

drophobic pocket with a correspondingly snug fit could assist in a strong complex between avidin and biotin.

**Acknowledgment.** We thank Professor N. J. Leonard for stimulating discussions on this problem. F.-T.L. was supported by a research grant (CHE-76-23543) from the National Science Foundation to Professor Leonard.

**Supplementary Material Available:** Tables of observed and calculated structure factors and thermal parameters for the crystals of **2** and **6** (42 pages). Ordering information is given on any current masthead page.

## References and Notes

- (1) This work was supported by NIH GM 19336.
- (2) N. M. Green, *Adv. Protein Chem.*, **29**, 84 (1975), and references cited therein.
- (3) F.-T. Liu and N. J. Leonard, *J. Am. Chem. Soc.*, preceding paper in this issue.
- (4) K. Mutai, B. A. Gruber, and N. J. Leonard, *J. Am. Chem. Soc.*, **97**, 4095 (1975), and references cited therein.
- (5) N. J. Leonard, R. S. McCredie, M. W. Logue, and R. L. Cundall, *J. Am. Chem. Soc.*, **95**, 2320 (1973).

- (6) (a) N. J. Leonard, K. Golankiewicz, R. S. McCredie, S. M. Johnson, and I. C. Paul, *J. Am. Chem. Soc.*, **91**, 5855 (1969); (b) J. K. Frank and I. C. Paul, *ibid.*, **95**, 2324 (1973); (c) P. L. Johnson, C. A. Maier, and I. C. Paul, *ibid.*, **95**, 5370 (1973); (d) P. L. Johnson, J. K. Frank, and I. C. Paul, *ibid.*, **95**, 5377 (1973); (e) J. K. Frank, N. N. Thayer, and I. C. Paul, *ibid.*, **95**, 5386 (1973).
- (7) (a) W. Traub, *Nature (London)*, **178**, 649 (1956); (b) *Science*, **129**, 210 (1959).
- (8) G. T. DeTitta, J. W. Edmonds, W. Stallings, and J. Donohue, *J. Am. Chem. Soc.*, **98**, 1920 (1976).
- (9) C. Bonnemere, J. A. Hamilton, L. K. Steinrauf, and J. Knappe, *Biochemistry*, **4**, 240 (1965); (b) J. Trotter and J. A. Hamilton, *ibid.*, **5**, 713 (1966).
- (10) C.-S. Chen, R. Parthasarathy, and G. T. DeTitta, *J. Am. Chem. Soc.*, **98**, 4983 (1976).
- (11) M. D. Glick, H. C. Wormser, and H. N. Abramson, *Acta Crystallogr., Sect. B*, **33**, 1095 (1977).
- (12) G. Germain, P. Main, and M. M. Woolfson, *Acta Crystallogr., Sect. A*, **27**, 368 (1971).
- (13) Virtually all crystallographic calculations were carried out on the Syntex EXTL structure determination system, provided by Syntex Analytical Instruments, Cupertino, Calif. 95014.
- (14)  $R = \sum |F_o| - |F_c| / \sum |F_o|$ ;  $R_w = [\sum w |F_o| - |F_c|]^2 / \sum w |F_o|^2]^{1/2}$ .
- (15) J. A. Ibers and W. C. Hamilton, Ed., "International Tables for X-ray Crystallography", Vol. IV, Kynoch Press, Birmingham, England, 1974, pp 71-147.
- (16) See paragraph at end of paper regarding supplementary material.
- (17) B. M. Craven, C. Cusatis, G. L. Gartland, and E. A. Vizzini, *J. Mol. Struct.*, **16**, 331 (1973).

# Nonoxidative Cyclization of Squalene by *Tetrahymena pyriformis*. Incorporation of a $3\beta$ Hydrogen (Deuterium) Atom into Tetrahymanol

D. John Aberhart and Eliahu Caspi\*

Contribution from The Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts 01545. Received September 5, 1978

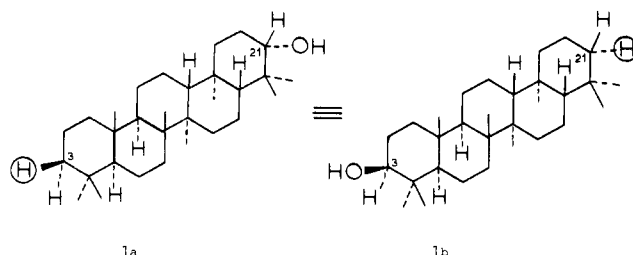
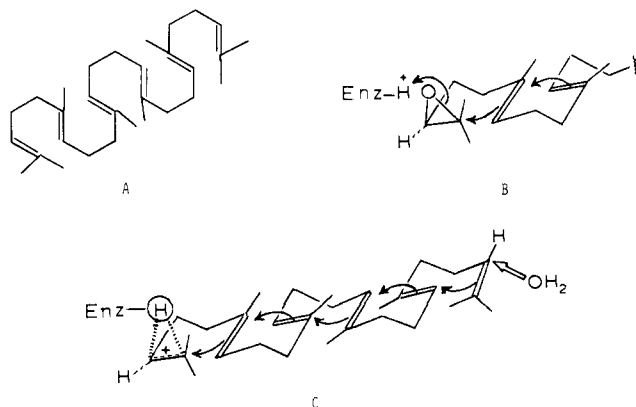
**Abstract:**  $3\alpha$ - and  $3\beta$ -[ $^2\text{H}_1$ ]- $5\alpha$ -cholestanes and  $3\alpha$ - and  $3\beta$ -[ $^2\text{H}_1$ ]-4,4-dimethyl- $5\alpha$ -cholestanes were synthesized, and their  $^2\text{H}$  NMR spectra were determined. The chemical shifts of the  $3\alpha$ - and  $3\beta$ -deuterio isomers were separated by 0.46 ppm in the cholestane isomers and by 0.23 ppm in the 4,4-dimethylcholestanes. The results were then used to establish that the nonoxidative cyclization of squalene by enzymes of *Tetrahymena pyriformis* in  $\text{D}_2\text{O}$  proceeds with the introduction of a  $3\beta$ -deuterium atom into tetrahymanol. The  $^2\text{H}$  NMR results were supported by comparison of the C- $^2\text{H}$  stretching patterns of  $3\alpha$ - and  $3\beta$ -[ $^2\text{H}_1$ ]-4,4-dimethyl- $5\alpha$ -cholestanes with the C- $^2\text{H}$  stretching pattern of biosynthetic tetrahymanol. The mechanism of the nonoxidative cyclization of squalene is also discussed.

## Introduction

It is now generally accepted that the biosynthesis of C-3 oxygenated triterpenes and sterols is an oxidative process involving the initial formation of 2,3(*S*)-oxidosqualene<sup>1</sup> (B). Enzymatic "cationic" cleavage of the oxirane ring is thought to generate an electron deficiency at C-2 which initiates the

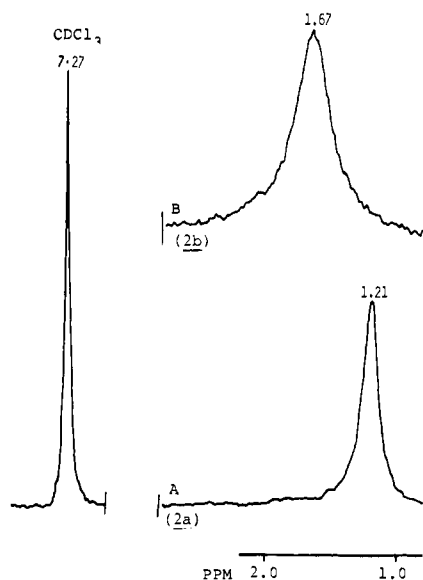
cyclization process. Depending on the species, different C-3 oxygenated products are then formed.<sup>1</sup>

In contrast, we have demonstrated that the biosynthesis of the triterpene tetrahymanol (**1**) by *Tetrahymena pyriformis* is not an oxygen-dependent process.<sup>2</sup> Accordingly, the biosynthesis of tetrahymanol (**1**) by an enzyme preparation of *T.*



Structures **1a** and **1b** are equivalent. For convenience of comparison of the  $^2\text{H}$  NMR spectra of [ $^2\text{H}$ ]tetrahymanol and the [ $^3\text{-}^2\text{H}_1$ ] model compounds, the numbering system (cf. ref 2) is used in the present paper. This is in contrast to the numbering system used in our paper, ref 3b.

Ⓜ: HYDROGEN (DEUTERIUM) ATOM  
INTRODUCED IN THE BIOSYNTHESIS  
FROM SQUALENE



**Figure 1.**  $^2\text{H}$  NMR spectra of (A)  $3\alpha$ -[ $^2\text{H}$ ]- $5\alpha$ -cholestane (**2a**) and (B)  $3\beta$ -[ $^2\text{H}$ ]- $5\alpha$ -cholestane (**2b**).

*pyriformis* proceeded in an atmosphere of nitrogen,<sup>3a</sup> in the absence of oxygen. In addition, when a mixture of [ $^3\text{H}$ ]-2,3-oxidosqualene and [ $^{14}\text{C}$ ]squalene was incubated *in vivo*<sup>4</sup> with *T. pyriformis*, or *in vitro*<sup>2,3a</sup> with an enzyme preparation of *T. pyriformis*, the *de novo* biosynthesized tetrahymanol was devoid of tritium, but contained  $^{14}\text{C}$ . This established that 2,3-oxidosqualene is not a precursor of tetrahymanol and that most likely the biosynthesis is initiated by a proton attack on a terminal double bond of squalene. Indeed, when squalene was incubated with the enzymes of *T. pyriformis* in a deuterium oxide medium, the tetrahymanol contained *one* atom of deuterium which was located in the portion of the molecule encompassing rings A and B.<sup>2,5</sup> The fact that only *monodeuterated* tetrahymanol was obtained precluded the possibility that this deuterium was introduced via *de novo* synthesis of deuterated squalene. Should indeed the mechanism proceed as shown in C, an electron deficiency will ultimately be generated at C-21. The C-21 cation should then be stabilized by the enzyme-mediated acquisition of a hydroxyl from the medium. In fact, when squalene (A) was incubated with an enzyme preparation of *T. pyriformis* in a medium enriched with  $^{18}\text{OH}_2$ , the biosynthesized tetrahymanol contained one atom<sup>2,6</sup> of  $^{18}\text{O}$ . Hence, the overall process of tetrahymanol formation was shown to be equivalent to the acquisition of a molecule of water by squalene<sup>2,5,6</sup> (A  $\rightarrow$  C).

It would seem reasonable to assume that the incoming hydrogen atom will attack the squalene molecule at one of the terminal double bonds of squalene (C). Therefore, it may be expected that the newly introduced hydrogen atom will be located at C-3 of tetrahymanol (**1**). Accordingly, we undertook to provide evidence in support of the stereochemistry and the C-3 location of the newly introduced hydrogen in tetrahymanol. A preliminary report of our results has been communicated.<sup>7,8</sup>

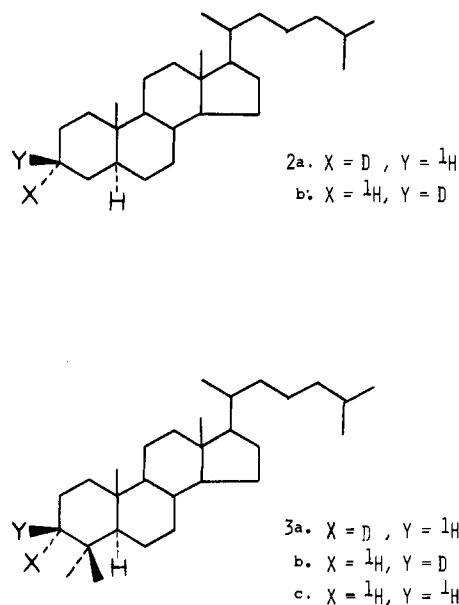
## Results

For the purposes stated above, we required a method of distinguishing between a  $3\alpha$  or  $3\beta$  (isotopic) hydrogen in tetrahymanol. Unfortunately, the absence of a reactive functionality in the vicinity of C-3 of tetrahymanol rendered it difficult to employ commonly used radioactive ( $^3\text{H}$ ) tracer techniques involving the stereospecific elimination of the  $3\alpha$  or  $3\beta$  (isotopic) hydrogen. Our limited attempts to effect microbiological hydroxylations at C-3 (or its vicinity) in repre-

sentative compounds such as  $5\alpha$ -cholestane or  $5\alpha$ -lanost-8-ene did not meet with success.<sup>9</sup> The recent reports on the uses of tritium<sup>10</sup> and deuterium<sup>11</sup> magnetic resonance attracted our attention as possible routes for the realization of our goal. It seemed likely that the chemical shifts of  $3\alpha$  (axial) and  $3\beta$  (equatorial) hydrogen atoms of  $5\alpha$ -steranes and  $5\alpha$ -triterpenes would be different, owing to the diamagnetic anisotropy of the ring system.<sup>12</sup> It has been shown that, under identical conditions, the chemical shifts of deuterons or tritons are the same as the chemical shifts of corresponding protons.<sup>13</sup> Thus, provided that the signals for the C-3 isotopic hydrogen atoms in the NMR spectra of 3-deoxy steroids or triterpenes could be unambiguously assigned, it would be possible to trace the stereochemistry of events occurring at the terminal double bond of squalene, in the enzymatic reactions leading to the formation of tetrahymanol.

We first set out to establish whether, in fact, there is an appreciable chemical shift difference between the C-3 hydrogen atoms of typical 3-deoxy- $5\alpha$ -steranes. It was of the utmost importance in our considerations to estimate the magnitude of such a chemical shift difference, since this would govern our choice of isotope ( $^3\text{H}$  or  $^2\text{H}$ ) to be used in biosynthetic investigations. A small (<0.1 ppm) difference would mandate the use of tritium as tracer rather than deuterium, owing to the broader line width of the deuterium signal. We have estimated that for tritium NMR, the compounds should contain at least ca. 0.2–0.4 mCi of tritium at the *pertinent site*. Because the synthesis of compounds containing large amounts of deuterium at the relevant carbon atoms is relatively easier and considerably less expensive, we first concentrated our efforts on the preparation of model compounds stereospecifically labeled with deuterium.

With this in mind, we prepared the ( $3\alpha$ )-[ $^2\text{H}$ ]- and ( $3\beta$ )-[ $^2\text{H}$ ]- $5\alpha$ -cholestanes (**2a** and **2b**) according to the method of

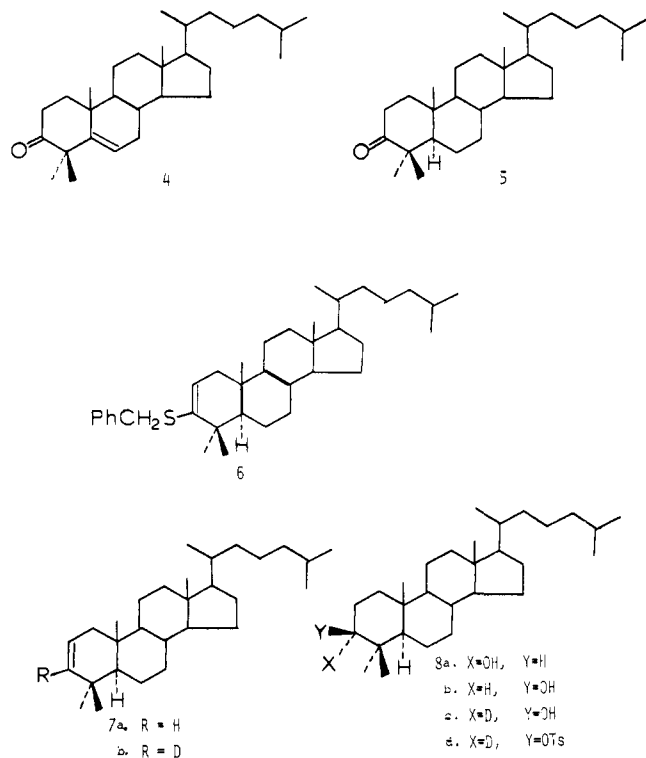


Corey et al.<sup>14</sup> The  $^2\text{H}$  NMR spectra of **2a** and **2b** are presented in Figure 1. The chemical shifts difference between the  $^2\text{H}$  signals of **2a** ( $\delta$  1.21) and **2b** ( $\delta$  1.67) was 0.46 ppm. Encouraged by these observations, we undertook to prepare compounds stereospecifically labeled with deuterium which could be used as models for tetrahymanol. Unfortunately, the most suitable models  $3\alpha$ - and  $3\beta$ -deuteriogrammaceranes or  $3\alpha$ - and  $3\beta$ -deuteriotetrahymanols could not be prepared because of the most limited availability of appropriate starting materials. We, therefore, considered the use of  $3\alpha$ - (**3a**) and  $3\beta$ - (**3b**) deuterio-4,4-dimethyl- $5\alpha$ -cholestanes. The structural environment of rings A and B of **3** and of tetrahymanol (**1**) is

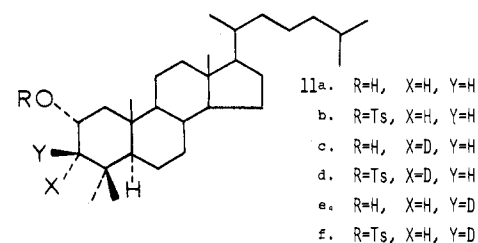
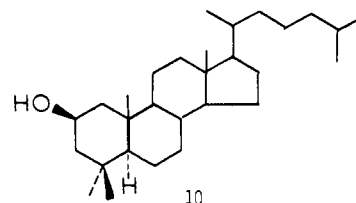
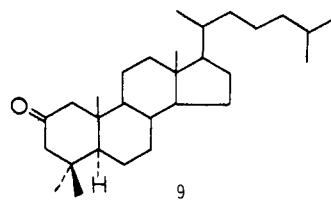
analogous. However, it is known that conformational variations of ring A may influence the chemical shifts of, e.g., C-3 hydrogen (deuterium) atoms. A single-crystal X-ray structure analysis of tetrahymanol (**1**) revealed that the compound exists in two crystallographically independent forms.<sup>15</sup> The two forms have all trans-anti conformations and show some distortion of bond lengths and bond angles. It is considered probable that, in solution, tetrahymanol will also exist in two conformationally isomeric forms. However, since the observed distortions of bond length and bond angles were relatively small, it was assumed that they would not influence, to any significant degree, the chemical shifts of the C-3 hydrogen (deuterium) atoms. Consequently, for our purposes, rings A and B of 4,4-dimethyl-5 $\alpha$ -cholestane (**3**) and tetrahymanol (**1**) were considered to be structurally and conformationally similar. Based on these considerations, the 3 $\alpha$ -deuterio- (**3a**) and 3 $\beta$ -deuterio- (**3b**) 4,4-dimethyl-5 $\alpha$ -cholestanes were accepted as appropriate models and were synthesized.

Cholest-4-en-3-one was converted to 4,4-dimethylcholest-5-en-3-one<sup>16</sup> (**4**), which was hydrogenated<sup>17</sup> to 4,4-dimethyl-5 $\alpha$ -cholest-3-one (**5**). Treatment of **5** with toluene- $\alpha$ -thiol<sup>18</sup> gave 3-benzylmercapto-4,4-dimethyl-5 $\alpha$ -cholest-2-ene (**6**). Hydrogenolysis<sup>18</sup> of **6** with P-2 nickel boride<sup>19</sup> in ethanol gave 4,4-dimethyl-5 $\alpha$ -cholest-2-ene<sup>20</sup> (**7a**).

We planned to prepare the stereospecifically C-3 deuterated 4,4-dimethylcholestanes via hydroboration of **7a** or its deuterated analogue, **7b**. Treatment of **7a** with borane in THF followed by oxidative workup<sup>21</sup> gave a mixture of four alcohols. The mixture was resolved by preparative TLC, and the alcohols were designated I, II, III, and IV in order of decreasing *R<sub>f</sub>* values. The identities of the alcohols in which I and IV predominated (I > IV) were secured in the following manner. Alcohol I [d, d,  $J_1 = J_2 = 3$  Hz, 3 $\beta$ (H)], upon treatment with Jones reagent, gave ketone **5**. Reduction of this ketone with sodium borohydride gave mainly alcohol III [ $W_{1/2} = 16$  Hz (broad multiplet), 3 $\alpha$ (H)]. The alcohol III was previously obtained (as the acetate) in the hydrogenation of ketone **4**. It follows that the major hydroboration product (alcohol I) is the 3 $\alpha$ -hydroxy isomer (**8a**), and the minor (alcohol III) is the 3 $\beta$  isomer (**8b**). The other major alcohol IV, on treatment with Jones reagent, gave a ketone differing from **5** and having



properties consistent with those reported<sup>20</sup> and expected for the 2-keto compound (**9**). Reduction of **9** with sodium borohydride gave predominantly alcohol II. Consequently, alcohol II is the 2 $\beta$  isomer<sup>20</sup> (**10**), and alcohol IV is the desired 2 $\alpha$  isomer<sup>22</sup> (**11a**). It is evident that, under these conditions, the

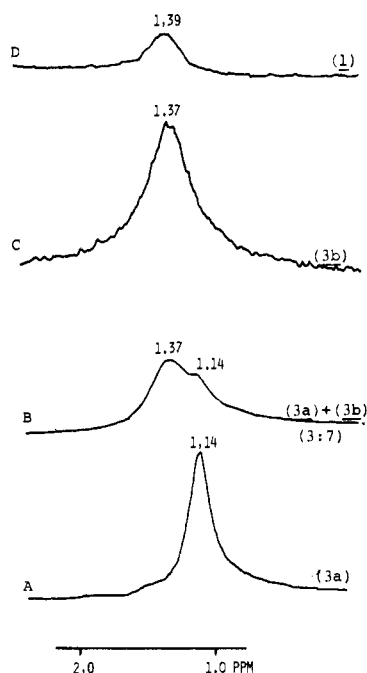


hydroboration gave greater quantities of the undesired isomer (**8a**). However, when the hydroboration was carried out with disiamylborane,<sup>21</sup> the required **11a** was obtained in moderate yield, and was accompanied by minor amounts of the other isomers **8a**, **8b**, and **10**. The tosylate **11b** derived from **11a**, on treatment with lithium aluminum hydride, gave a mixture of 4,4-dimethyl-5 $\alpha$ -cholestane<sup>23</sup> (**3c**) and 4,4-dimethyl-5 $\alpha$ -cholest-2-ene (**7a**).

In a similar manner, **7a** was treated with deuterated disiamylborane (prepared from  $\text{BD}_3$  in THF) to yield a mixture of products from which 3 $\alpha$ -deuterio-4,4-dimethyl-5 $\alpha$ -cholest-2-ol (**11c**) was isolated. Treatment of the tosyl ester (**11d**) with  $\text{LiAlH}_4$  resulted in a mixture of 3 $\alpha$ -deuterio-4,4-dimethyl-5 $\alpha$ -cholestane (**3a**) and the trans-elimination product (**7b**). The NMR spectrum of **7b** showed a signal for a *single vinylic* hydrogen ( $^1\text{H}$ ) atom.

For the synthesis of the 3 $\beta$ -deuterio isomer (**3b**), we required 3-deuterio-4,4-dimethyl-5 $\alpha$ -cholest-2-ene (**7b**). The small amount of **7b** obtained as a byproduct in the synthesis of **3a** was insufficient for our purposes. In an attempt to prepare larger amounts of **7b**, the thio enol ether **6** was treated with deuterated nickel boride generated by adding sodium borodeuteride to nickel chloride hexahydrate in deuterated ethanol.<sup>18</sup> The mass spectrum of the obtained **7b** ( $d_0$  13.0%,  $d_1$  56.3%,  $d_2$  28.5%,  $d_3$  1.4%,  $d_4$  0.8%) revealed significant scrambling of deuterium during hydrogenolysis. Consequently, this approach was abandoned, and **7b** was prepared by an alternative route.<sup>24</sup>

The ketone **5** was reduced with lithium aluminum deuteride to 3 $\alpha$ -deuterio-4,4-dimethyl-5 $\alpha$ -cholest-3-ol (**8c**). The corresponding tosylate (**8d**) was stirred with Woelm neutral alumina,<sup>24</sup> activity grade super I. The obtained mixture of products was resolved by argentation TLC to yield **7b** (52%).



**Figure 2.**  $^2\text{H}$  NMR spectra of (A)  $3\alpha$ - $[\text{2H}]$ -4,4-dimethyl- $5\alpha$ -cholestane (**3a**); (B) **3b** + **3a** at a ratio of 7:3; (C)  $3\beta$ - $[\text{2H}]$ -4,4-dimethyl- $5\alpha$ -cholestane (**3b**); (D)  $3\beta$ - $[\text{2H}]$ tetrahymanol (**1**).

For the preparation of the  $3\beta$ -deuterio (**3b**), hydrogenation of the C-3 deuterio olefin (**7b**) was attempted. Unfortunately, the hydrogenation proceeded only partially from the  $3\alpha$  side of the molecule and, as evidenced by deuterium NMR (Figure 2B), a 7:3 mixture of the  $3\beta$ -deuterio (**3b**) and  $3\alpha$ -deuterio (**3a**) was formed. Under the circumstances, the deuterated olefin **7b** was treated with  $[\text{1H}]$ disiamylborane. The resulting mixture of products was fractionated to yield  $3\beta$ -deuterio-4,4-dimethyl- $5\alpha$ -cholestan- $2\alpha$ -ol (**11e**). Treatment of the corresponding tosylate with  $\text{LiAlH}_4$  gave, after chromatographic purification, the required  $3\beta$ -deuterio-4,4-dimethyl- $5\alpha$ -cholestane (**3b**) (Figure 2C) and the 3-deuterio olefin **7b** [MS (%)  $d_0$  0.4,  $d_1$  96.5,  $d_2$  3.0,  $d_3$  0.2]. It is worthy of note that the  $\text{LiAlH}_4$  hydrogenolysis of  $3\alpha$ -deuterio- $2\alpha$ -tosyloxy (**11d**) and  $3\beta$ -deuterio- $2\alpha$ -tosyloxy (**11f**) gave 3-deuterio- $\Delta^2$  olefin (**7b**) as byproducts. It is apparent that elimination of the tosyloxy moiety in the case of **11d** proceeded via the loss of the  $3\beta$ -trans hydrogen atom, while in the case of **11f** the reaction proceeded with the elimination of the  $3\alpha$ -cis hydrogen atom. It appears that the isotope effect involved in the C-D bond breakage controls the steric course of the elimination reaction.

The  $^2\text{H}$  NMR spectrum of  $3\alpha$ -deuterio-4,4-dimethyl- $5\alpha$ -cholestane (**3a**) (Figure 2A, Table I) showed a single broad peak at 1.14 ppm, while  $3\beta$ -deuterio-4,4-dimethyl- $5\alpha$ -cholestane (**3b**) (Figure 2C) exhibited a broad peak at 1.37 ppm. The signals for the  $3\alpha$  and  $3\beta$  deuterons in **3a** and **3b** appeared at significantly higher field than was observed for the corresponding cholestanes. The upfield shifts may be attributed to the shielding effect of the C-CH<sub>3</sub> bonds. This is especially apparent in the  $3\beta$ -deuterio compound (**3b**), in which the  $3\beta$  deuterium appears 0.3 ppm upfield from its position in  $3\beta$ -deuteriocholestane (**2b**). It is worthy of note that the mixture of products of hydrogenation of **7b** showed the same two peaks at 1.37 and 1.14 ppm in a 7:3 ratio (Figure 2B). The two peaks were relatively well resolved and were used for determination of the composition of the mixture and for the initial assignment of the  $3\beta$ -deuterio signal<sup>7,8</sup> (1.37 ppm). In any event, the observed chemical shift difference, 0.23 ppm, between the  $3\alpha$  and  $3\beta$  deuterons of 4,4-dimethylcholestanes was ample for the

**Table I.**  $^2\text{H}$  NMR Chemical Shifts<sup>a</sup>

compd	chemical shift, ppm	
	$3\alpha$ - $^2\text{H}$	$3\beta$ - $^2\text{H}$
$5\alpha$ -cholestane ( <b>2</b> )	1.21	1.67
4,4-dimethyl- $5\alpha$ -cholestane ( <b>3</b> )	1.14	1.37
tetrahymanol (from squalene in $\text{D}_2\text{O}$ )		1.39

<sup>a</sup> The spectra were recorded on a Bruker HX-270 instrument at 41.44 MHz in  $\text{CHCl}_3$  with  $\text{CDCl}_3$  as internal reference, having a deuterium chemical shift of 7.27 ppm.

**Table II.** C- $^2\text{H}$  Stretching Frequencies<sup>a</sup>

compd	$\nu_{\text{max}}$ , $\text{cm}^{-1}$	
	$3\alpha$ - $^2\text{H}_1$	$3\beta$ - $^2\text{H}_1$
$5\alpha$ -cholestane ( $\text{CCl}_4$ )	2129 (s)	2170 (s)
	2153 (s)	2155 (s)
	2164 (w)	2160 (sh)
4,4-dimethyl- $5\alpha$ -cholestane ( $\text{CHCl}_3$ )	2128 (s)	2144 (s)
	2146 (w, sh)	
	2122 (w, sh)	
		2147 (s)
tetrahymanol (from squalene in $^2\text{H}_2\text{O}$ )		2164 (sh)
( $\text{CHCl}_3$ )		

<sup>a</sup> The spectra were recorded in the indicated solvents on a Perkin-Elmer 621 infrared spectrophotometer. s = strong; w = weak; sh = shoulder.

unambiguous assignment of the stereochemistry of C-3 deuterium atoms of samples with a similar structure and conformation of rings A and B.

These results established the feasibility of using  $^2\text{H}$  NMR for the determination of the C-3 stereochemistry of deuterium in tetrahymanol. We have, therefore, biosynthesized  $[\text{2H}]$ -tetrahymanol by incubating squalene with a homogenate of *T. pyriformis* in 99.5% deuterium oxide. The obtained tetrahymanol showed ca. 41%  $d_1$  and its  $^1\text{H}$  NMR spectrum was indistinguishable from that of an authentic sample. The deuterium NMR of the  $[\text{2H}_1]$ tetrahymanol (Figure 2D) showed a single deuterium peak at 1.39 ppm. The agreement of the deuterium chemical shifts of  $[\text{2H}]$ tetrahymanol (1.39 ppm) and of the  $3\beta$ -deuterio (**3b**) (1.37 ppm) is in accord with the  $3\beta$  location of the deuterium atom in tetrahymanol.

In addition to the deuterium NMR studies, we have also obtained infrared spectral evidence which fully supports the conclusions of the  $3\beta$  location of the deuterium in tetrahymanol. The rationale for considering the application of C- $^2\text{H}$  stretching frequencies for the stereochemical assignment was based on the observation that the axial and equatorial C-3 deuterons of cholestane and its analogues exhibited substantial infrared spectral differences.<sup>14</sup> We, therefore, examined the C- $^2\text{H}$  stretching region of the synthetic model compounds **2a**, **2b**, **3a**, and **3b** used in the  $^2\text{H}$  NMR studies, and of the biosynthesized  $[\text{2H}_1]$ tetrahymanol. The C-3  $^2\text{H}$  stretching frequencies of the reference compounds **2a**, **2b**, **3a**, and **3b** and of  $[\text{2H}_1]$ tetrahymanol (**1**) are summarized in Table II.

In accord with previous observations,<sup>14</sup> the  $3\alpha$ - (**2a**) and  $3\beta$ - (**2b**)  $[\text{2H}]$ cholestanes showed overlapping bands at 2153 and 2155  $\text{cm}^{-1}$  and distinct, characteristic bands at 2129 and 2170  $\text{cm}^{-1}$ , respectively. The  $3\alpha$ - and  $3\beta$ - $[\text{2H}]$ -4,4-dimethyl- $5\alpha$ -cholestanes, in addition to several minor bands, showed two strong characteristic bands, at 2128  $\text{cm}^{-1}$  for the  $3\alpha$ -deuterio (**3a**) (Figure 3A) and 2144  $\text{cm}^{-1}$  for the  $3\beta$ -deuterio (**3b**) (Figure 3B). It is apparent that the C- $^2\text{H}$  stretching vibration at 2147  $\text{cm}^{-1}$  of  $[\text{2H}_1]$ tetrahymanol (**1**) (Figure 3C) is essentially analogous to that of the  $3\beta$ -deuterio (**3b**) (Figure 3B). Also, the overall transmission pattern of  $[\text{2H}_1]$ tetrahymanol (Figure 3C) resembles that of **3b** (Figure 3B) and not that of

the 3 $\alpha$ -[<sup>2</sup>H] isomer (**3a**) (Figure 3A). The results support the conclusion derived from <sup>2</sup>H NMR that the C-3 deuterium of tetrahymanol, biosynthesized from squalene by *T. pyriformis* enzymes in <sup>2</sup>H<sub>2</sub>O, is located at the 3 $\beta$  position of **1**. It should be noted, however, that the infrared method of determining the position ( $\alpha$  or  $\beta$ ) of deuterium in such compounds is considerably less sensitive than the <sup>2</sup>H NMR. The transmission pattern of the 7:3 mixture of **3b** and **3a** (obtained by hydrogenation of **7b**) was very similar to the pattern of pure **3b** and had only a small amount of additional absorption in the region of 2140–2110 cm<sup>-1</sup>. In practice, the admixture of ca. 30% of the 3 $\alpha$ -[<sup>2</sup>H] (**3a**) in a sample of the 3 $\beta$ -[<sup>2</sup>H] (**3b**) isomer most probably would not have been detected.

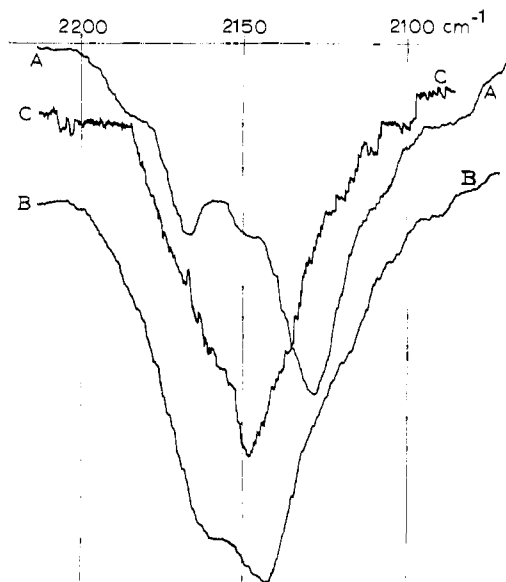
The X-ray structure determination<sup>15</sup> showed that tetrahymanol has the all-trans-anti chair structure, with a 10 $\beta$ -angular methyl and a 5 $\alpha$ -hydrogen atom. Based on the concepts of Eschenmoser et al.<sup>26</sup> and Cornforth,<sup>27</sup> it may be inferred that the biosynthesis of tetrahymanol involves the cyclization of squalene coiled in the all-chair form. Also, it is apparent that no rearrangement occurs in the process of the formation of the pentacyclic system. For the elaboration of the trans 10 $\beta$ -(CH<sub>3</sub>)-5 $\alpha$ (H) system of rings A and B of natural products, it is presumed that squalene is coiled in such a manner that its 5(6) double bond is located in a plane below the 2(3) double bond. Under these circumstances, generation of C-2 cation via a topside attack on the 2(3) double bond would allow the electron flow from the opposite side and the formation of the 4(5) bond of the triterpene from below.

This rationalization is used for the explanation of formation of 2,3(*S*)-oxidosqualene and its enzymatic cyclization to the 3 $\beta$ -hydroxy-5 $\alpha$ (*H*)-10 $\beta$ -methyl products (B). Hence, in analogy to the enzymatic cyclization of 2,3(*S*)-oxidosqualene, the proton attack on the terminal double bond could be expected to occur from the top and can be formally presented as shown in C. Our results demonstrate that the hydrogen (deuterium) atom introduced in the cyclization of squalene assumed the 3 $\beta$  configuration in tetrahymanol. This observation is in accord with the proposed rationalization of the mode of coiling and cyclization of squalene in the elaboration of tetrahymanol.

## Experimental Section

Infrared spectra were taken on a Perkin-Elmer Model 237 spectrometer. C-<sup>2</sup>H expanded scale infrared spectra were taken on a Perkin-Elmer 621 spectrometer. NMR spectra were taken on a Varian EM-360 or Varian HA-100 instrument. Deuterium NMR spectra were taken at the Southern New England High Field NMR facility, Chemistry Department, Yale University, at 41.44 MHz on a Bruker HX 270 instrument operating in the FT mode with proton decoupling. Samples were dissolved in degassed CHCl<sub>3</sub> with CDCl<sub>3</sub> as internal standard ( $\delta$  7.27). Mass spectra were recorded on a Nuclide 12-90-G mass spectrometer equipped with a Nuclide DA/CS 1.2 data acquisition system. Merck silica gel (HF 254 + 366) was used for TLC and for argentation TLC (10% silver nitrate w/w) in the indicated solvents. Melting points were taken on a hot stage, and are corrected. LiAlD<sub>4</sub> and BD<sub>3</sub>-THF were obtained from Alfa Inorganics. Dry pyridine was prepared by distillation from BaO. Tetrahydrofuran (THF) was dried by distillation from LiAlH<sub>4</sub>. Woelm neutral alumina, activity grade super I, was obtained from ICN Co., and was used as received.

**3-Benzylmercapto-4,4-dimethyl-5 $\alpha$ -cholest-2-ene (6).** To a solution of 4,4-dimethyl-5 $\alpha$ -cholestan-3-one (**5**, 2.20 g) in glacial acetic acid (220 mL), toluene- $\alpha$ -thiol (3.36 mL) and boron trifluoride etherate (10.4 mL) were added sequentially, and the mixture was kept (18 h) at 25 °C. The product began crystallizing after 5 min. The product was separated by filtration and washed with methanol. Crystallization (CHCl<sub>3</sub>-MeOH) gave **6** (1.80 g): mp 96.5–97 °C (fine needles);  $\nu_{\max}$  1220 cm<sup>-1</sup>; NMR  $\delta$  0.65 (3 H, s), 3.86 (2 H, s), 5.55 (1 H, dd,  $J_1 = 2$ ,  $J_2 = 7$  Hz), 7.27 (5 H, br s); mass spectrum  $m/e$  (rel intensity) 522 (15), 521 (44), 520 (M<sup>+</sup>, 100), C<sub>36</sub>H<sub>56</sub>S requires 520), 506 (3), 505 (6), 435 (4), 430 (11), 398 (5), 397 (6), 384 (4), 383 (11), 355 (8), 329 (3), 91 (50).



**Figure 3.** C-<sup>2</sup>H stretching patterns for deuterated model compounds and biosynthetic tetrahymanol: (A) 3 $\alpha$ -[<sup>2</sup>H]<sub>1</sub>-4,4-dimethyl-5 $\alpha$ -cholestane (**3a**); (B) 3 $\beta$ -[<sup>2</sup>H]<sub>1</sub>-4,4-dimethyl-5 $\alpha$ -cholestane (**3b**); (C) 3 $\beta$ -[<sup>2</sup>H]tetrahymanol (**1**).

**4,4-Dimethyl-5 $\alpha$ -cholest-2-ene (7a).** 3-Benzylmercapto-4,4-dimethyl-5 $\alpha$ -cholest-2-ene (**6**, 300 mg) and nickel chloride hexahydrate (1.8 g) were dissolved in warm 95% ethanol (200 mL), and the solution was cooled to room temperature. Then sodium borohydride (500 mg) in water (10 mL) was slowly added (5 min) to the rapidly stirred solution. The mixture was stirred and refluxed (2 h) under N<sub>2</sub>. The hot solution was filtered (Celite) and concentrated to ca. 25 mL under reduced pressure. Water (200 mL) was then added and the mixture was extracted with hexane (3 × 100 mL). The extract was dried (Na<sub>2</sub>SO<sub>4</sub>) and evaporated under reduced pressure to yield the crude **7a**. Argentation TLC (solvent hexane) showed a single band ( $R_f$  0.3). The oily product became crystalline on standing, and was recrystallized from CHCl<sub>3</sub>-MeOH (prisms): mp 88–89 °C (lit.<sup>20</sup> mp 89–90 °C); NMR  $\delta$  0.66 (3 H, s, 18-Me<sup>18b</sup>), 0.88 (3 H, s), 0.90 (3 H, s), 0.93 (19-Me<sup>22b</sup>), 5.41 (2 H, m).

**Hydroboration of 7a.** To a stirred (under N<sub>2</sub>) solution of **7a** (104 mg, 0.26 mmol) in dry THF (30 mL), a solution of borane in THF (1 M, 2.6 mL) was added in two portions during 5 min. The mixture was stirred at 25 °C for 2 h. Then water was added dropwise, followed by NaOH (3 M, 1 mL) and 30% hydrogen peroxide (1 mL). The mixture was stirred at room temperature for 2 h, diluted with water, and extracted with ether. The extract was washed (saturated NaCl), dried (Na<sub>2</sub>SO<sub>4</sub>), and evaporated to give a residue (121 mg). This was fractionated by preparative TLC (8% EtOAc-hexane) and the products from four main bands were recovered to give alcohol I (24 mg), alcohols II and III isolated in small amounts and incompletely resolved, and alcohol IV (8 mg). The four alcohols (I–IV) were identified as follows. Alcohol I (**8a**) had NMR  $\delta$  0.63 (3 H, s, 18-Me) five sharp lines, ca. 3 H each at 0.82, 0.85, 0.87, 0.90, and 0.92, and 3.40 (1 H, dd,  $J_1 = J_2 = 3$  Hz). Alcohol I (22 mg) in acetone (5 mL) was treated with Jones reagent (3 drops) at 25 °C for 10 min. The mixture was diluted with H<sub>2</sub>O and extracted with ether, and the extract was washed (dilute NaHSO<sub>3</sub>, saturated NaCl), dried (Na<sub>2</sub>SO<sub>4</sub>), and evaporated to yield 4,4-dimethyl-5 $\alpha$ -cholestan-3-one (**5**), identical with an authentic sample.<sup>17</sup>

A solution of ketone **5** (42 mg) in THF (3 mL) and MeOH (4 mL) was stirred (3.5 h) with sodium borohydride (100 mg) at 25 °C. Water was added and the solution was extracted with ether; the ether was washed (saturated NaCl), dried (Na<sub>2</sub>SO<sub>4</sub>), and evaporated to yield a residue (21 mg). The bulk of the residue (ca. 90%) showed an  $R_f$  value identical with that of alcohol III (**8b**).

Alcohol IV (**11a**, 15 mg) in acetone (2 mL) was treated with Jones reagent (5 drops) at 25 °C for 5 min. A diluted solution of NaHSO<sub>3</sub> was added and the mixture extracted with ether. The extract was washed (saturated NaCl), dried (Na<sub>2</sub>SO<sub>4</sub>), and evaporated to give nearly pure 4,4-dimethyl-5 $\alpha$ -cholestan-2-one (**9**, 10 mg). Following

purification by TLC (solvent 3% EtOAc-hexane), the product was crystallized (CHCl<sub>3</sub>-MeOH) (long prisms): mp 123–124 °C (lit.<sup>20</sup> mp 124–125 °C);  $\nu_{\max}$  1700 cm<sup>-1</sup>; NMR  $\delta$  0.63 (3 H, s, 18-Me), 0.86 (3 H, s, 4-Me), 0.88 (3 H, s, 4-Me), 1.01 (3 H, s, 19-Me).

A portion of **9** (4 mg) in THF (1 mL) and MeOH (2 mL) was stirred (2 h) with sodium borohydride (35 mg) at 25 °C. Water was added and the solution was extracted with ether; the ether was washed (saturated NaCl), dried (Na<sub>2</sub>SO<sub>4</sub>), and evaporated to yield a single product corresponding in *R<sub>f</sub>* value to alcohol **11** (**10**).

**Reaction of 7a with Disiamylborane.** A solution of borane in THF (1.0 M, 5 mL, 5 mmol) was cooled (-10 °C) and stirred under N<sub>2</sub>. Then a solution of 2-methyl-2-butene (700 mg, 10 mmol) in dry THF (10 mL) was added dropwise from a syringe (30 min) and the stirring was continued for 2 h at 0 °C. To the prepared reagent a solution of **7a** (246 mg, 0.62 mmol) in THF (5 mL) was added, and stirring was continued at 25 °C for 15 h. Water was added dropwise, followed by 3 M NaOH (1 mL) and 30% hydrogen peroxide (1 mL), and stirring was continued without external cooling for 2 h. The mixture was diluted with water and extracted with ether; the extract was washed (dilute NaCl, saturated NaCl), dried (Na<sub>2</sub>SO<sub>4</sub>), and evaporated. The main product (**11a**, 60 mg) was isolated by preparative TLC (solvent 3% EtOAc-hexane) and crystallized from MeOH (prisms): mp 123–124 °C, with softening at ca. 95–110 °C (lit.<sup>18</sup> mp 124–125 °C);  $\nu_{\max}$  3595, 3400 cm<sup>-1</sup> (br); NMR  $\delta$  0.63 (3 H, s, 18-Me), 0.88 (3 H, s, 4-Me), 0.91 (3 H, s, 4-Me), 0.93 (3 H, s, 19-Me), 3.9 (1 H, br m, 2 $\beta$ -H).

**4,4-Dimethyl-5 $\alpha$ -cholestane (3c).** A mixture of 4,4-dimethyl-5 $\alpha$ -cholestan-2 $\alpha$ -ol (**11a**, 30 mg) and *p*-toluenesulfonyl chloride (100 mg, freshly recrystallized) in dry pyridine (2 mL) was stored at 25 °C for 40 h. Water (25 mL) was added and the mixture was extracted with ether; the extract was washed with 1 M HCl and saturated Na<sub>2</sub>CO<sub>3</sub> (twice), dried (K<sub>2</sub>CO<sub>3</sub>), and evaporated to yield the chromatographically homogeneous tosylate (**11b**). This was treated in dry THF (10 mL) with LiAlH<sub>4</sub> (100 mg) at reflux for 48 h. The reaction was terminated with a saturated solution of Na<sub>2</sub>SO<sub>4</sub>, then ether (50 mL) was added and the mixture was filtered. Evaporation of the filtrate gave a residue (31 mg), which was passed through a column of silica gel (10 g, 0.063–0.2 mm, E. Merck). Elution with hexane (100 mL) gave a hydrocarbon (13 mg), which was resolved by argentation TLC (solvent hexane) into two products: 4,4-dimethyl-5 $\alpha$ -cholestane (**3c**, 8.4 mg) (*R<sub>f</sub>* 0.95) [prisms from CHCl<sub>3</sub>-MeOH, mp 85–86 °C (lit.<sup>19</sup> 86–87 °C); NMR  $\delta$  0.63 (3 H, s, 18-Me), 0.83 (6 H, s, 4 $\alpha$ , $\beta$ -Me<sub>2</sub>), 0.86 (3 H, s, 19-Me)] and 4,4-dimethyl-5 $\alpha$ -cholest-2-ene (**7a**, 4 mg) (*R<sub>f</sub>* 0.3), identical with a sample prepared earlier.

**3 $\alpha$ -Deuterio-4,4-dimethyl-5 $\alpha$ -cholestan-2 $\alpha$ -ol (11c).** A solution of borane-*d*<sub>3</sub> in THF (Alfa, 0.82 M, 24.4 mL, 20 mmol) at -10 °C under N<sub>2</sub> was treated dropwise over 30 min with 2-methyl-2-butene (2.80 g, 40 mmol) in dry THF (40 mL), and the resultant solution was stirred at 0 °C for 2 h. Then 4,4-dimethyl-5 $\alpha$ -cholest-2-ene (**7a**, 980 mg) was added and stirred at 25 °C for 48 h. Workup, as previously described, gave **11c** (348 mg); MS 8.3% *d*<sub>0</sub>, 85.4% *d*<sub>1</sub>, 3.6% *d*<sub>2</sub>, 2.7% *d*<sub>3</sub>.

**3 $\alpha$ -Deuterio-4,4-dimethyl-5 $\alpha$ -cholestane (3a).** Alcohol **11c** (310 mg) was treated with *p*-toluenesulfonyl chloride (1.0 g) in dry pyridine (20 mL) at 25 °C for 46 h. Water (200 mL) was added, and the mixture was extracted with ether. The extract was washed with 1 M HCl and saturated Na<sub>2</sub>CO<sub>3</sub>, dried (K<sub>2</sub>CO<sub>3</sub>), and evaporated to yield the crude tosylate **11d** (438 mg). The product was chromatographically homogeneous and was not further purified. Tosylate **11d** (400 mg) was treated with LiAlH<sub>4</sub> (600 mg) in dry THF (50 mL) at reflux under N<sub>2</sub> for 46 h. After cooling, saturated Na<sub>2</sub>SO<sub>4</sub> was added dropwise, ether (150 mL) was added, and the suspension was filtered and evaporated to yield a crude product (331 mg). This was triturated with hexane, and the hexane-soluble portion was filtered through a column of silica gel (0.063–0.200 mm, Merck). Elution with hexane (200 mL) gave a mixture of two products, separated by preparative argentation TLC (solvent hexane) to give 3 $\alpha$ -deuterio-4,4-dimethyl-5 $\alpha$ -cholestane (**3a**, 102 mg) [prisms from CHCl<sub>3</sub>-MeOH; mp 85–86 °C; MS 15.4% *d*<sub>0</sub>, 84.6% *d*<sub>1</sub>] and 3-deuterio-4,4-dimethyl-5 $\alpha$ -cholest-2-ene (**7b**, 106 mg), NMR as in **7a** except in the vinyl H region,  $\delta$  5.43 (1 H, dd, *J*<sub>1</sub> = 6, *J*<sub>2</sub> = 2 Hz).

**Hydrogenation of 7b.** The  $\Delta^2$  product **7b** (106 mg) in ethyl acetate (5 mL) with platinum oxide (110 mg) was hydrogenated at room temperature and atmospheric pressure for 3 h. The solution was filtered and evaporated to yield **3** [mixture of **3a** (30%) + **3b** (70%) as shown by <sup>2</sup>H NMR:  $\delta$  1.14 and 1.37 (ratio 3:7)]. The product gave

prisms from CHCl<sub>3</sub>-MeOH, mp 85–86 °C (lit.<sup>23</sup> mp 86–87 °C); mass spectrum indicated *d*<sub>0</sub> 9.4%, *d*<sub>1</sub> 86.9%, *d*<sub>2</sub> 2.1%, *d*<sub>3</sub> 1.6%.

**3-Deuterio-4,4-dimethyl-5 $\alpha$ -cholest-2-ene (7b).** 4,4-Dimethyl-5 $\alpha$ -cholestan-3-one (**5**, 2.17 g) in absolute ether (80 mL) was treated with LiAlD<sub>4</sub> (200 mg) at reflux under N<sub>2</sub> for 20 h. After cooling, saturated Na<sub>2</sub>SO<sub>4</sub> solution was added dropwise to destroy excess hydride. The mixture was filtered and the filtrate evaporated to yield 3 $\alpha$ -deuterio-4,4-dimethyl-5 $\alpha$ -cholestan-3 $\beta$ -ol (**8c**, 2.20 g). A portion of the product was recrystallized from CHCl<sub>3</sub>-MeOH, prisms: mp 155–156 °C (lit.<sup>25</sup> mp 156–157 °C); NMR  $\delta$  0.60 (18-Me), single peaks (ca. 3 H each) at 0.77, 0.80, 0.82, 0.86, 0.88, 0.93; no signal for 3 $\alpha$ -H was visible; mass spectrum indicated *d*<sub>0</sub> 1.1%, *d*<sub>1</sub> 93.0%, *d*<sub>2</sub> 3.4%, *d*<sub>3</sub> 2.5%.

The product **8c** (2.10 g) was then dissolved in dry pyridine (30 mL) and treated with *p*-toluenesulfonyl chloride (5 g, recrystallized from hexane) at room temperature for 20 h. Water (150 mL) was added and the mixture was stirred for 1 h at room temperature. The product was recovered with ether, and the extract was washed with 1 M HCl, 10% Na<sub>2</sub>CO<sub>3</sub>, and saturated NaCl, dried (Na<sub>2</sub>SO<sub>4</sub>), and evaporated to give tosylate **8d** as a glass (2.85 g); NMR  $\delta$  0.60 (3 H, s, 18-Me), 2.42 (3 H, s, aromatic Me), 7.30 and 7.76 (AB quartet, 4, *J*<sub>AB</sub> = 8 Hz); no signal for a 3 $\alpha$ -H was visible.

Tosylate **8d** (2.85 g) was then dissolved in absolute ether (200 mL) and shaken with Woelm neutral alumina, 150 g, activity grade super 1, on a gyrotory shaker (ca. 200 rpm) for 18 h at room temperature. The mixture was filtered under reduced pressure and the residue thoroughly washed with ether. The combined filtrates were evaporated to a glass (2.0 g). The product was fractionated by argentation TLC (10% AgNO<sub>3</sub>-silica gel HF254 + 366; solvent hexane) to yield 3-deuterio-4,4-dimethyl-5 $\alpha$ -cholest-2-ene (**7b**), *R<sub>f</sub>* 0.5 (1.31 g), and another product, *R<sub>f</sub>* 0.2 (0.38 g).

**3 $\beta$ -Deuterio-4,4-dimethyl-5 $\alpha$ -cholestane (3b).** 3-Deuterio-4,4-dimethyl-5 $\alpha$ -cholest-2-ene (**7b**, 604 mg) was treated with [<sup>1</sup>H]disiamylborane as described above. The crude reaction product was separated by preparative TLC (solvent 10% EtOAc-hexane) to yield 3 $\beta$ -deuterio-4,4-dimethyl-5 $\alpha$ -cholestan-2 $\alpha$ -ol (**11e**, 343 mg). A portion of the product was crystallized from CHCl<sub>3</sub>-MeOH, prisms: mp 109–113 °C; NMR  $\delta$  0.60 (3 H, s, 18-Me), 3.45 (1 H, s, OH), 3.84 (1 H, dd, *J*<sub>1</sub> = *J*<sub>2</sub> = 12 Hz with additional *J*<sub>3</sub> = 4 Hz, 2 $\beta$ -H).

The 2 $\alpha$  alcohol **11e** (270 mg) was dissolved in dry pyridine (5 mL), then *p*-toluenesulfonyl chloride (500 mg) was added and the mixture was stored at room temperature for 40 h. Water (100 mL) was added and the product was isolated as usual to give the tosylate **11f** (310 mg) as a glass, which gave a single spot (*R<sub>f</sub>* 0.8) on TLC (solvent 8% EtOAc-hexane). A mixture of the crude tosylate and LiAlH<sub>4</sub> (450 mg) in THF was refluxed under N<sub>2</sub> for 24 h. After workup, a crude product (208 mg) was obtained as a semicrystalline gum. This was separated by argentation TLC (solvent hexane) to give the  $\Delta^2$  compound **7b** (31 mg) (MS *d*<sub>0</sub> 0.4%, *d*<sub>1</sub> 96.5%, *d*<sub>2</sub> 3.0%, *d*<sub>3</sub> 0.2%) and 3 $\beta$ -deuterio-4,4-dimethyl-5 $\alpha$ -cholestane (**3a**, 67 mg) as a gum. The gum crystallized on standing and was recrystallized from CHCl<sub>3</sub>-MeOH, prisms: mp 85–86 °C; <sup>1</sup>H NMR identical with that of **3c**; mass spectrum indicated *d*<sub>0</sub> 1.8%, *d*<sub>1</sub> 97.4%, *d*<sub>2</sub> 0.7%.

**Biosynthesis of [3 $\beta$ -<sup>2</sup>H<sub>1</sub>]Tetrahymanol (1).** *Tetrahymana pyriformis*, type W (ATCC 10542), was grown in 25-L flasks, as previously described.<sup>2</sup> The cells were collected by continuous centrifugation. The combined pellets were suspended in water (100 mL) and divided into four equal fractions. Each fraction was placed in a disruption flask of a Braun homogenizer, to which glass beads (10 g) (0.17–0.18 mm diameter) were added. The bottles were closed and individually placed in a Braun Model MSK cell, homogenized, and cooled briefly (ca. 10 s) with liquid CO<sub>2</sub> from a gas cylinder (avoid freezing). The flasks were then shaken for 1 min with adiabatic cooling (liquid CO<sub>2</sub>). The beads were separated by decantation and the homogenates from the four flasks were combined. The cold homogenate was extracted gently with ice-cold ether (freshly distilled from FeSO<sub>4</sub>·5H<sub>2</sub>O) and the extraction was repeated four times (300 mL of ether total). Most of the remaining ether was removed with a stream of nitrogen and the homogenate was lyophilized (in a cold room) to give a powder (7.0 g).

A portion of the powder (2 g) was suspended in deuterium oxide (99.5% <sup>2</sup>H) (6 mL) and stirred (15 min) in a cold room. Then ether (freshly distilled from FeSO<sub>4</sub>·5H<sub>2</sub>O) (50 mL) was added and the mixture was gently swirled (10–15 min). The ether was decanted and the ether extraction was repeated for a total of five times. The [<sup>2</sup>H<sub>2</sub>O] homogenate was frozen and lyophilized to give a powder.

The deuterated powder was suspended in deuterium oxide (9 mL) and an emulsion of squalene (105 mg) prepared with Triton X-100 (30 mg) in deuterium oxide (1 mL) was added. The mixture was incubated at ambient temperature for 24 h. The reaction was terminated by the addition of a mixture of ether-ethanol (1:3, 100 mL). The denatured proteins were removed by filtration and the filtrate was concentrated to leave an aqueous phase. The aqueous phase was saturated with NaCl and extracted with freshly distilled, peroxide-free hexane (3 × 100 mL). The hexane extract was washed with a small amount of a saturated saline solution, dried, and concentrated to a residue. The residue was fractionated by TLC (EA-hexane, 3:17) and the recovered tetrahymanol was chromatographed in the same system. The samples of tetrahymanol isolated from these incubations contained ca. 40–50% of <sup>2</sup>H<sub>1</sub>. The MS of a sample of tetrahymanol pooled from several incubations (11.9 mg) showed a deuterium content of 41.0% d<sub>1</sub>.

**Acknowledgment.** This work was supported in part by NIH Grant RR05528 and by Institutional funds. The <sup>2</sup>H NMR spectra were recorded at the High Field NMR Facility, Yale University, supported in part by NIH Grant 1-P07-RR00798 from the Division of Research Resources. We thank Mr. Cliff McDonald and Dr. Warren G. Anderson for <sup>1</sup>H NMR spectra, and Dr. Thomas A. Wittstruck for mass spectra. We thank Dr. Satya P. Jindal for carrying out the conversion of squalene to [<sup>2</sup>H]tetrahymanol.

## References and Notes

- (1) For a review of the topic, see Nes, W. R.; McKean, M. L. "Biochemistry of Steroids and Other Isopentenoids", University Park Press: Baltimore, Md., 1977; pp 229–270.
- (2) Zander, J. M.; Greig, J. B.; Caspi, E. *J. Biol. Chem.* **1970**, *245*, 1247.
- (3) (a) Caspi, E.; Greig, J. B.; Zander, J. M. *Biochem. J.* **1968**, *109*, 931. (b) Wittstruck, T. A.; Caspi, E. *J. Chem. Res.* **1977**, (S), 180, (M), 2135.
- (4) Caspi, E.; Zander, J. M.; Greig, J. B.; Mallory, F. B.; Conner, R. L.; Landrey, J. R. *J. Am. Chem. Soc.* **1968**, *90*, 3563.
- (5) Caspi, E.; Greig, J. B.; Zander, J. M. *Chem. Commun.* **1969**, 28.
- (6) Zander, J. M.; Caspi, E. *Chem. Commun.* **1969**, 209.
- (7) Aberhart, D. J.; Jindal, S. P.; Caspi, E. *J. Chem. Soc., Chem. Commun.* **1978**, 333.
- (8) Caspi, E. Proceedings of the 11th International Symposium on Chemistry of Natural Products, Golden Sands, Bulgaria, 1978; Vol. 4, Part 1, p 166.
- (9) Gain, R. E.; Caspi, E. Unpublished results.
- (10) (a) Al-Rawi, J. M. A.; Bloxside, J. P.; O'Brien, C.; Caddy, D. E.; Elvidge, J. A.; Jones, J. R.; Evans, E. A. *J. Chem. Soc., Perkin Trans. 2* **1974**, 1635. (b) Al-Rawi, J. M. A.; Elvidge, J. A.; Jones, J. R.; Evans, E. A. *ibid.* **1975**, 449. (c) Al-Rawi, J. M. A.; Elvidge, J. A.; Jaiswal, D. K.; Jones, J. R.; Thomas, R. *J. Chem. Soc., Chem. Commun.* **1974**, 220. (d) Al-Rawi, J. M. A.; Elvidge, J. A.; Thomas, R.; Wright, B. *J. Chem. Soc., Chem. Commun.* **1974**, 1031. (e) Altman, L. J.; Silberman, N. *Steroids* **1977**, *29*, 557.
- (11) (a) Bycroft, B. W.; Wels, C. M.; Corbett, K.; Lowe, D. A. *J. Chem. Soc., Chem. Commun.* **1975**, 123. (b) Sato, Y.; Oda, T.; Saito, H. *Tetrahedron Lett.* **1976**, 2695. (c) *J. Chem. Soc., Chem. Commun.* **1977**, 415. (d) Dewick, P. M.; Ward, D. *ibid.* **1977**, 338. (e) Cane, D. E.; Buchwald, S. L. *J. Am. Chem. Soc.* **1977**, *99*, 6132. (f) Cane, D. E.; Murthy, P. P. N. *ibid.* **1977**, *99*, 8327.
- (12) Jackman, L. M.; Sternhell, S. "Applications of Nuclear Magnetic Resonance Spectroscopy in Organic Chemistry", Pergamon Press: Oxford, 1969; p 238.
- (13) Diehl, P. In "Nuclear Magnetic Resonance Spectroscopy of Nuclei Other Than Protons", Axenrod, T.; Webb, G. A., Ed.; Wiley-Interscience: New York, N.Y., 1974; p 275.
- (14) Corey, E. J.; Howell, M. G.; Boston, A.; Young, R. L.; Sneen, R. E. *J. Am. Chem. Soc.* **1956**, *78*, 5036.
- (15) Langs, D. A.; Duax, W. L.; Carrell, H. L.; Berman, H.; Caspi, E. *J. Org. Chem.* **1977**, *42*, 2134.
- (16) Woodward, R. B.; Patchett, A. A.; Barton, D. H. R.; Ives, D. A. J.; Kelly, R. B. *J. Chem. Soc.* **1957**, 1131.
- (17) Beton, J. J.; Halsall, T. G.; Jones, E. R. H.; Phillips, P. C. *J. Chem. Soc.* **1957**, 753.
- (18) Boar, R. B.; Hawkins, D. W.; McGhie, J. F.; Barton, D. H. R. *J. Chem. Soc., Perkin Trans. 1* **1973**, 654.
- (19) (a) Brown, H. C.; Brown, C. A. *J. Am. Chem. Soc.* **1963**, *85*, 1005. (b) Brown, C. A.; Ajuka, V. K. *J. Org. Chem.* **1973**, *38*, 2226.
- (20) Lablanche-Comblie, A.; Levisalles, J.; Pete, J. P.; Rudier, H. *Bull. Soc. Chim. Fr.* **1963**, 1689.
- (21) Brown, H. C. "Organic Syntheses via Boranes", Wiley-Interscience: New York, N.Y., 1975; p 23.
- (22) (a) Lablanche-Comblie, A.; Levisalles, J. *Bull. Soc. Chim. Fr.* **1964**, 2236. (b) Hemmert, F.; Lablanche-Comblie, A.; Lacoume, B.; Levisalles, J. *ibid.* **1966**, 982.
- (23) Shoppee, C. W.; Johnston, G. A. R. *J. Chem. Soc.* **1962**, 2684.
- (24) Posner, G. H.; Gurria, G. M.; Babiak, K. A. *J. Org. Chem.* **1977**, *42*, 3173.
- (25) Atwater, N. W. *J. Am. Chem. Soc.* **1960**, *82*, 2847.
- (26) Eschenmoser, A.; Ruzicka, L.; Jeger, O.; Arigoni, D. *Helv. Chim. Acta* **1955**, *38*, 1890.
- (27) Cornforth, J. W. *Angew. Chem., Int. Ed. Engl.* **1968**, *7*, 903.

## Specific Inclusion Catalysis by $\beta$ -Cyclodextrin in the One-Step Preparation of Vitamin K<sub>1</sub> or K<sub>2</sub> Analogues

Iwao Tabushi,\* Kazuo Yamamura, Kahee Fujita,<sup>1</sup> and Hiromu Kawakubo

Contribution from the Department of Synthetic Chemistry, Kyoto University, Yoshida, Kyoto 606, Japan. Received April 10, 1978

**Abstract:** Electrophilic allylation at the C<sub>3</sub> position of 2-methylhydronaphthoquinone-1,4 (**5**) with allyl (**6a**), crotyl (**6b**), methallyl (**6c**), or prenyl bromide (**6d**) was successfully developed to give a highly selective one-step preparation of the corresponding vitamin K<sub>1</sub> (or K<sub>2</sub>) analogue (**7a–d**) by the use of  $\beta$ -cyclodextrin in dilute aqueous alkaline solution. In order to elucidate the basis of this interesting inclusion catalysis which resembles "ligase and/or oxidase" reactions, mechanistic studies were carried out. The specific inclusion binding of the substrate, 2-methylhydronaphthoquinone (**5**), by  $\beta$ -cyclodextrin ( $K_a = 490 \text{ M}^{-1}$ , pH 3.55) facilitated the proton dissociation of **5**, resulting a decrease in its pK<sub>a</sub> value from 9.45 for uncomplexed **5** to 8.9 for complexed **5**. Based on kinetic results showing that the rate of allylation of  $\alpha$ -naphthol at pH 10.4 was enhanced by 2.5 to 3.5 times in the presence of  $\beta$ -cyclodextrin, it was concluded that the nucleophilic reactivity of the partially charged carbanion increased in the hydrophobic cavity and, therefore, the allylation reactions were accelerated by  $\beta$ -cyclodextrin. Another noteworthy aspect of the mechanism was seen in the interesting observation that 2-methylnaphthosemiquinone anion radical (detected by ESR) which was produced by oxidation of the allylated hydronaphthoquinone by molecular oxygen was strongly bound by  $\beta$ -cyclodextrin. This indicated either the possibility that the oxidation proceeds predominantly through the complexed form of the allylated hydroquinone or that the lifetime of the semiquinone anion radical is prolonged by the inclusion binding. Vitamin K analogues **7** and **8**, which were products, were found to be highly susceptible to oxidative degradation due to the attack of hydrogen peroxide, which was shown to be another product of the oxidation step.  $\beta$ -Cyclodextrin effectively protected those quinones from the attack by hydrogen peroxide, and their oxidation rates were from 1/9 to 1/17 of that for the uncomplexed quinone.

In the past decade there has been considerable interest in cyclodextrin inclusion catalysis and a wealth of information has been collected on mechanistic details, mostly with respect

to the use of cyclodextrin as hydrolase models.<sup>2</sup> However, no reported inclusion catalysis has been of practical use as a synthetic tool except for the interesting example of the exclu-