Communications to the Editor

Biosynthesis of Non-Head-to-Tail Isoprenoids. Synthesis of 1′−1 and 1′−3 Structures by Recombinant Yeast Squalene Synthase

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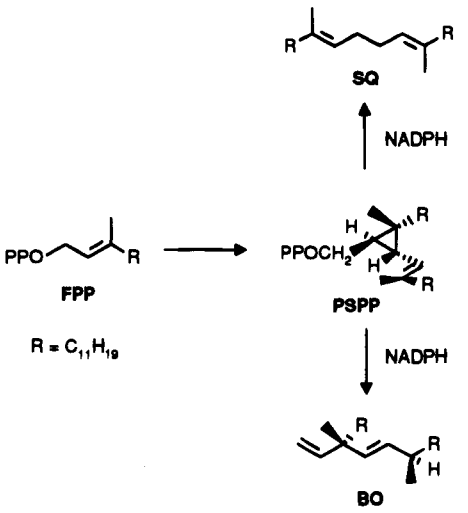
Although there are over 23 000 naturally occurring isoprenoid compounds, only 10 different ways for joining the five-carbon isoprene building blocks have been reported.[1] The most common attachment is a head-to-tail 1′−4 linkage, formed during the fundamental chain elongation process by condensation of isopentenyl diphosphate with an allylic isoprenoid substrate. Although less common, 1′−1 or 1′−3 linkages are found in several families of metabolites, including carotenoids, sterols, botryococcenes, and irregular monoterpenes. The 1′−1 and 1′−3 triterpene hydrocarbons squalene (SQ)[2,3] and botryococcene (BO)[4] are synthesized from farnesyl diphosphate (FPP) via presqualene diphosphate (PSPP) as outlined in Scheme 1. The respective biosynthetic enzymes, squalene synthase and botryococcene synthase, are highly selective for synthesis of their respective products.

Shortly after the discovery of the c1′−2−3 triterpene PSPP, several groups proposed mechanisms based on rearrangements of cyclopropylcarbinyl cations to explain the rearrangement of PSPP to SQ.[3,5−7] However, model studies of the c1′−2−3 → 1′−1 rearrangement met with limited success, giving only trace quantities (~0.04%) of 1′−1 products, although the c1′−2−3 → 1′−3 rearrangement was facile.[8,9] Attempts to study the rearrangements catalyzed by squalene synthase and botryococcene synthase have been hampered by the unavailability of pure preparations of the proteins. We recently constructed an *Escherichia coli* clone that produced an active soluble form of recombinant yeast squalene synthase and purified the enzyme to homogeneity.[10] We now describe experiments with squalene synthase that establish a direct mechanistic link between the biosyntheses of 1′−1 and 1′−3 isoprenoids.

FPP and NADPH were incubated with recombinant yeast squalene synthase[10] in 50 mM MOPS buffer, 10 mM $MgCl_2$, and 1 mM DTT (pH 7.2) for 1.5 h at 30 °C. Samples were removed periodically and analyzed by HPLC.[11] As expected, FPP was rapidly converted to PSPP and then to SQ with only a low steady-state concentration of PSPP seen during the course of the reaction. When FPP was incubated with squalene synthase in the absence of NADPH, PSPP again formed rapidly ($k_{cat} \sim 0.2\ s^{-1}$).[12] However, at longer reaction times, PSPP was converted to a mixture of three compounds (see Figure 1), ($k_{cat} \sim 2 \times 10^{-3}\ s^{-1}$). Control experiments established that FPP and PSPP were stable to the reaction conditions with heat-inactivated enzyme and that $MgCl_2$ was required for both reactions. Following a preparative scale incubation (14 mg of FPP and 42 mg of enzyme), the reaction mixture was extracted with methyl *tert*-butyl ether and the products were separated by HPLC to give, in order of elution, *cis*-dehydrosqualene[13] (DSQ, 13%), hydroxybotryococcene[14] (HBO, 26%), and hydroxysqualene[15] (HSQ, 61%). These three compounds accounted for >90% of the products from PSPP, and no other product constituted more than ca. 3% of the mixture.

Scheme 1. Biosynthesis of Squalene and Botryococcene



Other groups have reported experiments in which FPP was incubated with microsomal preparations of squalene synthase in buffers that did not contain NADPH.[16−19] When Mg^{2+} was present, FPP was converted to PSPP, which then was hydrolyzed to presqualene alcohol by phosphatases present in the crude

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(2) Abbreviations used are as follows: BO, botryococcene; DSQ, *cis*-dehydrosqualene; DTT, dithiothreitol; FPP, farnesyl diphosphate; HBO, hydroxybotryococcene; HSQ, hydroxysqualene; MOPS, 3-(*N*-morpholino)-propanesulfonic acid; PSPP, presqualene diphosphate; SQ, squalene.
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(13) UV (hexane), λ_{max} 285 nm. ^1H NMR ($CDCl_3$): 1.5−1.8 (24H, m), 1.9−2.2 (16H, br m), 5.04−5.14 (4H, br m), 6.1 (2H, br d), 6.3 (2H, br d). MS (EI, 70 eV): 69 (100), 408 (17.6). HRMS for $C_{30}H_{48}$: calcd 408.3756, found 408.3749.
(14) ^1H NMR ($CDCl_3$): 1.1 (3H, s), 1.26 (3H, s), 1.3−1.42 (2H, m), 1.52 (3H, s), 1.56 (9H, s), 1.65 (6H, s), 1.85−2.1 (14H, br m), 4.94 (1H, dd, J = 17.5, 1.4 Hz), 4.97 (1H, dd, J = 10.8, 1.4 Hz), 5.05−5.15 (4H, m), 5.43 (1H, d, J = 16.0 Hz), 5.6 (1H, d, J = 16.0 Hz), 5.79 (1H, dd, J = 17.5, 10.8 Hz). ^{13}C NMR ($CDCl_3$): 146.0, 135.4, 135.0, 134.3, 131.3, 124.6, 124.2, 111.6, 73.2, 42.7, 41.9, 41.2, 39.7, 28.5, 28.5, 26.8, 26.7, 25.7, 23.5, 23.4, 23.1, 22.9, 17.7, 16.0, 15.9. MS (EI, 70 eV): 69 (100), 203 (19), 408 (M $-H_2O$, 27), 426 (M, 2). HRMS for $C_{30}H_{50}O$: calcd 426.3861, found 426.3848.
(15) ^1H NMR ($CDCl_3$) 1.55−1.65 (24H), 1.90−2.15 (16H, br m), 2.17 (1H, m), 2.28 (1H, md), 4.33 (1H, dt, J = 7.4, 6.9 Hz), 5.07 (5H, br m), 5.18 (1H, d, J = 7.4 Hz). ^{13}C NMR ($CDCl_3$): 138.7, 138.3, 135.2, 131.3, 131.2, 127.2, 124.3, 124.0, 123.8, 119.6, 68.4, 40.0, 39.7, 39.6, 36.4, 26.8, 26.6, 26.4, 25.7, 17.7, 16.7, 16.4, 16.1, 16.0. MS (EI, 70 eV): 69 (69), 206 (100), 221 (37), 408 (M − H_2O, 21), 426 (M, 4.7). HRMS for $C_{30}H_{50}O$: calcd 426.3861, found 426.3890.
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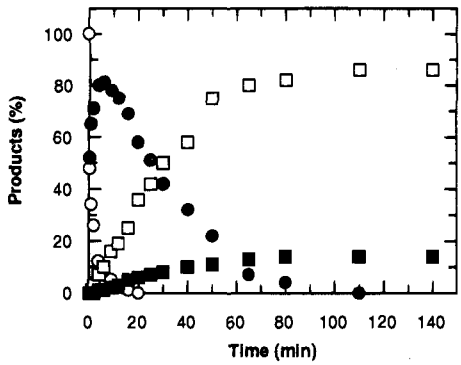
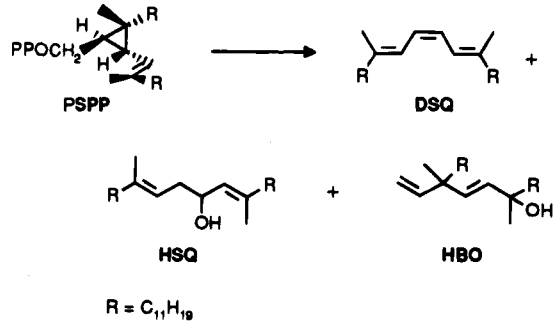


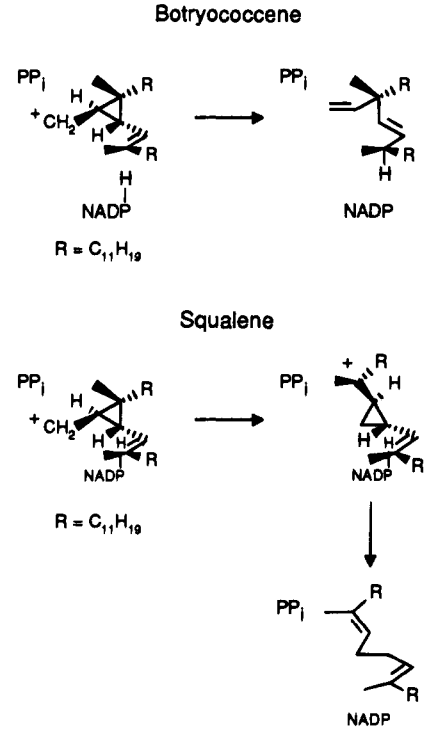
Figure 1. Products formed during incubation of FPP with recombinant yeast squalene synthase: FPP, ○; PSPP, ●; DSQ, ■; HBO and HSQ, □. Incubation was in 50 mM MOPS buffer, 10 mM $MgCl_2$, and 1 mM DTT (pH 7.2) at 30 °C. Samples were analyzed by reversed phase HPLC (ref 11). Under these conditions, BHO and HSQ eluted as a single peak. The isomeric alcohols were resolved on a 4.6 × 250 mm^2 10 μm silica column by elution with hexane (5 min), followed by a linear gradient of hexane to 7:3 (v/v) hexane:methyl *tert*-butyl ether.



microsomal preparations of yeast squalene synthase that were available at that time. When Mn^{2+} was substituted for Mg^{2+}, small amounts of DSQ and HSQ were also found in addition to presqualene alcohol.[18,19] By using purified recombinant enzyme, we were able to eliminate the competing hydrolysis reaction and reveal the competing enzyme-catalyzed solvolysis of PSPP in Mg^{2+}-containing buffers. As previously pointed out by Nishino *et al.*,[18,19] DSQ and HSQ are the apparent products of elimination and substitution, respectively, from the carbocation that is the immediate precursor of squalene in the normal reaction. HBO, which was not seen in previous studies, provides an obvious biosynthetic link between 1′−1 and 1′−3 isoprenoids and demonstrates that the catalytic machinery which evolved to synthesize squalene also synthesizes 1′−3-fused triterpenes.

Of the several mechanisms proposed for biosynthesis of 1′−1 and 1′−3 isoprenoids,[1,3,5−9] we favor the simple set of reactions shown in Scheme 2. In this scenario, PSPP is bound in the same conformation by both squalene synthase and botryococcene synthase, and the regiochemistry of the reaction is determined by the location of the nucleophile (NADPH) relative to PSPP in the enzyme−substrate−cofactor complex. In botryococcene synthase, NADPH is positioned to capture the primary-carbocation by addition to the double bond. In squalene synthase, the cofactor is positioned to transfer hydride to the cyclopropane carbon. A stereoelectronic barrier prevents addition of hydride to the cyclopropane ring in the primary cation with retention of configuration. The ensuing exothermic cyclopropylcarbinyl rearrangement gives the isomeric tertiary cation with NADPH now positioned to transfer hydride to the cyclopropane carbon with inversion. Without NADPH, squalene synthase is still capable of initiating the carbocationic reaction that converts PSPP to SQ. However, the nicotinamide binding pocket is now apparently occupied by water. Under these conditions, regioselectivity for addition of the nucleophile is lost and the primary carbocation is intercepted in competition with the c1′−2−3 → 1′−1 rearrangement to give both 1′−1 and 1′−3 products. This mechanism has predictable stereochemical consequences that are under investigation.

Scheme 2. Mechanism for Formation of Botryococcene and Squalene from Presqualene Diphosphate



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