PLANT STEROL METABOLISM

STUDIES ON THE SUBSTRATE SPECIFICITY OF AN ENZYME CAPABLE OF OPENING THE CYCLOPROPANE RING OF CYCLOEUCALENOL

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Summary

In a previous article we reported the existence in Bramble tissue cultures of an enzyme capable of opening the 9β ,19β -cyclopropane ring of cycloeucalenol (I). In this article we report the results obtained from a comparative study of this enzyme-mediated reaction using cycloartenol (II), 24-methylene cycloartanol (III) and cycloeucalenol, which are ubiquitous constituents of higher plants, as substrates. The 4,4-dimethyl sterols, cycloartenol and 24-methylene cycloartanol, are very poor substrates for this enzyme under the conditions used, whereas cycloeucalenol is converted relatively efficiently into obtusifoliol (IV).

Many studies have shown that the biosynthesis of sterols in higher plants probably involves the intermediacy of tetracyclic triterpenes possessing a cyclopropane ring (1). One biosynthetic pathway postulated the enzymic opening of this cyclopropane ring at three different points: cycloartenol, 24-methylene cycloartanol and cycloeucalenol (2). With this in mind, we have compared the ability of these three compounds to act as substrates for an enzyme, previously isolated from Bramble tissue cultures (3), which is capable of opening the cyclopropane ring of cycloeucalenol.

Experimental

3α -T cycloeucalenol (100mC/mmole) and 3α -T cycloartenol (65mC/mmole) were prepared as described previously (3). 24-methylene (28-^{14}C) cycloartanol (25mC/mmole) was prepared from the acetate of 4,4,14α -trimethyl 9β ,19β -cyclo

5α -cholest 24-one 3β -ol (4) using the Wittig reaction.

Microsomes were prepared from Bramble (Rubus fruticosus) tissue cultures as described previously (5). The incubation medium contained the following: 70mM Tris HCl, 5mM mercaptoethanol, 4mM $MgCl_2$, 2mM ATP, 1.25mM NADH and 4mM KOH. Incubations were carried out at 30°C for 5 hrs after the addition of one of the three labelled substrates. The incubations were terminated and the 4α-methyl or 4,4-dimethyl sterols isolated, acetylated and epoxidised as previously described (3). By T.L.C. the acetate monoepoxides of cycloartenol, 24-methylene cycloartanol and cycloeucalenol can be separated from the acetate diepoxides of lanosterol (V), 24-methylene lanostenol (VI) and obtusifoliol respectively. Radioactivity associated with the acetate diepoxides was determined by recrystallisation to constant specific radioactivity after addition of unlabelled carrier.

Results

Table I shows the results obtained after an incubation carried out with 24-methylene cycloartanol as substrate and a parallel incubation with cycloeucalenol as substrate. The radioactivity associated with the acetate diepoxide of 24-methylene lanostenol shows a large drop during chromatographic procedures whereas the radioactivity associated with the acetate diepoxide of obtusifoliol remains almost constant. Table II shows the results obtained after the addition of unlabelled carrier and several-fold recrystallisation. The specific radioactivity of the acetate diepoxide of 24-methylene lanostenol drops rapidly to a very low value after the fourth recrystallisation, whereas that of the acetate diepoxide of obtusifoliol shows only a relatively small decrease before reaching a constant value after four recrystallisations. Taking the value after the fourth recrystallisation, the quantity of radioactivity in the crystals, corresponding to the labelled product formed as a result of the enzymic reaction, can be calculated. In the case of the acetate diepoxide of obtusifoliol this value (200,000 dpm) represents the conversion of one

TABLE I

Radioactivity associated with the acetate diepoxides of 24-methylene lanostenol and obtusifoliol obtained after incubation with labelled 24-methylene cycloartanol and cycloeucalenol respectively.

Incubation of 24-methylene cycloart.	Radioactivity in recovered alcohol 29.1×10^6	Monoepoxide of 24-methylene cycloartanol acetate 13.5×10^6	Diepoxide of 24-methylene lanostenol acetate FIRST T.L.C. 0.085×10^6 SECOND T.L.C. 0.032×10^6
Incubation of cycloeucalenol	Radioactivity in recovered alcohol 6.3×10^6	Monoepoxide of cycloeucalenol acetate 1.66×10^6	Diepoxide of obtusifoliol acetate FIRST T.L.C. 0.262×10^6 SECOND T.L.C. 0.232×10^6

TABLE II

Recrystallisation of the acetate diepoxides

Recrystallisation	Specific activity dpm /mg	before crystallisation	1	2	3	4	Total radioactivity	Number of nmole of added substrate converted
Diepoxide of 24-methylene lanostenol acetate after addition of 14,5mg of unlabelled carrier	Crystals	2,180	1,200 ± 50	500 ± 25	185 ± 15	52 ± 5	754	0,013
	Mother liquor	2,180	2,700 ± 120	1,300 ± 55	700 ± 30	255 ± 20		
Diepoxide of Obtusifoliol acetate after addition of 12,2mg of carrier	Crystals	19,200	17,800 ± 900	16,900 ± 800	16,200 ± 800	16,500 ± 800	200,000	1
	Mother liquor	19,200	20,600 ±1,000	18,500 ± 850	17,000 ± 850	16,600 ± 850		

TABLE III

Radioactivity associated with acetates diepoxides of lanosterol and obtusifoliol obtained after incubation with labelled cycloartenol and cycloeucalenol respectively

Incubation of cycloartenol	Radioactivity in the recovered alcohol 3×10^6	Radioactivity in the recovered acetate 2.5×10^6	Monoepoxyde of cycloartenol acetate 0.55×10^6	Diepoxide of lanosterol acetate 0.015×10^6
Incubation of cycloeucalenol	Radioactivity in the recovered alcohol 6×10^6	Radioactivity in the recovered acetate 5×10^6	Monoepoxide of cycloeucalenol acetate 0.52×10^6	Diepoxide of obtusifoliol acetate 0.48×10^6

TABLE IV

Recrystallisation of the acetate diepoxides

Recrystallisation	Specific activity dpm /mg	Before crystallisation	1	2	3	4	Total radioactivity	Number of mmole of added substrate converted
Diepoxide of lanosterol acetate after addition of 14,5mg of unlabelled carrier	Crystals	1,030	580 ± 30	320 ± 20	250 ± 20	260 ± 20	3,625	0,025
	Mother liquor		2,700 ± 150	1,800 ± 100	1,000 ± 50	440 ± 30		
Diepoxide of obtusifoliol acetate after addition of 9 mg of unlabelled carrier	Crystals	53,000	51,500 ±2,600	50,500 ±2,600	51,000 ±2,600	51,500 ±2,600	463,500	2,08
	Mother liquor		54,000 ±2,700	52,000 ±2,600	51,000 ±2,600	50,500 ±2,600		

nmole of the added substrate. For the acetate diepoxide of 24-methylene lanostenol this value (750 dpm) represents a conversion of 0.013 nmole of the added substrate.

The results of a similar experiment carried out with cycloartenol and cycloeucalenol as substrates are shown in Table III. Clearly there is a very poor conversion of cycloartenol into lanosterol and this value decreases further after addition of unlabelled carrier and recrystallisation as shown in Table IV. On the other hand there is an important conversion of cycloeucalenol into obtusifoliol as shown in Table III by the radioactivity associated with the acetate diepoxide of obtusifoliol. Furthermore, Table IV shows that this latter radioactivity was retained throughout a fourfold recrystallisation. In the case of the acetate diepoxide of obtusifoliol the values obtained represent the conversion of 2.08 nmoles of the added cycloeucalenol into obtusifoliol. For the experiment with cycloartenol the equivalent value is 0.025 nmoles converted.

Discussion

The results obtained demonstrate that while cycloeucalenol is efficiently converted into obtusifoliol by the enzymic system used, both cycloartenol and 24-methylene cycloartanol are very poor substrates. In both cases their conversion into their respective products, relative to the conversion of cycloeucalenol into obtusifoliol, is very low, being of the order of 1%. The main structural difference between cycloeucalenol on the one hand and cycloartenol and 24-methylene cycloartanol on the other hand is the absence in cycloeucalenol of the 4β-methyl group common to the other two compounds. It may be that the 4β-methyl group present in 4,4 dimethyl sterols hinders the action of the enzyme in some way, either by interfering with the binding of the enzyme and the substrate or by hindering the approach of a chemical grouping essential for the opening of the cyclopropane ring.

The results we have obtained give us important knowledge concerning the biosynthesis of sterols in higher

STEROL BIOSYNTHESIS IN HIGHER PLANTS

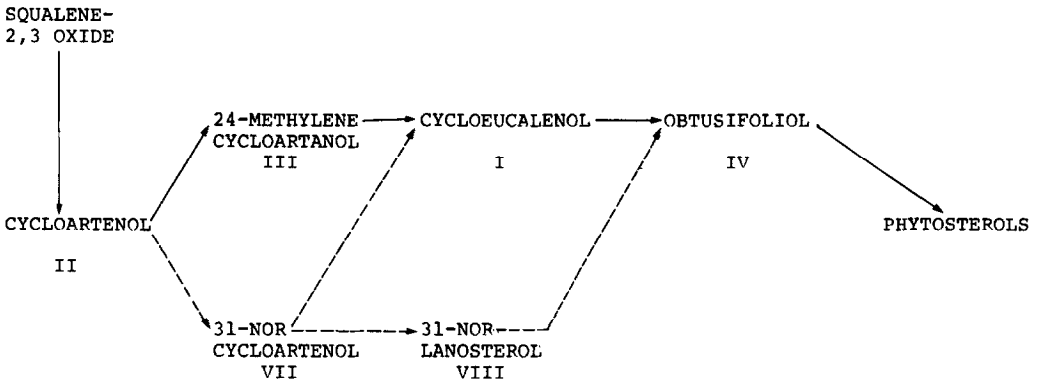

Fig.1 Hypothetical biogenetic scheme for phytosterol biosynthesis

plants. They suggest that neither lanosterol nor 24-methylene lanostenol play an intermediary role. Such a conclusion allows us to modify the ramified scheme previously proposed by Benveniste et al. (2) where the intermediacy of these compounds was proposed to explain the results obtained after kinetic studies using Tobacco tissue cultures. This modified scheme is shown in fig. 1. It can be seen that a branch point exists at the level of cycloartenol, which may be methylated to give 24-methylene cycloartanol following the pathway postulated by other workers (1), or demethylated to give 31-nor cycloartenol(VII). This latter compound may be methylated to give cycloeucalenol or may also be a substrate for the enzyme discussed in this article. We intend to pursue this latter point in our laboratory. The passage from the pathway represented by broken lines to the pathway represented by full lines involves the reaction of C-24 methylation which has been demonstrated *in vitro* with a microsomal system from Bramble tissue cultures (6). Finally this scheme is in agreement with the absence of lanosterol and the cooccurence of cycloeucalenol and obtusifoliol in higher plants.

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Trivial names

I: 4α,14α-dimethyl 9β,19β-cyclo 5α-ergost 24(28)-en 3β-ol
II: 4,4,14α-trimethyl 9β,19β-cyclo 5α-cholest 24-en 3β-ol
III: 4,4,14α-trimethyl 9β,19β-cyclo 5α-ergost 24(28)-en 3β-ol
IV: 4α,14α-dimethyl 5α-ergosta 8,24-dien 3β-ol
V: 4,4,14α-trimethyl 5α-cholesta 8,24-dien 3β-ol
VI: 4,4,14α-trimethyl 5α-ergosta 8,24(28)-dien 3β-ol
VII: 4α,14α-dimethyl 9β,19β-cyclo 5α-cholest 24-en 3β-ol
VIII 4α,14α-dimethyl 5α-cholesta 8,24-dien 3β-ol