THE SQUALENE SYNTHETASE ACTIVE SITE. CATALYTIC ACCEPTANCE OF 7- AND 11-DEMETHYLFARNESYL PYROPHOSPHATES

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SUMMARY: The substrate analogues 7-demethylfarnesyl pyrophosphate and 3,7-dimethylundeca-2,6,10-trienyl pyrophosphate are catalytically accepted at both substrate sites of yeast squalene synthetase, resulting in formation of 6,19-didemethylsqualene and terminal tetrademethylsqualene, respectively. The first product is formed at approximately one fourth, and the second at about one tenth, the rate of normal squalene synthesis. These results establish that the internal methyl groups are not specifically required for catalysis, but probably contribute to general lipophilic binding of the hydrocarbon chain.

The mechanism and active site topology of squalene synthetase, the enzyme which catalyzes formation of squalene (2a) from two farnesyl pyrophosphate (1a) units, has recently been investigated in this (1-4) and other (5-8) laboratories using substrate analogues as experimental probes. One important conclusion derived from our studies is that the 3-methyl group in each substrate unit is specifically bound in a sterically constrained region, and that this interaction of the enzyme with the first substrate unit is absolutely required for catalysis (2,4). This conclusion derives from the enzymatic acceptance of 3-demethylfarnesyl pyrophosphate only as a very poor substitute for the second substrate unit, and from the complete catalytic rejection of the 3-ethyl analogue despite its demonstrated binding at the active site. These results suggest that other methyl groups on the substrate might facilitate catalysis through specific interactions with the enzyme. We report here a search for specific involvement of the 7 and 11 methyl groups with the aid of the 7-demethyl (1b) and terminal didemethyl (1c) substrate analogues.

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Materials and Methods

Synthesis of 3,11-Dimethyl-2E,6E,10-dodecatrienyl Pyrophosphate ("7-Demethyl", 1b). Wittig-Schlosser reaction (9,10) of 5-methyl-4-hexenyltriphenyl phosphonium iodide (11) and 6-(2'-tetrahydropyranyloxy)-4-methyl-4E-hexenal (11) gave in 80% yield the tetrahydropyranyl (THP) derivative of 7-demethyl farnesol. Removal of the protecting group with p-toluenesulfonic acid (5 mM) in methanol (2 hr, room temperature) gave 7-demethylfarnesol in 91% yield: ir (neat) 3325 (OH), 1670 and 965 cm^{-1} (trans disubstituted double bond); NMR ($CDCl_3$) 1.60 and 1.68 (two, s, 9H, allyl methyl), 1.92-2.18 (m, 8H, allyl methylene), 4.12 (d, 2H, J=7Hz, CH_2O), 5.12 (broad s, 1H, vinyl H), and 5.18-5.53 ppm (m, 3H, vinyl H); cims m/e 209 (parent + 1) and 191 (loss of H_2O, base peak). Oxidation with activated manganese dioxide and reduction of the resulting aldehyde with tritium labeled $LiAlH_4$ gave [1-^{3}H]-7-desmethylfarnesol. This alcohol was phosphorylated (3) to give 1b in 10% isolated yield (specific activity 9.3 mCi/mmol). The structure was confirmed by quantitative phosphorus analysis (96% of theoretical) and by regeneration with bacterial alkaline phosphatase of the starting alcohol (3).

Synthesis of 3,7-Dimethyl-2E,6E,10-undecatrienyl Pyrophosphate ("terminal didemethyl", 1c). Ethyl 3,7-dimethyl-2E,6E,10-undecatrienoate was prepared in 69% yield by Wittig reaction of the previously described aldehyde ethyl 3,7-dimethyl-10-oxo-2E,6E-decadienoate (12) with methyl-enetriphenylphosphorane. Reduction of the ester with $LiAlH_4$ gave 3,7-dimethyl-2E,6E,10-undecatrien-1-ol (75% yield): ir (neat) 3320 (OH), 1669, and 1645 cm^{-1} (c=c); NMR ($CDCl_3$) 1.57 and 1.62 (singlets, 6H, allyl methyls), 1.95-2.18 (m, 8H, allyl methylene), 3.50 (broad s, 1H, exch. in D_2O, OH), 4.03 (d, 2H, J = 7 Hz, CH_2O), 4.77 (m, 1H, vinyl H), and 4.88-5.83 ppm (m, 4H, vinyl H); cims m/e 195 (parent + 1), 177 (loss of H_2O, base peak). Pyrophosphorylation (3) gave 1c in 19% isolated yield, the structure of which was confirmed as before by quantitative phosphorus analysis (99.4% of theoretical) and by regeneration of starting alcohol on reaction with bacterial alkaline phosphatase (3). Substitution of tritiated $LiAlH_4$ in the reduction step furnished specifically labeled [1-^{3}H]-1c (specific activity 2.11 mCi/mmol).

Synthesis of Authentic Squalene Analogues. Terminal tetrademethyl squalene (5,9,14,18-tetramethyl-1,5,9,13,17,21-docosahexaene, 2c) and 6,19-didemethylsqualene (2b) were synthesized from 3,7-dimethyl-2E,6E,10-undecatrien-1-ol and 7-demethylfarnesol, respectively, by conversion of the alcohols to bromides with PBr_3, followed by copper catalyzed dimerization of each bromide by the procedure of Yamamoto et al. (13). The principal product in each case (60-70%) was the desired all-E squalene analogue.

Incubations. The yeast squalene synthetase preparation employed in this study has been described (3), as have general procedures for assay of the enzymatic incorporation of labeled substrate analogues into nonpolar products (4). Similar incubations were employed here (labeled substrate concentration 10-20 μM), except that unlabeled farnesyl pyrophosphate was not added except where specifically indicated in the text. Hydrocarbon products, isolated from large scale (50 ml) incubations as described (4), were purified by sequential chromatography, first on an 0.6 x 5 cm column of neutral alumina (5% diethylether in hexane), and then on a similar silica gel column.

Product Characterization. Purified products were analyzed by gas chromatography, with liquid scintillation counting of effluent fractions, and by gas chromatography-mass spectrometric comparison with authentic samples using previously described procedures (1,2,4).

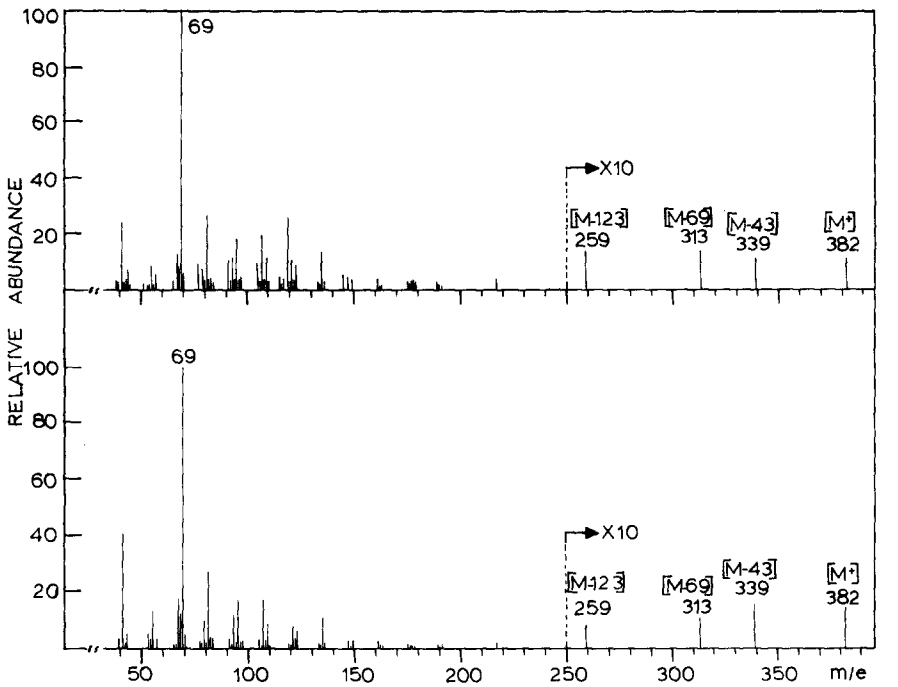


Figure 1. Electron impact mass spectra of biosynthetic (upper) and authentic (lower) 6,19-didemethylsqualene (2b).

Results and Discussion

Incubation of tritium labeled 1b or 1c with yeast squalene synthetase resulted in efficient incorporation of label into hydrocarbon products. Analysis of the hydrocarbon products obtained from 1b by gas chromatography revealed the presence of a single flame ionization detectable peak not also present in control incubations. This peak, associated with virtually all the radioactivity in the sample had a retention time identical with that of an authentic sample of 6,19-didemethylsqualene (2b). The identity of the biosynthetic product with 2b was confirmed by the close similarity between the mass spectra obtained from the enzymatic and synthetic samples (Figure 1).

Only one new peak was observed on gas chromatographic analysis of the hydrocarbon fraction from incubations of 1c. This peak, again

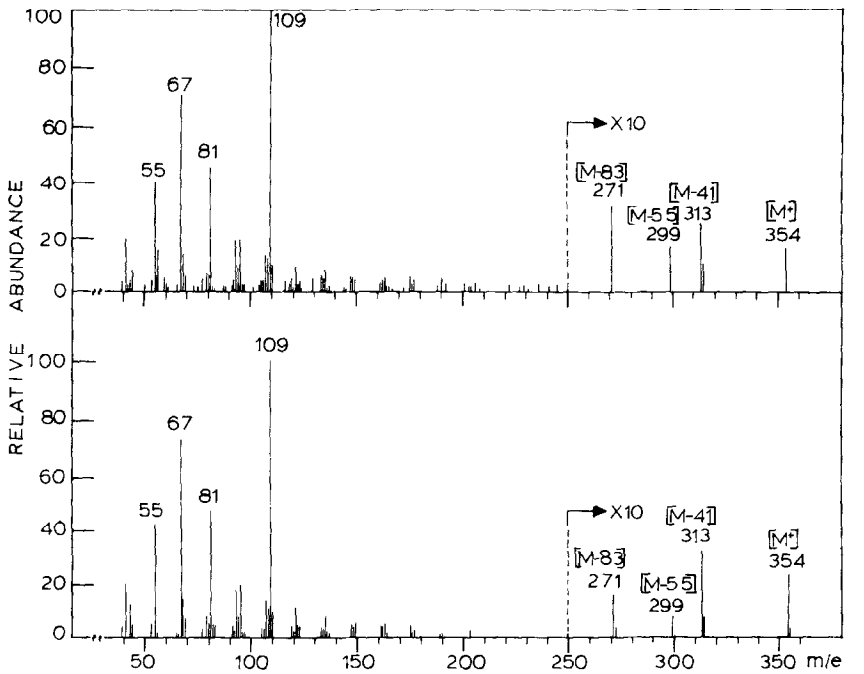


Figure 2. Electron impact mass spectra of biosynthetic (upper) and authentic (lower) tetrademethyl squalene analogue 2c.

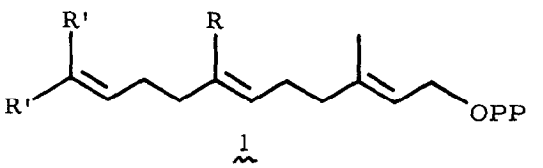
associated with most of the radioactivity detected by liquid scintillation counting of gas chromatographic effluent fractions, was firmly identified as the terminal tetrademethyl squalene analogue 2c by the identity of its retention time and mass spectrum (Figure 2) with those of an authentic sample. In this instance, about 5% of the gas chromatographic radioactivity emerged with a retention time similar to that of the asymmetric product expected from enzymatic cross condensation between endogenous farnesyl pyrophosphate (2, 4) and 1c, as established by correlation of the retention time with that of an authentic sample.

Formation of the symmetric condensation products 2b and 2c from 1b and 1c, respectively, clearly demonstrates that these substrate analogs

Table 1. Relative Rates of Hydrocarbon Product Formation

Substrate (10 μM)	Product	Rate (picomoles/mg protein/min)
1a	2a	200
1b	2b	46
1c	2c	2.1

are catalytically acceptable at both substrate sites of squalene synthetase.[1] A comparison of the rates of enzymatic hydrocarbon synthesis, summarized in Table I, shows that 2b is formed from 1b at about one fourth the rate of squalene synthesis from farnesyl pyrophosphate, while synthesis of 2c from 1c proceeds at about one tenth the rate of the normal reaction.



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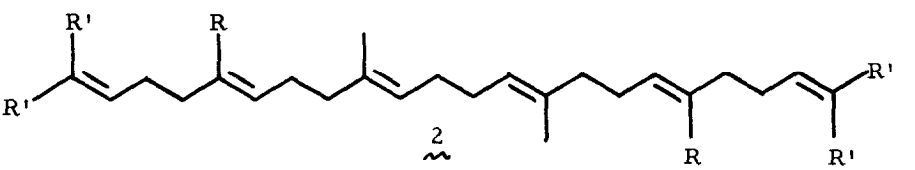
	R	R'
a)	Me	Me
b)	H	Me
c)	Me	H



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It is evident from the results that the internal methyl groups of farnesyl pyrophosphate are not specifically required for enzymatic catalysis, in contrast, with the 3-methyl group (2,4), although the rate of hydrocarbon formation is reduced in their absence. Since the conformation of the substrate is not likely to be altered by removal of the internal

[1] Dr. W. N. Washburn, University of California, Berkeley, has obtained similar results with the 7-demethyl analogue.

methyl groups, the decrease in reaction rate associated with their removal most probably reflects a general decrease in the lipophilicity of the hydrocarbon chain. The importance of such changes is impressively demonstrated by the extremely poor binding of geranyl pyrophosphate, a perfect "substrate analog", at the active site (3), and by the fact that this compound has never been observed to function as a substrate for the enzyme.

Acknowledgements

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