90056-31-8; 7 (isomer 1), 90129-48-9; 7 (isomer 2), 90056-32-9; 7 alcohol (isomer 1), 119637-88-6; 7 alcohol (isomer 2), 119717-48-5; 8 (isomer 1), 119619-14-6; 8 (isomer 2), 119717-34-9; 8 1-hydroxy derivative, 119619-13-5; 9 (isomer 1), 119619-15-7; 9 (isomer 2), 119619-16-8; 10, 119619-17-9; 11, 119619-18-0; 11 β,γ-unsaturated derivative, 119619-19-1; 11 hydroxy enone derivative, 119619-20-4; 11 epoxy alcohol derivative, 119619-21-5; 12, 119619-22-6; 13, 119717-35-0; 14, 119717-36-1; 15 (isomer 1), 119619-23-7; 15 (isomer 2), 119619-24-8; 15 7-alcohol derivative, 119619-25-9; 16, 119619-26-0; 16 diol mesylate derivative, 119637-89-7; 17, 119619-27-1; 17 aldehyde derivative, 119619-28-2; 18, 119619-29-3; 19, 119619-30-6; 19 diol derivative, 119619-31-7; (-)-20, 20744-71-2; 21 (isomer 1), 119678-62-5; 21 (isomer 2), 119678-63-6; 22 (isomer 1), 119619-32-8; 22 (isomer 2), 119678-64-7; $Na^+[PhSeB(OEt)_3]^-$, 117268-79-8.

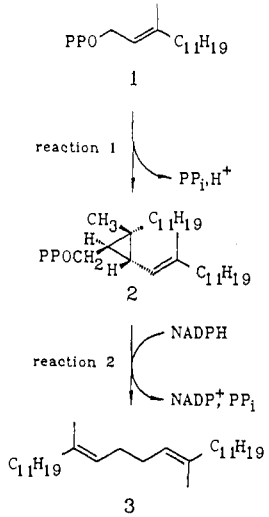
Squalene Synthetase. Inhibition by Ammonium Analogues of Carbocationic Intermediates in the Conversion of Presqualene Diphosphate to Squalene

C. Dale Poulter,* Todd L. Capson, Michael D. Thompson, and Ronda S. Bard

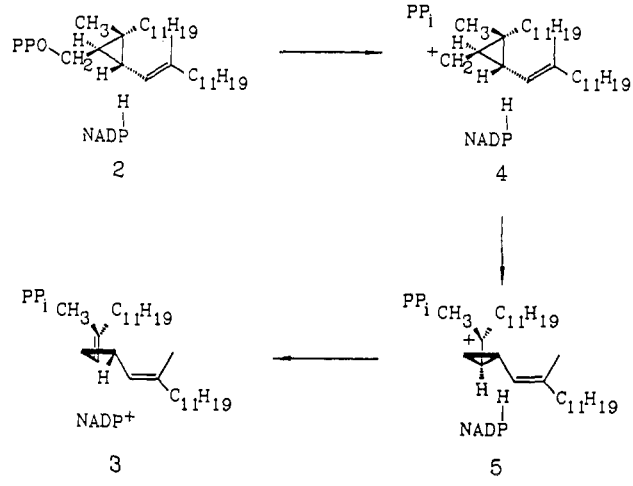
Contribution from the Departments of Chemistry and Medicinal Chemistry, University of Utah, Salt Lake City, Utah 84112. *Received September 16, 1988*

Abstract: Squalene synthetase (EC 2.5.1.21) catalyzes the formation of squalene (**3**) from farnesyl diphosphate (**1**) via presqualene diphosphate (**2**) in two steps. The mechanism of the rearrangement of **2** to **3** was studied with stable ammonium analogues **6** and **7** of primary and tertiary cyclopropylcarbinyl cations **4** and **5**, respectively, proposed as intermediates. In non-pyrophosphate-containing buffers, **6** and **7** were not inhibitors. However, the combination of **6** or **7** with PP_i produced potent synergistic inhibition of squalene synthesis from **1** and **2**. Amino acid **8**, an analogue in which a phosphonophosphate moiety was tethered to the amino group in **6**, was a potent inhibitor of squalene synthesis in pyrophosphate-free buffers. When synthesis of **2** and **3** from **1** was measured simultaneously in the presence of **8**, both rates were depressed in an identical manner. It was concluded that squalene synthetase has a single active site which catalyzes **1** → **2** and **2** → **3**. The mechanism of the second reaction is discussed.

Squalene synthetase (farnesyldiphosphate:farnesyldiphosphate farnesyl transferase, EC 2.5.1.21) catalyzes the formation of squalene from farnesyl diphosphate in two distinct steps.[1] In reaction 1, two molecules of farnesyl diphosphate (**1**) are condensed to form presqualene diphosphate (**2**), a prenyl transfer where the C1–C2 double bond of one farnesyl diphosphate serves as the prenyl acceptor for the farnesyl residue of the other.[2] Presqualene diphosphate is then converted to squalene (**3**) in reaction 2 by a rearrangement that cleaves the two newly formed cyclopropane bonds and joins the C1 carbons of the two original farnesyl residues to generate a 1′–1-fused isoprenoid.[3]



Scheme I. A Mechanism for Conversion of Presqualene Diphosphate to Squalene



In addition to conversion to squalene, whose sole fate is sterol synthesis, farnesyl diphosphate also serves as a primer for the prenyl transfers which generate 2,3-dehydrodolicyl diphosphate,[4] all-trans polyprenyl diphosphates for ubiquinone biosynthesis,[5] and perhaps the hydrophobic prenyl units involved in modification of nuclear proteins essential for cell division and maintenance of cellular morphology.[6] The 1′–1 condensation is the first pathway-specific reaction in sterol metabolism, and squalene synthetase

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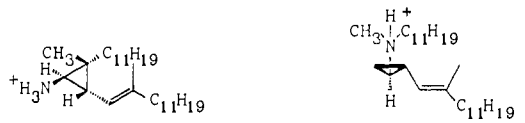
thus resides at an important branch point in the isoprenoid pathway. Rilling and co-workers recently solubilized and purified squalene synthetase to homogeneity.[7,8] The intrinsic microsomal protein had a molecular weight of 47 000, required Mg^{2+} and NADH[9] or NADPH as cofactors, and functioned independently of membrane-derived lipids. They also found that the catalytic activities for synthesis of presqualene diphosphate and squalene copurified at all stages of their procedure.

The mechanism of the 1′–1 coupling has been the subject of a considerable amount of speculation. With the elucidation of the structure of **2** and the recognition that **3** was synthesized in two distinct steps, attention was directed to the mechanisms of the individual transformations. Although several were proposed for reaction 1, there is little work which bears directly on this step.[1,10] In contrast, several groups recognized that the reductive rearrangement of **2** to **3** could be rationalized in terms of the bond reorganizations typically observed for cyclopropylcarbinyl cations.[11-15] Rilling and co-workers[13] originally proposed the sequence shown in Scheme I. Subsequently, Poulter et al.[16] discovered that the rearrangement of primary cation **4** to tertiary cation **5** was neither kinetically nor thermodynamically favored in nonenzymatic model studies and concluded that squalene synthetase must exert strict regiocontrol on the cationic intermediates if the high selectivity required for biosysthesis of squalene was to be realized. They suggested that regiocontrol was achieved through favorable electrostatic interactions between PP_i generated as a consequence of cleaving the C–O bond in **2** and carbocationic species along the reaction path to squalene.[16-18]

In several recent studies, ammonium[19-21] and sulfonium[21,22] analogues of carbocations proposed as reactive intermediates for reactions in the isoprenoid pathway proved to be potent inhibitors of the relevant enzymes. We now present a full account of experiments with ammonium analogues **6** and **7**.[23] The compounds were designed to mimic the electrostatic and topological properties of primary cation **4** and tertiary cation **5**, respectively. Although neither analogue was effective in standard assays, both became potent synergistic inhibitors in combination with PP_i, presumably by duplicating features of the related cyclopropylcarbinyl cation–PP_i ion pairs. Synergism between PP_i and sulfonium analogues of putative carbocationic intermediates in cyclizations of geranyl diphosphate to α-pinene and bornyl diphosphate was also recently reported by Croteau and co-workers.[21] In addition, we found that



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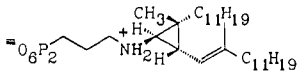

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phosphonophosphate **8**, a tethered analogue of the **6**–PP_i ion pair, was a potent inhibitor in the absence of PP_i. These results are discussed in terms of an ion pair mechanism for the rearrangement of presqualene diphosphate to squalene.

Experimental Section

Materials and General Methods. Unless stated otherwise all chemicals were purchased from Sigma or Aldrich. Cytochrome *c* reductase and glucose-6-phosphate dehydrogenase were from Sigma. Syntheses of ammonium analogues **6–8** were reported previously.[24] *endo*-Bicyclo-[2.2.1]heptane-2,3-dicarboxylic acid anhydride was synthesized.[25] BHDA buffer stocks were prepared by heating the anhydride with 1 equiv of 6 M KOH until the material dissolved. The solution was allowed to cool, and the pH was adjusted to 7.4. The solution was diluted to 1 M and stored until needed. [1-^{3}H]Farnesyl diphosphate was prepared according to the procedure of Davisson et al.[26] and diluted with cold material[27] to the desired specific activity. Fresh bakers' yeast (*Saccharomyces cerevisiae*) was purchased in 1-lb blocks from Westco Standard Co. (Salt Lake City, UT), stored at −20 °C, and used within 1 month.

Radioactivity was measured on a Packard Tri-Carb 4530 liquid scintillation spectrometer using 4% (w/v) Omnifluor in toluene, 0.4% (w/v) Omnifluor (Amersham) in 2:1 toluene/Triton-X (Amersham), Instagel (Packard), or Instafluor (Packard) cocktails. Protein concentrations were measured according to the procedure of Lowry.[28] Solutions of ammonium salts **6** and **7** for inhibition experiments were prepared by adding the corresponding amines to 100 mM BHDA containing 1% (v/v) Tween-80, pH 7.4 (approximately 1 mg/mL). The materials were mixed at high speed on a vortex mixer for 5 min, and the resulting slightly turbid suspensions were diluted to 5 mL with the above buffer to give clear solutions that were stored until needed.

[1-^{3}H]Presqualene Alcohol ([1-^{3}H]-12). In a flame-dried 2-mL vial containing 116 mg (0.27 mmol) of presqualene aldehyde,[24] a solution of sodium [^{3}H]borohydride (25 mCi, 500 mCi/mmol, Amersham) in 0.2 mL of basic methanol (one pellet of potassium hydroxide/30 mL) was added. The ampule that contained the sodium [^{3}H]borohydride was rinsed with two 0.1-mL portions of methanol, which were added to the reaction vial. The vial was capped, and the clear, light-yellow solution was stirred at room temperature for 18 h. Sodium borohydride (10 mg, 0.26 mmol) was added, and the cloudy solution was stirred for 2 h at room temperature. Excess sodium borohydride was quenched by a slow, dropwise addition of 0.05 mL of a saturated solution of ammonium chloride. The contents of the vial were transferred to a culture tube, and the vial was rinsed with 2 mL of pentane. Saturated sodium chloride (2 mL) was added, and the solution was carefully mixed. The pentane layer was removed and passed through a plug of magnesium sulfate. The aqueous layer was extracted with two 1-mL portions of pentane; each 1-mL portion was passed through magnesium sulfate. The material was purified by flash chromatography (2.5 × 13 cm column, 235–400-mesh silica gel, 1.8:8.2 ethyl acetate/hexanes) to yield a clear, viscous oil (12.9 mCi, 52% radiochemical yield) that comigrated with authentic material.[24] The alcohol was stored at −20 °C in 4.5 mL of purified hexanes.

Determination of Specific Activity for [1-^{3}H]Presqualene Naphthoate ([1-^{3}H]-13). In a flame-dried 1-mL vial containing a solution of 10 mg (0.08 mmol) of 4-(dimethylamino)pyridine and 34 mg of 2-naphthoic acid (0.2 mmol) in 0.5 mL of methylene chloride was added 56 μCi of [1-^{3}H]-**12** in 20 μL of pentane. The solution was stirred for 2 min before addition of 41 mg of 1,3-dicyclohexylcarbodiimide (0.20 mmol) dissolved

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in 0.5 mL of methylene chloride. The solution was stirred for 4 h at room temperature. Solvent was removed under a stream of nitrogen to afford a white residue that was suspended in 1 mL of hexanes. The solution was filtered, and the reaction vial was rinsed with four 1-mL portions of hexanes. The hexane extracts were combined, and solvent was removed under a stream of nitrogen to afford a white residue that was again treated four times with 1-mL portions of hexanes as previously described. Solvent was removed from the combined filtrates under a stream of nitrogen to afford a white residue that was suspended in 0.4 mL of hexanes. A 0.2-mL portion was applied to a 20 × 20 cm plate of silica gel 60, 0.25 mm thick, F 254 (EM reagents), and the plate was developed three times with 0.3:9.7 ethyl acetate/hexanes. The UV-active material that comigrated with authentic cold **13** prepared in an identical manner was dissolved in 10 mL of methylene chloride. Solvent was removed under reduced pressure, and the residue was dissolved in 1.0 mL of HPLC-grade acetonitrile. The radiochemical recovery was 11.8 μCi (21%). Analysis by UV and liquid scintillation spectrometry[26] gave a specific activity of 52.7 mCi/mmol.

In a previous experiment using the same procedure, a sample of cold **13** (5.3 mg, 25%) was prepared from 15 mg (0.036 mmol) of **12**: ^{1}H NMR (300 MHz, $CDCl_3$) 8.60 (br s, 1 H), 8.06(dd, J = 8.4 and 1.4 Hz, 1 H), 7.88 (m, 3 H), 7.54 (m, 2 H), 5.08 (m, 4 H, vinyl), 4.97 (d, J = 7.3 Hz, 1 H, vinyl coupled to C3′), 4.65 (m, 1 H, carbinyl), 4.19 (m, 1 H, carbinyl), 2.0 (m, 14 H, vinyl methylenes), 1.65 (s, 2 H, vinyl methylenes), 1.64 (s, 6 H, methyls), 1.56 (s, 15 H, methyls), 1.5–1.0 (unresolved cyclopropyl resonances), 1.20 ppm (s, 3 H, cyclopropyl methyl); IR (CCl_4) 2960, 2920, 2860, 1718, 1450, 1375, 1350, 1280, 1225, 1190, 1130, 1090 cm^{-1}; UV (λ_{max}, acetonitrile) 236 nm (ϵ, 6.3 × 10^4).

[1-^{3}H]Presqualene Diphosphate ([1-^{3}H]-2). To a flame-dried two-neck 15-mL round-bottom flask was added 6.3 mCi of [1-^{3}H]presqualene alcohol (0.12 mmol) in 2.2 mL of hexanes. Solvent was removed with a stream of nitrogen, and the flask was placed in an ice bath. Trichloroacetonitrile (163 mg, 1.13 mmol, freshly distilled) was added dropwise, and the solution was stirred under nitrogen at room temperature for 45 min at 0 °C. The reaction mixture was allowed to warm to room temperature, and a solution of 112 mg (0.38 mmol) of bis(triethylammonium) hydrogen phosphate in 2.7 mL of acetonitrile was added dropwise over a 4-h period. The yellow-green solution was allowed to stir for 26 h at room temperature. The reaction mixture was transferred to a 50-mL round-bottom flask, and solvent was removed under reduced pressure. The residue was dissolved in 1:25 (v/v) 2-propanol/25 mM ammonium bicarbonate buffer and applied to a 10-mL (17-mequiv) column of AG 50W-X8 ion-exchange resin (ammonium form). The column was slowly eluted with 4 column volumes of the same buffer. Solvent was removed by lyophilization. The residual fluffy, yellow-white solid was dissolved in 8 mL of chloroform and transferred to a culture tube. Ammonium bicarbonate (8 mL of a 0.1 M solution) was added, and the suspension was vigorously mixed for 10 s. The mixture was centrifuged, and the chloroform layer was removed. The aqueous layer was extracted with two 8-mL portions of chloroform, and solvent was removed from the combined chloroform layers at reduced pressure. The residue was dissolved in a minimal amount of 1:1:0.4 (v/v/v) butanol/tetrahydrofuran/0.1 M ammonium bicarbonate and applied to a 2.5 × 13 cm column of CF11 cellulose (Whatman). The column was eluted by gravity flow with the same solvent. Fractions that contained material that comigrated with an authentic sample of presqualene diphosphate were combined, and solvent was removed to give 0.403 mCi (6.4% radiochemical yield) of material that comigrated with authentic presqualene diphosphate on TLC [cellulose; 1:1:0.4 (v/v/v) 1-butanol/tetrahydrofuran/0.1 M ammonium bicarbonate, R_f = 0.3]. The material was stored at −78 °C.

In a previous experiment using the same procedure, a sample of cold **12** (59 mg, 0.14 mmol) was converted to **2** (20 mg, 22%): ^{1}H NMR (300 MHz, $CDCl_3$) 5.05 (br s, 4 H, vinyls), 4.87 (d, J = 8 Hz, vinyl adjacent to C3′), 4.0–2.6 (br, ammonium counterions, carbinyls), 1.96 (m, 16 H, vinyl methylenes), 1.64 (m, 9 H, vinyl methyls), 1.55 (s, 12 H, vinyl methyls), 1.4–0.8 ppm (unresolved resonances for the cyclopropyl methyl and C1′ and C3′ cyclopropyls); ^{31}P NMR (121 MHz, $CDCl_3$) −9.4 (br s, 1 P, P2), −11.3 (br s, 1 P, P1); negative ion FABMS, m/z (rel intensity) 585 (M − N_3H_{10}, 25), 189 (11), 181 (12), 177 (14), 159 (30), 97 (16).

Isolation of Yeast Microsomes. All procedures were at 4 °C. Yeast (30 g) was suspended in 30 mL of 100 mM BHDA containing 100 μM DTT, pH 7.4, and the cells were disrupted at 15000–18000 psi in a French pressure cell. The suspension was spun at 23000g for 30 min. The supernatant was filtered through six layers of gauze and spun again at 23000g for 30 min. The resulting supernatant was spun at 177000g for 1.5 h, and the pellet was resuspended in 24 mL of 100 mM BHDA, 10 μM DTT, 1 mM EDTA, and 10 mM $MgCl_2$, pH 7.4, by eight passes with a Dounce homogenizer. The suspension was spun and resuspended again as described above. The material was divided into 1-mL portions and frozen at −20 °C.

Assays. (A) Squalene Synthesis. Assays were in 500 μL of 50 mM BHDA, 11 mM KF, 5 mM $MgCl_2$, 50 μM DTT, 1 mM NADPH, and 0.2% Tween-80, pH 7.4, containing appropriate amounts of substrate (sp act. 18–200 μCi/μmol) and yeast microsomes (2–7 μg of protein) in 12 mm × 75 mm test tubes. Tubes containing all of the ingredients except for microsomes were flushed with nitrogen, sealed with cork stoppers, and equilibrated at 30 °C for 10 min. Microsomes were added, and the contents were mixed for 3 s. Incubations were for 10 min at 30 °C and were stopped by addition of 250 μL of 1:1 (v/v) 40% (w/v) aqueous KOH/ethanol, followed by mixing for 3 s. Squalene (2 μL) and solid NaCl (sufficient to saturate the solution) were added. The mixture was extracted with 1 mL of ligroin (bp 60–80 °C), and a 0.8-mL portion of the ligroin layer was loaded onto a column containing 2 mL of alumina (activity II) in a 10-mL disposable syringe barrel. The assay mixture was extracted with an additional 1-mL portion of ligroin, and 0.8 mL of the extract was added to the column. The combined extracts were eluted into a scintillation vial with 9 mL of toluene, 1 mL of 4% Omnifluor cocktail was added, and radioactivity was measured. All determinations were in triplicate against blanks. Calculations of specific activities (nmol min^{-1} mg^{-1}) were corrected for the stoichiometry of the condensation with respect to loss of a proton from C1 of one of the two farnesyl residues. The above assay was also used to measure squalene synthesis from [1-^{3}H]presqualene diphosphate.

(B) Presqualene Diphosphate Synthesis (Proton Release Assay). The proton release assay[7,29] was modified for the simultaneous evaluation of presqualene diphosphate and squalene synthesis. Assays were in 200 μL of 50 mM BHDA, 5 mM $MgCl_2$, 50 μM DTT, 1 mM NADPH, and 0.2% Tween-80, pH 7.4, with 48 μg of protein. Incubations were conducted as described above. The reaction was quenched with 80 μL of 1:1 (v/v) 40% (w/v) aqueous KOH/ethanol, and the resulting solution was saturated with NaCl. Squalene (2 μL) was added, and the solution was extracted three times with ligroin as before. The combined ligroin extracts were chromatographed, and radioactivity of the toluene eluate was determined to measure synthesis of squalene. The remaining ligroin was carefully removed from the aqueous layer, 2 mL of methanol was added, and the mixture was mixed vigorously for 1 min. The mixture was transferred to a 13 × 100 mM culture tube which was fitted with a glass tube elbow. The contents were distilled to near dryness at 78 °C with a block heater into a test tube cooled on ice. A 0.75-mL portion of the distillate was removed, and its radioactivity was determined in Instagel (10 mL).

In order to compare specific activities for the proton release and squalene assays, values determined by proton release were multiplied by a factor which reflected differences in proton stoichiometry and efficiencies of the two workups. With (±)-[1-^{3}H]farnesyl diphosphate, 25% of the total radioactivity was released in reaction 1, and the remaining 75% was incorporated into squalene. The squalene workup was greater than 95% efficient, while the proton release workup only measured approximately 33% of the released activity. When combined, these features give a correction factor of 8.6.

Results

Buffer Inhibition. Most investigators have used a 55 mM phosphate buffer for squalene synthetase assays. Since inorganic pyrophosphate (PP_i) is a product of both reactions catalyzed by the enzyme, we were concerned that the enzyme was susceptible to inhibition in P_i-containing buffers. An additional concern was that high concentrations of amine buffers might also inhibit the enzyme by binding to the region of the catalytic site that normally accommodates the carbocationic species proposed as intermediates during catalysis. Figure 1 shows the activity of squalene synthetase for conversion of **1** to **2** as a function of buffer concentration in P_i, Tris, and BHDA buffers at pH 7.4. The slight rise in activity between 10 and 25 mM in all three systems was perhaps the result of inadequate buffering at low concentrations. Above 25 mM, Tris and P_i both inhibited the enzyme, while BHDA did not. As concentrations increased, the differences became dramatic. In 100 mM buffers, the activity of squalene synthetase in Tris and P_i dropped to 19% and 2%, respectively, of the BHDA values. At 55 mM P_i, the concentration normally used to assay the enzyme, squalene synthetase had only 19% of the activity seen in BHDA.

A different pattern was seen for buffers containing Tris and P_i or PP_i. As shown in Figure 2, PP_i became a potent inhibitor

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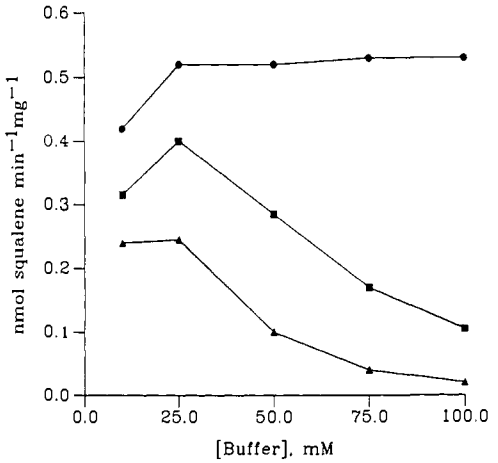

Figure 1. Rates for squalene synthesis from farnesyl diphosphate as a function of buffer concentration. All buffers contained 11 mM KF, 5 mM $MgCl_2$, 50 μM DTT, 1 mM NADPH, and 0.2% Tween-80. BHDA (●); Tris (■); phosphate (▲). pH 7.4.

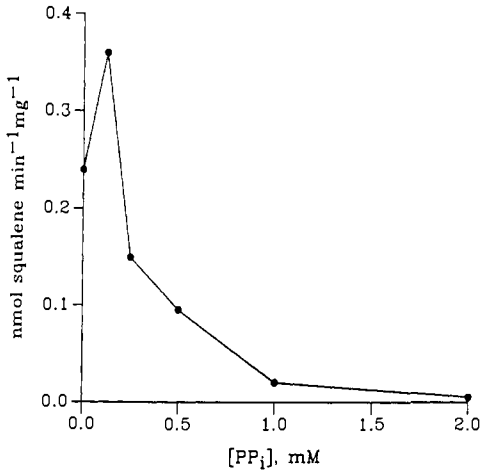

Figure 2. Rates for squalene synthesis from farnesyl diphosphate as a function of PP_i concentration in 100 mM Tris, 11 mM KF, 5 mM $MgCl_2$, 50 μM DTT, 1 mM NADPH, and 0.2% Tween-80.

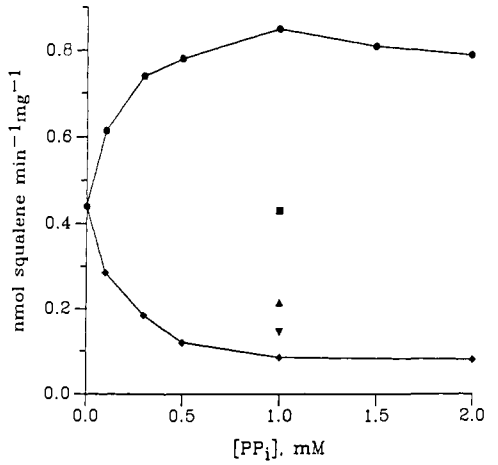

Figure 3. Rates for squalene synthesis from farnesyl diphosphate as a function of the concentrations of PP_i and 7: 0 (●), 3 (■), 5 (▲), 10 (▼), and 20 μM (◆).

in 100 mM Tris. There was a 45% increase in activity at 0.1 mM PP_i (vide infra), and activity was essentially abolished at 2 mM PP_i.

The individual effects of Tris and PP_i in 25 mM BHDA were substantially different. While there was no evidence for inhibition by 0–20 mM Tris, low concentrations of PP_i elicited a marked stimulation of squalene synthetase. As shown in Figure 3, the

Table I. Inhibition of Squalene Synthetase by Ammonium Analogues **6**, **7**, and **8**

analogue	substrate	[substrate] (μM)	[PP_i] (mM)	I_{50} (μM)
6	**1**	12	1	10
	2	2	1	5
7	**1**	1	1	3
8	**1**	4	0	5
	2	2	0	3

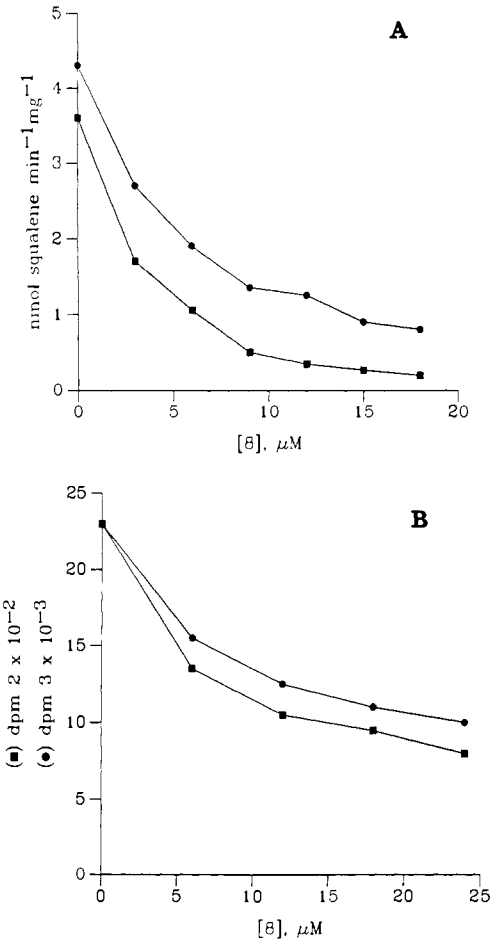

Figure 4. Inhibition of squalene synthetase by tethered ammonium analogue **8**. (A) Rate of squalene synthesis from **1** (●) and from **2** (■). (B) rates for synthesis of **2** (proton release, ■) and **3** (squalene assay, ●) from **1**.

rate of squalene synthesis almost doubled when 1 mM PP_i was added to 50 mM BHDA. At higher concentrations, PP_i slowed the rate of squalene synthesis, as expected for a product inhibitor. The magnitude of stimulation/inhibition varied with different preparations of microsomes. When a fresh preparation was allowed to stand at 5 °C, the activity of squalene synthetase in PP_i-free buffer slowly increased until, after 30 days, no additional stimulation was seen between 0 and 1 mM PP_i. As the microsomes aged further, the activity of the enzyme gradually decreased. The stimulation in activity elicited by PP_i in BHDA buffer was not seen for two other microsomal enzymes, cytochrome *c* reductase[30] and glucose-6-phosphatase,[31] and at this point we have no explanation to offer for the simulation.

Inhibition by Ammonium Analogues 6 and 7. In BHDA buffer containing chloride and fluoride as the only other anions, ammonium analogues **6** and **7** did not inhibit squalene synthetase at concentrations up to 170 μM. However, as illustrated in Figure 3, potent inhibition of enzymatic activity was noted when PP_i was

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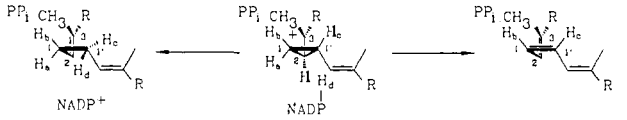
Scheme II. Mechanisms for Synthesis of Squalene and Phytoene



added to the buffer. A plot of the rate of squalene synthesis versus [PP_i] in the presence of 20 μM **7** decreased sharply as the concentration of PP_i increased from 0 to 1 mM. At [PP_i] = 1 mM, **7** was a potent inhibitor with I_{50} = 3 μM (the concentration of inhibitor required for 50% inhibition) in the presence of 1 mM NADPH. Similar profiles were observed for inhibition of synthesis of **3** from **1** and from **2** by primary ammonium analogue **6**. I_{50} values for the inhibitors are listed in Table I.

Inhibition by Tethered Ammonium Analogue 8. Figure 4A shows the results for inhibition of reaction 1 (**1** → **2**) and reaction 2 (**2** → **3**) by tethered ammonium analogue **8**. In contrast to experiments with analogues **6** and **7**, **8** was a potent inhibitor of the enzyme in the *absence* of PP_i and had a similar effect on both reactions. Figure 4B shows inhibition of reactions 1 and 2 in an experiment where the rates of proton release from C1 (**1** → **2**) and accumulation of radiolabeled **3** (**1** → **3**) were measured simultaneously. Tethered ammonium analogue **8** had similar effects on the rates of both reactions.

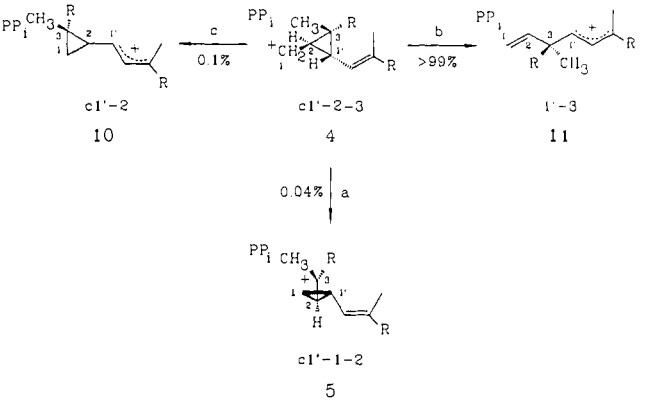
Discussion

Two major classes of isoprenoid metabolites, sterols[1] and carotenoids,[32] are derived from simple 1′–1 precursors. In sterol metabolism, the condensation of farnesyl diphosphate proceeds in two steps. The first yields (1*R*,2*R*,3*R*)-presqualene diphosphate[10] [(1*R*,2*R*,3*R*)-**2**, R = $C_{11}H_{19}$], a c1′–2–3-fused triterpene, and the second gives squalene (**3**, R = $C_{11}H_{19}$) with a saturated 1′–1 linkage. The second reaction proceeds with inversion of configuration at the C1′ and C1 centers as outlined in Scheme I. In carotenoid metabolism two molecules of geranylgeranyl diphosphate are condensed to give (1*R*,2*R*,3*R*)-prephytoene diphosphate[10] [(1*R*,2*R*,3*R*)-**2**, R = $C_{16}H_{27}$], which in turn rearranges to phytoene (**9**, R = $C_{16}H_{27}$) with a 1′–1 double bond. Since squalene synthetase requires NADPH and phytoene synthetase does not, it is resonable to assume that the 1′–1 condensations have similar mechanisms until the final stage of reaction 2 where reduction by NADPH produces the saturated 1′–1 linkage in **3** and elimination generates the central double bond in **9** (see Scheme II).

Several groups proposed that the c1′–2–3 to 1′–1 rearrangements catalyzed by squalene and phytoene synthetases involve cyclopropylcarbinyl cations.[11-18] The mechanisms suggested by Poulter and co-workers[17,18] in Schemes I and II proceed by isomerization of primary cation **4** to its more stable tertiary isomer **5** by a ring expansion/contraction sequence initiated by migration of the C2–C1′ cyclopropane bond. In sterol biosynthesis the tertiary C_{30} species (**5**, R = $C_{11}H_{19}$) is quenched by a hydride from NADPH to give **3** (R = $C_{11}H_{19}$), and in carotenoid biosynthesis its C_{40} (**5**, R = $C_{16}H_{27}$) counterpart loses a proton to give **9**. However, the rearrangements of cyclopropylcarbinyl cations are notoriously sensitive to substitution patterns on the cyclopropane ring. Poulter and co-workers[16] examined the solvolytic rearrangements of the chrysanthemyl cation (**4**, R = CH_3), a simple model for presqualene or prephytoene disphosphate (see Scheme III), and found the rearrangement **4** → **5** required for biosynthesis of 1′–1-fused isoprenoids was only a minor reaction channel (ca 0.04%). Two competing rearrangements, **4** → **10** and **4** → **11**, were observed, with the latter accounting for most of **4**. It became evident that the enzymes must exert strict regiocontrol if cyclopropylcarbinyl cations were indeed intermediates in the enzyme-catalyzed rearrangements.

A least-motion mechanism consistent with properties of cyclopropylcarbinyl cations and the stereochemical studies of

Scheme III. Rearrangements for Primary Cyclopropylcarbinyl Cation **4**



Cornforth and Popjak[1,33] was proposed by Poulter and co-workers.[17,18] The [1.2] sigmatropic shifts of carbon–carbon bonds during the ring expansion/contraction sequences of cyclopropylcarbinyl rearrangements are suprafacial, and the rotational barrier between the carbinyl carbon and the cyclopropane ring is too high to permit isomerization within the lifetimes of the carbocations.[34] Thus, for the rearrangement catalyzed by squalene synthetase, inversion at C1 during **4** → **5** requires that **2** be bound to the active site in a conformation where the C1–O bond is syn to the C2–C3 cyclopropane bond. The resulting conformation of the **4**–PP_i tight ion pair generated upon heterolysis of the C–O bond is optimal for stabilizing **4**–PP_i → **5**–PP_i with positive charge migrating from C1 (cation **4**) across C2 (species which interconnect **4** and **5**) to C3 (cation **5**). Rearrangements **4**–PP_i → **10**–PP_i and **4**–PP_i → **11**–PP_i are accompanied by large separations of positive and negative centers in the ion pairs with a resulting loss of electrostatic stabilization. A selective stabilization of **4** → **5** by approximately 8 kcal/mol would be sufficient to account for the high regioselectivity of the enzymatic reaction. This is a reasonable value to achieve in a tight ion pair given the distances between centers bearing charge in the various isomeric ion pairs.[35]

Following **4** → **5** (R = $C_{11}H_{19}$), the reaction is completed by addition of hydride from NADPH with concomitant opening of the cyclopropane ring. Stereoelectronic considerations dictate that the addition occurs with inversion at C1′.[36,37] Consequently, the cofactor must be bound in a conformation to be properly positioned for transfer of hydride following the rearrangement. Premature transfer of hydride from the cofactor to **4** is blocked because addition would require retention at C1′ and, thus, encounters a stereoelectronic barrier. It is only after rearrangement of **4** to **5** that the barrier vanishes.

Inhibition studies with ammonium analogues **6** and **7** constitute strong support for the ion-pair mechanism. It was clear that the inhibitors did not bind tightly to the active site of squalene synthetase in spite of their close resemblance to carbocations **4** and **5**. In fact, we were initially disappointed when 170 μM concentrations of the ammonium analogues failed to inhibit the enzyme. These concentrations exceeded S_{50} (substrate concentrations required for half-maximal activity in the presence of 1 mM NADPH) for **1** (10 μM) and **2** (8 μM) by approximately 15–20-fold.[8] However, 20 μM concentrations of the ammonium analogues, which failed to inhibit, and 1 mM PP_i, which stimulated the enzyme, combined to produce potent ion-paired inhibitors. For many enzymes which process isoprenoid diphosphates, the diphosphate moiety is a principal determinate for binding.[1,2] Presumably, complementary interactions between the hydrocarbon

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moieties of **6** and **7** and squalene synthetase were insufficient to overcome electrostatic repulsions between the unshielded ammonium moieties and the enzyme. I_{50}'s for **6** and **7** against **1** and **2** in buffer containing PP_i indicated that the diphosphate unit was particularly effective in promoting binding of the ammonium analogues, as opposed to other anions such as chloride or BHDA. Similar synergistic phenomena were observed, albeit at 10^4-fold higher concentrations, for mixtures of Tris and PP_i, where there is no apparent structural similarity between the ammonium species and the hydrocarbon regions of carbocations **4** and **5**. In addition to mechanistic insight, these studies emphasize the caution one must exercise in developing assays for squalene synthetase.

Analogue **8** was designed to increase the effective concentration of PP_i in the presence of **6** by tethering a phosphonophosphate moiety to the ammonium analogue. Examination of a space-filling model suggested that a two-carbon spacer between the nitrogen of **6** and a nonbridging oxygen in PP_i would permit the tethered molecule to fold into a relatively strain-free conformation with a nonbridging oxygen on the remote phosphorus near the positively charged ammonium center. Attempts to synthesize a tethered diphosphate analogue of **6** were unsuccessful, presumably because of the reactivity of the 2-aminoethyl diphosphate linkage, and phosphonophosphate **8** was prepared instead. The compound proved to be a potent inhibitor of squalene synthesis. I_{50}'s of 3–5 μM were found against **1** and **2**, values similar to those measured for **6** and **7** in the presence of 1 mM PP_i. As illustrated in Figure 3, the effectiveness of **6** and **7** decreased rapidly at lower concentrations of PP_i, and the analogues did not inhibit in buffers containing 3 μM PP_i. Thus, the tether increased the effective concentration of PP_i at least 300-fold, notwithstanding (1) an expected decrease in potency when the diphosphate moiety was replaced with a phosphonophosphate moiety and (2) steric and hydrophobic perturbations associated with introduction of a hydrophobic ethyl moiety into the diphosphate binding region of the catalytic site.

Rilling and co-workers[7,8] discovered that squalene synthetase is a relatively small protein composed of a single polypeptide chain which catalyzes synthesis of presqualene diphosphate and squalene. In microsomal preparations, **1** was converted to **3** with the accumulation of relatively little **2**. However, the activities for the two steps became clearly delineated when the enzyme was solubilized. Under these conditions the activity for reaction 1 was approximately 5-fold that of reaction 2.[8]

Rilling's observations raise the question of whether **2** and **3** are synthesized at the same or different catalytic sites. In earlier inhibition studies of squalene biosynthesis in rat liver microsomes, Corey and Volante[38] reported that a phosphonophosphate analogue of presqualene disphosphate blocked formation of squalene from mevalonate and **2** under conditions where some synthesis of **2** from mevalonate was seen. Although these results are consistent with a two-site hypothesis, such high levels of inhibitor (0.5–1 mM) were required to completely block squalene formation that nonspecific inhibition may have occurred. More recently, Bertolino et al.[39] discovered that a variety of detergent-like analogues were nonspecific inhibitors of squalene synthetase.

Agnew and Popjak[40] noticed severe inhibition of the second reaction, but not the first, when the rates of reactions 1 and 2 were measured simultaneously in microsomal preparations of squalene synthetase. The inhibition was transitory, and as **1** was consumed, the two activities, equalized. They proposed that the time course of the recovery indicated that the enzyme had a distinct active site for each reaction. We suggest that their data are also consistent with a single-site mechanism. Since the prenyl transfer step (reaction 1) utilizes two molecules of **1**, its rate depends on $[\mathbf{1}]^2$, and an increase in the concentration of **1** will only serve to accelerate reaction 1. However, at high concentrations, **1** can compete with **2** for binding and, thereby, inhibit reaction 2.

Our experiments with tethered analogue **8** support a single-site hypothesis. In the absence of the tethered inhibitor specifically designed to block reaction 2, the rates of the two reactions were almost equal. As illustrated in Figure 4A, **8** inhibited squalene synthesis from **1** and **2** to similar extents. In addition when the rates of both reactions were measured simultaneously with **1** as the initial substrate (Figure 4B), the extent to which reactions 1 and 2 were inhibited was almost identical. The simplest explanation of these results is that both are catalyzed at a single active site, and when **8** binds, both are blocked equally. However, more complicated scenarios which cannot be excluded at the present time include two spatially overlapping sites or nonoverlapping but strongly interacting sites where binding of **8** to one completely blocks catalysis at the other.

From the inhibition studies, we conclude that squalene synthetase has a single active site that catalyzes both reactions for synthesis of squalene from farnesyl diphosphate and that cyclopropylcarbinyl cations are reactive intermediates in the rearrangement of **2** to **3**. In particular, inhibitors whose topological and electrostatic properties mimic primary cation **4** and tertiary cation **5**, originally proposed as intermediates by Poulter and co-workers[13,16–18] bind tightly to the enzyme. However, binding of the positively charged species depends upon the availability of PP_i, and it is the cyclopropylcarbinyl cation (ammonium analogue)–PP_i ion pair that is tightly bound. We further suggest that PP_i also provides a negatively charged template which controls the regioselectivity of the carbocation fragment during the rearrangement.

A simple minimal mechanism can be proposed. Once squalene synthetase has sequestered presqualene diphosphate and NADPH in the proper orientation, catalysis is achieved by activation of the diphosphate moiety, presumably by neutralizing negative charge in the leaving group. By maintaining the original relative orientation of the positive and negative fragments during the rearrangements, the regioselective rearrangement of **4**–PP_i → **5**–PP_i which ensues is driven by electrostatic stabilization within enzyme-bound tight ion pairs. Similarities between the reactions catalyzed by squalene synthetase and phytoene synthetase suggest that the latter utilizes similar chemistry to convert prephytoene diphosphate to phytoene.

Acknowledgment. This work was supported by NIH Grants GM 21328 and GM 25521.

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