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Absolute Configuration of Hydroxysqualene. An Intermediate in Bacterial Hopanoid Biosynthesis

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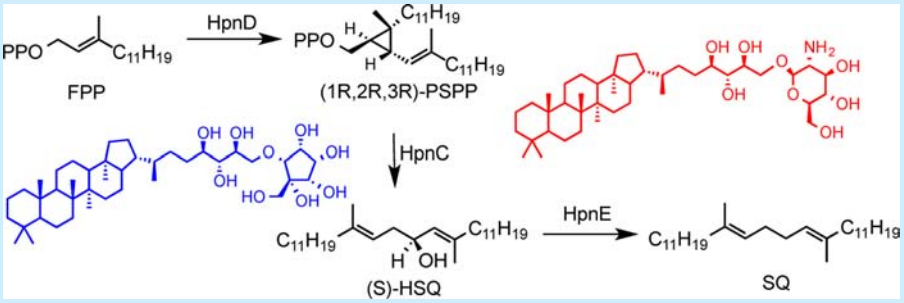
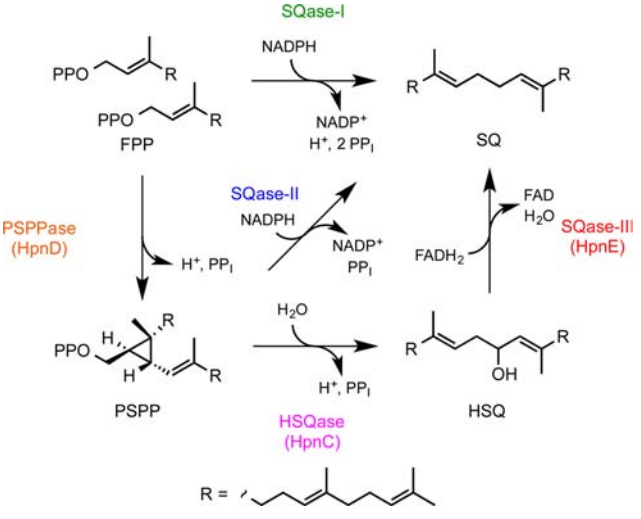
Supporting Information



ABSTRACT: Squalene (SQ) is a key intermediate in hopanoid biosynthesis. Many bacteria synthesize SQ from farnesyl diphosphate (FPP) in three steps: FPP to (1*R*,2*R*,3*R*)-presqualene diphosphate (PSPP), (1*R*,2*R*,3*R*)-PSPP to hydroxysqualene (HSQ), and HSQ to SQ. Chemical, biochemical, and spectroscopic methods were used to establish that HSQ synthase synthesizes (*S*)-HSQ. In contrast, eukaryotic squalene synthase catalyzes solvolysis of (1*R*,2*R*,3*R*)-PSPP to give (*R*)-HSQ. The bacterial enzyme that reduces HSQ to SQ does not accept (*R*)-HSQ as a substrate.

Squalene (SQ) is the first committed metabolite in the biosynthetic pathways for eukaryotic sterols and bacterial hopanoids. These polycyclic triterpenes are important molecules for maintaining the membrane rigidity and permeability and for formation of specialized lipid rafts.[1] In eukaryotes, the formation of SQ is typically catalyzed by a single enzyme: squalene synthase (SQase-I). The reaction proceeds in two distinct steps: condensation of two molecules of farnesyl diphosphate (FPP) to (1*R*,2*R*,3*R*)-presqualene diphosphate (PSPP), followed by rearrangement and reduction of (1*R*,2*R*,3*R*)-PSPP to give SQ.[2] Like other eukaryotes, the photosynthetic green algae *Botryococcus braunii* has a nicotinamide adenine dinucleotide phosphate (NADPH)-dependent SQase that converts FPP to SQ. In addition, the algae has a pair of enzymes, a PSPPase that catalyzes the condensation of FPP to PSPP and a NADPH-dependent SQase (SQase-II) that catalyzes the reduction and rearrangement of PSPP to SQ.[3] Recently, we discovered a new biosynthetic pathway for SQ in bacteria located in hopanoid biosynthesis clusters where FPP is converted to SQ in three steps by three enzymes.[4] The first enzyme, a PSPP synthase (HpnD, PSPPase), condenses two molecules of FPP to give (1*R*,2*R*,3*R*)-PSPP in analogy to the reaction seen in *B. braunii*. The second enzyme, a hydroxysqualene (HSQ) synthase (HpnC, HSQase), catalyzes solvolytic rearrangement of PSPP with quenching by water to give HSQ. The third enzyme, SQase-III (HpnE), catalyzes a flavin-dependent reduction of HSQ to SQ. The different pathways to SQ in eukaryotes and bacteria are shown in Scheme 1.

Scheme 1. Biosynthesis of Squalene in Bacteria and Eukaryotes



HSQ was first discovered when yeast SQase was incubated with FPP in the absence of NADPH.[5] Under these conditions, FPP was converted to (1*R*,2*R*,3*R*)-PSPP, followed by an enzyme catalyzed solvolysis reaction to give a mixture of products, including (*R*)-HSQ.[6,7] We now report that eukaryotic

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SQase-I and bacterial HSQase produce HSQ with opposite absolute configuration.

During experiments to establish the identity of HSQ as an intermediate in the bacterial pathway, we synthesized the alcohol using eukaryotic SQase-I or bacterial HpnC and HpnD. The regioselectivity for synthesis of HSQ by eukaryotic SQase-I was improved by adding dihydroNADPH ($NADPH_3$) to the incubation mixture.[7] In the experiments shown in Figure 1,

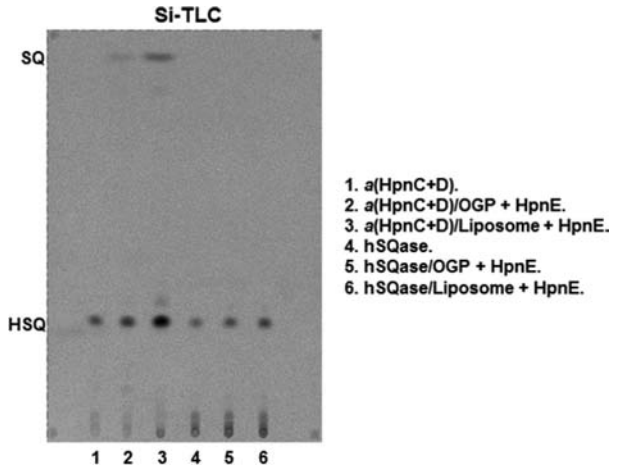

Figure 1. Radio-TLC on incubations of HSQ with *R. palustrius* HpnE. Lane 1: HSQ synthesized by *A. cellulolyticus* HpnC−D. Lane 2: acHSQ and rpHpnE with octyl-glucopyranoside (OGP). Lane 3: acHSQ and rpHpnE with POPC liposomes. Lane 4: HSQ synthesized by human SQase/$NADPH_3$. Lane 5: hHSQ and rpHpnE with octyl-glucopyranoside (OGP). Lane 6: hHSQ and rpHpnE with POPC liposomes.

[^{14}C]HSQ synthesized from [^{14}C]FPP using human SQase or *Acidothermus cellulolyticus* 11B HpnC−D was extracted from the incubation mixture with methyl *tert*-butyl ether (MTBE) and then incubated with *Rhodopseudomonas palustris* HpnE. Initial attempts to convert HSQ to SQ gave poor conversions, presumably because of the hydrophobicity of the substrate and the product. Although addition of octyl-glucopyranoside (OGP) gave some improvement, the best conversions to SQ were seen when HSQ from the HpnC−D incubation was incorporated into 1-palmitoyl-2-oleoyl-*sn*-3-phosphocholine (POPC) liposomes before incubation with HpnE. Related work with hydrophobic substrates for phytoene desaturase[8] and *cis*-carotene isomerase[9] showed substantial improvements in turnover when liposomes were included in the incubation buffer. Similar incubations with HpnE and (*R*)-HSQ synthesized by human SQase did not give SQ.

The results shown in Figures 1 and S9 suggest that HSQ synthesized from bacterial and eukaryotic enzymes have different absolute configurations. This hypothesis was verified by kinetic resolution of the enantiomers using the recently reported Competing Enantioselective Conversion (CEC) procedure, which is based on enantioselective acylation of alcohols using a chiral acylation catalyst, homobenzotetramisole (HBTM).[10] We found this method to have an exquisite level of sensitivity in our radio-TLC assays. [^{14}C]HSQ was synthesized from [^{14}C]FPP using HpnC−D from *Zymomonas mobiliz* (*zm*HSQ), *R. palustris* (*rp*HSQ), and *A. cellulolyticus* (*ac*HSQ) or SQase-I/$NADPH_3$[7] from *Saccharomyces cerevisiae* (*sc*HSQ) and *Homo sapiens* (*hs*HSQ). The latter two enzymes synthesize (*R*)-HSQ. The alcohol was extracted from the incubation mixtures with MTBE and acylated with propionic anhydride catalyzed by (*R*)- or (*S*)-HBTM. Identical acylations with the two enantiomers of HBTM were run to partial completion in parallel under a variety of conditions, portions of the mixtures were chromatographed in adjacent lanes on silica-TLC plates, and the amounts of HSQ and HSQ propionate were measured by autoradiography. The results are summarized in Table 1.

Table 1. HBTM-Catalyzed Acylation of HSQ by Propionic Anhydride[a]

			% conversion		
alcohol	*t* (°C)	time (h)	(*R*)-HBTM	(*S*)-HBTM	%*R*/%*S*
*zm*HSQ	22	2	27	17	1.6
	−20	22	85	72	1.2
	−20	15	87	67	1.3
	22	2	43	25	1.7
*rp*HSQ	22	2	27	17	1.6
	22	1	25	14	1.8
	−20	15	87	65	1.3
	−10	8	85	64	1.3
*ac*HSQ	−20	22	94	88	1.1
*sc*HSQ	22	2	16	18	0.89
	−20	15	53	58	0.91
	−20	15	45	58	0.78
*hs*HSQ	22	2	15	22	0.68
	−20	15	55	64	0.86

[a]Each *R*/*S* pair was run in parallel. Reaction conditions and radioTLC images are in the Supporting Information.

While the magnitudes of the kinetic resolutions were small, all of the bacterial HSQs were acylated faster by (*R*)-HBTM, while the eukaryotic (*R*)-HSQs were acylated faster by the (*S*) enantiomer of the catalyst. The small differences in the kinetic resolution are not surprising, considering the high similarity between the allylic and homoallylic substituents on the HSQ. First, it is known that the enantioselectivity tends to be low for allylic alcohols with similar amidine-based catalysts.[11] Second, HBTM has been shown to be effective in resolving both α- and β-aryl substituted alcohols,[12] indicating that the $\pi-\pi$/cation$-\pi$ interactions between the alcohol and HBTM are also present in the allylic and homoallylic substituents (as illustrated in Figure S10), and the interacting strengths are similar.[13] In addition, the long linear, and highly flexible substituents could further diminish the catalytic enantioselectivity.[12] Our ability to observe the small differences consistently throughout different experimental conditions clearly attests to the exquisite sensitivity of our radioactive assays. The mnemonic prediction for enantioselective acylation states that (*R*)-allylic alcohols are acylated faster than their (*S*)-enantiomers by (*S*)-HBTM.[10] This prediction correctly assigns the (*R*) configuration for *sc*HSQ[6] and *hs*HSQ and indicates that the bacterial HSQs have the opposite absolute configurations.

The absolute configuration for *zm*HSQ suggested by CEC analysis was verified by circular dichroic (CD) spectroscopy of its benzoate ester. UV and CD spectra for *zm*HSQ benzoate are shown in Figure 2. The UV spectrum is identical to that of a 91:9 (*R*/*S*) mixture of (*R*)-HSQ benzoate obtained from an incubation of *sc*SQase-I and FPP without NADPH.[14] Its CD spectrum, with peaks at 226 ($\Delta\varepsilon$, +3.6), 205 ($\Delta\varepsilon$, +9.3), and 192 ($\Delta\varepsilon$, −11.9) nm, is the mirror image of the spectrum for *sc*HSQ, with peaks at 226 ($\Delta\varepsilon$, −3.8), 205 ($\Delta\varepsilon$, −9.1), and 192 nm ($\Delta\varepsilon$, +8.0).[14] Thus, bacterial HSQases synthesize (*S*)-HSQ from (1*R*,2*R*,3*R*)-PSPP. The magnitudes of the Cotton effects

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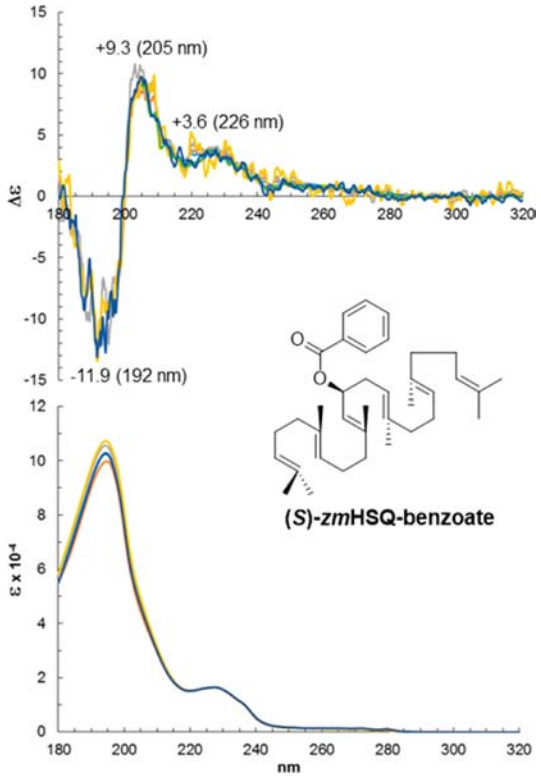
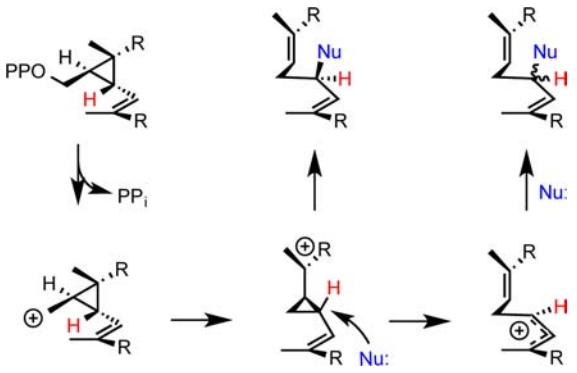


Figure 2. CD (top) and UV (bottom) spectra of *zm*HSQ-benzoate in hexane. The spectra were recorded in triplicate at concentrations of 0.66 (light blue), 0.34 (green), 0.25 (orange), 0.18 (dark blue), 0.13 (gray), and 0.08 mM (yellow). The averaged spectra were then normalized at 228 nm (ε = 16 400) for comparison.

measured at 192 and 205 nm for *zm*HSQ benzoate are slightly higher than those reported for *sc*HSQ benzoate, suggesting that the enantiomeric purity of the bacterial alcohol is >91:9.

The mechanism of the rearrangement/reduction of PSPP to SQ is shown in Scheme 2, where a hydride from NADPH adds

Scheme 2. Mechanisms for Conversion of PSPP to SQ and HSQ



to the final 3°-cyclopropylcarbinyl cationic intermediate with inversion at the cyclopropane carbon and concomitant opening of the cyclopropane ring.[15] While the stereochemistry of this step is undoubtedly controlled by the location of NADPH in the active site, addition of the nucleophile to the cyclopropane moiety with inversion is favored stereoelectronically.[16] Erosion of stereoselectivity in incubations without NADPH, when water is the nucleophile, can be attributed to a competing rearrangement, where the 3°-cyclopropylcarbinyl cation opens to an isomeric allylic carbocation with water adding to both faces of the allylic isomer.[17] The highly stereoselective synthesis of (*S*)-HSQ by the bacterial HSQases suggests that the reaction proceeds by the same cyclopropylcarbinyl−cyclopropylcarbinyl rearrangement catalyzed by eukaryotic SQase-I, followed by a cyclopropylcarbinyl to allylic rearrangement of the 3°-cyclopropylcarbinyl cation and stereoselective addition of water to the allylic cation to give (*S*)-HSQ, where the stereochemistry of water addition is controlled by HpnE.

The final reaction in bacterial SQ biosynthesis is the $FADH_2$-dependent reduction of (*S*)-HSQ to SQ. (*R*)-HSQ is not a substrate for the reductase. While there is no stereoelectronic "imperative" for addition of water to the allylic carbocation, it is reasonable that bacterial HSQases evolved the ability to position water in the active site to synthesize a single enantiomer in order to optimize the efficiency for utilization of HSQ. In this scenario, the preference for (*S*)-HSQ would have arisen from stereochemical demands of the reductase and the ability of an ancestral HSQase to initially provide a mixture of enantiomers from which the reductase could select the *S*-enantiomer.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.5b03546.

Experimental materials and methods, radio-TLC figures for CEC experiments (PDF)

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Notes

The authors declare no competing financial interest.

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