Biosynthesis of Squalene. Evidence for a Tertiary Cyclopropylcarbinyl Cationic Intermediate in the Rearrangement of Presqualene Diphosphate to Squalene

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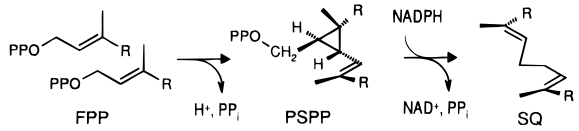
Received September 20, 1996

Squalene synthase (SQSase)[1] catalyzes the 1′-1 coupling[2] of two molecules of farnesyl diphosphate (FPP) to produce the triterpene squalene (SQ) as the first committed step in cholesterol biosynthesis.[3] The transformation occurs in two distinct steps (see Scheme 1). The first is a prenyl transfer reaction during which C(1) of one molecule of FPP is inserted into the C(2)−C(3) double bond of another to produce presqualene diphosphate (PSPP), a cyclopropylcarbinyl diphosphate with a c1′-2-3 structure. In the second reaction, PSPP rearranges to SQ in a series of steps that involve loss of inorganic pyrophosphate (PP_i), rearrangement of the hydrocarbon skeleton, and incorporation of a hydrogen from NADPH.[4] Simultaneous with the discovery of PSPP, several groups proposed mechanisms for the conversion of PSPP to SQ based on known rearrangements of cyclopropylcarbinyl cations.[5−8] One proposal,[4,7] which accounts for the regio- and stereochemical features of the enzyme-catalyzed reaction, is shown in Scheme 2. A key feature of the mechanism is the cyclopropylcarbinyl−cyclopropylcarbinyl rearrangement of carbocation **1** to **2**, followed by transfer of hydride from NADPH to C(1′) with inversion of configuration.

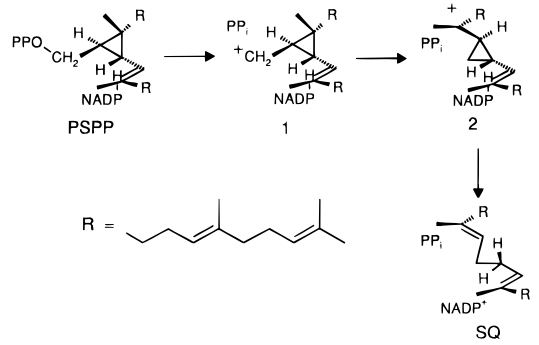
Zhang and Poulter[9] recently obtained direct evidence for carbocationic intermediates in the rearrangement of PSPP to SQ during studies with recombinant yeast SQSase.[10] Incubation of the enzyme with FPP in buffer lacking NADPH, resulted in the rapid conversion of the substrate to PSPP, followed by a substantially slower enzyme-catalyzed solvolysis of PSPP to give a mixture of (*Z*)-dehydrosqualene (DSQ, 24%) and two C_{30} alcohols, hydroxybotryococcene (HBO, 14%), a triterpene alcohol with a 1′-3 fusion of farnesyl residues, and hydroxysqualene (HSQ, 58%).[11] DSQ was formed by elimination of a proton from cation **2**, while the alcohols resulted from nucleophilic capture of cations **1** and **2**, respectively, by water (Scheme 3). The analogous naturally occurring triterpene hydrocarbons botryococcene and squalene are presumably formed from the same carbocationic intermediates by transfer of a hydride from NADPH.[4,12] Since the nucleophilic water presumably occupies the region in the catalytic site where the reduced nicotinimide ring of NADPH resides in the normal E•S complex, we thought it might be possible to shift the regioselectivity of the enzymatic reaction from alcohols to hydrocarbons by incubating the recombinant SQSase and FPP in the presence of a NADPH analog. Instead, incubation of the enzyme with $NADPH_3$, an unreactive dihydro analog of the cofactor, gave a C_{30} cyclopropylcarbinyl alcohol whose structure is identical to that originally proposed by Rilling[13] for presqualene alcohol (PSOH). The formation of this alcohol provides strong support for carbocation **2** as an intermediate in the rearrangement of PSPP to SQ.

$NADPH_3$ was synthesized by catalytic reduction of NADPH with $Pd/BaCO_3$ according to the procedure of Dave *et al*.[14] The analog was a competitive inhibitor against NADPH, $K_I = 40$ μM,[15] in the normal reaction catalyzed by recombinant yeast SQSase. Incubation of 15 μM enzyme at 30 °C in MOPS buffer

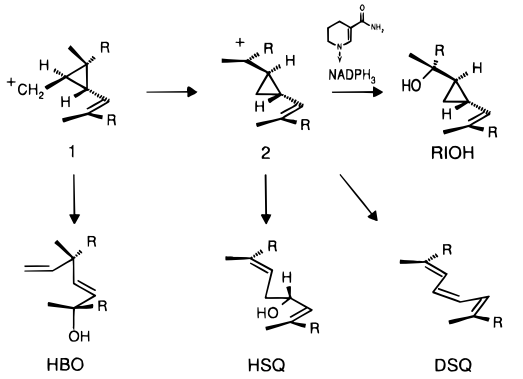
Scheme 1. Biosynthesis of Squalene



Scheme 2. Mechanism for Conversion of Presqualene Diphosphate to Squalene



Scheme 3. Mechanism for Formation of HBO, DSQ, HSQ, and RIOH



(1) Abbreviations used are as follows: DSQ, (*Z*)-dehydrosqualene; FPP, farnesyl diphosphate; HBO, hydroxybotryococcene; HSQ, hydroxysqualene; MOPS, 3-(N- morpholino)propanesulfonic acid; $NADPH_3$, dihydronicotinamide adenine dinucleotide phosphate, reduced form; PP_i, inorganic pyrophosphate; PSOH, presqualene alcohol; PSPP, presqualene diphosphate; RIOH, rillingol; SQ, squalene; SQSase, squalene synthase.

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(11) The product distribution is slightly different than originally reported because of improved HPLC conditions. We were able to detect several minor components that accounted for *ca*. 4% of the total.

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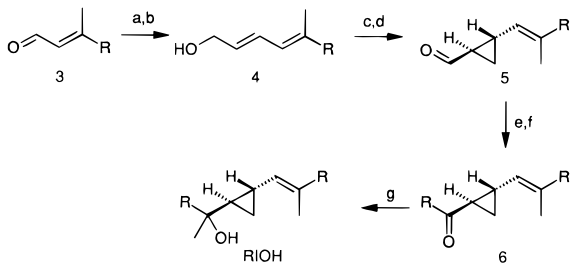
(15) Initial velocities were measured by the assay described by Zhang et al.[10] K_I was determined from measurements at four concentrations of NADPH (75, 150, 300, 600 μM) and $NADPH_3$ (50, 100, 200, 500 μM). Initial velocities were determined in duplicate and fit to the following equation for competitive inhibition: $V_{obs} = V_{max}$* [NADPH]/(K_M*(1 + [$NADPH_3$]/K_I) + [NADPH]).

S0002-7863(96)03308-2 CCC: $12.00

Scheme 4.[a] Synthesis of Rillingol



[a] (a) NaH, triethyl phosphonoacetate, 82%; (b) DIBALH, 99%; (c) D-dioxaborolane, $Zn(Et)_2$, CH_2I_2 (ref 21), 74%; (d) TPAP, NMO, 89%; (e) homogeranyl iodide, Mg, 85%; (f) TPAP, NMO, 96%; (g) CH_3MgBr, 88%.

containing 10 mM $MgCl_2$, 300 μM FPP, and 700 μM $NADPH_3$ resulted in rapid formation of PSPP ($k_{cat} \approx 0.2$ s^{-1}), followed by a slower conversion of PSPP ($k_{cat} \approx 0.002$ s^{-1}) to solvolysis products. HPLC analysis[16] revealed three major products: DSQ (39%), HSQ (31%), and a new compound not formed (<1%) in the absence of $NADPH_3$ with a retention time characteristic of a C_{30} alcohol (29%). No peak for HBO (<1%) was seen in the HPLC trace. Mass spectral and ^{1}H NMR data[17] for the new compound indicated that it was a tertiary cyclopropylcarbinyl alcohol isomeric with presqualene alcohol (PSOH), HBO, and HSQ. The carbon skeleton of the compound, including the relative stereochemistry in the cyclopropane ring, was confirmed by synthesis from farnesal as outlined in Scheme 4. Additional work is needed to establish the absolute stereochemistry of all three chiral centers in the molecule. The tertiary cyclopropylcarbinol has a c1′-1-2 fusion of isoprenoid units like that found in the naturally occurring monoterpene rothrockene and is identical to the structure originally proposed by Rilling for PSOH[13] that was subsequently synthesized by Ortiz de Montellano and Corey.[18] Hence, we name the new alcohol rillingol (RIOH).

Most of the mechanisms proposed for conversion of PSPP to SQ do not include **2** as an intermediate, and previous efforts to duplicate the rearrangement of **1** to **2** by solvolysis of C_{10} models for the presqualene system met with limited success.[2,19] Most of the products in the model studies were formed by nucleophilic capture of the primary cyclopropylcarbinyl cation or its open chain 1′-3 isomer, and less than 0.1% of the products had 1′-1 isoprenoid structures related to squalene. In contrast, approximately 80% of PSPP rearranged to 1′-1 products (DSQ and HSQ) during the SQSase-catalyzed solvolysis. Although this percentage decreased slightly to 71% when the incubation was conducted in the presence of $NADPH_3$, the remaining product was RIOH, which has a 1′-1-2 skeleton that readily rearranges to a 1′-1 structure.[20] Our results indicate that the catalytic machinery in SQSase promotes the formation of 1′-1 products by enhancing the rearrangement of **1** to **2** relative to capture of **1** by nucleophiles. We have previously proposed that PP_i released from PSPP serves as a template to direct the cyclopropylcarbinyl rearrangement within a highly ordered **1**:PP_i ion pair.[4,20] Formation of RIOH as a major product during the enzyme-catalyzed solvolysis of PSPP provides particularly strong support for the presence of cyclopropylcarbinyl cation **2** during the rearrangement of PSPP to SQ.

Acknowledgments. This study was supported by NIH grant GM25521. M.B.J. was an ACS Division of Organic Chemistry Graduate Fellow sponsored by Boehringer Ingelheim (1995−96) and a NIH trainee (GM 08537, 1993−95) and B.S.J.B. was supported by NIH training grant GM 08537 (1995−96).

JA963308S

(16) Products were chromatographed on a Microsorb-MV 5 μm silica gel column with isocratic elution using 1:20 (v/v) *tert*-butyl methyl ether/hexane.

(17) ^{1}H NMR (500 MHz, $CDCl_3$): 5.15 (t, 1H, 6.3 Hz), 5.11−5.07 (m, 3H), 4.61 (d, 1H, 9.3 Hz), 2.15−1.95 (m, 16H), 1.73 (s, 3H), 1.68 (s, 6H), 1.62 (s, 3H), 1.60 (s, 6H), 1.59(s, 3H), 1.47−1.41 (m, 1H), 1.13 (bs, 1H), 1.12 (s, 3H), 0.85−0.79 (m, 2H), 0.42−0.39 (m, 1H). LRMS (EI, 70 eV): 426 (M, 3), 408 (M − H_2O, 19), 271 (33), 149 (42), 81 (55), 69 (100). HRMS (EI, 70 eV) for $C_{30}H_{50}O$: calcd 426.3861, found 426.3865.

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