

Ambergris fragrances

Classical production of ambergris and chemical synthesis of Ambrox®

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Chemistry of Biotrasformations

Abstract

Ambergris, a waxy substance excreted from the sperm whale's intestinal tract, has been used for centuries to create quality perfumes, thanks to its sweet and delicate smell and to its fixative properties. As a consequence of its natural, a continuous and stable supply of this raw material is not possible without killing the animal. Due to the shortage and price inflation, grey amber has also been synthesized by alternative synthetic routes. One of the key olfactory components of ambergris is Ambrox® it is industrially obtained by semisynthesis from sclareol, a diterpene-diol isolated from *Salvia sclarea*, or by other alternative methods, like the biosynthesis with *Hyphozyma roseoniger* or *Cryptococcus albidus*.

The aim of this review is the comparison of the industrial synthesis of Ambrox® and some of innovative extraction processes, providing some examples of biotechnological methods that use microorganisms.

Keywords: Ambergris, Ambrein, Ambrox®, *Salvia sclarea*, sclareol, Diterpenoid, labdanolic acid, cis-abienol.

Introduction

Nowadays, demand and production of aromas and fragrances is considerable, and their use is extensive in pharmaceutical, cosmetics, food and textile industries. A product labeled as "natural" comes from biological processes, such as, extractions from plants and microorganisms and biocatalytic pathways of synthesis. Its cost however, is much higher than a product obtained with classical synthesis, even if the molecule obtained is the same. The impact of biosynthesis on the environment is much less severe, thanks to the use of renewable raw materials, on the reduced formation of unwanted by-products, on the use of less toxic and harmful reagents and the application of all "green chemistry" processes.

Today's legislation in the United States and the European Union is restrictive, flavors labeled as "natural", in fact, must be obtained from natural sources such as plants or animals or synthesized by precursors obtained in the same way and converted using only biological processes as enzyme reactions. Fragrances obtained from classical chemical synthesis, which do not show any difference in the structure of the product, must be classified as "nature-identical" and consequently their cost is lower.

The odorous compounds are mainly perceived with the sense of smell. The smell of a molecule is influenced by many factors, in particular by structural and stereochemical characteristics, functional groups and electronic properties. The odorous molecules interact with the human body and, if these are chiral, the interaction depends on their absolute configuration, for example the difference from the olfactory point of view between the (+)-limonene and the (-)-limonene is that the former has a characteristic orange smell, while the latter is typical of lemons. Small variations in the structure as the transpositions can lead to drastic changes in terms of smell and flavor of the fragrance.

The distinction between these two classes of products, "natural" and "identical to nature", can cause a major problem, since most natural aromas are present in an enantiomerically enriched form. The chemists studied their absolute configuration and tried to find a correlation between the geographical origin or the cultivar and the enantiomeric excess of a certain aroma. From these studies derive analytical methods, which consist in the development of new methods to discriminate against them, evaluating and demonstrating the authenticity of natural materials.

As already mentioned, it is essential to find alternative synthesis pathways to obtain fragrances and flavors that include the use of techniques such as fermentation and extraction from plants as well as the use of enzymes, living cells and microorganisms, to perform reactions generally conducted using solvents and chemicals. The substitution of these hazardous substances with environmentally friendly processes has been the main objective of scientists for decades. [Serra S, et al., 2005] [Vandamme EJ and Soetaert W, 2002] [Krings U. and Berger RG, 1998] [Berger RG, 2009]

A classic example of variability of these products that allows us to differentiate them in nature are the terpenoids (Mevalonate way), which constitute a large family of natural substances structurally different, derived from the C₅ isoprene units, joined head-to-tail.

The isoprene units are the diphosphate ethers: dimethylallyl diphosphate (DMAPP) and isopentenyl diphosphate (IPP) (Figure 1) and linking them produces the typical structures, classified in hemiterpenes (C₅), monoterpenes (C₁₀), sesquiterpenes (C₁₅), diterpenes (C₂₀), triterpenes (C₃₀) and tetraterpenes (C₄₀).

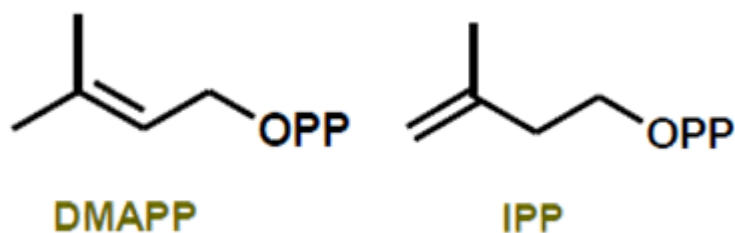


Figure 1: Structure of C₅ isoprene units

In plants the chemical diversity of terpenoids is exploited for multiple biological functions, in some cases these biologically active compounds can be produced by inducing biotic and abiotic stress factors.

Due to the different chemo-physical properties that vary from volatile compounds to semi-volatile and non-volatile compounds, they have a long history of practical uses such as aromas, fragrances and pharmaceutical products. Some compounds were used as fixatives of the perfumes, a historical example is the Ambrox® that is produced by the ambergris which in turn is produced by the sperm whale, and is a particular fragrance that is appreciated thanks to its scent of delicate amber, sweet and woody and its excellent fixative properties to preserve the scents of perfume.



Knowledge of grey amber dates to the time of the ancient Egyptians, who used it as ceremonial incense. From the X century AD, ambergris was collected on the banks or from the carcasses of sperm whales and traded throughout the Mediterranean and in Europe for their use in perfumery. It is the feminine aphrodisiac fragrance par excellence, its fragrance, unique in its kind, is intoxicating and possesses sweet tones of leather and sea. [Zerbe P. and Bohlmann J., 2015]

The Sperm Whale

The Sperm Whale (*Physeter macrocephalus*) it is the largest of all the Odontocetes and the largest living animal with teeth: it measures up to 18 meters in length and exceeds 45 tons in weight, one of the unmistakable characteristics of these animals is certainly the shape of the head, has a rounded shape and is of considerable size, occupies 1/3 of the length of the animal.

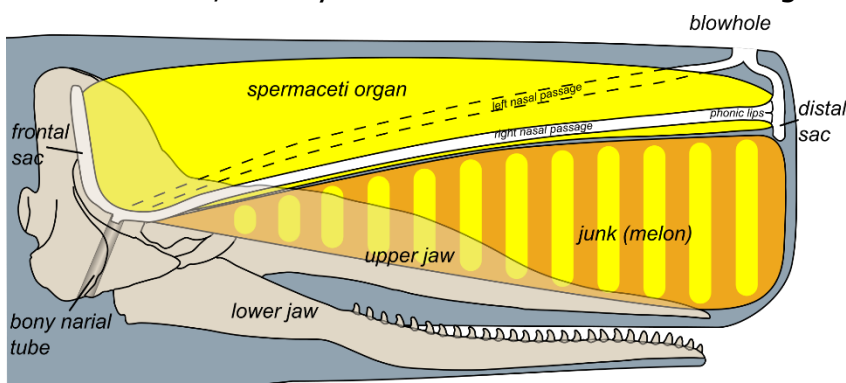
This particular form is due to the presence inside of the spermaceti organ, which is in front of and above the skull. This organ is filled with a substance, spermaceti, with a whitish waxy consistency, used by the animal to handle buoyancy during diving. Before diving, the cold water is transported through this organ and the wax solidifies. Raising the specific density generates a downward thrust (approximately 40 kg equivalent) and allows the sperm whale to sink effortlessly.



Figure 2: Sperm whales

The sperm whales are predators and their favorite dish is the giant squid. The preys live at great depths and the sperm whales dive up to 3000m to find them, holding their breath for more than an hour. Although the dimensions of these cetaceans exceed those of their preys, the hunt for squid is a difficult task.

As a matter of fact these creatures, do not surrender without fighting and, once ended up in the jaws of the Sperm Whale, try to get out of them with all their strength and windy, leaving obvious wounds around the mouth of the predator. After the hunt they take to the surface to rest, usually for about ten minutes. Since light is scarce at such depths, sperm



whales cannot count on their sight to detect their prey, but rely on echolocation, which allows them to receive information about the surrounding environment and the presence of potential preys. The sperm whales have been subject to fishing for almost two centuries, from

1800 to 1987, activity that drove these fantastic animals almost to the brink of extinction. The commercial value of sperm whales resided mainly in spermaceti, traditionally used as lamp oil, as lubricant and to produce candles. Fortunately, fishing for these animals is now prohibited and is no longer a threat to the survival of this species.

History of Ambergris

For almost a thousand years grey amber has been one of the most sought after and mysterious substances in Europe: the origin of this compound has only been discovered in relatively recent times and its properties have made it an extremely valuable ingredient in the cosmetic and medicinal field and sometimes also as a spice for food and drink.

The ancient Egyptians burned ambergris along with incense inside the temples or during religious ceremonies, while the Chinese used this substance as a medicine and as an aphrodisiac, whereas Arabs and Europeans use grey amber also as a valuable ingredient to enrich the aroma of some dishes.

One of the few positive aspects of the hunting of sperm whales begun in the 19th century was the discovery of the origin of ambergris. The nature and existence of this substance were almost completely unknown to the Greeks and Romans and ambergris became a much sought after substance in Europe only during the Middle Ages in the form of very rare and incredibly expensive spice imported from the Arab world.



Figure 3: Ambergris

According to the herbarium Hortus Sanitatis, the first encyclopedia of natural history, published and printed in 1491 in Germany by Jacob Meydenbach, the ambergris was not very different from the one generated by the fossilization of coniferous resin and was born from the latex of legendary marine trees.

At the beginning of the sixteenth century the Portuguese explorers began to introduce grey amber in Europe presenting it as an unknown and incredibly rare substance that could be retrieved during the expeditions on the Atlantic and on the Indian ocean. Some scholars, however, hypothesized that its origin was linked to sperm whales, others believed that it was a sort of fungus born on the seabed, or some hypotheses were often based on superstition, to explain the origin of ambergris. It was only at the end of the 17th century and with the beginning of the intensive whale hunting for the extraction of oil and fuel that sufficient evidence was found to prove the origin of this substance: the sperm whale.

The ambergris is the fruit of the digestive process of the sperm whale, feeding mainly of giant squids, the soft parts of the preys enter the intestinal tract whilst the hard and indigestible ones, like the beak of the squid, are usually regurgitated and expelled from the mouth.

Sometimes some bone fragments manage to reach the intestine, triggering an irritation of the walls of the digestive tract that gives rise to the process of ambergris' formation. During digestion and over several years, the intestine begins to form a cluster similar to a solid wax, formed by the accumulation of ambrein and other oily and odorous substances around

the hard-indigestible fragments. These ambergris blocks are generally ejected through the feces, but sometimes they can become so large that they have to be regurgitated.

The ambergris forms block of extremely variable dimensions, from small fragments of a few tens of grams up to compact clusters that can exceed the weight of 50 kg. As soon as expelled from the digestive apparatus of the sperm whales, amber has a strong smell of excrement, and having a lower density of water, it floats.

It is dark brown tending to black, this waxy floating mass exposed to the air, to the sun, to the action of the salinity of the sea, ripe in time and becomes of pale grey or straw yellow color, over time this excretion hardens and changes its organoleptic properties, giving it a musky, penetrating and pleasant smell, the longer the fragments float in the water and the finer the smell of ambergris, this is due to the degradation of the odorless Ambrein triterpene. In later crumbling gradually in smaller pieces is found mainly on the beaches of East and West Africa, in New Zealand, Australia and along the coasts of the Azores. [Cheng LP, et al., 2009]



Figure 4: Ambergris

To date, ambergris was considered as precious as gold. The scarcity of the resource and the animal origin has encouraged the chemists to look for alternative approaches, so as to obtain the same smell.

In 1820 two French chemists Joseph-Bienaimé Caventou (1795-1877) and Pierre-Joseph Pelletier (1788-1842) isolate and characterize Ambrein, the main component of grey amber, but its structure was described only in 1946 by Lederer. [Panten J, et al., 2014]

Components of Ambergris flavour

The main component of ambergris is a terpene known as Ambrein and, together with 24-methyl-5 β -cholestan-3 α -ol (Figure 5) represent about 70-90% of the unripe mass. The important olfactory components are generated during the maturation period at sea. Ambrein is probably produced by the intestinal microorganisms in sperm whales, by the partial cyclisation of squalene, and is known to be the active component that produces the alleged aphrodisiac effects, although it is odorless, it acts as a biological precursor for the synthesis of other compounds such as Ambroxan, which possesses fixative properties. The key reaction is photooxidation by oxygen (Figure 6).

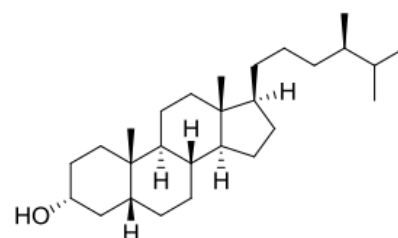


Figure 5: 24-methyl-5 β -cholestan-3 α -o

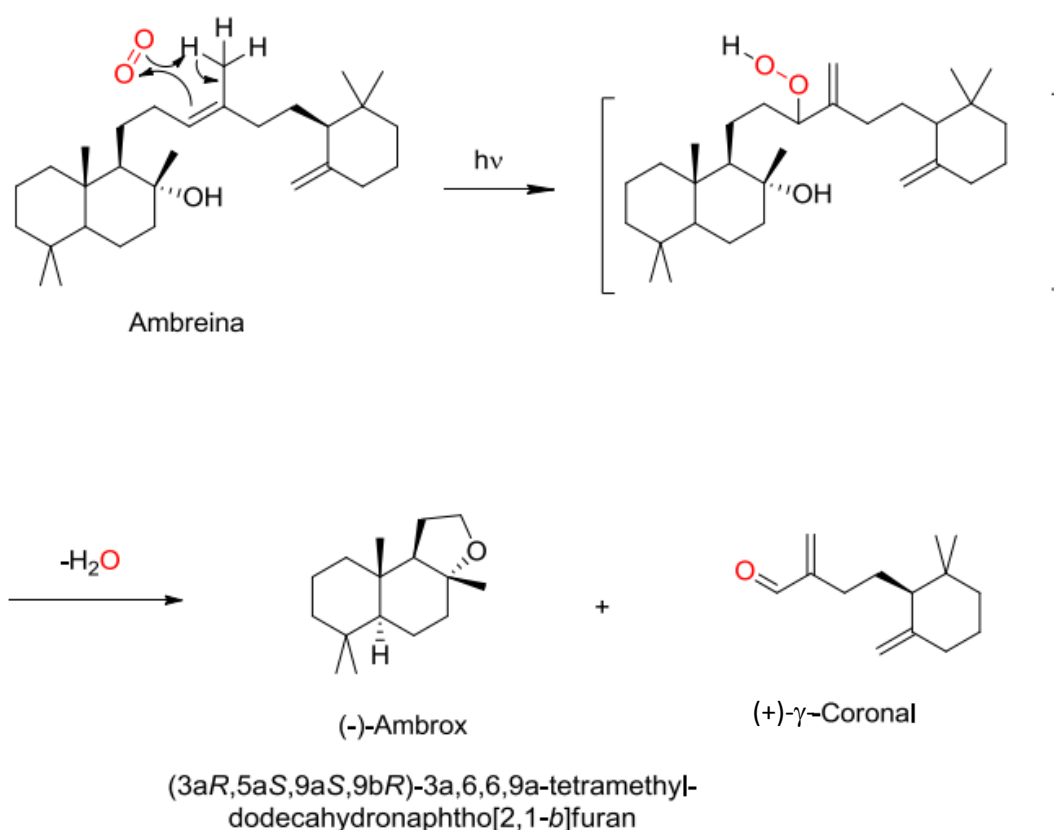


Figure 6: Biosynthesis of (-)-Ambrox

The characteristic scent of amber is also given by the presence of (+)- γ -Coronal. Due to the low availability of ambergris, scientists have discovered an alternative method to obtain Ambrein. [Rowland SJ & Sutton PA, 2017]

Biosynthesis of Squalene

Squalene, a C₃₀ triterpene, is a hydrocarbon originally isolated from shark liver, which was later found in rat liver and yeast. This approach was not widely used in the industry as squalene significantly increased process costs. On the contrary, however, yeasts naturally produce the precursor of triterpenoid squalene, through the biosynthesis of mevalonate, and moreover they can be easily manipulated genetically and, for these reasons they represent ideal hosts for terpenoid production. [Moser S, et al., 2018]

Farnesil diphosphate (FPP) is a sesquiterpene formed by three units C₅, joining these units tail-tail you get the squalene.

Here below is represented the mechanism (Figure 7), in which two units of farnesil PP react in the presence of an enzyme, that is the squalene synthetase, with loss of the diphosphate group and formation of the allyl cation and electrophilic addition leading to the formation of a tertiary cation; after which there is loss of a proton with formation of a cyclopropane ring and formation of an intermediate called also presqualene diphosphate; subsequently there is loss of the diphosphate leading to the formation of a primary cation, then shift Wagner-Meerwein 1,3 alkyl (transposition W-M where 1,2 or 1,3 of a hydride or methyl or alkyl is displaced to form more stable carbocations or to decrease ring voltages). The alkyl Shift 1,3 generates a new cyclopropane ring, which is a more stable tertiary cation. Subsequently there is a break of the ring that produces an alkene and an allyl cation, then with neutralization of the cation with a hydride attack leads to the formation of squalene. [Dewick PM, 2012]

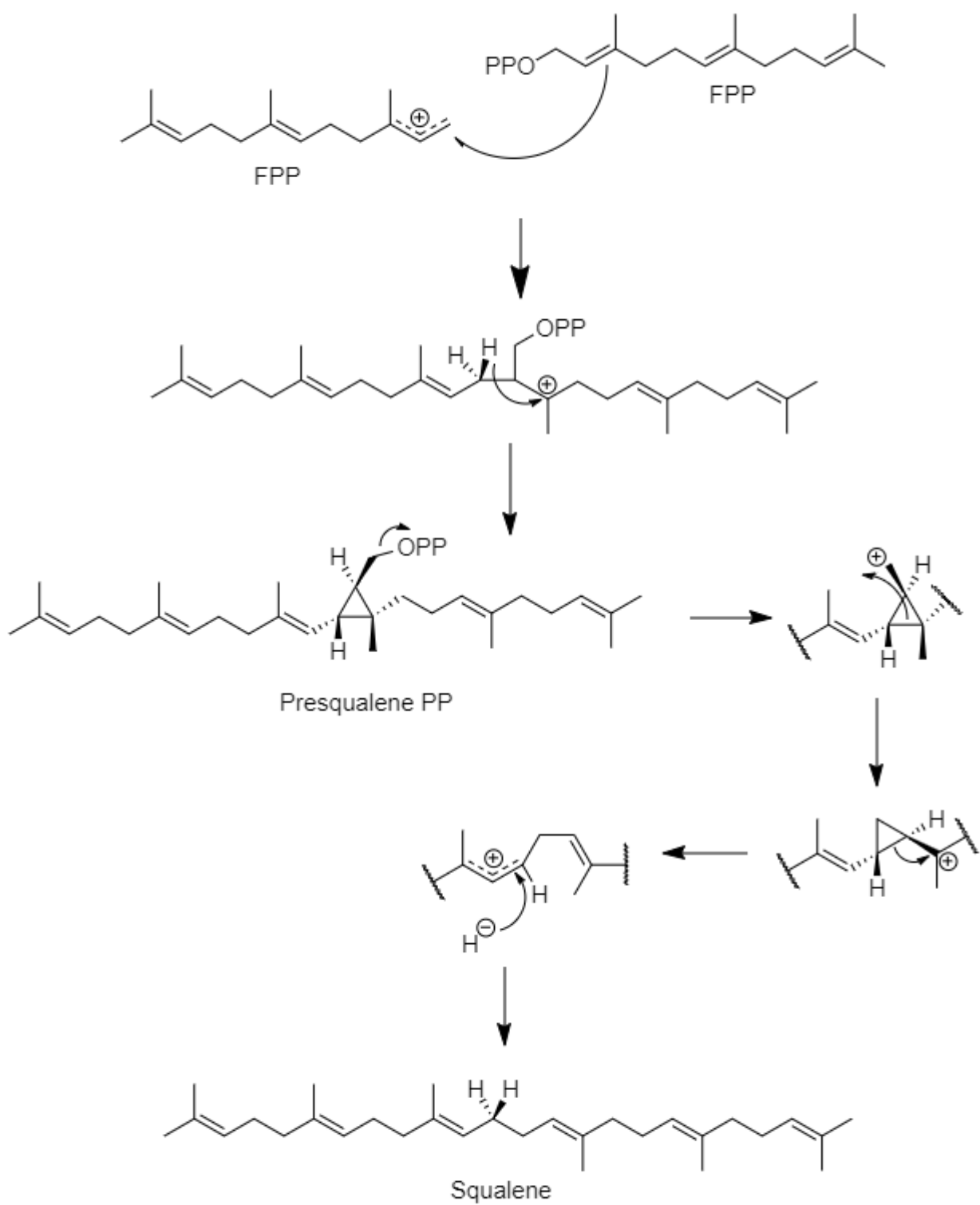


Figure 7: Reaction scheme for Squalene synthesis from farnesyl PP

Biosynthesis of Ambrein

Ambrein triterpene is an important component of amber, one of the most valuable animal resources for the production of perfumes. For its synthesis are used two enzymes: a variant of the squalene-hopene cyclase of *Alicyclobacillus acidocaldarius* (AaSHC D377C) and the tetraprenyl- β -curcumene cyclase by *Bacillus megaterium* (BmeTC).

The squalene-hopene cyclase is a key enzyme for the biosynthesis of triterpenoids, which converts acyclic precursors, such as squalene, into a polycyclic product, cyclisation is expected to occur through the formation of a series of carbocations, in which the substrate binds to the enzyme adopting a specific conformation, similar to the product and thus triggers cation formation by protonation of a double bond or an epoxide.

For the synthesis of the (+)-Ambrein, is used a variant, that is the D377C, that, as you can notice from the reaction represented below, does not go to cycle the squalene in order to form a hopanoid (natural pentacyclic compounds, containing five rings) (Figure 8), but goes to cycle only one ring by moving ties π , so as to form the 3-deoxyachilleol A (Figure 9). [Dang T. and Prestwich GD, 2000]

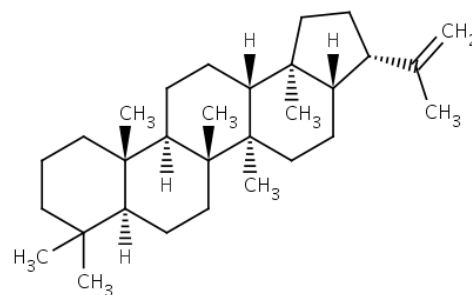


Figure 8: Structure of a hopanoid (Hopene)

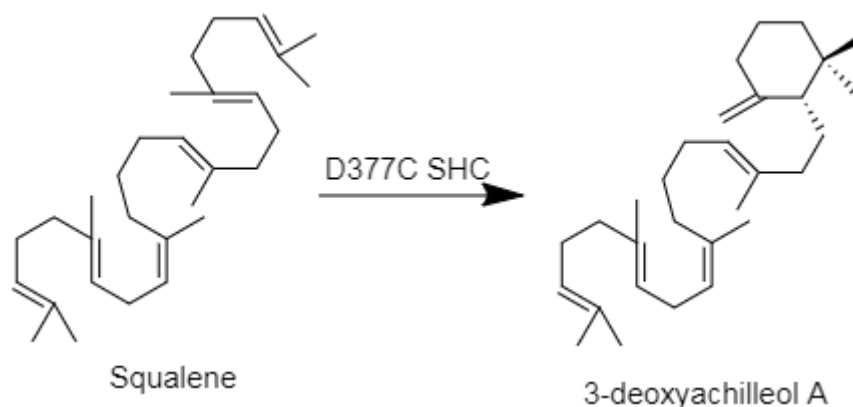


Figure 9: Synthesis mechanism of 3-deoxyachilleol A

In the second step the 3-deoxyachilleol is converted to (+)-Ambrein, using a second enzyme, namely the Tetraprenyl- β -curcumene cyclase by *Bacillus megaterium* (BmeTC), represented below (Figure 10).

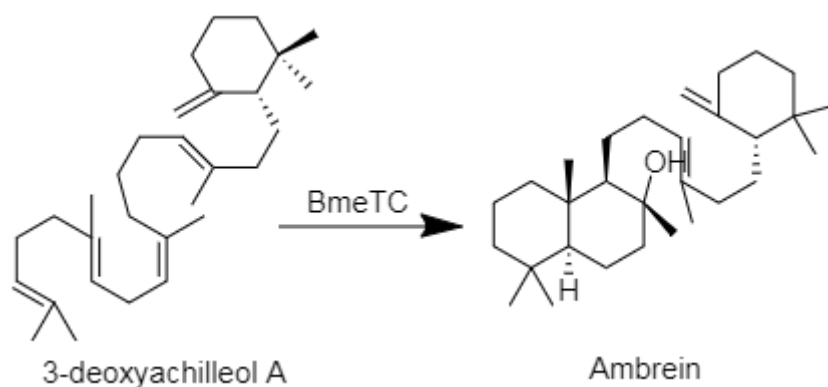


Figure 10: Synthesis of (+)-Ambrein

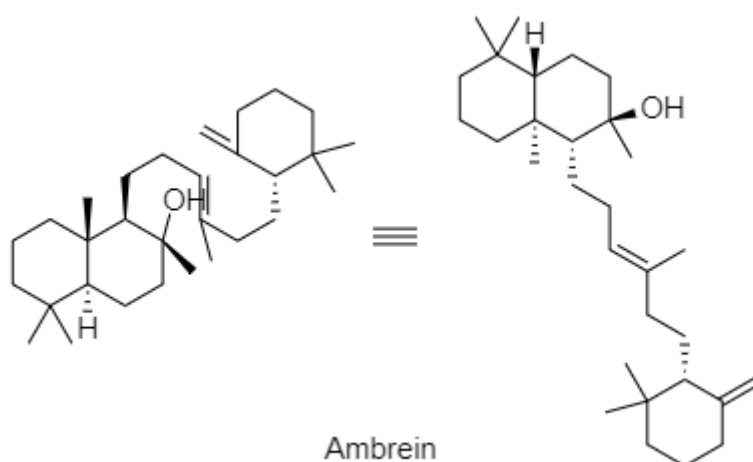


Figure 11: Structure of (+)-Ambrein

Chemical synthesis of Ambrox®

The main component of ambergris is the (+)-Ambrein, which is exposed to the sun, air and sea water degrades into (-)-Ambrox. Due to the lack of raw material and rising prices, a few of grey amber is developed by the fragrance industry. One of the key components responsible for the scent of ambergris is Ambrox®, used as a fixative for other fragrances. Ambroxide, also known under the brand name Ambroxan® or Ambrox®, is a terpenoid present in nature and is also produced industrially. The first chemical synthesis of Ambrox® was conceived by A. Hinder and A. Stoll from the company Firmenich in 1950. From the structural point of view, it is a terpenic furan, which is synthesized from the alcohol diterpenic natural sclareol, extracted from *Salvia sclarea* (Figure 12). [Vandamme EJ, Soetaert W, 2002]



Figure 12: Plant of Salvia sclarea

It is a biennial herb native to southern Europe and belongs to the family of Lamiaceae. This perennial evergreen plant, in two years reaches heights from 50 to 110 cm and grows on most of the countries bordering the Mediterranean.

In plants, diterpenes are derived from geranylgeranyl diphosphate (GGPP) (C_{20}), which is formed by addition of an additional molecule of isopentenyl diphosphate (IPP) to farnesyl diphosphate (FPP). The reaction takes place with the formation of an allyl cation and subsequent electrophilic addition, which leads to the formation of a tertiary cation, then there is stereospecific loss of a proton and thus the geranylgeranyl PP (GGPP) is obtained (Figure 13). [Dewick Paul M, 2012]

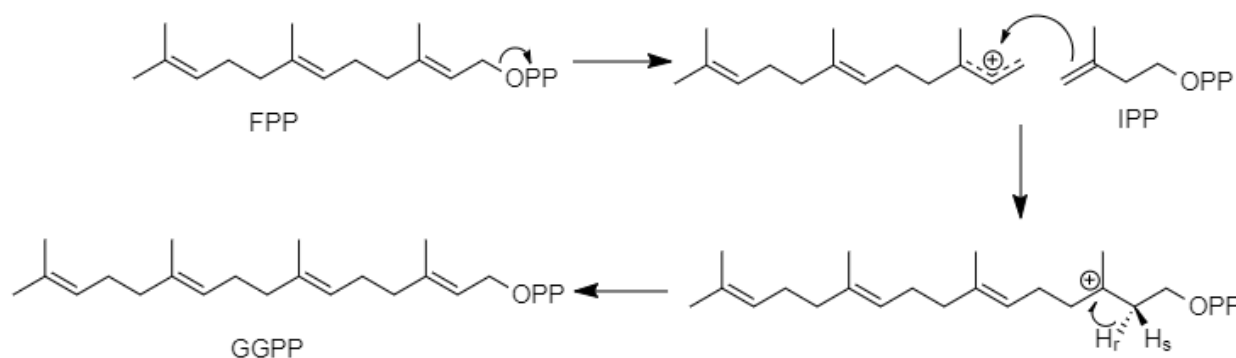


Figure 13: Biosynthesis of Geranylgeranyl PP (GGPP)

The cyclization reaction of the GGPP is catalyzed by synthesised diterpene (diTPS), and after a series of rearrangements leads to the formation of (-)-sclareol (Figure 14). [Schalk M, et al., 2012]

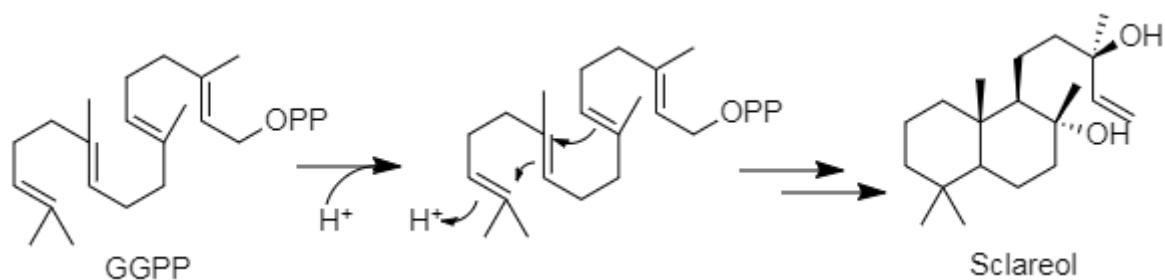


Figure 14: Synthesis of (-)-sclareol from GGPP

From the industrial point of view, the reaction mechanism most used for the synthesis of Ambrox® is the one reported below (Figure 15). This system is used despite the numerous disadvantages, such as expensive or toxic reagents, processed operations, long reaction times and massive discharges of pollutants. The Ambroxide is synthesized through three phases, in which there is an oxidation of the lateral chain of (-)-sclareol, that is the alcohol allyl, to give sclareolide, subsequent reduction of sclareolide to (-)-Ambradiol, and cyclodehydration to give Ambrox.

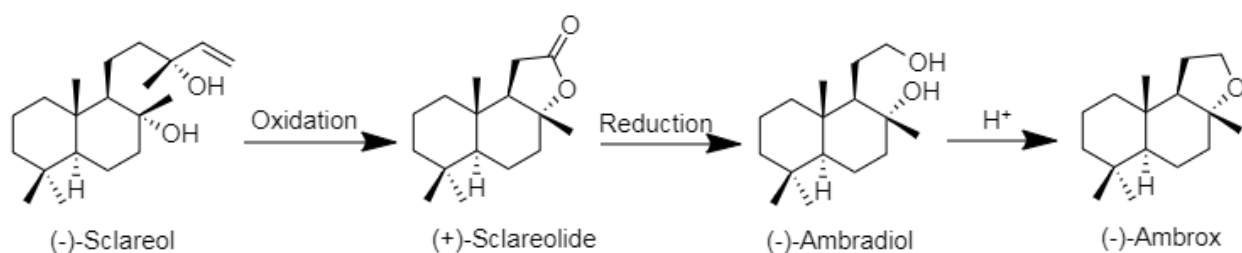


Figure 15: Synthesis of (-)-Ambrox from (-)-Sclareol

Other summaries have been carried out at an industrial level, but these processes are not very practical because they have a low effectiveness, and they have possible risks for safety and for the discharge of pollutants. [Yang S, et al., 2016]

Alternative synthesis

A particularly remarkable work belongs to Fritsche Dodge and Olcott at the International Flavors & fragrances, which have shown that the lateral chain of sclareol can also be degraded by fermentation with *Hyphozyma roseoniger* CBC 20624 or even better with *Cryptococcus albidus* ATCC 20918.

The microorganism *Hyphozyma roseoniger* CBC 214.83, exists both in filamentous form and in yeast form, has been isolated and is able to form a diol starting from sclareol, the conversion proceeds in a single step through a cascade of reactions with a yield >75% after 12 days of incubation; the yeast *Cryptococcus albidus* ATCC 20918 sclareol metabolizes to

ketonic lactone sclareolide with high yields, subsequently this last compound is converted to a diol to produce Ambrox® (Figure 16). [Sell Charles S, 2003]

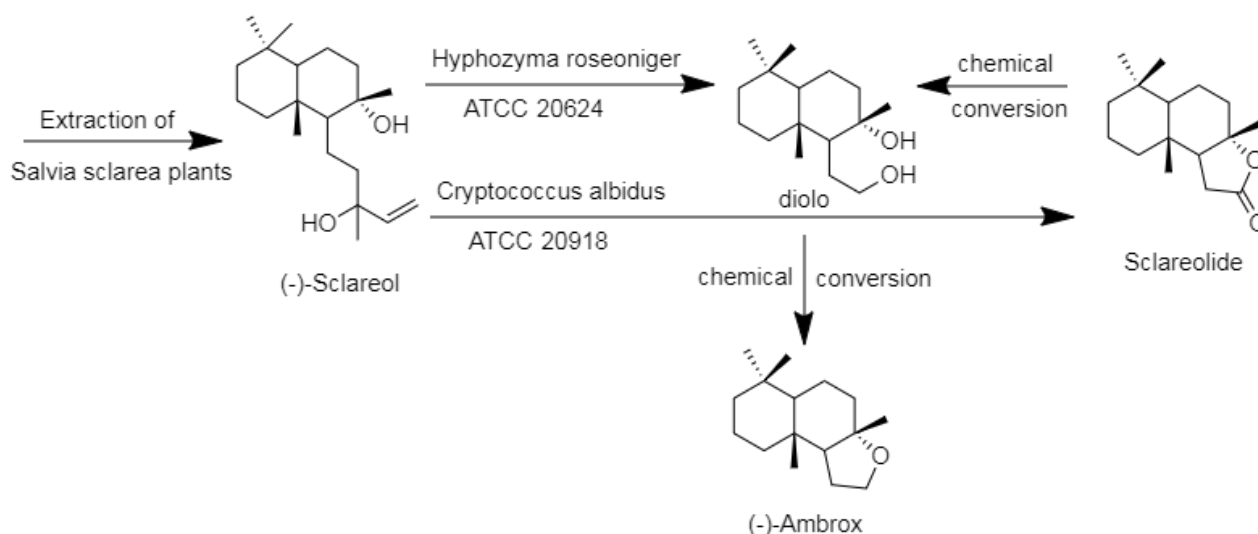


Figure 16: Biotransformation of Sclareol from *Hypozyma roseoniger* and *Cryptococcus albidus*

In addition to the negative aspects of the use of chromium (VI), potassium permanganate, sodium periodate or ozone, the disadvantages of these summaries are the unreliable situation of supply of Sclareol and therefore the prices often highly fluctuating. As an alternative to Sclareol, you can also use the (+)-cis-abienol, which is obtained from the fir balsam (*Abies balsamea*) of Canada. The resin is obtained by cutting the bark of the spruce growing in the north of the United States and Canada. By steam distillation, extraction and crystallization the (+)-cis-abienol is obtained with excellent purity. Supply is predictable and prices more stable. The Ambrox® is produced through two stages: one of ozonolysis and reductive workup that leads to the diol, which can then be cyclized to Ambrox® with tosyl chloride in pyridine with excellent yields (Figure 17). [Sell Charles S, 2003]

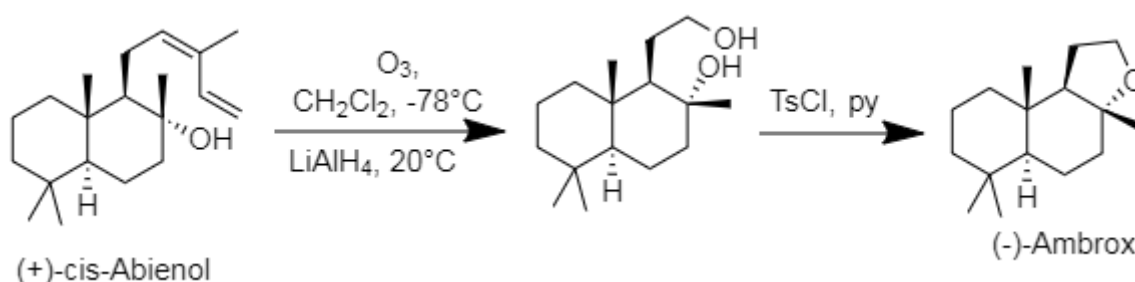


Figure 17: Synthesis of Ambrox from (+)-cis-Abienol

Labdanolic acid is a potential precursor of (-)-Ambrox and is found in large quantities in nature, because it comes from the acid fraction of the extract of *Cistus ladaniferus*, a plant belonging to the family of the *Cistaceae*. The extraction is made by soaking the twigs and leaves with n-hexane and evaporation of the solvent forms a sticky rubber. It is not easy to

oxidize the lateral chain of labdanolic acid because it has only one carboxylic group on the chain, but a possible synthetic strategy to obtain Ambrox® has been discovered, is iododecarboxylation. First the alcohol group on the ring must be protected with acetylation with AcCl, then IBDA (iodobenzenediacetate) and I₂ are the reagents used to replace the carboxylic group with iodide under radiation with a 100 W tungsten lamp. The iodide obtained is reactive and tBuOK (potassium tert-butoxide) in THF is added quickly to eliminate HI and deacetylate the hydroxyl group on the ring; ozonolysis is then performed in order to oxidise the double bond obtained on the side chain to ozonide and the reductive conditions of PPh₃ used in this reaction are useful for cyclizing methyl ketone in sclareol oxide. This compound is an enolic ether, which in the presence of O₃ reacts quickly to give the aldehyde group on the side chain and the acetyl group on the ring. Finally, aldehyde is reduced to alcohol with LiAlH₄ and then p-toluensulfonic acid leads to the formation of cyclic ether, the (-)-Ambrox (Figure 18).

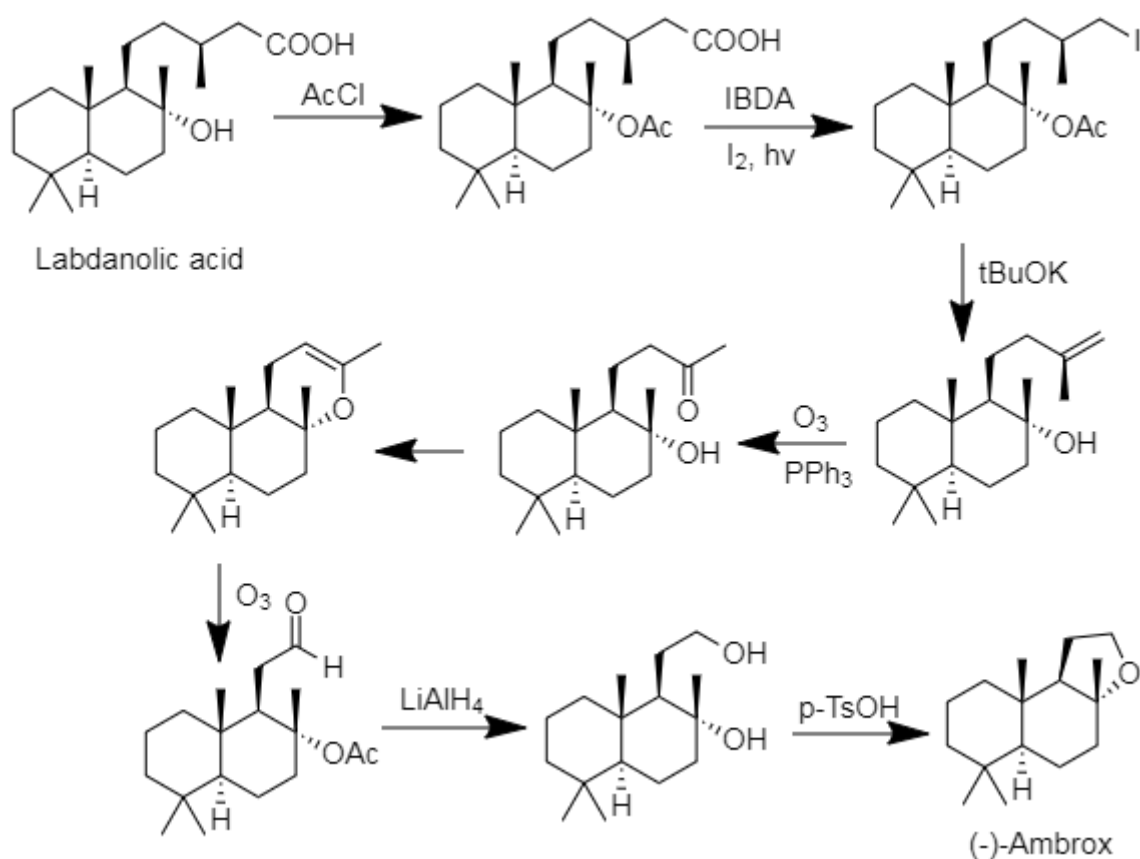


Figure 18: Synthesis of Ambrox from Labdanolic acid

After all, the above synthesis makes use of many chemicals, and it is therefore necessary to find other methods to synthesized the compounds of this category of fragrances using enzymes and microorganisms. [Bolster M, et al., 2001]

Natural Production: Biocatalysis

In recent years the biocatalytic production of quality chemicals has expanded rapidly and, flavours and fragrances are nowadays a stimulating goal for academic and industrial research. The following diagram (Figure 19) shows some of the different types of biocatalysis approaches using different organisms to produce the natural aroma of Ambergris.

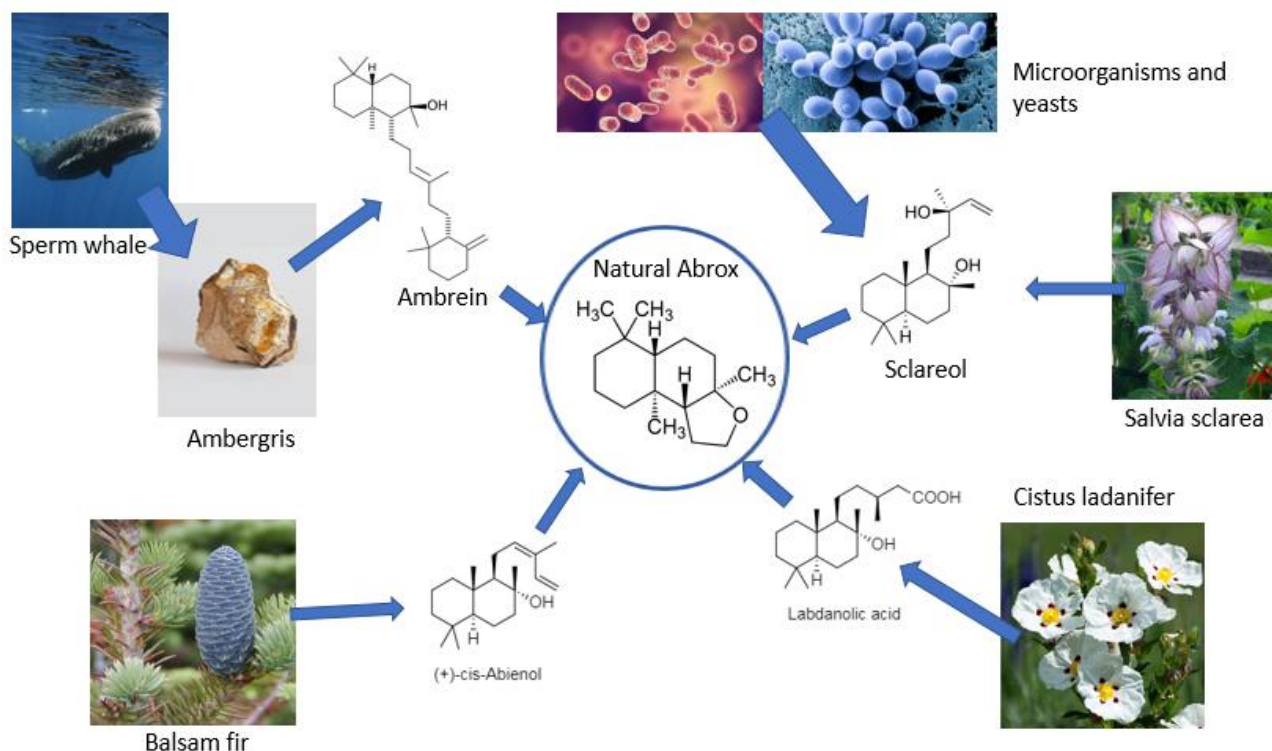


Figure 19: Summary scheme of the possible synthesis to obtain Ambrox®

Conclusions

Nowadays, the production of Ambroxide is not sufficient to satisfy the demand of customers. Natural products have a higher commercial value and are in high demand. These products can be obtained by extraction or biocatalysis with natural precursors as substrate. Biotechnology production of amber components through bio transformation is an interesting alternative to the exploitation of raw materials.

These alternative syntheses are taking hold in the whole production field of aromas and fragrances, due to all the attention paid to be environmentally friendly and animals and the health of who consumes them in food, pharmaceuticals and cosmetics.

The techniques identified go beyond extractions, reactions with enzymes and use of living cells and their objective is to ensure the application of the principles of "green chemistry".

The production of pollutant and hazardous compounds is gradually decreasing, as industries go beyond the methods of classical synthesis and are applying biotechnology.

Unfortunately, only a small amount of synthetic routes have been developed on an industrial scale and products labeled "natural" have a higher cost level than those referred as "identical to nature".

For this reason, alternative organic ways to get the fragrances that are rarely found in nature, from easily available precursors, such as amber, is the key to lower their price and increase their production.

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