Mechanism of Squalene Cyclization

THE BIOSYNTHESIS OF FUSIDIC ACID*

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SUMMARY

It is generally accepted that the C-20 cation or its stabilized equivalent is an intermediate in the biosynthesis of certain triterpenes and sterols. To evaluate this hypothesis the biosynthesis of the protosterol fusidic acid was investigated.

Fusidic acid was biosynthesized by incubating the fungus Fusidium coccineum on a medium containing (3RS,4R)-2,4-[2-14C,4-3H]-mevalonic acid (MVA). The obtained fusidic acid was shown to contain 6 14C atoms and 4 tritium atoms. It was proven that tritium atoms were located at the critically important 9α and 13α positions. Evidence was also adduced for the location of the remaining 2 tritium atoms at the 5α and 24 positions. The results show that the tritium atoms derived from 4-pro-R position of MVA are located at the theoretically predicted positions. Also this provides support for the view that fusidic acid is biosynthesized by stabilization of the C-20 cation (protosterol numbering) or its stabilized equivalent by elimination of the C-17 proton without backbone rearrangement.

The possible modes of stabilization of the C-20 cation and the formation of the C-17-C-20 double bond are discussed.

The reactions involved in the biosynthesis of sterols and triterpenes from mevalonic acid in plants and animals have been elucidated in considerable detail. In particular, it has been shown (1) that conversion of MVA1 to squalene proceeds with stereospecific retention of all six 4-pro-R protons and loss of the 4-pro-S protons of MVA. Conversion of squalene to triterpenes can proceed either by proton-initiated cyclization (2, 3) or, more frequently, by conversion to squalene-2,3-oxide (4, 5), which in turn undergoes cyclization.

According to present views the product of the cyclization is determined by the cyclase enzyme systems and by stereochemical interactions generated within the substrate (6). In the biosynthesis of cholesterol and the phytosterols squalene-2,3-oxide cyclization is thought to lead to the cation (Ia) (7, 8) or its stabilized equivalent (Ib) (9). Subsequent backbone rearrangement of this intermediate could account for the formation of lanosterol (II) cycloartenol (III) and other triterpenes. No direct evidence for this pathway has been obtained, although indirect evidence has been provided by examination of the elimination and migration of hydrogen atoms and methyl groups in the conversion of squalene to lanosterol and cho- lesterol mainly in rat liver (10-15). The results obtained are consistent with the initial formation of I followed by four concerted 1,2-hydrogen and methyl migrations and terminating in the loss of a proton from C-9.

In order to provide more direct evidence for the role of I as an intermediate in the cyclization of squalene-2,3-oxide we have examined the biosynthesis of fusidic acid2 (IVA), a member of the protosterol group of triterpenes produced by certain fungi (16, 17). The structure of fusidic acid was considered (16) to be formed by direct stabilization of the cation (Ia) or its equivalent (Ib) without backbone rearrangement. We have tested the hypothesis by investigation of the pattern of incorporation of (3R,4R)-[2-14C,4-3H]-MVA into fusidic acid (IVA) in Fusidium coccineum. This work has been the subject of a preliminary communication (18).

EXPERIMENTAL PROCEDURE

Materials—Samples of fusidic acid and F. coccineum were gifts from Dr. P. Diassi, Squibb Institute for Medical Research, New Brunswick, New Jersey, and Dr. W. O. Godtfredsen, Leo Pharmaceutical Products, Ballerup, Denmark.

Physical Measurements—Melting points were taken on a hot stage.


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1 The abbreviations used are MVA, mevalonic acid; TL.C. thin layer chromatography; NMR, nuclear magnetic resonance.

2 The rational nomenclature proposed for fusidic acid (15) is 2α,11α-dihydroxy 16β-acetoxyl fusidic acid [17β,20α(16,21)-cis]; 24-dione 21-oic acid. The numbering system is shown in structure IV.
stage apparatus and are corrected. Infrared spectra were recorded on a Perkin-Elmer 237 spectrophotometer as KBr discs. Proton magnetic resonance spectra were recorded on a Varian DA60 spectrometer at 60 MHz. Peaks are quoted in Hertz downfield from the tetramethylsilane internal standard. Mass spectra were measured on a Varian Associates M-66 instrument or on a Consolidated Electrodynamics Corporation 21-491 instrument.

Incubation of (3R,4R)-[2-14C,4-3H]-Mevalonic Acid with F. coccineum—A preliminary inoculum of F. coccineum was obtained by incubation of the organism in four 500-ml Erlenmeyer flasks each containing 100 ml of a medium composed of glucose (5 g per liter), yeast extract (2 g per liter), and micronutrients* (1 ml) at pH 6.5. The flasks were shaken at 26°C for 5 days.

A second medium (19) was prepared consisting of sucrose (60 g per liter), corn steep liquor (20 g per liter), KH2PO4 (10 g per liter), MgSO4·7 H2O (0.5 g per liter) at pH 6.5. Four 1-liter flasks, each containing 250 ml of this medium, were sterilized and the medium was supplemented with (3R,4R)-[2-14C,4-3H]-mevalonic acid dibenzylethylenediamine salt (60 μCi of 14C distributed equally among the flasks). The preliminary inocula were then added and the cultures shaken for 7 days at 26°C.

The mycelium was filtered off and the liquors were acidified to pH 5.5 and continuously extracted with ethyl acetate for 12 hours. The extract was washed, dried, and, after the addition of fusidic acid, concentrated to a residue. The residue was fractionated by thin layer chromatography in systems ethyl acetate-benzene (1:10) and the area corresponding to fusidic acid was removed and extracted. One aliquot of this extract was further diluted with nonradioactive fusidic acid (IVA) (400 mg) and crystallized twice from benzene to give the solvate (276 mg), m.p. 189-191°C. A further 60 mg of the solvate were obtained from the mother liquors.

3H14C4-Methyl Fusidate (IVA)—Fusidic acid-benzene solvate (IVA) (20 mg) was methylated with diazomethane in ethylacetate. The product was purified twice by thin layer chromatography in systems ethyl acetate-benzene (1:20) and ethyl acetate-benzene (1:1). The major band was extracted with ethyl acetate and crystallized from ether-hexane to constant 3H:14C ratio and 14C-specific activity giving IVA (15 mg) (m.p. 151-153°C) (specific activity 3.95 × 104 dpm of 14C per mmole; 3H:14C ratio 18.0).

3H14C4-Ethoxy Methyl Fusidate (IVb)—Fusidic acid-benzene solvate (IVA) (40 mg) was diluted with nonradioactive (IVA) (60 mg) and esterified with diazomethane in ether-methanol. The product was poured into water and the product was extracted with ethyl acetate. The organic phase was washed with water and dried. After removal of solvents and drying under reduced pressure the product (IVb) (55 mg) was purified by repeated preparative TLC (ethyl acetate-benzene (1:10)). This prepara-
tion could not be induced to crystallize (specific activity 1.55 × 104 dpm of 14C per mmole; 3H:14C ratio 17.0; vE: 1740, 1735, 1720, 1700 (C=O), 1250 cm-1. The crude product was acetylated by treatment with acetic anhydride (0.3 ml) and pyridine (0.3 ml) at 25°C for 18 hours. The solution was poured into water and the product was extracted with ethyl acetate. The organic phase was washed with water and dried. After removal of solvents and drying under reduced pressure the product (IVd) (55 mg) was purified by repeated preparative TLC (ethyl acetate-benzene (1:10)). This prepara-
tion could not be induced to crystallize (specific activity 1.55 × 104 dpm of 14C per mmole; 3H:14C ratio 17.0; vE: 1740, 1735, 1720, 1700 (C=O), 1250 cm-1). The product was treated with Jones reagent (3 drops) in acetone (5 ml) at 0°C for 15 min. The reaction was terminated by addition of methanol and water. The product was extracted with dichloromethane, washed, and dried. After removal of solvent the product was purified by repeated TLC (ethyl acetate-benzene, 1:10) to yield 15 mg (Va) (specific activity 3.88 × 104 dpm of 14C per mmole; 3H:14C ratio 18.1; vE: 1740, 1735, 1720, 1700 (C=O), 1240 cm-1); mass spectrum, m/e 526 (M-44) (specific activity 3.95 × 104 dpm of 14C per mmole; 3H:14C ratio 18.0).

3H14C4-Ethoxy-11-keto Methyl Fusidate (Va)—Fusidial acid benzene solvate (IVA) (25 mg) was converted to 3α-ace-
toxy methyl fusidate (IVd) described above and the product was treated with Jones reagent (3 drops) in acetone (5 ml) at 0°C for 15 min. The reaction was terminated by addition of methanol and water. The product was extracted with dichloromethane, washed, and dried. After removal of solvent the product was purified by repeated TLC (ethyl acetate-benzene, 1:10) to yield 15 mg (Va) (specific activity 3.88 × 104 dpm of 14C per mmole; 3H:14C ratio 18.1; vE: 1740, 1735, 1720, 1700 (C=O), 1240 cm-1).
(5 ml) and treated with Jones reagent (5 drops) at 0° for 10 min. 
Conventional work-up followed by TLC (ethyl acetate-benzene, 
(1:19)) gave a single product which was crystallized from ether-
hexane to give 13 mg of Vb; m.p. 129° (specific activity 4.17 × 10^4 
dpm of ^14C per mmole; ^1H:^14C ratio 18.1); \( r_{\text{max}}^{1746} \) (O CO-
CH₃), 1725 (CO·O·H₂), 1715 (C=O) cm⁻¹; NMR 62.5 
(doublt, 3H (30-CH₃, 6.0 Hz), 63.0, 69.0, 72.0 (singlets, each 3H (19-, 32-, and 18-CH₃)), 95.5, 100 (6H (26- and 27-CH₃)), 120 
(singlet, 3H (–O·CO·CH₃)), 218 (singlet, 3H (–CO·O·CH₃)), 302 
(multiplet, 1H (C-16)); mass spectrum, \( m/e \) 466 
(M-60), 431 (M-92), 420, 407, 397, 365, 246, 243, 219 (base 
peak), 177.

\[ ^{3}H_{4}^{14}C_{4}-3\alpha-\text{Acetoxy-\Delta^{11}} \text{Methyl Fusidate (VI)} \] —To a stirred 
and cooled solution (-30°) of 3a-acetoxy methyl fusidate (IVd) 
(50 mg) in pyridine (3 ml), a solution of thionyl chloride (0.2 ml) 
in pyridine (5 ml) was added dropwise over a period of 15 min. 
The mixture was stored for 1 hour at 0°, and the reaction was 
terminated by pouring onto ice. The product was extracted 
with dichloromethane and fractionated by TLC (ethyl acetate-
benzene, (1:9)) to give 25 mg of VI. The homogeneity of VI 
was assayed by repeated TLC (specific activity 1.76 × 10^4 
dpm of ^14C per mmole, ^1H:^14C ratio 14.9); \( r_{\text{max}}^{1745}, 1735, 1720 
(C=O), 1240, 1010, 900, 810, 790 \) cm⁻¹; NMR 59.5, 59.5, 71.0 
(singlets, each 3H (18, 19, - and 32-CH₃)), 95.5, 100.0 
(singlets, 6H (26 and 27 CH₃)), 110, 122 (singlets, 31H 
each (3a and 18β O·CO·CH₃)), 220 (singlet, 3H (CO·O·CH₃)), 
294 (multiplet, 1H (26H)), 305 (multiplet, 1H (C-15 proton)), 
328 (triplet, 1H (11-olefinic proton, 3.5 Hz)), 356.5 (doublet, 1H 
(24H)); mass spectrum, \( m/e \) 494 (M-60), 462, 425, 419, 365 
(base peak).

\[ ^{3}H_{4}^{14}C_{4}-\text{Dihydrofusidic Acid (VIIa)} \] —Fusidic acid-benzene 
solvate (IVA) (215 mg) was diluted with cold material (105 mg) 
and dissolved in ethanol (10 ml) and 5% palladium-calcium cera-
bonate (60 mg) was added (20). The mixture was stirred vigorously 
and hydrogenated until 1.1 moles of hydrogen had been 
asorbed. The catalyst was filtered off and the solution was 
diluted with water. The solid was filtered off and dried. The 
essentially quantitative conversion to VIIa was demonstrated by 
mass spectrometry (\( m/e \) 518).

\[ ^{3}H_{4}^{14}C_{4}-3\alpha-\text{Acetoxy-Methyl Dihydrofusidate (VIIb)} \] —Dihydrofusidic acid 
(VIIa) (25 mg) was esterified with diazomethane in ether-metha-
nol. The product was purified by TLC (ethyl acetate-benzene 
(1:9)) and crystallized from ether-hexane to give 17 mg of VIIb; 
m.p. 150° (literature (20), 150-150.5°) (specific activity 2.72 × 10^4 
dpm of ^14C per mmole; ^1H:^14C ratio 16.8).

\[ ^{3}H_{4}^{14}C_{4}-\text{Methyl Dihydrofusidic Acid (VIIc)} \] —To a solution of VIIa (60 mg) in dimethylformamide (0.2 ml), lithium 
chloride (15 mg) was added, and the mixture was heated at 140-
150° for 3 hours (21). The reaction mixture was cooled and 
diluted with water, and the product was recovered with ethyl 
acetate. The ethyl acetate solution was washed with water and
dried, and the solvent was removed under reduced pressure. The residue was dissolved in methanol and esterified by addition of diazomethane in ether. Separation of the products was achieved by TLC (ethyl acetate benzene (1:19), developed four times). The major product was identified as the \( \Delta^{14,17}(20) \)-diene (VIII) (25 mg) which was purified by repeated chromatography (specific activity \( 2.55 \times 10^{5} \text{ dpm of } ^{14}\text{C} \text{ per mmole}; \{ ^{3}\text{H}:^{14}\text{C} \text{ ratio 13.7} \}; \{ ^{3}\text{H} \} \text{max } 3450 \text{ (3x and 11a-0H), 1725} \text{ (CO O-Me) } \text{ cm}^{-1} ; \lambda_{\text{max } 252} \text{ nm} \)).

A second product from this reaction was identified as the isomeric \( \Delta^{14,17}(20) \)-diene (IX) (10 mg) and was purified as described above (specific activity \( 2.52 \times 10^{5} \text{ dpm of } ^{14}\text{C} \text{ per mmole}; \{ ^{3}\text{H}:^{14}\text{C} \text{ ratio 17.1} \}; \lambda_{\text{max } 271} \text{ nm} \)).

Ozonolysis of 4H-4C<sub>9</sub>-Methyl Dihydrofusidate (VIIb)—Dihydrofusidic acid (170 mg) was esterified with diazomethane and the resulting product was dissolved in dichloromethane (25 ml) containing pyridine (0.025 ml). The mixture was cooled to \(-75^\circ\) and a stream of ozonized oxygen was bubbled through the solution for 15 min. The excess ozone was removed in a stream of nitrogen, zinc (200 mg) and acetic acid (2.5 ml) were added, and the mixture was stirred briefly at \(0\) and then at \(25^\circ\) for 90 min. The filtered solution was washed with a dilute sodium bicarbonate solution and with water, then dried and the solvent was removed under vacuum.

The resulting gum was dissolved in a little ether and excess hexane was added. The supernatant liquid was decanted from the precipitated solid steroid material. The solution was diluted further with hexane to ensure complete removal of the steroidal product. This process gave a crude steroidal fraction (98 mg) while the hexane solution gave, on evaporation, methyl 2-hydroxy-6-methylheptanoate (Xa) (33 mg). 5H-4C<sub>9</sub>-p-Bromophenacyl-2-hydroxy-6-methyl Heptanoate (Xb) (30 mg) was hydrolyzed with potassium hydroxide (1.25 g, 11 moles) in aqueous methanol (1:2, 5 ml) at \(25^\circ\) for 4 hours, after which solvents were removed under reduced pressure. The brown gum was dissolved in dimethyl formamide (2 ml) and treated with triethylamine (0.2 ml) and p-bromophenacyl bromide (130 mg, \(6 \times 10^{5} \text{ dpm of } ^{14}\text{C} \text{ per mmole}) and was purified by TLC (ethyl acetate-benzene (1:10)).

The product was purified by repeated preparative TLC (ethyl acetate) to give (XV) (80 mg) (specific activity \( 0.81 \times 10^{5} \text{ dpm of } ^{14}\text{C} \text{ per mmole}; \{ ^{3}\text{H}:^{14}\text{C} \text{ ratio 17.2} \}). The lactone (XIII) was dissolved in methanol (1.4 ml) and treated with 5% aqueous sodium borohydride (1 ml) (26). After 30 min the reaction mixture was carefully acidified with acetic acid and water was added. The crude saturated lactone (XIV) was collected (150 mg) and was purified by TLC (ethyl acetate benzene (1:9); m.p. 173-174°) (literature (21)), \( 7/4-170^\circ \) (literature (21)). The mother liquors from crystallization of the \( ^{14}\text{C} \)-fusidic acid were combined with the remaining extract of the culture broth in which the biosynthesis was carried out. This was diluted with non-radioactive fusidic acid (220 mg), and crystallized from benzene to give the solvate (IVA) (290 mg). To a solution of IVA in aqueous methanol (1:4, 5 ml), 50% aqueous sodium hydroxide (0.6 ml) was added, and the mixture was heated for 15 min at \(75^\circ\) (22). The methanol was partially removed under a stream of nitrogen, and the aqueous solution was treated with glacial acetic acid (2.5 ml) on a water bath for 90 min. The cooled solution was diluted with water, and the product (XVIII) was collected (150 mg) and was purified by TLC (ethyl acetate benzene (1:9); m.p. 173-174°) (literature (21)), \( 7/4-170^\circ \) (literature (21)). The mother liquors from crystallization of the \( ^{14}\text{C} \)-fusidic acid were combined with the remaining extract of the culture broth in which the biosynthesis was carried out. This was diluted with non-radioactive fusidic acid (220 mg), and crystallized from benzene to give the solvate (IVA) (290 mg). 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The acid (XVIa) was esterified with diazomethane in ether-methanol, and the product was purified by TLC (ethyl acetate-benzene (1:1)) to yield 8 mg of the ester (XVIb) (specific activity 0.64 x 10^6 dpm of ^3H per mmole, ^1H: ^14C ratio 16.8); ^1H_NMR 3500, 2900, 1760, 1700, 1175, 1090, 950 cm⁻¹; NMR 61.0, 67.5, 74.5 (doublet and singlets (18-, 19-, 30-, and 32-CH₂)), 305 (multiplet, 1H (1α-H)); mass spectrum, m/e 444 (M⁺, base peak), 398, 384, 372, 265, 123.

The acid (XVII) was less than predicted for removal of 1 atom of tritium. However, the change in ^1H: ^14C ratio of VI was less than predicted for removal of 1 atom of tritium. This may have been due to the presence of small amounts of contaminants (e.g. 11-chloro or ∆¹¹ analogues), which could have cochromatographed with the major product. The presence of 1 atom of tritium at C-9β was confirmed by later results (see below).

For investigation of the presence of tritium at C-13α fusidic acid was hydrogenated over a deactivated catalyst (20) to give dihydrofusidic acid (VIIa) characterized as the methyl ester (VIIb) (^1H: ^14C ratio 16.8). The decreased ratio is consistent with the loss of some tritium from C-24 during hydrogenation. Deacetoxylation of (VIIa) with lithium chloride-dimethyl formamide (31) gave the ∆¹⁰,₁²(20)-dieno (VII) (^1H: ^14C ratio 13.7). The lower ratio indicates the removal of a tritium atom from C-13. It is noteworthy that the isomeric ∆¹⁰,₁²(20)-dieno (IX) (^1H: ^14C ratio 17.1) showed an unchanged tritium content.

Additional evidence for the presence of tritium atoms at C-9 and C-13 was obtained by a different route. Ozonolysis of VIIb gave a mixture of products from which the steroidal fragments were separated by precipitation with hexane. The crude solid material was oxidized with Jones reagent and resulting acetoxy-triketone (XVII) (16) was treated with zinc-acetic acid to give the 9β,13β (H)-triketone (XI) (22). The ^1H: ^14C ratio of the product (14.2) is consistent with the loss of 2 tritium atoms from C-13 and 24 and two ^14C atoms from C-22 and 26 of methyl dihydrofusidate (VIIb). The tritium atom originally present at C-13α would be expected to be lost during the epimerization of (XVII) on treatment with zim-acetic acid. Exposure of the 9β,13β-triketone (XI) to hydrogen chloride in dioxane resulted in epimerization (22) at C-9 and concomitant loss of tritium to give the 9α(H),13β (H)-triketone (XII) (^1H: ^14C ratio 7.0). This constitutes added evidence for the presence of a tritium atom at C-9β of fusidic acid. The location of a third tritium atom at a nonenolizable position in the nucleus of the triketone (XII) is also demonstrated by this result. Biogenetic considerations suggest its location at C-5α in fusidic acid (16, 17). The results are summarized in Table I.

The actual presence of the 4th tritium atom in the side chain of the antibiotic was proven by isolation of the hydroxy-ester.

### Table I

Summary of results of degradation of fusidic acids biosynthesized from (3R,4R)-(2^13C,4^14H)-mevalonic acid

<table>
<thead>
<tr>
<th>Product</th>
<th>Isotopic atoms removed</th>
<th>^1H: ^14C observed</th>
<th>^1H: ^14C (atomic)</th>
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<td>Methyl fusidate (IVb)</td>
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<td>Methyl fusidate-3-acetate (IVd)</td>
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<tr>
<td>11-Keto-3α-acetoxy ester (Va)</td>
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<tr>
<td>Methyl fusidate-3,11-diketone (Vb)</td>
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<tr>
<td>9(11),17(20),24-Triene (VI)</td>
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<tr>
<td>Methyl dihydrofusidate (VIIb)</td>
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<tr>
<td>9β,13β-Diene (VIII)</td>
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<tr>
<td>p-Bromophenacyl ester (Xb)</td>
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<tr>
<td>9α,13β-Triketone (XI)</td>
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<td>Lactone (XIV)</td>
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<tr>
<td>Diketo ester (XVIb)</td>
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* With reference to methyl fusidate (IVb).
overcome the internal repulsion. This is exemplified by the cy-
lanosterol. However, alternative routes of stabilization may
formed, respectively (30). These results were viewed (6) as evi-
XIXb and XIXc, the lanosterol analogues (XXIa and b) were
liver enzymes to the protosterol (XX) (27,29) was demonstrated.

evidence in support of the presence of the remaining
isotopic hydrogen atoms at C-9α and 13α has been demonstrated,
retention of 4 tritium atoms. The location of the important
atom at C-24.

In summary it may be concluded that the biosynthesis of fusi-
acid from (3R,4R)-(2-14C,4-3H) MVA proceeds with the
retention of 4 tritium atoms. The location of the important
isotopic hydrogen atoms at C-9β and 13α has been demonstrated,
and evidence provided in support of the presence of the remaining
tritium atoms at C-5α and 24, while no tritium was detected at
the C-3β position. This demonstrates that the 4-pro-R protons
of MVA retained in IVa occupy their original positions of attach-
ment to the carbon skeleton, as predicted for the conversion of
squalene, via the cation (I) to fusidic acid. Consequently, the
protosterol skeleton of IVa seems to be formed by elimination of a
proton from C-17 of I without rearrangement. In principle, our
results on the mode of formation of fusidic acid can be interpreted
as further evidence in favor of the biogenetic scheme proposed by
Eschenmoser et al. (7) for the formation of lanosterol.

It has been shown (25) that the protosterol (XVIII) can be re-
arranged in the presence of acid to lanosterol (II). This suggests
that lanosterol is the most energetically favored product of the
carbonium ion formed by protonation of the 13(17) double bond
of the protosterol. As a corollary to this, the idea was forwarded
(6) that, in lanosterol biosynthesis, the rearrangement of the cat-
ion (I) to lanosterol may be governed largely by thermodynamic
factors, with the enzyme having only a peripheral role. In addi-
tion the cyclization of the 10,15-di-nor-squalene (XIXa) by rat
liver enzymes to the protosterol (XX) (27,29) was demonstrated.
When only one of the methyl groups of squalene is removed, as in
XIXb and XIXc, the lanosterol analogues (XXia and b) were
formed, respectively (30). These results were viewed (6) as evi-
dence for the importance of intramolecular steric repulsion as
the driving force for the backbone rearrangement leading to
lanosterol. However, alternative routes of stabilization may
overcome the internal repulsion. This is exemplified by the cy-
clization of the conjugated analogue (XXII) which gives the
protosterol (XXIII) when cyclized with rat liver enzymes (28).
It is important to note, however, that the stereochemistry of
the products (XX) and (XXIII), particularly at C-17, was not
proven rigorously. The saturated analogue of XXXIII obtained
by catalytic hydrogenation, was recently converted by acid treat-
ment (24, 25) to dihydrolanosterol. This was interpreted as
evidence in support of the C-20 location of the hydroxyl group
and of the indicated stereochemistry of (XXIII) (29).

The current ideas on the cyclization of squalene fail to provide
a complete rationalization for the formation of the protosterols.
In F. coccineum two different pathways for stabilization of an
intermediate of type I appear to function in parallel (16). Our
results are consistent with the postulate that fusidic acid, and
hence the protosterols (XVIII and XXIV) are formed by direct
elimination of a proton from C-17 of I. On the other hand the
presence of ergosterol in the mold is clear evidence for the opera-
tion of the backbone rearrangement process (7) which terminates
in lanosterol (or cycloartenol).

The question arises as to why the C-20 cation (or its stabilized
analogue) should collapse to different end products in the same
species. The problem is mechanistically interesting and several
explanations for this divergence can be visualized. For instance,
it has been proposed (8) that the formation of lanosterol may re-
quire two enzymes, a cyclase which generates the intermediate
(1) and an isomerase which promotes rearrangement. Suppres-
sion of the latter enzyme might result in the activation of a shunt
mechanism leading to protosterols. Alternatively, cyclization and
isomerization may be a single enzyme process. The two
groups of compounds might then be formed on completely sepa-
rate enzyme systems, which would control the fate of the inter-
mediate (I) and hence the structure of the products. One exten-
sion of this idea leads to the postulate that the two cyclases could
produce different intermediates (XXVa and XXIVa) having
opposite stereochemistry about C-17. The trans relationship of the
13α and 17β protons of XXVa would facilitate backbone rear-
arrangement to lanosterol. The cis relationship of the 13α and
17α protons of XXVIa could prevent rearrangement and the most
favorable stabilization pathway under these circumstances may be elimination of the 17α proton.

A further observation of relevance to this discussion concerns the application of the nucleophilic stabilization (X group) mechanism (9) to the biosynthesis of the protosterols. It has been suggested that the involvement of an enzymic or chemical nucleophile in the conversion of squalene-2,3-oxide to lanosterol would lead to the transiently stabilized intermediate (XXVb). Rotation of the side chain of (XXVb) relative to the ring system would then permit backbone rearrangement to lanosterol via trans elimination of HX. This hypothesis circumvents some of the previously mentioned criticism of this hypothesis. On the other hand, to fit the facts, the X group theory would have to be modified or broadened. A basic tenet of the X group concept as it was formulated by Cornforth (9) is the trans elimination of the proton and of the prosthetic group. Obviously, a trans elimination will be thermodynamically favored over a cis elimination. To reconcile this requirement, it could be postulated that the “X” group in XXV or XXVI is displaced by another nucleophile “Y” with inversion of configuration.4 Thus in the newly formed Y-stabilized intermediate (corresponding to XXV or XXVI, but with inverted configuration of the C-X(Y) bond) the C-17 proton and the prosthetic Y moiety are positioned for a facile trans elimination, leading to protosterols with the correct geometry at the C-17-20 double bond. Finally, a broadening of the concept of the “X” group may also be considered. For example two types of “X” group may be invoked, one allowing a trans elimination-migration from XXVb leading to a lanosterol type side chain, and the other producing the protosterol (XXIVA) by cis elimination of HX from either XXVb or XXVIb. This mechanism is illustrated in XXVII and XXVIII in which the prosthetic group is shown as a phosphate ester. The indicated six-membered transition states would facilitate cis dehydration. It must be stressed, however, that the phosphate moiety is used only as an illustration and no experimental support for its participation is claimed.

Murata et al. (31–33) have recently isolated and determined the structure of several new protosterols which they named Alisols. Alisol A was shown by x-ray crystallography to have the structure of XXIX and the indicated 20R,23S,24R,23,24-threo configuration. In Alisol B the 24,25-diol of XXIX is replaced by a 24,25-epoxide; Alisol C has the structure of 16-keto-Alisol B. The Δ^{22,23}-20R-moiety common to the Alisol suggests their formation via an intermediate of type XXV. Indirectly therefore this provides some support for the two-enzyme concept of a squalene-oxide-cyclase and an isomerase in the biosynthesis of these triterpenes. In any event the actual nature of the factors governing the in vivo formation of the different triterpenes is not known and requires a detailed study.

Finally, the present results are of relevance to recent observations on the mechanism of elimination of the C-4 gem dimethyl group from triterpenes. It has been demonstrated that, in the formation of cholesterol in rat livers, the sequence involves oxidative loss of the 4α methyl group (via a 3-keto intermediate) and subsequent equilibration of the 4β methyl group to the 4α position (34–36). A similar sequence seems to be operative in some phytosterols (35). However, in fusidic acid the demethylation process appears to be different. Isolation of the triterpenes XVIII and XXIV from F. coccineum suggests that this group of compounds is formed from (3S)-squalene-2,3-oxide, as are lanosterol and other triterpenes (35, 36). Hence, the 4α-methyl group of XXIV and its congeners will originate from C-2 of MVA. The reported (24, 25) retention of deuterium in C-2 carbon atoms of MVA by fusidic acid (confirmed by the present investigation) and the absence of tritium from C-3 on incorporation of (3R,4R)-2H) MVA suggest that the formation of fusidic acid involves a direct loss of the 4β methyl group, presumably via a 3-keto analogue.

4 The displacement idea was formulated by Dr. J. W. Cornforth to whom we are indebted for a stimulating exchange of views.
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