Communications to the Editor

Biosynthesis of Fusidic Acid from Squalene 2,3-Oxide

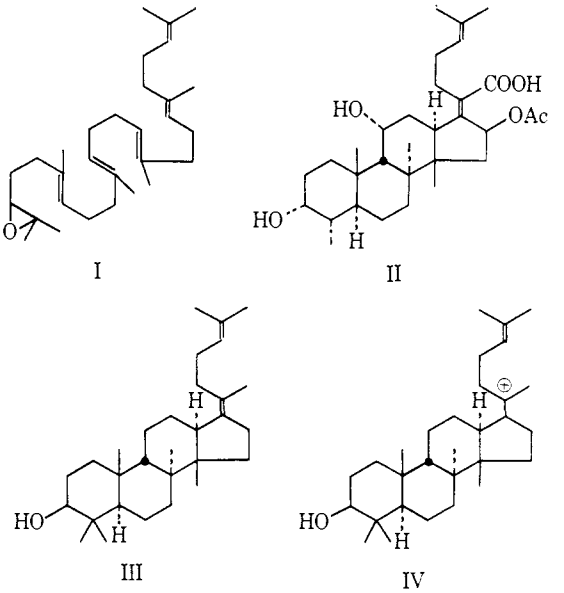
Sir:

Recent work carried out in our laboratories[1-3] and elsewhere[4,5] has established squalene 2,3-oxide (I) as an intermediate in the conversion of squalene to lanosterol and cholesterol. A further recent investigation[6] has also shown that squalene 2,3-oxide is converted to β-amyrin by an enzyme system prepared from germinating peas. In the context of earlier experimental studies[7] and theoretical considerations[8,9] of the relationship of squalene to the polycyclic terpenes and sterols, these findings strongly suggest that squalene 2,3-oxide has a general role in nature as the immediate acyclic precursor of polycyclic triterpenes that have a 3β-hydroxyl group.

A compound of particular significance in connection with theories of the mode of cyclization of squalene is fusidic acid (II),[10,11] the structure of which, it has been noted,[11] suggests a close relationship to a hypothetical tetracyclic precursor such as III. The precursor III could arise by a cyclization along the lines proposed by Eschenmoser, *et al.*,[8] with stabilization of the hypothetical intermediate carbonium ion IV by ejection of a proton from C_{17}, rather than by sequential hydride ion and methyl group shifts and loss of a proton from C_9, as proposed for the formation of lanosterol. Degradation of ^{14}C-labeled fusidic acid, prepared biosynthetically from [2-^{14}C]mevalolactone, has shown that the distribution of radioactivity conforms to the assumption of a squalene precursor,[12] and it was therefore of interest to examine the incorporation of squalene 2,3-oxide into fusidic acid by the mold *Fusidium coccineum* in order to ascertain whether, in fact, squalene 2,3-oxide served as a precursor of this antibiotic.

The preliminary results of this study, which gave clear evidence for the efficient incorporation of squalene 2,3-oxide into fusidic acid in *Fusidium coccineum*, are reported here.

[4,8,12,13,17,21-^3H]Squalene 2,3-oxide was prepared as described elsewhere,[1,3] from [^3H]squalene that had been obtained by the anaerobic incubation of [5-$^3H_2$]-mevalonic acid with the microsomal and supernatant



I II III IV

fractions of a homogenate of rat liver prepared according to Bucher and McGarrahan.[13] The [^3H]squalene 2,3-oxide was purified by tlc and thiourea clathrate formation, and 2 mg, which contained 6.43×10^6 dpm, was incorporated into 300 ml of an actively growing culture of *Fusidium coccineum*.

After 140 hr the mycelium was removed by filtration and the fusidic acid formed was isolated from the culture fluid as a benzene solvate as previously described.[10] The dried mycelium (4.2 g) was extracted with petroleum ether (bp 60–80°) and the extract was evaporated to leave an oily residue (1.05 g). This was saponified by refluxing with potassium hydroxide (10%) in 90% aqueous ethanol (5 ml) for 2 hr to yield 42 mg of an unsaponifiable residue.

Characterization of Labeled Fusidic Acid. The fusidic acid, mp 191–193° (160 mg), contained 861 dpm/mg. When 9.06 mg of this material was diluted with 11.47 mg of unlabeled fusidic acid benzene solvate and recrystallized twice, the products had 331 and 339 dpm/mg, respectively. Thus, the maximum specific activity of labeled fusidic acid is ~750 dpm/mg.

[^3H]Fusidic Acid Methyl Ester. Undiluted [^3H]fusidic acid (51.95 mg) was treated with excess ethereal diazomethane. Three crystallizations from ether–hexane gave 12.66 mg of methyl ester, mp 152–153°, having a constant specific activity (737 dpm/mg) in good agreement with the above result.

3,11-Dehydrofusidic Acid Methyl Ester. Labeled fusidic acid methyl ester (62.7 mg, 0.12 mmole, 767 dpm/mg) was oxidized in acetone at 10° with 0.26 mmole of chromic acid. After 5 min water and hexane were added. The hexane layer was washed with water, dried over sodium sulfate, and evaporated under nitrogen, giving 44.9 mg of diketo methyl ester, 682 dpm/mg. Further purification by tlc on silica gel G in 50% ethyl acetate–hexane (R_f 0.45) followed by sublimation at 125° (10^{-6} mm) gave a noncrystalline product of unchanged specific activity.

(1) E. E. Van Tamelen, J. D. Willett, R. B. Clayton, and K. E. Lord, *J. Am. Chem. Soc.*, **88**, 4752 (1966).
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(5) P. D. G. Dean, P. R. Ortiz de Montellano, K. Bloch, and E. J. Corey, *J. Biol. Chem.*, **242**, 3014 (1967).
(6) E. J. Corey and P. R. Ortiz de Montellano, *J. Am. Chem. Soc.*, **89**, 3362 (1967).
(7) For reviews see R. B. Clayton, *Quart. Rev.* (London), **19**, 168 (1965); J. A. Olson, *Ergeb. Physiol. Biol. Chem. Exptl. Pharmakol.*, **56**, 173 (1965).
(8) (a) L. Ruzicka, *Experientia*, **9**, 357, 362 (1953); (b) A. Eschenmoser, L. Ruzicka, O. Jeger, and D. Arigoni, *Helv. Chim. Acta*, **38**, 1890 (1955).
(9) G. Stork and A. Burgstahler, *J. Am. Chem. Soc.*, 77, 5068 (1955).
(10) W. O. Godtfredsen and S. Vangedal, *Tetrahedron*, **18**, 1029 (1962).
(11) W. O. Godtfredsen, W. von Daehne, S. Vangedal, A. Marguet, D. Arigoni, and A. Melera, *ibid.*, **21**, 3505 (1965).
(12) D. Arigoni, Conference on the Biogenesis of Natural Products, Accademia Nazionale dei Lincei, Rome, 1964.
(13) N. L. R. Bucher and K. McGarrahan, *J. Biol. Chem.*, **222**, 1 (1956).

The mass spectrum of the purified product showed the expected molecular ion at m/e 526 ($C_{32}H_{46}O_6$), with major fragments at m/e 466 (M − CH_3COOH), 457 (M − C_5H_9), 434, 397 (M − CH_3COOH, C_5H_9), 227, 219, 214, 205, 191, 177, 119, and 117; infrared $\nu_{max}^{CCl_4}$ 2980, 2955, 2940 (CH), 1745 (acetate), 1728 (methyl ester, α,β-unsaturated), 1712 (six-membered ring ketone), 1375, 1255, 1195, and 1165 cm^{-1}; nmr (100 Mc, CCl_4, TMS as internal reference) 5.80 (doublet, 1 H), 16β proton; 5.00 (multiplet, 1 H), vinyl proton of side chain; 3.55 (singlet, 3 H), OMe of methyl ester; 1.95 (singlet, 3 H), methyl of 16α-acetoxy group; 1.65 (broad doublet, 6 H), methyls of isopropylidene; 1.20 (doublet, 3 H), C-4 methyl; 1.09 (singlet, 3 H), tertiary methyls; 1.05 (singlet, 3 H), tertiary methyls; and 0.94 ppm (singlet, 3 H), tertiary methyls.

Hydrolysis and Isotopic Exchange. The foregoing diketo methyl ester (26 mg) was hydrolyzed by treatment for 15 hr at 25° with 3.1 ml of 1% potassium hydroxide in 95% methanol–water. The solvent was evaporated under nitrogen and the residue was dissolved in water. The solution was washed with ether and acidified with sulfuric acid and the product was extracted into ether, then converted to the methyl ester by treatment with excess diazomethane. The product (19.4 mg) had 351 dpm/mg. Oxidation of this material with an excess of chromic acid in acetone at 10° followed by standard isolation procedure gave a crude product having unchanged specific activity which, however, was not further characterized.

The mass spectrum of the hydrolysis product showed the expected molecular ion at m/e 484 ($C_{30}H_{44}O_5$), with major peaks at m/e 466 (M − H_2O), 452 (M − CH_3OH), 434 (M − H_2O − CH_3OH), 402, 384, 276, 219, 193, 177, and 164; infrared $\nu_{max}^{CCl_4}$ 3500 (broad, OH), 2980, 2960, 2940, 2880 (C–H), 1735, and 1710 cm^{-1}.

The nmr spectrum (100 Mc) was also consistent with the proposed structure, notably in the retention of the singlet methyl of the carbomethoxy group now at 3.75 ppm and the vinyl multiplet at 5.05 ppm (1 H) and the absence of the methyl of the acetoxy group at 1.95 ppm (3 H); the three singlet tertiary methyl groups at 0.95, 1.05, and 1.09 ppm can still be seen, but the methylene envelope is now more complex than in the case of the 16-acetoxy compound.

In the above conversions of fusidic acid methyl ester, tritium is lost in two stages. Based on the specific activity of the starting material (767 dpm/mg), 11% is lost on initial oxidation to the diketone and 54.2% is lost in the subsequent hydrolysis product. The predicted losses in these products are 10.5 and 58.0%, respectively. These values are calculated on the assumption of the proposed pattern of cyclization of squalene oxide and the incorporation of 6 moles of [5-$^3H_2$]mevalonate into each mole of squalene (from which the squalene oxide was synthesized) according to the established biosynthetic sequence.[7] The stereospecific loss of a tritium atom from one farnesyl residue in the course of incorporation into squalene[14] is also taken into account. Thus, only the 11β-^3H should be lost in the initial oxidation whereas the conditions of alkaline hydrolysis should remove further ^3H atoms at C_2 and C_{12} by enolization. The 16α-^3H should also be lost when the 16β-ol is exposed to alkali since epimerization of the OH group is known to occur under these conditions.[11] The observed retention of all radioactivity of the hydrolysis product on chromic acid oxidation supports the assumption that inversion has occurred at C_{16}. The loss of label in the oxidation and the final loss of label after alkaline exchange are in good agreement with the theoretical values and strongly support the identity of the labeled material with fusidic acid.

Nonsaponifiable Products. The examination of the radioactive components of the nonsaponifiable fraction is still in progress. Analysis of these materials by tlc and glpc reveals the presence of a complex mixture of labeled products among which ergosterol and lanosterol have been identified.

These results demonstrate the conversion of squalene 2,3-oxide to fusidic acid in *F. coccineum*, thereby establishing the biological importance of squalene 2,3-oxide as a precursor for the carbon skeleton of this class of antibiotics. The changes in ^3H content of the successive fusidic acid derivatives that are observed in our experiments are evidence for the intact incorporation of the squalene chain into the fusidane skeleton.

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A One-Step Synthesis of 1,5-Dienes Involving Reductive Coupling of Allyl Alcohols

Sir:

Preparation of specifically substituted and functionalized 1,5-dienes implies the need for a good method of bonding allyl units.[1] Following an earlier lead from this laboratory,[2] we have now developed a new, useful procedure for the reductive coupling of allyl alcohols, effected without isolation of intermediates through the combined action of titanium trichloride and alkyl- or aryllithium.[3]

In a typical experiment, 3.0 moles of methyllithium and 1.0 mole of titanium trichloride were mixed under nitrogen at −78° in ethylene glycol dimethyl ether. After a few minutes, 2.0 moles of geraniol was added to the solution of reagent maintained at −78°. Warming

(1) For an example of such a need, see E. E. van Tamelen, K. B. Sharpless, R. Hanzlik, R. B. Clayton, A. L. Burlingame, and P. C. Wszolek, *J. Am. Chem. Soc.*, **89**, 7150 (1967).

(2) E. E. van Tamelen and M. Schwartz, *ibid.*, **87**, 3277 (1965).

(3) Presentation of the coupling mechanism, a matter of interest in its own right, is deferred until the time of a later publication. Titanium trichloride solvate is reported to react between −50 and −80° with methyllithium in ether to give a dark green solution of unisolated product—presumed to be trimethyltitanium—which decomposes above −20° to gas and "black needles" and reacts with water to yield methane: K. Clauss and C. Beerman, *Angew. Chem.*, 71, 627 (1959).