Characterisation of sustainably produced aroma mixtures

Cumulative Dissertation

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“Wissenschaft fängt eigentlich erst da an interessant zu werden, wo sie aufhört.”

Justus von Liebig

(1803-1873)

Declaration

I declare that I have completed this dissertation single-handedly without the unauthorized help of a second party and only with the assistance acknowledged therein. I have appropriately acknowledged and cited all text passages that are derived verbatim from or are based on the content of published work of others, and all information relating to verbal communications. I consent to the use of an anti-plagiarism software to check my thesis. I have abided by the principles of good scientific conduct laid down in the charter of the Justus Liebig University Giessen "Satzung der Justus-Liebig-Universität Gießen zur Sicherung guter wissenschaftlicher Praxis" in carrying out the investigations described in the dissertation.

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Date, Place Signature

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Summary

In this work, new sources for sustainably produced flavourings were identified.

In the first part of the presented thesis, a fermentation medium of *Ashbya gossypii*, which is a waste product from the industrial biotechnological production of riboflavin (vitamin B_2), was investigated for the presence of aroma-active compounds, and more than 1 g/L of volatile aroma-active compounds were detected. For instance, 367 mg of 2- and 3-methylbutanol were determined per L fermentation medium. In addition, 2-phenylethanol was identified with a content of 473 mg/L. These three compounds are typical products of the Ehrlich pathway, which is a metabolic pathway of amino acids in yeasts. In addition, remarkable amounts of various γ-lactones were detected. These included γ-decalactone, γ-dodecalactone as well as some unsaturated derivatives thereof, such as (*Z*)-γ-dec-7-enlactone or (*Z*)-γ-dodec-6-enlactone. In nature, these unsaturated lactones occur in low concentrations in peaches or nectarines, for example, and are partly responsible for their typical smell. Sample preparation was carried out by means of Solvent Assisted Flavour Evaporation (SAFE). This method involves a high-vacuum distillation in which an organic extract of the sample is passed to a high-vacuum apparatus in which solvent and volatile compounds evaporate immediately and are cryofocused by liquid nitrogen in another part of the apparatus. The non-volatile components of the organic sample extract, such as triglycerides, collect in a vessel below the sample inlet. Subsequently, the volatile compounds are concentrated, and the obtained flavour extract is analysed by gas chromatography-mass spectrometry-olfactometry (GC-MS-O). During the analysis of the extract, some pleasant-smelling compounds were detected, although the comparison of their mass spectra with those of the database did not provide meaningful results in all cases. Manual interpretation of the mass spectra showed that the unknown compounds were γ-lactones, bearing a double bond in the side chain. The compounds were isolated by high-performance liquid chromatography (HPLC) in order to determine the position of the double bond using Paternò-Büchi reaction. In this reaction, a carbonyl compound, e.g., 3-acetylpyridine, is used to undergo a photo-induced [2+2] cycloaddition on the double bond. Subsequently, fragment ions can be generated by analysing the products by means of MS/MS, whereby the mass of the fragment ions can provide information about the position of the double bond. Based on these results, the substances were subsequently synthesised. The products obtained were converted to Mosher esters and the diastereomers were separated by preparative HPLC, so that after subsequent elimination of the Mosher acids, the enantiopure lactones were available. Thus, the enantiomeric distribution of the lactones in the investigated sample could be determined. In addition, the odour impressions and odour thresholds in air of the racemates and the pure enantiomers were determined by GC-O.

In the second part of the thesis, another fermentation medium of an industrial process was investigated. Culture media of the bacterium *Basfia succiniciproducens* were derived from the biotechnological production of succinic acid. The analysis of the medium by means of Headspace-Solid Phase Microextraction-GC-MS-O (HS-SPME-GC-MS-O) revealed mainly alkylated pyrazines as interesting odour impressions. Alkylated pyrazines are highly potent aroma substances that occur mainly in highly heated foods. For example, alkylated pyrazines are formed during roasting or frying at temperatures above 140 °C in Maillard-type reactions from reducing sugars and amino acids. They are mainly responsible for the smell of foods like coffee or chocolate. Since the preliminarily identified substances were not commercially available as reference compounds, they were chemically synthesised, and quantification could be carried out by means of standard addition. In total, about 10 mg/L of alkylated pyrazines were determined in the fermentation medium.

The third project of this thesis addressed the characterisation of aroma substances from the electrochemical oxidation of (*R*)-limonene. Therefore, (*R*)-limonene was dissolved in ethanol, and, after the addition of a conductive salt, a current was applied via graphite electrodes, so that various oxidation products of limonene were formed. The resulting mixture of aroma substances was analysed by GC-MS-O to describe the odour impressions of the respective substances. Since the comparison of the mass spectra with the database did not yield any useful suggestions for most of the compounds, attempts were made to isolate the individual oxidation products by column chromatography and subsequently by preparative HPLC. Using various combinations of solvents, 17 substances could be isolated, and the structure of the substances was elucidated by NMR experiments and mass spectrometry. The isolated compounds represented mainly mono- and diethoxyethers of limonene, but also several keto compounds and acetals. In total, the 17 isolated compounds made up more than 95% of the total peak area of the chromatogram of the sample. In addition to these 17 isolates, limonene, carvone and *p*-cymene were identified. The odour impressions of the isolated compounds were described as fruity, citrus-like, herbaceous, fresh and floral, with nuances in various directions. Most of the isolated compounds have not been described in the literature previously.

All of the flavour mixtures investigated in this study were derived from sustainable production processes. In the industrial production of riboflavin, biotechnology has now completely replaced chemical synthesis. As a result, the use of chemicals has been drastically reduced. Since biotechnological production produces large quantities of fermentation media, the extraction of natural flavouring substances from them is an alluring option. Currently, 2- and 3-methylbutanol are chemically synthesised from butenes, but can also be obtained as natural aroma compounds from fusel oils produced by yeasts. 2-Phenylethanol is mostly synthesised chemically, whereby mainly benzene or its derivatives are transformed. However, 2-phenylethanol can also be obtained naturally through fermentation with yeasts. Of the lactones detected in the fermentation medium, only γ-decalactone is produced commercially by biotechnological conversion of ricinoleic acid. The unsaturated lactones are not yet available commercially, so that a sustainable and natural source of these substances is even more desirable.

Similarly, the chemical synthesis of succinic acid is performed by oxidation of 1,4-butanediol or through the hydrogenation of other C_4 acids, such as maleic or fumaric acid. However, biotechnological production by various microorganisms is also possible. A special characteristic of *B. succiniciproducens* is that this bacterium also is able to use crude glycerol as a carbon source. Glycerol accrues in large quantities during the production of biodiesel. Thus, a good coupling with the biotechnological production of succinic acid is possible. The alkylated pyrazines formed during fermentation are hardly available as natural flavouring substances. In the industrial production of natural pyrazines, foodstuffs such as potatoes, nuts or coffee are extracted in most cases. Therefore, recovery from the resulting fermentation medium may represent a profitable and more sustainable alternative.

Both fermentation processes have currently not been designed nor optimised for the production of flavourings, so that by improving the processes, much higher quantities of natural flavourings could be produced as co-products.

The electrochemical oxidation of limonene also represents a sustainable process. Limonene is available in large quantities, as it can be obtained from the fruit juice industry. Furthermore, the chemical oxidation of terpenes often involves the use of heavy metal catalysts and toxic and/or hazardous chemicals. None of these is necessary in the electrochemical oxidation of terpenes. In addition, a wide variety of different aroma substances can be generated quickly and inexpensively, with diverse

combination possibilities by varying the parameters (duration, voltage, type of reactants (different alcohols in combination with different terpenes)).

Zusammenfassung

Im Rahmen dieser Arbeit wurde versucht, neue Quellen für nachhaltig produzierte Aromastoffe zu finden.

Im ersten Projekt wurde das Fermentationsmedium von *Ashbya gossypii*, welches bei der industriellen biotechnologischen Produktion von Riboflavin als Nebenstrom anfällt, auf das Vorhandensein von aromaaktiven Verbindungen untersucht. Dabei wurden mehr als 1 g/L an flüchtigen aromaaktiven Verbindungen detektiert. So wurden Gehalte an 2- und 3-Methylbutanol von in Summe 367 mg/L Fermentationsmedium ermittelt. Zudem konnte 2-Phenylethanol mit einem Gehalt von 473 mg/L quantifiziert werden. Diese drei Verbindungen sind typische Produkte des Ehrlich pathyway, der ein typischer Stoffwechselweg von Aminosäuren in Hefen ist. Neben diesen Verbindungen wurden verschiedene γ-Lactone nachgewiesen. Hierbei handelte es sich u.a. um γ-Decalacton, γ-Dodecalacton und einige ihrer ungesättigten Derivate, wie z.B. (*Z*)-γ-Dec-7-enlacton oder (*Z*)-γ-Dodec-6-enlacton. In der Natur kommen diese ungesättigten Lactone beispielsweise in Pfirsichen oder Nektarinen in geringen Konzentrationen vor und sind für deren typischen Geruch mitverantwortlich. Die Probenaufarbeitung erfolgte dabei mittels *Solvent Assisted Flavour Evaporation* (SAFE). Diese Methode eignet sich gut, um flüchtige Verbindungen aus komplexen Matrizes abzutrennen. Dabei handelt es sich um eine Hochvakuumdestillation, bei der ein organischer Extrakt der Probe einer Hochvakuumapparatur zugeführt wird, in der Lösemittel und flüchtige Verbindungen sofort verdampfen und in einem weiteren Teil der Apparatur mit flüssigem Stickstoff kryofokussiert werden. Die nicht-flüchtigen Bestandteile des organischen Probenextrakts, wie z.B. Triglyceride, sammeln sich in einem Gefäß unter dem Einlass der Probe. Die flüchtigen Verbindungen werden anschließend konzentriert und der erhaltene Aromaextrakt mittels Gaschromatographie-Massenspektrometrie-Olfaktometrie (GC-MS-O) analysiert. Bei der Analyse des Extrakts konnten einige wohlriechende Verbindungen wahrgenommen werden, wobei jedoch der Abgleich der Massenspektren mit der Datenbank nicht in allen Fällen sinnvolle Ergebnisse lieferte. Bei der manuellen Auswertung des Massenspektren zeigte sich, dass es sich bei den unbekannten Verbindungen u.a. um γ-Lactone handeln musste, die eine Doppelbindung in der Seitenkette trugen. Die Verbindungen wurden mittels Hochleistungs-Flüssigchromatographie (HPLC) isoliert, um anschließend die Position der Doppelbindung mittels Paternò-Büchi-Reaktion zu bestimmen. Hierbei wird mit einer Carbonylverbindung, z.B. 3-Acetylpyridin, eine photoinduzierte [2+2]-Cycloaddition an der Doppelbindung durchgeführt. Anschließend können durch Analyse der Additionsprodukte mittels MS/MS Fragmentionen generiert werden, deren Masse Auskunft über die Position der Doppelbindung gibt. Auf Grundlage dieser Ergebnisse wurden die identifizierten Substanzen chemisch als Referenzverbindungen synthetisiert. Die erhaltenen Produkte wurden zu Mosher-Estern umgesetzt und die Diastereomere mittels präparativer HPLC getrennt, sodass nach anschließender Abspaltung der Mosher-Säuren die enantiomerenreinen Lactone zur Verfügung standen. Somit konnte die Enantiomerenverteilung der Lactone in der untersuchten Probe ermittelt werden. Zudem wurden auch die Geruchseindrücke und Geruchsschwellen der Racemate und der reinen Enantiomere in Luft mittels GC-O ermittelt.

Im zweiten Projekt wurde ein weiteres Fermentationsmedium eines industriellen Prozesses untersucht. Hierbei handelte es sich um das Medium der biotechnologischen Produktion von Bernsteinsäure mit dem Bakterium *Basfia succiniciproducens*. Bei der Analyse des Mediums mittels *Headspace-Solid Phase Microextraction*-GC-MS-O (HS-SPME-GC-MS-O) wurden mehrere interessante Geruchseindrücke wahrgenommen. Bei den olfaktorisch wahrgenommenen Verbindungen handelte es sich hauptsächlich um alkylierte Pyrazine. Alkylpyrazine sind häufig potente Aromastoffe, die v.a. in erhitzten Lebensmitteln vorkommen. So entstehen sie z.B. beim Braten oder Rösten bei Temperaturen

von über 140 °C im Zuge der Maillard-Reaktion aus reduzierenden Zuckern und Aminosäuren. Sie sind hauptsächlich für den Geruch von Lebensmitteln wie z.B. Kaffee oder Schokolade verantwortlich. Da die vorläufig identifizierten Substanzen nicht als Standardsubstanzen kommerziell erhältlich waren, wurden sie chemisch synthetisiert. Somit konnte eine Quantifizierung mittels Standardaddition durchgeführt werden. Die ermittelten Gehalte an alkylierten Pyrazinen betrugen im untersuchten Fermentationsmedium in Summe etwa 10 mg/L.

Das dritte Projekt dieser Arbeit behandelte die Charakterisierung von Aromastoffen aus der elektrochemischen Oxidation von (*R*)-Limonen. Dabei wurde (*R*)-Limonen in Ethanol gelöst und nach Zugabe eines Leitsalzes eine Spannung über Graphitelektroden angelegt, sodass diverse Oxidationsprodukte des Limonens gebildet wurden. Die entstandene Mischung an Aromastoffen wurde mittels GC-MS-O analysiert und die Geruchseindrücke der jeweiligen Stoffe beschrieben. Da der Abgleich der Massenspektren mit der Datenbank für die meisten Verbindungen keine sinnvollen Vorschläge erbrachte, wurden die Substanzen säulenchromatographisch vorgetrennt und anschließend mittels präparativer HPLC isoliert. Durch diverse Laufmittelkombinationen konnten insgesamt 17 Substanzen isoliert werden. Die Struktur der Substanzen wurde anhand von NMR-Experimenten und massenspektrometrischen Daten aufgeklärt. Bei den isolierten Verbindungen handelte es sich hauptsächlich um Mono- und Diethoxyether des Limonens, aber auch um verschiedene Ketoverbindungen und Acetale. In Summe machten die 17 isolierten Verbindungen mehr als 95% der Gesamtpeakfläche des Chromatogramms der Probe aus. Neben diesen 17 Isolaten wurden zudem noch Limonen, Carvon und *p*-Cymol identifiziert. Die Geruchseindrücke der isolierten Verbindungen wurden vor allem als fruchtig, citrusartig, kräuterig, frisch und blumig, mit Nuancen in verschiedenste Richtungen beschrieben. Bei den isolierten Verbindungen handelte es sich vorrangig um Aromastoffe, die in der Literatur noch nicht beschrieben waren.

Bei allen drei Projekten lag ein besonderes Augenmerk auf der Nachhaltigkeit der Produktion der Stoffe. Bei der industriellen Produktion von Riboflavin hat die Biotechnologie mittlerweile die chemische Synthese komplett abgelöst. Damit wurde der Einsatz an Chemikalien drastisch reduziert. Da bei der biotechnologischen Produktion jährlich große Mengen des Fermentationsmediums anfallen und die Nachfrage nach Riboflavin anhält, ist die Gewinnung von natürlichen Aromastoffen daraus hochinteressant, insbesondere wenn es gute Alternativen zur bisherigen Produktion der Aromastoffe darstellt. 2- und 3-Methylbutanol werden chemisch aus Butenen synthetisiert, können aber auch als natürliche Aromastoffe aus durch Hefen produzierten Fuselölen gewonnen werden. 2-Phenylethanol wird hauptsächlich chemisch synthetisiert, wobei v.a. Benzol oder dessen Derivate umgesetzt werden. Auf natürlichem Weg kann 2-Phenylethanol auch durch Umsetzungen mit Hefen gewonnen werden. Von den im Fermentationsmedium detektierten Lactonen wird aktuell lediglich γ-Decalacton biotechnologisch durch Umsetzung von Ricinolsäure kommerziell hergestellt. Gerade die ungesättigten Lactone sind kommerziell nicht erhältlich, sodass eine nachhaltige und natürliche Quelle dieser Stoffe umso wünschenswerter ist.

Ein ähnliches Bild zeigt sich auch bei dem Fermentationsmedium der Bernsteinsäureproduktion. Die chemische Synthese von Bernsteinsäure erfolgt durch Umsetzung von 1,4-Butandiol oder durch Hydrierung von anderen C_4-Säuren, z.B. Malein- oder Fumarsäure. Jedoch ist auch die biotechnologische Produktion durch verschiedene Mikroorganismen möglich. Eine Besonderheit von *B. succiniciproducens* ist, dass dieses Bakterium auch Rohglycerol an Nährstoffquelle nutzen kann, wie es bei der Produktion von Biodiesel in großen Mengen anfällt. Somit ist eine gute Koppelnutzung zur biotechnologischen Produktion von Bernsteinsäure möglich. Die bei der Fermentation gebildeten alkylierten Pyrazine sind als natürliche Aromastoffe ebenfalls fast nicht erhältlich. Bei der industriellen Herstellung von natürlichen Pyrazinen werden zumeist Lebensmittel, wie etwa Kartoffeln, Nüsse oder

Kaffee, extrahiert. Von daher kann die Gewinnung aus dem anfallenden Fermentationsmedium eine lohnenswerte und nachhaltigere Alternative dazu sein.

Bei beiden Fermentationsprozessen muss zudem bedacht werden, dass sie aktuell weder auf die Produktion von Aromastoffen ausgerichtet noch dafür optimiert sind, sodass durch Optimierung der Prozesse noch weitaus höhere Mengen an natürlichen Aromastoffen als Koppelprodukte produziert werden könnten.

Bei der elektrochemischen Oxidation von Limonen handelt es sich ebenfalls um einen nachhaltigen Prozess. Limonen ist in großer Menge verfügbar, da es aus Nebenströmen der Fruchtsaftindustrie gewonnen werden kann. Des Weiteren werden bei der chemischen Oxidation von Terpenen häufig Schwermetallkatalysatoren und toxische und/oder gefährliche Chemikalien eingesetzt. Dies alles ist bei der elektrochemischen Oxidation der Terpene nicht nötig. Zudem kann schnell und kostengünstig eine Vielzahl an verschiedensten Aromastoffen generiert werden, wobei es durch Variation der Parameter (Dauer, Spannung, Art der Edukte (verschiedene Alkohole in Kombination mit unterschiedlichen Terpenen)) diverse Kombinationsmöglichkeiten gibt.

List of Publications

Peer-reviewed original publications as First Author:

1. Birk, F.; Fraatz, M. A.; Esch, P.; Heiles, S.; Pelzer, R.; Zorn, H. Industrial Riboflavin Fermentation Broths Represent a Diverse Source of Natural Saturated and Unsaturated Lactones. *J. Agric. Food Chem.* **2019**, 67, 13460–13469.

2. Birk, F.; Brescia, F. F.; Fraatz, M. A.; Pelzer, R.; Zorn, H. Aroma active alkylated pyrazines are produced by *Basfia succiniciproducens* as by-products of succinic acid production. *Flavour Fragr. J.* **2021**, 36, 605–612.

3. Birk, F.; Hausmann, H.; Fraatz, M. A.; Kirste, A.; Aust, N. C.; Pelzer, R.; Zorn, H. Generation of Flavor-Active Compounds by Electrochemical Oxidation of (*R*)-Limonene. *J. Agric. Food Chem.* **2022**, 70, 7220–7229.

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Patents:

Pelzer, R.; Zorn, H.; Birk, F.; Fraatz, M. A. Fermentative Production of Pyrazines Using Microorganisms of the Genus *Pasteurellaceae*. 19151402.5, Jan 11, 2019. **2019**.

Kirste, A.; Pelzer, R.; Birk, F.; Fraatz, M. A.; Zorn, H. Cyclic compounds as aroma chemicals. PCT/EP2021/070983, Jul 27, 2021. **2021**.

List of Abbreviations

AEDA	Aroma Extract Dilution Analysis
DMAPP	Dimethylallyl pyrophosphate
FD	Flavour Dilution
FID	Flame Ionisation Detector
GC	Gas Chromatography
GPP	Geranyl pyrophosphate
HS	Headspace
HPLC	High-Performance Liquid Chromatography
IPP	Isopentenyl pyrophosphate
MDGC	Multi-Dimensional Gas Chromatography
MEP	Methyl-D-erythritol phosphate
MS	Mass Spectrometry
MVA	Mevalonic Acid
NMR	Nuclear Magnetic Resonance
O	Olfactometry
OAV	Odour Activity Value
ODP	Olfactory Detection Port
OT	Odour Threshold
PDMS	Polydimethylsiloxane
SAFE	Solvent Assisted Flavour Evaporation
SPME	Solid Phase Microextraction

Chapter I

Flavours and fragrances

For thousands of years, people have been using herbs and spices to impart special aromatic properties to food. The oldest findings are over 20,000 years old.[1]

However, not only the flavouring of food has been known for a long time. Also extracts or distillates from plants, such as cypress, mimosa, roses, or lilies, were already produced by the ancient civilisations and used for fragrancing.[2,3]

Besides the addition of flavouring ingredients to food and beverages, the formation of flavours by microorganisms via fermentation is also a traditional method of improving the flavour of food. Well known examples include the production of vinegar, cheese, beer, wine, or bread. Fermentation can also increase the shelf life of food, e.g., by the formation of organic acids or ethanol.[4,5]

Aroma substances are the most important and key constituents of herbs, spices, and essential oils. Aroma substances can be described as volatile, mostly organic substances that create an olfactory impression. The odour is perceived with the nose, whereby orthonasal and retronasal perception are distinguished. In orthonasal perception, the aroma substances enter the nose directly with the breath and reach the olfactory epithelium. In retronasal perception, they enter the olfactory epithelium from the mouth via the pharynx. In both cases, biochemical processes can still take place before the perception itself, in which aroma substances can be produced or released, for example, by chewing or enzymatic processes. Enzymes in the mucosa or saliva play an important role in this process. In the olfactory epithelium, the aroma compounds interact with the respective olfactory receptors. In humans, about 400 different receptors have been described. By activating the various receptors, an individual profile is created for each olfactory impression.[6–11]

From antiquity until the 18th century, fragrances continued to be obtained mainly from plant sources, whereby the number of known sources for fragrances increased continuously. With the beginning of industrialisation and scientific achievements, aroma compounds such as cinnamaldehyde or vanillin were characterised for the first time, so that a chemical synthesis of these substances was possible. The invention of vanillin synthesis in 1874 is considered the beginning of the modern aroma industry.[5,12]

Since then, the number of known flavouring substances has been increasing steadily, regardless of whether the substances have been isolated and characterised from natural sources or whether they have been chemically synthesised and do not occur in nature. Of course, it is also possible to chemically synthesise naturally occurring flavouring substances. Thus, flavouring substances can be roughly divided into two groups: those that have been chemically synthesised or those that have been obtained naturally. In the latter case, a further differentiation can be made whether the substances are obtained directly from the natural material, e.g., from essential oils or from foods, or whether they are produced by microbial conversions, *de novo* or by biotransformation of certain substrates.

Regarding the European legislation for flavour compounds in and on foods, regulation (EC) No 1334/2008 is the central document. In this regulation, flavourings are divided into six categories: flavouring substances, flavouring preparation, thermal process flavourings, smoke flavourings, flavour

precursors, and other flavourings or mixtures thereof. In Art. 3 (2)(b), flavouring substances are defined as “a defined chemical substance with flavouring properties”. Art. 3 (2)(c) defines a natural flavouring substance as “a flavouring substance obtained by appropriate physical, enzymatic or microbiological processes from material of vegetable, animal, or microbiological origin either in the raw state or after processing for human consumption by one or more of the traditional food preparation processes listed in Annex II. Natural flavouring substances correspond to substances that are naturally present and have been identified in nature”.[13]

Based on this definition, chemically synthesised flavouring substances are declared as "flavouring" in foods, even if they have been described from natural sources, whereas flavouring substances derived from natural sources can be declared as "natural flavouring" in foods. This differentiation does have an impact on consumers' acceptance, which will be discussed below.

In contrast, there is no such differentiation by a regulation for fragrances for non-food products. Here, however, the safety of the product for the consumer must also be warranted.

As mentioned above, natural flavouring substances can be obtained directly from natural sources such as plants. A common class of flavouring substances found in plants, and especially in their essential oils, are terpenes, such as limonene. What all terpenes have in common is that their structure results from linkages of isopentenyl pyrophosphate (IPP) units and dimethylallyl pyrophosphate (DMAPP) units, respectively. In plants terpenes are formed via the mevalonic acid (MVA) pathway or via the methylerythritol phosphate (MEP) pathway (Figure 1). Both metabolic pathways start with compounds formed from pyruvate and end with IPP and DMAPP as products. From these two compounds, geranyl diphosphate (GPP) is synthesised by a geranyl diphosphate synthase. From this, all other monoterpenes are generated, e.g. limonene, myrcene, linalool, or α-terpineol.[14,15]

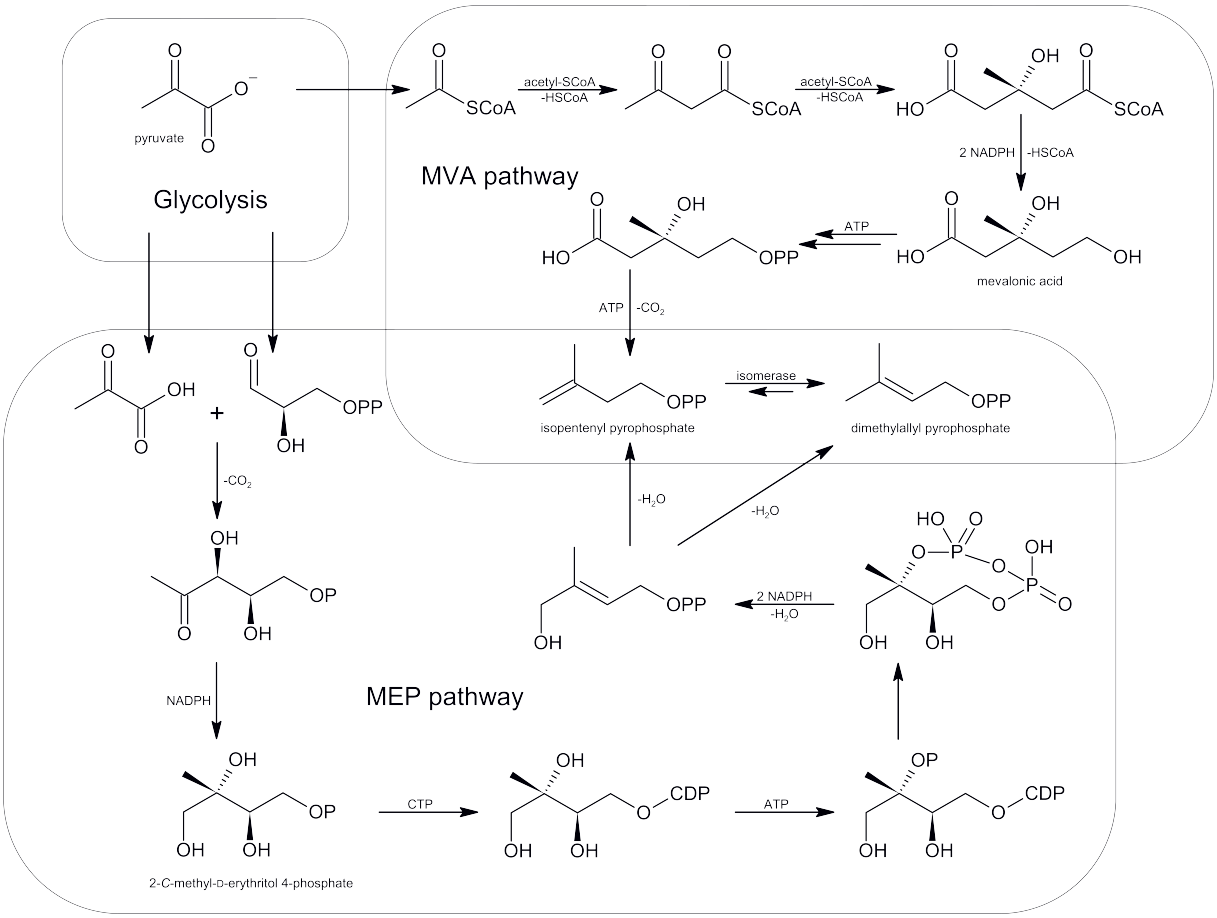

Figure 1: Formation of isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) via mevalonic acid pathway and methylerythritol pathway (according to Büttner, Handbook of Odor[16])

Besides extraction from plant sources, natural flavouring substances can also be obtained after processing according to the methods mentioned in the regulation. Flavour compounds that can be formed during processing are e.g. pyrazines during non-enzymatic browning reaction (Maillard reaction) or unsaturated aldehydes as breakdown products from fatty acids via autoxidation or enzymatic cleavage. In the Maillard reaction, pyrazines and other products are typically formed when heated to over 140 °C, for example during frying or roasting. They are formed from reducing sugars and amino acids (Figure 2) and are of great importance for the roasty character of these foods.[17,18]

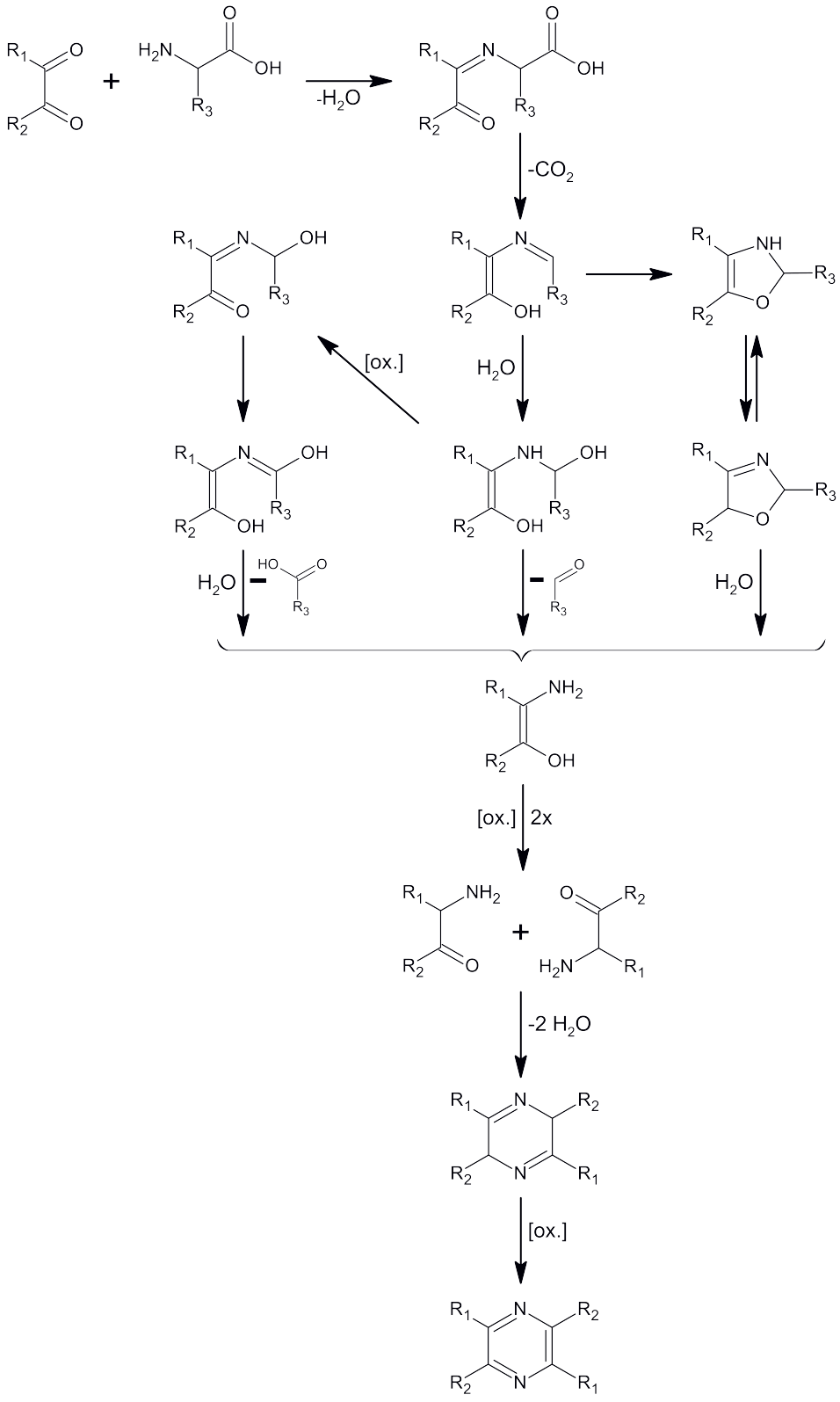

Figure 2: Formation of pyrazines and other products from 2,3-diketo compounds (generated from reducing sugars) and amino acids as part of the Maillard reaction (according to Büttner, Handbook of Odor[16])

In addition to direct extraction from natural sources, natural flavourings may also be produced biotechnologically. In the latter, a differentiation can be made between *de novo* synthesis and

biotransformation. *De novo* synthesised substances originate from the microorganism's metabolism, e.g., from glucose as a carbon source, whereas biotransformation describes the conversion of externally added precursors. An example of *de novo* synthesis is the formation of esters such as isoamyl acetate by yeasts, whereas the microbial conversion of ferulic acid or eugenol to vanillin e.g., by *Aspergillus niger* or *Bacillus subtilis* or the conversion of amino acids via the Ehrlich pathway are examples for biotransformations. The Ehrlich pathway is a typical metabolic pathway of yeasts, where amino acids are degraded by oxidative desamination, decarboxylation, and reduction to alcohols (Figure 3). Typical products of the Ehrlich pathway are e.g., 2-phenylethanol, derived from phenylalanine or 2- and 3-methylbutanol, which are formed from isoleucine or leucine, respectively. These compounds are found in alcoholic beverages that are fermented with yeast, like e.g. wine or beer.[19–24]

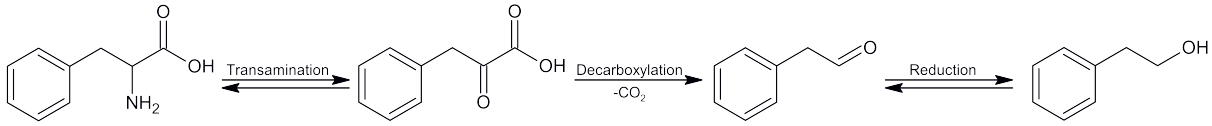

Figure 3: Degradation of phenylalanine via Ehrlich pathway to 2-phenylethanol via desamination and subsequent decarboxylation and reduction (according to Pires et al.[24])

However, not only naturally produced flavour and fragrance compounds are industrially used. In contrast to the above-mentioned ways of producing natural flavour and fragrance compounds, aroma active compounds are chemically synthesised as well. For chemically synthesised aroma compounds, two groups can be distinguished. One group comprises compounds that mirror their naturally occurring analogues, such as fruit esters or vanillin. The other group represents completely new flavouring substances that are not found in nature, e.g., ethylvanillin or ethyl methylphenylglycidate, which is also known as strawberry aldehyde.

The reasons for producing flavourings either synthetically or naturally can be various. On the one hand, consumers desire new flavours and fragrances, so that great efforts are made by the industry to develop new aroma substances that expand and enrich our known world of aroma and fragrances. Whether these are produced naturally or chemically depends on the feasibility, but also on the profitability and relevance of the respective process. On the other hand, however, chemically synthesised substances are usually cheaper than their natural counterparts, but they have a rather bad reputation among consumers, so they prefer naturally produced substances.

Manufacturers are meeting consumers' expectations and are producing the respective substances naturally wherever possible. In addition, manufacturers can also practice clean labelling, for example by promoting their products with the fact that no artificial flavourings have been added. As a result, the price is determined by the demand for the respective substances. For example, 1 kg of chemically synthesised vanillin costs about 15 US$, whereas biotechnologically produced vanillin, which can be declared as a "natural flavouring", costs about 1,000 US$. Vanillin extracted from vanilla beans costs about 1,200-4,000 US$, although rising demand and crop failures can increase the price many times over. 1 kg of synthetically produced 2,5-dimethylpyrazine costs about 200 US$, while its natural counterpart is currently sold for about 3,500 US$/kg.[18,20–22,25]

In addition to these aspects, there are also other reasons for choosing either natural or chemical production. For example, not all raw materials may be available at all times or the quality of natural raw materials may vary. Thus, it may happen that substances cannot be manufactured in appropriate

time, quantity, or quality. However, this may apply for natural as well as for chemical production of flavouring substances. Furthermore, environmental aspects play an increasingly important role.

Nevertheless, there may also be reasons for preferring chemical synthesis over the natural production. This is particularly the case when chemical synthesis also does not require the use of critical substances, can be carried out quickly and efficiently, and high-quality products can be produced from readily available substances.

Isolation and analysis of volatile compounds

For aroma analysis, gas chromatography (GC) is the technique of choice. Most aroma active compounds have a molecular mass $m \leq 300$ g/mol. Additionally, these compounds have a rather non-polar character, which means that they are not ionic and usually have no more than one or two polar functional groups, e.g., a carboxyl group or a hydroxy group. Due to this fact, the boiling point of these substances is comparatively low, which also goes along with the fact that they can be perceived olfactorily.

To make analysis by gas chromatography possible, the volatile compounds must be separated from the matrix. However, most matrices are aqueous. Since water is unsuitable as a solvent for GC analysis, however, separating the volatile compounds from the aqueous or non-volatile matrix is often challenging.

The easiest way to extract the desired aroma compounds from the matrix is to perform a liquid-extraction of a solid or liquid sample with an organic solvent, such as pentane/diethyl ether (1:1.12, v/v), hexane, isooctane, dichloromethane etc. In the case of aqueous matrices, the concentrated extract mostly may be used for analysis without further purification. In case of a more complex matrix like e.g., fatty samples, further purification steps can be necessary. An elegant way to remove undesired matrix compounds from the volatile fraction is Solvent Assisted Flavour Evaporation (SAFE). In this method, an organic extract of the sample is vaporised in a high vacuum. The solvents and the volatile compounds evaporate and are subsequently cryofocused by means of liquid nitrogen. Matrix compounds that are less volatile, like e.g., triglycerides or proteins, can't evaporate and are collected in a separate flask (Figure 4). This method was first developed by Engel, Bahr, and Schieberle in 1999.[26] The subsequently concentrated extract is free of matrix compounds and may be analysed immediately.

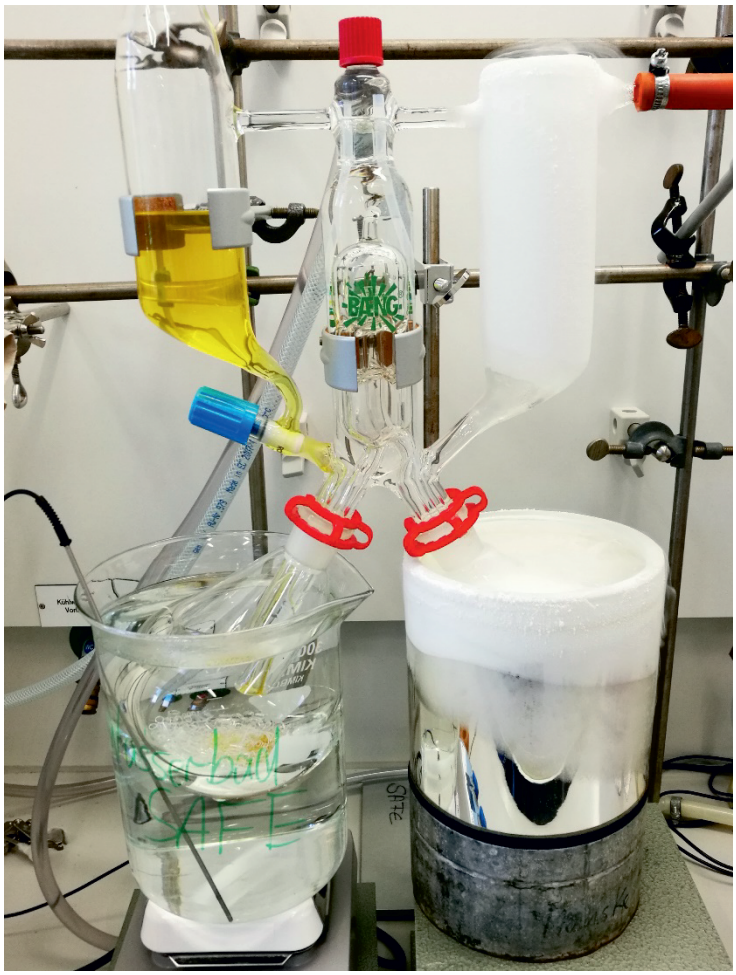

Figure 4: Solvent Assisted Flavour Evaporation (SAFE) of an organic extract from an industrial riboflavin fermentation broth (source: Florian Birk)

Another option is to analyse only the volatiles in the headspace of the sample. Therefore, a technique called Solid Phase Microextraction (SPME) was developed by Arthur and Pawliszyn.[27] A small fibre that is coated with an adsorbance material is inserted into the headspace over a sample. Volatile compounds can be adsorbed in the coating of the fibre. After sampling, the fibre is inserted into the GC's inlet and adsorbed volatile compounds are released from the coating by heating. Different coatings of the fibre are available, e.g., polydimethylsiloxane (PDMS), carboxen, polyacrylate, divinylbenzene, polyethylen glycol, or mixtures thereof. The choice of the coating depends on the analytes of interest, respectively their polarity.

With these techniques, it is possible to analyse the volatile compounds of a sample. However, being volatile does not necessarily mean being aroma active. Thus, a technique was developed where gas chromatography is coupled with olfactometry (O). Therefore, the GC's effluent is split after the GC column 1:1, with one half going to the chemical detector, e.g., a mass spectrometer (MS) or a flame ionisation detector (FID), and the other half going to an olfactory detection port (ODP) where trained panellist smell the effluent and describe the perceived odour impressions. This method was first used by Ullrich and Grosch.[28] To evaluate if an aroma active compound contributes to the overall aroma of a sample, an Aroma Extract Dilution Analysis (AEDA) can be performed. Therefore, an extract is diluted stepwise, e.g. 1+1, and each dilution is analysed by means of GC-O. The last dilution in which an aroma compound is still perceived corresponds to the Flavour Dilution (FD) factor. In the case of 1+1 dilutions, the FD factor is calculated as $FD = 2^n$, where *n* is the number of dilutions, when the compound was

perceived the last time. In order to make conclusions about whether an aroma compound contributes to the overall aroma of a sample, it is necessary to calculate Odour Activity Values (OAV). If the respective OAV is ≥ 1, it likely contributes to the overall aroma of the sample. If the OAV is < 1, it probably does not. The OAV is calculated according to the following formula, where *c* is the concentration and *OT* the odour threshold of the compound.

$$OAV = \frac{c_{compound}}{OT_{compound}}$$

The odour threshold (OT) of an aroma active compound can be calculated by the following formula, developed by Ullrich and Grosch as well. The OT is calculated referring to the odour threshold of an internal standard.

$$OT_{compound} = \frac{OT_{standard} \cdot c_{compound} \cdot FD_{standard}}{c_{standard} \cdot FD_{compound}}$$

In the case of chiral compounds, it is important to determine the odour impression and odour threshold for all isomers, as they typically differ. A prominent example is the different odour impressions of carvone, where (*S*)-carvone smells caraway-like while (*R*)-carvone exhibits a spearmint-like scent. The reason for this is that not only the aroma compounds are chiral, but also the olfactory receptors. Therefore, different enantiomers may interact with different receptors.[29–31]

Since the enantiomers cannot be separated on standard GC columns, special chiral separation phases, typically modified cyclodextrins, are used. Cyclodextrins consist out of six (α-phase), seven (β-phase), or eight (γ-phase) glucose units. These form hollow cylinders that can form inclusion complexes with the respective aroma compounds. To influence the separation properties, the cyclodextrins can be derivatised, e.g. by means of acetylation or alkylation.[32]

With these chiral columns, GC-O experiments can be performed as well to determine the odour impression and odour threshold of the different enantiomers. But in a complex sample with many aroma active compounds, it might be difficult to determine these properties due to e.g. co-elution with other compounds. Therefore, multidimensional GC (MDGC) systems have been developed. In these systems, an achiral column is used in the first dimension. After pre-separation on this column, the compounds of interest can be cut by means of a Deans switch to a second column. In this second dimension, a chiral column separates the enantiomers by an individual temperature program in the second oven (Figure 5).

Figure 5: Multidimensional gas chromatography system (Shimadzu MDGC-2010) with two dimensions (first dimension: right, second dimension: middle) and mass spectrometer (left) (source: Florian Birk)

Sometimes, it may be necessary to isolate a substance from a mixture of substances for structure elucidation. This is particularly the case if interesting odour impressions are perceived during the analysis by means of GC-O, but the comparison of the mass spectra with the database does not provide any reasonable results, or even the manual interpretation of the mass spectra does not allow any clear conclusions about the identity of the compound. The isolation of the compounds of interest may be achieved by means of preparative high-performance liquid chromatography (HPLC), preparative GC, or column chromatography. For the subsequently following structure elucidation, nuclear magnetic resonance (NMR) spectroscopy is a powerful tool. By means of different NMR experiments, the binding partners of, among others, hydrogen and carbon atoms can be determined, so that the structure of the analyte can be determined by combining the different experiments. A major disadvantage of NMR spectroscopy is the relatively large amount of about 1 mg of pure substance needed for NMR analysis to obtain signals of sufficient intensity. Although NMR spectroscopy is a non-destructive method and therefore no substance is lost during the analysis, it can sometimes be difficult to collect sufficient amounts of sample for NMR experiments.

If a sufficient amount of sample of the target analyte cannot be isolated for NMR experiments, other techniques can be used to determine the structure of an analyte that require less sample material. Mass spectra of analytes usually give important information about their structure. However, especially when the mass spectra are pretty complex, an additional functionalisation of the analyte can provide important information about the sample, e.g., the exact position of a C-C-double bond. A method used for the determination of the double bond's position is the Paternò-Büchi reaction coupled with mass spectrometric experiments. The Paternò-Büchi reaction is a photochemical [2+2] cycloaddition of an olefin with a carbonyl group, e.g., an aldehyde or a keto group, forming a four-membered oxetane ring. The light-catalysed reaction of ketones or aldehydes with substituted olefines was first described by Paternò et al. in 1909 (Figure 6).[33] Büchi et al. investigated the mechanism of this reaction.[34] The functionalisation of molecules by means of Paternò-Büchi reaction and subsequent tandem mass spectrometric analysis was developed by Xia and Ma.[35]

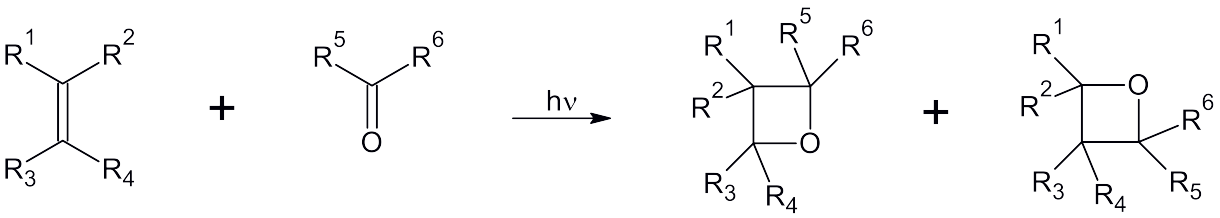

Figure 6: General scheme of Paternò-Büchi reaction of an olefin with a carbonyl group under UV radiation

According to this general reaction, an unsaturated γ-lactone with a double bond in the aliphatic side chain reacts with 3-acetylpyridine forming two different products (Figure 7).

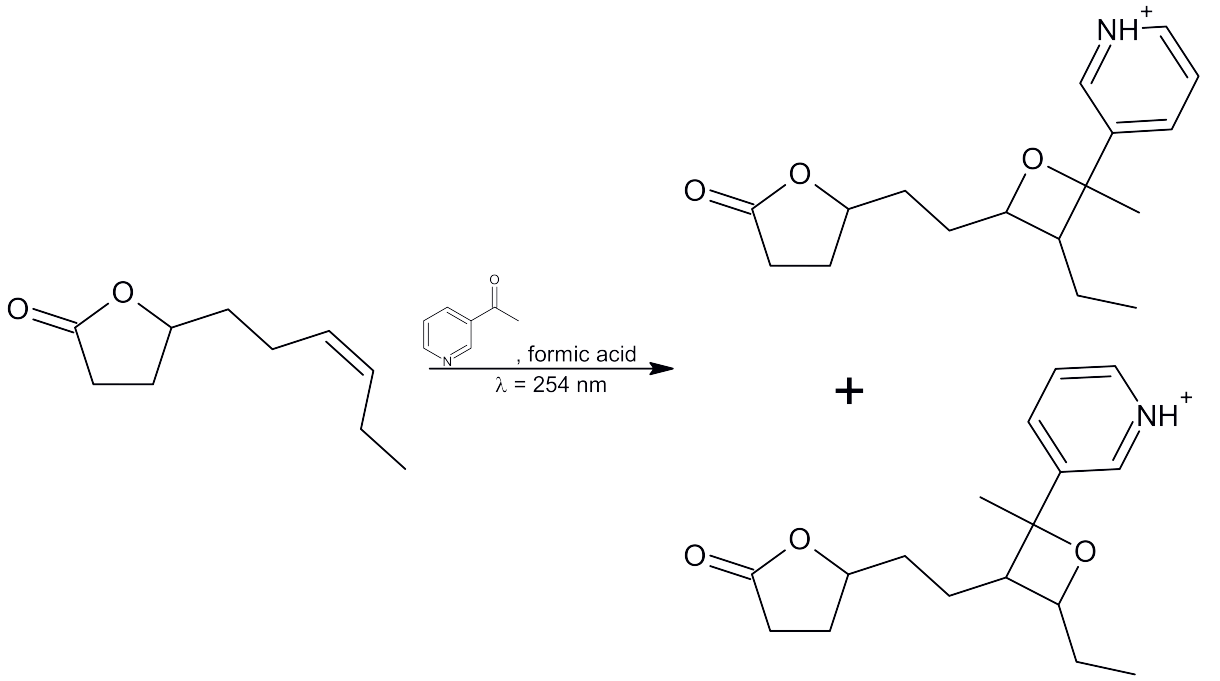

Figure 7: Reaction of an unsaturated γ-lactone with 3-acetylpyridine under UV radiation

These products can be analysed by means of MS/MS. Depending on the position of the double bond, specific ions are formed (Figure 8). Besides the formation of typical product ions, a retro-Paternò-Büchi reaction can take place as well, giving both starting molecules.

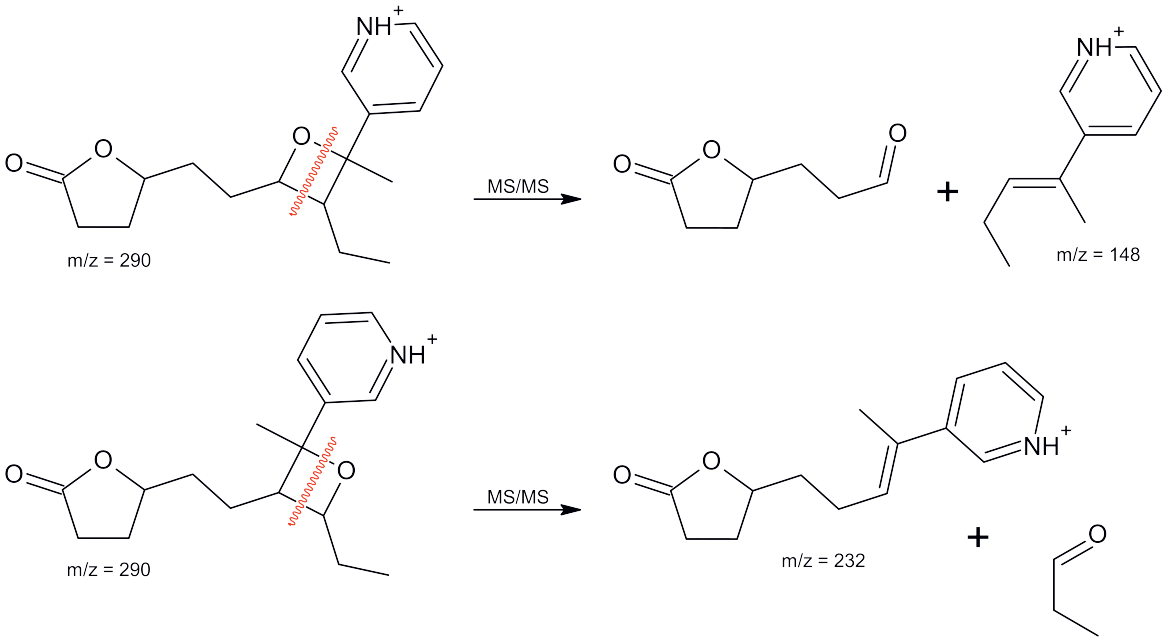

Figure 8: Formation of double-bond dependant ions by means of MS/MS from products formed in a Paternò-Büchi reaction

Sustainability and production of aroma compounds

Especially in view of today's problems, such as global warming or shortage of raw materials, the term sustainability is on everyone's lips. However, many people are not familiar with the definition of sustainability. In addition, it is also difficult to find an all-encompassing definition.

Hans Carl von Carlowitz is considered the creator of the term “sustainability”. In his book "Sylvicultura oeconomica - Anweisung zur Wilden Baum-Zucht" from 1713 for managing forests for sustainable yields, he wrote at that time

"*Wird derhalben die gröste Kunst / Wissenschafft / Fleiß / und Einrichtung hiesiger Lande darinnen beruhen / wie eine sothane Conservation und Anbau des Holtzes anzustellen / daß es eine continuirliche beständige und nachhaltende Nutzung gebe / weiln es eine unentberliche Sache ist / ohne welche das Land in seinem Esse nicht bleiben mag*"

which means that the greatest skill of a country will be based on how to establish such a conservation and cultivation of wood that there is a continuous constant and sustainable use, so one should harvest only the same amount of wood which equals to trees planted. These and similar terms were used until the middle of the 20th century. Then, however, the interests of economics and ecology collided and their goals, intentions and approaches seemed to be incompatible.[36–38]

This was followed by various conferences over the next decades, at which more than 100 nations agreed on common aims to protect the earth in a sustainable way. Furthermore, the two previous basic ideas of sustainability, environment and ecology, were expanded by another one. Thus,

sustainability is to be seen as the overlapping of social, ecological, and economic factors (Figure 9). Even if this model does not always meet all requirements, it is the most widely used concept today.[21,37]

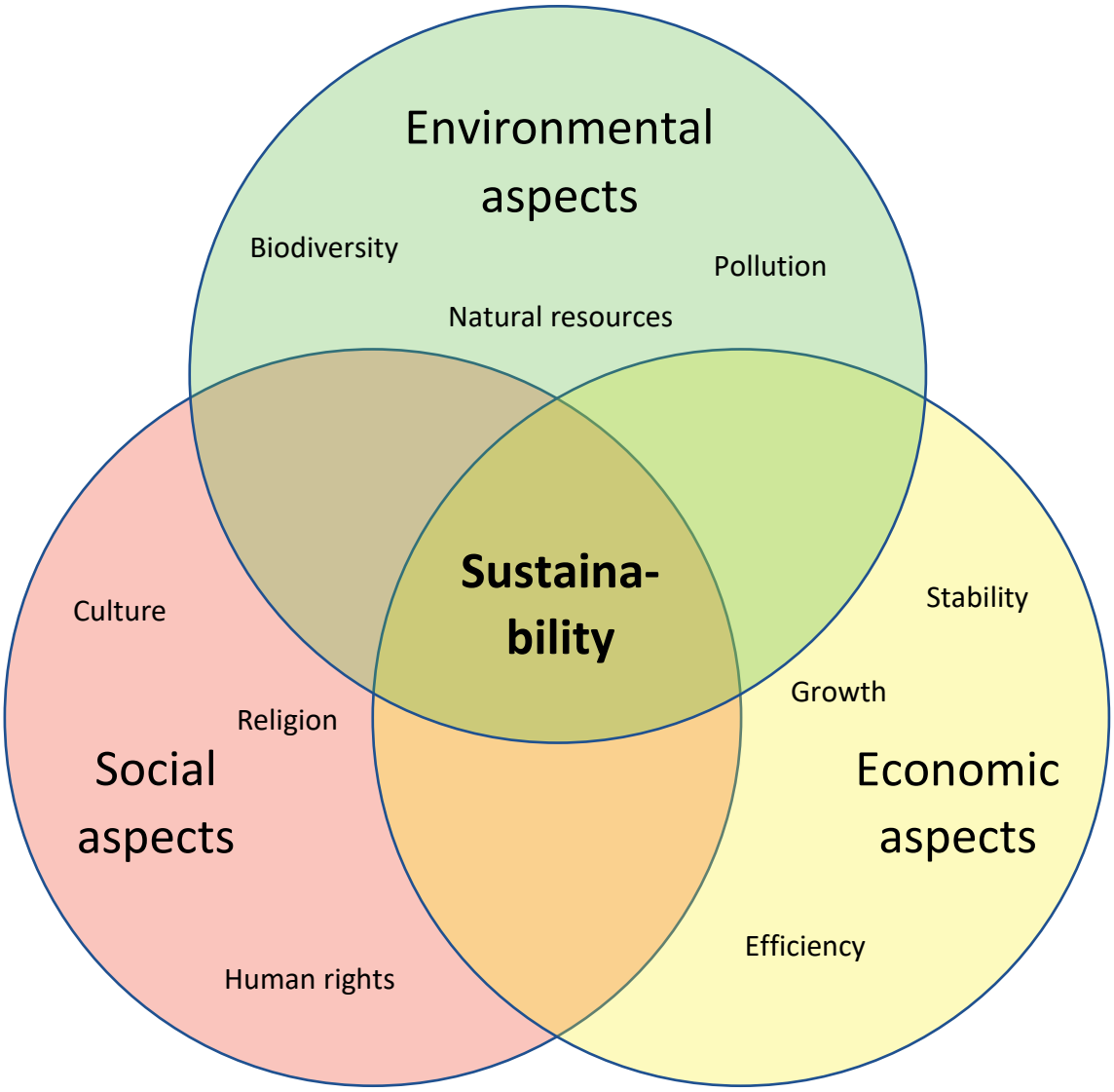

Figure 9: Graphical illustration of the term “sustainability” as intersection of its three fundamental aspects (based on Surampalli et al., Sustainability[37])

Ecological aspects are what comes to most people's minds when talking about sustainability. These are mainly aspects such as the preservation and promotion of biodiversity, minimisation of pollution, protection of natural resources, and minimisation of global warming.

Looking at economic aspects, growth, stability, profitability, efficiency, and progress are the most prominent and the most important for companies.

Health, safety, religions, cultural diversity and respect of human rights are the central points when talking about social aspects.[21,37,38]

All these and many other aspects must be considered if processes are to be designed sustainably. Looking at chemistry, a lot has already changed in the last decades.

In the past, industrial organic chemistry often used toxic catalysts or chemicals and large amounts of organic solvents, usually in combination with high temperatures and/or high pressure. Nowadays, biocatalysts, i.e. enzymes or microorganisms, such as fungi or bacteria, are increasingly used in the redesign or development of chemical processes. In these processes, the substrates are converted under mild reaction conditions, in an aqueous milieu and without the use of toxic catalysts or other safety risks. Further advantages of the use of biocatalysts can also be high conversion rates, chemoselective, regioselective, stereoselective, or enantioselective conversion. However, if conversion in an aqueous medium is not possible, e.g., due to the polarity of the substrate, the system can be modified accordingly. For this purpose, an enzyme can be e.g. immobilised or enclosed in micelles, or emulsifiers can be used, for example.[39–42]

However, there are also disadvantages in biocatalytic synthesis. Depending on the process, the amount of aqueous medium can be very high. Especially in the fermentative process, the purification of the product can also be difficult. Sometimes, however, conversion by means of an enzyme or microorganism is simply not possible because a respective reaction is not known. However, genetic modifications can be used to find a way to solve this problem.[40,42]

One of the industrial processes, which was carried out chemically for many decades and is nowadays completely replaced by a biocatalytic conversion, is the production of riboflavin. The classical chemical synthesis required up to eight steps from educt to product, starting from glucose, which is converted to ribose. In the next step, ribose reacts with 3,4-dimethylaniline and the resulting product is hydrogenated by means of a Pd/C-catalyst. After coupling with phenyldiazonium chloride, the resulting azo product reacts with barbituric acid to form riboflavin. Due to the release of aniline in the last step, trace amounts of aniline can always be found in chemically synthesised riboflavin. This process takes place under higher temperature and pressure and organic solvents and corrosive chemicals are used. In contrast, the fermentative process takes place at around 35 °C and no other harmful chemicals are needed (see below). Thus, the biocatalytic process is more economical and ecological compared to the chemical conversion.[5,43,44]

Another process that is now carried out biocatalytically is the production of (+)-nootkatone from (+)-valencene. (+)-Nootkatone is the key aroma component of grapefruit. Chemically, it can also be produced from (+)-valencene, but the regioselectivity of the oxidation is very poor, so that many by-products are produced. In the past, it was synthesised using carcinogenic compounds such as potassium dichromate, but this has now been replaced by non-toxic but still not uncritical substances such as *tert*-butyl peracetate with cobalt catalysts. In industrial biocatalysis, the reaction is carried out by whole-cell catalysis with e.g. *Pleurotus sapidus* or *Chaetomium globosum* or by reaction with purified enzymes, such as lipoxygenases.[45–47]

2-Phenylethanol can also be produced chemically or biocatalytically. In chemical synthesis, either benzene undergoes reaction with ethylene oxide in the presence of aluminium chloride, or styrene is epoxidised and subsequently hydrogenated. Alternatively, synthesis can also be carried out via a Grignard reaction of phenylmagnesium chloride with ethylene oxide. The biocatalytic conversion mainly is carried out by modified yeast, such as *Saccharomyces cerevisiae*. Although the chemical process is currently more efficient because the raw materials are still readily available in large quantities, the biocatalytic process is more lucrative on the one hand because of the much higher price of the product, and on the other hand, because of the use of safer substances, it is also more ecologically friendly.[3,5,20,48]

Besides the classic ecological and economic aspects, social aspects can also be essential for some population groups. For example, fragrances of animal origin, such as ambergris or musk, may be rejected because animals may suffer during their extraction. Ambergris is a waxy substance that is

produced in the intestinal tract of sperm whales. The smell of ambergris is described as warm, balsamic, and woody. Musk is extracted from a gland of the musk deer. It is an oily liquid, and the smell is described as sweetish, animalic. Both, ambergris and musk fragrances, are often used as base notes in perfumes. Nowadays, however, the fragrances are almost exclusively produced chemically. In the case of ambergris, the odour active substance (–)-ambrox is produced from sclareol, which can be obtained from clary sage. In the subsequent conversion, however, oxidising substances such as chromium salts or permanganates and ozone are used. Fortunately, there are now also enzymatic reactions that produce (–)-ambrox directly from homofarnesol. These processes thus fulfil all the basic principles of sustainability. In accordance with Jewish and Muslim laws, substances produced in this way can be marketed as kosher and halal and are thus available to a broader consumer group.[5,49–53]

Even if earlier biotechnological processes for the production of flavouring substances were not possible or not efficient enough, this does not mean that it has to stay that way. One reason is that genetic modifications can often be used to modify microorganisms or enzymes in such a way that the desired conversions can be carried out. Apart from that, new substrates may be used, since new material flows are being created, especially as a result of the replacement of petrochemical products that is currently in progress. It is of particular interest of sustainability if biotechnological products can be generated from resulting side streams or from waste materials that would otherwise have no further use. In this way, the efficiency of processes can be significantly increased through co-utilisation. For example, glycerol accruing from the production of biofuels can be converted by microorganisms to succinic acid, which is otherwise synthesised by petrochemically generated butane derivatives.[54,55]

Biotechnological production of riboflavin

Riboflavin, also known as vitamin B_2, is a yellow, fluorescent substance, which is used as an additive for food and feed (Figure 10). In the European Union, it is used as a food colorant (E101) and as a vitamin supplement.

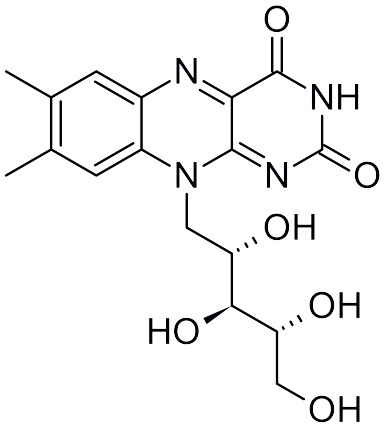

Figure 10: Structure of riboflavin

Riboflavin was already isolated at the end of the 19th century from milk in a material called "lactochrome", which contains different vitamins of the B-complex, such as thiamine, riboflavin, and niacin.[56] In the 1930s, riboflavin was isolated from the other compounds of the vitamin B-complex and its structure was elucidated by György et al. and Karrer et al.[57,58] Since it was known that riboflavin plays an important role as a growth factor in animal and human nutrition, attempts have been made to synthesise it. Chemical synthesis was carried out as a large-scale process until the early 1990s, when it was replaced by biochemical production using microorganisms. One of the microorganisms used for the industrial production of riboflavin is the filamentous fungus *Ashbya gossypii*. It was discovered by Ashby and Nowell as a pathogen of cotton plants.[59] It was found that it is a natural overproducer of riboflavin. Wild strains of *A. gossypii* are able to produce of about 100 mg riboflavin per gram biomass, whereas genetically optimised strains can produce up to 20 g/L fermentation medium. For the fermentative production of riboflavin with *A. gossypii*, soybean oil and soybean meal are used as carbon and nutrition source in fed-batch reactors with a volume of more than 100 m³.[5,43] Since only a small amount of the riboflavin formed is released into the medium, cell lysis takes place after fermentation. In this process, glucanases are formed by increasing the temperature to about 60 °C, which hydrolyse the cell walls.[5] Due to the relatively low solubility of riboflavin in water of about 70 mg/L, the crystalline riboflavin can be easily separated from the fermentation medium.[60]

Alternatively, a fermentative production of riboflavin can be performed with modified strains of *Bacillus subtilis*. With these two highly efficient microbial fermentation processes, 100% of the worldwide annual amount of nearly 10,000 tons of riboflavin are produced.[61]

When comparing the chemical synthesis of riboflavin with fermentative production using *A. gossypii*, it is noticeable that biotechnological production requires up to 75% less raw materials of fossil origin. In addition, the environmental impact is usually lower, and the operating costs are about 50% less, as the synthesis takes place in fewer steps and with cheaper starting materials (Table 1).[5,61,62]

Table 1: Relative comparison of the environmental impact of chemical riboflavin synthesis to the biotechnological production using *A. gossypii*[62]

	Chemical synthesis	**Fermentative production with *A. gossypii***
Production steps	7-8	1
Greenhouse potential	100%	67%
Acidification potential	100%	32%
Eutrophication potential	100%	115%
Formation of ozone	100%	29%

Biotechnological production of succinic acid

Platform chemicals are biobased chemical building blocks that are accessible from biomass precursors (carbohydrates, lignin, fats, proteins) through both biotechnological and chemical conversion.[63] Platform chemicals are e.g. glycerol, ethanol, or methanol, but also carboxylic acids such as fumaric acid, lactic acid, or succinic acid (Figure 11). Due to its bifunctionality, these acids are e.g. precursors for biodegradable polymers, but also for other C_4-compounds, e.g. 1,4-butanediol or tetrahydrofuran.[54,55] In terms of sustainability, the demand for these substances of non-petrochemical origin continues to rise steadily. Therefore, new ways are sought to produce these substances cost-efficiently and from readily available sources.

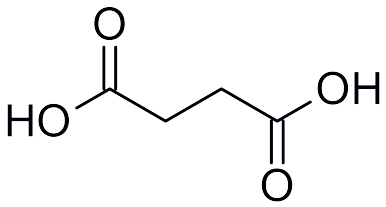

Figure 11: Structure of succinic acid (butanedioic acid)

Organic acids are typical products of microbial fermentations. For example, lactic acid is produced in dairy products and in industrial processes by *Lactococcus lactis*, or the bacterium *Acetobacter* is used to produce acetic acid.[64,65] A new way for the microbial production of succinic acid has been found in 2008. Scholten and Dägele isolated a new gram-negative bacterium from the genus *Pasteurellaceae* from rumen content of a cannulated Holstein cow, which was able to produce succinic acid. First called DD1, the strain was subsequently named *Basfia succiniciproducens*.[66,67]

The industrial microbiological production of succinic acid is mainly carried out with modified strains of *Escherichia coli* and *Saccharomyces cerevisiae*.[5] But also other microorganisms show promising results, e.g. *Anaerobiospirillum succiniciproducens* or *Actinobacillus succinogenes* with a production of succinic acid of about 32-106 g/L.[54] Wild types of *B. succiniciproducens* were able to produce amounts of about 6 g/L.[66] After strain optimisation 30-48 g/L were achieved.[68] A major advantage regarding *B. succiniciproducens* compared to most other microorganisms is the ability to convert glycerol, as mainly sugars, mostly glucose, serve as substrate for other microorganisms.[66,68] Glycerol is a by-product of the production of biofuel and is readily available. Especially in view of the fact that more and more fuels from plant-based sources are replacing fossil fuels, the production of glycerol will continue to increase in the coming years, so that the production of succinic acid from glycerol will become an increasingly attractive alternative.

Electrochemical oxidation of terpenes

In nature, terpenes are found in a large number of plants. Many terpenes, especially mono- and sesquiterpenes, have pleasant organoleptic properties. Therefore, depending on their concentration in the raw material, it may be lucrative to extract and work them up to obtain the essential oils of these plants. Examples for terpenes that are extracted from natural sources are α-pinene and β-pinene from turpentine or limonene from citrus fruits.[20,69] These compounds can be used as flavourings or as potential starting material for further reactions to generate new flavour compounds.

By means of an anodic oxidation in certain solvents, aliphatic olefins bearing at least one allylic hydrogen undergo a substitution reaction, in which the solvent works as a nucleophile (Figure 12).[70] The respective olefin is solved in the solvent and a conductive salt, e.g. ammonium salts like methyl-tri-*n*-butylammonium methylsulfate, is added.[71] Depending on the solvent used, a huge number of different products can be created from the same starting material.

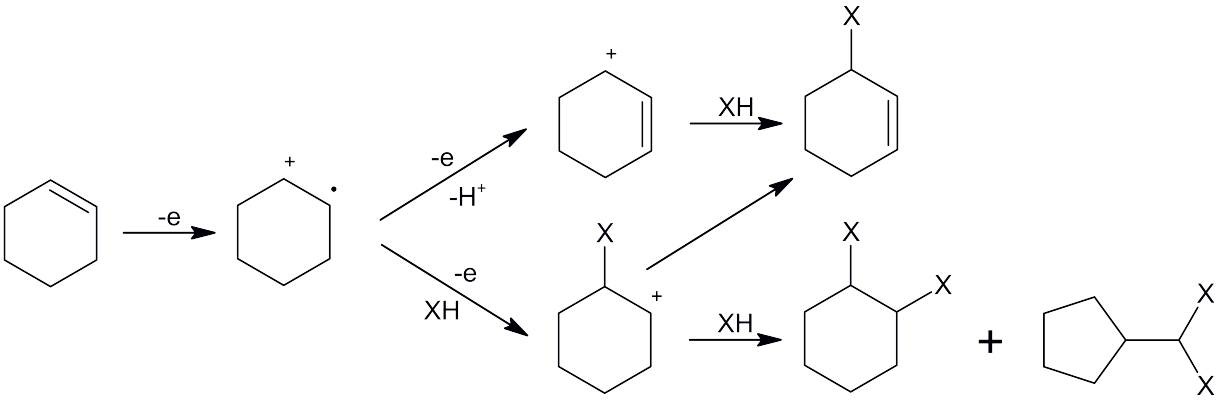

Figure 12: General reaction pathway of an aliphatic cyclic olefin in anodic oxidation with XH as solvent (according to Shono et al.[70])

A major advantage of electrochemical oxidation compared to “classical” chemical synthesis is that no harmful substances, e.g. heavy metals or their salts are used.[72] Additionally, a broad variety of products is produced in just one single step. Another advantage is the fast conversion within a few hours.[71] Thus, electrochemical oxidation offers an environment-friendly way of producing an olfactorily appealing mixture of different aroma substances from inexpensive and readily available starting materials within a short time.

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Chapter II

Industrial Riboflavin Fermentation Broths Represent a Diverse Source of Natural Saturated and Unsaturated Lactones

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Industrial Riboflavin Fermentation Broths Represent a Diverse Source of Natural Saturated and Unsaturated Lactones

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Supporting Information

ABSTRACT: Fermentation broths of *Ashbya gossypii* from the industrial production of riboflavin emit an intense floral, fruity, and nutty smell. Typical Ehrlich pathway products, such as 2-phenylethan-1-ol and 2-/3-methylbutan-1-ol, were detected in large amounts as well as some intensely smelling saturated and unsaturated lactones, e.g., γ-decalactone and γ-(*Z*)-dodec-6-enlactone. An aroma extract dilution analysis identified 2-phenylethan-1-ol and γ-(*Z*)-dodec-6-enlactone as the main contributors to the overall aroma, with flavor dilution factors of 32 768. The position of the double bonds of unsaturated lactones was determined by the Paternò−Büchi reaction, and reference compounds that were not available commercially were synthesized to elucidate the structures of the uncommon lactones. The absolute configuration and enantiomeric excess values of the lactones were determined by converting the lactones to their corresponding Mosher's esters. In addition, the odor impressions and odor thresholds in air were determined.

KEYWORDS: *Ashbya gossypii, flavor, Paternò−Büchi reaction, unsaturated lactones*

INTRODUCTION

Acceptance of food by consumers strongly depends upon its aroma, and natural flavors are preferred over synthetic compounds.[1,2] To meet the increasing demand for natural flavors by consumers and to minimize environmental pollution caused by the chemical synthesis of flavorings, biotechnological routes for the production of natural flavors have to be developed. Fermentation of apple pomace or molasses with basidiomycetes, for example, can produce appealing flavor mixtures.[3,4] Further potential sources for natural flavor compounds are industrial fermentation broths, which arise in large quantities as waste or side products from the microbial production of food additives.

Riboflavin, also known as vitamin B_2, is a common additive for food and feed. Industrially, riboflavin is mainly produced with *Ashbya gossypii* and *Bacillus subtilis*.[5] The filamentous fungus *A. gossypii*, first described as a pathogen of cotton plants, is a natural overproducer of riboflavin,[6,7] and fermentation broths of *A. gossypii* from the industrial production of riboflavin emit an intense floral, fruity, and nutty smell. The fruity smell is mainly caused by lactones. γ-Decalactone, the most important lactone for flavor applications, is typically obtained by conversion of ricinoleic acid (from castor oil) with the yeast *Yarrowia lipolytica*.[8] The stereospecific conversion of the fatty acid mainly leads to (*R*)-γ-decalactone. However, because no further hydroxy fatty acids occur in larger quantities in nature, the production of other γ-lactones as aroma compounds is still challenging. Likewise, there is currently no industrial process for the microbial production of unsaturated γ-lactones.

Considering the huge amounts of culture broth arising from the production of riboflavin by fermentation processes with *A. gossypii*, isolation of highly valuable natural flavor compounds from the culture broths is an alluring idea. This could contribute to meeting the steadily increasing industrial demand for natural flavor compounds.

The production of 2-phenylethan-1-ol and further aroma compounds derived from the Ehrlich pathway has been previously described for *A. gossypii* grown in a medium containing yeast extract, casein peptone, and glucose.[9] However, a systematic analysis of large-scale industrial fermentation broths as a potential source for natural aroma compounds, especially for saturated and unsaturated lactones, has not yet been reported. The aim of the current study was to structurally elucidate the aroma-active compounds in industrial riboflavin fermentation broths. Apart from that, the key aroma compounds were quantified and odor impressions and flavor threshold values of several unsaturated lactones in air have been described for the first time.

MATERIALS AND METHODS

Culture Broths. Culture supernatants of *A. gossypii* from the industrial riboflavin production process were provided by BASF SE (Ludwigshafen, Germany). The fermentation was carried out in a medium based on soybean oil and soybean meal. Data on the

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production strain and culture conditions have been reported in the literature.[10] The fermentation broths were colored dark orange to brown and exhibited an intense floral, fruity, and nutty smell.

Chemicals. (*S*)-(+)-α-Methoxy-α-(trifluoromethyl)phenylacetyl chloride (98%) and (*R*)-(−)-α-methoxy-α-(trifluoromethyl)-phenylacetyl chloride (98%) were purchased from abcr (Karlsruhe, Germany). (*Z*)-Hex-3-en-1-ol (98%) and sodium borohydride (99%) were purchased from Acros Organics (Geel, Belgium). Hept-1-yne (99%) and 3,4-dihydro-2*H*-pyran (99%) were purchased from Alfa Aesar (Karlsruhe, Germany). Ammonium sulfate (99.5%), Celite 545, chloroform (99%), chloroform-*d* [99.8 atom % D, with 0.03 vol % tetramethylsilane (TMS), stabilized with Ag], diethyl ether (99.5%), 1,4-dioxane (99.5%), hydrochloric acid (37%), sodium chloride (99.9%), sodium hydrogen carbonate (p.a.), sodium hydroxide (p.a.), sodium sulfate (p.a.), and sodium thiosulfate pentahydrate (p.a.) were purchased from Carl Roth (Karlsruhe, Germany). Lindlar catalyst (5% Pd on barium sulfate) was purchased from chemPUR (Karlsruhe, Germany). 4-Dimethylaminopyridine (99%), methanol [high-performance liquid chromatography (HPLC) grade], and pent-4-en-1-ol (99%) were purchased from Fisher Scientific (Darmstadt, Germany). *n*-Hexane (97%) was purchased from Honeywell (Seelze, Germany). Ammonium chloride (pure) was obtained from Labochem (Heidelberg, Germany), and silica gel 60 was from Macherey-Nagel (Düren, Germany). 2-Bromopropane (for synthesis), sodium carbonate decahydrate (p.a.), and succinimide (98%) were purchased from Merck (Darmstadt, Germany). 3-Acetylpyridine (98%), *m*-chloroperoxybenzoic acid (77%), dimethylformamide (99.8%), 3-phenylpropan-1-ol (98%), bromine (98%), (*E*)-hex-3-en-1-ol (97%), magnesium (turnings, 99.5%), sodium sulfite (p.a.), and silver nitrate (99.5%) were purchased from Sigma-Aldrich (Taufkirchen, Germany). 4,4′-Thiobis(6-*t*-butyl-3-*m*-cresol) (98%), *N*-chlorosuccinimide (98%), quinoline (97%), γ-undecalactone (98%), and triphenylphosphine (95%) were obtained from TCI (Eschborn, Germany). Methylene chloride (99.9%), *n*-pentane (99%), potassium hydroxide (99%), and tetrahydrofuran (99.9%) were purchased from Th. Geyer (Renningen, Germany). Hex-1-ene (pure) and *n*-butyllithium (2.5 M in *n*-hexane) were purchased from Thermo Fisher (Darmstadt, Germany). Dimethoxyethane (for synthesis), *n*-heptane (99%), and propan-2-ol were purchased from VWR (Darmstadt, Germany). Hydrogen (5.0) and helium (5.0) were obtained from Praxair (Düsseldorf, Germany), and nitrogen (5.0) was from Air Liquide (Düsseldorf, Germany). All numbers given in parentheses represent the minimum purity.

Reference Aroma Compounds. 2-Methylbutan-1-ol (98%) and 2-phenylethan-1-ol (99%) were purchased from Acros Organics. Benzaldehyde (for synthesis) was obtained from AppliChem (Darmstadt, Germany), and γ-nonalactone (98%) was from Merck. 3-Methylbutan-1-ol (98%), (+)-γ-decalactone (97%), γ-decalactone (98%), γ-dodecalactone (97%), (*E*)-dec-2-enal (95%), and phenylacetic acid (99%) were purchased from Sigma-Aldrich. 2-Methylbutyraldehyde (95%), 2-methylbutanoic acid (97%), 3-methylbutanoic acid (99%), *o*-aminoacetophenone (98%), and 5-ethyl-2,3-dimethylpyrazine (98%) were obtained from TCI.

Synthesis of Unsaturated Lactones. γ-(*Z*)-Dec-7-enlactone was synthesized according to the method of Miyakoshi and Tsukasa.[11] (*Z*)-1-Chlorohex-3-ene, which was used as a reagent for the synthesis of the lactone, was synthesized as described by Molander and Figueroa.[12] The reaction mixture was subsequently purified by means of preparative HPLC. The HPLC system used was a Young Lin Instrument (Anyang-si, South Korea) YL9110S with a quaternary pump (flow, 15 mL/min, eluents, *n*-hexane (A) and propan-2-ol (B); and gradient, 100% A and 0% B, ramped to 97.5% A and 2.5% B within 30 min, and ramped to 80% A and 20% B within 15 min) equipped with a polar column (guard column, Macherey-Nagel, Nucleodur 100-5, 10 × 16 mm; preparative column, Macherey-Nagel, Nucleodur 100-5, 250 × 21 mm) coupled with a YL9120S ultraviolet/visible (UV/vis) detector (wavelengths of 210 and 235 nm) and an Advantec (Dublin, CA, U.S.A.) CHF 112SC fraction collector. The structure was elucidated by gas chromatography−mass spectrometry (GC−MS) and nuclear magnetic resonance (NMR). The NMR experiments were performed on a Bruker (Rheinstetten, Germany) Avance II 400 MHz and a Bruker Avance III HD 400 MHz.

γ-(*E*)-Dec-5-enlactone was synthesized according to the method of Miyakoshi and Tsukasa.[11] (*E*)-1-Bromohex-1-ene was synthesized according to Tarchini et al.[13] and Tikhonov et al.[14] The reaction mixture was subsequently purified and analyzed as described above.

γ-(*Z*)-Dodec-6-enlactone was synthesized according to the method of Burger et al.[15] Ag_2CO_3 on Celite was prepared according to the method of Fétizon et al.[16,17] *n*-Heptane was used instead of benzene for the preparation of the reagent.

After purification by means of preparative HPLC, the structures were elucidated by means of GC−MS and NMR (Bruker Avance III 600 MHz).

Aroma Extract Dilution Analysis (AEDA). For AEDA, 10 mL of culture broth was diluted with 40 mL of distilled water and extracted 3 times with 50 mL of *n*-pentane/diethyl ether (1:1.12, v/v). The combined organic extracts were dried over anhydrous sodium sulfate and concentrated to approximately 1 mL by means of a Vigreux column at a water bath temperature of 42 °C. This extract was diluted stepwise 1 + 1 with *n*-pentane/diethyl ether (1:1.12, v/v). The dilutions were analyzed by means of a gas chromatograph coupled with a tandem mass spectrometer and an olfactory detection port (GC−MS/MS−O) until no odorants were perceived at the olfactory detection port. The gas chromatography system used was an Agilent (Waldbronn, Germany) 7890A gas chromatograph (GC) equipped with an Agilent VF-WAXms column [30 m × 0.25 mm; 0.25 μm film thickness; temperature program, 40 °C (3 min) at 5 °C/min to 240 °C (12 min); carrier gas, helium; 1.56 mL/min (constant)] and a split/splitless (S/SL) inlet (250 °C; splitless time, 1 min). After the column, the carrier gas was split 1:1 by a GERSTEL (Mülheim an der Ruhr, Germany) μFlowManager Splitter to an Agilent 7000B triple quadrupole detector (ionization energy, 70 eV; ion source, 230 °C; quadrupoles, 150 °C; transfer line, 250 °C; scan in q1, *m*/*z* 33−300; helium quench gas, 2.25 mL/min; and nitrogen collision gas, 1.5 mL/min) and a GERSTEL ODP3 olfactory detection port (transfer line, 350 °C; mixing chamber, 250 °C; and make up gas, nitrogen). The retention indices (RI) were calculated by linear interpolation from the retention times of *n*-alkanes (C_7−C_{30}).[18]

Perceived aroma compounds were identified by comparing the obtained mass spectra and RI to commercially available authentic standards or synthesized reference compounds on two columns of differing polarity {Agilent VF-WAXms and Agilent DB-5MS [30 m × 0.25 mm; 0.25 μm film thickness; temperature program, 40 °C (3 min) at 5 °C/min to 300 °C (12 min)]}.

Determination of the Position of the Double Bonds of Unsaturated Lactones. For determination of the position of the double bonds of unsaturated lactones, Paternò−Büchi (PB) functionalization was performed prior to mass spectrometric analysis. Therefore, 100 mL of fermentation broth was extracted 3 times with 100 mL of *n*-hexane, and the combined organic extracts were dried over anhydrous sodium sulfate. The organic extract was purified by means of solvent-assisted flavor evaporation (SAFE).[19] The obtained SAFE distillate was concentrated *in vacuo* to approximately 10 mL, and the compounds of the concentrate were separated by means of preparative HPLC (cf. synthesis of unsaturated lactones; eluents, *n*-hexane (A) and propan-2-ol (B); gradient, 100% A and 0% B, ramped to 97% A and 3% B within 40 min, and ramped to 90% A and 10% B within 20 min). The resulting HPLC fractions (7.5 mL per fraction) were stored at −20 °C until use. A total of 100 μL of each lactone-containing fraction was dried with nitrogen, and the residues were mixed with 45 μL of 3-acetylpyridine, 45 μL of methanol, and 10 μL of formic acid. In-house pulled nanospray capillaries (P-97, Sutter Instruments, Novato, CA, U.S.A.) with capillary diameters of 2 μm were loaded with 10 μL of sample solution. A home-built nanospray ionization source equipped with a low-pressure mercury UV lamp (UVP, Upland, CA, U.S.A.) with an emission maximum at 254 nm wavelength was used for all PB experiments as described previously.[20] To initiate nano-electrospray ionization (nanoESI), a voltage of +700 V was applied between the nanospray capillary and the inlet capillary of the mass spectrometer with an external high-voltage power supply.

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All samples were measured on a linear ion trap mass spectrometer in positive ion mode (LTQ, Thermo Fisher Scientific, San Jose, CA, U.S.A.), and collision-induced dissociation (CID) experiments were performed employing normalized collision energy values between 25 and 30 normalized collision energy (NCE).

Quantitation. The three compounds with the largest peak area in the GC−MS/MS chromatogram (2-/3-methylbutan-1-ol and 2-phenylethan-1-ol) and six lactones [γ-nonalactone (**1**; Figure 1), γ-decalactone (**2**; Figure 1), γ-(*Z*)-dec-7-enlactone (**3**; Figure 1), γ-(*E*)-dec-5-enlactone (**4**; Figure 1), γ-dodecalactone (**5**; Figure 1), and γ-(*Z*)-dodec-6-enlactone (**6**; Figure 1)] were quantitated. For this, 10 mL of fermentation broth was diluted with 40 mL of distilled water and pentan-1-ol (1.94 mg), 3-phenylpropan-1-ol (3.21 mg), and γ-undecalactone (0.54 mg) were added as internal standards. Pentan-1-ol, 3-phenylpropan-1-ol, and γ-undecalactone were chosen as internal standards as a result of their structural similarity to the target compounds. The mixture was extracted 3 times with 50 mL of *n*-pentane/diethyl ether (1:1.12, v/v) each, and the combined organic extracts were dried over anhydrous sodium sulfate. The extract was concentrated to approximately 1 mL by means of a Vigreux column. This extract (1 μL) was analyzed by means of an Agilent 7890A GC, equipped with a S/SL inlet (250 °C; splitless time, 1 min), a polar column [Agilent HP-INNOWAX; 30 m × 0.32 mm; 0.25 μm film thickness; temperature program, 40 °C (3 min) at 5 °C/min to 240 °C (12 min); carrier gas, hydrogen; 2.0 mL/min], and a flame ionization detector (FID; 250 °C; hydrogen, 40 mL/min; air, 400 mL/min; nitrogen, 30 mL/min). The corresponding response factors used for quantitation were determined as 1.00 for 3-methylbutan-1-ol (referred to pentan-1-ol), 1.08 for 2-phenylethan-1-ol (referred to 3-phenylpropan-1-ol), 1.06 for compound **1**, 1.03 for compounds **2**, **3***, and **4***, and 1.01 for compounds **5** and **6*** (referred to γ-undecalactone) (*, as a result of structural similarity to the corresponding saturated lactone).

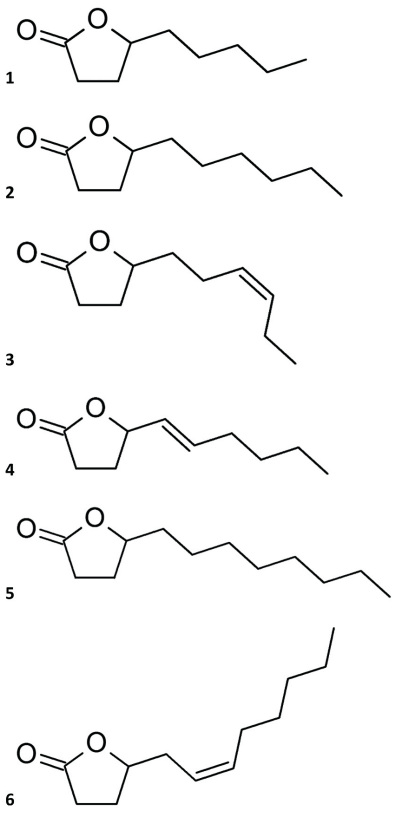

Figure 1. Structures of saturated and unsaturated lactones detected in the fermentation broth.

Determination of the Absolute Configuration. To determine the absolute configuration of the lactones, the structures were elucidated according to the method described by Dale and Mosher.[21] The lactones were converted into the corresponding Mosher's ester by the method described by Guichard et al.[22] Instead of the pure enantiomers, the racemates were converted and the mixtures of diastereomeric esters were separated by means of preparative HPLC (cf. PB reaction; flow, 15 mL/min; eluents, *n*-hexane (A) and diethyl ether (B), at 97.5% A and 2.5% B; 50 min; wavelengths, 210 and 266 nm). (*R*)-(+)-**2** was used as the enantiopure standard. Pure fractions were combined; the solvent was removed *in vacuo*; and the residue was dissolved in $CDCl_3$. The NMR experiments [^{1}H, ^{1}H correlation spectroscopy (COSY), and heteronuclear single-quantum correlation (HSQC)] were performed on Bruker Avance II 400 MHz and Bruker Avance III 400 MHz HD spectrometers. After the NMR experiments, the solvent was removed *in vacuo* and the esters were transferred to the corresponding enantiopure lactones. For this, the esters were hydrolyzed with KOH (1%) in methanol. After the solvent was removed *in vacuo*, the residue was treated with HCl.[23] The resulting lactones were analyzed by means of GC to determine the elution order on the chiral columns used in this study (Sigma-Aldrich Astec Chiraldex B-DM and Macherey-Nagel β-TBDAc; see the next sections).

Determination of Enantiomeric Excess (ee) Values. The ee values were determined by means of a Shimadzu (Duisburg, Germany) multidimensional gas chromatography (MDGC) system. This system consisted of a Shimadzu AOC-20i auto sampler, a Shimadzu GC-2010 Plus equipped with a polar column [Agilent VF-WAXms; 30 m × 0.25 mm; 0.25 μm film thickness; temperature program, 40 °C (3 min) at 5 °C/min to 220 °C (12 min)], a S/SL inlet [250 °C; carrier gas, helium; 208.1 kPa (constant); split ratio, 5:1; sample volume. 1 μL], a FID (250 °C; hydrogen, 40 mL/min; air, 400 mL/min; nitrogen, 30 mL/min), a Dean's switch (switching pressure of 129.1 kPa), and a second Shimadzu GC-2010 Plus equipped with a chiral column [Sigma-Aldrich Astec Chiraldex B-DM; 30 m × 0.25 mm; 0.12 μm film thickness; temperature programs, 40 °C (3 min) at 10 °C/min to 120 °C (55 min) for compounds **1**, **2**, and **4**; 40 °C (3 min) at 10 °C/min to 110 °C (80 min) for compound **3**; and 40 °C (3 min) at 10 °C/min to 130 °C (65 min) for compounds **5** and **6**] coupled to a Shimadzu QP2010 Ultra MS detector (transfer line, 220 °C; ion source, 200 °C; ionization energy, 70 eV; and scan mode, *m*/*z* 33−300).

Determination of Odor Impressions and Approximation of Odor Thresholds. Odor thresholds of racemic lactones were determined according to Ullrich and Grosch by means of GC−FID−O equipped with an achiral polar column.[24] An Agilent 7890A GC with a S/SL inlet (250 °C; splitless time, 1 min) equipped with an Agilent G4513A autosampler (sample volume of 1 μL) and a polar column (cf. quantitation; deviating heating rate, 15 °C/min) was used. The gas flow was split 1:1 by a GERSTEL μFlowManager Splitter to a FID (250 °C; hydrogen, 40 mL/min; air, 400 mL/min; nitrogen, 30 mL/min) and a GERSTEL ODP3 (transfer line, 250 °C; mixing chamber, 150 °C; and make up gas, nitrogen).

The odor thresholds of the racemic lactones were calculated using (*E*)-dec-2-enal (odor threshold of 2.7 ng/L air) as an internal standard.[25]

The odor thresholds of the enantiomers were calculated in relation to (+)-**2**. The threshold of the latter was first determined as described above. For the analyses of the enantiomers, GC−FID−O equipped with a chiral column (Macherey-Nagel β-TBDAc, 25 m × 0.25 mm)

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Table 1. Identified Odor-Active Compounds with Their Corresponding Retention Indices and Flavor Dilution (FD) Factors

compound	retention indices$_{sample}$ VF-WAXms	retention indices$_{sample}$ DB-5MS	retention indices$_{standard}$ VF-WAXms	retention indices$_{standard}$ DB-5MS	odor impression	FD factor
2-methylbutyraldehyde	929	<800	922	<800	green, cocoa-like	1
2-/3-methylbutan-1-ol	1207	<800	1206	<800	solvent-like, smelly, cocoa-like	64
5-ethyl-2,3-dimethylpyrazine	1449	1086	1456	1086	intense hazelnut-like	2048
benzaldehyde	1564	969	1516	966	sweetish, bitter almond-like	1
2-/3-methylbutanioc acid	1671	850	1669	860	sourish, smelly	1
phenylethyl acetate	1801	1286	1807	1256	sweetish, fruity	8
2-phenylethan-1-ol	1895	1112	1901	1113	floral, rose-like	32768
γ-nonalactone (**1**)	2005	1356	2007	1357	coconut-like	16
γ-decalactone (**2**)	2125	1464	2122	1463	fruity, peach-like, coconut-like	4096
γ-(Z)-dec-7-enlactone (**3**)	2156	1445	2158	1445	fruity, floral	2
γ-(E)-dec-5-enlactone (**4**)	2173	1460	2174	1460	sweetish, peach-like	1
o-aminoacetophenone	2194	1297	2207	1300	fatty, waxy, sweetish	2048
γ-dodecalactone (**5**)	2351	1675	2351	1675	fruity, peach-like, fatty	512
γ-(Z)-dodec-6-enlactone (**6**)	2372	1652	2371	1651	fruity, peach-like, green, pungent, fatty	32768

was used. Different from the setup above, the S/SL inlet (splitless time of 1 min) and the FID were operated at 220 °C. The oven temperature programs were as follows: 40 °C (3 min), 20 °C/min to 120 °C (33 min), 20 °C/min to 190 °C (2 min), and 20 °C/min to 220 °C (3 min) for compound **1**; 40 °C (3 min), 20 °C/min to 130 °C (33 min), and 20 °C/min to 220 °C (5 min) for compounds **2**, **3**, and **5**; 40 °C (3 min), 20 °C/min to 130 °C (28 min), 20 °C/min to 174 °C (1 min), 20 °C/min to 180 °C (5 min), and 20 °C/min to 220 °C (5 min) for compound **4**; 40 °C (3 min), 20 °C/min to 130 °C (33 min), 20 °C/min to 160 °C (1.5 min), 20 °C/min to 190 °C (2 min), and 20 °C/min to 220 °C (5 min) for compound **6**.

Statistics. AEDA was performed in triplicate by three trained persons. The quantitation experiments were also run in triplicates. The quantitated amounts are reported as means with standard deviation. Determination of the odor thresholds was performed in duplicates by a single trained male person.

RESULTS AND DISCUSSION

AEDA and Compound Identification. The odor of the fermentation broth was perceived as floral and fruity, with nutty nuances. To identify the main contributors to the overall aroma of the fermentation broth, an AEDA was performed. A total of 15 odor-active compounds were perceived and identified by GC−MS/MS−O. All of them were unambiguously identified by comparison of their retention indices (on two columns of different polarity), mass spectra, and their respective odor impressions to those of authentic standards (Table 1).

Apart from the expected and previously reported compounds derived from the Ehrlich pathway, some interesting and intense smelling γ-lactones were detected. For some compounds with appealing odor, no useful suggestions could be obtained from the mass spectra library. On the basis of their retention indices, odor, and mass spectra (cf. to the Supporting Information), it was assumed that these compounds were unsaturated lactones bearing a double bond in the aliphatic chain (for further discussion, cf. to the determination of ee values).

Mass Spectrometric C═C Double Bond Analysis by PB Functionalization. For determination of the position of the double bonds of unsaturated lactones, PB functionalization was performed prior to mass spectrometric analysis. Upon UV irradiation of filled nanospray capillaries, signals consistent with the formation of PB photoproducts between the lactone species and 3-acetylpyridine were detected. For example, two isomeric PB photoproducts with *m*/*z* 292 are expected for γ-dec-7-enlactone, as schematically shown in Figure 2. CID

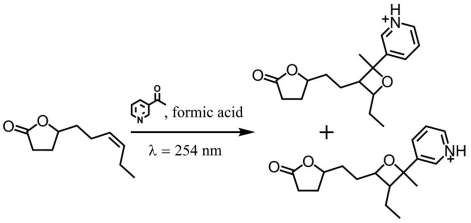

Figure 2. Reaction scheme for the PB reaction between γ-dec-7-enlactone and 3-acetylpyridine, resulting in oxetane formation.

activation of PB photoproducts leads to a formal retro-PB reaction and formation of double-bond-position diagnostic fragment ions. For γ-dec-7-enlactone, corresponding fragment ions are expected at *m*/*z* 232 and 148 (Figure 3). The CID tandem mass spectrum of the HPLC fraction tentatively assigned to γ-dec-7-enlactone is shown in Figure 4. Isolation and fragmentation of protonated PB photoproduct ions at *m*/*z*

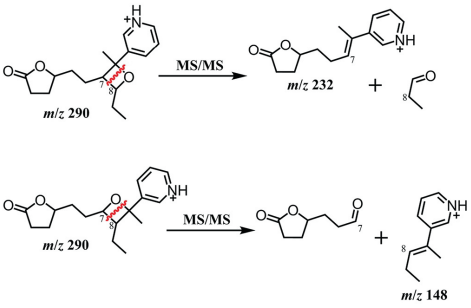

Figure 3. Retro-PB reaction for the cleavage of protonated PB-functionalized γ-dec-7-enlactone using CID. Other retro-PB reaction (not shown) products are γ-dec-7-enlactone and 3-acetylpyridine.

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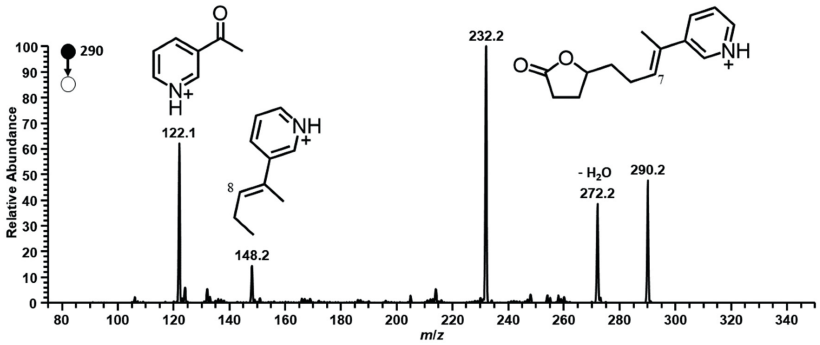

Figure 4. CID tandem MS spectrum of γ-dec-7-enlactone functionalized with 3-acetylpyridine. The fragment ions at *m/z* 148 and 232 are consistent with a C═C double bond at position 7.

292 give rise to the four most intense fragment ion signals at *m/z* 272, 232, 148, and 122. Whereas *m/z* 272 and 122 are assigned to neutral H_2O loss and protonated 3-acetylpyridine loss from the precursor ion, respectively, the signals at *m/z* 232 and 148 are consistent with a double bond at position 7 of the side chain of the lactone. Fragment ion signals that would indicate the presence of other double bond positional isomers were not detected. Analogous experiments were performed for all other lactones.

Thus, γ-dec-7-enlactone, γ-dec-5-enlactone, and γ-dodec-6-enlactone were tentatively identified in the sample (for further discussion on the respective lactones, cf. to the determination of ee values).

To the best of our knowledge, this is the first report on the use of MS/MS experiments after PB functionalization for assignment of the position of double bonds of lactones and in flavor analysis in general. This technique allows for analysis of complex samples with only little purification.[20] Even trace compounds may be analyzed by this sensitive method. The limit of detection, e.g., for oleic acid was determined to be 28 μg/L.[20]

Compound Identification by GC−MS and NMR Spectroscopy. *GC−MS and NMR Data of γ-(Z)-Dec-7-enlactone (**3**).* ^{1}H NMR ($CDCl_3$, 400 MHz): δ 5.43 (1 H, tdt, *J* = 1.5, 7.2, and 10.8 Hz), 5.31 (1 H, tdt, *J* = 1.5, 7.3, and 10.8 Hz), 4.50 (1 H, tdd, *J* = 5.1, 6.6, and 8.0 Hz), 2.54 (2 H, dd, *J* = 6.8 and 9.6 Hz), 2.34 (1 H, dq, *J* = 6.7 and 12.8 Hz), 2.19 (2 H, dq, *J* = 1.4 and 7.4 Hz), 2.05 (2 H, pd, *J* = 1.5 and 7.5 Hz), 1.84 (2 H, m), 1.65 (1 H, m), 0.96 (3 H, t, *J* = 7.5 Hz). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 177.14, 133.13, 127.11, 80.36, 35.63, 28.85, 27.99, 23.05, 20.52, 14.27. These data are in accordance with those reported in the literature.[26] The coupling constant of *J* = 10.8 Hz indicates a (*Z*) configuration of the double bond. The ^3J coupling constants of (*Z*)-configured compounds are always lower than those of the corresponding (*E*)-configured compounds.[27,28] GC−MS (EI, 70 eV): 68 (100), 85 (36), 67 (27), 41 (19), 79 (17), 55 (15), 108 (12), 81 (11), 39 (10), 69 (10), 168 ($M^{\bullet+}$, 1).

*GC−MS and NMR Data of γ-(E)-Dec-5-enlactone (**4**).* ^{1}H NMR ($CDCl_3$, 400 MHz): δ 5.81 (1 H, dtd, *J* = 0.6, 6.8, and 15.5 Hz), 5.49 (1 H, ddt, *J* = 1.6, 7.1, and 15.4 Hz), 4.89 (1 H, q, *J* = 7.3 Hz), 2.54 (2 H, ddd, *J* = 2.5, 6.9, and 12.8 Hz), 2.37 (1 H, m), 2.06 (2 H, qd, *J* = 1.7 and 7.0 Hz), 1.98 (1 H, dtd, *J* = 7.7, 9.2, and 12.7 Hz), 1.35 (4 H, m), 0.90 (3 H, t, *J* = 7.1 Hz). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 177.07, 135.72, 127.35, 81.16, 31.78, 30.91, 28.86, 28.75, 22.15, 13.88. These data are in accordance with those reported by Larock and Hightower.[29] The (*E*) configuration of compound **4** was thus confirmed.[27,28] GC−MS (EI, 70 eV): 111 (100), 41 (21), 55 (20), 125 (20), 98 (16), 68 (15), 56 (15), 85 (14), 57 (13), 83 (13), 168 ($M^{\bullet+}$, 2).

*GC−MS and NMR Data of γ-(Z)-Dodec-6-enlactone (**6**).* ^{1}H NMR ($CDCl_3$, 600 MHz): δ 5.58 (1 H, tdt, *J* = 1.6, 7.4, and 10.9 Hz), 5.36 (1 H, tdt, *J* = 1.6, 7.5, and 10.8 Hz), 4.53 (1 H, m), 2.52 (3 H, m), 2.41 (1 H, m), 2.30 (1 H, dddd, *J* = 5.6, 6.7, 8.6, and 12.6 Hz), 2.04 (2 H, dq, *J* = 1.6 and 7.3 Hz), 1.90 (1 H, dtd, *J* = 7.6, 9.4, and 12.8 Hz), 1.32 (6 H, m), 0.89 (3 H, t, *J* = 7.0 Hz). ^{13}C NMR ($CDCl_3$, 150 MHz): δ 177.12, 134.25, 122.15, 80.28, 32.90, 31.48, 29.14, 28.77, 27.44, 27.16, 22.54, 14.05. These data are in accordance with those reported in the literature.[30] On the basis of the observed coupling constant of 10.8 Hz, the (*Z*) configuration of the double bond of compound **6** has been confirmed.[27,28] GC−MS (EI, 70 eV): 85 (100), 96 (8), 41 (7), 55 (5), 57 (5), 86 (4), 81 (4), 67 (4), 54 (3), 39 (3), 196 ($M^{\bullet+}$, 2).

Quantitation. The three compounds with the largest peak area in the GC−MS/MS chromatogram (2-/3-methylbutan-1-ol and 2-phenylethan-1-ol) and six lactones (**1**−**6**) were quantitated after liquid−liquid extraction by means of GC−FID (Table 2). Large amounts of 2-/3-methylbutan-1-ol and 2-phenylethan-1-ol were detected. About 170 mg/L of lactones

Table 2. Quantitated Compounds in the Fermentation Broth

compound	concentration (mg/L) (σ)[a]	peak area (%)
2-/3-methylbutan-1-ol	366.6 (in total[b]) (5.1)	22.6
2-phenylethan-1-ol	473.3 (1.2)	43.8
1	2.0 (0.2)	0.1
2	76.0 (2.1)	5.8
3	4.4 (0.2)	0.3
4	1.5 (0.1)	0.1
5	36.9 (1.3)	2.9
6	46.8 (1.0)	3.8

[a]Numbers in parentheses give the standard deviation. [b]As a result of co-elution.

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Table 3. Chemical Shifts of the (αR,4R/S)-α-Methoxy-α-(trifluormethyl)-phenylacetoxy Fatty Acid Isopropyl Esters (I/II) and the (αS,4R/S)-α-Methoxy-α-(trifluormethyl)-phenylacetoxy Fatty Acid Isopropyl Esters (I′/II′) in the ^{1}H Spectra (400 MHz)

	C	δ(I) (ppm)/δ(I′) (ppm)	δ(II) (ppm)/δ(II′) (ppm)	Δδ(I−II) (ppm)/Δδ(I′−II′) (ppm)
esters of compound **1**	2	2.15	2.29	−0.14
	3	1.97/1.83	1.99/1.93	−0.02/−0.10
	4	5.12	5.12	0.00
	5	1.62	1.57	+0.05
	9	0.88	0.84	+0.04
esters of compound **2**	2	2.15	2.29	−0.14
	3	1.97/1.83	1.99/1.93	−0.02/−0.10
	4	5.12	5.12	0.00
	5	1.62	1.57	+0.05
	10	0.88	0.86	+0.02
esters of compound **3**	2	2.15	2.29	−0.14
	3	1.86/1.98	1.95/2.01	−0.09/−0.03
	4	5.14	5.13	+0.01
	5	1.97/1.66	1.93/1.63	+0.03/+0.03
	6	2.08	1.93	+0.15
	7	5.28	5.23	+0.05
	8	5.40	5.37	+0.03
	9	1.99	1.93	+0.06
	10	0.94	0.92	+0.02
esters of compound **4**	2	2.19	2.30	−0.11
	3	1.95	2.01	−0.06
	4	5.48	5.43	+0.05
	5	5.44	5.30	+0.14
	6	5.87	5.79	+0.08
	7	2.05	2.02	+0.03
esters of compound **5**	2	2.15	2.29	−0.14
	3	1.97/1.83	1.99/1.93	−0.02/−0.10
	4	5.12	5.12	0.00
	5	1.62	1.57	+0.05
	12	0.88	0.88	0.00
esters of compound **6**	2	2.15	2.32	−0.17
	3	1.98/1.84	2.02/1.95	−0.04/−0.11
	4	5.15	5.13	+0.02
	5	2.48/2.37	2.39/2.32	+0.09/+0.05
	6	5.33	5.22	+0.11
	7	5.54	5.46	+0.08
	8	2.01	1.95	+0.06

1−**6** were accumulated in the culture broth in total. Considering that the riboflavin process is currently neither designed nor optimized for the production of flavor compounds, these concentrations are surprisingly high and may offer options for a commercial utilization of this industrial side stream. Separation of the odor-active compounds could be achieved by fractionated distillation, which allows for separation of compounds with boiling point differences of less than 2 °C. Alternatively, extracts of the culture broth could be marketed as "flavoring preparation", which would not require an isolation of the single compounds.

Determination of the Absolute Configuration. To determine their absolute configuration, the lactones were transformed to their corresponding Mosher's esters, and the esters were analyzed by means of NMR spectroscopy (Table 3).

Albeit the orientation of the side chain at the stereo center as well as the optical rotation do not change, the priority (according to the Cahn−Ingold−Prelog sequence rules) of the aliphatic side chain changes if a double bond is located at position 5 or 6. The absolute configuration is thus inverted. On the basis of previous reports, lactones with the structure given in Figure 5A show a positive optical rotation, whereas lactones with the structure shown in Figure 5B show a negative optical rotation.[26,31] For this reason, in addition to their absolute

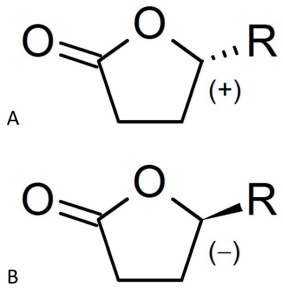

Figure 5. (A) Optically positively rotating (+)-lactones and (B) optically negatively rotating (−)-lactones.

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configuration, the unsaturated lactones are addressed as (+) and (−) enantiomers in the following sections.

Analysis by means of chiral GC revealed that the (+)-rotating lactones [(*R*)-**1**, **2**, **3**, **5**, (*S*)-**4**, and **6**] always eluted prior to their (−)-rotating antipodes on both chiral columns.

Determination of ee Values. The ee values were determined by means of MDGC (Table 4). Some of the analyzed lactones exhibited high ee values. Compounds **3** and **2** showed the highest ee values of 84.6 [92.3% (+) enantiomer] and 72.2 [86.1% (+) enantiomer], respectively. On the contrary, an excess of the (−) enantiomer (74%) was determined for compound **6**. Although no clear preference for the formation of either the (+) or (−) enantiomers was observed, the observed ee values allow for differentiation of the natural lactones from their synthetic analogues.

Table 4. Enantiomeric Excess of the Analyzed Lactones in the Fermentation Broth

compound	(+) enantiomer (%)	(−) enantiomer (%)	ee
1	49.4 (*R*)	50.6 (*S*)	1.2
2	86.1 (*R*)	13.9 (*S*)	72.2
3	92.3 (*R*)	7.7 (*S*)	84.6
4	64.7 (*S*)	35.3 (*R*)	29.4
5	57.5 (*R*)	42.5 (*S*)	15.0
6	26.0 (*S*)	74.0 (*R*)	48.0

In general, unsaturated lactones are formed from unsaturated fatty acids after *β*-oxidation. Especially, hydroxy fatty acids (e.g., ricinoleic acid) represent potential precursors. In feeding experiments with racemic 10-hydroxyhexadec-8-enoic acid, *Y. lipolytica* and *Pichia ohmeri* were able to produce compound **2**. *Y. lipolytica* produced compound **2** with an ee of 88 [in favor of the (−) enantiomer], while *P. ohmeri* formed compound **2** with an ee of 46 [in favor of the (+) enantiomer].[32]

The data presented in Table 4 reflect the enantiomeric distribution at the end of the fermentation process. Chalier and Crouzet showed that the ascomycetous fungus *Penicillium roqueforti* produces compounds **2**, **5**, and **6**. Compound **5** was formed with the highest ee of >99 [in favor of the (+) enantiomer] after 72 h of bioconversion, 90 after 96 h of bioconversion, and 70 after 120 h of bioconversion.[33] This might be caused by preferred conversion of one of the enantiomers of the hydroxy fatty acid. Alternatively, the fungus might be able to degrade the lactones or has an isomerase activity. It might thus be possible to produce lactones with *A. gossypii* in different enantiomeric compositions by isolating them after different times of cultivation.

By feeding *Y. lipolytica* with 10-hydroxyoctadec-8-enoic acid (formed by photooxidation from oleic acid), 10-hydroxyoctadec-8,12-dienoic acid (formed by photooxidation from linoleic acid), and 12-hydroxyoctadec-9,13-dieonic acid (also formed by photooxidation from linoleic acid), compounds **4**, **5**, and **6** were produced. Feeding *P. ohmeri* with 12-hydroxyoctadec-9,13-dienoic acid led to the production of compound **2**, which presumably results from the saturation of compound **4**.[32] Because *P. ohmeri* and *A. gossypii* belong to the same order (Saccharomycetales), they might have similar enzymes to catalyze this reaction. Compound **3** is assumed to be formed from 12-hydroxyoctadec-9,13,15-trienoic acid (from linolenic acid) similar to the other lactones after *β*-oxidation, followed by partial saturation of the double bonds.

Because soybean oil, which was used as a carbon source during the fermentative production of riboflavin, does not contain relevant amounts of hydroxy fatty acids,[34] *A. gossypii* must be able to hydrolyze triglycerides into free fatty acids and insert a hydroxyl group into the fatty acids. Analyses of soybean oil[34] showed that the main fatty acids are linoleic acid (54%), oleic acid (24%), palmitic acid (9%), and linolenic acid (8%). These fatty acids are all, except for palmitic acid, potential precursors of the above-mentioned hydroxy fatty acids.

As a result of the low amount of palmitoleic acid (0.1%) present in soybean oil, it seems to be likely that compound **2** is formed from compound **4** by saturation.

On an industrial scale, compound **2** is commonly produced by biotransformation of castor oil with yeasts, such as *Candida* or *Sporobolomyces*,[35] or conversion of ethyl dodecanoate with *Mucor circinelloides*.[8,36] Castor oil is the only natural oil available in larger quantities with a high amount of hydroxy fatty acids [ricinoleic acid and (9(*Z*),12*R*)-hydroxyoctadecenoic acid]. As a result of the (*R*) configuration of the hydroxyl group, only (*R*)-**2** can be produced from castor oil. Furthermore, ricinoleic acid bears no additional double bond after the hydroxyl group in the aliphatic chain. It is thus not possible to produce unsaturated lactones from castor oil. In the established industrial process for the production of compound **2**, the fermentation medium is acidified for lactonization.[8,36] This is not necessary in the production of lactones by *A. gossypii* because the pH of the culture broth is 6.3 at the end of the fermentation process. For some lactones, antimicrobial effects have been shown against a number of microorganisms. Therefore, *A. gossypii* might produce the lactones as protection against other competing microorganisms.[37]

Fungi, such as *Fusarium poae*, are no suitable alternatives for the production of lactones because of their formation of mycotoxins, e.g., fusarin C.[38] Other fungi that produce compound **2** *de novo*, e.g., *Bjerkandera adusta*, produce only minor amounts of this compound (0.3 mg/L).[39]

As a result of the steadily increasing demand for riboflavin as well as natural flavors, the simultaneous production of both with *A. gossypii* seems to be an alluring idea. All of these lactones, except for compound **2**,[9] have not been described for *A. gossypii*. Apart from that, the *de novo* formation of unsaturated and saturated lactones from non-hydroxy fatty acids by a filamentous fungus has not yet been reported.

All of the described lactones **1**−**6** naturally occur in plants, such as peaches, nectarines, and strawberries. In combination with other flavor compounds, they are responsible for the pleasant, fruity odor of fresh fruit.[40−42] The enantiomeric ratios of compound **1** reach from 28:72 (*R*/*S*) for raspberries to 93:7 (*R*/*S*) for passion fruits. For compound **2**, ratios of 49:51 (*R*/*S*) for raspberries to 100:0 (*R*/*S*) for strawberries have been reported. Racemic mixtures of compound **5** have been detected in raspberries, while enantiopure (*R*)-**5** was found in apricots, strawberries, and passion fruits.[43] On the basis of the overall intense and pleasant flavor impression imparted by the culture broth of *A. gossypii*, the formed enantiomeric composition of the lactones might well be suitable for the aromatization of food and beverages.

Determination of Odor Impressions and Approximation of Odor Thresholds. The odor impressions of the individual lactones and their odor thresholds in air were

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determined by means of GC−FID−O. The odor impressions of compounds **1**−**3**, **5**, and **6** were in agreement with those described previously.[22,23,44−46] Odor impressions of the individual enantiomers of compound **4** could not be found in the literature.

Until now, the odor thresholds of the racemic lactones **1**, **2**, **5**, and **6** were described in water and other matrices, e.g., oil.[40,47] Additionally, the odor threshold of racemic **5** has been determined in air (0.07−0.28 ng/L).[48] In addition, odor thresholds of both enantiomers of compounds **1**, **2**, and **5** were also determined in air [(*R*)/(*S*)-**1**, 4.5 ng/L; (*R*)/(*S*)-**2**, 12.5 ng/L; (*R*)-**5**, 0.8 ng/L; and (*S*)-**5**, 6.0 ng/L].[49] To the best of our knowledge, odor thresholds in air have not yet been described for compounds **3**, **4**, and **6** in the literature, neither for the racemates nor for the individual enantiomers.

For saturated lactones, decreasing odor thresholds are typically observed with an increasing length of the side chain.[50] This correlation was confirmed in the current study for the odor thresholds in air (Table 5). Similar results were

Table 5. Odor Impressions of the Racemic Lactones and Their Corresponding Enantiomers and Odor Thresholds in Air

	(+) enantiomer	(−) enantiomer	racemate
compound	odor impression/ odor threshold (ng/L)	odor impression/ odor threshold (ng/L)	odor impression/ odor threshold (ng/L)
1	coconut-like, fruity	coconut-like, creamy	coconut-like
	0.62	0.62	0.68
2	fruity, peach-like	coconut-like, fruity	fruity, peach-like, coconut-like
	0.30	0.61	0.74
3	fruity, floral	fruity, floral	fruity, floral
	3.36	3.36	2.90
4	sweetish, peach-like, fatty	sweetish, peach-like, green	sweetish, peach-like
	0.63	1.26	1.35
5	peach-like, fatty	peach-like, fatty	fruity, peach-like, fatty
	0.16	0.31	0.32
6	fruity, peach-like, green, soapy, pungent	fruity, peach-like, green, soapy, pungent	fruity, peach-like, green, pungent, fatty
	0.13	0.13	0.06

obtained for the unsaturated lactones. Because the increasing length of the saturated aliphatic chain is considered to cause decreasing odor thresholds of the lactones, this could also explain the observed trends with the unsaturated lactones. Compound **3**, bearing a *Z*-configured ω-3 double bond (which induces a kink in the side chain), showed the by far highest odor threshold of all analyzed lactones, followed by compound **4**, which bears an ω-5 double bond. Compound **6** (ω-6 double bond) showed the lowest odor threshold.

The comparison of the odor thresholds of the individual enantiomers to those of the corresponding racemates revealed no synergistic effects, and the odor qualities of the racemates mirrored the odor of the combination of the single enantiomers.

In summary, industrial riboflavin fermentation broths may represent an interesting source of natural lactones. Determination of odor thresholds in water as well as evaluation of a "flavoring preparation" will be the subject of a future study.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.9b01154.

Structure (top) and mass spectrum of compound (**1**) (middle) and the commercial standard (bottom) (S1), structure (top) and mass spectrum of compound (**2**) (middle) and the commercial standard (bottom) (S2), structure (top) and mass spectrum of compound (**3**) (middle) and the synthesized standard (bottom) (S3), structure (top) and mass spectrum of compound (**4**) (middle) and the synthesized standard (bottom) (S4), structure (top) and mass spectrum of compound (**5**) (middle) and the commercial standard (bottom) (S5), and structure (top) and mass spectrum of compound (**6**) (middle) and the synthesized standard (bottom) (S6) (PDF)

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS USED

AEDA, aroma extract dilution analysis; CID, collision-induced dissociation; ee, enantiomeric excess; ESI, electrospray ionization; FID, flame ionization detector; FD, flavor dilution; MDGC, multidimensional gas chromatography; NCE, normalized collision energy; NMR, nuclear magnetic resonance; O, olfactometry/olfactometric; ODP, olfactory detection port; PB, Paternò−Büchi; RI, retention index

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Industrial Riboflavin Fermentation Broths Represent a Diverse Source of Natural Saturated and Unsaturated Lactones

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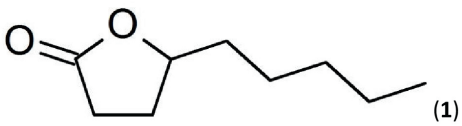
Supplementary information

Contents:

Mass spectra of the lactones found in the fermentation broth, analyzed by means of GC-MS (EI)



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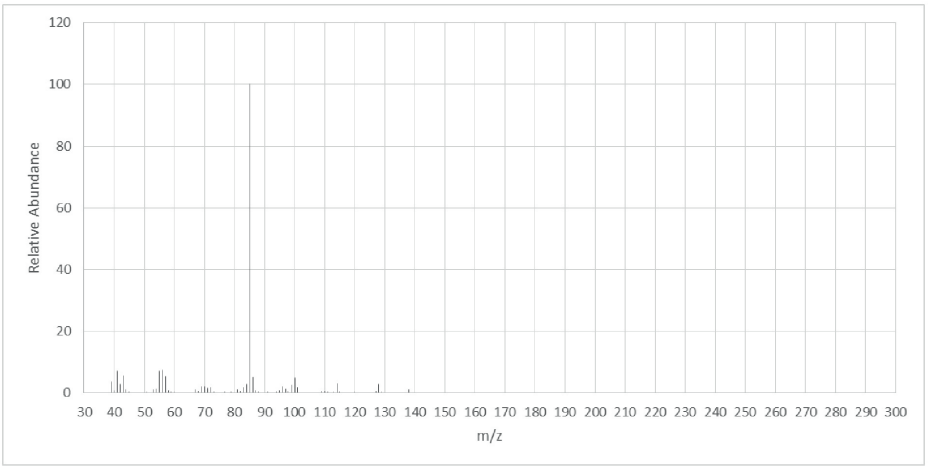

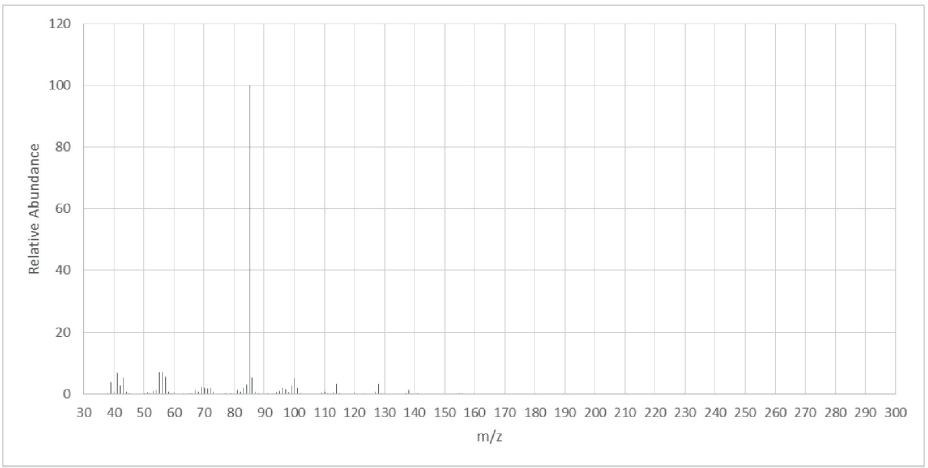

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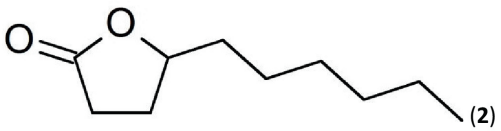

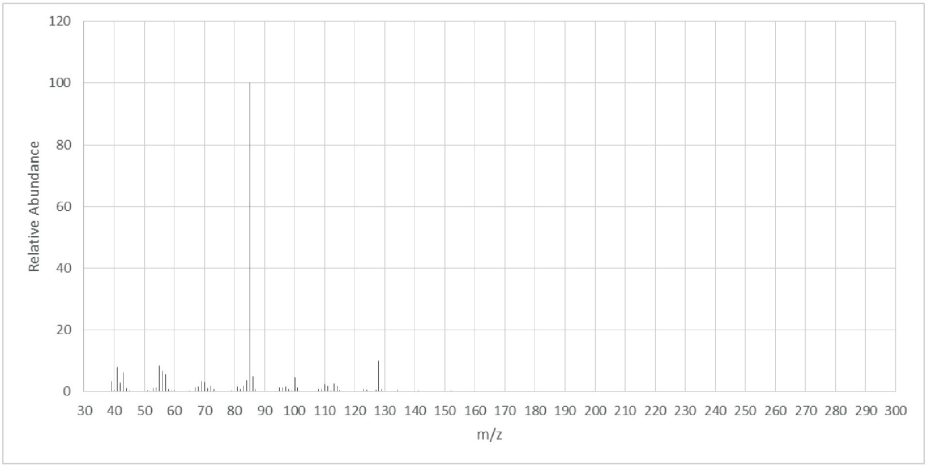

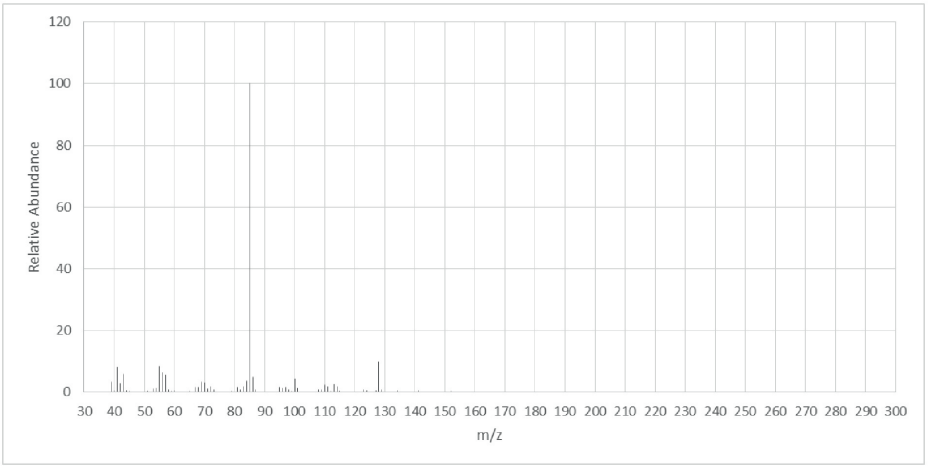

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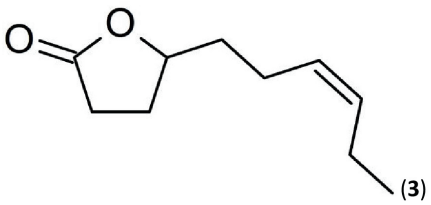

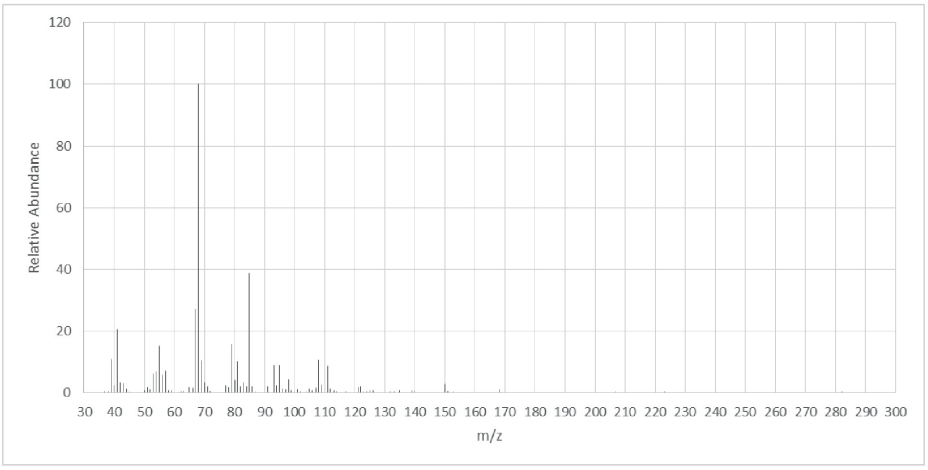

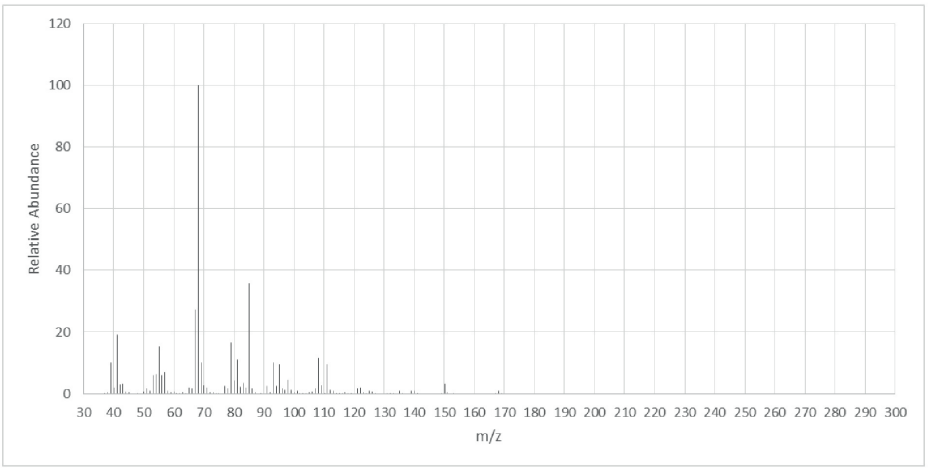

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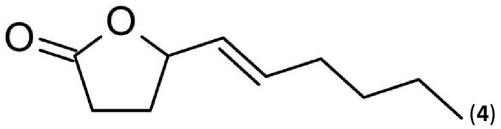

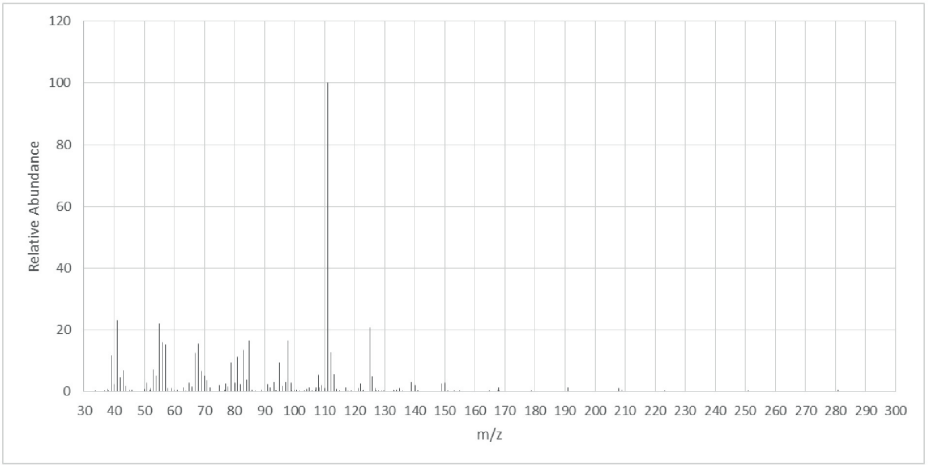

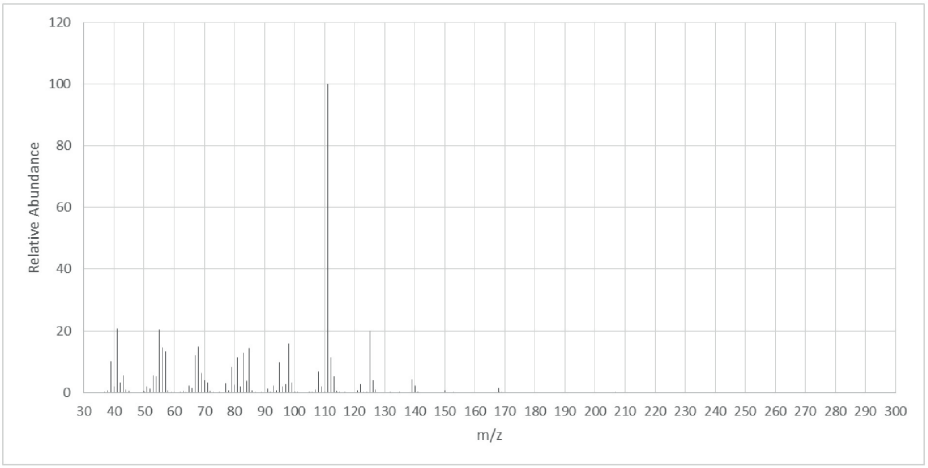

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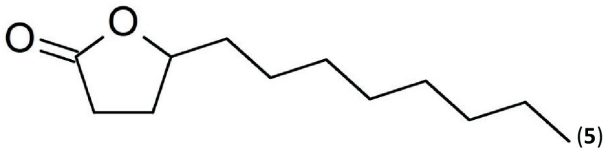

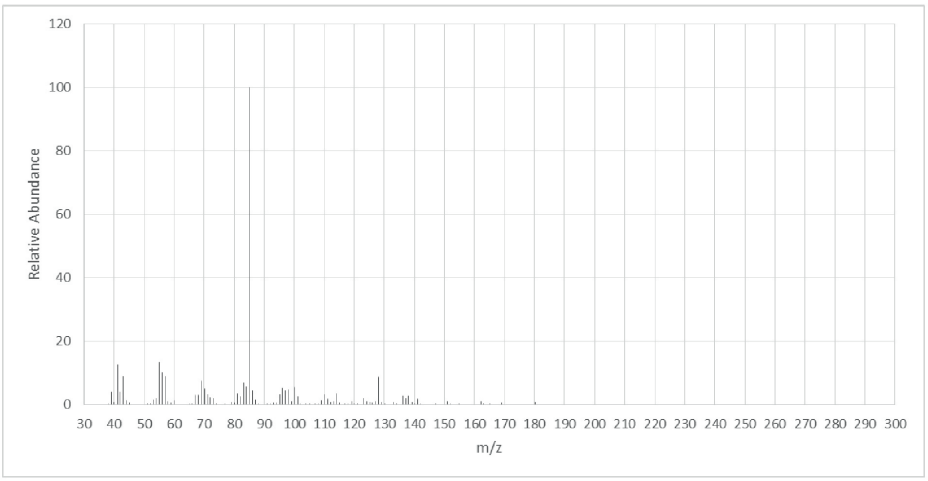

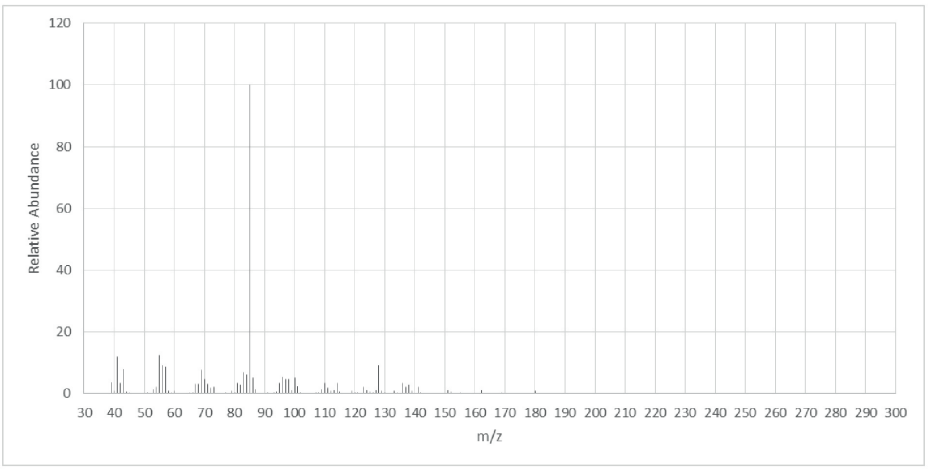

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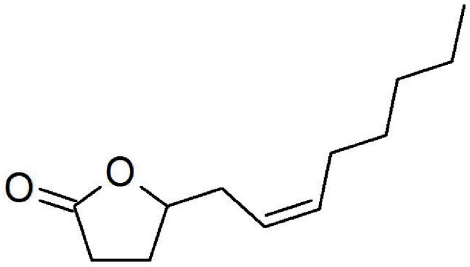
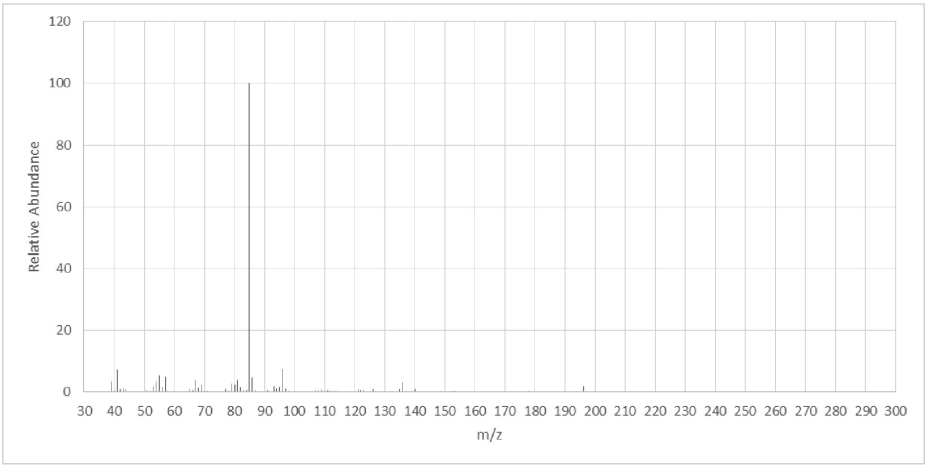
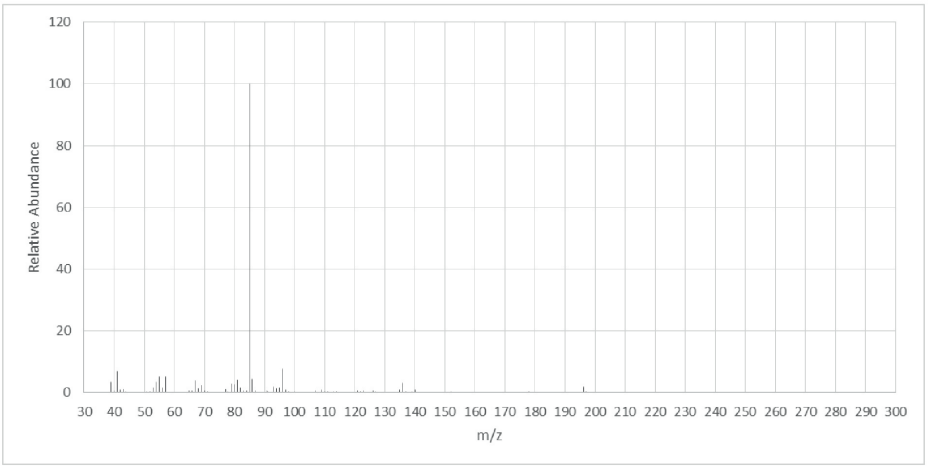


(**6**)





S6: structure (top) and mass spectrum of compound (**6**) (middle) and the synthesized standard (bottom)

Chapter III

Aroma active alkylated pyrazines are produced by Basfia succiniciproducens as by-products of succinic acid production

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RESEARCH ARTICLE

WILEY

Aroma active alkylated pyrazines are produced by *Basfia succiniciproducens* as by-products of succinic acid production

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Abstract

Culture supernatants of *Basfia succiniciproducens* derived from the industrial production of succinic acid exhibit an intense nutty, root vegetable-like, buttery and sourish smell. By means of headspace-gas chromatography-mass spectrometry-olfactometry (HS-GC-MS-O), 14 odour-active compounds were perceived and identified using two GC-columns of different polarity and comparison of retention indices and mass spectra to those of authentic reference compounds. Several alkylated pyrazines, including 2,3,5-trimethylpyrazine, 5-ethyl-2,3-dimethylpyrazine, 2,3,5,6-tetramethylpyrazine, 2,3,5-trimethyl-6-ethylpyrazine, 2,3,5-trimethyl-6-propylpyrazine and 2,3,5-trimethyl-6-butylpyrazine were found to contribute to the aroma of the culture supernatant. Quantitation of the pyrazines was performed by means of dynamic headspace-GC-MS after standard addition, and 2,3,5,6-tetramethylpyrazine (9.10 mg/L) was the most abundant compound. Waste streams of the biotechnological production of commodity chemicals may thus represent a sustainable resource for the isolation of aroma compounds.

KEYWORDS

alkylated pyrazines, dynamic headspace extraction, flavour, side stream, sustainability

1 | INTRODUCTION

Major advantages of the use of microorganisms for the industrial production of commodity chemicals include the mild reaction conditions, cheap and non-fossil fuel based reactants and the formation of the desired chirality.[1] Currently, succinic acid is biotechnologically produced with an annual production of several tens of thousands of tonnes, and the market is expected to further grow strongly.[2,3] The fermentative production of succinic acid is carried out with, *for example*, *Escherichia coli*, *Corynebacterium glutamicum* and *Basfia succiniciproducens*, using cheap, non-petroleum based and readily available raw materials, *for example*, glycerol (derived from the production of bio fuels) or glucose.[4-7] Succinic acid naturally occurs as an intermediate metabolite of the citric acid cycle. It is commercially used in food as acidifier and taste modifier. Due to its bifunctionality, it also represents a potential starting material for biodegradable polymers and many other C4 compounds.[2,7] *B succiniciproducens* is a Gram-negative bacterium which was first isolated in 2008 by Scholten and Dägele from bovine rumen.[4]

Abbreviations: CIS, cooled injection system; **DHS**, dynamic headspace; **GC**, gas chromatograph(y); **MS**, mass spectrometry; **NMR**, nuclear magnetic resonance; **O**, olfactometry; **RI**, retention index; **S/SL**, split/splitless; **SIM**, selected ion monitoring; **SPME**, solid phase microextraction; **TDU**, thermal desorption unit.

Surprisingly, industrial fermentation broths of *B succiniciproducens* from succinic acid production were observed to emit an intense, nutty, buttery, sourish and root vegetable-like smell. This odour could be attributed, amongst other compounds, mainly to alkylated pyrazines.

Alkylated pyrazines are broadly found in nature, for example, in bacteria, plants or insects but typically in low concentrations.[8] Some representatives are formed during preparation of food by, *for example*, deep frying or roasting from reducing sugars and amino acids via the Maillard reaction.[9] In thermally processed food, they contribute to their typical roasty and spicy odour properties.[10,11] In some of today's common food preparation methods, such as microwave cooking, these compounds are not formed due to the relatively low temperatures, so the demand for natural flavours with roasty odour properties is correspondingly high.[1] Some alkylated pyrazines show very low odour thresholds (<0.01 ng/L air).[10] Actually, 37 alkylated pyrazines are listed on the FEMA GRAS list.[12] Due to these characteristics, they are highly sought-after by the food industry and natural flavour compounds are preferred. They may be extracted from natural sources such as potatoes, coffee and nuts, but alkylated pyrazines do typically occur in trace amounts only in natural sources. Accordingly, the prices for natural pyrazines are high. For example, 1 kg of synthetically produced 2,5-dimethylpyrazine costs ~200 US$, while its natural counterpart is currently sold for ~3,500 US$/kg.[13]

Because of the immense amounts of fermentation broth resulting from the industrial succinic acid fermentation process, the extraction of valuable natural flavour compounds from this broth might be a promising idea. For the first time, an aroma analysis of a fermentation broth of this microorganism was carried out, whereby compounds of interest were subsequently quantitated.

2 | EXPERIMENTAL

2.1 | Culture broths

Culture supernatants of *B succiniciproducens*, deposited under the Budapest Treaty with DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Germany) under the deposit number DSM 18541, derived from the biotechnological succinate production, were provided by BASF SE (Ludwigshafen, Germany). Three batches were provided, and all analyses were performed with a representative sample. The microorganism was cultivated in a medium containing 50 g/L glycerol and 10 g/L glucose as carbon source, 5 g/L $(NH_4)_2SO_4$, 2 g/L Na_2CO_3, 1 g/L KH_2PO_4, vitamins, trace metals and osmolytes. The detailed cultivation conditions and medium composition have been described in the patent literature.[14] Besides the culture supernatant, the autoclaved non-inoculated medium was provided as a control.

2.2 | Chemicals

Petroleum ether (40-60°C; 95%) was purchased from Acros Organics (Geel, Belgium). 2,3-Hexanedione (94%) and 2,4,5-trimethyl oxazole (97%) were obtained from Alfa Aesar (Karlsruhe, Germany). Benzaldehyde (99%) and hydrogen peroxide (30%) were obtained from AppliChem (Darmstadt, Germany). Acetic acid (100%), chloroform-d (99.8 atom% D, with 0.03 vol% TMS, stabilized with Ag), diethyl ether (99.5%), iron(II) sulphate heptahydrate (99.5%), sodium carbonate decahydrate (98%), sodium sulphate (p.a.) and sulfuric acid (96%) were purchased from Carl Roth (Karlsruhe, Germany). Pentanal (97%) was obtained from Fisher Scientific (Darmstadt, Germany), Silica gel 60 was purchased from Macherey-Nagel (Düren, Germany) and 2,3-butanedione (97%) from Merck (Darmstadt, Germany). Acetoin (96%), 2,3-pentanedione (97%), 2,3,5,6-tetramethylpyrazine (98%) and 2,3,5-trimethylpyrazine (99%) were purchased from Sigma-Aldrich (Taufkirchen, Germany). 5-Ethyl-2,3-dimethylpyrazine (98%) was obtained from TCI (Eschborn, Germany). Butanal (97%) and propanal (97%) were purchased from Th. Geyer (Renningen, Germany) and ethyl acetate (98%) from VWR (Darmstadt, Germany). Helium (5.0) was obtained from Praxair (Düsseldorf, Germany) and nitrogen (5.0) from Air Liquide (Düsseldorf, Germany). All numbers given in parentheses represent the minimum purity.

2.3 | Methods

2.3.1 | Identification of odour-active compounds

The determination of odour-active compounds was performed by means of headspace-solid phase microextraction-gas chromatography-mass spectrometry-olfactometry (HS-SPME-GC-MS-O). For HS-SPME, 5 mL samples were added to a 20 mL-HS-vial. The samples were incubated and extracted on an agitator (15 min, 60°C, 250 rpm) of a GERSTEL MPS 2XL autosampler (Mülheim an der Ruhr, Germany), followed by an extraction with an SPME fibre for 30 min. The SPME fibre (Supelco, Steinheim, Germany) was coated with divinylbenzene/carboxen/polydimethylsiloxane (1 cm × 50/30 μm). The analytes were desorbed in the GC's inlet (250°C, splitless time: 1 min). The GC system used was an Agilent (Waldbronn, Germany) 7890A gas chromatograph equipped with either an Agilent VF-WAXms column (30 m × 0.25 mm, 0.25 μm film thickness; temperature programme: 40°C (3 min), 5°C/min to 240°C (12 min), carrier gas: helium, 1.56 mL/min (constant)) or an Agilent DB-5ms (deviating final temperature 300°C) and a split/splitless (S/SL) inlet. After the column, the carrier gas was split 1:1 by a GERSTEL μFlowManager splitter to an Agilent 7000B triple quadrupole detector (ionisation energy: 70 eV, ion source: 230°C, quadrupoles: 150°C, transfer line: 250°C, scan in q1: *m/z* 33-300, He quench gas: 2.25 mL/min, N_2 collision gas: 1.5 mL/min) and a GERSTEL ODP3 olfactory detection port (transfer line: 250°C, mixing chamber: 150°C, make up gas: N_2). Olfactometry was performed by three trained panelists. Compounds were considered odour-active if at least two panelists perceived and described the odour. The provided non-inoculated medium was analysed in the same way. The retention indices (RI) were calculated by linear interpolation from the retention times of *n*-alkanes (C_7-C_{30}).[15] The

odour-active compounds were identified by their mass spectra, their RIs on two columns of different polarities, their odour, and by comparison with authentic standards (commercially available or synthesized).

2.3.2 | Synthesis of 2,3,5-trimethyl-6-ethylpyrazine, 2,3,5-trimethyl-6-propylpyrazine and 2,3,5-trimethyl-6-butylpyrazine

The pyrazines were synthesized (Figure 1) based on a method described by Bohman *et al*.[16] One eq. 2,3,5-trimethylpyrazine and 0.4 eq. $FeSO_4$ x $7H_2O$ were solved in water and 4 eq. propanal, butanal or pentanal were added, respectively. At 0°C 50 eq. conc. sulfuric acid and 2.2 eq. H_2O_2 (30%) were added, and the solution was stirred for 3 h at room temperature. After 1 h and after 2 h 1.1 eq. H_2O_2 (30%) and 2 eq. of the respective aldehyde were added. Subsequently, the reaction mixture was washed with diethyl ether, the pH of the aqueous solution was adjusted to 8 with Na_2CO_3, and the mixture was extracted twice with diethyl ether. The combined organic extracts were dried over anhydrous Na_2SO_4 and the solvent was removed *in vacuo*. The residue was purified by means of column chromatography with SiO_2 using ethyl acetate and petroleum ether (1:1) as eluent. The structures of the isolated compounds were confirmed by means of nuclear magnetic resonance (NMR). The NMR-experiments were performed on a Bruker (Rheinstetten, Germany) Avance II 400 MHz and a Bruker Avance III HD 400 MHz.

NMR data of 2,3,5-trimethyl-6-ethylpyrazine

^{1}H NMR ($CDCl_3$, 400 MHz): δ 2.77 (2 H, q), 2.50 (3 H, s), 2.48 (3 H, s), 2.47 (3 H, s), 1.27 (3 H, t). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 153.0, 148.5, 148.2, 147.6, 28.0, 21.6, 21.5, 21.0, 13.0.

NMR data of 2,3,5-trimethyl-6-propylpyrazine

^{1}H NMR ($CDCl_3$, 400 MHz): δ 2.72 (2 H, m), 2.50 (3 H, s), 2.47 (3 H, s), 2.47 (3 H, s), 1.71 (2 H, m), 1.00 (3 H, t). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 151.9, 148.4, 148.1, 147.7, 36.6, 22.4, 22.2, 14.1, 14.1.

NMR data of 2,3,5-trimethyl-6-butylpyrazine

^{1}H NMR ($CDCl_3$, 400 MHz): δ 2.73 (2 H, m), 2.49 (3 H, s), 2.47 (3 H, s), 2.46 (3 H, s), 1.63 (2 H, m), 1.27 (2 H, m), 0.95 (3 H, t). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 152.2, 148.5, 148.2, 147.8, 34.6, 31.2, 22.9, 21.6, 21.5, 21.1, 14.1.

These data are in accordance with those described in the literature.[17] The NMR spectra can be found in the Figure SA1-SC2.

2.3.3 | Quantitation of alkylated pyrazines

Five alkylated pyrazines were quantitated by means of standard addition and dynamic headspace (DHS)-GC-MS (Table 3). 2,3,5-Trimethyl-6-butylpyrazine could not be quantitated due to its very low concentration in the culture broth. For quantitation, each standard compound was dissolved separately in water, a mixed stock solution was prepared and five standard solutions with differing concentrations were prepared. 100 μL of the standard solutions were added to 1 mL sample, each. Subsequently, 550 μL of these mixtures were extracted at room temperature (22 $\pm$ 1°C) by means of DHS with 3 L nitrogen and a flow rate of 100 mL/min. The analytes in the effluent were trapped on a TDU tube, filled with Tenax TA (GERSTEL). After DHS extraction, the Tenax tube was dried with 1 L nitrogen (flow rate 100 mL/min). Subsequently, the analytes were desorbed from the Tenax tube by means of a GERSTEL thermal desorption unit (TDU) (temperature programme: 30°C (0.5 min), 100°C/min to 240°C (3 min), transferline temperature: 250°C (fixed), split ratio: splitless, septum purge: 3 mL/min) coupled with a GERSTEL cooled injection system 4 (CIS 4) (temperature programme: 10°C (0.5 min), 12°C/s to 240°C (7 min), CIS glass liner packed with Tenax TA, split ratio: 10:1) and analysed by GC-MS. The GC system used was an Agilent 7890B gas chromatograph equipped with an Agilent VF-WAXms column (*cf.* 2.3.1; carrier gas: helium, 1.2 mL/min (constant)). The MS (Agilent 5877B quadrupole detector, ionisation energy: 70 eV, ion source: 230°C, quadrupole: 150°C, transferline: 250°C) was operated in selected ion monitoring-mode (SIM) (Table 1).

3 | RESULTS AND DISCUSSION

3.1 | Identification of odour-active compounds

The odour of the fermentation broths was perceived as sourish, buttery, nutty and root vegetable-like. By means of HS-SPME-GC-MS-O, 14 odour-active compounds were perceived and identified (Table 2,

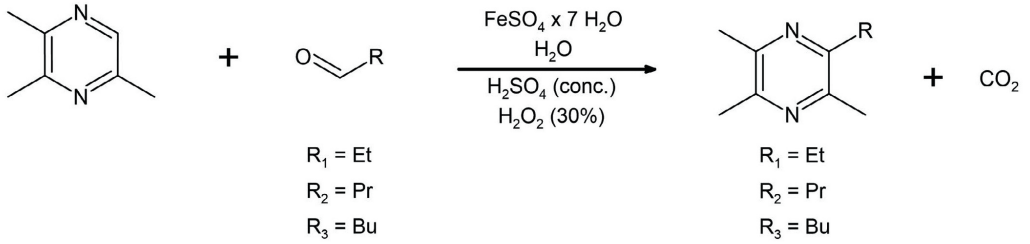

FIGURE 1 Synthesis of alkylated pyrazines *via* Minisci reaction (referring to Bohman *et al*)

TABLE 1 Quantifier ions and qualifiers ions of analytes used for quantitation and starting time of measurement in mass spectrometer

Starting time [min]	*m/z* quantifier ion	*m/z* qualifier ions
14.00	122	42, 81
15.60	135	54, 136, 137
15.60	136	54, 135, 137
17.00	149	122, 150
18.50	136	149, 164

Figure 2). The odour impressions perceived at the olfactory detector port well mirrored the overall impressions of the fermentation broths. Short chain 2,3-diketo compounds, *for example*, 2,3-butanedione and 2,3-pentanedione, are known for their buttery odour.[18] 3-Hydroxy-2-keto compounds (acyloins) are described in the literature as caramel-like, sweetish, buttery (3-hydroxy-2-pentanone) and earthy, mushroom-like (3-hydroxy-2-hexanone).[19] 2,4,5-Trimethyl oxazole is known for its fresh, mustard like[20] and acetic acid for its sourish, vinegar-like odour.[21] Alkylated pyrazines are aroma compounds which are typically formed by thermal food processing, *for example*, by roasting or frying, and exhibit an intense roasty, nutty or root vegetable-like odour.[10,22-24] The biosynthetic production of pyrazines has been described for some bacteria, *for example*, *Pseudomonas* and *Paenibacillus*.[25,26] In addition to their properties as potent aroma compounds, pyrazines are also known for their antimicrobial effects, which may explain their biosynthesis by bacteria to protect themselves from competitors.[27,28]

Diketo compounds, such as diacetyl, are constituents of many dairy products.[29] Diacetyl is produced by many different bacteria, *for example*, *Lactococcus lactis* or *Streptococcus diacetilactis*.[30,31] It is formed from 2-acetolactate, which is an intermediate in the biosynthesis of amino acids, such as valine, leucine, isoleucine, or aspartate.[32,33]

The biosynthesis of valine, leucine and isoleucine starts with the transfer of pyruvate to thiamine pyrophosphate under decarboxylation. In the case of leucine and valine, the resulting acetyl group is transferred to another pyruvate molecule, resulting in 2-acetolactate. In the biosynthesis of isoleucine, the acetyl group is transferred to 2-oxobutanoate instead of pyruvate, resulting in 2-aceto-2-hydroxybutanoate. The next step on the way to the amino acid is catalysed by a ketol-acid reductoisomerase. Dickschat *et al* proposed a pathway for the formation of acyloins in *C glutamicum*.[17] In this proposed pathway, acyloins are formed from 2-acetolactate and 2-aceto-2-hydroxybutanoate by decarboxylation resulting in acetoin or the respective elongated 3-hydroxy-2-keto compounds. By oxidation of the hydroxy group, 2,3-diketo compounds, *for example*, diacetyl, can be formed. A mutant of *C glutamicum* with deleted ketol-acid reductoisomerase activity showed a strongly increased formation of acyloins and 2,3-diketo compounds.[17] The production of acetoin by *C glutamicum* was already observed previously investigating a strain that was unable to form valine, leucine and isoleucine.[34] Furthermore, in both studies not only acyloins and 2,3-keto compounds were found but surprisingly also different alkylated pyrazines. Demain *et al* identified 2,3,5,6-tetramethylpyrazine in the fermentation broth of *C glutamicum*.[34] Dickschat *et al* additionally identified 2,3,5-trimethyl-6-ethylpyrazine, 2,3,5-trimethyl-6-prop

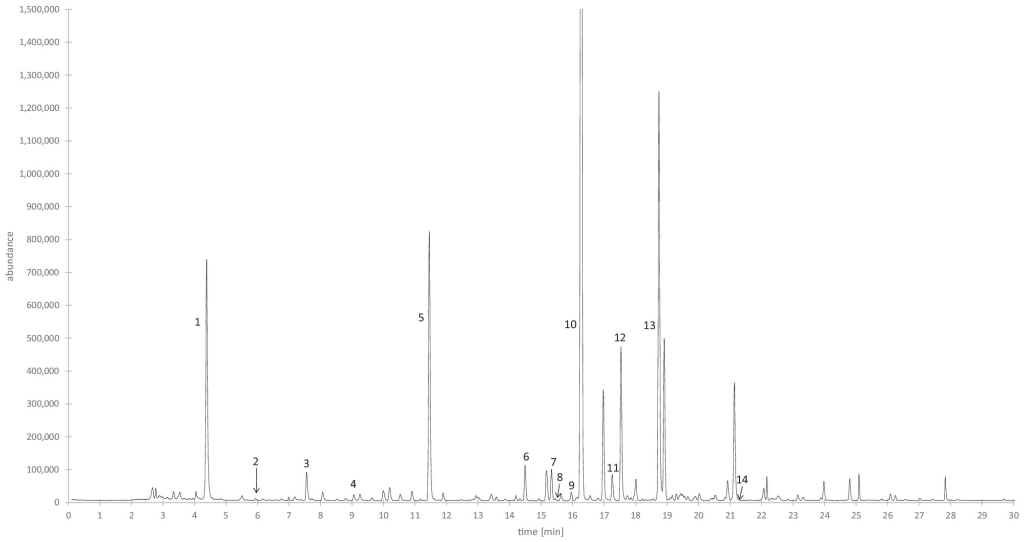

FIGURE 2 HS-SPME-GC-MS chromatogram (determined on a VF-WAXms column) of the analysed fermentation broth of *B succiniciproducens* with perceived and identified odour-active compounds on VF-WAXms column

TABLE 2 Perceived and identified odour-active compounds from the fermentation broth of *B succiniciproducens* analysed by means of HS-SPME-GC-MS-O with their corresponding retention indices (RI) on two columns of different polarity

			RI (VF-WAXms)		RI (DB-5ms)	
#	Compound	Odour impression	Sample	Standard	Sample	Standard
1	2,3-butanedione (diacetyl)	intense buttery	973	977	<700	<700
2	2,3-pentanedione	buttery, fresh	1054	1057	<700	<700
3	2,3-hexanedione	buttery, fruity, sweetish	1131	1131	791	793
4	2,4,5-trimethyl oxazole	green, fresh	1192	1196	833	841
5	3-hydroxybutan-2-one (acetoin)	buttery, green	1285	1284	712	711
6	2,3,5-trimethylpyrazine	nutty, musty	1400	1400	1002	1002
7	3-hydroxyhexan-2-one	roasty, earthy	1438	1434	892	890
8	acetic acid	vinegar	1443	1452	<700	<700
9	5-ethyl-2,3-dimethylpyrazine	cooked potatoes	1458	1458	1088	1087
10	2,3,5,6-tetramethylpyrazine	sweetish, nutty, musty	1470	1469	1085	1085
11	2,3,5-trimethyl-6-ethylpyrazine	spicy, vegetable stock like	1509	1509	1157	1156
12	benzaldehyde	bitter almond	1521	1521	964	964
13	2,3,5-trimethyl-6-propylpyrazine	sweetish, vegetable stock like	1577	1577	1237	1237
14	2,3,5-trimethyl-6-butylpyrazine	nutty, vegetable stock like	1674	1677	1330	1329

ylpyrazine and 2,3,5-trimethyl-6-butylpyrazine.[17] In the proposed pathway by Dickschat *et al*, the alkylated pyrazines are formed from acyloins or 2,3-diketo compounds. The first step is a transamination of these compounds, followed by a condensation reaction of two molecules and oxidation to give the final alkylated pyrazines (Figure 3). However, no quantitative data on the formation of alkylated pyrazines have been reported in this study.

Because of the similarities of the product patterns, it seems likely that *B succiniciproducens* might use analogous pathways to form acyloins, 2,3-diketo compounds and alkylated pyrazines. Therefore, it might be possible to delete the ketol-acid reductoisomerase activity, as described for *C glutamicum*, and to add the required amino acids to the fermentation medium to further increase the production of alkylated pyrazines.

Another *N*-containing heterocyclic compound identified in the samples was 2,4,5-trimethyl oxazole. It is known to be formed from a Schiff base (possibly derived from acyloins or 2,3-diketo compounds) in a condensation reaction with acetic acid.[17,35] Acetic acid was also perceived olfactorily during the analysis by means of GC-MS-O.

All of the described compounds, except for acetoin and acetic acid,[3] have not yet been described previously to be formed by *B succiniciproducens*.

Alkylated pyrazines are known for their intense odour impressions, and some representatives exhibit very low odour thresholds. By investigating the structure-odour-activity of more than 80 pyrazines, Wagner *et al* showed that trifunctionalized pyrazine stereoisomers bearing two methyl groups in *ortho* positions to one nitrogen atom show the lowest odour thresholds compared to isomers bearing two methyl groups in *ortho* and *meta* position.[10] All of the tetra-alkylated pyrazines analysed in this study bear two methyl groups in *ortho* positions. Therefore, it might be possible that the tetra-alkylated pyrazines detected in the fermentation broths of *B succiniciproducens* also show very low odour thresholds. However, the effect of the substitution pattern of tetrafunctionalized pyrazines on the respective odour threshold needs to be investigated to support this assumption.

Due to their desirable odour and low odour thresholds, alkylated pyrazines are important aroma compounds. Nevertheless, they are rarely found in higher amounts in nature and an economical extraction is thus difficult to achieve. 2,3,5-Trimethyl-6-butylpyrazine was only described a few times, *for example*, to be formed by *C glutamicum*,[17] in roasted cocoa,[36] or as a product of the Maillard reaction from wheat protein.[9] 2,3,5-Trimethyl-6-propylpyrazine occurs more often in natural sources. *For example*, it was described to be formed by *Pseudomonas* spp.,[25] in fermented soybean paste,[37] or as an aroma compound of gouda cheese.[22] 2,3,5-Trimethyl-6-ethylpyrazine has also been described as a Maillard product[38] and as an aroma compound found in chocolate.[39] The biosynthetic nature of the formed alkylated pyrazines was clearly shown, as no odour-active alkylated pyrazines could be detected in the non-inoculated media analysed as controls (cf. Figure SD).

3.2 | Quantitation of compounds of interest

Five alkylated pyrazines were quantitated by means of standard addition and DHS-GC-MS (Table 3, Figures SE1 and SE2). Amongst the analysed pyrazines, 2,3,5,6-tetramethylpyrazine showed by

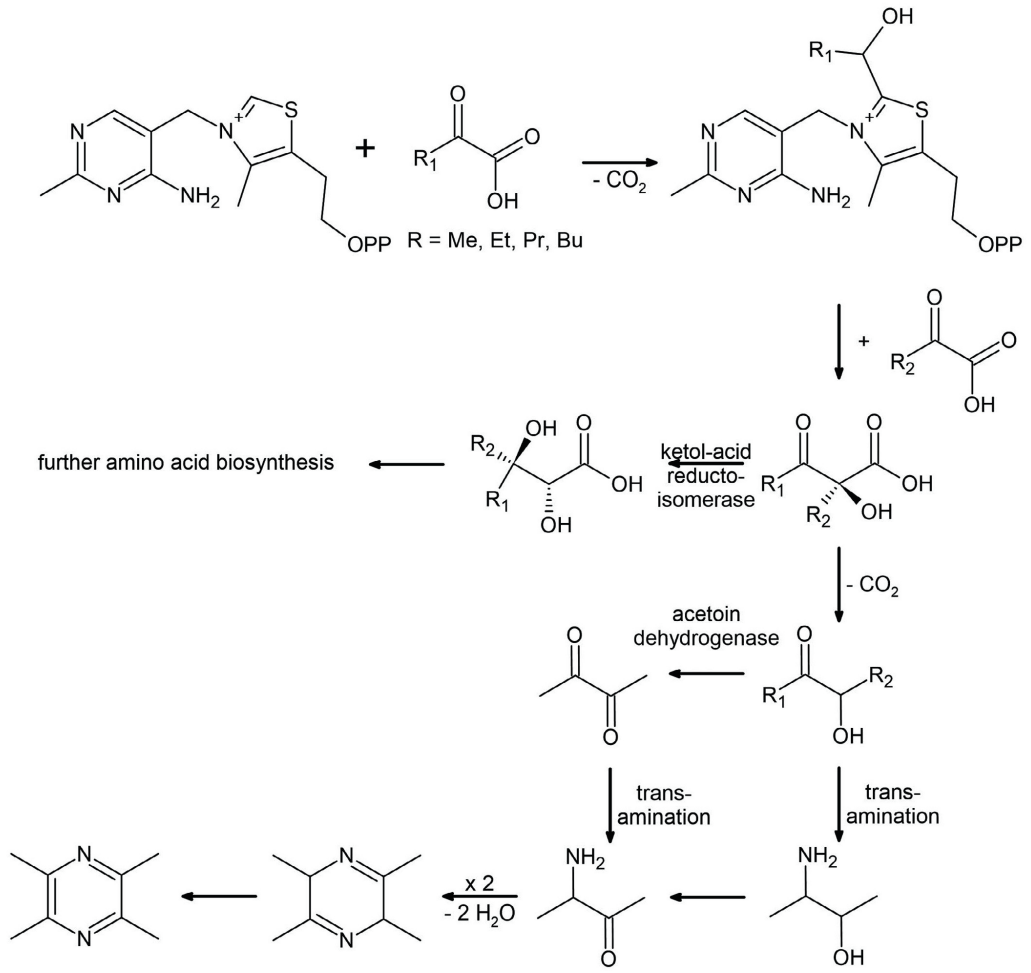

FIGURE 3 Proposed pathway for the formation of alkylated pyrazines according to Dickschat *et al* (here shown for 2,3,5,6-tetramethylpyrazine with $R_1 = R_2 = Me$)

TABLE 3 Quantitated amounts of the five compounds of interest by means of standard addition and DHS-GC-MS

Compound	Amount [μg/L]	R^2
2,3,5-trimethylpyrazine	179	0.998
5-ethyl-2,3-dimethylpyrazine	15	0.999
2,3,5,6-tetramethylpyrazine	9104	0.991
2,3,5-trimethyl-6-ethylpyrazine	36	0.999
2,3,5-trimethyl-6-propylpyrazine	233	0.993

far the highest concentration. The concentrations of the other alkylated pyrazines were much lower. Although the amounts of alkylated pyrazines were far away from other industrial processes for the fermentative production of odour-active compounds, the isolation from the fermentation broth could be achieved by concentration and distillation at reduced pressure or alternatively after acidification by cation exchangers.[14] Currently, natural alkylated pyrazines are typically extracted from food, such as potatoes, coffee or nuts.[13] To the best of our knowledge, there is no industrial production for these alkylated pyrazines based on fermentation. The succinic acid production process has actually neither been designed nor optimized for the production of alkylated pyrazines. Much higher amounts might thus be obtained, for example, by optimization of the fermentation medium or genetic optimization of the *B succiniciproducens* strain.

4 | CONCLUSION

A novel biotechnological access to alkylated pyrazines from culture supernatants of *B succiniciproducens* was investigated. The substrate used for the fermentation process is inexpensive glycerol, which is released in large quantities during the production of biofuels. Isolation of the flavour compounds from culture supernatants, a designated waste product produced during a large-scale process, may allow for a sustainable production of aroma compounds which do not occur in large quantities in nature. Alkylated pyrazines might be separated from the fermentation broth by distillation or cation exchangers. Depending on the cost of downstream processing, it might be economical to monetise the waste stream directly. Alternatively, the strain could be optimised for aroma production, *for example*, by deleting the ketol-acid reductoisomerase activity, or the amino acids needed as precursors could be added to the fermentation medium.

DECLARATION OF INTEREST

A patent application (Pelzer, R., Zorn, H., Birk, F., & Fraatz, M. A. (2019). Fermentative Production of Pyrazines Using Microorganisms of the Genus Pasteurellaceae (EP3680339A1)) has been filed by BASF SE. Ralf Pelzer is an employee of BASF SE. Apart from that, the authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this manuscript.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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Chapter IV

*Generation of Flavor Active Compounds by Electrochemical Oxidation of (*R*)-Limonene*

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Generation of Flavor-Active Compounds by Electrochemical Oxidation of (*R*)-Limonene

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ABSTRACT: Terpenes may be converted by electrochemical oxidation to various oxidized products with appealing aroma properties. In this study, (*R*)-limonene was anodically oxidized in the presence of ethanol, and the resulting mixture exhibited a pleasing fruity, herbal, citrus-like, and resinous odor. The aroma-active compounds were purified by means of preparative high-performance liquid chromatography, and their structures were elucidated by means of gas chromatography (GC)−mass spectrometry and nuclear magnetic resonance spectroscopy. In addition, the odor of the isolated compounds was determined by means of GC−olfactometry. Seventeen compounds were isolated, and for only four of them, analytical data had been reported previously in the literature. Furthermore, only for two of the compounds, an odor description had been available in the literature.

KEYWORDS: *aroma, terpenes, electrosynthesis, gas chromatography, structure elucidation*

INTRODUCTION

(*R*)-Limonene is the major constituent of orange oil, which can be obtained in huge amounts from the side streams of orange juice production by means of steam distillation or cold pressing.[1] The share of (*R*)-limonene in orange oil is up to 95%.[2] Besides the use of limonene as an important flavor and fragrance compound, it is also used as a platform chemical and extraction solvent.[3]

By natural oxidation reactions, further important aroma compounds may be generated from limonene, including carvone, limonene oxide, and menthol.[4,5] Other studies revealed that new flavor compounds may be generated by synthetic oxidation of terpenes.[6] Starting from, for example, linalool or citronellol, systematically oxidized derivatives thereof were produced, some of which exhibited highly interesting organoleptic properties. The odor impressions were sometimes fundamentally different from those of the original compounds.[7,8] Some of these compounds have not been described in the literature before.

Especially in view of the fact that (*R*)-limonene is obtained as a readily available starting material from a side stream of the food industry, it is alluring to generate new aroma substances from limonene. Therefore, in the current study, limonene was electrochemically oxidized in the presence of ethanol to create new aroma compounds with appealing olfactory impressions. Such an anodic oxidation of terpenes such as limonene is generally known.[9] Compared to other methods for the oxidation of terpenes, no environmentally harmful compounds, for example, aggressive chemicals or heavy metals or their respective salts, were used.[6,10−12] Therefore, the electrochemical oxidation of terpenes can be regarded as a sustainable alternative to generate valuable aroma compounds.

The aim of this study was to isolate and to structurally characterize the new aroma compounds prepared in a sustainable way by means of electrochemical oxidation of terpenes. This method has an enormous potential to expand the spectrum of currently known and available aroma compounds.

MATERIALS AND METHODS

Chemicals. *tert*-Butyl methyl ether (99.9%) and (*R*)-carvone (99%) were purchased from Acros Organics (Geel, Belgium). Chloroform-*d* [99.8 atom % D, with 0.03 vol % tetramethylsilane (TMS), stabilized with Ag] was obtained from Carl Roth (Karlsruhe, Germany). Methylene chloride (99.9%) was purchased from Fisher Scientific (Darmstadt, Germany). *n*-Hexane (97%) was obtained from Honeywell (Darmstadt, Germany). Silica gel 60 was purchased from Macherey-Nagel (Düren, Germany). Propan-2-ol (99.8%) was obtained from VWR (Darmstadt, Germany). (*R*)-Limonene (97%, analytical standard), (*R*)-limonene (94%, for synthesis), geranyl acetate (98%), dihydrocarvone (98%; mixture of isomers), and thin-layer chromatography (TLC) silica gel 60G plates were purchased from Sigma-Aldrich (Taufkirchen, Germany). *p*-Cymene was obtained from TCI (Eschborn, Germany). Hydrogen (5.0) and helium (5.0) were obtained from Praxair (Düsseldorf, Germany) and nitrogen (5.0) from Air Liquide (Düsseldorf, Germany). Numbers in parentheses are minimum purities.

Samples. Samples were prepared by BASF SE by electrochemical oxidation of (*R*)-limonene. Therefore, (*R*)-limonene (5%) and methyl-tri-*n*-butylammonium methylsulfate (12%) were dissolved in ethanol (83%). The solution was electrolyzed at 25 °C in a capillary gap cell. This lab cell resembles in principal BASF's capillary gap cell employed at the production scale.[12] It contains a stack of bipolar

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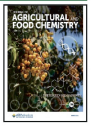

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Table 1. Overview over the Solvent Composition for the Isolation of the Respective Compounds by Means of Preparative HPLC and Their Odor Properties as Determined by GC−FID−O Analysis

isolate no.	preseparated fraction	eluents used for prep. HPLC (*n*-hexane = A, methylene chloride = B, *tert*-butyl methyl ether = C)	odor impression
1a	3	solvents: A + B; 0 min 100% A, 15 min 97% A, 30 min 90% A, 50 min 70% A, 60 min 50% A	herbal, parsley root-like
1b	3	solvents: A + B; 0 min 95% A, 20 min 95% A	herbal, parsley root-like
2a	4	solvents: A + C; 0 min 100% A, 30 min 98.75% A, 40 min 87.5% A, 50 min 75% A, 60 min 75% A	herbal, fresh, green, dill
2b	4	solvents: A + B; 0 min 100% A, 45 min 65% A, 55 min 40% A, 60 min 0% A	herbal, spicy, earthy, juniper
3a	6	see 2b	sweetish, fruity, anise, licorice
3b	6	solvents: A + B; 0 min 100% A, 20 min 97% A, 30 min 94% A, 40 min 90% A, 50 min 75% A, 55 min 50% A, 60 min 25% A	spicy, anise, cinnamon, clove
4a	6	solvents: A + B; 0 min 100% A, 20 min 96% A, 30 min 92% A, 50 min 75% A, 60 min 60% A, 65 min 45% A, 70 min 25% A, 75 min 0% A	herbal, green, floral, parsley root-like
4b	5/6	solvents: A + B; 0 min 100% A, 20 min 95% A, 30 min 90% A, 45 min 75% A, 55 min 50% A, 60 min 25% A	herbal, parsley root-like
5	5	solvents: A + B; 0 min 100% A, 48 min 0% A, 52 min 0% A	fruity, sweetish, green, coriander
6	5	see **2**b	herbal, earthy, parsley root-like
7	6	see **4**a	minerally, woody, earthy, spicy
8a	6	see **2**a	fresh, minty, herbal, minerally, caraway
8b	6	solvents: A + B + C; 0 min 99% A 0% B, 60 min 50% A 50% B	fresh, minty, herbal, minerally
9a	5	solvents: A + B + C; 0 min 100% A, 60 min 2% A 96% B	herbal, dill-like, earthy
9b	6	solvents: A + B; 0 min 90% A, 20 min 80% A, 40 min 55% A, 55 min 20% A, 60 min 0% A, 75 min 0% A	fresh, menthol like, floral, citrus-like
10a	6	see **9**a	minty, tart, floral, fruity, fresh, citrus-like
10b	6	see **9**a	minty, tart, resinous-like, green, spicy

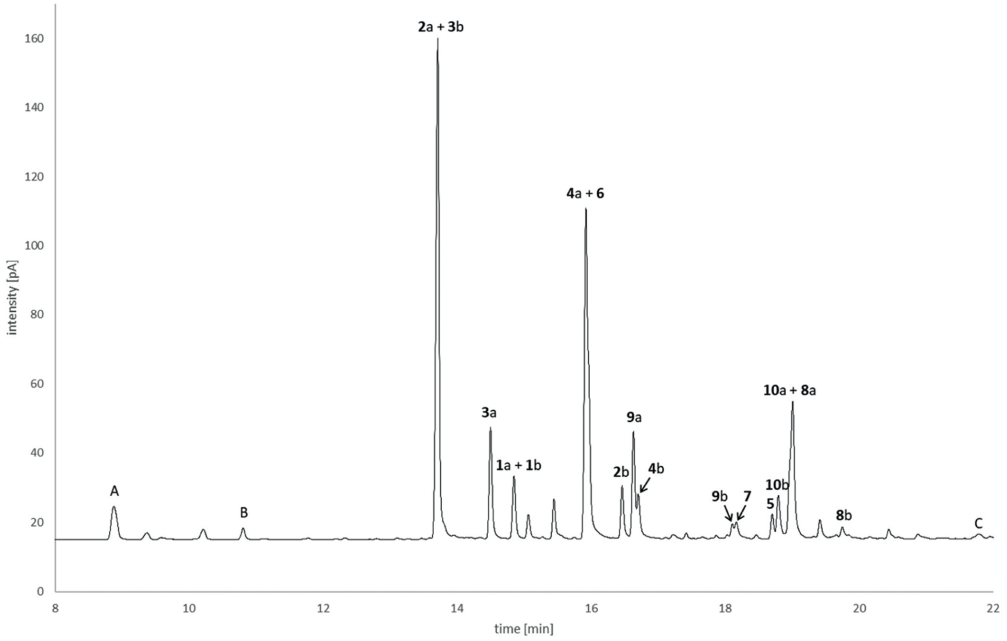

Figure 1. Representative GC−FID chromatogram of the diluted original sample (A: limonene; B: *p*-cymene; C: carvone) determined on an Agilent HP-INNOWAX column.

electrodes (147 cm^2 area per electrode). For this stack, Sigrafine MKUS (SGL Carbon, Wiesbaden) graphite electrodes were each covered on one side with a steel foil (25 μm) and then assembled with 1.5 mm spacers forming a stack with nine gaps. It was operated in the bipolar mode, resulting in a graphite anode and steel cathode at each gap. The cell was embedded in a circuit, and the electrolysis was conducted in a batch mode cycling the electrolyte. 3 F was applied at a constant current density of 17 mA/cm^2.[13]

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Preseparation of the Sample. Various solvent combinations were tested by means of TLC for the separation of the substances. Combinations of *n*-hexane and methylene chloride with a polar stationary phase were found to be suitable for an efficient separation of the substances. Subsequently, the aroma compounds were preseparated by means of column chromatography on silica gel 60 as a stationary phase. The mobile phase was composed of *n*-hexane (A) and methylene chloride (B) in different ratios (100% A; 80% A + 20% B; 60% A + 40% B; 40% A + 60% B; 20% A + 80% B; 100% B). Six fractions of approximately 100 mL each, respectively, and 200 mL for the last fraction were collected, and the solvent was removed under a stream of nitrogen.

Preparative High-Performance Liquid Chromatography. The six fractions were further subjected to preparative high-performance liquid chromatography (HPLC) according to a previously developed protocol.[14] Therefore, the samples were dissolved in the respective starting eluent (Table 1). The preparative HPLC system used was a Young Lin Instrument (Anyang-si, South Korea) YL9110S with a quaternary pump (flow: 15 mL/min) equipped with a polar column (guard column: Macherey-Nagel, Nucleodur 100−5, 10 × 16 mm; preparative column: Macherey-Nagel, Nucleodur 100−5, 250 × 21 mm) coupled with a YL9120S UV/Vis detector (wavelengths: 210 and 235 nm) and an Advantec (Dublin, CA) CHF 112SC fraction collector. 7.5 mL was collected per fraction.

Purity Check by Means of Gas Chromatography. Every fraction obtained from preparative HPLC was analyzed by means of gas chromatography (GC) coupled with a flame ionization detector (FID). The gas chromatographic system was an Agilent (Waldbronn, Germany) 7890A gas chromatograph equipped with an Agilent HP-INNOWAX column [30 m × 0.32 mm, 0.25 μm film thickness; temperature program: 40 °C (3 min), 20 °C/min to 240 °C (7 min); carrier gas: hydrogen, 2.0 mL/min, constant], a split/splitless inlet (250 °C; injection volume 1 μL; split ratio 1:20 or 1:50), and a FID (250 °C; hydrogen, 40 mL/min; air, 400 mL/min; nitrogen, 30 mL/min).

Description of Odor Impressions. In order to avoid a falsification of the odor impressions of the purified isolates due to possible traces of impurities, the odor impressions of the isolated compounds were determined by means of GC−FID−olfactometry (GC−FID−O). The gas chromatographic system used was an Agilent 7890A gas chromatograph equipped with an Agilent HP-INNOWAX column [30 m × 0.32 mm, 0.25 μm film thickness; temperature program: 40 °C (3 min), 5 °C/min to 240 °C (7 min); carrier gas: hydrogen, 2.2 mL/min, constant] and a split/splitless inlet (250 °C; injection volume 1 μL; split ratio 1:10 or splitless; splitless time: 1 min). After the column, the carrier gas was split 1:1 by a GERSTEL μFlowManager Splitter to a FID (250 °C; hydrogen, 40 mL/min; air, 400 mL/min; nitrogen, 30 mL/min) and a GERSTEL ODP3 olfactory detection port (transfer line, 250 °C; mixing chamber, 150 °C; make up gas nitrogen). A section of the chromatogram of the sample is shown in Figure 1; the odor impressions are presented in Table 1.

Structure Elucidation of the Isolated Compounds. Pure compounds obtained from preparative HPLC were analyzed by means of GC−mass spectrometry (MS) on two columns of different polarities. The first gas chromatographic system used was an Agilent 7890A gas chromatograph equipped with an Agilent VF-WAXms column [30 m × 0.25 mm, 0.25 μm film thickness; temperature program: 40 °C (3 min), 5 °C/min to 240 °C (7 min); carrier gas: helium, 1.2 mL/min, constant] and a split/splitless inlet (250 °C; injection volume 1 μL; split ratio 1:20 or 1:50) coupled to an Agilent 5975C quadrupole mass spectrometer (ionization energy: 70 eV; ion source: 230 °C; quadrupole: 150 °C; m/z 33−300). The retention indices were calculated by linear interpolation from the retention times of *n*-alkanes (C_7−C_{30}).[15] The second gas chromatographic system used for the determination of retention indices on a nonpolar column was an Agilent 7890B gas chromatograph equipped with an AgilentDB-5ms column [30 m × 0.25 mm, 0.25 μm film thickness; temperature program: 40 °C (3 min), 5 °C/min to 300 °C (7 min); carrier gas: helium, 1.2 mL/min, constant] and a split/splitless inlet (250 °C; split ratio 1:50 or 1:100) coupled to an Agilent 5977B quadrupole mass spectrometer (ionization energy, 70 eV; ion source, 230 °C; quadrupole, 150 °C; m/z 33−300).

Nuclear Magnetic Resonance Analyses of the Isolated Compounds. All isolated compounds were analyzed by means of nuclear magnetic resonance (NMR) spectroscopy. Therefore, the solvent of pure fractions was removed under a nitrogen stream, and the resulting residue was dissolved in $CDCl_3$. NMR spectra were recorded using a Bruker (Rheinstetten, Germany) Avance II 400 MHz [working at 400.130 MHz (^{1}H) and 100.613 MHz (^{13}C)] spectrometer equipped with a 5 mm inverse detection *z*-gradient BBI probe, a Bruker Avance III HD 400 MHz [working at 400.250 MHz (^{1}H) and 100.643 MHz (^{13}C)] spectrometer equipped with a 5 mm *z*-gradient PA TBO probe, or a Bruker Avance III HD 600 MHz [working at 600.050 MHz (^{1}H) and 150.883 MHz (^{13}C)] spectrometer equipped with a 5 mm *z*-gradient BBO probe at room temperature unless otherwise stated. The ^{1}H chemical shifts (δ) are reported in parts per million (ppm) relative to the TMS signal ($CDCl_3$: δ = 7.26 ppm relative to TMS δ = 0 ppm) and the ^{13}C chemical shifts corresponding to the deuterated solvent ($CDCl_3$: δ = 77.0 ppm). Coupling constants (J) are reported in hertz (Hz). ^{13}C NMR experiments (^{13}C{^{1}H} and DEPT) were proton-decoupled.

The complete ^{1}H and ^{13}C NMR assignments for the isolated compounds were achieved using a combination of 1D (^{1}H NMR, ^{13}C NMR, DEPT135) and 2D [^{1}H,^{1}H correlation spectroscopy (COSY), heteronuclear single-quantum correlation, heteronuclear multiple-bond correlation, and nuclear Overhauser effect spectroscopy] experiments using standard Bruker pulse programs. The data were collected and processed by TOPSPIN software (Bruker).

Semiquantitation of the Identified Components in the Original Samples. The amount of the isolated compounds in the original samples was determined semiquantitatively by means of GC−FID using geranyl acetate as an internal standard. Therefore, 84.7 mg of the sample and 11.1 mg of geranyl acetate were diluted to 20 mL with *n*-hexane. 1 μL thereof was analyzed by means of GC−FID. The gas chromatographic system was an Agilent 7890A gas chromatograph equipped with an Agilent HP-INNOWAX column [30 m × 0.32 mm, 0.25 μm film thickness; temperature program: 40 °C (3 min), 3 °C/min to 240 °C (7 min); carrier gas: hydrogen, 2.0 mL/min, constant], an Agilent DB-5 column [30 m × 0.32 mm, 0.25 μm film thickness; temperature program: 40 °C (3 min), 3 °C/min to 300 °C (7 min); carrier gas: hydrogen, 2.0 mL/min, constant], a split/splitless inlet (250 °C; different split ratios between 1:10 and 1:500), and a FID (250 °C; hydrogen, 40 mL/min; air, 400 mL/min; nitrogen, 30 mL/min). For the semiquantitative calculation, the response factor of the internal standard was assumed to be 1 (Table 2).

Statistics. The GC−O experiments were performed in triplicate by four trained panelists. The panelists, three men and one woman, were between 23 and 30 years old. A compound was considered to be odor active if at least three of the four panelists could perceive and describe the substance. The semiquantitative experiments were run in duplicate.

■ RESULTS AND DISCUSSION

The sample was prepared as described above. The resulting material was colored amber-like and exhibited a fruity, herbal, citrus, and resinous odor. From this sample, 17 compounds were isolated by means of preparative HPLC (Figure 2). Their structures were elucidated by means of NMR and GC−MS. Additionally, limonene, *p*-cymene, and carvone were identified by means of GC−MS by comparing their retention indices and mass spectra with those of commercially available standards on two columns of different polarities (Table 2). Thus, in total, 20 different compounds were identified in the oxidized (*R*)-limonene sample.

Compound Identification by GC−MS and NMR Spectroscopy. *NMR and GC−MS Data of Compound 1a.*

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Table 2. Determined Retention Indices of the Isolated and Identified Compounds on Two Columns of Different Polarities Compared with Those of Commercially Available Standards (n.a.: Not Available) and Their Determined Approximate Amounts in the Sample

	retention index$_{sample}$		retention index$_{standard}$		
compound/ isolate no.	VF-WAXms	DB-5	VF-WAXms	DB-5	approx. amount [mg/kg]
1a	1431	1226	n.a.	n.a.	69
1b	1431	1228	n.a.	n.a.	71
2a	1384	1192	n.a.	n.a.	1005
2b	1498	1241	n.a.	n.a.	122
3a	1418	1207	n.a.	n.a.	266
3b	1386	1188	n.a.	n.a.	337
4a	1477	1232	n.a.	n.a.	272
4b	1509	1259	n.a.	n.a.	86
5	1591	1313	n.a.	n.a.	123
6	1475	1234	n.a.	n.a.	782
7	1565	1373	n.a.	n.a.	41
8a	1617	1197	1612	1197	401
8b	1637	1204	1629	1203	11
9a	1501	1351	n.a.	n.a.	251
9b	1563	1369	n.a.	n.a.	31
10a	1611	1191	n.a.	n.a.	71
10b	1600	1186	n.a.	n.a.	9
limonene	1187	1029	1179	1028	136
p-cymene	1259	1026	1254	1023	8
carvone	1714	1243	1708	1243	5

^{1}H NMR ($CDCl_3$, 400 MHz): δ 4.75 (1H, m, H−C9), 4.73 (1H, m, H−C9), 4.44 (1H, d, J = 3 Hz, H−C2), 3.69 (2H, m, H−C11), 2.82 (1H, m, H−C3), 2.24 (1H, m, H−C6), 1.86 (1H, m, H−C5), 1.76 (1H, m, H−C4), 1.72 (3H, brs, H−C10), 1.36 (1H, m, H−C4), 1.28 (3H, t, J = 7 Hz, H−C12), 1.26 (1H, m, H−C5), 1.07 (3H, d, J = 7 Hz, H−C7).

^{13}C NMR ($CDCl_3$, 100 MHz): δ 158.7 (s (C−q), C1), 150.2 (s (C−q), C8), 109.8 (t (=CH_2), C9), 97.0 (d (=CH), C2), 61.8 (t (−O−CH_2), C11), 42.6 (d (CH), C3), 32.4 (d (CH), C6), 30.2 (t (CH_2), C5), 26.3 (t (CH_2), C4), 20.7 (q (CH_3), C10), 18.8 (q (CH_3), C7), 14.7 (q (CH_3), C12).

GC−MS (EI, 70 eV) m/z (%): 180 (100) [$M^{\bullet+}$], 165 (99), 43 (68), 137 (63), 109 (61), 95 (60), 81 (56), 123 (50), 41 (38), 67 (37).

NMR and GC−MS Data of Compound ***1b***. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 4.76 (1H, m, H−C9), 4.74 (1H, m, H−C9), 4.42 (1H, d, J = 3 Hz, H−C2), 3.70 (2H, m, H−C11), 2.83 (1H, m, H−C3), 2.21 (1H, sext, J = 6 Hz, H−C6), 1.73 (1H, m, H−C5), 1.72 (3H, brs, H−C10), 1.61 (1H, m, H−C4), 1.49 (1H, m, H−C5), 1.47 (1H, m, H−C4), 1.28 (3H, t, J = 7 Hz, H−C12), 1.09 (3H, d, J = 7 Hz, H−C7).

^{13}C NMR ($CDCl_3$, 100 MHz): δ 159.1 (s (C−q), C1), 150.3 (s (C−q), C8), 109.8 (t (=CH_2), C9), 96.9 (d (=CH), C2), 61.7 (t (−O−CH_2), C11), 42.9 (d (CH), C3), 31.8 (d (CH), C6), 29.2 (t (CH_2), C5), 24.6 (t (CH_2), C4), 20.4 (q (CH_3), C10), 19.1 (q (CH_3), C7), 14.7 (q (CH_3), C12).

GC−MS (EI, 70 eV) m/z (%): 180 (100) [$M^{\bullet+}$], 165 (99), 43 (68), 109 (64), 137 (63), 95 (62), 81 (57), 123 (51), 41 (38), 67 (38).

NMR and GC−MS Data of Compound ***2a***. ^{1}H NMR ($CDCl_3$, 600 MHz): δ 4.86 (1H, m, H−C7), 4.81 (1H, m, H−C7), 4.70 (2H, m, H−C9), 3.87 (1H, t, J = 3 Hz, H−C2), 3.43 (1H, dq, J = 10/7 Hz, H−C11), 3.29 (1H, dq, J = 10/7 Hz, H−C11), 2.53 (1H, tt, J = 12/3 Hz, H−C4), 2.33 (1H, tdt, J = 13/5/2 Hz, H−C6), 2.18 (1H, m, H−C6), 2.06 (1H, m, H−C3), 1.87 (1H, m, H−C5), 1.73 (3H, brs, H−C10), 1.48 (1H, ddd, J = 13/11/3 Hz, H−C3), 1.27 (1H, m, H−C5), 1.20 (3H, t, J = 7 Hz, H−C12).

^{13}C NMR ($CDCl_3$, 150 MHz): δ 149.8 (s (C−q), C8), 148.0 (s (C−q), C1), 110.6 (t (=CH_2), C7), 108.6 (t (=CH_2), C9), 79.0 (d (−O−CH), C2), 62.6 (t (−O−CH_2), C11), 38.6 (d (CH), C4), 38.2 (t (CH_2), C3), 32.9 (t (CH_2), C5), 30.4 (t (CH_2), C6), 21.0 (q (CH_3), C10), 15.4 (q (CH_3), C12).

GC−MS (EI, 70 eV) m/z (%): 134 (100), 119 (68), 91 (59), 93 (44), 137 (37), 41 (32), 83 (32), 79 (30), 67 (27), 92 (26), 180 (1) [$M^{\bullet+}$].

NMR and GC−MS Data of Compound ***2b***. ^{1}H NMR ($CDCl_3$, 600 MHz): δ 4.95 (1H, q, J = 2 Hz, H−C7), 4.76 (1H, q, J = 2 Hz, H−C7), 4.70 (2H, m, H−C9), 3.68 (1H, m, H−C2), 3.60 (2H, m, H−C11), 2.42 (1H, ddd, J = 13/4/3 Hz, H−C6), 2.17 (1H, m, H−C4), 2.15 (1H, m, H−C3), 2.02 (1H, m, H−C6), 1.79 (1H, m, H−C5), 1.71 (3H, brs, H−C10), 1.27 (1H, m, H−C3), 1.24 (1H, m, H−C12), 1.23 (3H, t, J = 7 Hz, H−C5).

^{13}C NMR ($CDCl_3$, 150 MHz): δ 148.9 (s (C−q), C8), 148.8 (s (C−q), C1), 109.1 (t (=CH_2), C9), 104.5 (t (=CH_2), C7), 79.6 (d (−O−CH), C2), 65.0 (t (−O−CH_2), C11), 44.4 (d (CH), C4), 39.7 (t (CH_2), C3), 34.1 (t (CH_2), C6), 33.1 (t (CH_2), C5), 20.6 (q (CH_3), C10), 15.6 (q (CH_3), C12).

GC−MS (EI, 70 eV) m/z (%): 137 (100), 93 (98), 91 (91), 119 (90), 83 (76), 79 (75), 134 (73), 41 (71), 67 (67), 55 (65), 180 (11) [$M^{\bullet+}$].

NMR and GC−MS Data of Compound ***3a***. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 5.66 (1H, m, H−C1), 5.65 (1H, m, H−C2), 4.76 (1H, m, H−C9), 4.70 (1H, m, H−C9), 3.43 (2H, qq, J = 9/7 Hz, H−C11), 2.75 (1H, m, H−C6), 1.90 (1H, m, H−C5), 1.86 (1H, m, H−C4), 1.72 (3H, brs, H−C10), 1.62 (1H, m, H−C4), 1.51 (1H, m, H−C5), 1.26 (3H, s, H−C7), 1.17 (3H, t, J = 7 Hz, H−C12).

^{13}C NMR ($CDCl_3$, 100 MHz): δ 148.1 (s (C−q), C8), 133.4 (d (=CH), C2), 132.0 (d (=CH), C1), 110.5 (t (=CH_2), C9), 73.4 (s (−O−C), C3), 57.2 (t (−O−CH_2), C11), 43.1 (d (CH), C6), 32.2 (t (CH_2), C4), 26.6 (q (CH_3), C7), 25.9 (t (CH_2), C5), 20.9 (q (CH_3), C10), 16.4 (q (CH_3), C12).

GC−MS (EI, 70 eV) m/z (%): 165 (100), 137 (60), 134 (60), 93 (52), 43 (51), 91 (47), 109 (47), 107 (46), 79 (38), 77 (33), 180 (1) [$M^{\bullet+}$].

NMR and GC−MS Data of Compound ***3b***. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 5.69 (1H, m, H−C1), 5.69 (1H, m, H−C2), 4.79 (1H, m, H−C9), 4.75 (1H, m, H−C9), 3.43 (2H, qd, J = 7/2 Hz, H−C11), 2.65 (1H, m, H−C6), 1.94 (1H, m, H−C4), 1.74 (3H, m, H−C10), 1.71 (2H, m, H−C5), 1.40 (1H, m, H−C4), 1.24 (3H, s, H−C7), 1.15 (3H, t, J = 7 Hz, H−C12).

^{13}C NMR ($CDCl_3$, 100 MHz): δ 148.1 (s (C−q), C8), 133.0 (d (=CH), C2), 132.6 (d (=CH), C1), 110.8 (t (=CH_2), C9), 71.9 (s (−O−C), C3), 57.4 (t (−O−CH_2), C11), 43.1 (d (CH), C6), 32.6 (t (CH_2), C4), 26.6 (q (CH_3), C7), 24.6 (t (CH_2), C5), 21.0 (q (CH_3), C10), 16.4 (q (CH_3), C12).

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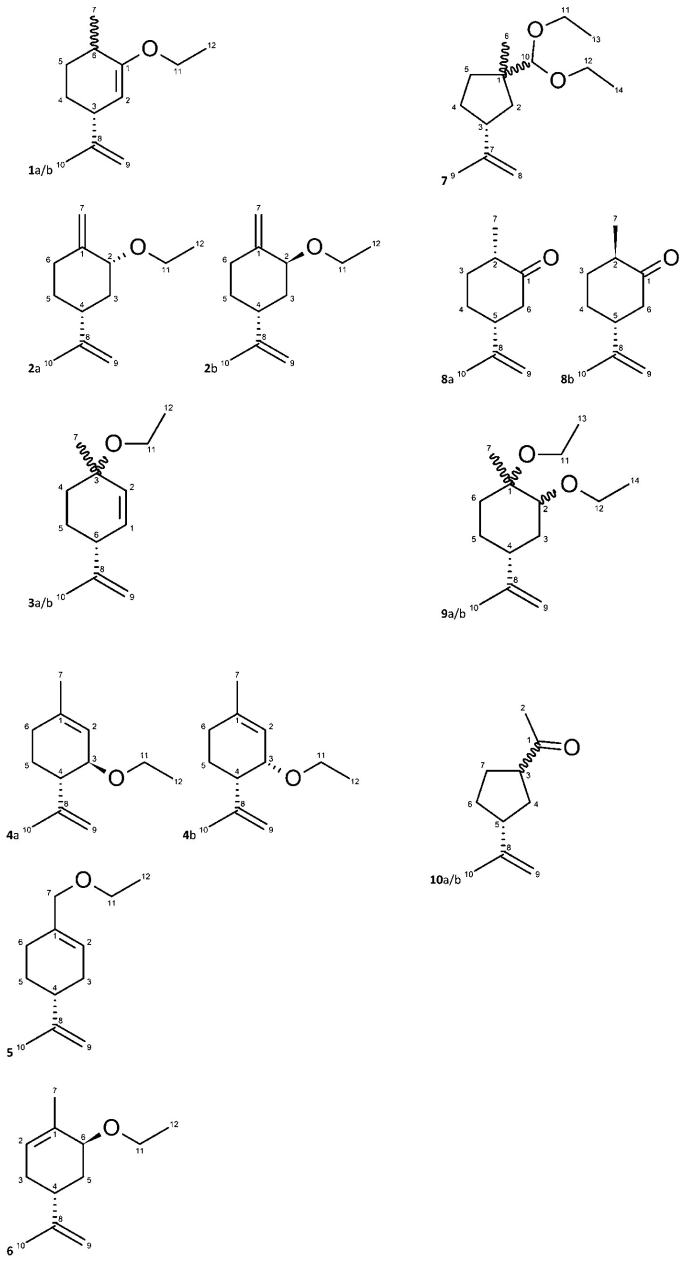

Figure 2. Structures of the isolated compounds {**1**a/b, 1-ethoxy-6-methyl-(*R*)-3-(prop-1-en-2-yl)cyclohex-1-ene; **2**a/b, 2-ethoxy-1-methylidene-(*R*)-4-(prop-1-en-2-yl)cyclohexane; **3**a/b, 3-ethoxy-3-methyl-6-(prop-1-en-2-yl)cyclohex-1-ene; **4**a/b, 3-ethoxy-1-methyl-(*R*)-4-(prop-1-en-2-yl)cyclohex-1-ene; **5**, 1-(ethoxymethyl)-(*R*)-4-(prop-1-en-2-yl)cyclohex-1-ene; **6**, 6-ethoxy-1-methyl-4-(prop-1-en-2-yl)cyclohex-1-ene; **7**, 1-(diethoxymethyl)-1-methyl-3-(prop-1-en-2-yl)cyclopentane; **8**a/b, 2-methyl-5-(prop-1-en-2-yl)cyclohexan-1-one; **9**a/b, 1,2-diethoxy-1-methyl-(*R*)-4-(prop-1-en-2-yl)cyclohexane; **10**a/b, 1-[3-(prop-1-en-2-yl)cyclopentyl]ethan-1-one}.

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GC−MS (EI, 70 eV) *m*/*z* (%): 165 (100), 93 (93), 137 (92), 107 (84), 91 (73), 94 (72), 43 (72), 109 (67), 79 (62), 77 (49), 180 (1) [$M^{\bullet+}$].

NMR and GC−MS Data of Compound ***4a****.* ^{1}H NMR ($CDCl_3$, 600 MHz): δ 5.48 (1H, m, H−C2), 4.80 (1H, m, H−C9), 4.76 (1H, m, H−C9), 3.84 (1H, m, H−C3), 3.58 (1H, dq, *J* = 9/7 Hz, H−C11), 3.47 (1H, dq, *J* = 9/7 Hz, H−C11), 2.22 (1H, ddd, *J* = 11/8/3 Hz, H−C4), 2.01 (1H, m, H−C6), 1.88 (1H, m, H−C6), 1.77 (3H, brs, H−C10), 1.72 (1H, m, H−C5), 1.68 (3H, brs, H−C7), 1.61 (1H, dddd, *J* = 13/11/10/5 Hz, H−C5), 1.17 (3H, t, *J* = 7 Hz, H−C12).

^{13}C NMR ($CDCl_3$, 150 MHz): δ 147.5 (s (C−q), C8), 137.4 (s (C−q), C1), 122.6 (d (=CH), C2), 110.6 (t (=CH_2), C9), 77.1 (d (−O−CH), C3), 63.7 (t (−O−CH_2), C11), 46.8 (d (CH), C4), 29.8 (t (CH_2), C6), 26.6 (t (CH_2), C5), 23.3 (q (CH_3), C7), 21.0 (q (CH_3), C10), 15.7 (q (CH_3), C12).

GC−MS (EI, 70 eV) *m*/*z* (%): 112 (100), 97 (89), 83 (80), 84 (42), 108 (35), 91 (19), 41 (18), 77 (14), 79 (12), 55 (11), 180 (1) [$M^{\bullet+}$].

NMR and GC−MS Data of Compound ***4b****.* ^{1}H NMR ($CDCl_3$, 600 MHz): δ 5.51 (1H, m, H−C2), 4.72 (2H, m, H−C9), 3.91 (1H, m, H−C3), 3.64 (1H, dq, *J* = 9/7 Hz, H−C11), 3.46 (1H, dq, *J* = 9/7 Hz, H−C11), 2.23 (1H, m, H−C4), 2.19 (1H, m, H−C5), 2.03 (1H, m, H−C6), 1.93 (1H, m, H−C6), 1.73 (3H, brs, H−C10), 1.72 (3H, m, H−C7), 1.46 (1H, ddd, *J* = 13/12/10 Hz, H−C5), 1.21 (3H, t, *J* = 7 Hz, H−C12).

^{13}C NMR ($CDCl_3$, 150 MHz): δ 149.3 (s (C−q), C8), 135.5 (s (C−q), C1), 124.4 (d (=CH), C2), 108.9 (t (=CH_2), C9), 78.0 (d (−O−CH), C3), 63.8 (t (−O−CH_2), C11), 40.8 (d (CH), C4), 34.4 (t (CH_2), C5), 31.0 (t (CH_2), C6), 20.4 (q (CH_3), C10), 19.2 (q (CH_3), C7), 15.7 (q (CH_3), C12).

GC−MS (EI, 70 eV) *m*/*z* (%): 84 (100), 112 (72), 134 (70), 119 (53), 55 (52), 79 (42), 41 (39), 95 (38), 77 (38), 83 (38), 180 (13) [$M^{\bullet+}$].

NMR and GC−MS Data of Compound ***5****.* ^{1}H NMR ($CDCl_3$, 400 MHz): δ 5.70 (1H, m, H−C2), 4.71 (2H, m, H−C9), 3.84 (2H, s, H−C7), 3.44 (2H, q, *J* = 7 Hz, H−C11), 2.16 (1H, m, H−C4), 2.15 (1H, m, H−C3), 2.10 (2H, m, H−C6), 1.97 (1H, m, H−C3), 1.84 (1H, m, H−C5), 1.74 (3H, brs, H−C10), 1.48 (1H, m, H−C5), 1.21 (3H, t, *J* = 7 Hz, H−C12).

^{13}C NMR ($CDCl_3$, 100 MHz): δ 150.0 (s (C−q), C8), 134.9 (s (C−q), C1), 124.0 (d (=CH), C2), 108.6 (t (=CH_2), C9), 75.0 (t (−O−CH_2), C7), 65.2 (t (−O−CH_2), C11), 41.2 (d (CH), C4), 30.5 (t (CH_2), C3), 27.5 (t (CH_2), C5), 26.5 (t (CH_2), C6), 20.8 (q (CH_3), C10), 15.2 (q (CH_3), C12).

GC−MS (EI, 70 eV) *m*/*z* (%): 93 (100), 91 (99), 119 (76), 79 (70), 137 (64), 67 (61), 83 (53), 92 (50), 68 (49), 134 (47), 180 (16) [$M^{\bullet+}$].

NMR and GC−MS Data of Compound ***6****.* ^{1}H NMR ($CDCl_3$, 600 MHz): δ 5.60 (1H, m, H−C2), 4.73 (2H, m, H−C9), 3.68 (1H, dq, *J* = 9/7 Hz, H−C11), 3.60 (1H, m, H−C6), 3.43 (1H, dq, *J* = 9/7 Hz, H−C11), 2.36 (1H, m, H−C4), 2.15 (1H, m, H−C3), 2.05 (1H, m, H−C5), 1.81 (1H, m, H−C3), 1.77 (1H, m, H−C7), 1.74 (3H, brs, H−C10), 1.40 (1H, ddd, *J* = 14/13/4 Hz, H−C5), 1.23 (3H, t, *J* = 7 Hz, H−C12).

^{13}C NMR ($CDCl_3$ with 0.05% v/v TMS, 150 MHz): δ 149.9 (s (C−q), C8), 133.2 (s (C−q), C1), 125.5 (d (=CH), C2), 108.6 (t (=CH_2), C9), 76.0 (d (−O−CH), C6), 64.8 (t (−O−CH_2), C11), 35.5 (d (CH), C4), 32.1 (t (CH_2), C5), 31.1 (t (CH_2), C3), 21.0 (q (CH_3), C7), 21.0 (q (CH_3), C10), 15.8 (q (CH_3), C12).

GC−MS (EI, 70 eV) *m*/*z* (%): 137 (100), 84 (94), 91 (76), 119 (66), 109 (63), 93 (61), 55 (47), 77 (46), 112 (45), 83 (42), 180 (18) [$M^{\bullet+}$].

NMR and GC−MS Data of Compound ***7****.* ^{1}H NMR ($CDCl_3$, 600 MHz): δ 4.69 (1H, m, H−C8), 4.65 (1H, m, H−C8), 4.11 (1H, s, H−C10), 3.81 (1H, m, H−C11 or H−C12), 3.81 (1H, m, H−C11 or H−C12), 3.54 (1H, m, H−C11 or H−C12), 3.54 (1H, m, H−C11 or H−C12), 2.52 (1H, m, H−C3), 1.96 (1H, ddd, *J* = 13/8/1 Hz, H−C2), 1.75 (1H, m, H−C5), 1.75 (1H, m, H−C4), 1.72 (3H, brs, H−C9), 1.50 (1H, m, H−C4), 1.37 (1H, m, H−C5), 1.22 (3H, t, *J* = 7 Hz, H−C13 or H−C14), 1.22 (3H, t, *J* = 7 Hz, H−C13 or H−C14), 1.11 (1H, dd, *J* = 13/11 Hz, H−C2), 1.03 (3H, s, H−C6).

^{13}C NMR ($CDCl_3$, 150 MHz): δ 149.0 (s (C−q), C7), 110.72 (d (−O−CH−O−), C10), 107.8 (t (=CH_2), C8), 65.9 (t (−O−CH_2), C11 or C12), 65.7 (t (−O−CH_2), C11 or C12), 47.4 (d (CH), C3), 47.2 (s (C−q), C1), 41.0 (t (CH_2), C2), 35.4 (t (CH_2), C5), 30.7 (t (CH_2), C4), 24.6 (q (CH_3), C6), 21.3 (q (CH_3), C9), 15.6 (q (CH_3), C13 or C14), 15.5 (q (CH_3), C13 or C14).

GC−MS (EI, 70 eV) *m*/*z* (%): 103 (100), 75 (40), 47 (33), 99 (13), 107 (9), 93 (8), 43 (8), 71 (8), 41 (7), 55 (7), 226 (1) [$M^{\bullet+}$].

NMR and GC−MS Data of Compound ***8a****.* ^{1}H NMR ($CDCl_3$, 400 MHz): δ 4.76 (1H, m, H−C9), 4.73 (1H, m, H−C9), 2.45 (1H, dt, *J* = 11/2 Hz, H−C6), 2.38 (1H, m, H−C2), 2.36 (1H, m, H−C5), 2.28 (1H, m, H−C6), 2.13 (1H, m, H−C3), 1.94 (1H, m, H−C4), 1.74 (3H, brs, H−C10), 1.65 (1H, m, H−C4), 1.38 (1H, qd, *J* = 13/4 Hz, H−C3), 1.04 (3H, d, *J* = 7 Hz, H−C7).

^{13}C NMR ($CDCl_3$, 100 MHz): δ 212.7 (s (C=O), C1), 147.7 (s (C−q), C8), 109.6 (t (=CH_2), C9), 47.0 (d (CH), C5), 46.9 (t (CH_2), C6), 44.8 (d (CH), C2), 34.9 (t (CH_2), C3), 30.8 (t (CH_2), C4), 20.5 (q (CH_3), C10), 14.4 (q (CH_3), C7).

GC−MS (EI, 70 eV) *m*/*z* (%): 67 (100), 95 (79), 68 (52), 81 (48), 82 (46), 109 (42), 41 (40), 69 (36), 55 (28), 39 (27), 152 (18) [$M^{\bullet+}$].

NMR and GC−MS Data of Compound ***8b****.* ^{1}H NMR ($CDCl_3$, 400 MHz): δ 4.83 (1H, m, H−C9), 4.69 (1H, m, H−C9), 2.60 (1H, m, H−C5), 2.55 (1H, m, H−C6), 2.42 (1H, m, H−C6), 2.40 (1H, m, H−C2), 1.85 (1H, m, H−C3), 1.85 (2H, m, H−C4), 1.73 (3H, brs, H−C10), 1.60 (1H, m, H−C3), 1.09 (3H, d, *J* = 7 Hz, H−C7).

^{13}C NMR ($CDCl_3$, 100 MHz): δ 214.0 (s (C=O), C1), 146.9 (s (C−q), C8), 111.5 (t (=CH_2), C9), 44.6 (d (CH), C2), 44.1 (t (CH_2), C6), 44.0 (d (CH), C5), 30.7 (t (CH_2), C3), 26.4 (t (CH_2), C4), 26.4 (q (CH_3), C10), 21.5, 15.6 (q (CH_3), C7).

GC−MS (EI, 70 eV) *m*/*z* (%): 67 (100), 95 (92), 68 (52), 82 (45), 41 (41), 69 (37), 81 (36), 152 (31) [$M^{\bullet+}$], 55 (29), 39 (27).

NMR and GC−MS Data of Compound ***9a****.* ^{1}H NMR ($CDCl_3$, 600 MHz): δ 4.70 (1H, m, H−C9), 4.68 (1H, m, H−C9), 3.61 (1H, dq, *J* = 9/7 Hz, H−C12), 3.37 (1H, m, H−C12), 3.37 (2H, m, H−C11), 3.23 (1H, brs, H−C2), 2.18 (1H, tt, *J* = 12/2 Hz, H−C4), 1.73 (1H, m, H−C3), 1.72 (3H, brs, H−C10), 1.67 (1H, m, H−C6), 1.53 (1H, m, H−C6), 1.44 (1H, m, H−C5), 1.37 (1H, m, H−C5), 1.26 (1H, m, H−

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C3), 1.17 (3H, t, J = 7 Hz, H−C14), 1.16 (3H, t, J = 7 Hz, H−C13), 1.16 (3H, s, H−C7).

^{13}C NMR ($CDCl_3$, 150 MHz): δ 150.8 (s (C−q), C8), 108.1 (t (=CH_2), C9), 79.8 (d (−O−CH), C2), 74.8 (s (−O−C), C1), 64.7 (t (−O−CH_2), C12), 55.5 (t (−O−CH_2), C11), 37.7 (d (CH), C4), 30.2 (t (CH_2), C6), 29.6 (t (CH_2), C3), 26.3 (t (CH_2), C5), 21.5 (q (CH_3), C7), 20.9 (q (CH_3), C10), 16.1 (q (CH_3), C13), 15.7 (q (CH_3), C14).

GC−MS (EI, 70 eV) m/z (%): 99 (100), 71 (36), 43 (19), 58 (11), 140 (8), 108 (7), 100 (7), 86 (6), 41 (6), 93 (6), 226 (1) [$M^{\bullet+}$].

NMR and GC−MS Data of Compound ***9b****.* ^{1}H NMR ($CDCl_3$, 400 MHz): δ 4.73 (1H, m, H−C9), 4.69 (1H, m, H−C9), 3.69 (1H, dq, J = 9/7 Hz, H−C12), 3.50 (1H, dq, J = 9/7 Hz, H−C11), 3.40 (1H, m, H−C11), 3.40 (1H, m, H−C12), 2.98 (1H, m, H−C2), 1.97 (1H, m, H−C6), 1.96 (1H, m, H−C4), 1.76 (2H, m, H−C3), 1.73 (3H, brs, H−C10), 1.42 (2H, m, H−C5), 1.23 (3H, s, H−C7), 1.18 (3H, t, J = 7 Hz, H−C14), 1.17 (3H, t, J = 7 Hz, H−C13), 1.12 (1H, m, H−C6).

^{13}C NMR ($CDCl_3$, 100 MHz): δ 149.8 (s (C−q), C8), 108.6 (t (=CH_2), C9), 85.0 (d (−O−CH), C2), 74.6 (s (−O−C), C1), 65.4 (t (−O−CH_2), C12), 56.6 (t (−O−CH_2), C11), 44.6 (d (CH), C4), 34.6 (t (CH_2), C6), 31.2 (t (CH_2), C3), 26.2 (t (CH_2), C5), 21.5 (q (CH_3), C7), 20.6 (q (CH_3), C10), 16.2 (q (CH_3), C13), 15.6 (q (CH_3), C14).

GC−MS (EI, 70 eV) m/z (%): 99 (100), 71 (38), 43 (19), 58 (10), 100 (7), 140 (7), 108 (6), 86 (6), 41 (6), 93 (6), 226 (1) [$M^{\bullet+}$].

NMR and GC−MS Data of Compound ***10a****.* ^{1}H NMR ($CDCl_3$, 600 MHz): δ 4.73 (1H, m, H−C9), 4.70 (1H, m, H−C9), 2.95 (1H, m, H−C3), 2.49 (1H, m, H−C5), 2.17 (3H, s, H−C2), 2.04 (1H, m, H−C4), 1.93 (1H, m, H−C7), 1.84 (1H, m, H−C7), 1.84 (1H, m, H−C6), 1.73 (3H, brs, H−C10), 1.61 (1H, ddd, J = 12/11/10 Hz, H−C4), 1.49 (1H, m, H−C6).

^{13}C NMR ($CDCl_3$, 150 MHz): δ 210.6 (s (C=O), C1), 147.4 (s (C−q), C8), 109.0 (t (=CH_2), C9), 51.9 (d (CH), C3), 47.7 (d (CH), C5), 31.2 (t (CH_2), C4), 30.5 (t (CH_2), C6), 28.8 (q (CH_3), C2), 27.3 (t (CH_2), C7), 21.0 (q (CH_3), C10).

GC−MS (EI, 70 eV) m/z (%): 43 (100), 109 (99), 67 (88), 137 (49), 71 (39), 41 (28), 79 (26), 93 (26), 55 (26), 82 (25), 152 (24) [$M^{\bullet+}$].

NMR and GC−MS Data of Compound ***10b****.* ^{1}H NMR ($CDCl_3$, 400 MHz): δ 4.70 (2H, m, H−C9), 3.00 (1H, dtd, J = 10/8/5 Hz, H−C3), 2.47 (1H, m, H−C5), 2.16 (3H, s, H−C2), 2.06 (1H, dddd, J = 13/8/5/1 Hz, H−C4), 1.98 (1H, m, H−C7), 1.88 (1H, m, H−C6), 1.78 (1H, m, H−C7), 1.73 (3H, brs, H−C10), 1.65 (1H, dt, J = 13/10 Hz, H−C4), 1.48 (1H, dtd, J = 12/10/8 Hz, H−C6).

^{13}C NMR ($CDCl_3$, 100 MHz): δ 210.9 (s (C=O), C1), 148.0 (s (C−q), C8), 108.7 (t (=CH_2), C9), 51.3 (d (CH), C3), 46.4 (d (CH), C5), 32.9 (t (CH_2), C4), 31.7 (t (CH_2), C6), 28.9 (q (CH_3), C2), 28.5 (t (CH_2), C7), 21.5 (q (CH_3), C10).

GC−MS (EI, 70 eV) m/z (%): 71 (100), 109 (99), 43 (78), 67 (67), 137 (65), 152 (44) [$M^{\bullet+}$], 41 (26), 39 (23), 79 (22), 82 (21).

1a and **1**b are the (*Z*)/(*E*)-isomers of 1-ethoxy-6-methyl-(*R*)-3-(prop-1-en-2-yl)cyclohex-1-ene. Both isomers have not been described in the literature so far. The odor of both isomers was perceived as herbal, parsley root-like.

2a and **2**b represent the (*Z*)/(*E*)-isomers of 2-ethoxy-1-methylidene-(*R*)-4-(prop-1-en-2-yl)cyclohexane, where **2**a is the (*Z*)-isomer and **2**b is the corresponding (*E*)-isomer. These compounds have been mentioned previously by Kergomard et al. while investigating the thermodynamics of the acetylation of carveol and by Gonçalves et al. while investigating the palladium-catalyzed oxidation of monoterpenes.[10,16] The recorded mass spectra are in accordance with those described in the literature, whereas the NMR data are only partly in accordance with those described in the literature for **2a**.[10,17] The ^{13}C values are identical to those described previously, but the assignment to the corresponding carbon atoms of the molecules made by Gonçalves et al. is not correct.[10] For both isomers, no odor impressions have been described in the literature. Furthermore, they have not yet been described in nature. In this study, the odor of **2**a was described as herbal, fresh, green, and dill-like and that of **2**b as herbal, spicy, earthy, and juniper-like.

3a and **3**b are the (*Z*)/(*E*)-isomers of 3-ethoxy-3-methyl-6-(prop-1-en-2-yl)cyclohex-1-ene. Both isomers have not been described in the literature yet. In this study, the odor of **3**a was described as sweetish, fruity, anise, and licorice-like and that of **3**b as spicy, anise, cinnamon, and clove-like.

4a and **4**b are the (*Z*)/(*E*)-isomers of 3-ethoxy-1-methyl-(*R*)-4-(prop-1-en-2-yl)cyclohex-1-ene, where **4**a represents the (*E*)-isomer and **4**b the (*Z*)-isomer. Both isomers have not been described in the literature yet. Because of the similarity in the structures of **4**a and **4**b, there is much similarity in the ^{1}H NMR spectra of the isomers. Unambiguous proton chemical-shift assignments of **4**a and **4**b were based on the multiplicity pattern of proton resonances and also on the use of homonuclear ^{1}H,^{1}H COSY spectra. The distinction between **4**a and **4**b was made mainly on the basis of the ^{1}H results, including the cross-peak between the methine protons 3-H and 4-H in the COSY spectra. The differentiation between **4**a and **4**b was based on rather different vicinal H−H couplings between the methine protons 3-H and 4-H. The magnitude of the vicinal coupling constant (J = 8 Hz) indicated a *trans*-diaxial relationship between 3-H and 4-H in the (*E*)-isomer **4**a and was fully consistent with the observed strong cross-peak in the COSY spectrum. The vicinal ^{1}H coupling constant is strongly dihedral angle-dependent. In order to determine dihedral angles for comparison of the different ^{1}H vicinal coupling constants in **4**a and **4**b, a conformer distribution analysis was performed to identify the low-lying conformations of **4**a with Spartan '20, Version 1.1.4, employing the Merck molecular force field.[18] The odor of **4**a was perceived as herbal, green, floral, and parsley root-like and that of **4**b as herbal, parsley root-like.

Analysis of NMR and mass spectrometric data revealed **5** as 1-(ethoxymethyl)-(*R*)-4-(prop-1-en-2-yl)cyclohex-1-ene. This compound has already been mentioned in the literature, but no NMR or MS data are available.[19,20] An odor impression could not be found in the literature as well. In this study, the odor was described as fruity, sweetish, green, and coriander-like.

Analysis of NMR and mass spectrometric data indicated **6** to be 6-ethoxy-1-methyl-4-(prop-1-en-2-yl)cyclohex-1-ene. By comparison of the NMR data with those described in the literature, **6** was identified as the (*E*)-isomer.[10] **6** was first mentioned by Kergomard et al.[16] The recorded mass spectra are in accordance with those described in the literature, whereas the NMR data are only partly in accordance with

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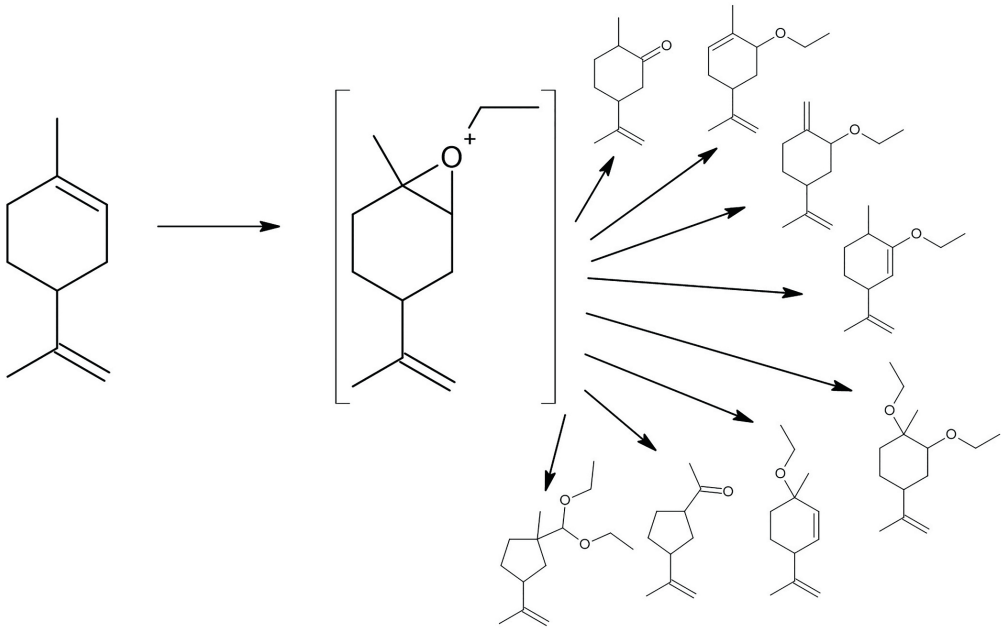

Figure 3. Proposed reaction for the formation of some of the isolated compounds.

those described in the literature studies.[10,21] The ^{13}C NMR data reported by Gonçalves et al. are approximately 1.1 ppm more positive, which may indicate an incorrect calibration in their experiments.[10] Additionally, the assignment to the corresponding carbon atoms of the molecules made by Gonçalves et al. is partly not correct. **6** represents one of the three isolated compounds for which an odor description has been reported in the literature previously. It has been described as carrot-like, parsley-like, herbal, earthy, and woody, which is in accordance with the olfactory impressions perceived in this study.[21] As indicated by the respective mass spectra, the other isomer was likely formed electrochemically as well, but it could not be isolated.

Analysis of NMR and mass spectra identified **7** as 1-(diethoxymethyl)-1-methyl-3-(prop-1-en-2-yl)cyclopentane. This compound has not been described in the literature yet. The odor of **7** was perceived as minerally, woody, earthy, and spicy.

8a and **8**b represent the (*Z*)/(*E*)-isomers of 2-methyl-5-(prop-1-en-2-yl)cyclohexan-1-one (dihydrocarvone), where **8**a is the (*Z*)-isomer and **8**b is the corresponding (*E*)-isomer. The determined NMR and mass spectra are in accordance with those described in the literature as well as with the data obtained for the commercially available standard.[22,23] The odor of **8**a has been described as musty and woody while that of **8**b as minty and caraway-like, which is mostly in accordance with the olfactory impressions perceived in this study.[24−26]

Analysis of NMR and mass spectra showed that **9**a and **9**b are isomers of 1,2-diethoxy-1-methyl-(*R*)-4-(prop-1-en-2-yl)-cyclohexane. Both compounds have not been described in the literature previously. The odor of **9**a was described here as herbal, dill-like, and earthy and that of **9**b as fresh, menthol-like, floral, and citrus-like.

10a and **10**b were identified by NMR and mass spectrometric data as isomers of 1-[3-(prop-1-en-2-yl)-cyclopentyl]ethan-1-one. **10**a and **10**b have been described in the literature as rearrangement products of limonene oxide, but neither analytical data nor an odor impression has been reported for them.[27−29] The perceived odor impressions for **10**a are minty, tart, floral, fruity, fresh, and citrus-like and those of **10**b are minty, tart, resinous-like, green, and spicy.

In summary, of the 17 compounds isolated in this study, the structures of **2**a/b, **5**, **6**, **8**a/b, and **10**a/b have been mentioned in the literature previously. NMR data are only available for **2**a, **6**, and **8**a/b, whereas MS data and odor descriptions are only given for **6** and **8**a/b. The retention indices (RIs) have been determined for **8**a.[25]

The electrochemical oxidation yields at first a radical cation.[9] It may be presumed that following this first step, the formation of a limonene oxide-like transition state (Figure 3) takes place. All of the isolated compounds, except for **4**a, **4**b, and **5**, are likely products of such a limonene oxide-like transition state. **7**, **8**a, **8**b, **10**a, and **10**b are products of an additional Meinwald rearrangement, where **8**a and **8**b are formed by a hydride shift and **7**, **10**a, and **10**b by an alkyl shift (Figure 4).[29−31] **4**a, **4**b, and **5** are assumably formed after abstraction of a hydrogen in the α position to the double bond due to a slightly increased acidity.

As indicated above, only for three of the isolated compounds, an odor impression was found in the literature. For these three compounds, the odor impressions perceived in this study matched those described in the literature well (Table

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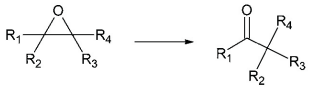

Figure 4. Meinwald rearrangement in a general reaction.

1). Unfortunately, there is a lack of systematically comparable compounds and their odor impressions in the literature. Thus, comparisons may only be made with few structurally similar compounds. For example, (*R*)-limonene is known for its typical orange-like odor, whereas carvone has a spearmint-like odor for the (*R*)-isomer and a caraway-like odor for the antipode. Linalool has a floral odor, estragole gives a licorice-like scent, and (+)-nootkatone is known for its grapefruit-like odor.[32−34]

On the basis of the semiquantitative analysis of the sample, it seems likely that products with lower steric hindrance, such as **4**a or **6**, are preferably formed, compared to those with higher steric hindrance, such as **4**b.

In general, oxidation of terpenes, either via natural processes or synthetically, represents a powerful tool to create novel aroma compounds with pleasing organoleptic properties. Some of the above-mentioned compounds are oxygenated terpenes which are well known and important aroma compounds that can be obtained from plants. Surprisingly, nonnatural, synthetically generated compounds were also revealed to have pleasant aroma properties in this study. The chemical oxidation may be performed either targeted to form specific compounds or nontargeted, like in this study, to generate a broad variety of compounds in just one step.[6,8]

An advantage of the electrochemical oxidation compared to other methods is the simple and efficient generation of new aroma compounds from inexpensive and easily available starting materials.[13] By altering some parameters such as the current density or the type of used electrodes, the composition of the created aroma compounds might be influenced. This needs to be further investigated in future studies.

Because of the pretty similar structures of the generated compounds, the preparative isolation of single compounds out of the mixture is difficult but, if necessary, can be achieved with the methods reported herein. Thus, a future industrial application of the generated mixture, after safety evaluation by the competent authorities, seems to be more likely.

Overall, 20 compounds could be structurally and sensorily characterized in electrochemically oxidized limonene, of which 11 had not been described in the literature before. In addition, a method for the sustainable production of aroma compounds is described that does not require the use of critical chemicals and which may quickly generate olfactorily appealing aroma mixtures.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.2c01301.

GC−FID chromatogram of the diluted sample determined on an Agilent HP-INNOWAX column (PDF)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

FID, flame ionization detector; GC, gas chromatograph(y); HPLC, high-performance liquid chromatography; MS, mass spectrometry; NMR, nuclear magnetic resonance; O, olfactometry; TLC, thin-layer chromatography; TMS, tetramethylsilane.

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Generation of Flavor Active Compounds by Electrochemical Oxidation of (*R*)-Limonene

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Supplementary information

Contents:

S1: GC-FID chromatogram of the diluted sample determined on a HP-INNOWAX column