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Biosynthesis of Tetrahymanol by *Tetrahymena pyriformis*: Mechanistic and Evolutionary Implications

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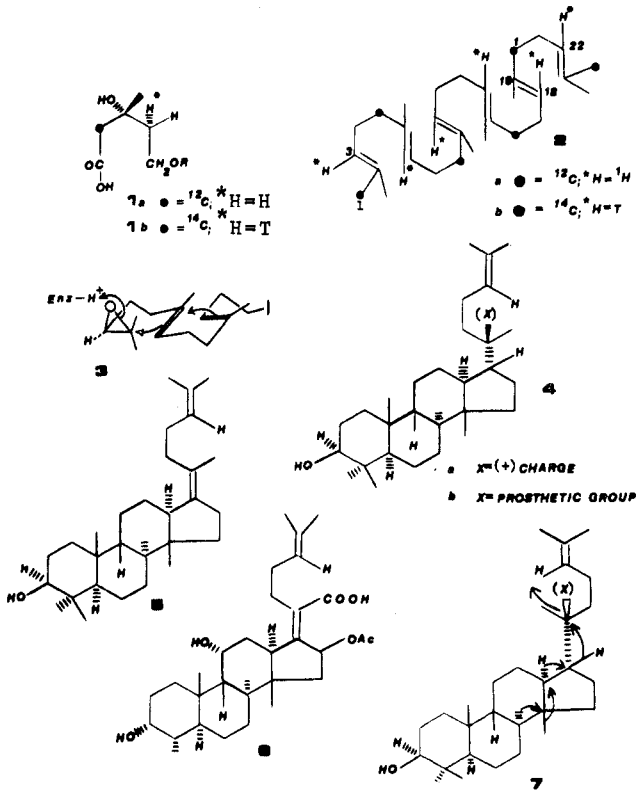
Polyprenoids constitute a diverse, widely distributed group of natural products derived biologically from (3*R*)-mevalonic acid[1,2] (1). Based on geochemical[3] and other studies,[4] it was inferred that the biosynthetic processes of polyprenoids evolved in the very early stages of evolutionary development.[5,6] Although polyprenoids play a vital role in numerous life processes, e.g., as sex or corticosteroidal hormones, as bile acids, in vision, in defense mechanisms, etc., it appears that their primary biological function is to provide a structural component(s) of cell membranes.[6]

Many eucaryotes, and particularly higher species, produce a variety of 3-oxygenated triterpenes and sterols.[1,2] The 3-oxygenated triterpenes are derived from squalene[1,2] (2) which is converted to 2,3(*S*)-oxidosqualene[7] (3). The enzymatic cationic cleavage of the oxygen–C-2 bond of 3 generates an electron deficiency at C-2 and precipitates the cyclization process. Depending on the characteristics of the enzyme system of the particular species, different products may be formed.[1,2,8,9] In *Fusidium coccineum*, cyclization[10] of 3 is presumed to result in the cation[8] **4a** or its transiently stabilized equivalent[9,11] **4b**, which loses the C-17 proton[12] to yield the parent protosterol[13] (5). The protosterol[13] (5) is then further metabolized to fusidic acid (6). This hypothesis is supported by the results of studies on the mode of incorporation of C-2,[12,14] C-4,[11] and C-5[15] hydrogen atoms of MVA into 6 and by the isolation from *Fusidium coccineum* of metabolites of the parent protosterol[13] (5). In the rat, yeast, etc., it is thought that cation 4, after rotating around the 17(20) bond, yields 7. Following the *four* 1,2 migrations, C-17β(H) → C-20(H), C-13α(H) → 17α(H), C-14β(CH_3) → 13β(CH_3), and C-8α(CH_3) → 14α(CH_3), indicated in 7, the C-8 cation 8 is obtained; this collapses with the loss of the C-9β hydrogen atom to yield lanosterol[8,9,16,17,18] (9). In plants (photosynthetic systems),



the rearrangement of the cation 8 proceeds one step further and the C-9β hydrogen shifts to C-8β to give 10

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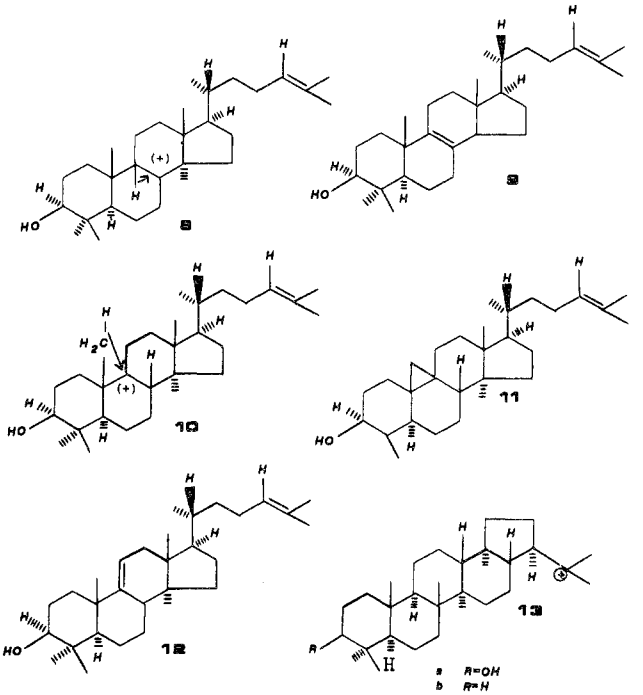
which is stabilized via the loss of a C-19 hydrogen to yield cycloartenol[19] (**11**). It is presumed that **10** is transiently stabilized by a 9α prosthetic group.[19] Alternatively, loss of a C-11 hydrogen will result in parkeol (**12**). It was proposed that cycloartenol (**11**) is the precursor of phytosterols.[20,21] Indeed, evidence was obtained that in peas cycloartenol or a C-19 carbanion is an intermediate in the elaboration of sitosterol.[22,23] In many organisms (e.g., plants, etc.) 2(3)-oxidosqualene can be cyclized to the pentacyclic hopenyl cation. The cation(s) **13a** may then undergo different rearrangements prior to stabilization and formation of a triterpene.[8,24,25]

The above indicates that, in general, the formation of the various types of triterpenes from oxidosqualene requires the participation of an *elaborate enzyme* system(s). The system(s) must have the capacity to hold squalene oxide (**3**) folded in the appropriate conformation for the cyclization to a particular cation and must then direct and control the complex rearrangements leading to the first stable product.[8,9]

It was established that the oxygen functions of squalene epoxide[26a] and of lanosterol[26b] are derived from



molecular oxygen. The question of the origin of terrestrial atmospheric oxygen is still being debated. Although most of the evidence favors a biotic–photosynthetic mechanism[27] of O_2 formation, arguments for a photodissociation[28] process have been presented. However, there appears to be a concensus evolving that the vast majority, or essentially all of the molecular oxygen in the atmosphere, is of photosynthetic origin.[27b] It is considered likely that squalene oxide was first biosynthesized when atmospheric oxygen became abundantly available.[6] This could occur only after the evolution of photosynthetic organisms, and particularly higher plants. Therefore, it may be inferred that the elaboration of the oxidative cyclization of squalene via 2,3-oxidosqualene is a relatively later, evolutionary development.

Geochemical studies of oils have revealed the existence in the anaerobic period of membraneous organisms containing polyprenoids and 3-deoxytriterpenes.[3,6] These organisms most likely biosynthesized 3-deoxyhopanoids and/or 3-deoxygammaceranes and presumably incorporated them into membranes.[6] The 3-deoxyhopanoids and -gammaceranes are thought to have been produced by an enzyme-mediated proton attack on a terminal double bond of squalene coiled in an all pro-chair conformation (**14**). The gammaceranyl cation (**15**) obtained could be stabilized in numerous ways. An enzyme-mediated acquisition of a hydroxyl or a hydride ion (from NADH or NADPH) would yield tetrahymanol (**16**) or gammacerane (**17a**), respectively. Elimination of a proton would result in dehydrogammacerane (**17b**). Alternatively, **15** could rearrange to the hopanoidyl cation **13b** which in turn could be stabilized in a number of ways. For example, loss of a proton would result in hop-22-ene (**18**), while acquisition of a hydroxyl would yield diplopterol (**26**). Obviously, the stabilization of the "gammacerane" and "hopane"

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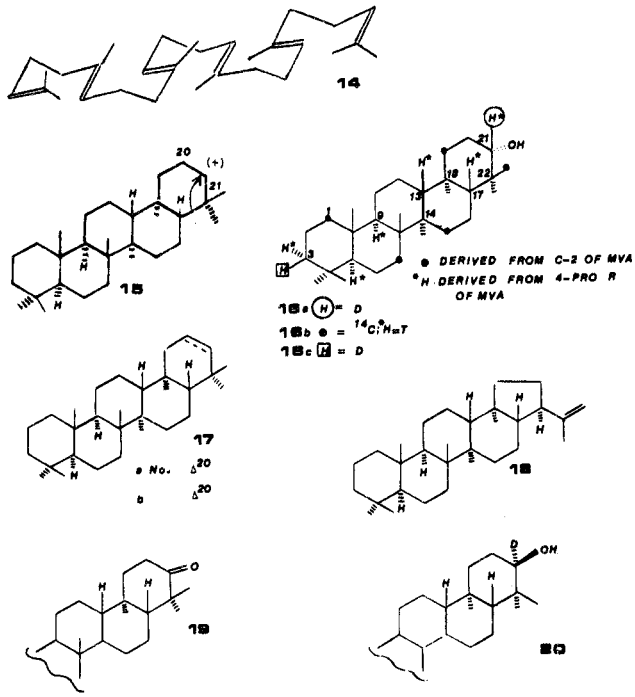
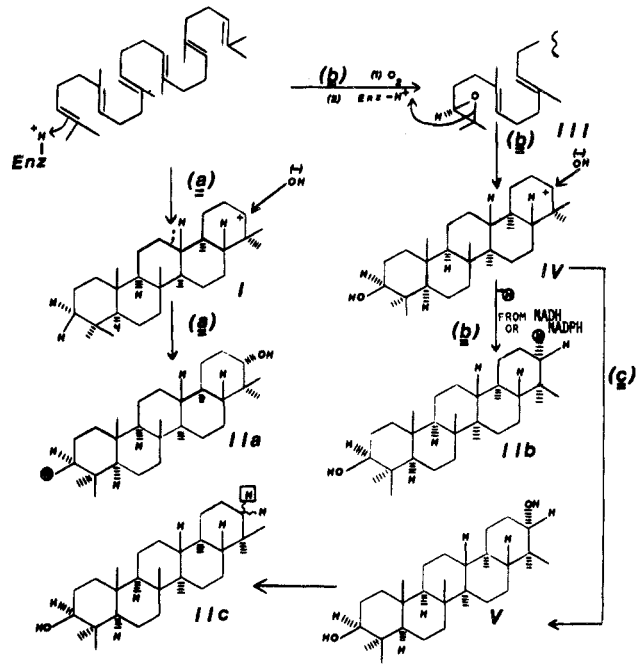


Scheme I



cations could involve additional rearrangements and result in a variety of other end products.[8,9b]

van Tamelen and his associates[29] have shown that in polar, water-containing systems, squalene is likely to be coiled in an all-pro-chair conformation with only the terminal double bonds exposed to an "external attack". It may therefore be inferred that in the aqueous environment of a cell, squalene is likely to assume a coiled, all-pro-chair conformation with no, or very little, enzymic assistance. Also, stabilization of the cation **15** to gammacerane- or hopane-type triterpenes is expected to require little enzymatic participation.[6] In summary, the elaboration of 3-deoxygammacerane and/or 3-deoxyhopane triterpenes is consistent with the enzyme capabilities of primitive organisms which are thought to have existed in the anaerobic period of evolutionary development.

In the early 1960s, when we first addressed the problem of the mechanism of nonoxidative cyclization of squalene, there was no evidence in support of the proposed biosynthetic hypothesis. For the studies, we required an organism in which the process could be tested, and we explored the use of certain primitive plants, ferns, lichens, or mosses which produce 3-deoxytriterpenes.[25,30] Fortunately, a lecture given by Professor R. B. Conner at a symposium attracted our attention to the protozoan *Tetrahymena pyriformis* which, in the absence of sterols, produces the pentacyclic triterpene tetrahymanol[31] (**16**). Subsequently, the triterpene was isolated from the fern *Oleandra walichi*.[32]

Initially, tetrahymanol was thought to be an isomer of cholesterol.[33] Later, Conner, Mallory, and their associates recognized that the product is a pentacyclic triterpene.[34] Finally, Tsuda, Mallory, et al.[35] defined its structure by correlating tetrahymanol with gammacerane and by comparison with a synthetic sample of **17a**. In cooperation with Drs. W. L. Duax, H. Berman, and their colleagues, we have carried out an X-ray crystal analysis of biosynthetic tetrahymanol and confirmed the proposed structure.[36] The tetrahymanol crystallized as a hemihydrate [$C_{30}H_{52}O{\cdot}0.5H_2O$] in two forms, both of which have the all *trans-anti-chair* structure. Steric overcrowding warps the gross conformation of the two molecules in the asymmetric unit and generates unusually long carbon–carbon single bonds. The C(8)–C(14) bond is particularly long (average length 1.61 Å) and, therefore, easily cleaved in the mass spectrometer (see below). The proton (^{1}H) 100-MHz spectra of tetrahymanol (**16**) and tetrahymanone (**19**) were recorded and the chemical shifts of their methyls were assigned.[37]

At the outset of our studies, we knew that [2-^{14}C]-acetate, [2-^{14}C]MVA, and [^{14}C]squalene are incorporated by *T. pyriformis* into tetrahymanol.[31] We assumed, therefore, that the biosynthesis of **16** proceeds in the conventional manner of polyprenoids, and that squalene is the key precursor. Also, it was tentatively assumed that the stereochemistry of the transformations of mevalonic acid to squalene will be the same as

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in rat liver, yeast, etc. (see below).

The biosynthesis of tetrahymanol from squalene can be rationalized in several ways, shown in Scheme I. If the process is initiated by an enzymatic cationic proton attack on a terminal double bond of squalene (path a), the resulting cation (I) could be stabilized by an enzyme-mediated acquisition of a hydroxyl to yield tetrahymanol (IIa). Alternatively, the squalene could be first oxidized to the epoxide III (path b), a step which would require molecular oxygen. Enzymatic cleavage of the oxygen–C(2) bond of the oxide (III) will initiate cyclization and give cation IV which could acquire a hydride ion from NADH or NADPH to yield tetrahymanol IIb. The possibility could also be considered that IV could acquire a hydroxyl group and yield the diol V (path c). The reductive elimination of one of the equivalent hydroxyl groups of the diol would then yield tetrahymanol IIc.

Several comments about the tetrahymanols obtained via routes a, b, and c are in order. Mechanistically, the oxygen atom of tetrahymanol IIa should originate from the water of the medium, while that of IIb from molecular (atmospheric) oxygen. In contrast, the origin of the oxygen atom in IIc cannot be predicted. The two hydroxyls of the intermediate V (pathway c), although chemically equivalent, are of different biosynthetic origins; one oxygen atom should originate from molecular oxygen, the other from water. It cannot be predicted a priori which of the two hydroxyls of V would be eliminated reductively and whether the elimination would be specific with respect to the origin of the oxygen function. In general, processes related to the biosynthesis of proprenoids have been shown to be stereospecific. Therefore, it is likely, but by no means certain, that the hydrogen atom incorporated in the course of elaboration of tetrahymanol from squalene would be introduced stereospecifically, irrespective of the biosynthetic pathway actually taken.

We first undertook to differentiate pathway a from pathways b and c. The approach we chose was to administer to a growing culture of *T. pyriformis* a mixture of 2,3(*RS*)-oxido[^{3}H]squalene (**3**) and [$^{14}C_6$]squalene (**2**; ●-^{14}C) (^{3}H:^{14}C ratio 14.2).[38] The biosynthesized tetrahymanol contained only ^{14}C (^{3}H:^{14}C ratio 0.025), indicating that the tritiated oxidosqualene was not utilized in the formation of the triterpene. The results were consistent with the hypothesis of a proton-initiated squalene cyclization (Scheme I, pathway a). However, since the study was carried out with live protozoans, it has all the drawbacks of an in vivo competitive incorporation experiment. It could be argued that the epoxide was not transported or was transported in insufficient amounts through the cell walls, that it did not reach the target organs, and/or that it did not mix with the pool of endogenous squalene oxide. Alternatively, it could be argued that the oxide could have been inactivated (reduced to an alcohol, hydrolyzed to a diol, etc.) prior to reaching the target organs. The fact that we have isolated from the processed residue of the tissues a small amount of material with chromatographic properties similar to those of oxido[^{3}H]squalene[38] could be interpreted as evidence that at least some oxide was ingested by *T. pyriformis*. However, unequivocal proof for the operation of the nonoxidative mechanism of squalene cyclization was still lacking.

To obviate the interpretational difficulties, we repeated the competitive incorporation studies with homogenates prepared from lyophilized *T. pyriformis*.[39,40] Again, the biosynthesized tetrahymanol contained ^{14}C only. At this point, it was evident that under the in vivo and in vitro conditions tested, squalene oxide was not incorporated into tetrahymanol.

If indeed the formation of tetrahymanol is a nonoxidative proton-initiated process, its biosynthesis should proceed under anaerobic conditions. Therefore, equal amounts of [^{14}C]squalene were incubated with aliquots of the same enzyme preparation under anaerobic and aerobic conditions. The [^{14}C]tetrahymanol recovered from the anaerobic and aerobic experiments contained 3% and 2.6% of the ^{14}C radioactivity of the added [^{14}C]squalene, respectively. Tetrahymanol isolated from a similar incubation of [^{14}C]squalene with a boiled (2 min) homogenate was essentially devoid of radioactivity (ca. 0.05% ^{14}C incorporation). The results establish that the cyclization of squalene to tetrahymanol by enzymes of *T. pyriformis* proceeds under anaerobic conditions and is not *oxygen dependent*. It is also evident that the anaerobic enzyme cyclase is heat labile.[39,40] The presented results are in full accord with the mechanism outlined in pathway a of Scheme I.

It may be reasonable to consider that the squalene proton cyclase and the hydroxylase of the intermediate cyclic cation are in a relatively rapid equilibrium with the medium. Should this be the case, then equilibrations of the proton cyclase and of the hydroxylase in deuterium oxide or $^{18}OH_2$ will result in labeling the enzymes with deuterium or oxygen-18, respectively. Consequently, incubation of squalene with the "deuterated" squalene proton cyclase should yield [$^2H_1$]tetrahymanol. Similarly, the incubation of squalene with "hydroxylase-^{18}OH" will result in [^{18}O]-tetrahymanol. It may be noticed that the proton-initiated mode of squalene cyclization (pathway a, Scheme I) provides for the obligatory incorporation into tetrahymanol of a single exogenous hydrogen (deuterium) atom. If the tetrahymanol will contain more than a single deuterium atom, most likely this will be derived from de novo formed deuterated precursors of polyprenoids (e.g., deuterioacetyl-CoA, etc.).

For the evaluation of the incorporation of a hydrogen (deuterium) atom from the medium into tetrahymanol, homogenates of *T. pyriformis* in deuterium oxide were prepared.[40-43] The highest incorporation of deuterium into tetrahymanol (40–50% of $^2H_1$) was obtained by incubating squalene with a deuterated-enzyme powder suspended in deuterium oxide. A sample of tetrahymanol **16** (11.9 mg) pooled from several experiments had 41.0% of monodeuterated molecules.

The mass spectrum of tetrahymanol **16** showed a peak for the molecular ion (*m/e* 428) (A-1) and two

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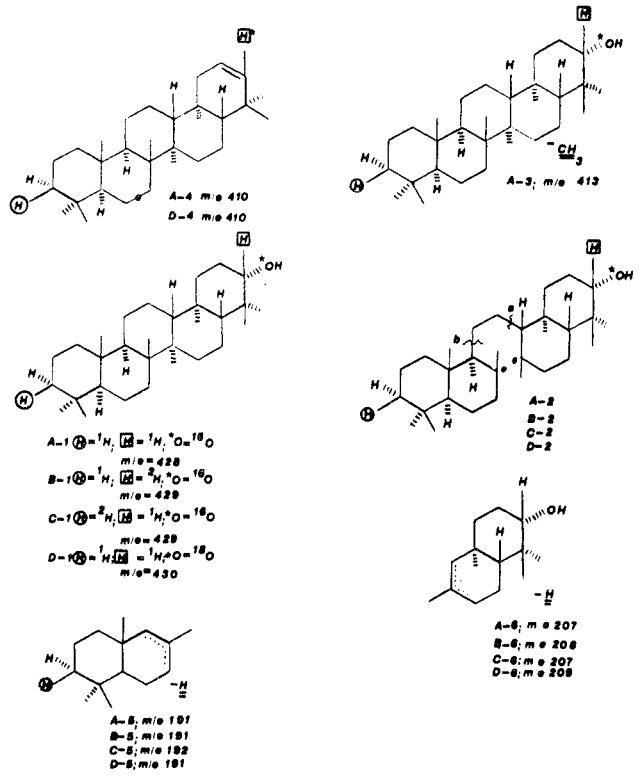


Figure 1.

diagnostic fragments at m/e 191 (A-5) and 207 (A-6) (Figure 1). Fragments m/e 191 (A-5) and 207 (A-6) are obtained through cleavages of ion A-2 at a and b (Figure 1) with the loss of a hydrogen atom from each particle, respectively. The interpretation of the mass spectrum is supported by the observation that the mass spectrum of tetrahymanone (**19**) (m/e 426; M^+) was devoid of the peak at m/e 207. Instead, a peak appeared at m/e 205 and the peak m/e 191 remained unchanged. Reduction of tetrahymanone (**19**) with $NaBD_4$ gave [21β-^{2}H]-tetrahymanol (**16a**) and [21α-^{2}H]isotetrahymanol (**20**). The mass spectrum of [21β-^{2}H]tetrahymanol (**16a**) showed the molecular ion at m/e 429 (B-1) (100% ^{2}H), a peak at m/e 208 (B-6) (100% ^{2}H), and an unchanged fragment at m/e 191 (B-5). The fragmentation pattern of the [21α-^{2}H]isotetrahymanol (**20**) was analogous to that of [21β-^{2}H]tetrahymanol (**16a**) except that the m/e 411 (429 – 18) peak was considerably more pronounced. Most likely this is due to the more facile elimination of the axial 21β-hydroxyl of the isotetrahymanol (**20**).

The mass spectrum of the pooled sample of [^{2}H]-tetrahymanol (**16c**) was recorded (Figure 1). The molecular ion m/e 429 (C-1) showed that the sample contained 41% of [$^2H_1$]tetrahymanol molecules. The m/e 207 (C-6) fragment was not deuterated (no increased intensity of the m/e 208) while all the excess isotope was found at m/e 192 (C-5). The results are fully consistent with the view that a single deuterium atom was incorporated in the course of the cyclization of squalene into tetrahymanol. Also, it is evident that all the isotopic hydrogen is located in the portion of the molecule representing rings A and B (m/e 191/192) (C-5).

The determination of the location and the stereochemistry of the hydrogen atom incorporated into tetrahymanol was more challenging. Mechanistic considerations of squalene cyclization require that this

Table I
^{2}H NMR Chemical Shifts[a]

compound	chemical shift, ppm	
	3α-^{2}H	3β-^{2}H
5α-cholestane	1.21	1.67
4,4-dimethyl-5α-cholestane	1.14 (21)	1.37 (22)
tetrahymanol (from squalene in D_2O)		1.39 (16c)

[a] The spectra were recorded on a Bruker HX-270 instrument at 41.44 MHz in $CHCl_3$ with $CDCl_3$ as internal reference, having a deuterium chemical shift of 7.27 ppm.

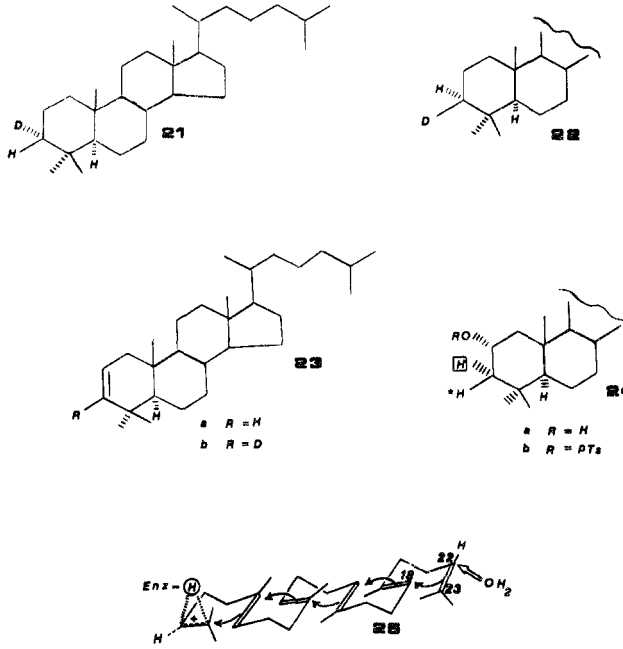
hydrogen be located at C-3 of the triterpene (Scheme I, pathway a). We have considered three approaches to the problem: microbial hydroxylation[44,45] of [$^3H_1$]-tetrahymanol, neutron diffraction,[46] and infrared spectroscopy of [$^2H_1$]tetrahymanol. Exploratory experiments indicated that the first two methods were not promising, while the infrared method proved to have only limited applicability (see below).

The actual breakthrough occurred with the development of deuterium[47a] and tritium[47b] NMR instrumentation. Evidence was obtained that, under identical conditions, the chemical shifts of deuterons and tritions are the same as the chemical shifts of the corresponding protons.[48]

First, we undertook to determine which hydrogen isotope, deuterium or tritium, should be employed. For practical reasons, deuterium seemed to be the isotope of choice. Unfortunately, the deuterium signal is much weaker and considerably broader than that of tritium. We have estimated that for tritium NMR studies, under most optimal conditions, we would require samples containing a *minimum* of 0.4 mCi of ^{3}H at the relevant site. Preparation of model compounds and particularly the biosynthesis of a sample of [3-$^3H_1$]tetrahymanol containing about 0.5 mCi of ^{3}H at the site under consideration was not a simple task. Consequently, we set out to determine whether an (axial) 3α-deuteron and (equatorial) 3β-deuteron in model steranes could be differentiated.[49] We have prepared 3α-deuterio and 3β-deuteriocholestanes[50] and recorded their deuterium NMR spectra[42,43] (Table I). The observed separation (0.46 ppm) of the axial 3α-^{2}H (δ 1.21) and equatorial 3β-^{2}H (δ 1.67) deuterium signals is sufficient for a distinct differentiation of the isomers.

(44) W. Charney and H. L. Herzog, "Microbial Transformations of Steroids", Academic Press, New York, 1967, Chapter II.
(45) R. E. Gain and E. Caspi, unpublished results.
(46) G. E. Bacon, "Neutron Diffraction", Oxford University Press, London, 1962.
(47) (a) B. W. Bycroft, C. M. Wels, K. Corbett, and D. A. Lowe, *J. Chem. Soc., Chem. Commun.*, 123 (1975); Y. Sato, T. Oda and H. Saito, *Tetrahedron Lett.*, 2695 (1976); *J. Chem. Soc., Chem. Commun.*, 415 (1977); P. M. Dewick and D. Ward, *ibid.*, 338 (1977); D. E. Cane and S. L. Buchwald, *J. Am. Chem. Soc.*, **99**, 6132 (1977); D. E. Cane and P. P. N. Murthy, *ibid.*, **99**, 8327 (1977); (b) J. M. A. Al-Rawi, J. P. Bloxsidge, C. O'Brien, D. E. Caddy, J. A. Elvidge, J. R. Jones, and E. A. Evans, *J. Chem. Soc., Perkin Trans. 2*, 1635 (1974); J. M. A. Al-Rawi, J. A. Elvidge, J. R. Jones, and E. A. Evans, *ibid.*, 449 (1975); J. M. A. Al-Rawi, J. A. Elvidge, D. K. Jaiswal, J. R. Jones, and R. Thomas, *J. Chem. Soc., Chem. Commun.*, 220 (1974); J. M. A. Al-Rawi, J. A. Elvidge, R. Thomas, and B. J. Wright, *ibid.*, 1031 (1974); L. J. Altman and N. Silberman, *Steroids*, **29**, 557 (1977).
(48) P. Diehl in "Nuclear Magnetic Resonance Spectroscopy of Nuclei Other Than Protons", T. Axenrod and G. A. Webb, Ed., Wiley-Interscience, New York, 1974, p 275.
(49) L. M. Jackman and S. Sternhell, "Applications of Nuclear Magnetic Resonance Spectroscopy in Organic Chemistry", Pergamon Press, Oxford, 1969, p 238.
(50) E. J. Corey, M. G. Howell, A. Boston, R. L. Young, and R. A. Sneen, *J. Am. Chem. Soc.*, **78**, 5036 (1956).

Encouraged by these results, we undertook the synthesis of deuterated model compounds suitable for comparison with tetrahymanol. We chose 3α- (21) and 3β-deuterio-4,4-dimethyl-5α-cholestanes (22) as models.[42,43] Our rationale for selecting these compounds was that rings A and B of the 4,4-dimethyl-5α-cholestane are structurally and environmentally analogous to rings A and B of tetrahymanol. Therefore, it was considered likely that the chemical shifts of the 3α- (21) and 3β-deuterons (22) of the model 4,4-dimethylcholestanes and of their 3-deuteriotetrahymanol counterparts would be very similar, if not identical.



Our plan for the syntheses of 21 and 22 was to prepare 4,4-dimethyl-5α-cholest-2-ene (23) which would then be hydroborated with disiamylborane and worked up oxidatively. On the basis of stereochemical considerations, it was expected that hydroboration of the C-2 olefin would yield 2α-hydroxy-4,4-dimethyl-5α-cholestane (24a) as a major product.[42,43] Since the four-centered mechanism of hydroboration of olefins is firmly established,[51] it follows that the hydrogen atom introduced in the elaboration of the 2α alcohol must have the 3α stereochemistry. Hydrogenolysis ($LiAlH_4$) of the 2α-tosyl ester (24b) should therefore yield the desired product. Thus, deuterioboration of the [3-^{1}H]-Δ^2-olefin 23a would yield finally [3α-^{2}H]-21, while ^{1}H hydroboration of [3-^{2}H]-Δ^2-olefin 23b would ultimately give [3β-^{2}H]-22. Accordingly, the required reference compounds [3α-$^2H_1$]-21 and [3β-$^2H_1$]-22 were synthesized and their $^2H_1$ NMR spectra recorded.[42,43]

We have also attempted to obtain the 3β-deuterio 22 via hydrogenation of the 3-deuterio-Δ^2-olefin 23b. However, when an ethyl acetate solution of 23b was hydrogenated over platinum oxide, the reaction proceeded only in part in the expected manner from the α side of the molecule. The product obtained was a (7:3) mixture of the 3β-deuterio (22) and 3α-deuterio (21) analogues, as shown by ^{2}H NMR (see below).

The results of deuterium NMR studies are summarized in Table I. The signals for the 3α-deuteron (21) and 3β-deuteron (22) of 4,4-dimethyl-5α-cholestanes appeared at δ 1.14 and 1.37, respectively. These two signals appeared also in the spectrum of the hydrogenation product of 3-deuterio-Δ^2-23b. Judging from the relative intensities of the δ 1.14 and 1.37 signals, the hydrogenation product consisted of a mixture of ca. 3:7 of the 3α-^{2}H (21) and 3β-^{2}H (22) compounds.[42,43] It is apparent that the signals for the axial 3α-^{2}H and equatorial 3β-^{2}H are sufficiently separated (0.23 ppm) to allow for an unambiguous assignment of the stereochemistry of the deuterons. Even in the mixture of products, the signals at δ 1.14 and 1.37 are sufficiently resolved. As a matter of fact, the δ 1.37 signal of the mixture was used in our initial assignment[42b,43] of the chemical shift of the 3β-deuteron of 22. Worthy of note is the upfield shift of the 3α (δ 1.21 → 1.14) and 3β (δ 1.67 → 1.37) deuteron signals in proceeding from the 5α-cholestanes to the 4,4-dimethyl-5α-cholestanes. This shift is especially pronounced (0.3 ppm) in the case of the 3β-deuteron. Presumably, the shift is due to a shielding effect of the 4,4-gem dimethyls and especially of the 4β-methyl.

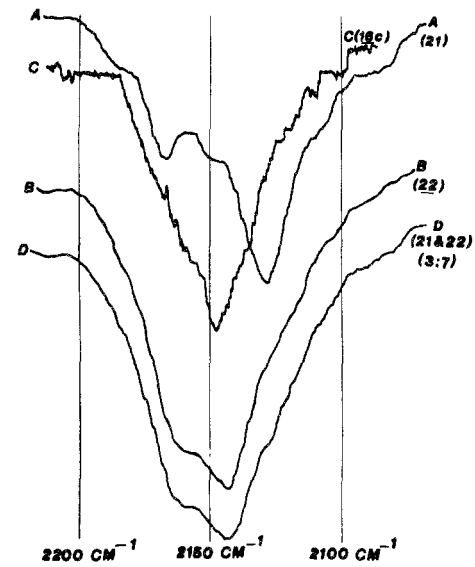


Figure 2. Stretching patterns for deuterated model compounds and biosynthesized [$^2H_1$]tetrahymanol: (A) [3α-^{2}H]-4,4-dimethyl-5α-cholestane (21); (B) [3β-^{2}H]-4,4-dimethyl-5α-cholestane (22); (C) [3β-^{2}H]tetrahymanol (16c); (D) mixture of [3α-^{2}H] (21) and [3β-^{2}H] (22) at ca. 3:7 ratio.

The ^{2}H spectrum of [3-$^2H_1$]tetrahymanol (16c) (41% $^2H_1$) showed a signal at δ 1.39 for a 3β-deuteron (Table I). It follows that the hydrogen (deuterium) atom introduced in the course of cyclization of squalenes by the *T. pyriformis* assumes the 3β configuration in the produced tetrahymanol.

Infrared studies of the carbon–deuterium stretching region of 21, 22, and [3-$^2H_1$]tetrahymanol support this conclusion. In accord with previous observations,[50] [3α-$^2H_1$]- and [3β-^{2}H]-5α-cholestanes showed overlapping bands at 2153 and 2155 cm^{-1} and characteristic bands at 2129 and 2170 cm^{-1}, respectively. The [3α-^{2}H]-(21) and [3β-^{2}H]-4,4-dimethyl-5α-cholestanes (22) in addition to several bands had pronounced diagnostic bands at 2128 (Figure 2A) and 2144 cm^{-1} (Figure 2B), respectively. [3-$^2H_1$]Tetrahymanol (16c) (Figure 2C) showed a strong band at 2147 cm^{-1} and a shoulder at 2164 cm^{-1}. Inspection of the tetrahymanol curve (Figure 2C) indicates that the overall shape of the curve and

(51) H. C. Brown, "Boranes in Organic Chemistry", Cornell University Press, Ithaca, NY, and London, 1972, pp 265–266.

the presence of the band at 2147 cm^{-1} are similar to that of the [3β-^{2}H]-4,4-dimethyl reference (**22**) (Figure 2B). Hence, the overall results of the infrared studies are in accord with the ^{2}H NMR investigations and show that tetrahymanol has a 3β-deuterium atom.

Except for rather minor differences, the infrared spectrum of the 3:7 mixture of 3α-deuterio (**21**) and 3β-deuterio (**22**) (Figure 2D) is very similar to that of the 3β-deuterio (**22**). In practice, the admixture of ca. 30% of the 3α-$^2H_1$ isomer (**21**) in **22** would not have been recognized. This, therefore, restricts considerably the applicability of the IR method in the studies of the stereochemistry of deuterium atoms and, particularly, in studies of this type of biosynthetic processes.

There remained the question of the origin of the oxygen atom of tetrahymanol.[40,52] In accord with the proposed mechanism of squalene cyclization (pathway a, Scheme I), the oxygen function should ultimately be derived from the water of the medium. [^{14}C]Squalene was incubated with an enzyme powder of *T. pyriformis* dissolved in $^{18}OH_2$ (62.4% excess ^{18}O) to test this hypothesis. The biosynthetic [^{18}O]tetrahymanol was analyzed for isotopic enrichment. The mass spectrum of the [^{18}O]tetrahymanol (Figure 1) showed peaks for a molecular ion (D-1) at *m/e* 430 [(M + 2); 30.5% ^{18}O enrichment]. Similarly, a peak for D-6 was present at *m/e* 209 [(207 + 2); 30% ^{18}O enrichment]. The peaks at *m/e* 191 (D-5) and 410 [(M + 2) − water] (D-4) were unchanged. The results show that ^{18}O from the medium was incorporated into tetrahymanol and that loss of water removed the ^{18}O-hydroxyl (fragment D-4; *m/e* 410).

The biosynthesis of tetrahymanol can now be mechanistically rationalized. In analogy to the 2(3)*S*-oxidosqualene cyclization, we suggest that a proton cyclase attacks a terminal double bond of all-pro-chair coiled squalene from the top site (**25**). For illustrative purposes, the attack is shown to generate a nonclassical cation (**25**) whose collapse would produce the required C-2 cation. This would precipitate the cyclization of the all-pro-chair coiled squalene to the required C-21 cation and formation of tetrahymanol. Should the electron flow occur from C-22 to C-18 of squalene (**2**) rather than C-23 to C-18, the cation **13b** will be obtained and diplopterol (**26**) will be formed. As indicated earlier, depending on the species, the cation **15** and/or **13b** could undergo additional rearrangements prior to stabilization. The outlined mechanism of the nonoxidative cyclization of squalene may be a general route of the biosynthesis of 3-deoxytriterpenes.[30] It should be indicated that Barton et al. have presented evidence consistent with the operation of a similar mechanism in the biosynthesis of fern-9-ene in the fern *Polypodium vulgare linn.*[25]

We have previously assumed that the stereochemical course of the elaboration of squalene from MVA by *T. pyriformis* is the same as in rat livers.[53] For example, it was proven that the biosynthesis of squalene by rat liver enzymes entails the stereospecific loss of six 4-*pro-S* hydrogen atoms of MVA and retention of six 4-*pro-R* hydrogen atoms of MVA.[17b,53] Accordingly, when (3*RS*,4*R*)-[2-^{14}C;4-^{3}H]MVA (**1b**) (atomic ratio 1:1) was incubated with a rat liver preparation, [$^3H_6$;$^{14}C_6$]-squalene (**2b**) was obtained.[53] The location of the ^{14}C (●) atoms and of the tritium atoms (*H) in the [^{3}H;^{14}C]squalene is indicated in **2b**.

Table II[a]
Biosynthesis[b] of Tetrahymanol (16b) and Diplopterol (26) from (3*RS*,4*R*)-[2-^{14}C;4-^{3}H]MVA by *T. pyriformis*

compound	^{3}H:^{14}C isotopic ratio	^{3}H:^{14}C atomic ratio exptl	^{3}H:^{14}C atomic ratio calcd
1b	4.88		
16b	4.79	5.89:6	6:6
26	4.86	5.98:6	6:6
19 and **19***	4.12	5.07:6	5:6
27	4.11	5.05:6	5:6
28	5.08	5.21:5	5:5
29	3.97	4.07:5	4:5

[a] Isotopic and atomic ratios of the triterpenes and of *certain* of their transformation products. [b] (i) Jones' oxidation; (ii) retropinacol rearrangement; (iii) (1) O_3; (2) Zn + AcOH; (iv) EtOH + aqueous NaOH.

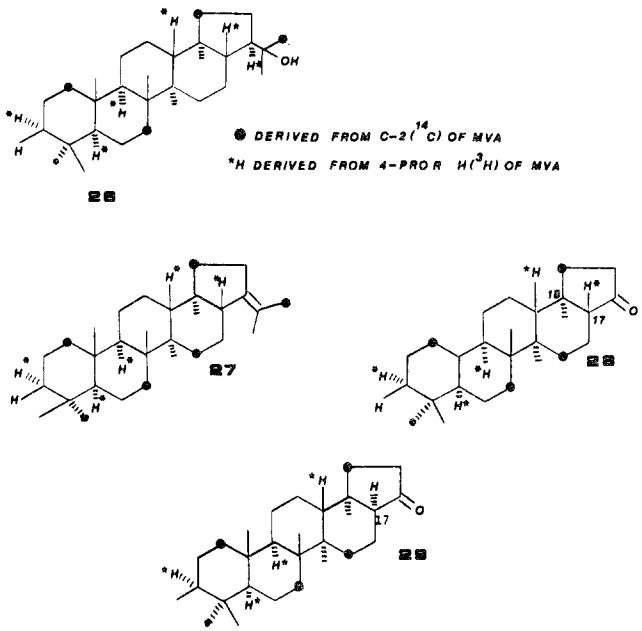
We thought that the fate of the 4-*pro-R* hydrogen atoms of MVA in the biosynthesis of tetrahymanol could be used for the evaluation of *one* facet of the stereochemistry of formation of polyprenoids by *T. pyriformis*. We reasoned that if the elaboration of squalene (**2b**) by *T. pyriformis* follows the same route as in rat livers, the tetrahymanol derived from the squalene will retain six C-2 (^{14}C) atoms and six 4-*pro-R* hydrogen (tritium) atoms of MVA. Hence, incubation of (3*RS*,4*R*)-[2-^{14}C;4-^{3}H]-MVA (**1b**) with *T. pyriformis* should yield [$^3H_6$;$^{14}C_6$]tetrahymanol which will be labeled as indicated in **16b**.

We have therefore incubated (3*RS*,4*R*)-[2-^{14}C;4-^{3}H]-MVA (**1b**) (isotopic ratio 4.88, atomic ratio 1:1) with *Tetrahymena pyriformis*[54] and isolated tetrahymanol (**16b**) and diplopterol (**26**). As can be seen from Table II, the isotopic ratios of **16b** and **26** are essentially the same as that of the parent [2-^{14}C;4(*R*)-^{3}H]MVA (**1b**). Since tetrahymanol (**16**) and diplopterol (**26**) are "primary" products of cyclization of squalene, they must contain six ^{14}C atoms originating from the [2-^{14}C]MVA and, hence, six tritium atoms derived from [4(*R*)-^{3}H]MVA.

The transformations summarized in the legend of Table II were carried out to test the distribution of the isotopic atoms in **16b**. As expected, tritium atoms were located at 21β and 17β positions, while one of the C-22 *gem*-dimethyls was labeled with ^{14}C. Based on these *limited results*,[54] it is likely that the biosynthesis of squalene by *T. pyriformis* follows the same overall route as that in rat livers.

Several ancillary observations related to the metabolism of sterols by *T. pyriformis* will be mentioned briefly. It was proven that cholesterol is metabolized by *T. pyriformis* to cholesta-5,7,22-trien-3β-ol.[55] We have shown that the protozoan will convert 5α-choles-

(52) J. M. Zander and E. Caspi, *Chem. Commun.*, 209 (1969).
(53) G. Popjak and J. W. Cornforth, *Biochem. J.*, 101, 553 (1966), and references therein.
(54) F. B. Mallory, R. L. Conner, J. R. Landrey, J. M. Zander, J. B. Greig, and E. Caspi, *J. Am. Chem. Soc.*, **90**, 3564 (1968).
(55) R. L. Conner, F. B. Mallory, J. R. Landrey, and C. W. L. Iyengar, *J. Biol. Chem.*, 244, 2325 (1969); F. B. Mallory, R. L. Conner, J. R. Landrey, and C. W. L. Iyengar, *Tetrahedron Lett.*, 6103 (1968).



tanol and 5α-cholest-7-en-3β-ol to the 5,7,22-triene. Desmosterol is dehydrogenated mainly to cholesta-5,7,22,24-tetraen-3β-ol and to a lesser extent to cholesta-5,7,24-trien-3β-ol.[56] The formation of the C-5(6)[56] and the C-7(8)[57-59] double bonds proceeds with the loss of the cis-5α,6α and the cis-7β,8β hydrogen atoms, respectively. The C-22(23) double bond formation involves the removal of the 22-*pro*-R[57] and 23-*pro*-S[58] hydrogen atoms. The protozoan is capable of dehydrogenating certain 24-alkylated steroids to the corresponding trienes.[60] Also, it has the capacity to remove a 24-ethyl but not a 24-methyl group of a steroid.[60]

Conclusion

The biosynthesis of tetrahymanol is an anaerobic process which most likely is initiated by an enzyme-mediated proton attack on a terminal double bond of all-pro-chair coiled squalene. Accordingly, a proton (deuterium) and a hydroxyl (^{18}OH) from the medium are incorporated into tetrahymanol. It follows that the overall process of the biosynthesis is equivalent to the acquisition by squalene of the equivalent of a molecule of water. The X-ray studies have confirmed that tetrahymanol has the all trans-anti-chair structure with a 10β-methyl and 5α-hydrogen stereochemistry.[36] Also, it was established with the use of deuterium NMR that the proton (deuteron) incorporated into tetrahymanol from the medium assumes the 3β stereochemistry in the triterpene. Studies of the C–deuterium stretching region supported the above results; however, it was demonstrated that the IR method is less sensitive and, therefore, has a limited applicability.

Based on the observations of van Tamelen,[29] it is considered likely that squalene could assume an all-pro-chair conformation in the polar aqueous environment of the cell with little or no enzyme assistance and then be cyclized to tetrahymanol. The overall process could be mediated by a rather simple enzyme system. This is consistent with the primitive enzymatic systems which are presumed to have existed in the aerobic stage of global development. The nonoxidative mode of squalene cyclization is probably an early evolutionary process of triterpene biosynthesis.

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(56) L. J. Mulheirn, D. J. Aberhart, and E. Caspi, *J. Biol. Chem.*, **246**, 6556 (1971).
(57) J. M. Zander and E. Caspi, *J. Biol. Chem.*, **245**, 1682 (1970).
(58) T. Bimpson, L. J. Goad, and T. W. Goodwin, *Biochem. J.*, **115**, 857 (1969).
(59) D. C. Wilton and M. Akhtar, *Biochem. J.*, **116**, 337 (1970).
(60) Reference 1, pp 419–420.