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STEROL METHYLTRANSFERASE FROM *UROMYCES PHASEOLI*: AN INVESTIGATION OF THE FIRST AND THE SECOND TRANSMETHYLATION REACTIONS*

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Key Word Index—*Uromyces phaseoli*; Pucciniaceae; Basidiomycete; phytosterol biosynthesis; sterol methyltransferase.

Abstract—The two-carbon unit at C-24 of many plant, algal and fungal sterols is known to be synthesized by two successive transmethylations with *S*-adenosylmethionine as the carbon donor. Enzyme(s) for the two transmethylations were isolated from *Uromyces phaseoli* in an attempt to determine if the two-step reaction is catalysed by separable enzymic activities. The sensitivity of the methyltransferase assay was improved and this modification removed the apparent inhibition of the enzyme(s) by detergents. Several substrates for the second transmethylation step were synthesized by a route developed to convert Δ^{24}-sterols to 24-methylene sterols. The enzymes for the two transmethylation reactions were solubilized and purified. Both activities were purified together to approximately the same degree, 155-fold. Degradation products from zymosterol were found to inhibit both reaction steps equally. The data suggest that a single enzyme or complex is responsible for the catalysis of both steps.

INTRODUCTION

The carbon atoms present at C-24 of major sterols in higher plants, algae and fungi arise by transfer of methyl groups from *S*-adenosylmethionine (SAM) to an acceptor sterol [1]. The acceptor sterol for the formation of a methylene group at C-24 is a Δ^{24}-sterol [2] and for the formation of a two-carbon chain a sequential transfer of two methyl groups occurs [1, 3, 4] with a 24-methylene sterol being the second acceptor [3, 5–9]. Lin and Knoche have demonstrated the second transmethylation step in cell-free preparations from uredospores of *Uromyces phaseoli* [10].

The work cited above does not indicate whether two separate enzymes or a single enzyme complex is involved in the synthesis of a two-carbon chain. The principal sterols in uredospores of *Uromyces phaseoli* are C-29 sterols [11], but cell-free preparations from this organism synthesize 24-methylene sterols primarily when Δ^{24}-sterols are provided as substrates [10].

For example, when zymosterol was used as a substrate, the ratio of 24-methylene to 24-ethylidene sterols produced was about 36:1. Further, the same preparation gave ^{14}C-incorporation rates from [^{14}C] SAM in a ratio of about 18:1 for zymosterol and 24-methylene cholesterol, respectively. The apparent disparity in activities for the two reactions suggests the presence of two separate enzymes.

The following studies were undertaken to explore the question of whether or not easily separable enzymes were responsible for the two-step biosynthetic sequence in the synthesis of 24-ethylidene sterols.

RESULTS

Effect of different assay conditions on methyltransferase activity

Detergents. Detergents in the assay have been observed to lower the methyltransferase activity [12] and the same effect of detergents was observed by us prior to improvement of the assay. As shown in Table 1, the

Table 1. Effect of Triton X-100 on the apparent methyltransferase activity

Triton X-100 concentration % (w/v)	DMSO treatment	Enzyme activity (dpm/40 min)
0	No	3907
1	No	1848
0	Yes	27118
1	Yes	31753

The methyltransferase assays were performed with zymosterol as the substrate as described in the Experimental with the following changes: Each incubation mixture contained 0.25 μCi [C^3H_3]SAM (sp. act. 9.1 Ci/mmol) and Triton X-100 as given in the table. After terminating the reaction with aq. KOH the products were isolated with or without DMSO pretreatment.

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Table 2. Effect of inhibitors isolated from aged zymosterol on methyltransferase activities

Sterol/inhibitor	Enzyme activity (dpm/40 min)	% Activity
Zymosterol		
Before purification	3823	13.1
After purification	29059	100.0
Pure + inhibitor No. 1*	10789	37.1
Pure + inhibitor No. 2*	7186	24.7
24,25-Dihydro-24-methylene zymosterol		
No inhibitor	1575	100.0
+ inhibitor No. 1*	562	35.6
+ inhibitor No. 2*	359	22.7

* Isolated from aged zymosterol by thin-layer chromatography as given in Results.

The methyltransferase assays were performed as described in the Experimental with the following changes: Each incubation mixture contained sterol, 333 nmol; [C^3H_3]SAM, 0.25 μCi (sp. act. 9.1 Ci/mmol) and as indicated in the table, an inhibitor, 25 μg, suspended in 0.05 ml water with the aid of Lubrol Px (4 mg/ml). The enzyme preparation was a 3000 g supernatant of the homogenate of *Uromyces phaseoli* uredospores.

apparent inhibition by detergents was abolished when the assay mixtures were treated with DMSO prior to extraction with petrol–diethyl ether (2:1).

Zymosterol. The activity of the methyltransferase with an 18-month-old sample of zymosterol (stored at 4°) was found to be only 13.4% of that for zymosterol freshly isolated from yeast. The melting point recorded initially (107–110°) dropped to 83–105° during the 18-month storage period. The activity was observed to decrease much more rapidly when the sterol was stored in the form of an aqueous suspension (suspended with Lubrol PX). Activity of the methyltransferase with a 14-day-old suspension of freshly isolated zymosterol was 13.8% of that for a fresh suspension of freshly isolated zymosterol.

Upon purification of the 18-month-old sample of zymosterol by TLC (diethyl ether–heptane–acetic acid, 70:30:1), the full activity was restored (Table 2) and the melting point elevated to 104–108°. Zymosterol had a TLC R_f of 0.51. Two appreciable bands of impurities were observed at R_f 0.44 and 0.2. These impurities were isolated separately and were recrystallized from MeOH–H_2O. Both impurities inhibited both transmethylation reactions and were referred to as inhibitor No. 1 (R_f 0.44) and inhibitor No. 2 (R_f 0.2).

Inhibitions for the first and the second transmethylation reactions were tested with zymosterol and 24,25-dihydro-24-methylene zymosterol as substrates. A 0.05 ml suspension containing 25 μg of the appropriate inhibitor (suspended in water with the detergent Lubrol PX, 4 mg/ml) was added to assay mixtures. Inhibitions caused by both inhibitors were similar for the first and the second transmethylation reactions (Table 2). The inhibition was found to be concentration dependent for zymosterol as a substrate and the double reciprocal plots indicated the inhibition to be of non-competitive type.

Compared to zymosterol, lanosterol was quite stable at 4°. Even after 22 months of storage at 4°, the melting point of lanosterol (137.5–138°) had not changed, no impurity was observed by TLC, and the activity of the methyltransferase with aged lanosterol as substrate had not changed. A similar high stability was observed for cycloartenol. The 4,4,14-trimethyl sterols were stored at 4° and the 4,4,14-tridesmethyl sterols were stored at −80°. However, the suspensions of all sterols were used within 10 days of their preparation and were preserved at −80°.

Salts. Inhibitions by Mg^{2+}, Mn^{2+} and Co^{2+} were found to be of the same order as reported by Lin *et al.* [12] but inhibitions by Na^+, K^+ and Ca^{2+} were found to be much less. At 0.02 M concentrations, $CaCl_2$ and NaCl were the least inhibitory (9 and 15% respectively) and were therefore tested further for their possible use in ion-exchange chromatography. At 0.1 M concentrations $CaCl_2$ inhibited by 46% and NaCl by 15%. Addition of EDTA (0.1 M) to the assay mixture did not relieve the inhibitions, in fact it inhibited the activity further by about 10–20%.

Substrate specificity. Zymosterol, desmosterol, lanosterol and cycloartenol, all possessing a Δ^{24}-bond, were considerably more active as substrates than 24,25-dihydro-24-methylene zymosterol, 24,25-dihydro-24-methylene lanosterol and 24-methylene cholesterol. The results are summarized in Table 3. Cholesterol, possessing no double bond at C-24, and 24-methyl sterols, ergosterol and campesterol were inactive substrates.

Zymosterol clearly was the best substrate among those investigated. The 4,4,14-trimethylsterols, lanosterol and cycloartenol, were also active, and it is interesting to note that cycloartenol, which apparently is not in the biosynthetic pathway of sterols in *Uromyces phaseoli* [13], was more active than lanosterol. For the second transmethylation, 24,25-dihydro-24-methylene zymosterol was a much more active substrate than the other two sterols tested.

The products of transmethylation reactions with zymosterol and 24,25-dihydro-24-methylene zymosterol as substrates were characterized by the ozonolysis and reductive cleavage method described by Lin and Knoche

Table 3. Substrate specificity of the methyltransferase

Substrate	Enzyme activity (dpm/40 min)	% Activity
Zymosterol	32 700	100.0
Desmosterol	19 600	59.9
Lanosterol	11 700	35.9
Cycloartenol	16 400	50.1
24,25-Dihydro-24-methylene zymosterol	2260	100.0
24,25-Dihydro-24-methylene lanosterol	390	17.3
24-Methylene cholesterol	180	7.9

Assays were performed as described in the Experimental. The amount of [C^3H_3]SAM used for each assay was 0.5 μCi (50 nmol). The enzyme preparation used for the assay was a 45 000 *g* supernatant of the Triton X-100 solubilized preparation (0.3 mg protein/assay).

[10]. Considering the yields of the cleavage reactions to be about 80% [10], the results indicated that the majority of the product with zymosterol as substrate was a 24-methylene sterol and with 24,25-dihydro-24-methylene zymosterol as substrate, a 24-ethylidene sterol.

Effect of SAM in the first and the second transmethylation reactions

K_m values for SAM were determined using zymosterol and 24,25-dihydro-24-methylene zymosterol as sterol substrates. Assays were performed as given in the Experimental except that the reaction time was 10 min. The K_m value for SAM for the first transmethylation reaction was 35.9 μM and for the second transmethylation reaction 158.7 μM. These K_m values indicate a requirement for a considerably higher concentration of SAM for the second transmethylation reaction.

To determine if a higher concentration of SAM relative to zymosterol would affect the distribution of products significantly, an experiment designed for the analysis of products was performed. Results of the product characterization are summarized in Table 4. When zymosterol was limiting (25 μM) and SAM in excess (400 μM), the ratio of 24-ethylidene to 24-methylene sterols produced was 0.131. This ratio decreased to 0.04 when the concentration of sterol was raised to 400 μM and SAM was kept at the same concentration. When the concentration of sterol was 400 μM and of SAM 70 nM this ratio fell to only 0.014. Thus a higher relative concentration of SAM caused the second reaction to proceed at a higher rate but still the rate of the second reaction is at best 13% of the first.

Attempted separation of a methyltransferase

Gel filtrations. Triton X-100 solubilized enzyme was applied to the gel-filtration column, described in the Experimental, and various fractions were assayed for the first and the second methyltransferase activities using zymosterol and 24,25-dihydro-24-methylene zymosterol as the substrates respectively. The results (Fig. 1) show that the two methyltransferase activities are present in the same fraction.

Ion-exchange chromatography. Ion-exchange chromatography was performed as described in the

Table 4. Alkylene group synthesized at C-24 of the sterol at different relative concentrations of zymosterol and SAM

Zymosterol (μM)	SAM (μM)	Sp. act. of dimedone derivatives* (dpm/μmol)			Ratio of 24-ethylidene- to 24-methylene-sterol
		Formaldehyde	Acetaldehyde	Theoretical†	
25	400	22.38	2.94	39.9	0.131
400	400	110.87	4.49	154.7	0.040
400	0.07	76.73	1.11	99.3	0.014

* Sp. act. of formaldehyde and acetaldehyde dimedone derivatives obtained by ozonolysis and reductive cleavage of the products isolated from enzyme incubations with zymosterol and SAM at concentrations given in the table. Each incubation mixture contained 0.5 μCi [C^3H_3]SAM (sp. act. 9.1 Ci/mmol). The enzyme preparation used for the assay was a 3000 *g* supernatant of the homogenate of *Uromyces phaseoli* uredospores and the enzyme assays were performed as given in the Experimental.

† Theoretical sp. act. is the total radioactivity of the sample divided by the quantity (1.0 mmol) of the aldehyde added as carrier.

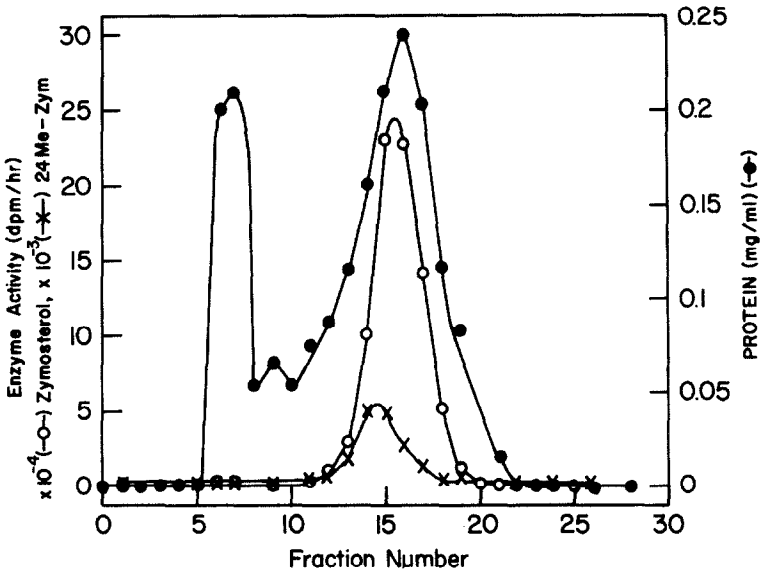

Fig. 1. Gel filtration of the methyltransferase(s): The chromatographic conditions are described in the Experimental. The first transmethylation reaction was assayed by zymosterol as substrate and the second by 24,25-dihydro-24-methylene zymosterol (24 Me-Zym) as substrate. Assays were performed as described in the Experimental. Enzyme activities are per 0.5 ml fraction.

Experimental. The Triton X-100 solubilized enzyme was applied to the column and various fractions were assayed for the first and the second transmethylation reactions using cycloartenol and 24,25-dihydro-24-methylene zymosterol as the substrates respectively. Again the two methyltransferases were not separated as shown in Fig. 2.

Purification of the enzyme

An enzyme purification procedure was performed with assays at various stages. Both zymosterol and 24,25-dihydro-24-methylene zymosterol were used individually as substrates for each enzyme preparation. One μCi of [C^3H_3]SAM (50 nmol) was used for each assay and other conditions were as described in the Experimental. Protein concentrations were determined after proper dilutions except for the fractions from ion-exchange chromatography which were concentrated 3,4-fold by lyophilization.

Specific enzyme activities of all these fractions are given in Table 5. An overall purification of 155-fold was achieved and at each step a similar purification factor, within the limits of experimental errors, was obtained for the transmethylation reactions with the two substrates.

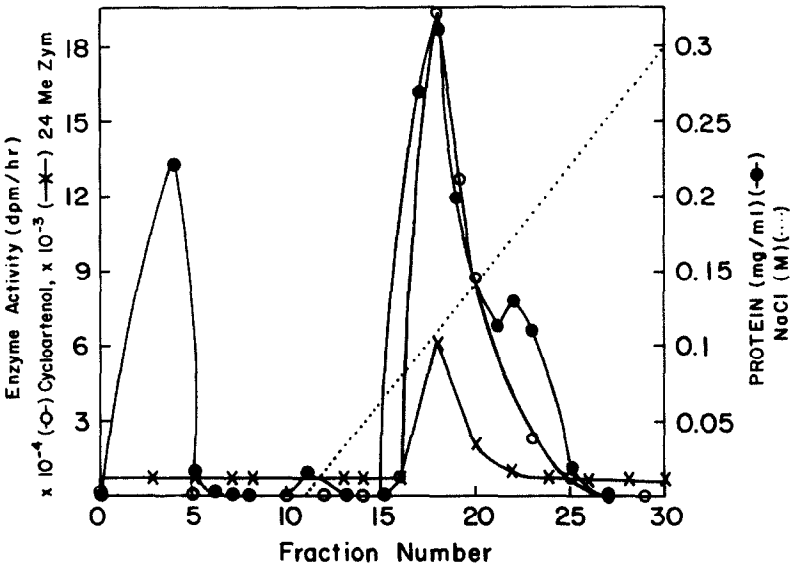

Fig. 2. Ion-exchange chromatography of the methyltransferase(s): The chromatographic conditions are described in the Experimental. The first transmethylation reaction was assayed by cycloartenol as substrate and the second by 24,25-dihydro-24-methylene zymosterol (24 Me-Zym) as substrate. Assays were performed as described in the Experimental. Enzyme activities are per 0.5 ml fraction.

Table 5. Summary of the purification of the methyltransferase(s)

Fraction	Protein		Zymosterol*		24,25-Dihydro-24-methylene zymosterol†	
	Total (mg)	Concentration (mg/ml)	Sp. act.‡	Purification	Sp. act.‡	Purification
Crude homogenate	215.0	21.50	7.94	1	0.359	1
4300 g supernatant of homogenate	82.6	10.33	24.93	3.1	0.729	2.0
Solubilized enzyme, 45000 g supernatant	8.69	1.58	200.00	25.2	4.928	13.7
Pooled gel-filtration fraction	3.28	0.158	442.53	55.7	23.57	65.6
Pooled ion-exchange fraction	0.598	0.0272	1236.70	155.8	57.26	159.5

* Zymosterol as substrate.
† 24,25-Dihydro-24-methylene zymosterol as substrate.
‡ pmol/min/mg protein.

DISCUSSION

Lin and Knoche [10] observed much higher activities for the first transmethylation reaction than the second in their enzyme preparations. When a Δ^{24}-sterol zymosterol was supplied as a substrate the principal product was a 24-methylene sterol, and using 24-methylene cholesterol as a substrate the activity was about 5% of that for zymosterol at equivalent substrate concentrations. The lack of activity for the synthesis of the 2-carbon side-chain appears in other organisms that produce C-29 sterols *in vivo* [14, 15].

Several explanations of these results were considered. Perhaps two separable enzymes exist and the one for the second step is lost in preliminary purification procedures. A higher lability for the enzyme of the second step and a lack of knowledge concerning the actual sterol intermediates involved in the synthesis of 24-ethylidene sterols may contribute to inappropriate comparison of activities.

The task of synthesizing all the plausible intermediates was not undertaken but 24-methylene sterols analogous to lanosterol, zymosterol and desmosterol were prepared and tested. In all cases each 24-methylene sterol was much less active than its Δ^{24}-counterpart as a substrate. The testing of $\Delta^{5,7}$- or Δ^{7}-sterols would be desirable but different results would not be anticipated in light of these results.

Factors that were observed to affect transmethylase activity were investigated. Two degradation products of zymosterol inhibited both steps equally; salts had similar effects on both steps but there was some difference between the K_m values for SAM in the two reactions. While extreme ratios of SAM to sterol did increase the yield of 24-ethylidene products to about 13%, the difference is still large.

Purification of the transferase activities about 155-fold showed parallel purification for both. Thus the presence of both activities in a single complex seems more likely from the data that include consistent differences in activities between the two steps for several analogous sterol substrates, effects of inhibitors and salts, and various states of purity.

Being membrane-bound enzymes and the requirement for sterol substrates to be suspended in detergent solutions undoubtedly creates a highly unnatural environment for enzymatic action *in vitro*, therefore solutions to the remaining questions may require an *in vivo* approach.

EXPERIMENTAL

General procedures. TLC was performed with 0.75 mm thick Si gel or 15% $AgNO_3$–Si gel plates. The solvent systems are given in the text. For detection, the TLC plates were sprayed with a 0.02% soln of Rhodamine 6G in 95% EtOH and viewed under UV light. The components from the TLC absorbent were extracted with $CHCl_3$.

GLC analyses were performed with a 1.5% OV-17 column (2 m × 3 mm). The operating conditions were: column temp., 270°; injector temp., 280–290°; detector temp., 280–290°; the carrier gas was N_2 at a flow rate of 60 ml/min. IR spectra were recorded using the KBr pellet technique. Mass spectra were determined with a high resolution mass spectrometer by electron impact at 70 eV. Source temp. was 150°. All reported mps are uncorr.

The sterols were acetylated by refluxing them for 30 min with a mixture of dry pyridine and Ac_2O (2:3). Transesterification was accomplished by refluxing the steryl ester with 0.02 N NaOMe in MeOH for 1 hr. Steryl acetates were saponified by refluxing them with 15% KOH in 95% aq. EtOH for 1.5–2 hr. *N*-Bromosuccinimide was purified by recrystallization and stored over P_2O_5 as described by Dauben and McCoy [16].

Materials. Bean rust uredospores *Uromyces phaseoli* (pers.) Wint. var. typica Arth. were produced as described by Trocha and Daly [17]. [C^3H_3]-*S*-Adenosylmethionine was purchased from Amersham, Illinois, U.S.A.

24,25-Dihydro-24-methylene zymosterol: A methylene group was introduced at C-24 of zymosterol according to the following procedure. Zymosterol was acetylated and the acetylated sterol was converted to 3β-acetoxy-24ξ-bromo-25-hydroxy-cholest-8-ene (bromohydrin) essentially by the procedure van Tamelen and Curphey [18] used for squalene. Reacting 555 mg zymosteryl acetate with recrystallized *N*-bromosuccinimide (245 mg) in a mixture of glyme (245 ml) and water (40 ml) yielded a product

having a mp of 143–151°. The bromohydrin was converted to 3β-acetoxy-24ξ,25-epoxy-cholest-8-ene according to the procedure of Willet *et al.* [19]. IR characterization of the product showed that the 3β-acetoxy group had been hydrolysed during the reaction since the absorption band of the carbonyl group at 1735 cm^{-1} was missing. The product was therefore reacetylated and recrystallized. The yield of the product 3β-acetoxy-24ξ.25 epoxycholest-8-ene was 450 mg and the mp 109–119°.

The epoxide group of 3β-acetocy-24ξ,25-epoxy-cholest-8-ene was isomerized to a keto group by the reaction described by Bethell *et al.* [20]. The products were separated by TLC (C_6H_6–EtOAc, 9:1). On the TLC plate a number of bands were observed and only the major ones were characterized by IR spectroscopy. The second band from the top, R_f of 0.5, was identified as 3β-acetoxy-24-keto-cholest-8-ene. IR absorption bands at 1735 and 1265 cm^{-1} of 3β-acetoxy-24-keto-cholest-8-ene were attributed to the carbonyl of the acetoxy group and the absorption band at 1720 cm^{-1} to the 24-keto group since a band at 1720 cm^{-1} was present in the IR spectrum of the hydrolysed product while the bands at 1735 and 1265 cm^{-1} were absent. The 3β-acetoxy-24-keto-cholest-8-ene upon recrystallization from MeOH–H_2O had mp 108–113°.

The 3β-acetoxy-24-keto-cholest-8-ene was converted to 3β-acetoxy-24-methylene-cholest-8-ene by the Wittig reaction as described by Idler and Fagerlund [21]. The product was reacetylated and purified by TLC (hexane–Et_2O, 7:3). Upon recrystallization from MeOH–H_2O, the plate-like crystals of 3β-acetoxy-24-methylene-cholest-8-ene had mp 134–138°. GLC indicated greater than 95% purity. Transesterification converted this product into 24,25-dihydro-24-methylene zymosterol which also formed plate-like crystals (mp 131–135°) upon recrystallization from MeOH–H_2O. An IR spectrum revealed absorption bands at 885 and 1645 cm^{-1}, characteristic of the 24-methylene group [22, 23]. The MS analyses of 3β-acetoxy-24-methylene-cholest-8-ene and 24,25-dihydro-24-methylene zymosterol, previously unreported, are given in Table 6 and confirm their structures.

24,25-Dihydro-24-methylene lanosterol: Lanosterol was converted to 24,25-dihydro-24-methylene lanosterol by the same synthetic procedure as given for the conversion of zymosterol to 24,25-dihydro-24-methylene zymosterol. The TLC R_f values were the same for lanosterol derivatives as for the corresponding zymosterol derivatives and all the reactions were similar except for the following differences.

During the conversion of 3β-acetoxy-24ξ-bromo-25-hydroxy-lanost-8-ene to 3β-acetoxy-24ξ,25-epoxy-lanost-8-ene the 3β-acetoxy group was not hydrolysed as indicated by IR spectroscopy of the product. The product therefore was not reacetylated at this step. The other difference was that for the conversion of 3β-acetoxy-24-methylene-lanost-8-ene to 24-25-dihydro-24-methylene lanosterol saponification rather than transesterification was required.

The physical properties of various intermediates isolated during the conversion of lanosterol to 24,25-dihydro-24-methylene lanosterol are as follows: 3β-acetoxy-24ξ-bromo-25-hydroxy-lanost-8-ene, mp 143–150°; 3β-acetoxy-24ξ,25-epoxy-lanost-8-ene, long plates, mp 183–190°; 3β-acetoxy-24-keto-lanost-8-ene, mp 132–134°, (lit. value 135–137°, ref. 24), IR absorption band at 1712 cm^{-1} due to 24-keto group; 3β-acetoxy-24-methylene-lanost-8-ene, plates, mp 141–145° (lit. 147–150°,

Table 6. Mass spectroscopic characterization of synthesized 24,25-dihydro-24-methylene sterols

Description/ molecular fragmentation*	24,25-Dihydro-24-methylene zymosteryl acetate	24,25-Dihydro-24-methylene zymosterol	24,25-Dihydro-24-methylene lanosteryl acetate	24,25-Dihydro 24-methylene lanosterol	Comments
	(*m/z*)	(*m/z*)	(*m/z*)	(*m/z*)	
Observed mol. ion	440.3648	398.3546	482.4122	440.3999	Indicates acetate of a triterpene alcohol
Calculated: MW	440.3654	398.3548	482.4122	440.4017	
Empirical formula	$C_{30}H_{48}O_2$	$C_{28}H_{46}O$	$C_{33}H_{54}O_2$	$C_{31}H_{52}O$	
Molecular ion	440 (100)	398 (100)	482 (76.7)	440 (37.8)	
M^+ – Me	425 (29.6)	383 (56.0)	467 (100)	425 (100)	
M^+ – ROH	380 (7.6)	380 (13.3)	422 (3.2)		
M^+ – ROH – Me	365 (8.8)	365 (11.5)	407 (47.4)	407 (32.6)	
M^+ – SC – 42	273 (3.3)	231 (14.8)	315 (2.7)	273 (3.1)	Shows SC has 9 C-atoms and 1 double bond. Fragment (–42) involves carbons 15, 16 and 17 with their hydrogens plus one (ref. [32])
M^+ – SC – 42 – ROH	213 (17.0)	213 (22.9)	255 (5.5)	255 (4.8)	
M^+ – SC – 2H	313 (13.4)	271 (27.4)			
M^+ – Me – 84	341 (2.0)	299 (4.2)	383 (5.6)	341 (6.7)	Indicates presence of $\Delta^{24(28)}$-bond (ref. [33, 34])
M^+ – Me – 84 – ROH	281 (1.6)		323 (6.7)	323 (3.7)	
M^+ – SC – 56			301 (10.4)	259 (11.9)	Indicates presence of 14α-methyl group (ref. [35])
M^+ – SC – 56 – ROH			241 (9.8)	241 (9.3)	

* R = H in free sterol and MeCO in steryl acetate; SC = side-chain at C-17.

† Numbers in parentheses indicate relative abundances.

ref. [24]), at least 98% pure by GLC; 24,25-dihydro-24-methylene lanosterol, needles, mp 166–170° (lit. 157.5–159°, ref. [24]).

Although the mp of 24,25-dihydro-24-methylene lanosterol is significantly higher than the reported value, other analytical data confirmed its structure and purity. The IR spectrum of 24,25-dihydro-24-methylene lanosterol revealed absorption bands at 885 and 1645 cm^{-1}, characteristic of the 24-methylene group [22, 23] and the MS data (Table 6) is consistent with the reported structure.

24-Methylene cholesterol: 3β-Hydroxy-5-cholenic acid was acetylated and converted to 3β-acetoxy-5-cholenyl chloride by the action of oxalyl chloride in the presence of pyridine [25]. The latter compound was converted to 3β-acetoxy-24-keto-cholest-5-ene by a Grignard reaction [26] and then converted to 24-methylene cholesterol by the Wittig reaction [21]. The purity of 24-methylene cholesterol was at least 95% by GLC and its mp was 135°.

Other sterols: Zymosterol and lanosterol were obtained as described by Lin and Knoche [10]. Cycloartenol was isolated from *Strychnos nux-vomica* seeds [13]. Desmosterol, ergosterol, cholesterol and campesterol were obtained commercially.

Enzyme isolation and solubilization. All operations were performed at 4° and the buffer used was 0.01 M Tris–maleate, pH 7.3. When the enzyme was to be purified by any of the chromatographic processes, 2 mM 2-mercaptoethanol was added to the buffer.

Glass beads (1 mm diameter) (5 g), uredospores (250 mg) and buffer (2.5 ml) were placed in a 15 ml-pyrex glass test-tube cooled on ice. The uredospores were disrupted by agitating the tube with a vortex mixer at high speed. Disruption was conducted in a cold room and after each minute of disruption the tube was cooled on ice. Disruption was continued for a total of 220 sec, after which the contents of the tube were filtered with a pre-chilled Buchner funnel. The tube and the glass beads were washed with 1 ml buffer and the wash was added to the homogenate. The homogenate was centrifuged at 4300 *g* for 20 min and the supernatant was again centrifuged at 45 000 *g* for 30 min. The resulting pellet was dispersed in about 5 ml buffer and centrifuged at 45 000 *g* for 30 min. The pellet from the latter centrifugation was dispersed in a soln of Triton X-100 (1.5%) in buffer. For every 250 mg uredospores 1 ml of the Triton X-100 soln was used. The suspension was stirred on a magnetic stirrer at moderate speed for 10 min and then very slowly for another 20 min. Finally, the suspension was centrifuged at 45 000 *g* for 30 min. The supernatant from this centrifugation contained the solubilized enzyme.

Enzyme assay. Both the first and second transmethylation reaction were assayed by measuring the amount of incorporation of radioactivity from methyl-labelled *S*-adenosylmethionine (SAM) to the acceptor sterol. The assay procedure described previously [10] was used initially but it was found that the assay did not give the precision desired. Work by Park's group [27] suggested that product extraction was not complete in that method, therefore a procedure involving the use of dimethylsulfoxide (DMSO) was devised. Table 7 illustrates the improvement obtained in product extraction. To determine if the extra radioactivity isolated after the DMSO treatment was due to the product only, the Et_2O extract was applied to TLC and after development with Et_2O–hexane–HOAc (70:30:1) the TLC strip was scanned for radioactivity. Only one radioactive spot was observed which had the same mobility as sterols like

Table 7. Effect of DMSO on the extraction of sterol products from enzyme assay mixtures

Radioactive substrate	Treatment	Enzyme activity (dpm/40 min)	
		Sample	Blank
[$^{14}CH_3$]SAM	1 ml of 10% aq. KOH	1056	146
	Saponified by heating at 100° for 30 min	1126	125
	2 ml of DMSO added and heated at 100° for 10 min	11 761	277
[C^3H_3]SAM	1 ml of 10% aq. KOH	19 626	1164
	2 ml of DMSO added and heated at 100° for 10 min	163 291	1428

The methyltransferase assays were performed with zymosterol as the sterol substrate and ^{14}C- or ^3H-labelled SAM as described in the Experimental with the following changes. The amount of ^{14}C-labelled SAM was 0.18 μCi (sp. act. 0.5 Ci/μmol) and of ^3H-labelled SAM 0.8 μCi (sp. act. 9.1 Ci/mmol). After terminating the reaction by adding 1 ml of 10% aq. KOH the products were extracted with petrol–diethyl ether (2:1) (PE–ether). The residue was then saponified by heating at 100° for 30 min. The products were again extracted with PE–ether. To the resulting residue, DMSO was added to a 50% concentration. The mixtures were heated at 100° for 10 min and the products were extracted with PE–ether. Blanks were treated the same way except that the sterol was omitted. Saponification prior to addition of DMSO was omitted from the assay with [C^3H_3]SAM.

stigmasterol. Further confirmation that the radioactivity was due to the product only is provided by characterization of the products as given in the Results.

Incubations were carried out in 16 mm × 150 mm test-tubes. The standard assay incubation medium contained the following: 400 nmol of sterol dispersed in 0.3 ml of Lubrol PX soln (4 mg Lubrol PX/ml water), 10 μmol of Tris–maleate (pH 7.3) and 0.5 μCi of [C^3H_3]-*S*-adenosylmethionine (sp. act. 9.1 Ci/mmol unless otherwise indicated). Where indicated, non-radioactive SAM (usually 50 nmol) was added to the incubation mixture. The total incubation vol. after addition of enzyme was 1.0 ml. Incubations were carried out for 40 min or for the time indicated. The reaction was stopped by addition of 1.0 ml 10% aq. KOH and 2 ml DMSO was added to each of the tubes which were then heated at 100° for 10 min. The tubes were cooled to room temp. and 5 ml petrol–Et_2O (2:1) was added to each tube. The contents of the tubes were shaken and centrifuged at low speed. The tubes were placed in a dry ice–EtOH bath for 2 min to congeal the lower layer. The upper Et_2O phase was decanted carefully into a scintillation vial. The lower phase was warmed and two more extractions were carried out with the petrol–Et_2O mixture. The combined Et_2O extracts were dried on a warm water bath and 10 ml of scintillation liquid (4 g PPO/l. toluene) was added and the radioactivity was determined. The efficiency of radioactivity counting was determined by sample channels ratio method and was about 80%.

Protein assay. Since Triton X-100 was used to solubilize the enzyme, it was present in many samples. Triton X-100 interferes with the Lowry procedure of protein determination, therefore a modification suggested by Wang and Smith [28] with some changes was used. An alkaline copper reagent was prepared by adding 1 ml 1% crystalline $CuSO_4$ soln and 1 ml 2% Na–K tartarate soln to a stirred 100 ml soln of 2% Na_2CO_3 in 0.1 N NaOH. To a 0.2 ml sample in a test-tube, was added 1 ml of this freshly prepared alkaline copper reagent. The soln was mixed thoroughly and kept at room temp for exactly 10 min. At the end of 10 min 1 ml 10% sodium dodecyl sulfate was added, mixed thoroughly and, with continued mixing, 0.1 ml 1 N Folin–Ciocalteu reagent (diluted fresh from the 2 N stock soln) was added. The samples were allowed to stand for 30 min and analysed spectrophotometrically at 730 nm. The colour was stable for up to 2 hr. Bovine serum albumin dissolved in a soln of Triton X-100 (0.1%) in buffer was used as the standard.

Ion-exchange chromatography. An affinity column according to Kim *et al.* [29] was prepared in an attempt to purify the methyltransferase. For this affinity resin, *S*-adenosyl-L-homocysteine (SAH) was attached to activated ω-aminohexyl Sepharose 4B and then an excess of 2-mercaptoethanol was added to mask any remaining active sites [29]. The methyltransferase was found to bind to the resin quantitatively and, surprisingly, the resin was active. Both transmethylation reactions were observed with approximately the same sp. act. as that of the solubilized enzyme. However, all attempts to elute the enzyme by various concentrations of SAM, SAH, different pH buffers or detergents were unsuccessful. Later it was found that the enzyme adsorbed to the resin equally well when SAH was omitted and the activated ω-aminohexyl Sepharose 4B was completely reacted with 2-mercaptoethanol or glycine. This finding led to the preparation of the resin as described below for purification of the enzyme by ion-exchange chromatography.

The preparation of the resin was performed by the combined procedures of Cuatrecasas and Parikh [30, 31]. According to their procedure, succinyl aminohexyl Sepharose 4B was prepared, activated and added to a rapidly stirred ice-cold soln of 1 M glycine in 0.2 M phosphate buffer, pH 6.4 (5 ml glycine soln per g moist gel). The suspension was stirred for 30 min and kept overnight at 4°. After this, the gel was washed with 50 vols of 0.2 M NaCl and thoroughly with 0.01 M Tris–maleate buffer, pH 7.3 (containing 2 mM 2-mercaptoethanol).

Column dimensions were 0.6 cm × 20 cm. After application of the sample the column was washed with 0.01 M Tris–maleate, pH 7.3 (containing 2 mM 2-mercaptoethanol). After about 20 ml of the buffer had passed through, elution was continued with a linear NaCl gradient in the above buffer. The flow rate of the column was 11.2 ml/hr and fractions were collected over 15 min periods.

Gel filtration. Bio gel A-15m was used for gel filtration. Column dimensions were 1.5 cm × 55 cm and the buffer was 0.01 M Tris–maleate, pH 7.3 (containing 2 mM 2-mercaptoethanol). Flow rate of the column was 18 ml/hr and fractions were collected over 15 min periods.

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