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Review

Yeast metabolic engineering – Targeting sterol metabolism and terpenoid formation

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ABSTRACT

Terpenoids comprise various structures conferring versatile functions to eukaryotes, for example in the form of prenyl-anchors they attach proteins to membranes. The physiology of eukaryotic membranes is fine-tuned by another terpenoid class, namely sterols. Evidence is accumulating that numerous membrane proteins require specific sterol structural features for function. Moreover, sterols are intermediates in the synthesis of steroids serving as hormones in higher eukaryotes. Like steroids many compounds of the terpenoid family do not contribute to membrane architecture, but serve as signalling, protective or attractant/repellent molecules. Particularly plants have developed a plenitude of terpenoid biosynthetic routes branching off early in the sterol biosynthesis pathway and, thereby, forming one of the largest groups of naturally occurring organic compounds. Many of these aromatic and volatile molecules are interesting for industrial application ranging from foods to pharmaceuticals. Combining the fortunate situation that sterol biosynthesis is highly conserved in eukaryotes with the amenability of yeasts to genetic and metabolic engineering, basically all naturally occurring terpenoids might be produced involving yeasts. Such engineered yeasts are useful for the study of biological functions and molecular interactions of terpenoids as well as for the large-scale production of high-value compounds, which are unavailable in sufficient amounts from natural sources due to their low abundance.

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Abbreviations: ABC, ATP-binding cassette; ADH, alcohol dehydrogenase; ADR, adrenodoxin reductase; ADX, adrenodoxin; ARE2, sterol acyltransferase; ARV1, mediator of sterol homeostasis; BTS1, GGPP synthase; CPR, cytochrome P450 reductase; CRT, carotenogenic (genes); CYP, cytochrome P450 enzymes; DCW, dry cell weight; DGPP, diacylglycerol diphosphate; DHCR7, dehydrocholesterol 7-reductase; DHCR24, dehydrocholesterol 24-reductase; DMAPP, dimethylallyl diphosphate; DPP1, DGPP phosphatase; ER, endoplasmic reticulum; ERG1, squalene epoxidase; ERG2, C-8 sterol isomerase; ERG3, C-5 sterol desaturase; ERG4, C-24(28) sterol reductase; ERG5, C-22 sterol desaturase; ERG6, C-24 sterol methyl transferase; ERG7, lanosterol synthase; ERG8, phosphomevalonate kinase; ERG9, squalene synthase; ERG10, acetoacetyl-CoA thiolase; ERG11, C-14 sterol demethylase; ERG12, mevalonate kinase; ERG13, hydroxymethylglutaryl-CoA synthase; ERG19, mevalonate diphosphate decarboxylase; ERG20, geranyl/farnesyl diphosphate synthase; ERG24, C-14 sterol reductase; ERG25, C-4 methyl sterol oxidase; ERG26, C-3 sterol dehydrogenase; ERG27, 3-keto sterol reductase; FF-MAS, follicular fluid meiosis-activating sterol; FPP, farnesyl diphosphate; FPPS, farnesyl diphosphate synthase (ERG20); GGPP, geranylgeranyl diphosphate; GPCR, G-protein coupled receptor; GPP, geranyl diphosphate; HEM1, 5-aminolevulinate; HFA, hydroxylated fatty acids; HMG1/HMG2, hydroxymethylglutaryl-CoA reductase 1/2; HOG1, high osmolarity glycerol response; HPO, *Hyoscyamus muticus* premnaspirodiene oxygenase; HXT1, hexose transporter 1; HXT2, hexose transporter 2; IDI1, isopentenyl diphosphate isomerase; IPC, inositol phosphorylceramide; IPP, isopentenyl diphosphate; LPP1, lipid phosphate phosphatase 1; MAP, mitogen activated protein; MAS, meiosis-activating sterol; MK, mevalonate kinase; NADPH, reduced nicotinamide adenine dinucleotide phosphate; PHA, polyhydroxyalkanoates; PLC1, phospholipase C; PMK, phosphomevalonate kinase, see ERG8; PTC1, protein phosphatase type 2C; PUFA, polyunsaturated fatty acids; SMT1, sterol methyltransferase 1; SMT2, sterol methyltransferase 2; SUE, selection for aerobic uptake of exogenous ergosterol; TAT2, tryptophan permease; tHMG1, truncated 3-hydroxy-3-methylglutaryl coenzyme A reductase; T-MAS, testicular meiosis-activating sterol.

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1. Introduction

Sterols are essential lipid constituents of eukaryotic membranes. The structural features of terminal sterols differ among species. Whereas mammalian and fungal cells generally contain one major sterol, cholesterol and ergosterol, respectively, plants have complex sterol profiles, dominated by sitosterol, stigmasterol and campesterol [1,2]. The structural differences between these sterols are highlighted in the sterol biosynthetic pathways shown in Fig. 1. Common to all sterols is the double bond at C-5 and the 3β-hydroxyl group, enabling proper orientation and anchoring in the membrane [3]. In yeast as in higher eukaryotes, free sterols are predominantly present in the plasma membrane [4]. As "bulk" membrane components, sterols fulfill a major structural function of controlling the physical state of the plasma membrane by modulating its bilayer fluidity and permeability [5]. On the other hand, molecular interaction with specific sterols governs membrane protein stability and function [6,7]. These diverse roles of sterol molecules imply that eukaryotes had to develop elaborate control of sterol structures and sterol abundance. Beside regulating de novo synthesis accordingly, excessive sterols can be acylated and stored in lipid droplets [8,9]. Like higher eukaryotes, yeasts are equipped to acylate elevated amounts of sterols and re-mobilize them by lipases if needed [10–12]. Remarkably high portions of total cellular sterols can thus be sequestered from membranes sustaining their functionality. High metabolic flux rates through the sterol biosynthesis pathway indicate a substantial pool of isoprene units and render yeast an interesting production organism for sterols and other terpenoid compounds. Furthermore, sterol and other lipid biosynthetic pathways in yeasts, especially in *Saccharomyces cerevisiae*, have been intensively studied [13], making yeast an ideal host system for metabolic engineering approaches.

Metabolic engineering of microorganisms is developing into a major field in biotechnology. Metabolic analyses of cells to identify most promising targets for manipulation are followed by genetic engineering of cells [14–17]. The goals of metabolic engineering are the optimization of strains for overproducing recombinant proteins and small molecule chemicals, the extension of substrate range, enhanced productivity and yield, elimination of by-products, improvement of process performance and of cellular properties [18,19]. Regardless of the desired product, the common aim of metabolic engineering is to transfer product-specific enzymes to microorganisms in order to generate an optimized biosynthetic pathway in terms of minimal cost and maximum productivity [20].

Yeast, especially *S. cerevisiae,* has been successfully used as microbial system in metabolic engineering approaches for the

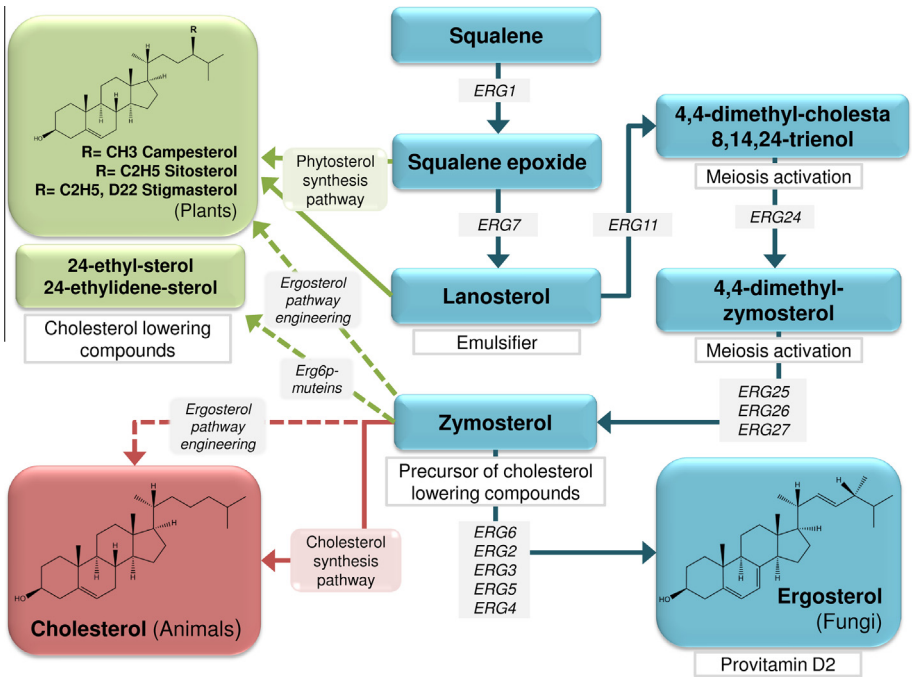


Fig. 1. Sterol biosynthesis pathways starting from squalene with sterol intermediates and end products in fungi (blue), plants (green) and animals (red). Solid arrows indicate naturally occurring reactions while dashed arrows denote engineering approaches described for yeasts. *ERG1*, Squalene epoxidase; *ERG2*, C-8 sterol isomerase; *ERG3*, C-5 sterol desaturase; *ERG4*, C-24(28) sterol reductase; *ERG5*, C-22 sterol desaturase; *ERG6*, C-24 sterol methyl transferase; *ERG7*, Lanosterol synthase; *ERG11*, C-14 sterol demethylase; *ERG24*, C-14 sterol reductase; *ERG25*, C-4 methyl sterol oxidase; *ERG26*, C-3 sterol dehydrogenase; *ERG27*, 3-keto sterol reductase.

production of high-value pharmaceuticals, industrially relevant enzymes and vitamins, and in recent years as well for the synthesis of lipid compounds of high commercial interest including sterols, steroid hormones and polyunsaturated fatty acids (PUFAs) [21–23]. In respect of the production of natural, value-added terpenoid compounds, metabolic engineering of the ergosterol biosynthesis (mevalonate) pathway in yeast has emerged as promising approach in synthetic biology [15,24–26]. Yeasts with modified sterol patterns and/or overproducing terpenoids have already been successfully applied in basic and applied research [22]. There are profound reasons to assume that their importance will grow in the near future. In basic research, molecular functions and interactions should be proven by reconstitution in heterologous systems to minimize the probability of overlooking a redundancy effect in the original host. For example, mammalian membrane proteins can be expressed and studied in sterol-modified yeasts [27]. Terpenoids obtained by fermentation of engineered yeasts are likely to provide a more consistent and uniform product quality than when extracted from natural sources. This is highly relevant as the uniformity of product quality and composition is a cornerstone of all approval procedures in the food and pharmaceutical industries. In particular, sterol-engineered yeasts have been useful for both, research and industrial production. Yeast synthesizing cholesterol instead of ergosterol aided in the understanding of the diverse sterol functions [28] and provides non-animal derived cholesterol for any commercial application representing a safe alternative to the risky animal-derived cholesterol, which is prone to contamination by viruses and prions [29].

2. Sterol-engineered yeast – basic research and applications

2.1. Sterol homeostasis

Numerous reviews describe how ergosterol is produced from acetyl-CoA in wild-type yeasts, particularly in *S. cerevisiae*. It is well-known which enzymes are involved, in which compartments of the cells the conversions take place and frequently also how they are regulated [4,13,30–32]. For some of the reactions, and their counterparts in higher eukaryotes, the reaction mechanisms have been clarified in detail [3]. The enzymes of ergosterol biosynthesis in *S. cerevisiae* appear to have been identified by 2001 [33]. Therefore, the recent years of yeast sterol research have been mainly devoted to investigating uptake from the culture medium, intracellular transport and storage/mobilization upon acylation/de-acylation [34–38]. Mass balances and even mathematical models of the ergosterol pathway in *S. cerevisiae* have been published [39]. Thus, it is not the goal of this work to give an extensive overview on ergosterol metabolism in wild-type strains of yeast, which is very well documented. Rather, we focus on yeasts with qualitatively and/or quantitatively modified sterol patterns.

Owing to the essential membrane-related functions of sterols in yeast, membrane sterol content and composition are highly preserved and tightly controlled, which is usually referred to as 'sterol homeostasis' [40]. Consequently, manipulation of the yeast sterol patterns will be counteracted by the mechanisms of sterol homeostasis. While the plasma membrane contains approximately 90% of the cellular free sterols, the endoplasmic reticulum (ER), despite being the site of sterol synthesis, has a low membrane sterol concentration [4,30,34,41,42]. This imbalance is preserved partially by sterol acyltransferases creating steryl ester pools in the ER [43]. Remarkably, the *S. cerevisiae* enzymes Are1p and Are2p seem to have some, but no strict sterol specificity suggesting that even sterols atypical to yeast will be recognized [44]. If required, sterols can be re-mobilized from the steryl ester pools of lipid droplets for membrane formation by the action of lipases [10–12]. Another decisive control mechanism of sterol homeostasis is intracellular sterol transport which occurs both vesicle-mediated and vesicle-independent [34–38,45,46].

2.2. Sterol-engineering in yeast

Superficially seen, the easiest way to modify the sterol patterns of yeast is to enforce uptake of externally added sterols. *S. cerevisiae* synthesizes ergosterol under aerobic conditions and becomes auxotrophic for sterols when molecular oxygen is not available [47]. Under these conditions, sterols from the environment can be taken up by the cells to compensate for the sterol biosynthetic defect [48,49]. The molecular mechanisms of anaerobic sterol uptake are not well understood, but could be governed by the organisation of the cell wall that might prevent exogenous sterols from reaching the plasma membrane [42]. Hypoxic or even anoxic conditions elicit many further cellular responses beside sterol uptake [50,51] and, thus, might not be the method of choice to incorporate foreign sterols into yeast. Alternatively, heme-deficiency triggers a similar sterol-uptake response as anaerobic conditions because endogenous sterol synthesis is blocked in both situations [49,52]. Screens have been performed on *S. cerevisiae* single-gene knockout collections to elucidate the mechanisms of sterol uptake and transport [53,54]. Uptake assays based on radio- and fluorescently-labelled cholesterol identified 17 yeast knockout strains strongly affected in uptake and/or esterification of exogenously supplied cholesterol. Using heme-deficient cells, mutations were identified which affected the expression and localisation of the ABC transporters Aus1p and Pdr11p. The latter two proteins had already been shown to be involved in sterol uptake under anaerobic conditions [55,56]. A second class of sterol uptake mutants affected mitochondrial functions, indicating a not yet identified mitochondrial role in the uptake of sterols under anaerobic conditions. Interestingly, several of the sterol uptake mutants as well as an *aus1Δpdr1Δ* double mutant have electron-dense mitochondrial inclusions, suggesting that the aberrant mitochondria may result from impaired sterol uptake [53,54]. The precise nature of sterol transport mediated by Aus1p and Pdr11p is still a major question [57]. Involvement of non-vesicular movement of plasma membrane sterol to the endoplasmic reticulum (ER) mediated by both transporters has been suggested for *S. cerevisiae* [56]. In contrast, a fluorescence study showed that Aus1p and Pdr11p are required for the entry of external sterol molecules into the yeast plasma membrane and that the yeast cell wall is actively involved in binding and uptake of ergosterol-like sterols [58]. Another screen identified 56 mutants from the yeast non-essential deletion collection that differed in their susceptibility to both lovastatin and nystatin as compared to the wild-type control. While lovastatin inhibits sterol synthesis at the early stage of mevalonate formation, nystatin complexes ergosterol with high specificity. Yeast mutants susceptible to both drugs are supposedly affected in sterol homeostasis. Remarkably, mutations in phospholipase C (Plc1), protein phosphatase type 2C (Ptc1), and Arv1, resulted in elevated levels of free sterols in the cell [42,59].

A more direct way to modify sterol composition in yeast is to interfere with ergosterol biosynthesis by eliminating or suppressing Erg protein functions. This can be achieved either by manipulating or deleting the respective coding sequences in the genome or by applying inhibitors of ergosterol biosynthesis [30]. These measures lead to yeast strains that accumulate high amounts of compounds that are otherwise only precursors of ergosterol, e.g. zymosterol and cholesta-5,7,24-trienol (*erg6Δ* knockout) and ergosta-5,7-dienol (*erg5Δ* knockout) [28,60,61]. Sterols barely detectable in wild-type yeast may become major sterols, e.g. ergosta-8-enol (*erg2Δerg3Δ* double knockout). Furthermore, sterol-converting enzymes from other organisms can be recombinantly expressed yielding sterol structures atypical of yeasts

[22,28]. The best-studied genetic manipulation to increase the abundance of total yeast sterols is the overexpression of HMG-CoA reductase (*HMG*) either as full length form leading to stacked ER membranes, so-called 'karmellae' [62], or preferably as truncated HMG (*tHMG*) [63]. On the other hand, the yeast total sterol content can be reduced by downregulation of ergosterol biosynthesis. It was observed that hyperosmotic stress leads to transcriptional downregulation of *ERG* genes via the Hog1 MAP kinase and the Mot3 and Rox1 transcription factors [64]. Literature apparently lacks examples of intentionally downsizing the yeast sterol pool by genetically manipulating the regulatory machinery. However, metabolic engineering projects aiming at increasing the production of plant sesquiterpenes and other terpenoids in *S. cerevisiae* have reduced the total sterol pools by downregulating particularly *ERG9*, thereby diverting farnesyl diphosphate (FPP) building blocks [25,26,65,66]. By cleverly devised genetic engineering approaches, the sterol patterns of yeast can be altered quantitatively and/or qualitatively.

Sterol biosynthesis pathways have common steps in fungi, plants, and animals (see Fig. 1). Besides the conserved reactions, fungi have an additional branch that adds a methylidene/methyl group at position C-24 and desaturates position C-22 to form ergosterol, while mammals saturate double bonds at C-7 and C-24, to give cholesterol. Yeast has been proposed to have sterol and sphingolipid-dependent membrane domains which are important for protein sorting [67–69]. Both, cholesterol and ergosterol can form liquid ordered domains in artificial membranes [70]. Therefore, structurally different sterols might have relatively similar impact on the physical properties of membranes, but nevertheless their specific structures can be highly relevant for distinct proteins and, therefore, for certain cellular processes in yeast. In a recent study, yeast strains were created substituting ergosterol by cholesterol in order to be able to distinguish between raft-dependent and structure-dependent sterol functions [28]. To construct the cholesterol producing strain, the *ERG5* and *ERG6* genes were deleted, which are responsible for introducing the yeast specific changes into the sterol side chain. The *erg5Δerg6Δ* strain accumulated cholesta-5,7,24(25)-trienol as the dominant free sterol which was converted to cholesterol by the heterologous expression of dehydrocholesterol C-7 reductase (DHCR7) and dehydrocholesterol C-24 reductase (DHCR24) from *Danio rerio* in the *erg5Δerg6Δ* strain background. GC–MS analysis of total sterols from a stable, cholesterol producing strain harbouring codon optimized DHCR7 and DHCR24 genes revealed a sterol composition of 96% cholesterol. Applying the same genetic elements, yeast strains harbouring 7-dehydrocholesterol, campesterol (ergosta-5-enol) and desmosterol (cholesta-5,24(25)-dienol) as predominant sterols were obtained as well [28]. These and further sterol-engineered strains may (i) serve as sources to obtain high amounts of otherwise inaccessible sterols and (ii) can be applied to study the functions and molecular interactions of defined sterol species.

2.3. The impact of sterol structure on membrane proteins and cellular transport processes

Screening for yeast genes required for fluid phase endocytosis had yielded the first indication that this vesicular transport process is affected by specific membrane sterol structures [71]. In detail, a non-functional *erg2* allel was found to impair endocytosis, which triggered thorough studies on the endocytic phenotypes of yeast strains with altered sterol patterns [60,61]. The importance of the desaturation state of the sterol B-ring and of the side-chain methylation by C-24 methyl transferase (*ERG6*) was observed when analysing – among a collection of other *erg* mutant strains – *erg2-Δerg6Δ* and *erg3Δerg6Δ* double mutants for their endocytic capabilities [72]. Both double mutants were defective in the ligand-induced internalisation of Ste2p underscoring the importance of specific sterol structural features in the endocytic pathway.

Erg4p, C-24(28) sterol reductase, catalyses a final step in the ergosterol biosynthesis pathway converting ergosta-5,7,22,24(28)-tetraenol, which accumulates in *erg4Δ* cells, to ergosterol. Erg4p has been reported to play a major role in mating and cell fusion [73–75]. Recently, a genetic screen identified mutations in the *ERG4* gene, revealing the importance of structural features of sterols in polarized cell growth and cell fusion [75]. During mating conditions the shape of the cell changes to a pear-like shape or "shmoo", enabling direct contact with the mating partner. In an *erg4Δ* strain, defects in shmoo formation, but not in cell fusion were partially abolished by exogenously supplied ergosterol or cholesterol. Interestingly, deletion of *ERG5* in the *erg4Δ* background suppressed these defects. It was suggested that wild-type, *erg5Δ* or *erg4Δerg5Δ* cells containing sterols with zero or one double bond in their aliphatic chains lead to proper shmoo formation. Conversely, *erg4Δ* mutants accumulate ergosta-5,7,22,24(28)-tetraenol featuring two conjugated double bonds in the side chain. Either the more rigid aliphatic chain alters the biophysical properties of the lipid bilayer and, thus, prevents polarised cell growth and cell fusion events, or the sterol species accumulated in the *erg4Δ* strain does not meet the requirements of proteins involved in mating and cell fusion.

Not only cell fusion events but also the reverse processes are influenced by sterols of specific structure. It has been shown that meiosis can be activated by specific methylated sterol intermediates, so-called meiosis activating sterols (MAS). The accumulation of MAS has been described in yeast cells lacking sterol C-14 reductase (*ERG24*), C-4 oxidase (*ERG25*) and 5-aminolevulinate synthase (*HEM1*). The latter mutation allowed sterol import and rendered the strain viable under aerobic conditions. The *erg24Δerg25Δhem1Δ* triple mutant accumulated 4,4-dimethyl-cholesta-8,14,24-trienol (FF-MAS) and the *erg25Δhem1Δ* mutant produced 4,4-dimethyl-cholesta-8,24-dien-ol (T-MAS) when ergosterol and heme were supplemented [76]. MAS represent a class of potent regulators of reproductive processes, and their pharmacological activity can be applied in the control of reproduction and in vitro fertilisation.

The lipid composition of yeast membranes was known to have a major impact on compartmentalisation of solute transporters Can1p, Tat2p and Pdr12p, and their sorting to the cell periphery [69,77,78]. Thus, these proteins were the primary targets to elucidate the influence of ergosterol replacement by cholesterol on plasma membrane protein localisation and functionality in yeast [28]. Can1p is an arginine permease of the plasma membrane, and its localisation is dependent on the presence of phosphatidyl ethanolamine in the membrane [78,79]. Can1p is sensitive to the toxic arginine analogue canavanine, which is used to test the activity of the protein in yeast. The second transporter, Tat2p is the high affinity tryptophan permease, present in the plasma membrane only under low tryptophan concentrations [80]. The third transporter analysed was the ABC transporter Pdr12p, responsible for the resistance of yeast to weak organic acids. In the presence of weak organic acids, Pdr12p is highly expressed, localises to the plasma membrane and serves to efflux weak organic anions from the cell [81]. Mutants in the ergosterol biosynthesis pathway, like *erg6Δ*, have been found by screening the yeast deletion mutant collection for hypersensitivity to weak organic acids [82], suggesting that ergosterol plays a structural role in the resistance to weak organic acids. The activities of Can1p, Tat2p and Pdr12p were analysed by stressing wild-type, cholesterol-containing and other sterol-engineered yeast strains with canavanine, low tryptophan concentrations or sorbic acid. All three solute transporters seem to have different sterol requirements for their activity [28]. While ergosterol replacement by cholesterol showed almost no effect on targeting, localisation and activity of Can1p and Tat2p, the

solute transporter of the multidrug resistance family, Pdr12p, was not functional. Pdr12p activity was, furthermore, impaired in the presence of the sterols of the *erg5Δerg6Δ* mutant, implying a specific molecular interaction between the transporter protein and sterols, which is necessary for solute transport. Reduced association of Pdr12p with detergent resistant membranes (DRMs) in the cholesterol strain, despite its proper localisation to the plasma membrane, suggested that a direct interaction between Pdr12p and sterol molecules is required for fractionation into DRMs. It seemed that side-chain methylation and B-ring desaturation have a major impact on Pdr12p activity. An indirect effect via other membrane lipids was excluded, due to unchanged glycerophospholipid and sphingolipid profiles in the sterol-modified strains.

A more global model of sterol-membrane protein interactions in yeast and fungal plasma membranes in general was recently presented by te Welscher et al. [83,84]. Based on the observations that natamycin binds ergosterol without permeabilising the fungal plasma membrane, immediately inhibits amino acid and sugar import, and induces transcription of a plenitude of fungal plasma membrane transporters, it was proposed that many, if not all, transporters require proper sterol-interaction, even if they do not reside in sterol-enriched domains of the plasma membrane. This is a strong indication that many more molecular interactions between sterols and membrane proteins await their characterisation. Sterol-engineered yeast strains will be a promising tool for these studies.

Table 1
Sterol responsive membrane proteins (adapted version from [85]).

	Membrane protein	Sterol dependency	Effect	Refs.
G – Protein Coupled Receptors (GPCRs)	Serotonin (1A) receptor	Cholesterol	Cholesterol depletion leads to reduction in specific ligand binding and G-protein coupling to serotonin(1A) receptor	[243,244]
		Desmosterol	Replaces cholesterol for ligand binding function	[245]
	Rhodopsin	Cholesterol	Stabilizes rhodopsin; high cholesterol levels inhibit rhodopsin	[246–248]
			Cholesterol and docosahexaenoic acid (DHA) acyl chain-containing phospholipids influence rhodopsin function and stability	[249]
	Oxytocin receptor	Cholesterol	Acts as general stabilizer of the receptor	[250,251]
			Conformational changes leading to more compact receptor state	[252]
	Metabotropic glutamate receptors (*Drosophila melanogaster*)	Ergosterol	Glutamate binding to the receptor is dependent on the presence of ergosterol	[253]
	Human β3-adrenergic receptor	Cholesterol/ cholesterol-like sterols	Enhanced GPCR production in yeast with cholesterol-like sterols	[27]
	μ-opioid receptor	Cholesterol	Protein activity depends on cholesterol	[254]
ABC – Transporters	P-glycoprotein/MDR1	Cholesterol	Alters P-gp function; increases ATPase activity	[255,256]
		Cholesterol, stigmasterol, sitosterol, campesterol	Cholesterol increases the binding affinity of small drugs; sterols, but not ergosterol, enhance drug stimulated ATPase activity	[257]
	Pdr5p (*S. cerevisiae*)	Ergosterol	Reduced efficiency of Pdr5p (drug efflux pump) in *erg* mutants	[258]
	Cdr1p (*C. albicans*)	Ergosterol	Sphingolipid-ergosterol interactions are important in drug susceptibilities of yeast	[259–261]
	Pdr12p (*S. cerevisiae*)	Ergosterol	Exchange of ergosterol to cholesterol leads to impaired Pdr12p activity	[28]
Transporters	$Ca^{2+}/Mg^{(2+)}$-ATPase	Cholesterol	Increased thermal stability of the protein	[262]
	Na^+/K^+-ATPase	Cholesterol	Regulates expression of protein	[263]
			Cholesterolstabilizes the protein in presence of PtdSer	[264,265]
			Increases protein activity (in combination with phospholipids)	[266–268]
	γ-Aminobutyric acid transporter	Cholesterol	Stimulates transport up to 20-fold	[269]
	Dopamine transporter (DAT)	Cholesterol	Modulates protein conformation and alters cocaine binding	[270]
	Serotonin transporter (SERT)	Cholesterol	Modulates function by stabilizing protein	[271,272]
	Arginine permease (Can1p)	Ergosterol	Interaction of ergosterol with nystatin uncouples the permease from proton motive force	[273]
	Glucose/H^+ symporter (Hup1p)	Ergosterol	The presence of ergosterol resulted in an increased activity of the protein in vitro	[274]
	Tryptophan permease (Tat2p)	Ergosterol	Tryptophan uptake deficiency in yeast *erg6Δ* cells due to Tat2p sorting defect	[69,275]
			Sorting defects in *erg2Δ* mutant	[276]
			erg9 deletion promotes vacuolar Tat2p degradation	[277]
	Plasma membrane H^+-ATPase (*Zea mays L.*)	Cholesterol/ stigmasterol	Cholesterol and stigmasterol stimulate H^+ pumping	[278]
Channels	Nicotinic acetylcholine receptor (AChR)	Cholesterol	Key modulator of AChR function and dynamics	[279,280]
	Voltage-dependent anion-selective channel (Phaseolus coccineus)	Phytosterols	Selectivity and voltage-dependence are differentially altered by phytosterols	[281]

Apart from studying yeast protein function in sterol-modified strains, sterol-engineered yeast strains are finding a new field of application, lately. Sterol-engineered yeast may be beneficial for the heterologous expression of membrane proteins requiring specific sterol species, e.g. cholesterol, for localisation, stability and function [28]. Kitson and co-workers have enhanced the formation of mammalian G-protein coupled receptors (GPCRs), human β3-adrenergic and μ-opioid receptors, in an engineered *S. cerevisiae* strain producing cholesterol-like sterols [27]. They genetically modified the strain by replacing three genes coding for enzymes of the ergosterol biosynthesis pathway (*ERG6*, *ERG5*, *ERG4*) with cDNAs encoding human C-8 sterol isomerase, C-24 sterol reductase and C-7 sterol reductase, respectively. The cholesterol intermediates cholesta-5,7,24-trienol and cholesta-5,24-dienol, but no cholesterol were produced by the newly created strain, predominantly due to inefficient C-24 and C-7 sterol reductase activities. The influence of cholesterol on the function of membrane proteins, especially membrane receptors, has been described in several other studies, e.g. for Na/K–ATPase, rhodopsin or the oxytocin receptor [85]. Table 1 gives an overview of different membrane proteins, e.g. GPCRs and membrane transporters, which have been shown to be responsive to membrane sterol structure. The use of sterol-modified yeast strains in expression studies of sterol-dependent membrane proteins may serve a dual purpose. On the one hand, this approach will be revealing the sterol requirements for protein stability and function without the need to purify the proteins. On the other hand, sterol-engineered yeasts will provide access to substantial amounts of recombinant membrane protein that can be used for structural studies and the subsequent development of pharmaceuticals, many of which usually target membrane proteins.

2.4. Sterol-engineering may influence overall membrane lipid composition and membrane permeability

Significant evidence has been provided over the last two decades that proteins and lipids of the plasma membrane are not equally distributed, but that the layer is laterally segregated into dynamic micro-/nanodomains. These postulated membrane rafts are enriched in sterols and sphingolipids, which cluster and interact with each other and with specific proteins, affecting their function and/or localisation. The lipids in these subcompartments are thought to be enriched in saturated and longer hydrocarbon chains and hydroxylated ceramide backbones [86–88]. The structure of sterols and sphingolipids can greatly influence the tendency of lipid bilayers to form lipid domains/rafts, making them key players in the regulation of raft properties and/or dynamics [70]. Studies on molecular dynamics and biophysical behaviour have shown that sterols and sphingolipids can interact in artificial membranes [89–93].

Besides the biophysical interaction of sterols and sphingolipids in membranes, a close genetic interaction of the biosynthetic pathways of both lipid classes has been suggested by several studies using different yeast mutants [94]. Swain et al. investigated the sterol-dependent regulation of the sphingolipid pathway in an *erg26* mutant strain. This *erg26* mutation led to an accumulation of zymosterol instead of ergosterol. The loss of proper Erg26p (4α-carboxylsterol C-3 dehydrogenase) activity also leads to changes in sphingolipid levels and this sterol-dependent regulation most likely occurs at the points of ceramide biosynthesis and IPC hydroxylation [95]. Eisenkolb et al. provided further evidence for the cross-talk of both biosynthetic pathways [96]. The group compared the lipid composition of *erg6Δ*, *elo3Δ*, and double mutant strains and revealed compensatory changes in sterol and sphingolipid synthesis. The absence of ergosterol in the *erg6Δ* mutant was compensated by elevated levels of total sterols and sphingolipids, and mutations in *ELO3* were synthetically lethal with mutations in *ERG6*. Elo3p is a component of the ER-associated fatty acid elongase complex and required for the synthesis of C_{26} long chain fatty acids. The lethality of an *elo3Δ erg6Δ* double mutant was rescued by supplementation with ergosterol but not with cholesterol, indicating a structural requirement for the ergosterol-specific methyl group introduced by Erg6p in wild-type cells.

Valachovic et al. showed that a mutation in the *ERG2* gene is synthetically lethal with mutations in the transcription factors encoded by *UPC2* and *ECM22*. They identified a mutation in the *ELO3* gene, which acted as a suppressor of the lethal phenotype of the *erg2upc2ecm22* mutant [97]. Another systematic analysis of yeast mutants showed that cells have a mechanism to sense and react to changes in their membrane sterols by modifying their membrane lipid compositions affecting mainly sphingolipids. Combinations of mutations in sterol and sphingolipids biosynthesis resulted in a large number of synthetic and suppression phenotypes, providing evidence that both lipid classes cooperate functionally [98]. Yeast genome expression studies using antifungals targeting sterol biosynthesis have revealed that many genes involved in lipid metabolism are transcriptionally regulated in response to changes in sterol levels, among these were *SUR2* and *LCB1*, genes required for the production of phytosphingosine and 3-ketosphinganine, respectively [99,100].

Nystatin-resistant *erg* mutants were used in the study by Sharma [101] to investigate the in vitro effects of altered sterol structure on membrane lipid composition, fluidity, and asymmetry of phospholipids. Sharma suggested in this work that adaptive response in yeast mutants with altered sterol structures (*erg2Δ*, *erg3Δ*, *erg6Δ*) is manifested through changes in the membrane lipid composition and fluidity and not through transbilayer rearrangement of aminophospholipids. Recent work by Abe and Kiraki [102], focusing on the role of ergosterol on integrity and dynamics of the yeast plasma membrane, showed that the interfacial region of the plasma membrane is highly rigid and that altered sterols in *erg* mutants, particularly in the *erg2Δ* mutant, compromises rigidity. The altered sterol structures possibly fail to pack lipid acyl chains and thereby create voids within the plasma membrane leading to enhanced drug permeability. The hypersensitivity to cycloheximide is ascribed to enhanced passive diffusion of this lipophilic compound across the plasma membrane when the sterol composition is altered. The study revealed that the *erg6Δ* mutation diminished the rigidity of the membrane less than the *erg2Δ* mutation. Previous studies had shown that *erg6Δ* mutants contain equivalent amounts of zymosterol and cholesta-5,7,24-trienol which has a C-7,8 double bond [61]. Thus, sterols with properly conjugated double bonds in the B-ring like ergosterol or cholesta-5,7,24-trienol likely contribute to moderate packing of the plasma membrane.

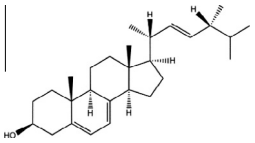
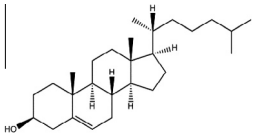
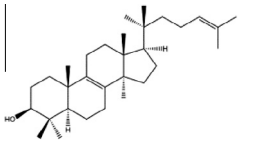
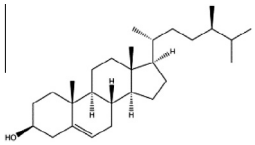
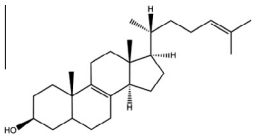
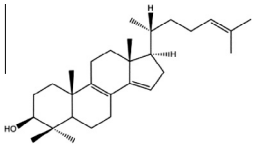
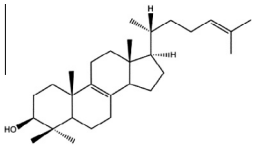
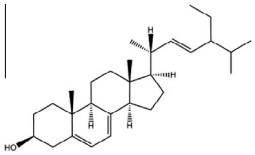
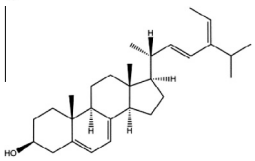
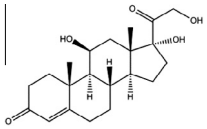
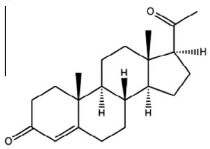
A study by Dupont et al. comparing the membrane behaviour during the dehydration–rehydration cycle between wild-type *S. cerevisiae* and a *erg6Δ* mutant strain suggested that the nature of membrane sterols influences the mechanical properties of the plasma membrane during hyperosmotic perturbation [103]. The mutant strain, which accumulated zymosterol and cholesta-5,7,24-trienol was more sensitive to osmotic fluctuations than the ergosterol producing strain. The hypersensitivity of *erg6Δ* was linked to modifications of the membrane properties, such as stretching resistance and deformation, which led to plasma membrane permeabilisation during the dehydration–rehydration process.

3. Metabolic engineering of yeast for terpenoid synthesis

3.1. Modification of cellular sterol metabolism for enhanced production of industrially relevant lipid compounds

Ergosterol is an economically important product, as it is a precursor of vitamin D2 or cortisone [104], but also has potential in

Table 2
List of some industrially relevant sterols and steroids produced in yeast.

	Produced compound		Genetic modifications	Refs.
STEROLS		Ergosterol (Ergosta 5,7,22-trienol)	Overexpression of *tHMG1*, *ERG1* and *ERG11*, overexpression of *ERG4* and *ARE2*	[63,108–110,161]
		Cholesterol (Cholesta-5-enol)	*erg5Δerg6Δ*, overexpression of DHCR7 and DHCR24	[28]
		Lanosterol (4,4, 14-trimethylcholesta-8,24- dienol)	Overexpression of *tHMG1* and *ERG1*	[63]
		Campesterol (Ergosta-5-enol)	*erg5Δ*, overexpression of DHCR7	[28]
		Zymosterol (Cholesta-8, 24-dienol)	*erg6Δerg2Δ*	[114]
		4,4-Dimethylcholesta-8,14, 24-trienol	*erg24Δ erg25Δhem1Δ*	[76]
		4,4-Dimethylzymosterol	*erg25Δhem1Δ*	[76]
		24-Ethyl sterol	Mutations in *ERG6*	[115] [282]
		24-Ethylidene sterol	Expression of SMT from *N. tabacum* or *A. thaliana* in *erg6Δ* mutant	[283] [284]
STEROIDS		Hydrocortisone (Cortisol) (11β,17α,21-trihydroxy-4-pregnene-3,20-dione)	Expression of CYP11A1, ADX, ADR, mitochondrial ADX and CYP11B1, 3β-HSD, CYP17A1, CYP21A1 and ARH1 in *atf2Δgcy1Δypr1Δ* strain	[126]
		Progesterone (Pregn-4-ene-3,20-dione)	Expression of *At*C-7 reductase, CYP11A1, ADX, ADR, 3β-HSD3 in *erg5Δ* strain	[122]

the development of anticancer drugs [105]. Various strategies have been applied to enhance the native sterol content of yeast, i.e. ~0.6% of the dry cell weight (DCW), for commercial application of the sterols, including screening for high-sterol strains, manipulation of the ergosterol biosynthesis pathway or the optimisation of fermentation conditions [63,106,107]. Combined overexpression

of *tHMG*, *ERG1* and *ERG11* genes [63] or overexpression of sterol C-24(28) reductase (Erg4p) [108] and the sterol acyltransferase (Are2p) [109] have been tested. He and co-workers [110] successfully developed a strategy combining overexpression of *ERG4* and *ARE2* genes with optimisation of fermentation conditions by using cane molasses as inexpensive carbon source. Ethanol accumulation during yeast fermentation is an important parameter in ergosterol production. The increase of ethanol concentration to 15% in the cultivation medium leads to an accumulation of ergosterol and squalene, but causes a decrease in cell growth due to reduced membrane integrity of the cells [111]. Nitrogen limitation during the fermentation process has also been shown to have a stimulating effect on yeast sterol content. Limiting the nitrogen supply results in reduction of biomass formation but ongoing lipid biosynthesis [112,113].

Besides ergosterol, the sterol pathway in yeast harbours also other economically interesting sterol metabolites, such as lanosterol (4,4,14-trimethyl-cholesta-8,24-dienol) or zymosterol (cholesta-8,24-dienol) (Table 2). Lanosterol is added to cosmetic products as an emulsifier. Genetic modification of the ergosterol pathway by overexpression of *ERG1* (squalene epoxidase) and *tHMG1* resulted in accumulation of lanosterol [63]. Zymosterol, which is used as precursor for the production of cholesterol lowering substances, could be accumulated in a yeast strain deleted for *ERG2* and *ERG6* genes [114]. Nes et al. modified the C(1)-transfer activity of the fungal C-24 sterol methyltransferase (SMT1, Erg6p) by site-directed mutagenesis leading to a switch to plant-like C(2)-transfer activity (SMT2) [115,116]. Plant 24-ethyl sterols (phytosterols) are industrially important substances due to their cholesterol lowering and anti-inflammatory properties [117–119].

3.2. Biosynthesis of steroids in engineered yeast cells

Steroids are a large family of structurally similar compounds responsible for multiple and diverse actions in the body, including anti-inflammatory, sex-determining and growth-regulating activities [22]. As natural steroids are derived from squalene by cyclisation, desaturation and demethylation, they may be considered as modified triterpenes, like ergosterol, cholesterol and phytosterols (see Fig. 1). Steroid hormones are complex molecules composed of a four ring gonan system with a multitude of chiral centers, which makes chemical synthesis difficult. Hydrocortisone (11β,17α,21-trihydroxy-4-pregnene-3,20-dione), a member of the glucocortico-steroids, is the major steroid in mammals and is of great biotechnological interest due to its use as starting material for synthesis of drugs with anti-inflammatory, abortive or anti-proliferative effects [120]. Several mammalian cytochrome P450 enzymes (CYPs) were functionally expressed in *S. cerevisiae*, and hydrocortisone precursors could be produced in bioconversion experiments using diverse substrates [121,122]. Other yeasts such as *Schizosaccharomyces pombe* or *Yarrowia lipolytica* have also been documented to be valuable hosts for CYP expression and bioconversion experiments leading to hydrocortisone production [123–125].

Szczebara et al. generated a *S. cerevisiae* strain capable of hydrocortisone biosynthesis from a simple carbon source by an artificial and fully self-sufficient pathway involving 13 engineered genes [126]. The strategy was based on in vivo production of ergosta-5-enol as substrate for the bovine mitochondrial CYP11A1 enzyme by overexpressing a plant C-7 reductase and a yeast NADPH-reductase encoded by *NCP1* [122]. Mammalian mitochondrial proteins are supplied with electrons via NADPH by two associated proteins, NADPH adrenodoxin oxidoreductase (Adrp) and adrenodoxin (Adxp). Additionally, an artificial electron transfer chain was generated by combining the natural yeast reductase Arh1p and the bovine Adxp electron carrier. Unwanted side reactions like the esterification of pregnenolone by Atf2p (acetyl transferase) and the 20-keto reduction of 17α-hydroxyprogesterone catalysed by the gene products of *GCY1* (galactose-inducible crystalline-like yeast protein) and *YPR1* (aldo–keto reductase), were abolished by inactivation of the respective genes. Finally, the biosynthesis pathway involved eight mammalian proteins, i.e. mature forms of CYP11A1 (P450 side chain-cleaving), adrenodoxin (ADX) and adrenodoxin reductase (ADR), mitochondrial forms of ADX and CYP11B1 (11β-steroid hydroxylase), 3β-hydroxy steroid dehydrogenase, CYP17A1 (17α-steroid hydroxylase) and CYP21A1 (21-steroid hydroxylase). This engineered yeast strain is an outstanding example of the heterologous reconstitution of a complex and mainly membrane bound biosynthetic pathway in *S. cerevisiae* [22,126,127]. Numerous steroid biotransformations are described for fungal systems, however only a limited number involves engineered yeasts [32]. Lately, particularly the fission yeast *S. pombe* has been applied to produce or convert steroids with the potential for human application [123,124,128–130].

3.3. Metabolic engineering of yeast for terpenoid production

Terpenoid biosynthetic routes are at the centre of interest in synthetic biology as relevant genes from different biological sources (e.g. plants, bacteria, and fungi) can be reassembled in recombinant microorganisms yielding in a plenitude of natural, high-value compounds. Terpenoids belong to the largest class of natural products with important medical and industrial properties. They are used as flavours and fragrances or food additives, as they are primary constituents of the essential oils of plants and flowers, e.g. (−)-limonene, (+)-valencene, menthol and zingiberene. Furthermore, terpenoids serve as pharmaceuticals, e.g. artemisinin, taxol and prostratin, and are known for their various biological functions, such as their use as hormones, e.g., steroids, gibberellins and abscisic acid, and their roles in membrane fluidity maintenance (sterols), respiration (quinones) or in photosynthesis in plants (carotenoids) [24,131,132]. Extraction of terpenoids like artemisinin or taxol from natural sources is often low-yielding, inefficient, and dependent on unpredictable fluctuations in environmental conditions or external circumstances. Development of synthetic microbial production platforms for the biosynthesis of complex terpenoids provides a good alternative to the standard extraction methods, due to large-scale and cost-effective industrial production via fermentation, which is independent from climate and cultivation risks. Fig. 2 shows an overview of the terpenoid biosynthesis pathway in yeast and latest cell engineering approaches for the production of industrially relevant compounds.

The biosynthesis of terpenoid compounds in yeast starts from isopentenyl diphosphate (IPP), an intermediate of the ergosterol biosynthetic pathway (mevalonate pathway). Several metabolic engineering approaches have been published in the last years for enhancing the production of mono-, sesqui- or diterpenoids in different host systems [24,133,134]. The yeast *S. cerevisiae* has been used in several studies for modification of the terpenoid biosynthesis pathway, thus enhancing production of these compounds [14,24,25,131,134,135]. Engineering of the pyruvate dehydrogenase bypass in *S. cerevisiae* resulted in general enhancement of acetyl-CoA supply in the cells, an early precursor of all terpenoid compounds. The simultaneous overexpression of an acetaldehyde dehydrogenase (*ALD6*) and a *Salmonella enterica* acetyl-CoA synthetase gene led to increased carbon flux into the mevalonate pathway, and therefore higher levels of terpenoids [136].

3.3.1. Monoterpenoids

Wild-type *S. cerevisiae* strains, with the notable exception of a few winemaking strains [137], do not produce monoterpenoids (C_{10}-isoprenoids). Winemaking yeast strains were found to

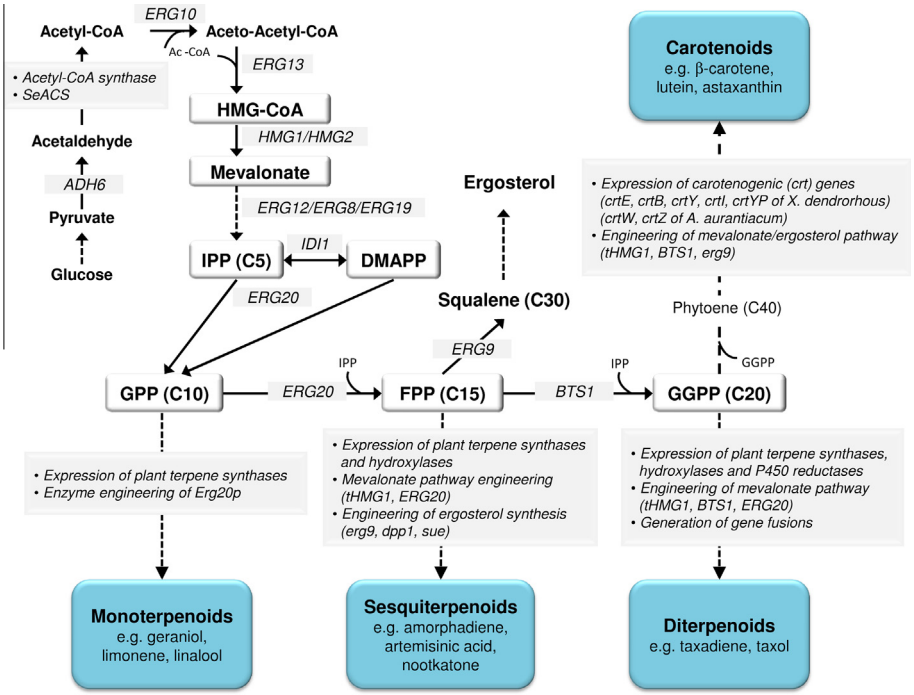


Fig. 2. Isoprenoid pathway in yeast and metabolic engineering strategies for production of interesting terpenoids. *ADH6*, alcohol dehydrogenase; *ERG10*, acetyl-CoA C-acetyltransferase; *ERG13*, hydroxymethylglutaryl-CoA synthase; *HMG1/HMG2*, hydroxymethylglutaryl-CoA reductase 1/2; *ERG12*, mevalonate kinase; *ERG8*, Phosphomevalonate kinase; *ERG19*, Mevalonate diphosphate decarboxylase; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; GPP, geranyl diphosphate; FPP, farnesyl diphosphate; GGPP, geranylgeranyl diphosphate; *IDI1*, isopentenyl diphosphate isomerase; *ERG20*, geranyl/farnesyl diphosphate synthase; *BTS1*, GGPP synthase; *ERG9*, squalene synthase; *Se*ACS, Acetyl CoA synthase from *Salmonella enterica*.

accumulate traces of linalool, α-terpineol, citronellol and geraniol. As monoterpenoid accumulation in these yeasts was fostered by microaerobic conditions and enhanced supply of nitrogen source including amino acids, it was speculated that mitochondrial rather than glycolysis-based isoprene formation delivers the building blocks for terpenoid biosynthesis [137]. In laboratory strains of *S. cerevisiae*, both geranyl- (GPP) and farnesyl-diphosphate (FPP) synthase activities are shared by one enzyme termed farnesyl diphosphate synthase (FPPS, Erg20p). For a long time it was thought that GPP is tightly bound to FPPS and cannot be released from the catalytic site leading to complete conversion to FPP [138]. Analysis of a yeast strain with a K197E mutation in the catalytic site of Erg20p, however, exhibited unusual properties such as excreting prenyl alcohols like geraniol and linalool (Fig. 3) [139,140], and

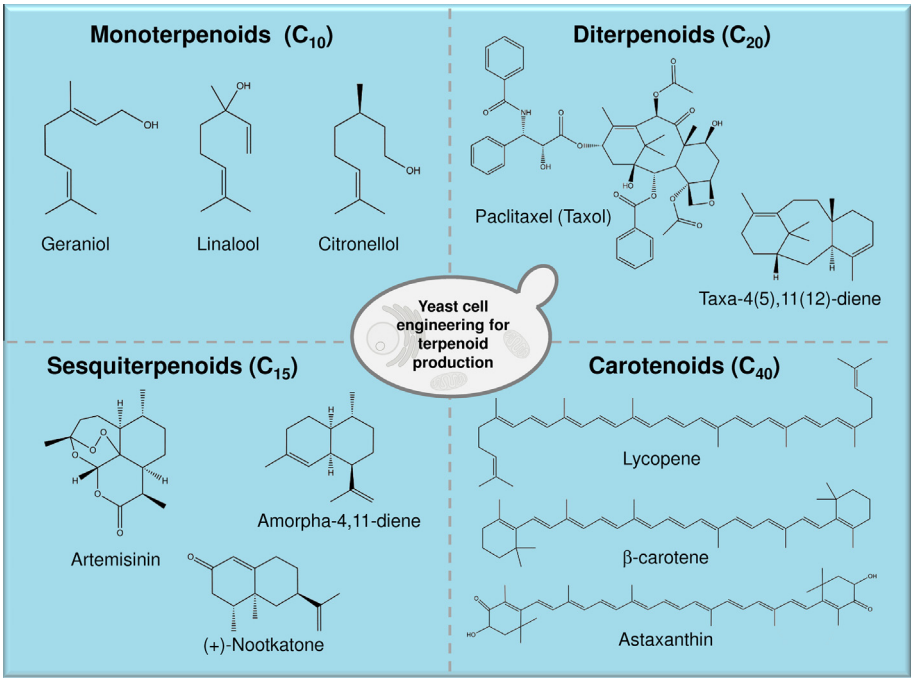


Fig. 3. Structures of some industrially interesting terpenoids or terpenoid precursors biosynthetically or semi-synthetically produced by engineered yeast strains.

synthesizing significantly longer dolichols than the wild-type strain [141]. Further amino acid exchanges in Erg20p (K197R, K197E, and K197V) highlighted the correlation between the FPPS activity, the amount of ergosterol synthesized and cell growth [142]. Monoterpenoid production capability of *S. cerevisiae* has been accomplished by co-expression of – for example – geraniol or linalool synthases (see Fig. 2) [143,144]. Oswald et al. demonstrated that, in addition to the *ERG20* mutations described above, monoterpenoid production capacities could be further increased by co-expressing geraniol synthase (GES) from *Ocimum basilicum* (sweet basil) in *S. cerevisiae* [143,145]. Analysis revealed that the expression of GES had a suppressing effect on sterol biosynthesis, which was dependent on the genetic background of the wild-type strains. Furthermore, the Karst group showed that blocking ergosterol biosynthesis downstream from GPP synthesis by amorolfine strongly inhibited ergosterol formation but did not affect terpenoid production [143]. Therefore, geraniol formation could not further be enhanced by blocking the synthesis of ergosterol.

Another study showed that linalool yields were improved by deregulated overexpression of a *tHMG* allele in *S. cerevisiae*, although the levels were still too low for industrial applications and further strain development was suggested to be beneficial [146]. A major drawback in high-level monoterpenoid production by engineered cell systems is the toxicity of the C_{10} compounds in microorganisms, which is caused by the interference of the lipophilic compounds with cell membranes [147,148]. The use of an extractive solvent in a bi-phasic biotransformation reaction, e.g. dodecane, is a common approach to remove toxic products. However, biocompatibility of organic solvents with the host cells, monoterpenoid distribution, phase separation and the costs have to be considered when choosing a solvent. Recently, Brennan et al. analysed five monoterpenoid products regarding their inhibitory limits in *S. cerevisiae* and showed that the toxicity is likely a phase effect [149]. The application of bi-phasic fermentation systems significantly enhanced monoterpene production in *S. cerevisiae*. It was demonstrated that the yeast product farnesene can be used as extractant and co-product for microbial terpene-based jet fuel production, which seems to be an attractive bioprocessing option.

3.3.2. Sesquiterpenoids

Sesquiterpenoids (C_{15}-isoprenoids) are a large and highly diverse class of isoprenoids, naturally occurring in plants and insects. Besides their characteristic flavours and aromas they also show anticancer, antitumor, cytotoxic and antibiotic properties, making them industrially relevant compounds (see Fig. 3) [133,150–153]. For example, amorpha-4,11-diene is a precursor of artemisinin, a sesquiterpene endoperoxide lactone, which is a highly effective anti-malarial drug [154,155] and a potential anticancer agent [150,156,157]. The phytoalexin capsidiol is a bicyclic, dihydroxylated sesquiterpene produced by numerous solanaceous species, e.g. *Nicotiana tabacum* in response to fungal elicitation. It is formed via the isoprenoid pathway from 5-*epi*-aristolochene [158,159].

Jackson et al. reported higher yields of the sesquiterpenoid epicedrol, by overexpressing the corresponding synthase gene and *tHMG1* in the presence of a mutation conferring sterol uptake (*upc2–1*) from the exogenous media [160]. Overexpression of the catalytic domain of Hmg1p in yeast results in accumulation of squalene in the cells [25,63,161]. Disruption or downregulation of the squalene synthase gene (*ERG9*) minimized the FPP flux towards sterol biosynthesis and more FPP was available for other terpenoid producing enzymes such as heterologously expressed sesquiterpene synthases, e.g. valencene, cubebol, patchoulol or amorphadiene synthases [25,26,65,66,135,162,163]. Ro et al. showed that sesquiterpene production in yeast could be dramatically improved with a combination of alterations, including overexpression of *tHMG1*, downregulation of *ERG9*, introduction of the *upc2-1* allele and overexpression of FPP synthase (Erg20p) [65]. These modifications led to enhanced levels of amorphadiene, which could be further oxidized to the antimalarial drug precursor artemisinic acid by the cytochrome P450 monooxygenase CYP71AV1. Recently, Westfall et al. [164] communicated a semi-synthetic route for production of the anti-malaria agent artemisinin. They engineered the mevalonate pathway in *S. cerevisiae* by overproducing every enzyme involved and further overexpressed *ERG20* thereby doubling artemisinic acid production compared to the reference strain. The production level of amorpha-4,11-diene at > 40 g/L was even clearly higher than artemisinic acid yield. Thus, amorpha-4,11-diene was used as precursor for chemical synthesis of artemisinin.

In the work of Takahashi et al. the carbon flux through the mevalonate pathway was enhanced and FPP was accumulated by impairing Erg9 function, thereby obtaining a mutant capable of efficient, aerobic uptake of ergosterol from the culture media [135]. Simultaneously, endogenous dephosporylation of FPP was limited by knocking out a phosphatase activity encoded by *DPP1* known to contribute to the hydrolysis of FPP in yeast [165]. The high FPP pool permitted high-level production of sesquiterpene hydrocarbons and hydroxylated products by co-expressing terpene synthase genes, cytochrome P450 hydroxylases and cytochrome P450 reductases [135]. In another study by Takahashi et al. they characterized the *Hyoscyamus muticus* premnaspirodiene oxygenase (HPO), which catalyses the oxidation of various sesquiterpenes, including (+)-valencene, which is an aroma compound of the essence oil of Valencia orange (*Citrus sinensis*) and precursor of the highly demanded grapefruit flavour compound (+)-nootkatone [166]. Engineering of the substrate recognition sites of HPO enhanced the formation of the intermediate product nootkatol, but HPO failed to synthesize (+)-nootkatone. Especially, plant derived P450 enzymes have been shown to be capable of oxyfunctionalisation of (+)-valencene to (+)-nootkatone. Recently, cytochrome P450 monooxygenase CYP71AV8 from chicory (*Cichorium intybus* L.) was identified to be able to hydroxylate (+)-valencene, but the expression of the enzyme in a (+)-valencene producing *S. cerevisiae* strain yielded only limited amounts of (+)-nootkatone [167].

Enhanced production levels of α-santalene, a precursor of α-santalol being one of the main components of East Indian sandalwood oil, could be accomplished by metabolic engineering of *S. cerevisiae* coupled with fermentation optimisation [168,169]. The promoters of the hexose transporter genes *HXT1* and *HXT2* were chosen to govern expression of *ERG9* and antisense-*ERG9*, respectively, in a fed-batch process. The aim was to couple squalene synthase activity to glucose concentration, i.e. to achieve maximal repression during the feed phase when glucose is limiting. Using the *HXT1* promoter to control *ERG9* expression, it was possible to divert the carbon flux from sterol synthesis towards α-santalene improving the productivity by 3.4-fold. Additionally, overexpression of *tHMG1*, deletion of *LPP1* (lipid phosphate phosphatase), and *DPP1* (diacylglycerol diphosphate phosphatase) generated a strain with a final α-santalene productivity of 0.21 mg g^{-1} DCW h^{-1}.

Recently, a carotenoid-based phenotypic screen for visual selection of improved isoprenoid producing strains was described [170]. The group expressed carotenogenic genes in a *S. cerevisiae* single-knockout collection and strains which showed improved carotenoid levels were further screened for the production of the sesquiterpene bisabolene, which is an alternative to petroleum-derived diesel. The carotenoid-based pre-screening in combination with other mevalonate pathway engineering approaches led to a final bisabolene production level of 800 mg/L in shake flask cultures.

3.3.3. Diterpenoids

Diterpenoids (C_{20}-isoprenoids) are produced from geranylgeranyl diphosphate (GGPP) by cyclisation and further metabolism

yielding a variety of important compounds used as pharmaceuticals, agrichemicals or herbal medicines, e.g. paclitaxel (Taxol) which is used as efficient drug in cancer chemotherapy [171]. Paclitaxel is a complex diterpenoid containing three benzene rings in the molecule and was first isolated from the bark, roots, and branches of western yew, *Taxus brevifolia* (Fig. 3). Due to inefficient and environmentally costly extraction of paclitaxel from natural sources, major focus in research was put on alternative methods, including chemical synthesis, plant cell culture strategies, microbial fermentation using endophytic fungi or metabolic engineering approaches using *Escherichia coli* or *S. cerevisiae* [172–179] .

Taxol biosynthesis involves 19 enzymatic steps and parts of the involved terpenoid pathway could be transferred into bacteria or yeast. Engels et al. [177] engineered the yeast *S. cerevisiae* towards the enhanced production of taxa-4(5),11(12)-diene (taxadiene), a precursor of Taxol. The group expressed the *Taxus chinensis* taxadiene synthase and *tHMG* genes in combination with a GGPP synthase gene from *Sulfolobus acidocaldarius (Sa*GGPS) in *S. cerevisiae.* Strain engineering led to a 40-fold increase in taxadiene level yielding 8.7 mg/L. Dejong et al. described the independent functional expression of eight Taxol biosynthetic genes in *S. cerevisiae*, and reported on the installation of five sequential steps leading from primary metabolism to taxadiene-5α-acetoxy-10β-ol synthesis [175]. Cytochrome P450 taxoid hydroxylases have been described to act on taxadiene to make very low levels of taxadiene-5α-ol. The activity was improved 7-fold by co-expression of a *Taxus* cytochrome P450 reductase [180].

Tanshinones are a group of active diterpenoids found in Chinese medicinal herb *Salvia miltiorrhizha*, which exhibit diverse pharmacological activities including antibacterial, anti-inflammatory, antioxidant, neuroprotective, cardioprotective, and antitumor effects [181]. Recently, the production of miltiradiene, which is the common precursor in the tanshinone biosynthetic pathway, has been improved by metabolic engineering in *S. cerevisiae*. Active site engineering and fusions of the copalyl diphosphate synthase (*Sm*CPS) and CPP kaurene synthase-like enzyme (*Sm*KSL) as well as of Bts1p (GGPP synthase) and Erg20p (farnesyl diphosphate synthase), led to significantly enhanced miltiradiene production, yielding 365 mg/L in a 15 L bioreactor and concomitantly reduced byproduct accumulation [182]. Dai et al. showed a similar engineering approach including combinatorial over-expression of fusion genes *tHMG1/upc2-1* and *ERG20/BTS1* together with *Sa*GGPS leading to 488 mg/L miltiradiene in 5 L fed-batch fermentation [183]. Co-expression of a fusion of the *BTS1* and farnesyl diphosphate synthetase (*ERG20*) genes along with the *HMG1* gene had already been published as successful strategy for the production of geranylgeraniol, an acyclic diterpene alcohol, used as valuable starting material for perfumes and pharmaceutical products [184,185]. Engineering terpenoid synthases and the precursor supplying enzymes as well as the generation of gene fusions appear to be advantageous for efficient heterologous production of diterpenoids in *S. cerevisiae.*

3.3.4. Tetraterpenoids (Carotenoids)

Carotenoids are mainly C_{40}-isoprenoid pigments, synthesized in all phototrophic organisms such as plants, algae and cyanobacteria, but also in some non-phototrophic bacteria and fungi. Carotenoids with 30 or 50 carbon atoms can be produced by certain bacteria via different intermediates. C_{40}-derived pigments contribute to the red, yellow and orange colours found in many flowers, fruits and vegetables. More than 700 carotenoid structures are known within this large group of terpenoids, which comprises two subclasses: (i) carotenes being pure hydrocarbons such as lycopene, α-carotene and β−carotene and (ii) xanthophylls such as canthaxanthin, zeaxanthin, astaxanthin and lutein as oxygen-containing carotenoids (Fig. 3) [186]. In photosynthetic organisms, carotenoids are essential components of the photosynthetic complex, they play an important role in preventing photooxidative damage, function as precursors for phytohormones (strigolactones and abscisic acid) and are important for attracting pollinators and seed dispersers to coloured flowers and fruits [187–189]. Across all kingdoms, the most important common functions of carotenoids are related to their photoprotective and antioxidative properties [190]. Furthermore, carotenoids are responsible for many of the bright colours of animals, but due to their inability of carotenoid biosynthesis they have to obtain them from the diet [191]. In humans, a carotenoid-rich diet leads to several health benefits, like protection of cells from oxidative damage caused by reactive oxygen species, reduced age-related macular degeneration of the eye, and prevention of prostate cancer or cardiovascular disease [192–195]. Furthermore, carotenoids act as precursors for vitamin A production [196–199].

Due to their colours and health related benefits carotenoids are of high commercial interest as colorants, pharmaceuticals, and nutritional supplements or cosmetic additives. Some of the commercially used carotenoids, e.g. β-carotene, astaxanthin and canthaxanthin, are mainly produced by chemical synthesis, whereas both lycopene and lutein are predominantly extracted from natural sources such as tomato, marigold or microalgae [200,201]. However, the increasing industrial demand on carotenoids has led to extensive efforts in finding suitable natural sources and developing biotechnological processes, e.g. metabolic pathway engineering in plants [202–204], or carotenogenic or non-carotenogenic microorganisms [205–207] for efficient production of novel carotenoids. Elucidation of a number of carotenoid biosynthetic pathways at a molecular level has provided a toolbox of carotenogenic (*crt*) genes that can be used to engineer microorganisms for carotenoid production.

Most *crt* genes and gene clusters have been cloned and expressed in the non-carotenogenic host *E. coli*. By a synthetic biology approach, combining the genes from organisms with different carotenoid biosynthetic branches, novel carotenoids could be synthesized [208]. However, the supply of the common isoprenoid precursors IPP, DMAPP and GGPP, needed for carotenoid biosynthesis is limited in the *E. coli* system. Hence, the production levels of carotenoids in *E. coli* are low compared to commercially employed carotenogenic microbial strains such as *Dunalliella*, *Haematococcus* and red yeast *Xanthophyllomyces dendrorhous* [209]. Metabolic engineering approaches in *E. coli* led to an increase in carotenoid production levels, but limits were reached possibly due to reduced carotenoid storage capacity in *E. coli* [205,207,210]. In contrast, yeasts have an efficient isoprenoid pathway and are capable of accumulating large quantities of ergosterol. Thus, the ergosterol biosynthesis pathway turned out to be a major engineering target for the successful production of carotenoids in the yeasts *S. cerevisiae* and *Candida utilis* [163,205,211–213]. In a first approach, Yamano et al. used episomal vector systems to overexpress the bacterial genes *crtE*, *crtB*, *crtI*, and *crtY* yielding very low levels of β-carotene [212]. Verwaal et al. combined the over-expression of genes from the isoprenoid and carotenoid pathways for creating a stable, β-carotene producing *S. cerevisiae* strain [211]. Therefore, carotenogenic genes *crtE*, *crtI*, and *crtYP,* encoding a bifunctional phytoene synthase and lycopene cyclase from *X. dendrorhous*, were integrated into the yeast genome. Over-expression of GGPP synthase (*BTS1*) and *tHMG1* from *S. cerevisiae* resulted in higher carbon flux and further improved β-carotene production. The β-carotene concentration reached 5.9 mg/g DCW in the final strain. A similar approach was applied in *C. utilis* by overexpressing carotenoid genes *(crtE, crtB,* and *crtI*) and the catalytic domain of HMG-CoA reductase in combination with disruption of the *ERG9* gene. These engineering steps yielded lycopene at 7.8 mg/g DCW [214]. The synergistic effect of

overexpressing *HMG* and blocking ergosterol biosynthesis on β-carotene production was also shown in *S. cerevisiae* by adding ergosterol synthesis inhibitor ketoconazole instead of disrupting the *ERG9* gene yielding 6.29 mg/g DCW of β-carotene [215]. Recently, the non-carotenogenic yeast *Pichia pastoris* has been described as alternative host system for the production of lycopene, β-carotene and astaxanthin [216,217]. A *P. pastoris* strain heterologously expressing β-carotene-pathway enzymes from *Erwinia uredovora* and *Ficus carica* was created and demonstrated to produce β-carotene. In a subsequent study, this strain was further engineered by introducing the β-carotene ketolase (*crtW*) and β-carotene hydroxylase (*crtZ*) genes from the marine bacterium *Agrobacterium aurantiacum*. This resulted in recombinant *P. pastoris* which synthesized astaxanthin as well as pathway intermediates such as lycopene, β-carotene and canthaxanthin. However, the astaxanthin yield is lower than previously reported for heterologous astaxanthin production in *C. utilis* [213] and *S. cerevisiae* [218]. Further strain engineering or optimisation of fermentation conditions would be necessary to improve astaxanthin production to an industrially-relevant yield. The oleaginous yeast *Y. lipolytica* has emerged as another host system for the production of carotenoids [219,220]. The LipoYeasts project (http://www.lipoyeasts.ugent.be/) dealt with the transformation of *Y. lipolytica* into a versatile and high-throughput microbial host for lipid pathway engineering towards production of industrially relevant compounds like wax esters, terpenoid compounds, e.g. carotenoids and carotenoid ester, polyhydroxyalkanoates (PHAs) and free hydroxylated fatty acids (HFAs) [221,222]. Different lipid stocks, such as petroleum, alkane, vegetable oil or fatty acid have been evaluated as substrates in combination with genetically modified *Y. lipolytica* strains, in order to produce high-value products at industrial scale [219,223].

The carotenogenic red yeast *X. dendrorhous* (*Phaffia rhodozyma*) naturally synthesizes astaxanthin as its main carotenoid. Several studies have been performed with the goal to establish microbial astaxanthin production as competitive alternative to industrial chemical synthesis [224–228]. Further approaches comprised engineering of sterol pathway to enhance metabolite flux towards carotenoid biosynthesis [229] or random mutagenesis [230,231]. The potential of *X. dendrorhous* for production of astaxanthin in relation to other natural sources, i.e. microalgae *Haematococcus pluvialis*, diverse fungi and transgenic plants, and to chemical synthesis has been extensively reviewed [232,233].

4. Conclusions

Versatility of host cell systems is an important aspect in basic and applied science, because it helps to safeguard time and financial budgets. Cultivation of yeasts is highly reproducible and cheap. Moreover, yeasts can be genetically manipulated to produce various compounds only occurring naturally in higher eukaryotes. Lately, the high capacities of *S. cerevisiae* to form isoprene building blocks and the adducts thereof, i.e. geranyl- and farnesyl-diphosphate, have rendered this particular yeast a favourite commercial production platform for sterols, steroids and other terpenoids [22,65,168,170]. Thereby, yeast has acquired a prominent position in synthetic biology beside *E. coli*. Concerning availability of building blocks for terpenoids, yeast is still superior to *E. coli*, even if the latter has been engineered to produce isoprene-derived compounds [234]. A further advantage of yeasts over bacteria in terpenoid biosynthesis is that the eukaryotic membranes are usually better suited to accommodate substantial amounts of mammalian or plant terpenoid converting enzymes, e.g. cytochrome P450s, than the prokaryotic cytoplasmic membrane. However, there are some terpenoids, mainly monoterpenoids, that are clearly detrimental to yeast fitness [147,148], which can be counteracted by extracting the terpenoids into a water-immiscible organic phase [149]. Yeast-derived terpenoids are preferred by the food, flavour & fragrance and pharmaceutical industries over extracts from natural sources like plants and cell cultures, because the yields are more predictable, are not subjected to seasonal or regional variation, and the products can be obtained without an elevated risk for contaminations by for example viruses and herbicides. For the time being, *S. cerevisiae* is by far the most established yeast in terpenoid research, but *S. pombe*, *Y. lipolytica* and *P. pastoris* are catching up for different reasons. In terms of flux through the mevalonate pathway towards FPP, baker's yeast is undoubtedly superior to other yeasts. However, regarding heterologous (membrane) protein expression (*S. pombe* and *P. pastoris*), growth in the presence of hydrophobic compounds (*Y. lipolytica* and *P. pastoris*) and high cell density growth (*P. pastoris*) the alternative yeasts have their benefits [128,235,236].

In contrast to metabolic engineering of yeast for producing steroids or plant terpenoids, which is clearly driven by commercial interests, sterol-engineering of yeast carries vast potential in gaining knowledge on the cellular functions and interactions of sterols. While biophysical approaches and lipid analyses of *erg* mutants have already uncovered numerous aspects of sterol-lipid interactions [60,61,70,89,98], much less solid evidence exists concerning sterol-protein molecular interactions. Besides the identification of sterol binding and transporting proteins in yeasts and mammals [36,37,45,237,238], particularly the increasing percentage of database entries of membrane protein structures stabilised by interaction with specific sterols suggest a potential future line of research on sterol-modified yeast(s) [239–242]. It is feasible to generate yeast strain collections containing various main sterols, and to express diverse membrane proteins in this collection to study protein folding, stability, transport and function [27]. Enhancing the knowledge on sterol/lipid – membrane protein relationship will have a positive feed-back effect on metabolic engineering of yeast, e.g. for terpenoid biosynthesis, because effective membrane protein expression is still a limiting factor in many projects.

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