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Triterpenoid Biosynthesis in *Euphorbia Lathyris* Latex

D.R. Hawkins
(Ph.D. Thesis)

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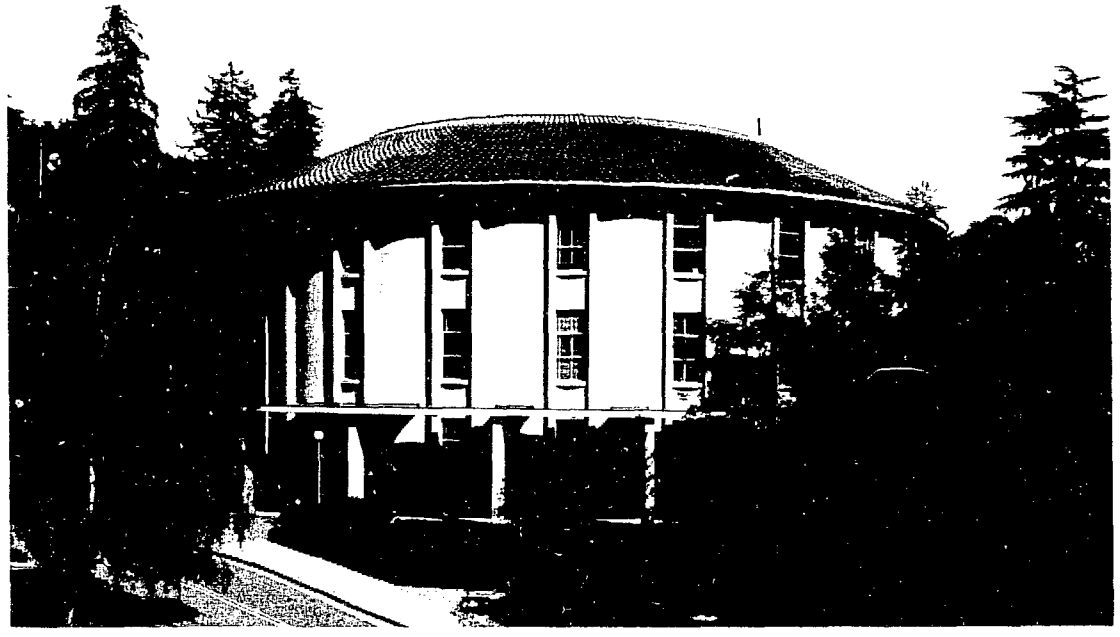

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ABSTRACT

The structures of those tetracyclic triterpenols in Euphorbia lathyris latex which had not previously been known were elucidated. The latex was found to contain lanosterol, cycloartenol, 24-methylenelanosterol, 24-methylenecycloartenol, and butyrospermol. Small amounts of a compound which is probably a pentacyclic triterpenol were also isolated from the latex. A method for quantifying very small amounts of these compounds was developed.

A wide variety of experiments were performed concerning the biochemistry of the latex. The addition of various cofactors and cations was examined. In general, no exogenous cofactors were required for the biosynthesis and the addition of compounds such as NADPH and ATP did not stimulate the biosynthesis. The addition of DTE or a similar anti-oxidant was found to help reduce the oxidation of the latex, thus increasing the longevity of the latex . The requirement of a divalent cation and the preference for Mn in the pellet was observed. Many attempts were made to observe the incorporation of squalene into the triterpenoids. The fact that none of these provided any incorporation is attributed to the compartmentation of the latex and the absence of any system for the transport of squalene across the membrane.

Many time dependent incubations were done, investigating the stability of the whole latex system and the re-suspended pellet systems. The effects of centrifugation on the biosynthesis were examined. The ability of the pellet to synthesize the triterpene esters was found to be much lower than its ability to synthesize the free triterpenols (as compared to the whole latex). The partitioning of the triterpenoid pool as a function of when the compounds were made was examined. The most recently synthesized triterpenoids are concentrated in the pellet. Localization of the acetate-to-triterpene and the mevalonate-to-triterpene activities by centrifugation was accomplished, with the pellet not having the ability to utilize acetate, but a reconstitued system showing good incorporation from both substrates. Several attempts to

delipidate the latex while maintaining some biosynthetic activity were tried. The only method which provided any delipidation while maintaining some biosynthetic activity was centrifugation.

A number of incubations were performed in the hopes of observing some interconversion of the individual triterpenols. No interconversion was ever observed, even from lanosterol to 24-methylenelanosterol or from cycloartenol to 24-methylenecycloartenol. Also, no interconversion of the triterpenols and the triterpene esters was ever observed.

The effect of several inhibitors on the biosynthesis of the triterpenoids was examined. Mevinolin was found to inhibit the biosynthesis of the triterpenoids from acetate, but not mevalonate. A Dixon plot of the inhibition of acetate incorporation showed an I_{50} concentration of 3.2 μM. The effects of the morpholine based fungicides, tridemorph and fenpropimorph were examined. Fenpropimorph was found to have little or no effect on the biosynthesis. Tridemorph was found to inhibit the biosynthesis of all of the triterpenoids with an I_{50} of 4 μM. It was also observed that the cyclopropyl-containing triterpenols, cycloartenol and 24-methylenecycloartenol were inhibited much more strongly than those containing an 8-9 double bond, lanosterol and 24-methylenelanosterol. The evidence indicated that lanosterol and 24-methylenelanosterol are **not** made from cycloartenol and 24-methylenecycloartenol via a ring-opening enzyme such as cycloeucalenol-obtusifoliol isomerase.

The possibility that cycloartenol is made via lanosterol was investigated by synthesizing 4-R-4-^{3}H-mevalonic acid and incubating the latex with a mixture of this and ^{14}C-mevalonic acid. From the $^{3}H/^{14}C$ ratio it was shown that cycloartenol and 24-methylenecycloartenol are **not** made via an intermediate containing an 8-9 double bond.

The question of whether lanosterol is made via cycloartenol or not was investigated by incubating the latex with 3'-D_3-mevalonic acid. The substrate was synthesized in good yield and the isotopic enrichment was found to be virtually 100 atom %. Several incubations with this substrate were performed. The major obstacle was trying to get as much of the labelled material synthesized in the presence of the smallest possible amount of the endogenous triterpenoids. None of the attempts at delipidation were successful in obtaining a product whose mass

spectrum showed any signs of incorporation of the deuterium label.

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INTRODUCTION

Euphorbia lathyris has been considered for use as an energy crop [1-3] and its white, milky latex has been examined as a model system for triterpenoid biosynthesis in plants [4-8]. During the course of these studies there have been several reports concerning the identity of the triterpenols in E. lathyris and its latex [9-14]. Most of these reports either failed to mention the presence of certain compounds or left others unidentified. In order to attempt a very thorough study of triterpenoid biosynthesis in the latex, we felt it was necessary to know the identities of the products. The results of our structural elucidation study are reported in chapter 1.

Euphorbia lathyris contains high percentages of hydrocarbon-like materials (mostly triterpenols, the fatty acid esters of those triterpenols, and other fatty acid derived lipids) and sugars [2,3,9]. Both of these components are very desirable because they can ultimately be used to replace some portion of petroleum in our energy use. Both simple sugars and polysaccharides can be used for fermentation to ethanol. The hydrocarbon-like materials of E. lathyris can be cracked catalytically to form a crude oil which is similar to that obtained from petroleum [14]. After the extraction of the hydrocarbon and sugar fractions one is left with a biomass which can be dried and burned. The energy yields from E. lathyris are roughly comparable to the yields obtained from energy cane [3] and E. lathyris is not as limited in its growth environment. Unfortunately, the amount of energy obtained does not make the crop economically viable at today's oil prices. If the amount of hydrocarbons and sugars could be increased in some manner, it could provide a reasonable alternative to petroleum products in the event of another "oil crisis". Wtih the advances taking place in the field of biotechnology, it is possible to imagine the development of a plant which shows huge increases in certain compounds. Such advances require a considerable knowledge of the biochemistry involved in the biosynthesis of the compounds. Our intention was to investigate the biosynthesis of terpenoids in plants, using the latex of E. lathyris as a model system for our in vitro studies.

Chapter 2 reports some of the results we have obtained concerning the compartmentation, regulation, and control of the triterpenoid biosynthesis in latex.

The biosynthesis of triterpenes has been very thouroughly studied in nonphotosynthetic organisms, due in large part to a great interest in the biosynthesis of cholesterol [15]. Virtually all of the steps involved in the pathway in plants, from pyruvate to squalene oxide have been found to be identicle to those in nonphotosynthetic organisms [16,17]. The cyclization of squalene oxide is the one major point at which the pathway in plants diverges from that in animal systems. In plants, squalene oxide is cyclized to form cycloartenol; while in nonphotosynthetic organisms it is cyclized to form lanosterol [18,19]. Lanosterol is not normally thought of as being found in plants. There are a few plants which contain lanosterol [20]. E. lathyris happens to contain substantial amounts of lanosterol, especially in the latex [10-14]. We were very curious as to the origin of this lanosterol and the details of its biosynthesis. The results of our attempts at elucidating the biosynthesis of lanosterol in the latex are decribed in chapter 3.

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CHAPTER ONE: Triterpenols of Euphorbia lathyris latex

During the course of the many studies of Euphorbia lathyris, there have been several reports concerning the identity of the triterpenes in E. lathyris and its latex [1-6]. Ponsinet and Ourisson first identified the triterpenoid constituents on the basis of comigration on TLC and argentation TLC. They reported the presence of euphol(1), lanosterol(2), cycloartenol(3), and 24-methylenecycloartenol(4) [2]. In more recent investigations, the latex was reported to contain a compound which was referred to as "lanosterol isomer" [1,6]. Nishimura and coworkers [5] identified this compound as butyrospermol(5) on the basis of its behavior on GC. Using the same methods, they also reported finding euphorbol(6).

We found that it was possible to separate the triterpenols from E. lathyris latex by HPLC on an analytical reverse phase system. Using authentic samples supplied by Ourisson we tentatively confirmed the presence of euphol(1), lanosterol(2), cycloartenol(3), and 24-methylenecycloartenol(4). We also observed three other peaks in the chromatogram. Although chromatographic behavior can yield a considerable amount of information about a compound it is usually not the method of choice for structure determination and should not be relied upon without some other evidence.

In order to verify the identity of the triterpenoids found in E. lathyris latex we wanted to obtain spectroscopic evidence. The mass spectra of many triterpenoids and sterols have been examined in very great detail [7-11]. The quantity of material needed to obtain a mass spectrum is very small; we were able to separate the triterpenols from E. lathyris latex by HPLC in sufficient quantities to obtain mass spectra for each of the seven compounds. The mass spectra of 9,19-cyclopropyl sterols show a very characteristic fragmentation which is not observed in the spectra of any of the other tetracyclic triterpenols (fig. 2) [10,11]. By their behavior on HPLC and from the characteristic mass spectra it was possible to verify the identities of cycloartenol (3) and 24-methylenecycloartenol (4). The mass spectra of euphol (1) and

lanosterol (2) were identical to those of authentic samples, verifying their presence in latex.

FIGURE 1

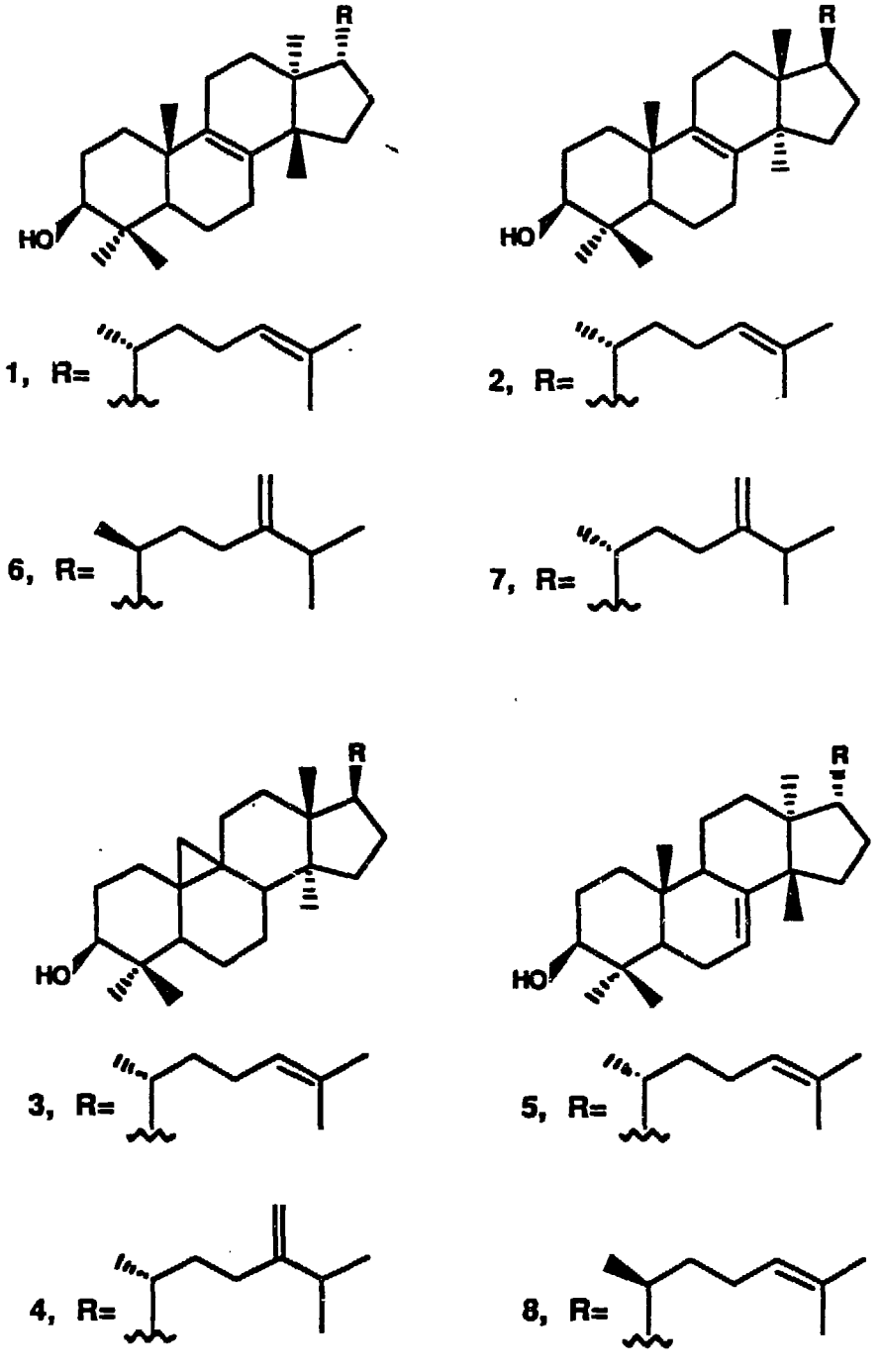
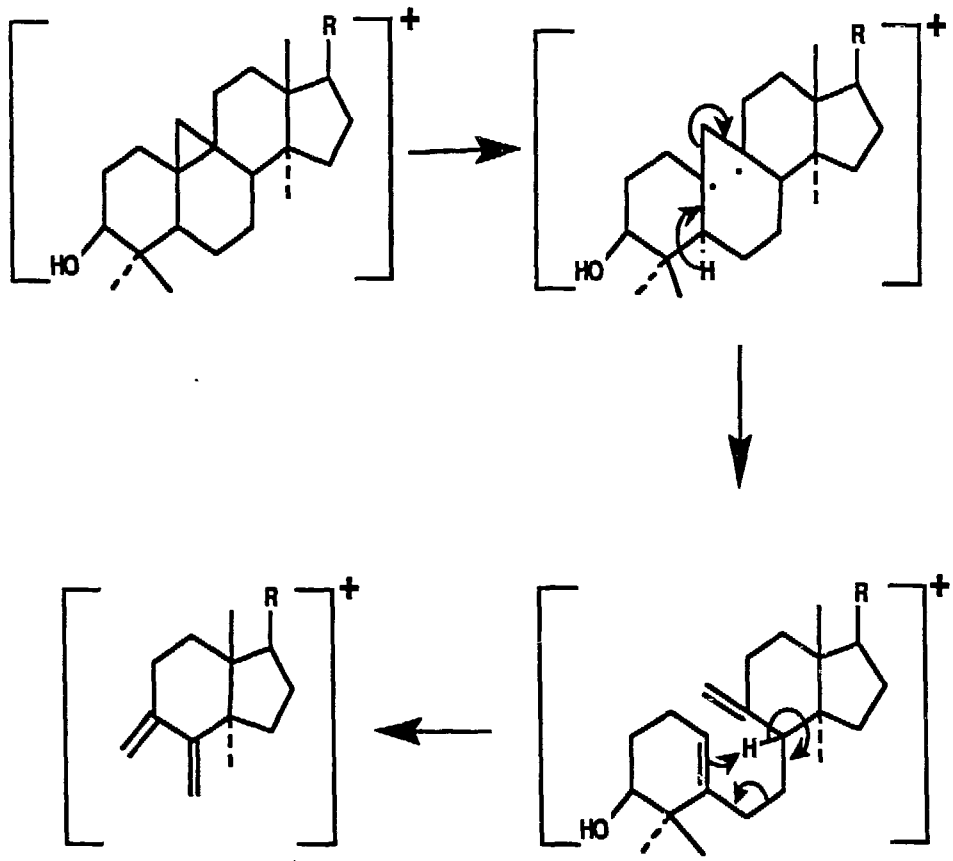


FIGURE 2



The mass spectrum of the compound tentatively identified as butyrospermol (5) is very unremarkable. One difficulty with mass spectroscopy is that diastereomers give either identical or very similar spectra. Because of this and the lack of an authentic sample of butyrospermol we could not positively identify this compound without further evidence. Using a semi-preparative column on the HPLC system it was possible to collect several mg of the compound This was enough material for a ^{1}H-NMR spectrum to be obtained. In general, the only features of the spectra of triterpenols that are reported in the literature are the chemical shifts of the methyl groups, any vinyl protons, and the 3-α-CH. It was readily apparent from the vinyl region of the

proton NMR spectrum that there were two trisubstituted double bonds. Comparing the ^{1}H-NMR spectrum to literature values [12] for **5** and similar structures led to the conclusion that the compound was either **5** or tirucala-7,24-dienol (**8**).

Considerable work has been published concerning the ^{1}H-NMR of triterpenols in the presence of lanthanide isotope shift reagents [12-15]. Itoh and coworkers have shown that in the presence of lanthanide shift reagents the allylic methyl groups (C26,C27) of **8** are shifted downfield to 1.74 and 1.80 while in compound **5** they do not shift downfield and are observed at 1.62 and 1.68 [12]. The chemical shifts observed for the compound from E. lathyris latex were 1.64 and 1.67. These are within experimental error of the chemical shifts reported for **5**. Examination of the spectra in the presence of Eu(fod)$_3$ thus made it clear that the compound was indeed butyrospermol (**5**).

We then examined the compound previously identified as euphorbol. The mass spectrum showed a molecular ion at m/e 440 which is expected for a 24-methylene triterpenol. Other than this molecular ion the mass spectrum yields little information concerning the structure of this compound. It could very easily have been euphorbol (**6**), 24-methylenelanosterol (**7**), or 24-methylene euphol. Again, further evidence was needed in order to be certain of the identity of this compound. A sufficient quantity for ^{1}H-NMR work was collected using the semipreparative column on the HPLC. The farthest upfield chemical shift of a methyl group in the spectrum was 0.693. The farthest upfield shift for a methyl group of eupha-8-enol is reported to be 0.77 (no data was available for 24-methylene euphol) while that of euphorbol is reported to be 0.77 [12]. The farthest upfield chemical shift of a methyl group in 24-methylenelanosterol (**7**) is reported to be at 0.70 [12]. A closer examination of the proton spectrum shows an excellent agreement with that of **7**.

To further verify the identity as **7**, an authentic sample was synthesized by the method of Barton, et al [16] using commercially available lanosterol as the starting material (see scheme 1). Lanosterol is first acetylated using acetic anhydride and pyridine. The side chain of the acetate is then epoxidized via the bromohydrin and rearranged with boron trifluoride etherate complex

to a mixture of 24-oxo-lanosterol and an isomeric aldehyde. The mixture is subjected to oxidation with pyridinium dichromate which converts the aldehyde to the corresponding acid. This undesired acid is removed from the mixture by extraction with base. The Wittig reaction then converts the ketone to 24-methylenelanosteryl acetate. The acetate is hydrolyzed with potassium hydroxide in methanol to yield 24-methylenelanosterol. The final product was examined by HPLC, mass spectroscopy, and ^{1}H-NMR spectroscopy. Other than a slight amount of the Wittig product resulting from some unoxidized aldehyde, it matches the literature values reported by Barton [16]. The natural product from latex has identical mass and proton NMR spectra to those of this synthetic standard. On this basis we believe that this compound is 24-methylenelanosterol (7), not euphorbol (6).

The remaining peak observed in the chromatogram of the triterpenols from E. lathyris latex eluted much later than the others. This compound was called "unknown E". The mass spectrum of this compound is very uninformative. It shows a molecular ion at m/e 426 ($C_{30}H_{50}O$) which is much more intense than the molecular ions observed for the other triterpenols from latex. There are only minor peaks at m/e 411 (M+-CH_3), m/e 357 (M+-C_5H_9) and m/e 315 (M+-C_8H_{15}). Slightly larger peaks are observed for m/e 218 (M+-208) and m/e 207. This type of mass spectrum is sometimes observed for a pentacyclic triterpenol [7]. Triterpenoids of the amyrin type with a double bond in the 12 position generally show a very distinctive fragmentation corresponding to a retro Diels-Alder reaction in the C-ring (see figure 3) [17]. If unknown E was either α-amyrin (9) or β-amyrin (10) one would expect to see a very prominent ion at m/e 203 due to this type of fragmentation. The mass spectrum is very similar to those observed for pentacyclic triterpenes of the lupane type (11) [7]. It was not possible on the basis of this mass spectrum alone to assign a structure to E.

SCHEME 1

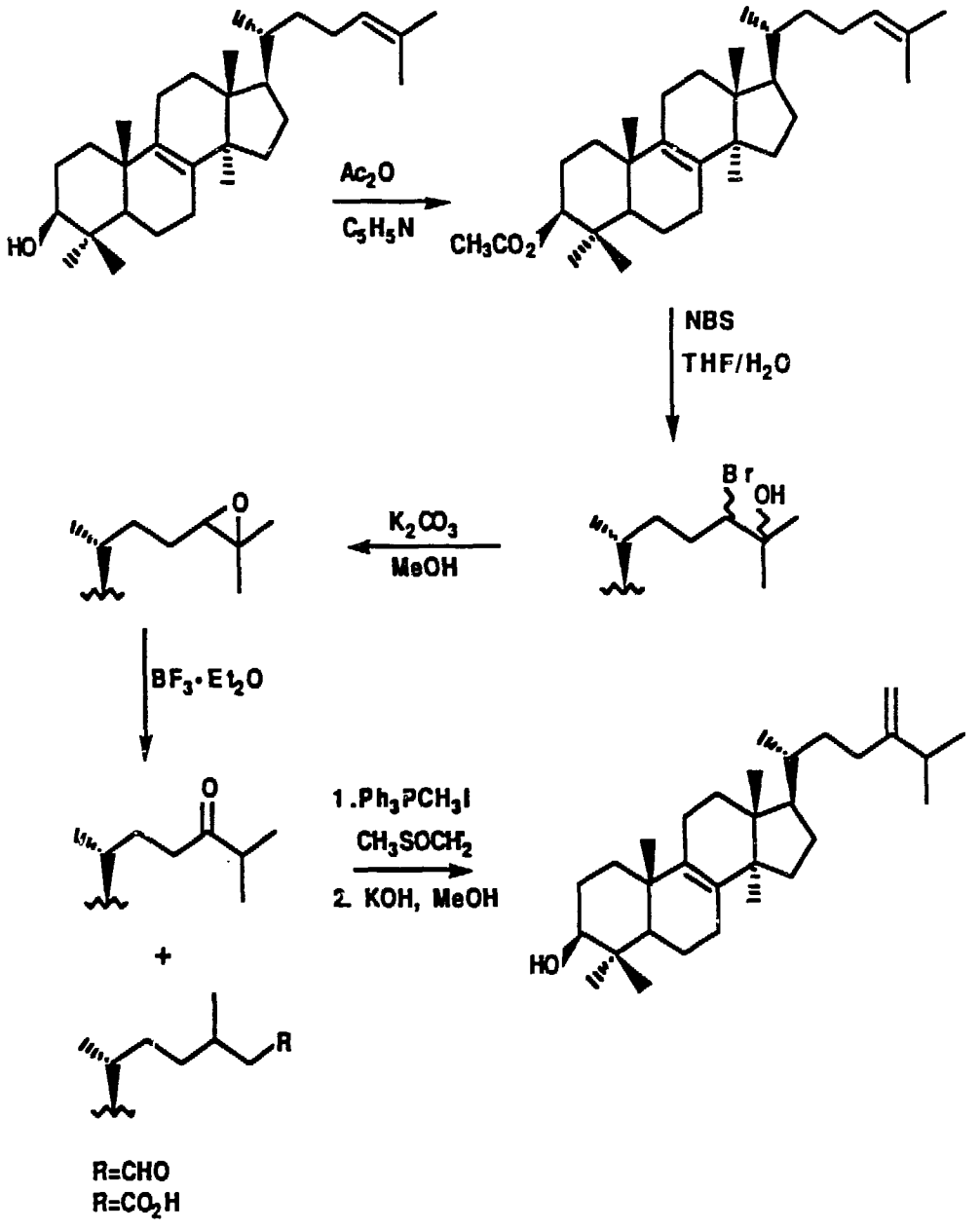
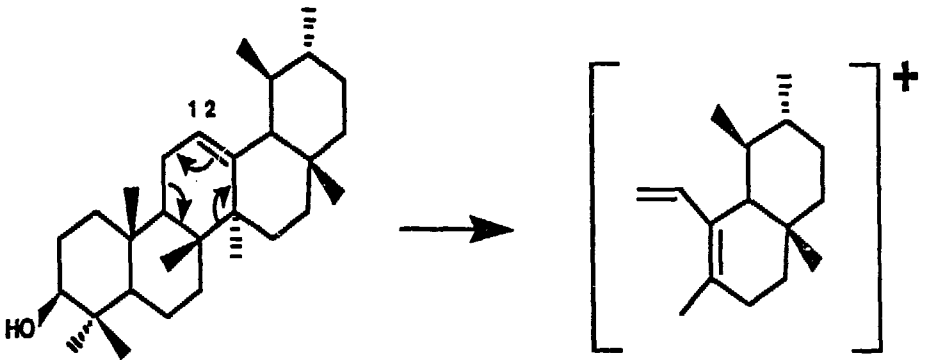

HO
Ac2O
C5H5N
CH3CO2
NBS
THF/H2O
Br
OH
O
K2CO3
MeOH
BF3·Et2O
O
1. Ph3PCH3I
CH3SOCH2
2. KOH, MeOH
HO
+
R
R=CHO
R=CO2H

FIGURE 3



α-amyrin m/e 218

A sufficient quantity of E was collected to obtain a ^{1}H-NMR spectrum. The most striking feature of the spectrum is a two proton broad singlet at δ 4.7. Some of the other features which were discernable were that there was one allylic methyl group and a one proton multiplet at ca. 2.7. These signals could be caused by an isopropylidene group, such as is found in lupeol. Also there are only six signals for methyl groups in the region 0.7-1.0 ppm. Normally, triterpenols such as lanosterol or even the amyrins have a total of eight methyl signals (including allylic methyls). Comparison of the ^{1}H-NMR spectrum of E to that of lupeol (11) shows them to be very similar but not identical. Where E has one 2 proton peak at 4.7, lupeol has two separate signals for one proton each. Also, the one proton multiplet at 2.7 in E is at 2.38 in lupeol. The similarity of the two spectra suggests that although E is not lupeol, it must be very closely related. There are reports of a wide variety of pentacyclic triterpenols of the B':A'-Neogammacerane (hopane) (12) and A'-Neogammacerane (13) families [18-21]. To determine the exact identity of E would have required a fair amount of effort; it constitutes only about one percent of the triterpenol mixture from latex and can only be separated from the others by HPLC. Sufficient material would have to have been isolated to obtain a ^{13}C-NMR

spectrum, a 2-dimensional ^{1}H-NMR spectrum, and possibly a circular dichroism spectrum. Our primary interest was in the tetracyclic triterpenols; **E** is a minor component of the total triterpenoids and we did not feel that it was worth the time that it would have involved to determine its exact structure.

During the course of our investigations of the biosynthesis of the triterpenols found in Euphorbia lathyris we were interested in the quantities of the individual triterpenols that we were isolating off of the HPLC column. In order to accurately determine these quantities on the µg level it was necessary to calibrate the HPLC detector. This was accomplished by injecting known amounts of the individual compounds under set conditions of wavelength and flow rate. The peaks produced from these injections were integrated and a calibration constant for each compound was found. Shown in the table are the calibration constants determined for absorbance at 214 nm with a flow rate of one mL/min.

compound	constant ($mm^2/\mu g$)
lanosterol	3.6
butyrospermol	2.5
cycloartenol	0.94
24-methylene lanosterol	0.94
24-methylene cycloartenol	0.28

As it was difficult to separate butyrospermol (**5**) in sufficient quantities from E. lathyris latex for the ^{1}H-NMR work using the lanthanide shift reagent, we decided to examine the latex of a closely related specie: Euphorbia characias. The triterpenol fraction from the latex of E. characias was examined by HPLC and was found to be >90% one compound. This compound was identicle in all respects (elution time, mass spectrum and ^{1}H-NMR spectrum) to the butyrospermol that we isolated from E. lathyris latex. The purification of butyrospermol from the latex of E. characias was much easier and made it possible to obtain several mg of pure compound in a reasonable amount of time.

EXPERIMENTAL

Euphorbia lathyris latex was obtained as described previously [8]. The lipid fraction was

extracted out with hexanes and chromatographed on preparative silica gel TLC. The triterpenols isolated from the TLC plate were then separated by HPLC using an ODS column (4.6x500mm) eluted with methanol. Peaks were detected by measuring absorbance at 214 nm using a Hitachi variable wavelength detector. In order to obtain sufficient quantities for NMR spectra a semi-preparative 10x250mm ODS column was used with multiple injections.

^{1}H-NMR spectra were recorded at 250 MHz in $CDCl_3$ with TMS as internal standard. Only the chemical shifts of the methyl groups and any other distinguishable signals are reported. The spectra recorded in the presence of the lanthanide shift reagent were obtained by incremental additions of $Eu(fod)_3$ to the NMR sample. MS were obtained at 70 eV; high resolution data were within 5ppm of calculated values.

HPLC elution volumes: The most effective system for the separation of the triterpenols from E. lathyris latex was 2 ODS column in series (each 4.6x250 mm) eluted with methanol. By changing the solvent to 90:10 or 80:20 methanol:acetonitrile a better separation between 24-methylenelanosterol and butyrospermol was obtained. However, these solvents did not separate 24-methyelelanosterol from cycloartenol. The volumes shown are for two columns, eluted with 100% methanol as measured from the injection to the top of the peak. The numbers given are in mL.

compound	elution volume
euphol	31.0
lanosterol	35.5
butyrospermol	37.0
24-methylenelanosterol	38.2
cycloartenol	39.2
24-methylenecycloaretenol	41.8
unknown E	48.8

Determination of the calibration constants: 24-methylenecycloartenol(4) isolated from E. lathyris and purified by HPLC was used to make a solution of known concentration in methanol. Using a 50 µL injection loop, 100 µg of 4 was injected onto two ODS columns in series (each being 4.6x250 mm) with the detector set at 214 nm. The resulting peak had an area of 27 mm^2

which yields a calibration constant of 0.27 $mm^2/\mu g$. Two more injections of **4** at lower concentrations gave calibration constants of 0.30 and 0.26 $mm^2/\mu g$. The average of the three determinations giving a constant of 0.28 $mm^2/\mu g$. A similar set of injections was used to determine the constants for 24-methylenelanosterol and for cycloartenol. For lanosterol, a commercially available (Sigma) sample was used. This sample was only 50-60% lanosterol, the remainder being mainly 24,25-dihydrolanosterol. Previous experience with this sample had shown it to be approximately 50% lanosterol. Injection of 24 µg of this mixture produced a lanosterol peak with an area of 56.55 mm^2. As this was actually produced by 12 µg of lanosterol, the calibration constant for lanosterol was found to be 4.71 $mm^2/\mu g$. Using these constants, a sample that was mainly butyrospermol and lanosterol was injected. The masses of the non-butyrospermol compounds were calculated from their calibration constants and subtracted from the total mass injected. In this manner it was possible to inject an accurately weighed sample of butyrospermol and obtain a calibration constant of 2.5 $mm^2/\mu g$. No constant was calculated for euphol as it is present in very small quantities and its constant would be expected to be very similar to that of lanosterol.

Butyrospermol(5): ^{1}H-NMR: ∂ obs for the methyl groups: 0.742; 0.803; 0.847, d; 0.806; 0.970; 0.970; 1.683; 1.60. Other signals: 5.27, m (vinyl); 5.11, m (vinyl); 3.25, m (C3α-H). Literature values for the methyl groups[12]: 0.75, 0.81, 0.85, 0.99, 0.99, 1.69, 1.63 (note: no value was reported for the C-20 methyl group). Using 7.29 mg of **5** a series of ^{1}H-NMR spectra were recorded after incremental additions of Eu(fod)$_3$. The chemical shift data were then analyzed by a least squares regression and normalized as suggested by Itoh [12] for comparison to literature results. Chemcal shifts observed (for 1:1 molar ratio of **5** to Eu): 11.00, 10.20, 4.66, 2.14, 1.53, 1.13d, 1.64, 1.67. Lit. values[16]: 11.00, 10.18, 4.51, 2.10, 1.58, 1.16d, 1.62, 1.68. $[\alpha]_D(CHCl_3, c\ 0.52)$=-11.9°.

Lanosteryl acetate: Lanosterol (10.05 g)(Sigma, 50-60%) was placed in a 250 mL flask and 125 mL of pyridine was added. Acetic anhydride (50 mL) was added and the resulting solution heated to 70° for one hour. The reaction was cooled and ether was added followed by water.

The aqueous layer was extracted three times with ether. The ether extract was washed with a copper sulfate solution to remove the pyridine, then washed three times to remove any the blue color. The combined ether extracts were then dried and evaporated in vacuo. The product was crystallized from methanol yielding 9.42 g (85%). This material showed no unreacted starting material by HPLC, but is still a mixture of lanosteryl acetate and 24,25-dihydrolanosteryl acetate.

24-bromo-25-hydroxylanosteryl acetate: Recrystallized N-bromosuccinimide (2.08 g) was added to a THF (1400mL): water (700mL) solution of lanosteryl acetate (5.00 g) over a 40 minute period with vigorous stirring. The solution was stirred at room temperature for one hour. The volume was then reduced by about two thirds in vacuo and extracted with ether. The ether extract was washed with a sodium sulfite solution followed by water. The ether extract was dried and the solvents removed in vacuo. The TLC of the crude product showed mainly two spots (product and 24,25-dihydrolanosteryl acetate). This mixture was easily separated by preparative HPLC using the Waters Prep 500A with a silica cartridge eluted with 90:10 hexane:ethyl acetate. This yielded about 4 g of the bromohydrin and 1.7 g of the dihydrolanosteryl acetate.

24,25-epoxylanosteryl acetate: The crude bromohydrin was dissolved in methanol (100mL) and K_2CO_3 (1.6 g) was added. The reaction was stirred for 3 hours at room temperature. Water was added and the mixture was extracted with ether. The ether extract was dried and evaporated in vacuo and the residue crystallized from methanol, yielding 2.04 g. This product was >95% pure by HPLC. ^{1}H-NMR was identicle to that reported by Barton [16].

24-oxo-lanosteryl acetate: BF_3•etherate (1.3 mL) was added to the epoxide (1 g) in benzene (65 mL) at room temperature and allowed to react for 5 minutes. The solution was washed with sodium carbonate solution then with water. The benzene was then dried and evaporated in vacuo. The product is a mixture of the desired ketone and the isomeric aldehyde. The aldehyde was oxidized to the corresponding acid by PDC in DMF for 9 hours at room temperature. This acid was then removed by a basic extraction of an ether solution of the product mixture. The remaining ketone was crystallized from methanol yielding 0.52 g (55%).

Examination of this product by HPLC shows that there is <4% of the aldehyde.

24-methylenelanosteryl acetate: Triphenyl methyl phophonium iodide was synthesized by the method of Wittig [22] and was recrystallized just before use. The Wittig reagent was prepared from this using sodium hydride in DMSO. A THF (10 mL) solution of 24-oxo-lanosteryl acetate (380 mg) was added to the Wittig reagent under nitrogen and the solution heated to 50° for 22 hours. The reaction mixture was added to water then extracted with hexane. The hexane was back extracted with water then dried and evaporated in vacuo. The residue was subjected to PTLC on a 2 μm silica gel plate developed in benzene. The band at R_f=0.6 was scraped, eluted and crystallized yielding 75 mg of the desired product. ^{1}H-NMR was identicle to that described by Barton [16].

24-methylenelanosterol: The acetate (50 mg) was hydrolyzed in a refluxing solution of 2% KOH in methanol (20 mL) for 2 hours. The product was isolated by the addition of water and extraction with hexane. The hexane was evaporated in vacuo and the residue was crystallized from methanol and dried in a vacuum desiccator overnight. The final product (42 mg, 88%) showed a proton NMR spectrum identicle to that reported by Barton [16], with no sign of the acetate. HPLC of this material using an ODS column eluted with methanol shows that it contains a small amount of an impurity (the Wittig product resulting from unreacted aldehyde). The retention time for this synthetic product is identicle to that of the compound from E. lathyris and upon coinjection they appear to be one peak.

24-methylenelanosterol(7) from E. lathyris: ^{1}H-NMR δ obs for the methyl groups: 0.693; 0.811; 0.881; 0.982; 1.001; 1.012, d, J=6.9Hz; 1.040, d, J=6.9Hz. Other signals: 4.71, 1H, broad s (vinyl); 4.66, 1H, broad s (vinyl); 3.24, dd, J=11,4.8 Hz (C3α-H); part of a septet is visible at 2.21, J=6-7 Hz (C25-H). Literature values for the methyl groups[16]: 0.70; 0.81; 0.88; 0.99; 0.99; 0.99, d; 1.08, d.

Lupeol: ^{1}H-NMR, δ obs for the methyl groups: 0.759, 0.787, 0.828, 0.943, 0.966, 1.028, 1.680. Other signals: 4.690, 1H, d, J=2.0 Hz, (vinyl); 4.682, 1H, d, J=1.0 Hz, (vinyl);

3.19, 1H, m (C3α-H); 2.38, m (C19-H).

Unknown Compound E: MS (m/e, %RIC): 426, 0.71; 411, 0.10; 393, 0.05; 383, 0.09; 370, 0.06; 357, 0.12; 315, 0.16; 379, 0.11; 219, 0.19; 218, 0.32; 217, 0.15; 207, 1.26; 189, 2.70; base peak, 44. ^{1}H-NMR, δ obs for the methyl groups: 0.720, 0.760, 0.817, 0.932, 0.959, 0.970, 1.750. Other signals: 4.781, 2H, broad s (vinyl); 3.19, 1H, m (C3α-H); 2.67, 1H, m.

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CHAPTER TWO: Triterpenoid Biosynthesis in Latex

INTRODUCTION

Triterpenoid biosynthesis in Euphorbia lathyris latex has been studied by several workers [1-8]. In general, most of these papers report that acetate and mevalonate are good precursors for the in vitro biosynthesis of the triterpenoids. A wide variety of other substrates have been tried with limited success. Some in vivo studies have been attempted using the defoliated stems of seedlings or in the cotyledons [6,7]. Triterpenoid biosynthesis in E. lathyris latex is very different from rubber biosynthesis in Hevea brasiliensis. In Hevea latex, pyruvate, acetate, acetoacetate, mevalonate, hydroxymethylglutaryl-CoA (HMG-CoA), and isopentenyl pyrophosphate (IPP) were all found to be good precursors for rubber biosynthesis [9,10]. In E. lathyris latex, only pyruvate, acetate, and mevalonate were found to be good precursors for triterpenoid biosynthesis [4]. Very little is known about the regulation of the biosynthesis; there are indications that HMG-CoA reductase may be an important regulating enzyme in latex [5].

Euphorbia lathyris latex is a suspension of organelles, starch grains, and lipids [1-8]. The pH of the latex as tapped from the plant is 5.5. Whether this is the pH of the latex in vivo or not is unknown. When the latex is tapped, one is withdrawing the cell contents, or a portion thereof. The laticifer is surrounded by the vacuole which may be adding organic acids to the latex which would not normally be present in vivo. The triterpenoids can constitute up to 50 % of the dry weight of the latex [12]. The two major classes of triterpenoids in the latex are free triterpenols and the fatty acid esters of those triterpenols [4, 8,]. The structures of the triterpenols were discussed in chapter 1. The fatty acids which are found esterified to the triterpenols have been identified as palmitic, dodecanoic, decanoic, and 2,4-decadienoic [11]. It has been shown that the fatty acid portion of the triterpene esters is not labelled during incubations with acetate or pyruvate [4]. The latex also contains polyisoprenes, protein, phospholipids, inorganic salts, and starch grains [4-5]. The phospholipids were found to contain the same fatty acids as were found esterified to the triterpenols and were found to be effective donors of their fatty acids for

triterpene ester synthesis [4]. A more recent paper on the biosynthesis of the triterpene esters reported a much lower concentration of the phospholipids and suggested that the esterification reaction plays an important role in the transport of the triterpenoids into and out of the latex particles [8].

Ourisson and coworkers were the first to notice that the fraction of latex which is responsible for the biosynthesis of the triterpenoids can be pelletted fairly easily [2,3]. This method of separating the latex was further developed by Nemethy et al [4]. In this chapter we report the results obtained from a variety of biosynthetic studies ranging from the effect of various cofactors to the effects observed when the latex is treated in various ways.

RESULTS AND DISCUSSION

Centrifugation of latex at 5000xg for 10 min. produces a white pellet which is a heterogeneous mixture of various organelles and plastids [5]. As a part of our investigation of the biosynthesis of triterpenoids by latex we were interested in what biological activity this pellet contained. We were most interested in whether the pellet was capable of converting any of the early precursors such as acetate or mevalonic acid to the triterpenoids.

We attempted to answer this question by examining the incorporation of ^{14}C-sodium acetate and ^{3}H-mevalonic acid in whole latex, a 5000xg pellet, a 5000xg supernatant, and a reconstituted system made by mixing a 5000xg pellet and supernatant. The results of this experiment are summarized in table 1.

From this experiment it was concluded that the pellet does not have the ability to metabolize acetate to the triterpenoids, but it does have the ability to metabolize mevalonic acid to both the triterpenols and their esters. One must assume, therefore, that the pellet lacks the ability to convert acetate to mevalonic acid. The very small activities demonstrated by the 5000xg supernatant are probably due to incomplete pelleting during the centrifugation. The

reconstituted system lost roughly equal amounts of activity from the two substrates: having 41% of the mevalonic acid to triterpenoid activity and 45% of the acetate to triterpenoid activity of whole latex. The supernatant seems to be required to complete the acetate to triterpenoid pathway. The aqueous products from the incubation of the supernatant were examined in the hopes of finding that some portion of the acetate was metabolized to some precursor previous to mevalonic acid in the pathway. The products were examined by HPLC using both ion exchange and reverse phase columns. No radioactivity was observed in the regions corresponding to acetoacetate, hydroxymethylglutarate, or mevalonic acid.

TABLE 1

Incorporation (pmoles x mL^{-1} latex x hr^{-1})

		Acetate		MVA	ratio (MVA incorp./Ac incorp)
Whole latex	TE	21.7	TE	462	21.3
	TOH	2.5	TOH	68.3	27.3
SN	TE	5	TE	5.7	1.1
	TOH	2.5	TOH	3.3	(1.3)
Pellet	TE	0	TE	70	–
	TOH	0	TOH	87.5	–
Mixed	TE	10	TE	188	18.8
	TOH	0.8	TOH	28.3	34

TE=Triterpene Esters, TOH=Triterpenols

The 5000xg pellet loses a large amount (85%) of the ability to metabolize mevalonic acid to the triterpene esters, but actually produces more of the triterpenols. In the reconstituted system the ratio of esters to the free alcohols is returned to near the ratio observed for the whole latex control. This indicates that the supernatant contains something required for the esterification. The acyl donor for the esterification is thought to be phospholipids [4]. It was suggested that the loss in the esterification activity could be due to depletion of S-adenosyl methionine (SAM) [13]. The loss of esterification ability of the pellet could be due to either loss of SAM or depletion of a phospholipid pool. In either case, the activity is restored by the addition of the supernatant.

During our centrifugation studies, we noticed that less than 10% of the endogenous triterpenoids are normally found in the 5000xg pellet, but virtually all of the remaining biosynthetic activity is found in the pellet. In order to determine if this was due to the presence of separate sites for synthesis and storage, we incubated whole latex with ^{3}H-mevalonic acid for various times, centrifuged and looked for radioactive triterpenoids in the pellets and the supernatants. The results of this experiment are shown in table 2.

TABLE 2

Nmoles incorporated.
Rate is expressed in nmoles x mL^{-1} x hr^{-1}.

Time		Pellet	SN	Total	Rate
2.33 hrs	TE	1.7	0.39	2.09	4.48
	TOH	0.34	0.075	0.415	0.89
	TE/TOH	5.0	5.2	5.04	
6 hrs	TE	3.62	1.11	4.73	3.94
	TOH	0.54	0.21	0.75	0.6
	TE/TOH	6.70	5.29	6.31	
23 hrs	TE	8.14	1.85	9.99	2.17
	TOH	0.77	0.38	1.15	0.25
	TE/TOH	10.57	4.87	8.69	

TE=Triterpene Esters, TOH=Triterpenols

From this experiment it is clear that the majority of the newly synthesized triterpenols and their esters are in the 5000xg pellet, not the supernatant. The other interesting result is that the ratio of the esters to the free alcohols increases with time. Also a greater percentage of the triterpenols is found in the supernatant as opposed to the pellet as a function of time. One possibile explanation for this might be that the esterification process is involved in some way with transport out of the organelle which is synthesizing the triterpenoids. Another possible explanation is that there is a hydrolase present in latex that is isolated from the triterpenoids in whole latex but is exposed to them due to breakage of organelles during centrifugation. These burst organelles would not pellet at 5000xg and their contents would be found in the supernatant. This possibility would not be supported by the reconstitution experiment (see

table 1) in which the ratio of triterpenols to the triterpene esters in the reconstituted incubation was virtually the same as in whole latex, even though more than half of the total activity was lost, probably due to disruption of the organelles.

The organelle responsible for the biosynthesis appears to be the site of storage, at least for times less than 24 hours. The fact that the supernatant contains most of the endogenous triterpenols could be due to breakage of a large number of the synthetic organelles or could represent the result of a transport process much slower than we could measure. The firm conclusion to be drawn from this experiment is that the most recently synthesized triterpenoids are pelletted most readily.

It is possible to directly compare the biosynthetic rates of a sample of whole latex and the corresponding 5000xg pellet that one obtains from this sample by using both ^{14}C- and ^{3}H-mevalonic acid. Two latex samples were incubated with ^{14}C-mevalonic acid for two hours then centrifuged. The control pellet was quenched. The second pellet (B) was incubated with ^{3}H-mevalonic acid for one hour then centrifuged again. The results of this are shown in table 3.

The first point to notice is that the label used in whole latex was not further metabolized during the incubation of the pellet. The second point is that the pellet retains over 60% of the activity of whole latex with respect to the conversion of mevalonic acid to the triterpenols but shows a much lower activity for the production of the triterpene esters (ca. 35%). This is reflected in the ratio of the triterpene esters (TE) to the triterpenols (TOH) which is 5.3 for the material made in whole latex but drops to 3.0 for the material made in the pellet. This is consistent with the results shown in table 1, apparently indicating that the supernatant contains an important component for the synthesis of the triterpene esters.

During our studies of the mechanism of the formation of lanosterol and cycloartenol it was desirable to obtain labelled triterpenols in high specific activity. This can be achieved by either reducing the amount of endogenous triterpenoids that are present or by increasing the biosynthesis, or a combination of both. The centrifugation of latex that has been incubated with a radiolabelled substrate is the easiet means for removing the endogenous triterpenoids from

the majority of the labelled products. This removal of the endogenous material can be further improved by resuspending then centrifuging the resulting pellet. As the pellet itself is capable of converting mevalonic acid to the triterpenoids, the total biosynthesis can be increased by incubating the pellet for some period before centrifuging again.

TABLE 3

triterpene esters (nmoles x mL latex^{-1} x hr^{-1})

	control	B (carbon)	B(Tritium)
SN1	1.12	1.12	–
SN2	–	0.68	0.40
Pellet	3.20	2.45	1.10
Total	4.32	4.25	1.50

triterpenols (nmoles x mL latex^{-1} x hr^{-1})

SN1	0.20	0.20	–
SN2	–	0.12	0.10
P	0.60	0.48	0.40
Total	0.80	0.80	0.50
TE/TOH	5.40	5.31	3.00

SN1, SN2= first and second supernatants. TE=Triterpene esters, TOH=Triterpenols

Whole latex can carry out the conversion of mevalonic acid to the triterpenoids very effeciently for up to 12 hours and at a slower rate for up to 24 hours [4]. The pellet has a much shorter life-time with biosynthesis being linear with respect to time for 2-3 hours and slowing rapidly, ceasing at between 4 and 6 hours. A series of incubations were conducted to try to find the maximum specific activity one could obtain by varying the amount of time spent incubating as whole latex and as the pellet.

A sample of latex was divided amongst six tubes containing ^{14}C-mevalonic acid. Three of the tubes were incubated for two hours and then centrifuged. The pellets of these were then resuspended and incubated with ^{3}H-mevalonic acid for various times. The remaining three tubes were allowed to incubate for six hours before being centrifuged. Their pellets were then resuspended and incubated with ^{3}H-mevalonic acid for various times. At the end of the desired

incubation period each tube was centrifuged, the supernatant was removed and both the pellet and the supernatant were quenched. For each tube there was a first supernatant (SN1) which contained only ^{14}C, a second supernatant (SN2), and a pellet (P). The results are shown in tables 4, 5, 6.

TABLE 4

Pmoles of ^{14}C-mevalonic acid incorporated

Tube:	1	2	3	4	5	6
Times:	2+1	2+2	2+4	6+1	6+2	6+4
SN1						
TE	315	293	312	689	628	603
TOH	70	57	71	110	115	103
SN2						
TE	188	350	158	750	384	658
TOH	45	81	56	139	42	137
Pellet						
TE	356	481	725	863	1429	1067
TOH	114	95	176	146	247	206
Total						
TE	859	1125	1195	2302	2441	2328
TOH	230	233	303	395	405	446

TABLE 5

Pmoles of ^{3}H-mevalonic acid incorporated

Tube:	1	2	3	4	5	6
Times:	2+1	2+2	2+4	6+1	6+2	6+4
SN2						
TE	68	234	182	90	94	181
TOH	22	54	58	27	13	40
Pellet						
TE	126	360	1048	99	314	299
TOH	65	82	184	46	91	77
Total						
TE	194	594	1230	189	408	480
TOH	86	136	243	73	104	117

TABLE 6

Total pmoles of mevalonic acid incorporated (whole+pellet activity)

Tube:	1	2	3	4	5	6
Times:	2+1	2+2	2+4	6+1	6+2	6+4
SN2						
TE	256	584	340	840	478	839
TOH	67	135	114	166	55	177
TE+TOH	323	719	454	1006	533	1016
Pellet						
TE	482	841	1773	962	1743	1366
TOH	179	117	360	192	338	283
TE+TOH	661	1018	2133	1154	2081	1649
Total incorporation into all fractions:						
TE	1053	1719	2425	2491	2849	2808
TOH	316	369	546	468	509	563
TE+TOH	1369	2088	2971	2959	3358	3371

The first observation one can make from this experiment concerns the variability from tube to tube. In this experiment there is very little variation in the incorporation into the first supernatant of each set of three tubes. Also the total incorporations from whole latex in the six hour group (tubes 4-6) are surprisingly similar to one another. The average ^{14}C incorporation for those three tubes is 2772 pmoles. For the 2 hour set (tubes 1-3) the average total ^{14}C incorporation is 1315 pmoles. The rates of biosynthesis calculated for these incubations are shown in table 7 .

TABLE 7

Tube	1	2	3	4	5	6
whole latex	544	679	749	450	474	462
pellet	280	365	369	262	256	149
% of whole	51	54	49	58	54	32

Biosynthetic rates ($pmoles x hr^{-1} x mL\ latex^{-1}$) for whole latex and the corresponding pellets.

There is a slight variation in the biosynthetic rates of the two hour time points. This is

probably due to uneven distribution of the latex as can be seen by the fact that the rates observed for the pellets has an almost identicle variation. The percent recovery of activity for each tube is very good and fairly constant except for tube 6; each pellet has almost exactly half of the activity of the whole latex from which it was made. One can conclude from this that after sitting for six hours as whole latex, the activity of the pellet drops off more dramatically as a function of time; being drastically reduced after two hours.

One of the possible explanations for the lower ratio of triterpene esters to free triterpenols observed in the pellets as opposed to whole latex involved the depletion of SAM. In this experiment all of the pellets were incubated in the presence of 50 μM SAM. Table 8 shows the ratios (TE/TOH) observed for the material made by whole latex and by the pellet.

TABLE 8

TE/TOH ratios for whole latex synthesis and for pellet synthesis

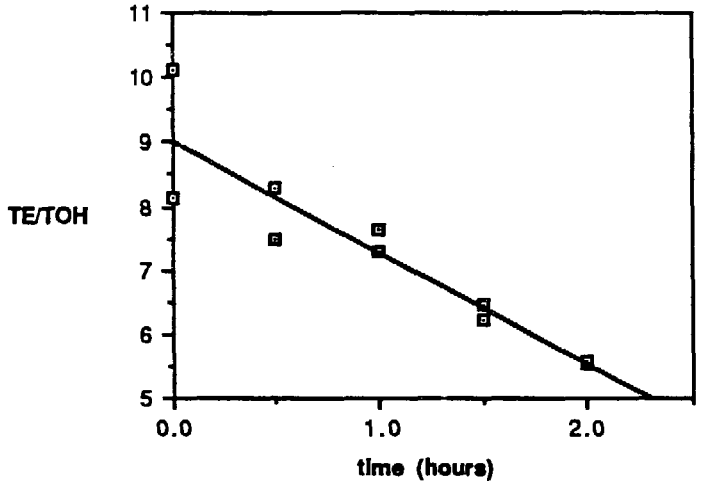
tube	1	2	3	4	5	6
Whole latex	3.73	4.83	3.94	5.83	6.03	5.22
Pellet	2.26	4.37	5.06	2.59	3.92	4.10

The TE/TOH ratios for the whole latex material are not as high as one might normally find but lie within the range observed in numerous incubations. Previously we have seen that the TE/TOH ratio for pellet tends to be considerably lower than that of whole latex (see tables 1 and 2). In this experiment most of the pellets maintain fairly good ratios. The pellet of tube 3 even has a higher ratio than that of the whole latex. This would seem to indicate that the addition of SAM does help to stimulate the conversion of mevalonic acid to the triterpene esters. It is also of interest that the TE/TOH ratios from whole latex of the six hour tubes are much higher than those of the two hour tubes. This could have many explanations. Perhaps the most simple explanation is that the organelles making the triterpenes are losing activity with time faster than the esterification enzyme.

The biosynthesis of the triterpenoids in latex was shown to be sensitive to changes in the osmotium; the effect of the osmoticum was shown to be greater for the TE than for the TOH [4].

An experiment was conducted to examine the effect of osmoticum on the TE/TOH ratio of triterpenoids that were made in whole latex, then pelleted and exposed to very low osmotic pressure for various times. The latex was centrifuged in buffer containing osmoticum but the pellets were solubilized into medium without osmoticum. The resulting TE/TOH ratios as a function of the time of incubation of the pellets are plotted in figure 1.

FIGURE 1



The labelled triterpenols produced from incubations of the latex can be separated by HPLC and the radioactivity in each compound can be determined by scintillation counting. We attempted to study the mechanism by which the individual triterpenols were made by determining the amount of label present in each compound as a function of time. It was hoped that one would be able to observe a product-precursor relationship for certain compounds. One might expect that 24-methylenecycloartenol, being made from cycloartenol, would show a lag time before it appearred to be labelled and that cycloartenol would be quickly labelled at first, then reach some steady-state level. A number of time dependent incubations were performed

using whole latex and 5000xg pellets. In the incubations in whole latex using mevalonic acid as the substrate it was observed that the individual compounds are linear with time; the percent distribution of the label did not change as a function of time [13]. Similar results were obtained when these experiments were repeated in both whole latex and in the 5000xg pellet. We assumed this was due to the presence of a rate-limiting step prior to the cyclization of squalene oxide. Thus we were observing a rate due to this slow step followed by a distribution into the various compounds which was much faster and was not subject to kinetic control.

In order to obtain more meaningful kinetic evidence, several pulse-chase type experiments were performed. In some of these we were interested in the TE/TOH ratio as well as the distribution into the individual TOH. One of the experiments involved incubating five samples of latex for one hour with ^{3}H-mevalonic acid then centrifuging. The pellets produced from these centrifugations were washed with fresh buffer, centrifuged again, resuspended and allowed to incubate for various times. The supernatants of each tube were combined and analysed in the same manner as the pellets. The results of the TLC analysis are shown in table 9.

Perhaps the most striking feature in table 9 is the wide variation in the total activity of each sample. This was not uncommon in latex experiments. We have attributed this to the difficulties involved in pipetting small volumes of a heterogeneous mixture. Some tubes will receive a higher concentration of the particles than others. In this case it appears that tube three has substantially higher activity while tube four is quite low. Variations as large as this make it difficult to analyze the data directly. If one plots the pmoles of triterpenols in each pellet as a function of time it simply reflects the variation in activity. The TE/TOH ratio provides a simple normalization of the individual tubes. Plotting this ratio as a function of time for the pellets provides a more coherent picture of what is taking place (se figure 2). From figure 2 it is readily apparent that the amount of triterpene esters is dropping compared to the free alcohols.

TABLE 9

Tube	1	2	3	4	5
Time	0	0.5	1	1.5	2
Supernatant (pmoles):					
TE	30.0	65.1	124	17.2	36.9
TOH	8.4	10	21.4	4.9	7.0
Total TE+TOH	38.4	65.1	145	22.1	43.9
TE/TOH	3.57	6.38	5.79	3.51	5.27
Pellets (pmoles):					
TE	125	110	187	66.4	97.1
TOH	15.4	14.7	24.5	10.7	17.4
Total TE+TOH	140	125	212	77.1	114
TE/TOH	8.12	7.48	7.63	6.21	5.58
Total incorporation (pmoles):					
TE	155	175	311	83.6	134
TOH	23.8	24.9	45.9	15.6	24.4
Total (TE+TOH)	179	200	357	99.2	158
TE/TOH	6.51	7.03	6.78	5.36	5.49
% of total in the pellet:	78	62	59	78	72

The triterpenols from this experiment were eluted from the silica gel and examined by HPLC. The amount of radioactivity in each compound was quantified and is expressed as a percent of the total radioactivity in that tube (see table 10). It is difficult to determine if there is any interconversion of the triterpenols taking place in this experiment. The percentage of the label in lanosterol increases with time but it is unclear what is decreasing. One would expect based upon Ourrison's work that cycloartenol would decrease with time as lanosterol and 24-methylenecycloartenol increase [3]. It is most likely that the changes in distribution observed are due to variations in the five samples and are not due to interconversion.

FIGURE 2

Effect of time on the TE/TOH ratio

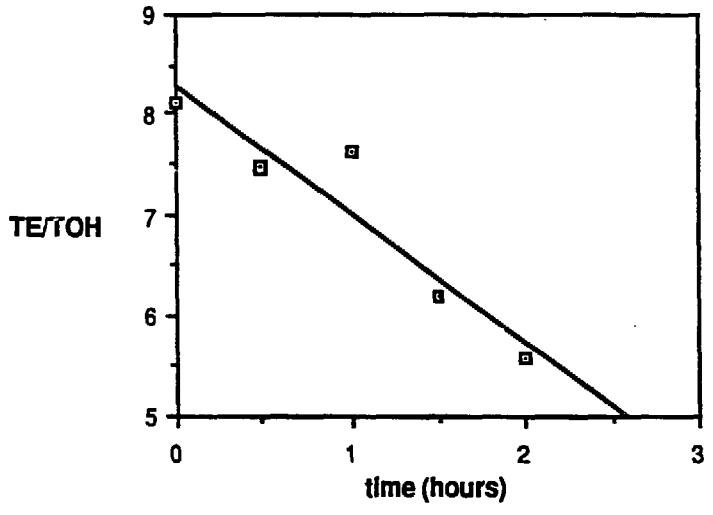


TABLE 10

Percent Distribution of Label in the Triterpenols

Time:	0	0.5	1.0	1.5	2.0
Lanosterol	7.7	10.7	10.9	13.0	15.0
Butyrospermol	26.2	20.8	20.8	17.3	27.0
24-mL	6.3	6.2	6.3	4.8	5.2
Cycloartenol	6.5	7.0	7.6	8.2	8.0
24-mCA	42.3	43.6	43.9	42.6	32.8
Unknown E	11.0	11.6	10.6	14.2	12.1

A series of similar experiments was conducted in which the whole latex was labelled with one isotope (^{3}H or ^{14}C) of mevalonic acid then centrifuged and the pellet incubated with the other isotope. Each experiment was designed to test the effect of one or more variables on the distribution of label in the triterpenols. The first experiment involved three samples of latex each of which was incubated with ^{14}C-mevalonic acid for the first two hours of the experiment. To one tube was added ^{3}H-mevalonic acid. This tube was allowed to incubate for another two hours. As no attempt was made to remove the ^{14}C-mevalonic acid it was present for the entire four hours. The other two tubes were centrifuged and their supernatants removed. One pellet

was resuspended and incubated with ^{3}H-mevalonic acid. The other was first washed with fresh buffer and centrifuged again before being resuspended and incubated with ^{3}H-mevalonic acid. All three tubes were quenched at four hours. The effect of these treatments on the percent of the labels found in each triterpenol are shown in table 11.

TABLE 11

Percent of each label [Carbon(C) or Tritium(T)] in the individual triterpenols

	whole		pellet		washed Pellet	
	^{14}C	T	^{14}C	T	^{14}C	T
Lanosterol	8.8	5.4	18.0	5.2	16.3	7.0
Butyrospermol	24.8	25.1	29.1	27.5	22.8	25.6
24-mL	2.1	3.4	4.7	4.9	2.1	1.2
Cycloartenol	10.9	12.9	7.0	6.8	6.9	23.7
24-mCA	47.6	49.8	33.8	47.6	45.3	38.5
Unknown E	5.7	3.2	7.5	7.9	6.5	4.0

The results of this experiment show several interesting features. First, comparing the carbon and tritium distributions for each sample one finds a striking difference in the amount of lanosterol present; the levels produced from the tritiated mevalonic acid being much lower than those of the ^{14}C-mevalonic acid. Secondly, comparing the level of ^{14}C-labelled lanosterol in the three tubes one finds it is much higher in the material obtained from centrifugation. From this particular experiment it is not possible to say whether that is due to there being more lanosterol in the organelles that precipitate or if it is due to some interconversion of the other triterpenols to lanosterol that takes place more rapidly in the pellet. This latter hypothesis seems unlikely in light of the low levels of lanosterol being synthesized by the pellet (as seen from the tritiated label). The effect of washing the pellet seems to have a small effect on the triterpenols made in the whole latex which are pelleted. We observed an increase in the level of 24-methylenecycloartenol as compared to the other triterpenols with butyrospermol showing the greatest decrease. The major change which is observed in the pellet biosynthesis in the washed and unwashed pellets is the levels of cycloartenol and 24-methylenecycloartenol. In each pellet the two compounds account for over 50% of the total label. In the unwashed pellet most of the material is in 24-methylenecycloartenol. In the washed pellet the level of

24-methylenecycloartenol is much lower while that of cycloartenol is much higher. If one of the cofactors that is present in the supernatant is SAM then these results can easily be interpretted as showing the effect of two levels of depletion of the SAM pool. The unwashed pellet has enough SAM present to maintain its normal methylation rate; the washed pellet has much less SAM and shows an accumulation of cycloartenol and a lowering of the amount of 24-methylenecycloartenol. These results are parallelled by those for lanosterol and 24-methylenelanosterol.

The second experiment of this type involved five samples of latex incubated for two hours with ^{3}H-mevalonic acid then centrifuged and their pellets incubated for various times with ^{14}C-mevalonic acid. In the short time points there was not enough label present from the pellet biosynthesis to examine the individual triterpenols; only the longest time point, four hours, had sufficient ^{14}C label to allow the individual compounds to be analyzed. The percent distribution determined from these samples is shown in table 12.

TABLE 12

Percent Distribution as a Function of Time

time (hours):	0.75	1.5	2.5	4	4 (pellet)
lanosterol	14.1	15.0	14.1	14.8	5.7
Butyrospermol	24.4	22.6	22.2	21.5	27.2
24-mL	3.2	3.7	4.0	3.6	1.9
Cycolartenol	6.0	5.8	4.7	5.8	15.0
24-mCA	40.4	41.2	41.2	41.0	42.1
Unknown E	11.8	11.7	13.7	13.2	8.1

There is no real change in the distribution pattern observed in this experiment as a function of time. The compounds which were made in whole latex and pelletted have retained the same ratio over the duration of this experiment. The triterpenols made by the pellet are, on the other hand, quite different. The amount of lanosterol produced is considerably lower while the amount of cycloartenol present has increased. The mechanism by which the relative amounts of the triterpenols is controlled is not known. It is apparent that centrifugation can have a pronounced effect on these relative amounts.

The third experiment of this set examined the effect of addition of supernatant to the

pellets. This was done by labelleing whole latex with ^{14}C-mevalonic acid for two hours then centrifuging. Cold 100,000xg supernatant was added to half of the pellets and all of the pellets were incubated with ^{3}H-mevalonic acid for various times. Table 13 shows the relative amounts of the triterpenols which were made in whole latex. Table 14 shows the relative amounts of the triterpenols made in the pellet.

TABLE 13

Percent Distribution of the Triterpenols Made in Whole latex

time (hours):	0.5		1.0		1.5		2.0	
	P+SN	P	P+SN	P	P+SN	P	P+SN	P
Lanosterol	11.6	10.6	10.3	13.2	12.2	12.8	10.5	11.9
Butyrospermol	21.6	18.8	24.9	21.1	21.0	18.5	19.9	15.6
24-mL	3.3	2.8	2.3	3.3	3.8	3.3	3.6	4.3
Cycolartenol	6.0	5.7	6.5	6.2	5.5	5.9	5.6	5.4
24-mCA	43.5	47.7	42.6	43.8	43.7	45.2	46.1	49.2
Unknown E	14.0	14.5	13.3	12.5	13.7	14.3	14.3	13.6

TABLE 14

Percent Distribution of the Triterpenols Made by the Pellet

time:	0.5		1.0		1.5		2.0	
	P+SN	P	P+SN	P	P+SN	P	P+SN	P
lanosterol	4.8	2.3	3.2	2.7	4.3	8.3	3.9	6.0
Butyrospermol	24.6	22.0	28.7	23.5	23.8	20.5	19.1	17.9
24-mL	4.4	3.1	2.7	4.4	4.7	4.9	5.4	4.6
Cycolartenol	5.1	7.5	5.1	6.2	6.0	6.5	4.5	6.9
24-mCA	54.1	56.7	50.7	55.1	54.1	50.0	54.0	55.8
Unknown E	7.0	8.4	9.6	8.1	7.1	9.8	13.0	8.7

In the previous experiments we did not observe any interconversion taking place in the pellet. That same trend holds true for the results shown in table 13. The addition of cold 100,000xg supernatant seems to have no effect on that aspect. The differences between the pellets alone and the pellets with the cold supernatant added to them are very slight and are simply due to minor variations in the latex samples. The distribution of the triterpenols made by the pellet is quite different from that of those made by the whole latex, just as we have seen

previously. The level of lanosterol is considerably lower. In these samples the pellet appears to have maintained a very good methylating activity. The levels of 24-methylenecycloartenol are higher in the pellet synthesized material than in that of the whole latex.

A number of attempts were made to isolate the organelle which is responsible for the biosynthesis of the triterpenoids in latex. Most of the methods utilized involved centrifugation, either in buffer with an osmotic pressure of 0.4 M or through a medium containing various amounts of Percoll. Percoll is a suspension of PVP coated silica. Percoll can be used to form a continous gradient, a step gradient, or just as a "cushion" at the bottom of a centrifuge tube to prevent irreversible adsorbtion on the bottom of the centrifuge tube. Dr. G. Piazza tried several gradients both step and continous which provided the basis for our current attempts. The above section dealt with the effects of centrifugation at 5000xg for short times.

Several of our earliest attempts to isolate the organelle were assayed using radioactive acetate as the substrate. As can be seen from table 1, acetate is not incorporated into the triterpenols in the pellet. These experiments were of no real value other than to prove that acetate is not utilized by the pellet and will not be discussed here.

Experiments performed by other workers had shown that the organelle which is responsible for the biosynthesis would not pellet through 55% Percoll but would sediment through 18% Percoll. In an attempt to test these earlier results and determine if they were reproducible we attempted to repeat a procedure used by Dr. G. Piazza. Latex was mixed with 90% Percoll to form a suspension of about 57% Percoll. This was transferred to a centrifuge tube and 55% Percoll, 18% Percoll, and 0% Percoll (centrifugation buffer) were layered on top. This tube was centrifuged at 4000xg and each layer was removed starting at the top of the tube. From the region near the 18/55 interface, 200 µL was removed. As Percoll had been shown to inhibit the biosynthetic activity, this sample was diluted with 800 µL of reaction buffer and incubated with ^{3}H-mevalonic acid for one hour. The TLC results of the incubation are shown in table 15.

TABLE 15

TLC Band	dpms present
TE	11580
TE-	540
TOH	1460
TOH-	412

The radioactivity in the triterpene ester peak corresponds to 1.05 nmoles. This would be a very good incorporation. The presence of radioactivity on the TLC plate in the triterpenol or triterpene ester region is usually a reliable measure of the incorporation into those compounds. When using an unusual method for handling the latex it is best if one examines the radioactivity more closely. The triterpenols from this experiment were examined by HPLC and the radioactivity was found to coelute with the mass peaks of the individual compounds. The triterpene esters were hydrolyzed and the resulting triterpenols were purified by TLC and examined by HPLC to verify that the radioactivity in this band also coeluted with the mass peaks of the triterpenols. This experiment verified that a fraction having very little protein [14] and very small quantities of the triterpenoids (as observed by HPLC calibration) can be isolated that maintains a fair amount of biosynthetic activity.

We had shown that the 5000xg pellet retains the majority of the biosynthetic activty while having only a small fraction of the endogenous triterpenols. If this pellet could be purified on the Percoll step gradient in the same manner as whole latex, it might prove to have an even lower level of endogenous triterpenoids. This was of interest to us for our investigation into the mechanism of the formation of lanosterol. An experiment to test the activity of the material at the 18/55 interface of a Percoll gradient formed from a 5000xg pellet was conducted. The TLC results of this experiment are shown in table 16.

TABLE 16

TLC Band	dpms present
TE	632
TE-	142
TOH	2330
TOH-	222

The incorporation into the triterpene esters was extremely low and that into the triterpenols is not much higher. It is in cases such as this that one should be very suspicious of the TLC results. The triterpenol region of the TLC plate was scraped and eluted and examined by HPLC. The mass peaks contained no radioactivity. The 18/55 interface of the gradient had no biosynthetic activity.

Another experiment to try to purify the triterpenoid biosynthetic activity using a Percoll step gradient was attempted. A 5000xg pellet was suspended in 90% Percoll as 55%, 18% and 0% Percoll were layered on top. This step gradient was centrifuged and the region at the interface of the 55% Percoll and the 18% Percoll was removed and divided in half. One portion was mixed with reaction buffer to dilute the Percoll and centrifuged again. The bottom portion of that tube was then mixed with reaction buffer and incubated with mevalonic acid. The other portion of the interface was incubated with mevalonic acid directly without a second centrifugation or any dilution of the Percoll. The TLC results of these two incubations are given in table 17.

TABLE 17

	18/55 Interface	Pellet from 18/55 Interface
TE	999	10,480
TE-	126	681
TOH	414	4297
TOH-	138	161

The 18/55% interface from the Percoll gradient in which the Percoll was not diluted had very poor activity (on the order of 74 pmoles). The aliquot of the interface which was centrifuged a third time and the resulting fraction diluted with reaction buffer did much better (total biosynthesis of 260 pmoles). As we had had problems with the reliability of our TLC results when using Percoll, the triterpenene esters from this experiment were eluted, hydrolyzed, and examined by HPLC. The triterpenols resulting from the hydrolysis were found to contain no label. There was virtually no biosynthetic activity in either fraction. One of the conclusions drawn from this experiment is the confirmation that Percoll has an inhibitory effect

on the biosynthesis of the triterpenoids.

Although we were having difficulty in isolating any activity from the Percoll gradient we thought it might be possible to isolate a storage organelle containing the recently synthesized triterpenoids. Two samples of latex were incubated with ^{14}C-mevalonic acid for one hour. They were then centrifuged at 5000xg and the supernatants removed. The pellet from tube 1 was resuspended in reaction buffer and incubated with tritiated mevalonic acid. The pellet of tube 2 was resuspended in 90% Percoll and placed at the bottom of a step gradient of 55%, 18%, and 0% Percoll. This gradient was centrifuged. The 18/55% interface was removed, diluted with reaction buffer, and incubated with tritiated mevalonic acid. The results of this experiment are given in table 18.

TABLE 18
pmoles incorporated

	Tube 1		Tube 2	
	^{14}C		^{3}H	^{14}C
^{3}H				
TE	1126	4640	256	491
TOH	880	380	300	200
Total	2006	5020	556	691
5000xg Supernatants:				
TE	313		268	
TOH	81		55	
Total	394		323	

Hexane Solubles From the Percoll Gradient:

Top	247
Middle	70
Lower	17
Pellet	767
Total	1101

There are several interesting points raised by this experiment. First, the two latex samples had very similar biosynthetic activities and the cuts from the gradient show how that activity was distributed. The pellet has most of the activity with the top and 18/55% interface having smaller, about equal amounts of the hexane soluble label. The aqueous soluble ^{14}C label was

distributed fairly equally between the top, middle and lower regions with a smaller amount in the pellet. If the organelle responsible for the biosynthesis is at the 18/55% interface, a large portion of those organelles must not be in tact and their contents are either floating or are staying at the bottom.

The results shown in table 18 are based upon counting of the TLC bands. The triterpenols from both tubes were examined by HPLC in order to verify that the radioactivity was indeed in the triterpenols. The triterpene esters were also hydrolyzed and the resulting triterpenols were examined by HPLC to verify that they too were labelled. The most striking difference between the pellet and the 18/55% interface, both in the presence of the compounds made in whole latex and in the biosynthetic activity, is in the triterpene esters. With respect to the compounds made in whole latex the interface has lost about half of the triterpenols, but has lost more than 75% of the triterpene esters (compared to the 5000xg pellet). The difference in the compounds synthesized by these two tubes after their treatments is even more dramatic. The interface made about 20% of the triterpenols of the pellet, but only about 6% of the triterpene esters.

After many attempts at obtaining a fraction off of the Percoll gradient that would retain a large amount of activity, we have only been able to get very poor activities. Percoll itself seems to be inhibitory to the biosynthesis. For our purposes, the 5000xg pellet was a more useful fraction. The pellet normally retains 40-60% of the activity of the whole latex and is viable for at least four hours. It contains much less that 10% of the endogenous triterpenols (often less than 5%) and is the site of storage for recently made triterpenoids. Further attempts at using Percoll as the medium for the purification of the organelle responsible for the biosynthesis did not seem warranted.

The latex of Euphorbia lathyris when tapped from the plant has a pH of 5.5 [1,4]. During our early centrifugation studies, phthalic acid was chosen as a buffer to keep the pH at 5.5. After a considerable number of experiments we began to suspect that phthalate might have some inhibitory effect. We replaced phthalate with MES and tested whether MES had an

inhibitory effect on the biosynthesis in the 5000xg pellet. A pellet without a pH buffer in the reaction buffer was compared to one with 20 mM MES. The tube without buffer produced 2.8 nmoles, that with the MES produced 8 nmoles. In all experiments thereafter, MES was the buffer used.

The reaction buffer used for most of the latex incubations in this investigations contained some divalent cation. For most of the work, Mg^{++} and/or Ca^{++} was used. It was suggested by Dr. G. Piazza that Mn^{++} might be stimulatory as compared to Ca^{++}. One method for removing Ca^{++} from biological reactions is to chelate it with EGTA. The effect of the divalent cations added to the incubation was examined by centrifuging three latex samples in buffers containing different divalent cations. The control tube and the second tube had only 5 mM $MgCl_2$, the third tube had 30 mM Ca^{++} during centrifugation. The pellet from the second tube was taken up in buffer containing 10 mM Mn^{++} ion. The third pellet was taken up in buffer containing 10 mM Mn^{++} ion and 10 mM EGTA to chelate any remaining Ca^{++} ions. Using the first tube as the control and expressing the incorporation into the other two tubes as a percent of this control, the second tube, Mn^{++} alone, had 183% of the control. The tube which was centrifuged in the presence of Ca^{++} and incubated in the presence of EGTA had only 51% of the incorporation of the control. Whether this was due to the presence of Ca^{++} in the centrifugation, or the inhibition by EGTA was not of interest to us. We were only interested in the increased activity with the addition of Mn^{++} ion. All incubations of the pellet thereafter were done in the presence of Mn^{++}.

The osmotic sensitivity of the biosynthetic activity found in latex has been observed many times during the course of this and other studies [2,4]. The conversion of mevalonic acid to the triterpenoids requires several enzymes and cofactors. All of these must be inside the organelle or capable of being transported inside in order to protect the phosphorylated intermediates from the high levels of phosphatase activity found in the latex. It is reasonable to assume that when the organelle is ruptured either osmotically or by some other physical means that the organization which is maintained in the organelle is lost. The activity of the individual enzymes

may not be destroyed by the disruption of the organelle. We were interested in the conversion of squalene to the triterpenols. Incubations of squalene in the whole latex and in 5000xg pellets had not been successful. Even with the addition of detergents to the medium, there was no incorporation into the triterpenols. The lack of incorpoartion of squalene into the triterpenols may have been due to a transportation problem. Squalene would not normally be found in significant levels as a free compound in the medium and there is no reason to expect that the organelle would have a mechanism to transport squalene in from the medium. If the organelle were burst open that might make the enzyme responsible for the epoxidation-cyclisation accessible to the squalene. This was attempted using 5000xg pellets which were burst either osmotically or by sonication. Neither treatment produced a pellet which showed any incorporation into the triterpenoids from either squalene or mevalonic acid.

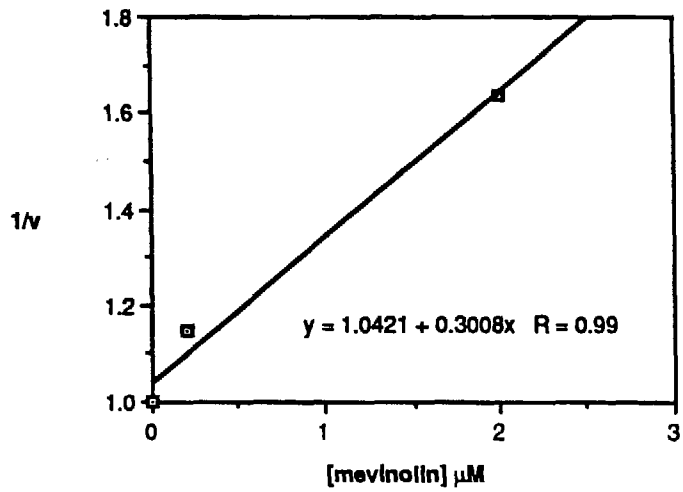
The 5000xg pellet contains a very small percentage of the total terpenoids of the whole latex. This was the most simple form of delipidation that we were able to find which gave a viable biosynthetic pellet. We attempted several other methods of delipidation to remove as much as possible of the endogenous triterpenoids from the biosynthetic organelle. Centrifugation in medium other than 0.4 M osmoticum had a destructive effect. An osmoticum of 0.8 M is totally destructive to the biosynthetic activity.

There are several methods of delipidation in the literature which involve non-aqueous techniques. We attempted one method which involved iso-propyl ether and n-butanol as solvents for the removal of lipids. A sample of latex was divided into four microfuge tubes and diluted with reaction buffer. Tube one was the control, diluted latex. Tube two was extracted with 80:20 diisopropyl ether: butanol and the remaining aqueous layer was incubated with mevalonic acid. Tube three was the 5000xg pellet control; it was simply centrifuged, solubilized, centrifuged again, and incubated with mevalonic acid. Tube four was centrifuged, the pellet was extracted with 80:20 diisopropyl ether: butanol, and the aqueous layer centrifuged again. The resulting pellet was then incubated with mevalonic acid. The washed pellet had 46% of the activity of whole latex with respect to the triterpenols and 13% with

respect to the triterpene esters. Neither of the tube which were exposed to the organic solvents showed any incorporation into the triterpenoids.

The enzyme HMGCoA Reductase has been found to play an important role in regulating terpenoid biosynthesis in most organisms examined so far [15]. In Euphorbia lathyris, C. Skrukrud has found evidence of this enzyme being important for the regulation of triterpenoid biosynthesis [5,16]. HMGCoA reductase can be inhibited by the compound mevinolin. We tested the effect of mevinolin on the biosynthesis of triterpenoids from acetate in whole latex. Three latex samples were incubated with ^{14}C-acetate and ^{3}H-mevalonic acid and varying concentrations of mevinolin. The incorporation into the triterpenols and the triterpene esters of each tube from acetate and from mevalonic acid were expressed as a ratio. The ratio of each tube was then compared to the ratio found in the control tube and a Dixon plot was made of 1/relative velocity from acetate vs. the concentration of mevinolin. The resulting plot is shown in figure 3.

FIGURE 3



The concentration at which one would observe 50% inhibition, the I_{50} was found to be 3.2 µM from the best-fit line. This is quite a bit higher than the values found in several other systems, but as this is actually a comparison of the activity of mevalonic acid going to the

triterpenoids vs the activity of acetate going to the triterpenoids, this is different from most inhibitor studies that have been done using mevinolin [15]. What we are actually observing is a rate for several steps that are undergoing overall inhibition by mevinolin.

CONCLUSION

We have learned a great deal about the biochemical properties of the latex with respect to the biosynthesis of triterpenoids. Our primary purpose was to further our understanding of the biosynthesis of the triterpenoids in latex. We discovered that many things, both chemical and physical, were inhibitory to that biosynthesis. The 5000xg pellet has a much shorter lifetime of its biosynthetic capability than the whole latex. This pellet does not possesses the ability to convert acetate to the triterpenoids. There seems to be something in the supernatant which is very important for the biosynthesis of the triterpene esters. This factor could sometimes be replaced by SAM. We were unable to observe any interconversion of the triterpenols and the triterpene esters in either the pellet or in whole latex. We were also unable to observe any interconversion of the individual triterpenols in whole latex or in the 5000xg pellet. In our experiments with non-traditional biochemical techniques we learned that it was important to verify incorparation by some means more definite than TLC. Lastly, it was possible to show that the biosynthesis of the triterpenes from acetate is subject to inhibition by mevinolin; showing that HMG-CoA reductase is accessible to mevinolin.

EXPERIMENTAL

General: The method for obtaining latex, the sources of the substrates and materials used, and the chromatographic techniques have all been described elsewhere [4,5]. Incubations were quenched by the addition of acetone and the aqueous solution was extracted with hexanes several times. The combined hexane extracts were evaporated to dryness under a stream of nitrogen and chromatographed by TLC. The solvent system used for TLC was 3:1 ether:petroleum ether unless otherwise noted. For determination of the results by TLC alone,

the band corresponding to the desired compounds was scraped from the plate into a scintillation vial, water (5 mL) was added and the vial was sonicated. Aquassure (NEN) was added to the suspension of silica in water and the resulting suspension was shaken before being counted. All radioactive assays were performed using liquid scintillation counting in Aquassure. The counting efficiency was determined using an externel standard and a calibration curve generated using quenched standards of both ^{3}H and ^{14}C obtained from Packard Instruments. Centrifugation was at 5000xg unless otherwise noted and was normally performed using a Beckmann benchtop microfuge with 1.5 mL microfuge tubes. Centrifugation buffer was a solution of 0.4 M sorbitol; 50 mM phthalate or MES, pH=5.5; 5 mM $MgCl_2$; 5 mM DTE; and, in the later experiments, 10 mM $MnCl_2$.

Incubation of Acetate in the Pellet: Four reaction tubes each containing U-^{14}C-sodium acetate (0.4 µmole, 3.3 µCi) and ^{3}H-mevalonic acid (0.3 µmole, 10 µCi) were prepared and labelled A, B-SN, B-P, and C. Latex (200 µL) and centrifugation buffer (200 µL) were added to each of three centrifuge tubes. Two of these (tubes B and C) were centrifuged (5000xg, 10 min.) while the third (tube A) was allowed to sit at room temperature. The supernatant of tube B was transferred to a reaction tube (B-SN). The pellet was solubilized in buffer (400 µL) and transferred to a reaction tube (B-P). The pellet and supernatant of tube C were mixed and transferred to a third reaction tube (C). The whole latex which was sitting in tube A was then transferred to reaction tube A and all four tubes were allowed to incubate for 3 hours. The incubations were quenched and worked up in the usual manner. Half of each of the bands corresponding to the triterpenols and the triterpene esters was counted in the gel phase, the other half was left in tact in case it was needed for HPLC analysis. The results of the counting are shown in table 1.

The aqueous phase from the 5000xg supernatant was acidified with conc. HCl to a pH less than one and blown to dryness under a stream of nitrogen. One would expect that a considerable portion of any acetic acid present would evaporate under these circumstances. This was desirable in order to reduce the high background produced by the ^{14}C-acetate used.

Most of the mevalonolactone should not be lost during this procedure. The residue which remained in the tube was spotted onto a TLC plate and developed in 2:1 $CHCl_3$:acetone. This is a TLC system in which mevalonolactone is known to have an R_f of about 0.3-0.4. The plate was cut into bands and virtually all of the ^3H was found in band 3, comigrating with mevalonolactone. There was a very small amount of ^{14}C radioactivity at that same position. Whether or not the dpms detected in the ^{14}C channel were actually from ^{14}C or if they were from ^3H spillover is difficult to determine. There were 958,173 dpm in the ^3H channel and only 1098 dpm in the ^{14}C channel. The dpm in the ^{14}C channel represent only 0.1 % of those in the ^3H channel. To try to answer the question an aliquot of this fraction was chromatography using an ODS column eluted with 10mM KH_2PO_4 at pH=2.6. The column effluent was collected using a fraction collector. The ^3H eluted mainly in three fractions around the mass peak of mevalonolactone. The ^{14}C channel of the scintillation counter showed less than background dpm. The program which was attempting to correct for the ^3H dpm spillover into the ^{14}C channel had overcorrected. It was not possible to definitely state that there was no conversion of acetate to mevalonate, if there was any conversion it must have been very slight.

Distribution of Labelled Triterpenoids by centrifugation as a function of time: Three centrifuge tubes were prepared with ^3H-mevalonic acid (50 µCi/µmole, 0.2 µmole) and DTE (4.0 µmole). Latex (200 µL) was added to each tube and they were allowed to incubated for 2.33 hrs., 6 hrs., and 23 hrs. At the end of the incubation period each tube was centrifuged and the supernatant removed. Acetone was added to the supernatant and the pellet was solubilized in acetone and water. After the normal extraction and TLC the triterpenols from the pellets were examined by HPLC in order to quantify the specific activity of the products. The areas of the absorbance peaks of the HPLC detector were determined and multiplied by the calibration constant for each compound in order to determine the mass present. The eluent from these peaks was radioassayed to determine the amount of radioactivity incorporated into the triterpenols. From these two values one can obtain a ratio of the mass of newly synthesized material to the endogenous material. The results for this experiment are shown in table 19.

TABLE 19

Time (Hours)	Total Mass Synthesized (ng)	Total Mass Endogenous (µg)	Ratio $x10^3$
2.33	103	520	0.20
6.0	185	800	0.23
23	258	550	0.47

Dual Label Comparison of Whole Latex and Pellet Activities: Three incubation tubes were prepared with ^{14}C-mevalonic acid (0.40 µmole, 10 µCi/ µmole) and DTE (2.0 µmole). Latex (200 µL) was added to each tube and they were allowed to incubate for 2 hours. The tubes were then centrifuged and the supernatants removed. The pellet of tube A was cut out and quenched with acetone. The pellet of tube B was solubilized in centrifugation buffer (200 µL), transferred to a clean centrifuge tube and centrifuged again. The resulting pellet was cut out and quenched with acetone. The pellet of tube C was solubilized in centrifugation buffer (200 µL) and transferred to a tube containing ^{3}H-mevalonic acid (0.4 µmole, 25 µCi/µmole). This tube was incubated for one hour, centrifuged and the supernatant removed. The pellet was cut out and quenched. Each pellet and supernatant was worked up in the usual manner with the triterpenols and the triterpene esters being eluted from the silica gel of the TLC plates and the radioactivity determined by counting an aliquot. The triterpenols were examined by HPLC in order to determine the ratios of new biosynthesis/old endogenous triterpenols. The ratios found were $2.5x10^{-4}$ and $3.6x10^{-4}$ for tubes one and two, respectively. For tube three the ratio for the ^{14}C labelled material was $2.4x10^{-4}$. The ratio for the ^{3}H labelled material was $1.3x10^{-4}$ for a combined ratio (^{3}H and ^{14}C) of $3.7x10^{-4}$.

Dual Label Time Course in Whole Latex and Pellets: Six incubation tubes were charged with ^{14}C-mevalonic acid (0.30 µmole, 10 µCi/ µmole) and DTE (2.5 µmole). Latex (150 µL) was added to each tube and they were incubated at room temperature. Tubes 1-3 were centrifuged after 2 hours. The pellets were solubilized in centrifugation buffer (150 µL) and transferred to

prepared tubes containing ^{3}H-mevalonic acid (0.30 µmole, 33 µCi/ µmole) and SAM (7.5 nmole) and incubated for 1, 2 and 4 hours. At the end of each incubation period the tube was centrifuged, the supernatant was removed, and both the pellet and the supernatant were quenched with acetone. Tubes 4-6 were centrifuged after incubating for 6 hours as whole latex. They were then treated exactly as tubes 1-3; being incubated with ^{3}H-mevalonic acid for 1, 2, and 4 hours, centrifuged and quenched. For each tube, the first and second supernatants and the pellet were worked up in the ususal manner. The results shown in tables 4, 5, 6 , 7, and 8 were determined by counting the bands from the TLC plates.

Tube (Total)	Total Mass Synthesized (ng)		Total Mass Endogenous	Ratio x10^{3} (µg)
	^{14}C	^{3}H		
1	26	12	64	0.6
2	30	22	37	1.4
3	56	66	88	1.4
4	37	7.3	23	2.0
5	55	16	113	0.6
6	50	16	32	2.0

Time Course of Triterpenoids Made by Whole Latex in an Osmotically Burst Pellet: Latex was divided into five samples (200 µL each) and incubated with ^{3}H-mevalonic acid (0.05 µmole, 470 µCi/ µmole) for one hour. The tubes were then centrifuged (15 min. 15,000x*g*), the supernatants removed, the pellets washed with fresh buffer (containing osmoticum) and pelleted again. These washed pellets were then resuspended into medium containing ATP, NADPH, DTE, and BSA but no osmoticum. The pellets were then quenched at time zero and each half hour therafter. The products from each pellet were obtained in the usual manner and the incorporation determined by counting the TLC bands corresponding to the triterpenols and the triterpene esters.

Time Course of Triterpenoids Made by Whole Latex in Pellets and Supernatants: Latex was

divided into five samples (175 µL each) and incubated with ^{3}H-mevalonic acid (0.05 µmole, 470 µCi/ µmole) for one hour. The tubes were then centrifuged and the supernatants removed. The pellets were allowed to sit in the centrifuge tubes for various times (0-2 hours). At the time each pellet was quenched, the corresponding supernatant was also quenched. The triterpenol and triterpene ester bands from the TLC plates were eluted and an aliquot (10%) counted. The triterpenols were examined by HPLC. The triterpene esters from the pellets were hydrolyzed with 10% KOH in methanol and chromatographed again, using TLC plates impregnated with 0.1 N NaOH (250 µm, Analtech). The recovery of radioactivity in the triterpenols ranged from 58-69% with from 7-17% of the radioactivity remaining as unhydrolyzed triterpene esters.

Pulse-Chase Experiments Using Dual Labelled Mevalonic Acid in Whole Latex and Pellets: A set of three experiments were done in collaboration with Dr. C. Skrukrud. Experiment 1: Three samples of latex were incubated with ^{14}C-mevalonic acid for two hours. Two of the tubes were then centrifuged, one of the resulting pellets was washed (solubilized in buffer and pelletted again) the other was not. These pellets were then incubated with ^{3}H-mevalonic acid for two hours. The third tube was not centrifuged. At two hours ^{3}H-mevalonic acid was added and the tube allowed to incubate two more hours, for a total incubation period of four hours. The incubations were quenched and chromatographed in the usual manner. The resulting triterpenols were examined by HPLC. The results of this experiment are given in table 11.

Experiment 2: Four samples of whole latex were incubated for 2 hours with ^{3}H-mevalonic acid. They were then centrifuged, the supernatants removed, and the pellets incubated with ^{14}C-mevalonic acid for various times (0.75- 4 hours). The resulting triterpenols were examined by HPLC and the incorporation into each compound was determined. The specific activity of the ^{14}C-mevalonic acid was not very high which made it difficult to determine the incorporation into the individual triterpenols for the first three time points.

Experiment 3: Eight samples of latex were incubated with ^{14}C-mevalonic acid for 2 hours. They were then centrifuged and the supernatants removed. Half of the pellets were solubilized in centrifugation buffer and incubated with ^{3}H-mevalonic acid for various times. The other half

of the pellets were solubilized in centrifugation buffer and unlabelled 100,000xg supernatant was added. They were then incubated with ^{3}H-mevalonic acid for various times. The triterpenols resulting from these incubations were separated using HPLC and the incorporation of each label into each compound was determined.

Isolation of Biosynthetic Activity from Whole Latex on a Percoll Gradient: Latex (175 µL) was added to 90% Percoll (300 µL) and mixed in a microfuge tube. On top of the resulting solution were carefully layered 55% Percoll (250 µL), 18% Percoll (400 µL) and centrifugation buffer (200 µL). This tube was centrifuged for 10 min. at 4000xg. From the interface of the 18% and the 55% Percoll layers, 200 µL was removed and added to a tube containing 800 µL of centrifugation buffer (to dilute the Percoll) and ^{3}H-mevalonic acid (2 µmole, 5 µCi/ µmole). This tube was allowed to incubate for one hour then quenched and worked up as usual. The incorporation was determined by scraping half of the silica gel from the plate and counting it in the gel phase. The triterpenols and the triterpene esters from the remaining half of the plate were eluted. The triterpenols were examined by HPLC to confirm that the radioactivity coeluted with the triterpenols. The triterpene esters were hydrolyzed with 10% KOH in methanol, the resulting products chromatographed (TLC), and the triterpenols isolated from them were examined by HPLC. The radioactivity was found to coelute with the triterpenes produced from the triterpene esters. The ratio of newly synthesized material to endogenous mass was determined to be 2.5×10^{-4} as opposed to 3.3×10^{-5} for the triterpenols themselves.

Attempted Isolation of Biosynthetic Activity from a Pellet on a Percoll Gradient: Latex (200 µL) was diluted with centrifugation buffer (200 µL) and centrifuged for 10 min. at 5000xg. The pellet was washed with fresh centrifugation buffer (400 µL) and centrifuged again. The resulting pellet was resuspended in 90% Percoll (300 µL) and transferred to a clean microfuge tube. A step gradient of Percoll was then carefully formed by layering on 55% Percoll (300 µL), 18% Percoll (500 µL), and centrifugation buffer (300 µL). This gradient was centrifuged for 10 min. at 4000xg. The upper portion of the gradient was removed to near the interface of the 18% and the 55% Percoll. The region near the interface was removed and added to a tube

containing centrifugation buffer (800 µL) and ^{3}H-mevalonic acid (1 µmole, 10 µCi/ µmole). This tube was allowed to incubate for 1.5 hours. The usual work up and chromatography yielded a TLC plate which showed very little incorporation into the triterpenoids. The triterpenols from this plate were eluted and examined on HPLC. There was no radioactivity detectable in the region where the triterpenols eluted.

Second Attempted Isolation of Biosynthetic Activity from a Pellet on a Percoll Gradient: The interface of the 18% and the 55% Percoll was obtained as above, but starting with 600 µL of latex. This interfacial region was divided in half. One half was diluted with centrifugation buffer (500 µL) and incubated with ^{3}H-mevalonic acid (0.6 µmole, 16.6 µCi/ µmole) for two hours. The other half was diluted with centrifugation buffer (500 µL) and centrifuged for 10 min. at 5000xg. There was no visible pellet, only a small cushion of concentrated Percoll. This bottom region was removed, diluted with centrifugation buffer (100 µL), and incubated with ^{3}H-mevalonic acid (0.1 µmole, 50 µCi/ µmole) for two hours. The TLC bands corresponding to the triterpenols and the triterpene esters were eluted. The triterpenols were examined by HPLC and no radioactivity was found to coelute with the triterpenols. When the triterpene esters were hydrolyzed with KOH in methanol, the recovery of radioactivity in the triterpenols was very low. These triterpenols were not examined by HPLC, but it was presumed that there was no real incorporation into the triterpenoids.

Attempted Isolation of the Site of Storage or Biosynthetic Activity on a Percoll Gradient: Two samples of latex (500 µL each) were incubated with ^{14}C-mevalonic acid (1.0 µmole, 10 µCi/ µmole) for one hour. Each sample was diluted with centrifugation buffer (500 µL each) and centrifuged (5000xg, 10 min). The pellet from one of the tube was resuspended in 90% Percoll (400 µL) and transferred to a clean microfuge tube. A step gradient was layered on top of this using 55 % Percoll (500 µL), 18% Percoll (250 µL), and centrifugation buffer (200 µL). This gradient was centrifuged (4000xg, 6 min). The region (200 µL) near the 18% and 55% Percoll interface was removed, combined with a portion of the 18% to 0% interface (100 µL), diluted with centrifugation buffer (700 µL), and incubated with ^{3}H-mevalonic acid (2 µmole, 15 µCi/

μmole). The other pellet was used as a control. It was allowed to sit at room temperature for about half an hour while the other pellet was being manipulated. It was then solubilized in centrifugation buffer (200 μL) and incubated with ^{3}H-mevalonic acid (0.4 μmole, 25 μCi/ μmole). The incubations were quenched and chromatographed as usual. The results from the counting of the bands from the TLC plates are given in table 18. The triterpenols were eluted and examined by HPLC to verify that the radioactivity coeluted with the triterpenols. The triterpene esters were hydrolyzed using 10% KOH in methanol and the resulting triterpenols were examined by HPLC to verify that they were actually labelled.

Testing if MES is Inhibitory to Biosynthesis: Two samples of latex (150 μL each) were placed in microfuge tubes. To one of these samples was added a solution (150 μL) of 0.4 M sorbitol, 5 mM $MgCl_2$, and 5 mM DTE. To the other was added a solution (150 μL) of a solution with the same constituents plus 20 mM MES, pH 5.5. The samples were mixed and centrifuged, each pellet was washed with the same solution it had been diluted in and centrifuged again. The pellets from this centrifugation were solubilized in their respective solutions and incubated for 2.5 hours with ^{3}H-mevalonic acid (0.2 μmole, 50 μCi/ μmole each). The results were determined by counting half of the TLC plate in the gel phase.

The Effect of Divalent Cations on the Biosynthetic Activity of the Pellet: Three samples of latex (200 μL each) were placed in microfuge tubes. Samples one and two were diluted with buffer (200 μL each) containing 0.4 M sorbitol, 60 mM MES (pH 5.5), 5 mM $MgCl_2$, and 5 mM DTE. Sample number three was diluted with buffer (200 μL) containing 0.4 M sorbitol, 60 mM MES (pH 5.5), 5 mM $MgCl_2$, 5 mM DTE, and 30 mM $CaCl_2$. The pellet from sample number one was solubilized in the same buffer it was centrifuged in with the addition of 50 μM SAM. The pellet from sample number two was solubilized in the same buffer as was used to solubilize number one with the addition of 10 mM $MnCl_2$. The pellet from sample number three was solubilized in the same buffer that was used to solubilize number two with the addition of 10 mM EGTA (pH 5.5). The solubilized pellets were then incubated with ^{3}H-mevalonic acid (2.0 μmole, 10 μCi/

μmole) for 2 hours. The results were determined by counting half of the bands from the TLC plates in the gel phase.

Attempted Recovery of Biosynthetic Activity from an Osmotically Burst Pellet: Three samples of latex (175 μL each) were placed in microfuge tubes. Two of these samples were diluted with centrifugation buffer and centrifuged. The supernatants were removed, the pellets washed with fresh buffer, and centrifuged again. The resulting pellets were solubilized in buffer (200 μL) without osmoticum. The remaining sample of whole latex was incubated with ^{3}H-mevalonic acid (0.05 μmole, 15 μCi/ μmole), ATP, and NADPH. One of the burst pellets was incubated in the presence of Tween 20 (ca. 0.15% w/v), ^{3}H-mevalonic acid (0.05 μmole, 15 μCi/ μmole), ATP, and NADPH. The remaining burst pellet was incubated in the presence of Tween 20 (ca. 0.15% w/v), ^{3}H-squalene (0.05 μmole, 40 μCi/ μmole), ATP, and NADPH. All of the samples were incubated for 2 hours then quenched by boiling. The tubes incubated with mevalonic acid were worked up as usual. The incubation using squalene was blown to dryness under a stream of nitrogen then solubilized in acetone and spotted onto a TLC plate. The TLC plate was developed in 80:20 cyclohexane:ethyl acetate then allowed to dry and developed in100% cyclohexane (in which only squalene shows any significant mobility). The incorporation in the mevalonic acid incubations were determined by counting the bands from the TLC plate. The triterpenols from the burst pellet were eluted and examined by HPLC. There was no radioactivity in the region where the triterpenols eluted. The TLC plate from the incubation with squalene showed a very high background. This is to be expected when there is a large amount of tritium in a compound with a high R_f. The tailing by the squalene obscurred the region of the TLC plate which contained the triterpene esters. The triterpenol band showed a very small incorpoation over background (6250 dpm vs 3850 in the adjacent band). When this band was examined by HPLC, no radioactivity was found to coelute with the triterpenols.

Attempted Nonaqueous Delipidation: Four samples of latex (100 μL each) were prepared. Two of these were diluted with centrifugation buffer and centrifuged. The supernatants were

removed and the pellets were solubilized in fresh centrifugation buffer. One of these was transferred to a tube containing 80:20 diisopropyl ether: butanol (1 mL) and inverted several times. The tube was centrifuged for 7 min. at 5000xg and the separate layers removed. The other pellet was washed with centrifugation buffer as usual. Each of the pellets was rinsed with fresh centrifugation buffer, centrifuged a third time, and the resulting pellets solubilized and incubated with ^{3}H-mevalonic acid (0.2 µmole, 25 µCi/ µmole). One of the remaining two latex samples was diluted with centrifugation buffer (100 µL) and added to 80:20 diisopropyl ether: butanol (1 mL), mixed briefly and centrifuged for 1 min. at 5000xg. The organic layer was removed and the aqueous layer used to solubilize the pellet. This aqueous layer was then incubated with ^{3}H-mevalonic acid (0.2 µmole, 25 µCi/ µmole). The final sample of latex was allowed to sit during the 30 minutes that these manipulations took. It was then incubated with ^{3}H-mevalonic acid (0.2 µmole, 25 µCi/ µmole). The incubations were quenched, worked up, and chromatographed as usual.

Inhibition of Triterpenoid Biosynthesis from Acetate by Mevinolin: Three tubes were prepared, each containing ^{3}H-mevalonic acid (0.2 µmole, 25 µCi/ µmole), ^{14}C-acetate (0.4 µmole, 50 µCi/ µmole), DTE (2 µmole), and either 0.4 nmole, 0.04 nmole, or no mevinolin. Latex (200 µL) was added to each tube and they were allowed to incubate for two hours. The incorporation of each isotope into the triterpenoids was determined by counting the bands from the TLC plates. The incorporation of acetate was expressed as a percent of the incorporation of mevalonate for each tube. This percent activity of the tubes with inhibitor was then expressed as a percent of that of the control. The reciprocals of these values were plotted against the concentration of inhibitor. The resulting graph is shown in figure 3.

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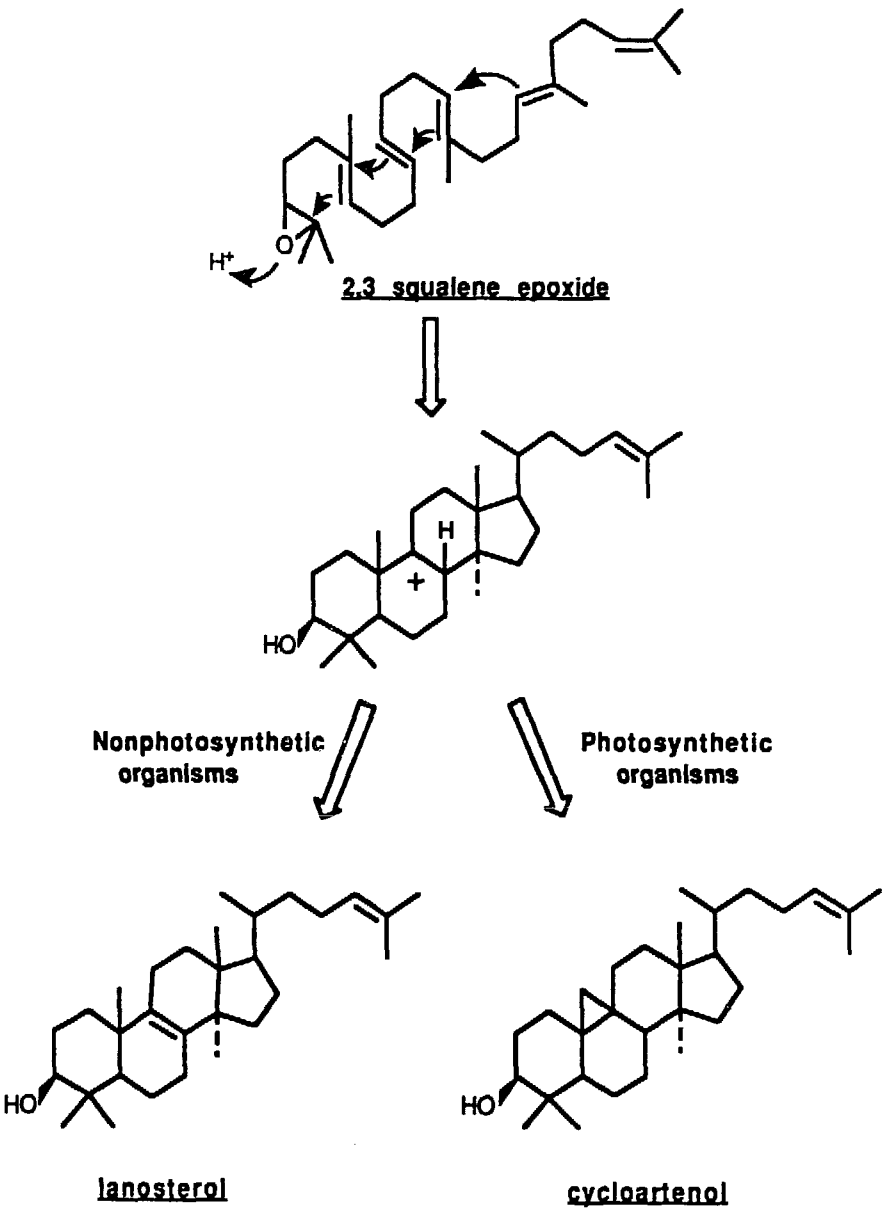
CHAPTER THREE: The Formation of Lanosterol and Cycloartenol in latex

The biosynthesis of steroids in non-photosynthetic organisms, most notably liver tissue, has been studied quite extensively [1-3]. The biosynthesis of steroids in photosynthetic organisms has not been studied as thoroughly, but the pathway has been examined in a number of species with varying degrees of examination focusing on the many steps involved in the pathway [3]. Most of the enzymatic steps found in the pathway utilized by liver are also found in plants. The most significant difference lies in the cyclization of squalene oxide. In virtually all non-photosynthetic organisms, squalene oxide is cyclized to lanosterol. In tissue from plants, squalene oxide is cyclized to cycloartenol, without the intermediacy of lanosterol [4,5]. This bifurcation is shown in figure 1.

Cycloartenol is further metabolized in plants to a number of different sterols. It is first methylated by S-adenosyl methionine: cycloartenol methyl transferase to form 24-methylenecycloartenol which then undergoes oxidative loss of the 4β-methyl group to form cycloeucalenol. The cyclopropane ring of cycloeucalenol is then opened to form obtusifoliol. From obtusifoliol, the pathway in various plants diverges to form a wide variety of steroids. Most of the common steroids are derived from demethylations at the 4 and 14 positions, double bond migration to the 5 position, and further modifications of the side chain. Most of the modifications to the triterpenoid nucleous which occur in plants after the formation of obtusifoliol parrallel the steps involved in the metabolism of lanosterol to cholesterol in liver. The two pathways diverge at the cyclization of squalene oxide and rejoin after the formation of obtusifoliol [3].

FIGURE 1


H+
O
2,3 squalene epoxide
H
+
HO
Nonphotosynthetic organisms
Photosynthetic organisms
HO
HO
lanosterol
cycloartenol

The enzyme, cycloeucalenol-obtusifoliol isomerase (COI), obtained from bramble and maize has been examined very thoroughly [6-8]. The substrate specificity is very high, especially with respect to substitution at the 4ß position. The enzyme system would convert cycloeucalenol to obtusifoliol but would not metabolize cycloartenol or 24-methylenecycloartenol to their corresponding ring-open compounds, lanosterol and 24-methylenelanosterol.

There are some plants which have been found to contain lanosterol; in some of these it is a major component of the triterpenoids found in the plant [9]. Euphorbia lathyris happens to be one such plant [10-13]. As discussed above, plants do not normally produce lanosterol, the presence of lanosterol in these plants is, therefore, of some interest. The major question concerning the presence of lanosterol in plants is its biosynthesis. Is it made directly from the cyclization of squalene oxide as it is in liver? And, if it is made directly from squalene oxide, is cycloartenol then made from lanosterol? Or is lanosterol made from cycloartenol via a ring opening enzyme which is capable of accepting cycloartenol as a substrate?

The latex of Euphorbia lathyris contains a considerable amount of triterpenoids, of which lanosterol is a major component [10-13]. Ourrison and coworkers studied this biosynthetic question earlier [14,15]. They attempted to answer the question by incubating the latex in the presence of labelled cycloartenol and observing the formation of labelled lanosterol. This work was done at a time when analytical methods were not as developed as they are today. They were unaware of the presence of several compounds in the latex due to their inability to separate the complex mixture. The result that they observed was the appearance of a very small quantity of labelled lanosterol. We felt that their result might not be valid due to the difficulties that they had in separating the triterpenoid products. We felt that the question was still unanswered and we hoped that a careful examination of this biosynthetic system might yield some answers to the question of the biosynthetic origin of lanosterol in the latex.

We first attempted to approach this problem using kinetics. Unfortunately, as was discussed in chapter 2, the most advanced substrate in the terpenoid pathway for which we

were able to observe incorporation was mevalonic acid. This is quite far removed from the step that we were attempting to study; namely, the cyclization of squalene oxide. The kinetics observed for the incorporation into the individual triterpenols from mevalonic acid were all found to be linear with respect to time. This was true for compounds such as 24-methylenelanosterol and 24-methylenecycloartenol which are presumably made from the unmethylated triterpenols, lanosterol and cycloartenol. We did not observe a product-precursor relationship which one would expect to see for these compounds. This was readily interpreted as evidence for the presence of a rate-limiting step somewhere after the formation of mevalonic acid but before the methylation and possibly before the cyclization. If this were true, then we were observing the result of kinetics controlling the total formation of the triterpenoids, but with the distribution of the individual compounds was being controlled by factors other than kinetic.

One means of avoiding this rate-limiting step would be to follow the incorporation of a substrate that occurs later in the pathway. We decided to try to obtain incorporation into the triterpenols from ^{3}H-squalene. The radiolabelled squalene was incubated with latex under a variety of conditions; as whole latex (both with and without detergents to assist in the solubilisation of the squalene), in a 5000xg pellet, with an osmotically burst pellet (at two pH's), and in a disrupted and resolubilized system using deoxycholate to solubilize the membrane fragments. The details of these experiments are described in the experimental section, although no incorporation into the triterpenoids was ever observed from squalene.

The conversion of cycloeucalenol to obtusifoliol is proposed to be an acid catalysed process which passes through an intermediate with some cationic character at C-9, followed by the loss of the proton at C-8 to give the 8,9 double bond (see figure 2). Benveniste and his coworkers have studied a number of compounds which inhibit a specific steps in the biosynthesis of steroids. Several of these compounds are in use commercially as fungicides. The morpholine-based fungicides, tridemorph (1) and fenpropimorph (2) (see figure 3) have been shown to inhibit, both <u>in vivo</u> and <u>in vitro</u>, the ring-opening enzyme, COI [16-19]. The effectiveness of these compounds as inhibitors is usually expressed as an I_{50} value; the

concentration of inhibitor at which the enzymatic activity is 50% of the uninhibited level. For both tridemorph and fenpropimorph, the I_{50} values for COI are 0.4 μM [16]. Tridemorph was also shown to inhibit the cyclization of squalene oxide to cycloartenol in a microsomal suspension from maize seedlings, for which it had an I_{50} value of 50 μM [20]. The authors did not report whether tridemorph had any inhibitory effect on the squalene oxide-lanosterol cyclase from rat liver. They did examine a very similar compound for which they found an I_{50} value of 1 μM for the cycloartenol cyclase and an I_{50} value of 2 μM for the lanosterol cyclase; showing the lanosterol cyclase to be inhibited to only a slightly lesser degree than the cycloartenol cyclase [20].

If Euphorbia lathyris latex utilizes a ring-opening enzyme to convert cycloartenol to lanosterol, it should be possible to inhibit this enzyme with tridemorph or fenpropimorph. One would expect, if this were the case, that the relative amount of lanosterol would decrease in the presence of these inhibitors, with little or no effect on the absolute amount of cycloartenol and 24-methylenecycloartenol. This same strategy was attempted by Berg in an examination of sterol biosynthesis in Saprolegnia ferax [21]. Depending on the biological controls, the total amount of these cyclopropyl sterols could be increased in the presence of the inhibitors.

A number of experiments were conducted using varying concentrations of tridemorph and examining the relative distribution of the label incorporated into the individual triterpenols. These experiments are more difficult to interpret than standard in vitro enzyme inhibition studies. Normally, one would like to examine only the enzymatic step of interest. In our latex experiments we are actually looking at a series of enzymatic steps in a fairly complex system. We suspected that between mevalonic acid and the cyclization of squalene oxide there was a rate-limiting step that would probably not be inhibited by these compounds. In order for the inhibitory effect of these compounds to be noticed, their effect must be large enough to slow the step they are inhibiting to approximately the same rate or slower than this rate-limiting step. Also, one must realize that for the inhibitors to have any activity they must reach the enzyme that they are to inhibit. Latex is an organellar system with the triterpenoid biosynthesis contained

inside of some organelle. In such a case, where we knew nothing of the transport properties of the inhibitors, all that we could hope to determine was an apparent I_{50}, the concentration present in the bulk solution needed to obtain 50% inhibition.

FIGURE 2

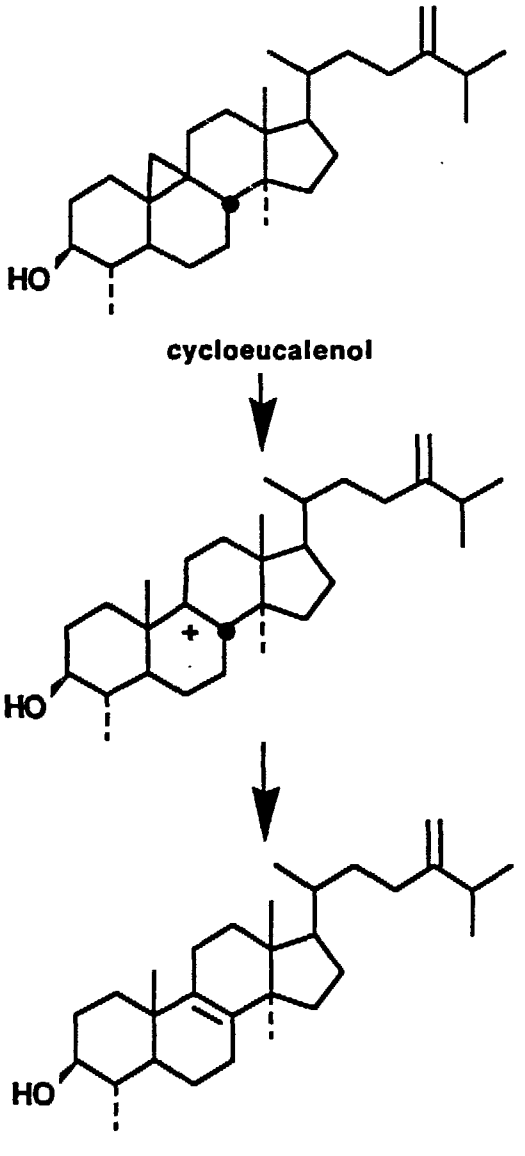
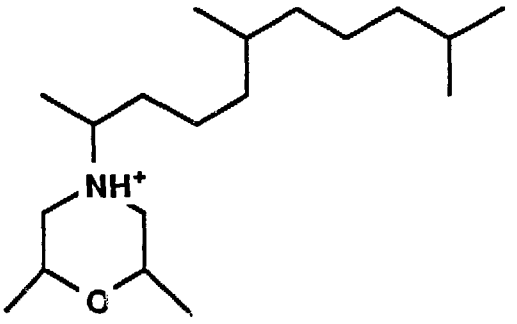
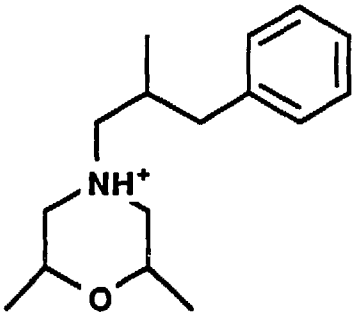


FIGURE 3



TRIDEMORPH

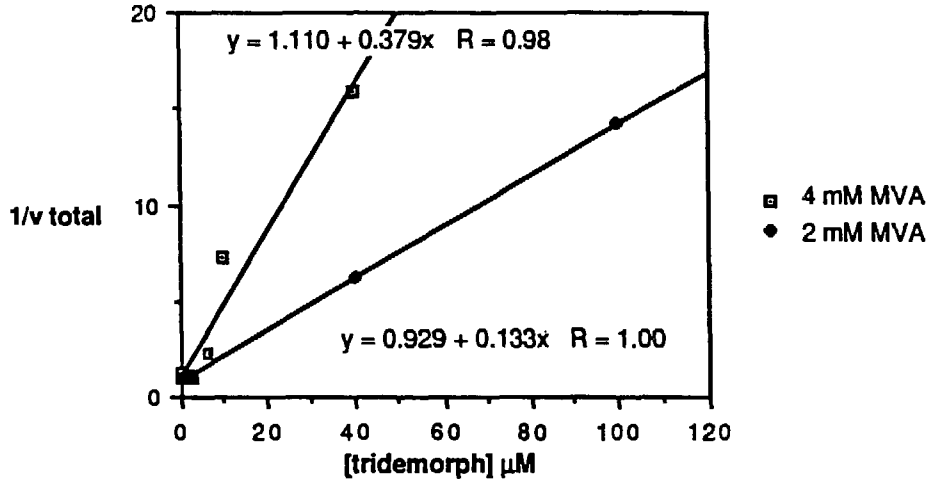


FENPROPIMORPH

The effect of tridemorph on the biosynthesis of triterpenoids from mevalonic acid was determined by dividing a sample of latex into several separate tubes and incubating them with concentrations of tridemorph ranging from zero to as high as 100 μM and with the substrate (mevalonic acid) concentration at two different levels, while all other parameters were kept constant. The incorporation into the triterpenoids was determined for each of the incubations, and the level of incorporation in each of the tubes containing tridemorph was compared to that in the control sample. In order to collect sufficient data to accurately determine the effect of

tridemorph, it was necessary to perform several incubations, on several days, using different latex for each group of incubations. Each sample of latex is very different from any other sample of latex. In order to compare the data collected from various samples of latex, the activity observed for each sample was compared to the control tube from the same latex and expressed as a percent of control. A Dixon plot was then constructed by plotting 1/the velocity relative to the control vs. the inhibitor concentration (see figure 4).

FIGURE 4

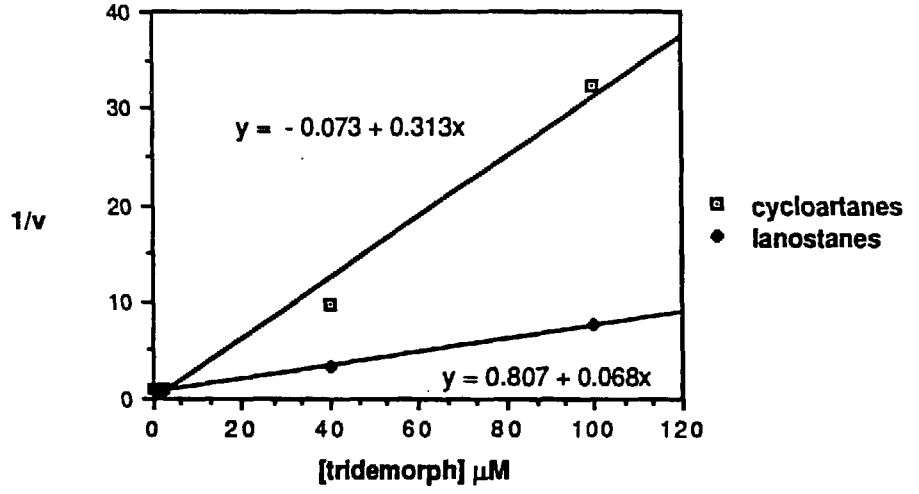


It is apparent from figure 4 that the biosynthesis of the triterpenoids is inhibited by tridemorph. The I_{50} concentrations determined from the "best-fit" lines shown in figure 4 are 8 µM for those experiments using a mevalonic acid concentration of 2 mM and 2.3 µM for those using a mevalonic acid concentration of 4 mM. These values are much higher than the reported values for the inhibition of COI by tridemorph (I_{50}=0.4 µM) [16], but are lower than the reported value for the inhibition of squalene oxide-cycloartenol cyclase (I_{50}=50 µM) [20].

The triterpenols from these incubations in the presence of tridemorph were isolated from the TLC plates and examined by HPLC to determine the incorporation into the individual compounds. If tridemorph was inhibiting the conversion of cycloartenol to lanosterol, one

would expect the amount of label going into lanosterol to decrease in the presence of the inhibitor. The percent of the radioactivity found in each compound from each incubation was compared to the percent of the same compound found in the control from that incubation. The plot of 1/ this % of the percentages in the control vs. the concentration of tridemorph is basically a Dixon plot and is shown in figure 5. Cycloartenol and 24-methylenecycloartenol have been combined as have lanosterol and 24-methylene lanosterol. Butyrospermol, euphol and the unknown triterpenol have been omitted, in order to focus on the cyclopropyl sterols and their ring-open isomers.

FIGURE 5



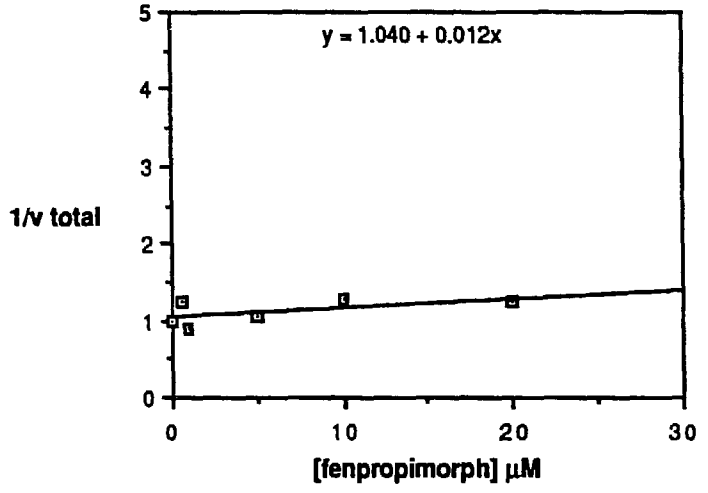
The percentage of the incorporation going into the cyclopropyl sterols decreases as a function of the concentration of tridemorph, while that of the lanostane-type triterpenols increases. It is clear from these results that if there is a ring-opening that accounts for the presence of lanosterol and 24-methylenelanosterol by opening the cyclopropyl ring of cycloartenol and 24-methylenecycloartenol, it is not being inhibited by tridemorph. Tridemorph is inhibiting the biosynthesis of the triterpenoids in some manner. While this does not prove that it is taken up inside of the organelle which is carrying out that biosynthesis, it does seem to

indicate that it is capable of reaching some point from which it has the ability to interfere with the biosynthetic pathway. Given that tridemorph is probably reaching the area in which a ring-opening enzyme would exist and the fact that tridemorph has inhibited similar steps in other systems; it seems unlikely that lanosterol and 24-methylenelanosterol are produced from cycloartenol and 24-methylecycloartenol via a ring-opening enzyme.

Fenpropimorph is a morpholine-based fungicide that is very similar to tridemorph both in chemical structure and in its ability to inhibit COI. One of the major differences in their biological activities is that fenpropimorph has not been shown to inhibit the cyclization of squalene oxide. If the inhibitions observed for tridemorph were due to the inhibition of the cyclization of squalene oxide, then one would not expect to observe the same inhibition from fenpropimorph. The biosynthesis of the triterpenoids from mevalonic acid in the presence of various concentrations (0 to 20 μM) of fenpropimorph was determined. The activity of each tube containing fenpropimorph was compared to the activity found in the control and the results of 1/activity relative to control were plotted vs. the concentration of fenpropimorph (see figure 6). the resulting figure can be directly compared to figure 4. From the very small slope of the line from the fenpropimorph experiment, it can be seen that fenpropimorph is a vey weak inhibitor (if at all) of the biosynthesis of the triterpenoids from mevalonic acid. The inhibition observed from fenpropimorph is certainly much less than that observed from tridemorph. The I_{50} value one obtains for fenpropimorph is 80 μM; about a factor of ten greater than the concentration of tridemorph needed to achieve the same level of inhibition. One interpretation of these results is that the inhibition observed from tridemorph is due to inhibition of the cyclization which is not as strongly inhibited by fenpropimorph. This would indicate that tridemorph has been translocated to the site at which the biosynthesis is taking place. If this is correct, then one must assume that if there is a ring-opening enzyme which is converting cycloartenol to lanosterol it is not being inhibited by tridemorph. The other possibility is that there is no ring-opening enzyme operating which converts cycloartenol to lanosterol.

FIGURE 6

Effect of Fenpropimorph



The results from our inhibitor studies indicated but did not prove that there was no enzymatic conversion of cycloartenol into lanosterol in Euphorbia lathyris latex. They did not rule out the possiblity that cycloartenol could come from lanosterol. Indeed, the fact that the cyclopropyl sterols were more inhibited by tridemorph than the lanostanes could be interpretted as being due to inhibition of the conversion of the lanostanes into the cyclopropyl sterols. This possiblity was considered unlikely due to the fact that the triterpenoid biosynthetic pathway has been examined in quite a few photosynthetic organisms and it is always cycloartenol that is proposed as the primary cyclization product from squalene oxide [22-24]. It was decided that we should repeat the method which was used to verify that cycloartenol was not produced via lanosterol to verify that this was true for the pathway in E. lathyris latex.

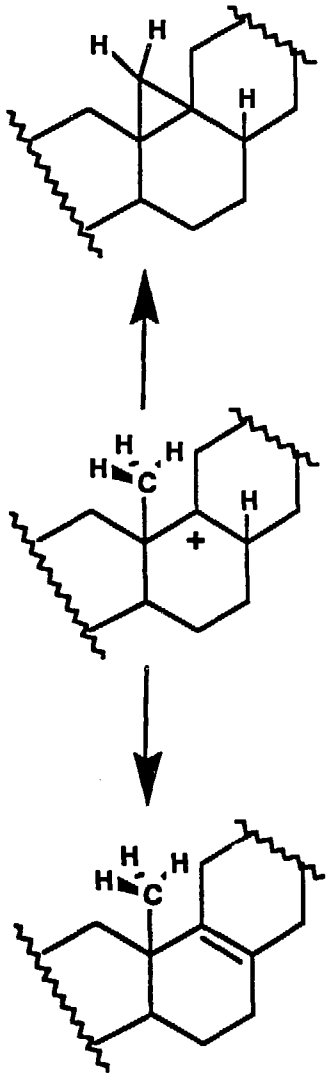
The method employed involved incubating the latex with mevalonic acid that was labelled with tritium in the 4-R position. It has been shown that the 4-R proton is incorporated into squalene while the 4-S proton is lost. It is a 4-R proton which is present at carbon 9 of the protosteroid cation and which is subsequently lost in the formation of the 8-9 double bond to form lanosterol. In peas, this same proton migrates to carbon 8 and is still present in cycloartenol [23]. The 4-R-4-$^{3}H_{1}$-mevalonic acid is incubated together with ^{14}C-mevalonic acid. By

comparing the ratio of tritium to ^{14}C in the starting material and in the products, one can determine if any of the tritium atoms were lost during the biosynthesis. The details of the mechanism are given in figure 7.

It was first neccessary to synthesize the desired tritiated substrate. This was done following the method of Cornforth, et. al. [5,24]. The synthesis is outlined in scheme 1. Complete details of the experimental procedures and the characterisation of the intermediates and the final product can be found in the experimental section. The final product was purified by reverse phase HPLC for which the detector was calibrated in order to accurately determine the total mass of the product. An aliquot was assayed by LSC to determine the total radioactivity from which the specific activity of the ^{3}H-mevalonic acid was determined. An aliquot was then mixed with a known amount of ^{14}C-mevalonic acid and a sample of this starting mixture was counted. The ratio of $^{3}H/^{14}C$ was determined and defined as being 1/1. On this basis one would expect that cycloartenol would show a ratio of 6/6, while lanosterol would show a ratio of 5/6.

Latex was then incubated with the mixed $^{3}H/^{14}C$-mevalonic acid and the triterpenols from the incubation were collected and purified by HPLC. The chemical purity of each triterpenol was then checked by HPLC to avoid cross-contamination of the individual compounds. The radioactivity present in each isotope in each of the compounds was then determined by LSC in the dual isotope mode. The results for cycloartenol and 24-methylenecycloartenol were within 3% of the expected value of 6/6 for the ratio of tritium to ^{14}C. This is in agreement with what has been observed by other workers in plant systems: that cycloartenol is made without the presence of an intermediate containing an 8-9 double bond (such as lanosterol). Unfortunately, the results for lanosterol and 24-methylenelanosterol were not as we had expected. The amount of radioactivity present in 24-methylenelanosterol was extremely small. When one attempts to count a small amount of ^{14}C in the presence of a larger amount of tritium, the accuracy of the counting suffers due to overlap of the two isotope windows and difficulties associated with background subtraction. The combination of errors made the results for

FIGURE 7



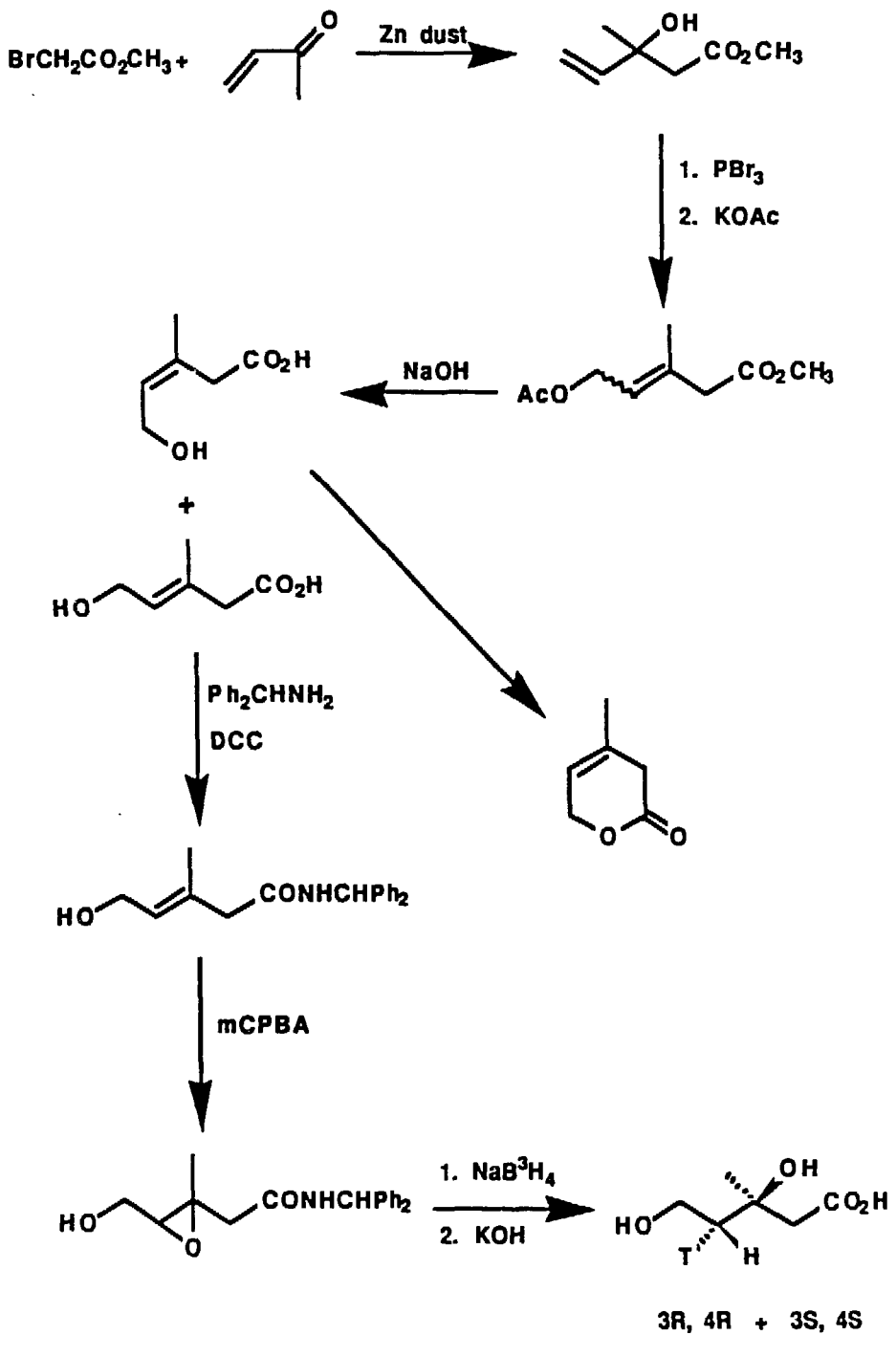
24-methylenelanosterol meaningless. The results of the ratio of the two isotopes present in lanosterol are quite difficult to interpret. The ratio was virtually 6/6. The most reasonable explanation for the ratio observed for lanosterol is that there is some tritiated impurity which

coelutes with lanosterol off the HPLC. The only other explanations involve contradictions of the details of the biosynthesis of lanosterol from mevalonic acid that are found in the literature. One such possibility is that the 8-9 double bond is formed from a compound with a double bond elsewhere, a 9-11 double bond, for example, via an intramolecular proton shift in which the proton that is normally lost is retained. Such a possibility must be considered with a bit of skeptacism and would require a much more detailed study to verify.

The results obtained for cycloartenol and 24-methylenecycloartenol show that these two compounds are not made from lanosterol. This leaves only two options: either lanosterol is made from cycloartenol or the two triterpenols are made separately. It was decided that this question could best be answered by performing an experiment in which the protons of the C-10 methyl group would be labelled. It is this methyl group which loses a proton in the process of forming the cyclopropyl ring of cycloartenol. If the lanosterol isolated from this incubation retained all three of the original protons, it could not have been made from cycloartenol. If it was made via cycloartenol, it should retain only two of the original three protons.

Our first thought in designing the experiment to label the methyl group of interest was to use tritium. A tritium to ^{14}C ratio could be used to determine if any of the tritium atoms were lost. We soon realised that this experiment would be complicated by the large isotope effect observed for tritium in reactions involving the cleavage of the carbon-tritium bond. The deuterium isotope effect for the formation of cycloartenol from squalene oxide containing a chiral methyl group in the position of interest by squalene oxide:cycloartenol cyclase from a microsomal preparation from Ochromonas malhamensis was observed to be ca. 4 [25]. One can calculate from this an expected tritium isotope effect of nearly 10:1. Given such an isotope effect and the fact that we must use mevalonic acid as our precursor (thus incorporating six methyl groups, only one of which might lose a tritium) it was decided that the experiment would not be feasible unless a methyl group containing virtually a 100 % enrichment of tritium could be used. At the time that we were considering the experiment, the ability to synthesize a methyl group containing 100 % tritium did not exist.

SCHEME 1



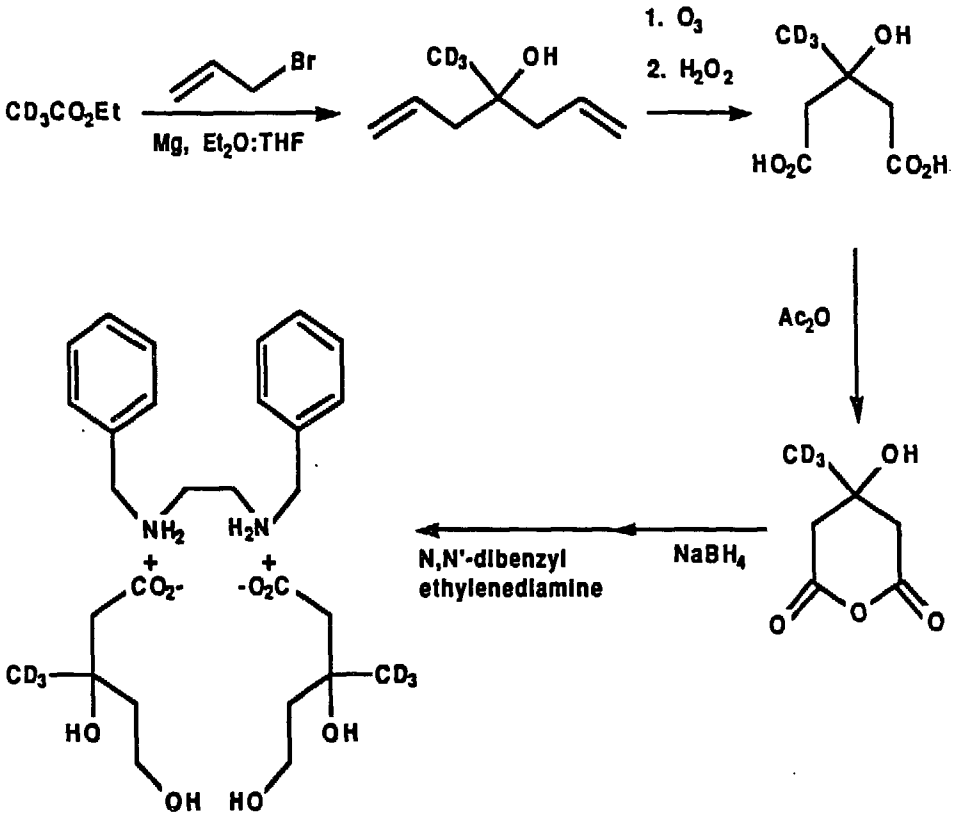
It was decided that in order to obtain a 100 % isotopically enriched methyl group, we would

have to use deuterium as the isotope. The interpretation of the experiment would then have to rely on mass spectroscopy, a technique which, unlike radioactivity, is sensitive to unlabelled "impurities" and requires a fairly substantial quantity of material by biological standards. It was difficult to predict if the results of an incubation with mevalonic acid labelled in the methyl group with three deuterium atoms would be interprettable or not. We decided to synthesize the precursor and try the experiment.

There have been many syntheses of carbon- and hydrogen-labelled mevalonic acids [5, 26-29]. Very few of these were aimed at labelling the methyl group. There are two reports of the synthesis of 3'-D- or 3'-T-mevalonic acid [27], a synthesis of mevalonic acid with a chiral methyl group [28], and a communication reporting a very good synthesis of 3'-^{13}C-mevalonic acid [29]. The previous syntheses of the hydrogen-labelled compounds are not regiospecific, do not produce 100 atom %, or are very inefficient. We decided to modify the method used by Scott, et al [29] to synthesize the desired substrate.

The precursor, 3'-D_3-mevalonic acid, was synthesized by the route shown in scheme 2. Deuterated acetic acid is inexpensive and is available with 99.96 atom% deuterium, making it the ideal starting material for the synthesis. Labelled ethyl acetate was prepared just prior to running the Grignard reaction and was kept at 0° until used. The Grignard reaction with allyl bromide works quite well, forming allyl magnesium bromide in situ. As long as the work-up is kept cool one does not observe any appreciable elimination. The diallyl methyl carbinol thus formed undergoes oxidative ozonolysis to give good yields of methyl-labelled 3-hydroxy-3-methyl glutaric acid and it is fairly simple to prepare 5-10 grams of this labelled material. The major obstacle in the synthesis of mevalonate via hydroxy methyl glutaric acid is the reduction of only one of the two carboxyl groups. This was achieved via the cyclic anhydride.

SCHEME 2



The formation of the cyclic anhydride is quantitative and the crude product is suitable for the reduction with sodium borohydride. The reduction of the anhydride with sodium borohydride produces a crude lactone which could be purified as the lactone; however, we found reduced yields upon purification on silica gel which we attributted to irreversible adsorption. Also, in our enzymatic system we have found that the lactone is not utilized. The final product can be easily purified by crystallization as the DBED salt and is then very easily characterized. Examination of the final product by ^{1}H- and ^{13}C-NMR spectroscopy and by mass spectroscopy showed the methyl group to be greater than 99 atom% labelled.

Several incubations with the deuterated mevalonic acid were conducted. The main

problem was, as we had expected, trying to find the small quantities of labelled triterpenols in the comparatively large masses of the endogenous triterpenols. The incubations with the deuterated substrate were conducted in the presence of a small amount of tritated mevalonic acid. The radioactivity was then used to determine the incorporation into the triterpenoids. The mass present in each of the triterpenols was normally too small to be accurately weighed, but could be determined by observing the detector response on the HPLC and comparing the peak area of each compound to the calibration constant which was determined in chapter 1. As was discussed in chapter 2, centrifugation of the latex yields a pellet which retains about half of the biosynthetic activity of the whole latex. This pellet contains only a small amount of the endogenous triterpenoids. By centrifuging the latex and working with the pellet, one sacrifices a portion of the biosynthesis in order to obtain a matrix which has a much lower endogenous mass of triterpenoids. We had also observed that newly synthesized triterpenoids can be pelleted preferentially to the endogenous pools of triterpenoids. We attempted to maximize our incorporation while minimizing the quantities of endogenous triterpenoids by first incubating the deuterated substrate with whole latex, then centrifuging. This pellet was then incubated again with the deuterated substrate, then centrifuged once more. This final pellet was found to contain triterpenoids with the highest percentage of labelled (newly synthesized) compounds in the total mass of the compounds isolated. Following this procedure, we were able to obtain triterpenols which contained as much as ca. 0.1 % labelled compounds. Unfortunately, this small amount of labelled compound did not produce any ions visible by mass spectroscopy which corresponded to incorporation of the deuterated substrate.

The endogenous mass of the triterpene esters is about one third that of the triterpenols. The incorporation of labelled substrates such as acetate and mevalonate into the triterpene esters is much faster than into the triterpenols. We attempted to take advantage of these facts by isolating the triterpene esters, hydrolyzing them with KOH in methanol, and isolating the resulting triterpenols. These triterpenols were then found to contain higher percentages of the labelled compounds than the free triterpenols. We were able to isolate lanosterol hydrolyzed

from the triterpene esters which contained ca. 0.27 % labelled material. Unfortunately, this material, when examined by mass spectroscopy, did not show any ions correponding to the incorporation of the deuterated substrate.

The rate of biosynthesis of the triterpenoids by the latex is not easily increased. A number of cofactors and biological perturbations had been used in attempts to improve the biosynthesis. None of the many things we tried had any great impact on the biosynthetic rate. If the rate of biosynthesis could not be increased, our only other alternative was to obtain a more thouroughly delipidated system. Some of our attempts at delipidation were described in chapter 2. The only delipidation method which provided a system with biosynthetic activity was centrifugation. All of our other attempts produced biologically inactive systems. Despite our best attempts at determining whether lanosterol is derived from cycloartenol via the use of the deuterated mevalonic acid, we were unable to obtain a definite answer. The deuterated experiment will probably not deliver a definitive answer until a mass spectral method capable of detecting very minor quantities of a labelled compound in the presence of large amounts of unlabelled material is developed.

The work we have done with tridemorph and fenpropimorph suggests, but does not prove, that lanosterol is not made via cycloartenol. Our results from the incubation with 4-R-4-^{3}H-mevalonic acid seem to indicate that cycloartenol is not made via lanosterol. The most reasonable explanation for all of the data is that cycloartenol and lanosterol are made independent of one another, probably by two different cyclization enzymes.

EXPERIMENTAL

General: All radioactive substrates were obtained from New England Nuclear. Tridemorph and fenpropimorph were obtained from Dr. Pommerer at BASF. Latex was tapped from the plants as previously described [30]. Incubations with whole latex were conducted as previously described [30] unless otherwise noted. All TLC plates are Analtech silica gel G, 20 cm x 20 cm, 500 µm thick, unless otherwise specified. All HPLC was performed on Altex C-18 columns, 5

μm spherical particles, 4.6 mm I.D. x 25 cm. Normally, two columns were used in series. For semi-preparative HPLC, a 10 mm I.D. x 25 cm column was used. ^{1}H-NMR spectra were recorded at 250 MHz in $CDCl_3$ with TMS as internal standard, unless otherwise noted. Mass spectra were obtained at 70 eV.

Attempted incubations with squalene: A. in whole latex: ^{3}H-Squalene (0.05 μmole, 40 μCi/μmole), $MgCl_2$ (1.4 μmole), and DTE (1 mg) were placed in an incubation tube and blown to dryness under a stream of nitrogen. Latex (200 μL) was added and the tube was allowed to incubate for seven hours. The incubation was quenched by the addition of hexanes. Water (ca. 2 mL) was added and the mixture extracted 3 times with hexanes. The hexanes extract was evaporated under a stream of nitrogen and spotted onto a TLC plate which was developed once in 80:20 cyclohexane:ethyl acetate and once in cyclohexane. The resulting TLC plate was cut into 1 cm wide bands; the band which contained the triterpenols was eluted and an aliquot counted. The remaining bands were counted in the gel phase. Approximately 75 % of the initial radioactivity was recovered unchanged. With the very large amount of radioactivity in the squalene band, there was quite a bit of tailing of the radioactivity which left a "background" of 16000 dpm in the band directly above the triterpenols. From the counting of the aliquot of the triterpenol band it was calculated that there was 16000 dpm in the triterpenol band. Due to the presence of a considerable amount of endogenous triterpenols and the relatively low level of radioactivity, it was not possible to examine this material by HPLC. This experiment was repeated and no significant radioactivity was found in the triterpenol band of the TLC plate (the band below had 19000 dpm, the triterpenol band had 22000 dpm, and the band above had 29000 dpm).

B. in a 15000xg pellet, with detergent: ^{3}H-squalene (0.24 μmole, 2.46 Ci/mmole) was placed in a small test tube and 25 μL of a solution of Tween 80 in acetone (1 mg/mL) was added. To a second tube was added ^{3}H-mevalonic acid (0.06 μmole, 0.24 Ci/mmole) and 25 μL of Tween 80 in acetone (1 mg/mL) and the solvents in each tube were evaporated under a stream of nitrogen. A third tube was prepared for use as a control, with ^{3}H-mevalonic acid (0.06 μmole,

0.24 Ci/mmole), but no detergent. A sample of latex (600 µL) was divided into thirds and centrifuged for 15 minutes at 15,000xg. The supernatants were removed and the pellets solubilized in centrifugation buffer (200 µL) containing ATP, NADPH, and DTE. The resulting solutions were transferred to the prepared reaction tubes containing the substrates. Each centrifugation tube was rinsed with 100 µL of fresh buffer which was also added to the reaction tubes. The tubes were gently agitated to facilitate the solubilisation of the substrates and were allowed to incubate for one hour. The incubations were quenched and extracted with hexanes as usual. The hexane extracts from the two tubes with mevalonic acid as the substrate were chromatographed in the usual TLC system of 3:1 ether: petroleum ether. The control incubation and the incubation of mevalonic acid with the Tween 80 showed virtually identical incorporations; thus indicating that Tween 80 did not inhibit the biosynthesis at the level used. The hexane extract from the tube with squalene as the substrate was spotted onto a TLC plate and developed in cyclohexane. The unreacted squalene was recovered at an R_f of 0.3. The origin was scraped and eluted. From an aliquot of this eleunt it was determine_ that there was 21000 dpm present. This material was spotted onto a fresh TLC plate and developed in the ether: petroleum ether system. The band containing the triterpenols was scraped, eluted and examined by HPLC; from which fractions were collected and counted. Virtually all of the radioactivity injected eluted in the first 30 minutes. There was no radioactivity found in the fractions taken from the 50-75 minute region in which the triterpenols eluted.

C. in a burst pellet: Reaction buffer without osmoticum was prepared at two pH's: 5.5, buffered by phthalate, and 7.2, buffered by MOPS. Two reaction tubes were prepared as above with ^{3}H-squalene (0.24 µmole, 2.46 Ci/mmole) and Tween 80 in acetone (25 µL, 1 mg/mL) blown to dryness under a stream of nitrogen. A sample of latex (400 µL) was divided in half and centrifuged for 15 minutes at 15,000xg. To each pellet was added 200 µL of buffer without osmoticum (one at pH 5.5, one at pH 7.2). The pellets were solubilized, transferred to the prepared reaction tubes and incubated for 2 hours. Acetone was added and the acetone solubles were transferred to a clean vial and taken to dryness under a stream of nitrogen. The

resulting material was spotted onto TLC plates which were developed twice in cyclohexane. From a marker spot on the side, it was determined that squalene was at R_f 0.6. The squalene bands were scraped from the plates. The triterpenols were still at the origin of these plates which were then developed in 90:10 toluene: ethyl acetate. From the pH 5.5 plate, the bands corresponding to the triterpenols and to the triterpene esters were scraped and eluted. The adjacent bands were counted in the gel phase. From the counting of these bands, there was no significant radioactivity present in the triterpenoid bands when compared to the levels present in the adjacent bands. This result was also observed for the pH 7 incubation. From that TLC plate, one cm wide bands were cut along a portion of the length along which the sample was streaked. The dpm present in each band are shown in table 1.

TABLE 1

Band	dpm x 10^{-3}
7	32
6	19
5	15
4	12
3	12
2	10
1	10
origin	19

D. Attempted Recovery of Biosynthetic Activity from an Osmotically Burst Pellet: Three samples of latex (175 µL each) were placed in microfuge tubes. Two of these samples were diluted with centrifugation buffer and centrifuged. The supernatants were removed, the pellets washed with fresh buffer, and centrifuged again. The resulting pellets were solubilized in buffer (200 µL) without osmoticum. The remaining sample of whole latex was incubated with ^{3}H-mevalonic acid (0.05 µmole, 15 µCi/ µmole), ATP, and NADPH. One of the burst pellets was incubated in the presence of Tween 20 (ca. 0.15% w/v), ^{3}H-mevalonic acid (0.05 µmole, 15 µCi/ µmole), ATP, and NADPH. The remaining burst pellet was incubated in the presence of Tween 20 (ca. 0.15% w/v), ^{3}H-squalene (0.05 µmole, 40 µCi/ µmole), ATP, and NADPH. All of the samples were incubated for 2 hours then quenched by boiling. The tubes incubated with

mevalonic acid were worked up as usual. The incubation using squalene was blown to dryness under a stream of nitrogen then solubilized in acetone and spotted onto a TLC plate. The TLC plate was developed in 80:20 cyclohexane:ethyl acetate then allowed to dry and developed in 100% cyclohexane (in which only squalene shows any significant mobility). The incorporations from the incubations with mevalonic acid were determined by counting the bands from the TLC plates. The whole latex control showed normal incorporation. The triterpenols from the burst pellet were eluted and examined by HPLC. There was no radioactivity in the region where the triterpenols eluted. The TLC plate from the incubation with squalene showed a very high background. This is to be expected when there is a large amount of tritium in a compound with a high R_f. The tailing by the squalene obscurred the region of the TLC plate which contained the triterpene esters. The triterpenol band showed a very small incorpoation over background (6250 dpm vs 3850 dpm in the adjacent band). When this band was examined by HPLC, no radioactivity was found to coelute with the triterpenols.

E. in a disrupted, solubilized system: A sample of latex (600 µL) was divided in half, centrifuged for 10 minutes at 5000xg and the supernatants removed. To one of the pellets was added 200 µL of a solution of 2 % deoxycholate, 100 mM MES, and 10 mM DTE (pH=7.1). To the other pellet was added 200 µL of a solution containing 0.5 % deoxycholate, 100 mM MES, and 10 mM DTE (pH=6.75). Each of the tubes was then vortexed for ca. 2 minutes. The resulting milky suspensions were then centrifuged for 10 minutes at 5000xg. Most of the visible mass pelletted again. The resulting supernatants were removed and added to reaction tubes which containED ^{3}H-squalene (0.4 µmole, 2.5 µCi/µmole), Tween 80 (0.4 mg), and NADPH. The tubes were incubated for one hour and quenched by the addition of hexane. Water was added and the tubes extracted several times with hexane. The hexane extracts were then spotted on TLC plates which were developed in cyclohexane. The origins of the TLC plates were scraped and eluted. These eluents were then spotted onto fresh TLC plates which were developed in 1:1 ether: petroleum ether. There was not a significant amount of radioactivity above background in the triterpenol region of these TLC plates. There was a small

amount radioactivity in the triterpene ester region which was scraped and eluted. This material was hydrolyzed and rechromatographed to obtain the resulting triterpenols. When examined by HPLC, no radioactivity was found to be associated with the mass peaks of the triterpenols.

Inhibition by tridemorph: General: Latex was incubated in the presence of 5-^{3}H-mevalonic acid and various concentrations of the inhibitors for 2-3 hours. The enzymatic reactions were stopped by the addition of acetone and the hexane soluble products isolated by extraction. The triterpenols and the triterpene esters were isolated by TLC (developed in 3:1 ether:petroleum ether). The incorporation into each group of products was determined by scintillation counting of aliquots of each. The incorporation by each of the inhibited tubes was compared to that of a control sample from the same latex, incubated over the same time period without inhibitor. This relative activity was then analysed by the method of Dixon; plotting the inverse of the relative activity vs. the concentration of the inhibitor.

Experiment 1: Three tubes were prepared containing 0, 0.04, and 0.4 nmoles of tridemorph and ^{3}H-mevalonic acid (0.8 µmole, 18.75 µCi/µmole). Latex (200 µL) was added to each tube (to give tridemorph concentrations of 0, 0.2 and 2.0 µM). The tubes were incubated for three hours then quenched and worked up as usual. Based on the results from the counting of half of the TLC plates of these incubations, the incorporation in the inhibitor tubes was 81 % of the control for the 0.2 µM and 8 % of the control for the 2.0 µM. The triterpenols from the remaining half of each TLC plate were scraped, eluted and examined by HPLC to verify that the radioactivity observed by TLC was indeed associated with the triterpenols. The percentage of the radioactivity associated with each of the triterpenols from the three tubes are given in table 2. The level of radioactivity present in the 2 µM tube was very low which introduces a substantial error into the percentages obtained from that tube.

TABLE 2

Compound	Control	0.2 μM	2μM
lanosterol	8.6	10.5	**
butyrospermol	41.6	40.1	49.5
24-methylenelanosterol	4.0	3.5	8.4
cycloartenol	15.1	14.8	15.0
24-methylenecycloartenol	24.7	24.6	13.1
unknown E	6.0	6.6	14.0

**note: lanosterol and butyrospermol were collected together. Their combined incorporation is represented by the percentage given under butyrospermol.

Experiment 2: Four tubes were prepared containing ^{3}H-mevalonic acid (0.2 μmole, 75 μCi/μmole), DTE (1 μmole), and 0, 0.2, 4.0, and 10 nmoles of tridemorph. Latex (100 μL) was added to each tube and they were incubated for 2 hours. After work-up and TLC as usual, the results were obtained by counting half of each triterpenol and triterpene ester band from the TLC plates in the gel phase. The incorporation observed in the inhibited tubes as a percent of control was: 93.8 % (2 μM), 16.3 % (40 μM), and 7.4 % (100 μM). The remaining halves of the triterpenol bands were scraped, eluted and examined by HPLC. The percentage of the radioactivity associated with each of the triterpenols is given in table 3.

TABLE 3

Compound	Control	2 μM	40 μM	100 μM
lanosterol	8.4	**	17.4	19.7
butyrospermol	21.8	33.6	32.7	38
24-methylenelanosterol	4.1	5.3	7.1	5.3
cycloartenol	24.9	20.4	12.0	9.2
24-methylenecycloartenol	34.8	34.3	22.9	17
unknown E	6.0	6.4	7.8	10.5

**note: lanosterol and butyrospermol were collected together. Their combined incorporation is represented by the percentage given under butyrospermol.

Experiment 3: Four tubes were prepared containing ^{3}H-mevalonic acid (0.2 μmole, 75 μCi/μmole), DTE (1 μmole), and 0, 0.4, 0.8, or 1.2 nmoles of tridemorph. Latex (100 μL) was added to each tube. The tubes containing tridemorph immediately began changing colors, turning purple. An addition of DTE (1 μmole) reversed this for the 4 μM tube. The higher tridemorph concentrations turned to a light gray and remained that color throughout the

incubation (2 hours). After the usual quenching, work-up, and chromatography it was clear that the biosynthesis in the colored tubes was very strongly inhibited. The tube with 4 μM tridemorph showed 81.4 % of the incorporation of the control. The 8 μM and 12 μM tridemorph tubes had incorporations of 8.0 % and 1.5 % of the control by TLC and had nearly a 1:1 ratio of label in the triterpenols and the triterpene esters (which we have learned is a good indication that something was drastically wrong). There was not enough radioactivity on the TLC plates from the two strongly inhibited incubations to permit an examination by HPLC. The results produced from these two tubes were discarded. The triterpenols from the control and the 4 μM tridemorph tube were srcaped, eluted, and examined by HPLC. The percentage of the radioactivity found in each of the triterpenols from these incubations are given in table 4.

TABLE 4

compound	control	4 μM
lanosterol	7	9
butyrospermol	37	42
24-methylenelanosterol	3	2
cycloartenol	27	21
24-methylenecycloartenol	15	16
unknown E	10	9

Experiment 4: Conducted in the same manner as experiment 3 with the exception that 20 mM MES, pH 5.5, was added to maintain the normal pH of the latex and the concentrations of tridemorph used were 0, 4, 10, and 20 μM. The color changed was again observed when the latex was added. The addition of more DTE (to a concentration of 30 mM) kept the color from going too dark. From the TLC results, the incorporations observed in each of the inhibited tubes (as a percent of the control) was 165 % for the 4 μM, 51 % for the 10 μM, and 33 % for the 20 μM. This sample of latex had poor activity which made it impossible to examine the label in the individual triterpenols by HPLC.

Experiment 5: Six reaction tubes were prepared containing MES, pH 5.5 (1 μmole), DTE (1 μmole), ^{3}H-mevalonic acid (0.4 μmole), and tridemorph (0, 0.2, 0.6, 1.0, 2.0, or 4.0 nmole) blown to dryness under a stream of nitrogen. Latex (100 μL) was added to each tube and they were allowed to incubate for 2 hours. The incorporation in each of the inhibited tubes

(expressed as a percentage of that observed in the control) is given in table 5. The triterpenols from these TLC plates were scraped, eluted and examined by HPLC. No radioactivity was found to coelute with the triterpenol mass peaks from the 20 μM tridemorph incubation. The percent distributions of the radioactivity coeluting with the individual triterpenols from the other five tubes are given in table 6 (compound unknown E was omitted from these calculations).

TABLE 5

[tridemorph]	incorporation (%)
2	99.4
6	43.5
10	13.7
20	5.2
40	6.3

TABLE 6

Compound	Control	2 μM	6 μM	10 μM	40 μM
lanosterol	10.7	11.0	14.1	14.5	16.6
butyrospermol	31.5	29.8	23.8	32.4	31.4
24-methylenelanosterol	6.5	5.6	4.8	8.5	9.8
cycloartenol	17.2	19.1	13.3	14.8	14.9
24-methylenecycloartenol	34.0	34.4	44.0	29.9	27.4

Effect of Fenpropimorph on Triterpenoid Biosynthesis in latex: Six tubes were prepared containing MES, pH 5.5 (1 μmole), DTE (1 μmole), ^{3}H-mevalonic acid (0.4 μmole, tubes 1-5 at 75 μCi/μmole, tube 6 at 150 μCi/μmole), and fenpropimorph (0, 0.5, 1.0, 5.0, 10, or 20 nmole). Latex (100 μL) was added to each and the tubes were incubated for 2 hours. The incubations were quenched and work-up in the same manner as the tridemorph experiments. The results from the counting of the TLC plates are shown in table 7; expressing the incorporation into the triterpenols and the triterpene esters by each tube as a percentage of the incorporation observed for the control. The triterpenols from the control and the incubation with the highest concentration (20 μM) of fenpropimorph were scraped, eluted and examined by HPLC. The distribution of the radioactivity associated with each of the individual triterpenols is given in table 8. Due to the very minor differences in these two distributions, the triterpenols from the

remaining incubations were not examined.

TABLE 7

[fenpropimorph] (μM)	activity
0.5	81 %
1.0	113 %
5.0	95 %
10	79 %
20	80 %

TABLE 8

compound	control	20 μM
lanosterol+butyrospermol	38.4	35.8
24-methylenelanosterol	3.3	3.4
cycloartenol	24.9	21.8
24-methylenecycloartenol	26.6	31.0
unknown E	6.9	8.0

Synthesis of 4-R-4-^{3}H-Mevalonic Acid: Methyl 3-hydroxy-3-methyl-4-pentenoate: Methyl vinyl ketone (18.6 g) and methyl bromoacetate (38 g) were dissolved in benzene (100 mL). A portion (30 mL) of this solution was added to zinc dust (20 g) in benzene (100 mL) under nitrogen with vigorous stirring. This solution was refluxed for one hour before the remainder of the methyl vinyl ketone and methyl bromoacetate solution was added dropwise to the reaction flask. The reaction was refluxed for an additional hour then allowed to cool to room temperature. Ice cold aqueous acetic acid (20 mL in 180 mL of water) was added and the layers were allowed to separate. The aqueous layer was saturated with NH_4Cl and was extracted several times with ether. The combined organic extracts were washed with saturated sodium bicarbonate and saturated NaCl and dried over $MgSO_4$. The solvents were removed under reduced pressure and the product distilled (83-85°, 18 mm). Yields ranged from 32 to 46 %. The highest yields were obtained when all of the starting materials were rigorously purified. NMR: 1.33, s, 3H; 2.59, d , 2H; 3.69, s, 3H; 3.91, br. s., 1H (OH); 5.06, dd, J=0.7, 10.68, 1H; 5.27, dd, J=0.7, 17.3, 1H; 5.93, dd, J=10.65, 17.3, 1H.

Mixture of cis and trans methyl 5-acetoxy-3-methyl-3-pentenoate: methyl 3-hydroxy-3-methyl-4-pentenoate (9.0 g) was dissolved in petroleum ether (18 mL) and pyridine (1.5 mL) and cooled to -7°. A solution of PBr_3 (2.7 mL) in petroleum ether (10 mL) was added dropwise over half an hour. The reaction was stirred for 2 hours during which time the temperature was allowed to climb to 5°. Ice water was added and the reaction was stirred for an additional 15 minutes. The layers were separated and the aqueous layer was extracted with fresh petroleum ether. The combined organic extracts were washed with saturated sodium bicarbonate and saturated NaCl and dried over $MgSO_4$. The petroleum ether was evaporated in vacuo. Dry acetone (250 mL) was used to dissolve the crude bromide which was then added to freshly powdered potassium acetate (36 g). This suspension was either stirred vigorously or shaken for at least 48 hours (better yields were obtained from those reactions which were shaken). The reaction was then filtered and the solvents removed in vacuo. The residue was dissolved in ether, washed with saturated sodium bicarbonate and dried. The ether was removed in vacuo and the product vacuum distilled. A large amount of the dienes resulting from elimination were distilled under aspirator vacuum (60-70°) before the desired product mixture was collected using a vacuum pump (87-90°, 1 mm). The dienes produced appear by NMR to be about a 1:1 mixture of cis and trans methyl 3-methyl-2,4-pentadienoate. The desired product is about a 2:1 mixture of the trans:cis acetate. Yields ranged from 23 % to 33 %. NMR (trans): 1.79, s, 3H; 2.06, s, 3H; 3.06, s, 2H; 3.69, s, 3H; 4.6, m, 2H; 5.6, m, 1H. NMR (cis): 1.85, s, 3H; 2.05, s, 3H; 3.16, s, 3H; 3.69, s, 3H; 4.6, m, 2H; 5.5, m, 1H.

Hydrolysis of the mixture of cis and trans methyl 5-acetoxy-3-methyl-3-pentenoate: The starting material (11.7 g) was dissolved in 0.1 N NaOH (1 L) and stirred for 24 hours at room temperature. The solution was neutralized with sulfuric acid and concentrated in vacuo to a volume of 50 mL. This concentrated solution was acidified (to pH 1), saturated with NH_4Cl and repeatedly extracted with ether. The combined ether extracts were dried and the solvents were removed in vacuo. The products were distilled under reduced pressure. Upon heating, the cis acid lactonizes and was readily distilled (86-88°, 1 mm). The oil bath was then lowered and

preheated to 150° before being raised to distill the trans acid (125-140°, 1 mm). The fraction containing the lactone was found to be ca. 90 % lactone and 10 % free acid by ^{1}H-NMR. ^{1}H-NMR, lactone: 1.78, s, 3H; 2.96, br. s, 2H; 4.78, br. s, 2H; 5.67, br. s, 1H. ^{1}H-NMR, cis acid: 1.67, s, 3H; 3.0, s, 2H; 3.62, br. s, 2H; 5.4, br. s, 1H. The fraction containing the trans acid required further purification. It was found to be contaminated with a variety of partial hydrolysis prdoucts. All of the partial hydrolysis products were much less polar than the desired acid and were easily removed by flash chromatography, eluting with a gradient of $CHCl_3$:CH_3OH (from 100:0 to 50:50). ^{1}H-NMR, trans-5-hydroxy-3-methyl-3-pentenoic acid: (acetone-d_6) 1.71, s, 3H; 2.99, s, 2H; 4.10, d, J=6.1, 2H; 5.48, t, J=6, 1H.

Trans-N-diphenylmethyl-5-hydroxy-3-methyl-3-pentenamide: DCC (0.78 g) and diphenylmethyl amine (0.70 mL) were dissolved in methylene chloride and heated to reflux. Trans-5-hydroxy-3-methyl-3-pentenoic acid (0.5 g) was dissolved in methylene chloride, heated to reflux and added rapidly with shaking to the refluxing solution of the amine. The reaction is very rapid and very exothermic. The flask was shaken until the reaction subsided, then filtered to remove the dicyclohexyl urea. The methylene chloride was removed on a steam bath and the crude amide was recrystallized from benzene. The pure product was obtained after a second recrystallization in 68 % yield. Anal. Calc for $C_{19}H_{21}NO_2$: C, 77.26; H, 7.17; N, 4.74. Found: C, 77.07; H, 7.29; N, 4.88. ^{1}H-NMR: 1.74, s, 3H; 3.03, s, 2H; 4.185, t, J=5.9, 2H; 5.59, t, J=6, 1H; 6.25, br. s, 2H; 7.18-7.36, m, 10H.

N-diphenylmethyl-trans-3,4-epoxy-5-hydroxy-3-methylpentamide: A $CHCl_3$ (6 mL) solution of trans-N-diphenylmethyl-5-hydroxy-3-methyl-3-pentenamide (0.4 g) was cooled in an ice bath while a solution of mCPBA (0.26 g) in benzene (30 mL) was added. The reaction was allowed to warm to almost room temperature and was allowed to sit overnight at 4°. The reaction was extracted twice with 1 M KOH then twice with saturated NaCl. The organic layer was then dried and the solvents removed in vacuo. The product was isolated as an oil which was very hygroscopic. ^{1}H-NMR: 1.36, s, 3H; 2.42, d, J=14.7, 1H; 2.60, d, J=14.7, 1H; 3.066, dd, J=4.9, 6.1, 1H; 3.70, dd, J=6.2, 12.1, 1H; 3.81, dd, J=4.8, 12.1, 1H; 6.25, d, J=8.3, 1H;

6.80,br. d, J=8.2, 1H; 7.2-7.4, m, 10H. This product was not entirely pure, for use in the reaction with the NaB^3H_4 a sample was purified by PTLC (1mm silica, developed in 1:1 toluene: acetone).

N-diphenymethyl-mevalonamide: N-diphenylmethyl-trans-3,4-epoxy-5-hydroxy-3-methyl-pentamide (50 mg) was placed in a small round bottom flask under nitrogen. $LiBH_4$ (2 mg) was dissolved in THF (0.8 mL), which had been freshly distilled from sodium/benzophenone under nitrogen, and was added to the flask with the epoxide. The $LiBH_4$ was rinsed in with an additional 0.8 mL of THF and the reaction was refluxed for 1.5 hours. Methanol (0.2 mL) was added and the solution was refluxed for an additional 15 minutes. Water (2 mL) was added and the mixture was acidified to pH 2 with dilute phosphoric acid. The organic solvents were removed under a stream of nitrogen, more water was added and the flask was allowed to stand overnight. The product crystallized and was collected and dried. ^{1}H-NMR: 1.31, s, 3H; 1.6, m, 2H; 2.338, d, J=14.7, 1H; 2.614, d, J=14.7, 1H; 3.81-3.92, m, 2H; 6.28, d, J=8.1, 1H; 6.75, br. d, J=7.9, 1H; 7.2-7.4, m, 10H. For reduction with $NaBH_4$, the same procedure was followed, but the reaction was refluxed for 4 hours instead of 1.5.

N-diphenylmethyl-4R-4-^{3}H$_1$-mevalonamide: The vial containing NaB^3H_4 (NEN, 25 mC_i, 319 mC_i/mmole) was rinsed three times with a total of 12 mL of THF which was added to N-diphenylmethyl-trans-3,4-epoxy-5-hydroxy-3-methylpentamide (44 mg) in 4 mL of THF. The vial was rinsed with Aquassure and an aliqout of this was counted. It was determined that $3.8x10^9$ dpm (1.7 mC_i) had been left in the vial. The reaction was refluxed for 10 hours, methanol was added and the reaction was refluxed for an additional 20 minutes. Water was added and the mixture acidified using dilute phosphoric acid. The organic solvents were removed on a hot plate and the solution allowed to cool. No crystals were observed. The solution was then extracted with ether and the ether extracts were evaporated under a stream of nitrogen. The ether solution was then chromatographed (PTLC) and the pure product isolated in 22 % yield. The 9.7 mg of product were found to contain $3.22x10^9$ dpm for a specific activity

of 47 mCi/mmole. The chemical and radiopurity of this product was verified by HPLC.

4-R-4-$^{3}H_1$-mevalonic acid: To N-diphenylmethyl-4R-4-$^{3}H_1$-mevalonamide (9.7 mg) was added a solution (1.5 mL) of 2.5 N NaOH in 50 % aqueous methanol. The reaction was stirred for 30 minutes at room temperature, then heated to reflux and stirred for an additional 1.5 hours. The reaction was cooled, water (2 mL) was added and the mixture was extracted several times with ether. N,N-bis benzyl ethylenediammine (3.77 mg) in methanol (3 mL) was added to the aqueous layer. This solution was stirred at room temperature for 18 hours, then blown to dryness under a stream of nitrogen. The residue was taken up in methanol and an attempt was made to crystallize the product. No crystallization was observed. The tritiated MVA was purified on the HPLC using an ODS column (analytical) eluted with a phosphate buffer (10 mM, pH=2.6) with a flow rate of 1 mL/min. Under these conditions the free acid elutes at about 14 min. The lactone elutes at about 17 min. Having first injected known amouts of cold potassium mevalonate and measured the responses, it was possible to determine the mass of the ^{3}H-mevalonic acid that was injected. Virtually all of the radioactivity was associated with the peak corresponding to mevalonic acid, with a small amount eluting as the lactone. The labelled mevalonic acid was collected, basified using KOH and blown down to a small volume under a stream of nitrogen. An aliqout was removed, diluted several times and counted. From this it was determined that the vial contained 50 µCi. From the calibration of the HPLC detector it was determined that the specific activity of the ^{3}H-mevalonic acid was 33 µCi/µmole.

Incubation with latex: Ten µCi of ^{14}C-mevalonic acid was added and a portion of the solution was transferred to a vial for use in an incubation. Ten percent of this material was removed and 5% of that material was counted. From the results of this counting it was determined that there was 33 µCi of ^{3}H and 6.27 µCi of ^{14}C actually in the incubation tube. The exact ratio of $^{3}H/^{14}C$ was determined by this scintillation counting to be 4.96. Cold mevalonic acid (1 µmole) was added to the prepared $^{3}H/^{14}C$-mevalonic acid in the incubation tube to give a total mass of mevalonic acid of 2 µmole. Latex (0.5 mL) and DTE (2 µmole) were added to the tube and the tube was incubated for 5 hours at room temperature. The incubation was

quenched and worked up like normal. The triterpenols and triterpene esters were eluted from the silica gel and an aliquot of the triterpenols was counted. There was a much higher ratio of $^{3}H/^{14}C$ than expected (19:1 instead of 4.9:1). The triterpenols were then analysed by HPLC (using the semi-preperative ODS column) and the $^{3}H/^{14}C$ ratios of the individual compounds was determined. The expected value for cycloartenol and 24-methylenecycloartenol is 4.96. The values determined from the counting of the HPLC purified products were 4.81 for cycloartenol and 5.10 for 24-methylenecycloartenol (only 3 % off from the expected value). There was much less radioactivity in the lanosterol peak from the HPLC which made it very difficult to obtain an accurate count of the dpm present in each isotope. With the small number of dpm present, the subtraction of the background and the overlap of the two windows introduced a considerable error. The "best" value for the $^{3}H/^{14}C$ ratio for lanosterol was determined to be 5.10. This value would be correct if lanosterol did not lose the proton of interest (C-8 of cycloartenol). A value of 4.2 would be expected for a compound which lost one tritium atom.

Synthesis of 3'-D_3-mevalonic acid: Ethyl D_3-acetate: A solution of absolute ethanol (5 mL) and conc. sulfuric acid (5 mL) was heated to near reflux under nitrogen. A solution of ethanol (20 mL) and D_3-acetic acid-D (19g) (Aldrich) was added dropwise. The reaction was refluxed for one hour then cooled to room temperature. The product was distilled along with some of the ethanol. The distillate was then washed successively with water, satd. bicarbonate and 40% calcium chloride solution. The product was then dried over $MgSO_4$; yielding 25.1g (93%).

3-(D_3-methyl)-1,4-pentadien-3-ol: Using the method described by Tschesche [31], the product was obtained from ethyl D_3-acetate in a 65% yield. Magnesium turnings (18.0g) in a 3-necked round-bottom flask were dried in an oven then cooled to room temperature under nitrogen. To the turnings was added a solution of dry ether:THF 1:1 (60mL). In an addition funnel were placed allyl bromide (52mL), ethyl D_3-acetate (17.6g), and ether:THF 1:2 (165mL). This solution was added dropwise to the turnings at a rate which kept the vigorous reaction at a

mild reflux. After the addition was completed the funnel was rinsed with ether:THF 1:2 (60mL) and this rinse was added to the reaction which was stirred for an additional hour, during which time the reaction mixture solidified. The reaction was cooled to 0°, quenched with cold water and acidified with cold 6 M H_2SO_4. After extraction, the ether layer was dried and the solvent removed in vacuo. The product was distilled (bp 72-75, 21mm) yielding 21.4g (85%). ^{1}H-NMR ($CDCl_3$) 2.21, d, J=7.5, 4H; 5.1, m, 4H; 5.9, m, 2H. ^{13}C-NMR ($CDCl_3$) 25.49, septet, J_{C-D}=19.4 Hz, 45.90, 71.39, 118.24, 133.78.

3-Hydroxy-3-(D_3-methyl)-glutaric acid: A solution of 3-(D_3-methyl)-1,4-pentadien-3-ol (5.26g) in CH_2Cl_2:acetic acid 10:1 (200mL) was cooled to -78°. Ozone was bubbled through the solution until a deep blue color persisted. A stream of oxygen was bubbled into the solution to remove the excess ozone and the CH_2Cl_2 was removed at room temperature in vacuo. Hydrogen peroxide, 30% (40mL) and acetic acid (15mL) were added to the remaining solution and it was then refluxed overnight. The solvents were removed in vacuo and the crude product crystallized. This material was recrystallized from acetone:benzene yielding 5.14g (76%). The product was characterized by ^{1}H-NMR, ^{13}C-NMR, elemental analysis and mass spectroscopy.

3-Hydroxy-3-(D_3-methyl)-glutaric anhydride. 3-Hydroxy-3-(D_3-methyl)-glutaric acid was reacted with an excess of acetic anhydride under nitrogen at room temperature overnight. Removal of the solvent at room temperature in vacuo gave a quantitative yield of the anhydride. This material could either be used as is or could be recrystallized from $CHCl_3$. ^{1}H-NMR (d_6-acetone): 2.85, d, J=16, 2H; 3.2, d, J=16, 2H. ^{13}C-NMR (CD_3COCD_3): 44.13, 67.58, 167.3 (the methyl carbon was obscured due to C-D splitting; it appeared at 27.7 in the unlabelled compound). Analysis: Calc'd for $C_6H_5D_3O_4$: C,43.63; H,6.10; found: C,43.81; H,6.16. MS 148(M+1), 1.1%; 103, 2.9%; 85, 2.4%; 75, 21.4%; 61, 27.3% b.p.

N,N'-dibenzylethylenediammonium bis-(3,5-dihydroxy-3-(D_3-methyl)-pentanoate). 3-Hydroxy-3-(D_3-methyl)-glutaric anhydride (0.89g) was dissolved in 30 mL of isopropanol.

$NaBH_4$ (0.33g) was added and the reaction was stirred at room temperature for 2 days. Water (10 mL) was added and the solution acidified to pH 1. After stirring at room temperature for 30 min. the solution was freeze dried. The residue was washed with hot $CHCl_3$ which was then filtered to remove the insolubles and dried over $MgSO_4$. Removal of the $CHCl_3$ in vacuo gave a viscous oil. This crude lactone was then treated with N,N'-dibenzylethylenediamine as described by Hoffman (6), by dissolving the lactone in water (2.5 mL) and adding N,N'-dibenzylethylenediamine (0.5g) in methanol (2.5 mL) and allowing the solution to stand overnight. The methanol was removed in vacuo and the remaining solution was extracted with $CHCl_3$. The aqueous layer was freeze dried and the residue dissolved in hot methanol (2 mL). Ether (20 mL) was added and the product was allowed to crystallize. The mother liquor and the $CHCl_3$ layer were combined and again treated with N,N'-dibenzylethylenediamine and was worked up in the same manner. The combined, crystallized yield was 0.66g (40%). ^{1}H-NMR (CD_3OD): 1.82, t, J=7.4, 4H; 2.36, s, 4H; 3.40 , s, 4H; 3.73, t, 7.4, 4H; 4.25, s, 4H; 7.5, m, 10H. ^{13}C-NMR (D_2O): 43.1, 43.5, 48.3, 52.1, 58.5, 71.3, 129.9, 130.0, 130.3, 131.3, 180.7 (the methyl group was obscured due to C-D splitting; it appeared at 29.9 in the unlabelled sample). Analysis: Calc'd for $C_{28}H_{38}D_6N_2O_8$: C,61.97; H,8.17; N,5.16. Found: C,61.80; H,8.12; N,5.15. FAB mass spectrum showed a negative ion of mass 150 (labelled mevalonate) and the positive ion of mass 241 ($C_{16}H_{21}N_2^+$).

Incubation of D_3-mevalonic acid with latex: In a 5000xg pellet: Initially, the substrate used was isolated as the lactone and opened with KOH just prior to use in the incubation. Latex (1 mL) was centrifuged for 5 minutes at 5000xg. The pellet was solubilized in 1 mL of centrifugation buffer and transferred to a vial containing NADPH (0.3 mg) and ATP (0.95 mg). To this solution was added 5 µL of a 1 M solution of the labelled mevalonic acid. The incubation was allowed to proceed overnight. It was then quenched and worked up in the usual manner. The triterpenols were isolated from the TLC plate (4.21 mg) and were separated into the individual compounds on the HPLC using a semi-preparative ODS column. The lanosterol isolated from this

incubation was examined by probe mass spectroscopy using EI (70 eV). A close examination of the region from mass 426 to 444 showed some traces of hydrocarbon impurities, but did not show any ions corresponding to an incorporation of 17 or 18 deuterium atoms. The triterpene esters from this incubation were hydrolyzed and the resulting triterpenols were separated by HPLC. The lanosterol resulting from the triterpene esters was examined by mass spectroscopy. Again, no sign of a mass due to the presence of the deuterium atoms could be found.

Incubation with whole latex and the pellet: 3'-D_3-mevalonic acid (2 µmole, purified by two PTLC plates as the lactone, opened the lactone using KOH, 40° for 4 hours) and DTE (2.5 µmole) were placed in a tube and blown to dryness under a stream of nitrogen. Latex (1 mL) was added and the tube was allowed to incubate for 6 hours. The latex was then centrifuged (5000xg, 5 min.). The pellet was solubilized in centrifugation buffer (200 µL) and incubated with fresh 3'-D_3-mevalonic acid (0.4 µmole) for 4 hours. This suspension was then centrifuged again (5000xg, 5 min.), the pellet was removed and quenched with acetone. The acetone solubles were then partitioned into water and hexane and the hexane solubles were chromatographed (TLC, 3:1 ether:petroleum ether). The triterpenols were isolated from the TLC plate and separated by HPLC. The lanosterol isolated from the HPLC was then re-injected and the peak re-collected to be certain that there was no contamination of the lanosterol by the neighboring compounds. The lanosterol thus isolated was examined by mass spectroscopy. Unfortunately, there was still no sign of any peak corresponding to the incorporation of the deuterated substrate.

Incubation in combination with ^{3}H-mevalonic acid: In order to determine the actual incorporation, it was decided to add a small amount of ^{3}H-mevalonic acid to the incubation. A tube was prepared containing ^{3}H-mevalonic acid (5 µCi), 3'-D_3-mevalonic acid (3.75 µmole), and DTE (2 µmole). Latex (1 mL) was added and the tube was incubated for 4 hours at room temperature. Centrifugation buffer (1 mL) was added to the tube and the contents were centrifuged (5000xg, 10 min.). The supernatant was removed and hexane was added to it. The pellet was solubilized in centrifugation buffer (500 µL) and transferred to a tube containing

3'-D_3-mevalonic acid (1 µmole) and ^{3}H-mevalonic acid (5 µC_i). This solution was allowed to incubate for 3.5 hours. It was then centrifuged (5000xg, 10 min.) and the supernatant was removed and added to the previous supernatant. The pellet was cut out of the microfuge tube and rinsed out with acetone. The acetone solubles were then partitioned into water and hexane and the hexane solubles were chromatographed. The triterpenols and the triterpene esters were eluted from the silica gel and an aliqout of each was counted. From the counting of the aliquouts it was determined that the incubation had produced 2.0 nmoles of triterpenols and 2.5 nmoles of the triterpene esters. The triterpenols were then separated on the HPLC using two analytical ODS columns. The mass of the triterpenols was calculated from the detector response observed (using the calibration given in chapter 1). The total mass of the triterpenols was found to be 168 µg. Using a molecular weight of 430 for the triterpenols, 2 nmoles represents 860 ng, or 0.09 % of the total mass.

The mass spectrum of the lanosterol isolated from this incubation was examined very closely. There was a small amount of an impurity of mass 440 which showed the usual M+1 and M+2 peaks which made it difficult to determine if there was an ion of mass 443 present above the background noise level. Small peaks were visible at 443.8 and 444.6, but each of these was more than a mmu off from the calculated values for lanosterol with 17 or 18 deuterium atoms. The triterpene esters from this incubation were hydrolyzed and the resulting triterpenols were isolated and separated on the HPLC. The total mass observed on the HPLC was 0.6 µg. From the radioactivity present in the sample it was determined that there was 7.8 ng of labelled triterpenols present, or 0.2 % of the total mass (the values for the individual compounds were not determined). The mass spectrum of the lanosterol isolated from this incubation showed a large amount of contamination. There were many hydrocarbon impurities and a contaminant of mass 440 (which was almost 20% of the intensity of the 426 ion). It was hoped that these contaminations could be dealt with through the use of GC-MS. Unfortunately, there was not enough material left from this incubation to attempt a derivatization and examination by GC-MS.

The incubation was repeated in exactly the same manner. This incubation yielded 1.3

nmoles of triterpenols and 4.6 nmoles of the triterpene esters. The triterpenols were separated on the HPLC and the mass of each compound was determined from the detector response. An aliquout of the material collected from each peak eluting from the HPLC was counted to determine the exact amount of incorporation in each compound. The incorporation into each of the triterpenols was below 0.1 %. The triterpene esters from this incubation were hydrolyzed and the resulting triterpenols were separated on the HPLC in the same manner. The radioactivity present in the lanosterol peak was found to represent 0.17 % of the total mass. All attempts at probe mass spectroscopy failed to show any ions corresponding to an incorporation of any deuterium. The remaining mass was derivatized using BSTFA to the trimethyl silyl ether. This material was examined by GC-MS. The peak for lanosterol was easily detected but the instrument lacked the sensitivity to detect the minor ions which would have been produced by the incorporation observed.

Incubation with the DBED salt: A reaction tube was prepared containing ^{3}H-mevalonic acid (20 μCi) and N,N'-dibenzylethylenediammonium bis 3'-D_3-mevalonate (5 μmole). Latex (1 mL) from young plants (less than 2 mo.) was added and the tube was incubated for 2 hours. The latex was then diluted 1:1 with centrifugation buffer (0.4 M sorbitol, 60 mM MES, pH 5.5, 5 mM DTE, 5 mM $MgCl_2$) and centrifuged (5000xg, 10 min.). The supernatant was removed and the pellet was solubilized in fresh buffer (500 μL). The pellet was then transferred to a tube containing ^{3}H-mevalonic acid (10 μCi) and DBED bis 3'-D_3-mevalonate (2.5 μmole). This incubation was allowed to proceed for 3 hours before being centrifuged (5000xg, 10 min.). The supernatant was removed and the pellet was cut out of the tube and solubilized in acetone. The acetone solubles were partitioned into water and hexane and the hexane extract was chromatographed on TLC. The triterpenols and the triterpene esters were isolated from the TLC plate and an aliquot of each was counted. The triterpenols were then separated on HPLC. The mass of the individual triterpenols was calculated from the detector response and the incorporation into each triterpenol was determined from the radioactivity present. The highest percentage of labelled material in the total mass was found to be less than 0.03 %. This was far

below the noise level we had observed from the mass spectrometer and was therefore not examined by mass spectroscopy. The triterpene esters from this incubation were hydrolyzed and the resulting triterpenols were separated on HPLC. The amount of labelled compound in the total mass was determined in the same manner as for the free triterpenols. The percentage of labelled mass in the total mass of lanosterol from the triterpene esters was found to be 0.27%. We felt this was high enough that it might be observable by mass spectroscopy. Unfortunately, there were no visible ions corresponding to incorporation of the deuterium label.

This experiment was repeated with slightly longer incubation times. That sample of latex produced more of the labelled products, but was not as well delipidated and showed the same percentage of labelled material in the total mass.

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CONCLUSION

During the course of our study of triterpenoid biosynthesis in Euphorbia lathyris latex we have tried many different approaches to investigating several aspects of the process. We successfully elucidated the structures of those triterpenols which had not previously been known. A method for quantifying very small amounts of these compounds was developed. The chemistry of the latex is now fairly well understood.

A wide variety of experiments were performed concerning the biochemistry of the latex. The addition of various cofactors and cations was examined. In general, no exogenous cofactors are required for the biosynthesis and the addition of compounds such as NADPH and ATP do not stimulate the biosynthesis. The addition of DTE or a similar anti-oxidant was found to help reduce the oxidation of the latex, thus increaing the length of time that the latex remains active. The requirement of a divalent cation and the preference for Mn in the pellet was observed. Many attempts were made to observe incorporation of squalene. The fact that none of these provided any incorporation is probably due to the compartmentation of the latex and the absence of any system for the transport of squalene across the membrane.

Many time dependent incubations were done, investigating the stability of the whole latex system and the re-suspended pellet systems. The effects of centrifugation on the biosynthesis were examined. The ability of the pellet to synthesize the triterpene esters was found to be much lower than its ability to synthesis the free triterpenols (as compared to the whole latex). The partitioning of the triterpenoid pool as a function of when the compounds were made was examined. The most recently synthesized triterpenoids are concentrated in the pellet as compared to those which were synthesized earlier which tend to be found at the top of the supernatant. Localisation of the acetate-to-triterpene and the mevalonate-to-triterpene activities by centrifugation was accomplished, with the pellet not having the ability to utilize acetate, but a reconstitued system showing good incorporation from both substrates. Several

attempts to delipidate the latex while maintaining some biosynthetic activity were tried. The only method which provided any delipidation that also maintained some biosynthetic activity was centrifugation.

A number of incubations were performed in the hopes of observing some interconversion of the individual triterpenols. No interconversion was ever observed, even from lanosterol to 24-methylenelanosterol or from cycloartenol to 24-methylenecycloartenol. Also, no interconversion of the triterpenols and the triterpene esters was ever observed.

The effect of several inhibitors on the biosynthesis of the triterpenoids was examined. Mevinolin was found to inhibit the biosynthesis of the triterpenoids from acetate, but not mevalonate. A Dixon plot of the inhibition of acetate incorporation showed an I_{50} concentration of 3.2 μM. The effects of the morpholine based fungicides, tridemorph and fenpropimorph were examined. Fenpropimorph was found to have little or no effect on the biosynthesis. Tridemorph was found to inhibit the biosynthesis of all of the triterpenoids with an I_{50} of 4 μM. It was also observed that the cyclopropyl containing triterpenols, cycloartenol and 24-methylenecycloartenol were inhibited much more strongly than those containing an 8-9 double bond, lanosterol and 24-methylenelanosterol. The evidence indicates, but does not definetely prove, that lanosterol and 24-methylenelanosterol are **not** made from cycloartenol and 24-methylenecycloartenol via a ring-opening enzyme such as cycloeucalenol-obtusifoliol isomerase.

The possibility that cycloartenol is made via lanosterol was investigated by synthesizing 4-R-4-^{3}H-mevalonic acid and incubating latex with a mixture of this and ^{14}C-mevalonic acid. From the $^{3}H/^{14}C$ ratio it was shown that cycloartenol and 24-methylenecycloartenol are **not** made via an intermediate containing an 8-9 double bond.

We attempted to obtain a definite answer to the question of whether lanosterol is made via cycloartenol by incubating the latex with 3'-D_3-mevalonic acid. The substrate was synthesized in good yield and the isotopic enrichment was found to be virtually 100 atom %. Several incubations with this substrate were performed. The major obstacle was trying to get as much of

the labelled material synthesized in the presence of the smallest possible amount of the endogenous triterpenoids. None of our attempts at delipidation was successful in obtaining a product whose mass spectum showed any signs of incorporation of the deuterium label. While this question was left without a definitive answer, it appears from all the evidence that lanosterol and cycloartenol are made independently of one another in the latex of Euphorbia lathyris.