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)- (2-14C,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 9a and 13a positions. Evidence was also adduced for the location of the remaining 2 tritium atoms at the 5a 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 retention of six 14C-atoms in the antibiotic confirms the view that in this fungus the transformation of the parent 4-gem dimethyl protosterol to fusidic acid involves the loss of the 4β-methyl derived from 3- of MVA. The absence of isotopic hydrogen at C-3, proven by us, indicates that the removal of the 4β-methyl probably proceeds through a 3-keto intermediate.

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

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Proton magnetic resonance spectra were recorded on a Varian spectrometer apparatus and are corrected. Infrared spectra were recorded on a Perkin-Elmer 237 spectrophotometer as KBr discs. Mass spectra were measured on a Varian Associates M-66 instrument or on a Consolidated Electrodynamics Corporation 21-491 instrument.

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 (50 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 TLC (methanol-dichloromethane (1.12)), 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.

³H₄-¹⁴C-Methyl Fusidate (IVb)—Fusidic acid-benzene solvate (IVA) (20 mg) was methylated with diazomethane in ether-methanol. The product was purified twice by thin layer chromatography in systems ethyl acetate-benzene (1.20) and ethyl acetate-benzene (1.7). The major band was extracted with ethyl acetate and crystallized from ether-hexane to constant ¹⁴C:²H₄-ratio and ²H₄-specific activity giving IVb (15 mg); m.p. 151-155°C (literature (20), 153.5-154°C) (specific activity 3.95 × 10⁴ dpm of ¹⁴C per mmole; ¹⁴C:²H₄-ratio 18.0).

³H₄-¹⁴C-3α-Acetoxy Methyl Fusidate (IVd)—Fusidic acid-benzene solvate (IVA) (40 mg) was diluted with nonradioactive (IVA) (60 mg) and esterified with diazomethane in ether-methanol. 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 preparative could not be induced to crystallize (specific activity 1.55 × 10⁴ dpm of ¹⁴C per mmole; ¹⁴C:²H₄-ratio 17.0); ν_max 3500 (OH), 1740, 1735, 1720, 1700 (C=O), 1250 cm⁻¹.

Another method for the isolation and purification of fusidic acid benzene solvate (IVA) (25 mg) was converted to 3α-acetoxy 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 preparative TLC (ethyl acetate-benzene (1: 19)) to yield 15 mg (Va) (specific activity 3.88 × 10⁴ dpm of ¹⁴C per mmole; ¹⁴C:²H₄-ratio 18.1); ν_max 3500 (OH), 1740, 1735, 1720, 1700 (C=O), 1250 cm⁻¹; mass spectrum, m/e 526 (M₄⁺), 380 (M+), 294, 170 (M-OH), 169 (M-H₂O), 168 (M-OH), 167 (M-H₂O), 166 (M-OH), 165 (M-H₂O), 164 (M-OH), 163 (M-H₂O), 162 (M-OH), 161 (M-H₂O), 160 (M-OH), 159 (M-H₂O), 158 (M-OH), 157 (M-H₂O), 156 (M-OH), 155 (M-H₂O), 154 (M-OH), 153 (M-H₂O), 152 (M-OH), 151 (M-H₂O), 150 (M-OH), 149 (M-H₂O), 148 (M-OH), 147 (M-H₂O), 146 (M-OH), 145 (M-H₂O), 144 (M-OH), 143 (M-H₂O), 142 (M-OH), 141 (M-H₂O), 140 (M-OH), 139 (M-H₂O), 138 (M-OH), 137 (M-H₂O), 136 (M-OH), 135 (M-H₂O), 134 (M-OH), 133 (M-H₂O), 132 (M-OH), 131 (M-H₂O), 130 (M-OH), 129 (M-H₂O), 128 (M-OH), 127 (M-H₂O), 126 (M-OH), 125 (M-H₂O), 124 (M-OH), 123 (M-H₂O), 122 (M-OH), 121 (M-H₂O), 120 (M-OH), 119 (M-H₂O), 118 (M-OH), 117 (M-H₂O), 116 (M-OH), 115 (M-H₂O), 114 (M-OH), 113 (M-H₂O), 112 (M-OH), 111 (M-H₂O), 110 (M-OH), 109 (M-H₂O), 108 (M-OH), 107 (M-H₂O), 106 (M-OH), 105 (M-H₂O), 104 (M-OH), 103 (M-H₂O), 102 (M-OH), 101 (M-H₂O), 100 (M-OH), 99 (M-H₂O), 98 (M-OH), 97 (M-H₂O), 96 (M-OH), 95 (M-H₂O), 94 (M-OH), 93 (M-H₂O), 92 (M-OH), 91 (M-H₂O), 90 (M-OH), 89 (M-H₂O), 88 (M-OH), 87 (M-H₂O), 86 (M-OH), 85 (M-H₂O), 84 (M-OH), 83 (M-H₂O), 82 (M-OH), 81 (M-H₂O), 80 (M-OH), 79 (M-H₂O), 78 (M-OH), 77 (M-H₂O), 76 (M-OH), 75 (M-H₂O), 74 (M-OH), 73 (M-H₂O), 72 (M-OH), 71 (M-H₂O), 70 (M-OH), 69 (M-H₂O), 68 (M-OH), 67 (M-H₂O), 66 (M-OH), 65 (M-H₂O), 64 (M-OH), 63 (M-H₂O), 62 (M-OH), 61 (M-H₂O), 60 (M-OH), 59 (M-H₂O), 58 (M-OH), 57 (M-H₂O), 56 (M-OH), 55 (M-H₂O), 54 (M-OH), 53 (M-H₂O), 52 (M-OH), 51 (M-H₂O), 50 (M-OH), 49 (M-H₂O), 48 (M-OH), 47 (M-H₂O), 46 (M-OH), 45 (M-H₂O), 44 (M-OH), 43 (M-H₂O), 42 (M-OH), 41 (M-H₂O), 40 (M-OH), 39 (M-H₂O), 38 (M-OH), 37 (M-H₂O), 36 (M-OH), 35 (M-H₂O), 34 (M-OH), 33 (M-H₂O), 32 (M-OH), 31 (M-H₂O), 30 (M-OH), 29 (M-H₂O), 28 (M-OH), 27 (M-H₂O), 26 (M-OH), 25 (M-H₂O), 24 (M-OH), 23 (M-H₂O), 22 (M-OH), 21 (M-H₂O), 20 (M-OH), 19 (M-H₂O), 18 (M-OH), 17 (M-H₂O), 16 (M-OH), 15 (M-H₂O), 14 (M-OH), 13 (M-H₂O), 12 (M-OH), 11 (M-H₂O), 10 (M-OH), 9 (M-H₂O), 8 (M-OH), 7 (M-H₂O), 6 (M-OH), 5 (M-H₂O), 4 (M-OH), 3 (M-H₂O), 2 (M-OH), 1 (M-H₂O), 0 (M-OH).

A third medium (20) 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), and micronutrients* (1 ml) at pH 6.5. The flask were shaken at 26°C for 5 days.

*Micronutrient solution: FeSO₄·7 H₂O (0.5 g per liter), ZnCl₂ (0.21 g per liter), MnSO₄·H₂O (0.15 g per liter) and CaCl₂·2 H₂O (18.5 g per liter).
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(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 × 10^6 dpm of 14C per mmole; 3H : 14C ratio 18.1); ν_max 1745 (O = CO - CH_3), 1725 (ν = CO - CH_3), 1715 (C = O) cm^-1; NMR δ 2.5 (doublet, 3H (3α-CH_3, 6.0 Hz)), 5.0, 6.0, 7.0 (singlets, each 3H (19-32, and 18-CH_3)), 9.5, 10.0 (6H (26- and 27-CH_3)), 120 (singlet, 3H (ν = CO - CH_3)), 218 (singlet, 3H (ν = CO - CH_3)), 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.

3H_4-14C_3-3α-Acetoxy-A_9(11) Methyl Fusidate (VI) — To a stirred and cooled solution (-30°) of 3α-acetoxy methyl fusidate (IVd) (50 mg) in pyridine (3 ml), a solution of thionyl chloride (0.2 ml) in pyridine (3 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, 1:19) to give 25 mg of VI. The homogeneity of VI was assayed by repeated TLC (specific activity 1.76 × 10^6 dpm of 14C per mmole; 3H : 14C ratio 14.9); ν_max 1745, 1735, 1720 (C = O), 1240, 1010, 900, 810, 790 cm^-1; NMR δ 9.5 (doublet, 5H (C-3)), 52.5, 59.5, 71.0 (9H (18, 19, and 32-CH_3)), 95.5, 100.0 (6H (26- and 27-CH_3)), 110, 122 (singlets, 3H each (3α and 16β O = CO - CH_3)), 220 (singlet, 3H (ν = CO - CH_3)), 224 (multiplet, 1H (3H)), 305 (multiplet, 1H (C-16 proton)), 328 (triplet, 1H (α olefinic proton, 3.5 Hz)), 356.5 (doublet, 1H (24H)); mass spectrum, m/e 494 (M-60), 462, 425, 419, 365 (base peak).

3H_4-14C_3-Dihydrojusidic 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 carbonate (60 mg) was added (20). The mixture was stirred vigorously and hydrogenated until 1.1 moles of hydrogen had been absorbed. 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).

Deacetoxylation of 3H_4-14C_3-Methyl Dihydrofusidic Acid (VIIb) — Dihydrofusidic acid (VIIa) (25 mg) was esterified with diazomethane in ether-methanol. 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 × 10^6 dpm of 14C per mmole; 3H : 14C ratio 16.8).

Deacetoxylation of 3H_4-14C_3-Dihydrofusidic Acid (VIIa) — To a solution of VIIa (80 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-n-butanol-water, 9:7:3). The major product was identified as the \( \Delta_{17(20)} \)-dienoate (VIII) (25 mg) which was purified by repeated chromatography (specific activity 2.55 \( \times 10^3 \) dpm of \(^{14} \)C per mmol; \( \Delta_{14} \):\(^{14} \)C ratio 13.7); \( p_{\text{max}} \) 3450 (singlet, 1H, \( \Delta_{17(20)} \)-dienoate (VIII)).

A second product from this reaction was identified as the \( \Delta_{14,17(20)} \)-dienoate (IX) (10 mg) and was purified as described above (specific activity 2.52 \( \times 10^3 \) dpm of \(^{14} \)C per mmol; \( \Delta_{14} \):\(^{14} \)C ratio 17.1); \( p_{\text{max}} \) 271 nm (~16,000).

**Epimerization of XI to \( \Delta_{14,17(20)} \)-9P,13R-Triketone (XII)—**The triketone (XI) (35 mg) was dissolved in dioxane (8 ml) previously saturated with dry hydrogen chloride. After 18 hours at 25° the solution was diluted with water and concentrated by evaporation at 60° under vacuum. The cooled solution was extracted with dichloromethane which was then washed with water and dried. After removal of solvents under vacuum, the crude product was purified by TLC (ethyl acetate-benzene, 1:19) and crystallized from ether-hexane to give 60 mg of XI; m.p. 174°-175° (literature (22), 171°-174°) (specific activity 1.77 \( \times 10^3 \) dpm per mmol; \( \Delta_{14} \):\(^{14} \)C ratio 14.2); \( p_{\text{max}} \) 1735, 1710, 1700 (C=O), 1220, 1190, 1150, 1120, 990 cm\(^{-1}\); NMR 49.5, 74.0, 88.0 (singlets, each 3H; 18, 19, 32-CH\(_3\)): 62.0 (doublet, 3H, 30-CH\(_3\)); mass spectrum, \( m/e \) 330 (M\(^+\)), 302 (M-17), 273, 234, 219, 205, 191, 179, 137, 97 (base peak).

**Sidechain Degradation of \( \Delta_{14,17(20)} \)-Fusidyl Acid (IVa)—**The mother liquors from crystallization of the \( \Delta_{14,17(20)} \)-fusidyl acid were combined with the remaining extract of the culture broth in which the biosynthesis was carried out. This was diluted with non-radioactive fusidyl acid (220 mg), and crystallized from benzene to give the solvate (IVa) (230 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° (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 (XIV) was collected (\( \Delta_{14} \):\(^{14} \)C ratio 17.2). The lactone (XIII) was dissolved in methanol (10 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, 1:19). A homogenous XIV was treated with osmium tetroxide (120 mg, 1.2 moles) in ether-pyridine (5:4; 6 ml) with stirring at 25° for 36 hours. TLC indicated the absence of starting material. A solution of sodium bisulphite (250 mg) in pyridine-water (1:1, 9 ml) was added, and the mixture was stirred for 30 min. The resulting product was extracted with dichloromethane, and the organic phase was washed with water and dried. The product and traces of pyridine were removed under reduced pressure. The product was purified by repeated preparative TLC (ethyl acetate-n-butanol-water, 9:7:3) to give (XV) (80 mg) (specific activity 1.89 \( \times 10^3 \) dpm of \(^{14} \)C per mmol; \( \Delta_{14} \):\(^{14} \)C ratio 7.0); \( p_{\text{max}} \) 1740, 1710, 1690 (C=O), 1320, 1105, 1130, 1100 cm\(^{-1}\); NMR 62.5, 88.0, 70.5 (singlets, each 3H; 18, 19, and 32-CH\(_3\)): 62.0 (doublet, 3H, 30-CH\(_3\)); mass spectrum, \( m/e \) 330, 179 (base peak), 135, 123, 110.

The homogenous XIV was treated with osmium tetroxide (120 mg, 1.2 moles) in ether-pyridine (5:4; 6 ml) with stirring at 25° for 36 hours. TLC indicated the absence of starting material. A solution of sodium bisulphite (250 mg) in pyridine-water (1:1, 9 ml) was added, and the mixture was stirred for 30 min. The resulting product was extracted with dichloromethane, and the organic phase was washed with water and dried. The product and traces of pyridine were removed under reduced pressure. The product was purified by repeated preparative TLC (ethyl acetate) to give (XV) (80 mg) (specific activity 0.81 \( \times 10^3 \) dpm of \(^{14} \)C per mmol; \( \Delta_{14} \):\(^{14} \)C ratio 16.7); \( p_{\text{max}} \) 3450, 1750, 1575, 1625, 1515, 1190, 1060, 1010, 965, 925 cm\(^{-1}\).

**Isolation of the \( \Delta_{14,17(20)} \)-Triketone (XT)—**The triketone portion from the ozonolysis described above (98 mg) was dissolved in acetone and treated with Jones reagent for 25° for 15 min. After conventional work-up the crude product was treated with zinc (600 mg) and glacial acetic acid (15 ml) under reflux for 30 min. Following filtration and washing of the zinc with chloroform the solvents were removed under vacuum, and the product was purified by TLC (ethyl acetate-benzene, 1:10). Crystalization from ether-hexane gave 60 mg of XI; m.p. 174°-175° (literature (22), 171°-174°) (specific activity 1.77 \( \times 10^3 \) dpm per mmol; \( \Delta_{14} \):\(^{14} \)C ratio 14.2); \( p_{\text{max}} \) 1735, 1710, 1700 (C=O), 1220, 1190, 1150, 1120, 990 cm\(^{-1}\); NMR 49.5, 74.0, 88.0 (singlets, each 3H; 18, 19, 32-CH\(_3\)): 62.0 (doublet, 3H, 30-CH\(_3\)); fs mass spectrum, \( m/e \) 330 (M\(^+\)), 302 (M-17), 273, 234, 219, 205, 191, 179, 137, 97 (base peak).
0.65 \times 10^5 \text{ dpm of } ^{14}\text{C per mmole, } ^{1}\text{H:}^{14}\text{C ratio 16.8): } \chi_{\text{max}}^{14}\text{C} 3500, 2900, 1700, 1700, 1175, 1090, 950 \text{ cm}^{-1}; \text{NMR } 61.0, 67.5, 74.5 (\text{doublet and singlet (18-19, 30-31, and 32-C\_H}_2)) 305 (\text{multiplet, } 1H (\text{10-xH})); \text{mass spectrum, } m/e 444 (M^+, \text{base peak}), 398, 384, 372, 205, 123.

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 \times 10^5 \text{ dpm of } ^{14}\text{C per mmole, } ^{1}\text{H:}^{14}\text{C ratio 16.5): } \chi_{\text{max}}^{14}\text{C} 1760, 1740, 1700, 1690, 1215, 1175, 1075, 1050 \text{ cm}^{-1}; \text{mass spectrum, } m/e 488 (M^+, \text{base peak}), 437 (M-1), 366, 336, 307, 304, 275, 225, 219, 200, 193, 123.

RESULTS AND DISCUSSION

Fusidic acid has been shown to retain six C-2 carbon atoms of MVA (24, 25) and to be derived from squalene via squalene-2, 3-oxide (26). The enzymatic cyclization of the epoxide to the cat-
ion (Ia) or its stabilized equivalent (Ib) should proceed with the retention of six 4-pro-R protons of MVA at C-3, C-5, C-9, C-13, C-17, and C-24 as shown in I (1). Conversion of I to fusidic acid would be expected to result in the retention of the protons at C-5a, C-9a, C-13a, C-24, and possibly C-3β while at C-17 should be lost (16).

In order to evaluate this hypothesis F. cocceum was grown in a medium (19) containing (3R,4R)-2-[14C, 4-3H] MVA (50 μCi of 14C) and the fusidic acid isolated showed 0.2% incorporation of 14C. The actual presence of the 4th tritium atom in the side chain of fusidic acid was proven by isolation of the hydroxy-ester (XVIa) which had an unchanged 3H:14C ratio (16.8), indicating that no loss of tritium from C-24 had occurred during hydrogenation. Deacetoxylation of (VIIa) with lithium chloride-dimethylform-
amide (21) gave the 9a,13β-triketone (XII) (3H:14C ratio 17.0). This constitutes added evidence for the presence of a tritium atom at C-9 of fusidic acid. The location of a third tritium atom at a position of fusidic acid was then examined. The diacetate (IVD) was dehydrated (16) to give the triene (VI). The decreased 3H:14C ratio (14.9) revealed the presence of tritium at C-9 of fusidic acid, which is removed on introduction of the Δ9\(11\) double bond. However, the change in 3H: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 Δ13 analogues), which could have cochromatographed with the major product. The presence of 1 atom of tritium at C-9β of fusidic acid 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) (3H: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 form-
amide (21) gave the Δ10(17),20(22)-diene (VIIa) (3H:14C ratio 13.7). The lower ratio indicates the removal of a tritium atom from C-13. It is noteworthy that the isomeric Δ10,17(20)-diene (IXb) (3H:14C ratio 11.7) 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 acetooxy-
triketone (XVII) (16) was treated with zinc-acetic acid to give the Δ9β,13β- (H)-triketone (XI) (22). The 3H: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 dihy-
drofusidate (VIIb). The tritium atom originally present at C-12β was expected to be lost during the epimerization of (XVII) on treatment with zinc-acetic acid. Exposure of the 9β, 13β-triketone (XI) to hydrogen chloride in dioxide resulted in epimerization (22) at C-9 and concomitant loss of tritium to give the 9αβ,13β(3H)-triketone (XII) (3H: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 nonenomizable position in the nucleus of the triketone (XII) is also demonstrated by this result. Biogenetic considerations suggest its location at C-5a 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)-(2^14C, 4^3H)-mevalonic acid*

<table>
<thead>
<tr>
<th>Product</th>
<th>Isotopic atoms removed*</th>
<th>3H:14C observed</th>
<th>3H:14C (atomic)</th>
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<tr>
<td></td>
<td></td>
<td>3H</td>
<td>14C</td>
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<tr>
<td><strong>Methyl fusidate (IVb)</strong></td>
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<td>18</td>
<td>0</td>
</tr>
<tr>
<td><strong>Methyl fusidate-3α-acetate (IVd)</strong></td>
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<td>6</td>
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<td><strong>11-Keto-3α-acetoxy ester (Va)</strong></td>
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<td>1</td>
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<tr>
<td><strong>Methyl fusidate-3,11-diketone (Vb)</strong></td>
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<td>18</td>
<td>1</td>
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<tr>
<td><strong>9(11),17(20),24-Triene (VI)</strong></td>
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<td>14</td>
<td>9</td>
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<td><strong>Methyl dihydrofusidate (VIIb)</strong></td>
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<td><strong>13(17),20(22)-Diene (VIII)</strong></td>
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<td>24</td>
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<td><strong>9αβ,13β-Triketone (XII)</strong></td>
<td>9αβ, 13α, 24</td>
<td>16</td>
<td>9</td>
</tr>
<tr>
<td><strong>Lactone (XIV)</strong></td>
<td></td>
<td>14</td>
<td>10</td>
</tr>
<tr>
<td><strong>Diketo ester (XVIIa)</strong></td>
<td>24</td>
<td>16</td>
<td>5</td>
</tr>
</tbody>
</table>

*With reference to methyl fusidate (IVb).*
(Xa) obtained by ozonolysis of methyl dihydrofusidate (VIIb) (zinc-acetic acid work-up (20)). The ester (Xa) was hydrolyzed and the resulting hydroxy acid was characterized as the p-bromophenacyl ester (Xb) (H:14C ratio 9.8). The isotopic ratio and 14C specific activity of this product were compatible with the presence of 1 tritium and two 14C atoms in the side chain of fusidic acid. For a more precise definition of the location of the tritium atom a degradation of the side chain was performed. Fusidic acid (IVA) was transformed to the lactone (XIII) which was reduced with sodium borohydride (23) to the saturated lactone (XIV) (H:14C ratio 16.9). Exposure of XIV to cesium tetrafluoroborate provided the glycol (XV) which was converted to the diketo acid (XVIa) (H:14C ratio 16.8) by treatment with Jones reagent. The H:14C ratios of the acid (XVIa) and its methyl ester (XVIb) (H:14C ratio 16.5) showed the loss of one tritium and one 14C atom, a result consistent with the predicted presence of a tritium atom at C-24.

In summary it may be concluded that the biosynthesis of fusidic acid from (3H,4H)-(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 attachment to the carbon skeleton, as predicted for the conversion of squalene, via the cation (I) to lanosterol. Consequently, the protosterol skeleton of IVA seems to be formed by the conversion 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 rearranged 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 cation (I) to lanosterol may be governed largely by thermodynamic factors, with the enzyme having only a peripheral role. In addition the cyclization of the 10,15-di-nor-squalene (XIa) by rat liver enzymes to the protosterol (XX) (27, 29) was demonstrated. When one of the methyl groups of squalene is removed, as in XIb and XIc, the lanosterol analogues (XXIa and b) were formed, respectively (30). These results were viewed (6) as evidence 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 cyclization 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 XXIII obtained by catalytic hydrogenation, was recently converted by acid treatment (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 operation 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 require two enzymes, a cyclase which generates the intermediate of type I and an isomerase which promotes rearrangement. Suppression 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 separate enzyme systems, which would control the fate of the intermediate (I) and hence the structure of the products. One extension of this idea leads to the postulate that the two cyclases could produce different intermediates (XXXa) and (XXVa) having opposite stereochemistry about C-17. The trans relationship of the 13α and 17β protons of XXVa would facilitate backbone rearrangement to lanosterol. The cis relationship of the 13α and 17α protons of XXVla 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 difficulties inherent in the nonstop carbonium ion theory, particularly with regard to the spatial requirements of the enzyme. The invoked "stable" intermediate also allows accommodation of the "X" group theory that two enzymes may be involved in the production of lanosterol. However, this concept appears not to be directly applicable to the production of the protosterols, since, irrespective of whether the side chain of the nucleophile-stabilized intermediate has configuration (XXVb) or (XXVIIb), a cis elimination of the elements of HX (as required by the theory) from C-17 and 20 would lead to a protosterol having the wrong geometry about the Δ[20] bond.

Several interpretations can be projected which would accommodate this discrepancy. First it is possible that a free carbonium ion is indeed involved in the biosynthesis of protosterols. Unfortunately, however, acceptance of this view would leave unanswered 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 XXXa leading to a lanosterol type side chain, and the other producing the protosterol (XXIV) 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 structure 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[20]-20R-moiety common to the Alisols 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 phytoesterols (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 (38)-squalene-2,3-oxide, as are lanosterol and other triterpenes (38, 39). Hence, the 4α-methyl group of XXIV and its congeners will originate from C-2 of MVA. The reported (24, 25) retention of six 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)- (24C,4-H) 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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