1.21 Sterol and Steroid Biosynthesis and Metabolism in Plants and Microorganisms

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1.21.1 Introduction

The diversity of sterol and steroid chemical structures across kingdoms and the broad outline of corresponding biosynthetic schemes have been depicted.[1] The structural elucidation and biosynthesis of cholesterol was recently summarized from a historical prospect.[2] Sterols are isoprenoid lipids essential to cell membrane structure and function, and to a further metabolism into steroidal hormones in eukaryotes. Recently, phytosterol derivatives used as food additives were shown to be efficient cholesterol-lowering agents.[3] Plant sterol biosynthesis and its peculiarities were described,[4–6] as well as its general terpenoid metabolic context.[7] This chapter reviews the current state of knowledge on sterol and steroid metabolism in bacteria, fungi, and plants. Particular focus is on

functional gene discovery for a comprehensive understanding of biosynthetic pathways of steroids and the function of the latter in physiology and development. The nomenclature of sterol, sterol conjugates, steroids, steroidal saponins and glycoalkaloids, and cardenolides is given elsewhere.[8] Analysis of sterols has also been comprehensively reported elsewhere.[9,10] This chapter describes sterol and steroid metabolism across diverse organisms for which progress in the field has been achieved in recent years owing to molecular genetic approaches. Aspects of animal steroid (hormones) metabolism and action are not included here.

1.21.2 Sterol and Steroid Biosynthetic Pathways

1.21.2.1 Squalene Metabolism

1.21.2.1.1 Squalene or 2,3-oxidosqualene cyclizations

Squalene (**1**) is the C_{30} precursor of steroids (**Figure 1**). It is produced in prokaryotes and eukaryotes by squalene synthases. These enzymes are prenyl transferases that catalyze catalyzing the head-to-head condensation of two farnesyl diphosphates to yield presqualene diphosphate as an intermediate of squalene.[11] Prokaryotes generally cyclize squalene into hopane triterpenes, although it is known that a few species, such as *M. capsulatus*, can produce steroids in addition to hopane triterpenes and bacteriohopanols.[12] *M. capsulatus* contains squalene hopane cyclase and squalene oxide lanosterol cyclase activities.[13] Genomic analysis of such methanotrophic bacterium indicates the presence of functional genes encoding squalene epoxidase (SQE), the product of which is (3*S*)-2,3-oxidosqualene (**2**) and (3*S*)-2,3-oxidosqualene cyclase (OSC), the product of which is lanosterol (**3**).[14] Genome mining identified the planctomycete *Gemmata obscuriglobus* as another bacterial species containing the biosynthetic sequence squalene to lanosterol, which requires an SQE and an OSC.[15] Although *M. capsulatus* or the tubercle bacillus *Mycobacterium tuberculosis* metabolizes lanosterol into 4α-methyl-$\Delta^{8(14)}$-sterols or cholesterol, *G. obscuriglobus* synthesizes lanosterol (**3**) and parkeol (**4**) as end products with no downstream modifications. Phylogenetic and biochemical data suggest that sterol biosynthetic pathways might have been exchanged through gene transfer between bacteria and early eukaryotes. *Stigmatella aurantiaca*, another species from the myxobacteria, produce cycloartenol (**5**) as a sterol precursor. The similarity of *S. aurantiaca* and eukaryotic cycloartenol synthase gene products may also indicate an evolutionary relationship between these organisms.[16] Coexistence of squalene and squalene oxide cyclization products is not restricted to prokaryotes. *Adiantum capillus-veneris* and *Dryopteris crassirhizoma* are two fern species from the polypodiale order from which squalene cyclases (SQCs) have been cloned and shown to possess 35–40% identity with prokaryotic SQC. Functional analysis in the heterologous host *Saccharomyces cerevisiae* demonstrated that the *D. crassirhizoma* encoded a dammaradiene synthase, converting squalene (**1**) into dammara-18(28),21-diene (**6**).[17] Genes encoding SQEs from higher plants have been isolated in *Arabidopsis thaliana*,[18] *Artemisia annua*,[19] *Panax*

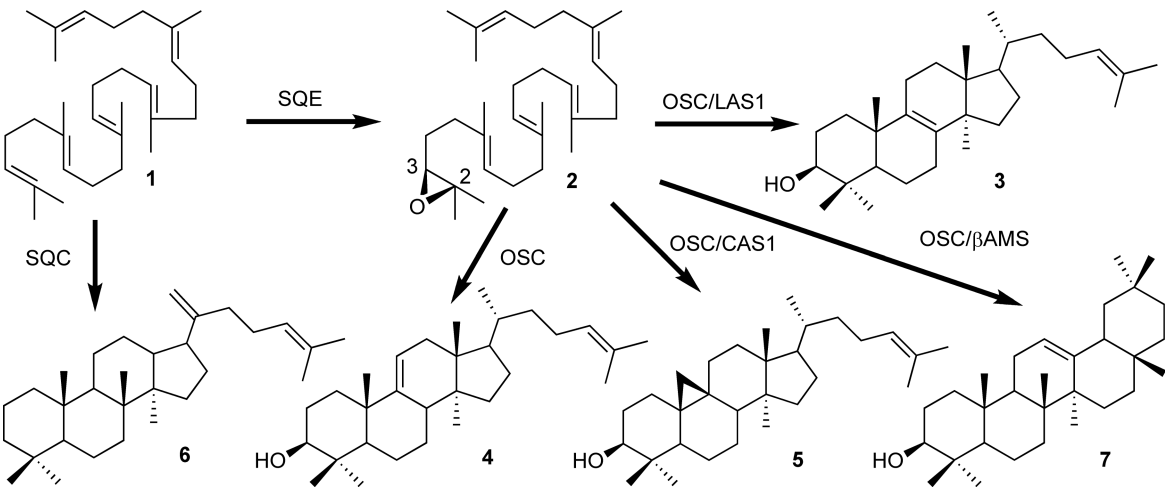

Figure 1 Squalene or 2,3-oxidosqualene cyclizations.

notoginseng,[20] and *Euphorbia tirucalli*.[21] *In situ* hybridization experiments with antisense probes of *E. tirucalli* pointed out a strong expression of *SQE* in parenchyma cells adjacent to laticifers. The Arabidopsis genome contains six genes encoding putative SQEs, among which three were shown to functionally complement a yeast mutant deficient in the endogenous SQE. Among the possibly redundant *SQE* genes of *A. thaliana*, *SQE1* was shown to be essential for triterpene and sterol biosynthesis because T-DNA (transfer DNA) insertional mutants of *SQE1* accumulated squalene. Consequently, root and seed development were impaired in these plants.[18] SQEs contain conserved flavin adenine dinucleotide (FAD)-binding domains. SQE enzymatic activity studied in yeast required molecular oxygen, nicotinamide adenine dinucleotide phosphate, reduced form (NADPH), and FAD.[22] The substrate-binding site of a mammalian SQE was identified by photoaffinity labeling.[23]

1.21.2.1.2 2,3-Oxidosqualene cyclization in cycloartenol or lanosterol in eukaryotes

Enzymatic transformation of (3*S*)-2,3-oxidosqualene (**2**) into cycloartenol (**5**), lanosterol (**3**), parkeol (**4**), or polycyclic triterpenoids such as β-amyrin (**7**) (**Figure 1**) involves carbocationic cyclizations and 1,2 rearrangements reviewed in Abe *et al.*[24] The diversity of the chemical structures produced from (3*S*)-2,3-oxidosqualene (**2**) is well illustrated when considering the 13 genes from *A. thaliana* encoding OSCs and the corresponding products formed upon expression of these genes in yeast.[25] The biodiversity in nonsteroidal triterpenes cyclization has been recently summarized.[26] Cyclization of 2,3-oxidosqualene into lanosterol (**3**) and cycloartenol (**5**) or into β-amyrin (**7**) were differentiated biochemically by the use of the inhibitor *N*-alkyl-4α,10-dimethyl-8-aza-*trans*-3β-ol, designed to mimic the high-energy carbocationic intermediates of the reaction.[27] A benzophenone photophore-containing nonterpenoid inhibitor, used as a photoaffinity label, mapped common binding sites of the mammalian lanosterol synthase and squalene hopene cyclase from *Alicyclobacillus acidocaldarius*.[28] *A. thaliana* cycloartenol synthase *CAS1* (2,3-oxidosqualene cycloartenol synthase) was isolated by metabolic interference in a yeast mutant lacking lanosterol synthase (strain *erg7*) and was transformed from being an auxotroph to being an ergosterol. A cDNA library cloned into a yeast expression plasmid vector was used to transform *erg7*. A chromatographic screen was then implemented to detect the expected formation of cycloartenol synthase in *erg7*.[29] Equivalent functional complementation strategies were developed to isolate the *ERG7* gene encoding the fungal lanosterol synthase.[30] In addition to the growing number of plant cycloartenol synthases and to the few bacterial ones, cycloartenol synthases have been characterized in the amoebas *Acantamoeba polyphaga* and *Dictyostelium discoidum*, and in the flagellate euglenoid *Astasia longa*.[31] Parasite protists from the kinetoplastideae, such as *Trypanosoma brucei*, use lanosterol synthase to produce their sterols.[32] An *A. thaliana* CAS1 mutant enzyme, bearing a valine residue at position 481 instead of an isoleucine residue, was able to produce lanosterol (**3**) and parkeol (**4**) in addition to cycloartenol (**5**).[33] Other amino acid residues important for the enzymatic formation of cycloartenol (**5**) or lanosterol (**3**) by the respective cyclases were disclosed in molecular evolution experiments.[34,35] The chemistry–biology interdisciplinary study, including site-directed mutagenesis and structural approaches of the yeast and mammalian lanosterol synthase, has been comprehensively described.[36] Position 481 of *A. thaliana CAS1* resides in the protosteryl cation-binding domain. Alignment of other OSCs with *CAS1* indicates that plant enzymes exhibit an isoleucine at position 481, whereas fungal and mammalian enzymes have a valine. *S. aurantiaca*, which uses a cycloartenol synthase, has an isoleucine at position 481, whereas *M. capsulatus* and *G. obscuriglobus*, which use lanosterol synthase, have a valine. This was crucial for the assignment of the function of an *A. thaliana* gene (At3g45130), whose deduced polypeptide sequence had 62% identity with CAS1. This protein, after being expressed in yeast, was shown to catalyze the synthesis of lanosterol (**3**),[37,38] as were the *Lotus japonicus* orthologs.[39] Although the presence of lanosterol (**3**) in certain plant species (e.g., from the genus *Euphorbia*) has been known for a long time, the fact that lanosterol synthases (LAS1) are apparently widely distributed in dicotyledonous plants raises the question of their physiological significance. In *A. thaliana*, the essential function of CAS1 has been demonstrated by a genetic approach. Complete loss-of-function of *CAS1* was lethal. Plants that were characterized by a weak allele, and therefore had a reduced transcription of the gene, accumulated 2,3-oxidosqualene (**2**). They were characterized by albino inflorescence shoots.[40] A conditional *CRE/loxP* (cyclization recombination locus of X-over P) recombination-dependent mutant allele also showed an albino phenotype at the seedling stage shortly after the *CRE/loxP*-induced onset of *CAS1* loss of function. In addition, these seedlings, which also accumulated 2,3-oxidosqualene, finally arrested their growth. It was concluded that there was no redundancy in *CAS1* for the synthesis of sterol precursors.[40] A dual biosynthetic pathway to phytosterols through cycloartenol (**5**) and lanosterol (**3**) was

investigated in *A. thaliana* seedlings overexpressing *LAS1* or deficient in *LAS1* expression using labeled mevalonate (6-$^{13}C^{2}H_3$), fed in the presence of an inhibitor of the enzyme 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR), which catalyzes the production of mevalonate. The synthesis of small amounts of lanosterol-derived sitosterol (**10**) was detected by nuclear magnetic resonance (NMR) of deuterium at carbon 19.[41] However, the fact that *CAS1*-deficient mutants are lethal indicates that *LAS1* cannot exert a compensation effect in plants on sterol biosynthesis. Lanosterol synthase might appear to be an evolutionary remnant, or it might have been recruited for a specific steroid pathway, for example, in secondary metabolism (as was proposed but not demonstrated).[41]

1.21.2.1.3 Metabolization of the cyclopropane ring of 9β,19-cyclopropyl sterols

Plants and some protists use an apparently complicated pathway to produce tetracyclic sterols via the pentacyclic triterpene cycloartenol (**5**), instead of the tetracyclic lanosterol (**3**). This implies the existence of a cyclopropane-opening enzyme or cyclopropyl isomerase (CPI), **Figure 2**). The enzymatic activity has been originally described in microsomal fractions of plant cell suspensions[42] and was later characterized thoroughly in subcellular fractions of maize. This enzyme catalyzes the isomerization of cycloeucalenol (**8**) into obtusifoliol (**9**), according to a carbocationic mechanism.[43] Obtusifoliol (**9**) (the substrate of CYP51, see Section 1.21.2.3.2)

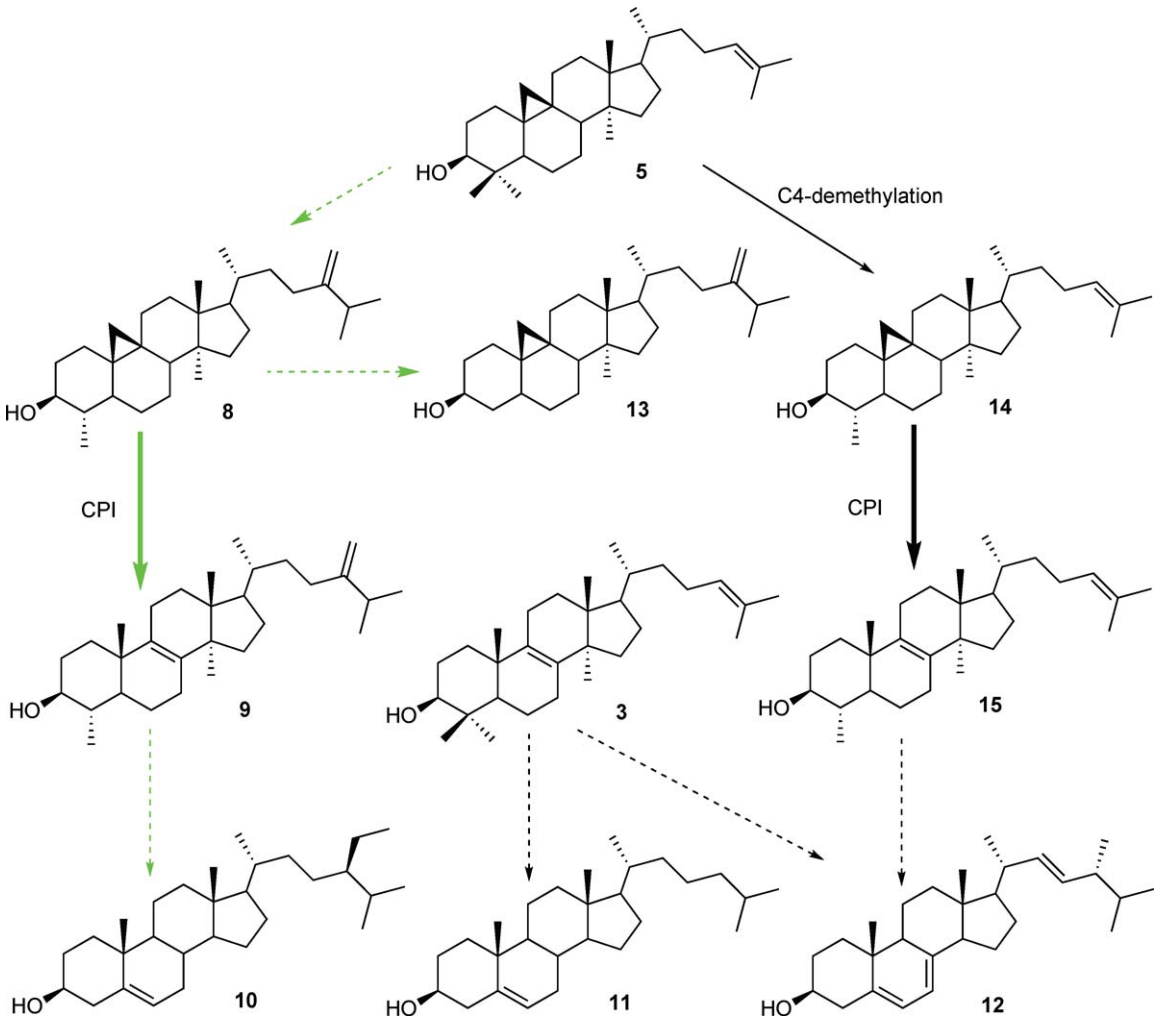

Figure 2 Metabolization of the 9β,19-cyclopropanic ring during the course of sterol biosynthesis in plants (green arrows) and yeast (black arrows).

then undergoes C14 demethylation. One can observe that downstream of obtusifoliol (**9**), the plant sterol biosynthetic pathway (dashed green arrow from obtusifoliol (**9**) to sitosterol (**10**) in **Figure 2**) is very similar to the vertebrate and fungi pathway (dashed black arrows to cholesterol (**11**) and to ergosterol (**12**), respectively, in **Figure 2**). Cycloeucalenol (**8**) or its C4-demethylated derivatives pollinastanol or 24-alkyl-pollinastanol (e.g., 24-methylene pollinastanol (**13**)) do not usually accumulate in cells except in the pollen.[44] However, cycloeucalenol (**8**) and other 9β,19-cyclopropyl sterols (**13**) may accumulate in plants[45] or protists[46] after the cyclopropyl sterol isomerase (CPI, formerly designated COI for cycloeucalenol–obtusifoliolisomerase) has been inhibited by *N*-alkyl-morpholine fungicides (tridemorph, fenpropimorph) and other C9 carbocationic transition state analogs. Chemical inhibition in growing seedlings of wheat[47] or in tobacco callus cultures isolated in a somatic genetic approach of sterol biosynthesis[48] resulted in an almost complete replacement of pathway end products by cycloeucalenol derivatives. In these experiments, cells or organisms have been viable with unusual sterols in their membranes. The significance of cycloartenol (**8**) and 9β,19-cyclopropylsterols as mandatory intermediates in sterol biosynthesis of some organisms has therefore been discussed in terms of surrogates of Δ^5-sterols. Molecular cloning of the *A. thaliana* CPI was achieved by metabolic interference in *S. cerevisiae*.[49] An ergosterol auxotroph *erg*7 strain, expressing the *A. thaliana* CAS1, and therefore accumulating 9β,19-cyclopropylsterols, was transformed with a cDNA library cloned into a yeast expression vector. A yeast transformant capable of ergosterol prototrophy was isolated. Prototrophy was most probably conferred by a protein able to open the cyclopropane of 31-nor cycloartenol (**14**) to yield 31-nor lanosterol (**15**).[8,50] This metabolite was a precursor for ergosterol biosynthesis in that yeast transformant.

Genetic inhibition of *CPI* in plants has been documented. An *A. thaliana* mutant carrying a transposable DNA element inserted into the *CPI* gene was characterized by a sterol profile fully consistent with those of plants treated with CPI inhibitors. This resulted in the accumulation of cycloeucaleneol and its derivatives, especially 24-alkyl pollinastanol.[51] Interestingly, this mutant had a severly hampered growth and development. The correct membrane sterol composition was shown to be essential for polar localization of auxin transporters.[51] This study pointed out a possible mechanism for sterol action on establishing asymmetric protein localization. From the overall *in vivo* studies, it is interesting to note apparent conflicting interpretations on the physiological significance of 9β,19-cyclopropane sterol intermediacy in plants. Genetic inhibition indeed is not compatible with the accomplishment of a complete life span,[51] whereas chemical somatic inhibition does not prevent cellular growth.[48]

1.21.2.2 Structural Diversity of Sterols: The C17 Side Chain

1.21.2.2.1 Sterol-C24-methyltransferases

Besides the lanosterol–cycloartenol bifurcation, alkylation of the sterol side chain is the other prominent peculiarity of sterol biosynthesis, which confers diversity in this pathway. Sterols from fungi and plants possess an alkyl group at C24 that sterol from vertebrate do not possess (**Figure 3**). A tremendous array of sterol structures with different side chains and the occurrence of such compounds have been described extensively.[1] Alkylation at C24 is performed by *S*-adenosyl-methionine-sterol-C-methyltransferases (SMTs), which generate 24-alkyl-sterols and *S*-adenosyl-homocysteine. These reactions have been initially studied in yeast and in the chlorophyte *Trebouxia* spp.[52] Methylation of zymosterol (**16**) into fecosterol (**17**) and methylation of cycloartenol (**5**) into 24-methylene cycloartanol (**18**) in these species were inhibited by azasterols.[53] In plant microsomal fractions, synthetic 25-azacycloartanol was described as a carbocationic transition state analog that inhibited cycloartenol C24-methyltransferase (SMT1) and 24-methylene lophenol C24^1-methyltransferase (SMT2).[54] *S. cerevisiae* contains a single SMT encoded by *ERG6*. This gene was described as nonessential because yeast strains containing *erg6* knockout alleles had a normal vegetative growth.[55] The essentiality or dispensability of ERG6 genes was challenged in a growth competition experiment. Isogenic strains differing by mutations in stereoidogenic genes were grown together with the wild type in order to test the possible competitive advantage of sterol biosynthetic mutants over ergosterol-producing strains. The conclusion was that the earlier the mutation in the biosynthetic scheme, the less able the strain was to compete with the wild type; therefore, no ergosterol biosynthetic gene/enzyme could be considered as nonessential.[56] A detailed analysis of the intracellular distribution of sterol biosynthetic enzymes was conducted with yeast. This analysis showed that SMT/ERG6 was localized almost exclusively

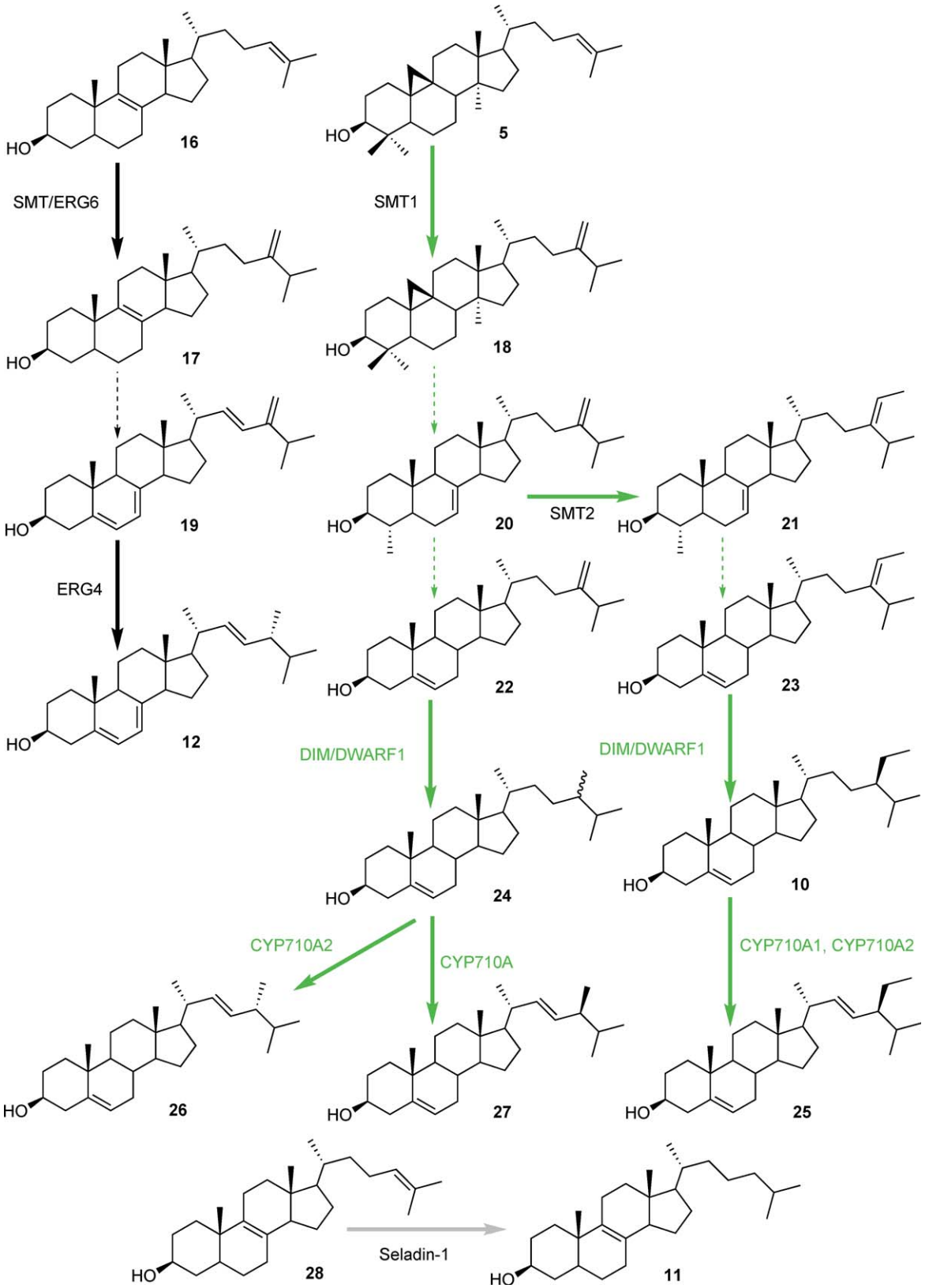

Figure 3 Sterol side-chain metabolism in yeast, plants, and vertebrates (black, green, and gray arrows).

in lipid particles and therefore was possibly implicated in sterol translocation.[57] Other sterol biosynthetic enzymes in yeast were shown to have a dual localization in the endoplasmic reticulum and in lipid particles.[58] Extensive chemical and enzymological studies have defined the mechanism and structural requirements for sterol side-chain alkylation by SMTs.[59] Sterol biosynthesis, and particularly the SMTs, have been considered as chemotherapeutic targets in trypanosomes and other kinetoplastid parasites.[60] In plants, *SMTs* have been studied mostly in *A. thaliana*, *Glycine max*, *Nicotiana tabacum*, and *Zea mays*. The capability of plants to produce alkylated sterols is due to the existence of two distinct and biosynthetically nonconsecutive sterol-C24-methyltransferases. Biosynthetic studies, including incorporation of radiolabeled precursors or treatment of cells with inhibitors, proposed that a first methylation reaction should be applied to cycloartenol to yield 24-methylene cycloartanol, then a second methylation reaction should be applied to 24-methylene lophenol to yield 24-ethylidene lophenol. A soybean gene *SMT1* was isolated by screening a cDNA library with antiplasma membrane serum. The corresponding protein expressed in *Escherichia coli* possessed lanosterol-C24-methyltransferase activity.[61] A homology-based approach led to the isolation of an *A. thaliana* SMT showing a 38% identity with the ERG6. Transformation of the wild-type and *erg6* mutants with this gene led to the synthesis of 24-ethyl sterols, indicating that this plant SMT could perform two sequential methylations in the sterol side chain.[62] Identification of two types of SMTs in plant genomes indicated that there were different biochemical functions or a (partial) redundancy of functions. The yeast mutant *erg6* provided the demonstration of the distinct reactions catalyzed by distinct SMT1 and SMT2. Transformed *erg6* strains were used to prepare delipidated microsomes to determine the substrate specificities of the SMT1 and SMT2 enzymes encoded by respective genes. Catalytic efficiencies measured in a study with *N. tabacum* enzymes indicated that SMT1 converted cycloartenol (**5**) into 24-methylene cycloartanol (**18**) but did not convert 24-methylene lophenol (**20**) into 24-ethylidene lophenol (**21**).[63] The case was slightly different with SMT2, which had a catalytic efficiency of 17 times higher with 24-methylene lophenol (**20**) (converted into 24-ethylidene lophenol (**21**)) than with cycloartenol (**5**).[63] This study concluded that the identity of SMT1 or SMT2 proteins with each other was close to 80%, whereas the identity of SMT1 and SMT2 was close to 40%. Conserved domains of the SMTs include a sterol-binding site and an *S*-adenosylmethionine-binding pocket.[64,65] In plants, SMT2 defines a branching point between the 24-methyl sterol and the 24-ethyl sterol biosynthetic segments. Transgenic approaches reported in *N. tabacum* indicated specific biochemical effects of *SMT1* or *SMT2* following up- or downregulated expression;[66] in particular, *SMT1* was shown to control the flux of carbon into sterol biosynthesis in tobacco seeds.[67] *SMT2* had a considerable impact on controlling the ratio of 24-methyl cholesterol to sitosterol.[68] A cholesterol-rich profile characterized an *A. thaliana* mutant impaired in one of its three genes (At5g13710) encoding SMTs. This *smt1* mutant was identified in a visual screen of a transposon-mutagenized population for root sensitivity to calcium.[69] A Series of allelic *smt1* mutants were characterized by slow overall growth, severely hampered embryogenesis, and altered root gravitropism.[70] In addition to an elevated cholesterol level, these *smt1* mutants had an almost-unchanged 24-methyl cholesterol but strongly reduced sitosterol levels. Such a chemical phenotype of mutants confirmed an overlap in the substrate specificity of SMT enzymes, *in vivo* particularly the fact that SMT2 can methylate cycloartenol, although with a low efficiency.[63] *A. thaliana*, overexpressing *SMT2* constitutively, accumulates more sitosterol than the wild type at the expense of campesterol and were smaller in size, most probably due to a reduced pool of campesterol at the entry of the brassinosteroid pathway (see Section 1.21.2.4.3).[71] Other transgenic lines showed cosuppression of *SMT2* and, therefore, contained four- to fivefold more 24-methyl cholesterol than the wild type at the expense of sitosterol. Pleiotropic effects on development such as reduced growth, increased branching, modified flower morphology, and low fertility were associated with modified sterol composition.[71] The *cvp1* (cotyledon vein patterning) insertional mutant of *SMT2*, displaying similar biochemical traits, was identified in a screen for novel cotyledon vascular patterns.[72] The allele *fril1* of *SMT2* had serrate petals and sepals due to ectopic endoreduplication in petal tips, suggesting a possible link between sterol composition and suppression of endoreduplication.[73] Cholesterol is naturally present in high proportions in species from the Solanaceae, which contain steroidal glycoalkaloids (see Section 1.21.2.5.2). Cholesterol has been suggested to be a precursor of solanidin in potato or tomatidin in tomato. Transgenic *Solanum tuberosum* plants expressing a *SMT1* from *G. max* had a decreased level of cholesterol associated with decreased levels of glycoalkaloids.[74]

1.21.2.2.2 Sterol Δ^{24}-isomerase/reductase

This biosynthetic step exists in all eukaryotes (**Figure 3**). Under normal conditions, *S. cerevisiae* converts ergosta-5,7,22,24(24^1)-tetraen-3β-ol (**19**) into ergosterol. The reductase encoded by the gene *ERG4* is located in the endoplasmic reticulum.[75] This enzyme in yeast was inhibited by azasteroids.[76] Besides biosynthetic aspects, ERG4 appeared to play a role in cell polarity, apical bud growth, cell wall assembly, mating, and invasive growth due to its interaction with p21-activated kinase Ste20.[77] Identification of the plant enzyme originated from a genetic approach using *A. thaliana*. This is no doubt a good example of serendipity in plant metabolic biology. The *diminuto* mutant was isolated among T-DNA transformed lines for its poor growth due to a defect in regulating cell elongation at the level of tubulin gene expression.[78] Molecular characterization of the mutated locus showed that *DIM* encoded a sterol Δ^{24}-isomerase/reductase due to the accumulation of 24-methylene cholesterol (**22**) and isofucosterol (**23**) in plants.[79] Expression of GFP fusion protein indicated an endoplasmic reticulum localized sterol Δ^{24}-isomerase/reductase. The allele *dwarf1* of a series of dwarf mutants was, in fact, the first *A. thaliana* mutant generated by T-DNA insertional mutagenesis, displaying a morphological phenotype (dwarfism) inherited as a single recessive nuclear mutation, which cosegregated with the associated marker gene (kanamycin antibiotic resistance) and the T-DNA insert.[80] In this *dwarf1* mutant, the lack of campesterol resulted in a reduced amount of bioactive brassinosteroids (see Section 1.21.2.4.3), causing dwarfism and altered development.[81] The plant sterol Δ^{24}-isomerase/reductase contains a flavin adenine dinucleotide (FAD)-binding domain indicative of a flavoenzyme. It also contains a Ca^{2+}/calmodulin-binding domain, which is essential for its function.[82] The sequence of reactions leading from $\Delta^{24(241)}$ to a C24(24^1) saturated bond through $\Delta^{24(25)}$ catalyzed by such a multifunctional enzyme was reported earlier.[83] The human desmosterol (**28**) Δ^{24}-isomerase/reductase was isolated in a mRNA differential display experiment designed to compare different brain regions in the context of neurodegeneration associated with Alzheimer's disease and was named seladin-1 for *sel*ective *A*lzheimer *d*isease *in*dicator-1 (Seladin).[84,85] This enzyme is an ortholog of the plant DIM/DWARF1, which is not the case of the yeast ERG4. Seladin-1 was also implicated in adrenocortical tumorigenesis[86] and is highly expressed in melanoma cell lines derived from cutaneous metastases.[87]

1.21.2.2.3 Sterol-22-desaturase

Cytochrome P-450 oxygenases are responsible for sterol-C22(23) desaturation in fungi[88] and plants.[8] The microsomal enzyme in yeast was purified, and its activity was reconstituted in an assay, including an animal NADPH-P-450 reductase.[89] The gene *ERG5* encoding this enzyme was cloned by functional complementation of a yeast mutant using negative selection for nystatin-sensitive transformants, which indicated the presence of ergosterol.[90] A dual biochemical function of *ERG5*/*CYP61* was considered because it showed xenobiotic metabolism, particularly with benzopyrene in yeast genotoxicity assays.[91] The *ERG5* orthologs of yeast-like symbiots, which synthesize sterols used by rice planthopper hosts (otherwise called sterol auxotrophs), had a different exon–intron organization compared to that of the *S. cerevisiae* gene.[92] The ciliated protozoan *Tetrahymena thermophila* was able to transform exogenous cholesterol into $\Delta^{7,22}$ derivatives in a cell-free assay, which required molecular oxygen, cytochrome b_5, and reduced cofactors (NADH or NADPH), pointing out an implication of cytochrome b_5 reductase in the (non-P-450 dependent) desaturation reaction.[93] *A. thaliana* has four genes encoding sterol-22-desaturases. These genes form the family *CYP710A* of cytochrome P-450 oxygenases that were isolated based on the sequence analysis and partial identity with the fungal sterol-22-desaturase encoded by *ERG5*/*CYP61*. *A. thaliana* contains multiple sterol products with a C22(23) saturation (see **Figure 3**). CYP710A1 and CYP710A2, expressed as recombinant proteins in insect cells, converted sitosterol (**10**) into stigmasterol (**25**) and converted the epimer of 24-methyl cholesterol (24) 24-*epi*-campesterol into brassicasterol (**26**).[94] Recruitment of a specific CYP710A isoform for the conversion of campesterol (**24B**) into crinosterol (**27**) was not detailed. Transgenic plants overexpressing CYP710A1 had over 30-fold increased levels of stigmasterol[94], and transgenic plants overexpressing CYP710A4 also increased their levels of stigmasterol.[95] This was associated with the esterification of Δ^5-sterols.[95] The apparent dispensability (because of highly variable levels) of stigmasterol (**25**) in plants is not well understood. The moss *Physcomitrella patens* also has *CYP710A* ortholog. Disruption of that gene by homologous recombination did not affect the viability of protonema, chloronema, or caulonema,[96] as was the case for *A. thaliana* knocked out in one of the *CYP710A*, which developed identically to the wild type.[94]

1.21.2.3 Biosynthesis of Δ^5-Sterols: Metabolism of the Tetracyclic Skeleton

1.21.2.3.1 The C4-demethylation complex

Cycloartenol (5) and lanosterol (3) are triterpene precursors that undergo a succession of enzymatic transformations, ultimately leading to functional sterols (e.g., cholesterol (11), ergosterol (12), and 24-alkyl cholesterol (10, 24)). Triterpene–sterol conversion implies oxidative removal of two methyl groups at C4 and another methyl group at C14. The succession of these steps varies, depending on the organism being considered. In animal or fungal sterol biosynthetic pathways, the two methyl groups at C4 are removed sequentially by the same enzymatic complex (**Figure 4**).[97,9] This complex has been identified genetically via transcriptome analysis in *S. cerevisiae* and isolated in classical genetic approaches and in two-hybrid analysis experiments.

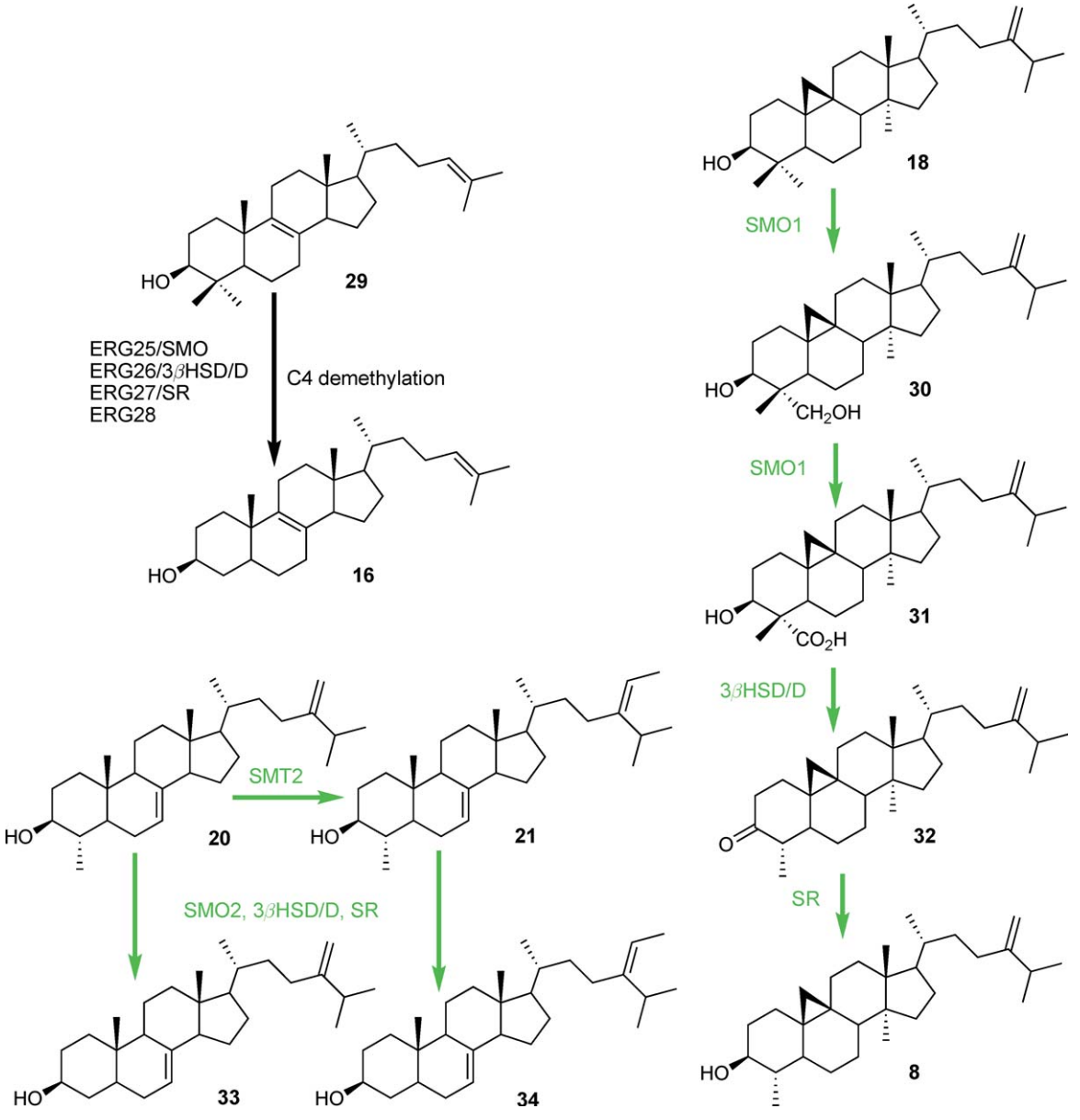

Figure 4 Sterol-C4-demethylation.

The enzymatic complex consists of ERG25, a sterol-4α-methyl-oxidase (SMO), isolated by functional complementation of the ergosterol auxotroph *erg25*, which accumulates 4,4-dimethyl-zymosterol (**29**).[98,99] The *erg25* was also complemented with the human ortholog of ERG25.[100] The ERG25-catalyzed reaction yields a 4α-hydroxymethyl sterol (**30**), then a 4α-carboxy-sterol intermediate (**31**),[101] is further used as a substrate by ERG26, a second component of the complex, which is a bifunctional 4α-carboxysterol-3β-hydroxysteroid dehydrogenase/C4-decarboxylase (3βHSD/D), belonging to the family of short-chain dehydrogenase/reductase (SDR)[102] whose product is a 3-oxosteroid (**32**). A third component of the C4-demethylation complex, ERG27, is a sterone reductase (SR) whose product is a 4-desmethyl sterol.[103] In addition to these enzymes, the C4-demethylation complex includes ERG28, a transmembrane protein[104,105] essential for the activity of the complex. A model multienzymatic membrane-bound sterol-C4-demethylation complex included cytochrome b_5 reductase and cytochrome b_5 in addition to ERG25, ERG26, ERG27, and ERG28.[9] Enzymological studies of ERG25 confirmed 4,4-dimethyl-zymosterol (**29**) as the preferred substrate of the microsomal enzyme[101] and were in full accordance with previous biochemical characterization of the reaction (in the animal and yeast system).[106,107] ERG25/SMOs are phylogenetically related to other membrane-bound nonheme iron hydroxylases widely distributed as like sterol-C5(6)-desaturases, cholesterol-25-hydroxylases, sphingolipid hydroxylases, and fatty acid desaturases.[9]

In plants, there are two distinct C4-demethylation reactions (**Figure 4**) implicated in two nonconsecutive reactions in the pathway, as is C24 alkylation of the C17 sterol side chain by SMTs (see Section 1.21.2.2.1). The first C4-demethylation applies to 24-methylene cycloartanol (**18**) which is transformed into cycloeucalenol (**8**). The second C4-demethylation applies to 24-methylene lophenol (**20**) and 24-ethylidene lophenol (**21**), which are transformed into episterol (**33**) and Δ7-avenasterol (**34**), respectively.[108] This peculiarity of the plant sterol pathway, compared to the yeast pathway, was demonstrated in *Z. mays* by the enzymology of microsomal sterol C4-methyl oxidases, 4α-carboxysterol-3β-hydroxysteroid/C4-decarboxylase (3βHSD/D), and NADPH-dependent-3-oxosteroid reductase (SR).[108–110] Virus-induced gene silencing (VIGS) in *Nicotiana bethamiana* proved that plants have *SMO1* and *SMO2* organized in small gene families, most probably implicating the redundancy of their biochemical functions.[111] Functional identification of plant genes coding for 3βHSD/D was done by via ERG26 homology-based searches for orthologs and expression in yeast.[112] Two *A. thaliana* cDNAs encoding 3βHDS/D restored ergosterol prototrophy in *erg26*. Finally, VIGS reduced the expression of *3β-HSD/D* in *N. benthamiana* and triggered the accumulation of 3β-hydroxy-4β-14-dimethyl-5α-ergosta-9β,19-cyclo-24(24^1)-en-4α-carboxylic acid (**31**).[112] The murine gene encoding NSDHL (sterol dehydrogenase) can also complement *erg26*.[113]

1.21.2.3.2 CYP51

In animals and fungi sterol biosynthesis, oxidative removal of the 14α-methyl group by the cytochrome P-450 oxidase CYP51 proceeds immediately after cyclization of 2,3-oxidosqualene into lanosterol (vertebrate and yeasts demethylate lanosterol (**3**), filamentous fungi demethylate 24-methylene lanosterol also called eburicol (**38**), **Figure 5**).[114] In plants, oxidative removal occurs at a later stage (obtusifoliol (**9**)), between the first and the second C4-demethylation reactions (**Figure 5**). CYP51 is the only known P-450 distributed in all organisms with conservation of function. CYP51s, including plant or animal pathogens, are target sites for azole inhibitors.[115] Site-directed mutagenesis, combined with genetic screens for azole-resistant mutants, indicated that azole and substrate binding had different structural requirements.[116] The pathogen *M. tuberculosis* has a soluble CYP51 ortholog that demethylates lanosterol and obtusifoliol.[117] This enzyme was expressed in *E. coli* then and crystallized in the presence of the antifungal agent fluconazole. Its structure at 2.2 Å (the first reported for a P-450 oxidase), and a mapping analysis of *Candida albicans* azole-resistant mutants, showed that drug resistance in pathogenic fungi mapped to protein regions required for catalysis rather than an azole-binding domain.[118] The proteobacterium *M. capsulatus* revealed in its genome a novel type of CYP51 bearing a ferrodoxin domain at the C-terminus and producing lanosterol-14-demethylase activity when expressed and purified from *E. coli*.[119] Trypanosomes (kinetoplastids) have CYP51s that display a strong substrate preference to obtusifoliol (**9**).[120] Plant CYP51 was purified and then cloned from *Sorghum bicolor*.[121,122] Such orthologs could complement *erg11*(*cyp51*) defective yeast mutants. Microsomal fractions from these yeasts permitted us to measure the binding constants of azole herbicides, ranging to a micromolar order of magnitude.[123] A somatic genetic approach in *N. tabacum*

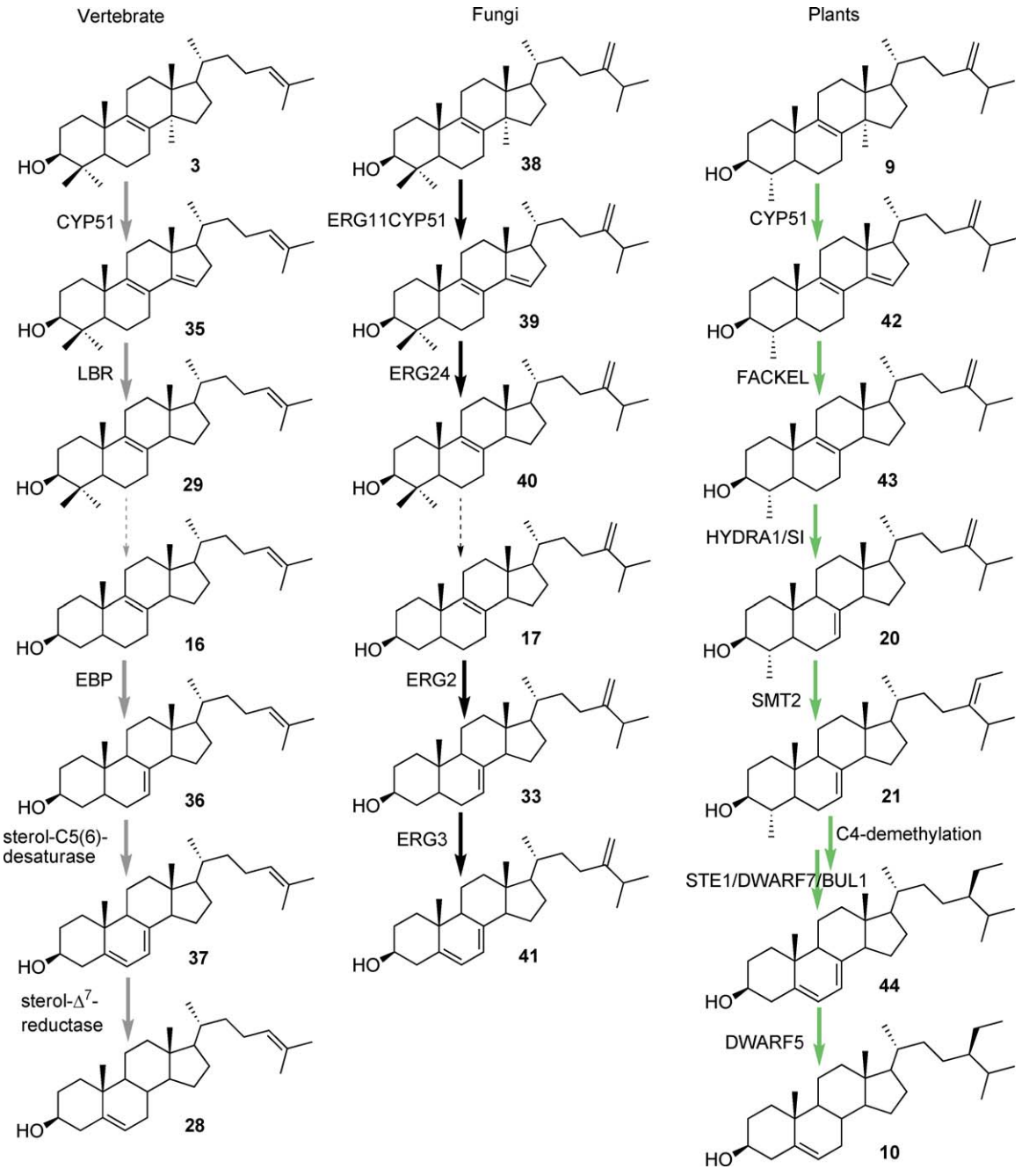

Figure 5 Comparative sterol biosynthesis in vertebrate, fungi, and plants.

led to the screening of biochemical sterol mutants presenting a marked azole-resistance phenotype.[124] Lethality characterized *A. thaliana* mutants carrying loss-of-function T-DNA alleles of CYP51, as were yeast strains deficient in ERG11/CYP51.[125] *CYP51* from *N. tabacum* mimicked the effect of inhibitors of obtusifoliol-14-demethylase in a VIGS approach, that is, accumulation of obtusifoliol (**9**), 24-dihydroobtusifoliol, and corresponding C4-demethylated and/or Δ^{24}-reduced metabolites (14α-methyl-fecosterol and 14α-methyl-24(24^1)-dihydrofecosterol).[126]

1.21.2.3.3 A common trunk of genes implicated in isomerization, desaturation, and reductions on the B and D rings

Comparative analysis of vertebrate, fungal, and plant sterol biosynthesis (**Figure 5**) led to the observation that the bioconversion of sterol intermediates possessing a cholesta-8,14-dien-3β-ol, an ergosta-8,14-dien-3β-ol, or a stigmasta-8,14-dien-3β-ol into Δ^5-sterols required enzymes that are functionally interchangeable across kingdoms in most, if not all, cases. The yeast gene *ERG24* encoding a $\Delta^{8,14}$-sterol-Δ^{14}-reductase was isolated by homologous functional complementation.[127] The *erg24* produced ignosterol (ergosta-8,14-dien-3β-ol instead of ergosterol), the C-4 demethylated substrate (**39**) of ERG24, and this did not affect viability.[128] However, in the case of *erg24* mutants of *C. albicans*, such a biochemical phenotype was shown to reduce the pathogenicity of an inoculum of fungal cells intravenously injected into mice.[129] This reinforced the potency of inhibitors of sterol-Δ^{14}-reductase as antifungals.[130,131] A 15-azasteroid (15-aza-24-methylene-D-homocholesta-8,14-dien-3β-ol), produced by the soil fungus *Geotrichum flavobrunneum*, was a strongly specific inhibitor of ERG24.[132] Enzymology and inhibition studies of the sterol-Δ^{14}-reductase, done with microsomal fractions of *Z. mays* coleoptiles, showed that this 15-azasteroid iminium analog behaved as a carbocationic transition state mimic, and this was also true for the *N*-alkyl morpholine fungicides.[133] Homology-based searches have led to the identification of a plethora of *ERG24* orthologs and of a human membrane lamin B receptor (LBR).[134,135] LBRs possess a sterol reductase domain and was therefore functional in a sterol-Δ^{14}-reductase complementation assay of *erg24*. The LBR from *Drosophila melanogaster*, a sterol auxotroph organism, did not encode a sterol-Δ^{14}-reductase function because it could not restore ergosterol prototrophy in *erg24*,[136]; therefore, it was most probably an evolutionary variant. The plant gene encoding a $\Delta^{8,14}$-sterol-Δ^{14}-reductase was isolated by positional cloning of a mutated allele called *fackel* in a genetic screening for *A. thaliana* that affected embryo development.[137] This ortholog had a 33% identity with ERG24 and also the typical sterol reductase and LBR motifs. It was able to rescue growth of *erg24* in the presence of high calcium concentrations, otherwise deleterious to these latter yeast cells.[138] *A. thaliana* embryo-defective *fackel* homozygotes cultivated on a synthetic medium contained elevated levels of $\Delta^{8,14}$-sterols, a chemotype in agreement with the mapped mutation.

Δ^8-Sterol intermediates are isomerized into Δ^7 isomers by a Δ^8-sterol-Δ^8-Δ^7-sterol isomerase (SI). In plants, Δ^8-sterols replace Δ^5-sterols in cells grown on the inhibitor AY9944.[7] The preferred substrate of the plant enzyme is 4α-methyl-5α-ergosta-8,24(24^1)-dien-3β-ol (**43**) (**Figure 5**), which accumulates upon treatment with AY9944, and is further metabolized into 4-desmethyl-Δ^8-sterol including 24-ethyl-Δ^8-sterols as alternative end products of the pathway. This is a good example of the plasticity of the sterol pathway, due to the relatively low specificity of enzymes localized downstream of the target site of an enzyme inhibitor (i.e., compound **43** may be demethylated at C4 by SMO2 and other enzymes of the C4-demethylation complex, methylated at C24 most probably by SMT2, and reduced at $\Delta^{24(241)}$ by DWARF1/DIM). This has been clearly explained in grids of alternative biosynthetic routes that parallel the main one.[7,8] The *S. cerevisiae ERG2* gene, encoding the Δ^8-sterol-Δ^8-Δ^7-sterol isomerase, was cloned by homologous functional complementation.[139] *ERG2* orthologs of plant pathogenic fungi such as *Magnaporthe grisea* (causing rice blast disease) and *Ustilago maydis* (causing corn smut disease) were also isolated.[140] ERG2 is indeed an important target for antifungal commercial compounds from the *N*-alkyl-morpholine group.[141] During the characterization of immunosuppressant compounds in the model *S. cerevisiae*, a genetic screening of UV-induced mutants that were resistant to an immunosuppressant molecule SR31747 (known to block the proliferation of lymphocytes) identified an allelic series of mutations in two *erg2* lethality suppressor genes, which conferred resistance to many structurally different sterol biosynthesis inhibitors. In the same study, overexpression in yeast of ERG2 conferred resistance to SR31747, indicating that Δ^8-sterol-Δ^8-Δ^7-sterol isomerase was the primary target of SR31747. This study discussed the relationship between immunosuppressants, sigma receptors, and Δ^8-sterol-Δ^8-Δ^7-sterol isomerase.[142] Mammalian and plant enzymes were also isolated by functional complementation of the yeast *erg2*.[143,144] In mice and humans, the ERG2 counterparts are bifunctional proteins. One function is Δ^8-sterol-Δ^8-Δ^7-sterol isomerization and the other is a pharmacological isomerization, that is, emopamil-binding proteins (EBP). Mouse and human orthologs have only 12% identity with the yeast ERG2 (at the peptidic sequence level), whereas plant and fungal isomerases share about 35% identity.[145] Sigma1 receptors in animals have a 30% identity with the yeast sterol-Δ^8-isomerase but do not possess the corresponding enzyme activity.[146,147] Yeasts expressing the plant isomerase in the *erg2* background had an ergosterol biosynthesis blocked by sigma ligands, for example, haloperidol and verapamil.[144] *Arabidopsis thaliana hydra* mutants are a

class of severe dwarfs that were identified in a genetic screen for defective embryogenesis and cell patterning in seedlings. Molecular analysis of the mutations showed that *HYDRA1* encoded the plant sterol-Δ^8-isomerase.[148] A deficiency of the sterol biosynthetic pathway at this nonredundant step led to a severe depletion of Δ^5-sterols and an accumulation of (yet nonelucidated) ergostenol and stigmastenol derivatives.[149] The physiological implications of *hydra1* (and *hydra2/fackel/sterol;-$\Delta^{8,14}$-reductase*) genetic defects in embryonic and postembryonic development were discussed in terms of sterol signaling. Most importantly, the modified sterol composition of *hydra* mutants affected auxin and ethylene signaling.[148] This showed that an appropriate sterol composition is essential for the activity of membrane-bound proteins and for membrane biology in general, as was previously indicated in the case of ATPases localized in the plasma membrane of plants.[150]

The Δ^7-sterol intermediates lathosterol (Δ^7-cholestenol) or $\Delta^{7,24}$-cholestadienol (**36**), episterol (**33**), and Δ^7-avenasterol (**34**) are the substrates of Δ^7-sterol-C5(6)-desaturases in animals, fungi, and plants (**Figure 5**). The gene *ERG3* was isolated by functional complementation of an *erg3* mutant.[151] The situation at the Δ^7 sterol intermediacy in plants was different from that occurring with Δ^8-sterol intermediates with respect to cell viability and development in autotrophic conditions. Phytochemical analyses have revealed that species from many families, including Cucurbitaceae and Chenopodiaceae, contain Δ^7-sterols as major sterols.[152,153] Nes and McKeen[1] listed plant species producing Δ^7-sterols as pathway end products. In a genetic approach of sterol biosynthesis, a chromatographic screen was applied to pooled individuals from populations of EMS (ethyl methane sulfonate)-mutagenized *A. thaliana* in order to isolate biochemical mutants. This led to the isolation of the *ste1* mutant, which accumulated about 70% of (24ξ)-24-methyl-5α-cholest-7-en-3-ol (Δ^7-campesterol) and (24*R*)-24-ethyl-5α-cholest-7-en-3-ol (Δ^7-sitosterol), at the expense of the Δ^5-sterols campesterol and sitosterol.[154] The mutation had almost no effect on morphogenesis and growth. An allelic series of *STE1*, *dwarf7* and *bul1*, was characterized by an extremely dwarf phenotype of plants. Such dwarfism and biosynthetic defects could be complemented biochemically by feeding exogenous brassinosteroids to growing seedlings[155,156] and genetically by the expression of *ERG3*.[154] In the tiny dwarf *bul1*, a cellular analysis showed that microtubule polymerization/depolymerization was compromised,[156] just like in the *diminuto/dwarf1 (sterol-Δ^{24}-isomerase/reductase)* mutant. These plants expressing strong alleles of *STE1* contained Δ^7-sterols and as little as 2% of residual Δ^5-sterols. This indicated that the dwarfism was due to the lack of a sufficient pool of campesterol that served as a precursor for the synthesis of brassinosteroids, and that in Arabidopsis at least, Δ^7-sterols cannot prime the synthesis of brassinosteroids (see Section 1.21.2.4.3). The *A. thaliana* sterol-C5(6)-desaturase *STE1* was cloned by functional complementation of the yeast *erg3* mutant. A plant cDNA expression library was transformed in *erg3* and transformants were screened for cycloheximide resistance, nystatin sensitivity, and sterol content.[157] One cDNA expressed in the *ste1* mutant restored a wild-type sterol composition and was therefore a Δ^7-sterol-C5(6)-desaturase. A second gene encoding a putative Δ^7-sterol-C5(6)-desaturase located beside *STE1* was found in the genome of *A. thaliana*: the encoded protein shared 80% identity with STE1. The functional analysis of this gene was not reported. Molecular analysis of *ste1* showed that the encoded polypeptide contained a single amino acid substitution T114I.[158] Δ^7-sterol-C5(6)-desaturase enzymatic activities were characterized in rat liver microsomes[159] and in *Z. mays* coleoptile microsomes.[160] The human gene *SC5DL* complemented the mutant erg3.[161] The plant Δ^7-sterol-C5(6)-desaturase STE1, studied by functional expression in *erg3*, is a nonheme iron oxygenase, requiring cytochrome b_5 as an electron carrier from the reductant NADPH to the Δ^7-sterol-C5(6)-desaturase, via cytochrome b_5 reductase. Site-directed mutagenesis identified the histidine-rich motifs of the protein as ligands for a catalytic Fe center, as was the case for membrane-bound fatty acid desaturases.[162] The plant enzyme carrying the T114I mutation expressed in *erg3* had a higher K_m and a lower catalytic efficiency, in agreement with the biochemical phenotype of *ste1*. Interestingly, the conservative T114S mutation had a 28-fold higher V_{max} value and an increased catalytic efficiency compared to the wild type, indicating that this amino acid residue played an essential role in the catalytic process.[162] The molecular mechanism of sterol C5(6) desaturation was performed either with C5α- or C6α-deuterated Δ^7-cholestenol analogs as mechanistic probes. These substrates showed deuterium kinetic isotope effects in accordance with the chemical activation of the C6α–H bond prior to its cleavage by the enzyme as a rate-limiting step in the desaturation reaction.[163]

The last enzymes of the sterol pathway that modify the tetracyclic skeleton are $\Delta^{5,7}$-sterol Δ^7-reductases. These reductases in plants and animals but not in yeasts (**Figure 5**). In plants, the reduction of $\Delta^{5,7}$-cholestadienol into cholesterol was enzymatically characterized in microsomal preparations from

Z. mays coleoptiles.[164] This reduction reaction is NADPH dependent and is strongly inhibited by carbocationic transition state substrate analogs. In agreement with such a carbocationic mechanism of the reduction of the Δ^7 double bond, synthetic azasteroids, including 6-aza-B-homo-5α-cholest-7-en-3β-ol, were particularly efficient in inhibiting the *Z. mays* microsomal $\Delta^{5,7}$-sterol Δ^7-reductase *in vitro*. *In vivo*, 6-aza-B-homo-5α-cholest-7-en-3β-ol–treated *Rubus fruticosus* cells contained (24*R*)-24-ethyl-5α-cholest-5,7-dien-3β-ol (**44**).[165] In mammals, inhibition by a piperazine derivative of the $\Delta^{5,7}$-sterol Δ^7-reductase caused an accumulation of $\Delta^{5,7}$-cholestadienol.[166] A plant cDNA encoding the $\Delta^{5,7}$-sterol Δ^7-reductase was isolated from an *A. thaliana* library expressed in wild-type yeast. With this strategy of metabolic interference of the reductase with the ergosterol biosynthetic pathway, a transformant displaying nystatin resistance was isolated due to the absence of ergosterol that was further metabolized into Δ^5-sterols. The cloned protein presented sequence similarities with other sterol reductases.[167] The efficient expression of the *A. thaliana* $\Delta^{5,7}$-sterol Δ^7-reductase (DWARF5) in yeast supported a further biotechnological strategy designed to produce mammalian steroids.[168] Functional analysis of the plant $\Delta^{5,7}$-sterol Δ^7-reductase was done in *A. thaliana*. An allelic series of *dwarf5* mutants was characterized by knockout mutations or other null mutations (deletion, splice-site, missense, and nonsense mutations). This resulted in an accumulation of $\Delta^{5,7}$-sterols at the expense of campesterol and sitosterol.[169,170] Consequently, strong mutations in *DWARF5* (as in the Δ^7-*sterol-C5(6)-desaturase/STE1/DWARF7* or in the Δ^{24}-*sterol isomerase/reductase DIMINUTO/DWARF1* of *A. thaliana*) result in a brassinosteroid deficiency and, therefore, are a typical dwarfism. Biochemical and genetic complementation of the *dwarf5* mutants with exogenous brassinosteroids and with an expressed cDNA encoding DWARF5, respectively, were in full accordance with the crucial role of Δ^5-sterols as precursors of plant steroid hormones and as structural components of membranes.[8,169,170]

1.21.2.4 Polyoxidized Derivatives

1.21.2.4.1 Steroidal hormones in fungi

Antheridiol (**46**) and oogoniol (**47**) are derivatives of fucosterol (**45**) (**Figure 6**) acting as pheromones in the oomycetes of *Achlya ambisexualis* (water mold). These compounds control sexual morphogenesis.[171] Their action on cellular metabolism includes ribosomal RNA or protein synthesis.[172,173] Biochemical analysis of a high-affinity steroid-binding protein suggested that it could function as a steroid receptor.[174] An antheridiol-induced chaperone HSP90 heat-shock protein was shown to be associated with the steroid receptor complex.[175,176] Molecular and genetic aspects of the biosynthesis of antheridiol and oogoniol have not been investigated in detail.

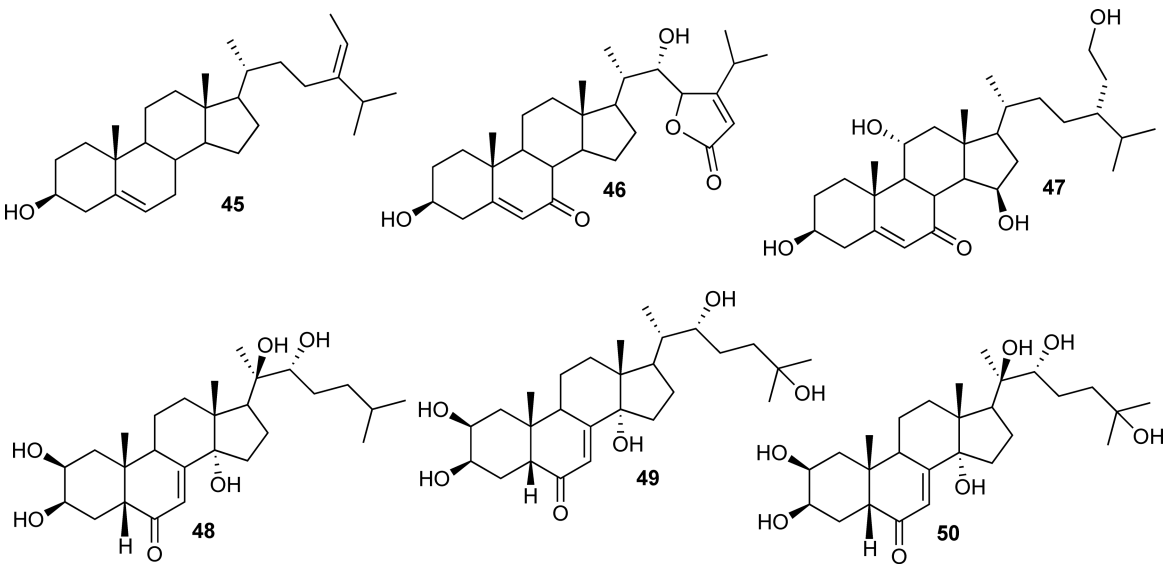

Figure 6 Structures of steroid hormones in Oomycetes and of some ecdysteroids.

1.21.2.4.2 Phytoecdysteroids

Phytoecdysteroids are distributed in a large number of land plants[177] including polypodine B (**48**) in ferns[178] and α-ecdysone (**49**) or 20-hydroxyecdysone (**50**) (**Figure 6**) in Chenopodiaceae.[179,180] Mycoecdysteroids have been described in fungi.[181] These compounds have the same structural features as ecdysteroids found in insects or other arthropods, for instance, α-ecdysone (**49**) or 20-hydroxyecdysone (**50**), which are hormones acting in important developmental cellular processes such as molting.[182] The biology and molecular regulation of this process in insect development has been reviewed.[183] It is assumed that plant ecdysteroids could exert deterrent or antifeedant effects on predators[184] or develop interfering effects in molting.[185] Although the phytochemical diversity of ecdysteroids has largely been covered, the biosynthesis of this class of compound is still largely unknown. Hundreds of C27, C28, or C29 5β-steroids collectively named 'phytoecdysteroids' have been reported.[186] Conjugated forms of ecdysteroids are known in plants and animals.[187] Ecdysteroid biosynthesis and transport have been studied in spinach leaves. Radiolabeled [^{14}C]-mevalonate was efficiently incorporated into 20-hydroxecdysone[188], therefore cholesterol might be a precursor. A possible biosynthetic intermediate between cholesterol (or lathosterol in Caryophyllaceae that do not produce Δ^5-sterols) is a 14α-hydroxy-7-en-6-one derivative.[186] Structural characteristics of ecdysteroids, in addition to the chromophore in the B ring and hydroxyl groups at 3β and 14α, typically show a high degree of oxidation (additional double bonds, hydroxyl or oxo groups). Biotransformations of putative phytoecdysteroid precursors were studied in tissue cultures of *Polypodium vulgare*. Incubation of calli or prothalli with various labeled ecdysteroids led to the detection of C2-hydroxylase enzyme activity when compounds with a hydroxyl group at C3 were tested as substrates. The same material was used to study the stereospecificity of the enzymatic conversion of 22-hydroxycholesterol or 25-hydroxycholesterol into ecdysone and 20-hydroxyecdysone.[189] In ecological approaches, plant families have been surveyed for the presence of ecdysteroid agonist or antagonist activities using *D. melanogaster* cellular bioassays.[190] Biological activities of ecdysteroids and brassinosteroids have been compared using this insect cell bioassay as well as the rice lamina inclination assay, classically used in brassinosteroid biology. There was no interference of brassinosteroid with ecdysteroid signaling in insects observed, nor was any ecdysteroid with brassinosteroid signaling in plants observed.[191]

1.21.2.4.3 Brassinosteroids

Brassinolide was discovered in rape pollen in 1979[192] and castasterone in chestnut insect gall in 1982.[193] An array of plant steroids named brassinosteroids were subsequently described, and their natural occurrence and biosynthesis was documented extensively.[194] Chemical analysis of brassinosteroids, present in plants at the nanomolar range, first included bioassays and radioimmunoassays, as well as methods in GC–MS (gas chromatography–mass spectrometry). Many aspects of the physiological effects of brassinosteroids were studied, and their possible applications in agriculture were described.[194] The elucidation of the biosynthetic pathway and the characterization of molecular regulation of brassinosteroid signaling was achieved within a decade, mostly through the use of genetic approaches with the model plant *A. thaliana*. Oxidative conversion of sterols into brassinosteroids was shown to consist of a grid of multiple pathways. Arabidopsis mutants showing impaired skotomorphogenesis were characterized as small dwarfs whose growth can be restored to the wild-type level by exogenous brassinosteroids.[195,196] The gene *DET2s*, isolated through this mutational approach, was shown to encode a protein of 40% identity with the mammalian steroid-5α-reductase. Interestingly, it was shown that plant *DET2* orthologs of the DET2 can substitute for each other, indicating a structural and functional conservation of steroid hormone signaling to a certain extent.[197] DET2 was shown to catalyze the reduction of (24*R*)-24-methylcholest-4-en-3-one (**51**) to (24*R*)-24-methyl-5α-cholestan-3-one (**52**) en route to campestanol (**53**) (**Figure 7**) in *A. thaliana* and in *Pisum sativum*.[198,199] The DET2 ortholog of *Gossypium hirsutum* was shown to play an important role in the basipetal growth of cotton fiber.[200] In *Solanum malacoxylon*, two isozymes of DET2 were identified by comparing the metabolization of 5α-campestanone to that of progesterone in different tissues.[201] The occurrence of DET2s was extended to fungi. A DET2 fungal ortholog was isolated from *U. maydis* and was shown to be induced in plant–host parasitic interaction (corn smut disease).[202] The fungal DET2 was expressed in the Arabidopsis *det2-1* mutant, and this was sufficient to restore a wild-type phenotype in the plant mutant. Consequently, fungal, plant, and mammalian DET2s are most probably evolutionarily related. A series of dwarf Arabidopsis mutants isolated in a T-DNA insertional mutagenesis approach was also considered in the elucidation of the brassinosteroid biosynthetic pathway. The conversion of

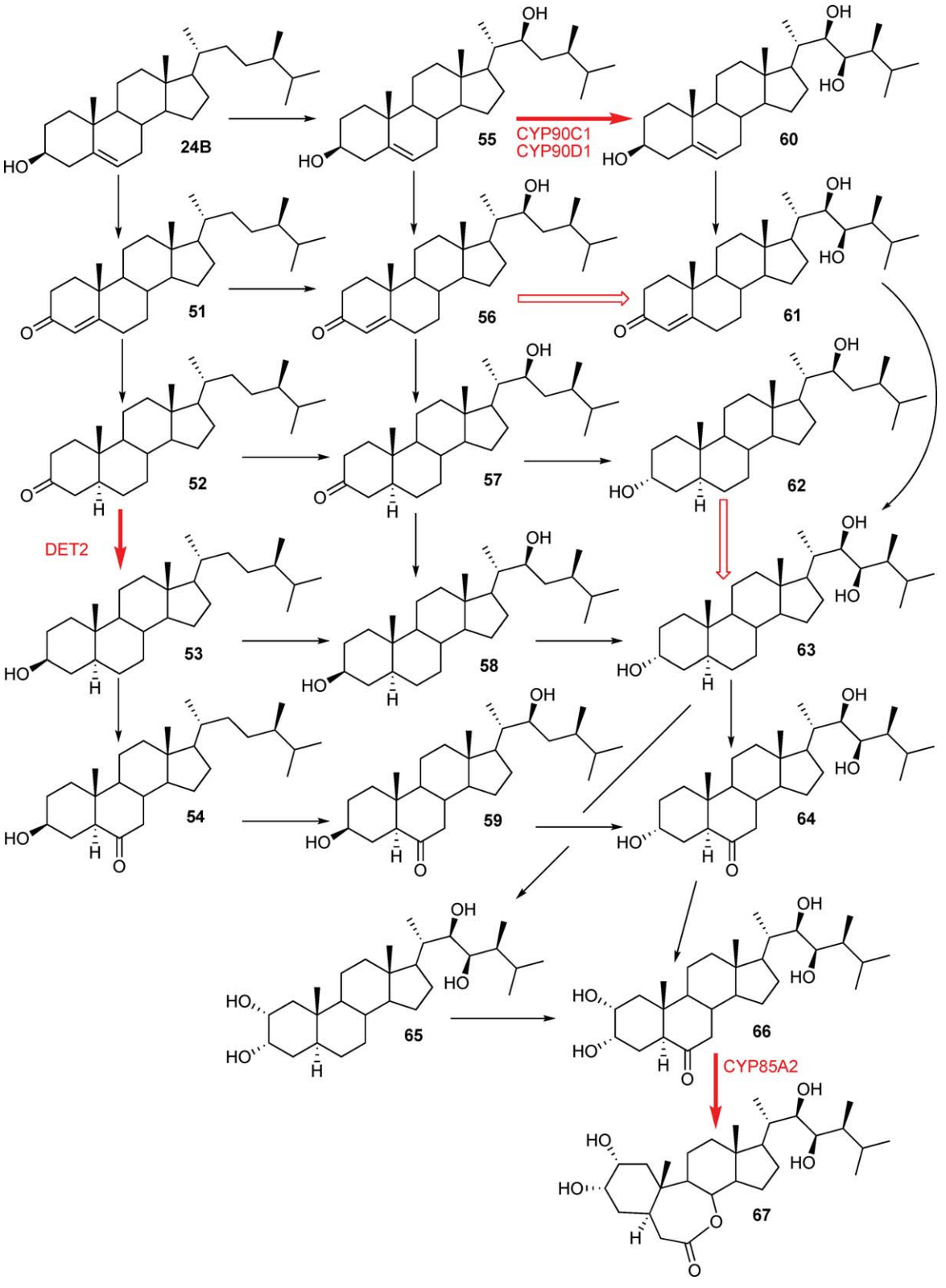

Figure 7 Brassinosteroid biosynthesis emphasizing short cuts of the C23-hydroxylation (large red arrows). Adapted from T. Ohnishi; A. M. Szatmari; B. Watanabe; S. Fujita; S. Bancos; C. Koncz; M. Lafos; K. Shibata; T. Yokota; K. Sakata; M. Szekeres; M. Mizutani, *Plant Cell* **2006**, *18*, 3275–3288. **56**, 22-hydroxy-ergost-4-en-3-one; **57**, 22-hydroxy-5α-ergost-3-one; **58**, 6-deoxo-cathasterone; **59**, cathasterone; **60**, 22,23-dihydroxy-campesterol; **61**, 22,23-dihydroxy-ergost-4-en-3-one; **62**, 3-*epi*-6-deoxo-cathasterone; **63**, 6-deoxo-typhasterone; **64**, typhasterone; **65**, 6-deoxo-castasterone.

campesterol into C28 brassinosteroid intermediates, and then into catasterone (**66**) and brassinolide (**67**) after Baeyer–Villiger C6 oxidation to form a 7-oxolactonic B ring typical of brassinolide, was depicted in detail in *A. thaliana* and *Catharanthus roseus*.[194,203] Biosynthetic segments that form an intricate network of alternate routes between campesterol (**24**), (22*S*)-22-hydroxycampesterol (**55**), campestanol (**53**), 6-oxo-campestanol (**54**), 6-oxo-brassinosteroid intermediates, or 6-deoxo-brassinosteroid intermediates have been described as an early C22 oxidation branch,[204] a late C6 oxidation pathway, or an early C6 oxidation pathway. C23-hydroxylation shortcuts interspersed into those segments, which allow conversion of C22-hydroxylated intermediates into 6-deoxo intermediates,[205] are summarized in **Figure 7**. Cytochrome P-450 oxidases are involved not only in biosynthesis but also in the catabolism of brassinosteroids. Hydroxylation at C26 described in *A. thaliana* and *Lycopersicon esculentum* represents a possibility for the plant cell to inactivate brassinosteroids.[206] The essential functions of brassinosteroids in the regulation of biological processes are mediated by a signaling pathway whose components and molecular mechanisms include, BRI1, a plasma membrane receptor kinase, the transcription factors BES1 and BRZ1, and kinases and phosphatases at play in this signaling pathway.[207–209]

1.21.2.5 Steroids with Heterocycles in the Side Chain

1.21.2.5.1 Steroidal saponins

Two types of steroidal saponins are distributed within monocotyledones from the Asparagaceae, Costaceae, Poaceae, Dioscoreaceae, and Liliaceae and dicotyledones from the Solanaceae or Fabaceae.[210] Structurally, these compounds are spirostan (diosgenin (**68**) or furostan (nuatigenin (**69**)) sapogenins of which the hydroxyl at C3 is linked to an oligosaccharide (**Figure 8**). These compounds have been studied from the phytochemical and pharmacological aspects due to their medicinal properties and widespread uses. The occurrence and structural elucidation of steroidal saponins from *Dioscorea* species has driven several lines of research because of the industrial interest of diosgenin for steroid production. Steroidal saponins are described as anticancer agents along with other pharmacological properties.[211] Dioscin (**70**, **Figure 8**) from the yam[212] has antifungal activity against the human pathogenic yeast *C. albicans*. Other derivatives have antiallergic activity as was monitored using biochemical markers from specific test cell lines.[213] Molecular and genetic aspects of steroidal saponin biosynthesis are scarce. In *Avena sativa*, avenacosides (**71**) were converted into the fungicidal 26-desglucoavenacosides (**72**) upon infection by pathogens, and the hydrolytic fungal enzymes implicated in this process were isolated and described.[214] This type of hydrolytic enzyme, the 26-*O*-β-glucosidase, was purified from *Costus speciosus*. Its activity was high in stored rhizomes, where saponins underwent the transformation of spirostanol glycosides to furostanol glycosides.[215] Glycosylation of steroidal sapogenins and saponins is of

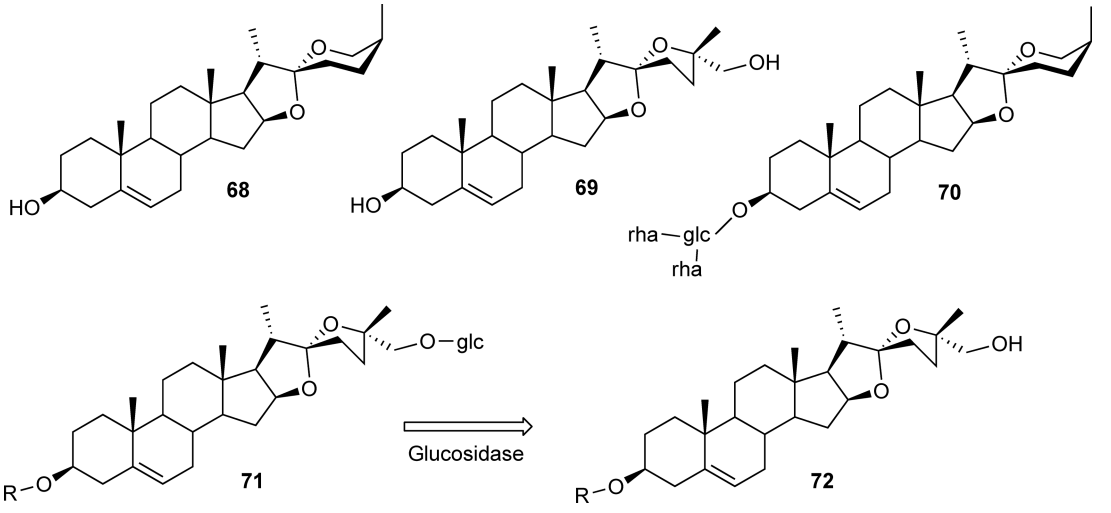

Figure 8 Sapogenins and steroidal saponins.

considerable importance for pharmacological activity. Glucosyltransferases involved in diosgenin (**68**), nuatigenin (**69**), and tigogenin metabolism have been cloned from *Solanum aculeatissimum*.[216] Their physiological role in plant biology has been related to plant defense. A functional study of these glycosyltransferases expressed in *E. coli* led to the identification of essential residues from the donor–sugar recognition domain of the protein.[217] One of these glycosyltransferases from *S. aculeatissimum* was shown to glucosylate steroidal alkaloids, in addition to saponins.

1.21.2.5.2 Steroidal glycoalkaloids

Steroidal glycoalkaloids are secondary metabolites found mainly in species belonging to the Solanaceae family. The structure is based on aglycones of the solanidane (e.g., solanidine (**73**)) or of the spirosolane (e.g., tomatidine (**74**)) types (**Figure 9**). Structural elucidation of these metabolites was documented over 50 years ago,[218] and structures continue to be reported.[219] Steroidal glycoalkaloids have been shown to play a role in plant–pathogen interactions. Resistance to bacterial and fungal diseases[220,221] and to insects[222,223] were considered in relation to the high contents of glycoalkaloids. Similarly, the tomato steroidal glycoalkaloid α-tomatine was shown to exert an antifungal activity associated with membrane permeabilization.[224] The well-known toxicity of α-chaconine and α-solanine (**75**) present in potato tubers produced for human consumption has also been linked to cell membrane permeabilization.[225] Upregulation of cholesterol biosynthetic genes in Caco-2 intestinal epithelial cells after treatment with α-chaconine was also reported.[226] The biosynthesis of steroidal glycoalkaloids has been studied in *Solanum melongena* and *S. tuberosum*. Biochemical studies support the view that cholesterol is the most probable precursor for the biosynthesis of solanidine.[227–229] A UDP-glucose:solasodine glucosyltransferase was partially purified from *S. melongena* leaves. The glucosyltransferase was able to glucosylate aglycones from the spirosolane type but not from the solanidane type.[230] In *S. tuberosum*, the biosynthesis of α-solanine and α-chaconine downstream to solanidine includes an UDP-galactose:solanidine galactosyltransferase (SGT1) catalyzing the conversion of solanidine into γ-solanine.[231] This gene was originally cloned as a UDP-glucose glucosyltransferase expressed from a potato cDNA library, transformed in yeast.[232] Other glucosyltransferases were isolated: a UDP-glucose:solanidine glucosyltransferase (SGT2) was implicated in the production of α-chaconine. In tubers of potato plants expressing an antisense SGT2 construct, the accumulation of α-solanine was increased and α-chaconine was reduced.[232] Another transferase, SGT3, was identified as a glycosterol rhamnosyltransferase that was able to catalyze the terminal step in the formation of the triose side chain of α-solanine and α-chaconine from β-solanine and β-chaconine, respectively.[233] Regulatory aspects of potato steroidal glycoalkaloid biosynthesis have been addressed in the context of the isoprenoid metabolism. A high steroidal glycoalkaloid content in tissues was associated with high expression levels of HMGR and squalene synthase.[234] The biosynthetic link between cholesterol and glycoalkaloid biosynthesis was addressed in the transgenic potato overexpressing a cycloartenol-C24-methyltransferase from *G. max* (*Gm*SMT1). The expression of *GmSMT1* led to increased levels of isofucosterol and sitosterol at the expense of cholesterol. Shortage of cholesterol resulted in an associated reduction of steroidal glycoalkaloids in leaves and tubers,[235] reinforcing the role of cholesterol as biosynthetic precursor of α-solanine and α-chaconine.

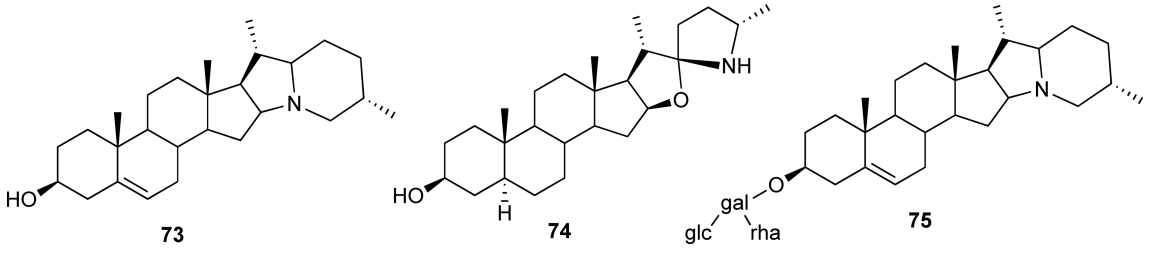

Figure 9 Steroidal glycoalkaloids.

1.21.2.5.3 Cardiotonic steroidal glucosides

These compounds are produced by plant species from diverse families such as Apocynaceae or Plantaginaceae but are also produced by amphibians. Historically, they have attracted considerable interest due to their medicinal properties. An epoxybufenolide series displaying a growth inhibition effect on the cancer cell line KB was described.[236] Recently, a series of plant cardiotonic steroids were shown to behave like potent splicing modulators in a test system (a reporter gene construct) designed for screening chemical libraries.[237] The steroidal aglycones of these compounds are cardenolides, bearing a lactone ring of five atoms at C17, or bufenolides, bearing a lactone ring of six atoms at C17.[2] *Digitalis purpurea* accumulates high levels of the cardenolides digitoxigenin (**76**) and digoxigenin (**77**) and high levels of the cardenolide glycosides (e.g., digoxin (**78**)) in leaves, with a diversity of sugar residues.[238,239] Cardenolide formation has been studied in *Digitalis lanata*, *D. purpurea*, and *Asclepias incarnata*, with an important focus on the activity of pregnane-modifying enzymes.[240–243] These steroids are thought to originate from cholesterol or possibly from 24-alkyl-sterols.[244] Cardenolide and pregnane biosynthesis requires a 3α-hydroxysteroid-5β-reductase.[245] A cDNA encoding a progesterone 5β-reductase (5β-POR) was cloned from *Digitalis lanata* leaves and functionally expressed in *E. coli* (**Figure 10**).[246] When progesterone was used as a substrate in this assay, the 5β isomers were formed exclusively. The crystal structures of 5β-POR in complexes containing progesterone indicated an architecture of the active site similar to that of SDR.[247] Other plant orthologs of 5β-POR were identified in *Isoplexis canariensis*,[248] another cardenolide-rich plant species, and in the model *A. thaliana*, which has not been reported as producing cardenolide.[249] The Arabidopsis protein functionally expressed in *E. coli* stereospecifically reduced progesterone to 5β-pregnane-3, 20-dione.[249] The corresponding gene was strongly transcribed in leaves. This Arabidopsis 5β-POR gene was originally described as a mutant allele negatively affecting cotyledon and leaf *vein patterning*, therefore called VEP1, required for normal vascular strand development.[250] A set of enzymes implicated in the bioconversion of pregnenolone into cardenolides in the model *D. lanata* included a Δ^5-3β-hydroxysteroid dehydrogenase (βHSD) and a Δ^5-3-oxosteroid isomerase. The *D. lanata* 3βHSD expressed in *E. coli* uses pregnenolone but not cholesterol as a substrate (**Figure 10**).[246] A malonyl-coenzymeA:21-hydroxypregnane 21-*O*-malonyltransferase involved in the formation of the butenolide ring of digitoxigenin was reported in leaves of *D. lanata*.[251] Cardenolides, produced in laticifers of Apocynaceae,[252] have been looked at in ecological approaches of plant–insect interactions to illustrate the defense-escalation theory and the evolutionary trends of secondary metabolism moving toward a decline of plant chemical defence strategies.[253]

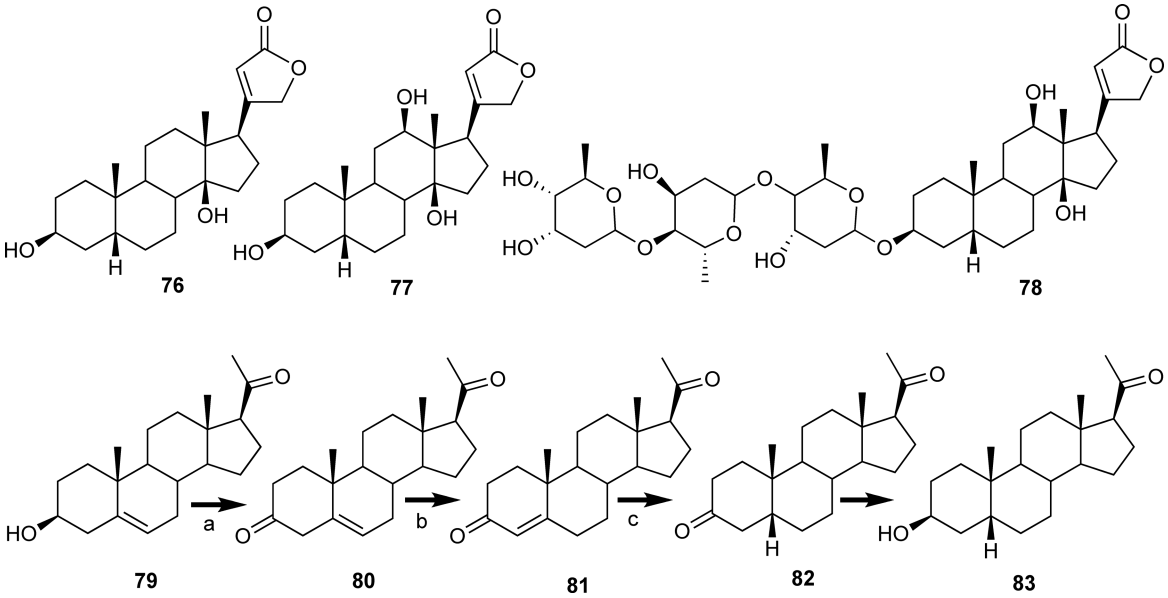

Figure 10 Cardenolide structure (**76–78**) and biosynthesis (**79–83**). a = 3β HSD, b = Δ^5-3-oxosteroid isomerase, and c = 5βPOR. 79, pregnenolone; 80, isoprogesterone; 81, progesterone; 82, 5β-pregnane-3, 20-dione; 83, 5β-pregnane-3β-ol-20-one.

1.21.3 Sterol and Steroid Conjugates

1.21.3.1 Steryl Esters

Steryl esters are ubiquitous sterol conjugates.[254] The fatty acyl moieties of these conjugates are usually representative of abundant compounds in a given organism such as C16 or C18 fatty acids. A diversity of sterol esters has been described in mammals,[255] in fungi,[256] and in plants.[257] Cholesterol is esterified in mammals by two distinct types of enzymes. Membrane-bound acyl-coA:cholesterol acyltransferases (ACAT) catalyze acyl-coA-dependent acylations in cells.[258] Soluble lecithin:cholesterol acyl transferase, a circulating enzyme present in the bloodstream, is evolutionarily unrelated to the ACAT type of enzymes.[259] Ergosterol is esterified in yeast by two ACAT-related enzymes (AREs), ARE1 and ARE2. These two proteins share a 49% identity, and their functional redundancy is indicated by the fact that sterol ester biosynthesis is not affected in an *are1* mutant, is reduced to 75% of its physiological level in an *are2* mutant, but is totally abolished in a double mutant *are1 are2*.[260] The absence of steryl ester-forming enzymes has no effect on cell viability in laboratory conditions. Enzyme activity measurements and fluorescence microscopy of proteins fused to the green fluorescent protein (GFP) indicated that both ARE1 and ARE2 proteins were localized in the endoplasmic reticulum. Are1p was shown to esterify the precursor and the end product, namely lanosterol and ergosterol, whereas are2p has a strong substrate preference for ergosterol.[261] Steryl esters are stored in lipid droplets or particles in yeast. Sterols in this storage form may be mobilized by the action of steryl ester hydrolases.[262] Three enzymes in yeast contribute to this process.[263] Experiments performed with *S. cerevisiae* support storage and mobilization of sterols as a dispensable process. However, enzymes implicated in there are the key elements of sterol homeostasis. Acetylation of sterols and steroids in yeast has been described as a detoxification pathway, including also a deacetylase. The acetyltransferase ATF2 and the steryldeacetylase SAY1 have been functionally characterized in a steroid export process.[264] In plants, the situation resembles that of mammals. Although biochemical studies performed with subcellular fractions have described sterol and acyl donors implicated in the reactions, molecular characterization of sterol ester-forming enzymes is recent. In the model *A. thaliana*, two genes encoding steryl ester-forming enzymes have been reported. One is related to the mammalian (lecithin cholesterol acetyltransferase) LCAT. Leaf microsomal membranes enriched with phospholipid:sterol acyltransferase PSAT1 (phospholipid sterol acyltransferase) catalyze the transacylation of fatty acyl moieties from the *sn-2* position of phosphatidylethanolamine.[265] The implication of this enzyme in regulatory aspects of sterol metabolism was indicated by the fact that sterol intermediates were preferentially esterified by the PSAT-rich fraction in the presence of pathway end products. Arabidopsis mutant lines deficient in PSAT1 are strongly depleted in steryl esters, but this has no effect on viability. Another sterol acyltransferase belongs to the family of plant membrane-bound *O*-acyltransferases related to the ACATs of yeast and animal. This plant sterol-*O*-acyltransferase, ASAT1, when expressed in yeast, catalyzes the production of lanosterol esters. Similarly, enzyme assays performed with subcellular fractions of this yeast indicated a substrate preference for cycloartenol as acyl acceptor and saturated fatty acyl coenzyme A as acyl donor. A seed-specific overexpression of *ASAT1* in *A. thaliana* resulted in elevated amounts of cycloartenol esters.[266] The sites of steryl esters accumulation in plants are of two types. Steryl esters accumulate in cellular lipid droplets when the amount of free sterol synthesized is higher than that normally required to build membrane structures. This has been shown with the tobacco mutant *sterov* isolated in a somatic genetic approach[267–268] and in plant cell cultures fed with the upstream sterol precursor mevalonate.[269] Steryl esters are also deposited in the lipid bodies of elaioplasts in the tapetum. Tapetal cells in developing anthers of *Brassica napus* contain tapetosomes and elaioplasts, two types of organelles that are required for the pollen coat elaboration. Comparative analysis of sterols from the sterol ester-rich lipid bodies of elaioplasts and from the pollen coat indicated that a lipid coating of pollen is made with neutral lipid produced in the tapetum.[270,271] Species from the Poaceae (corn, wheat, rice, triticale, barley, and oat) contain steryl ferulates and other esters of phenylpropanoids localized in aleurone cells.[272] Cycloartenyl ferulates present in rice bran oil revealed their anti-inflammatory properties during pharmacological studies.[273]

1.21.3.2 Sterol Glucosides

Uridine diphospho (UDP)-glucose sterol-β-D-glucosyltransferase (USGT) is a plasma membrane–bound enzyme.[274] Sterols recovered from the acid hydrolysis of sterol glucoside fractions are usually Δ^5-sterols (cholesterol, ergosterol, campesterol, and sitosterol),[254] but others have been reported, such as Δ^7-sterols in Leguminosae.[275] The USGT from *A. sativa* etiolated shoots was solubilized and purified up to 12,500-fold using a sepharose-based chromatographic process[276], and a cDNA was functionally expressed in *E. coli*.[277] Genes encoding USGT were isolated from *S. cerevisiae*, *C. albicans*, *Pichia pastoris*, and *D. discoidum* and functionally expressed.[278] The characterization of a yeast mutant deficient in sterol glucosylation indicated that this biochemical process was apparently dispensable because the lack of USGT had no effect on cell viability. An alternative pathway for the synthesis of sterol glucosides has been proposed in Arabidopsis, based on the use of a mutant of *P. pastoris* deficient in the production of glucosylceramides and of sterol glucosides. A plant glucosylceramide synthase was expressed in this double mutant. This resulted in the synthesis of glucosylceramides and of sterol glucosides, therefore indicating the presence of multiple pathways for sterol glucoside synthesis in plants.[279] Sterol glucosides in *P. pastoris* have been described as enhancers for the autophagic process of peroxisome degradation.[280] Sterol glucoside biosynthetic capability has been reported for prokaryotes. *Helicobacter pylori*, the pathogen responsible for gastric ulcers and carcinoma, is a sterol auxotroph but contains a gene Hp0421 that encodes cholesterol-α-glucosyltransferase.[281] A bacterial strain lacking a functional Hp0421 was generated and used to point out the important role of sterol glucosides in the interaction of bacteria with the host. It was concluded that cholesterol glycosylation promotes immune evasion by *H. pylori*.[282] Plasma membrane lipid alterations, and particularly the sterol glucosides alterations, were shown to be associated with cold acclimatization of plants.[283] Biophysical studies have addressed the functional role of sterol glucosides in membrane models.[284,285] The possible role of sterol glucosides in plants as primers for cellulose biosynthesis has been addressed experimentally. Microsomes prepared from cotton fibers synthesize sitosterol-cellodextrins from sitosterol glucoside and UDP-glucose under conditions that favor cellulose synthesis.[286] Studies on plant–microbe interaction revealed that USGT is required for pathogenicity. Indeed, conidia of *Colletotrichum gloeosporioides* use it as a virulence factor whose synthesis is induced by surface contact.[287]

1.21.3.3 Acylated Sterol Glucosides

The synthesis of acylated steryl glucosides has been reported in plants.[254] Fatty acyl moieties have been identified as well as acyl donors.[288] A membrane-bound phospholipid:steryl glucoside acyltransferase from *Solanum melalonga* was partially purified and shown to acylate phytosteryl glucosides. The preferred acyl donors were phosphoglycerolipids compared to 1,2-diacylglycerols, particularly 1,2-dimirystoylphosphatidylic acid, providing the acyl moiety from the *sn-1* position.[289] An acylated sterol glucoside, β-sitosteryl-3-*O*-β-D-glucopyranosyl-6′-*O*-palmitate, exhibited high anticomplementary activity following an activity-guided isolation from *Orostachys japonicus* extracts. Interestingly, the corresponding nonacylated sterol glucoside had no pharmacological activity.[290]

1.21.3.4 Steroid Sulfates

Sulfate conjugation by sulfotransferases is a process that participates in regulating the biological activity of steroid hormones in mammals,[291] a process that has been described in plants.[292] In *B. napus*, a steroid sulfotransferase catalyzes an O-sulfonation of brassinosteroids and also of animal steroids. This plant enzyme is specific for the hydroxyl group at C22 and preferentially uses biosynthetic intermediates. It has been shown that sulfonation of 24-epibrassinolide abolishes its activity in a functional assay. Hormone inactivation by sulfonation is, therefore, a common mechanism distributed in eukaryotes.[293] As in the case of *B. napus*, *A. thaliana* contain two distinct brassinosteroid sulfotransferases that share a 44% identity and defined distinct plant sulfotransferase families. These enzymes exhibited partial overlapping functions with human dehydroepiandrosterone sulfotransferase. Molecular characterization of the plant steroid sulfotransferases revealed transcription specificities, particularly induction by cytokinins. The reaction catalyzed by the sulfotransferase *AtST1* was shown to be stereospecific for 24-epibrassinosteroids.[294] The expression of the sulfotransferase in *B.*

napus was induced by salicylic acid, indicating that steroid-dependent biological response may be at play in plant defense processes.

1.21.4 Sterol Degradation

Sterol degradation is known to occur in actinobacteria such as *Nocardia rhodochrous*. The potential for bacteria to provide genetic resources for steroid biotechnology has recently driven more research. Cholesterol oxidases produce cholest-4-en-3-one, as they were shown to have both 3β-hydroxysteroid dehydrogenase and 3-oxo-steroid Δ^4–Δ^5-isomerase activities in acellular fractions.[295] *Sterolibacterium denitrificans* is a proteobacteria from the Rhodocyclaceae that can also degrade cholesterol to carbon dioxide under anoxic conditions. A cholest-4-en-3-one-Δ^1-dehydrogenase partially purified, sequenced as tryptic peptides, then cloned and expressed in *E. coli*, accepted cholest-4-en-3-one and other mammalian steroids as substrates.[296] This FAD-dependent enzyme is similar to 3-oxo-steroid-Δ^1-dehydrogenase (and to other enzymes from the SDR group), which is involved in aerobic degradation of steroids by another proteobacteria *Pseudomonas testosteroni*.[297] A bioinformatic-based study of an actinobacteria from the *Rhodococcus* genus (also known for plasiticity in bioconversion/biodegradation processes) provided a compendium of genes responsible for catabolism of cholesterol to propionyl coenzyme A and pyruvate.[298] The related pathogenic genus *Mycobacterium* displayed a conserved cholesterol catabolic pathway that enabled *M. tuberculosis*, for example, to grow on cholesterol *in vitro* and, most importantly, to survive in macrophages.

1.21.5 Transport

Aspects of steroid transport at the cellular or organism levels are known across prokaryotes and eukaryotes. Actinomycetales (*Mycobacterium*, *Rhodococcus* for instance) possess *mce* loci (mammalian cell entry), on which *mce* genes are arranged in operons.[299] These genes are upregulated during growth on cholesterol. A genetic approach using *Rhodoccocus jostii* deletion mutants of genes belonging to the *mce4* locus, combined with cholesterol and other steroid uptake assays, demonstrated that *mce4* encodes a sterol uptake system that is a steroid transporter of the ABC type.[300] Cholesterol metabolism and circulation in mammals is closely linked to its transport across cell membranes toward lipoproteins of the bloodstream, and this is mediated by ABC transporters.[301] In human enterocytes, transporters ABC G5 and G8 are responsible for 24-alkyl-sterols (plant sterols from the diet) efflux to the intestinal lumen. Mutations in these transporters cause the rare disease sitosterolemia.[302] Sterol carrier proteins (SCP) in animals are described as intracellular transporters of lipids with important roles in membrane biology and have been extensively studied over the past 30 years.[303] Structure–activity of the human SCP2, which facilitated such lipid transport, was determined using an assay of cholesterol and phosphatidylcholine transferred from small donor unilamellar vesicles to acceptor membrane of bacterial protoplasts.[304] Binding experiments suggested an interaction of cholesterol or fatty acids with SCP2, consistent with the role of an intracellular aqueous carrier or of an enhancer of sterol desorption from membranes.[305,306] The crystal structure of an ortholog of SCP2, determined at 1.8 Å resolution, revealed a hydrophobic tunnel suitable for lipid binding. Closely similar cavities were found in plant lipid transfer proteins (LTPs).[307] An ortholog of SCP2 was found in insects.[308] Another ortholog of SCP2 identified in *A. thaliana* showed lipid transfer activity.[309] The *A. thaliana* SCP2 is a ubiquitous peroxisomal protein essential for seed morphology and germination.[310] The evolution of SCP2 indicated a large distribution among living organisms and essential functions.[311] This is also true for oxysterol-binding proteins, which are implicated in sterol metabolism, transport, trafficking, and signaling, and which are found in animals, yeast, and plants (reviewed in Fairn and McMaster[312] and Javitt[313]). An *o*xysterol-binding protein-*r*elated *p*rotein (ORP) from *Petunia inflata* was shown to interact with a receptor kinase in pollen. This ORP allowed the detection of 12 genes encoding ORPs in *A. thaliana*, which were not yet assigned to physiological functions.[314] In oomycetes from the *Phytophthora* genus, a secreted protein called cryptogein was able to bind a sterol and to transfer it between phospholipidic bilayers.[315] The function of such proteins was related to plant–pathogen interactions.[316]

1.21.6 Molecular Regulation of Sterol Biosynthesis

Although cholesterol and structurally related sterols are distributed in bacteria, fungi, plants, and animals, the molecular regulation of the biosynthesis and accumulation of these compounds in different organisms may be diverse.

Cholesterol homeostasis in mammalian cells is controlled by feedback mechanisms acting at the transcriptional and the posttranscriptional levels of genes implicated in the synthesis of steroidogenic or lipidogenic enzymes or in the production of regulatory elements. Transcription of genes encoding HMGR, HMGcoA synthase, and the LDL (low-density lipoprotein) membrane-bound receptor increases when there is a cellular need for sterol.[317] Common *cis*-elements found in promoter sequences of these genes named SRE[318] were found to be the binding sites of a class of transcription factors, the SREBP (sterol regulatory element-binding proteins).[319] SREBP precursors of 125 kDa are composed of an N-terminal domain, which is the transcription factor itself, a member of the basic helix–loop–helix leucine zippers (bHLHZ), a central hydrophobic region composed of two transmembrane domains, and a C-terminal domain implicated in SREBP processing.[320] SREBPs are anchored in the endoplasmic reticulum and in the nuclear envelope. In the absence of cholesterol, SREBPs undergo intramembrane proteolytic cleavage, and the transcription activator bHLHZ translocates to the nucleus where it increases expression of target genes.[321] The proteolysis of SREBP implicates a SREBP cleavage activation protein (SCAP) in physical interaction with SREBP. SCAP–SREBP interaction enables the action of two distinct proteases S1P and S2P (sites 1 or 2 proteases).[322,323] HMGR has a central regulatory role in cholesterol homeostasis at the transcriptional but also at the translational levels. The mammalian HMGR contains eight helical membrane-spanning domains essential to sterol-mediated proteolysis. This was shown using reporter proteins whose half-life was monitored in the presence of cholesterol.[324] Sterol-sensing domains are present in HMGR and in SCAP and consist of approximately 180 amino acids forming five membrane-spanning domains. These domains have also been observed in other proteins implicated in lipid trafficking such as the Niemann–Pick disease type C1 protein (NPC1).[325] INSIG-1 (INSulin-Induced Gene) is another membrane protein that binds the sterol-sensing domain of SCAP in order to facilitate the retention of SCAP–SREBP complex in the ER.[326] In the absence of cholesterol, SCAP escorts SREBP toward the Golgi bodies proteolytic cleavage, thereby stimulating cholesterol synthesis.

Different types of ergosterol regulatory biosynthetic schemes are known in yeasts. A similar SREBP pathway was described in *Schizosaccharomyces pombe*.[327] It contains functional homologs of the SREBP pathway such as SREBP and SCAP. A microarray analysis has demonstrated that these regulatory elements enhance the sterol biosynthetic enzymes (as is the case in the animal system) but also functions as an oxygen sensor because these regulatory gene products activate hypoxia marker genes and, therefore, mediate oxygen-dependent sterol synthesis.[327] The pathogenic yeast *Cryptococcus neoformans* (causing meningoencephalitis) also possesses an SREBP, which is activated under low oxygen and triggers ergosterol biosynthesis.[328] Although insects are sterol auxotrophs, the genome of *D. melanogaster* contain all components of the SREBP pathway and were shown to be functional in Drosophila cells in culture.[329] Processing of the transcription factor is regulated by fatty acids, and not by sterols, and therefore favors transcription of genes implicated in fatty acid synthesis. From an evolutionary point of view, these observations underline the essential role of SREBP pathways in membrane integrity in the first place, and not only in cholesterol biosynthesis. Insects also share other components with mammals that are implicated in the regulatory effect of cholesterol synthesis and in neurodegeneration.[330]

The yeast model *S. cerevisiae* does not contain the SREBP pathway. However, it controls the biosynthesis of ergosterol at the HMGR level and other mevalonate pathway enzymes.[331,332] Additionally, another rate-limiting biosynthetic step was identified beyond the committed precursor squalene: the overexpression of a cytosolic HMGR led indeed to high squalene accumulation,[333] therefore identifying SQE as a bottleneck. This is not the case for other organisms, for example, plants in which HMGR represents a major bottleneck: its overexpression results in the accumulation of high amounts of pathway end product as steryl esters stored in cytosolic lipid droplets.[334] The regulated degradation of HMGR is an element of ergosterol homeostasis.[335] Levels of ergosterol have been implicated in the transcriptional regulation of sterol biosynthetic genes such as ERG10[336] or ERG3.[337] In the latter experiments, the promoter sequence of ERG3 that was fused to the bacterial reporter lacZ drove increasing levels of β-galactosidase activity, which was inversely proportional to

the quantity of ergosterol. A regulatory protein SUT1 of *S. cerevisiae* was involved in sterol uptake under hypoxia.[338] A transcriptional regulatory mechanism alternative to the SREBP pathway at play in *S. pombe* was described in *S. cerevisiae*. It is made up of two transcription factors upc2p and ecm22p, which are members of the fungal Zn2-Cys(6) binuclear cluster family, and bind to promoter sequences of *ERG* genes[339,340] and other regions of the same ERG genes.[341] *S. cerevisiae* also has a transcriptional repressor mot3, which interacts with ergosterol biosynthetic genes and plays a role in vacuolar and membrane transport.[342]

A series of oxysterols have been implicated in cellular cholesterol homeostasis. The oxidases that catalyze the synthesis of 7-hydroxycholesterol, 24-hydroxycholesterol, and 27-hydroxycholesterol are cytochrome P-450 oxygenases, whereas 25-hydroxycholesterol is made by a nonheme iron oxygenase. Among this series of molecules, 25(*R*)-hydroxycholesterol was the most potent regulator of HMGR in hepatic cells.[343] Oxysterol-binding proteins have been recently described as possible key elements of nonvesicular sterol transport.[344] Liver X receptors are nuclear proteins that bind oxysterols and act as transcription factors of genes implicated in sterol and lipid biosynthesis.[345] Pharmacological studies have shown that 7-hydroxycholesterol shows antitumor activity.[346] Interestingly, oxysterols were shown to be also nonenzymatic products in food due to autooxidation. The consequence of this process in functional foods was discussed.[10]

Plants must regulate their sterol biosynthesis and cellular lipid homeostasis by alternate systems because they do not have a SREBP pathway (as indicated by genome data mining). These alternate pathways are neither understood nor identified. However, the key regulatory role of the enzyme HMGR in the production and accumulation of steady-state levels of phytosterols is a trait that plants share with other eukaryotes. Arabidopsis or tobacco plants overexpressing HMGR, or deficient in the expression of HMGR, consequently have a higher or lower amount of sterol.[334,347] The cellular machinery linking a putative sensing system in membranes with action on gene activity is unknown. Light has a probable role in this process. It was indeed shown that phytochrome A, B, C, and D, which are major light receptors in plants, act as negative regulators of HMGR in Arabidopsis.[348]

1.21.7 Functions of Steroids

Major functions of sterols and steroids are related to cell membrane structure and hormonal functions and, are, therefore, typical of essential metabolites. Steroidal saponins, steroidal glycoalkaloids, and cardenolides belong to the category of natural products (secondary metabolites), which possess various biological and pharmacological activities linked to environmental interactions.

The crucial importance of sterols in regulating physical properties of membranes has long been known.[349] The formation of membrane microdomains, also known as lipid rafts[350] was shown to be favored by cholesterol, ergosterol, or plant sterols, together with sphingolipids.[351] These membrane domains have been described over the last 15 years as platforms supporting signal transduction[352] and host–pathogen interactions[353] in mammals. Membrane microdomains have been operationally characterized by their insolubility in nonionic detergents.[354] Such lipid rafts have been isolated and characterized in plant cells[355] and in yeast.[356] In the latter, ergosterol and membrane microdomains have an essential role in generation and maintainance of cell polarity during mating.[357] In the case of higher plants that are sessile organisms, the multiplicity of pathway end products (24-alkyl-Δ^5-sterols), compared to the uniqueness of cholesterol in vertebrates or ergosterol in fungi, has been discussed as a way to regulate membrane thermal shocks.[358] Indeed, ordering/disordering properties of membrane models made of sterols, sphingolipids, and deuterated dipalmitoylphosphatidylcholine (therefore mimicking natural rafts) were analyzed by solid-state ^{2}H-NMR in this study. Raft mimics with plant sterols showed less sensitivity to temperature variations than mimics with cholesterol or ergosterol.

The essentialness of Δ^5-sterols (therefore considered as primary metabolites) in multicellular organisms has been illustrated over the last decade by mostly biological and genetic approaches. The nematode *Caenorhabditis elegans*, which is an auxotroph to cholesterol, was grown on cholesterol or on synthetic *ent*-cholesterol for comparison. The enantiomer of cholesterol was unable to support development of *C. elegans*, a result which demonstrated that the absolute configuration of cholesterol, in addition to its biophysical properties, was essential.[359] In the model plant *A. thaliana*, the characterization of sterol biochemical mutants, which were affected in biosynthetic steps between squalene and the end products campesterol and sitosterol, clearly showed

that sterol intermediates are unable to support normal growth and development in replacing Δ^5-sterols structurally or as precursors of brassinosteroids (see Section 1.21.2.4.3). The same consideration is true in humans in the case of loss-of-function mutations that affect cholesterol biosynthetic genes: mutations in NSDHL (3β-hydroxysteroid dehydrogenase) cause the CHILD syndrome,[360] mutations in the Δ^8–Δ^7-sterol isomerase cause the Conradi–Hünermann–Happle syndrome,[361] mutations in the human CYP51 cause the Antley–Bixler syndrome,[362] and mutations in the Δ^7-reductase cause the Smith–Lemli–Opitz syndrome (SLOS).[363] All these syndromes consist of severe developmental defects.

Cholesterol also exerts a crucial role in animal (insect and vertebrates) development as part of its signaling processes. In fact, cholesterol covalently modifies HEDGEHOG-secreted signaling proteins that are essential in embryogenesis.[364–366] Covalent binding of cholesterol to the N-terminal end of the signaling protein is achieved by the cholesterol transferase activity of its C-terminal end. In the signaling process, HEDGEHOG binds its PATCHED receptor, which contains a conserved sterol-sensing domain.[367] Interestingly, mammalian meiosis was activated by intermediates of the cholesterol pathway MAS (meiosis-activating sterols).[368]

Abbreviations

ABC	ATP-binding cassette
ACAT	acyl-coA-cholesterol acyltransferase
ARE	ACAT-related enzymes
bHLHZ	basic helix–loop–helix leucine zipper
BRI	brassinosteroid insensitive
BZR	brassinazole resistant
CPI	cyclopropylsterol isomerase
CRE/*lox*P	cyclization recombination locus of X-over P
CVP	cotyledon vein patterning
CYP51	sterol-14-demethylase
CYP710	sterol-22-desaturase
CYP85A2	7-oxolactone synthase (brassinolide synthase)
CYP90C1	brassinosteroid-C23-hydroxylase
CYP90D1	brassinosteroid-C23-hydroxylase
DET2	steroid-5α-reductase
DIMINUTO/DWARF1	sterol-Δ^{24}-isomerase/reductase
DWARF5	$\Delta^{5,7}$-sterol Δ^7-reductase
EBP	emopamil-binding protein
EMS	ethyl methane sulfonate
ERG11/CYP51	lanosterol-14-demethylase
ERG11/CYP51	sterol-14-demethylase
ERG2	Δ^8-sterol-Δ^8-Δ^7-sterol isomerase
ERG24	$\Delta^{8,14}$-sterol-Δ^{14}-reductase
ERG25/SMO	sterol C4-methyl oxidase
ERG26/ 3βHSD/D	4α-carboxysterol-3β-hydroxysteroid/C4-decarboxylase
ERG27/SR	3-oxosteroid reductase
ERG3	Δ^7-sterol-C5(6)-desaturase
ERG4	sterol-Δ^{24}-isomerase/reductase
ERG5/CYP61	sterol-22-desaturase
ERG7	lanosterol synthase
FACKEL	$\Delta^{8,14}$-sterol-Δ^{14}-reductase
FAD	flavin adenine dinucleotide
GC–MS	gas chromatography–mass spectrometry
GFP	green fluorescent protein
HMGR	3-hydroxy-3-methylglutaryl coenzyme A reductase

HSP	heat-shock protein
HYDRA1/SI	Δ^8-sterol-Δ^8-Δ^7-sterol isomerase
LBR	lamin B receptor
LCAT	lecithin cholesterol acetyltransferase
LDL	low-density lipoprotein
LTP	lipid transfer protein
NADH	nicotinamide adenine dinucleotide, reduced form
NADPH	nicotinamide adenine dinucleotide phosphate, reduced form
NMR	nuclear magnetic resonance
NSDHL	3β-hydroxysteroid dehydrogenase
ORP	oxysterol-binding protein-related protein
OSC/CAS1	oxidosqualene cyclase/cycloartenol synthase
OSC/LAS1	oxidosqualene cyclase/lanosterol synthase
OSC/βAMS	oxidosqualene cyclase/β-amyrin synthase
PSAT	phospholipid sterol acyltransferase
SCAP	SREBP cleavage activation protein
SCP	sterol carrier protein
SDR	short-chain dehydrogenase/reductase
Seladin	selective Alzheimer's disease indicator
SGT	solanidine glucosyl transferase
SMO	sterol-4α-methyl-oxidase
SMT/ERG6	zymosterol-C24-methyltransferase
SMT1	cycloartenol-C24-methyltransferase
SMT2	24-methylene-C24^1-methyltransferase
SQC	squalene cyclase
SQE	squalene epoxidase
SR	sterone reductase
SRE	sterol regulatory element
SREBP	sterol regulatory element-binding proteins
STE1/DWARF7/BUL1	Δ^7-sterol-C5(6)-desaturase
T-DNA	transfer DNA
UDP	uridine diphosphate
USGT	(UDP)-glucose sterol-β-D-glucosyltransferase
VEP	vein patterning
VIGS	virus-induced gene silencing
3βHSD/D	4α-carboxysterol-3β-hydroxysteroid dehydrogenase/C4-decarboxylase
5βPOR	progesterone 5β-reductase

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