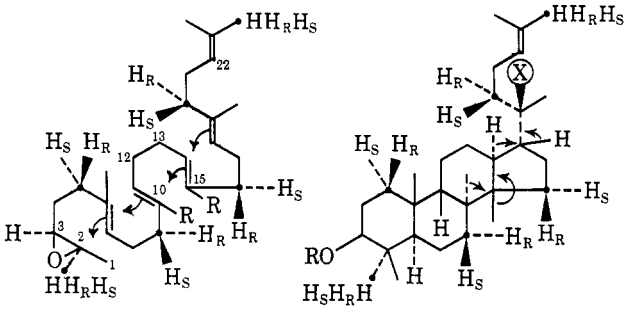
Mechanism of Oxidative Cyclization of Squalene. Concerning the Mode of Formation of the 17(20) Double Bond in the Biosynthesis of Fusidic Acid by *Fusidium coccineum*[1,2]

Richard C. Ebersole,[3a,b] W. O. Godtfredsen,[3c] S. Vangedal,[3c] and Eliahu Caspi*[3a,d]

Contribution from the Worcester Foundation for Experimental Biology, Incorporated, Shrewsbury, Massachusetts 01545, and Leo Pharmaceutical Products, DK. 2750 Ballerup, Denmark. *Received May 8, 1974*

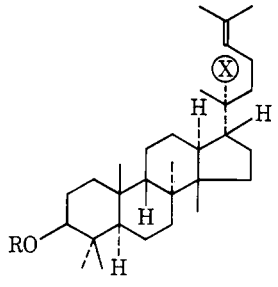
Abstract: Specimens of (*R*)- and (*S*)-fusidic acid were biosynthesized by incubating (3*RS*,2*R*)-[2-^{14}C,2-^{3}H]-mevalonic acid (MVA) and (3*RS*,2*S*)-[2-^{14}C,2-^{3}H]-MVA with *Fusidium coccineum*, respectively. It was shown that each specimen had six carbon atoms labeled with ^{14}C and ^{3}H. Evidence was presented that the (*R*)- and (*S*)-methyl fusidates have a tritium atom in the 22-pro-*R* and 22-pro-*S* positions, respectively. This essentially precludes the possibility that the biosynthesis of fusidic acid by *F. coccineum* proceeds through a stable intermediate with a 20(22) double bond.

The enzymatic cyclization of squalene to triterpenes can be initiated by a proton[4] or oxidative[5-7] attack on a terminal double bond of squalene. It is now recognized that the oxidative cyclization of squalene is not a concerted process, as originally considered,[5] but involves at least one stable intermediate, (3*S*)-2,3-oxidosqualene[6,7] (**1a**). The cyclization is thought to occur on the squalene cyclase enzyme and apparently is initiated by the opening of the oxirane ring of **1a**. This is presumed to generate an electron deficiency at C-2 and thus precipitate the "nonstop" cyclization indicated in **1a**. In many species (*e.g.*, rat, yeast, *Fusidium coccineum*, *Digitalis lanata*, etc.) it is considered likely that the cyclization proceeds through the C-20 cation[5] **2**. Indirect evidence consistent with the participation of **2** in the biosynthesis of fusidic acid (**6a**) was obtained.[8]



1a, R = CH_3
b, R = H

2a, Ⓧ ≡ (+) charge
b, Ⓧ ≡ prosthetic group



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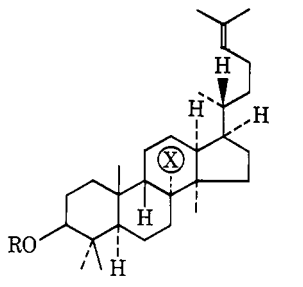

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For the biosynthesis of lanosterol (**5**) a backbone rearrangement involving the concerted migration of the 17β- and 13α-hydrogens and of 14β- and 8α-methyls as indicated in **2** and leading to cation **4** is postulated. Stabilization of the cation **4** *via* the loss of the 9β-hydrogen results then in lanosterol[9] (**5**).

Eschenmoser, *et al.*,[5] in formulating their generalized scheme of the biosynthesis of polyisoprenoids, postulated that the atoms or groups participating in the rearrangements and/or eliminations must have an axial antiparallel orientation.[5,10] Indeed, the hydrogen atoms and methyl groups involved in the backbone rearrangement of **2** *via* **4** to lanosterol (**5**) fulfill this requirement. Also, according to the hypothesis, the transformations from epoxide **1a** to lanosterol (**5**), which take place on the squalene cyclase, are a nonstop sequence of events.[5] This, therefore, precludes the formation of stable intermediates[5] between **1a** and **5**.

In discussing the biosynthetic reactions from epoxide

(1) The very generous support of this work at the Worcester Foundation for Experimental Biology by the National Institutes of Health (Grants AM 12156, CA 13369, and GM 19882) and by the National Science Foundation (Grant GB 36201) is gratefully acknowledged.

(2) The following abbreviations were used in this paper: yeast alcohol dehydrogenase (YADH); β-diphosphopyridine nucleotide (NAD); fusidic acid biosynthesized from (3*RS*,2*R*)-[2-^{14}C,2-^{3}H]-MVA [(*R*)-fusidic acid]; fusidic acid biosynthesized from (3*RS*,2*S*)-[2-^{14}C,2-^{3}H]-MVA [(*S*)-fusidic acid]; mevalonic acid (MVA); fusidic acid (Fu); lithium aluminum hydride ($LiAlH_4$); thin-layer chromatography (tlc); preparative tlc (ptlc); gas–liquid chromatography (glc); preparative glc (pglc).

(3) (a) Worcester Foundation for Experimental Biology, Shrewsbury, Mass. (b) Postdoctoral Fellow, 1970–1973. (c) Leo Pharmaceutical Products, DK. 2750 Ballerup, Denmark. (d) Except for the microbial incubation experiments, the work was carried out at the Worcester Foundation for Experimental Biology.

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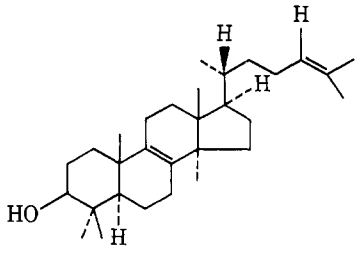

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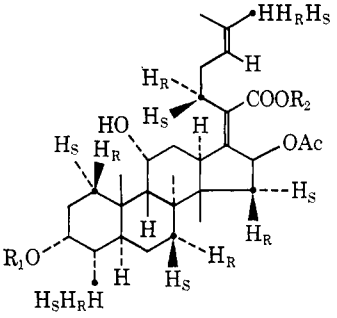

6a, $R_1 = H$; $R_2 = H$
b, $R_1 = H$; $R_2 = CH_3$ from (*R*)-fusidic acid
c, $R_1 = H$; $R_2 = CH_3$ from (*S*)-fusidic acid
d, 24-dihydro "*b*" from (*R*)-fusidic acid
e, 24-dihydro "*c*" from (*S*)-fusidic acid

1a to lanosterol (**5**) Cornforth[10] pointed out the difficulties of visualizing a *single enzyme* capable of accommodating all the proposed biochemical steps. To obviate this difficulty, he suggested the possible formation of transiently stabilized intermediates and the participation of more than one enzyme in the process.[10] Thus, the C-20 cation could be transiently stabilized by a nucleophilic prosthetic group to yield **2b**. At this point, a partial or complete release of **2b** from the enzyme could take place. Rotation through 120° around the C-17(20) bond of **2b** of the side chain or of the tetracyclic moiety would result in **3** in which the X group, as well as the hydrogens at C-17β, -13α, and -9β and the methyls at C-14β and -8α, has the required axial antiparallel orientations.[5,10] The obtained **3** could remain attached to the same enzyme or be transferred to another enzyme and yield, after the backbone rearrangement and elimination of the 9β-hydrogen, lanosterol (**5**).

We pointed out that although **2b** satisfactorily explains the transformations leading to lanosterol, because of the geometry of the X group and of the 17β-hydrogen it fails to provide a plausible rationalization of the biosynthesis of certain protosterols such as fusidic acid[8b] (**6a**). Fusidic acid and related natural products[11] are presumed to be metabolites of the parent protosterol[12] **7** which has the *Z* geometry at the 17(20) double bond.

It is worthy of note that the mold *F. coccineum* which produces fusidic acid also biosynthesizes ergosterol, which is a metabolite of lanosterol. Hence, both pathways, the backbone rearrangement and the direct stabilization of **2**, are functioning in this organism.

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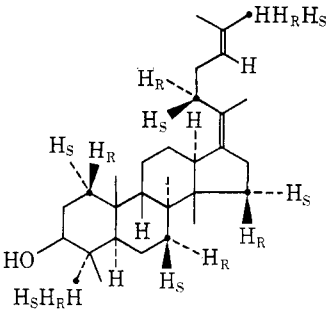
Because of this difficulty, we suggested several alternative ways of stabilization[8b] of **2b** which could yield **7**.

For example, the X group of **2b** could be displaced enzymatically with inversion of configuration to yield **8**. The 17β-hydrogen and the Y moiety in **8** have the required antiparallel orientation[5,10] for trans elimination and formation of **7**. Broadening of the nature of the prosthetic group, which would allow a cis elimination, was also considered.[8b] For illustrative purposes, such a pathway *via* a phosphate (or pyrophosphate) ester is shown in **9**.

Corey, *et al.*,[13] observed that cyclization of the 10,15-dinorsqualene (**1b**) by rat liver enzymes resulted in the dinorprotosterol **10a** having a 20(22) double bond. This suggested the possibility that the biosynthesis of the protostanol **7** could proceed through the initial formation of the protostanol **10b** having a 20(22)



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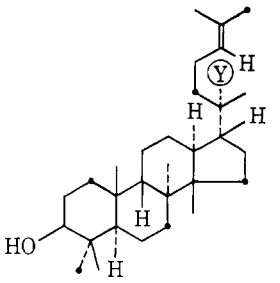

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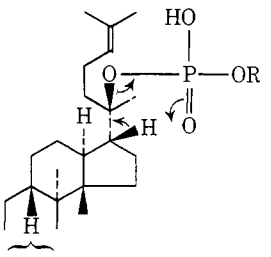

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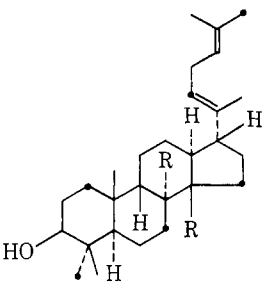

10a, R = H
b, R = CH_3

double bond. Enzyme mediated allylic rearrangement of **10b** would then give **7**.

Biosynthetic allylic rearrangements of double bonds are known and are well documented. One of the reactions in the multistep transformation of lanosterol to cholesterol is the isomerization of the 8(9) double bond to the 7(8) position.[14] This reaction proceeds with the loss of a C-7 hydrogen[15,16] and acquisition of a hydrogen (proton) from the water of the medium[17] at C-9. In contrast, the isomerization of 5-en-3-one to 4-en-3-one may involve the intramolecular transfer of the 4β-hydrogen to the 6β position.[18]

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We have previously shown that fusidic acid biosynthesized from (3*RS*,4*R*)-[2-^{14}C,4-^{3}H]-MVA retains four tritium atoms at the 5α, 9β, 13α, and 24 positions.[8] Consequently, an isomerization of **10b** to **7** would have to proceed with the loss of the 17β hydrogen which originates from the 4-pro-*R* hydrogen to MVA.

The exploration of the pathway from MVA *via* **10** to fusidic acid is the subject of this paper. A preliminary communication of these results was published.[19]

Experimental Section

Gas–Liquid Chromatography (Glc). Glc was carried out on an F and M Model 720 instrument equipped with a thermal conductivity detector. Helium (60 ml/min) was the carrier gas. For analytical and preparative work, $^1/_8$ in. × 6 ft columns packed with (i) 3% SE-30 on Gas-Chrom Q (80–100 mesh), (ii) 3% OV-17 on Gas-Chrom Q (60–80 mesh), and (iii) 3% OV-101 on Gas-Chrom Q (80–100 mesh) were used. Compounds were identified by the peak enhancement technique with authentic samples and quantitated by the cut and weigh method. For preparative collection of samples the glc carrier gas was exited through a sintered glass tube immersed in ether cooled to −70°.

Radioactivity. Radioactive samples were counted on a Nuclear Chicago Mark I liquid scintillation counter as solutions in scintillation fluid containing 2,5-diphenyloxazole (4 g) and 1,4-bis[2-(5-phenyloxazolyl)]benzene (0.1 g) in toluene (1 l.). Samples of (3*RS*)-[2-^{14}C]mevalonic acid lactone, (3*RS*,2*R*)-[2-^{3}H]mevalonic acid lactone, and (3*RS*,2*S*)-[2-^{3}H]mevalonic acid lactone were purchased from Amersham-Searle Corp., Arlington Heights, Ill.

The radioactivity of the side-chain fragments derived from the specimens of (*R*)- and (*S*)-fusidic acid was determined on the indicated solid derivatives.

Physical Measurements. Melting points (mp) were taken on a hot stage and are corrected. Infrared spectra (ir) were recorded on a Perkin-Elmer Model 237 spectrophotometer and peaks are reported in reciprocal centimeters. Ultraviolet (uv) measurements were made with a Perkin-Elmer 202 spectrophotometer. Mass spectra were obtained on a Varian Associates M-66 instrument or on a Du Pont Corp. 21-491 instrument. Proton magnetic resonance spectra (nmr) were recorded on a Varian DA-60 spectrometer at 60 MHz with samples dissolved in $CDCl_3$. Peaks are reported in τ units downfield from tetramethylsilane.

Thin-Layer Chromatography (Tlc). Tlc was carried out on plates coated with silica gel HF (254,366) (E. Merck, A.G., Darmstadt, Germany). Compounds were visualized with ultraviolet light of wavelengths 254 or 366 nm and/or by spraying the plates with phosphomolybdic acid (20% in ethanol). Solvent systems used were (a) hexane–acetone (9:1, v/v), (b) hexane–acetone (7:3, v/v), (c) benzene, (d) hexane–ethyl acetate–acetic acid (50:49:1, v/v).

Model Synthetic Studies. 6-Methylheptan-1-ol-2-one Acetate (11b). To a solution of 6-methyl-2-heptanone (**11a**) (5.00 g, purchased from K and K Laboratories, Inc.) in benzene (760 ml) was added boron trifluoride etherate (100 ml), methanol (70.0 ml), and lead tetraacetate (26.0 g).[20,21] After stirring for 4 hr the reaction was poured onto ice chips. The recovered benzene solution was washed with aqueous $NaHCO_3$ and water and then dried (Na_2SO_4). Removal of the solvent afforded an oil (6.98 g) consisting mainly of **11b**. The product was purified by preparative glc (column i, 130°, retention time 22 min) to yield **11b** as a colorless oil (2.78 g): ir 1740, 1227, 1190, 1165 cm^{-1}; nmr 9.08 [d, 6 H, J = 6 Hz, $(CH_3)_2CH$], 7.83 (s, 3 H, OAc), 7.59 (m, 2 H, $-CH_2CH_2-CO-$), 5.37 (s, 2 H, $-COCH_2OAc$); mass spectrum 186 (M^+).

6-Methylheptane-1,2-diol (12a). The crude acetoxy ketone **11b** (4.00 g) was added to a suspension of lithium aluminum hydride (6.00 g) in ether (200 ml). After refluxing for 16 hr, water was added dropwise until a granular precipitate was obtained. The solid was removed by filtration through Celite and washed with a small amount of ether. The combined ether filtrate was dried (Na_2SO_4) and concentrated. The obtained colorless oil (3.68 g) was dissolved in hexane and placed on a column of neutral alumina (90 g). The column was eluted with ether (1 l.), ether–ethyl acetate (1:1, 1 l.), and methanol (1 l.). On concentration of the methanol eluate, 6-methylheptane-1,2-diol (**12a**, 1.96 g) was obtained as a colorless oil. The specimen was 90% pure by glc analysis (column i, 145°). Additional purification by pglc (column i, 145°) afforded **12a** as a homogeneous oil: ir 3350 (OH), 1130, 1065, 1025 cm^{-1}; nmr 9.10 [d, 6 H, J = 5.5 Hz, $(CH_3)_2CH$], 6.38 (m, 3 H, $CHOH$), 6.34 (m, 2 H, OH); mass spectrum 115 ($M^+ - CH_2OH$), 97 (115 − H_2O).

5-Methylhexan-1-al (13a). To a solution of the freshly prepared CrO_3–pyridine complex[22] (13.4 g) in 100 ml of CH_2Cl_2 (distilled from P_2O_5), 5-methylhexan-1-ol (purchased from Sapon Laboratories, 1.16 g) in CH_2Cl_2 (10 ml) was added with stirring at room temperature. The stirring was continued for 5 min, then the volatile components were distilled at room temperature *in vacuo*, and the distillate was collected in a flask cooled in liquid nitrogen. The distillate was washed with 0.1 *N* HCl and H_2O and dried (Na_2SO_4) and the solvent was distilled through a 10-cm Vigreux column. By glc (column iii, 83°) the concentrate contained 5% starting alcohol. Distillation of the total reaction products afforded 0.45 g of homogeneous 5-methylhexan-1-al (**13a**): bp 95° (16 cm); ir 2710 (–CHO), 1718 cm^{-1} (C=O); nmr 9.08 [d, 6 H, J = 5.5 Hz, $(CH_3)_2CH-$], 7.60 (t, 2 H, J = 6 Hz, $-CH_2CHO$), 0.65 (t, 1 H, J = 2 Hz, CHO); retention time 4 min (column iii, 83°).

4-Methylpentan-1-al (16a). 4-Methylpentan-1-ol (**14a**, purchased from Aldrich Chemical Co.) was treated with a 6 mol excess of Collins reagent in CH_2Cl_2 as above to yield 4-methylpentan-1-al (**16a**): bp 76° (16 cm); ir 2710 (CHO), 1720 cm^{-1} (C=O); nmr 9.06 [d, 6 H, J = 5 Hz, $(CH_3)_2CH-$], 8.39 (2 H, J = 4.5 Hz, CH_2), 7.60 (2 H, $-CH_2CHO$); retention time 3 min (column iii, 75°).

Biosyntheses[23] of (*R*)- and (*S*)-[$^{14}C_6$,$^{3}H_6$]Fusidic Acids. To a 24-hr culture of *Fusidium coccineum* in 225 ml of a synthetic medium was added a filtered, sterile aqueous solution (6 ml) of (3*RS*,2*R*)-[2-^{14}C,2-^{3}H]mevalonic acid lactone (20 μCi of ^{14}C, ^{3}H:^{14}C ratio 5.15). The culture was shaken for an additional 144 hr. The mycelium was harvested, by filtration, and washed with water. The combined filtrate and washings were adjusted to pH 3 and extracted with ether. The ether solution was extracted with aqueous KOH (pH 10.5). The alkaline aqueous phase was immediately acidified with dilute hydrochloric acid to pH 3 and extracted with ether. After washing and drying, the ether was removed. Trituration of the residue with benzene gave 101 mg of crystalline (*R*)-fusidic acid as a benzene solvate (*ca.* 2 μCi of ^{14}C) (Table I).

The incubation of the (3*RS*,2*S*)-[2-^{14}C,2-^{3}H]-MVA lactone (20 μCi of ^{14}C, ^{3}H:^{14}C ratio 5.26) was carried out as above and afforded (*S*)-fusidic acid as a benzene solvate (*ca.* 1.8 μCi of ^{14}C) (Table II).

For counting purposes aliquots of the (3*RS*,2*R*)-[2-^{14}C,2-^{3}H]-mevalonic acid lactone and of the (3*RS*,2*S*)-[2-^{14}C,2-^{3}H]mevalonic acid lactone used in the incubations were diluted with nonradioactive MVA and converted to the corresponding diphenylmethylamides as previously described.[23]

Studies of (*R*)-[$^{14}C_6$,$^{3}H_6$]Fusidic Acid Derived from (3*RS*,2*R*)-[2-^{14}C,2-^{3}H]Mevalonic Acid Lactone. Dilution of (*R*)-Fusidic Acid. To an ethanol solution (27 ml) of nonradioactive fusidic acid (968 mg), 36.8 mg of (*R*)-fusidic acid benzene solvate (2.0 × 10^6 dpm ^{14}C) was added. The resulting solution was then warmed to 40° and stirred (15 min).

An aliquot of the solution was taken to dryness and the residue (26 mg) was treated with an ether solution of diazomethane. The obtained methyl fusidate (**6b**) was purified by tlc (hexane–acetone, 4:1) and crystallized from ether–hexane to constant specific activity of ^{14}C and constant ^{3}H:^{14}C ratio (see Table I): mp 151° (lit.[24] 153–154.5°).

(*R*)-[$^{14}C_6$,$^{3}H_6$]**Methyl 24-Dihydrofusidate (6d).** To an ethanolic solution of the diluted (*R*)-fusidic acid, 5% palladium on calcium carbonate catalyst (202 mg, Engelhard, Newark, N. J.) was added and the mixture was stirred vigorously in an atmosphere of hydrogen.[8,24] In 2 hr *ca.* 50 ml of hydrogen was absorbed. The catalyst was removed by filtration on Celite and washed with ethanol. Removal of the solvent afforded the 24-dihydro acid as a colorless residue (1.2 g). The product was homogeneous by argentation tlc [silica gel, 10% $AgNO_3$; hexane–ethyl acetate–acetic acid (50:49:1) developed three times].

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(20) D. M. Piatak and E. Caspi, *Tetrahedron*, **22**, 2823 (1966); *Chem. Commun.*, 501 (1966).

(21) J. D. Cocker, H. B. Henbest, G. H. Phillips, G. P. Slater, and D. A. Thomas, *J. Chem. Soc.*, 6 (1965).

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(24) W. O. Godtfredsen and S. Vangedal, *Tetrahedron*, **18**, 1029 (1962).

Table I. ^{14}C Specific Activities and $^{3}H:^{14}C$ Ratios of Fusidic Acid and Its Degradation Products Biosynthesized from (3*RS*,2*R*)-[2-^{14}C,2-^{3}H]Mevalonic Acid

Entry	Compound	Product counted	Specific act., ^{14}C[a] Crystn 1	Crystn 2	Crystn 3	$^{3}H:^{14}C$ ratio, isotopic Crystn 1	Crystn 2	Crystn 3	Specific act.[a]	$^{3}H:^{14}C$ ratio Isotopic	Atomic
1	(3*RS*,2*R*)-[2-^{3}H,2-^{14}C]-Mevalonic acid	Benzhydryl-amide	8.80	8.79		5.18	5.15		8.79	5.15	
2	Methyl fusidate	**6b**	89.5	86.0	84.6	5.00	5.07	5.06	86.7	5.04	6.00:6[b]
3	Methyl dihydrofusidate	**6d**	85.6	85.9	85.4	5.03	5.05	5.06	85.6	5.05	6.00:6
4	6-Methylheptane-1,2-diol	**12c**	30.2	30.5	30.1	4.89	4.92	4.79	30.2	4.92	1.95:2
5	5-Methylhexan-1-al	**13c**	30.0	30.1	31.1	5.01	5.04	5.04	30.4	5.04	2.00:2
6	4-Methylpentan-1-ol	**14d**	2.74	2.73	2.73	4.83	4.99	5.02	2.73	5.02	1.99:2
7	4-Methylpentan-1-al	**16c**	1.52	1.55		3.15	3.02		1.54	3.02	1.20:2

[a] Specific activity in dpm/mmol × 10^4; the specific activity of the products was determined at different dilutions. [b] Based on methyl fusidate.

Table II. ^{14}C Specific Activity and $^{3}H:^{14}C$ Ratios of Fusidic Acid and Its Degradation Products Biosynthesized from (3*RS*,2*S*)-[2-^{14}C,2-^{3}H]Mevalonic Acid

Entry	Compound	Product counted	Specific act., ^{14}C[a] Crystn 1	Crystn 2	Crystn 3	$^{3}H:^{14}C$ ratio, isotopic Crystn 1	Crystn 2	Crystn 3	Specific act.[a]	$^{3}H:^{14}C$ ratio Isotopic	Atomic
1	(3*RS*,2*S*)-[2-^{3}H,2-^{14}C]-Mevalonic acid	Benzhydryl-amide	4.96	4.52	4.77	5.18	5.44	5.16	4.75	5.26	
2	Methyl fusidate	**6c**	84.4	81.7	81.2	4.61	4.65	4.70	82.4	4.65	6.00:6[b]
3	Methyl dihydrofusidate	**6e**	82.8	83.5	81.2	4.63	4.57	4.66	82.5	4.62	5.96:6
4	6-Methylheptane-1,2-diol	**12e**	29.0	28.5	29.6	4.52	4.52	4.58	29.0	4.58	1.97:2
5	5-Methylhexan-1-al	**13e**	29.5	28.6	28.9	4.67	4.66	4.66	29.0	4.66	2.00:2
6	4-Methylpentan-1-ol	**14g**	3.99	4.06	4.05	4.55	4.52	4.60	4.03	4.60	1.98:2
7	4-Methylpentan-1-al	**16e**	4.63	4.74		4.54	4.47		4.86	4.54	1.95:2
			10.1	10.1	10.2	4.17	4.23	4.27	10.1	4.27	1.84:2
8	Methyl 4-methylpentan-1-oate	**19b**								2.38	1.02:2

[a] Specific activity in dpm/mmol × 10^4; the specific activity of the products was determined at different dilutions. [b] Based on methyl fusidate.

Treatment of the residue with ethereal diazomethane afforded 1.03 g of the methyl ester **6d**: mp 150–151° (lit.[24] 150–151°); *m/e* 532 (M^+). A portion (23 mg) of **6d** was further purified by tlc and crystallized (ether–hexane) to constant specific activity of ^{14}C and constant $^{3}H:^{14}C$ ratio (see Table I).

(3*R*)-[3,7-$^{14}C_2$,3,7-$^{3}H_2$]-6-Methylheptane-1,2-diol (12b). A mixture of (*R*)-methyl 24-dihydrofusidate (**6d**, 950 mg) and pyridine (0.4 ml) in dry dichloromethane (40 ml) was cooled to −70° and treated with a stream of ozonized oxygen (20 ml/min) for 30 min.[24] The excess ozone was removed in a stream of nitrogen, then a slurry of lithum aluminum hydride (1.74 g) in ether (300 ml) was added, and the stirred mixture was allowed to warm up to room temperature. The reduction was continued at reflux (18 hr) and was terminated by dropwide addition of an aqueous solution of Na_2SO_4 until a filterable precipitate was formed. The solid was removed by filtration and washed with ether. The ethereal filtrate was dried (Na_2SO_4) and removal of the solvent afforded a noncrystalline residue (1.06 g).

The residue (1.06 g) was dissolved in chloroform (5 ml), Celite filter-aid (5 g) was added, and the solvent was removed. The dried Celite was placed in a Soxhlet apparatus and extracted with hexane (20 hr). The hexane extract was concentrated to a small volume (20 ml), stored at 0° (4 hr), and filtered. Removal of the hexane afforded 175 mg of (3*R*)-[3,7-$^{14}C_2$,3,7-$^{3}H_2$]-6-methylheptane-1,2-diol (**12b**). This specimen was identical with the authentic **12a** by glc (column ii, 120°; retention time 12 min), ir, nmr, and mass spectra.

A sample (17 mg) of the above [$^{14}C_2$,$^{3}H_2$]diol **12b** was refluxed (12 hr) with phenyl isocyanate (110 mg) in benzene (10 ml) containing pyridine (0.12 ml). Removal of volatile components under reduced pressure (1 mm) yielded an oil (62.4 mg) which was purified by ptlc [hexane–acetone (8:2) developed three times]. The isolated bis(phenylurethane) **12c** (18.2 mg) was crystallized from hexane–ether to constant specific activity of ^{14}C and constant $^{3}H:^{14}C$ ratio (see Table I): mp 83.5–86°; ir (KBr) 3330 cm^{-1}; nmr 9.08 [d, 6 H, J = 6 Hz, $(CH_3)_2CH$], 5.73 (d, 2 H, J = 5 Hz, CH_2O), 4.90 (m, 1 H, CHO), 2.80 (m, 12 H); mass spectrum 384 (M^+), 265 ($M^+ - C_6H_5NCO$), 248.

(2*R*)-[2,6-$^{14}C_2$,2,6-$^{3}H_2$]-5-Methylhexan-1-al (13b). The [$^{14}C_2$,$^{3}H_2$]-6-methylheptane-1,2-diol **12b** (158 mg) in 6 ml of CH_2Cl_2 was added to a stirred solution of H_5IO_6 (310 mg) in water (2 ml) at room temperature. After 15 min the aqueous layer was removed, and the organic phase was washed with aqueous $NaHCO_3$ and water until the washes gave no reaction with a starch–iodide solution. [99 mol % pure methylene chloride (Fisher Co.) was utilized both for the cleavage of the diol and the Baeyer–Villiger oxidation of **13**. Other grades of methylene chloride contained higher boiling impurities which interfered with the glc analysis and the purification of the products.]

A portion of the solution of the aldehyde **13b** was added to a mixture of 5,5-dimethyl-1,3-cyclohexanedione (12 mg), ethanol (0.2 ml), and phosphate buffer (pH 6.8, 0.2 ml). The mixture was warmed (50°, 20 min) and then additional buffer was added until turbid. The solution was stored at 0° for 16 hr and filtered. The obtained solid (23 mg) was washed with water and digested with several portions (1 ml) of hot ethanol. The combined extract was filtered and concentrated. The **13c** was crystallized from aqueous ethanol (thin plates) (12.8 mg): mp 125.0–126.5°; ir (KBr) 1605, 1378, 1365, 1248, 1168, 1152, 882 cm^{-1}; nmr 9.14 [d, 6 H, J = 5.5 Hz, $(CH_3)_2CH$–], 8.92 [s, 12 H, $(CH_3)_2C$], 8.10 (2 H, OH), 7.72 (s, 8 H, CH_2), 6.10 (t, 1 H, J = 7 Hz, $CHCH_2$–); mass spectrum 376 (M^+), 305 [$M^+ - (CH_3)_2CHCH_2CH_2$], 291 ($M^+ - C_6H_{13}$); tlc [hexane–acetone (9:1), R_f 0.45]. The **13c** was counted and the results are given in Table I.

(1*R*)-[1,5-$^{14}C_2$,1,5-$^{3}H_2$]-4-Methylpentan-1-ol (14a). The above solution of the (2*R*)-[2,6-$^{14}C_2$,2,6-$^{3}H_2$]-5-methylhexan-1-al (**13b**, 108 mg) in methylene chloride (25 ml) was dried (Na_2SO_4) for 3 hr and decanted into a flask containing disodium hydrogen phosphate (0.625 g) previously heated over a flame for 15 min. A solution (3.2 ml) of trifluoroperacetic acid was then added dropwise (3 min) with stirring.[25] The trifluoroperacetic acid solution was prepared immediately before use by adding trifluoroacetic an-

(25) J. B. Greig, K. R. Varma, and E. Caspi, *J. Amer. Chem. Soc.*, **93**, 760 (1971).

hydride (1.89 g) to a suspension of 90% hydrogen peroxide (0.285 g, FMC Corp., Buffalo, N. Y.) in methylene chloride (5 ml). The peracid mixture was then vigorously shaken (2 min) and diluted to 10 ml with methylene chloride.[25]

The disappearance of the aldehyde **13b** was monitored by glc and usually the oxidation was completed within 2 hr. The reaction was terminated with a saturated solution of sodium carbonate (5 ml) and the stirring continued (2 hr). Anhydrous sodium sulfate (30 g) was added, and after 1 hr the solid was removed by filtration and washed with small portions of methylene chloride. The volume of filtrate was reduced to 6 ml by distillation through a Vigreux column (10 cm). Glc analysis (column iii at 83°) revealed the presence of a small amount of starting material and of two major components, 5-methylhexan-1-oic acid (**15a**) and 4-methylpentan-1-ol formate (**14c**). The formate **14c** showed bp 94° (17 cm): ir (film) 2980 (CH), 1725 (C=O), 1460, 1175, and 1160 cm^{-1} (CO–); nmr 9.12 [d, 6 H, J = 6 Hz, $(CH_3)_2CH$–], 5.82 (t, 2 H, J = 6 Hz, $-CH_2OOCH$), 1.94 (s, 1 H, $HCOO$); glc retention time 13 min (column i at 100°).

The concentrated methylene chloride solution (6 ml) was added dropwise to a suspension of $LiAlH_4$ (0.133 g) in ether (25 ml) and the mixture was stirred for 3 hr. Water (0.1 ml), 2 *N* KOH (0.1 ml), and more water (0.5 ml) was added dropwise. The resulting solid was removed by filtration on Celite and washed with ether. The filtrate was dried (Na_2SO_4) and concentrated to 2 ml by distillation through a Vigreux column. Glc of the concentrated solution (column iii at 80°) indicated the presence of (1*R*)-[1,5-$^{14}C_2$,1,5-$^3H_2$]-4-methylpentan-1-ol (**14b**, ~14 mg) and of (2*R*)-[2,6-$^{14}C_2$,2,6-$^3H_2$]-5-methylhexan-1-ol (**15b**, ~15 mg).

The mixture containing **14b** and **15b** was resolved by preparative glc (column iii at 68°) and each of the alcohols was trapped in ether at −70°. The recovered **14b** (*ca.* 1.3 × 10^4 dpm of ^{14}C) was homogeneous by glc analysis.

To a portion of the **14b** (2.1 × 10^3 dpm of ^{14}C) in benzene (4 ml), nonradioactive **14a** (5 mg), phenyl isocyanate (60 mg), and pyridine (20 mg) were added and the mixture was refluxed for 18 hr. Removal of the volatile components under reduced pressure afforded a residue from which 4-methylpentan-1-ol phenylurethane (**14d**, 11 mg) was isolated by tlc [hexane–acetone (9:1), developed three times]. Recrystallization from hexane afforded **14d** as white needles of constant specific activity of ^{14}C and constant ^3H:^{14}C ratio (Table I): mp 44.9–45.5°; ir (KBr) 3325 (NH), 3060, 2955 (CH), 1705 (C=O), 1604, 1525, 1442, 1315, 1205, 1078, 755 cm^{-1}; nmr 9.14 [d, 6 H, J = 5.5 Hz, $(CH_3)_2CH$], 5.87 (t, 2 H, J = 6.5 Hz, $-CH_2O$), 3.22 (1 H, NH), 2.72 (m, 5 H, aromatic); mass spectrum 221 (M^+), 137 [$M^+ - (CH_3)_2CHCH_2CH_2CH_2$].

[1,5-$^{14}C_2$,5-^3H]-4-Methylpentan-1-al (16b). A stock solution of 0.25 *M* glycine–sodium hydroxide buffer (pH 9.8, 25 ml), NAD^+ (214 mg, 290 μmol of 98% pure β-NAD), and crystalline YADH (21.2 mg, activity 370 units/mg, Sigma Chemical Co.) was prepared and chilled in ice. An aliquot of this solution (0.1 ml) was diluted with the glycine buffer to 3 ml and served as a blank for uv measurements.

A solution of the homogeneous (1*R*)-[1,5-$^{14}C_2$,1,5-$^3H_2$]-4-methylpentan-1-ol (**14b**, 1.1 × 10^4 dpm of ^{14}C) in ether (0.5 ml) was placed in a test tube (10 ml) and the solvent was removed in a gentle stream of nitrogen. Then the buffered NAD–YADH stock solution (3.5 ml) was added[25] and the reaction was stirred at 4°. The extent of oxidation was followed by measuring the optical density at 340 nm of appropriately diluted aliquots. After 18 hr the optical density became constant during which time 4.5 μmol of NADH had formed. Nonradioactive aldehyde **16a** (1.8 mg) and 5,5-dimethyl-1,3-cyclohexanedione (10 mg) were added and the pH of the reaction mixture was carefully adjusted to 6.8 with aqueous acetic acid (10%, added from a microsyringe). A solution of 5,5-dimethyl-1,3-cyclohexanedione (10 mg) in hot water (1.3 ml) was added; the mixture was heated at 50° (30 min), then left at room temperature (4 hr), and finally stored at 0° (16 hr). The obtained solid (11 mg) was collected by filtration, washed with water, and digested several times with portions (0.5 ml) of warm ethanol. The combined ethanol solution was filtered, concentrated (0.3 ml) under N_2, and diluted with a phosphate buffer (pH 6.8) until turbid. On cooling [5-^3H,1,5-$^{14}C_2$]-4-methylhexan-1-al dimedone (**16c**) was obtained as colorless plates (6.8 mg). Several recrystallizations from hexane afforded **16c**: mp 123.5–125.0°; ir (KBr) 2960 (CH), 1595, 1385, 1365, 1250, 1170, 1150, 1128 cm^{-1}; nmr 9.13 [d, 6 H, J = 5.5 Hz, $(CH_3)_2CH$], 8.92 [s, 12 H, $(CH_3)_2C$–], 8.15 (2 H, –OH), 7.72 (s, 8 H, $-CH_2$–), 6.10 (t, 1 H, J = 7 Hz, $CHCH_2$–); mass spectrum 362 (M^+), 291 [$M^+ - (CH_3)_2CHCH_2CH_2$], 305 [$M^+ - (CH_3)_2CHCH_2$]. The radioactivity measurements are summarized in Table I.

Studies of (*S*)-[$^{14}C_6$,$^3H_6$]Fusidic Acid. The (*S*)-fusidic acid (23.0 mg, 1.15 × 10^6 dpm of ^{14}C) biosynthesized from the (3*RS*,2*S*)-[2-^{14}C,2-^3H]mevalonic acid lactone was diluted with 975 mg of nonradioactive **6a**. A portion (20 mg) of the mixed material was treated with ethereal diazomethane, and the obtained **6c** was purified, crystallized, and counted (Table II).

The degradation was carried out in the same manner as described above for the (*R*)-fusidic acid. The Δ^{24} double bond of the *S* acid was hydrogenated. The resulting (*S*)-24-dihydrofusidic acid (0.940 g) was esterified with ethereal diazomethane to yield **6e**. A sample (18 mg) of **6e** was purified by tlc, recrystallized, and counted (Table II).

The sterol side chain was removed by ozonization of a solution of **6e** in methylene chloride–pyridine (99:1) for 35 min at −70°. This was followed by refluxing with $LiAlH_4$ (1.68 g) in ether (300 ml) for 20 hr. The recovered products (0.80 g) were adsorbed on Celite (5 g) and extracted with hexane to yield 238 mg of diol **12d**.

A mixture of **12d** (21 mg), phenyl isocyanate (100 mg), pyridine (0.11 ml), and benzene (10 ml) was refluxed (18 hr) to yield the bis-(phenylurethane) (**12e**). The **12e** was purified by tlc, crystallized from a hexane–ether mixture and counted (Table II).

Periodic acid (465 mg) oxidation of **12d** in methylene chloride (7 ml) and water (2.1 ml) afforded aldehyde **13d**. The aldehyde solution was dried over Na_2SO_4 (3 hr) and then utilized for Baeyer–Villiger oxidation. An aliquot (10 mg) of the aldehyde **13d** was converted to the dimedone derivate **13e**, crystallized, and counted (Table II).

The bulk of the dry CH_2Cl_2 solution of [$^{14}C_2$,$^3H_2$]-5-methylhexan-1-al (**13d**) was treated with trifluoroperacetic acid (0.10 g), disodium hydrogen phosphate (0.625 g), and methylene chloride (30 ml). The recovered reaction products consisting of **14e**, **14f**, and 5-methylhexan-1-oic acid (**15c**) were reduced with $LiAlH_4$ (0.133 g) in ether (25 ml). The recovered mixture of **14e** and **15d** was resolved by glc to yield (1*S*)-[1,5-$^{14}C_2$,1,5-$^3H_2$]-4-methylpentan-1-ol (**14e**).

An aliquot of the homogeneous (by glc) **14e** was diluted with 5.3 mg of nonradioactive **14a** and converted to the phenylurethane **14g** (19 mg). The derivative was purified by tlc and recrystallized (hexane) to constant specific activity of ^{14}C and ^3H:^{14}C ratio (Table II).

A stock solution of 0.25 *M* glycine–sodium hydroxide buffer (pH 9.8, 25 ml), NAD^+ (214 mg), and YADH (20.3 mg) was prepared and chilled in ice. The enzyme solution (6.5 ml) was then added to two tubes each containing purified (1*S*)-[1,5-$^{14}C_2$,1,5-$^3H_2$]-4-methylpentan-1-ol (**14e**, 1.0 × 10^4 dpm of ^{14}C, 7.7 mg) and the reaction was stirred at 4° for 17 hr. The experiments were separately processed in an identical manner. Each oxidation gave *ca.* 30 μmol of NADH. During the reaction 5,5-dimethyl-1,3-cyclohexanedione (10 mg) was added after 4 and 17 hr. Nonradioactive 4-methylpentan-1-al (**16a**, 2.1 mg) was added to the solution, the pH adjusted to 6.7 with acetic acid (10%), and additional 5,5-dimethyl-1,3-cyclohexanedione (10 mg) in hot water (1 ml) was added. The mixture was warmed (55° for 20 min) and then stored at 4° for 12 hr. The solid (11.2 mg) was digested with warm ethanol, and the extract was diluted with buffer (pH 6.8) to yield 10.8 mg of dimedone (**16e**) containing 3.0 × 10^3 dpm of ^{14}C (*ca.* 33% oxidation). The results from both experiments are given in Table II.

(*S*)-$\Delta^{13(17),20(22)}$-Diene (17). A mixture of (*S*)-[$^{14}C_6$,$^3H_6$]-24-dihydrofusidic acid (562 mg, *ca.* 1.2 × 10^4 dpm of ^{14}C), lithium chloride (126 mg), and dimethylformamide (2 ml) was stirred and heated (140–150°) for 3 hr.[26] After cooling to room temperature, water (20 ml) was added and the product was recovered with ethyl acetate. The ethyl acetate solution was washed with water and dried (Na_2SO_4) and the solvent removed under reduced pressure. The residue was dissolved in ether and treated with ethereal diazomethane. Fractionation by preparative tlc [ethyl acetate–benzene (1:8), developed five times] afforded the $\Delta^{13(17),20(22)}$-diene (**17**, 140 mg, homogeneous oil): ir (film) 3455, 1728 cm^{-1} (–COOMe); uv (ethanol) 252 nm; nmr 6.28 (m, 1 H, 3β-H), 6.23 (s, 3 H, $COO-CH_3$), 5.73 (m, 1 H, 11β-H), 4.20 (t, 1 H, J = 8 Hz, 22-H).

A second product (28.7 mg) was identified as the $\Delta^{15,17(20)}$-diene (**18**): mp 163–164° (from ether–hexane); ir (KBr) 3450, 2935 (CH), 1710 (C=O), 1665, 1610 (C=C–), 980 cm^{-1} (–CH=CH–); nmr 9.18 (d, 3 H, J = 5 Hz, 4α-CH_3), 9.16, 9.02, 8.60, 8.59 (s, 9 H, 18, 32 and 19-CH_3), 8.03 (m, 2 H, –OH), 6.60 (d, 1 H, J = 12 Hz, 13α-H), 6.29 (s, 3 H, $COOCH_3$), 5.57 (s, 1 H, 3β-H), 3.62, and 3.10

(26) W. O. Godtfredsen, W. V. Daehne, and S. Vangedal, *Chem. Commun.*, 638 (1966).

(d, 1 H, J = Hz); mass spectrum ($M^+ - CH_3OH^+H_2O$), 278, 247 (base peak); uv (ethanol) 274 mμ (ε 1720).

Methyl [1,5-$^{14}C_2$,5-^{3}H]-4-Methylpentanoate (19) from 17. Ruthenium tetraoxide was generated by stirring ruthenium dioxide (60 mg) in acetone (8 ml) with sodium metaperiodate (450 mg) in water (3 ml).[27] The diene **17** (86.8 mg) in acetone (5 ml) was added dropwise to the stirred yellow solution of the ruthenium tetraoxide.[25,27] As the reaction mixture darkened a solution of sodium metaperiodate (690 mg) in acetone (3 ml) and water (3 ml) was added as needed. After 4 hr at room temperature, the reaction was terminated with isopropyl alcohol. The mixture was acidified with phosphoric acid (10%) to pH 3, poured on a column of Celite (1 g), and eluted with acetone (40 ml). The pH of the filtrate was adjusted to 8 with 1 N potassium hydroxide and the acetone removed under reduced pressure. Water (5 ml) was added and the mixture was distilled *in vacuo* (2×10^{-5} Torr) into a flask cooled in liquid nitrogen. The distillate was discarded. The nonvolatile residue was dissolved in water (5 ml), acidified to pH 2 with phosphoric acid (10%), and distilled (2×10^{-5} Torr) as above. The distillate containing the acid **19a** was treated with ethereal diazomethane at room temperature. After 20 min the excess diazomethane was carefully removed in a slow stream of nitrogen. The ether solution was separated, dried (Na_2SO_4), and concentrated (1 ml) by distillation through a Vigreux column (10 cm). By glc the concentrated solution contained *ca.* 28 mg of **19b**. The radioactive **19b** was purified by preparative glc (column iii at 70°) and collected in dry toluene (10 ml) cooled in Dry Ice–acetone.

A fivefold concentrated scintillating solution[25] was prepared by diluting 21 ml of "Liquifluor" (New England Nuclear) with toluene (100 ml). For determination of $^3H:^{14}C$ ratio, 5 ml of the above solution of **19b** was introduced into a counting vial containing the phosphor solution (2 ml) and toluene (3 ml).

An authentic sample of **19b** showed bp 94° (16.5 cm): ir (film) 1735 (C=O), 1370, 1330 [$(CH_3)_2CH$], 1170, 1110 cm^{-1}; nmr 9.12 [d, 6 H, J = 5.5 Hz, $(CH_3)_2CH$], 7.70 (t, 2 H, J = 7 Hz, methylene), 6.38 (s, 3 H, $COOCH_3$); retention time 4.3 min (column iii at 70°).

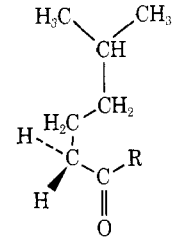
Results and Discussion

Cornforth, *et al.*,[28] and Popjak and Cornforth[29] have shown that the C-2 hydrogens of MVA are incorporated into farnesyl-OPP and then into squalene without scrambling and with retention of their stereochemical integrity.[30] Although the sequence from MVA to squalene involves the loss of asymmetry of MVA, each step of the process is stereospecific.[28,29] It was proved that the 2-pro-*R* and 2-pro-*S* of MVA assume the pro-*R* and pro-*S* configurations at C-4 and C-8 of farnesyl-OPP, respectively.[28-30] Coupling of two molecules of farnesyl-OPP results in squalene in which the hydrogens derived from the 2-pro-*R* and 2-pro-*S* of MVA are located and have the stereochemistry shown[30] in **1a**. Oxidative cyclization of squalene by *F. coccineum* is thought to proceed through[8,12] **2–7**. Should the biosynthesis of **7** from **2** involve the intermediate formation of **10**, it follows that only *one proton* derived from C-2 of MVA will be present at C-22 of **7** and hence at C-22 of fusidic acid (**6a**).

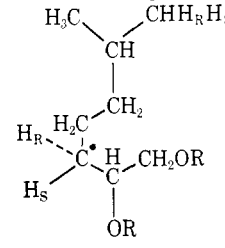
With this in mind, we have investigated the incorporation of (3*RS*,2*R*)-[2-^{14}C,2-^{3}H]-MVA and (3*RS*,2*S*)-[2-^{14}C,2-^{3}H]-MVA into fusidic acid (**6**). Incubation of (3*RS*,2*R*)-[2-^{14}C,2-^{3}H]-MVA [$^3H:^{14}C$ ratio 5.15, atomic ratio (a.r.) 1:1] with the mold *Fusidium coccineum* gave (*R*)-[$^{14}C_6$,$^3H_6$]fusidic acid. The (*R*)-fusidic acid was treated with diazomethane and the resulting (*R*)-methyl fusidate (**6b**) was purified and showed a $^3H:^{14}C$ ratio 5.04 which corresponded to an a.r. of 6:6 (Table I). When (3*RS*,2*S*)-[2-^{14}C,2-^{3}H]-MVA ($^3H:^{14}C$ ratio 5.26, a.r. 1:1) was incubated with *F. coccineum* and the recovered acid was processed as above, the resulting (*S*)-methyl fusidate (**6c**) showed a $^3H:^{14}C$ ratio 4.65. The lower isotopic ratio was calculated to correspond to an atomic ratio of 5.3 atoms of tritium and six atoms of ^{14}C. The loss of nearly an atom of tritium in this experiment enhanced the feasibility of the pathway (**1a** → **2** → **10** → **7** → **6**). Obviously, if in fact the biosynthesis proceeded through a $\Delta^{20(22)}$ intermediate, the results suggested that a 22-pro-*S* hydrogen of **2**, derived from 2-pro-*S* of MVA, was abstracted in the course of formation of **10**.

In the postulated enzyme-mediated allylic rearrangement of $\Delta^{20(22)}$ of **10** to $\Delta^{17(20)}$ of **7** reprotonation could occur on either side of C-22. Thus, depending on the mode and the stereospecificity of addition of the hydrogen at C-22, the hydrogen originating from 2-pro-*R* of MVA which seemingly was retained at C-22 of **6** could have the 22-pro-*R* or the 22-pro-*S* configuration. However, if the postulated reprotonation at C-22 is not stereospecific, equal amounts of tritium will be located at 22-pro-*R* and 22-pro-*S* positions. Hence, for the evaluation of the possible intermediacy of **10** in the biosynthesis of fusidic acid (**6a**) it was necessary to determine the "amount" and the stereochemistry of the tritium atoms at C-22 in specimens of the (*R*)- and (*S*)-fusidic acid.

For this purpose (*R*)-fusidic acid was first hydrogenated over a 5% palladium on calcium carbonate catalyst and then treated with diazomethane to yield methyl 24-dihydrofusidate (**6d**) ($^3H:^{14}C$ 5.05, a.r. 6.0:6).[8,24] The side chain of **6d** was cleaved with ozone and the resulting mixture of products was reduced with lithium aluminum hydride ($LiAlH_4$). The recovered alcohols were adsorbed on Celite from which (3*R*)-[$^{14}C_2$,$^3H_2$]-6-methylheptane-1,2-diol (**12b**) was extracted



11a, R = CH_3
b, R = CH_2OAc



12a, R = H; H_R = H; H_S = H
b, R = H; H_R = ^{3}H; H_S = H; • = ^{14}C
c, R = CONHPh of "*b*"
d, R = H; H_R = H; H_S = ^{3}H; • = ^{14}C
e, R = CONHPh of "*d*"

(Soxhlet) with hexane. The diol **12b** was counted as the 1,2-bis(*N*-phenylurethane) (**12c**) (Table I).

Authentic nonradioactive 6-methylheptane-1,2-diol (**12a**) was prepared by treating a methanolic solution of 6-methylheptan-2-one (**11a**) with lead tetraacetate and boron trifluoride etherate[20,21] to yield 6-methylheptan-2-

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(28) J. W. Cornforth, R. H. Cornforth, G. Popjak, and L. Yengoyan, *J. Biol. Chem.*, **241**, 3970 (1966).

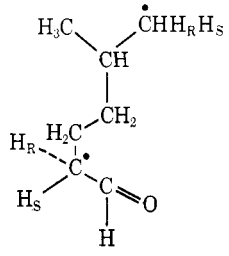
(29) G. Popjak and J. W. Cornforth, *Biochem. J.*, **101**, 553 (1966).

(30) It should be kept in mind that the *R* and *S* assignments of the discussed hydrogens of MVA, farnesyl-OPP, and squalene relate to the order of priorities in these molecules [R. S. Cahn, Ch. Ingold, and V. Prelog, *Angew. Chem., Int. Ed. Engl.*, **5**, 385 (1966)]. However, it is of great importance to note that *each* of the *discussed hydrogens* of farnesyl-OPP and squalene is derived from a *specific* 2-pro-*R* or 2-pro-*S* hydrogen of MVA. The expression "with retention of their stereochemical integrity" is used deliberately to stress this point.

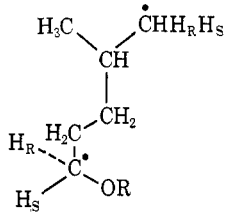
on-1-ol acetate (**11b**, *m/e* 186). The acetoxy ketone was reduced with $LiAlH_4$ to yield the required 6-methylheptane-1,2-diol [**12a**, *m/e* 115 ($M^+ - CH_2OH$)]. The authentic diol **12a** and the diol **12b** derived from (*R*)-fusidic acid were identical by glc, ir, nmr, and mass spectrum.

The [$^{14}C_2$,$^3H_2$]diol **12b** was dissolved in methylene chloride and stirred for 15 min with an aqueous solution of periodic acid to yield (2*R*)-[2,6-$^{14}C_2$,2,6-$^3H_2$]-5-methylhexan-1-al (**13b**). The radioactive aldehyde **13b** was identical with an authentic sample of the aldehyde **13a** prepared by the oxidation[22] of 5-methylhexan-1-ol. The radioactivity of **13b** was determined on the dimedone derivative **13c** (Table I).

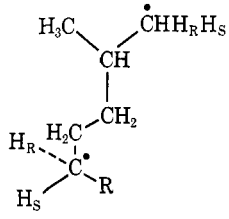
The bulk of the methylene chloride solution of the aldehyde **13b** was treated with trifluoroperacetic acid.[25] After the usual work-up the recovered products were analyzed by glc. The residue contained 4-methylpentanol (**14b**), the formate ester **14c**, and a considerable amount of the acid **15a**. The crude mixture was reduced with $LiAlH_4$ and the resulting **14b** and **15b** were resolved by glc. A portion of the homogeneous (1*R*)-[1,5-$^{14}C_2$,1,5-$^3H_2$]-4-methylpentan-1-ol (**14b**) was converted to its *N*-phenylurethane (**14d**) and counted (Table I). At this point, it should be recalled that the Baeyer–Villiger reaction proceeds with retention of configuration.[31]



13a, $H_R = H$; $H_S = H$
b, $H_R = {}^3H$; $H_S = H$; $\bullet = {}^{14}C$
c, dimedone of "*b*"
d, $H_R = H$; $H_S = {}^3H$; $\bullet = {}^{14}C$
e, dimedone of "*d*"



14a, R = H; $H_R = H$; $H_S = H$
b, R = H; $H_R = {}^3H$; $H_S = H$; $\bullet = {}^{14}C$
c, R = OCH of "*b*"
d, R = CONHPh of "*b*"
e, R = H; $H_R = H$; $H_S = {}^3H$; $\bullet = {}^{14}C$
f, R = OCH of "*e*"
g, R = CONHPh of "*e*"



15a, $H_R = {}^3H$; $H_S = H$; $\bullet = {}^{14}C$; R = COOH
b, $H_R = {}^3H$; $H_S = H$; $\bullet = {}^{14}C$; R = CH_2OH
c, $H_R = H$; $H_S = {}^3H$; $\bullet = {}^{14}C$; R = COOH
d, $H_R = H$; $H_S = {}^3H$; $\bullet = {}^{14}C$; R = CH_2OH

Initially, the cleavage of the glycol was carried out by the method of Ireland[32] whereby an ether solution of **12b** was added to an ether solution of periodic acid. Then after the usual work-up, as much ether as possible was removed and the concentrated solution of the aldehyde **13b** was diluted with methylene chloride. Treatment of this methylene chloride solution of the aldehyde **13b** with trifluoroperacetic acid gave essentially only the acid **15a**. Apparently the small amount of ether left in the medium influenced the course of the Baeyer–Villiger oxidation and the C–H rather than the C–C bond was cleaved. It is of interest that in the larger scale model experiments in which the ether was removed completely and the aldehyde was redistilled prior to oxidation, the Baeyer–Villiger reaction proceeded as expected.

The (1*R*)-[1,5-$^{14}C_2$,1,5-$^3H_2$]-4-methylpentan-1-ol (**14b**) was oxidized with NAD^+ and yeast alcohol dehydrogenase to yield the aldehyde **16b**. The dimedone derivative **16c** was purified and crystallized to constant specific activity of ^{14}C and constant $^3H:{}^{14}C$ ratio (Table I). Clearly the (3*RS*,2*R*)-[2-^{14}C,2-^3H]-MVA, (*R*)-methyl fusidate (**6b**), and the fragments derived from the side chain of the (*R*)-methyl 24-dihydrofusidate (**6d**) retained tritium and ^{14}C in the same isotopic ratio (entries 1–6, Table I). This indicates that the biosynthesis of fusidic acid from the (3*RS*,2*R*)-[2-^{14}C,2-^3H]-MVA proceeded without loss of isotopic hydrogen.

It is also apparent that the relative amount of tritium per ^{14}C atom in the (3*RS*,2*R*)-[2-^{14}C,2-^3H]-MVA, (*R*)-methyl fusidate (**6b**), and in the side chain of **6d** are the same and correspond to an atomic ratio (1:1). Subsequently, we have proved that the amount of tritium per ^{14}C atom at C-7 and -15 of **6b** is the same as at C-22.[33]

Oxidation of the alcohol **14b** with NAD^+ and YADH to aldehyde **16b** entailed the loss of nearly a whole atom of tritium (0.8 atom). The NAD^+–YADH dehydrogenation of primary alcohols proceeds with the stereospecific abstraction of the 1-pro-*R* hydrogen.[25,32] This, therefore, establishes the (1*R*)-[1,5-$^{14}C_2$,1,5-$^3H_2$]-4-methylpentan-1-ol structure of **14b**.

The carbon-1 of **14b** together with the tritium atom and the hydrogen atom attached to it correspond to the 22-carbon, tritium, and hydrogen atoms of fusidic acid. In the process of degradation of methyl fusidate (**6b**) *via* **6d** to **14b**, the stereochemistry of these hydrogens was not changed. Also, the order of priorities at C-1 of **14b** is the same as that at C-22 of **6d**. It follows, therefore, that the 22-tritium atom originating from 2-pro-*R* of MVA is in the 22-pro-*R* position. Consequently (*R*)-methyl fusidate (**6b**) has the 22-*R* stereochemistry.

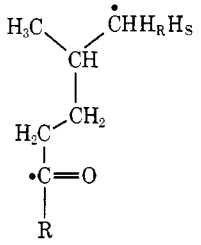
The processing and degradation of the (*S*)-methyl fusidate (**6c**) were carried out in the same manner as described for the (*R*)-methyl fusidate (**6b**). Thus the (*S*)-fusidic acid was converted to methyl 24-dihydro-

(31) K. R. Varma and E. Caspi, *J. Org. Chem.*, **34**, 2489 (1969), and references cited therein.
(32) L. F. Fieser and M. Fieser, "Reagents for Organic Chemistry," Wiley, New York, N. Y., 1967, p 817.
(33) E. Caspi, R. C. Ebersole, L. J. Mulheirn, W. O. Godtfredsen, and W. V. Daehne, *J. Steroid Biochem.*, **4**, 433 (1973).

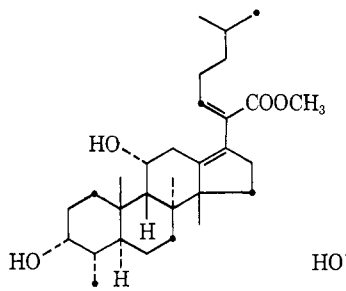
fusidate (**6e**). Cleavage of the side chain of **6e** and reduction with $LiAlH_4$ provided the 1,2-glycol **12d** which in turn on treatment with periodic acid gave the aldehyde **13d**. The aldehyde **13d** was oxidized with CF_3COOOH and the resulting products were reduced with $LiAlH_4$. Following glc purification the homogenous alcohol **14e** was obtained. Incubation of **14e** with NAD^+ and YADH resulted in aldehyde **16d**. The dehydrogenation of the alcohol **14e** to the aldehyde **16d** proceeded without loss of tritium. The results are summarized in Table II (entries 2–7).

The retention of all the tritium in the aldehyde **16d**, obtained by NAD^+–YADH oxidation of **14e**, posed the question whether the alcohol is indeed asymmetric and has a tritium atom at C-1. It should be recalled that C-1 of **14e** corresponds to C-22 of (*S*)-fusidic acid. Obviously, the presence and the stereochemistry of a tritium atom at C-22 in (*S*)-fusidic acid, derived from 2-*S* tritiated MVA, are of significance for the interpretation of the mechanism of the biosynthesis of this protosterol. This is particularly important in the evaluation of the possible biosynthetic intermediacy of **10** and we therefore undertook to verify the presence of a tritium atom at C-22 of the (*S*)-fusidic acid.

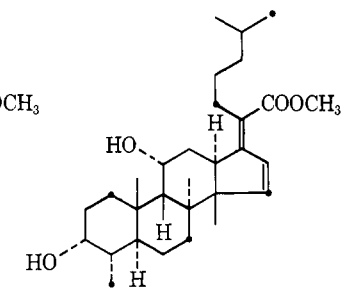
The (*S*)-24-dihydrofusidic acid was treated with lithium chloride in dimethylformamide[26] to yield after work-up and esterification (CH_2N_2) the 13(17),20(22)-diene ester **17** and the 15(16),17(20)-diene ester **18**. The noncrystalline, homogeneous **17** was oxidized with ruthenium tetraoxide.[27] The acid **19a** was recovered



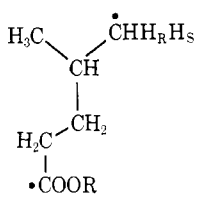
16a, R = H; H_R = H; H_S = H
b, R = H; H_R = ^3H; H_S = H; • = ^{14}C
c, dimedone of "*b*"
d, R = ^3H; H_R = H; H_S = ^3H; • = ^{14}C
e, dimedone "*d*"



17



18



19a, R = H
b, R = CH_3

from the resulting mixture of products by vacuum line distillation. The obtained **19a** was treated with diazomethane and the ester **19b** was purified by glc. The homogeneous **19b** was counted and showed a ^3H:^{14}C ratio of 2.38 which corresponds to an atomic ratio of 1.02:2.

These results confirm the presence of an isotopic hydrogen atom at C-22 of the (*S*)-fusidic acid and hence at C-1 of the alcohol **14e**. Since NAD^+–YADH dehydrogenation of **14e** yielded the aldehyde **16d** without loss of tritium, it follows that the alcohol has the (1*S*)-[1,5-$^{14}C_2$,1,5-$^3H_2$]-4-methylpentan-1-ol structure. The degradation of the (*S*)-methyl fusidate (**6c**) *via* **6e** to **14e** proceeded with retention of the stereochemistry of the 22-tritium atom and the 22-hydrogen atom.[25,31] In addition, the order of priorities at C-22 of **6c** is the same as at C-1 of **14e**. Thus, it can be concluded that the 22-tritium atom derived from (3*RS*,2*S*)-[2-^{14}C,2-^{3}H]-MVA has the 22-pro-*S* configuration and the (*S*)-methyl fusidate (**6c**) has the 22-*S* stereochemistry.

Inspection of the results in Table II (entries 2–7) shows that the isotopic ratio (^3H : ^{14}C) of the fusidates **6c** and **6e** is the same as that of the side-chain fragments **12e**, **13e**, **14g**, and **16e**. On the other hand, the isotopic ratio of methyl 4-methylpentan-1-oate (**19b**), which is obviously devoid of a C-1 tritium atom, was 50% lower (entry 8, Table II). This indicates that equal amounts of tritium are present at C-22 and at the 26-terminal methyl of **6c**. We have proven that in the biosynthesis of fusidic acid by *F. coccineum*, epoxidation and hence cyclization of squalene take place equally from both ends of the squalene molecule.[23] It follows, therefore, that the amounts of tritium at the 4α-methyl of the (*S*)-methyl fusidate (**4c**) should be equal to that at C-26. Finally, we have also determined[33] that the amounts of tritium at C-7 and at C-15 are equal to those at C-22 and at C-26. It is very likely therefore that *equal amounts of tritium* are located at the *six carbon* atoms derived from C-2 of MVA of **6c**.

The presented results show that incubation of (3-*RS*,2*R*)-[2-^{14}C,2-^{3}H]-MVA and (3*RS*,2*S*)-[2-^{14}C,2-^{3}H]-MVA with *F. coccineum* yielded (*R*)- and (*S*)-fusidic acids, each of which contained an atom of tritium at C-22. This in effect excludes **10** as a stable intermediate in the biosynthesis of fusidic acid.

A comparison of the isotopic ratios (^3H:^{14}C) of the (3*RS*,2*S*)-[2-^{14}C,2-^{3}H]-MVA (5.26) and of the (*S*)-methyl fusidate (4.65) (**6c**) (entries 1 and 2, Table II) reveals a significant discrepancy. The relative amount of tritium per ^{14}C atom in the (3*RS*,2*S*)-[2-^{14}C,2-^{3}H]-MVA is considerably higher than in **6c**. As shown above, the amounts of *tritium located at each of the six carbon atoms*, derived from C-2 of MVA of the (*S*)-methyl fusidate (**6e**) are *equal but lower than in the MVA*. It is feasible that the discrepancy is due to a persistent tritiated impurity not removed in the multiple purification steps (tlc, crystallization, etc.) of the benzhydrylamide of the (3*RS*,2*S*)-[2-^{14}C,2-^{3}H]-MVA. Alternatively, if it is assumed that the 2*S*-tritiated MVA was radiochemically homogeneous, some tritium could have been lost in the reversible isomerization[34,35]

(34) G. Popjak, D. S. Goodman, J. W. Cornforth, R. H. Cornforth, and R. Ryhage, *J. Biol. Chem.*, **236**, 1934 (1961).
(35) D. C. Wilton, K. A. Munday, S. J. M. Skinner, and M. Akhtan, *Biochem. J.*, **106**, 803 (1968).

of isopentenyl-OPP and dimethylallyl-OPP. However, the drawback of the latter hypothesis is the fact that tritium was lost only in the biosynthesis of fusidic from the 2*S*-tritiated MVA and not from the 2*R*-tritiated MVA. Clarification of these points requires additional experimentation.

Communications to the Editor

Emission Characteristics of Camphorquinone[1]

Sir:

The literature on the luminescence of camphorquinone is confusing. The purpose of this note is to resolve some of that confusion.

Total luminescence spectra of camphorquinone are displayed in Figure 1. Fluorescence occurs in the 490–550-mμ region and phosphorescence in the 550–660-mμ range. The fluorescence spectrum and all phosphorescence spectra are characterized by a set of three band heads (*i.e.*, the sets {1, 2, 3}, {4, 5, 6}, and {7, 8, 9} of Figure 1) which correspond to the set {origin band, one quantum of a >C—C< stretch mode of the carbonyl carbons, and one quantum of a >C=O stretch mode}.

The phosphorescence spectrum of a glassy solution is shown in Figure 1B and is consistent with previous reports[2] which place the origin band at ~556 mμ. Kuboyama and Yabe,[2a] unable to detect any >C=O stretching activity in the phosphorescence spectrum, concluded that the phosphorescence was an exception among those of the α-diketones; the observation here of a common ~1700-cm^{-1} vibronic interval removes this exceptional status.

The luminescence spectrum of Figure 1A typifies that found for all solid forms of camphorquinone (*i.e.*, microcrystalline suspensions in glasses, thin films obtained from a melt, powdered solid, and single crystal) at 77°K. The phosphorescence of Figure 1A is characterized by an intense origin band at 575 mμ; this spectrum is identical, in most regards, to that observed for the solid state at 77°K by Kuboyama and Yabe[2a] (KY) and Ford and Parry[3] (FP) but quite different from that observed for the solid state at ~300°K by Tsai and Charney (TC). However, as the crystal is warmed to 300°K, the intensity distribution among the 575-mμ peak and 556-mμ shoulder inverts until, at room temperature, the dominant band lies at 556 mμ and the 575-mμ band is either absent or present only as a very slight inflection. Indeed, the room temperature, solid-state phosphorescence is indistinguishable from the phosphorescence of the glassy solution at 77°K shown in Figure 1B which, in turn, is identical with the spectrum reported by TC.[4] Thus, the interrelatedness of the results of all previous authors[2a,3,4] is now obvious.

It is clear from the vibronic analyses given in Figure 1A that the phosphorescence peaks 7, 8, and 9 refer to a defect emission which is ~600 cm^{-1} lower in energy than the T_1 state of camphorquinone, as this state is found in camphorquinone solution at 77°K or in the solid state at 300°K. It is also clear from the vibronic analysis that this defect is itself camphorquinone or some molecule very closely related to camphorquinone. This view obtains further corroboration from the fact that the phosphorescence lifetimes at 77°K are 2.64×10^{-3} sec for the 556-mμ shoulder and 1.07×10^{-3} sec for the 575-mμ peak. We tend toward the view that the defect emitter is camphorquinone and that the 600-cm^{-1} energy decrement is associable with a greater coplanarity of the carbonyl units in the T_1 state of the defect molecule and/or relief of steric strain. This view, in part anyway, is suggested by the observation that in the relatively strain-free 3,3,5,5-tetramethylcyclopentanedione[5] the 0,0 absorption peak occurs at ~530 mμ whereas, in camphorquinone, it is observed at ~478 mμ. The dominance of the intrinsic 556-mμ phosphorescence at 300°K is, then, the result of either thermal trap depopulation or reduced exciton motion at higher temperatures.

Tsai and Charney[4] have recorded the phosphorescence excitation spectrum of the powdered solid at ~300°K. Their results were quite novel and were interpreted as follows: (i) the T_1–T_2 separation is about 6700 cm^{-1}, (ii) the efficiency for phosphorescence excitation is greater upon excitation into S_2 than into S_1, (iii) item (ii) was used to infer that intersystem crossing $S_2 \rightsquigarrow T_2$ more efficient than $S_1 \rightsquigarrow T_1$. The phosphorescence excitation spectrum of a thin film of camphorquinone at 77°K is shown in Figure 2. This spectrum is of higher resolution[6] than that of TC, being a rather faithful replica of the $S_1 \leftarrow S_0$ and, in part, of the $S_2 \leftarrow S_0$ absorption bands[7] of camphorquinone. In fact, the vibronic structure of the absorption spectrum duplicates that present in the excitation spectrum. Contrary to the observations of TC, we did not detect any significant $T_1 \leftarrow S_0$ excitation process in the thin film system; however, the excitation spectrum of the powdered solid at 77°K, as shown in Figure 3, does exhibit some minimal structure in the 550–500-mμ region and one might cautiously suppose that this structure could be representative of an $T_1 \leftarrow S_0$ excitation event. The excitation efficiency in the $S_2 \leftarrow S_0$ region is higher than that in the $S_1 \leftarrow S_0$ absorption

(1) Research supported by contract between the United States Atomic Energy Commission—Division of Biomedical and Environmental Research—Physics and Technological Program and the Louisiana State University.

(2) (a) A. Kuboyama and S. Yabe, *Bull. Chim. Soc. Jap.*, **40**, 2475 (1967); (b) T. R. Evans and P. A. Leermakers, *J. Amer. Chem. Soc.*, **89**, 4380 (1967).

(3) R. A. Ford and F. Parry, *Spectrochim. Acta*, **12**, 78 (1958).

(4) L. Tsai and E. Charney, *J. Phys. Chem.*, **73**, 2462 (1969). The 525-mμ shoulder in the spectrum reported by these authors is presumably fluorescence.

(5) C. Sandris and G. Ourisson, *Bull. Chim. Soc. Fr.*, **23**, 958 (1956).

(6) The excitation monochromator was a Cary 15 spectrometer, the emission monochromator a Jarrell-Ash 0.5 m spectrometer, the light source a 500-W Sun Gun, and the detection system was phase sensitive.

(7) As obtained on a Cary 14 spectrophotometer.