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Inhibitors of 2,3-Oxidosqualene Cyclase as Tools for Studying the Mechanism and Function of the Enzyme

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CONTENTS

I. INTRODUCTION

Searching for enzyme inhibitors is a rational and efficient approach to drug discovery. Nevertheless, it is very difficult for an enzyme inhibitor to become a practical drug, since too many requisites, such as specificity, lack of toxicity and acceptable pharmacokinetic features, must be met. Indeed, relatively few enzyme inhibitors have become marketed drugs. Another purpose in designing enzyme inhibitors is to obtain more information on the mechanism and active site involved in catalysis. This in turn should help the medicinal chemist to redesign new and more specific enzyme inhibitors, so rationalizing the effort to synthesize new drugs.

Our interest in studying new inhibitors of 2,3-oxidosqualene cyclase (OSC), a key enzyme in sterol and triterpenoid biosynthesis, is twofold: (1) to obtain significant and specific activity suitable for antifungal, hypocholesterolemic and phytotoxic drugs; (2) to gain insight into the mechanism of oxidosqualene cyclization in different tissues, and to obtain information on the function and regulation of sterols.

In this review we will focus on the last point, as we have previously discussed the importance of OSC as target enzyme for developing new drugs. In particular, we would like to show that the discovery of new OSC inhibitors has been useful in better understanding both the mechanism of OS cyclization, and the possible role of some amino acids present in the enzyme.

II. OXIDOSQUALENE CYCLASE: STATE OF THE ART

2,3-oxidosqualene cyclases (OSC) are important enzymes in the biosynthesis of animal, plant and fungal sterols. They catalyze the conversion of (3*S*)-2,3-oxidosqualene **1** to lanosterol **8** in mammals and fungi (Figure 1) and to cycloartenol **9** in algae and higher plants.[1–7]

The formation of lanosterol and cycloartenol is initiated in the pre-chair-boat-chair-boat conformation of **1** and starts from an initial protonation of the epoxide by a suitable electrophilic residue present in the enzyme[8] to give a first C-2 carbonium ion intermediate **2**. It proceeds through the formation of a series of discrete conformationally rigid carbonium ion intermediates, such as the C-10 monocyclic intermediate **3**, the C-8 bicyclic **4**, the C-13 tricyclic **5**, and the C-20 tetracyclic carbonium ion **6** or **7**.[9–17] This latter undergoes backbone rearrangements to yield either lanosterol **8** in animals and yeasts or cycloartenol **9** in higher plants.

The stereochemistry of the C-20 protosteryl ion has been long discussed.[18,19] Following the "biogenetic isoprenic rule," the C-20 cation has a 17α oriented side chain.[18] However, Corey and coworkers recently demonstrated[15,16] that the 20-oxa analogue of OS, **10** (first synthesized as 22,23-dihydro derivative **11** in our laboratory[20]) and the (20*E*)-20,21-dehydro-2,3-oxidosqualene **12** are enzymatically transformed to protosterol derivatives **13** or **14** having a 17β side chain (Figure 2). In this case, a smaller (60°) instead

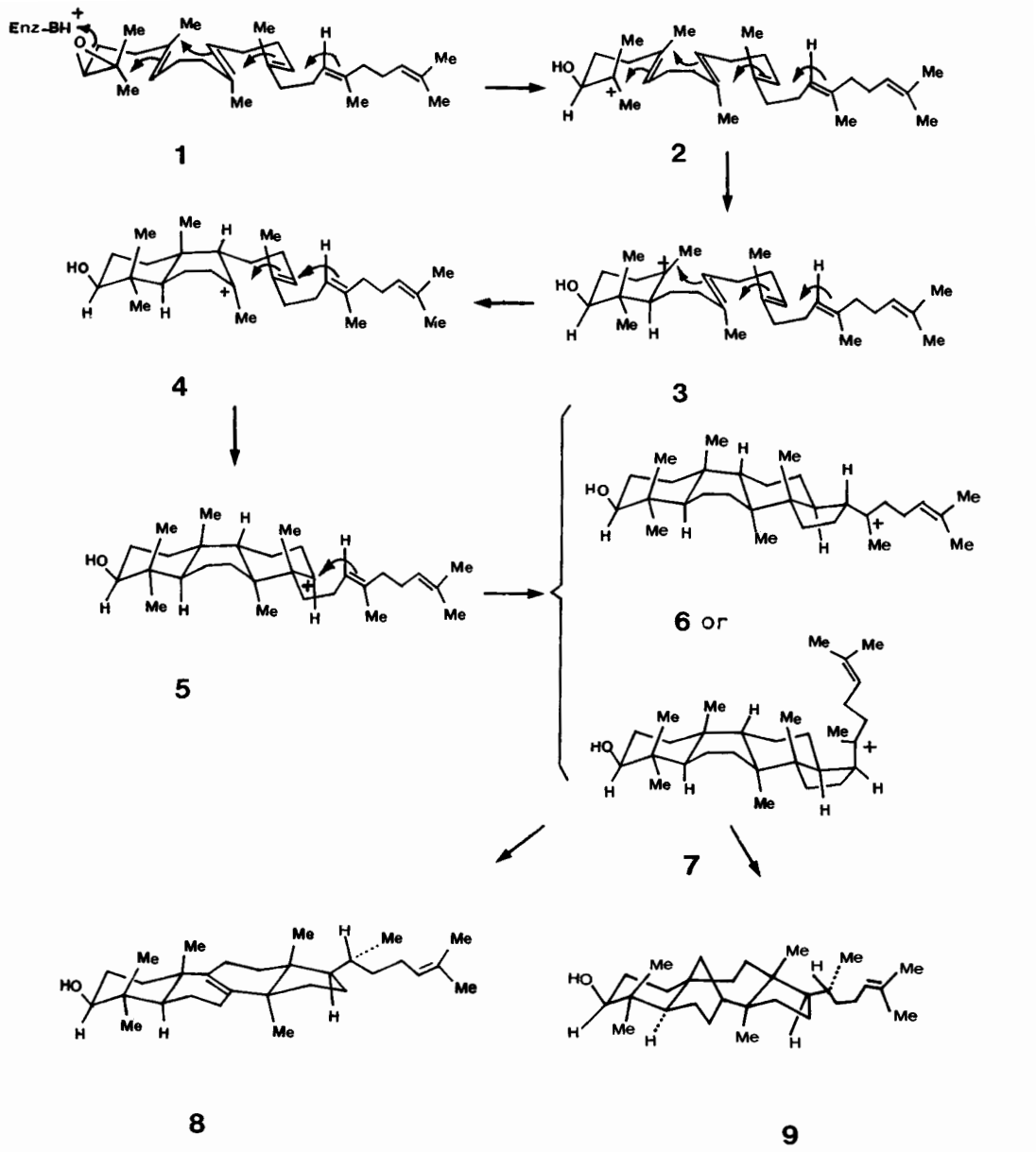


Figure 1 Mechanism of cyclization of 2,3-oxidosqualene to lanosterol and cycloartenol.

of a 120° previously postulated rotation of the side chain around the C-17-20 bond in the enzyme is required prior to the antiparallel 1,2 hydride migration from C-17 to C-20.

In higher plants, OSC is also responsible for the formation of a variety of tetra- or pentacyclic triterpenoids (Figure 3) such as α- or β-amyrin **15** or **16**.[21,22] The proton-initiated cyclization of OS in all chair conformation produces the tetracyclic dammarenyl cation **17**, and the subsequent rearrangement leads to the pentacyclic oleanyl cation **18** via the baccharenyl **19**, and lupenyl **20** ion intermediates. In some bacteria and protozoa, squalene can be directly cyclized by squalene cyclase (SC) to pentacyclic 3-deoxytriterpenes with the hopane and gammacerane skeleton such as diploptene, diplopterol and tetrahymanol.[7]

Oxidosqualene cyclase is associated with the endoplasmic reticulum in eukaryotic cells. For this reason and for its intrinsic instability it was completely characterized only in the last years. Several OSC have been purified to homogeneity (Table 1) from vertebrate, plant and yeast sources.[23,34] Duriatti and

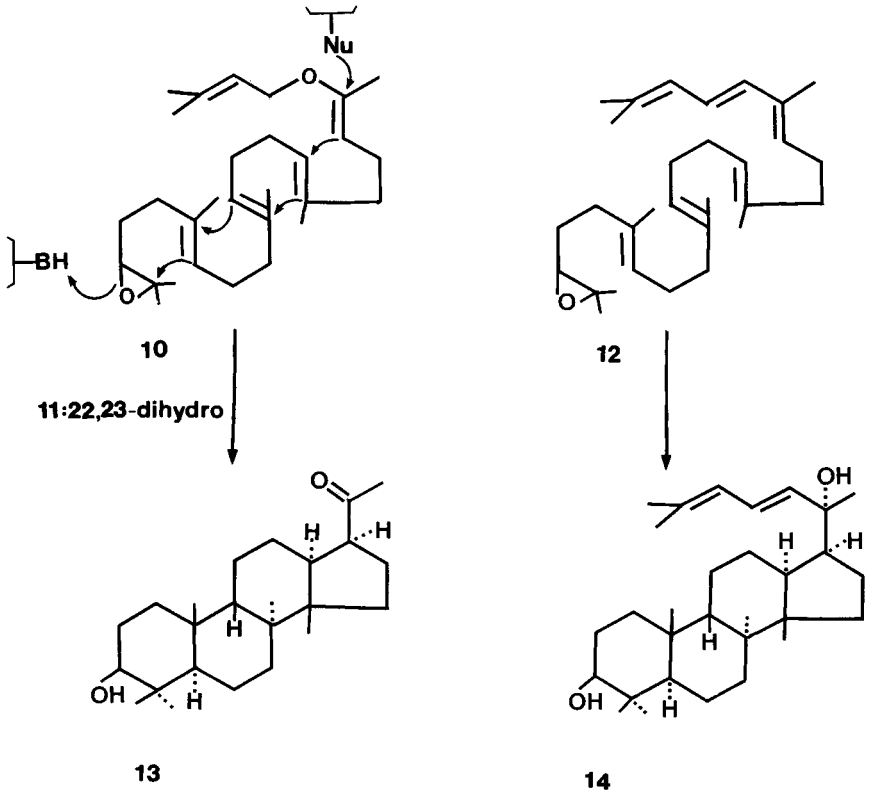


Figure 2 Enzymatic transformation of 20-oxa- and (20*E*)-20,21-dehydro-2,3-oxidosqualene derivatives to protosterol derivatives.

Schuber first developed an efficient method for solubilization and 140-fold purification of pig liver OSC.[23] Later, Abe et al. achieved a final 441-fold purification of OSC from pig liver.[25] By performing chemical affinity labeling of OSC from pig and rat liver, as well as dog and human OSC, they found a molecular mass which varied from 73 kDa (human and dog) to 75 kDa (pig) and 78 kDa (rat). By contrast, neither yeast nor pea OSC were labeled, revealing differences in the active site of the animal, plant and fungal enzymes. At the same time Moore and Schatzman[26] reported a rapid and simple purification of OSC from rat liver by selective solubilization of microsomes with lauryl maltoside, followed by chromatography with a strong anion exchange resin (mono Q). The purified enzyme consists of a single subunit with a molecular mass of 65 kDa.

Yeast oxidosqualene cyclase was initially suggested to be a soluble enzyme optimally active in solutions of low ionic strength and stimulated by Triton X-100.[27] Instead, the particulate nature of the enzyme was demonstrated in our laboratory by comparing the properties of the enzyme recovered from both the microsomes and the soluble fraction of yeast homogenate.[28] We also succeeded in a 140-fold enrichment of the enzyme by extracting it from integral protein fractions with octyl glucoside and chromatographing on DEAE Bio-Gel. In parallel, Hoshino et al.[29] realized a 112-fold purification of yeast OSC by applying finally a DEAE Sephacel chromatographic step on the solubilized enzyme, and a subsequent HPLC purification. Finally, Corey and Matsuda[30] described the complete purification of the yeast enzyme by using as a crucial step a rationally designed affinity chromatography resin prepared by amide formation between the activated ester Affigel-15 and a squalene diamine derivative. The OSC showed a molecular weight of 26 kDa, as estimated by SDS-PAGE.

Cycloartenol and β-amyrin cyclases, which are two OSC from higher plants, were purified from pea seedlings and from suspension cultures of *Rabdosia japonica*.[31–34] The cyclases were purified to homogeneity by solubilization with Triton X-100, chromatography on hydroxylapatite and DEAE-cellulose, isoelectric focusing and gel filtration. A crucial step was the use of isoelectric focusing,[34] which enabled the separation of cycloartenol cyclase (pI = 5.1) from β-amyrin cyclase (pI = 5.6). Each gave a single band on SDS-PAGE with a molecular mass of 55 and 35 kDA, respectively.

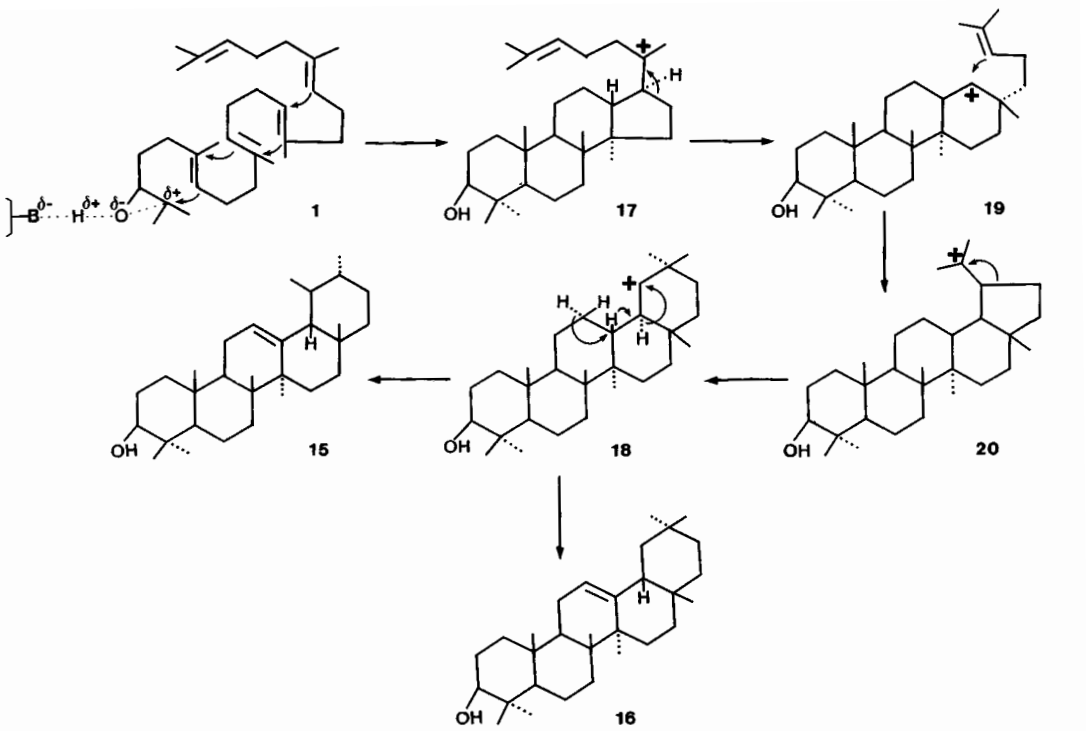


Figure 3 Mechanism of cyclization of 2,3-oxidosqualene to α- and β-amyrin.

Table 1 Purification and Properties of 2,3-oxidosqualene Cyclases from Vertebrates, Higher Plants, and Yeasts

Source	Ref.	Mol Wt, kDa	Purif. Factor from Microsomes	Spec. Activity
Vertebrates				
Pig liver	23		138	256[a]
Pig liver	25	75	441	2643[a]
Rat liver	25	78		
Dog liver	25	73		
Human liver	25	73		
Rat liver	24	75	1863	436[b]
Rat liver	26	65	128[c]	58[d]
Yeasts				
S. cerevisiae	28		142	84[a]
S. cerevisiae	29		112	40[e]
S. cerevisiae	30	26	160	4400[f]
Higher Plants				
R. japonica				
Cycloartenol	33	54	139	16[b]
β-amyrin	33	28	541	41[b]
Pea				
Cycloartenol	31	55	235	167[g]
β-amyrin	32	35	1072	28[b]

[a] nmol/h/mg; [b]μkat/kg; [c]from homogenate; [d]pmol/min/mg; [e]mg protein necessary for 20% conversion ratio; [f]units/mg; [g]pkat/mg.

Very recently numerous cyclase genes were cloned and sequenced, making it possible to deduce the primary amino acid sequences of the enzyme.[35–41] The gene encoding the *Candida albicans* OSC was initially cloned by Kelly et al.[35] on the basis of its ability to complement cyclase deficient (erg7) strains of *Saccharomyces cerevisiae*. This gene was sequenced,[36,37] showing that it consists of 2184 nucleotides and encodes a predicted protein of 728 amino acids with molecular weight 83.7 kDa. This size is similar to the relative Mr above reported for purified vertebrate OSC (73-78 kDa).[25] A hydropathic plot indicated that the enzyme is a moderately hydrophilic protein with two notable hydrophobic regions which may be involved in anchoring the enzyme to membranes. There was good homology between the predicted amino acid sequence of the *C. albicans* OSC and the predicted amino acid sequence of the squalene hopene cyclase of *Alicyclobacillus acidocaldarius*.[38] Four regions of notable similarity varied from 28% identity over 77 residues to 46% identity over 37 residues. The cloning of the ERG7 gene encoding *S. cerevisiae* cyclase, using genetic complementation was also reported.[39] The DNA and derived amino acid sequences of this gene predict that the *S. cerevisiae* cyclase is a protein of 731 amino acids with a molecular mass of 83.4 kDa, substantially larger than that assigned to the yeast OSC purified by Corey (26 kDa).

The predicted amino acid sequence of *S. cerevisiae* cyclase[40] shows 63% identity with that of *C. albicans* cyclase,[36] 39% identity with that of cycloartenol cyclase from *Arabidopsis thaliana*[41] and 16% identity to the predicted amino acid sequence of squalene-hopene cyclase from *A. acidocaldarius*.[38] Corey recently isolated an *A. thaliana* gene encoding cycloartenol cyclase.[41] A yeast mutant lacking OSC was transformed with an *A. thaliana* cDNA yeast expression library and transformants assayed for the ability to cyclize OS to cycloartenol. This activity was found to be dependent on a plasmid capable of encoding an 86 kDa protein with significant homology to OSC either from *C. albicans* (34%) or *A. acidocaldarius* squalene hopene cyclase (18%). Later, Corey et al.[40] were able to clone, characterize and overexpress the ERG7 *S. cerevisiae* gene encoding OSC. The functional reconstructed gene contains a 2196 bp open reading frame capable of encoding an 83 kDa protein similar to that found by Griffin et al.[39] Purification of OSC from an overexpressing strain gave an 80 kDa protein. Further effort succeeded in separating the 26 kDa band from the enzymatic activity in OSC purification from wild-type yeast. Consequently, it is possible that the OSC purified by Corey could be a degradation product.

In conclusion, comparison of the different results obtained by enzyme purification and by cloning and sequencing OSC genes of different origin, shows that the various OSC are very similar. This provides support for the postulated model for divergent evolution of cyclase genes from a common ancestral cyclase.[42–45]

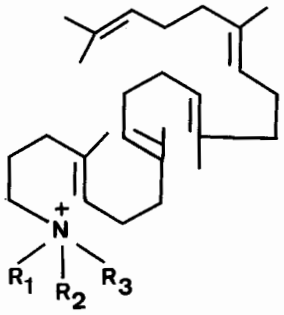
III. OXIDOSQUALENE CYCLASE INHIBITORS AFFECTING THE CYCLIZATION PROCESS

The enzymatic cyclization of 2,3-oxidosqualene to tetra- or pentacyclic triterpenes may be ideally separated into three distinct steps: (1) acid-catalyzed opening of the epoxide ring; (2) concerted or not concerted cyclization to give a tetra- or pentacyclic carbonium ion (i.e. protosteryl, dammarenyl or oleanyl ions); (3) the concerted backbone rearrangement to lanosterol, cycloartenol or similar tetra- or pentacyclic triterpenes. This step is characterized by the final abstraction of a proton by a suitable basic or nucleophilic group in the enzyme.

Each enzymatic step could be affected by certain OSC inhibitors, thus shedding light on the precise mechanism of action of the enzyme. Our initial interest in the study of OSC was the development of new inhibitors for potential use as hypocholesterolemic, antifungal or phytotoxic drugs.[13,46–48] To prepare the new inhibitors, we applied a general strategy[9] mimicking the above described carbonium ions by replacing the positively charged carbon atom in the structure with a nitrogen (protonated at physiological pH). These inhibitors are called analogues of the enzyme-bound high energy intermediates (HEI).[49–53]

Initially we focused our attention on the mechanism of opening of the oxirane ring, initiated by an electrophilic attack of a proton on OS, which implies the formation of a series of dipoles, leading to the C-2 carbocation.[12] Following Van Tamelen, this mechanism can be viewed as an SN_2 or SN_1-like mechanism.[54] Thus, we synthesized a series of molecules such as 2-aza-2,3-dihydrosqualene **21** and related compounds, such as **22-26** (Figure 4), in order to mimic the C-2 transient carbocation. We tested their effect *in vitro*[46,47,55–59] on OSC from different sources (microsomes or purified enzymes from rat liver, pig liver, higher plants and yeasts), as well as *in vivo* in rats, higher plants, yeasts and fungi. As expected, compounds **21-26** were potent inhibitors of OSC from animals, yeasts or higher plants (IC_{50} ranged from 0.15 to 16 μM) (Table 2), as well as inhibitors of squalene epoxidase, an enzyme that

transforms squalene to 2,3-oxidosqualene.[6,60] They did not inhibit specifically, since they also affected similarly the OSC of animals, fungi or higher plants. The obvious conclusion is that the different OSC may possess similar structures in that part of the enzyme responsible for protonation of the substrate and the following formation of the first C-2 HEI. Moreover, these C-2 HEI analogue inhibitors do not strictly need a geometrically correct folded squalene skeleton, since very different compounds (Figure 5) such as U-18666A **27** and derivatives,[61–65] *N*-dodecylimidazole **28**,[66] 2,3-iminosqualene **29**,[67] and *N*,*N*-dimethyldodecylamine **30** are equally active on higher plant and yeast OSC.[13,55]



21 $R_1=R_2=Me$, $R_3=H$
22 $R_1=R_2=Et$, $R_3=H$
23 $R_1=R_2=Me$, $R_3=O^-$
24 $R_1=R_2=Et$, $R_3=O^-$
25 $R_1=R_2=R_3=Me$
26 $R_1=R_2=Et$, $R_3=Me$

Figure 4 Structures of 2-aza-2,3-dihydrosqualene and derivatives.

Table 2 I_{50} Values[a] (m*M*) of *In Vitro* Inhibition of 2,3-oxidosqualene Cyclases by 2-aza-2,3-dihydrosqualene **21** and Derivatives **22-25**

Compound	Rat Microsomes	Pig Microsomes	Solubilized Pig Enzyme	*S.cerevisiae* Microsomes	*C. albicans* Microsomes
21	7.5	2.3	0.15	10	6.5
22	3.2	ND	ND	12.5	ND
23	3.7	7	3.3	16	ND
24	1.5	ND	ND	14	ND
25	5.1	ND	ND	ND	13.3

[a] Values are means of two different experiments. ND, not determined.

Recently we were able to suppress inhibition of squalene epoxidase, without impairing potency towards OSC (Figure 6), by introducing a polar group, such as epoxy or hydroxy in the squalene skeleton,[68–70] as in compounds **31-34** (Table 3). Azasqualene alcohol **33** proved to be the most specific compound both in microsomes (from animal liver, higher plants and yeasts), and cell cultures. Failure to inhibit squalene epoxidase may be explained by the fact that introduction of a polar function makes it more difficult to bind the hydrophobic active site of the enzyme.

The next step in our strategy was to mimic the different conformationally rigid carbonium ion intermediates, such as the C-8 carbonium ion and the C-20 carbonium ion (Figure 7), by designing the corresponding acyclic azasqualenoid derivatives **35** and **36**. Various groups have synthesized cyclic aza derivatives (Figure 8), with a nitrogen corresponding to the C-10 HEI as in **37**, to the C-8 HEI as in the azadecaline derivatives **38** and **39**, to the C-13 HEI as in the aza tricyclic derivative **40**, and to the C-20 HEI as in the azadammarenol **41**.[71–77] The mono- and bicyclic derivatives were shown to be potent inhibitors of OSC, whereas the tricyclic and tetracyclic ones showed only modest inhibition *in vitro*. We believe that the acyclic azasqualenoid derivatives, conformationally more flexible than the cyclic ones, should not interfere with other sterol biosynthesis enzymes, such as Δ^8-Δ^7 sterol isomerase or Δ^{14} sterol

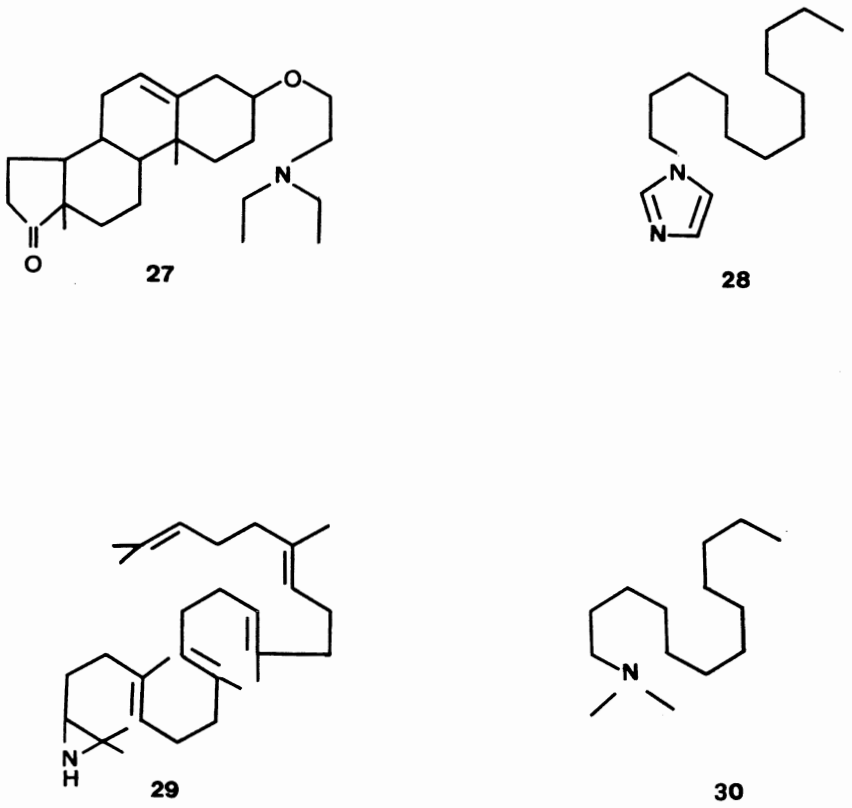


Figure 5 Structures of U-18666A, *N*-dodecylimidazole, 2,3-iminosqualene and *N,N*-dimethyldodecylamine.

demethylase, sometimes inhibited by the cyclic azasqualenoid derivatives.[74,78–80] It is also known that some cyclic uncharged inhibitors, such as 4,4,10β-trimethyl-*trans*-decal-3β-ol (TMD) **42**, are potent inhibitors of OSC. Recently it was shown that some amide and thioamide derivatives (Figure 9), such as compounds **43-45** were also potent inhibitors of OSC.[81,82] In addition, a series of sulfur-containing oxidosqualene analogues **46** and **47** showed a significant inhibition of mammalian OSC.[72]

The first internal acyclic azasqualene derivative synthesized in our laboratory was the C-8 HEI analogue, (6*E*)-10-aza-10,11-dihydrosqualene 2,3-epoxide **35**.[83,84] We found that inhibition of OSC was strictly dependent on the overall conformation of the azasqualene derivative. In fact, by comparing the activity of the two different geometrical isomers (Figures 7 and 9) (6*E*) **35** and (6*Z*) **48**, only **35**, the carbonium ion analogue, corresponding to the natural all *E* OS, was active in all the biological systems tested: the I_{50} values varied from 3–5 μ*M* in yeasts to 5 μ*M* in animal OSC (Table 4). Moreover, both isomers (6*E*) **35** and (6*Z*) **48**, as opposed to 2-aza-2,3-dihydrosqualene **21** were inactive on squalene epoxidase from rat liver. The *in vivo* results obtained by studying the activity of the two isomers in 3T3 fibroblast cell cultures confirmed the potency and selectivity of (6*E*)-10-aza-10,11-dihydrosqualene 2,3-epoxide **35**. In contrast **35** failed to inhibit OSC from maize,[85] whereas the azadecalines and the 2-aza-2,3-dihydrosqualene derivatives proved to be potent inhibitors.

Thus, the noncyclized azasqualene analogues of the C-8 HEI must possess the all-*trans* squalene geometry of the natural substrate. Moreover, the pro C-8 HEI inhibitor **35** appeared to be more selective than the C-2 HEI azasqualene-type inhibitors such as **21**, since it was able to inhibit OSC from animals and yeasts, but not from higher plants.

The next step in our program was the synthesis of acyclic azasqualenes mimicking the C-20 carbonium ion intermediate,[86] such as 19-aza-18,19,22,23-tetrahydrosqualene-2,3-epoxide **36**, its *N*-oxide **49** and 19-aza-18,19,22,23-tetrahydrosqualene **50** (Figure 10). The most active compounds, 19-aza-18,19,22,23-tetrahydrosqualene-2,3-epoxide **36**, and its *N*-oxide **49** showed *in vivo* (Hep G2 hepatoma cultures) and *in vitro* an inhibitory activity (microsomes from pig and rat liver and partially purified OSC from pig liver) similar to that found for the C-2 HEI analogue inhibitor 2-aza-2,3-dihydrosqualene **21** and its

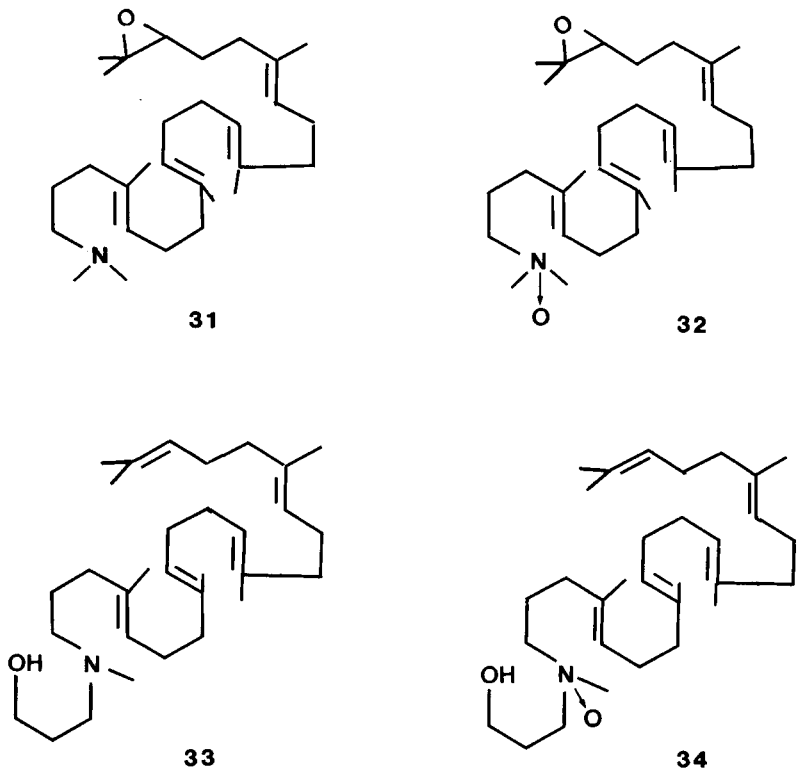


Figure 6 Structures of 22,23-epoxy-2-aza-2,3-dihydrosqualene, azasqualene alcohol and their *N*-oxides.

Table 3 Inhibition of Squalene Epoxidase in Rat Liver Microsomes

Inhibitor	Concentration (μ*M*)	Inhibition (%)
Azasqualene **21**	10	70
Azasqualene epoxide **31**	10	8
Azasqualene epoxide *N*-oxide **32**	10	14
Azasqualene alcohol **33**	10	2
Azasqualene alcohol *N*-oxide **34**	10	13

N-oxide **23** (Table 5). The presence of an epoxide ring is essential for the activity, since 19-aza-18,18,22,23-tetrahydrosqualene **50** was not active under the same conditions. The two 19-azasqualene-2,3-epoxide derivatives **36** and **49** were not effective inhibitors of OSC from yeast microsomes or from higher plants such as cycloartenol OSC or β-amyrin OSC[85] and they did not inhibit animal squalene epoxidase. Consequently, 19-aza-18,19,22,23-tetrahydrosqualene-2,3-epoxide **36** is a more selective inhibitor than 10-aza-10,11-dihydrosqualene 2,3-epoxide **35** and 2-aza-2,3-dihydrosqualene **21**, since it inhibited mainly mammalian OSC, rather than yeast or higher plant OSC.

Thus, it was confirmed that the internal azasqualenes, provided with the correct all *E* geometry and bearing an epoxide terminal ring, could mimic the corresponding HEI formed by cyclization of OS, as shown by their good inhibitory activity. This can be considered indirect evidence of the Ruzicka, Cornforth and Van Tamelen studies which postulate the cyclization of a correctly folded OS to tetra- or pentacyclic triterpenes through the formation of a series of conformationally rigid carbonium ion intermediates (from C-2 to C-20). This theory was further developed by Johnson and colleagues,[14,87,88] who postulated that the C-8, C-13 and C-20 cationic HEI formed by cyclization of OS would be stabilized by a suitable external negative point charge present in the enzyme by ion pairing (Figure 11).

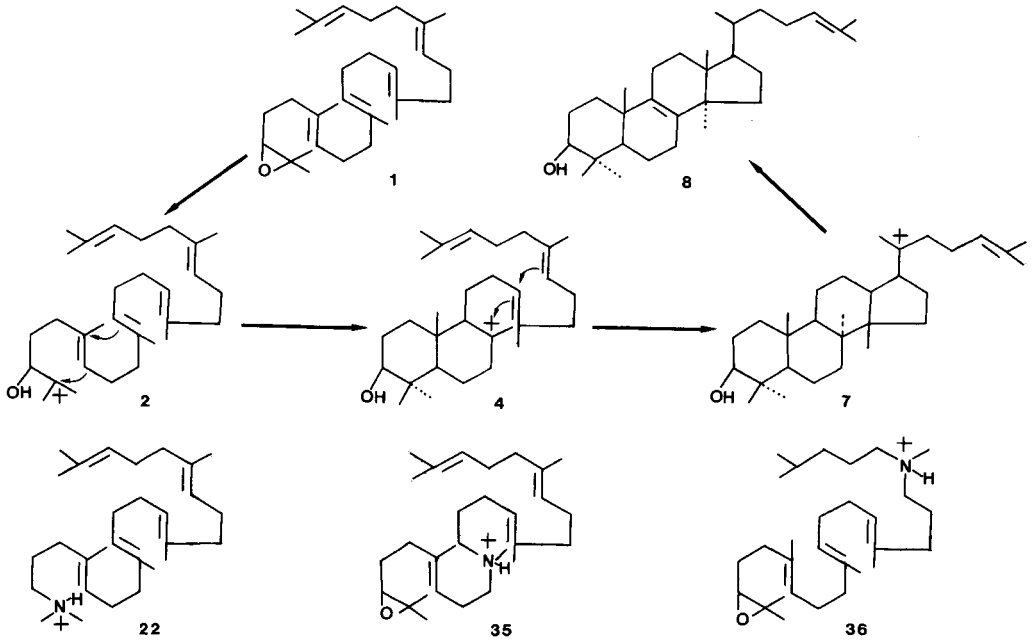


Figure 7 The acyclic azasqualenes mimicking the C-2, C-8 and C-20 carbonium ion intermediates formed during cyclization of 2,3-oxidosqualene to lanosterol.

Later, Poralla and coworkers,[89,90] comparing the amino acid sequences of bacterial OSC of *A. acidocaldarius* and *Zymomonas mobilis* with those of *C. albicans* and *A. thaliana*, proposed the QW (tryptophan-glutamine) motif theory (Figure 12), on the basis of the evolutionary considerations of Nes, Rohmer and Ourisson.[5,42–45] In particular, Poralla found a specific 16 amino acid repeat (QW motif) occurring mainly within the carboxy terminal part in aligned positions. One alignable QW motif also occurs at the extreme amino terminus and one additional nonalignable motif can be found at the amino terminus of the bacterial cyclase. The consensus structure of the QW motif is Arg/Lys Gly/Ala X_{2-3} Tyr/Phe/Trp Leu X_3 Gly X_{2-5} Gly X Trp. Part of the QW motif also occurs in one peptide obtained by proteolysis and cyanogen bromide digestion of rat liver OSC (Figure 13) covalently modified by 29-methylidene-2,3-oxidosqualene **51**, a suicide substrate for vertebrate OSC.[91,92] The QW motif contained mainly hydrophobic aromatic-rich amino acids such as tryptophan or phenylalanine or tyrosine, which may correspond to the "sites of negative point charges" postulated by Johnson for the stabilization of the carbocation during cyclization of OS. In this case, the carbocation intermediates would be stabilized through π-cation interactions, as suggested for receptor-ligand and enzyme-substrate complex as found in biomimetic catalyst systems.[93–97] Shi et al., following Poralla's postulate, assumed the "aromatic" hypotheses for cyclase active site structure[39] (Figure 14). Following this hypothesis, the electron-rich indole and phenol side chain of tryptophan and tyrosine residues, respectively, present in OSC stabilize the positively charged carbocationic intermediates through cation π-interactions. Probably almost two cation π-interactions, one from each face of the folded squalene, are necessary to assist the correctly folded stereospecific cyclization of OS.

Returning to our internal azasqualene derivatives, we can affirm that their proposed mode of action as HEI analogues is in line with the theories of Poralla and Griffin. Indeed, in order to rationalize the evolutionary QW motif theory, they supposed the occurrence of almost 5-6 and 6-7 "motifs", respectively, for tetracyclic and pentacyclic triterpene formation from OS. This is not completely in agreement with the hypothesis of Johnson, who proposed the occurrence of three sites of interaction between the negatively charged points and the intermediate carbocations (i.e. C-8, C-13 and C-20). Our work and that of Rahier described new azasqualene OSC inhibitors mimicking the ions C-2 as in **21**, C-10 as in **37**, C-8 as in **35**, **38** and **39**, C-13 as in **40** and C-20 as in **36** and **41**. This confirms the formation of five carbocationic intermediates in accord with the QW motif theory (Figure 15).

To inhibit OSC, we showed that both (6*E*)-10-aza-10,11-dihydrosqualene 2,3-epoxide **35** and 19-aza-18,19,22,23-tetrahydrosqualene 2,3-epoxide **36** must possess a correct all-*E* geometry as well as an

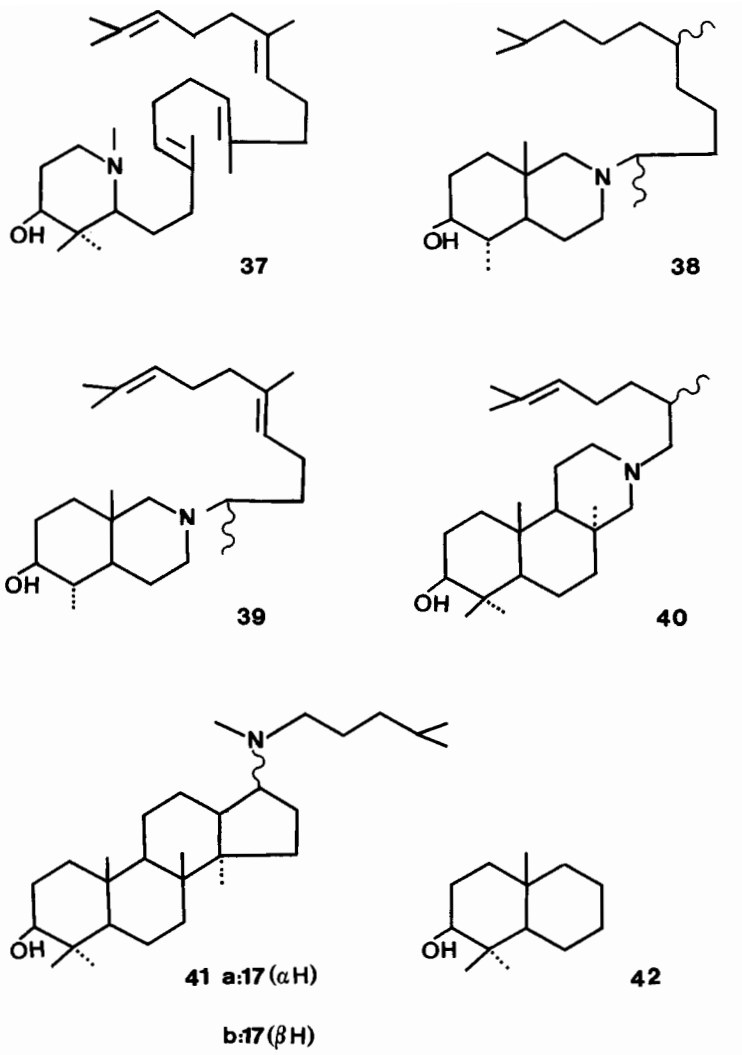


Figure 8 Structures of various monocyclic, bicyclic, tricyclic and tetracyclic aza derivatives and of 4,410β-trimethyl-*trans*-decal-3β-ol.

epoxide terminal ring to obtain the best conformation mimicking the pro C-8 and pro C-20 carbocationic intermediates. At this point two mechanisms can be envisaged: (1) acyclic internal azasqualenes are only recognized, and not cyclized by OSC through potent ion pair interactions (ammonium vs. electron-rich residues of the enzyme); (2) acyclic azasqualenes are recognized and cyclized by OSC to form an intermediate which can be covalently or noncovalently bound to the enzyme. To clear this problem, we made an accurate kinetic study utilizing microsomal or partially purified animal OSC.

It was earlier found that both acyclic azasqualene derivatives and cyclic aza derivatives apparently behaved as noncompetitive inhibitors.[68,70] Generally, this feature has been explained by the highly anisotropic OSC membrane-bound enzyme faced with the highly hydrophobic substrate or inhibitors. Surprisingly, both (6*E*)-10-aza-10,11-dihydrosqualene 2,3-epoxide **35** and 19-aza-18,19,22,23-tetrahydrosqualene 2,3-epoxide **36** (Figure 16) were able to inactivate OSC from pig liver in a time-dependent manner, while 19-aza-18,19,22,23-tetrahydrosqualene **50** was ineffective.[98] At the moment we cannot definitively decide about the type of inhibition (whether tight-binding reversible or site-directed irreversible) and the nature of the inhibition involved (the acyclic aza derivative or a cyclized intermediate). Some preliminary results seem to indicate a truly irreversible inhibition through cyclization, giving a cyclic reactive intermediate. So we can explain now why the C-2 HEI analogues did not require an

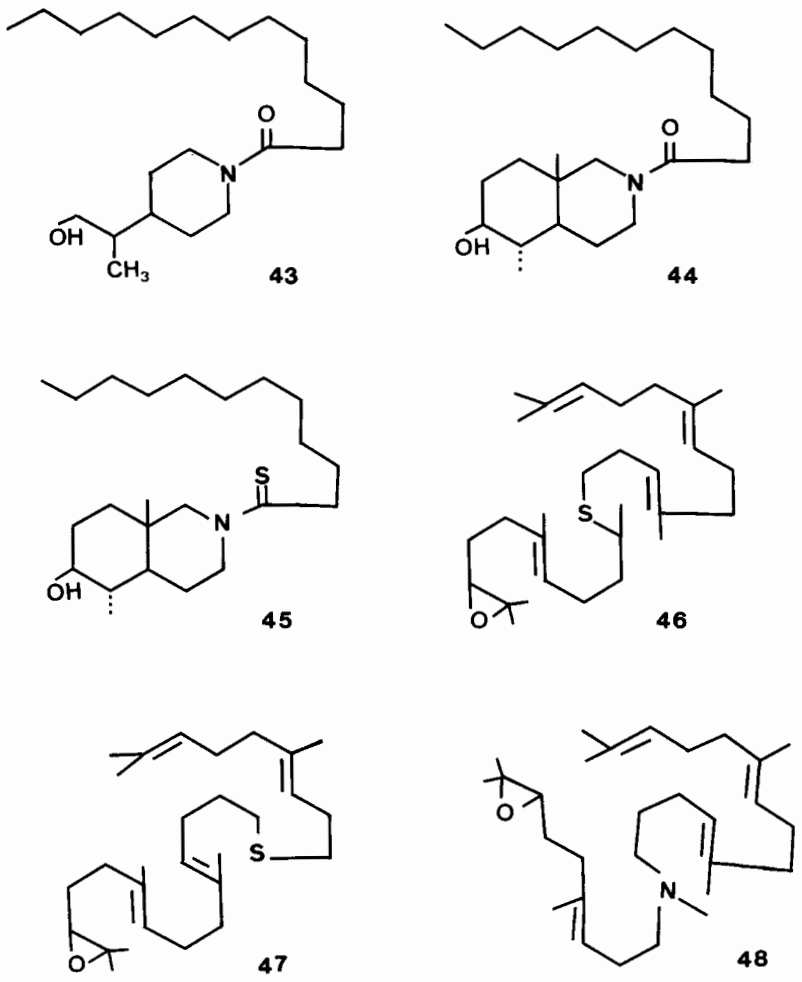


Figure 9 Structures of various monocyclic and bicyclic amides and thioamides, 11-thio- and 15-thio-2,3-oxidosqualene analogues, and (6*Z*)-10-aza-10,11-dihydrosqualene-2,3-epoxide.

Table 4 I_{50} Values[a] (μ*M*) of Inhibition of Microsomal 2,3-oxidosqualene Cyclase by (6*E*)- **35** and (6*Z*)-10-aza-10,11-dihydrosqualene-2,3-epoxide **48**

Type of Microsomes	Protein Conc. mg/ml	Isomer *E* 35	Isomer *Z* 48
Rat Liver	5	4.8	>20
Pig liver	1	5	ND
S. cerevisiae	2	5	>100
C. albicans	3	3	>100

[a] Values are means of two different experiments. ND, not determined.

all-*E* folded squalene analogue (i.e. *N,N*-dimethyl and *N,N*-diethyldodecylamine were also effective), while the internal azasqualenes needed a terminal epoxide function and a strict all-*E* conformation.

As postulated by the QW motif theory, OSC of different origins have to be very similar. Nevertheless, some differences exist between crucial sequences of the different isoenzymes especially in those responsible for interaction with carbocationic intermediates. We found progressive selectivity on the inhibition of different OSC going from C-2 to pro C-8 and pro C-20 HEI. This could mean that the OSC active site complementary to the C-8 and C-20 HEI differs in mammalian, yeast and higher plant enzymes.

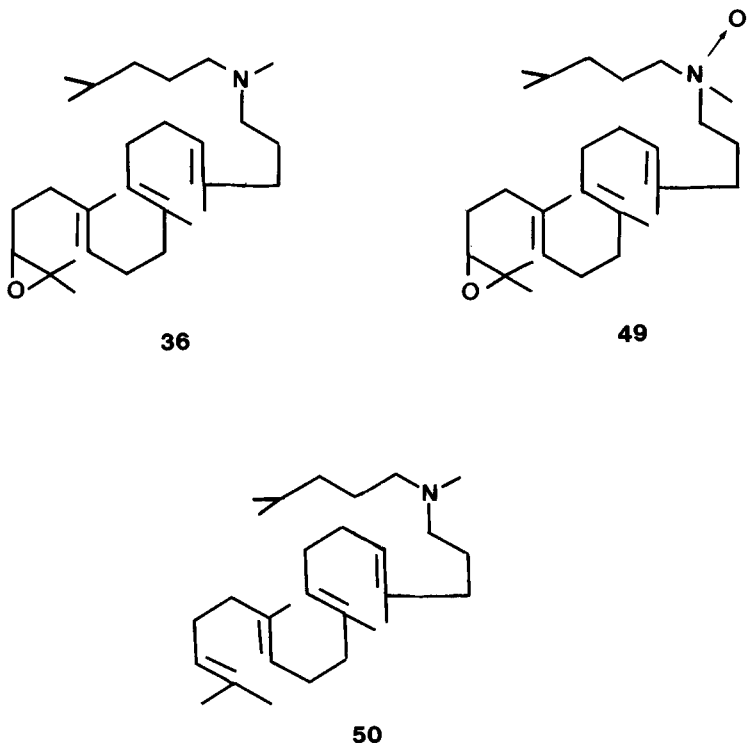


Figure 10 Structures of 19-aza-18,19,22,23-tetrahydrosqualene-2,3-epoxide, its *N*-oxide and 19-aza-18,19,22,23-tetrahydrosqualene.

Table 5 IC_{50} Values[a] (μM) of Inhibition of 2,3-oxidosqualene Cyclase by 19-aza-18,19,22,23-tetrahydrosqualene-2,3-epoxide **36** and its *N*-oxide **49**

Enzymatic Assay	Protein Conc. mg/ml	Compounds 36	49
Partially purified pig enzyme		1.7	7
Rat liver	5	7.5	ND
Pig liver	1	1.5	ND
S. cerevisiae	2	35	100
C. albicans	3	22	55

[a] Values are means of two different experiments. ND, not determined.

The vertebrate OSC active site covalently attached to the site-directed inhibitor 29-methylidene-2,3-oxidosqualene **51** contains the conserved sequence Asp-Asp-Thr-Ala-Glu-Ala (DDTAEA) (Figure 13).[25,91,92] Based on the observed labeling by [^{3}H]-29-methylidene-2,3-oxidosqualene **51**, the two aspartate residues appeared responsible for the nucleophilic attack of the enzyme to the C-20 ion intermediate arising by cyclization of OS. In contrast to animal OSC, neither yeast nor plant OSC were labeled, indicating that in these cases stabilization is not attained by covalent binding, but probably by electrostatic interactions, giving rise probably to a slow tight-binding inhibition. By comparison of the compound **51** binding site sequence with fungal, higher plant and bacterial OSC/SC, Corey et al.[41] observed a SDCTAE consensus sequence in *A. thaliana* and *C. albicans* OSC in which a cysteine residue substituted an aspartate residue. For this reason, we can speculate why compound **36** was mainly active on the animal OSC, compound **35** showed activity in yeast and mammalian OSC, and finally the "external" azasqualene **21** was active in all tested OSC (animal, yeast and higher plant).

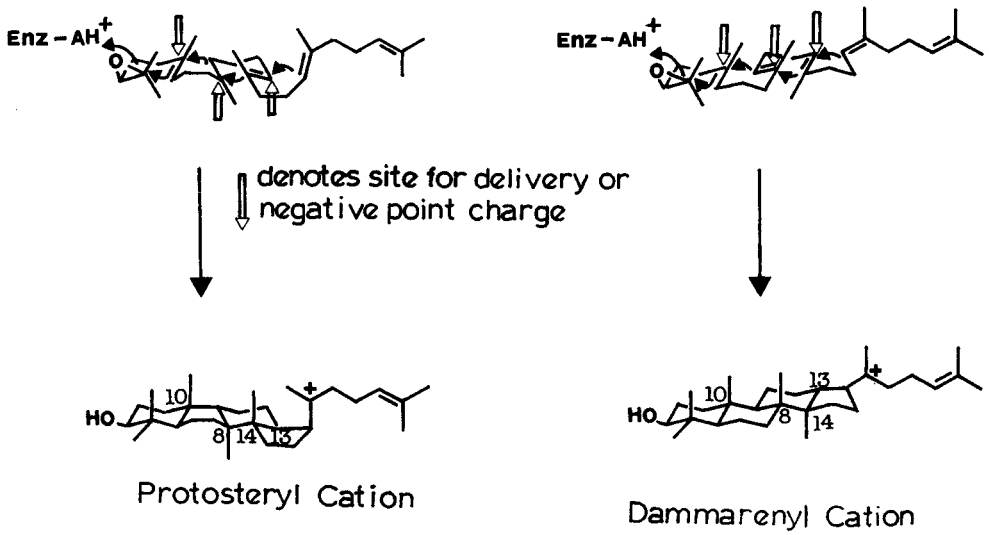


Figure 11 Proposed mechanism for cyclization of 2,3-oxidosqualene to protosteryl and dammarenyl cations involving stabilization by suitable external negative point charges by ion pairing. (From Abe, I., Rohmer, M., and Prestwich, G.D., *Chem. Rev.*, 93, 2189, 1993. With permission.)

A.a.	QW_1	572	RGVQYLVETQRPDGGW	Z.m.	QW_1	595	KGINWLAQNQDEEGLW
C.a.	QW_1	671	RGIQFLMKRQLPTGEW	A.t.	QW_1	702	RAARYLINAQMENGDF
A.a.	QW_2	514	KAALWVEQHQNPDGGW	Z.m.	QW_2	536	KAVAWLKTIQNEDGGW
C.a.	QW_2	613	RGCDFLISKQLPDGGW	A.t.	QW_2	640	KACEFLLSKQQPSGGW
R.r.	peptide		YLRSVQLPDGGW				
A.a.	QW_3	465	RA.VEYLQREQ.KPDGSW	Z.m.	QW_3	487	KAAVDYLLKEQ.EEDGSW
C.a.	QW_3	562	SSAIQYILDSQDNIDGSW	A.t.	QW_3	591	KAVK.FIESIQ.AADGGW
A.a.	QW_4	398	KGFRWIVGMQSSNGGW	Z.m.	QW_4	420	RAMEWTIGMQSDNGGW
C.a.	QW_4	482	DAVEVLLQIQN.VGEW	A.t.	QW_4	514	EAVNYIISLQNADGGL
A.a.	QW_5	332	KAGEWLLDRQI.TVPGDW	Z.m.	QW_5	350	SALSWLKPQQILDVKGDW
A.a.	QW_6	17	RAVEYLLSCQKDEGYW	Z.m.	QW_6	24	KATRALLEKQQQDGHW
C.a.	QW_6	72	KGADFLKLLQLDNGIF	A.t.	QW_6	99	RGLDFYSTIQAHDGHW

Figure 12 Alignable QW motifs (1–6) of eukaryotic and bacterial triterpenoid cyclases. The peptide fragment from rat liver lanosterol cyclase aligns best to QW motifs 1 and 2. The numbers indicate the sequence position of the first amino acid of the motif. A.a., *Alicyclobacillus acidocaldarius*; Z.m., *Zymomonas mobilis*; C.a., *Candida albicans*; A.t., *Arabidopsis thaliana*; R.r., *Rattus rattus*. The rat liver OSC peptide is also shown for comparison. (From Poralla, K., Hewelt, A., Prestwich, G. D., et al., *TIBS*, 19, 157, 1994. With permission.)

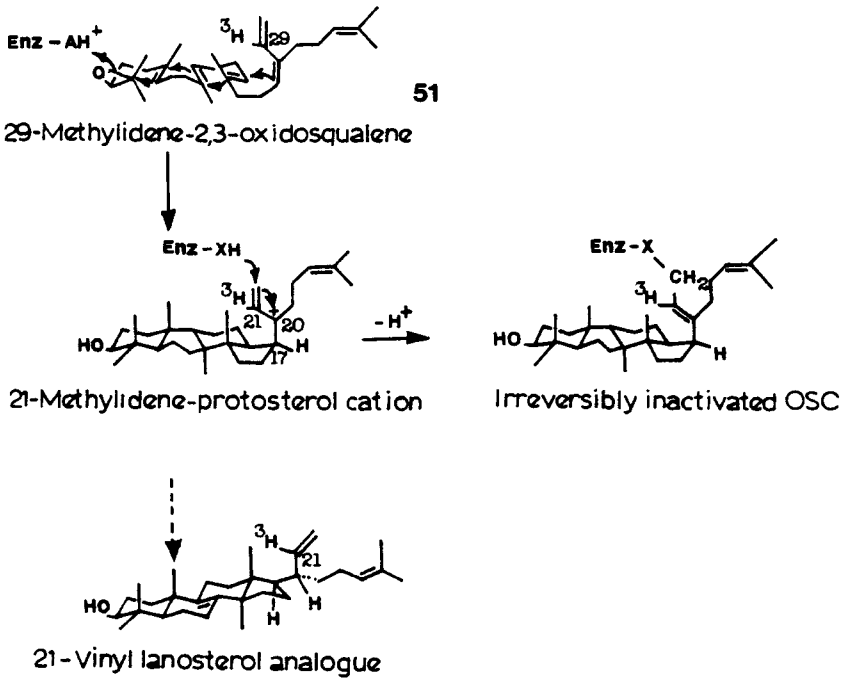


Figure 13 Affinity labeling experiments of OSC using the potent mechanism-based inhibitor, 29-methylidene-2,3-oxidosqualene and comparison of its binding site sequence in rat OSC with fungal and bacterial OSC/SC. (From Abe, I., Rohmer, M., and Prestwich, G. D., *Chem. Rev.*, 93, 2189, 1993. With permission.)

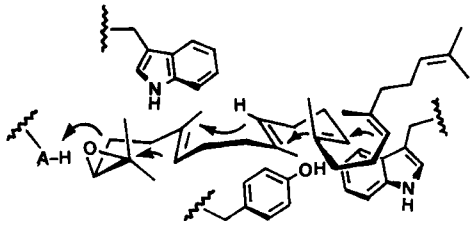
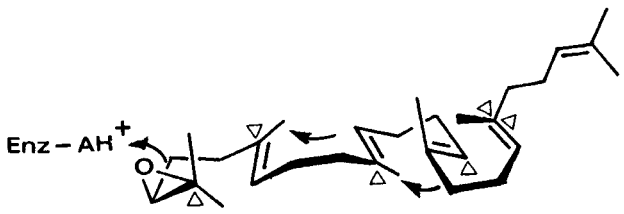


Figure 14 Proposed stabilization of the carbonium ions arising during cyclization of 2,3-oxidosqualene to the protosterol cation. (From Shi, Z., Buntel, C. J., and Griffin, J. H., *Proc. Natl. Acad. Sci. U.S.A.*, 91, 7370, 1994. With permission.)

IV. OXIDOSQUALENE CYCLASE INHIBITORS AS THIOL-MODIFYING AGENTS

Synthesis and study of the biological action of new site-directed irreversible OSC inhibitors may help to understand the enzyme's structure and mechanism of action. It has been suggested that both prokaryotic SC and eukaryotic OSC are sensitive to thiol reagents. *p*-Chloromercuribenzenesulfonic acid and *N*-ethylmaleimide strongly inhibited squalene-hopene cyclase from *A. acidocaldarius*[99] and OSC from yeast [29] and hog liver.[23] In particular, Duriatti and Schuber, using inactivation experiments with *N*-ethylmaleimide,[23] showed that a sulfydryl group is essential for the activity of mammalian liver cyclase.



△ denotes possible sites of interaction of the QW motifs with the carbonium ion intermediates.

Figure 15 Supposed positions of interaction of the QW motifs of OSC that are implied in the stabilization of the carbonium ions and in the correct cyclization of 2,3-oxidosqualene to the protosterol cation.

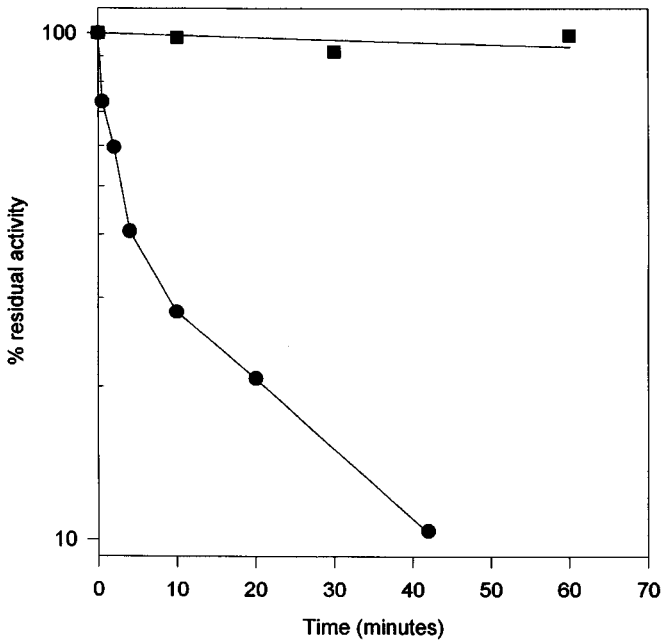


Figure 16 Time-dependent inhibition of 2,3-oxidosqualene cyclase by 19-aza-18,19,22,23-tetrahydrosqualene-2,3-epoxide (5 μ*M*) (●) compared with that of 19-aza-18,19,22,23-tetrahydrosqualene (50 μ*M*) (■). OSC was preincubated at 37°C in phosphate buffer in the presence of the inhibitors. The enzymatic activity was determined as percent of the activity obtained after preincubation in the absence of inhibitors.

Search for more specific inactivators of yeast OSC, which would be able to enter and modify the active site of the enzyme, led us to design new thiol-modifying reagents possessing a squalene skeleton. With this in mind, a series of potential irreversible inhibitors of OSC carrying a squalenoid or dodecyl skeleton (Figure 17) were synthesized.[100] They consisted of Michael-type inhibitors such as squalene maleimide **52** and the α,β-unsaturated nitrile **53** or Ellman-type squalene inhibitors such as the 2-nitro-5-dithiobenzoic derivative **54** (Squalene Ellman) and the 2-dithiopyridyl derivative **55**. By comparing the activity of such inhibitors we found that the maleimide derivatives **52** and **56** were the most active inhibitors of mammalian OSC, followed by the Ellman-type compounds **54** and **57**. By evaluating the time-dependent inactivation of these compounds following their incubation with the mammalian enzyme,

we found that only the maleimide derivatives, such as squalene maleimide **52** were effective as irreversible inhibitors (Figure 18). In particular, we found that the substrate OS was not able to protect the enzyme from inactivation by *N*-dodecylmaleimide **56**, but partially protected the enzyme after incubation with squalene maleimide **52**.

Our results confirmed the presence of different thiol groups essential for the activity of mammalian OSC. One of these groups could be present at the active site of the enzyme. Surprisingly, the squalene Ellman derivative **54** (3-carboxy-4-nitrophenyldithio-1,1′,2-tris-nor-squalene; CNDT-squalene) did not

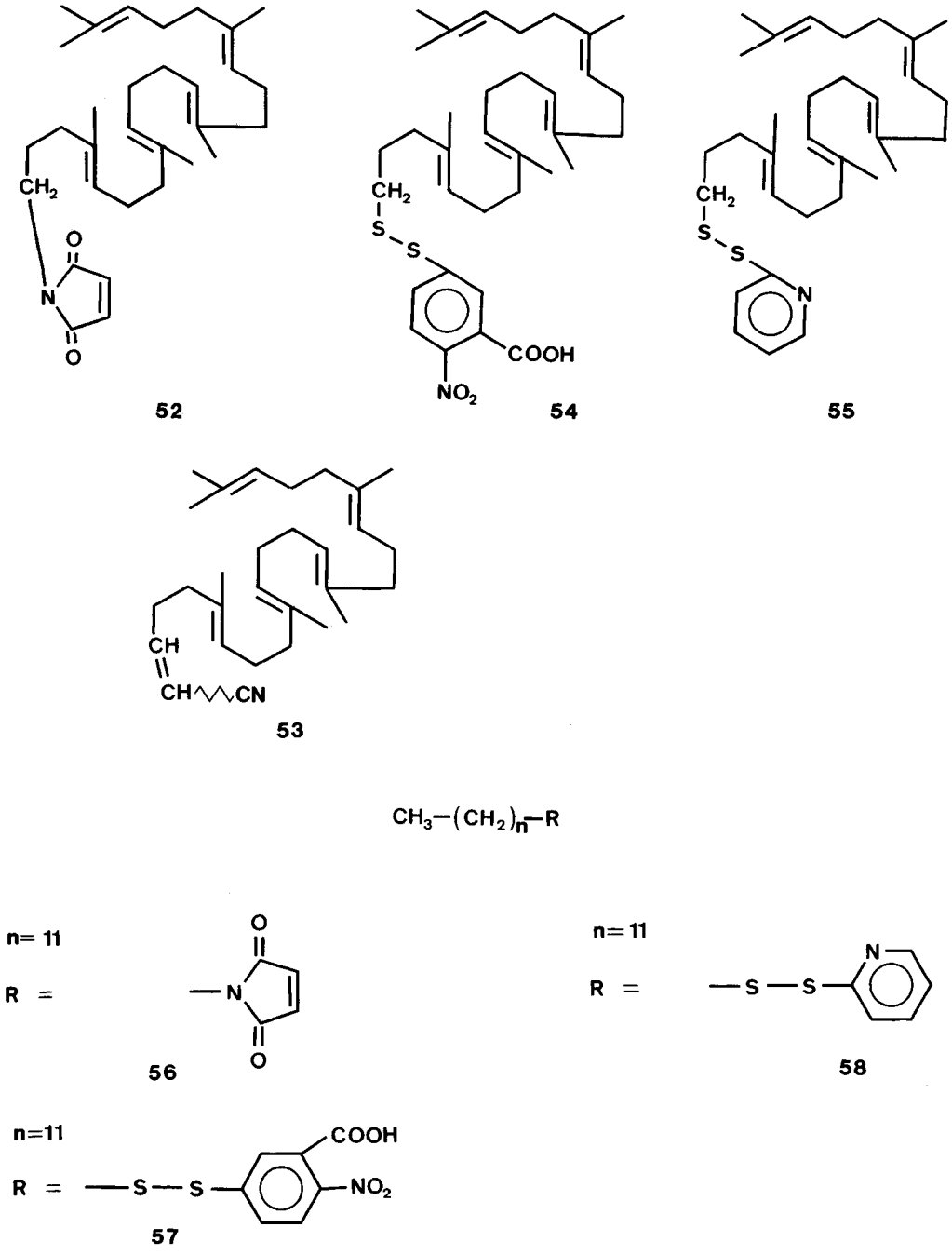


Figure 17 Structures of various squalene and dodecyl derivatives as thiol-modifying agents.

behave as a time-dependent irreversible inhibitor of liver OSC. This compound was found to be an irreversible inhibitor of OSC from *S. cerevisiae*.[101] In order to localize the thiol groups involved in the inactivation of OSC, substrate protection experiments were performed in the presence of inactivator **54**. Substrate, at a saturating concentration, clearly protected the yeast OSC against inactivation (Figure 19).

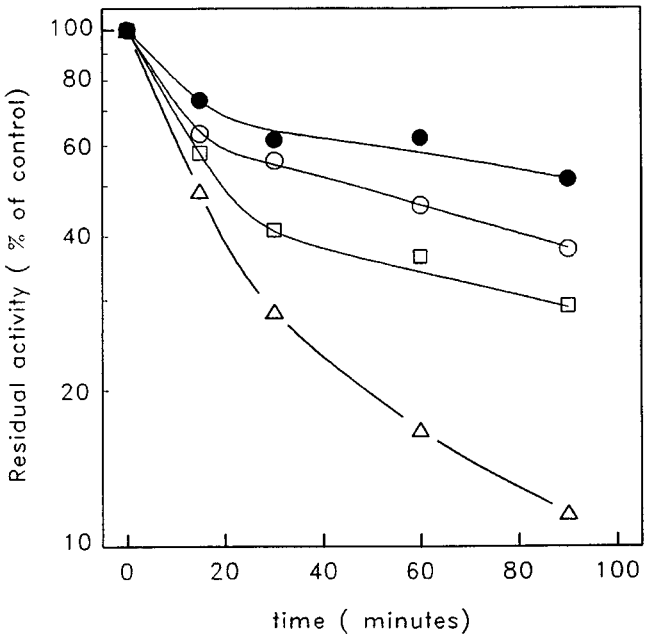


Figure 18 Time-dependent inhibition of 2,3-oxidosqualene cyclase with different concentrations of squalene maleimide. Residual activity (expressed as percent of controls without inhibitor) was determined after preincubation of solubilized OSC with squalene maleimide 50 μ*M* (○), 100 μ*M* (□), and 200 μ*M* (△). The black circles indicate the residual activity after preincubation of 50 μ*M* of the inhibitor in the presence of 500 μ*M* of OS.

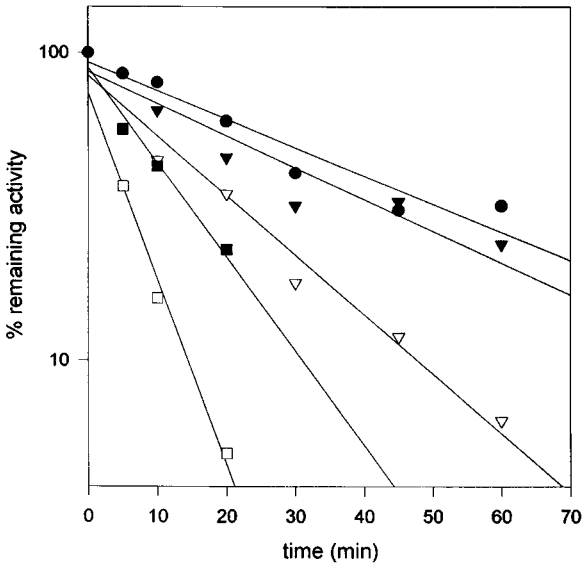


Figure 19 Protection by 2,3-oxidosqualene (substrate) against CNDT-squalene inactivation of yeast OSC. CNDT-squalene concentrations were 0.0 (●), 0.1 (▼), and 0.5 m*M* (■) in experiments with substrate (0.5 m*M*) as a protecting agent, and 0.1 (▽) and 0.5 m*M* (□) in experiments with no substrate as protecting agent.

By contrast, the Ellman reagent 5,5′-dithio-bis-(2-nitrobenzoic) acid (DTNB) proved to be effective as a time-dependent inactivator, but failed to be affected by the presence of the substrate. These results were confirmed by following photometrically the release of TNB (thio nitro benzoate) from CNDT-squalene or DTNB in the presence or absence of substrate. While the release of TNB from DTNB was not affected by the substrate, there was a significant reduction of TNB release from CNDT-squalene under the same conditions. Replacement of the squalene moiety by a dodecyl residue also made the inhibition less specific and less active. The squalene group of **54** acted as a carrier able to lead the inhibitor into the active site of the enzyme.

Much more work will be necessary to make a definite statement on the role of the single amino acid residues in the overall enzymatic catalysis leading to the cyclization of OS to tetracyclic or pentacyclic triterpenes. For example, it would be nice to do some more affinity labeling experiments in order to isolate the radioactive protein fragment containing the covalently linked inhibitor and identify the possible nucleophilic groups of the enzyme. Moreover, cloning and expressing of the OSC genes together with site-directed mutagenesis experiments would give more information on the enzyme, awaiting the final tridimensional crystallographic structure.

At the moment, on the basis of our experiments, we can assume the existence of a set of thiol groups outside and within the OSC active site (in mammals and yeasts). The internal site can potentially interfere in each step of OS transformation: initial protonation, cyclization and the following rearrangement, and the final removal of a proton that leads to the final product.

ACKNOWLEDGMENTS

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