scan rates many orders of magnitude greater than was previously possible. The advantages of the use of high-speed electrochemistry at ultramicroelectrodes for the examination of heterogeneous and homogeneous kinetic phenomena have been established. Particularly advantageous is the ability to make voltammetric measurements at high speeds in solvents that are too resistive for use at large electrodes, thus opening new areas of study. The use of even smaller electrodes in the future should allow shorter time scales to be explored.[37] Currently, the limitation to the use of even faster scan rates, or shorter time scales, is the lack of current transducers with sufficient sensitivity and bandwidth to allow the use of smaller electrodes.

We are indebted for the support of this research by the NSF (Chemical Analysis and Behavior and Neural Sciences Programs) and NIH.

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Biosynthesis of Non-Head-to-Tail Terpenes. Formation of 1′–1 and 1′–3 Linkages

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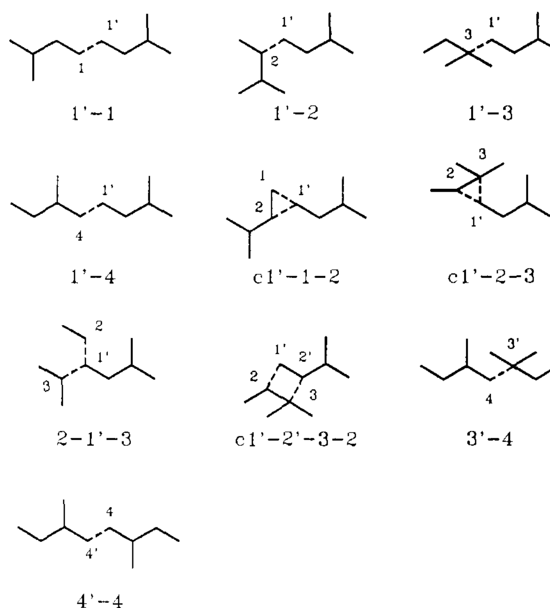
When Ruzicka proposed the biogenetic isoprene rule in 1922, he recognized that individual isoprene units can be joined to one another at different positions.[1–4] The five-carbon segments are attached by a "regular" or head-to-tail fusion in the vast majority of cases, and numerous naturally occurring terpenes have been identified that contain this linkage. Isoprene units joined by "irregular" or non-head-to-tail bonds are encountered less frequently. Prior to the use of radioisotopes to establish biosynthetic pathways, the most prominent non-head-to-tail isoprenes were carotenes, although as early as 1934 Robinson[5] had pointed out a structural resemblance between sterols, as typified by cholesterol, and squalene. Similar analogies were also drawn between squalene and polycyclic triterpenes.[2–4] In 1952 Bloch and co-workers established that squalene was an intermediate in cholesterol biosynthesis by incorporation experiments with radiolabeled acetate.[6] Subsequent work from a number of laboratories has demonstrated that non-head-to-tail compounds are important intermediates in several branches of the isoprene biosynthetic pathway. This Account briefly reviews naturally occurring non-head-to-tail linkages and addresses mechanistic questions concerning biosynthesis of isoprenes with 1′–1 and 1′–3 bonds.

Structure of Non-Head-to-Tail Isoprenes. Only a limited number of fusions between isoprene units are found in nature. These are illustrated in Scheme I. Inspection of the structures reveals a common motif. If the isoprene units are numbered to reflect the original location of the diphosphate moiety in dimethylallyl diphosphate or isopentenyl diphosphate, the five-carbon building blocks for all higher isoprenes, C(1′) of one isoprene residue is usually attached to or embedded in the other. The only exceptions are the 3′–4 attachment found in artemone[7] and the 4′–4 fusion in the isoprene residues of archaebacterial tetraethers.[8] As previously noted, the 1′–4 (heat-to-tail) linkage is most common. It is generated during the fundamental polymerization reaction of isoprene metabolism where successive

Scheme I
Linkages for Non-Head-to-Tail Isoprenes



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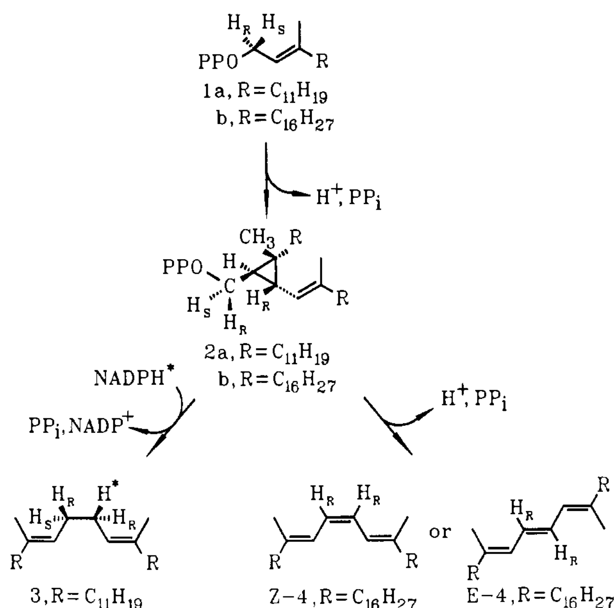
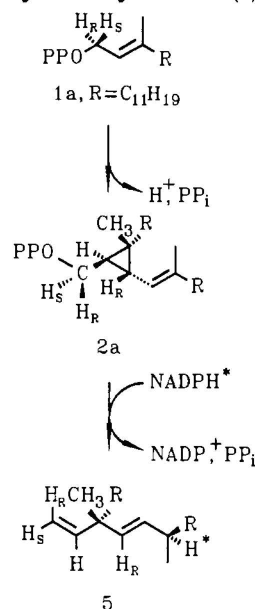
Scheme II
Stereochemistry of Squalene (3) and Phytoene (4) Biosynthesis



Scheme III
Stereochemistry of Botryococcene (5) Biosynthesis



molecules of isopentenyl diphosphate are attached to growing allylic diphosphate polyisoprene chains.[9] The remaining entries are collectively referred to as non-head-to-tail structures.

Unlike 1′-4-fused isoprenes, many non-head-to-tail structures are only synthesized by a few closely related organisms. The major exceptions are sterols and carotenes, metabolites derived from the 1′-1 isoprenes squalene and phytoene, respectively. The greatest variety of non-head-to-tail structures are found in the essential oils of closely related members of sagebrush indigenous to the Great Basin of the western United States.[10] These plants produce monoterpenes with 1′-3,[10,11] c1′-1-2,[11] c1′-2-3,[12] and 2-1′-3[10,11,13-15] skeleta. The most prolific producer of non-head-to-tail isoprenes is a colonial photosynthetic alga *Botryococcus braunii*. The B-race of *B. braunii* synthesizes massive quantities of 1′-3-fused triterpenes constructed from two C_{15} farnesyl residues.[16] The parental C_{30} compound is subsequently methylated at the trisubstituted double bonds to generate a complex family of acyclic and cyclic triterpene hydrocarbons called botryococcenes.[17,18]

Precursors for 1′-1 and 1′-3 Structures. Squalene (3) and phytoene (4) are constructed from two molecules of farnesyl diphosphate (**1a**) and geranylgeranyl diphosphate (**1b**), respectively, during a condensation that joins the C(1) carbons of the substrates.[19] These transformations are, in fact, the result of the two independent reactions illustrated in Scheme II. The first is a prenyl-transfer step during which C(1′) of one of the allylic substrates is bonded to the C(2)-C(3) double bond of the other to produce a cyclopropylcarbinyl diphosphate with a c1′-2-3 structure.[20] In this manner, farnesyl diphosphate (**1a**) yields presqualene diphosphate (**2a**)[21,22] and geranylgeranyl diphosphate (**1b**) yields prephytoene diphosphate (**2b**).[23] In the second reaction, **2a** rearranges to a 1′-1 structure with a saturated linkage between the two original farnesyl residues by a series of steps that involves loss of inorganic pyroposphate and incorporation of a hydride from NADPH[24] to yield squalene (3). A similar second reaction occurs during biosynthesis of phytoene (4) from **2b**. However, in this case the geranylgeranyl residues are joined by a double bond, pyrophosphate is expelled, a proton is eliminated, and pyridine nucleotide cofactors are not required.

We recently reported feeding experiments consistent with a similar two-step sequence for biosynthesis of the 1′-3-fused triterpene botryococcene (5) outlined in Scheme III.[16] Although attempts to obtain a cell-free system capable of utilizing **1a** or **2a** were not successful, we discovered that *B. braunii* readily incorporated [5-^{3}H,1-^{14}C]farnesol into **5** without loss of either label. In subsequent feeding experiments with [1-^{2}H]- and [2-^{2}H]farnesol, ^{2}H NMR spectra of the botryococcenes clearly demonstrated that the farnesyl residue in the

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(24) Abbreviations used are as follows: NAD^{+}, nicotinamide dinucleotide; NADH, reduced nicotinamide dinucleotide; $NADP^{+}$, nicotinamide dinucleotide phosphate; NADPH, reduced nicotinamide dinucleotide phosphate; PP_i, inorganic pyrophosphate.

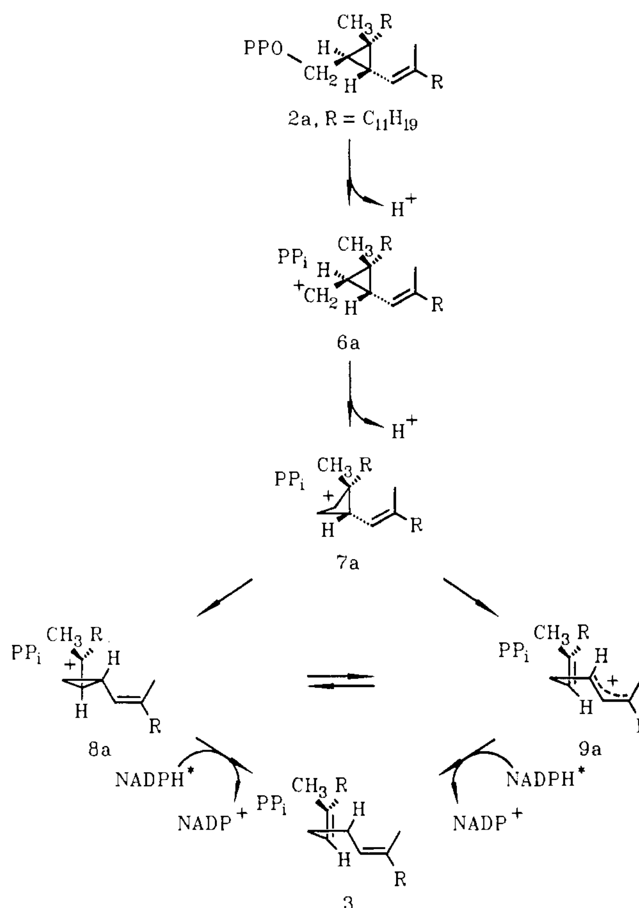
1′-branch of the 1′-3 linkage lost a single deuterium from C(1′), while C(1) of the residue in the 3-branch was incorporated without loss of deuterium. If one assumes that the hydrogen at C(3′) in botryococcene is supplied by NADH or NADPH, a plausible sequence of reactions leading from farnesyl diphosphate to botryococcene is (1) formation of presqualene diphosphate followed by (2) loss of PP_i, cleavage of the C(2)–C(1′) cyclopropane bond, and addition of hydrogen from the reduced nicotinimide cofactor to C(3′).

Stereochemistry of 1′-1 and 1′-3 Condensations. The stereochemistries of both steps in the synthesis of squalene and phytoene have been elucidated. Only the *all-R* enantiomers of **2a** and **2b** have been found in nature.[25,26] They are formed from **1a** and **1b**, respectively, with inversion at C(1′) and concomitant loss of the *pro-S* hydrogen at that center.[25,27] Upon rearrangement of **2a** to squalene (**3**), C(1) and C(1′) are both inverted, as illustrated in Scheme II.[28,29] Gregonis and Rilling found two different results for phytoene biosynthesis, depending on the stereochemistry of the C(1)–C(1′) double bond.[27] (*Z*)-Phytoene ((*Z*)-**4**) is synthesized with stereoselective loss of the *pro-S* hydrogen at C(1) in *Phycomyces blakesleeanus*, while (*E*)-phytoene ((*E*)-**4**) is formed with loss of the *pro-R* hydrogen at C(1) in *Mycobacterium* sp.

The stereochemistry for biosynthesis of botryococcene from farnesol is also consistent with formation of (*R*,*R*,*R*)-**2a** as an intermediate. During feeding experiments, we found botryococcene from (*R*)-[1-^{2}H]farnesol incorporated deuterium at C(1) and C(1′) while that from (*S*)-[1-^{2}H]farnesol only had deuterium at C(1).[16] Thus, the *pro-S* hydrogen was selectively lost from a farnesyl residue in analogy to formation of (*R*,*R*,*R*)-presqualene diphosphate during squalene biosynthesis (see Scheme III). We also deduced from ^{1}H–^{13}C HETCOR experiments that deuterium from (*S*)-[1-^{2}H]farnesol was incorporated into the *E* position at C(1) stereoselectively.

Enzymology. Progress in the purification and characterization of the enzymes that catalyze formation of non-head-to-tail isoprenes has been painfully slow. Thus far, only two, squalene synthetase from *Saccharomyces cerevisiae*[30] and phytoene synthetase from *Capsicum annum*,[31] have been purified to homogeneity. Yeast squalene synthetase is a monomeric microsomal protein, M_R 47 000, with a pH optimum at 7.4 and requirements for Mg^{2+} and reduced pyridine nucleotides. Although NADPH is reported to be preferred in microsomal preparations, Sasiak and Rilling found no substantial preference for NADPH over NADH with solubilized enzyme.[30] They also discovered that the activities for synthesis of presqualene diphosphate and squalene copurified at all stages of the isolation. The Michaelis constants for farnesyl diphosphate and

Scheme IV
Mechanism for Conversion of Presqualene Diphosphate (2a) to Squalene (3)



presqualene diphosphate are 10 and 8 μM, respectively.

Dogbo et al. purified a soluble form of phytoene synthetase from *Capsicum* chloroplast stroma using affinity chromatography on an aminophenethyl diphosphate–Sepharose column as a key step.[31] The monomeric enzyme, M_R 47 500, has a pH optimum at 7.6, but unlike squalene synthetase, prefers Mn^{2+} as a cofactor. Michaelis constants for geranylgeranyl diphosphate and prephytoene diphosphate were both 0.3 μM, and both activities copurified, indicating that, like squalene synthetase, the enzyme catalyzed both reactions required for 1′-1 coupling.

Mechanistic Considerations and Model Studies. The mechanism of 1′-1 coupling has been of continuing interest since farnesyl diphosphate was established as a precursor for squalene in 1958.[32,33] Early proposals for squalene biosynthesis were rendered untenable when the structure of presqualene diphosphate, initially discovered by Rilling[34] in 1966, was firmly established.[21,35–38] At that point, several groups were quick to recognize that the skeletal rearrangements required to convert c1′-2-3 to 1′-1 structures were similar to

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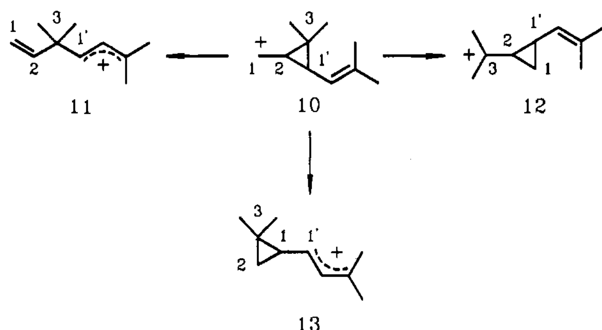
Scheme V
Summary of Model Studies



those seen for cyclopropylcarbinyl cations.[21,36,39] A few years earlier, Bates and co-workers[40,41] had also proposed a related mechanism for biosynthesis of 1′–3 artemisia monoterpenes by a cationic rearrangement of chrysanthemyl diphosphate, the 10-carbon equivalent of presqualene diphosphate.

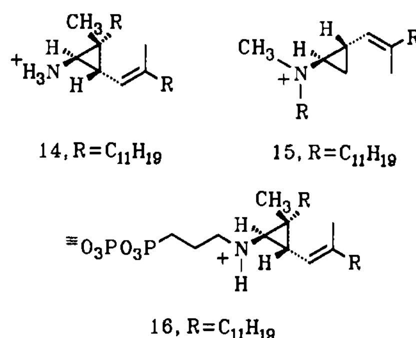
Salient features of the mechanisms suggested for squalene biosynthesis are summarized in Scheme IV, where primary cyclopropylcarbinyl cation **6a** rearranges to tertiary cation **8a** or allylic cation **9a** via cyclobutyl species **7a** followed by nucleophilic addition of hydride from NADPH to C(3′) of **8a** or **9a** to give squalene. Since [1.2] sigmatropic shifts are suprafacial in cyclopropylcarbinyl cations, inversion of configuration at C(1) during rearrangement of **6a** to **7a** predicts a conformation for **2a** in the *E*·*S* complex with the C(1)–oxygen bond antiperiplanar to the C(2)–C(1′) cyclopropane bond.[42,43] C(1′) is also inverted, which could be a consequence of stereoelectronically enforced transfer of hydride to tertiary cation **8a** from the favored direction (inversion of configuration) or a topologically induced stereoselective transfer to allylic cation **9a**. Gregonis and Rilling[27] pointed out a similar mechanism also suffices to explain the stereochemistry of phytoene biosynthesis. Elimination of a proton from tertiary cyclopropylcarbinyl cation **8b** predicts stereoselective removal of the *pro-S* hydrogen to form (*Z*)-phytoene and removal of the *pro-R* hydrogen to form (*E*)-phytoene. If elimination occurs from the allylic cation, topological constraints are required to obtain the observed stereoselectivities.

The precedents initially used to develop a mechanism for the c1′–2–3 → 1′–1 rearrangement were based on simple alkyl-substituted cyclopropylcarbinyl systems. Subsequent model studies designed to duplicate the rearrangements of the presqualene system with chrysanthemyl derivatives bearing the appropriate pattern of substituents on the cyclopropane ring met with mixed success, and results from several solvolysis experiments are summarized in Scheme V.

In aqueous solvents only 6% of chrysanthemyl cation **10** is captured before it partitions among **11**, **12**, and **13** irreversibly.[44] Solvolysis products from chrysanthemyl precursors of cation **10** are mostly derived from 1′–3 cation **11**, and the mixture contains only trace quantities of compounds derived from c1′–1–2 cation **12** and cation **13**. When **12** is generated directly from covalent precursors, excellent yields of 1′–1 products are obtained.[42,44,45] Attempts to generate or capture a C_{10} model of cyclobutyl cation **7a** failed.[42,44] Either the cation was too unstable to be intercepted before rearrangement, or covalent cyclobutyl precursors rearranged directly to tertiary cyclopropylcarbinyl cation **12** upon solvolysis. Despite the abysmally low proportion of c1′–2–3 → c1′–1–2 rearrangement detected during model studies, the stereochemical features of squalene biosynthesis are nicely reproduced. Chrysanthemyl precursors to **10** prefer to solvolyze in an antiperiplanar conformation like that shown for **2a** in Scheme IV,[46] and that small proportion of **10** which rearranges to **11** does so with inversion at C(1).[47] In addition, nucleophilic attack at C(1′) of **12** also occurs with inversion.[47]

Although model studies with the c1′–2–3 system demonstrate the feasibility of rearrangement of 1′–1, 1′–3, 2–1′–3, and c1′–1–2 structures, they raise serious questions with respect to regiocontrol in the enzyme-catalyzed reactions. The major channel for **10** is an irreversible c1′–2–3 → 1′–3 rearrangement to **11**. Only a small proportion is diverted to **12**, a c1′–2–3 → 1′–1 rearrangement, or to **13**. From product ratios, we estimate that the barrier from **10** to **11** lies approximately 4.6 kcal/mol below those to **12** and **13**.[44] To attain the level of regioselectivity required during biosynthesis of squalene (ca. > 99.9%), the barriers separating **10** and its isomers must be adjusted so the barrier between **10** and **12** within the catalytic site of squalene synthetase is at least 4 kcal/mol lower than the other two.

Reactive Intermediate Analogues for Squalene Synthetase. It is sometimes possible to design potent inhibitors for enzymatic reactions by duplicating structural features of transition states or reactive intermediates. We synthesized two inhibitors, **14** and **15**,



to mimic the topological and electrostatic properties of cyclopropylcarbinyl cations **5a** and **7a**.[48] In each instance, the trigonal carbocationic center was replaced by a tetrahedral positively charged nitrogen. Although we did not precisely duplicate the topology of the carbocations, the steric demands of the extra proton and the differences between trigonal and tetrahedral geom-

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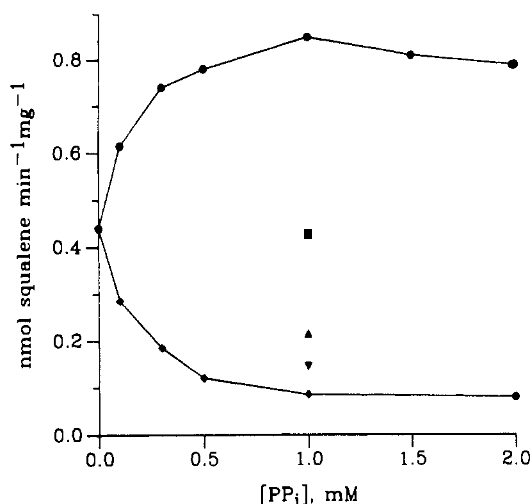

Figure 1. Rates of squalene synthesis from farnesyl diphosphate as a function of the concentration of PP_i and **15**: 0 (●), 3 (■), 5 (▲), 10 (▼), 20 μM (♦).

etries at the charged atom are relatively small. The flexibility in the remainder of the structures does not transmit these distortions beyond the immediate vicinity of the substitution.

During preliminary experiments with squalene synthetase, we were disappointed to discover that **14** and **15** were not inhibitors at concentrations as high as 170 μM, more than 15-fold above K_M for either farnesyl or presqualene diphosphate![49] Upon reflection, we realized that the initial species produced upon heterolysis of the C(1)–oxygen bond in **2a** is an intimate ion pair and that inhibition by **14** or **15** might require PP_i or a structurally related species as a coinhibitor. The results of inhibition experiments in the presence of PP_i are summarized in Figure 1. As the concentration of PP_i was increased in standard assay buffer, the activity of squalene synthetase increased to a maximal 2-fold stimulation at 1 mM PP_i. At higher concentrations, PP_i was a product inhibitor. The stimulation of activity by PP_i was not seen for two other microsomal enzymes in the preparation, cytochrome *c* reductase and glucose-6-phosphatase, and we have no explanation for the selective stimulation of squalene synthetase. However, as illustrated in Figure 1, **15** became a potent inhibitor when PP_i was added to the buffer. A plot of the rate of squalene synthesis versus [PP_i] in the presence of 20 μM **15** decreased sharply as the concentration of PP_i increased from 0 to 1 mM. In buffer containing 1 mM PP_i, the I_{50} (the concentration of inhibitor required for 50% inhibition in the presence of 1 mM NADPH) for **15** was 3 μM. Similar inhibition profiles were seen for synthesis of squalene from farnesyl diphosphate or presqualene diphosphate in the presence of either the primary or the tertiary ammonium analogue.

From these results it is clear that **14** and **15** are not bound tightly by squalene synthetase in spite of their close resemblance to **6a** and **8a**, respectively. However, at 20 μM **14** or **15**, a concentration that failed to inhibit in PP_i-free buffer, and 1 mM PP_i, a concentration that elicited a 2-fold stimulation of activity, the positively and negatively charged "fragments" combined to generate potent ion-pair inhibitors. The diphosphate moiety of **2a** is a major factor in substrate binding, and presumably complementary interactions between the hydrocarbon moieties of the ammonium analogues and squalene synthetase are insufficient to overcome electrostatic repulsions between the unshielded ammonium moieties in **6a** or **8a** and the enzyme. Similar synergistic phenomena were observed, albeit at 10^4-fold-higher concentrations, for mixtures of Tris and PP_i.[49] In this case there is no apparent structural similarity between the ammonium species and the hydrocarbon regions of **6a** or **8a**. It is interesting to note that Dogbo et al.[31] reported that PP_i also strongly inhibits phytoene synthetase in Tris buffer.

The synergistic interaction between the ammonium analogues and PP_i suggested it might be possible to construct an ion-pair analogue by joining the two charged fragments with a short tether. Examination of space-filling models indicated that a molecule containing a two-carbon bridge between the nitrogen in **14** and a PP_i oxygen could fold into relatively unstrained conformations with the nonbridging phosphate near the nitrogen. We employed an isosteric phosphonophosphate moiety to link the fragments in order to improve the chemical stability of the compound.[48] Tethered analogue **16** proved to be a potent inhibitor of squalene synthetase.[49] I_{50}'s of **16** for inhibition of squalene synthesis from **1a** or **2a** measured in PP_i-free buffer were similar to those determined for **14** and **15** in the presence of 1 mM PP_i. Thus, the effective concentration of PP_i is increased by at least 300-fold when tethered to an ammonium moiety, notwithstanding expected decreases in potency associated with (1) replacement of PP_i with a phosphonophosphate unit and (2) steric and hydrophobic interactions introduced with the side chain. In addition, both steps were inhibited to similar degrees when synthesis of **2a** and **3** from **1a** was assayed simultaneously.[49]

This result suggests that both reactions in the conversion of farnesyl diphosphate to squalene occur at the same or strongly overlapping active sites. In an earlier study of squalene biosynthesis in rat liver microcomes, Corey and Volante found that a phosphonophosphate analogue of presqualene diphosphate blocked formation of squalene from mevalonate and from **2a** under conditions where some synthesis of **2a** from mevalonate was seen.[50] Although these results are more consistent with unique catalytic sites for the two reactions leading from farnesyl diphosphate to squalene, such high levels of inhibitor (0.5–1.0 mM) were required to substantially block squalene formation that nonspecific inhibition may have occurred.[51]

Our inhibition studies provided strong support for a mechanism involving cyclopropylcarbinyl cationic intermediates in the rearrangement of **2a** to squalene. In particular, inhibitors whose topological and electrostatic properties mimic primary cation **6a** and tertiary cation **8a**, originally proposed by us as intermediates in the reaction, bind tightly to the enzyme. The observation that binding of the ammonium analogues depends on the availability of PP_i suggests that the cyclopropylcarbinyl cation–PP_i intimate ion pairs generated upon cleavage of the C(1)–oxygen bond in **2a** are bound throughout the rearrangement.

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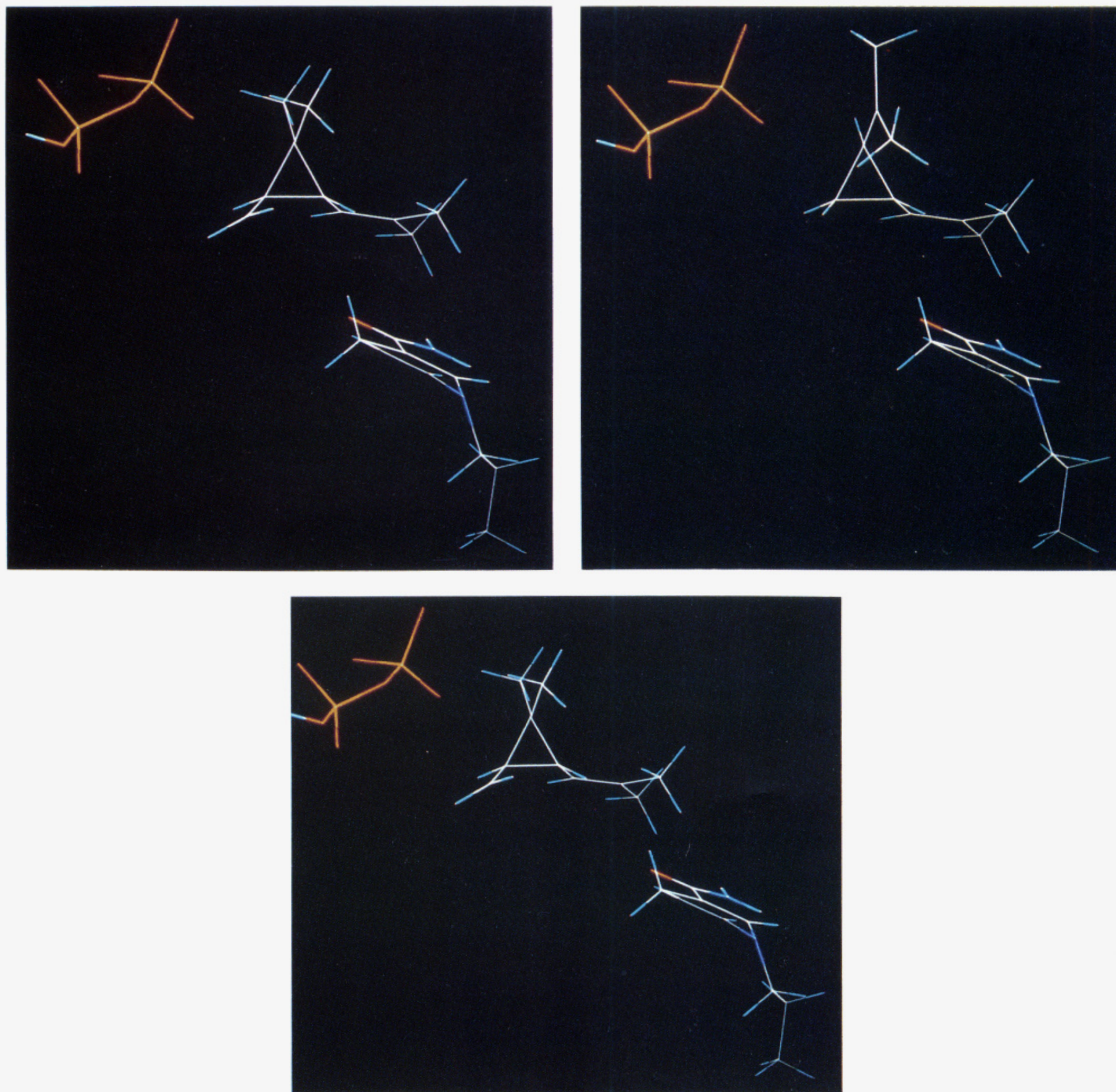

Figure 2. Relative positions of **6a** (part a, top left), **8a** (part b, top right), PP_i, and NADPH in a least-motion mechanism for formation of squalene (**3**). Relative positions of **6a**, PP_i, and NADPH for formation of botryococcene (**5**) (part c, bottom).

Regiocontrol in the Enzymatic Synthesis of 1′–1 and 1′–3 Isoprenes. If c1′–2–3 isoprenes are precursors for 1′–1- and 1′–3-fused compounds, the enzymes mediating the reactions must be able to exert strict regiocontrol on the cyclopropylcarbinyl rearrangements. In particular, the regiochemical diaster encountered in model studies of squalene biosynthesis must be reversed by squalene synthetase. We proposed this can be achieved by a simple least-motion ion-pair mechanism.[42,43,49] As discussed earlier, stereochemical considerations dictate that the C(1)–oxygen bond and C(2)–C(1′) cyclopropane bond in presqualene diphosphate be antiperiplanar in the *E*·*S* complex. This conformer happens to be 6 times more reactive than its periplanar counterpart.[46] Upon cleavage of the C(1)–oxygen bond in **2a**, PP_i is located on the C(1)–C(2)–C(3) face of the cyclopropylcarbinyl cation, as illustrated in Figure 2a. In this orientation, PP_i is in position to electrostatically stabilize primary cation **6a**, cyclobutyl species **7a** interconnecting **6a** and tertiary cation **8a**, and **8a**. However, a c1′–2–3 → 1′–3 rearrangement or a [1.2] sigmatropic shift of the C(2)–C(3) bond in **6a** separates the charged centers with a concomitant destabilization of the intimate ion pairs. We estimate that the transition state for c1′–2–3 → c1′–1–2 rearrangement can be selectively stabilized by up to 10 kcal/mol over the c1′–2–3 → 1′–3 isomerization or the C(2)–C(3) bond shift leading to structures like **13**. This is a sufficient adjustment of barrier heights to achieve the level of regioselectivity required for squalene biosynthesis. In the ion-pair mechanism, PP_i serves as a template to direct the c1′–2–3 → 1′–1 rearrangement. Ion pairs are also well-documented intermediates in enzyme-catalyzed cyclizations of mono- and sesquiterpene allylic diphosphates where the pyrophosphate partner plays crucial roles in promoting isomerization and exerting

regio- and stereocontrol within the active site of the cyclases.[52-55]

Coates and Robinson[45] suggested that regiocontrol of the c1′–2–3 → c1′–1–2 rearrangement could be maintained by twisting about the C(1′)–C(2′) bond in **6a** to minimize overlap between C(1′) and the π orbitals of the C(2′)–C(3′) double bond. Although 1′–3 rearrangement will be blocked in the twisted conformer, a mechanism based solely on conformational control has some disadvantages. The twisted carbocation is less reactive and must be precisely aligned and rigidly held in the *E·S* complex to prevent opening to a 1′–3 structure. Twisting should also reduce the reactivity of C(1′) in relation to C(3) in **6a**.[46] Although twisting about the C(2′)–C(3′) bond in **2a** may augment the regioselectivity of the ion-pair mechanism, it is not sufficient to explain the regioselectivity of the enzyme-catalyzed reaction, nor is it a required feature.

Another important aspect of the ion-pair mechanism is the final transfer of hydride to **8a** following rearrangement. We propose a least-motion process for this step as well with NADPH initially bound in an orientation compatible with inversion at C(1′) in **8a** following the c1′–2–3 → 1′–1 rearrangement.[42,44,49] As shown in Figure 2, parts a and b, the rearrangement occurs with minimal change in the Cartesian coordinates of the carbons constituting the cyclopropylcarbinyl core of **6a** and **8a**. Thus, it is reasonable to assume that hydride is adjacent to C(1′) in **6a** as well as **8a**. Furthermore, it should be noted that our model studies show that the homoallylic C(1′) positions in both cyclopropylcarbinyl cations react with nucleophiles, and one might anticipate the possibility of premature transfer of hydride to C(1′) in **6a**. However, a least-motion mechanism requires that the transfer to C(1′) occur with retention of stereochemistry. Since reaction at the cyclopropane carbons normally proceeds with inversion,[56] we suggest a stereoelectronic barrier precludes premature attack with retention at C(1′) until the c1′–2–3 → c1′–1–2 rearrangement has taken place, whereupon the transfer can occur with inversion.

Our stereochemical studies support the contention that botryococcene is also synthesized from presqualene diphosphate. As mentioned previously, the quaternary C(3) centers in presqualene diphosphate and botryococcene have the same absolute stereochemistry. Also, the *pro-S* hydrogen is lost from C(1′) during biosynthesis in both **3** and **5**. During squalene biosynthesis, this loss occurs during formation of the cyclopropane ring in (*R*,*R*,*R*)-**2a**. A similar result is expected if botryococcene is derived from (*R*,*R*,*R*)-**2a**. The stereochemical analogy also extends to C(1) in **3** and **5**. For stereoelectronic reasons discussed above, presqualene diphosphate must adopt a conformation with its C-(1)–oxygen bond antiperiplanar to the C(2)–C(1′) cyclopropane bond for C(1) to be inverted during the c1′–2–3 → 1′–1 rearrangement. Although there is no apparent stereoelectronic imperative for botryococcene

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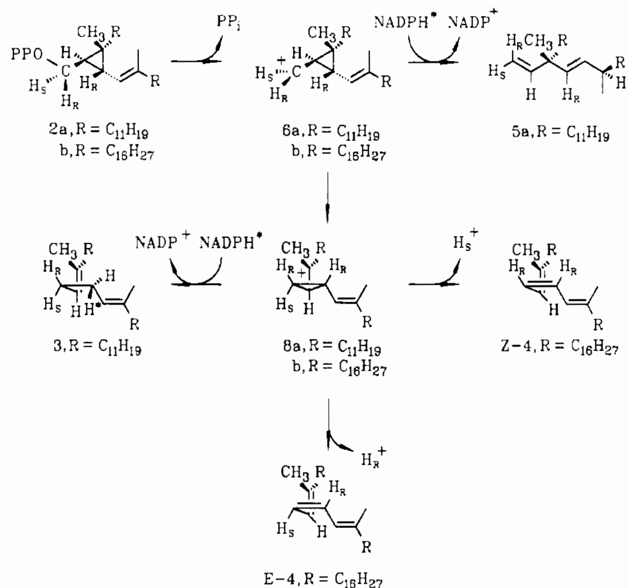
Scheme VI
Mechanisms for the Stereoselective Biosynthesis of Botryococcene (5), Phytoene (4), and Squalene (3)



other than the 6-fold increase in reactivity for the antiperiplanar conformer, stereoselective formation of (*E*)-[^{2}H]-**5** from (*S*)-[1-^{2}H]farnesol demands a similar conformation for the C(1)–C(2) bond of **2a** in the *E·S* complex of botryococcene synthetase. Thus, the stereochemical and mechanistic studies summarized in Scheme VI suggest that squalene synthetase and botryococcene synthetase bind similar conformers of **2a**.

Congruent conformations for presqualene diphosphate in the catalytic sites of the two enzymes again raise questions about how regiocontrol is maintained during the 1′–1 and 1′–3 rearrangements since the topologies of the primary cyclopropylcarbinyl–PP$_i$ ion pairs are similar. We suggest that the precise location of the hydride donor may be crucial for maintaining regiocontrol.[16] As noted previously, premature capture of **6a** can be thwarted during biosynthesis of squalene by binding NADPH near C(1′) in an orientation where a stereoelectronic barrier precludes transfer of hydride prior to rearrangement. If the cofactor is moved to a position where transfer to C(3′) is possible, as shown in Figure 2c, the stereoelectronic barrier vanishes, and reduction can occur with concomitant opening of the C(2)–C(1′) bond to give a 1′–3-fused structure.

Finally, we can deduce the conformation of the C-(1′)–C(2′) bond for **2a** in the *E·S* complex of botryococcene synthetase. Since the C(1′)–C(2′) double bond in botryococcene is trans, a least-motion mechanism suggests that the dihedral angle between the C–H bonds at C(1′) and C(2′) is near 180°. Also note that the *R* stereocenter at C(3′) is formed by transfer of hydride to the *si* face of C(3′). If one assumes squalene synthetase binds a similar conformer of presqualene diphosphate, the major difference between the active-site topology of the two enzymes is the location of NADPH. A relatively small displacement of the hydrogen in the cofactor by approximately 2.5 Å along the bottom face of the substrate and sufficient flexibility to accommodate the products suffice to explain the regioselective formation of 1′–1 or 1′–3 triterpenes. In view of the stereochemical analogies between the re-

actions catalyzed by the two enzymes, it is quite possible that botryococcene synthetase is derived from squalene synthetase, and the first step in this process was a mutation that repositioned the cofactor.

Conclusions. From a combination of model studies, stereochemical experiments, and inhibition kinetics, we have made process in elucidating the mechanisms for biosynthesis of 1′–1 and 1′–3 isoprenes. A central role for c1′–2–3 cyclopropylcarbinyl diphosphates is indicated for non-head-to-tail isoprenes with 1′–1, 1′–3, 2–1′–3, and c1′–1–2 fusions, with the regioselective formation of metabolites such as squalene, botryococcene, or phytoene by their respective synthetases primarily a consequence of electrostatic interactions in enzyme-bound intimate ion pairs. Much less is known about the cyclopropanation step. Ortiz de Montellano and co-workers[57] have evidence for distinct donor and acceptor sites for the two molecules of farnesyl diphosphate participating in the reaction. Although the regioselectivity they observed for their analogues is suggestive of an electrophilic cyclopropanation, the mechanism of the reaction remains to be established. Further work in this area will be difficult until a reliable source of soluble purified enzyme is secured.

I express my sincere gratitude to the dedicated students and postdoctorals in my group whose enthusiastic contributions made this work possible, and to Hans Rilling and Bill Epstein for sharing their interests in isoprenes with me. I also thank the National Institutes of Health (GM 21328 and GM 25521), the National Science Foundation (DCB 8803825), and the Amoco Corporation for support.

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The Shroud of Turin: Blood or Artist's Pigment?

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The scientific controversy over the Shroud of Turin is unusual in its disparity of opinions, the one-sidedness of those opinions, and its nearly dozen-year length. Although the 1988 carbon-14 date confirms a medieval origin, I reached that conclusion a decade earlier on the basis of polarized light microscopy.[1–3] I hope that a review of this evidence will not only reemphasize the importance of objectivity in scientific research but also serve as a reminder of the unique capabilities of this "mature" technique. The light microscope was invented in the 1600s, and it became a useful chemical analytical tool in the 1800s.

The Shroud of Turin (Figure 1), a linen cloth measuring 1.1 × 4.3 m, depicts two images, back and front views, of a naked crucified man. A beautiful image that fits perfectly the New Testament version of the crucifixion, it has been an object of veneration since it first appeared in history in 1356—this, in spite of the fact that Bishop Henri of Troyes, in whose diocese it was first exhibited, said it was "cunningly painted as attested by the artist who had painted it."

A group of scientists from the Shroud of Turin Research Project (STURP) spent five days and nights in Turin in October 1978 examining the Shroud. They studied it visually, photographically, and spectroscopically and took sticky-tape lift samples (18 × 37 mm); 18 from body- and blood-image areas, and 14 from nonimage areas as controls (Figure 1).

The STURP scientists identified the Turin Shroud image as blood and oxidized/dehydrated cellulose.[1] I, instead, found no blood and established the presence of $Fe_2O_3{\cdot}xH_2O$ and HgS corresponding to two common artist's pigments of the 14th century, red ochre and vermilion, with a collagen (gelatin) tempera binder. My microscopical studies were made on thousands of fibers and particles on the 32 Shroud tapes.[2,3]

Characteristics of the Image

To the unaided eye, the Shroud image is yellow in most body-image areas (Figure 2), but red in the blood-image areas (Figure 3). Microscopically, the image consists of yellow fibers (Figure 4) and red particles (Figure 5); the red particles are more abundant in the red blood images, and the yellow fibers are the major colored substance in the body image.

A careful microscopical survey of the 22 image tapes and 10 nonimage tapes shows, without exception, tiny red particles in body- and blood-image areas but no red particles on the fibers in nonimage areas. They are readily apparent as easily visible red incrustations in most blood-image areas, but more widely dispersed red particles in the body-image areas. The red particles require careful high-magnification light microscopy (600–1000×) to see and identify.

A representative group of 12 of the 32 tapes were also carefully examined for the presence of yellow fibers, and

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