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PHYTOSTEROL BIOSYNTHESIS FROM LANOSTEROL IN *EUPHORBIA PEPLUS*

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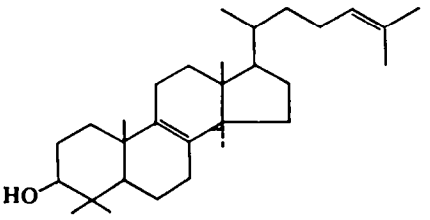
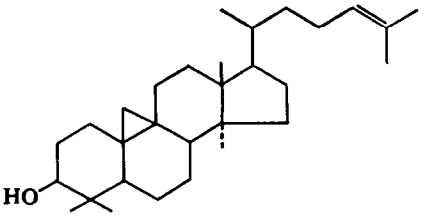
Abstract—Lanosterol-^{14}C was administered to the leaves of two *Euphorbia peplus* plants and radioactive sterol was isolated by extraction, saponification and chromatography. Radioactivity was retained in this fraction after acetylation, chromatography on silver nitrate-impregnated silicic acid, crystallization, hydrolysis and further crystallization. The isolated triterpene was acetylated, epoxidized and chromatographed on thin-layer plates. Radioactivity in zones corresponding to the epoxy-acetates of the major triterpenes of *E. peplus*, cycloartenol and 24-methylenecycloartanol, was immeasurably small on the chromatoplate.

INTRODUCTION

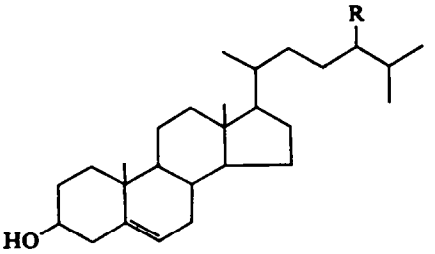
LANOSTEROL (I) is a key intermediate in the biosynthesis of cholesterol.[1] It has been found in few plants[2] and it has been suggested[3] that the pathway leading to phytosterols (e.g. III) does not involve lanosterol but the closely related triterpene, cycloartenol (II). The postulate



(I) Lanosterol



(II) Cycloartenol



(III) R = CH_3, Campesterol
R = CH_2CH_3, β-Sitosterol
R = =$CHCH_3$, Fucosterol

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[1] R. B. CLAYTON, *Quart. Rev. Chem. Soc. Lond.* **19**, 168 (1965); **19**, 201 (1965).

[2] (a) G. OURISSON, P. CRABBÉ and O. RODIG, *Tetracyclic Triterpenes*, Holden-Day, U.S.A. (1964); (b) G. PONSINET and G. OURISSON, *Phytochem.* **6**, 1235 (1967).

[3] P. BENVENISTE, L. HIRTH and G. OURISSON, *Compt. Rend.* **259**, 2284 (1967].

has recently been discussed and amplified by Goad and Goodwin.[4] The utility of lanosterol or cycloartenol as substrates for *in vivo* sterol biosynthesis in tissues of green plants has not yet been tested, though lanosterol[5] and 24-methylenedihydrolanosterol[6,7] have been shown to serve as precursors of ergosterol in whole yeast cells. Furthermore, lanosterol and cycloartenol have been converted to their corresponding 24-methylene derivatives in pea-seed homogenates.[8,9] The purpose of this paper is to describe the incorporation of lanosterol, biosynthsized from mevalonate 2-^{14}C by rat-liver homogenate, into the sterols of *Euphorbia peplus*.

RESULTS

Lanosterol-^{14}C (811×10^3 dpm; 0·57 mg) in 95 per cent ethanol (0·4 ml) was applied to the mature leaves of two *Euphorbia peplus* plants which had previously been exposed to 48 hr continuous illumination in a light box with a light intensity of 55000 lux at shelf level. After 62 hr in the dark the leaves were briefly washed with 95 per cent ethanol to remove non-absorbed lanosterol (514×10^3 dpm). The plants, which must have absorbed 297×10^3 dpm, were then extracted with acetone. The extract (212×10^3 dpm) was evaporated and saponified. The sterol and 4,4'-dimethylsterol fractions were isolated from the non-saponifiable fraction (183×10^3 dpm) by preparative thin-layer chromatography (TLC). The sterol fraction (5600 dpm; 5·5 mg) was diluted with carrier mono-unsaturated sterol (15 mg) previously isolated from *E. peplus* and the mixture acetylated. Mono-unsaturated sterol acetate was isolated by preparative TLC on silica gel G impregnated with silver nitrate. Crystallization to constant specific activity was carried out as shown in Table 1. The acetate

TABLE 1. RECRYSTALLIZATION OF *E. peplus* STEROL AND ITS ACETATE

	Crystallization solvent	m.p.°	dpm/mmole*	dpm/mg
Sterol acetate	Methanol	124–5	22,800 ± 900†	
	Methanol	125–4	21,900 ± 1,050	
	Ethanol	125–6	23,550 ± 1,050	
Sterol‡	Methanol–H_2O	137–9	7,420 ± 185	
	Methanol	138–9	7,000 ± 330	51·6§
	Ethanol–H_2O	138–9	7,160 ± 330	

* Radioactivity was determined on a Packard Tri-Carb Liquid Scintillation Spectrometer, Model 574, using the fluors 2,5-diphenyloxazole and p-bis[2-(5-phenyloxazolyl)]-benzene in toluene. Counter efficiency was 85 per cent. The molecular weight of the sterol was based on that of β-sitosterol.

† 90 per cent confidence level.

‡ Obtained by hydrolysis of the crystallized acetate. The resulting sterol was diluted with twice its weight of carrier.

§ The figure in parentheses is calculated from the average of the last two crystallizations of free sterol and represents the specific activity in the original undiluted sterol. This figure is used to calculate the total radioactivity associated with the mono-unsaturated sterol fraction.

[4] L. J. GOAD and T. W. GOODWIN, *Biochem. J.* **99**, 735 (1966).
[5] M. AKHTAR, P. F. HUNT and M. A. PARVEZ, *Chem. Commun.* 565 (1966).
[6] M. AKHTAR, M. A. PARVEZ and P. F. HUNT, *Biochem. J.* **100**, 38c (1966).
[7] D. H. R. BARTON, D. M. HARRISON and G. P. MOSS, *Chem. Commun.* 595 (1966).
[8] W. R. NES and P. T. RUSSELL, *Federation Proc.* **25**, 222 (1966).
[9] P. T. RUSSELL, R. T. VAN ALLER and W. R. NES, *J. Biol. Chem.* **242**, 5802 (1967).

was hydrolysed and the free sterol, after dilution with twice its weight of carrier, was crystallized to the same molar specific activity, taking into account the dilution factor. A total of 1030 dpm calculated to be associated with the mono-unsaturated sterol fraction (see Discussion) represents an incorporation of 0·4 per cent based on lanosterol-^{14}C absorbed.

The triterpene fraction from the first TLC was acetylated, epoxidized, chromatographed on silica gel G plates and the plates were then scanned for radioactivity. Radioactivity in zones on the chromatoplate corresponding to the epoxyacetates of the two major triterpenes of *E. peplus*,[10] cycloartenol and 24-methylenecycloartanol, was minimal (<450 dpm, see Experimental).

DISCUSSION

A gas chromatographic study[10] of the sterol fraction of *Euphorbia peplus* reveals it to be 90 per cent mono-unsaturated sterol consisting of cholesterol, campesterol and β-sitosterol in the ratio 1:6:93 respectively. In our experiment we recovered 5·5 mg of total sterol which we diluted with 15 mg of mono-unsaturated sterol. Consequently, the total mono-unsaturated sterol crystallized to constant specific activity is 19·95 mg. The total radioactivity incorporated into this sterol is therefore 1030 dpm based upon the average specific activity of the last two crystallizations. This represents an incorporation of 0·4 per cent* based on lanosterol absorbed. This is a minimum figure as some lanosterol was recovered in the triterpene fraction from the first TLC.

The observation that the radioactivity in the triterpenes other than lanosterol was small compared with that found in the sterols indicates that no significant randomization has occurred. This conclusion is made on the basis of time-course studies of the distribution of radioactivity from mevalonate-2-^{14}C into the triterpene and sterol fractions of *E. peplus*: radioactivity in the triterpene fraction is considerably higher than that in the sterol fraction during the early stages.[10] Similar findings have also been made with tissues of other plants.[11,12]

In previous experiments we isolated by TLC a radioactive zone corresponding to 24-methylenecycloartanyl acetate from the acetylated triterpene fraction. The material could not be crystallized to constant specific activity, and during several recrystallizations it fell progressively to a low value (<8 per cent of original activity) after which insufficient material prevented further crystallizations. We suspect the radioactivity in this triterpene zone arises from 24-methylenedihydrolanosteryl acetate.

The case for the intermediacy of cycloartenol in phytosterol biosynthesis is based upon its ubiquity whereas the case against lanosterol is its rare occurrence. If lanosterol occurred as an enzyme-bound intermediate, or had a turnover rate comparable with its rate of formation, then difficulty in detecting it would not be surprising. Our own observations do not settle the question of the intermediacy of lanosterol or cycloartenol in phytosterol biosynthesis except to show that exogenous lanosterol can serve as a sterol-precursor in *E. peplus*. It can however be argued that a relatively non-specific enzyme is present in plants which is capable

* Calculated from:

$$\frac{\text{radioactivity in mono-unsaturated sterols}}{\text{radioactivity in lanosterol absorbed}} \times \frac{6}{5} \times 100$$

The factor 6/5 arises from the fact that one of the six radioactive carbon atoms in lanosterol, labelled from mevalonate-2-^{14}C, is lost during the transformation to sterol.

[10] D. J. Baisted. Unpublished results.

[11] (a) D. J. Baisted, E. Capstack, Jr. and W. R. Nes, *Biochemistry* **1**, 537 (1962); (b) D. J. Baisted and W. R. Nes, *J. Biol. Chem.* **238**, 1947 (1963).

[12] (a) H. J. Nicholas, *J. Biol. Chem.* **237**, 1477 (1962); (b) H. J. Nicholas, *J. Biol. Chem.* **237**, 1481 (1962).

of transforming lanosterol and cycloartenol to sterols. In support of non-specificity in this pathway is the finding by Nes *et al.*[8,9] that lanosterol, cycloartenol and desmosterol can accept the methyl group of methionine in a cell-free system from peas. These findings also rule out the possibility that the 9,19-cyclopropane ring system is an essential requirement for side-chain alkylation. There is a second alternative however in which two pathways may be functioning, one involving lanosterol as an intermediate and the other cycloartenol. In regard to this last possibility it is noteworthy that cholesterol has been found to be widely occurring in plant tissues, though generally in trace amounts, and in animals the route to cholesterol is known to involve lanosterol.

Although we have only quantitated the mono-unsaturated sterol in the present experiment, we also found radioactivity in a di-unsaturated sterol of *E. peplus*. This sterol we have found to be 29-isofucosterol but we had insufficient material to carry out a specific activity determination.

We are currently examining the metabolic relationship of this di-unsaturated sterol to β-sitosterol biosynthesis.

EXPERIMENTAL

Materials and Methods

All thin-layer chromatography (TLC) was carried out on 20 × 20 cm plates with a 375 micron layer of silica gel G activated at 100° for 30 min unless otherwise indicated.

Lanosterol was obtained free of dihydrolanosterol by column chromatography of a mixture of the acetates on a 1:2 mixture of Celite and silicic acid (Mallinckrodt, 100 mesh) containing 40 per cent w/w $AgNO_3$.[13] The two compounds were separated by elution with increasing amounts of benzene in hexane. Lanosterol was obtained from the separated acetate by base hydrolysis and extraction with ether.

Lanosterol-^{14}C was prepared biosynthetically from a rat-liver homogenate prepared according to the procedure described by Bucher and McGarrahan.[14] The incubation was carried out with homogenate (60 ml) and mevalonate-2-^{14} (13·5 μc) in the presence of lanosterol (3·2 mg) dispersed in propyleneglycol (2 ml). The cofactors, conditions of incubation and isolation of non-saponifiable material have been described.[15] Lanosterol-^{14}C was separated from the non-saponifiable material by TLC in ether–hexane (1:1) in which cholesterol and lanosterol had R_f's 0·40 and 0·58 respectively. Zones were located using a $KMnO_4$ spray (0·2 per cent (w/v) $KMnO_4$ in 0·04 per cent (v/v) H_2SO_4) on the sides and center of the plate where a mixture of the two compounds had been chromatographed. The locations were verified by scanning the plate for radioactivity using a procedure previously described.[16] The areas successively counted from origin to solvent front were 18 × 10 mm. With the Tracer-lab thin-window counter, we found that it was possible to detect at least 50 dpm in such an area. The zone corresponding to lanosterol was scraped from the plates, the scrapings packed into a small tube and the lanosterol eluted with ether–ethanol (9–1). Re-chromatography in a dichloromethane–acetone (99–1) system and isolation of the lanosterol in the same way gave lanosterol-^{14}C (897,000 dpm). Purity of the sample was established by crystallization of an aliquot (13,285 cpm) with unlabelled carrier (25 mg). After two crystallizations its specific activity was $232 \pm 5.5 \times 10^3$ cpm/mmole. Acetylation and further crystallization led to a specific activity of $229 \pm 8.5 \times 10^3$ cpm/mmole. These data are in good agreement with the expected specific activity of 226×10^3 cpm/mmole for the free alcohol if all the radioactivity in the aliquot were associated with the carrier.

The specific activity of the isolated lanosterol-^{14}C was determined by obtaining a radioactivity measurement on an aliquot using a liquid scintillation spectrometer. All samples for specific activity measurement were counted in vials containing 10 ml of a mixture of 2,5-diphenyloxazole (4 g) and p-bis-[2-(5-phenyloxazolyl)]-benzene (30 mg) in toluene (1 l.). The weight determination for lanosterol was made on an aliquot using a Beckman GC 4 gas chromatograph equipped with a flame detector. The column used was 1·8 m × 6·5 mm glass-tubing packed with water-washed Anakrom A* pre-coated with dichlorodimethylsilane then coated with SE 30 to a loading of 1 per cent. The column was maintained at 220° with a helium flow rate of 20ml/min. Peak area measurement was made using a recorder equipped with a Disc integrator.

* Perco Supplies, Anaheim, California, U.S.A.

[13] B. DEVRIES, *J. Am. Oil Chem.* **40**, 124 (1963).
[14] N. L. R. BUCHER and K. MCGARRAHAN, *J. Biol. Chem.* **222**, 1 (1956).
[15] D. J. BAISTED, S. R. KLEMP and M. L. SMITH, *Biochem. Biophys. Res. Commun.* **29**, 258 (1967).
[16] D. J. BAISTED, *Phytochem.* **6**, 93 (1967).

Euphorbia peplus plants* were grown from seed in a greenhouse. Lanosterol-^{14}C (811,000 dpm; 0·57 mg) dissolved in 95 per cent ethanol (0·4 ml) was applied to the mature leaves of two bushy plants, each about 8 in. high. The plants had been exposed to continuous illumination in a light box for 48 hr before the application. The leaves were then sprayed with a solution of silicone oil in hexane taking care to avoid spraying the immature parts of the plant. These latter areas slowly darkened after treatment with the reagent but the mature leaves showed no adverse effect. Absorption of the substrate should be enhanced by this technique.[17] The experiment was conducted for 62 hr in the dark so as to reduce to a minimum the possibility of light-catalyzed changes of the substrate on the leaf surfaces. Such changes could produce $^{14}CO_2$, some of which might be re-incorporated into the terpene pathway. The plants were cut at the soil level and the leaves washed briefly with 95 per cent ethanol (514,000 dpm). The cuttings (20 g) were ground in a mortar, extracted with hot acetone and the evaporated extract (212,000 dpm) saponified with 130 ml of 10 per cent (w/v) KOH in ethanol–water (80–20) for 3 hr. The volume of the mixture was reduced on the rotary evaporator and the non-saponifiable material (183,000 dpm) extracted with 5 × 250 ml portions of ether. The evaporated extract was submitted to TLC in a hexane–ether (1:1) system. The triterpene and sterol fractions were isolated as described above. To the sterol fraction (5600 dpm; 5·5 mg) was added 15 mg of mono-unsaturated sterol previously isolated from *E. peplus* and the mixture was acetylated. The acetates were chromatographed on two plates spread with a 375 micron layer of silica gel G containing 8 per cent (w/w) $AgNO_3$. The chromatoplates were run in a benzene–hexane (1:1) system. A non-radioactive sample of *E. peplus* sterol acetate was used as a reference in strips at the edges and center of the plates. Two distinct regions were observed on spraying the reference strips with the $KMnO_4$ spray. A band with R_f 0·50 consists of the acetates of the mono-unsaturated sterol fraction and one at R_f 0·32 contains the acetate of a di-unsaturated sterol (29-isofucosterol). Stigmasterol acetate in this system has R_f 0·47 so that only the front of the upper band was removed for elution of sterol acetate. The isolated material from the front of the band with R_f 0·50 (406 dpm) was repeatedly crystallized to constant specific activity (Table 1). To the recovered material (3·14 mg), calculated from a radioactivity measurement, 6·28 mg of the acetate of mono-unsaturated sterol carrier was added. The mixture was hydrolysed and the extracted sterol again recrystallized (Table 1).

The triterpene material (67,000 dpm) from the first TLC was acetylated and epoxidized[18] with p-nitroperbenzoic acid in $CHCl_3$. The product was applied as a band 16 cm wide across a TLC plate. Epoxy-acetates of lanosterol, cycloartenol and 24-methylenecycloartanol were used as reference compounds. The TLC was carried out in a cyclohexane–ethyl acetate (4–1) system. The radioactive area of the plate was covered with a plastic wrap held down with elastic bands and the reference material visualized in a tank saturated with I_2 vapor. The plate was then scanned for radioactivity by the method earlier indicated. Radioactivity was concentrated in a band associated with the acetate di-epoxide of lanosterol with some radioactivity distributed between the origin and this band. No radioactivity could be detected in the zones corresponding to the epoxy acetates of the other two reference compounds.

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Note added in proof—Ponsinet and Ourisson have recently[19] published the results of a chemical examination of seventy-five *Euphorbia* species. They found lanosterol to be present as a minor constituent in several species including *E. Peplus*. More recently, M. Hewlins, P. Benveniste, L. Hirth and G. Ourisson (private communication) have shown that both lanosterol and cycloartenol can be incorporated by tobacco tissue cultures into phytosterols. However, lanosterol does not trap ^{14}C from acetate when it is used as a scavenger, which leaves little doubt that cycloartenol is the normal precursor, lanosterol being only a usable substrate.

* Harry E. Saier, Diamondale, Michigan, U.S.A.

[17] R. D. Bennett and E. Heftmann, *Phytochem.* 4, 577 (1965).
[18] G. Ponsinet and G. Ourisson, *Phytochem.* 4, 799 (1965).
[19] G. Ponsinet and G. Ourisson, *Phytochem.* 7, 89 (1968).