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Biocatalytic production of flavors and fragrances*

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Abstract: The preparation of flavors and fragrances has to be done with great care. When these compounds are meant for consumption, no toxic chemicals are allowed during their preparation. For use in non-food additives, a high purity is required. Both constraints can be easily fulfilled when enzymes are used as catalyst during the production of these compounds. This paper summarizes the work that we have done regarding the preparation of branched-chain fatty acids (sheep flavors) using lipases, the production of glucosides (controlled-release flavors) using glucosidases, the formation of terpene alcohols and grapefruit flavor using enzymes from chicory, and the formation of phenolic antioxidants using lipases.

INTRODUCTION

Nature is full of flavors and fragrances, present in many living organisms such as flowers, fruits, nuts, and animals [1]. Traditionally, the flavor and fragrance chemicals were obtained by isolation from natural sources (plants, meat), chemical synthesis, or fermentations. Nowadays, consumers' demand for the production of natural flavors has stimulated industries to use enzymes or micro-organisms for the production of these chemicals, since (in principle!) in those cases no toxic chemicals are applied, but only "Nature's own catalysts" [2].

In case compounds are used to give a certain odor to a product, one has to be sure that only the compound(s) that leads to the desired odor perception is added. This means that highly pure compounds have to be produced. Also, here biocatalysis is very beneficial since enzymes are renowned for their high selectivity and are therefore able to perform very clean conversions.

A wide range of chemicals is known to be a flavor or fragrance compound, ranging from terpenes (e.g., geraniol) to phenols (e.g., eugenol), and from aldehydes (e.g., benzaldehyde) to esters (e.g., isoamyl acetate). In addition to the diversity in chemical structures, chirality is important. A large number of flavors and fragrances are chiral, and often the enantiomers have different sensory properties [3]. Enzymes are not only regio-, but also highly stereoselective and are, therefore, the catalysts *par excellence* for the preparation of enantiopure flavors and fragrances.

In this paper, we wish to report on the enzyme-catalyzed production of a number of flavor and fragrances (derivatives), which has been carried out at the first author's laboratory. The preparation of the following compounds will be treated: enantiopure branched-chain fatty acids (BCFAs), using lipases; glucosylated alcohols as controlled-release flavors, using glucosidases; terpene alcohols and ke-

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tones, using enzymes from plants. At the end, some attention will be given to the enzymatic preparation of phenolic antioxidants using lipases, since antioxidants are also important food additives.

BRANCHED-CHAIN FATTY ACIDS

Chiral BCFAs are abundant in Nature [4]. They have been recognized as important flavor components. We focused on the kinetic resolution of 4-methyloctanoic acid since this is an essential constituent of the characteristic flavor of mutton and sheep's cheese. During initial screening studies on the transesterification of 4-methyloctanoic acid methyl ester with *n*-butanol in an organic solvent, only 5 out of 25 hydrolases in our collection were able to catalyze the reaction [5]. Of these 5, only *Candida antarctica* lipase B (CALB) gave a reasonable reaction rate and selectivity (E = 8 at 45 °C). Optimization of the reaction conditions revealed that the best system to resolve 4-methyloctanoic acid in a "food grade" way is a direct esterification with ethanol in a solventless system, at 35 °C [6], see Scheme 1.

Scheme 1 Kinetic resolution of racemic 4-methyloctanoic acid by immobilized *C. antarctica* lipase B (Novozym 435®).

The enantioselectivity of the esterification reaction increased with increasing ethanol concentration. The best results (E = 56) were obtained using an acid/ethanol ratio of 1:8. The explanation lies in the fact that water acts as a lubricant in the enzyme; when it is replaced by a polar organic solvent like ethanol, the enzyme loses some of its flexibility leading to a decreased ability to transform the slow-reacting enantiomer [7]. The rate by which the "fast" enantiomer is transformed hardly changes so the net result is an increased E-value. Various reactor concepts for the CALB-mediated enantioselective esterification of 4-methyloctanoic acid with ethanol have been evaluated, and the reaction can be easily performed on a 100 g scale [8].

The observed enantioselectivity in this reaction was much higher than anticipated. Namely, in 4-methyloctanoic acid, the chiral center is quite remote from the reaction center, which may enable facile binding of both enantiomers in different conformations in the active site, leading to a low E-value. Apparently, this is not the case, so we wondered what the enantioselectivities would be for other positional isomers of methyloctanoic acids.

For this reason, all these isomers have been synthesized and subjected to CALB-mediated esterification under the optimal reaction conditions (acid:ethanol = 1:8, 35 °C). The observed initial rates, enantioselectivities, and stereopreferences for *n*-methyloctanoic acid (n = 2–7), as well as for nonanoic acid, are listed in Table 1. The results clearly show that an increased distance between the chiral center and the reaction center does *not* necessarily lead to a decreased enantiomeric recognition by the enzyme. Furthermore, it appears that BCFAs are hindered compounds: as soon as the methyl group is placed close to the carbonyl function, the reaction rate strongly decreases. Currently, molecular modeling studies are performed to rationalize our results.

Table 1 Initial rates, stereoselectivity, and stereopreference of the CALB-mediated esterification of methyloctanoic acids with ethanol (acid: ethanol = 1:8, 35 °C).

Position of methyl group	v_{ini} (mmol min^{-1} g^{-1})	*E*-value	Enantiopreference
2	0.008	1.72	*R*
3	0.08	38	*S*
4	0.3	56	*R*
5	0.6	1.64	*S*
6	0.7	1.43	*S*
7	1.3	–	–
(8)[a]	1.8	–	–

[a]nonanoic acid

GLUCOSIDES AS CONTROLLED-RELEASE FLAVORS

Ideally, flavors in food should be present in the right concentration at or shortly before the moment of consumption. This is sometimes difficult to control since volatile flavors will evaporate from the food product, even during storage in the freezer. In those cases, it would be helpful if the flavor is present in a bound, nonvolatile form, which allows the liberation of the flavor upon heating in the oven. Such a nonvolatile derivative could be a glycoside, since glycosides have very low vapor pressures and are "natural", i.e., as long as they are prepared in a "food grade" manner. Nature makes glycosides using glycosyl transferases, but they are not very practical in synthetic chemistry [9]. We decided to make glycosides using β-glucosidases since they are cheap and readily available, from almond meal or from thermophilic Archaea [10]. The natural reaction of these enzymes is the *hydrolysis* of glucosides, but this reaction can be reversed by working at low water activity and high concentrations of glucose and alcohol. When the alcohol is water-soluble, a homogeneous system is obtained, and for these cases reasonable yields can be obtained [11]. However, for two-phase systems, the equilibrium is unfavorable, leading to a yield of only 5–8 % [12]. Therefore, we devised a system in which the product is continuously separated from the reaction mixture, see Fig. 1.

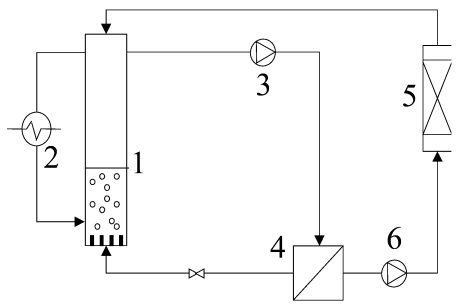


Fig. 1 Schematic presentation of a reactor for the continuous enzymatic production and downstream processing of glucosides: (1) spray column reactor, (2) water bath (50 ºC), (3) gear pump, (4) membrane module, (5) adsorption column, (6) plunger pump.

The system consists of a spray column, which provides a low shear environment for the enzyme (half-life time of almost 30 days). The continuous phase consists of the alcohol (hexanol or geraniol) in which the glucoside accumulates. The stationary, aqueous, enzyme-containing phase does not enter the downstream process due to a hydrophobic microfiltration membrane between the spray column reactor

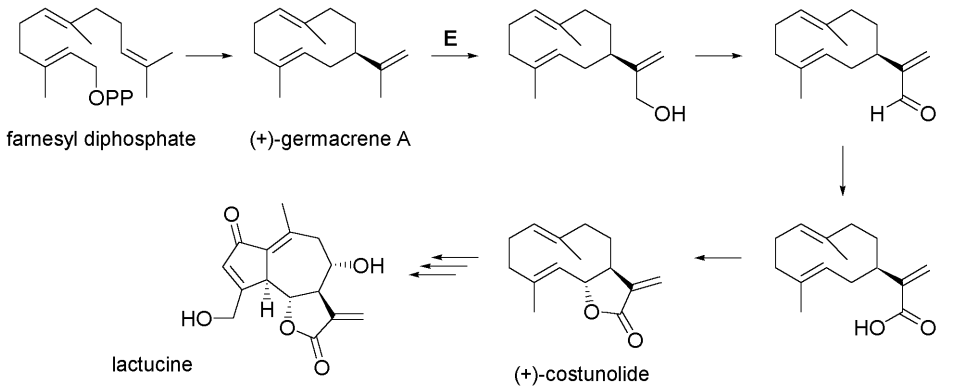
and the downstream process. The membrane allows permeation of the alcohol phase, which contains the glucoside, but retains the dispersed aqueous phase with the enzyme. An additional benefit is the retention of excess water and glucose, which interferes with the downstream process. To prevent breakthrough of water the membrane was pretreated with a block copolymer. The glucoside product is selectively adsorbed on an alumina column, while the excess of alcohol is pumped back to the column.

With this bioreactor system, 100 g of hexyl [13] and geranyl glucoside [14] have been produced using almond β-glucosidase with an initial production rate of 2.24 mg U^{-1} h^{-1} and 0.58 mg U^{-1} h^{-1}, respectively. Glucosides of phenols and enols cannot be made in this way due to the fact that the oxygen atom of these molecules is not nucleophilic enough [15].

OXIDATION OF TERPENES BY PLANT ENZYMES

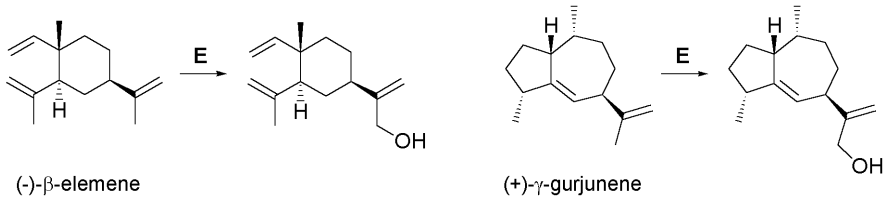
Wild chicory (*Cichorium intybus* L.) is a blue-flowered composite plant that has spread all over the world from the Mediterranean. Sprouts of chicory var. *foliosum* Hegi that are grown in the dark became popular as a vegetable (Belgian endive) halfway through the 19th century. Nowadays, it is a common crop in Belgium, northern France, and the Netherlands.

The well-known bitter taste of chicory is associated with the presence of sesquiterpene lactones of which lactucin, a guaianolide, is one of the most abundant compounds. The sesquiterpene lactones in chicory act as feeding deterrent toward insects, but may have an antifungal and antibacterial function as well. We have been studying the biosynthesis of these compounds in detail [16–18], see Scheme 2 below.



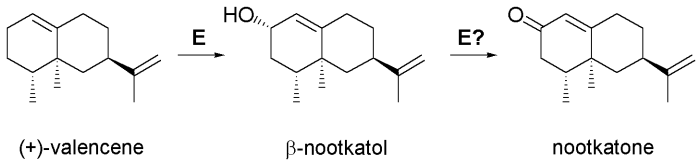
Scheme 2 Biosynthesis of lactucin, a major bitter principle in chicory. The first five steps have been proven, the conversion of costunolide to lactucin is postulated.

In our search for novel enzyme activities that can be useful to the organic chemist, we decided to study the substrate specificity of the (+)-germacrene A hydroxylase, an enzyme of the cytochrome P450 monooxygenase type. This enzyme appeared to accept a broad range of sesquiterpenes, giving exclusively hydroxylation at the isopropenyl group of the side chain [19,20]. See the examples in Scheme 3.



Scheme 3 Hydroxylation of some sesquiterpenes by the (+)-germacrene A hydroxylase from chicory roots (E), present in a 100 000 g pellet.

During the incubation of (+)-valencene, however, we did not obtain the expected valencen-12-ol, but we found a quite efficient formation of nootkatone, a compound much sought after which is responsible for the flavor of grapefruit. We could establish β-nootkatol as an intermediate (see Scheme 4). All conversions are strongly inhibited by the natural substrate (+)-germacrene A; yet, it is unclear how this enzyme could catalyze the ring hydroxylation of valencene and especially the subsequent oxidation of β-nootkatol to nootkatone. We hope to be able to study this reaction in more detail once we have the corresponding genes in our hands.



Scheme 4 Bioconversion of (+)-valencene into nootkatone via β-nootkatol, presumably catalyzed by the (+)-germacrene A hydroxylase from chicory roots (E).

PREPARATION OF PHENOLIC ANTIOXIDANTS BY LIPASES

Polyhydroxylated aromatic compounds are reported to have antioxidant properties. Antioxidants are able to inhibit oxidative reactions and thus can improve the shelf life of food products containing unsaturated fats. These kinds of antioxidants are rather water-soluble, and this hydrophilic nature might be a restriction to application in hydrophobic media. Therefore, the attachment of a fatty acid chain via an esterification reaction would make them sufficiently apolar to be used as protective agent.

In order to increase the lipophilic character of the compounds **1–6** (see Fig. 2), an esterification reaction catalyzed by immobilized lipase were carried out using different fatty acid vinyl esters as acyl donor, according to the procedure described by Nicolosi et al. [21] using *Pseudomonas cepacia* lipase adsorbed on Celite and a mixture of cyclohexane and *t*-amyl alcohol as the solvent. Care has to be taken that only one hydroxyl group is modified, since diesters are much weaker antioxidants (J. P. H. Linssen and M. C. R. Franssen, unpublished results).

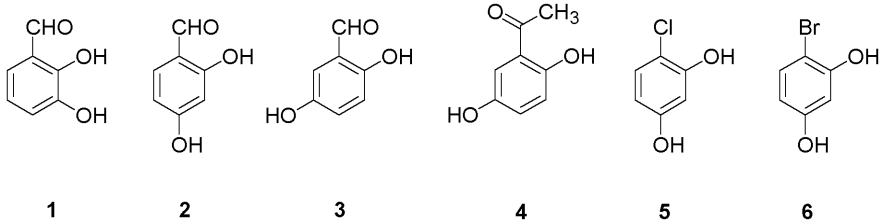


Fig. 2 Substrates for the lipase-catalyzed acylation with vinyl esters in an organic solvent.

The procedure described in [21] appeared to be exactly reproducible for vinyl acetate and was also very useful for vinyl laurate, myristate, palmitate, and stearate. The rates and yields obtained were not very different for the different chain lengths of the acyl donor. Compounds **1**–**4** always yielded the corresponding monoester, whereas **5** and **6** gave mixtures of two isomeric monoesters and a diester. We are now working on a modified system in which the solvents and the vinyl esters are replaced by "food grade" solvents and reagents.

CONCLUSIONS

In this paper, we have given several examples where the use of biocatalysts can be beneficial for the production of pure flavors, fragrances, and antioxidants that can be used as food additives without problems. The clean conversions and high yields make these reactions also very suitable for other applications. It is, therefore, to be expected that the number of biocatalytic processes in food and chemical industry will increase in the near future.

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