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CYCLISATION OF 1-*TRANS*-1′-NORSQUALENE-2,3-EPOXIDE AND 1-*CIS*-1′-NORSQUALENE-2,3-EPOXIDE BY A CELL FREE SYSTEM OF CORN EMBRYOS

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Key Word Index—*Zea mays*; Gramineae; plant sterol biosynthesis; squalene-2,3-epoxide-cycloartenol cyclase; cycloeucalenol–obtusifoliol isomerase; squalene-2,3-epoxide analogs.

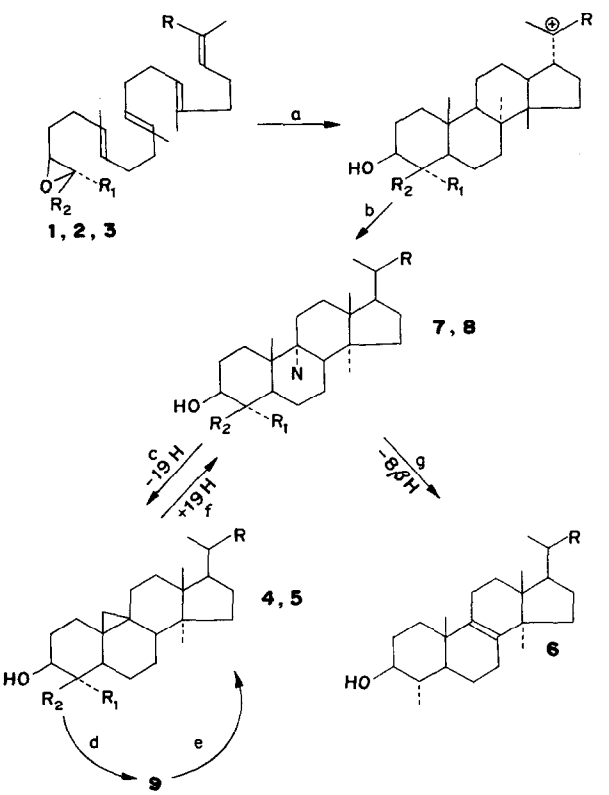
Abstract—Squalene-2,3-epoxide-cycloartenol cyclase and cycloeucalenol–obtusifoliol isomerase activities were found in microsomal fractions of corn (*Zea mays*) embryos. Squalene-2,3-epoxide, 1-*trans*-1′-norsqualene-2,3-epoxide and 1-*cis*-1′-norsqualene-2,3-epoxide were incubated. Squalene-2,3-epoxide was cyclized giving only cycloartenol, whereas 1-*trans*-1′-norsqualene-2,3-epoxide gave 31-norcycloartenol and 31-norlanosterol with a reduced yield, 1-*cis*-1′-norsqualene-2,3-epoxide was not significantly cyclized.

INTRODUCTION

As shown previously in two different laboratories [1,2], comparative incubation in a cell free extract from rat liver of squalene-2,3-epoxide (**1**) and of 1-*trans*-1′-norqualene-2,3-epoxide (**2**) gave respectively lanosterol and 31-norlanosterol, whereas incubation of 1-*cis*-1′-norsqualene-2,3-epoxide (**3**) gave no detectable cyclization product.

It has been suggested [3,4] that in higher plants, reactions catalyzed by the squalene epoxide-cycloartenol (**4**) cyclase and the cycloeucalenol (**5**)-obtusifoliol (**6**) isomerase involve the two closely related intermediates **7** and **8** (Scheme 1) and it has been proposed [4] that the number and stereochemistry of methyl groups at the C-4 position of intermediates **7** (two methyls) and **8** (one 4 α-methyl) could play a key role in orienting the reactions respectively towards closure of the 9β,19β-cyclopropane ring with cyclopropyl sterol formation or cleavage of the cyclopropane ring and Δ^8-sterol formation.

To test this hypothesis we have performed comparative incubations of **1**, **2** and **3** with a cell free system derived from corn embryos. Whereas incubation of **1** was expected to give cycloartenol, through **7** and subsequent attack of a 19-H, incubation of **2** could directly give an intermediate lacking the 4β-methyl group, thus being very similar to **8**, and which could give other products than the expected 31-norcycloartenol. We decided therefore to investigate very carefully cyclization products of modified squalene epoxides **2** and **3**.



1,4,7,9 $R_1 = R_2 = Me$ **1,2,3,4,7** R =
2,5,8 $R_1 = Me$, $R_2 = H$
3 $R_1 = H$, $R_2 = Me$ **5,6,8,9** R =

Scheme 1. Cyclization of squalene-2,3-epoxide in higher plants. Letters a, b, etc... represent successive steps leading finally to obtusifoliol (**6**).

RESULTS

Assay of squalene-2,3-epoxide-cycloartenol cyclase and cycloeucalenol–obtusifoliol isomerase in corn seedlings microsomes

Table 1 shows that microsomes from corn embryos actively transformed **1** into cycloartenol and cycloeuca-

Table 1. Squalene-2,3-epoxide-cycloartenol cyclase and cycloeucalenol-obtusifoliol isomerase activities in corn embryo microsomes

Substrate	Radioactivity of substrate (dpm)	Substrate concentration (μM)	Epoxide of cycloartenyl acetate formed (dpm)	(nmol)	Diepoxide of obtusifoliyl acetate formed (dpm)	(nmol)	Substrate transformed (nmol/10 g embryos)
Squalene-2,3-epoxide	4×10^6	27	6.3×10^5	21	—	—	17.5
Cycloeucalenol	3.2×10^6	3.6	—	—	6×10^5	2.5	1.4

Table 2. Comparative incubation of squalene-2,3-epoxide (**1**), 1-*trans*-1′-norsqualene-2,3-epoxide (**2**) and 1-*cis*-1′-norsqualene-2,3-epoxide (**3**) in a cell free system from corn embryos

Substrate	**1**	**2**	**3**
Radioactivity in the substrate (dpm)	3.2×10^6	16.5×10^6	16×10^6
Substrate concentration (μM)	16.3	68	66
Epoxide of cycloartenyl acetate formed (dpm)	2×10^5 (2.5)*	—	—
Diepoxide of 31-norlanosteryl acetate formed (**a**) (dpm)	—	1×10^4 (0.17)*	0.04×10^4
Epoxide of 31-norcycloartenyl acetate formed (**b**) (dpm)	—	0.66×10^4 (0.11)*	0.03×10^4
Epoxides of endogenous phytosteryl acetates (dpm)	0.03×10^4	0.03×10^4	0.03×10^4
Total substrate transformed (nmol/10 g embryos)	—	0.17	—
a/**b**	—	1.5	—
Percentage transformation = (product/substrate) × 100	12.5	0.1	—

* Values in parentheses are the nmol formed/10 g embryos.

lenol into obtusifoliol. The percentage conversion found in the case of the incubation of cycloeucalenol may be explained by the lower concentration of substrate used in this case.

Comparative incubations of **1**, **2** *and* **3**

The results are given in Table 2. As expected **1** was efficiently cyclised giving only cycloartenol (12.5% yield) whereas **2** was cyclized with a much reduced yield (0.1%) presumably giving 31-norcycloartenol and 31-norlanosterol identified respectively as their epoxy and diepoxyacetates. By contrast, **3** was not significantly cyclized to any detectable 4-methyl sterols since radioactivities measured either in the 24,25-epoxide of 31-norcycloartenyl acetate or in the 8,9;24,25-diepoxide of 31-norlanosteryl acetate were of the same order of magnitude as radioactivity measured in the epoxides of phytosteryl acetates which under our experimental conditions could not be labelled [5]. To confirm these results, the epoxyacetates were crystallized to constant specific radioactivity as shown in Table 3. In the case of the incubation of the *cis*-isomer (**3**), the specific radioactivity of the 8,9;24,25-diepoxide of 31-norlanosteryl acetate reached a very low and insignificant value. In the case of the incubation of the *trans*-isomer (**2**), a constant specific radioactivity was reached after the first crystallization in the epoxide of 31-norcycloartenyl acetate and after the second crystallization in the diepoxide of 31-norlanosteryl acetate. The loss of radioactivity observed in the case of the latter product was not observed in two other experiments. Preparative GLC confirmed these results by showing significant radioactivity associated with both 31-norlanosteryl acetate and 31-norcycloartenyl acetate after their unambiguous GLC separation. In all experiments (Table 2), 31-norlanosterol was shown to be formed to a higher extent than 31-norcycloartenol since the ratio of the former to the latter varied from 1.3 to 1.9.

Time course of the enzymic cyclization of **2**

Table 4 shows the results of the incubation of **2** using different incubation times (1,4 and 24 hr). The radioactivity associated with the 8,9;24,25-diepoxide of 31-norlanosteryl acetate and with the 24,25-epoxide of 31-nor-

Table 3. Crystallization to constant specific radioactivity of the epoxide of 31-norcycloartenyl acetate and the diepoxide of norlanosteryl acetate obtained after incubation of 1-*trans*-1′-norsqualene-2,3-epoxide

	diepoxide of 31-norlanosteryl acetate	epoxide of 31-norcycloartenyl acetate
Amount of unlabelled product added (mg)	6	6
Radioactivity before crystallization (dpm)	20×10^3	40×10^3*
1st recrystallization (dpm/mg)	3265 ± 320	7600 ± 750
2nd recrystallization (dpm/mg)	1680 ± 170	7680 ± 750
3rd recrystallization (dpm/mg)	1590 ± 160	7500 ± 750
4th recrystallization (dpm/mg)	1570 ± 160	7400 ± 750

* Products from two experiments were mixed to obtain sufficient radioactivity.

Table 4. Time course of the cyclization of 1-*trans*-1'-norsqualene-2,3-epoxide. Incubations were carried out as described in the experimental. Microsomes (2 ml) were incubated in the presence of **2** (7×10^6 dpm, 57 μM

Time of incubation (hr)	1	4	24
Diepoxide of 31-norlanosteryl acetate (dpm) **a**	1550	6250	12000
Epoxide of 31-norcycloartenyl acetate (dpm) **b**	850	3400	6300
a/b	1.80	1.85	1.90

cycloartenyl acetate increased with the incubation time whereas the ratio of the former to the latter remained constant (1.8–1.9).

Effect of addition of unlabelled 31-*norcycloartenol upon the conversion of labelled* **2** *into* 31-*norcycloartenol and* 31-*norlanosterol*

Compound **2** was incubated under standard conditions either alone or in the presence of unlabelled 31-norcycloartenol at different concentrations. As shown in Table 5, the amount of labelled 31-norlanosterol formed (measured as its diepoxy-acetate) was not significantly modified whether the trapping agent was present or not. Also no accumulation of label in 31-norcycloartenol occurred so that the ratio of 31-norlanosterol to 31-norcycloartenol remained constant. Under the same conditions, unlabelled cycloeucalenol and 31-norcycloartenol inhibited conversion of labelled cycloeucalenol to obtusifoliol (Table 6), showing that in the experiment reported in Table 5 the trapping agent saturated the enzymatic system at the isomerase level.

DISCUSSION

Following incubation of 1-*cis*-1'-norsqualene-2,3-epoxide (**3**) no cyclization product was detectable in agreement with results obtained with a rat liver homogenate [1,2]. By contrast 1-*trans*-1'-norsqualene-2,3-epoxide (**2**) was cyclized and in addition to the expected 31-norcycloartenol gave 31-norlanosterol, the ratio of the latter to the former product being greater than 1. Unlabelled 31-norcycloartenol exogenously added failed to trap radioactivity from **2** since the label did not accumulate in 31-norcycloartenol and since formation of 31-norlanosterol was not inhibited. However, unlabelled 31-norcycloartenol considerably inhibited conversion of labelled cycloeucalenol into obtusifoliol. These results suggest that transformation of intermediately formed 31-norcycloartenol into 31-norlanosterol does not occur and that formation of this latter product following incubation of **2** is catalyzed by the cyclase. This conclusion is strengthened by the results of the incubation of **2** for different times which show (Table 4) that the ratio between 31-norlanosterol and 31-norcycloartenol remains constant when the incubation time increases. Such a result suggests that there are no product-to-precursor relationships between the two compounds in our incubation experiments. Therefore, it can be assumed that both 31-norcycloartenol and 31-norlanosterol are formed directly by the squalene-2,3-epoxide-cycloartenol cyclase through intermediate **7'** similar to **7** but lacking the 4β-methyl (Scheme 2). The increased molecular freedom caused by the absence of a 4β-methyl in **7** makes possible the approach of the basic enzymatic site towards

Table 5. Effect of addition of unlabelled 31-norcycloartenol upon conversion of labelled 1-*trans*-1'-norsqualene-2,3-epoxide (**2**) into 31-norcycloartenol and 31-norlanosterol

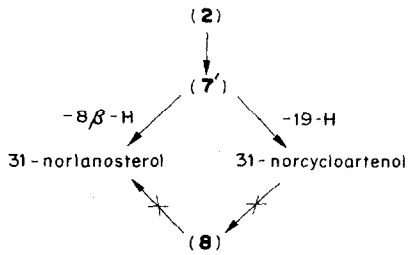
Substrate	Radioactivity of **2** (dpm $\times 10^{-6}$)	31-norcycloartenol concentration (μM)	Epoxide of 31-norcycloartenyl acetate **a** (dpm)	(μM*)	Diepoxide of 31-norlanosteryl acetate **b** (dpm)	**a/b**
2	10	—	10800	0.040	14000	1.28
2 + 31-norcycloartenol	12.5	55	11800	0.045	15800	1.30
2 + 31-norcycloartenol	13	550	17000	0.063	21500	1.27

* Concentration of endogenously formed labelled 31-norcycloartenol.

Table 6. Effect of addition of unlabelled cycloeucalenol and 31-norcycloartenol upon conversion of labelled cycloeucalenol into obtusifoliol

Incubation	Radioactivity of substrate (dpm $\times 10^{-6}$)	Substrate concentration (μM)	Competitor concentration (μM)	Epoxide of cycloeucalenyl acetate **a** (dpm)	Diepoxide of obtusifoliyl acetate **b** (dpm)	**a/b**
Cycloeucalenol-[3-^{3}H] only	4.8	14	—	8.4×10^5	1.7×10^6	2.09
Plus unlabelled cycloeucalenol	4.8	14	1,300	2.1×10^6	5.6×10^5	0.27
Plus unlabelled 31-norcycloartenol	5.0	14	1,300	8.0×10^6	8.0×10^5	0.30

either 19-H or 8β-H leading respectively to 31-norcycloartenol and 31-norlanosterol.



Scheme 2.

The biosynthesis of obtusifoliol (**6**) from squalene-2,3-epoxide is summarized in Scheme 3. Formation of cycloartenol would involve intermediate **7**, previously proposed by Rees *et al.* [3]. The following molecular interactions may be involved in **7**: neutralization of the carbocation at C-9 by a nucleophilic site N of the enzyme; possible interaction between the OH group and a hydrophilic site of the enzyme and interaction between the two methyl groups at C-4 and a hydrophobic part of the enzyme. Attack of a proton at C-19 by the basic group B is the lone possibility since lanosterol has never been detected [6,7]. By contrast when **2** is incubated in place of **1** giving intermediate **7'** lacking the 4β-methyl group, molecular freedom would increase and therefore, the attack of the C-8β proton by the basic site B becomes possible. This latter possibility would be favoured by the absence of a 4β-methyl 10-methyl diaxial interaction in 31-norlanosterol. Finally, cycloartenol is transformed into cycloeucalenol (**5**) which binds to the enzyme giving intermediate **8**. Attack of the C-8β proton gives obtusifoliol which is continuously transformed into phytosterols in nature [7].

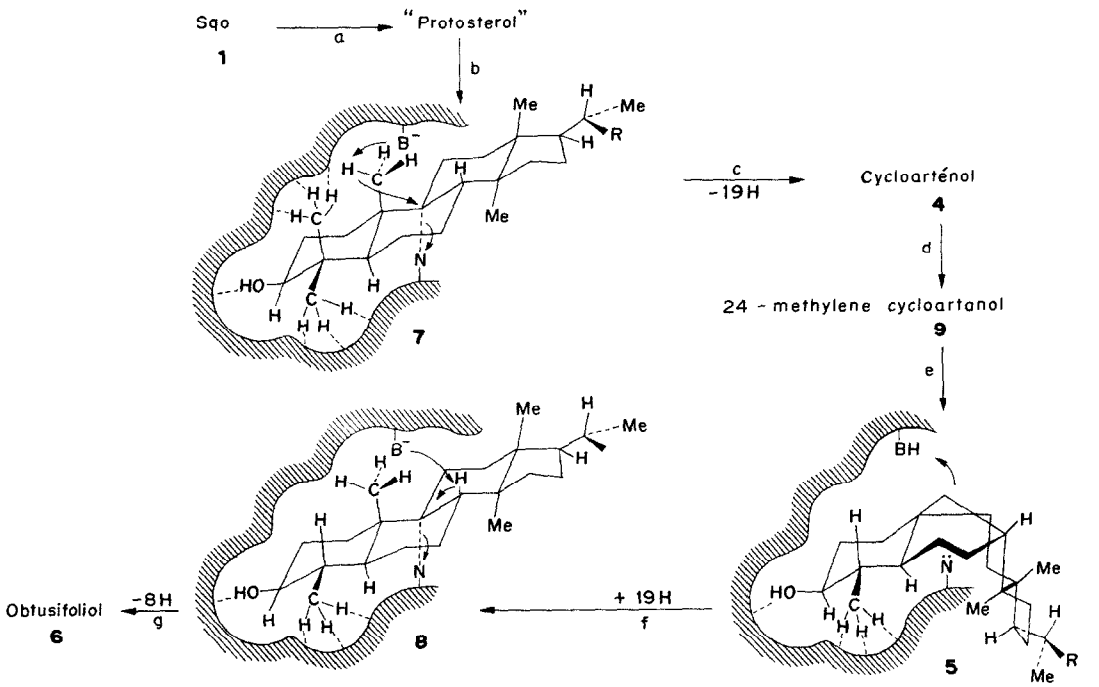
EXPERIMENTAL

TLC was carried out on Merck HF 254 plates (0.2 mm). After spraying with 0.1% berberin–HCl in EtOH, the products were observed under UV (340 nm). GLC employed a GC fitted with two FID and two glass columns (1.50 m × 3 mm) packed with either 1% SE 30 or 1% OV-17 and N_2 at 30 ml/mn. Preparative GLC was performed using a glass column (1.50 m × 3 mm) packed with 1% OV-17 and an effluent splitter. The products were collected into a capillary tube and analyzed on OV-17 taking squalene as internal reference. Radioactivity on the TLC plates was detected by a thin layer scanner. Radioactivity was assayed by liquid scintillation counting. NMR spectra were determined at 60 MHz, using $CDCl_3$ as solvent.

Authentic materials. 31-Norlanosterol was kindly supplied by Prof. R. Goutarel (Gif sur Yvette, France); 31-norcycloartenol was extracted from *Carnegia gigantea* pollen kindly supplied by Dr. L. N. Standifer (Tucson, Arizona, U.S.A.). Cycloeucalenol was extracted as described previously [4] from a piece of eucalyptus wood (*Eucalyptus robusta*) kindly supplied by Dr. E. B. Huddlerton (Sydney, Australia). Obtusifoliol was extracted as described previously [4] from the latex of *Euphorbia obtusifolia* which was kindly supplied by Prof. Gonzales Gonzales (Las Palmas, Las Canarias, Spain).

Radiochemicals. Tritiated H_2O (5 Ci/ml) was supplied by the Commissariat à l'Energie Atomique (Gif sur Yvette, France). Cycloeucalenol [3-^{3}H] (100 μCi/μmol) was prepared as described previously [4].

1,1',2-trinorsqualene-3-aldehyde-[4-^3H]. Squalene-2,3-epoxide (**1**) was prepared as described previously[8]. To **1** (200 mg) dissolved in 2 ml dioxane–H_2O (7:3), 8 μl of 0.06 N $HClO_4$ was added. After 1 hr of stirring at 100°, TLC (EtOAc–cyclohexane, 15:85) showed complete disappearance of the epoxide (R_f = 0.60). Routine work up gave 125 mg (60% yield) of squalene-2,3-glycol. Squalene-2,3-glycol was then dissolved in 5 ml dioxane–H_2O (7:3), $NaIO_4$ (130 mg in 0.4 ml H_2O) was added and the mixture allowed to stand overnight with stirring. Routine work up gave 70 mg (63% yield) of pure 1,1',2-trinorsqualene-3-aldehyde. The aldehyde (70 mg) was dissolved in a small vol of C_6H_6–cyclohexane (1:1), applied to a column of basic



Scheme 3. Postulated mechanism involved during formation and opening of the 9β,19-cyclopropane ring of cyclopropyl sterols.

alumina (10 g Merck act. I) previously deactivated with 0.15 ml tritiated H_2O (5 Ci/ml) and left on the alumina for 1 hr [9]. The tritiated products were then eluted with C_6H_6–cyclohexane (1:1). TLC of the mixture using EtOAc–cyclohexane (15:85) as developing solvent gave the 1,1′,2-trinorsqualene-3-aldehyde-[4-^{3}H] (23 mg, 30 mCi/mmole, $R_f = 0.41$) and two unidentified products (35 mg $R_f = 0.53$ and 4 mg, $R_f = 0.16$).

1-*trans*-1′-*norsqualene*-2,3-*epoxide*-[4-^{3}H] (**2**) *and* 1-*cis*-1′-*norsqualene*-2,3-*epoxide*-[4-^{3}*H*] (**3**) [10]. 25 mg (0.08 mmol) of finely powdered diphenyl ethyl sulphonium tetrafluroborate [11] were suspended in 2 ml THF and cooled with stirring under Ar in a dry ice–Me_2CO bath (−78°). 50 μl of a 2 M pentane soln of butyl lithium (0.08 mmol) were added by syringe to the suspended salt. After 20 min 28 mg (0.077 mmol) of 1,1′,2-trinorsqualene-3-aldehyde-[4-^{3}H] dissolved in 0.5 ml THF was introduced to the mixture. The reaction was stirred for 15 min at −78° and then allowed to come to room temp (20 min). Addition of hexane (10 ml) and H_2O (5 ml) and routine work up gave a mixture containing the epoxides. This mixture was dissolved in EtOH (2 ml) and treated with $NaBH_4$ in order to reduce unreacted aldehyde. TLC of the mixture using EtOAc–cyclohexane (15:85) gave 12 mg of a mixture of diastereoisomeric epoxides. IR: ν_{max}^{CHCl}, cm^{-1}: 1640, 1500, 1375 (no evidence for an aldehyde band at 1715 cm^{-1}) NMR (60 MHz, $CDCl_3$): δ 1.25 (3H, *d*, *J* = 5 Hz, C-1), 1.58 (6 vinyl methyls), 1.97 (methylene envelope), 2.50–2.90 (2H, *m*, C-2 and C-3). The mixed isomers (12 mg) were separated by TLC by continuous development for 6 hr using hexane–EtOAc (97:3). Two isomers were separated into two narrow bands separated by a thin line ($R_f = 0.75$). To obtain the absolutely pure samples required for the incubation experiments, each epoxide isomer had to be subjected to continuous TLC 3 more times until the peaks of radioactivity were perfectly symmetrical. IR, NMR and MS of the two isomeric epoxides were indistinguishable [12]. According to Clayton *et al.* [13] the more polar epoxide was 1-*cis*-1′-norsqualene-2,3-epoxide-[4-^{3}H] (**3**) and the less polar compound was 1-*trans*-1′-norsqualene-2,3-epoxide-[4-^{3}H] (**2**). Finally 2.9 mg of **2** (28 mCi/mmol) and 1.4 mg of **3** (28 mCi/mmol) were obtained.

Preparation of subcellular fractions. Corn seeds (*Zea mays* var. INRA 170) were soaked for 12 hr in tap water before planting in moist vermiculite. They were grown in darkness for 60 hr at 25°. The embryos (70 g) were ground in a mortar at 0° with 3 vol of medium containing: 0.1 M tris–HCl, 0.3% bovine serum albumin, 10 mM mercaptoethanol, 0.5 M sucrose, 4 mM $MgCl_2$, final pH 7.5. The homogenate was squeezed through two layers of cheese cloth and centrifuged at 2000 *g* for 5 min. The supernatant was centrifuged at 105000 *g* for 60 min. Soluble supernatant was removed and the microsomal pellets were suspended in 16 ml of medium containing: 0.1 M Tris–HCl, 2 mM mercaptoethanol, 2 mM $MgCl_2$, final pH 7.5 and dispersed in a Potter–Elvehjem homogenizer. All these operations were performed at 4°. Because of progressive acidification the pH was adjusted to 7.5 with a 1 M Tris soln.

Enzymatic assays. The dispersed microsomal pellets (1.5–5 ml) were incubated with the appropriate substrate dissolved in 50–100 μl 0.5% Tween-80 in Me_2CO and in the presence of 0.1–0.4 ml of 0.4 M Tris–HCl pH 7.6 for 4 hr at 31°. The reaction was terminated by the addition of 20% KOH in EtOH (1 vol). In one experiment a duplicate consisting of particles boiled for 30 min was used. When competitors were incubated, they were mixed with the substrate and dissolved in the same vol of solvent as recommended by Schroepfer *et al.* [14]. Protein was quantitated by the procedure of Lowry *et al.* [15].

Analytical procedure. The incubation mixture was extracted 5× with petrol (5 ml). Combined extracts were dried over Na_2SO_4, evaporated under red. pres. and authentic unlabelled 31-norcycloartenol and 31-norlanosterol (0.5–1 mg each) were added before TLC. TLC of the crude extract using EtOAc–cyclohexane (15:85) gave radioactive 4α-methyl sterols and the starting material. Each band was scrapped off and products eluted with CH_2Cl_2. After solvent evaporation an aliquot (1/20) was transferred to a scintillation vial and counted. Alcohols were acetylated at room temp. for 14 hr using a mixture of C_5H_5N (50 μl) and Ac_2O (100 μl) dissolved in C_6H_6 (100 μl). The reagents were lyophilized and crude acetates separated on TLC using EtOAc–cyclohexane (1:9). Purified acetates were epoxidized with *p*-nitroperbenzoïc acid (40 mg) in dry Et_2O (1 ml) as described earlier [6]. Epoxides were separated on TLC using EtOAc–cyclohexane (1:9, two migrations) as developing solvent. Under these conditions the 24,25-epoxide of 31-norcycloartenyl acetate could be easily separated from 8,9;24,25-diepoxide of 31-norlanosteryl acetate and similarly the 24,28-epoxide of cycloeucalenyl acetate and the 8,9;24,28-diepoxide of obtusifoliyl acetate were separated. Each band was scrapped off and the products worked up as usual. An aliquot (1/20) was transferred to a scintillation vial and counted, taking phytosteryl acetate epoxides as a control. After addition of 6–7 mg of carrier, the epoxide acetates were recrystallized in MeOH.

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