Phytochemistry, 1976, Vol. 15, pp. 683–687. Pergamon Press Printed in England.

A STEROL METHYLTRANSFERASE FROM BEAN RUST UREDOSPORES, *UROMYCES PHASEOLI**

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(*Revised received* 8 *October* 1975)

Key Word Index—*Uromyces phaseoli*; bean rust; phytosterols, biosynthesis; methyltransferase, S-adenosylmethionine

Abstract—A methyltransferase(s) that catalyzes the transfer of the methyl group from S-adenosylmethionine to a sterol acceptor was solubilized with Triton X-100 and partially purified from bean rust uredospores (*Uromyces phaseoli*). Zymosterol was the most active substrate tested while desmosterol and lanosterol exhibited good activity. The products were sterols with either a methylene or ethylidene group at the C-24 position. Direct evidence for the synthesis of the ethylidene group was obtained by using 24-methylenecholesterol as a substrate.

INTRODUCTION

Evidence has been presented that 24-ethyl or 24-ethylidene groups of phytosterols are formed by two successive methylation reactions. Thus the pathway commonly accepted is Δ^{24}-sterol → 24-methylene sterol → 24-ethylidene sterol → 24-ethyl sterol [1–4]. The donor of the one carbon unit in the synthesis of ergosterol has been shown to be S-adenosylmethionine (SAM) and S-adenosylmethionine: Δ^{24}-sterol methyltransferases (EC 2.1.41) have been partially purified and investigated [5–11]. The immediate product has been shown to be a 24-methylene derivative [7,8]. The formation of 24-methylene sterols by cell free extracts from pea seedlings [12] *Rubus fruticosus* [13] and algae [14] has been demonstrated and the utilization of 24-methylene sterols for the synthesis of a two carbon side chain at C-24 has been shown *in vivo* with *Pinus pinea* [3] and *Ochromonas malhamenses* [4]. Nevertheless the conversion of 24-methylene sterols to 24-ethylidene sterols by cell free preparations does not appear to have been demonstrated. Furthermore, the major sterol of germinating peas has a 24-ethyl group but the major product isolated from incubations of cell-free preparations with Δ^{24}-sterols and methionine was a 24-methylene sterol [12].

It was the purpose of this study to investigate the enzymatic formation of the 24-ethylidene group, and the organism selected, *Uromyces phaseoli* (bean rust), has been shown to produce a 24-ethylidene sterol, stigmasta-7,(*Z*)-24(28)-dien-3β-ol [15,16]. The question of whether the two methyl transfers are catalyzed by the same enzyme, an enzyme complex or by two separate enzymes may be approached after the demonstration of enzymatic synthesis of the ethylidene group.

*Published with the approval of the Director as Paper No. 4025, Journal Series, Nebraska Agricultural Experiment Station. Research reported was conducted under project 15–18. This study was supported by the Nebraska Wheat Division.

RESULTS

Methyltransferase purification. Methyltransferase activity was determined from the incorporation of ^{14}C into sterols when S-adenosylmethionine-[methyl-^{14}C] (SAM) and Δ^{24}-sterols were used as substrates. This enzyme activity is called methyltransferase in this report although it may represent more than one enzyme. The methyltransferase was isolated from bean rust uredospores by centrifugation and the major portion of the activity was associated with a 45000 *g* pellet. Triton X-100 was used to solubilize the methyltransferase, which was then passed through a column of Bio-gel A-15 M. An approximate purification of 14-fold was observed. Details of the purification procedure are given in the Experimental and a summary is given in Table 1. Further attempts to purify were unsuccessful, partly because methods requiring even moderate salt concentrations could not be used due to the sensitivity of the preparation to salt. Activity was inhibited by 80% with 0.1 M NaCl and at 0.02 M, inhibition was about 10–20% for NaCl and KCl. Other methods for purification that were tried included, chromatography with DEAE-Cellulose, CM-Cellulose, and three different affinity columns with lanosterol attached to the resin. Several electrophoretic and centrifugal methods were also attempted.

Some properties of the methyltransferase

The effect of enzyme concentration on the velocity of the reaction was linear throughout the concentration range investigated and the rate of transmethylation remained essentially constant during the first 20 min of incubation time.

The optimum pH of the methyltransferase was determined with Tris–HCl buffers and found to be pH 7.3. The activities at pH 8.0 and 6.6 were about 62 and 43% of the optimum respectively. Half optimal activities were indicated by a pH-activity curve at pH 8.1 and 6.7.

The temperature optimum of the methyltransferase was about 23°. At the lowest temperature tested, 15°,

Table 1. A summary of the purification of methyltransferase

Fraction	Protein (mg)	Radioactivity (dpm)	Specific radioactivity (dpm/mg protein)	Purification
1000 *g* supernatant	134.0	195040	1.45	1
45000 *g* pellet	58.8	155030	2.65	1.8
Solubilized enzyme 45000 *g* supernatant	9.9	109220	10.93	7.5
Bio-Gel A-15M Chromatography,	4.6	91420	20.05	13.8

and at 30° the activity was about one-half of the optimum. Only slight activity (5% of optimum) was observed at 40°.

The K_m for zymosterol as determined by a Lineweaver–Burk plot was 55 μM. The K_m for SAM was 33.4 μM. Because Lubrol PX, a detergent used to suspend the sterol substrate, was an inhibitor of the methyltransferase, the K_m values varied when different amounts of detergent were used. For example the K_m for zymosterol was determined to be 75 μM when the detergent concentration was 0.8 mg/ml instead of 0.4 mg/ml. With Tween-80 (4.0 mg/ml) in place of Lubrol PX the K_m was determined to be 62 μM. The optimum activity was observed when the concentration of the detergent was a minimum yet was sufficient to yield a clear preparation without visible particles of the sterol substrate. Below 4 mg/ml concentration of the detergent particles of the sterol were present.

None of the cofactors tested such as NADP, NAD, NADPH and NADH stimulated the methyltransferase activity. At 2.4 mg/ml the inhibition by NAD and NADP (more than 80%) was much stronger than that of NADH and NADPH (about 30%). This high inhibitory effect of the oxidized cofactors may be explained by their influence on the hydrogen migration from C-24 to C-25 [17]. The ionic effect of the cofactors may also be a factor in their inhibition of the methyltransferase. Glutathione and dithiothreitol did not stimulate this methyltransferase preparation.

Triparanol has been shown to inhibit the reduction at the C-24 position and the Δ^{24}-alkylation of sterols [17,18]. When triparanol was incubated with the enzyme preparation at a concentration of 100 μg/ml, it inhibited the enzyme activity by about 70%.

Substrate specificity of methyltransferase

Sterols that lack a double bond or possess an alkane function at C-24 such as stigmasterol, cholesterol and sitosterol, were not acceptors of the ^{14}C-group from SAM, whereas Δ^{24}-sterols were effective substrates. Zymosterol appeared to be the best substrate of those investigated. Compared to zymosterol, desmosterol and lanosterol had activities of about 50% and 20%, respectively. The only 24-methylene sterol tested, 24-methylenecholesterol, was found to serve as a substrate. The activity with this substrate was about 5% of that observed with zymosterol in two experiments.

Characterisation of products

The radioactive material extracted from the methyltransferase reaction mixture was subjected to TLC then radioautography was performed. Only one dark spot was observed on the radioautograph and its R_f corresponded to that of other sterols such as stigmasterol or lanosterol. The petrol soluble radioactive material was essentially completely precipitated by digitonin.

To determine if either a 24-methylene or 24-ethylidene group was present at the C-24 position the sterol product from a particular substrate was divided into equal samples and both were treated with ozone and cleaved. To one of the samples formaldehyde was added and to the other acetaldehyde was added. Then dimedone derivatives of each of the aldehydes were prepared and crystallized to constant specific radioactivity. When desmosterol was used as a substrate the specific radioactivity of the formaldehyde derivative was about 10 times greater than that of the acetaldehyde derivative thus indicating a mole ratio of 20:1 for the 24-methylene to 24-ethylidene product. For lanosterol the mole ratio was near 10:1 and about 40:1 for zymosterol. Varying the ratio of the substrates ^{14}C-SAM to Δ^{24}-sterol changed the ratio of the products only slightly. For example, a 10-fold increase in ^{14}C-SAM did not double the amount of 24-ethylidene product formed.

When 24-methylenecholesterol was used as a substrate the assay conditions were altered by increasing the specific radioactivity of the ^{14}C-SAM to 51 μCi/mmol and incubating for 60 min. Zymosterol was also tested under these modified conditions. As shown in Table 2 the amount of ^{14}C-activity incorporated into the products when zymosterol was used as a substrate was about 19 times greater than that obtained when 24-methylenecholesterol was used as a substrate. Nevertheless, the 24-ethylidenesterol did appear to have been synthesized. The products from these incubations were extracted and subjected to TLC. Single radioactive spots were detected by scanning the plates and the spots corresponded to the position of the reference sterols, fucosterol and 24-methylenecholesterol which had identical mobilities in the TLC system employed. For each product the absorbent containing the radioactivity was scraped from the plate, extracted with $CHCl_3$, and assayed for radioactivity. The radioactivity incorporated into products shown in Table 2 is 95% of the total radioactivity after TLC purification. Two aliquots (2.3×10^4 dpm) of the product from the incubation with zymosterol were mixed with 24-methylenecholesterol (0.5 mg) and fucosterol (0.5 mg) respectively. Fucosterol (0.5 mg) was also added to the product from the incubation with 24-methylenecholesterol. The samples were then subjected to ozonolysis as described in the Experimental. After ozonolysis and reductive cleavage formaldehyde was added as a carrier to the sample to which 24-methylenecholesterol had been added. Acetaldehyde was added to the other tubes to which fucosterol had been added. The preparation of the dimedone derivatives was performed as described in the

Table 2. The incorporation of ^{14}C from S-adenosylmethionine into sterol products by an enzyme preparation from *U. phaseoli*

Substrate	^{14}C incorporation* (dpm)	^{14}C-recovered from ozonolysis of products	
		Acetaldehyde (%)	Formaldehyde (%)
24-methylenecholesterol	9880	77	—
Zymosterol	182000	2	72

* After purification by TLC.

Experimental. Between 17 to 25 mg (60–85 μmol) of the dimedone derivatives were used for each determination of specific radioactivity. Table 3 gives the results obtained for these specific radioactivity measurements. Neglecting the contribution of aldehyde due to ozonolysis of the sample and carrier sterols, considering that 1 mmol of the appropriate aldehyde was added to each derivative preparation, assuming the ozonolysis and reductive cleavage reaction gave 100% yields, and assuming that all of the radioactive aldehyde released by ozonolysis and reductive cleavage was the same type as the aldehyde added as carrier, the theoretical specific radioactivities were calculated and are given in Table 3. When the products from the zymosterol incubation were analyzed, 74% of the ^{14}C-activity was released as formaldehyde while only about 2% was released as acetaldehyde. For the products from the 24-methylenecholesterol incubation, about 77% of the ^{14}C was released as acetaldehyde (Table 2). Since ozonolysis procedures generally are not quantitative and yields in the range of 80% are common, it appears that the principle product when zymosterol was used as a substrate was a sterol with a 24-methylene group and that the major product when 24-methylenecholesterol was used as substrate was a sterol with a 24-ethylidene group. The 24-ethylidene group appears to be formed when Δ^{24}-sterols are used as substrates also, but the extent of synthesis is small compared to the synthesis of the 24-methylene group. The relative ratios of products did not appear to change appreciably when incubations were performed with crude homogenates instead of the partially purified methyltransferase.

DISCUSSION

Although the 24-methylene compounds are presumed to be precursors of the 24-ethylidene sterols, previously no cell free system has been successfully isolated. A fraction from macerated pea seedlings was shown to be capable of introducing ^{14}C from methyl-labeled methionine into such substrates as lanosterol, desmosterol and cycloartenol [5]. However this cell-free system did not carry the biosynthesis further than the 24-methylene stage although peas do contain 24-ethylsterols.

With methyltransferases prepared from *Trebouxia* sp. and *Scenedesmus obliquus* the 24-methylene sterols tested were not active substrates for the presumed second transmethylation step leading to C_{29} sterols [14], although *in vivo*, a methylene sterol, cycloeucalenol, was converted to 24-ethyl sterols [19].

The methyltransferase isolated from bean rust uredospores in this study could carry the biosynthesis further to obtain C_{29} sterols when zymosterol, desmosterol, lanosterol and 24-methylenecholesterol were supplied as substrates. Between 2–5% of the product appeared to contain an ethylidene group when Δ^{24}-sterols were used as substrates. With 24-methylenecholesterol as a substrate the ethylidene group was formed but the enzyme activity was about 5% of that when zymosterol was used as a substrate. The lack of knowledge with this organism concerning the reaction sequence for the conversion of the first cyclized product of squalene to the major sterol, stigmasta-7,(*Z*)-24(28)-dien-3β-ol, makes conclusions based on relative activities of the substrates used difficult. Because 24-methylene sterols are not detected in the bean rust uredospores, the addition of the second methyl group should be very direct. Yet low activity for the second transmethylation step was observed. Several explanations for these observations are possible. One is that there are two separable enzymes and during purification the enzyme catalyzing the second methylene transfer was partially lost. However, in our hands when crude homogenates were used with Δ^{24}-sterols as substrates the ratio of products with methylene and ethyli-

Table 3. Specific radioactivities of formaldehyde and acetaldehyde dimedone derivatives obtained by ozonolysis and reductive cleavage of products isolated from enzyme incubations with zymosterol and 24-methylenecholesterol

Derivative Recrystallization	Substrate: Zymosterol Formaldehyde (dpm/μmole)	Zymosterol Acetaldehyde (dpm/μmole)	24-Methylenecholesterol Acetaldehyde (dpm/μmole)
Fourth	16.9	0.432	7.62
Fifth	16.9	0.430	7.59
Sixth	17.2	0.438	7.62
Seventh	16.9	0.429	7.62
Theoretical	23.0	23.0	9.88

dene groups was about the same as that observed for the partially purified preparation. Another possibility with two enzymes or a complex is that the second enzyme is more labile and conditions for optimum incorporation of the methyl group of SAM into 24-methylenesterol substrates are quite different than those for the first step. The possibility of the ethylidene group being derived from a C_2-donor has been considered. In the investigations of others [2,20] the radioactivity of ethionine-[^{14}C-ethyl] was not incorporated into sterols under conditions that did allow incorporation from methionine-[^{14}C-methyl]. Further studies will be required to explain the enzymatic synthesis of the ethylidene group.

EXPERIMENTAL

Bean rust uredospores, *Uromyces phaseoli* (pers.) Wint. var. *typica* Arth., were produced as described by Trocha and Daly [21].

Enzyme assays. All incubations were carried out in 16 × 150 mm test tubes. The standard reaction mixture contained 10 μmol Tris–maleate–NaOH, pH 7.3, 160 nmol of sterol substrate suspended in H_2O with 4 mg Lubrol PX per ml, 20 nmol EDTA and 10 μl of a soln containing 100 nmol of S-adenosyl-L-methionine-[methyl^{14}C] (0.14 μCi). The addition of H_2O and the enzyme soln made a total vol of 1.0 ml. The reaction mixture was preincubated 5 min at 23°, then enzyme was added to start the incubation and the samples were incubated for 20 min at 23°. The reaction was stopped by the addition of 5 ml of extraction soln (petrol–Et_2O, 2:1). The soln was mixed well and centrifuged to aid in layer separation. The organic layer was transferred directly into scintillation vials and evaporated to dryness. This extraction procedure was repeated 2×. Radioactivity was determined with a liquid scintillation spectrometer. The scintillation soln was 4 g PPO/l. toluene.

Enzyme isolation and purification. Cell-free extracts were prepared with a Braun model MSK mechanical homogenizer. Between 0.5–1.0 g of spores and 25 g of 1.0 mm glass beads were weighed into the homogenizer vessel and 15 ml 0.01 M Tris–maleate buffer, pH 7.3 was added. The mixture was shaken for 50 sec at 400 oscillations per min while being cooled with CO_2 [22]. Homogenate was removed from vessel and remaining beads were rinsed 2× with 3 ml of the same buffer. This crude homogenate was centrifuged at 1000 *g* for 20 min and the supernatant fraction was centrifuged at 45000*g* for 30 min. The resulting pellet was resuspended in 25–30 ml 0.01 M Tris–maleate buffer, pH 7.3, that also contained 0.1% Triton X-100. After gently stirring for 40 min the soln was centrifuged at 45000 *g* for 30 min. The supernatant fraction containing the solubilized enzyme was then concentrated with a Diaflo-filter with XM-100A ultrafiltration membrane. The filter removed part of the Triton X-100. The condensed, solubilized enzyme was added to a column (85 × 2 cm) of Bio-Gel A-15M which had been equilibrated in the cold with 0.01 M Tris–maleate buffer, pH 7.3. The flow rate of the column was 0.4 ml/min and fractions were collected over 12 min intervals. Effluent was assayed for enzyme activity within 12 hr of sample addition. All enzyme operations except the assays were performed at 0–4°. Protein was determined by the method of Lowry *et al.* [23] using bovine serum albumin as a standard.

Ozonolysis. The following procedure was adapted from a method by Pappas *et al.* [24]. Products from enzyme incubations were isolated by TLC, extracted from the absorbent with $CHCl_3$ (3×) and transferred to a 16 × 150 mm test tube. Carrier sterols, 0.5 mg fucosterol or 24-methylenecholesterol, were added and the solvent was evaporated under N_2. 2.0 ml CH_2Cl_2–MeOH (1:1) was added to dissolve the sterols and the tube was cooled to −30°. Ozone was bubbled through the soln for 5 min, then the temp. was lowered to −65° and bubbling continued for 10 min. After inspection of the sample for the presence of a light blue color indicating an excess of O_3, the O_3 line was disconnected and dry N_2 was bubbled through the soln to remove the excess O_3. When the sample was colourless 0.5 ml $(Me)_2$ S and a small magnetic stirring bar were added. The tube was closed with a tight fitting serum bottle stopper that was secured with wire. The tube was placed in a brine-ice bath maintained at −10° and the contents were stirred continuously for 1 hr. Stirring was continued at 0° for 1 hr and finally at room temp. for 1 hr. With a syringe 3.8 ml of a warm 20% soln of dimedone in EtOH, and 1 ml H_2O was added. This mixture was stirred for 15 min. Again with a syringe, 2.0 ml of the appropriate aldehyde soln (either formaldehyde or acetaldehyde in H_2O, 0.5 M) was added and the sample was stirred for 15 min with intermittent warming in a 60° bath. The tube was cooled, vented with a hypodermic needle, then 3 ml H_2O was injected which caused a phase separation. The stopper was removed and with vigorous stirring and gentle warming the lower phase (CH_2Cl_2) was evaporated. After adding more H_2O (7 ml) the sample was heated to 60° and allowed to stand overnight. The crystalline dimedone derivative was collected by centrifugation. When acetaldehyde had been added as the aldehyde carrier the crystals were dissolved in 80% EtOH (minimum volume at 60°) and 2 drops of conc HCl were added. The sample was heated for 10 min at 60°, and an equal vol of H_2O was added. The crystalline ppt. collected was the dehydrated dimedone derivative (derivative II) [25]. It was then subjected to recrystallizations with EtOH–H_2O once and thereafter with a mixture of xylene–hexane (1:4). Specific radioactivity measurements were not made until after 4 recrystallizations. When formaldehyde was added the dimedone derivative was not dehydrated but was crystallized from a EtOH–H_2O mixture twice and subsequently with a mixture of xylene–hexane (1:4). Specific radioactivity measurements were not made until after 4 crystallizations of this derivative. The mp's of the derivatives were found to be identical to those given by Vogel [25] (acetaldehyde 174°, formaldehyde 189°). Acetaldehyde was purified by distillation just before the preparation of the two aldehyde solns which was done about 1 hr before their use. To determine if cross contamination of the aldehydes would be eliminated by recrystallizations acetaldehyde–formaldehyde mixtures with molar ratios of 100:6 and 6:100 were prepared. When dimedone derivatives (derivative I) were prepared with 6:100 acetaldehyde to formaldehyde mixtures, recrystallization yielded a product that had the mp of the formaldehyde derivative (189°). Tracer radioactivity added as acetaldehyde-[^{14}C] was lost by recrystallizations. Because of the high solubility of the acetaldehyde derivative (as derivative I) difficulty was encountered in purifying samples with a molar ratio of 100:6 (acetaldehyde to formaldehyde). However it was found that purification could be achieved if the sample was converted to the dehydrated dimedone derivatives (II). Criteria for purity was mp (174°) and the loss of radioactivity from tracer formaldehyde-[^{14}C].

Materials. Zymosterol was isolated from Fleishman dry yeast using the method described by Thompson *et al.* [10]. Ergosterol was removed by the ergosterol–maleic anhydride adjunct formed according to Schwenk *et al.* [26]. Purity of the zymosterol was determined by the presence of a single spot on TLC (R_f 0.32) [10] and a mp of 107° which agrees closely with reported literature values [8,26] and by GLC with a QF-1 liquid phase. Commercial lanosterol was contaminated with about 40% of 24,25-dihydrolanosterol as determined by GLC. Pure lanosterol was prepared from the commercial material by recrystallizations from MeOH–$CHCl_3$ followed by acetylation. The acetylated products were separated on TLC plates impregnated with $AgNO_3$. The purified lanosterol acetate was saponified, and the lanosterol was recrystallized 2× from MeOH–$CHCl_3$. The mp of purified lanosterol was 138–140° (lit [10] 138–139°). The purity was over 95% as determined by GLC (3% QF-1). Desmosterol (95% purity) was

obtained from Schwarz/Mann Company, Orangeburg, N.Y. 24-Methylenecholesterol was synthesized using 24-ketocholesterol as a starting compound and a synthetic method reported by Idler and Fagerlund [27]. The product was purified by TLC and recrystallizations gave the expected mp of 135°. S-adenosyl-L-methionine-[methyl-^{14}C] (51 mCi/mmol) was purchased from Nuclear Dynamics Inc., ElMonte, California and from New England Nuclear, Boston, Massachusetts. Triparanol was kindly provided by Dr. A. Richardson, Jr., Wm. S. Merrill Co., Cincinnati, Ohio.

Chromatography. TLC was performed with Si gel G, 250 μm, and a solvent of Et_2O–heptane–HOAC (70:30:1) or with Si gel G, 250 μm, impregnated with $AgNO_3$ and a solvent of $CHCl_3$–HOAC (200:1). GLC procedures have been reported [15].

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