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P. J. Krusic,* U. Klabunde
Contribution No. 2341
Central Research and Development Department
E. I. du Pont de Nemours and Company
Wilmington, Delaware 19898

C. P. Casey, T. F. Block
Department of Chemistry, University of Wisconsin
Madison, Wisconsin 53706
Received December 15, 1975

Squalene Synthetase. Differentiation between the Two Substrate Binding Sites by a Substrate Analogue[1]

Sir:

Squalene (**1a**) is synthesized biologically from two farnesyl pyrophosphate (**2a**) residues via intermediate **3a**.[2,3] Squalene synthetase, the membrane-bound enzyme which catalyzes this complex transformation in the presence of NADPH and Mg^{2+}, has recently been solubilized from yeast.[4,5] Kinetic studies indicate that, in forming **3a**, the pyrophosphate moiety of the farnesyl residue which is bound first to the enzyme is released before the second farnesyl unit is bound.[4] Little beyond this is known, however, about the enzyme mechanism or the active site topology.[2-4] We now report our studies with 2-methyl farnesyl pyrophosphate (**2b**), a substrate analogue, which for the first time demonstrates a difference in the tolerance to substrate variation of the two farnesyl pyrophosphate binding sites.[6]

Wittig condensation of geranyl acetone with diethyl-1-carboethoxyethyl phosphonate yielded ethyl 2-methyl-farnesoate (84%, *E*:*Z* ratio by GLC 54:46).[7,8] The *E,E* isomer, isolated by spinning-band distillation, was reduced with $LiAlH_4$, and the alcohol was pyrophosphorylated to give **2b**.[8,9] The structure of **2b** was confirmed by quantitative phosphorus analysis[10] and by regeneration of 2-methyl farnesol on treatment with bacterial alkaline phosphatase.[11] Substitution of ^3H-$LiAlH_4$ into the synthetic sequence gave [1-^{3}H]**2b** (sp act. 0.5 mCi/mmol).

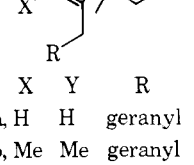
Standard incubations in this study contained the following (concentration units): substrate(s), 20 μM; $MgCl_2$, 10 mM, NADPH, 1.6 mM; NH_4OH, 0.8 mM; protein, 1.34 mg/ml;[12] and potassium phosphate buffer (pH 7.5), 50 mM. Incubations (37°, 1-10 min) were terminated with ethanol and the hydrocarbon products, extracted with petroleum ether, were chromatographed on silica gel or neutral alumina before liquid scintillation counting or structural studies.

Incubation of [^{3}H]**2b** gave a radiolabeled product which behaved like squalene on silica gel TLC (hexane, R_f 0.37). Its formation was absolutely dependent on the presence of NADPH and active enzyme, while it was stimulated by addition of unlabeled **2a** to the incubation. GLC analysis showed approximately equal amounts of radioactivity associated with two peaks, one coincident with endogenous squalene (system A, 232 °C, 7.6 min; system B, 260 °C, 14.8 min) and the other at longer retention time (system A, 232 °C, 10.0 min; system B, 260 °C, 19.35 min).[13] Both peaks were formed whether the incubation was supplemented with unlabeled **2a** or not. Control experiments showed that squalene, and probably **2a**, were present in small amounts in the enzyme preparation.

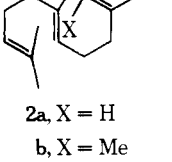
Unambiguous chemical routes were used to prepare the all-*E* isomers of 11,14-dimethylsqualene (**1b**)[14] and 11-methylsqualene (**1c**).[17] The GLC retention time of authentic **1b** (system A, 232 °C, 13.3 min) did not correspond to either labeled peak in the biosynthetic sample, while authentic **1c** had the same retention time as the slower radioactive peak. The identity of this second GLC peak as **1c** was firmly established by GLC–mass spectrometry,[20] since the fragmentation patterns of both samples were identical.[21]

Monomethylsqualene (**1c**) can be formed by distinct pathways in which **2b** replaces **2a** in either the first or second catalytic step. These two alternatives are distinguishable, since a proton is exchanged on the first but not the second farnesyl residue.[2,4] Deuterated **1c**, prepared by incubation of approximately equimolar amounts of 1-dideuterio-**2a**[22] and unlabeled **2b**, was analyzed by GLC–mass spectrometry.[20] The parent ion and all fragment ions retaining the central carbons were shifted upward by one (not two) mass units relative to the corresponding peaks in the spectrum of unlabeled **1c**.[21] This implies that only **2a** is acceptable at the first site and, therefore, **2b** can only replace the second farnesyl moiety.

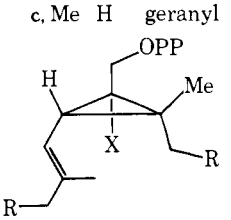
The inability of **2b** to function as a first substrate in synthesis of **1c** can result from failure to form a Michaelis complex, or, if bound, failure to meet catalytic requirements. To investigate if **2b** is bound at the first substrate site, this analogue was evaluated as an inhibitor of squalene biosynthesis from [1-^{3}H]**2a** (sp act. 6 mCi/mmol).[22] Standard 2-min incubations were carried out in which the concentration of [1-^{3}H]**2a** was varied from 1.25 to 5.00 μM in



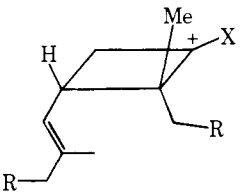
X Y R
1a, H H geranyl
b, Me Me geranyl
c, Me H geranyl



2a, X = H
b, X = Me



3a, X = H; R = geranyl
b, X = Me; R = geranyl



4a, X = H; R = geranyl
b, X = Me; R = geranyl

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

the presence of either 0, 1, or 3 μM concentrations of **2b**. The results, analyzed as Lineweaver-Burk plots, indicate that **2b** is a strongly bound ($K_I = 0.5$ μM), strictly competitive inhibitor of squalene synthesis.[23] This result is compatible with binding of **2b** at the first site, although it is not unequivocal proof, since competitive inhibition can also be explained by exclusive binding of **2b** at the second site when it is much poorer as a cosubstrate than **2a**.

The enzymatic formation of **1c**, with **2b** replacing the second farnesyl residue, requires that presqualene analogue **3b** also be a substrate.[3] This is somewhat surprising, because the extra methyl introduces a strong perturbation, both steric and electronic, into the postulated rearrangements which normally convert **3a** into **1a**.[24] The stability of cation **4a**, for example, a possible transient species in squalene formation,[24] is enhanced by methyl substitution (**4b**). It may be that the still unidentified radioactive product with the retention time of squalene results from abnormal drainage of some such intermediate into a chemical pathway made competitive by methyl substitution. Investigation of this point, as well as related studies with other substrate analogues, are continuing in our laboratory.

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(13) GLC on a Varian 2100 with flame ionization detectors, 6 ft × 2 mm i.d. glass columns, N_2 carrier gas (18 ml/min): system A, 3% OV-225 on 100–200 mesh Varoport 30; system B, 2.5% Dexyl 300 on 80–100 Chromosorb GHP.
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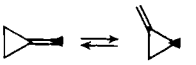
Paul R. Ortiz de Montellano,* Rafael Castillo[25]
Wayne Vinson, Jeng Shu Wei
Department of Pharmaceutical Chemistry
School of Pharmacy, University of California
San Francisco, California 94143
Received January 12, 1976

Maintenance of Chirality in a Photochemical Methylenecyclopropane Rearrangement

Sir:

Although there exists extensive literature related to the thermally induced automerization of methylenecyclopropanes (eq 1),[1] there is a notable paucity of data regarding the corresponding photochemically promoted process.



(1)

Kende et al.[2] as well as ourselves[3] have observed that in addition to suffering photofragmentation, aryl- or alkylmethylenecyclopropanes rearrange photochemically. Furthermore, the former investigators concluded that a singlet excited state is responsible for the reaction because the process could be neither triplet sensitized nor quenched by triplet acceptors.

Even though further mechanistic details for the photochemical rearrangement are lacking, it is already clear that the automerization of methylenecyclopropanes affords the rare opportunity to study a hydrocarbon reaction that can be initiated both thermally and photochemically. As one part of a program designed to exploit this potential, we have investigated the stereochemical fate upon rearrangement of an optically active methylenecyclopropane activated in these two significantly different ways.

Synthesis of a suitable methylenecyclopropane,[4] 2-phenyl-3-methylmethylenecyclopropane (**1**), is outlined in Scheme I; all transformations, save for the final one, proceeded in an unexceptional fashion.[6] The strictly cis stereochemistry of the phenyl and methyl groups, which had been maintained up to this point,[6,7] was lost owing to the lability of the benzylic hydrogen of the methylenecyclopropane in the basic medium in which the elimination was performed.[8,9] Although it was not possible to effect the separation of the two isomers by GLC techniques, the mixture exhibited a rotation, $[\alpha]^{20}D$ +2.0°,[10] of sufficient magnitude for our purposes. It is to be noted that the base-catalyzed geometric isomerization has the effect of racemizing one of the two chiral centers in **1** and, because the asymmetry of the second center is lost during the expected methylenecyclopropane rearrangement (vide infra), a cis to trans ratio in **1** of 1:1 would have proven disastrous to our goals; fortunately, the trans isomer predominates by a factor of about three over the cis in the isolated mixture.

Thermolysis of the optically active mixture of *cis*- and

Scheme I. Synthesis of a Chiral Methylenecyclopropane