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Sustainable (–)-Ambrox Production: Chemistry Meets Biocatalysis

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Sandmeyer Award 2023

Abstract: (–)-Ambrox, the most prominent olfactive component of ambergris, is one of the most widely used biodegradable fragrance ingredients. It is traditionally produced from the diterpene sclareol chemically modified and cyclized into (–)-ambrox. The availability of the new feedstock (*E*)-β-farnesene produced by fermentation opened new routes to (*E,E*)-homofarnesol as a precursor to (–)-ambrox. Combining the chemical transformation of (*E*)-β-farnesene to (*E,E*)-homofarnesol and its enzymatic cyclization with an engineered Squalene Hopene Cyclase provided a new sustainable route for the production of (–)-ambrox at industrial scale. Compared to the traditional synthesis from sclareol, the new and innovative route from (*E*)-β-farnesene improves atom and step economy, reduces waste production, solvent and energy consumption.

Keywords: (–)-Ambrox · Biocatalysis · Farnesene · Homofarnesol · Squalene Hopene Cyclase

Photo taken at the Swiss Chemistry Science Night 2023 on the occasion of the celebration of the **Sandmeyer Award 2023**. From left to right: Eric Eichhorn, Boris Schilling, Agnes Bombrun, and Fridtjof Schroeder. Photo Andre Maurer. © SCS.

Dr. Eric Eichhorn is a senior research scientist in process research in the Fragrance & Beauty division of Givaudan in Kemptthal, Switzerland. He studied biology at Université Louis Pasteur in Strasbourg and obtained his Bioengineer's degree from the Ecole Supérieure de Biotechnologie de Strasbourg (Eucor). After his PhD in microbial metabolism with Prof. Thomas Leisinger, postdoctoral work in protein crystallography (Prof. Timothy Richmond) and biotechnology (Prof. Bernard Witholt) at ETH Zurich, he was project leader biotransformations at Lonza, Visp. In 2008 he joined Givaudan as a Research Scientist applying biocatalysis to the discovery and production of fragrance ingredients.

Dr. Boris Schilling is currently head of external opportunities in ingredients research in the Fragrance & Beauty division of Givaudan in Kemptthal, Switzerland - and involved in open innovation programs. He started more than 30 years ago as laboratory head in charge of flavor and fragrance projects at Givaudan Corporate Research and later became head of the biotechnology department. Boris studied biochemistry and molecular biology and got a PhD from the University of Zurich. He completed his postdoctoral training at Harvard Medical School in Boston prior to joining the Givaudan research organization.

Dr. Agnes Bombrun has been in charge of the ingredients research in the Fragrance & Beauty division of Givaudan in Kemptthal, Switzerland, since 2014. Her main responsibility is to drive dynamically a rich and competitive pipeline of sustainable innovation for the perfumers, delivering the best fragrance molecules and processes by green chemistry and biotechnology. Agnes started out her career in the pharmaceutical industry. By training she is a chemical engineer (Lyon, France) and an organic chemist with a PhD (Emory University, Atlanta). Agnes was Director of Medicinal Chemistry for Merck Serono and also worked for GSK and Affymax.

Dr. Fridtjof Schroeder is a research fellow in process research in the Fragrance & Beauty division of Givaudan in Kemptthal, Switzerland. He completed his studies at the Freie Universität Berlin with a PhD in synthetic organic chemistry in the group of Prof. Johann Mulzer, followed by postdoctoral years with Prof. Oppolzer (Geneva) and Prof. Eschenmoser (Frankfurt). After two years of research in pharmaceutical chemistry at Cilag AG (Johnson & Johnson) he joined Givaudan in 1999. His current process research activities are dedicated to innovative techniques in the area of homogeneous catalysis, cyclopropanation, flow chemistry and photochemistry for the development of fragrance ingredients.

1. Introduction

Finding novel, innovative and sustainable routes to manufacture iconic molecules using state of the art technologies is at-

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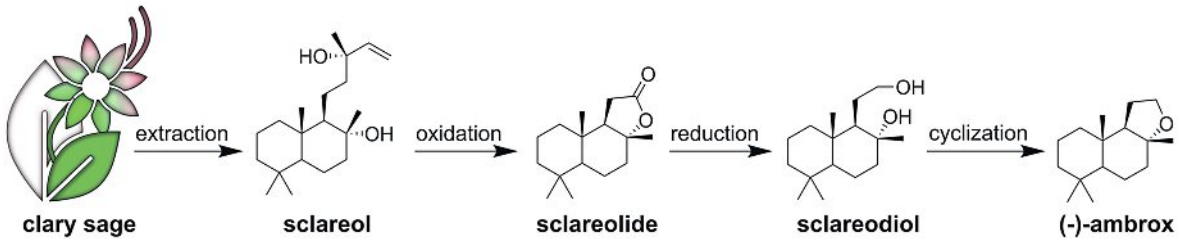


Scheme 1. (–)-Ambrox production from clary sage.

tractive. The perfumery ingredient (–)-ambrox has been the gold standard for a much-appreciated olfactive note that is widely used in perfumery creation work and has kept generations of chemists, and more recently biochemists on their toes.

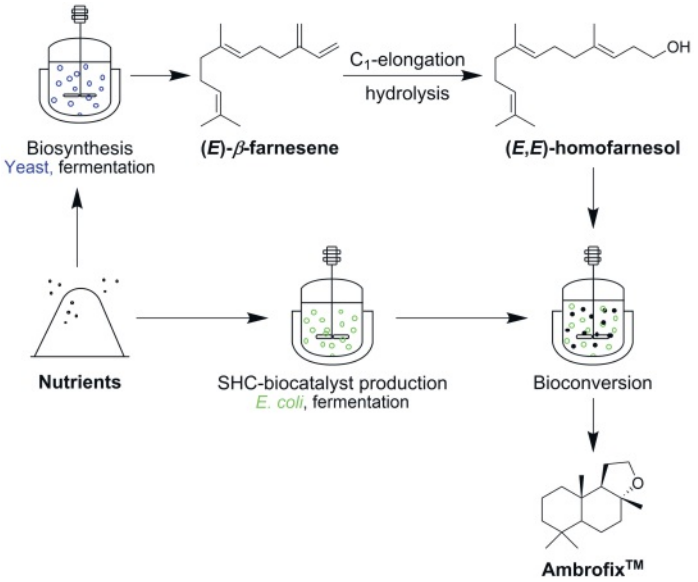
(–)-Ambrox was originally identified as the main odour vector in ambergris, a coprolith of the sperm whale that is produced as a response to irritation of its intestines. When the solid matter is eventually expelled it is believed that the odourless terpene (–)-ambrein is subject to oxidative degradation where (–)-ambrox, the odorous principle of the much praised ambergris is generated.[1] The first synthesis of (–)-ambrox using the diterpene sclareol as the starting material was reported in 1950.[2,3] Access to large quantities of sclareol extracted from clary sage (*Salvia sclarea*) triggered exploration of various chemistry routes for (–)-ambrox production leading to the first and well established industrial route shown in Scheme 1.[1,4] With the growing use of the biodegradable fragrance ingredient, which adds woody, ambery, tobacco, animalic, musky and warm earthy facets in perfume creations, numerous alternative approaches to this material were considered, mainly through new chemical synthesis routes.[1]

The discovery of a suitable biocatalyst to produce the tricyclic (–)-ambrox compound has been the centerpiece of the innovative route described in this paper. Historically, the enzyme Squalene Hopene Cyclase (SHC) was extensively studied as it catalyses a very complex reaction when converting the C_{30} squalene to the pentacyclic hopene, which is widespread in microorganisms.[5-9] Back in 1986, research teams isolated for the first time an SHC enzyme and demonstrated that it was able to cyclize (*E,E*)-homofarnesol (EEH), an alternative substrate with a C_{16} skeleton to (–)-ambrox, albeit at very low rate.[10,11] A multi-year and iterative research program at Givaudan allowed to evolve the wild-type SHC enzyme from the thermophilic Archaea *Alicyclobacillus acidocaldarius* into a highly efficient and selective biocatalyst for the cyclization of EEH to (–)-ambrox, produced as Ambrofix™ at Givaudan. Whole-cell biotransformation with *Escherichia coli* cells producing the desired SHC enzyme worked very well both in the lab and at industrial scale.[12,13]

An efficient access to EEH was a prerequisite to consider upscaling the new route. The availability of the sesquiterpene (*E*)-β-farnesene from a yeast fermentation process[14] was the starting point to investigate a plethora of chemical synthesis routes in order to identify an environmentally conscious process, where cyclopropanation of (*E*)-β-farnesene followed by rearrangement and hydrolysis resulted in the formation of EEH.[1] Continuous improvement of this process to meet sustainability criteria as well as quality and efficiency targets involved the development of an elegant and scalable multistep flow process, which is further described herein.

Industrialization of the SHC biocatalyst production, the synthesis of EEH from (*E*)-β-farnesene, and the whole-cell biotransformation process for EEH cyclization was orchestrated in a synchronized manner with the invaluable support of a team of experts. While this new and innovative process is currently the most sustainable and carbon efficient in the market, it was also crucial to demonstrate that the purified Ambrofix™ can directly replace the material produced *via* the traditional route from sclareol.

In 2016, we published for the first time the biocatalytic process for the production of Ambrofix™ in which EEH produced from (*E*)-β-farnesene is cyclized in a single-step with an engineered SHC enzyme (Scheme 2).[12]



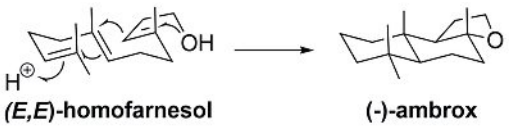
Scheme 2. Ambrofix™ production from (*E,E*)-homofarnesol with Squalene Hopene Cyclase.

Today, (–)-ambrox is one of the most widely used biodegradable fragrance ingredients and it occurs in about 30 % of all fragrance creations globally. Ambrofix™ is produced at Givaudan with the innovative approach described in this article, combining the use of a new starting material with breakthrough biotechnology and a state-of-the-art green chemistry process. Recent perfumes featuring Ambrofix™ are for example *Paradoxe* by Prada, *MYSLF* by YSL and *Eau Capitale* by Diptyque.

The successful industrialization of the new route to (–)-ambrox and the fruitful collaboration of Givaudan teams internally and with external project partners has been acknowledged with the prestigious **Sandmeyer Award**, which was presented to Givaudan at the occasion of the Swiss Chemistry Science Night 2023.

2. Synthesis of the (*E,E*)-Homofarnesol Precursor

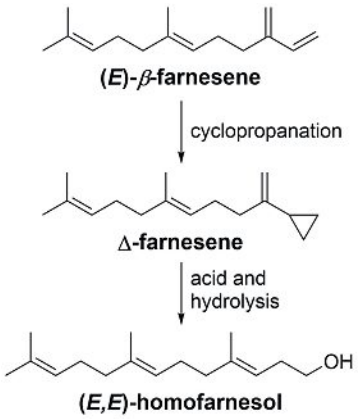
To access the desired stereoisomer (–)-ambrox, which is formed through a chair–chair configurated transition state according to the Stork-Eschenmoser principle (Scheme 3),[15,16] synthetic efforts were directed towards the synthesis of predominantly *E*-configurated homofarnesol.



Scheme 3. Cyclization of (*E,E*)-homofarnesol to (–)-ambrox.

After an in-depth exploration of many potential routes,[1] the one shown in Scheme 4 was prioritized due to the availability of (*E*)-β-farnesene through sugar fermentation,[14] a highly selective Pd-catalyzed cyclopropanation of (*E*)-β-farnesene at the monosubstituted double bond, and an *E*-selective smooth cyclopropyl carbinyl rearrangement of Δ-farnesene. In addition, the processing of liquid long-chain polyenes (of Scheme 4) are of industrial advantage preventing from the tedious use and transfer of solids such as sclareol, sclareolide and sclareodiol (as in Scheme 1).

Furthermore, cyclopropanation and rearrangement to non-cyclopropane entities turned out to be the most straightforward preparation of EEH and benefited from our earlier studies in that area.[17]



Scheme 4. (*E,E*)-Homofarnesol synthesis from (*E*)-β-farnesene.

2.1 *Cyclopropanation of (E)-β-Farnesene*

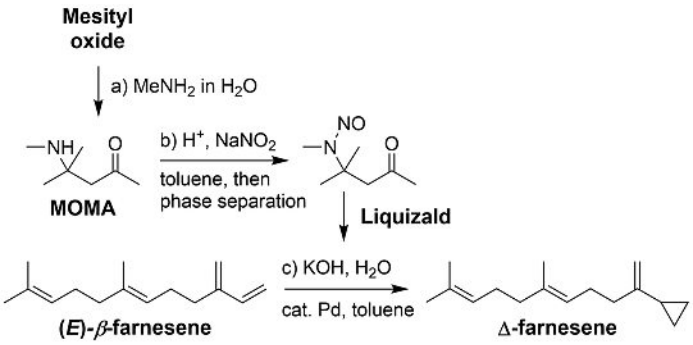
The first challenge of the cyclopropanation step was the regioselective functionalization of the *mono*-substituted double bond of (*E*)-β-farnesene, which was best achieved with diazomethane (DAM) under palladium catalysis in analogy to earlier studies by others on isoprene and other alkene substrates.[18] This method also allowed the *in situ* generation and immediate consumption of the highly toxic and explosive DAM, dose-controlled by the addition of the *N*-methyl-*N*-nitroso precursor to (*E*)-β-farnesene and catalytic amounts of Pd(II) in a two-phase system of toluene and aqueous KOH. Pd(II) catalysts such as $Pd(OAc)_2$, $Pd(acac)_2$ or Herrmann's catalyst were best suited for this purpose, whereas iron(III) tetraphenylporphyrine chloride[19] for example catalyzed preferentially the cyclopropanation of the *exo*-methylene group under these conditions.[20]

The handling of diazomethane and its *N*-methyl-*N*-nitroso precursor, however, posed safety challenges, including the safe synthesis of the latter. Only *N*-methyl-*N*-nitroso urea (NMU) had been described for the generation of DAM in Pd-catalyzed cyclopropanation reactions at this time.[18,21] The low initiation temperature of NMU (T_{ex} 20 °C) and toxic by-products expected from a potential explosion of this precursor rendered NMU a 'no go' for further development at Givaudan. In addition, the processing of solid NMU was seen as problematic as that of solid Diazald and recently proposed analogues.[22] They were therefore not considered any further.

Whereas DAM can be generated from other liquid *N*-methyl-*N*-nitroso precursors, Liquizald[23] was finally chosen as the safest reagent (T_{ex} 75 °C). Advantageously, the preparation of Liquizald and recycling of mesityl oxide formed after cyclopropanation does not involve isolation and transportation of solids.

Liquizald was prepared from the instable mesityloxide-methylamine adduct (MOMA) by nitrosation in the presence of simple carboxylic acids (Scheme 5).[24] Because the cyclopropanation of (*E*)-β-farnesene occurs in basic medium, separation of Liquizald from the acid reaction medium used for its formation is necessary. Dose-controlled addition of Liquizald to (*E*)-β-farnesene keeps the DAM level of this reaction at a minimum, as followed online by carefully adjusted process analytical technology (PAT), which also controls all other reaction components and by-products. Thus, generation and consumption of Liquizald and DAM are safely monitored, keeping the critical DAM headspace concentration during reaction at zero and producing Δ-farnesene without traces of Liquizald.

The sequence of Scheme 5 was engineered into a multistep-flow process for a continuous separation of Liquizald (after step b) to avoid its accumulation and possible explosive decomposition. Additionally, we realized a better control and reproducibility of all other steps, *e.g.* the formation of instable MOMA and the cyclopropanation step. Such engineering for high flow-through volumes represented a significant logistic effort (Scheme 6), all the more because the next best flow cyclopropanation procedure known at the beginning of this endeavour was the one of styrene with *N*-methyl-*N*-nitroso urea.[21] Safe handling of the C_1 reagent in flow was the key criteria for the move from batch to flow.

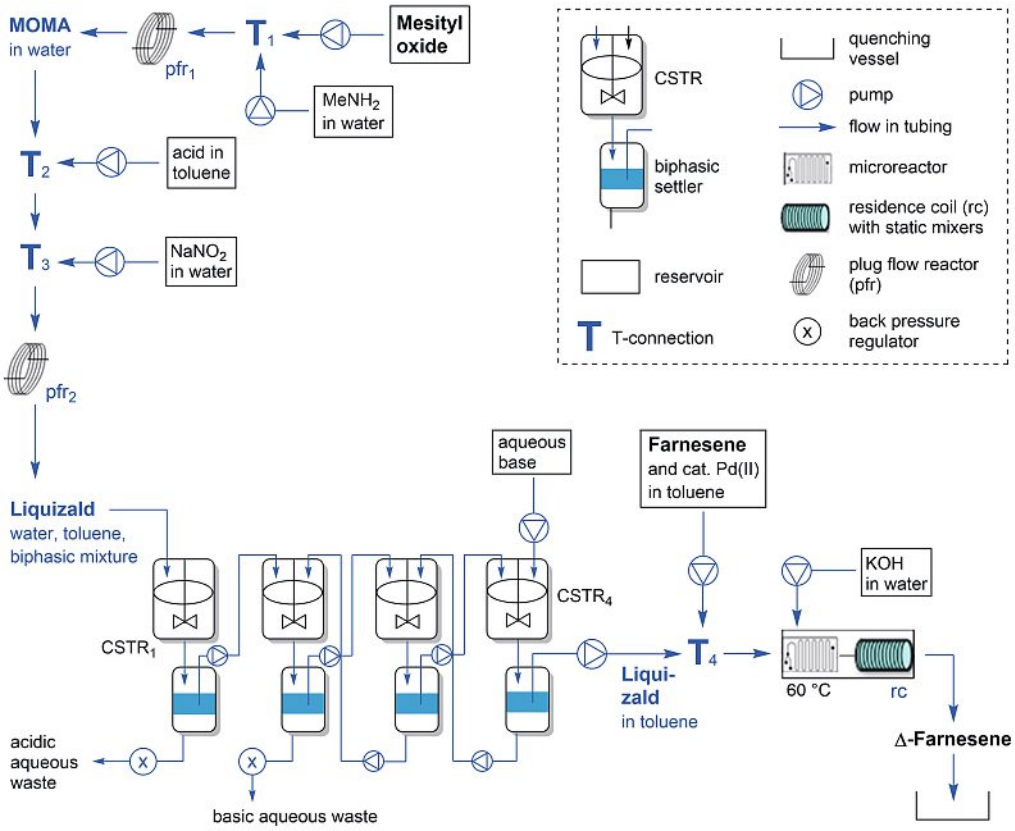


Scheme 5. Cyclopropanation of (*E*)-β-farnesene with Liquizald.

On top of the better safety control, the yield of Liquizald was significantly improved in flow *versus* batch. The cyclopropanation was realized with similar efficiency in batch and flow, with initial TON's of 1600 at 0.06 mol-% catalyst concentration, independent from microreactor fine structures and with complete conversion through residence units with static mixers. Partial and full flow processing of all steps (of Scheme 5) resulted in a multistep flow process,[25] with continuous separation units of Liquizald as centerpiece, including the controlled generation and reaction of instable MOMA, the controlled generation, separation, and reaction of explosive Liquizald (without accumulation and storage) and the final generation of Liquizald- and DAM-free Δ-farnesene (Scheme 6).[26]

2.2 *Cyclopropylcarbinyl Rearrangement of Δ-Farnesene*

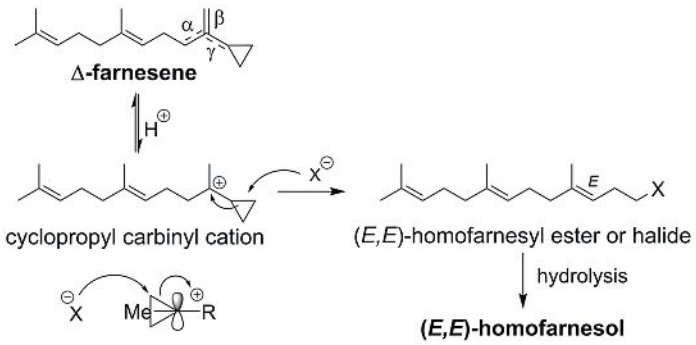
The β-isomer of Δ-farnesene was rearranged under carbocationic conditions, producing after hydrolysis, EEH almost quantitatively and with good 3,4-*E,Z*-ratios (Scheme 7).[27] This selectivity follows from a Newman projection where the less hindered methyl group adopts a staggered position *versus* the cyclopropyl group.[28] The cyclopropylvinyl rearrangement of the Δ-farnesene isomers occurs in counter clockwise formation *via* $\gamma \rightarrow \beta \rightarrow \alpha$ according to the $\alpha > \beta > \gamma$ stabilities of these isomers. Practically, the β-isomer of Δ-farnesene upon reaction with a Brønsted acid, forms the corresponding (*E,E*)-homofarnesyl ester or halide with slightly better yields and selectivities than the corresponding γ-isomer claimed elsewhere,[29] which isomeriz-



Scheme 6. Continuous multistep flow synthesis of Δ-farnesene. General chemical scheme and structures: see Scheme 5. This scheme does not include further variations *e.g.* additional pumps for pressure maintenance, additional units for Pd-recycling, *etc.*

es to the β- and α-isomers before rearrangement. Accordingly, only the α-isomer but never the γ-isomer was detected during the rearrangement of the isolated β-isomer selectively accessed through our route.

Regarding process mass intensity (PMI) the synthesis of EEH from (*E*)-β-farnesene was superior to other processes. These and other advantages such as a shorter reaction sequence without redox chemistry rendered the route shown in Scheme 4 as a highly efficient sequence, which was selected for industrialization.[1]



Scheme 7. Cyclopropyl carbinyl rearrangement of Δ-farnesene, cyclopropyl carbinyl cation and (*E,E*)-homofarnesyl ester or halide. X = Brønsted acid anion.

3. SHC Enzyme Evolution

Efficient and rapid enzyme engineering transforms wild-type enzymes into tailor-made biocatalysts.[30,31] Enzymes with novel or improved catalytic activities are designed and enzyme engineering provides the synthetic industry with stable, selective, and productive biocatalysts operating under the desired process conditions. Biocatalysis is a standard approach in route scouting in chemical synthesis and industrial applications, when looking for exquisite specificity and selectivity in chemical synthesis.[32]

3.1 *Random Mutagenesis*

Random mutagenesis was used for the first and second SHC evolution rounds. The first enzyme evolution provided approx. 10'000 variants of *A. acidocaldarius* wild-type SHC, which were screened for improved activity in microtiter plate reactions with 4 g/l EEH at reaction conditions defined as optimal for the parent wild-type enzyme.[12,13] 90 hits were selected and validated at millilitre scale. Three variants were identified where initial rates of (–)-ambrox formation were significantly improved over the parent enzyme, uncovering eight distinct amino acid mutations distributed across the three variants. Based on these mutations, 28 additional variants were constructed (single or combined mutations) to identify mutations responsible for activity improvement, and to combine beneficial mutations for a possible further increase in performance.[12,13] Reaction conditions were set to individual optima for a final performance ranking of the variants and selection of a best performer. A benchmark process was set up with the 1st generation SHC variant for full conversion of 125 g/l EEH in 3 days with 250 g/l of *E. coli* cells producing this variant (Fig. 1).[12,13]

The same procedure was applied using the 1st generation SHC variant as the parent enzyme.[33] The same optimal mutational load as determined for the SHC enzyme during the first enzyme evolution was targeted. Three libraries were produced and screened for improved SHC variants in reactions with an EEH concentration increased to 34 g/l, and at reaction conditions defined as optimal for

the 1st generation SHC variant parent enzyme. The screening of approx. 9'900 variants identified three improved variants with 2 or 3 new mutations each. A best performer was selected after the identification of beneficial mutations and a final ranking of the variants as described above. The 2nd generation variant reduced significantly reaction time in bioconversions run at the benchmark substrate and cells concentrations of the first generation process (Fig. 1).

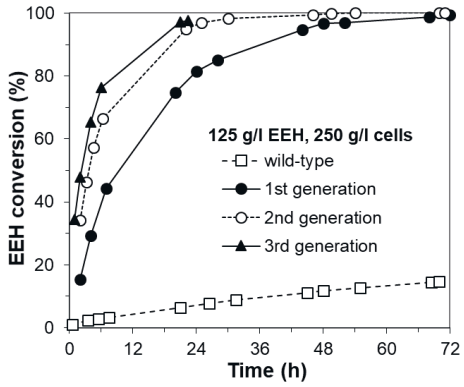


Fig. 1. Wild-type, 1st, 2nd, and 3rd generation SHC biocatalysts in bioconversions with 125 g/l EEH and 250 g/l cells run at reaction conditions individually optimized for each SHC enzyme.

3.2 *Rational Design*

Random mutagenesis was not used for the third enzyme evolution. Instead, mutations were addressed at positions corresponding to substrate-interacting residues conserved in more than 70 or 90 % of SHC amino acid sequences.[34] These mutations control cationic cyclization cascades by introducing or removing hydrogen bonds between a functional group of the substrate and surrounding polar amino acids of the active site.[35]

Substrate-docking studies identified 8 distinct amino acid residues in vicinity to the alcohol, which were randomized by site-saturation mutagenesis, and the EEH cyclization properties of the resulting total 900 variants were analysed. Positions 600 and 169 appeared most relevant to the cyclization.[36]

Variants were correspondingly constructed in *A. acidocaldarius* wild-type SHC, 1st and 2nd generation SHC variants. The characterization of 8 variants at millilitre scale in reactions with 16 g/l EEH allowed reaction conditions to be adjusted to new individual *optima* for a final ranking and selection of the best performer(s) in reactions run with 125 g/l EEH and 250 g/l cells.[37] The 3rd generation variant reduced further reaction time in bioconversions run at the benchmark substrate and cells concentrations of the first generation process (Fig. 1).

3.3 *Mutations*

The SHC variant produced in three evolution rounds caries a total of seven mutations, with a maximum of 3 mutations introduced per evolution round (Fig. 2). Two of the mutations identified during the first enzyme evolution (Fig. 2, red) are located in a region designated as gate keeper and controlling substrate passage in the entrance channel.[38] They are postulated to increase catalytic performance by promoting substrate trafficking and binding in the active site (positions 132 and 432), enhancing this effect by means of network interactions and amino acid side-chain reorganizations.

The two mutations in the active site at positions 169 and 600 (Fig. 2, green) expand size and shape of the active site and/or play a role in the formation/stabilization of cationic intermediates.[36] These mutations increase both the substrate specificity and product selectivity of the enzyme: the 3rd generation SHC variant leaving (*E,Z*)-homofarnesol almost untouched is highly selective towards the (*E,E*)-isomer of homofarnesol, from which it produces only or almost only (–)-ambrox.[37]

Altogether, a better substrate flow and/or pre-folding is postulated to be responsible for the improved catalytic properties of the designed SHC variants.

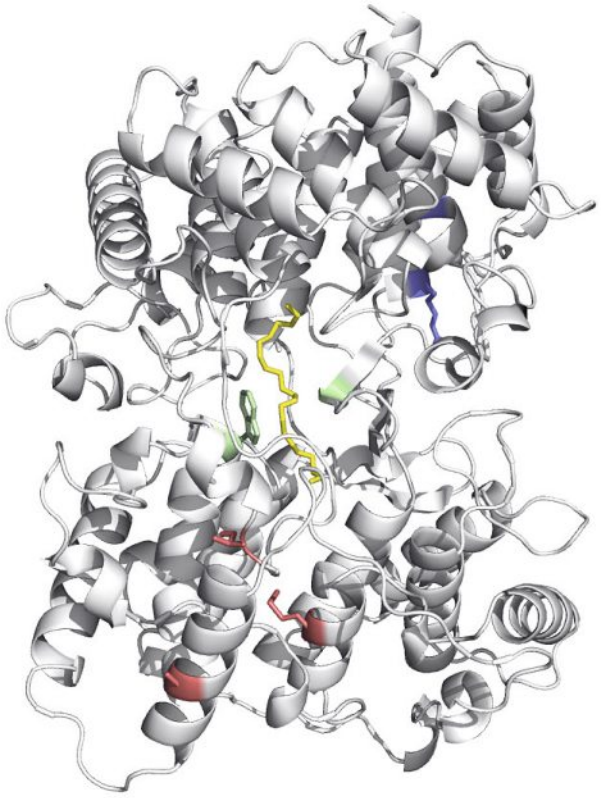


Fig. 2. Amino acids mutated in the 1st (red), 2nd (blue) and 3rd (green) enzyme evolution round. Prepared with the crystal structure of *A. acidocaldarius* SHC (PDB 1SQC) co-crystallized with a substrate analogue (yellow) using PyMOL (The PyMOL Molecular Graphics System, version 2.4.0 Schrödinger, LLC).

4. Ambrofix™ Production from (*E,E*)-Homofarnesol with Squalene Hopene Cyclase

4.1 *Biotransformation*

Ambrofix™ production from EEH is based on biotransformation systems with whole cells of *E. coli* producing an SHC enzyme variant. The reactions are run in a succinate buffer. They require pH and temperature control, and some surfactant according to the specific requirements of the individual SHC variants.

The biotransformation is responsive to three interconnected parameters: substrate, cells and surfactant concentrations. The sensitivity of the SHC variants towards changes in pH, temperature and surfactant concentration was recognized at the early stages of variant characterization. Optimal reaction conditions for SHC variants differed significantly from the ones of the parent enzymes applied during screening, although only a few mutations were introduced. Setting reaction parameters to individual *optima* was a prerequisite for the selection of best performer(s), and for optimization toward increased space-time yield.[12,13,33,37]

The low substrate concentration conditions applied during the initial screening for SHC variants with improved EEH cyclization properties prevented Ambrofix™ production at multi-ton scale. Setting viable process-relevant conditions with the 1st generation

SHC variant was done by means of a DOE to increase substrate concentration and optimize the biocatalyst to substrate ratio. Key process metrics were defined regarding substrate conversion, product titre, biocatalyst load and space time yield to evolve the reaction conditions, targeting high productivity for setting up the first generation process.[12,13] Investigations with the 2nd and 3rd generation variants aimed at increasing space time yield, targeting higher substrate concentrations and lowest possible cells:substrate ratio for full EEH conversion, and also confirming the robustness of the new process conditions.

The initial Ambrofix™ production process allowed for full conversion of 125 g/l EEH in 72 h with 250 g/l cells producing the 1st generation SHC variant (Figs. 1 and 3A). The product titre achieved was well positioned among reported biocatalytic processes and was already cited as impressive.[39] Increasing volumetric productivity was possible: 188 g/l EEH were fully cyclized maintaining a cells:substrate ratio of 2:1.[12,13]

The second generation SHC variant enabled full EEH conversion at the benchmark substrate and cell concentrations of the 1st generation process in approx. 1/3rd of the time (24 *vs*. 72 h, Figs. 1, and 3A). It also allowed an increase in the volumetric productivity by a factor of roughly 2.4 with full conversion of 300 g/l EEH in 72 h at a cells:substrate ratio reduced by half (1:1) with 300 g/l cells (Fig. 3B).[33]

The third generation SHC variant reduced further the time for full EEH conversion under first generation process benchmark conditions (Figs. 1, and 3A). With this variant, 450 g/l EEH are cyclized in 72 h at a further reduced cells:substrate ratio of 0.4:1 with 180 g/l cells (Fig. 3B).[37] This represents a 1.5-fold, respectively 3.6-fold volumetric productivity increase compared to the 2nd and 1st generation processes.

The improvement achieved during the process intensification chain can be measured in terms of gram EEH converted per gram of cells. An up to 5-fold increase between 1st and 3rd generation process was achieved (Fig. 3C).

4.2 *Product Recovery*

The homofarnesol feedstock used for Ambrofix™ production consists of a mixture of (*E,E*)- and (*E,Z*)- homofarnesol isomers in a ratio of approximately 80:20. The reaction products resulting from the cyclization of this homofarnesol feedstock with *A. acidocaldarius* SHC identified during our investigations[12,13] are shown in Scheme 8.

The reaction products, other than (–)-ambrox can vary depending on the wild-type or variant SHC used. In this regard, we demonstrated that certain wild-type and variant SHC enzymes show different substrate specificity towards different homofarnesol isomers.[33] Given that an *A. acidocaldarius* SHC variant was identified with improved substrate specificity towards the *E,E*-isomer of homofarnesol, and product selectivity towards (–)-ambrox,[37] it is anticipated that combining genetic diversity and engineering of SHC enzymes may create SHC variants of absolute substrate specificity and product selectivity, producing only (–)-ambrox from EEH in mixtures with one or more homofarnesol isomers.

In our new Ambrofix™ production process (–)-ambrox directly crystallizes out of the reaction independently of the process considered. (–)-Ambrox is the only solid product out of the four reaction products arising from the cyclization of an *EE:EZ* homofarnesol mixture (Scheme 8), the other three are oily liquids. This finding was surprising because at least two of the other three products are very similar in structure to (–)-ambrox. This feature allowed for an efficient Ambrofix™ recovery in high yields from the reaction mixture by means of solid-liquid separation (*e.g.* filtration) in the 1st generation process.[40] This property allows for an environmentally-friendly product recovery, avoiding the use of any kind of solvents for extraction. Olfactive purity can be obtained with thorough washing of the recovered Ambrofix™ crystals (Fig. 4).[41]

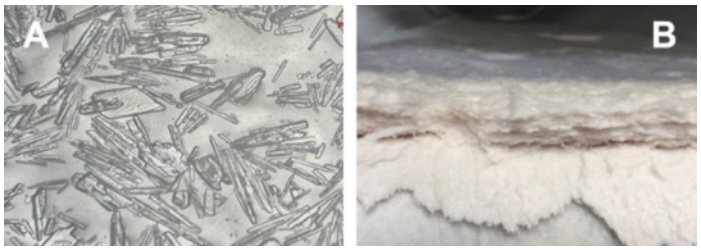


Fig. 4. Ambrofix™ crystals in the reaction broth (**A**) and Ambrofix™ filter cake (**B**) during product recovery.

5. Sustainability of Ambrofix™ Production from (*E*)-*β*-Farnesene

(–)-Ambrox produced as Ambrofix™ from (*E*)-*β*-farnesene *via* the cyclopropanation/biotech route (Scheme 2) or the traditional route from clary sage (Scheme 1) is naturally derived and readily biodegradable. However, when starting from sclareol, parts of the side chain are lost as organic waste. Furthermore, it is estimated that the production of one kilogram of Ambrofix™ from sugar requires 100 times less land compared to the clary sage route.[42]

The concept of sustainable fragrances and the subsequent need for their environmentally and socially responsible production was recently discussed, and the new process for Ambrofix™ production developed by Givaudan (Scheme 2) was judged as being amongst the most prominent examples with regard to sustainability.[43] Significant milestones are the use of (*E*)-*β*-farnesene made by sugar fermentation for the production of EEH, and the fact that in this process (–)-ambrox directly crystallizes out from the reaction, allowing its green and efficient recovery.

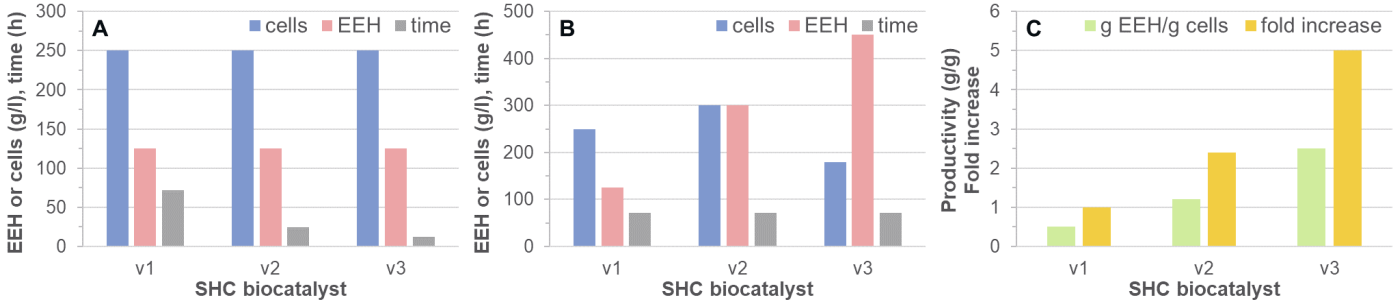
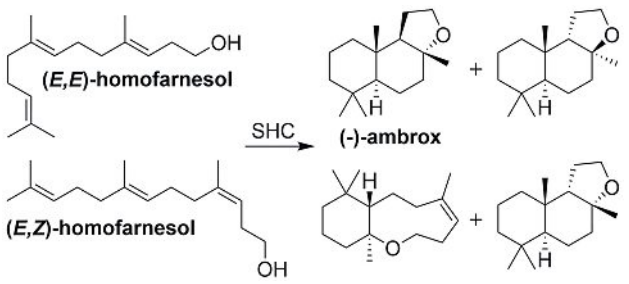


Fig. 3. Milestones achieved with the three generations of SHC biocatalysts for Ambrofix™ production. **A**, reduction in time for full conversion of 125 g/l EEH with 250 g/l cells. **B**, increased volumetric productivity in 72 h with 125/250 (v1), 300/300 (v2), and 450/180 (v3) g/l EEH and cells. **C**, gram EEH converted per gram of cells.



Scheme 8. Cyclization of a mixture of (*E,E*)- and (*E,Z*)-homofarnesol isomers with *A. acidocaldarius* SHC.

The milestones and improvements achieved during SHC enzyme evolution and biotransformation optimization (Fig. 3) as well as engineering the cyclopropanation of (*E*)-*β*-farnesene (Scheme 5) into a multistep flow process at industrial scale are advantageous not only because of increased volumetric productivity, but also due to a strong reduction in raw material consumption, waste production and energy consumption resulting altogether in a more efficient, sustainable and environmentally friendly process.

Material consumption and waste production are reduced due to the strong reduction of the amount of cells required for the production of one kilogram of Ambrofix™ along the process intensification chain. A faster EEH cyclization and/or lower temperatures significantly reduce energy consumption. As an illustration, a 3-fold productivity increase of the bioconversion step reduced the energy consumption by 40 % over the whole Ambrofix™ production process starting from the initial fermentation producing (*E*)-*β*-farnesene. Starting with a 1st generation process with a carbon efficiency already improved over the standard route from clary sage, sustainability was increased further with the 2nd and 3rd generation process.

Whereas the criteria of sustainability have been discussed for the new process to Ambrofix™ according to Givaudan's Five Carbon Path,[1] the long established *12 principles of green chemistry* are another useful set of metrics regarding the assessment of the sustainability of industrial processes.[44]

In this context we especially realized *principles 3, 11 and 12* in the multistep flow cyclopropanation sequence, e.*g. principle 3 - less hazardous chemical synthesis,* through *N*-methyl-*N*-nitroso precursor Liquizald and *in situ* DAM generation with immediate consumption, *principle 11 - real-time analysis for pollution prevention*, through PAT, and *principle 12 - inherently safer chemistry for accident prevention*, through the multistep flow mode.

Applied to the whole sequence from sugar to Ambrofix™ (Scheme 2), the other principles were also obeyed, especially when compared to the classical sclareol process (Scheme 1), *e.g. principle 1 - waste prevention* by avoiding the organometallic waste of the sclareolide reduction step, and *principle 2 - atom economy,* by avoiding loss of four carbon atoms in the clary sage route. The new process also strongly adheres to *principle 5 - safer solvents and auxiliaries*, through (*E*)-*β*-farnesene production *via* fermentation, Δ-farnesene rearrangement and SHC cyclization of EEH being mostly carried out in aqueous media. *Principle 6 - design for energy efficiency*, is adhered to at its maximum because all steps of the sequence are processed at or close to ambient temperature without energy-intensive cooling or heating. *Principle 7 - use of renewable feedstocks,* was realized through the use of renewable (*E*)-*β*-farnesene and methylamine for the cyclopropanation step, methylamine being mass-balanced with the methanol production from CO_2 side streams at the producer. *Principle 9 – catalysis*, was realized through combination of catalysis and biocatalysis. *Green chemistry principles 4 and 10* are related to product qualities such as toxicity and degradation, which are identical to the ones of Ambrofix™ produced from sclareol and are therefore also adhered to.

6. Conclusion

Towards the sustainable production of Ambrofix™ a key determinant factor was the joint efforts between the teams working on the two pillars of the project: biocatalyst and substrate. Success was the result of a holistic approach that tackled all aspects in the early stages of the project: enzyme evolution, engineering of bioconversion reaction and process conditions, supply of feedstock and substrate synthesis, product recovery and purification. This integrative approach enabled the production of a substrate suited for the enzymatic reaction and an efficient biocatalytic process for its conversion to a perfumery grade Ambrofix™.

With our new process for the production of the iconic fragrance ingredient (–)-ambrox as Ambrofix™, we were first in demonstrating that SHC enzymes are amenable to industrial scale biocatalysts applicable at viable process-relevant conditions. Due to their high plasticity and ability to be evolved, the use of SHC enzymes in chemical synthesis will certainly expand, taking advantage of intrinsic or designed properties such as their ability to discriminate between geometric isomers of a substrate, for the production of other cyclic terpenoids and derivatives of interest.

The chemistry for the synthesis of EEH including a sustainable multistep-flow cyclopropanation will set a standard as well, likely finding other industrial applications.

Our new route to Ambrofix™ demonstrates that biocatalysis and chemical synthesis are complementary tools. Their combination enabled us to develop and implement an efficient, sustainable and environmentally friendly production process at a multi-metric ton scale.

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