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Pyrazine Biosynthesis in *Corynebacterium glutamicum*

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The volatile compounds released by *Corynebacterium glutamicum* were collected by use of the CLSA technique (closed-loop stripping apparatus) and analysed by GC-MS. The headspace extracts contained several acyloins and pyrazines that were identified by their synthesis or comparison to commercial standards. Feeding experiments with [$^2H_7$]acetoin resulted in the incorporation of labelling into trimethylpyrazine and tetramethylpyrazine. Several deletion mutants targeting genes of the primary metabolism were constructed to elucidate the biosynthetic pathway to pyrazines in detail. A deletion mutant of the ketol-acid reductoisomerase was not able to convert the acetoin precursor (*S*)-2-acetolactate into the pathway intermediate (*R*)-2,3-dihydroxy-3-methylbutanoate to the branched amino acids. This mutant requires valine, leucine, and isoleucine for growth and produces significantly higher amounts and more different compounds of the acyloin and pyrazine classes. Gene deletion of the acetolactate synthase (AS) resulted in a mutant that is not able to convert pyruvate into (*S*)-2-acetolactate. This mutant also requires branched amino acids and produces only very small amounts of pyrazines likely from valine via the valine biosynthetic pathway operating in reverse order. A ΔASΔKR double mutant was constructed that does not produce any pyrazines at all. These results open up a detailed biosynthetic model for the formation of alkylated pyrazines via acyloins.

Introduction

Pyrazines occur frequently in Nature and are produced by several bacteria.[1] The bacterium *Paenibacillus polymyxa* releases several alkylated pyrazines with 2,5-diisopropylpyrazine (**1**) as the major compound (Figure 1).[2] The myxobacterium *Chondromyces crocatus* emits an exceptionally complex pattern of volatile products composed of several alkylated and methoxypyrazines. The major components of headspace extracts are **1**, 2-methoxy-3-(1-methylethyl)-6-(2-methylpropyl)pyrazine (**15**), and 2-methoxy-3-(1-methylpropyl)-6-(2-methylpropyl)pyrazine (**16**) in strain-specific proportions.[3] Several bacteria from the marine *Roseobacter* clade also produce pyrazines. In the volatile fraction from *Sulfitobacter pontiacus* a diverse array of pyrazines such as 3,6-dimethyl-2-(2-methylpropyl)pyrazine (**7**), 3,6-dimethyl-2-butylpyrazine (**8**), 3,6-dimethyl-2-(3-methylbutyl)pyrazine (**9**), 3,6-dimethyl-2-(2-methylbutyl)pyrazine (**10**), and the unusual compound 3,6-dimethyl-2-(methylsulfanyl)pyrazine (**17**) have been detected.[3] The compounds **8** and **9** are also released by cultures of *Loktanella* sp.,[4] while a marine arctic bacterium from the *Cytophaga-Flexibacter-Bacteroides* (*CFB*) phylum produces 2-ethyl-3,6-dimethylpyrazine (**2**) and 2-ethyl-3,5-dimethylpyrazine (**3**).[5] Alkylpyrazines such as **7**, **9**, and **10** also occur in insects, e.g. in the defensive spray of the phasmid insect *Phyllium westwodii*.[6] They are furthermore known as ant trail pheromones represented by **2** in *Atta sexdens rubropi-*

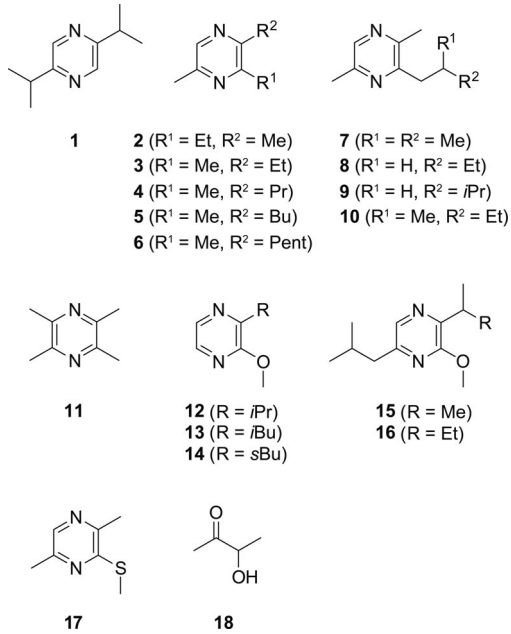

Figure 1. Structures of known pyrazines and acetoin.

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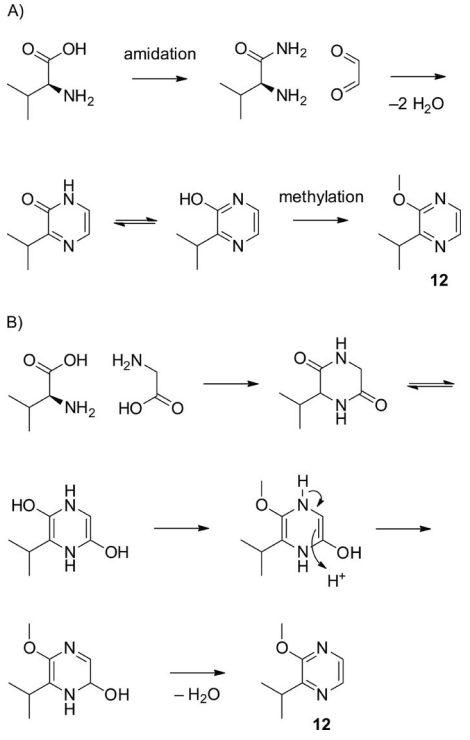
losa[7] or alarm pheromones such as **9** in *Odontomachus hastatus*, or **3**, 3,5-dimethyl-2-propylpyrazine (**4**), 2-butyl-3,5-dimethylpyrazine (**5**), and 3,5-dimethyl-2-pentylpyrazine (**6**) in *O. brunneus*.[8] Methoxypyrazines such as 2-methoxy-3-(1-methylethyl)pyrazine (**12**), 2-methoxy-3-(2-methylpropyl)pyrazine (**13**), and 2-methoxy-3-(1-methylpropyl)-pyrazine (**14**) have been reported from several aposematic insects including *Coccinella septempunctata*.[9] The methoxypyrazines **12**–**14** are also found in some plants, e.g. in peas (*Pisum sativum*).[10]

Knowledge about the biosynthesis of pyrazines is very limited, although this topic has long been questioned and controversally discussed in the literature. For the biosynthesis of 2-methoxy-3-alkylpyrazines the condensation of an α-amino acid amide such as valine amide with glyoxal followed by *O*-methylation was suggested (Scheme 1).[10] In contrast, Gallois et al. proposed valine and glycine as building blocks. However, feeding experiments with ^{13}C-labelled precursors demonstrated the incorporation of valine, but not of glycine.[11] Cheng et al. used [2-^{13}C]pyruvate and [3-^{13}C]pyruvate resulting in the incorporation of the isotopic labelling in specific positions that is in accordance with a biosynthetic pathway via valine, glycine, and *S*-adenosylmethionine (SAM) for *O*-methylation.[12] The suggested pathway proceeds via a cyclic dipeptide (dioxopiperazine), *O*-methylation, and elimination of water, and is more plausible than any other published route (Scheme 1).



Scheme 1. Proposed biosynthetic pathways to 2-methoxy-3-alkylpyrazines. A: Biosynthesis of **12** from valine and glyoxal as suggested by Murray et al.[9] B: Biosynthetic pathway to **12** after Cheng et al. from glycine, valine, and SAM.[12]

Early investigations by Kosuge et al. resulted in the identification of tetramethylpyrazine (**11**) and acetoin (**18**) in *Bacillus natto*.[13] Although no experimental proof was given, the authors speculated about a biosynthesis of **11** from **18**. Demain et al. demonstrated a direct biosynthetic link between pyrazines and the amino acids valine, leucine, and isoleucine.[14] A genetically uncharacterised *C. glutamicum* mutant was isolated that produced large amounts of **11** and was only able to grow with the supply of these amino acids. The biosynthesis of **11** was also shown to be thiamine-dependent, and based on these data a biosynthetic pathway via **18** was suggested. Rizzi demonstrated the spontaneous formation of **11** from **18** in the presence of ammonium acetate or ammonium citrate at neutral to slightly acidic pH (pH 5.14), albeit at high non-physiological salt concentrations.[15] Inspite of these investigations a detailed understanding of the biosynthetic pathway to pyrazines was missing.

Since the unravelling of the complete genome sequence of *C. glutamicum*[16] a targeted gene deletion is possible. Here we describe the identification of volatiles released by *C. glutamicum*. The headspace extracts contain several pyrazines and acyloins in different concentrations. Feeding experiments with isotopically labelled precursors and gene knockout experiments are described giving rise to a biosynthetic pathway from glycolytic intermediates via acyloins to a variety of alkylated pyrazines.

Results and Discussion

Volatile Products from *Corynebacterium glutamicum*

The volatiles released by agar plate cultures of the wild-type strain of *C. glutamicum* have been collected in four separate experiments by use of the CLSA (closed-loop stripping apparatus) headspace technique and analysed by GC-MS. The results of these analyses are presented in Table 1 and the total ion chromatogram of one representative extract is shown in part A of Figure 2. Pyrazines formed the predominant compound class represented by methylpyrazine (**27**), 2,5-dimethylpyrazine (**28**), 2,3-dimethylpyrazine (**29**), large quantities of trimethylpyrazine (**30**), 2-ethyl-3,6-dimethylpyrazine (**2**), 2-ethyl-3,5-dimethylpyrazine (**3**), 2-propyl-3,5-dimethylpyrazine (**4**), tetramethylpyrazine (**11**), ethyltrimethylpyrazine (**31**), 2-(hydroxymethyl)-5-methylpyrazine (**34**), and 2-(hydroxymethyl)-5,6-dimethylpyrazine (**35**, Figure 3). The pyrazines were accompanied by acetoin (**18**) and its derivatives 3-hydroxypentan-2-one (**19**), 2-hydroxypentan-3-one (**20**), 2-hydroxyhexan-3-one (**21**), and 2,3-butanediol (**23**). The pyrazines **2**, **11**, **29**, and **30** were consistently found in all four replicates, whereas the other volatiles were only released by one or a few cultures. The identity of all these compounds was confirmed by comparison of the mass spectra (see Supporting Information, Figures SI-1 and SI-2) and retention indices to those of commercially available standards (**11**, **18**, **23**, and **27**–**30**), or to synthetic references.

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Table 1. Compounds identified in the headspace extracts of *C. glutamicum* wild-type strain (WT) and mutant strains described in the text (ΔKR, ΔAS, ΔKRΔAS).

Compound[a]	*I*[b]	Ident.[c]	WT (1)	WT (2)	WT (3)	WT (4)	ΔKR	ΔAS	ΔKRΔAS
Acetoin (**18**)	762	ms, com	+	+	++		+++		
2,3-Butanediol (**23**)	792	ms, com				++	+		
2,3-Hexanedione (**24**)	795	ms, com					+		
3-Hydroxypentan-2-one (**19**)	800	ms, syn	+	+	+		+		
2-Hydroxypentan-3-one (**20**)	818	ms, syn			+		+		
Methylpyrazine (**27**)	821	ms, com		+	+				
Trimethyloxazol (**26**)	848	ms, com					+		
2,3-Heptanedione (**25**)	888	ms, com					+		
2-Hydroxyhexan-3-one (**21**)	900	ms, syn	+++		++		++		
2,5-Dimethylpyrazine (**28**)	912	ms, com		+	+				
2,3-Dimethylpyrazine (**29**)	914	ms, com	++	++	++	++	++	+	
2-Hydroxyheptan-3-one (**22**)	999	ms, syn					+		
Trimethylpyrazine (**30**)	1002	ms, com	+++	+++	+++	+++	+++	+	
2-Ethyl-3,6-dimethylpyrazine (**2**)	1077	ms, syn	+	++	++	+	+		
2-Ethyl-3,5-dimethylpyrazine (**3**)	1082	ms, syn		+					
Tetramethylpyrazine (**11**)	1087	ms, com	++	++	+++	+	+++	+	
2-(Hydroxymethyl)-5-methylpyrazine (**34**)	1137	ms, syn				++			
Ethyltrimethylpyrazine (**31**)	1162	ms, syn		+	+		++		
2-Propyl-3,5-dimethylpyrazine (**4**)	1169	ms, syn			+				
2-(Hydroxymethyl)-5,6-dimethylpyrazine (**35**)	1231	ms, syn				+			
Trimethylpropylpyrazine (**32**)	1243	ms, syn					+		
Butyltrimethylpyrazine (**33**)	1338	ms, syn					+		

[a] Relative amounts of the volatile components in one extract are indicated by +: 0–2%, ++: 2–8%, +++: >8% of total area in the gas chromatograms. Note that these indications for one specific sample are not comparable to the indications of another extract, i.e. the indication of e.g. ++ for **21** in the ΔKR mutant represents a higher absolute amount than the indication +++ in the first wild-type extract (compare Figure 1). [b] Retention indices *I* were determined from a homologous series of alkanes (C_8–C_{36}) on a HP-5 GC column. [c] Compound identification was based on the mass spectrum (ms), comparison to a commercially available standard (com) or a synthetic reference (syn, this work).

Compound **2** was synthesised from **28** (Scheme 2). Pyrazines can be transformed into their *N*-oxides by using the peroxide reagent $NaBO_2{\cdot}H_2O_2{\cdot}H_2O$ (simply named "$NaBO_3$" in Scheme 2) in refluxing glacial acetic acid.[17] This method was adopted to **28** to obtain 2,5-dimethylpyrazine *N*-oxide (**36**) in good yield (85%) that was treated with $POCl_3$ to result in 2-chloro-3,6-dimethylpyrazine (**37**).[18] Fürstner and Leitner reported a method for the iron-catalysed cross-coupling of aryl chlorides with Grignard reagents[19] that is also successful for the alkylation of chloropyrazines.[3] The cross-coupling of **37** and EtMgBr yielded the desired pyrazine **2**. The pyrazines **3** and **4** were equally synthesised from 2-chloro-3,5-dimethylpyrazine (**39**) and EtMgBr or PrMgBr, respectively. The mass spectra and gas chromatographic retention indices of the regioisomeric alkylpyrazines **2** and **3** are very similar, indicating the need for synthetic reference material of positional isomers. To rule out a confusion between **4** and its regioisomer 3,6-dimethyl-2-propylpyrazine (**38**), the latter compound was synthesised by the iron-catalysed alkylation of **37** with PrMgBr. Comparison of both alkylpyrazines with the natural material clearly established the structure of **4** for the volatile product released by *C. glutamicum*. Trimethylpyrazine (**30**) served as starting material for the synthesis of **31**. The *N*-oxidation yielded two regioisomeric *N*-oxides **40a** and **40b** in a ratio of 2:3 not separable by column chromatography. Therefore, the mixture was used in the chlorination step to give chlorotrimethylpyrazine (**41**) that was alkylated with EtMgBr resulting in **31**. The (hydroxymethyl)pyrazine **34** was synthesised from 5-methylpyrazinecarboxylic acid (**42**) that was transformed into the respective methyl ester **44**. The reduction of this ester to **34** proved to be difficult. While $LiAlH_4$ reduction failed, the usage of $NaBH_4$ gave **34** in poor yield. A known condensation/oxidation sequence was carried out for the synthesis of **35**.[20] The reaction of diacetyl with 2,3-diaminopropionic acid proposedly resulted in a diimine intermediate that was oxidised by the passage of an airstream through the reaction mixture to give the heteroaromatic compound **43**. Esterification to **45** and $NaBH_4$ reduction furnished **35**.

The acetoin derivatives **19**–**21** were synthesised as depicted in Scheme 3. Acetaldehyde was converted into 2-methyl-1,3-dithiane (**46**) that was used in a Seebach umpolung with propanal to yield 2-(1-hydroxypropyl)-2-methyl-1,3-dithiane (**50**). Cleavage of the dithiane moiety with $AgNO_3$ in water, extraction of the reaction products with diethyl ether, and analysis of the dried organic layer by GC-MS demonstrated the quantitative conversion of **50** to the hydroxy ketone **19**. However, removal of the ether by distillation resulted in a significant loss of the highly volatile product and only small amounts of the pure product could be obtained. Similarly, the hydroxy ketones **20** and **21** were synthesised from propanal and acetaldehyde, or butanal and acetaldehyde, respectively.

Pyrazine Biosynthesis

The observations made by Demain et al.[14] together with the results of our analyses showing the concomitant occur-

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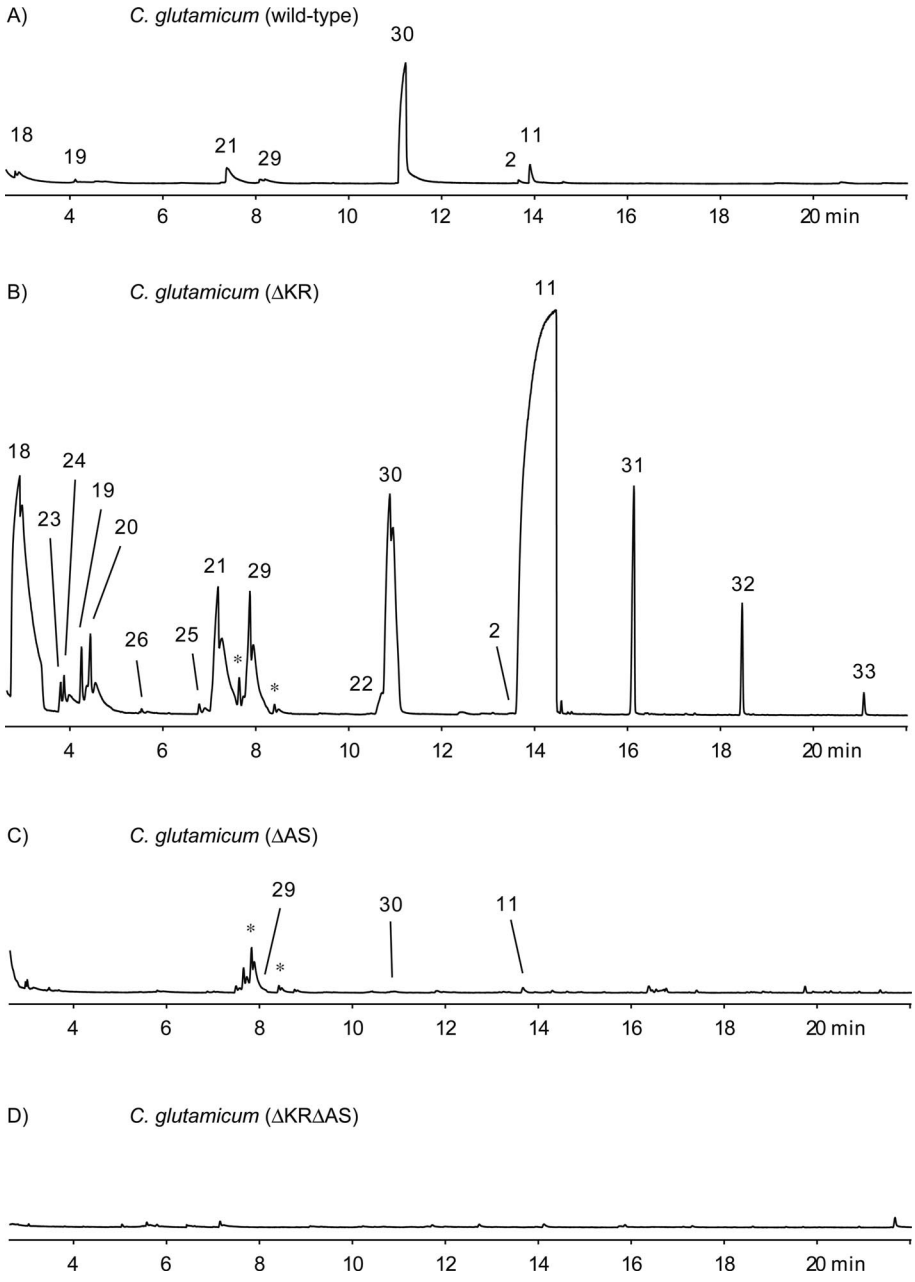

Figure 2. Total ion chromatograms of *Corynebacterium glutamicum* headspace extracts obtained by CLSA. (A) *C. glutamicum* wild-type strain, (B) mutant strain with a deletion of the ketol-acid reductoisomerase (ΔKR), (C) mutant strain with a deletion of the acetolactate synthase (ΔAS), and (D) double mutant strain with a deletion of both the ketol-acid reductoisomerase and the acetolactate synthase (ΔKRΔAS). To allow for comparison of the wild-type and the mutant strains scaling of the ordinates is the same for all chromatograms (A–D). Asterisks indicate artifacts also present in headspace extracts of agar plates containing the medium alone.

rence of acyloins and pyrazines in the headspace of *C. glutamicum* suggest that the biosynthesis of several different alkylated pyrazines proceeds via acyloin building blocks (Scheme 4). To test this hypothesis, deuterated [$^2H_7$]acetoin was synthesised from [$^2H_4$]acetaldehyde in an umpolung reaction using 3-benzyl-5-(2-hydroxyethyl)-4-methyl-1,3-thiazolium chloride and triethylamine. The labelled [$^2H_7$]-**18** was fed to the wild-type strain of *C. glutamicum*. Incorporation of the deuterium label was observed into the pyrazines **30** and **11** indicated by a shift of the molecular ions of **30** from *m*/*z* = 122 to *m*/*z* = 128 and of **11** from *m*/*z* = 136 to *m*/*z* = 142 (Figure SI-3 of Supporting Information). These data are in accordance with two deuterated methyl groups. However, the incorporation proceeded with low rates (**30**: 0.5%, **11**: 1.0%; incorporation rates were determined from the ratio of the peak areas of the molecular ions of the deuterium labelled and the unlabelled isotopomers). The doubled incorporation rate observed for **11** compared to **30** reflects the statistically more likely uptake of one labelled acetoin molecule into **11** as it is formed from two acetoins, whereas **30** requires only one acetoin unit (see discussion below).

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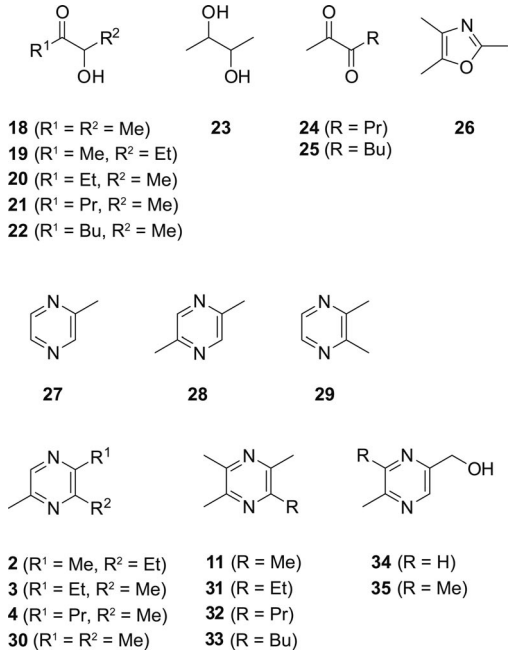
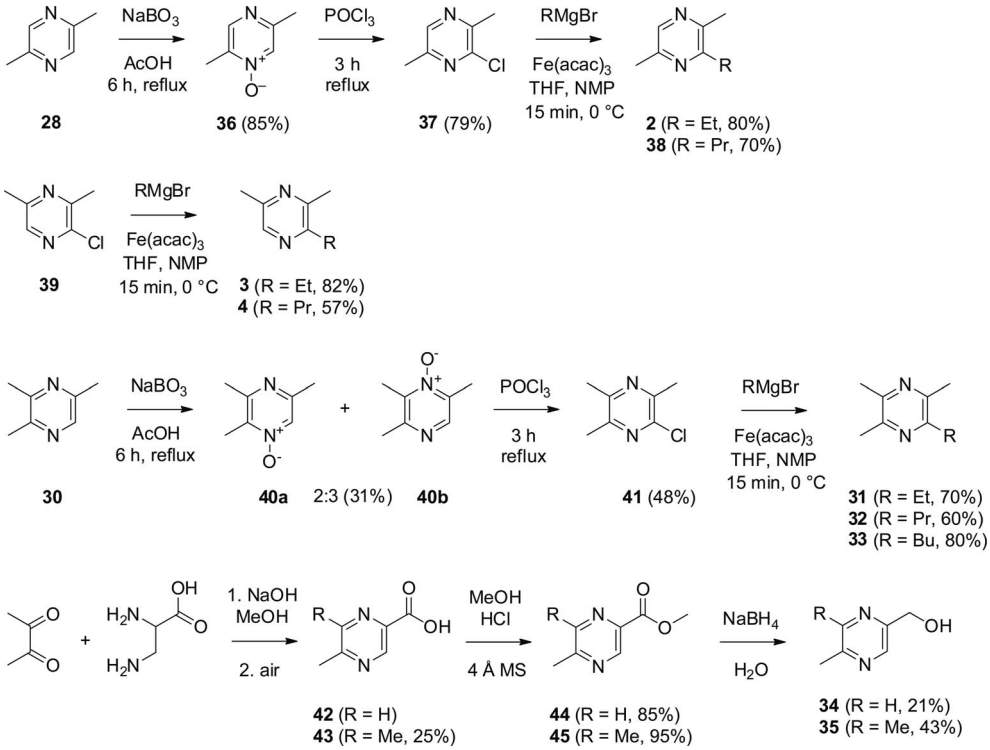
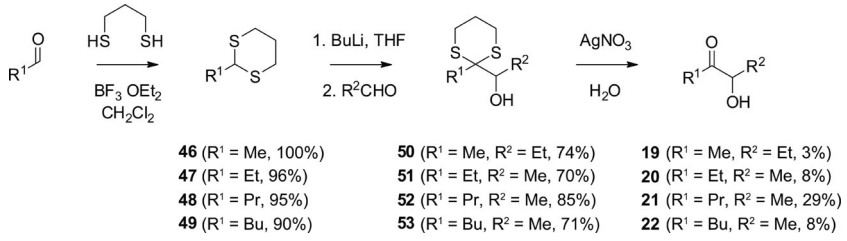


Figure 3. Volatile products from *Corynebacterium glutamicum*.

According to textbook biochemistry[21] the biosynthesis of amino acids valine and leucine needs two pyruvate units (**54a**), one of which is transferred to the thiamine diphosphate (TPP, **55**) cofactor under decarboxylation to give 2-(1-hydroxyethyl)thiamine diphosphate (**56a**, Scheme 4). This compound adds an acetyl group to the second pyruvate unit to (*S*)-2-acetolactate (**57a**) in a reaction catalysed by the acetolactate synthase (AS). The ketol-acid reductoisomerase (KR) catalyses the reaction of **57a** to (*R*)-2,3-dihydroxy-3-methylbutanoate (**58a**) that is an intermediate to valine and leucine. The AS and KR enzymes are also involved in similar reactions in the biosynthesis of isoleucine. The addition of an acetyl group from **56a** to 2-oxobutanoate (**54b**) under catalysis of the AS results in (*S*)-2-aceto-2-hydroxybutanoate (**57b**) that is converted by the KR to (2*R*,3*R*)-2,3-dihydroxy-3-methylpentanoate (**58b**) en route to isoleucine. Some organisms use an acetolactate decarboxylase to catalyse the formation of (*R*)-acetoin from (*S*)-2-acetolactate, but this enzyme is not encoded in the genome of *C. glutamicum*, and therefore spontaneous decarboxylation of **57a** to acetoin (**18**) or the presence of an unknown type of acetolactate decarboxylase must be assumed.



Scheme 2. Synthesis of pyrazines from *C. glutamicum*.



Scheme 3. Synthesis of acyloins **19**–**22** from *C. glutamicum*.

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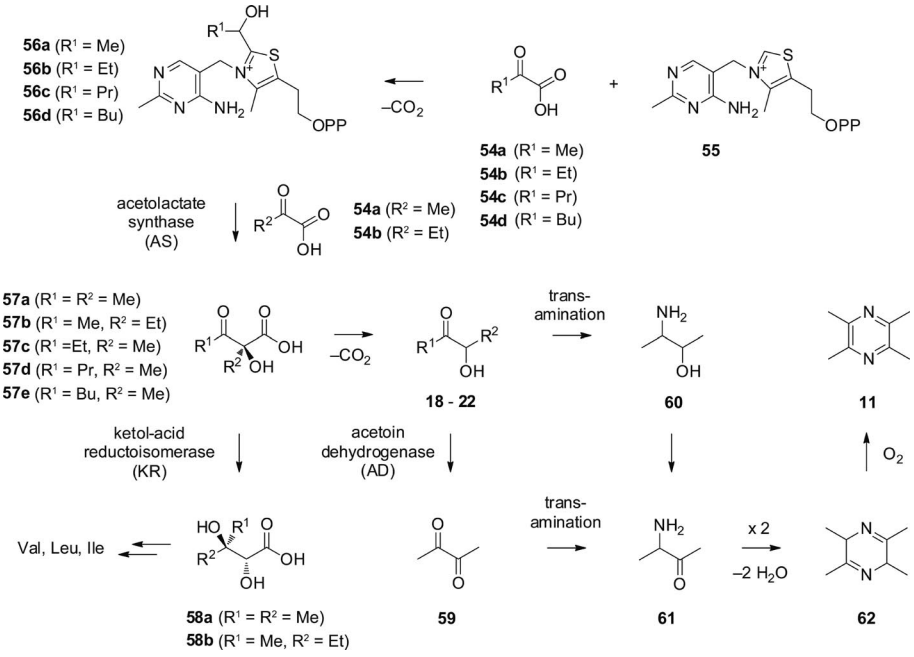

Scheme 4. Proposed biosynthetic pathway to tetramethylpyrazine (**11**) via acetoin (**18**).

Equally **57b** is converted to **19**. The common biosynthesis of the intermediates **57a** and **57b** by the AS and of **58a** and **58b** by the KR demonstrates some structural flexibility of the enzyme system with respect to their substrates. Therefore it seems reasonable that the action of the AS-KR system also accounts for the biosynthesis of **20** and **21**. The reaction of 2-(1-hydroxypropyl)thiamine diphosphate (**56b**) derived from **54b** with **54a** yields (*S*)-2-hydroxy-2-methyl-3-oxopentanoate (**57c**) that decarboxylates to **20**. Equally, 2-oxopentanoate (**54c**) is transferred to the TPP cofactor resulting in 2-(1-hydroxybutyl)thiamine diphosphate (**56c**), which is together with **54a** the precursor of (*S*)-2-hydroxy-2-methyl-3-oxohexanoate (**57d**); decarboxylation of the latter results in **21**.

The downstream steps from the acyloins to the pyrazines are exemplarily shown for the formation of the D_{2h}-symmetrical compound **11** from two **18** molecules. The pathway to **11** might include the oxidation of **18** to butanedione (**59**) by the acetoin dehydrogenase (AD) and transamination to 3-aminobutanone (**61**) by an unknown aminotransferase. Alternatively, a transamination reaction with **18** may first proceed to 3-aminobutan-2-ol (**60**) that can be oxidised to **61**. Condensation of two units of **61** to tetramethyldihydropyrazine (**62**) and spontaneous oxidation in the presence of oxygen can result in **11**. As will be discussed below a detailed analysis of our data suggest an enzymatic rather than a non-enzymatic formation of the pyrazines from the acyloin derivatives.

In analogy to the biosynthesis of **11** the pyrazine **30** requires an acyloin building block in addition to a C_2 building block such as glycolaldehyde or glyoxal (see Scheme SI-1, Supporting Information). These metabolites are both not found in the headspace extracts because they coelute with the solvent in GC-MS. Compound **30** may be formed from **18** and a C_3 unit such as hydroxyacetone/lactaldehyde or methylglyoxal that also coelute with the solvent. These C_2 and C_3 building blocks can also serve for the formation of **27** (C_2 + C_3) and **28** (C_3 + C_3). The compounds **2** and **3** can be generated from the respective C_3 unit and **19** or **20**, **4** can arise from a C_3 unit and **21**, whereas **32** can be formed from **18** and **19** or **20**.

The origin of the (hydroxymethyl)pyrazines **34** and **35** found in one wild-type sample remains elusive. They may either be generated by the incorporation of hydroxyacetone or glycerinaldehyde as exemplarily shown for **35** in Scheme SI-1 (see Supporting Information). Alternatively, these hydroxylated pyrazines are formed from **28** and **30**, respectively, in a reaction catalysed, for instance by a monooxygenase. Another possibility is the spontaneous oxidation in the presence of oxygen, maybe via radical intermediates, although this mechanism seems unlikely, because the formation of several additional products could be expected. The oxidation of any methyl group of **28** with molecular oxygen results in **34**, whereas the oxidation of **30** should result in three different products represented by **35**, 2-(hydroxymethyl)-3,6-dimethylpyrazine, and 2-(hydroxymethyl)-3,5-dimethylpyrazine, but only **35** is found.

Volatiles from the ΔKR Mutant

To investigate the suggested pathway several deletion mutants were constructed and analysed for their production of acyloin and pyrazines. A mutant with a gene deletion of the gene encoding the ketol-acid reductoisomerase (ΔKR) was constructed. This mutant required valine, leucine, and isoleucine for growth and produced significantly higher

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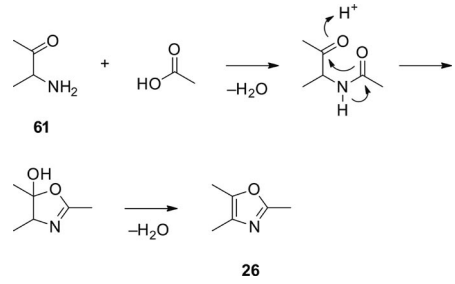
amounts of acyloins and pyrazines compared to the wild-type strain (Table 1 and Figure 2, B), because acetolactate cannot be directed towards the amino acids valine and leucine. In consequence, a large acetolactate pool for the formation of **18** and the pyrazines derived thereof is available. The derivatives **19**–**21** and **23** were also found, in addition to 2-hydroxyheptan-3-one (**22**) not present in the wild-type strain (mass spectra are depicted in Figure SI-2 of Supporting Information). For unambiguous identification compound **22** was synthesised using the same approach as for the shorter homologues from pentanal and acetaldehyde (Scheme 3).

Similar to the proposed biosynthesis of the other acyloin derivatives, **22** is presumably made from 2-oxohexanoate (**54d**) by decarboxylation and addition to TPP (Scheme 4). The reaction of 2-(1-hydroxypentyl)thiamine diphosphate (**56d**) with pyruvate yields the (*S*)-2-hydroxy-2-methyl-3-oxoheptanoate (**57e**) precursor of **22**. Furthermore, the related compounds 2,3-hexanedione (**24**), and 2,3-heptanedione (**25**) were found. The presence of **24** and **25** supports the biosynthetic pathway from the acyloin derivatives via oxidation to the dicarbonyl compounds as depicted in Scheme 4. The respective oxidation products of **18** (butanedione) as well as **19** and **20** both yielding 2,3-pentanedione cannot be found in the headspace extracts, because these compounds coelute with the solvent.

The pyrazines produced by the ΔKR mutant included the wild-type compounds **2**, **11**, **29**, **30**, and **31**, accompanied by trimethyl-propylpyrazine (**32**) and butyl-trimethylpyrazine (**33**). The identity of these volatiles was established by synthesis from the chloropyrazine **41** using Fürstner's and Leitner's alkylation[19] with PrMgBr or BuMgBr, respectively (Scheme 1). These pyrazines can be generated from the acyloin derivatives produced by the ΔKR mutant. Compound **32** can arise from **18** and **21**, and **33** can originate from **18** and **22** (Scheme 4). Further pyrazines such as 3,6-dimethyl-2,5-dipropylpyrazine that could be formed by a dimerisation of **21** present in large quantities in the headspace extracts of the ΔKR mutant were not found, pointing to an enzymatic formation of the pyrazines. The enzyme seems to be able to catalyse the condensation of the proposed aminocarbonyl building blocks derived from **18** and **21**, whereas the condensation of two such intermediates from **21** may exceed the limits of the enzyme. This is in contrast to the suggested non-enzymatic formation of pyrazines such as **11** from acetoin and ammonium salts at neutral to slightly acidic pH.[15] To exclude a non-enzymatic mechanism for the formation of pyrazines in the agar plate cultures of *C. glutamicum* the CG12½ medium plates containing $(NH_4)_2SO_4$ (1 g L^{-1}) at different pH (pH 5.2, 6.5, and 7.2) were spiked with **18** (1 mM). Analysis of the volatiles released from these plates did not show the production of the expected pyrazine **11** or any other pyrazine derivatives (data not shown). In case of a non-enzymatic condensation of the acyloin derivatives and ammonium salts a complex pattern of several additional pyrazines would be expected, formed from all possible combinations of the acyloins **18**–**22**.

Another volatile component found in traces in the headspace extract of the ΔKR mutant was trimethyloxazol (**26**) that is the formal condensation product of **61** and acetic acid (Scheme 5), corroborating the proposed biosynthetic pathway to pyrazines via α-aminocarbonyl intermediates. Instead of acetic acid the more reactive acetyl-CoA may serve as the true reactant.



Scheme 5. Formation of trimethyloxazol (**26**) from 3-aminobutanone (**61**) and acetic acid.

Volatile Products from the ΔAS and ΔASΔKR Mutants

A *C. glutamicum* mutant with a deletion of the acetolactate synthase (ΔAS) was constructed and analysed for the production of acyloin and pyrazine derivatives (Table 1 and Figure 2, C). This mutant required the addition of the amino acids leucine, valine, and isoleucine to the growth medium. The headspace extract contained only traces of the pyrazines **11**, **29**, and **30** that are the most abundant pyrazines found in the wild-type strain, whereas all other pyrazines and the acyloin derivatives **18**–**22** produced by the wild-type strain were absent in the ΔAS mutant. Since the formation of acetolactate and **18** from pyruvate is impaired in this mutant, the biosynthesis of these pyrazines may proceed via the valine and leucine biosynthetic pathway operating in reverse order. It is remarkable that only the pyrazines derived from **18** (i.e., **11**) and from **18** and a C_2 or C_3 building block (**29** and **30**) were emitted, but no other pyrazines originating from the acyloin derivatives **19**–**22** were found, pointing to the formation of all acyloins by the acetolactate synthase in the wild-type strain.

A *C. glutamicum* ΔASΔKR double mutant was constructed and analysed for its profile of volatiles (Table 1 and Figure 2, D). This mutant also required supply of the branched amino acids and produced neither acyloin nor pyrazine derivatives. Complementation of this mutant with acetoin (1 mM) resulted in the restored production of **11** and **30** (data not shown).

Occurrence of Identified Pyrazines in Nature

Alkylated pyrazines have frequently been described from processed food. Their formation has been related to roasting processes in which they are formed from amino acids and sugars in the Maillard reaction.[22,23] Several pyrazines such as **28** can be formed in this reaction during the autoclavation process from medium ingredients,[1] but this was

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ruled out for the pyrazines discussed here by analysis of the headspace compounds released from medium plates alone; no pyrazines were found in the blank analysis. A hazelnut, cocoa, chocolate, rum, or roasted aroma has been attributed to several alkylated pyrazines such as **11** and **27**–**30** found in dark chocolate.[24] Several pyrazines described in this article can also be found in insects, e.g. the mixture of **2**, **28**, and **30** is the trail pheromone of ants of the *Pogonomyrmex* genus.[25,26] Among bacteria, *Sulfitobacter pontiacus* produces a blend of several pyrazines including **2**, **11**, and **31**.[3] Besides the compounds **2** and **3** already mentioned in the introduction a marine arctic bacterium from the *CFB* phylum produces **11**, **28**, **30**, and **31**.[5] The hydroxylated pyrazine **34** has been reported from the bat *Tadarida brasiliensis mexicana*,[27] whereas its counterpart **35** and the alkylpyrazines **32** and **33** are new natural products.

Conclusions

The profile of volatiles released by *Corynebacterium glutamicum* is composed of several pyrazine and acyloin derivatives with trimethylpyrazine (**30**), tetramethylpyrazine (**11**), and acetoin (**18**) as main compounds. Several additional pyrazine and acyloin derivatives were identified from their mass spectra and by comparison to commercially available or synthetic references. Feeding experiments with [$^2H_7$]-**18** clearly demonstrated that the pyrazines **11** and **30** are derived from acetoin. Gene knockout mutants of relevant genes of the primary metabolism have been constructed to further investigate the underlying pathways. Deletion of the ketol-acid reductoisomerase (KR) of the biosynthetic pathway to the branched amino acids resulted in a mutant that required addition of valine, leucine, and isoleucine to the medium and produced significantly higher amounts and more different compounds of the pyrazine and acyloin classes. Gene inactivation of the acetolactate synthase (AS) gave a mutant that also required supply of the branched amino acids and produced only minute amounts of the pyrazines **11**, **29**, and **30** explainable by their formation from valine by the valine biosynthetic pathway operating in reverse order. The ΔASΔKR double mutant is impaired in the production of pyrazines and acyloins, but the production of **11** and **30** could be restored by the addition of acetoin to the culture medium. These data strongly support the biosynthesis of alkylated pyrazines from acyloin derivatives. The formation of **11** from **18** and ammonium salts has been reported to proceed non-enzymatically under neutral to slightly acidic pH and high salt concentrations. A similar reaction was ruled out for the generation of pyrazines from *C. glutamicum* by the addition of acetoin to agar plates with medium alone at different pH values. Downstream steps from the acyloin derivatives to the pyrazines likely proceed via α-aminocarbonyl compounds. An indicator for this assumption is the presence of trimethyloxazol (**26**) as a trace component in the headspace extract of the ΔKR mutant that likely arises from 3-aminobutanone (**61**) and acetyl-CoA.

Further investigations will be carried out in our laboratories to identify the genes and enzymes of the downstream steps of the biosynthetic pathway from the acyloins to the pyrazine derivatives.

Experimental Section

Microorganisms: In the present work, the wild-type *Corynebacterium glutamicum* ATCC 13032 (American Type and Culture Collection, Manassas, USA) and specifically designed mutants, constructed on basis of the wild-type, were investigated (Table SI-1 of Supporting Information). *Escherichia coli* DH5α and NM522 were obtained from Invitrogen (Karlsruhe, Germany) and used for plasmid amplification and DNA methylation, respectively.

Nucleic Acid Isolation: Cells were maintained at 30 °C on agar plates, harvested after 2 d with a sterile inoculation loop and suspended in 500 μL of sterile water. Cell disruption was performed for 1 min at 30 Hz in a ribolyzer (MM301, Retsch, Haan, Germany) after addition of glass beads (0.1–0.25 mm, Retsch, Haan, Germany) and a mixture of phenol/chloroform/isoamyl alcohol (700 μL, Carl-Roth GmbH, Karlsruhe, Germany). After separation of aqueous and organic phase (10,000 × g, 5 min), DNA from the aqueous phase was precipitated by addition of sodium acetate (65 μL, 3 M, pH 5.5) and ethanol (100%, 1.3 mL) and a centrifugation step (10,000 × g, 10 min). Subsequently, the supernatant was removed. The precipitated genomic DNA was dissolved in sterile water (100 μL). Isolation of plasmid DNA from *E. coli* DH5α and NM522 was performed using the DNA isolation kits GFX Micro Plasmid Prep (GE Healthcare, Piscataway, NJ, USA) and HiSpeed Plasmid Midi Prep (Qiagen, Hilden, Germany), respectively, according to the instruction manual given by the manufacturer.

Strain Construction: Gene deletion was achieved by allelic replacement of the wild-type gene by a shortened DNA fragment. The knockout is usually carried out by a deletion of at least 300 bp to be able to distinguish wild-type and knockout gene in gel electrophoresis. Here, 500 bp were deleted for single knockout of the ketol-acid reductoisomerase. In the genome the large and small subunit of the acetolactate synthase as well as the ketol-acid reductoisomerase are located next to each other. Due to this, the corresponding knockouts were carried out by deletions containing more base pairs. In the case of the large and small subunit of the acetolactate synthase 1500 bp were deleted and in the case of the double knockout of the acetolactate synthase and the ketol-acid reductoisomerase 2500 bp were deleted. The desired DNA fragment was obtained in three steps by PCR. Sequences of the respective site-specific primers P1-P4 for the deletions are given in Table SI-2 (see Supporting Information). The obtained DNA fragments were subsequently inserted into the vector pClik int *sacB* via two restriction sites. Vector amplification was performed in *E. coli* DH5α and NM522, carrying a plasmid-encoded copy of the *C. glutamicum* specific DNA methyltransferase gene, to enable the appropriate DNA methylation pattern. *C. glutamicum* was transformed by electroporation as described previously.[28] Gene deletion was validated by PCR analysis. As the genetic modification was restricted to the internal coding sequence of each enzyme, no other genes were affected.

Media and Growth Conditions: Cells were maintained at 30 °C on agar plates containing complex medium with glucose (10 g L^{-1}), NaCl (2.5 g L^{-1}), urea (2 g L^{-1}), yeast extract (5 g L^{-1}), beef extract (5 g L^{-1}), polypeptone (5 g L^{-1}) and agar (20 g L^{-1}). Afterwards, cells were harvested by centrifugation (10,000 × g, 15 min) and

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washed two times with 2.6% NaCl. Subsequently, cells were plated on agar plates with CG12½ minimal medium that contained per litre: glucose (10 g), K_2HPO_4 (16 g), KH_2PO_4 (4 g), $(NH_4)_2SO_4$ (5 g), 3,4-dihydroxybenzoic acid (300 mg), $CaCl_2$ (10 mg), $MgSO_4 \cdot 7H_2O$ (250 mg), $FeSO_4 \cdot 7H_2O$ (10 mg), $MnSO_4 \cdot H_2O$ (10 mg), $ZnSO_4 \cdot 7H_2O$ (2 mg), $CuSO_4 \cdot 5H_2O$ (200 μg), $NiCl_2 \cdot 6H_2O$ (20 μg), $Na_2MoO_4 \cdot 2H_2O$ (20 μg), cyanocobalamin (100 μg), thiamine (300 μg), pyridoxal phosphate (4 μg), biotin (100 μg), and agar noble (15 g). Additionally, the medium was supplemented with valine, leucine, and isoleucine to a final concentration of 1 mM due to auxotrophy for these amino acids of the designed deletion mutants. Agar plates were incubated for 3 d at 30 °C prior to CLSA and GC-MS analyses.

Sampling of Volatile Compounds: The volatile products emitted by the agar plate cultures were collected by using the CLSA headspace technique.[29] Briefly, in a closed apparatus containing the agar plates a circulating air stream was passed through a charcoal filter (Chromtech GmbH, Idstein, Precision Charcoal Filter, 5 mg) for 24 h. The absorbed volatiles were eluted from the charcoal with analytically pure dichloromethane (30 μL). The obtained solutions were immediately analysed by GC-MS and stored at –80 °C. For comparison agar plates containing the medium alone were investigated. None of the volatiles shown in Table 1 have been detected in these experiments.

GC-MS: GC-MS analyses were carried out on an Agilent 7890A connected with an Agilent 5975C inert mass detector fitted with a HP-5 (30 m, 0.25 mm i. d., 0.25 μm film) or BPX5 (25 m, 0.25 mm i. d., 0.25 μm film) fused silica capillary column. Conditions were as follows: inlet pressure: 77.1 kPa, He 23.3 mL min^{-1}; injection volume: 2 μL; transfer line: 300 °C; electron energy: 70 eV. The GC was programmed as follows: 5 min at 50 °C increasing at 5 °C min^{-1} to 320 °C, and operated in splitless mode (60 s valve time). The carrier gas was He at 1.2 mL min^{-1}. Retention indices *I* were determined from a homologous series of *n*-alkanes (C8–C38). Identification of compounds was performed by comparison of mass spectra and retention indices with synthetic standards.

Supporting Information (see also the footnote on the first page of this article): Experimental procedures for the synthesis of compounds and full characterisation data.

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