Novel approaches to generate alkylpyrazine derivatives

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Zusammenfassung

Die vorliegende Arbeit beschreibt zwei neue Wege, um geruchsaktive Pyrazinderivate herzustellen. Der erste Prozess war zweistufig und bestand aus einer Biotransformationsreaktion, an die eine chemische Reaktion unter milden Bedingungen gekoppelt war. 27 Aliphatische Acyloine wurden durch Biotransformation von aliphatischen Aldehyden und 2-Oxocarboxylaten mit Hilfe intakter Bäckerhefezellen synthetisiert. Sechs dieser Acyloine wurden erstmals synthetisiert und charakterisiert. Hohe Ausbeuten bis zu 55 % und das breite Substratspektrum zeigten, dass das Potential intakter Bäckerhefezellen zur Bildung von aliphatischen Acyloinen aus Aldehyden, bisher unterschätzt worden war. Die Carboligation lieferte überwiegend Acyloine der (*R*)-Konfiguration, wobei der Enantiomerenüberschuss von den strukturellen Eigenschaften der Aldehydsubstrate abhängig war. Die chemische Reaktion dieser Acyloine mit 1,2-Propandiamin führte bei Raumtemperatur zu der Produktion von 14 5,6-Dihydropyrazinen und 10 Tetrahydropyrazinen in Ausbeuten bis zu 50 %. 18 dieser Moleküle wurden zum ersten Mal beschrieben. Die Aromaqualitäten wurden charakterisiert, und 11 der Verbindungen zeigten bemerkenswerte röstige oder erdige Geruchseigenschaften. Mit Geruchsschwellenwerten im pg Bereich gehören einige der produzierten Verbindungen zu den geruchsintensivsten Pyrazinderivaten, die in der Literatur beschrieben worden sind.
Der zweite Prozess beschreibt eine neue chemische Gesamtsynthese für 2-Ethenyl-3,5-dimethylpyrazin und 3-Ethenyl-2,5-dimethylpyrazin, zwei erdig riechende Substanzen. Die vier ersten Schritte der Synthese führten zu der Bildung zweier neuer Pyrazinverbindungen. Letztere fungierten als Vorläufer der Zielmoleküle, welche in einer anschliessenden Retro-Diels-Alder Reaktion mit hoher Reinheit (>99 %) erhalten wurden. Die Gesamtausbeute betrug 7.0 % für jedes der Moleküle. Dies ensprach der höchsten Ausbeute, die in der Literatur bekannt ist.

Schlagwörter: Acyloine, Pyrazine, Aroma.

Summary

The present work describes two new ways to produce aroma-active pyrazine derivatives. The first process consisted of a biotransformation reaction, followed by a chemical reaction under mild conditions. 27 aliphatic acyloins were generated by biotransformation of aliphatic aldehydes and 2-oxocarboxylates using whole cells of baker's yeast as catalyst. Six of these acyloins were synthesized and characterized for the first time. High yields up to 55 % and the broad substrate range showed that the potential of whole cells of baker's yeast to catalyze the formation of aliphatic acyloins from aldehydes had been underestimated before. The carboligation reaction afforded predominantly acyloins in (*R*)-configuration. The enantiomeric excess was dependent on the structural properties of the aldehyde substrate. The chemical reaction of these generated acyloins with 1,2-propanediamine under mild conditions allowed to produce 14 5,6-dihydropyrazines and 10 tetrahydropyrazines with yields up to 50 %. 18 of these molecules were reported for the first time. The odour qualities were evaluated, and 11 compounds showed pronounced roasted or earthy aroma characteristics. With odour thresholds in pg range, some of the produced compounds can be placed among the pyrazine derivatives with the lowest threshold values described in literature.

The second process describes the chemical synthesis of two earthy smelling compounds, 2-ethenyl-3,5-dimethylpyrazine and 3-ethenyl-2,5-dimethylpyrazine. Steps 1 to 4 of the new pathway allowed to prepare novel ethenylpyrazine precursors, which upon subsequent Retro-Diels-Alder reaction were transformed into the mixture of the target molecules with high purity (>99 %). The total yield of 7.0 % for each molecule was the highest one reported in literature.

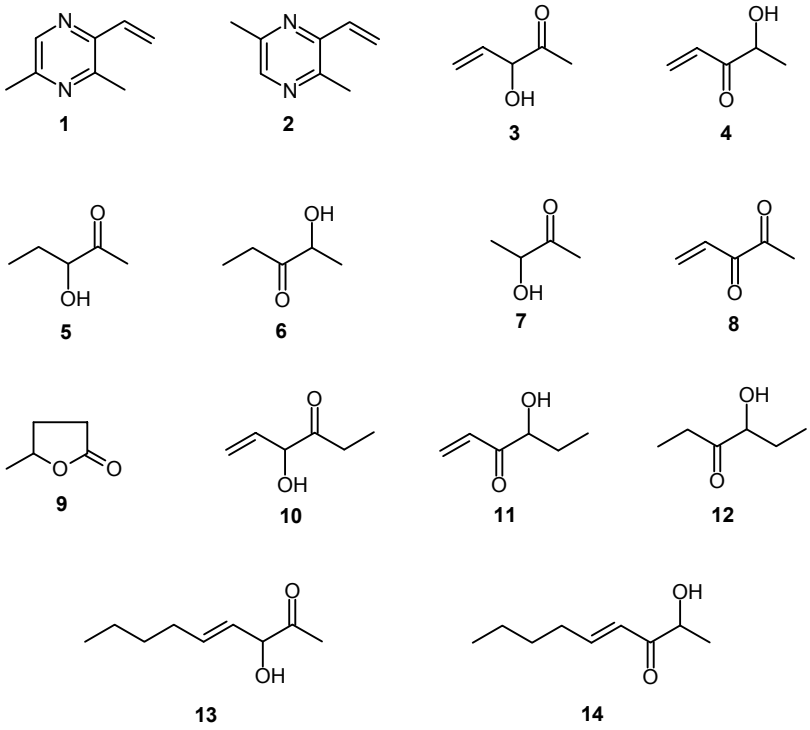
Keywords: Acyloins, pyrazines, aroma.

TABLE OF CONTENTS

Figures

Tables

Important molecules (A)



1. 2-Ethenyl-3,5-dimethylpyrazine

2. 3-Ethenyl-2,5-dimethylpyrazine

3. 3-Hydroxy-1-penten-4-one

4. 4-Hydroxy-1-penten-3-one

5. 3-Hydroxy-2-pentanone

6. 2-Hydroxy-3-pentanone

7. 3-Hydroxy-2-butanone

8. 1-Penten-3,4-dione

9. γ-Pentalactone

10. 3-Hydroxy-1-hexen-4-one

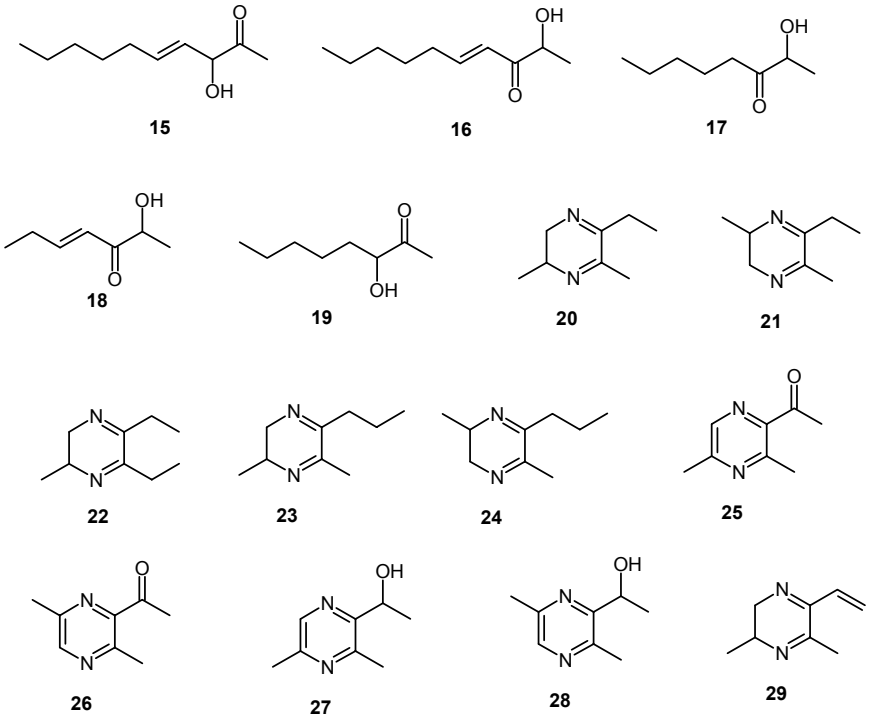
11. 4-Hydroxy-1-hexen-3-one

12. 4-Hydroxy-3-hexanone

13. 3-Hydroxy-4*E*-nonen-2-one

14. 2-Hydroxy-4*E*-nonen-3-one

Important molecules (B)



15. 3-Hydroxy-4*E*-decen-2-one

16. 2-Hydroxy-4*E*-decen-3-one

17. 2-Hydroxy-3-octanone

18. 2-Hydroxy-4*E*-hepten-3-one

19. 3-Hydroxy-2-octanone

20. 2-Ethyl-3,5-dimethyl-5,6-dihydropyrazine

21. 3-Ethyl-2,5-dimethyl-5,6-dihydropyrazine

22. 2,3-Diethyl-5-methyl-5,6-dihydropyrazine

23. 2-Propyl-3,5-dimethyl-5,6-dihydropyrazine

24. 3-Propyl-2,5-dimethyl-5,6-dihydropyrazine

25. 2-Acetyl-3,5-dimethylpyrazine

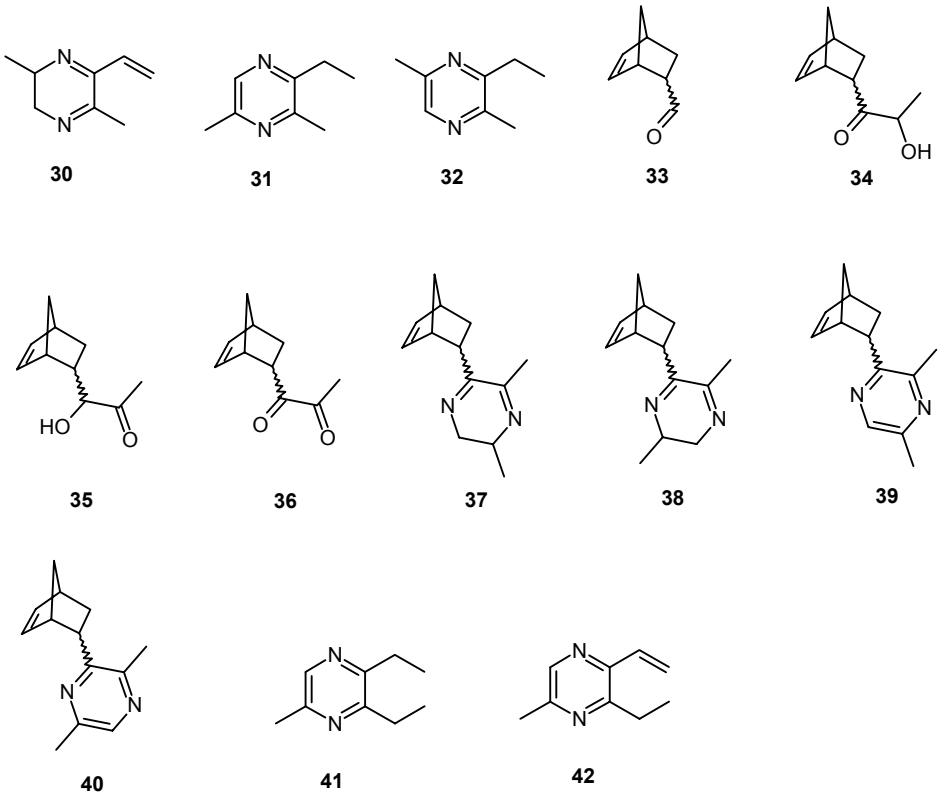
26. 3-Acetyl-2,5-dimethylpyrazine

27. 2-(1-Hydroxyethyl)-3,5-dimethylpyrazine

28. 3-(1-Hydroxyethyl)-2,5-dimethylpyrazine

29. 2-Ethenyl-3,5-dimethyl-5,6-dihydropyrazine

Important molecules (C)



30. 3-Ethenyl-2,5-dimethyl-5,6-dihydropyrazine

31. 2-Ethyl-3,5-dimethylpyrazine

32. 3-Ethyl-2,5-dimethylpyrazine

33. 5-Norbornen-2-carboxaldehyde

34. 1-[Bicyclo[2.2.1]5-hepten-2-yl]-2-hydroxy-1-propanone

35 1-[Bicyclo[2.2.1]5-hepten-2-yl]-1-hydroxy-2-propanone

36. 1-[Bicyclo[2.2.1]5-hepten-2-yl]-1,2-propanedione

37. 2-[Bicyclo[2.2.1]5-hepten-2-yl]-3,5-dimethyl-5,6-dihydropyrazine

38. 3-[Bicyclo[2.2.1]5-hepten-2-yl]-2,5-dimethyl-5,6-dihydropyrazine

39. 2-[Bicyclo[2.2.1]5-hepten-2-yl]-3,5-dimethylpyrazine

40. 3-[Bicyclo[2.2.1]5-hepten-2-yl]-2,5-dimethylpyrazine

41. 2,3-Diethyl-5-methylpyrazine

42. 2-Ethenyl-3-ethyl-5-methylpyrazine

Abbreviations

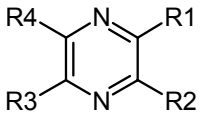
AEDA	Aroma extract dilution analysis
A.C.	After Christ
B.C.	Before Christ
CI	Chemical Impact
deg	Degree
ee	Enantiomeric excess
EI	Electronic Impact
eV	Electron Volt
FID	Flame ionisation detector
HPLC	High Performance Liquid Chromatography
GC	Gas Chromatography
HMBC	Heteronuclear Multi-Bond Connectivity
MS	Mass Spectrometry
m/z	Mass-charge ratio
NMR	Nuclear Magnetic Resonance
PAC	Phenylacetylcarbinol
PAGE	Polyacrylamid gel electrophoresis
PDC	Pyruvate decarboxylase
PDMS-DVB	Polydimethylsiloxane-divinylbenzene
SDS	Sodium dodecyl sulfate
SPME	Solid Phase Microextraction
RI	Linear retention index
TPP	Thiamine pyrophosphate
U	Unit
wt	Wildtype

It is not because things are out of reach that we do not risk them, it is because we do not risk them that we do not reach them.

Seneca (4 B.C. – 65 A.C.)

1 Introduction

Alkylpyrazines are nitrogen containing heterocyclic molecules bearing alkyl residues as substituents (Figure 1). Their aroma tonalities can, with a few exceptions, be considered as nutty, roasted, or earthy.[1 2 3]



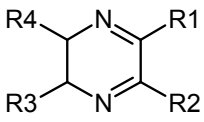
R1 to R4 = Alkyl

Figure 1. General molecular structure of alkylpyrazines.

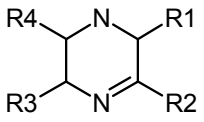
Two independent investigations, published in 1879, mark the birth of pyrazine chemistry. Gutknecht[4] synthesized tetramethylpyrazine, while Schrötter[5] isolated two basic compounds from a fusel oil of the beet molasses which are strongly suggested to have been tetramethylpyrazine and a diethyldimethylpyrazine. Since then, many research groups have devoted considerable efforts to the identification, characterization, and synthesis of pyrazines, and several reviews have been published.[6 7 8 9] The importance of alkylpyrazines as flavour components is evidenced by hundreds of publications and by many patents granted for their use in food products.[6 7 10]

Various alkylpyrazines have been reported in foods as impact compounds where they significantly contribute to the overall aroma profile. They have been identified in plants[8], insects[11], processed and fermented foods[9]. Processed foods, which have been shown to contain a multitude of alkylpyrazines, include chocolate, cocoa, coffee, heated eggs, roasted filberts, cooked Macademia nuts, baked potatoes, and cooked rice.[9] Pyrazines in fermented foodstuffs, including soy sauce, sake, and vinegar, arise from microbial processes.[12] Today, pyrazines of natural origins are of special interest since the flavour industry can label those compounds as natural.[13]

Closely related derivatives are 5,6-dihydropyrazines and tetrahydropyrazines (Figure 2), which have been less extensively studied . 5,6-dihydropyrazines have been produced from diketones and diamines,[14] but their structural diversity is relatively low, at least partially, because of the limited commercial availability of diketones. Furthermore, the aroma thresholds of these compounds have not been investigated. Tetrahydropyrazines have never been reported as aroma compounds so far.



5,6-Dihydropyrazines



Tetrahydropyrazines

Figure 2. General structure of 5,6-dihydropyrazines and tetrahydropyrazines.

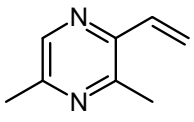
1.1 Aims of the Ph.D. thesis

a) Chemoenzymatic synthesis of pyrazine derivatives

It was attempted in the first part of the study to generate 5,6-dihydropyrazines or tetrahydropyrazines by a two-step process, combining biotransformation with chemical reaction under mild conditions. In the first step, a pool of acyloins should be produced. In a previous study, 34 acyloins were synthesized by incubation of different aldehydes and 2-oxo acids with PDC from *Zygosaccharomyces bisporus*.[15] Especially the transformation of this broad range of aliphatic aldehydes into acyloins had never been reported before. Refering to these results, the capability of baker's yeast, to produce these and other aliphatic acyloins from aliphatic aldehydes and 2-oxocarboxylates should be investigated. Moreover, the substrate specificity as well as the stereoselectivity of the carboligation should be determined. In the second step, the acyloins should be reacted with 1,2-propanediamine to yield pyrazine derivatives. The organoleptic properties of the pyrazine derivatives should be determined.

b) Chemical synthesis of ethenylpyrazines

There was further interest in the synthesis of 2-ethenyl-3,5-dimethylpyrazine ***(1)*** and 3-ethenyl-2,5-dimethylpyrazine ***(2)***. Their molecular structures are shown in Figure 3.



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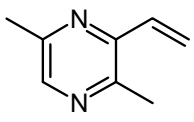


2

Figure 3. Molecular structures of 2-ethenyl-3,5-dimethylpyrazine and 3-ethenyl-2,5-dimethylpyrazine.

Both molecules have been reported to be earthy smelling compounds,[16] which are present in food and commercially not available. As the syntheses of ethenylpyrazines described in literature had been performed with low yields,[17] [18] a new and more efficient pathway should be developed in this study.

2 Materials and Methods

2.1 Materials

2.1.1 Microorganisms and enzymes

Dried baker's yeast was purchased from Hefe Schweiz. Pyruvate decarboxylase (PDC) from baker's yeast was purchased from Sigma Aldrich Chemical Co. Recombinant PDC from *Zymomonas mobilis* with his-tag was a gift from Prof. R. M. Kula (Heinrich Heine University of Düsseldorf). The enzyme was a lyophilisate containing 16 % of protein. The enzyme had a specific activity of 110 U/mg protein.

2.1.2 Chemicals

The mixture of 2-acetyl-3,5-dimethylpyrazine ***(25)*** and 3-acetyl-2,5-dimethylpyrazine ***(26)*** was purchased from Pyrazine Specialties (Atlanta, USA), and 5-norbornen-2-carboxaldehyde ***(33)*** from Lancaster Synthesis (Strasbourg, France). All other chemicals were from Sigma Aldrich Chemical Co. and of analytical purity unless otherwise indicated. Diethyl ether and dichloromethane were distilled prior to use.

2.2 Analytical techniques

2.2.1 Thin Layer Chromatography (TLC)

Where indicated, product mixtures were analyzed by thin layer chromatography (TLC) using Silica Gel 60 F_{254} (Merck). The revelation of spots was done by spraying a solution of 6 g vanillin, 197 mL ethanol, and 3 mL sulphuric acid (97 %, Merck), followed by subsequent heat treatment at 120 °C.

2.2.2 Column Chromatography

A cylindric glass column (3 cm x 30 cm) was filled with silica gel 60 (70-230 mesh, Merck) and equilibrated with an eluent. The separation was followed by TLC (see 2.2.1). The eluent used for each procedure is specified where molecules are purified.

2.2.3 Gas Chromatography (GC)

GC-analyses were performed on a Agilent 6890 Series equipped with a splitless injector and a flame ionisation detector (FID, 250 °C). Separation of volatiles was performed using helium as carrier gas (1.5 mL min^{-1}) and nitrogen (45 kPa) as make-up gas for the FID. For the separation of the compounds, either a DB-1 or a DB-WAX capillary column (both 30 m x 0.25 mm, film thickness 0.25 μm, J & W Scientific) was used. The chosen capillary column and the temperature programs are described where molecules are separated. Data acquisition was done with the Software GC ChemStation Rev. A. 08.03 (Agilent Technologies).

2.2.4 Gas Chromatography-Olfactometry (GC-O)

GC-O analyses were performed on a Agilent 6890 Series equipped with a splitless injector and a sniffing port. Separation of volatiles was performed using helium as carrier gas (1.5 mL min^{-1}) and nitrogen (45 kPa) as make-up gas for the flame ionisation detector (FID, 250 °C). The gas flow was divided at a point 200 mm before reaching the detector. A part of the gas flow was deviated to the FID, while the other part was led to a sniffing port. The sniffing port was heated to 200 °C in order to prevent condensation of the molecules, and ended into a glass funnel. For the separation of the pyrazine derivatives, a DB-1 capillary column (30 m x 0.25 mm, film thickness 0.25 μm, J & W Scientific) was used. The temperature program was 5 min isothermal at 20 °C, then raised to 240 °C at 4 °C min^{-1}, and kept at 240 °C for 5 min. Data acquisition was done with the Software GC ChemStation Rev. A. 08.03 (Agilent Technologies).

2.2.5 Gas Chromatography-Mass Spectrometry (GC-MS)

GC-MS analyses were performed on a Finnigan MAT-8430 mass spectrometer combined with an HP 5890 gas chromatograph equipped with a splitless injector. Separation of volatiles was performed using helium as carrier gas (1.5 mL min^{-1}). The MS-EI spectra were generated at 70 eV and MS-CI spectra at 150 eV with ammonia as reagent gas. The mass range was selected between *m/z* 33 and 300. For the separation of the compounds, either a DB-1 or a DB-WAX capillary column (both 30 m x 0.25 mm, film thickness 0.25 μm, J & W Scientific) was used. The chosen capillary column and the temperature programs are specified where molecules are separated. Data acquisition was done with Mass Lib V8.3C-1 (Max-Planck-Institut für Kohlenforschung, Mühlheim a. d. Ruhr, Germany).

2.2.6 Chiral Gas Chromatography

Chiral gas chromatography was performed using a Sichromat double oven gas chromatograph (Siemens, Germany). The device was equipped with a Carbowax 20M capillary column (30 m x 0.32 mm, film thickness 0.38 μm, CS Chromatographie Service) in oven 1 and a life t-switching device to cut into a chiral β-cyclodextrin capillary column (Cyclosil-B, J&W Scientific), which was situated in oven 2. Separation of volatile compounds was performed using hydrogen as carrier gas (1.5 mL min^{-1}) and nitrogen (45 kPa) as make-up gas for the FID. Data acquisition was done with a Shimazu Integrator R5A. Several temperature programs of the ovens were individually applied to achieve best separation of the compounds.

2.2.7 Preparative Gas Chromatography

Preparative GC was performed on a Agilent 5890 Series GC equipped with a KAS-3 cold injection system (Gerstel) and Multi Column Switching System (MCS, Gerstel). The MCS allowed to selectively cut compounds from a short capillary OV 1 fused silica precolumn (3 m x 0.53 mm, 2 μm, Leupold) into a CW 20M capillary column (25 m x 0.53 mm, film thickness 2 μm, Leupold). The temperature program was 5 min isothermal at 40 °C, raised to 70 °C at 5 °C min^{-1}, kept at 70 °C for 10 min, raised to 220 °C at 10 °C min^{-1}, and kept at 220 °C for 5 min. Separation of volatiles was performed using hydrogen as carrier gas (5 mL min^{-1}), counter-gas (10 mL min^{-1}), and support gas (1 mL min^{-1}), and nitrogen (45 kPa) as make-up gas for the flame ionisation detector (FID, 250 °C). The gas flow was divided before reaching the detector. The minority of the gas flow was led to the FID, while the other part was deviated to a heated transfer line (220 °C). The transfer line was connected with a GERSTEL PFC device that automatically collected compounds after gas chromatographic separation. The PFC was equipped with six sample traps of 100 µL volume and one waste trap. For optimum compound recovery, the PFC was equipped with a cryostatic trap cooling system at –5 °C. The PFC was microprocessor controlled, and trap switching times could be selected to within 0.01 min. The system was controlled by GERSTEL-Master software.

2.2.8 Nuclear Magnetic Resonance (NMR)

NMR spectra were acquired on a Bruker DPX-360 spectrometer (360.13 MHz proton frequency), equipped with a selective 5 mm ^{1}H probehead or a 5 mm quadrinuclear (QNP) probehead. The substances were dissolved in 0.7 mL of 99.8 % deuterated solvent and transfered to Wilmad 728-PP 5 mm pyrex NMR tubes. Tetramethylsilane (TMS) vapor phase was added as internal shift standard (20 μL for ^{1}H-NMR and 150 μL for ^{13}C-NMR). The

solvents used to dissolve the compounds are specified together with the NMR data of molecules.

2.3 Biogeneration of 3-hydroxy-1-penten-4-one

2.3.1 Biogeneration of 3-hydroxy-1-penten-4-one using pyruvate decarboxylase

2.3.1.1 Influence of acrolein concentration on formation of 3-hydroxy-1-penten-4-one

2.3.1.1.1 Biotransformation

To 20 mL of 0.1 M citrate buffer (pH 6.0), containing 2 mM thiamine pyrophosphate, 20 mM magnesium sulfate, and 1 U mL^{-1} PDC, in shaking flasks, were added 674 mg of sodium pyruvate (6 mmol; Aldrich, 98 %), while acrolein quantities (Aldrich, 90 %) were varied as follows: 0.6 mg (0.01 mmol), 3 mg (0.05 mmol), 6 mg (0.1 mmol), 12.5 mg (0.2 mmol), 25 mg (0.4 mmol), 75 mg (1.2 mmol), 149 mg (2.4 mmol), 187 mg (3 mmol), 249 mg (4 mmol), 311 mg (5 mmol), or 373 mg (6 mmol). The mixtures were incubated at 23 °C and after 15 min, 30 min, 45 min, 60 min, 2 h, 3 h, and 4 h of incubation, 2 mL samples were withdrawn, filtered (Schleicher and Schuell folded filters), and 11.6 μg of 4-hydroxy-4-methyl-2-pentanone (100 nmol) were added as internal standard.

2.3.1.1.2 Work-up and analysis

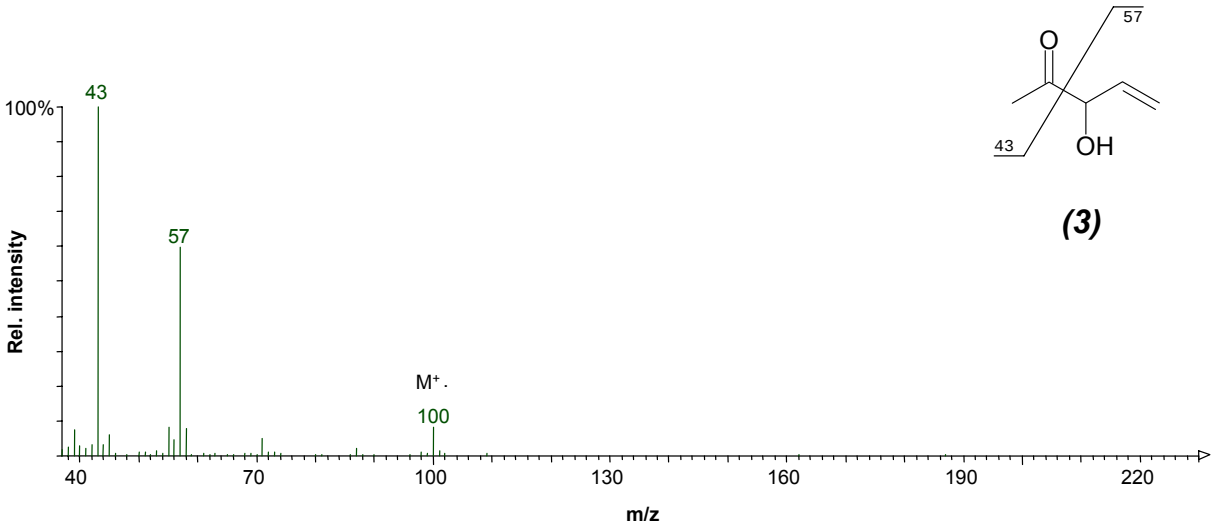
The aqueous phases were extracted twice with diethyl ether. The combined etheral solutions were dried over Na_2SO_4 and concentrated using a Vigreux column at 40 °C. The extracts were analyzed by GC (see 2.2.3) and GC-MS (see 2.2.5) using a DB-WAX capillary column. The temperature program was 5 min isothermal at 40 °C, then raised to 220 °C at 5 °C min^{-1}, and kept at 220 °C for 5 min. Relative quantification was done by comparing the area of 3-hydroxy-1-penten-4-one ***(3)*** with that of the internal standard, assuming similar FID response factors. It was shown that the FID response factors of commercial 1-hydroxy-2-propanone, 3-hydroxy-2-butanone ***(7)***, and 4-hydroxy-4-methyl-2-pentanone showed only small deviations (<10 %).

The following compound was identified:

- 3-Hydroxy-1-penten-4-one ***(3)***

RI: 1381.

MS-EI:



Reference spectrum: unknown.

2.3.1.2 Influence of pH on formation of 3-hydroxy-1-penten-4-one

20 mL of 0.1 M citrate buffer, containing 2 mM thiamine pyrophosphate, 20 mM magnesium sulfate, and 1 U mL^{-1} PDC, in shaking flasks, were equilibrated at different pH values (4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, and 8.0). 45 mg of sodium pyruvate (0.4 mmol) and 25 mg of acrolein (0.4 mmol) were added, and the mixtures were incubated at 23 °C. After 3 h of incubation, 2 mL samples were withdrawn, filtered, and 11.6 µg of 4-hydroxy-4-methyl-2-pentanone (100 nmol) were added as internal standard. The samples were further treated as described in 2.3.1.1.2.

2.3.1.3 Influence of temperature on formation of 3-hydroxy-1-penten-4-one

20 mL 0.1 M citrate buffer (pH 6.0), containing 2 mM thiamine pyrophosphate, 20 mM magnesium sulfate, and 1 U mL^{-1} PDC, in shaking flasks, were incubated at different temperatures (4 °C, 14 °C, 23 °C, 34 °C, 50 °C, and 60 °C). 45 mg of sodium pyruvate (0.4 mmol) and 25 mg of acrolein (0.4 mmol) were added, and after 3 h, 2 mL samples were withdrawn, filtered, and 11.6 µg of 4-hydroxy-4-methyl-2-pentanone (100 nmol) were added as internal standard. The samples were further treated as described in 2.3.1.1.2.

2.3.1.4 Sodium dodecyl sulfate-polyacrylamid gel electrophoresis (SDS-PAGE)

To 20 mL of 0.1 mM citrate buffer (pH 6.0), containing 2 mM thiamine pyrophosphate, 20 mM magnesium sulfate, and 10 U mL^{-1} PDC, in shaking flasks, 45 mg of sodium pyruvate (0.4 mmol) and 25 mg of acrolein (0.4 mmol) were added. The mixtures were incubated at 23 °C and samples of 500 μL were withdrawn after 15 min, 30 min, 45 min, 60 min, 75 min, 90 min, 120 min, and 150 min. Acrolein was evaporated using a vacuum centrifuge and the samples were desalted (Microcon Centrifugal Filter Devices, Millipore). After final concentration to 100 μL in the vacuum centrifuge, 1 Vol of a denaturation mix (125 mM Tris-HCl pH 6.8 / 10 % 2-mercaptoethanol / 10 % SDS / 10 % glycerol/ trace of bromophenol blue) was added. After 5 min of incubation at 95 °C, 10 μL of each sample were loaded on a SDS-Gel. Mark12 Unstained Standard was used as molecular weight marker. Denaturing SDS gelelectrophoresis was performed using Novex Pre-Cast 10 % Tris-Glycine Gels (Invitrogen life technologies) in combination with a XCell SureLock Mini-Cell. The method of Laemmli[19] under reducing conditions was applied. The buffers were prepared following the manufacturer's protocols. Proteins were visualized by silver-staining[20].

2.3.2 Biogeneration of 3-hydroxy-1-penten-4-one using whole cells of baker's yeast

2.3.2.1 Influence of acrolein concentration on formation of 3-hydroxy-1-penten-4-one

Biotransformation studies were carried out with commercially-available dried baker's yeast in shaking flasks. To 50 mL of 0.1 M citrate buffer (pH 6.0), containing 10 g baker's yeast, 2 mM thiamine pyrophosphate, and 20 mM magnesium sulfate, was added 1 g of glucose (5.6 mmol). Sodium pyruvate and acrolein were then added to give 5, 10, 25, 50, 100, and 150 mM initial concentrations. The mixtures were incubated at 23 °C and after 15 min, 30 min, 45 min, 60 min, 2 h, 3 h, 4 h, and 24 h, 5 mL samples were withdrawn, centrifuged, and 11.6 μg of 4-hydroxy-4-methyl-2-pentanone (100 nmol) were added to the supernatant as internal standard. The supernatants were further treated as described in 2.3.1.1.2.

The following compounds were identified:

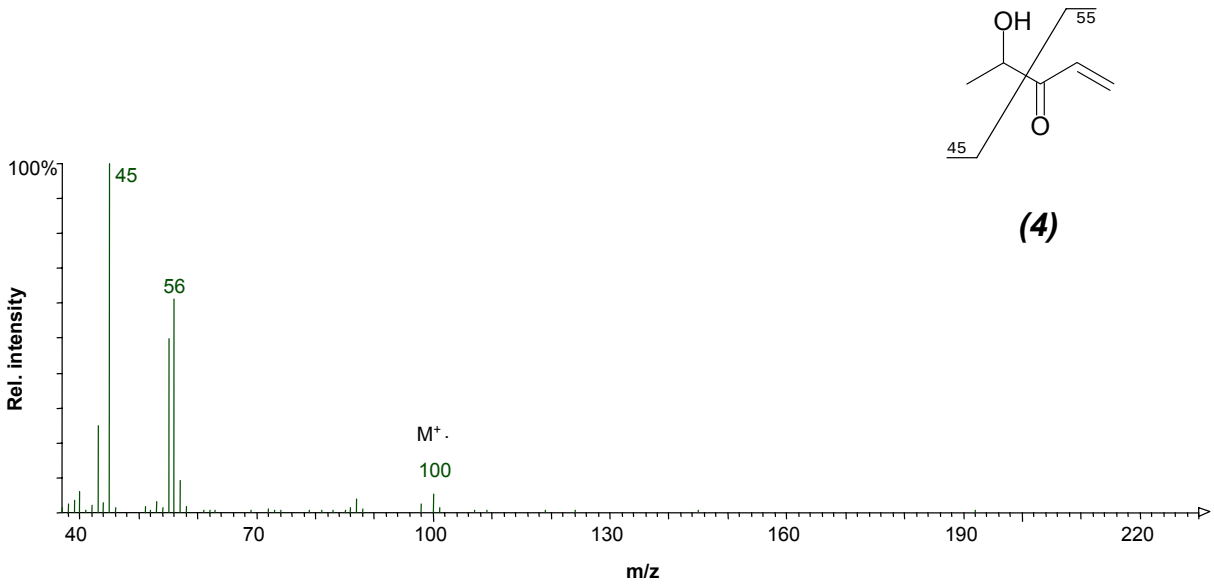
- 3-Hydroxy-1-penten-4-one ***(3)***

RI and **MS-EI** data agreed with those described in 2.3.1.1.2.

- 4-Hydroxy-1-penten-3-one ***(4)***

RI: 1372.

MS-EI:



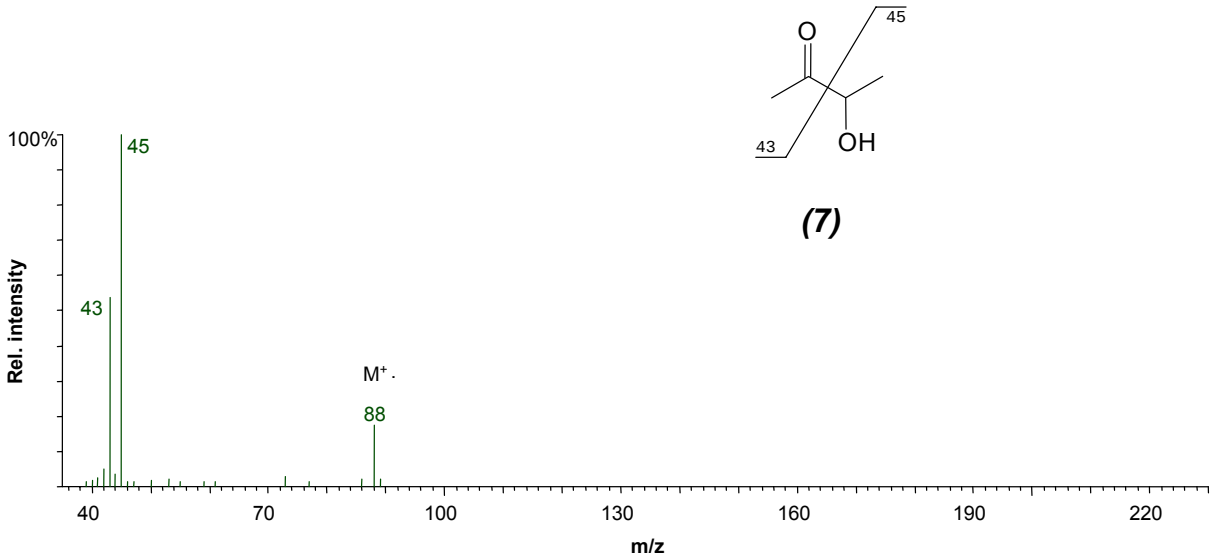
(4)

Mass spectrum agreed with that that described by Stammen *et al*.[21]

- 3-Hydroxy-2-butanone ***(7)***

RI: 1262.

MS-EI:



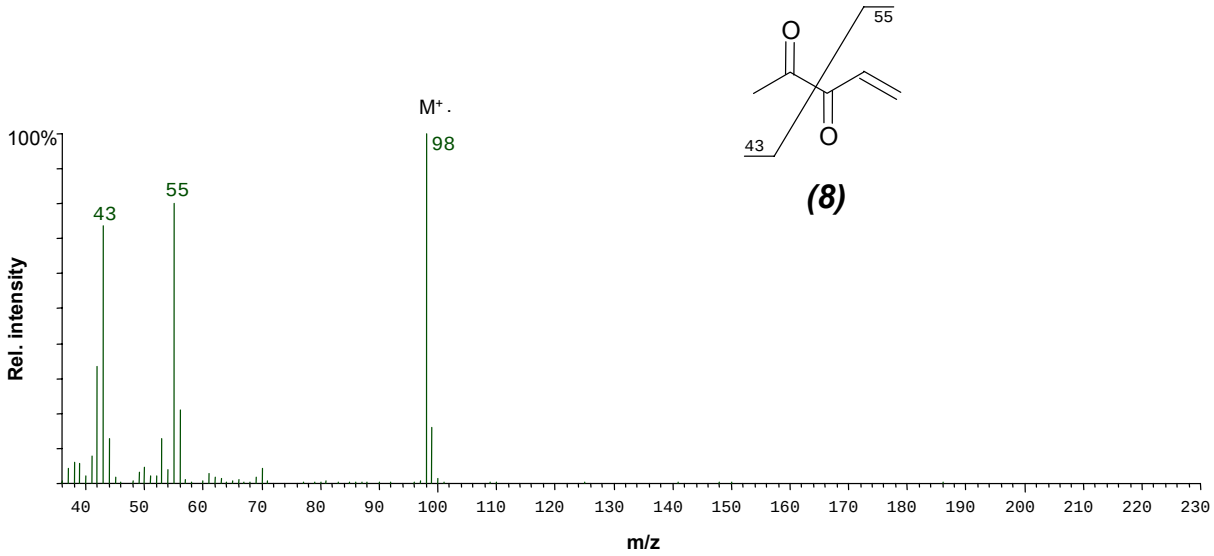
(7)

RI and mass spectrum agreed with those of the injected commercial compound.

- 1-Penten-3,4-dione ***(8)***

RI: 1097.

MS-EI:



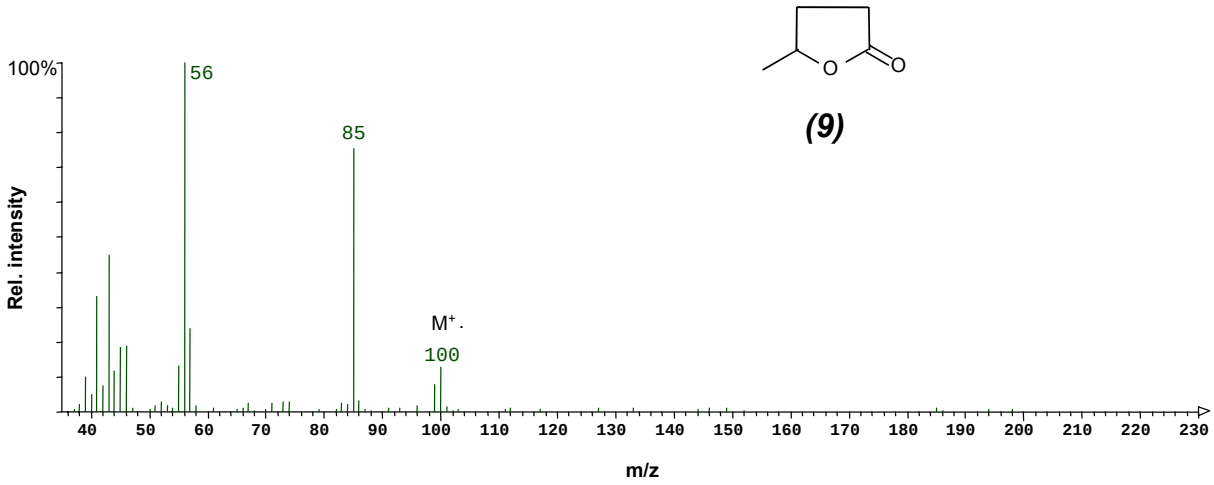
(8)

RI and mass spectrum agreed with those of injected 1-penten-3,4-dione ***(8)***, produced according to Kramme *et al*.[22]

- γ-Pentalactone ***(9)***

RI: 1594.

MS-EI:



(9)

RI and mass spectrum agreed with those of the injected commercial compound.

2.3.2.2 Characterization of 3-hydroxy-1-penten-4-one and 4-hydroxy-1-penten-3-one

2.3.2.2.1 Isolation of acyloins

To 500 mL of 0.1 M sodium citrate buffer (pH 6.0), containing 100 g baker's yeast, 2 mM thiamine pyrophosphate, and 20 mM magnesium sulfate, were added 10 g glucose

(56 mmol), 2.8 g sodium pyruvate (25 mmol), and 1556 mg of acrolein (25 mmol). The mixture was stirred at 23 °C and after 1 h of incubation, the reaction mixture was centrifuged, and the supernatant was extracted continuously with 800 ml pentane/CH_2Cl_2 (2:1) overnight. The organic phase was dried over Na_2SO_4 and concentrated using a Vigreux column at 40 °C.

2.3.2.2.2 Confirmation of molecular structures

3-Hydroxy-1-penten-4-one ***(3)*** and 4-hydroxy-1-penten-3-one ***(4)*** were produced as described in 2.3.2.2.1 and were purified by High Performance Liquid Chromatography (HPLC) using a Hewlett Packard series 1050 device, equipped with a quaternary microflow pump, a diode array detector (DAD), and an autosampler. The mobile phase for the gradient HPLC separation of the compounds was pentane (solvent A) and diethyl ether (solvent B). Separation of the compounds was achieved at a flow rate of 1 mL min^{-1} on a normal phase diol column (Nucleosil 100-7-OH, 4x250 mm, Macherey Nagel) using a linear gradient from 0-30 % of solvent B in 30 min followed by a 20 min wash step at 100 % of solvent B. UV-detection was done at 210 nm and 280 nm. 3-Hydroxy-1-penten-4-one ***(3)*** was repetitively collected between 21.0 and 23.0 min retention time, and 4-hydroxy-1-penten-3-one ***(4)*** between 23.0 min and 25.5 min. Both compounds were obtained in 90 % purity and contained 10 % of the other isomer.

- 3-Hydroxy-1-penten-4-one ***(3)***

^{1}H-NMR ($CDCl_3$) δ 5.84 (m, H-2), 5.56 (dt, J_1=17.0 Hz/J_2=10.2 Hz/J_3=6.7 Hz, vinyl H_{trans}), 5.37 (dt, J_1=17.0 Hz/J_3=1.4 Hz, vinyl H_{cis}), 4.62 (d, J_4=6.3 Hz, H-3), 3.74 (s, OH), 2.24 (s, H-1).

^{13}C NMR ($CDCl_3$) δ 119.4 (C-1), 134 (C-2), 79.2 (C-3), 206.9 (C-4), 25.2 (C-5).

Reference spectra: unknown.

- 4-Hydroxy-1-penten-3-one ***(4)***

^{1}H-NMR ($CDCl_3$) δ 6.48 (m, vinyl H_{trans}, vinyl H_{cis}), 5.92 (m, H-2), 4.51 (m, H-4), 3.50 (OH), 1.39 (d, H-5).

NMR spectrum agreed with the one described by Stammen *et al*.[21]

2.3.2.2.3 Determination of enantiomeric excess and absolute configuration

50 mg of a crude mixture of 3-hydroxy-1-penten-4-one ***(3)*** and 4-hydroxy-1-penten-3-one ***(4)***, produced as described in 2.3.2.2.1, were dissolved in 10 mL of methanol, and after 10 min stirring, 1 mg of palladium catalyst (30 wt. % on activated carbon) was added. The reaction vessel was three times evacuated and refilled with hydrogen in order to completely remove

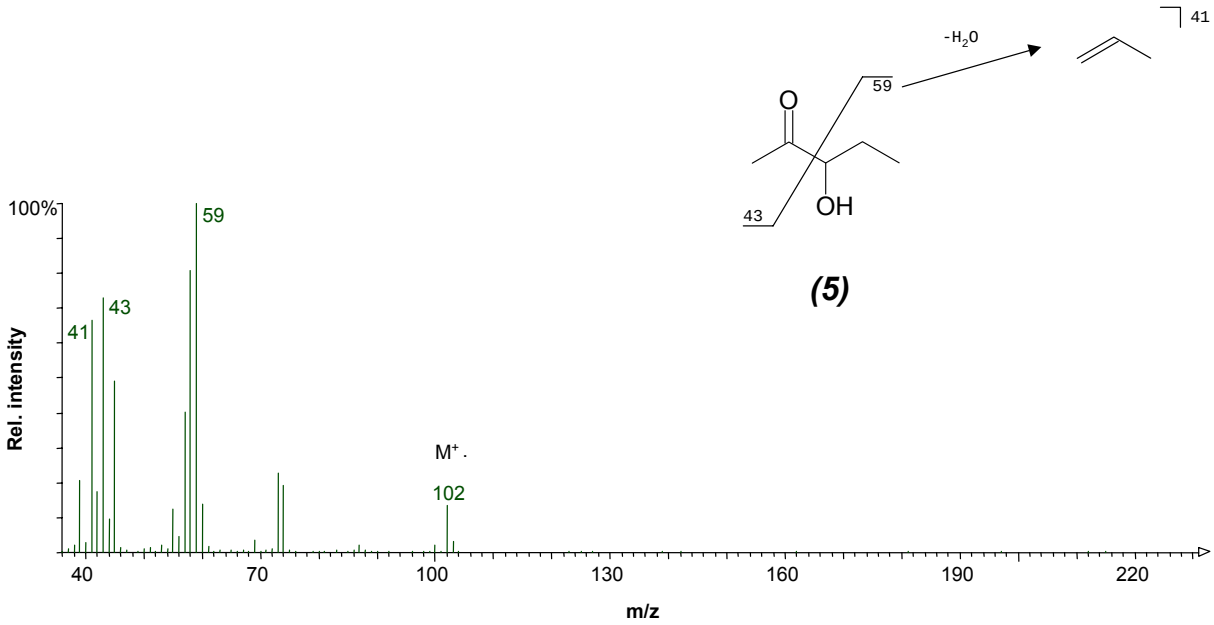
the oxygen. The solution was restirred again and the reaction was run until the hydrogen consumption was complete (1 h). The vessel was evacuated, the catalyst filtered, and the methanol solution was directly analyzed by GC-MS (2.2.5) and chiral gas chromatography (2.2.6). The temperature program for GC-MS was 5 min isothermal at 40 °C, then raised to 220 °C at 5 °C min^{-1}, and kept at 220 °C for 5 min. For chiral gas chromatography, the temperature program of oven 1 was 3 min isothermal at 40 °C, then raised to 90 °C at 5 °C min^{-1}, kept at 90 °C for 30 min, raised to 220 °C at 10 °C min^{-1}, and kept at 220 °C for 3 min. The temperature program of oven 2 was 20 min isothermal at 40 °C, then raised to 180 °C at 2 °C min^{-1}.

The ee values and the absolute configurations of 3-hydroxy-1-penten-4-one ***(3)*** and 4-hydroxy-1-penten-3-one ***(4)*** were deduced from those of the corresponding hydroxy-pentanones. The stereochemistry of the latter was determined by comparison to reference compounds. The ee values were confirmed by direct chiral GC analysis of the hydroxy-pentenones.

- (*R*)-3-Hydroxy-2-pentanone ***(5)***. ee=72 %**.**

RI: 1344.

MS-EI:

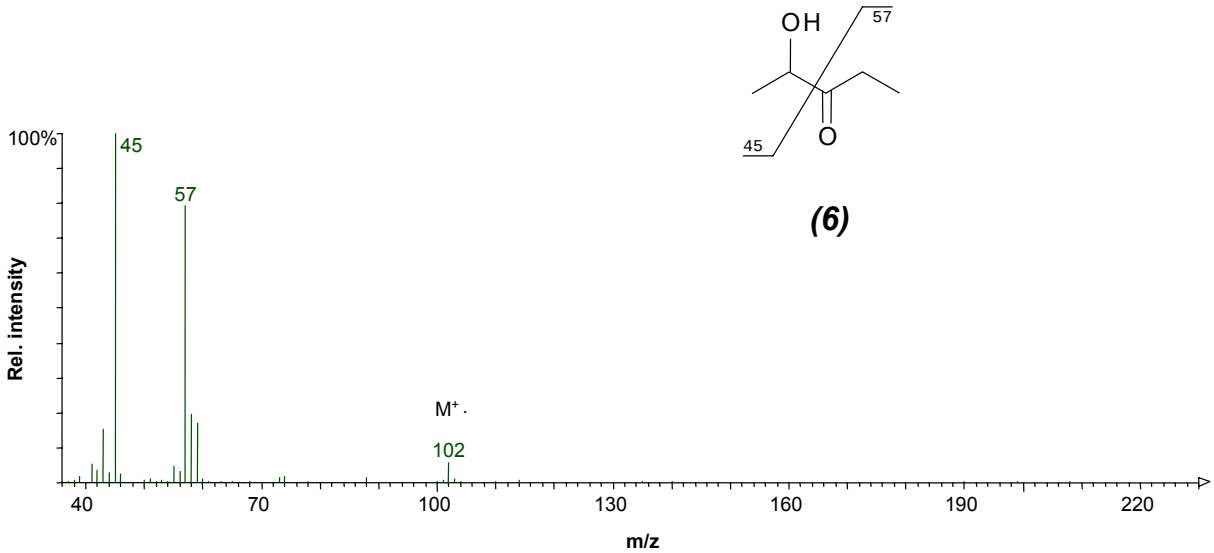


RI and mass spectrum agreed with those of injected (*S*)-3-hydroxy-2-pentanone ***(5)***, produced according to Bel Rhlid *et al*. [23]

- (*R*)-2-Hydroxy-3-pentanone ***(6)***. ee=92 %.

RI: 1361.

MS-EI:



(6)

RI and mass spectrum agreed with those of injected (*S*)-2-hydroxy-3-pentanone ***(6)***, produced according to Besse *et al*.[24]

- (*R*)-3-Hydroxy-1-penten-4-one ***(3)***. ee=72 %**.**

RI and mass spectrum agreed with those described in 2.3.1.1.2.

- (*R*)-4-Hydroxy-1-penten-3-one ***(4)***. ee=92 %.

RI and mass spectrum agreed with those described in 2.3.2.1.

2.3.2.2.4 Determination of specific optical rotation

3-Hydroxy-1-penten-4-one ***(3)***, produced as described in 2.3.2.2.1, was purified by preparative gas chromatography (2.2.7) in the course of approximately 200 injections. The compound was dissolved in 1 ml $CHCl_3$, and the optical rotation was measured at 589 nm on a Perkin-Elmer 241 polarimeter using a quartz cell of 10 cm length and 1 ml volume.

- (-)-(*R*)-3-Hydroxy-1-penten-4-one ***(3)***

Specific optical rotation: $[\alpha]^{25}_{J}$ =–75° (c=0.06 in $CHCl_3$), ee=72 %.

2.4 Biogeneration of 3-hydroxy-1-hexen-4-one and 4-hydroxy-1-hexen-3-one

2.4.1 Biotransformation

To 50 mL of 0.1M citrate buffer (pH 6.0), containing 10 g baker's yeast, 2 mM thiamine pyrophosphate, and 20 mM magnesium sulfate, were added 1 g of glucose (5.6 mmol), 155 mg of sodium 2-oxobutyrate (1.25 mmol), and 78 mg of acrolein (1.25 mmol). The mixture was incubated at 23 °C and after 1 h, the reaction mixture was centrifuged.

2.4.2 Work-up and analysis

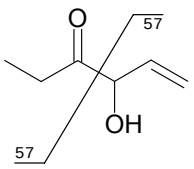
After centrifugation, 145 μg of 4-hydroxy-4-methyl-2-pentanone (1.25 μmol) were added to the supernatant as internal standard. The supernatant was then extracted continuously with 150 ml pentane/CH_2Cl_2 (2:1) overnight. The organic phase was dried over Na_2SO_4 and concentrated using a Vigreux column at 40 °C. The extracts were analysed by GC (see 2.2.3) and GC-MS (see 2.2.5) using a DB-WAX capillary column (30 m x 0.25 mm, film thickness 0.25 μm, J & W Scientific). The temperature program was 5 min isothermal at 40 °C, then raised to 220 °C at 5 °C min^{-1}, and kept at 220 °C for 5 min. Relative quantification was done by comparing the area of acyloins with that of the internal standard, assuming that the FID response factors were similar. It was shown that the FID response factors of commercial 1-hydroxy-2-propanone, 3-hydroxy-2-butanone ***(7)***, and 4-hydroxy-4-methyl-2-pentanone showed only small deviations (<10 %).

The following compounds were identified:

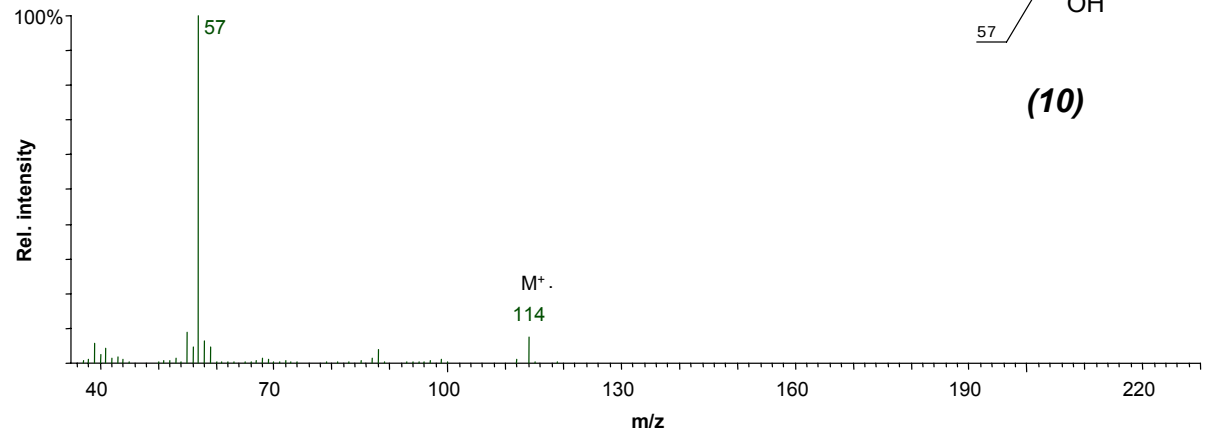
- 3-Hydroxy-1-hexen-4-one ***(10)***

RI: 1453.

MS-EI:



(10)

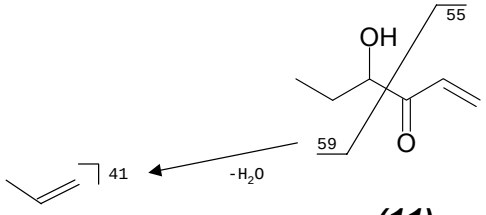


Reference spectrum: unknown.

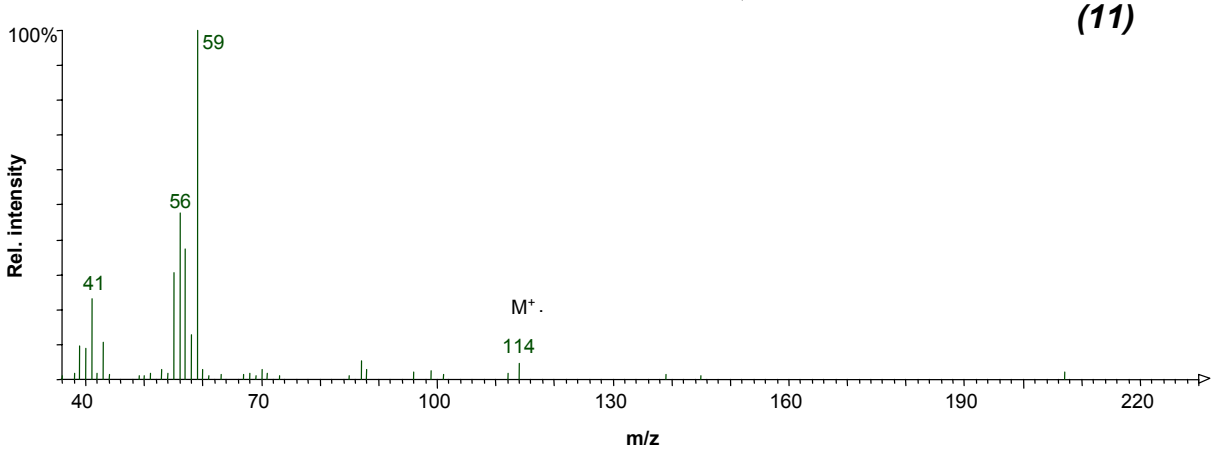
- 4-Hydroxy-1-hexen-3-one ***(11)***

RI: 1430.

MS-EI:



(11)

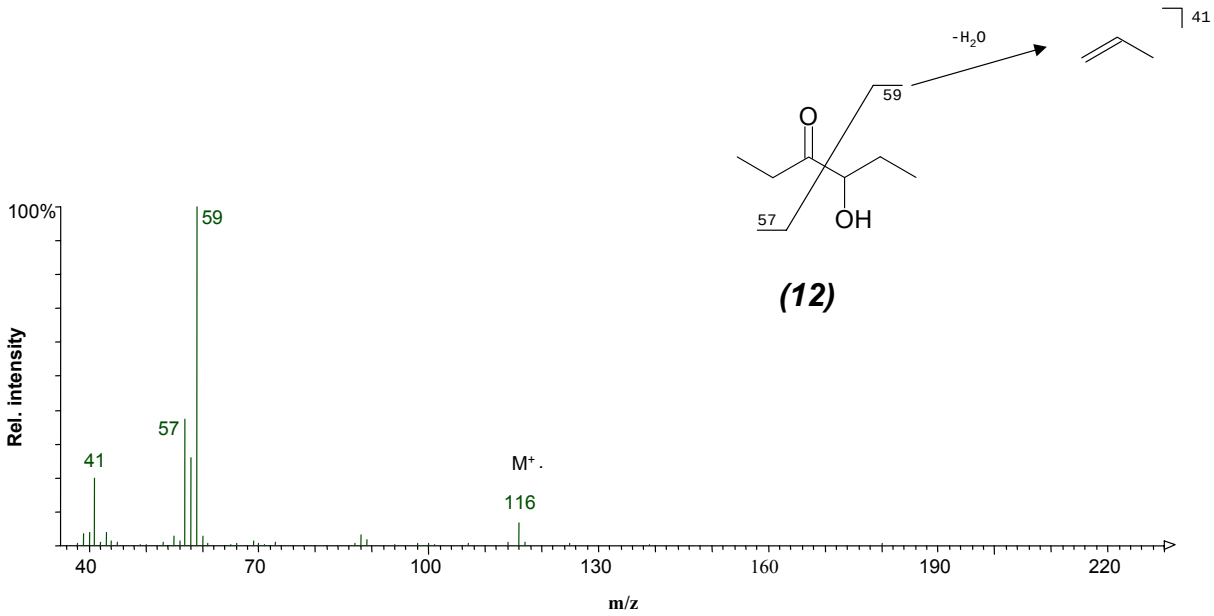


Reference spectrum: unknown.

4-Hydroxy-3-hexanone ***(12)***

RI: 1389

MS-EI:



(12)

RI and mass spectrum agreed with those of injected (*S*)-4-hydroxy-3-hexanone (***12***), produced according to Bel Rhlid *et al*.[25]

- 3-Hydroxy-2-pentanone ***(5)***

RI and mass spectrum agreed with those described in 2.3.2.2.3.

- 2-Hydroxy-3-pentanone ***(6)***

RI and mass spectrum agreed with those described in 2.3.2.2.3.

- 3-Hydroxy-1-penten-4-one ***(3)***

RI and mass spectrum agreed with those described in 2.3.1.1.2.

- 4-Hydroxy-1-penten-3-one ***(4)***

RI and mass spectrum agreed with those described in 2.3.2.1.

The enantiomeric excesses were determined by chiral GC (see 2.2.6). The temperature program of oven 1 was 3 min isothermal at 40 °C, then raised to 90 °C at 5 °C min^{-1}, kept at 90 °C for 30 min, raised to 220 °C at 10 °C min^{-1}, and kept at 220 °C for 3 min. The temperature program of oven 2 was 20 min isothermal at 40 °C, then raised to 180 °C at 2 °C min^{-1}.

2.5 Biogeneration of other acyloins using whole cells of baker's yeast

2.5.1 Biotransformation

To 50 mL of 0.1 M citrate buffer (pH 6.0), containing 10 g baker's yeast, 2 mM thiamine pyrophosphate, and 20 mM magnesium sulfate, were added 1 g of glucose (5.6 mmol) and 140 mg of sodium pyruvate (1.25 mmol). One of several aldehydes (1.25 mmol) was added per assay. The reaction was incubated at 23 °C and after 1 h, the reaction mixture was centrifuged.

2.5.2 Work-up and analysis

After centrifugation, 145 μg of 4-hydroxy-4-methyl-2-pentanone (1.25 μmol) were added as internal standard to the supernatant. The mixture was then extracted continuously with 150 ml pentane/CH_2Cl_2 (2:1) overnight. The organic phase was dried over Na_2SO_4 and concentrated using a Vigreux column at 40 °C. The extracts were analyzed by GC (see 2.2.3) and GC-MS (see 2.2.5) using a DB-WAX capillary column (30 m x 0.25 mm, film thickness 0.25 μm, J & W Scientific). The temperature program was 5 min isothermal at 40 °C, then raised to 220 °C at 5 °C min^{-1}, and kept at 220 °C for 5 min. Relative quantification was done by comparing the area of acyloins with that of the internal standard, assuming similar FID response factors. It was shown that the FID response factors of commercial 1-hydroxy-2-propanone, 3-hydroxy-2-butanone ***(7)***, and 4-hydroxy-4-methyl-2-pentanone showed only small deviations (<10 %).

The following compounds were identified:

From Propanal:

- 3-Hydroxy-2-pentanone ***(5)***

RI and mass spectrum agreed with those described in 2.3.2.2.3.

- 2-Hydroxy-3-pentanone ***(6)***

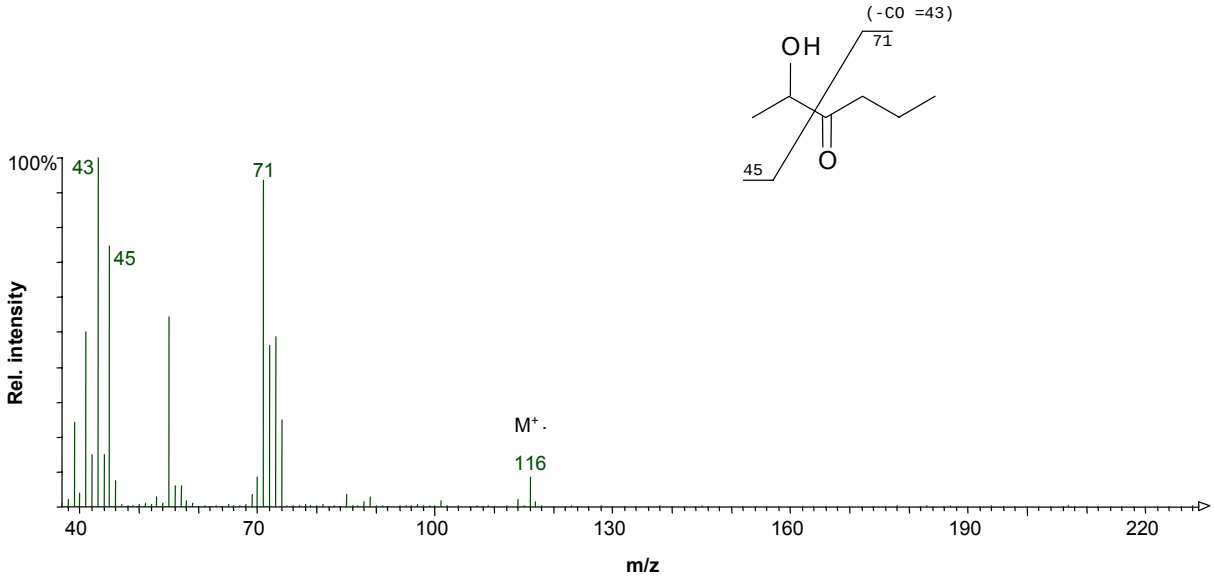
RI and mass spectrum agreed with those described in 2.3.2.2.3.

From Butanal:

- 2-Hydroxy-3-hexanone

RI: 1423.

MS-EI:

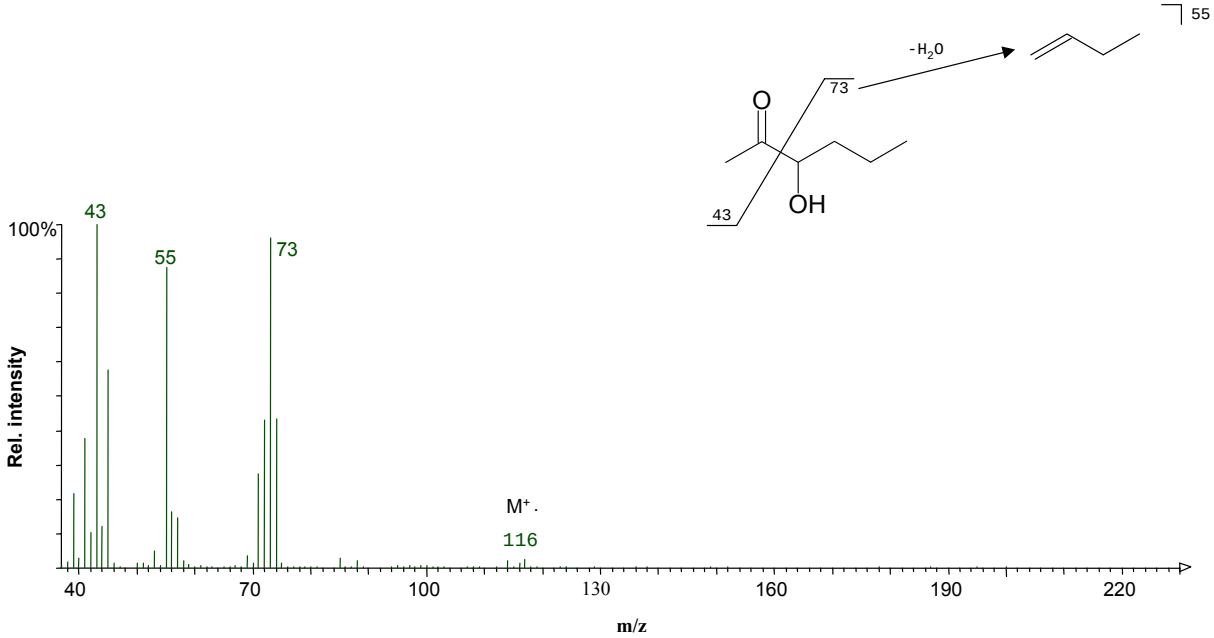


Mass spectrum agreed with that described by Neuser[26].

- 3-Hydroxy-2-hexanone

RI: 1430.

MS-EI:



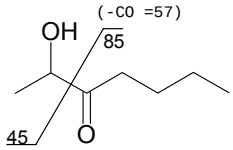
Mass spectrum agreed with that described by Neuser[26].

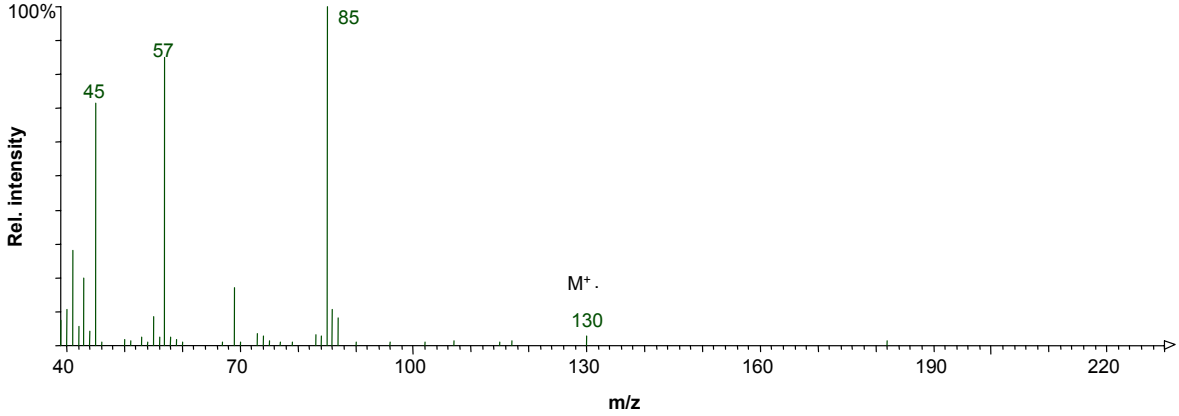
From pentanal (97 %):

- 2-Hydroxy-3-heptanone

RI: 1551.

MS-EI:



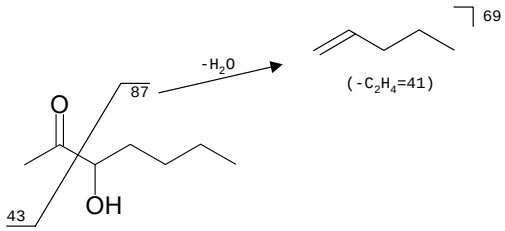


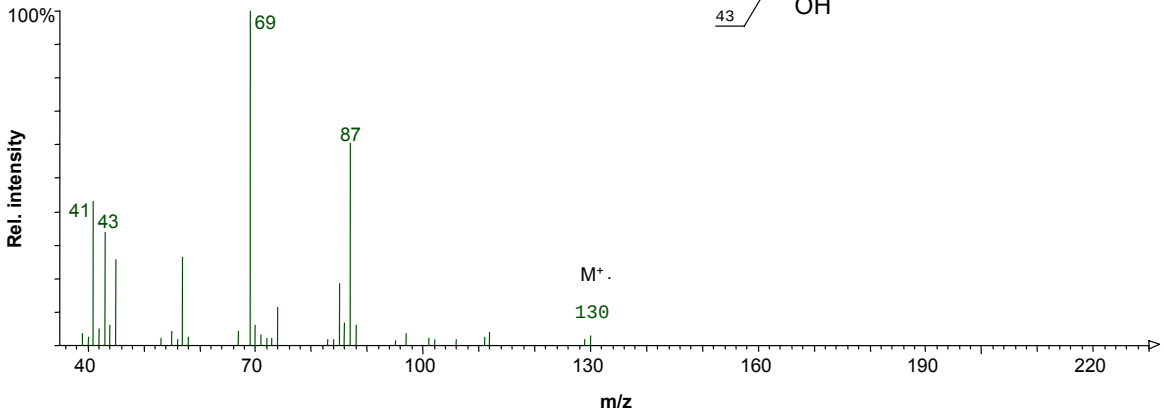
Mass spectrum agreed with that described by Neuser[26].

- 3-Hydroxy-2-heptanone

RI: 1560.

MS-EI:





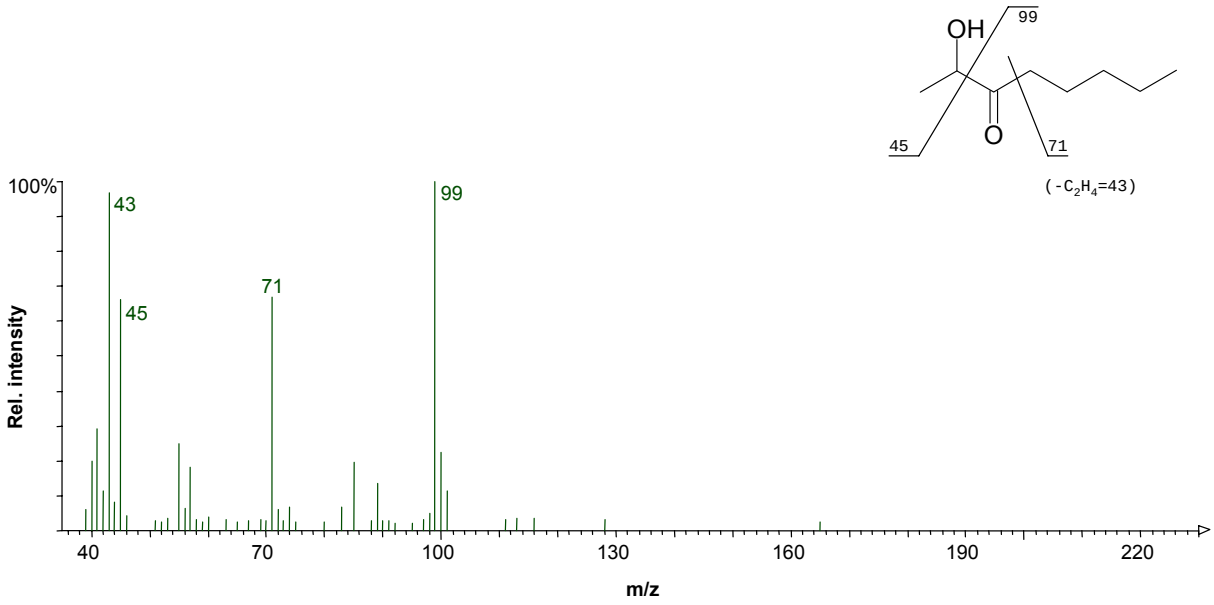
Mass spectrum agreed with that described by Neuser[26].

From hexanal (98 %):

- 2-Hydroxy-3-octanone ***(17)***

RI: 1647.

MS-EI:

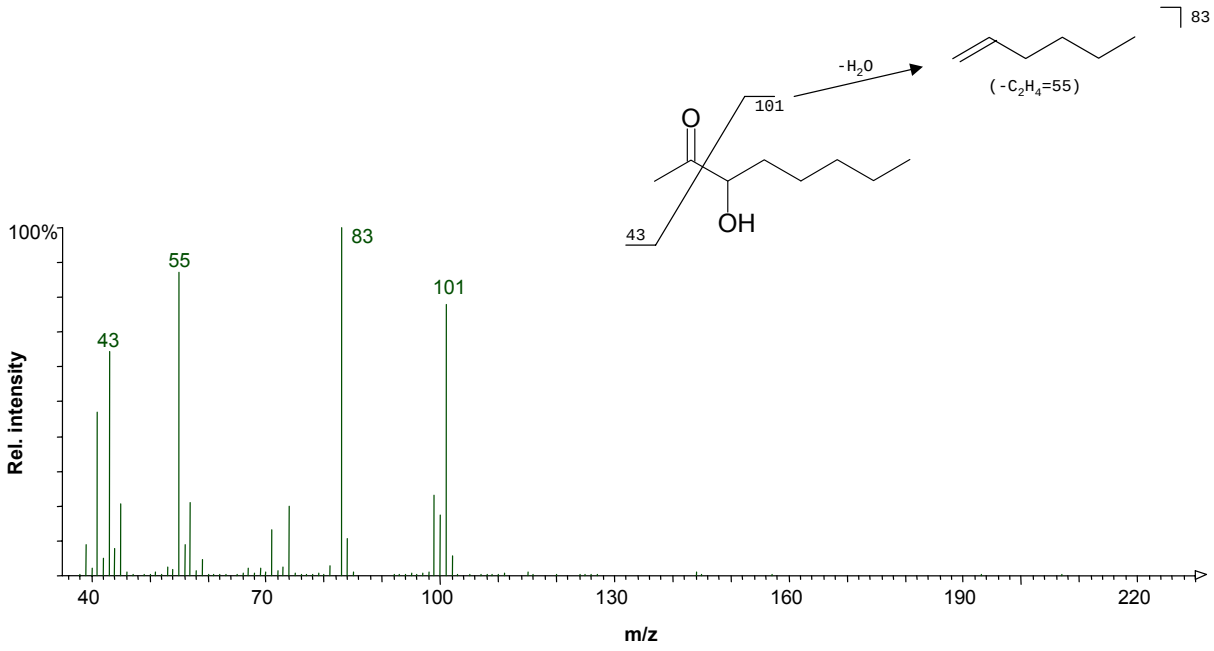


Mass spectrum agreed with that described by Neuser[26].

- 3-Hydroxy-2-octanone ***(19)***

RI: 1655.

MS-EI:



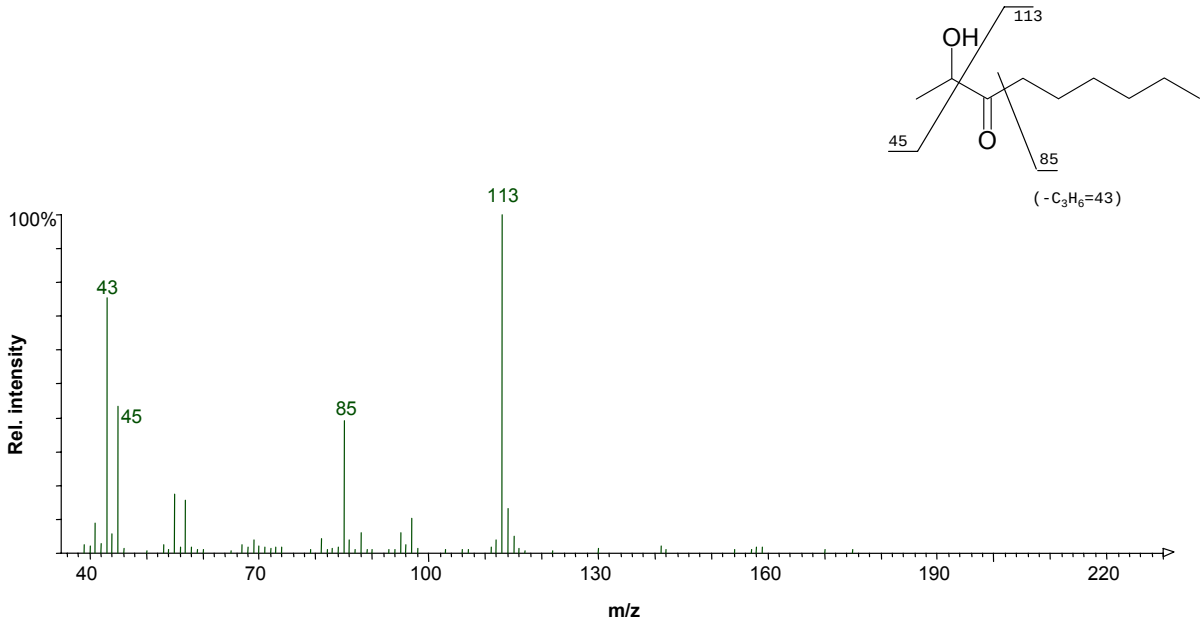
Mass spectrum agreed with that described by Neuser[26].

From heptanal (95 %):

- 2-Hydroxy-3-nonanone

RI: 1757

MS-EI:

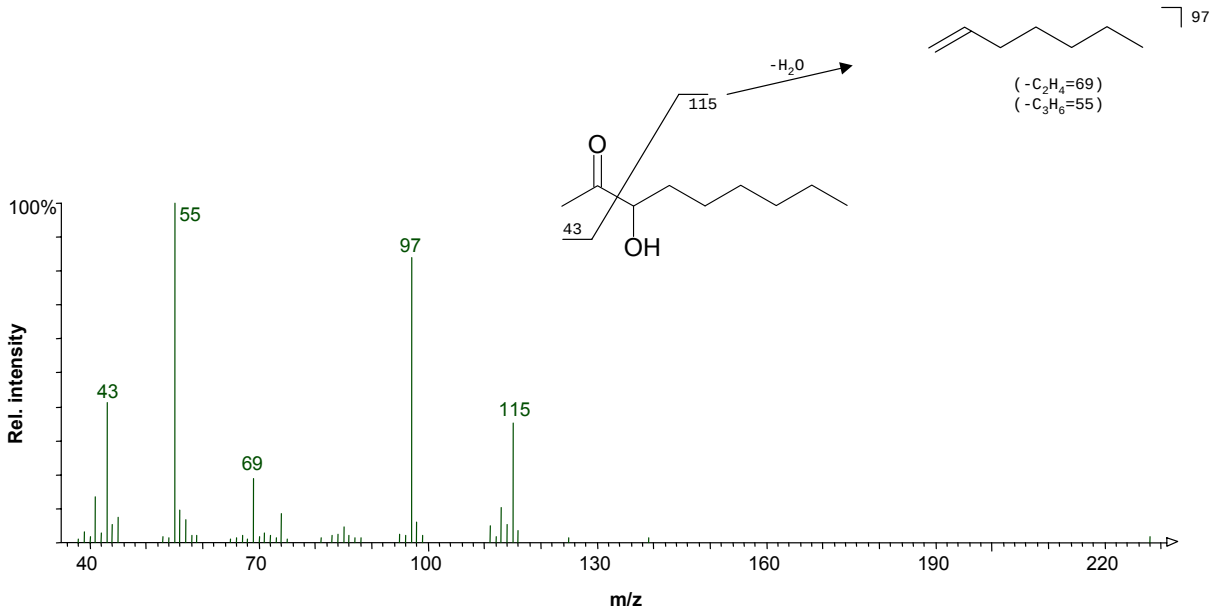


Mass spectrum agreed with that described by Neuser[26].

- 3-Hydroxy-2-nonanone

RI: 1768

MS-EI:



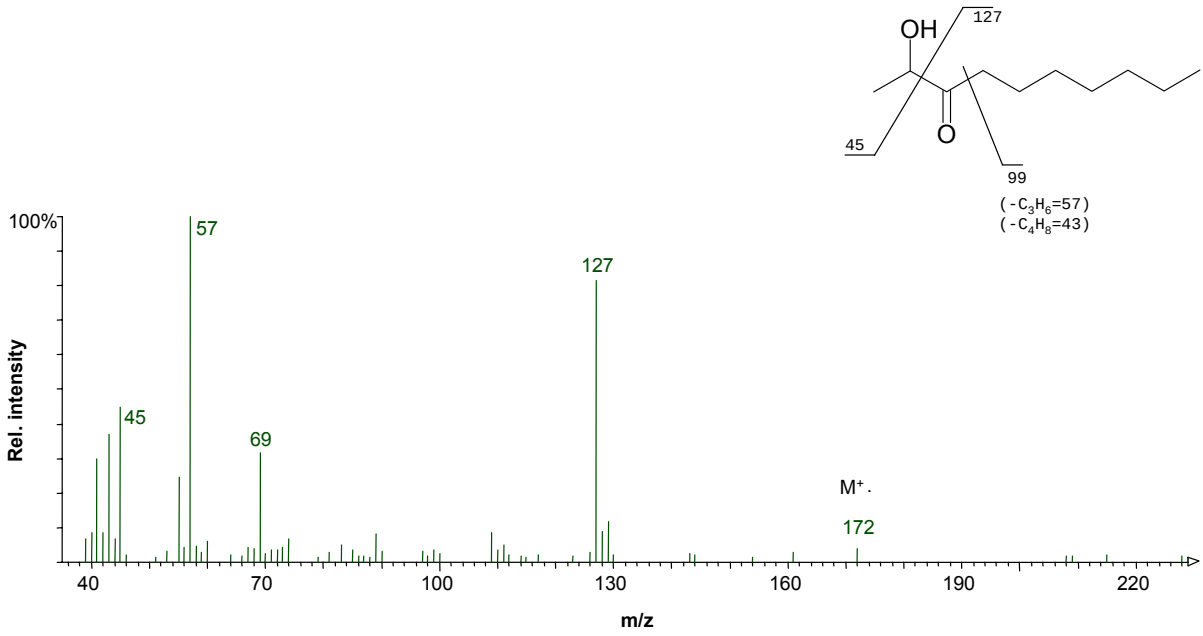
Mass spectrum agreed with that described by Neuser[26].

From octanal:

- 2-Hydroxy-3-decanone

RI: 1874.

MS-EI:

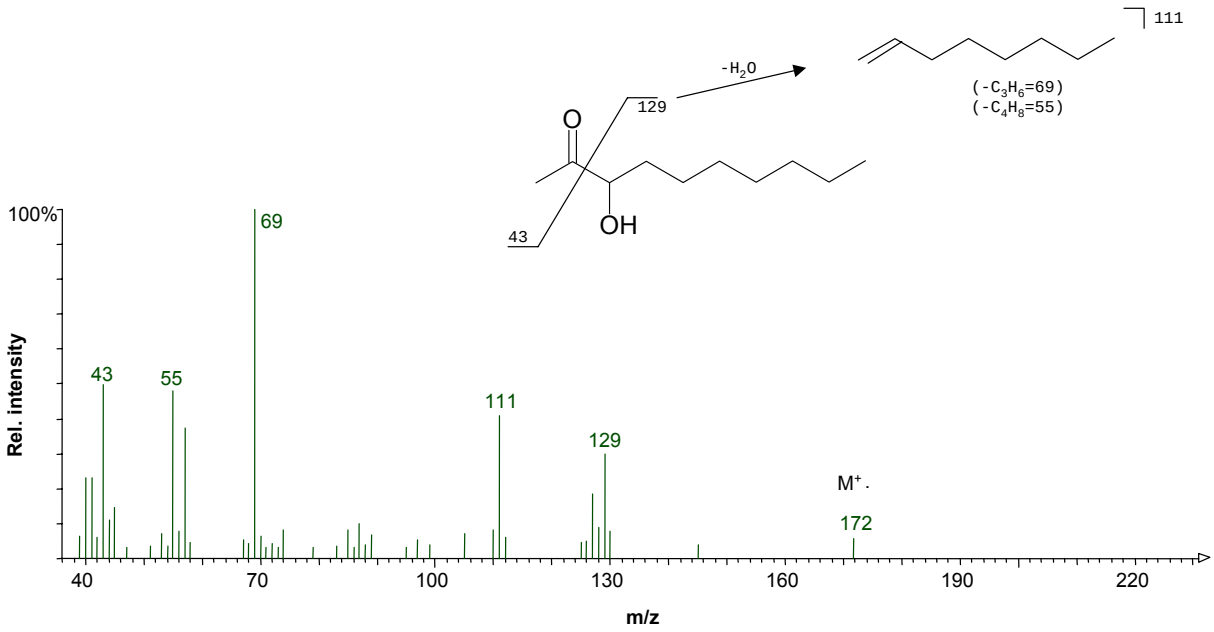


Mass spectrum agreed with that described by Neuser[26].

- 3-Hydroxy-2-decanone

RI: 1889.

MS-EI:



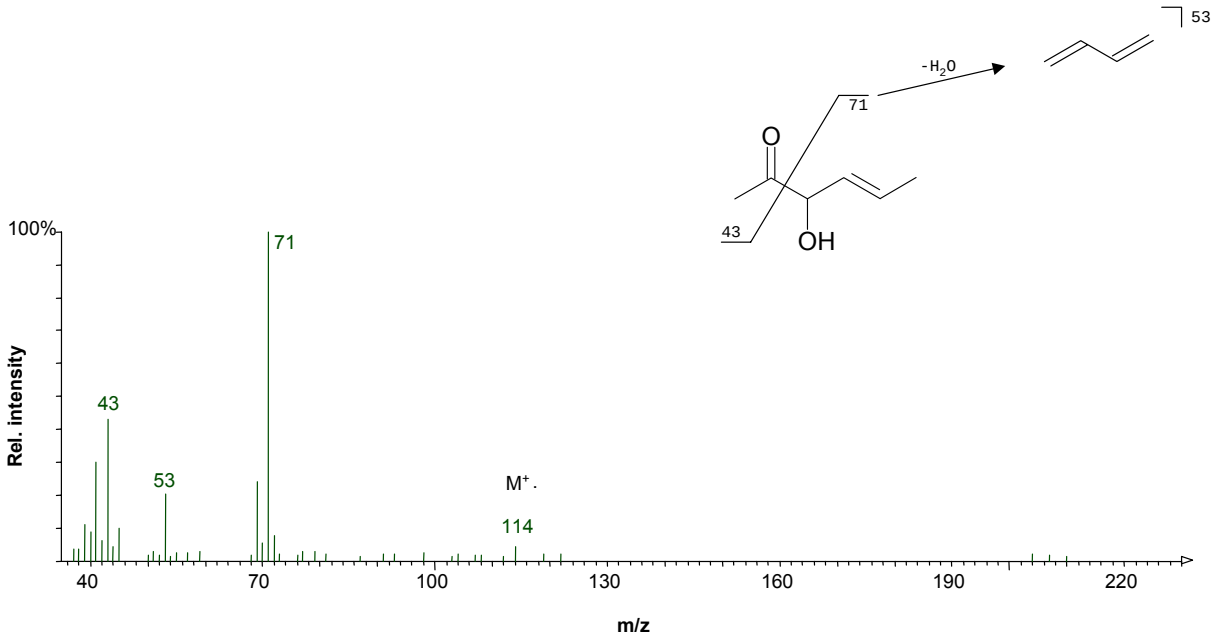
Mass spectrum agreed with that described by Neuser[26].

From 2*E*-butenal (≥97 %):

- 3-Hydroxy-4*E*-hexen-2-one

RI: 1481.

MS-EI:



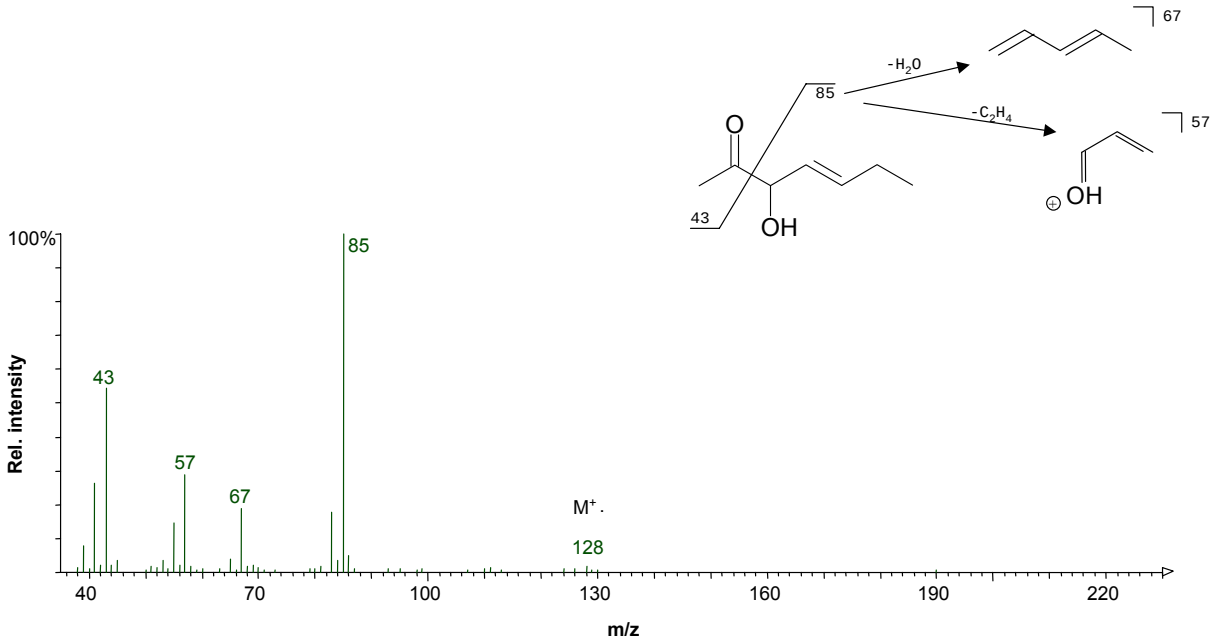
Mass spectrum agreed with that described by Neuser[26].

From 2*E*-pentenal (95 %):

- 3-Hydroxy-4*E*-hepten-2-one

RI: 1586.

MS-EI:

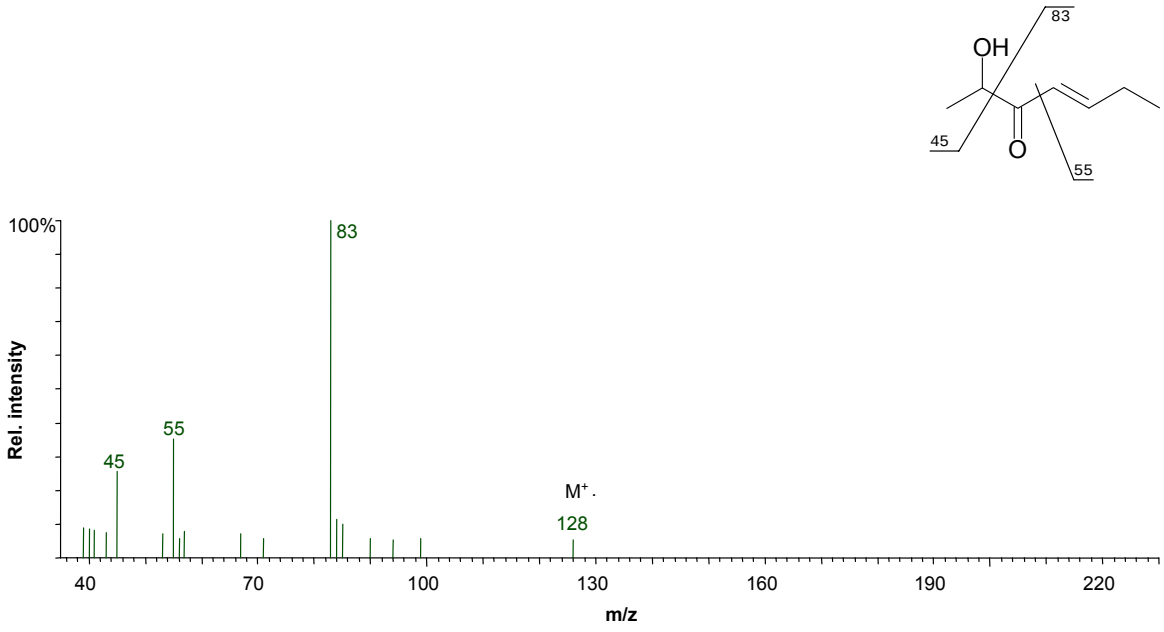


Mass spectrum agreed with that described by Neuser[26].

- 2-Hydroxy-4*E*-hepten-3-one

RI: 1619.

MS-EI:



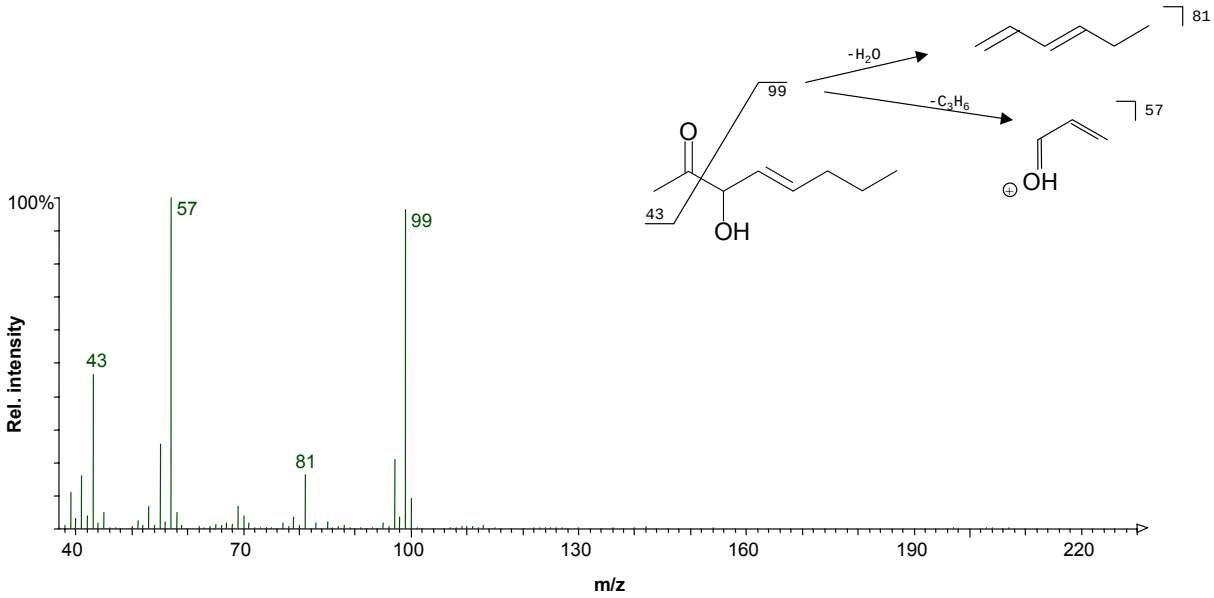
Mass spectrum agreed with that described by Neuser[26].

From 2*E*-hexenal:

- 3-Hydroxy-4*E*-octen-2-one

RI: 1683.

MS-EI:

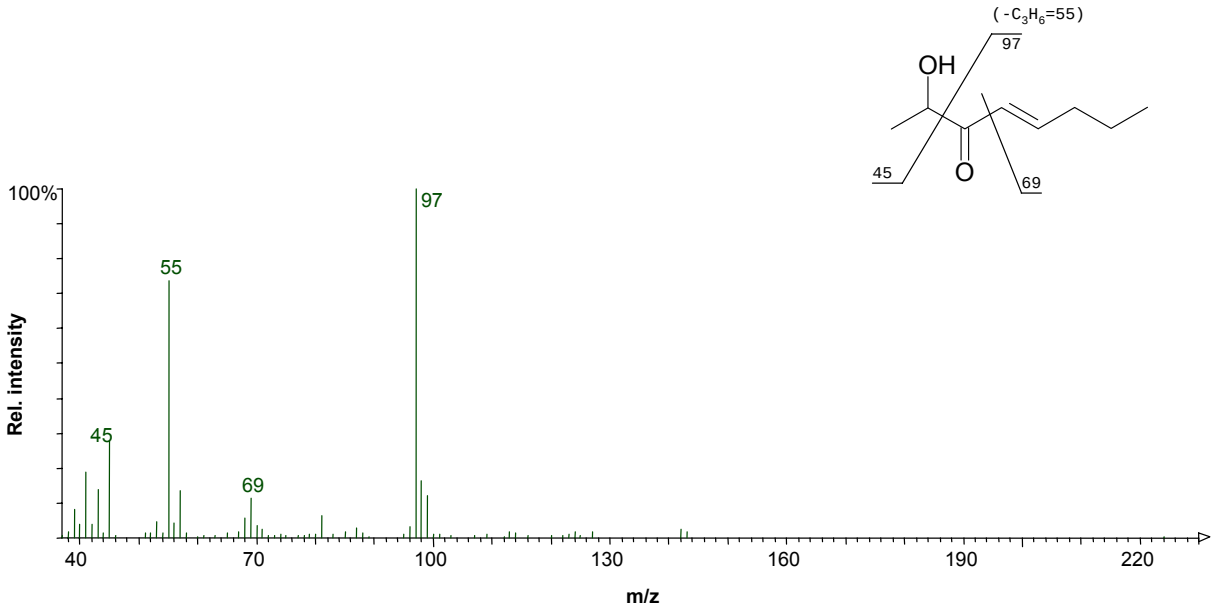


Mass spectrum agreed with that described by Neuser[26].

- 2-Hydroxy-4*E*-octen-3-one

RI: 1698.

MS-EI:



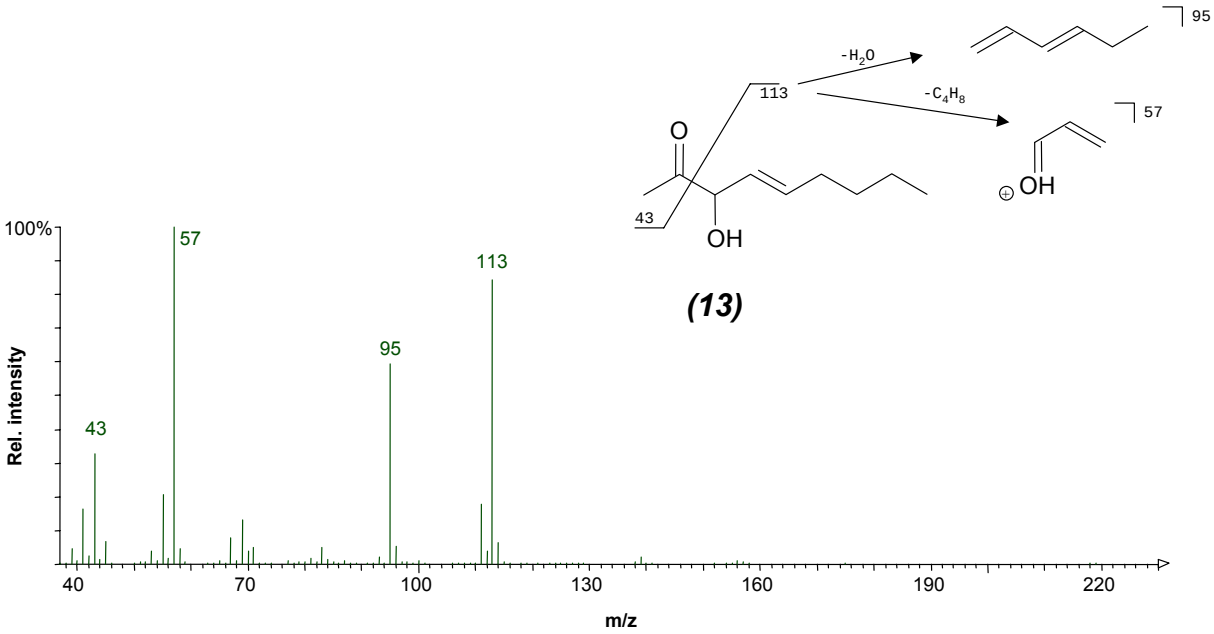
Mass spectrum agreed with that described by Neuser[26].

From 2*E*-heptenal (97 %):

- 3-Hydroxy-4*E*-nonen-2-one ***(13)***

RI: 1776.

MS-EI:



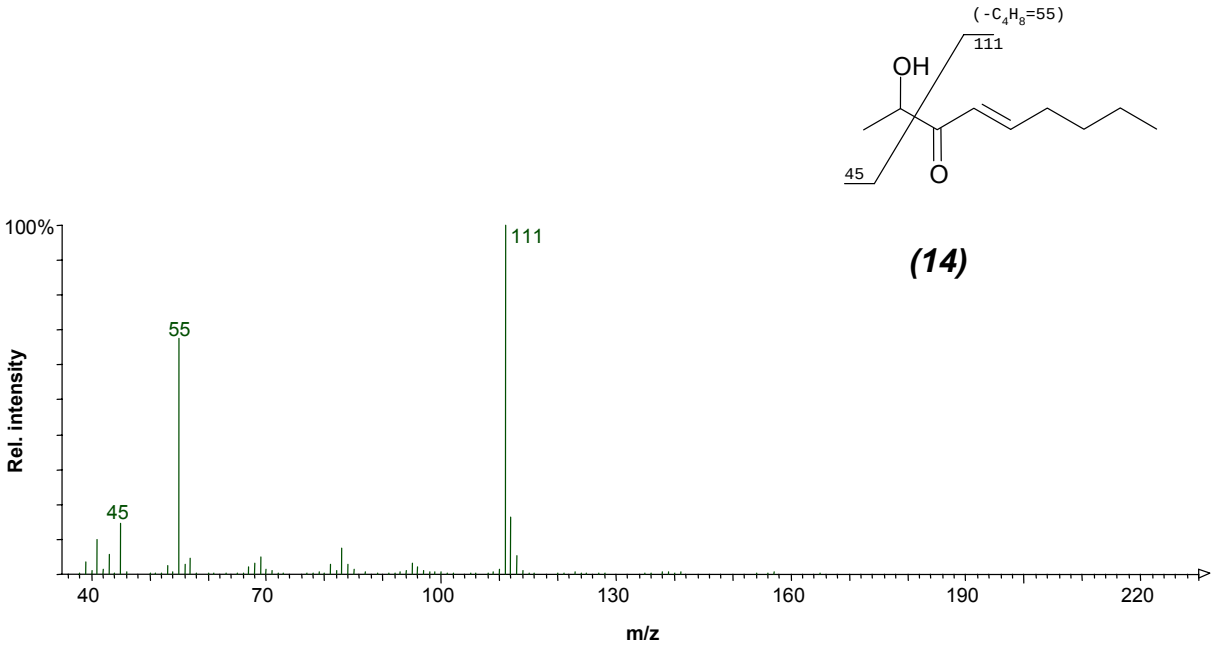
(13)

Reference spectrum: unknown.

- 2-Hydroxy-4*E*-nonen-3-one ***(14)***

RI: 1796

MS-EI:



(14)

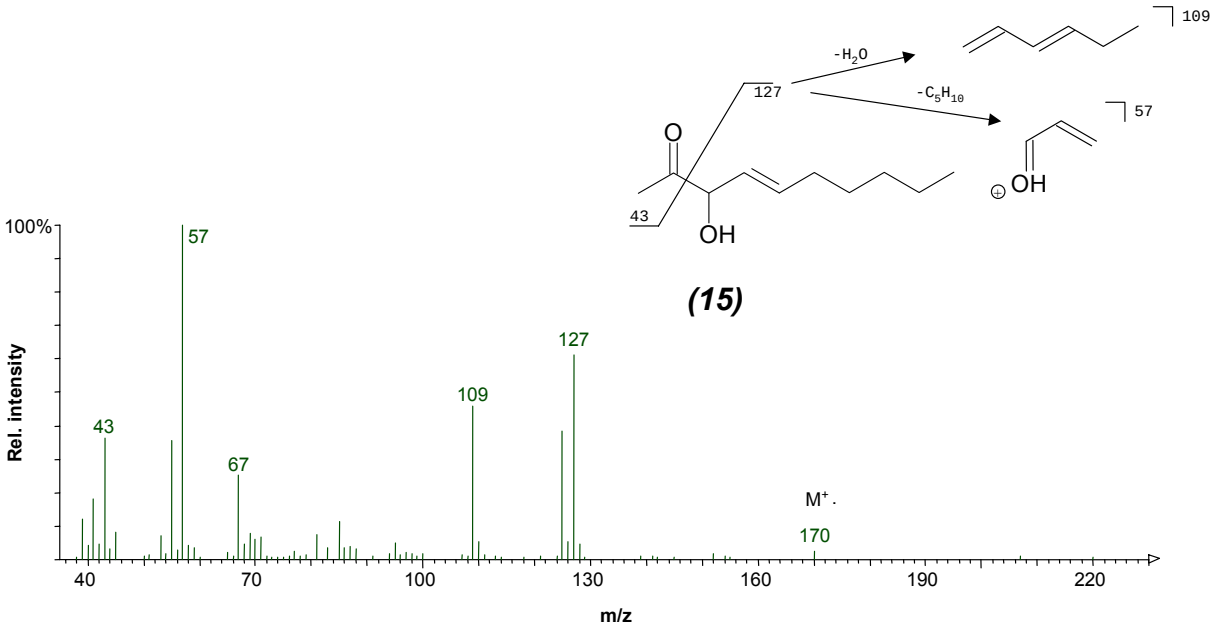
Reference spectrum: unknown.

From 2*E*-octenal (94 %):

- 3-Hydroxy-4*E*-decen-2-one ***(15)***

RI: 1946.

MS-EI:



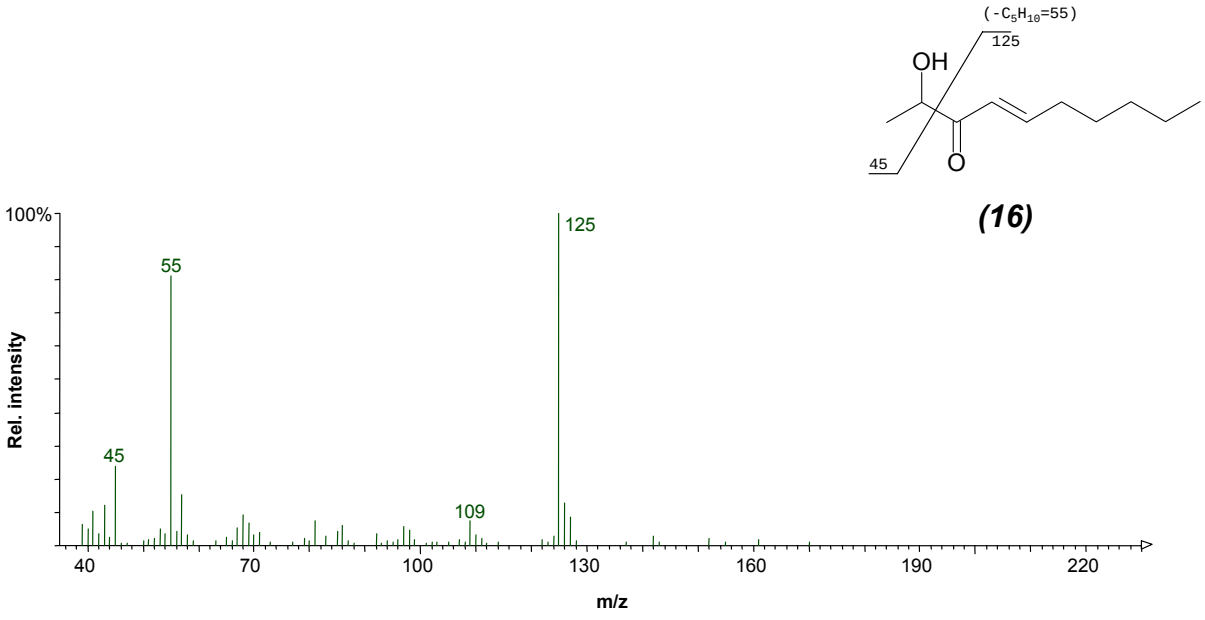
(15)

Reference spectrum: unknown.

- 2-Hydroxy-4*E*-decen-3-one ***(16)***

RI: 1959.

MS-EI:



(16)

Reference spectrum: unknown.

The enantiomeric excesses were determined by chiral gas chromatography (see 2.2.6). For the analysis of saturated acyloins, the temperature program of oven 1 was 3 min isothermal at 40 °C, then raised to 220 °C at 2 °C min^{-1}, and kept at 220 °C for 5 min. The temperature

program of oven 2 was 50 min isothermal at 40 °C, then raised to 220 °C at 2 °C min^{-1}. For the analysis of desaturated acyloins, the temperature program of oven 1 was 3 min isothermal at 40 °C, then raised to 220 °C at 3 °C min^{-1}, and kept at 220 °C for 5 min. The temperature program of oven 2 was 20 min isothermal at 40 °C, then raised to 220 °C at 2 °C min^{-1}.

2.6 Chemoenzymatic synthesis of pyrazine derivatives

2.6.1 Synthesis of 5,6-dihydropyrazines from 2*E*-alkenals

To 50 mL of 0.1 M citrate buffer (pH 6.0), containing 10 g baker's yeast, 2 mM thiamine pyrophosphate, and 20 mM magnesium sulfate, were added 1 g of glucose (5.6 mmol) and 140 mg of sodium pyruvate or 155 mg of sodium 2-oxobutyrate (1.25 mmol). One of several 2*E*-alkenals (1.25 mmol) was added per assay. The reaction was incubated at 23 °C and after 1 h, 925 mg of 1,2-propanediamine (12.5 mmol) were added. After further incubation of 90 min, the reaction mixture was centrifuged, and 10 mg of trimethylpyrazine (82 μmol) were added to the supernatant as internal standard. The mixture was extracted twice with diethyl ether. The combined etheral solutions were dried over Na_2SO_4 and concentrated using a Vigreux column at 40 °C. The extract was analyzed by GC (2.2.3) and GC-MS (2.2.5), using a DB-1 capillary column (30 m x 0.25 mm, film thickness 0.25 μm, J & W Scientific). The temperature program was 5 min isothermal at 40 °C, then raised to 260 °C at 4 °C min^{-1}, and kept at 260 °C for 5 min. Relative quantification was done by comparing the area of the pyrazine derivatives with that of the internal standard, assuming similar FID response factors. It was shown that the FID response factors of commercial trimethylpyrazine, 2-ethyl-3,5-dimethylpyrazine ***(31)***, and 2,3-diethyl-5-dimethylpyrazine ***(41)*** showed only small deviations (<10 %).

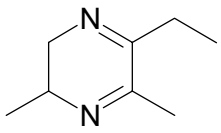
The following compounds were identified:

From acrolein and pyruvate:

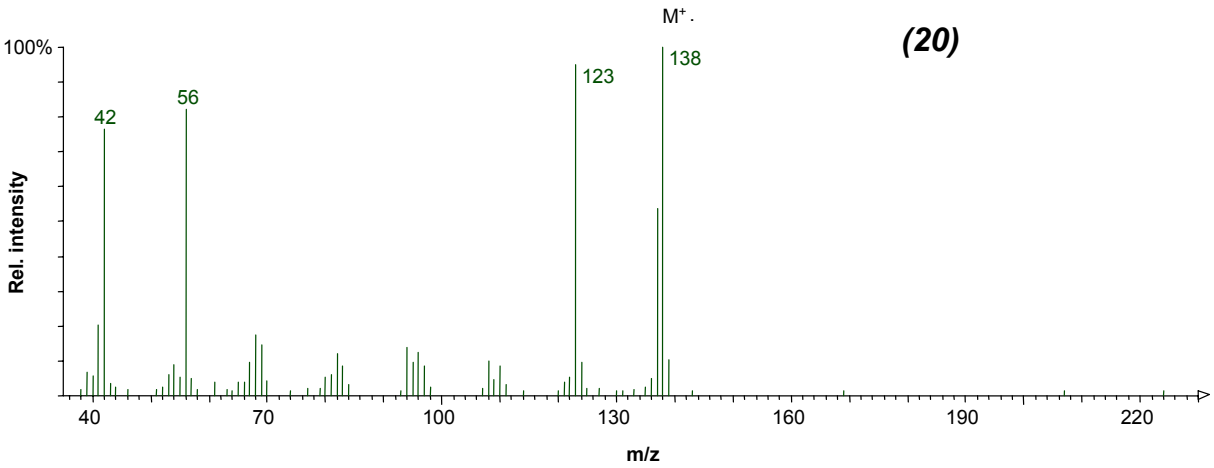
- 2-Ethyl-3,5-dimethyl-5,6-dihydropyrazine ***(20)***

RI: 1038

MS-EI:



(20)

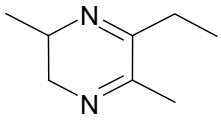


Mass spectrum agreed with that described by HAG AG[27].

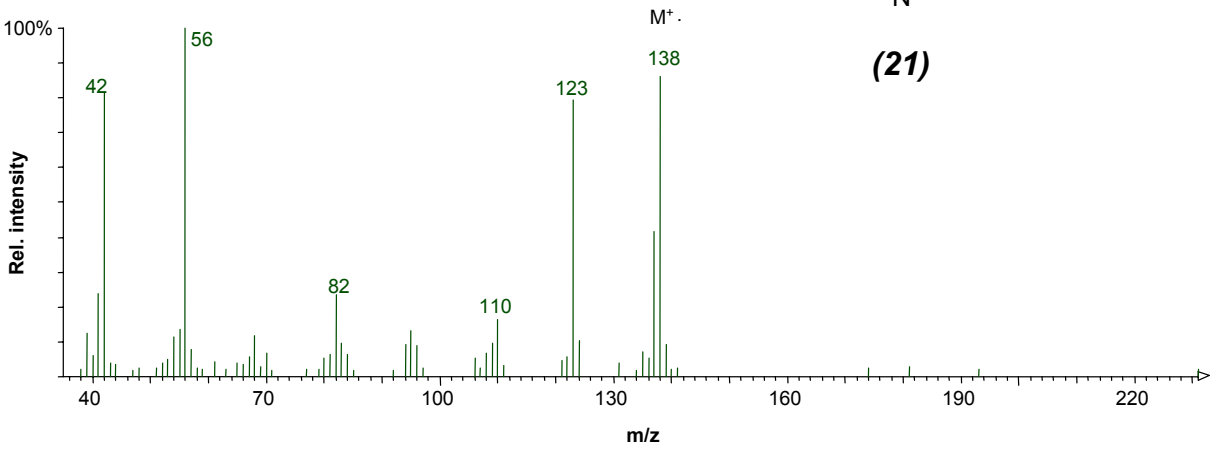
- 3-Ethyl-2,5-dimethyl-5,6-hydropyrazine ***(21)***

RI: 1031.

MS-EI:



(21)



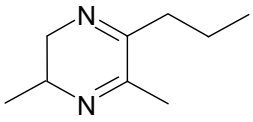
Reference spectrum: unknown.

From 2*E*-butenal and pyruvate:

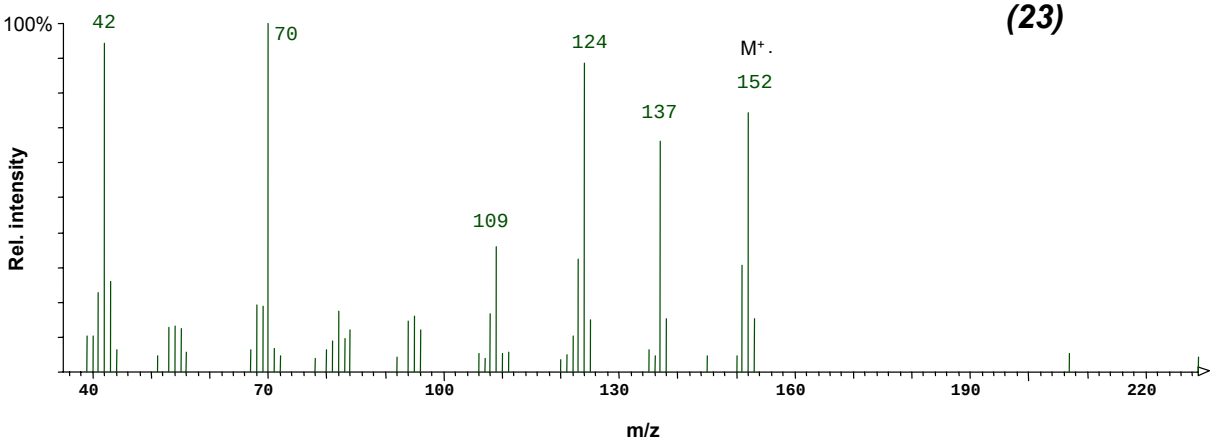
- 2-Propyl-3,5-methyl-5,6-dihydropyrazine ***(23)***

RI: 1110.

MS-EI:



(23)

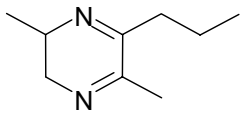


Reference spectrum: unknown.

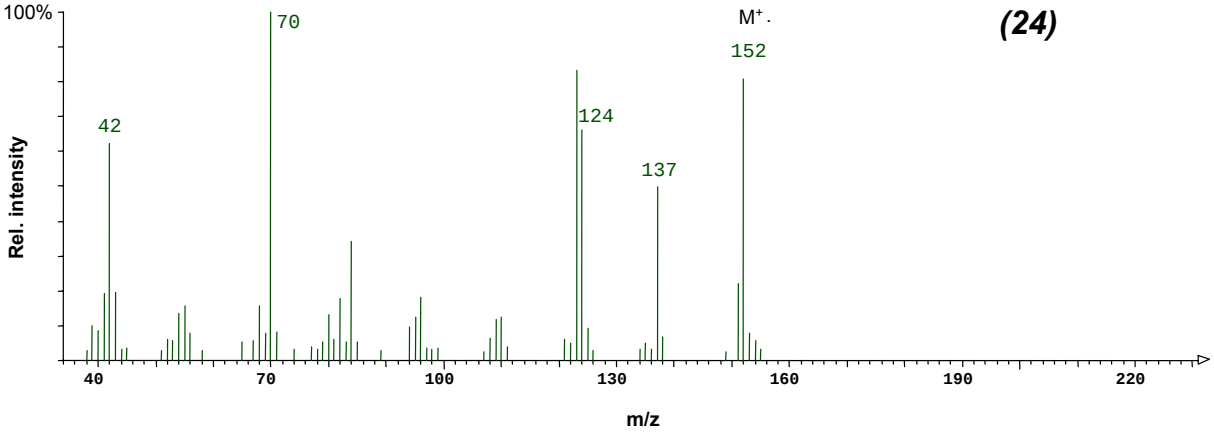
- 3-Propyl-2,5-methyl-5,6-dihydropyrazine ***(24)***

RI: 1100.

MS-EI:



(24)



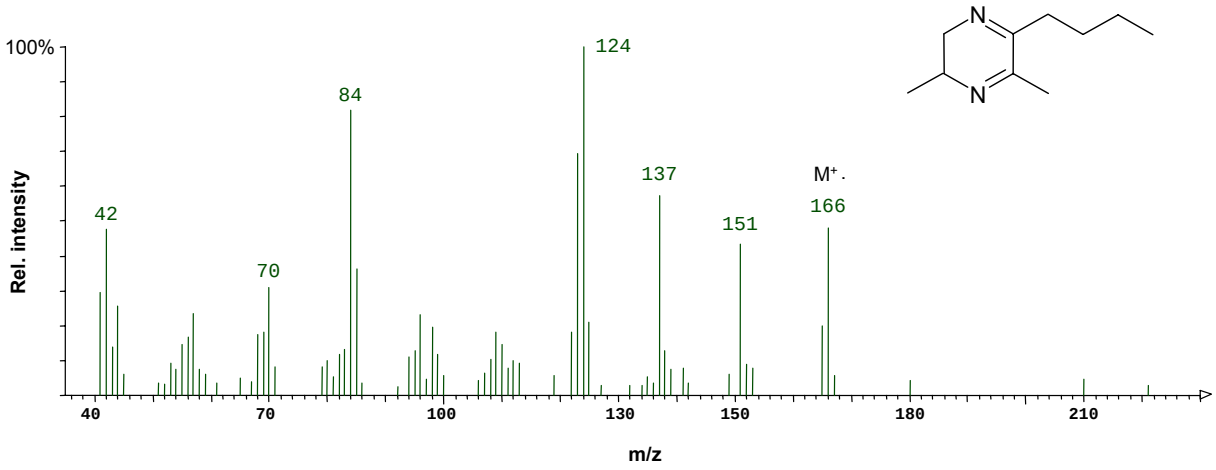
Reference spectrum: unknown.

From 2*E*-pentenal and pyruvate:

- 2-Butyl-3,5-methyl-5,6-dihydropyrazine

RI: 1215.

MS-EI:

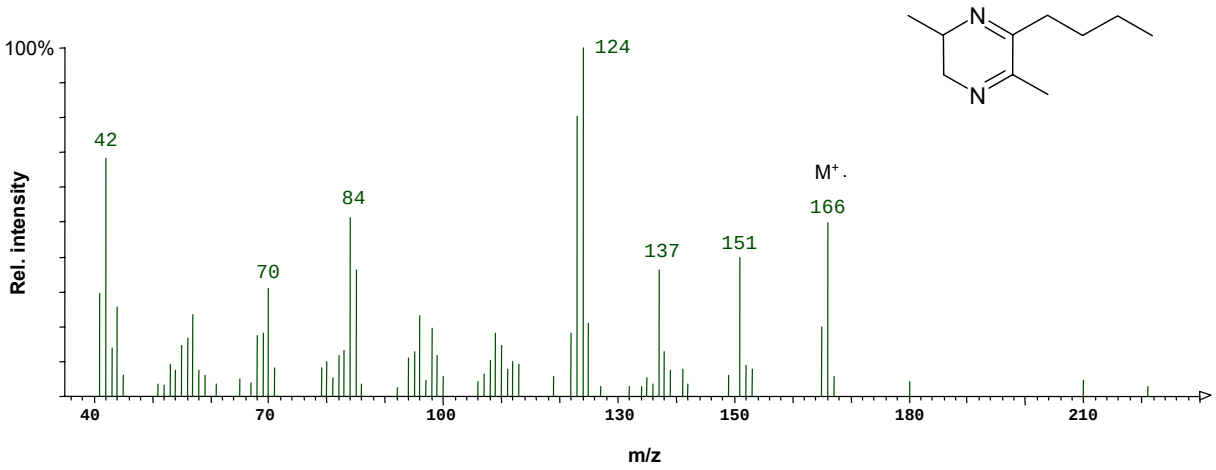


Reference spectrum: unknown.

- 3-Butyl-2,5-methyl-5,6-dihydropyrazine

RI: 1198.

MS-EI:



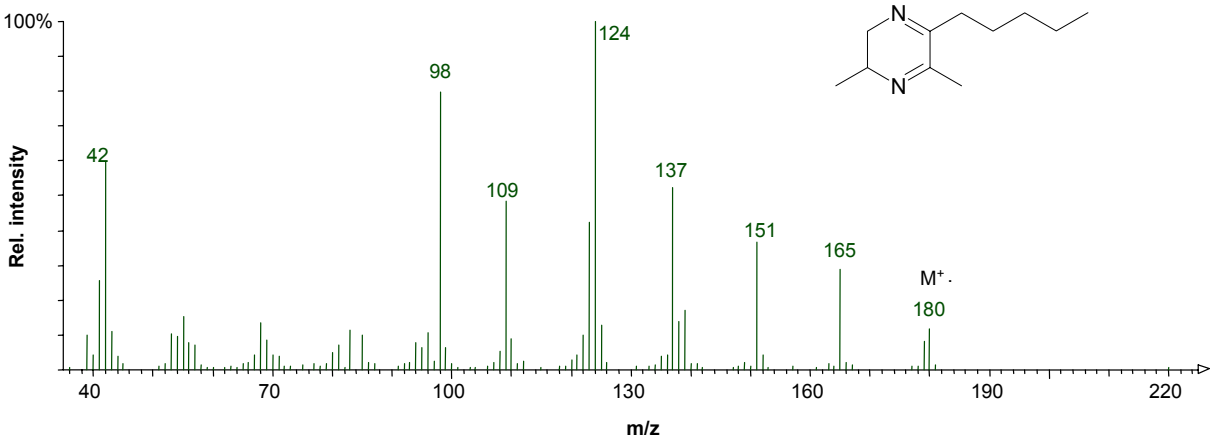
Reference spectrum: unknown.

From 2*E*-hexenal and pyruvate:

- 2-Pentyl-3,5-methyl-5,6-dihydropyrazine

RI: 1311.

MS-EI:

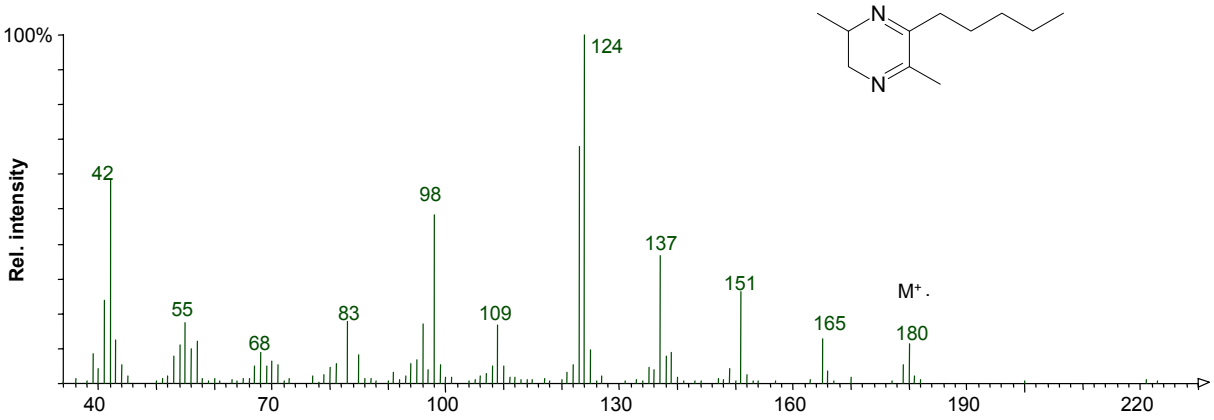


Reference spectrum: unknown.

- 3-Pentyl-2,5-methyl-5,6-dihydropyrazine

RI: 1298.

MS-EI:



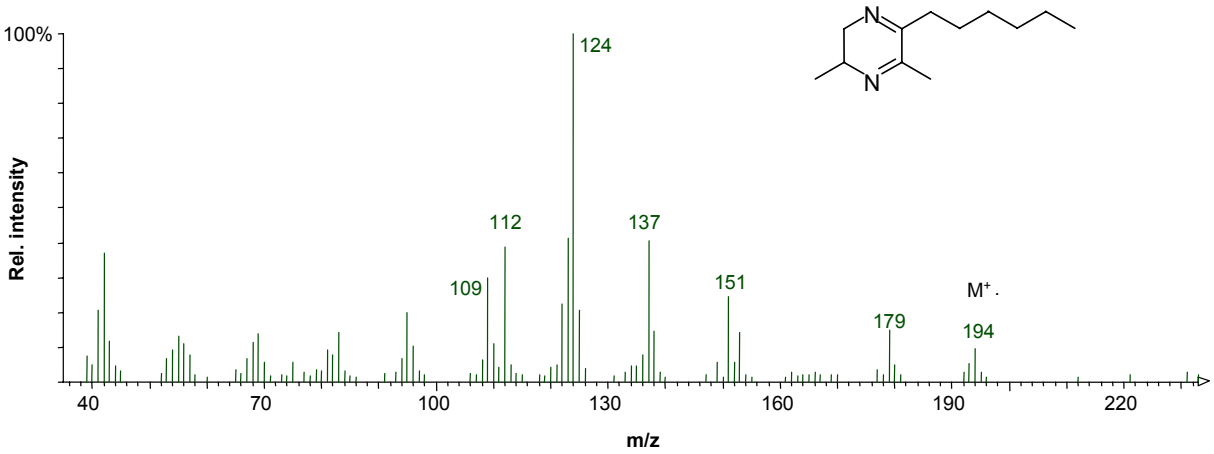
Reference spectrum: unknown.

From 2*E*-heptenal and pyruvate:

- 2-Hexyl-3,5-methyl-5,6-dihydropyrazine

RI: 1393.

MS-EI:

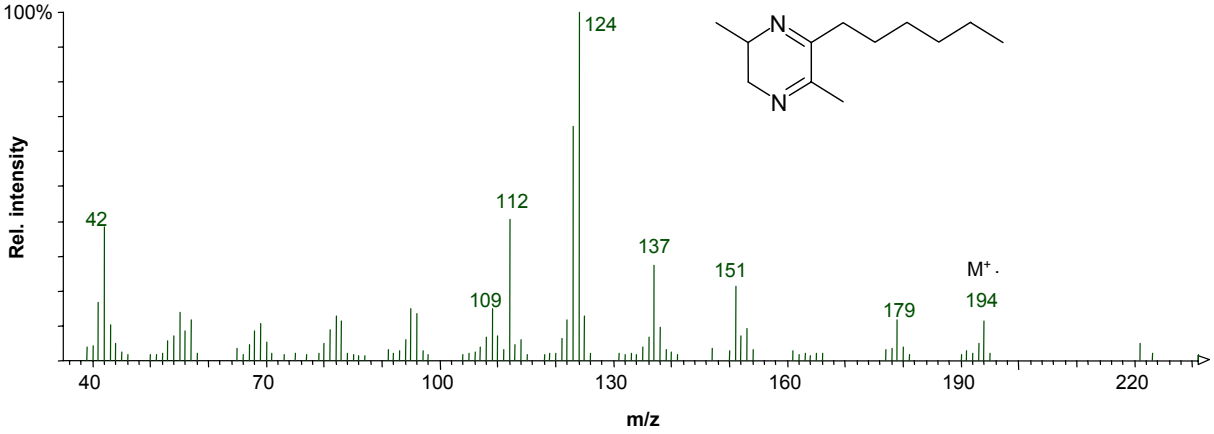


Reference spectrum: unknown.

- 3-Hexyl-2,5-methyl-5,6-dihydropyrazine

RI: 1380.

MS-EI:



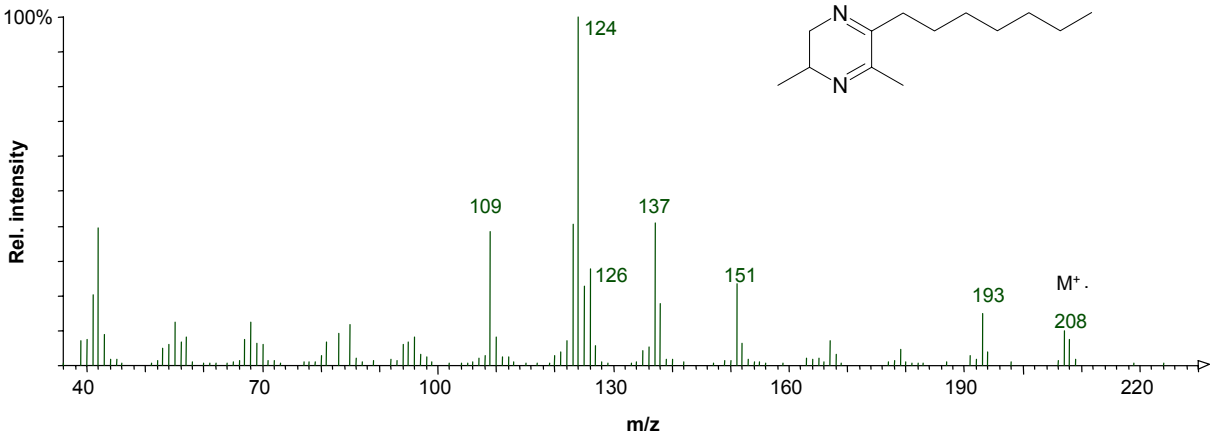
Reference spectrum: unknown.

From 2*E*-octenal and pyruvate:

- 2-Heptyl-3,5-methyl-5,6-dihydropyrazine

RI: 1511.

MS-EI:

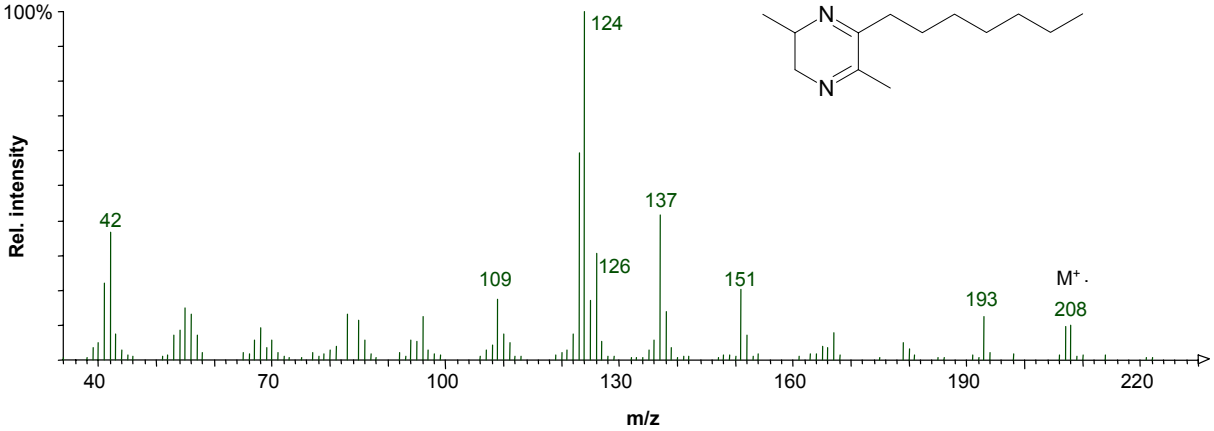


Reference spectrum: unknown.

- 3-Heptyl-2,5-methyl-5,6-dihydropyrazine

RI: 1498.

MS-EI:



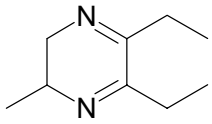
Reference spectrum: unknown.

From acrolein and 2-oxobutyrate:

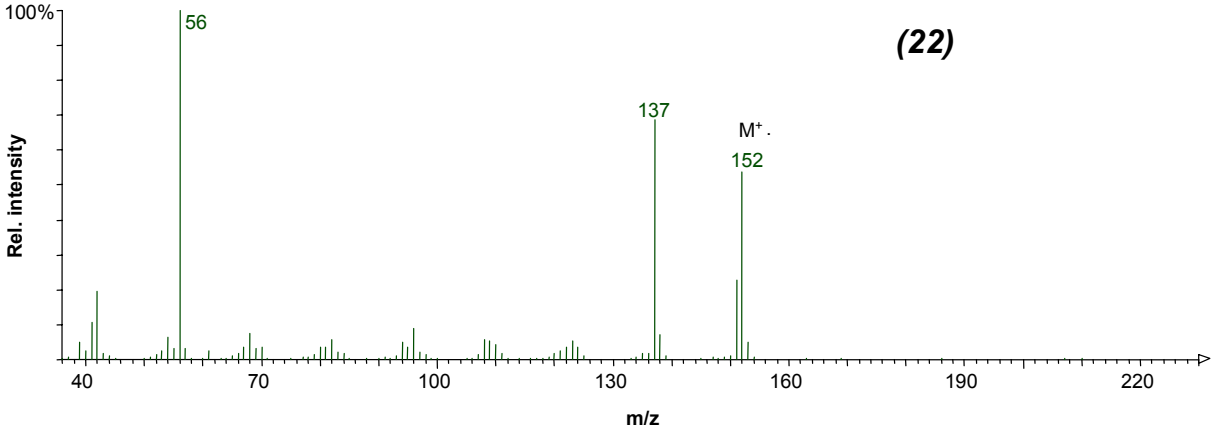
- 2,3-Diethyl-5-methyl-5,6-dihydropyrazine

RI: 1090.

MS-EI:



(22)



Mass spectrum agreed with that described by Tsai *et al*. [28]

2.6.2 Synthesis of tetrahydropyrazines from alkanals

Biotransformation of alkanals and sodium pyruvate using whole cells of baker's yeast was carried out as described in 2.5.1. After centrifugation and extraction of the supernatant with diethyl ether, the organic phase was dried over Na_2SO_4 and concentrated using a Vigreux column at 40 °C to a final volume of 5 mL. 925 mg of 1,2-propanediamine (12.5 mmol) were slowly added and the mixture was further reacted for 90 min. The etheral phase was washed 2 times with water, and 10 mg of trimethylpyrazine (82 μmol) were added as internal standard. The extracts were analyzed by GC (2.2.3) and GC-MS (2.2.5), using a DB-1 capillary column. The specification of the column, the temperature program, and the relative quantification were described in 2.6.1.

For none of the tetrahydropyrazines produced, literature mass spectra were available. Each given MS spectrum is probably the sum of the spectrum of a 1,2,5,6-tetrahydropyrazine and the corresponding 2,3,5,6-tetrahydropyrazine, which could not be separated by gas chromatography, neither on DB-Wax nor on DB-1 columns. For tetrahydropyrazines with a butyl, pentyl, hexyl, and heptyl group in position 2, only one peak was observed.

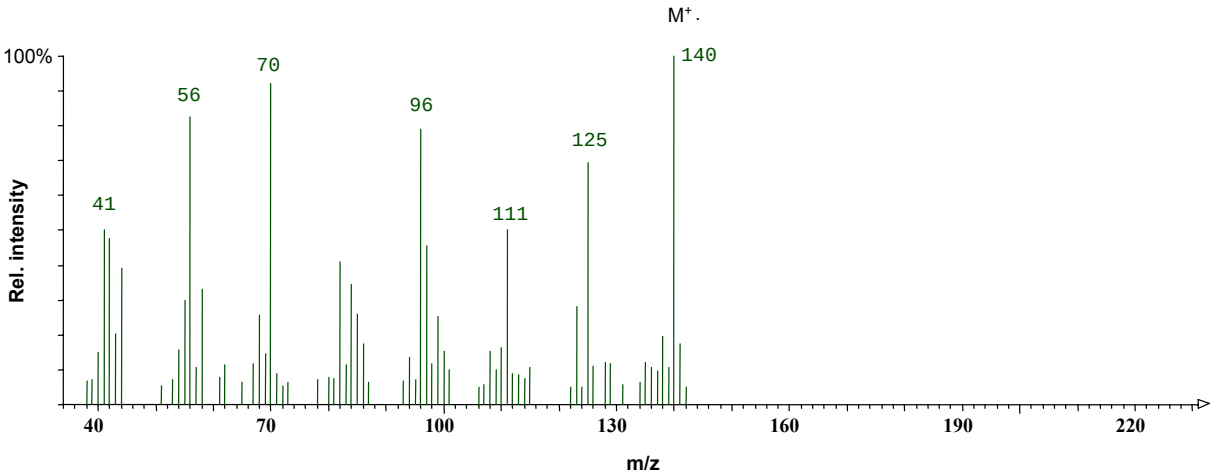
The following compounds were identified:

From propanal:

- 2-Ethyl-3,5-dimethyltetrahydropyrazine

RI: 1174.

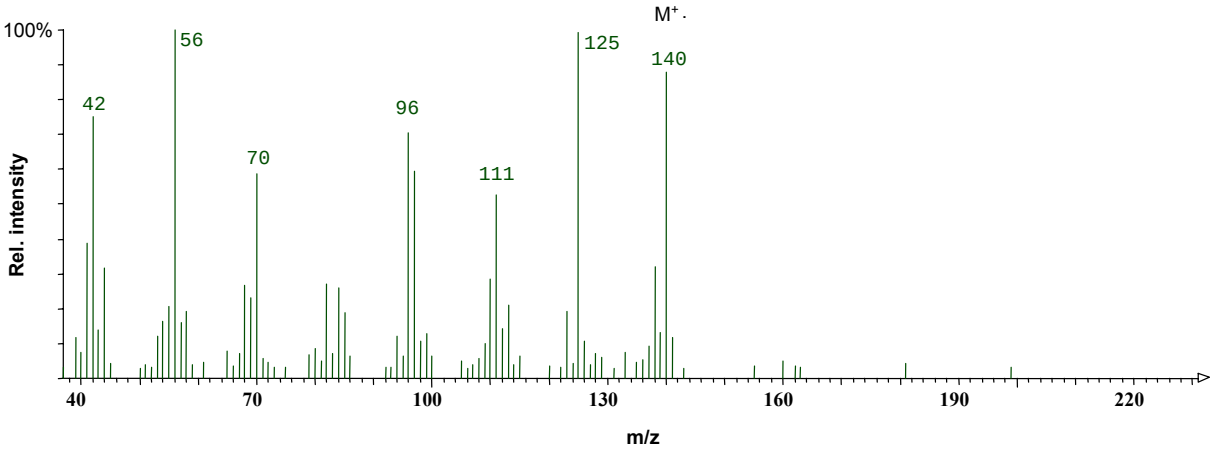
MS-EI:



- 2-Ethyl-3,6-dimethyltetrahydropyrazine

RI: 1159.

MS-EI:

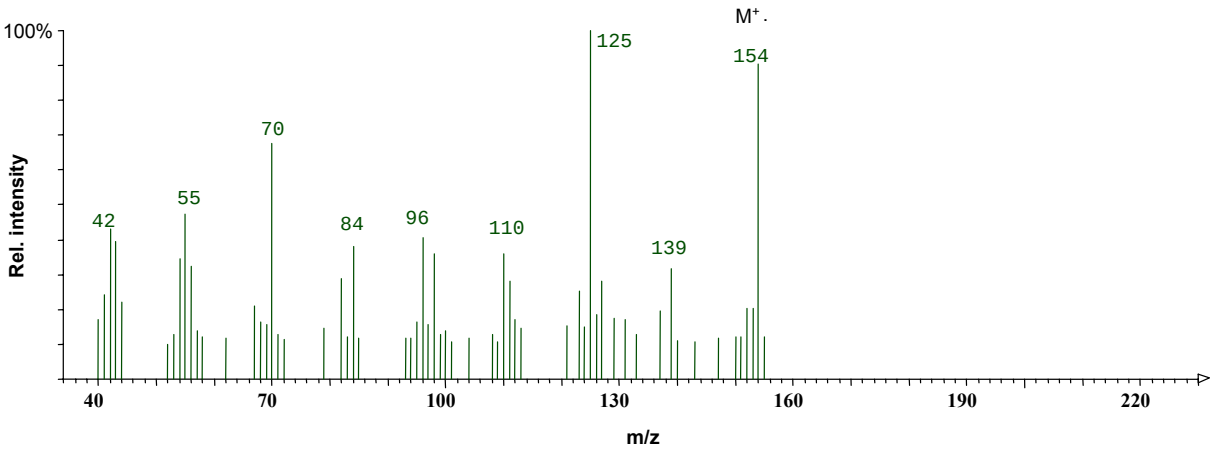


From butanal:

- 2-Propyl-3,5-dimethyltetrahydropyrazine

RI: 1268.

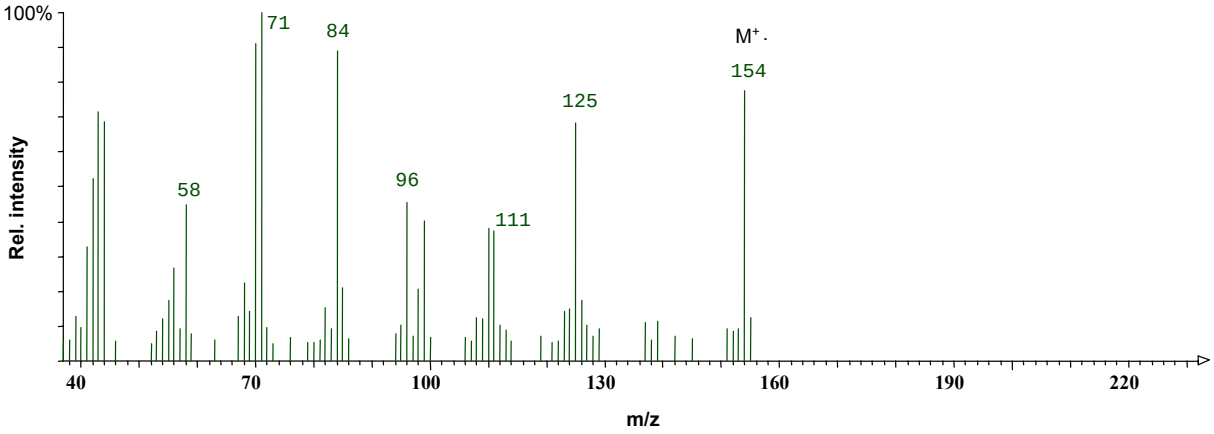
MS-EI:



- 2-Propyl-3,6-dimethyltetrahydropyrazine

RI: 1245.

MS-EI:

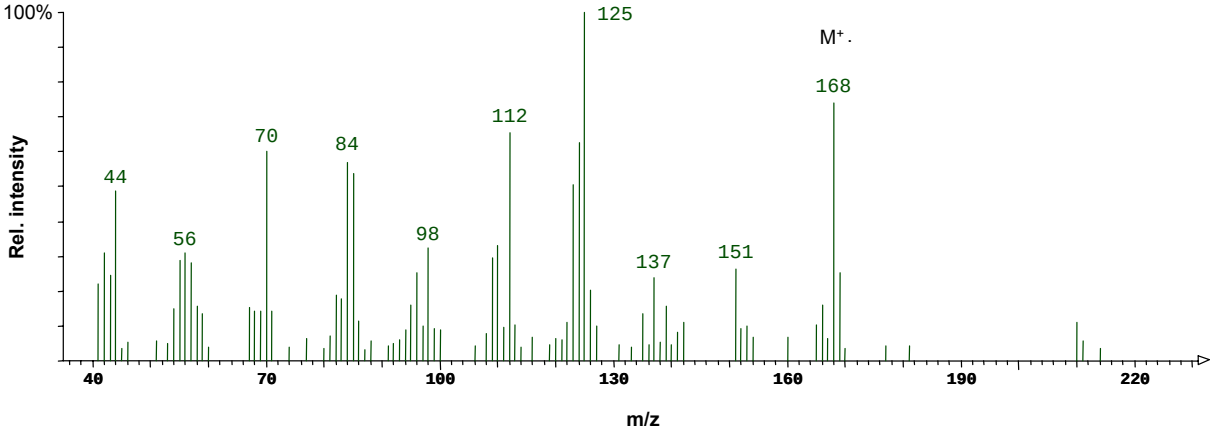


From pentanal:

- 2-Butyldimethyltetrahydropyrazine

RI: 1341.

MS-EI:

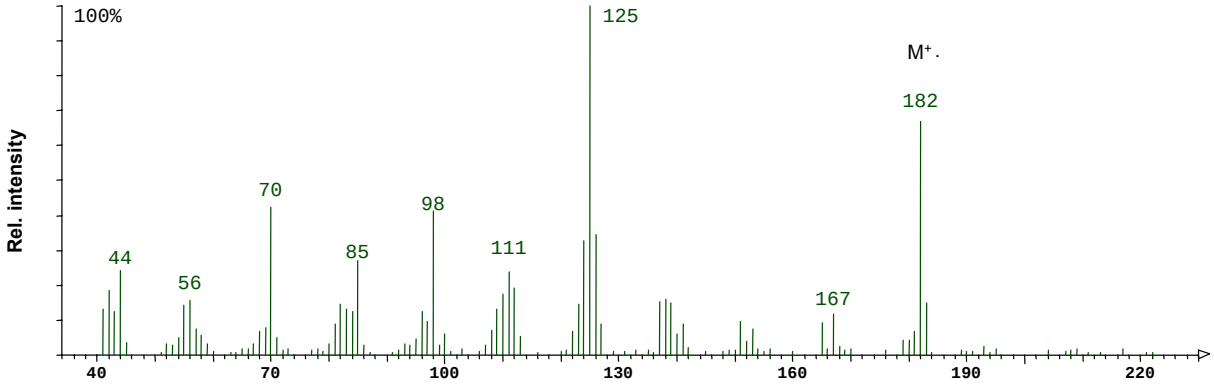


From hexanal:

- 2-Pentyldimethyltetrahydropyrazine

RI: 1431.

MS-EI:

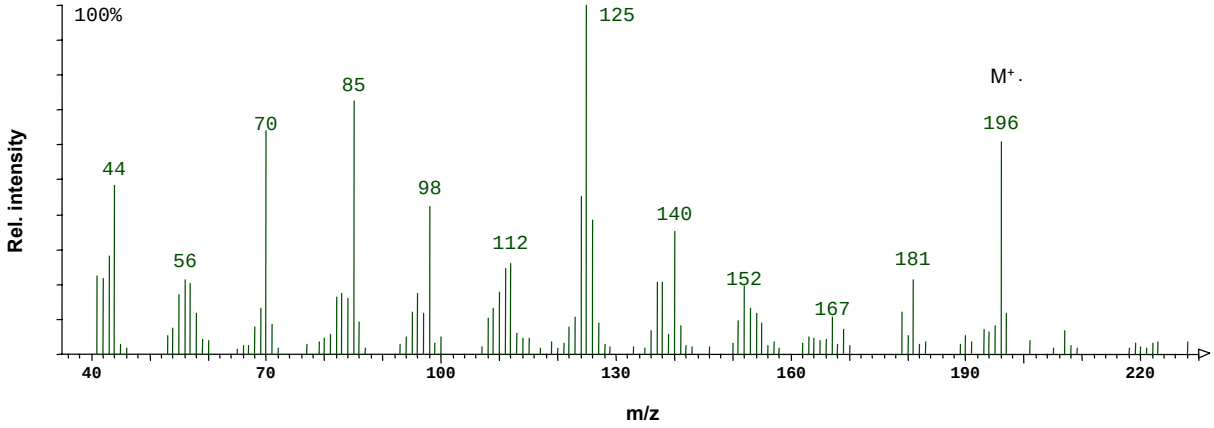


From heptanal:

- 2-Hexyldimethyltetrahydropyrazine

RI: 1525.

MS-EI:

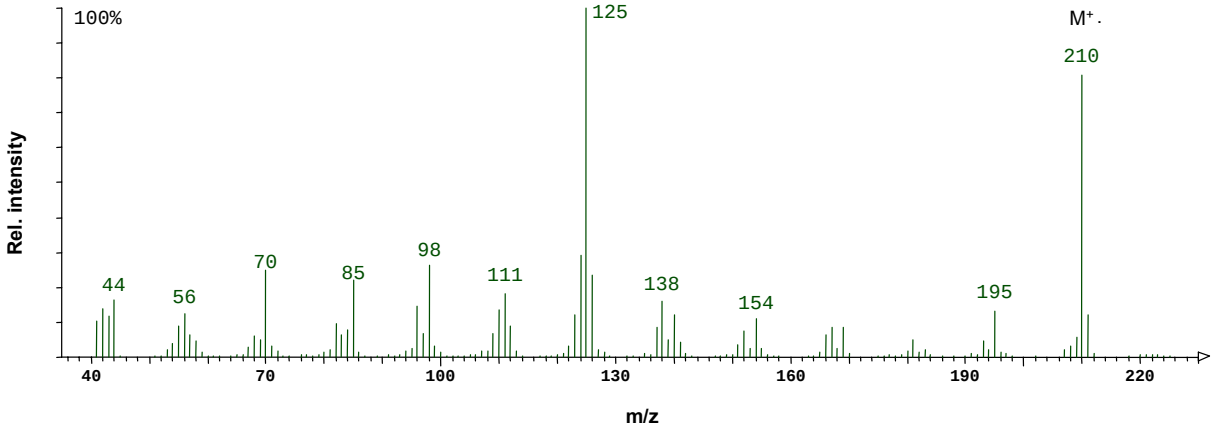


From octanal:

- 2-Heptyldimethyltetrahydropyrazine

RI: 1633.

MS-EI:



2.7 Chemical syntheses

2.7.1 Chemical synthesis of 3-hydroxy-1-penten-4-one and 4-hydroxy-1-penten-3-one

2.7.1.1 Acyloin condensation

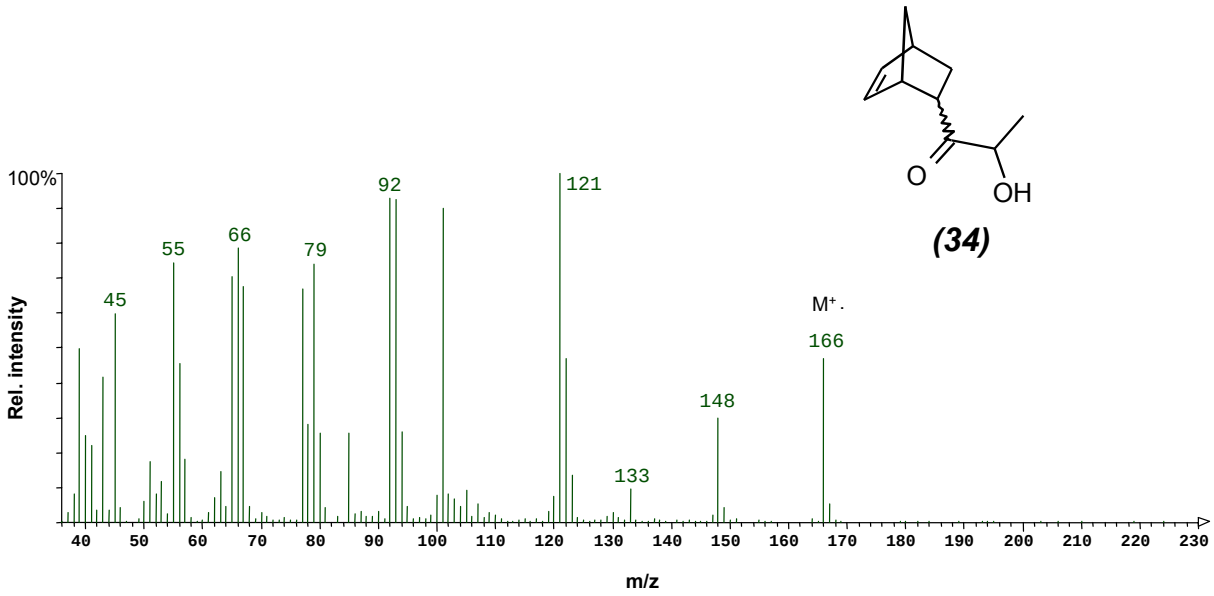
The acyloin condensation of 5-norbornen-2-carboxaldehyde ***(33)*** and acetaldehyde was realized according to the protocol described by Stetter and Dämbkes[29]. The procedure was

slightly modified in order to increase the yields, notably by the addition of excesses of acetaldehyde at several time points.

To 75.3 g of 5-norbornen-2-carboxaldehyde ***(33)*** (0.616 mol) in EtOH (158 mL), were added 104 mL of acetaldehyde (1.85 mol) and 37.8 g 3,4-dimethyl-5-(2-hydroxyethyl)-thiazolium iodide (0.616 mol). Under strong agitation in an argon atmosphere, 49.3 mL of triethylamine (0.355 mol) were added dropwise. The reaction mixture was then shaken and heated to 65 °C. After 4 h and 8 h, respectively, 51.8 mL of acetaldehyde (0.927 mol) were added and heating was continued until a total duration of 24 h. The mixture was cooled to room temperature, poured in ice (250 mL), and extracted with CH_2Cl_2 (3x75 mL). The organic phase was washed with an aqueous solution of 1 M HCl (100 mL) and with a saturated aqueous solution of $NaHCO_3$ (100 mL). The extract was dried over $MgSO_4$, the solvent was evaporated, and the residue was distilled under reduced pressure (76-84 °C / 6 Pa). The crude mixture of 1-[bicyclo[2.2.1]5-hepten-2-yl]-2-hydroxy-1-propanone ***(34)*** and 1-[bicylo[2.2.1]5-hepten-2-yl]-1-hydroxy-2-propanone ***(35)*** was directly used in further reactions without purification. The products were analysed by GC (see 2.2.3) and GC-MS (see 2.2.5) using a DB-WAX capillary column. The temperature program was 5 min isothermal at 40 °C, then raised to 220 °C at 5 °C min^{-1}, and kept at 220 °C for 5 min.

- 1-[Bicyclo[2.2.1]5-hepten-2-yl]-2-hydroxy-1-propanone ***(34)***

MS-EI:

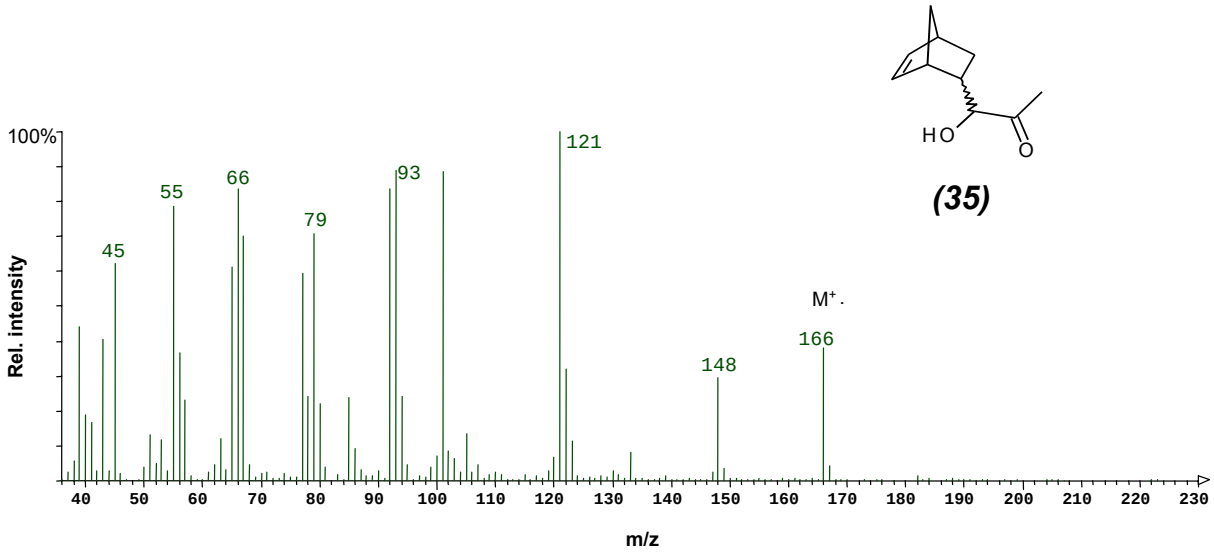


(34)

Reference spectrum: unknown.

- 1-[Bicylo[2.2.1]5-hepten-2-yl]-1-hydroxy-2-propanone ***(35)***

MS-EI:



Reference spectrum: unknown.

2.7.1.2 Pyrolysis

Retro-Diels Alder reaction of 5.4 g of the mixture of the protected acyloins 1-[bicyclo[2.2.1]5-hepten-2-yl]-2-hydroxy-1-propanone ***(34)*** and 1-[bicylo[2.2.1]-1-hydroxy-2-propanone ***(35)*** (32.5 mmol) was performed by gas phase pyrolysis at 600 °C and 1 – 2 Pa, according to Kramme *et al*. [22] The compounds were vaporized by heat treatment to 80 °C. The vapors penetrated in a quartz tube that had been heated to 600 °C. After pyrolysis, the products were collected in traps which were connected to the quartz tube. The first trap, cooled at –78 °C, contained the products, while in the second trap, which was cooled with liquid nitrogen, cyclopentadien condensed. One isolated 2.14 g of a mixture of 3-hydroxy-1-penten-4-one ***(3)*** and 4-hydroxy-1-penten-3-one ***(4)*** (66 %).

^{1}H-NMR data of these compounds agreed with those obtained in 2.3.2.2.

2.7.2 Chemical synthesis of 1-penten-3,4-dione

2.7.2.1 Oxidation with Bi_2O_3

The synthesis of the protected diketone 1-[bicyclo[2.2.1]5-hepten-2-yl]-1,2-propanedione ***(36)*** was realized according to the protocol described by Stetter and Dämbkes[29] The 50 g of the crude mixture of the protected acyloins 1-[bicyclo[2.2.1]5-hepten-2-yl]-2-hydroxy-1-propanone ***(34)*** and 1-[bicylo[2.2.1]-1-hydroxy-2-propanone ***(35)*** (0.3 mol), obtained from 75.3 g of 5-norbornen-2-carboxaldehyde ***(33)*** (0.616 mol), were dissolved in ethoxyethanol (250 mL), and 75 mL of acetic acid were added. Under agitation, the mixture was heated to

105 °C, and 50 g Bi_2O_3 (0.107 mol) were added. Agitation and heat treatment were performed for 5 h, until the complete consumption of the acyloins. The reaction mixture was cooled to room temperature, filtered, and washed several times with CH_2Cl_2 (100 mL). The solvents were eliminated under reduced pressure (1.3 kPa) and the protected diketone ***(36)*** was distilled (55-65 °C under 6 Pa). One obtained 40.4 g of a yellow oil (40 %).

- 1-[Bicyclo[2.2.1]5-hepten-2-yl]-1,2-propanedione ***(36)***

^{1}H-NMR ($CDCl_3$) δ 6.2-6.4 (m, *endo*: 5'-H, *exo*: 5'-H, 6'-H), 5.8 (dd, *endo:* 6'-H), 3.5-3.8 (dt, *endo*: 2'-H), 3.30 (s, *endo:* 1'-H), 2.9-3.2 (m, *endo*: 4'-H; *exo*: 1'-H, 2'-H, 4'-H), 2.37 (s, *exo*: CH_3), 2.33 (s, *endo*: CH_3), 1.7-2.1 (m, *endo:* 3'-H_x; *exo:* 3'-H_n), 1.2-1.6 (m; *endo:* 3'-H_n, 7'-H_a, 7'-H_b; *exo:* 3'-H_n, 7'-H_a, 7'-H_b).
NMR data of this compound agreed with those described by Kramme *et al.*[22]

2.7.2.2 Pyrolysis

Retro-Diels Alder reaction of 3.7 g of 1-[bicyclo[2.2.1]5-hepten-2-yl]-1,2-propanedione ***(36)*** (22.5 mmol) was performed by gas phase pyrolysis at 600 °C and 1 – 2 Pa, according to Kramme *et al*.[22] The compound was vaporized by heat treatment to 80 °C. The vapors penetrated in a quartz tube that had been heated to 600 °C. After pyrolysis, the products were collected in traps which were connected to the quartz tube. The first trap, cooled at –78 °C, contained the product, while in the second trap which was cooled with liquid nitrogen, cyclopentadien condensed. One isolated 1.4 g of 1-penten-3,4-dione ***(8)*** (66 %).

- 1-Penten-3,4-dione ***(8)***

^{1}H-NMR ($CDCl_3$) δ 6.0-7.3 (m; 3 H, vinyl-H), 2.47 (s; 3 H, CH_3).
NMR data of this compound agreed with those described by Kramme *et al.*[22]

2.7.3 Chemical synthesis of pyrazine alcohols

To 113 mg of $NaBH_4$ (3 mmol) in methanol, 900 mg of a mixture of 2-acetyl-3,5-dimethylpyrazine ***(25)*** and 3-acetyl-2,5-dimethylpyrazine ***(26)*** (6 mmol) were slowly added with intense cooling from an ice bath. After 1 h of reaction, 30 mL of ice-cold water were added to hydrolyse the excess of $NaBH_4$ within 30 minutes. The mixture was then extracted three times with diethyl ether, dried over Na_2SO_4, and the ether was evaporated under reduced pressure to obtain 711 mg of a pale yellow oil (78 %). The products were analysed by GC (see 2.2.3) and GC-MS (see 2.2.5) using a DB-WAX capillary column. The temperature program was 5 min isothermal at 40 °C, then raised to 220 °C at 5 °C min^{-1}, and

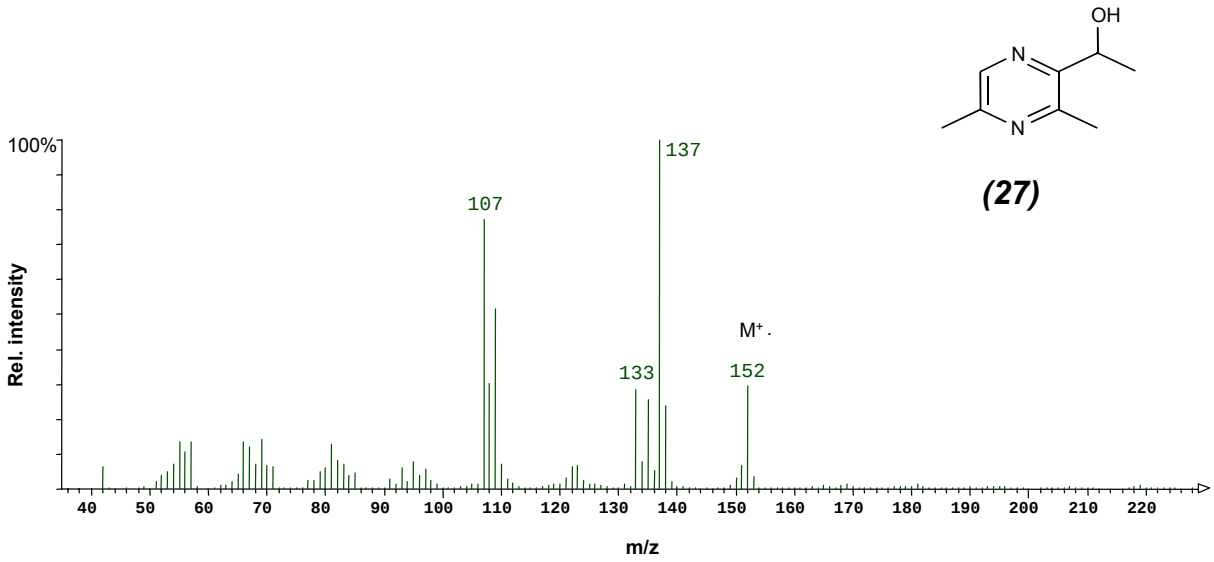
kept at 220 °C for 5 min. ^{1}H-NMR signals of 2-(1-hydroxyethyl)-3,5-dimethylpyrazine ***(27)*** and 3-(1-hydroxyethyl)-2,5-dimethylpyrazine ***(28)*** could be differentiated, due to the ratio of the pyrazine alcohol isomers (30:70), which was reflected in the ratio of corresponding ^{1}H-NMR signal areas.

- 2-(1-Hydroxyethyl)-3,5-dimethylpyrazine ***(27)***

^{1}H-NMR (CD_2Cl_2) δ 8.20 (m, H-6), 4.94 (m, C*H*OH-CH_3), 4.09 (d, 7.8 Hz, OH), 2.49 (m, C-3-CH_3), 2.45 (m, C-5-CH_3), 1.38 (d, 6.4 Hz, CHOH-C*H*$_3$).

RI: 1840.

MS-EI:



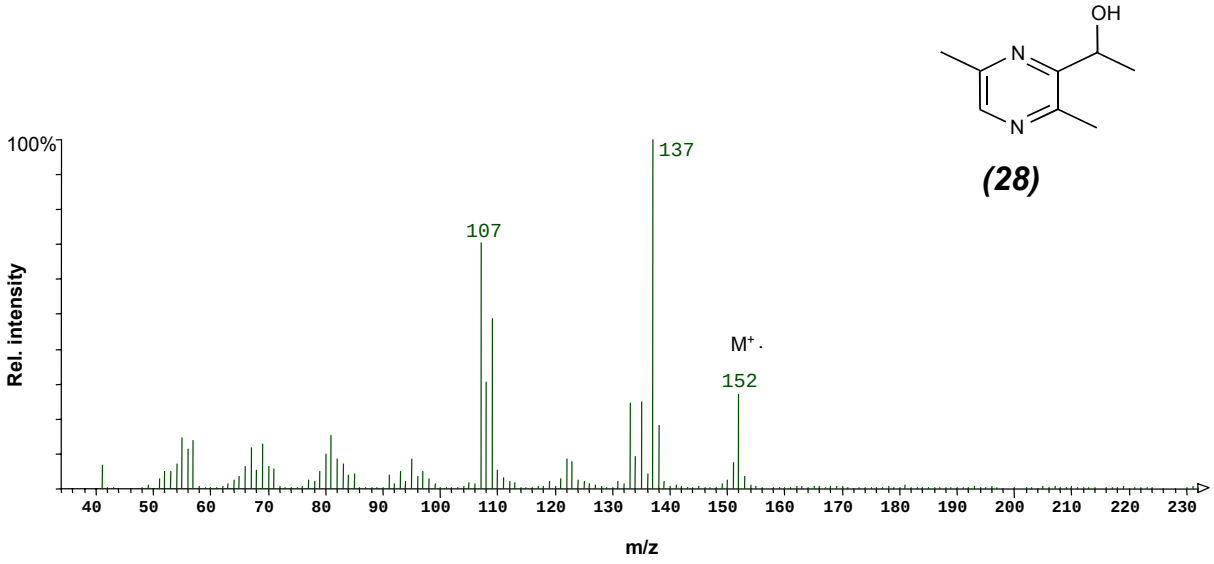
Reference spectra: unknown.

- 3-(1-Hydroxyethyl)-2,5-dimethylpyrazine ***(28)***

^{1}H-NMR (CD_2Cl_2) δ 8.24 (m, H-6), 4.95 (m, C*H*OH-CH_3), 4.33 (d, 7.7 Hz, OH), 2.50 (m, C-2-C*H*$_3$), 2.47 (m, C-5-C*H*$_3$), 1.38 (d, 6.4 Hz, CHOH-C*H*$_3$).

RI: 1811.

MS-EI:



Reference spectra: unknown.

2.7.4 Dehydration of pyrazine alcohols

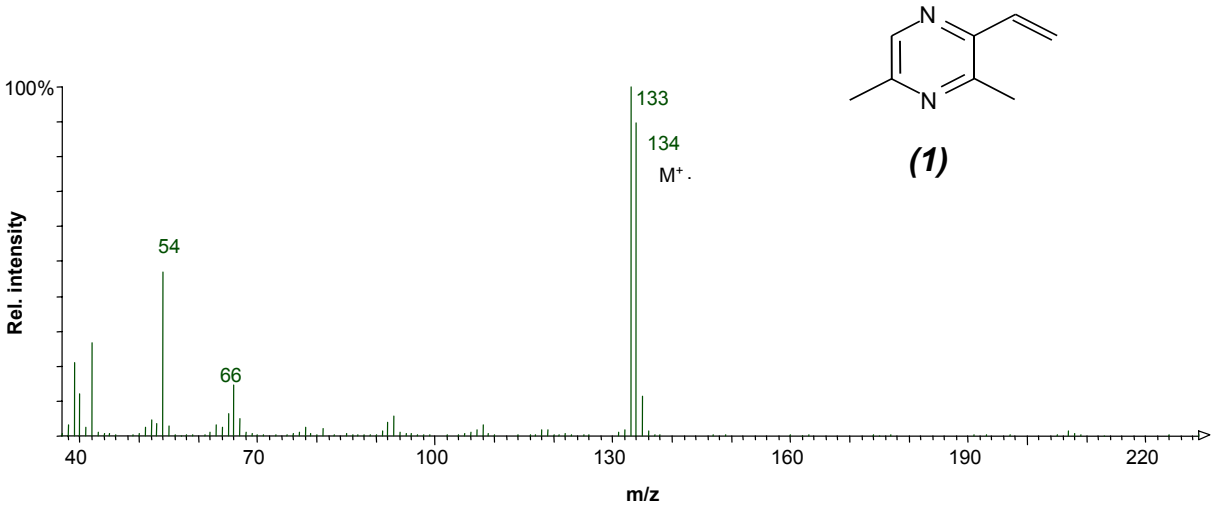
To 15.2 mg of 2-(1-hydroxyethyl)-3,5-dimethylpyrazine ***(27)*** and 3-(1-hydroxyethyl)-2,5-dimethylpyrazine ***(28)*** (100 μmol) were added 15.2 mg of anhydrous oxalic acid (169 μmol) and the dry mixture was heated at 220 °C for 20 minutes. After the reaction was cooled down to room temperature, 2 mL of water (containing 136 μg tetramethylpyrazine as internal standard) were added, and the solution was extracted twice with distilled diethyl ether and dried over Na_2SO_4. The volatile compounds were analysed by GC (see 2.2.3) and GC-MS (see 2.2.5) using a DB-WAX capillary column. The temperature program was 5 min isothermal at 40 °C, then raised to 220 °C at 5 °C min^{-1}, and kept at 220 °C for 5 min.

The following compounds were identified:

- 2-Ethenyl-3,5-dimethylpyrazine ***(1)***

RI: 1531.

MS-EI:



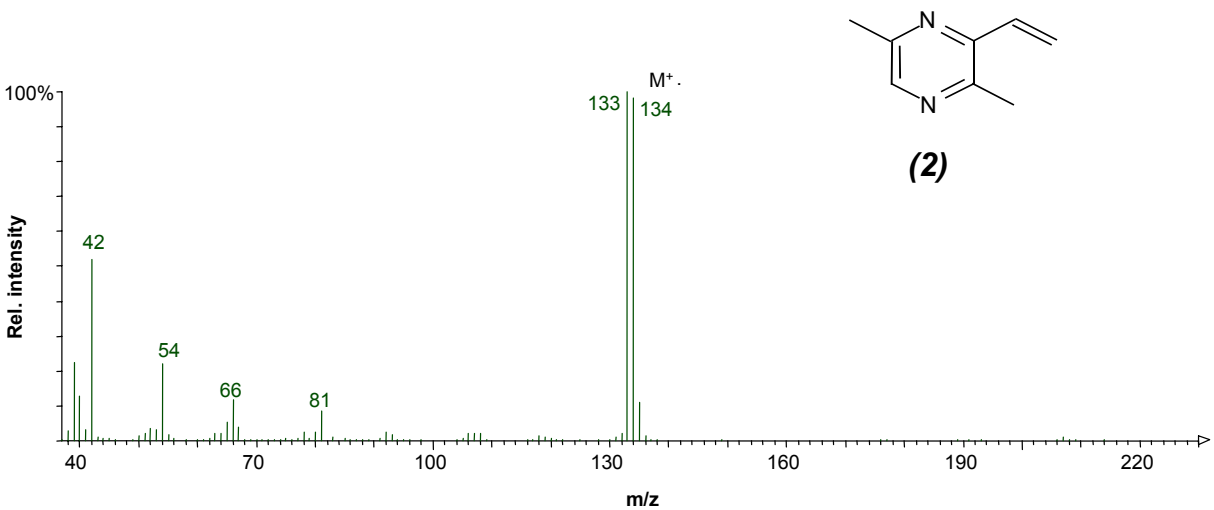
(1)

Mass spectrum agreed with that described by Czerny *et al*.[17]

- 3-Ethenyl-2,5-dimethylpyrazine ***(2)***

RI: 1513.

MS-EI:



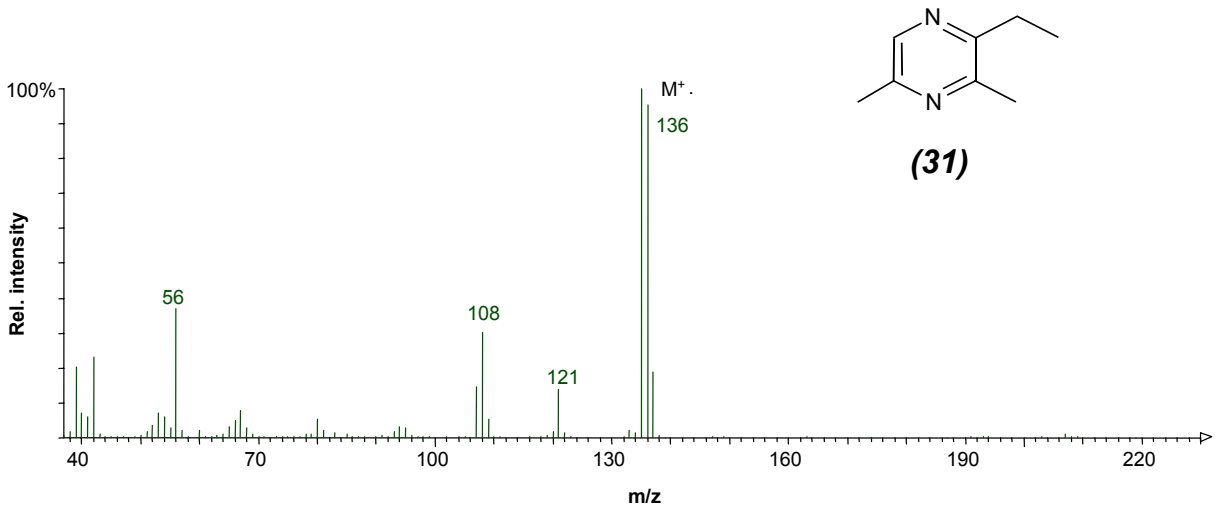
(2)

Mass spectrum agreed with that described by Buttery *et al*.[30]

- 2-Ethyl-3,5-dimethylpyrazine ***(31)***

RI: 1439.

MS-EI:



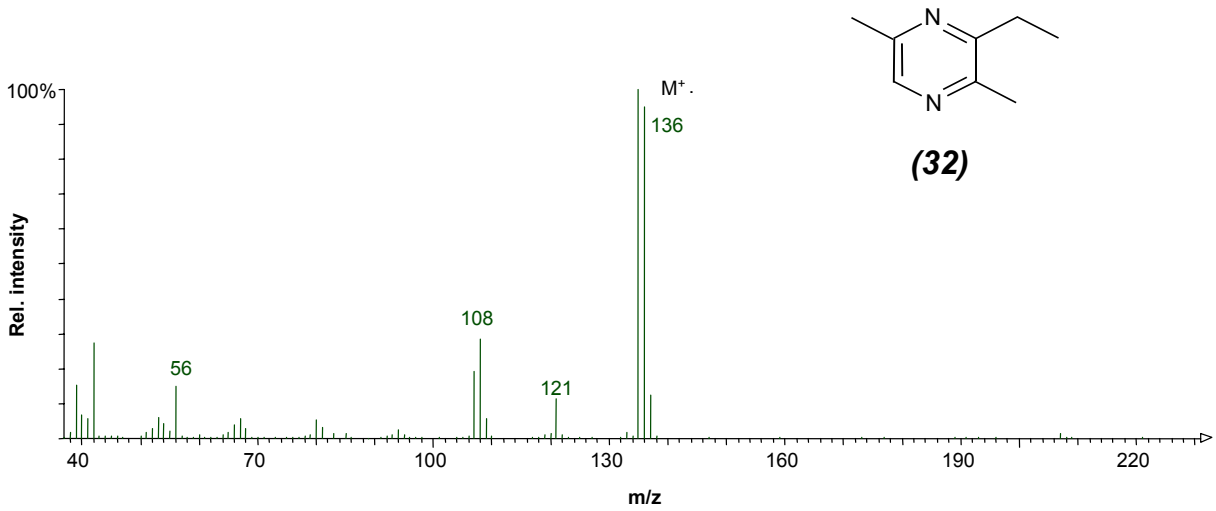
(31)

RI and mass spectrum agreed with those of the injected commercial compound.

- 3-Ethyl-2,5-dimethylpyrazine ***(32)***

RI: 1424.

MS-EI:



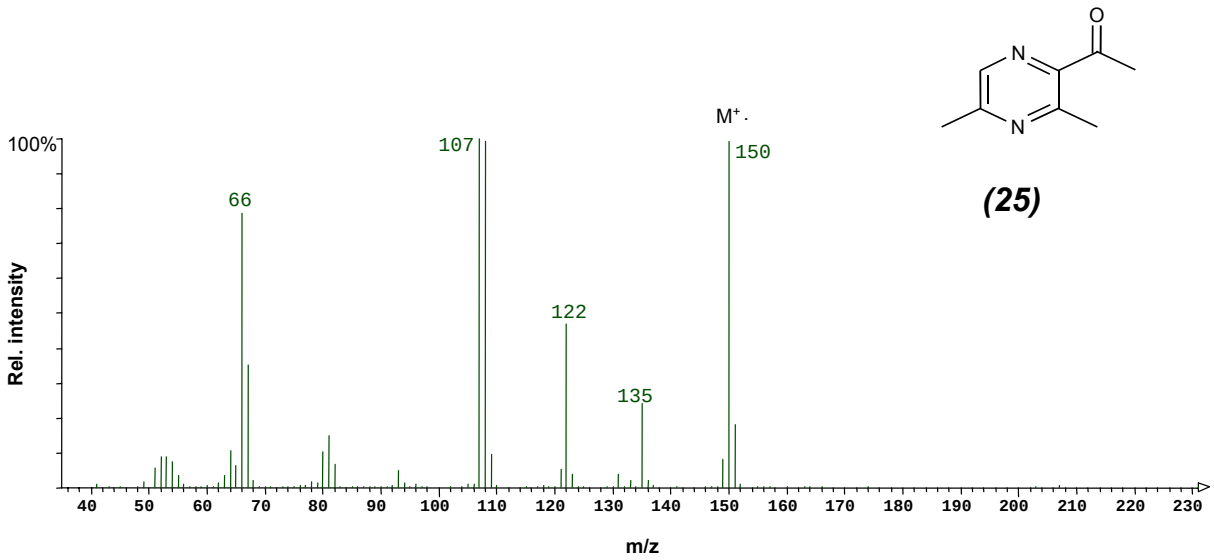
(32)

RI and mass spectrum agreed with those of the injected commercial compound.

- 2-Acetyl-3,5-dimethylpyrazine ***(25)***

RI: 1680.

MS-EI:



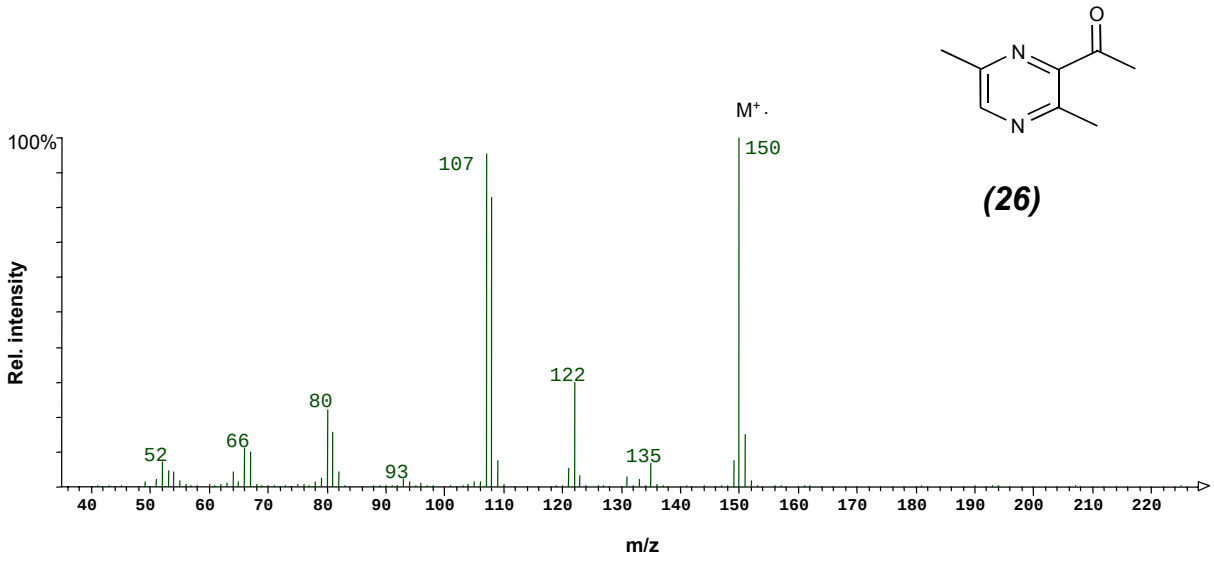
(25)

RI and mass spectrum agreed with those of the injected commercial compound.

- 3-Acetyl-2,5-dimethylpyrazine ***(26)***

RI: 1670.

MS-EI:



(26)

RI and mass spectrum agreed with those of the injected commercial compound.

2.7.5 Chemical synthesis of 5,6-dihydropyrazines

To 81.4 mg of 1,2-propanediamine (1.1 mmol) in 5 ml ether, 1 mmol of one of several diketones was slowly added. After 1 h of reaction at room temperature, 68 mg of tetramethylpyrazine (0.5 mmol) were added as internal standard, the ether phase was

washed twice with one volume water and dried over Na_2SO_4. The concentrated organic phases were analysed by GC (2.2.3) and GC-MS (2.2.5), using a DB-1 capillary column. The temperature program was 5 min isothermal at 40 °C, then raised to 260 °C at 4 °C min^{-1}, and kept at 260 °C for 5 min. Relative quantification was done by comparing the area of the pyrazine derivatives with that of the internal standard, assuming similar FID response factors.

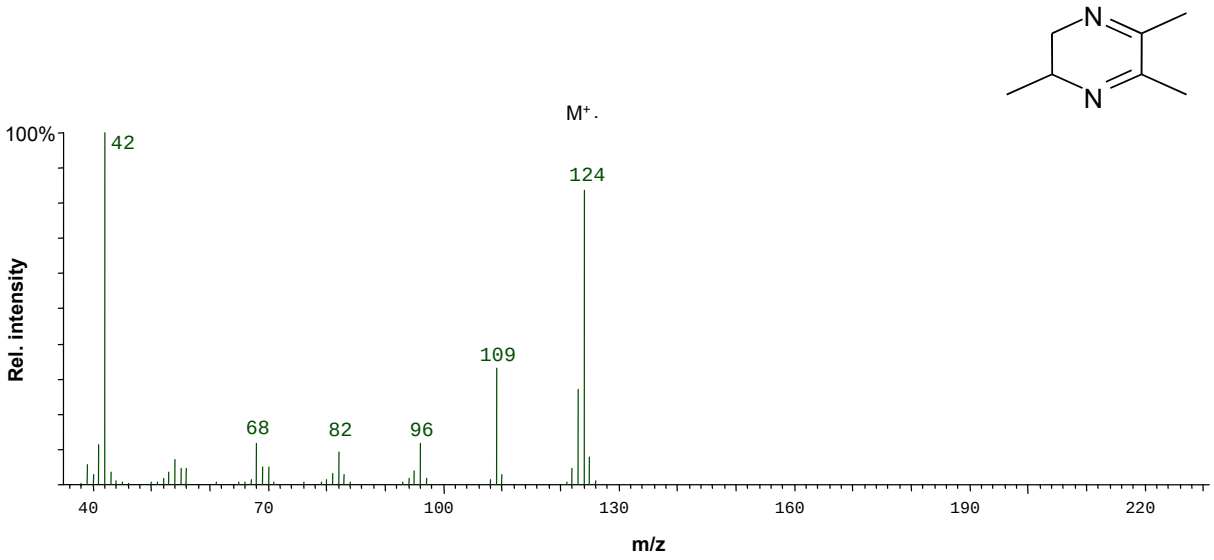
The following 5,6-dihydropyrazines were produced:

From 2,3-butanedione:

- 2,3,5-Trimethyl-5,6-dihydropyrazine

RI: 940.

MS-EI:



Mass spectrum agreed with that described by HAG AG.[27]

From 2,3-pentanedione:

- *2-Ethyl-3,5-dimethyl-5,6-dihydropyrazine* ***(20)***

Mass spectrum and RI agreed with those described in 2.6.1.

- *3-Ethyl-2,5-dimethyl-5,6-dihydropyrazine* ***(21)***

Mass spectrum and RI agreed with those described in 2.6.1.

From 2,3-hexanedione:

- *2-Propyl-3,5-dimethyl-5,6-dihydropyrazine* ***(23)***

Mass spectrum and RI agreed with those described in 2.6.1.

- *3-Propyl-2,5-dimethyl-5,6-dihydropyrazine* ***(24)***

Mass spectrum and RI agreed with those described in 2.6.1.

From 3,4-hexanedione:

- *2,3-Diethyl-5-methyl-5,6-dihydropyrazine **(22)***

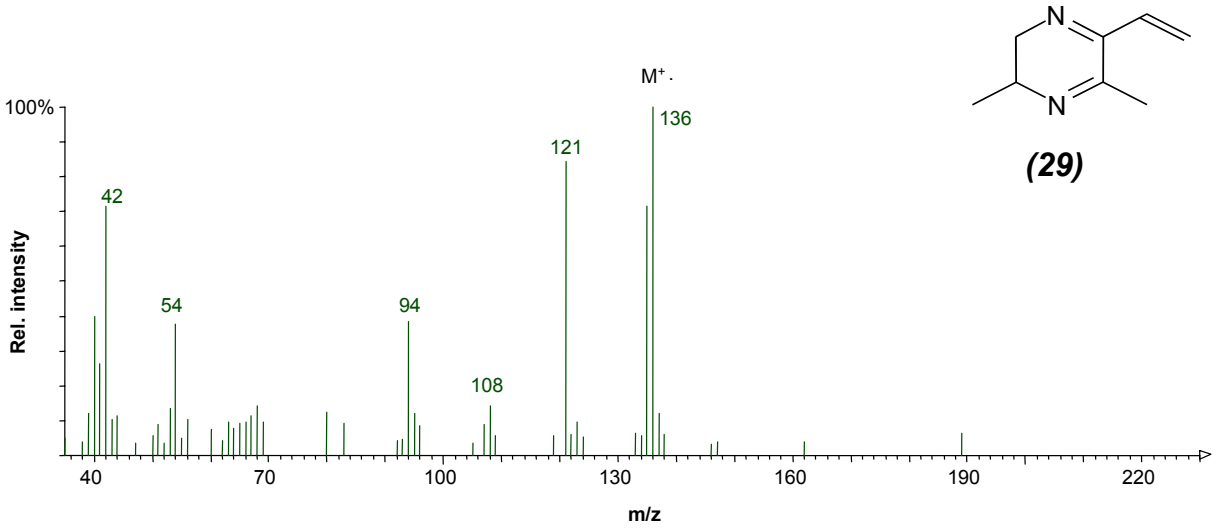
Mass spectrum and RI agreed with those described in 2.6.1.

From 1-penten-3,4-dione:

- *2-Ethenyl-3,5-dimethyl-5,6-dihydropyrazine **(29)***

RI: 1037.

MS-EI:



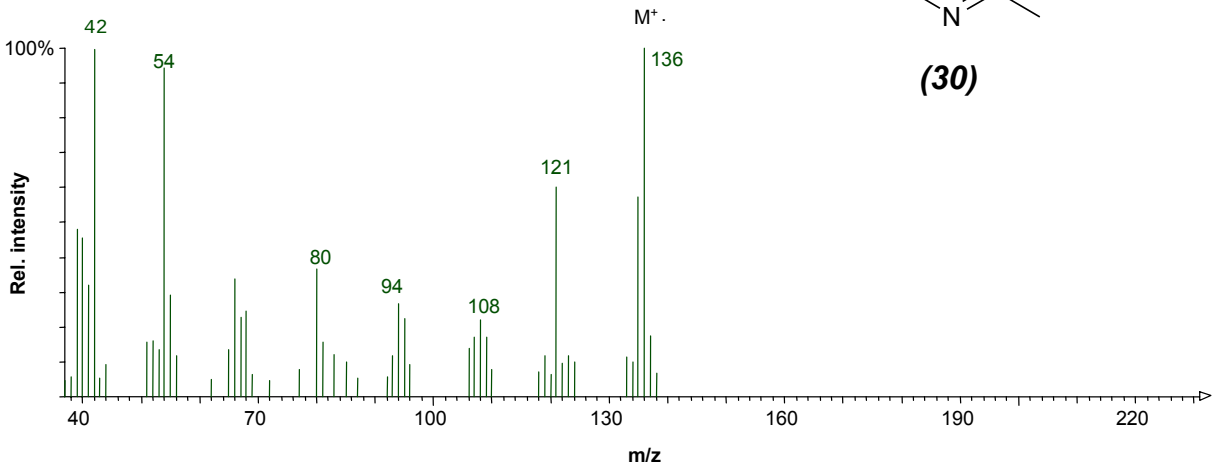
(29)

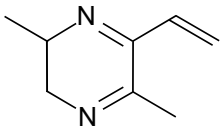
Reference spectrum: unknown.

- *3-Ethenyl-2,5-dimethyl-5,6-dihydropyrazine **(30)***

RI: 1034.

MS-EI:





(30)

Reference spectrum: unknown.

2.7.6 Chemical synthesis of tetrahydropyrazines

To 1 mmol of 3-hydroxy-2-butanone ***(7)*** or 4-hydroxy-3-hexanone ***(12)*** in 5 ml ether, 81.4 mg of 1,2-propanediamine (1.1 mmol) were added and stirred at room temperature. After 8 h of incubation, 68 mg of tetramethylpyrazine (0.5 mmol) were added as internal standard. The products were analysed by GC (2.2.3) and GC-MS (2.2.5), using a DB-1 capillary column. The MS spectra of the tetrahydropyrazines, mentioned below, is the sum of the spectrum of an 1,2,5,6-tetrahydropyrazine and a 2,3,5,6-tetrahydropyrazine derivative, which could not be separated neither on DB-Wax nor on DB-1 columns. The temperature program was 5 min isothermal at 40 °C, then raised to 260 °C at 4 °C min^{-1}, and kept at 260 °C for 5 min. Relative quantification was done by comparing the area of the pyrazine derivatives with that of the internal standard, assuming similar FID response factors.

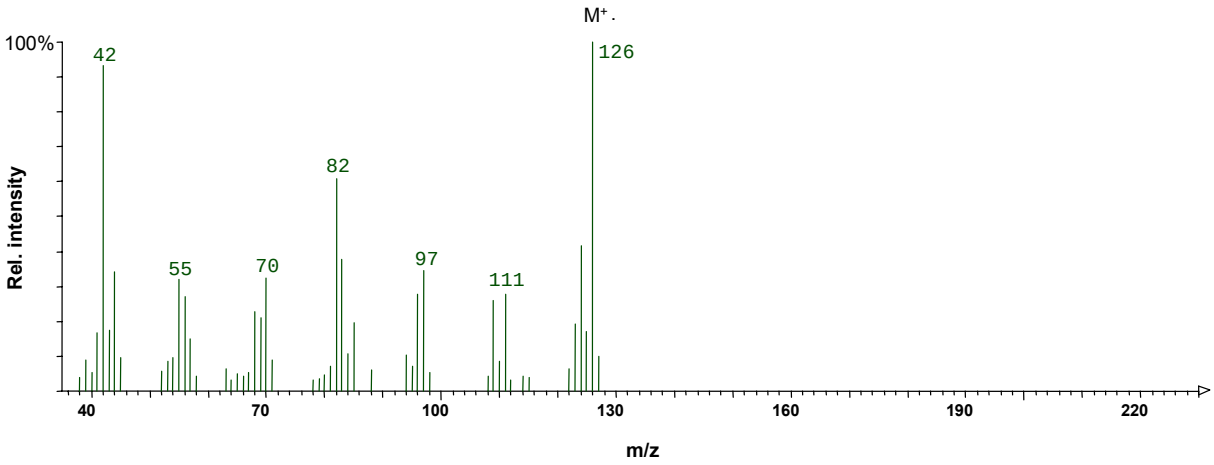
The following compounds were identified:

From Acetoin:

- 2,3,5-Trimethyltetrahydropyrazine

RI: 1070.

MS-EI:



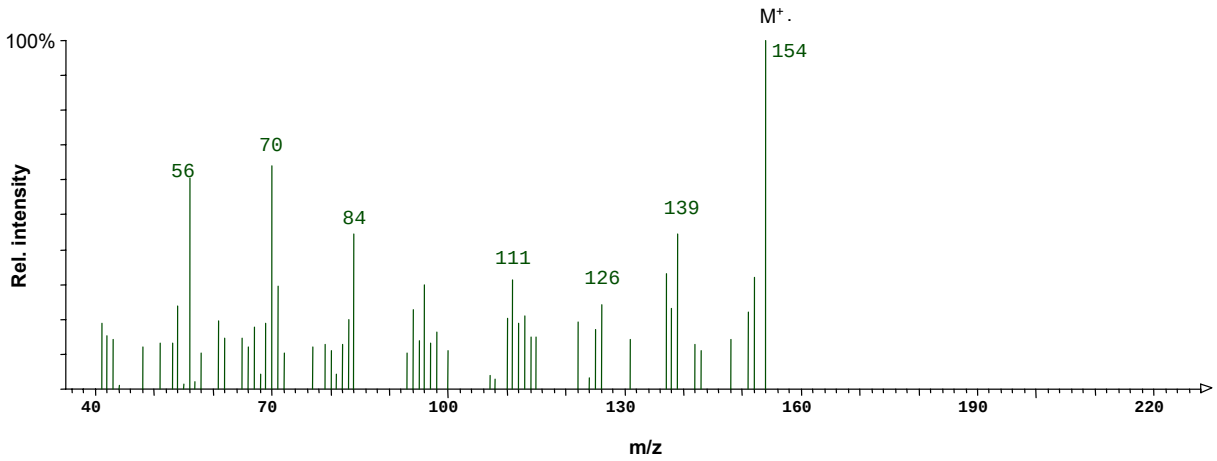
Reference spectrum: unknown.

From 4-hydroxy-3-hexanone:

- 2,3-Diethyl-5-methyl-tetrahydropyrazine

RI: 1255.

MS-EI:



Reference spectrum: unknown.

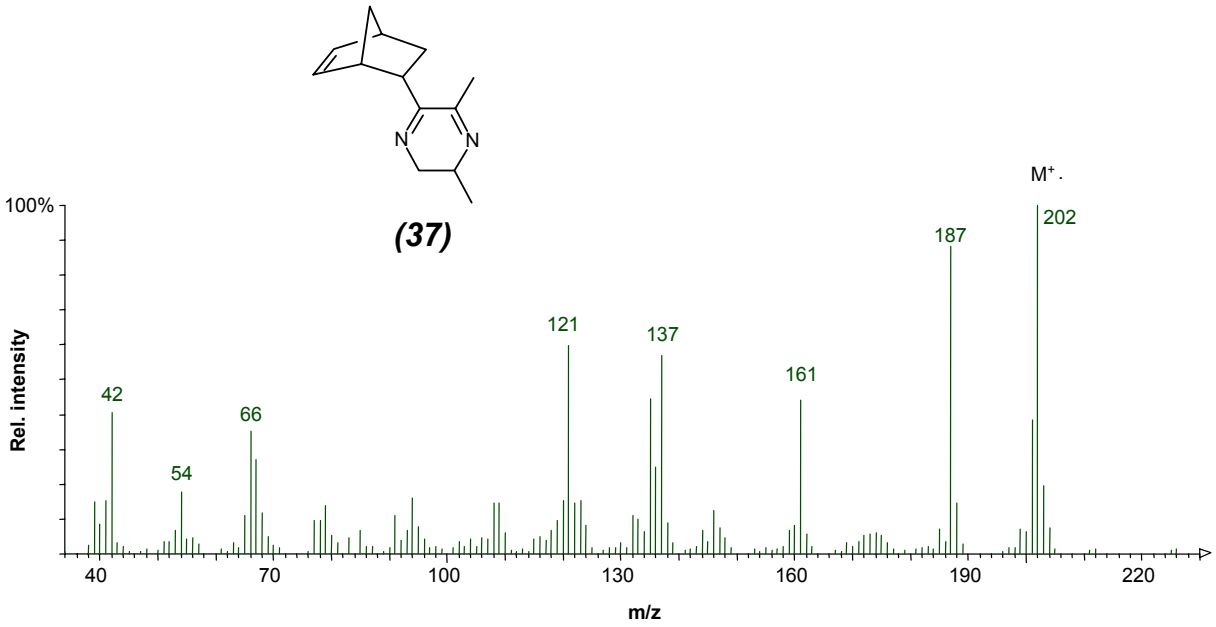
2.7.7 Total synthesis of 2-ethenyl-3,5-dimethylpyrazine and 3-ethenyl-2,5-dimethylpyrazine

2.7.7.1 Ring closure using 1,2-propanediamine

To 5.0 g of 1-[bicyclo[2.2.1]5-hepten-2-yl]-1,2-propanedione ***(36)*** (30.5 mmol) in dried diethyl ether (100 mL), were added 3 g of 1,2-propanediamine (40.5 mmol). The reaction was performed at ambient temperature for 16 h until the complete consumption of the substrates. After dilution with diethyl ether (100 mL), the reaction mixture was washed with brine (100 mL), dried over $MgSO_4$, and the solvent was evaporated under reduced pressure. The crude mixture of 2-[bicyclo[2.2.1]5-hepten-2-yl]-3,5-dimethyl-5,6-dihydropyrazine ***(37)*** and 3-[bicyclo[2.2.1]5-hepten-2-yl]-2,5-dimethyl-5,6-dihydropyrazine ***(38)*** was directly used in further reactions without purification. The products were analysed by GC (see 2.2.3) and GC-MS (see 2.2.5) using a DB-1 capillary column. The temperature program was 5 min isothermal at 40 °C, then raised to 260 °C at 4 °C min^{-1}, and kept at 260 °C for 5 min.

- 2-[Bicyclo[2.2.1]5-hepten-2-yl]-3,5-dimethyl-5,6-dihydropyrazine ***(37)***

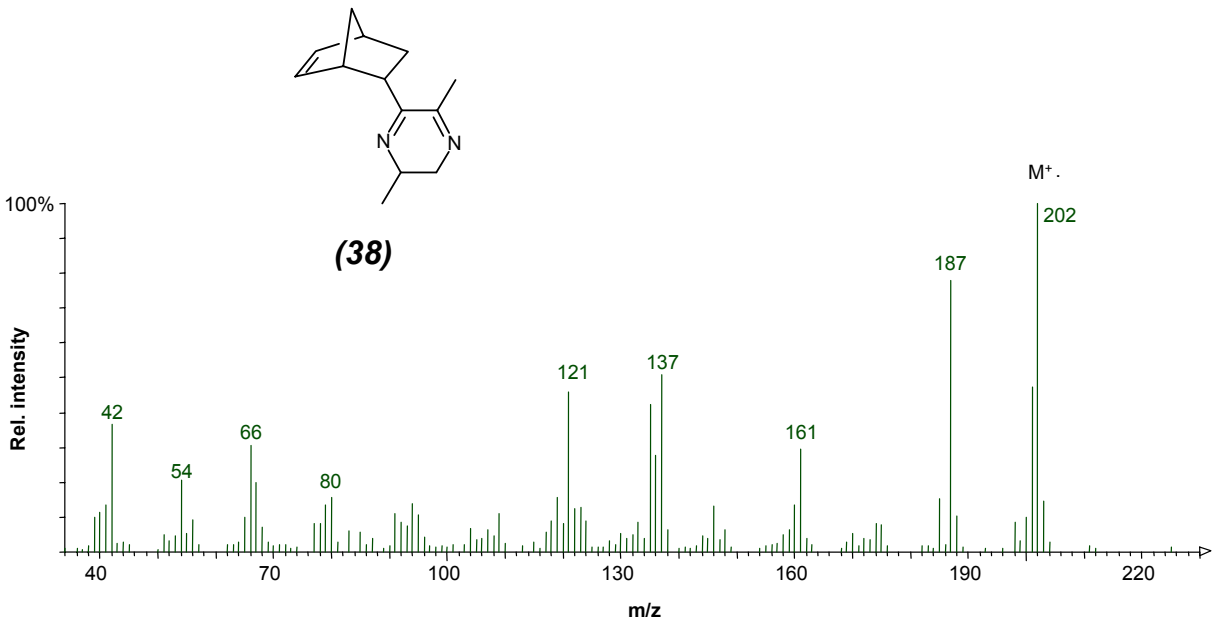
MS-EI:



Reference spectrum: unknown.

- 3-[Bicyclo[2.2.1]5-hepten-2-yl]-2,5-dimethyl-5,6-dihydropyrazine ***(38)***

MS-EI:



Reference spectrum: unknown.

2.7.7.2 Aromatization using MnO_2

The crude mixture of 2-[bicyclo[2.2.1]5-hepten-2-yl]-3,5-dimethyl-5,6-dihydropyrazine ***(37)*** and 3-[bicyclo[2.2.1]5-hepten-2-yl]-2,5-dimethyl-5,6-dihydropyrazine ***(38)***, obtained from 5 g

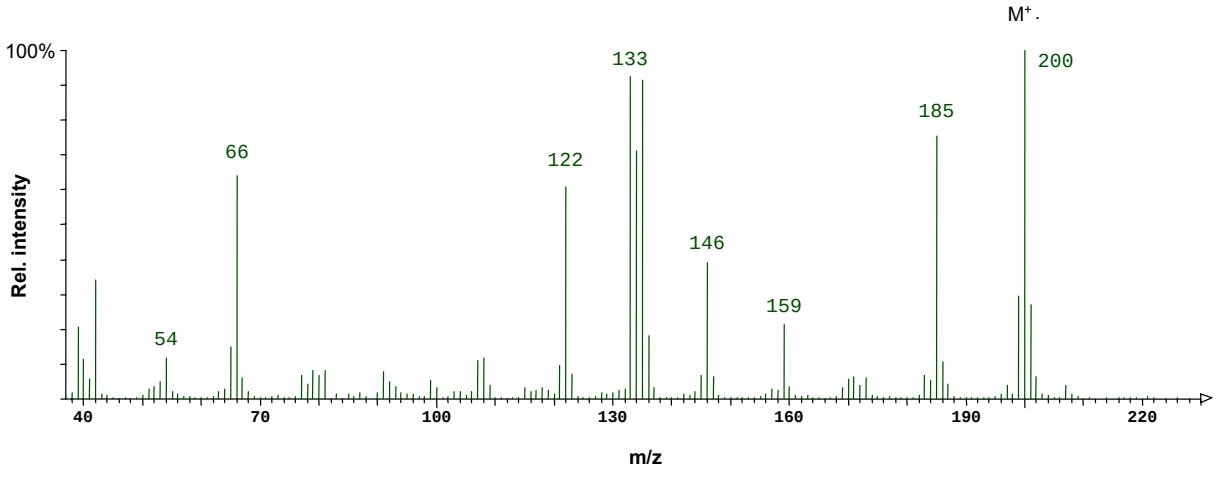
of 1-[bicyclo[2.2.1]5-hepten-2-yl]-1,2-propanedione ***(36)*** (30.5 mmol), was dissolved in 100 mL EtOH containing KOH (1.9 g, 33.5 mmol), and 8.0 g of MnO_2 (92 mmol) were added. The mixture was refluxed for 10 h until the complete consumption of the substrates. The reaction mixture was cooled to room temperature and filtered. The solvent was evaporated under reduced pressure, and the pyrazines were purified by column chromatography (eluent: cyclohexane/EtOAc: 9/1). A first fraction (1.75 g, 29 %) corresponded to an equimolar mixture of *exo*-2-[bicyclo[2.2.1]5-hepten-2-yl]-3,5-dimethylpyrazine ***(39)*** and *exo*-3-[bicyclo[2.2.1]5-hepten-2-yl]-2,5-dimethylpyrazine ***(40)***. A second fraction (0.75 g, 12 %) corresponded to the *endo*-pyrazines with a ratio of 80:20 in favour of the 3,5-dimethylpyrazine regioisomer. Both fractions were isolated as pale yellow oils. The products were analysed by GC (see 2.2.3) and GC-MS (see 2.2.5) using a DB-1 capillary column. The temperature program was 5 min isothermal at 40 °C, then raised to 260 °C at 4 °C min^{-1}, and kept at 260 °C for 5 min.

- *Exo*-2-[bicyclo[2.2.1]5-hepten-2-yl]-3,5-dimethylpyrazine ***(39)*** and *exo*-2-[bicyclo[2.2.1]5-hepten-2-yl]-3,6-dimethylpyrazine ***(40)***

^{1}H NMR ($CDCl_3$) δ 8.20 (s, 1H), 8.10 (s, 1H), 6.25 (m, 2H), 3.00 (m, 1H), 2.96 (m, 1H), 2.92 (m, 1H), 2.85 (m, 1H), 2.55 (s, 3H), (s, 3H), 2.49 (m, 3H), 2.47 (m, 3H), 2.15 (m, 1H), 2.05 (m, 1H), 1.79 (m, 1H), 1.53 (m, 1H), 1.52 (m, 1H), 1.40 (m, 1H).

^{13}C NMR ($CDCl_3$) δ 157.2, 155.2, 151.4, 149.4, 148.9, 148.9, 140.2, 139.6, 138.4, 136.7, 47.3, 47.1, 45.6, 45.5, 42.2, 41.9, 31.5, 31.1, 21.8, 21.3, 21.2, 20.8.

MS-EI:



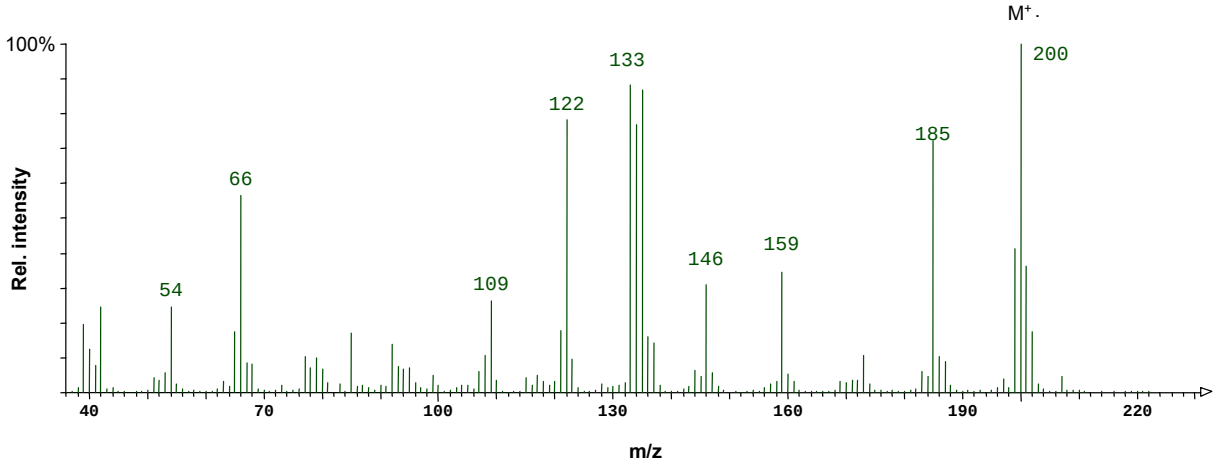
Reference spectra: unknown.

- *Endo*-2-[bicyclo[2.2.1]5-hepten-2-yl]-3,5-dimethylpyrazine ***(39)*** and *endo*-3-[bicyclo[2.2.1]5-hepten-2-yl]-2,5-dimethylpyrazine ***(40)***

^{1}H NMR ($CDCl_3$) δ 8.04 (s, 1H), (s, 1H), 6.14 (m, 1H), 6.05 (m, 1H), 5.69 (m, 1H), 5.62 (m, 1H), 3.50 (m, 1H), 3.18 (m, 1H), 2.89 (m, 1H), 2.53 (s, 3H), 2.49 (s, 3H), 2.37 (s, 3H), 2.34 (s, 3H), 2.00 (m, 1H), 1.63 (m, 1H), 1.44 (m, 2H).

^{13}C NMR ($CDCl_3$) δ 155.4, 153.7, 151.4, 149.0, 139.9, 139.59, 136.7, 136.1, 132.9, 132.4, 50.2, 49.8, 46.5, 46.2, 43.0, 42.9, 42.8, 42.3, 30.7, 30.6, 21.9, 21.4, 21.1, 20.9.

MS-EI:



Reference spectra: unknown.

2.7.7.3 Pyrolysis

Retro-Diels-Alder reaction of 1 g of the mixture of *exo*-2-[bicyclo[2.2.1]5-hepten-2-yl]-3,5-dimethylpyrazine ***(39)*** and *exo*-3-[bicyclo[2.2.1]5-hepten-2-yl]-2,5-dimethylpyrazine ***(40)*** (6.9 mmol) was performed by gas phase pyrolysis at 600 °C and 1–2 Pa, according to Kramme *et al*.[22] The compounds were vaporized by heat treatment to 80 °C. The vapors penetrated in a quartz tube that had been heated to 600 °C. After pyrolysis, the products were collected in traps which were connected to the quartz tube. The first trap, cooled at –78 °C, contained an lightly amber oil with pronounced odour while, in the second trap which was cooled with liquid nitrogen, cyclopentadien condensed. One isolated 570 mg of a mixture of 2-ethenyl-3,5-dimethylpyrazine ***(1)*** and 3-ethenyl-2,5-dimethylpyrazine ***(2)*** (85 %).

- Mixture of 2-ethenyl-3,5-dimethylpyrazine ***(1)*** and 3-ethenyl-2,5-dimethylpyrazine ***(2)***

^{1}H-NMR ($CDCl_3$) δ 8.1-8.2 (s, 2H, H-6), 6.8-6.9 (m, 2H, C*H*=CH_2), 6.2-6.4 (m, 2H, $vinyl_{trans}$), 5.4-5.55 (m, 2H, $vinyl_{cis}$), 2.4-2,5 (s, 12H, CH_3).
NMR data of compound ***(1)*** agreed with that reported in the literature.[17]
NMR data of compound ***(2)***: unknown.

2.7.8 Stability study of ethenylpyrazines in acetate buffer and coffee solution

A coffee solution was prepared by dissolving 0.4 g coffee powder (Nestlé, KS-UK 10638.02, spray-dried coffee UK) in 20 mL of acetate buffer (0.02 M, pH 5.2) and filtering it (Millipore, 0.45 μm). To 3 mL of this coffee solution as well as to 3 mL of acetate buffer in dark brown vials, 2-ethenyl-3,5-dimethylpyrazine ***(1)*** and 3-ethenyl-2,5-dimethylpyrazine ***(2)*** (dissolved in 3 mL acetate buffer) were added to reach a final concentration of 5 mg L^{-1}. After stirring for 20 min at 25 °C, 800 μL of the solution were transferred into vials which were closed. After 1 h, 3 h, 5 h, 7 h, 9 h, and 11 h the headspace was analysed by Solid Phase Microextraction-GC-MS (SPME-GC-MS).
SPME was performed using a PAL autosampler, thermostated at 25 °C. The fiber was coated with polydimethylsiloxane-divinylbenzene (PDMS-DVB) and had a diameter of 65 μm (Supelco 57345-U). The vial penetration was at 22 mm, extraction time 1 min, injection penetration 54 mm, and desorption time 5 min. Directly coupled GC-MS analysis was performed with the conditions described in 2.2.5 using a DB-WAX capillary column. The temperature program was 5 min isothermal at 40 °C, then raised to 220 °C at 5 °C min^{-1}, and kept at 220 °C for 5 min.

2.8 Determination of organoleptic properties

The odour qualities and odour thresholds of the synthesized compounds were determined according to Christoph[31] by GC-O (see 2.2.4) by one person. To begin with, concentrated extracts of the compounds were injected (>2 mg) and sniffed in order to determine the odour quality. The odour thresholds were then estimated by aroma extract dilution analysis (AEDA). The odour threshold of a compound was the minimal amount at which its characteristic odour properties were still unequivocally recognized. The concentration of extracts was estimated by the use of trimethylpyrazine as internal standard, assuming similar FID factors. It was shown that the FID response factors of commercial trimethylpyrazine, 2-ethyl-3,5-dimethylpyrazine ***(31)***, and 2,3-diethyl-5-dimethylpyrazine ***(41)*** showed only small deviations (<10 %).

The odour thresholds were calculated using the following formula:

$M = \frac{c \cdot V}{2}$, with

M=quantity of compound at sniffing port [ng]

c=concentration of extract [ng μL^{-1}]

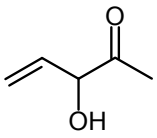
V=injection volume [μL].

3 Results

The chemoenzymatic synthesis of pyrazine derivatives was investigated. The first step consisted of a biotransformation reaction, in order to produce various aliphatic acyloins, followed by chemical reaction with 1,2-propanediamine.

3.1 Biogeneration of acyloins

It was initially aimed to produce 3-hydroxy-1-penten-4-one ***(3)***, because this particular acyloin had never been characterized nor synthesized in literature before (Figure 4).



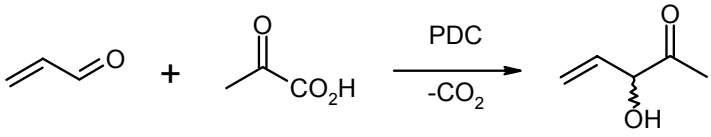
3

Figure 4. Molecular structure of 3-hydroxy-1-penten-4-one.

Furthermore, it was supposed that acyloin ***(3)*** is a potential precursor of interesting pyrazine derivatives.

3.1.1 Biogeneration of 3-hydroxy-1-penten-4-one

3.1.1.1 Biogeneration of 3-hydroxy-1-penten-4-one using pyruvate decarboxylase (E.C. 4.1.1.1)



3

Figure 5. Biogeneration of 3-hydroxy-1-penten-4-one using pyruvate decarboxylase.

As depicted in Figure 5, PDC from baker's yeast was tested for the ability to catalyze the formation of ***(3)*** from acrolein and pyruvate. After 4 h of incubation (for details, see 2.3.1.1), acyloin ***(3)*** was identified as judged by comparison of its RI and GC-MS data with those of a chemically synthesized reference compound (see 2.7.1). Figure 6 shows the MS-EI spectrum with mass fragment, characteristic for the acetyl group.

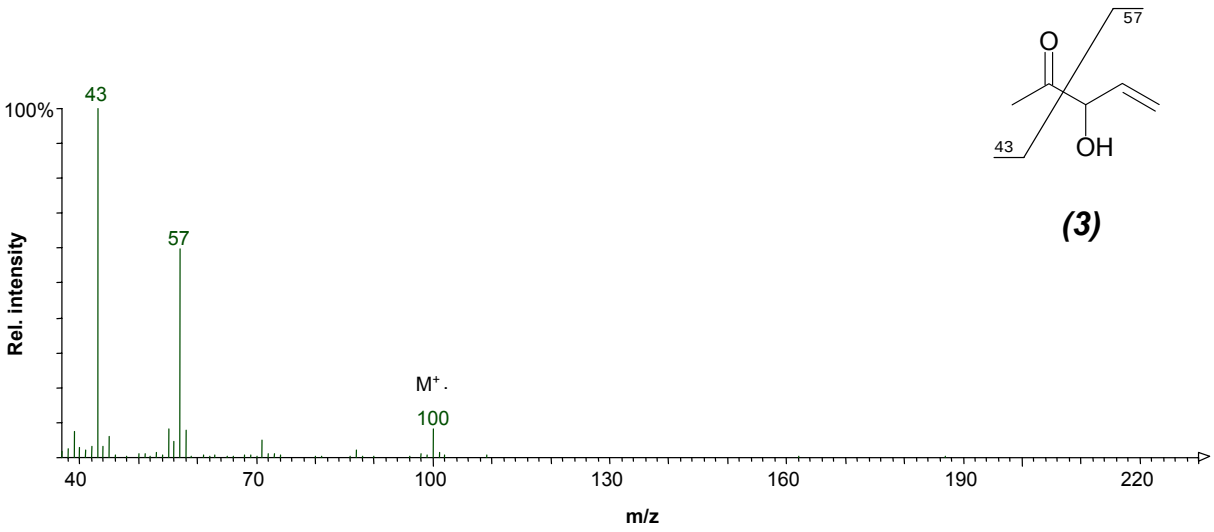


Figure 6. GC-MS/EI spectrum of 3-hydroxy-1-penten-4-one.

The major product in the mixture, however, was acrolein dimer, which was probably formed by Diels-Alder-type reaction of two acrolein molecules (Figure 7).

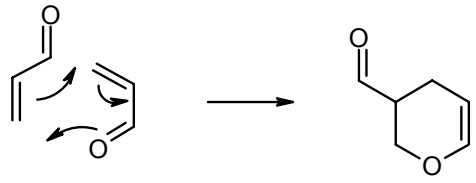


Figure 7. Proposed formation of acrolein dimer from acrolein.

Due to the low yield of acyloin ***(3)*** (<1 %), the biotransformation parameters, such as acrolein concentration, pH, and incubation temperature, needed to be optimized in order to get larger amounts of product.

Influence of acrolein concentration on formation of 3-hydroxy-1-penten-4-one

The influence of initial acrolein concentration on the production of compound ***(3)*** was investigated (see 2.3.1.1). At acrolein concentrations between 0.5 mM and 200 mM, ***(3)*** was produced during the first 2 h. After that, no more acyloin was formed, and a plateau was reached. At acrolein concentrations of 300 mM, no production of ***(3)*** was observed at all. The time courses for six different acrolein concentrations are shown in Figure 8.

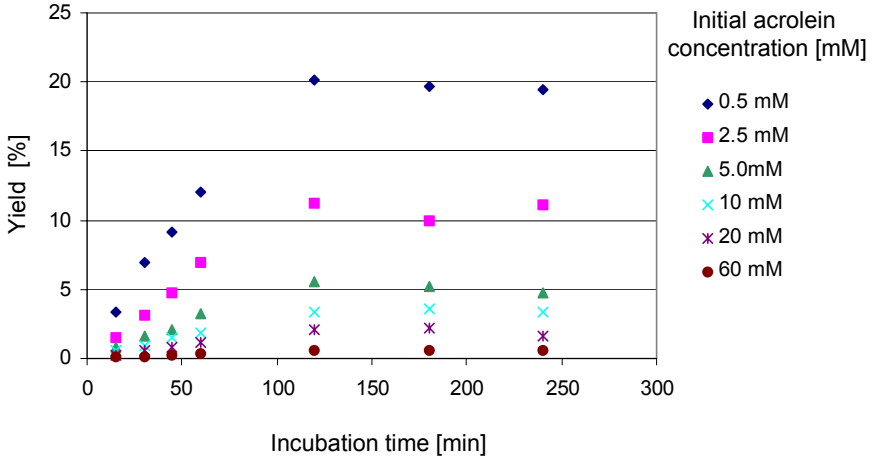


Figure 8. Yield of 3-hydroxy-1-penten-4-one as function of incubation time.

During the reaction, a white precipitate, possibly denatured PDC, was formed in solution. Plotting the yields of acyloin ***(3)*** after 2 h of incubation against initial acrolein concentrations showed that the highest yield of 20 % was obtained at 0.5 mM of acrolein (Figure 9).

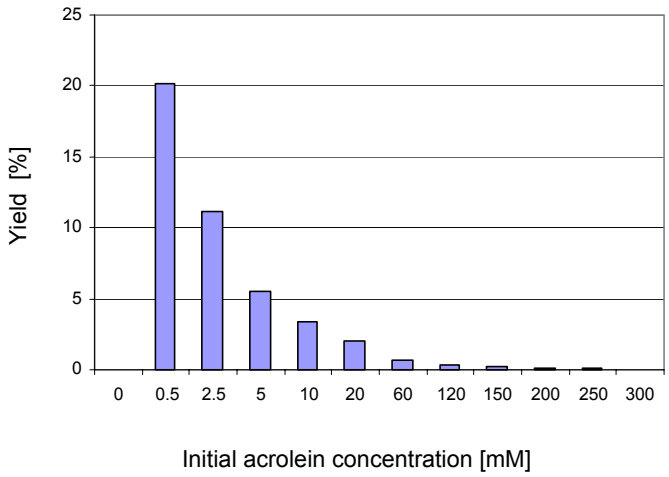


Figure 9. Yield of 3-hydroxy-1-penten-4-one after 2 h of incubation as function of acrolein concentration.

The highest concentration of acyloin ***(3)*** was from 35 to 40 mg L^{-1}. These quantities were obtained at acrolein concentrations between 20 mM and 150 mM (Figure 10). In this concentration range, it seemed that, regardless of the acrolein quantity, PDC can catalyze a certain number of turnovers before becoming inactive. For acrolein concentrations above 200 mM, substrate inhibition is likely to cause lower product concentrations. For further experiments, 20 mM acrolein was used.

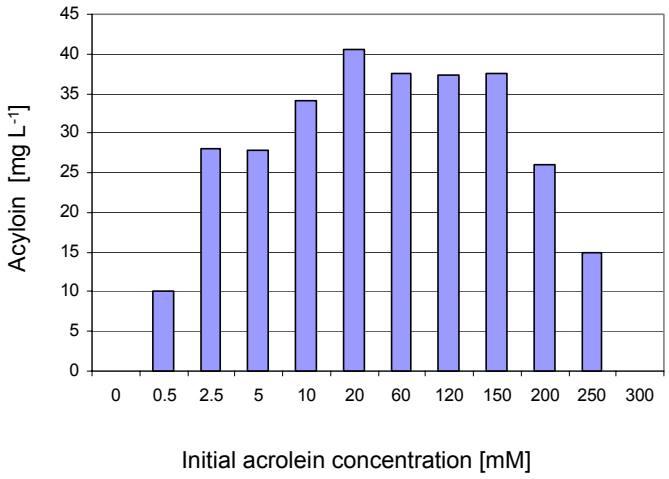


Figure 10. Influence of acrolein concentration on concentration of 3-hydroxy-1-penten-4-one after 2 h of incubation.

Influence of pH and temperature on formation of 3-hydroxy-1-penten-4-one *(3)*

The influence of the pH and temperature on the production of compound ***(3)*** was investigated (see 2.3.1.2 and 2.3.1.3). Under the tested conditions, highest concentrations of ***(3)*** were obtained at pH 6.0 (Figure 11) and at temperatures of 23 °C or 34 °C (Figure 12). Higher as well as lower temperatures led to less product, even when incubation times were extended.

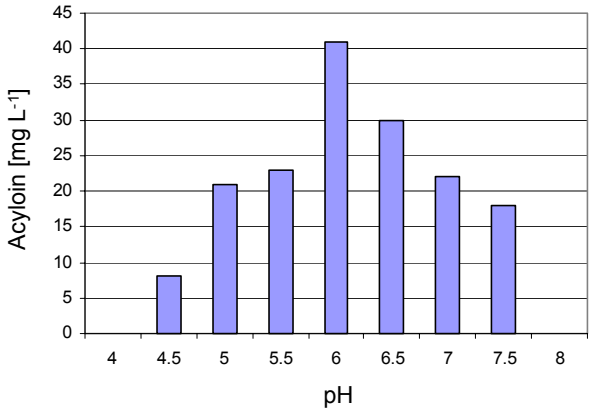


Figure 11. Influence of pH on concentration of 3-hydroxy-1-penten-4-one after 2 h of incubation.

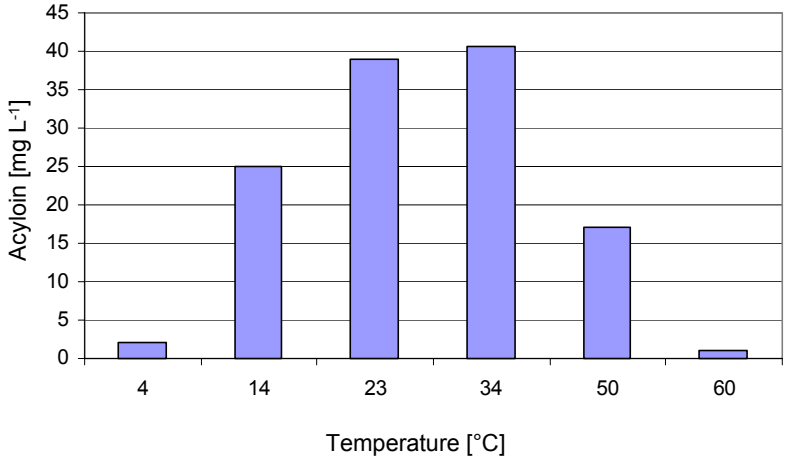
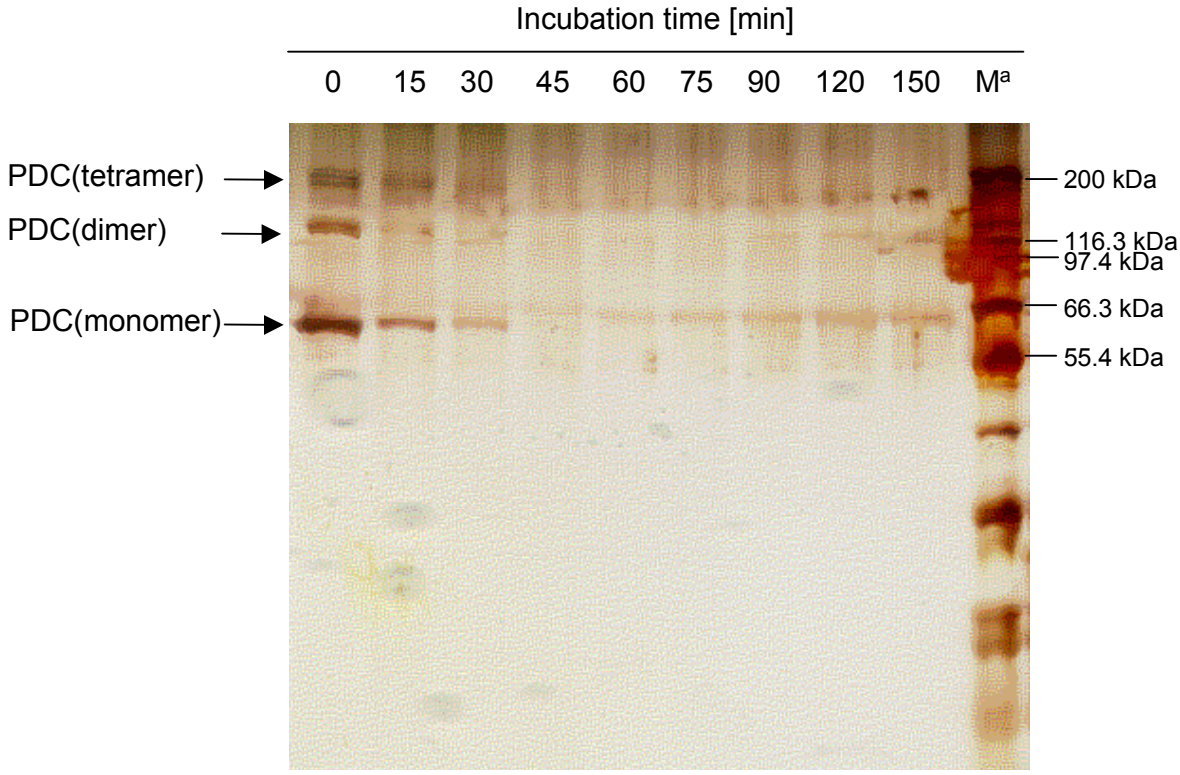


Figure 12. Influence of temperature on concentration of 3-hydroxy-1-penten-4-one after 2 h of incubation.

Inactivation of PDC

In order to understand the inactivation of PDC, samples from different time points of biotransformation were subjected to denaturing SDS-PAGE (2.3.1.4) under reducing conditions. After staining, the bands which corresponded to native PDC enzymes disappeared with incubation time (Figure 13). These results suggest that acrolein probably reacts unspecifically with nucleophilic amino acids. Then, either intramolecular crosslinking of amino acids of individual PDC holoenzymes and/or intermolecular oligomerization of PDC holomers are suggested to lead to the denaturation of PDC. The detailed molecular events, however, which lead to PDC inactivation remain unclear.



M^a=Molecular weight marker

Figure 13. SDS PAGE of samples containing PDC after different incubation time with acrolein.

3.1.1.2 Biogeneration of 3-hydroxy-1-penten-4-one using whole cells of baker's yeast

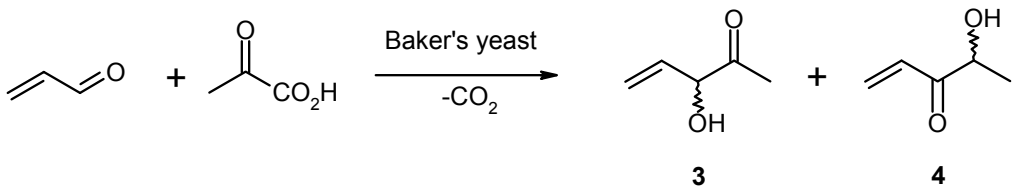


Figure 14. Biogeneration of 3-hydroxy-1-penten-4-one and 4-hydroxy-1-penten-3-one using whole cells of baker's yeast.

In order to produce higher quantities of compound ***(3)***, biotransformation of acrolein and pyruvate was next carried out with whole cells of baker's yeast under non-fermenting conditions as depicted in Figure 14 (for details, see 2.3.2). After 1 h of incubation, a mixture of ***(3)*** and its tautomer, 4-hydroxy-1-penten-3-one ***(4)***, was obtained (Table 1).

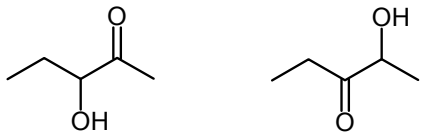
Table 1. Biotransformation of acrolein and pyruvate using baker's yeast.

Acyloin	Absolute configuration	mg L^{-1}	$[\alpha]^{25}_{J}$ [deg]	ee [%]	RI[a]
3-Hydroxy-1-penten-4-one ***(3)*****	*R*	150	-75	72	1372
4-Hydroxy-1-penten-3-one ***(4)***	*R*	30	n.d.	92	1381

[a] Linear retention index on a DB-Wax column.

**Compound never synthesized nor characterized before.

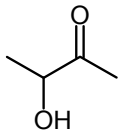
The molecular structures of both acyloins were determined by comparison of their ^{1}H NMR spectra with those of chemically derived substances (see 2.7.1). The enantiomeric excesses (ee 72 % for ***(3)*** and 92 % for ***(4)***) were determined after chemical reduction to 3-hydroxy-2-pentanone ***(5)*** and 2-hydroxy-3-pentanone ***(6)*** (Figure 15), followed by chiral GC analysis. The absolute configurations of both acyloins have been assigned as (*R*), by comparison with authentic (*S*)-***5*** and (*S*)-***6*** (see 2.3.2.2.3), which were produced as described by Bel Rhlid and coworkers[23] and Besse and coworkers[24], respectively. Acyloins ***(3)*** and ***(4)*** were shown to be odourless molecules at quantities up to 2000 ng as judged by GC-O (see 2.8).



5 **6**

Figure 15. Molecular structures of 3-hydroxy-2-pentanone and 2-hydroxy-3-pentanone.

In addition to the above mentioned acyloins, 3-hydroxy-2-butanone ***(7)***, having the (*R*)-configuration, was identified in the reaction mixture by comparison of its RI and GC-MS data with those of the commercial reference compound (Figure 16).

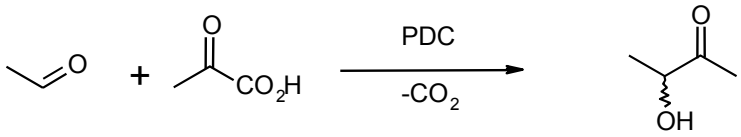


7

Figure 16. Molecular structure of 3-hydroxy-2-butanone.

The enantiomeric excess (ee 46 %) was determined by chiral GC, the retention times of the enantiomers being compared to those of the commercial racemic 3-hydroxy-2-butanone ***(7)***.

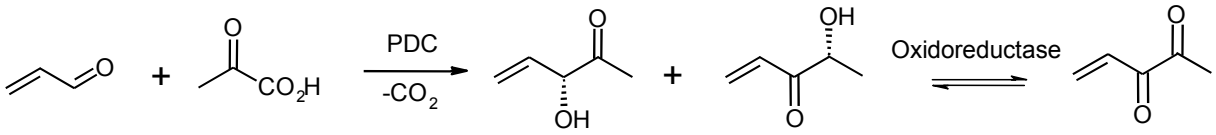
Compound ***(7)*** is thought to be produced by carboligation reaction between pyruvate and acetaldehyde catalyzed by PDC (Figure 17). Acetaldehyde is formed from decarboxylation of pyruvate.



7

Figure 17. Suggested formation of 3-hydroxy-2-butanone from pyruvate and acetaldehyde.

Low quantities of 1-penten-3,4-dione ***(8)*** were also identified in the reaction mixture by comparison of its RI and GC-MS spectrum with those of a chemically derived compound (see 2.7.2). Its formation is likely to be mediated by a dehydrogenase confering oxidation of ***(3)*** and ***(4)*** (Figure 18).



(*R*)-**3** (*R*)-**4** **8**

Figure 18. Suggested biochemical pathway leading to the production of 1-penten-3,4-dione from acrolein and pyruvate.

A further molecule, formed in small amounts, was γ-pentalactone ***(9)***, which was characterized by GC-MS (2.3.2). As depicted in Figure 19, the formation of ***(9)*** is thought to consist of five steps. A carboligase is first suggested to catalyze an enzymatic Michael-type reaction between pyruvate and acrolein to yield 4-oxopentanal. The 4-oxoaldehyde is suggested to be oxidized to 4-oxopentanoic acid. After esterification to ethyl pentanoate, reduction to ethyl-4-hydroxy-pentanoate is likely to be followed by ring closure which leads to the formation of the aroma-active lactone ***(9)***. The initial carboligation event is probably catalyzed by a carboligase distinct from PDC because 4-oxopentanal could not be identified when PDC was used instead of whole cells. However, the possibility remains that the special conditions in the cells, which can not be reproduced *in vitro*, allow PDC to catalyze the Michael-type reaction.

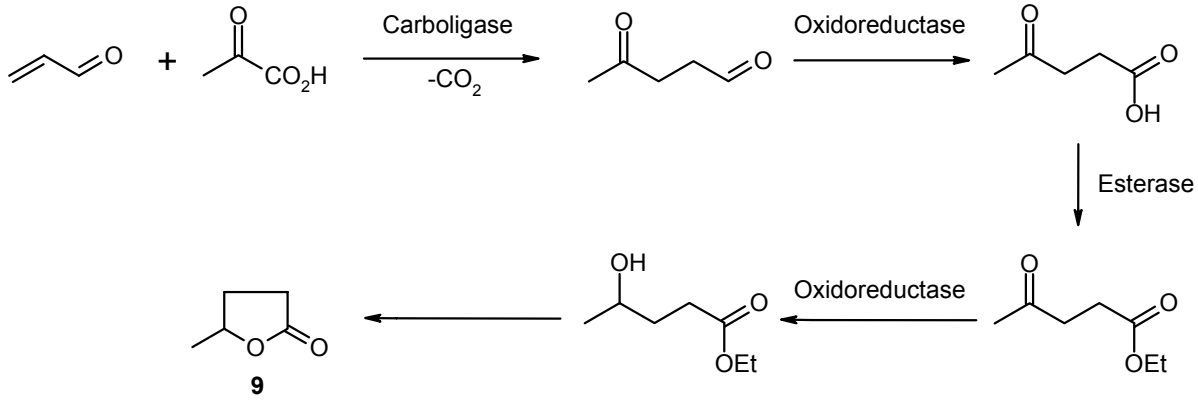


Figure 19. Suggested biochemical pathway for γ-pentalactone production from acrolein and pyruvate.

Influence of acrolein concentration on formation of 3-hydroxy-1-penten-4-one

The influence of initial acrolein concentration on the production of 3-hydroxy-1-penten-4-one ***(3)*** was investigated (see 2.3.2.1). As Figure 20 shows, at substrate concentrations of 5.0 and 10 mM, compound ***(3)*** was produced during the first 50 min and was then degraded. The degradation was faster at 5.0 mM, which resulted in total disappearance of acyloin ***(3)*** after 2 h. Using 25 mM to 150 mM substrate concentrations led to production of ***(3)*** during the first 1 to 2 hours. After that, no more acyloin was formed, and a plateau was reached. The higher the substrate concentration, the faster the plateau was reached. The maximal product concentration, corresponding to the plateau, however, was for all the latter substrate concentrations comparable (~150 mg L^{-1}).

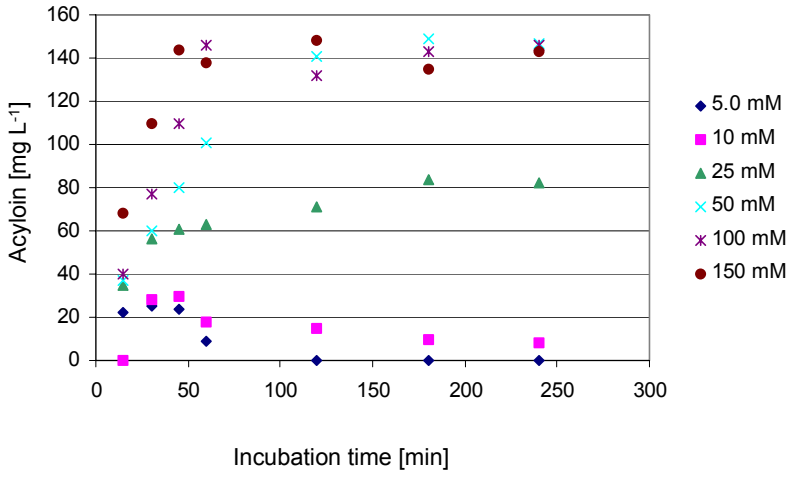


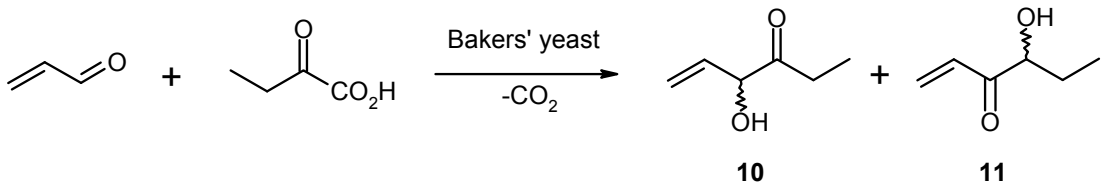
Figure 20. Generation of 3-hydroxy-1-penten-4-one using baker's yeast as function of time.

From 25 mM to 150 mM substrate concentration, regardless of the substrate concentration, baker's yeast catalyzed a limited number of turnovers before becoming inactive. These results are similar to those obtained using PDC (3.1.1.1). The biotransformation with the commercial enzyme PDC, however, was less effective, at least partially because of less PDC

enzymes. It could also be possible that the PDC enzymes are better protected in the cytoplasm against the inhibitory action of acrolein. Another reason might be, that other carboligases are present in the cells which catalyze the same reaction.

All in the whole, the new acyloin ***(3)***, produced by whole cells of baker's yeast, was characterized for the first time by GC-MS, ^{1}H-NMR, and ^{13}C-NMR. The acyloin was of the (*R*)-configuration and was stereoselectively produced with an enantiomeric excess of 72 %. Its specific optical rotation at 592 nm was –75 deg. As the production of ***(3)*** was more efficient with whole cells (max. ~150 mg L^{-1}) than with PDC (max. ~40 mg L^{-1}), further carboligation experiments were conducted with whole cells.

3.1.2 Biogeneration of 3-hydroxy-1-hexen-4-one



10 **11**

Figure 21. Biogeneration of 3-hydroxy-1-hexen-4-one and 4-hydroxy-1-hexen-3-one using whole cells of baker's yeast.

In order to synthesize another new acyloin, 3-hydroxy-1-hexen-4-one ***(10)***, which could also be a precursor of interesting pyrazine derivatives, further biotransformation reactions with whole cells of baker's yeast were performed using acrolein and 2-oxobutyrate as substrates, as illustrated in Figure 21 (see 2.4). After 1 h of incubation, a mixture of (*R*)-3-hydroxy-1-hexen-4-one ***(10)***, the tautomeric acyloin (*R*)-4-hydroxy-1-hexen-3-one (***11***), and five other acyloins with (*R*)-configuration were obtained (Table 2).

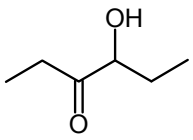
Table 2. Biotransformation of acrolein and 2-oxobutyrate using baker's yeast.

Acyloin	Absolute configuration	mg L^{-1}	ee [%]	RI[a]
3-Hydroxy-1-hexen-4-one ***(10)*****	*R*	18	72	1453
4-Hydroxy-1-hexen-3-one ***(11)***	*R*	12	92	1430
3-Hydroxy-1-penten-4-one ***(3)*****	*R*	14	78	1381
4-Hydroxy-1-penten-3-one ***(4)***	*R*	15	96	1372
4-Hydroxy-3-hexanone ***(12)***	*R*	15	50	1389
3-Hydroxy-2-pentanone ***(5)***	*R*	14	43	1344
2-Hydroxy-3-pentanone ***(6)***	*R*	13	45	1361

[a] Linear retention index on a DB-Wax column.

**Compound never synthesized nor characterized before.

The molecular structures of acyloins ***(3)***, ***(4)***, ***(5)***, and ***(6)*** (Figure 14, Figure 15), as well as that of ***(12)*** (Figure 22) were determined by comparison of their RI and GC-MS data with those of synthesized reference compounds.



12

Figure 22. Molecular structure of 4-hydroxy-3-hexanone.

The molecular structures of the acyloins ***(10)*** and ***(11)*** were suggested by interpretation of GC-MS data (Figure 23). The GC-MS/EI spectrum of ***(10)*** shows almost exclusively fragment 57 which arises from fragmentation between C3 and C4. The spectrum of ***(11)*** shows, in analogy, mass fragments of 55 and 59, as well as an additional fragment 41 which arises from dehydration of fragment 59 (Figure 24).

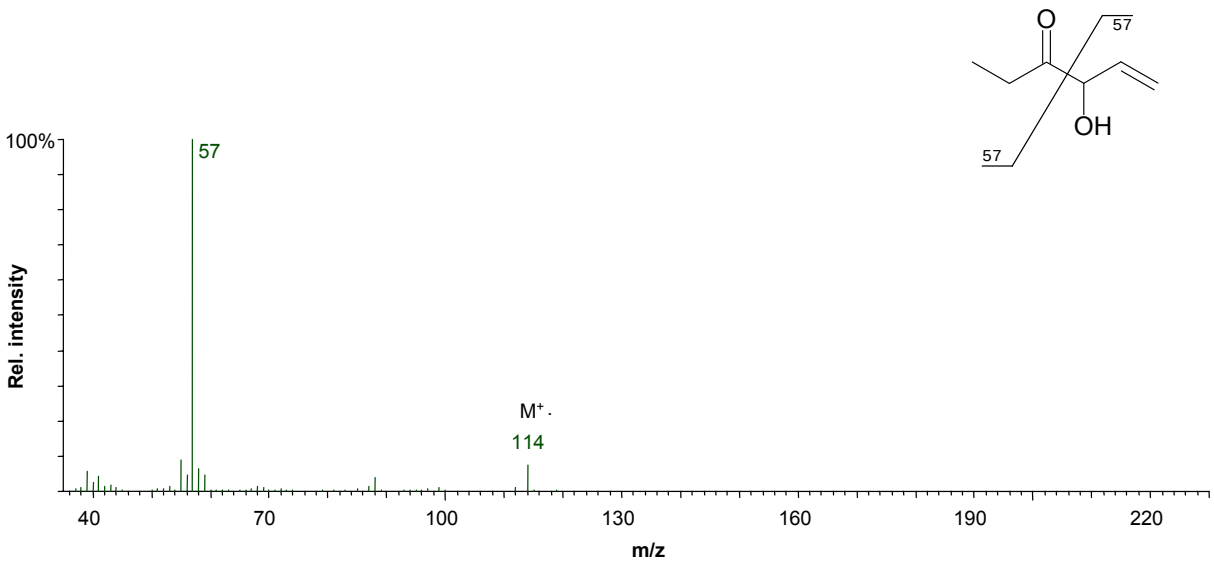


Figure 23. GC-MS/EI spectrum of 3-hydroxy-1-hexen-4-one.

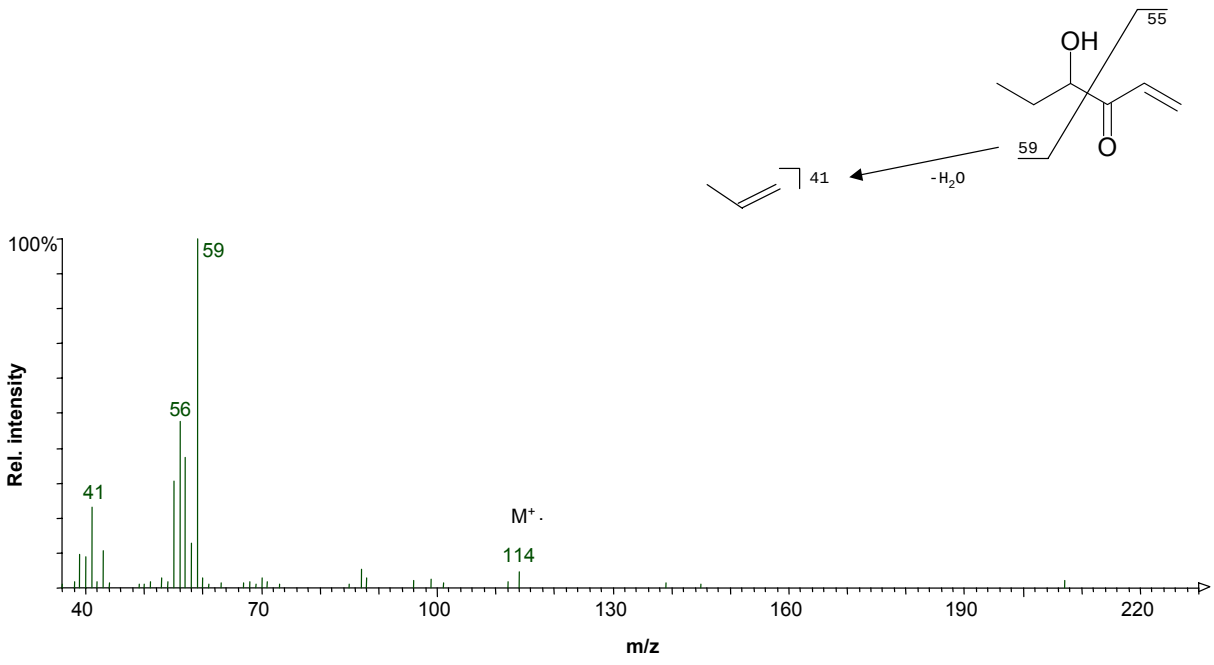


Figure 24. GC-MS/EI spectrum of 4-hydroxy-1-hexen-3-one.

The enantiomeric excesses and the stereochemistry of acyloins ***(3)***, ***(4)***, ***(5)***, ***(6)***, and ***(12)*** were determined by GC analysis on a chiral phase, the retention times of the ketols being compared with those of reference substances of known stereochemistry (see 2.4.2).

The enantiomeric excesses of acyloins ***(10)*** and ***(11)*** were putatively determined by GC analysis on a chiral phase without the use of reference compounds. The determination of the stereochemistry was based on the assumption that (*R*)-acyloins had lower retention times

than (*S*)-acyloins on a chiral phase. This assumption should be valid, because with all saturated and unsaturated acyloins, where the absolute configurations had been proven before, this retention order had been observed, and due to structure similarity, no difference from this retention behaviour should be expected. Acyloins ***(3)*** and ***(4)*** were identified with comparable ee values as those obtained in paragraph 3.1.1.2. Their presence in this reaction can be explained by the fact that endogenous pyruvate, *per se*, exists inside baker's yeast cells. The presence of the saturated acyloins ***(5)***, ***(6)***, and ***(12)*** point to an oxidoreductase activity that confers the reduction of the unsaturated counterparts. The suggested reduction step results in compounds of moderate enantiomeric excesses (Table 2). A biochemical pathway which illustrates the production of all the above mentioned acyloins is depicted in Figure 25.

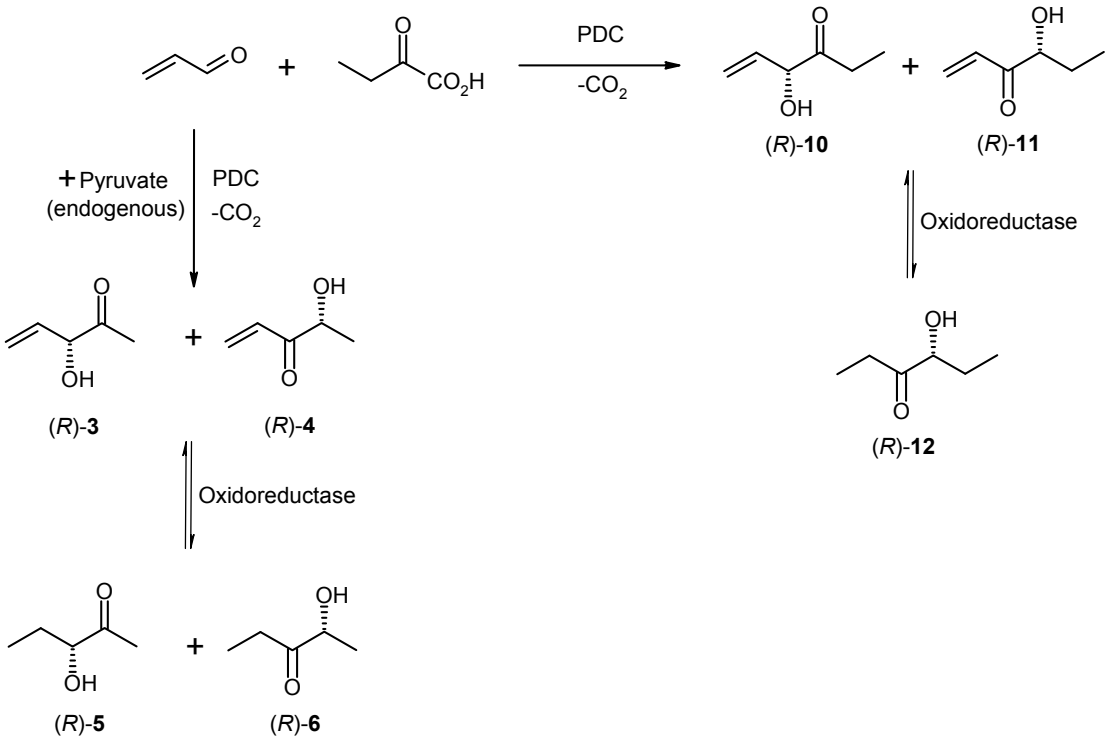
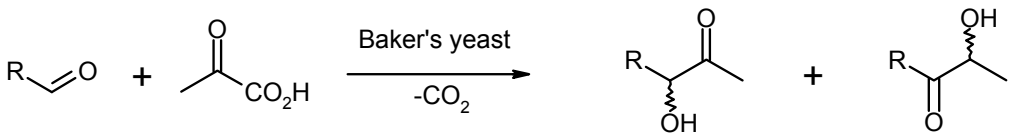


Figure 25. Suggested biochemical pathway leading to the production of acyloins from acrolein and 2-oxobutyrate.

To summarize, seven acyloins of the (*R*)-configuration were produced from biotransformation of acrolein and 2-oxobutyrate using baker's yeast as catalyst. The new acyloin ***(10)*** was characterized for the first time by GC-MS and was formed with an enantiomeric excess of 72 %. The acyloin condensation obviously could be accomplished with 2-oxobutyrate instead of pyruvate as substrate. Further reactions were performed with other aldehydes than acrolein as substrates for biotransformation.

3.1.3 Biogeneration of other acyloins



R=alkyl (C1-C7), alkenyl (C1-C7)

Figure 26. Biogeneration of a pool of acyloins using whole cells of baker's yeast.

In order to determine the scope and limitations of baker's yeast mediated carboligation reaction, other aldehydes and pyruvate were subjected to biotransformation as illustrated in Figure 26 (for experimental details, see 2.5). After 1 h of incubation, with all aldehydes except 2*E*-butenal, a pair of acyloins was identified (Table 3).

Table 3. Biotransformation of pyruvate and various aldehydes using baker's yeast.

Aldehyde	Acyloin	Absolute configuration	Yield [%]	ee [%]	RI[a]
2*E*-Butenal	3-hydroxy-4*E*-hexen-2-one	*R*	4.9	75	1481
2*E*-Pentenal	3-hydroxy-4*E*-hepten-2-one	*R*	21	79	1586
	2-hydroxy-4*E*-hepten-3-one	*S*	14	48	1619
2*E*-Hexenal	3-hydroxy-4*E*-octen-2-one	*R*	28	60	1683
	2-hydroxy-4*E*-octen-3-one	*R*	31	72	1698
2*E*-Heptenal	3-hydroxy-4*E*-nonen-2-one ***(13)*****	n.d.	7.2	n.d.	1776
	2-hydroxy-4*E*-nonen-3-one ***(14)*****	n.d.	4.5	n.d	1796
2*E*-Octenal	3-hydroxy-4*E*-decen-2-one ***(15)*****	n.d.	6.1	n.d.	1946
	2-hydroxy-4*E*-decen-3-one ***(16)*****	n.d.	3.7	n.d.	1959
Propanal	3-hydroxy-2-pentanone	n.d.	0.5	n.d.	1344
	2-hydroxy-3-pentanone	*R*	3.9	83	1361
Butanal	3-hydroxy-2-hexanone	*R*	3.8	77	1430
	2-hydroxy-3-hexanone	*R*	1.6	57	1423
Pentanal	3-hydroxy-2-heptanone	*R*	9.1	70	1560
	2-hydroxy-3-heptanone	*R*	3.8	38	1551
Hexanal	3-hydroxy-2-octanone (***19***)	*R*	55	85	1655
	2-hydroxy-3-octanone (***17***)	*S*	19	80	1647
Heptanal	3-hydroxy-2-nonanone	n.d.	21	n.d.	1768
	2-hydroxy-3-nonanone	n.d.	9.2	n.d.	1757
Octanal	3-hydroxy-2-decanone	n.d.	4.5	n.d.	1889
	2-hydroxy-3-decanone	n.d.	1.7	n.d.	1874

[a] Linear retention index on a DB-Wax column.

** Compound never synthesized nor characterized before.

As it can be seen from Table 3, with 2-butenal, only the 3-hydroxy tautomer could be detected. The corresponding 2-hydroxy compound was probably formed in too small quantities to be identified. Most structures were determined by comparison of their GC-MS data with those described in literature (see 2.5.2). As exceptions, the spectra of the new acyloins 3-hydroxy-4*E*-nonen-2-one ***(13)***, 2-hydroxy-4*E*-nonen-3-one ***(14)***, 3-hydroxy-4*E*-decen-2-one ***(15)***, and 2-hydroxy-4*E*-decen-3-one ***(16)***, were suggested by interpretation of their GC-MS spectra. The GC-MS/EI spectra of the 3-hydroxy tautomers (Figure 27, Figure 28) show that, after loss of a fragment with mass 43, characteristic for the acetyl

group, the remaining fragment either loses water (-18) or the alkyl moiety adjacent to the double bond to form a protonated acrolein fragment (mass 57).

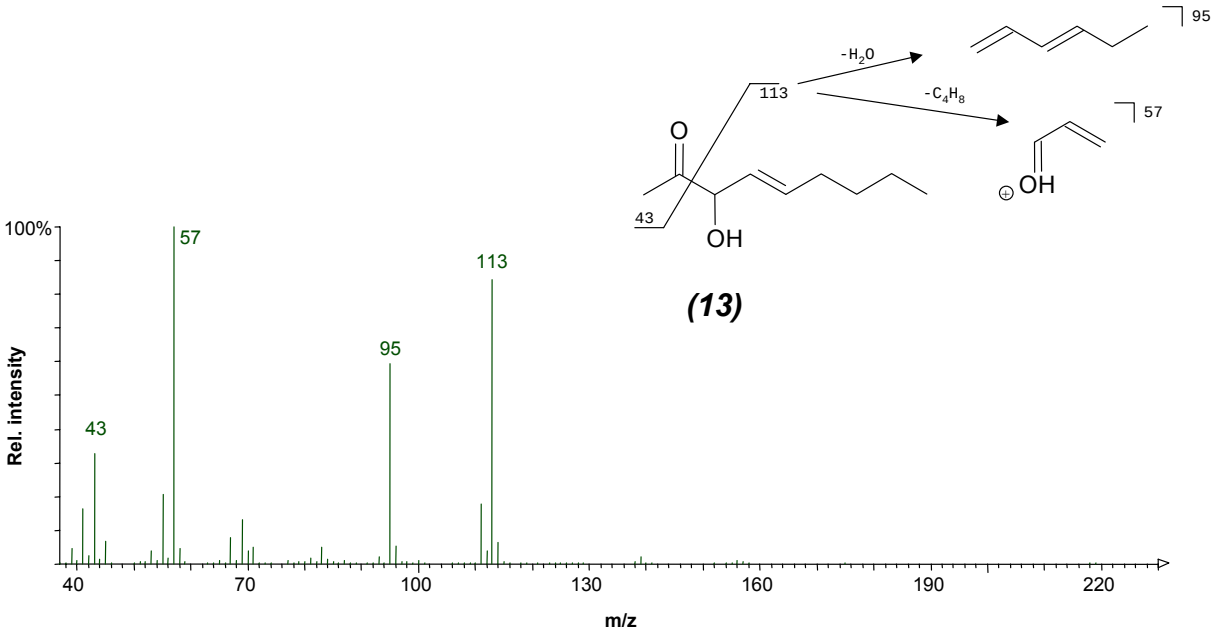


Figure 27. GC-MS/EI spectrum of 3-hydroxy-4*E*-nonen-2-one.

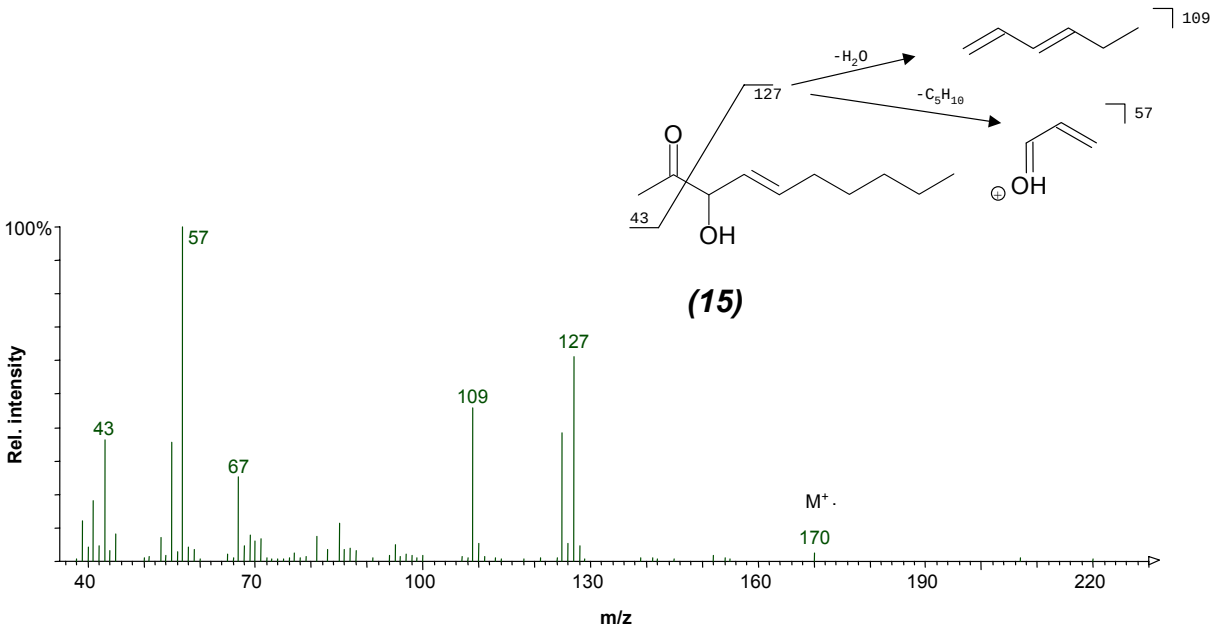


Figure 28. GC-MS/EI spectrum of 3-hydroxy-4*E*-decen-2-one.

The spectra of the 2-hydroxy ketones (Figure 29, Figure 30) show that, after loss of a fragment with 45, characteristic for the hydroxyethyl functionality, the remaining fragment loses the alkyl moiety adjacent to the double bond to form a deprotonated acrolein fragment (mass 55).

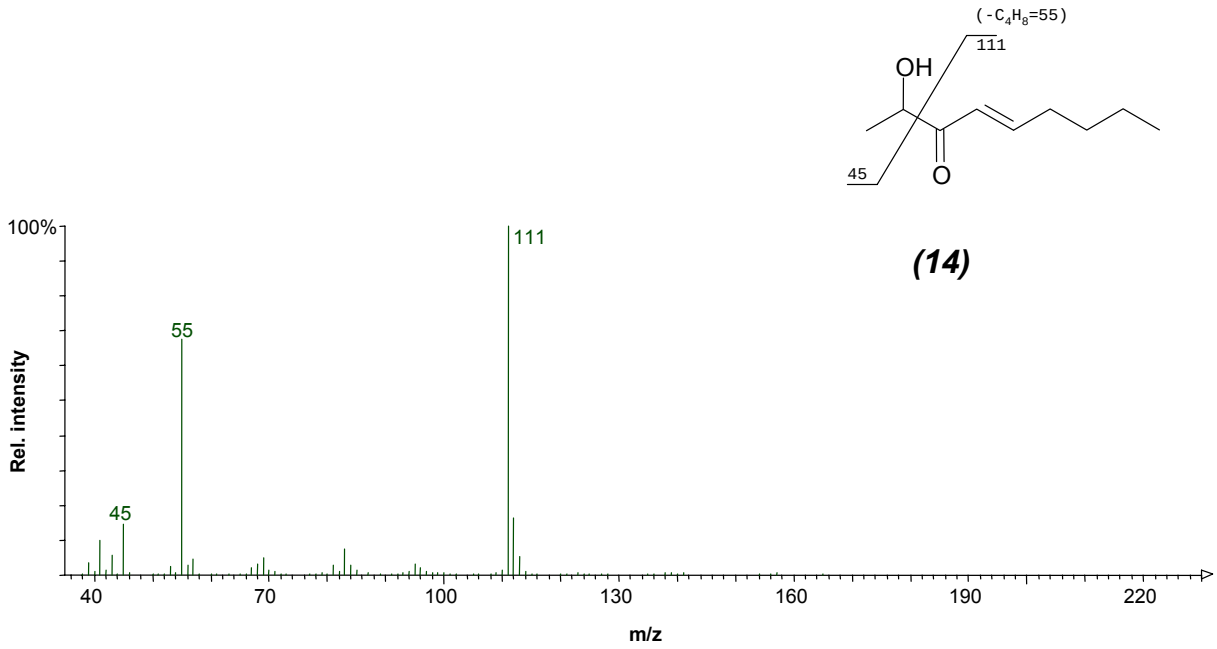


Figure 29. GC-MS/EI spectrum of 2-hydroxy-4*E*-nonen-3-one.

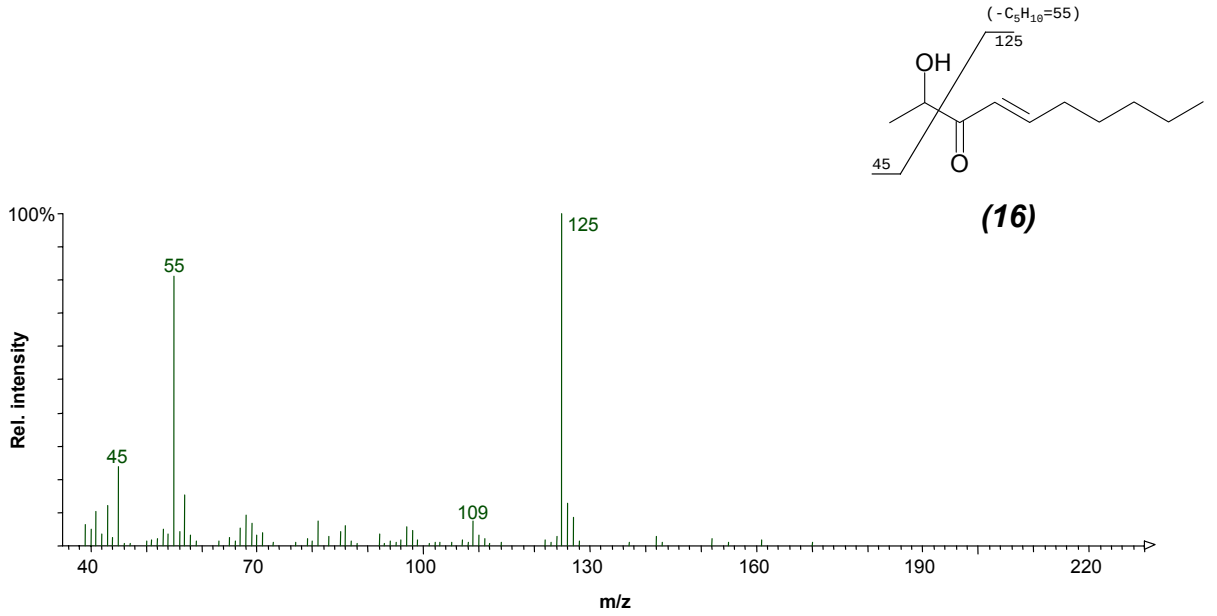


Figure 30. GC-MS/EI spectrum of 2-hydroxy-4*E*-decen-3-one.

In the reaction mixtures containing the unsaturated C7, C8, C9, and C10 acyloins, the corresponding saturated acyloins could also be identified. They are suggested to be produced by enzymatic reduction of the unsaturated counterparts which goes in line with what was already found in paragraph 3.1.2.

The product concentrations of all obtained acyloins from biotransformation of aldehydes and pyruvate were next compared and summarized in Figure 31. Under the used conditions, the

C8 acyloins, formed from hexanal and 2*E*-hexenal, were formed with highest total yields of approximately 75 % and 60 %, respectively.

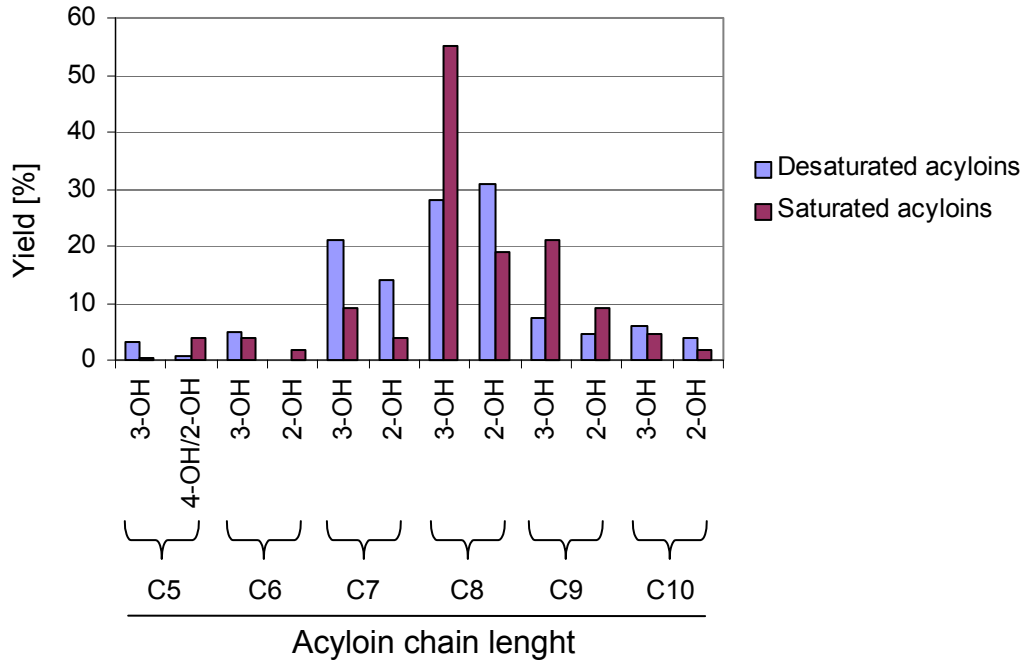
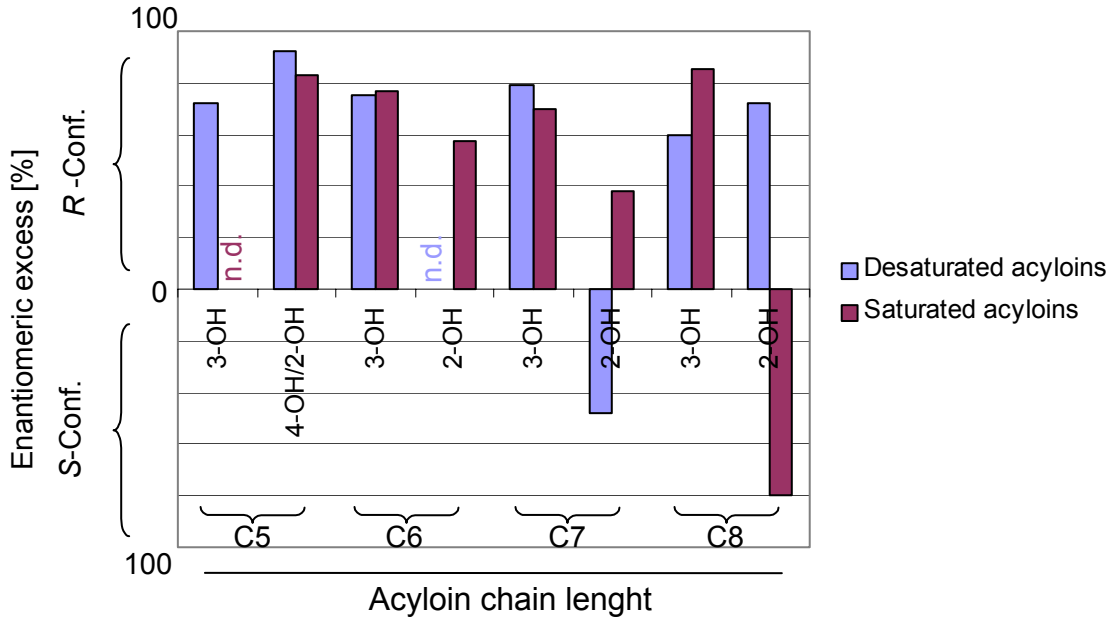


Figure 31. Yields of acyloins produced by biotransformation of aliphatic aldehydes and pyruvate using whole cells of baker's yeast.

Acyloins with one carbon atom less or more, C7 and C9 acyloins, were generated with moderate total yields of 10 % to 30 %. Shorter acyloins than C7 or longer than C9 were produced the least effectively and gave yields of less than 10 %. All in the whole, desaturated acyloin pairs were generated in comparable quantities, while for the majority of the saturated ones, more 3-hydroxy tautomer was produced.

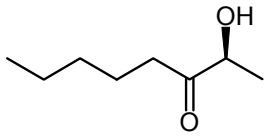
The enantiomeric excesses of the C5 to C8 acyloins were putatively determined by GC analysis on a chiral phase without the use of reference substances. The determination of the stereochemistry was based on the assumption that (*R*)-acyloins had lower retention times than (*S*)-acyloins. All tested acyloins, except for two compounds, were exclusively transformed to acyloins of the (*R*)-configuration (Figure 32).



n.d. not determined.

Figure 32. Putatively identified enantiomeric excesses and absolute configurations of acyloins produced by biotransformation of aliphatic aldehydes and pyruvate using whole cells of baker's yeast.

The two exceptions were (*S*)-2-hydroxy-3-octanone ***(17)*** (Figure 33) and (*S*)-2-hydroxy-4*E*-hepten-3-one ***(18)***.



(*S*)-**17**

Figure 33. Molecular structure of (*S*)-2-hydroxy-3-octanone.

The opposite configurations are probably the consequence of different structural binding conditions of the substrates to the active site. As shown in Figure 34, (*R*)-3-Hydroxy-2-octanone ***(19)*** is synthesized from a TPP-bound acetaldehyde carbanion intermediate, the so-called "active acetaldehyde", and hexanal as acceptor aldehyde molecule. The small "active acetaldehyde" is preferably attacked from one side by hexanal, which leads to the formation of the (*R*)-enantiomer. (*S*)-2-Hydroxy-3-octanone ***(17)***, however, is thought to be formed from a TPP-bound hexanal-carbanion intermediate, or "active hexanal", and acetaldehyde. It is thought that the larger "active hexanal" is, due to specific interactions with amino acids in the active site, preferably attacked by acetaldehyde from the opposite side, and therefore yields the (*S*)-enantiomer. Analogue explanations could be done for 2*E*-pentenal as substrate, because of its comparable size to hexanal. The other 2-hydroxy

tautomers are of the (*R*)-configuration, because the corresponding alkanal- carbanion intermediates probably do not interact with the amino acids of the active site in a similar manner as with hexanal or 2*E*-pentenal.

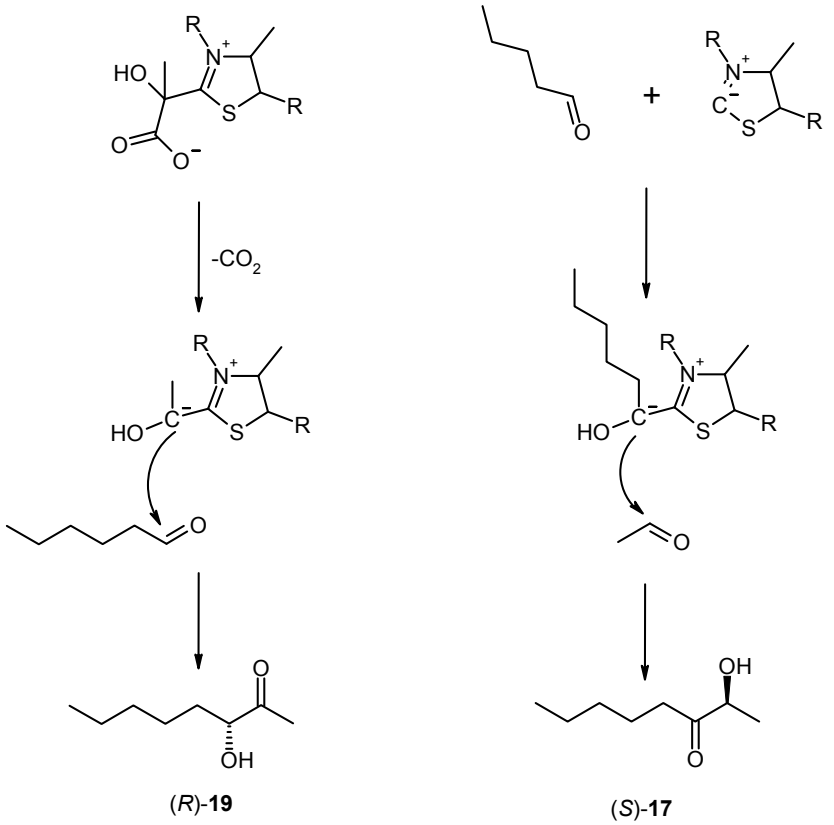


Figure 34. Suggested mechanisms leading to the formation of (*R*)-3-hydroxy-2-octanone and (*S*)-2-hydroxy-3-octanone.

Independent from the absolute configuration, the ee values of corresponding desaturated and saturated acyloins were always comparable (Figure 32. The ee values varied from 38 to 92 %.

3.1.4 Main findings

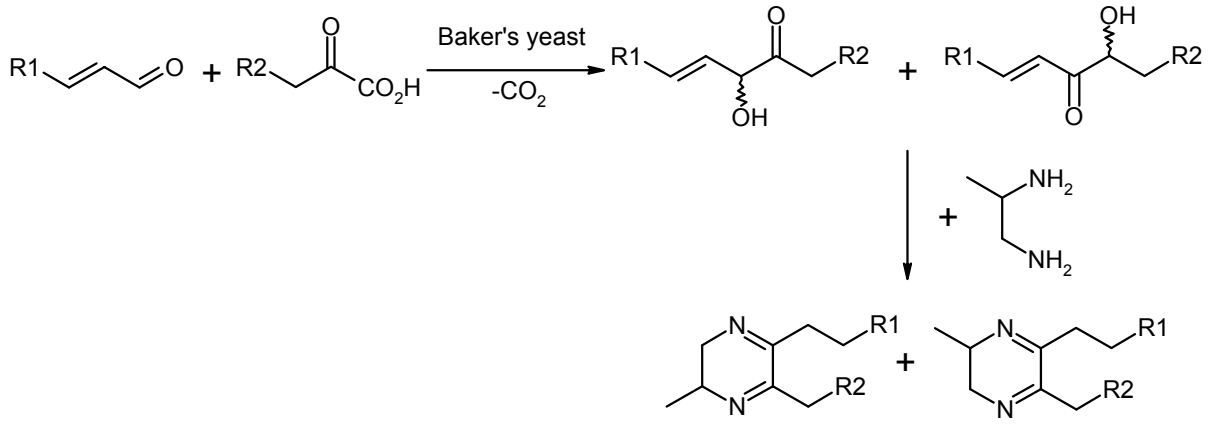
All in the whole, a pool of 27 aliphatic acyloins was synthesized by biotransformation of aliphatic aldehydes and 2-oxocarboxylates using baker's yeast as biocatalyst. Six of them, compounds ***(3)***, ***(10)***, ***(13)***, ***(14)***, ***(15)***, and ***(16)***, were synthesized and characterized for the first time. Yields up to 55 % and the broad substrate spectrum showed that the capability of whole cells of baker's yeast to catalyze the formation of aliphatic acyloins from aldehydes had been understimated before. Stereochemical investigations revealed that 15 out of 17 tested acyloins had the (*R*)-configuration, while 2 of them had the (*S*)-configuration. The enantiomeric excesses varied from 38 to 92 %. The highest enantiomeric excesses were

observed for (*R*)-4-hydroxy-1-hexen-3-one ***(11)*** and (*R*)-4-hydroxy-1-penten-3-one ***(4)***. Both acyloins were synthesized from acrolein. The nature of the 2-oxocarboxylate (pyruvate or 2-oxobutyrate) had no influence on the ee values. In the next paragraph, the reaction of the pool of acyloins with 1,2-propanediamine was investigated.

3.2 Chemoenzymatic synthesis of pyrazine derivatives

The pool of acyloins produced in paragraph 3.1 was tested in further reactions with 1,2-propanediamine to produce pyrazine derivatives. Because of their expected different behaviour, 2-alkenals and alkanals were separately investigated.

3.2.1 2*E*-Alkenals as substrate for chemoenzymatic synthesis



R1=H, alkyl
R2=H, methyl

Figure 35. Chemoenzymatic synthesis of 5,6-dihydropyrazines.

Biotransformation of 2*E*-alkenals and pyruvate (2-oxobutyrate) with whole cells of baker's yeast was carried out as described in paragraph 3.1, and an excess of 1,2-propanediamine was added as depicted in Figure 35 (for experimental details, see 2.6.1). After 150 min of total incubation time, for each studied combination of 2*E*-alkenal and pyruvate (2-oxobutyrate), a mixture of two isomeric 5,6-dihydropyrazines was obtained (Table 4) in yields from 44 to 55 % (based on concentration of intermediate acyloins).

Table 4. Biotransformation of 2*E*-alkenals and pyruvate (2-oxobutyrate) using baker's yeast and subsequent chemical reaction with 1,2-propanediamine.

Acyloin[a]	5,6-Dihydropyrazine derivative	Yield [%][b]	RI[c]	MS-EI, m/z (relative intensity)
(3), ***(4)***	2-Ethyl-3,5-dimethyl- ***(20)***	24	1038	138 (100) $[M]^+$, 123 (75), 68 (19), 56 (83), 42 (70).
	3-Ethyl-2,5-dimethyl- ***(21)***	24	1031	138 (100) $[M]^+$, 123 (67), 83 (15), 56 (79), 42 (74)
(10), ***(11)***	2,3-Diethyl-5-methyl- ***(22)***	44	1090	152 (43) $[M]^+$, 137 (68), 56 (100), 42 (20).
Hydroxy-4*E*-hexenones	2-Propyl-3,5-dimethyl- ***(23)***	21	1110	152 (75) $[M]^+$, 137 (67), **124** (89), 109 (36), 70 (100), 42 (94).
	3-Propyl-2,5-dimethyl- ***(24)***	19	1100	152 (81) $[M]^+$, 137 (51), **124** (67), 123 (85), 70 (100), 42 (63).
Hydroxy-4*E*-heptenones	2-Butyl-3,5-dimethyl- **	23	1215	166 (48) $[M]^+$, 151 (44), 137 (57), **124** (100), 123 (69), 84 (82), 42 (47).
	3-Butyl-2,5-dimethyl- **	23	1198	166 (50) $[M]^+$, 151 (40), **124** (100), 123 (80), 84 (50), 42 (68).
Hydroxy-4*E*-octenones	2-Pentyl-3,5-dimethyl- **	24	1311	180 (10) $[M]^+$, 137 (52), **124** (100), 123 (42), 109 (49), 98 (80), 42 (60).
	3-Pentyl-2,5-dimethyl- **	22	1298	180 (12) $[M]^+$, 157 (38), **124** (100), 123 (67), 98 (48), 42 (58).
(13), ***(14)***	2-Hexyl-3,5-dimethyl- **	24	1393	MS: 194 (10) $[M]^+$, 137 (40), **124** (100), 123 (41), 112 (39), 42 (37).
	3-Hexyl-2,5-dimethyl- **	24	1380	194 (12) $[M]^+$, 151 (22), 137 (28), **124** (100), 123 (66), 112 (40), 42 (38)
(15), ***(16)***	2-Heptyl-3,5-dimethyl- **	27	1511	208 (12) $[M]^+$, 137 (41), 126 (28), **124** (100), 123 (41), 109 (38), 42 (40).
	3-Heptyl-2,5-dimethyl- **	26	1498	208 (11) $[M]^+$, 151 (20), 137 (42), 126 (31), **124** (100), 123 (59), 42 (37).

[a] Intermediate α,β-desaturated acyloins formed from 2*E*-alkenals and 2-oxocarboxylates.

[b] Yields based on concentration of acyloins.

[c] Linear retention index on a DB-1 column.

Right column, **bold** type: characteristic mass fragment 124.

Right column, underlined: characteristic mass fragment 27+x.

**Compound never synthesized nor characterized before.

The molecular structures of 2-ethyl-3,5- (***20***), 3-ethyl-2,5- (***21***), 2-propyl-3,5- (***23***), and 3-propyl-2,5-dimethyl-5,6-dihydropyrazines (***22***) as well as that of 2,3-diethyl-5-methyl-5,6-dihydropyrazine (***24***) (Figure 36) were determined by comparing RI and GC-MS spectra with those of chemically-synthesized reference substances (see 2.7.5).

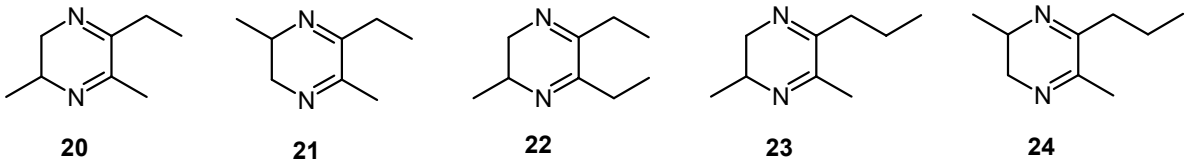


Figure 36. Molecular structures of five 5,6-dihydropyrazines.

The structures of the butyl-, pentyl-, hexyl-, and heptyl pyrazine derivatives were suggested on the basis of GC-MS data. The MS/EI spectra of these pyrazine derivatives, together with those of the propyl derivatives, contain a prominent mass fragment of 124 (Table 4, bold type). The fragment can be explained by transfer of the γ-hydrogen from the alkyl chain to the pyrazine nitrogen, which leads to the formation of reactive intermediate A, that easily fragments to the allyl radical-stabilized intermediate B (Figure 37). In agreement with this mechanism, 2-ethyl-3,5- and 3-ethyl-2,5-dimethyl-5,6-dihydropyrazine as well as 2,3-diethyl-5-methyl-5,6-dihydropyrazine do not have a mass fragment 124, due to the missing γ-hydrogen of the alkyl chain.

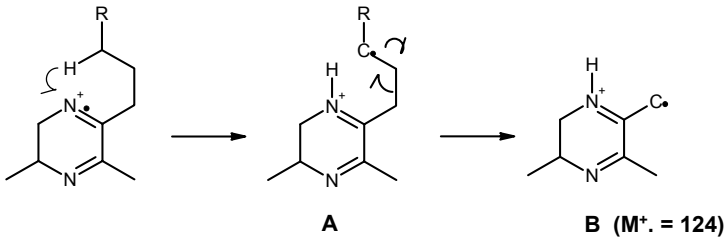
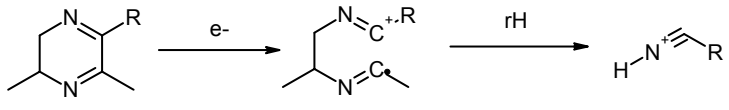


Figure 37. Suggested mechanism leading to mass fragment 124 in GC-MS/EI spectra of 5,6-dihydropyrazines.

Furthermore, the MS/EI spectra of all 5,6-dihydropyrazines contain mass fragments of 27+x, where x stands for the mass of the alkyl chain (Table 4, underlined). 2-Ethyl-3,5- and 3-ethyl-2,5-dimethyl-5,6-dihydropyrazine (x=29), thus, fragment to mass 56, and 2-propyl-3,5- and 3-propyl-2,5-dimethyl-5,6-dihydropyrazine (x=43), fragment to mass 70, and so and forth. It is suggested that ring opening of 5,6-dihydropyrazines upon electron impact is followed by a

rearrangement (rH), leading to these characteristic mass fragments (Figure 38).



R=CH3	x=15	M=42
R=C2H5	x=29	M=56
R=C3H7	x=43	M=70
R=C5H11	x=71	M=98
R=C6H13	x=85	M=112
R=C7H15	x=99	M=126

Figure 38. Suggested mechanism leading to characteristic mass fragments in GC-MS/EI spectra of 5,6-dihydropyrazines.

In order to explain, how the 5,6-dihydropyrazines were formed, a reaction mechanism for each acyloin tautomer was suggested (Figure 39).

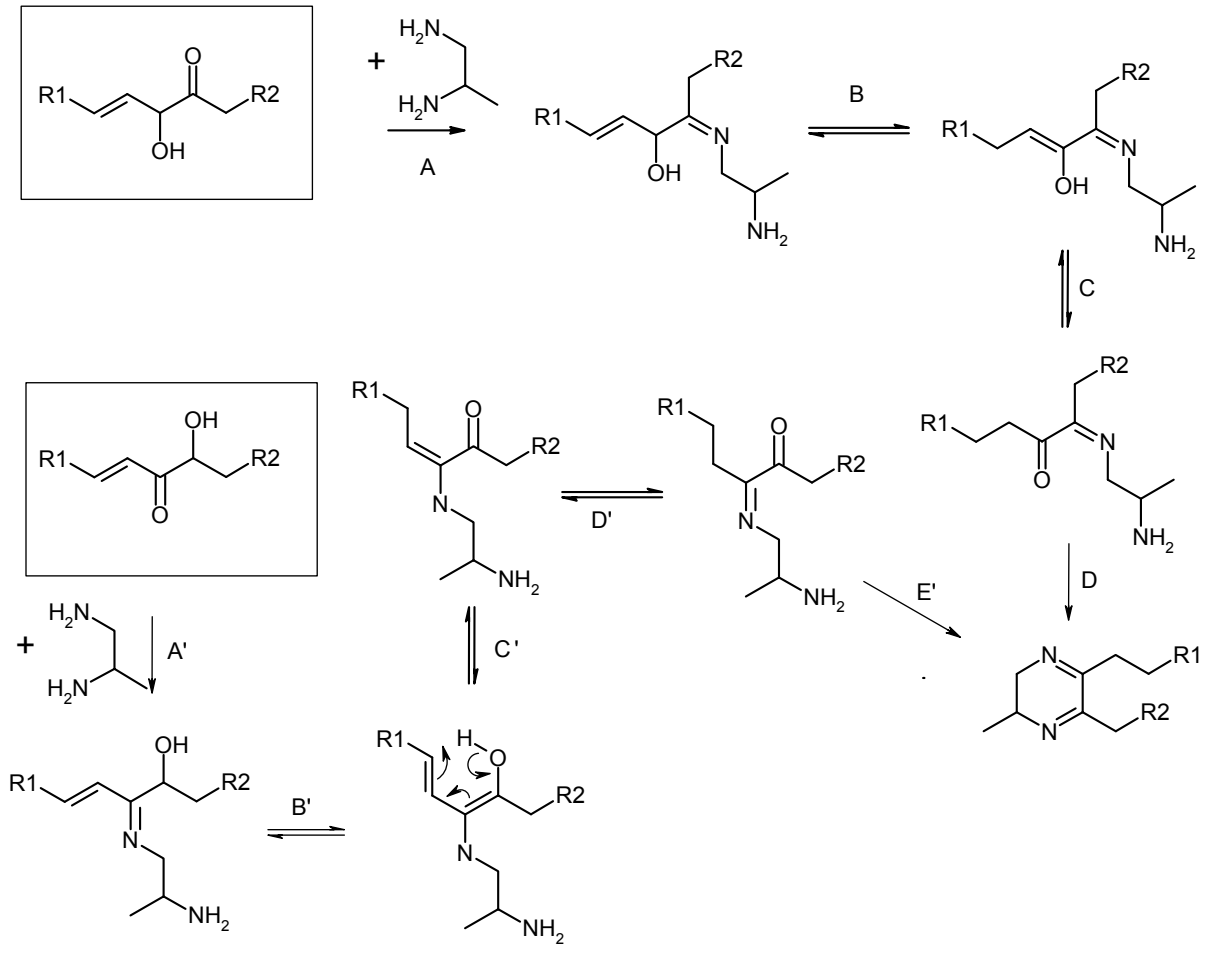


Figure 39. Suggested mechanisms for the formation of 5,6-dihydropyrazines from α,β-desaturated acyloins and 1,2-propanediamine.

Concerning the 3-hydroxy tautomer, it is thought that the keto group of desaturated 3-hydroxyketones reacts with one of the amino groups of 1,2-propanediamine to form a Schiff base (A). It is further suggested that a double bond shift occurs which results in the formation of a energetically more favourable conjugated π system (B). The resulting molecule, has an enol function, which is in tautomeric equilibrium with a keto function (C). The keto group reacts with the other free amino group of 1,2-propanediamine to give a 5,6-dihydropyrazine (D). The second regioisomer is formed when the other amino group of 1,2-propanediamine attacks first. The possibility that baker's yeast might be necessary for the proposed double bond migration, was excluded by reactions in the absence of cells: Chemically synthesized compounds ***(3)*** and ***(4)*** (see 2.7.1) reacted with 1,2-propanediamine in water to the same products. Concerning the 2-hydroxy tautomer, Schiff base formation (A') is suggested to be followed by a double bond migration (B'), a rearrangement (C'), a second double bond migration (D'), and dihydropyrazine formation (E'). Furthermore, it can not be excluded that tautomerization between the acyloin tautomers occurs.

3.2.2 Alkanals as substrate for chemoenzymatic synthesis

Unlike with 2*E*-alkenals (see 3.2.1), no pyrazine derivatives were obtained when alkanals were used instead of them in analogous carboligation reactions (for experimental details, see 2.6.1). This observation can at least partially be explained by the low reactivity of the Schiff base which is formed in the first step. In order to increase the rate of the following reaction step between an alcohol and an amino group, the chemical reaction with 1,2-propanediamine was carried out in an aprotic solvent, diethyl ether, after extraction of the saturated acyloins. (Figure 40) (for details see 2.6.2). The symmetrical acyloins, 3-hydroxy-2-butanone ***(7)*** and 4-hydroxy-3-hexanone ***(12)***, were also reacted with 1,2-propanediamine.

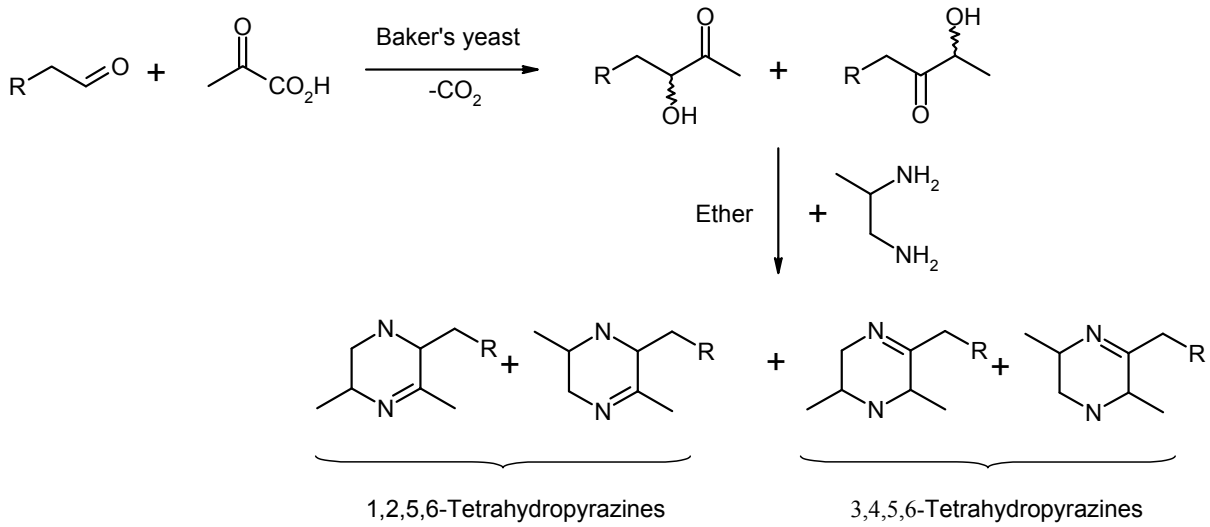


Figure 40. Chemoenzymatic synthesis of tetrahydropyrazines.

After 90 min incubation time, the reactions yielded tetrahydropyrazines, which had all never been described before (Table 4).

Table 5. Chemical reaction of saturated acyloins with 1,2-propanediamine in diethyl ether.

Acyloin(s)	Tetrahydropyrazine	Yield[a] [%]	RI[b]
3-Hydroxy-2-butanone ***(7)***	Trimethyl-**	24	1070
4-Hydroxy-3-hexanone ***(12)***	2,3-Diethyl-5-methyl-**	21	1255
3-Hydroxy-2-pentanone ***(5)***/ 2-hydroxy-3-pentanone ***(6)***	2-Ethyl-3,5-dimethyl-**	11	1174
	2-Ethyl-3,6-dimethyl-**	10	1159
3-Hydroxy-2-hexanone/ 2-hydroxy-3-hexanone	2-Propyl-3,5-dimethyl-**	11	1268
	2-Propyl-3,6-dimethyl-**	11	1245
3-Hydroxy-2-heptanone/ 2-hydroxy-3-heptanone	2-Butyldimethyl-**	20	1341
3-Hydroxy-2-octanone/ 2-hydroxy-3-octanone	2-Pentyldimethyl-**	21	1431
3-Hydroxy-2-nonanone/ 2-hydroxy-3-nonanone	2-Hexyldimethyl-**	25	1525
3-Hydroxy-2-decanone/ 2-hydroxy-3-decanone	2-Heptyldimethyl-**	23	1633

[a] Yields based on concentrations of acyloins.

[b] Linear retention indices on a DB-1 column.

**Compound never synthesized nor characterized before.

Interpretation of the GC-MS spectra as well as comparison of the retention indices with the predicted values suggested the structures of the tetrahydropyrazine derivatives (for data see 2.6.2). Due to theretical considerations, it had been expected before that for each pair of acyloins, 4 tetrahydropyrazines would be obtained. As Figure 41 shows, a 3-hydroxy-2-ketone was thought to form a 1,2,5,6-tetrahydropyrazine, while a 2-hydroxy-3-ketone was thought to yield a 3,4,5,6-tetrahydropyrazine. Additionally, two regio isomers of each tetrahydropyrazine should be obtained, depending on the orientation of 1,2-propanediamine attack.

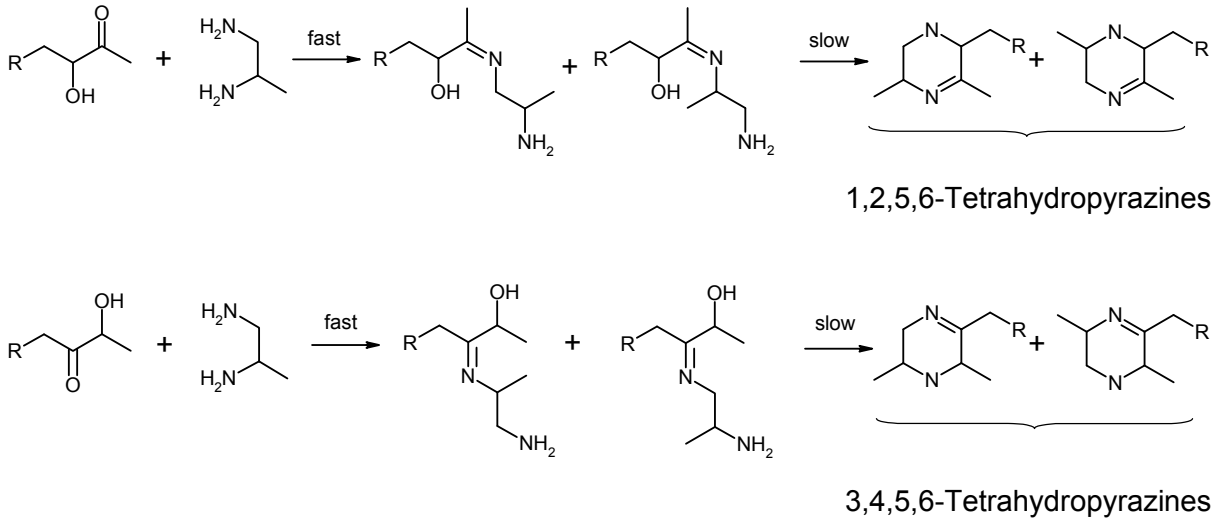


Figure 41. Reaction of 3-hydroxy-2-ketones or 2-hydroxy-3-ketones with 1,2-propanediamine.

However, the GC trace revealed only two peaks for the 2-ethyldimethyl- and 2-propyldimethyltetrahydropyrazines, and only one wide peak for the higher substituted tetrahydropyrazines. Although more compounds should be represented by one peak, each peak was in the following only counted as one compound. The identification of the corresponding 5,6-dihydropyrazines suggested that tetrahydropyrazines were partially oxidized. Furthermore, the presence of the two 5,6-dihydropyrazine regioisomers indicated the existence of at least two tetrahydropyrazines under the wide peak of the higher substituted tetrahydropyrazines. The instability of the tetrahydropyrazines may explain why lower yields were obtained (around 20 %) than for the dihydropyrazines in paragraph 2.6.1.

3.2.3 Determination of organoleptic properties of pyrazine derivatives

Due to their structural similarity to odourous pyrazines, it was expected that at least a part of the pyrazine derivatives from chemoenzymatic synthesis might have interesting aroma-active properties. The odour qualities were characterized by GC-O (2.2.4), and the odour thresholds were estimated by aroma extract dilution analysis (AEDA) (see 2.8). Two of the most potent pyrazines described in literature, 2,3-diethyl-5-methylpyrazine and 2-ethyl-3,5-dimethylpyrazine,[16] were also analyzed as reference substances. The results are summarized in Table 6 for the 5,6-dihydropyrazines (incl. reference compounds), and Table 7 for the tetrahydropyrazines.

Table 6. Organoleptic properties of 5,6-dihydropyrazines and the reference compounds.

5,6-Dihydropyrazines	Odour quality	Odour threshold [ng][a]
2,3,5-Trimethyl-	roasted, sweet	10
2-Ethyl-3,5-dimethyl- ***(20)***	roasted, nutty	0.002
3-Ethyl-2,5-dimethyl- ***(21)***	roasted, popcorn-like	0.227
2,3-Diethyl-5-methyl- ***(22)***	roasted	0.004
2-Propyl-3,5-dimethyl- ***(23)***	roasted	2.5
3-Propyl-2,5-dimethyl- ***(24)***	no odour	>2000
2-Butyl-3,5-dimethyl-	roasted	16.8
3-Butyl-2,5-dimethyl-	no odour	>2000
2-Pentyl-3,5-dimethyl-	roasted	3.2
3-Pentyl-2,5-dimethyl-	no odour	>2000
2-Hexyl-3,5-dimethyl-	no odour	>2000
3-Hexyl-2,5-dimethyl-	no odour	>2000
2-Heptyl-3,5-dimethyl-	no odour	>2000
3-Heptyl-2,5-dimethyl-	no odour	>2000
Reference pyrazines	Odour quality	Odour threshold [ng][a]
2-Ethyl-3,5-dimethyl- ***(31)***	roasted	0.002
2,3-Diethyl-5-methyl- ***(41)***	roasted	0.002

[a]Odour threshold values (in absolute ng of each odour impression)

Table 7. Organoleptic properties of tetrahydropyrazines.

Tetrahydropyrazines	Odour quality	Odour threshold [ng][a]
Trimethyl-	roasted	135
2-Ethyl-3,5-dimethyl-	bread crust-like	1.9
3-Ethyl-2,5-dimethyl-	cooked rice-like	70
2,3-Diethyl-5-methyl-	roasted	4.3
2-Propyl-3,5-dimethyl-	no odour	>2000
3-Propyl-2,5-dimethyl-	no odour	>2000
Butyldimethyl-	no odour	>2000
Pentyldimethyl-	no odour	>2000
Hexyldimethyl-	no odour	>2000
Heptyldimethyl-	no odour	>2000

[a]Odour threshold values (in absolute ng of each odour impression)

Some of the 5,6-dihydropyrazines had pleasant roasted odour properties. The lowest odour thresholds in pg range were determined for 2-ethyl-3,5-dimethyl-5,6-dihydropyrazine ***(20)*** and 2,3-diethyl-5-methyl-5,6-dihydropyrazine ***(22)***. These values were as low as those of the reference pyrazine compounds. The different electronic properties at carbons 5 and 6 as well as the slightly different geometry of 5,6-dihydropyrazines in comparison with that of pyrazines (change of hybridization at carbon 5 and 6 from sp^3 to sp^2) obviously had no significant influence on the odour thresholds. Related tetrahydropyrazines, however, had about 1000 times higher thresholds. The higher polarities and the significantly different cyclohexen-like structure of tetrahydropyrazines could lower the affinity to the receptors. Several tetrahydropyrazines, however, imparted interesting odour characterstics. 3-Ethyl-2,5-dimethyltetrahydropyrazines, for example, had the odour of cooked rice. Seven 5,6-dihydropyrazines and six tetrahydropyrazines, all of them with longer alkyl substituents, showed no characteristic odour at concentrations up to 2000 ng.

3.2.4 Main findings

In summary, a new chemoenzymatic route to produce pyrazine derivatives was presented. Biotransformation of aldehydes and 2-oxocarboxylates using whole cells of baker's yeast and subsequent chemical reaction with 1,2-propanediamine yielded 14 5,6-dihydropyrazines and 10 tetrahydropyrazines with yields up to 50 % (based on concentration of the intermediate acyloins). The odour qualities of the compounds were evaluated, and 11 of them showed

pronounced roasted or earthy aroma characteristics. With odour thresholds in pg range, two of the 5,6-dihydropyrazines, compounds ***(20)*** and ***(22)***, can be placed among the pyrazine derivatives with the lowest threshold values described in literature. These results show that the potential of 5,6-dihydropyrazines as aroma compounds has been underestimated before. Furthermore, the aroma properties of tetrahydropyrazines were shown for the first time. 4 of the 10 new compounds exhibited roasted, bread crust-like, or cooked rice-like aroma tonalities. The odour thresholds of the most potent tetrahydropyrazines were in ng range.

3.3 Chemical synthesis of 2-ethenyl-3,5-dimethylpyrazine and 3-ethenyl-2,5-dimethylpyrazine

The second part of this work aimed at the chemical generation of 2-ethenyl-3,5-dimethylpyrazine ***(1)*** and 3-ethenyl-2,5-dimethylpyrazine ***(2)*** Two hypotheses were developed. In the first hypothesis, acetylpyrazines ***(25)*** and ***(26)*** should be reduced to the corresponding pyrazine alcohols. Dehydration was thought to generate the target compounds (Figure 42).

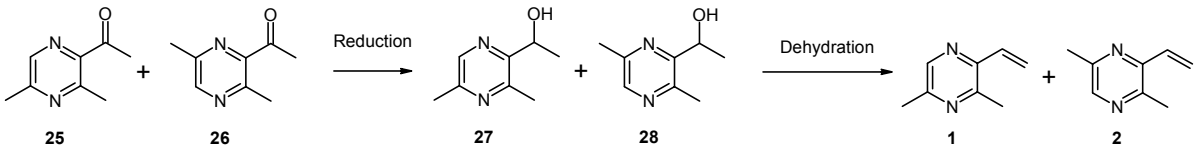


Figure 42. Proposed chemical pathway to generate 2-ethenyl-3,5-dimethylpyrazine and 3-ethenyl-2,5-dimethylpyrazine from acetylpyrazines.

In the second hypothesis, 1-penten-3,4-dione ***(8)*** should be reacted with 1,2-propanediamine to produce 2-ethenyldimethyl-5,6-dihydropyrazines ***(29)*** and ***(30)***, and oxidation was thought to generate the target pyrazines ***(1)*** and ***(2)*** (Figure 43).

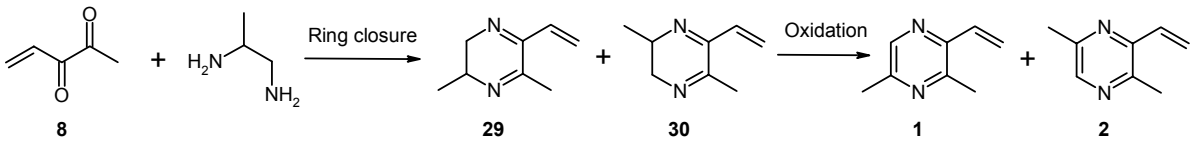


Figure 43. Proposed chemical pathway to generate 2-ethenyl-3,5-dimethylpyrazine and 3-ethenyl-2,5-dimethylpyrazine from 1-penten-3,4-dione.

3.3.1 Chemical synthesis of ethenylpyrazines starting from acetylpyrazines

Following the first hypothesis, a mixture of acetylpyrazines ***(25)*** and ***(26)*** was chemically reduced to the corresponding secondary alcohols (Table 8) (for details see 2.7.3).

Table 8. Chemical reduction of 2-acetyl-3,5-dimethylpyrazine and 3-acetyl-2,5-dimethylpyrazine.

Pyrazine	RI[a]	Yield [%][b]
2-(1-Hydroxyethyl)-3,5-dimethylpyrazine ***(27)*** **	1811	78
3-(1-hydroxyethyl)-2,5-dimethylpyrazine ***(28)*** **	1840	

[a]Linear retention indices on a DB-Wax column.

[b]Yields based on concentrations of ***(25)*** and ***(26)***.

**Compound never synthesized nor characterized before.

The molecular structures of compounds ***(27)*** and ***(28)*** were determined by interpretation of ^{1}H NMR data. To test whether the pyrazine alcohols act as precursors of the corresponding ethenylpyrazines, the mixture of ***(27)*** and ***(28)*** was heated at 220 °C in the presence of oxalic acid for 20 minutes. As Table 9 reveals, three pairs of pyrazines were produced.

Table 9. Heat treatment of pyrazine alcohols in presence of oxalic acid.

Pyrazine	RI[a]	Yield [%][b]
2-Ethyl-3,5-dimethylpyrazine ***(31)***	1424	2.2
3-Ethyl-2,5-dimethylpyrazine ***(32)***	1439	2.2
2-Ethenyl-3,5-dimethylpyrazine ***(1)***	1513	<0.1
3-Ethenyl-2,5-dimethylpyrazine ***(2)***	1531	<0.1
2-Acetyl-3,5-dimethylpyrazine ***(25)***	1670	8.1
3-Acetyl-2,5-dimethylpyrazine ***(26)***	1680	8.0

[a]Linear retention indices on a DB-Wax Column.

[b]Yields based on concentrations of ***(27)*** and ***(28)***.

The structures of the molecules were determined by comparison of their RI and GC-MS spectra with those of reference substances. Target pyrazines ***(1)*** and ***(2)***, however, were produced only in trace quantities. The acetylpyrazines together with the ethylpyrazines are suggested to be formed by redox reaction of ethenylpyrazines and unreacted pyrazine alcohols. The proposed reaction pathway is depicted in Figure 44.

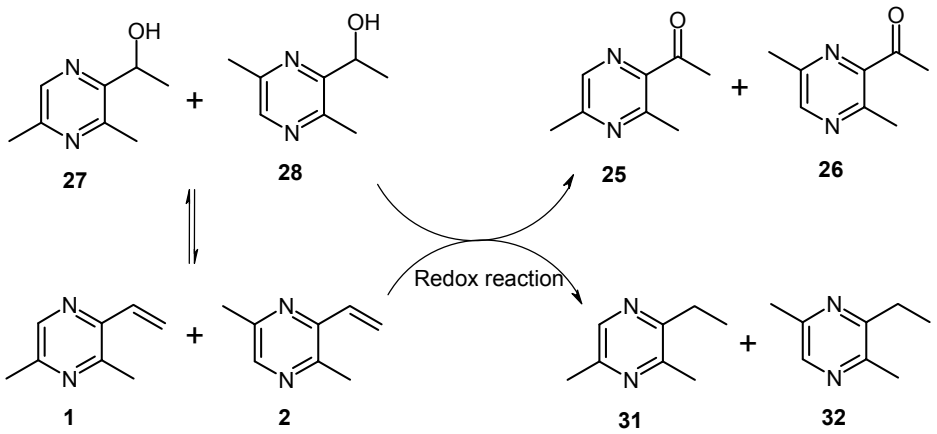


Figure 44. Suggested pathway for the formation of acetyl- and ethylpyrazines from the dehydration of pyrazine alcohols.

As replacing oxalic acid by other organic acids (malonic acid, fumaric acid, phophoric acid) and modifying the heating temperature could not improve the yields of ***(1)*** and ***(2)***, this approach was given up, and the second one (Figure 43) was investigated.

3.3.2 Chemical synthesis of ethenylpyrazines starting from 1-penten-3,4-dione

The second hypothesis to synthesize pyrazines ***(1)*** and ***(2)*** started with the reaction of 1-penten-3,4-dione ***(8)*** and 1,2-propanediamine. After 1 h of incubation, 2-ethenyl-3,5-dimethyl-5,6-dihydropyrazine ***(29)*** and regio isomer 3-ethenyl-2,5-dimethyl-5,6-dihydropyrazine ***(30)*** were produced (Table 10.). At the same time a white precipitate formed in solution.

Table 10. Reaction of 1-penten-3,4-dione with 1,2-propanediamine.

5,6-Dihydropyrazine	RI[a]	Yield [%][b]	Odour quality	Odour threshold [ng]
2-Ethenyl-3,5-dimethyl- ***(29)***	1037	3.4	earthy	0.001
3-Ethenyl-2,5-dimethyl- ***(30)***	1034	3.4	earthy	8

[a]Linear retention indices on a DB-1 Column.

[b]Yields based on concentrations of ***(8)***.

The structures of molecules ***(29)*** and ***(30)*** were proposed by interpretation of GC-MS/EI data. It was suggested that polymerization of 1-pentene-3,4-dione, which probably led to the formation of a white precipitate, was the reason for the low yields (~7 %). Molecules ***(29)*** and ***(30)*** have both never been reported in literature. The organoleptic properties were determined by GC-O and AEDA analysis (see 2.8). Both compounds have pronounced

earthy aroma characteristics and odour thresholds of 0.001 ng and 8 ng, respectively. Thus, molecule ***(29)*** is among the pyrazine derivatives with the lowest odour thresholds. The mixture of ***(29)*** and ***(30)*** was then refluxed in EtOH/KOH. After 3 h, ethenylpyrazines ***(1)*** and ***(2)*** were formed together with ethylpyrazines ***(31)*** and ***(32)*** (Table 11).

Table 11. Heat treatment of ethenyl-5,6-dihydropyrazines in KOH/EtOH.

Pyrazine	RI[a]	Yield [%][b]
2-Ethenyl-3,5-dimethylpyrazine ***(1)***	1081	9.0
3-Ethenyl-2,5-dimethylpyrazine ***(2)***	1077	8.8
2-Ethyl-3,5-dimethylpyrazine ***(31)***	1067	9.8
3-Ethyl-2,5-dimethylpyrazine ***(32)***	1063	8.8

[a]Linear retention indices on a DB-1 Column.

[b]Yields based on concentrations of ***(29)*** and ***(30)***.

The structures of the molecules were determined by comparison of RI and GC-MS spectra with those of reference substances and literature data. The formation of the molecules ***(31)*** and ***(32)*** give rise to suggest that the compounds ***(1)*** and ***(2)*** are likely to be reduced to the ethylpyrazines. The reducing redox partner could be ***(29)*** and ***(30)***.

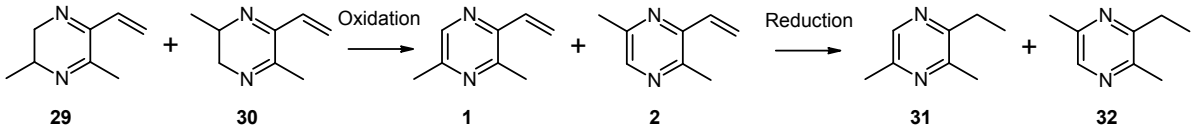


Figure 45. Suggested reaction mechanism leading to ethenyl- and ethylpyrazines from ethenyl-5,6-dihydropyrazines.

In order to favour the oxidation reaction, molecules ***(29)*** and ***(30)*** were again refluxed in EtOH/KOH, however, this time in the presence of the oxidant MnO_2. After 6 h of reaction, exclusively ethenylpyrazines ***(1)*** and ***2)*** were formed in 38 % yield each (Table 12).

Table 12. Heat treatment of ethenyl-5,6-dihydropyrazines in KOH/EtOH/MnO_2.

Pyrazine	RI[a]	Yield [%][b]
2-Ethenyl-3,5-dimethylpyrazine	1081	38
3-Ethenyl-2,5-dimethylpyrazine	1077	38

[a]Linear retention indices on a DB-1 Column.

[b]Yields based on concentrations of ***(29)*** and ***(30)***.

The structures of the molecules were determined by comparison of RI and GC-MS spectra with those of reference substances and literature data. The presence of MnO_2 not only prevented the formation of ethylpyrazines, but also significantly increased the yields. The cycloaddition reaction, however, has clearly to be considered as the bottle neck of this approach, leading to low total yields for the generation of ***(1)*** and ***(2)***. Based on all the results obtained so far, a five-step approach for the total chemical synthesis of ***(1)*** and ***(2)*** was proposed.

3.3.3 Total chemical synthesis of 2-ethenyl-3,5-dimethylpyrazine and 3-ethenyl-2,5-dimethylpyrazine

The low yields of the reaction between 1,2-propanediamine and 1-penten-3,4-dione ***(8)***, probably due to self polymerization of the latter, gave rise to apply protection chemistry. The modified synthetic route for the total synthesis of ***(1)*** and ***(2)*** is described in Figure 46 (for experimental details, see 2.7.1.1, 2.7.2.1, and 2.7.7.

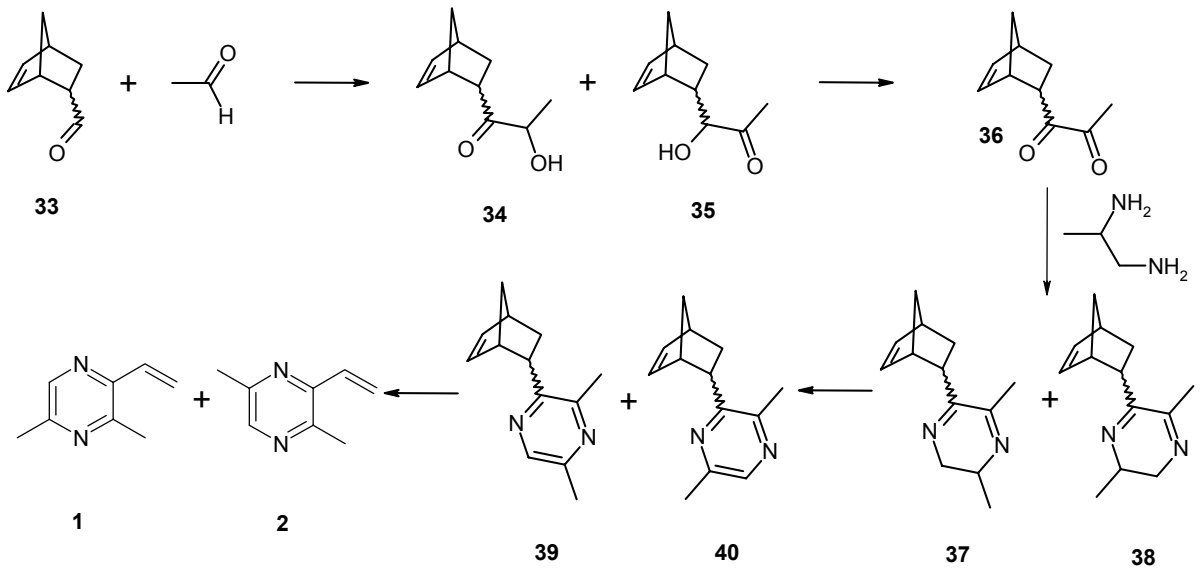


Figure 46. Total synthesis of 2-ethenyl-3,5-methylpyrazine and 3-ethenyl-2,5-dimethylpyrazine.

Acyloins ***(34)*** and ***(35)*** were prepared by the condensation reaction of 5-norbornen-2-carboxaldehyde ***(33)*** and acetaldehyde in the presence of a thiazolium catalyst. As this type of reaction had been reported in literature by pure organic chemistry, it was prefered to conduct the acyloin condensation by this reported way. The oxidation of ***(34)*** and ***(35)*** with Bi_2O_3 led to the formation of compound ***(36)*** as a 70:30 mixture of *exo* and *endo* isomers. The two steps were performed with an overall yield of 40 %. Diketone ***(36)*** was then reacted

with 1,2-propanediamine and afforded 5,6-dihydropyrazines ***(37)*** and ***(38)***. These molecules were oxidized to pyrazines ***(39)*** and ***(40)*** in EtOH/KOH in the presence of MnO_2. An overall yield of 41 % was achieved for these two steps. At this stage, *exo* and *endo* isomers could be separated by preparative chromatography on silicagel. Although the *exo* fraction (70 % of the total pyrazines) was an equimolar mixture of ***(39)*** and ***(40)***, the *endo* fraction was composed of ***(39)*** and ***(40)*** in a 80:20 molar ratio as determined by heteronuclear multi-bond connectivity-NMR (HMBC-NMR) analysis. In experimental conditions for which only geminal and vicinal coupling constants are observed, the major compound shows an intense correlation between its aromatic hydrogen and the norbornenyl substituted C_2 of the pyrazine ring. These results show that the reaction between *endo*-***(36)*** and 1,2-propanediamine proceeds regioselectively. The Retro-Diels-Alder reaction of *exo*-***(39)*** and *exo*-***(40)*** was realized by gas phase pyrolysis at 600 °C and afforded ***(1)*** and ***(2)*** in 1:1 ratio and 85 % yield (Figure 47).

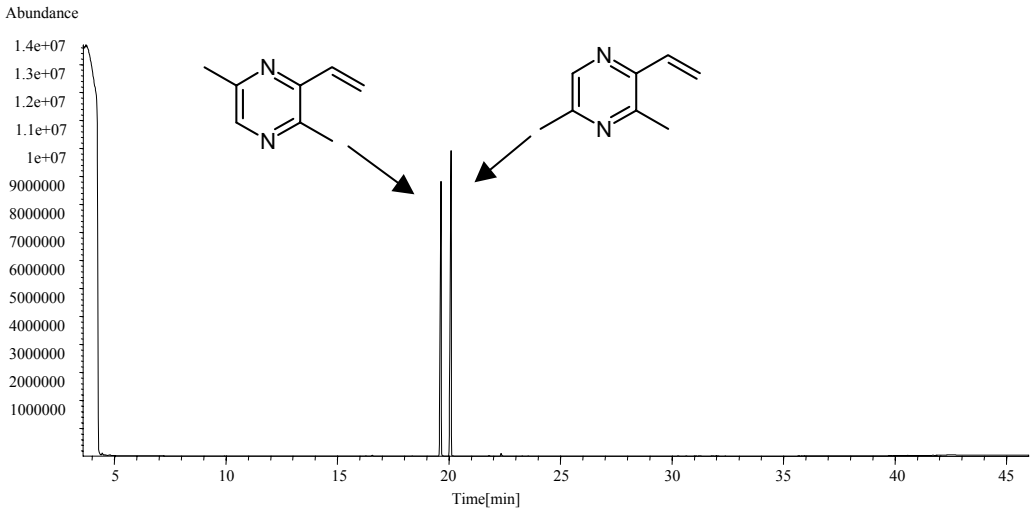


Figure 47. GC-MS chromatogram of 2-ethenyl-3,5-dimethylpyrazine and 3-ethenyl-2,5-dimethylpyrazine produced by Retro-Diels-Alder reaction.

3.3.4 Stability of ethenylpyrazines

The use of aroma compounds as additives in food products demands their stability in the food matrix until consumption. Due to the potential for their use in coffee beverages, preliminary investigations studied the stability of ***(1)*** and ***(2)*** in a 2 % coffee solution (KS-UK 10638.02, spray-dried coffee UK) as well as in a 0.01 M acetate buffer (pH 5.2). In both cases, after 11 h of incubation, no loss of both compounds was observed as judged by SPME-GC-MS (see 2.7.8).

3.3.5 Main findings

In the course of the investigations to find an efficient way to produce the target pyrazines ***(1)*** and ***(2)***, two new ethenylpyrazine derivatives, compound ***(29)*** and ***(30)*** were synthesized with 7 % total yield. Both compounds have pronounced earthy aroma characteristics and odour thresholds of 0.001 ng and 8 ng, respectively. Thus, molecule ***(29)*** is among the pyrazine derivatives with the lowest odour thresholds. Furthermore, a new chemical synthesis of 2-ethenyl-3,5-dimethylpyrazine ***(1)*** and 3-ethenyl-2,5-dimethylpyrazine ***(2)*** was accomplished in five steps giving a total yield of 14 %. The approach described here could be used to synthesize other alkenylpyrazines. ***(1)*** and ***(2)*** are stable in a 2 % coffee matrix and, thus, could probably be added to coffee-based beverages without the risk of degradation.

4 Discussion

4.1 Acyloins

4.1.1 Natural occurrence of acyloins

Several acyloins have been identified in foodstuffs. Most frequently, 3-hydroxy-2-butanone ***(7)*** has been reported. The compound has a buttery aroma and taste and is used as flavour compound in margarine and bakery products, for example.[32] [33] 3-Hydroxy-2-pentanone ***(5)*** and 2-hydroxy-3-pentanone ***(6)*** were found in cheese[34], honey[35], wine[36], butter,[37] sherry[38], and soy sauce[38]. Further food sources of acyloins are summarized in Table 13.

Table 13. Food sources of acyloins as described by Watanabe *et al.*[39], Moio *et al.*[34], Burdock[32], Nijssen *et al.*[40], Brock *et al.*[41], and Neuser *et al.*[38]

Acyloin	Natural occurrence in food
3-Hydroxy-2-pentanone ***(5)***	Cheese, durian, wine, aspargus, honey, tea, butter, sherry, soy sauce
2-Hydroxy-3-pentanone ***(6)***	Cheese, durian, coffee, wine, honey, butter, sherry, soy sauce
2-Hydroxy-3-hexanone	Wine
4-Hydroxy-3-hexanone ***(12)***	Durian
3-Hydroxy-5-methyl-2-hexanone	Mozzarella cheese
2-Hydroxy-5-methyl-3-hexanone	Sherry
3-Hydroxy-2-octanone ***(19)***	Beef and mutton fat (heated)
5-Hydroxy-4-octanone	Cocoa
3-Hydroxy-4-phenyl-2-butanone	Wine, sherry, honey

The formation of acyloins in food is mainly confered by microorganisms. The generation of compound ***(7)*** is done by two enzymes. Acetolactatsynthase catalyzes the formation of acetolactat from pyruvate and hydroxyethyl-TPP (produced by PDC). The subsequent decarboxylation is catalyzed by a second enzyme, acetolactat decarboxylase, and yields compound ***(7)***.[42]

In wine, acyloins ***(5)***, ***(6)***, and ***(7)*** are accompanied by the corresponding diols and diketones. The diketones are mainly formed outside the cell from spontaneous degradation of acetolactate or 2-aceto-2-hydroxybutyrate, respectively, and can be reduced to the acyloins.[43]

4.1.2 Importance of acyloins as aroma compounds

For a long time, just a few acyloins had been characterized in terms of their organoleptic properties. Only recently, Neuser and coworkers[38] synthesized 34 acyloins and systematically evaluated their odour qualities and threshold values. 23 of them possessed pronouced aroma characteristics (Table 14).

Table 14. Odour qualities and threshold values (in absolute ng of each odour impression) of acyloins as reported by Neuser *et al.*[38]

Acyloin	Odour qualities	Odour threshold [ng]
3-Hydroxy-2-pentanone ***(5)***	Caramel-sweet, buttery	500-600
2-Hydroxy-3-pentanone ***(6)***	Buttery, hay-like	250–300
2-Hydroxy-3-hexanone	Green, hay-like, sour milk	500-600
3-Hydroxy-2-hexanone	Earthy, mushroom-like	900-1000
2-Hydroxy-3-heptanone	Floral, buttery, mushroom-like	100-150
3-Hydroxy-2-heptanone	Earthy, hay-like, herbaceous	400-500
2-Hydroxy-3-octanone ***(17)***	Floral-sweet, buttery	40-50
3-Hydroxy-2-octanone ***(19)***	Earthy, mushroom-like, herbaceous	250-300
2-Hydroxy-3-nonanone	Buttery, sweet	100-120
3-Hydroxy-2-nonanone	Mushroom-like, buttery	500-600
2-Hydroxy-3-decanone	Fruity-sweet, floral, green	100-120
3-Hydroxy-2-decanone	Green, herbaceous, hay-like	700-800
2-Hydroxy-5-methyl-3-hexanone	Cheese, sour milk	400-500
3-Hydroxy-5-methyl-2-hexanone	Cheese, sour milk	800-900
2-Hydroxy-4-methyl-3-heptanone	Floral-green, hay-like	80-120
3-Hydroxy-4-methyl-2-heptanone	Floral-earthy, hay-like	200-250
2-Hydroxy-4*E*-hepten-3-one ***(18)***	Floral, spicy, earthy	50-80
3-Hydroxy-4*E*-hepten-2-one	Earthy, mushroom-like	250-300
2-Hydroxy-4*E*-octen-3-one	Floral, green, woody	20-30
3-Hydroxy-4*E*-octen-2-one	Earthy, floral, mushroom-like	80-100
2-Hydroxy-5-(methylthio)-3-pentanone	Spicy, meaty, sulfurous	15-20
3-Hydroxy-5-(methylthio)-2-pentanone	Spicy, alliaceous, sulfurous	5-10
3-Hydroxy-4-phenyl-2-butanone	Floral-Sweet	75-100

The odour qualities were found to differ considerably within this class of compounds. The two components of an isomeric pair often showed similar sensory characteristics. The 2-hydroxy-

3-oxo-acyloins displayed distinctly lower odour thresholds than the respective 3-hydroxy-2-oxo isomers. The only exception in this respect were the methylthio-acyloins, which, moreover, were the most powerful flavours examined. The pleasant odour properties, along with low odour threshold values of some of the acyloins, reflected the potential of this class of compounds as flavour ingredients in food. Five acyloin pairs, however, were odourless under the conditions applied. These were short-chain acyloins (max. C_6) that were branched and/or unsaturated (Table 15).[26]

Table 15. Odourless acyloins as described by Neuser[26].

Acyloin	Odour qualities	Odour threshold [ng]
2-Hydroxy-4-methyl-3-pentanone	Odourless	≥1500
3-Hydroxy-4-methyl-2-pentanone	Odourless	≥1500
2-Hydroxy-4-methyl-3-hexanone	Odourless	≥1500
3-Hydroxy-4-methyl-2-hexanone	Odourless	≥1500
2-Hydroxy-4*E*-hexen-3-one	Odourless	≥1500
3-Hydroxy-4*E*-hexen-2-one	Odourless	≥1500
2-Hydroxy-4-methyl-4*E*-hexen-3-one	Odourless	≥1500
3-Hydroxy-4-methyl-4*E*-hexen-2-one	Odourless	≥1500
2-Hydroxy-5-methyl-4*E*-hexen-3-one	Odourless	≥1500
3-Hydroxy-5-methyl-4*E*-hexen-2-one	Odourless	≥1500

In this work, the new acyloin 3-hydroxy-1-penten-4-one ***(3)*** and its tautomer, 4-hydroxy-1-penten-3-one ***(4)***, were shown to be odourless under the conditions applied. The determination of the organoleptic properties of the new unsaturated acyloins ***(10)***, ***(13)***, ***(14)***, ***(15)***, and ***(16)*** should be evaluated in near future. It can be expected that especially the longer ones have interesting aroma properties.

4.1.3 Importance of acyloins as aroma precursors

In addition to their potential as aroma compounds *per se*, acyloins have been investigated in their role as aroma precursors. Apart from pyrazines, other aroma-active heterocycles, such as oxazoles, oxazolines, thiazoles, and thiazolines have been produced from acyloins (Figure 48).

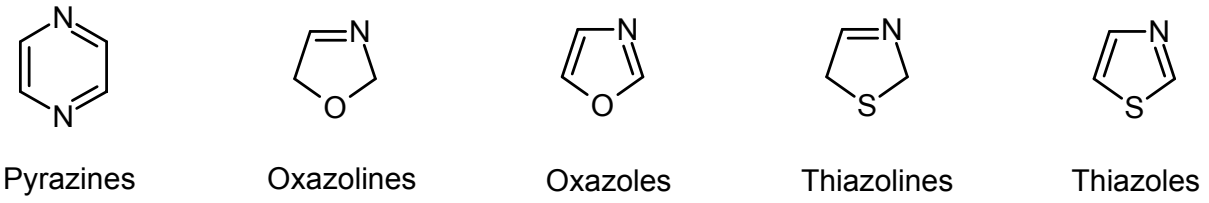


Figure 48. Five classes of heterocyclic compounds with acyloins as precursors.

Maga has reviewed the occurrence of oxazoles and oxazolines in a variety of processed food systems.[44] Most of them possess green, sweet, and nutty aroma qualities and have been identified in coffee[45 46], soy sauce[47], wheat[48], and cooked beef[49], and some of them have very low odour thresholds, The occurrence of thiazoles and thiazolines has also been intensively reviewed.[50 51] They are generally described as having green, nutty, and vegetable-like aroma characteristics and have been found in heated foods such as baked potato[52], roasted peanuts[53], peanut butter[54], cocoa butter[55], and fried chicken[56].

Acyloins as precursors of pyrazines

Griffith and Hammond [57] studied the generation of Swiss cheese flavour by the reaction of amino acids with carbonyl compounds and reported that several alkylpyrazines can be formed from a mixture of lysine-acetoin, lysine-acetol, or lysine-dihydroxyacetone at room temperature (Figure 49). They suggested that the final stages of flavour formation in Swiss cheese were dominated by chemical reactions rather than enzymatic or microbiological processes.

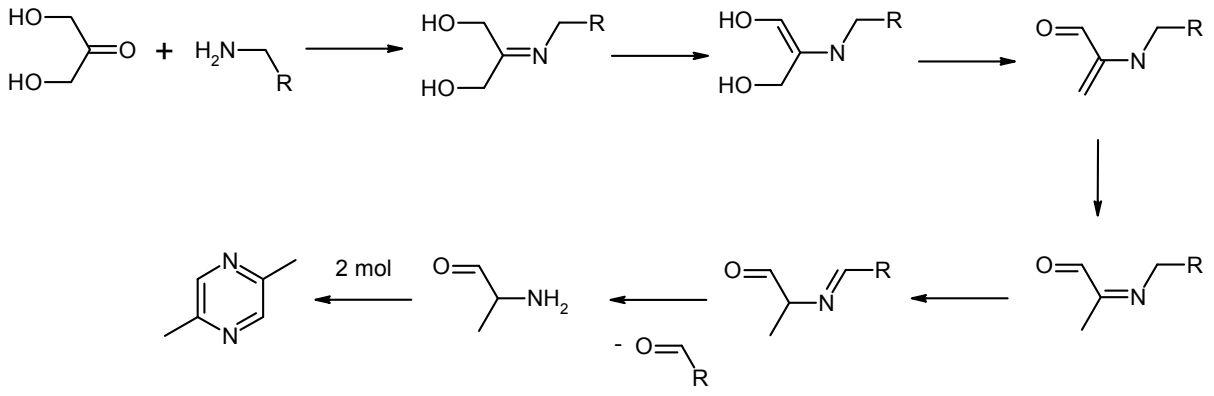


Figure 49. Pathway to generate 2,5-dimethylpyrazine from lysine and dihydroxyacetone at ambient temperature as suggested by Griffith and Hammond[57].

Furthermore, Rizzi[58] found that alkylpyrazines can be produced from concentrated solutions of acyloins and ammonium salts (3.3 M for the acyloin) at room temperature.

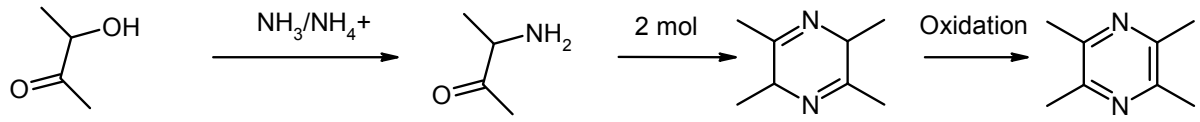


Figure 50. Generation of alkylpyrazines from acyloins and ammonium precursors as described by Rizzi[58].

Tetramethylpyrazine was formed from acetoin and ammonium acetate at room temperature with a yield of 13.0 %. (Figure 50) Increasing temperature could improve the yields up to 58.0 %. Dilution of reactants with water (0.4 M for acetoin) led to greatly reduced pyrazine yields (0.2 %). With these diluted conditions, Huang and coworkers[59] observed a significant enhancement of tetramethylpyrazine formation under high hydrostatic pressure.

Acyloins as precursors of oxazolines and oxazoles

In 1995, Shu and Lawrence[60] found that tetramethylpyrazine is not the only compound formed in acetoin/ammonia systems. They identified 2,4,5-trimethyl-2-(1-hydroxyethyl)-3-oxazoline, which was described as nutty and breat-crust-like. It was proposed that condensation of acetoin and ammonia generates an α-hydroxyimine and an α-amino ketone by tautomerism (Figure 51). The latter forms tetramethylpyrazine as already proposed by Rizzi (see Figure 50). The α-hydroxyimine was proposed to condense with a second molecule of acetoin and then cyclize by Schiff base formation to the oxazoline.

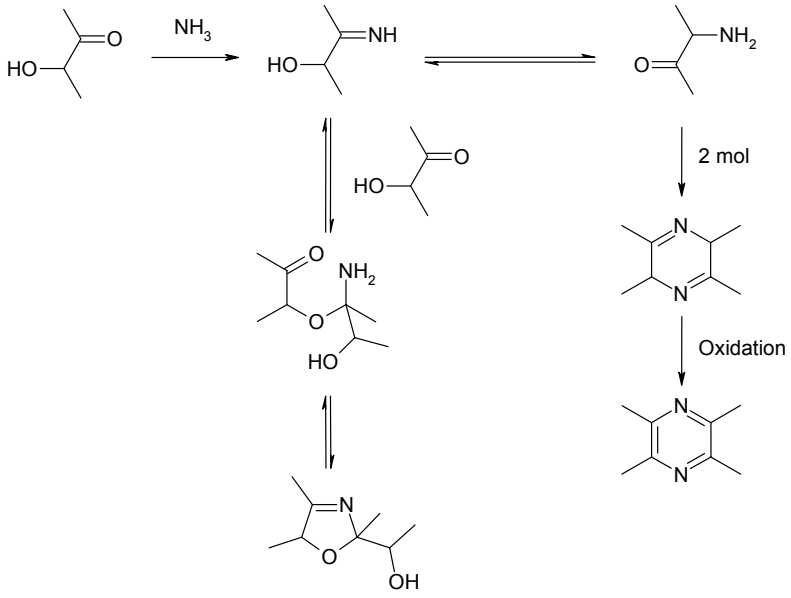


Figure 51. Formation mechanism of 2,4,5-trimethyl-2-(1-hydroxyethyl)-3-oxazoline as suggested by Shu and Lawrence[60].

Two years later, Fu and Ho[61] identified 2,4,5-trimethyloxazole in the 3-hydroxy-2-butanone/ammonium acetate model system and suggested its formation by oxidation of the 3-oxazoline.

Acyloins as precursors of thiazolines and thiazoles

Recently, Xi and coworkers[62] studied the reactions between 3-hydroxy-2-butanone and ammonium sulfide at 25 °C. Four well known flavour compounds, 2,4,5-trimethyloxazole, 2,4,5-trimethyl-3-oxazoline, 2,4,5-trimethyloxazole, and 2,4,5-trimethyl-3-thiazoline, were identified. Two further interesting oxazoline and thiazoline compounds were also identified by GC-MS (Table 16).

Table 16. Volatile compounds generated from 3-hydroxy-2-butanone/ammonium sulfide model system at 50 °C as reported by Xi *et al.*[62]

Compound	Quantity [mg/g of acetoin]
2,4,5-Trimethyl-3-oxazoline	0.101
2,4,5-Trimethyloxazole	0.113
2,4,5-Trimethylthiazole	0.086
2,4,5-Trimethyl-3-thiazoline	0.018
2-(1-Hydroxyethyl)-2,4,5-trimethyl-3-oxazoline	21.394
2-(1-Mercaptoethyl)-2,4,5-trimethyl-3-oxazoline	0.710
2-(1-Hydroxyethyl)-2,4,5-trimethyl-3-thiazoline	1.230
2-(1-Mercaptoethyl)-2,4,5-trimethyl-3-thiazoline	1.849

3-Hydroxy-2-butanone was suggested to react with ammonia and hydrogen sulfide, generated from ammonium sulfide, to form 2-amino-3-butanone, 3-mercapto-2-butanone, ans 3-mercapto-2-iminobutane. These compounds are thought to react which each other to form the 2-(1-hydroxyethyl)- and 2-(1-mercaptoethyl)-substituted oxazoline and thiazoline compounds. At higher temperatures, these intermediates might undergo further transformation to the corresponding oxazoles and thiazoles.

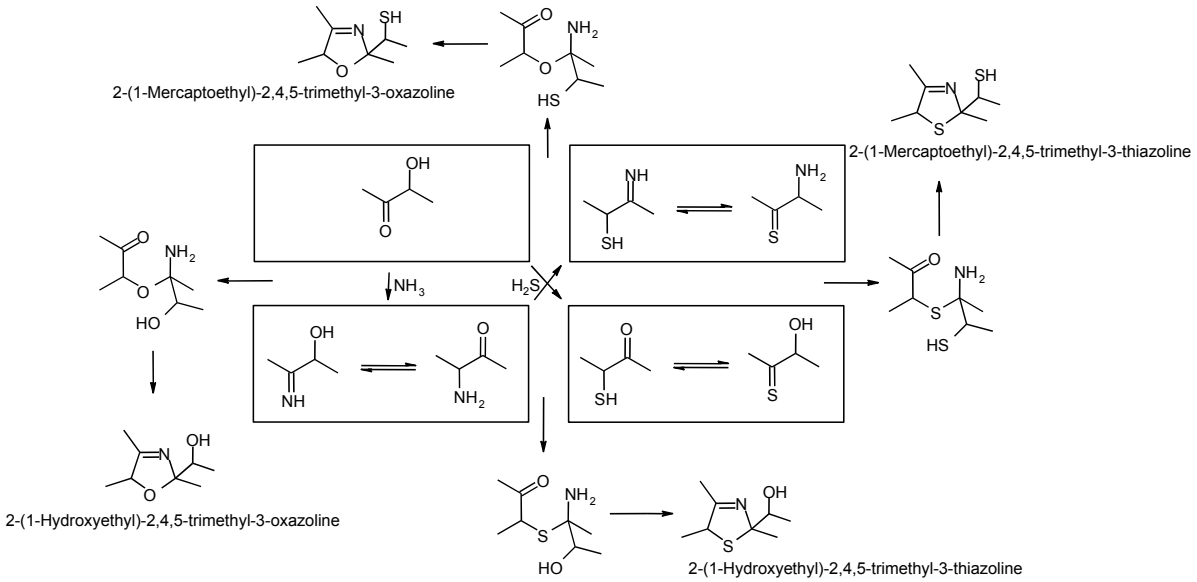


Figure 52. Formation of oxazolines and thiazolines in 3-hydroxy-2-butanone/ammonium sulfide model system as suggested by Xi *et al.*[62]

A row of other thiazolines and thiazoles were produced by reaction of acyloins with ammonium sulfide in the presence of aliphatic aldehydes (Table 17).[63]

Table 17. Thiazolines and thiazoles produced from the reactions of acetoin and ammonium sulfide in the presence of several aliphatic aldehydes as described by Elmore and Mottram[63].

Aldehyde	3-Thiazolines	Yield [%][a]	Thiazoles	Yield [%][a]
2-Methylpropanal	4,5-Dimethyl-2-isopropyl	26.0	4,5-Dimethyl-2-isopropyl	0.6
3-Methylbutanal	4,5-Dimethyl-2-isobutyl	15.2	4,5-Dimethyl-2-isobutyl	0.2
Pentanal	2-Butyl-4,5-dimethyl	18.2	2-Butyl-4,5-dimethyl	0.2
Hexanal	4,5-Dimethyl-2-pentyl	36.7	4,5-Dimethyl-2-pentyl	1.7
Heptanal	4,5-Dimethyl-2-hexyl	41.8	4,5-Dimethyl-2-hexyl	0.3
Octanal	4,5-Dimethyl-2-heptyl	33.5	4,5-Dimethyl-2-heptyl	0.5
Nonanal	4,5-Dimethyl-2-octyl	31.4	4,5-Dimethyl-2-octyl	0.4
Decanal	4,5-Dimethyl-2-nonyl	26.2	4,5-Dimethyl-2-nonyl	0.7

[a] relative amounts as percentage of total chromatogram peak area

The 2-position of the heterocycles was occupied by an alkyl group derived from the aliphatic aldehyde, while the substituents in the 4- or 5-position were from the acyloin. The proposed mechanism is shown in Figure 53.

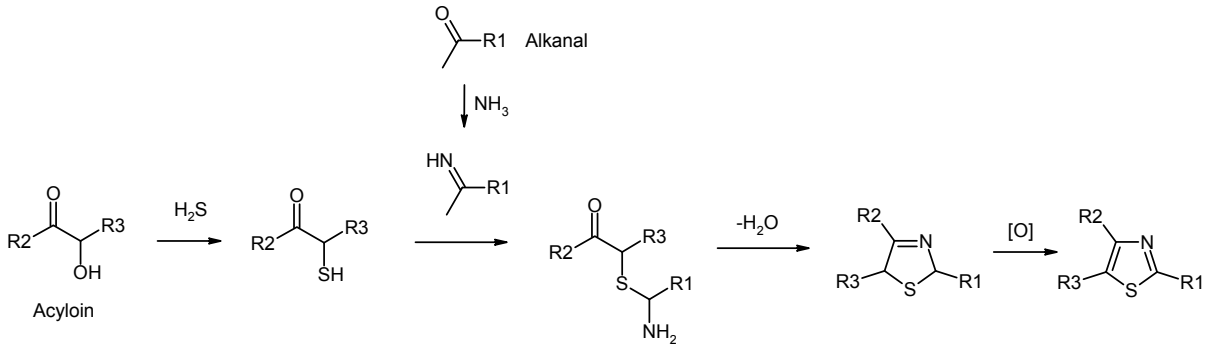


Figure 53. Formation of alkyl-3-thiazolines and alkylthiazoles from the reaction of acyloins with ammonium sulfide in the presence of aliphatic aldehydes as suggested by Elmore and Mottram.[63]

Substitution of the hydroxy group with a thiol group is thought to be followed by nucleophilic attack by the sulfur at the carbon atom of an imine intermediate formed by the reaction between ammonia and aldehyde. Subsequent ring closure, with the elimination of water, yields the 3-thiazoline. Thiazoles could result from the oxidation of the thiazolines.

4.1.4 Biochemical generation of acyloins

Two major approaches to generate acyloins are well documented in literature. Bel Rhlid and coworkers reported the enantiogenic microbiological reduction of acyclic 2,3-diketones[23] and 3,4-diketones[25] using the microorganisms *Aspergillus niger*, *Beauveria sulfurescens*, *Geotrichum candidum*, *Dipodascopsis uninucleata*, *Rhodotorula rubra, and Saccharomyces cerevisiae*. The reactions led to the formation of acyloins in good yields (up to 50 %) and were accompanied by the production of the corresponding diols. For most of the acyloins, high enantiomeric excesses (up to enantiopurity) were observed.

On the other hand, the generation of acyloins using a carboligase activity was already reported in 1921, when Neuberg and Hirsch[64] found that fermenting yeast could catalyze the C-C bond formation. At first, this reaction was not considered as a side reaction of PDC, until Dirscherl[65], in 1930, formulated this hypothesis. Singer and Pensky[66] supported the hypothesis by showing acetoin production from pyruvate and acetaldehyde with PDC from wheat germs. The PDC-catalyzed acyloin production was much later unequivocally proven by several groups who used isolated pyruvate decarboxylases for their biotransformation assays.[67 68 69]

Various laboratories have tried to optimize the effiency of the acyloin condensation, by searching for better catalysts [70], applying several fermentation techniques [71 72], immobilizing PDC enzymes or cells [73 74 74 75 76] or applying site-directed mutagenesis to the *Zymomonas*

mobilis enzyme [77]. Recently PDC from *Zygosaccharomyces bisporus* was isolated, characterized, and further investigated.[78]

The scientific discovery of the asymmetric generation of acyloins has from early on attracted interest in the pharmaceutic industry. Since 1930, the most commonly used method for the production of L-phenylacetylcarbinol (L-PAC) has been the acyloin condensation of pyruvate and benzaldehyde using *S. cerevisiae* as catalyst [79], as shown in Figure 54. L-PAC is used as substrate in a subsequent chemical reaction which results in the production of L-ephedrine. The latter is used in the treatment of conditions including hypotension and asthma.

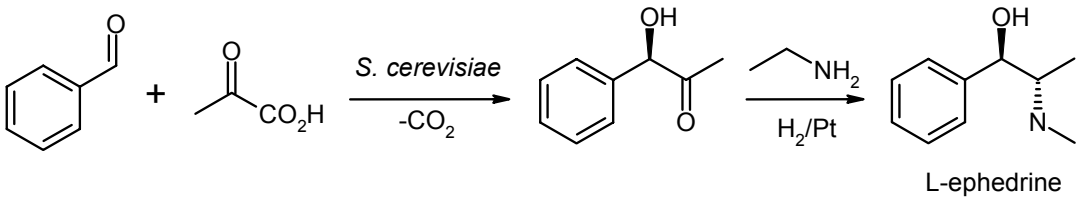


Figure 54. Synthesis of L-ephedrine from L-PAC prepared by biotransformation with *S. cerevisiae*.

4.1.4.1 Pyruvate decarboxylase

Pyruvate decarboxylase (E.C. 4.1.1.1) is a key enzyme in glycolysis and ethanol fermentation. It catalyzes the irreversible non-oxidative decarboxylation of pyruvate to acetaldehyde and CO_2. The enzyme employs thiaminpyrophosphate (TPP) and Mg^{2+} ions as cofactors[80 81], which dissociate from the protein at pH values above 7.5.[82] Since its first detection in yeast extracts[83], the enzyme has been found in many other fungi[84], plants[85 86 87], and bacteria[88 89].

All characterized PDCs have an oligomeric structure and in most cases they are composed of four identical or almost identical subunits with a molecular mass of 60 kDa per subunit.[90] The subunit of PDC consists of three distinct structural domains.[80] The amino- and carboxy-terminal domains, which are also called α-domain and γ-domain, respectively, are similar in structure and have the same fold as the corresponding domains of pyruvate oxidase and transketolase.[91] These two domains are involved in binding of the cofactors and pyruvate. The β-domain, located in the middle of the polypeptide chain, contains a seven-stranded mixed sheet with five parallel and two antiparallel strands. This domain is involved in activation of the enzyme by the pyruvate and it has been proposed that it contains the binding site for activators such as pyruvamide.[80 92] Two of the subunits associate tightly to form a dimer. As in other TPP dependent enzymes the cofactors are bound between the α- and γ-domains from two different subunits at the subunit-subunit interface.[91] Two of these

dimers assemble to a tetramer, which can be described as dimer of dimers.[80 93] The dimer-dimer interactions are formed almost exclusively through residues from the β-domain, the most important being the extension of the seven-stranded β-sheet in the β-domains across the subunit-subunit interface to a large 14-stranded β-sheet. Farrenkopf and coworkers separated three different isoenzymes (α_4, $(\alpha\beta)_2$, β_4) of PDC from *S. cerevisiae* by anion exchange chromatography.[94] The subunits of the isoforms slightly differ in size. The kinetic data of the decarboxylation reaction for the isoenzymes were similar.

Genes for pyruvate decarboxylases have been isolated from yeasts and fungi such as *Saccharomyces cerevisiae*, *Zygosaccharomyces bisporus*, *Hanseniaspora uvarum*, *Klyveromyces marxianus*, *Klyveromyces lactis*, *Neurospora crassa*, *Aspergillus parasitus*, from plants, like maize, rice, tomato, pea, and tobacco, and from the bacterium *Zymomonas mobilis*.[95 78] These PDC sequences enoced subunits of about 562-610 amino acids. Enzymes from plants (pea, maize, rice) have longer chains compared to the enzymes from yeasts, fungi, and bacteria. Six genes have been reported for PDC from *Saccharomyces cerevisiae*, three of which are structural ones.[96 97 98 99 100] PDC1 codes for the major isoenzyme and is reponsible for most of the wt-PDC activity.[99 100 98] The expression of either PDC1[98] or PDC5[101] in *E. coli* resulted in the formation of active homotetramers. It is thought that the partial proteolytic digestion of one single gene product is the origin of the different isoforms with slightly different subunit sizes. This assumption has been supported by the isolation of active homotetrameric PDC from a protease-deficient *S. cerevisiae* strain, which was composed of only the larger subunit.[96]

An extraordinary property of all PDC's characterized so far, except the enzyme from *Zymomonas mobilis*, is the allosteric substrate activation process first described by Davies[102] in 1967. Substrate analogues such as pyruvamide[103] also activate the enzyme. Based on X-ray structure[80] and biochemical investigations[92] it has been proposed that substrate activation occurs by binding to the regulatory β-domain, specifically to Cys221, which induces a confirmational transition from the inactive to the active conformation of the enzyme.

PDC increases the rate of decarboxylation of pyruvate by thiamine alone by a factor 3×10^{12} at pH 6.2 an 30°C.[104] The structure of the cofactor is shown in Figure 55.

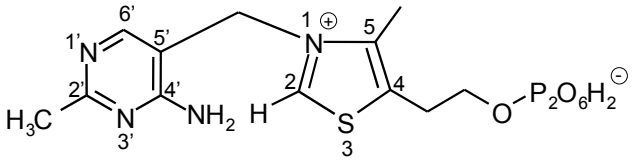


Figure 55. Molecular structure of thiamin pyrophosphate.

The different steps which are relevant for the thiamine-catalyzed decarboxylation and the formation of acyloins are depicted in Figure 56.

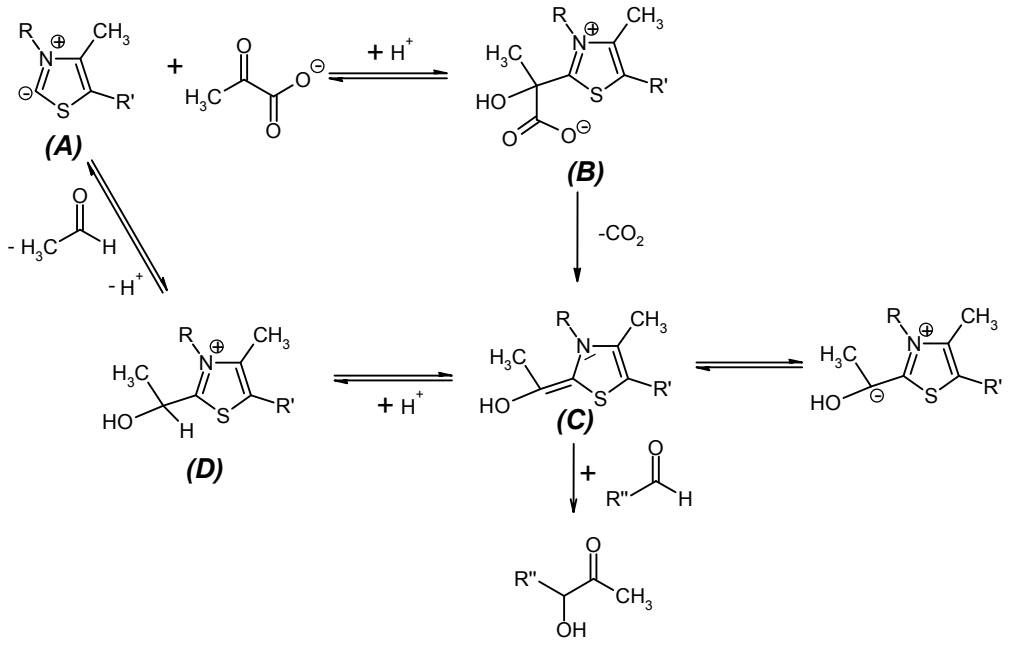


Figure 56. Reaction path of enzymatic pyruvate decarboxylation and formation of acyloins.

The reaction cycle is started with the formation of a TPP carbanion ***(A)***. Recent NMR-investigations showed that the C2 of TPP is undissociated in the enzyme-bound state and does not exist as a discrete carbanion.[105] The fast deprotonation occurs upon addition of the substrate pyruvate and is accelerated in the substrate activated state of PDC from *S. cerevisiae*. In PDC from *Z. mobilis*, which is not activated by the substrate, no differences in the deprotonation step have been detected in the presence or absence of the substrate pyruvate.

In the next step, the negative charge at C2 of TPP ***(A)*** performs a nucleophilic attack on the α-carbonyl group of pyruvate. Stabilization of the resulting double negatively charged species by proton transfer to the former carbonyl group yields α-lactyl-TPP ***(B)***. The decarboxylation of α-lactyl-TPP results in the formation of an α-carbanion/enamine, which is also known as "active acetaldehyde" ***(C)***. The latter is subsequently protonated to give

hydroxyethyl-TPP ***(D)***. The cleavage of acetaldehyde from hydroxyethyl-TPP followed by deprotonation results in the regeneration of the TPP-carbanion ***(A)***.[106] [107] It is important to mention that the last steps of this reaction cycle are reversible.[69] Thus, "active acetaldehyde" ***(C)*** may also be obtained upon binding of acetaldehyde to TPP and subsequent deprotonation. Instead of being protonated, "active acetaldehyde" ***(C)*** may also react with a second aldehyde cosubstrate to form acyloins.[95] [108]

4.1.4.2 Biogeneration of acyloins with baker's yeast

The capability of PDC to synthesize acyloins from pyruvate and a wide range of aromatic and α,β-unsaturated aldehydes has been intensively studied. Most of these studies were performed using baker's yeast under fermenting conditions. Fuganti and Grasselli [109] [110] incubated α,β-unsaturated aromatic aldehydes such as cinnamaldehyde derivatives with baker's yeast, and identified, the unsaturated diols, extended by a C2 unit, which were derived from reduction of the acyloins. Stumpf and coworkers[111] [112] as well as Ohta and coworkers[113] also isolated diols, and not acyloins, from acyclic α,β-unsaturated aldehydes or aromatic aldehydes, respectively. The only systematic study which investigated the formation of aliphatic saturated and α,β-desaturated acyloins was recently investigated by Neuser[38], who observed the generation of numerous acyloins by incubation of different aldehydes and 2-oxo acids with PDC from *Zygosaccharomyces bisporus.*

Refering to the latter results, the capability of PDC from baker's yeast to produce those and other new aliphatic acyloins from aliphatic aldehydes and 2-oxocarboxylates was investigated in this work. Special attention was paid to the biotransformation of acrolein and 2-oxocarboxylates. First of all, acrolein has, probably due to its known toxicity, never been used as substrate in enzymatic reactions before. Secondly, the products, 3-hydroxy-1-penten-4-one ***(3)*** and 3-hydroxy-1-hexen-4-one ***(10)*** have never been produced nor characterized in literature before. The industrial interest for these compounds can obviously be seen by the fact, that their use as substrates for the enzymatic transformation into the corresponding amino alcohol has already been patented.[114]

4.1.4.2.1 Biogeneration and characterization of (*R*)-3-hydroxy-1-penten-4-one

Maximal product concentrations for ***(3)*** were obtained with whole cells of baker's yeast and amounted to 150 mg L^{-1}, what corresponded to about 3 % yield at 50 mM substrate concentrations (see 3.1.1.2). Acrolein probably reacts not only unspecifically with amino acid residues of PDC, but rather also with other proteins, and other polar compounds, thereby leading to low total yield. Similar suggestions were made in studies on the inactivation of the *Escherichia coli S*-Adenosylmethionine (AdoMet) decarboxylase.[115] Added acrolein inactivated AdoMet decarboxylase, and there was only limited protection from acrolein

inactivation by the competitive inhibitor methylglyoxyl-*bis*-guanylhydrazone.[115] This suggested that acrolein did not specifically react with the active site. Because of the limited tolerance of PDC to acrolein, various physical methods could be applied to reduce the exposure to the enzyme or yeast. The conventional methods used include dosing regimes, immobilization of biomass, biphasic bioconversions and the use of additives.

The absolute configuration of ***(3)*** was assigned as *R* and the specific optical rotation was determined as minus 75 degrees. This data goes in line with what is described in literature. All published aliphatic acyloins of the (*R*)-configuration have negative optical rotation values, while those of the (*S*)-configuration show positive values. Examples are given in Table 18.

Table 18. Specific optical rotations of some aliphatic acyloins.

Acyloin	Absolute configuration	Ref	$[\alpha]^{25}_{J}$ [deg]	ee [%]
4-Hydroxy-1-penten-3-one	*S*	[21]	+62.0 (c 1.02 in $CHCl_3$)	>99
3-Hydroxy-2-butanone	*S*	[23]	+96.0 (c 0.05 in $CHCl_3$)	≥98
3-Hydroxy-2-octanone	*R*	[23]	-74 (c 0.02 in $CHCl_3$)	80
3-Hydroxy-2-pentanone	*S*	[23]	+15 (c 0.02 in $CHCl_3$)	30

The tautomer of acyloin ***(3)***, 4-hydroxy-1-penten-3-one ***(4)*** was also identified in the reaction medium as a minor component. The predominant formation of the 3-hydroxy isomers was also observed by Neuser [26], when pyruvate was reacted with aldehydes. The fact that the (*R*)-enantiomer of ***(4)*** was also identified with an enantiomeric excess of 92 % showed that both tautomers are enzymatically produced, and tautomerization is unlikely to be involved.

The formation of the acyloins ***(3)*** and ***(4)*** was furthermore accompanied by small amounts of γ-pentalactone ***(9)***, which was suggested to be formed from reduction of ethyl pentanoate and subsequent ring closure. These steps of lactone formation have already been reported in literature, where baker's yeast stereospecifically reduced ethyl pentanoate to (*S*)-ethyl-4-hydroxypentanoate, and yielded (*S*)-***(9)*** (Figure 57).[116]

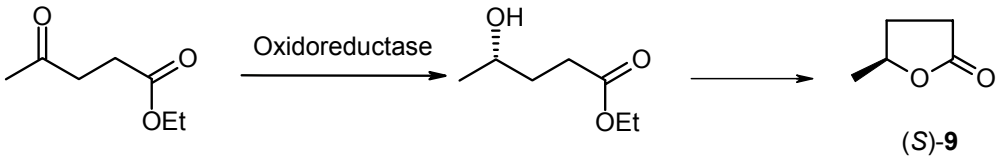


Figure 57. Formation of (*S*)-γ-pentalactone by baker's yeast as described by Manzocchi *et al.*[116]

The formation of ethyl pentanoate in the present study was thought to be initiated by enzymatic Michael-type reaction between TPP-bound "active acetaldehyde" and acrolein

yielding 4-oxopentanal, followed by oxidation and esterification (Figure 19). Michael-type addition, mediated by baker's yeast, was observed by Kitazume and Ishikawa[117] using trifluoroethanol and α,β-unsaturated carbonyl compounds as substrates (Figure 58). It was suggested that trifluoroethanol is enzymatically oxidized to trifluoroacetaldehyde before formation of the putative thiamin pyrophosphate adduct. Then the acyl anion is enzymatically added across the double bond of the α,β-unsaturated carbonyl compound to form trifluoromethyl ketones as intermediates. The latter are stereoselectively reduced by dehydrogenases to form chiral trifluoromethyl carbinols or the corresponding lactones, respectively. The authors suggested PDC as the enzyme that catalyzes the Michael addition.

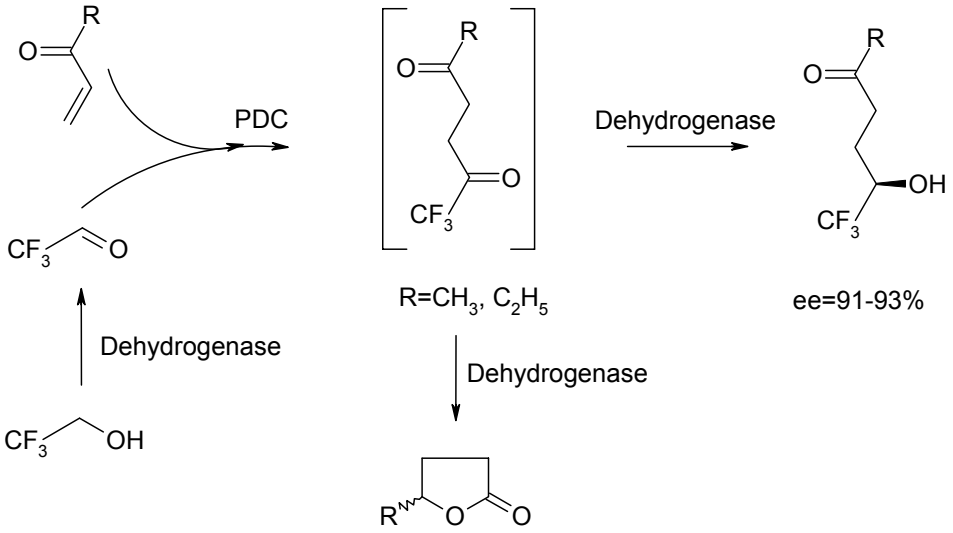


Figure 58. Michael-type addition catalyzed by baker's yeast as described by Kitazume and Ishikawa[117].

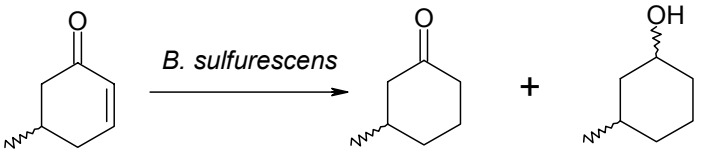
4.1.4.2.2 Biogeneration and characterization of (*R*)-3-hydroxy-1-hexen-4-one

The new acyloin (*R*)-3-hydroxy-1-hexen-4-one ***(10)*** was produced together with tautomer *(R)*-4-hydroxy-1-hexen-3-one ***(11)*** with enantiomeric excesses of 78 % and 92 %, respectively, by biotransformation of acrolein and 2-oxobutyrate using whole cells of baker's yeast (see 3.1.2). The yield (0.3 %) of ***(10)*** was around 10 times lower than that for ***(3)***. Neuser[26] obtained a 5 times decrease of the yields, when 2-oxobutyrate instead of pyruvate was reacted with benzaldehyde. The phenomenon could be explained by the proven lower decarboxylation rates of 2-oxobutyric acid[118], and possible additional substrate inhibition of 2-oxobutyrate.

4.1.4.2.3 Simultaneous formation of α,β-desaturated acyloins and saturated acyloins from 2*E*-alkenals

In most cases, where 2*E*-alkenals and 2-oxocarboxylates were subjected to biotransformation using baker's yeast, the formation of the expected α,β-desaturated

acyloins was accompanied by the saturated counterparts (see 3.1.2 and 3.1.3). This pointed to the presence of an oxidoreductase confering the reduction of the double bond. Diols were not produced, because the reduction of the carbonyl function is probably much slower than that of the double bond. Similar results were obtained by Kergomard and coworkers [119]. Reduction of 5-methylcyclohex-2-en-1-one using *Beauveria sulfurescens* afforded 3-methylcyclohexanone as the only reaction product after 5 h of incubation. The corresponding alcohol was only obtained in small quantities after 9 h (Figure 59).



Time 0 h	100 %	0	0
Time 2 h	75 %	25 %	0
Time 5 h	0	100 %	0
Time 9 h	0	90 %	10 %

Yield

Figure 59. Reduction of 5-methylcyclohex-2-en-1-one by *Beauveria sulfurescens* as described by Kergomard *et al.*[119]

Also Kawai and coworkers[120] reported that baker's yeast reduction of α,β-unsaturated ketones afforded optically active saturated ketones contaminated by allylic and saturated alcohols as minor components.

4.1.4.2.4 Biogeneration of a pool of acyloins

In the present study, it was shown that baker's yeast accepts a wide range of aliphatic aldehyde substrates and produces the corresponding acyloins. Highest yields (up to 55 %) were obtained for the conversion of saturated and unsaturated C5, C6, and C7 aldehydes. Shorter and longer aldehydes were less effectively transformed. In order to give an explanation for this observation, some further structural properties of PDC have to be considered. The TPP binding site of PDCs of different origins as well as that of other TPP-dependent enzymes is highly conserved. The cofactor environment and the walls of the cavity leading to the reaction center are lined with hydrophobic residues.[80] [121] These structural features could explain why the yields for shorter, and thereby more polar, aldehydes were lower, in this study. Neuser[26] observed the same tendency and obtained highest yields for the conversion of hexanal and heptanal. C8 aldehydes might somehow be

too long and thereby not match well into the active site. This could explain the low yields obtained in Neuser's and this work.

There are, however, some differences in the quantitative consideration of acyloin formation between the present study and that of Neuser[38]. For all the α,β-desaturated acyloins obtained in the present study, 3-hydroxy tautomers and 2-hydroxy tautomers were produced in comparable quantities, while for saturated acyloins more 3-hydroxy tautomer was formed relatively to 2-hydroxy ones. Neuser[26], however, obtained systematically for both saturated and α,β-desaturated acyloins an excess of the 3-hydroxy tautomer when pyruvate and aldehydes were subjected to biotransformation with PDC from *Z. bisporus*. Furthermore, in the present work, the yields for corresponding saturated and desaturated counterparts were comparable. Neuser, however, reported that α,β-desaturated acyloins were systematically less efficiently produced than their saturated counterparts. The different observations can at least partially be explained by the fact that different microorganisms with different enzymes were used. Sequence comparisons between the PDCs from *S. cerevisiae* and *Z. bisporus* revealed 81 % identity at the amino acid level.[78] The formation of the pair of hydroxy-4*E*-nonenones ***(13)*** and ***(14)*** as well as the hydroxy-4*E*-decenones ***(15)*** and ***(16)*** was reported for the first time in this study.

4.1.4.2.5 Stereoselectivity of acyloin formation

Enzyme-catalyzed reactions hold several advantages over chemical reactions, particularly in that they often show stereospecificity when chiral compounds are produced. Many studies in different systems have shown that two enantiomers frequently display different biological activities. In the past ten years the chemical community has realized that the preparation of enantiopure materials is critically important to mankind, and many research groups have devoted a considerable amount of time to the development of new asymmetric synthesis methods.[122] The biotransformation of benzaldehyde and several other aromatic aldehydes using PDC from *Z. mobilis* afforded (*R*)-aycloins with enantiomeric excesses of more than 97 % [123]. (*R*)-Acyloins with high ee values were also obtained with PDC from *S. cerevisiae,* or whole cells of baker's yeast.[124] Lower ee values were described when aliphatic aldehydes were transformed. PDC of *S. cerevisiae* produced (*R*)-acetoin in about 50 % enantiomeric excess, while the *Z. mobilis* enzyme synthesized (*S*)-acetoin in about 25 % enantiomeric excess.[125] [69] These differences in the control of the product stereochemistry have been investigated by molecular modeling techniques.[123] [107] The relevance of the side-chain of isoleucine 476 from PDC of *S. cerevisiae* was suggested, because this side chain may protect one side of the TPP-acetaldehyde carbanion-enamine against the bulky aromatic cosubstrate. In the case of acetoin formation, the small co-substrate acetaldehyde can bind to both sites of the carbanion, thereby probably leading to lower ee values.

Highest ee values (>90 %) in this work were obtained when acrolein was used as substrate in combination with both pyruvate and 2-oxobutyrate. For the other tested acyloins, the ee values were lower, with minimum ee values of about 40 % for *(R)*-2-hydroxy-3-heptanone. These results show that the stereoselectivity of the reaction was dependent on the structural properties of the aldehyde substrate.

The observation, that for all tested 3-hydroxy- and for 2-hydroxy-acyloins, one enantiomer was produced in excess, strongly suggests that the both isomers were enzymatically formed. As described above (see 4.1.4.1), the relevant steps for the formation of acyloins in the reaction mechanism are reversible. The reactive carbanion-enamine-intermediate, or "active aldehyde", can also be formed by addition of an aldehyde to the TPP-carbanion and subsequent deprotonation. In fact, Neuser identified 5-hydroxy-4-octanone as product from the incubation of butanal and pyruvate with PDC from *Z. bisporus*, which was explained by reaction of "active butanal" and another butanal molecule. [26]

However, it can not be excluded that the lower ee values for some of the acyloins are the consequence of partial isomerisation or racemisation. Crout *et al.*[67], for example, explained the formation of tautomeric acyloins by chemical isomerisation (Figure 60).

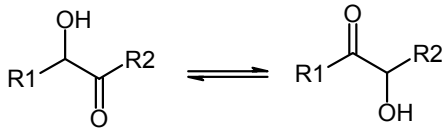
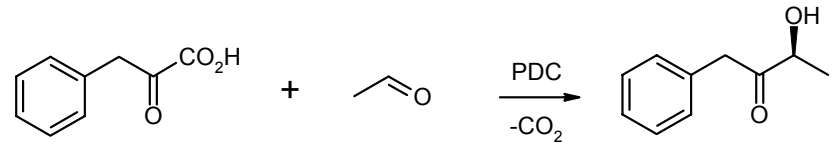


Figure 60. Isomerisation equilibrium of acyloins.

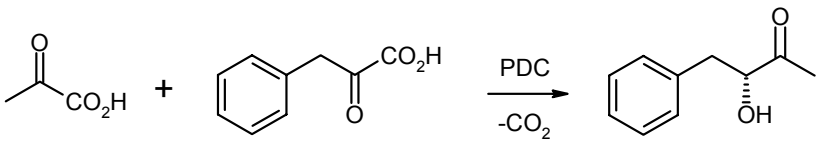
The keto-enol conversion is favoured in the presence of several catalysts such as acids and bases as well as by heating. Neuser[26] supported the hypothesis of partial tautomerisation by reporting that (*S*)-3-hydroxy-1-phenyl-2-butanone, which was formed from phenylalanine by fermentation with *Z. bisporus*, had an ee value of 84 %, while the acyloin synthesized with isolated PDC from phenylpyruvate and acetaldehyde had only an ee of only 30 %. This observation strongly indicated that with the isolated enzyme, chemical racemisation due to isomerisation had occurred.

The acyloins produced in the present work were predominantly of the (*R*)-configuration. This result is in agreement with the stereoselectivity of acyloins formed by PDC from *S. cerevisiae* reported in literature.[125] The exceptions in this study were (*S*)-2-hydroxy-3-octanone ***(17)*** and (*S*)-2-hydroxy-4*E*-hepten-3-one ***(18)***. Acyloins of the (*S*)-configuration have never been reported as products of carboligation reactions with the *S. cerevisiae* enzyme. The differences of absolute configurations could be explained by different mechanisms of 2-hydroxy and 3-hydroxy tautomer formation (see 3.1.3).

There is only one other report, where related tautomeric acyloins were produced with opposite absolute configurations[78]. Phenylglyoxylate and acetaldehyde reacted to (*S*)-3-hydroxy-1-phenyl-2-butanone with 30 % ee, while pyruvate and phenylacetaldehyde formed (*R*)-3-hydroxy-4-phenyl-2-butanone with 58 % ee (Figure 61).



(*S*)-3-hydroxy-1-phenyl-2-butanone



(*R*)-3-hydroxy-4-phenyl-2-butanone

Figure 61. Formation of acyloin pairs of opposite absolute configuration using PDC from *Z. bisporus* as described by Neuser *et al*.[78]

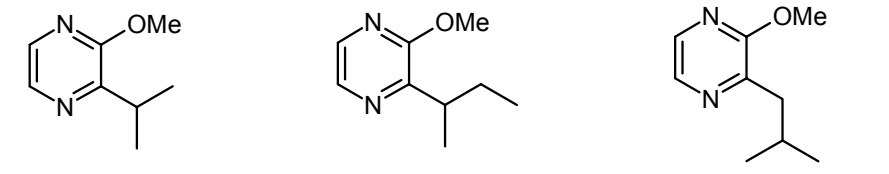
4.2 Pyrazines and derivatives

4.2.1 Natural occurrence of pyrazines

Pyrazines found in nature are predominantly alkylpyrazines or methoxypyrazines. Although the present work focused on the generation of alkylpyrazines, some features about methoxypyrazines will also be concisely given in order to impart a global insight to the most important pyrazines.

Methoxypyrazines occur widely in nature, but in contrast to alkylpyrazines, their structural diversity is limited to a few compounds.[9] The first methoxypyrazine which was identified in food was 2-isobutyl-3-methoxypyrazine.[126] It has the typical aroma character of freshly chopped green bell peppers (*Capsicum annuum*) from which it was first isolated.[127] Its odour threshold amounts to 0.002 ppb in water, a value placing it among the most potent flavour constituents and even in front of dienals which are other strong and characteristic flavour constituents of bell peppers.[128] The same compound has been detected in other raw vegetables such as green peas[129 130] and fruits as grape C.V. Cabernet Sauvignon[131]. Apart from 2-isobutyl-3-methoxypyrazine, two other methoxypyrazines have frequently been described in raw vegetables, 2-isopropyl-3-methoxypyrazine and 2-sec-butylmethoxypyrazine (Figure 62). The presence of methoxypyrazines in nature and their

limitation to certain derivatives strongly suggest that enzymatic processes are involved in the biosynthesis of these compounds. The biosynthetic pathways of methoxypyrazines in plants, however, have not yet been investigated.



2-isopropyl-3-methoxypyrazine | 2-sec-butyl-3-methoxypyrazine | 2-isobutyl-3-methoxypyrazine

Figure 62. Most frequently encountered methoxypyrazines in raw vegetables.

Alkylpyrazines have been found in a wide variety of foods and impart nutty, roasted and toasty aroma tonalities.[7] [8] [9] These characteristics make them interesting for the application as additives for flavourings in the savoury flavour area. Unlike methoxypyrazines a vast number of alkylpyrazines and related compounds have been detected in various foods.[7] Some representative examples illustrate this diversity of compounds which have been found in roasted almonds[132] [133] (Figure 63), grilled beef[134] [135] (Figure 64) and beer[136] (Figure 65).

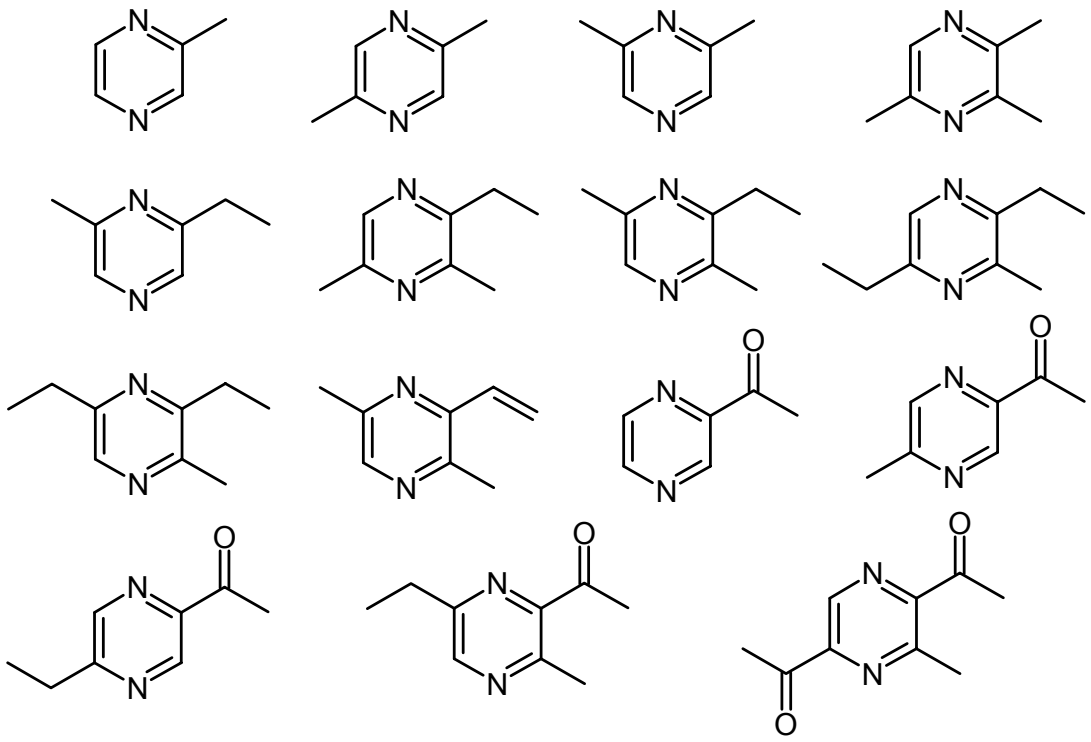


Figure 63. Pyrazines in roasted almonds.

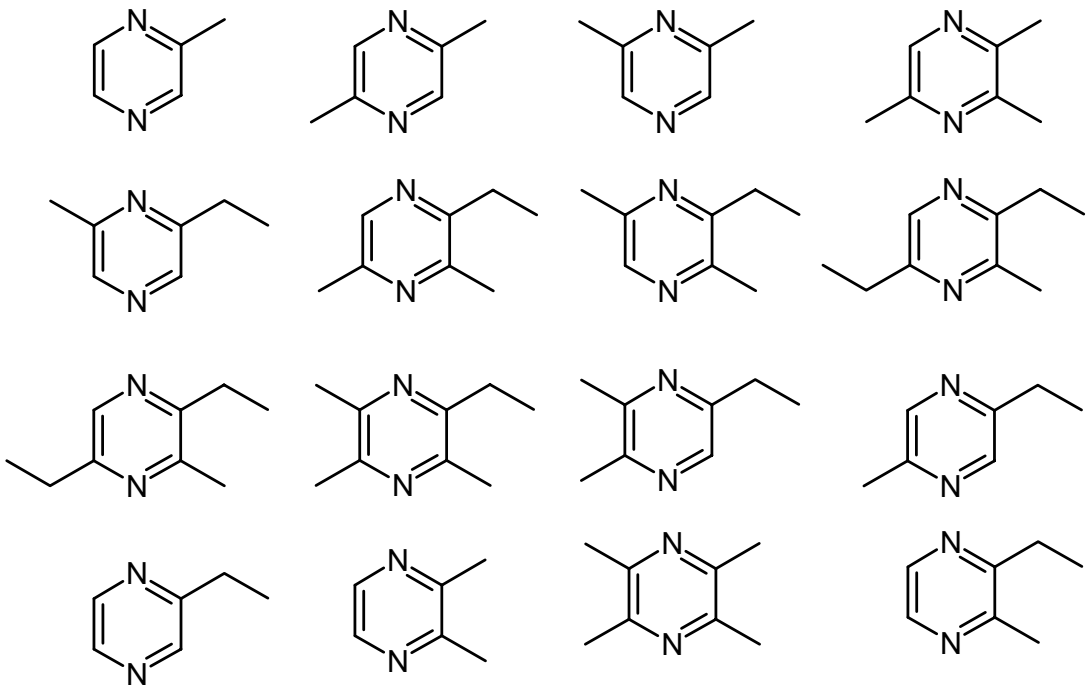


Figure 64. Pyrazines in grilled beef.

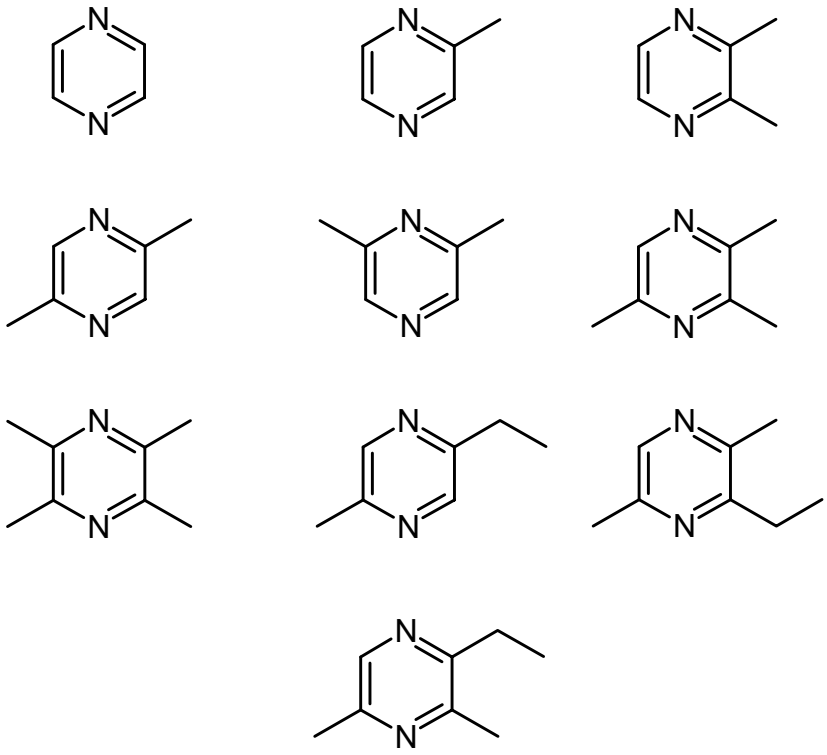
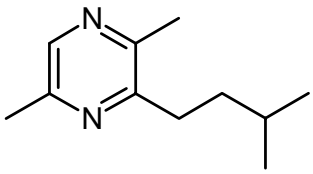


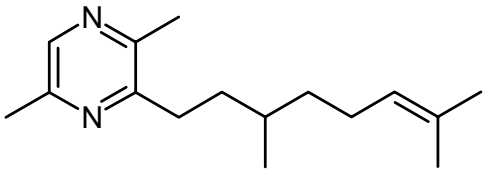
Figure 65. Pyrazines in beer.

Other consumer goods which have been shown to contain a multitude of alkylpyrazines include chocolate, cocoa, coffee, heated eggs, roasted filberts, cooked Macademia nuts, baked potatoes, cooked rice, black tea, green tea and tobacco smoke.[7] [137] [138] [139]

Furthermore, alkylpyrazines have been found in small quantities in insects and their function in these species may be as alarm or trail pheromone.[11] The presence of the isopentyl and citronellyl side-chain in pyrazines from the Australian Ponerine ant (*Rytidoponera metallica*) suggests that they might be partially formed from terpenoid precursors (Figure 66). However, no work has been reported on the biosynthetic pathways of pyrazines in any insect.



3-isopentyl-2,5-dimethylpyrazine



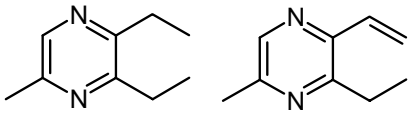
3-citronellyl-2,5-dimethylpyrazine

Figure 66. Identified pyrazines in *Rytidoponera metallica.*

4.2.2 Importance of pyrazines as aroma compounds

The industrial interest of methoxypyrazines can be seen by patents describing their synthesis and their use as flavouring ingredients.[140] [141] [142] Some compounds confer nutty and roasted peanut-like or almond-like notes, but the majority of them is known to have green aroma tonalities. The effect of the size of the alkyl group on the flavour intensity of various 2-alkyl-3-methoxypyrazine was determined by Seifert and coworkers.[143] 2-Methoxypyrazine has an odour threshold 175 times higher than that of 2-methoxy-3-methylpyrazine, 1650 times higher than that of 2-ethyl-3-methoxypyrazine and over 10^5 times higher than that of its superior homologs, 2-isobutyl-3-methoxypyrazine and 2-isopropyl-3-methoxypyrazine. The effect extends to 2-hexyl-3-methoxypyrazine which has an odour threshold of 0.001 ppb. Substitutions in other positions are postulated to be unfavourable for bell-pepper odour. More recently, Mihara and Masuda[144] mentioned that besides the hydrophobic interaction stemming from the alkyl group, hydrogen bonding between the nitrogen atoms of the pyrazine ring and the heteroatom as electron pair donors on one hand, and acceptors from receptor-pocket on the other hand, should be important for bell-pepper flavour.

In another comparative systematic study of the organoleptic properties of alkylpyrazines, it was shown by Wagner[16] that out of 80 tested alkylpyrazines only four, namely 2-ethyl-3,5-dimethylpyrazine ***(31)***, 2-ethenyl-3,5-dimethylpyrazine ***(1)***, 2,3-diethyl-5-methylpyrazine ***(41)***, and 2-ethenyl-3-ethyl-5-methylpyrazine ***(42)*** (Figure 67), possessed lowest odour thresholds of around 0.01 ng/l air as determined by gas chromatography-olfactometry .



41 **42**

Figure 67. Molecular structure of 2,3-diethyl-5-methylpyrazine and 2-ethenyl-3-ethyl-5-methylpyrazine.

As Figure 68 shows, a propyl and a butyl group in position 2 was too bulky, and the odour threshold increased from 0.01 ng L^{-1} to 23 ng L^{-1} and 180 ng L^{-1}. Elongation of the alkyl group to give a C5 group lowered the threshold to that of the propyl-substituted pyrazine, and the odour changed to "peasy". An analogous change was reported by Shibamoto[145] when the butyl group in 5-butyl-2,3-dimethylpyrazine was replaced by a pentyl group. A further prolongation of the pentyl group in position 2 to give a hexyl group drastically increased the odour threshold again.

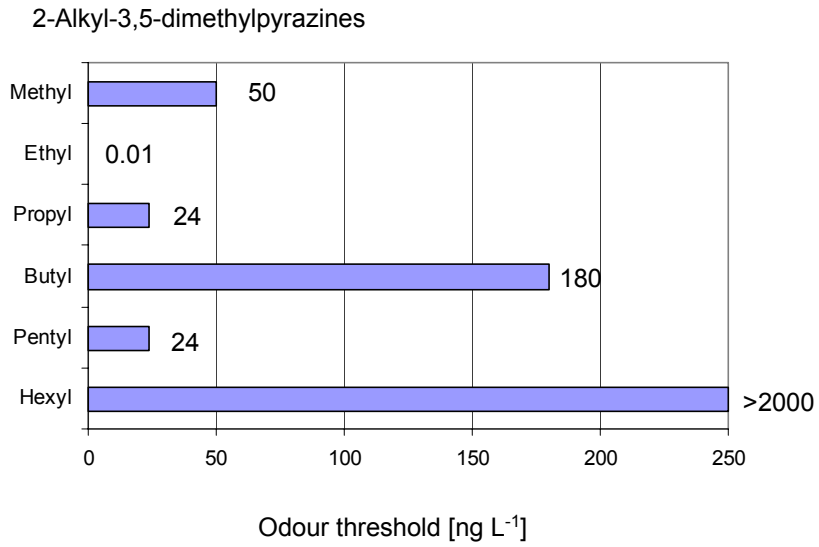


Figure 68. Odour thresholds of 2-alkyl-3,5-dimethylpyrazines as described by Wagner *et al.*[16]

Exchange of the 3-ethyl group of ***(41)*** by an ethenyl group increased the odour threshold by a factor of nearly 8000, while an analogous exchange at position 2 was tolerated. The study confirmed that both steric and electrostatic interactions with a receptor are the basis of odour recognition. The theoretical receptor was modeled by Wagner[16] who superimposed step by step the five pyrazines with the lowest odour thresholds.

4.2.3 Generation of alkylpyrazines

4.2.3.1 Generation of alkylpyrazines by Maillard reaction

The Maillard or non-enzymatic browning reaction has generated much interest over the past 60 years. The Maillard reaction mainly involves the reaction of free amino groups of amino acids and reducing sugars.[146] The principal chemistry of this reaction was reviewed by Hodge[147] in 1953. The aromas in most thermally processed foods, such as bread, cereal products, roasted peanuts, and roasted coffee, are generated during Maillard reaction.[8] Numerous heterocyclic compounds have been identified in food and model systems. These heterocycles include furans, thiazoles, thiophenes, oxazoles, pyrroles, pyridines, and pyrazines.[146] Several mechanisms have been proposed for the formation of pyrazines in food flavours. Strecker degradation is one of the most important reactions which involves the oxidative deamination and decarboxylation of an α-amino acid in the presence of a dicarbonyl compound.[148] This leads to the formation of an aldehyde, the Strecker aldehyde, containing a carbon atom less than the original amino acid, and an α-amino ketone. Self-condensation of two amino ketone molecules, affords 1,4-dihydropyrazines which readily oxidize to pyrazines (see Figure 50). The Maillard reaction, however, generates a mixture of numerous compounds with very low yields (<0.01 %). Thus, this type of generation is not the method of choice to produce particular pyrazines in large amounts, e.g. for the use as additives in food products.

4.2.3.2 Generation of alkylpyrazines by fermentation

An increase in the production of processed food by industrial methods has resulted in an expanding market for additives such as flavourings. In this area, consumers generally prefer additives exhibiting a natural label.[13] Aromas can in principal be classified as natural when obtained from extraction of plants or fermentation processes. The first evidence that microorganisms were able to synthesize pyrazines was provided in 1962 by Kosuge and coworkers[149] who showed that tetramethylpyrazine could be produced by *Bacillus subtilis*. Since this observation, several other microorganism able to synthesize different alkylpyrazines have been discovered[1 3]. In a solid state fermentation process on soybeans with *B. subtilis*, addition of threonine was shown to increase the yield of 2,5-dimethylpyrazine, while acetoin led to enhancement of tetramethylpyrazine production.[150] Nevertheless, fermentation incubation times have been too long and final product concentrations too low to allow an industrial application of this process.[151] Furthermore, several authors think that the majority of pyrazines associated with microbial fermentations are not exclusively formed by enzyme-catalyzed reactions and that the last steps are chemical reactions.[152]

4.2.3.3 Chemoenzymatic synthesis of pyrazine derivatives

As the generation of pyrazine derivatives by Maillard reaction or by fermentation hamper from low yields and/or long incubation times, a new chemoenzymatic route was presented in this study. As relatively little information about 5,6-dihydropyrazines and tetrahydropyrazines was found in literature, these classes of compounds were defined as the target compounds. Biotransformation of aliphatic aldehydes and 2-oxocarboxylates using whole cells of baker's yeast and subsequent chemical reaction with 1,2-propanediamine under mild conditions generated 14 5,6-dihydropyrazines and 10 tetrahydropyrazines with yields up to 50 % (based on concentration of the intermediate acyloins). These results show for the first time that acyloins can not only act as precursors of oxazolines, oxazoles, thiazolines, or thiazoles, (see 4.1.3) but also as those of 5,6-dihydropyrazines and tetrahydropyrazines.

For several decades, a limited number of 5,6-dihydropyrazines have been synthesized from corresponding dicarbonyls and diamines with comparable yields.[153] Thus, the chemoenzymatic synthesis discovered in this work is particularly interesting for the synthesis of 5,6-dihydropyrazine compounds, for which no diketone precursors are available, and might open possibilities to produce a wider range of new 5,6-dihydropyrazine derivatives.

All 10 tetrahydropyrazines were reported for the first time. The yields, however, accounted to only about 20 %. This was mainly due to the instability of tetrahydropyrazines which were partially oxidized to the corresponding 5,6-dihydropyrazines.

4.2.4 Organoleptic properties of 5,6-dihydropyrazines and tetrahydropyrazines

A systematic evaluation of the organoleptic properties of 5,6-dihydropyrazines was never described in literature. Therefore, the odour qualities and threshold values of the 5,6-dihydropyrazines and tetrahydropyrazines, obtained from the chemoenzymatic synthesis, were determined. 11 of them showed pronounced roasted or earthy aroma characteristics which showed that the potential of 5,6-dihydropyrazines and tetrahydropyrazines as aroma compounds had been underestimated before. Two of the 5,6-dihydropyrazines, compounds ***(20)*** and ***(22)***, had lowest threshold values in pg range, and can be placed among the most potent pyrazine derivatives. Moreover, a similar influence of the length of the alkyl group in position 2 of the synthesized 2-alkyl-3,5-dimethyl-5,6-dihydropyrazines on the threshold values was observed as for the corresponding pyrazines (see 4.2.2) (Figure 69). A propyl and a butyl group, instead of an ethyl group, increased the odour thresholds by a factor of about 1000 or 10000, respectively. Elongation to a C5 group lowered the threshold to that of the propyl-substituted 5,6-dihydropyrazine. A further prolongation of the pentyl group in

position 2 to give a hexyl group or heptyl grop drastically increased the odour threshold again.

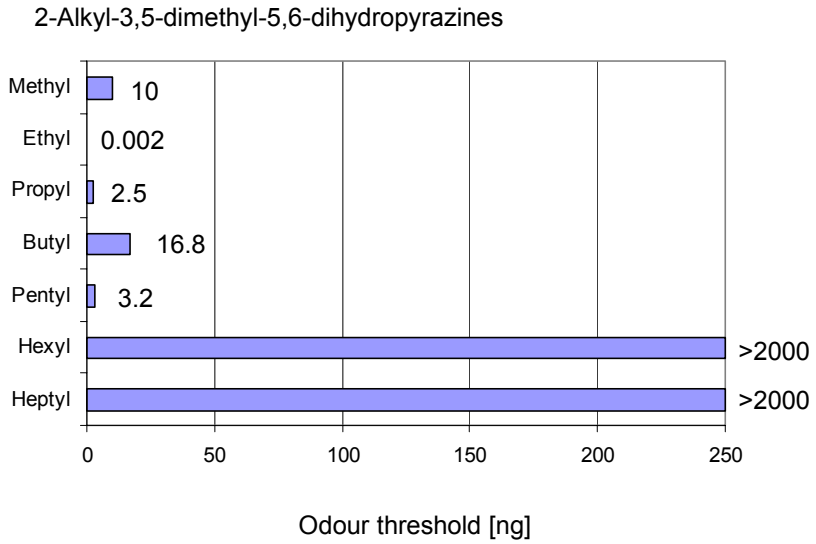


Figure 69. Odour thresholds of 2-alkyl-3,5-dimethyl-5,6-dihydropyrazines.

These similar results suggest that 5,6-dihydropyrazines and pyrazines are probably recognized by the same receptor. The odour thresholds of the synthesized tetrahydropyrazines, however, were signifcantly higher. A comparison of the odour thresholds of related 2-ethyl-3,5-dimethylpyrazine derivatives, revealed about 1000 times less aroma-intensity for the tetrahydropyrazine (Table 19).

Table 19. Comparison of odour thresholds of homologue pyrazine derivatives.

Pyrazine derivative	Odour threshold [ng][a]
2-Ethyl-3,5-dimethylpyrazine ***(31)***	0.002
2-Ethyl-3,5-dimethyl-5,6-dihydropyrazine ***(20)***	0.002
2-Ethyl-3,5-dimethyltetrahydropyrazine	1.9

[a] Odour threshold values (in absolute ng of each odour impression)

Tetrahydropyrazines might also interact with the same receptor. The higher polarities and the significantly different cyclohexen-like structure of tetrahydropyrazines could be the reason for the lower affinity to the receptors.

4.2.5 Chemical synthesis of 2-ethenyl-3,5-dimethylpyrazine and 3-ethenyl-2,5-dimethylpyrazine

In the second part of this work, a new total chemical synthesis for ethenylpyrazines ***(1)*** and ***(2)*** was reported. Both molecules are earthy smelling compounds with lowest odour thresholds.[16] Compound ***(1)*** was identified as one of the odorants in freshly ground Brazilian coffee[154], coffee powder and coffee brew[155], roasted seeds of wild mango[156], while molecule ***(2)*** was found in aroma volatiles of roasted peanut[157], corn torilla chips[158], and green kohlrabi[159]. Two routes have been described in literature for the synthesis of ethenylpyrazines (Figure 70).

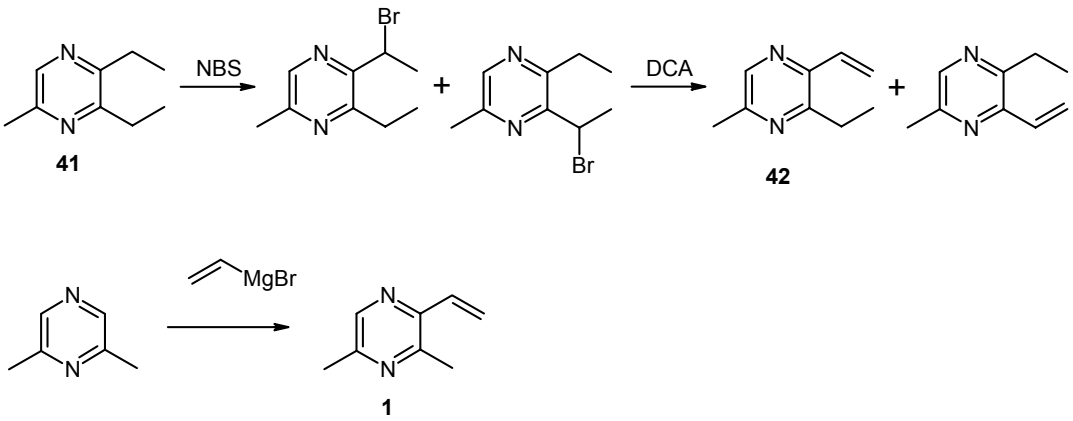


Figure 70. Syntheses of ethenylpyrazines as reported by Czerny[17] and Lambrecht and Kaulen[18].

The synthesis of 2-ethenyl-3-ethyl-5-methylpyrazine and 3-ethenyl-2-ethyl-5-methylpyrazine was done by bromination of 2,3-diethyl-5-methylpyrazine using *N*-bromo-succinimide (NBS) followed by elimination of hydrogen bromide with potassium *tert*-butoxide or dicyclohexylamine (DCA) [18]. The total yield of this method was lower than 1 %. Furthermore, compound ***(1)*** was synthesized by alkylation of 2,6-dimethylpyrazine using vinylmagnesium bromide with 5 % yield.[17]

In the present work, a new synthesis of a mixture of 2-ethenyl-3,5-dimethylpyrazine ***(1)*** and 3-ethenyl-2,5-dimethylpyrazine ***(2)*** was presented (see 3.3.3). Both pyrazines were produced with a total yield of 7.0 % each. The yield could further be increased by the optimization of reaction procedures and conditions. For example, the acyloin condensation of the protected compounds ***(34)*** and ***(35)*** could be more efficiently performed from 5-norbornen-2-carboxaldehyde ***(33)*** and pyruvate using PDC or whole cells of baker's yeast. In order to increase the yield of ***(1)*** relatively to that of ***(2)***, further efforts should focus on the generation of intermediate *endo*-[1-bicyclo[2.2.1]5-hepten-2-yl]-1,2-propanedione ***(36)***. This can be achieved by Diels-Alder reaction of 1-penten-3,4-dione and cyclopentadien, which yields 90 % of endo-***(36)*** as described by Kramme and coworkers.[22]

5 General Conclusion and Outlook

27 Aliphatic acyloins were produced in yields up to 55 % by biotransformation of aliphatic aldehydes and 2-oxocarboxylates using whole cells of baker's yeast as biocatalyst. The results show that baker's yeast is a versatile biocatalyst which is capable of accepting several aliphatic aldehydes and at least two 2-oxocarboxylates as substrates for acyloin generation. Six out of the 27 generated acyloins, compounds ***(3)***, ***(10)***, ***(13)***, ***(14)***, ***(15)***, and ***(16)***, were new and characterized for the first time. Other substrates could be tested in future in order to fully exploit the scope of this reaction. In this study, all acyloins were characterized by GC-MS, and the structure of the new acyloin, 3-hydroxy-1-penten-4-one ***(3)***, was confirmed by ^{1}H and ^{13}C NMR. Although the identification of known acyloins by comparison of RI values and GC-MS data with literature values should be reliable, new molecules should in future generally be purified, and their structures be confirmed by NMR analysis. As all of the tested acyloins were produced stereoselectively, new ones should also be investigated in terms of enantiomeric excess, absolute configuration, and specific optical rotation. The aim of the present work was to transform acyloins into alkylpyrazine derivatives, however, new acyloins, which would be synthesized in future, could also be reacted with other bidental nucleophiles. First investigations show promising results. The reaction of the new acyloin, 3-hydroxy-1-penten-4-one ***(3)***, with cysteamine led to the production of two roasted compounds, 2-acetyl-2-ethylthiazolidine and 3-acetyl-2-methyl-2,3,5,6-tetrahydro-1,4-thiazine (identified by interpretation of GC-MS), the latter being a new molecule with pronounced roasted aroma properties. Furthermore, the reaction of new acyloins with ammonium sulfide should be performed, which could yield numerous new oxazolines, oxazoles, thiazolines, and thiazoles.

In this study, the acyloin formation by whole cells of baker's yeast was followed by chemical reaction with 1,2-propanediamine under mild conditions and led to the production of 14 5,6-dihydropyrazines and 10 tetrahydropyrazines with yields up to 50 % (based on concentration of the intermediate acyloins). While 6 of the 10 new tetrahydropyrazines were odourless under the conditions applied, 4 of them exhibited roasted, bread crust-like, or cooked rice-like aroma tonalities. All tetrahydropyrazines, however, were to some degree oxidized to their 5,6-dihydropyrazine counterparts, which led to lower yields (~ 20 %). Further efforts should be done to stabilize them, e.g. by the use of inert atmospheres, such as nitrogen. This might open possibilities to use them as aroma precursors, which only in the presence of oxygen (e.g. by opening a flask) are transformed into the more intense 5,6-dihydropyrazines. Three of the synthesized 5,6-dihydropyrazines, compounds ***(20) and (22)*** from chemoenzymatic synthesis as well as the new compound ***(29)*** from chemical synthesis,

can be placed among the pyrazine derivatives with the lowest threshold values described in literature. These results demonstrate the great potential of 5,6-dihydropyrazines as aroma compounds. For the future, the search for new aroma-active molecules should be continued. The possibilities of their future application in food products, however, are limited, as long as new compounds have not been detected in food systems. This would render them natural-identical and facilitate a potential use.

In the second part of this work, an elegant route to obtain a mixture of pure 2-ethenyl-3,5-dimethylpyrazine ***(1)*** and 3-ethenyl-2,5-dimethylpyrazine ***(2)*** was developed. The total yields, the highest ones reported in literature, accounted to 7.0 % for each molecule. The chemical synthesis described in this work is powerful, because it will allow to produce other alkenylpyrazines. Further investigations should focus on the synthesis of 2-ethenyl-3-ethyl-5-methylpyrazine ***(42)***.

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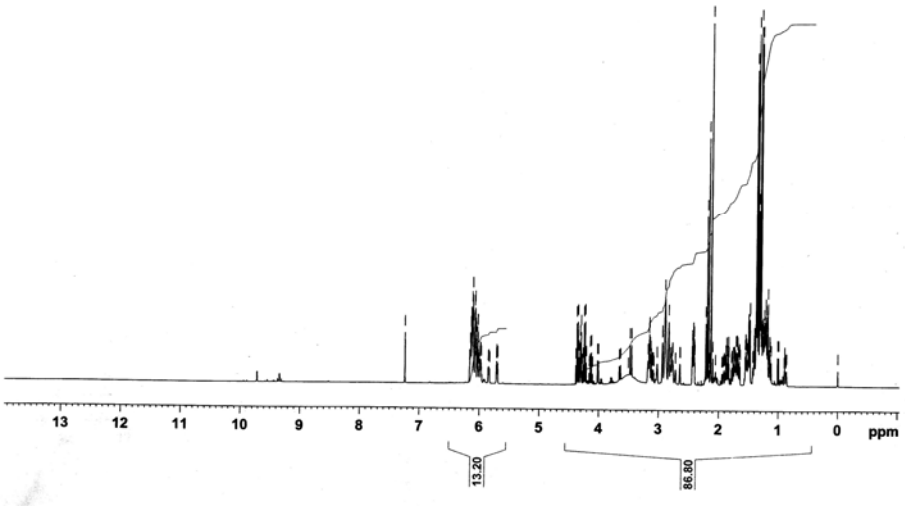
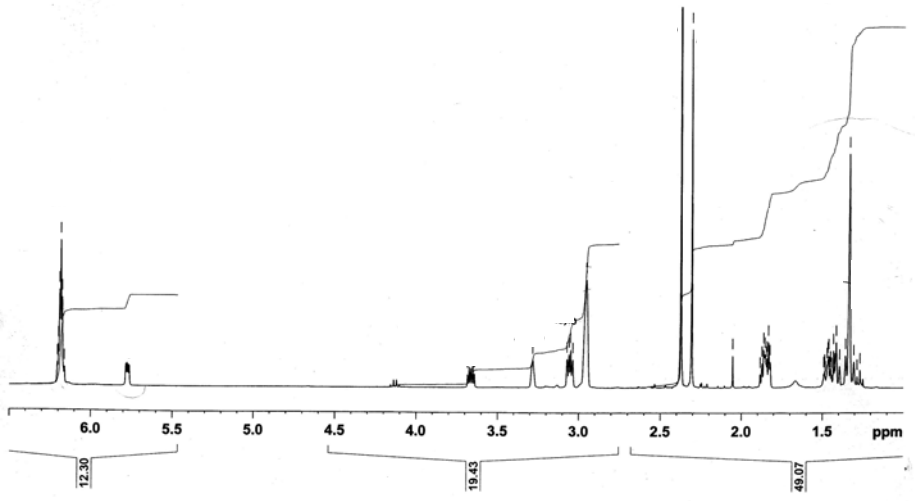
7 Appendix

7.1 NMR spectra

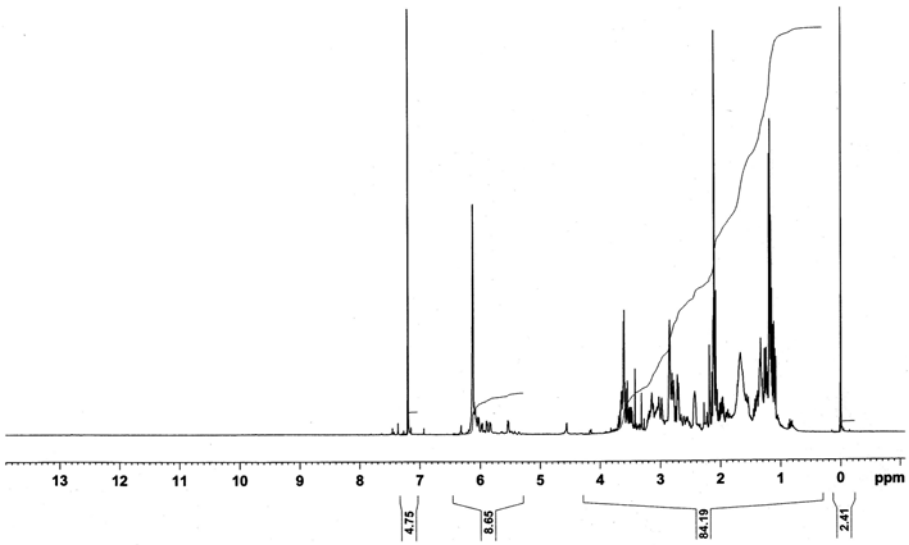
- Mixture of 1-[bicyclo[2.2.1]5-hepten-2-yl]-2-hydroxy-1-propanone ***(34)*** (*exo*+*endo*) and 1-[bicyclo[2.2.1]5-hepten-2-yl]-1-hydroxy-2-propanone ***(35)*** (*exo*+*endo*):



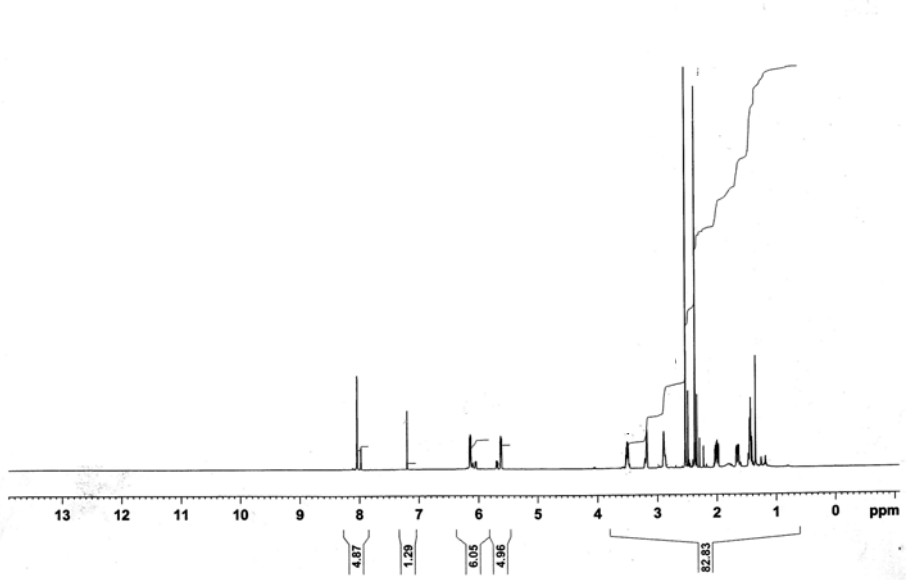
- 1-[Bicyclo[2.2.1]5-hepten-2-yl]-1,2-propanedione ***(36)*** (*exo*+*endo*):



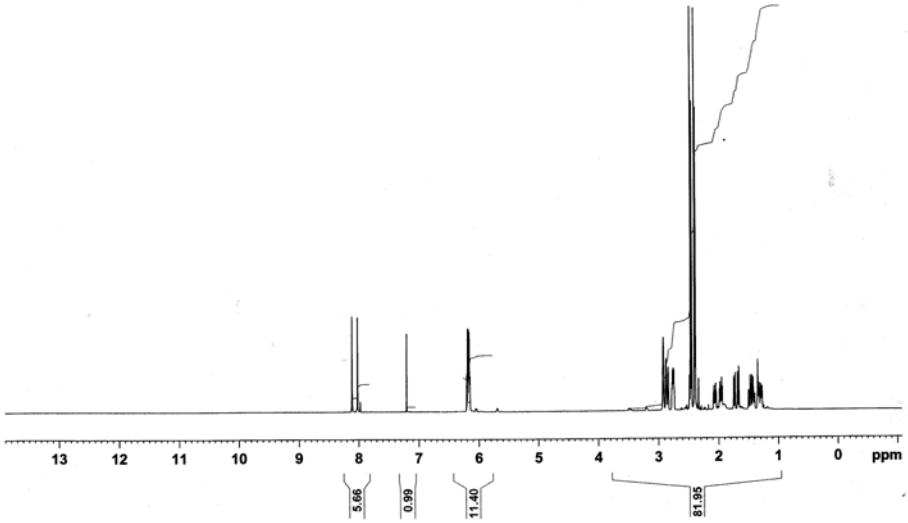
- Mixture of 2-[bicyclo[2.2.1]5-hepten-2-yl]-3,5-dimethyl-5,6-dihydropyrazine ***(37)*** (exo+endo) and 3-[bicyclo[2.2.1]5-hepten-2-yl]-2,5-dimethyl-5,6-dihydropyrazine ***(38)*** (*exo*+*endo*):



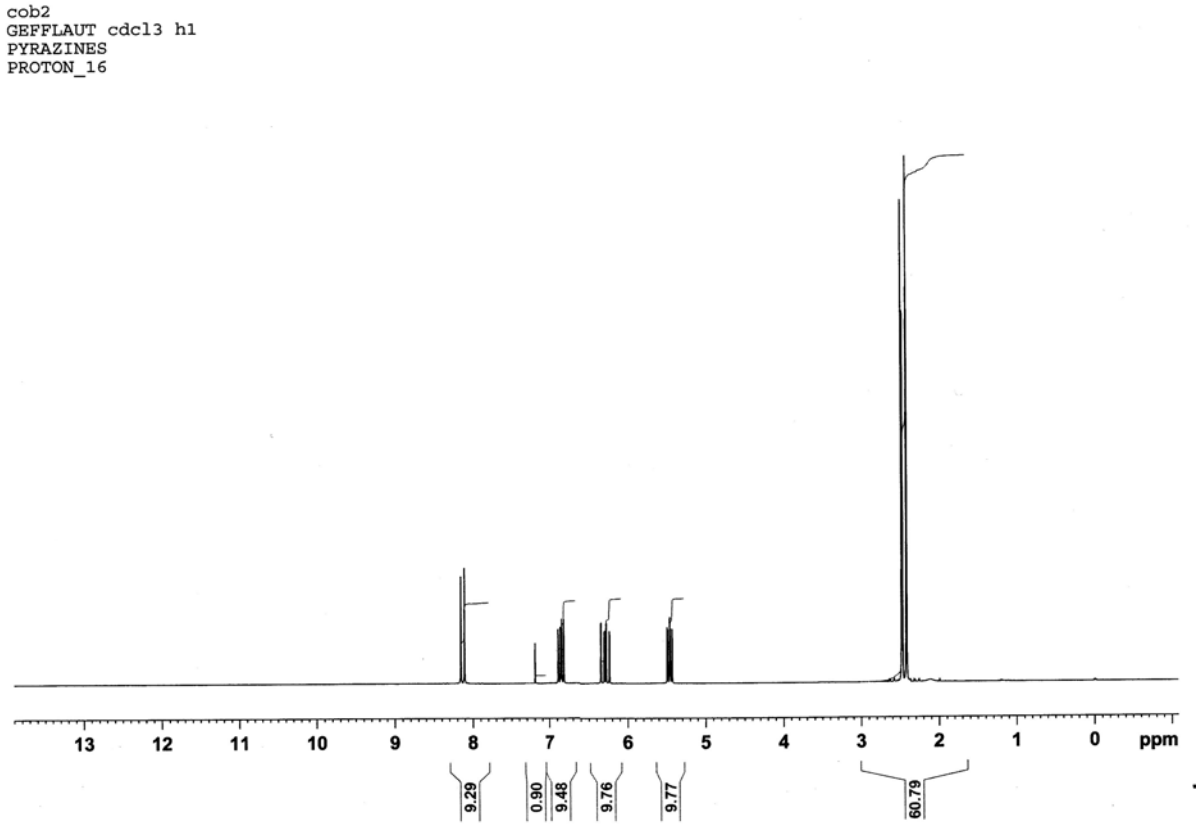
- Mixture of *endo*-2-[bicyclo[2.2.1]5-hepten-2-yl]-3,5-dimethylpyrazine ***(39)*** and *endo*-3-[bicyclo[2.2.1]5-hepten-2-yl]-2,5-dimethylpyrazine ***(40)***:



- Mixture of *exo*-2-[bicyclo[2.2.1]5-hepten-2-yl]-3,5-dimethylpyrazine ***(39)*** and *exo*-3-[bicyclo[2.2.1]5-hepten-2-yl]-2,5-dimethylpyrazine ***(40)***:



- Mixture of 2-ethenyl-3,5-dimethylpyrazine ***(1)*** and 3-ethenyl-2,5-dimethylpyrazine ***(2)***:



Resumé

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EXPERIENCE

Nestlé Research Center (NRC), Lausanne, Switzerland, 2000-2002
Ph.D. student in Bioorganic chemistry

- Discovered and developed new biotechnological and chemical routes to produce novel, proprietary flavour molecules for Nestlé products.
- 2002 Inventor of 3 Patent Applications (EP02076573.1, EP02076574.9, EP02078060.7).
- 2002 Awarded: Invitation to 52nd Meeting of Nobelpricewinners organized by the Foundation Lindau Nobelpricewinners.

EDUCATION

Ludwig-Maximilians-Universität, Munich, Germany, 1995-1999

- 1999 Diplom (equiv. to Master's Degree) in Biology.
- 1998 Vordiplom (equiv. to Bachelor's Degree) in Chemistry.
- 1997 Vordiplom (equiv. to Bachelor's Degree) in Biology.

Final grades: 1.3* (Biology), 1.4* (Chemistry).

Heinrich-Heine-Gymnasium, Munich, Germany, 1987-1995

- 1995 Abitur (equiv. to A-levels). Grade: 1.2*.
- 1994 Awarded: Invitation to summer course for "Highly talented students" organized by the Bavarian Ministry.

*German grades: 1.0=Very good 2.0=good 3.0=satisfactory 4.0=sufficient 5.0=poor 6.0=unsatisfactory

ACTIVITIES

Leader of Ph.D. students and Post-docs, Nestlé Research Center (NRC), 2002

- Elected in January 2002. Introduced new seminar system in agreement with Nestlé management. Organize and lead monthly seminars presenting projects of Ph.D. students and post-docs to Nestlé management. Represent Ph.D. students and post-docs to the outside.

Tutor, Ludwig-Maximilians-Universität, Munich, Germany, 1997-1999

- Taught students in mathematics, physics and chemistry. During season commitment of 15 hours per week.

SKILLS

- Languages: German, English, French (fluent). Spanish (basic).
- Programming: C.

HOBBIES

- Play piano and compose (ranked 2nd in national competition for young composers 1994), chess (ranked 1st in Munich Junior Chess Competition 1986) and basketball.