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Nootkatone

Robin-Hagen Leonhardt and Ralf G. Berger

Abstract The continuing interest in the sesquiterpene ketone (+)-nootkatone is stimulated by its strong grapefruit-like odor and numerous further bioactivities. Also numerous were the attempts to chemosynthesize or biotechnologically produce the compound. Cytochrome P_{450} enzymes from bacteria and fungi were intensively studied and expressed in *Escherichia coli* and in more food compatible hosts, such as *Saccharomyces cerevisiae*. The lipoxygenase-catalyzed generation was demonstrated using an enzyme from several *Pleurotus* species. Laccases required artificial mediators for an efficient catalysis. More recently, plant valencene synthases were expressed in microbial hosts. Combined with an endogenous farnesyl diphosphate delivery pathway and a valencene oxidase, this approach opened access to high yields of nootkatone possessing the appreciated attribute of "natural" according to present food legislation. Little biochemical engineering was carried out on the novel recombinant strains, leaving many options for future improved bioprocesses.

Keywords Natural flavor · Insecticide · CYP450 · Lipoxygenase · Laccase · Valencene synthase · Valencene oxidase

Contents

R.-H. Leonhardt (✉) · R.G. Berger
Institut für Lebensmittelchemi, Gottfried Wilhelm Leibniz Universität Hannover,
Callinstraße 5, 30167 Hannover, Germany
e-mail: rg.berger@lci.uni-hannover.de

1 Introduction

The compound (+)-nootkatone [2(3H)-Naphthalenone, 4,4a,5,6,7,8-Hexahydro-4,4a-dimethyl-6-(1-methylethenyl)-, (4R,4aS,6R)–eremophila-1(10),11-dien-2-one; CAS # 4674-50-4; Fig. 1] is a flavor compound with a grapefruit-like odor, an odor perception threshold in the range of 1 μg/L water, and a slightly bitter taste. First isolated from the heartwood of Alaska cedar [*Cupressus nootkatensis* (D. Don 1824); syn. *Chamaecyparis nootkatensis* (D. Don) Spach 1841, *Callitropsis nootkatensis* (D. Don) Oersted 1864, *Xanthocyparis nootkatensis* (D. Don) Farjon & Hiep 2002], nootkatone was found in essential oils of the genera *Citrus* (Rutaceae), *Alpinia* (*Zingiberaceae*) and other *Pinales*; in Java (*Cyperus rotundus*) and Vetiver grass (*Vetiveria* sp.); and in many other plant essential oils.The biogenetic precursor of nootkatone, (+)-Valencene, is just two steps away from the sesquiterpene starter, the farnesyl cation, and is quickly produced through a germacryl intermediate (Fig. 1). This explains the widespread occurrence of the (+)-valencene bicyclus and its accompanying allylic oxidation products, the stereoisomer α- and β-nootkatols, and (+)-nootkatone. Valencene shows a distinct and pleasant orange odor, whereas

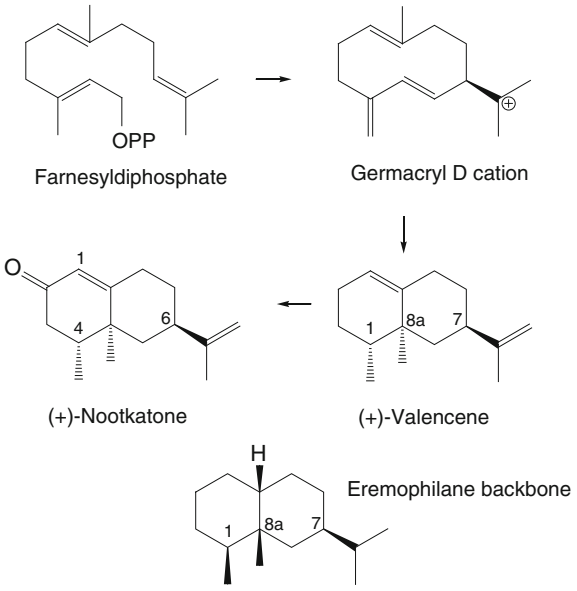

Fig. 1 The proposed biogenetic path from farnesyldiphosphate to the eremophilanes (+)-valencene and (+)-nootkatone

α- and β-nootkatol (= 2*R* and 2*S*-hydroxyvalencene), with lower odor intensity, are reminiscent of grapefruit. Single compounds or mixtures of this group can be used to flavor food, fragrances, cosmetics, and detergents. The worldwide consumption of flavors and fragrances is increasing, as indicated by almost doubled total sales in the past decade. The estimated turnover of the flavor and fragrance industries in 2013 amounted to around $24 billion (www.leffingwell.com/top_10.htm). Patents also claim the use of (+)-nootkatone as an insecticide and repellent.

Because (−)-nootkatone shows an odor threshold at least 1,000 times higher than the (+)-isomer and has less overall bioactivity, it is not of commercial interest. In this chapter, the term *nootkatone* refers only to the more bioactive (+)-form of the molecule.

2 Chemical Analyses

Nootkatone is quite amenable to both gas chromatography (GC) and liquid chromatography (LC). A thorough gas chromatographic reanalysis of an Alaska cedar steam distillate identified additional nootkatone-related compounds, such as the cycloheptatrienone nootkatin and the triene nootkatene [1]. Apart from its better resolution, GC offers the advantage of delivering sensory data on each separated compound, if coupled with a sniff-port for online olfactometric assessment. Ultrafast LC coupled to a time-of-flight mass spectrometer (MS) identified nootkatone in *Citrus grandis* (i.e., *Citrus maxima*, pomelo) [2], whereas classic reverse-phase LC was applied to analyze fruits of *Alpinia oxyphylla* (a relative of blue ginger) [3]. A particularly detailed picture of complex essential oils was obtained by the combination of high-resolution LC for prefractionation and two-dimensional GC coupled to MS [4]. This kind of approach produces a number of less crowded gas chromatograms and creates better spectra for a safer identification of compounds, which is a generally difficult task for samples rich in oligoisoprenoids with often very similar fragmentation patterns. Quantitative analyses have confirmed that the concentration of nootkatone in essential oils is low. The total yield of hydro-distilled oil obtained from various *Citrus* fruits ranged from 0.13 to 0.53 %, and nootkatone had a share of 1.6–2.5 % thereof [5]. A concentration of roughly 100 mg of nootkatone per kilogram of fresh fruit at best is calculated from these data. Because of the high sensory potency, even ultralow concentrations impart the typical grapefruit impression. For commercial production, however, natural plant sources of nootkatone are not rich enough.

3 Biological Activities

Apart from the sensory properties, *Citrus* essential oils were associated with numerous biological activities, such as antioxidant, antimicrobial, anti-inflammatory, and more [6]. Frequently, whole distillates or extracts were applied, making it impossible to confidently ascribe the activity observed to a single constituent of the

sample. The case is different for nootkatone and some of its derivatives and analogues. A recent patent claimed various nootkatone carriers and their application as an insecticide [7]. The efficiency of nootkatone against termites [8] and ticks (arachnid parasites) [9] was experimentally proven, explaining the superior tolerance of cedar wood against insect infestation. Because nootkatone is regularly consumed by mammals, it was concluded that its application would pose no threat to livestock or human consumers. It was suggested to inhibit an insect infestation by onsite inoculation of a nootkatone-transforming microorganism, such as *Paenibacillus alginolyticus,* exploiting this "fermentation" to produce advanced oxidation products of nootkatone with anti-insecticidal activities [8].

The primary mode of action of nootkatone remains uncertain. A recent study [10] disproved earlier assumptions that nootkatone could inhibit acetylcholinesterase (ACE). When compared to the known ACE inhibitor carbaryl, neither nootkatone nor carvacrol, a monoterpenoid also abundant in cedar wood, showed more than a weak activity in the ACE assay.

Human metabolic pathways appear to be affected by nootkatone because of its cardiovascular [11], antiproliferative [12], and antiplatelet effects [13]. The positive effect on survival of septic mice was attributed to the induction of a heme oxygenase [14]. The expression of this enzyme inhibits, in turn, nitric oxide (NO) synthase and NO production, eventually resulting in the observed anti-inflammatory effect. The stimulation of an Adenosin monophosphate-activated protein kinase (AMPK) was recently substantiated [15]. The resulting acceleration of the energy metabolism was supposed to aid in the treatment of human obesity. Upstream of AMPK lays liver kinase B1 (LKB1) or renal carcinoma antigen NY-REN-19, another protein kinase involved in regulating cell polarity and development. LKB1 exerts growth suppressing effects by activating an entire group of kinases, and it is thus a key player in preventing carcinogenesis. Nootkatone also activated this kinase [16].

4 Chemosynthesis

Chemosyntheses of nootkatone were developed to meet industrial demand. Because the immediate precursor valencene is abundant in orange peel oil, its allylic oxidation using chromate (VI) or manganate (VII) ions provided easy access to the target compound. Advanced versions are still under investigation using phase transfer catalysis [17] or a metal mixed oxide catalyst and *tert*-butyl hydroperoxide as a less problematic oxidant [18]. Introducing two of the three isoprene units with the substrate molecule, routes from the easily available substrate β-pinene were also explored [19]. Among the hazardous chemicals required for this synthesis were alkenyl chlorides, benzene, heavy metal salts, and again manganate (VII).

Increasing environmental concerns and costs for the handling and disposal of toxic chemicals are considered in novel approaches that rely on less critical oxidants, with molecular oxygen being one of those. Orange juice develops a grapefruit note after opening and prolonged exposure to air, indicating that autoxidation

of valencene occurred in the acidic medium. Accelerated autoxidation of valencene was achieved in a continuous-flow microreactor [20]. The reactor consisted of heated Pyrex spiral glass tubing, and the reaction proceeded rapidly with high yields and in the absence of a catalyst, radical initiator, or solvent.

5 Biosynthesis

Nootkatone produced by a concerted and deliberate chemical reaction must not be labelled "natural", but this would be the highly preferred quality for food flavors. Effective European law (EG 1334/2008) defines a "natural flavouring substance" as a compound "obtained by appropriate physical, enzymatic or microbiological processes from material of vegetable, animal or microbiological origin." The U.S. Food and Drug Administration Code of Federal Regulation likewise emphasizes the need for the presence of a biocatalyst, using the terms "enzymolysis" and "fermentation."

The distinction between a naturally generated chemical with oxidizing properties and an enzyme with a metal ion coordinated in a high positive redox state is not sharp. However, processes based on an enzymatically generated oxidant are more likely to become approved. Molecular oxygen is a natural co-substrate of dioxygenases and laccases; hydrogen peroxide, the natural co-substrate of peroxidases and peroxygenases, is easily generated, such as by using glucose oxidase in glucose-containing foods. Is a linoleic acid hydroperoxide formed by a soybean lipoxygenase a natural oxidant? This reagent was generated in the presence of valencene, and a subsequent co-oxidation of the hydrocarbon in excellent yields was reported [21]. While the first step is clearly enzyme catalyzed, the second is an inevitable, but deliberate, chemical reaction. Both the forced autoxidation and the lipoxygenase-mediated process benefit from the preferred reactivity of the allylic C3 of valencene (Fig. 1), which helps to prevent the formation of larger amounts of unwanted side products.

5.1 Biosynthesis Based on CYP450

Cytochrome P450 enzymes (CYP450) are a large group of hemoproteins occurring in animals, plants, fungi, bacteria, archaea, and even in viruses. The name-giving property is an absorption maximum at $\lambda = 450$ nm when in the reduced state and complexed with CO. Lipophilic compounds, such as lipids and steroids, and also terpenes are preferably accepted. The typical reaction catalyzed is mono-oxygenation—that is, the introduction of an oxygen atom into the hydrocarbon part of the substrate (HC), while a second oxygen atom is reduced to water:

$$\mathrm{HC} + \mathrm{O_2} + \mathrm{NADPH} + \mathrm{H^+} \xrightarrow{\text{Monooxygenase}} \mathrm{HC-OH} + \mathrm{H_2O} + \mathrm{NADP^+}$$

High catalytic efficiency and pronounced substrate promiscuity of CYP450 suggested these enzymes for the conversion of valencene to hydroxyvalencene. A subsequent oxidation yielded the desired carbonyl nootkatone. In fact, many of the successful transformations reported may be attributed to CYP450 activity [22], although in most cases its actual involvement was merely concluded from the structure of the products. Cellular systems, rather than purified enzymes, were used to meet the demand for protein co-factors. The (often) soluble bacterial enzymes, for example, depend on a ferredoxin reductase and a ferredoxin to deliver the electrons for the final reduction of molecular oxygen.

Among the enzymes used for the hydroxylation of valencene is CYP450 $_{\text{BM-3}}$ from *Bacillus megaterium*. Originally found involved in terminal or subterminal hydroxylations of long-chain fatty acids, it presents the particular feature of being a fusion protein between a CYP domain and an electron donor. Mutants of this enzyme were created and compared with the wild-type enzyme [23]. Activity as measured by the consumption of NADPH and the generation of nootkatone were evaluated. Another versatile CYP450 was found in *Bacillus subtilis* [24]. Here, the CYP450 activity was obtained by co-expression with genes for a putidaredoxin reductase (PdR) and putidaredoxin (Pdx) from *Pseudomonas putida* in *E. coli* to obtain both nootkatols and nootkatone. Typical of many CYP450 catalysed reactions, a large number of side products were observed (Fig. 2). A better product yield of 120 mg/L of odor-active compounds was finally achieved in a two-phase system that dissolved the primary products into the organic phase, thus protecting them from further oxidation. A CYP450 from *Nicotiana* and a P450-reductase from *Arabidopsis* were cloned and expressed in *Saccharomyces cerevisiae* [25]. Product yields were low, but the chances to transfer such a process based on a food-grade recombinant host into industrial application are higher than with *E. coli* or other potentially toxin-forming bacteria. Consumers know and appreciate bakers′ yeast; although nootkatone produced by a recombinant cell will quite obviously not contain remains of inactivated or living genetically modified organisms, the food industry has set out to deal respectfully with public concerns.

While products of valencene overoxidation are too polar to be of interest as flavors, they may be useful for therapeutic applications. A CYP450 from *Sorangium cellulosum*, a myxococcus, accepted nootkatone as a substrate, and also the norisoprenoids α- and β-ionone [26]. Antiproliferative properties of the nootkatone derivatives were examined, and the structural background of the regioselective hydroxylation was elucidated using a homology model. A similar study supplemented cell cultures of Ascomycetes, such as *Aspergillus*, *Chaetomium* or *Fusarium*, with nootkatone. The product profile showed mono- and diols, triols, and epoxides indicating active CYP450s, but the identity of the fungal enzymes was not elucidated [12].

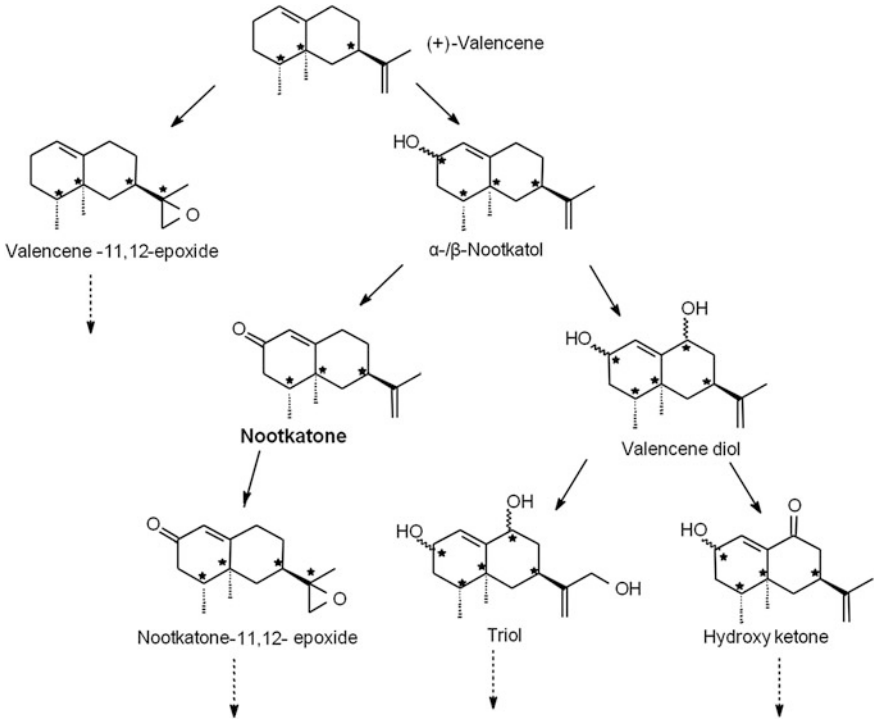

Fig. 2 Overoxidation of nootkatone yields multiple nonflavor compounds with antiproliferative and possibly other bioactivities

It was estimated that product peak yields of 1 g/L or productivities of 0.1 g/L per hour will be required to cross the profitability threshold for a food flavor. Bioprocesses based on CYP450 have not yet reached this stage. The reasons are numerous:

- The terpene hydrocarbon substrate is often insufficiently soluble in water.
- Emulsions complicate the system due to the presence of the cytotoxic emulsifier.
- Both the terpene hydrocarbon substrate and terpenoid product may be toxic for the native or recombinant producer cell or inhibit the enzymes involved.
- Reactive primary metabolites, such as epoxides, give reason to side-product formation.
- Substrate and product accumulate in lipophilic cell compartments and are not secreted.
- Substrate and product will be stripped out of aerated bioreactors.
- The second oxidation step to the often more odor-active carbonyl is not catalyzed by CPY450s.

5.2 Biosynthesis Based on Lipoxygenases

During extended screenings for microbial producers of nootkatone, the basidiomycete *Pleurotus sapidus*, a member of the order *Agaricomycetes* and a close relative of the edible oyster mushroom *Pleurotus ostreatus*, stood out for a selective and highly efficient oxidation of valencene to nootkatone [27]. Although the occurrence of both nootkatols suggested another CYP450-driven reaction, the responsible enzyme, after laborious purification, showed homologies of around 50 % to putative lipoxygenases from *Aspergillus fumigatus* and *Laccaria bicolor*, and 26 % homology to the well-known commercial lipoxygenase-1 of soy bean (*Glycine max*). Because no lipoxygenase from a basidiomycetous source was known on the molecular level, detailed metabolite analyses were carried out using cold on-column GC-MS and APCI-LC-MS methods. The data suggested a lipoxygenase-type oxidation of valencene via secondary and tertiary hydroperoxides [28]:

$$\text{Valencene} + O_2 \xrightarrow{\text{Dioxygenase}} \text{4a-}(R,S)\text{-Val-OO-H} \xrightarrow{\text{Schenkrearrangement (Peroxo-shift)}} 3-(R,S)\text{-Val-OO-H} \xrightarrow{\text{Disproportionation}} \text{Val-OH} + \text{Nootkatone}$$

The gene was cloned and heterologously expressed in *E. coli* [29]. Soluble recombinant protein was gained by cold shock expression, chaperone co-expression, and employment of mutant *E. coli* strains. The expression in *P. pastoris* SMD1168 was carried out using a pPIC9K vector construct [30]. In this construct, the α-factor signal sequence provided by the original vector was replaced by insertion of a second Kozak sequence between the signal sequence and the lipoxygenase gene [30]. The recombinant enzymes, both purified using the N-terminal His tag, showed the catalytic properties of the wild-type enzyme, as was confirmed by the LC-MS analysis of hydroperoxide intermediates and GC-MS analysis of the volatile products. As with the wild-type lipoxygenase, optimal activity was found at pH 7 at 30–35 °C. Conversion of linoleic acid gave high yields of 13*S*-hydroperoxy-9*Z*,11*E*-octadecadienoic acid (94 % ee), as was confirmed by chiral high-performance LC analysis of the hydroperoxides [30, 31].

A follow-up study on a number of *Pleurotus* species resulted in lipoxygenase genes from five strains. The genes were amplified, functionally expressed in the proven *E. coli* system, and characterized [32]. All sequences coded for proteins of 643 amino acids, sharing similarities greater than 95 % among each other. The ligands that determined iron coordination and stereochemistry were fully conserved. Similar lipoxygenase activities were found when linoleic acid served as the substrate. The conversion of valencene, however, differed between two clusters of highly homologous sequences. The future engineering of these dioxygenases could create catalysts accepting other terpenes and alkenes as precursors for novel flavor compounds and possibly bioactives. In clear contrast to CYP450 enzymes, oxygen is the only co-substrate required. Moreover, the situation regarding substrate and product inhibition as well as side product formation appears to be more favorable with these fungal dioxygenases.

5.3 Biosynthesis Based on Laccases

Laccases comprise a large group of well-described oxidases that are frequently used in different applications and processes throughout the food industry [33]. These enzymes are easily available, either by purification from culture supernatants, or in larger amounts by heterologous expression of the respective genes in host strains, such as *P. pastoris*. A patent from 2001 [34] described the use of laccases for the industrial-scale production of nootkatone by oxidation of valencene. The work focused on a mediator-based oxidation, with most of the mentioned mediators being nonnatural laccase substrates (i.e. 1-hydroxybenzotriazole or 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid). This conflicts with the legal definition of natural flavors; however, optimal reaction conditions gave an impressive molar conversion rate of around 40 %. Because laccases preferably oxidize diphenolic compounds [35], the exact reaction mechanism responsible for the oxidation of the sesquiterpene valencene is disputable. Due to the radicals formed during the enzymatic reaction and the various possible oxidations sites of the precursor molecule, side reactions to other products could be envisaged, as they were found for chemosynthetic or CYP450 catalyzed reactions (Fig. 2). Similar processes based on natural precursors and mediators were successfully optimized [36], but no further data on the mediated oxidation of valencene have been published since.

5.4 Biogenesis Based on Plant Enzymes Involved in Terpene Metabolism

There is no generally accepted definition of the term *synthetic biology*, but a short definition could be "to make use of the advantages of biocatalysis using engineering concepts." Because the easily cultivated pro- and eukaryotic cells of classical bioprocesses do not possess a pronounced terpene metabolism, plant genes have to be implanted into recombinant "engineered" hosts. The concept has been patented several times by flavor, food, and even rubber companies to produce terpene hydrocarbons; sometimes terpenoids were also claimed. A recent example was a patchoulol synthase from *Pogostemon cablin* (Indian patchouli) cloned in *E. coli* [37]. To prevent formation of inclusion bodies, the protein of interest was fused to a thioredoxin-tag. Codon optimization improved the yield of soluble enzyme significantly. The product spectrum as analyzed by GC-MS showed farnesol isomers from the hydrolysis of the substrate and germacra-3,9,11-triene A as the major components and the target compound (−)-patchoulol.

The building block molecule of Amyris Inc. for flavors is β-(*E*)-farnesene, called "biofene." One of the company's fundamental patents described the engineering of isoprenoid pathways in host cells [38]. By incorporation of an acetoacetyl-CoA synthase gene and other enzymes of the mevalonate pathway, the host cell became independent from the activated diphosphate precursors used, such as in the patchoulol

study [37]. Branching pathways leading to unwanted intermediates were blocked by disrupting the responsible genes. Also considered was the issue of redox balance in the heterologous host [38].

Another option is to equip the heterologous host with an additional synthase gene, which is a strategy followed by Allylix (www.allylix.com) and Isobionics (http://www.isobionics.com). The cyclisation of farnesyl diphosphate (FPP) to valencene was achieved using enzymes from *C. nootkatensis* [39–41], from *Citrus* species [42] or from *Vitis vinifera* (grape). According to the patent [42], the genes may have been engineered by changing, adding, or deleting codons to obtain variants that are more suitable than the wild-type enzyme. Once the genes were optimized, they were introduced into a tailored yeast strain. This strain was engineered to produce endogenous FPP. The enzyme from Alaska cedar was robust and efficient in vitro, but not well expressed in the usual microbial hosts. When incorporated in *Saccharomyces cerevisiae*, a valencene concentration of around 1.4 mg/L was obtained; a heterologous *Rhodococcus sphaeroides*, under an n-dodecane accumulation layer and upon co-expression of a mevalonate operon, eventually reached remarkable 352 mg valencene per liter [40].

To arrive at nootkatone, a valencene converting CPY450 enzyme from *Cichorium intybus* (chicory) was co-expressed with a synthase gene in a nonspecified yeast [43]. Because chicory contains neither valencene nor nootkatone, this activity can again be related to the frequently observed substrate promiscuity of this type of enzyme. However, the nootkatone levels were low, and it was not clear if this second oxidation step was the result of the activity of other enzymes [Alcohol dehydrogenases (ADHs)] present in the host cell. *Pichia pastoris* was complemented using the premnaspirodiene oxygenase of *Hyoscyamus muticus* (a member of the *Solanaceae*) and the *Arabidopsis thaliana* reductase to hydroxylate valencene [44]. Intracellular production of valencene was achieved by co-expression of valencene synthase from *C. nootkatensis*. Biphasic cultivations of *P. pastoris* resulted in the accumulation of trans-nootkatol, which was oxidized to nootkatone by a genuine activity of *P. pastoris*. Further genetic fine-tuning enhanced the nootkatone yield to 208 mg/L in bioreactor cultivations.

The two approaches are different, but both suffer from inherent problems: To establish an entirely new pathway in a heterologous host is feasible, but the additional load of new genes and enzymes may overburden a simple prokaryotic host. Eukaryotes, on the other hand, are more complicated to transform, grow more slowly, and might more certainly recognize the new genes as "foreign," resulting in the rapid degradation of transcribed or translated metabolites. To implement one or two key enzymes (valencene synthase and oxidase) appears to be more straightforward, but it must cope with the high hydrolytic sensitivity of the obligatory precursor FPP. This activated compound cannot be isolated in sufficient yields from natural sources; thus, the conversion of the chemosynthetic precursor by cell cultures or enzymes cannot yield legally "natural" valencene and nootkatone. As with all enzyme-based attempts, cytotoxic or inhibitory effects of metabolites accumulating in abundance must be expected. Moreover, the poor qualitative and quantitative predictability of heterologous attempts presents a severe technical drawback.

Table 1 Chemical and biochemical generation of nootkatone

Process/organism	Nootkatone (maximum yield)	References
Cr^{VI} oxidation of valencene	47 % from valencene	[48]
Oxidation using lipoxygenases (patented)	60 g L^{-1} (24 h)	[49]
Laccase-mediated oxidation (patented)	50 % from valencene	[34]
Transformation using *Chlorella spec.*	~330 mg L^{-1} (7 d)	[50]
Cultured plant cells (*Gynostemma pentaphyllum*)	~650 mg L^{-1} (20 d)	[51]
CYP450 expression in *E. coli*/two-phase system	120 mg L^{-1} (8 h)	[24]
Oxidation using a lipoxygenase from *Pleurotus sapidus*	~300 mg L^{-1} (16 h)	[28]
	~250 mg L^{-1} (4 h)	[R-HL unpublished]
CYP450 expression in *P. pastoris*	7 mg L^{-1} (24 h)	[25]
Autoxidation in a microreactor	316 g L^{-1} h^{-1}	[20]
Cultivation of recombinant *P. pastoris*	208 mg L^{-1} (15 h)	[44]

In this situation, the idea to engineer a transgenic plant is self-suggesting [45]. Essential oil plants are distinguished by special morphological features, such as storage hairs or cavities, which provide protection to terpenoid compounds from evaporation and overoxidation. Moreover, any inhibitory effects are minimized due to the spatial compartmentalization of producing enzyme and enzyme product. A severe argument speaks against this solution: the continuing aversion of the public toward transgenic plants.

6 Outlook

Significant progress was made in recent years to characterize and apply biocatalysts for the production of the sought-after flavor compound and insecticide nootkatone [46]. However, the former molecule of the month [47] still remains a kind of "holy grail" of flavor biotechnology because the maximum yields reported for processes resulting in a product categorized as "natural" are not yet sufficient to set up an industrial process [48–51] (Table 1). Novel and more efficient enzymes and improved systems to heterologously express them are the focus of current research. The development of alternative bioengineering solutions is a neglected field. The tremendous progress achieved with simple two-phase systems [24, 43] shows that the hidden potential is far from being exhausted. New impetus for the field could come from medical research. If one of the claimed bioactivities on human metabolism, such as antiproliferative, could be verified, the intensified interest in a high-quality nootkatone would induce increased efforts for its cost-effective production through bioprocesses.

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References

1. Khasawneh MA, Xiong Y, Peralta-Cruz J, Karchesy JJ (2011) Biologically important eremophilane sesquiterpenes from Alaska cedar heartwood essential oil and their semi-synthetic derivatives. Molecules 16:4775–4785
2. Li P, Liu M, Hu J, Su W (2014) Systematic chemical profiling of *Citrus grandis* 'Tomentosa' by ultra-fast liquid chromatography/diode-array detector/quadrupole time-of-flight tandem mass spectrometry. J Pharm Biomed Anal 90:167–179
3. Li Y, Lai W, Huang S, Wang J, Wang Y, Chen F, Song W, Zhang J (2013) RP-HPLC determination of nootkatone and yakuchinone A in fruits of *Alpinia oxyphylla* at different growth phases. Yaowu Fenxi Zazhi 33:808–811
4. Tranchida PQ, Zoccali M, Bonaccorsi I, Dugo P, Mondello L, Dugo G (2013) The off-line combination of high performance liquid chromatography and comprehensive two-dimensional gas chromatography-mass spectrometry: a powerful approach for highly detailed essential oil analysis. J Chromatogr A 1305:276–284
5. Rana V, Blazquez MA (2012) Compositions of the volatile oils of *Citrus macroptera* and *C. maxima*. Nat Prod Commun 7:1371–1372
6. Hamdan D, Ashour ML, Mulyaningsih S, El-Shazly A, Wink M (2013) Chemical composition of the essential oils of variegated pink-fleshed lemon (*Citrus* × *limon* L. Burm. f.) and their anti-inflammatory and antimicrobial activities. Z Naturforsch C: J Biosci 68:275–284
7. Goldblum S, Warren CB (2014) Nootkatone in various carriers and formulations as an insecticide and insect repellent. PCT Int Appl, WO 2014031790 A1 20140227. Assignee: Allylix, Inc., USA
8. Zhao K (2013) A nootkatone-producing *Bacillus alginolyticus*. Faming Zhuanli Shenqing CN 103289923 A 20130911
9. Flor-Weiler LB, Behle RW, Stafford KC (2011) Susceptibility of four tick species, *Amblyomma americanum, Dermacentor variabilis, Ixodes scapularis*, and *Rhipicephalus sanguineus* (Acari: Ixodidae), to nootkatone from essential oil of grapefruit. J Med Entomol 48:322–326
10. Anderson JA, Coats JR (2012) Acetylcholinesterase inhibition by nootkatone and carvacrol in arthropods. Pestic Biochem Physiol 102:124–128
11. Jung IS, Seo EJ, Lee DU (2012) Nootkatone for preventing and treating cardiovascular disease. Korean Kongkae Taeho Kongbo KR 2012021545 A 20120309
12. Gliszczynska A, Lysek A, Janeczko T, Switalska M, Wietrzyk J, Wawrzenczyk C (2011) Microbial transformation of (+)-nootkatone and the antiproliferative activity of its metabolites. Bioorg Med Chem 19:2464–2469
13. Seo EJ, Lee DU, Kwak JH, Lee SM, Kim YS, Jung YS (2011) Antiplatelet effects of *Cyperus rotundus* and its component (+)-nootkatone. J Ethnopharmacol 135:48–54
14. Tsoyi K, Jang HJ, Lee YS, Kim YM, Kim HJ, Seo HG, Lee JH, Kwak JH, Lee DU, Chang KC (2011) (+)-Nootkatone and (+)-valencene from rhizomes of *Cyperus rotundus* increase survival rates in septic mice due to heme oxygenase-1 induction. J Ethnopharmacol 137:1311–1317
15. Murase T, Misawa K, Haramizu S, Minegishi Y, Hase T (2010) Nootkatone, a characteristic constituent of grapefruit, stimulates energy metabolism and prevents diet-induced obesity by activating AMPK. Amer J Physiol 299:E266–E275
16. Misawa K, Murase T (2010) Nootkatone as LkB1 activator. Jpn. Kokai Tokkyo Koho JP 2010280598 A 20101216

17. Mucha JK, Munck M, Grant KE, Karchesy J (2013) Oxidation of valencene to nootkatone with potassium permanganate (KMnO4) facilitated by crown ether phase transfer catalysis. In: Abstracts, 68th northwest regional meeting of the American Chemical Society, Corvallis, Or, United States, 21–24 July, NORM-186
18. Garcia-Cabeza AL, Marin-Barrios R, Moreno-Dorado FJ, Ortega MJ, Massanet GM, Guerra FM (2014) Allylic oxidation of alkenes catalyzed by a copper-aluminum mixed oxide. Org Lett 16:1598–1601
19. Sauer AM, Crowe WE, Henderson G, Laine RA (2009) An Efficient and Economic Asymmetric Synthesis of (+)-Nootkatone, Tetrahydronootkatone, and Derivatives. Org Lett 11:3530–3533
20. Neuenschwander U, Jensen KF (2014) Olefin autoxidation in flow. Ind Eng Chem Res 53:601–608
21. Djuris M, Stefuca V, Eghbaldar M, van den Schaft P. Edited by Blank I, Wuest M, Yeretzian C (2010) Biocatalysis based transformation of valencene into nootkatone. In: Expression of multidisciplinary flavour science, proceedings of the weurman symposium, 12th, Interlaken, Switzerland, 1–4 July 2008, pp 332–335
22. Asakawa Y, Hashimoto T, Noma Y, Furusawa M (2013) Modification of valencene by bio- and chemical transformation. Nat Prod Commun 8:859–862
23. Meng F, Yu C, Li H, Xie T (2012) Production of (+)-nootkatone from (+)-valencene by biocatalysis. Shengwu Jishu Tongbao 8:194–198
24. Girhard M, Machida K, Itoh M, Schmid RD, Arisawa A, Urlacher VB (2009) Regioselective biooxidation of (+)-valencene by recombinant E. coli expressing CYP109B1 from *Bacillus subtilis* in a two-liquid-phase system. Microb Cell Fact 8:0. doi:10.1186/1475-2859-8-36
25. Gavira C, Hofer R, Lesot A, Lambert F, Zucca J, Werck-Reichhart D (2013) Challenges and pitfalls of P450-dependent (+)-valencene bioconversion by *Saccharomyces cerevisiae*. Metab Eng 18:25–35
26. Ly TTB, Khatri Y, Zapp J, Hutter MC, Bernhardt R (2012) CYP264B1 from *Sorangium cellulosum* So ce56: a fascinating norisoprenoid and sesquiterpene hydroxylase. Appl Microbiol Biotechnol 95:123–133
27. Fraatz MA, Riemer SJL, Stoeber R, Kaspera R, Nimtz M, Berger RG, Zorn H (2009) A novel oxygenase from *Pleurotus sapidus* transforms valencene to nootkatone. J Mol Cata B: Enzym 61:202–207
28. Kruegener S, Krings U, Zorn H (2010) Berger RG (2009) A dioxygenase of *Pleurotus sapidus* transforms (+)-valencene regio-specifically to (+)-nootkatone via a stereo-specific allylic hydroperoxidation. Bioresour Technol Volume Date 101:457–462
29. Zelena K, Krings U, Berger RG (2012) Functional expression of a valencene dioxygenase from *Pleurotus sapidus* in *E. coli*. Bioresour Technol 108:231–239
30. Kelle S, Zelena K, Krings U, Linke D, Berger RG (2014) Expression of soluble recombinant lipoxygenase from *Pleurotus sapidus* in *Pichia pastoris*. Protein Expr Purif 95:233–239
31. Plagemann I, Zelena K, Arendt P, Ringel PD, Krings U, Berger RG (2013) LOXPsa1, the first recombinant lipoxygenase from a basidiomycete fungus. J Mol Cata B: Enzym 87:99–104
32. Leonhardt RH, Plagemann I, Linke D, Zelena K, Berger RG (2013) Orthologous lipoxygenases of *Pleurotus* spp.—A comparison of substrate specificity and sequence homology. J Mol Cata B: Enzym 97:189–195
33. Minussi RC, Pastore GM, Duran N (2002) Potential applications of laccase in the food industry. Trends Food Sci Technol 13:205–216
34. Huang R, Christenson PA, Labuda IM (2001) Process for the preparation of nootkatone by laccase catalysis. Google Patents, US5847226 A
35. Calcaterra A, Galli C, Gentili P (2008) Phenolic compounds as likely natural mediators of laccase: a mechanistic assessment. J Mol Cata B: Enzym 51:118–120
36. Canas AI, Camarero S (2010) Laccases and their natural mediators: Biotechnological tools for sustainable eco-friendly processes. Biotechnol Adv 28:694–705

37. Hartwig S, Frister T, Alemdar S, Li Z, Krings U, Berger RG, Scheper T, Beutel S (2014) Expression, purification and activity assay of a patchoulol synthase cDNA variant fused to thioredoxin in *Escherichia coli*. Protein Expr Purif 97:61–71
38. Gardner TS, Hawkins KM, Meadows AL, Tsong AE, Tsegaye Y (2013) Fermentation of acetyl-coenzyme A-derived isoprenoids using transgenic microorganisms. US 8415136 B1 20130409
39. Sonke T, De Jong RM (2012) Valencene synthase from *Chamaecyparis nootkatensis* with improved activity and specificity for use in converting farnesyl diphosphate to valencene. PCT Int. Appl., WO 2012177129 A2 20121227
40. Beekwilder J, van Houwelingen A, Cankar K, van Dijk ADJ, de Jong RM, Stoopen G, Bouwmeester H, Achkar J, Sonke T, Bosch D (2014) Valencene synthase from the heartwood of Nootka cypress (*Callitropsis nootkatensis*) for biotechnological production of valencene. Plant Biotechnol J 12:174–182
41. Achkar J, Sonke T. (2011) Valencene synthase from Chamaecyparis nootkatensis with improved activity and specificity for use in converting farnesyl diphosphate to valencene. PCT Int Appl WO 2011074954 A2 20110623
42. Amick JD, Park E, Julien Bryan N, Burlingame RP (2012). Modified valencene synthase polypeptides, encoding nucleic acid molecules and uses for terpene biosynthesis. PCT Int Appl WO 2012058636 A1 20120503
43. Cankar K, van Houwelingen A, Bosch D, Sonke T, Bouwmeester H, Beekwilder J (2011) A chicory cytochrome P450 mono-oxygenase CYP71AV8 for the oxidation of (+)-valencene. FEBS Lett 585:178–182
44. Wriessnegger T, Augustin P, Engleder M, Leitner E, Muller M, Kaluzna I, Schurmann M, Mink D, Zellnig G, Schwab H, Pichler H (2014) Production of the sesquiterpenoid (+)-nootkatone by metabolic engineering of *Pichia pastoris*. Metab Eng 24:18–29
45. Lange BM, Rios-Estepa R, Turner GW (2010) Production of terpenes and terpenoids in transgenic glandular trichome-bearing plants. PCT Int. Appl., WO 2010111571 A1 20100930
46. Fraatz MA, Berger RG, Zorn H (2009) Nootkatone—a biotechnological challenge. Appl Microbiol Biotechnol 83:35–41
47. Zviely M (2009) Molecule of the month: nootkatone. Perfumer Flavorist 34(20):22
48. Wilson CW, Shaw PE (1978) Synthesis of nootkatone from valencene. J Agric Food Chem 26:1430–1432
49. Muller B, Dean C, Schmidt C, Kuhn JC (1998) Process for the preparation of nootkatone. Google Patents, US5847226 A
50. Furusawa M, Hashimoto T, Noma Y, Asakawa Y (2005) Highly efficient production of nootkatone, the grapefruit aroma from valencene, by biotransformation. Chem Pharm Bull 53:1513–1514
51. Sakamaki H, Itoh K, Taniai T, Kitanaka S, Takagi Y, Chai W, Horiuchi CA (2005) Biotransformation of valencene by cultured cells of *Gynostemma pentaphyllum*. J Mol Catal B: Enzym 32(3):103–106