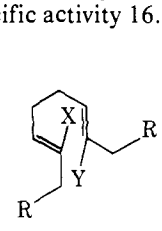
Squalene Biosynthesis. Role of the 3-Methyl Group in Farnesyl Pyrophosphate[1]

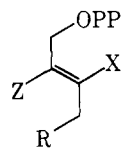
Sir:

The formation of squalene (**1a**) from two farnesyl pyrophosphates (**2a**) occurs in two distinct stages, separated by the isolable intermediate presqualene pyrophosphate (**3a**), both of which are catalyzed by the membrane-bound enzyme squalene synthetase.[2-4] The unit of **2a** which binds first to the enzyme loses its pyrophosphate moiety and a C_1 proton in the synthesis of **3a,** while the second farnesyl pyrophosphate is incorporated intact.[2,3,5] Conversion of **3a** to squalene results from pyrophosphate loss to give a carbonium ion, which, after multiple rearrangements, is neutralized by transfer of a hydride from NADPH.[3,6,23] We have recently reported that the substrate analogue 2-methylfarnesyl pyrophosphate (**2b**) is only accepted by the enzyme as a substitute for the unit of **2a** *not* undergoing proton exchange, a distinction thus being made between the two farnesyl binding sites.[7] We now show that 3-desmethylfarnesyl pyrophosphate (**2c**) also acts only as a cosubstrate, pointing to a critical role for the 3-methyl group of **2a** in the functioning of the enzyme.

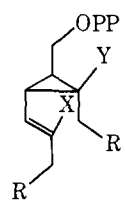
Pyrophosphorylation[8] of 3-desmethylfarnesol, prepared as previously described,[9] yielded **2c,** whose structure was confirmed by quantitative phosphorus analysis[10] and by regeneration of 3-desmethylfarnesol (as judged by TLC and GLC) on treatment with bacterial alkaline phosphatase.[11] Similar pyrophosphorylation of radiolabeled 3-desmethylfarnesol, obtained by oxidation to the aldehyde with activated MnO_2[12] followed by reduction with [^{3}H]$LiAlH_4$, gave [1-^{3}H]**2c** (specific activity 16.2 mCi/mmol).



1a, X = Me; Y = Me
b, X = Me; Y = H
c, X = H; Y = H



2a, X = Me; Z = H
b, X = Me; Z = Me
c, X = H Z = H



3a, X = Me; Y = Me
b, X = Me; Y = H

R = $(CH_3)_2C{=}CHCH_2CH_2(CH_3)C{=}CHCH_2$

Standard incubations of the analogue with an insoluble yeast enzyme preparation, as described before,[7] contained the following (concentration units): substrate(s), 20 μM; $MgCl_2$, 10 mM; NADPH, 1.6 mM; NH_4OH, 0.8 mM; protein 1.34 mg/ml; and potassium phosphate buffer (pH 7.5), 50 mM. Incubations of 1–100 ml volume (37°, 10–30 min) were terminated by addition of ethanol (2 ml/ml incubation), and the hydrocarbon products were extracted with petroleum ether. The extracts, after chromatography on neutral alumina, were either assayed by liquid scintillation counting or used for structural elucidation studies.

Incubation of [1-^{3}H]**2c** gave a radiolabeled hexane-extractable product which behaved like squalene on silica gel TLC and gave only one radioactive peak on gas chromatography (230 °C, retention time 6.11 min).[13] Incorporation of [1-^{3}H]**2c** into the hydrocarbon product[14] was absolutely dependent on the presence of NADPH and active enzyme, while it was stimulated approximately twofold by addition of an equimolar amount of unlabeled **2a** to the incubation mixture. The same product was formed, however, whether unlabeled **2a** was added or not.[15] The structure of this biosynthetic hydrocarbon was unambiguously established as all-E 10-desmethylsqualene (**1b**) by the fact that it had the same gas chromatographic retention time, and an identical mass spectral fragmentation pattern,[16] as an authentic sample prepared by synthesis.[17] The product to be expected from enzymatic self-condensation of **2c,** 10,15-didesmethylsqualene (**1c**), was also synthesized[20] and used to specifically establish the absence of any radioactive incubation product with the same GLC retention time (230 °C, 5.10 min).[13]

The formation of **1b** but not **1c** in incubations of **2c** shows that the analogue is catalytically acceptable in place of only one of the two farnesyl units incorporated into squalene. The farnesyl moiety that is replaced by **2c** can be identified, since only the first molecule of **2a** bound to the enzyme undergoes proton exchange during the condensation reaction.[2,3] Incubations essentially as above of equimolar amounts of [1-^{3}H]**2c** and unlabeled **2a** were carried out, except that part of the ethanol was distilled from the incubations after extraction of the hydrocarbons with petroleum ether.[2b] An aliquot of the alcohol distillates was assayed by liquid scintillation counting to determine the amount of tritium present,[2b] while the hydrocarbon product was measured as usual. Parallel control incubations were also carried out with ethanol inactivated enzyme. Essentially the same amount of label (6500 cpm) was found in the distillate from the normal and control incubations, whereas radiolabeled hydrocarbon (115 000 cpm) was only obtained in the normal incubation. Exclusive acceptance of [1-^{3}H]**2c** as a first substrate would be expected to release as much tritium into the medium as is found in the hydrocarbon product (115 000 cpm), since the substrate is racemically labeled and it has been shown that normal enzymatic proton removal occurs stereospecifically without internal isotope effects.[24] The fact that no enzymatic tritium loss is observed (same amount of label in control and normal incubations) and the sensitivity limits of the assay, indicate that no more than 0.2% (230 cpm) of the hydrocarbon product (if any) was formed with **2c** as a first substrate. Analogue **2c** is therefore acceptable only at the second binding site.

Discrimination by the enzyme against **2c** indicates that the 3-methyl group excised from **2a** *is essential for enzymatic squalene formation.* The methyl group may be involved in "anchoring" the substrate by specific binding, in electronic or steric stabilization of an intermediate, or in an as yet undetected chemical reaction. The first alternative seems the most likely, since there is no tritium release from **2c** and the enzyme is not irreversibly inhibited by the analogue.[22] These observations suggest that the 3-methyl group is essential prior to or during the first committed step in squalene synthesis, the irreversible loss of the pyrophosphate moiety from the first substrate unit.[3,5] The synthesis of **1b** from **2c,** by way of intermediate **3b,**[2,5] furthermore requires that the sequence of carbonium ion rearrangements leading from **1a** to squalene[23] not involve any species whose inherent stability is so altered by replacement of the methyl with hydrogen that normal reaction is frustrated.

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References and Notes

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(13) GLC was performed on a Varian Model 2100 (flame ionization detectors, N_2 carrier gas at 18 ml/min flow rate), fitted with glass 6 ft × 2 mm i.d. columns packed with 3% OV-225 on 100–200 mesh Varoport 30. At 230 °C, squalene had a retention time of 7.39 min.
(14) The amount of incorporation in a standard 1-ml, 10-min incubation varied from 0.25 to 1.0%, depending on the specific enzyme preparation.
(15) The enzyme preparation itself apparently contains low levels of **2a**, as well as squalene. The stimulation of the reaction by added **2a** is restricted by the fact that squalene synthetase is inhibited by high substrate (and substrate analogue) concentrations.[3]
(16) GLC–mass spectrometry was performed on an AEI MS-12 equipped with a Biemann-Watson molecular separator and an Infotronics Model 2400 gas chromatograph fitted with 6 ft × 0.25 mm i.d. columns. The stationary phase was 2% Dexsil 300 on 80–100 Chromosorb GHP. Mass spectrum of **1b** at 50 eV ionization potential, *m/e* (% relative intensity): 396 (1.6), 327 (0.8), 285 (0.9), 259 (0.6), 205 (0.7), 204 (0.9), 203 (1.4), 191 (2.0), 189 (2.1), 177 (1.7), 163 (2.2), 149 (5.0), 137 (10.0), 123 (9.0), 121 (9.0), 109 (10.2), 107 (8.8), 95 (20.0), 81 (56.0), 69 (100). The peaks at 396 (M^+), 327 ($M^+ - 69$), 285 ($M^+ - 43 - 68$), and 259 ($M^+ - 68 - 69$), highly characteristic fragmentations of squalene and its analogs, occur here 14 mass units lower than the corresponding peaks in squalene itself.[25]
(17) Condensation of 3-desmethylfarnesyl bromide[9a] with the sodium salt of 2-mercapto-2-thiazoline (THF, −78°) gave 2-(3-desmethylfarnesylthio)-2-thiazoline (85% yield).[18,19] Reaction of this intermediate with BuLi and farnesyl bromide (−78°, THF), followed by Raney nickel desulfuration (EtOH, 0°), gave **1b** as a mixture of isomers.[18,19] The desired all-E isomer was isolated by chromatography on basic alumina.[19] The stereochemical assignment was established by the presence of an infrared band at 10.3 μ (trans disubstituted double bond)[26] and by the highly preferential formation of a thiourea clathrate.[27]
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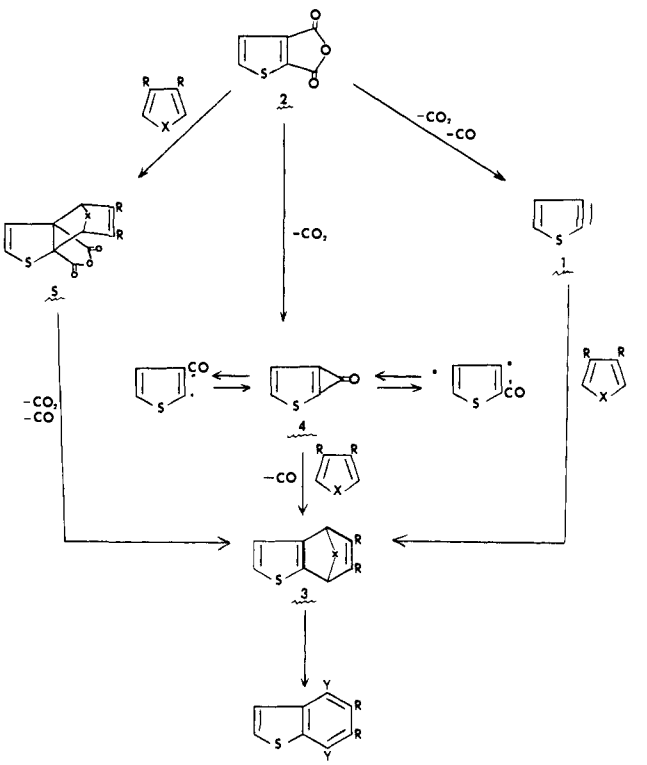
2,3-Thiophyne

Sir:

It is ironic that the existence of the very first type of aryne intermediate to be formulated,[1] the five-membered hetaryne, remains in doubt[2b,d] after 75 years. The many attempts to generate such species by methods successfully applied to the six-membered carbocyclic and heterocyclic analogues[2] either fail to give typical "aryne" products of cine-substitution and cycloaddition[2,3] or do so by demonstrated[4] nonaryne pathways. Although it has been claimed[2c] that this failure is due to "the prohibitive ring strain that would be associated with a dehydro bond in a five-membered heterocycle" the fact that cyclopentyne has been generated[5,6] suggests that this reason is insufficient. An additional factor is probably the ability of five-membered heterocycles to avoid aryne formation by undergoing unique reactions[4] unavailable to the electronically dissimilar[8] six-membered carbocyclic and heterocyclic analogues.



	X =	R =	Y =
A:	-S-	H	H
B:	-H,H-	CH_3	H
C:	$-CH_2CH_2-$	H	H
D:	-O-	H	H, OH
E:	$-CH_2-$	H	H, CH_3

A well-known method for generating arynes which might be expected to minimize this latter problem is the thermolytic elimination of CO and CO_2 from cyclic anhydrides.[9] Previous attempts to apply this method to five-membered hetarynes have led to either no reaction in the case of the anhydride of thiophene-3,4-dicarboxylic acid[3f] or a variety of nonaryne, oxygen-containing, condensation products from the anhydrides of the *N*-phenylpyrrole dicarboxylic acids.[3b] These results suggest that the probability of aryne formation would be optimized under high-temperature, vapor-phase conditions so that both CO and CO_2 are lost before any bimolecular reactions occur. The anticipated reactivity of the aryne[2c] and the probable instability of its dimerization products[10] under such conditions further dictates that a large excess of an aryne trapping agent be present. Accordingly, the flash vacuum thermolysis (FVT)[11] of the readily available[12] anhydride of thiophene-2,3-dicarboxylic acid (**2**) was carried out in the presence of several trapping agents as summarized in Table I.

The products from experiments A–E strongly suggest the intermediacy of an adduct **3** which aromatizes by desulfurization (A), dehydrogenation (B), a retro-Diels–Alder reaction (C), and carbon–oxygen (D) or carbon–carbon bond cleavage (E). Analogies for each of these processes in related systems are well known.[13] The cyclopentenothiopenes found in D are probably secondary reaction products of the thianaphthols analogous to the conversion of naphthols to indene.[14]

The most obvious origin of the adduct **3** would be a Diels–Alder reaction between the diene trap and the thiophyne **1.** Although such evidence ordinarily would be considered adequate to support claims for the generation of an aryne,[2] the known formation of thiophyne adducts via nonaryne mechanisms[4b] dictates that additional evidence or arguments be presented.

First of all, the only reports of the formation of "aryne ad-