Enzymatic Cyclization of Squalene and Oxidosqualene to Sterols and Triterpenes

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Contents

I. Introduction

The enzymatic cyclizations of squalene (1) and oxidosqualene (2) (Chart I) are the most remarkable steps in the biosynthesis of steroids and triterpenoids. Woodward and Bloch first proposed a hypothesis concerning the course of cyclization of squalene followed by rearrangement to lanosterol in 1953,[1] and the stereochemical implications of the cyclization were addressed independently in 1955 by Stork and Burgstahler[2] and by the ETH team of Eschenmoser, Ruzicka, Jeger, and Arigoni.[3] For almost 40 years, the mechanistic and evolutionary aspects of squalene and oxidosqualene cyclization reactions have elicited intense chemical and biochemical interest. The first direct evidence for 1,2-methyl and hydride shifts during lanosterol formation was obtained from incorporation experiments by Bloch and by Cornforth.[4,5] Intermediacy of 2,3-oxidosqualene in lanosterol biosynthesis was first demonstrated by Corey and by van Tamelen,[6-8] and many substrate analogue compounds were synthesized and tested in their laboratories. Further, Barton verified that eukaryotic oxidosqualene cyclases only accepted the (3*S*)- and not the (3*R*)-enantiomer of oxidosqualene as a substrate.[9]

It is now accepted that the formation of lanosterol (3) in animals and fungi and of cycloartenol (4) in plants proceeds *via* the so-called "chair–boat–chair" conformation of (3*S*)-oxidosqualene, and that the cyclization is initiated by a acid-catalyzed oxirane ring opening with participation by a neighboring π-bond. The cyclization proceeds to give a protosteryl C-20 cation, which then undergoes a series of 1,2-methyl and hydride shifts with proton elimination to yield either the lanosterol or cycloartenol skeleton (Scheme I).[10-12] Similarly, cyclization to pentacyclic triterpenes such as β-amyrin (5) appears to proceed *via* the corresponding "chair–chair–chair" conformation of the substrate. A tetracyclic dammarenyl C-20 cation is the first "stopping point" for the cyclization, and subsequent rearrangements lead to the pentacyclic β-amyrin *via* the baccharenyl, lupenyl, and oleanyl cationic intermediates (Scheme VI).[13] Tetracyclic and pentacyclic triterpenoids derived from these cationic intermediates are also widespread in nature. In some bacteria and protozoa, squalene can be cyclized directly (*without* prior epoxidation) to pentacyclic 3-deoxytriterpenes with the hopane and gammacerane skeletons such as diploptene (hop-22(29)-ene) (10), diplopterol (hopan-22-ol) (11), and tetrahymanol (9). Some of these, *e.g.* tetrahymanol, are regarded as sterol surrogates in these organisms.[14,15] In this case, the cyclization reaction is relatively more simple, and proceeds in all prechair conformation without carbon skeletal rearrangement (Scheme VII).

Squalene and oxidosqualene are therefore converted to various skeletal types of sterols and triterpenes by different enzyme systems. The relationship between the cyclization mechanism and enzyme structure is extremely interesting. In principle, such variations could be attained by only small modification of the structure of the active site of the enzyme. A hypothesis concerning the possible molecular evolution from the "primitive" squalene cyclases to oxidosqualene cyclases in higher organisms has been proposed by Ourisson and co-workers.[15-17] Recent molecular information on several of the cyclases have led Poralla *et al.* to further hypothesize how specific repeating motifs in the cyclase protein structures may account for functional differences in the enzymes.[18]

In the past five years, there have been several remarkable advances in the study of squalene cyclase (SC) and oxidosqualene cyclase (OSC) enzymes. First, although SC and OSC enzymes had remained incompletely characterized until recently because of their unstable, membrane-bound nature, several cyclases were purified to homogeneity from vertebrate,[19,20] plant,[21-24] yeast,[25] and bacterial[26,27] sources for the first time (Table I). Second, it has now become feasible to clone the cyclase genes (or cDNAs) and to deduce

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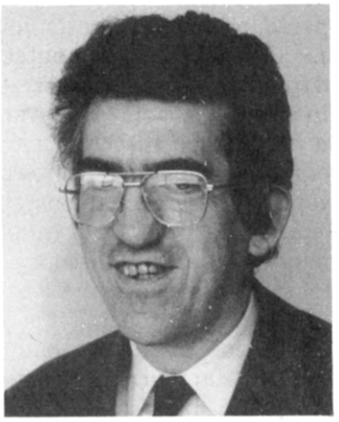
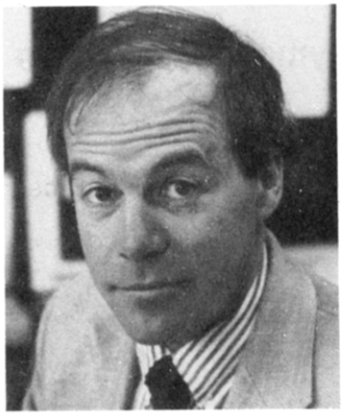
Ikuro Abe was born in Tokyo, Japan, in 1960. He received his Ph.D. in 1989 from The University of Tokyo, Faculty of Pharmaceutical Sciences, under the direction of Dr. Yutaka Ebizuka. Then he went to France as a Boursier du Gouvernement Français to work with Professor Guy Ourisson at the CNRS Institut de Chimie des Substances Naturelles, and mostly with Professor Michel Rohmer at the Ecole Nationale Supérieure de Chimie de Mulhouse. In 1991, he joined Professor Glenn D. Prestwich's group at the University at Stony Brook, where he is now Senior Research Associate. His main research interests include the biosynthesis of natural products, enzyme mechanism, protein chemistry, and molecular biology of sterol synthesis. He is especially interested in molecular evolution of the cyclases.



Michel Rohmer was born in Strasbourg, France, in 1948. He received his Diplôme d'Ingénieur Chimiste from the Ecole Nationale Supérieure de Chimie de Strasbourg in 1970 and his Doctorat d'Etat in 1975 from the Université Louis Pasteur in Strasbourg, studying with Professors Guy Ourisson and Pierre Benveniste. From 1974 to 1979, he was Maître-Assistant in Pharmacognosy at the Université Louis Pasteur, and after one year as post-doctoral fellow at Stanford University with Professor Carl Djerassi, he joined the Ecole Nationale Supérieure de Chimie de Mulhouse/Université de Haute-Alsace where he is now Professor of Organic Chemistry. His major fields of interest include the chemistry of natural products (sterols, triterpenoids of the hopane series, carbohydrate derivatives) from microorganisms (protozoa, unicellular algae, fungi and mainly bacteria) and the elucidation of metabolic pathways. His research group is currently involved in the elucidation of a novel biosynthetic route for the formation of isoprenic units in eubacteria. He received the Vaillant Award from the French Académie des Sciences and the Alfred Valentine Award (1993, Mulhouse).



Glenn D. Prestwich is an addictive peripatetic. Born in Panama in 1948, raised in California, upstate New York, and Maryland, he graduated from Caltech with a B.Sc. in 1970 and received his Ph.D. in Chemistry from Stanford University (with Prof. W. S. Johnson) in 1974. After three years postdoctoral experience (6 months in Cornell University with Prof. J. Meinwald), mostly at the International Centre for Insect Physiology and Ecology in Nairobi, Kenya, he settled down at the State University of New York at Stony Brook in 1977 as an Assistant Professor of Chemistry. Now Professor of Chemistry and Professor of Biochemistry and Cell Biology, his research incorporates synthetic chemistry and molecular biochemistry in the quest of understanding how insects smell, how insects molt, how brain receptors work, how platelets aggregate, how hyaluronic acid can be converted to a gel, and how squalene is turned into cholesterol. In September 1993, he added the role of Director, Center for Biotechnology at the University at Stony Brook and now spends half his time in fostering technology transfer and economic development. Dr. Prestwich has been the recipient of both NIH Postdoctoral and Senior Fellowships, a Camille and Henry Dreyfus Teacher–Scholar Grant, and an Alfred P. Sloan Foundation Fellowship.

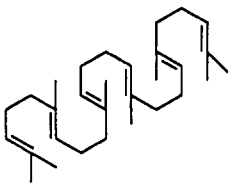
the primary amino acid sequences of the enzymes. So far two cyclases, *Bacillus acidocaldarius* SC[28] and *Candida albicans* OSC[29,30] have been cloned and sequenced. Indeed, sequences for the rat liver OSC[31] and for the *Zymomonas mobilis* SC[32] will be reported soon. Third, several potent cyclase inhibitors, including the first mechanism-based irreversible inhibitor,[33] were developed. These inhibitors will produce more detailed information about the active sites of the cyclases. Fourth, it was demonstrated for the first time that only a small modification of the substrate can cause a dramatic change in the cyclization mechanism, suggesting that the geometry of the active site of some cyclases has been potentially prepared for other cyclization products. This finding is of great importance from the viewpoint of the molecular evolution of triterpene and sterol cyclases.[34] Finally, comprehensive reexamination of the cyclization mechanism by Scott Virgil of E. J. Corey's laboratory clearly established the structure of the protosterol intermediate cation and gave new mechanistic and stereochemical insights on the cyclization reaction.[11,35] The studies of SC and OSC have now entered a new epoch in which chemistry and molecular biology can interact to reveal the intimate three-dimensional structural details of the enzyme-catalyzed processes.

II. Animal and Fungal Oxidosqualene Cyclases

A. General Considerations for Cyclization Mechanism

According to Ruzicka's "biogenetic isoprene rule", the cyclization of all *trans* squalene takes place *via* a well-defined sequence of prechair and preboat conformations. The transformation was proposed to proceed according to the rules of antiperiplanar cationic 1,2-addition, 1,2-rearrangement, and 1,2-elimination; all steps on the route from squalene to the final product would proceed in a "non-stop" reaction.[3] However, the

Chart I



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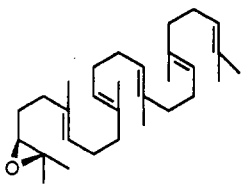

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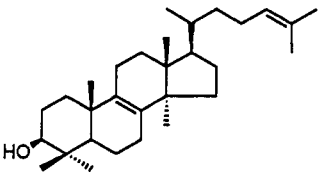

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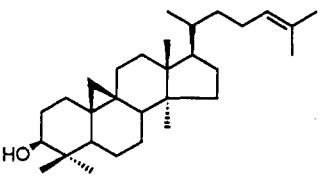

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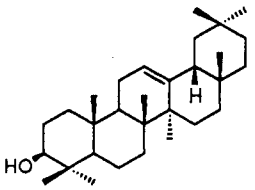

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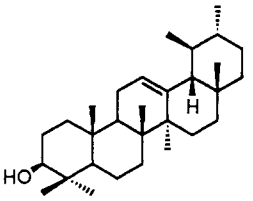

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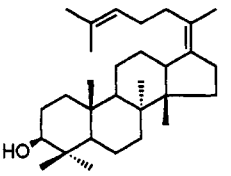

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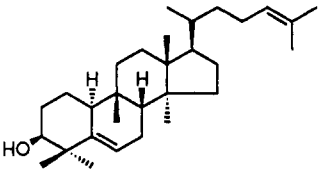

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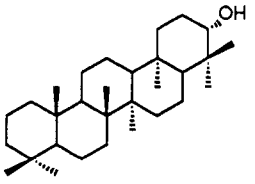

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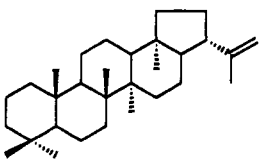

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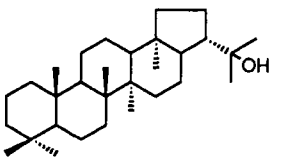

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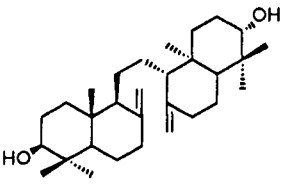

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Table I

source	cyclization product	molecular weight (kDa)	purification methods	purification (*n*-fold)
		Oxidosqualene Cyclase		
vertebrate				
pig liver	lanosterol	75	Triton X-100 solubilization, DEAE–cellulose, hydroxylapatite	441
rat liver	lanosterol	78	Triton X-100 solubilization, hydroxylapatite, isoelectric focusing, Q-sepharose, gel filtration	1863
yeast				
Saccharomyces cerevisiae	lanosterol	26	Triton X-100 solubilization, DEAE–sepharose (×2), affinity column	160
plant				
Rabdosia japonica (cultured cell)	cycloartenol	54	Triton X-100 solubilization, hydroxylapatite (×2), isoelectric focusing, gel filtration	139
	β-amyrin	28	Triton X-100 solubilization, hydroxylapatite (×2), DEAE–cellulose, isoelectric focusing, gel filtration	541
Pisum sativum (seedlings)	cycloartenol	55	Triton X-100 solubilization, hydroxylapatite, isoelectric focusing, DEAE–cellulose, gel filtration	471
	β-amyrin	35	Triton X-100 solubilization, hydroxylapatite (×2), DEAE-cellulose, isoelectric focusing, gel filtration	4290
		Squalene Cyclase		
protozoan				
Tetrahymena thermophila	tetrahymanol	72	octylthioglucoside solubilization, DEAE–trisacryl, hydroxylapatite, mono Q	297
bacteria				
Bacillus acidocaldarius	hopene	75	Triton X-100 solubilization, DEAE–cellulose, phenyl-sepharose, gel filtration (×2)	886

concertedness of the overall ring-forming process is a matter of debate. For entropic reasons and on the basis of experimental evidence, van Tamelen suggested that a single transition state for a complex polycyclization is less likely and that the cyclization proceeds through a series of discrete conformationally rigid, partially cyclized carbocationic intermediates (Scheme I).[10,36] Indeed, monocyclic and bicyclic triterpenes, which are

Scheme I

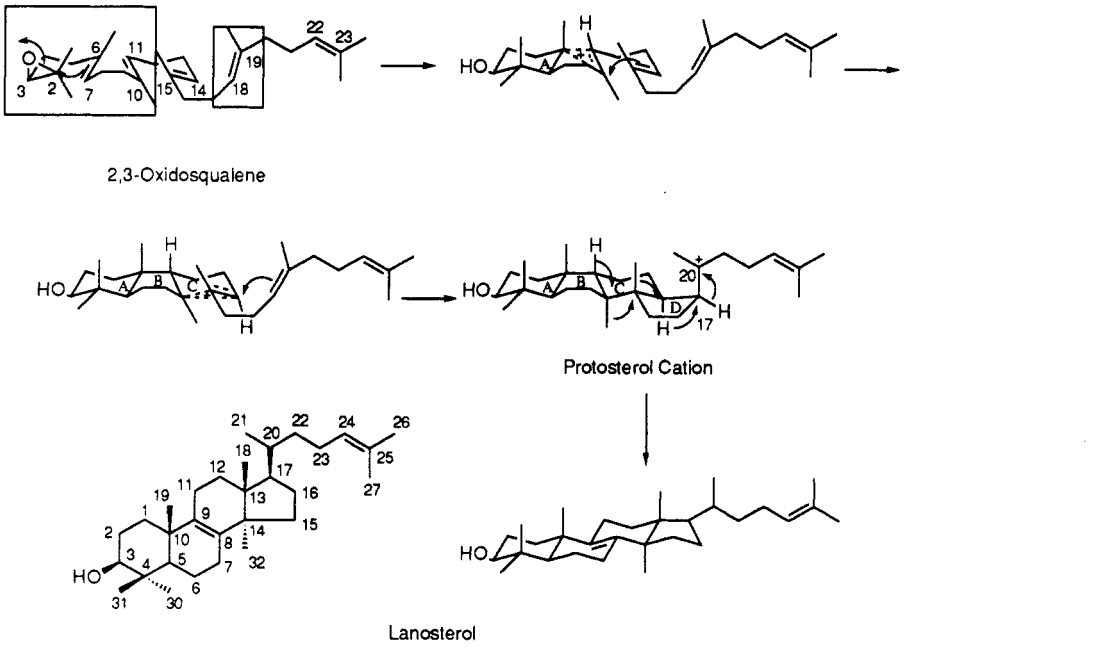
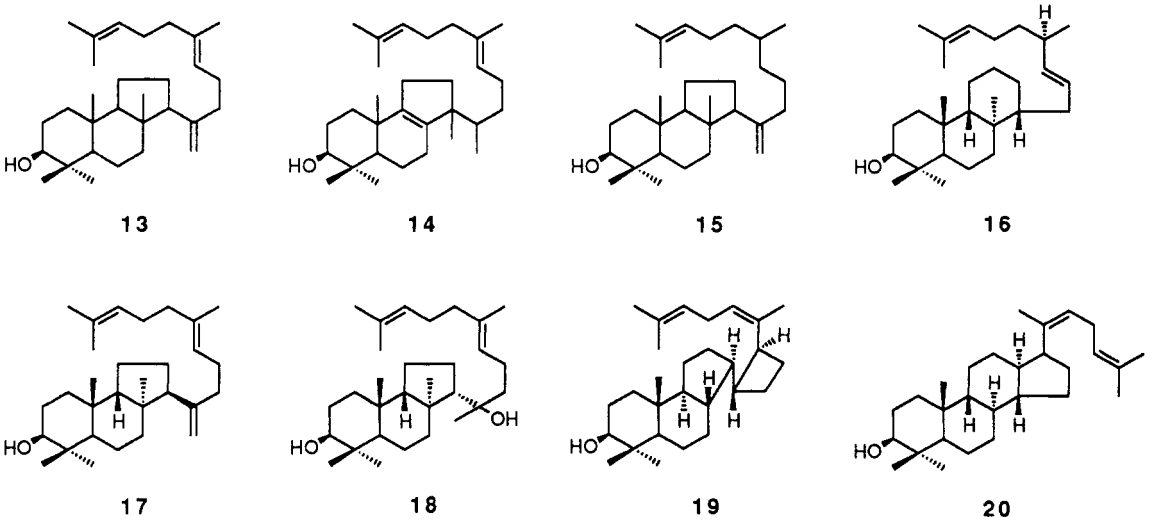


Chart II

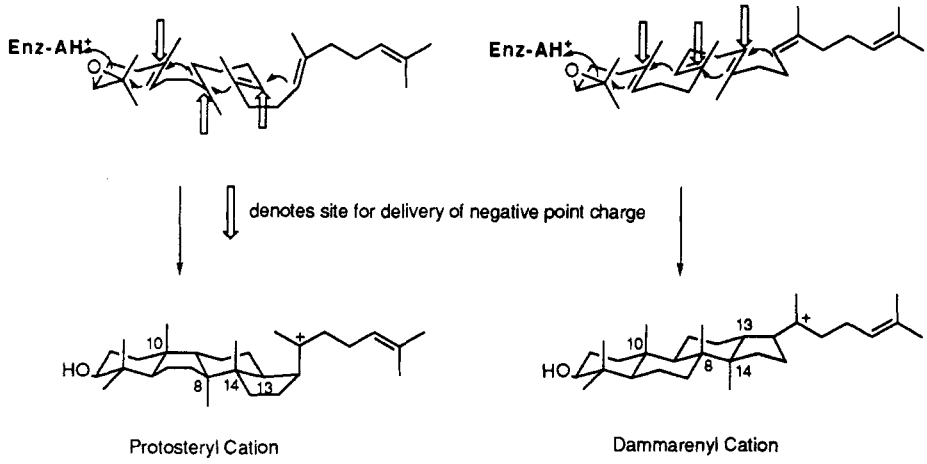


thought to be "trapped", partially cyclized intermediates, have been isolated from nature.[37,38] Further, as we will discuss later, when incubated with hexaprenyl ether, squalene:tetrahymanol cyclase produces bi-, tri-, tetra-, and pentacyclic skeletons corresponding to all possible cationic intermediates of a possible stepwise cyclization process.[39]

During the enzymatic cyclization of oxidosqualene, the six-membered C-ring is formed *via* a thermodynamically unfavorable secondary ("6.6.6-fused") tricyclic cation in a formally anti-Markovnikov addition. In contrast, non-enzymatic cyclization of oxidosqualene affords 6.6.5-fused tricyclic products (**13** and **14**, Chart II), which are formed from a thermodynamically favored tertiary cation with a five-membered C-ring.[40] Such a 6.6.5-fused tricyclic product (**15**) is also obtained by enzymatic conversion of 18,19-dihydro-2,3-oxidosqualene (**21**) (Chart III), a substrate lacking the Δ^{18} double bond.[41] The liver enzyme cyclizes 15-nor-2,3-oxidosqualene (**40**) (Chart IV) to 13-norlanosterol almost as efficiently as it cyclizes the natural substrate.[42] On the other hand, 15-nor-18,19-dihydro-2,3-oxidosqualene (**41**) is enzymatically transformed to a tricyclic product with a six-membered C-ring (**16**), a reaction which apparently involves hydrogen transfer from the side chain to the cationic C-ring intermediate.[43] This suggests carbons C-14 and C-18 in the substrate must be held in close proximity by the enzyme. Such constraints should also operate on the natural substrate so that C-14 and the Δ^{18} double bond would be similarly juxtaposed, thereby ensuring bond formation between these centers in an anti-Markovnikov sense.[10] Van Tamelen proposed that such an intermediate cation should be formulated as a nonclassical species to best rationalize the partitioning between five- and six-membered rings, to maintain the enzyme-enforced proximity of carbons, and to preserve stereochemistry during cyclization.[10]

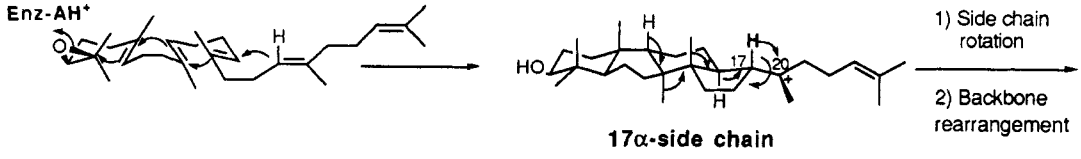
Recently, Johnson proposed a new mechanism for the action of oxidosqualene cyclase which involves axial delivery of negative point charges by the enzyme so as to form ion pairs which could stabilize the developing cationic centers on the cyclizing substrate (Scheme II).[44-47] This model has the advantage that no particular

Scheme II

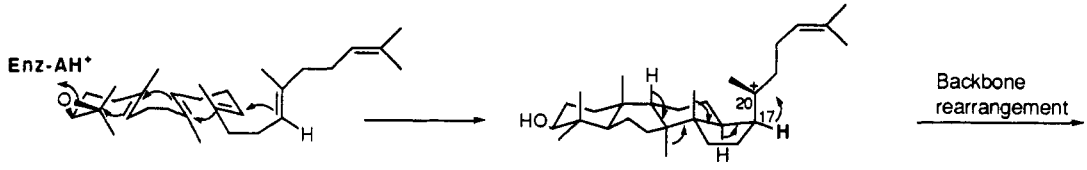


Scheme III

A. Cornforth (1968)



B. Corey & Virgil (1991)

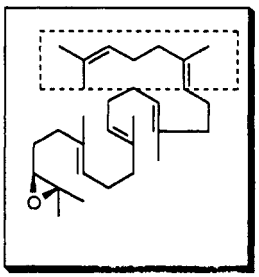


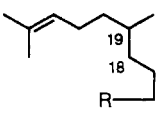
conformational control is required by the enzyme and the expected transition-state stabilization accounts for the boat ring B as well as the anti-Markovnikov closure of ring C. Thus, the boat closure of ring B could be promoted by delivery of a point charge to the α-face at *pro*-C-8, thereby lowering the activation energy of the boat relative to the chair closure. Another point charge proximal to the α-face at *pro*-C-13 but not to *pro*-C-14 could guide the course of reaction in the anti-Markovnikov manner. It has been further postulated that such charge delivery to the β-face at *pro*-C-10 may be important in enhancing the rate and efficiency of the overall cyclization process. However, other steric and electrostatic factors such as hydrophobic interaction and cation-π interaction could also shelter incipient cations, and there is reason to believe aromatic amino acid residues at the active site could play a crucial role in the cyclization reaction.[18]

How oxidosqualene cyclase achieves the C-20*R* stereochemistry found in lanosterol and cycloartenol has been long debated. According to the "biogenetic isoprene rule",[3] the first formed protosterol C-20 cation has 17α-oriented side chain, and in order to produce the natural C-20*R* configuration, a 120° rotation of the side chain around the C-17–C-20 bond is required prior to proton migration from C-17 to C-20. For this reason, the tetracyclic intermediate was believed to be stabilized by the covalent attachment of nucleophilic group of the enzyme, an "X-group", to the *re* face of C-20, while the rest of the molecule is reoriented in the configuration favorable to subsequent rearrangement (Scheme IIIA).[48,49] However, Corey and Virgil recently demonstrated that the 20-oxa analogue of 2,3-oxidosqualene (**22**) and (20*E*)-20,21-dehydro-2,3-oxidosqualene (**23**) (Chart III) are enzymatically transformed to protosterols having a 17β side chain, clearly demonstrating that the ring-D closure during lanosterol formation produces a protosterol with the 17β-oriented side chain (Scheme IIIB).[11,35] In this case, the initially formed conformation of the protosteryl C-20 cation can lead to the natural C-20*R* configuration *via* a least motion pathway involving only a small (<60°) rotation about the C-17–C-20 axis. Further, the β-orientation of the side chain at C-17 is thought to also facilitate the control of configuration at C-20 in lanosterol formation, since the protosterol cation is generated in the correct geometry for C-17–C-20 hydride migration and since C-17–C-20 bond rotation is restricted by the strong steric interaction between the side chain and the *cis*-14β-methyl substituent.

For the enzymatic formation of the protosterol intermediate, it has been demonstrated that a mi-

Chart III





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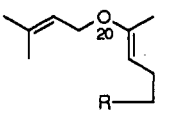

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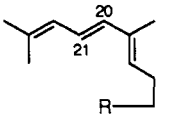

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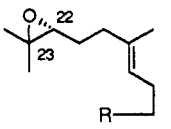

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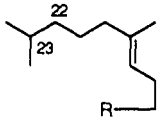

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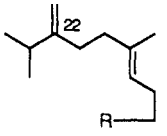

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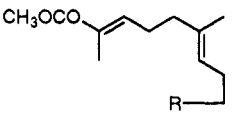

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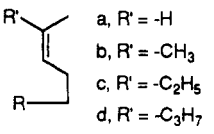
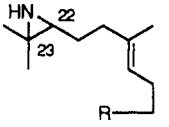
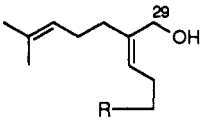


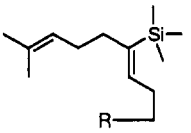
28a-28d (+)



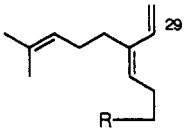
29 (+)



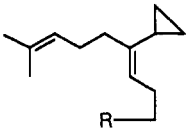
30 (+)



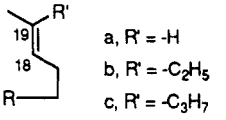
31 (+)



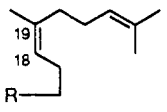
32 (+), Inhibitor



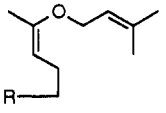
33 (-)



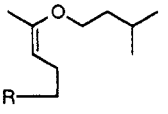
34a-34c (+)



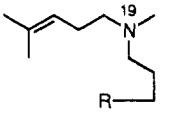
35 (+)



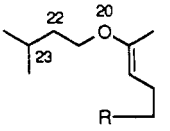
36 (-)



37 Inhibitor



38 Inhibitor



39 Inhibitor

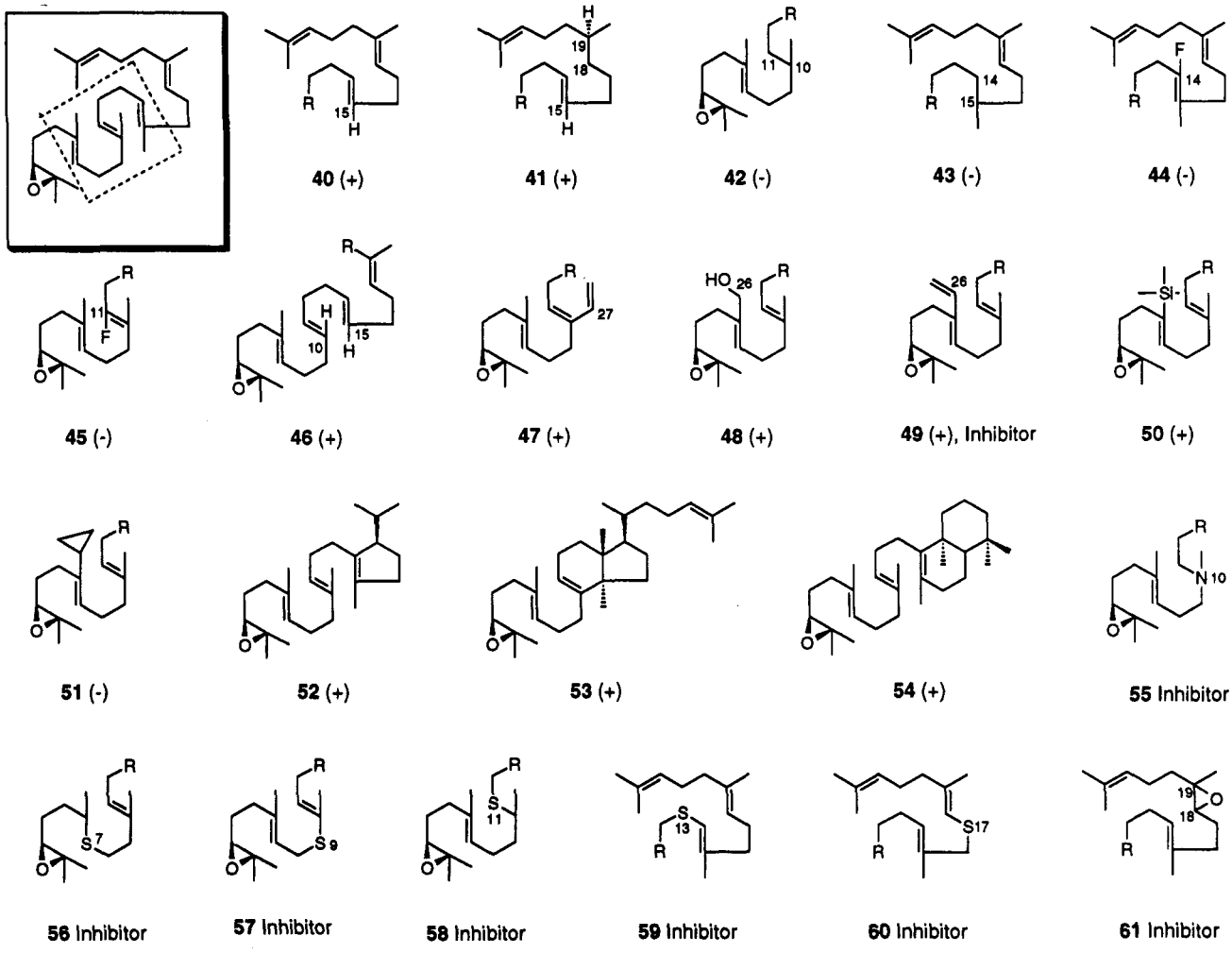
crosomal preparation from the fungus *Cephalosporium caerulens* can cyclize oxidosqualene into lanosterol and 3β-hydroxyprotosta-17(20)[16,21-*cis*],24-diene (**7**).[50] The latter compound is a biosynthetic precursor of an antibiotic, fusidic acid and possesses the unique protosterol skeleton.[51] In this case, cyclization of oxidosqualene proceeded *without* the backbone rearrangement, and the first formed C-20 protosterol cation was stabilized by proton elimination of H-17 to form a double bond between C-17 and C-20. This hypothesis was confirmed by the incorporation of (3*RS*,4*R*)-[2-^{14}C,4-^{3}H]mevalonic acid (MVA) into fusidic acid in the fungus *Fusidium coccineum*, since the tritium atom derived from 4-*pro*-*R* position of MVA and located at C-17 of the protosterol cation was lost *via* an elimination during fusidic acid formation.[51,52]

B. Substrate Specificity

The substrate specificity of oxidosqualene:lanosterol cyclase has been probed with a variety of substrate analogues, as summarized in Charts III–V. Each chart shows substrates which test a specific region of the 2,3-oxidosqualene structure. Each compound prepared is annotated as to whether or not it is a substrate for the enzyme (or an inhibitor in some cases).

The Chart III shows substrates with modification in the *pro*-side chain region of 2,3-oxidosqualene. The enzyme accepted most modifications of this terminal side chain region; 2,3:22,23-dioxidosqualene (**24**),[53,54] 22,23-dihydro-2,3-oxidosqualene (**25**),[53] 22,23-dihydro-22-methylene-2,3-oxidosqualene (**26**),[55] 30-acetyl-2,3-oxidosqualene (**27**),[56] and oxidosqualene analogues with truncated side chains (**28a–d**)[57,58] could all be cyclized. Even 2,3-iminosqualene (**75**) (Chart V), which is known as a potent inhibitor of the cyclase,[59] has been shown to be metabolized to 24,25-iminolanosterol [presumably *via* 22,23-imino-2,3-oxidosqualene (**29**)] in fungi.[60] Certain modifications at the C-29 methyl group were also acceptable. 29-Hydroxy-2,3-oxidosqualene (**30**),[61] and 29-(trimethylsilyl)-2,3-oxidosqualene (**31**)[62] were transformed to lanosterol analogues. 29-Methylidene-2,3-oxidosqualene (**32**), the first developed potent irreversible mechanism-based inhibitor, could also be cyclized if concentration was less than K_I.[33] However, 29-cyclopropyl-2,3-oxidosqualene (**33**) was poorly accepted.[62] The truncated oxidosqualene analogues possessing Δ^{18} double bonds with the unnatural *Z* stereochemistry (**34a–c**) produced the norlanosterols having the unnatural 20*S* stereochemistry.[57,63,64] In contrast, the 2,3-oxidosqualene with the unnatural *Z* stereochemistry at Δ^{18} (**35**) was cyclized to the mixture of 6.6.5-fused tricyclic products (**17** and **18**), both having *trans/syn/trans* A/B/C ring junctions.[65] The 20-oxa analogues with the *Z* stereochemistry at Δ^{18} (**36** and **37**) were not accepted as substrates.[35,66]

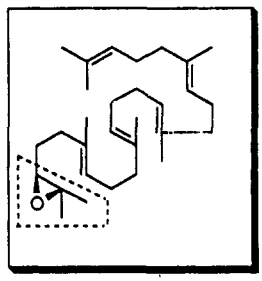
Chart IV

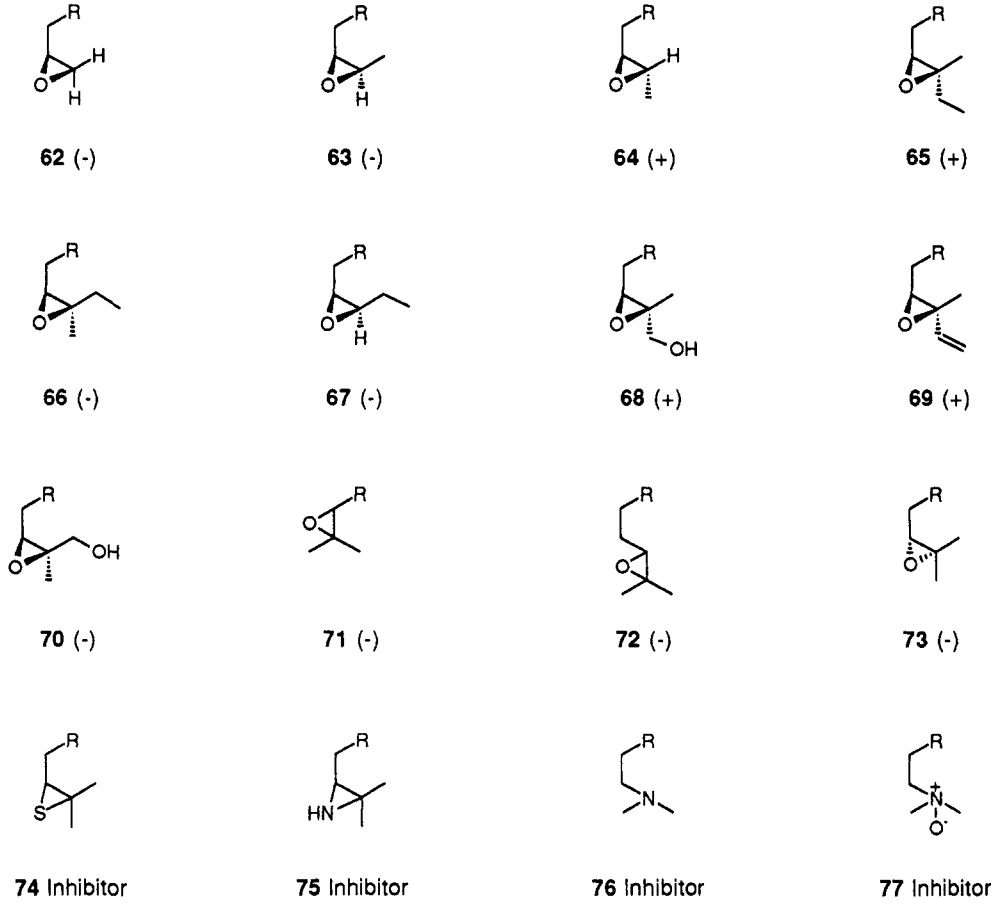


In Chart IV, the modifications of the internal polycyclic region of 2,3-oxidosqualene are explored. The presence of the Δ^{10} and Δ^{14} double bonds of 2,3-oxidosqualene are essential for the overall cyclization process. Neither 10,11-dihydro-2,3-oxidosqualene (**42**) or 14,15-dihydrooxidosqualene (**43**) afforded any cyclization products.[67] Further, 14-fluoro-2,3-oxidosqualene (**44**) and 11-fluoro-2,3-oxidosqualene (**45**) were poorly accepted as substrates.[62] The product of the enzymatic cyclization of 10,15-didesmethyl-2,3-oxidosqualene (**46**) was recently revised to be a unique tetracyclic compound with 6.6.5-fused A/B/C ring system and a pendant four-membered "D" ring (**19**), instead of the unrearranged protosterol derivative structure (**20**).[68,69] Interestingly, this substrate also functioned as a very effective time-dependent irreversible inhibitor of the yeast cyclase.[69] It has been demonstrated that an "ultrasonically stimulated" suspension of bakers' yeast (*Saccharomyces cerevisiae*) can be employed for the gram-scale enzymatic cyclization of oxidosqualene and selected analogues.[56] By using this preparation, 27-methylidene-2,3-oxidosqualene (**47**) was converted to a 30-methylidene lanosterol analogue, which was the first demonstration of the remarkable ability of the lanosterol cyclase to rearrange a substituent other than a hydrogen or methyl group.[70] 26-Hydroxy-2,3-oxidosqualene (**48**),[61] 26-methylidene-2,3-oxidosqualene (**49**),[33] and 26-(trimethylsilyl)-2,3-oxidosqualene (**50**)[62] were also accepted as substrates; in contrast, 26-cyclopropyl-2,3-oxidosqualene (**51**) was not cyclized.[62] Even partially cyclized mono- and bicyclic substrate anlogues (**52** and **53**) could be enzymatically transformed to pentanorlanosterol and dihydrolanosterol, respectively;[71] in contrast, *nonenzymic* transformation of these compounds afforded pentanoreuphenol and Δ^{8}-dihydrolanosterol, respectively.[72] Furthermore, it has been shown that lanosterol cyclase did induce cyclization of the bicarbocyclic epoxide (**54**) to the onocerin skeleton.[73]

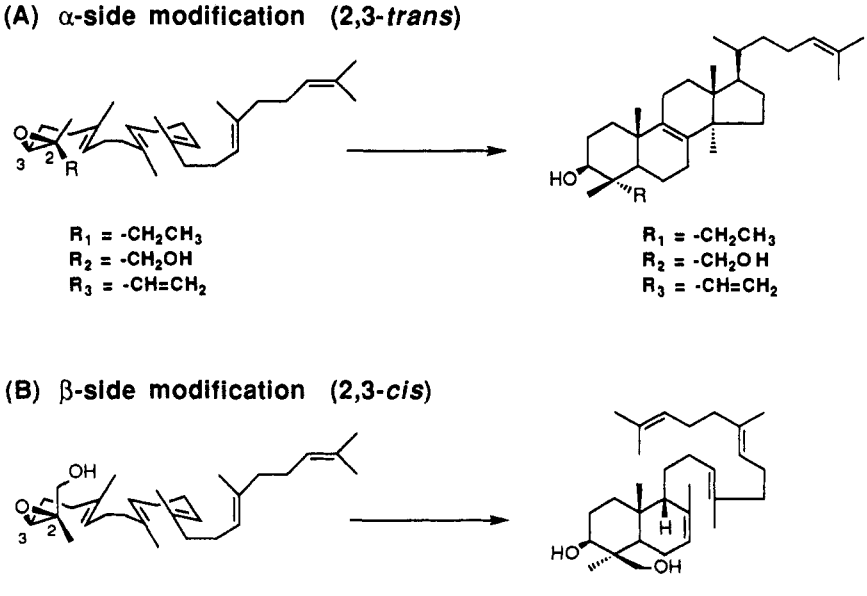
In Chart V, modifications of the cyclization-initiating oxirane ring moiety of 2,3-oxidosqualene are explored. First, 1,1′-bisnor-2,3-oxidosqualene (**62**),[74] and 2,3-*cis*-1′-nor-2,3-oxidosqualene (**63**)[75] were not cyclized, while 2,3-*trans*-1′-nor-2,3-oxidosqualene (**64**) was acceptable.[75] Surprisingly, 2,3-*trans*-1-methyl-2,3-oxidosqualene (**65**) was much more efficiently cyclized to 4α-ethyl lanosterol analogue than the normal substrate.[74,76] However, 2,3-*cis*-1-methyl-2,3-oxidosqualene (**66**) and 2,3-*cis*-1-methyl-1′-nor-2,3-oxidosqualene (**67**) were not cyclized.[74,76] Recently, 2,3-*trans*-1-hydroxy-2,3-oxidosqualene (**68**),[77] and 2,3-*trans*-1-methylidene-2,3-oxidosqualene (**69**)[78] were found to be enzymatically cyclized to 4α-hydroxymethyl and 4α-vinyl analogue of lanosterol, respectively. In contrast, an entirely different cyclization pathway was found for 2,3-*cis*-1-hydroxy-2,3-oxidosqualene (**70**) and only a partially cyclized bicyclic product was obtained (Scheme IV).[77] Kyler suggested that although the enzyme easily

Chart V





Scheme IV

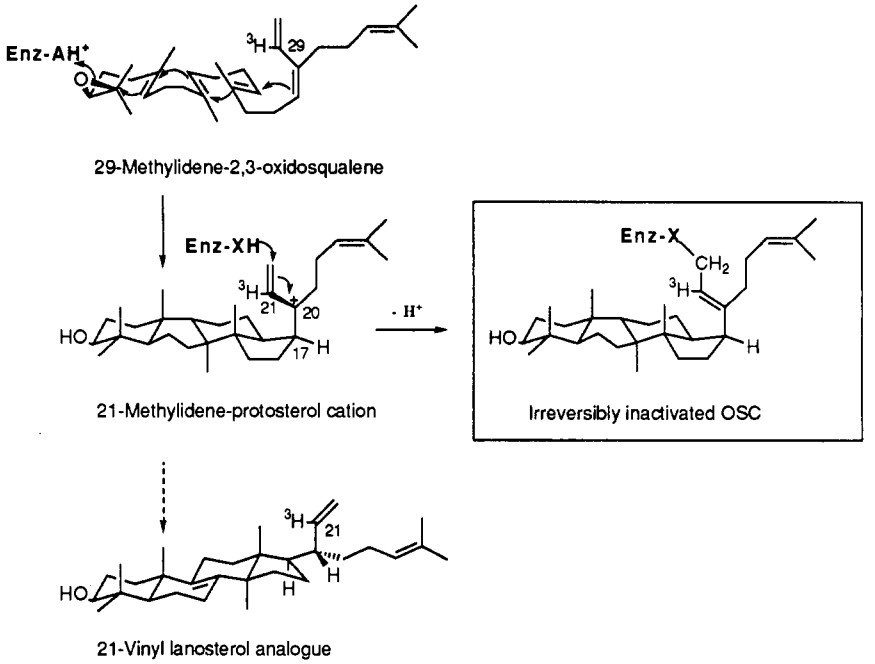


tolerated modifications at the *pro*-α-side of the incipient sterol "plane", stereoelectronic effects operating at the β-face of the conformationally-folded substrate dramatically altered the course of the cyclization pathway. Furthermore, 4-nor-2,3-oxidosqualene (**71**) and homooxidosqualene (**72**) did not afford any detectable cyclization products.[79] As described before, eukaryotic OSCs do not accept (3*R*)-2,3-oxidosqualene (**73**) as a substrate.[9] 2,3-Sulfidosqualene (**74**) has been reported as a weak inhibitor of OSC.[59]

C. Enzymology

Although many investigations have been performed on the mechanistic aspects of oxidosqualene cyclization reactions, the cyclase itself had remained incompletely characterized until recently. Membrane-bound sterol and triterpene cyclases have been found in many organisms; however, attempts to purify cyclases have met with difficulties in obtaining a solubilized enzyme in an active form. In particular, cyclase activities

Scheme V



seemed to depend not only on detergents but also on the salt concentration, making the purification difficult. For example, it had been reported that the solubilized hog liver cyclase requires both anionic detergent, deoxycholate, and high salt concentration for its active form,[80] whereas soluble yeast lanosterol cyclase is active in solution of low ionic strength and stimulated by nonionic detergent, Triton X-100.[81] Further, since the solubilized enzyme was rather unstable, it was therefore essential to find enzyme stabilizing conditions.

The breakthrough in purification of the oxidosqualene cyclases (OSCs) was first achieved by the purification of several plant triterpene cyclases.[21-24] The enzyme activities were efficiently solubilized by Triton X-100 and purified to homogeneity in the presence of detergent and stabilizing reagents. By employing essentially the same methods, rat liver oxidosqualene:lanosterol cyclase was for the first time completely purified up to 1863-fold with 28% recovery.[19] The purified rat liver enzyme showed a single band on SDS-PAGE with molecular mass of 75 kDa, an isoelectric point of 5.5, and an apparent K_M value for (3*S*)-oxidosqualene of 55 μM. Recently, we found that vertebrate OSCs can be rather easily purified by employing a simple combination of DEAE-cellulose followed hydroxylapatite column chromatography after solubilization of the cyclase with Triton X-100.[20] A rapid purification of rat liver OSC using another detergent, lauryl maltoside, has also been reported, but the homogeneity of this preparation was less acceptable.[82] Finally, affinity labeling experiments using the first potent mechanism-based irreversible inactivator of the cyclase, 29-methylidene-2,3-oxidosqualene (29-MOS) (Scheme V),[33] demonstrated that the vertebrate OSCs were specifically labeled with [^{3}H]29-MOS and gave single bands with molecular masses ranging from 70 to 80 kDa (rat, 78 kDa; dog, 73 kDa; pig, 75 kDa; and human, 73 kDa).[20]

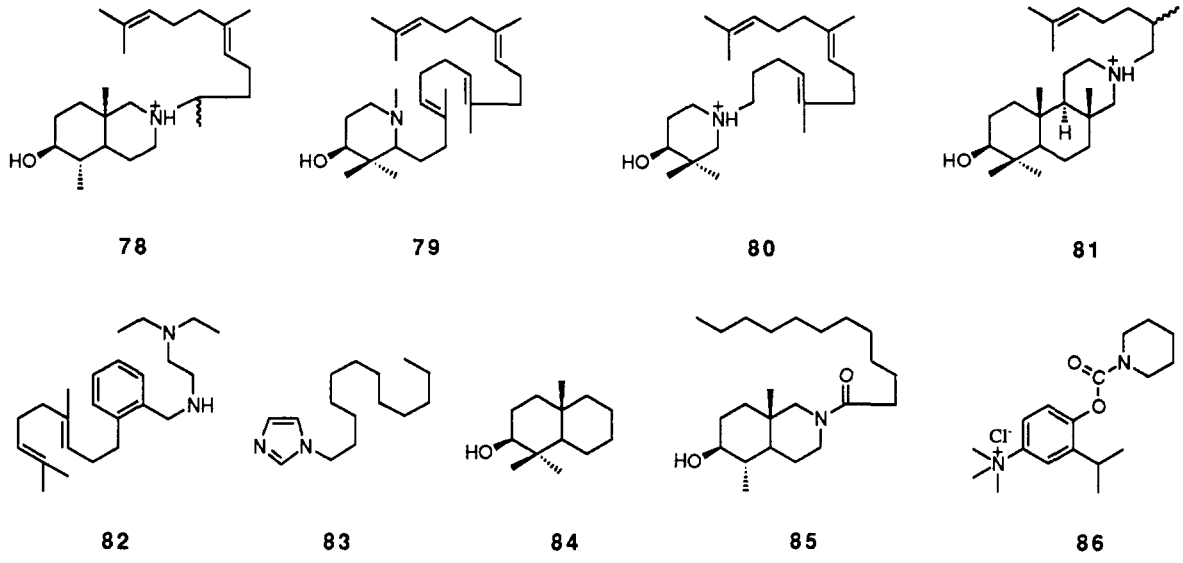
Yeast (*S. cerevisiae*) lanosterol cyclase was purified by affinity chromatography and was reported to have rather small molecular mass of 26 kDa,[25] but the possibility that this is a degradation product cannot be ruled out. The yeast cyclase cannot be labeled with 29-MOS,[20] which suggests a subtle species difference in the structures of the active site as well as global differences. Other examples of partial purification of lanosterol cyclases have been also reported from hog liver[83] and yeast.[84,85] Very recently, an oxidosqualene:lanosterol cyclase from the fungus *Candida albicans* was for the first time cloned by complementation of a *S. cerevisae* OSC deficient mutant (*erg* 7).[29,30,86] The open reading frame consists of 2187 nucleotides and encodes a predicted protein of 728 amino acids with molecular mass of 83.7 kDa. A hydropathy plot indicates that the enzyme is a moderately hydrophilic protein with two notable hydrophobic regions.[29] However, no enzyme activity has yet been reported for this protein. Although no significant DNA similarities were found with sequences contained in GenBank 71 or EMBL, significant similarity at the level of the predicted amino acid sequence was obtained with the sequence of *Bacillus acidocaldarius* squalene:hopene cyclase gene, which had been also cloned and sequenced too recently to have been included in these data bases.[28] Four regions of notable similarity varied from 28% identity over 77 residues to 46% identity over 37 residues.[29] The finding of these identities provides support Ourisson's model for divergent evolution of cyclase genes.[15,16]

D. Enzyme Inhibitors

It has been reported that both pig liver cyclase and yeast cyclase were efficiently inhibited by *p*-(chloromercuri)benzenesulfonic acid and *N*-ethylmaleimide, suggesting a cysteinyl residue essential for catalysis in the active site of the enzyme.[83,84] Diethyl pyrocarbonate, a histidyl-selective reagent, did not inhibit these eukaryotic OSCs.[83,84]

Besides the above mentioned 29-methylidene-2,3-oxidosqualene (**32**), the first mechanism-based potent irreversible inhibitor (IC_{50} = 0.5 μM, K_I = 4.4 μM, k_{inact} = 221 min^{-1} for liver OSC),[33] a variety of inhibitor

Chart VI



78 79 80 81

82 83 84 85 86

compounds have been synthesized and reported, which are also summarized in Charts III–V. 2,3-Iminosqualene (**75**) (IC_{50} = 0.4 μM for liver cyclase),[59,87] 2-aza-2,3-dihydrosqualene (**76**) (IC_{50} = 7.5 μM for liver cyclase), 2-aza-2,3-dihydrosqualene *N*-oxide (**77**) (IC_{50} = 3.7 μM for liver cyclase), and their derivatives have been reported as potent cyclase inhibitors.[87,88] The secondary and tertiary amines are protonated at physiological pH and would present some structural and charge similarities to the incipient C-2 carbocation generated by the oxirane ring opening of 2,3-oxidosqualene. The degree of their inhibitory activity was reported to be greater in higher plants than in rat liver or fungi.[89] A bicyclic 8-azadecalin derivative, *N*-(1,5,9-trimethyl decyl)-4α,10-dimethyl-8-aza-*trans*-decal-3β-ol (**78**) (IC_{50} = 2 μM for liver cyclase) was designed to mimic a high-energy intermediate bearing a positive charge at C-8; this material was a strong inhibitor of plant cycloartenol cyclase, but did not inhibit β-amyrin cyclase.[90]

Similarly, acyclic, monocyclic, and tricyclic compounds possessing nitrogen atoms situated at positions corresponding to the carbenium ion of high energy intermediates or transition states involved during cyclization of oxidosqualene have been shown to inhibit the cyclase activity. Monocyclic 4-hydroxy-2,3-substituted-4-piperidine (**79**) (IC_{50} = 0.23 μM for fungal cyclase)[91,92] and *N*-alkylhydroxypiperidine (**80**) (IC_{50} = 1 μM for liver cyclase)[93] showed strong inhibition (Chart VI). In contrast, 13-aza tricyclic derivatives **81** displayed little inhibition for oxidosqualene cyclases.[93] The acyclic analogues 10-aza-10,11-dihydro-2,3-oxidosqualene (**55**) (IC_{50} = 4.8 μM for liver cyclase),[94,95] 19-aza-18,19-dihydro-2,3-oxidosqualene (**38**) (IC_{50} = 1.5 μM for liver cyclase),[96] the 20-oxa vinyl ether analogue of 2,3-oxidosqualene (**39**)(IC_{50} = 80 μM for liver cyclase)[66] and several sulfur-containing oxidosqualene analogues **56–60**[97] also showed significant inhibition of cyclase activity. Other potent inhibitors such as 2,3:18,19-dioxidosqualene (**61**) (IC_{50} = 0.11 μM for liver cyclase),[98,99] compound **82** (IC_{50} = 0.7 μM for fungus cyclase),[100] *N*-(1-*n*-dodecyl)imidazole (**83**) (IC_{50} = 3.9 μM for liver cyclase) and its derivatives,[101] *trans*-decalin derivatives (4,4,10β-trimethy-*trans*-decal-3β-ol (**84**); IC_{50} = 9 μM for liver cyclase),[102,103] and *N*-(1-oxododecyl)-4α,10-dimethyl-8-aza-*trans*-decal-3β-ol (**85**) (IC_{50} = 0.1 μM for HepG2 cyclase)[104] have been also reported. A comprehensive review of inhibitors of squalene synthase, squalene epoxidase, and oxidosqualene cyclase activities will be presented elsewhere.[105]

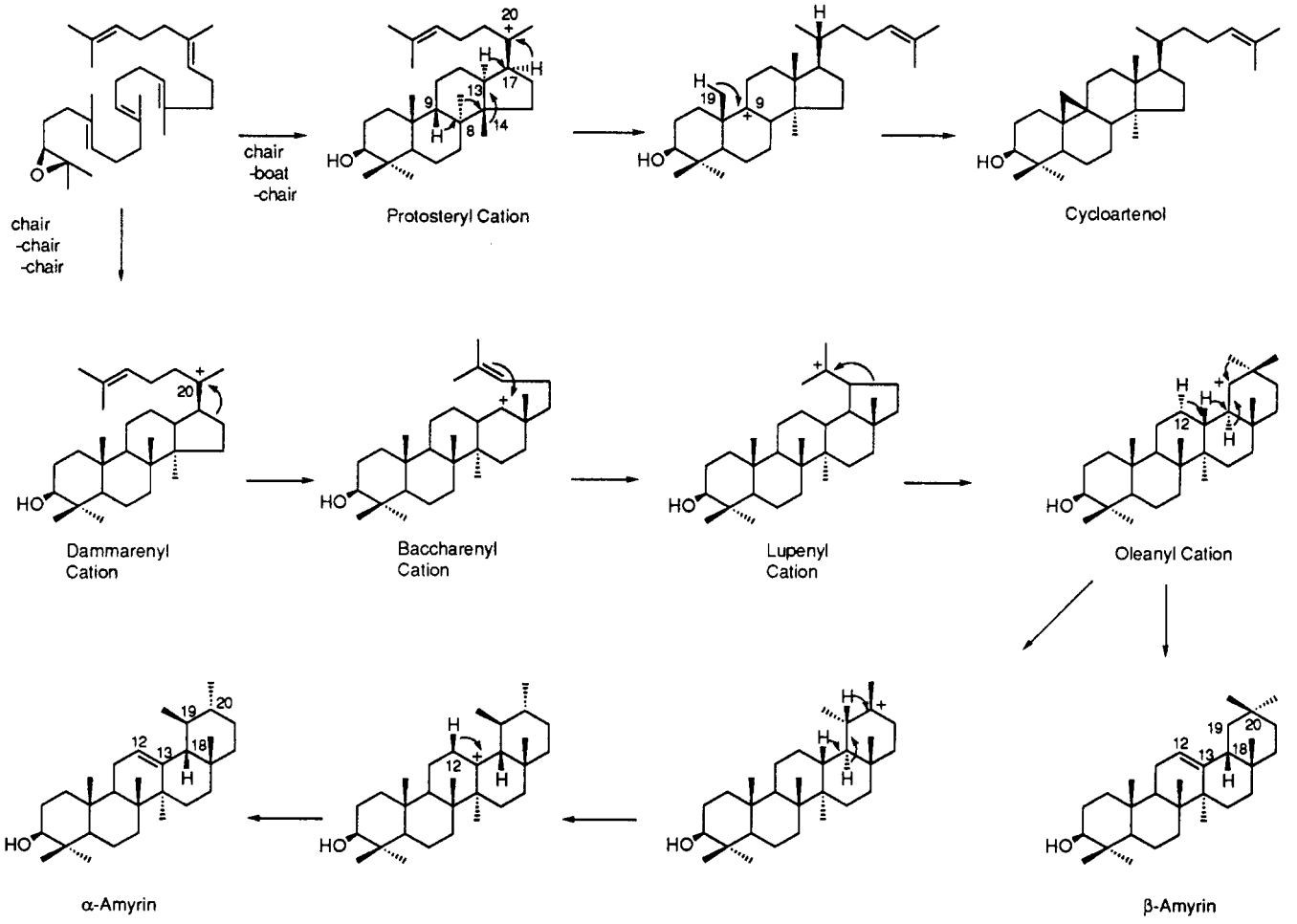
III. Plant Oxidosqualene Cyclases

A. Cyclization Mechanism

In higher plants, oxidosqualene is the biosynthetic intermediate common to both steroids and triterpenoids.[13] During phytosterol biosynthesis, it is cyclized to cycloartenol (**4**), which corresponds to lanosterol in animals and fungi.[106] A variety of tetracyclic and pentacyclic triterpenes widely distributed in plants are also cyclization products of oxidosqualene. These terpenoids are further converted into a variety of steroid and triterpenoid saponins and steroidal alkaloids; in addition, some plants contain large quantities of triterpenes in their latexes and resins. These latter compounds are regarded as "secondary metabolites", and their physiological function is generally ascribed to chemical defense against pathogens and herbivores. The *raison d'être* and regulatory mechanisms for multiple cyclase enzymes in plants are incompletely understood.

The postulated mechanism of cyclization of oxidosqualene into cycloartenol is essentially the same as that for lanosterol cyclization, except for the final 9β,19-cyclopropane ring closure instead of C-9 proton elimination (Scheme VI).[12] The final migration of a hydrogen of the C-19 methyl group would be *cis* to the preceeding C-9 hydrogen migration. Elegant studies by Altman and co-workers showed that the stereochemistry of the cyclopropane ring closure proceeds with retention of configuration.[107] Thus, 2,3-oxidosqualene bearing a chiral methyl group (D, H, T) at C-6 was synthesized and cyclized with the phytoflagellate *Ochromonas malhamensis* oxidosqualene:cycloartenol cyclase. Then, the NOE suppressed, proton-decoupled ^{3}H NMR spectrum of tritium-labeled biosynthetic cycloartenol showed that the predominant

Scheme VI



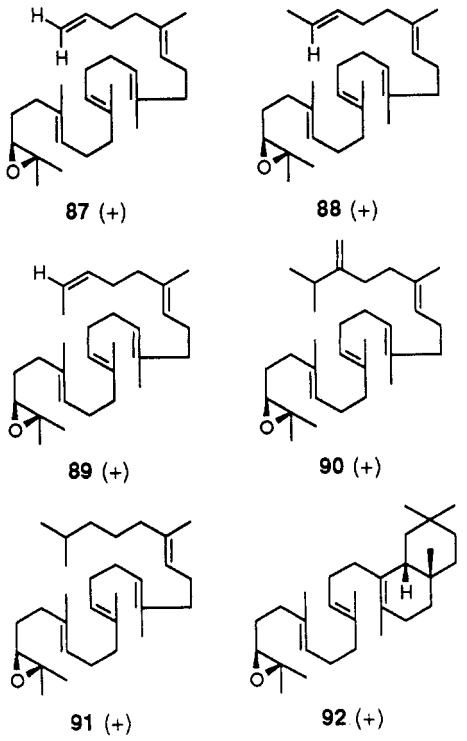
species contained an *exo*-cyclopropyl tritium in molecules which also have an *endo*-deuterium, clearly indicating retention of configuration. Recently, direct evidence for the postulated two 1,2-methyl migrations (from C-14 to C-13 and from C-8 to C-14) and three 1,2-hydride shifts (20-H from C-17, 17-H from C-13, and 8-H from C-9) was obtained by incorporation experiment of [2-$^{13}C^2H_3$]acetate using cultured cells of higher plants, *Rabdosia japonica*[108] and *Physalis peruviana*.[109]

Cyclization to pentacyclic triterpenes such as β-amyrin (olean-12-en-3β-ol) (**5**) and α-amyrin (urs-12-en-3β-ol) (**6**) probably proceeds *via* the all prechair conformation of oxidosqualene.[3] In principle, the proton-initiated cyclization first produces the tetracyclic dammarenyl C-20 cation, and the subsequent rearrangement leads to the pentacyclic oleanyl cation *via* the baccharenyl and lupenyl cationic intermediates. Finally, a series of 1,2-hydride shifts with elimination of the H-12 proton gives the β-amyrin or α-amyrin framework with Δ^{12} double bond. Two 1,2-hydride shifts in β-amyrin formation (one from C-18 to C-19 and one from C-13 to C-18) and three 1,2-hydride shifts in α-amyrin formation (from C-19 to C-20, from C-18 to C-19, and from C-13 to C-18) have been postulated (Scheme VI).[3,110,111] The mechanism was also recently confirmed by incorporation experiment of [2-$^{13}C^2H_3$]acetate and [5-$^{13}C^2H_3$]mevalonolactone into oleanolic acid and ursolic acid using cell suspension cultures of *R. japonica*, by using the β-deuterium isotope effect.[112,113] The proton and deuterium simultaneously decoupled ^{13}C NMR spectra of the labeled compounds verified that the 12-*pro*-*S* (H-12α) proton was eliminated to form β-amyrin, while the 12-*pro*-*R* (H-12β) proton was eliminated (1,2 *cis* elimination) in α-amyrin formation. Moreover, squalene, an achiral and symmetrical molecule, was released from squalene synthase; thus, the subsequent squalene epoxidase reaction did not distinguish the two terminal double bonds of squalene. This has been also observed with lanosterol[114] and cycloartenol formation.[108]

B. Substrate Specificity

Pea (*Pisum sativum*) seedling β-amyrin cyclase was shown to cyclize 24,30-bisnor-2,3-oxidosqualene (**87**) (Chart VII) to 29,30-bisnoramyrin.[115] In this case, it should be noticed that in ring-E formation, the postulated lupanyl cation becomes a primary cation or its equivalent. In the same way, (*E*)- and (*Z*)-30-nor-2,3-oxidosqualene (**88** and **89**) were cyclized to 30-nor-β-amyrin and 30-nor-α-amyrin, respectively.[116] Further, 22,23-dihydro-22-methylene-2,3-oxidosqualene (**90**) was transformed to 29,29-dimethyl-30-nor-β-amyrin, implying that ring-E formation in this case could be rationalized by purely chemical factors (Markovnikov manner).[117] 22,23-Dihydro-2,3-oxidosqualene (**91**), a substrate lacking the terminal double bond of oxidosqualene, was enzymatically cyclized to bacchar-12-en-3β-ol.[118] Finally, like lanosterol cyclase, β-amyrin cyclase cyclized a bicyclic analogue of 2,3-oxidosqualene **92**.[119] In contrast, chemical cyclization of the same bicyclic substrate afforded δ-amyrin and not β-amyrin.[120]

Chart VII



Further, corn (*Zea mays*) embryos cycloartenol cyclase cyclized 2,3-*trans*-1′-nor-2,3-oxidosqualene (**64**) (Chart V) to 31-norcycloartenol and 31-norlanosterol with a reduced yield, whereas it did not cyclize 2,3-*cis*-1′-nor-2,3-oxidosqualene (**63**).[121] Bramble (*Rubus fruticosus*) cycloartenol cyclase was shown to cyclize 22,23-dihydro-22-methylene-2,3-oxidosqualene to 24-methylenecycloartanol.[55]

C. Enzymology

The first purification of plant OSC activities was achieved using cell suspension cultures of *R. japonica* which produce olean-12-ene- and urs-12-ene-type triterpenes as well as phytosterols.[23] Three different cyclase activities were present in the microsomal preparation which respectively catalyzed the cyclization of oxidosqualene into cycloartenol, β-amyrin (olean-12-en-3β-ol) and α-amyrin (urs-12-en-3β-ol). All three OSC activities were efficiently solubilized by Triton X-100 treatment. Then, cycloartenol cyclase (p*I* = 6.1) and β-amyrin cyclase (p*I* = 5.4) were completely separated and purified to homogeneity by chromatography on hydroxylapatite, DEAE–cellulose, gel filtration, and isoelectric focusing. The α-amyrin cyclase activity was very low compared to those of the other two cyclases. The purified cycloartenol cyclase showed a single band on SDS-PAGE with molecular mass of 54 kDa, while β-amyrin cyclase gave a single band of 28 kDa. It has been also reported that the purified cycloartenol cyclase obtained from pea (*P. sativum*) seedlings in a similar way had a different molecular mass (55 kDa) from that of β-amyrin cyclase (35 kDa).[21,22,24] The apparent K_M values for (3*S*)-oxidosqualene were 50 μM for cycloartenol cyclase and 40 μM for β-amyrin cyclase, while the reported values for pea cyclases were 25 μM (cycloartenol cyclase) and 50 μM (β-amyrin cyclase). The cyclases required the presence of a nonionic detergent such as Triton X-100 for their highest activity, and each enzyme showed a broad pH optimum within the range of pH 6.5–7.5.

Pea (*P. sativum*) seedlings have been used as an enzyme source of β-amyrin cyclase.[122,123] Production of β-amyrin is very high in pea seeds during development and just after germination, whereas phytosterol synthesis becomes active several days after germination.[124,125] This dramatic shift from triterpenoid to steroid biosynthesis during germination might be related to a change in electrolyte concentration *in vivo*.[124] One hypothesis is that a single "switched" protein might be responsible for the two cyclase activities. Modification of such a protein by proteolytic action, oxidation of SH groups, or change of salt concentration could alter product distributions.[126] As described above, the cycloartenol cyclase and β-amyrin cyclase have been purified as two different proteins having molecular masses of 55 and 35 kDa, respectively.[21,22,24] The structural relationship between the two cyclases is however not clear at the moment. Although similar changes in triterpenoid and steroid biosynthesis in developing seed have been reported with several plants such as *Sorghum bicolor*,[127] it is not known whether this is a common phenomenon in nature.

Oxidosqualene:α-amyrin cyclase activity has also been reported from bramble (*R. fruticosus*) cell suspension culture,[128] and oxidosqualene:10α-cucurbita-5,24-dien-3β-ol (**8**) cyclase activity from *Cucurbita maxima* seedlings.[129] Both cyclase activities are found in microsomal preparations. Further, a cell-free extract of *Ononis spinosa* has been shown to convert squalene-2(3),22(23)-diepoxide to α-onocerin (**12**).[130] Partial purification of cycloartenol cyclase from phytoflagellate, *O. malhamensis* has also been reported.[131]

D. Enzyme Inhibitors

Both cycloartenol cyclase and β-amyrin cyclase were efficiently inhibited by the SH reagents *p*-(chloromercuri)benzenesulfonic acid and *N*-ethylmaleimide and enhanced by the addition of DTT, suggesting the presence of an essential SH group in the active site of the enzyme, as has been postulated for several cyclases.[24] AMO 1618 (**86**), a potent growth retardant which is known to be an inhibitor of OSCs from rat liver and tobacco seedlings[132,133] and of bacterial SC,[134] strongly inhibited cycloartenol cyclase, but showed only partial inhibition of β-amyrin cyclase.[24]

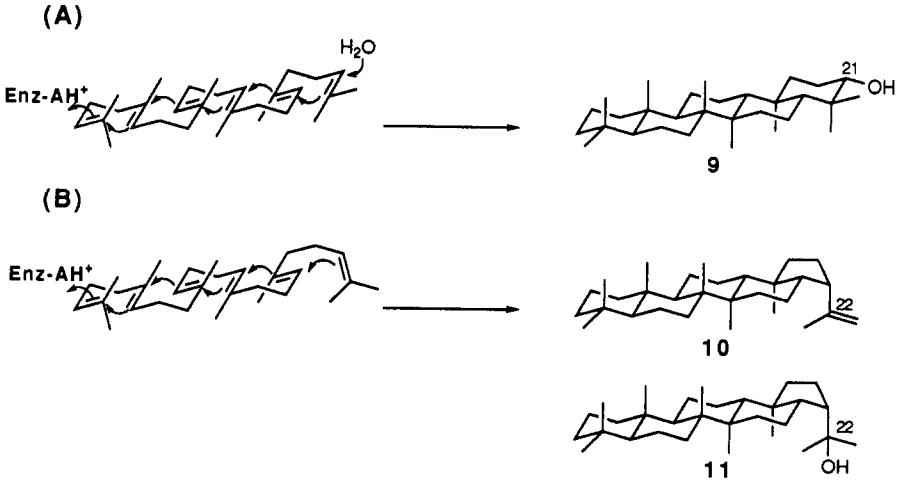
Like lanosterol cyclase, pea seedlings β-amyrin cyclase was strongly inhibited by 2,3-iminosqualene (**75**) (Chart V) (IC_{50} = 0.2 μM), 2-azasqualene (**76**) (IC_{50} = 1 μM), and its derivatives such as **77**.[87–89] Further, it has been demonstrated that the bicyclic 8-azadecalin analogue, *N*-(1,5,9-trimethyl decyl)-4α,10-dimethyl-8-aza-*trans*-decal-3β-ol (**78**) strongly inhibited cycloartenol cyclase (IC_{50} = 1 μM), whereas it did not inhibit β-amyrin cyclase.[90] A monocyclic *N*-alkylhydroxypiperidine (**80**) has been also reported to be a strong inhibitor of cycloartenol cyclase (IC_{50} = 1 μM), but much less effective on β-amyrin cyclase.[93]

IV. Bacterial Squalene Cyclases

A. Cyclization Mechanism

In bacteria, sterols are usually absent, but some bacteria and protozoans produce pentacylic triterpenes

Scheme VII



(3-deoxyhopanoids and/or 3-deoxygammaceranes) which are regarded as sterol surrogates in these organisms.[15,17,135,136] For example, the protozoan *Tetrahymena pyriformis* produces a gammacerane triterpene, tetrahymanol (**9**).[14] The bacterium *Acetobacter pasteurianum* and the thermoacidophilic bacterium *Bacillus acidocaldarius* produce diploptene (hop-22(29)-ene) (**10**) which has been postulated to be a biosynthetic precursor of the ubiquitous bacterial hopanoids.[15,136,137] Interestingly, the obligate methylotroph *Methylococcus capsulatus* contains both 3-deoxyhopanoids and 3β-hydroxy derivatives of lanosterol.[138] The biosynthesis of tetrahymanol has been well studied, and it was established that the formation of tetrahymanol does not proceed *via* the cyclization of oxidosqualene, but *via* a nonoxidative enzyme-catalyzed cyclization of squalene as found for bacterial hopanoids.[139,140] Isotopic labeling experiments with ^2H_2O and $H_2{}^{18}O$ have proved the acquisition from the medium of one hydrogen in the 3β position[141] and one hydroxyl group at C-21 derived from water and not from molecular oxygen.[142] Thus, the cyclization of squalene, folded in its all prechair conformation, is initiated by a proton attack on a terminal double bond and followed by addition of H_2O at the resulting gammaceranyl C-21 cationic center without carbon skeletal rearrangement (Scheme VII).[14] In the case of hopanoid formation, the hopanyl C-22 carbocation with a five-membered E-ring is formed and then followed by either H-29 proton elimination or addition of H_2O at the cationic center yielding diploptene or diplopterol (hopan-22-ol) (**11**), respectively (Scheme VII).[14]

The bacterial SCs have been considered to be rather primitive compared to the eukaryotic OSCs. First, the direct cyclization of squalene in bacteria is an anaerobic process, while, in eukaryotes, squalene is first oxidized by squalene epoxidase, using molecular oxygen. Presumably, the prokaryotic SCs already existed in the anaerobic period of evolutionary stage. Elaboration of the oxidative cyclization of squalene *via* oxidosqualene is a relatively later evolutionary development which could occur only after the evolution of photosynthetic organisms.[14,15]

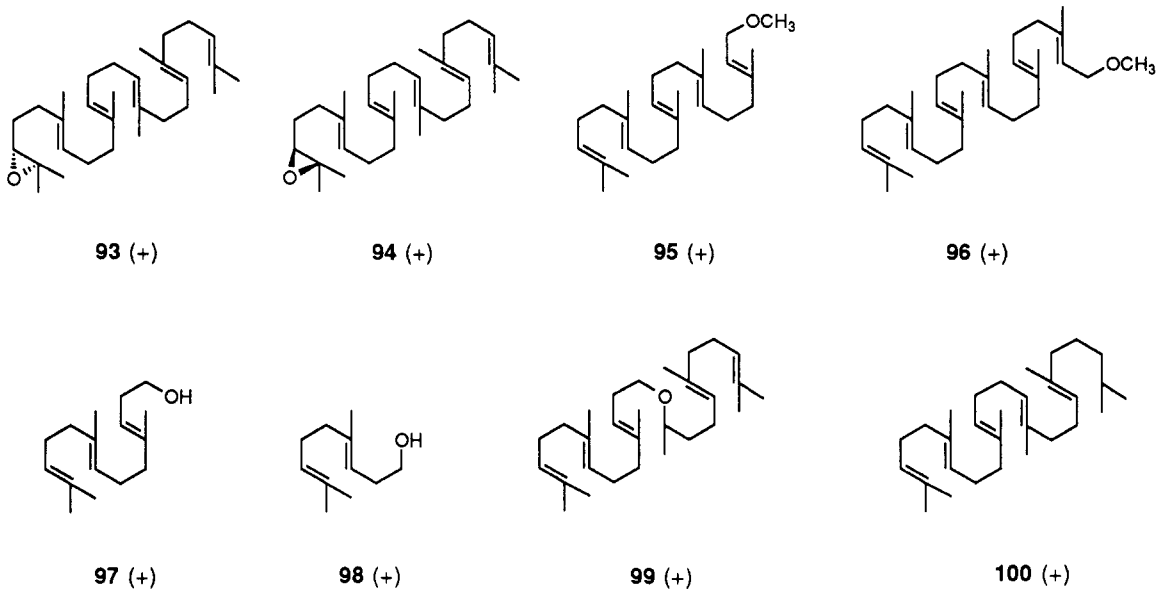
Secondly, SCs catalyze mechanistically and conformationally "simpler" processes. The folding of the squalene molecule in the less strained all "prechair" conformation would require minimal "enzymatic assistance" in the aqueous environment of a cell.[14,17] Further, the cyclization reaction into tetrahymanol or diploptene/diplopterol proceeds without carbon skeletal rearrangement. This might not require such an elaborate enzyme system like eukaryotic OSCs which have to direct and control the apparently complex rearrangement reactions. Finally, as described below, the bacterial SCs display a very low substrate specificity.[15,136] They can cyclize not only the natural substrate squalene, but also both enantiomers of oxidosqualene,[138,143,144] and regular polyprenols.[39,145] In contrast, the eukaryotic OSCs have a rigorous substrate specificity as they do not accept intact squalene or its (3*R*)-epoxide, but cyclize specifically the (3*S*)-oxidosqualene.[9]

B. Substrate Specificity

The tetrahymanol cyclase of *T. pyriformis* cyclized (3*R*)-oxidosqualene (**93**) (Chart VIII) into gammacerane-3α,21α-diol (**101**), while its (3*S*)-enantiomer **94** was cyclized to gammacerane-3β,21α-diol (**102**) (Scheme VIII).[144,146] Cyclization of the isotopically substituted oxidosqualene where the 1-C^2H_3 and the 2-H were in *cis* position showed that during the enzymatic cyclizations of both enantiomers of oxidosqualene, the labeled methyl group completely maintained its stereochemical integrity. Further, it has been also suggested that (3*S*)-oxidosqualene is cyclized in an all prechair conformation, whereas its (3*R*)-enantiomer is cyclized in a preboat conformation for ring A. This is consistent with the observation that in gammacerane-3β,21α-diol, the equatorial methyl group at C-4α was labeled, while in gammacerane-3α,21α-diol, the axial methyl group at C-4β was labeled[144] (Scheme VIII). This conformational versatility is characteristic of bacterial SCs, but has been lost in the eukaryotic OSCs. The hopanoid cyclase of *A. pasteurianum* also showed the same lack of substrate specificity and yielded the 3α-hydroxy- and 3β-hydroxyhopanoids from (3*R*)- and (3*S*)-2,3-oxidosqualene, respectively.[143] A cell-free system from *M. capsulatus*, which produced both 3-deoxyhopanoids and 3β-hydroxy derivatives of lanosterol, did not convert squalene into 3-deoxylanosterol, but cyclized racemic oxidosqualene into a mixture of 3β- and 3α-lanosterol.[138]

A cell-free system from *T. pyriformis* cyclized *all-trans*-pentaprenyl methyl ether (**95**) to a scalarane-

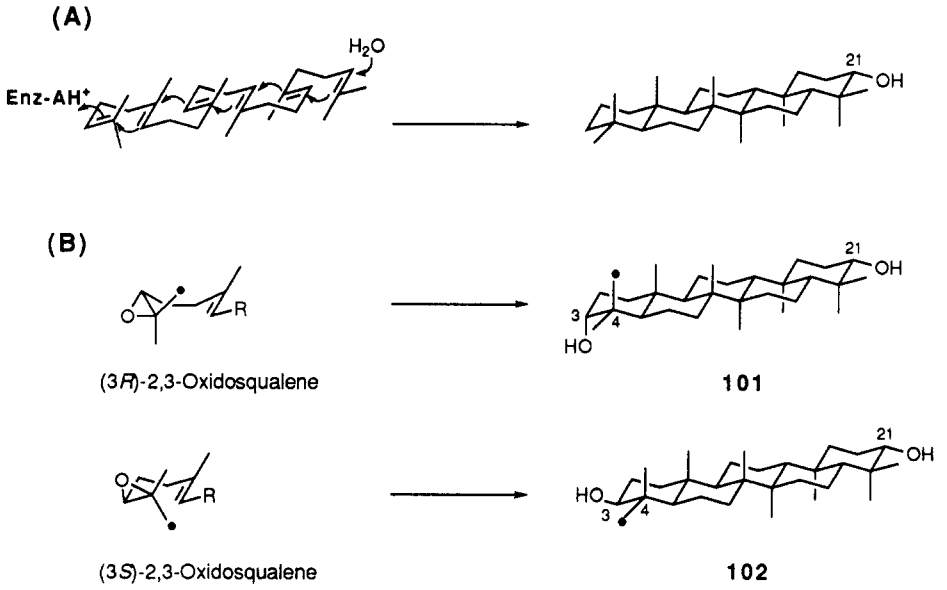
Chart VIII



93 (+) 94 (+) 95 (+) 96 (+)

97 (+) 98 (+) 99 (+) 100 (+)

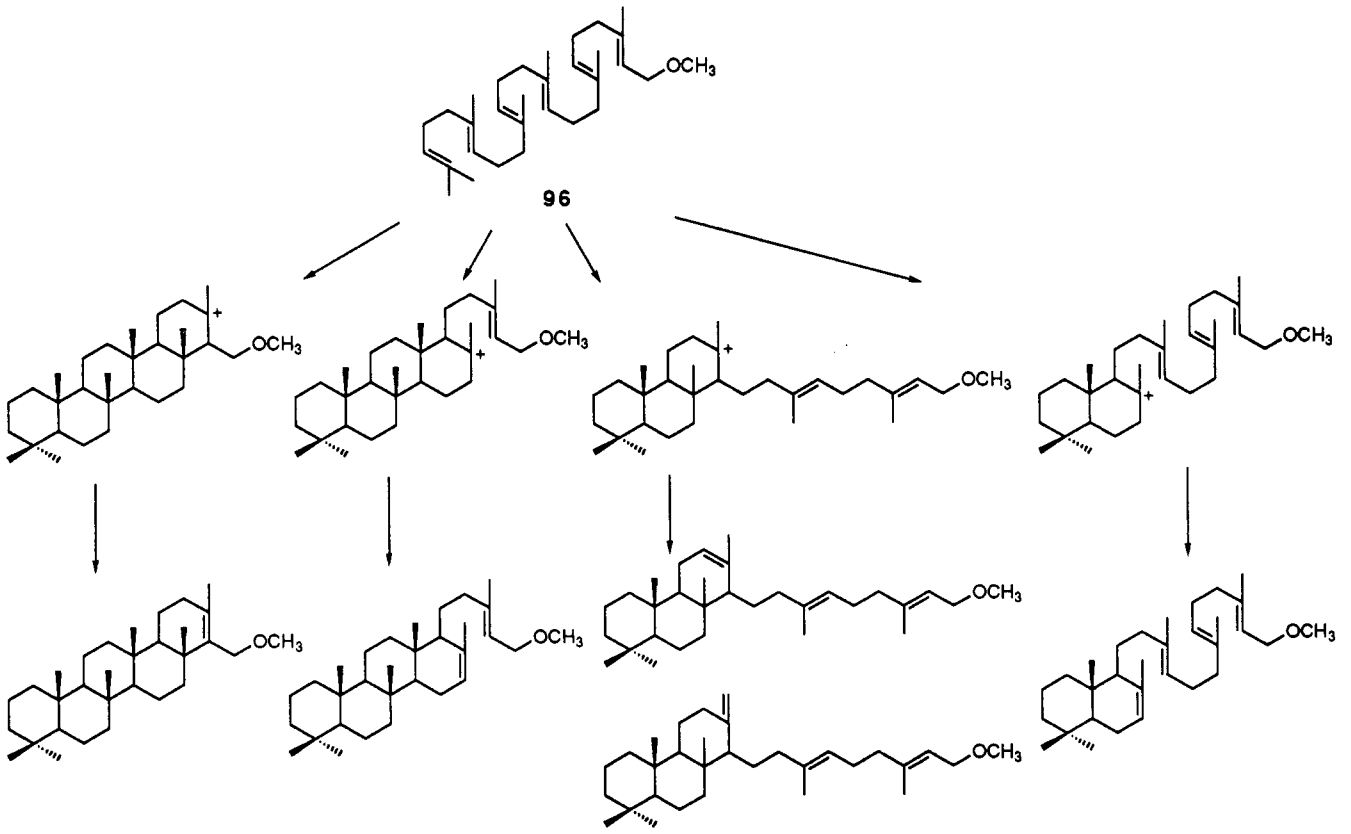
Scheme VIII



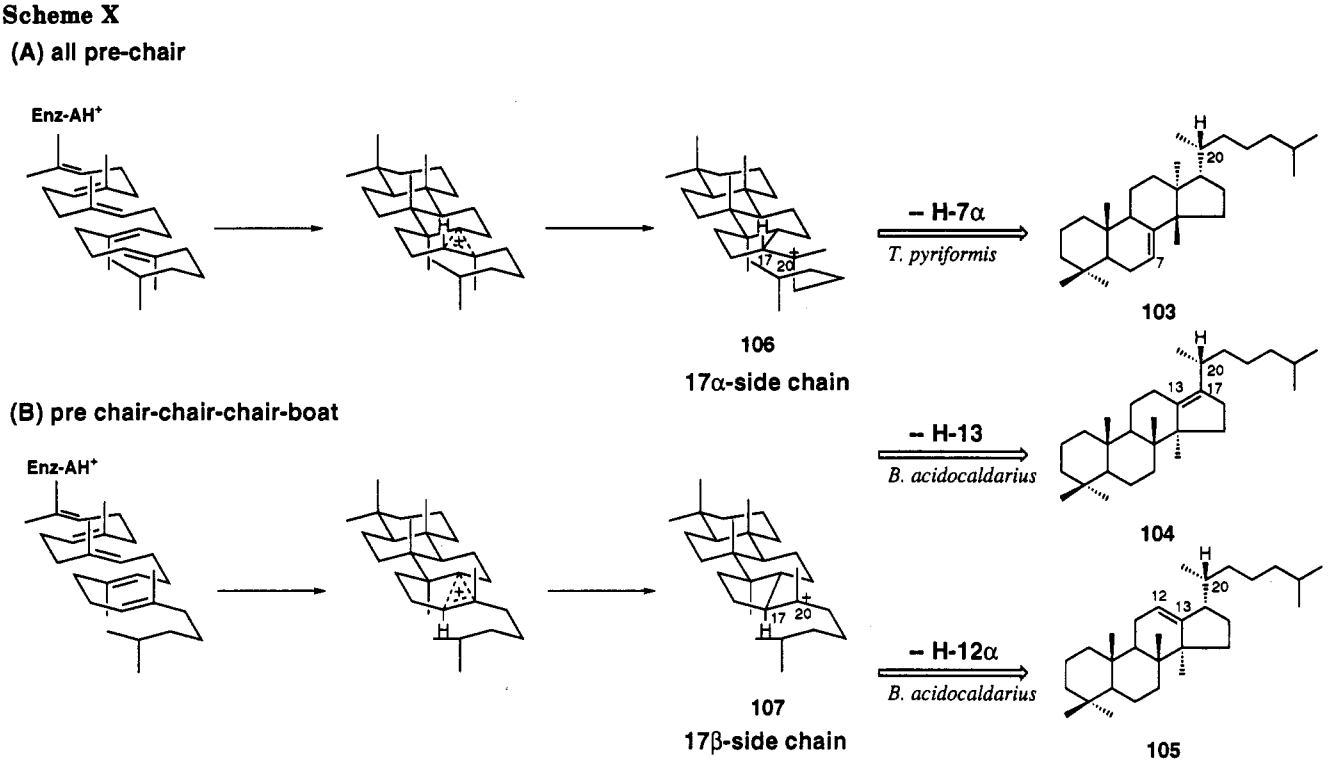
type sesterterpene and *all-trans*-hexaprenyl methyl ether (**96**) to bicyclic, tricyclic, tetracyclic, and pentacyclic methyl ethers[39] (Scheme IX). Similarly, *B. acidocaldarius* SC converted *all-trans*-farnesol (**97**), homogeraniol (**98**), and homofarnesyl (1,5,9-trimethyl-4,8-decadienyl) ether (**99**) into tricyclic and bicyclic ethers.[145] In these polyprenyl ethers, the isoprenoid units are linked head-to-tail, whereas squalene is a symmetrical molecule with a central head-to-head linkage. Assuming that the cyclization occurs at the same active site of the cyclase, the enzyme should encounter difficulty in cyclizing the C-ring since the methyl groups of hexaprenyl ether are on the β-side instead on the α-side as in the cyclization of squalene. This would account for incomplete cyclizations leading to a series of polycyclic skeletons corresponding to all cationic intermediates of a possible stepwise cyclization process. Here it should be noted that the reaction is an "all-Markovnikov" process. Interestingly, tricyclic polyprenyl derivatives have been identified in the organic matter from numerous sediments, and they have been interpreted as chemical fossils produced by as yet unidentified ancient microorganisms.[15]

2,3-Dihydrosqualene (**100**) is a molecule lacking one of the terminal double bonds of squalene, therefore making it impossible to form the pentacyclic framework. A cell-free system from *T. pyriformis* converted 2,3-dihydrosqualene to tetracyclic triterpene, euph-7-ene (**103**) with unexpected skeleton and backbone rearrangement as a single product in fairly good yield.[34] On the other hand, the hopene cyclase of *B. acidocaldarius* cyclized this dihydrosqualene into a 1:1 mixture of tetracyclic (20*R*)-dammar-13(17)-ene (**104**) and (20*R*)-dammar-12-ene (**105**).[146] In both cases, the cyclization produced only transposed dammarenes with a five-membered D-ring, and all cyclization products had only the 20*R* configuration, suggesting that the stereochemistry of the rearrangement reactions must be strictly controlled by the enzymes. Most likely, cyclization of 2,3-dihydrosqualene occurred at the same SC active site, folded in an all prechair conformation to generate a tetracyclic cation with a six-membered D-ring. In

Scheme IX



Scheme X



the absence of the terminal double bond, this intermediate evolves spontaneously toward the most stable 17-isodammaranyl tertiary cation (**106**) (Scheme XA). A different folding of the precursor in a "chair–chair–chair–boat" conformation would yield an alternative dammaranyl tertiary cation with a 17β side chain (**107**) (Scheme XB). However, this second pathway seems less likely as it implies a folding of the precursor different from that adopted for squalene cyclization. (Although, in this case, a 1,3-diaxial interaction between the two methyl groups in pre ring D is released and the following rearrangement reaction can be explained in all antiparallel manner.)

The differences between these two pathways are similar to those discussed for the formation of lanosterol, which also has the 20*R* configuration, from a protosterol cationic intermediate with 17α side-chain as proposed by Cornforth[48] or with a 17β side chain as recently

suggested by Corey and Virgil.[35] Subsequent hydride shifts, backbone rearrangement, and proton elimination will lead to euph-7-ene (**103**) of the 20*R* configuration (Scheme X). The formation of different cyclization products by the two cyclases might reflect differences in the geometry of their active sites. Note that eupha-7,24-diene and (20*R*)-dammar-13(17),24-diene have been isolated as fern constituents from *Polypodium* species.[147] On the other hand, tetrahymanol has been reported from another fern *Oleandra wallichii*,[148] and hydrocarbons derived from the hopanyl or the isomeric moretanyl C-22 cation are widespread in these lower plants; indeed, fern-7-ene derives from a similar backbone rearrangement as euph-7-ene.[149] The occurrence of all these triterpenic skeletons in the Pteridophyta suggests a close relationship between the cyclases leading either tetracyclic or pentacyclic frameworks.

The versatility of the above-described SCs is of great interest from the viewpoint of the molecular evolution of triterpenes and sterols. According to Ruzicka's "biogenetic isoprene rule",[3] squalene is converted to various skeletal types of triterpenes by different enzymes which employ only small variations of a single catalytic mechanism. Such variations are considered to be attained by only a small modification of the structure of the active site of the enzyme, which made possible the evolution from primitive triterpenes to sterols in higher organisms.[15] The above described results demonstrated a further possibility: the geometry of the active site of the "primitive" squalene cyclases has been already "prepared" for other cyclization products. Formation of a five-membered D-ring instead of a six-membered D-ring is apparently only induced in the cyclization process by the lack of participation of the π-system from the terminal double-bond. The "primitive" tetrahymanol cyclase normally catalyzing the formation of a pentacyclic framework without carbon skeletal rearrangement could also accomplish the apparently complex cyclization reaction which resembles that postulated for lanosterol formation. Indeed the cyclization to the tetracyclic skeletons would appear to be a spontaneous sequence of events dictated primarily by a folding conformation of the polyene in which the terminal double bond is sufficiently distant from the pre ring D.

For the formation of novel and unexpected products by the cyclases, it is also possible that the active sites are simply permissive and therefore do not intervene completely in the folding and cyclization of substrates which they do not normally encounter. The role of the cyclase is most likely to involve initiation of cyclization and preventing alternative modes of cyclization. However, at least, in the case of euph-7-ene formation, the enzyme seems to play a more active role in order to guide the course of reaction by strictly controlling the backbone rearrangement to yield the euphene skeleton with the C-20*R* stereochemistry and Δ^7 double bond rather than the more thermodynamically stable $\Delta^{13(17)}$ double bond.[146]

C. Enzymology

Squalene cyclase from the thermophilic and acidophilic bacterium *B. acidocaldarius* has been purified,[26,134,145] cloned, expressed, and sequenced.[28] The membrane-bound enzyme was solubilized with Triton X-100 and purified up to 886-fold by chromatography on DEAE–cellulose, phenyl sepharose, and two gel-filtration columns. The purified enzyme showed a single band on SDS-PAGE with molecular mass of 75 kDa and gave two cyclization products, diploptene (hop-22 (29)-ene) and diplopterol (hopan-22-ol) in a fixed molar ratio of 5:1.[26,134] The enzyme showed an optimal conversion rate at pH 6 and 60 °C, and the apparent K_M value was 3 μM.[26] Tetrahymanol cyclase has been also purified to homogeneity from *Tetrahymena thermophila*.[27] In this case, Triton X-100 was proved to be a strong inactivator of the enzyme, and β-octylthioglucoside was used for solubilization and purification. The purified cyclase has a molecular mass of 72 kDa and the apparent K_M value was 18 μM.

Both bacterial SCs are efficiently inhibited by *p*-(chloromercuri)benzenesulfonic acid and diethyl pyrocarbonate, suggesting a functional SH group at the catalytic center and the possible participation of a histidine residue in catalysis.[27,134] 2-Aza-2,3-dihydrosqualene (**76**) (Chart V) (IC_{50} = 0.1 μM for *Methylobacterium organophilum* cyclase) and its derivatives, as well as 2,3-iminosqualene (**75**) (IC_{50} = 0.1 μM for *M. organophilum* cyclase) have been reported to be potent inhibitors of both bacterial SCs.[150,151] Moreover, *n*-alkyldimethylammonium halides with alkyl chain lengths between 12 and 18 carbon atoms strongly inhibited *B. acidocaldarius* cyclase activity (IC_{50} = 0.2 μM for dodecyltrimethylammonium bromide),[26] and *T. thermophila* cyclase was especially inhibited by 2,3-iminosqualene (IC_{50} = 0.05 μM) and *N,N*-dimethyldodecylamine *N*-oxide (IC_{50} = 0.03 μM).[27]

Cloning, expression, and sequencing of *B. acidocaldarius* squalene:hopene cyclase has been achieved by Poralla and co-workers.[28] The cloned cyclase is expressed in *Escherichia coli*. Activity was found only in the cytoplasmic membrane, and enzyme activity was observed only after enzymatic lysis or sonication of cells. According to the DNA sequence, the cyclase contained 627 amino acids with a molecular mass of 69.5 kDa. As described above, the sequence shows similarity with that of *Candida albicans* oxidosqualene:lanosterol cyclase.[29,30]

V. Concluding Remarks

Bioorganic chemistry "began" when synthetic organic chemists interested themselves in the remarkable reactions catalyzed by OSC and SC some 40 years ago. Here was a veritable treasure chest for studying sigmatropic shifts, carbon–carbon bond-forming reactions, anchimeric assistance, and the influence of conformation on stereochemical outcome. At the time, the tools of synthetic chemistry offered the most powerful technology for asking questions about the active site and the catalytic mechanism. Now, the tools of protein chemistry, molecular biology, and structural biology offer the next opportunity to unravel the still cryptic interactions between oxidosqualene and its cyclase which lead to such exquisite complexity in triterpenoid natural products. Using such tools, we can begin to address many exciting mechanistic and evolutionary questions which remain: What are the protein–substrate interactions involved in the initiation and termination of cyclization? Are the carbocationic transition states and high-energy intermediates stabi-

lized by hydrophobic isolation, negative point charges, or π-stacking interactions? What structural motifs in the protein determine the panoply of products generated? How has nature modified and reshuffled structural motifs in the evolution of squalene and oxidosqualene cyclases during 500 million years?

Our quest is not yet over, but the long-sought answers to these questions are now within our grasp. The answers will provide landmarks worth celebrating in the history of bioorganic chemistry.

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VI. References

(1) Woodward, R. B.; Bloch, K. *J. Am. Chem. Soc.* **1953**, *75*, 2023–2024.
(2) Stork, G.; Burgstahler, A. W. *J. Am. Chem. Soc.* **1955**, *77*, 5068–5077.
(3) Eschenmoser, A.; Ruzicka, L.; Jeger, O.; Arigoni, D. *Helv. Chim. Acta* **1955**, *38*, 1890–1904.
(4) Maudgal, R. K.; Tchen, T. T.; Bloch, K. *J. Am. Chem. Soc.* **1958**, *80*, 2589–2586.
(5) Cornforth, J. W.; Cornforth, R. H.; Donninger, C.; Popják, G.; Shimizu, Y.; Ichii, S.; Forchielli, E.; Caspi, E. *J. Am. Chem. Soc.* **1965**, *87*, 3224–3228.
(6) Corey, E. J.; Russey, W. E.; Ortiz de Montellano, P. R. *J. Am. Chem. Soc.* **1966**, *88*, 4750–4751.
(7) van Tamelen, E. E.; Willett, J. D.; Clayton, R. B.; Lord, K. E. *J. Am. Chem. Soc.* **1966**, *88*, 4752–4754.
(8) Willett, J. D.; Sharpless, K. B.; Lord, K. E.; van Tamelen, E. E.; Clayton, R. B. *J. Biol. Chem.* **1967**, *242*, 4182–4191.
(9) Barton, D. H. R.; Jarman, T. R.; Watson, K. C.; Widdowson, D. A.; Boar, R. B.; Damps, K. *J. Chem. Soc., Perkin Trans. 1* **1975**, 1134–1138.
(10) van Tamelen, E. E. *J. Am. Chem. Soc.* **1982**, *104*, 6480–6481.
(11) Corey, E. J.; Virgil, S. C.; Sarshar, S. *J. Am. Chem. Soc.* **1991**, *113*, 8171–8172.
(12) Rees, H. H.; Goad, L. J.; Goodwin, T. W. *Biochem. J.* **1968**, *107*, 417–426.
(13) Goodwin, T. W. In *Biosynthesis of Isoprenoid Compounds*; Porter, J. W., Spurgeon, S. L. Eds.; Wiley: New York, 1980; Vol. 1, pp 443–480.
(14) Caspi, E. *Acc. Chem. Res.* **1980**, *13*, 97–104.
(15) Ourisson, G.; Rohmer, M.; Poralla, K. *Annu. Rev. Microbiol.* **1987**, *41*, 301–333.
(16) Ourisson, G.; Rohmer, M.; Anton, R. *Recent Adv. Phytochem.* **1979**, *13*, 131–162.
(17) Rohmer, M.; Bouvier, P.; Ourisson, G. *Proc. Natl. Acad. Sci. U.S.A.* **1979**, *76*, 847–851.
(18) Poralla, K.; Hewelt, A.; Prestwich, G. D.; Abe, I.; Reipen, I.; Sprenger, G. Submitted for publication.
(19) Kusano, M.; Abe, I.; Sankawa, U.; Ebizuka, Y. *Chem. Pharm. Bull.* **1991**, *39*, 239–241.
(20) Abe, I.; Bai, M.; Xiao, X.-y.; Prestwich, G. D. *Biochem. Biophys. Res. Commun.* **1992**, *187*, 32–38.
(21) Abe, I.; Ebizuka, Y.; Sankawa, U. *Chem. Pharm. Bull.* **1988**, *36*, 5031–5034.
(22) Abe, I.; Sankawa, U.; Ebizuka, Y. *Chem. Pharm. Bull.* **1989**, *37*, 536–538.
(23) Abe, I.; Ebizuka, Y.; Seo, S.; Sankawa, U. *FEBS Lett.* **1989**, *249*, 100–104.
(24) Abe, I.; Sankawa, U.; Ebizuka, Y. *Chem. Pharm. Bull.* **1992**, *40*, 1755–1760.
(25) Corey, E. J.; Matsuda, S. P. T. *J. Am. Chem. Soc.* **1991**, *113*, 8172–8174.
(26) Ochs, D.; Tappe, C. H.; Gärtner, P.; Kellner, R.; Poralla, K. *Eur. J. Biochem.* **1990**, *194*, 75–80.
(27) Saar, J.; Kader, J.-C.; Poralla, K.; Ourisson, G. *Biochim. Biophys. Acta* **1991**, *1075*, 93–101.
(28) Ochs, D.; Kaletta, C.; Entian, K.-D.; Beck-Sickinger, A.; Poralla, K. *J. Bacteriol.* **1992**, *174*, 298–302.
(29) Buntel, C. J.; Griffin, J. H. *J. Am. Chem. Soc.* **1992**, *114*, 9711–9713.
(30) Roessner, C. A.; Min, C.; Hardin, S. H.; Harris-Haller, L. W.; McCollum, J. C.; Scott, A. I. *Gene* **1993**, *127*, 149–150.
(31) Abe, I.; Prestwich, G. D. Unpublished results.
(32) Reipen, I.; Sprenger, G. Submitted for publication.
(33) Xiao, X.-y.; Prestwich, G. D. *J. Am. Chem. Soc.* **1991**, *113*, 9673–9674.
(34) Abe, I.; Rohmer, M. *J. Chem. Soc. Chem. Comm.* **1991**, 902–903.
(35) Corey, E. J.; Virgil, S. C. *J. Am. Chem. Soc.* **1991**, *113*, 4025–4026.
(36) van Tamelen, E. E.; James, D. R. *J. Am. Chem. Soc.* **1977**, *99*, 950–952.
(37) Boar, R. B.; Couchman, L. A.; Jaque, A. J.; Perkins, M. J. *J. Am. Chem. Soc.* **1984**, *106*, 2476–2477.
(38) Arai, Y.; Hirohara, M.; Ageta, H.; Hsü, H. Y. *Tetrahedron Lett.* **1992**, *33*, 1325–1328.
(39) Renoux, J.-M.; Rohmer, M. *Eur. J. Biochem.* **1986**, *155*, 125–132.
(40) van Tamelen, E. E.; Willet, J.; Schwartz, M.; Nadeau, R. *J. Am. Chem. Soc.* **1966**, *88*, 5937–5938.
(41) van Tamelen, E. E.; Sharpless, K. B.; Hanzlik, R.; Clayton, R. B.; Burlingame, A. L.; Wszolek, P. C. *J. Am. Chem. Soc.* **1967**, *89*, 7150–7151.
(42) van Tamelen, E. E.; Hanzlik, R. P.; Sharpless, K. B.; Clayton, R. B.; Richter, W. J.; Burlingame, A. L. *J. Am. Chem. Soc.* **1968**, *90*, 3284–3286.
(43) van Tamelen, E. E.; Leopold, E. J.; Marson, S. A.; Waespe, H. R. *J. Am. Chem. Soc.* **1982**, *104*, 6479–6480.
(44) Johnson, W. S.; Telfer, S. J.; Cheng, S.; Schubert, U. *J. Am. Chem. Soc.* **1987**, *109*, 2517–2518.
(45) Johnson, W. S.; Lindell, S. D.; Steele, J. *J. Am. Chem. Soc.* **1987**, *109*, 5852–5853.
(46) Johnson, W. S. *Tetrahedron* **1991**, *47*, xi-l.
(47) Johnson, W. S.; Buchanan, R. A.; Bartlett, W. R.; Tham, F. S.; Kullnig, R. K. *J. Am. Chem. Soc.* **1993**, *115*, 504–515.
(48) Cornforth, J. W. *Angew. Chem., Int. Ed. Engl.* **1968**, *7*, 903–911.
(49) Nes, W. R.; Varkey, T. E.; Krevitz, K. *J. Am. Chem. Soc.* **1977**, *99*, 260–262.
(50) Kawaguchi, A.; Kobayashi, H.; Okuda, S. *Chem. Pharm. Bull.* **1973**, *21*, 577–583.
(51) Godtfredsen, W. O.; Lorck, H.; van Tamelen, E. E.; Willett, J. D.; Clayton, R. B. *J. Am. Chem. Soc.* **1968**, *90*, 208–209.
(52) Mulheirn, L. J.; Caspi, E. *J. Biol. Chem.* **1971**, *246*, 2494–2501.
(53) Corey, E. J.; Gross, S. K. *J. Am. Chem. Soc.* **1967**, *89*, 4561–4562.
(54) Boutaud, O.; Dolis, D.; Schuber, F. *Biochem. Biophys. Res. Commun.* **1992**, *188*, 898–904.
(55) Anding, C.; Heintz, R.; Ourisson, G. *C. R. Acad. Sci. Paris D* **1973**, *276*, 205–207.
(56) Bujons, J.; Guajardo, R.; Kyler, K. S. *J. Am. Chem. Soc.* **1988**, *110*, 604–605.
(57) van Tamelen, E. E.; Sharpless, K. B.; Willet, J. D.; Clayton, R. B.; Burlingame, A. L. *J. Am. Chem. Soc.* **1967**, *89*, 3920.
(58) Anderson, R. J.; Hanzlik, R. P.; Sharpless, K. B.; van Tamelen, E. E.; Clayton, R. B. *Chem. Comm.* **1969**.
(59) Corey, E. J.; Ortiz de Montellano, P. R.; Lin, K.; Dean, P. D. G. *J. Am. Chem. Soc.* **1967**, *89*, 2797–2798.
(60) Nes, W. D.; Parish, E. J. *Lipids* **1988**, *23*, 375–376.
(61) Xiao, X.-y.; Prestwich, G. D. *Tetrahedron Lett.* **1991**, *32*, 6843–6846.
(62) Xiao, X.-y. Ph.D. Thesis, State University of New York, Stony Brook, 1991.
(63) Hérin, M.; Sandra, P.; Krief, A. *Terahedron Lett.* **1979**, *33*, 3103–3106.
(64) Krief, A.; Pasau, P.; Quéré, L. *Bioorg. Med. Chem. Lett.* **1991**, *1*, 365–368.
(65) Krief, A.; Schauder, J.-R.; Guittet, E.; Herve du Penhoat, C.; Lallemand, J.-Y. *J. Am. Chem. Soc.* **1987**, *109*, 7910–7911.
(66) Ceruti, M.; Viola, F.; Dosio, F.; Cattel, L.; Bouvier-Navé, P.; Ugliengo, P. *J. Chem. Soc., Perkin Trans. 1* **1988**, 461–469.
(67) Corey, E. J.; Russey, W. E. *J. Am. Chem. Soc.* **1966**, *88*, 4751–4752.
(68) Corey, E. J.; Ortiz de Montellano, P. R.; Yamamoto, H. *J. Am. Chem. Soc.* **1968**, *90*, 6254–6255.
(69) Corey, E. J.; Virgil, S. C.; Liu, D. R.; Sarshar, S. *J. Am. Chem. Soc.* **1992**, *114*, 1524–1525.
(70) Medina, J. C.; Guajardo, R.; Kyler, K. S. *J. Am. Chem. Soc.* **1989**, *111*, 2310–2311.
(71) van Tamelen, E. E.; Freed, J. H. *J. Am. Chem. Soc.* **1970**, *92*, 7206–7207.
(72) van Tamelen, E. E.; Milne, G. M.; Suffness, M. I.; Rudler Chauvin, M. C.; Anderson, R. J.; Achini, R. S. *J. Am. Chem. Soc.* **1970**, *92*, 7202–7204.

(73) van Tamelen, E. E.; Hopla, R. E. *J. Am. Chem. Soc.* **1979**, *101*, 6112–6114.
(74) Corey, E. J.; Lin, K.; Jautelat, M. *J. Am. Chem. Soc.* **1968**, *90*, 2724–2726.
(75) Clayton, R. B.; van Tamelen, E. E.; Nadeau, R. G. *J. Am. Chem. Soc.* **1968**, *90*, 820–821.
(76) Crosby, L. O.; van Tamelen, E. E.; Clayton, R. B. *Chem. Comm.* **1969**, 532–533.
(77) Medina, J. C.; Kyler, K. S. *J. Am. Chem. Soc.* **1988**, *110*, 4818–4821.
(78) Xiao, X.-y.; Sen, S. E.; Prestwich, G. D. *Tetrahedron Lett.* **1990**, *31*, 2097–2100.
(79) van Tamelen, E. E.; Pedlar, A. D.; Li, E.; James, D. R. *J. Am. Chem. Soc.* **1977**, *99*, 6778–6780.
(80) Yamamoto, S.; Lin, K.; Bloch, K. *Proc. Natl. Acad. Sci. U.S.A.* **1969**, *63*, 110–117.
(81) Shechter, I.; Sweat, F. W.; Bloch, K. *Biochim. Biophys. Acta* **1970**, *220*, 463–468.
(82) Moore, W. R.; Schatzman, G. L. *J. Biol. Chem.* **1992**, *267*, 22003–22006.
(83) Duriatti, A.; Schuber, F. *Biochem. Biophys. Res. Comm.* **1988**, *151*, 1378–1385.
(84) Hoshino, T.; Williams, H. J.; Chung, Y.; Scott, A. I. *Tetrahedron* **1991**, *47*, 5925–5932.
(85) Balliano, G.; Viola, F.; Ceruti, M.; Cattel, L. *Arch. Biochem. Biophys.* **1992**, *293*, 122–129.
(86) Kelly, R.; Miller, S. M.; Lai, M. H.; Kirsch, D. R. *Gene* **1990**, *87*, 177–183.
(87) Cattel, L.; Ceruti, M.; Viola, F.; Delprino, L.; Balliano, G.; Duriatti, A.; Bouvier-Navé, P. *Lipids* **1986**, *21*, 31–38.
(88) Duriatti, A.; Bouvier-Navé, P.; Benveniste, P.; Schuber, F.; Delprino, L.; Balliano, G.; Cattel, L. *Biochem. Pharmacol.* **1985**, *34*, 2765–2777.
(89) Delprino, L.; Balliano, G.; Cattel, L.; Benveniste, P.; Bouvier, P. *J. Chem. Soc., Chem. Commun.* **1983**, 381–382.
(90) Taton, M.; Benveniste, P.; Rahier, A. *Biochem. Biophys. Res. Comm.* **1986**, *138*, 764–770.
(91) Dodd, D. S.; Oehlschlager, A. C. *J. Org. Chem.* **1992**, *57*, 2794–2803.
(92) Dodd, D. S.; Oehlschlager, A. C.; Georgopapadakou, N. H.; Polak, A.-M.; Hartman, P. G. *J. Org. Chem.* **1992**, *57*, 7226–7234.
(93) Taton, M.; Benveniste, P.; Rahier, A.; Johnson, W. S.; Liu, H.-t.; Sudhakar, A. R. *Biochemistry* **1992**, *31*, 7892–7898.
(94) Ceruti, M.; Balliano, G.; Viola, F.; Grosa, G.; Rocco, F.; Cattel, L. *J. Med. Chem.* **1992**, *35*, 3050–3058.
(95) Balliano, G.; Milla, P.; Ceruti, M.; Viola, F.; Carrano, L.; Cattel, L. *FEBS Lett.* **1993**, *320*, 203–206.
(96) Ceruti, M.; Rocco, F.; Viola, F.; Balliano, G.; Grosa, G.; Dosio, F.; Cattel, L. *Eur. Med. Chem.*, in press.
(97) Oehlschlager, A. C. Personal communication.
(98) Abad, J.-L.; Casas, J.; Sanchez-Baeza, F.; Messeguer, A. *Bioorg. Med. Chem. Lett.* **1992**, *2*, 1239–1242.
(99) Abad, J.-L.; Casas, J.; Sánchez-Baeza, F.; Messeguer, A. *J. Org. Chem.* **1993**, in press.
(100) Jolidon, S.; Polak, A.-M.; Guerry, P.; Hartman, P. G. *Biochem. Soc. Trans.* **1989**, *18*, 47–48.
(101) Mercer, E. I.; Morris, P. K.; Baldwin, B. C. *Comp. Biochem. Physiol. B* **1985**, *80*, 341–346.
(102) Nelson, J. A.; Czanny, M. R.; Spencer, T. A.; Limanek, J. S.; McCrae, K. R.; Chang, T. Y. *J. Am. Chem. Soc.* **1978**, *100*, 4900.
(103) Chang, T. Y.; Schiavoni, E. S., Jr.; McCrae, K. R.; Nelson, J. A.; Spencer, T. A. *J. Biol. Chem.* **1979**, *254*, 11258.
(104) Wannamaker, M. W.; Waid, P. P.; Van Sickle, W. A.; McCarthy, J. R.; Wilson, P. K.; Schatzman, G. L.; Moore, W. R. *J. Med. Chem.* **1992**, *35*, 3581–3583.
(105) Abe, I.; Tomesch, J. C.; Wattanasin, S.; Prestwich, G. D. *Natl. Prod. Rep.*, in preparation.
(106) Heintz, R.; Benveniste, P. *Phytochemistry* **1970**, *9*, 1499–1503.
(107) Altman, L. J.; Han, C. Y.; Bertorino, A.; Handy, G.; Laugani, D.; Muller, W.; Schwartz, S.; Shanker, D.; de Wolf, W. H.; Yang, F. J. *J. Am. Chem. Soc.* **1978**, *100*, 3235–3237.
(108) Seo, S.; Uomori, A.; Yoshimura, Y.; Takeda, K.; Seto, H.; Ebizuka, Y.; Noguchi, H.; Sankawa, U. *J. Chem. Soc., Perkin Trans. 1* **1988**, 2407–2414.
(109) Seo, S.; Uomori, A.; Yoshimura, Y.; Takeda, K.; Seto, H.; Ebizuka, Y.; Noguchi, H.; Sankawa, U. *J. Chem. Soc. Perkin Trans. I* **1989**, 261–263.
(110) Rees, H. H.; Britton, G.; Goodwin, T. W. *Biochem. J.* **1968**, *106*, 659–665.
(111) Barton, D. H. R.; Mellows, G.; Widdowson, D. A.; Wright, J. J. *J. Chem. Soc. (C)* **1971**, 1142–1148.
(112) Seo, S.; Tomita, Y.; Tori, K. *J. Am. Chem. Soc.* **1981**, *103*, 2075–2080.
(113) Seo, S.; Yoshimura, Y.; Uomori, A.; Takeda, K.; Seto, H.; Ebizuka, Y.; Sankawa, U. *J. Am. Chem. Soc.* **1988**, *110*, 1740–1745.
(114) Etemadi, A. H.; Popják, G.; Cornforth, J. W. *Biochem. J.* **1969**, *111*, 445–451.
(115) Corey, E. J.; Gross, S. K. *J. Am. Chem. Soc.* **1968**, *90*, 5045–5046.
(116) Brunner, H. G. Thesis, ETH, Zürich, Switzerland, 1976.
(117) Dietsch, A.; Delprino, L.; Benveniste, P.; Cattel, L. *J. Chem. Res. (S)* **1980**, 60–61.
(118) Dietsch, A. Thesis, Université Louis Pasteur, Strasbourg, France, 1980.
(119) Horan, H.; McCormick, J. P.; Arigoni, D. *J. Chem. Soc., Chem. Commun.* **1973**, 73–74.
(120) van Tamelen, E. E.; Seiler, M. P.; Wierenga, W. *J. Am. Chem. Soc.* **1972**, *94*, 8229.
(121) Cattel, L.; Anding, C.; Benveniste, P. *Phytochemistry* **1976**, *15*, 931–935.
(122) Corey, E. J.; Ortiz de Montellano, P. R. *J. Am. Chem. Soc.* **1967**, *89*, 3362–3363.
(123) Dean, P. D. G. *Methods Enzymol.* **1969**, *15*, 495–501.
(124) Baisted, D. J. *Biochem. J.* **1971**, *124*, 375–383.
(125) Fang, T.-Y.; Baisted, D. J. *Biochem. J.* **1975**, *150*, 323–328.
(126) Goad, L. J. *Biochem. Soc. Trans.* **1983**, 548–552.
(127) Palmer, M. A.; Bowden, B. N. *Phytochemistry* **1977**, *16*, 459.
(128) Elder, J. W.; Benveniste, P.; Fonteneau, P. *Phytochemistry* **1977**, *16*, 490–492.
(129) Balliano, G.; Caputo, O.; Viola, F.; Delprino, L.; Cattel, L. *Phytochemistry* **1983**, *22*, 915–921.
(130) Rowan, M. G.; Dean, P. D. G.; Goodwin, T. W. *FEBS Lett.* **1971**, *12*, 229–232.
(131) Beastall, G. H.; Rees, H. H.; Goodwin, T. W. *FEBS Lett.* **1971**, *18*, 175–178.
(132) Douglas, T. J.; Paleg, L. G. *Phytochemistry* **1978**, *17*, 705.
(133) Nes, W. D.; Douglas, T. J.; Lin, J.-T.; Heftmann, E.; Paleg, L. G. *Phytochemistry* **1982**, *21*, 575.
(134) Seckler, B.; Poralla, K. *Biochim. Biophys. Acta* **1986**, *881*, 356–363.
(135) Rohmer, M.; Bouvier-Navé, P.; Ourisson, G. *J. Gen. Microbiol.* **1984**, *130*, 1137–1150.
(136) Ourisson, G.; Rohmer, M. *Acc. Chem. Res.* **1992**, *25*, 403–408.
(137) Rohmer, M. *Pure Appl. Chem.* **1993**, *65*, 1293–1298.
(138) Rohmer, M.; Bouvier, P.; Ourisson, G. *Eur. J. Biochem.* **1980**, *112*, 557–560.
(139) Caspi, E.; Zander, J. M.; Creig, J. B.; Mallory, F. B.; Conner, R. L.; Landrey, J. R. *J. Am. Chem. Soc.* **1968**, *90*, 3563–3564.
(140) Anding, C.; Rohmer, M.; Ourisson, G. *J. Am. Chem. Soc.* **1976**, *98*, 1274–1275.
(141) Aberhart, D. J.; Caspi, E. *J. Am. Chem. Soc.* **1979**, *101*, 1013–1019.
(142) Zander, J. M.; Greig, J. B.; Caspi, E. *J. Biol. Chem.* **1970**, *245*, 1247–1254.
(143) Rohmer, M.; Anding, C.; Ourisson, G. *Eur. J. Biochem.* **1980**, *112*, 541–547.
(144) Bouvier, P.; Berger, Y.; Rohmer, M.; Ourisson, G. *Eur. J. Biochem.* **1980**, *112*, 549–556.
(145) Neumann, S.; Simon, H. *Biol. Chem. Hoppe-Seyler* **1986**, *367*, 723–729.
(146) Abe, I.; Rohmer, M. *J. Chem. Soc., Perkin Trans. 1* submitted.
(147) Arai, Y.; Masuda, K.; Ageta, H. *Chem. Pharm. Bull.* **1982**, *30*, 4219–4221.
(148) Zander, J. M.; Caspi, E.; Pendley, G. N.; Mitra, C. R. *Phytochemistry* **1969**, *8*, 2265–2267.
(149) Barton, D. H. R.; Mellows, G.; Widdowson, D. A. *J. Chem. Soc. (C)* **1971**, 110–115.
(150) Flesch, G.; Rohmer, M. *Arch. Microbiol.* **1987**, *147*, 100–104.
(151) Renoux, J. M. Thesis, Université de Haute Alsace, Mulhouse, France, 1986.