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THE MODE OF INCORPORATION OF C-2 HYDROGEN ATOMS OF MEVALONIC ACID INTO PROTOSTEROLS AND STEROLS

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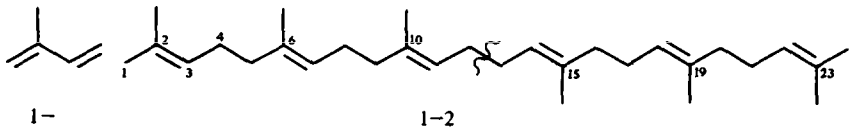
INTRODUCTION

The years 1950–1975 were a period of unprecedented developments in the fields of chemistry and biochemistry of steroids, steroid hormones and polyprenoids in general. The groups of Woodward *et al.* and of Robinson and Cornforth completed the first two total syntheses of cholesterol. At the same time, Bloch, Lynnen, Cornforth, Popjak and many others labored on problems of biosynthesis of steroids. Pincus *et al.* explored the mode of formation and role of corticosteroids. Ruzicka and his associates in Zurich developed a set of mechanistic concepts which allowed the rationalization of the mode of elaboration of polyprenoids. Barton introduced the concept of axial and equatorial bonds which changed our understanding of bond reactivity. The observations of the Upjohn group on the utility of microorganisms in the synthesis of corticosteroids was of great technological, scientific and economic importance. Then, the discovery of mevalonic acid by the Merck group revolutionized our understanding of the routes of biosynthesis of polyprenoids. From this moment on, information on the biosynthesis of polyprenoids developed in an explosive manner and continues unabated.

I was privileged to participate in a small manner in the efforts of the period. In this report, I wish to summarize a segment of our work dealing with the mode of incorporation of C-2 hydrogen atoms of mevalonic acid into protosterols and steroids. To facilitate the understanding of the problems, a brief review of the earlier work on the origin of carbon atoms of polyprenoids is included. This report is a rather personalized account and no attempt was made to cover all the literature.

ON THE ORIGIN OF CARBON ATOMS OF POLYPRENOIDS

Early in the nineteenth century it was realized that the atomic composition of rubber could be expressed in multiples of [C_5H_8] units.[1] With the discovery of other terpene hydrocarbons and carotenes, it was noted that their structures were better presented as multiples of [C_5] units. Later in the nineteenth century, isoprene (1-1) was proven to be a product of rubber pyrolysis.[2–5] Wallach realized that the structures of most of the then known terpenes could be arrived at through head to tail or some alternative coupling of isoprene units.[6] Subsequently, many new polyprenoid compounds were isolated, the structures of which could not be rationalized in the simple terms of Wallach's concepts. The composition of numerous newly-isolated compounds did not correspond to multiples of [C_5] units and



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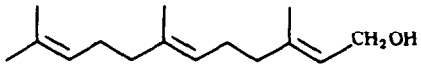

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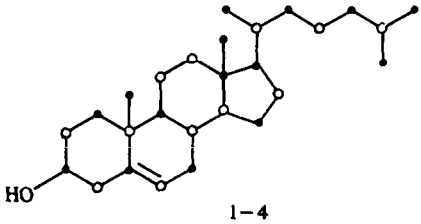
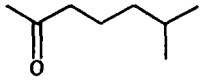


1–4

• and ○ carbon atoms derived from methyl and carboxyl moieties of acetate respectively



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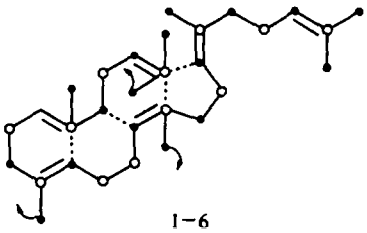

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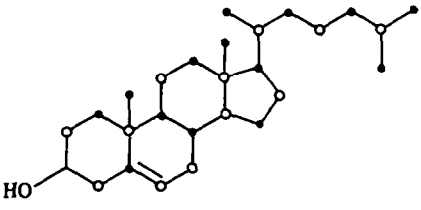

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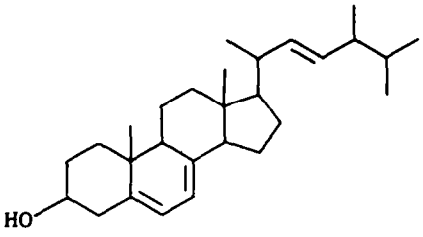


1–8

Chart 1.

their structures were the results of complicated biosynthetic rearrangements. Confronted with these problems, Ruzicka and his associates in Zurich developed an ingenious concept, the "biogenetic isoprene rule", which allowed them to interpret mechanistically the biosynthesis of polyprenoids.[7,8] The Zurich group proposed, *inter alia*, that the biosynthesis of cyclic polyprenoids was initiated by an electrophilic attack on a double bond of a linear polyprenoid substrate to give a cyclic (or polycyclic) intermediate "cation" which, in turn, can then undergo various transformations and rearrangements. Ultimately, the cation can be stabilized through the acquisition of a nucleophilic (anionic) moiety (e.g. $^-$OH) or loss of a proton to form a double bond, a cyclopropane ring, etc.

Squalene (**1-2**) was isolated by two groups[9,10] in 1916–17 and was identified as a $C_{30}H_{50}$ linear polyprenoid hydrocarbon resulting from head-to-head coupling of two farnesyl moieties[11] (**1-3**). It was noted that oxidation of cholesterol (**1-4**) gave a ketonic fragment $CH_3CO\cdot(CH_2)_3CH(CH_3)_2$ (**1-5**), which was recognized as a compound of polyprenoid origin.[13] When squalene (**1-2**) was fed to rats, an increase of liver cholesterol was observed.[12] The two observations, and the results of feeding squalene *in vivo* to rats,[12] linked linear polyprenoids and particularly squalene (**1-2**) with the polycyclic cholesterol (**1-4**). Based on these observations, and even before the structure of cholesterol was established, Heilbron and co-workers suggested that cholesterol was a polyprenoid.[11] Soon after the establishment of the structure of cholesterol,[14] Robinson realized that cholesterol could be derived from squalene and

proposed an ingenious hypothetical scheme for the process.[15] Accordingly, cyclization of squalene, folded as shown in **1-6**, could be initiated by an oxidative attack of a hypothetical $[OH]^+$ moiety. Following the elimination of the indicated (with arrows) three methyl groups, cholesterol (**1-7**) would be formed.[15]

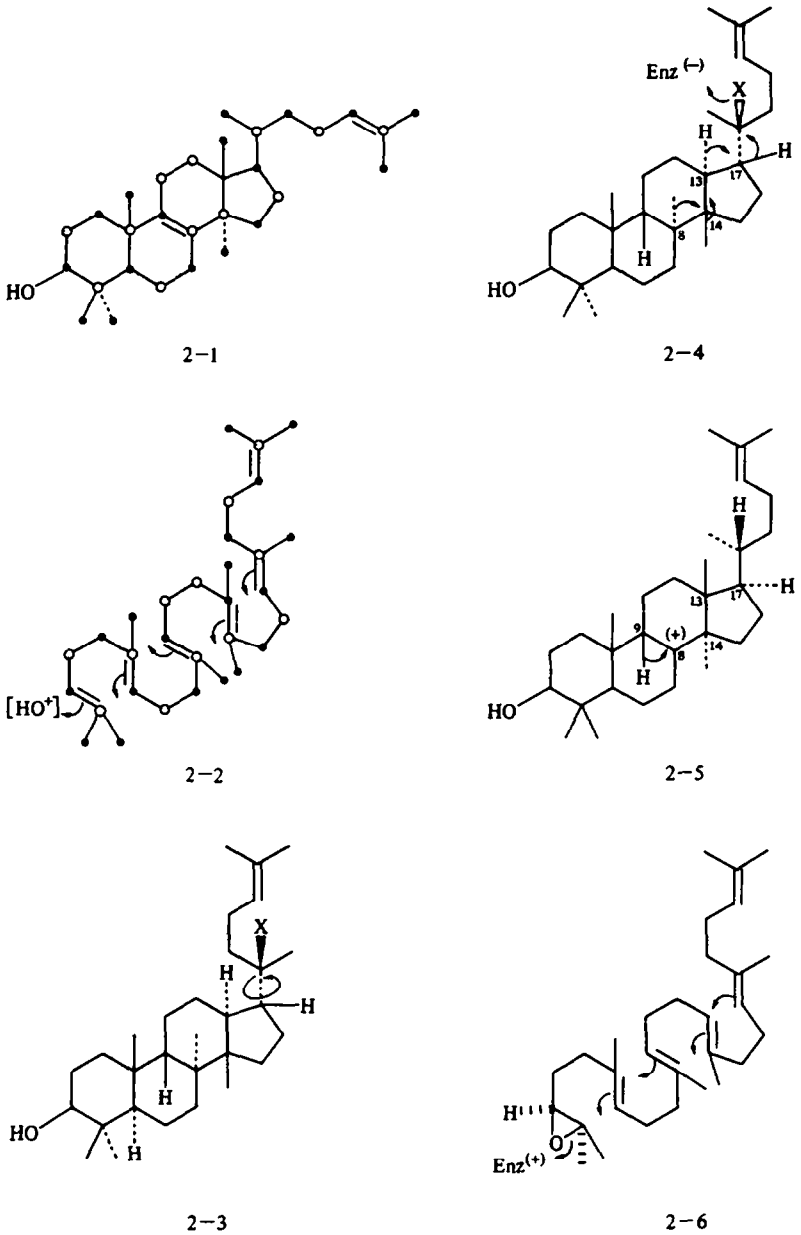
The proposed mode of squalene cyclization, although later proven incorrect,[16] set the stage for the exploration of the mechanism of the biosynthesis of sterols. Sonderhoff and Thomas[17] grew yeasts on deuterated sodium acetate and isolated an unsaponifiable residue containing mainly ergosterol (**1-8**). The residue was enriched in deuterium, thus showing for the first time that acetate was a precursor of steroids.[17] Similar experiments in animal tissues confirmed the role of acetate as the precursor of cholesterol[18,19] and showed that both carbon atoms of acetate are incorporated into steroids.[20] Soon thereafter, a direct link between acetate, squalene and cholesterol was established. ^{14}C-Squalene produced from ^{14}C-acetate in rat liver,[21] when fed to mice, gave ^{14}C-cholesterol.[22] These observations stimulated intensive efforts to determine the mode of incorporation of the *methyl* and *carboxyl* carbon atoms of acetate into squalene[22] and cholesterol.[23–25] It was found that the carboxyl and methyl carbon atoms were incorporated into squalene[22] in a manner expected for coupling of isoprenoid units.[23] In cholesterol, the acetate carbon atoms were located as shown in **1-4**.

It then became possible to evaluate Robinson's hypothesis of squalene cyclization.[15] Cyclization of squalene (**1-6**) in the Robinson mode would result in cholesterol in which methyl and carboxylic carbon atoms of acetate would be distributed as shown in **1-7**. However, the experimentally determined distribution of the methyl and carboxylic carbon atoms (**1-4**) differed at C7, 8, 12 and 13 from that expected according to Robinson's hypothesis (**1-7**). That both, carbon[26,27] 7 and carbon[16] 13 originated from methyl carbons of acetate and not from carboxylic carbons of acetate was proven (loc. cit.), thus making Robinson's hypothesis untenable. Although disproven in mechanistic details, Robinson's contribution was enormously valuable since it provided a conceptual link between squalene, steroids, and triterpenes.

When the structure of lanosterol (**2-1**) was established,[28] Woodward and Bloch[16] suggested that lanosterol (**2-1**) was the primary cyclization product of squalene en route to cholesterol. They proposed that oxidative cyclization of squalene folded as in **2-2** will give the C-20 cation **2-3a**. Following 120° rotation around the 17(20) bond of **2-3a**, cation **2-4a** will be formed. Cation **2-4a** may undergo the indicated four 1 → 2 shifts, whereby C-17β(H) → C-20(H); C-13α(H) → C-17α(H); C-14β(CH_3) → C-13β(CH_3); and C-8α(CH_3) → C-14α(CH_3) to give the C-8 cation **2-5**. Following the loss of the C-9β-hydrogen atom of **2-5**, lanosterol (**2-1**) will be formed which, in turn, will be metabolized to cholesterol (**1-4**). The distribution of methyl and carboxylic carbon atoms of acetate in squalene[22] (**1-2**; **2-2**) and in the cholesterol[23–27] (**1-4**) derived via lanosterol (**2-1**) will be in accord with that found experimentally. The biosynthetic conversion of squalene to lanosterol[29] and of the latter to cholesterol[30] was then proven.

The cyclization of squalene was thought to be a non-stop process initiated by an attack of a hypothetical oxidative entity $[OH]^+$ on C-3 of squalene (**2-2**). Accordingly, it was proven that molecular oxygen [$^{18}O_2$] and not oxygen from the water of the medium [$H_2{}^{18}O$] was the source of the oxygen of the 3β-hydroxy group of lanosterol.[31] Later, it was shown that cyclization of squalene is preceded by the formation of the stable intermediate 2,3(*S*)-oxido squalene[32–34] (**2-6**). The oxygen atom of the epoxide is derived from molecular oxygen[35] and is incorporated as the C-3β-hydroxyl in lanosterol.[36]

It had been presumed that cyclization of oxido squalene (**2-6**) was started by the enzymatic cleavage of the carbon-2 and oxygen bond which generates an electron deficiency at C-2 and this initiates the non-stop cyclization yielding the hypothetical C-20 cation **2-3a** which, following rearrangements to **2-4a** and **2-5**, gives lanosterol (**2-1**). However, Cornforth considered it rather unlikely that all these complex transformations could occur as a non-stop process on a single enzyme.[37] He therefore suggested the possible involvement of several enzymes and the "stepwise" formation of transiently stabilized intermediates. Thus, after the "stepwise" elaboration of the tetracyclic moiety, the evolving C-20 cation could be transiently stabilized (**2-3b**) by a nucleophilic prosthetic group [X] which could either be "free" or be part of the enzyme system.[37] Thus the transiently stabilized intermediate could be partially or completely released from the enzyme surface. Assuming a partial desorption and a retained attachment of the intermediate to the enzyme via the prosthetic group [X] (**2-3b**), the side chain or the tetracyclic moiety could now rotate (120°) around the C-17(20) bond to give the reoriented intermediate (**2-4b**). It may be noticed that in **2-4b**, the C-20 prosthetic group [X], the 17β- and 13α-



a, X = (+) charge
b, X = prosthetic group

Chart 2.

hydrogen atoms and the 14β- and 8α-methyl groups are positioned "antiparallel" for facile Meerwein–Wagner rearrangements, as required by the hypotheses of polyprenoids' elaboration.[7,8,37]

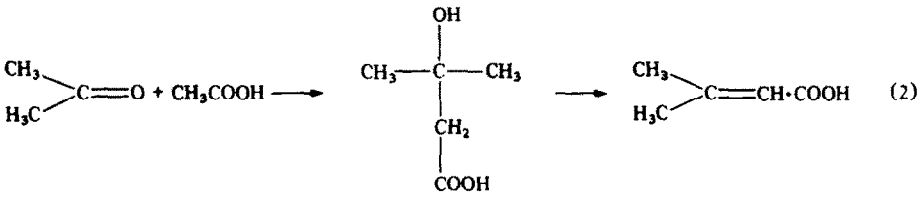
In any event, evidence in support of the formation of a C-20-cationic intermediate has been obtained.[38] The migration of methyl groups as proposed by Woodward and Bloch[16] was proven and it was shown that the rearrangements are intramolecular and involve two 1 → 2 migrations of methyls and not a single 1 → 3 methyl migration.[39,40] The expected sequential 1 → 2 shifts of hydrogen atoms from 17β to 20 and 13α to 17α were confirmed.[41–43]

Then the sequence of metabolic transformation of lanosterol to cholesterol started to unravel rapidly (see below). In contrast, the understanding of the events between *acetate* and squalene was stalled. That acetate is a precursor of many natural products was known and, therefore, it was not surprising that its incorporation into steroids was low. It was realized that acetate is very likely

metabolized first to a larger entity which is the actual precursor of polyprenoids. Unfortunately, the search for that intermediate remained elusive. The transformation of acetate into acetoacetate and to 3-hydroxy-3-methyl glutaric acid, which was thought to be a precursor of polyprenoids, was proven. However, the incorporation of 3-hydroxy-3-methyl glutaric acid into steroids was only of the order[48a] of 0.16%.

Based on their work on the biosynthesis of rubber, Bonner and Arreguin[44] proposed that acetoacetate could undergo decarboxylation to yield acetone and carbon dioxide (Eq. 1). The acetone could then condense with another molecule of acetate and the resulting product, following the loss of water, would give β-methylcrotonate (Eq. 2). The β-methylcrotonate could be the precursor of the C_5-isoprene unit (Eq. 3).

$$2CH_3COOH \longrightarrow CH_3 \cdot CO \cdot CH_2 \cdot COOH \longrightarrow CH_3 \cdot CO \cdot CH_3 + CO_2 \qquad (1)$$



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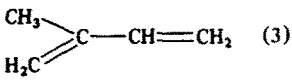


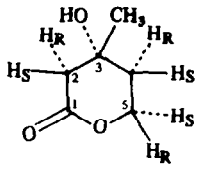
(3)

Again, the likelihood of β-methylcrotonate being a precursor of polyprenoids was minimal, since it was poorly incorporated (3.8%) into cholesterol.[48a]

The solution to the problem of the precursor of polyprenoids came from an unrelated, fortuitous study of microbial metabolism. A Merck group[45–47] identified mevalonic acid (**3-1**) as the acetate-replacing factor which promoted the growth of the acetate-dependent *Lactobacillus acidophilus*. The isolated optically active mevalonic acid (**3-1**) ($C_6H_{10}O_3$) had 3*R*-stereochemistry. Its gross similarity to 3-hydroxy-3-methyl glutaric acid was apparent and, therefore, its role as a steroid precursor was tested.[48] When (3*RS*) [1-^{14}C]-MVA was incubated with a rat liver preparation, it was noted that the isolated cholesterol was not radioactive,[48b] but instead $^{14}CO_2$ was produced which contained *ca* 50% of the incubated radioactivity.[48b] Since racemic (3*RS*) [1-^{14}C]-MVA was the substrate, it follows that only one of the optical isomers was metabolized and that its carboxylic moiety was eliminated as CO_2. The remaining C_5-unit could be the long sought for biological "isoprene-entity".

When (3*RS*) [2-^{14}C]-MVA was incubated with a rat liver homogenate, the isolated cholesterol contained *ca* 41.7% of the incubated radioactivity.[48a,b,49] Considering that only 50% of the (3*RS*) [2-^{14}C]-MVA was biologically active, it follows that *ca* 83.3% of the radioactivity was incorporated into cholesterol. Since only five of the six carbon atoms of MVA are incorporated into polyprenoids, hence squalene ($C_{30}H_{50}$) derived from 2-^{14}C-MVA will contain six C_5-units and will have six ^{14}C-carbon atoms. Therefore, the incorporation of 83.3% of ^{14}C into cholesterol revealed that 5-^{14}C atoms derived from the biologically active [2-^{14}C]-MVA were present in the cholesterol. This is in accord with the sequence 6[2-^{14}C]-MVA → [$^{14}C_6$]-squalene → [$^{14}C_6$]-lanosterol → [$^{14}C_5$]-cholesterol. Indeed, it was proven that squalene[49–52] (**3-2**) (lanosterol) and cholesterol[53] (**3-3**) biosynthesized from [2-^{14}C]-MVA were labeled as shown.

The question of the structure of the C_5-units derived from MVA and the mode of coupling of such units into linear polyprenoids still remained. It was Bloch *et al.*[54] and Lynen *et al.*[55] who showed that decarboxylation of [2-^{14}C]-MVA-pyrophosphate results in isopentenyl-pyrophosphate (**3-4**) in which all the ^{14}C is located at the methylene carbon.[55] The isopentenyl-pyrophosphate (**3-4**) is then isomerized to dimethylallyl-pyrophosphate[56,57] (**3-5**) and the two are coupled to give geranyl-OPP (**3-6**). The coupling of geranyl-OPP (**3-6**) with an additional molecule of isopentenyl-OPP (**3-4**) yields farnesyl-OPP (**3-7**) (see e.g. Ref. 58). Eventually, two farnesyl-OPP molecules (**3-7**) combine in an asymmetric process[58–60] to give squalene (**3-2**). The formation of squalene proceeds with the loss of a



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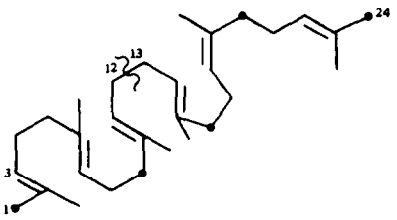
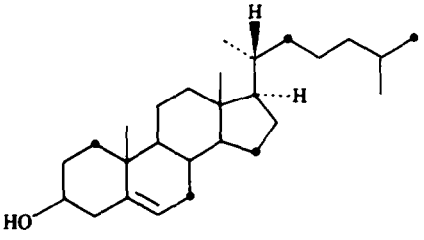


3-2

• ≡ Carbons [^{14}C] derived from C-2 of MVA



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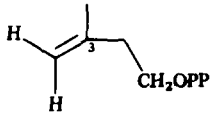

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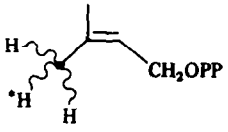

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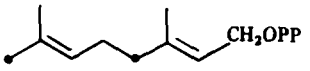

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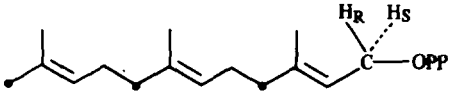


3-7

Chart 3.

1 pro *S* hydrogen atom of one of the farnesyl-OPP esters (**3-7**) and inversion of configuration of C-1 protons of the other farnesyl-OPP ester. The removed hydrogen atom is replaced in the course of squalene elaboration by a 4 pro *S* hydrogen atom of NADPH which assumes the 12 pro *R* configuration in squalene.[58–60] The coupling proceeds via presqualene-OPP[61] apparently as shown (Chart 4). The distribution of C-2(C) carbon atoms of MVA in squalene and cholesterol was proven to be in accord with the Woodward–Bloch scheme.[16] This essentially completed the century-old search for the origin of carbon atoms of steroids.

THE MODE OF COUPLING OF C_5-UNITS

Except where necessary to understand the biosynthetic sequence, we will limit our discussion to the events taking place at carbon atoms derived from C-2 of MVA. It may be pointed out that C-1 and C-4 of IPP (**3-4**) and DMAPP (**3-5**) are derived from C-5 and C-2 of MVA (**3-1**), respectively. Studies of the biosynthesis of squalene[49–52] indicated that carbon atoms derived from C-2 of MVA are located at both termini of the molecule (**3-2**). The remaining four carbon atoms derived from C-2 of MVA are coupled to carbon atoms derived from C-5 of MVA.[58]

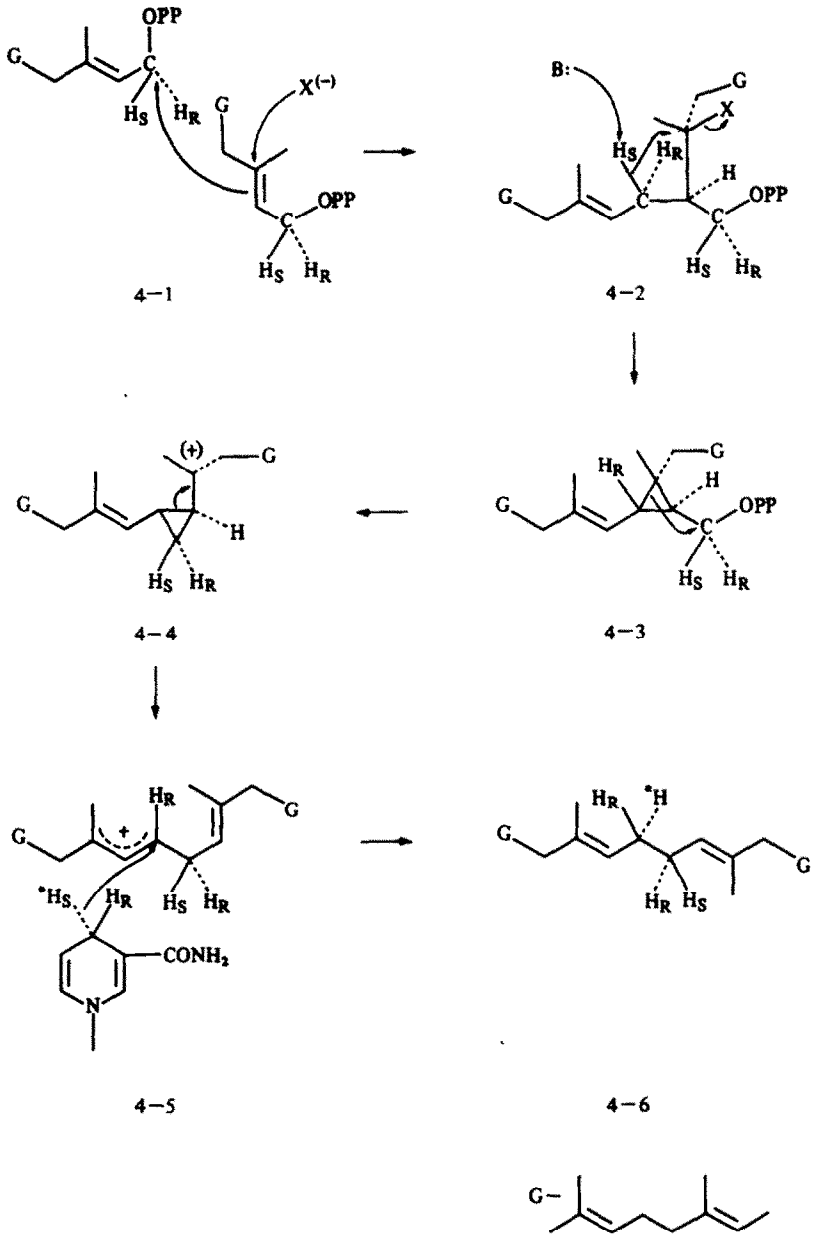


Chart 4.

It was previously mentioned that isopentyl-OPP (**3-4**) is isomerized to dimethylallyl-OPP (**3-5**) and then the two are coupled to give geranyl-OPP (**3-6**). In the context of these transformations, two questions had to be answered: (1) the manner in which a proton is added at C-4 of isopentenyl-OPP (**3-4**) during its isomerization to dimethylallyl-OPP (**3-5**), and (2) the stereochemical consequences of coupling of the C-4 methylene carbon atoms of isopentenyl-OPP (**3-4**) to C-1 carbon atoms of dimethylallyl-OPP (**3-5**) (or geranyl-OPP (**3-6**)).

Answers to these questions were made possible through the elegant efforts of Popjak and Cornforth and their associates.[58] These authors realized that the study of the stereochemistry of elaboration of polyprenoids can be best carried out with the use of mevalonic acid stereospecifically labeled with isotopic hydrogen atoms at the relevant carbon atoms[58,60,62,63] (4, 2 or 5). Accordingly, they devised the syntheses of two racemic mixtures of (3*RS*)-MVA (**5-1**; **5-2** and **5-3**; **5-4**), the components of which were stereospecifically labeled with tritium (or deuterium)[60] at C-4. Since only the 3*R* isomers are enzymatically phosphorylated and metabolized,[64,65] it follows that each of the two MVA racemates contains a single (3*R*)-active species (**5-1** and **5-3**) stereospecifically labeled with tritium at C-4. Hence,

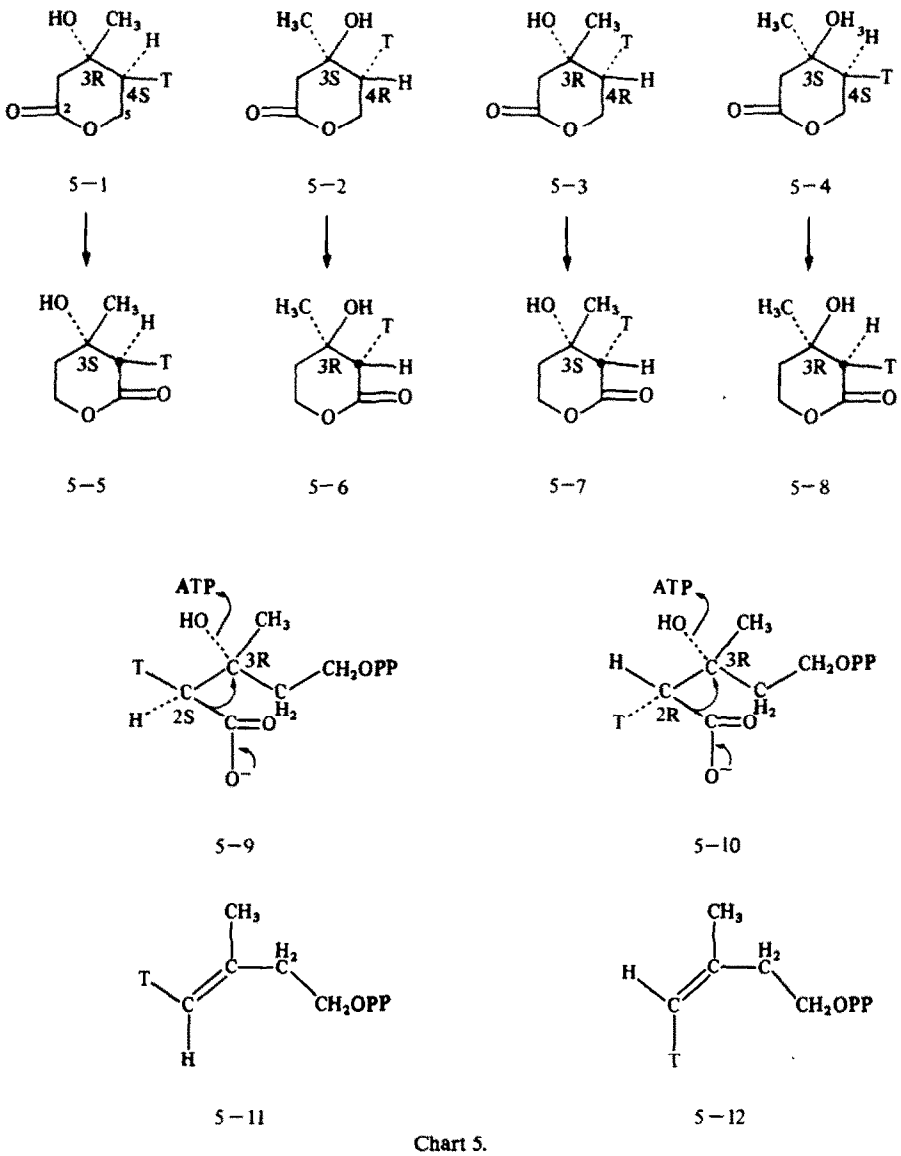
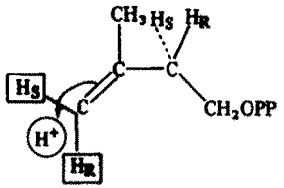


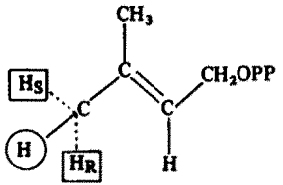
Chart 5.

only the [3*R*; 4*R*-^{3}H]-MVA (**5-3**) and [3*R*; 4*S*-^{3}H]-MVA (**5-1**) will be metabolized, while the [3*S*; 4*S*-^{3}H]-MVA (**5-4**) and [3*S*; 4*R*-^{3}H]-MVA (**5-2**) can be disregarded. It should be pointed out that in the rat, yeast, *F. coccineum* and *T. pyriformis*, the isomerization of isopentyl-OPP (**6-1**) to dimethylallyl-OPP (**6-2**) proceeds with the stereospecific *elimination* of the 2 pro *R* hydrogen atom of isopentenyl-OPP (**6-1**) derived from 4 pro *S* of MVA and *retention* of the 2 pro *S* hydrogen atom of IPP (**6-1**) derived from 4 pro *R* of MVA.[60] The same steric pattern of elimination of hydrogen atoms derived from 4 pro *S* of MVA and retention of hydrogen atoms derived from 4 pro *R* of MVA persists in the formation of geranyl-OPP, farnesyl-OPP[60] and squalene.[58,60,66,67] Hence squalene biosynthesized from (3*R*; 2-^{14}C; 4*R*-^{3}H) retained six tritium atoms (**6-4**), while squalene biosynthesized from (3*R*; 2-^{14}C; 4*S*-^{3}H) was devoid of tritium.[58,60,66] Accordingly, lanosterol (**6-5**) and cholesterol (**6-6**) derived from [3*R*; 4*R*-4-^{3}H]-MVA retained five and three tritium atoms, respectively.[66]

The rationalization[7,8,37] of the formation of lanosterol and cholesterol rests on the logical assumption that the C-20 cation (**2-3**) rearranges to **2-4** and **2-5**, to yield lanosterol[16] (**2-1**). However, direct evidence for the intermediacy of the C-20 cation was lacking. The fungus *Fusidium coccineum* biosynthesizes the protosterol fusidic acid (**7-2**), small amounts of other protosterols and ergosterol.[68] The biosynthesis of the protosterol fusidic acid (**7-2**) can be rationalized in terms of stabilization of the C-20 cation (**2-3**) through the loss of the C-17 hydrogen atom to give protosterol (**7-1**) which is then



6–1



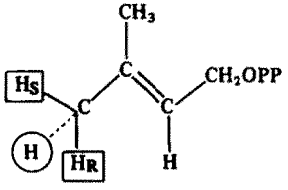
6–2

H_R and H_S indicate hydrogen atoms derived from 2 pro R and 2 pro S of MVA.

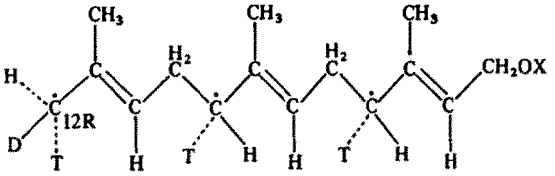
(H) proton from the medium.

H_R and H_S denote the prochirality at C-2 of IPP. H_R originated from 4 pro S and H_S from 4 pro R of MVA

• Carbon atoms derived from C-2 of MVA T = ^3H



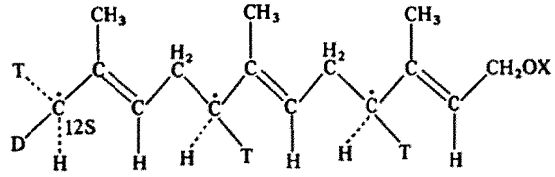
6–3



6–7

a X = OPP

b X = H



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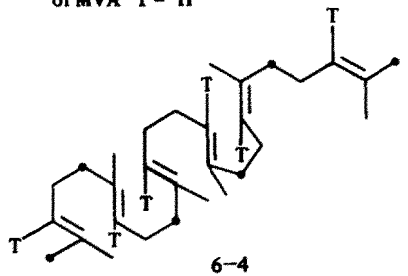

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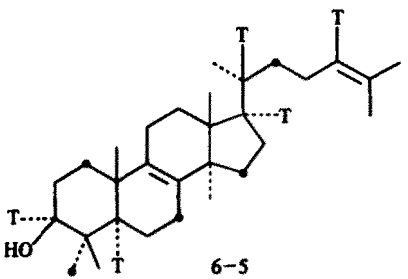
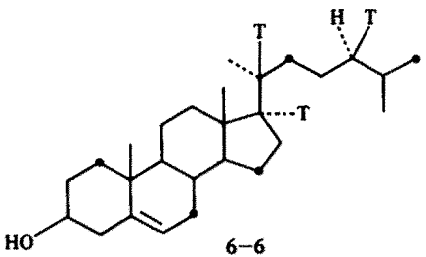
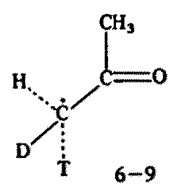


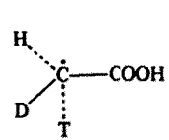
6–5



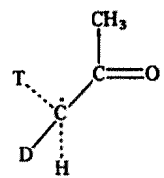
6–6



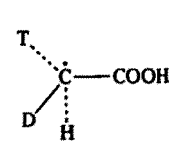
6–9



6–11

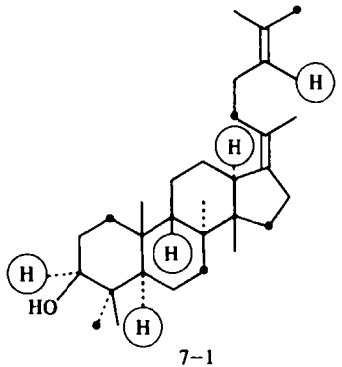


6–10

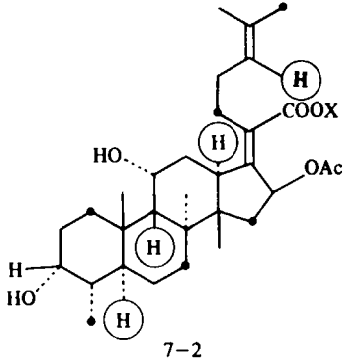


6–12

Chart 6.

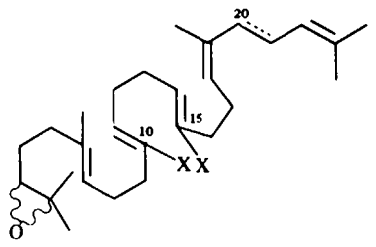


7-1



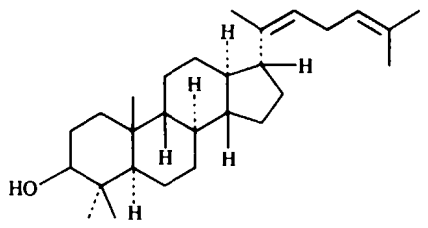
7-2

a (H) = H, X = H
b (H) = T, X = H

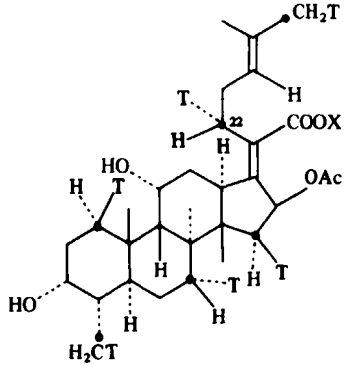


7-5

a X = H, No Δ^{20}
b X = CH_3, Δ^{20}

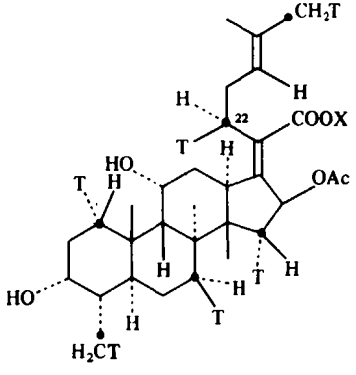


7-6

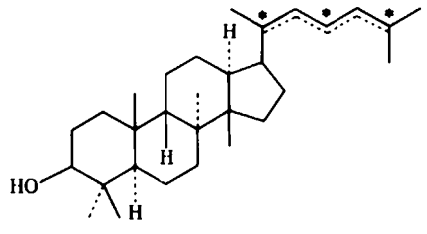


7-3

a X = H
b X = CH_3

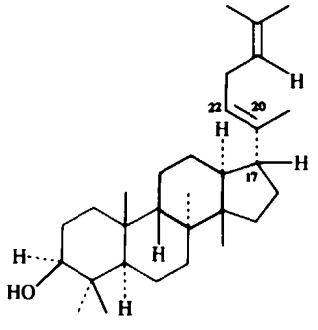


7-4



7-7

• Possible locations of a hydroxyl moiety



7-8

Chart 7.

metabolized to fusidic acid (**7-2**). Accordingly, the 3α-, 5α-, 9β-, 13α- and 24-hydrogen (tritium) atoms of the protosterol (**7-1**) should be derived from the 4 pro *R* hydrogen (tritium) of MVA.[38] The metabolic transformation of **7-1** to fusidic acid (**7-2**) involves, among others, the removal of the 4β-methyl[68] (derived from 3′ of MVA) and of the 3α-hydrogen (tritium) atoms (derived from 4 pro *R* of MVA).[38] It follows that if indeed the C-20 cation is involved in the biosynthesis of protosterols and steroids (ergosterol), the fusidic acid (**7-2b**) biosynthesized from (3*RS*, 4*R*) [2-^{14}C; 2-^{3}H]-MVA should retain four tritium atoms, two of which must be located at the critically important 9β- and 13α-positions and two at 5α- and 24-positions.[38] Indeed, we have proven that the fusidic acid (**7-2b**) retained four tritium atoms, two of which were located at the mechanistically important 9β- and 13α-positions and two at 5α- and 24-positions.[38] These findings support the biosynthetic participation of the C-20 cation (**2-3**).

For the synthesis of mevalonic acid stereospecifically labeled at C-2 (Chart 5), Cornforth *et al.*[62] took advantage of the (3*S*; 4*R*-^{3}H) (**5-2**) and (3*S*; 4*S*-^{3}H) (**5-4**) which accompanied the (3*R*; 4*S*-^{3}H) (**5-1**) and (3*R*; 4*R*-^{3}H) (**5-3**) isomers.[58,60] By transposing the carboxylic and the primary hydroxy moieties, the C-3 chiralities of all four enantiomers were inverted and the isotopic hydrogens were now located at C-2.

Thus, oxidation of the C-5 hydroxyls to carboxylic groups and selective reduction of the initial carboxylic moieties (as methyl-esters) of the specimens derived from (3*R*)-MVA (**5-1** and **5-3**) resulted in their inactivation,[64,65] since they acquired the 3*S*-stereochemistry (**5-5** and **5-7**). In contrast, analogous treatment of (3*S*; 4*R*-^{3}H) (**5-2**) and (3*S*; 4*S*-^{3}H) (**5-4**) gave the biologically active (3*R*; 2*S*-^{3}H)-MVA (**5-6**) and (3*R*; 2*R*-^{3}H)-MVA (**5-8**), respectively.[58,62]

The availability of MVA stereospecifically labeled at C-2 with *deuterium* or *tritium* allowed the determination of geometry of the elimination of the carboxylic moiety and of the C-3 hydroxyl of MVA-OPP.[58,62] It was shown that the reaction is an anti-elimination process whereby the (2*R*; 2-^{3}H)-MVA-OPP (**5-10**) gave the *Z*-(**5-12**), while the (2*S*; 2-^{3}H)-MVA-OPP (**5-9**) gave the *E*-(**5-11**) isopentenyl-OPP.[62] Based on the proven geometry of the isotopic hydrogen atoms at the C-4-methylene carbons of **5-12** and **5-11**, it was possible to define the steric mode of protonation at C-4 in the isomerization of IPP (**5-11** and **5-12**) to dimethylallyl-OPP.[69] Stereospecific protonation at C-4 of **6-1** could proceed to give **6-2** or **6-3**. Incubation of samples of [3*RS*; 2-^{14}C; 2*R*-2-^{3}H]- (**5-7** + **5-8**) and [3*RS*; 2-^{14}C; 2*S*-2-^{3}H]-MVA (**5-5** + **5-6**) (● = ^{14}C) with pig liver preparations in *deuterium oxide* gave samples of "*R*"- and "*S*"-farnesyl-OPP. The "*R*"- and "*S*"-farnesyl-OPPs (**6-7a** and **6-8a**, respectively) were saponified and the resulting alcohols (**6-7b** and **6-8b**) were ozonized. The obtained samples of acetone (**6-9** and **6-10**) were oxidized (iodoform reaction) to yield two specimens of chiral acetic acid (**6-11** and **6-12**). The *chiral acetic acids* were derived from the terminal carbon atoms of the farnesols which must have originated from C-2 of MVA. "*R*"-acetic acid[70,71] was obtained from farnesol (**6-7**) derived from incubation of (2*R*) [2-^{3}H]-MVA,[69] while "*S*"-acetic acid[70,71] was obtained from farnesol (**6-8**) derived from incubation of (2*S*) [2-^{3}H]-MVA. The results lead to the conclusion that a deuteron was added from the 3 *re*–4 *re* face of the double bond of isopentenyl-OPP.[69]

STEREOCHEMISTRY OF HYDROGEN (TRITIUM) ATOMS ORIGINATING FROM 2 pro *R* AND 2 pro *S* OF MVA IN FUSIDIC ACID

Earlier, we indicated that the organism *F. coccineum* produces protosterols (e.g. fusidic acid (**7-2**)) and sterols (e.g. ergosterol)[68] from a common precursor, the C-20 cation[38,72,73] (**2-3**). The biosynthesis of protosterols (e.g. **7-1**) proceeds through the loss of the 17-hydrogen atom of the C-20 cation[38,72,73] (**2-3**). In contrast, the elaboration of ergosterol requires the rearrangement of the C-20 cation (**2-3**), first to **2-4**, then to **2-5** which, in turn, following the loss of the C-9 hydrogen atom, gives lanosterol. Subsequently, lanosterol is metabolized to ergosterol. It is apparent that in *F. coccineum*, two routes of biosynthetic stabilization of the C-20 cation function in parallel.

Considering that lanosterol and fusidic acid are biosynthesized from MVA via squalene and the C-20 cation (**2-3**), it may be accepted with certainty that the distribution and orientation of the C-2 hydrogen atoms of MVA in *lanosterol*, and *fusidic acid* are the same. For the projected studies on the modes of elaboration of cholesterol and ergosterol from lanosterol and of phytosterols from cycloartenol, we required *reference* information on the stereochemistry of the incorporated C-2 hydrogen atoms of MVA into the respective parent triterpenes. We considered fusidic acid an appropriate model for the "base line" determination and we undertook to define the stereochemistry of the tritium atoms in fusidic acid derived from 2 pro *R* and 2 pro *S* hydrogens of MVA.

To this end, (3*RS*, 2*R*) [2-^{14}C; 2-^{3}H]-MVA and (3*RS*, 2*S*) [2-^{14}C; 2-^{3}H]-MVA were incubated with *F. coccineum* and "*R*"-fusidic acid (**7-3a**) and "*S*"-fusidic acid (**7-4a**) were isolated.[72,73] Based on the ratio of tritium to ^{14}C of the derived methyl fusidates (**7-3b**; **7-4b**) and knowing that 6-^{14}C carbons were incorporated,[68–74] it was calculated that "*R*"-fusidic acid contains *ca* 6 tritium atoms while "*S*"-fusidic acid contains only 5.3 tritium atoms.[72,73] While the formation of "*R*"-fusidic acid proceeded as expected with the incorporation of *ca* 6 tritium atoms, the loss of 0.7 atom of tritium in the biosynthesis of *S*-fusidic acid was rather unexpected.

Corey *et al.* noted that cyclization of 2,3(*RS*)-oxido-10,15-bis-nor-squalene (**7-5a**) with hog liver oxidosqualene cyclase produced the $\Delta^{20(22)}$-protosterol[75] (**7-6**). Similarly, incubation of 2,3(*RS*)-oxido-20-dehydro-squalene (**7-5b**) with the hog liver oxido cyclase resulted in protosterol (**7-7**) having a hydroxy-dien side chain.[76] In view of these observations, we considered the possibility that the C-20 cation (**2-3**) could initially give a $\Delta^{20(22)}$-protosterol (**7-8**) which would then rearrange to yield the 17(20)-isomer (**7-1**). Abstraction of a hydrogen derived from 2 pro *S* of MVA from C-22 of cation **2-3** in the elaboration of $\Delta^{20(22)}$ could account for the observed loss of tritium.

Enzyme-mediated allylic rearrangements were reported on numerous occasions. In the biosynthesis of cholesterol, isomerization of $\Delta^{8(9)} \rightarrow \Delta^{7(8)}$ (see below) proceeds with the loss of a C-7 allylic hydrogen atom,[77] while isomerization of $\Delta^{5(6)} \rightarrow \Delta^{4(5)}$ involves (partial) transfer of 4β(H) to 6β(H) position.[78] Based on our results on the incorporation of (3*RS*, 4*R*) [2-^{14}C; 4-^{3}H]-MVA into fusidic acid it may be concluded that if indeed a $\Delta^{20(22)}$ intermediate (**7-8**) is formed, its rearrangement to $\Delta^{17(20)}$ (**7-1**) must proceed with the loss of the C-17 hydrogen atom derived from 4 pro *R* of MVA.[38] In the hypothetical $\Delta^{20(22)} \rightarrow \Delta^{17(20)}$ shift, reprotonation at C-20 could occur stereospecifically from the *re* or *si* site or be non-stereospecific and take place from both sites.

To assess the hypothesis of a $\Delta^{20(22)}$ intermediate, it was necessary to evaluate the situation at C-22. To this end, the biosynthesized "*R*"- and "*S*"-fusidic acids (**8-1a** and **8-2a**, respectively) were hydrogenated and the resulting 24(25)-dihydro-acids (**8-1b** and **8-2b**) were treated with diazomethane. The obtained "*R*"- and "*S*"-methyl 24(25)-dihydrofusidates (**8-1c** and **8-2c**) were ozonized to yield upon reductive workup ($LiAlH_4$) a mixture of alcohols from which the "*R*"- and "*S*"-[3,7-$^{14}C_2$; 3,7-$^{3}H_2$]-6-methyl-hepta-1,2-diols (**8-3** and **8-4**, respectively) were isolated.[72,73] The diols were cleaved (HIO_4) and the obtained *R*-(**8-5**) and *S*-(**8-6**) aldehydes were submitted to Baeyer–Villiger oxidation (trifluoroperacetic acid). The resulting mixtures of products were reduced (LAH) to give, following GLC fractionation, homogeneous samples of "*R*"- and "*S*"-[1,5-$^{14}C_2$; 1,5-$^{3}H_2$]-4-methylpentan-1-ols (**8-7** and **8-8**, respectively). It may be recalled that Baeyer–Villiger oxidations of carbonyls proceed with retention of configuration.[79] It follows therefore that the hydroxyl moieties of the "*R*"- and "*S*"-alcohols (**8-7** and **8-8**, respectively) have the orientation of the cleaved C-1(2) bonds of their parent aldehydes (**8-5** and **8-6**). Results of the above transformations are summarized in Table 1.

Oxidation of the "*R*"-alcohol (**8-7**) (Table 1, entry 6A) with yeast alcohol dehydrogenase (YAD) and NAD proceeded with the loss of tritium to give 4-methylpentanal (**8-9**) (Table 1, entry 7A). Since the NAD–YAD oxidation proceeds with abstraction of 1 pro *R* hydrogen atom, the results establish the (1*R*)-chirality of **8-7** derived from "*R*"-fusidic acid. In contrast, analogous oxidation of the "*S*"-alcohol (**8-8**) (Table 1, entry 6B) to the aldehyde (**8-10**) (Table 1, entry 7B) proceeded without loss of tritium. However, retention of the tritium by the "*S*"-aldehyde (**8-10**) does not constitute proof of the presence of tritium at C-1 of the "*S*"-alcohol (**8-8**) and, hence, at C-22 of the parent "*S*"-fusidic acid. To confirm the presence of tritium at C-22, the dihydro-"*S*"-fusidic acid (**8-2b**) was converted (LiCl–DMF) to the *E*-diene-ester (**8-11**). Oxidation of the diene (**8-11**) (RuO_4) gave after treatment with diazomethane and vacuum line distillation methyl[1,5-$^{14}C_2$; 5-$^{3}H_1$] 4-methyl-1-pentanoate (**8-12**) which retained only one tritium atom (**8-8**) (Table 1, entry 8B). The loss of 50% tritium indicates that tritium is distributed equally at C-1 and C-5 of **8-8** and hence at C-22 and C-26 of "*S*"-fusidic acid (**8-2a**). The results establish the presence of tritium at C-1 of **8-8** and the (1*S*)-chirality of the alcohol. It may therefore be concluded that the 2 pro *R* and 2 pro *S* protons of MVA were incorporated at C-22 of fusidic acid without scrambling. These observations exclude the participation of a stable C-20(22) olefinic intermediate (**7-8**) in the elaboration of the protosterol (**7-1**). However, the question of the observed loss of tritium still remained and we will return to this topic later (see below).

The methyl groups derived from C-2 of MVA have the *E*-geometry at the terminal isopropylidene moieties of farnesol,[80] squalene oxide and lanosterol.[81] Since fusidic acid is biosynthesized from squalene, via squalene oxide,[82] it follows that the C-25-*E*-methyl of fusidic acid must originate from C-2 of MVA and, therefore, C-26 of "*R*"- and "*S*"-fusidic acids (**8-1a** and **8-2a**, respectively) will be labeled

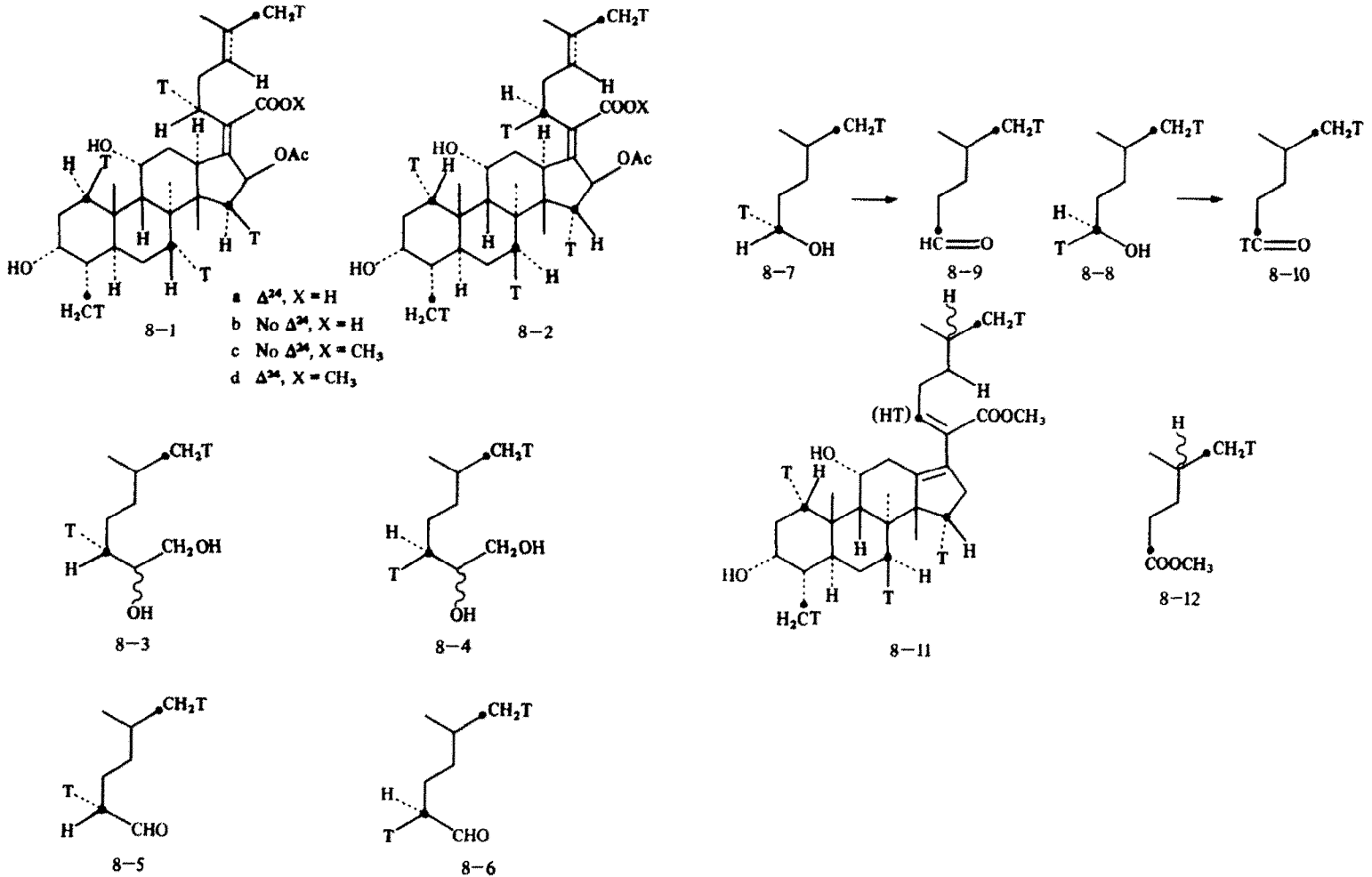

8–1
8–2
a Δ24, X = H
b No Δ24, X = H
c No Δ24, X = CH3
d Δ24, X = CH3
8–3
8–4
8–5
8–6
8–7
8–8
8–9
8–10
8–11
8–12

Chart 8.

Table 1. Distribution and stereochemistry of tritium atoms in fusidic acid biosynthesized from (3*RS*, 2*R*)- and (3*RS*, 2*S*) [2-^{14}C; 2-^{3}H]-MVA by *Fusidium coccineum*

		A "R"-products		B "S"-products	
		$^{3}H:^{14}C$ Ratio			
NO	Compound	Isotopic	Atomic	Isotopic	Atomic
1	MVA-benzhydrylamide	5.2	1.0:1	5.3	1.0:1
2	Methyl fusidate (8-1d or 2d)	5.1	6.0:6	4.7	6.0:6
3	Methyl dihydro fusidate (8-1c or 2c)	5.1	6.0:6	4.6	6.0:6
4	6-methyl heptane-1,2-diol* (8-3 or 4)	4.9	2.0:2	4.6	2.0:2
5	5-methyl hexane-1-al** (8-5 or 6)	5.0	2.0:2	4.7	2.0:2
6	4-methyl pentanol* (8-7 or 8)	5.0	2.0:2	4.6	2.0:2
7	4-methyl pentanal** (8-9 or 10)	3.0	1.2:2	4.5	1.95:2
8	Methyl 4-methyl pentanoate (8-12)			2.4	1.0:2
9	26-nitrile (9-4)	4.5	5.3:6		
10	26-nor-Me fusidate (9-5)		5.3:6		
11	7α-hydroxy-Me-fusidate (10-2 or 5)	4.3	5.1:6	4.8	6.2:6
12	3,7,11-triketone (10-3 or 6)	4.2	5.0:6	3.9	5.0:6
13	15,17(20)-diene	5.0	6.0:6	4.0	5.1:6

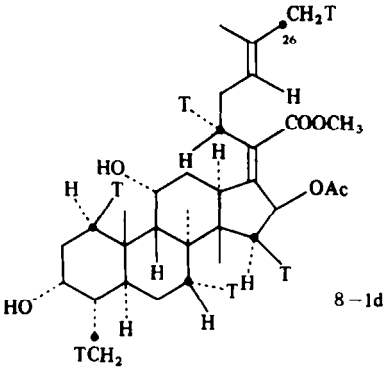
In all tables, the tritium atomic factors were rounded up to the nearest decimals.

* Counted as phenylurethanes

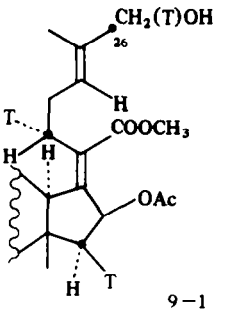
** Counted as dimedone derivatives

with ^{14}C and tritium. To confirm the presence of ^{14}C and of tritium at the C-26 carbon atom, "*R*"-methyl fusidate (**8-1d**) (Table 1, entry 2A) was treated with selenium dioxide in ethanol[83] to yield the *E*-26-alcohol (**9-1**) admixed with *E*-26-aldehyde (**9-2**) (unpublished results). Subsequently, the 26-alcohol was oxidized (MnO_2) to the 26-aldehyde. The obtained *E*-26-aldehyde (**9-2**) was converted to the 26-oxime (**9-3**) which, on treatment with cyanuric chloride,[84] gave the 26-nitrile (**9-4**) (Table 1, entry 9). Alternatively, decarbonylation[85] of the *E*-26-aldehyde (**9-2**) (specific activity 7.5×10^4 dpm of ^{14}C per mmol) gave methyl 26-nor-fusidate (**9-5**) with a specific activity of 6.6×10^4 dpm of ^{14}C per mmol (Table 1, entry 10). The removal of the 26-carbon atom of **8-1d** to yield **9-5** proceeded with a decrease of ^{14}C-specific activity equivalent to the loss of 0.7 atom of ^{14}C. Similarly, the conversion of "*R*"-methyl fusidate (**8-1d**) to methyl-25-cyanofusidate (**9-4**) proceeded with the loss of 0.7 atom of tritium. It is thus apparent that the carbon-26 of "*R*"-methyl fusidate (**8-1d**) is labeled with both ^{14}C and tritium. The reason for the loss of only 0.7 atom, rather than 1 atom each of ^{14}C and tritium is most likely related to the *mechanism* of selenium dioxide oxidation of an isopropyledine methyl group.[83] The end product of the oxidation reaction, which is presumed to proceed through a single bond intermediate, is usually the *E*-alcohol and/or *E*-aldehyde. However, due to possible rotation around the single bond of the hypothetical intermediate,[83] some scrambling of the C-26 and C-27 carbon atoms can take place.

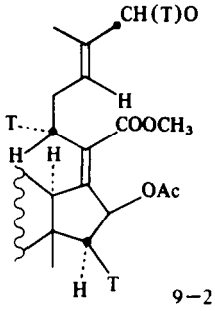
For the determination of the presence and chirality of tritium atoms at C-7 of "*R*"- and "*S*"-fusidic acids (**10-1** and **10-4**, respectively), we took advantage of microbial hydroxylation which proceeds in the retention mode.[86,87] The helvolic acid producing strain of *Acremonium persicinum* hydroxylates fusidic acid at the 7α-position[88] and the transformation should therefore proceed with the abstraction of the 7α-hydrogen atom.[86,87] Incubation of "*R*"-[$^{14}C_6$; $^{3}H_6$]-fusidic acid (**10-1**) (Table 1) with *Acremonium persicinum*,[89] gave "*R*"-7α-hydroxy fusidic acid which was converted to the methyl ester (**10-2**). The ester (**10-2**) contained only five tritium atoms [$^{14}C_6$; $^{3}H_5$] (Table 1, entry 11A). Oxidation (CrO_3–Py) of the "*R*"-[$^{14}C_6$; $^{3}H_5$]-7α-hydroxy (**10-2**) to 3,7,11-trione (**10-3**) proceeded without loss of tritium (Table 1, entry 12A). This establishes the presence of a 7α-tritium in the "*R*"-fusidic acid (**10-1**). In contrast, analogous microbial hydroxylation of "*S*"-[$^{14}C_6$; $^{3}H_6$]-fusidic acid (**10-4**) gave, after esterification, methyl-"*S*"-[$^{14}C_6$; $^{3}H_6$]-7α-hydroxy fusidate (**10-5**) (Table 1, entry 11B) which retained all the tritium.



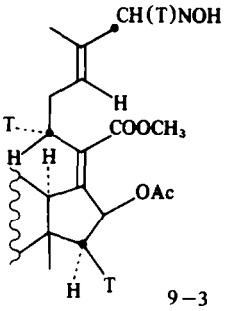
8–1d



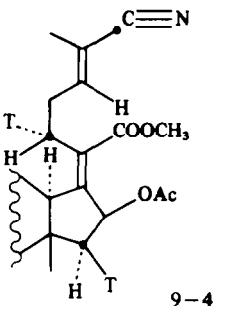
9–1



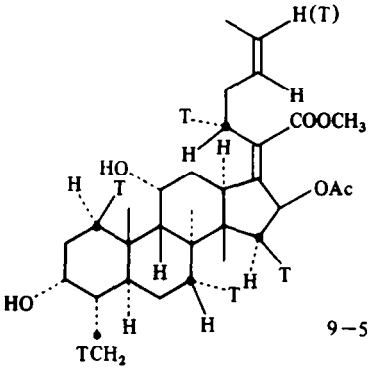
9–2



9–3



9–4



9–5

Chart 9.

However, oxidation of the derived methyl-"*S*"-7α-hydroxy fusidate (**10-5**) to the "*S*"-3,7,11-triketone (**10-6**) (Table 1, entry 12B) proceeded with the loss of tritium which must have been located at the 7β-position.

We then addressed the question of stereochemistry[89] of tritium atoms at C-15. The approach we chose was based on the premise that base-catalyzed elimination of a 16β-mesyloxy moiety (**11-4**) will proceed through *trans* elimination of the 15α-hydrogen atom to yield the 15(16); 17(20)-diene (**11-5**). Consequently, "*R*"-24-dihydro fusidic acid (**11-1b**) was treated with dihydropyran and *p*-TsOH to give the 3,11-bis-THP ether (**11-2**). Brief exposure of the bis-THP ether (**11-2**) to LAH in THF yielded the 16β-hydroxy-21-acid (**11-3a**) which was immediately treated with diazomethane to yield the methyl ester (**11-3b**). The 16β-hydroxy (**11-3b**) was mesylated (CH_3SO_2Cl–pyridine), and the resulting "*R*"-16β-mesyl ester (**11-4**) was exposed to collidine to give the diene (**11-5a**) which, upon removal of the THP groups, gave the "*R*"-dien-diol (**11-5b**). The sequence of reactions from **11-1a** via the 16β-mesylate to the diene (**11-5b**) proceeded without loss of tritium[89] (Table 1, entry 13A).

The "*S*"-dihydro fusidic acid was converted in an analogous manner to give "*S*"-16β-mesylate (**11-6**). Treatment of the "*S*"-mesylate (**11-6**) with collidine and removal of the 3,11-THP groups gave the

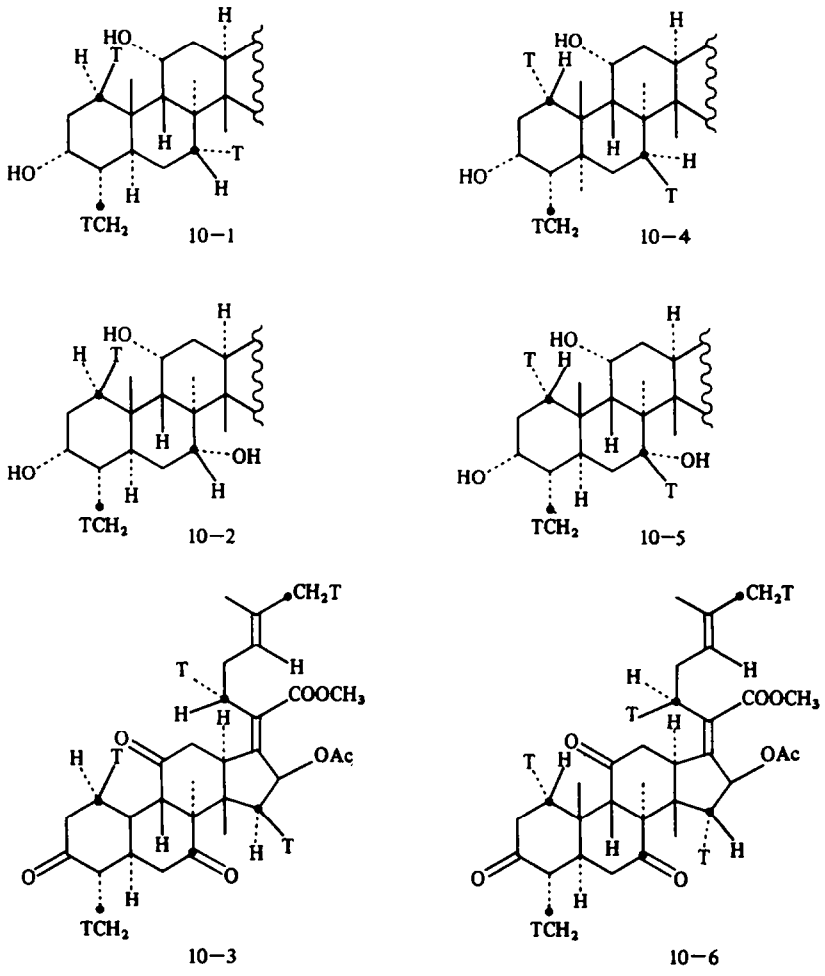
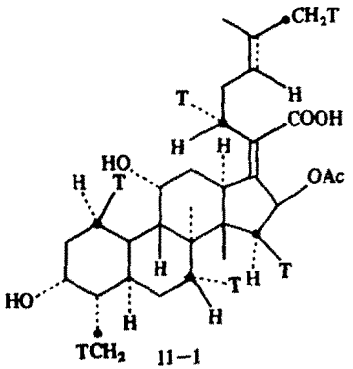


Chart 10.

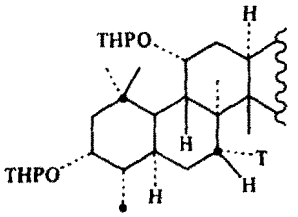
"*S*"-15(16); 17(20)-diene (**11-7**). The formation of the "*S*"-15(16) double bond proceeded with the loss of an atom of tritium (Table 1, entry 13B). It follows therefore that dihydro "*R*"- (**11-1b**) and dihydro "*S*"-fusidic acids and, hence, the parent "*R*"- and "*S*"-fusidic acids have 15β- and 15α-tritium atoms, respectively.[89]

Similarly, when a benzene solution of "*R*"-16β-hydroxy-3,11-bis-THP ether (**11-3**) was treated with methyl(carboxysulfamoyl)triethylammonium hydroxide inner salt ($CH_3O_2C\bar{N}SO_2\overset{+}{N}(C_2H_5)_3$), the obtained "*R*"-diene (**11-5b**) *retained all the tritium.*[90] We have proven that the dehydration with the inner salt is a *trans* elimination reaction.[90] The results corroborate the conclusion that the "*R*"-fusidic acid (**11-1a**) has a 15β-tritium atom.

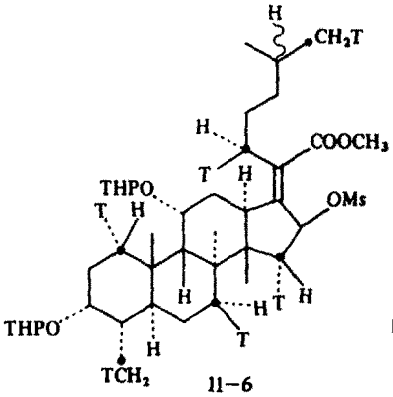
Several observations on the distribution of ^{14}C and tritium atoms in fusidic acids derived from (2*R*)- and (2*S*) [2-^{14}C; 2-^{3}H]-MVA seem in order. We have proven that the biosynthesis of fusidic acid proceeds via cyclization of squalene from either end of the squalene molecule.[91] It follows, therefore, that the three pairs of carbon atoms [26 and 30]; [1 and 22] and [7 and 15] of fusidic acid (**11-1a**) are equivalent. Accordingly, degradation of the fusidic acid side chain showed that equal amounts of ^{14}C and tritium are present at C-22 and 26 (Table 1, entries 6 and 7). Consequently, symmetry of squalene incorporation implies that the amounts of tritium and ^{14}C at C-1, 30, 22 and 26 must be equal. The amounts of tritium at C-7 and 15 were shown to be the same and equal to those at other labeled positions. It may be assumed that the amounts of ^{14}C at C-7 and 15 are also equal to those at the other ^{14}C-labeled positions. Our results show that, while the absolute amounts of ^{14}C and tritium in the "*R*" and "*S*" samples of fusidic acids were different, within each sample the isotopes were distributed about equally over the six carbon atoms derived from C-2 of the respective parent mevalonates. Considering that C-1 corresponds to C-22, it may be inferred that the 2 pro *R* and 2 pro *S* hydrogen (tritium) atoms of MVA must have the 1β- and 1α-stereochemistry, respectively. In summary, it may be concluded that



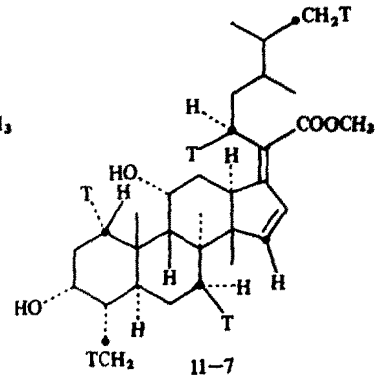
11–1
a Δ^{24}
b No Δ^{24}



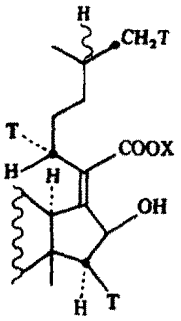
11–2



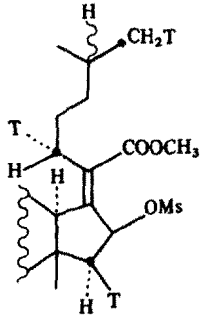
11–6



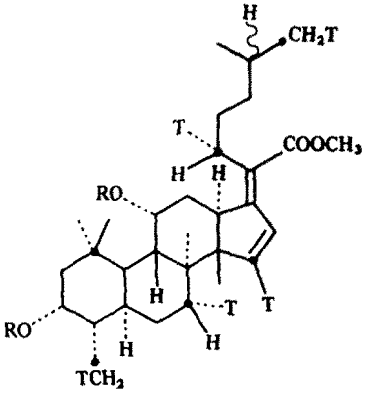
11–7



11–3
a X = H
b X = CH_3



11–4



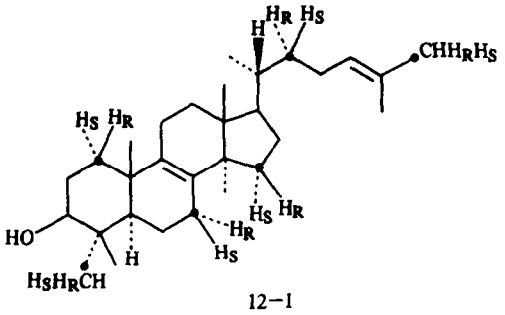
11–5
a R = THP
b R = H

Chart 11.

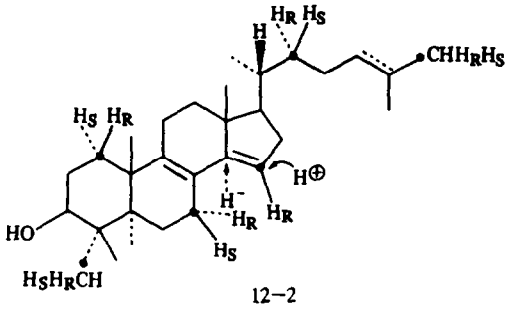
C-2(^{14}C) carbon atoms of MVA were incorporated, as expected, and that the 2 pro *R* and 2 pro *S* hydrogen (tritium) atoms of MVA were incorporated stereospecifically essentially without scrambling at C-1, 7, 15, 22, 26, and 30. In addition, the results show that cyclization of squalene to the protosterol (**7-1**), which is the apparent precursor of fusidic acid, proceeds via the loss of the C-17 hydrogen of the C-20 cation.[38]

DISPOSITION AND STEREOCHEMISTRY OF THE C-2 HYDROGEN ATOMS OF MVA IN CHOLESTEROL

We will now address the problem of the stereochemical changes of hydrogen atoms derived from C-2 of MVA taking place in the course of the metabolic transformations of lanosterol (**12-1**) to cholesterol. The biosynthetic reactions leading from lanosterol to cholesterol were reviewed[92,93] and will not be



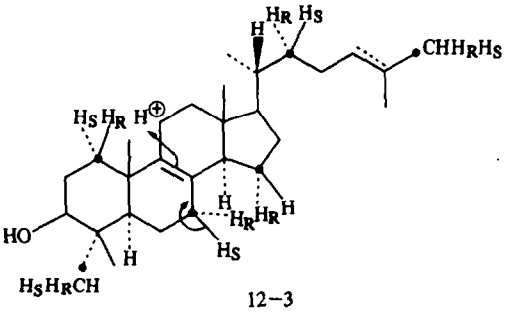
12–1



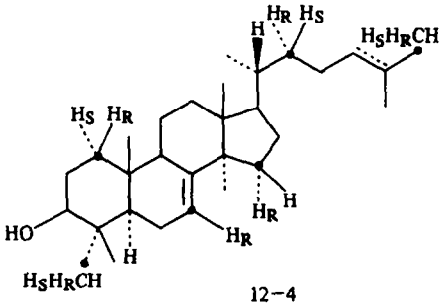
12–2

a, H_R and H_S hydrogen atoms derived from 2 pro R and 2 pro S hydrogens of MVA

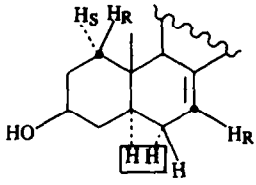
b, R = T; • = ^{14}C



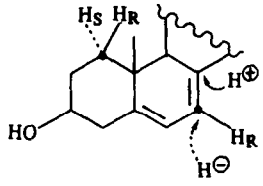
12–3



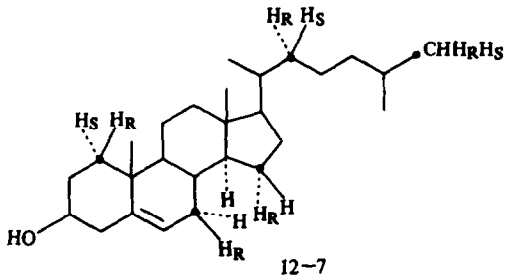
12–4



12–5



12–6



12–7

Chart 12.

recapitulated, except for the steps relevant to the present discussion. The removal of the 14α-methyl of lanosterol (**12-1**) proceeds with the extrusion of formic acid[94] and, very likely, leads to the formation of the 8(9), 14(15)-diene[95,96] (**12-2**). Reduction of the 14(15) double bond yields the 8(9) mono-ene[97,98] (**12-3**) which in turn is isomerized to the C-7(8) mono-ene[77,99–101] (**12-4**). Following the oxidative removal of the C-4 gem dimethyl groups,[102,103] the resulting **12-5** is dehydrogenated to give the 5,7-diene[101,104,105] (**12-6**). Reduction of the C-7 double bond[106,107] of **12-6** and of C-24 double bond of lanosterol (**12-1**), which may take place at different stages of the metabolic sequence,[99] will yield cholesterol (**12-7**).

In rat livers, the formation of the 14(15) double bond (**12-2**) proceeds with the retention at C-15 of the 2 pro *R* hydrogen (tritium) atom of MVA[108–110] and loss of the 2 pro *S* hydrogen (tritium) atom of MVA.[108] Similarly, the allylic rearrangement of the C-8(9) double bond (**12-3**) to the C-7(8) double bond (**12-4**) proceeds with retention at C-7 of the 2 pro *R* hydrogen of MVA[110–113] and the loss of the 2 pro *S* hydrogen of MVA.[111,113]

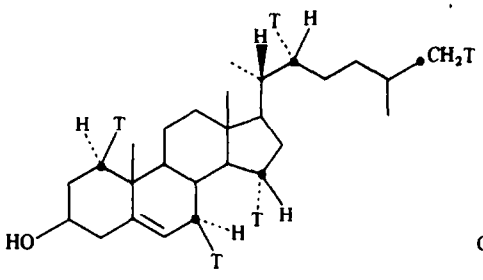
Based on the proposed mode of squalene cyclization (Chart 2) to lanosterol (**12-1**) and on the proven distribution and stereochemistry of C-2 protons of MVA in fusidic acid (Charts 10 and 11), it follows that the hydrogen atoms *eliminated* in the elaboration of cholesterol had the 15α- and 7β-stereochemistry in lanosterol (**12-1**).

In considering the biosynthesis of cholesterol from lanosterol (Chart 12), it is expected that "*R*"-cholesterol derived from [2*R*; 2-^{3}H]-MVA will retain five tritium atoms which will be located at C-1β, 7(?), 15(?), 22*R* and 26. In contrast, the "*S*"-cholesterol obtained from [2*S*; 2-^{3}H]-MVA will have only three tritium atoms at C-1α, 22*S* and 26. Our objective was to confirm the location and the stereochemistry of the tritium atoms in "*R*"-cholesterol. We were particularly interested in the orientation of tritium atoms at C-7 and 15 whose stereochemistry depended on the mode of reduction (*cis* or *trans*) of the C-7(8) double bond of **12-6** and the C-14(15) double bond of **12-2**, respectively.

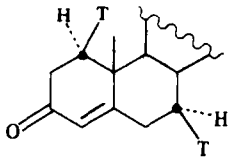
The required "*R*"-cholesterol was prepared by incubating (3*RS*, 2*R*) [2-^{14}C; 2-^{3}H]-MVA with a rat liver microsomal fraction[109,110,112] (**13-1**). For the determination of the tritium content and stereochemistry at C-1, the "*R*"-cholesterol (**13-1**) was oxidized by the Oppenauer procedure and the resulting cholest-4-en-3-one (**13-2**) was reduced with lithium in liquid ammonia.[109,110] The recovered mixture of products was reoxidized to yield 5α-cholestan-3-one (**13-3**) which was dehydrogenated with dichlorodicyanobenzoquinone[114] (DDQ) to give 5α-cholest-1-en-3-one (**13-4**). The introduction of the C-1(2) double bond via dehydrogenation with DDQ involves the abstraction of the 1α- and 2β-hydrogen atoms.[114] The sequence of transformations: "*R*"-cholesterol **13-1** → **13-2** → **13-3** → **13-4** proceeded *without* loss of tritium (Table 2, entries 2–5) which showed that tritium was not present at the

Table 2. Distribution and stereochemistry of tritium atoms in cholesterol biosynthesized from (3*RS*, 2*R*) [2-^{14}C; 2-^{3}H]-MVA by *rat liver enzymes*

NO	Compound	^{3}H:^{14}C Ratio	
		Isotopic	Atomic
1	Squalene	10.7	
	Squalene-6HCl	10.4	6.0:6
2	Cholesterol (13-1)	10.1	5.0:5
3	Cholest-4-en-3-one (13-2)	9.7	4.8:5
4	5α-cholestan-3-one (13-3)	9.7	4.8:5
5	Cholest-1-en-3-one (13-4)	10.0	5.0:5
6	1,2-seco-di-acid (13-5)	8.2	4.1:5
7	5α-hydroperoxy-cholest-6-en-3β-ol (13-6)	9.8	4.9:5
8	7α-hydroperoxy-cholest-5-en-3β-ol (13-7)	9.7	4.8:5
9	Cholest-5-en-3β-ol-7-one (13-8)	8.1	4.0:5
10	Pregnenolone (14-2)	9.8	2.9:5
11	Progesterone (14-3)	9.1	2.7:3
12	Pregn-4-en-12β,15α-diol-3,20-dione (14-4)	6.2	1.8:3
13	Pregn-4-en-12β-ol-3,15,20-trione (14-5)	6.6	2.0:3
14	Pregn-4-en-3,12,15,20-tetra-one	6.4	1.9:3



13–1



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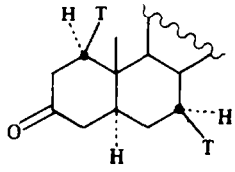

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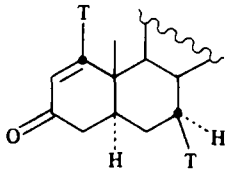

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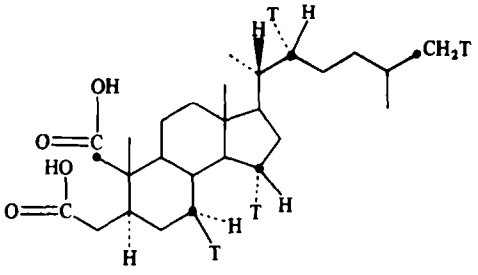
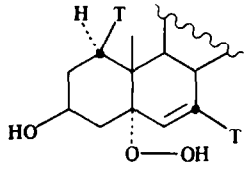


13–5



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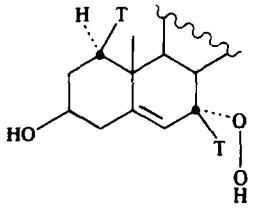

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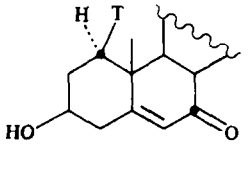


13–8

Chart 13.

1α-position. In contrast oxidation of **13-4** with ruthenium tetraoxide[115] gave 1,2-seco-A-norcholestan-1,2-dioic acid (**13-5**) with the loss of *ca* 19% of tritium (Table 2, entry 6). These results show that, as expected, "*R*"-cholesterol has a tritium atom at the 1β-position. In addition, considering that about 20% of tritium was lost in the conversion of **13-4** to the seco-diacid (**13-5**), it can be inferred that "*R*"-cholesterol indeed has five tritium atoms, one of which is located at the 1β-position, as expected.

Nickon and Bagli have proven that photolysis of cholesterol in the presence of the sensitizer hematoporphyrin and oxygen gives 5α-hydroperoxycholest-6-en-3β-ol[116] (**13-6**). The reaction proceeds with the stereospecific elimination of the 7α-hydrogen atom of cholesterol.[116] In chloroform, the Δ^6-5α-hydroperoxide (**13-6**) rearranges to Δ^5-7α-hydroperoxide[117] (**13-7**). Alternatively, the hydroperoxide (**13-6**) can be converted by treatment with $CuCl_2 \cdot 2H_2O$ in pyridine to 7-keto-cholesterol[117a] (**13-8**).

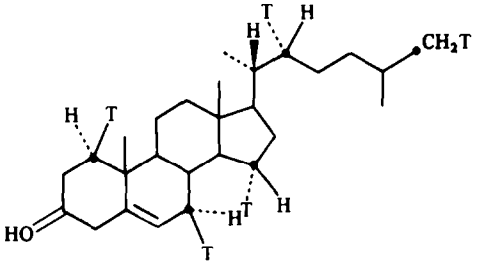
When "*R*"-cholesterol was oxidized[116] to "*R*"-Δ^6-5α-hydroperoxide (**13-6**) and then rearranged to "*R*"-Δ^5-7α-hydroperoxide[117] (**13-7**), the two reactions proceeded without loss of tritium (Table 2, entries 7 and 8) revealing the absence of a 7α-tritium atom.[109,110] In contrast, the transformation of the "*R*"-Δ^5-7α-hydroperoxide (**13-7**) to the "*R*"-7-ketocholesterol (**13-8**) involved the loss of 20% of total tritium[109,110] (Table 2, entry 9). This establishes the presence of a 7β-tritium atom in "*R*"-cholesterol (**13-1**) and confirms the elimination of a hydrogen (tritium) atom derived from 2 pro *S* of MVA from C-7 in the C-7(8) double bond formation.[111] That the 7β-hydrogen atom of lanosterol, derived from 2 pro *S* of MVA, is lost in the elaborations of cholesterol was confirmed with the use of "*S*"-[$^{14}C_5$; $^3H_3$]-cholesterol.[111,113,118]

Considering that the hydrogen (tritium) derived from 2 pro *R* of MVA has the 7α-configuration in

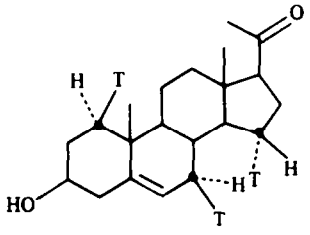
fusidic acid[88] (**10-1**), and in lanosterol while in cholesterol it has the 7β-configuration, shows that the hydrogen (tritium) atom has undergone inversion of configuration.[109,110] This obviously occurred in the reduction of the Δ^7-bond of the intermediate of 5,7-diene-ol (**12-6**) which must have proceeded via the addition of 7α-hydrogen atom.[109,110] Since cholesterol has the 8β(H)-stereochemistry, the saturation of the 7(8) double bond of **12-6** is a *trans* reduction process. Gibbons *et al.*[113] arrived at a similar conclusion. Akhtar and co-workers[107] showed that a 4 pro *R* hydrogen of NADPH is added at 7α and a proton from the medium at 8β.

The determination of the stereochemistry of tritium at C-15 of "*R*"-cholesterol was more challenging because of the absence of a facile access to this carbon atom. Ultimately we opted for a route based on the observation that progesterone can be microbially hydroxylated at C-15. As indicated earlier, microbial hydroxylations at secondary carbon atoms proceed stereospecifically with retention of configuration.[86,87]

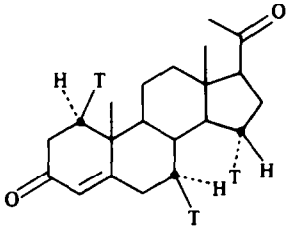
Our plan was to cleave the side chain of "*R*"-cholesterol (**14-1**) with bovine adrenal enzymes and then oxidize the resulting "*R*"-pregnenolone (**14-2**) to "*R*"-progesterone (**14-3**). The "*R*"-progesterone would then be microbially hydroxylated at C-15. Unfortunately, this route had some inherent drawbacks. The specific activity of the "*R*"-cholesterol biosynthesized in a rat liver homogenate was relatively low, due to unavoidable dilution with endogenous cholesterol. Therefore, to produce sufficient amounts of "*R*"-pregnenolone, it was necessary to incubate rather large amounts of low specific activity "*R*"-cholesterol with the bovine adrenal homogenates. Under these conditions, the side chain cleavage proceeded poorly and resulted in low yield(s) of "*R*"-pregnenolone. Also the Oppenauer oxidation of small amounts of pregnenolone gave progesterone in relatively low yield. Consequently, the overall yield of conversion of "*R*"-cholesterol to "*R*"-progesterone was poor. Under the circumstances, a highly efficient procedure for microbial hydroxylation at C-15 was required. Of the available microbial methods of C-15 hydroxylation of progesterone the most efficient seemed to be incubation with *Calonectria decora* which gives 12β,15α-dihydroxy-progesterone[119] (**14-4**) in up to



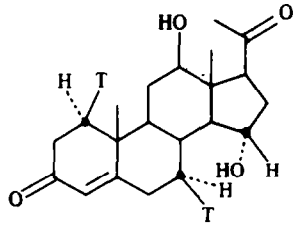
14–1



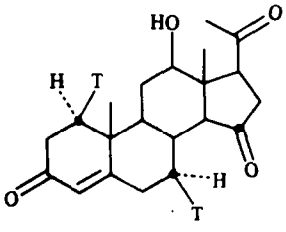
14–2



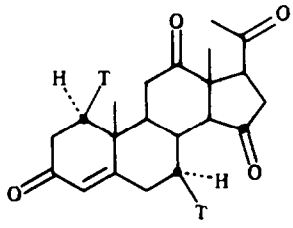
14–3



14–4



14–5



14–6

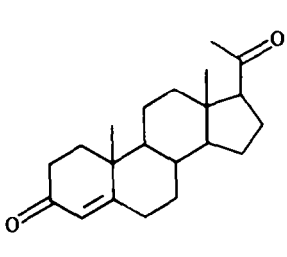
Chart 14.

90% yield. However, there was some uncertainty concerning the stereochemistry of the crucial 15-hydroxyl which was initially thought to have the 15β-orientation.[119b] Since the problem was of paramount importance to us, we decided to define unambiguously the stereochemistry of the C-15 hydroxyl.

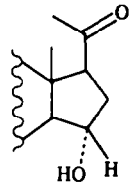
Our plan was to synthesize progesterone stereospecifically labeled with tritium at C-15β and submit it to microbial hydroxylation with *Calonectria decora*. From the loss or retention of tritium in the derived 12β,15-dihydroxyprogesterone (**15-7**), the stereochemistry of the C-15 hydroxyl will be deduced. For the preparation of the required [15β-^{3}H]-progesterone, progesterone (**15-1**) was incubated with *Penicillium* sp.[120] (ATCC 11598) to yield 15α-hydroxyprogesterone[110,120] (**15-2**) in low yield (5–10%). The 15α-hydroxy product (**15-2**) was ketalized and the 3,20-diketal (**15-3a**) was treated with *p*-toluenesulfonyl chloride in pyridine. Hydrogenolysis of the 15α-tosylate (**15-3b**) with $LiAlH_4$(^{3}H) gave, after the removal of the 3,20-ketals, [15β-^{3}H]-progesterone[109,110] (**15-4**). The 15β-stereochemistry of the tritium atom was inferred on the basis of the proven inversion of stereochemistry in the course of hydrogenolysis of tosyloxy esters with metal hydrides.[121,122]

To confirm the 15β-stereochemistry of the tritium atom, the tritiated progesterone (**15-4a**) was mixed with [4-^{14}C]-progesterone and the doubly labeled material (**15-4b**) was incubated with *Penicillium* sp. (ATCC 11598) to yield 15α-hydroxyprogesterone (**15-5**). The obtained **15-5** retained all the tritium. Oxidation of the 15α-alcohol (**15-5**) to the 15-ketone (**15-6**) proceeded with the loss of tritium which provided conclusive evidence for the 15β-configuration[109,110] of the tritium in **15-4**.

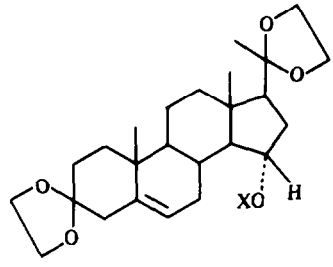
When the [15β-^{3}H; 4-^{14}C]-progesterone (**15-4b**) was incubated with *Calonectria decora*, the produced [4-^{14}C; 15β-^{3}H]-12β,15α-dihydroxy-progesterone (**15-7**) retained all the tritium. Controlled



15–1

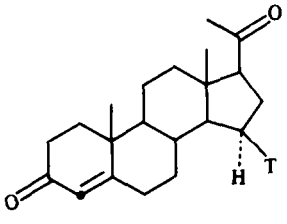


15–2



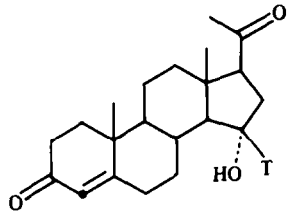
15–3

a X = H
b X = pTs

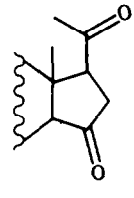


15–4

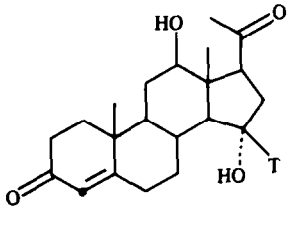
a • ≡ ^{12}C
b • ≡ ^{14}C



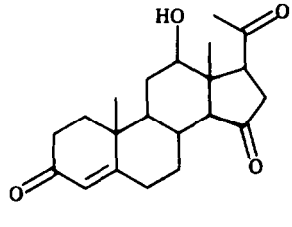
15–5



15–6



15–7



15–8

Chart 15.

oxidation of the diol with restricted amounts of Jones' reagent gave 12β-hydroxy-pregn-4-ene-3,15,20-trione (**15-8**) which was devoid of tritium. This sequence of reactions proved the 15α-stereochemistry of the hydroxyl of the 12,15-diol (**15-7**).

Having confirmed the 12β,15α-dihydroxy-progesterone (**15-7**) structure of the product of incubation of progesterone with *C. decora*, work on "*R*"-cholesterol (**14-1**) was undertaken. Incubation of "*R*"-[$^{14}C_5$; $^{3}H_5$]-cholesterol (**14-1**) with a bovine adrenal preparation gave "*R*"-[$^{14}C_3$; $^{3}H_3$]-pregnenolone (**14-2**) (Table 2, entry 10), which was then oxidized by the Oppenauer procedure to "*R*"-[$^{14}C_3$; $^{3}H_3$]-progesterone (**14-3**) (Table 2, entry 11). The resulting "*R*"-progesterone (**14-3**) was incubated with *C. decora* to give "*R*"-[$^{14}C_3$; $^{3}H_2$]-12β,15α-dihydroxy-progesterone (**14-4**) which retained only two tritium atoms (Table 2, entry 12). Oxidation of **14-4**, first to the 12-hydroxy-15-ketone (**14-5**) and then to the tetra-one (**14-6**) did not involve additional loss of tritium (Table 2, entries 13 and 14). It is clear therefore, that "*R*"-progesterone (**14-3**) and hence the parent "*R*"-cholesterol (**14-1**) have 15α-tritium atoms which originated from 2 pro *R* hydrogen (tritium) atom of MVA. The 2 pro *R* hydrogen (tritium) atom of MVA had the 15β-stereochemistry in "*R*"-fusidic acid and in "*R*"-lanosterol and it has the 15α-orientation in "*R*"-cholesterol (**14-1**). The observed inversion of stereochemistry must have occurred in the course of *trans* reduction of the C-14(15) double bond[109,110] which involves the addition of a hydrogen derived from 4 pro *R* of NADPH at 14α and of a proton from the medium at 15β.[98]

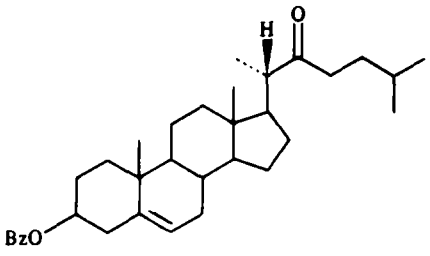
We now turned our attention to the hydrogen atoms at C-22 of cholesterol. To gain access to C-22, we utilized the protozoan *Tetrahymena pyriformis* which converts cholesterol to cholesta-5,7,22-trien-3β-ol.[123–125] For our purposes, it was necessary to determine first which of the hydrogen atoms (22 pro *R* or 22 pro *S*) is abstracted in the C-22(23) double bond formation. The syntheses required for these studies (22*R*)- and (22*S*)[2-^{3}H]-cholesterols was therefore undertaken[122] (Chart 16). The starting material for the syntheses, 22-keto-cholest-5-en-3β-ol 3-benzoate (**16-1**), was prepared and treated with $NaBH_4(^{3}H)$ to yield a mixture of [22-^{3}H]-22-diols (**16-2a** and **16-3a**). The diols were resolved by a combination of column chromatography and multiple TLC on alumina oxide. The tritiated 22*R*- and 22*S*-alcohols (**16-2a** and **16-3a**, respectively) were obtained in a 1 : 10 ratio. The chirality of the 22-alcohols was determined by the Horeau and Prelog methods.[122] Subsequently the 22-alcohols were mesylated and the mesyl esters were hydrogenolyzed with $LiAlH_4$. Thus, [22*R*-^{3}H]-mesylate (**16-2b**) and [22*S*-^{3}H]-mesylate (**16-3b**) gave [22*R*-^{3}H]- and [22*S*-^{3}H]-cholesterols (**16-4a** and **16-5a**), respectively. The samples were mixed with 4-^{14}C-cholesterol to give doubly labeled [22-^{3}H; 4-^{14}C] **16-4b** (Table 3, entry 1) and **16-5b** (Table 3, entry 4).

Incubation of [4-^{14}C; 22*R*-^{3}H]-cholesterol (**16-4b**) with *Tetrahymena pyriformis* gave [4-^{14}C]-cholesta-5,7,22-trien-3β-ol (**16-6**) (Table 3, entry 3) with loss of the C-22 tritium.[124,125] In contrast, the formation of [4-^{14}C; 22-^{3}H]-cholesta-5,7,22-trien-3β-ol (**16-7**) (Table 3, entry 6) from [4-^{14}C; 22*S*-^{3}H]-cholesterol (**16-5b**) (Table 3, entry 4) proceeded with retention of tritium.[124,125] It is thus clear that elaboration of the C-22(23) double bond by *T. pyriformis* proceeds with the abstraction of the 22 pro *R* hydrogen atom of cholesterol.[124,125]

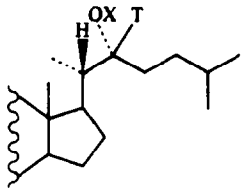
With this information at hand, we proceeded with the determination of the steric mode of incorporation of the 2 pro *R* and 2 pro *S* hydrogen (tritium) atoms of MVA at C-22 of cholesterol. Biosynthetic "*R*"-[$^{14}C_5$; $^{3}H_5$]-cholesterol (**17-1**) (Table 3, entry 7) (derived from (2*R*) [2-^{3}H; 2-^{14}C]-MVA) was incubated with *T. pyriformis* to give [$^{14}C_5$; $^{3}H_3$]-cholesta-5,7,22-trien-3-β-ol (**17-3**) (Table 3, entry 9). The introduction of the 7 and 22 double bonds in "*R*"-cholesterol (**17-1**) entailed the loss of two tritium atoms. Since "*R*"-cholesterol (**17-1**) has a tritium at the 7β-position, it follows that the introduction of the 7 and 22 double bonds involved the abstraction of 7β- and 22*R*-tritium atoms (**17-3**) (Table 3, entry 9). Hence, the tritium atom at C-22 derived from (2*R*) [2-^{3}H]-MVA has the predicted 22*R*-stereochemistry. Analogous dehydrogenation of (*S*)[$^{14}C_5$; $^{3}H_3$]-cholesterol (**17-2**) (Table 3, entry 10) (derived from (2*S*) [2-^{3}H; 2-^{14}C]-MVA) with *T. pyriformis* gave [$^{14}C_5$; $^{3}H_3$]-cholesta-5,7,22-trien-3-β-ol (**17-4**) (Table 3, entry 12) with retention of tritium. This therefore proves that "*S*"-cholesterol has the (22*S*) [22-^{3}H]-chirality.

BIOSYNTHESIS OF CHOLESTEROL IN THE BRAIN OF RATS

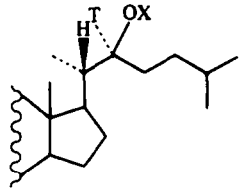
In the course of studies of the impact of a prenatal and continuous postnatal protein restricted diet on the development of rats,[126] we investigated the *in vivo* biosynthesis of cholesterol in the brains of these rats.[127] Virgin female rats were maintained for 35 days on isocaloric diets containing 8 or 25% of



16–1

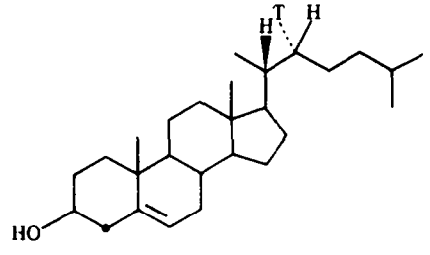


16–2

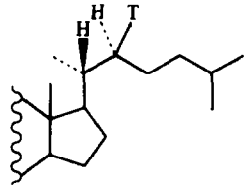


a X = H
b X = Ms

16–3

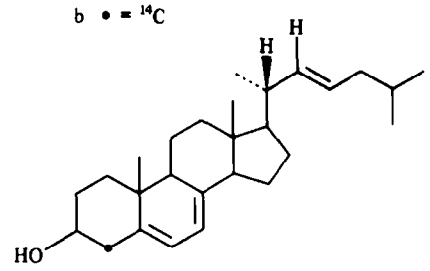


16–4

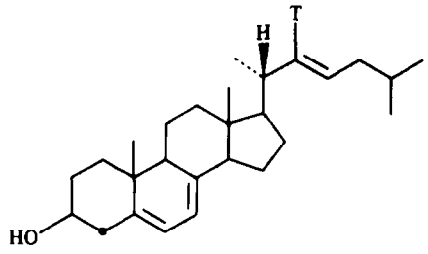


16–5

a • = ^{12}C
b • = ^{14}C



16–6



16–7

Chart 16.

casein and mated.[126] The dams were fed their respective diets during gestation and subsequent lactation. It was noted that the offspring of mothers kept on the protein restricted diet (8% casein) had 50% lower body weight and 10–15% lower brain weight than the corresponding controls (25% casein diet). In addition, there was a significant delay in the age at which the maximal rate of *de novo* biosynthesis of steroids from 2-^{14}C-MVA was achieved in the cerebellum, but not in the thalamic region.[126]

To determine the pathways of cholesterol biosynthesis in the brains of the rats, (3*RS*, 2*R*) [2-^{14}C; 2-^{3}H]-MVA was administered into the midline thalamic region of the brains of 13 day-old rats born and nursed by mothers on the 8 and 25% casein-containing diets. After 4.5 hr, the rats were killed, the brains were removed, saponified and the biosynthesized cholesterol recovered.[127] It was found that the overall outcome of biosynthetic events at C-7 and C-15 of cholesterol produced in the brains of the two groups of animals was the same as in cholesterol biosynthesized by rat liver enzymes.[127]

BIOSYNTHESIS OF YEAST STEROLS

The pathway from lanosterol to cholesterol was investigated almost exclusively with the use of rat liver enzymes. It was therefore of obvious interest to determine whether the biosynthesis of sterols from

Table 3. Dehydrogenation of cholesterol to cholesta-5,7,22-trien-3β-ol by *Tetrahymena pyriformis*

NO	Compound	$^3H:^{14}C$ Ratio Isotopic	Atomic
1	[22R;22-^{3}H;4-^{14}C]-cholesterol$^+$ (16-4b)	9.8	1.0:1
2	[22R;22-^{3}H]-acetate (recovered)$^{++}$	9.7	1.0:1
3	[4-^{14}C]-triene acetate (from 1) (16-6)	1.2	0.1:1
4	[22S,22-^{3}H;4-^{14}C]-cholesterol$^+$ (16-5b)	9.65	1.0:1
5	[22S,22-^{3}H;4-^{14}C]-acetate (recovered)$^{++}$	9.2	1.0:1
6	[22-^{3}H;4-^{14}C]-triene acetate (16-7)	9.2	1.0:1
7	"R"-[$^{14}C_5$;$^3H_5$]-cholesterol* (17-1)	9.8	5.0:5
8	"R"-cholesterol acetate (recovered)$^{++}$	9.9	5.0:5
9	"R"-[$^{14}C_5$;$^3H_3$]-triene acetate (17-3)	6.1	3.1:5
10	"S"-[$^{14}C_5$;$^3H_3$-cholesterol** (17-2)	5.6	3.0:5
11	"S"-cholesterol acetate (recovered)$^{++}$	5.45	3.0:5
12	"S"-[$^{14}C_5$;$^3H_3$]-triene acetate (17-4)	5.2	2.9:5

$^+$ Synthetically prepared[122]

$^{++}$ Recovered from incubation with T. pyriformis

* Biosynthesized from (3RS;2R) [2-^{3}H;2-^{14}C]-MVA in rat liver homogenate

** Biosynthesized from (3RS;2S) [2-^{3}H;2-^{14}C]-MVA in rat liver homogenate

lanosterol in other species follows the pathways observed in rat livers. To this end, we investigated the biosynthesis of yeast sterols known to proceed via the initial cyclization of squalene to lanosterol. Samples of (3*RS*,2*R*)[2-^{14}C;2-^{3}H]-MVA and (3*RS*,2*S*)[2-^{14}C;2-^{3}H]-MVA were therefore incubated with homogenates of mechanically disrupted yeast cells.[128–131] At the termination of the incubation, the mixtures were processed to yield "*R*" and "*S*" unsaponifiable residues. Upon chromatographic fractionation, in addition to squalene and lanosterol, two major chemically and radioactively homogeneous metabolites were isolated from each unsaponifiable residue. The major metabolites proved to be "*R*"-(**18-1a**)- and "*S*"-(**18-2a**)-3β-hydroxycholesta-5,7,24-triene.[129,131] The

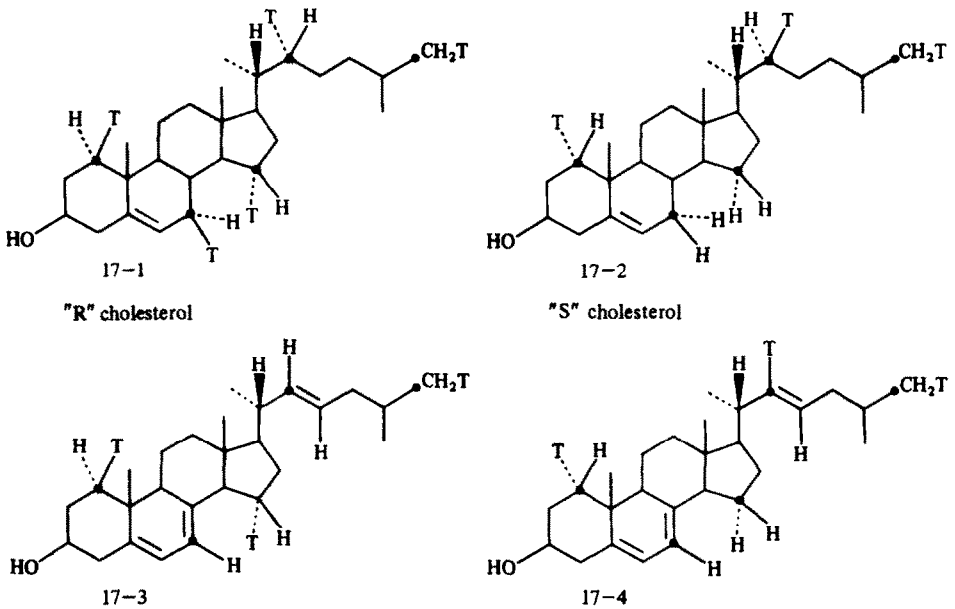
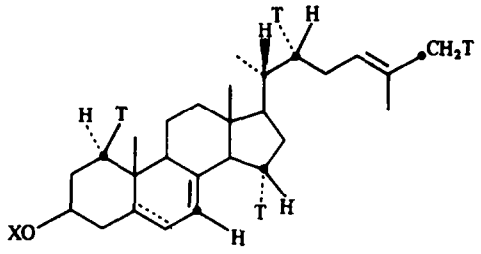
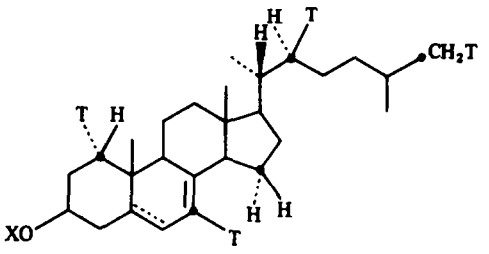


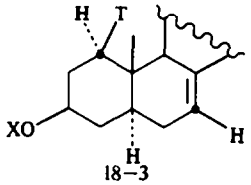
Chart 17.



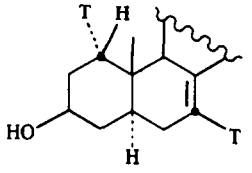
18–1
a Δ^5, X = H
b No Δ^5, X = H, 5d-H
c Δ^5, X = Ac



18–2



18–3
a X = Ac
b X = H



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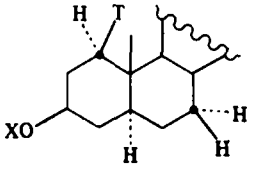

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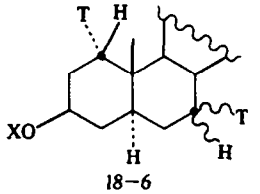

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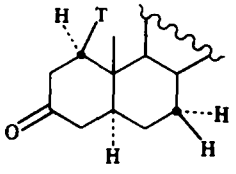

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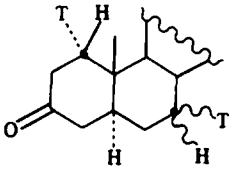

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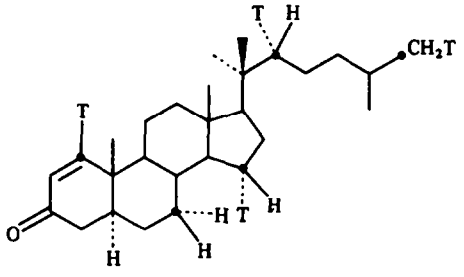

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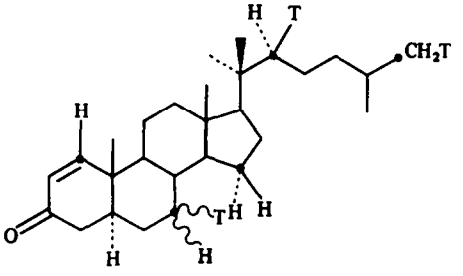

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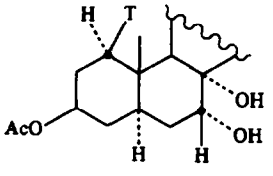

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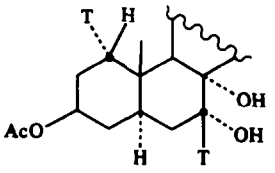
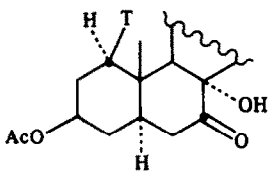


18–12

Chart 18.



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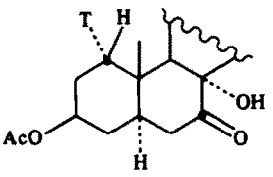

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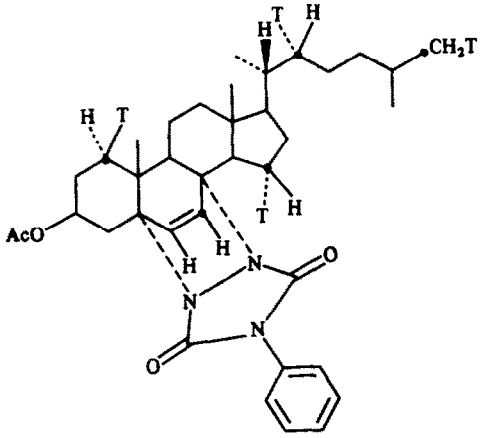

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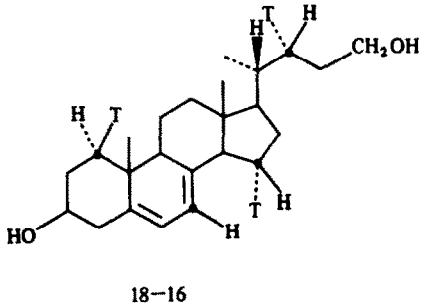

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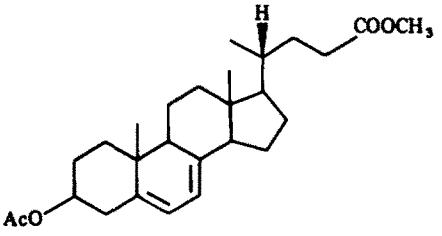


18–17

Chart 18—*continued.*

Table 4. Distribution and stereochemistry of tritium atoms at C-7 and C-1 in sterols biosynthesized from (3*RS*, 2*R*)- and (3*RS*, 2*S*) [2-^{14}C; 2-^{3}H]-MVA in a yeast homogenate

		A "R"-products		B "S"-products	
		$^{3}H:^{14}C$ Ratio			
NO	Compound	Isotopic	Atomic	Isotopic	Atomic
1	MVA-benzhydrylamide	8.7	1.0:1	8.5	1.0:1
2	Squalene	8.4	6.0:6	7.4	6.0:6
3	Squalene 6HCl	8.4	6.0:6	7.2	5.8:6
4	Lanosterol	8.4	6.0:6	7.4	6.0:6
5	5α-cholestan-3β-ol (18-5b or 6b)	6.4	3.8:5	5.7	3.9:5
6	5α-cholestan-3-one (18-7 or 8)	6.5	3.9:5	5.8	3.9:5
7	5α-cholest-1-en-3-one (18-9 or 10)	6.5	3.9:5	4.6	3.1:5
8	5α-cholest-7-en-3β-ol Ac (18-3a or 4a)	6.4	3.8:5	5.8	3.9:5
9	5α-cholesta-3β,7α,8α-triol 3-Ac (18-11 or 12)	6.5	3.9:5	5.6	3.8:5
10	5α-cholesta-3β,8α-diol-7-one 3-Ac (18-13 or 14)	6.5	3.9:5	4.4	3.0:5
11	Cholestesta-5,7,24-trien-3β-ol Ac (18-1c)				
	a. from glc purification	7.8	4.0:5	-	-
	b. co-crystallized*	7.75	4.0:5	-	-
	c. recovered from adduct++	7.7	4.0:5	-	-
12	Chole-5,7-dien-3β,24-diol (18-16)	7.3	3.0:4	-	-

* Cocrystalized with an authentic sample[132]

++ Recovered from (18-15) by treatment with LAH.

minor products were "*R*"- and "*S*"-3β-hydroxycholesta-7,24-diene (**18-1b** and **18-2b**), respectively.[129,131] The metabolites were identified by chemical transformations and by comparison with authentic samples.[132] No radioactive *de novo* biosynthesized ergosterol was found in the unsaponifiable residues which indicated that, in the homogenates of mechanically disrupted yeast cells, the C-24 methyl transferase and C-22 dehydrogenase were impaired.

Rather unexpectedly, the tritium to ^{14}C atomic ratios of the "*R*" and "*S*" yeast metabolites were the same, revealing that each metabolite incorporated four tritium atoms and five ^{14}C atoms[128,131] [$^3H_4$; $^{14}C_5$] (Table 4). The incorporation of the same number of tritium atoms into the "*R*" and "*S*" yeast metabolites was most surprising and differed markedly from the situation in cholesterol. It may be recalled that liver "*R*"-cholesterol had five tritium atoms at C-1β, 7β, 15α, 22*R* and 26, while the "*S*"-cholesterol had only three tritium atoms at C-1α, 22*S* and 26. At the time, the variation(s) of biosynthetic pathways was unexpected and necessitated a study of the distribution of tritium atoms in the yeast metabolites.

For the determination of tritium at C-1 of yeast sterols, we used "*R*"-(**18-5**)- and "*S*"-(**18-6**)-3β-acetoxy-(5α)-cholestanes which were obtained as minor *by-products*[128] of hydrogenation of "*R*"-(**18-1c**)- and "*S*"-(**18-2c**)-3β-acetoxycholesta-5,7,24-trienes by the method of Cornforth *et al.*[25] The derived cholestanols (**18-5b** and **18-6b**) were oxidized to "*R*"-(**18-7**)- and "*S*"-(**18-8**)-(5α)-cholestan-3-one and dehydrogenated with DDQ to "*R*"-(**18-9**)- and "*S*"-(**18-10**)-(5α)-cholest-1-en-3-one, respectively. It may be recalled that the dehydrogenation with DDQ proceeds via the abstraction of the 1α- and 2β-hydrogen atoms.[114]

When yeast "*R*"-cholestatrienol (**18-1a**) was submitted to the above sequence of transformations, the "*R*"-(5α)-cholestanol (**18-5b**) (Table 4, entry 5A), "*R*"-(5α)-cholestan-3-one (**18-7**) (Table 4, entry 6A) and "*R*"-(5α)-cholest-1-en-3-one (**18-9**) (Table 4, entry 7A) retained all the tritium. This demonstrates that the "*R*"-cholestanol is devoid of a 1α-tritium and it may be inferred that the tritium at C-1 has the 1β-stereochemistry.

In the case of "*S*"-cholestatrienol (**18-2a**), the transformations to **18-6b** (Table 4, entry 5B) and **18-8** (Table 4, entry 6B) again proceeded without loss of tritium. However, introduction of the C1(2) double bond via DDQ dehydrogenation to yield **18-10** (Table 4, entry 7B) proceeded with the loss of a 1α-tritium. The results show that in **18-1** and **18-2**, the 2 pro *R* and 2 pro *S* hydrogen (tritium) atoms of MVA assumed 1β- and 1α-orientations, respectively, as expected.

The main products of hydrogenation of 3β-acetoxy-cholesta-5,7,24-triens (**18-1c** and **18-2c**) in ethyl acetate over nickel catalyst[25,128] were "*R*"-(**18-3a**)- and "*S*"-(**18-4a**)-3β-acetoxy-(5α)-cholest-7-ene which were used for the determination of tritium content[128] at C-7.

The "*R*"-7-ene-3-acetate (**18-3a**) (Table 4, entry 8A) was treated with osmium tetraoxide in pyridine to yield "*R*"-3β-acetoxy-7α,8α-dihydroxy-(5α)-cholestane (**18-11**) (Table 4, entry 9A) which, in turn, was oxidized to "*R*"-3β-acetoxy-8α-hydroxy-(5α)-cholestane-7-one (**18-13**) (Table 4, entry 10A). The "*R*"-7-ketone (**18-13**) (Table 4, entry 10A) retained all the tritium initially present in the parent "*R*"-trien-3β-ol (**18-1a**) which proved the absence of tritium at C-7.

"*S*"-3β-acetoxy-cholesta-5,7,24-trien (**18-2c**) was converted in a similar manner to 3β-acetoxy-7α,8α-diol (**18-12**) (Table 4, entry 9B). Oxidation of **18-12** to "*S*"-8α-hydroxy-7-one (**18-14**) (Table 4, entry 10B) proceeded with the loss of tritium located at C-7. It is therefore apparent that while the isomerization of the 8(9) double bond to the 7(8) double bond by rat liver enzymes proceeds with the loss of a 7β-hydrogen atom derived from 2 pro *S* of MVA of the parent lanosterol, the isomerization by yeast enzymes involves the elimination of the 7α-hydrogen atom derived from 2 pro *R* of MVA.[128]

Considering that yeast sterols contained four tritium atoms originating from stereospectrally labeled [2-^{3}H]-MVA, the results suggested that "*S*"-metabolites are labeled at C-1α, 7, 22*S* and 26, while "*R*"-metabolites are labeled at C-1β, 15(?), 22*R* and 26.

The presence of tritium and ^{14}C at C-26 was proven in the course of structure determination of the "*R*"-metabolites.[131] To demonstrate the presence of the C-24(25) double bond, "*R*"-[$^{15}C_5$; $^3H_4$]-triene acetate (**18-1c**) was converted to the 4-phenyl-1,2,4-triazoline-3,5-dione (**18-15**) and the triazoline was ozonized.[131] The reaction mixture was treated with $LiAlH_4$ and the resulting "*R*"-[$^{14}C_4$; $^3H_3$]-chole-5,7-diene-3β,24-diol (**18-16**) was recovered and proven to be identical to an authentic sample prepared by $LiAlH_4$ reduction of methyl 3β-acetoxy-chol-5,7-diene-24-oate[131] (**18-17**). The transformation of the "*R*"-[$^{14}C_5$; $^3H_4$]-C_{27}-triene (**18-1c**) (Table 4, entry 11) to "*R*"-[$^{14}C_4$; $^3H_3$]-C_{24}-dien-diol (**18-16**) (Table 4, entry 12) involved the loss of an atom of tritium and an atom of ^{14}C. This confirmed the presence of a C-24(25) bond and of tritium and ^{14}C at a terminal methyl group.

Based on the results of studies of fusidic acid and cholesterol, it could be assumed with certainty that the 2 pro *R* and 2 pro *S* hydrogen (tritium) atoms of MVA were incorporated at C-22 of yeast sterols without scrambling. However, the situation at C-15 was not clear. To account for the incorporation of four tritium atoms in "*R*"-(**18-1**)- and "*S*"-(**18-2**)-metabolites and in view of the presence of a C-7 tritium atom in the "*S*"-metabolites, the "*R*"-metabolites would have to retain a tritium atom at C-15. This suggested that the 14α-methyl of lanosterol is eliminated by yeast enzymes with the formation of a 8(9); 14(15)-diene in a manner similar to that of rat liver enzymes. Therefore, elaboration of the 14(15) double bond would have to proceed with the retention of a tritium atom derived from [2*R*; 2-^{3}H]-MVA at C-15.

Then, there was the question of the mode ("*cis*" or "*trans*") of reduction of the C-14(15) double bond. The yeast metabolites, 5,7,24-cholestatrienols and 7,24-dienols, have the 14α(H)-stereochemistry, hence the stereochemistry of the reduction of $\Delta^{14(15)}$ could be deduced from the stereochemistry of the C-15 tritium atom of the "*R*"-metabolite(s). Thus, a 15α-tritium will prove that the reaction is a *trans* reduction, whereby a 14α-hydrogen (from NADPH)[98] and a 15β-hydrogen from the medium were acquired. A 15β-tritium will demonstrate that the process is a *cis* reduction since, in this instance, a 14α-hydrogen and a 15α-hydrogen had to be added.

STEREOCHEMISTRY OF PHOTOCHEMICAL C-14(15) DOUBLE BOND FORMATION[133,134]

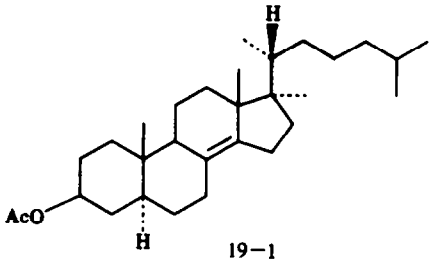
The determination of the stereochemistry of C-15 tritium atom posed several problems. The procedure previously used in the determination of tritium at C-15 of cholesterol[109,110] was long, required relatively large amounts of biosynthetic cholesterol, preferably with high specific activity, and the yields, except in the last step of microbial hydroxylation, were low. We therefore decided to develop an alternative general method for a stereospecific attack at C-15 of steroids.

In our search for an alternative method, we turned our attention to the photochemical dehydrogenation procedure of Breslow and co-workers.[133,134] They showed that irradiation of a mixture of 5α-cholestanyl acetate and $C_6H_5ICl_2$ in benzene, followed by treatment of the intermediate chlorides with $AgClO_4$, yields 5α-cholest-14-en-3β-ol acetate and 5α-cholest-9(11)-en-3β-ol acetate as the main products, and a small amount of cholesteryl acetate. They inferred that the formation of the 14(15) double bond may involve the overall *cis* abstraction of the 14α- and 15α-hydrogen atoms.[134] The simplicity of the dehydrogenation procedure was most attractive, but to be applicable for our purposes, it was necessary to prove its stereospecificity with respect to the abstraction of the C-15-hydrogen atom.

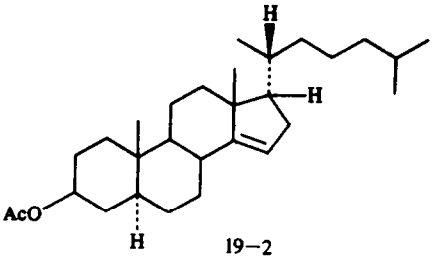
Our approach to the investigation of the stereochemistry of the removal of a C-15-hydrogen atom in the C-14(15) double bond formation was to use samples of (5α)-cholestanyl acetates stereospecifically labeled with deuterium at C-15. From the loss or retention of deuterium in the photochemical dehydrogenation of the model compounds, the overall stereochemistry of the C-14(15) double bond formation would then be deduced.[135]

In the course of other studies, we have investigated the $HCl/CHCl_3$ catalyzed backbone rearrangement of 17α(H)-$\Delta^{8(14)}$-sterols (**19-1**) to 17β(H)-$\Delta^{14(15)}$-sterols (**19-2**).[136,137] When the isomerization was carried out at −78°, the 17β(H)-$\Delta^{14(15)}$-sterol (**19-2**) was obtained in 80–90% yield. However, in the range of temperatures from −30° to *ca* 6°, the isomerization proceeded mainly without the backbone rearrangement to yield 17α(H)-$\Delta^{14(15)}$-sterols (**19-3**). To determine the time course of the reaction, we carried out the isomerization of **19-1** in $C^2HCl_3/^2HCl$ at 5°. With passage of time, the initially-formed 8β-monodeuterated Δ^{14}-product (**19-4**) was slowly converted to a dideuterated olefin (**19-5**). The NMR spectrum revealed the C-15 location of the second deuterium atom, as evidenced by the decreased intensity of the signal for the C-15 vinylic proton at 5.12 ppm relative to the intensity of the signal for the 3α-hydrogen at 4.7 ppm used as reference (1H). After 8 hr, the mixture contained *ca* 85% of the dideuterated 14(15) olefin (**19-5**). The experiment was then repeated on a preparative scale and the mass spectrum (MS) of the recovered 14-olefin indicated that the product was a mixture of *ca* 80% of dideutero [8β,15]-$^2H_2$ molecules (**19-5**) and *ca* 20% of monodeutero [8β]-$^2H_1$ molecules (**19-4**). The proton NMR spectrum was in accord with this conclusion, since the signal for the C-15 vinylic hydrogen was equivalent to 0.17 atom of hydrogen, in comparison to the 3α-hydrogen (Fig. 1(A)).

Anastasia *et al.*[138] independently investigated the transformations associated with the $HCl/CHCl_3$ catalyzed isomerization of 5α-cholest-8(14)-en-3β-ol acetate (**19-1**) and of the 7(8) analog to the 14(15)-



19–1



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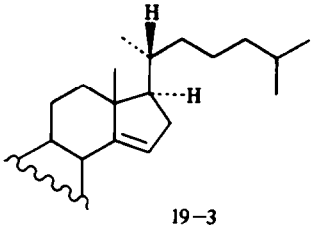

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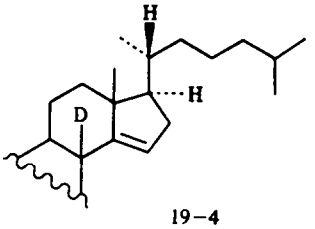
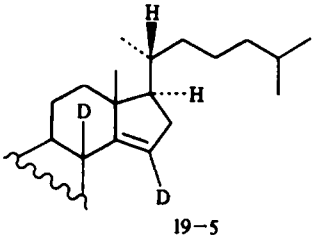


19–4



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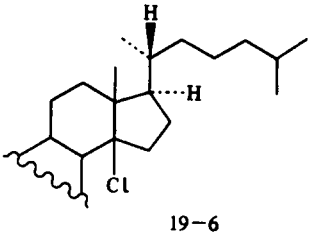

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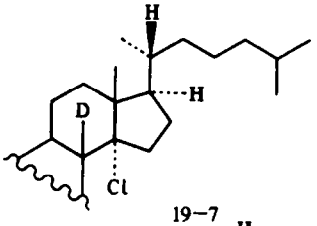

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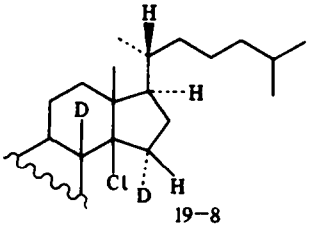
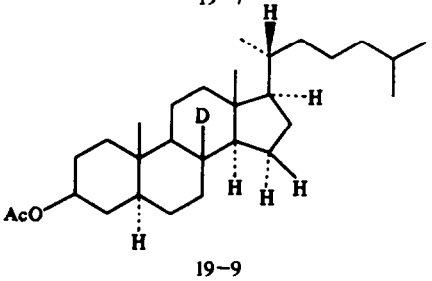
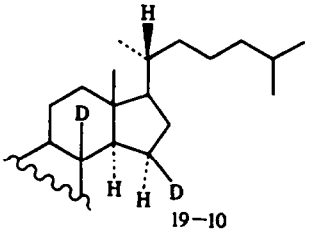


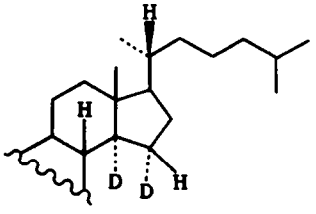
19–8



19–9



19–10



19–11

Chart 19.

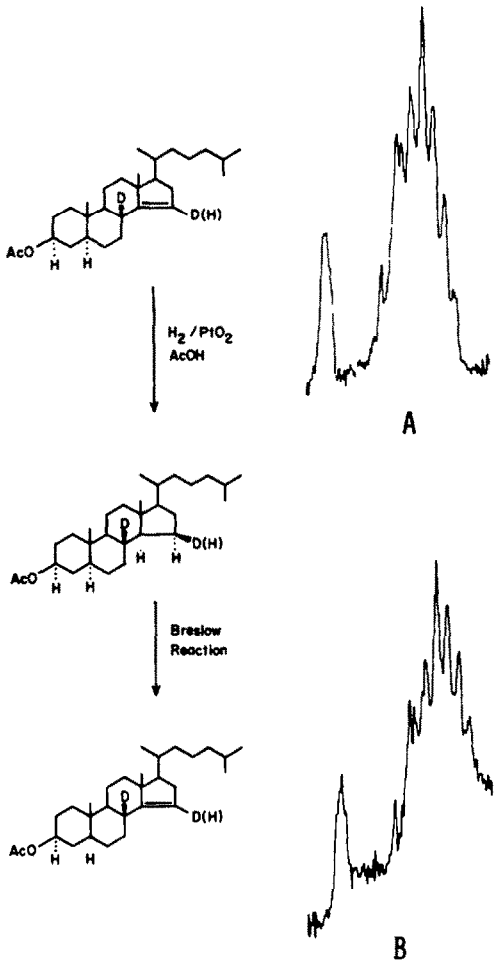


Fig. 1.

olefin. They isolated, from the reaction mixture, 14β-chloro-(5α)-cholestan-3β-ol acetate (**19-6**) and postulated that the chloride is formed via the addition of HCl to the 14(15)-olefin[138] (**19-3**). Assuming that the acquisition of HCl by a double bond is a *trans* addition reaction, the addition of ^{2}HCl to **19-3** could result in the acquisition of a 14β-chloride and a 15α-deuterium. Based on these hypotheses, the elaboration of the 8,15-dideutero-olefin (**19-5**) can be tentatively rationalized as follows. Exposure of **19-1** to ^{2}HCl may yield 8β-deutero-14α-chloride (**19-7**), which could collapse to **19-4**. The isolation from the isomerization reaction of an unidentified 14ξ-chloride (which could be the 14α-chloride) and which, on treatment with $NaHCO_3$ gave the 14(15)-olefin (**19-3**), was reported.[25] Subsequent acquisition[138] by **19-4** of ^{2}HCl should give the 14β-chloride 15α-^{2}H-(**19-8**). The *cis* elimination of HCl (14β-Cl and 15β-^{1}H) would yield the [8β,15]-dideutero olefin (**19-5**). The proposed *cis* HCl elimination is based on Breslow *et al.*'s hypothesis for the introduction of the 14(15) double bond.[134] According to Breslow *et al.* irradiation of cholesterol acetate with $C_6H_5ICl_2$ gives 14α-chlorocholestanol acetate which is dehydrohalogenated ($AgClO_4$) with the loss of the *cis* 15α-hydrogen atom.[134]

In any event, the formation of the [8β,15]-dideutero-14(15)-olefin (Fig. 1(A)) was most welcomed since it provided a facile access to one of the required substrates, stereospecifically labeled with deuterium. Hydrogenation (PtO_2–AcOH) of the mixture of mono and dideutero (**19-4**; **19-5**) gave cholestanyl acetate consisting of 21.8% of the 8β-monodeutero (**19-9**) and 78.2% of [8β,15β]-dideutero (**19-10**) molecules. The assignment of the 15β-^{2}H-stereochemistry is based on the known course of *cis* addition of 14α- and 15α-hydrogen atoms in the (PtO_2–AcOH) hydrogenation of the 14-olefin.

A solution of the [8β]-mono and [8β,15β]-dideutero (**19-9**; **19-10**) and $C_6H_5ICl_2$ in benzene was irradiated and the recovered steroids were treated with $AgClO_4$. The residue was fractionated by argentation TLC to yield a mixture of 14(15)-olefins (**19-5** and **19-4**) (Fig. 1(B)) and the 9(11)-olefin. The

MS of the recovered Δ^{14}-compound indicated that it contains *ca* 22% of the 8β-monodeuterated (**19-4**) and 78% of the [8β,15β]-dideuterated (**19-5**) olefins. The proton NMR spectrum of the product shows a signal for the 15 vinylic proton equivalent to 0.2 of a hydrogen atom (Fig. 1(B)). It follows that the formation of the 14(15) double bond proceeded without loss of the [15β]-deuterium.

The mass spectrum of the 9(11)-olefin showed that it is a mixture of the mono-8β-[$^2H_1$]-$\Delta^{9(11)}$(19%) and the dideutero-[8β,15β]-[$^2H_2$]-$\Delta^{9(11)}$ (81%). It is evident that the introduction of the 9(11) double bond did not involve the loss of 8β- and/or 15β-hydrogen atoms.

The results indicated that the $C_6H_5ICl_2/h\nu/AgClO_4$ introduction of the 14(15) double bond seems to proceed with the *overall* loss of the *cis* 14α- and 15α-hydrogen atoms. However, the possibility could not be excluded that the observed overall *cis* rather than *trans* elimination occurred because the abstraction of the 15β-deuterium atom involved a large isotope effect. We felt, therefore, that the stereochemistry of the dehydrogenation must be confirmed with the use of 15α-deutero labeled substrate.

Accordingly, the Δ^{14}-olefin (**19-3**) (Fig. 2(A)) was deuterated ($^2H_2$–PtO_2–AcO^2H) to yield (14α,15α)-[$^2H_2$] cholestanyl acetate (**19-11**) (d_0 = 7.8%; d_1 = 2.1; d_2 = 89.2%). The photochemical $C_6H_5ICl_2/h\nu/AgClO_4$ dehydrogenation of **19-11** gave the olefin (**19-3**) as the main product (Fig. 2(B)). The MS of the recovered **19-3** indicated that it was devoid of deuterium and the proton NMR showed a signal for the 15-vinylic proton equivalent to one hydrogen atom (Fig. 2(B)). It can thus be concluded that the 14,15 dehydrogenation involves the overall abstraction of the *cis* 14α- and 15α-hydrogen (deuterium) atoms. The obtained 9(11)-olefin (d_0 = 8.1%; d_1 = 1.2%; d_2 = 90.4%) retained all the deuterium initially present in **19-11**.

In the course of the above described studies of the biosynthesis of cholesterol, we have proven with

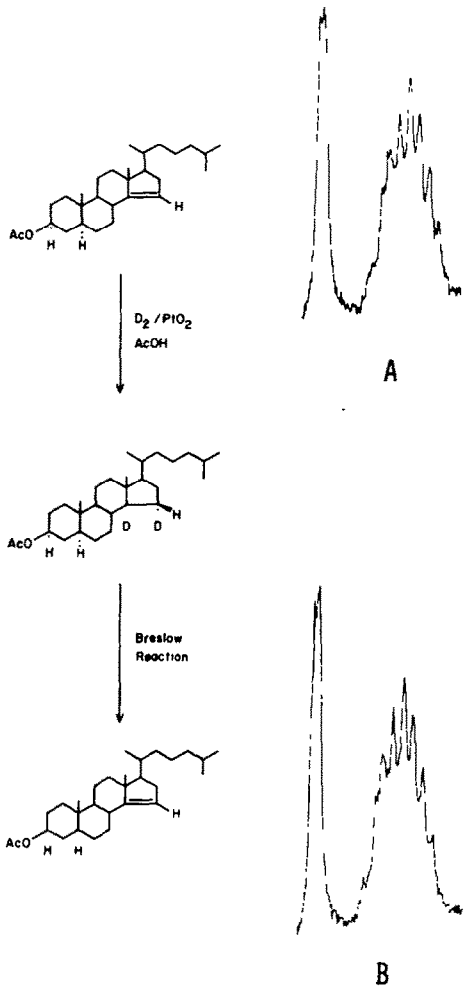
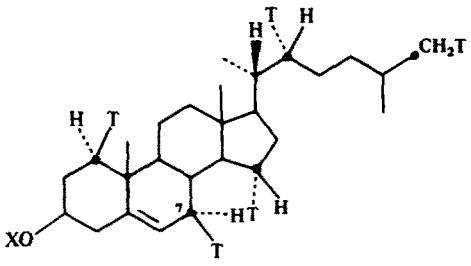
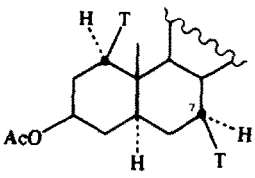


Fig. 2.

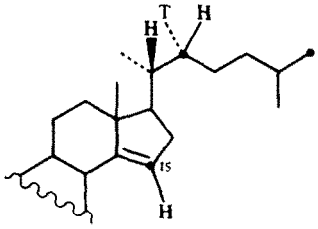


20–1

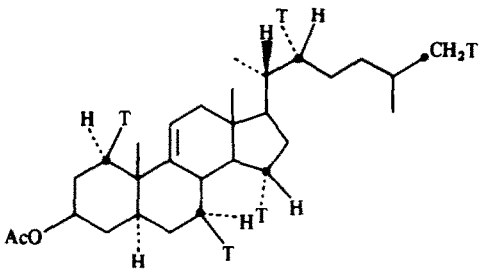
a X = H • = ^{14}C
b X = Ac



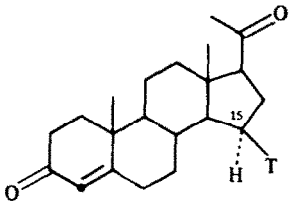
20–2



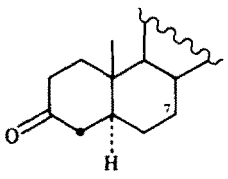
20–3



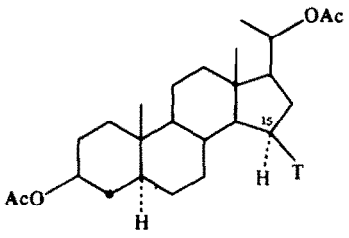
20–4



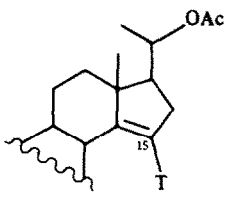
20–5



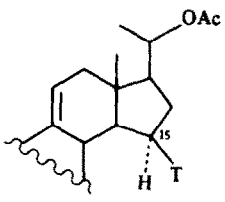
20–6



20–7



20–8



20–9

Chart 20.

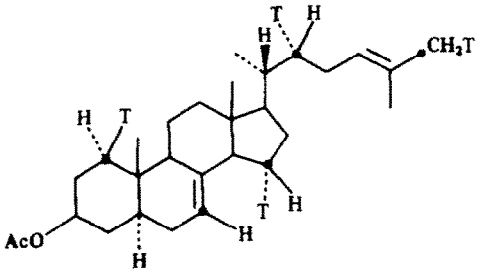
the use of enzymatic procedures that "*R*"-[$^{14}C_5$; $^{3}H_5$]-cholesterol (**20-1a**) biosynthesized by rat liver enzymes from (3*RS*; 2*R*; 2-^{3}H)-MVA has a 15α-tritium atom. We therefore tested the $C_6H_5ICl_2/h\nu/AgClO_4$ dehydrogenation procedure with the use of the "*R*"-[$^{3}H_5$; $^{14}C_5$]-cholesterol of rat origin. Accordingly, "*R*"-[$^{3}H_5$; $^{14}C_5$]-cholesteryl acetate (**20-1b**) (^{3}H : ^{14}C isotopic 8.7; atomic 5.0 : 5) was hydrogenated (EtOAc–PtO_2–$HClO_4$) to give "*R*"-[$^{3}H_5$; $^{14}C_5$]-(5α)-cholestanyl acetate (**20-2**) (^{3}H : ^{14}C isotopic 8.7). Dehydrogenation of **20-2** with $C_6H_5ICl_2/h\nu/AgClO_4$ gave "*R*"-[$^{3}H_4$; 5-$^{14}C_5$]-5α-cholest-14(15)-en-3β-ol acetate (**20-3**) (^{3}H : ^{14}C isotopic 6.7; atomic 3.9 : 5) and "*R*"-[$^{3}H_5$; $^{14}C_5$]-5α-cholest-9(11)-en-3β-ol acetate (**20-4**) (^{3}H : ^{14}C isotopic 8.7). The results clearly establish that the $C_6H_5ICl_2/h\nu/AgClO_4$ dehydrogenation of "*R*"-cholestanyl acetate to "*R*"-(5α)-cholest-14-en-3β-ol acetate (**20-3**) proceeded with the loss of the 15α-tritium atom. This confirmed the stereo-

specificity of the dehydrogenation process which is in accord with the hypotheses of Breslow *et al.*[134] No involvement of the 8β-, 14α-, and 15β-hydrogen atoms was noted in the course of the 9(11) double bond introduction.

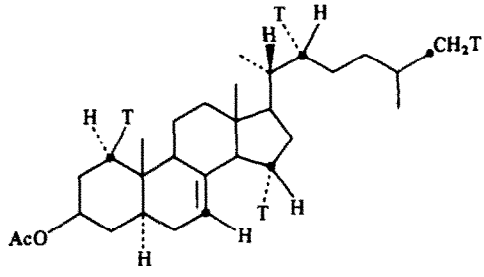
We also tested the applicability of the dehydrogenation procedure on a C-21 steroid. To this end, the previously prepared [4-^{14}C; 15β-^{3}H]-progesterone[110,109] (**20-5**) (^{3}H : ^{14}C atomic 1:1) was reduced with lithium in liquid ammonia and the recovered residue was oxidized (Jones' reagent) to yield 5α-pregnan-3,20-dione (**20-6**) admixed with some starting material. The crude residue was dissolved in methanol, treated with sodium borohydride and the resulting alcohols were acetylated. The acetates were treated with *m*-chloroperbenzoic acid to give, after chromatographic purification, homogeneous [4-^{14}C; 15β-^{3}H]-3,20-diacetate (**20-7**). Dehydrogenation of **20-7** ($C_6H_5ICl_2/h\nu/AgClO_4$) gave the 14(15)-olefin (**20-8**) and the 9(11)-olefin (**20-9**). The atomic ^{3}H : ^{14}C ratio of **20-8** was 0.84 : 1 indicating a loss of *ca* 16% of tritium present in the starting material. No loss of tritium was observed in the 9(11)-olefin (**20-9**). The reason for the partial loss of (15β)-tritium in **20-8** is not clear. It might be due to the involvement of the 20β-acetate in the reaction.

STEREOCHEMISTRY OF THE C-15 TRITIUM ATOM IN YEAST STEROLS[130,135]

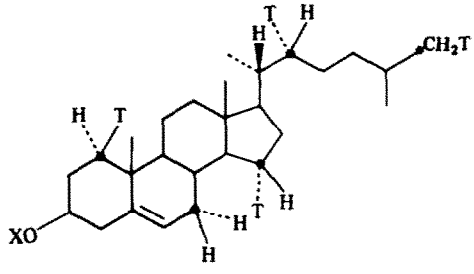
We could now turn our attention to the determination of the stereochemistry of tritium at C-15 of yeast metabolites. Our first objective was the conversion of the "*R*"-yeast metabolites to the required "*R*"-(yeast)-[$^{14}C_5$; $^{3}H_4$]-cholestanol. In the absence of an appropriate chemical method of reduction



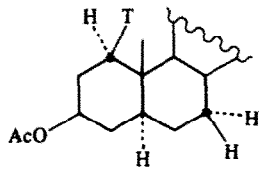
21–1



21–2

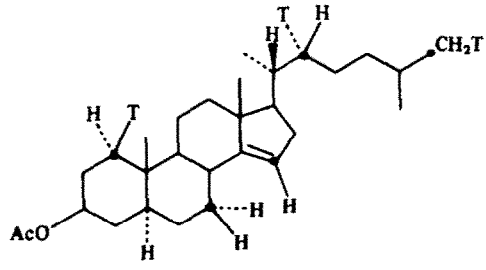


21–3



21–4

a X = H
b X = Ac



21–5

Chart 21.

Table 5. Stereochemistry of tritium atom at C-15 in sterols biosynthesized from (3*RS*, 2*R*) [2-^{14}C; 2-^{3}H]-MVA in a yeast homogenate

NO	Compound	$^{3}H:^{14}C$ Ratio Isotopic	Atomic
1	MVA-benzhydrylamide	10.7	1.0:1
2	Squalene HCl	10.3	5.8:1
3	5α-cholest-7-en-3β-ol Ac[a] (21-2)	8.7	4.1:1
4	Cholesterol[b] (21-3a)	8.65	4.0:1
5	Cholesteryl acetate (21-3b)	8.7	4.0:1
6	5α-Cholestanyl acetate[c] (21-4)	8.7	4.0:1
7	5α-Cholest-14-en-3β-ol Ac[d] (21-5)	6.7	3.1:1
8	Recovered[e] (21-4)	8.6	4.0:1

[a] Obtained by hydrogenation of the "R"-yeast metabolite (see text)

[b] From incubation of "R"-(yeast)-7-en-3β-ol (21-2) with rat liver enzymes

[c] Hydrogenation of (21-3b)

[d] Photochemical dehydrogenation of (21-4)

[e] Starting material (21-4) recovered from dehydrogenation

of the C-7(8) double bond of the yeast metabolites, we opted for a combination of biosynthetic and chemical procedures. The *yeast* "*R*"-[$^{14}C_5$; $^{3}H_4$]-3β-acetoxy-(5α)-cholesta-7,24-diene (**21-1**) (note no tritium at C-7) was mixed with 3β-acetoxycholesta-5,7-diene and the mixture was hydrogenated (EA–Raney Ni) to yield after saponification "*R*"-[$^{14}C_5$; $^{3}H_4$]-3β-hydroxy-(5α)-cholest-7-en (**21-2**) (Table 5, entry 3). The obtained 7-en (**21-2**) was incubated with a rat liver homogenate and the resulting "*R*"-(yeast)-[$^{14}C_5$; $^{3}H_4$]-cholesterol (**21-3a**) (Table 5, entry 4) was acetylated. The acetate (**21-3b**) (Table 5, entry 5) was hydrogenated to give the required "*R*"-(yeast)-[$^{14}C_5$; $^{3}H_4$]-3β-acetoxy-(5α)-cholestane (**21-4**) (Table 5, entry 6). It may be recalled that the conversion of a (5α)-Δ^7-cholestanol precursor to cholesterol proceeds via the intermediate formation of a 5,7-dien-ol and subsequent reduction of the 7(8) double bond[99] (see Chart 12). The "*R*"-(yeast)-[$^{14}C_5$; $^{3}H_4$]-3β-acetoxy-(5α)-cholestane (**21-4**) was dehydrogenated ($C_6H_5ICl_2$/*hv*/$AgClO_4$) to yield, following chromatographic fractionation, "*R*"-(yeast)-[$^{14}C_5$; $^{3}H_3$]-3β-acetoxy-cholest-14-ene (**21-5**) (Table 5, entry 7). The introduction of the 14(15) double bond proceeded with the loss of tritium revealing the presence of a 15α-tritium atom in the yeast metabolites. It may therefore be concluded that in the yeast, the elimination of the 14α-methyl (e.g. of lanosterol) involves the formation of a 14(15) double bond with the loss of a 15α-hydrogen derived from 2 pro *S* of MVA. The 14-olefin is then reduced through a *trans* acquisition of a 14α-hydrogen from NADPH and a 15β-hydrogen from the water of the medium. While in the yeast the events at C-7(8) proceed in a different manner than in rat livers, the events at C-14(15) follow the same route in the two species.

In summary, the results show that the yeast "*R*"-cholesta-5,7,24-trien-3β-ol (and "*R*"-7,24-dien-ol) have tritium atoms at 1β-, 15α-, 22*R*- and 26-carbon atoms, while the "*S*"-cholesta-5,7,24-trien-3β-ol (and "*S*"-7,24-dien-ol) have tritium atoms at 1α-, 7-, 22*S*- and 26-carbon atoms.

BIOSYNTHESIS OF PLANT STEROLS

At the time, the observed stereochemical differences in the metabolism of lanosterol in tissues of different species (rat livers and yeast preparations) were unexpected. The situation was further complicated by a report which was interpreted as indicative of the retention of both the 2 pro *R* and 2 pro *S* hydrogen atoms of MVA at C-15 of phytosterols biosynthesized by petals of marigold flowers (*Calendula officinalis*).[139] The proposed retention of both C-2 hydrogen atoms of MVA at C-15 of plant sterols was in sharp contrast to the situation in rat livers and yeasts and suggested that the removal of the 14α-methyl in plants proceeds via the formation of the 8(14)-olefin[140–142] and not of a 8,14-dien.

The likelihood of an alternative mechanism was further enhanced by the isolation of 5α-stigmasta-8(14),22-dien-3β-ol from rayless goldenrod.[143]

The observations in marigold flowers,[139] if true, had much broader ramifications. The available evidence indicated that the biosynthesis of plant sterols proceeds via the initial cyclization of squalene to cycloartenol (**22-1**), rather than to lanosterol, and that the cycloartenol is then metabolized to phytosterols.[144–147] This suggested that the mechanism of metabolic transformations of cycloartenol to plant sterols differs from that of lanosterol in rats and yeast.

The hypothesis that cycloartenol is the key triterpene precursor of plant sterols was based on indirect evidence which can be summarized as follows. Cycloartenol was present in relatively large amounts in plants, while lanosterol was found rarely and then in minor amounts.[147,148] Both [1-^{14}C]-acetate[145b] and [2-^{14}C]-MVA[149] were much more efficiently incorporated into cycloartenol than into lanosterol by plants and plant tissues. It was noted also that (radioactive) cycloartenol was more efficiently metabolized to phytosterols than (radioactive) lanosterol.[146]

In view of the importance of the problem, we wished to evaluate the hypothesis and eventually establish a direct link between cycloartenol and phytosterols.[150] The rationale of our approach was based on the premise that if cycloartenol is an obligatory precursor of phytosterols, then at a certain stage of the biosynthetic transformations the C-19(9) bond of the cyclopropane ring of (**22-1**) will be cleaved[7,8] to an intermediate with a cationic terminus at C-9 and an anionic terminus at C-19 (**22-2**). The C-9 cation will be stabilized via the loss of an, e.g. C-8 (or C-11) proton to give the C-8(9) double bond (or C-9(11) double bond), while the C-19 anion will acquire a proton to give a 10β-methyl group (**22-3**). The same outcome may be expected if the reaction is concerted. It follows, therefore, that if cycloartenol is an obligatory biosynthetic intermediate, the derived phytosterols will have a proton originating from the water of the medium at C-19, but not at C-18. Hence, if the biosynthesis of a phytosterol, e.g. sitosterol, is carried out in deuterium oxide, deuterium will be incorporated[150] at C-19 and not at C-18 (**22-4**). In contrast, it was proven that cholesterol biosynthesized from squalene or lanosterol in ^2H_2O did not incorporate deuterium[31,81,151] at C-19 or C-18. This hypothesis will remain valid as long as no significant amounts of endogenously produced [^{2}H]-acetyl-CoA is incorporated via MVA → squalene → cycloartenol into phytosterols which would then be labeled with deuterium at both C-19 and C-18.

Peas contain relatively large amounts of cycloartenol and can be germinated in deuterium oxide.[152] Hence, if cycloartenol is an obligatory precursor of plant sterols, then, e.g. sitosterol, biosynthesized in peas germinated in deuterium oxide [^2H_2O], will incorporate a maximum of one deuterium atom at C-19, but none at C-18. To evaluate the pathway, we have germinated peas in deuterium oxide and then processed their embryos and cotyledons separately.[150] Following saponification and preliminary

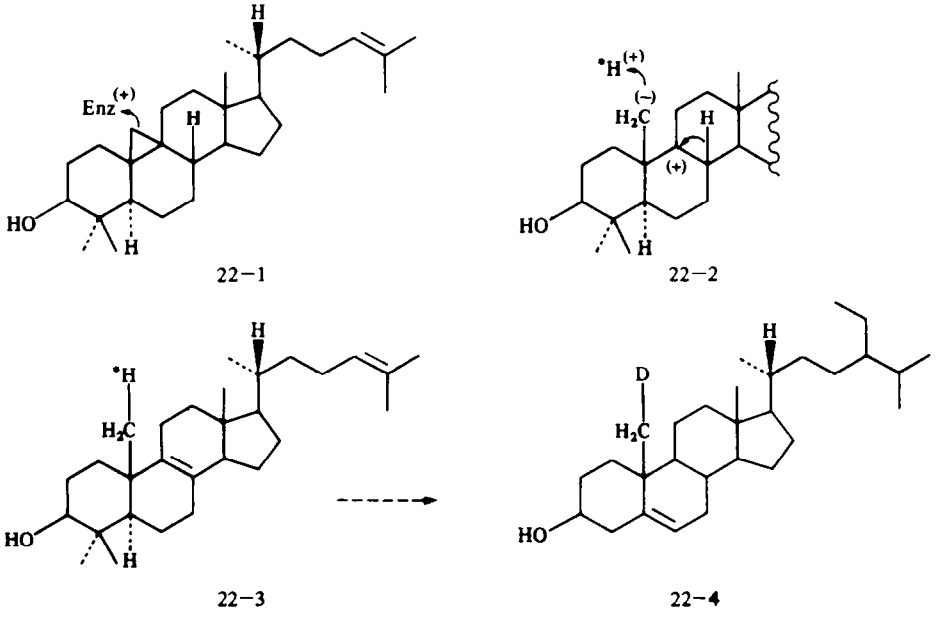


Chart 22.

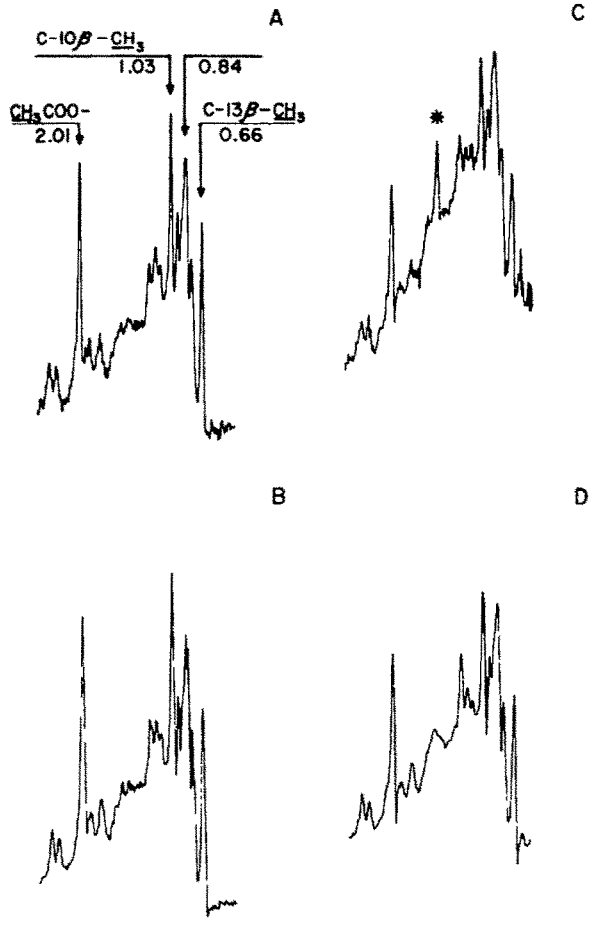


Fig. 3.

chromatographic fractionation of the unsaponifiable residues, the appropriate steroidal zones were isolated and acetylated. The acetates were then extensively purified (TLC, argentation TLC, GLC) until homogeneous samples of [^{2}H]-sitosterol acetates (from embryos and cotyledons) were obtained. In addition reference samples were obtained from embryos of peas germinated in water and from extensively purified authentic sitosterol. The proton NMR spectra of the acetates of the four samples were recorded (Fig. 3). The authentic sitosteryl acetate shows a singlet for the three C-19 protons at 1.03 ppm which is visibly "higher" than the multiplet at 0.84 ppm (Fig. 3(A)). The same applies also to the spectrum of the sitosteryl acetate isolated from the embryos of peas germinated in H_2O (Fig. 3(B)). The signals for the 10β-methyl (1.03 ppm), 13β-methyl (0.66 ppm) and methyl of the acetate (2.01 ppm) of expanded spectra (4 : 1) were integrated. The signals of the methyls of the acetates (2.01 ppm) were used as internal standards equivalent to three hydrogens. The ratios of the C-10β and C-13β methyls to the methyl of the acetate were 1 : 1 : 1. The spectra of the [^{2}H]-sitosteryl acetates (Figs 3(C) and (D)) show that the signals for the 10β methyls at 1.03 ppm are visibly less intense than those of the multiplets at 0.84 ppm. Integration of the C-10β methyl signals of the [^{2}H]-sitosteryl acetates from embryos (Fig. 3(C)) and cotyledons (Fig. 3(D)) showed that they correspond to 85 and 87% of the intensity of the acetate methyls, respectively. In contrast, the intensities of the 13β-methyl signals (Figs 3(C) and (D)) were the same as those of the acetates (1 : 1). The results indicate that 0.45 and 0.4 atoms of deuterium were incorporated at C-19 of the [^{2}H]-sitosterols isolated from embryos and cotyledons, respectively. No detectable amounts of deuterium were incorporated at C-18.

The relatively high incorporation of deuterium at C-19 (40–45% of theoretically possible) is in accord with the view that the biosynthesis of plant sterols proceeds via an obligatory intermediate having an "anionic terminus" at C-19. The C-19 anionic intermediate is derived from cycloartenol through the cleavage of the C-19(9) bond of the cyclopropane ring.

BIOSYNTHESIS OF SITOSTEROL FROM (3*RS*, 2*R*)- AND (3*RS*, 2*S*)[2-^{14}C; 2-^{3}H]-MVA

Having confirmed the key role of cycloartenol (or of an intermediate with an anionic terminus at C-19) in the biosynthesis of phytosterols, we proceeded with studies of the mode of incorporation of the C-2 proton of MVA into plant sterols. To this end we again used peas which were germinated in aqueous solutions of either (3*RS*, 2*R*)- or (3*RS*, 2*S*)[2-^{14}C; 2-^{3}H]-mevalonic acid.[153–155] The peas were processed in the usual manner and, after chromatographic fractionation, samples of "*R*"- and "*S*"-squalenes and sitosterols were isolated. The squalenes were first purified by TLC, then via thiourea adducts and finally were converted to "*R*"- and "*S*"-squalene · 6HCl and crystallized (× 3). The sitosterols were acetylated and then extensively purified until chemically and radiochemically homogeneous "*R*"- and "*S*"-sitosteryl acetates were obtained.[154] The squalene · 6HCl's and sitosteryl acetates were counted (Table 6, entries 2 and 3). The parent [2*R*; 2-^{14}C; 2-^{3}H]- and [2*S*; 2-^{14}C; 2-^{3}H]-mevalonic acids were converted to the respective benzhydrylamides and the amides were also counted (Table 6, entry 1). We were surprised to notice that the biosynthesis of squalene from the (2*R*; 2-^{14}C; 2-^{3}H)- and (2*S*; 2-^{14}C; 2-^{3}H)-MVA proceeded with very large losses of tritium, as evidenced by the unusually large decreases of the isotopic ^{3}H : ^{14}C ratios of the "*R*"- and "*S*"-squalenes vs their parent [2*R*; 2-^{14}C; 2-^{3}H] and [2*S*; 2-^{14}C; 2-^{3}H] samples of mevalonic acids.

In the context of other studies (see below), we have investigated the biosynthesis of squalene and sitosterol from (3*RS*, 2*R*)- and (3*RS*, 2*RS*)[2-^{14}C; 2-^{3}H]-MVA in petals of marigold flowers (*Calendula officinalis*) and, again, massive losses of tritium were observed in the biosynthesis of squalene. In contrast, in our previous studies in yeast, *F. coccineum* and rat livers, considerably smaller losses of tritium were observed.

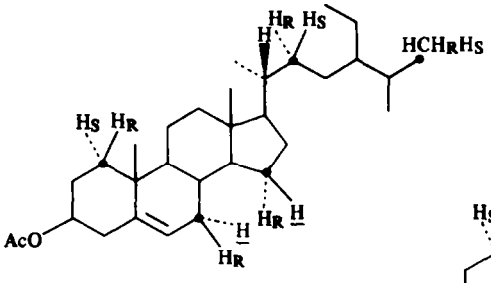
In any case, for the current discussion of the distribution and stereochemistry of tritium atoms in "*R*"- and "*S*"-sitosterols, we will accept the premise that the respective "*R*" and "*S*" (plant) squalenes contained six atoms of tritium and six atoms of ^{14}C. In other words the squalenes derived from (2*R*; 2-^{14}C; 2-^{3}H)- and (2*S*; 2-^{14}C; 2-^{3}H)-MVA each have six carbon atoms labeled with ^{14}C and the carbons labeled with ^{14}C also contain carbon atoms labeled with tritium. To facilitate discussion, we will

Table 6. Distribution and stereochemistry of ^{3}H at C-1, C-7 and C-15 of (*R*)- and (*S*)-sitosterols biosynthesized in the pea from (3*RS*, 2*R*)[2-^{14}C; 2-^{3}H]- and (3*RS*, 2*S*)[2-^{14}C; 2-^{3}H]-MVA, respectively

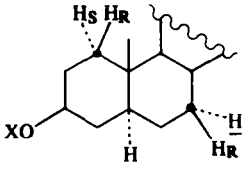
		A "R"-products ^{3}H:^{14}C Ratio		B "S"-products ^{3}H:^{14}C Ratio	
NO	Compound	Isotopic	Atomic	Isotopic	Atomic
1	MVA benzhydrylamide	10.0	1.0:1	3.1	1.0:1
2	Squalene 6HCl	5.2	6.0:6	2.0	6.0:6
3	Sitosteryl acetate (23-1)	5.2	5.1:5	1.3	3.2:5
4	5α-stigmastanone (23-3)	5.1	5.0:5	1.3	3.2:5
5	5α-stigmastanone equil. with base (23-3)	5.2	5.1:5	1.3	3.2:5
6	5α-stigmast-1-en-3-one (23-4)	5.0	4.9:5	1.0	2.5:5
7	1,3-seco-2-nor-5α-stigmasta-1,3-dioic acid (23-5)	4.3	4.1:5	0.9	2.3:5
8	Sitosterol (23-1; 3β-OH)	5.2	5.0:5	1.3	3.2:5
9	5α-hydroperoxystigmast-6-en-3β-ol (23-6)	5.2	5.0:5	1.2	3.1:5
10	3β-hydroxystigmast-5-en-7-one (23-7)	4.3	4.2:5	1.2	3.0:5
11	5α-stigmastanyl acetate (23-2)	5.2	5.0:5	1.2	3.0:5
12	5α-stigmast-14-en-3β-ol acetate (24-1)	4.5	4.4:5	1.2	3.0:5
13	5α-stigmasta-3β,14α,15β-triol 3-acetate (24-3)	4.5	4.4:5	1.2	3.0:5
14	3β,14α-dihydroxy-5α-stigmast-15-one 3-acetate (24-4)	4.5	4.4:5	1.2	2.9:5

assume that "*R*"- and "*S*"-sitosterols retained 5 and 3 tritium atoms, respectively, and each has 5-^{14}C atoms.[153–155]

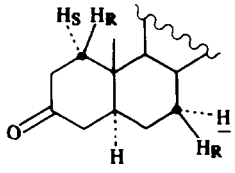
The determination of the location and stereochemistry of tritium in the sitosterols[153–155] was carried out by methods developed in the course of studies of cholesterol and yeast sterols. The sequence of reactions employed is summarized in Chart 23. Sitosteryl acetate (**23-1**) was hydrogenated (EtOAc; $HClO_4$; PtO_2; H_2) and the resulting (5α)-stigmast-3β-ol acetate (**23-2**; X = acetyl) was saponified (**23-2**; X = H) and oxidized (Jones' reagent) to yield (5α)-stigmastan-3-one (**23-3**). Dehydrogenation of **23-3** with DDQ, involving the abstraction of 1α- and 2β-hydrogen atoms, gave (5α)-stigmast-1-en-3-one (**23-4**). The obtained Δ^1-(**23-4**) was oxidized (RuO_4)[115] to 1,3-seco-2-nor-5α-stigmasta-1,3-dioic acid (**23-5**). To confirm the absence of tritium at C-2 and C-4, the (5α)-stigmast-3-one (**23-3**) was equilibrated with base (KOH/ether–MeOH; reflux).



23–1



23–2



23–3

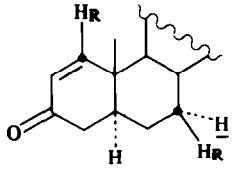
H_R and H_S protons derived from 2 pro R and 2 pro S hydrogens of MVA.

H: protons derived from water of the medium

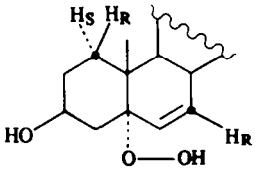
• Carbon atoms (^{14}C) derived from C-2 of MVA

a H_R = T, H_S = H; b H_R = H, H_S = T

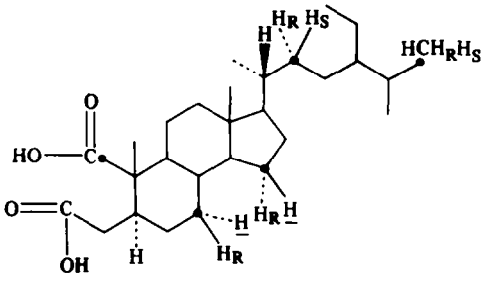
• ≡ ^{14}C



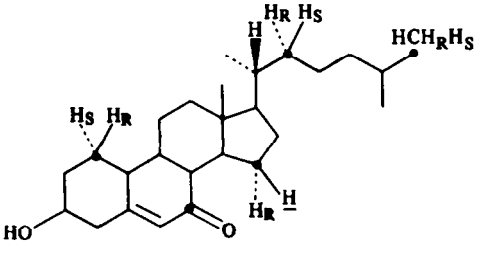
23–4



23–6



23–5



23–7

Chart 23.

When "*R*"-sitosterol (**23-1a**) ($H_R = {}^3H$; $H_S = H$; ● = ^{14}C) (Table 6, entry 3A) was submitted to the sequence of transformation **23-1a** → **23-2a** → **23-3a** → **23-4a** (Table 6, entries 3A–6A), no loss of tritium was observed. However, when **23-4a** was oxidized to the di-carboxylic (**23-5a**) (Table 6, entry 7A), tritium was lost. Since base-catalyzed equilibration of the 3-ketone (**23-3a**) proceeded without loss of tritium (Table 6, entry 5A), the tritium lost in formation of the di-carboxylic acid (**23-5a**) must have been located at C-1. Considering that the C-1(2) dehydrogenation with DDQ involved the removal of the 1α-hydrogen atom, it follows that the "*R*"-sitosterol has a 1β-tritium atom.[154]

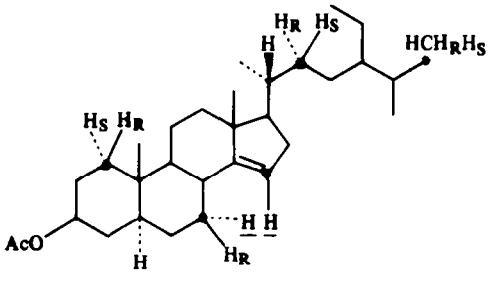
When "*S*"-sitosteryl acetate (**23-1b**) (Table 6, entry 3B) ($H_R = H$; $H_S = {}^3H$; ● = ^{14}C) was processed as shown **23-1b** → **23-2b** → **23-3b** (Table 6, entries 3B–5B) no loss of tritium was observed. However, DDQ-dehydrogenation of **23-3b** gave **23-4b** (Table 6, entry 6B) with loss of tritium. No additional loss of tritium occurred on oxidation of **23-4b** to the diacid (**23-5b**) (Table 6, entry 7B). The results show that "*S*"-sitosterol has 1α-tritium atoms.[154]

As before[110] for the determination of the situation at C-7, the Nickon–Bagli photochemical oxygenation method was employed.[116]

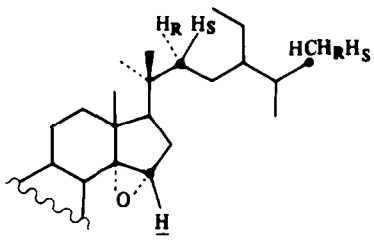
Photochemical oxygenation of sitosterol (**23-1**) (pyridine; hematoporphyrin; *hν*; air)[116] gave 5α-hydroperoxystigmast-6-en-3β-ol (**23-6**) which was rearranged (pyridine; $CuCl_2 \cdot H_2O$) to 3β-hydroxy-stigmast-5-en-7-one (**23-7**). The formation of the 5α-hydroperoxy-6-en (**23-6**) proceeds with the abstraction of the 7α-hydrogen atom.[116]

Conversion of "*R*"-sitosterol (**23-1a**) (Table 6, entry 8A) to the hydroperoxide (**23-6a**) (Table 6, entry 9A) did not involve loss of tritium. In contrast, rearrangement of **23-6a** to the 7-ketone (**23-7a**) (Table 6, entry 10A) proceeded with the loss of tritium. It follows therefore that "*R*"-sitosterol (**23-1a**) has a 7β-tritium atom.[154] The transformation of "*S*"-sitosterol (**23-1b**) via **23-6b** to the 7-ketone (**23-7b**) (Table 6, entries 8B–10B) did not entail losses of tritium which showed that "*S*"-sitosterol is devoid[154] of tritium at C-7.

The analysis of the stereochemistry of the tritium atom at C-15 was once more carried by the method of Breslow and co-workers.[134,135] Stigmastanyl acetate (**23-2**; X = acetate) was dehydrogenated photochemically (benzene; $C_6H_5ICl_2/h\nu/AgClO_4$) to yield 5α-stigmast-14-en-3β-ol

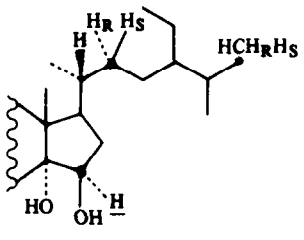


24–1

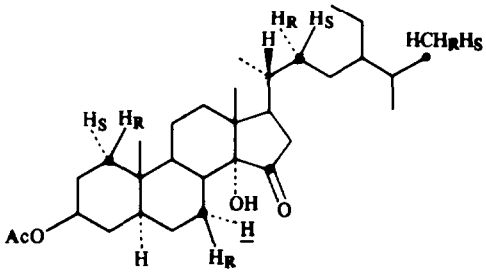


24–2

a H_R = T, H_S = H, ● ^{14}C
b H_R = H, H_S = T, ● ^{14}C
c H_R = T, H_S = T, ● ^{14}C



24–3



24–4

Chart 24.

acetate (**24-1**), with the loss of the *cis* 14α- and 15α-hydrogen atoms.[135] The Δ^{14}-olefin was treated with *m*-chloroperbenzoic acid and the resulting 14α,15α-epoxide (**24-2**) was hydrolyzed (acetone; water; periodic acid) to 5α-stigmasta-3β,14α,15β-trihydroxy 3-acetate (**24-3**). Oxidation of **24-3** (pyridine–CrO_3) resulted in 5α-stigmasta-3β,14α-dihydroxy-15-one 3-acetate (**24-4**).

The dehydrogenation of "*R*"-stigmastanyl acetate (**23-2a**; X = acetate) resulted in "*R*"-Δ^{14}-stigmastanyl acetate (**24-1a**) (Table 6, entries 11A and 12A) and proceeded with the loss of tritium. Subsequent transformations of **24-1a** → **24-2a** → **24-3a** → **24-4a** (Table 6, entries 12A–14A) did not involve additional loss of tritium. When "*S*"-stigmastanyl acetate (**23-2b**) was processed in a similar manner **24-1b** → **24-2b** → **24-3b** → **24-4b** (Table 6, entries 11B–14B) no loss of tritium was observed. The results show that "*R*"-sitosterol (**23-1a**) has tritium in the 15α-position while "*S*"-sitosterol (**23-1b**) has no tritium at C-15.

In view of the uncertainty concerning the incorporation of both the 2 pro *R* and 2 pro *S* hydrogen (tritium) atoms of MVA at C-15 of sitosterol biosynthesized in marigold flowers,[139] we have reinvestigated the problem.[155] The excised petals of marigold flowers were inserted in aqueous solutions of (3*RS*, 2*R*) [2-^{14}C; 2-^{3}H]-MVA and (3*RS*, 2*RS*) [2-^{14}C; 2-$^{3}H_2$]-MVA for 144 hr and then processed to yield "*R*"- and "*RS*"-sitosterols.[155]

Using methods described above, the recovered marigold-"*R*"-sitosteryl acetate (Table 7, entry 3A) was converted to "*R*"-Δ^{14}-stigmastanyl acetate (**24-1a**) (Table 7, entry 5A) with the loss of a 15α-tritium. Subsequent transformations of "*R*"-stigmastanyl acetate **24-1a** → **24-2a** → **24-3a** → **24-4a** (Table 7, entries 6A and 7A) proceeded without loss of tritium. Similarly the "*RS*"-sitosteryl acetate (Table 7, entry 3B) was converted to "*RS*"-stigmastanyl acetate (Table 7, entry 4B) and the latter, on dehydrogenation to "*RS*"-Δ^{14}-stigmastanyl acetate (**24-1c**), lost a 15α-tritium (Table 7, entry 5B). The transformations of **24-1c** → **24-2c** → **24-3c** → **24-4c** (Table 6, entries 6B and 7B) proceeded without additional loss of tritium. The results show that only the 2 pro *R* hydrogen (tritium) atom of MVA is retained at the 15α-position of sitosterol biosynthesized in the petals of marigold flowers.[155] It may therefore be concluded that the previous report was in error.[139]

Analysis of the results for plant sterols indicates that tritium atoms at C-1, 7 and 15 of pea sitosterol and at C-15 of sitosterol of *C. officinalis* were incorporated in a manner analogous to that of cholesterol biosynthesized by rat liver enzymes. However, it seems that in contrast to the fusidic acid, cholesterol and yeast sterols, the tritium atoms in plant sterols apparently are not distributed equally over the appropriate carbon atoms. Insofar as C-7 and C-15 are concerned, no significant scrambling of tritium was observed, since the isotopic hydrogen atoms were located at 7β- and 15α-positions, as expected. The pea-derived "*S*"-sitosterol (**23-1b**) showed the presence of *ca* 0.9 atom of tritium at C-1, of which *ca* 0.7 atom was at the expected 1α-position. However, the implied presence of 0.2 atom of tritium at the 1β-position is very likely a counting error, due to the very small amount of diacid (**23-5b**) which was available for counting.[154] The "*S*"-sitosterol was devoid of tritium at C-7 and C-15.

Table 7. Distribution and stereochemistry of ^{3}H at C-15 of (*R*)- and (*RS*)-sitosterols biosynthesized in excised *Calendula officinalis* petals from (3*RS*, 2*R*) [2-^{14}C; 2-^{3}H]- and (3*RS*, 2*RS*) [2-^{14}C; 2-^{3}H]-MVA, respectively

		A "R"-products		B "RS"-products	
		$^{3}H:^{14}C$ Ratio			
NO	Compound	Isotopic	Atomic	Isotopic	Atomic
1	MVA-amide	11.1		20.8	
2	Squalene 6HCl	9.1		13.3	
3	Sitosteryl acetate	8.9	5.0:5	14.3	8.0:5
4	5α-stigmastanyl Ac	8.9	5.0:5	14.4	8.1:5
5	5α-stigmast-14-en 3β-ol Ac (24-1)	7.6	4.3:5	12.9	7.3:5
6	5α-stigmast-3β,14α,15β-triol 3-Ac (24-3)	7.5	4.2:5	13.0	7.3:5
7	5α-stigmast-3β,14α-diol-15-one 3-Ac (24-4)	7.4	4.1:5	12:75	7.2:5
8	Stigmasteryl acetate			13.0	7.3:5

The results show that "*S*"-sitosterol has three tritium atoms at C-1α, 22*S* and 26 positions, while "*R*"-sitosterol has five tritium atoms at 1β, 7β, 15α, 22*R* and 26 positions.

CONCERNING THE LOSS OF C-2 TRITIUM IN THE BIOSYNTHESIS OF SQUALENE

We have pointed out that losses of tritium occur in the biosynthesis of squalene from mevalonic acid labeled with *tritium* at C-2. A summary of the relevant results is given in Table 8. It may be noted that in *F. coccineum*, yeast homogenates, and in the S_{10} fraction of rat liver homogenates, the losses were of the order of 2–13%. In contrast, in the plants the losses were significantly greater and varied from a low of *ca* 18–20% to about 50% of the C-2 tritium initially present in the parent MVA. Large losses of tritium were also observed by Goodwin and co-workers in the biosynthesis of ergosterol by *Aspergillus fumigatus* Fred,[156] and the biosynthesis of phytosterols by *Ochromonas malhamensis*.[157] In these two instances, some *minor scrambling* of the C-2-hydrogen (tritium) atoms of MVA was claimed.[156] Occasionally, we noted some additional losses of tritium between the *de novo* obtained squalene and the derived sterols, however, these were small and not significant. Except for sitosterol, in the biosynthesized cholesterol, yeast sterols and fusidic acid, the tritium atoms derived from (2-^{3}H-MVA) were distributed equally over the relevant carbon (^{14}C) atoms derived from C-2 of MVA. In all the products, including sitosterol, the 2 pro *R* and 2 pro *S* hydrogen (tritium) atoms of MVA were incorporated stereospecifically, essentially without randomization and retained their stereochemical integrity in relation to the parent C-2 hydrogen atoms of the precursor MVA.

It is now recognized that events leading to polyprenoids (see above) require the transformation of MVA-OPP to isopentenyl-OPP[54,55] which in turn is isomerized to dimethylallyl-OPP.[56,57,158] The isomerization is reversible and the equilibrium is shifted to dimethylallyl-OPP[57] (DMAPP : IPP (9 : 1)). It was proposed that the losses of the C-2 tritium (hydrogen) atoms of MVA in the biosynthesis of polyprenoids are due to the reversible IPP $\rightleftharpoons$ DMAPP isomerization.[156–159]

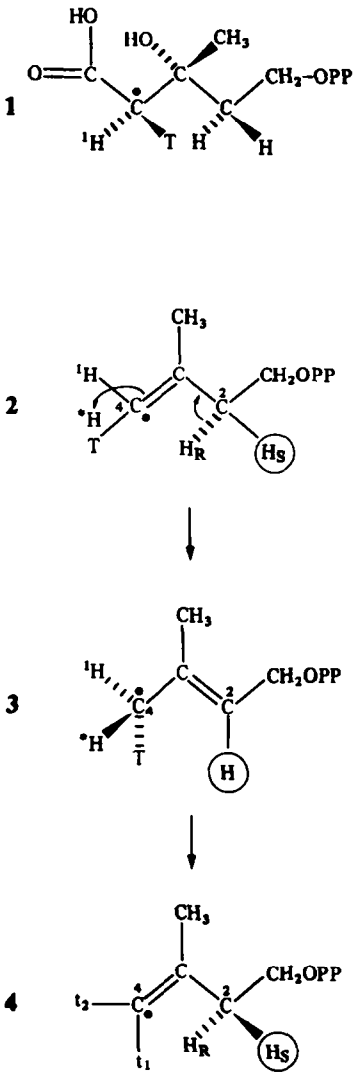
Popjak and co-workers[58,59] showed that the 2 pro *R* and 2 pro *S* hydrogen atoms of MVA assume the [*Z*] and [*E*] geometry in the derived isopentenyl-OPP (IPP). Therefore, the enzymatic transformation of [2*R*; 2-^{14}C; 2-^{3}H]-MVA (Scheme 1, **1**) will yield the [*Z*]-[4-^{3}H]-IPP (Scheme 1, **2**). The "*Z*"-[4-^{3}H]-IPP (Scheme 1, **2**) will be transferred to the prenyl isomerase and isomerized to [4-^{3}H]dimethylallyl-OPP (DMAPP) (Scheme 1, **3**). In the isomerization reaction, a proton from the medium will be added at C-4 from the 3 *re* : 4 *re* face[69] of the double bond and a 2 pro *R* hydrogen of the IPP (Scheme 1, **2**) will be eliminated.[58] Subsequently, the biosynthesis will proceed via coupling of C_5-units to give geranyl-PP → farnesyl-PP → squalene, etc.[58]

Table 8. Observed losses of tritium from C-2 of MVA in the biosynthesis of polyprenoids in the indicated systems

Biosynthetic System	(3RS;2X)[2-^{3}H]-MVA	Biosynthesized Polyprenoids	% Loss of Tritium
Yeast Homogenates[131]	R	"R"-squalene	3.5
	S	"S"-squalene	13.0
Peas (germinating)[154,155]	R	"R"-squalene	48.2
	S	"S"-squalene	32.8
Calendula officinalis[156]	R	"R"-squalene	17.6
	RS	"RS"-squalene	35.8
Calendula officinalis[139]	R	"R"-squalene	20.0
	S	"S"-squalene	22.0
F. coccineum[71,72]	R	"R"-Me-fusidate	2.1
	S	"S"-Me-fusidate	11.6
Rat liver S_{10} fraction[110,125]	R	"R"-squalene	5.0
	S	"S"-cholesterol* [125]	8.7

* Corrected for the incorporation of 3 tritium and 5-^{14}C atoms

The samples of mevalonic acid were extensively purified prior to use. On several occasions, parallel incubation experiments of the purified MVA with liver homogenates were carried out and the biosynthesized cholesterol was recovered. The loss of tritium in the produced cholesterol was low, of the order of 5-8%.



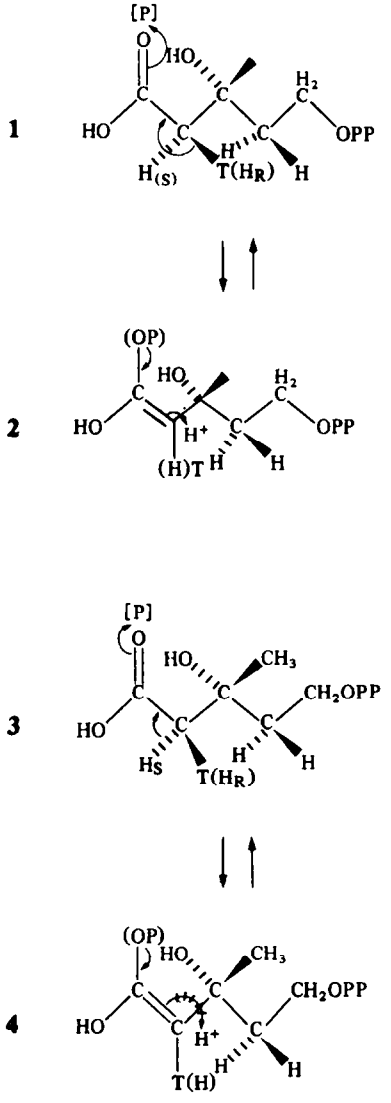
Scheme 1.

To explain the observed losses of tritium derived from C-2 of MVA in the reversible IPP $\rightleftharpoons$ DMAPP isomerization,[157-159] the methyl group of DMAPP (Scheme 1, 3) derived from C-2 of MVA (indicated with a ●) must be able to rotate freely. This could be achieved by partial or complete release of the DMAPP (Scheme 1, 3) from the isomerase.[37] Assuming a partial release which would allow the methyl (●) to rotate it is *most unlikely that the reverse isomerization DMAPP → IPP will give the [Z]-IPP* (Scheme 1, 2). On the other hand, if the DMAPP remains fully attached to the isomerase, the methyl (●) will be stationary and no loss of tritium will occur. Under these circumstances, the isomerization will proceed back and forth via the addition and/or abstraction of hydrogens from the same prochiral positions. In this instance, only hydrogens from the 4 pro *re* and 2 pro *R* positions will participate in the reversible DMAPP $\rightleftharpoons$ IPP isomerizations without loss of tritium. This reasoning is based on the premise that the reversible removal and/or addition of hydrogens at C-4 and C-2 of the "immobilized" DMAPP and IPP is stereospecific.

Consideration of numerous enzymatic reactions indicates that normal isotope effects are involved in the cleavage of carbon–tritium bond.[160,161] Therefore, it is likely that the IPP $\rightleftharpoons$ DMAPP isomerization will also involve a normal kinetic isotope effect. Thus, should the methyl group (●) of the DMAPP be able to rotate, it is much more probable that one of the two available hydrogens rather than a tritium atom will be abstracted from C-4 of (Scheme 1, 3). Hence, the product of the reverse isomerization of DMAPP (Scheme 1, 3) will not be IPP (Scheme 1, 2), but the IPP (Scheme 1, 4) in which *statistically* tritium (t_1) and (t_2) ($t_1 \neq t_2$) will be present at the [*Z*] and [*E*] positions. In actuality the IPP

(Scheme 1, **4**) will consist of two tritiated species (*Z*) [4-^{3}H]-IPP; (*E*) [4-^{3}H]-IPP. The accompanying large amount of [4-$^1H_2$]-IPP, which includes also the small amounts of the [4-$^1H_2$]-IPP formed through loss of tritium are disregarded since they have no bearing on the results. However, for the present discussion, we will consider only the tritiated "statistical" sample. Additional reversible isomerization of IPP (Scheme 1, **4**) to DMAPP will tend to equalize t_1 and t_2. Should the isomerization proceed through a sufficient number of cycles, it will result in the (statistical) complete scrambling of the tritium atoms ($t_1 = t_2$). Under these circumstances the 2 pro *R* and 2 pro *S* hydrogen atoms of MVA will be incorporated into polyprenoids (squalene, steroids, etc.) without preservation *of the steric integrities.* Obviously, regardless of the anticipated operation of an isotope effect, some tritium will be lost during the (multiple) reversible isomerization cycles. It follows that the more C-2 tritium atoms of MVA are lost, the more scrambled will be the tritium atoms in the biosynthesized polyprenoids.

However, our results and the results of other investigators indicate that, despite the significant losses of ^{3}H, the C-2 tritium atoms of MVA are incorporated into polyprenoids essentially with *retention of their "steric" integrity and with no (or very little) scrambling.* These observations argue against the hypothesis that the reversibility of IPP $\rightleftharpoons$ DMAPP isomerization is the main factor responsible for the loss of tritium derived from C-2 of MVA. Unless some unusual properties are ascribed to the prenyl isomerase and/or to the interaction of IPP and DMAPP with the enzyme, we feel that the reversibility of the isomerization does not provide a satisfactory rationalization of the observations.



Scheme 2.

It was argued that the loss of tritium increases with the length of the biosynthetic experiments.[159] However, we have carried out *in vivo* incubation experiments of (2*R*)- and (2*S*) [2-^{14}C; 2-^{3}H]-MVAs with *F. coccineum* for 144 hr and the losses of tritium in the biosynthesis of "*R*"- and "*S*"-fusidic acids were 2.1 and 11.6%, respectively (Table 8). No detectable scrambling of the isotopic hydrogen (tritium) atoms was observed.

The available data suggest that the results could be better rationalized in terms of events involving MVA (or MVA-OPP) prior to conversion to IPP. To illustrate the point, we will consider the possibility that C-2 hydrogen atoms of MVA can be *abstracted* and then *replaced with retention of configuration* by a proton from the medium. For the sake of argument, we will assume that a C-2 hydrogen of MVA can be removed, e.g. by enolization. The enolization could be facilitated by a transient interaction of the MVA-OPP, MVA-acid or MVA-lactone with a prosthetic group (e.g. of a carrier protein, an enzyme or another entity). The prosthetic group [P] (Scheme 2, **1**) responsible for the activation of the carboxylic group of MVA could interact with MVA-OPP (Scheme 2, **1**) (or another form of MVA) to promote enolization. The enolization of MVA-OPP could involve the loss of, e.g. the 2 pro *R* hydrogen (^{1}H) to give an enolate (Scheme 2, **2**). Reprotonation of **2** with *retention of configuration* will give MVA-OPP (Scheme 2, **1**). If the enolization proceeds with the loss of tritium, the recovered MVA-OPP (Scheme 2, **1**) will be devoid of tritium and will cease to be chiral at C-2. If, on the other hand, ^{1}H is abstracted during the enolization, the chirality of the regenerated [2-^{3}H]-MVA-OPP (Scheme 2, **1**) will be unaffected.

The enolization of MVA-OPP can also proceed with the loss of the 2 pro *S* hydrogen (as in Scheme 2, **3**) to give the *tritiated* enol (Scheme 2, **4**). Protonation of the enolate (Scheme 2, **4**) from the side from which the hydrogen was abstracted will result in regeneration of MVA-OPP (Scheme 2, **3**), which will retain its initial chirality.

The outlined hypothesis provides a satisfactory rationalization of the facts associated with the loss of C-2 tritium and the incorporation of C-2 hydrogen atoms of MVA into polyprenoids without (significant) scrambling of tritium.

The hypothesis is amenable to experimental evaluation with the use of (3*R*, 2*R*)- and (3*R*, 2*S*) [2-^{14}C; 2-^{3}H]-MVA which can be prepared from the parent (3*RS*, 2*R*)- and (3*RS*, 2*S*)-MVA,[162] respectively. The chiral (3*R*, 2*R*)- and (3*R*, 2*S*) [2-^{14}C; 2-^{3}H]-MVA could be administered to, e.g. peas, and at the termination of the experiments the excess mevalonate, and the biosynthesized squalene and sitosterol could be recovered. The ^{3}H : ^{14}C ratios of the parent MVA, squalene and sitosterol could then be compared with the ^{3}H : ^{14}C ratio of the MVA recovered from the incubation. This will allow the evaluation of the proposed mechanism.

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