ACS Partner Journal

CHEM & BIO ENGINEERING

pubs.acs.org/ChemBioEng

Review

Efforts toward Ambergris Biosynthesis

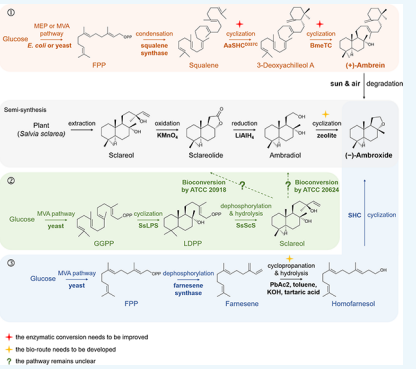
Ni He, Hongwei Yu, and Lidan Ye*

Cite This: *Chem Bio Eng.* 2024, 1, 91−98

Read Online

ACCESS | Metrics & More | Article Recommendations

ABSTRACT: Ambergris is a very rare and highly valued fauna natural perfume. Its main component, ambrein, undergoes oxidative degradation to produce ambroxide, forming the unique ambergris fragrance. To meet the market demand while not offending the law of protecting sperm whales, ambrein and ambroxide are chemically synthesized. Recently, the biosynthesis of these compounds has been explored as a green and sustainable production route to ensure the safety of use. The ambrein biosynthesis pathway has been successfully constructed in model microorganisms, leading to *de novo* biosynthesis of ambrein from glucose and glycerol. In addition, partial biosynthesis of ambroxide has been achieved by modular co-culture of engineered sclareol-producing yeast and a natural fungus converting sclareol to ambradiol, which can be further converted to ambroxide by zeolite. Alternatively, ambroxide can be produced by the chemical transformation of biosynthesized farnesene, followed by enzymatic cyclization. In this paper, the efforts toward biosynthesis of ambrein and ambroxide as representative compounds to substitute the natural ambergris are reviewed, and the challenges and prospects are discussed.



KEYWORDS: *ambergris, ambrein, ambroxide, biosynthesis, synthetic biology*

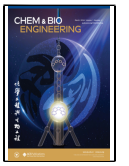
INTRODUCTION

Ambergris is a waxy substance, prized for over a thousand years as medicine, condiment, and perfume. The biological origin of ambergris was claimed in the 19th century, and it is now generally accepted that it is a pathological gut secretion of sperm whales (*Physeter microcephalus*) arising from undigested horny squid beaks. After the death of the sperm whale, ambergris floats on the sea, and its color becomes lighter while its odor improves under the action of air and sun.[1] The smell of ambergris is described as well-balanced with soft musky, animalic, marine, sweet, earthy, and woody facets.[2]

Ambergris is a very rare natural product, because it only occurs in about one in a hundred sperm whales, and the ban on commercial whaling by many countries has increasingly limited its sources.[3] Studies have shown that the main constituents of ambergris are ambrein, *epi*-coprosterol, coprosterol, cholesterol, and coprostane-3-one.[1] Ambrein ($C_{30}H_{52}O$) was first isolated from ambergris in 1820.[4] Its chemical structure is a tricyclic triterpene alcohol containing one tertiary hydroxyl group and two double bonds. As a major constituent of ambergris, (+)-ambrein was used as a perfume additive for the tenacity or persistence of its odor, and has been reported to possess anti-inflammatory, anti-nociceptive, and aphrodisiac activities.[5] The first total synthesis of a diastereomeric mixture of ambrein was achieved using farnesylacetic acid and 1-bromomethyl-3,3-dimethyl-1-cyclohexene as raw materials.[6] Recrystallization of the mixture by seeding with natural ambrein gives (+)-ambrein. In the same year, the total synthesis of (+)-ambrein was accomplished in 0.38% overall yield from geranylacetone through 22 steps or in 1.1% yield from (*S*)-3-hydroxy-2,2-dimethylcyclohexanone through 21 steps.[7] (+)-Drimane-8,11-diol has also been used for the synthesis of enantiomerically pure (+)-ambrein.[8]

Ambrein is odorless, while its degradation generates a complex mixture of products, among which (−)-ambroxide is recognized as the prototype of all ambergris odorants. (−)-Ambroxide shows a characteristic and memorable ambery and woody smell historically linked to ambergris, and reminiscent of tobacco and clary sage.[9] The yearly use of (−)-ambroxide as the most appreciated substitute of ambergris is estimated to be more than 100 t.[2] (−)-Ambroxide is commercially available from Firmenich SA or Kao Kabushiki Kaisha as Ambroxor Ambroxan and is produced by Givaudan SA as Ambrofix.[9] The chemical synthesis route of (−)-ambroxide from diterpenoid sclareol was first proposed in 1950.[10] Other natural monoterpenes, sesquiterpenes and diterpenes, e.g., (+)-carvone,[11] *β*-ionone,[12] nerolidol,[12] *β*-farnesene,[13] abietic acid[14] and labdanolic acid,[15] were also used for semi-synthesis of (−)-ambroxide. Among all these starting materials, sclareol is the most promising due to its structural similarity to (−)-ambroxide, both having a labdane carbon skeleton. This

Received: October 31, 2023
Accepted: December 12, 2023
Published: January 4, 2024



https://doi.org/10.1021/cbe.3c00083
Chem Bio Eng. 2024, 1, 91−98

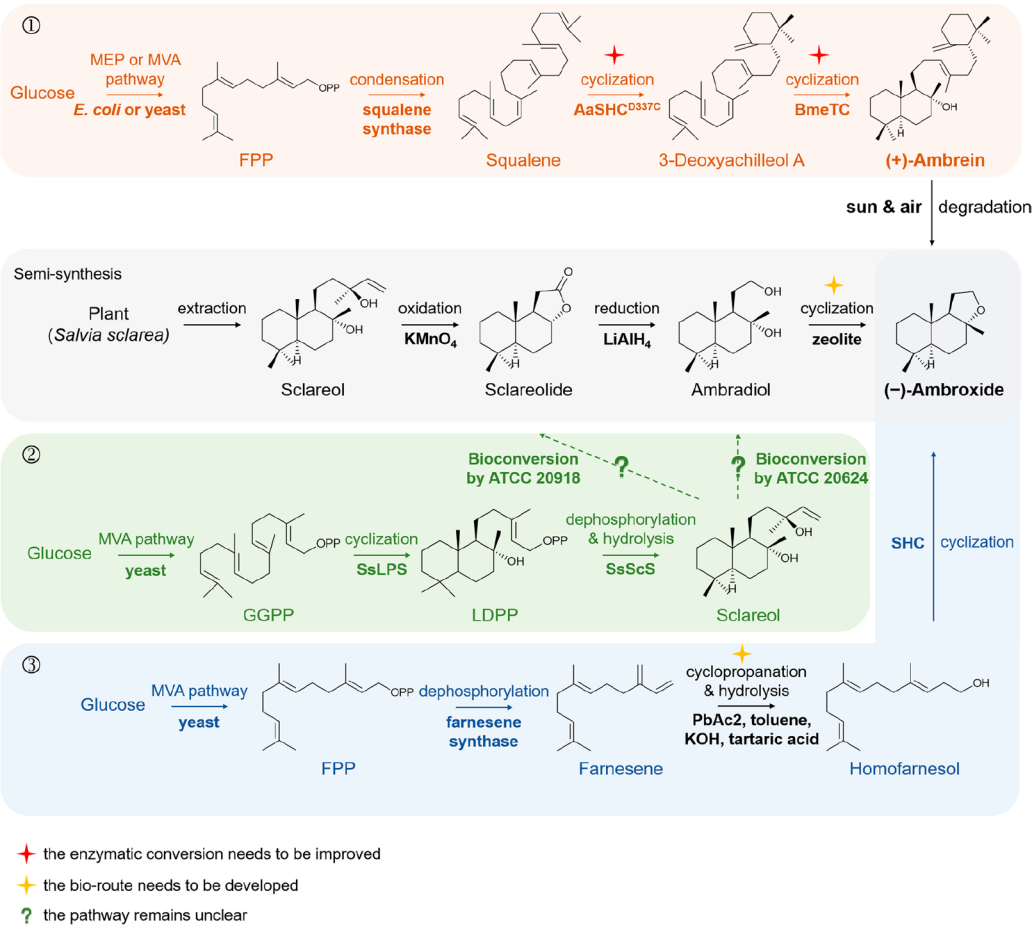


Figure 1. Synthetic pathways of ambrein and ambroxide. ① Engineering of enzymes for converting biosynthesized squalene to (+)-ambrein; ② ambroxide biosynthesis based on sclareol bioconversion; ③ biosynthetic pathway based on (*E,E*)-homofarnesol. The different biosynthetic routes are marked by orange, green, and blue, respectively. The semi-synthesis route is marked in gray. The pathways from sclareol to sclareolide and ambradiol catalyzed by ATCC 20918 and ATCC 20624, respectively, remain unclear. The chemical or biological catalysts are marked in bold. FPP, farnesyl pyrophosphate; GGPP, geranylgeranyl diphosphate; LDPP, (13*E*)-8α-hydroxylabden-15-yl diphosphate; AaSHC^{D337C}, the D337C variant of squalene-hopene cyclase from *Alicyclobacillus acidocaldarius*; BmeTC, tetraprenyl-β-curcumene cyclase from *Bacillus megaterium*; SsLPS, LDPP synthase from *Salvia sclarea*; SsScS, sclareol synthase from *S. sclarea*.

production route has been industrialized for a long time and is composed of four steps: extraction of (−)-sclareol from the plant *Salvia sclarea*, oxidation of the (−)-sclareol side chain to yield (+)-sclareolide, reduction of (+)-sclareolide to (−)-ambradiol, and finally cyclodehydration of (−)-ambradiol to give (−)-ambroxide. For this semi-synthesis process, research has been mostly focused on the development of appropriate oxidants and reducing agents.[16−18] However, other challenges remain, for example, the long growth cycle of the plant and its susceptibility to climate changes, the environmental issue associated with the chemical waste, and the chemical residues from the oxidoreduction reactions which would affect the aroma characteristics and the safety of (−)-ambroxide.[19]

In recent years, biosynthesis of valuable compounds has attracted increasing attention due to its advantages of short production cycles, mild and green production conditions, and the specificity of enzyme-catalyzed reactions. Efforts have been made toward biosynthesis of (+)-ambrein and (−)-ambroxide, paving the way for green and sustainable production of ambergris. This paper aims to provide an overview of the recent research progress in the biological production of these valuable chemicals by describing the different synthetic pathways (Figure 1), summarizing the advances in each synthetic pathway, and discussing the remaining challenges and future perspectives.

Biosynthesis of Ambrein. *Engineering of Enzymes for Converting Squalene to (+)-Ambrein.* Biocatalytic synthesis of (+)-ambrein was first achieved using squalene as the substrate (Figure 2), under the cascade catalysis of the D377C mutant of squalene-hopene cyclase from *Alicyclobacillus acidocaldarius* (AaSHC) and the tetraprenyl-β-curcumene cyclase from *Bacillus megaterium* (BmeTC).[20] By substitution of the last aspartic acid in the DXDD motif with cysteine, the product profile of AaSHC was altered. Squalene was converted by AaSHC^{D377C} to the monocyclic 3-deoxyachilleol A instead of the pentacyclic hopene,[21] and 3-deoxyachilleol A was then further converted to (+)-ambrein by BmeTC. Based on this, *de novo* biosynthesis of (+)-ambrein was realized for the first time by introducing AaSHC^{D377C} and BmeTC into a squalene-producing *Escherichia coli* strain which was constructed by expressing the *ScERG9* gene encoding the squalene synthase from *Saccharomyces cerevisiae*. The final strain produced 2.6 mg·L^{-1} (the mass of product per liter of culture) of (+)-ambrein from glycerol.[22] However, this yield is too low to meet the needs of industrial production.

https://doi.org/10.1021/cbe.3c00083
Chem Bio Eng. 2024, 1, 91−98

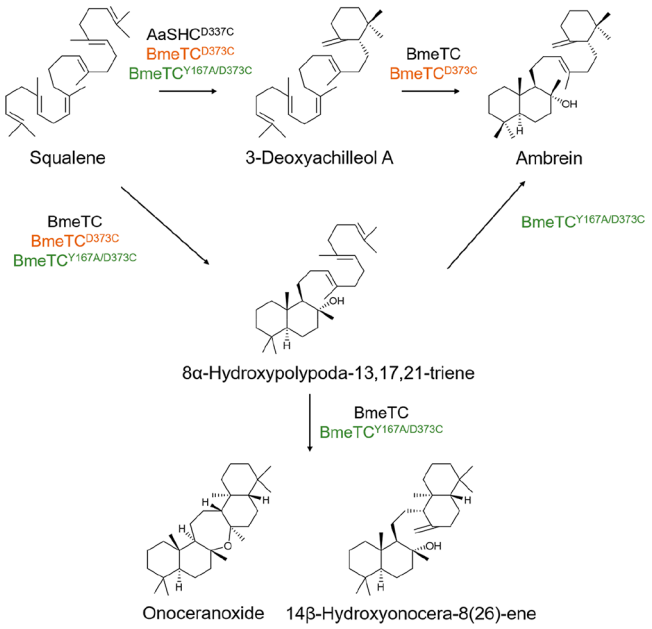


Figure 2. Routes for the biocatalytic conversion of squalene to ambrein. Route 1: Squalene is converted by AaSHC^{D377C} to the monocyclic 3-deoxyachilleol A and is then further converted to ambrein by BmeTC. BmeTC could also utilize squalene as a substrate to convert it to three byproducts, including 8α-hydroxypolypoda-13,17,21-triene, onoceranoxide, and 14β-hydroxyonocera-8(26)-ene. Route 2: The BmeTC^{D373C} mutant catalyzes the reactions from squalene to ambrein; meanwhile, 8α-hydroxypolypoda-13,17,21-triene is formed as a byproduct. Route 3: The double mutant BmeTC$^{Y167A/D373C}$ produces ambrein from squalene via 8α-hydroxypolypoda-13,17,21-triene, forming 3-deoxyachilleol A, onoceranoxide, and 14β-hydroxyonocera-8(26)-ene as byproducts. Route 4: A dual enzyme system composed of wild-type BmeTC and BmeTC$^{Y167A/D373C}$ produces ambrein from squalene via 8α-hydroxypolypoda-13,17,21-triene and 8α-hydroxypolypoda-13,17,21-triene, with onoceranoxide and 14β-hydroxyonocera-8(26)-ene as byproducts.

Yeast strains can produce squalene or 2,3-oxidized squalene through the intrinsic mevalonate (MVA) pathway and sterol biosynthesis pathway. In *Pichia pastoris* strain engineered by increasing intracellular squalene level and introducing AaSHC^{D377C} and BmeTC, 2 mg·L^{-1} of (+)-ambrein was produced.[23] Meanwhile, large amounts of the intermediate 3-deoxyachilleol A and the byproduct 8α-hydroxypolypoda-13,17,21-triene were also detected, which were generated from squalene by the bifunctional cyclase BmeTC. To further increase the production of (+)-ambrein, BmeTC was engineered by mutating the last aspartic acid in the DXDD motif to cysteine.[23] Surprisingly, the BmeTC^{D373C} mutant efficiently catalyzed the whole reaction from squalene to (+)-ambrein at a titer of 15 mg·L^{-1} [0.8 mg·g^{-1} dry cell weight (DCW)] in shake flasks and 105 mg·L^{-1} in fed-batch fermentation.[23] When *S. cerevisiae* was used as the chassis to produce (+)-ambrein by the above strategy, a (+)-ambrein titer of 2.9 mg·L^{-1} (0.7 mg·g^{-1} DCW) was achieved in shake flasks.[24] The difference in (+)-ambrein titers between these two yeast chasses was mainly derived from cell density, with approximately 5-fold higher OD_{600} for *P. pastoris* than that for *S. cerevisiae*.

To further improve the reaction efficiency of mutant BmeTC^{D373C}, alanine screening was conducted for the six residues presumed to be located near the intermediates during the reaction based on the modeling structure of BmeTC. The resulting double mutant BmeTC$^{Y167A/D373C}$ produced (+)-ambrein from squalene via 8α-hydroxypolypoda-13,17,21-triene with a yield of 21.5%, approximately 10-fold higher than that of BmeTC^{D373C} which produced (+)-ambrein from squalene via 3-deoxyachilleol A (2.2%).[25] However, 3-deoxyachilleol A was accumulated as a byproduct during the biocatalysis of BmeTC$^{Y167A/D373C}$. To accelerate squalene conversion to 8α-hydroxypolypoda-13,17,21-triene and meanwhile to reduce 3-deoxyachilleol A accumulation, a dual enzyme system was constructed by coupling the wild-type BmeTC and BmeTC$^{Y167A/D373C}$ (Figure 2), showing 20 times higher yield than using BmeTC^{D373C} alone (46.0% vs. 2.2%).[25] The authors anticipated the replacement of BmeTC^{D373C} with this dual enzyme system in the above-mentioned engineered *P. pastoris* strain may lead to an (+)-ambrein titer of 2 g·L^{-1}.[25] However, the performance of the dual enzyme system was evaluated using purified enzymes *in vitro*, and a large amount of the intermediates 3-deoxyachilleol A and 8α-hydroxypolypoda-13,17,21-triene as well as the byproducts onoceranoxide and 14β-hydroxyonocera-8(26)-ene were accumulated. How efficient it could be when constructed in *P. pastoris* remains a question.

Biosynthesis of Squalene as the Precursor of Ambrein. Besides engineering the two key enzymes (AaSHC and BmeTC) in the (+)-ambrein biosynthesis pathway, strengthening the supply of squalene as the key precursor is another premise for efficient ambrein biosynthesis. Squalene is a triterpenic hydrocarbon synthesized in all types of cells as a key intermediate in the formation of eukaryotic sterols and bacterial hopanoids. Currently, the primary commercial source of squalene is the liver of deep-sea sharks, which is challenged by rising cost because of diminishing supply and concerns about environmental sustainability.[26] Due to the high value of squalene, many efforts have been made to construct efficient squalene cell factories for low-cost and sustainable production.

Squalene is biosynthesized via the head-to-head condensation of two molecules of farnesyl diphosphate (FPP) under the catalysis of squalene synthase. In *E. coli*, the precursor FPP is produced through the methylerythritol 4-phosphate (MEP) pathway, where 1-deoxyxylulose-5-phosphate synthase (DXS) and isopentenyl diphosphate isomerase (IDI) are known as rate-limiting enzymes.[27] The overexpression of *E. coli dxs* and *idi*, in the presence of *hopA* and *hopB* (encoding squalene synthase) together with *hopD* (encoding farnesyl diphosphate synthases) from *Streptomyces peucetius*, elevated squalene yield by 2-fold.[28] To establish an efficient *E. coli*-based system for squalene production, two different squalene synthases in combination with precursor pathways was tested. By overexpressing the endogenous IDI (*idi*) and farnesyl diphosphate synthase (*ispA*) genes in the MEP pathway and the endogenous acetyl-CoA acetyltransferase (*atoB*) together with the heterologous mevalonate (MVA) pathway from *S. cerevisiae* and the squalene synthase from human or *Thermosynechococcus elongatus* in *E. coli*, up to 230 mg·L^{-1} (54 mg·g^{-1} DCW) or 150 mg·L^{-1} (55 mg·g^{-1} DCW) of squalene was produced in shake-flask cultures.[29] Recently, extension of the cell membrane of a squalene-producing *E. coli* strain to provide more storage room for this lipophilic product by overexpression of Tsr, a protein induced invagination of the inner membrane to form multilayered structure, resulted in squalene production of 612 mg·L^{-1}.[30]

https://doi.org/10.1021/cbe.3c00083
Chem Bio Eng. 2024, 1, 91−98

Table 1. Microorganisms Naturally Metabolizing Sclareol

Organism	Product	Description	Reference
Cryptococcus albidus ATCC 20918	sclareolide	60 g·L^{-1} sclareol was converted to 43 g·L^{-1} sclareolide within 5 d in fermenter	36
Filobasidium magnum JD1025	sclareolide	30 g·L^{-1} of sclareol was converted to 21.62 g·L^{-1} sclareolide within 72 h in flasks	37
Bensingtonia ciliata ATCC 20919	ambradiol	8.42 g·L^{-1} sclareol was converted to 5.35 g·L^{-1} ambradiol within 15 d in fermentor	36
Hyphozyma roseonigra ATCC 20624	ambradiol	50 g·L^{-1} of sclareol was converted to 35.5 g·L^{-1} ambradiol with within 13 d in flasks	38
		Proteomics was performed to preliminarily elucidate the molecular mechanism of sclareol metabolism	39
		Metabolite profiling was performed to analyze the biocatalytic conversion of sclareol to ambradiol by ATCC 20624	40,41
		The dynamic time course of sclareol biotransformation was explored by ATCC 20624 resting cell assays and several intermediates produced during biotransformation were identified	42

In yeast, squalene is an intrinsic intermediate in the ergosterol pathway and is produced through the MVA pathway. Strengthening the yeast MVA pathway by overexpressing the truncated form of HMG-CoA reductase (tHMG1) as the well-recognized rate-limiting enzyme increased the squalene production by 10-fold.[31] By upregulation of the structural genes, addition of the squalene monooxygenase inhibitor, and downregulation of the ethanol production pathway, a squalene titer of 304.49 mg·L^{-1} was achieved.[32] Other metabolic engineering strategies include improving NADPH and acetyl-CoA supply, pathway compartmentalization, promoter engineering, and optimization of fermentation conditions, as reviewed recently by Paramasivan and Mutturi.[33] At present, the highest squalene titer in yeast is 21.1 g·L^{-1} (437.1 mg·g^{-1} DCW) in a two-stage fermentation process, achieved by a combinatorial strategy of cytoplasmic and mitochondrial engineering to alleviate the metabolic burden and improve cell growth.[34]

In recent years, Thraustochytriaceae has emerged as another promising sustainable source of squalene due to their fast growth and high squalene contents (10−317.74 mg·g^{-1} DCW).[35] However, genetic manipulation of the native biochemical pathways in these marine protists for further improvement of squalene production remains challenging.

The high production of squalene in the model microorganisms implies the potential of (+)-ambrein bioproduction from squalene. By screening novel enzymes capable of efficiently converting squalene to (+)-ambrein and enzyme modification to improve their activities, the (+)-ambrein titer may be enhanced to the g·L^{-1} level or even higher.

Biosynthesis of Ambroxide. As a substitute for natural ambergris, ambroxide has received extensive attention. Recently, two major approaches were proposed to achieve green and sustainable production of ambroxide using sclareol and (*E*,*E*)-homofarnesol as the key precursors, respectively (Figure 1).

Ambroxide Biosynthesis Based on Sclareol Bioconversion. Some natural microbial strains have been reported to metabolize sclareol to ambroxide precursors, although the metabolic pathways remain unknown (Table 1). *Cryptococcus albidus* ATCC 20918 and *Filobasidium magnum* JD1025 can transform sclareol to sclareolide, while *Bensingtonia ciliata* ATCC 20919 and *Hyphozyma roseonigra* ATCC 20624 can convert sclareol to ambradiol.[36−38] This lays the foundation for development of an ambroxide production route by microbial conversion of sclareol to replace the current semi-synthesis route.

Among the above natural producers, *H. roseonigra* ATCC 20624 has been the most extensively studied. Proteomics of the cells cultivated using sclareol as the sole carbon source, compared to those cultivated without sclareol, revealed upregulation of 18 aldehyde dehydrogenases. This result suggested possible roles of aldehyde dehydrogenases in the metabolism of sclareol,[39] in accordance with the semi-synthesis route, which involves chemical oxidation and reduction reactions. Metabolite profiling showed that sclareolide may be an intermediate during the conversion of sclareol to ambradiol.[40,41] However, a recent study explored the dynamic time course of sclareol biotransformation by resting cells of *H. roseonigra* and proposed a different metabolic pathway where ambradiol and sclareolide are not interconverted. The authors suggested that ambradiol and sclareolide may derive from different metabolic pathways. In addition, several putative intermediates featured with the same labdane carbon backbone, including isomerization and oxidation in the side chain, were found.[42] However, the metabolic pathway has still not been fully elucidated, and the genes responsible for this process remain unrevealed. The lack of knowledge on sclareol metabolism hinders the reconstruction of the ambradiol formation pathway in a model microorganism. In contrast, the well-studied sclareol synthetic pathway enabled the construction of sclareol-producing microbial cell factories (Table 2), which provides the possibility of changing the sclareol supply method from plant extraction to fermentation.

Sclareol is naturally derived from the flowers and leaves of *S. sclarea* (known as Clary sage). Since the elucidation of the enzymes (13*E*)-8α-hydroxylabden-15-yl diphosphate (LDPP) synthase (SsLPS) and sclareol synthase (SsScS) responsible for sclareol biosynthesis in 2012,[43] heterologous sclareol production has been realized in *E. coli* and *S. cerevisiae*. By introducing the exogenous MVA pathway and sclareol synthetic genes into *E. coli*, 1.5 g·L^{-1} of sclareol was produced in two-phase high-density fermentation with dodecane as the organic phase.[43] More efforts have been made to construct a sclareol-producing yeast. By strengthening the precursor supply and fusion expression of *BTS1* encoding geranylgeranyl diphosphate (GGPP) synthase and *ERG20* encoding FPP synthase as well as *LPPS* encoding SsLPS and *TPS* encoding SsScS for substrate channeling, 408 mg·L^{-1} of sclareol was produced by the diploid yeast in fed-batch fermentation.[44] Episomal expression of *Cistus creticus* 8-hydroxy copalyl diphosphate synthase (CcCLS) and SsScS in *S. cerevisiae* together with the deletion of six endogenous genes (*rox1*, *dos2*, *yer134c*, *vba5*, *ynr063w* and *ygr259c*) resulted in sclareol production of up to 750 mg·L^{-1} in shake-flask cultivation.[45] Genomic integration of SsLPS

https://doi.org/10.1021/cbe.3c00083
Chem Bio Eng. 2024, 1, 91−98

Table 2. Metabolic Engineering of Microorganisms for Sclareol Biosynthesis

Organism	Yield	Description	Fermentation type	Reference
E. coli	1.5 g·L^{-1}	overexpression of *CrtE* from *Pantoea agglomerans*, *atoB* from *E. coli*, *mvaA*, and *mvaS* from *Staphylococcus aureus*, *mvaK1*, *mvaK2*, *mvaD*, and *fni* from *Streptococcus neumoniae*, *ERG20* from *S. cerevisiae*, *SsLPS* and *SsScS* from *S. sclarea*	fed-batch in 3.7L bioreactor	43
S. cerevisiae	8.96 mg·L^{-1}	fusion expression of BTS1 and ERG20 as well as LPPS and TPS, overexpression of tHMG1	shake flask	48
S. cerevisiae	408 mg·L^{-1}	BY4741 with fusion expression of BTS1 and ERG20 as well as LPPS and TPS, overexpression of tHMG1, crossed with BY4742 to form diploids	fed-batch in 3L bioreactor	44
S. cerevisiae	750 mg·L^{-1}	fusion expression of *Cc*CLS and ERG20 (F96C), overexpression of the catalytic domain of HMG2(CD-HMG2) and SsScS, deletion of *rox1*, *dos2*, *yer134c*, *vba5*, *ynr063w*, and *ygr259c*	shake flask	45
S. cerevisiae	357 mg·L^{-1}	genomic integration of *tHMG1*, *ERG20 (F96C)*, and *CrtE03M* (encoding the C81T variant of GGPP synthase from *Xanthophyllomyces dendrorhous*), two copies of *SsLPS* and *SsScS*, deletion of *rox1*, downregulation of *ERG9* using P_{HXT1}	shake flask	46
S. cerevisiae	11.4 g·L^{-1}	MBP (maltose-binding protein from *E. coli*) fused with SsLPS and SsScS, overexpression of *ERG20 (F96C)*, *tHMG1*, and *HMGR* from *Silicibacter pomeroyi*, *ERG10*, *LAC1*, *OYE3*, three copies of *HMG2(K6R)*, BTS1-PaGGPPS (from *Phlomopsis amygdali*) fusion protein, downregulation of *ERG9* using P_{HXT1}, deletion of *rox1*, *dos2*, *yer134c*, *vba5*, *ynr063w*, and *ygr259c* in the central metabolism engineered strain Y&Z036	fed-batch in 1L bioreactor	47

and SsScS together with enhancing the precursor supply, weakening the competitive pathway and balancing the metabolic flux, led to the production of 357 mg·L^{-1} sclareol.[46] Recently, a modular approach, where the global metabolism was divided into three modules: central metabolism for the supply of acetyl-CoA, isoprenoid biosynthesis pathway, and regulation factor modules, was used to improve sclareol production in *S. cerevisiae*, resulting in the highest ever reported sclareol production of 11.4 g·L^{-1}.[47]

The progress in sclareol biosynthesis provides the possibility for fermentative production of ambroxide using a mixed culture of a sclareol-producing engineered strain and a sclareol-converting natural microorganism. By dividing the biosynthetic pathway of ambradiol into a sclareol-producing engineered yeast as the upstream pathway module and the sclareol-converting *H. roseonigra* ATCC 20624 strain as the downstream pathway module, a modular co-culture system was established to enable ambradiol biosynthesis (Figure 3). Subsequent optimization of inoculation ratio, carbon source supplementation, and sclareol secretion promotion led to production of 493.1 mg·L^{-1} of ambradiol in an induction-free co-culture system.[46] This is the first example of the complete biosynthesis of ambradiol from glucose, which can be easily converted to ambroxide by a cyclodehydration process,[49,50] leading to a new green route of (−)-ambroxide production.

Biosynthetic Pathway Based on (E,E)-Homofarnesol. Enzymatic synthesis of (−)-ambroxide from homofarnesol is another attractive approach, where a SHC cyclizes the C16 terpene (*E,E*)-homofarnesol to (−)-ambroxide in a single step.[49] In 1986, it was first reported that AaSHC could yield (−)-ambroxide from (*E,E*)-homofarnesol by enzymatic cyclization. However, the conversion rate was very low (3%).[49] Later, a novel SHC from *Zymomonas mobilis* (ZmoSHC1) was found, which exhibited over 50-fold higher conversion of homofarnesol in comparison to squalene conversion.[50] The highest conversion of the enzyme toward homofarnesol (10 mM) reached approximately 40%,[50] which is, however, still too low to meet the industrial requirement. By random mutagenesis of AaSHC, the best variant 215G2 (M132R/A224V/I432T) was obtained, which allowed the conversion of 125 g·L^{-1} (*E,E*)-homofarnesol with a cells-to-substrate ratio of 2:1 in 72 h.[9] The volumetric productivity increased when the cells-to-substrate ratio was changed to 1:1, fully converting up to 300 g·L^{-1} (*E,E*)-homofarnesol in 3 days.[2] Based on this, Givaudan has developed a cost-effective process for the production of Ambrofix™ (Figure 4).[2]

For massive preparation of the key intermediate (*E,E*)-homofarnesol, biobased (*E*)-*β*-farnesene commercially produced by fermentation can serve as a sustainable feedstock. (*E*)-*β*-Farnesene can be chemically converted to (*E,E*)-homofarnesol in two steps by selective cyclopropanation at the terminal double bond with *in situ* generated diazomethane followed by rearrangement under carbocationic conditions, leading to hydrolysis into (*E,E*)-homofarnesol with good 3,4-*E,Z*-ratios (around 80:20).[2]

CONCLUSION AND FUTURE PERSPECTIVES

For thousands of years, the only source of ambergris was the rare and mysterious secretion of sperm whales. Since the last century, the chemical synthesis of ambrein and ambroxide has largely expanded the application of ambergris, which, however, raises environmental issues as well as safety risk and unfavorable aromatic characteristics due to impurities. In

https://doi.org/10.1021/cbe.3c00083
Chem Bio Eng. 2024, 1, 91−98

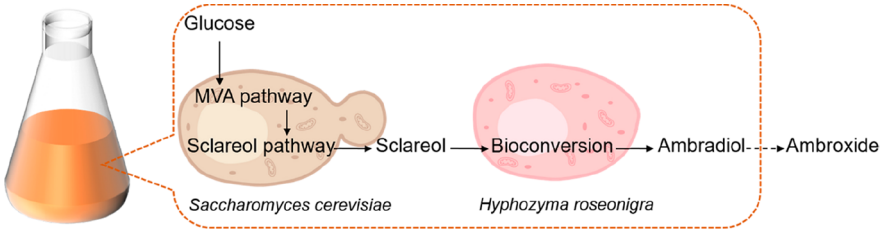


Figure 3. Flowchart of the artificial microbial consortium. The solid-line arrows represent biological reactions, and the dotted-line arrow represents the non-biological cyclodehydration reaction.

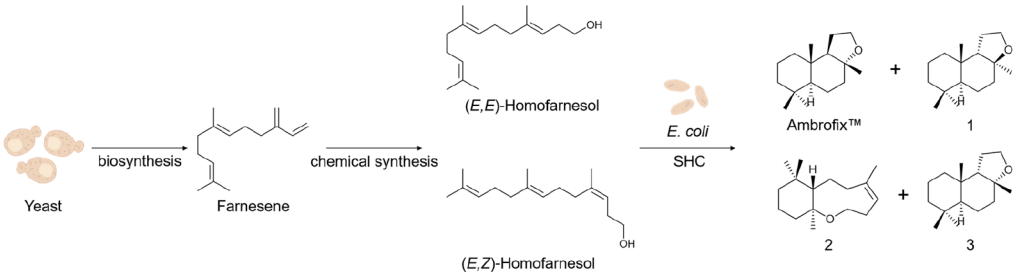


Figure 4. Production route of Ambrofix™ by Givaudan. Byproduct 1 is (3a*S*,5a*S*,9a*S*,9b*S*)-3a,6,6,9a-tetramethyldodecahydronaphtho[2,1-*b*]furan, byproduct 2 is (7a*S*,11a*S*,*Z*)-5,8,8,11a-tetramethyl-2,3,6,7,7a,8,9,10,11,11a-decahydrobenzo[*b*]oxonine, and byproduct 3 is (−)-9b-*epi*-ambrox.

recent years, the rapid development of biotechnology has enabled the development of biosynthetic routes for these ambergris-related compounds.

Using squalene as the key intermediate, *de novo* biosynthesis of ambrein has been realized in *E. coli*, *S. cerevisiae*, and *P. pastoris* cell factories by introducing and engineering of TC and SHC. However, the highest ambrein production of only 105 mg·L^{-1} is still too low to meet the industrial requirement. Considering that the highest fermentative production of precursor squalene reached 21.1 g·L^{-1}, the low yield of ambrein should be caused by the insufficient catalytic efficiency of the enzymes converting squalene to ambrein. Screening and engineering of TC and SHC for improved substrate specificity and activity, followed by introduction of their encoding genes into high-squalene producing strains as well as balancing the metabolic flux, is expected to further increase ambrein production.

As the prototype of all ambergris odorants, ambroxide is naturally derived from ambrein degradation via complex reactions in the presence of air and sun. To date, *de novo* biosynthesis of ambroxide has not been accomplished, although two partial bioproduction routes have been developed, using sclareol and (*E*,*E*)-homofarnesol as key intermediates, respectively. In the former route, plant-extracted or fermentatively produced sclareol could be converted to ambradiol by a natural fungus, followed by chemical cyclodehydration to yield ambroxide using catalysts, such as zeolite. If the molecular mechanism of sclareol metabolism in the fungal strain could be revealed using comparative transcriptomics and metabolomics in the presence and absence of sclareol, it would be possible to introduce the encoding genes into previously constructed sclareol-producing cell factories for *de novo* biosynthesis of ambradiol in a fermentative process. In the latter route, the bio-based farnesene should be converted to (*E*,*E*)-homofarnesol via a chemical process, which could then be catalyzed to ambroxide enzymatically. This route is efficient, sustainable, and environmentally friendly. However, if not carefully separated, the mixtures of *E*/*Z* homofarnesol isomers generated during the chemical process would give complex mixtures of cyclization products that harm the product aromatic properties. Future attempts may be focused on improving the ability of SHC to distinguish the geometric isomers of the substrate or developing a green route with better 3,4-*E*,*Z*-ratios.

AUTHOR INFORMATION

Corresponding Author

Lidan Ye − *Key Laboratory of Biomass Chemical Engineering (Education Ministry), College of Chemical and Biological Engineering and Institute of Bioengineering, College of Chemical and Biological Engineering, Zhejiang University, Hangzhou 310058, China;* orcid.org/0000-0002-6248-8457; Email: yelidan@zju.edu.cn; Fax: +86-571-88273997

Authors

Ni He − *Key Laboratory of Biomass Chemical Engineering (Education Ministry), College of Chemical and Biological Engineering and Institute of Bioengineering, College of Chemical and Biological Engineering, Zhejiang University, Hangzhou 310058, China*

Hongwei Yu − *Key Laboratory of Biomass Chemical Engineering (Education Ministry), College of Chemical and Biological Engineering and Institute of Bioengineering, College of Chemical and Biological Engineering, Zhejiang University, Hangzhou 310058, China;* orcid.org/0000-0002-9144-4496

Complete contact information is available at: https://pubs.acs.org/10.1021/cbe.3c00083

Author Contributions

N.H.: methodology, investigation, writing original draft. H.Y.: funding acquisition; project administration. L.Y.: conceptualization, funding acquisition, project administration, supervision, writing—review and editing.

Notes

The authors declare no competing financial interest.

https://doi.org/10.1021/cbe.3c00083
Chem Bio Eng. 2024, 1, 91−98

■ ACKNOWLEDGMENTS

This work was supported by the National Key Research and Development Program of China (Grant No. 2021YFC2103700), the National Natural Science Foundation of China (Grant No. 32171412), and the Fundamental Research Funds for the Central Universities (Grant No. 226-2022-00055).

■ REFERENCES

(1) Lederer, E. Chemistry and biochemistry of some mammalian secretions and excretions. *J. Chem. Soc.* **1949**, 2115−2125.
(2) Eichhorn, E.; Schroeder, F. From ambergris to (−)-ambrox: chemistry meets biocatalysis for sustainable (−)-ambrox production. *J. Agric. Food Chem.* **2023**, *71* (13), 5042−5052.
(3) Rowland, S. J.; Sutton, P. A.; Knowles, T. D. The age of ambergris. *Nat. Prod. Res.* **2019**, *33* (21), 3134−3142.
(4) Pelletier, P.; Caventou, J. Sur la nature de la substance adipocireuse de l'ambre gris et sur l'origine de ce produit. *Journal de Pharmacie* **1820**, 49−58.
(5) Taha, S. A.; Islam, M.; Ageel, A. Effect of ambrein, a major constituent of ambergris, on masculine sexual behavior in rats. *Arch. Int. Pharmacodyn. Ther.* **1995**, *329* (2), 283−294.
(6) Oritani, T.; Yamashita, K.; Matsui, M. Synthesis of ambrein. *Agric. Biol. Chem.* **1990**, *54* (2), 571−573.
(7) Mori, K.; Tamura, H. Triterpenoid total synthesis, I. Synthesis of ambrein and Ambrox®. *Liebigs Ann. Chem.* **1990**, *1990* (4), 361−368.
(8) Tanimoto, H.; Oritani, T. Synthesis of (+)-ambrein. *Tetrahedron* **1997**, *53* (10), 3527−3536.
(9) Eichhorn, E.; Locher, E.; Guillemer, S.; Wahler, D.; Fourage, L.; Schilling, B. Biocatalytic process for (−)-ambrox production using squalene hopene cyclase. *Adv. Synth. Catal.* **2018**, *360* (12), 2339−2351.
(10) Stoll, M.; Hinder, M. Odeur et constitution III. Les substances bicyclohomofarnésiques. *Helv. Chim. Acta* **1950**, *33* (5), 1251−1260.
(11) Verstegen-Haaksma, A. A.; Swarts, H. J.; Jansen, B. J.; de Groot, A.; Bottema-MacGillavry, N.; Witholt, B. Application of *S*-(+)-carvone in the synthesis of biologically active natural products using chemical transformations and bioconversions. *Ind. Crops Prod.* **1995**, *4* (1), 15−21.
(12) Barrero, A. F.; Altarejos, J. n.; Alvarez-Manzaneda, E. J.; Ramos, J. M.; Salido, S. a. Synthesis of (±)-ambrox from (*E*)-nerolidol and *β*-ionone via allylic alcohol [2, 3] sigmatropic rearrangement. *J. Org. Chem.* **1996**, *61* (6), 2215−2218.
(13) Chapuis, C. Enantioselective access to (−)-Ambrox® atarting from *β*-farnesene. *Helv. Chim. Acta* **2014**, *97* (2), 197−214.
(14) Koyama, H.; Kaku, Y.; Ohno, M. Synthesis of (−)-ambrox from L-abietic acid. *Tetrahedron Lett.* **1987**, *28* (25), 2863−2866.
(15) Bolster, M. G.; Jansen, B. J.; de Groot, A. The synthesis of (−)-Ambrox® starting from labdanolic acid. *Tetrahedron* **2001**, *57* (26), 5657−5662.
(16) Moulines, J.; Lamidey, A.-M.; Desvergnes-Breuil, V. A practical synthesis of Ambrox® from sclareol using no metallic oxidant. *Synth. Commun.* **2001**, *31* (5), 749−758.
(17) Moulines, J.; Bats, J.; Lamidey, A.; Da Silva, N. About a practical synthesis of Ambrox® from sclareol: a new preparation of a ketone key intermediate and a close look at its Baeyer-Villiger oxidation. *Helv. Chim. Acta* **2004**, *87* (10), 2695−2705.
(18) Barrero, A. F.; Alvarez-Manzaneda, E. J.; Chahboun, R.; Arteaga, A. F. Degradation of the side chain of (−)-sclareol: a very short synthesis of *nor*-ambreinolide and ambrox. *Synth. Commun.* **2004**, *34* (19), 3631−3643.
(19) Yang, S.; Tian, H.; Sun, B.; Liu, Y.; Hao, Y.; Lv, Y. One-pot synthesis of (−)-ambrox. *Sci. Rep.* **2016**, *6* (1), No. 32650.
(20) Ueda, D.; Hoshino, T.; Sato, T. Cyclization of squalene from both termini: identification of an onoceroid synthase and enzymatic synthesis of ambrein. *J. Am. Chem. Soc.* **2013**, *135* (49), 18335−18338.
(21) Sato, T.; Hoshino, T. Functional analysis of the DXDDTA motif in squalene-hopene cyclase by site-directed mutagenesis experiments: initiation site of the polycyclization reaction and stabilization site of the carbocation intermediate of the initially cyclized A-ring. *Biosci., Biotechnol., Biochem.* **1999**, *63* (12), 2189−2198.
(22) Ke, D.; Caiyin, Q.; Zhao, F.; Liu, T.; Lu, W. Heterologous biosynthesis of triterpenoid ambrein in engineered *Escherichia coli*. *Biotechnol. Lett.* **2018**, *40*, 399−404.
(23) Moser, S.; Strohmeier, G. A.; Leitner, E.; Plocek, T. J.; Vanhessche, K.; Pichler, H. Whole-cell (+)-ambrein production in the yeast *Pichia pastoris*. *Metab. Eng. Commun.* **2018**, *7*, No. e00077.
(24) Moser, S.; Leitner, E.; Plocek, T. J.; Vanhessche, K.; Pichler, H. Engineering of *Saccharomyces cerevisiae* for the production of (+)-ambrein. *Yeast* **2020**, *37* (1), 163−172.
(25) Yamabe, Y.; Kawagoe, Y.; Okuno, K.; Inoue, M.; Chikaoka, K.; Ueda, D.; Tajima, Y.; Yamada, T. K.; Kakihara, Y.; Hara, T. Construction of an artificial system for ambrein biosynthesis and investigation of some biological activities of ambrein. *Sci. Rep.* **2020**, *10* (1), No. 19643.
(26) Pollier, J.; Vancaester, E.; Kuzhiumparambil, U.; Vickers, C. E.; Vandepoele, K.; Goossens, A.; Fabris, M. A widespread alternative squalene epoxidase participates in eukaryote steroid biosynthesis. *Nat. Microbiol.* **2019**, *4* (2), 226−233.
(27) Matthews, P.; Wurtzel, d. E. Metabolic engineering of carotenoid accumulation in *Escherichia coli* by modulation of the isoprenoid precursor pool with expression of deoxyxylulose phosphate synthase. *Appl. Microbiol. Biotechnol.* **2000**, *53*, 396−400.
(28) Ghimire, G. P.; Lee, H. C.; Sohng, J. K. Improved squalene production via modulation of the methylerythritol 4-phosphate pathway and heterologous expression of genes from *Streptomyces peucetius* ATCC 27952 in *Escherichia coli*. *Appl. Environ. Microbiol.* **2009**, *75* (22), 7291−7293.
(29) Katabami, A.; Li, L.; Iwasaki, M.; Furubayashi, M.; Saito, K.; Umeno, D. Production of squalene by squalene synthases and their truncated mutants in *Escherichia coli*. *J. Biosci. Bioeng.* **2015**, *119* (2), 165−171.
(30) Meng, Y.; Shao, X.; Wang, Y.; Li, Y.; Zheng, X.; Wei, G.; Kim, S.; Wang, C. Extension of cell membrane boosting squalene production in the engineered *Escherichia coli*. *Biotechnol. Bioeng.* **2020**, *117* (11), 3499−3507.
(31) Donald, K.; Hampton, R. Y.; Fritz, I. B. Effects of overproduction of the catalytic domain of 3-hydroxy-3-methylglutaryl coenzyme A reductase on squalene synthesis in *Saccharomyces cerevisiae*. *Appl. Environ. Microbiol.* **1997**, *63* (9), 3341−3344.
(32) Rasool, A.; Ahmed, M. S.; Li, C. Overproduction of squalene synergistically downregulates ethanol production in *Saccharomyces cerevisiae*. *Chem. Eng. Sci.* **2016**, *152*, 370−380.
(33) Paramasivan, K.; Mutturi, S. Recent advances in the microbial production of squalene. *World J. Microbiol. Biotechnol.* **2022**, *38* (5), 91.
(34) Zhu, Z.-T.; Du, M.-M.; Gao, B.; Tao, X.-Y.; Zhao, M.; Ren, Y.-H.; Wang, F.-Q.; Wei, D.-Z. Metabolic compartmentalization in yeast mitochondria: Burden and solution for squalene overproduction. *Metab. Eng.* **2021**, *68*, 232−245.
(35) Patel, A.; Bettiga, M.; Rova, U.; Christakopoulos, P.; Matsakas, L. Microbial genetic engineering approach to replace shark livering for squalene. *Trends Biotechnol* **2022**, *40*, 1261.
(36) Farbood, M. I.; Morris, J. A.; Downey, A. E. Process for producing diol and lactone and microorganisms capable of same. US Pat. US4970163 1990.
(37) Fang, Y.; Wang, Z.; Shi, Y.; Liu, F.; Wang, J.; Yang, T.; Xin, Y.; Gu, Z.; Zhang, L. Efficient biotransformation of sclareol to sclareolide by *Filobasidium magnum* JD1025. *Appl. Biochem. Biotechnol.* **2023**, *195* (2), 1184−1196.
(38) Farbood, M. I.; Willis, B. J. Process for producing diol and furan and microorganism capable of same. US Pat. US4798799 1989.

https://doi.org/10.1021/cbe.3c00083
Chem Bio Eng. 2024, 1, 91−98

(39) Wang, X.; Zhang, X.; Yao, Q.; Hua, D.; Qin, J. Comparative proteomic analyses of *Hyphozyma roseonigra* ATCC 20624 in response to sclareol. *Braz. J. Microbiol* **2019**, *50* (1), 79−84.
(40) Ncube, E. N.; Steenkamp, P. A.; van der Westhuyzen, C. W.; Steenkamp, L. H.; Dubery, I. A. Metabolomics-guided analysis of the biocatalytic conversion of sclareol to ambradiol by *Hyphozyma roseoniger*. *Catalysts* **2022**, *12* (1), 55.
(41) Ncube, E. N.; Mathiba, K.; Steenkamp, L. H.; Dubery, I. A. Gas chromatographic profiling of the biocatalytic conversion of sclareol to ambradiol by *Hyphozyma roseoniger*. *Biocatal. Biotransform.* **2022**, *40* (4), 308−312.
(42) Diao, M.; Li, C.; Li, J.; Lu, J.; Xie, N. Probing the biotransformation process of sclareol by resting cells of *Hyphozyma roseonigra*. *J. Agric. Food Chem.* **2022**, *70*, 10563.
(43) Schalk, M.; Pastore, L.; Mirata, M. A.; Khim, S.; Schouwey, M.; Deguerry, F.; Pineda, V.; Rocci, L.; Daviet, L. Toward a biosynthetic route to sclareol and amber odorants. *J. Am. Chem. Soc.* **2012**, *134* (46), 18900−18903.
(44) Song, Y.; Shen, H.; Yang, W.; Yang, X.; Gong, Z.; Zhao, Z. K. High cell density culture of an engineered yeast strain for sclareol production. *Shengwu Gongcheng Xuebao* **2015**, *31* (1), 147−151.
(45) Trikka, F. A.; Nikolaidis, A.; Athanasakoglou, A.; Andreadelli, A.; Ignea, C.; Kotta, K.; Argiriou, A.; Kampranis, S. C.; Makris, A. M. Iterative carotenogenic screens identify combinations of yeast gene deletions that enhance sclareol production. *Microb. Cell Fact.* **2015**, *14* (1), 1−19.
(46) He, N.; Li, D.-F.; Yu, H.-W.; Ye, L.-D. Construction of an artificial microbial consortium for green production of (−)-ambroxide. *ACS Sustainable Chem. Eng.* **2023**, *11* (5), 1939−1948.
(47) Cao, X.; Yu, W.; Chen, Y.; Yang, S.; Zhao, Z. K.; Nielsen, J.; Luan, H.; Zhou, Y. J. Engineering yeast for high-level production of diterpenoid sclareol. *Metab. Eng.* **2023**, *75*, 19−28.
(48) Yang, W.; Zhou, Y.; Liu, W.; Shen, H.; Zhao, Z. K. Engineering *Saccharomyces cerevisiae* for sclareol production. *Shengwu Gongcheng Xuebao* **2013**, *29* (8), 1185−1192.
(49) NEUMANN, S.; SIMON, H. Purification, partial characterization and substrate specificity of a squalene cyclase from *Bacillus acidocaldarius*. *Biol. Chem.* **1986**, *367* (2), 723−730.
(50) Seitz, M.; Klebensberger, J.; Siebenhaller, S.; Breuer, M.; Siedenburg, G.; Jendrossek, D.; Hauer, B. Substrate specificity of a novel squalene-hopene cyclase from *Zymomonas mobilis*. *J. Mol. Catal. B: Enzym.* **2012**, *84*, 72−77.

https://doi.org/10.1021/cbe.3c00083
Chem Bio Eng. 2024, 1, 91−98