Novel biocatalytic strategies for the sustainable production of flavour compounds

Cumulative Dissertation

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für Opa

Abstract

Due to rising consumers' environmental and health consciousness and therewith an increasing trend towards natural flavour ingredients, the industry constantly seeks for alternative production methods to fulfil these demands. Biocatalytic strategies are hereby playing an increasingly prominent role, as they enable ecologically and economically sustainable production of flavour chemicals in line with the principles of 'Green Chemistry' and meet consumers' demand for products of natural origin.

Fatty aldehydes represent a class of highly demanded odour-active compounds contributing to the smell of many foods including fruits, herbs, and meat products, and are therefore important ingredients for respective flavourings. Moreover, they are applied in a variety of cosmetic products including highly valuable fragrances. In nature, fatty aldehydes are biosynthesised mainly from fatty acids via enzymatic cascade reactions. These involve lipoxygenases together with hydroperoxide lyases, carboxylic acid reductases or α-dioxygenases, which are key enzymes of the plant α-oxidation pathway. Recently, a cyanobacterial α-dioxygenase from *Crocosphaera subtropica* (*Cs*α-DOX) has been identified to be a highly promising candidate as biocatalyst. It enables the production of unusual odd-chain numbered, mono- or polyunsaturated fatty aldehydes from naturally abundant fatty acids in the presence of molecular oxygen as sole cofactor. In the present study, a recombinant, cyanobacterial fatty aldehyde dehydrogenase from *Vibrio harveyi* (*Vh*FALDH) has been jointly applied with *Cs*α-DOX in a coupled-enzyme reaction to produce a series of carbon chain shortened fatty aldehydes. The biocatalytic approach was systematically established and optimised to increase the production efficiency towards the desired C_{n-x} aldehydes. It was subsequently used to convert various single fatty acids as well as hydrolysed lipid extracts with unusual fatty acid profiles obtained from plant and fungal sources. The resulting aldehydes were quantified and characterised by means of gas chromatography coupled with mass spectrometry and olfactometry alongside sensory evaluations of the aldehyde mixtures. Thereby, odours of numerous (*Z*)-unsaturated fatty aldehydes have been described for the first time.

Apart from the generation of natural odourants, the field of non-volatile flavour compounds is equally in the spotlight. With the growing trend of sugar reduced foods and those enriched

with protein or amino acids, artificial sweeteners are usually applied to compensate for the lack of sugar and to mask off-flavours. However, with lately accumulating studies on health concerns regarding such sweeteners, alternatives are highly demanded. A number of *O*-methylated flavonoids, such as neohesperidin dihydrochalcone, homoeriodictyol, and hesperetin have been described as taste-active, with sweetening, bitter-masking or sweet-enhancing properties. Direct catecholic precursors of such flavonoids e.g. eriodictyol are abundantly found in nature. Thus, a suitable *O*-methyltransferase activity was sought to efficiently produce the corresponding products. The mycelia of several fungi from the phylum Basidiomycota were screened for the targeted enzyme activity, and *Lentinula edodes* (syn. shiitake) was identified as the most promising candidate with the targeted biocatalytic activity against eriodictyol [dihydrochalcone], resulting in considerable product concentrations of homoeriodictyol [dihydrochalcone] and hesperetin [dihydrochalcone]. In addition, other catecholic compounds were enzymatically converted into the corresponding *O*-methylated analogues, including industrially highly relevant flavouring substances such as vanillin. By means of a bottom-up proteomics approach, genes of *L. edodes* encoding for the responsible *O*-methyltransferases were successfully identified and functionally expressed in *Escherichia coli*. The purified enzymes were systematically characterised including reaction conditions, enzyme kinetic parameters, and activity against numerous phenolic substrates. Thereby, high product yields of up to 100% were achieved.

The present work demonstrates the successful enzymatic production of odour-active fatty aldehydes and flavour-active *O*-methylated flavonoids from natural sources. In contrast to chemical synthetic methods and the elaborate sourcing of naturally scarce substances, this represents a sustainable and environmentally friendly production strategy and is therefore of major relevance to the flavours & fragrances industry.

Zusammenfassung

Angesichts des kontinuierlich wachsenden Umwelt- und Gesundheitsbewusstseins in der Bevölkerung, sowie der damit verbundenen steigenden Nachfrage nach natürlichen Aroma- und Geschmacksstoffen werden fortlaufend alternative Produktionsmethoden erforscht, um diesen Bedarf zu decken. Biokatalytische Strategien spielen dabei eine zunehmend wichtige Rolle, da sie eine ökologisch und ökonomisch nachhaltige Produktion von geruchs- und geschmacksaktiven Verbindungen nach den Prinzipien der ‚Grünen Chemie' ermöglichen und die Nachfrage nach Produkten natürlichen Ursprungs erfüllen können.

Mittel- und langkettige (bis ~C_{17}) Fettaldehyde tragen zum Geruch vieler Lebensmittel, einschließlich Früchten, Kräutern und Fleischerzeugnissen bei und sind daher wichtige Inhaltsstoffe entsprechender Aromen. Außerdem werden sie in einer Vielzahl von kosmetischen Produkten, wie etwa hochwertigen Parfums eingesetzt. In der Natur werden Fettaldehyde hauptsächlich aus Fettsäuren über enzymatische Reaktionen u.a. mittels Lipoxygenasen, Carbonsäurereduktasen oder α-Dioxygenasen synthetisiert. Eine aus dem Cyanobakterium *Crocosphaera subtropica* stammende α-Dioxygenase (*Cs*α-DOX) wurde als vielversprechender Kandidat für die Herstellung ungeradzahliger und ungesättigter Fettaldehyde aus Fettsäuren identifiziert. In der vorliegenden Studie wurde eine Fettaldehyd-Dehydrogenase des Cyanobakteriums *Vibrio harveyi* (*Vh*FALDH) heterolog exprimiert und mit *Cs*α-DOX als gekoppelte Enzymreaktion eingesetzt, um eine Serie von kettenverkürzten Fettaldehyden zu generieren. Der biokatalytische Ansatz wurde systematisch optimiert, um die Produktionseffizienz der gewünschten C_{n-x}-Aldehyde zu erhöhen. Anschließend wurde die Enzymkaskade in Gegenwart einer Reihe einzelner Fettsäuren sowie komplexer Fettsäuremischungen aus hydrolysierten Pflanzen- und Pilzextrakten mit interessanten Fettsäureprofilen angewendet. Die Produkte wurden mittels Gaschromatographie-Massenspektrometrie gekoppelt mit Olfaktometrie sowie sensorischen Untersuchungen der Aldehydgemische charakterisiert. Dabei wurden Geruchseindrucke zahlreicher (*Z*)-ungesättigter Fettaldehyde erstmals beschrieben.

Neben der Erzeugung natürlicher Geruchsstoffe nimmt der Bereich nichtflüchtiger Geschmacksstoffe eine zunehmend bedeutende Rolle ein. Einhergehend mit dem Trend hin zu zuckerreduzierten und mit Protein oder Aminosäuren angereicherten Lebensmitteln ist es

üblich, künstliche Süßstoffe einzusetzen, um die fehlende Süße und Fehlgeschmäcker solcher Produkte auszugleichen bzw. zu maskieren. Angesichts der sich in den letzten Jahren häufenden Studien über potentiell negative gesundheitliche Auswirkungen einiger Süßstoffe, wird intensiv nach Alternativen gesucht. Einige *O*-methylierte Flavonoide wie z. B. Neohesperidin-Dihydrochalkon, Homoeriodictyol, oder Hesperetin sind geschmacksaktiv mit süßenden, bittermaskierenden oder süßmodulierenden Eigenschaften. Direkte catecholische Vorstufen solcher Flavonoide wie z. B. Eriodictyol kommen in der Natur in höheren Konzentrationen vor. Deshalb wurde in dieser Studie das Ziel verfolgt, geeignete Biokatalysatoren mit *O*-Methyltransferase-Aktivität zu finden, um entsprechende Produkte effizient auf natürlichem Wege zu generieren. Dazu wurde mittels Submerskultivierung erzeugtes Myzel verschiedener Pilze der Abteilung Basidiomycota auf die angestrebte Enzymaktivität hin untersucht. Insbesondere das Myzel des Speisepilzes *Lentinula edodes* (Shiitake) wurde als vielversprechender Kandidat mit der angestrebten biokatalytischen Aktivität gegenüber Eriodictyol[-Dihydrochalkon] identifiziert, mit der die Produkte Homoeriodictyol[-Dihydrochalkon] und Hesperetin[-Dihydrochalkon] erfolgreich generiert werden konnten. Darüber hinaus wurden weitere Verbindungen mit Catechol-Motiv enzymatisch in die entsprechenden *O*-methylierten Analoga umgesetzt, darunter auch industriell hochrelevante Aromastoffe wie z.B. Vanillin. Mit Hilfe eines *bottom-up proteomics*-Ansatzes wurden die Gene aus *L. edodes*, die für die verantwortlichen *O*-Methyltransferasen kodieren, erfolgreich identifiziert und in *Escherichia coli* funktionell exprimiert. Die gereinigten Enzyme wurden systematisch charakterisiert und die Biokatalyse einschließlich der Reaktionsbedingungen, der enzymkinetischen Parameter und der biokatalytischen Aktivität gegenüber weiteren phenolischen Substraten optimiert, wobei hohe Produktausbeuten von bis zu 100% erzielt wurden.

In der vorliegenden Arbeit wird die erfolgreiche enzymatische Produktion geruchsaktiver Fettaldehyde und geschmacksaktiver *O*-methylierter Flavonoide aus natürlichen Quellen demonstriert. Im Gegensatz zu chemisch-synthetischen Methoden und der aufwändigen Bereitstellung von natürlich nur in geringen Mengen vorkommenden Substanzen stellt dies eine nachhaltige und umweltfreundliche Produktionsstrategie dar und ist daher für die *F*&*F*-Industrie von großer Bedeutung.

List of publications

Peer-reviewed original articles

1. Kanter, J.-P.; Honold, P. J.; Lüke, D.; Heiles, S.; Spengler, B.; Fraatz, M. A.; Harms, C.; Ley, J. P.; Zorn, H.; Hammer, A. K. An enzymatic tandem reaction to produce odor-active fatty aldehydes. *Appl. Microbiol. Biotechnol.* **2022**, *106*, 6095–6107.
2. Kanter, J.-P.; Honold, P. J.; Luh, D.; Heiles, S.; Spengler, B.; Fraatz, M. A., Zorn, H.; Hammer, A. K. Biocatalytic Production of Odor-Active Fatty Aldehydes from Fungal Lipids. *J. Agric. Food Chem.* **2023**, *71*(21), 8112-8120.
3. Kanter, J.-P.; Milke, L.; Metz, J. K.; Biabani A.; Schlüter, H.; Gand, M.; Ley, J. P.; Zorn, H. Novel Catechol *O*-Methyltransferases from *Lentinula edodes* Catalyze the Generation of Taste-Active Flavonoids. *J. Agric. Food Chem.* **2024**, *72*(19), 11002-11012.

Conference contributions

Oral presentations

1. Kanter, J.-P.; Honold, P. J.; Hammer, A. K.; Harms, C.; Gross, E.; Geißler, T.; Bornscheuer, U. T.; Ley, J. P.; Zorn, H. Biotechnologische Generierung von aromaaktiven Fettaldehyden durch eine Kaskaden-Reaktion der Oxidoreduktasen α-Dioxygenase und Aldehyd-Dehydrogenase. *Lebensmittelchemie* 2021, *75*, 22. 49. Deutscher Lebensmittelchemikertag, online, **2021**.
2. Kanter, J.-P.; Hammer, A. K.; Honold, P. J.; Fraatz, M. A.; Lüke, D.; Harms, C.; Gross, E.; Bornscheuer, U. T.; Ley, J. P.; Zorn, H. An enzyme cascade system for biotechnological generation of odour-active fatty aldehydes. 11th Workshop on Fats and Oils as Renewable Feedstock for the Chemical Industry, Dortmund (Germany), **2022**.
3. Kanter, J.-P.; Hammer A. K.; Honold, P. J.; Bornscheuer, U. T.; Ley, J. P.; Fraatz, M. A.; Zorn, H. Biotechnological generation of odour-active fatty aldehydes by means of single or coupled enzyme applications. Bioflavour, Frankfurt/Main (Germany), **2022**.
4. Kanter, J.-P.; Milke, L.; Groß, E.; Harms, C.; Schwarze, E. C.; Fraatz, M. A.; Ley, J. P.; Zorn, H. GMO-free biocatalytic approach for the production of odour-active fatty aldehydes using rice seedling α-dioxygenase. Young Scientists Conference Food Biotechnology Summer School, Geisenheim (Germany), **2023**.

5. Kanter, J.-P.; Milke, L.; Ley, J. P.; Zorn, H. Biotransformation of flavonoids by fungal mycelium from edible *Lentinus* spp. to produce taste-active flavonoids with isovanilloid moiety. 13th Wartburg Symposium on Flavor Chemistry & Biology, Eisenach (Germany), **2023**
6. Kanter, J.-P.; Milke, L.; Metz, J. K.; Ley, J. P.; Zorn, H. Novel *O*-methyltransferases from Basidiomycota for the generation of flavour compounds. 17th International Weurman Flavour Research Symposium (The Netherlands), **2024**

Poster presentations

1. Kanter, J.-P.; Honold, P. J.; Hammer, A. K.; Lüke, D.; Harms, C.; Bornscheuer, U. T.; Ley, J. P.; Zorn, H. Biotechnologische Generierung von aromaaktiven Fettaldehyden mittels einer Enzymkaskade. *Lebensmittelchemie* 2022, *76*, 2-257. 50. Deutscher Lebensmittelchemikertag, Hamburg (Germany), **2022**.
2. Kanter, J.-P.; Milke, L.; Ley, J. P.; Zorn, H. Biotransformation von Flavonoiden durch Pilzmyzel aus *Lentinula edodes* zur Herstellung von geschmacksaktiven Flavonoiden mit Isovanilloyl-Motiv. *Lebensmittelchemie* 2023, *77*, 3-185. 51. Deutsche Lebensmittelchemietage, Bonn (Germany), **2023**.

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List of abbreviations

ADA	aroma dilution analysis
ADH	alcohol dehydrogenase
AEDA	aroma extract dilution analysis
AMP	adenosine 5′-monophosphate
API	active pharmaceutical ingredient
CAR	carboxylic acid reductase
CHI	chalcone isomerase
CPR	cytochrome P450 reductase
Csα-DOX	*α*-dioxygenase from *Crocosphaera subtropica*
DHS	dynamic headspace extraction
DVB	divinylbenzene
EG	ethylene glycol
ERED	enoate reductase
F3′H	flavonoid 3′-hydroxylase
FID	flame-ionisation detector
GC	gas chromatograph(y)
GC-O	GC-Olfactometry
GMO	genetically modified organism
GPCR	G-protein-coupled receptor
HIS	high-intensity sweetener
LOX	lipoxygenase
metK	*S*-adenosyl methionine synthase
MS	mass spectrometry
NDC	naringin dihydrochalcone
NHDC	neohesperidin dihydrochalcone
OAV	odour activity value
ODP	olfactory detection port
OMT	*O*-methyltransferase
OSN	olfactory sensory neuron
PA	polyacrylate
PAM	positive allosteric modulator
PDMS	polydimethylsiloxane
(a)SAFE	(automated) solvent assisted flavour evaporation
SBSE	stir bar sorptive extraction
SPE	solid-phase extraction
SPME	solid-phase microextraction
T1R2	taste 1 receptor family member 2
T1R3	taste 1 receptor family member 3
T2R	taste 2 receptor family
TRPM5	transient receptor potential protein 5
*Vh*FALDH	fatty aldehyde dehydrogenase
α-DOX	*α*-dioxygenase

1. Introduction

1.1 Flavour

Flavour has always played a central and multifaceted role in human culture. From early on, plants and other natural materials have been explored to be used as spices to enhance the palatability of foods, or were used as preservatives for such. Already the early Mesopotamian and Egyptian civilisations developed techniques to extract the flavourful fractions of materials by, for instance, liquid extraction using oils and even distillation techniques.[1] In the course of the rapidly increasing trade between the continents in the Middle Ages, the variety of herbs and spices, used for culinary and cosmetic purposes, rose accordingly. By the 17th century it had become common to apply odorous extracts to aromatise foods and drinks or scent materials such as often unpleasant smelling leather.[1] A milestone in flavour and fragrance history was the invention of chemical techniques to identify and synthesise single odorous molecules. In the course of Liebig & Wöhlers investigations on their 'Radical Theory' in the 1830s, they succeeded in isolating benzaldehyde, the most important aroma compound of *Prunus* plants such as cherry, apricot, or almond.[2] In 1833, Dumas & Péligot identified cinnamic aldehyde as the main contributor to the smell of cinnamon oil, and its first synthesis was achieved by Chiozza in 1856.[3] Industrial flavour compound production traces back to 1874 when Tiemann & Haarmann successfully synthesised vanillin from coniferin. This achievement served as the initial spark for establishing Haarmann & Reimer (now Symrise AG) later that year, with a focus on large-scale production of vanillin and other aroma chemicals.[4]

In the area of tastants, such as non-caloric sweeteners, scientific studies started relatively late and accelerated rapidly with the serendipitous discovery of saccharin by Fahlberg & Remsen in 1879.[5] As a result, other now common synthetic sweeteners such as salts of cyclamic acid (1958), neohesperidin dihydrochalcone (1963), aspartame (1981), acesulfame K (1988), and sucralose (1998) were developed during the 20th century.[6] Some plants with sweetening properties have received renewed attention within the past decade. These include stevia (*Stevia rebaudiana*), liquorice root (*Glycyrrhiza glabra*), monk fruit (*Siraita gosvernori*), katamfe (*Thaumatococcus daniellii*) and pentadiplandra (*Pentadiplandra brazzeana*), of which the compounds responsible for the sweetness have been identified and most of which are

authorised for use in foods in the European Union.[7,8] Only a few substances with a bitter taste have become established as flavourings over time. The most notable example is quinine, originally extracted from the bark of the *Cinchona* tree for the use as a remedy for malaria.[9] Quinine remains relevant in the food industry due to its use in popular soft drinks such as tonic water. However, excessive bitterness in food products naturally leads to customer rejection. Therefore, a number of taste-modifying compounds with bitter-masking or sweet-enhancing properties have been discovered over the past decades, including a range of flavanones and dihydrochalcones, which are particularly considered in the present work (see section 1.3).[10]

1.1.1 Legal framework in the European Union

In the European Union, flavour-active compounds are regulated in two different legal texts. EU Regulation (EC) No. 1334/2008 covers the categories 'flavourings', 'flavouring substances', 'flavouring preparations', 'thermal process flavourings', 'smoke flavourings', and 'food ingredients with flavouring properties'.[11] It should be noted that the term flavouring includes not only volatiles, but also non-volatile compounds in the sense of taste-active compounds, including substances with e.g. taste-modifying properties. However, substances with exclusively sweet, sour, or salty taste are explicitly excluded. For example, the bittering agent quinine, is covered by the Regulation (EC) No. 1334/2008, whereas sugar substitutes such as the earlier mentioned high-intensity sweeteners are not part of it. These are covered by the EU Regulation (EC) No. 1333/2008 on food additives.[12] A special case is the compound neohesperidin dihydrochalcone, which is included in both, Regulation (EC) No. 1334/2008 (FL No. 16.061) and Regulation (EC) No. 1333/2008 (E959). On the other hand, the structurally related compounds hesperetin, naringin dihydrochalcone, and trilobatin are only listed in Regulation (EC) No. 1334/2008 (FL No. 16.097, 16.110, 16.112) since they are considered solely as taste-modifying compounds.

In the light of sustainability and consumers' demand for natural products, the definition of natural flavourings plays an important role. Reason for this is that it determines the extent to which manufacturers are permitted to declare flavourings or products in which they are applied. According to Regulation (EC) No. 1334/2008, a 'natural flavouring substance' is a "... flavouring substance obtained by appropriate physical, enzymatic or microbiological

processes from materials of vegetable, animal or microbiological origin ...".[11] Moreover, the legislation clearly points out that the substance can only be named natural if it is present in nature. Thus, biosynthetic production of a compound alone would not be sufficient to label the resulting flavouring as natural. To give a recent example, ethyl vanillin is a widely used flavouring agent with a strong vanilla odour that has so far only been available on the market as a purely synthetic flavouring. As it does not occur naturally, it cannot be declared as such, even though it was produced by e.g. a biotechnological process. Recently, ethyl vanillin was discovered for the first time in a natural source (*Fragaria* × *ananassa*), which could now lead to it being authorised as natural, provided it was prepared by one of the methods described initially.[13]

If the compound occurs naturally and is produced by a suitable biotechnological process using genetically modified organisms (GMOs), the resulting substance can still be declared as natural.[14] However, this poses a problem as consumers are often concerned about the use of genetic engineering. As a consequence, food manufacturers are trying to meet the demand for GMO-free products. In EU Regulation (EC) No. 1829/2003 it is specified how a product that has been produced using genetic engineering must be declared.[15] This also includes flavourings. With regard to the declaration as 'genetically modified', a distinction is made between production from a GMO and with a GMO. If all or part of the flavouring is produced from a GMO, and the whole organism, or parts of it (e.g. DNA) is still detectable in the product, it must be declared accordingly.[14] Since flavourings are usually highly purified, e.g. by solvent extraction, filtration, and distillation, residues of GMOs will not enter the product and are therefore not affected by the declaration as genetically modified.[16]

1.1.2 Odour

Odour-active compounds, also known as odourants, are volatile and mainly semi- to non-polar compounds with a low molecular mass (< 300 Da) that are responsible for the smell of *inter alia* foods. The significance of a single aroma compound to the overall odour impression is mainly driven by the relationship of its concentration present in the analysed matrix and its odour threshold, commonly described as the odour activity value (OAV).[17] The compounds which contribute most to the overall odour impression are known as character impact compounds. Prominent examples are vanillin, which shapes the smell of vanilla pods,

eugenol, which imparts the typical clove-like character, or diacetyl, which is abundantly found in butter.[18–20] Although every food product contains a wide variety of odour-active compounds, only a limited set, present in a distinct proportion, creates the food's characteristic smell. For example, fresh strawberries usually get their characteristic odour profile not from 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone (strawberry, caramel-like) alone, but also from the presence of (*Z*)-3-hexenal (green, leaf-like) and a number of esters such as methyl butanoate or ethyl butanoate (fruity).[21]

1.1.2.1 Aroma analysis

In order to identify, isolate, and evaluate the responsible odour-active compounds from usually complex food matrices, reliable and sensible analytical tools are essential. Therefore, aroma analysis plays a central role in the flavour research and food industry.

The process starts with an appropriate extraction of the target compounds. A number of methods can be used for this purpose. Since odourants are usually characterised by their non- to semipolar properties, they can be isolated from their matrix using organic solvents or non-polar adsorbents. In liquid extraction, the sample for analysis is mixed with organic solvents or a combination thereof. The resulting extract can then be analysed directly or subjected to further fractionation steps. Thereby, separation from abundant polar by-products such as carbohydrates and proteins can be achieved. As the sample may also contain highly non-polar interfering substances, in particular lipids and their derivatives, modern flavour analysis makes use of the volatility of flavour compounds. By means of the solvent assisted flavour evaporation (SAFE) method, the organic extract is gently separated into volatile and non-volatile substances under vacuum at moderate temperatures. The target substances are then collected in a container placed in a bath of liquid nitrogen.[22] Although recently a semi-automated version (aSAFE) has been developed, the extraction proved very laborious and time-consuming.[23]

Since organic solvents pose the risk of unwanted chemical reactions (depending on the solvent) and rise safety and environmental concerns, a number of solvent-free methods using sorbents to extract flavours have been developed over the past decades. Thereby, compounds from the matrix selectively bind to coatings, most often non-polar polymers such as polydimethylsiloxane (PDMS), divinylbenzene (DVB) and (semi-)polar materials such as

polyacrylate (PA) and PDMS/ethylene glycol (PDMS/EG) based coatings. Frequently used methods are solid-phase extraction (SPE), stir bar sorptive extraction (SBSE), dynamic headspace extraction (DHS), and solid-phase microextraction (SPME), which offer several advantages. Apart from the absence of potentially harmful solvents, the sample can be utilised for extraction without requiring labour-intensive preparation. Additionally, volatile compounds can be selectively separated using a technique known as headspace extraction. For the headspace-SPME method, a thin, one to two centimetres long, fibre coated with a sorbent is placed in the headspace of an airtight container, and the volatile compounds are then extracted until an equilibrium of the compounds inside the sample, in the headspace, and bound to the sorbent is reached.

Following extraction, the sample can be subjected to gas chromatographic (GC) analysis, in which the extracted analytes are separated and commonly detected using a flame-ionisation detector (FID), and/or mass spectrometer (MS). By incorporating an olfactory detection port (ODP), the analytes can not only be detected as a signal in the chromatogram and identified by comparison with analytical standards or MS spectra, but the odour impression can be characterised simultaneously using the user's own sense of smell. In addition, GC-Olfactometry (GC-O) can be used to approximate the odour threshold of a compound.[24] Moreover, the impact of an analyte on the overall odour profile can be determined using a so-called aroma dilution analysis (ADA) or aroma extract dilution analysis (AEDA) through determination of the flavour-dilution factor (FD-factor) and the resulting OAV.[25–27]

1.1.2.2 Olfactory system

Although human olfaction is generally considered inferior to that of many other mammals, it remains highly sensitive and, as described in the previous section, can serve as a powerful ‘analytical tool’.[28] Commonly, the literature claims, that humans can discriminate around 10,000 different odours; however, recent studies suggest that the human sense of smell can distinguish over a trillion different olfactory stimuli.[29,30] The complexity of the olfactory system is already demonstrated by the fact, that even minor changes in molecular structure or concentration can result in a significantly different odour perception.[28,31] Odour-active compounds can be perceived either by smelling through the nostrils (ortho-nasal olfaction) or via transportation from the mouth and throat to the back of the nasal cavity. The detection

process begins at the top of the nasal cavity in the olfactory epithelium, which is composed of various cell types including sustentacular cells, basal cells, and olfactory sensory neurons (OSNs).[28] The cilia of the OSNs are situated in a mucus layer on the surface of the olfactory epithelium and carry G protein-coupled receptors (GPCRs).[31] There have been around a thousand genes identified to encode different odour receptors, however only 300–400 are functionally expressed in humans.[30] When odourants reach the epithelium through the respiratory air, they dissolve in the mucus, allowing them to bind to specific GPCRs. Each OSN expresses only a single GPCR type yet each GPCR can bind to multiple odourants with different affinities.[32,33] The bound receptor-odourant complex leads to a signal transduction cascade inducing an action potential. The electrical signal moves along the axon through the cribriform plate leading to the olfactory bulb. In the glomerular layer, axons of multiple OSNs terminate in their specific glomeruli, where the signal is transmitted to mitral cells through various olfactory bulb projection neurons and glomerular interneurons. Eventually, the signal reaches the amygdala, where odour perception is processed and integrated with emotional and behavioural responses.[32]

1.1.3 Taste

In contrast to the above-described odourants, taste-active compounds are perceived via the gustatory system in the oral cavity exhibiting sensations such as sweet, sour, salty, bitter, and umami alongside less explored taste properties such as kokumi, fatty, and metallic.[34] Also, oral somatosensory sensations such as spiciness, astringency, tickling, and lingering are usually associated with taste.[35] As this is a very broad topic, the focus here will be on the taste sensations of sweet, bitter and their modulation.

The sweetness of a compound can be determined using a numerical value, whereby a corresponding sucrose solution is rated as 1, and is therefore described as ‘relative sweetness’.[36] The monosaccharide glucose is slightly less sweet (~0.7) while fructose exhibits a higher sweetness (~1.2).[37] Sugar alcohols, also known as polyols, are sugar substitutes widely applied in confectionery and baked goods, and are often used for ‘tooth-friendly’ chewing gum and pastilles due to their decreased cariogenicity in comparison to common sugars. Most polyols have a relative sweetness lower than 1, with only xylitol being as sweet as sucrose (1.0).[37] In contrast, so-called high-intensity sweeteners (HIS) have a significantly higher relative

sweetness with values ranging from 30 for cyclamate up to 200,000 for guanidine-based compounds such as carrelame and luguname.[38] High-intensity sweeteners (HIS) are generally regarded as non-caloric because they are mostly unabsorbed by the human body. However, certain HIS, such as aspartame and thaumatin, have a calorific value because of their peptide or protein nature, respectively. Nevertheless, they are still considered as non-caloric as they are typically applied in very low concentrations.[36] Due to the unfavourable taste sensations associated with HIS, such as bitter and metallic aftertastes, or delayed sweet-taste onset, there are multiple strategies to enhance palatability. A common approach is to blend multiple HIS to make use of synergistic effects, enabling the use of lower overall compound concentrations and consequently minimising undesirable off-flavours.[36] An alternative approach involves using substances that have no taste on their own but can modify the taste perception of another compound.[39]

Bitterness in particular is a major problem for the food and pharmaceutical industries. As mentioned above, this is due to *inter alia* bitter-tasting compounds being present in raw food materials, intermediate products and, most problematically, in finished products. Current strategies to reduce the bitterness include:

- Reducing presence of bitter-tasting compounds in plant-based materials by breeding
- Selective removal of bitter tastants from the product
- Adjustment of technological processes, which are sensible to formation of bitter compounds (e.g. heat treatment of milk products)
- Physical separation within the product by encapsulation or emulsion
- Masking bitterness by increasing dosage of sweeteners, salt, or strong "sweetish" volatile flavours (vanilla, caramel etc.) to cover up bitterness
- Introduction of taste-modifying compounds directly interacting on a molecular level[39]

There are a number of compounds reported which have the ability to mask bitterness e.g. adenosine 5'-monophosphate (AMP), cyclodextrins, lipoproteins, and the HIS thaumatin and neohesperidin dihydrochalcone (NHDC).[40–44] Moreover, a number of flavonoids structurally related to NHDC were identified to mask bitterness such as homoeriodictyol and modified hydroxylated benzoic acid amides as their structural analogues.[45]

1.1.3.1 Gustatory system

Sweet, bitter, and umami taste perception is based on binding and activation of GPCRs assembled in the taste buds, which are found across the tongue papillae.[34] Human sweet taste recognition is mediated by a single heterodimeric GPCR composed of the taste 1 receptor family member 2 (T1R2) and taste 1 receptor family member 3 (T1R3).[46] Bitter taste recognition is a far more complex task due to the evolutionarily crucial defence against potentially toxic compounds. As these have a significantly greater structural diversity than sweet and umami-tasting compounds, around 25–30 different receptors of the taste 2 receptor family (T2R) take this function.[47] The signal transduction is initiated by the binding of the taste-active molecule to the corresponding GPCR, which leads to the dissociation of the G protein heterotrimer which ultimately stimulates the release of intracellular calcium, thus opening the transient receptor potential protein 5 (TRPM5) ion channels leading to membrane depolarisation and release of ATP.[48,49] The resulting neural signals are subsequently transmitted to the central nervous system via the *chorda tympani* of the facial nerve, as well as the glossopharyngeal nerve and the vagus nerve.[35]

In contrast to the activation of the above described GPCRs, there is a number of compounds known to influence bitter perception by interfering with the signalling process, which leads to the masking of the corresponding taste sensations. Such compounds belong to a group known as taste-modifiers, and in this case to the subgroup bitter-blockers.[50] There are different targets of the signalling pathway for them to interact with. The most obvious mechanism is the direct interaction as an antagonist or modulator at the T2R binding sites. However, also other parts of the signal transduction may be influenced, such as the TRPM5 channels or even the subsequent neurotransmitter release.[39]

Conversely, there is a number of compounds known to augment a taste sensation rather than preventing or reducing it. So-called positive allosteric modulators (PAMs) usually do not possess a taste of their own, but are capable of increasing another tastant's perception.[51] These compounds can bind to allosteric sites of the T1R2/T1R3 heterodimer, which causes a conformational change of the receptor and eventually leads to an increased receptor-affinity towards the agonist (sugar, sweetener) in the 'active pockets' of the receptors.[52,53] Consequently, the compounds are perceived as sweeter than their concentration would

suggest, opening up possibilities for the production of sugar-reduced foods without undesirable side effects such as loss of mouthfeel or off-tastes.

1.1.4 Biocatalytic flavour production

While pure chemical synthesis is a simple and viable process for many flavours, with high yields and good reproducibility, it inherits a number of disadvantages, including the use of energy-intensive petrochemicals, which are often toxic and environmentally harmful. Moreover, process conditions mostly involve high temperatures and high pressures. Not least, consumers' rejection of artificial flavourings makes it increasingly unattractive for manufacturers to (exclusively) produce chemo-synthetic products.[54] At the other end of the spectrum, natural flavourings are frequently produced by extraction directly from natural sources, predominantly plant materials. This can be a viable method to efficiently obtain a target compound in a cost-effective, environmentally friendly way, and allowing the flavour to be labelled as natural. Yet, this does not hold true for many flavour compounds demanded by the market. If the desired compound is not readily available in nature, if there is a lack of suitable extraction and purification methods, or if it has poor chemical stability, the processes involved are typically inefficient and therefore not considered worthwhile to pursue. Moreover, agricultural sourcing goes along with unpredictable factors such as weather conditions and plant diseases.[55] Alternatively, there are plenty of examples for naturally abundant compounds that are structurally very similar to scarcely available flavour-active compounds. Such flavour precursors can be modified chemically, which in turn goes along with the above-mentioned disadvantages of chemical synthetic products and disqualifies the product to be labelled as natural. Another major downside is the lack of selectivity, which can result in a complex mixture of structurally similar compounds or even isomers, which are challenging to purify.

In contrast, enzymatically catalysed reactions are characterised by a high degree of chemoselectivity, regioselectivity, and enantioselectivity, which enables a highly specific modification of a target substrate.[56] In addition, biocatalytic processes are usually conducted under mild reaction conditions, often in the neutral pH range and at moderate temperatures and atmospheric pressure. There are several production strategies involving biocatalytic reactions. Fermentative processes are performed by using living organisms, which harbour a

targeted set of enzymes to catalyse the desired reactions. In modern biotechnology, genetically modified microorganisms such as *Escherichia coli*, *Saccharomyces cerevisiae*, or *Komagataella phaffii* are commonly used for this purpose, as they are characterised by short doubling times and inexpensive growth media.[57] Moreover, they are convenient hosts for genetic modification as compared to higher organisms. In particular, *E. coli* is highly efficient at incorporating foreign DNA and expressing genes coding for recombinant proteins at a very high rate.[58] In fermentative processes, it is also common to simultaneously express multiple genes encoding the corresponding target enzymes. This allows for the generation of an entire enzyme cascade or artificial biosynthetic pathways to produce target compounds from e.g. readily available platform chemicals. Application of single enzymes or a combination of purified enzymes are also common practices. The benefit of this approach is that it eliminates metabolic by-products, cell compartments, and unwanted native enzyme catalytic activity from the host organism.[59] This significantly improves the recovery of the target compound and prevents it from undesirable modification or degradation. On the downside, enzymes tend to be unstable outside their natural environment and should be stored under appropriate conditions to preserve their structural integrity and catalytic function. In solution, enzymes generally remain stable only briefly, but can be stabilised by storage at low temperatures, adjusting the pH and adding stabilisers like glycerol, EDTA, sodium chloride, or antioxidants. Alternatively, enzymes can be lyophilised, encapsulated or immobilised to improve their stability.[59]

1.2 Odour-active fatty aldehydes

1.2.1 Structure and natural appearance

Fatty aldehydes belong to the structural class of aliphatic and acyclic aldehydes with a carbon chain length of typically higher than six. Representatives with a length of up to ~C_{17} are regarded as odour-active, and are frequently used and highly demanded due to their diverse odour properties and wide field of applications in flavourings and scents for the food industry, cosmetic industry and pharmaceutical industry. The odour attributes described for fatty aldehydes are manifold including green, herbaceous, waxy, soapy, citrus-like, fatty, and meaty. [60]

A structurally diverse set of odour-active fatty aldehydes are found in nature and shape the flavour of many plants, in particularly of their foliage, flowerings and fruits.[60] Citrus fruits are an important source of saturated odourous aldehydes, such as octanal, decanal and dodecanal.[61] Although fatty aldehydes only make up about 1% of the essential oil, their low odour thresholds – typically < 100 ng/L (air) – make them crucial for the aroma profile of citrus fruits.[62] In addition to the abundant saturated straight-chain aldehydes, highly sought-after unsaturated and methyl-branched aldehydes are also found in nature. A well-known example is 2-methylundecanal, which is a popular ingredient in fragrances, but occurs naturally only in small amounts in e.g. kumquat peel oil and in some meat products.[63,64] Another key flavour compound, (*E*,*E*)-2,4-decadienal, is responsible for the smell of roasted or cooked chicken and is particularly interesting due to its relatively low odour threshold of 0.04–0.16 ng/L (air).[65] It is abundant in chicken meat products, but is also reported to be present in a number of filamentous fungi.[66,67] A selection of industrially relevant fatty aldehydes and their natural occurrence is summarised in Table 1.

Table 1 Examples of fatty aldehydes used for flavourings and fragrances and their respective natural sources and odour attributes.

compound	CAS No.	natural occurrence	odour attributes
saturated (straight-chain) aldehydes			
octanal	124-13-0	*Citrus* spp.[61]	citrus-like[61]
nonanal	124-19-6	*Citrus* spp.[68]	soapy, citrus-like[61]
decanal	112-31-2	coriander[69]	green, soapy[70]
(poly)unsaturated aldehydes			
(*Z*)-4-heptenal	557-48-2	fish oil[71]	sweet, fishy[72]
(*E*,*Z*)-2,6-nonadienal	557-48-2	cucumber[73]	cucumber-like[74,75]
(*E*,*E*)-2,4-decadienal	25152-84-5	chicken meat[76], *Laetiporus* spp.[66]	fatty, deep-fried, chicken[77]
branched-chain aldehydes			
8-methylnonanal	3085-26-5	yuzu peel oil[78]	fatty, melon-like, soapy[79]
2-methylundecanal	110-41-8	kumquat peel oil[63], dry-cured ham[64]	herbaceous, citrus-like, ambergris-like[80]
12-methyltridecanal	75853-49-5	beef[81]	tallowy, stewed beef[82]

1.2.2 Biocatalytic production strategies

As mentioned earlier, the conventional method for the production of natural flavourings is direct extraction from natural sources. However, the concentrations of many desired fatty aldehydes are low, leading to cost inefficiency. Hence, biotechnological methods have emerged as a viable alternative, primarily due to the high natural abundance of fatty acids,

which serve as direct biosynthetic precursors for fatty aldehydes. Plants host numerous biosynthetic pathways that generate aldehydes as either intermediates or final products. These aldehydes play a crucial role in plant defence mechanisms against pathogens, and are involved in the signalling for attracting or repelling insects.[83] Over recent years, various biocatalytic techniques have been developed based on these biosynthetic pathways to produce fatty aldehydes (summarised in Figure 1).

One approach involves the oxidation of fatty alcohols to the respective aldehyde catalysed by alcohol dehydrogenases (ADH).[84] Due to the limited catalytic activity against alcohols with carbon chain lengths longer than six, and the scarce availability of unsaturated and branched-chain alcohols, this approach has proven unsuitable for the industrial production of the respective fatty aldehydes.[85] Due to the widespread presence of fatty acids in nature, primarily bound as triglycerides, and their ability to undergo diverse structural alterations, research has mostly focused on enzymatic processes for converting fatty acids into aldehydes.

The lipoxygenases (LOX) 9-LOX and 13-LOX in combination with hydroperoxide lyases (HPL) catalyse the generation of C_6- (green-leaf volatiles) and C_9-aldehydes from α-linoleic acid [18:2(9*Z*,12*Z*)] and α-linolenic acid [18:3(9*Z*,12*Z*,15*Z*)] in plants.[86] This enzyme combination has been successfully applied using plant tissue extracts containing the desired catalytic activities and also by heterologous expression of the enzymes and subsequent biotransformations.[87–89]

The direct reduction of fatty acids by means of carboxylic acid reductases (CAR) has been applied to convert a number of fatty acids to the respective aldehydes.[90] A CAR from *Mycobacterium marinum* has been extensively characterised and revealed a broad substrate spectrum with high catalytic activity against saturated short-, middle- and long-chain fatty acids, but also a series of ω1-unsaturated and methyl-branched fatty acids.[91–93] On the downside, the CAR-mediated reactions require costly cofactors such as ATP and NAD(P)H. Thus, a biosynthetic production strategy with a suitable cofactor recycling system is crucial for a viable industrial process.

In recent years, another group of dioxygenases found in plants has been investigated. α-Dioxygenases (α-DOX) catalyse the stereospecific peroxidation at the C_2 position (α-C-atom) of a fatty acid, resulting in an unstable (2*R*)-hydroperoxy fatty acid intermediate.[94,95] This either

reacts to a (2*R*)-hydroxy fatty acid in the presence of a peroxidase and/or spontaneously decarboxylates, producing the corresponding aldehyde, shortened by one carbon atom (C_{n-1}).[96] The main advantage in comparison to the above-mentioned enzymes is that α-DOX do not require any cofactor besides molecular oxygen. Additionally, given the scarcity of odd-chain aldehydes in nature and their high demand, the conversion of easily accessible straight-chain fatty acids presents a particularly appealing mechanism for industrial applications. α-DOX are found in many plants and have been characterised for *inter alia* peanut, cucumber, tobacco, pea, rice, and *Arabidopsis*.[97–100,96,101,102] Despite the fact that numerous biotechnological approaches utilising α-DOX worked in principle, the majority of them exhibited low catalytic activity or were not evaluated for their activity against unsaturated or branched-chain fatty acids. Apart from terrestrial plants, only one α-DOX from the algae *Ulva pertusa* was known until recently, when three α-DOX of cyanobacterial origin (*Crocosphaera subtropica*, *Calothrix parietina*, and *Leptolyngbya* sp.) have been discovered and functionally characterised.[103,60,104] Remarkably, they exhibited a wide substrate spectrum, converting not only naturally common fatty acids like palmitic acid (16:0) but also various unsaturated and methyl-branched fatty acids into C_{n-1} aldehydes such as 13-methyl-tetradecanoic acid (*a*15:0), palmitoleic acid [16:1(9*Z*)], and α-linolenic acid.

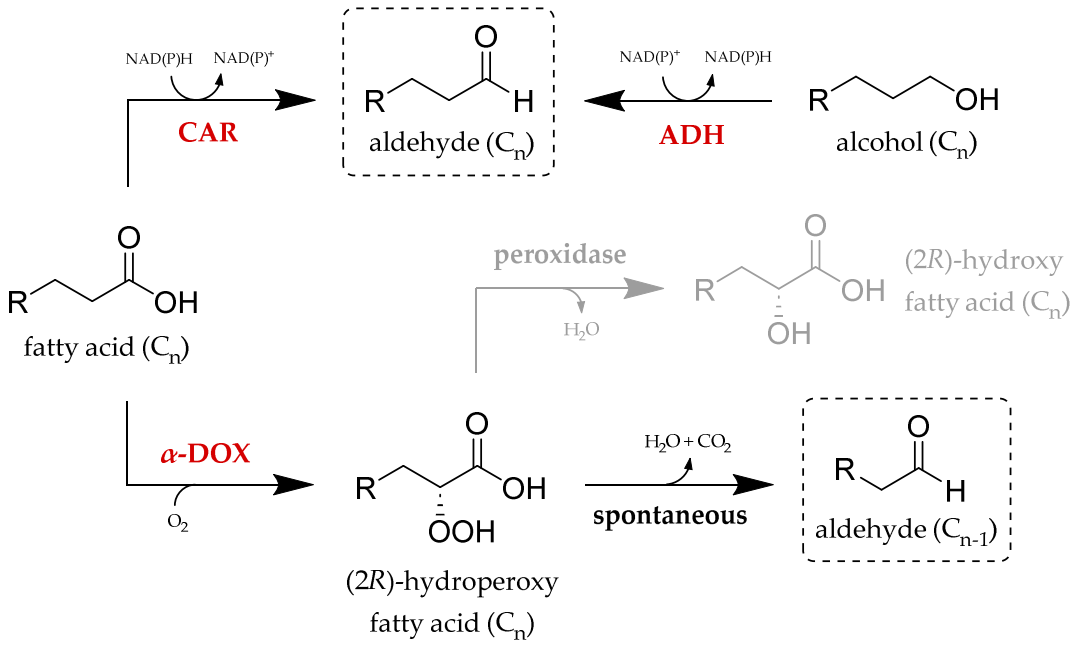

Figure 1 Examples of enzyme catalytic reactions for the synthesis of fatty aldehydes. Reduction of a fatty acid to an aldehyde by carboxylic acid reductase (CAR), oxidation of a primary alcohol to an aldehyde by alcohol dehydrogenase (ADH), and α-dioxygenase catalysed peroxidation of a fatty acid yielding a hydroperoxy fatty acid, which either oxidises to a hydroxy fatty acid or spontaneously decarboxylates resulting in the C_{n-1} aldehyde.

Convenient and easily accessible sources of fatty acids are essential to make the above discussed biocatalytic approaches an economically viable and sustainable process. Regarding (poly)unsaturated fatty acids, plant seed oils from rapeseed, sunflower or linseed are rich sources of e.g. α-linoleic acid or α-linolenic acid.[105] However, when it comes to odd-chain fatty acids or branched-chain fatty acids, there is only a limited availability among commonly used plants. Therefore, other natural sources, such as fungi, have proven to be promising alternatives. For example, several oleaginous yeasts, such as *Yarrowia lipolytica,* have been investigated and genetically modified to increase lipid contents and/or alter the fatty acid profiles.[106–108] The lipids of the filamentous fungus *Conidiobolus heterosporus* showed extraordinary high lipid contents with up to 30% of the dry biomass consisting of the unusual methyl-branched fatty acids 12-methyltridecanoic acid (*i*14:0) and 13-methyltetradecanoic acid.[92] Closely related *Mortierella* spp. have been reported to accumulate considerable amounts of the polyunsaturated fatty acids arachidonic acid [20:4(5*Z*,8*Z*,11*Z*,14*Z*)] and eicosapentaenoic acid [20:5(5*Z*,8*Z*,11*Z*,14*Z*,17*Z*)] as well as naturally uncommon odd-chain fatty acids.[109,110]

1.3 Taste-active flavonoids

1.3.1 Structure and natural appearance

Flavonoids are a diverse class of compounds widely found in nature, especially in plant-derived foods like fruits, vegetables, herbs, and grains. They belong to the class of polyphenols and serve as plant secondary metabolites. These are crucial for protecting plants against various abiotic and biotic stress factors, such as UV-irradiation, extreme temperatures, pathogens or animal predators.[111,112] In recent years, flavonoids have gained attention for their health-promoting properties, attributed to their antioxidative, anti-inflammatory, anti-mutagenic, anti-carcinogenic, and antiviral characteristics.[113] The basic carbon framework of flavonoids consists of 15 carbon atoms arranged in three rings (C_6-C_3-C_6), where the two benzene rings A and B are interconnected by a pyran heterocycle (C-ring) (Figure 2). Although the C-ring of (dihydro)chalcones is not configured as a heterocycle but as a linear carbon chain, they are classified as flavonoids since they fulfil the C_6-C_3-C_6 rule. In plants, flavonoids are mainly present as glycosides, with several mono-, di- and trisaccharides attached to the hydroxy groups, such as D-glucose, L-rhamnose, and combinations thereof.[114]

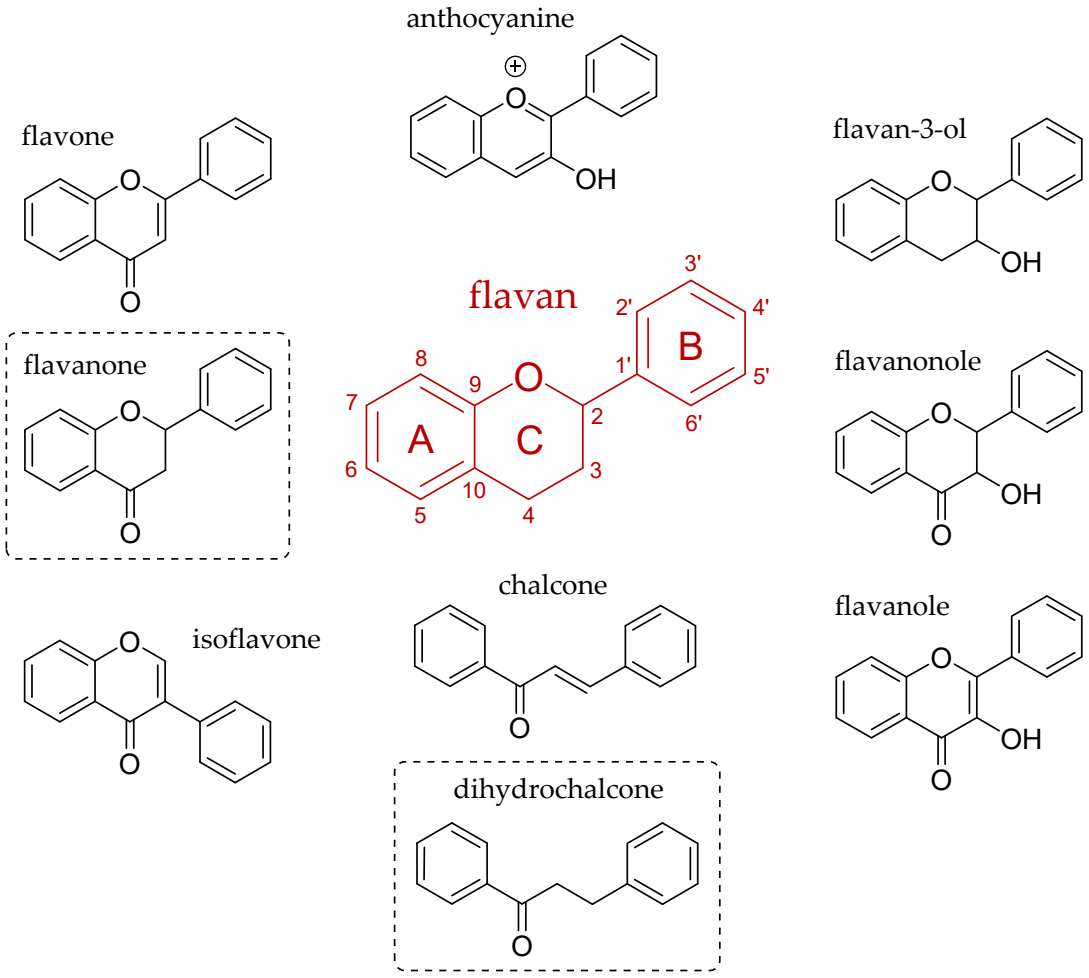

Figure 2 Basic structures of flavonoids (flavan) and naturally occurring variations. Compounds framed by dashed lines represent the most important structural classes for taste-active flavonoids.

While the majority of known flavonoids have a bitter or neutral taste, others exhibit a sweet taste or act as taste modifiers, masking bitterness or enhancing sweetness.[115] This makes them highly valuable to the food and pharmaceutical industries for improving the palatability of products, such as bitter-tasting foods or active pharmaceutical ingredients (APIs).

Long before taste-active flavonoids were identified, people were already making use of them. For example, in the 19th century, the extract from the evergreen shrub known as 'yerba santa' (*Eriodictyon californicum*), was discovered to mask the bitterness of quinine, which was commonly used as a remedy for malaria.[116] Later, the taste-modifying properties were assigned to the flavanones eriodictyol, homoeriodictyol and sterubin (Table 2).[116] In experiments utilising the pure compounds, the bitter taste of caffeine was decreased by 43% with eriodictyol, by 46% with homoeriodictyol (sodium salt), and by 20% with sterubin.[116]

Although hesperetin – a regioisomer of homoeriodictyol and sterubin – did not show a significant bitter-masking effect in this study, a later report demonstrated that it exhibits sweet-enhancing properties.[117] Supplementation of a sucrose solution with hesperetin increased the sweetness by 41%, indicating that it acts as a PAM. Neodiosmin, a closely related flavone, was among the earliest flavonoids identified for its taste-modifying properties, particularly for masking bitterness. Guadagni et al. (1979) demonstrated that neodiosmin was capable of increasing the taste threshold of caffeine by almost 80%.[118] An intensely sweet taste has also been observed for structurally more distant flavonoids, such as various acetylated dihydroflavonols.[119,120] A number of naturally occurring dihydrochalcones are known to exhibit taste-active properties. In a study reported by Ley et al. (2012), phloretin showed considerable masking properties of caffeine-induced bitterness (−30%).[121] Phlorizin, the 2′-O-glucoside of phloretin, and trilobatin are the major dihydrochalcones in *Lithocarpus litseifolius* leaves (~1.5% w/w), which are used to prepare a traditional 'sweet tea' in China.[122] In a sensory study, trilobatin revealed bitter-masking properties, reducing the bitterness of a naringin solution by 24%, but it had no effect on the bitterness of quinine.[115] Interestingly, the acetylated analogue of trilobatin, trilobatin 2′′-acetate has a sweet taste.[123] The most prominent taste-active flavonoids neohesperidin dihydrochalcone (NHDC) and naringin dihydrochalcone (NDC) are high-intensity sweeteners first synthesised in 1963 and are often described as 'semi-synthetic' sweeteners because of their minor chemical modification from the naturally occurring compounds neohesperidin and naringin.[124] Apart from its high relative sweetness (250 times as sweet as sucrose), NHDC exhibits bitter-masking properties in concentrations below its sweet taste threshold.[125,126] The closely related aglycone hesperetin dihydrochalcone is also sweet-tasting.[127]

While taste-active flavanones are particularly abundant in citrus fruits, their dihydrochalcone analogues are mainly found in the vegetative tissues, such as seeds, bark, and leaves of e.g. apple trees (*Malus* spp.).[115,128] Side streams from the citrus and apple production therefore represent valuable sources of desired taste-active compounds or precursors thereof and may contribute to a sustainable production strategy of natural flavours while fulfilling the principles of 'Green Chemistry'.

Table 2 Examples of flavonoids with taste-active properties. NHDC, neohesperidin dihydrochalcone.

compound	CAS No.	natural occurrence	flavour attributes
flavanones			
naringin	10236-47-2	grapefruit[129]	bitter[124]
eriodictyol	552-58-9	*E. californicum*[130]	bitter-masking[116]
sterubin	51857-11-5	*E. californicum*[130]	bitter-masking[116]
homoeriodictyol	446-71-9	*E. californicum*[130]	bitter-masking[116]
hesperetin	520-33-2	orange[131]	sweet-enhancing[117]
dihydrochalcones			
trilobatin	4192-90-9	*Malus* spp.[132], *L. litseifolius*[122]	bitter-masking[115]
trilobatin 2''-acetate	647853-82-5	*Lithocarpus pachyphyllus*[123]	sweet[123]
phloretin	60-82-2	*Malus* spp.[132]	bitter-masking[121]
NHDC	20702-77-6	not found in nature, precursor neohesperidin in *Citrus* spp.[133]	sweet[124], bitter-masking[125]
others			
neodiosmin	38665-01-9	bergamot[134]	bitter-masking[118]
taxifolin 3-O-acetate	78834-97-6	*Tessaria* sp.[119]	sweet[119]

1.3.2 Biocatalytic production strategies

In recent years, several approaches for the biocatalytic generation of taste-active flavonoids have emerged. The natural sources of the previously described target substances (see section 1.3.1) usually accumulate structurally similar compounds or metabolic precursors like phenylpropanoids.[115] These compounds are often present in significantly higher concentrations, and are therefore attractive substrates for the biocatalytic generation of the targeted taste-active products. Therefore, a variety of strategies, including enzymes from the flavonoid biosynthetic pathways in plants as well as from other organisms like bacteria and fungi, have proven to be promising tools, paving the way for industrial bioprocesses.[135] Thereby, genetic engineering including several mutagenesis techniques are employed to modify native enzymes, thus enhancing their catalytic activity and chemoselectivity.[136,137]

There are numerous biocatalytic strategies to obtain taste-active flavonoids, including single enzyme conversions, sequential enzyme catalyses, biotransformations, and even *de novo* generation by using artificial biosynthetic pathways. In the following, a selection of different approaches is described (summarised in Figure 3). Gall et al. (2013) characterised a chalcone isomerase (CHI) and an enoate reductase (ERED) from the gut bacterium *Eubacterium ramulus*. The heterologously expressed enzymes were capable of converting the flavanones naringenin, eriodictyol, and homoeriodictyol efficiently into their respective dihydrochalcone analogues.[138] A recent study demonstrated the generation of homoeriodictyol and hesperetin

from the phenylpropanoids ferulic acid and isoferulic acid via an engineered biosynthetic pathway by heterologous co-expression in *E. coli*.[139] In a similar manner, Hanko et al. (2023) produced homoeriodictyol and hesperetin starting from caffeic acid.[140] Thus, an additional *O*-methylation step catalysed by an *O*-methyltransferase (OMT) from *Arabidopsis thaliana* was crucial to obtain the (iso)vanilloic motifs at the B-ring of the flavonoid. In the biocatalytic approach reported by Liu et al. (2022), the sweet-enhancing hesperetin was produced from bitter-tasting naringenin.[141] Therefore, an *E. coli* consortium carrying modules for the overexpression of a flavonoid 3'-hydroxylase (F3'H) (*Gentiana trifloral*), a cytochrome P450 reductase (CPR) (*A. thaliana*), and a 4'-OMT (*Mentha × piperita*) were produced. While the conversion of naringenin to the intermediate eriodictyol was efficient, the final *O*-methylation step, catalysed by this mint OMT, proved less effective due to the OMT's limited acceptance of the catechol motif of eriodictyol. However, the yield of hesperetin was increased by co-expressing a gene encoding an *S*-adenosyl methionine synthase (metK), which ensured the availability of the methyl donor. Kunzendorf et al. (2023) recently discovered an OMT from the bacterium *Zooshikella ganghwensis* with considerable *O*-methylation activity against eriodictyol and eriodictyol dihydrochalcone, the catecholic precursors of the sweet/sweet-enhancing compounds hesperetin and hesperetin dihydrochalcone.[136] Thereby, a directed evolution approach led to an altered regioselectivity, diminishing the presence of undesired regioisomers. In another study, a rational design approach was used to genetically modify a strict 3'-selective OMT from *Mesembryanthemum crystallinum* (ice plant), shifting its catalytic regioselectivity towards the 4'-hydroxy group. This modification enabled the improved conversion of eriodictyol into hesperetin.[137] Evidently, *O*-methylation is a key step in many approaches for the generation of highly sought-after flavour-active flavonoids and is therefore of great interest to the research community and the industry.

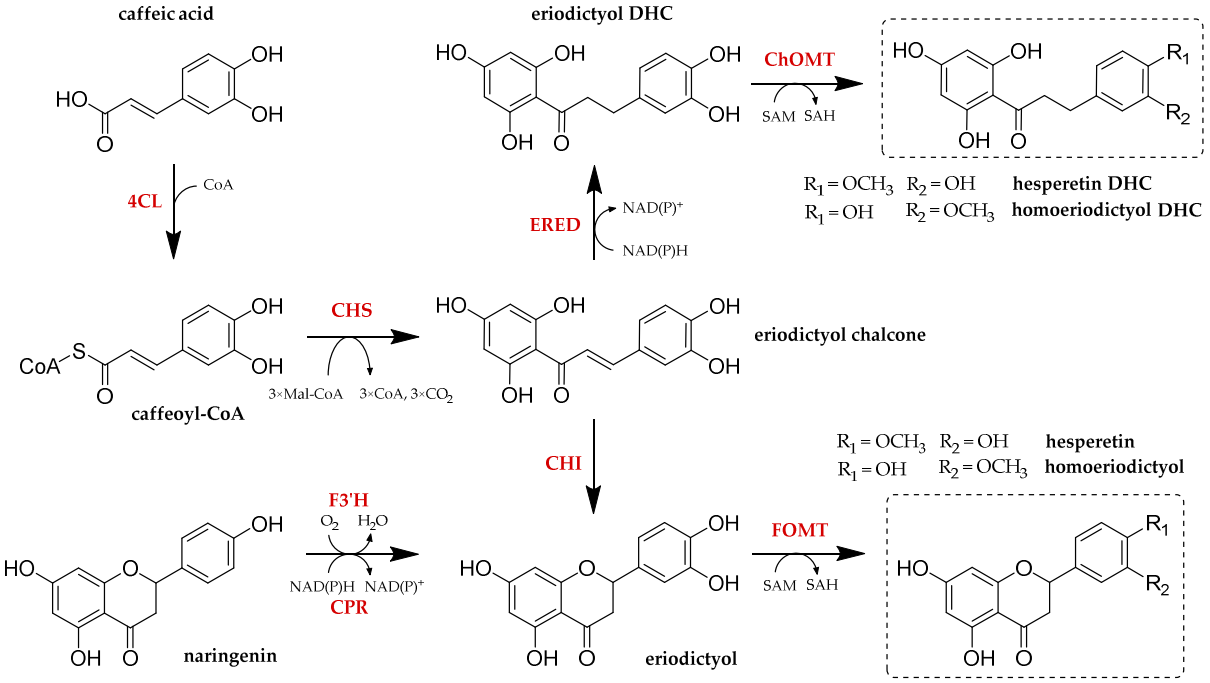

Figure 3 Biocatalytic strategies for the production of taste-active flavonoids, exemplified by the target products homoeriodictyol [dihydrochalcone] and hesperetin [dihydrochalcone] (dashed line). DHC, dihydrochalcone; CoA, coenzyme A, 4CL, 4-coumarate-CoA ligase; CHS, chalcone synthase; Mal-CoA, malonyl-CoA; F3′H, flavonoid 3′-hydroxylase; CPR, cytochrome P450 reductase; ERED, enoate reductase; CHI, chalcone isomerase; ChOMT, chalcone *O*-methyltransferase; FOMT, flavonoid *O*-methyltransferase; SAM, *S*-adenosyl-L-methionine.

2. Objectives of the study

The present work should demonstrate novel biocatalytic strategies to obtain highly demanded flavour compounds, such as odour-active fatty aldehydes and taste-active flavonoids.

To produce a series of carbon chain shortened fatty aldehydes, an enzymatic tandem reaction with an α-dioxygenase and a fatty aldehyde dehydrogenase should be established by means of a one-pot reaction and optimised to increase the yields of the aldehydes. In the following, several different medium-chain and long-chain fatty acids should be applied for the biocatalysis to evaluate the enzymes' substrate specificity and characterise odour attributes of the generated carbon chain shortened aldehydes by means of GC-Olfactometry. Apart from single fatty acids, lipid extracts of plant and fungal origin with interesting fatty acid profiles should be investigated and subsequently applied for the coupled enzyme reaction. The resulting complex fatty aldehyde mixtures should be analysed for their aldehyde yields, aldehyde distributions, and sensory characteristics.

The second part of the project should address the biocatalytic production of *O*-methylated flavonoids with taste-active properties, such as sweetening, bitter-masking, and sweet-enhancing. Therefore, a number of Basidiomycota should be cultivated submerged, and the produced mycelia should be tested for inherent *O*-methyltransferase activity against catecholic precursors to generate taste-active flavonoids with an (iso)vanilloid motif at the B-ring. The biocatalytic concept using fungal mycelia should be optimised, including fungal cultivation conditions, enzyme extraction techniques, and enzyme reaction parameters. In order to identify the enzymes responsible for the observed *O*-methyltransferase activity in *Lentinula edodes*, a bottom-up proteomics approach should be applied. The identified *O*-methyltransferases should be heterologously expressed in *E. coli* and subsequently characterised for their biochemical properties including enzyme kinetics, chemoselectivity, and regioselectivity.

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4. Coupled Enzyme Reaction to produce odour-active fatty aldehydes

An enzymatic tandem reaction to produce odor-active fatty aldehydes

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BIOTECHNOLOGICALLY RELEVANT ENZYMES AND PROTEINS

An enzymatic tandem reaction to produce odor-active fatty aldehydes

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Abstract
Aldehydes represent a versatile and favored class of flavoring substances. A biocatalytic access to odor-active aldehydes was developed by conversion of fatty acids with two enzymes of the α-dioxygenase pathway. The recombinant enzymes α-dioxygenase (α-DOX) originating from *Crocosphaera subtropica* and fatty aldehyde dehydrogenase (FALDH) from *Vibrio harveyi* were heterologously expressed in *E. coli*, purified, and applied in a coupled (tandem) repetitive reaction. The concept was optimized in terms of number of reaction cycles and production yields. Up to five cycles and aldehyde yields of up to 26% were achieved. Afterward, the approach was applied to sea buckthorn pulp oil as raw material for the enzyme catalyzed production of flavoring/fragrance ingredients based on complex aldehyde mixtures. The most abundant fatty acids in sea buckthorn pulp oil, namely palmitic, palmitoleic, oleic, and linoleic acid, were used as substrates for further biotransformation experiments. Various aldehydes were identified, semi-quantified, and sensorially characterized by means of headspace–solid phase microextraction–gas chromatography–mass spectrometry–olfactometry (HS–SPME–GC–MS–O). Structural validation of unsaturated aldehydes in terms of double-bond positions was performed by multidimensional high-resolution mass spectrometry experiments of their Paternò–Büchi (PB) photoproducts. Retention indices and odor impressions of *inter alia* (*Z*,*Z*)-5,8-tetradecadienal (*Z*,*Z*)-6,9-pentadecadienal, (*Z*)-8-pentadecenal, (*Z*)-4-tridecenal, (*Z*)-6-pentadecenal, and (*Z*)-8-heptadecenal were determined for the first time.

Key points
- *Coupled reaction of Csα-DOX and VhFALDH yields chain-shortened fatty aldehydes.*
- *Odors of several Z-unsaturated fatty aldehydes are described for the first time.*
- *Potential for industrial production of aldehyde-based odorants from natural sources.*

Keywords α-Dioxygenase (α-DOX) · Fatty aldehyde dehydrogenase (FALDH) · Biotransformation · Fatty aldehydes · Flavoring production

Introduction

Medium- and long-chain fatty aldehydes represent an important class of substances, widely applied for flavor and fragrance applications (Truong et al. 2017; Kim et al. 2022a). Saturated and unsaturated fatty aldehydes with carbon chain lengths between 11 and 18 exhibit floral, soapy, citrus-like, and waxy odors (Guadagni et al. 1963; Buttery et al. 1988). Even though fatty aldehydes are found in a wide variety of organisms, their concentrations are typically rather low and extraction from, *e.g.,* citrus peel lacks economic efficiency. Besides, due to their similar physicochemical properties, fractionation and separation of saturated and unsaturated fatty aldehydes requires costly and process-intensive

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techniques. On the other hand, chemical synthesis, as a rather traditional and convenient industrial method, increasingly fades from the spotlight due to several aspects. One of the main drawbacks is the trend toward natural ingredients caused by the rising consumer awareness for sustainability, ecology, and health issues. Apart from considerable skepticism for chemically synthesized food and cosmetic ingredients, such production methods still typically require augmented amounts of chemicals, often originating from petroleum and its derivatives (Burger et al. 2019).

In order to find alternative ways for the synthesis of fatty aldehydes, several approaches in the field of biotechnology have aroused. However, they mostly showed limited yields or restricted substrate specificity (Buchhaupt et al. 2012; Kerler et al. 2005). Among them, multiple enzymatic systems were applied. Lipoxygenase (LOX), which is commonly present in plant tissue, catalyzes the conversion of fatty acids to short- to medium-chained aldehydes. Kerler et al. (2005) applied a soy-derived LOX for biotransformation of hydrolyzed triglycerides or free fatty acids to short-chain aldehydes via hydroperoxides and thermal treatment under acidic conditions. Zhu et al. (2018) applied a multifunctional LOX from the algae *Pyropia haitanensis* expressed in *E. coli* for the production of C_5–C_9 aldehydes. Buchhaupt et al. (2012) obtained ~ 60 mg/L of C_6-aldehydes from biotransformation of fatty acids via co-expression of a recombinant LOX and hydroperoxide lyase in *Saccharomyces cerevisiae*. Another approach makes use of direct reduction of fatty acids to the corresponding aldehydes by means of carboxylic acid reductase (CAR) (Fraatz et al. 2018; Horvat and Winkler 2020; Hammer et al. 2021). The opposite reaction from alcohols to aldehydes catalyzed by alcohol dehydrogenases has been established as well (Berger 1995). Alcohol dehydrogenases are dependent on the cofactor NAD^+ and CARs require NADPH and additionally ATP. Therefore, cofactor regeneration is essential for large-scale biotechnological applications. In contrast, α-dioxygenase solely requires molecular oxygen for the catalytic α-oxidation of fatty acids. The resulting 2-hydroperoxy fatty acid either reacts to a 2-hydroxy fatty acid (C_n) or spontaneously decarboxylates, forming a C_{n-1} aldehyde (Hamberg et al. 2002; Kim et al. 2022a). Several plant-derived α-dioxygenases have been described and applied for the production of aliphatic aldehydes, *e.g.,* from cucumber (Galliard and Matthew 1976), tobacco (Kawasaki et al. 1998; Hamberg et al. 1999), rice (Koeduka et al. 2002; Kaehne et al. 2011), *Arabidopsis thaliana* (Hamberg et al. 1999; Liu et al. 2006), and algae (Kajiwara et al. 1989; Akakabe et al. 1999). More recently, α-dioxygenases were identified in the cyanobacteriae *Crocosphaera subtropica* (Hammer et al. 2020), *Calothrix parietina*, and *Leptolyngbya* sp. (Kim et al. 2022b).

The herein described α-dioxygenase reaction is naturally linked to a further oxidation of the aldehyde to the corresponding fatty acid by an aldehyde dehydrogenase (Fig. 1) (Hamberg et al. 2005). This reaction cycle was already assumed to be present in plants by Shine and Stumpf (1974) and is known to act as a defense mechanism against environmental stress and pathogen infections (Hamberg et al. 2002). The α-dioxygenase reaction needs oxygen as a co-substrate, while aldehyde dehydrogenases are usually dependent on $NAD(P)^+$ (Buchhaupt

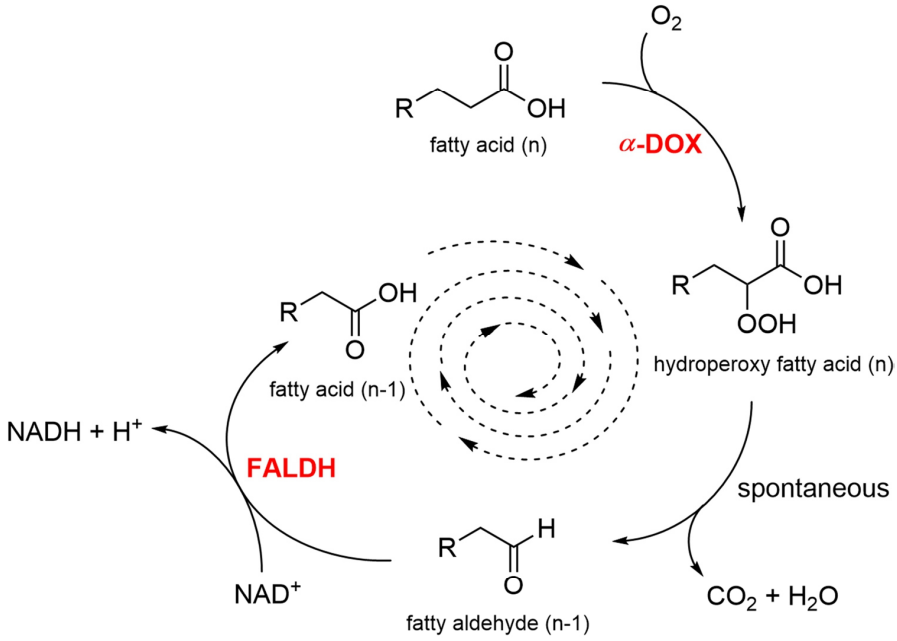

Fig. 1 Catalytic cycle yielding chain-shortened fatty aldehydes and acids by oxidative decarboxylation via α-dioxygenase (α-DOX) and successive oxidation via fatty aldehyde dehydrogenase (FALDH)

et al. 2013). In the context of industrial biotechnology, there is a high demand for readily available and inexpensive substrates to raise profitability. Thus, naturally abundant materials with valuable contents are of significant interest. For the purpose of aldehyde biosynthesis, organisms rich in lipids might prove beneficial for the generation of complex aldehyde mixtures applying the biotechnological methods described.

Sea buckthorn (*Hippophae rhamnoides*) is a deciduous shrub distributed across Eurasia (Wang et al. 2014). The lipid fraction of sea buckthorn could serve as a promising candidate for aldehyde synthesis, since the pulp and seeds of the berries are relatively rich in lipids. The seeds are reported to contain up to 15% and the pulp up to 34% lipids in dry matter (Yang and Kallio 2002). Sea buckthorn seed oil mainly consists of polyunsaturated linoleic [18:2(9Z,12Z)] and α-linolenic acid [18:3(9Z,12Z, 15Z)], while the pulp's predominant fatty acids are palmitic [16:0], palmitoleic [16:1(9Z)], oleic [18:1(9Z)], and linoleic acid [18:2(9Z,12Z)] (Yang and Kallio 2001). Sea buckthorn stands out for its high lipid contents and interesting fatty acid profile, wide distribution in nature, extreme temperature tolerance of –43 °C to + 40 °C, and drought resistance (Koskovac et al. 2017). These features make sea buckthorn oil an interesting candidate for industrial applications.

In this study, a repetitive tandem reaction of an α-dioxygenase from *Crocosphaera subtropica* (*Cs*α-DOX) (Hammer et al. 2020) in combination with a fatty aldehyde dehydrogenase from *Vibrio harveyi* (*Vh*FALDH) (Buchhaupt et al. 2013) was developed to produce odorous carbon chain shortened fatty aldehydes from the corresponding fatty acids (Fig. 1). By using a combination of these two enzymes, it was possible to produce multiple different aldehydes with carbon chains shortened by one C-atom per reaction cycle in a one-pot reaction. The enzymatic tandem reaction demonstrated in the present work was optimized to obtain higher quantities of odor-active fatty aldehydes. Application of a lipid extract from sea buckthorn was chosen as an exemplary natural substrate for the reaction cycle. Upon lipid hydrolysis, the free fatty acids serve as substrates for the generation of complex odorous aldehyde mixtures that could be used as natural flavoring/fragrance ingredients. To identify the major aldehydes formed unambiguously, analytical standards of the most predominant fatty acids of sea buckthorn pulp oil were biotransformed and sensorially characterized. Molecular structures of the generated aldehydes in terms of the double-bond positions were verified, and the efficiency and substrate specificity of the process were estimated by semi-quantitation.

Materials and methods

Chemicals

Acetone (99.8%) and palmitoleic acid [16:1(9Z)] (99%) were obtained from Acros (Fair Lawn, NJ, USA). Nitrogen was purchased from Air Liquide (Düsseldorf, Germany). Decanal (96%), dodecanal (95%), and tridecanal (90%) were supplied by Alfa Aesar (Ward Hill, MA, USA). Coomassie Brilliant Blue R250 and sodium dodecyl sulfate (99%) were purchased from AppliChem (Darmstadt, Germany). Disodium hydrogen phosphate (99.5%), glycine (99%), imidazole (99.5%), lysogeny broth (LB) medium, potassium dihydrogen phosphate (98%), tris(hydroxymethyl) aminomethane (TRIS) (99%), and Triton X-100 were obtained from Carl Roth (Karlsruhe, Germany) and *iso*-octane from Merck (Darmstadt, Germany). Helium (5.0) was supplied by Praxair (Düsseldorf, Germany). Isopropyl β-D-1-thiogalactopyranoside (IPTG) (99%) and kanamycin sulfate (> 750 I.U./mg) were purchased from Serva (Heidelberg, Germany). 3-Acetylpyridine (98%), (*Z*)-7-decenal (97%), nicotinamide adenine dinucleotide (NAD^+), oleic acid [18:1(9Z)] (99%), palmitic acid [16:0] (99%), and linoleic acid [18:1(9Z, 12Z)] (99%) were obtained from Sigma Aldrich (St. Louis, MO, USA). Heptadecanal (97%), hexadecanal (97%), (*Z*)-11-hexadecenal (95%), pentadecanal (97%), tetradecanal (95%), undecanal (97%), and decanal (97%) were purchased from TCI (Tokyo, Japan). Hydrochloric acid 25% (HCl) was obtained from Th. Geyer (Renningen, Germany). Acetonitrile (99.9%) was supplied by VWR Chemicals (Radnor, PA, USA).

Enzymes, heterologous expression, and purification

A pETDuet vector with a codon-optimized gene insert encoding for *Vh*FALDH was produced by GENEART (Regensburg, Germany) (GenBank accession number: ON677428) (Buchhaupt et al. 2013). This gene was transferred to a pET28a-vector to add an N-terminal HIS-Tag using restriction enzymes *Nde*I and *Xho*I (Thermo Scientific) and T4-Ligase (Thermo Scientific). The construct was validated by sequencing (Eurofins, Luxemburg) using a T7-primer. *E. coli* W3110 (DE3) cells were transformed with this vector. Preparation of *Cs*α-DOX was performed as described by Hammer et al. (2020) (GenBank accession number: ON711410). Both *E. coli* strains were cultivated in LB medium with 30 µg/mL kanamycin to an OD_{600} of 1.4–1.6 at 37 °C in baffled shake flasks. Induction was initiated by addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) (0.5 mM). Expression was conducted at 21 °C (*Cs*α-DOX) and 18 °C (*Vh*FALDH) overnight, after which the cells were harvested by centrifugation.

For enzyme purification, the cells were mixed with extraction buffer (25% (w/v), cell wet weight, 50 mM phosphate buffer (pH 7.5), 20 mM imidazole), and lyzed by a sonifier (Bandelin Sonopuls, Berlin, Germany). One percent of Triton X-100 was added to the buffer to enhance extraction performance. After centrifugation, the enzymes were purified by means of a nickel loaded nitrilotriacetic acid column

(Ni–NTA) (Macherey–Nagel, Düren, Germany). The His-tagged enzymes were eluted with 50 mM phosphate buffer (pH 7.5) containing 250 mM imidazole, concentrated by centrifugal filter devices (Merck, Darmstadt, Germany) with molecular mass cutoff of 30 kDa for *Vh*FALDH and 50 kDa for *Csα*-DOX, and desalted using a PD-10 column (GE Healthcare, Buckinghamshire, UK).

Enzyme concentrations were determined after purification by photospectroscopy using an Implen NanoPhotometer© P300 (Munich, Germany) with 5 µL sample volume. The specific ε values used were 34,295 L/(mol • cm) for *Vh*FALDH (MW 56,649.95) and 96,433 L/(mol • cm) for *Csα*-DOX (MW 69,894.92). The values were calculated based on the respective amino acid sequences using the ProtParam calculator of the Swiss Institute of Bioinformatics (Walker 2005).

Enzyme expression was checked by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli 1970) with 4% stacking and 12% resolving gel and Coomassie R250 staining.

Enzyme activity

To determine *Csα*-DOX activity, the consumption of oxygen in the reaction mixture was measured by use of an optical oxygen probe (Microx TX3, PreSens, Regensburg, Germany). The total reaction volume in the microtiter plate was 300 µL containing 5 mM dodecanoic acid as a fatty acid standard substrate and 10 µg/mL of *Csα*-DOX. The probe was calibrated with saturated sodium dithionite solution and with double distilled water, saturated with compressed air for at least 5 min. The measurements were performed in microtiter plates at 25 °C and 250 rpm stirrer speed every 10 s. The values from 20–120 s after addition of the enzyme solution were used for calculation. Fivefold determinations were conducted to calculate enzyme activity.

Since the reaction of *Vh*FALDH is NAD(P)$^+$-dependent, enzyme activity was measured spectrophotometrically via absorbance of NAD(P)H. The measurements were performed in microtiter plates at 340 nm and 25 °C. The total volume per well was 200 µL containing 50 µM NAD$^+$, 1 µg/mL *Vh*FALDH, and 50 µM undecanal were used. NAD$^+$ was applied instead of NADP$^+$ due to a higher enzyme activity observed in the presence of the former (supplementary Fig. S1). Triplicates were measured in each case. The extinction coefficient used was determined as 3328 L/(mol • cm). The measurement was immediately started after addition of NAD$^+$, and activity was determined within the first 60 s.

Application of the coupled enzyme reaction

Method optimization with model substrate oleic acid

Biotransformation experiments were performed in 20 mL headspace vials (Th. Geyer, Renningen, Germany) containing 5 mg oleic acid, dispersed in 2 mL phosphate buffer (50 mM, pH 7.5) by means of an ultrasonic bath (Bandelin Sonorex, Berlin, Germany) for 5 min. Two hundred µL cofactor NAD$^+$ (5 mM) and purified *Csα*-DOX and *Vh*FALDH enzyme solutions were added to a final reaction volume of 4 mL. ~ 20 glass beads (Ø 3 mm) were added to increase dispersion. Incubation was performed in a rotary shaker (40 rpm, Stuart Rotator SB3, Merck, Darmstadt, Germany) at 24 °C in the dark. Incubation time (1 h, 4 h, 8 h), enzyme ratios of *Csα*-DOX to *Vh*FALDH (4:1, 8:1, 12:1, and 16:1, where *Vh*FALDH was kept constant at a concentration corresponding to an activity of 12.5 U/L), and total enzyme activity with constant relative enzyme ratios of 8:1 (100:12.5 U/L, 50:6.25 U/L, 150:18.75 U/L) were compared. After incubation, the reaction mixtures were cooled in an ice bath and the reaction was stopped by addition of 200 µL of 4 M HCl.

Instrumental analysis was performed by means of HS–SPME–GC–MS–O. SPME extraction was executed with a polydimethylsiloxane/divinylbenzene (PDMS/DVB) coated fiber (1 cm length, 65 µm) (Merck, Darmstadt, Germany). Samples were incubated for 10 min at 60 °C and extracted for 30 min at 60 °C at 250 rpm agitation rate using a GERSTEL MPS2 XL autosampler (Mülheim/Ruhr, Germany). Analytes were desorbed in an SPME liner within the inlet of an Agilent (Waldbronn, Germany) A7890 GC system, equipped with an Agilent VF-WAXms column (30 m L × 0.25 mm ID × 0.25 µm film thickness) at 250 °C for 90 s and injected with a split ratio of 10:1. Helium (5.0) was used as a carrier gas with a constant flow rate of 1.56 mL/min. The oven was programmed with an initial temperature of 40 °C (3 min) and heating with 5 °C/min to 240 °C (12 min). Detection was performed with an Agilent 7000B triple quadrupole tandem mass spectrometer with the following parameters applied: electron ionization energy, 70 eV; ion source temp., 230 °C; scan range, *m/z* 33–300; quadrupoles temp., 150 °C. The GC system was equipped with a GERSTEL olfactory detection port ODP 3 (transfer line temp., 250 °C; mixing chamber temp., 150 °C; makeup gas, N_2).

Biotransformation of sea buckthorn oil and standard fatty acids

Sea buckthorn pulp oil (obtained from Henry Lamotte Oils GmbH, Bremen, Germany) was enzymatically hydrolyzed. Therefore, 5 mg oil was dispersed in 2 mL phosphate buffer (50 mM, pH 7.5) by means of an ultrasonic bath for 5 min. Lipase (6 U, E.C. 3.1.1.3) from *Candida rugosa* (Sigma Aldrich) was added and the mixture was incubated in a rotary shaker (40 rpm) at 24 °C for 3 h in the dark.

Besides the hydrolyzed lipid extract, standard fatty acids palmitic, palmitoleic, oleic, and linoleic acid were prepared as indicated above. Biotransformation experiments were

executed under optimized conditions. 150 U/L *Csα*-DOX and 18.75 U/L *Vh*FALDH were added to the substrate-cofactor dispersion. After incubation for 4 h, the reaction was stopped by addition of HCl as described above, and the samples were stored at –20 °C until analysis.

Compound identification and GC–olfactometry

HS–SPME–GC–MS analysis for determination of retention indices (van den Dool and Kratz 1963) and GC–MS–olfactometry were carried out as described under "Method optimization with model substrate oleic acid." Olfactometric assessment was executed by two trained assessors.

Retention indices on a nonpolar GC column were determined after SPME extraction on an Agilent 7890B GC system equipped with an Agilent DB-5 ms column (30 m L × 0.25 mm ID × 0.25 µm film thickness). The flow rate of the carrier gas helium (5.0) was set to 1.2 mL/min (constant flow). The initial temperature was held at 40 °C for 3 min, heated to 320 °C with 5 °C/min and held for 12 min. Detection was performed with an Agilent 5977B mass spectrometer (electron ionization energy, 70 eV; ion source temp., 230 °C; quadrupole temp., 150 °C).

The reaction products were identified by comparison of mass spectra to those of the NIST MS library (NIST MS Search 2011, National Institute of Standards and Technology, Gaithersburg, MD, USA) and of retention indices calculated from nonpolar (DB-5 ms) and polar (VF-WAXms) GC columns with published retention indices or analyzed authentic standards.

For determination of double-bond positions, enzymatic reaction mixtures of palmitoleic, oleic, and linoleic acid as well as incubated blanks (either without substrates or enzymes) were extracted three times with 4 mL of *n*-pentane. The extracts were concentrated under a gentle stream of nitrogen to a volume of approximately 10 µL and diluted with 500 µL of acetonitrile. Five µL of the resulting solutions was mixed with 93 µL of acetonitrile and 1 µL of acetylpyridine and 1 µL of formic acid to allow for nano-electrospray ionization–online PB functionalization–tandem mass spectrometric experiments (nanoESI–online–PB–MS/MS). Nanospray capillaries (2 µm ID, in-house pulled; P-97, Sutter Instruments, Novato, CA, USA) were loaded with 10 µL of sample. A home-built nanoESI source with a voltage of 700 V between nanospray capillary and mass spectrometer inlet was applied. The emitting sample was exposed to the light of a low-pressure mercury UV lamp (254 nm emission maximum; UVP, Upland, CA, USA) as described previously (Esch and Heiles 2018). All measurements were performed with an orbital trapping mass spectrometer in positive-ion mode (Q Exactive™ HF-X, Thermo Scientific, San Jose, CA, USA), and higher energy collisional dissociation (HCD) experiments by employing 25 to 30 normalized collision energy (NCE) values.

Semi-quantitation

Saturated aldehydes with corresponding carbon chain lengths were used for semi-quantitation of not commercially available unsaturated fatty aldehydes. As a proof of concept for this approach, signal intensities of two saturated fatty aldehydes and available authentic unsaturated counterparts were compared by calculating relative response factors (supplementary Fig. S2).

Stock solutions of analytical standards of C_{11}–C_{18} saturated aldehydes (~ 1 g/L dissolved in acetone) were diluted to a final concentration of 100 µg/L in 50 mM phosphate buffer (pH 7.5). Biotransformation samples were diluted 1:500 (linoleic acid: 1:100) in phosphate buffer (50 mM, pH 7.5) to a final volume of 4 mL and spiked with 200 µg/L (*Z*)-7-decenal as internal standard.

Instrumental analysis was performed by means of HS–SPME–GC–MS with nonpolar DB-5 ms column as stated above. Semi-quantitation of fatty aldehydes from biotransformation experiments was carried out with biological duplicates and triplicate analytical measurements.

Results

Establishment of the coupled enzyme reaction

Both enzymes were successfully expressed and purified to electrophoretic homogeneity. The enzymatic tandem reaction was successfully applied for the biotransformation of oleic acid as a model substrate. In order to increase the yield of the target aldehydes and extend the number of reaction cycles, the parameters incubation time, enzyme ratio, and concentration were optimized.

Biotransformation mixtures incubated for 1 h showed the highest signal intensity for the primary reaction product (*Z*)-8-heptadecenal. However, metabolites of further reaction cycles were less abundant in comparison with longer incubation times (Fig. 2a). While incubation for 1 h and 8 h resulted in 3 reaction cycles, however samples incubated for 4 h showed a 4th cycle exhibiting a remarkably high signal intensity for (*Z*)-5-tetradecenal and even minor amounts of (*Z*)-4-tridccenal resulting from a 5th reaction cycle. With prolonged incubation, the activity of the *Csα*-DOX decreased compared to that of *Vh*FALDH, leading to reduced aldehyde concentrations.

A *Csα*-DOX to *Vh*FALDH ratio of 8:1 (corresponding to 100:12.5 U/L) was found to be most efficient (Fig. 2b). With this ratio, 5 reaction cycles were observed, while other ratios resulted in a maximum of 4 cycles and significantly lower signal intensities of the aldehydes (*Z*)-6-pentadecenal (3rd cycle) and (*Z*)-5-tetradecenal (4th cycle). With the optimized

parameters of 8:1 enzyme ratio and 4 h incubation time, total enzyme activity was varied to investigate effects on cycle number and aldehyde yields (Fig. 2c). Reduced enzyme concentrations, corresponding to 50 U/L *Csα*-DOX and 6.25 U/L *Vh*FALDH, showed an insufficient efficacy regarding the reaction cycle numbers. Conversely, the concentration of the 1st cycle reaction product (*Z*)-7-heptadecenal was higher in comparison with higher doses applied. An increase of enzyme concentration by 50% (150:18.75 U/L) resulted in twofold abundance of 4th cycle product (*Z*)-5-tetradecenal in comparison with initial concentration.

Production of fatty aldehydes

Biotransformation of sea buckthorn oil

The biotransformation of hydrolyzed sea buckthorn oil resulted in the generation of numerous fatty aldehydes (Table 1, supplementary Fig. S3). In order to investigate the substrate spectrum in correlation to the aldehydes generated, the fatty acid profile of sea buckthorn oil was determined (Table S1). The most abundant fatty acids were palmitic, palmitoleic, and oleic acid. In smaller amounts, vaccenic, linoleic, linolenic, and stearic acid were detected. GC–MS–O analyses demonstrated that most of the aldehydes formed by biotransformation of hydrolyzed sea buckthorn oil were olfactorily perceived with manifold odor impressions (Table 1). The most abundant odor attributes were soapy, waxy, and green. Furthermore, citrus-like, metallic, and fatty odors were detected. Upon biotransformation, a total yield of ~ 203 mg aldehydes per gram employed oil was obtained. Pentadecanal and (*Z*)-8-pentadecenal were the most abundant fatty aldehydes, followed by (*Z*)-8-heptadecenal, tetradecanal, and (*Z*)-7-tetradecenal.

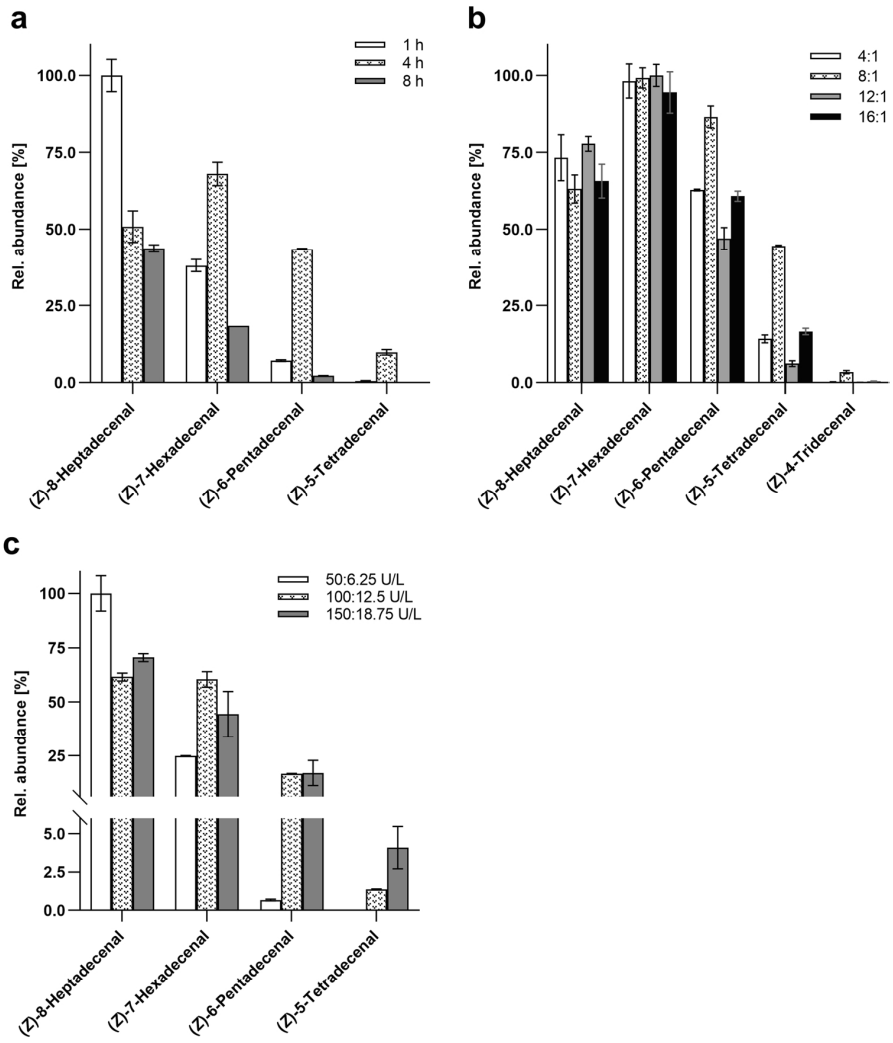

Fig. 2 Optimization of biotransformation: **a** Comparison of incubation times, **b** enzyme unit ratios *Csα*-DOX:*Vh*FALDH [U/L] (4:1 = 50:12.5, 8:1 = 100:12.5, 12:1 = 150:12.5, 16:1 = 200:12.5), **c** total enzyme activity concentrations. Error bars indicate standard deviations

Table 1 Products of the biotransformation of hydrolyzed sea buckthorn oil with retention indices, odor impressions, and approximated yields (mg aldehyde per g lipid extract). Retention indices in parentheses are calculated from aldehydes obtained upon biotransformation of single fatty acids (cf. Tables 2, 3, 4, and 5) or from literature, whenever a reference is cited. Errors are given as standard deviations. References: a — Choi (2005), b — Choi (2006), c — Eyres et al. (2005), d — Chisholm et al. (2003), e — Marques et al. (2000), f — Miyazawa et al. (2007), g — Kajiwara et al. (1989), h — Hamberg et al. (1999)

Retention index		Compound	Odor impression		Probable precursor [number of cycles]	Yield [mg/g]
DB-5 ms	VF-WAXms		GC–O	Literature		
1408 (1408)	1703 (1705)	dodecanal	green, citrus, waxy	green[a], citronellol-like[c]	16:0 [4]	0.4 ± <0.1
1510 (1511)	1810 (1811)	tridecanal	green, waxy, soapy, textile	fresh, green[a]	16:0 [3]	1.9 ± 0.3
1491 (1491)	1843 (1842)	(*Z*)-6-tridecenal	soapy, waxy	n.r	16:1(9*Z*) [3]	0.2 ± 0.0
1614 (1613)	1917 (1919)	tetradecanal	green, soapy, floral, citrus	fresh, herbaceous[a]	16:0 [2]	10.6 ± 1.6
1593 (1591)	1947 (1948)	(*Z*)-5/**7**-tetradecenal*	soapy, fresh, green, grapefruit	(5*Z*): soapy, green[d]	18:1(9*Z*) [4]/ **16:1(9*Z*) [2]**/ 18:1(11*Z*) [4]	7 ± 1
1722 (1717)	2025 (2024)	pentadecanal	soapy, waxy, brothy	pungent, spicy, woody[c]	16:0 [1]	35.8 ± 5.2
1697 (1693)	2052 (2053)	(*Z*)-6/**8**-pentadecenal*	green, herbaceous, metallic	n.r	**16:1(9*Z*) [1]**/ 18:1(11*Z*) [3]	21 ± 2
1793 (1792)	2139 (2139)	(*Z*)-7-hexadecenal		n.r	18:1(9*Z*) [2]	2 ± 0
1795 (1800)[e]	2142 (2147)[e]	(*Z*)-9-hexadecenal		n.r	18:1(11*Z*) [2]	1 ± 0
1785 (1785)	2220 (2225)	(*Z*,*Z*)-7,10-hexadecadienal	waxy, fatty	n.r	18:2(9*Z*,12*Z*) [2]	0.7 ± 0.1
1918 (1919)[f]	2234 (2221)[f]	heptadecanal		sweet[b]	18:0 [1]	2.3 ± 0.4
1897 (1894)	2240 (2238)	(*Z*)-8-heptadecenal		n.r	18:1(9*Z*) [1]	83 ± 6
1900	2242	(*Z*)-10-heptadecenal		n.r	18:1(11*Z*) [1]	27 ± 3
1886 (1887)	2330 (2330)	(*Z*,*Z*)-8,11-heptadecadienal	fatty, brothy, tallow	green, algae-like[g]	18:2(9*Z*,12*Z*) [1]	10 ± 1
n.d (1895)[f]	2373 (2358)[f]	(*Z*,*Z*,*Z*)-8,11,14-heptadecatrienal	fatty, waxy	seaweed[h]	18:2(9*Z*,12*Z*,15*Z*) [1]	1 ± 0

n.r. — not reported, n.d — not detected

Unsaturated compounds were semi-quantified using authentic saturated homologues (cf. supplementary Fig. S2 for general validation of this procedure). Results for these compounds are therefore only given without decimal digits, if larger than 1

* Co-eluting compounds. Double-bond position marked in **bold** is considered as expected main constituent/precursor

Biotransformation of single fatty acids

GC–MS–O analyses of biotransformed standard fatty acids revealed a wide variety of odor impressions within the formed fatty aldehydes (Table 2, 3, 4 and 5). Odors described as soapy, waxy, and green were most abundant and not clearly limited to a specific structural feature. However, a tendency of fatty odor perception with increase in chain length could be observed. Results of semi-quantitation (Fig. 3) clearly demonstrated a substrate specificity toward saturated fatty acid palmitic acid, which showed the highest total yield with roughly 430 mg aldehyde per gram substrate. The biotransformation of unsaturated fatty acids resulted in significantly lower yields, with oleic acid yielding the highest aldehyde concentrations of ~ 260 mg/g, followed by palmitoleic acid with ~ 67 mg/g. The conversion of polyunsaturated linoleic acid was even lower with a yield of ~ 30 mg/g.

Compound identification

Fatty aldehydes formed during biotransformation via the proposed enzyme tandem reaction were tentatively identified by means of MS data and retention indices, calculated from analyses on polar and nonpolar GC columns and comparison to published data. As several compounds had not been reported previously, structure elucidation was performed via

Table 2 Products of the biotransformation of palmitic acid [16:0] with retention indices, odor impressions, and yields. Retention indices in parentheses are from cited literature. Errors are given as standard deviations. References: a — Chisholm et al. (2003); b — Mahattanatawee et al. (2005); c — Choi (2005); d — Sukhonthara et al. (2009); e — Eyres et al. (2005)

Retention index		Compound	Odor impression		Yield [mg/g]
DB-5 ms	VF-WAXms		GC–O	Literature	
1307 (1307)[d]	1598 (1610)[a]	undecanal	citrus, soapy, fresh, metallic	citrus[a], fruity, floral, spicy[e]	0.3 ± 0.0
1408 (1401)[c]	1705 (1718)[c]	dodecanal	waxy, soapy, green, coriander	herbaceous, waxy[a], soapy[b], green[c], pungent, spicy, floral, citronellol-like[e]	2.4 ± 0.4
1511 (1503)[c]	1811 (1824)[c]	tridecanal	green, soapy, grapefruit	fresh, green[c]	8.9 ± 1.2
1613 (1613)[d]	1919 (1924)[c]	tetradecanal	soapy, fatty, metallic	fresh, herbaceous[c]	100.7 ± 12.2
1717 (1710)[d]	2024 (2030)[d]	pentadecanal	waxy	pungent, spicy, woody[e]	315.9 ± 41.3

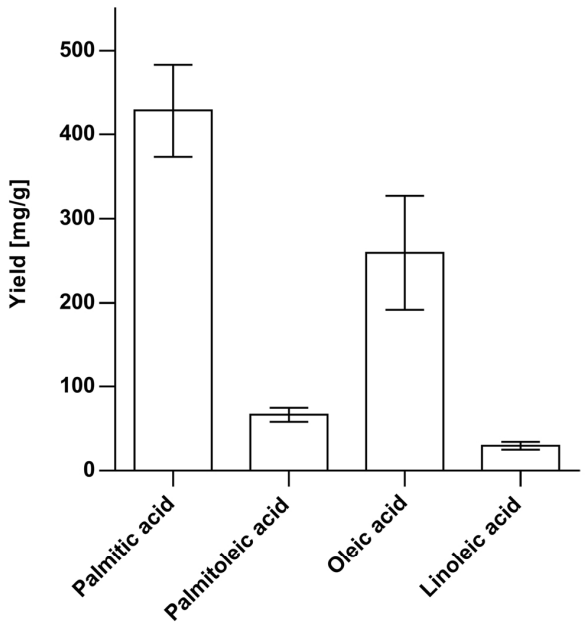

Fig. 3 Yield of aldehydes per gram substrate supplemented for biotransformation experiments with *Cs*α-DOX and *Vh*FALDH. Error bars indicate standard deviations

PB reaction (Fig. 4). The observed MS signals indicated the successful generation of adducts of 3-acetylpyridine and a series of expected unsaturated fatty acids and aldehydes for all three samples. The reaction mixture of oleic acid showed *m/z* values of 404, 390, 376, 374, 362, 360, 348, 346, 334, 332, and 318. For instance, *m/z* 374 resulted from the two isomeric oxetanes (PB products) of heptadecanal and 3-acetylpyridine. Retro-PB reaction, initiated during HCD experiments, revealed the double-bond position between C_8 and C_9, as characteristic fragments of *m/z* 232.1696 (α-ion) and 232.2060 (ω-ion) were detected. On the other hand, PB adducts with *m/z* 376, 362, 348, 346, 334, 332, 320, 318, and 304 were detected in the palmitoleic acid, *m/z* 402, 388, 374, 372, 360, 358, 346, 344, and 330 in the linoleic acid sample. Diagnostic fragmentation signals of all PB products are reported in supplementary Tables S2–S4. While ω-ions were always detectable, signals of α-ions were found only in some cases. Apart from not transformed substrate acids, blanks contained no or more than 1000-fold lower signal intensities of the relevant *m/z* values.

Discussion

In the current study, a biotechnological approach yielding numerous targeted odor-active fatty aldehydes with promising efficiency was developed. Enzyme activity and resulting coupled reaction of *Cs*α-DOX and *Vh*FALDH were shown to be well suitable for the efficient production of targeted fatty aldehydes from various long-chain fatty acids.

The efficacy of fatty aldehyde generation was optimized by varying reaction parameters by use of oleic acid as a model substrate. One of the main challenges when dealing with coupled enzyme reactions in a one-pot bioprocess is to assure the *quasi* co-working of enzymes in order to maximize the yield of target compounds. Since enzyme activities of the applied enzymes are likely to differ from each other over time, the generation of aldehydes was investigated for varying incubation times. The highest signal intensities were obtained after 4 h. Shorter incubation resulted in the highest concentration of the C_{n-1} aldehyde (*Z*)-7-heptadecenal, whereas product yields of the following reaction cycles were considerably lower. For targeted applications in industrial processes, the incubation time might serve as powerful tool for controlling the product pattern, and thus overall odor impression of the resulting aldehyde

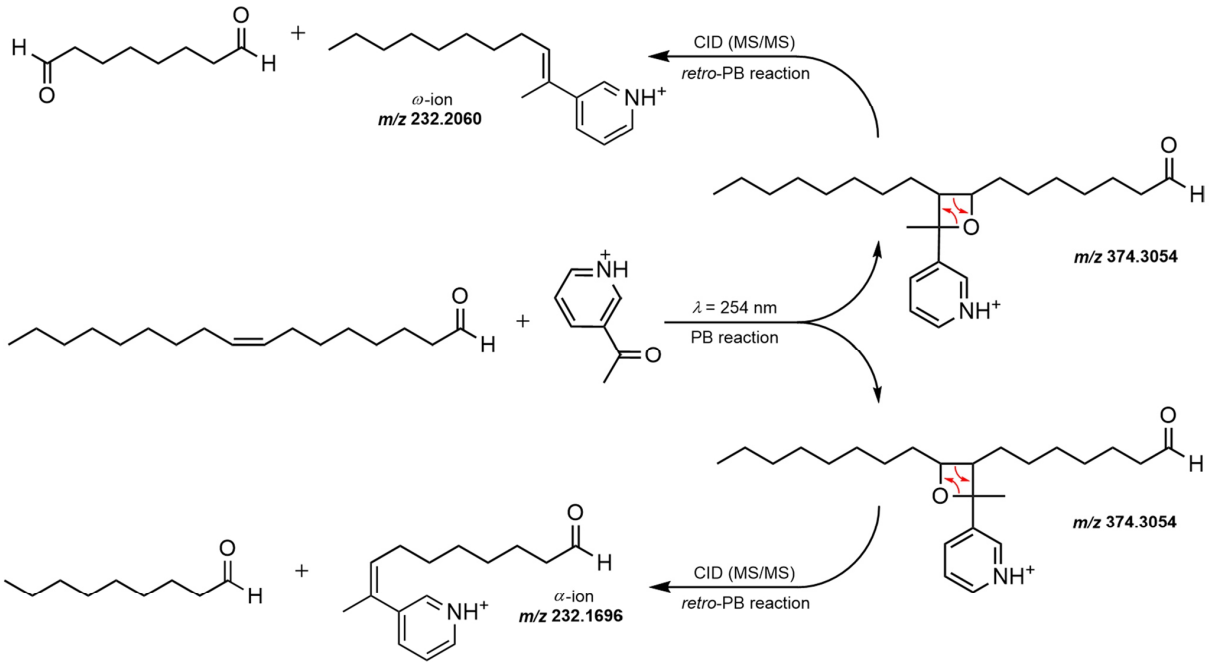

Fig. 4 Exemplary PB reaction, of the analyte (*Z*)-8-heptadecenal with 3-acetylpyridine. HCD experiments initiated fragmentation of PB-products yielding diagnostic α- and ω-ions confirming the double-bond position of the aldehyde between C_8 and C_9

mixture. However, with prolonged incubation times of more than 4 h, a decrease of total aldehyde yield was observed. A lower enzyme stability of *Csα*-DOX might well explain this observation. As a result, an excess *Vh*FALDH activity catalyzes the oxidation of aldehydes as the terminal reaction step. The optimal enzyme activity ratio of *Csα*-DOX and *Vh*FALDH was thus crucial for the purpose of fatty aldehyde generation. Throughout biotransformation, *Csα*-DOX should exceed *Vh*FALDH activity to avoid augmented oxidation of aldehydes to acids. An activity ratio of 8:1 (*Csα*-DOX:*Vh*FALDH) was identified as most suitable for generating an excess of fatty aldehydes. The effect of total enzyme concentration in the reaction mixture on aldehyde production is an important parameter, especially when it comes to bioprocess upscaling to an industrial scale. Hence, varying total enzyme concentrations were applied for biotransformation trials. The results of laboratory scale experiments revealed that increased enzyme concentrations resulted in higher aldehyde yields, thus no needless excess of enzyme introduction was observed. Nevertheless, results indicated that varying enzyme concentrations could serve as a tool for regulation of aldehyde distribution in the resulting reaction mixture, *e.g.,* if C_{n-1} aldehyde was aimed as predominant target compound, a lower enzyme concentration would be most suitable.

The optimized enzymatic tandem reaction was successfully applied for biotransformation of a hydrolyzed lipid extract from sea buckthorn pulp and single aliphatic fatty acids yielding odor-active fatty aldehydes. The fatty acid profile of sea buckthorn oil was in accordance with published data (Yang and Kallio 2001; Zielińska and Nowak 2017). The predominant fatty acids palmitoleic, oleic, and linoleic acid are of particular interest, since their chain-shortened fatty aldehydes produced via the coupled enzyme reaction have been scarcely described as aroma ingredients and their chemical synthesis is highly complex. GC–MS–O analyses revealed most of them being odor-active, exhibiting even auspicious smells. Most of the *Z*-unsaturated aldehydes have not yet been described in terms of their odorant properties.

Biotransformation of the most abundant fatty acids present in sea buckthorn pulp oil indicated a clear substrate preference toward the saturated fatty acid palmitic acid in comparison with unsaturated substrates. Earlier studies on the substrate specificity of the well described α-dioxygenase from *Oryza sativa* (*Osα*-DOX) did not show a clear preference for palmitic acid compared to unsaturated oleic or palmitoleic acids (Koszelak-Rosenblum et al. 2008). Furthermore, investigations on *Arabidopsis thaliana* α-dioxygenase (*Athα*-DOX) even showed opposite results with substrate preference toward unsaturated oleic and palmitoleic acid compared to saturated stearic and palmitic acid (Liu et al. 2006; Koszelak-Rosenblum et al. 2008). Surprisingly, the biotransformation of the polyunsaturated linoleic acid

resulted in lowest yields of aldehydes. This likely results from the steric hindrance caused by the two *Z*-configured double bonds, which might prevent a proper docking of the ligand into the active site. HOwever, this seems to be quite specific for the herein applied *Csα*-DOX since earlier studies on *α*-dioxygenases of various other organisms showed a high acceptance toward linoleic acid (Kajiwara et al. 1989; Koeduka et al. 2002; Koszelak-Rosenblum et al. 2008; Bannenberg et al. 2009). Recently, a study on the substrate specificity of a cyanobacterial *α*-dioxygenase (Kim et al. 2022b) showed results that are in good agreement with our findings. Thus, low substrate acceptance for polyunsaturated fatty acids is possibly a cyanobacteria-specific trait. Interestingly, palmitoleic acid gave lower aldehyde yields in comparison with oleic acid even though the double bond of both is located at C_9. The coupled enzyme reaction terminated when reaching the cycle with products exhibiting a double bond at C_5. Nevertheless, for oleic acid a 5th reaction cycle, yielding a product with the unsaturated bond at C_4, could be observed. The termination at these positions could be a result of steric hindrance within the binding site of the enzymes. This would be in accordance with findings that indicated a drastic decrease of *Osα*-DOX activity for substrates with unsaturated bonds within the first 6 carbon atoms (Koszelak-Rosenblum et al. 2008). Further investigations suggest that the first 7 carbon atoms are pivotal for substrate binding within the active site of *Osα*-DOX and related *Athα*-DOX, and therefore for an efficient catalysis (Goulah et al. 2013).

To verify the structural properties of reaction products, mass spectrometric fragmentation analyses of PB photoproducts of unsaturated compounds enabled the determination of their double-bond positions. This targeted approach renders elaborate purification procedures unnecessary and allows for an investigation of different compounds in parallel. Typically, it is employed for the characterization of different types of lipids (Esch and Heiles 2018; Wäldchen et al. 2019), but was already applied for structure elucidation in flavor research as well (Birk et al. 2019). By means of nanoESI–online–PB–MS/MS experiments, unsaturated positions of all fatty aldehydes, assigned in (Tables 3, 4, and 5), and their intermediate fatty acids were verified (see Supplementary Tables S2–S4 for assignments of all *m/z* signals and fragmentation). While the applied SPME–GC–MS technique was well suitable for the analysis of volatile fatty aldehydes, the corresponding fatty acids could not be analyzed simultaneously due to their high boiling points as well as inefficient desorption resulting in persistent memory effects on the SPME fibers. Thus, PB-MS/MS was also employed to unambiguously prove the presence of fatty acids.

In the literature, *Z*-unsaturated fatty aldehydes are predominantly discussed in the context of insect pheromones (Swedenborg and Jones 1992; Teal et al. 1995; Silva et al. 2016; Becher et al. 2018) and to a lesser extent in the field of flavors and fragrances (Shi et al. 2013; Lorber et al. 2018). Odor impressions of numerous unsaturated fatty aldehydes like (*Z*)-6-tridecenal, (*Z*)-7-tetradecenal, and (*Z*)-8-heptadecenal or dienals like (*Z,Z*)-5,8-tetradecadienal, (*Z,Z*)-6,9-pentadecadienal, and (*Z,Z*)-7,10-hexadecadienal are described here for the first time (Tables 3, 4, and 5). Only few of the produced aldehydes have already been sensorily characterized (Kajiwara et al. 1989; Wakamura et al. 1999; Chisholm et al. 2003; Choi 2005; Eyres et al. 2005). The majority of the aldehydes showed pleasant odor impressions with fresh, green, and soapy notes, which are of great interest to the flavor and fragrance industry. By subjecting naturally occurring oils and fats—such as the herein applied buckthorn-derived oil—to the developed biotechnological approach, the production of appealing aroma mixtures seems possible. Moreover, this approach includes solely NAD^+ as a cofactor, which is relatively inexpensive, in comparison with cofactors such as NADH or NADPH. However, addition of stoichiometric amounts of cofactors to the reaction lacks economic efficiency. Hence, to increase the total turnover number, a suitable cofactor recycling system would be crucial for an efficient large-scale production approach. Therefore, the introduction of a NADH oxidase (E.C 1.6.99), *e.g.*, isolated from *Lactobacillus brevis* as proposed by Geueke

Table 3 Products of the biotransformation of palmitoleic acid [16:1(9*Z*)] with retention indices, odor impressions, and approximated yields. Retention indices in parentheses are from cited literature. Errors are given as standard deviations. References: a — Chisholm et al. (2003); b — Wakamura et al. (1999)

Retention index		Compound	Odor impression		Yield [mg/g]
DB-5 ms	VF-WAXms		GC–O	Literature	
1388 (1364)[a]	1749 (1753)[a]	(*Z*)-5-dodecenal	waxy, musty	piney, waxy[a]	< 0.1
1491 (1471)[a]	1842	(*Z*)-6-tridecenal	waxy, green	n.r	0.2 ± 0.0
1591 (1609)[a]	1948 (1962)[b]	(*Z*)-7-tetradecenal	soapy, coriander	n.r	15 ± 2
1693	2053	(*Z*)-8-pentadecenal	green, fatty, chicken	n.r	52 ± 6

n.r. — not reported

Unsaturated compounds were semi-quantified using authentic saturated homologues

Table 4 Products of the biotransformation of oleic acid [18:1(9Z)] with retention indices, odor impressions, and approximated yields. Retention indices in parentheses are from cited literature. Errors are given as standard deviations. References: a — Chisholm et al. (2003); b — Wakamura et al. (1999)

Retention index		Compound	Odor impression		Yield [mg/g]
DB-5 ms	VF-WAXms		GC–O	Literature	
1492	1841	(Z)-4-tridecenal	n.d	n.r	0.3 ± 0.1
1590 (1565)[a]	1948 (1962)[a]	(Z)-5-tetradecenal	soapy, waxy	soapy, green[a]	3 ± 1
1692	2043	(Z)-6-pentadecenal	soapy, waxy	n.r	27 ± 9
1792 (1798)[b]	2139 (2144)[b]	(Z)-7-hexadecenal	waxy	n.r	83 ± 24
1894	2238	(Z)-8-heptadecenal	waxy	n.r	147 ± 33

n.r. — not reported, n.d. — not detected

Unsaturated compounds were semi-quantified using authentic saturated homologues

Table 5 Products of the biotransformation of linoleic acid [18:2(9Z,12Z)] with retention indices, odor impressions, and approximated yields. Retention indices in parentheses are from cited literature. Errors are given as standard deviations. References: a — Xu et al. (2020); b — Miyazawa et al. (2007); c — Kajiwara et al. (1989)

Retention index		Compound	Odor impression		Yield [mg/g]
DB-5 ms	VF-WAXms		GC–O	Literature	
1580	2019	(Z,Z)-5,8-tetradecadienal	sweet, waxy, citrus	n.r	0.3 ± 0.1
1683	2125	(Z,Z)-6,9-pentadecadienal	sweet, waxy, fatty	n.r	3 ± 0.6
1785	2225 (2225)[a]	(Z,Z)-7,10-hexadecadienal	soapy, herbaceous, spicy	n.r	11 ± 2
1887 (1889)[b]	2330	(Z,Z)-8,11-heptadecadienal	soapy, herbaceous, fatty, tallowy	green, algae-like[c]	16 ± 5

n.r. — not reported

Unsaturated compounds were semi-quantified using authentic saturated homologues

et al. (2003) or applications of advanced enzyme immobilization techniques (Twala et al. 2012) are likely suitable for the herein described approach due to similar reaction conditions. In particular, immobilized enzymes would be of special interest for industrial processes due to their higher stability, re-usability and easy handling (Sheldon 2007). However, since NADH oxidases require molecular oxygen as electron acceptor, additional gassing would have to be considered in the experimental design.

The coupled one-pot enzyme reaction process with *Csα*-DOX and *Vh*FALDH was shown to be highly efficient to produce various homologous series of fatty aldehydes from the corresponding long-chain fatty acids. Gas chromatography–olfactometric analyses provided deeper insights into previously not reported odor impressions of *Z*-unsaturated fatty aldehydes. Aldehyde mixtures obtained from the biotransformation of buckthorn pulp or other oils could be of great interest to the food and cosmetic industry as flavorings or fragrances and represent a promising start for further screening of natural lipid extracts as substrates for bioprocess developments.

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Author contributions JPK, PJH, and DL conducted experiments, JPK and DL analyzed data. The manuscript was written by JPK, PJH, and AKH. PJH, JPK, MAF, HZ, and AKH conceived and designed research. JPL, SH, BS, and CH gave intellectual input. All authors read and approved the manuscript.

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Data availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval This article does not contain any studies with animals performed by any of the authors.

Conflicts of interest Authors declare no competing interests.

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5. Biocatalytic conversion of fungal lipids to fatty aldehydes

Biocatalytic Production of Odour-active Fatty Aldehydes from Fungal Lipids

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Biocatalytic Production of Odor-Active Fatty Aldehydes from Fungal Lipids

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ABSTRACT: Odor-active fatty aldehydes are important compounds for the flavor and fragrance industry. By a coupled enzymatic reaction using an α-dioxygenase (α-DOX) and an aldehyde dehydrogenase (FALDH), scarcely available aldehydes from the biotransformation of margaroleic acid [17:1(9Z)] were characterized and have shown highly interesting odor profiles, including citrus-like, soapy, herbaceous, and savory notes. In particular, (Z)-8-hexadecenal and (Z)-7-pentadecenal exhibited notable meaty odor characteristics. Submerged cultivation of *Mortierella hyalina* revealed the accumulation of the above-mentioned, naturally uncommon fatty acid 17:1(9Z). Its production was significantly increased by the modulation of culture conditions, whereas the highest accumulation was observed after 4 days at 24 °C and L-isoleucine supplementation. The lipase-, α-DOX-, and FALDH-mediated biotransformation of *M. hyalina* lipid extract resulted in a complex aldehyde mixture with a high aldehyde yield of ∼50%. The odor qualities of the formed aldehydes were assessed by means of gas chromatography−olfactometry, and several of the obtained fatty aldehydes have been sensorially described for the first time. To assess the aldehyde mixture's potential as a flavor ingredient, a sensory evaluation was conducted. The obtained product exhibited intense citrus-like, green, and soapy odor impressions.

KEYWORDS: *Mortierella hyalina, fungal lipids, fatty aldehydes, odorants, α-dioxygenase (α-DOX), fatty aldehyde dehydrogenase (FALDH), biotransformation*

INTRODUCTION

Fatty aldehydes are character-impact compounds of various foods and fragrances and are thus highly sought-after by the flavor and fragrance industry. Well-known representatives are decanal, undecanal, dodecanal, and undec-10-enal, which are important ingredients of popular perfumes like Chanel No 5 and Rallet No 1.[1,2] Long-chain fatty acids are the main precursors for producing such aldehydes. They are traditionally sourced from plants or animals. In recent years, microbial production approaches have been studied extensively, in which a variety of fungi were found to be rich sources of oils containing, inter alia, valuable unsaturated fatty acids.[3−5] Yeasts, including *Saccharomyces cerevisiae* and especially oleaginous yeasts, have been intensely investigated concerning fatty acid production and modulation of the fatty acid pattern by, e.g., breeding or metabolic engineering.[6−8] Filamentous fungi have been less well studied in this context. However, representatives belonging to the genus *Mortierella* were reported to accumulate considerable amounts of desired polyunsaturated fatty acids (PUFA), such as arachidonic acid [20:4(5Z,8Z,11Z,14Z)] and eicosapentaenoic acid [20:5-(5Z,8Z,11Z,14Z,17Z)], alongside naturally scarce odd-chain fatty acids.[9−12] Fermentative approaches were undergone to optimize and modulate fatty acid production. Totani and Oba observed the formation of PUFAs during cultivation on agar-plates,[9] whereas later studies focused on industrially more favorable submerged cultivation to accumulate target compounds in the mycelium. Alteration of the cultivation parameters was shown to drastically modulate lipid yields as well as the fatty acid composition of various *Mortierella* strains.[5,13−16] Media supplementation of *Mortierella* and other fungi such as *Entomophtora* or *Conidiobolus* spp. led to increased yields of unsaturated fatty acids.[11,17−19] Such mono- or polyunsaturated fatty acids from fungi are of particular interest for the fine chemical industry as they can serve as natural compounds for medicinal, nutritional, or cosmetic applications as well as precursors for uncommon unsaturated fatty aldehydes.

With rising demand for naturally sourced food ingredients, biotechnological processes have been proven to be highly suitable to bypass the limited availability of target compounds in nature and their energy intensive chemical syntheses. Various biocatalytic approaches for the synthesis of unsatu-

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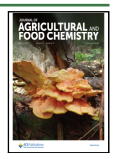

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rated and methyl-branched fatty aldehydes from fatty acids have been reported. The reduction of fatty acids by a recombinant carboxylic acid reductase (CAR) from *Mycobacterium marinum* was successfully applied for the generation of saturated C_4 to C_{18} aldehydes and unsaturated C_{18} analogues.[20] The same enzyme was later applied to obtain 12-methyltridecanal from its corresponding fatty acid. Since *Conidiobolus* spp. were identified as a rich source of methyl-branched fatty acids,[21−23] a hydrolyzed lipid extract was also biotransformed, resulting in a complex aldehyde-mixture.[23] Similarly, several terminally monounsaturated C_7−C_{13} acids and a fungal lipid extract of *Flammulina velutipes* served as substrates for CAR-mediated biotransformation experiments.[24] Another well-known oxidoreductase is the α-dioxygenase (α-DOX) that plays an essential role in the α-oxidation pathway described for plants and bacteria. It catalyzes the introduction of molecular oxygen to the α-carbon of fatty acids. The chemically unstable hydroperoxy fatty acid spontaneously decarboxylates, resulting in a carbon chain-shortened aldehyde.[25−27] A recombinant α-DOX from *Oryza sativa* was applied for the biotransformation of several saturated and unsaturated medium- and long-chain fatty acids to the corresponding C_{n-1} chain-shortened aldehydes.[28,29] Recently, several cyanobacterial α-DOXs were identified and successfully applied for the generation of various fatty aldehydes from fatty acids.[30,31] By a coupled enzymatic reaction using a cyanobacterial fatty aldehyde dehydrogenase (FALDH) and an α-DOX, C_{16}−C_{18} fatty acids were efficiently converted to a series of homologous carbon chain-shortened aldehydes.[32]

In the current study, the latter biocatalytic approach was applied to characterize aldehydes from the biotransformation of the uncommon odd-chain fatty acid margaroleic acid [17:1(9*Z*)] that are of potential interest as odorants. *Mortierella hyalina* (MHY) was found to accumulate considerable amounts of margaroleic acid in its mycelium. Thus, it was chosen as a promising feedstock for the production of desired aldehydes. To elevate margaroleic acid yields in the fungus, cultivation parameters were optimized. Subsequently, the hydrolyzed lipid extract was subjected to the coupled-enzyme reaction of α-DOX and FALDH to produce a complex odorous mixture rich in sensorially interesting, naturally uncommon unsaturated aldehydes.

MATERIALS AND METHODS

Chemicals. Acetone (99.8%) and L-threonine (98%) were obtained from Acros Organics (Fair Lawn, NJ, US). Nitrogen (5.0 purity grade, 99.999%) was purchased from Air Liquide (Düsseldorf, Germany). Decanal (96%), dodecanal (95%), and tridecanal (90%) were bought from Alfa Aesar (Ward Hill, MA, US). L-Valine (99%) was obtained from AppliChem (Darmstadt, Germany). Disodium hydrogen phosphate (99.5%), glucose monohydrate, imidazole (99.5%), L-isoleucine (99%), potassium dihydrogen phosphate (98%), potassium hydroxide (85%), L-methionine (99%), sodium sulfate (99%), tris(hydroxymethyl)aminomethane (99%), and triton X-100 (99.9%) were purchased from Carl Roth (Karlsruhe, Germany). *n*-Hexane was obtained from Honeywell (Charlotte/NC, USA). Margaroleic acid (99.5%) and its methyl ester (99%) were bought from Larodan (Solma, Sweden). *iso*-Octane (99.8%) was obtained from Merck (Darmstadt, Germany) and helium (5.0 purity grade, 99.999%) from Praxair (Düsseldorf, Germany). Isopropyl β-D-1-thiogalactopyranoside (IPTG) (99%) and kanamycin sulfate (>750 IU/mg) were purchased from Serva (Heidelberg, Germany). 3-Acetylpyridine (98%), (*Z*)-7-decenal (97%), and nicotinamide adenine dinucleotide (NAD^+) were bought from Sigma-Aldrich (St. Louis, MO, US). A 37-component fatty acid methyl ester (FAME) mix was used for the identification of FAMEs (Supelco, Bellefonte, Pennsylvania). Heptadecanal (97%), hexadecanal (97%), pentadecanal (97%), tetradecanal (95%), and undecanal (97%) were obtained from TCI (Tokyo, Japan). Hydrochloric acid 25% (HCl) was purchased from Th. Geyer (Renningen, Germany), and acetonitrile (99.9%) was bought from VWR Chemicals (Radnor, PA, USA). All numbers given in parentheses represent the minimum purity.

Cultivation of *M. hyalina*. The fungus MHY was collected in a forest nearby Giessen (Germany), isolated, and identified by internal transcribed spacer sequencing. The strain was deposited in the strain collection (collection number 115433) of the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (Brunswick, Germany).

MHY was grown on YD agar (10 g/L yeast extract, 22 g/L glucose monohydrate, and 15 g/L agar−agar; glucose was autoclaved separately from other medium components) for 7 days at 24 °C. Approximately 2 cm^2 of fresh mycelium of the surface culture was transferred to Erlenmeyer-flasks (500 mL) containing 200 mL of liquid medium and homogenized (30 s at 10,000 rpm with an Ultra-Turrax T25 (IKA, Staufen, Germany). After 3 days of growth, the pre-cultures were homogenized again, and an aliquot of 40 mL was used for inoculating 400 mL of liquid main culture medium in Erlenmeyer flasks (1 L). Submerged pre- and main cultures were incubated on a rotary shaker (150 rpm, 25 mm shaking diameter, Orbitron, Infors, Einsbach, Germany) at 24 °C in the absence of light. The same medium composition as for the surface cultures was used (without agar−agar). Mycelium was harvested after 6 days by vacuum filtration with a Buchner funnel, rinsed with deionized water, and stored at −20 °C.

Growth and lipid and fatty acid production of MHY were investigated in detail by either varying main culture temperatures (16, 24, or 37 °C) or cultivation times (2, 4, 6, 10, and 14 d). The concentration of glucose was monitored over the cultivation period by means of a Biosen C-Line biosensor (EKF-Diagnostics, Barleben, Germany). 1 mL of the culture supernatant was sampled, centrifuged (16,000*g*, 30 min, 4 °C), diluted (1:5, v/v), and directly measured. Furthermore, supplementation of the YD main culture medium with different amino acids (L-isoleucine, L-methionine, L-threonine, and L-valine, each 1 g/L) was tested (4 d, 24 °C).

Lipid Extraction and Fatty Acid Profiling. 3−4 g of freeze-dried (Alpha 1−2 LD plus freeze dryer from Christ (Osterode am Harz, Germany), 37 Pa, 3−5 d, −30 °C) mycelium was ground in a mortar, and lipids were extracted (2 h) with 160 mL of *n*-hexane by means of a Soxtherm apparatus [Gerhardt, Königswinter, Germany, 165 °C, 6× reduction interval (3 min 30 s, 2 s impulse)]. For the determination of the lipid contents, the remaining extraction solvent was removed by gently flushing the Soxtherm beakers with nitrogen. Beakers were dried to a constant weight at 105 °C for a maximum of 45 min.

Fungal lipids were analyzed for their fatty acid compositions via their methyl esters, as described by Hammer et al.[24] The free *cis*-vaccenic acid reference compound was methyl esterified as described by Fraatz et al.[23] Analysis was performed with an Agilent (Waldbronn, Germany) 7890B gas chromatograph, equipped with a MPS robotic multipurpose autosampler (GERSTEL, Mülheim an der Ruhr, Germany) and a split/splitless (S/SL) inlet (250 °C). Helium was used as a carrier gas in constant flow mode in combination with different columns and temperature programs: analysis using an Agilent VF-WAXms column (30 m length × 0.25 mm inner diameter; 0.25 μm film thickness) was performed with a carrier gas flow of 1.2 mL/min. The initial GC-oven temperature was held at 40 °C for 3 min, subsequently increased from 3 °C/min to 240 °C, and finally held for 12 min. An Agilent Select FAME column (100 m length × 0.25 mm inner diameter, 0.25 μm film thickness) was applied with a carrier gas flow of 1.0 mL/min. The initial temperature of 80 °C was held for 1 min, increased from 20 °C/min to 145 °C, and held for 30 min. Then, the temperature was raised to 198 °C with 1 °C/min and finally increased to 250 °C with 5 °C/min and held for 15 min. The mass spectrometer Agilent 5977B MSD (ionization energy, 70 eV; ion

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source, 230 °C; quadrupole, 150 °C; transfer line, 250 °C; scan mode, m/z 33−425) was used for analyte detection.

***E. coli* Strains, Heterologous Expression, and Enzyme Purification.** The gene encoding an α-dioxygenase from *Crocosphaera subtropica* (*Cs*α-DOX) (GenBank accession number: ON711410) was synthesized, subcloned into a pET-28a plasmid, and transferred to *Escherichia coli* strain BL21(DE3) as described before.[30] A recombinant FALDH from *Vibrio harveyi* (*Vh*FALDH) (GenBank accession number: ON677428) was prepared by the transformation of the *E. coli* strain W3110 (DE3) with a pET28a plasmid harboring the gene encoding *Vh*FALDH.[32,33] *E. coli* strains BL21(DE3)−pET28a−*Cs*α-DOX and W3110(DE3)−pET28a−*Vh*FALDH were grown in lysogeny broth (LB) medium containing 30 μg/mL of kanamycin at 37 °C. Expression was induced at an OD_{600} of 0.4−0.6 by the addition of β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mmol/L. *E. coli* cells were further cultivated for 18 h at 18 °C, followed by collecting cells by means of centrifugation. Cell pellets were suspended in extraction buffer (50 mmol/L phosphate buffer, pH 7.5, 20 mmol/L imidazole) to a final concentration of 25% w/v (cell wet mass) and lysed with an ultrasonic probe (Bandelin Sonopuls, Berlin, Germany) while being kept on ice. Cell-free extracts were obtained by centrifugation (4000*g*, 15 min, 4 °C), and target enzymes were purified by means of immobilized metal affinity chromatography using a nickel-loaded nitrilotriacetic acid column (Ni-NTA) (Macherey-Nagel, Düren, Germany), as described before.[30,32]

Enzyme Activity and Biotransformation Experiments. Enzyme activity of *Cs*α-DOX was determined by an oxygen consumption assay applying an optical oxygen probe (Microx TX3, PreSens, Regensburg, Germany) according to Hammer et al.[30] Enzyme activity of *Vh*FALDH was measured spectrophotometrically via absorbance of produced NADH according to Kanter et al.[32] The corresponding values were calculated as units per liter (U/L). Prior to biotransformation, 5 mg of pure margaroleic acid or MHY lipid extract was dispersed (ultrasonic bath, 5 min) in 2 mL phosphate buffer (50 mmol/L, pH 7.5). Subsequently, the fungal oil was enzymatically hydrolyzed (3 h incubation, 40 rpm, 24 °C) by a lipase from *Candida rugosa* (6 U, E.C. 3.1.1.3) to obtain free fatty acids. Biotransformations were initiated by the addition of 200 μL co-factor NAD^+ (5 mmol/L) and enzyme solutions to a final concentration corresponding to 150 U/L for *Cs*α-DOX and 18.75 U/L for *Vh*FALDH. Ultimately, buffer was added to a final reaction volume of 4 mL. Biotransformation experiments were performed in 20 mL headspace vials (PTFE sealed screw caps) containing ∼2 g of pyrolyzed glass beads (3 mm diameter) to increase dispersion during incubation. Reactions were performed at 24 °C under vigorous shaking (40 rpm, rotary shaker) for 4 h in the absence of light. Subsequently, the samples were placed in an ice bath, and 200 μL HCl (4 M) was added to stop the reaction.

Compound Identification and Semi-quantitation. Fatty aldehydes produced during the coupled enzymatic reaction were identified and semi-quantified by means of headspace-solid-phase microextraction−gas chromatography−mass spectrometry (HS-SPME−GC−MS). A polydimethylsiloxane/divinylbenzene (PDMS/DVB) coated fiber (1 cm length, 65 μm) (Merck, Darmstadt, Germany) was applied for the extraction of volatile compounds. Therefore, biotransformed samples were diluted 1:1000 in deionized water to a final volume of 4 mL in a 20 mL-headspace vial. Incubation was performed for 10 min at 60 °C prior to extraction for 30 min at 60 °C under agitation (250 rpm), using a GERSTEL MPS autosampler. An Agilent 7890A gas chromatograph equipped with a VF-WAXms column (30 m length × 0.25 mm inner diameter × 0.25 μm film thickness) was applied. Helium (5.0 purity grade, 99.999%) was used as a carrier gas with a flow rate of 1.2 mL/min. The temperature program started with 40 °C for 3 min, followed by heating to 240 °C with 5 °C/min, and it was held at 240 °C for 12 min. An Agilent 5975C mass spectrometer was used for detection with the following settings: ionization energy, 70 eV; ion source temperature, 230 °C; scan range, m/z 33−300; and quadrupole temperature, 150 °C.

Retention indices were determined according to van den Dool and Kratz[34] on polar and non-polar columns. For the latter, an Agilent DB-5ms column (30 m length × 0.25 mm inner diameter × 0.25 μm film thickness) was installed into the same GC−MS setup. The carrier gas flow was set to 1.56 mL/min. The initial temperature of 40 °C was held for 3 min, followed by heating with 5 °C/min to 320 °C to be held for 12 min. The formed aldehydes were identified by a comparison of retention indices to those found in the literature and by the interpretation and alignment of mass spectra using NIST MS library software (NIST MS Search 2011, National Institute of Standards and Technology, Gaithersburg, MD, USA). Additionally, the reaction products from the biotransformation of pure margaroleic acid were analyzed for their double-bond position by means of electrospray ionization-online Paternò−Büchi functionalization-tandem mass spectrometric (nanoESI-online-PB-MS/MS) experiments according to the method described previously.[32]

The concentrations of the obtained aldehydes were semi-quantified by means of response factors (Rf), calculated from saturated aliphatic C_{10}−C_{18} aldehyde standards and the internal standard (*Z*)-7-decenal. Therefore, stock solutions of standard substances (∼1 g/L in acetone) were diluted to a final concentration of 100 μg/L in deionized water in a total volume of 4 mL. Standards as well as diluted samples from biotransformation (1:1000 in deionized water) were spiked with the internal standard (*Z*)-7-decenal to a final concentration of 40 μg/L. Instrumental analysis by HS-SPME−GC−MS was performed as described above (polar column: VF-WAXms). Yields of aldehydes were calculated as mg of produced aldehyde per gram of employed margaroleic acid or lipid extract as the substrate in biotransformations.

Gas Chromatography−Mass Spectrometry−Olfactometry. Sensory evaluation of unsaturated aldehydes from the biotransformation of margaroleic acid as well as aldehydes obtained from the biotransformation of fungal lipids was executed by means of sniffing via HS-SPME−GC−MS−olfactometry. Therefore, SPME-extracted samples (cf. "compound identification and semi-quantitation") were desorbed by means of a split/splitless injector (split ratio 1:10) into an Agilent A7890 gas chromatograph equipped with an Agilent VF-WAXms column (30 m length × 0.25 mm inner diameter × 0.25 μm film thickness). Helium (5.0 purity grade, 99.999%) with a flow rate of 1.56 mL/min was used as a carrier gas. The initial oven temperature was 40 °C (3 min), followed by heating to 240 °C with 5 °C/min, and the temperature was held at 240 °C for 12 min. An Agilent 7000B triple quadrupole tandem mass spectrometer was used for detection with the following settings: ionization energy, 70 eV; ion source temperature, 230 °C; scan range, m/z 33−300; quadrupole temperature, 150 °C. An olfactometric detector port (ODP 3, GERSTEL) was connected to the GC system (transfer line, 250 °C; mixing chamber, 150 °C; makeup gas, nitrogen). Sniffing was executed by three trained panelists.

Sensory Evaluation. The reaction mixture obtained from the biotransformation of MHY oil was diluted 1:10 with deionized water, and 1 mL of this dilution was transferred in a 20 mL glass vial with a lid. In the same manner, control samples containing solely enzyme solutions or lipase-treated hydrolyzed MHY lipid extracts were prepared. Freshly prepared samples were presented to weekly trained assessors (four males and four females, 24 to 33 of age) in a random order. Descriptors were defined by asking the panelists to list attributes associated with the samples. In a second assessment, the panel rated the odor qualities, named at least twice in the previous session, on a scale from 0 (not perceivable) to 5 (very strong perception).

Statistics. The determination of yields of biomass and lipids, as well as fatty acid profiling, was at least performed for two independent biological samples. Aldehyde concentrations from biotransformations were determined from at least biological duplicates and analytical triplicates. Results are given as mean values and corresponding standard deviations, unless stated otherwise.

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Table 1. Fatty Acids (>0.5%) of Fungal Lipids Obtained from the Mycelia of *M. hyalina* (for the Complete Profile cf. Supporting Information, Table S1)[a]

	retention indices[c]			
fatty acid (methyl ester)[b]	VF-WAX	select FAME	relative share [%]	approximated yield [mg/L][d]
14:0	2002 (2002)	2115 (2114)	1.7 ± 0.1	36 ± 2
15:0	2105 (2105)	2225 (2225)	0.7 ± 0.1	16 ± 1
16:0	2209 (2209)	2335 (2334)	28.5 ± 0.5	608 ± 10
17:0	2311 (2312)	2454 (2451)	1.6 ± 0.2	34 ± 5
17:1(9Z)	2331 (2332)	2506 (2506)	1.0 ± 0.1	20 ± 2
18:0	2415 (2416)	2553 (2552)	5.2 ± 0.6	112 ± 13
18:1(9Z)	2433 (2433)	2602 (2601)	49 ± 2[f]	1042 ± 42[f]
18:1(11Z)	2441 (2441)	2611 (2611)	0.8 ± 0.1[f]	17 ± 3[f]
18:2(9Z,12Z)	2478 (2479)	2696 (2692)	3.5 ± 0.4	74 ± 8
18:3(6Z,9Z,12Z)	2509 (2510)	2738 (2737)	1.0 ± 0.2[f]	21 ± 3[f]
19:1[e]	2533	2723	0.6 ± 0.1[f]	13 ± 2[f]
20:4(5Z,8Z,11Z,14Z)	2738 (2738)	3006 (3004)	3.4 ± 0.5	72 ± 11
24:0	3036 (3037)	3220 (3215)	0.8 ± 0.1	16 ± 2

[a]Compounds were identified by retention indices and mass spectra of authentic reference compounds. Values represent means of two biological replicates with standard errors. [b]Abbreviations give the carbon number, position, and configuration of double bonds of the corresponding fatty acids. [c]RIs were calculated according to van den Dool and Kratz;[34] and numbers in parentheses give the RIs of the reference compounds. [d]Milligram per liter main culture, calculated by a relative share of the fatty acid profile and lipid content. [e]Suggested substance due to retention behavior and MS spectrum. [f]Quantitative data approximated due to not completely separable peaks.

RESULTS AND DISCUSSION

Fungal Lipid Production and Fatty Acid Profiling. MHY yielded comparatively high amounts of biomass and lipids within the short cultivation time of 6 days. It produced 3.5 ± 0.1 g of dry biomass per liter with a lipid content of 29.0 ± 1.0 g per 100 g dry matter and contained more than 55% of mono-, di-, and PUFAs, with oleic acid (49.0 ± 2.0%) being the most abundant representative, followed by α-linoleic acid (3.5 ± 0.4%), and arachidonic acid (3.4 ± 0.5%), a typical PUFA found in *Mortierella* spp.[5] Interestingly, notable amounts (1.0%, approximately 20 ± 2 mg/L) of the very scarcely occurring ω8-unsaturated margaroleic acid were found as well (Table 1).

Variation of Culture Parameters. Fungal lipid biosynthesis is well-known to be modulated by culture parameters[3,4,35] which allows for a targeted synthesis of desired compounds. Therefore, the growth and lipid and fatty acid production of MHY were investigated in detail. As the fatty acid 17:1(9Z) is an interesting candidate for the biosynthetic production of valuable fatty aldehydes, cultivation was modulated toward its increased accumulation.

MHY was cultivated (6 days) at different temperatures (16, 24, or 37 °C). Partly significant, but not noteworthy, differences regarding the yields of biomass, lipid contents, and the targeted margaroleic acid were found for the mycelia cultured at 16 and 24 °C (data not shown). In contrast to earlier findings for other fungi,[3,4,36,37] a reduced cultivation temperature did not lead to an increased production of unsaturated fatty acids in the present study. At 37 °C, no growth was observed. Therefore, cultivation was pursued at 24 °C.

When supplied with glucose as a carbon source for lipid biosynthesis, lipids were mainly formed within the first 4 days and biomass within the first 6 days (Figure 1). At day 14, a slight decrease of biomass was observed, which might indicate the beginning of autolysis due to an insufficient nutrient supply. MHY lipids showed the highest shares of 17:1(9Z) in the fatty acid profile in the early growth phase, declining from 2.6 ± 0.0% (day 2) to 1.6 ± 0.0% (day 4) and 1.0 ± 0.1% (day 6) to 0.6 ± 0.0% (day 14). Due to the inverse development of the lipid yield, the highest amounts of 17:1(9Z) accumulated at day 4 (22 ± 0 mg/L culture broth) were chosen as the main culture period for further studies.

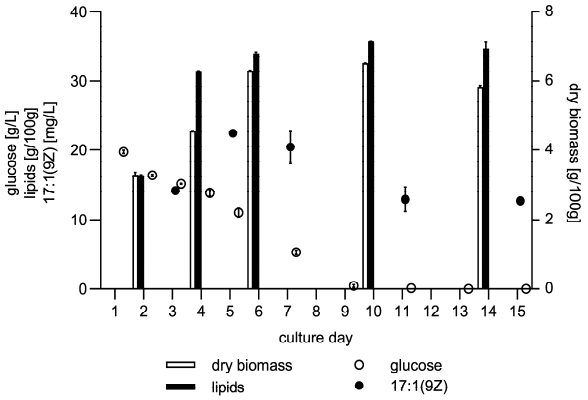

Figure 1. Influence of culture time on yields of dry biomass, lipid content, and 17:1(9Z) fatty acid, produced by *M. hyalina*. Values represent means of two biological replicates with standard errors.

To increase the yields of 17:1(9Z), the YD culture broth was supplemented with potential precursors of fatty acids with odd-numbered carbon chain lengths, namely L-isoleucine, L-methionine, L-threonine, and L-valine. In the literature, there are inconsistent data on inhibiting or promoting effects of free amino acids on microbial or fungal growth.[38,39] In the present study, the supplementation of amino acids did not influence the production of biomass or lipids by MHY significantly (Table 2). L-Methionine and L-threonine caused a significant increase in biomass but did not improve the yields of 17:1(9Z). However, supplementation with L-valine, and especially with L-isoleucine, affected the fatty acid distribution in favor of margaroleic acid, resulting in fourfold higher yields (7.1 ± 0.1% of the fatty acid profile; 72 ± 0.0 mg/L culture broth).

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Table 2. Influence of Supplemented Amino Acids on Yields of Dry Biomass, Lipid Content, and 17:1(9Z) Fatty Acid Produced by *M. hyalina*[a]

medium[b]	dry biomass [g/L]	lipid content [g/100 g dry biomass]	17:1(9Z) share of the fatty acid profile [%]	approximated yield of 17:1(9Z)[c]
YD	3.79 ± 0.06	28.1 ± 0.3	1.7 ± 0.1	18 ± 1
YD + Ile	3.56 ± 0.03[d]	28.5 ± 0.1	7.1 ± 0.1[d]	72 ± 1[d]
YD + Met	4.16 ± 0.03[d]	24.7 ± 0.1[d]	0.6 ± 0.0[d]	7 ± 0[d]
YD + Thr	4.08 ± 0.05[d]	27.9 ± 0.0	1.3 ± 0.1[d]	15 ± 1
YD + Val	3.65 ± 0.04	29.4 ± 0.2[d]	4.2 ± 0.1[d]	45 ± 1[d]

[a]Values represent means of three biological replicates with standard errors. [b]YD—yeast dextrose, Ile—L-isoleucine, Met—L-methionine, Thr—L-threonine, Val—L-valine, each amino acid supplemented with 1 g/L. [c]mg per liter main culture, calculated by the relative share of the fatty acid profile and lipid content. [d]Significantly different (α = 5%) from the results of not supplemented YD culture (1st line).

Table 3. Aldehydes Produced by Biotransformation of Margaroleic Acid [17:1(9Z)] with Retention Indices, Odor Impressions, and Yields (mg Aldehyde per g Fatty Acid)[a]

retention index			odor impressions		
DB-5ms	VF-WAXms	compound	GC–O	literature	yield [mg/g]
1489	1834	(Z)-5-tridecenal	green, herbaceous, waxy, citrus peel	n.r.	2 ± 0
1593 (1572[b])	1946 (1964[b])	(Z)-6-tetradecenal	waxy, sweet, floral, coriander soapy	soapy, green[b]	21 ± 2
1695	2049	(Z)-7-pentadecenal	citrus peel, soapy, fatty, brothy	n.r.	73 ± 4
1799 (1798[c])	2154 (2144[c])	(Z)-8-hexadecenal	citrus peel, waxy, fatty, tallowy, meat broth	n.r.	130 ± 7
					∑ 226 ± 13

[a]Compound identification was performed by mass spectra alignment with the NIST database, comparison of retention indices found in the literature, and structure verification by means of nanoESI-online–PB–MS/MS–analysis (Table 4). Values represent means of three biological replicates with standard deviations. [b]Chisholm et al.[41] [c]Marques et al.,[42] n.r.—not reported.

Biotransformation of Single Fatty Acids. *Cs*α-DOX as well as *Vh*FALDH were successfully produced by the heterologous expression in *E. coli* and subsequently purified to electrophoretic homogeneity. The biotransformation of margaroleic acid, as an interesting precursor of unsaturated fatty aldehydes, yielded high concentrations of the respective chain-shortened aldehydes. Upon bioconversion, four reaction cycles were observed (Table 3) by which the aldehydes (Z)-8-hexadecenal, (Z)-7-pentadecenal, (Z)-6-tetradecenal, and (Z)-5-tridecenal were formed. The total aldehyde yield was estimated at 226 ± 14 mg/g of employed fatty acid. This is well in accordance with an earlier application of the coupled enzymatic reaction with the structurally similar oleic acid, for which ∼260 mg of total aldehydes per gram substrate was reached.[32] As two different enzymes were applied, substrate specificity is likely to differ for *Cs*α-DOX and *Vh*FALDH. An earlier study on the biocatalytic activity of *Vh*FALDH indicated no carbon chain length specificity. However, Z-unsaturated homologues showed slightly lower enzymatic conversion rates compared to saturated analogues. (Z)-8-Hexadecenal represented the main transformation product of margaroleic acid with 130 ± 7 mg/g employed fatty acid, followed by the aldehyde from the second reaction cycle (Z)-7-pentadecenal with around 73 ± 4 mg/g. Despite rather low concentrations of the 3rd and 4th cycle aldehydes (<5% yield), all four reaction products were clearly perceived olfactometrically. The odors of (Z)-8-hexadecenal, (Z)-7-pentadecenal, and (Z)-5-tridecenal are described here for the first time. Predominant odor attributes were "citrus-like" and "waxy", but savory notes were also perceived. In particular, (Z)-8-hexadecenal exhibited an intense odor reminiscent of inter alia cooked meat and tallow. Interestingly, with decreasing carbon chain length, the odor impressions shifted toward more typical odor attributes of unsaturated fatty aldehydes, such as green, citrus-like, soapy, or waxy.

As mass spectra are rather ambiguous when it comes to isomers with solely different double bond positions, Paternò–Büchi reaction, coupled to a high-resolution mass spectrometer, was used to verify the reaction products' identities (Table 4). Unsaturated compounds from the biotransformation of margaroleic acid react with 3-acetylpyridine under UV-light irradiation inside a borosilicate capillary connected to a nanoESI–MS/MS instrument. The photochemical reaction yielded two isomeric cycloadducts containing an oxetane function at the position of the C=C double bond. Cycloadducts containing C_{16} (m/z 360) down to C_{12} (m/z 304) unsaturated aldehydes were detected. Fragmentation induced by higher-energy collisional dissociation (HCD) resulted in diagnostic fragments, specific for the compounds'

Table 4. Identified PB Oxetane Adducts and Their Deduced Diagnostic Fragments (α- and ω-ions) from nanoESI-Online-PB-MS/MS-Analysis of the Biotransformed Margaroleic Acid (Deviation from Theoretical Masses <2 ppm)

compound	m/z [PB adduct][a]	m/z [α-ion]	m/z [ω-ion]
heptadec-9-enoic acid	390.3003	262.1802	218.1903
hexadec-8-enoic acid	376.2846	248.1645	218.1903
pentadec-7-enoic acid	362.2690	234.1489	218.1903
tetradec-6-enoic acid	348.2533	220.1332	218.1903
tridec-5-enoic acid	334.2377	206.1176	218.1903
dodec-4-enoic acid	320.2220	192.1019	218.1903
hexadec-8-enal	360.2897	232.1696	218.1903
pentadec-7-enal	346.2741	218.1539	218.1903
tetradec-6-enal	332.2584	204.1383	218.1903
tridec-5-enal	318.2428	190.1226	218.1903
dodec-4-enal	304.2271	176.1070	218.1903

[a]Corresponds to the photoproduct of acetylpyridine and fatty acid/aldehyde.

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Table 5. Aldehydes Obtained from the Biotransformation of Hydrolyzed Lipid Extracts of MHY[a]

retention index			odor impressions			
DB-5ms	VF-WAXms	compound	GC−O	literature	probable precursor [number of cycles]	yield [mg/g]
1306 (1307[b])	1597 (1613[b])	undecanal	green, citrus, metallic	citrus[d]	16:0 [5]/15:0 [4]/14:0 [3]	<1
1408 (1409[b], 1408[c])	1702 (1716[b], 1703[c])	dodecanal	waxy, soapy, green, coriander, citrus	green, citrus, waxy[c]; herbaceous, waxy[d]; pungent, spicy/floral, citronellol-like[e]	16:0 [4]/15:0 [3]/14:0 [2]	1 ± 0
1508 (1510[b], 1511[c])	1809 (1824[b], 1810[c])	tridecanal	green, soapy, floral, grapefruit	fresh, green[b]	16:0 [3]/15:0 [2]/14:0 [1]	8 ± 1
1612 (1613[b], 1613[c])	1916 (1932[b], 1919[c])	tetradecanal	soapy, fatty, metallic, floral	fresh, herbaceous[b]	16:0 [2]/15:0 [1]	73 ± 12
1590 (1590[c], 1565[d])	1938 (1948[c], 1962[d])	(Z)-5-tetradecenal	soapy, waxy, herbaceous	soapy, green[e]	18:1(9Z) [4]	1 ± 0
1591 (1593)	1946 (1946)	(Z)-6-tetradecenal	soapy, waxy, herbaceous, coriander	soapy, green[e]	17:1(9Z) [3]	1 ± 0
1712 (1710[b])	2022 (2030[b])	pentadecanal	waxy, sweetish	pungent, spicy/woody[e]	16:0 [1]	128 ± 25
1690 (6Z: 1692) (7Z: 1695[c])	2049 (6Z 2043[c]) (7Z: 2049)	(Z)-6/7-pentadecenal	soapy, waxy, citrus, green, metallic	green, herbaceous, metallic[c]	17:1(9Z) [2]/18:1(9Z) [3]	15 ± 2
1816 (1814[b], 1793[d])	2128 (2140[b], 2146[d])	hexadecanal	n.d.	woody[d]	17:0 [1], 18:0 [2]	112 ± 25
1792 (7Z: 1792[c]) (8Z: 1799)	2153 (7Z: 2139[c]) (8Z: 2154)	(Z)-7/8-hexadecenal	waxy, soapy, herbaceous, fatty, brothy	waxy[c]	17:1(9Z) [1]/18:1(9Z) [2]	54 ± 9
1782 (1785[c])	2200 (2225[c])	(Z,Z)-7,10-hexadecadienal	soapy, herbaceous, spicy	soapy, herbaceous, spicy[c]	18:2(9Z,12Z) [2]	3 ± 1
1917 (1918[c], 1919[f])	2234 (2234[c], 2221[f])	heptadecanal	soapy, sweetish	sweet[c]	18:0 [1]	19 ± 5
1894 (1894[c])	2257 (2238[c])	(Z)-8-heptadecenal	green, metallic, soapy	waxy[c]	18:1(9Z) [1]	81 ± 15
1884 (1887[c], 1889[f])	2339 (2330[c], 2348[f])	(Z,Z)-8,11-heptadecadienal	soapy, herbaceous, fatty, tallowy	green, algae-like[h]	18:2(9Z,12Z) [1]	2 ± 1
1993 (9Z: 1995)[g]	2364	octadecenal[h]	citrus, sweetish	n.r.	19:1 [1]	10 ± 2
						∑ 509 ± 98

[a]Retention indices, odor impressions, and approximated yields (mg aldehyde per g of the lipid extract). Retention indices in parenthesis are from cited literature or calculated from biotransformation experiments of margaroleic acid (Table 3). Saturated aldehydes were identified via retention indices and mass spectra of authentic reference compounds. Identification of unsaturated fatty aldehydes was performed by comparison with the mentioned transformation products of margaroleic acid as well as NIST database and retention indices from the literature. Values represent means of three biological replicates with standard deviations. [b]Sukhonthara et al.[43] [c]Kanter et al.[32] [d]Chisholm et al.[41] [e]Eyres et al.[44] [f]Miyazawa et al.[45] [g]Samadi et al.[46] [h]Suggested substance due to retention behavior and MS spectrum, n.r.—not reported, n.d.—not detected.

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double-bond position. For example, fragmentation of (*Z*)-8-hexadecenal resulted in the characteristic α-ion with m/z 232 along with a second non-specific ω-ion (m/z 218), which applies equally for all detected compounds (Supporting Information, Figure S2). Besides the targeted aldehydes, the corresponding fatty acids from the oxidation reaction catalyzed by *Vh*FALDH were detected as well, further confirming the reaction mechanism. Surprisingly, not only the reaction products of four reaction cycles from the coupled-enzyme reaction detected by GC-analyses but also fragments with m/z values corresponding to reaction products of a fifth reaction cycle were found. The Paternò–Büchi method proved to be a suitable tool to confirm the identity of the proposed biocatalytic reaction products. However, additional analytical methods, such as gas chromatography, are indispensable for unequivocal compound identification, as double bond configurations cannot be elucidated by the Paternò–Büchi reaction.

Biotransformation of Fungal Lipid Extracts. The extracted lipid fraction from the mycelium of MHY with elevated margaroleic acid contents was applied for the production of aldehyde mixtures as potential flavor ingredients. The total aldehyde yield from the bioconversion of the fungal lipids was reproducibly high, with around 500 mg of total formed aldehydes per gram of fungal lipids (Table 5). The aldehyde mixture showed a high share of saturated fatty aldehydes (~50%). The predominance of pentadecanal—the primary reaction product of palmitic acid—is in accordance with previous studies that demonstrated a high biocatalytic activity of cyanobacterial α-DOXs toward saturated medium- and long-chain fatty acids.[31,32] The remaining 50% of the formed aldehydes consisted almost entirely of monounsaturated reaction products. Analogous to the biotransformation of the single fatty acid margaroleic acid, the carbon chain-shortened aldehydes (*Z*)-8-hexadecenal, (*Z*)-7-pentadecenal, and (*Z*)-6-tetradecenal were detected. Since oleic acid [18:1(9*Z*)] was the main fatty acid in the lipid extract of MHY, the aldehydes (*Z*)-8-heptadecenal, (*Z*)-7-hexadecenal, (*Z*)-6-pentadecenal, and (*Z*)-5-tetradecenal were detected as well. With only <1% share, polyunsaturated fatty aldehydes played a minor role. This is probably, on the one hand, due to the low contents of PUFAs in the fungal lipid extract (~8%), and on the other hand, due to the low acceptance of PUFAs as substrates by *Cs*α-DOX.[31,32] Odor impressions of most aldehydes were in accordance with those described in the literature. Aldehydes deduced from margaroleic acid, for which no aroma description had been published previously, were found in both single fatty acid and MHY-lipid biotransformations. The predominant descriptors were citrus-like, green, herbaceous, soapy, and waxy. Additionally, unsaturated C_{15}-aldehydes exhibited interesting savory attributes such as meaty, brothy, and tallowy. These attributes are commonly known to be characteristic for methyl-branched fatty aldehydes such as 12-methyltridecanal.[40]

In order to evaluate the overall olfactory impression of this reaction mixture, a sensory evaluation was conducted with a panel of eight trained assessors. The formed mixture emitted strong citrus-like, fruity, green, and coriander-like odor impressions (Figure 2). However, the savory notes such as meaty or metallic (as a descriptor for, i.e., raw meat) from the C_{15}- and C_{16}-aldehydes increased only to a minor degree. This could be explained by a relatively low vapor pressure of the rather long carbon chain lengths of the responsible fatty aldehydes. On the other hand, "fatty" odor impressions decreased distinctly upon biocatalysis, which is likely due to a discriminating effect in the presence of strongly odor-active aldehydes.

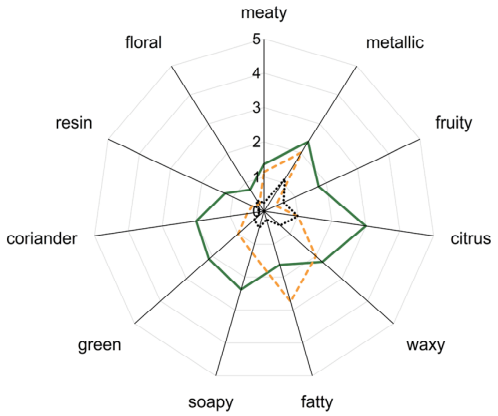

Figure 2. Sensory evaluation of the biotransformed lipid extract of MHY (green, solid line), non-biotransformed lipid extract (orange, dashed line), and enzyme solution without lipid supplementation (black, dotted line). The radar chart shows mean scores for each sensory odor quality rated by eight trained assessors on a scale from 0 (no perception) to 5 (very strong perception).

In conclusion, the naturally uncommon unsaturated fatty acid margaroleic acid was efficiently biotransformed through a coupled enzyme reaction by means of an α-dioxygenase and an aldehyde dehydrogenase to produce a series of novel odorous carbon chain-shortened fatty aldehydes. The fungus *M. hyalina* was found to be a rich source of lipids, including the valuable margaroleic acid. Biotransformation of its lipids yielded complex aldehyde mixtures that could serve as sustainable ingredients in future flavor applications.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.3c01972.

Fatty acid profile of lipid extract from MHY; mass spectra of main unsaturated fatty aldehydes produced by biotransformation of margaroleic acid; exemplary Paternò–Büchi adduct of (*Z*)-8-hexadecenal and corresponding diagnostic fragments from nanoESI-online-PB-MS/MS analysis; and GC–MS chromatograms of biotransformation experiments with the MHY lipid extract (PDF)

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Notes

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■ ABBREVIATIONS

PUFA, polyunsaturated fatty acid; CAR, carboxylic acid reductase; α-DOX, α-dioxygenase; FALDH, fatty aldehyde dehydrogenase; ITS, internal transcribed spacer; MHY, *M. hyalina*; YD, yeast extract dextrose; YM, yeast extract malt extract; LB, lysogeny broth; IPTG, β-D-1-thiogalactopyranoside; IMAC, immobilized metal affinity chromatography; Ni-NTA, nickel-loaded nitrilotriacetic acid; HS-SPME, headspace solid-phase microextraction; PDMS, polydimethylsiloxane; DVB, divinylbenzene; PB, Paternò−Büchi; Rf, response factor; FAME, fatty acid methyl ester; S/SL, split/splitless; ODP, olfactory detection port; O, olfactometry; HCD, higher-energy collision dissociation

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6. Shiitake *O*-methyltransferases to produce taste-active flavonoids

Novel Catechol *O*-methyltransferases from *Lentinula edodes* Catalyze the Generation of Taste-Active Flavonoids

Kanter, J.-P.; Milke, L.; Metz, J. K.; Biabani, A.; Schlüter, H.; Gand, M.; Ley, J. P.; Zorn, H.

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Novel Catechol *O*-methyltransferases from *Lentinula edodes* Catalyze the Generation of Taste-Active Flavonoids

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Jean-Philippe Kanter, Lars Milke, Judith K. Metz, Ali Biabani, Hartmut Schlüter, Martin Gand, Jakob P. Ley, and Holger Zorn*

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ABSTRACT: Due to the increasing demand for natural food ingredients, including taste-active compounds, enzyme-catalyzed conversions of natural substrates, such as flavonoids, are promising tools to align with the principles of Green Chemistry. In this study, a novel *O*-methyltransferase activity was identified in the mycelium of *Lentinula edodes*, which was successfully applied to generate the taste-active flavonoids hesperetin, hesperetin dihydrochalcone, homoeriodictyol, and homoeriodictyol dihydrochalcone. Furthermore, the mycelium-mediated OMT activity allowed for the conversion of various catecholic substrates, yielding their respective (iso-)vanilloids, while monohydroxylated compounds were not converted. By means of a bottom-up proteomics approach, three putative *O*-methyltransferases were identified, and subsequently, synthetic, codon-optimized genes were heterologously expressed in *Escherichia coli*. The purified enzymes confirmed the biocatalytic *O*-methylation activity against targeted flavonoids containing catechol motifs.

KEYWORDS: *basidiomycota, shiitake, mycelium, O-methyltransferase, flavonoid, homoeriodictyol, hesperetin, hesperetin dihydrochalcone, flavor, biocatalysis*

1. INTRODUCTION

Flavonoids are a highly diverse group of compounds, including *inter alia* flavanols, flavones, flavanones, isoflavones, and (dihydro-)chalcones. Many of their representatives are found in plants and are considered to exhibit multifaceted bioactivities, including the potential to maintain human health and prevent several diseases when consumed with a corresponding diet.[1−3] While common representatives, such as certain catechins, e.g., epigallocatechin gallate, several proanthocyanidins, some flavanone glycosides (e.g., naringin), and polyhydroxylated flavonols (e.g., rutin or kaempferol 3-*O*-(2‴-*O*-sinapoyl-*β*-sophoroside)), exhibit bitter and/or astringent sensory impressions,[4−6] the majority of flavonoids are not described with a noticeable taste effect.

However, some flavanones and dihydrochalcones show remarkable bitter-masking or sweet-tasting properties.[7−11] Naringin dihydrochalcone and, especially, neohesperidin dihydrochalcone (NHDC) are well-known taste-active flavonoids widely applied in foods. Interestingly, the aglycone of NHDC, hesperetin dihydrochalcone, and the corresponding flavanone analogue hesperetin along with its regioisomer homoeriodictyol have been reported to possess taste-active properties as well.[12−15,10] NHDC is a semisynthetic compound, which is commonly produced by hydrogenation of the naturally occurring neohesperidin under alkaline conditions.[7] Through subsequent hydrolysis of the neohesperidose sugar moiety, hesperetin dihydrochalcone can be formed. In nature, hesperetin occurs predominantly in citrus fruits as its rutinoside, hesperidin; however, the concentration of the free aglycone is rather low.[16] Homoeriodictyol as an aglycone can be found in larger amounts in the *Eriodictyon* ssp. called *Yerba santa*, growing in the wild in arid areas of Northwest Mexico or Southwest US.[10] Recently, a number of studies demonstrated promising approaches to obtain hesperetin dihydrochalcone, hesperetin, or homoeriodictyol by selective *O*-methylation of their respective precursors, eriodictyol dihydrochalcone and eriodictyol.[12,17,18]

As flavonoids usually possess multiple hydroxy groups at various positions, efficient chemical methylation is difficult, as it generally lacks the required chemo- and regioselectivity. Furthermore, highly toxic and environmentally harmful chemicals, such as methyl halides and dimethyl sulfate, are commonly used for such reactions.[12] In contrast, biocatalytic approaches are usually highly chemo- and regioselective and mostly environmentally friendly.[19] Therefore, *O*-methyltransferases (OMTs, EC 2.1.1.x) represent a promising class of enzymes for targeted flavonoid modification. OMTs catalyze the selective transfer of the methyl group from the sulfonium moiety of *S*-adenosyl-L-methionine (SAM) to a hydroxy group.

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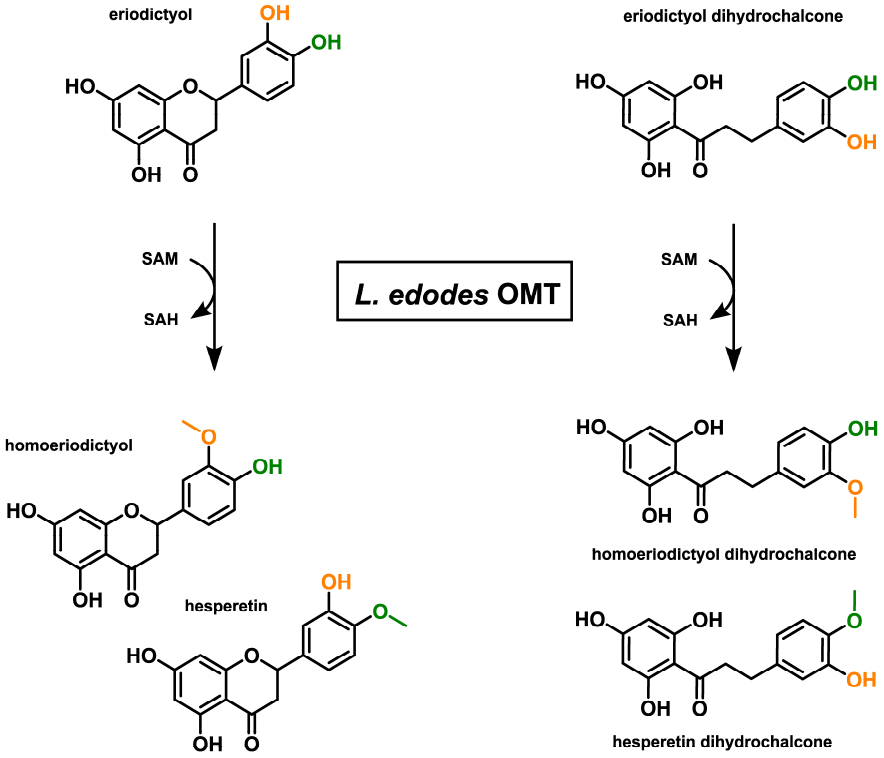

Figure 1. Enzyme-catalyzed *O*-methylation of eriodictyol [dihydrochalcone] to homoeridictyol [dihydrochalcone] or hesperetin [dihydrochalcone] in the presence of the methyl-group donor *S*-adenosyl-L-methionine (SAM), which results in *S*-adenosyl-L-homocysteine (SAH), following methyl transfer.

In plants, OMTs play pivotal roles in processes such as flower pigmentation, defense against phytopathogens, and ultraviolet (UV) protection.[20] The majority of reported flavonoid-specific OMTs were described for plants, such as soybean (*Glycine max*), rice (*Oryza sativa*), or periwinkle (*Catharanthus roseus*), which showed biocatalytic activities for the modification of *i.a.* naringenin, quercetin, and eriodictyol.[21−23] Moreover, synthetic pathways have recently been developed by heterologous expression of plant genes in bacterial cell hosts to produce *O*-methylated flavonoids.[24,25] Flavonoid-specific OMTs outside the plant kingdom have been reported only to a lesser extent.[26−29,12,30,31] In particular, the presence of OMTs from higher fungi, such as Basidiomycota, has been scarcely described. However, results from genome sequencing of various white-rot fungi revealed the presence of genes putatively encoding OMTs, which may be involved in the *O*-methylation of monolignols to inhibit oxidative stress from their free hydroxy groups and facilitate peroxidase activity for lignin degradation.[32] The biocatalytic *O*-methylation of phenolic compounds by a Basidiomycota species was first described for *Neolentinus lepideus* in 1975.[33] Up to date, further OMTs have been characterized from *Phanerochaete chrysosporium* and *Flammulina velutipes* for the *O*-methylation of numerous phenolic compounds, including acetovanillone, isovanillic acid, protocatechuic acid, *p*-coumaryl alcohol, *p*-coumaric acid, and the flavonoids epigallocatechin gallates, myricetin, luteolin, and butein.[34−38,32] Nonetheless, targeted *O*-methylation of the flavonoids eriodictyol and its corresponding dihydrochalcone eriodictyol dihydrochalcone by Basidiomycota has not been investigated so far.

Lentinula edodes—commonly known as shiitake—is the world's leading cultivated edible mushroom, accounting for over 26% of the world's supply (2019).[39] Besides its popularity as a food, it is regarded as a medicinal fungus rich in nutritionally valuable vitamins (pro-vitamin D_2, vitamin B_1, B_2, B_6, B_{12}, and niacin), health-beneficial phenolics, and polysaccharides, such as lentinan.[40] As *L. edodes* is a well-documented white-rot fungus and is closely related to previously identified Basidiomycota species harboring OMTs, the objective of the current study was to investigate and characterize the OMT activity of *L. edodes* mycelium obtained from submerged cultivation. Specifically, we aimed to determine its potential to catalyze the conversion of eriodictyol and eriodictyol dihydrochalcone to the taste-active compounds homoeriodictyol, hesperetin, homoeriodictyol dihydrochalcone, and hesperetin dihydrochalcone (Figure 1). Besides *L. edodes*, mycelia from other Basidiomycota species, which have been earlier described to provide an OMT activity (*F. velutipes* and *P. chrysosporium*) alongside close relatives of *L. edodes* (*Lentinula lateritia*, *Neolentinus squarrosulus*, and *Neolentinus cladopus*), and strains that were chosen based on genomic data with putatively OMT-encoding genes (*Hypsizygus tessulatus*, *Pleurotus sapidus*, and *Sparassis crispa*) were tested for the desired OMT activity. Furthermore, the capability to transform a range of other phenolic compounds, including protocatechuic aldehyde, the direct catecholic precursor of vanillin, was sought to be explored. A bottom-up proteomics approach was applied to identify the corresponding OMTs present in the mycelia of *L. edodes*. Subsequently, synthetic, codon-optimized genes encoding for the putative OMTs were heterologously

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expressed, and the enzymes were tested for their catalytic activity.

2. MATERIALS AND METHODS

2.1. Chemicals. *m*-Coumaric acid (98%), dihydroferulic acid (95%), and tamarixetin (99%) were obtained from abcr GmbH (Karlsruhe, Germany). Coomassie Brilliant Blue R-250 (100%), dithiothreitol (99.5%), iodoacetamide (98%), and magnesium chloride (98%) were obtained from AppliChem (Darmstadt, Germany). Bovine serum albumin (98%), Bradford solution (5× ROTIQuant), bromophenol blue (99%), caffeic acid (98%), disodium hydrogen phosphate (99.5%), ethanol (99.5%), glycine (99%), imidazole (99.5%), isovanillin (98%), β-mercaptoethanol (99%), potassium dihydrogen phosphate (98%), protocatechuic aldehyde (98%), sodium dodecyl sulfate (99%), sodium sulfate (99%), tris(hydroxymethyl)aminomethane (99%), and vanillin (99%) were purchased from Carl Roth (Karlsruhe, Germany). Butein, eriodictyol dihydrochalcone (98%), homobutein (99%), homoeriodictyol (98%), and homoeriodictyol dihydrochalcone (98%) were bought from Extrasynthese (Genay, France). *S*-Adenosyl-L-methionine disulfate tosylate (95%) was purchased from Fluorochem (Hadfield, U.K.), and dimethyl sulfoxide (99.7%) was obtained from Honeywell (Charlotte, NC). Ammonium hydrogen carbonate (99%) was obtained from Merck (Darmstadt, Germany). Dihydrocaffeic acid (98%) was purchased from Santa Cruz Biotechnology (Dallas, TX). Formic acid (99%) and isopropyl β-D-1-thiogalactopyranoside (IPTG) (99%) were purchased from Serva (Heidelberg, Germany). Acetic acid (100%), *p*-coumaric acid (98%), eriodictyol (95%), ferulic acid (98%), glycerol (100%), methanol (99.8%), and neomycin trisulfate (99%) were bought from Sigma-Aldrich (St. Louis, MO). Symrise AG (Holzminden, Germany) provided hesperetin dihydrochalcone (95%). Dihydroisoferulic acid (95%) and trifluoroacetic acid (99%) were obtained from Thermo Fisher Scientific (Waltham, MA). (−)-Epicatechin (97%), isoferulic acid (98%), and isorhamnetin (95%) were obtained from TCI (Tokyo, Japan). Hydrochloric acid (25%) was purchased from Th. Geyer (Renningen, Germany). 3′-*O*-Methylepicatechin and 4′-*O*-methylepicatechin were purchased from Toronto Research Chemicals (Toronto, Canada). Sodium chloride (100%) and acetonitrile (99.9%) were purchased from VWR Chemicals (Radnor, PA). All numbers given in parentheses represent the minimum purity.

2.2. Cultivation and Isolation of Mycelium from *L. edodes*. A *L. edodes* culture was obtained from the Westerdijk Fungal Biodiversity Institute (Utrecht, The Netherlands, collection number: CBS 389.89). The fungus was grown on malt extract agar (15 g/L malt extract, 15 g/L agar−agar in deionized water (dH_2O)) for 7 days at 24 °C until the Petri dish was overgrown. 100 mL of sterile malt extract peptone (MEP) medium (30 g/L malt extract, 3 g/L peptone from soy in dH_2O) in a 250 mL shaking flask was inoculated with ∼0.5 cm^2 of grown mycelium and homogenized for 30 s at 10,000 rpm (Ultraturrax, IKA, Staufen, Germany). After 6 days of incubation at 24 °C on a rotary shaker (150 rpm, 25 mm shaking diameter; Orbitron, Infors, Einsbach, Germany) in the dark, the preculture was homogenized, and 20 mL of the homogenate was transferred to 200 mL MEP medium (in 500 mL shaking flasks). Main cultures were initially incubated for 7 days. To evaluate the optimal cultivation time for the targeted OMT activity, main cultures were incubated for up to 26 days, and mycelia were harvested every day (starting from the second day after inoculation). For successive experiments, mycelia from main culture day 10 were used. Mycelia were separated from the culture supernatant by means of vacuum filtration, washed twice with dH_2O, and subsequently freeze-dried for 3−4 days (1 mbar, −50 °C; α 1−2 LD Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany). The cultivation of further Basidiomycota (*F. velutipes*, *H. tessulatus*, *L. edodes*, *L. lateritia*, *N. cladopus*, *N. squarrosulus*, *P. chrysosporium*, *P. sapidus*, *S. crispa*) was performed as described above (for collection numbers of fungi, see Supporting Information Table S1).

2.3. Biotransformation and Extraction. Lyophilized mycelium was mortared to a fine powder and suspended in Tris-HCl buffer (50 mM, pH 8.0, 5% glycerol, 2 mM $MgCl_2$) to a final concentration of 10 mg/mL. After vigorous mixing for 60 s, the insoluble cell fraction was removed by centrifugation (14,000*g*, 15 min, 4 °C). Enzymatic reactions were carried out with 980 μL of cell-free extract and 200 μM substrate (eriodictyol or eriodictyol dihydrochalcone; substrate screening: protocatechuic aldehyde, caffeic acid, dihydrocaffeic acid, *m*-coumaric acid, *p*-coumaric acid, piceatannol, butein, (−)-epicatechin, or quercetin) and 400 μM *S*-adenosyl-L-methionine (SAM) (final reaction volume: 1 mL) in 2 mL microcentrifuge tubes on an orbital shaker (180 rpm) at 36 °C for 1 h, if not stated otherwise. The reaction was terminated by addition of 10 μL of HCl (4 M). For product extraction, samples were supplemented with 100 mg of NaCl and 5 μL of internal standard (−)-epicatechin (20 mM in DMSO) and extracted twice with 800 μL of ethyl acetate. The dehydrated (with Na_2SO_4) organic phase was evaporated under a gentle stream of nitrogen and the residue was dissolved in 1:1 methanol/ultrapure H_2O (0.1% formic acid), membrane-filtered (0.4 μm, nylon), and transferred to a 1.5 mL glass vial.

2.4. Identification of *O*-methyltransferases in *L. edodes* Mycelium by Bottom-up Proteomics. Proteins of the mycelial cell-free extract from *L. edodes* (prepared as described above, 20 mg/mL from cultivation day 10) were purified by means of FPLC using a HiPrep DEAE FF column 16/10 (Cytiva, Marlborough, MA) for anion-exchange (AEX) chromatography with a column volume (CV) of 160 mL and a HiLoad 16/600 Superdex 200 column (Cytiva) for size-exclusion (SEC) chromatography. AEX chromatography was performed at a flow rate of 1 mL/min with a gradient elution of buffer A (50 mM Tris-HCl, pH 8.0) and buffer B (50 mM Tris-HCl, pH 8.0, 500 mM NaCl): 0 min, 0% B; 50 min, 0% B; 130 min, 100% B; and 150 min, 100% B. The collected protein fractions were desalted (using buffer A) and concentrated using Amicon Ultra-15 centrifugal filters (10 kDa MWCO; Merck). OMT activity assays were performed with each fraction as described in Section 2.6. Fractions of interest were applied for SEC chromatographic purification with an isocratic elution with Tris-HCl buffer (50 mM, pH 8, 100 mM NaCl) at a flow rate of 0.5 mL/min. The obtained protein fractions were desalted and assayed for the OMT activity. Protein molecular masses and purity of the fractions were assessed by means of SDS-PAGE analyses according to Laemmli.[41]

The protein fractions from AEX and SEC chromatography were digested with trypsin.[42] The peptides were desalted and concentrated using C_{18} ZipTip pipet tips (Merck) according to the manufacturer's instructions. The samples were analyzed by LC-MS/MS according to Brandt and Brognaro et al.[43] The obtained data from peptide analysis by means of LC-MS/MS measurements were analyzed with Proteome Discoverer 3.0 software (Thermo Fisher Scientific), including the search engine Sequest HT. Thereby, sequences of *L. edodes* genome (GenBank assembly: GCA_002003045.1) were used to identify the mycelial proteins.

2.5. Heterologous Expression of *Le*OMT-Encoding Genes in *E. coli* and Protein Purification. Synthetic, codon-optimized (for expression in *Escherichia coli*) genes encoding for the putative *O*-methyltransferases *Le*OMT1 (GenBank accession number: XP_046089355), *Le*OMT2 (XP_046082518), and *Le*OMT3 (XP_046081978) from *L. edodes* were synthesized and each subcloned with an *N*-terminal His_6-tag into a pET28a(+) vector (pET28a(+)-*Le*OMT1, pET28a(+)-*Le*OMT2, pET28a(+)-*Le*OMT3; TWIST Bioscience, South San Francisco, CA). Electrocompetent *E. coli* BL21(DE3) cells were transformed using pET28a(+)-*Le*OMT1, pET28a(+)-*Le*OMT2, and pET28a(+)-*Le*OMT3 by electroporation. Integrity of the generated strains was confirmed by sequencing. For heterologous expression, transformed cells were grown in 1000 mL baffled shake flasks (37 °C, 180 rpm) containing 200 mL of LB medium (30 μg/mL neomycin) until an OD_{600} of 0.6−0.7 was reached. Expression of the *Le*OMT-encoding genes was then induced by addition of 1 mM IPTG. The cultures were incubated for 18 h (18 °C, 180 rpm), and subsequently, cells were harvested by centrifugation (4200*g*, 15 min, 4 °C) and stored at −20 °C. The

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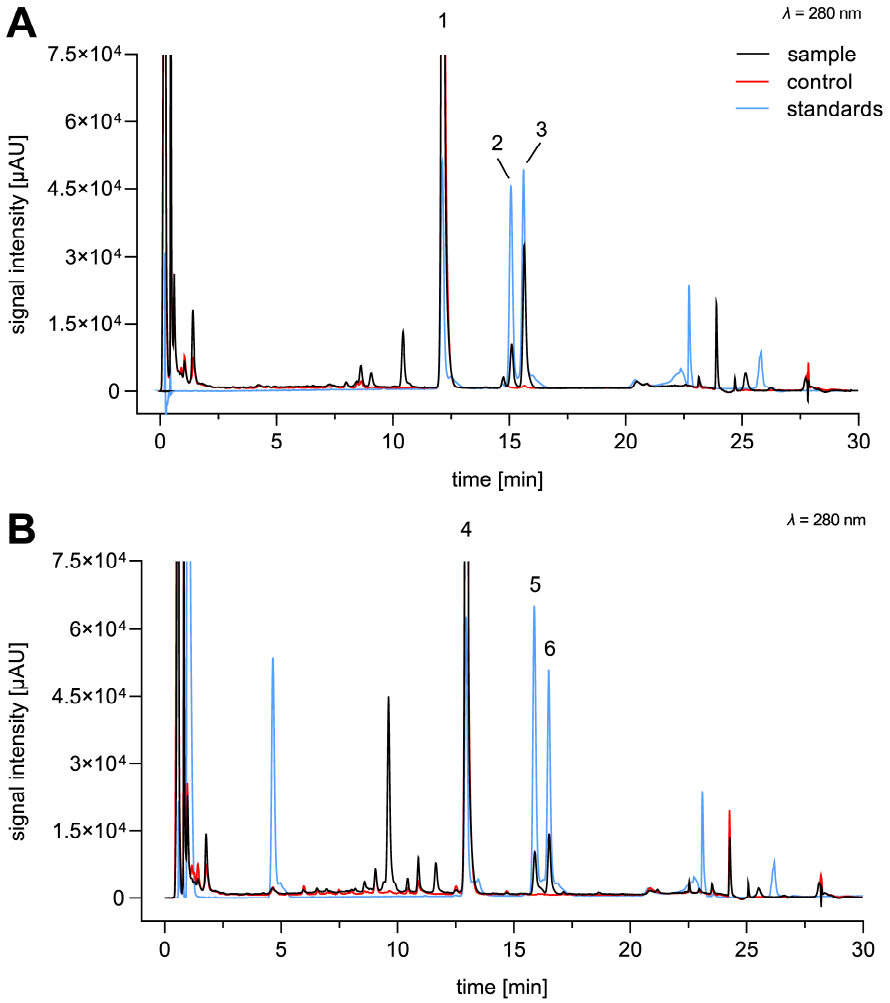

Figure 2. Chromatograms (RP-HPLC-DAD) of reaction products from biotransformation of eriodictyol (A) or eriodictyol dihydrochalcone (B) by means of mycelial extracts of *L. edodes* (extraction of 10 mg per mL of 50 mM PO_4^{3-} buffer pH 7.5, 5% glycerol, 2 mM $MgCl_2$). Reactions were performed with 200 μM substrate and 400 μM SAM at 24 °C for 18 h. Control variants were prepared with heat-inactivated mycelial extract (20 min, 95 °C). Standards = each 12.5 μM authentic standards. 1 = eriodictyol, 2 = homoeriodictyol, 3 = hesperetin, 4 = eriodictyol dihydrochalcone, 5 = homoeriodictyol dihydrochalcone, 6 = hesperetin dihydrochalcone.

recombinant enzymes were purified using immobilized metal ion affinity chromatography (IMAC), applying a fast protein liquid chromatography (FPLC) system (NGC Quest 10, Bio-Rad Laboratories, Hercules, CA) equipped with a HisTrap High-Performance Ni-NTA column (5 mL; Cytiva). *E. coli* cells were thawed on ice and resuspended in a mixture of 92% binding buffer (50 mM phosphate buffer, pH 7.4, 300 mM NaCl) and 8% elution buffer (binding buffer including 500 mM imidazole) to a final cell concentration of 25% (w/v). Cells were disrupted by means of an ultrasonic probe (MS72, Bandelin Sonopuls, Berlin, Germany) and centrifuged for 15 min at 14,000*g* and 4 °C. The resulting cell-free extract was subjected to FPLC purification. After a washing step with five CVs of 8% elution buffer with a flow rate of 1.5 mL/min, elution of the target protein was performed with three CVs of 100% elution buffer. The protein-containing fractions were pooled and desalted using three connected HiTrap Desalting Sephadex G-25 columns (3 × 5 mL; Cytiva) with a flow rate of 2 mL/min (desalting buffer: 50 mM Tris-HCl, pH 8.0, 5% glycerol, 2 mM $MgCl_2$). For quality control of the expression and purification, SDS-PAGE analysis was conducted according to Laemmli.[41] Protein concentrations were determined by means of Bradford assay using bovine serum albumin (BSA) as the reference protein.[44]

2.6. Biocatalysis with Purified Enzymes. For determination of the enzyme activity against targeted flavonoids and further substrates, 200 μg/mL purified enzyme was assayed with 200 μM substrate and 400 μM SAM in 500 μL of total reaction volume (50 mM Tris-HCl, pH 8.0, 5% glycerol, 2 mM $MgCl_2$) for 1 h at 36 °C with shaking at 180 rpm. The reaction was terminated by addition of 5 μL of HCl (4 M) and 500 μL of methanol and stored on ice for 1 h to precipitate proteins. The samples were centrifuged (14,000*g*, 15 min, 4 °C), membrane-filtered (0.45 μm), and transferred to 1.5 mL glass vials.

2.7. Chromatographic Analysis and Product Quantitation. Analytes were measured by means of reversed-phase high-performance liquid chromatography coupled to a diode array detector (RP-HPLC-DAD) using a modular instrumental setup by Shimadzu (Nakagyo-ku, Japan), consisting of a CBM-20A communication bus module, an LC-20AD pump unit, an SIL-20AC HT autosampler, a CTO-20AC column oven, and an SPD-M20A detector. Extracts obtained from biotransformations were quantified by means of response factors calculated from authentic standards and the spiked internal standard (−)-epicatechin (or eriodictyol for the quantitation

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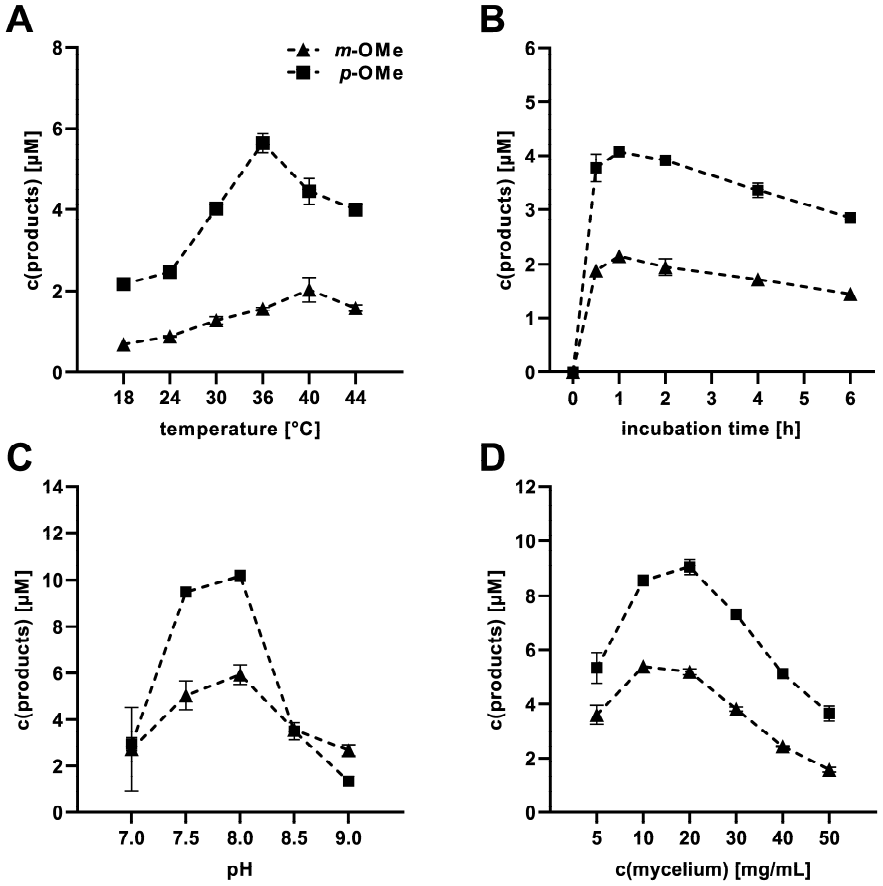

Figure 3. Optimal reaction parameters for mycelial-mediated *O*-methylation of eriodictyol dihydrochalcone (200 μM). Temperature (A): 10 mg/mL mycelium, 50 mM PO_4^{3-} pH 7.5, 18 h; incubation time (B): 10 mg/mL mycelium, 50 mM PO_4^{3-} pH 7.5, 36 °C; pH conditions (50 mM PO_4^{3-} for pH 7.0, 50 mM Tris-HCl for pH 7.5–9.0) (C): 10 mg/mL mycelium, 36 °C, 1 h; and concentrations used for production of mycelial crude extracts (D): 50 mM Tris-HCl pH 8, 36 °C, 1 h. Values represent means and standard deviations of triplicate experiments.

of (−)-epicatechin). Reactions utilizing purified *Le*OMTs were quantified by means of external calibration. RP-HPLC-DAD measurements were conducted using an Atlantis T3 column (Waters, Milford, MA) with dimensions 100 mm × 3 mm × 5 μm and a gradient elution using the following mobile phases: A, ultrapure H_2O + 0.1% formic acid; B, acetonitrile starting with 10% B for 1 min followed by a linear increase to 40% B within 20 min. The flow rate was set to 1 mL/min, the column oven temperature was 30 °C and the injection volume was 20 μL. The DAD monitored the absorbance between λ = 230 and 500 nm. Analytes were quantified at λ = 280 nm. Identities of the biotransformation products were confirmed by means of LC-HR-MS measurements and a comparison to authentic reference standards. Therefore, an Acquity Iclass plus UPLC system (Waters) coupled to a Bruker impact II (Bruker Corporation, Billerica, MA) was employed, equipped with a Kinetex RP-C18 column (100 × 2.1 × 1.2 μm^3; Phenomenex, Aschaffenburg, Germany). Analyses were performed using a gradient elution starting with 100% buffer A (ultrapure H_2O + 0.05% formic acid) and a linear increase of eluent B (acetonitrile + 0.05% formic acid) to 95% within 22 min. The flow rate was set to 0.55 mL/min, the column temperature to 50 °C, and the injection volume to 0.5 μL. The electrospray ionization (ESI) parameters were as follows: capillary voltage, 4.5 kV (negative mode) and 3.0 kV (positive mode); end plate offset, 500 V; nebulizer, 4 bar; source temperature, 220 °C; and desolvation gas flow rate, 8 L/min. Mass spectra were obtained in the scan range of 50–1600 m/z and a spectra rate of 12 Hz.

3. RESULTS AND DISCUSSION

3.1. *O*-methyltransferase Activity in Mycelial Extracts.

An initial screening for *O*-methylating activity in mycelia of various Basidiomycota (*F. velutipes*, *H. tessulatus*, *L. edodes*, *L. lateritia*, *N. cladopus*, *N. squarrosulus*, *P. chrysosporium*, *P. sapidus*, and *S. crispa*) revealed that only *L. edodes* mycelia showed significant activity against the target substrates eriodictyol and eriodictyol dihydrochalcone. Minor *O*-methylation activity was observed in the mycelia of *H. tessulatus*, *L. lateritia*, *N. squarrosolus*, and *N. cladopus*. No signals were detected corresponding to dimethylated products or for modifications other than those on the B-ring.

As *L. edodes* was the most promising candidate to produce the target compounds, further experiments were conducted solely by using this fungus. Bioconversions of eriodictyol and eriodictyol dihydrochalcone led to the appearance of the *meta*-*O*-methylated (*m*-OMe) regioisomers homoeriodictyol and homoeriodictyol dihydrochalcone, as well as the *para*-*O*-methylated (*p*-OMe) products hesperetin and hesperetin dihydrochalcone (Figure 2). The ratio of *m*-OMe to *p*-OMe products for eriodictyol was 1:3 and that for eriodictyol dihydrochalcone was 1:2, indicating a preference toward the *para*-hydroxy group. However, it is important to note that a crude mycelial extract was applied, in which multiple OMTs

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with different chemo- and regiospecific properties may be present. While OMTs are generally considered to be regiospecific,[45] the observed variability is not uncommon, as most of the previously described OMTs showed considerable activity on more than one hydroxy group or were even able to catalyze multiple *O*-methylation reactions within the same molecule.[35,19,46] Here, no dimethylated products were detected and *O*-methylation exclusively appeared on OH-groups of the B-ring.

3.2. Optimization of Reaction Conditions and Fungal Cultivation. In order to characterize the OMT activity found in the cultivated mycelium of *L. edodes* and to optimize its biocatalytic efficiency, several reaction parameters were investigated including temperature, incubation time, pH, and mycelium concentration (Figure 3). The optimum reaction temperature was 36 °C and the maximum product concentration was reached after 60 min and decreased with prolonged incubation. This is possibly due to side reactions catalyzed by flavonoid-degrading enzymes, such as laccases or peroxidases, which could be present in the crude mycelial extract, as they are known to be key enzymes of lignin-degrading Basidiomycota.[47]

The highest product yield was observed at pH = 8.0. Remarkably, the regioisomeric ratio of the products was found to change depending on the pH value. At pH 7.5 and pH 8.0, the ratio of *m*- to *p*-OMe products was 1:2, while it was equimolar at pH 8.5 and even resulted in an excess of the *m*-OMe product at pH 9.0. These observations align with reported findings that the protonation state of the acceptor group plays a crucial role in catalytic activity; variations in the protonation of hydroxy groups can significantly alter the binding and spatial orientation of the substrate, ultimately influencing the regioselectivity.[48] The optimum mycelial concentration for the production of the crude extract was determined to be 20 mg of mycelium per mL of extraction buffer. Higher concentrations led to decreased product yields (up to −60%) compared to the optimum, potentially due to proportionally elevated input of, e.g., interfering enzymes, such as peptidases, or product degrading laccases and peroxidases. Fungal growth and OMT activity were observed to be a function of the cultivation time (Figure 4). Growth kinetics of the fungus were monitored by assessing the dry fungal biomass during the cultivation period. The strongest fungal biomass formation was observed between days 2 and 14, after which there was only a minimal increase in biomass, and later on, a decrease was observed. Mycelium isolated on day 10 exhibited the highest OMT activity, resulting in total product yields of 20.8 ± 0.7 μM from bioconversion of eriodictyol dihydrochalcone, which represented a 3-fold increase compared to mycelium isolated on day 6 during the rapid growth phase. Subsequently, enzyme activity decreased by approximately 50% and remained relatively constant for the subsequent time points studied.

3.3. Chemo- and Regioselectivity of Mycelial OMT Activity. To get a deeper insight into the chemo- and regioselectivity of the newly discovered OMT activity of *L. edodes* mycelia, in addition to eriodictyol and eriodictyol dihydrochalcone, a number of structurally diverse phenolic/catecholic substrates were tested. Besides further flavonoids (butein, epicatechin, quercetin), a stilbene (piceatannol), phenylpropanoids (caffeic acid, *m*-/*p*-coumaric acid), and protocatechuic aldehyde were subjected to substrate screening (Figure 5).

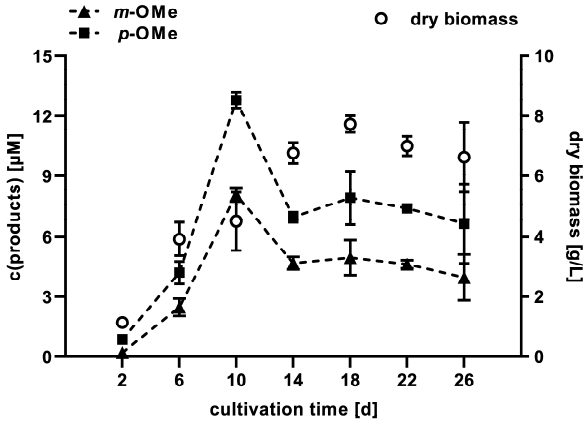

Figure 4. Growth profile (determined as dry biomass of isolated mycelium per liter culture medium) and *O*-methyltransferase activity of the mycelium (determined as product concentrations from biotransformation of eriodictyol dihydrochalcone using the respective mycelial extracts) in the course of submerged cultivation of *L. edodes*. Reactions were performed with 1 mL cell-free mycelial extract, 200 μM substrate, and 400 μM SAM for 1 h at 36 °C (n = 3). Values represent means of biological triplicates with standard deviations.

Both the herein identified OMT activity from the crude extract of *L. edodes* and the *F. velutipes* OMT (*Fv*OMT) previously characterized by Kirita et al.[35] showed catalytic activity exclusively toward substrates with vicinal hydroxy groups; thus, they can be classified as catechol *O*-methyltransferases (COMTs). The *L. edodes* OMT activity showed a high degree of substrate promiscuity, which is in line with reports from *Fv*OMT.[35] Both enzyme activities were capable of transforming a variety of catechol-moiety-containing substance classes. The substrate scope of the OMT activity found in *L. edodes* mycelium revealed a considerable conversion rate for protocatechuic aldehyde to its *m*-OMe product vanillin (22.3 ± 0.5% yield). Interestingly, the *p*-OMe product isovanillin was not detected at all, which indicates strict regioselectivity for the *meta*-position. The conversion of caffeic acid resulted in similar yields (15.0 ± 0.4%) with a high selectivity for the *meta*-position with a regioisomeric ratio of 95:5 (*m*-OMe/*p*-OMe). Among the flavonoids, biotransformation of chalcone butein showed the highest product yield (20.0 ± 0.5%). Conversely, for the structurally closely related eriodictyol dihydrochalcone, the yield was 2.5-fold higher and the regiospecificity differed clearly, exhibiting a surplus of the *m*-OMe product homobutein (65:35), while eriodictyol dihydrochalcone was mainly converted to the targeted *p*-OMe hesperetin dihydrochalcone (36:64). The *m*-OMe product homoeriodictyol dihydrochalcone, however, has been reported to show bitter-masking properties, which makes it an interesting product as well.[49] The conversion of eriodictyol resulted in a total product yield of 14.9 ± 0.2% with a ratio of 24:76 of the taste-active reaction products hesperetin and homoeriodictyol. Interestingly, the transformation of the structurally similar flavonoids, such as (−)-epicatechin and quercetin, resulted in low product yields (<5%). Signals corresponding to dimethylated products were not detected for any of the substrates.

3.4. Identification of *O*-methyltransferases in *L. edodes* Mycelium Extract by Bottom-up Proteomics. To identify potential OMT candidates, the crude extract

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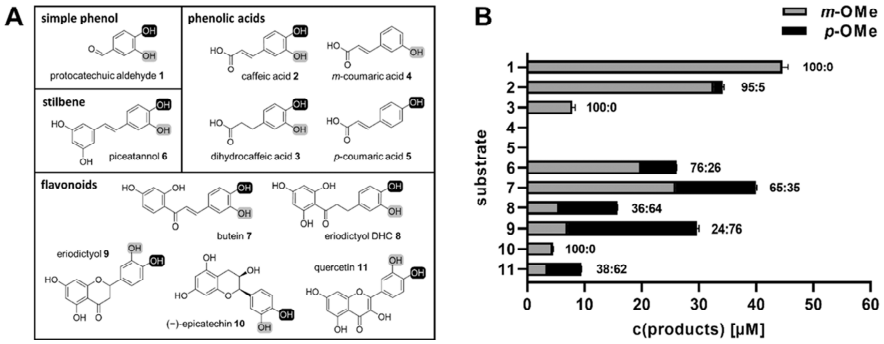

Figure 5. Product concentrations and regioisomeric ratios (% *m*-OMe/*p*-OMe) (B) from biotransformations of phenolic compounds (A) by *O*-methyltransferase activity from *L. edodes* mycelium. Reactions were performed with 1 mL of cell-free mycelial extract containing 200 μM substrate and 400 μM SAM for 1 h at 36 °C (n = 3) (180 rpm shaking). Products were quantified by means of RP-HPLC-DAD and compounds' identities were verified by means of LC-HR-MS analyses; DHC, dihydrochalcone.

obtained from *L. edodes* lyophilized mycelium was subjected to anion-exchange chromatography (Supporting information Figure S1 A). Following desalting and concentration, each of the six obtained fractions was tested for the OMT activity. Fraction 1 (AEX-1) revealed the targeted OMT activity in the presence of the model substrate caffeic acid, resulting in the formation of ferulic acid and a minor signal for isoferulic acid (Figure 6B). A single intense band corresponding to a protein with a molecular mass between 25 and 30 kDa was detected by SDS-PAGE analysis (Figure 6A).

For additional purification, fraction 1, providing the desired OMT activity, was subjected to size-exclusion chromatography (Supporting Information Figure S1 B), which resulted in separation of three fractions (SEC-1 to SEC-3). SDS-PAGE analysis revealed a single band at ~25 kDa in SEC-1 and SEC-3 but no visible band in SEC-2 (Figure 6A). OMT activity was detected only in SEC-1 (Figure 6C).

Tryptic digestion was performed in-solution using desalted and concentrated fractions AEX-1, SEC-1, and SEC-3. For in-gel digestion, the corresponding bands were excised from the SDS-PAGE (Supporting Information Figure S2). By mass spectrometric analysis, unique peptides of the putative OMT encoded by the genes annotated in the GenBank database XP_046089355 (*Le*OMT1), XP_046082518 (*Le*OMT2), and XP_046081978 (*Le*OMT3) (see Supporting Information Tables S2−S4) were identified in all analyzed fractions (AEX-1, SEC-1, and SEC-3). All tested protein fractions showed a predominance of peptide signals corresponding to *Le*OMT3, which suggests a major contribution to the detected overall OMT activity in the purified protein fractions and thus the *L. edodes* mycelium itself. However, as considerable signals corresponding to *Le*OMT1 and *Le*OMT2 specific peptides were also detected, all of the identified OMT candidates were taken into account for further investigations.

3.5. Heterologous Expression and Catalytic Activity of *L. edodes* OMTs. The synthetic, codon-optimized genes encoding for the identified OMT candidates *Le*OMT1, *Le*OMT2, and *Le*OMT3 were successfully expressed in *E. coli* and purified to electrophoretic homogeneity (Supporting Information Figure S3). Results from SDS-PAGE analysis revealed a single intense band for each variant corresponding to molecular masses of 25−30 kDa, which is in accordance with the expected masses of the proteins.

The purified *Le*OMT candidates were assayed for their biocatalytic activity against the target compounds eriodictyol and eriodictyol dihydrochalcone. All three recombinant enzymes revealed *O*-methylation activity in the presence of the substrates eriodictyol and eriodictyol dihydrochalcone as well as the model substrate caffeic acid (Table 1). Interestingly, all three enzymes were unable to convert *p*-coumaric acid to its *O*-methylated analogue, which is in accordance with the level of the OMT activity found in the mycelium. This substantiates the assumption that they represent the key enzymes of the mycelial OMT activity. *Le*OMT1 reached total product yields (*m*-OMe + *p*-OMe) of around 2% for eriodictyol (2.2 ± 0.2%), eriodictyol dihydrochalcone (1.7 ± 0.1%), and caffeic acid (3.0 ± 0.2%). *Le*OMT2 revealed a clearly higher catalytic activity for the substrates, with product yields of 80.1 ± 2.7% for eriodictyol, 68.4 ± 2.5% for eriodictyol dihydrochalcone, and 91.5 ± 1.0% for caffeic acid. *Le*OMT3 catalyzed reactions resulted in product yields of 38.1 ± 1.0% for eriodictyol, 42.0 ± 0.9% for eriodictyol dihydrochalcone, and 47.3 ± 0.1% for caffeic acid. Interestingly, the regiospecificity of all tested recombinant OMTs *Le*OMT1, *Le*OMT2, and *Le*OMT3 differed from the mycelial extract's OMT activity. The regioisomeric ratios (*m*-OMe/*p*-OMe) of the reaction products with eriodictyol were 46:54 (*Le*OMT1), 51:49 (*Le*OMT2), and 43:57 (*Le*OMT3), with eriodictyol dihydrochalcone 39:61 (*Le*OMT1), 44:56 (*Le*OMT2), and 35:65 (*Le*OMT3) and with caffeic acid 100:0 (*Le*OMT1), 78:22 (*Le*OMT2), and 87:13 (*Le*OMT3). Thereby, *Le*OMT3 showed the highest similarity with the regiospecificity of the mycelial OMT activity, which suggests its native appearance in the mycelium to play a major role in the observed OMT activity. In order to improve the regioselectivity toward the target flavonoids, a protein engineering approach could be a viable tool, as has been recently demonstrated.[12] As *Le*OMT2 showed the highest catalytic activity against the targeted substrates, and it was regarded as the most promising candidate for future applications as a recombinant enzyme. Therefore, further biochemical and kinetic parameters were evaluated (Supporting Information Figure S4). Interestingly, the results showed comparable values to the OMT activity found in the fungal mycelium with a pH optimum at pH 8.0 and a temperature optimum of 36 °C. In both cases, the enzymatic conversions reached maximum product concentrations after 60 min. However, other than those observed for the biotransformations using mycelial extracts, product concentrations did not decrease but remained constant with prolonged incubation. Enzyme kinetic values for *Le*OMT2

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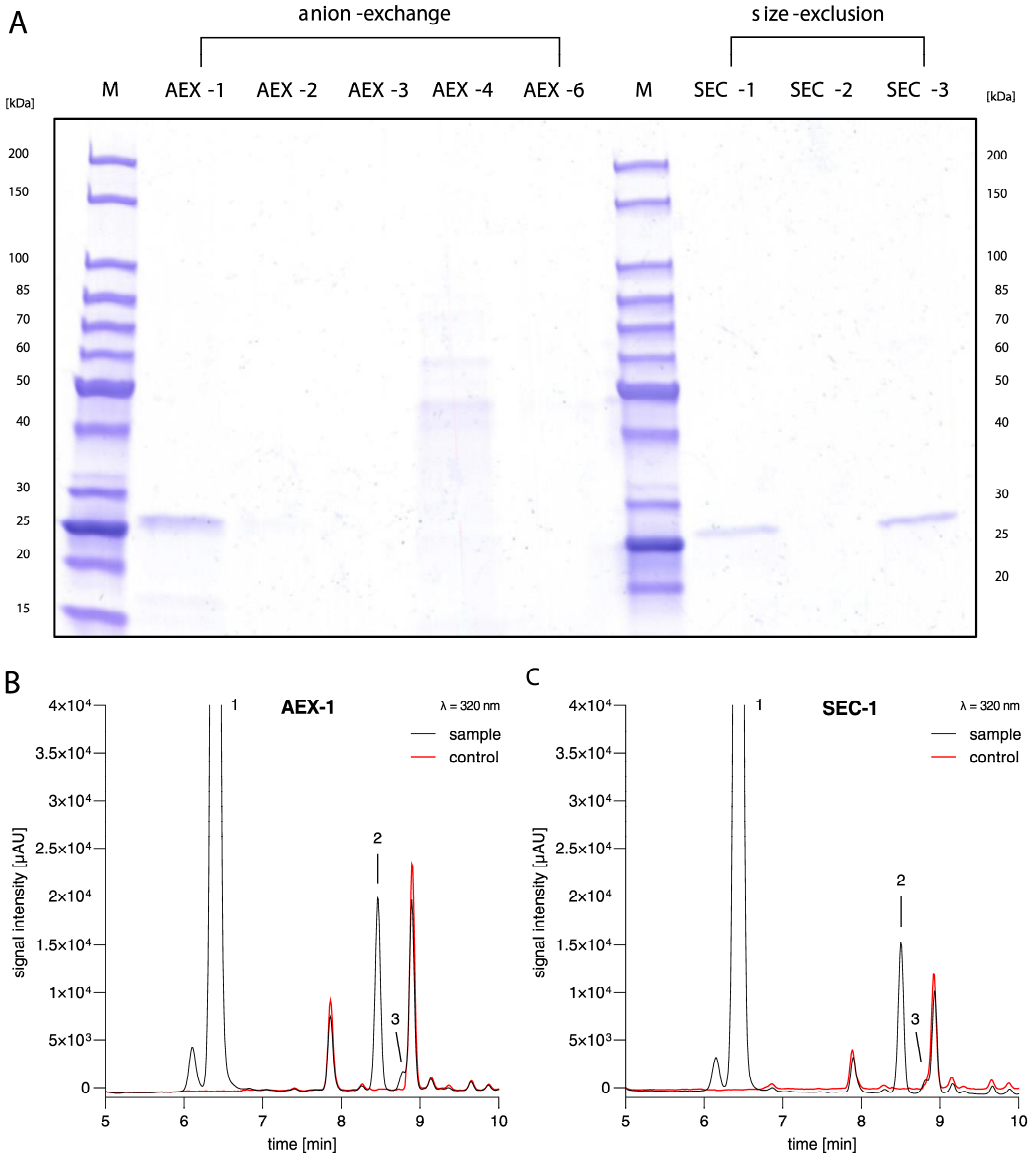

Figure 6. Protein fractions obtained from *L. edodes* mycelial extract by anion-exchange (AEX) and subsequent size-exclusion (SEC) chromatography. Desalted and concentrated fractions were applied for SDS-PAGE (A) and biotransformation of 200 μM caffeic acid (model substrate), 400 μM SAM, 1 h, 36 °C, followed by HPLC analysis (B,C).

Table 1. Product Concentrations (*meta*/3′-OMe and *para*/4′-OMe Products) and Regioisomeric Ratios (*m*-OMe/*p*-OMe) Determined from Biocatalyses of 200 μM Eriodictyol, Eriodictyol Dihydrochalcone, or Caffeic Acid with Purified Recombinant OMTs from *L. edodes* (*Le*OMT1, *Le*OMT2, and *Le*OMT3)[a]

[μM]	*Le*OMT1			*Le*OMT2			*Le*OMT3		
substrate	*m*-OMe	*p*-OMe	ratio [%]	*m*-OMe	*p*-OMe	ratio [%]	*m*-OMe	*p*-OMe	ratio [%]
eriodictyol	2.0 ± 0.2	2.4 ± 0.1	46:54	81.9 ± 2.6	78.2 ± 2.7	51:49	32.8 ± 1.0	43.8 ± 1.1	43:57
eriodictyol dihydrochalcone	1.4 ± 0.1	2.2 ± 0.0	39:61	60.3 ± 1.9	76.5 ± 3.0	44:56	30.2 ± 0.8	55.5 ± 1.0	35:65
caffeic acid	6.0 ± 0.3	<0.1	100:0	142.2 ± 1.5	40.8 ± 0.6	78:22	78.4 ± 0.0	11.8 ± 0.1	87:13

[a]Shown are average values and standard errors of at least two enzyme reaction replicates.

against eriodictyol, eriodictyol dihydrochalcone, and caffeic acid were determined by means of Michaelis−Menten function (Table 2, Supporting Information Figure S6).

The present study highlights a novel *O*-methyltransferase activity in the mycelium of the edible fungus *L. edodes*, which is capable to transform a variety of phenolic compounds with the catechol moiety to their *O*-methylated analogues. In particular,

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Table 2. Michaelis–Menten Kinetic Constants of the Recombinant OMT from *L. edodes* *Le*OMT2 against Several Substrates[a]

substrate	K_m [μM]	V_{max} [μM/s]	k_{cat} [1/s]	(k_{cat}/K_m)
eriodictyol	87.7 ± 18.3	0.2437 ± 0.0152	0.0337	3.84×10^{-4}
eriodictyol dihydrochalcone	136.4 ± 15.4	0.2954 ± 0.0116	0.0408	2.99×10^{-4}
caffeic acid	184.7 ± 32.3	0.4482 ± 0.0308	0.0620	3.35×10^{-4}

[a]Shown are average values and standard errors of at least two reaction replicates.

taste-active flavonoids containing an isovanillyl motif, such as hesperetin and hesperetin dihydrochalcone, were successfully generated. The newly developed biocatalytic approach using fungal mycelium as a biocatalyst is a viable alternative to chemical methylation by reducing the reliance on synthetic additives and adhering to Green Chemistry principles. Furthermore, it aligns with the growing preference for food ingredients and additives of a natural origin. By means of a bottom-up proteomics approach, three OMTs were identified and heterologously expressed and showed high biocatalytic activity, which offers the possibility to use the isolated enzymes as a biocatalyst for the production of valuable flavor ingredients.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.4c01514.

Screening for OMT activity in mycelia of various basidiomycota; proteomics results from purified mycelial extract of *L. edodes*; results from LC-MS analyses of reaction products from *L. edodes* mycelial OMT activity and recombinant *Le*OMT2; FPLC chromatograms of purification of OMTs from *L. edodes* mycelial extract; SDS-PAGE of obtained fractions from FPLC purification and recombinant *Le*OMT1–3; optimization of reaction conditions of recombinant *Le*OMT2; chromatograms of reaction products from recombinant *Le*OMT1–3; Michaelis–Menten kinetics plot of *Le*OMT2 against eriodictyol, eriodictyol dihydrochalcone, and caffeic acid (PDF)

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■ ABBREVIATIONS

NHDC, neohesperidin dihydrochalcone; OMT, *O*-methyltransferase; SAM, *S*-adenosyl-L-methionine; dI_2O, deionized water; MEP, malt extract peptone; IMAC, immobilized metal ion affinity chromatography; FPLC, fast protein liquid chromatography; CV, column volume; RP-HPLC, reversed-phase high-performance liquid chromatography; DAD, diode array detector; LC-HR-MS, liquid chromatography-high resolution-mass spectrometry; ESI, electrospray ionization; AEX, anion-exchange; SEC, size-exclusion chromatography; *m*-OMe, *meta*-*O*-methylated; *p*-OMe, *para*-*O*-methylated; COMT, catechol *O*-methyltransferase

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