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MECHANISM OF THE BIOSYNTHESIS OF SQUALENE FROM FARNESYL PYROPHOSPHATE

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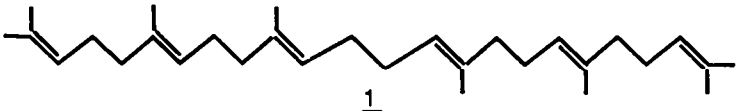
Abstract: The biosynthesis of squalene (1) from farnesyl pyrophosphate (2) has been studied by carrying out calculations for models. The suggested mechanism involves initial allylic attack on 2 by the enzyme, probably by the trapping of farnesyl cation, followed by S_N2' reaction with a second molecule of 2 to form a π complex which is deprotonated to presqualene pyrophosphate (3). Ionization of 3, followed by cyclopropylcarbinyl rearrangement and hydride reduction, gives 1. The rearrangement does not involve a cyclobutyl cation (bicyclobutanium ion) as an intermediate.

Introduction

An important precursor in cholesterol biosynthesis is squalene (1).



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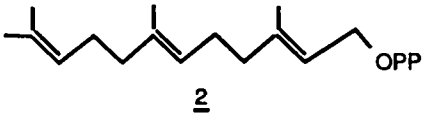
The biosynthesis of the symmetric molecule 1 occurs by an asymmetric process. The first stage in the enzyme catalyzed reaction is the condensation of two molecules of farnesyl pyrophosphate (2) to form presqualene pyrophosphate (3). In this stage, one of the two reacting



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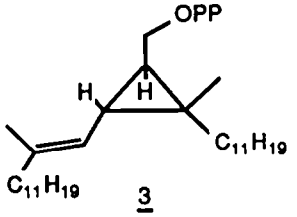


3

molecules loses a proton stereospecifically[1] at carbon 1. In the next stage, 3 ionizes, rearranges, and is reduced by a reductive pyridine nucleotide to produce 1. This interesting transformation has been the subject of much experimental research[2] and a variety of mechanisms have been proposed for each of the two stages in the biosynthesis of 1. Mechanisms of enzyme reactions are difficult to determine by experiment. In the case of squalene synthetase, the problem is compounded by lack of information concerning the structure of the enzyme, it being only recently partially purified[3].

The mechanisms that have been suggested are open to criticism, either on the basis of experiment or because they involve the postulation of reactions for which there are no analogies. Criticisms of this kind have already been directed[2b] at some[4-9] of the mechanisms that have been proposed for the formation of 3 from 2. A third (Figure 1a), proposed by Altman et al.[10], is open to similar objections. In the first place, no case seems to have been reported where an olefin acts as the nucleophile in an S_N2 reaction; see Figure 1a. Secondly, if the initial species were in fact a free carbenium ion, the

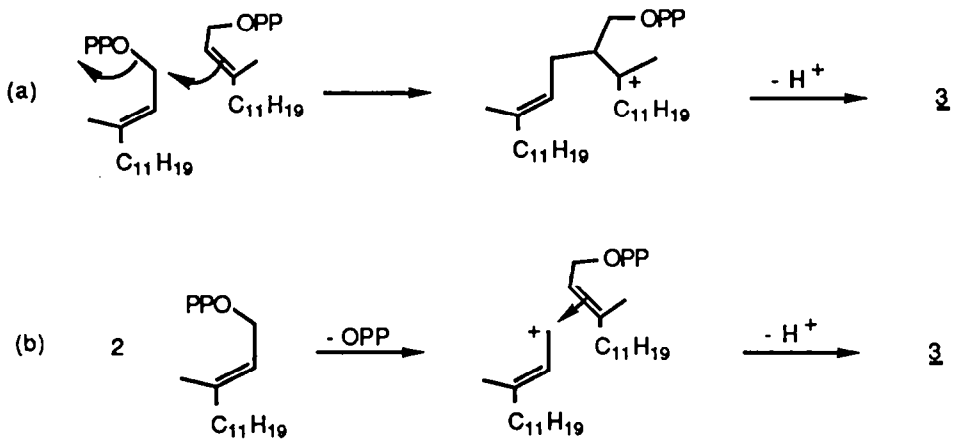


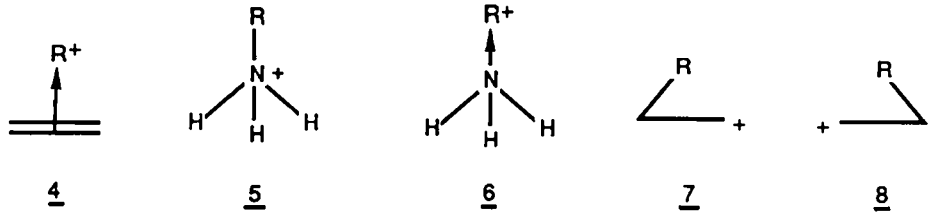
Figure 1. Formation of Presqualene Pyrophosphate (3) (a) via a tertiary cation; (b) via a π complex.

retention of regiospecificity about the resulting cyclopropane ring would require special explanation. Thirdly, there seems to be no analogy for the formation of a cyclopropane by cyclization of a tertiary carbenium ion.

Here we present simple mechanisms for the formation of 1 and 3 based upon π complex theory and supported by theoretical calculations. The possibility that a "corner protonated" species, i.e. a π complex, might be an intermediate (see Figure 1b) has been suggested previously[2b] but for no reason other than simplicity.

Although the π complex theory was introduced long ago[11], its ability to describe the chemistry of "nonclassical" carbocations has been largely ignored. The majority of such ions can be regarded, and are best regarded, as π complexes and it has become clear in recent years that such species are involved as intermediates in many reactions involving carbocations[12,13,14].

A π complex is formed when a filled π MO from a CC double bond interacts with an empty valence orbital of another atom, forming a dative bond. An alkyl cation (R^+), having a high electron affinity, can couple with an olefin to form such a complex (4). This is similar to the addition of R^+ to an amine to form an ammonium ion (5), as becomes apparent when it is written in the equivalent notation (6).



4 5 6 7 8

Such a π complex can be just as stable as, or even more stable than, its classical isomers (7 and 8). In 4, two CC single bonds are replaced with a CC double bond and a dative bond between the π MO and

R^+. The energy of the dative bond need not be large to make 4 more stable than 7 or 8, since the bond energy of the double bond is only ~20 kcal/mol less than the sum of the two single bond energies. The three-center dative bond in 4 is qualitatively the same as a two-center covalent bond, the only difference being that in 4 the interaction involves a π MO instead of an AO. Given that even the weakest covalent bond (F-F) has a bond energy greater than ~30 kcal/mol, 4 should be at least as stable as its isomers 7 and 8. This has been borne out by calculations[15-21] and experiments[22] done on simple carbocations.

Nonclassical cations such as π complexes, are usually not included in mechanistic schemes because of the lesser role they play in solution. It is thought that the classical isomers are energetically more stable in solution relative to their nonclassical isomers because of differences in solvation. In the classical ions, the charge is more localized and so the ions can be better solvated, whereas in the nonclassical ions, the charge is more diffuse and thus the ion is solvated less effectively.

In enzyme reactions, however, this is not the case. Recently, an explanation for the effectiveness of enzymes as catalysts has been attributed to the absence of solvent from the enzyme active site[23]. When the proper substrates are adsorbed, they exclude all solvent molecules, so the subsequent reactions take place in the same way that they would in the gas phase. It is well known that reactions often behave differently in the gas phase and in solution. The effectiveness of enzymes as catalysts can be explained in this way[23]. In the present case, since there is no solvent in the enzyme site to stabilize any ions present, π complexes will be favored over the classical carbocations (see above). Indeed, proper positioning of charge stabilizing groups in the active site may stabilize the π complexes even further relative to their classical isomers.

Because enzyme reactions occur *as if* in the gas phase, their mechanisms can be probed effectively by quantum mechanical calculations since these refer to isolated molecules in the absence of solvent. Calculations using MINDO/3[24] are especially useful in dealing with carbocations, both classical and nonclassical. The results are similar to those from "state of the art" *ab initio* methods and likewise consistent with the available experimental evidence[6,17,18,19,25], and MINDO/3 can be used to study large molecules properly at reasonable cost in computing time.

Procedure

The calculations were carried out using the standard MINDO/3[24] and MNDO[26] procedures as implemented in the AMPAC program[27]. All geometries, except those of transition states, were found by minimizing the energy with respect to all parameters using the Davidon-Fletcher-Powell algorithm[28]. Transition states were located by the reaction coordinate method[29], refined by minimizing the norm of the gradient[30], and characterized by calculating force constants[30].

Results and Discussion

The route used by nature to convert 2 to 1 seems devious by current organic standards. In the laboratory, reactions of this kind are normally carried out using organometallic reagents or by coupling

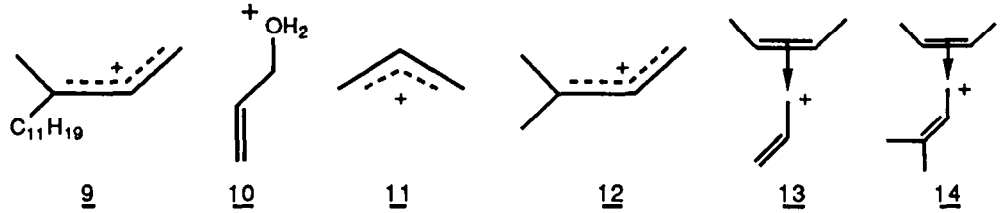
of radicals, expedients that are inapplicable in biological systems. Biological reactions of this kind are usually effected by formal electrophilic addition of carbenium ions to carbon-carbon double bonds and the conversion of 2 to 3 follows this pattern, formally involving addition of farnesyl cation (9) to the terminal double bond of a molecule of 2. A molecular rearrangement is then required to convert the branched carbon chain in the adduct to the linear one in 1.

A. Conversion of Farnesyl Pyrophosphate (2) to Presqualene Pyrophosphate (3). The first question concerns the manner in which the original coupling of two molecules of 2 is carried out. Does it take place by an S_N2 reaction, the double bond of the first farnesyl moiety acting as the nucleophile (*cf.* Figure 1), or by an S_N1 reaction, involving ionization of 2 to 9 ?

We studied the feasibility of such an S_N2 reaction by examining the corresponding reactions of ethylene with allyloxonium ion (10). Since H_2O is a better leaving group than pyrophosphate, this reaction should take place at least as easily as that of 2 with itself. The activation energy calculated for this model reaction by MINDO/3 was, however, greater than 30 kcal/mol. The S_N2 route therefore seems to be eliminated. This result is not surprizing because S_N2 reactions are rare in biochemistry, being autoactivated[31] processes with relatively large activation barriers. The barrier to an analogous enzymatic process should be equally large[23].

We will therefore start by assuming the obvious alternative, i.e. an S_N1 reaction involving ionization of 2 to 9 followed by addition of 9 to the relevant C=C bond in the second molecule of 2. According to π complex theory, the resulting adduct should be a π complex (Figure 1b), deprotonation of which should give 3.

To probe the feasibility of this mechanism, we studied the adducts from allyl (11) and 1,1-dimethylallyl (12) cations with cis-2-butene to model the formation of 3. The results are listed in Table I. In both cases, the π complex adducts, 13 and 14, were very close in energy to the acyclic cations, 15 and 16, the former being slightly higher in energy. Indeed, 13 and 14 were found to be transition states rather than minima. Comparison with *ab initio* results[17,32,33] does suggest , however, that MINDO/3 underestimates the stabilities of π complexes relative to the isomeric classical carbenium ions while overestimating those of analogous edge-protonated cyclopropanes. There is therefore good reason to believe that the initial product from an olefin and a carbenium ion is in general a π complex and that this is true in any case for the adduct from 9 and 2. It also seems likely that 13 and 14 are in fact more stable than their classical counterparts.

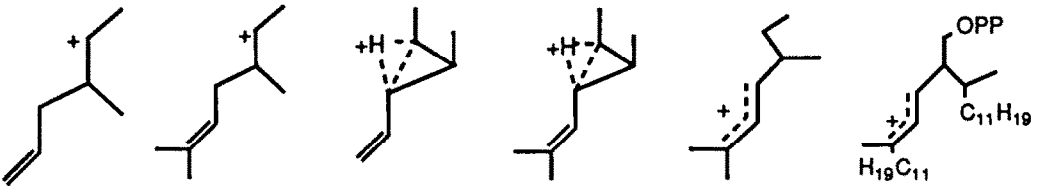


9 10 11 12 13 14

As a further check, we examined the alternative mechanism proposed by Altman et al[10] (Figure 1a) where the initial adduct is a carbenium ion. Cyclization of 15 gave the edge-protonated cyclopropane 17. Attempts to cyclize 16 failed, however, to give the corresponding

edge-protonated product 18. Indeed, 18 could not be found as a stationary point on the C_9H_{17} potential energy surface. The product from the attempted cyclization was instead the substituted allyl cation 19, the added methyl groups favoring its formation.

The situation in the case of the ion from 2 should be similar. Here, because it is a tertiary carbenium ion, its cyclization should be more hindered. The ease of rearrangement of its corresponding edge-protonated cyclopropane, however, should not be significantly altered. The latter contains a localized three-electron two-center (CHC) bond whose strength should not be significantly affected by an additional alkyl substituent. Since MINDO/3 is moreover likely to have overestimated the stability of the edge-protonated species 18, it seems likely that isomerization of the ion from 2 by interaction of the cationic center with the relevant methylene would lead by direct proton transfer to the allyl cation (20) which could not be converted to 3.



15 16 17 18 19 20

Our calculations do suggest that the initial π complex from 9 and 2 will be relatively unstable, also undergoing rearrangement to 20 very easily. The conversion to 3 requires deprotonation to be fast compared with rearrangement. Indeed, since rotation of the apical group in a π complex involves only a small barrier, the fact that deprotonation is stereospecific, only *one* of the methylene protons being lost, requires deprotonation to be very fast. This suggests that there must be a base in the active site of the enzyme appropriately placed to deprotonate the π complex stereospecifically. Note that transfer of the proton to a base of adequate strength is expected to be very rapid since only a small change in geometry is involved. The formation of cyclopropanes by deprotonation of π complexes has ample precedent[12,13,14].

Table I. MINDO/3 Heats of Formation (ΔH_f) of Presqualene Model Compounds.

Compound	ΔH_f (kcal/mol)
9	222.0
10	189.3
11	196.7
12	180.4
13	194.8
14	178.7
15	187.8
17	157.7

The suggestion that 9 is an intermediate in the conversion of 2 to 3 presents problems. If 2 can ionize to 9 in the enzyme, why does it not do so in aqueous solution, given that water is an excellent ionizing medium? The stability of 2 in water is not hard to explain. The OPP group, being already doubly negatively charged, would be expected to resist acquiring additional negative charge. It should therefore be a very poor leaving group. As Figure 2a shows, ionization leads to two additional repulsions between neighboring negatively charged oxygen atoms whereas the additional attractions between the farnesyl cation and oxygen anions will be decreased by the polar medium (water). Why then should 2 ionize when adsorbed in the active site of the enzyme?

It is known[7] that squalene synthetase can function only in the presence of a divalent cation (Mg^{2+} or Mn^{2+}), this presumably being adsorbed in the active site and used to bind 2. As Figure 2b shows, ionization of 2, when adsorbed in this way, should lead to a strong attractive interaction between the metal cation and the new anionic center. The absence of intervening water will moreover strengthen this stabilizing interaction. It is therefore quite reasonable that 2 should undergo rapid ionization on the enzyme while remaining stable in water.

Now, however, another problem emerges. If 2 ionizes easily under the influence of the metal cation, why does it not do so as soon as 2 is adsorbed, before adsorption of the second molecule of 2 ? If this happened, the resulting cation would react with water to form farnesol. There seem to be only two reasonable explanations. One would require the *second* molecule of 2 to be adsorbed first. This is certainly possible. The other involves an interesting possible alternative, i.e. that 9 is not formed as a free ion but is trapped immediately by some nucleophilic group in the active site.

Sulfur inhibition studies[7] have indeed shown that a sulfur-containing group (SCG) in the active site plays a vital role in the conversion of 2 to 3 and 1. It has been suggested[7] that the SCG reacts with 2 to form a farnesyl derivative of the enzyme, which in turn undergoes an S_N2 reaction with the second molecule of 2; see Figure 3a. The first step could involve an S_N1 reaction, 9 being formed and reacting with the sulfur. The second step must, however, be of S_N2 type because the whole object is to trap 9 in a form that cannot react prematurely with water before the second molecule of 2 has been adsorbed. The arguments and calculations discussed above make it very unlikely that such an S_N2 process can be involved in the conversion of 2 to the π complex 21.

The essential features of this mechanism are, however, retained in a seemingly feasible alternative involving a formal S_N2' displacement of pyrophosphate from 2 by the sulfur-containing group in the enzyme. A second S_N2' reaction on the resulting nerolidyl derivative of the enzyme and the second molecule of 2 could then lead to 21; see Figure 3b. The first step could be of S_N1 type, involving immediate trapping of 9. However, the second must be a genuine S_N2' reaction to account for the fact that no reaction occurs until the second molecule of 2 is in place.

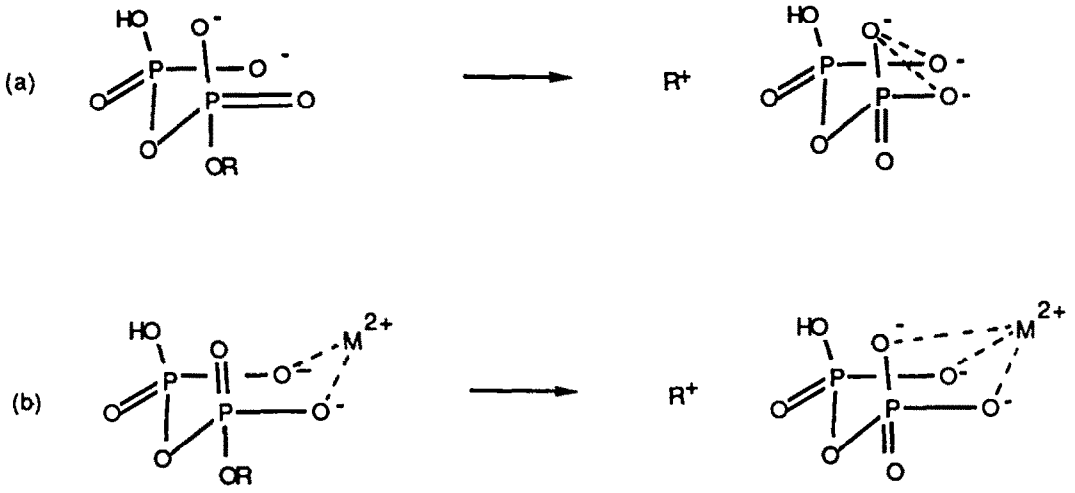


Figure 2. (a) Unfavorable repulsions produced by ionization of pyrophosphate ion. (b) Enhancement of ionization by an electrostatic interaction with a metal cation.

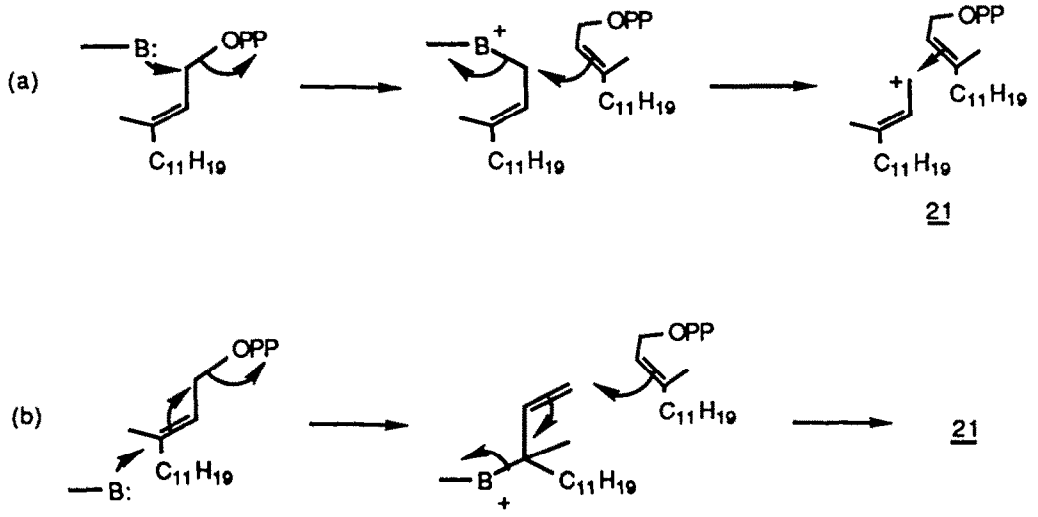


Figure 3. Formation of the Presqualene π complex (21) via (a) an S_N2 reaction displacing a sulfur containing group of the enzyme; and (b) an S_N2' reaction displacing a sulfur containing group of the enzyme.

The situation concerning S_N2' reactions is still unclear. In solution, such processes are normally much slower than their S_N2 counterparts. MNDO[26] calculations[34] have, however, indicated that in the reaction of chloride ion with allyl chloride in the gas phase, the S_N2' process should be much the faster. The slowness of such S_N2' reactions in solution can be reasonably attributed to the greater energy needed to desolvate the anionic reagent, given that it has to approach the nonpolar end of the substrate. It is interesting to note that most of the facile S_N2' reactions that have been reported in solution have involved neutral nucleophiles.

It seems clear[34,35] that the activation barriers in S_N2 reactions are essentially steric in origin, the small size of the carbon atom making it difficult to pack five groups round it in the corresponding TS. Anions are predicted[32] to add exothermically and without activation to silyl halides to form analogous pentacovalent adducts, silicon being large enough to form the latter without excessive strain. Anionic addition to carbonyl, a reaction formally analogous to a corresponding S_N2 displacement, is also predicted[31,36] to take place exothermically and without activation. Here the central carbon atom is linked only to four groups in the adduct. Since the latter situation also holds for the S_N2' reaction, steric strain should play only a minor role in it. The prediction[34], that S_N2' reactions should be more facile than S_N2 ones, therefore seems very reasonable. While *ab initio* calculations[37] have led to the opposite conclusion for the reaction of chloride ion with allyl chloride, these were not carried out at a high enough level to be in any way definitive. Indeed, tests[38] have shown that MNDO is at least comparable in reliability and accuracy with the procedures used.

As a check, we carried out MINDO/3 and MNDO calculations for the S_N2 and S_N2' reactions of ammonia with allyl dimethylsulfonium ion (22), dimethylsulfide serving as a model of the sulfur-containing group in the enzyme;

The MNDO calculations were included to insure that the results were not unique to MINDO/3. The results are shown in Tables II and III. Both methods give similar estimates for the activation energy of the S_N2' reaction and both predict the S_N2 activation energy to be greater. Indeed, no S_N2 TS could be found using MNDO, presumably because of the known tendency of MNDO to overestimate nonbonded repulsions[39]. On the other hand, the difference (6 kcal/mol) given by MINDO/3 may well be too small because MINDO/3 tends to underestimate activation energies for S_N2 reactions.

MINDO/3 calculations were also carried out for the analogous reactions of ethylene with 22; see Table III. Here again the S_N2' reaction was predicted to be the more facile. While the calculated activation energy was rather large, that for the analogous reaction of 2 should be much smaller because of the electron-releasing effect of the substituents, in particular the negatively charged $-CH_2OPP$ group.

Table II. Heats of Formation (ΔH_f) of S_N2 and S_N2' Reactants and Products

Compound	MINDO/3[a]	MNDO[a]
22	164.9	178.1
NH_3	-9.1	-6.3
$CH_2=CH_2$	19.2	15.3
$S(CH_3)_2$	-11.6	-17.1
$CH_2=CHCH_2NH_3^+$	161.3	177.4

[a] ΔH_f (kcal/mol)

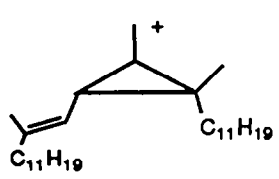
TABLE III. Heats of Reaction (ΔH) and Activation ($\Delta H^{\ddagger}$) for S_N2 and S_N2' Reactions[a]

		$\Delta H^{\ddagger}$			ΔH
Reactants	Method	S_N2	S_N2' (anti)	S_N2' (syn)	
(22) + NH_3	MINDO/3	15.5	9.1	9.2	-6.1[b]
	MNDO	-	11.0	-	-11.5
(22) + $CH_2=CH_2$	MINDO/3	33.3	23.6		

[a] Enthalpies are in kcal/mol. [b] This is the energy of the isolated reactants going to isolated products. In the S_N2 reaction, a charge-dipole complex is formed first (ΔH=-13.1 kcal/mol).

The substituents in 2 would equally hinder S_N2' attack by the sulfur containing group in the enzyme. The alternative mechanism indicated above, involving an initial S_N1 reaction of 2 with the enzyme to form a nerolidyl derivative which then undergoes a S_N2' reaction with the second molecule of 2, thus seems entirely reasonable.

B. Conversion of Presqualene Pyrophosphate to Squalene. Two mechanisms have been proposed for the formation of 1 from 3. Both involve the ionization of 3 to a primary cyclopropylcarbinyl cation (23), followed by rearrangement to an isomeric allyl cation (24) and hydride reduction. In the first[6], 23 rearranges to a tertiary cyclopropyl carbinyl cation (25) which undergoes ring opening to 24. In the second[8], the intermediate is a cyclobutyl cation (26) rather than an isomeric cyclopropylcarbinyl cation.



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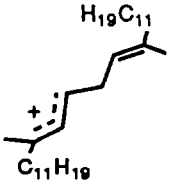

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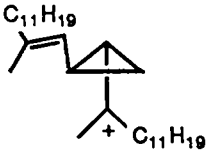

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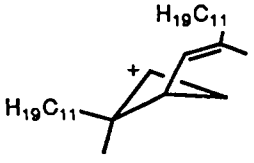

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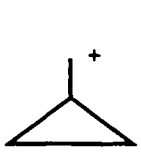
All information concerning the feasibility of these mechanisms has come from model studies[40,41,42]. These were hampered by the occurence of unwanted side reactions, leading to low yields of the

desired products. While the results showed that the proposed sequence of reactions could lead to the stereochemistry found in 1[42], they could not differentiate between the two proposed intermediates, 25 and 26. The fact that a nitrogen analog of the tertiary cyclopropylcarbinyl cation inhibits[43] formation of squalene does suggest, however, that the intermediate is 25 rather than 26.

A previous theoretical study of the parent system[21] indicated that cyclopropylcarbinyl cation (27) is best described as a π complex (28) derived from vinyl cation and ethylene and stabilized by back coordination[19] from the filled π MO of the vinyl cation to the antibonding π MO of ethylene; see 29. The isomeric ion formed by rearrangement of 27 and commonly formulated as cyclobutyl cation was



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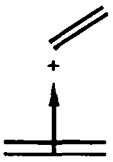

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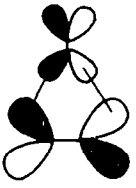

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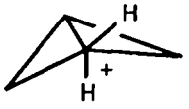

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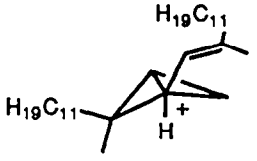

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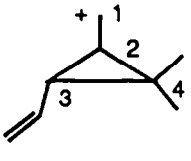
also predicted to be a nonclassical species, being best represented as protonated bicyclobutane (30), with a short transannular bond (1.71 Å). Of the two isomers, 30 was found to be the lower in energy, in agreement with the available experimental evidence, the calculated heat of isomerization being 5.6 kcal/mol. A recent high level *ab initio* study[44] confirms the stability and nonclassical nature of 30. These results suggested that the corresponding protonated bicyclobutyl cation (31) might be involved in the formation of 1.

Our object was to establish the role, if any, of 26, or the corresponding bicyclobutanium ion (31), in the conversion of 3 to 1. The first step was to find out whether substituents in 27, analogous to those in 3, would hinder its conversion to 31. We chose as our model for 23 the vinyldimethyl derivative (32) of 27, which again is best described as a π complex (33). The bond length between C_1 and C_2 was very short (1.36 Å), as was also the bond between C_3 and C_4 (1.48 Å). It is clear from the π complex description that rotation about the CC double bond of the apical vinyl group in 33, interchanging the terminal hydrogens, will involve a large activation barrier. Once ionization has occurred, the stereochemistry of the terminal carbon is thus locked. The orientation of the leaving pyrophosphate group in the enzyme thus determines the stereochemistry at C_1.



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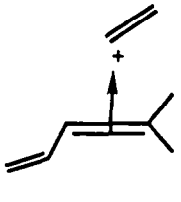
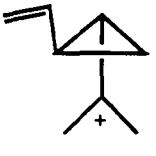
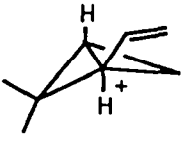


33



34



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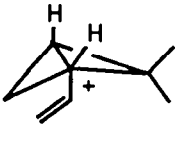
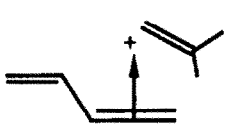


36

We found that 33 rearranges only to a tertiary cyclopropylcarbinyl cation, 34. The reaction profile for the interconversion is given in Figure 4. No stationary point could be found on the potential energy surface for the isomeric bicyclobutanium ion (35). A minimum was found, however, for the isomeric bicyclobutanium ion (36) where the configuration at the site of

protonation is inverted. This suggests that the apparent instability of 35 may be due to unfavorable steric interactions between the *cis* vinyl and H substituents.

Our results indicate that 26 is not involved in the formation of 1. This is consistent with the fact that a squalene analog is produced from 2-methylfarnesyl pyrophosphate[45]. Such a methyl substituent would alter the reactivity of the bicyclobutanium ion if it were an intermediate[45].



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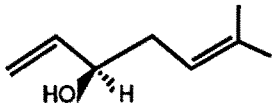

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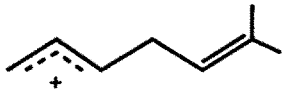

39

The final step in the formation of 1 involves reduction and ring opening of 25. As a π complex (37), this is easily achieved with the correct stereochemistry by hydride acting as a nucleophile[12,20]. Such reactions[20] have been shown to occur in biomimetic cyclization reactions[20]. Indeed, when a calculation was carried out for 34 with a hydroxide ion adjacent to the carbon bearing the vinyl group and separated from it by 2.5 Å, the squalene analog 38 was produced exothermically and without activation. An activation barrier of 6.5 kcal/mol was found for the ring opening of 34 to 39. In the absence of reductive nucleotide, this is the favored pathway producing, in conjunction with proton elimination, didehydrosqualene[46].

Conclusions

Simple mechanisms for the biosynthesis of squalene from farnesyl pyrophosphate have been proposed on the basis of π complex theory and supported by theoretical calculations. The arguments used illustrate the advantages of π complex theory as a basis for interpreting the reactions of nonclassical carbocations.

The first step involves an allylic displacement of pyrophosphate by some group in the enzyme, probably by an S_N1 mechanism followed by trapping of the ion. The resulting intermediate undergoes another

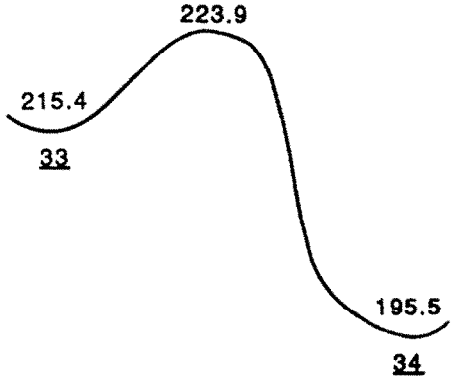

Figure 4. Minimum Energy Reaction Path (MERP) of the conversion of 33 to 34 (ΔH_f in kcal/mol).

allylic displacement, by the second molecule of farnesyl pyrophosphate, to produce the π complex. The second step is probably a genuine S_N2' reaction.

The pathway to squalene from presqualene pyrophosphate does not involve a bicyclobutanium ion as an intermediate. Presqualene cation, a primary cyclopropylcarbinyl cation, rearranges directly to a tertiary cyclopropylcarbinyl cation which undergoes hydride reduction to squalene.

This work illustrates the potential of theoretical calculations, using our procedures, as an aid in the elucidation of enzyme mechanisms. Current theoretical procedures are unable to give *a priori* predictions because even the best are far too inaccurate. Experimental studies are also inconclusive because chemical reactions take place too rapidly for their course to be observed, a restriction that applies equally to enzymatic processes. The best approach currently available to problems of this kind lies in a combination of experiment with theory. Such an approach is particularly apposite to enzyme reactions because they effectively take place in the absence of solvent. Theoretical calculations refer of course to such a situation, dealing as they do with reactions of isolated molecules.

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