

Chemistry of biotransformations

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## **Biotechnological production of flavours and fragrances**

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## 1. Introduction

In this review, we will focus on the application of biochemistry to the production of flavours and fragrances, with particular attention on menthol. Compounds like this find many uses in our every day's life like food, cosmetics and pharmaceuticals. A product labelled as "natural" derives from biological processes as extractions from plants and microorganisms and biocatalytic paths of synthesis, its cost is much higher than a product obtained with classical synthesis even if the molecule is the same. The impact that biosynthesis has on the environment is much less heavier due to the use of non-toxic solvents and reactants and the application of all the "Green Chemistry" principles and atom economy to the process. Legislation in USA and EU these days is restrictive on the flavours labelled as "natural", in fact, it is necessary that the molecule is obtained by extraction from natural sources as plants or animals, or synthesized from precursors obtained in the same way and converted using only biological processes like enzymatic reactions. Fragrances obtained by classic chemical synthesis do not report any difference in the structure of the products, but they must be classified as "nature-identical", and therefore their prices are lower. This distinction between these two classes of products can cause a big problem, which is faking the ones produced with chemical synthesis as "naturals", and for this reason an achievement of analytical chemistry is to develop new methods to discriminate them, for example studies on stable isotopes characterization applied to prove authenticity. Biotechnological methods for synthesis can also be divided in biocatalysis with enzymes and whole cells, and fermentation with more complex living beings such as fungi, moulds and bacteria. It is known that many compounds obtained by extraction from plants give a very low yield of the process, and this was a thrust to biochemical processes' development given when scientists noticed that microbial pathways were giving higher yields, for example the application of *Aspergillus Niger* to the synthesis of 2-phenylethanol, obtained by deamination, decarboxylation and reduction. The raspberry ketone from raspberries is an example too, which the condensation of p-hydroxy-benzaldehyde with acetone gives only 3.7 mg of product from 1 kg of berries, but the hydrolysis of betuloside, a compound found in rhododendron, with a  $\beta$ -glucosidase extracted from the same plant can improve the yield at very high levels. Chirality is also fundamental in this field because many flavours occur as single enantiomers or regioisomers, in fact interactions between sensorial receptors and these molecules are specific and give different signals if the stereochemistry of the compound is not correct. An example is (+)-limonene and (-)-limonene, which give respectively smell to oranges and lemons. Fragrances are always volatile with a low molecular weight and usually made up of a big hydrophobic region and a small polar group like hydroxyls and carbonyls. Little variations in structure like transpositions can lead to drastic changes in terms of smell and taste of the fragrance.<sup>[1], [2], [3], [4]</sup>

## 2. Biotechnological methods

As already said, it is fundamental to find alternative pathways of synthesis to obtain fragrances and flavours that includes use of techniques like fermentation and extraction from plants and use of enzymes, living cells and microorganisms to perform reactions usually conducted using solvents and chemicals. Substitution of these hazardous substances with environmental friendly processes is the focus of scientists since decades.

Talking about biocatalysis, enzymes convert substances with a high stereo specificity (products have a particular stereochemistry that depends on the geometry of the reagents) and, for this reason, the reactions that they catalyse give only small percentages of secondary products preventing the problem of separation and waste emission. They also work at low temperature and pressure allowing saving a lot of energy, but their use at industrial scale is hard to manage. Separation from products require slow filtration, the peptide used can unfold and lose its activity if conditions like pH and temperature deviate from its optimal range. It is necessary then an immobilization on a solid support to prevent these and other problems.

Another tool previously mentioned that found application in bioconversions are whole cells. Their use is appreciated for the capacity of performing multiple steps of a synthesis and avoiding the necessity of enzymes' isolation. Yet they cannot work with high substrate concentrations and organic solvents, able to dissolve precursors of fragrances, because they do not have a high affinity with cells. For these and other problems, like formation of secondary products due to the presence of many enzymes inside them, operating conditions have to be kept under control.

The majority of volatile flavours contained in food is produced using enzymes of the lipase/esterase family able to perform enzymatic kinetic resolutions (EKR), fundamental to obtain the correct stereochemistry of the product. An example is the acylation of ethanol with phenylacetic acid, where only one of the two enantiomers of the acid must react to give the ester, and the synthesis is conducted using enzymes derived from *Aspergillus* and *Rhizopus*. The product is ethyl phenylacetate, an ester used in the food industry as natural aroma. Many flavours are simple molecules like esters, ketones, aldehydes and alcohols, containing stereogenic centres and this particularity, together with the necessity of using only bioprocesses in flavour's industry, makes enzymes play a very important role.

Another class of reactions used in aroma biosynthesis is oxyfunctionalisation. Enormous amounts of hydrocarbons, like terpenes, are thrown away because of their low aroma value, but oxidizing these molecules by addition of hydroxylic and carbonylic groups can give products with excellent aroma properties. The focus on the production of derivates of hydrocarbons was to use enzymes that catalyse oxidations but also whole cells in order to find a more efficient way to obtain these flavours. Reactions that occur in cells have, as target, the detoxification when hydrophobic substrates are added but their mechanisms are still not clear at all. However, it has been

frequently suggested that the catalysts where oxidations take place are cytochrome P450, since these proteins have the task of making toxic molecules safer by adding polar groups that grant solubility in water and possibility of being eliminated with metabolism. Substrates like these are preferred from cytochromes due to their hydrophobicity. Nowadays, only a small part of all P450 were characterized, but a big amount of genes codifying for them are known, and therefore eukaryotic species are rich in cytochromes inside their cells, especially plants. An example of isolated and purified protein is P450cam, obtained from *Pseudomonas putida*, capable of converting (+)-valencene, a sesquiterpene, in a nootkatone, a commercial natural aroma. These and other enzymes catalyse redox reaction by using co-factor such as NADH or FADH<sub>2</sub>, which are consumed during the reaction course. Their cost is high and it can be useful to regenerate them by adding an enzyme that restores the original form of the co-factor. This coupling is used to catalyse a reaction without consuming a large amount of oxidizing agents, and an example is coupling of P450cam with an alcohol dehydrogenase in the conversion of cinnamaldehyde in cinnamic acid and cinnamyl alcohol, where NADH is continually converted between its reduced and oxidized form giving the two products, both utilized in the flavours' industry. Involving cells in synthesis helps to deceive the co-factor regeneration problem, because we have all enzymes necessary to close this cycle of redox reactions.<sup>[1], [2], [3], [4]</sup>

### 3. Flavours and fragrances

#### 3.1. Vanillin

Vanillin is the major component of natural vanilla and the most used flavouring compound. It is found in trace in other plants like tobacco, but the only commercial source of natural vanillin is the Vanilla orchid, where it constitutes about 2% of total weight of this plant. Only 1% of vanillin is produced by extraction from orchids and its cost is much higher than the one produced by chemical synthesis (1200/4000 \$/kg against 15 \$/kg). The main constituents of vanilla flavour are 4-hydroxy-3-methoxybenzaldehyde or vanillin, vanillic acid, vanillic alcohol, p-hydroxybenzaldehyde, p-hydroxybenzoic acid and p-hydroxybenzoyl alcohol. About 50% of produced vanillin is exploited in pharmaceutical industries as an intermediate for production of drugs, such as L-dopa and trimethoprim, for its antimicrobial properties against Gram-(+) and Gram-(-) bacteria, moulds and yeasts.

Vanillin can be extracted from *Vanilla planifolia*, but this process requires months for beans to growth after pollination and the flavour is conjugated to  $\beta$ -glucoside. Only after  $\beta$ -glucosidases, produced during fermentation, has hydrolysed these molecules, vanilla compounds can be released.

Talking about biological methods for production of vanillin, a famous pathway is converting eugenol in ferulic acid first, and in vanillin in a second moment. Eugenol is found in clove oil, and some microorganisms can catalyse with their enzymes the reactions steps needed to obtain the aldehydic group, *Pseudomonas strains* for example. Eugenol is converted in coniferyl alcohol by a hydroxylase, in coniferyl aldehyde and ferulic acid by two dehydrogenase. Ferulic acid is the precursor to form vanillin, as it is esterificated with CoA with a CoA-ligase obtaining feruloyl-CoA. The 4-hydroxycinnamoyl-CoA hydratase/lyase (HCHL) catalyse an hydration reaction on the double bond giving 4-hydroxy-3-methoxyphenyl- $\beta$ -hydroxypropionyl-CoA which is presumed to be an enzyme-bound intermediate finally converted in vanillin by the same enzyme.<sup>[5]</sup>

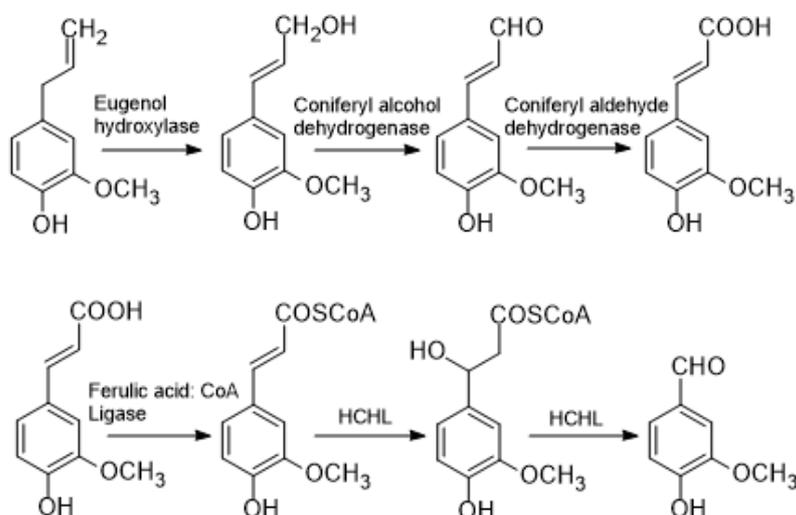


Fig.1: Synthesis of vanillin from eugenol.

Esters of coniferyl alcohol can be converted in vanillin with the action of soybean lipoxygenase. Another biotransformation to synthesize this flavour involves phenolic stilbenes as raw material, which are found in spruce bark. A dioxygenase is the enzyme that catalyses the oxidative cleavage of the stilbenes' double bond between the aromatic cycles giving vanillin and ferulic acid. This pathway has been found serendipitously during studies on *Pseudomonas putida* metabolism, where the dehydroconiferyl alcohol is the substrate for the production of the stilbene whose oxidation gives vanillin. [6]

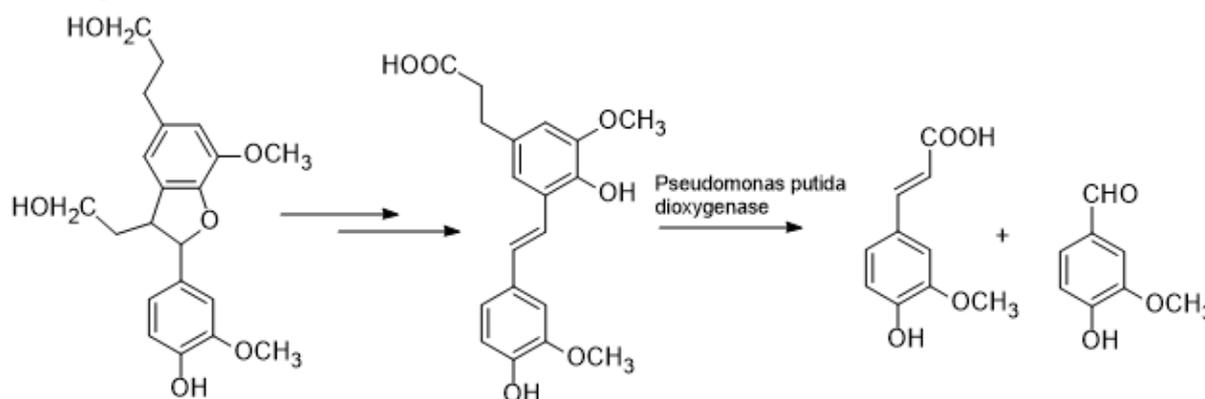


Fig.2: Synthesis of vanillin from coniferyl alcohol.

Isorharpontin is a stilbene contained in spruce bark, present in this plant in its monoglucosylated form. It can be used as a precursor for biotechnological production of vanillin because it undergoes to cleavage reaction, always catalysed by a dioxygenase in various *Pseudomonas* strains.

Van den Heuvel discovered an enzymatic synthesis starting from vanillylamine (or vanillyl alcohol), using vanillyl alcohol oxidase (also named VAO) contained in *Penicillium simplicissimum*. This precursor is not commonly found in nature, and its production is thus needed in order to perform this enzymatic reaction. Capsaicin, contained in red pepper, can be converted to vanillylamine with a simple hydrolysis involving the amidic group of this molecule, catalysed by hydrolases of rat liver microsomes (fragments of ribosomes and rough endoplasmic reticulum). Vanillylamine obtained, is now converted to vanillin with VAO. The reaction mechanism comprehends a first conversion to vanillylimine, which is an intermediate that gives in a second hydrolytic step vanillin. Studies on the dependence of this conversion from pH showed that velocity increases exponentially in a range of pH from 9 to 10, but overcoming this value of basic pH was not possible due to instability of hydrolase. VAO is also able to convert creosol in vanillin, but, in this case, optimum pH is between 7 and 8. The mechanism is divided in two steps. Creosol is first hydroxylated to vanillyl alcohol, and kinetic studies show that this is the rate-determining step. VAO uses a flavin cofactor for catalysis and hydroxylation involves its reduction. This reaction occurs with formation of a complex between reduced enzyme and a *p*-quinone methide intermediate. Oxygen regenerates oxidized form of flavin and water attacks methide to give vanillyl alcohol, which is finally oxidized to vanillin in the second step. [7]

## 3.2. Irones

Irones are a category of flavours found in *Iris* flowers, with a very high cost of production. The first irone found in nature was the phenyl-hydrazone of a smelly ketonic compound. Ten stereoisomers of this ketone were soon discovered, and Italian iris oil contained four of these molecules. [8]

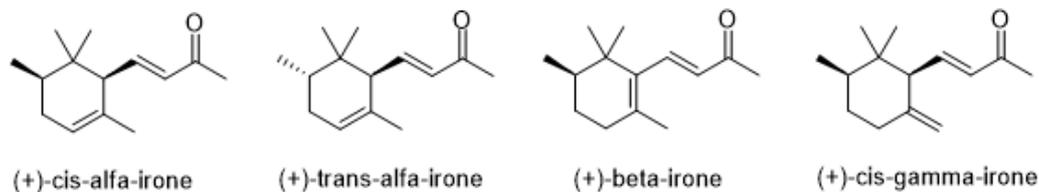


Fig.3: Four isomers of irone.

This category of fragrances has two licensed procedures that involve use of enzymes and bacteria for the synthesis. First, we have an enzymatic oxidation of terpenic precursors using proteins such as lipoxygenases or peroxidases, second we can use rhizomes from iris' extract and treating them with microorganisms like *Pseudomonas maltophilia* and *Serratia liquefaciens*.

There are other biotransformations that require the use of enzymes to obtain enantiomerically pure irones like (+) or (-)-cis- $\alpha$ -irones. Iroles, precursors of irones were first prepared with an enantioselective Diels-Alder reaction between 2,4-dimethyl-1,3-pentadiene and acrolein followed by reduction of the aldehydic group with  $\text{LiAlH}_4$ . Another way to proceed is an enzymatic resolution using hydrolytic enzymes to catalyse transesterifications with vinyl acetate as acetyl donor. Use of Porcine Pancreatic Lipase gives moderate enantiomeric excesses in formation of acetate but higher values in hydrolysis. However, it is resolution using a resolving agent that permits to obtain almost enantiomerically pure iroles. In this resolution, aldehydic group has been oxidized to carboxylic acid and then converted to amide with (S)-phenylethylamine to obtain two diastereoisomers, separated by fractionated recrystallization in HCl, converted to methyl esters with diazomethane and reduced to irones with  $\text{LiAlH}_4$ . [9]

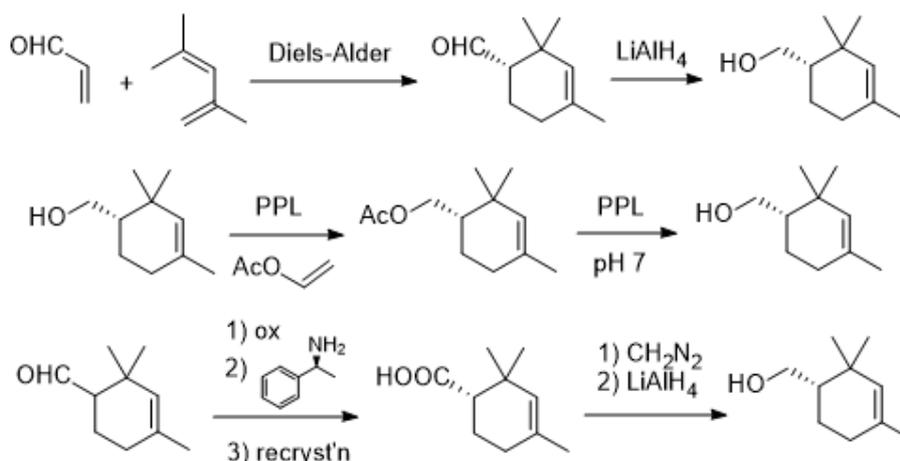


Fig.4: Synthesis of irole.

Iroles are transformed in a cyclic ether with an intramolecular reaction of a vinyl ether, previously formed with an alkyne. Methyl ester formed in the first step was then converted in carboxylic acid,

and finally in methyl ketone, giving the (+) and (-)-*cis*- $\alpha$ -irones. The same synthetic pathway has been used to produce (+) and (-)-*cis*- $\gamma$ -irones.<sup>[9]</sup>

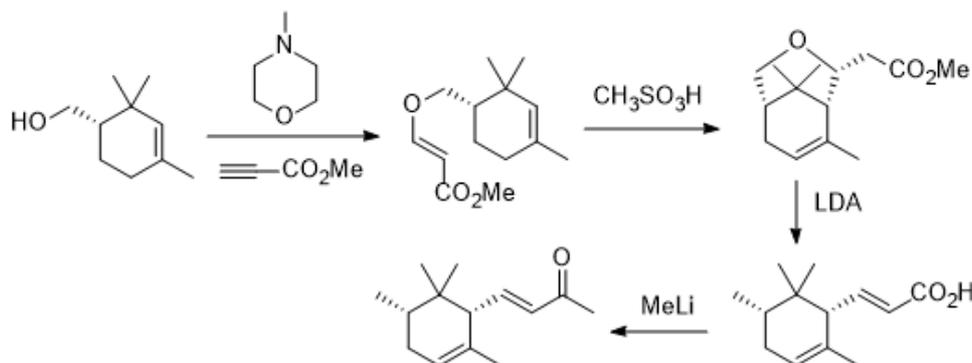


Fig.5: Synthesis of *cis*- $\alpha$ -irones.

### 3.3. Grey amber

Grey amber is a solid secretion found in the intestinal tract of the sperm whale. Its content includes a 25% of (+)-ambreine, which exposed to sun, degrades in (-)-Ambrox, (+)- $\gamma$ -coronal and (+)- $\gamma$ -dihydroionone (these last two compounds are similar in structures to irones). Its cost is high because of its rarity in nature. Capturing whales is fortunately a practice in decline, but to prevent this loss of grey amber industry focused on biosynthetic ways to produce its odorous components. All grey amber compounds have a base structure of a substituted bicyclo[4.4.0]decane with substituents in trans on the bridge bond and other stereocentres. This kind of stereochemistry is needed for the interaction with the receptor inside the nose.

The (-)-Ambrox is the most important fragrance in this category of compounds, and it is obtained from sclareol, a diterpene, extracted from *Salvia sclarea*. Sclareol has an allylic alcohol on the side chain, which can be oxidized to obtain (+)-sclareolide and (-)-isosclareolide, reduced to diastereoisomeric diols and cyclized to (-)-Ambrox and its 8-epimer.<sup>[10]</sup>

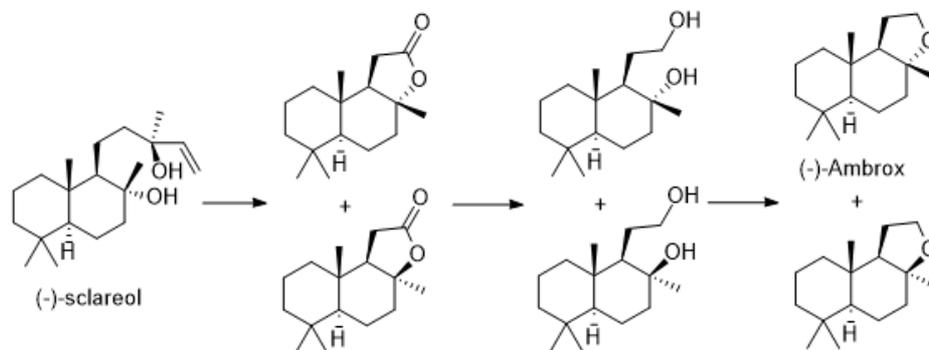


Fig.6: Synthesis of (-)-Ambrox from (-)-sclareol.

Labdanolic acid is a potential precursor of (-)-Ambrox and it is found in large quantities in nature, because it derives from acidic fraction of the extract of *Cistus ladaniferus*. It is not simple to oxidize the side chain of labdanolic acid because it only has a carboxylic group on the side carbon chain, but a synthetic strategy discovered to obtain Ambrox is the iododecarboxylation. First

alcoholic group on the first ring needs to be protected by acetylation with AcCl. Then IBDA (iodobenzenediacetate) and I<sub>2</sub> are the reagents used to substitute carboxylic group with iodide under radiation with a 100W tungsten lamp. The iodide obtained is reactive and tBuOK in THF is quickly added to eliminate HI and deacetylate hydroxylic group on the ring. Ozonolysis is then performed to oxidize at ozonide the double bond obtained on the side chain and reductive conditions by PPh<sub>3</sub> used in this reaction are useful to cyclize the methyl ketone into sclareol oxide. This compound is an enol ether, which in presence of O<sub>3</sub> quickly reacts to give aldehydic group on the side chain and acetyl group on the ring. Finally, aldehyde is reduced to alcohol with LiAlH<sub>4</sub> and *p*-toluenesulfonic acid gives the formation of the cyclic ether, characteristic of (-)-Ambrox.<sup>[11]</sup>

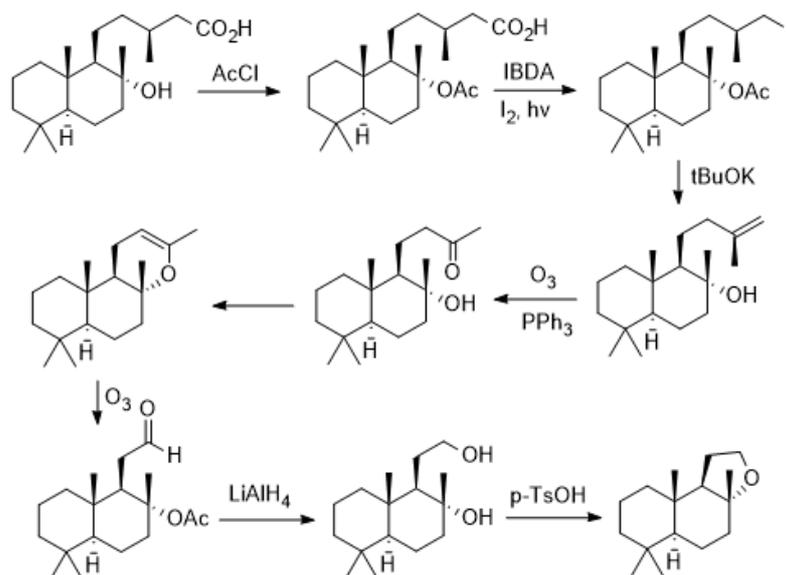


Fig.7: Synthesis of (-)-Ambrox from labdanolic acid.

However, the synthesis explained before makes use of many chemicals. It is then important to find other ways to synthesize compounds of this category of fragrances using enzymes and microorganisms. An example are fungi and yeasts, like *Hyphozyma roseoniger*, that is able to convert sclareol, extracted from clary sage plants, in a diol that easily reacts to give (-)-Ambrox. Other microorganisms, such as *Cryptococcus albidus*, which converts sclareol into sclareolide ( $\gamma$ -lactone), and then, this molecule, is chemically reduced to diol and cyclized to Ambrox. This kind of synthesis is the approach already cited in the first section of this paragraph. We also have other examples of biotransformation for the production of Ambrox, but this time enzymes are involved. Lipase are commonly used to perform kinetic enzymatic resolution, like lipase PE-266 extracted from *Alcaligenes*, lipase PS, and acylase from *Aspergillus melleus*. Starting from racemic mixtures of precursors of Ambrox, such as (+)-albicanol or its hydrated form, we can obtain, with a high enantioselectivity, only (-)-Ambrox. Other compounds fundamental to give grey amber its characteristic smell are (+)- $\gamma$ -dihydroionone, (+)- $\gamma$ -coronal, and also (+)- $\alpha$ -ambrinol, obtainable through resolution by fractionated crystallization of two esters of *S*-(-)-camphanic acid.

## 4. Menthol

Menthol is widely used in the fragrances and flavours industry and it is therefore found in many products, as food additive, flavouring agent for pharmaceuticals, toothpastes, and pesticides. Annually over 30 tons of menthol are consumed for all of these applications. Menthol can be extracted from many plants, widely spread all over the world, such as *Lamiaceae*, a family of flowering plants also known as mints where we can also find rosemary, sage, lavender, oregano and so on. In Italy, we can easily find plants with typical menthol smell like *Mentha piperita*, used to extract peppermint oil with a content of 50% of menthol, and *Mentha arvensis*.

It has three asymmetric carbons in its cyclohexane ring and, for this reason, we have 8 optical isomers: (+)-menthol, (+)-neomenthol, (+)-isomenthol, (+)-isoneomenthol, (-)-menthol, (-)-neomenthol, (-)-isomenthol, (-)-isoneomenthol.<sup>[12]</sup>

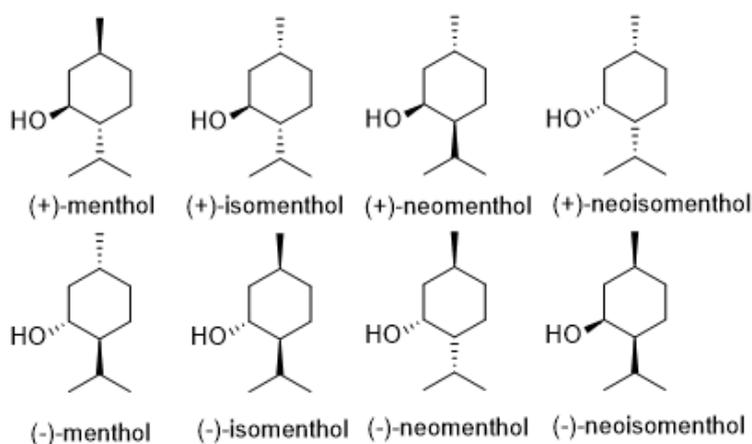


Fig.8: Eight isomers of menthol.

As we can see, menthol is a cyclic terpenic alcohol, with a base structure commonly found in nature, but only a small part of these compounds has flavouring properties. (-)-Menthol is the isomer named simply menthol because it is present in plants in higher quantities, and it is able to give a cooling sensation when applied to skin and mucosal surfaces. Because of its hydrophobicity, metabolism of menthol involves the formation of glucuronide compounds to make it water-soluble, thanks to glucuronyltransferases that act on monoterpene alcohols. This molecule is transported to the liver where it is hydroxylated by oxidative enzymes to *p*-menthane-3,8-diol (or 3,9-diol in small quantities), successively it is converted to 3,8-dihydroxy-*p*-menthane-7-carboxylic acid by oxidation of methyl group, and hydroxyl groups are also converted to ether forming a bicyclic compound with an oxetane group. Enzymes used for these oxidations are cytochrome P450 that use NADPH as co-factor.

Menthol is produced by plants with the mevalonate pathway, a part of the metabolism used to synthesize monoterpenes. In addition, the MEP (methylerythritol phosphate) pathway in some plants can be useful to the production of the precursor of all isoprenoids, the IPP (isopentenyl diphosphate). IPP can be isomerized to DMAPP (dimethylallyl diphosphate) with the Isopentenyl-

diphosphate Delta Isomerase (IDI), and the reaction is an equilibrium, particularly shifted to DMAPP. Geranyl diphosphate synthase (GPPS) is the enzyme that catalyses the reaction between IPP and DMAPP that gives GPP (geranyl diphosphate), used for the synthesis of monoterpenes, like limonene, carvone and camphor. If more isoprenic units are added, by using the diphosphate as leaving group to make GPP react with DMAPP, we can obtain farnesyl diphosphate (FPP), from which sesquiterpenes derive, that present a characteristic carbon skeleton with a  $\beta$ -lactone in a tricyclic system, and then geranylgeranyl diphosphate (GGPP), precursor of diterpenes, like cafestol, present in coffee.

To synthesize menthol, GPP is converted in (-)-limonene by (-)-limonene synthase (LS), that is a monoterpene cyclase, and catalyses the reaction with a cationic mechanism where diphosphate is shifted to bring double bond in a terminal position for the attack, as nucleophile, of the other double bond converted to carbocation. (-)-Limonene is then hydroxylated with (-)-limonene-3-hydroxylase (L3OH), using NADPH and O<sub>2</sub>, obtaining (-)-*trans*-isopiperitenol. In spearmint pathway hydroxylation takes place in position 6 with L6OH, bringing to (-)-*trans*-carveol, that is then oxidized to (-)-carvone with a dehydrogenase (CDH). (-)-*trans*-isopiperitenol is oxidized to ketone with a dehydrogenase (IPDH) with NAD<sup>+</sup>, and hydrogenated on the endocyclic double bond to (-)-isopiperitenone with NADPH, with a reductase, giving (+)-*cis*-isopulegone. Previous hydroxylation and this last step are both highly stereospecific reactions because two stereocentres are formed. Now terminal double bond on the unsaturated ramification must be moved in exocyclic position, and this isomerization is catalysed with a histidine of (+)-*cis*-isopulegone isomerase (IPGI), that gives a proton transfer passing by the enolic form of the ketone group. (+)-Pulegone, synthesized in this step, can be transformed with (+)-menthofuran synthase (MFS) into (+)-menthofuran in a secondary metabolic way and stop the synthesis here. This happens when plant is under stress conditions like low temperature and lack of light, water and food. To produce menthol, instead, another reduction is required, and (+)-pulegone is converted in (-)-menthone with (+)-pulegone reductase (PGR) and NADPH. This reaction does not have a very high enantioselectivity and for this reason small quantities of (+)-isomenthone are produced. As final step, we have reduction of (-)-menthone in (-)-menthol, converting ketone into alcohol and forming the third stereocentre. (-)-Menthone reductase (MMR) and (+)-neomenthol reductase (MNMR) are the enzymes that can reduce not only (-)-menthone, but also (+)-isomenthone, giving 4 of all the optical isomers that menthol has.<sup>[13], [14]</sup>

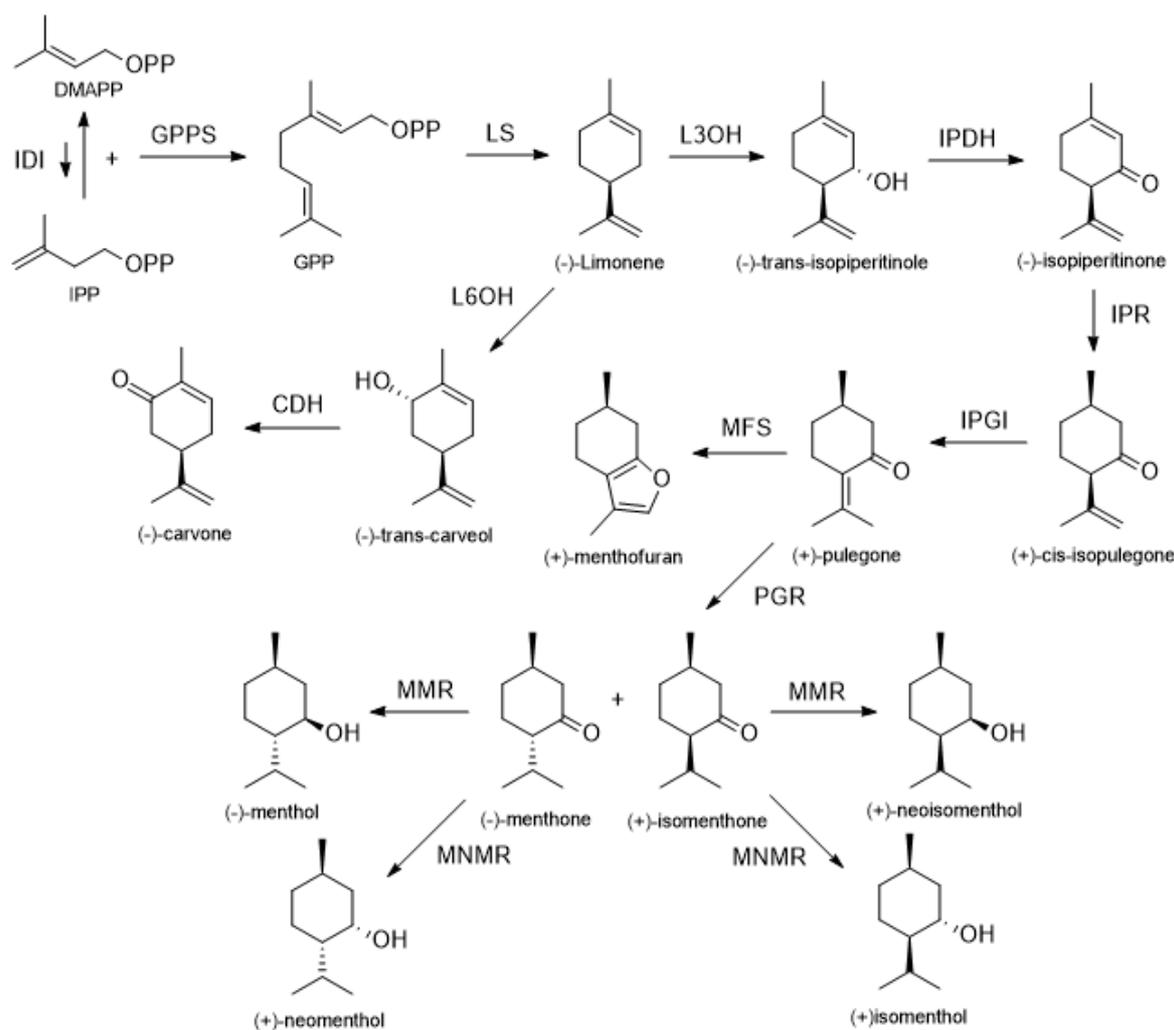


Fig.9: Peppermint metabolic pathway for the production of menthol.

Before talking about biotechnological methods for the production of menthol, we are going to introduce a couple of synthetic approaches, used to produce this flavouring agent in large quantities, mainly destined to the alimentary industry. Takasago has developed first method, and precursor of menthol is  $\beta$ -pinene. First pinene is heated to give myrcene, then diethylamine is added and reacts with the conjugated double bonds, in a 1,4-addition mechanism, to obtain *N,N*-diethylgeranylamine. Now a complex of rhodium  $[\text{Rh}(\text{BINAP})_2]\text{ClO}_4$  catalyses an isomerization to stabilize the molecule at enamine, successively hydrolyzed at aldehyde (+)-citronellal. Next step is cyclization at (-)-isopulegol with  $\text{ZnCl}_2$  that increases, by coordination of oxygen, the electrophilicity of carbonylic carbon, attacked by the double bond. Finally hydrogenation with NiRaney catalysts gives (-)-menthol.<sup>[15]</sup>

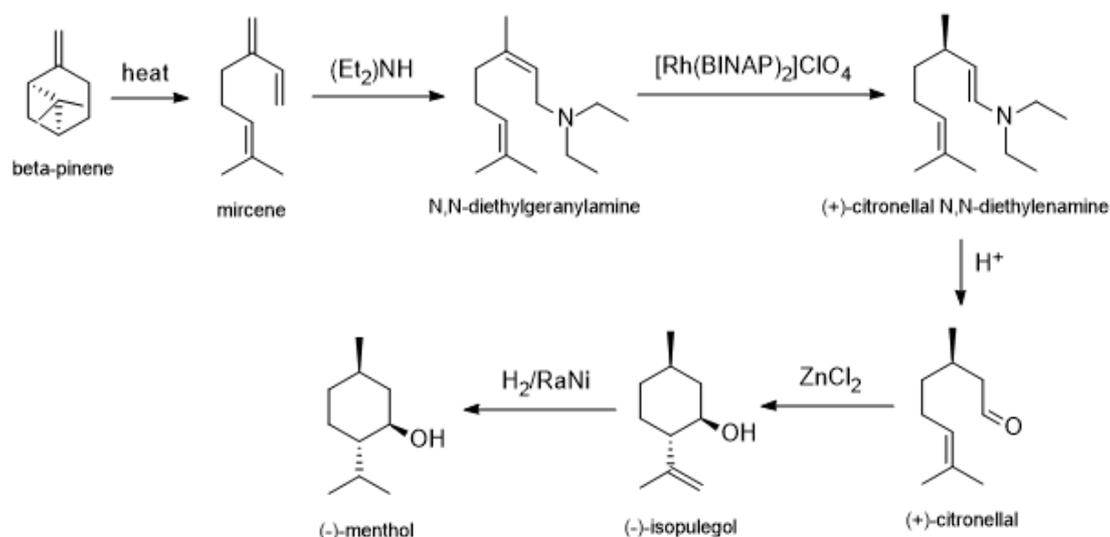


Fig.10: Synthesis of (-)-menthol from  $\beta$ -pinene.

Second method, Haarmann & Reimer, is economic because *m*-cresol and propylene are easy to produce. First reaction is a Friedel-Crafts, where thymol is produced. Catalytic hydrogenation of benzene ring gives a racemic mixture of all isomers of menthol, and for this reason, a first separation step is needed obtain only menthol. Fractional distillation is used to separate isomenthol, neomenthol and neoisomenthol from menthol, and recycle them, epimerizing their stereocenters to obtain more racemic menthol. Last reaction is an esterification with benzoic acid, to obtain both enantiomers of menthyl benzoate that can be separated by crystallisation. (-)-Menthyl benzoate precipitates and can be hydrolysed to (-)-menthol, (+)-menthyl benzoate stays in solution and is recycled by hydrolysis to the epimerisation step.<sup>[16]</sup>

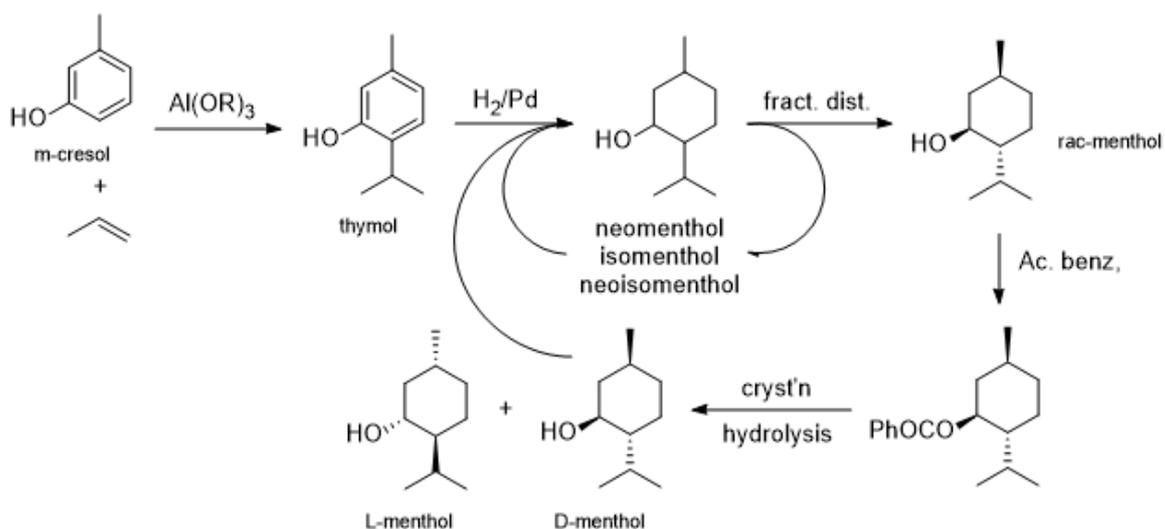


Fig.11: Synthesis of menthol from *m*-cresol.

As always, it is important to find alternative pathways to obtain a higher quality menthol with biotechnological processes like extraction from plants or reaction with enzymes.

Extraction is the easiest way of producing (-)-menthol, in fact the majority of this flavouring agent is taken from *Mentha arvensis* by freezing of extracted oil and crystallisation of menthol.

Harrmann and Reimer discovered also the first biocatalytic route, consisting of an enzymatic resolution of racemic menthyl benzoate, using for example *Candida rugosa* lipase that catalyses an enantioselective hydrolysis giving enantiomerically pure (-)-menthol. Another method is used by AECl Ltd and involves the total acetylation of a mixture of all 8 isomers of menthol using lipases obtaining only (-)-menthyl acetate thanks to the high enantioselectivity of the enzymes that consent to achieve 96% of enantiomeric excess of the ester. This is separated from all the unreacted isomers by distillation and finally hydrolysed to (-)-menthol. All other isomers can be recycled by epimerization.<sup>[17]</sup>

All synthetic route described until now can be summarized in a general scheme.

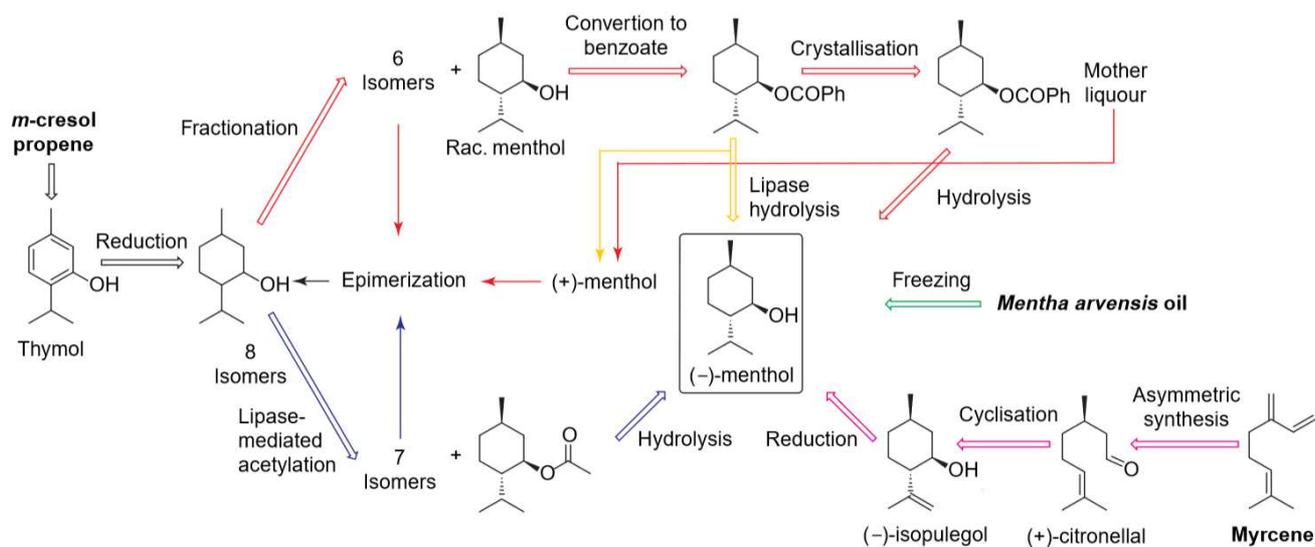


Fig.12: Summary of most famous synthetic pathways of (-)-menthol.

The *p*-menthane is an important molecule in this class of flavours. It is the only monocyclic monoterpene and it is commonly used to produce menthol. The metabolism of this ketone in peppermint leaves has been shown to involve the reduction to (-)-menthol, some of which is subsequently acetylated, and to (+)-neomenthol mostly glucosylated. This reduction has been developed also in vitro, extracting the enzyme from leaves and adding NADPH, utilised as cofactor, to the solution of *p*-menthane.<sup>[18]</sup>

## 5. Conclusions

Biotechnological methods are catching on all over the field of flavours and fragrances production, due to all the attention paid on the respect for the environment and for the health of people who consumes them in food, pharmaceuticals and cosmetics. Techniques identified range over extractions, reactions with enzymes and use of living cells and their focus is to guarantee the application of the principles of "Green chemistry". Production of pollutants and hazardous substances is gradually decreasing because industries are going beyond classical synthesis methods and are applying biotechnologies. Unfortunately, only a small amount of synthetic pathways has been developed at industrial scale, and products labelled as "natural" have a higher cost than "nature-identical". For this reason, the majority of these compounds are still produced in the classical way. Menthol and vanillin have a high annual demand and bio techniques displayed in this review still cannot supply all of it. On the other hand, alternative biological ways to obtain fragrances rarely found in nature, like grey amber, from precursors easily available is the key to lower their price and increase their production.

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