Dilithium Benzophenone Dianion. Butyllithium in hexane (6.0 mmol) was added slowly to 1,2-dibromoethane (0.957 g, 5.1 mmol) in a 40-ml centrifuge tube capped with a No-Air stopper. The resulting precipitate of anhydrous lithium bromide was washed four times with 20-ml portions of pentane, dissolved in 7 ml of hot DME, and added to 2 mmol of dipotassium benzophenone dianion in 30 ml of DME containing 0.1534 g of *n*-nonadecane as internal standard in a stoppered 40-ml centrifuge tube. The solution was thoroughly mixed by shaking and the precipitate of potassium bromide separated by centrifugation. The resulting solution of dilithium benzophenone[79] was added to a solution of α,α-diphenyl-α-hydroxyacetophenone (0.0967 g, 0.336 mmol) in 10 ml of DME. The red color of dilithium benzophenone persisted after the addition. After stirring at room temperature for 2 hr, the reaction mixture was hydrolyzed with aqueous potassium hydroxide solution; the aqueous layer was separated and extracted with one 50-ml portion of ethyl ether, and the organic layers were combined and dried (Na_2SO_4). Analysis by glpc indicated the presence of α,α-diphenylacetophenone (72%) and α,α-diphenyl-α-hydroxyacetophenone (9%).

Preparation of Other Anions. Lithium phenyl acetylide and lithium butyl acetylide were prepared by reaction of the corresponding acetylene with methyllithium in ether solution. Lithium diphenyl phosphide was prepared by the reaction of diphenylphosphine with *n*-butyllithium in THF. Lithium diphenylamide was prepared by the reaction of diphenylamine with methyllithium in THF. Sodium dimsylate was prepared as described by Greenwald, Chaykovsky, and Corey.[80] All of the above were reacted with carbon monoxide at 200 psi; only sodium dimsylate took up an appreciable quantity of carbon monoxide (0.5 equiv of carbon monoxide per equivalent of dimsylate over a 24-hr period).

(79) Solutions of **18** prepared using this procedure were analyzed for potassium by hydrolysis and treatment with lithium tetraphenylborate: *cf.* D. N. Bhattacharyya, C. L. Lee, J. Smid, and M. Szwarc, *J. Phys. Chem.*, **69**, 608 (1965). Less than 1% of the potassium originally present as dipotassium benzophenone remained in solution after addition of the lithium bromide.

Acknowledgment. F. R. Koeng carried out preliminary experiments in this problem. This work was assisted substantially by a grant from the Research Corporation for the purchase of glpc equipment. Dr. Charles Hignite, Mr. Brian Andresen, and Professor Klaus Biemann generously provided us with high-resolution spectra and help with glc–mass spectra on several occasions; this assistance was provided under National Institutes of Health Research Grant No. RR00317 from the Division of Research Facilities and Resources. Professor Barry Sharpless was helpful in suggesting techniques for isolation and preparation of certain of the compounds of interest in this work. Professor Ronald Breslow made several useful criticisms of our initial mechanistic hypotheses.

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Mechanism of Oxidative Cyclization of Squalene. Evidence for Cyclization of Squalene from Either End of the Squalene Molecule in the *in Vivo* Biosynthesis of Fusidic Acid by *Fusidium coccineum*[1a]

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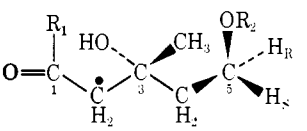
Contribution from the Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts 01545, and Leo Pharmaceutical Products, DK 2750 Ballerup, Denmark. *Received March 2, 1973*

Abstract: Fusidic acid biosynthesized by *F. coccineum* from (3*RS*,5*S*)-[2-^{14}C,5-^{3}H]mevalonic acid was shown to contain 0.5 atom of tritium at the C-11β and C-12 positions. From the known mechanism of squalene formation the 0.5 atom of tritium at C-12 must have the α configuration. Our results indicate that either one of the terminal double bonds of squalene is epoxidized to an equal degree, and that the ensuing cyclization to prosterol occurs from either end of the squalene molecule. This shows that the geometrical asymmetry imparted to the squalene on the squalene synthethase is not retained during the conversion to oxidosqualene. These observations are consistent with the hypothesis of the release of squalene into a free squalene pool prior to epoxidation.

The biosynthetic steps involved in the formation of squalene from mevalonic acid (MVA) (**1a**) are now well understood.[2] Mevalonic acid is first converted to the C-5 pyrophosphate (**1b**), which undergoes in the presence of ATP a concerted decarboxylation and elimination of the C-3 tertiary hydroxyl to yield[3] isopentenyl pyrophosphate (**2**). This process involves a trans elimination of the two participating moieties.[4]



1a, R_1 = OH; R_2 = H; H_R = 5-pro-*R* hydrogen
H_S = 5-pro-*S* hydrogen
b, R_1 = OH; R_2 = pyrophosphate (PP)
c, R_1 = $NHCH(C_6H_5)_2$; R_2 = H; C ≡ ^{14}C; H_S ≡ ^{3}H

(1) (a) The results described in this paper were presented at the 9th International Congress of Biochemistry, Stockholm, 1973, Abstract 9f6, and in a series of lectures at the Bar-Ilan University, Hebrew University, Jerusalem, University of the Negev, Beer-Sheva, and the Weizmann Institute, Rehovoth, during the tenure by E. Caspi of a visiting professorship at the Bar-Ilan University in Ramat Gan, Israel. (b) Worcester Foundation for Experimental Biology. (c) Postdoctoral Fellow, 1970–1973. (d) Except for the microbial incubation experiment, the work was carried out at the Worcester Foundation for Experimental Biology. (e) Leo Pharmaceutical Products.

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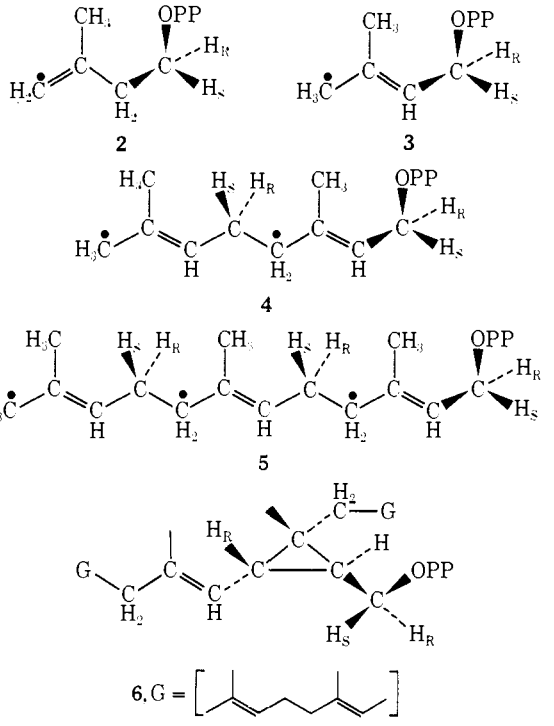
The isopentenyl pyrophosphate (**2**) isomerizes to dimethylallyl pyrophosphate (**3**) which in turn condenses sequentially with two molecules of the isopentenyl pyrophosphate to yield first geranyl pyrophosphate (**4**) and then farnesyl pyrophosphate (**5**). The coupling of two farnesyl pyrophosphate molecules to squalene is an asymmetric process.[2,5] Whether the coupling of two farnesyl pyrophosphate molecules to squalene proceeds through the obligatory intermediacy of presqualene pyrophosphate[6] (**6**) is unsettled.[7] In the



process of squalene formation a 1-*pro-S* hydrogen (originating from 5-*pro-S* of MVA) is lost from one of the two participating farnesyl pyrophosphate molecules[2,5] and is replaced by a 4-*pro-S* hydrogen of NADPH.[8] The newly introduced (from NADPH) hydrogen assumes the *pro-R* configuration[9] at C-12 (or C-13) of the thus formed squalene **7a**. Evidence was provided that squalene biosynthesized from (5*R*)-[5-$^2H_1$]MVA has the configuration[10] as in **7b**. This indicates that formation of each new C–C bond proceeded with inversion of configuration[2,10] of the protons derived from C-5 of MVA.

It is apparent that from biosynthetic point of view the two halves of squalene are not equivalent.[2] While one half retained both hydrogens derived from C-5 of MVA at its central carbon (*e.g.*, C-13), the other retained only the 5-*pro-R* proton of MVA at its central carbon (*e.g.*, C-12). As mentioned earlier, the second hydrogen at this carbon (*e.g.*, C-12) originates from 4-*pro-S* of NADPH and assumed the pro *R* configuration (*e.g.*, 12-*pro-R*) in squalene.

The question was raised whether this asymmetricity of squalene is retained in the subsequent biosynthetic conversions of squalene.[11] We present evidence that in the biosynthesis of fusidic acid by the mold *Fusidium coccineum* the cyclization of squalene occurs to an equal extent from either end of the molecule.

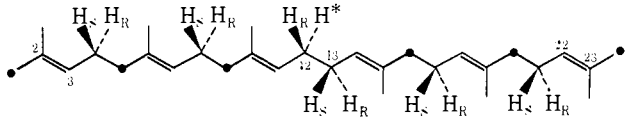
Experimental Section

Radioactivity. Specific activities and $^3H:^{14}C$ ratios were determined on samples which were first purified by thin-layer chromatography and then repeatedly crystallized from a suitable solvent to constant specific activity of ^{14}C and constant $^3H:^{14}C$ ratio. Radioactive samples were counted on either a Nuclear Chicago Mark I or Mark II liquid scintillation counter. The samples were dissolved in a scintillation solution (10 ml) prepared by adding 126 ml of Liquifluor (purchased from New England Nuclear, Boston, Mass.) to 3000 ml of scintillation grade toluene.

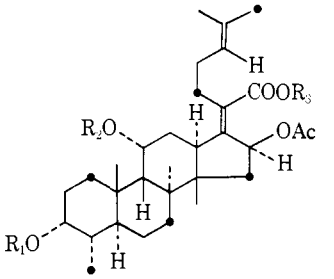
Chromatography. Gas chromatographic (glc) analyses were performed on a Hewlett-Packard Model 7620A gas chromatograph equipped with a flame ionization detector. A 6-ft silanized glass column of 1% SE-30 on gas-chrom Q was used for all analyses. Silica gel (Merck HF $-254+366$) was used for preparative and analytical thin-layer chromatography (tlc) in the indicated solvent systems. The products were detected under ultraviolet light and by color reactions with either phosphomolybdic acid (20% w/v ethanol solution) or sulfuric acid (10% v/v methanol solution).

Physical Measurements. Melting points were taken on a hot-stage and are corrected. Infrared spectra were recorded on solids incorporated into microdisks[12] on a Perkin-Elmer Model 237 spectrophotometer and peaks are reported in cm^{-1}. Ultraviolet measurements were made with a Perkin-Elmer 202 spectrophotometer. Mass spectra were obtained on a Du Pont 21-491 instrument. Proton magnetic resonance spectra (nmr) were recorded on a Varian DA60 spectrometer at 60 MHz with samples dissolved in $CDCl_3$. Peaks are reported in τ units downfield from tetramethylsilane.

Biosynthesis of [$^{14}C_6$,$^3H_4$]Fusidic Acid (8a) from a Mixture of (3*RS*,5*S*)-[5-^{3}H]Mevalonic Acid and (3*R*)-[2-^{14}C]Mevalonic Acid Po-



7a, • ≡ originating from C-2 of MVA;
H* = hydrogen derived from 4-pro-*S* of NADPH
b, $H_R \equiv {}^2H$



8a, R_1 = H; R_2 = H; R_3 = H
b, R_1 = H; R_2 = H; R_3 = CH_3
c, R_1 = THP; R_2 = THP; R_3 = H
d, R_1 = THP; R_2 = H; R_3 = H
e, R_1 = Ac; R_2 = H; R_3 = CH_3

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tassium Salts. Benzene solutions containing (3*RS*,5*S*)-[5-^{3}H]mevalonolactone and (3*RS*)-[2-^{14}C]mevalonolactone (1.96 μCi of ^{14}C; New England Nuclear) were mixed and stirred (15 min) at room temperature. A portion of the [2-^{14}C,5-^{3}H]mevalonolactone solution was utilized for preparation of mevalonic acid diphenylmethylamide for determination of isotopic content (see below). To the remaining solution of [2-^{14}C,5-^{3}H]mevalonolactone, ethanolic KOH (0.1 *N*) was added until a pH of 9 was obtained and this was maintained for 2 hr using an external indicator. The benzene was then removed under a stream of nitrogen.

A synthetic medium containing 50 g of D-sucrose, 5 g of glycerol, 0.5 g of KCl, 10 g of K_2HPO_4, 0.5 g of $MgSO_4$, 0.01 g of $FeSO_4$, 0.001 g of $ZnSO_4$, 1.5 g of NH_4NO_3, and 1 ml of a 0.1% solution of biotin per 1000 ml was prepared. An aliquot of the sterilized medium (120 ml) was inoculated with *F. coccineum* and the organism was grown for 24 hr at 28°. To that an aqueous solution of the potassium salt of mevalonic acid (sterilized by filtration) was added and the incubation continued with shaking for 168 hr. Then the mycelium was filtered and washed with water, and the filtrate was acidified to pH 3 and extracted with ether. The ether extract was washed, dried, concentrated to a small volume, and diluted with benzene. Removal of the residual ether gave a crystalline fusidic acid benzene solvate (26 mg). Evaporation of the filtrate gave a residue (29 mg) which was saved.

[2-^{14}C,5-^{3}H]Mevalonic Acid Diphenylmethylamide (1c). DL-Mevalonic acid lactone (90 mg, Sigma Chem. Co.) was added to a benzene solution (4 ml) containing a portion (0.08 μCi of ^{14}C) of the mixed radioactive mevalonic acid lactones. To the solution benzhydrylamine (300 mg) was added and the mixture was stirred at room temperature for 40 hr in an atmosphere protected from CO_2 by a drying tube filled with Ascarite (A. H. Thomas Co.). Ether (60 ml) was added and the solution washed with 1 *N* HCl and water (3 × 25 ml), dried (Na_2SO_4), and concentrated to a residue (105 mg). Fractionation of the residue by tlc (hexane–acetone, 7:3; developed three times) afforded 52 mg of homogeneous **1c** which was crystallized from ether as needles of constant specific activity (30.8 × 10^4 dpm of ^{14}C/mmol) and ^{3}H:^{14}C ratio (2.88).

Methyl [$^{14}C_6$,$^3H_4$]Fusidate (8b). Biosynthesized [$^{14}C_6$,$^3H_4$]fusidic acid (**8a**) (8.5 mg, 7.7 × 10^4 dpm of ^{14}C) was added to a stirred ethanolic solution (10 ml) of nonradioactive fusidic acid (510 mg). The ethanol was removed under reduced pressure and an aliquot (290 mg) of the diluted sample **8a** was treated with ethereal diazomethane. After the yellow color persisted (10 min), the excess diazomethane and the solvent were removed in a stream of N_2. A sample of the ester **8b** (20 mg) was purified by tlc (8:2 hexane–acetone, developed three times) and then repeatedly crystallized from an ether–hexane solution to constant specific activity (see table).

[$^{14}C_6$,$^3H_4$]Fusidic Acid 3α,11α-Bistetrahydropyranyl Ether (8c). A mixture of freshly distilled dihydropyran (200 mg), *p*-toluenesulfonic acid monohydrate (3.2 mg), and [$^{14}C_6$,$^3H_4$]fusidic acid **8a** (279 mg) in dry benzene (20 ml) was stirred for 4.5 hr at room temperature. The reaction was terminated by the addition of ice and aqueous $NaHCO_3$ (30 ml) and the mixture diluted with ether (150 ml). The organic layer was separated, washed with water and dried (Na_2SO_4), and the solvent was removed under reduced pressure. From the resulting viscous residue (398 mg), [$^{14}C_6$,$^3H_4$]fusidic acid 3α,11α-bistetrahydropyranyl ether (**8c**, 114 mg) was isolated by preparative tlc (hexane–acetone, 8:2, developed four times) as a homogeneous oil: 100 × 10^4 dpm ^{14}C/mmol and a ^{3}H:^{14}C ratio of 2.17 (atomic ratio, 4.10:6); ir 3140 (COOH), 2945 (C–H), 1740 and 1703 cm^{-1} (C=O); nmr ($CDCl_3$), τ 9.08 (m, 12 H, 18-, 19-, 30-, and 32-CH_3), 8.42 and 8.34 (singlets, each, 3 H, 26- and 27-CH_3), 8.04 (s, 3 H, 16β-OAc), 6.44 (m, 1 H, 3β-H), 6.20 and 5.67 (multiplets, THP protons), 5.32 (m, 1 H, 11β-H), 4.93 (m, 1 H, 24-H), 4.10 (d, 1 H, J = 7 Hz, 16α-H), 2.12 (m, 1 H, COOH).

A small amount of the 3-monotetrahydropyran ether **8d** was also isolated.

Methyl [$^{14}C_6$,$^3H_4$]-16-Deacetoxy-16-ketofusidate (9c). The [$^{14}C_6$,$^3H_4$]bistetrahydropyran acid **8c** (110 mg) in dry tetrahydrofuran (3 ml) was added rapidly to a stirred slurry of $LiAlH_4$ (85 mg) in 38 ml of tetrahydrofuran (reagent grade, distilled from $LiAlH_4$). The stirring was continued for 2 min and the reaction was terminated with ice–water (6 ml). More water (25 ml) was added and the reaction mixture was transferred to a separatory funnel and mixed with ether (90 ml) and 3–5 drops of a 2% aqueous solution of methyl orange was added. Hydrochloric acid (1 *N*) was added dropwise (*ca.* 4 ml) to the end point of methyl orange, and the aqueous phase was removed. The ether solution was washed with water (4 × 30 ml), treated with an excess of ethereal diazomethane, and the volatile components were removed in a stream of nitrogen to leave a residue (98 mg). The residue was fractionated by tlc (hexane–acetone, 9:1, developed three times) and was shown to consist mainly of **9a**. The product **9a** undergoes facile lactonization even on standing at room temperature.

To a solution of **9a** (96 mg) in pyridine (0.5 ml), 2 ml of Cornforth's reagent[13] (5.0 g of chromium trioxide in 5.0 ml of H_2O and 59 ml of pyridine) was added and the mixture was stirred at ambient temperature for 3.5 hr. Ethyl acetate (25 ml) was then added; the obtained solid was removed by filtration on Celite, then washed with ethyl acetate. The solution and the washings were combined and concentrated *in vacuo* to a dark residue (94 mg). The gum was purified by tlc (hexane–acetone, 9.2:1; developed three times) to yield **9b** as an oil (74 mg): ir (film) 2950 (C–H), 1750 and 1730 (C=O), 1642 (C=C), 1080, 1040, and 1005 cm^{-1} (THP ether bands); uv (ethanol) 255 mμ. For counting, a sample of **9b** was repeatedly chromatographed and showed a specific activity of 98.4 × 10^4 dpm of ^{14}C/mmol and a ^{3}H:^{14}C ratio of 2.25 (atomic ratio, 4.25:6).

A solution of **9b** (65 mg), acetone (4.5 ml) and concentrated HCl (0.06 ml) was refluxed for 50 min and then poured into ice and saturated aqueous $NaHCO_3$ (20 ml). The reaction products were recovered with ether and after a conventional work-up a crystalline solid (55 mg) was obtained. The solid was fractionated by preparative tlc (hexane–acetone, 1:3) and from the slowest migrating zone methyl [$^{14}C_6$,$^3H_4$]-16-deacetoxy-16-ketofusidate **9c** (24.2 mg) was obtained. The product **9c** was crystallized from ether–hexane to give colorless needles: mp 116–118°; uv (ethanol) 253 mμ (ϵ 7400), ir (KBr) 3465 (OH), 2920 (C–H), 1728 (C=O), 1640 (C=C), 1275, 1215, and 1150 cm^{-1}; nmr ($CDCl_3$) τ 9.05 (m, 9 H, 19-, 30-, and 32-CH_3), 8.54 (s, 3 H, 18-Me), 8.39 and 8.34 (singlets, each, 3 H, 26- and 27-CH_3), 7.72 (3- and 11-OH), 6.51 (broad doublet J = 11 Hz, 22-H), 6.27 (m, 1 H, 3β-H), 6.22 (s, 3 H, $COOCH_3$), 5.64 (m, 1 H, 11β-H), 4.90 (t, 1 H, 24-H); *m/e* 486 (2%, M$^-$), 468 (11. M$^+$ − H_2O), 436 (12, 468 − H_2O), 399 (10, 468 − $(CH_3)_2C{=}CHCH_2\cdot$), 368 (5), 367 (4, 436 − $(CH_3)_2C{=}CHCH_2$), 157 (33), 149 (base peak) (see table for radioactivity determination).

Methyl [$^{14}C_6$,$^3H_4$]Fusidate 3-Acetate (8e). A mixture of methyl [$^{14}C_6$,$^3H_4$] fusidate (**8b**, 360 mg), acetic anhydride (3.5 ml), and pyridine (5 ml) was stored at room temperature for 4.6 hr. The volatile components were then removed under reduced pressure (0.1 mm) and the residue was dissolved in ether (150 ml). The ether solution was washed successively with 1 *N* HCl, saturated aqueous $NaHCO_3$, and water, dried (Na_2SO_4), and concentrated. The obtained residue (403 mg) was resolved by tlc (hexane–acetone, 7:3, developed three times), and from the faster moving zone, 300 mg of homogeneous [$^{14}C_6$,$^3H_4$]acetate **8e** was obtained. A sample was repeatedly crystallized (hexane–ether) and showed mp 119–121°; ir (KBr) 3490 (OH), 2940 (C–H), 1728 and 1698 (C=O), and 1245 cm^{-1} (C–O); nmr ($CDCl_3$) τ 9.08 (m, 9 H, 18-, 19-, and 30-CH_3), 8.62 (s, 3 H, 32-CH_3), 8.39 and 8.32 (singlets, each, 3 H, 26- and 27-CH_3), 7.92 and 8.03 (singlets, each, 3 H, 3- and 16-OAc), 6.35 (s, 3 H, $COOCH_3$), 5.70 (m, 1 H, 11β-H), 5.08 (m, 1 H, 3β-H), 4.95 (m, 1 H, 24-H), 4.15 (d, 1 H, J = 7.5 Hz, 16α-H); *m/e* 512 (15, M$^+$ − HOAc), 503 (20, M$^+$ − $(CH_3)_2C{=}CHCH_2\cdot$), 494 (29, M$^+$ − (HOAc + H_2O)), 443 (16, 503 − HOAc), 434 (18, 494 − HOAc), 425 (20, 494 − $(CH_3)_2C{=}CHCH_2$), 414 (7, 434 − CH_3), 365 (53, 425 − HOAc), 207 (91), 177 (base peak) (see table for radioactivity determination).

Methyl 3-Acetoxy-11-ketofusidate (10). To a stirred and cooled in ice solution of **8e** (45 mg) in acetone (8 ml), Jones reagent (0.04 ml) was added. After 10 min the reaction was terminated with cold saturated aqueous $NaHCO_3$ and the product was recovered with ether. The ether solution was washed with water and dried and the solvent removed. The resulting solid (52 mg) was purified by tlc (hexane–acetone, 85:15; developed three times) to yield homogeneous **10**: nmr ($CDCl_3$) τ 8.98 and 8.83 (s, 18-, 19-, 30-, 32-CH_3), 8.39 and 8.33 (singlets, each, 3 H, 26- and 27-CH_3), 8.00 and 7.94 (singlets, each, 3 H, 16β- and 3α-OAc), 7.38 (broad singlet, 1 H, 9β-H), 7.21 (broad singlet, 2 H, 12α- and 12β-H), 4.12 (d, 1 H, J = 7 Hz, 16α-H).

8e from Ketone 10. A mixture of **10** (42 mg), methanol (4 ml), and aqueous $NaBH_4$ (0.2 ml of a 20% w/v solution) was stirred at ice bath temperature for 15 min. Glacial acetic acid (0.08 ml) was added and the product was recovered with ether. After a conventional work-up, **8e** (38 mg) was obtained. The solid was purified

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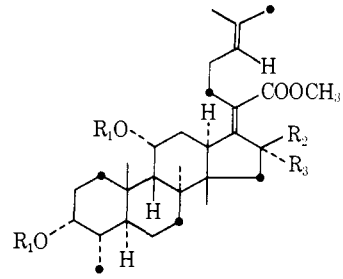
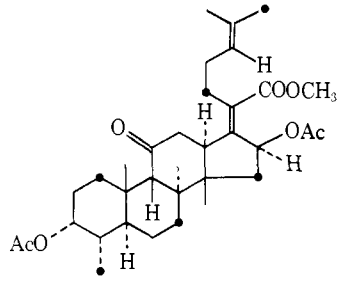


9a, R_1 = THP; R_2 = OH; R_3 = H
b, R_1 = THP; R_2 = R_3 = O
c, R_1 = H; R_2 = R_3 = O



10

by tlc (hexane–acetone, 7:3; developed three times) and then crystallized several times (ether–hexane) to constant specific activity of ^{14}C and constant ^{3}H:^{14}C ratio (see table for radioactivity determination).

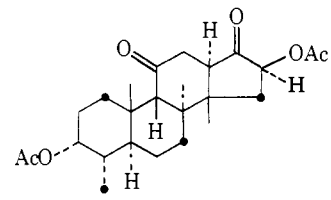
The compound was identical with **8e** obtained by 3-monoacetylation of methyl fusidate **8b**.

[$^{14}C_4$,$^{3}H_2$]-3α-Acetoxy-4α,8,14-trimethyl-18-nor-5α,8α,9β,13β,14β-androstane-11,17-dione (12). Ruthenium tetroxide was generated[14] by treating a stirred suspension of ruthenium dioxide (96 mg) in acetone (12 ml) with aqueous sodium periodate (720 mg in 3.6 ml of H_2O). To the stirred yellow solution of the reagent a solution of **8e** (240 mg) in acetone (12 ml) was added dropwise. The yellow color of the reaction was maintained by adding a solution of sodium periodate (1.0 g) in acetone–water (1:1), as needed. After 5 hr isopropyl alcohol (1 ml) was added to destroy excess reagent and the mixture was poured on a column of Celite (2 g). The column was percolated with $CHCl_3$–ethyl acetate (1:1; 300 ml) and the combined eluate was concentrated to yield a dark oil (280 mg). The residue was fractionated by preparative tlc (hexane–acetone, 8:2; developed twice) to afford a residue (102 mg) from which a small amount of **11** crystallized; mp 144–146°; *m/e* 372 (M^+ − HOAc).

The residue (100 mg) recovered from tlc was stirred in refluxing glacial acetic acid (15 ml) with zinc dust (604 mg) for 2 hr. The reaction mixture was filtered and the collected solid washed with $CHCl_3$ (20 ml). Concentration of the combined filtrate and wash solutions *in vacuo* (0.1 mm) afforded a noncrystalline residue (117 mg) from which **12** was isolated by preparative tlc (hexane–acetone, 8:2; developed three times). Crystallization from hexane–chloroform gave **12** (35 mg) as homogeneous needles: mp 167–170°; ir (KBr) 2940 (C–H), 1730 and 1718 sh (C=O), 1245 cm^{-1} (OAc); nmr ($CDCl_3$) τ 9.18 (d, 3 H, J = 6 Hz, 4α-CH_3), 9.17, 8.89, and 8.47 (singlets, each, 3 H, 14β-, 19-, and 8α-CH_3), 7.88 (s, 3 H, 3-OAc), 5.02 (m, 1 H, 3β-H); *m/e* 374 (3, M^+), 314 (base peak, M^+ − HOAc), 299 (40, 314 − CH_3), 296 (9, 314 − H_2O), 285 (6) 259 (15), 179 (68), 97 (90).

The isolated **12** [specific activity of ^{14}C of 67.0 × 10^4 dpm/mmol and a ^{3}H:^{14}C ratio of 1.76 (atomic ratio, 2.21:4)] was then equilibrated. A mixture of **12** (8 mg), glacial acetic acid (1.0 ml), and zinc dust (55 mg) was refluxed with stirring for 5.0 hr. The reaction mixture was filtered through cotton and the solids were washed with $CHCl_3$ (5 ml). The combined wash and filtrate was concentrated and the steroidal residue isolated by preparative tlc as described above. The obtained homogeneous **12** was repeatedly crystallized from a $CHCl_3$–hexane mixture and counted (see table for radioactivity determination).

(14) D. M. Piatak, G. Herbst, J. Wicha, and E. Caspi, *J. Org. Chem.*, 34, 116 (1969); D. M. Piatak, H. B. Bhat, and E. Caspi, *ibid.*, 34, 112 (1969).



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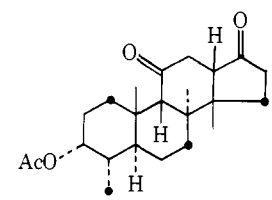

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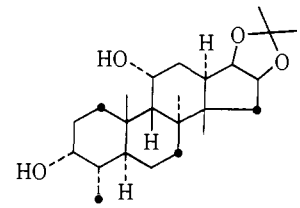


13

[$^{14}C_4$,$^{3}H_{2.5}$]Acetonide 13. The ruthenium tetroxide oxidation of **8e** (280 mg) was repeated as described above.

A solution of the resulting oxidation residue (284 mg) in ether (3 ml) was added to a suspension of $LiAlH_4$ (201 mg) in ether (30 ml). The reaction mixture was stirred for 18 hr at room temperature, then water (0.8 ml) was added. The solid was removed by filtration through Celite, washed with tetrahydrofuran (100 ml), and discarded. The combined filtrate and wash was concentrated to a noncrystalline residue (198 mg) from which the tetrol was isolated by tlc (ethyl acetate–hexane, 8:2) and crystallized from tetrahydrofuran–hexane: mp 227–228°; *m/e* 352 (M^+), 334 (M^+ − H_2O), 319 (334 − CH_3), 316 (334 − H_2O).

The tetrol (109 mg) was dissolved in 21 ml of acetone containing 0.12 ml of perchloric acid (62%). After 10 min at room temperature the solution was diluted with water and extracted with two 30-ml portions of methylene chloride. The methylene chloride extract was washed with saturated aqueous $NaHCO_3$ and saturated NaCl solution and dried over Na_2SO_4. Removal of the solvent afforded a residue (171 mg) from which 53 mg of acetonide **13** was isolated by preparative tlc (hexane–acetone, 7:3, developed twice). Crystallization from tetrahydrofuran–hexane afforded homogeneous **13** as prisms: mp 227–228°; ir (KBr) 3530 and 3425 (OH), 2925 (C–H), 1370, 1196, 1063, 965 cm^{-1}; *m/e* 392 (0.5, M^+), 377 (100, M^+ − CH_3), 374 (1, M^+ − H_2O), 359 (2, 377 − H_2O), 299 (16, 377 − $C_3H_6O_2$) (see table for radioactivity determination).

Results and Discussion

In many organisms biosynthesizing triterpenes (*e.g.*, rat, yeast, plants, etc.) the *in vivo* pool of squalene seems to be relatively small. Apparently the produced squalene is fairly rapidly cyclized and then metabolized to the different products in various species. The cyclization may be initiated by a proton attack[15] or by an oxidative attack[16,17] on a terminal double bond of the squalene. The nonoxidative, proton initiated mechanism was shown to operate in the biosynthesis of tetrahymanol[15] **14**. The overall process of tetrahymanol **14** formation is considered to be equivalent to the acquisition of the elements of water by squalene.[15]

The oxidative mechanism of cyclization begins with the epoxidation[16,17] of a terminal double bond of squalene to yield the stable intermediate 2,3-(*S*)-oxidosqualene (**15**). In many species the enzymatic opening of the oxide and the ensuing cyclization is thought to lead to the free[18] or transiently stabilized[19] cation **16**. The cation may then rearrange as indicated in **16** to yield

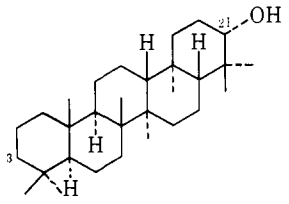
(15) J. M. Zander, J. B. Greig, and E. Caspi, *J. Biol. Chem.*, **245**, 1247 (1970).

(16) E. E. Van Tamelen, J. D. Willett, R. B. Clayton, and K. E. Lord, *J. Amer. Chem. Soc.*, **88**, 4752 (1966).

(17) E. J. Corey, W. E. Russey, and P. R. Ortiz de Montellano, *J. Amer. Chem. Soc.*, **88**, 4750 (1966).

(18) A. Eschenmoser, L. Ruzicka, O. Jeger, and D. Arigoni, *Helv. Chim. Acta*, **38**, 1890 (1955).

(19) J. W. Cornforth, *Angew. Chem., Int. Ed. Engl.*, 7, 903 (1968).



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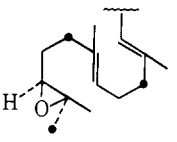

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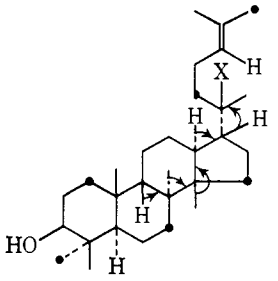
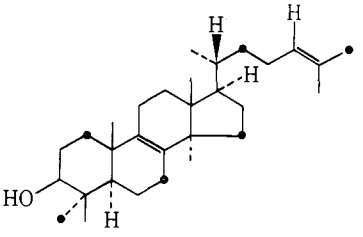


16, X = + charge or prosthetic group

lanosterol **17** after the loss of the 9β hydrogen (*e.g.*, rat, yeast, etc.). Alternatively, stabilization of **16** without rearrangement *via* the loss of the 17β hydrogen apparently occurs in *F. coccineum* and results in the protosterol[20] **18**.



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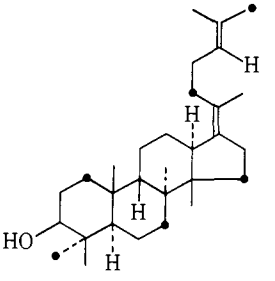

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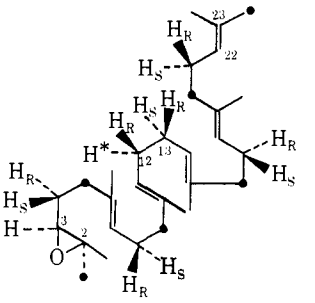
Epoxidation of squalene takes place on the oxidosqualene enzyme system.[21] Hence, for the oxidative cyclization to be initiated, the squalene should be first transferred from the synthethase to the epoxidase. A direct transfer of the squalene from the synthethase to the epoxidase or a transfer by other means not involving randomization should proceed with the retention of the geometrical orientation and thus align the molecule in a particular manner with respect to the active site of the epoxidase. This will then result in the exposure of *one specific* terminal double bond of the squalene, whether the 2(3) of **19** or 22(23) of **23**, to epoxidation. On the other hand, should the squalene be released from the synthethase into a free pool of squalene, the specific geometrical orientation of the molecule will be lost. The transfer of the squalene from the pool and the ensuing adsorption on the epoxidase will be at random. Consequently, *either one* of the terminal double bonds will become available for epoxidation[11] and equal amounts of **19** and **23** will be formed.

Inspection of the epoxides **19** and **23** indicates that the selectivity of the epoxidation or lack of it could be established by defining the location (with respect to the oxygen function) of the hydrogen originating

(20) G. Visconti, Ph.D. Thesis, Eidg. Technische Hochschule, Zurich, 1968.

(21) H. H. Tai and K. Bloch, *J. Biol. Chem.*, **247**, 3767 (1972); S. Yamamoto and K. Bloch in "Natural Substances Formed Biologically from Mevalonic Acid," T. W. Goodwin, Ed., Academic Press, New York, N. Y., 1970, p 35.



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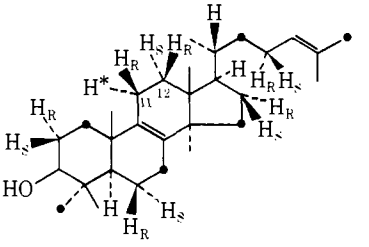

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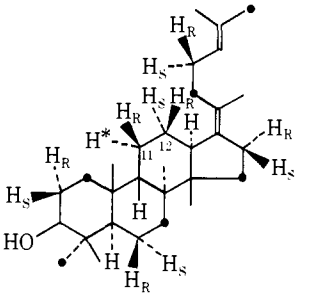

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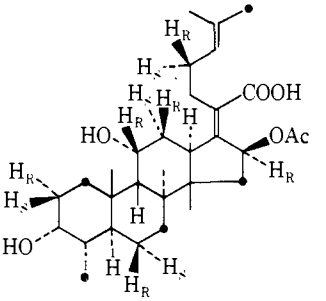

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from the 1-*pro-S* position of farnesyl pyrophosphate or of the hydrogen [H*] derived from the 4-*pro-S* position of NADPH. A minimal and obligatory requirement for this type of experiment is that the biosynthetic sequences from farnesyl pyrophosphate *via* squalene to squalene epoxide be uninterrupted and *identical* with those in nature. Interference with the continuity of the enzymatic reactions could cause an unwanted release of the squalene from the synthethase and lead to erroneous conclusions. For this reason *in vivo* studies in which the enzymatic sequences occur in the normal ambiance of the cells should, in our view, be carried out prior to *in vitro* investigations. An *in vitro* experiment by necessity employs a disrupted enzyme system and therefore *may not necessarily* reflect the actual situation prevailing in the normally functioning cells.

There are several routes for the stereospecific labeling of the squalene at a central carbon atom with isotopic hydrogen. The squalene could be biosynthesized *in vitro* from farnesyl pyrophosphate in the presence of NADPH labeled with tritium or deuterium at the 4 position. Alternatively, the squalene could be derived biosynthetically from farnesyl pyrophosphate labeled stereospecifically with isotopic hydrogens at the 1-*pro-S* position. Incubation of (5*S*)-[5-^{3}H or 5-^{2}H]MVA with yeast or rat liver enzymes should result in (1*S*,5*S*,-9*S*)-[$^3H_3$ or $^2H_3$]farnesyl pyrophosphate.[22] Consequently, MVA stereospecifically labeled at the 5-*pro-S* position with tritium could be used and the biosynthesis allowed to proceed to squalene oxide. In practice,

(22) C. Donninger and G. Popják, *Proc. Roy. Soc., Ser. B*, **163**, 465 (1966).

rather than to focus on the small amounts of the difficult to isolate squalene oxide, it is preferable to utilize *an appropriate* more accessible squalene cyclization product which accumulates in the system.

Two groups[23,24] have investigated the oxidative cyclization of asymmetrically labeled squalene by incubating farnesyl pyrophosphate and NADP^{3}H *in vitro* with tissue homogenates. Samuelson and Goodman[23] used cholesterol biosynthesized in two stages as the model in their investigation. In the first stage the farnesyl pyrophosphate and (4*R*,*S*)-[4-$^3H_2$]NADPH were incubated anaerobically with a rat liver squalene synthesizing system. Subsequently, a soluble supernatant fraction of the rat liver homogenate was added and the incubation continued anaerobically for 5 min until the tritium of the (4*R*,*S*)-[4-$^3H_2$]NADPH was exchanged with the water of the medium. Then oxygen was admitted and the accumulated, asymmetrically labeled squalene was biosynthetically converted to cholesterol. The derived cholesterol was injected intraperitoneally into a rat with a bile fistula. From the collected bile, 3α,7α,12α-cholanic acid was isolated and the bile acid was shown to contain about equal amounts of tritium at the 11 and 12β positions. The results were then interpreted as indicative of the cyclization of squalene from either end of the molecule.[23]

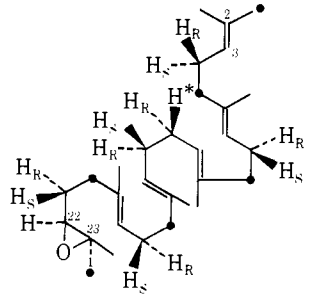
It is obvious that in the experiment described above the biosynthetic sequences were blocked on the level of formation of squalene,[23] which was forced to accumulate in the system. The blocking of the biosynthetic pathway and the forcible accumulation of the squalene could have caused an *abnormal release* of the squalene from the synthethase into a free squalene pool, thus the conclusions of Samuelson and Goodman[23] may not represent the *in vivo* situation.[11,24]

In the second investigation Etemadi, *et al.*,[24] have incubated farnesyl pyrophosphate and (4*S*)-[4^{3}H]-NADPH with a pig liver homogenate and isolated lanosterol (^{3}H:^{14}C ratio, 7.76). The lanosterol was acetylated, then hydrogenated and oxidized to yield lanost-8(9)-ene-7,11-dion-3β-ol acetate (^{3}H:^{14}C, 4.04). Equilibration with base provided 3β-hydroxylanost-8(9)-ene-7,11-dione (^{3}H:^{14}C ratio, 1.07). They found[24] that the ratio of tritium at C-11 to that at C-12 was 1.28 indicating a certain inequality of labeling. Moreover the presence of a significant amount of residual tritium in the equilibrated 8(9)-ene-7,11-dione, which could not be accounted for, also complicated matters. These observations rendered the results inconclusive.

When we approached the problem, for reasons given earlier, an *a priori* decision was made to use an *in vivo* system. In a search for an appropriate model, we concentrated on fusidic acid which is efficiently biosynthesized from MVA by the mold *F. coccineum*.[25,26] The use of fusidic acid presented certain additional distinct advantages. First it is apparently a metabolite of the protosterol (**18**) which most probably is formed by the stabilization of the cation **16** without a backbone rearrangement.[25] Secondly, it has an 11α-hydroxy group which provides an easy entry for the evaluation of the labeling pattern at C-11 and 12.

The rationale of our approach was that *in vivo* incubation of (3*RS*,5*S*)-[2-^{14}C,5-^{3}H]MVA with *F. coccineum* will yield (1*S*,5*S*,9*S*)(4,8,12)[$^{14}C_3$,$^3H_3$]farnesyl pyrophosphate which will be converted to [$^{14}C_6$,$^3H_5$]squalene asymmetrically labeled at a central carbon. The *uninterrupted* biosynthetic processes will then continue *via* 2,3-(*S*)-oxidosqualene to the protosterol **18** and then to fusidic acid **8a**.

Three distinct options were available for the course of the biosynthesis of the "*S*"-fusidic acid ($H_S \equiv {}^3H$). If epoxidation of the intermediate asymmetrically labeled squalene will occur at the C-2(3) double bond as shown in **19**, the labeling pattern of the fusidic acid will be as in **22** and a tritium atom will be present at the 12α position. Alternatively, epoxidation of squalene at the 22(23) double bond of **23** will result in fusidic acid labeled as in **26** and having a tritium atom at the



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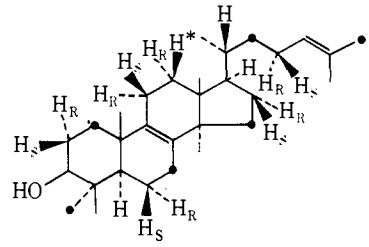

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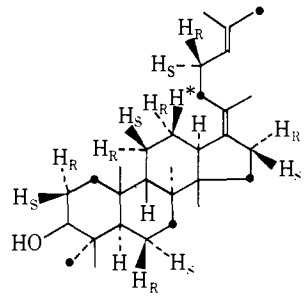

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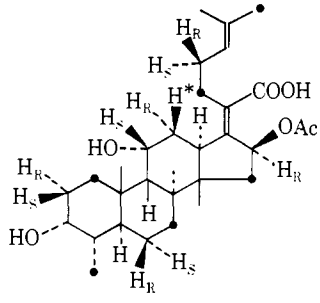

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11β position. Finally, should epoxidation occur equally at the C-2(3) and C-22(23) double bonds of the squalene molecule, equal amounts of **19** and **23** and hence of **22** and **26** will be formed. Consequently, the resulting fusidic acid will have 0.5 atom of tritium at the 11β and 12α positions. These three possibilities could be differentiated through a selective attack at C-11 and 12 of the biosynthesized "*S*"-fusidic acid.

Through the kind cooperation of Professor J. W. Cornforth we obtained a sample of (3*RS*,5*S*)-[5-^{3}H]MVA which was mixed with (3*RS*)-[2-^{14}C]MVA (1.96 μCi of ^{14}C). The double labeled specimen (^{3}H:^{14}C ratio, 2.88) was added to an actively growing culture of *F.*

(23) B. Samuelson and D. S. Goodman, *J. Biol. Chem.*, **239**, 98 (1964).
(24) A. H. Etemadi, G. Popják, and J. W. Cornforth, *Biochem. J.*, 111, 445 (1969).
(25) L. J. Mulheirn and E. Caspi, *J. Biol. Chem.*, **246**, 2494 (1971).
(26) W. O. Godtfredsen, W. v. Daehne, S. Vangedal, A. Marquet, D. Arigoni, and A. Melera, *Tetrahedron*, **21**, 3505 (1965).

Table I. ^{14}C Specific Activity and $^{3}H:^{14}C$ Ratio of Products Derived from Fusidic Acid Biosynthesized from (3*RS*,5*S*)-[2-^{14}C,5-^{3}H]Mevalonic Acid

Compound	^{14}C specific activity[a] Crystallization 1	Crystallization 2	Crystallization 3	$^{3}H:^{14}C$ ratio isotopic Crystallization 1	Crystallization 2	Crystallization 3	Results Sp act.	Results $^{3}H:^{14}C$ ratio Isotopic	Results $^{3}H:^{14}C$ ratio Atomic
Methyl fusidate (**8b**)	101	100	101	2.14	2.10	2.12	101	2.12	4.00:6
Methyl deacetoxy-16-ketofusidate (**9c**)	94.5	97.8	98.5	2.19	2.08	2.14	98.2	2.14	4.03:6
Methyl fusidate 3α-acetate (**8e**)	99.6	98.9	99.6	2.09	2.09	2.08	99.4	2.09	3.95:6
Methyl fusidate 3α-acetate (after oxidation and reduction with $NaBH_4$) (**8e**)	96.8	95.0	96.9	1.81	1.81	1.82	96.2	1.81	3.42:6
11,17-Dione-3α-acetate (after Zn–acetic acid equilibration) (**12**)	66.2	64.5		1.59	1.61		65.3	1.60	2.02:4
Acetonide (**13**)	58.2	58.9	58.9	1.96	1.97	1.96	58.7	1.96	2.47:4

[a] ^{14}C specific activity $\times 10^4$ dpm/mmol.

coccineum. At the termination of the incubation the [$^{14}C_6$,$^{3}H_4$]fusidic acid was recovered and crystallized (25.7 mg (benzene solvate); 0.1 μCi of ^{14}C total). Based on the administered [2-^{14}C]MVA the incorporation of MVA into fusidic acid was about 5.4%.

An aliquot of the [$^{14}C_6$,$^{3}H_4$]fusidic acid (8.5 mg; *ca.* 7.7×10^4 dpm of ^{14}C) was diluted with 510 mg of nonradioactive material. A portion of the diluted specimen was treated with diazomethane and the resulting ester **8b** showed a specific activity of 101×10^4 dpm/mmol of ^{14}C and a $^{3}H:^{14}C$ ratio of 2.12.

Our first objective was to determine if tritium is present at C-16 of the biosynthetic "*S*"-fusidic acid. The parent protosterol (**21** or **25**) should have a 16β-tritium. If the enzymatic introduction of the 16-oxygen function proceeded in the usual manner, with retention of configuration, this tritium atom should be absent in fusidic acid. For this purpose the fusidic acid was treated with dihydropyran and *p*-toluenesulfonic acid to yield a noncrystalline 3,11-ditetrahydropyran ether **8c**. A tetrahydrofuran solution of the diether **8c** was treated for 2 min with $LiAlH_4$, then the reaction was terminated with water and diluted with ether. Immediately after workup excess diazomethane was added and the 16β-hydroxy-3,11-dihydropyran methyl ester (**9a**) was isolated. The obtained alcohol **9a** was treated with Cornforth's reagent,[13] and after the removal of the THP groups from **9b** by acid hydrolysis, the 3α,11α-dihydroxyl-16-keto methyl ester **9c** was obtained. A comparison of the $^{3}H:^{14}C$ ratios of methyl fusidate (**8b**) and of the 16-keto ester (**9c**) (see Table I) reveals no loss of tritium. This indicates that the "*S*"-fusidic acid does not contain tritium at C-16.

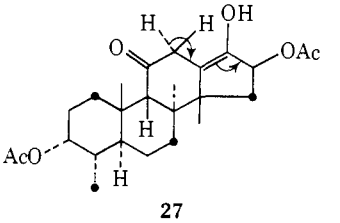
For the definition of the tritium content at C-11, methyl fusidate (**8b**) was selectively acetylated at C-3 and the resulting **8e** was oxidized with Jones' reagent. To minimize the possibility of loss of tritium from C-12 through enolization, the crude 3-acetoxy keto ester **10** was treated with sodium borohydride (15 min at 0°) to yield back **8e**. The transformation of the 11α-hydroxyl *via* the 11-ketone back to the 11α-hydroxyl was accompanied by the loss of *0.5 atom of tritium* which must have had the *11β stereochemistry* (see Table I). It is also apparent that the enzymatic 11α-hydroxylation proceeded with retention of configuration as expected.

The determination of the location of the 0.5 atom of tritium at the 12α position was carried out on the tetracyclic moiety obtained by oxidative cleavage of the C-17 side chain with ruthenium tetroxide.[14] The removal of the side chain of the methyl [$^{14}C_6$,$^{3}H_4$]fusidate 3-acetate by ruthenium tetroxide entails the loss of two atoms of ^{14}C (C-22 and -26) and 1.5 atoms of tritium (one atom from C-23 and 0.5 atom from the 11β position). The residual tetracyclic moiety should therefore retain 2.5 atoms of tritium and four atoms of ^{14}C. Because of the importance of these values in the interpretation of the results we decided to determine the $^{3}H:^{14}C$ content of the tetracyclic residue.

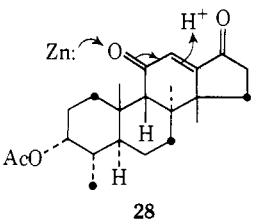
Oxidation of methyl fusidate 3-acetate with ruthenium tetroxide resulted in a mixture of products. Chromatographic fractionation of the residue led to the isolation of a small amount of the diacetoxy diketone **11**. The product proved difficult to purify and apparently decomposed on chromatography. Consequently, another ruthenium tetroxide oxidation experiment was carried out and the crude reaction residue was treated with lithium aluminum hydride in ether to yield after preparative thin-layer chromatography the 3α,11α,16β,17β-tetrol as the main product; mp 243–245°; *m/e* 352 (M^+). Exposure of the tetrol to acetone and perchloric acid gave the 3α,11α-dihydroxy-16β,17β-acetonide (**13**); mp 227–228°; *m/e* 392 (M^+). The readily crystallizing acetonide was counted and showed a $^{3}H:^{14}C$ ratio of 1.96 corresponding to an atomic ratio of 2.47 atoms of tritium and four atoms of ^{14}C as expected (see Table I).

We now proceeded with the determination of the C-12 location of the other half of a tritium atom. The approach chosen was to prepare and equilibrate the 3-acetoxy 11,17-diketone **12** by treatment of the 3,16-diacetoxy 11,17-diketone **11** with Zn in boiling acetic acid.[25,26]

The mechanism of this reaction is thought to proceed[26] as indicated in **27** and **28**. The 16β-deacetoxyla-



27



28

tion and formation of the 12(13)-ene-11,17-dione (**28**) occurs first. Subsequently the ene-dione **28** is reduced

to yield **12**. At this point it is worthy to recall that the biosynthesized **8a** contained 0.5 atom of tritium at the 11β position and that it was devoid of tritium at C-16. Furthermore, previously we have proven that the C-9β and C-13α hydrogens of **8a** originate from the 4-*pro-R* hydrogen of MVA.[25] Consequently, should **12** after equilibration contain less tritium than **13**, the removed isotopic hydrogen must have been located at C-12.

With this in mind the residue of the oxidation of 3-acetoxymethyl fusidate with ruthenium tetroxide was fractionated by preparative tlc and the fraction corresponding to 3,16-diacetoxy-11,17-dione (**11**) was treated with Zn in boiling acetic acid for 1 hr. After purification of the residue by chromatography, **12** (mp 167–170°; *m/e* 374 (M^+)) was obtained. ($^3H:^{14}C$, 1.76, atomic ratio 2.21:4). Complete equilibration of **12** with Zn in boiling acetic acid (5 hr) gave after work-up unchanged **12** which showed a $^3H:^{14}C$ ratio of 1.60 corresponding to an atomic ratio of 2.02 atoms of tritium and four atoms of ^{14}C (see Table I).

Our results show that 0.5 atom of tritium is located at each of the 11β and 12 positions. From the known mode of the biosynthesis of squalene[2] the tritium at C-12 must have the α configuration. Since we proved that equal amounts of tritium are present at C-11β and -12α positions, it follows that in the *in vivo* biosynthesis of fusidic acid by *F. coccineum*, the epoxidation and the ensuing cyclization occur to an equal degree from either end of the squalene molecule. These observations are consistent with the hypothesis of the release of squalene from the synthethase into a free squalene pool prior to its transfer to the epoxidase. The unlikely possibilities of scrambling of the squalene on the synthethase or of its being held by the synthethase in such a manner that both terminal double bonds are equally available for epoxidation are not excluded by our results.

Extrapolation of results from various species and different biosynthetic systems (*in vivo* and *in vitro*) is at best very tenuous. However, to the extent that such extrapolations are permissible, certain tentative conclusions can be drawn from our observations. As a *first approximation* it can be inferred that the results on the stepwise biosynthesis of cholesterol[23] from farnesyl pyrophosphate and (4*R*,*S*)-[4-$^3H_2$]NADPH by a rat liver enzyme preparation, in which equal amounts of tritium were found at C-11 and -12, may reflect the situation in the intact cell. Similarly the observations on the incorporation of [1-$^3H_2$]farnesyl pyrophosphate into eburicoic acid by *Polyporus sulphureus*[27] are also consistent with the cyclization of squalene from either end of the molecule. It would seem therefore that the geometrical asymmetry of squalene acquired on the synthethase is not retained in the course of the epoxidation.

In general terms the observations of Etemadi, *et al.*,[24] also fit this hypothesis. However, their observation on a certain inequality of labeling with tritium (derived from (4*S*)-[4-^{3}H]NADPH) at C-11 and -12 of lanosterol and the presence of unaccounted for tritium in other parts of the lanosterol is rather difficult to rationalize.

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