypothesis required for a biosynthetic interpretation of the presence of 24-methylene cycloartanol, cycloeucalenol and obtusifoliol in many plants. It is now demonstrated ; and this clearly provides further strong evidence for the genuine intermediary of these substances in sterol biosynthesis.

Furthermore, such a finding clearly argues against the operation, in bramble tissue cultures, of a ramified biosynthetic pathway of the type suggested by Benveniste et al. for tobacco tissue cultures (12) and in favour of a branched linear pathway as formulated by Goad et al. (13).

Acknowledgements

This work was supported by a grant from the D.G.R.S.T.

We thank Profs L. HIRTH and G. OURISSON, and Dr. B. FRITIG for discussion and encouragement and Mrs P. SCHMITT and Mrs. P. FUND for technical assistance.

One of us (T.B.) was receipt of a Royal Society European Fellowship.

REFERENCES

1) P. Benveniste, L. Hirth and G. Ourisson ; Phytochemistry, 9, 1073 (1970).

2) a) H.H. Rees, L.J. Goad and T.W. Goodwin ; Tetrahedron Letters, 723 (1968)
b) H.H. Rees, L.J. Goad and T.W. Goodwin ; Biochem.Biophys. Acta, 176, 892 (1969).
3) R. Heintz and P. Benveniste ; Phytochemistry, 9, 1499 (1970).
4) R. Heintz and P. Benveniste ; submitted for publication.
5) P.T. Russel, R.T. van Aller and W.R. Nes ; J.Biol.Chem., 242, 5802 (1967).
6) G. Ponsinet and G. Ourisson ; Phytochemistry, 4, 799 (1965).
7) a) B.L. Williams, L.J. Goad and T.W. Goodwin ; Phytochemistry 6, 1137 (1967).
b) B.L. Williams, L.J. Goad and T.W. Goodwin ; Europ.J. Biochem., 3, 232 (1967).
8) P. Benveniste, L. Hirth and G. Ourisson ; Phytochemistry, 5, 31, 45 (1966).
9) G. Ponsinet and G. Ourisson ; Phytochemistry, 7, 757 (1968).
10) G. Ponsinet, Thèse de Doctorat es-Sciences, 1967.
11) R. Heintz ; unpublished observations.
12) P. Benveniste, M.J.E. Hewlins and B. Fritig ; Europ.J. Biochem. 9, 526 (1969).
13) L.J. Goad and T.W. Goodwin ; Europ.J.Biochem. 1, 357 (1967).