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 OXIDE ANALOGS.

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ENZYMIC AND ACID CYCLIZATIONS OF SQUALENE OXIDE ANALOGS

A DISSERTION

SUBMITTED TO THE DEPARTMENT OF CHEMISTRY

AND THE COMMITTEE ON THE GRADUATE DIVISION

OF STANFORD UNIVERSITY

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

By

Robert Paul Hanzlik

May 1970

I certify that I have read this thesis and that in my
opinion it is fully adequate, in scope and quality, as
a dissertation for the degree of Doctor of Philosophy.

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ACKNOWLEDGMENTS

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INTRODUCTION

One of the longest continuous threads in the fabric of organic chemistry and biochemistry is spun from the long and intricate chain of events related to the biosynthesis of triterpenes and sterols from the humble precursor acetate. Although several excellent reviews of these processes are available,[1] a brief summary of the major steps serves as a background for, and an introduction to, the area in which the research described in this Thesis was conducted.

Acetate $\longrightarrow$ Mevalonate $\longrightarrow$ Isopentenyl pyrophosphate

Squalene $\longleftarrow$ Farnesyl pyrophosphate

Squalene oxide **1** $\longrightarrow$ Lanosterol **2**

Natural processes are frequently models of simplicity and efficiency. It therefore behooves one to study and imitate these processes. This is the essence of the concept of biogenetically-patterned organic chemical syntheses,[2] a concept which was successfully applied in the alkaloid field by Professor van Tamelen and his research group starting more than a decade ago. His extension of this reasoning to some of the later steps in sterol biosynthesis led to the discovery of several new and important chemical reactions. Included here are (i) the reductive dimerization of farnesyl units to form squalene, (ii) the selective terminal epoxidation of squalene, and (iii) the conversion of this epoxide to polycyclic triterpenes. The laboratory counterparts of these reactions are found in (i) the titanium-based coupling reaction,[2,3] (ii) the selective action of N-bromosuccinimide in terminal oxidations,[2,4] an important discovery which preceded the finding that squalene oxide was a natural intermediate in sterol biosynthesis, and (iii) the proton- and Lewis acid -catalyzed cyclizations of polyolefin terminal epoxides.[2,5]

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The discovery that living systems first epoxidized squalene and then in a separate step converted the epoxide to polycyclic triterpenes was revealed simultaneously by two research groups, E. E. van Tamelen, R. B. Clayton, and co-workers at Stanford,[6] and by E. J. Corey and W. E. Russey at Harvard.[7] In the few short years since this disclosure, the squalene oxide story has taken two distinct courses, one extensive and one intensive.

Thus squalene oxide has been found common to numerous diverse living systems which produce myriad tetra- and pentacyclic triterpenes as well as sterols. Although the recent hypothesis that lanosterol biosynthesis in yeast might proceed via proton-initiated cyclization of squalene followed by subsequent hydroxylation has been refuted by its original proponent,[8] a protozoan organism has been shown to form tetrahymanol from squalene via a proton-initiated cyclization with subsequent trapping of the pentacyclic cation by water.[9]

Also very intriguing from an evolutionary point of view is the fact that a common species of fern has been shown to form β-sitosterol from squalene oxide, while forming fern-9-ene by direct cyclization of squalene.[10]

Squalene oxide ◄──────── Squalene

HO

β -Sitosterol   Fern-9-ene

The widespread distribution of squalene oxide both beautifully exemplifies

the simplistic economy of nature ("Nature doth not that by many things

that may be done by few"[11]), and extends the generality of the Eschen-

moser-Stork scheme which related all polycyclic triterpenes to squalene

on theoretical grounds more than a decade before the discovery of squa-

lene oxide.[12,13]

On the other hand, the discovery of squalene oxide and of a key

enzyme in mammalian steroidogenesis, squalene-2,3-oxide lanosterol cyclase,

started a cascade of experiments aimed at understanding how the enzyme

catalyses the impressive isomerization of an acyclic epoxide with one

asymmetic center into a tetracyclic molecule possessing seven centers

of asymmetry. Work on this aspect has been carried out successfully at

both Stanford and Harvard Universities. Analogs of the natural substrate

were presented to a partially-purified cyclase system in the hope that

the normal process would be modified in a way that would reveal something

of the inner workings of the enzyme - i.e., the active site behavior and/or

the reaction mechanism.

Shortly after Jim Willet finished the initial studies with squalene

oxide, Barry Sharpless prepared synthetic analogs which incorporated

a reduced terminal double bond (22,23-dihydrosqualene-2,3-oxide (3)), and

a shortened hydrocarbon chain (23,24,24'-trisnorsqualene-2,3-oxide (5)).

He showed that these artificial substrates were converted by the cyclase

to lanosterol analogs 4 and 6.[14] These results were very encouraging

in that they showed that the enzyme displayed some tolerance for unnat-

ural substrates.
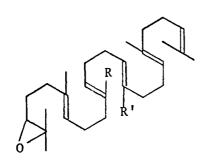
It was at about this time that I came to Stanford and decided to join Professor van Tamelen's research group, which had recently begun collaborating with R. B. Clayton at the Stanford Medical Center. With Ronn Nadeau and Barry Sharpless, I embarked on what was expected, and later found, to be an exciting chemical search for the mechanism of action of squalene-2,3-oxide lanosterol cyclase.

DISCUSSION

After considering the possible enzymic consequences, we decided to use two basic types of substrate alterations. Barry Sharpless would prepare and test 18,19-dihydrosqualene-2,3-oxide as a substrate. This modification would certainly prevent the formation of a tetracyclic product and might provide some insight into the nature of the cyclization, particularly in C-D ring forming steps.

On the other hand, I would work on altering the pattern of the methyl substituents in the substrate. In addition to altering various inter- and intramolecular steric interactions, these modifications would influence the nucleophilicity (reactivity) of the double bonds and the relative stabilities of potential carbonium ion centers in the molecule. These points were of interest for two reasons. First, because of the comparisons that could be made between enzymic- and acid-catalyzed cyclizations of epoxides, and second, because there was no evidence available which tended to indicate whether or not the enzymic process actually was cationic, although it was reasonable to assume so.

In particular, the series 15'-nor- ,10'-nor- , and 10',15'-bisnor-squalene-2,3-oxide appeared interesting and invited us to devise a flexible synthetic method with which to generate these and possibly other potential substrates.

Modified Squalene Oxides

	R	R'	
7,	CH_3	H	15'-nor
8,	H	CH_3	10'-nor
9,	H	H	10',15'-bisnor

10

The titanium coupling reaction was an obvious choice for flexibility since an endless variety of allylic alcohols could be cross-coupled in all combinations. In general however, a cross-coupling of two alcohols A and B produces three hydrocarbons A-A, A-B, and B-B.[15] Furthermore, unless the given hydrocarbon is symmetrical, terminal monoepoxidation produces two epoxides which are not easily separable (for example see Corey, et al.[16]). Reasoning that this difficulty might be overcome if one of the two alcohols was functionalized terminally before coupling, I prepared 10,11-epoxyfarnesol 10 using the standard terminal oxidation method.[4] Barry Sharpless subjected a mixture of epoxy-alcohol 10 and farnesol to the coupling conditions, but the epoxide moiety did not survive. Next I converted the acetate 10a to acetal-alcohol 11 by a sequence of epoxide hydrolysis, sodium periodate cleavage, acetal formation, and saponification. We then found that 11 cross-coupled efficiently with geraniol (12) to produce acetal 13.[3] This experiment was doubly rewarding because while showing that a general coupling scheme was workable, it also provided a precursor for a modified substrate (page 96).

Synthesis of the 15'-norepoxide required the coupling of 11 with 3'-norfarnesol 16. The latter alcohol was synthesized using a method adapted from the synthesis of 3'-norfarnesic acid used by Eschenmoser, et.al.[17] Geraniol was converted to its chloride using triphenylphosphine and carbon tetrachloride (the Lee reagent).[18]

10, R=H
10a, R=OAc

11

12

13

10-OAc

14

15

11

Treatment of this chloride with propargyl Grignard effected a three-carbon homologation and produced geranyl propyne (**17**) in good yield. This chlorination-homologation sequence was repeated with a sample of nerol (**19**) furnished by John McCormick. By comparing the respective products using a DC550 glc column, it was shown that virtually no cis-trans isomerization occurred in either case. In both cases varying amounts of other non-terminal-acetylene hydrocarbons formed, but they were not removed since they would not react further.

The next homologation involved formation of the lithium acetylide

by treating the crude geranyl propyne in THF with methyl lithium. Next,

gaseous formaldehyde (from pyrolysis of paraformaldehyde at 180°) was

swept into the flask containing the acetylide solution, using a stream

of dry nitrogen. The acetylenic alcohol 18 was isolated by extraction

with ether. The unreacted hydrocarbons were removed by distillation

. at reduced pressure. The crude alcohol was then reduced by refluxing

with lithium aluminum hydride in THF. This reduction was later studied

in detail by Corey, et al.,[19] who showed that an organo-aluminum com-

plex was involved, and attributed the specificity of the trans reduction

to the nature of this complex. A small amount of allenic hydrocarbon

(IR 1950 cm^{-1}) also formed during this reduction. The 3'-norfarnesol

was purified by distillation at reduced pressure.

Alcohols 16 and 11 were coupled in a ratio of 10:1 using the standard

titanium system. The hydrocarbon fraction, after thiourea clathrate formation, consisted of two components present in equal amounts. One of these was presumably the all-trans isomer of 10',15'-bisnorsqualene. The monoacetal fraction was found to consist of five components upon glc analysis. After thiourea clathrate purification only three components remained. Their retention times on glc (DEGS, 200°) were 4.65, 3.82, and 3.26 relative to squalene. The 4.65 isomer was isolated by chromatography over silver nitrate-impregnated silica gel. Spectral data confirmed the all-trans structure 20: 970 cm^{-1} for trans -HC=CH-; one trans vinyl methyl at 1.68 ppm and four cis vinyl methyls at 1.60 ppm.

The aldehyde was obtained by hydrolysis of the acetal with dilute perchloric acid in aqueous tetrahydrofuran. Following standard procedures[20] it was labeled by exchange in tetrahydrofuran containing acidic tritiated water, and treated with diphenylsulfoniumisopropylide in tetrahydrofuran at -78°, giving labeled 15'-norepoxide 7 with a specific activity of 62,150 dpm/μg.

20

epoxide 7

14

Test incubation of the 15'-norepoxide was done using an enzyme preparation which converted squalene oxide to lanosterol in 28.8% yield. Under identical conditions, each of four 100 μg samples of epoxide $\underline{7}$ was incubated with 3 ml of the cyclase, and two similar incubations were done using 3 ml of boiled enzyme as a control. The tubes were worked up using the saponification method, and the recovered material was resolved by tlc into two radioactive bands, epoxide (0.66-0.74, 25%), and a band with the same Rf as lanosterol, (0.42-0.51, 25%). (For tlc notation see page 71.) The average conversion, determined by eluting the bands and counting aliquots, was 20.6% in the incubations and 5.4% in the boiled controls. The sterol fractions were pooled, and aliquots were silylated for glc analysis. It was found by glc that in the case of the incubations, 80% of the radioactivity was associated with a peak having a retention time that was 0.58 that of lanosterol TMSE. In the case of the controls all of the radioactivity was found to have short retention times. Thus the 15'-nor substrate was converted to a single enzymic product, hereafter called "A," which was ultimately shown to have the structure $\underline{21}$.

$\underline{21}$ = "A"

15

Two large scale incubations using a more active enzyme resulted in very good yields of product A. One involved incubation of 2.46 mg of 15'-norepoxide with 123 ml of cyclase, and gave a 55.2% yield (680μg) of A, which was found to be a crystalline solid. Another 200 μg sample of the epoxide was incubated with 11 ml of the same cyclase preparation and gave 52 μg (52% yield) of A. With the same batch of enzyme squalene oxide gave a 70-75% yield of lanosterol. If the 15'-norepoxide were to undergo enzymic cyclization in a manner exactly analogous to squalene oxide, the product expected would be 18-norlanosterol (21). It was therefore natural to begin a structure determination for enzyme product A by comparing it with lanosterol (2). This was accomplished by using spectral as well as chemical means.

Time-averaged 100MHz nmr spectra of A and its acetate were obtained, as were single scan spectra of lanosterol and lanosteryl acetate. The spectrum of A was strikingly similar to that of lanosterol. Furthermore upon acetylation of A, the saturated methyl resonances shifted in the way observed for many A:B-trans-4,4-dimethyl-3β-hydroxy steroids.[21] The assignments for the lanosterol methyls in Table I are taken from Cohen and Rosenthal,[21] and from Stone, et al.[22] Of particular interest is the fact that there is no high-field resonance in the spectra of A attributable to a 13β (C-18) methyl group.

Table I: NMR Data for Lanosterol, Sterol A, and Their Acetates (ppm).

		LanOH	LanAc	A-OH	A-Ac
vinyl	:	5.12		5.12	
vinyl methyls:		1.69, 1.60		1.69, 1.60	
	4α	1.01	0.89	1.00	0.89
	10β	0.99	1.01	0.97	1.00
saturated	14α	0.89	0.89	0.80	0.81
methyls	4β	0.82	0.89	0.80	0.89
	13β	0.70	0.70		

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A mass spectrum of A-TMSE gave the expected molecular ion ($M^+ = 484.4100$, calc. for $C_{32}H_{56}OSi$, 484.4112), and had the same general appearance as a spectrum of lanosterol-TMSE, _i.e._, relatively simple and lacking any pronounced fragmentation pattern, except for the usual silyl-ether peaks.

When A-TMSE was hydrogenated over platinum in ethyl acetate for 30 minutes, a new product was formed with a glc retention time 0.69 that of A-TMSE. Its mass spectrum was similar to the relatively plain spectrum produced by dihydrolanosterol TMSE, and showed it to be a dihydro derivative, H_2A-TMSE, as suggested by its glc retention time. Notably absent in the mass spectrum of H_2A-TMSE was a peak due to loss of the side chain which would be expected for a tetracyclic $\Delta^{13(14)}$ sterol.

At this point, the available information did not rule out the possibility that A might be 18-norlanosterol but in general it only indicated that A was tetracyclic with only one double bond in the side chain and one tetra-substituted double bond in the ring system. Further insight into the location of the methyl groups and the endocyclic double bond came from the high-resolution mass spectrum of the _seco_-diketone obtained by treating H_2A-Ac with ruthenium tetroxide in carbon tetrachloride solution. Quoting from a previously published report of these results,[23]

> "The high-resolution mass spectral fragmentation pattern was
> compatible with the expected structure X and corresponded
> well with the spectral data of the homologous _seco_ product
> XI, resulting from H_2Lan-Ac upon treatment with the same
> reagent.[23a] Transannular aldolization of these 1,6-diketones
> was avoided by careful handling, and mass spectra of the in-
> tact diketones were obtained _via_ direct sample introduction.
> Major fragmentations which furnish evidence for the position

16

16

"of the carbonyl functions (and hence, the original double bond in A), are denoted in X as overall cleavages, not including associated hydrogen-transfer reactions.

Thus the fragment at m/e 305 represents the rather characteristic behavior of a cyclic ketone and limits, together with the highly saturated fragment at m/e 193, the possible positions of the carbonyl groups to the original rings B and C. Corresponding fragments occur at m/e 319 and 207 in the spectrum of the higher homolog XI.

m/e 305
($C_{20}H_{33}O_2$)

Another prominent fragment of the composition $C_{18}H_{32}O_2$ at m/e 280 (m/e 294 in XI) confines the possible carbonyl locations to a sufficiently small area of the B-C ring moiety

"to permit exclusion of alternative positions based on possible chemical structures of the olefinic precursor (A). Finally an abundant fragment recorded at m/e 279 ($C_{17}H_{27}O_3$) must comprise such a portion of the molecule as to include C-14 and C-15, since it appears at the same mass in the spectrum of the lanosterol derivative XI. An alternative formation of this fragment ion would have to include C-17 in the latter case, and thus would require the highly unlikely rupture of two bonds attached to the same carbon atom (XI). Genesis of this important ion may be initiated by cleavage ∝ to the carbonyl group with reverse charge distribution, followed by radical induced fragmentation of the 15,16 and 13,14 bonds. From the abundant fragment at m/e 279 and associated high-resolution data it is also apparent that C-14 bears the methyl group which migrated during the bio-synthesis of A."

(m/e 279)

Other evidence supporting the 8,9 placement of the double bond in A comes from acid-catalyzed isomerization results. When H_2A-Ac was treated under conditions which equilibrate Δ^8- and Δ^7- H_2Lan-Ac (HCl/CHCl$_3$ for 48 hr), a single new isomer, H_2B-Ac, formed in 90% yield, with 10% H_2A-Ac remaining. The new isomer (TMSE) had a longer glc retention time than did H_2A-TMSE, and in its mass spectrum, in contrast to that of H_2A-TMSE, there appeared an intense peak at m/e 274 and a very prominent peak at m/e 375. The 274 peak occurring at even-mass can be attributed to a

19

retro-Diels-Alder fragmentation initiated by a 7,8 double bond. This position of the double bond would also activate the molecule for the side-chain cleavage depicted in 22. Analogous prominent peaks appeared in the spectrum of Δ^7 - H_2Lan - TMSE at m/e 288 and m/e 387, respectively.

H$_2$B-TMSE, R=H

Δ^7-H$_2$Lan-TMSE, R=CH$_3$

22

R=H, m/e 274
R=CH$_3$, m/e 288

R=H, m/e 373
R=CH$_3$, m/e 387

In addition to indicating a 8(9) double bond in A, the Δ^7 / Δ^8 equilibration supports the placement of the 14-methyl in the α position, analogous to lanosterol, since Δ^8- and Δ^7- 14 β -methyl compounds undergo fairly extensive rearrangements when treated under acidic conditions.[24]

Final confirmation of the 8(9) placement of the double bond in A, as well as further evidence that A was stereochemically analogous to lanosterol, came from the uv spectrum of the 7,9(11)-diene 23-a derived from A. Synthesis of this derivative, as well as the lanosterol derivative 23-b (dihydroagnosteryl acetate) was accomplished by epoxidation of the 8,9 position of the respective dihydrosterols using meta-chloroperbenzoic acid in methylene chloride,[25] followed by dehydration of the epoxides in benzene/acetic acid containing a trace of perchloric acid.

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23a,R=H
23b,R=CH₃

24

The $8\alpha,9\alpha$-epoxide products from both H_2A-Ac and H_2Lan-Ac had the same Rf on tlc. The epoxide product ($47\mu g$) from $78\mu g$ of H_2A-Ac, dissolved in 0.80 ml of ethanol, gave a uv spectrum (Figure la) consisting of only a sloping baseline and end-absorption. After exposure of this epoxide to perchloric acid in benzene/acetic acid, the diene product ($37\mu g$) was dissolved in ethanol to successive dilutions of 0.95, 1.20, and 2.00 ml, (8.35-, 6.56-, and 3.97 X 10^{-4} M, respectively) and uv spectra were recorded (Figures lb,c,d). Three absorption maxima were apparent at 235 243, and 252 mμ, but the calculated ϵ values were too high. However, using the uv spectrum of the epoxide as a baseline, the ϵ values were found to be in the ratio 1.00:1.12:0.75, respectively. Dihydro-agnosteryl acetate 23b had uv maxima in ethanol of 237, 244, and 253 mμ, with ϵ ratio 1.00:1.22:0.84, respectively. The euphol analog of dihydro-agnosteryl acetate 24, has significantly different uv maxima in ethanol: 230, 240, and 247 mμ.[26]

a) oxide, $47\mu g/0.80$ ml
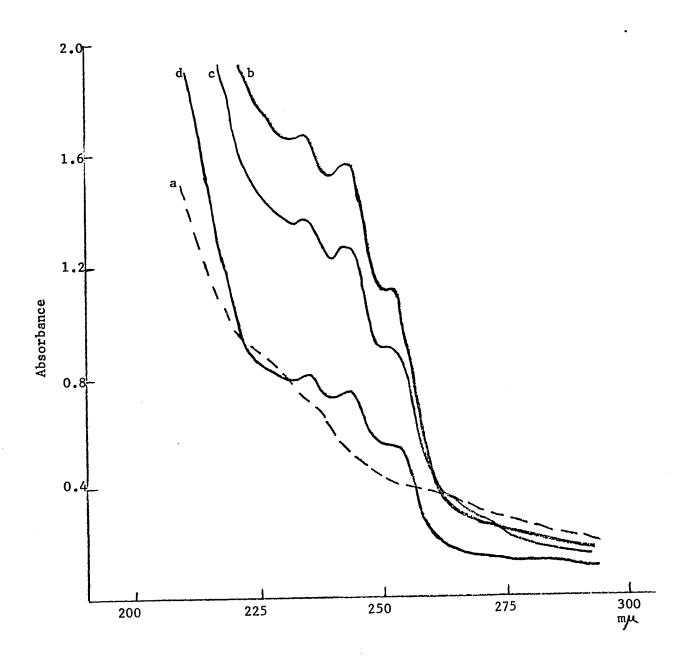b) diene, $37\mu g/0.95$ ml
c) diene, $37\mu g/1.20$ ml
d) diene, $37\mu g/2.00$ ml

Figure I. U V Spectra

Dienes 23a and 23b were also compared by high-resolution mass spectroscopy and found to behave similarly. In each case molecular ions indicating the correct compositions were observed (for 23a, M^+ = 454.3812, calc. for $C_{31}H_{50}O_2$, 454.3810; for 23b, M^+ = 468.3970, calc. for $C_{32}H_{52}O_2$, 468.3967), along with the usual M-15, M-60, and M-75 fragments typical of sterol acetates. Both spectra also contained prominent peaks which had compositions indicating that they formed by simple loss of C_8H_{17} side chains (M-113), and by further loss of acetic acid (M-173). Most characteristic of these diene systems however, was the loss, in both cases, of a $C_{11}H_{17}$ fragment ($C_{13}H_{27}O_2$ with associated loss of acetic acid), the formation of which is depicted in 25 and 26 as occurring in the same way as proposed for the pentacyclic triterpene-7,9(11)-dienes.[27]

The two dienes 23a and 23b, as TMSE derivatives, had glc retention times in the ratio 23a/23b = 0.58, the same relationship as observed for the other silyl-ether pairs: i.e., A-TMSE/Lan-TMSE and H_2A-TMSE/H_2Lan-TMSE. Along with the uv data, this suggests that diene 23a derived from A is probably in the same sterochemical series as dihydroagnosterol, and hence A is probably exactly analogous to lanosterol.

25

26a, R=H, m/e 299
26b, R=CH$_3$, m/e 313

The $M-C_{11}H_{17}$ fragmentation also indicates the probable attachment of the side chain to C-17 in the normal steroid fashion. For reasons to be discussed later (pp.41-43) it was desirable to confirm this hypothesis. Enzyme product A was converted to tetrahydro derivative 27a by hydrogenation of its acetate over platinum in acetic acid containing a trace of perchloric acid.[28] After tlc purification the product gave a mass spectrum which showed the correct molecular ion (M^+ = 458) and the usual peaks at M-15, M-60, and M-75. In addition, peaks were recorded at m/e 345 and 303 indicating loss of side chain and loss of the entire D-ring (285 and 243 with corresponding loss of acetic acid), respectively. The latter fragmentation, typical of saturated sterols, has been studied in detail by Djerassi, et al.[29] and shown to involve initial rupture of the 13-17 bond. Since the common sterols all possess a methyl substituent at C-13, the tetrahydro derivative of dammara-20(21),24-dienyl acetate (27b) was used as a model to gage the effect of a missing angular methyl on this fragmentation. Apparently there was no significant effect as the mass spectrum of 27b showed a prominent peak at m/e 317, homologous to the 303 peak in the spectrum of 27a.

27a, R=H

27b, R=CH$_3$

Enzyme product A therefore was a lanosterol analog in terms of gross structural features, and very probably in terms of stereochemistry as well, as suggested by the glc correlations and by the uv and nmr spectral data. In order to determine if the formation of A was mechanistically analogous to that of lanosterol, unlabeled 15'-norepoxide (0.96 mg) was incubated in an enzyme solution which contained tritiated water (1.4 ml, 0.235 Curie/ml). By means of tlc and glc product A was isolated and shown to be devoid of radioactivity. Thus, as in the case of lanosterol biosynthesis[30] no incorporation of a proton from the medium was observed in the biosynthesis of A.

These experiments demonstrated that the replacement of the 15'-methyl of squalene-2,3-oxide by a hydrogen had no effect on the conversion of this modified substrate to a lanosterol analog other than a small diminution in the rate of conversion relative to that of squalene oxide. This effect is discussed further on pages 29-35.

At this point, although 10',15'-bisnorsqualene was produced by the coupling of 3'-norfarnesol with itself during the synthesis of the 15'-norepoxide, it seemed more appropriate to study single substrate modifications before attempting double-modifications. Therefore I went ahead with the synthesis of the 10'-norepoxide 8. Synthesis of this compound was parallel to that of the 15'-norepoxide. Acetal-alcohol 28 was prepared from 3'-norfarnesol in a fashion similar to the synthesis of 11 from farnesol. Coupling of alcohol 28 with farnesol produced acetal 32, which was converted to its parent aldehyde by hydrolysis. The aldehyde was labeled by exchange and converted to epoxide 8 (38,700 dpm/μg) by treatment with diphenylsulfoniumisopropylide.

16 ⟶

29

30

31

28

32 ⟶ epoxide 8

While work on the synthesis of epoxide 8 was in progress, Corey,
Montellano, and Yamamoto [31] reported the synthesis and enzymic testing
of the 10',15-bisnorepoxide 9. Interestingly, their synthesis also in-
volved a symmetical reductive coupling of a 3'-norfarnesyl precursor,
followed by terminal epoxidation. Most interesting, however, was their
finding that although this substrate was tetracyclized, it failed to
undergo the normal rearrangement process; instead, a side chain double
bond was formed by the proton loss.

epoxide 9

Their result provided further encouragement and incentive for the testing

of the 10'-norepoxide with the cyclase. This was done by incubating the

10'-norepoxide, along with squalene oxide, at the usual concentration of

25 μg/ml of enzyme. The reactions were worked up using the methanol

precipitation method and the recovered material was resolved into three

radioactive bands on tlc: epoxide (0.54-0.69, 20%), the sterol band

(0.31-0.41, 20%), and a band called "J" (0.41-0.54, 20%), which was not

observed with other substrates. The bands were eluted and aliquots were

counted. The results, expressed as percent of total tritium recovered,

but not doubled to correct for <u>d,l</u>-epoxide, were:

	Oxide	Sterol	"J"
10'-nor Incubations:	77%	10.2%	12.7%
	75	11.1	13.8
10'-nor Boiled controls:	84	2.6	12.7
	85	3.5	11.2
Squalene oxide:	52	48	-
	53	47	-

Other incubations gave similar results, except as discussed below. A large-scale incubation of 0.95 mg of the 10'-norepoxide with 80 ml of the cyclase gave a 23% (corrected) yield of sterol product which could be separated from the J material by careful tlc.

The appearance of the J-band was unique to incubations of this epoxide. It was not formed by the cyclase since it was present in equal amounts in the boiled controls. In one set of controls wherein the enzyme was boiled vigorously for 30 min, the J material was nearly absent. It may have been formed by another enzyme which was relatively heat-stable. J was an alcohol since it easily formed a non-polar TMSE derivative (Rf 0.85-0.95, 20%). This TMSE gave a single sharp peak on glc having Rc 0.86. Since J was not a product formed by action of the cyclase, it was not studied further.

Material in the sterol band from the boiled controls was silylated and analyzed by glc. It was found to consist mainly of the same material as was found in J -- evidently the tlc separation had not been complete. Material from the sterol band of the incubations had a similar amount of contaminant J, but most of the radioactivity was associated with a peak

of retention time 4.05 relative to cholestane. This value was 1.42 times
that found for the TMSE of 4,4-dimethylcholest-8-enol (34), (Rc 2.85), and
suggested that the enzymic product might differ from sterol 34 only by
the presence of a 24-25 double bond. When the enzymic product was re-
duced with W-2 Raney Nickel (prepared by L. O. Crosby), the reduction
product TMSE had retention time Rc 2.85, and both TMSE's gave identical
high-resolution mass spectra which were similar to that of dihydrolanos-
terol-TMSE.

$$34, \Delta^8$$
$$35, \Delta^{8,24}$$

$$36$$

Raney Nickel was used for the above reduction because it was known[32]
that platinum catalysts cause the isomerization of sterol 34 to its 8(14)
double bond isomer (36). This was verified by exposing the TMSE of sterol
33 to platinum oxide and hydrogen in ethyl acetate and comparing the pro-
duct by glc with the authentic 8(14) isomer (Rc 2.70). The enzymic
product showed similar behavior when exposed to the platinum/hydrogen
system in that the resultant dihydrosterol TMSE had the same retention
time as the authentic 8(14) isomer TMSE.

The enzymic product from the 10'-norepoxide therefore was 4,4-dimethylcholest-8,24-dienol 35. Again, as in the case of the 15'-norepoxide, the substitution of a hydrogen for the C-10 methyl had no effect on the enzymic reaction other than a small diminution in the rate of conversion to sterol.

The rates at which substrates were converted to sterols by the cyclase were not measured directly. For a number of reasons, the percent yield of a sterol product was used to approximate its integrated rate of formation.
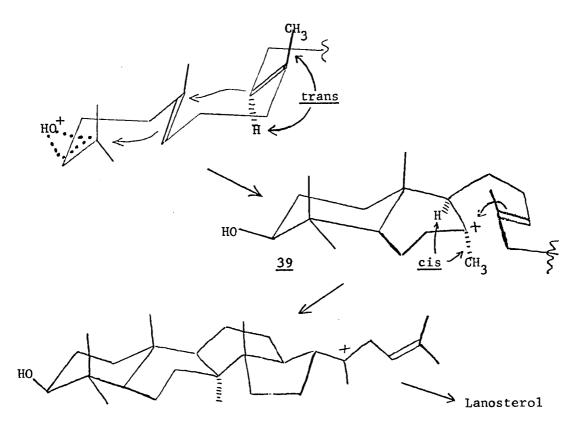
While the cyclase preparations which were used gave reproducible results, they contained other enzymic activities as well (c.f., glycol formation[33] and the J-band formation discussed above). Since the rat liver cyclase was never purified, its concentration was never known. Furthermore, even in the partially purified form in which it was used (page 73), the cyclase was not particularly stable at 37° in solution. The products, therefore, were formed to an extent which was a complex function of (i) the time-varying concentration of the substrate, (ii) the rate at which the incubation mixture warmed up in the incubator, and (iii), the gradual denaturation or inactivation of the enzyme, which was usually complete within about thirty minutes. Nevertheless, the effects of these unpredictable and rather uncontrollable factors were experimentally circumvented by testing substrates under standardized conditions and by running parallel tests with squalene oxide. The results of several controlled incubations were similar, if not identical within experimental error, and therefore the modified substrates could confidently be compared vis a vis squalene oxide as a "least common denominator."

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30

Such a comparison is found in Table II where, for each racemic substrate, the corrected yield of the sterol product is given as a range taken from at least three sets of duplicate controlled experiments using different batches of cyclase for each. The Corey-Montellano-Yamamoto result with the bisnorepoxide is included because this result (i.e., the observed failure to undergo rearrangement after tetracyclization) contrasts sharply with the others. Although the stereochemistry of the unrearranged bisnor-sterol was not determined, they assumed it to be in the protosterol or fusidane series,[31] as has also been assumed for the case of squalene oxide.[1]

	R	R'
37a, 38a,	CH$_3$	CH$_3$
37b, 38b,	CH$_3$	H
37c, 38c,	H	CH$_3$
37d, 38d,	H	H (*)

Table II: Percent-Conversions for Oxides 1, 7, 8, and 9.
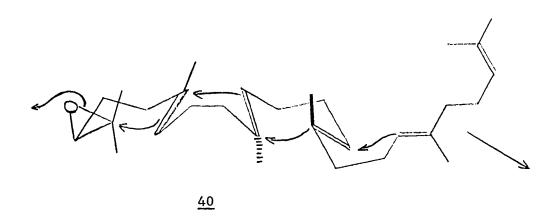
Oxide	%-Conversion	Decrease in Non-bonding Interactions		
		Proto (37) → Δ^8-Sterol (38),		Decrease
Squalene	70-80% (38a)	8	2	6
15'-nor	40-50% (38b)	5	1	4
10'-nor	18-24% (38c)	3	1	2
10',15'-bisnor	38% (33) (*)	0	0	0

(*) see ref. 31.

This assumption is a necessary consequence of the (so-called but unstated) Stork-Eschenmoser Hypothesis[34,35] which assumes that the stereospecific cyclization of polyolefins is concerted or synchronous starting (in this example) with protonation-and-opening of the oxide function and culminating in the formation of a polycyclic ion whose stereochemistry was predestined when the acylic polyolefin folded prior to the cyclization. The essence of this hypothesis is that the formation of a cyclic σ bonded framework from an acyclic polyolefin may be viewed as just another example of the well known phenomenon of trans-addition of X-Y to a C=C double bond. This explains the retention of cis- or trans- character as a C=C double bond becomes a ring junction, provided that (i) no carbonium ion center so formed inverts an odd number of times before the next double bond (or other nucleophile) traps it, and (ii) that elimination-reprotonation does not occur so as to generate a different ion.

39

cis

Lanosterol

Stork and Burgstahler originally assumed [35] that the former process was operative in lanosterol biosynthesis at the bicyclic stage as shown by 39. However, a slight revision of this scheme, proposed by Eschenmoser, et.al., [34] obviates this elimination-reprotonation. Their theory involves folding the acyclic precursor (originally considered to be squalene) in the chair-boat-chair-boat conformation in 40.

40

If this process were operative and if it were relatively synchronous, or "nonstop" as pictured, there would be little charge accumulation at any of the centers which were originally π bonds, except possibly for those carbon atoms which become C-4 and C-20 of the sterol, and one would not expect to effect dramatically the cyclization process per se by removing either or both of the methyl groups at C-10 and C-15 of squalene oxide. If anything, the decreased steric bulk might facilitate the juxtaposition of the π-bonds for a concerted reaction.
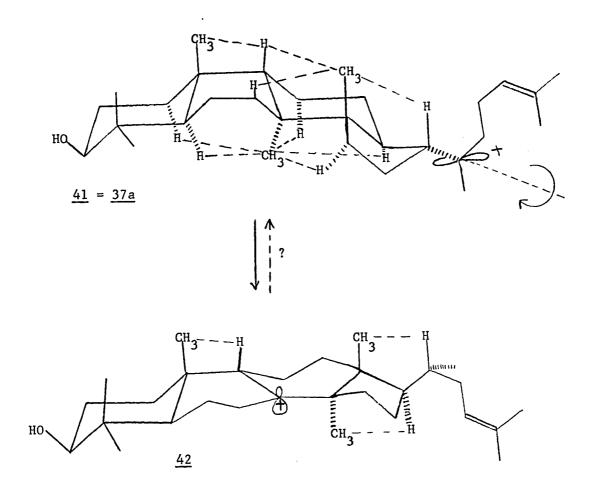
Alternatively, if the process were stepwise, methyl removal might have profound effects on the cyclization. In several cases, both enzymic [33,36] and non-enzymic, [36,37] a clear preference has been established

for the formation of tertiary, as opposed to secondary carbonium ions, and the necessity of a tertiary center at C-2 of squalene oxide has been clearly demonstrated enzymically.[33,38]

Of the two alternatives, I think that the idea of a concerted or synchronous B→C→D annelation better agrees with the observed facts (i.e., that all of the nor-substrates in Table II were tetracyclized). This same reasoning also implies that A-ring formation might not be as nearly concerted with epoxide protonation-and-opening as the B→C→D annelation steps, since removal of a methyl from C-2 of squalene oxide results in a very drastic reduction in substrate efficiency, and 1,1'-bisnorsqualene-2,3-oxide is evidently inert towards the cyclase.[39] This effect must be electronic (inductive) rather than steric in origin.

The major difference in the enzymic behavior of the substrates manifested itself in the rearrangement process after the cyclization. A tremendous number of chemical studies of triterpene rearrangements have been made, and many of these have been discussed in terms of "driving forces" for the elimination of intramolecular steric strain.[40] Assuming that enzymic cyclization of epoxides 1,7,8, and 9 leads to their corresponding cations of the protosterol or fusidane skeleton (37), one can correlate (Table II) the yields of the various products (and hence their overall rates of formation) with the decrease in the number of non-bonded axial interactions as the rearrangement proceeds from the protosterol form (37) to the lanosterol analog. Those steric interactions which do not appear or disappear as the rearrangement proceeds (e.g., the 1,3-diaxial methyl groups at C-4 and C-10) are not counted. Drawings made from Dreiding models compare the important steric

interactions in cation <u>41</u> with those in cation <u>42</u>,

$$\underline{41} = \underline{37a}$$

$$\underline{42}$$

the rearranged skeleton bearing a positive charge at C-8 penultimate to

elimination of the 9-β proton. Ion <u>42</u> rather than the neutral sterol <u>38</u>

was chosen for comparison to ion <u>41</u> because suggestions have been made [41]

that these potentially concerted rearrangements may involve equilibration

of carbonium ions prior to the trapping of one form by elimination or

attack by a nucleophile.

Considering the results in Table II, we see that an equilibrating

ion mechanism can not accommodate both the rearrangement and accompanying elimination of a 9-β proton in three cases, and the elimination of a C-22 proton in the other case. Such an argument would depend on the methyl substituents affecting the position of the equilibrium, so that in three cases a C-8 ion form was favored while in the other case, a C-20 ion would predominate, thus giving rise to three cases of C-9 elimination and one case of C-22 elimination. This argument, however, would be in violation of the Curtin-Hammett principle,[42] and would therefore be untenable.

A more acceptable explanation that accommodates all the results in Table II relates the yield of enzymic product (rate of product formation) to the rate at which the rearrangement takes place once tetracyclization is complete. Whatever the transition state for rearrangement is, it presumably has an activation energy requirement. Any perturbation of the enzyme-substrate system which raises this energy requirement will reduce the rate at which the rearrangement occurs, and a relative stabilization of the unrearranged ion will result in a similar delay of the rearrangement. (As used here, the term "rearrangement" refers to the transformation of ion 41 to 42, irrespective of whether this process is concerted, stepwise, spontaneous, or enzyme-mediated.) A delayed rearrangement would then be reflected in a decreased yield of enzyme product because the enzyme would cycle (turn over) more slowly. The crucial assumption is that the removal of the methyl substituents from the precursor epoxides, and hence from the unrearranged tetracyclic ion, provides relative stabilization of that ion in proportion to the number and severity of the non-bonding interactions relieved.

This hypothesis predicts that any situation which gives rise to stabilization of the C-20 ion should cause the rate of rearrangement to either decrease or vanish entirely. Another way to stabilize a carbonium ion is to link it to a conjugated system which can effectively delocalize the charge. Corey, Lin, and Yamamoto have studied an example of such a system using the unnatural substrate 22,23-dehydrosqualene-2, 3-oxide.[43] They found that this compound was enzymically tetracyclized to an air-sensitive diol, formulated as 43. Oxidative cleavage produced compound 44, the product which they finally characterized. (The stereochemistry was again assumed to be in the protosterol or fusidane series, but now there is an excellent opportunity to verify this assumption.)

Evidently the stabilization of the C-20 ion by the dienyl system was sufficient to overcome the "push" of the two methyl substituents since the rearrangement did not occur. While there may be another explanation for this result, the one given here is predicted by, and consistent with, the quasi transition-state theory I have presented.

As Corey, et al., have pointed out, the appropriate Grignard reagent

will convert ketone 44 into the tertiary alcohol related to cation 41.
Acid treatment of that alcohol, or solvolysis of an appropriate deriv-
ative, should produce cation 41 having both methyl groups but no com-
pensating stabilization provided by a conjugated side chain. According
to my hypothesis, the ion thus generated should rearrange and eliminate
giving lanosterol. However, since the initiation of rearrangement de-
pends on the conformation of the side chain with respect to rotation
about the 17-20 bond, it will not be surprising if some direct elimin-
ation from cation 41 occurs without rearrangement in the absence of the
enzyme. This would indicate that a necessary function of the enzyme
is one of protecting the reacting species from solvent and nucleophiles,
and possibly one of restricting the rotation of the side chain, although
some rotation about the 17-20 bond must occur as a separate step between
the cyclization and rearrangement steps. When cation 37 forms via
cyclization, the p-orbital of C-20 is initially parallel to the newly-
formed 13-17 bond. The rotation indicated in 41 is needed to allign
this orbital with the 17 β C-H bond for rearrangement to begin.

Another easily overlooked feature of the rearrangement is the
specificity of the elimination which terminates it. Only the 9 β proton
is lost, and apparently no Δ^7 isomer is formed directly, even though
it could arise by concerted loss of the axial 7 β proton from cation 42.
There are two mechanisms which may be operative in controlling the
specificity of the 9 β proton elimination. First, there may be a basic
functional group in the enzyme which assists removal of the 9 β proton
preferentially. A second rationale is that the B-boat geometry of the
proto skeleton together with the eclipsed 10 β methyl and 9 β proton,
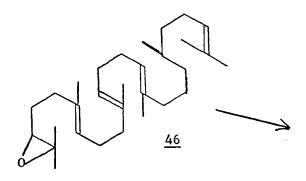
fortifies the rearrangement ensuring its completion and termination in the normal sense.   If the 7β proton were eliminated, ring C would remain locked in a boat form (45) with a severe interaction between the 9β hydrogen and the 13β methyl.

45

The arguments which I have used in deriving what may be called the "ion-stabilization hypothesis" have not considered any possible inter-molecular interactions between the substrate and the enzyme, but it is possible to speculate about these.   The protosterol form (37) has a generally curved shape which nearly completely surrounds the 8$\propto$ methyl group, so it is hard to imagine any further significant interaction with an enzymic group.   On the other hand the 14β methyl group in cation 37 is not surrounded intramolecularly, and interaction with an enzymic group on its less-hindered side is conceivable and would "give it a push" in the direction it normally migrates.   The 8$\propto$ methyl, of course, ex-periences a tremendous "push" in the direction of normal migration from the 5$\propto$ hydrogen situated at nearly bonding distance to the carbon atom of that methyl.   Another factor not included in the analysis is the

relative stabilities of the two carbon-frameworks (e.g., 37 and 38), irrespective of substituents, which depends on torsional and ring-fusion strain. Finally, it should be noted that in terms of gross molecular shape (i.e., viewing the substituted tetracycles 37 and 38 as smooth three-dimensional solids), there is only a slight overall · change. A minimum of atomic motion takes place during the rearrangement, and the entire process may follow a concerted reaction path because of the favorable mutual dispostions of the migrating groups.

While I worked on the 15'-norepoxide, Barry Sharpless was studying the chemical and enzymic cyclization of 18,19-dihydrosqualene-2,3-oxide.[46] He found that two enzymic products were formed by the cyclase. The major product had the tricyclic structure 47, and could be isomerized by treatment with acid to a rearranged Δ^8-compound (48). The minor enzymic product was thought to be the diol 49.[44]

When epoxide 46 was cyclized with stannic chloride in bezene, Sharpless found that a stereoisomer of 47 was formed, in addition to a larger amount of a stereoisomer of 48. Thus, here, as in other chemical cyclizations,[37,45] the greater stability of tertiary, as opposed to secondary carbonium ions determined the structure of the product.

46

46

47, XY = CH$_2$
49, X=CH$_3$, Y=OH

48
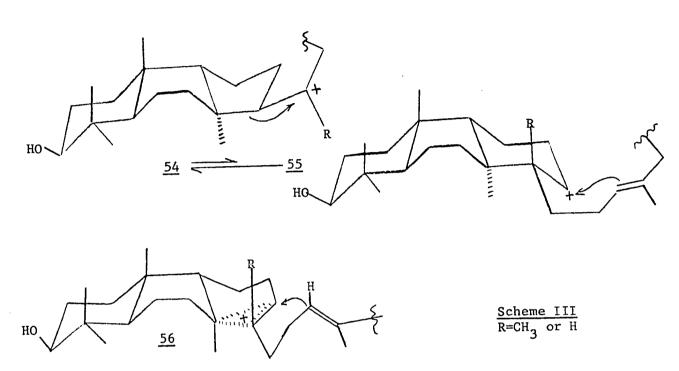
It is now necessary to broaden the perspective of this discussion to include two more recently proposed mechanisms for the enzymatic synthesis of lanosterol. The first of these (Scheme II), a radical departure from the Stork-Eschenmoser mechanism (Scheme I), was envisioned by Professor van Tamelen.[46] He reasoned that since the chemical cyclization of squalene oxide always led to tricyclic material with a 6-6-5 skeleton due to the preferred formation of a tertiary carbonium ion, the enzyme might show a similar preference for a cyclization pathway involving only tertiary carbonium ions. The overall Scheme II is depicted on page 41, including the novel rearrangement sequence (1,2,3 in **52**) necessitated by the spiro-structure.

Oxides **1** or **7**

Enzyme
or
Acid

50, R = H or CH₃

Enzyme only →

51, R = H or CH₃

52

R=CH₃
(1,2,3)

R=H
(1,2')

Lanosterol

————————————— Scheme II ———————— ————

53

Another mechanism, proposed by Barry Sharpless (Scheme III), differs from Scheme I only with respect to the cyclization of squalene oxide to the cation 37. The key to this scheme is the assumption that equilibrating carbonium ions 54 and 55, or their bridged equivalent, 56, are involved.

54 ⇌ 55

Scheme III
R=CH$_3$ or H
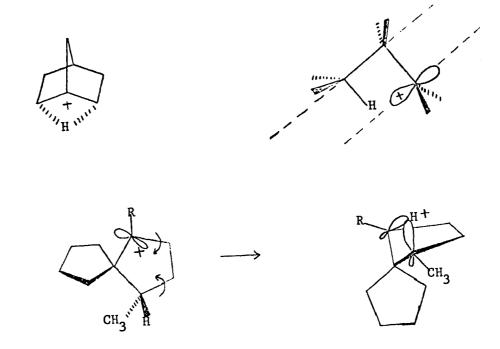
In light of either of these two new schemes however, we are still faced with the inescapable facts of Table II and the conclusion that aside from minor effects on the cyclization step, the major effect of the various nor- and bisnor- substrate modifications was reflected in the rearrangement step. Obviously then, whtever the mechanism for the cyclization is, it is one which is relatively insensitive to the secondary or tertiary nature of carbonium ions during intermediate stages. Although this result does not refute schemes II and III, it indicates that their main purpose, the deliberate avoidance of secondary carbonium ions, is not necessarily relevant to the normal enzymic process.

Scheme III explains well Sharpless' enzymic result with 18,19-dihy-drosqualene-2,3-oxide, but when applied to the normal substrate the idea of the 18,19-double bond anchimerically assisting the collapse of an unsymmetrical bridged ion such as 56 is more a variation on the idea of a concerted cyclization than an actual new mechanistic proposal since formation of a a 6-membered C ring depends on the assistance of the 18(19) double bond.

Several comments concerning Scheme II may also be made. Whether or not the enzymic reaction $\underline{1} \to \underline{50}$ is concerted, the reaction $\underline{1} \to \underline{51}$ cannot be, and thus would require in the 15'-nor case, the very early formation of a secondary carbonium ion. Once at the stage of the spiro-cation 52, the "ion-stabilization hypotheses" discussed for the Scheme I intermediate 37 can be applied to the initiation of the rearrangement (1,2,3 of 52) to lanosterol. However, in the case of the 15'-norepoxide, a new and possibly more reasonable rearrangement mode is conceivable. This is pictured in Scheme II as the occurrence of an alternate ring-expansion (1,2' of 52) instead of the 1,3-hydride transfer. Were this to occur, the enzymic product would be a Δ^8 sterol with the side chain attached to C-13 instead of C-17 as in 53. This possibility was definitely ruled out by mass spectral data, and probably by the nmr evidence as well.
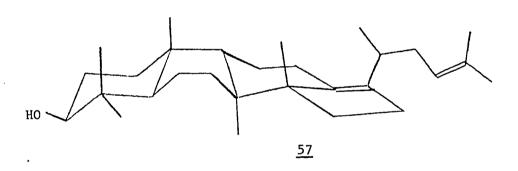
The 1,3-hydride transfer, while not entirely unobserved, is not a common molecular rearrangement process. In the special case of the nor-bornyl cation,[47] and in the n-propyl cation,[48] the 1,3-hydride transfer presumably occurs only because of the ability of these systems to assume a special geometry favorable to the transfer. However for a 1,3-shift to occur suprafacially on a five-membered ring (e.g., 2 in 52),

the ring would have to undergo considerable distortion to achieve a fav-
orable overlap situation.

In a terpene case, a 1,3-hydride shift has been ruled out by deuterium
labeling in the formic acid conversion of manool to 14 α -hibol.[49]

Finally, the Scheme II mechanism involves considerably more atomic motion in the formation of <u>52</u> and its subsequent rearrangement to lanosterol than does the concerted route of Scheme I. This may be an important distinction since an enzyme cavity which is large enough to accommodate structures which differ widely in overall shape and size would not be well suited to careful control of the stereochemistry of folding of the acyclic precursor. The existence of this enzymic specificity is deduced from two lines of evidence. First, only one product is formed by the enzyme, even when modified substrates are used. Second, information obtained in Dr. Clayton's laboratory suggests that protolanosterol, (<u>57</u>), is a much better inhibitor of the cyclase than is lanosterol.

<u>57</u>

The question ultimately arises, why are secondary carbonium ions apparently allowed during the "normal-type" enzymic tetracyclizations, and yet not allowed during abnormal enzymic tricyclizations, or during acid-catalyzed cyclizations of squalene oxide analogs? Since we also wanted to differentiate more clearly between Schemes I, II, and III, two additional experiments were planned, which we felt might help to

answer both questions. One experiment involved cyclizing the 15'-nor-epoxide under acid conditions. The other involved the synthesis and enzymic testing of 15'-nor-18,19-dihydrosqualene-2,3-oxide.[58] The absence of the 15'-methyl would decrease the energy difference between the 6-6-5 and the 6-6-6 ring system ions 54 and 56 since both cases would now involve secondary ions, and possibly the enzyme product would more accurately reflect the true nature of the tricyclic stage of lanosterol biosynthesis.

The 15'-norepoxide (7) was cyclized in the usual way with one equivalent of stannic chloride in benzene, and also in nitromethane, with ten equivalents of stannic chloride. The "sterol-like" products were isolated by tlc, silylated, and analyzed by glc on Warner-Chilcott DEGS columns (see Experimental for explanation). The benzene cyclization gave three products (Rc 2.95, 2.68, and 2.34), and the nitromethane reaction gave four (Rc 2.98, 2.58, 2.36, and 1.77). On this column the 15'-nor enzyme product A-TMSE had Rc 3.06, and although this is close to the 2.95-2.98 value, the difference was consistently observed. On Applied Science brand DEGS columns, the three components of the benzene reaction did not separate well so retention time correlations could not be made accurately. The Rc 2.68 isomer is probably the 15'-nor analog of the rearranged bicyclic compounds 65,66, and 67. One result appears certain however: no significant amount of the enzyme product 21 is formed in the acid cyclizations of this epoxide.

The synthesis of epoxide 58 was very similar to that of the 15'-norepoxide. Nordihydrofarnesol 59 was synthesized starting with citronellal (60), since one attempt to alkylate citronellyl magnesium bromide

with propargyl bromide failed to produce any C_{13} acetylenic products.
Citronellal was treated with propargyl Grignard, and the resultant
alcohol was converted to its tosylate (61) with pyridine and tosyl
chloride. This tosylate was reduced with lithium aluminum hydride in
refluxing tetrahydrofuran.

The resulting acetylene (62) was treated in succession with methyl
lithium and formaldehyde, producing alcohol 63, which was reduced
to nordihydrofarnesol 59 with lithium aluminum hydride in refluxing
tetrahydrofuran.

After the critical step of cross-coupling alcohols 11 and 59, the crude monoacetal was isolated and purified via the thiourea clathrate method. The purified material consisted of three components with glc retention times 3.52, 2.86, and 2.46 relative to squalene. These retention times relate to those for the three components of the 15'-nor mixture by a constant factor of 1.27, identical to the 1.25 factor* relating the derivatives of squalene and 18,19-dihydrosqualene observed by Sharpless. (It is interesting to note that this factor is much less than the 1.42-1.45 value observed for the reduction of the terminal double bond, both with squalene derivatives and tetracyclic compounds.)

The 3.52 isomer was isolated by silver nitrate chromatography, and spectral data were obtained which confirmed the all-trans structure 64, IR: 970 cm^{-1}, trans - HC=CH-; NMR: 1.68, trans vinyl methyl, and 1.60, three cis vinyl methyls. The acetal was hydrolyzed, and the resulting aldehyde was labeled and converted to epoxide 58 (42,900 dpm/μg) by the standard methods.

Several test incubations were run using the nordihydro epoxide 58. In the best case, a substrate concentration of 30 μg/ml was used. After saponification, the material was resolved by tlc into two radioactive bands: epoxide (0.51-0.68, 20%), and sterol (0.29-0.48, 20%). Lanosterol had Rf 0.29-0.35 in this system. In the case of the boiled-enzyme controls the radioscan of the tlc plate showed a low broad peak in the sterol region, but in the case of the incubations, the sterol peak was larger and was partly resolved into a main peak "C" (0.29-0.38, 20%) and a less-polar shoulder "D" (0.38-0.48, 20%). The corrected percent conversions were 4.26% for C, 0.84% for D, and 0.70% for the boiled

* (Within experimental error)

control sterol region. Squalene oxide, incubated at a concentration of 25 μ g/ml, gave a 62% yield of lanosterol.

The material from band C was silated and analyzed by glc. Two radioactive peaks were observed above a relatively high radioactive background, a major peak "F" (Rc 1.97) and a minor peak "G" (Rc 1.29) in about an 8:1 ratio. Material from tlc band D and from the boiled controls gave similar glc results. In both cases, most of the radio-activity had short retention times, and in both cases there was a radio-active peak at Rc 1.29 corresponding to the minor component G of band D. Unfortunately, even after several attempts it was not possible to decide whether this compound was or was not a product of cyclase action. Al-though G was always present in the controls, its formation appeared to be slightly enhanced in the presence of the active cyclase preparation.

Numerous large scale incubations were run with the nordihydro epoxide. The average corrected yield of band C material was about 2% after careful tlc purification. Since the conversion was so small, recovered epoxide was re-incubated several times to accumulate product F.
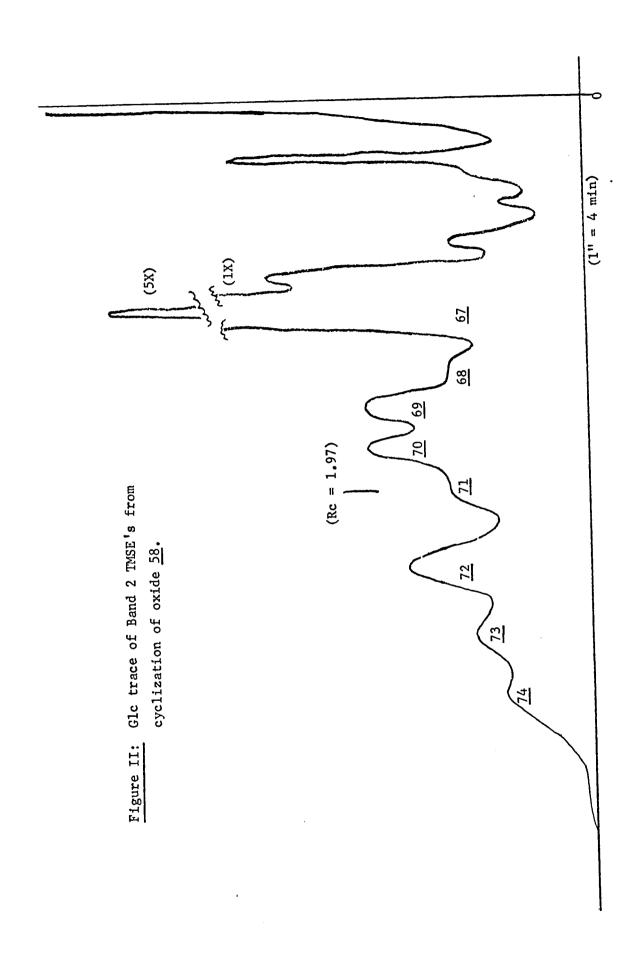
Nordihydro epoxide 58 was also cyclized chemically in four different experiments, and the results were all identical. For example, 6.05 mg of oxide 58 was dissolved in 1 ml of dry benzene and stirred magnetically (either at room temperature or cooled with an ice bath). Ten minutes after the addition of 1.2 equivalents of stannic chloride (0.176 ml of a 0.1 M benezene solution), 1 ml of sodium bicarbonate was added and the mixture was extracted.

After preparative tlc, two bands containing cyclized material were isolated; Band 2 (1.26 mg, Rf 0.43-0.48, 25%) and Band 3 (1.15 mg, Rf 0.48-0.54, 25%), with lanosterol having Rf 0.38-0.44 in this system.

Samples of bands 2 and 3 were silylated and analyzed by glc.

Band 2 showed (Figure II) one main peak (ca. 75%) at Rc 1.07, and several smaller peaks at longer retention times. Band 3 showed the same main peak as band 2, (>85%) and only two other peaks, which also appeared in the glc of band 2. Thus, this main product had formed in ca. 37% total · yield from starting epoxide. On the basis of the following considerations, and by analogy with the chemical cyclizations of squalene oxide and 18,-19-dihydrosqualene oxide, the bicyclic structure 67 was assigned to the main product in this case.(44)

65, R=

66, R=

67, R=

The first information linking 67 with the other similar compounds 65 and 66 was their relative retention times on glc: compounds 65, 66, and 67, had Rc values of 1.50, 1.24, and 1.07 respectively. The same series of values may be arrived at by using the relative retention time factors which relate the acyclic acetal or aldehyde precursors to the respective epoxides. Since the factor for the 18,19-double bond was 1.25, and that for the 15'-methyl was 1.09, we then had the series (1.07)(1.09)= 1.18, and (1.18)(1.25)=1.47. This showed that the only differences between 65, 66, and 67 resided in the acyclic part of the molecules.

Figure II: Glc trace of Band 2 TMSE's from cyclization of oxide 58.

(Rc = 1.97)

(5X)

(1X)

67

68

69

70

71

72

73

74

(1" = 4 min)

Structure <u>67</u> was also supported by (time averaged) nmr data for the band 3 alcohol and its acetate. The axial nature of the hydroxyl was inferred by the fact that acetylation produced no shift in the saturated methyl absorbances, as is observed in normal A:B-<u>trans</u> - 3 β -hydroxy compounds,[21] and by the fact that <u>67</u> was much more difficult to acetylate than lanosterol.

alcohol (9 scans)	acetate (25 scans)		
5.32 ppm	5.33 ppm	3H	vinyl
5.05	5.04	1H	vinyl
3.41	4.67	1H	O-C-H
1.69, 1.61	1.68, 1.60	6H	vinyl methyls
1.32, 1.28, 1.25	1.29, 1.26, 1.23	9H	unsplit methyls
1.15, d,J=5	1.15, d,J=5	3H	split methyl (ring)
0.88, d,J=5	0.88, d,J=5	3H	split methyl (chain)
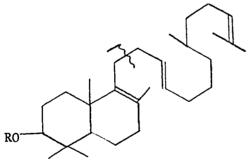
An infrared spectrum of the acetate of <u>67</u> (CCl$_4$ solution) showed a peak at 972 cm^{-1} for the <u>trans</u>-disubstituted double bond; other major peaks were 2925, 1730, 1450, 1370, and 1245 cm^{-1}.

In contrast to the mass spectra of <u>65</u> and <u>66</u> (as TMSE's), m/e 135 (C$_{10}$H$_{15}$) rather than m/e 129 (C$_6$H$_{13}$OSi) was the base peak in the mass spectrum of <u>67</u>-TMSE. However, when the TMSE's of <u>67</u> and <u>65</u> (sample furnished by Barry Sharpless) were hydrogenated over platinum in ethyl acetate (0.5 hr or 24 hr gave the same result), their mass spectra showed similar fragmentation patterns with m/e 129 as base peak in both cases since the Δ^5 double bond was not reduced. Both reduction products showed strong retro-Diels Alder peaks at m/e 134 and only weak peaks at 135.
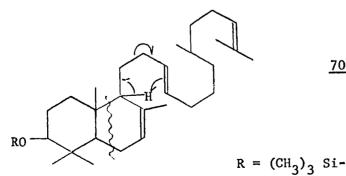
In an attempt to identify the minor components of this cyclization mixture, the material from tlc bands 2 and 3 was silylated and subjected to preparative glc. High resolution mass spectra were obtained for seven

components of the mixture. Although the glc trace (Figure II) did not

indicate perfect separation of some of these components, their respective

mass spectra (at least two scans per component) showed significant and

characteristic differences indicating minimal cross-contamination. While

it was not possible, on the basis of mass spectral evidence alone, to

identify the components conclusively, it was possible to make some

reasonable tentative structure assignments. A list of these structures,

numbered to correspond to the glc trace, follows along with key infor-

mation from the high-resolution mass spectra.

68, base peak is $M-C_3H_{10}OSi$, 189,190 ($C_{14}H_{21-22}$) very prominent.

69, base peak is 202 ($C_{15}H_{23}$) 189,190 reduced in intensity.

70, base peak is 292 ($C_{18}H_{32}OSi$), 274 ($C_{20}H_{34}$) RDA cleavage.

$R = (CH_3)_3 Si-$

71, same glc retention time as enzymic product F.

73, base peak 243 ($C_{18}H_{27}$), 189,190 reduced in intensity.

74, base peak 190 via RDA, 243 very prominent

R = $(CH_3)_3Si-$

Because of the quality and quantity of the mass spectral data collected, and the availability of model compounds, the assigned structures for **73** and **74** are on secure ground, except for stereochemistry. Another of the cyclization products, **71**, had the same retention time (Rc 1.95) as the enzyme product "F" from the nordihydro epoxide, and their high-resolution mass spectra were similar. Unfortunately, these spectra did not provide a great deal of useful structural information, and the identity of **71** with the enzyme product remains unproven.

A sample of F-TMSE was purified by glc and was used to obtain three high-resolution mass spectra. A tabulation of the key fragments (*) is given along with other major peaks which frame the picture.

Observed Mass	Relative Intensity	C-H-OSi	
486.4249	2842	32-58-1	M^+
396	7382	29-48	M-90 (base peak)
357	4048	26-45	M-129
335	574	21-39-1	(245 + 90)
333	836	21-37-1	(243 + 90)
279	1156	17-31-1	(189 + 90)
260	1062	19-32	*
257	524	19-29	* M-90-allylic side chain
245	5002	18-29	*⎫
244	1334	18-28	*⎬ M-90-$C_{11}H_{19-21}$
243	5722	18-27	*⎭
233	1516	17-29	* Ring C + side chain
229	1054	17-27	*
190	2862	14-22	*⎫ Rings A + B
189	4096	14-21	*⎭

Some of the general aspects of the structure of F are apparent from these data. For instance, the m/e 243-245 series indicates that F is very probably a tricyclic molecule with a 6-6-6 skeleton. Furthermore, the m/e 189 and 190 peaks which comprise rings A and B indicate that C-8 of F bears a methyl substituent and that therefore F is most likely a perhydrophenanthrene derivative. The spectrum also shows that it is very unlikely that F has a $\Delta^{12(13)}$ double bond, since these compounds are well known to give very strong m/e 190 peaks via RDA cleavage. The most logical unrearranged 6-6-6 skeleton then is 75. It is possible to rationalize most of the peaks in the mass spectrum of F-TMSE on the basis of this assumption. It was not possible to accommodate the mass spectral data with a 6-6-5 structure such as 76.

Further work on the structure of F was impeded by the difficulty of obtaining enough material. Several large incubations finally gave about 200 μ g of an impure sample of F. Attempts to obtain time-averaged nmr spectra were without success, so a combination of chemical, chromatographic, and mass-spectral techniques was finally resorted to.

The first of these involved attempted acid-catalyzed isomerization. F-TMSE was treated in benzene with excess stannic chloride (30 min), or in chloroform saturated with hydrogen chloride (24 hr). In each case the material was re-silylated and analyzed by glc on both DEGS and XE-60 columns. It was found that F-TMSE had the same retention time (Rc 2.50 at 190° in 1% XE-60) before and after treatment with acid, which indicated

that apparently no isomerization had occurred.

Next, F-TMSE was reduced with W-2 Raney Nickel, and with platinum, for reaction times between 30 min and 24 hr, and in all cases the same new product was formed. Its glc retention time was less than that of F by a factor of 1/1.30, indicating that more than the terminal isopropylidene group had been reduced. The mass spectrum of the reduction product showed that two double bonds in F had been reduced. Thus two double bonds in F are easily reduced, and only two double bonds are reduced by prolonged hydrogenation. The mass spectrum of the reduction product also showed strong m/e 189 and 190 peaks confirming the earlier suggestion that C-8 had a methyl substituent. The spectra were somewhat complicated by small peaks due to hydrocarbon fragments at virtually every composition, but in ten scans of the spectrum, there was consistently a greater abundance of the two ions indicating a 6-6-6 structure than those which would arise from a 6-6-5 skeleton.

$$(FH_4\text{-}TMSE)^{+\bullet}$$

? /-90   ? \-90

245   155   231   159

The problem could be resolved if the double bonds in F could be located. Oxidative cleavage combined with mass spectroscopy was chosen, as this approach had served well in the 15'-nor case. To this end, the acetate of F was treated for 24 hr with excess osmium tetroxide in benzene/pyridine, and then with sodium bisulfite solution. The Rf of the product (0.38-0.53, 60%) indicated that it was a mono-glycol acetate. After cleavage with sodium periodate in aqueous t-butyl alcohol the product had the Rf of a mono-carbonyl acetate (0.43-0.50, 35%). This result was initially attributed to osmylation and cleavage of only the isopropylidene group. Extended osmylation of F-OAc for seven days produced in 80% yield a material which was obviously a bis-glycol acetate (Rf 0.00-0.08, 60%). However when this material was cleaved with periodate, the product again had the tlc properties of a mono-carbonyl acetate.

The cleavage results, and to some extent the hydrogenation results, suggest that possibly F has two double bonds in the side chain as in 76. Another possibility is that since the cleavage of the bis-glycol was done in the presence of silica gel, sodium periodate, and periodic acid, some type of aldol condensation might have affected the initial cleavage product forming a less polar secondary product such as shown for 79-80a.

High-resolution mass spectra were obtained with the periodate-cleaved material from the bis-glycol acetate. The highest reasonable mass peak was m/e 428.3295 ($C_{28}H_{44}O_3$). Other higher peaks were observed, but these were seen only in the later scans and must have arisen via sample decomposition on the heated probe. The m/e 189 series was prominent in all the spectra indicating that the A and B rings remained intact during the osmylation and cleavage sequence. Obviously then, F was not

a rearranged compound and the double bond in question was in ring C. Of the other ions in the spectrum of the cleavage product, m/e 57 (C_3H_5O), m/e 75 (C_5H_9O), and m/e 145 ($C_9H_{15}O_2$) could have arisen from a cleaved side chain such as in 80, although other possibilities also exist. A search was made through the spectra for various ions corresponding to possible chemical structures for the cleavage product, but except for the m/e 57, 75, and 145 fragments, no fragments suggestive of the following possible structures were found.

At this point, it is certain that F is an unrearranged tricyclic compound and the structure of F is tentatively formulated as 75. It would be very interesting now, if the "minor enzymic product G" referred to earlier were a 6-6-5 compound. This appears unlikely, however, since the mass spectrum of G-TMSE also had major fragments at m/e 243-245. The base peak of the spectrum was m/e 116 ($C_5H_{12}OSi$, the composition of trimethylsilyl vinyl ether) and the usual m/e 129 and M-129 silyl ether fragmentations were very minor. These results are suggestive of a $\triangle^{5(10)}$ double bond in G. Furthermore, platinum hydrogenation of G-TMSE produced a tetrahydro product which no longer displayed the 116 fragmentation. These unusual characteristics, coupled with the fact that G was formed in the boiled controls, suggest that G was probably not a product of cyclase action.

Obviously, the nordihydro epoxide did not give us a clear-cut method for differentiating between the mechanisms of Schemes I, II, and III. That the tricyclic products from this epoxide which were identified were all apparently 6-6-6 structures might be taken to favor Scheme I, or possibly Scheme III, if the assumption is made that without the 15'-methyl the bridged ion 56 prefers to react from the cyclohexyl rather than the cyclopentyl form. This preference might be due to the difference in strain energy of 5- vs. 6-membered rings.

Throughout the preceding discussion, only the electronic (inductive) effects of altering the methyl substituent pattern have been considered; the steric effects have only been alluded to. An experimental probe into the latter area was made using the easily-accessible substrate 3-methyl-squalene-2,3-oxide 81. This substrate provided an interesting compliment

to three other detailed studies of C-2 modifications carried out at Stanford.

Initially, Ronn Nadeau prepared the _trans_ and _cis_ isomers of 1'-norsqualene-2,3-oxide, <u>82</u> and <u>83</u>. He found that the cyclase converted the _trans_ isomer in very low efficiency to 4α, 14α-dimethylcholest-8,24-dienol, and that the _cis_ isomer (<u>83</u>) and 1,1'-bisnorsqualene-2,3-oxide (<u>84</u>) were not substrates for the cyclase.[33,39] He went on to show that no product was formed with a 5-membered A ring. This was considered significant since in theory, epoxide opening at either C-2 or C-3 would produce a secondary carbonium ion, which in either case, might then cyclize. Similar behavior was observed by John McCormick who showed that the non-enzymic cyclization of epoxides <u>87</u> and <u>88</u> produced no 5-membered ring products.[55]

	R	R'	R₃
<u>81</u>, 3-methyl	CH_3	CH_3	CH_3
<u>82</u>, _trans_-1'-nor	H	CH_3	H
<u>83</u>, _cis_-1'-nor	CH_3	H	H
<u>84</u>, 1,1'-bisnor	H	H	H
<u>85</u>, _trans_-1'-homo	CH_3	C_2H_5	H
<u>86</u>, _cis_-1'-homo	C_2H_5	CH_3	H

The second study in this area of the substrate was carried out by John Stone, who showed that during the enzymic cyclization of squalene oxide , there was no rotation about the C-2/C-3 bond so that the cis methyl became the 4β -methyl, and the trans methyl became the 4α - methyl, of lanosterol.[51] Stone, and co-worker Christine Weintjes, also showed that the unnatural enantiomer of squalene oxide is not an inhibitor of the cyclase.

A third significant finding was made by L. O. "Os" Crosby, III. He showed that of the two homo-squalene oxides 85 and 86, only the trans isomer 85 was a substrate for the cyclase, and that the product was the expected lanosterol analog (4α ethyl).[52]

3-Methylsqualene-2,3-oxide (80,500 dpm/μg) was synthesized from squalene trisnoraldehyde, 89, via 1) addition of methyl lithium, 2) Jones oxidation of the alcohol, 3) exchange labeling of the ketone, and 4) the usual sulfonium ylid reaction.
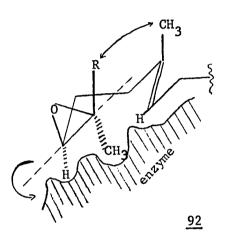
The 3-methyl epoxide was incubated, along with squalene oxide, at a concentration of 22 μg/ml, and worked up by saponification. The following results, expressed in terms of percent conversion, were obtained: Squalene oxide, 62% and 58%; 3-methyl oxide, 1.66%, 1.24%, and 1.58%; boiled-enzyme controls for the 3-methyl oxide, 1.18% and 1.48%. These results were essentially duplicated by another trial with another active batch of cyclase. In all cases, a broad sterol region (Rf 0.37-0.59, 25%), was eluted from the tlc plates for counting and in all cases total recoveries of tritium label were high.

The difference between the controls and incubations indicates an upper limit of about 0.2% utilization of the 3-methyl oxide under conditions which promote conversion of squalene oxide to lanosterol in 60% yield. Stated another way, the 3-methyl oxide is about 300 times less efficient a substrate than is squalene oxide.

Stone's results may be explained in two ways. Either A-ring formation is concerted with epoxide protonation-and-opening, or there are intramolecular (enzyme + substrate) steric interactions which prevent rotation about the C-2/C-3 bond as the epoxide is opening. As Barry Sharpless pointed out (group seminar, Spring 1968), the potential 1,3-diaxial interaction across the developing A ring pushes the cis methyl (R in 92) tending to cause a rotation as indicated in 92. With squalene oxide, but not with Nadeau's cis epoxide 83, there is a trans methyl that can interact with the enzyme to counter the rotation. Nadeau's cis epoxide gave a much larger amount of glycol product than did the trans isomer. However, there are other enzymes in the crude cyclase preparation which converts mono- or disubstituted oxirane compounds to glycols.[38,53]

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Whether Nadeau's result is due to the presence of one of these hydrases and its exertion of cis-trans selectivity, or the induced rotation bringing the C-2 p-orbital away from the next double bond and toward solvent, is not really known for our system. With the cis-homoepoxide 86, the 1,3-diaxial interaction (92, R=Et, R'=Me) may be too great to be counteracted by the normally-sufficient trans methyl vs. enzyme interaction.

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The results with the 3-methyl epoxide support the idea that the cyclase has highly sensitive steric requirements in the vicinity of the epoxide end of the substrate. In fact, of all the modified substrates tested, the most dramatic effects have been due to modifications at the oxirane centers.

In light of these ideas, one further interesting substrate to test would be the 2,3-cis-1',6'-bisnorsqualene-2,3-oxide 93. Since the 1,3-interaction developing across ring A would be relieved by the absence of

the 6'-methyl, there would be no need for a <u>trans</u>-1'-methyl to serve as a handle for the enzyme to use to prevent the rotation, and the substrate should cyclize a sterol product. Experiments in this direction are being carried out by J. A. Smaal, who has found that the 6'-norepoxide <u>94</u> does cyclize to give a sterol whose structure is as yet unknown. These results will also be interesting in terms of the rearrangement process which follows tetracyclization. The removal of the C-10 angular methyl from ion <u>41</u> (or <u>42</u>) might have an effect on the ultimate termination of the rearrangement.

<u>93</u>,   R = CH$_3$,   R' = H

<u>94</u>,   R = R' = CH$_3$

These studies have been a part of a larger effort designed to "trace the exact course of squalene oxide in its journey to lanosterol," and ultimately to present "a detailed, cinematographic-like view of how enzyme molecules act on squalene oxide."[2] Such a picture, based on experimental results as well as theoretical considerations, and subject

to the important qualification <u>in vitro</u>, is assembled for the first time in the conclusion of this Thesis. An outline form is used for convenience in future modification.

I. The Enzyme and Substrate in Solution: Mutual Recognition.

The lipid substrate is suspended in an aqueous buffer and is probably highly coiled or "internally solvated."[2] Therefore there is probably only a small entropy change as the substrate coils into the active site. In fact, in attempting to maximize hydrogen bonding, the aqueous phase may literally inject the coiled molecule into the enzyme, the latter serving to reorganize a random coil into a specifically coiled structure. The active site area is probably a cavity or pocket, rather than a surface, which can impose a particular folding pattern on the substrate and protect intermediates from solvents and nucleophiles. Except for modifications at C-2 and C-3 of the oxirane moiety, the enzyme is relatively non-specific in its recognition of a wide variety of structures as substrates or inhibitors.

II. Initiation and Propagation of Cyclization.

It has always been an assumption that a proton from an acidic source in the active site initiates epoxide opening. Since squalene oxide is stable toward acetic acid and reacts only slowly with monochloroacetic acid, the active site acid must be a protonated phosphate or sulfate group, <u>i.e.</u>, a <u>strong</u> <u>acid.</u> Furthermore, the active site must be extremely well-protected from the <u>slightly</u> <u>basic</u> buffer medium.

A tertiary center at C-2 of the epoxide is necessary for efficient enzymic cyclization. This is presumably related to the ease of attaining carbonium ion character at C-2 prior to ring A formation, rather than concerted with it. The cyclization process was rather less sensitive to the introduction of secondary centers by methyl removal at C-6, C-10, or C-15 of the substrate.

As the annelation proceeds there is a migration of positive charge away from the negatively charged conjugate base near ring A. Presumably the endothermic process of charge separation is offset by the energy release accompanying conversion of π bonds to σ bonds. Perhaps this "ionic bond" is responsible for holding the substrate in the active site until the process is complete.

Once the tetracyclization is complete, there may be a negative enzymic group (e.g., a phosphate) which stabilizes, either ionically or covalently, the positive charge at C-20 allowing rotation of the side chain to occur before the rearrangement begins.

III. The Rearrangement Process.

Once the tetracyclic stage is attained, those steric interactions which have been built up, both intra- and intermolecularly, provide a "driving force" for a cationic rearrangement leading to their relief. Reduction of this "driving force" by removing certain methyl substituents, or otherwise stabilizing the C-20 ion by linking it to a conjugated system, delays or entirely prevents the rearrangement. This is observed as a reduction in the amount of rearranged sterol product formed under standard conditions. There probably is no equilibration of rearranged

and unrearranged carbonium ions prior to elimination of a proton. The rearrangement proceeds in a unique way because of the favorable disposition of the migrating groups, and because of the tendency to maximize strain relief.

IV. Termination of Rearrangement and Proton Re-cycling.

The elimination of the 9 β proton, which locked ring B in a boat form is possibly concerted with the other rearrangement steps and there may be a basic group in the enzyme which accepts this proton. The conjugate base at the active site must be reprotonated, and any basic group which accepted a proton from C-9 must be deprotonated. The enzyme is now ready to accept and to act upon another molecule of substrate.

EXPERIMENTAL

During the planning and execution of the experiments described in this thesis, an effort was made to establish and to adhere to standardized laboratory procedures and chemical manipulations. This served two purposes: it formed a uniform basis for comparing varying experimental data by minimizing the number of (potential) variables, and it reduced the task of describing hundreds of incubations, tlc/glc analyses, hydrogenations, etc., to that of describing the standard form. The first part of the Experimental Section of this Thesis is therefore devoted to the latter purpose.

Spectral data are reported for compounds only in so far as it is pertinent to structural analysis of functional group characterization. This is especially true for high-resolution mass spectral data, a tremendous quantity of which was obtained during various parts of this study.

Infrared spectra were recorded on Perkin-Elmer Infracord or Model 421 instruments. Nuclear magnetic resonance spectra were determined on Varian HA-100, A-60, and T-60 instruments, and time-averaging was done with a C-1024 computer connected to the HA-100 instrument. Values are reported as parts per million (ppm) vs internal tetramethylsilane in deuteriochloroform or carbon tetrachloride.

Microanalyses were performed by Messrs. E. Meier and J. Consul in the Stanford Microanalytical Laboratory.

Column chromatographies were done on Davison silica gel deactivated with water (usually 12%), and all solvents were distilled. Hexane and 60-68° petroleum ether were used interchangeably as available. Silver nitrate-impregnated silica gel was made by adding a solution of silver nitrate (10 g) in distilled water to a slurry of silica gel (90 g) in distilled water, mixing, and then removing the excess water on a rotovap

and drying the mixture at 130° overnight. Olefin-free hexane with varying percentages (0-15%) of ethyl acetate was used for elutions.

Solvents for the biochemical work were very carefully purified as follows. Benzene or hexane was stirred overnight with concentrated sulfuric acid, washed with water, and distilled from calcium hydride. Ethyl acetate was stirred with and distilled from anhydrous potassium carbonate. Acetone was distilled from a trace of potassium permanganate after drying with potassium carbonate. Methylene chloride was extracted with aqueous sodium thiosulfate, dried with potassium carbonate, and distilled. Tetrahydrofuran (THF), ether, and glyme, were distilled from lithium aluminum hydride.

Tlc plates were prepared with Merck silica gel G that had been prewashed with methanol in a Soxhlet extractor. In some cases, a 0.05% solution of Rhodamine-6G was used to slurry the silica gel for spreading. These plated dried pink and most compounds appeared as darker pink spots when the plate was developed and dried in air. Plates were made 5 X 20 cm, usually 0.35 mm thick, and were channeled into three parallel sections. The outer two strips were used for standards and the center section for the compounds being investigated. Tlc data are reported as follows: in parentheses, two Rf values mark the limits of the band or spot, followed by a number indicating the volume-percent of ethyl acetate mixed with the hexane in the binary solvent system. For example, cholesterol (0.30-0.35, 25%).

Measurements of radioactivity were made with a Packard liquid scintillation spectrometer operating at an efficiency of about 40% for tritium. Samples were dissolved in 7-10 ml of toluene scintillant (containing 5.0 g of PPO and 0.3 g of POPOP per liter of toluene) in 20 ml glass vials

Tlc plates were scanned for radioactivity with a Packard recording rate-
meter using a scan rate between 0.5 and 2.0 cm/min depending on the activ-
ity of the plate.

Since most of the work with labeled compounds involved microgram
amounts of material, reaction conditions were chosen, whenever possible,
which involved completely volatile, highly purified reagents so that normal
workup could be replaced by a simple evaporation. Thus, silylation was
done by dissolving the sample in one drop of pyridine, adding one drop of
trimethylchlorosilane, heating at 60° for 15 min, and evaporating the re-
agents with a stream of nitrogen. The TMSE derivative was washed away
from the white residue with hexane and filtered through sodium sulfate.
Acetylation was accomplished by heating the sample in pyridine/acetic an-
hydride (1:1) and evaporating with a nitrogen stream. Hydrolysis of TMSE
derivatives was most easily done by dissolving the sample in 2 ml of
ethanol, adding one drop each of water and acetic acid, heating to 70° for
1 hr, and evaporating to dryness. Similar treatment with ethanol and aq-
ueous potassium hydroxide (but not with ammonium hydroxide or tiethyl-
amine/water) also cleaved TMSE's, but here normal extractions were em-
ployed for workup. Cleavage of acetates was accomplished by dissolving
the sample in ether, adding a tiny amount of lithium aluminum hydride,
shaking for 30 sec, and then extracting with water and hexane.

Microhydrogenations were performed using 2 ml of solvent in a 13 X
100 mm culture tube with a Teflon-lined screwcap. The catalyst was added
(ca. 2 mg of platinum dioxide in ethyl acetate or 10 mg of W-2 Raney
Nickel in ethanol) and hydrogen was bubbled through the mixture from a
glass capillary. If platinum was being used the tube was filled with

hydrogen and allowed to stand, but with Raney Nickel continuous saturation with hydrogen gas was maintained during the entire reaction. Workup consisted of flushing the tube with nitrogen, filtering to remove the catalyst, washing the catalyst, and evaporating the solvents. TMSE derivatives were stable to these procedures.

For acid-catalyzed isomerization, the sterol (or its acetate or TMSE derivative) was dissolved in 1 ml of chloroform and the solution was saturated with hydrogen chloride gas. After 24-48 hr, the mixture was evaporated with a nitrogen stream. Sterols and acetates were stable to this treatment but TMSE's were partly cleaved and had to be re-silylated prior to glc. Alternatively the sterol TMSE was dissolved in benzene, treated with excess stannic chloride for 30 min, and recovered by extracting with hexane and sodium bicarbonate solution. The material was re-silylated, although it is not known if the TMSE group was cleaved off.

Exchange labeling of aldehydes and ketones was done using acidic tritiated water.[20] One-half milliliter of water (0.5 Curie) was frozen and 120 mg of phosphorus pentachloride was added. After careful thawing this solution was approximately five-molar in hydrochloric acid and three-normal in phosphoric acid.

Squalene-2,3-oxide lanosterol cyclase was prepared as follows. Male (and sometimes female) rats were decapitated and their livers were removed and chilled in ice-cold pH 7.4, 0.08 $\underline{M}$ phosphate buffer. After this point all operations were carried out keeping the biological materials in the range of 0-4°, except for freezing or incubating. When about 50 g of liver was obtained it was chopped with a razor blade on a glass plate and then homogenized with buffer (2 ml/ g of liver) for 60-90 sec .

Two of these batches were pooled and centrifuged for 20 min at 30,000 Xg. The supernatant from two such runs was pooled and spun for 3-4 hr at 105,000 Xg to isolate the microsomes. In this way twelve microsome pellets, each weighing 1.8-2.0 g , were obtained from 200-230 g of liver. The pellets could be frozen in the tubes using a Dry-Ice acetone bath, and could be stored frozen for 4-6 weeks without much apparent loss of activity.

Frozen pellets were thawed in buffer (3 ml/pellet) in a homogenizer tube kept in a refrigerator. When thawed, or when fresh pellets were used, the microsomes were re-suspended by homogenizing briefly. Then for each pellet, 3 ml of cold 3% sodium deoxycholate solution was added and the mixture was homogenized intermittantly for 20-30 min. Next the deoxycholate was precipitated by adding a 0.085 $\underline{M}$ solution of calcium chloride (3 ml per pellet = 10% excess of calcium). The volume was then measured and solid potassium chloride was added to produce a 0.4 $\underline{M}$ concentration.

The resulting milky-pink solution was centrifuged at 105,000 Xg for at least 3.5 hr and sometimes for as long as 12 hr. The supernatant, clear and yellowish or pinkish, was gently extracted with freshly distilled cold ether. The extracted enzyme was frequently turbid. It was placed in a large Erlenmeyer flask packed in ice, and a stream of nitrogen was blown across the surface until the ether odor was gone. After clarification by centrifuging for 10 min at 30,000 Xg, the solution was ready for use in incubations. Activity remained high during storage at 0-3° for 24 hr but dropped off after longer storage times.

Since racemic epoxides were used, the convention of expressing percent-conversions in terms of a single optical isomer was arbitrarily adopted. Percent-conversions were calculated using the formula S/(S+E) X 200% , where S is the amount of sterol formed and E is the

amount of epoxide recovered after incubation. In some cases where multiple identical incubations were run on the same substrate, the percent-recovery of tritium label from the mixtures would vary over the range 70-100%. Even when this occurred, the percent-conversions determined after tlc analysis by using the S/(S+E) formula agreed well. This finding led to the interesting and important conclusion that there was no partitioning or selection in favor of either the epoxide or the sterol during the workups.

With each unnatural substrate, initial enzymic studies were done in multiples on an analytical scale, along with boiled-enzyme controls. Parallel incubations of squalene oxide were also run under the same conditions of time, temperature, and concentration along with these test incubations.

Test incubations were done in 15 ml round-bottom glass-stoppered test tubes. When more than 5 ml of cyclase was to be used, 25 ml Erlenmeyer flasks were employed. An appropriate amount of the substrate, usually 25 μg/ml of cyclase, in a few ml of hexane was mixed in the tube with a solution of an equal weight of Tween-80 in 1-2 ml of acetone. The solvents were evaporated under a nitrogen stream, the walls of the tube were rinsed down with a small volume of acetone, and the rinse solvent was evaporated leaving a film of organic material in the bottom. Next 40-60 μl of buffer was added and the tube was vibrated with a Vortex mixer to disperse the substrate into the aqueous phase. The tube was then cooled in an ice bath and the cold enzyme (native or boiled) was added. At time zero, the tubes were transferred to a rack in a Dubnoff shaker-incubator already equilibrated at 37°. Small test incubations were usually carried out for 30 min, whereas large-scale preparative incubations were done for one hour.

For stopping the incubations, two different methods were used on various occasions. In earlier work, an equal volume of a methanolic 15% solution of potassium hydroxide was added and incubation-saponification

was continued for several hours. Then after cooling, hexane was added and the tube was closed with the glass stopper and shaken vigorously. When the phases separated, the hexane was removed by pipet and more was added for the next extraction. Several hexane and several ether extracts were combined and dried over sodium sulfate. The material in these extracts was then used for tlc analysis.

Another workup method suggested by R. B. Clayton was adopted for later work and was found to be simpler and more efficient, especially for large-scale incubations. The incubations were stopped by the addition of 2-3 volumes of methanol. Some precipitate formed and was removed by suction filtration with Celite, or by centrifugation, depending on the scale. The solids were washed several times with ether/methanol and finally with ether. All the liquid phases were combined and concentrated, and the aqueous phase which remained was then extracted with ether in the normal way. This method had the advantages that emulsions were not encountered during the extractions and the recoveries were high.

Each incubation and boiled-control was worked up in one of these two ways. The next step in the analysis involved tlc separation of the components of the extract. After development, the plates were scanned for radioactivity. Active areas of the plate were scraped off and the silica gel was placed for elution in a short filter-column over a layer of sodium sulfate. Usually only two bands, epoxide and sterol, accounted for all of the radioactivity on the plate. Only in the case of the 10'-nor-epoxide was a third band observed. Corresponding areas of the boiled control plates were scraped as well.

The next step in the analytical process was the examination of an aliquot of the material from the sterol band by means of glc. In all

cases, a Perkin-Elmer Model 881 instrument was used. It was equipped with a pair of 6' X 2mm glass columns packed with 5% diethyleneglycol succunate (DEGS) on 100-120 mesh silanized Chromosorb-G (obtained from Applied Science Labs as 5% HI-EFF-1BP) and operated at 200° with a 90 ml/min flow of nitrogen carrier gas. A 4:1 (collect:detect) stream-splitter was used with flame detection. Collections were made with about 60% efficiency by inserting Pyrex capillaries into the exit port of the glc. Unless specifically stated otherwise, the term glc refers to this instrument operated with HI-EFF-1BP columns. Cholestane was used as a standard and other retention times were referred to its retention time defined as 1.00. Another source (Warner-Chilcott Labs) supplied 5% DEGS on 100-120 mesh AW Chromosorb-W, but the glc characteristics of this material were quite different from those of the HI-EFF-1BP material. In general, separations were much cleaner on the Warner-Chilcott material, but the HI-EFF-IBP gave retention times that could be correlated with earlier data of Clayton and Sharpless.

The existence of an enzymic product was inferred by comparing the tlc/glc data for the incubations with that of the boiled controls.

Preparative scale incubations were done in a similar way. For 100 ml of cyclase, the substrate and a slight excess of Tween-80 were evaporated from acetone in the bottom of a 500 ml Erlenmeyer flask. A small volume of buffer was used to get the material up into suspension by gently swirling the flask. Then the cold enzyme solution was added and the incubation was started. After one hour at 37° the enzyme solution usually was turbid so the reaction was stopped and worked up.

11,12,12'-Trisnorfarnesol-10-carboxaldehyde Ethylene Acetal (11).

11,12,12'-trisnorfarnesol-10-carboxaldehyde acetate was prepared according to Stormi, et.al.[4] Farnesyl acetate (65% trans, trans/35% cis, trans, 24 g, 0.11 mole), was dissolved in 1000 ml of t-butyl alcohol and cooled to 12-15° while saturating with water (1200 ml). N-bromosuccinimide (21.4 g, 0.12 mole) was added to the stirred solution and when it had dissolved (about 1.5 hr) the butanol was removed on a rotovap. The oily bromohydrin was recovered by ether extraction and was purified by chromatography on silica gel (12% water). The bromohydrin-acetate was treated in succession with potassium carbonate in methanol and then with pyridine/acetic anhydride (1:1) for re-acetylation. The yield of epoxy-acetate was 55% based on farnesyl acetate. The epoxy-acetate was next dissolved in 500 ml of THF and saturated with water (450 ml). Then 50 ml of THF was added followed by 10 ml of 3% perchloric acid. After standing overnight, 15 g of sodium periodate was added. After several more hours of stirring, the white precipitate was removed by filtration, excess bicarbonate was added, the THF was removed on a rotovap, and the aldehyde-acetate was recovered in 80% yield by extraction with ether. IR neat: 3450, 2920, 2750, 1740, 1660, 1240, and 1020 cm^{-1}.

For acetal formation the aldehyde-acetate was stirred in 100 ml of benzene and 20 ml of ethylene glycol in a 250 ml flask set up to reflux through a Dean-Stark trap. About 20 mg of oxalic acid hemihydrate was added and the mixture was stirred and refluxed for six hours. Since the starting aldehyde and the acetal had similar Rf's on tlc, the reaction was monitored by removing a tiny aliquot and spotting it on the tlc, then reducing it with borohydride and spotting this mixture alongside.

By this means it was determined that the reaction was complete in about four hours. Water and hexane were added for extraction; final extractions were made with ether. The extracts were combined, dried ($MgSO_4$), and evaporated to give a high yield of acetal-acetate. This material was dissolved in 100 ml of methanol and stirred with an excess of potassium carbonate. Extraction with ether after removal of most of the methanol on a rotovap recovered the acetal-alcohol in 40% yield based on farnesyl acetate. IR neat: 3450, 2900, 1655, 1450, 1410, and 1140 cm^{-1}. NMR: 5.45 (t,J=7,1H, vinyl), 5.18 (t,J=7,1H, vinyl), 4.88 (t,J=4,1H, dioxolane), 4.13 (d,J=8,2H, $-CH_2O-$), 3.90 (m, 4H, dioxolane), 2.32-2.00 (7H, hydroxyl and methylene), 1.90-1.75 (m,2H, CH_2), 1.68 and 1.61 (6H, vinyl methyl). Analysis: found, 70.22%C, 10.23%H; calc. for $C_{14}H_{23}O_3$, 70.00%C, 10.00%H.

Diphenylisopropylsulfonium fluorobate. Diphenyl sulfide was stirred in a 100 ml flask cooled with an ice bath, and 5.5 g of silver fluoroborate was added, followed by 4.76 g of isopropyl iodide. The flask was closed with a drying tube filled with Drierite. After a few minutes the flask was removed from the bath and the stirring was continued until all of the lumps had broken up. For workup, methylene chloride was added and the yellow silver iodide was removed by filtration. Evaporation of the filtrate produced a brown viscous oil which was extracted several times with ether. As the excess sulfide was removed, a white salt began to solidify. It was obtained in 47% yield by recrystallization from methylene chloride/ether, and it melted at 117-120°. NMR: 8.40-8.10 (4H, aromatic), 7.90-7.60 (6H, aromatic), 5.05 (septet, J=7, 1H, CH), 1.53 (d,J=7,6H, methyls). Analysis: found 57.00%C, 5.47%H; calc. for $C_{15}H_{17}BF_4S$, 56.80%C, 5.37%H.

Geranyl chloride. Commercial geraniol (200 g) was dissolved in 500 ml of 30-60° petroleum ether in a 2000 ml flask fitted with a sturdy mechanical stirrer. Powdered anhydrous calcium chloride (250 g) was added in small portions while stirring. After three hours more solvent was added and the mixture was filtered. The solid was transferred into a separatory funnel containing 2000 ml of ice water and 1000 ml of petroleum ether. After standing for three hours the complex dissolved. The organic phase was dried with sodium sulfate and evaporated to give 128 g of geraniol.

To a refluxing solution of triphenylphosphine (73 g, 0.28 mole) in 300 ml of carbon tetrachloride was added a solution of 35 g geraniol (0.228 mole) in 100 ml of CCl_4. This mixture was refluxed at 80° for 15 hr. When cooled to room temperature much precipitate formed and was removed by filtration. The filtrate was concentrated, cooled, and filtered (several cycles), to remove more phosphine oxide. Finally, distillation (45°, 0.3 torr) afforded a 57% yield of geranyl chloride. IR neat: 2940, 1675, 1460, 1388, 1255 cm^{-1}.

Geranyl propyne (17). Propargyl magnesium bromide in ether was formed by initiating in refluxing ether, the reaction of magnesium with a few drops of propargyl bromide and ca. 10 mg of mercuric chloride, and then cooling rapidly to 10-12°.[50] At this temperature the rest of the bromide was added slowly, carefully maintaining the temperature, until all of the magnesium had reacted. Following this procedure with 3.4 g (0.14 mole) of magnesium in 150 ml of ether and 16.7 g (0.14 mole) of propargyl bromide produced after 2 hr, a murky gray solution of Grignard reagent. This was cooled to 0° and a solution of geranyl chloride (22.3 g,

0.13 mole) in 50 ml of ether was added slowly. After 2 hr at this temperature magnesium salts had precipitated so a few ml of water was added and the mixture was filtered, dried with calcium chloride, and evaporated.

A small sample of the geranyl propyne was purified via the silver acetylide complex as follows. A 10% solution of the crude acetylene in ethanol was added slowly to a rapidly-stirred 5% solution of silver nitrate in 95% ethanol. The off-white crystals were collected after a few hours but were not dried. They were decomposed in hexane/ethanol (9:1) using excess dilute hydrochloric acid. Recovery was not good but purity was high. IR neat: 3330, 2920, 2130, 1670, 1450, 1370 cm^{-1}. NMR: 5.20 (2H, vinyl), 2.15-2.00 (8H, methylene), 1.78 (1H, acetylene), 1.68, 1.62, 1.60 (9H, vinyl methyls).

trans-trans-7,11-Dimethyldodeca-6,10-dien-2-yn-1-ol (18). The crude geranyl propyne (30.6 g, 0.17 mole) was dissolved in 300 ml of dry THF in a 1000 ml three-necked flask fitted with a rubber septum, an inlet for nitrogen, and nitrogen outlet connected to a bubbler. This solution was titrated by addition of methyllithium (1.5 M in ether). The evolution of methane was rapid and when complete, a 10% excess of methyllithium was added. Gaseous formaldehyde was generated by pyrolyzing 1.2 equivalents of paraformaldehyde at 180°. Here it was convenient to use a small two-necked flask with a nitrogen inlet and a short glass tube of a large diameter with a 24/40 ground-joint end to replace the nitrogen inlet on the flask with the acetylide solution. As the polymer decomposed, the formaldehyde flowed with the nitrogen over to the ice-cold acetylide solution and reacted. For workup a few ml of water was added and the volatile solvents were

removed on a rotovap. Ether and water were added for extraction. The alcohol was isolated by distilling off the hydrocarbons at very low pressures (e.g., 27, 0.005 torr). The purified alcohol had these spectral characteristics. IR neat: 3300, 3000, 2920, 2225, 1660, 1450, 1135, 1020 cm^{-1}. NMR: 5.18 (2H, vinyl), 4.18 (2H, $-CH_2O-$), 3.52 (1H, hydroxyl), 2.25-1.95 (8H, methylene), 1.68, 1.62, 1.60 (9H, vinyl methyls). Analysis: found 81.11%C, 10.80%H; calc. for $C_{14}H_{22}O$, 81.50%C, 10.70%H.

3'-Norfarnesol (16). To a stirred suspension of lithium aluminum hydride (5.7 g, 0.15 mole) in 450 ml of THF in a 1000 ml flask, a solution of alcohol 18 (21.7 g, 0.106 mole) in 100 ml of THF was added dropwise. After the addition was complete the mixture was refluxed for 6 hr. It was then cooled in an ice bath, water was added, the mixture was filtered, and the solution was dried ($MgSO_4$). The alcohol was isolated by distillation of the hydrocarbons (45-95°, 0.05 torr) and then the alcohol (98-99°, 0.05 torr), through a Vigreux column. IR neat: 3350, 2930, 1670, 1450, 970 cm^{-1}. NMR: 5.62 (2H, vinyl), 5.20 (2H, vinyl), 4.00 (d,J=5,2H, $-CH_2O-$), 2.20 (1H, hydroxyl), 2.15-1.95 (8H, methylene), 1.68 and 1.60 (3H and 6H, vinyl methyl). Analysis: found 80.47%C, 11.49%H; calc. for $C_{14}H_{22}O$, 80.71%C, 11.61%H.

1,1',2,15'-Tetranorsqualene-3-carboxaldehyde Ethylene Acetal (20). Titanium trichloride (7.85 g, 0.051 mole) was added to 175 ml of glyme at -78° and stirred under nitrogen in a three-necked flask equipped with a nitrogen inlet, a rubber septum, and a reflux condenser with a nitrogen outlet. Next, a solution of methyllithium (95 ml, 1.6M in ether, 0.153 mole) was added via syringe. The Dry Ice bath was removed and stirring was continued until the frost on the sides of the flask melted. Then

the temperature was raised rapidly using an oil bath, allowing the ether vapors to be swept out with nitrogen. When the reflux temperature reached 65-70°, the water flow through the condenser was started and refluxing was continued for 45 min. The workup consisted of cooling, adding a few ml of water, stirring 15 min, filtering with Celite, and removing the solvents with a rotovap. The monoacetal fraction was isolated by column chromatography over silica gel and purified further by means of thiourea clathrate formation. Ten milliliters of a 10% solution of the crude monoacetal in benzene was added dropwise to a stirred saturated solution of thiourea in methanol (100 ml). The mixture was slowly cooled to -15° and the crystals were collected by rapid suction filtration of the cold mixture. The clathrate was decomposed by shaking with water and hexane in a separatory funnel. After final purification of the isomer with retention time 4.65 relative to squalene (DEGS, 200°) by silver nitrate-silica gel chromatography, the all-_trans_ acetal had the following spectral characteristics. $IR(CCl_4)$: 2920, 1450, 970 cm^{-1}. NMR: 5.30 and 5.20 (2H and 4H, vinyl), 4.85 and 3.90 (t,J=5,1H, and m, 4H, dioxolane), 2.15-1.80 (20H, methylene), 1.68 and 1.60 (3H and 12H, vinyl methyls). Mass spectrum: $M^+ = 414$.

$4-(^3H)-1,1',2,15'$-Tetranorsqualene-3-carboxaldehyde. For hydrolysis, 10 mg of acetal 20 was dissolved in 10 ml of THF and 8 ml of 3% perchloric acid was added. After 48 hr at room temperature, the mixture was extracted with hexane. The aldehyde had the following spectral characteristics. NMR: 9.66 (1H, aldehyde), 5.38 and 5.10 (2H and 4H, vinyl), 2.50-2.25 and 2.15-1.90 (4H and 16H, methylene), 1.67 and 1.60 (3H and 12H, vinyl methyls). Mass spectrum: $M^+ = 370.3230$; calc. for

$C_{26}H_{42}O$, 370.3235.

For labeling, 10 mg of the aldehyde was dissolved in 1 ml of THF and 20 μl of acidic tritiated water (page 73) was added. The exchange was stopped after 48 hr by extracting with hexane and water. The hexane solution was dried with sodium sulfate and the aldehyde was used immediately for conversion to the epoxide.

4-(^{3}H)-15'-Norsqualene-2,3-oxide (7). A solution of diphenyl-sulfoniumisopropylide was generated by stirring diphenylisopropylsulfonium fluoroborate (31 mg, 0.1 mmole) in 8 ml of dry THF under nitrogen at -78° and adding phenyllithium (55 μl, 1.8$\underline{M}$ in benzene, 0.1 mmole). The ylid was bright yellow but its color faded as a solution of the labeled aldehyde (17.7 mg, 0.048 mmole) in 2 ml of THF was added via syringe. After 10 minutes water and hexane were added for extraction. The hexane was dried (Na_2SO_4), and the epoxide was purified by tlc (0.30-0.40, 7%). It had a specific activity of 62,150 dpm/μg. IR(CCl_4): 2930, 1455, 1380, 970 cm^{-1}. NMR: 5.40 and 5.15 (2H and 4H, vinyl), 2.70 (t,J=7,1H oxirane), 2.20-1.90 (20H, methylene), 1.68 and 1.60 (3H and 12H, vinyl methyls), 1.30, 1.25 (6H, oxirane methyls).

Cocrystallization of A-Ac with Lanosteryl Acetate. A 3 μg sample of enzyme product A was mixed with 68 mg of lanosterol and the mixture was acetylated. This acetate had a specific activity of 855 cpm/mg. It was dissolved in a small amount of warm acetone in a tared tube and allowed to cool. The excess solvent was removed from the crystals with a capillary pipet. The tube and contents were dried under a vacuum and weighed. Acetone was then added so that a known aliquot could be removed for counting. After three such crystallizations from

acetone, discarding the mother liquors each time, the specific activity dropped successively to 625, 343, and 252 cpm/mg, respectively.

Preparation of Ruthenium Tetroxide and Use in Cleavage Reactions.

Ruthenium dioxide on charcoal (0.66 g, 59% RuO_2) was stirred with 50 ml of CCl_4 and 50 ml of water containing 3.2 g of sodium periodate and cooled to 0° for 1 hr. The CCl_4 phase was isolated, filtered through glass wool, and stored under a layer of sodium periodate solution in the refrigerator.

Dihydrolanosteryl acetate (280 mg) was dissolved in 20 ml of CCl_4 and 20 ml of the above solution of RuO_4 was added. After two hours at room temperature, 1 ml of 2-propanol was added, and the mixture was filtered. The solution was concentrated to a yellow tar, which after preparative tlc (0.54-0.64, 25%) gave 122 mg of the pale yellow crystalline seco-diketo acetate XI mp 164-165.[23a]

A 78 μg sample of enzyme product A was acetylated, reduced over platinum in ethyl acetate, and treated for 8 hr with 1 ml of the RuO_4 solution. The recovered material was analyzed by tlc and a single radioactive band (0.55-0.65, 25%) was found. After eluting the band, 38 μg of the product X was isolated.

Formation of Steroid 7,9(11)-Dienes (23a) and (23b). As described by Fried et.al.,[25] dihydrolanosteryl acetate (700 mg) was treated in 35 ml of methylene chloride with 3 g of disodium phosphate and 1.1 g of meta-chloroperbenzoic acid for 20 hr. Extraction with hexane and 5% sodium carbonate recovered 1.05 g of a white solid which gave fine white needles from acetone (Rf 0.50-0.61, 15%). The epoxide (100 mg) was dissolved in 10 ml of benzene/acetic acid (1:1) and treated for

two hours with one drop of 3% perchloric acid before extracting with hexane and water. The product, 90 mg, (0.60-0.70, 15%), mp 167° from acetone, gave the expected uv spectrum and IR(CCl$_4$): 2950, 1738, 1468, 1370, 1345 cm^{-1}. The epoxide precursor gave a high-resolution mass spectrum identical to that obtained from the diene, indicating that dehydration had occurred during sample introduction or ionization.

A 78 μg sample of H$_2$A-Ac was dissolved in 1 ml of methylene chloride and stirred overnight with 10 mg of disodium phosphate and 5 mg of meta-chloroperbenzoic acid. The crude product was subjected to tlc analysis and 47 μg of the epoxy-acetate was isolated (0.54-0.63, 15%). This epoxy-acetate was then dissolved in 1 ml of benzene/acetic acid (1:1) and treated with 30 μl of 3% perchloric acid for 2 hr before adding hexane and water for extraction. The product consisted of a single peak on tlc (0.63, 0.72, 15%).

Tetrahydro A-Ac. Following a procedure described for tetrahydro-lanosteryl acetate,[28] 80 μg of enzyme product A was acetylated and then dissolved in 0.8 ml of acetic acid. About 2 mg of platinum dioxide was added, followed by 5 μl of 60% perchloric acid. The catalyst was activated by bubbling hydrogen gas through the mixture for 10 min and the reaction was sealed under an atmosphere of hydrogen for 4 days. After hexane extraction, the major product on tlc (50 μg, 0.37-0.60, 8%), was used to obtain a mass spectrum.

3'-Norfarnesyl Acetate Terminal Bromohydrin (29). The acetate from 7.0 g (0.0336 mole) of 3'-norfarnesol was dissolved in 500 ml of t-butyl alcohol and cooled to 10° while saturating with water (850 ml). N-bromosuccinimide (6.55 g, 0.0369 mole) was added and the mixture

was stirred while it warmed to room temperature. The butanol was re-
moved on a rotovap and the crude bromohydrin was then isolated by
extraction with ether giving 10.2 g of a yellowish oil. Chromatography
on silica gel (12% water) gave 5.30 g (46% yield) of the pure bromo-
hydrin acetate. IR neat: 3520, 3450, 1740, 1235, 966 cm^{-1}. NMR: 5.60
and 5.19 (2H and 1H, vinyl), 4.43 (d,J=5,2H, -CH$_2$O-), 3.80 (d,J=10,HCBr),
3.46 (1H, hydroxyl), 2.10 (m,10H, methylene), 2.00 (3H, acetate), 1.60
and 1.32 (3H and 6H, methyls). Analysis: found 55.41%C, 7.78%H, 22.98%Br;
calc. for C$_{16}$H$_{27}$BrO$_3$, 55.45%C, 7.79%H, 23.05%Br.

10,11-Epoxy-3'-Norfarnesyl Acetate (30). Bromohydrin 29 (5.3 g)
was dissolved in 75 ml of methanol and stirred under nitrogen with 3 g
of potassium carbonate for 1 hour. Extraction with hexane and water
recovered 3.1 g (90%) of the epoxy-alcohol, which was reacetylated
immediately with pyridine and acetic anhydride (10 ml each). A small
sample of the epoxy alcohol gave the following spectral data: IR neat:
3450, 2940, 1670, 1375, 970 cm^{-1}. NMR: 5.68 and 5.18 (2H and 1H, vinyl),
4.07 (d,J=5,2H, -CH$_2$O-), 2.71(t,J=6,1H, oxirane), 2.20-2.00(7H, hydroxyl
and methylene), 1.83-1.50 (2H. methylene), 1.62 (3H, vinyl methyl), 1.25
and 1.29 (6H, oxirane methyls). Analysis: found 74.76%C, 10.69%H;
calc. for C$_{14}$H$_{24}$O$_2$, 75.00%C, 10.71%H.

trans,trans-7-Methyldeca-2,6-dien-10-aldehyde-1-ol Acetate (31).
For hydrolysis, 3.5 g of epoxide 30 was dissolved in 300 ml of THF and
saturated with 375 ml of water. Then another 20 ml of THF was added,
followed by 10 ml of 3% perchloric acid. After stirring for 2 hr,
excess sodium bicarbonate was added and the THF was removed on a roto-
vap. Extraction with ether after saturation with salt gave 3.12 g of

pure glycol-acetate. IR neat: 3450, 2970, 1745, 1240, 968 cm^{-1}.

NMR: 5.62 and 5.18 (2H and 1H, vinyl), 4.42 (d,J=5,2H, -CH$_2$O-), 3.13 (m, hydroxyls and HCO), 2.20-1.95 (8H, methylene), 2.00 (3H, acetate), 1.60 (3H, vinyl methyl), 1.12 and 1.18 (6H, saturated methyls).

The glycol-acetate (3.12 g) was dissolved in 200 ml of THF and 100 ml of water, and 6 g of sodium periodate was added. After stirring for 3 hr, the mixture was filtered. The filtrate was concentrated on a rotovap and extracted with ether, recovering 2.49 g of aldehyde-acetate. IR(CCl$_4$): 2920, 2710, 1735, 1727 sh, 1230, 970 cm^{-1}. NMR: 10.00 (t,J=1.5,1H, aldehyde), 5.70 and 5.16 (2H and 1H, vinyl), 4.55 (d,J=6,2H, -CH$_2$O-), 2.55-2.30 and 2.25-2.00 (4H and 6H, methylene), 2.06 (3H, acetate), 1.62 (3H, vinyl methyl). Analysis: found 69.32%C, 9.01%H; calc. for C$_{13}$H$_{20}$O$_3$, 69.63%C, 8.92%H.

3',11,12,12'-Tetranorfarnesol-10-carboxaldehyde Ethylene Acetal (28).

Aldehyde 31 (2.49 g) was dissolved in 90 ml of benzene and 14 ml of ethylene glycol, and ca. 20 mg of oxalic acid hemihydrate was added. The mixture was stirred and refluxed vigorously through a Dean-Stark trap for 11 hours. Water and hexane were then added for extraction; final extractions were made with ether. The extracts were dried (MgSO$_4$), and evaporated. The acetal-acetate thus obtained was dissolved in 70 ml of methanol and saponified with 3 g of potassium carbonate for one hour. After concentration of the methanol using a rotovap, the acetal-alcohol was recovered by ether extraction. The yield was 2.2 g (29% overall from 3'-norfarnesol). NMR: 5.67 (2H, vinyl), 5.18 (1H, vinyl), 4.86 (t,J=5,1H, dioxolane), 4.08 (d,J=6,2H, -CH$_2$O-), 3.90 (m,4H, dioxolane), 2.18-1.95 (6H, methylene), 1.95-1.68 (3H, hydroxyl and CH$_2$),

Mass spectrum: M^+ = 414.3487; calc. for $C_{28}H_{46}O_2$, 414.3497.

4-(^{3}H)-1,1',2,10'-Tetranorsqualene-3-carboxaldehyde. Acetal 20

(4.0 mg) was dissolved in 15 ml of THF and 10 ml of 3% perchloric acid

was added. After 48 hr at room temperature, the aldehyde was recovered

by hexane extraction. IR(CCl$_4$): 2925, 2860, 2720, 1730, 1450, 970 cm^{-1}.

NMR: 9.70(1H, aldehyde), 5.40-5.00 (6H, vinyl), 2.20-1.90 (20H, methylene),

1.66 (3H, trans vinyl methyl), 1.58 (12H, cis vinyl methyls). Mass

spectrum: M^+ = 370.3252; calc. for $C_{26}H_{42}O$, 370.3235. The aldehyde was

labeled by dissolving it in 0.5 ml of THF and adding ca. 25 μl of acidic

tritiated water. After 48 hr the aldehyde was again recovered by ex-

traction with hexane and water, dried (Na$_2$SO$_4$), and used immediately for

conversion to the epoxide.

4-(^{3}H)-10'-Norsqualene-2,3-oxide (8). A solution of diphenylsul-

foniumisopropylide was prepared under nitrogen at -78° in a test tube

sealed with a rubber septum by adding 60 μl of a 1.75 molar solution

of phenyl lithium (ca. 0.1 mmole) to 65 mg of isopropyldiphenlysulfonium

fluoroborate in 2 ml of THF. The solution was intensely yellow, and as

the aldehyde was added via syringe, the color lightened. After 20 min-

utes at -78°, water was added and the mixture was extracted with hexane.

The extracted material was subjected to preparative tlc and the labeled

epoxide (1.78 mg) (0.35-0.45,6%) was found to have a specific activity

of 38,700 dpm/μg. NMR: 5.40 and 5.20 (2H and 4H, vinyls), 2.70 (t,

J=6,1H, oxirane), 2.15-1.95 (20H, methylene), 1.68 and 1.60 (3H and 12H

vinyl methyls), 1.28 and 1.24 (6H, oxirane methyls). Mass spectrum:

M^+ = 412.3694; calc. for $C_{29}H_{48}O$, 412.3704.

6,10-Dimethylundec-9-en-1-yn-4-ol (61). Propargyl magnesium bromide

was prepared (see page 80) from propargyl bromide (64 g, 0.54 mole) and

magnesium (13.1 g, 0.54 mole) in 300 ml of ether. After one hour at

5° all the magnesium had reacted, so addition of citronellal (55 g,

0.358 mole) in 125 ml of ether was begun, keeping the reaction flask

packed in an ice water slush. After one hour water was added to de-

compose the excess Grignard reagent. The mixture was then acidified

with dilute sulfuric acid and extracted several times with ether and

the extracts were dried ($MgSO_4$). Distillation (94°, 1.25 torr) gave

49 g of pure alcohol (70% yield). IR neat: 3400, 3300, 2900, 2130,

1450, 1370 cm^{-1}. NMR: 5.12 (t,J=7,1H, vinyl), 3.87 (quintet,J=5.5,

1H, OC-H), 2.50-1.15 (9H, methylene and methine), 1.68 and 1.61 (6H,

vinyl methyl), 0.90 (d,J=5,3H, secondary methyl). Analysis: found

80.05%C, 11.39%H; calc. for $C_{13}H_{22}O$, 80.45%C, 11.39%H.

Citronellyl propyne (62). Alcohol 61 (49 g) was tosylated with

75 g of p-toluenesulfonyl chloride in 100 ml of pyridine. The reaction

was left in a refrigerator for 26 hr, poured onto 600 g of ice and

400 ml of water, and extracted with hexane. The extracts were washed

with cold dilute sulfuric acid and dried ($MgSO_4$). Recovery was essenti-

ally quantitative and tlc showed the reaction to be complete. IR neat:

3300, 2900, 2120, 1600, 1500, 1355, 1190, 1175, 1100, 900 cm^{-1}. This

tosylate (87 g) was dissolved in 180 ml of THF and was added dropwise

to a stirred suspension of lithium aluminum hydride (24 g, 0.62 mole)

in a 1000 ml flask. After addition, the temperature was slowly increased

and refluxing was continued for 8 hr. The flask was then cooled with

an ice bath and 80 ml of ethyl acetate was added dropwise. This mixture,

which was semisolid, was poured onto a mixture of ice and hexane and
cold dilute sulfuric acid was added to dissolve the aluminum residue.
Hexane extraction and distillation (72-73°, 1.20 torr) afforded 36 g
of a colorless oil. IR neat: 3300, 2900, 2125, 1450, 1370 cm^{-1}. NMR:
5.10 (t,J=7,1H, vinyl), 2.30-1.80 and 1.50-1.20 (11H,CH$_2$ and CH), 1.67
and 1.60 (6H, vinyl methyl), 0.85 (d,J=5,3H, secondary methyl). Analysis:
found 87.40%C, 12.36%H; calc. for C$_{13}$H$_{22}$, 87.63%C, 12.37%H.

trans-6,7-Dihydro-3'-norfarnesol (59). This one-carbon homolo-
gation and reduction sequence was completely analogous to that described
previously for geranylpropyne. Thus, 32 g of acetylene 62 in 400 ml
of THF was converted to the acetylide anion by titration to 10% excess
with methyl lithium in ether. Then gaseous formaldehyde, from pyrolysis
of 8 g of paraformaldehyde at 185°, was swept over the cold, rapidly-
stirred acetylide solution. After concentrating the mixture on a roto-
vap, workup with water and ether afforded 44 g of a yellowish oil,
which after distillation (92-93°, 0.05 torr) gave 21.7 g of acetylenic
alcohol 63 (58% yield, uncorrected for recovered acetylene). NMR: 5.12
(t,J=7,1H, vinyl), 4.25 (t,J=2,2H, -CH$_2$O-), 2.80-1.90 (5H, methylene and
hydroxyl), 1.68 and 1.60 (6H, vinyl methyl), 1.50-1.20 (6H, methylene),
0.87 (d,J=5,3H, secondary methyl). Analysis: found 80.50%C, 11.65%H;
calc. for C$_{14}$H$_{24}$O, 80.75%C, 11.51%H. Reduction was accomplished by
slowly adding a solution of the acetylenic alcohol (21.7 g, 0.105 mole)
in 100 ml of THF to a stirred suspension of lithium aluminum hydride
(5.7 g, 0.15 mole) in 450 ml of THF. After addition, the mixture was
refluxed slowly for 4 hr, cooled with an ice bath, and quenched by the
addition of water. Filtration with Celite and removal of solvents on

a rotovap afforded 21.7 g of an oil, which after distillation (98-99°, 0.05 torr) gave 15.5 g of the allylic alcohol $\underline{59}$. IR neat: 3350, 2900, 1680, 1450, 1370, 1090, 1000, 972 cm^{-1}. NMR: 5.78 and 5.13 (2H and 1H, vinyl), 4.10 (d,J=4,2H, -CH$_2$O-), 2.25-1.80 and 1.50-1.10 (12H, hydroxyl and methylene), 1.68 and 1.60 (6H, vinyl methyl), 0.87 (d,J=5, 3H, secondary methyl).

1,1',2 ,15'-Tetranor-18,19-dihydrosqualene-3-carboxaldehyde Ethylene Acetal (<u>64</u>). The coupling and subsequent steps were carried out in the same way as described for the 15'-nor sequence. Thus 6.2 g (0.04 mole) of titanium trichloride was added to 200 ml of dry glyme under nitrogen at -78°. This was followed by 92 ml of a 1.65 $\underline{M}$ solution of methyl lithium (0.152 mole), and 15 minutes later by the addition of a mixture 3.0 g of the acetal alcohol $\underline{11}$ and 14.0 g of nordihydrofarnesol $\underline{59}$ (total alcohol, 0.079 mole). The stirred mixture was warmed to room temperature, heated with an oil bath distilling out the ether, and refluxed for 45 minutes at 70-80°. After cooling with ice, a few ml of water was added and the mixture was filtered with Celite, and dried (NaSO$_4$). This material (16 g) was chromatographed on silica gel (10% water) and the coupled monoacetal (<u>ca</u>. 2.0 g) was isolated. Glc showed this material to consist of 5 components, just as in the 15'-nor case. The thiourea clathrate was formed, isolated, and decomposed as before, giving <u>ca</u>. 500 mg of a mixture which now consisted of only 3 components whose retention times on glc (DEGS, 200°) relative to squalene = 1.00, were 3.52, 2.86, and 2.46. The Rc 3.52 isomer was isolated by chromatography on silica gel impregnated with 10% silver nitrate. After several such chromatographies, 30 mg of pure material

was obtained and characterized spectrally. IR(CCl$_4$): 2990, 1450, 1275,

1140, 970 cm^{-1}. NMR: 5.40 and 5.12 (2H and 3H, vinyl), 4.83 and 3.80

(t,J=5,1H, and m,4H, dioxolane), 2.20-1.80 and 1.50-1.10 (23H,CH$_2$ and CH),

1.68 and 1.60 (3H and 9H, vinyl methyl), 0.85 (d,J=5,3H, secondary methyl).

Mass spectrum: M$^+$ = 416.3666; calc. for C$_{28}$H$_{48}$O$_2$, 416.3654. Analysis:

found 80.56%C, 11.61%H; calc., 80.60%C, 11.52%H.

4-(^{3}H)-15'-Nor-18,19-dihydrosqualene-2,3-oxide (58). Hydrolysis,

labeling, and conversion to epoxide were all done in sequence. The

acetal, (ca. 25 mg) was dissolved in 30 ml of THF, 20 ml of 3% perchloric

acid was added, and the mixture was kept at 40° for 15 hours. For workup

sodium bicarbonate was added, the THF was removed on a rotovap, and the

mixture was extracted with hexane. This material was completely hy-

drolyzed as shown by glc (DEGS, 200°, retention time relative to squalene,

1.87), and by IR and NMR. IR(CCl$_4$): 2920, 2850, 2705, 1730, 1450, 1375,

970 cm^{-1}. NMR: 9.70 (1H, aldehyde), 5.40 and 5.15 (2H and 4H, vinyl),

2.40, 2.10-1.80, and 1.60-1.10 (29H,CH$_2$ and CH), 1.68 and 1.60 (3H and

9H, vinyl methyls), 0.85 (d,J=6,3H, secondary methyl). Analysis: found

84.07%C, 11.98%H; calc. for C$_{26}$H$_{44}$O, 83.90%C, 11.82%H. This aldehyde

was stored for 24 hr in 1 ml of THF containing 20μl of acidic tritiated

water, recovered by hexane extraction, and dried over sodium sulfate.

Diphenylsulfoniumisopropylide was prepared at -78° under nitrogen

by adding 0.07 mmole of phenyl lithium (37μl, 1.85M) to 63 mg (0.2 mmole)

of isopropyldiphenylsulfonium fluoroborate stirred in 8 ml of THF. To

this bright yellow solution the labeled aldehyde was added using 2 ml

of THF. After 15 minutes, water and hexane were added for extraction.

After drying over sodium sulfate, the epoxide was purified by tlc, first

in 7% ethyl acetate (Rf 0.50) and then with 4% (Rf 0.37). The isolated

epoxide (11.20 mg) had a specific activity of 42,900 dpm/ g, and was

characterized spectrally. IR(CCl$_4$): 2920, 1450, 1370, 1248, 1120,

970 cm^{-1}. NMR: 5.40 and 5.12 (2H and 3H, vinyl), 2.70 (t,J=6,1H

oxirane), 2.20-1.80 and 1.50-1.10 (23H, CH$_2$ and CH), 1.68 and 1.60

(3H and 9H, vinyl methyl), 1.29 and 1.25 (6H, oxirane methyls), 0.85

(d,J=5,3H, secondary methyl). Mass spectrum: M$^+$ = 414.3890; calc.

for C$_{29}$H$_{50}$O, 414.3861.

3-Hydroxy-(and 3-Keto-) 1,1'-Bisnor-2,3-dihydrosqualene. A solu-

tion of squalene trisnoraldehyde (200 mg, 0.52 mmole) in 5 ml of THF

was added dropwise to a solution of 0.7 mmole of methyl lithium in 5

ml of ether. Water and hexane were then added for extraction. The

recovered alcohol was purified by tlc (0.50-0.60, 40%) in 70% yield.

IR(CCl$_4$): 3620, 3500, 2920, 1670, 1450, 1370 cm^{-1}. NMR: 5.15 (5H,

vinyl), 3.77 (sextet,J=7,1H, OC=H), 2.20-1.85 (21H, hydroxyl and methylene),

1.67 and 1.59 (3H and 15H, vinyl methyl), 1.16 (d,J=7, secondary methyl).

Mass spectrum: M$^+$ = 400.3712; calc. for C$_{28}$H$_{48}$O, 400.3705. For oxida-

tion 50 mg of the alcohol was dissolved in 8 ml of acetone, cooled in

an ice bath, and titrated with Jones' reagent (200 g of chromium trioxide

and 192 ml conc. sulfuric acid diluted to 1000 ml with water). One

drop was added past the equivalence point. This solution was filtered

and extracted with hexane and water. After drying (MgSO$_4$) and purifi-

cation by tlc (0.50-0.60, 20%), the ketone was obtained in ca. 90%

yield. IR(CCl$_4$): 2915, 1720, 1450, 1370, 1355, 1155 cm^{-1}. NMR: 5.15

(5H, vinyl), 2.50-2.20 (4H, methylene), 2.13 (3H, Ch$_3$CO-), 2.10-1.90

(16H, methylene), 1.68 and 1.61 (3H and 15H, vinyl methyls). Analysis:

found 84.07%C, 11.47%H, calc. for $C_{28}H_{46}O$, 84.48%C, 11.54%H.

4-(^{3}H)-3-Methylsqualene-2,3-oxide (81). Ketone 91 (20 mg, 0.05 mmole), was dissolved in 2 ml of THF and ca. 50 μl of acidic tritiated water was added. After standing for six days, the labeled ketone was recovered by extraction with hexane and dried ($MgSO_4$). Next a solution of diphenyl-sulfoniumidopropylide was prepared at -78° under nitrogen by treating a solution of diphenylisopropylsulfonium fluoroborate (80 mg, 0.25 mmole) in 8 ml of THF with 0.1 ml of a 1.9 $\underline{M}$ solution of phenyl lithium. The labeled ketone, in 2 ml of THF, was added slowly via syringe. The yellow color faded slowly during the next hour, indicating that the ylid reacted more slowly with ketones that with aldehydes. Before workup with hexane and water, the solution was treated with 10 mg of potassium borohydride for ten minutes to reduce any unreacted ketone, thereby rendering it more easily separable from the epoxide. The recovered material was purified by tlc, and the epoxide (11.10 mg, Rf 0.39-0.45, 6%) had a specific activity of 80,500 dpm/μg and had the following spectral characteristics: IR (CCl_4): 2920, 1665, 1450, 1375, 1120 cm^{-1}. NMR: 5.15(5H, vinyl), 2.20-1.85(20 H, methylene), 1.68 and 1.60(3H and 15H, vinyl methyl), 1.30(9H, oxirane methyls). Analysis: found 84.14%C, 11.83%H, calc. for $C_{31}H_{52}O$, 84.50%C, 11.82%H.

Incubation of Pentanorsqualene-2,3-oxide. The pentanor epoxide (synthesis described by B. Sharpless[44b]) was incubated in triplicate, with boiled controls in duplicate, using 100 μg of oxide with 10 ml of cyclase for 30 min at 37°. After saponification and tlc separation the epoxide region (0.54-0.74, 25%) and the sterol region (0.36-0.48, 25%) were eluted and counted for radioactivity. The percent conversions for the incubations were 58.4%, 56.6% and 51.6%, and for the controls, 17.6% and 17.8%.

A sample of authentic pentanorlanosterol,95, had been prepared by Richard Anderson[54] and was used for characterizing the enzymic material. When a portion of the crude sterol product from tlc was silylated and examined by glc, approximately 45-50% of the radioactivity was associated with a peak having the same retention time as the authentic pentanorsterol (Rc 0.91, DEGS, 190°). Some of this silyl ether, collected from the glc, was hydrolyzed using ethanolic potassium hydroxide. This material was then injected on an XE-60 glc column at 180°. In this case, 91% of the radioactivity was in a fraction with the same retention time as the authentic pentanorsterol (Rc 1.91).

Another portion of the crude sterol product (154,000 dpm) was mixed with 39.2 mg of sterol 95 to give an expected specific activity of 3920 dpm/mg. After each of four successive recrystalizations from methanol, the specific activity was found to be 3370, 2950, 3025, and 2860 dpm/mg, respectively. This alcohol was then acetylated and further recrystalized from acetone giving values of 2340, 2100, 1950, 2020, and 1950 dpm/mg, respectively. The last three values average to 1973 dpm/mg which represents a total decrease of 44% in specific activity. This result is about that expected since the glc examination indicated that the material in the crude sterol fraction was only about 50% pure. A neater result would have been obtained if the sterol were purified by glc before the recrystalizations.

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SUMMARY

The objective of these studies was to determine the detailed mechanism of squalene-2,3-oxide lanosterol cyclase action. The approach consisted of synthesizing radiolabeled analogs of squalene oxide (1) and comparing their enzymic and acid cyclization behavior.
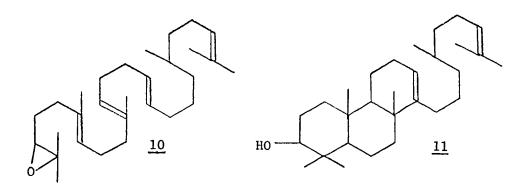
Oxides 2 and 3 were synthesized and found to undergo efficient enzymatic conversion to lanosterol analogs 7 and 8. Concurrently, E. J. Corey, et al., reported that although oxide 4 was enzymically tetracyclized, the product, (9), was an unrearranged sterol formed by direct elimination from C-22 in 5. In all cases only one enzymic product was formed.

	R	R'
1,6,	CH_3	CH_3
2,7,	CH_3	H
3,8,	H	CH_3
4,	H	H

Although the cyclase was only partially purified and rigorous kinetic studies were not possible, under standardized conditions the yields of enzymic products reflected the rates of the overall enzymic reaction. The fact that substrates 1 - 4 tetracyclized, but one of the four cases failed to rearrange, suggested that the substrate-recognition and cyclization steps per se, are not much affected by removal of methyl substituents. Implicit in this argument is the conclusion that the B→C→D annelation steps are relatively insensitive to the secondary or tertiary nature of the carbonium ion centers involved. This was interpreted as evidence that the B→C→D annelation is relatively "nonstop" or concerted, as originally supposed by the Eschenmoser-Stork Hypothesis.

It was further noted that the enzymic yields of rearranged Δ^8 sterol products (and hence their rates of formation) correlated with the amount of intramolecular steric strain relieved during the rearrangement. The specificity of the elimination which produces the Δ^8 sterol was attributed to the release of the ring B boat geometry of the protosterol form 5. Thus the rearrangement and elimination behavior observed with oxides 1 - 4 may be explained without invoking any enzymic participation other than protection of intermediates from solvent or nucleophiles. However, an obligatory function of the enzyme is one of controlling the stereochemistry of chain folding and cyclization.

Oxide 10 was also synthesized, and was cyclized both enzymatically and chemically. The enzymic product, obtained in very low yield, was tricyclic and unrearranged, and was tentatively formulated as 11. A small amount of 11, along with four bicyclic and two other 6-6-6 tricyclic alcohols, was formed by cyclization of 10 with stannic chloride in benzene. Similar cyclization

of oxide <u>2</u> produced no detectable amount of <u>7</u>. Finally 3-methylsqualene-2,3-oxide was prepared and was found to be inert toward the cyclase.

<u>10</u>

HO ⸻   <u>11</u>

A mechanistic picture of lanosterol biosynthesis, based on experimental results and theoretical considerations, was assembled for the first time. The overall enzymic process was discussed in four sections entitled

I - The Enzyme and Substrate in Solution: Mutual Recognition

II - Initiation and Propagation of Cyclization

III - The Rearrangement Process

IV - Termination of Rearrangement and Proton-Recycling.

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