Biotechnology - Conquests and Challenges in Flavors & Fragrances

by

John C. Leffingwell[*1] *& Diane Leffingwell*[1]

[1] *Leffingwell & Associates, Canton, GA 30115 USA*

Summary

In this review we will discuss biotechnological progress for the following ingredients: amino acid derived flavor enhancers (*umami* - monosodium glutamate and *kokumi* – γ-glutamyl-valyl-glycine), Patchouli oil (patchoulol), Vanilla (natural vanillin), Sclareol (an ambergris scent intermediate) and Sandalwood oil (β-santalol).

Background:

Since early in history, humans utilized what is now called biotechnology using fermentation techniques for producing beer and to make dough rise during the making of leavened bread. But the scientific basis for these was only deduced in the 19th century.

Louis Pasteur is perhaps best known to chemists because of his studies on the optical resolution of tartaric acid, i.e., the discovery of chemical chirality in 1848[1]. Pasteur was also the first to prove that fermentation was caused by microorganisms. His studies of microbiology led to methods (e.g. pasteurization) for preventing spoilage and to many improvements in the processing of wine, beer and milk. During his work on the pathogenic bacteria responsible for chicken cholera, he discovered that by partially inactivating the bacteria, upon injecting this in chickens, the weakened bacteria caused the chickens to become immune. Similarly, he extended this to vaccines for anthrax immunization in cattle and for rabies in dogs and humans. In addition, Pasteur developed the 'Germ theory' which explained that communicable diseases were spread by germs (e.g. bacterial infections). These discoveries revolutionized work in infectious diseases. Certainly, Pasteur must be considered one of the 'fathers of biochemistry'. It was he that laid the groundwork for modern fermentations in that he showed "it is necessary to supply the correct organism to provide the correct type of fermentation". As Asimov stated – "In biology it is doubtful that anyone but Aristotle and Darwin can be mentioned in the same breath with him"[2].

Historically, we should also mention a few of the important commercial fermentation products that were developed before the era of metabolic engineering: a) Lactic acid (using *Lactobacillales*) by Louis Pasteur[3] in 1857 and commercialized by Boehringer Ingelheim in 1895; b) Citric acid (using *Aspergillus niger*) by James Currie[4] in 1917 and commercialized by Pfizer in 1919; c) Penicillin (using *Penicillium notatum*) by Alexander Fleming[5] in 1944 and commercialized by the U.S.A. War board consortium in 1944; d) Streptomycin (using *Streptomyces griseus*) by Selman Waksman[6] in 1944 and commercialized by Merck in 1944.

The Nobel Prize was awarded to Fleming in 1945 for Penicillin[7] and to Waksman in 1952 for Streptomycin[8].

Amino acid derived flavor enhancers:
The flavor enhancer mono-Sodium glutamate (MSG) is the largest production volume flavoring ingredient with an estimate of 2.96 million metric tons[9] in 2013. Discovered by Kikunae Ikeda at Tokyo Imperial University in 1907 as the unique flavor enhancing character (now called *umami*) in kelp (*kombu*). Ikeda patented a process for manufacturing this from hydrolyzed protein in 1908[10] and in 1909 production began by Saburosuke Suzuki at the Suzuki Seiyakusho Co. (now Ajinomoto). The product was introduced in Japan on May 20, 1909 with the trademark AJI-NO-MOTO® which means the "essence of flavor".

In 1956, Asai et al.[11] at Tokyo University and Kinoshita et al.[12] at Kyowa Hakko Kogyo (now Kyowa Kirin), found that fermentation of carbohydrate and ammonia sources in a strain of a *Micrococcus glutamicus* (now *Corynebacterium glutamicum*[13]) produced L-glutamic acid in good yields.

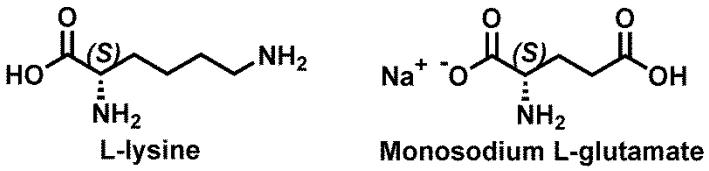
By the 1960's, glutamate production was rapidly moving to the fermentation method with the *coryneform* bacteriums (*Corynebacterium glutamicum* and *Brevibacterium* species) being preferred[14-17]. By 1966, work on mutant strains was well underway with the first patents appearing in the early 1970's[18-19]. Since that time, over 4000 patents and patent applications have appeared that use mutant microorganisms for producing amino acids, nucleosides and nucleotides. Publications have recently appeared on the metabolic engineering of glutamate in *C. glutamicum*[20] and specifically for the genome sequence of a *C. glutamicum* mutant used in the commercial production of glutamic acid[21].

Major producers of MSG are Ajinomoto, Fufeng, Vedan and CJ CheilJedang.

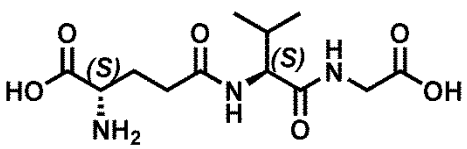
In addition to MSG, L-lysine is an approved flavorant which is also manufactured using *C. glutamicum* strains. However, the main use is as an animal feed additive. Production of L-lysine in 2013 was estimated at 2.1 million metric tons[9].

L-γ-Glutamyl-L-valyl-glycine is a newly discovered "*kokumi*" flavor enhancer[22]. *Kokumi* is sometimes translated as "heartiness" or "mouth fullness" and can enhance the intensities of salty, sweet and umami tastes. In January 2015, it was reported that γ-Glu-Val-Gly can also enhance thick flavor, aftertaste and oiliness in reduced-fat peanut butter, ***suggesting that addition of γ-Glu-Val-Gly can improve the flavor of low-fat foods***[23]. γ-Glu-Val-Gly is prepared from Val-Gly with a γ-glutamyl group donor in the presence of a mutant γ-glutamyltransferase[24].

Figure 1



L-lysine Monosodium L-glutamate



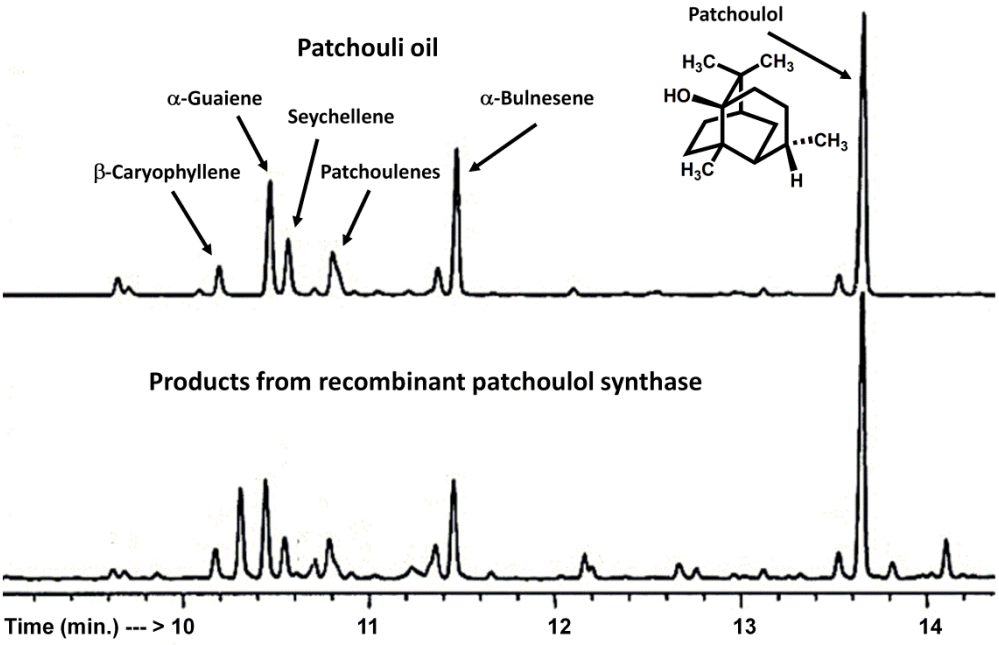
L-γ-Glutamyl-L-valyl-glycine

L-γ-Glutamyl-L-valyl-glycine is FEMA GRAS No. 4709 & JECFA No. 2123.

Recent Developments:
2014 was a breakthrough year for the flavor and fragrance industries quest for sustainability and cost reduction for a number of important ingredients. It was a year where the value of biotechnical partnerships with companies like Amyris and Evolva began to payoff. Also, in December 2014 Evolva acquired Allylix, a biotech producer of the important grapefruit ingredient nootkatone used extensively in both flavors and fragrances.

Patchouli Oil (Patchoulol):
In 2014, the seminal work of Firmenich on patchoulol synthase[25-31] resulted in the introduction of its CLEARWOOD™ patchouli oil substitute, described as a "Soft, clean version of Patchouli without the earthy, leathery and rubbery notes found in the natural oil".[31] This resulted from the scale-up and production of the Firmenich technology by their Amyris partner[32]. Figure 2 (adapted from Deguerry et al.[27]) provides the chromatogram comparisons of patchouli oil with that produced via a recombinant patchoulol synthase.

Figure 2



In addition, as part of their overall ingredient sustainability program, Firmenich is working with farmers in both Indonesia and Guatemala on the production of patchouli oil to improve both their supply chain and revenues for farmers[33]. Givaudan's patchouli oil sustainability programs in Indonesia and Borneo also reflects this type of industry commitment.

Vanilla (Natural vanillin):
Vanillin was first isolated from vanilla extract in 1858[34]. In 1874, Tiemann and Haarmann were able to prepare vanillin by oxidation of coniferin (Coniferyl alcohol β-D-glucoside) present in soft wood pine species[35]. This led to the formation in 1875 of Haarmann & Reimer (now Symrise) for the commercial production of vanillin. By 1876,

they had developed syntheses from both eugenol[36] and guaiacol[37-38]. Subsequently, a number of other processes have been employed to produce synthetic vanillin from eugenol, lignin and guaiacol-glyoxalic acid[39]. It should be noted that, because of environmental concerns, most of the vanillin producing facilities using lignin-containing waste liquor from paper plants have ceased operation. The guaiacol-glyoxalic acid route is now used extensively in China[40].

Vanilla and vanillin are among the most important fragrance and flavoring products in the world, with a total market value of about USD 600 million (and a total volume of about 18,000 metric tons). Only a small fraction of this volume consists of natural vanilla or natural vanillin.[41]

The ice cream and chocolate industries together comprise 75% of the market for vanillin as a flavoring, with smaller amounts being used in confections and baked goods[42]. In the last two decades, consumers and marketers have increasingly expressed the desire for natural flavors in their products. For example, recently (February 2015), Nestlé USA announced that it will eliminate all artificial colours and flavors from its chocolates by the end of 2015, in response to consumer preference for natural ingredients[43]. This follows similar moves by Nestlé in other parts of the world.

But in the case of vanilla, and vanillin, the very high cost of supplying "natural" has been a challenge for the flavor industry. In the case of vanilla extract, the price is dramatically affected by the cost of vanilla beans. As Madagascar exports about 80% of the world's supply, a number of factors (cyclones, price to farmers, political turmoil, and crop disease) have historically caused some major price fluctuations[44-45]. This was especially true in 2003 when vanilla bean prices rose to about US $400/kg and then dropped to $32/kg in 2005. As cured vanilla beans contain about 2% vanillin, even at a more normal $25/kg the natural vanillin portion would cost at least $1250/kg. This compares to the synthetic vanillin price of about $16/kg. Thus, the interest in bio-based natural vanillin production – at a reasonable price.

In 1977, Tasada reported that a soil microorganism, tentatively identified as a *Corynebacterium* sp.[46], and a *Pseudomonas* sp.[47] converted eugenol to ferulic acid and vanillin. The first patent on production of natural vanillin appeared in 1991 from Haarmann & Reimer[48], which described a process of converting isoeugenol or eugenol to vanillin using microorganism strains of *Serrati* sp., *Enterobacter* sp. or *Klebsiella* species. Numerous publications and patents on bio-based vanillin from either ferulic acid or eugenol soon followed these discoveries. For a recent review, see that of Gallage & Møller[49].

In 2000, Rhodia (now Solvay) introduced Rhovanil® Natural, a natural vanillin produced from ferulic acid based on technology from Givaudan[50-51]. Although priced at ~$700/kg, the timing was perfect considering the disastrous vanilla bean crisis of 2003.

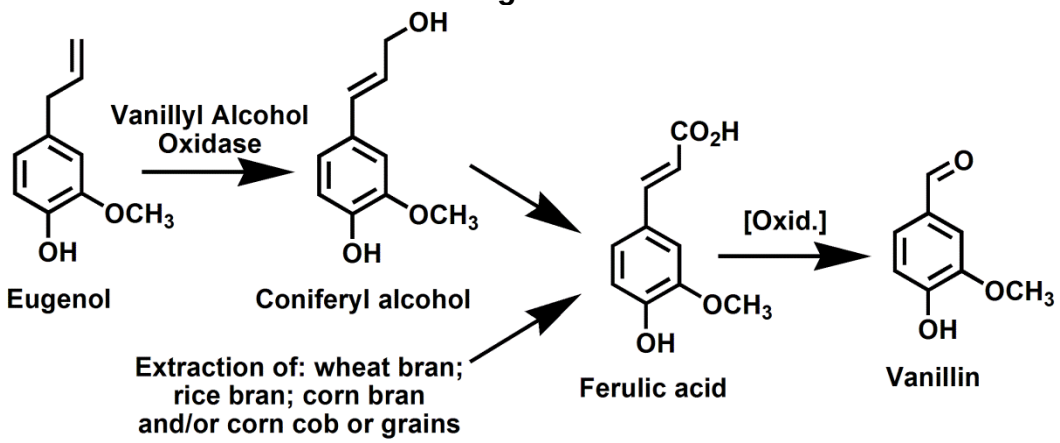
In 2015, a Conagen/Givaudan patent application appeared that disclosed a new process for producing ferulic acid used for vanillin biosynthesis. In this case, ferulic acid is made from p-coumaric acid by a two-step enzymatic process, encompassing hydroxylation and O-methylation, with caffeic acid as an intermediate metabolite[42]. This is potentially a significant advancement as p-coumaric acid at high levels accompanies ferulic acid in waste products such as maize cobs, Brewer's Spent Grain and rice

husks[52-53]. Further, in 2015 BASF disclosed an improved bio-catalytic process for producing vanillin from ferulic acid based on genetically engineered *Pseudomonas* strains[54] and in 2009 Shanghai Apple developed an improved process from Ferulic acid using a new a *Streptomyces* strain[55].

Also, in 2014, Mane S.A. introduced its Sense Capture™ Vanillin obtained by an improved bioconversion of eugenol[56-57].

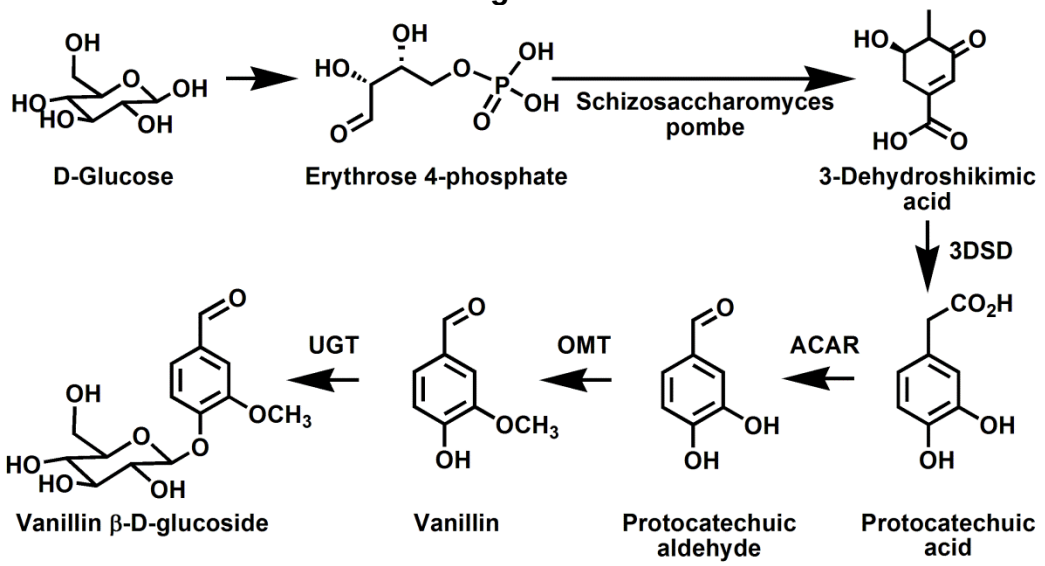
Figure 3 illustrates the eugenol and ferulic acid routes.

Figure 3



In 1998, John Frost's group at the University of Michigan published the *Synthesis of vanillin from glucose*[58]. This laid the groundwork for the Evolva - International Flavors & Fragrances (IFF) collaboration for the production of natural vanillin, which process is shown in Figure 4[59-60]. As vanillin is toxic to the microorganisms employed, the process forms vanillin β-D-glucoside which is later hydrolyzed to vanillin. IFF began use of commercial quantities of this material in vanilla flavored products in 2014.

Figure 4



ACAR = aromatic carboxylic acid reductase; 3DSD = a 3-dehydroshikimate dehydratase; UGT = a uridine 5'-diphosphoglucosyl transferase; OMT = O-methyltransferase.

In addition, companies such as Symrise, Firmenich, Givaudan and Takasago have active sustainability programs with local vanilla bean farmers to improve both agricultural practices and wages in key producing areas because natural vanillin does not totally replicate the characteristic flavor profiles of high quality vanilla extracts.

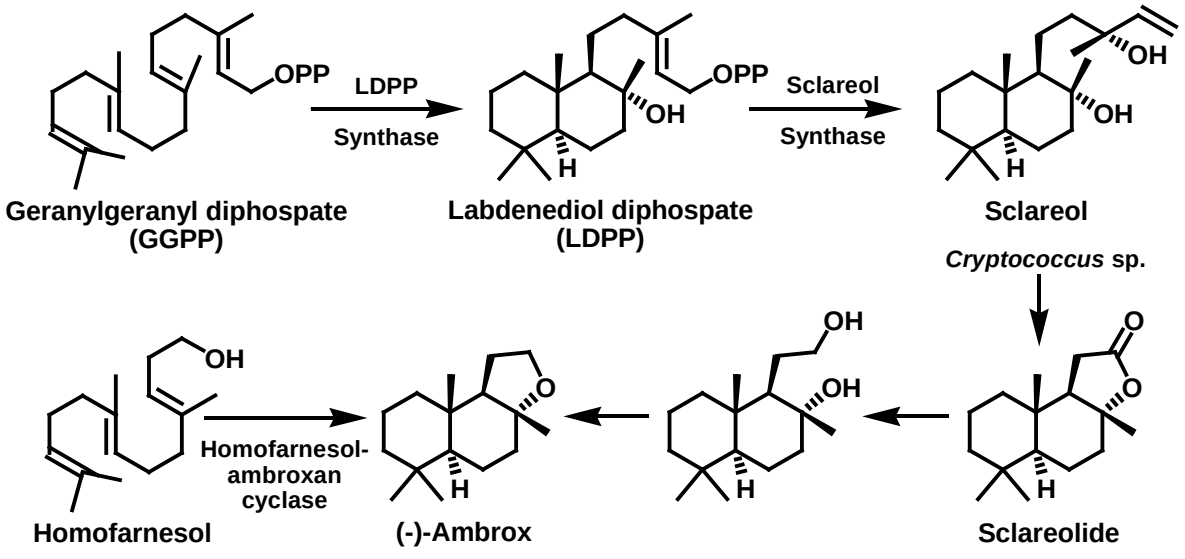
Sclareol (and Homofarnesol) – Precursors to the important ambergris odorant Ambrox®:

(-)-Ambrox® was first disclosed as one of the most important odor constituents in ambergris by Max Stoll's group at Firmenich in 1950, which determined both its structure and a process for producing it from sclareol[61].

Sclareol is produced from the Clary sage plant (*Salvia sclarea* L.). Normally, clary sage oil, which also is a valuable perfume ingredient, is first steam distilled from the plant material, after which sclareol is extracted with a hydrocarbon solvent and then separated by methanol extraction with subsequently conversion to sclareolide[62] (which is the key intermediate for the classic Ambrox product). In 1988, a shortage of sclareol/sclareolide occurred and work began on the development of alternative synthetic routes, which we have previously reviewed[63].

Avoca, in Merry Hill, NC (USA) is the world's largest producer of sclareol and sclareolide. Originally, Avoca utilized a permanganate oxidation of sclareol to produce sclareolide[64], but today use a biochemical process[65]. As both sclareol and sclareolide are relatively expensive, and are subject to potential volatility in availability, investigations on the biosynthesis have been underway for over a decade. Both Firmenich[66] and Allylix[67] (now Evolva) appear to have developed viable biosynthetic routes for production of sclareol from geranylgeranyl diphosphate via a labdendiol intermediate which can be converted to Ambrox by classical procedures as shown in Figure 5. Another approach to Ambrox is the partial biosynthesis using homofarnesol as recently developed by both KAO and BASF. The KAO process[68] uses a squalene-hopene cyclase while the BASF process[69] employs a polypeptide with the activity of a homofarnesol-ambroxan cyclase (also shown in Fig. 5). These type processes may eventually be scaled up for commercialization.

Figure 5

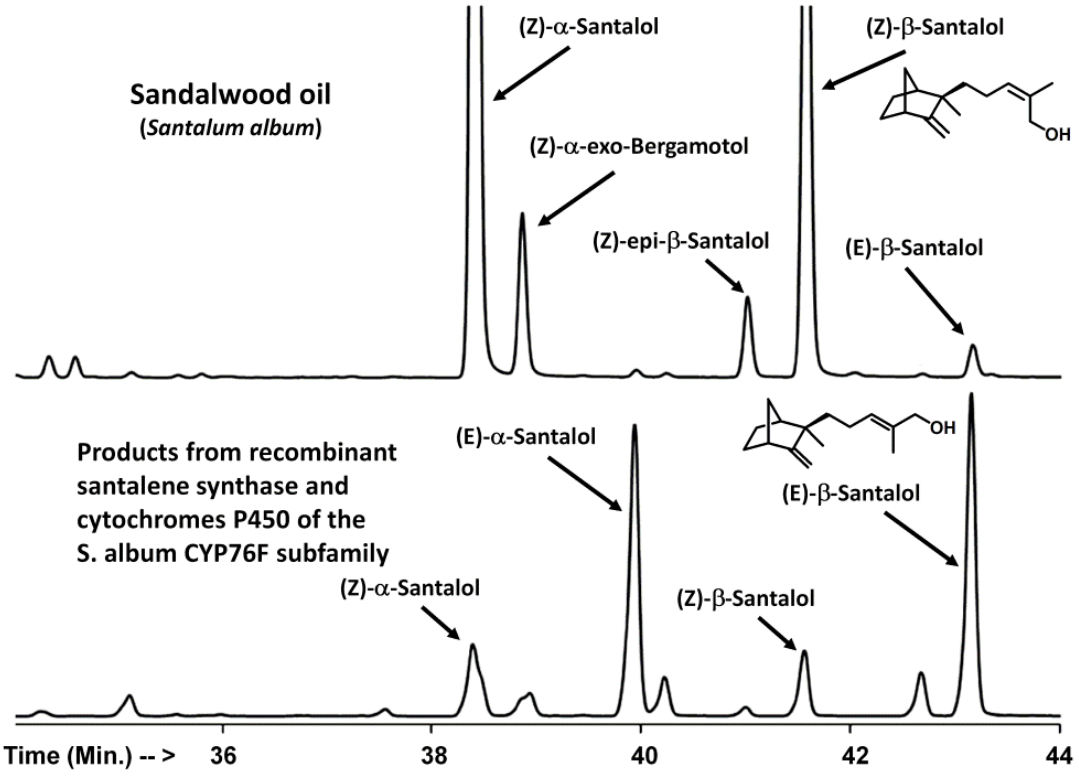


Sandalwood (β-Santalol):

Sandalwood odorants are widely used in fragrances. The benchmark for the fragrance industry is East Indian sandalwood oil (*Santalum album*). Four sesquiterpenols, (Z)-α-santalol, (Z)-β-santalol, (Z)-epi-β-santalol and (Z)-α-exo-bergamotol, make up approximately 90% of the oil of *Santalum album*. These compounds are the hydroxylated analogues of α-, β-, epi-β-santalene and α-exobergamotene.

(-)-(Z)- β-santalol, which comprises about 18-24% of the oil, is the most important constituent from an odor standpoint, being considered as the "gold standard". To date, with the possible exception of Fehr's recent work[70], no economically synthesis of (-)-(Z)- β-santalol is yet available[71]. With the limited availability and price of E. I. sandalwood oil being about US$2500/kg[72], the biosynthesis of β-santalol and/or biosynthetic sandalwood oil are being investigated by both Firmenich[73-78] and by Allylix (Evolva) and their consultants[77-82]. This involves the preparation using santalene synthases from which α- & β-santalenes and exo-bergomotene are formed. In theory, it should be possible by a hydroxylation procedure to produce either (Z)-β-santalol or a bio-sandalwood like oil. Figure 6 (adapted from Diaz-Chavez et al.[80]) represents the results of such a bio-oil from a recombinant santalene synthase and oxidation with cytochromes P450 of the S. album CYP76F subfamily.

Figure 6



As will be noted from the chromatogram, instead of the desired (Z)-isomers the predominant products are (E)-isomers, which are much inferior in odor. In addition,

process yields from the santalene synthases are low. Never-the-less, we expect these types of problems to be solved over time.

Historically, India produced about 90% of the world's supply of E. I. Sandalwood oil, producing over 100 metric tons in the 1970's[83]. Thereafter, Indian production rapidly declined due to over-exploitation, misguided government policies and illegal smuggling. Until 2005, India was a net exporter of sandalwood oil, but as of 2013-14 legal exports had fallen to 420 Kg while imports were 34.4 metric tons[84] (mainly of *Santalum spicatum* oil). Similarly, Indonesia, which at one time produced about 25 metric tons, now markets less than a metric ton.

Worldwide demand for this oil is estimated at 80-120 metric tons per year. About 15-20 metric tons of Australian Sandalwood oil (*Santalum spicatum* normally with >5-20% β-santalol) along with 1-2 metric tons of New Caledonian Sandalwood oil (*Santalum austrocaledonicum* with 18-22% β-santalol) are used as replacements. As demand far exceeds supply, several thousand metric tons of synthetic sandalwood odor chemicals are employed to fill the void. But hope of a comeback for the oil of *Santalum album* is rising. Two Australian groups with established, genetically selected, *S. album* plantations have begun processing small amounts of oil harvested from trees planted 14-15 years ago. TFS Corporation[85] manages about 9600 hectares and Santanol about 2100 hectares[86]. TFS acquired Mt. Romance in 2008, which is the currently the world's largest distiller of sandalwood oils and a sustainability partner with Givaudan. TFS expects that it will be the largest supplier of *S. album* oil by 2020. Santanol, a direct competitor of TFS, is a partner with KKR.

Conclusion:

Finally, in September 2014, at the International Federation of Essential Oils and Aroma Trades (IFEAT) convention in Rome, the role of biotechnology and its importance to the flavor & fragrance industry was discussed extensively. Panchapagesa Murali (Evolva) pointed out that the biotech sector is currently addressing the "low hanging fruit", but complex offerings may logically follow. Toine Janssen of Isobionics (which produces the important natural citrus ingredients valencene and nootkatone via biotech) indicated that fermentation technologies could produce many of the terpenes and sesquiterpenoids used in the industry, including the carvones, menthol and the pinenes. Pascal Longchamp (Evolva) mentioned that new projects to produce agarwood (*Oud*) and saffron were now underway. Jason Kelly (Ginkgo BioWorks) indicated that many of the new biotech companies are in partnerships not only with F&F companies but also with those in fuel/biofuel and that the number of entries is expanding. Relative to the types of products that can be produced; Longchamp said 'The world is the limit' [87].

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Ambrox is a registered trademark of Firmenich SA
Rhovanil is a registered trademark of Rhodia (now Solvay)

Note: The authors declare no competing interests.

Corresponding Author
John C. Leffingwell, 4699 Arbor Hill Road, Canton, Georgia 30115 (USA)
*Phone: +01-770-889-5111 * Fax: +01-770-887-0089 * Email: leffingwell@leffingwell.com

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