Biosynthesis of Cholesterol and Other Sterols

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1. INTRODUCTION

Cholesterol and its relatives possessing the 1,2-cyclopentanoperhydrophenanthrene ring system (Figure 1) form the sterolome, which comprises a chemical library of more than 1000 natural products found in all forms of eukaryotes and some prokaryotes that serve a myriad of biological functions.[1,2] The structural and stereochemical commonality of these compounds derive in large part from the action of oxidosqualene synthases (formerly cyclases) that generate the parent sterol frame. In their 1985 Nobel lecture, Brown and Goldstein stated

that "cholesterol is the most highly decorated small molecule in biology", a comment supported by the many Nobel prizes awarded to individuals who devoted a large part of their lives to research one or more aspects of the chemistry and chemical biology of sterols, their metabolites, or other isoprenoids.[2,3]

The first known sterol, cholesterol, was discovered by French chemists as a crystalline component of human gallstones over 230 years ago. In 1789, Francois Poulletier de La Salle observed an alcohol-soluble portion of bile stones, which 10 years later was reported by De Fourcroy to be identical to a waxy material in the fat of putrefied corpses referred to as adipocire.[4] Shortly thereafter, Michel Chevreul established that the crystalline component of bile stones, which gave a melting point of 137 °C (accepted mp for cholesterol is 149 °C), was distinct from adipocire or that of another waxy material from whale, spermaceti (their mp ranged 44−68 °C), and named it "cholesterine" from two Greek words: chole, meaning bile and stereos, meaning solid.[4,5] In English-speaking countries, the name cholesterin was replaced with cholesterol after recognition that the substance was as a secondary alcohol. The correct formula ($C_{27}H_{45}O-$, which here shows that it has a hydroxyl group although it contains 46 hydrogen atoms) of cholesterol was proposed in 1888 by F. Reinitze, yet it took another 30 years to establish the exact steric representation of the molecule, efforts that led to the Nobel Prizes in chemistry for Wieland (1927) and Windaus (1928). The first connection between cholesterol and human health appeared in 1843 as Vogel showed that cholesterol was present in arterial plaques.[5] Cholesterol was subsequently determined to be widely distributed in the animal kingdom and its isomeric forms, termed "cholesterol bodies" or phytosterol, were shown to frequent the vegetable kingdom.[6] These early attempts at natural product surveys of sterols were based on measurements of melting point, color reaction, optical rotation, and crystalline form.

Sterol research changed in the mid- to late 20th century and centered on biomimetic chemistry, tracer work, enzymology, and structure determination using high-field NMR and X-ray diffraction methods, culminating with a broad outline for cholesterol synthesis and the partial or complete purification of all the microsomal-bound enzymes that act on sterols between lanosterol and cholesterol.[7−13] Ergosterol, then cholesterol, was discovered to play multiple cellular roles associated with membrane (bulk role) and signal (sparking) functions, which could be differentiated by structure and amount of compound;[14−17] notably sterols accumulate in cultured plant and animal cells at approximately 3000 fg/cell and in yeast at 20 fg/cell.[18] During

this time, there was a renewed focus on regulation of cholesterol synthesis and human physiology.[19] These studies, which involved oxysterols, led to the development of an inhibitor of hydroxymethyl glutaryl CoA reductase (HMGR), atorvastatin (Lipitor), which in 2006 was the best-selling drug in the world grossing more than $12 billion dollars in sales.[20−22] Further work on sterol biosynthesis demonstrated that lanosterol is the product of squalene-oxide cyclizaton in organisms of a nonphotosynthetic lineage and cycloartenol is the product of squalene-oxide cyclization in organisms of a photosynthetic lineage.[23−25] These findings show that sterol biosynthesis can proceed by phyla-specific pathways.

Central to the advances of the past two decades is the development of molecular genetic approaches that have witnessed the cloning, primary amino acid sequences, and functional characterization of a large number of enzymes that act on sterol and revealed unexpected inborn errors of cholesterol metabolism. The full exploitation of these genes lies in medical diagnostics, treatment, and the ability to ultimately engineer phytosterol pathways to generate plants with tailored sterol profiles for commercial production. Another spectacular discovery involving ^{13}C-isotopically labeled compounds supplied to microorganisms and plants was the demonstration that the classic acetate−mevalonate pathway to animal cholesterol can be replaced in the biosynthesis of algal sterols and other isoprenoids by a mevalonate-independent pathway.[26,27] The work on the biosynthesis of cholesterol and other sterols is ongoing, and many talented investigators all over the world are contributing to the latest surge of biochemical investigation. Their efforts constitute a collective undertaking of significant importance to several remarkable advances toward the completion of the enzymatic inventory of sterol synthesis, and these new findings, together with a brief examination of prior art in the field of sterols, are discussed in this review.

2. STRUCTURE AND DISTRIBUTION

2.1. Sterol Frame and Functional Domains

Sterols are amphipathic compounds that originate in isoprenoid biosynthesis with the main frame composed of a nucleus and side chain (Figure 2). Accordingly, the sterol molecule possesses four indispensible domains. In domain A, the polarity and tilt of the C3 OH-group contribute functionally to hydrogen-bond
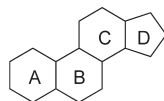
interactions. In domain B, the C4 and 14-methyl groups can affect the A ring conformation and back face planarity, respectively. Alternatively, the number and position of double bonds in the nucleus can affect the shape of the sterol and tilt of the 17(20)-bond. In domain C, the natural configuration at C20, R, determines the conformation of the side chain to orient into a "right-handed" side chain. In domain D, the conformation and length of the side chain, in addition to the stereochemistry of the C24-alkyl group in phytosterols, are critical to intermolecular contacts. These molecular features are crucial for the Δ^5-sterol molecule to function in membranes as a flat, elongated compound of approximate volume 794 A^3; notably, the cholesterol shape can be mimicked and its structure replaced by sterol-like pentacylic compounds (hopanoids) in bacteria having a smaller volume of 745 A^3.[28] Cholesterol has eight stereocenters that give rise to 2^8 stereoisomers (256), yet only one of them, the natural enantiomer with the 3R, 20R configurations, is utilized as a membrane insert.[17] On the other hand, the size and direction of the 24-alkyl group have phylogenetic significance; 24β-methyl sterols predominate in less-advanced organisms, including many fungi and protozoa, and 24α-ethyl sterols populate the sterol mixture of advanced organisms, typified by vascular plants.[29−31]

2.2. Nomenclature, Stereochemistry, and the Isoprene Rule

The earliest chemical definition for a sterol was provided by Fieser and Fieser. These substances will possess the characteristic perhydro-1,2-cyclopentanophenanthrene ring skeleton (Figure 1).[32] The revised 1989 nomenclature system recommended by the International Union of Pure and Applied Chemistry (IUPAC) and the International Union of Biochemistry (IUB) for the naming of sterols and related isoprenoids permits unambiguous assignments of configuration and introduces a convenient and logical uniformity to the nomenclature of all tetracyclic triterpenoids (Figure 3B).[33] However, the recommendations can cause confusion with regard to the phytosterol side chain structure and nomenclature, particularly with respect to the application of the R and S designation, and in NMR assignments. The revised system has further complications and becomes cumbersome as the complexity of side-chain modifications by biosynthetic alkylations increase.[34,35] We have adopted the older convention (Figure 3A) and rely on the biosynthetic side chain rule, which, for instance, states that the E-methyl group (C26) associated with the $\Delta^{24(25)}$-double bond in the sterol side chain can be shown to originate in C2-mevalonic acid and the Z-methyl group (C27) to arise from C6 (C3′) of mevalonic acid with the numbering of these methyl groups retained in the case of the cholesterol side chain.[2,36] This subtle differentiation between the chemically equivalent but biosynthetically distinct terminal methyl groups can be lost in

Figure 1. Common tetracyclic steroid frame containing the 1,2-cyclopentanoperhydrophenanthrene ring skeleton.
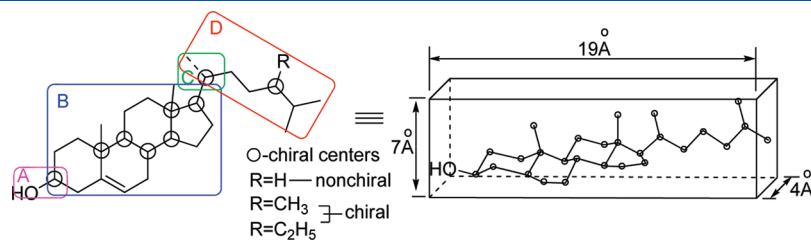

Figure 2. Perspective drawings of the cholesterol molecule showing four domains of functional importance (left) and the flat elongated structure presumed to form in the membrane (right).

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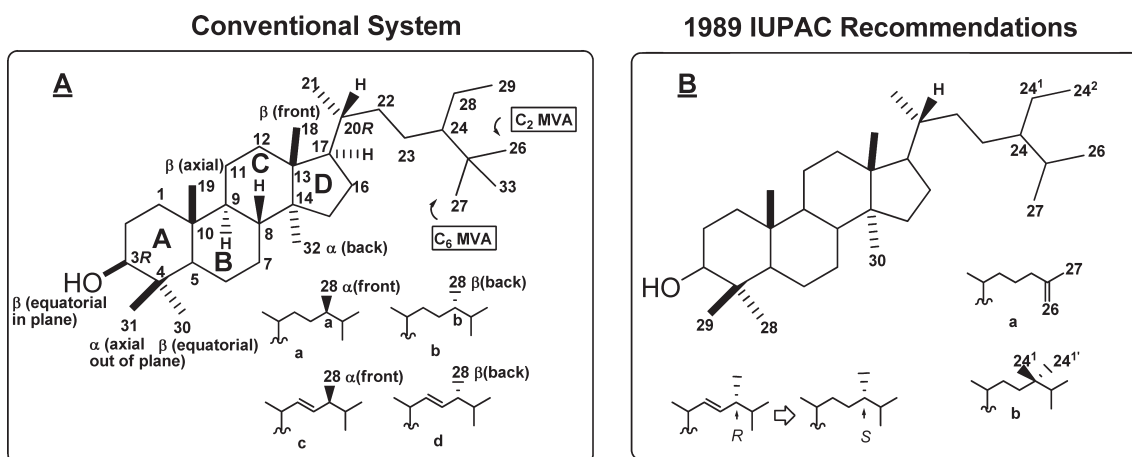dx.doi.org/10.1021/cr200021m |Chem. Rev. 2011, 111, 6423–6451

Figure 3. Two systems recognized for numbering of carbon atoms of the sterol nucleus and side chain. The conventional system, which incorporates the α/β-chiral descriptors in nucleus and side chain is based on the Fieser and Fieser book *Steroids* and biosynthetic considerations as discussed in refs 1, 2, and 5 (panel A), versus the 1989 IUPAC recommendations (panel B).

the numbering procedure adopted by the IUPAC—IUB recommendations. Another convention relevant to this rule involves chiral substituents at C24 to be designated α and β, but this α/β notation is unrelated to the similar notation for substituents on the ring structure. The corresponding family of phytosterols are then related to structures based in ergostane (24β-methyl), campestane (24α-methyl), and stigmastane (24α-ethyl) or poriferstane (24β-ethyl). The systematic names of the sterols are treated as derivatives of 5α-cholestan-3β-ol or that of a related stanol. As relevant, the corresponding trivial name for a sterol may be used in place of the systematic name for its familiarity with the reader such as cholesterol rather than cholest-5-en-3β-ol.[1,2,5,12]

Because sterols are derived from the C_{30} squalene, they are a class of triterpenoids. These tetracycles are generated from the linear combinations of the C_5-isoprenoid building block, isopentyl diphosphate. In order to conform to the empirical isoprene rule, triterpenes (C_{30}) are the products of joining 6 × 5 C_5-units.[37] Thousands of triterpene products have been reported from plants, and they arise from more than 80 different carbon skeletons; the triterpene cyclase—baruol synthase recently cloned can generate as many as 23 products.[38] Sterols therefore form a unique family of triterpenes that can be defined on biosynthetic reasonableness. For this alternate definition, the focus is on the reaction mechanism and ignores the precise isoprenoid character of the cyclization product, assuming only that the intermediate adopts steroidal character during cyclization to produce a tetracycle compound of specific structure and stereochemistry. Thus, a true sterol is formed by the electrophilic cyclization reactions that pass through a transition state similar to the *trans—syn—trans—anti—trans—anti* configuration affording a protosteroid C20 cation.[2] The cyclization product(s), lanosterol or cycloartenol and in rare cases parkeol, should contain absolute configurations at C3 and C20 of the *R*-orientation. In light of recent work from Corey's laboratory, the intermediate C20 cation is now known to assume the 17β-side chain (pseudoaxial side chain generated by a 17α-hydrogen atom) required of the synthase to establish the natural C20 configuration of the sterol product.[39,40] The structure and stereochemistry for several structural isomers in the tetracyclic triterpene series, including lanosterol, cycloartenol, euphol, tirucallol, and cucurbitacin, have been established by X-ray crystallography.[41—44] The lanostane and cycloartane sterol skeletons

possess relative stereochemistry of 5α, 8β, 10β, 13β, 14α, 17α, and 20α configurations, consistent with their stereospecific formation involving the protosteroid cation, which agree with the stereochemistry in the cholestane frame resulting from their metabolism (Figure 4).

2.3. Variation in Sterol Construction

Principal differences in cholesterol and related phytosterols are in the side chain (C_{20}—C_{26}/C_{27}), which has different degrees of substitution and unsaturation. Chemical surveys of the sterol composition of prokaryotes, eukaryotes, and sedimentary organic matter show that there are at least 250 sterols and related steranes; in corn, 60 different sterols have been characterized.[45—47] Higher plants are a mixture of 24-alkylated sterols, 24-ethyl and 24-methyl sterols generally accounting, respectively, for more than 70% and less than 30% of the total sterol. Djerassi and his associates, using a computer-assisted program, calculate that natural sterols may have as many as 1778 different structures; many of them may be found in marine organisms, which are known to synthesize highly bioalkylated sterol side chains.[48] The number of cholesterol variants can increase by an order of magnitude by including cholesterol/24-alkyl sterol derivatives to esters, glycosides, and sulfates and those metabolites that retain the perhydro-1,2-cyclopentano-phenanthrene skeleton. In animals, cholesterol is converted to sex hormones, bile acids, vitamin D, and different classes of "oxysterols", in insects to ecdysteroids, and in nematodes to dauer steroids, whereas in plants, cholesterol is converted to C_{27}-spirostanols to form glycoalkaloids and saponins or C_{23} cardenolides.[1,2,49] Alternatively, the C_{28}- and C_{29}-phytosterols metabolize to the plant defense withanolides (from 24(28)-methylene cholesterol), to plant growth hormone brassinosteroids (from campesterol), or to fungal sex hormones antheridiol and oogoniol (from fucosterol) in the Oomycetes.[50—52]

3.0. BIOGENETIC CONSIDERATIONS: HISTORICAL PEDAGOGY

3.1. Ionic Reactions

According to the currently accepted hypothesis, the formation of steroids proceeds by a cationic cyclization process. This theory
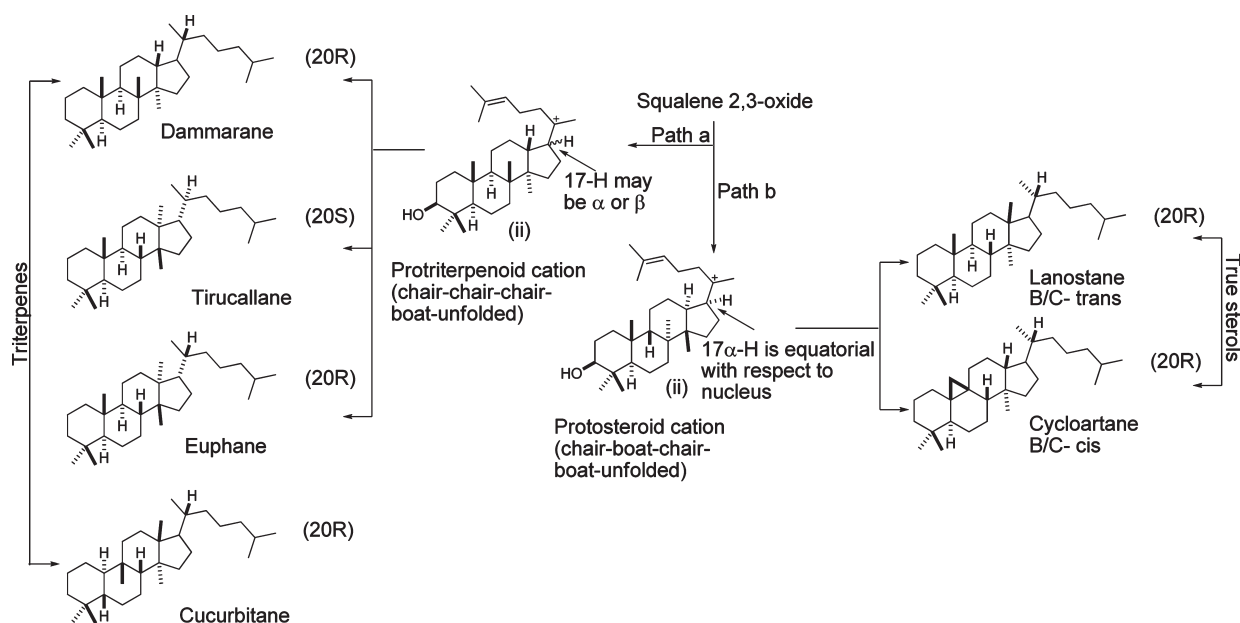
Figure 4. The cyclization of squalene-2,3-oxide (i) folded in either the chair−chair−chair−boat−unfolded (ii) or chair−boat−chair−boat−unfolded (ii) conformations to yield a cation (ii), which can stabilize to produce the dammarane, tirucallane, euphane, and cucurbitane skeletons (via path a) or cycloartane and lanostane skeletons (via path b). Generally, the cyclization products contain a 3β-OH group and a Δ^8-bond or in the case of cucurbitacin a Δ^5-bond.
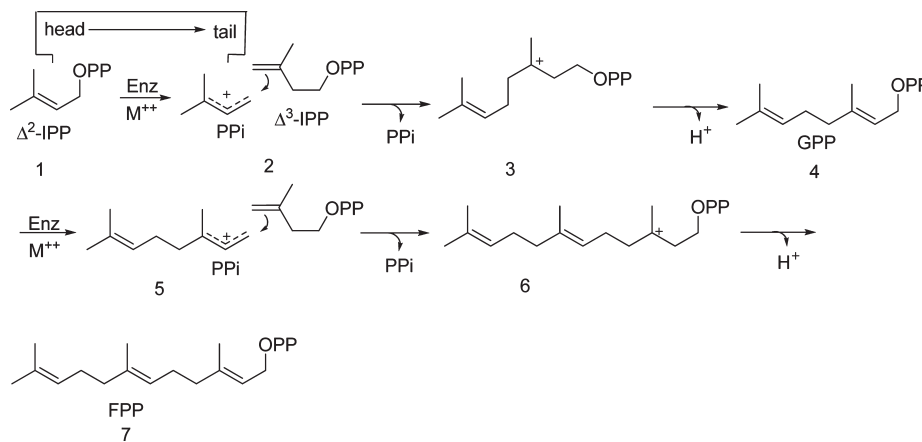

Figure 5. The prenyl transferase reaction and formation of farnesyl diphosphate (FPP). Prenyl (C$_5$) units composed of isopentyl diphosphates (IPP) are assembled in a head to tail fusion to yield C$_{10}$ (geranyl diphosphate, GPP) and in repeat fashion to C$_{15}$ (FPP) isoprenoids. Alternate isoprene unit assemblies yield a variety of different structures notable in terpene metabolism. Enz = enzyme and M^{++} can be Mn^{2+} or Mg^{2+} ions.

has it roots in the biogenetic isoprene rule of Ruzicka and his associates in Zurich who considered biosynthesis of triterpenoids was initiated by an electrophilic attack on a double bond of a linear polyprenoid substrate forming a cyclic (or polycyclic) intermediate cation, which, in turn, can then undergo various transformations and rearrangements.[37] Carbocationic intermediates can also arise in the presterol segment of isoprenoid biosynthesis, and they include allylic pyrophosphates that generate allylic carbocations on elimination of pyrophosphate. Ionization of the allylic pyrophosphate leads to formation of a charge-stabilized allylic cation. The cations can react with alkenes to form new carbon−carbon bonds using Δ^2-IPP (dimethyl allyl pyrophosphate) as a source of allylic carbocations, such that after *trans* elimination of a proton, a new prenyl diphosphate is generated that is five carbons larger (Figure 5).[5,53] For these biochemically rare electrophilic additions to a double bond to

proceed, the divalent metal ion binds to the pyrophosphate moiety of the allylic cosubstrate so as to make it a better leaving group in the ionization step. Two structurally unrelated classes of isopentyl diphosphate isomerase (IDI) are known. Type I IPP (IDI-1) utilizes a divalent metal in a protonation−deprotonation reduction. In contrast, the type II enzyme (IDI-2) requires reduced flavin, raising the possibility that the reaction catalyzed by IDI-2 involves the net addition or abstraction of a hydrogen atom.[54] Other coupling reactions contribute to squalene biosynthesis, the most notable being that involved in the two-step reaction yielding squalene from farnesyl diphosphate (FPP).[55,56] First, one molecule undergoes loss of PPi and addition of the allylic carbocation to the alkene end of the other molecule of FPP, accompanied by loss of a proton, to form presqualene pyrophosphate. In the second step, presqualene pyrophosphate (PSPP) loses PPi, and the presumptive cyclopropylcarbinyl
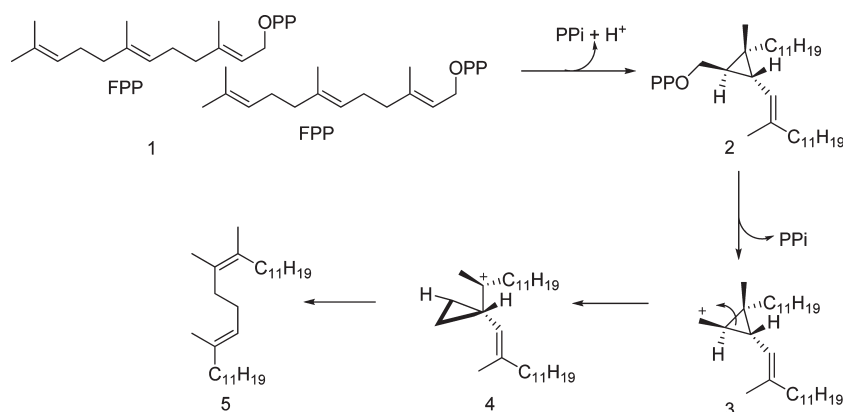
Figure 6. Proposed mechanism for the coupling of farnesyl diphosphate (FPP) to form squalene. Adapted with permission from ref 53. Copyright 2009 American Chemical Society.

Table 1. Electrophilic Reactions Catalyzed by Sterol Biosynthesis Enzymes

carbocation undergoes ring opening and reduction by NADPH to squalene (Figure 6).

Rahier and co-workers have drawn attention to ionic processes that generate sterol cations concomitant with allylic rearrangements, double bond C-methylations, or reduction reactions in the postsqualene segment of sitosterol biosynthesis (Table 1).[11] The most celebrated and best characterized of these electrophilic processing enzymes has been the sterol C24-methyltransferase (24-SMT), attracting justly deserved attention, not only for its centrality in phytosterol diversity but for possible inactivation by catalyst-specific drugs in ergosterol biosynthesis to treat human infections. A broad unifying mechanistic framework for the origin of all phytosterol side chains that included the intermediacy of a cationic intermediate at C25 in the conversion of $\Delta^{24(5)}$- to $\Delta^{24(28)}$-sterols was provided by Castle et al. in their demonstration of the incorporation of the *S*-methyl of methionine into sitosterol in *Pisum sativum*.[57]

3.2. Formation of Steroidal Backbone

The usual pathway to the C_{30} primary sterols proceeds along the well-established isoprenoid trail that leads from the "active

isoprene unit", isopentenyl diphosphate, to the C_{30} triterpenoid squalene. The biosynthesis of C_{30} sterols from squalene and thence to cholesterol can be outlined in three major stages as envisioned by Bloch:[58] acetate $\rightarrow$ isoprenoid intermediate $\rightarrow$ cyclization product $\rightarrow$ cholesterol. Stage 1 involves (i) conversion of acetyl CoA into acetoacetyl CoA catalyzed by acetoacetyl CoA thiolase (AACT), (ii) conversion of acetoacetyl CoA into 3-hydroxyl-3-methylglutaryl CoA by hydroxyl-3-methylglutaryl CoA synthase (HMGS), (iii) conversion of 3-hydroxy-3-methylglutaryl CoA into mevalonic acid (MVA) by 3-hydroxy-3-methylglutaryl CoA reductase (HMGR), (iv) conversion of MVA into phosphomevalonate by phosphomevalonate kinase (MK), (v) conversion of phosphomevalonate into diphosphomevalonate by phosphomevalonate kinase (PMK), and (vi) conversion of diphosphomevalonate into isopentyl diphosphate (Δ^3-IPP) by mevalonate diphosphate decarboxylase (MVD). Thus, in the first stage MVA is transformed into IPP by two phosphorylation steps at C5 of MVA and a decarboxylation/ elimination step; IPP, the basic C_5 building block, is then added to prenyl diphosphate cosubstrates to form longer chains. Δ^3-IPP itself is insufficiently reactive to undergo ionization to initiate the
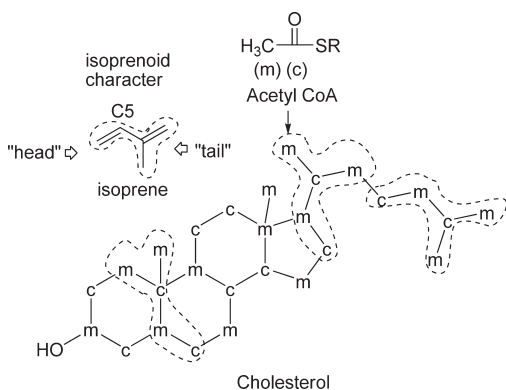
Figure 7. Distribution of acetate carbon atoms found in cholesterol; a repeating pattern of five carbon atoms (isoprene unit), surrounded by dotted lines, is recognizable in three places in the molecule.
Adapted with permission from ref 5. Copyright 1986 Plenum Press, New York.

condensation of higher isoprenoids. Therefore, it is first isomerized to the allylic ester Δ^2-IPP through an antarafacial rearrangement followed by head to tail condensation of Δ^2- and Δ^3-IPP to form geranyl diphosphate by geranyl diphosphate synthase (DPS). In the second stage, the condensation reaction is repeated by the addition of Δ^3-IPP producing the C_{15} allylic product farnesyl diphosphate. Two molecules of farnesyl diphosphate condense tail to tail to the C_{30} acyclic polyene squalene by the action of squalene synthase (SQS). The C_{30} symmetric olefin undergoes oxidation to form S-oxidosqualene via an NADPH-dependent mono-oxygenase reaction catalyzed by squalene epoxidase (SQE), and this substrate can be cyclized by an oxidosqualene—sterol synthase to yield the steroidal backbone structure represented in lanosterol. In stage 3, lanosterol is converted to cholesterol (section 4.4).

In the early phase of cholesterol research, it was not immediately apparent that the C_{27} structure of cholesterol was related to lanosterol, since it failed to be divisible by C_5 units. To establish isoprenoid character, several groups incubated [1-^{14}C]acetate and [2-^{14}C]acetate with liver slices affording a decisive pattern in the distribution of acetate carbon atoms in the labeled cholesterol. As shown in Figure 7, where "M" denotes an acetate methyl and "C" an acetate carboxyl carbon, there are three places in the molecule where a repeating C_5 pattern typical of isoprene can be recognized. These tracer studies also provided the foundation for the acetate—mevalonate pathway in sterol biosynthesis.[1,2,5] The stereochemistry of the enzymatic reactions involved with biosynthetic steps that lead to squalene, lanosterol, and cholesterol have been completely elucidated by the work of Popjak and Cornforth using six species of MVA stereospecifically labeled with either ^{2}H or ^{3}H at C2, C4, or C5.[5,59] Seo and co-workers complemented this work by feeding [5-^{13}C^{2}H$_2$]MVA together with [2-^{13}C^{2}H$_3$]acetate or [1,2-^{13}C$_2$]acetate to yeast and cultured plant cells.[60] Using ^{13}C NMR spectroscopy, they confirmed the fate of all the relevant hydrogen atoms comprising MVA incorporated into ^{13}C-labeled ergosterol, cycloartenol, and sitosterol.

Since the late 1960s, it has been known that two major cyclization pathways exist for the conversion of oxidosqualene to steroidal tetracycles; lanosterol is formed in organisms of a nonphotsynthetic lineage, and cycloartenol is formed in organisms of a photosynthetic lineage by independent synthase

enzymes.[2,9] This biosynthetic bifurcation in sterol biosynthesis is one of the most interesting phylogenetic markers available because it has no apparent influence on the structure of the functional steroid at the end of the pathway and the enzymes themselves are membrane-bound though not in plastids (e.g., cycloartenol).

By carrying out experiments with doubly labeled [2-^{14}C(4R)4-^{3}H$_1$]MVA fed to animal and plant tissues, Goad and Goodwin demonstrated that cycloartenol having a ^{3}H/^{14}C atomic ratio of 6:6 is not produced by rearrangement of lanosterol having a ^{3}H/^{14}C atomic ratio of 5:6, consistent with retention of label at C19 in the biosynthetically formed cycloartenol.[61] Altman and co-workers studied cycloartenol biosynthesis in the alga *Ochromonas malhamensis*. Incubation of oxidosqualene bearing a chiral methyl group (H,^{2}H,^{3}H) at C6 with the plant synthase revealed that the stereochemistry of the cyclopropane ring closure proceeds with retention of configuration.[62] These variant synthase-mediated substrate transformations were formerly postulated to involve a concerted ring annulation that led to a C20 protosterol intermediate in which the cationic side chain at C17 is α-oriented (C17β-hydrogen), affording the unnatural C20S arrangement. To form the natural 20R configuration, it was postulated that intervention of an electron-donating "X-group" on the synthase takes place to transiently neutralize the C20 charge.[63,64] Utilization of the X$^-$-group permits rotation about the 17(20)-bond of 120° prior to hydride migration. A final elimination reaction takes place at C9α to produce lanosterol.[65,66] In contrast to formation of a "flat" $\Delta^{8(9)}$-sterol, formation of "bent" cycloartenol requires the product bend through almost 90° to accommodate the 9β,19-cyclopropane-8β-H bridgehead in which the 19-CH$_3$ and 8H-atom are *cis* to one another.[5,14,24,67,68] For "bent" cycloartenol formation, a distinct enzyme—substrate interaction from the one involved at C20 is considered to intercept the positive charge generated at C9α (Figure 8).[9,67] Ring construction in this pathway requires a 1,3-*anti* elimination of a proton from the C19 angular methyl group and the added nucleophile. If this is not done, the final step, migration of a hydrogen from C19-methyl, will be *cis* to the C9 hydrogen transfer instead of *trans* in order to conform with the biogenetic isoprene rule. Subsequent withdrawal of the X$^-$ then permits closure of the cyclopropane ring in a *trans* manner with concomitant removal of the C19 proton, which is tantamount to a double inversion mechanism. These postulates are revisited in section 4.

4. RECENT ADVANCES IN STEROL BIOSYNTHESIS

4.1. The Genome—Sterol Metabolome Congruence

The different molecular libraries that constitute isoprenoid—sterol metabolomes across Kingdoms are organized through a series of discrete assemblies of enzymatic reactions, which are characterized compartmentally. The acetate—MVA pathway to squalene oxide is considered to be the main route to the production of steroidal backbones. Recent international efforts have resulted in the complete sequencing of the model plant *Arabidopsis*, nonpathogenic fungus *Saccharomyces*, and human genomes.[69−71] In the entirely sequenced genomes of these organisms, homologues for all genes of the acetate—MVA pathway to sterols are present. The functional genomics approach together with the establishment of defective biosynthesis steps in humans and generation of yeast and plant mutants in sterol biosynthesis enabled the elucidation of structural genes
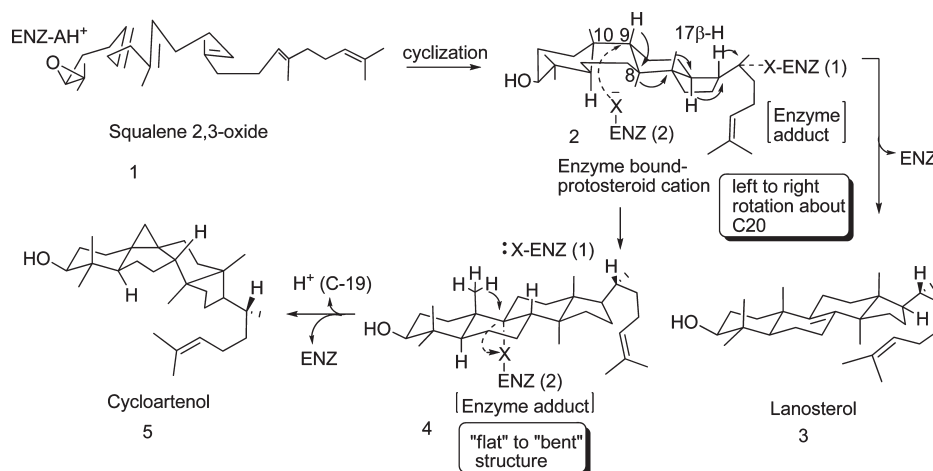
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dx.doi.org/10.1021/cr200021m |*Chem. Rev.* 2011, 111, 6423–6451

Figure 8. Interpretation of the mechanism of squalene-2,3-oxide cyclization to lanosterol and cycloartenol according to refs 61, 63, and 64. The cyclization mechanisms are hypothesized to require "X⁻ group" (any electron-donating group on the enzyme) participation to generate the C20R configuration, which requires the side chain of the X⁻ group bound protosteroid to rotate from left to right about C20 and formation of "bent" cycloartenol from a "flat" 9β,19-cyclosteroid intermediate. See text.
Adapted with permission from refs 61 and 64. Copyright 1977 American Chemical Society and 1968 Biochemical Journal.
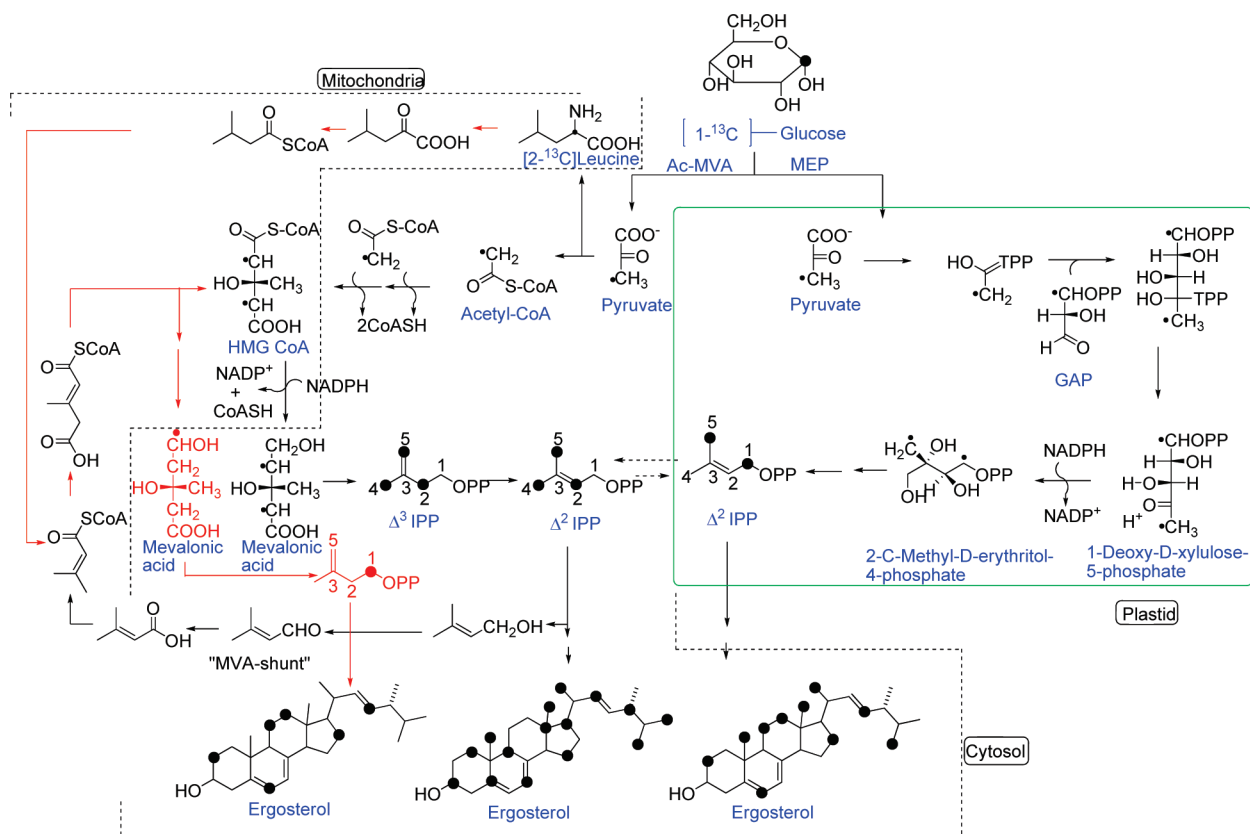

Figure 9. Overview of compartmentalized isoprenoid–sterol biosynthesis pathways. Feeding studies of [2-^{13}C]leucine or [1-^{13}C]glucose distinguish the leucine–HMGCoA, acetate–mevalonic acid (MVA) or the 2-C-methyl-D-erythritol-4-phosphate (MEP) pathways that contribute carbon to isopentenyl diphosphate (IPP). The ^{13}C-labeling pattern of ergosterol (synthesized in all major eukaryote Kingdoms) reveals which of the pathways operate in a given organism and whether cross-talk between the pathways exist. In the forward direction, IPP converts to ergosterol or IPP can retroconvert via the MVA shunt to HMGCoA and to acetate. See text.

for the individual enzymes in sitosterol, ergosterol, and cholesterol formation.[72−74] Notably, sterol biosynthesis in plants and fungi differ markedly from that in animals since these organisms contain more sterol genes than do animals. In nonanimal

systems different sterolic genes can encode for similar reaction steps, for example, sterol C24-methyltransferase, sterol C14-demethylase, or sterol methyl oxidase, whereas mammals generally have only a single gene for each enzymatic step. The best
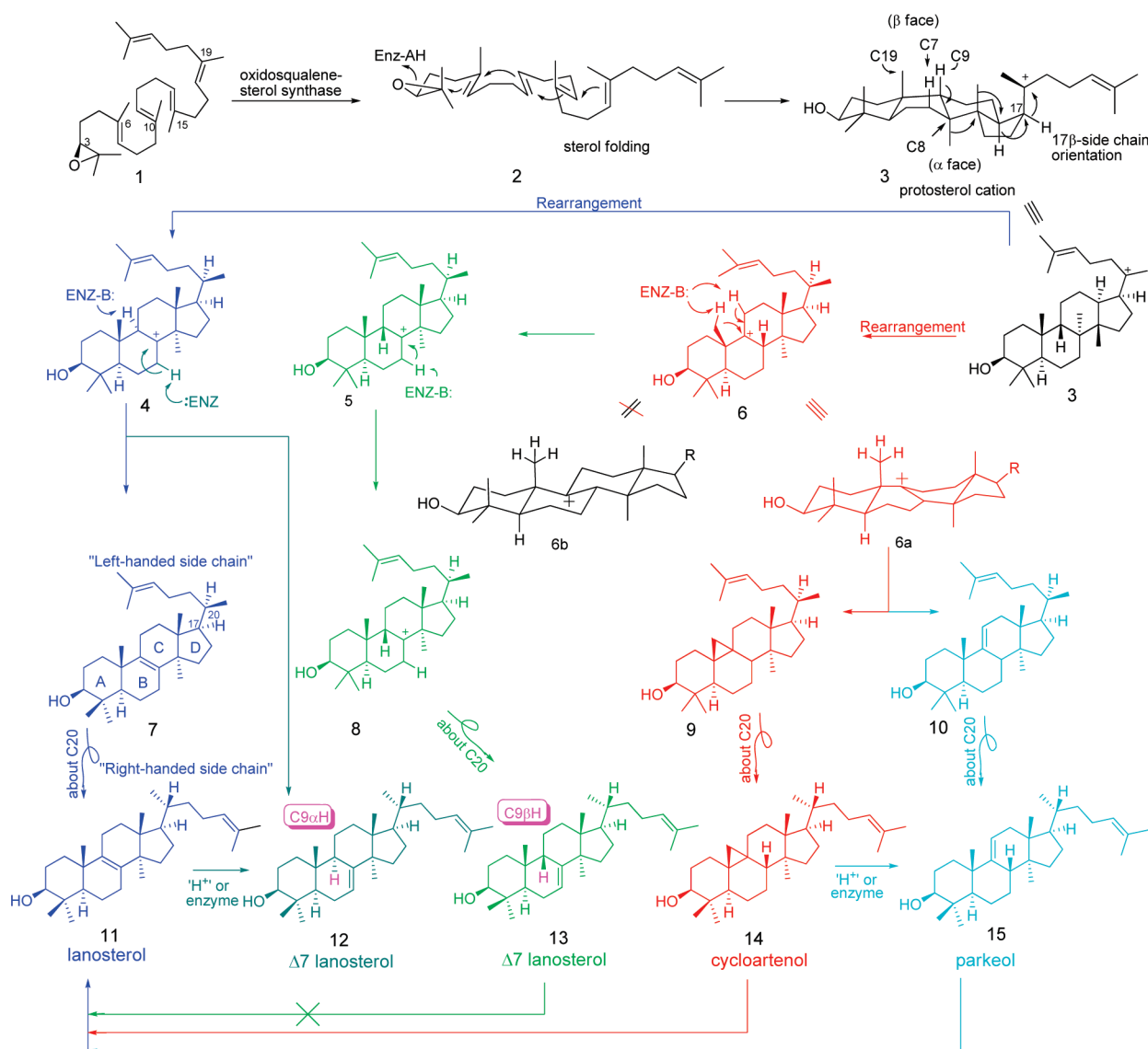
Figure 10. The cyclization of squalene-2,3-oxide (**1**) catalyzed by the lanosterol synthase (LAS) or cycloartenol synthase (CAS) to give true sterols. Ring annulation proceeds by synthase (cyclase)-specific cationic mechanisms involving a common protosteroid cation (17α-H/17β-side chain orientation, **3**). The cyclization cascade terminates with formation of either (i) intermediate **4** (LAS) followed by its deprotonation at C9 or C7 to form lanosterol **11** or lanosta-7,24-dienol **12**, respectively or (ii) intermediate **6** (CAS), which depending on the position of deprotonation at C7, C19, or C11 yields lanosta-7,24-dienol **13**, cycloartenol **14**, or parkeol **15**, respectively; *trans*-cyclization of the chair—boat type of system in **6a** (natural intermediate compared with **6b** as discussed in the text) affords the 9β-H instead of a 9α-H, which is necessary to complete biosynthesis of cholesterol. The asymmetric conformation of bound intermediates **4** and **6** with a 20α-H-atom ($20R$-configuration) eclipsed to 17α-H-atom produce an enzyme-bound "left-handed" rotamer at C20. After release of true sterols **7**—**10** from the active site, the sterol side chain can rotate about C20, such that C21 and C22 lies in a 1,3-diaxial relationship with C18 to form the energetically favorable "right-handed" conformation of **11** to **15** utilized in membranes.

described gene—gene product pairing is in the yeast pathway for which every relevant gene in the conversion of lanosterol to ergosterol has been identified. The principal enzymes of lanosterol conversion to cholesterol are coded for by nine genes (section 4.4). The fungal 24-alkyl sterol pathway has acquired at least four additional genes from the human pathway that give rise to a sterol C24-methyltransferase, sterol C22-desaturase, sterol C24—24(28)-reductase and sterol $\Delta^{24(28)}$- to $\Delta^{24(25)}$-isomerase as reported in the ascomycetes, *Saccharomyces*, and *Gibberella*.[18] Plants, variably from algae to tracheophytes, have evolved all 11 of the fungal/animal sterol genes and can code for two more of them, the sterol $\Delta^{25(27)}$-reductase

and 9,19-cyclopropyl to Δ^8-isomerase. To date, the number of sterol genes involved in conversion of lanosterol or cycloartenol to Δ^5-sterol is 15. Isoforms can exist to alter the number of enzymatic reactions in a specific pathway.

4.2. Biosynthesis of Squalene: MVA versus MVA-Independent Pathways

At least two alternative biosynthetic pathways to Δ^2-(dimethyl allyl pyrophosphate) and Δ^3-isopentenyl diphosphate (formerly pyrophosphate) have been shown, by biosynthetic labeling studies, that can supply prenyl units to squalene, which in turn is converted to sterols (Figure 9). One involves acetate directly
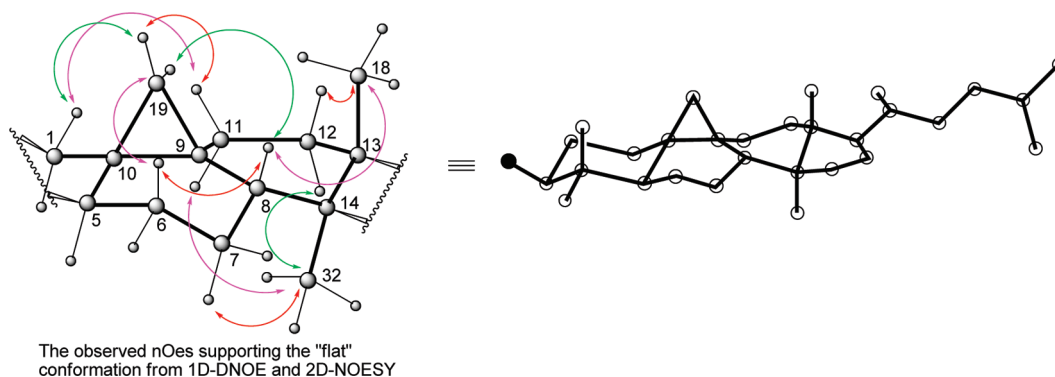
The observed nOes supporting the "flat"
conformation from 1D-DNOE and 2D-NOESY

Figure 11. Structure of "flat" cycloartenol established by NMR (left) and X-ray (right) measurements. Reprinted with permission from ref 44a. Copyright 1998 American Chemical Society.

incorporated into the MVA pathway after activation into acetyl CoA. The other, known as the methyl-D-erythritol 4-phosphate (MEP) pathway or mevalonate-independent pathway, utilizes triose phosphate units as the precursor and is located in the plastid of plants.[75-77] The former pathway uses seven enzymes, some of which have crucial isoforms that contribute to drug sensitivities, compared with the latter pathway that consists of eight reactions catalyzed by nine enzymes. Many of these enzymes have been characterized structurally. In the newly discovered mevalonate-independent pathway reported independently by Rohmer who detected it in eubacteria and Arigoni who detected it in higher plants,[75,76] Δ^2-IPP (dimethyl allyl diphosphate) is formed directly from pyruvate rather than via acetyl CoA—MVA and Δ^3-IPP. Terpenoids of the C_{10}, C_{15}, C_{20}, and C_{40} skeletons are generally synthesized within the plastid and sterols synthesized in the cytosol of higher plants. Goad and co-workers reported that mitochondria of some parasitic protozoa have the capability to convert leucine to MVA, which then converts to IPP and ergosterol via the cytosol.[78] Alternatively, isopentenyl diphosphate can undergo catabolism through an "MVA shunt" pathway (also known as the Popjak shunt") that forms a non-sterol-forming pathway to redirect flux to small molecules or fatty acids in animal, fungal, or plant tissues.[79-81] The MVA shunt, a multidirectional pathway, can allow carbon from amino acids to be incorporated into IPP; isovaleric acid has in its metabolism intermediates common to those in the shunt pathway.

It has been possible to establish the contribution of these variant isoprenoid pathways to sterol biosynthesis in plants and microorganisms by the retrobiosynthetic approach. When, for example, [1-^{13}C]glucose, [2-^{13}C]acetate, L-[2-^{13}C]leucine, [2-^{13}C]MVA, or [2,3,4,5-^{13}C$_4$]1-deoxy-D-xylulose is used, the MVA and MEP pathways yield distinctive labeling patterns in IPP and sterols. Thus, in higher plants, sitosterol can be made by either the MVA or MEP routes; the MVA pathway is always senior to the MEP pathway. In the diatom *Rhizosolenia setigera* that can synthesize 24-ethyl sterol, the MEP pathway is favored.[27] In the photosynthetic alga *Scenedesmus obliquous* and nonphotosynthetic yeast-like *Prototheca wickerhamii* (cycloartenol route) that synthesize 24-methyl sterols, the MEP pathway operates to the exclusion of the acetate—MVA as shown by the distinctive ^{13}C-labeling pattern of ergosterol, which possesses 10 rather 15 of its carbons enriched with ^{13}C after incubation with [1-^{13}C]glucose (Figure 9).[82,83]

4.3. Cyclization of Squalene Oxide to Lanosterol or Cycloartenol

By far the best studied of the enzymes involved in sterol biosynthesis are the lanosterol and cycloartenol synthases. For cyclization, the reaction requires (3S)-2,3-oxidosqualene first to adopt a preorganized chair—boat—chair conformation. Protonation of the epoxide ring then triggers a cascade of stereospecific ring-forming reactions to protosterol 3 channeled to specific outcomes for the different synthases (Figure 10). Recent findings of the Corey group, studying lanosterol formation, demonstrated that the initial substrate cation in the chair—boat—chair—boat ring conformer 3 generates a 17β-side chain (17α-hydrogen) that connects to the sterol side chain at C20; the resulting *cis* C17H and C20H geometry orients the side chain into a "left-handed" structure.[83] The protosterol cation converts to lanosterol 11 by elimination of the 9α-H atom (originally the 9βH of the protosteroid cation), which requires nuclear rearrangements that involve a series of 1,2-shifts of hydride and methyl groups in an antiparallel manner coupled to quenching of the positive charge at C20. The C9β-hydrogen on the protosteroid cation is then lost to the medium as a proton when the 8α-methyl migrates to C14 to form intermediate 5, which undergoes proton abstraction at C9α to form the 8,9-double bond in 7.[66] Lanosterol cyclization may be considered complete when sterol 7 assumes the physiological conformation of the energetically favored "right-handed" side chain (C17α-H and C20R-H *trans* to one another in the usual view of the molecule) after product release (Figure 9).[43]

The postulated mechanism of cyclization of squalene oxide into cycloartenol (and parkeol) is essentially the same as that for lanosterol, except for the final 9β,19 cyclopropane ring closure with the 9β-H atom migrating to C8 instead of C9α proton elimination. Nes found that the solid state and solution studies unambiguously show that cycloartenol is "flat" of A/B/C-rings in the chair/half-chair/twist-chair conformer (Figure 11).[44a] An alternative reaction sequence to generate cycloartenol from intermediate 3 (C9β) is to proceed to 6b (C8β) of constrained boat structure (chair—boat—chair) and to 14, rather than to 6a of chair—chair—chair conformation, which has resemblance to 4 to 12 (Figure 10); noticeably, the C9-cations in 6a and 6b considered in the final cyclopropane formation are on opposite sides of the reaction intermediate.

Cloning and mutagenesis studies of cycloartenol and lanosterol squalene oxide (= oxidosqualene) synthases [OSS] suggest that the native enzymes in plants and fungi are catalytically and

structurally similar to the human oxidosqualene—lanosterol and bacterial squalene—hopene cyclases.[65,84—86] The cloned and purified human oxidosqualene cyclase is active as a monomer, whereas the corresponding purified enzyme from bovine liver exhibited active forms of 70 and 140 kDa, suggesting dimer organization.[87,88] Insight into lanosterol scaffold formation from the recently determined structure of human oxidosqualene synthase reveals the presence of a cavity, shaped so as to accept the substrate prefolded product-like conformation.[86] Structural analysis, mutagenesis, and inhibition experiments have shown that the protruding part of the protein contains a lipophilic channel leading to the active-site cavity that can influence substrate recognition (squalene versus oxidosqualene) and access to the active site.[89] Directed mutagenesis of cycloartenol and lanosterol synthases have shown that point mutations in the active site can alter the cyclase product specificity. Mutations of aliphatic residues in the cycloartenol synthase of *Arabidopsis thaliana* and *Dictyostelium discoideum* resulted in a partitioning shift from cycloartenol formation in the wild-type to lanosterol, parkeol, and 9β-lanosta-7,24-dienol.[68] In a study on cycloartenol synthase redesign, Matsuda and co-workers achieved the nearly complete shift from cycloartenol to lanosterol (99%) formation through engineering a double mutant of His[477]Asn and Ile[481]Val.[90] These results show that only small changes in the active site topography of these enzymes are required to fashion a fungal/animal synthase from a plant synthase. Lanosterol, parkeol, and lanostane derivatives with the 9βH-sterochemistry have recently been found to occur naturally in plants,[12,91] raising questions of their biosynthetic origin, that is, from preformed cycloartenol. Detailed investigations of the steric and electrostatic effects of conserved aromatic residues through site-directed mutagenesis experiments have revealed cation—π interactions that may influence the product outcome. Examination of yeast *ERG*7 homology structure and human OSC structure suggest that Tyr[707] and Tyr[99] residues in the *Saccharomyces cerevisiae ScERG*7 might play an important role in stabilizing the C8 cation during the formation of the second cyclohexyl ring and the final lanosterol C9 cation.[65] On the other hand, Thomas and colleagues suggest the relevant active site amino acid in the human synthase occurs at His[232], which is a strictly conserved residue among the oxidosqualene cyclases,[89] whereas Christianson noted that Tyr[503] could possibly accept this proton because it is closer and better-oriented with regard to the C9 proton that is ultimately eliminated to form lanosterol.[92]

Although cycloartenol is the first cyclized product in plants, lanosterol has been considered as an intermediate in phytosterol biosynthesis. Several reports involving plant sterol biosynthesis show similar rates of [2-^{3}H] (or ^{14}C)-lanosterol and [2-^{3}H] (or ^{14}C)-cycloartenol incorporation into phytosterols suggesting that cycloartenol might transform to lanosterol via a 9β,19-cyclopropane to Δ^8-isomerase catalyzed reaction as a preliminary step to phytosterol formation.[93—95] This reaction would allow for the cyclosterol intermediate (cycloartenol or some other cyclosterol) to rearrange into the more stable structure that can yield products having the C9α-H in Δ^5-sterols. However, primary sequences of lanosterol synthase were discovered recently in three different laboratories from dicotyledonous plant species, including *Arabidopsis thaliana*, *Panax ginseng*, and *Lotus japonica*, using a yeast expression system that suggest that lanosterol can be synthesized directly and independently from cycloartenol.[96—98] Conservative patterns suggest that the Ile481 residue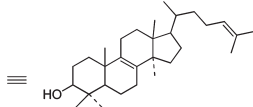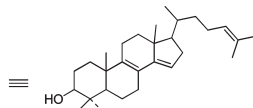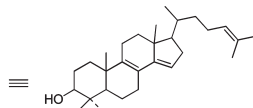 is partly respons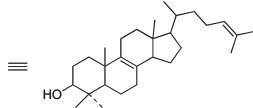ible for the catalytic differences between the lanosterol and cycloartenol synthases.[68] These findings together with reports showing that radiolabeled squalene oxide converts to lanosterol in a cell-free latex preparation of *Euphorbia lathyris* and intact *Arabidopsis thaliana* converts [6[13]C[2]H$_2$]MVA to lanosterol and phytosterol with retention of three [2]H-atoms at C19 in the labeled products show the cycloartenol—lanosterol bifurcation in sterol biosynthesis is not absolute.[99,100] Moreover, the cycloartenol and lanosterol synthases have been cloned and functionally expressed from the same bacterium *Stigmatella aurantiaca*, and the lanosterol synthase has been cloned from other primitive organisms, including *Methylococcus capsulatus* and *Gemmata obscruiglobus*.[101—104]
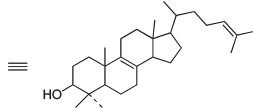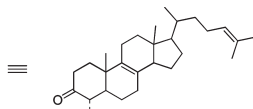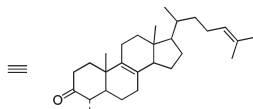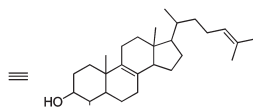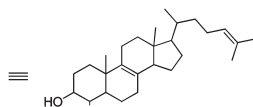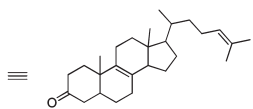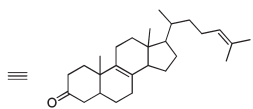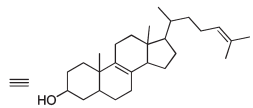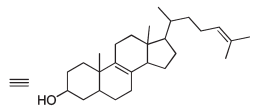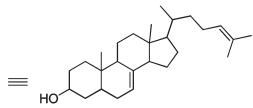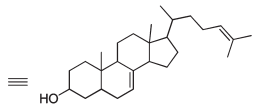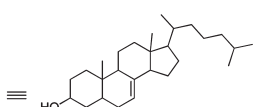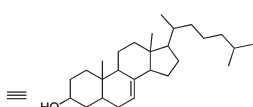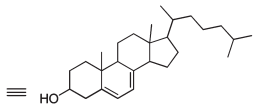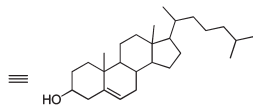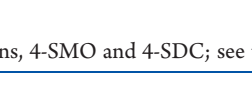
4.4. The Core Pathway: Lanosterol Conversion to Cholesterol

Three different approaches have been used to study the nature and sequence of steps involved with sterol biosynthesis: organic, enzymatic, and genetic approaches. The enzymatic approach to understand and control formation of the sterol structure was hampered by the low abundance of sterol enzymes in cell-free preparations, as well as difficulties associated with purifying microsomal proteins to homogeneity. By the early 1980s, it was evident primarily from the pioneering efforts of Gaylor and his co-workers at cataloging the properties of enzymes that act on sterols that an accurate sequence of 10 enzymatic reactions performed by nine distinct enzymes can be written for the lanosterol conversion to cholesterol as outlined in Table 2.[10a] These enzymes were shown to be integral membrane-bound proteins of the endoplasmic reticulum.

Cholesterol synthesis requires one molecule of C_{30}-4,4,14-trimethyl sterol converted to C_{27}-4,4,14-trisdesmethyl sterol followed by the formation of a saturated isooctyl side chain and Δ^5-bond in the final product. The stoichiometry of cholesterol synthesis from lanosterol is therefore lanosterol + 15NADPH + 4H$^+$ + 10O$_2$ → cholesterol + 2CO$_2$ + HCOOH + 15NADP$^+$. This process can be divided into two stages: stage 1 (reactions 1—6) represents the nuclear demethylation reactions that fashion the lanosterol frame into the cholesterol structure. The product of reaction 6 lacks methyl groups at C4 and C14 and contains a 3β-OH group that is distinct from the one generated by the cyclization of squalene-2,3-oxide to lanosterol. During these conversions of the C4-sterol to a C4-desmethyl sterol, a stable 3-keto sterol intermediate is formed. For stage 1, the process consumes 12 NADPH, 2 NAD$^+$, 9 O$_2$, and 1 H$^+$. Reactions 3 and 4 are enzymically coupled and repeated with the resulting C4 methyl product catalyzed in reactions 5 and 6; in both sets of reactions two distinct enzymes, sterol C4-methyl oxidase (4-SMO) and sterol C4-decarboxylase (4-SDC, also referred to as 3β-hydroxysteroid dehydrogenase/C4-decarboxylase), are utilized to carry out the overall conversion of the sterol C4-demethylation reaction. Key to this series of reactions is that the equatorial (in the plane of the sterol nucleus) methyl group of the 4,4-dimethyl and 4-monomethyl substrates is recognized for catalysis. To maintain stereochemical consistency in 4,4-dimethyl substrate recognition by 4-SDC, during decarboxylation the methyl group that occupies the 4β-(axial) position of the C4-dimethyl sterol epimerizes to the more stable 4$\al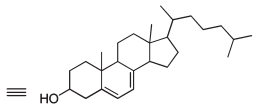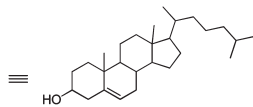pha$-(equatorial) position in the 4-methyl 3-ketosteroid product and, in doing so, re-establishes the C4α configuration. Thus, the natural occurrence of C4β-methyl sterols seems unlikely. Stage 2 (reactions 7—10) involves the rearrangement of the Δ^8-bond to the Δ^5-position and saturation of the side chain double bond. Reactions in stage 2 consume 3 H$^+$, 3 NADPH, and 1 O$_2$, and the

Table 2. Biosynthesis Steps Involved in the Conversion of Lanosterol to Cholesterol^a

Biosynthesis pathway	Favored substrate	Enzyme commission number/ Catalyst name [alternate name, corresponding ergosterol protein]
Stage I		
Lanosterol 4,4,14-trimethylcholesta-8,24-dien-3β-ol	≡	1.14.17.70/C14-Sterol demethylase, 14-SDM [CYP51; Erg11p]
① 3 O$_2$/3 NADPH → 3 NADP$^+$		
4,4,-dimethylcholesta-8,14(15), 24-trien-3-ol	≡	
② H$^+$/NADPH → NADP$^+$		1.3.1.70/C14-Sterol reductase, 14-SR [human lamin B receptor; Erg24p]
4,4,-dimethylcholesta-8,24-dien-3β-ol	≡	
③ 3 O$_2$/3 NADPH/NAD$^+$ → 3 NADP$^+$		1.14.13.72/C4-Sterol methyl oxidase, 4-SMO, Erg25p* and [C4 sterol decarboxylase, 4-SDC; 3β-hydroxysteroid-dehydrogena-se/C4-decarboxylase,Erg26p]
4α-methyl--cholesta-8,24-dien-3–one	≡	
④ NADPH → NADP$^+$		1.1.1.270/C3-Sterol reductase, 3-SR [Ketosteroid reductase; Erg27p]
4α-methyl-cholesta-8,24-dien-3β-ol	≡	
⑤ 3 O$_2$/3 NADPH/NAD$^+$ → 3 NADP$^+$		1.14.13.72/4-SMO and 4-SDC
cholesta-8,24-dien-3-one	≡	
⑥ NADPH → NADP$^+$		1.1.1.270/3-SR
cholesta-8,24-dien-3β-ol	≡	
Stage II		
⑦ H$^+$		5.3.3.5/C8-Sterol isomerase, 8-SI [Emopamil binding protein; Erg2p]
cholesta-7,24-dien-3β-ol	≡	
⑧ H$^+$/NADPH → NADP$^+$		1.3.1.72/C24-Sterol reductase, 24-SR [Seladin-1; Erg4p]
cholesta-7-en-3β-ol	≡	
⑨ O$_2$/NADPH → NADP$^+$		1.14.21.6/C5-Sterol desaturase, 5-SD [Lathosterol oxidase; Erg3p]
cholesta-5,7-dien-3β-ol	≡	
⑩ H$^+$/NADPH → NADP$^+$		1.3.1.21/C7-Sterol reductase, 7-SR
cholesta-5-en-3β-ol cholesterol	≡	

^{a} Steps 3 to 4 and 5 to 6 involve two discrete enzymatic reactions, 4-SMO and 4-SDC; see text.

enzymes act sequentially to convert the ring structure from a Δ^8 to Δ^7 to $\Delta^{5,7}$ to Δ^5. Two enzymatic studies completed on the cloned human sterol C8—C7 isomerase and microsomal rat C24(25)-reductase after Gaylor postulated a lanosterol—choles-terol pathway clarified the later sequence of reactions to cholesterol.[105,106] These enzymatic studies reveal that the $\Delta^{24(25)}$-double bond of zymosterol is reduced prior to Δ^5-desaturation.

The nine catalysts that convert lanosterol to cholesterol may be considered the core enzymes of Δ^5-sterol biosynthesis. As more enzymes have been isolated from an increasing number of sources, it has become clear that they fall into only a small
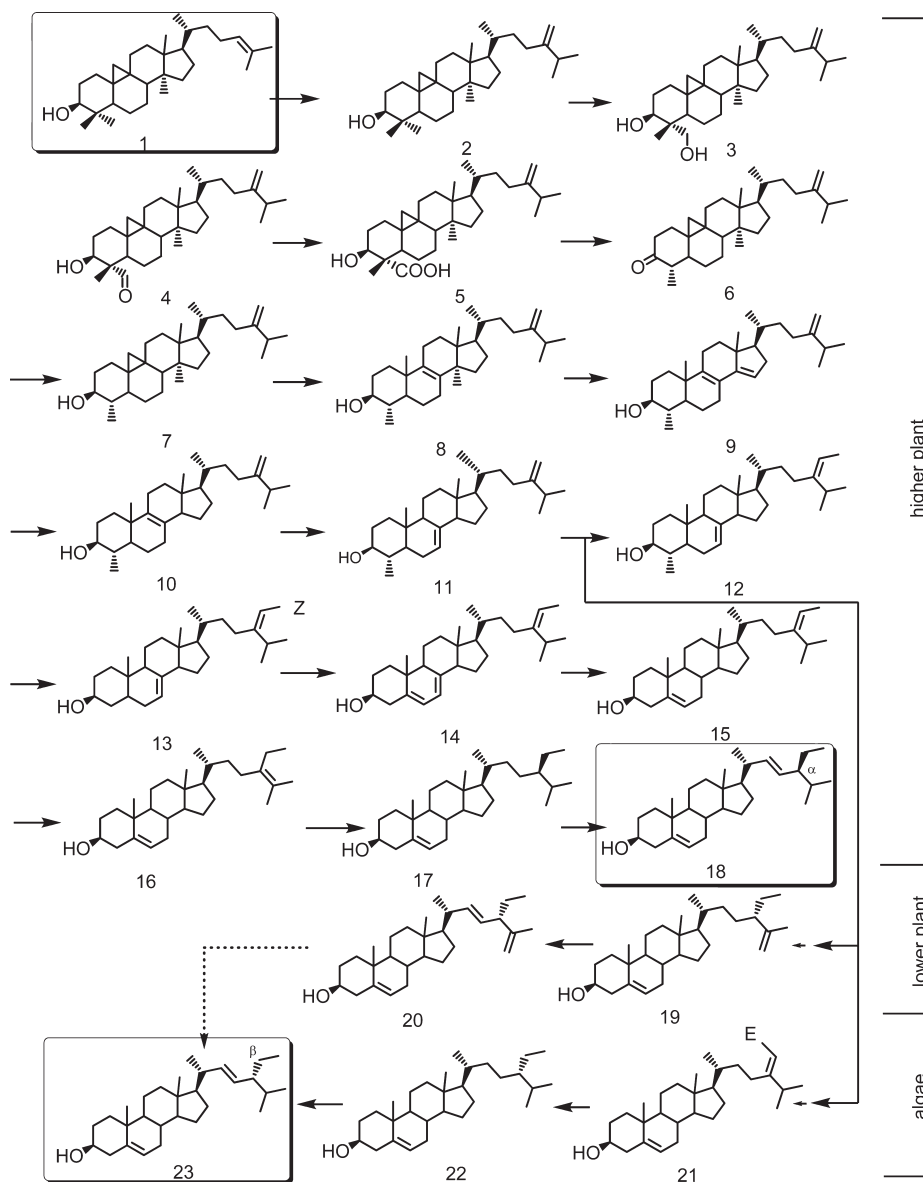
Figure 12. A generalized route from cycloartenol **1** to Δ^5-24α/β-ethyl sterols, stigmasterol **18** and poriferasterol **23**, respectively.

number of reaction types and that the chemical names give varied indication of this. In an attempt to rationalize this situation, Table 2 includes trivial names of the parent catalysts and a related system of abbreviations proposed by the author. Although cholesterol biosynthesis is often linked to the formation and metabolism of C_5 (isoprenoid = isopentenoid = terpenoid)-units, the "sterol biosynthesis pathway" can be defined as that set of enzymes that act on the sterol structure. The most direct route to cholesterol will be dependent upon the relative specificities of the enzymes for a particular sterol substrate thereby giving rise to the kinetically favored pathway. When multiple routes are postulated, it has been possible to draw a sterol biosynthesis matrix for an organism, which can predict the main or trace sterols which might be present, particularly after exposure to a sterol biosynthesis inhibitor or genetic defect.

In the case of cholesterol biosynthesis from lanosterol, two intersecting routes have been postulated. The choice of pathway is determined by the stage at which the double bond at C24 in the

sterol side chain is reduced. If C24 double bond reduction is retained until the last reaction, cholesterol synthesis proceeds via cholesta-5,24-dienol (desmosterol) (Bloch pathway). On the other hand, early Δ^{24}-reduction involving lanosterol can proceed to cholesta-5,7-dienol (7-dehydrocholesterol) and cholesterol (Kandutsch–Russell pathway). A common interpretation regarding which pathway is utilized involves the positioning of the Δ^{24}-reductase in cholesterol biosynthesis such that skin and intestines, which have higher sterol C24-reductase activities than liver or brain, proceed via the C_{24}–C_{25}-terminal intermediates.[10b–d] Regardless of tissue specificity, the kinetically favored pathway for cholesterol biosynthesis appears to involve the Kandutsch–Russell pathway.

The relevant committed step that distinguishes sterol from isoprenoid–triterpenoid biosynthesis occurs at the cyclization of oxidosqualene. Major control points in sterol biosynthesis may arise in the primary pathway before squalene formation at
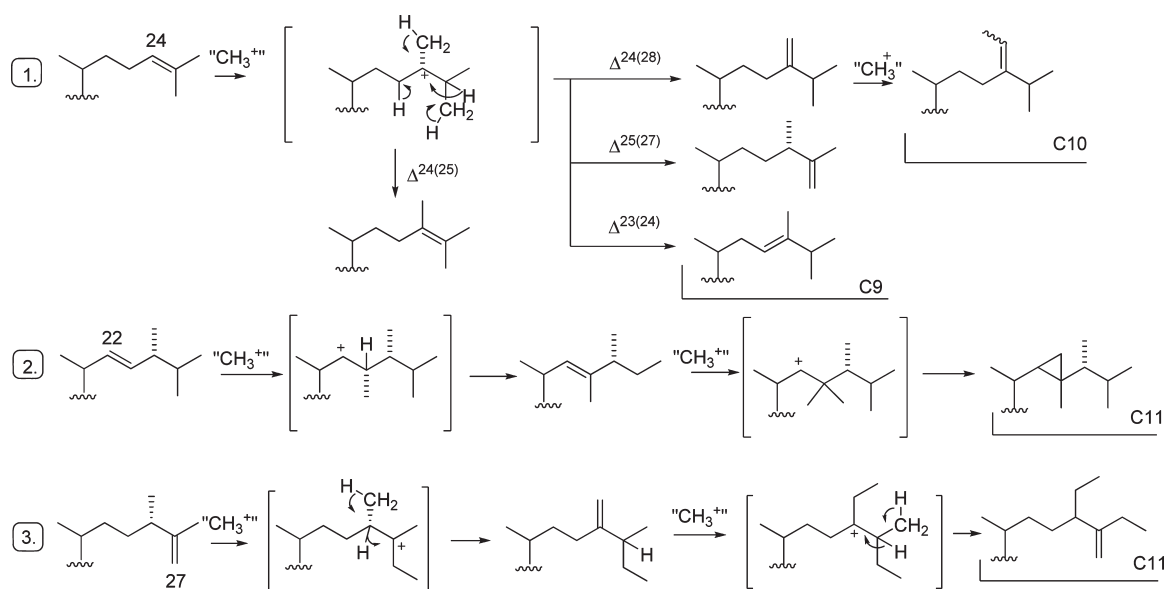
Figure 13. The sterol alkylation reaction pathways operating in different organisms; routes 1 in fungi, protozoa, and plants to form C_9 and C_{10} side chains and routes 2 and 3 to form C_{11} extended side chains in marine organisms.

hydroxymethyl-glutaryl-CoA reductase (HMGR) (coarse control)[3] or after squalene formation at the sterol C24-methyltransferase (24-SMT) step (fine control)[107] specific to organisms other than animals. Cofactor control by differential allocation of oxygen, NADPH, and AdoMet can further influence reaction rates and product distributions in cholesterol or phytosterol biosynthesis.

4.5. C24-Alkylation—Reduction Bifurcation in Phytosterol Synthesis

Early attempts to deduce sterol biosynthetic pathways in systems other than animals were based largely on indirect approaches that included structural and stereochemical correlations of co-occurring metabolites and *in vivo* tracer studies. Using microsome preparations of corn, Rahier, Benveniste, and their co-workers systematically worked through the major enzymatic reactions from cycloartenol to sitosterol and established substrate preference for many of these enzymes.[11] The accumulated evidence supports the kinetically favored pathway to Δ^5-sterol synthesis from cycloartenol illustrated in Figure 12.[2,11,72] Cholesterol can accumulate in plants by reduction of the Δ^{24}-bond depending on the expression of the sterol C24-methyltransferase enzyme, as demonstrated in genetically modified *Arabidopsis* plants.[108] The 24-SMT is recognized first in the phytosterol reaction sequence in plants and in some fungi. The typical products of transmethylation of the substrate Δ^{24}-bond are the C24-methyl $\Delta^{24(28)}$- and $\Delta^{25(27)}$-olefins, or in rare cases they become the 24-methyl $\Delta^{23(24)}$ (corn)- or $\Delta^{24(25)}$ (protozoa)-olefins (Figure 13).[109−111] Terrestrial organisms, as well as many pathogenic fungi and protozoan parasites synthesize 24-alkyl sterols, whereas animals, especially humans, lack the 24-SMT gene. Biomimetic studies of electrophilic alkylations of a remote double bond support the detection of multiple outcomes of enzyme-generated 24-alkyl sterol olefins[112] Separate biosynthetic routes, which have the following differences—major sterols are 24α-ethyl sterols or 24β-ethyl sterols or cholesterol and cyclopropyl or 24-methyl(ene) sterols replace 24-ethyl sterols as the major compounds in the sterol mixture, can be

generally categorized and result in the production of diverse sterol compositions.

In organisms that live in the marine world, sterol methylation patterns vary greatly and reflect the complexity of mixtures of sterols arising through the food chain. The 24-SMT of these organisms catalyze branched and highly alkylated side chains of distinct stereo- and regiochemistry, including tris- or quadruple alkylation of the C_{11} side chain, not typically observed in the sterol methyltransferase catalyzed products of terrestrial organisms that produce C_8 and C_9 side chains (Figure 13). Whereas plants can synthesize as many as 60 sterols, marine organisms have been shown to contain as many as 74 sterols in a single organism.[113] Dinoflagellate algae that form the ocean ecosystem are different from other algae and plants in their ability to synthesize 22,23-cyclopropyl sterols, effectively from an ergosterol-containing side chain. Biosynthesis of many other nonconventional phytosterols are postulated to result from nucleophiles for these reactions that originate in Δ^{22}-, Δ^{23}-, Δ^{24}-, and Δ^{25}-olefin-containing side chains. The stereochemistry of the transmethylation reactions and subsequent side chain modifications by reduction have been subjected to detailed investigation.[114] The translational significance for unearthing unconventional sterols, such as the 24-isopropyl steranes, in fossil remains is that their chemical fingerprint provides relevant biomarker information about sterol evolution and its relationship to speciation.[115,116]

5. STEROL ENZYME ACTION

5.1. C24 Methylation

In reviewing the action of enzymes that catalyze sterol formation, it has been found convenient to divide topics according to the properties of the enzyme that include its specificity, mechanism, inhibition, and where evidence is available results of mutagenesis experiments. We start with the enzymatic C24-methylation reaction, where progress has been made in identifying the genes and catalytic properties of the corresponding proteins. Four different sterol C24-methyltransferase enzymes
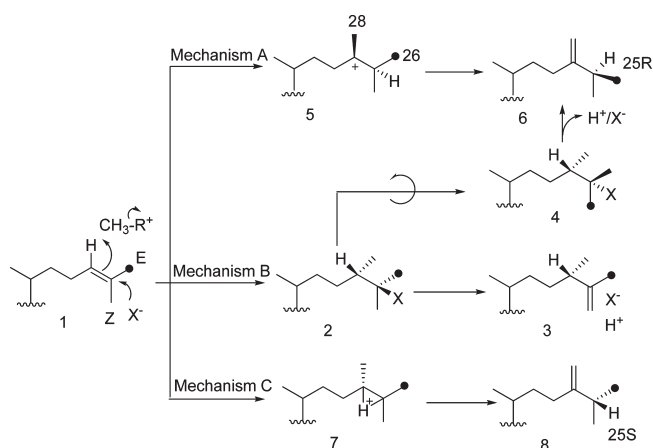
Figure 14. Mechanisms for methyl transfer to $\Delta^{24(25)}$-substrate in the action of sterol C24-methyltransferase; dot shows to ^{13}C-labeled carbon. In mechanisms A and C, the carbocationic intermediates are discrete species, and an important difference between the two mechanisms involves the S_N2 (Si, β)-attack of methyl cation on the substrate double bond coupled to the regiospecific deprotonation at C28 to yield the 25S stereochemistry typical of 24-alkyl sterols of the ergostane and stigmastane family. In mechanism B, the formation of carbocation intermediates is avoided by postulating the participation of an enzymatic nucelophile-X to form covalent bonds with incipient cations to yield the C25R stereochemistry typical of animal cholesterol.

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(24-SMT) have been detected and classified according to the substrate favored by the enzyme for catalysis. Plants synthesize two 24-SMTs, SMT1, which prefers cycloartenol ((EC 2.1.1.142), and SMT2, which prefers 24(28)-methylene lophenol (EC 2.1.1.143); some fungi and protozoa synthesize 24-SMT1 of substrate preference for zymosterol (EC 2.1.1.410), and other fungi also synthesize 24-SMT with a substrate preference for lanosterol.[111,117−120] In all cases studied, the catalysts shared similar properties of subunit organization as homotetramers of M_r 38 000−43 000, as determined by SDS-PAGE and gel permeation, and each contained a single active site for sterol and AdoMet. An interesting feature of several of the cloned 24-SMTs is their ability to produce multiple and distinct product sets at either the C_1- or C_2-stage of methylation.[111,120,121]

So far as is known, 24-SMTs vary in sequence identity (49−77%), the whole family containing only about 63 completely conserved residues (less than 20% of an average of *ERG6*). Twenty five of these residues lie in the substrate binding segments, and another 38 lie outside of these areas.[122] The cloned enzymes share similar slow turnover numbers of $k_{cat} = 0.01 \text{ s}^{-1}$ and pH optima of 7.5. Steady-state kinetic studies show fungal SMT1 (*S. cerevisiae*) AdoMet and zymosterol bind randomly to the enzyme. Alternatively, the plant SMT1 (*G. max*) exhibits an ordered binding mechanism, AdoMet binds first, followed by the methyl acceptor cycloartenol.[118,123]

The structure of 24-SMT has not been established. In the absence of atomic coordinates, the general characteristics of the 24-SMT active site have been probed by means of photoaffinity labeling using AdoMet, suicide substrates using 26,27-dehydrozymosterol and site-directed mutagenesis experiments. These studies, focused on the *S. cerevisiae* 24-SMT, have identified several conserved amino acids that cluster in sets of

approximately 11 residues in four substrate binding segments of the protein; homology modeling shows that region II contains the Rossmann fold, which consists of central β-sheets flanked by α-helices, and the experimental data shows contact amino acids that can interact with AdoMet and regions I, III, and IV. The sterol binding segments were defined through mutagenesis experiments to give rise to gain-in-function activities that upon mutation can lead to the second C_1-tranfer reaction characteristic of plant 24-SMTs.[121,124,125]

The 24β-chirality in algal ergosterol and the 24α-chirality of vascular plant campesterol and sitosterol is considered to arise from direct C24-alkylation or through a $\Delta^{24(28)}$-sterol intermediate, respectively. To account for the product diversity, ionic and X$^-$ group C24-alkylation mechanisms have been proposed as shown in Figure 14.[125−127] The different stereochemical outcomes observed in ergosterol and sitosterol were proposed to involve a C24-methylation reaction that led to a C24S-methyl group (fungi) or C24R-methyl/ethyl group (plant). In either case, C24-methylation was thought to generate the C25R-H chirality, which is analogous structurally to the stereochemistry at C25 in animal cholesterol. As envisioned in the mechanism for squalene oxide cyclization to sterols, the C24-alkylation mechanism should include a nucleophilic center on the 24-SMT (X$^-$, unidentified amino acid) to form a covalent bond with the C25 or C24 carbocation resulting from the destruction of the $\Delta^{24(25)}$- or $\Delta^{24(28)}$-bonds. The covalent species are shown in mechanism b, and the formation of such intermediates would obviate the necessity to form discrete carbocations. The X$^-$ group mechanism considered in 24-alkyl sterol biosynthesis is based entirely on stereochemical considerations that relate to changes in the conformation of covalent-bound sterol-24-SMT of different organisms directing the chirality in enzyme-generated products.

In the ionic mechanisms, illustrated in paths a and c (see Figure 14) the biomethylation reaction involves discrete carbocationic intermediates that can be the result of synchronous changes in bonding that occur in a concerted fashion to form a single product or involve a stepwise process where topology is maintained between the initiation and termination steps affording multiple products. Ammonium and sulfonium analogs of the hypothetical 24-methyl sterol C25/C24 cation intermediates have been found to inhibit the C24-methylation, consistent with a cationic reaction mechanism in sterol alkylations.[128] Equilibrium binding of the transition state analog 25-azalanosterol (charged at physiological pH) to yeast 24-SMT indicated that its K_d was similar to that of the sterol acceptor and AdoMet, ca. 4 μM, whereas the K_i for 25-azalanosterol against zymosterol ($K_{mapp} = 35 \, \mu$M), determined by steady-state kinetic analysis, is ca. 20 nM. These results suggest that the 24-SMT undergoes a conformational change upon inhibitor binding for the substrate analog to interrupt catalysis.[130,131]

Arigoni and colleagues reported the preparation of [^{1}H^{2}H^{3}H]AdoMet (only one enantiomer of the chiral methyl group was present) and have used it to probe the stereochemical course of a methyl transfer to carbon in fungal production of ergosterol. The results obtained indicate that the chirality of the acetate derived from the methyl group at C24 in ergosterol was the same as that of the methyl group of methionine but with a reduction in chiral purity.[132] This addition−elimination reaction proceeds with inversion at the transferring, chiral methyl center yielding an S_N2 mechanism for the methyl transfer. The question as to which side of the double bond of the Δ^{24}-sterol acceptor the

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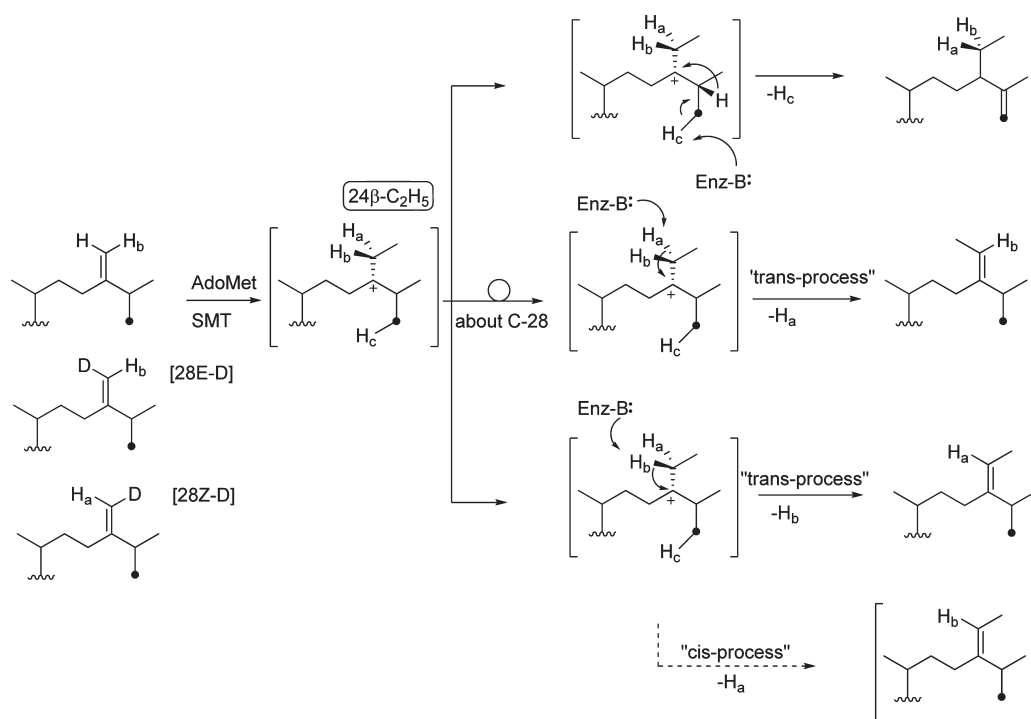dx.doi.org/10.1021/cr200021m |*Chem. Rev.* 2011, 111, 6423–6451

Figure 15. C_1-Transfer mechanisms in the conversion of $\Delta^{24(28)}$-substrate into 24-ethyl(idene) products. In each case, the isotopically labeled substrate contains a ^{13}C-27 atom (dot) and a Z- or E-^{2}H-atom selectively introduced at C28. The "cis-process" has been eliminated by experiment. Adapted from ref 123. Copyright 2003 The American Society of Biochemistry and Molecular Biology.

incoming methyl group is added was resolved by studying the metabolic fate of the pro-Z methyl of various Δ^{24}-^{13}C27-sterols incorporated into ergosterol and comparing the isotopically labeled natural product, found to have the 25S-configuration, to chemically synthesized 25-S-[^{13}C]ergosterol.[133,134] The results show that the first C_1-transfer reaction mediated by the plant and fungal 24-SMTs proceeds by alkylation from the Si-face of the substrate $\Delta^{24(25)}$ (yielding β-methyl stereochemistry) followed by migration of a hydrogen from C24 to C25 across the Re-face.

Yagi et al. investigated the second C_1-transfer reaction by feeding a set of [26-^{13}C], [27-^{13}C], and [26,27-^{13}C$_2$]-desmosterols (cholesta-5,24-dienol) to cultures of *Ajuga reptans* and found that the C24-alkylation takes place in a specific manner wherein the C26- and C27-methyl groups of the substrate become C26 (vinyl methyl) and C27 (exomethylene carbon), respectively of the 24β-ethyl $\Delta^{25(27)}$-sterols synthesized by the plant.[135] Regiospecificity directed at $\Delta^{25(27)}$-olefin formation was confirmed using microsomal preparations of *Prototheca wickerhamii* by the demonstration that ^{13}C-label at C27 (pro-Z methyl) in the lanosterol substrate is retained in the enzyme-generated 24β-methyl $\Delta^{25(27)}$-product.[136] Using cloned *Arabidopsis* 24-SMT1, we subsequently demonstrated that conversion of [27-^{13}C]-fecosterol (C_1) to [27-^{13}C]24(28)Z-ethylidenefecosterol (C_2) takes place with retention of configuration at C25.[135] Studies of synthesized [28E-^{2}H]- and [28Z-^{2}H]-24-methylene sterol acceptors transmethylated to give 24-ethyl(idene) sterol products established that the second methylation proceeded in such a manner that addition of the methyl group and proton loss occur on opposite faces of the original $\Delta^{24(28)}$-double bond.[123,137] On the basis of these observations and recognizing that 24-SMTs can be bifunctional and capable of operatong successive transfer reactions to yield multiple products, we proposed the C24-

methylation reaction illustrated in Figure 15, in which the C28 elimination occurring on the isofucosterol cation during the second C_1-transfer reaction occurs by an *anti*-mechanism.[123]

Kinetic studies involving ^{2}H-substrates have revealed another important feature of the reaction catalyzed by the soybean 24-SMT enzyme. Thus, incubation of AdoMet versus [*methyl*-^{2}H$_3$]AdoMet as substrate against saturating concentrations of cycloartenol afforded a kinetic isotope effect of V_{CH_3}/V_{CD_3} close to unity.[123] Similarly, incubation of [24-^{2}H]cycloartenol failed to produce any apparent isotopes effects related to the 1,2-hydride shift of H24 to C25, consistent with a concerted mechanism in the first C_1-transfer reaction. However, substrate binding experiments of [28E-^{2}H]- and [28Z-^{2}H]-24-methylene sterol acceptors to determine isotope effects associated with formation of multiple product resulting from the second C_1-transfer reaction demonstrated that [28E-^{2}H]-olefin afforded a KIE ((k_H/k_D) observed) of 0.92 for the deprotonation step whereas for the [28Z-^{2}H]-olefin a KIE of 1.23 was obtained. The inverse value established by the isotoptically sensitive branching experiments for the second C_1-transfer reaction is strong evidence that the proton transfer from the substrate to the active site base has to come to equilibrium prior to the rate-determining transition state and supports the proposal of a discrete carbocation intermediate.

We reasoned further that it would be possible to differentiate between the X-group and ionization mechanisms by selectively substituting hydrogen with fluorine or other atoms in the vinylic substrate. By changing the nucleophilicity of the sterol Δ^{24}-bond by substitution of methyl or fluorine for hydrogen significantly decreases the rate at which soybean 24-SMT catalysis proceeds without affect on the binding properties of the substrate appreciably. The observed order of V_{max}/K_m values, H (34) < CH$_3$(6)

Table 3. Sterol Analogs Tested with Sterol C-24-Methyltransferase Enzymes[a]

No.	Substrate	Product (s)	Byproduct (s)
1		13a, 13b	ND**
2		14	ND
3		15a, 15b, 15c	ND
4		ND	ND
5		16a, 16b	16c*
6		ND	ND
7		17a	17b, 17c
8		18a	18b*
9		20	21
10		ND	ND
11		19a	19b*
12		ND	ND

[a] An * on the compound number indicates putative. ND = not detected. Compounds **5**, **7**, **8**, and **11** have been shown to be mechanism-based inactivators. See text.

< F (0.9), clearly correlates with the carbocation stabilizing ability of the C25 group, where catalysis of the 24-methyl and 24-fluoro derivatives afforded putative 25-hydroxysterols (Table 3). The 24-bromo derivative **12** was not an acceptable substrate although it could serve as a competitive inhibitor (K_i approximately 32 μM) of the C24-methylation reaction of soybean 24-SMTs.[138–141]

The trapping of intermediary carbocations as diol structures obtained by saponfication of the sterol–enzyme complex has been accomplished by incubation of **17a** and recovering of **17b** (Table 3).[124,131,141] The diol **17b** was shown to be bound in an ester linkage to the E68 residue of the yeast 24-SMT. The trapping of a sterol methyl cation **17b** is the first example of an intermediate of the 24-alkylation reaction from any source. The structure and stereochemistry of the 24β-hydroxyl group was proven by extensive NMR analysis and the addition of the C28 methyl group to C26 (rather than to C24) confirmed by the incorporation of three deuterium atoms derived from incubation with [methyl-^{2}H$_3$]AdoMet. Also notable is the formation of a diene structure in **17a** in which double bonds are produced at C23 and C25 in the sterol side chain, suggesting that cryptic nucleophiles are present in the active site of these enzymes and which readily explains the operation of the $\Delta^{23(24)}$-route by the corn 24-SMT. Affinity labeling experiments using [3-^{3}H]26,27-dehydrozymosterol or the methylenecyclopropane derivatives paired with [methyl-^{3}H$_3$]AdoMet, which forms the first potent mechanism-based irreversible inactivator of the 24-SMT, demonstrated that the fungal and plant 24-SMTs were specifically labeled and gave single bands on fluorogram with apparent molecular masses that matched the predicted molecular masses

of the primary structures of the corresponding enzyme. Substrates bearing a conjugated double bonds system in the side chain at $\Delta^{22,24}$ or $\Delta^{24,26}$ are catalyzed by 24-SMT to product pairs, which after saponification of the enzyme preparation yield the predicted C24(28)-sterol product and an uncommon C24-methylated sterol with a hydroxyl group at C22 **21** or C26 **18b** (Table 3).[142] These analogs, like those formed by 24-SMT catalysis of 26,27-deydrozymosterol, may also serve as suicide substrates of the enzyme. The ability of 24-SMT to catalyze the 26-homosterol-$\Delta^{22,24(26)}$-**9** and 26,27-dehydro **7** analog show that lengthening of the side chain by one carbon atom is accepted by 24-SMT.[117,118,131,142]

Because aromatic amino acid residues are crucial for the oxidosqualene cyclization reaction catalyzed by lanosterol and cycloartenol synthases, their possible role in related electrophilic alkylation reactions catalyzed by 24-SMTs has been studied. It is expected that the stereochemical course of the C24-methylation reactions would be subject to thermodynamic control, which is a measure of the energy needed to overcome the activation barrier of sterol alkylation, and kinetic control, which is a measure of the site-specific proton eliminations (e.g, at C27 or C28) that govern product diversity.[131] Based on the information from homologous alignment of the primary structures of 24-SMTs that show that region I is very rich in aromatic amino acids, the highly conserved Tyr[81] (*ERG*6 nomenclature) in the fungal Erg6p was replaced by phenylalanine or tryptophan and mutated to a series of aliphatic residues of different size (glycine, alanine, leucine, and isoleucine).[124,125,130,131] The substrates zymosterol and 26,27-dehydrozymosterol (**1** and **7** in Table 3) were tested against the native and mutant yeast 24-SMTs. In the enzyme−substrate complex, these chemically distinct substrates catalyze to C24- or C26-methylated sterols. When Tyr[81] in Erg6p is substituted with phenylalanine, the enzyme becomes plant-like in its ability to recognize $\Delta^{24(28)}$-sterols and convert the exocyclic methylene side chain of fecosterol to the same set of 24-ethyl(idene) products **15a**, **15b**, and **15c** (Table 3) that the soybean 24-SMT normally can produce. Multiple products can also arise from incubation of 26,27-dehydrozymosterol with the wild-type Erg6p; the products are shown in Table 3 as **17a**, **17b**, and **17c**. Mutations of Erg6— at Tyr[81] involving the replacement to aromatics or aliphatics had noticeable affects on the partitioning outcome of 26,27-dehydrozymosterol; mutating to aromatically favored C26 monol **17a** formation whereas mutating to favored aliphatic of smaller size C26 diol **17b** and **17c** formation (effecting enzyme alkylation and inactivation). In no case was zymosterol converted to an intermediate that included a 25-hydroxy sterol (i.e., a diol). Tyr[81] replacement of related aromatic amino acids to 24-SMT and testing either substrate increased the rate from 0.04 min^{-1} to as much as 0.20 min^{-1}, and substitution of aliphatic residues led to decreased rates from 0.04 min^{-1} to as little as 0.007 min^{-1}. In the thermodynamic analysis, there was no evidence for a change in the product ratio as a function of temperature. This effect is strongly suggestive of thermodynamic control of the C24-methylation reaction. The energetic costs associated with the enzyme−substrate complex in the transition state was found to be +13.7 kcal for zymosterol and +15.6 kcal for 26,27-dehydrozymosterol. the activation differences (E_a) relative to wild-type of +0.9 to −1.0 kcal for the aromatics, in contrast to a difference in the E_a of +2.3 kcal for the aliphatics. The difference in energy between the two substrates in catalysis by the wild-type enzyme is +1.9 kcal
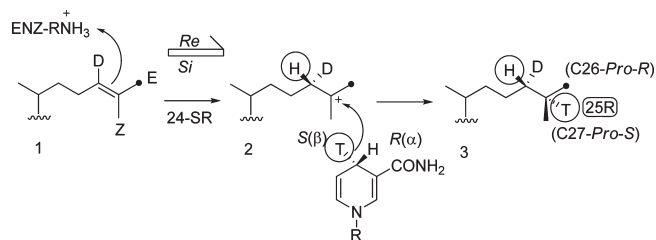

Figure 16. Revised mechanism for the reduction of the sterol Δ^{24}-double bond by the action of sterol C24-reductase (24-SR), which uses NADPH as coenzyme. The new hydrogen atoms (as hydrogen or tritium) introduced are marked with circles, and ^{13}C-labeled carbon is shown with a dot. The mechanism requires introduction of a proton from an active site donor, for example, lysine, to the *re*-face of the substrate double bond at C24 (**1**) followed by *trans*-addition of the hydride ion to eliminate the carbonium ion at the *si*-face of C25 (**2**) thereby forming the C26 *Pro-R* stereochemistry typical of animal cholesterol (**3**).

indicating that a higher energy route is required to reach the transition state for 26,27-dehydrozymosterol catalysis consistent with the topography of these interactions. It would appear that the relative positioning of Tyr[81] or its variants with respect to the different intermediates of zymosterol and 26,27-dehydrozymosterol catalysis in the activated complex of a tight or loosely fit structure almost certainly underlies the direction of partitioning of the 24-SMT catalyzed reaction.

5.2. C24-Reduction

Sterol side chain C24-reductases (SR) are a family of NADPH-dependent catalysts that transfer a hydride ion (H$^-$) to olefinic centers at C24 or C25 of the sterol side chain. Genes corresponding to the $\Delta^{24(25)}$-reductase (24-SR) in humans (*DHCR*24 = seladin-1), $\Delta^{24(28)}$-reductase in fungi (28-SR, *ERG*24), and $\Delta^{24(25)}$-reductase in plants (*DIM/DWF*1) have been identified and modeled to analyze its binding properties but in no case has the corresponding enzyme been cloned and purified to evaluate the enzyme's properties.[143−145] Interestingly, in the pathogenic bacterium *Coxiella burnetii* that lacks *de novo* sterol biosynthesis, the organism encodes a eukaryote-like Δ^{24}-sterol reductase homologue that is absent in other prokaryotes.[146] The predicted molecular weight of the human sterol 24-SR is 60.1 kD and fungal 28-SR is 56.1 kDa. The enzymes themselves require no cofactors other than NADPH. The conversion of fungal $\Delta^{24(28)}$-methylene sterol to 24β-methyl sterol has been found to proceed in *S. cerevisiae* under strict anaerobic culture conditions,[147] suggesting that sterol C24-reductases are not oxygen-dependent and that these enzymes cannot be operationally a flavin dinucleotide (FAD)-dependent oxidoreductase-type enzymes as reported for the human sterol C24-reductase.[145]

The substrate specificity of the sterol C24-reductase has been examined in two cases, in rat and insect (*Manduca sexta*) microsomal preparations.[105,148] The catalytic competence for the two enzymes differ markedly in their recognition of the cholestane and lanostane frame. The apparent V_{max}/K_m for lanosterol and desmosterol are for rat 0.361 nmol/(min·mg) and 109 μM and 3 nmol/(min·mg) and 163 μM compared with the insect 3 pmol/(min·mg) and 18 μM and 1 pmol/(min·mg) and 3 μM, respectively. For rat, cholesta-7,24-dienol is the optimal substrate for the enzyme yielding kinetic constants of 2 nmol/(min·mg) and 37 μM. Complicating the isolation and study of these proteins from natural sources is the fact that many

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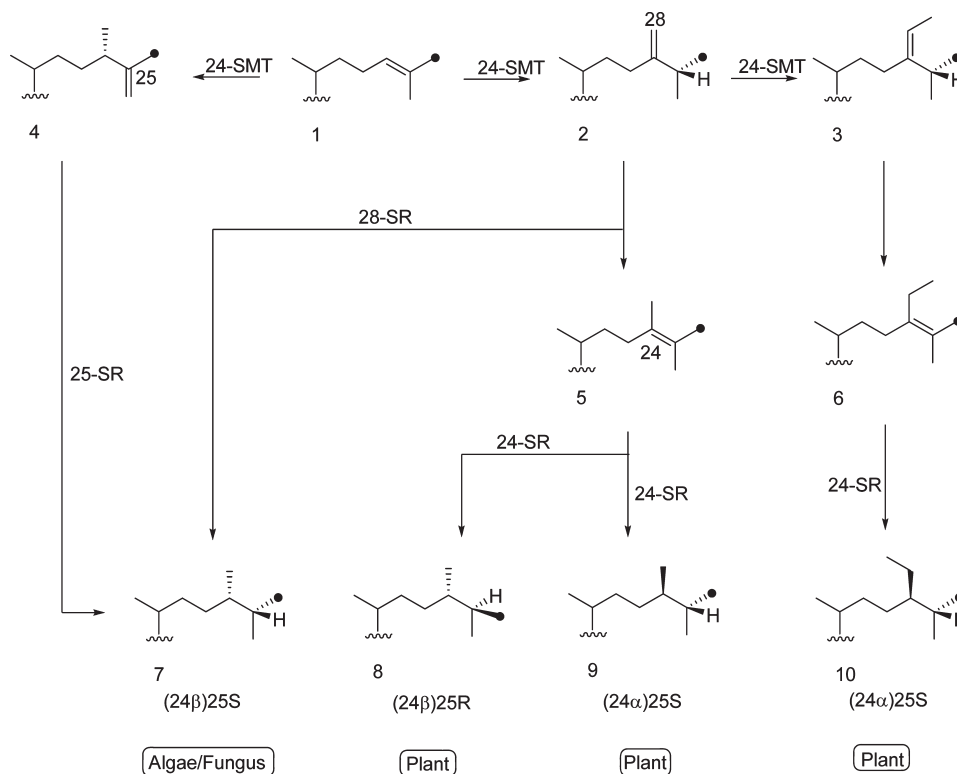dx.doi.org/10.1021/cr200021m |*Chem. Rev.* 2011, 111, 6423−6451

Figure 17. Phyla-specific pathways for the regio- and stereospecific reduction of a remote double bond in 24-alkyl sterol side chains. Three distinct sterol reductases are considered in the formation of the C24-methyl/ethyl and C25-H stereochemistry, sterol C28-reductase (28-SR), sterol C25-reductase (25-SR), and sterol C24-reductase (24-SR). Products **8**, **9**, and **10** can originate by the action of the same plant 24-SR. Dot indicates ^{13}C27-labeled atom. See text.

appear to be present in low abundance in the host organisms, and for cloned enzymes, the membrane-bound nature of them can interfere with the purification process. Bioinformatic analysis and homology modeling of human DHC24R indicates an N-terminal segment (Leu[23]-Trp[55]), which may act as a membrane spanning domain and a set of apolar residues at Ile[363], Leu[366], His[382], Val[384], Trp[411], Ile[437], Ala[439], and Pro[443], affording hydrophobic contacts with the substrate, whereas Ser[291] and Ser[295] provide contacts to hydrogen bond with the sterol C3-hydroxyl group.[145b] Thermodynamic analyses that take into consideration docking scores suggest that the human sterol C24-reductase binds more stably to desmosterol, −30.18 kcal/mol, compared with lanosterol, −12.87 kcal/mol, consistent with the substrate preferences noted in the structure—activity kinetic studies.[145b]

Concerning the mechanism of the reduction, it was reported to occur by the addition of a hydrogen from the medium at C24 and another from NADPH at C25.[149] Support for the cationic nature of the enzymic reaction intermediate has been obtained through observation that mimics of these species are effective inhibitors of the rat 24(25)-reductase and 24(28)-reductase of yeast.[150,151] The stereochemistry of the reduction of the 24(25)-double bond in lanosterol or desmosterol synthesis was originally studied by Caspi and his colleagues.[59] They deduced that in the reduction of the 24(25)-double bond, both hydrogens are added to the *re*-face of the double bond, equivalent to a *cis* addition of the hydride ion to C25 from NADPH and a proton (from water) to C24. As Popjak and co-workers observed, in the ^{13}C NMR of [^{13}C$_{10}$]cholesterol derived from [3′,4-^{13}C$_2$]mevalonate, C26 and C27 of lanosterol (or of desmosterol) become, respectively, the pro-R (C26) and

the pro-S (C27) of methyl groups in the side chain of cholesterol, affording the 25R-stereochemistry for the reduction of the 24(25)-double bond.[152] Takahashi et al., 30 years later, reexamined the stereochemistry of the C24,25-double bond in the conversion of desmosterol to cholesterol in rat liver, silkworm, and plant (*Oryza sativa*) preparations by incubation of [24-^{13}C,24-^{2}H]desmosterol.[153] In contrast to the original findings where the hydrogen atom from the medium should be β-oriented, the Japanese group determined, by using deuterium-decoupled ^{1}H,^{13}C shift correlation NMR analysis of the biosynthesized cholesterol, that the stereospecific incorporation of hydrogen atoms occurs from the *re*-face of the C24-position of the $\Delta^{24(25)}$-substrate generating an α-oriented hydrogen. It was also found using intact *Manduca sexta,* that conversion of [24-^{2}H]lanosterol to [24-^{2}H]dihydrolanosterol proceeds with retention of ^{2}H at C24 and that incubation of [4-*pro-S*-^{3}H]NADPH paired with desmosterol leads to the incorporation of tritium into cholesterol.[148] The summation of the stereochemical experiments are illustrated in the revised mechanism of sterol C24-reduction illustrated in Figure 16, which shows that the addition of hydrogen on the substrate 24(25)-double bond takes place in *anti* fashion from the *re*-face of C24 and the *si*-face of C25 and that the pro-S hydrogen is transferred from a nicotamide cofactor to the C25 cation intermediate. The only other enzymatic studies of sterol side chain reductase beyond those involving $\Delta^{24(25)}$-reductases have targeted the yeast sterol 28-SR, which has been found to prefer ergosta-5,7,22,24(28)-tetraenol as the optimal substrate and to require NADPH in conversion of the 24(28)-double bond to 24β-methyl sterol.[154]

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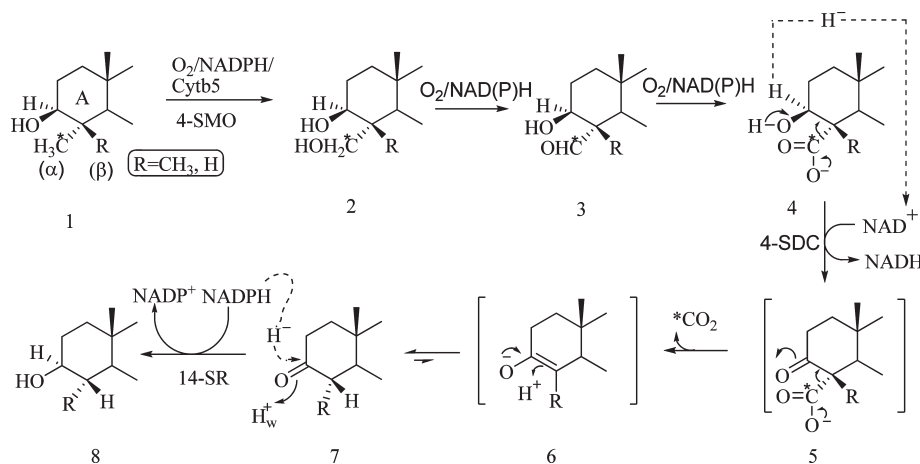dx.doi.org/10.1021/cr200021m |*Chem. Rev.* 2011, 111, 6423–6451

Figure 18. Stereochemical scheme for the stepwise enzymatic removal of C4α-methyl group and reduction of C3 ketone to produce a 3β-hydroxyl group. The first set of enzymatic reactions (**1**−**5**) is catalyzed by C4-sterol methyl oxidase (4-SMO) in complex with O_2, NADPH, and Cytb$_5$ yielding a C4-oxygenated intermediate. The second set of reaction steps (**5**−**7**) involves C4-sterol decarboxylase (4-SDC) [3β-hydroxysteroid dehydrogenase/C4-decarboxylase (4-HSD/D)] and NAD$^+$ catalyzing sterol 3-ketone formation; 4-SMO and 4-SDC are coupled but separate reactions. The third enzymatic reaction (**7**−**8**) involves sterol 3-reductase (3-SR) in complex with NADPH yielding 3β-hydroxy sterol. In the conversion of a 4,4-dimethyl sterol (e.g., lanosterol) to a C4-mono methyl sterol (e.g., 31-norlanosterol), the C4β-methyl group in the substrate **1** epimerizes to become the C4α-methyl group **8** in the product as shown. See text.

The formation of 24β- or 24α-alkyl sterols can be the result of stepwise involvement of three enzymes, the sterol C24-methyltransferase (24-SMT), the sterol C24(28)- to C24(25)-isomerase (24-ISO), and one or another C24(25)- or C25(27)-sterol reductase enzymes (C24-SR or C25-SR).[155−160] The mechanism for the double bond isomerization from $\Delta^{24(28)}$- to $\Delta^{24(25)}$-sterol was established by feeding [28-^{13}C,28-^{2}H]isofucosterol to cell cultures of *Oryza sativa* and analysis of sitosterol formed from 24-ethyldesmosterol; the double bond isomerization takes place in a *syn*-SE-2′ manner wherein the pro-*S* hydrogen at C28 of sitosterol is introduced from the 28-*si* face of isofucosterol. A similar isomerization mechanism was demonstrated in the biosynthesis of campesterol and 24-epicampesterol from 24(28)-methylene cholesterol.[161]

As evident in the labeling of the sterol side chain C26 (or C27) with ^{13}C, the two common sterol alkylation routes involving the $\Delta^{24(28)}$ and $\Delta^{25(27)}$-pathways (Figure 17) are now known to proceed to 24β-methyl sterols of opposite C25 stereochemistry (compare **7** and **8** in Figure 17).[155] Moreover, the C25 stereochemistry of fungal and algal ergosterol and plant campesterol and sitosterol are 25*S*, which is opposite to the C25*R* stereochemistry of cholesterol synthesized in plants or animals.[132,152,156−160] The stereochemistry of the reduction of 24-methyl/ethyl $\Delta^{24(25)}$-sterol intermediates to form 24α- and 24β-methyl/ethyl sterols has been investigated. To explain the formation of the two epimers at C24 for C24-methyl sterols and the formation a single 24α-ethyl sterol, one proposal suggests the reaction to consist of an addition of a H$^+$ on the *re*-face of the $\Delta^{24(25)}$ leading to a three-center carbocation and then an attack of H$^−$ from NADPH would then occur either at C24 or C25 leading to the formation of 24-methyl sterols of opposite stereochemistry. To explain the differences of regiospecificity, Bladocha and Benveniste suggested that steric hindrance caused by the bulky ethyl group at C24 would favor H$^−$ addition to C25, whereas no such constraints would exist in the case of C24-methyl sterols, allowing attack of H$^−$ at both C24 and C25.[163] A more probable mechanism has been elucidated by Fujimoto by feeding [26-^{13}C]- and [27-^{13}C]-

labeled 24-methyldesmosterol to cultured plant cells. The stereochemistry of reduction of **5** to **7** and **8** (Figure 17) could arise by either a *syn* or *anti* mechanism, as was discussed in the reduction of the $\Delta^{24(25)}$-bond in cholesterol synthesis.[159,160] The results revealed that stereospecific hydrogen attack on 24-*si*,25-*re* face of 24-methyldesmosterol affords campesterol, whereas 24-epicampesterol is produced by 24-*re*,25-*si* attack of hydrogen on the same $\Delta^{24(25)}$-olefin. The reductions that lead to the 24-methyl group of opposite stereochemistry evolve from the same enzyme and proceed in an *anti*-mode of hydrogen addition in both cases. The 24α-ethyl stereochemistry likely is formed by the same *anti* mechanism. The stereochemistry of algal ergosterol synthesized by the $\Delta^{25(27)}$-pathway is reported to be 25*S* analogous to fungal ergosterol, which obtains the C25 stereochemistry by the 1,2-hydride shift of H24 to C25 during the C24-alkylation reaction. In algal ergosterol, the reduction performed by a 25-SR likely proceeds by an *anti* mechanism as well. Still unclear is the ability of the $\Delta^{24(25)}$-reductase to discriminate substrates of 24-H, 24-methyl, and 24-ethyl ranking reflected mechanistically in the enzyme's ability to catalyze epimeric mixtures utilizing 24-methyl- but neither 24-desakyl or 24-ethyl $\Delta^{24(25)}$-sterol substrates.

5.3. Removal of Nuclear Methyl Groups at C4

The geminal methyls at C4 initially associated with lanosterol and cycloartenol are eliminated in two separate processes: an O_2- and NADPH-dependent formation of a 4α-carboxysterol metabolite followed by an O_2-independent but NAD(P)$^+$-dependent oxidative decarboxylation of this intermediate. In contradistinction to C32 removal, which occurs as formic acid, C4 methyl removal occurs as CO_2. The loss of the 3α-hydrogen atom during C4 demethylation is universal, consistent with the intermediacy of a β-keto acid at C3. Notably, during decarboxylation the methyl group that occupies the 4β (axial) position of **1** epimerizes to the more stable 4α (equatorial) position as shown **7** and, in doing so, re-establishes the chirality at C4 as α-oriented (Figure 18).

Recent study of cloned enzymes showed that the overall C4 demethylation process involves (i) the sequential reaction of C4α monohydroxylation of the 4,4-dimethyl substrate and oxidation of the CH$_2$OH group to the carboxyl stage catalyzed by sterol C4α-methyl oxidase (4-SMO), (ii) oxidative decarboxylation by sterol C4α-decarboxylase (4-SDC) [also known as 3β-hydroxysteroid-dehydrogenase/C4-deacarboxylase, 3βHSD/D)] complexed with NAD$^+$ followed by tautomerism, and then (iii) stereospecific reduction by a NAD(P)H-dependent sterone reductase of the 4-monomethyl-3-ketone to the 4-monomethyl-3β-alcohol catalyzed by sterol 3-reductase (3-SR).[10,164,165] The second demethylation in which the latter C4α-sterol intermediate is converted into a 4-desmethyl sterol is mechanistically identical and is catalyzed by the same membrane-bound cytochrome b_5 pathway that participates in the electron carrier from NADPH to the demethylation complex.[10,166,167]

A sterol C4α-methyloxidase gene has been identified in fungi, animals, and plants, and the deduced human protein contains 293 amino acids and at least four transmembrane loops.[72,167−169] These proteins share a set of conserved four histidine clusters typically associated with metal binding. Based on tracer work and limited specificity studies, it is generally accepted that 14-desmethyl lanosterol or 24(28)-methylene-24,25-dihydrolanosterol (trivial name, eburicol) are recognized initially by 4-SMO in animals and fungi, whereas the first substrate bound productively by the plant 4-SMO is 24(28)-methylene-24,25-dihydrocycloartanol. Three genes encoding sterol C4 demethylation components in higher plants have been characterized, and the corresponding gene products exhibit distinct substrate specificities: SMO1p has an apparent substrate specificity for 4,4-dimethyl-9β-cyclopropyl sterols and SMO2p shows specificity for 4α-methyl-Δ^7-sterols. The 3βHSD/D enzyme system, used to carry out the second phase of C4-demethylation, recognizes either products of SMO1p or SMO2p catalysis. Homology modeling and site-directed mutagenesis of the 3βHSD/D from *A. thaliana* indicate that Ser^{133} is in close proximity to the C4 domain of the bound sterol. The proposed reaction mechanism for this catalyst involves the Tyr^{159}, Lys^{163}, and Arg^{326} residues that contribute to the decarboxylation process of C4α-methyl 3-keto sterol formation from a C4β-methyl C4α-carboxy intermediate.[170]

5.4. Removal of Nuclear Methyl Group at C14

Sterol C32 removal from C14 is catalyzed by the oxidative action of sterol 14α-methyl demethylase (14-SDM), a member of the cytochome P450 superfamily (P450-DM, CYP51, Erg11p) and a drug target for many diseases caused by fungi and protozoa.[171,172] There is also evidence in support of the P450-obtusifoliol-14α-demethylase to be a herbicidal target.[173] Lanosterol 14-demethylase (also CYP51) is a class II enzyme requiring the FAD−FMN-containing NADPH−cyctochrome P450 reductase as redox partner.[174] This remarkable sterol metabolizing enzyme, in contrast to the sterol C4-demethylase complex, engenders three distinct oxidation activities and a fourth activity to promote the difficult removal of the elements of formic acid. As of 2007, the CYP51 family has been shown to distribute in 82 organisms from all biological kingdoms. Humans contain only one CYP51 gene, whereas in some plants and fungi, multiple CYP51 genes are detected.[172] The human CYP51 encodes for a protein of 503 amino acids that possesses a molecular weight of 56 806 Da. These catalysts have been identified in several prokaryotes, not necessarily with an intact

cholesterol pathway, suggesting that it represents an ancient metabolic activity.[175] The average amino acid sequence identity in the CYP51 family is about 30% across biological Kingdoms with evolutionarily close species, such as mammals, showing 95% identity.

The Erg11p catalyzes the removal of the 14α-methyl group from the sterol ring via three NADPH−O$_2$-dependent steps organized by a single cytochrome P450. The substrate specificity for sterol differs markedly among CYP51 enzymes: the optimal substrate for enzymes of yeast and animals is lanosterol; for filamentous fungi, it is 24(28)-methylene-24,25-dihydrolanosterol; for higher plants, it is 31-nor 24(28)-methylene lanosterol (obtusifoliol); and for protozoa, it is 31-norlanosterol.[172] These findings indicate that depending on the source of enzyme, the proximal end bearing the polar group of the sterol is critical in substrate recognition, and the C4β (axial)-methyl group is a disfavored structural feature in Δ^8-sterol binding to the plant and protozoan CYP51.[172,176] The importance of the nuclear double bond position in the C14-demethylation reaction has been examined in cell-free fungal and bacterial preparations. The naturally occurring Δ$^{8(9)}$- and Δ$^{7(8)}$-bonds are recognized equally well by CYP51s. However, neither 9β,19-cyclopropyl sterols, or 6-lanostene-3β-32-diol or lanost-3β-32-diol are bound productively by these enzymes, suggesting that the 8-double bond of sterols play an important role in the enzyme−substrate interactions.[177,178] The Erg11p from *Candida albicans* has been purified, and the turnover number of the heterologously expressed protein is 2.5 nmol of Erg11p/mg of protein. Similar slow turnover numbers have been reported for other cloned and wild-type purified 14-SDMs.[179,180]

The mechanism of sterol C14-demethylation utilizes cytochrome P450, oxygen, and reducing equivalents to perform regioselective and regiospecific oxygen insertion into the molecule followed by methyl removal and double bond formation. Formation of the 14α-carboxyaldehyde from a 14α-methyl Δ^8-sterol is followed by an acyl−carbon bond cleavage reaction that involves release of formic acid accompanied by the stereospecific loss of the 15α-hydrogen atom (H$_{2S}$ originated in MVA) with creation of the Δ$^{14(15)}$-bond.[5] Thus, in proceeding from lanosterol where the 15α-hydrogen at C15 is lost to the Δ^{8,14}-sterol, the 15β hydrogen is retained and epimerized. The reaction sequence can be interrupted by inhibitors that are substrate-based sterol analogs that contain a functional group at C32 (usually nitrogen) or nonsteroidal compounds commonly known as azole drugs, such as fluconazole (a triazole), and other promising scaffolds of nonazole structures typified by VNI.[181,182] The N-4 (or N-3 of imidazole) of the azole can bind to the heme of the enzyme as a sixth ligand with the N-1 substituent group binding to the apoprotein.[183] Indeed, the X-ray structures of 14-SDM crystallized from human and human parasite *Trypanosma brucei* show tight binding of these drugs in the active site by coordination to the heme iron.[184,185] The crystallographic work also shows the subunit organization of these enzymes to be monomers. The mode of binding of sterol substrate and azole antifungal agent with fungal (*Cryptococus neoformans*) or bacterial (*Mycobacterium tuberculosis*) CYP51 was identified by flexible molecular docking and through site-direct mutagenesis experiments.[181,186,187] Important residues that may form contacts with sterol C3-hydroxyl group are the carbonyl oxygen of Met^{433} or the hydroxyl groups of Ser^{388} or Thr^{315}. Other critical residues of phylogenetic significance have been identified by substitution of animal/fungi-like Ile^{105} in the B′ helix with phenylalanine. The Ile^{105}Phe mutant

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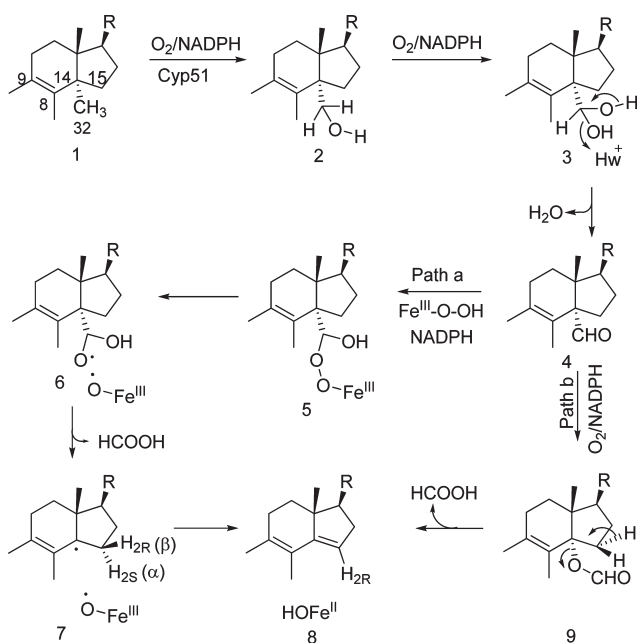dx.doi.org/10.1021/cr200021m |*Chem. Rev.* 2011, 111, 6423−6451

Figure 19. Possible pathways for oxidative removal of the 14α-methyl group in sterol biosynthesis (R = sterol side chain; Hw$^+$ is proton from water) according to Akhtar, Galylor, and cowokers.[193–197] The reaction is carried out by 14α-sterol demethylase (14-SDM, CYP51) and proceeds by oxidation of the 14α-methyl group to alcohol **2** and thence to the C32-aldehyde **4**. During cholesterol biosynthesis in rat liver the loss of C32 as formaldehyde is accompanied by loss of the 15α-hydrogen to generate a Δ^{8,14}-sterol **8**. Akhtar et al.[190] proposed two possible concepts for the elimination of the 32-aldehyde shown in paths a and b, with path b considered to act under physiological conditions.

protein dramatically alters substrate preference of *T. cruzi* CYP51, converting it into a more plant-like enzyme.[181]

The reaction pathway for the coupled removal of the C32-group and C15α-hydrogen atom has been a matter of controversy.[1,2,5,188–190] Enigmatic is whether an 8(14)-sterol is an obligatory intermediate in the 14-demethylation process and the nature of the C—C bond cleavage reaction that results in the formation of the 8,14-diene system. For various reasons, the 8(14)-pathway has been deemed either physiologically not significant or a primitive pathway that has been supplanted by the Δ^{8,14}-diene pathway in advanced organisms.[2,191] The recent demonstration of the *Saccharomyces cerevisiae* GL7 sterol auxotroph to convert cycloartenol to 24-methylene 30,31-dinor cycloartenol (24-methylene pollinastanol) and to the 24(28)-methylene cycloart-8(14)-dienol product via a presumptive Δ$^{8(14)}$-cyclopropyl sterol intermediate suggests that the Δ$^{8(9)}$-bond in the starting substrate is not obligatory for sterol 32-demethylation in the yeast system.[192] For the elimination of the 32-aldehyde group, three possible cleavage routes, two of which proceed through a peroxyhemiacetal intermediate by an ionic or radical process, have been considered: (i) The first involves 15α-hydroxylation followed by nucleophile-facilitated elimination, which has been discounted by Fisher et al., who have shown that synthetic 3β,15α-dihydroxylanost-8-en-32-al is not metabolized to 4,4-dimethyl cholesta-8,14-dien-3β,ol under conditions that promote demethylation of lanost-8-en-3,32-diol and 3β-hydroxylanost-8-en-al.[193] (ii) The second involves a reaction analogous to a Baeyer—Villiger oxidation at C14 to produce a formate ester, which then, through a *syn*-elimination reaction,
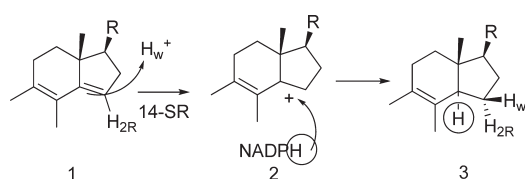

Figure 20. Chemical steps in the reaction of sterol 14(15)-reductase (14-SR) in complex with NADPH to form a Δ^8-sterol. Reduction of the Δ^{14}-bond introduces the hydride from NADPH into the 14α-position and a proton from water (Hw$^+$) into the 15β-position of **3**. This results in an inversion of the 15β-H derived from the original 14α-methyl sterol precursor (Figure 19) into the 15α-position of the product Δ^8-sterol.
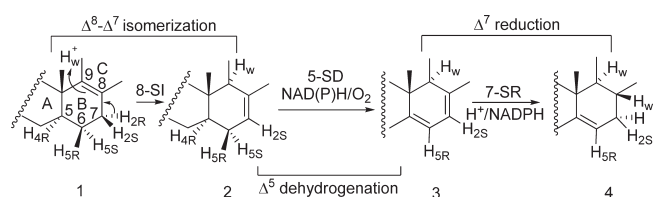

Figure 21. Enzymatic processing of sterol 8(9)-bond to the 5-position in rings A and B of the sterol nucleus. The sequence considered is based on the catalytic competence of the sterol C8-to-C7 isomerase (8-SI), sterol-5-desaturase (5-SD), and sterol C7-reductase (7-SR) enzymes tested from animal, plant, and fungi. The stereospecificities of the hydrogen losses and additions in the reactions shown have been elucidated in rat liver and a few other organisms with regard to both the hydrogen of the sterol substrate and the NADPH coenzyme. Some variations not shown in the mechanism may exist. See text.

yields the final Δ^{8,14}-sterol.[194,195] This pathway has been dismissed based on circumstantial evidence using structure—activity and modeling data of related CYP50 enzymes (e.g., sterol aromatase) that lend support to a direct decomposition β-scission mechanism.[196,197] The preponderance of evidence supports the sterol C14-oxidation—decarbonylation path a, shown in Figure 19, in which an intermediate alkoxy radical **6** converts to Δ^{8,14}-diene product **8**.

5.5. Shift of Δ^8 to Δ^5-Position

The formation of the final sterol in the pathway is usually taken to be generation of the Δ^5-bond. The typical pathway following the formation of the Δ^{8,14}-sterol by C32 elimination proceeds as Δ^{8,14} → Δ^8 → Δ^7 → Δ^{5,7} → Δ^5. However, the well-described presence of sterols with an unconjugated Δ$^{5,8(9)}$-diene system in fungi and algae and in humans with certain genetic disorders suggests an alternative route of Δ^{8,14} → Δ^8 → Δ^{5,8} → Δ^{5,7} → Δ^5,[2,12,72] but not one of Δ^{8,14} (with or without the 14αCH$_3$ group) → Δ$^{8(14)}$ → Δ^7 → Δ^{5,7} → Δ^5. The enzymatic reactions will be discussed in order of their appearance in the sterol biosynthesis pathway starting with the sterol Δ$^{14(15)}$-reductase (14-SR). The reduction of the 14(15)-double bond is catalyzed by a microsomal 14-SR with NADPH as coenzyme in accordance with the outline described for sterol C24-reduction.[190] The mechanism is triggered by an electrophilic attack by a proton derived from an active site residue of the 14-SR on the sterol substrate that results in the C14 intermediate cation shown in Figure 20. The suggested formation of a high-energy intermediate was confirmed by incubation with 15-azasterols, one of which is a natural product, 15-aza-24-methylene-D-homocholesta-8,14-dien-3β-ol.[198,199] The proton

is added to C15 at the β-position, and the hydride ion from the coenzyme becomes the 14α-hydrogen. The only reported purification of the enzyme comes from the work of Paik and Gaylor who found it possessed a turnover number of 40 nmol/(min·mg) for 4,4-dimethyl cholesta-7,14-dienol-3β-ol and was strongly inhibited by AY-9944.[200] Cloning of the 14-SR gene has been accomplished in yeast and other systems.[201,202] Analysis of the *ERG*24 gene indicated a 1316-bp open reading frame encoding a 438 amino acid protein of a predicted molecular mass of 50 612; similar values have been reported for other cloned 14-SR proteins.[201,202] In addition to its role in sterol catalysis, the 14-SR appears to function as a human lamin B receptor.[203]

Isomerization of sterol Δ^8 bond to the Δ^7 position carried out by sterol 8-to-7 isomerase (8-SI) is triggered by an α-protonation of the Δ^8 double bond from an active site general acid residue, giving a C8 carbocation that is quenched by the elimination of an appropriate hydrogen atom at C7 by a general base residue (Figure 21). Studies of MVA labeled with tritium at C2, C4, and C5 show that loss of 7β-H occurs in animals and plants (H$_{2R}$) whereas in fungi the enzyme proceeds with elimination of the 7α-hydrogen (H$_{2S}$).[23,204,205] Consistent with the involvement of a high-energy intermediate in the conversion of Δ^8 to Δ^7 sterol, various nitrogen-containing drugs that target breast cancer (tamoxifen) or ischemia (emopamil) or act as antifungals (tridemorph) inhibit the sterol isomerization reaction *in vitro* exhibiting dissociation constants in the low nanomolar range.[206−208] By examining a cationic azadecalin and the corresponding neutral amide analog of the intermediate formed in the 8-SI reaction, Rahier and co-workers demonstrated that the two inhibitors yielded IC$_{50}$ values of 0.5 μM and more than 100 μM, respectively, which confirmed that the latter set of drugs function with a charged ammonium group.[209] The 8-SI generates a single product, although a small percent of reversibility of the $\Delta^8−\Delta^7$ cholesterol isomerase reaction in cholesterol biosynthesis has been demonstrated; at equilibrium, the Δ^7-isomer is predominant (19/1).[210] Kinetic analyses of the cloned human enzyme revealed that the free energy ($\Delta G^{o\prime}$) of the forward isomerization reaction was calculated to be −6.5 kJ/mol.[208] In contrast, bimimetic studies of sterol Δ^8-isomeration reactions that show acid-promoted isomerization of the nuclear double bond in zymosterol yields a combination of Δ^7 and $\Delta^{8(14)}$-products. The purified enzyme from rat and human failed to convert a 8(14)-sterol to Δ^7- or Δ^8-sterol.[208,210] Treatment of *Chlorella sorokinia* with tritemporph results in the accumulation of ergost-8(14)-enol and 9,19-cyclopropyl sterols and loss of cellular ergosterol.[211] In structure−metabolism studies using cultures of sterol auxotrophic yeast GL7, under conditions that exogenous $\Delta^{8(9)}$-sterols convert to $\Delta^{5,7}$-sterols, 8(14)-sterol was not further transformed in the ring system.[192] These results are consistent with the proposal that $\Delta^{8(14)}$-sterols are neither formed nor metabolized by the same microsomal enzymes that catalyze transformation of lanosterol to cholesterol.[210]

Detailed studies of the substrate specificity of the human 8-SI indicated that the presence of C4 and C14 methyl groups, typical of lanosterol, were harmful to activity; the optimal substrate is one that possesses a 3β-hydroxyl group, a $\Delta^{8(9)}$-double bond, and an intact C8-side chain with a remote 24(25)-double bond.[208] However, humans, fungi, and plant 8-SI differ in their substrate specificity for cholesta-8,24(25)-enol, cholesta-8,24(28)-enol (fecosterol), and stigmast-8-enol, respectively.[207−212] The 8-SI enzyme has been purified from

rat and human. Properties of cloned human 8-SI are K_m of 50 μM, turnover of $k_{cat} = 0.423$ s^{-1}, apparent molecular mass of 26.7 kDa, and tetrameric subunit organization. The 8-SI has been cloned from several sources, the first one from yeast given the *ERG*2 designation. The predicted molecular weight for animal, plant, and fungal proteins vary by only 2 kDa, whereas homology in their primary sequences is low, less than 85% identity and 50% similarity across kingdoms with plant and animal sharing a closer relationship in sequence identity to each other than either group to fungi. Mutagenesis experiments of the human and plant (*Zea mays*) 8-SI have revealed that conserved acidic and aromatic residues are involved in catalysis. Moebius et al. reported that several amino acids at His[77], Glu[81], Thr[126], Asn[194], and Trp[197] of human 8-SI are required for the isomerization reaction.[206] Rahier et al, studying the *Zea mays* 8-SI, came to similar conclusions for equivalently spaced residues in the plant enzyme and established further that trytophan residues at positions 66 and 193 play crucial roles, perhaps in cation−π interactions during catalysis, and that Thr[124] may be involved in hydrogen bonding interactions with the sterol C3-hydroxyl group. It would appear that a core of critical residues exist in the plant and animal enzyme that are similarly important to binding and catalysis, whereas they are either not essential or not conserved in the yeast 8-SI. For this reason, the high sequence relatedness of human and plant 8-SI compared with their catalytic competence may explain their divergence with the yeast 8-SI that recognizes different substrates and stereochemistry of their mechanisms.[209]

Insertion of the Δ^5-bond into a Δ^7-sterol substrate has been shown to involve molecular oxygen and cytochrome b5 as the carrier of electrons from NADPH via NADH-cytochrome b5 reductase and to proceed stereospecifically in the removal of the 5α (4-Pro*R* in MVA) and 6α hydrogen (5-Pro*S* in MVA) atoms from the Δ^7-sterol during sterol biosynthesis in animals, plants, and fungi (Figure 17).[23,213,214] The enzyme sterol 5-desaturase (5-SD, E.C. 1.14.21.6) has substrate specificity that differs in animals, fungi, and plants for cholest-7-enol, ergosta-7,22-dienol, and campest-7-enol or stigmast-7-enol, respectively.[214−217] The gene coding for 5-SD has been identified in animals, plants, fungi, and protozoa (*Tetrahymena thermophila*); the yeast gene is known as ERG3. These proteins differ slightly in size, yet their amino acid sequences show low relatedness across kingdoms (<35% identity). On the other hand, they resemble each other in having three conserved histidine-rich motifs and Thr[114] in the *A. thaliana* 5-SD was found to be important in substrate binding and catalysis.[218,219]

Mechanistically, the C5(6)-double bond could be introduced into the Δ^7-sterol by a hydroxylation−dehydration reaction.[2,220] However, recent work by Rahier using 6-aza-B-homosteroid as an inhibitor of the reaction failed to provide evidence for a discrete carbocation intermediate in the plant 5-SD reaction, and neither synthetic 5α-cholest-7-en-3β,6α-diol nor 5α-cholest-7-en-3β,6β-diol were converted to diene product in the standard microsomal preparation from wild-type *A. thaliana* 5-SD.[221] An alternative mechanism was considered involving a stepwise removal of the C6α-hydrogen by an iron-bound oxygen to generate a carbon-centered radical at C6, which furnishes the Δ^5-product by a disproportionation reaction.[221]

The 7-dehydrocholesterol reductase (7-SR, EC 1.3.1.21) is the terminal enzyme in the pathway of cholesterol biosynthesis;
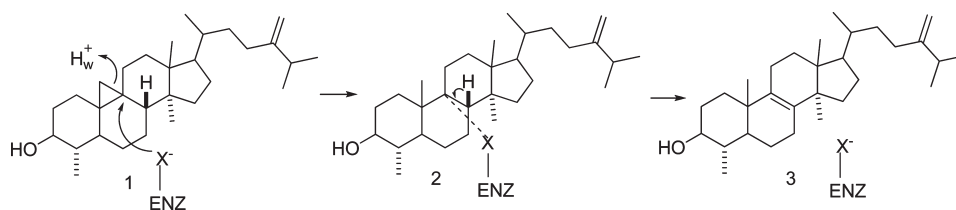
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Figure 22. Cleavage of the 9β,19-cyclopropane ring system to form a Δ^8-sterol by sterol 9β,19-cyclopropane mutase (19-SCM) according to Rahier and co-workers.[229] In order for the reaction to proceed in a 1,2-*trans* manner, it has been postulated that a negatively charged group (X$^-$) associated with the cycloeucalenol **1** to obtusifoliol **3** isomerase (COI; 19-SCM) may participate as shown.[12,67] The preferred substrate recognized by COI is a 4α-methyl 24(28)-methylene sterol, such as cycloeucalenol. The loss of a proton from C11 instead of 8β-H would produce a $\Delta^{9(11)}$-14α-methyl ring system, and this may also be a route to this type of sterol in plants and marine organisms.[12]

however in phytosterol synthesis additional reaction steps may come after the formation of the Δ^5-monoene ring, including Δ^{22}-desaturation. The 7-reduction of the 7(8)-double bond of steroidal dienes, investigated in animal and plants, entails the *trans*-addition of a proton added from the medium in the 8β-position and the hydride ion from NADPH being added to the 7α-position.[59] A microsomal preparation from seedlings of *Zea mays* catalyzed the NADPH-dependent reduction of the Δ^7 bond to $\Delta^{5,7}$-cholestadienol.[222] Evidence for the intermediacy of a 7(8)-cation occurring during the reaction was provided by testing 6-aza-B-homosteroids in the maize microsomal assay system, which strongly inhibited the conversion of a $\Delta^{5,7}$-sterol to Δ^5-sterol.[223] A deficiency of this enzyme activity due to genetic mutation in humans has been shown to cause Smith−Lemli−Opitz syndrome (SLOS).[72,224] The gene encoding Δ^7-reductase has been cloned and characterized from the plant *Arabidopsis thaliana* and fungus *Mortierella alpina*.[225,226] The predicted molecular weight of the *M. alpina* 7-SR is 53 965, similar to other 7-SRs. Functional analysis in a yeast expression system revealed that the recombinant 7-SR has broad substrate specificity for steroidal $\Delta^{5,7}$-dienes. The fungal 7-SR exhibits 39−51% homology with other 7-SR proteins at the amino acid level and shows two typical sterol family signatures.

5.6. 9β,19-Cyclopropane Ring Opening

A key enzyme in the biosynthesis of phytosterols, absent from organisms of a nonphotosynthetic lineage, is the sterol 9β,19-cyclopropane mutase (S-CM), which is often referred to as the cycloeucalenol−obtusifoliol isomerase (COI, EC 5.5.1.9). This membrane-bound microsomal enzyme is responsible for the cleavage of the 9β,19-cyclopropane ring of cycloeucalenol yielding the Δ^8-sterol obtusifoliol.[227] Although to date the enzyme has not been purified, structure−activity tests of the microsomal enzymes from bramble (*Rubus fruticosis*) and corn (*Zea mays*) have provided much information regarding sterol specificity; the crucial domains of the sterol substrate for catalysis are the A- and B-rings.[227−229] The cycloeucalenol structure, which appears after formation of 24-alkyl sterols in phytosterol biosynthesis, lacks the C4β-methyl group attached to cycloartenol or 24(28)-methylene cycloartanol. It would appear that the configuration of the methyl group at C4 controls the reaction process since 4β-methyl-cycloeucalenol was not transformed by a cell-free preparation containing COI, and this selectivity may explain the absence of lanosterol and eburicol in plants generally.[228]

Mechanistic studies have shown the reaction proceeds through an acid-catalyzed mechanism, that is, protonation of

the C9−C19 bond by an acidic subsite of the active site situated over the C-ring of the substrate followed by a *cis* region-specific elimination of the 8β-H affording the Δ^8-double bond (Figure 22).[229] In D$_2$O, the COI adds a deuterium to C19 and abstracts hydrogen from C8, consistent the proposed regiospecificity of the reaction and involvement of a carbonium ion generated at C9 following proton attack on the nucleophilic 9,19-cyclopropane ring system.[230,231] N-substituted azadecalins, designed as high-energy intermediate analogs of the COI catalyzed reaction, have been shown to be potent inhibitors of this reaction.[232] The nonenzymatic acid-catalyzed isomerization of cycloartenol can proceed to a myriad of rearranged tetracyclic products via eliminations at C8, C11, and other sites, testifying to the conformational control imposed on the isomerization reaction by the isomerase.[233] The COI gene from *Arabidopsis thaliana* has been cloned and shown to encode a 36 kDa protein.[234] Notably, the COI cDNA compared with other plant sequences revealed that it was present in photosynthetic organisms. However, in no organism from prokaryotes or eukaryotes of a nonphotosynthetic lineage was significant identity to the plant gene evident.

5.7. C22 Desaturation

The introduction of the C22(23)-double bond occurs at the antepenultimate step in the ergosterol biosynthetic pathway and probably the last step of the plant sterol pathway.[2] It, like the CYP51, is sensitive to inhibition by azole drugs and, like the 24-SMT, has no counterpart in humans. This reaction has been shown to involve molecular oxygen and NADPH and is inhibited by CO and metyrapone, consistent with a cytochrome-P450-dependent species.[235] The substrate specificity of the sterol C22-desaturase (22-SD) has been studied using cell-free preparations of *Saccharomyces cerevisiae*, the sterol yeast mutant GL7 auxotrophic for sterol and microsomal preparations of cloned *Arabidopsis thaliana* and *Physcomitrella patens*.[236−239] Ergosta-5,7,24(28)-trienol is considered to be the natural substrate for the yeast 22-SD and is converted to ergosta-5,7,22,24(28)-tetraenol *in vitro*. Sterol structure−activity tests using yeast 22-DES are most detailed and show that the size and direction of the substituent at C24 affects the percentage of Δ^{22}-bond formation ranked as follows based on GL7 studies: 24β-C$_2$H$_5$ (65%) < 24β-CH$_3$ (50%) < 24α-C$_2$H$_5$ (10%) = 24α-CH$_3$ (5%) = 24H (7%). 24-Methyl desmosterol, the substrate for the 24-SR in phytosterol synthesis, was converted to the Δ^{22}-derivative in 15% yield, showing that the neighboring double bond at C24 is not a determent for activity.[237] In contrast, the higher plant 22-SD recognizes the 24α-ethyl and 24β-methyl group equally well and the 24α-methyl sterol is not a suitable substrate, whereas in
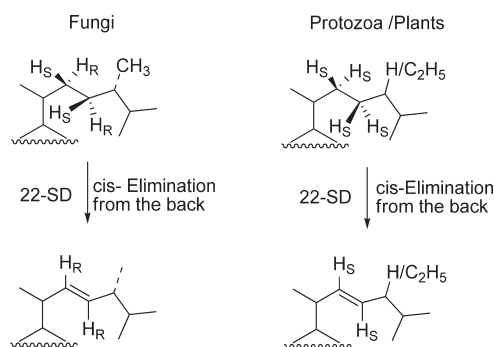
Figure 23. Two mechanisms for the *cis*-dehydrogenation reaction catalyzed by sterol C22-desaturase (22-SD), also known as CYP51A1 for the fungal enzyme and CYP107A for the plant enzyme, that leads to the introduction of the C22(23)-*E*-double bond in the sterol side chain; H_R and H_S refer to hydrogen atoms originally part of the MVA molecule.

the less-advanced 22-SD from moss (*P. patens*), 24α-ethyl sterol was a preferred substrate, but neither 24α- nor 24β-methyl sterols were acceptable substrates for the C22-desaturation reaction.[238,239] In studies of the protozoan *Tetrahymena pyriformis*, additional details regarding the substrate specificity for 22-SD were established, and rules of substrate recognition that govern the introduction of the Δ^{22}-bond were described.[240] The protozoan 22-SD is different from the plant and fungal 22-SD in that cholesterol is the optimal substrate for the *T. pyriformis* enzyme, and campesterol, compared with cholesterol, metabolism is about 50% as effective in conversion to campesta-5, 22-dienol.

The 22-SD has been purified from *Saccharomyces cerevisiae* (CYP51A1) and found to possess an apparent molecular weight of 58 000. The Michaelis constant for cloned 22-SD from plants (CYP107A) was for sitosterol, K_m of 1.0 μM and V_{max} of 0.0027 nmol/(nmol P450·min). The kinetic mechanism of cloned CYP61 from yeast has been investigated by Kelly and co-workers.[241] Catalytic competence for the conversion of ergosta-7,22-dienol to ergosta-5,7,22*E*-trienol yielded $K_m = 25 \mu$M and $V_{max} = 3.1$ nmol/(ergosterol formed·min·nmol CYP61). Kinetic studies of sterol and NADPH as cosubstrates revealed a random bi—bi mechanism with NADPH donating electrons directly to NADPH—cytochrome P450 oxidoreductase to produce a reduced intermediary form of the enzyme. The 22-SD has been cloned from fungi and plants, and the molecular mass for the *A. thaliana* mature protein was reported to be 56 344 D. The fungal gene is *ERG5*.[242] These sterol desaturase enzymes are characterized by general features common to most cytochrome P450-enzymes, have identity across plants and fungi (>43% identity) and possess a unique stretch, FLFASQ-DASS, which contains a highly conserved alanine residue considered to be a contact amino acid in the substrate binding recognition site.[238,239]

The mechanism of the 22-SD has been studied using MVA labeled with tritium at C2 and C5 and with synthetic substrates incubated with cell-free preparations of 22-SD.[240,243,244] These studies revealed that stereoselectivity of removal of hydrogen from C22 and C23 varies between fungi and plants and protozoa (Figure 23); in fungi two hydrogen atoms are removed from the α-face, whereas in plants and protozoa the two hydrogen atoms are removed from the β-face. In addition, a hydroxylation—dehydration mechanism for the *cis*-removal of

hydrogen atoms, considered based on metabolite profiling,[249] was discounted by Giner and Djerassi who incubated 23*R*- and 23*S*-hydroergostenols with the yeast 22-SD and found no evidence of metabolism.[236] The different sterospecificities and substrate preferences in the 22-SD catalyzed reaction among fungi and plants and protozoa appear influenced by specific amino acids whose positions and orientation are responsible for interacting with distinct side chain variants that determine the level of substrate discrimination that occurs with each enzyme.

6. CONCLUDING REMARKS

We have seen that the outline for similarity and differences in sterol biosynthesis across kingdoms is now available; these originate in the cloning and characterization of enzymes that form lanosterol and cycloartenol, elucidation of the pathways to cholesterol and 24-alkylation leading to ergosterol and sitosterol and the emergence of cloned enzymes that can be overexpressed affording an exacting analysis of native and mutant protein properties. Yet, the mechanistic details involved in the reaction course of many enzymes that catalyze sterols still need to be worked out. In all the years of pharmaceutical research, sterol biosynthesis has been a major source for new drug discoveries. These studies have contributed to our basic knowledge concerning regression of cholesterol biosynthesis in general. With the increase in fungal resistance and the opportunity to develop novel means for controlling sterol biosynthesis pathways that are absent from humans, the development of new and more potent antifungal and antiparasitic drugs has been one of the impetuses of recent biomedical research. Thus, the search for phyla-specific pathways that contain unconventional reaction sequences to 24-alkyl sterols are currently being pursued in a number of laboratories. In addition, with the recent determination of the X-ray structure of sterol enzymes and the finding that azole drugs used to treat diseases of ergosterol biosynthesizing organisms can bind differentially to human and trypanosome 14α-demethylase enzymes opens the door for a new era of rational drug design.[184] Understanding and treating genetic modifications in sterol biosynthesis that affect human health and researching genetically modified sterol pathways for improvement of specific traits or the addition of new traits to economically important plants is another major worldwide objective.

Finally, a word on sterol evolution. Bloch gave us the first insight into the role of oxygen and sterol features affecting sterol competency and biosynthesis, while Ourisson and Rohmer provided a blue print for a rational progression in time for the biosynthesis of terpenes to hopanoids to true sterols, which starts in prebiotic systems.[245,246] However, the recent demonstration of horizontal gene transfer complicates our view for connections to bacterial origins in sterol biosynthesis.[247] There is a correlation between the presence of the nervous system (animals) and the absence of certain biosynthetic steps, notably the absence of sterol C24-alkylation.[29] It is clear that convergent evolution in ergosterol biosynthesis exists since the ergosterol synthesized in *Prototheca wickerhamii* occurs by the MVA-independent pathway and the cycloartenol route utilizing the $\Delta^{25(25)}$-alkylation path to 24β-methyl group synthesis, whereas the ergosterol formed in fungi occurs by the classic acetate—mevalonate pathway and the lanosterol route utilizing the $\Delta^{24(28)}$-alkylation path to 24β-methyl group synthesis. Equally intriguing to consider are the prospects that sterol biosynthesis and kinetic control of it evolved

by a patchwork assembly with the individual members having undergone a duplication event followed by divergent evolution to give rise to the unique specificities. In this regard, we have recently shown that the SMT2 with a distinct product set and substrate specificity likely originates from SMT1 in phytosterol biosynthesis and that a correlation exists between the activation barrier (measured as E_a) to sterol C24-methylation and the product outcome; a higher energy route (and therefore more primitive) exists to form $\Delta^{25(27)}$-24β-methyl sterol compared with the formation of $\Delta^{24(28)}$-methylene sterols.[248,249]

Although many of the membrane-bound enzymes of sterol biosynthesis continue to be recalcitrant to crystallization and they occur in low abundance in tissues, with tools of protein chemistry, mechanistic enzymology, and structural biology, it is now possible for a renewed sustained attack on the of the individual steps in the pathway to address questions that remain: What about the protein structure that governs product diversity or recognizes one substrate better than another? Are there any allosteric modulators that can affect activity? What changes occurred in enzymes of the oxidosqualene—lanosterol and cycloartenol pathways and of 24-alkyl sterol biosynthesis that fashioned distinct stereospecificity of reactions that are of phylogenetic and functional significance.

There is still much to learn about sterol biosynthesis and the enzymes involved in the pathway. A determination of the structure and function of all the sterol catalysts is now within our grasp. The forthcoming decades should be an exciting time for basic bioorganic chemistry and biochemistry regarding sterol biosynthesis and production.

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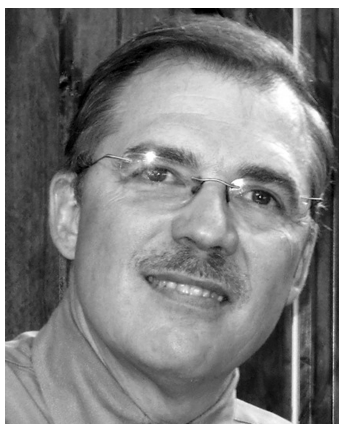
W. David Nes, born in Bethesda, Maryland, and educated at Gettysburg College, is the Paul Whitfield Horn Professor and Chair of Biochemistry Division at Texas Tech University. He received a M.S. at Drexel University in 1977 with his father, William R. Nes, with whom he published a series of research papers and a book on sterols, and a Ph.D. at the University of Maryland in 1979 where he studied plant biochemistry. In 1980, he began his career at the ARS-USDA Western Regional Research Center in Albany, CA, and after relocation to the Russell B. Research Center in Athens, GA, was promoted to Lead Scientist in 1988. He joined the Chemistry and Biochemistry faculty at Texas Tech University, Lubbock, Texas, in 1993. From 2003 to 2005, he was Visiting Scientist and Program Director at the National Science Foundation, Molecular and Cellular Biosciences Division, and Visiting Professor in 2008 at the Max Planck Institute for Chemical Ecology, Jena, Germany. His research broadly in natural products chemistry with a major focus on enzyme mechanisms, biosynthesis, and function of phytosterols has resulted in 165 publications and 9 books.

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