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Identification and synthesis of volatiles released by the myxobacterium *Chondromyces crocatus*

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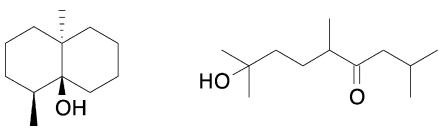
Abstract—Cultures of the myxobacterium *Chondromyces crocatus* on agar plates were analysed by closed-loop-stripping analysis or solid phase micro extraction. The odour profiles consist mainly of pyrazines, sesquiterpenoids and some aromatic compounds, summing up to more than 50 components. Several new pyrazines as 2-(1-hydroxy-1-methylethyl)-3-methoxypyrazine (**9**), 2-(1-hydroxy-1-methylpropyl)-3-methoxypyrazine (**10**), and 2-(1-hydroxy-2-methylpropyl)-3-methoxypyrazine (**11**) were identified besides several known pyrazines. A major pyrazine occurring in most samples was 2,5-bis-(1-methylethyl)pyrazine (**3**). While the well known sesquiterpenoid geosmin (**1**) was present in low amounts, the related compound (1(10)*E*,5*E*)-germacradien-11-ol (**21**) was identified in most samples in larger quantities. Other prominent sesquiterpenoids not reported before from microorganisms were (6*S*,10*S*)-6,10-dimethylbicyclo[4.4.0]dec-1-en-3-one (**16**), which was accompanied by smaller amounts of several derivatives. The biosynthesis of these compounds is discussed in relation to the recently proposed biosynthetic pathways to **1** and **21**.

1. Introduction

Myxobacteria are a unique group of bacteria, characterised by the formation of multicellular fruiting bodies, the most complex behaviour so far observed in any procaryote. They are also known to produce a wide variety of secondary metabolites which often show high pharmacological or fungicidal activity, e.g., the epothilones and myxalamides.[1] In contrast, the analysis of volatile compounds from myxobacteria has received relatively little attention. The widespread bacterial product geosmin (**1**) has been identified in *Nannocystis exedens* (Scheme 1),[2] and is present in many other species, detectable by its characteristic odour. Stigmolone (**2**) has been isolated from fruiting *Stigmatella aurantiara* and is believed to be the pheromone responsible for the attraction of the cells in the fruiting body formation process of this species.[3,4] In an attempt to further characterise the metabolic spectrum of the myxobacteria, we started to investigate the volatiles released from cultures of several myxobacteria. The results obtained from *C. crocatus* are presented here. Cultures of this organism produce a very characteristic odour unlike that of any other myxobacterium, which allows to recognise the bacterium simply by smelling.



1 **2**

Scheme 1.

2. Results

For the analyses of the volatiles, *C. crocatus* was grown on agar plates on different media. Cultures between 15 and 24 days old were then sampled by headspace methods and the obtained volatiles analysed by GC–MS. Two different methods were used for the headspace analysis. Agar plates were introduced in a custom made sample holder of a closed-loop-stripping apparatus (CLSA).[5] Air was circulated for 6–8 h over the culture, and the volatiles trapped by a charcoal filter were extracted with 20 μl of CH_2Cl_2 (see Fig. 1). This procedure allowed storage of the solution for later handling. In an alternative procedure, a small hole was drilled into the Petri dish just prior to analysis, a solid-phase micro-extraction (SPME) syringe was introduced through this hole, and the SPME fibre exposed to the volatiles. After 30 min, the SPME syringe was retracted and introduced into a GC–MS system, where the trapped volatiles were directly analysed by desorption from the fibre (see Fig. 1).[5]

Keywords: Myxobacteria; Ketones; Pyrazines; Degraded sesquiterpenes; Sesquiterpenes; CLSA analysis.
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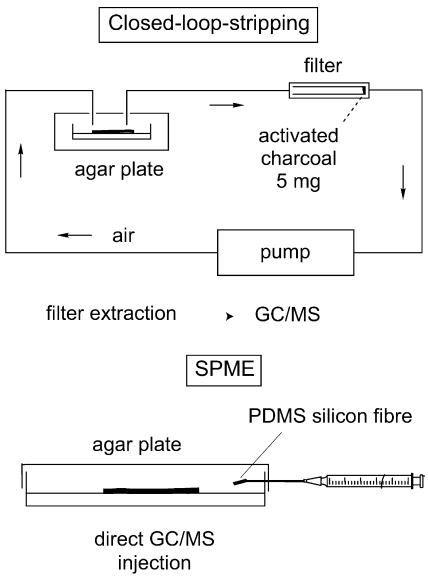


Figure 1. Methods used for volatile collection. The CLSA method furnished storable solutions, while the SPME procedure was used for direct analysis.

Compounds were identified by GC–MS analysis and comparison of retention times with synthetic reference samples. Unknown compounds were synthesised to confirm the results. Control experiments were performed with sterilised agar plates without bacteria to exclude compounds emanating from the agar or the Petri dishes.

Our results indicate that two major compound classes, pyrazines and sesquiterpenoids, are formed by the bacteria. Smaller amounts of aromatic compounds were also found. The identified compounds are listed in Table 1.

Both alkylpyrazines and alkylmethoxypyrazines are produced by *C. crocatus*. In several samples a major pyrazine with a molecular ion of *m*/*z*=164 was present. The spectrum was very similar to that of 2,5-bis-(1-methylethyl)pyrazine published recently by Zilkowski et al.[6] and Beck et al.[7] To confirm the identification, the different regioisomers of bis-(1-methylethyl)pyrazine were synthesised. The reaction of isopropylmagnesium bromide with pyrazine[8] afforded (1-methylethyl)pyrazine, also present in the extracts. This compound was reacted again with isopropylmagnesium bromide to furnish a 76:20:4 mixture of the 2,6, 2,5, and 2,3-bis-(1-methylethyl)pyrazines, which could be assigned by their NMR spectra. The major compound produced by *C. crocatus* proved to be identical to 2,5-bis-(1-methylethyl)pyrazine (**3**), while 2,6-bis-(1-methylethyl)pyrazine (**4**) was a minor component in the samples (Scheme 2). Comparison with a synthetic sample also confirmed the presence of 2,5-bis-(2-methylpropyl)pyrazine (**7**) and other alkylmethylpyrazines (see Table 1). Two further compounds exhibited a molecular ion of *m*/*z*=162, 2 amu less than the molecular ion of **3** and **4**. The spectra showed a base peak at M−15 (*m*/*z*=147), and an intense M−28 ion at (*m*/*z*=134), typical for isopropyl branched pyrazines. Furthermore, an intense ion at M−1 occurs, often observed in vinylpyrazines. We therefore conclude that these compounds are 2-(1-methylethenyl)-5-(1-methylethyl)pyrazine (**5**) and 2-(1-methylethenyl)-6-(1-methylethyl)pyrazine (**6**).

A second group of pyrazines consists of methoxypyrazines. The well known 2-methoxy-3-(1-methylethyl)pyrazine, 2-methoxy-3-(2-methylpropyl)pyrazine, and 2-methoxy-3-(1-methylpropyl)pyrazine were identified in varying amounts in the samples, but the most prominent member of this compound class was always 2-methyl-3-methoxypyrazine.

Three unknown compounds **9**, **10**, and **11** showed related mass spectra with low intensity ions below *m*/*z*=100, typical for pyrazines, and a base peak at *m*/*z*=153 or 139 (see Fig. 2). The shift of 16 amu compared to both the molecular ion and the *m*/*z*=137 ion of 2-methoxy-3-(1-methylethyl)-pyrazine in compound **9** indicated the presence of an additional oxygen atom outside the aromatic nucleus. Furthermore, the additional ion at *m*/*z*=59 amu can be attributed to a $[C_3H_6OH]^+$ fragment. The compound was easily silylated with *N*-trimethylsilyltrifluoroacetamide (MSTFA), consistent with the presence of a hydroxy function. We concluded, that the structure of **9** was 2-(1-hydroxy-1-methylethyl)-3-methoxypyrazine. This assignment was confirmed by synthesis starting from methoxypyrazine (**8**), which can be selectively deprotonated at C-3 with lithium tetramethylpiperidide.[9] Reaction with acetone furnished the desired compound, which proved to be identical with natural **9**.

In a similar manner, compound **10** was identified as 2-(1-hydroxy-1-methylpropyl)-3-methoxypyrazine and **11** as 2-(1-hydroxy-2-methylpropyl)-3-methoxypyrazine. Both compounds were also synthesised as shown in Scheme 3 and exhibited identical mass spectra and retention times as the natural compounds. To further strengthen our identification, a positional isomer of **10** was synthesised. Starting from 2,6-dichloropyrazine (**12**),[10] transformation into 2,6-diiodopyrazine (**13**) with NaI, followed by treatment with sodium methoxide according to Turck et al.[11] furnished 2-iodo-6-methoxypyrazine (**14**). After transmetallation with *tert*-butyllithium according to Street et al.,[12] reaction with butanone yielded 6-(1-hydroxy-1-methylpropyl)-3-methoxypyrazine (**15**). Its mass spectrum and retention time showed slight differences to the natural compound **10**, thus confirming our initial assignment of the natural compound (Scheme 3).

Sesquiterpenoids represent the second major group of compounds. The well known characteristic odorous compound geosmin (**1**) was present in the samples in varying amounts, sometimes not detectable by GC–MS, but by the human nose. A major constituent in most analyses was (1(10)*E*,5*E*)-germacradien-11-ol (**21**), which was identified by comparison with a synthetic sample.

Furthermore, a major component **16** with a molecular ion at *m*/*z*=178 occurred. Its mass spectrum (Fig. 2) was similar, but not identical, to several spectra of dimethylbicyclo[4.4.0]decenones and methyloctahydrobenzocycloheptanones present in the Wiley and NIST databases. Therefore this compound was believed to represent a degradation product of **21**, related to **1**, namely 6,10-dimethylbicyclo[4.4.0]dec-1-en-3-one (**16**). To prove our assignment, **16** was synthesised by Robinson annellation of 2,6-dimethylcyclohexanone and butenone according to

Table 1. Compounds identified in *Chondromyces crocatus* headspace

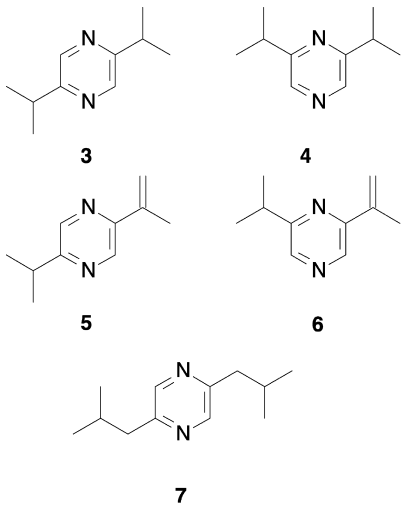
No	Compound	Sample					
		CLSA	SPME	Cm c5 y	Cm c5 p	Cm c2 y	Cm c2 p
1	2-(Methoxymethyl)furan	x					
2	Anisol	Trace				xx	x
3	2,5-Dimethylpyrazine			Trace	x		
4	2-Methoxy-3-methylpyrazine	xx		xx	xxx	xx	x
5	(1-Methylethyl)pyrazine			x		x	
6	Benzyl alcohol				x		xx
7	2-Methyl-6-(1-methylethyl)pyrazine			x	x		
8	3-Methoxy-2,6-dimethylpyrazine	x				x	x
9	3-Methoxy-2,5-dimethylpyrazine	x				x	x
10	2-Methyl-5-(1-methylethyl)pyrazine			x	x	x	
11	1-Phenylethanol	x		Trace	x		x
12	2-Methoxy-3-(1-methylethyl)pyrazine	x	x	x	x	xx	
13	Nonanal	Trace		Trace	x	Trace	x
14	2-Phenylethanol	Trace		Trace	x		xx
15	Dimethyl-(1-methylethyl)pyrazine			x	x	x	
16	2,6-Bis(1-methylethyl)pyrazine (**4**)	x	x	x	xx	x	
17	2-Methoxy-3-(1-methylpropyl)pyrazine	x	x	x	x	Trace	
18	1,4-Dimethoxybenzol			x	x	xx	x
19	2-Methoxy-3-(2-methylpropyl)pyrazine	x	x			Trace	
20	2,5-Bis(1-methylethyl)pyrazine (**3**)	xx	xx	xxx	xxx	xx	x
21	Methyl salicylate	x			x	Trace	x
22	2-(1-Hydroxy-1-methylethyl)-3-methoxypyrazine (**9**)	xx		x	xx	Trace	
23	2-(1-Methylethenyl)-6-(1-methylethyl)pyrazine (**6**)			x			
24	Benzothiazol	x			x		Trace
25	2-(1-Methylethenyl)-5-(1-methylethyl)pyrazine (**5**)	x		x	Trace		
26	*endo*-Bornyl acetate		xx				
27	2-(1-Hydroxy-1-methylpropyl)-3-methoxypyrazine (**10**)	x		x	xx	x	
28	2-Aminoacetophenone				x		x
29	Bicycloelemene		x				
30	2-(1-Hydroxy-2-methylpropyl)-3-methoxypyrazine (**11**)	Trace			Trace		
31	Methyl anthranilate				x		
32	2,5-Bis(2-methylpropyl)pyrazine	xx	xxx	Trace		x	x
33	α-Elemene	Trace	x				
34	Geosmin (**1**)	x	Trace				x
35	Methyl 2-methoxybenzoate			Trace	x	x	
36	β-Ylangene	Trace	xx				
37	β-Copaene	Trace	x				
38	*iso*-Germacrene D		x				
39	6,10-Dimethylbicyclo[4.4.0]decan-3-ol (**18**)	x					
40	Eremophilene	Trace	xx				
41	6,10-Dimethylbicyclo[4.4.0]decan-3-ol (**18**)	x					
42	(1*R**,6*R**,10*R**)-6,10-Dimethylbicyclo[4.4.0]decan-3-one (**17**)	Trace			x		
43	(1*S**,6*R**,10*R**)-6,10-Dimethylbicyclo[4.4.0]decan-3-one	x					x
44	(−)-Germacrene D	x	xxx				
45	α-Muurolene	x	xx				
46	Zonarene	x	xxx		x	x	
47	1,4-Cadinadiene		x				
48	(6*R**,10*S**)-6,10-Dimethylbicyclo[4.4.0]dec-1-en-3-one (6*S**,10*R**-**16**)	Trace					
49	(6*S*,10*S*)-6,10-Dimethylbicyclo[4.4.0]dec-1-en-3-one (6*S*,10*S*-**16**)	xxx	x	x	xxx		xxx
50	2-Benzyl-3-methoxypyrazine				x	Trace	
51	Cubenol	xx	xx	x	x	x	x
52	1(10)*E*,5*E*-Germacradien-11-ol (**21**)	xxx	xxx	xx	xxx	xx	xxx

xxx, xx, x, trace: relative proportion of component in an extract. SPME: sample analysed by SPME; CLSA: sample analysed by CLSA; Cm c2 and Cm c5: strain names; y: yeast medium; p: peptone medium. Compounds found in blank runs are not shown.

Ziegler and Hwang.[13] The resulting 1:1 mixture of the *cis* and *trans*-diastereomers 6*R**,10*S**-**16** and 6*R**,10*R**-**16** was transformed into the thermodynamically more stable *trans*-isomer by treatment with base. This isomer proved to be identical with natural major component **16**. The *cis*-isomer occurs also naturally, but only in trace amounts.

We then synthesised an enantiomerically enriched sample of 6*S*,10*S*-**16** (60% ee) according to the procedure of Revial[14] which was used to determine the absolute configuration of the naturally occurring compound using GC–MS with chiral phases. The racemate is well separated on a chiral cyclodextrin phase. As can be seen in Figure 5, only the 6*S*,10*S*-enantiomer occurs naturally. It exhibits the same absolute configuration at C-6 and C-10 as natural geosmin produced by several microorganisms.[15]

Hydrogenation of (6*R**,10*R**)-**16** with Pd/C furnished preferentially the *cis*-fused (1*R**,6*R**,10*R**)-6,10-dimethylbicyclo[4.4.0]decan-3-one **17**, but also minor amounts of the *trans*-fused (1*R**,6*S**,10*S**)-diastereomer,[16] which



Scheme 2.

were both present as minor components in the samples. The relative configuration of the major synthetic product (1*R**,6*R**,10*R**)-**17** was established by comparison of its ^{13}C NMR data with the published ones of all four diastereomers of **17**.[16] Its preferred conformation, depicted in Scheme 4, was deduced by analysis of the ^{1}H NMR spectrum. One of the two protons on C-2 show two large coupling constants which can only be explained by its axial position and an antiperiplanar arrangement to the vicinal proton at C-10. This conformation is opposite to the one recently observed in the related 10-nor derivative of **17**.[17] The reduction of the mixture of **17** with $LiAlH_4$ gave four 6,10-dimethylbicyclo[4.4.0]decan-3-ols (**18**). At least two of these alcohols were also present in trace amounts in some samples, but the relative configuration of them was not determined because of extensive peak overlapping in GC and difficulties in separating the synthetic mixture.

Considerable differences especially in the relative amount of the compounds produced were found between different experiments. In one experiment the same agar plate was investigated by both the SPME and the CLSA technique (see Table 1 and Fig. 3). The SPME technique seems to be very good for sampling of less volatile material, which can be seen by the total absence of compounds **1**–**12** in Table 1. The technique is very sensitive for hydrocarbon sesquiterpenes, which are the peaks of highest abundance in the gas chromatogram. Oxygenated compounds are less well captured, as can be seen by the total absence of **10** and the reduced amount of **16**. In contrast, the CLSA method disfavoured hydrocarbon sesquiterpenes and seems to give a more total view of the compounds produced by *C. crocatus*.

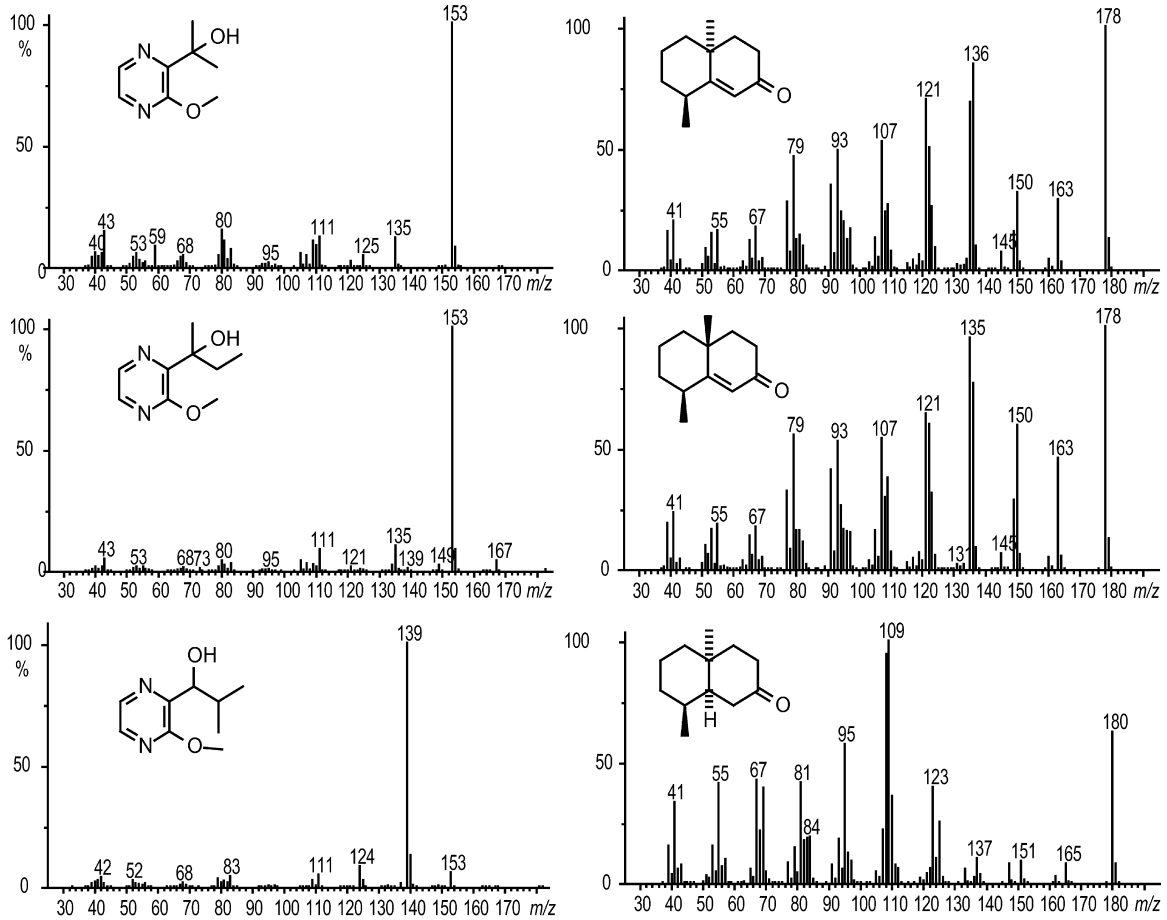
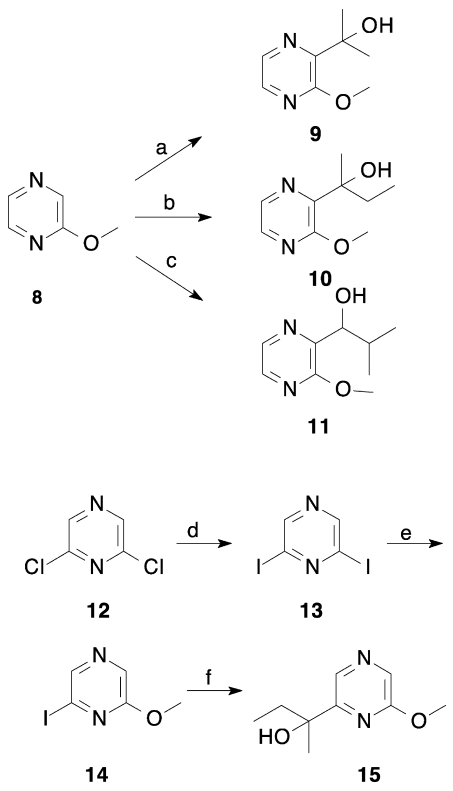


Figure 2. Mass spectra of 2-(1-hydroxy-1-methylethyl)-3-methoxypyrazine (**9**), 2-(1-hydroxy-1-methylpropyl)-3-methoxypyrazine (**10**), 2-(1-hydroxy-2-methylpropyl)-3-methoxypyrazine (**11**), (6*R**,10*R**)-6,10-dimethylbicyclo[4.4.0]dec-1-en-3-one (6*R*,10*R*-**16**), (6*R**,10*S**)-6,10-dimethylbicyclo[4.4.0]dec-1-en-3-one (6*R*,10*S*-**16**), and (1*R**,6*R**,10*R**)-6,10-dimethylbicyclo[4.4.0]decan-3-one (**17**).



Scheme 3. Synthesis of pyrazines. Reagents and conditions: (a) LiTMP, acetone; (b) LiTMP, butanone; (c) LiTMP, isobutanal; (d) sulfolane, *p*-TsOH, NaI, 15-crown-5, 4 h, 150 °C; (e) NaOMe, MeOH; (f) −80 °C, *t*-BuLi, butanone, then to rt.

The two different *C. crocatus* strains Cm c2 and Cm c5 also showed some differences in their profile of the volatiles (see Table 1 and Fig. 4).

The amount of the compounds identified varied considerably between the two different culture media used. Sesquiterpenoids were present predominantly on the peptone medium, while they were markedly reduced on the yeast medium. In contrast, the pyrazines were always present as major components on the yeast medium, while different cultures on peptone furnished different results ranging from low to major amounts. Furthermore, considerable quantitative variability occurs between different experiments performed with the same strain.

3. Discussion

Major components of the headspace of most *C. crocatus* samples were the pyrazines 2-methoxy-3-methylpyrazine and **9** as well as the sesquiterpenoids **16**, **21**, and occasionally **1**. Both mono- and dialkylpyrazines as well as alkylmethoxypyrazines occur. Pyrazines are important aroma compounds and have been found abundantly in several foodstuff.[18] Furthermore, several pyrazines are known to be produced by microorganisms,[7,19] but so far they have not been reported from myxobacteria. The 2,5-bis-(1-methylethyl)pyrazine (**3**) has been previously identified as an attractant of *Carpophilus* beetles to oranges upon which the beetles fed. It is believed to be of microbial origin.[6] To the best of our knowledge, the

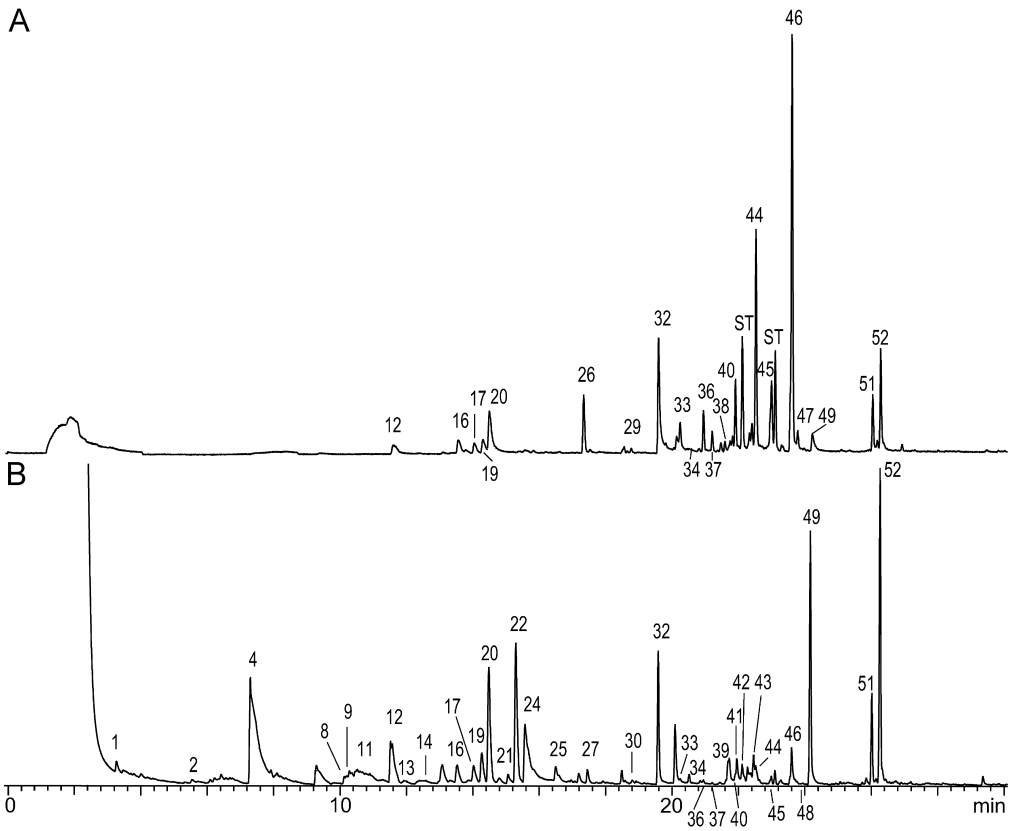


Figure 3. Gas chromatograms of volatiles collected from the same culture by SPME (A) or CLSA (B) methods. 25 m BPX-5, 60 °C, 5 min isothermal, then with 5 °C/min to 300 °C.

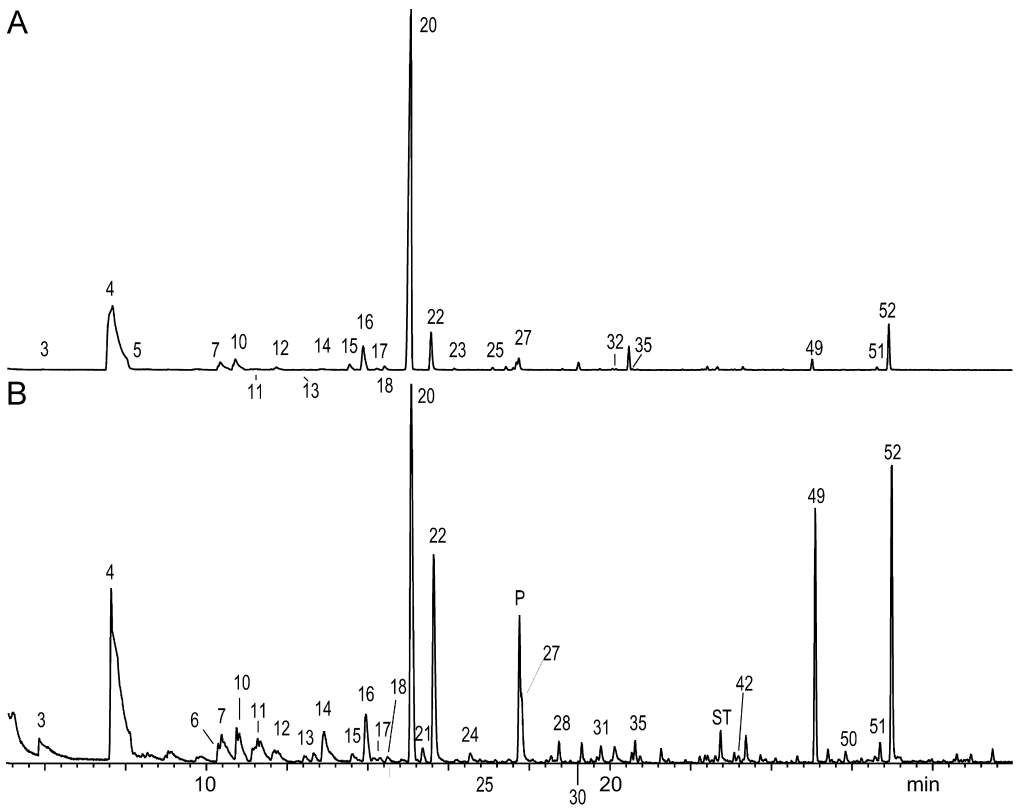


Figure 4. Gas chromatograms of volatiles collected from the same strain (Cm c5) of *Chondromyces crocatus* cultured on peptone (A) or yeast (B) medium. 25 m BPX-5, 60 °C, 5 min isothermal, then with 5 °C/min to 300 °C.

hydroxyalkyl-methoxypyrazines **9**, **10**, and **11** have not been reported before from nature.

While geosmin (**1**) is widespread in microorganisms, the germacradienol **21** has been isolated before only from *Streptomyces coelicor*,[20] where it occurs together with **1**, and from the liverwort *Dumortiera hirsuta*.[21] The *trans*-bicyclodecanone **16** has been previously identified in the plant *Vetiver zizanioides*,[22,23] and is structurally closely related to **1**. It has been patented. The derivatives **17** and **18** have so far not been reported as natural products.

Recently the gene responsible for the production of **1** and **21** in *Streptomyces coelicor* has been identified.[24,25] The protein it codes for has two domains. While one domain is needed for production of **1**, the other one is required for the production of **21**. It has been proposed by Crane and Watt that **21** is an intermediate in the biosynthesis of **1**,[24] while Boland and co-workers independently postulated the isomer 1(10),4-germacradien-11-ol (hedycaryol) (**20**) as

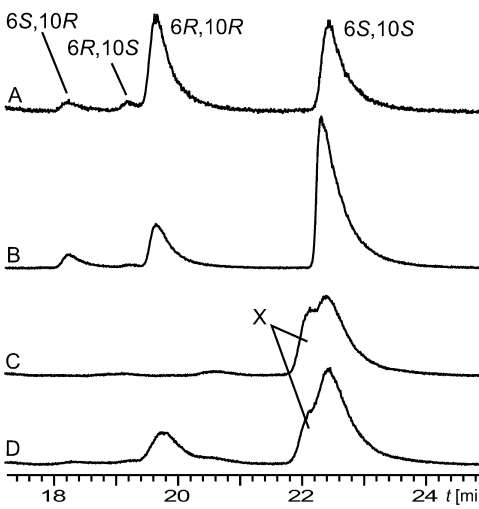
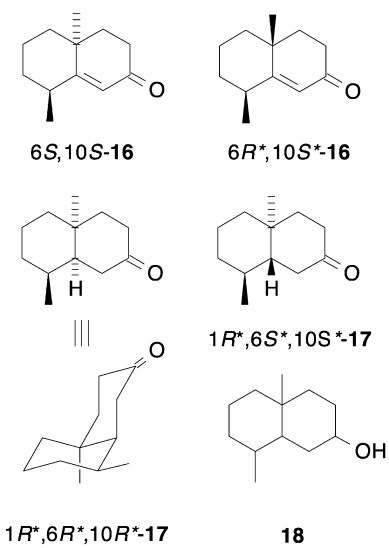
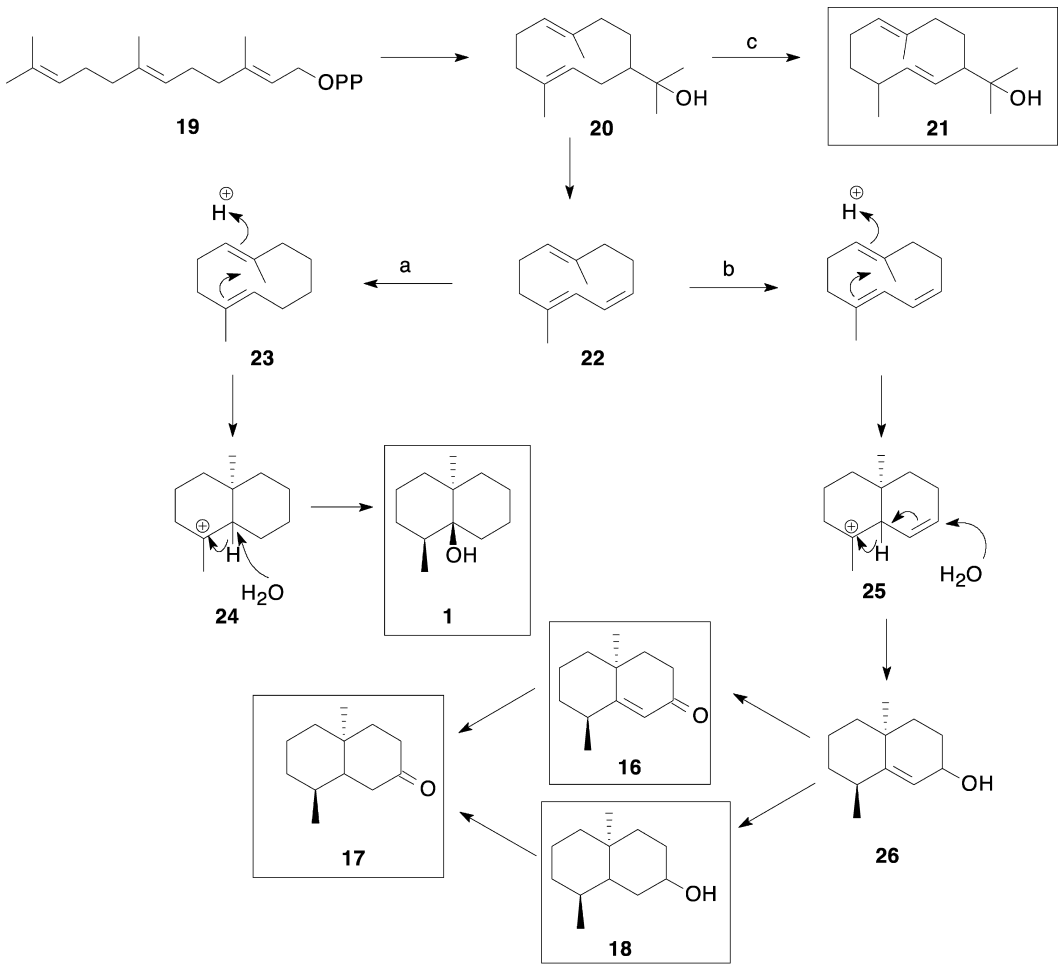


Figure 5. Gas chromatographic separation of **16**. A: racemate; B: synthetic 6*S*,10*S*-**16**; C: natural sample (Cm c2); D: coinjection racemate and natural sample; X: **21**. 15 m Hydrodex-6-TBDMS programmed as follows: 3 min isothermal at 110 °C, then heated with 1 °C/min to 140 °C.



Scheme 4.



Scheme 5. Proposed biosynthetic pathway to the sesquiterpenoids **1**, **16**, **17**, **18**, and **21**. Compounds identified in *C. crocatus* are shown in boxes.

precursor.[15] The latter hypothesis was supported by feeding studies with labelled precursors with a *Streptomyces* strain and MS analysis of the products. It is presented in Scheme 5 as path a. The labelling patterns observed by Boland et al. are not consistent with the proposed pathway by Crane and Watt.[24] It seems likely that **21** is formed by the independent pathway c in Scheme 5 via isomerisation.

The formation of the degraded sesquiterpenoids **16**, **17** and **18** can be explained by a modification of the geosmin pathway. According to Boland, the geosmin biosynthesis proceeds via the proposed intermediate **22**. After hydrogenation of the C-9–C-10 double bond to the intermediate **23**,† cyclisation furnishes the cation **24**, which finally forms **1** after addition of water. If the hydrogenation step is omitted and **22** directly cyclised via path b, the homoallylic cation **25** is formed. This cation can be attacked by water to form the alcohol **26** as depicted in Scheme 5. In both the biosynthesis of **1** and **26** a 1,2-H shift occurs, which is completed by attack of water on C-10 in the case of **1**, and on C-2 in the case of **26**. The latter compound can then be transformed into the derivatives **16**, **17**, and **18** by oxidation and reduction.

† For convenience, the numbering scheme used is based on the numbering of the germacrane skeleton.

4. Conclusion

In essence we have shown that efficient analysis of the volatiles produced by myxobacteria can be performed by analysis of the headspace of simple agar plate cultures. Several new pyrazines and sesquiterpenoids could thus be found and the complex odour bouquet of *C. crocatus* with more than 50 components delineated.

5. Experimental

5.1. General remarks

^{1}H and ^{13}C NMR spectra were obtained with Bruker AC-200 and AMX-400 instruments. For NMR experiments, $CDCl_3$ was used if not mentioned otherwise; the internal standard was tetramethylsilane. GC–MS investigations were carried out with a Hewlett–Packard model 5973 mass selective detector connected to a Hewlett–Packard model 6890 gas chromatograph. Analytical GLC analyses were performed with a CE instruments GC 8000 gas chromatograph equipped with a flame ionisation detector and split/splitless injection. An apolar BPX-5 (SGE) capillary column was used with H_2 as the carrier gas. All reactions were carried out under an inert atmosphere of N_2 in oven-dried glassware. Dry solvents: dry toluene was

distilled from Na, CH_2Cl_2 from CaH_2, THF from K and Na. All other chemicals were commercially available (Fluka, Aldrich) and used without further treatment, if not stated otherwise. All reactions were monitored by thin layer chromatography (TLC) carried out on Macherey-Nagel Polygram SIL G/UV_{254} silica plates visualised with heat gun treatment with 10% molybdato phosphoric acid in ethanol. Column chromatography was performed with Merck silica gel 60 (70–200 mesh). All new compounds were determined to be >95% pure by HPLC, GLC, or ^{1}H NMR spectroscopy. Identification of known compounds was performed by comparison of mass spectra with those in the databases Wiley7, NIST 2.0, and MassFinder 2.3 (Essential Oils), taking their retention indices into account[26] when no reference sample was available. Identification of known sesquiterpenes was performed by comparing mass spectra and retention times with critical evaluated data.[27] Chiral GC was performed using a 15 m Hydrodex-6-TBDMS column (Macherey and Nagel) with a constant flow of 1 ml/min He installed into the GC–MS system to allow peak determination. The oven was programmed as follows: 3 min isothermal at 110 °C, then heated with 1 °C/min to 140 °C.

5.2. Microorganisms and culture conditions

Strains Cm c2 and Cm c5 of *C. crocatus* were isolated at the GBF in 1982 and 1985 from soil samples with decaying plant material collected on the island of Madeira, Portugal and near Iguaçu, Brazil, respectively. While Cm c2 requires a bacterial symbiont for growth, Cm c5 is a pure strain. The organisms were cultivated either on VY/2-(yeast-) agar (bakers'yeast, 0.5%; $CaCl_2{\cdot}2H_2O$, 0.1%; cyanocobalamin, 0.5 mg/l; agar, 1.5%; pH 7.2, autoclaved) or on CY-(peptone-) agar (peptone from casein, tryptically digested, 0.3%; yeast extract, 0.1%; $CaCl_2{\cdot}2H_2O$, 0.1%; agar, 1.5%; pH 7.2, autoclaved). Cultures in 8 cm Petri dishes were incubated at 30 °C the dark.

5.3. Headspace analysis

The sample vessel of a commercial CLSA system (Brechbühler) was replaced by a custom made glass chamber. The chamber consisted of two parts connected by a planschliff; a standard agar plate fits in the lower part, while the upper part developed conical into a NS 29 female joint, in which the CLSA adapter fits. Before every headspace sampling from bacteria, the apparatus was thoroughly cleaned with CH_2Cl_2 and dried in an oven. Blank runs were performed with and without sterile agar plates to identify the compounds emanating from the CLSA system, the agar, and the polystyrene Petri dishes. Samples from bacteria were obtained by running the apparatus for 8 h. Then the 5 mg charcoal filter was extracted two times with 15 μl CH_2Cl_2, and the extract stored in ampoules at low temperature until analysis.

5.4. General procedure for the preparation of hydroxyalkylpyrazines (9–11)

A mixture of 2,2,6,6-tetramethylpiperidine (0.7 ml, 4.1 mmol), 1.6 M *n*-butyllithium in hexane (2.6 ml, 4.2 mmol) and diethyl ether (6 ml) was stirred for 1 h at 0 °C then cooled to −70 °C. A solution of methoxypyrazine (0.27 ml, 2.7 mmol) in diethyl ether (2 ml) was added and the mixture stirred for 30 min. Then the corresponding ketone or aldehyde was added dropwise and the mixture stirred overnight, during which time it was allowed to warm to room temperature. Then 10 ml water was added, the mixture extracted twice with CH_2Cl_2, the organic phase dried with $MgSO_4$ and the solvent removed. The residue was purified by column chromatography (silica, petrol ether/diethyl ether 5:1 containing 0.5% triethylamine).

5.4.1. 2-(1-Hydroxy-1-methylethyl)-3-methoxypyrazine (9). Using the general procedure with abs. acetone (0.3 ml, 4.1 mmol) gave a colourless oil in 23% (105 mg) yield. Odour: strong herbaceous and root like smell.

^{1}H NMR (400 MHz, C_6D_6) δ 1.80 (s, 6H, $2CH_3$), 3.64 (s, 3H, OCH_3), 5.52 (s, 1H, OH), 7.65 (d, J=2.7 Hz, 1H, ArH), 7.70 (d, 1H, ArH); ^{13}C NMR (100 MHz, C_6D_6) δ 28.1 (2C, C-2′), 53.1 (H_3CO), 71.3 (C-1′), 134.1 (C-6), 139.6 (C-5), 150.8 (C-2), 157.5 (C-3); HR-EI-MS calculated for $C_8H_{12}N_2O_2$ 168.0899. Found 168.0903.

5.4.2. 2-(1-Hydroxy-1-methylpropyl)-3-methoxypyrazine (10). Using the general procedure with butanone gave a colourless oil. Yield 43%. Odour: herbaceous, root like.

^{1}H NMR (400 MHz, $CDCl_3$) δ 0.67 (t, 3H, H-3′), 1.55 (s, 3H, C-1′–CH_3), 1.87 (dq, $J_{2b',3'}$=7.4 Hz, 1H, H-2b′), 2.10 (dq, $J_{2a',3'}$=7.4 Hz, $J_{2a',2b'}$=14.8 Hz, 1H, H-2a′), 4.01 (s, 3H, OCH_3), 5.27 (s, 1H, HO), 8.05 (d, J=2.8 Hz, 1H, ArH), 8.06 (d, J=2.8 Hz, 1H, ArH); ^{13}C NMR (100 MHz, $CDCl_3$) δ 8.1 (C-3′), 26.1 (C-1′–CH_3), 32.3 (C-2′), 53.5 (H_3CO), 73.6 (C-1′), 133.8 (C-6), 139.5 (C-5), 149.3 (C-2), 157.4 (C-3); HR-EI-MS calculated for $C_9H_{14}N_2O_2$ 182.1055. Found 182.1064.

5.4.3. 2-(1-Hydroxy-2-methylpropyl)-3-methoxypyrazine (11). Similar to the synthesis of **9**, using methylpropanal instead of acetone. Yield 32%. Odour: vetiver root, potato peels.

^{1}H NMR (400 MHz, $CDCl_3$) δ 0.72 (d, $J_{2',3b'}$=6.8 Hz, 3H, H-3b′), 1.08 (d, $J_{2',3a'}$=6.9 Hz, 3H, H-3a′), 2.16 (m, 1H, H-2′), 3.88 (d, 1H, OH), 3.99 (s, 3H, OCH_3), 4.76 (dd, $J_{1',OH}$=7.1 Hz, $J_{1',2'}$=3.7 Hz, 1H, H-1′), 8.03 (d, J=2.8 Hz, 1H, ArH), 8.08 (d, 1H, ArH); ^{13}C NMR (100 MHz, $CDCl_3$) δ 15.5 (C-3b′), 19.8 (C-3a′), 32.5 (C-2′), 53.6 (H_3CO), 73.0 (C-1′), 134.5 (C-6), 139.3 (C-5), 147.3 (C-2), 157.4 (C-3); HR-EI-MS calculated for $C_9H_{14}N_2O_2$ 182.1055. Found 182.1061.

5.4.4. 2-(1-Hydroxy-1-methylpropyl)-6-methoxypyrazine. Pyrazine was transformed into **12** according to a published procedure.[10] Treatment with NaI gave **13**,[11] which was monomethoxylated by treatment with NaOMe to furnish **14**.[11]

A solution of 81 mg (0.34 mmol) **14** in 5 ml diethyl ether was added at −80 °C 0.42 ml of a 1.7 M *tert*-butyllithium solution in pentane.[12] After stirring for 30 min, 0.1 ml (1.1 mmol) butanone was added and the mixture stirred for

an additional 2 h at room temperature. Then 10 ml water was added and the mixture extracted three times with diethyl ether. The combined organic phases were dried with Na_2SO_4, the ether removed by evaporation, and the residue purified by chromatography (silica, diethyl ether/petroleum ether 3:1). A colourless oil was obtained in 41% yield (26 mg, 0.14 mmol). Odour: potato like, earthy.

^{1}H NMR (400 MHz, $CDCl_3$) δ 0.82 (t, 3H, H-3′), 1.55 (s, 3H, H_3C–C-1′), 1.85 (q, $J_{2',3'}$=7.4 Hz, 2H, H-2′), 3.73 (s, 1H, OH), 3.99 (s, 3H, OCH_3), 8.13 (s, 1H, ArH), 8.25 (s, 1H, ArH); ^{13}C NMR (100 MHz, $CDCl_3$) δ 8.0 (C-3′), 28.0 (H_3C–C-1′), 35.8 (C-2′), 53.5 (H_3CO), 73.6 (C-1′), 132.7 (d, C-Ar), 133.3 (d, C-Ar), 157.0 (q, C-Ar), 158.9 (q, C-Ar); HR-EI-MS calculated for $C_9H_{14}N_2O_2$ 182.1055. Found 182.1070.

5.5. General procedure for alkylation of pyrazines

A Grignard solution was prepared from 0.121 g (5.0 mmol) magnesium and 0.5 ml (5.3 mmol) 2-bromopropane in 5 ml THF at 0 °C. After 1 h 0.2 g (2.5 mmol) of the appropriate pyrazine in 2 ml THF were added dropwise and the solution stirred for further 15 min. Longer reaction times resulted in lower yields. The reaction was quenched with ammonium chloride solution and the mixture extracted with THF. The organic phases were dried with $MgSO_4$, the solvent removed and the residue purified by column chromatography (petroleum ether/diethyl ether 2:1). The spectroscopical data are identical to those reported in the literature.[10]

5.5.1. (1-Methylethyl)pyrazine. Prepared according to the general procedure for the alkylation of pyrazines starting from pyrazine. Yield 20% (61 mg). The spectroscopical data are identical to those reported in the literature.[10]

5.5.2. Bis-(1-methylethyl)pyrazine. Prepared according to the general procedure for the alkylation of pyrazines starting from (1-methylethyl)pyrazine in 14% yield. The reaction time after addition of the pyrazine was reduced to 5 min. A mixture of the 2,3-isomer (4%), the 2,5-isomer (20%), and the 2,6-isomer (76%) was obtained.

2,3-Bis-(1-methylethyl)pyrazine: ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.34 (s, 2H, ArH); ^{13}C NMR (100 MHz, $CDCl_3$) δ 22.12 (C-2′); MS (70 eV) 164 (M^+, 81), 149 (100), 136 (24), 135 (53), 134 (16), 133 (13), 121 (19), 80 (14), 52 (13), 41 (17).

2,5-Bis-(1-methylethyl)pyrazine (**3**): ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.32 (d, $J_{1',2'}$=6.9 Hz, 12H, H-2′), 8.39 (s, 2H, ArH); ^{13}C NMR (100 MHz, $CDCl_3$) δ 22.2 (C-2′), 33.5 (C-1′), 141.8 (C-3, C-6), 159.3 (C-2, C-5); MS (70 eV) 164 (M^+, 25), 163 (12), 150 (10), 149 (100), 136 (42), 134 (16), 133 (10), 121 (17), 53 (9), 41 (10).

2,6-Bis-(1-methylethyl)pyrazine (**4**): ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.32 (d, 12H, H-2′), 3.06 (sept, $J_{1',2'}$=6.9 Hz, 2H, H-1′), 8.27 (s, 2H, ArH); ^{13}C NMR (100 MHz, $CDCl_3$) δ 22.1 (q, C-2′), 34.0 (C-1′), 139.8 (C-3, C-5), 161.0 (C-2, C-6); MS (70 eV) 164 (26, M^+), 163 (21), 150 (10), 149 (100), 136 (55), 134 (18), 133 (10), 53 (11), 41 (10), 39 (8).

5.6. Synthesis of sesquiterpenoids

5.6.1. 6,10-Dimethylbicyclo[4.4.0]dec-1-en-3-one (16). 2,6-Dimethylcyclohexanone and methylvinylketone were reacted according of the procedure of Ziegler and Hwang[13] to yield a 1:1 mixture of *cis*- and *trans*-**16**. Treatment of this mixture with 5% KOH in ethanol furnished a 60:1 *trans*/*cis* or 6*R**,10*R**/6*R**,10*S**-mixture of **16**.

^{1}H NMR (400 MHz, $CDCl_3$) δ 1.07 (d, $J_{CH_{3,10}}$=6.5 Hz, 3H, H_3C–C-10,), 1.24 (s, 3H, H_3C–C-6), 1.95–1.15 (m, 8H), 2.43–2.30 (m, 2H, H-4b, H-10), 2.49 (ddd, $J_{4a,4b}$=16.9 Hz, $J_{4a,5a}$=13.2 Hz, $J_{4a,5b}$=6.0 Hz, 1H, H-4a), 5.79 (d, $J_{2,10}$=1.5 Hz, 1H, H-2); ^{13}C NMR (100 MHz, $CDCl_3$) δ 18.1 (CH_3), 21.7 (CH_2), 23.0 (CH_3), 33.9 (CH_2), 34.2 (C-10), 36.3 (CH_2), 38.4 (CH_2), 41.9 (CH_2), 121.6 (C-2), 173.9 (C-1), 200.2 (C-3).

5.6.2. 6,10-Dimethylbicyclo[4.4.0]decan-3-one (17). To a solution of 6*R**,10*R**-**16** (20 mg, 0.11 mmol) in 1 ml CH_2Cl_2 was added 3 mg 5% Pd/C and the mixture stirred under an H_2 atmosphere for 30 min. The mixture was filtered over a short plug of silica and the solvent evaporated. The product (20 mg, 97% yield) consisted of a 6:1 mixture of (1*R**,6*R**,10*R**)- and (1*R**,6*S**,10*S**)-**17**.

^{1}H NMR (400 MHz, $CDCl_3$) δ 0.80 (d, $J_{CH_{3,10a}}$=6.8 Hz, 3H, H_3C–C-10), 1.04 (s, 3H, H_3C–C-6), 1.16–1.07 (m, 2H, H-9, H-7e), 1.44 (m, 1H, H-9), 1.69–1.58 (m, 4H, H-1a, H-5a, H-8a, H-8e), 1.73 (ddd, $J_{5e,5a}$=13.9 Hz, $J_{4a,5e}$=6.3 Hz, 1H, H-5e), 1.87 (m, 1H, H-7a), 1.98 (m, 1H, H-10a), 2.16 (ddd, $J_{2e,1a}$=4.8 Hz, $J_{4e,2e}$=2.3 Hz, 1H, H-2e), 2.20 (ddt, $J_{4e,5a}$=4.8 Hz, $J_{4e,5e}$=2.8 Hz, 1H, H-4e), 2.29 (t, $J_{2a,2e}$=$J_{2a,1a}$=13.9 Hz, 1H, H-2a), 2.49 (dt, $J_{4a,4e}$=$J_{4a,5a}$=14.4 Hz, 1H, H-4a); ^{13}C NMR (100 MHz, $CDCl_3$) δ 19.4 (CH_3–C-10), 21.7 (C-8), 26.4 (CH_3–C-6), 27.8 (C-9), 28.9 (C-7), 30.1 (C-10), 33.3 (C-6), 37.1 (C-4), 37.7 (C-5), 41.3 (C-2), 47.8 (C-1), 214.0 (C-3).

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