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Stereochemistry of the Biosynthesis of Presqualene Alcohol[1]

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Current hypotheses of the biosynthesis of presqualene pyrophosphate were tested by the examination of presqualene alcohol biosynthesized from [1*R*,5*R*,9*R*-1,5,9-D_3]farnesyl pyrophosphate and from [1-^{18}O]farnesyl pyrophosphate. Nuclear magnetic resonance spectrometry showed that the octet of the two cyclopropylcarbinyl protons seen in the spectrum of protio-presqualene alcohol, centered at τ 6.35, was replaced by a broad doublet of one proton (τ, 6.23; *J*, 6.2 Hz), which became sharpened after deuterium decoupling and was reduced to a singlet after deuterium and proton decoupling. Also the doublet of a single olefinic proton adjacent to the cyclopropane ring, seen in the spectrum of protio-presqualene alcohol at τ 5.08 (*J*, 8.5 Hz), was reduced to a broad singlet. The presqualene alcohol biosynthesized from the [1-^{18}O]farnesyl pyrophosphate contained the same isotopic concentration as its precursor. The observations, taken together with previous results, are interpreted to mean that the pyrophosphate-bearing group of one farnesyl pyrophosphate molecule appears without change of configuration, and without previous cleavage of the C–O bond of farnesyl pyrophosphate, in presqualene pyrophosphate and that the pro-*R* hydrogen atom at C-1 of the second farnesyl pyrophosphate molecule appears at C-3 of the cyclopropane ring *anti* to the vinylic substituent. The observations support the view that presqualene pyrophosphate is not an artifact, but a true intermediate in the biosynthesis of squalene.

INTRODUCTION

The various mechanisms proposed for the biosynthesis of presqualene-PP (**1a**; $R = P_2O_6^{3-}$) require that the absolute configuration at its pyrophosphate-bearing group remain the same as at C-1 of farnesyl-PP[2] from which it is synthesized (*1–4*), and that the C–O bond of the farnesyl-PP molecule furnishing the pyrophosphate-bearing group of presqualene-PP remains unbroken. It was probably a tacit assumption in all these hypotheses that presqualene-PP was a normal, or obligatory, intermediate between farnesyl-PP and squalene. A mechanism different from the hypotheses cited was presented by J. W. Cornforth (*5*) in his Robert Robinson Lecture to the British Chemical Society, in which he implied that presqualene-PP need not be an obligatory intermediate in the biosynthesis of squalene, but that it may be an artifact resulting from withholding NADPH from the enzyme catalyzing the conversion of farnesyl-PP into squalene. Cornforth (*6*), embodying in his hypothesis part of a mechanism of squalene biosynthesis proposed much earlier, showed that it was logically possible to formulate presqualene-PP as a reversibly formed by-product, in the absence of NADPH, through

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[2] Abbreviations used: presqualene-PP, presqualene pyrophosphate; farnesyl-PP, farnesyl pyrophosphate.

the rearrangement of a homoallylic to a cyclopropyl carbinyl cation which is then stabilized to presqualene-PP by the transfer of a pyrophosphate anion from the enzyme. This enzyme-bound pyrophosphate is, supposedly, the one originally attached to that molecule of farnesyl-PP whose hydrogen atoms at C-1 are retained in squalene, but with an inversion of steric position [cf. Scheme 4 in Ref. (*5*)]. If presqualene-PP were formed by this mechanism, the C–O bond in both farnesyl-PP molecules condensing should be cleaved and hence the C–O bond found in presqualene-PP would be a new one and not likely to contain the same O-atom as the one contained in the starting farnesyl-PP. A second possible, though not necessary, consequence of the mechanism proposed by Cornforth (*5*) might be a racemization, at least partial, of the configuration around C-1 of farnesyl-PP after conversion to presqualene-PP.

We have examined these possibilities by two sets of experiments. In one we have examined the absolute configuration at the carbinyl carbon of presqualene alcohol biosynthesized from [1*R*-1-D_1]farnesyl-PP and in another the ^{18}O-content of presqualene alcohol biosynthesized from [1-^{18}O]farnesyl-PP. The results showed complete retention of the absolute configuration of C-1 of farnesyl-PP and of the ^{18}O at the carbinyl carbon of presqualene alcohol. Details of these experiments, reported briefly before (*7*), are being presented here.

EXPERIMENTAL

[1R,5R,9R-1,5,9-D_3-4,8,12-$^{14}C_3$]farnesyl pyrophosphate. This substrate was made biosynthetically from mevalonate as described by Popják et al. (*8*). The mevalonate, made by the reduction of mevaldate (3-hydroxy-3-methyl-5-oxovalerate) with [4*R*-4-D_1]NADH (*9*, *10*), was a mixture of 40 mg of 3*R*[2-^{14}C-5*R*-5-D_1]- and 125 mg of 3*RS*[2-^{14}C-5*R*-5-D_1]mevalonate, in which about 80% of the molecules were deuterated. The specific activity of each preparation was 21×10^3 dpm/μmol. These amounts of mevalonate were contained in a 1500-ml incubation with the soluble multienzyme system of pig liver (*8*) supplemented with 7 m*M* ATP, 5 m*M* $MgCl_2$, and 2 m*M* $MnCl_2$, and buffered at pH 7.5 with 0.1 *M* Tris–HCl. The [2-^{14}C]mevalonate was added to the D-labeled substrate in order to aid quantitation of yields of products and their identification in chromatography. The particular preparations of mevalonate used and the proof for their absolute configuration at C-5 being *R* were described in another communication (*11*). After a 3-hr incubation under N_2 and at 37°C the farnesyl-PP was purified as described previously (*8*) up to and including the step of its adsorption onto the Abmerlite XAD-2 resin (Rohm and Haas) and elution from it by ammoniacal methanol. Addition of 3 vol of diethyl ether to the methanolic eluate precipitated overnight at 4°C the farnesyl-PP and left unreacted mevalonate and lipids in the supernatant. After collection and washing of the precipitate repeatedly with ice-cold diethyl ether, 180 μmol of radiochemically pure farnesyl-PP were obtained (yield 69%).

[1-^{18}O]farnesyl pyrophosphate. This specimen, also, was made biosynthetically from [5-^{18}O]mevalonate. The [5-^{18}O]mevalonolactone was synthesized for us at the Milstead Laboratory of Chemical Enzymology, Shell Research Ltd., Sittingbourne, Kent, England. The method of synthesis will be published elsewhere from that laboratory. Ninety percent of the molecules in the [5-^{18}O]mevalonolactone specimen contained one atom of ^{18}O, the position of which at C-5 followed not only from the method of its

synthesis, but also from its mass spectral fragmentation. The mass spectrum of this lactone obtained by us was identical with that reported recently by Gray (*12*). The [5-^{18}O]mevalonolactone specimen, 257 mg (= 1.95 mmol), was converted to the potassium salt from which a 0.25 *M* solution was made. For the synthesis of [1-^{18}O] farnesyl-PP, two 500-ml incubations were set up with the multienzyme system prepared from pig liver as described (*8*) except that 500 μmol of *RS*-[5-^{18}O]mevalonate and 1 μmol of *RS*-[2-^{14}C]mevalonate (21.30×10^6 dpm) were the substrates. Analysis of a 1-ml sample (*13*), taken from the incubation mixtures 200 min from the start, indicated a quantitative conversion of the utilizable 3*R*-[^{14}C]mevalonate added to the incubation into farnesyl-PP [$2.229 \times 10^4 \times 500$ dpm found in nerolidol + farnesol after acid hydrolysis of the products of the incubation (*13*)]. The farnesyl-PP synthesized was then purified as described in the preceding paragraph for the [D_3]farnesyl-PP. Contrary to the very satisfactory yields of farnesyl-PP obtained by this procedure from the large-scale preparations made with the [5-D_1]mevalonate, in the syntheses with the [5-^{18}O]mevalonate the final yields of the pure farnesyl-PP were disappointingly low, 6.5 and 8 μmol (in spite of quantitative conversions of the mevalonates to farnesyl-PP). Thin-layer chromatographic examination of the diethyl ether supernatants in both instances showed that in these relatively small-scale incubations the farnesyl-PP was incompletely precipitated by diethyl ether from the methanolic eluate of the XAD-2 resin. However, the specimens obtained from both incubations were chromatographically pure: on thin-layer plates of silica-gel H, developed with *n*-propanol-concd ammonia–water (6:3:1, by volume) only one radioactive component (R_f 0.2) was found which coincided with a spot stainable by I_2-vapor and which corresponded with the chromatographic behavior of specimens of farnesyl-PP made in our laboratory over several years. Hydrolysis by intestinal alkaline phosphatase (cf. below) released [^{18}O]farnesol, which was identified by mass spectrometry.

Hydrolysis of labeled farnesyl pyrophosphate specimens. Samples of the [D]- and [^{18}O]-farnesyl-PP were hydrolyzed with intestinal alkaline phosphatase as follows. One micromole of either deuterium- or ^{18}O-labeled farnesyl-PP was added to a 3-ml (final volume) reaction mixture containing 0.1 *M* Tris–HCl, pH 9.5, 5 m*M* $MgCl_2$ and 552 units (1.0 mg) of intestinal alkaline phosphatase. After 2 hr incubation at 37°C and under N_2 the reaction flasks were left at room temperature overnight. The free farnesol was obtained by four extractions with diethyl ether, 5 ml each. The combined extracts were dried with $MgSO_4$ and were concentrated to about 0.1 ml. The farnesol was then purified on plates coated with 0.3–0.5 mm layers of silica-gel H and developed twice with benzene and once with 10% ethylacetate in benzene (*2*). The farnesols, being labeled also with ^{14}C in each case, were located on the plates by a Packard radiochromatogram scanner. The radioactive bands were scraped off the thin-layer plates and were eluted over a sintered glass funnel with diethyl ether. After concentration of this eluate to 0.1–0.2 ml with a gentle stream of N_2, samples containing 5–10 μg of farnesol were evaporated onto the direct insertion probe of the mass spectrometer.

Biosynthesis of presqualene alcohols. The presqualene alcohols were biosynthesized either from the [1,5,9-D_3-4,8,12-$^{14}C_3$]farnesyl-PP or from the [1-^{18}O-4,8,12-$^{14}C_3$]-farnesyl-PP with yeast microsomes as described (*2*) except that the buffer of incubations was 0.1 *M* potassium phosphate, pH 7.35, and that the concentration of farnesyl-PP was 0.1 m*M*. The use of the phosphate buffer, instead of Tris–HCl, very much decreased

the hydrolysis of the farnesyl-PP and increased the yield of presqualene alcohol. The [D]presqualene alcohol was made in a 65% yield on a large-scale from 174 μmoles of the [D]farnesyl-PP.

In the case of the [1-^{18}O]farnesyl-PP, the yields of presqualene alcohol in two incubations (one of 1 ml and the other 60 ml) were 50 and 70%, respectively.

The presqualene alcohols were extracted from the reaction mixtures and were purified by preparative thin-layer chromatography as described (*2*). About one-half of the D-labeled specimen (giving 7.5 mg [D]presqualene alcohol) and all of the ^{18}O-labeled specimens were processed.

Instruments and supplies. All physical measurements, mass, and nmr spectra, and ^{14}C-determinations, localization of ^{14}C-labeled compounds on thin-layer plates, and gas–liquid radiochromatographic analyses were made with instruments described previously (*2*, *8*). The sources of [^{14}C]mevalonate and of other reagents were also described (*2*, *11*).

RESULTS

[D_3]*Farnesol and* [D_6]*Presqualene Alcohol*

Mass spectral analysis of the farnesol, liberated from the farnesyl-PP biosynthesized from the [5*R*-5-D_1]mevalonate, showed the specimen to contain 2.8% D_0-, 8.9% D_1-,

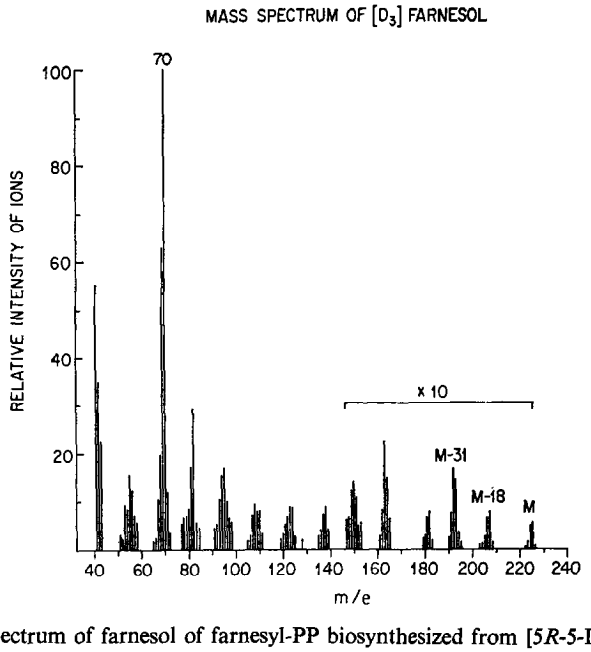


FIG. 1. Mass spectrum of farnesol of farnesyl-PP biosynthesized from [5*R*-5-D_1]mevalonate. The symbols, M, M-18, and M-31 are written over the regions of ions found in the spectrum of non-isotopic farnesol. Instead of the molecular ion at *m/e* 222, the most intense ions in the molecular ion region were at *m/e* 224 and 225. Similarly, the $[M-18]^+$ ions at 204 in the nonisotopic specimen were replaced by ions at *m/e* 205, 206, and 207. In the M-31 region, normally at *m/e* 191, the most intense ions were at *m/e* 192 and 193 indicating the loss of [CHDOH]. from the molecular ions. The base peak, found in nonisotopic farnesol at *m/e* 69 ($[CH_3C(CH_3)—CH=CH_2]^+$) was replaced by ions at *m/e* 70 ($[CH_3C(CH_3)—CH=CHD]^+$).

44.2% D_2-, and 43.9% D_3-molecules (Fig. 1). The molecular ions seen between *m/e* 222 and 226 were used for the calculation of isotopic abundances. The absolute configuration of such [D_3]farnesol has been established before (*9*, *14*) as 1*R*, 5*R*, 9*R*.[3]

The presqualene alcohol biosynthesized from the [D_3]farnesyl-PP contained, according to mass spectral analysis, variously deuterated molecules in the proportions: D_1, 1.7%; D_2, 6.4%; D_3, 12.8%; D_4, 26.8%; D_5, 33.1%; and D_6, 19.2% (Fig. 2). The proportion of the nondeuterated molecules could not be measured accurately, as

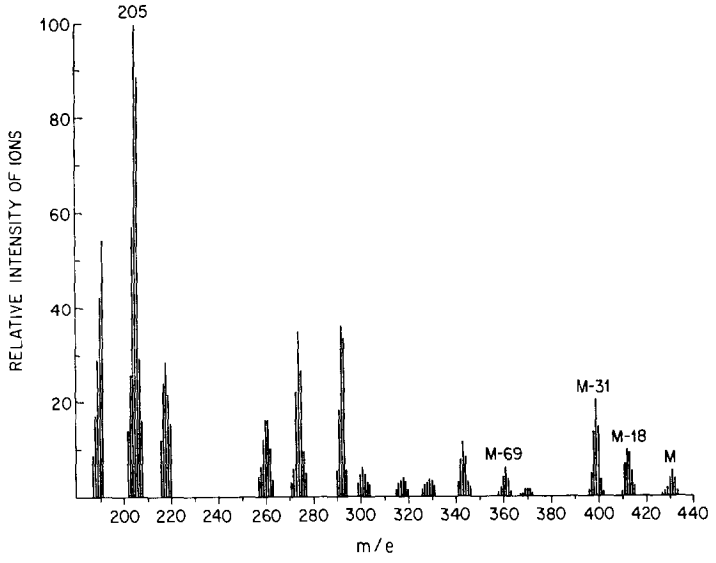


FIG. 2. Partial mass spectrum of presqualene alcohol biosynthesized from [1,5,9-D_3]farnesyl-PP. The symbols M, M-18, M-31, and M-69 indicate the regions of ions found in the spectrum of the unlabeled alcohol at *m/e* 426, 408, 395, and 357, respectively. The intensities of ions in the molecular ion region indicated the preponderance of tetra- to hexadeuterio molecules in the specimen. The presence of a deuterium atom at the carbinol carbon was shown by the dominant loss of 32 mass units ([CHDOH]) from the molecular ions (cf. also text).

these amounted to less than 1% of the ion current in the region of the molecular ions between *m/e* 426 and 433. Even the $[M-18]^+$ ions, seen in the spectrum of the protio-presqualene alcohol at *m/e* 408, with an intensity usually higher than that of the molecular ion (*2*), were just detectable.

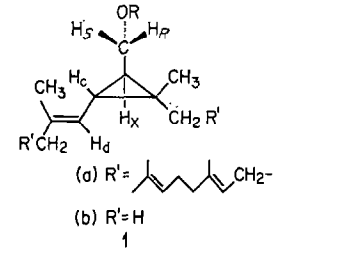
The proportions of the deuterated molecules in both farnesol and presqualene alcohol were in close agreement with the values calculated on the assumption that only 75% (not 80%) of the molecules in the starting mevalonate were deuterated,[4] and that not only the labeled and unlabeled molecules of mevalonate were used randomly in

[3] Although the assignment of the absolute configuration of this farnesyl-PP at all three of its asymmetric centers is *R* by the sequence rule, the spatial orientation of the deuterium atoms at C-5 and C-9 is opposite to that seen at C-1 (cf. **2**).

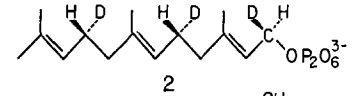
[4] There is some uncertainty in the determination of deuterated molecules in a specimen of mevalonolactone by mass spectrometry because lactones tend to give an $[M+1]^+$ ion of higher intensity than is predictable from the natural abundance of the heavy isotopes of C, H, and O. An ion–molecule reaction, depending on pressure in the ion source, may account for varying and abnormally high $[M+1]^+$ ions.

the biosynthesis of farnesyl-PP but that the variously deuterated farnesyl-PP molecules also were treated indiscriminately by the presqualene-PP synthetase. The calculated values for farnesol were: D_0, 1.56%; D_1, 14.06%; and D_2 and D_3, 42.19% each; and for the presqualene alcohol they were: D_0, 0.04%; D_1, 0.56%; D_2, 3.64%; D_3, 13.44%; D_4, 29.40%; D_5, 35.28%; and D_6, 17.64%. It can be calculated further, on the same assumptions as used above, that the percentage of the molecules of presqualene alcohol that contained one deuterium atom at any one of the predictable positions should be the same as the percentage of the deuterated molecules in the [5-D_1]mevalonate used as the starting substrate. Thus, unless isotopic molecules were being discriminated against during the biosyntheses, 75% of all the molecules in the specimen of presqualene alcohol should contain one deuterium atom at the carbinol carbon, and at five other predictable positions.

It has been demonstrated by Edmond et al. (*2*) that the carbinol group of presqualene alcohol (**1a**, R = H) was derived from C-1 of one of the two farnesyl-PP molecules condensing without the loss of the hydrogen atoms attached to C-1 of the precursor.



1



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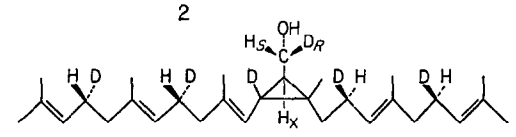

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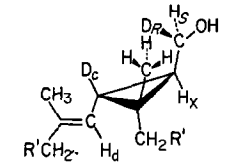

4

It was shown further that the two hydrogen atoms on the carbinol carbon were magnetically nonequivalent and gave a characteristic octet of an ABX system. Thus it was expected that the absolute configuration around the carbinol carbon of the [D_6]presqualene alcohol would be revealed by the nmr spectrum of the compound. The nmr spectra, taken at 100 MHz in $CDCl_3$, of the [D_6]presqualene alcohol showed (Figs. 3 and 4) that the octet of the ABX system, given by the carbinyl protons in the protio-presqualene alcohol (τ_A, 6.21; τ_B, 6.50; J_{AB}, 11.5 Hz; J_{AX}, 6.2 Hz; J_{BX}, 8.3 Hz), was

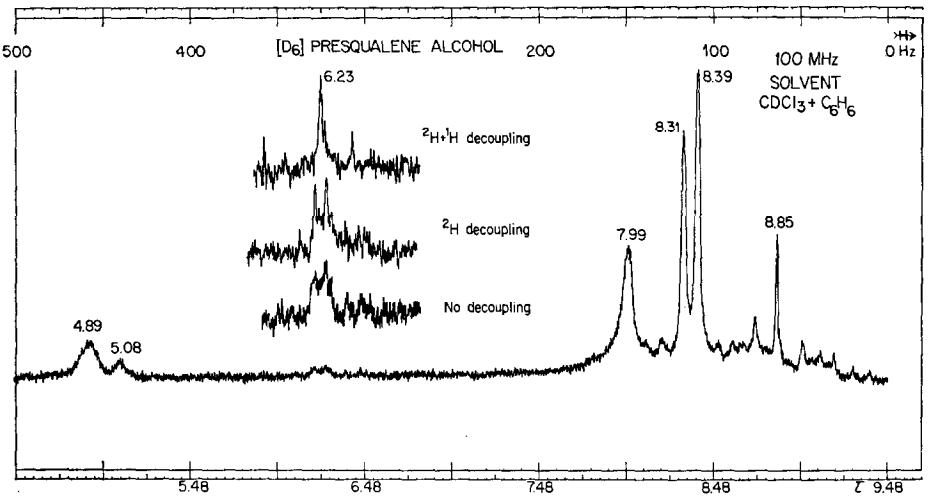

FIG. 3. Nuclear magnetic resonance spectrum of presqualene alcohol biosynthesized from [1*R*,5*R*,9*R*-1,5,9-D_3]farnesyl pyrophosphate.

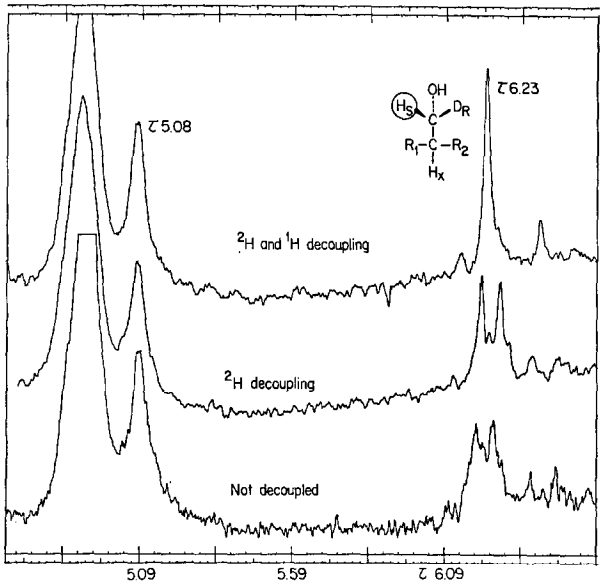

FIG. 4. Low-field portion of the spectrum shown in Fig. 3 and obtained by summation of nine scans by the use of a computer of average transients, before spin–spin decoupling and after deuterium, and deuterium + proton decoupling with the frequency of H_x (cf. formula **4**) at τ 9.12.

replaced in the spectrum of this specimen by a broad doublet at τ 6.23 (J_{AX}, 6.2 Hz), which became sharpened after deuterium decoupling and became a singlet after deuterium and proton decoupling with the frequency of H_x (τ_x, 9.12) at C-1 of the cyclopropane ring (cf. **1a**). The octet of nondeuterated molecules was just perceptible in the spectra taken without accumulation of repeated scans. As will be discussed, the

observation showed that the steric individuality of the two hydrogen atoms at C-1 of farnesyl-PP was retained at the carbinol carbon of presqualene alcohol.

Another important feature of the nmr spectrum of [D_6]presqualene alcohol was the singlet at τ 5.08 of one olefinic proton distinct from the broad resonances of the four other olefinic protons seen at τ 4.88 (cf. Discussion).

[1-^{18}O]Farnesyl Pyrophosphate and [^{18}O]Presqualene Alcohol

The mass spectrum of the farnesol obtained by the hydrolysis of [1-^{18}O]farnesyl-PP was very similar to that of normal farnesol except that the specimen contained two molecular ions: one at *m/e* 222 and another at 224. The molecular weight of the ion at *m/e* 224, determined at a resolving power of 1:15 000, was 224.202514 which differs by less than 1 ppm from 224.202603 calculated for $C_{15}H_{26}[^{18}O]$. It was further calculated from the ratios of the intensity of ions between *m/e* 221 and 225 that the specimen contained 81% ^{18}O-labeled and 19% unlabeled molecules. The somewhat lower ^{18}O-content of the farnesol than that of the starting mevalonolactone might be attributed to some exchange during the preparation of the specimen. Nevertheless, the data showed that hydrolysis of the pyrophosphate ester with phosphatase did not cause a cleavage of the C–O bond.

Mass spectra of two specimens of presqualene alcohol, biosynthesized in two separate experiments from [1-^{18}O]farnesyl-PP, were taken; they were identical and differed from the mass spectrum of unlabeled presqualene alcohol only in that a molecular ion of greater intensity than that at *m/e* 426 (M_1^+) was found also at *m/e* 428 (M_2^+) and also that the spectrum contained an ion at *m/e* 359 (absent in the spectrum of unlabeled presqualene alcohol) with an intensity five times greater than that at *m/e* 357. The latter two ions are attributed to the elimination of 69 mass units, $[CH_3C(CH_3)=CH—CH_2]\cdot$, from the two molecular ions. Measurements made, at a resolving

TABLE 1

INTENSITIES OF IONS IN MASS SPECTRUM OF [^{18}O]PRESQUALENE ALCOHOL RELATIVE TO INTENSITY OF ION AT *m/e* 203

Ion *m/e*	Origin of ion	Relative intensity[a]
429	$[M_2 + 1]$	2.4
428	M_2	7.0
427	$[M_1 + 1] + [M_2 - 1]$	0.8
426	M_1	1.6
408	$[M_1 - 18] + [M_2 - 20]$	8.8
395	$[M_1 - 31] + [M_2 - 33]$	12.0
359	$[M_2 - 69]$	1.0
357	$[M_1 - 69]$	0.2
339	[408 − 69]	10.0
271	[339 − 68]	35.2
203	[271 − 68]	100.0

[a] The base peak was at *m/e* 69 (intensity relative to *m/e* 203: 1680); the relative intensity of another characteristic ion at *m/e* 81 was 640.

power of 1:15 000, of the molecular weights of the ions at *m/e* 426 and 428 by the peak-matching technique against a reference ion at *m/e* 425.977510 gave values of 426.385171 (calcd for $C_{30}H_{50}O$: 426.386144) and 428.390247 (calcd for $C_{30}H_{50}[^{18}O]$: 428.390395). These measurements left little doubt that the ion at *m/e* 428 was indeed the ^{18}O-substituted presqualene alcohol.

The quantitative relationship between the molecular ions at *m/e* 426 and 428 (Table 1) showed that 81.4% of the molecules in the two specimens contained ^{18}O; this value is nearly identical with the value determined for the ^{18}O-content, 81%, of the farnesyl-PP (measured on the farnesol liberated from farnesyl-PP by intestinal alkaline phosphatase).

DISCUSSION

The data obtained by the use of ^{18}O-labeled substrates make it unlikely that presqualene pyrophosphate is synthesized by a mechanism that involves the cleavage of the C–O bond at C-1 of the molecule of farnesyl-PP that furnishes the carbinyl-group of presqualene pyrophosphate (or alcohol). Although the ^{18}O-content of the farnesol (obtained from the hydrolysis of the farnesyl-PP with intestinal alkaline phosphatase) was somewhat lower than that of the [5-^{18}O]mevalonate used for its synthesis, we have no doubt that intestinal alkaline phosphatase cleaves an O–P rather than the C–O bond in a phosphate or pyrophosphate ester. The same is true for the microsomal phosphatase that hydrolyzes presqualene-PP since the ^{18}O-content of the presqualene alcohol derived from the [^{18}O]farnesyl-PP was the same, within narrow experimental error, as that of its precursor. Thus the experiments with the ^{18}O-labeled substrates leave Cornforth's hypothesis (*5*) in a weak position. It was expected, if that hypothesis were valid, that the ^{18}O-content of presqualene alcohol should be substantially lower than that of the farnesyl-PP from which it was synthesized. It is conceivable, though improbable, that the pyrophosphate anion cleaved off from farnesyl-PP by the break of the C–O bond, as Cornforth's hypothesis (*5*) demands, could be stabilized on the enzyme, e.g., by complexing with Mg^{2+} ions, so that it would reassociate with the cyclopropylcarbinyl cation through the same oxygen atom as the one attached originally to C-1 of farnesyl-PP.

The simplest interpretation of our data is that the C–O bond of the farnesyl-PP molecule that furnishes the carbinyl group of presqualene alcohol is not broken during synthesis. If this interpretation is correct, then it also follows that the absolute configuration around the carbinyl carbon of presqualene alcohol must be the same as around C-1 of farnesyl-PP. The nmr spectra of the [D_6]presqualene alcohol support this conclusion as these showed the presence of only one protium atom at the carbinyl carbon; the pro-*S* position can be assigned to this atom. Poulter (*15*) made a specimen of *trans*-1*R*,3*R*-[1′S-1′-D_1]chrysanthemol (**1b**; R = H, H_S = D) and found that the octet of the carbinyl protons of the protio-compound in the nmr spectrum, taken in CCl_4, was reduced to a doublet at the high-field part of the octet (τ_B, 6.78; J_{BX}, 8.3 Hz).[5]

[5] Poulter (*15*) recorded the chemical shifts of the pro-*R* (H_B) and pro-*S* (H_A) carbinyl protons in *trans*-chrysanthemol, measured in CCl_4, as $\tau_B = 6.78$ and $\tau_A = 6.53$, J_{AX} 6.2 Hz. Our measurements, made in $CDCl_3$ + benzene and at 100 MHz, gave for the corresponding protons in a specimen of *trans*-(1*R*,3*R*)-chrysanthemol (**1b**; R = H) values of $\tau_B = 6.47$, and $\tau_A = 6.23$ (J_{AB} 11.4 Hz, J_{AX} 6.2 Hz J_{BX} 8.3 Hz).

The observations thus leave no doubt that the stereochemical individuality of the two hydrogen atoms at C-1 of farnesyl-PP was retained at the carbinol carbon of presqualene alcohol. This observation, by itself, is not contrary to Cornforth's hypothesis which permits also the retention of absolute configuration at the carbinyl carbon of presqualene alcohol, but only after two inversions at around the C-1 of that farnesyl-PP residue which furnishes the carbinyl carbon of presqualene alcohol and provided that a free rotation around the C-1 and C-α bond in an intermediary cyclopropyl carbinyl cation does not occur.

Our observations, coupled with those of Poulter (*15*), give evidence also as to the predominant conformation in solution of presqualene alcohol and of *trans*-chrysanthemol and indicate that the oxygen of the alcohol group lies preferentially away from the cyclopropane ring, thus bringing in eclipse the pro-*R* hydrogen atom on the carbinol carbon of both substances with the methyl group at C-2 of the cyclopropane ring (cf. formula 4). The shielding effect of the protons at the latter group accounts for the chemical shift of the pro-*R* hydrogen atom being at a higher field than that of the pro-*S* hydrogen atom.

The nmr spectrum of the deuterio-presqualene alcohol confirmed also, as could be inferred from previous studies in our laboratory (*2*), that the pro-*R* hydrogen atom at C-1 of one of the two farnesyl-PP molecules appears at C-3 of the cyclopropane ring (**1a**, H_c). It has been recorded (*2*) that the one vinylic proton (**1a**, H_d), *anti* to the proton at C-3 of the cyclopropane ring of presqualene alcohol, gave a doublet at τ 5.06 (J, 8.5 Hz) distinct from the broad resonances of the four other olefinic protons seen in the spectrum of presqualene alcohol at τ 4.88. It was shown also (*2*) that in the nmr spectrum of [D_3]presqualene alcohol, biosynthesized from [1-D_2]farnesyl-PP, the resonance of this vinylic proton was reduced to a singlet. We now find that the doublet of this one vinylic proton was similarly replaced by a singlet at τ 5.08. The stereospecific loss of the pro-*S* hydrogen atom and retention of the pro-*R* hydrogen atom at C-1 of one of the two farnesyl-PP molecules during synthesis of squalene was demonstrated some time ago (*9*). The loss of one hydrogen atom from C-1 of one farnesyl-PP during biosynthesis of presqualene-PP was also shown (*1*, *2*). Our data show unequivocally, in harmony with the earlier work on squalene biosynthesis (*6*, *9*), that the pro-*R* hydrogen atoms at C-1 of the farnesyl-PP molecules are retained in presqualene-PP and one pro-*S* hydrogen atom is lost. Thus, taking the evidence from the present investigation together with the results of previous studies (*2*, *9*, *14*, *16*), and, particularly, that the absolute configuration of protio-presqualene alcohol at its three asymmetric centers is *R* (*16*), we conclude that the absolute configuration of presqualene alcohol (and hence of presqualene pyrophosphate) biosynthesized from [1*R*,5*R*,9*R*-1,5,9-D_3]farnesyl-PP is that shown in (**3**) and (**4**).

Our experiments do not support the idea that presqualene-PP is an artifact resulting from the rearrangement of a "true," though unstable, intermediate of squalene synthesis in the absence of NADPH. Cornforth's hypothesis (*5*), although not supported by our observations, has the virtue that it can be tested further experimentally, but only after highly purified preparations of squalene synthetase, free of phosphatase and inorganic pyrophosphatase, become available. If Cornforth's hypothesis is correct, squalene synthetase should catalyse, in the absence of NADPH, a very substantial pyrophosphate exchange between inorganic pyrophosphate and presqualene-PP. An argument against

the hypothesis that formation of presqualene-PP is merely the product of NADPH deprivation of the enzyme is the observation, reported recently from Rilling's laboratory (*17*), that synthesis of presqualene-PP can be demonstrated in yeast microsomes even in the presence of NADPH and even *in vivo* in rat liver. Enzymic studies made with solubilized and partially purified squalene synthetase (W. Agnew and G. Popják, unpublished) suggest also that synthesis of squalene from farnesyl-PP is a two-step reaction: One of presqualene synthesis followed by the reduction of presqualene-PP to squalene.

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