DOI: 10.1002/cbic.200500174

Biosynthesis and Identification of Volatiles Released by the Myxobacterium *Stigmatella aurantiaca*

Jeroen S. Dickschat,[a] Helge B. Bode,[b] Silke C. Wenzel,[b] Rolf Müller,[b] and Stefan Schulz*[a]

The volatiles released by agar plate cultures of two strains of the myxobacterium Stigmatella aurantiaca *(strains Sg a15 and DW4/3-1) were collected in a closed-loop stripping apparatus (CLSA) and analyzed by GC-MS. Large numbers of substances from different compound classes (ketones, esters, lactones, terpenes, and sulfur and nitrogen compounds) were identified; several of them are reported from natural sources for the first time. The volatiles 2-methyltridecan-4-one (**17**), its isomer 3-methyltridecan-4-one (**20**), and the higher homologue 2-methyltetradecan-4-one (**18**) were identified in the extracts of both strains and were synthesized. In addition, strain Sg a15 produced 2,12-dimethyltridecan-4-one (**19**), 2-methyltridec-2-en-4-one (**23**), and a series of phenyl ketones, among them 1-phenyldecan-1-one (**14**) and 9-methyl-1-phenyldecan-1-one (**16**), whereas strain DW4/3-1 emitted traces of 10-methylundecan-2-one (**21**). The biosynthesis of **14** and **16** was examined in feeding experiments with deuterated precursors carried out on agar plate cultures. The leucine-derived starter unit isovalerate was shown to be incorporated into **16**, as was phenylalanine-derived benzoic acid into both **14** and **16**. The results point to formation both of the phenyl ketones and of the structurally related aliphatic ketones through an unusual head-to-head coupling between a starter unit such as benzoyl-CoA and a fatty acyl-CoA, followed by decarboxylation.*

Introduction

In recent studies we have reported on the profiles of volatiles released by different bacteria such as the myxobacteria *Chondromyces crocatus* and *Myxococcus xanthus*,[1,2] bacteria of the *Roseobacter* clade,[3] *Flavobacteriaceae*,[4] and *Streptomyces* sp.[5] by use of a modified closed-loop stripping apparatus (CLSA). In this study, the volatiles emitted by two strains of the myxobacterium *Stigmatella aurantiaca* (strains Sg a15 and DW4/3-1) were investigated. Myxobacteria are of special interest, because the cells aggregate under starvation conditions to form fruiting bodies. A pheromone mediating this process in *S. aurantiaca* DW4/3-1 was identified as the volatile stigmolone.[6,7] Our investigations have focused on the vegetative growth phase in order to provide better understanding of volatile formation in myxobacteria.

Results

Major volatiles

The cultures were grown on agar plates, and headspace samples were collected by the CLSA method on charcoal filters, which were extracted with dichloromethane as reported earlier.[2] The extracts were immediately analyzed by GC-MS. The results of the analyses are summarized in Table 1 and total ion chromatograms are shown in Figure 1. Three to four analyses of each strain were performed in order to check the reproducibility of the emitted profiles. Some differences were observed in the number of compounds and in the total amounts present in the extracts. One experiment with strain Sg a15 (experiment 1) and one experiment with strain DW4/3-1 (experiment 5) yielded significantly higher numbers of volatiles. Most of the compounds emitted by the bacteria were readily identified by their GC retention indices and mass spectra, through reference to spectral databases. Both strains of *S. aurantiaca* turned out to be producers of methyl benzoate (**1**) as a major compound, whereas 2-phenylethanol (**2**) and acetophenone (**3**) were present in only some samples of both strains (Scheme 1). Furthermore, the extracts were also characterized by terpenoids, two of which—(−)-geosmin (**4**) and (1(10)*E*,5*E*)-germacradien-11-ol (**5**)—were present in all samples as major components, while (−)-germacrene D (**6**) was produced in smaller amounts. The absolute configurations of **4** and **6** were determined by GC on a chiral stationary phase by use of enantiomerically enriched samples. The stereochemistry of **5** re-

[a] *Dr. J. S. Dickschat, Prof. Dr. S. Schulz*
Technische Universität Braunschweig, Institut für Organische Chemie
Hagenring 30, 38106 Braunschweig (Germany)
Fax: (+49) 0531-391-5272
E-mail: stefan.schulz@tu-bs.de

[b] *Dr. H. B. Bode, Dipl.-Chem. S. C. Wenzel, Prof. Dr. R. Müller*
Institut für Pharmazeutische Biotechnologie, Universität des Saarlandes
Im Stadtwald, Geb. 8.1, 66123 Saarbrücken (Germany)

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Table 1. Compounds identified in the headspace extracts of two different strains of *Stigmatella aurantiaca* (strains Sg a15 and DW4/3-1).[a]

GC	Compound	*I*	Identification	Sg a15			DW4/3-1			
				1.	2.	3.	4.	5.	6.	7.
	butyl acetate		syn					x		
	5-methylhexan-3-one		ms, inc	x				xx		
	methyl 3-methylcrotonate		ms	x						
a	methyl 2-methylcrotonate		ms	x				xxx		x
	3-methylbutyric acid		ms					x		
	2-methylbutyric acid		ms					x		
	butyl propionate	920	syn					x		x
b	2-acetylfuran	930	ms	xx			xx	xx		
	butan-4-olide	938	syn				x			
	unknown (B: 56, M: 140)	960		xx						
c	pentan-4-olide	976	syn	x			xx	xx		
	benzaldehyde	982	syn	xx			xx			
	dimethyl trisulfide	983	ms	x			x			
	methyl 5-methylhexanoate	993	syn						xx	
	6-methylhept-5-en-2-one	999	syn	x			x	x		
	N-isopentylidene isopentylamine (**10**)	1147	ms	x				x		
	benzyl alcohol	1051	syn	x	xx	x	xx	x		
d	pent-2-en-4-olide	1063	ms	xx			x	xx		
e	acetophenone (**3**)	1080	syn	xx	xx	xx	x	x		
	S-methyl methanethiosulfonate	1081	ms				x	x		
f	methyl benzoate (**1**)	1104	syn	xx	xxx	xxx	xx	xx	xxx	xxx
	N-isopentylformamide (**11**)	1112	ms	x				x		
g	2-phenylethanol (**2**)	1125	syn	x		x		x		
	unknown (B: 71, M: 169)	1147		xx						
	N-isopentylacetamide (**12**)	1150	ms	x				x		
	menthol	1188	ms					x		
	p-menth-1-en-3-ol	1201	ms						xx	
	α-terpineol	1206	ms		xx		x	x		
	methyl salicylate	1208	syn	x						
	unknown (B: 149, M: 164)	1239		x			x	xx		
i	6,10-dimethylbicyclo[4.4.0]dec-1-ene	1241	syn	x			x	x		
	unknown (B: 149, M: 164)	1259		x						
	unknown (B: 108, M: 164)	1263		x				x		
j	unknown (B: 43, M: 134)	1277						x		
k	undecan-2-one	1302	syn	x			x	x		
	undecan-2-ol	1310	syn					x		
l	10-methylundecan-2-one (**21**)	1363	syn					x		
	10-methylundecan-2-ol (**22**)	1373	syn					x		
	unknown (B: 81, M: 182)	1375						x		
m	methyl 9-methyldecanoate (**8**)	1393	syn, inc				x	xx	xx	xx
	dodecan-3-one	1396	syn				x	x		
	4-methylquinoline	1396	syn							x
n	*N*-(2-phenylethylidene)isopentylamine (**9**)	1396	ms	xx	xx	xx				
	dodecan-3-ol	1407	syn	x				x		
	β-ylangene (lit: $I = 1420$)	1425	ms	x				x		
o	(−)-geosmin (**4**)	1430	syn, chgc	xxx	xxx	xxx	xx	xx	xx	xx
	β-copaene (lit: $I = 1430$)	1437	ms	x				x		
	unknown (B: 82, M: 204)	1445		x						
	isogermacrene D (lit: $I = 1445$)	1452	ms	x						
	unknown (B: 191, M: 204)	1453		x						
p	geranylacetone (**7**)	1457	syn	x			x	xxx		x
	6,10-dimethylundeca-5,9-dien-2-ol	1459	ms					x		
	unknown (B: 149, M: 182)	1459		x						
q	(−)-germacrene D (**6**)	1490	syn, chcg	x	x	x	x	x		
	unknown (B: 107, M: 204)	1507		x			x	x		
	unknown (B: 151, M: 222)	1509								
	guaioxide	1521	ms	x			x	x		
	unknown (B: 149, M: 222)	1523					x			
r	2-methyltridecan-4-one (**17**)	1527	syn, inc	xx				x		
	3-methyltridecan-4-one (**20**)	1532	syn, inc	x				x		
	unknown (B: 149, M: 222)	1536		x				x		
	unknown (B: 237, M: 250)	1543		x						
	dihydroactinidiolide	1554	ms				x			
	nerolidol (lit: $I = 1553$)	1567	ms	x				x		
	2-methoxy-1,1′-biphenyl	1574	ms				x	x		

Table 1. (Continued)

GC	Compound	*I*	Identification	Sg a15			DW4/3-1			
	unknown (B: 183, M: 183)	1575		x				x		
s	2,12-dimethyltridecan-4-one (**19**)	1589	syn, inc	x						
	2-methyltridec-2-en-4-one (**23**)	1593	syn	x						
	rosifoliol (lit: $I=1599$)	1602	ms	x			x	x		
	unknown (B: 159, M: 201)	1612		x				x		
	unknown (B: 82, M: 222)	1624		x				x		
	2-methyltetradecan-4-one (**18**)	1628	syn, inc	x						
	unknown (B: 281, M: 294)	1635		x						
	β-eudesmol (lit: $I=1641$)	1637	ms	x			x	x		
	valerianol (lit: $I=1655$)	1650	ms	x						
t	(1(10)*E*,5*E*)-germacradien-11-ol (**5**)	1656	syn	xxx	xx	xxx	xxx	xxx	xx	xxx
u	tetradecan-1-ol (**24**)	1687	syn	xx				x		
	(*E*,*E*)-farnesol (lit: $I=1722$)	1726	syn	x						
v	13-methyltetradecan-1-ol (**27**)	1750	syn	xx						
	pentadecan-1-ol (**25**)	1787	syn	x						
	1-phenylnonan-1-one (**13**)	1794	ms	x						
	hexahydrofarnesylacetone	1847	syn, inc	x						
	unknown (B: 197, M: 212)	1864						x		
	hexadecan-1-ol (**26**)	1892	syn	x						
w	1-phenyldecan-1-one (**14**)	1903	ms, inc	xxx	xxx	xxx				
	tetradecan-4-olide	1912	ms, inc	x				x		
x	9-methyl-1-phenyldecan-1-one (**16**)	1966	syn	xx	x	x				
y	1-phenylundecan-1-one (**15**)	2005	syn	x	xx	x				
z	nonadecan-10-one (**54**)	2082	ms	x						

[a] For unidentified compounds the base peaks (B) and molecular ions (M) occurring in the EI mass spectra are given. Artifacts originating from the medium are not mentioned. GC: marker in TIC (Figure 1), *I*: retention index, 1.–7.: different experiments with the strains Sg a15 or DW4/3-1, ms: mass spectrum, inc: retention index increment system, syn: synthetic sample, chgc: GC on chiral stationary phase. The relative amounts of the compounds are noted. x: trace compound (0–2%), xx: minor compound (2–8%), xxx: main compound (>8% of total area in GC).

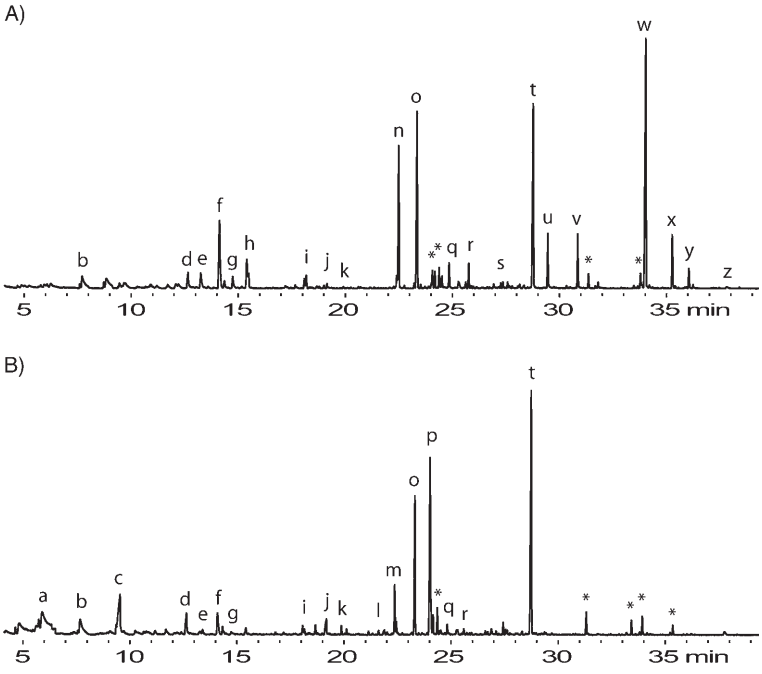


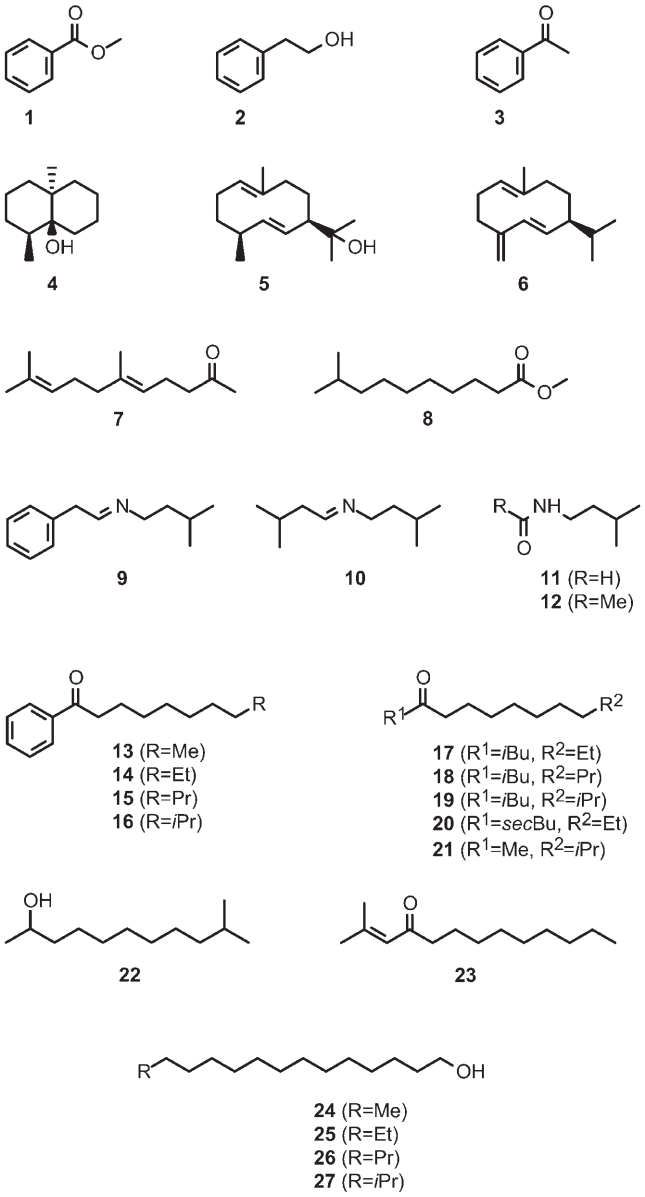
Figure 1. Total ion chromatograms of two extracts of *Stigmatella aurantiaca*, strain Sg a15 (A, experiment 1 in Table 1) and strain DW4/3-1 (B, experiment 5). Letters refer to compounds in Table 1. Artifacts are indicated by asterisks.

mains unknown, due to the lack of an enantioenriched sample, but the absolute configuration shown in Scheme 1 seems to be most likely since a common biosynthesis of these compounds can be assumed.[8] In addition, this stereochemistry has been reported for **6** isolated from *Streptomyces citreus*.[9] Geranylacetone (**7**) was found in only some of the samples, but in large amounts in one experiment with strain DW4/3-1. This combination of terpenoid compounds has also been identified in the headspaces of other myxobacteria investigated by us (*M. xanthus* and *C. crocatus* (without **7**)).[1,2]

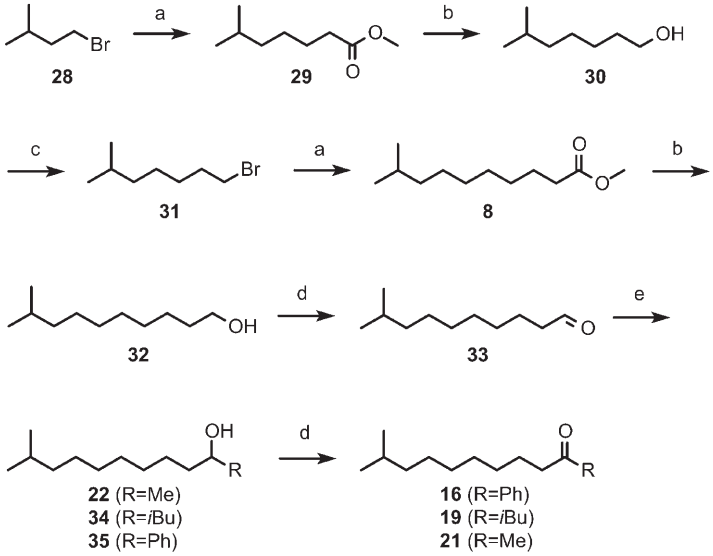
Some nitrogen-containing compounds were also released by both strains, among them the amides *N*-isopentylformamide (**11**) and *N*-isopentylacetamide (**12**), as well as the imines *N*-isopentylideneisopentylamine (**10**) and *N*-(2-phenylethylidene)isopentylamine (**9**), which was emitted in substantial amounts only by strain Sg a15.

Identification of fatty acid derivatives

Strain DW4/3-1 emitted a compound that showed the fragmentation pattern typical of methyl esters ($m/z=74$ and 87).[10,11] The molecular ion at $m/z=200$ corresponds to the formula $C_{12}H_{24}O_2$, but its gas chromatographic retention index of $I=1393$ did not match that of methyl undecanoate ($I=1432$) and the mass spectra also showed slight differences, so the ester seemed to be methyl-branched. As reported previously, the retention indices of methyl-branched compounds can be calcu-



Scheme 1. Volatiles released by *Stigmatella aurantiaca*. The relative configuration of **5** is given.



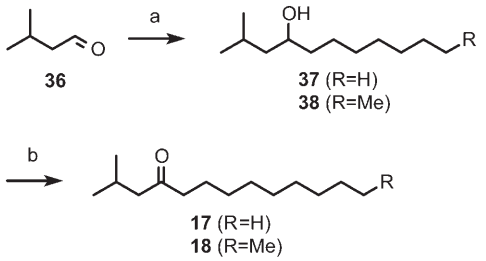
Scheme 2. Synthesis of ester **8**, alcohol **22**, and ketones **16**, **19**, and **21**. a) 1. Mg, 2. CuI, Me_2S, DMAP, methyl acrylate, TMSCl, 81–85%. b) $LiAlH_4$, 90–94%. c) Br_2, PPh_3, 88%. d) PCC, 74–99%. e) RMgBr, 84–86%.

lated by an empirical numerical model.[2,12] The functional group increment for methyl esters is *FG*(ME) = 332, which, together with 1000 for the C_{10} chain and $Me_{(\omega-1)} = 60$, sums up to a calculated value of $I_c = 1392$ for methyl 9-methyldecanoate (**8**). This assignment was made because the difference (ΔI) between unbranched and the corresponding iso compounds is 40.[2,12]

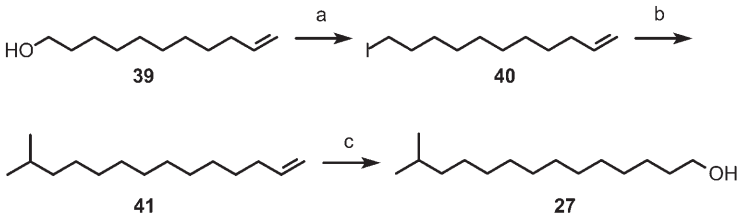
To test this proposal, a synthesis starting with the alkyl bromide **28** was carried out (Scheme 2). Copper-catalyzed Michael addition of its Grignard reagent to methyl acrylate furnished **29**.[13] Standard reduction with $LiAlH_4$ and bromination of the alcohol **30** gave the bromide **31**,[14] which was transformed into **8** by further alkylation of methyl acrylate. This ester proved to be identical with the corresponding volatile component of strain DW4/3-1.

Strain Sg a15 consistently emitted a series of phenyl ketones, with 1-phenyldecan-1-one (**14**) as a major compound, together with 1-phenylundecan-1-one (**15**) and occasionally 1-phenylnonan-1-one (**13**). These ketones were readily identified by their mass spectra. The latest eluting phenone **15** ($I = 2005$) was identical to a commercially available sample. One isomeric phenyl ketone eluted slightly earlier than **15** ($I = 1966$, $\Delta I = 39$), and was suggested to be an $\omega-1$ methyl-branched compound: 9-methyl-1-phenyldecan-1-one (**16**). This ketone was then synthesized from **8**, which was transformed into the corresponding aldehyde **33** by $LiAlH_4$ reduction and PCC oxidation (Scheme 2).[15,16] Addition of phenylmagnesium bromide and oxidation with PCC provided **16** in high yield, confirming the identity of the volatile.

Further ketones were present in one sample of strain Sg a15. Their mass spectra showed strong fragment ions at $m/z = 85$ and $m/z = 100$, pointing to a butyl, isobutyl, or *sec*-butyl ketone. Furthermore, the molecular ion of the first eluting compound of this class at $m/z = 212$ was consistent with the molecular formula $C_{14}H_{28}O$, but the retention index of tetradecan-5-one ($I = 1581$) did not match that of the unknown volatile ($I = 1527$). Because of strong similarities with the mass spectrum of 2-methyldecan-4-one the unknown compound was assumed to be the isobutyl ketone 2-methyltridecan-4-one (**17**). The synthesis of **17** was carried out, starting with nonyl bromide (Scheme 3). The corresponding Grignard reagent was added to **36** to give 2-methyltridecan-4-ol (**37**). Subsequent PCC oxidation afforded **17**, identical to the natural compound. Its homologue 2-methyltetradecan-4-one (**18**) was also released by the bacteria and its structure was verified by synthesis. Furthermore, an isomer of **18** with a retention index of $I = 1589$ was also emitted by this strain. Again, the decrease in the retention index ($\Delta I = 39$) pointed to a second methyl branch in the chain at the $\omega-1$ position. The aldehyde **33** was



Scheme 3. Synthesis of the ketones **17** and **18**. a) Nonyl/decylmagnesium bromide, 83–89%. b) PCC, 82–90%.



Scheme 4. Synthesis of **27**. a) PPh_3, imidazole, I_2, 86%. b) *i*BuMgBr, $Li_2[CuCl_4]$, 98%. c) 9-BBN, NaOH, H_2O_2, 91%.

used for a synthesis of **19** by the route described above (Scheme 2), establishing the presence of 2,12-dimethyltridecan-4-one (**19**) in the bacteria. Interestingly, the different aliphatic chains found in the phenyl ketones are the same as in the isobutyl ketones, pointing to a related biosynthesis (see below).

Another unknown volatile eluted slightly later than **17** ($I=1532$). The mass spectrum suggested a very similar structure, because of the same molecular ion at $m/z=212$ and major fragment ions at $m/z=57$, 85, and 155. This compound was proposed to be the *sec*-butyl ketone **20**. A synthesis through a Grignard reaction with decanal and *sec*-butylmagnesium bromide and PCC oxidation confirmed this suggestion.

Furthermore, a related compound, also eluting slightly later then **17** and showing strong fragment ions at $m/z=83$ and 98 with a molecular ion at $m/z=210$, was also present. Conclusively, this volatile seemed to be an α,β-unsaturated counterpart of **17**. A synthesis through a Grignard reaction with nonylmagnesium bromide and 3-methylcrotonaldehyde, followed by oxidation with PCC, confirmed this compound as 2-methyltridec-2-en-4-one (**23**).

Additional ketones were detected in one extract of strain DW4/3-1. The first was characterized by a mass spectrum with major fragment ions at $m/z=43$ and 58, as is expected for methyl ketones, and a molecular ion at $m/z=184$. From retention index calculations the presence of an $\omega-1$ methyl branch was suggested (*FG*(2-one)$=200$).[4] Furthermore, the corresponding methyl carbinol was also present, eluting slightly later than the methyl ketone (*FG*(2-ol)$=210$)[4]. Its mass spectrum showed a strong fragment ion at $m/z=45$, whereas the molecular ion was not detected. Both compounds were synthesized by addition of methylmagnesium bromide to the aldehyde **33** to furnish 10-methylundecan-2-ol (**22**), which was converted into the corresponding methyl ketone **21** by PCC oxidation (Scheme 2). The synthetic compounds were identical to the bacterial volatiles.

In addition, a series of 1-alcohols was present in the extracts of strain Sg a15. The unbranched compounds tetradecan-1-ol (**24**), pentadecan-1-ol (**25**), and hexadecan-1-ol (**26**) were readily identified by comparison with authentic standards. Another alcohol eluting shortly earlier than **25** ($\Delta I=37$) was thus suggested to be 13-methyltetradecan-1-ol (**27**). This was therefore synthesized from undec-10-en-1-ol (**39**) (Scheme 4), with transformation into the corresponding iodide **40**[17,18] and alkylation with isobutylmagnesium bromide in the presence of $Li_2[CuCl_4]$ (Kochi's catalyst) affording **41**.[19] Highly regioselective hydroboration with 9-borabicyclo[3.3.1]nonane (9-BBN)[20] and oxidative workup then gave **27**, which proved to be identical to the volatile emitted by *S. aurantiaca*.

Biosynthesis

We recently showed that investigations on the biosynthesis of volatiles emitted by bacteria can be carried out easily by feeding labeled precursors to cultures grown on agar plates,[2] thus avoiding the lavish extraction and workup procedures normally performed with liquid cultures. We applied this method to investigation of the unknown biosynthetic pathway(s) to the phenyl ketones. The double methyl branching in **19** suggested that two isovaleryl-CoA (IV-CoA) units might be involved in the biosynthesis of this compound. Similarly, we assumed that the phenyl analogue **16** might arise from one benzoyl-CoA unit and one IV-CoA unit. Chain-extension of IV-CoA with three acetate-derived malonyl-CoA units (or degradation of an iso-odd fatty acid such as iso-17:0) would generate 9-methyldecanoyl-CoA. Head-to-head fusion either with benzoyl-CoA or with IV-CoA should then produce branched β-keto thio esters, subsequent decarboxylation of the free acids finally affording **16** and **19**, respectively. The volatiles **13**–**15**, **17**, and **18** could similarly be derived from unbranched acids and a head-to-head fusion-decarboxylation process.

To elucidate whether the proposed biosynthetic pathways were indeed operative in *S. aurantiaca,* [$^2H_5$]benzoic acid ([$^2H_5$]-**42**) was fed to strain Sg a15 and the profile of the released volatiles was analyzed (Scheme 5). Deuterium-labeled compounds elute earlier than their unlabeled counterparts from the GC column (retention indices decrease about one unit per deuterium atom),[2] so pure mass spectra of labeled compounds were obtained even in the event of low incorporation rates. These rates were determined from the corresponding peak areas in the total ion chromatogram. Labeled [$^2H_5$]-**42** was incorporated into **16** (90% incorporation) as indicated by a shift of the molecular ion from $m/z=246$ to 251 (Figure 2A and B). Furthermore, the fragment ions at $m/z=77$, 105, 120, and 133 were shifted to $m/z=82$, 110, 125, and 138, respectively, which is in accordance with the labeling of five deuteriums present in the phenyl ring. The incorporation of [$^2H_5$]-**42** was also found for **14** and **15** (89% and 90%, respectively). Benzoic acid itself

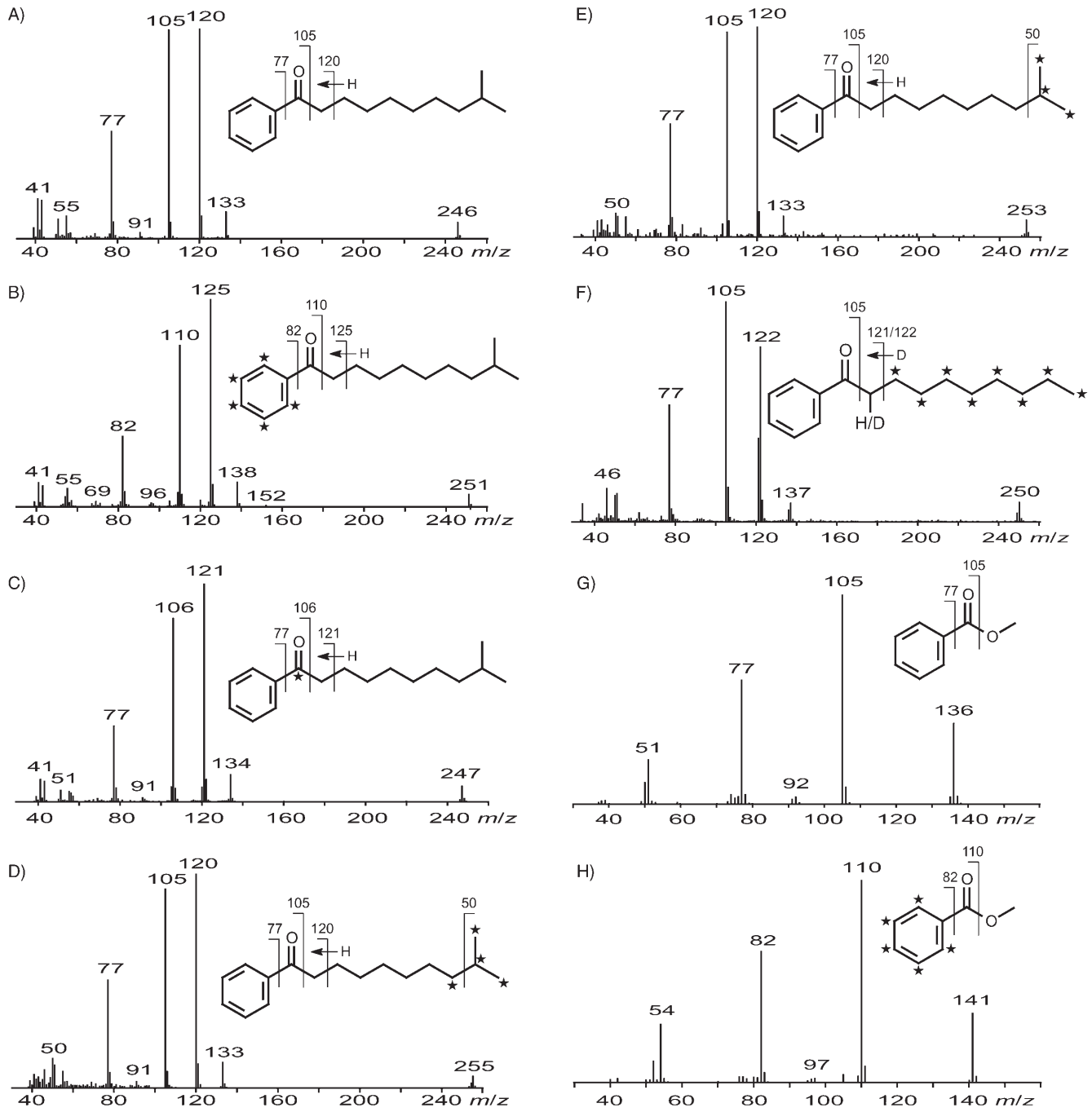


Figure 2. Mass spectra of labeled and unlabeled 9-methyl-1-phenyldecan-1-one (**16**), 1-phenyldecan-1-one (**14**), methyl benzoate (**1**), and acetophenone (**3**) obtained in experiments with strain Sg a15: **16** (A), [$^2H_5$]-**16** after feeding of [$^2H_5$]benzoate (B), [^{13}C]-**16** after feeding of [^{13}C]-benzoate (C), [$^2H_9$]-**16** after feeding of [$^2H_{10}$]leucine (D), [$^2H_7$]-**16** after feeding of [$^2H_7$]-15-methylhexadecanoate (E), coeluting [$^2H_{17}$]-**14** and [$^2H_{18}$]-**14** after feeding of [$^2H_{31}$]hexadecanoate (F), **1** (G), [$^2H_5$]-**1** after feeding of [$^2H_5$]benzoate (H), [^{13}C]-**1** after feeding of [^{13}C]-benzoate (I), **3** (J), [$^2H_5$]-**3** after feeding of [$^2H_5$]benzoate (K), [^{13}C]-**3** after feeding of [^{13}C]-benzoate (L).

might be derived from phenylalanine through degradation catalyzed by phenylalanine ammonium lyase. Feeding of [$^2H_5$]cinnamic acid ([$^2H_5$]-**43**), which is formed through the ammonium lyase reaction,[21,22] also resulted in labeled **16** (Scheme 5, 11% incorporation).

The carbonyl function in the phenyl ketones was assumed to arise from benzoate, but the position of this oxygenated carbon would also be explainable (although unlikely) by the incorporation of 9-methyldecanoyl-CoA and transfer of the phenyl group from benzoate. Therefore, [^{13}C]-**42** was fed to strain Sg a15 (Scheme 5). The resulting ^{13}C labeling is not associated with any significant decrease in retention times relative to the unlabeled counterpart, so the mass spectra of labeled and unlabeled volatiles are superimposed.[2] The incorporation of [^{13}C]benzoic acid into **16** (89% incorporation rate) clearly indicated that the carbonyl carbon is derived from this precursor

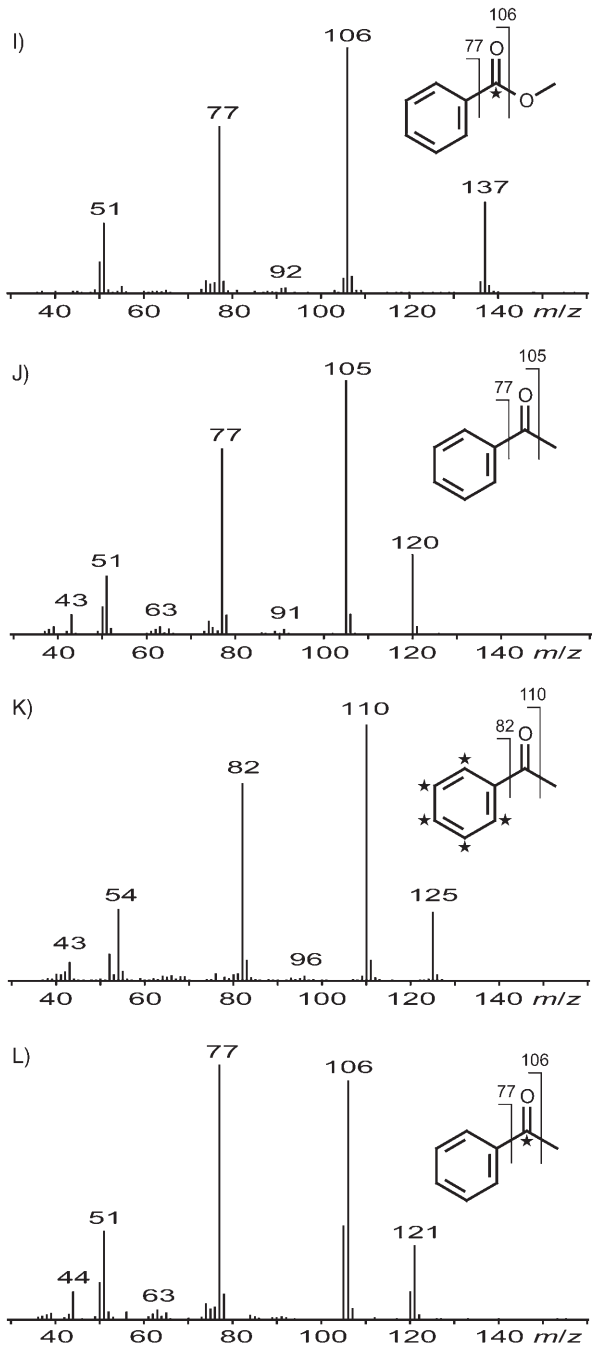


Figure 2. (Continued).

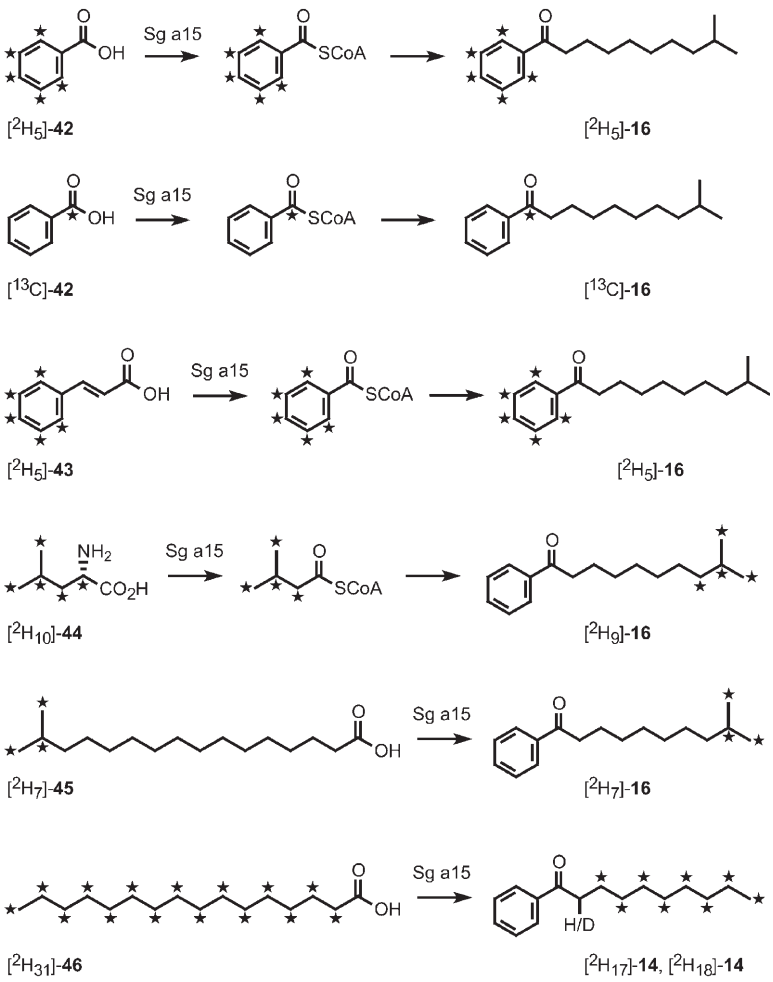
(Figure 2C). The molecular ion increased from $m/z=246$ to 247, and the fragment ions at $m/z=105$, 120, and 133 were shifted to $m/z=106$, 121, and 134, whereas the fragment ion at $m/z=77$ originating solely from the unlabeled phenyl group did not increase. Corresponding results were obtained for **14** (90%) and **15** (91%).

Furthermore, the incorporation of both [$^2H_5$]-**42** and [^{13}C]-**42** into **1** and **3** (Figure 2G, H, J, and K) was observed. Incorporation rates between 72% and 88% were found, elucidated through the use of the ions at $m/z=105$ and 120, respectively, as references.

In a following experiment, [$^2H_{10}$]leucine ([$^2H_{10}$]-**44**) was fed to strain Sg a15 (Scheme 5). Because leucine is transformed by transamination and oxidative decarboxylation into IV-CoA and one deuterium is lost in the transamination step, incorporation of [$^2H_{10}$]-**44** into **16** (17%) was indicated by a shift of the molecular ion from $m/z=246$ to 255 (Figure 2D), reflecting the incorporation of nine deuterium atoms. The fragment ions at $m/z=77$, 105, 120, and 133 did not change, but $m/z=50$ showed that the isopropyl group was completely deuterium-labeled.

The remaining carbons of **16** were believed to originate from the well known elongation of the IV-CoA starting unit with three malonyl-CoA building blocks to form the fatty acid 9-methyldecanoic acid or its CoA-thioester. In experiments on the biosynthetic pathways to iso fatty acids in *S. aurantiaca*,[23] synthetic [$^2H_7$]-15-methylhexadecanoic acid ([$^2H_7$]-**45**), with a completely labeled isopropyl moiety, was fed to this myxobacterium (Scheme 5). The fatty acid extracts also contained trace amounts of **16** and [$^2H_7$]-**16** (62% incorporation rate), showing that the remaining carbon atoms must indeed be derived from malonyl-CoA. The mass spectrum of [$^2H_7$]-**16** (Figure 2E) is characterized by a molecular ion of $m/z=253$, consistently with the presence of seven deuterium atoms. The fragment ion at $m/z=50$ points to a labeled isopropyl moiety. Obviously, 15-methylhexadecanoic acid can be degraded to 9-methyldecanoyl-CoA, the putative precursor of the phenyl ketones. The fatty acid 15-methylhexadecanoic acid plays a central role in the fatty acid metabolism of *S. aurantiaca* and is a precursor of shorter acids derived by α- or β-oxidation.[23]

In order to obtain insight into whether the medium-chain fatty acyl precursors—9-methyldecanoyl-CoA in the case of **16** or decanoyl-CoA for **14**—are activated by carboxylation to give (7-methyloctyl)malonyl-CoA or octylmalonyl-CoA, respectively, [$^2H_{31}$]hexadecanoic acid ([$^2H_{31}$]-**46**) was fed to *S. aurantiaca* (Scheme 5). The incorporation of 18 deuterium atoms was indicated by a shift of the molecular ion to $m/z=250$, whilst the incorporation of only 17 deuterium atoms also occurred, as indicated by $m/z=249$ (Figure 2F). Additionally, major fragment ions were found at $m/z=105$ and 121/122, indicating that the deuterium labeling is located in the alkyl chain. A completely deuterated alkyl chain, however, would require $m/z=123$, corresponding to 19 deuterium atoms. These data therefore reveal single or double loss of deuterium in the position α to the carbonyl group. In other feeding experiments performed with the myxobacterium *Myxococcus xanthus* to probe the biosynthesis of fatty acids, [$^2H_{31}$]-**46** was fed to that species. In these experiments the fatty acid extracts were methanolyzed under acidic conditions and the obtained methyl esters were analyzed by GC-MS. Under these conditions the fatty acids derived from **46** were predominately composed of acids with no deuterium loss at C-2 (e.g., 82%), together with minor amounts of acids with one deuterium loss (e.g., 17%) and only traces of acids lacking both α deuterium atoms (e.g., 2%). This indicates that almost no H/D exchange had taken place. It can be assumed that H/D exchange α to the carbonyl group under physiological conditions should be even lower. These data therefore strongly suggest the activation of



Scheme 5. Feeding experiments with *Stigmatella aurantiaca* strain Sg a15.

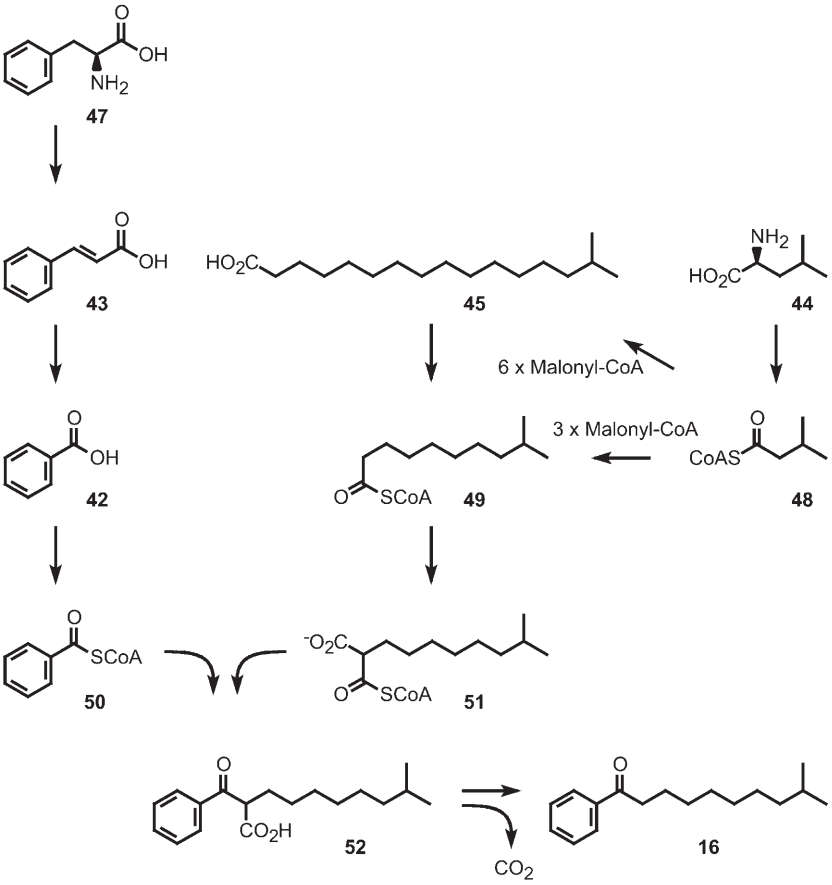
decanoyl-CoA by carboxylation, resulting in octylmalonyl-CoA with increased C,H-acidity. Accordingly, 9-methyldecanoyl-CoA might be activated as (7-methyloctyl)malonyl-CoA prior to its incorporation into **16**.

Discussion

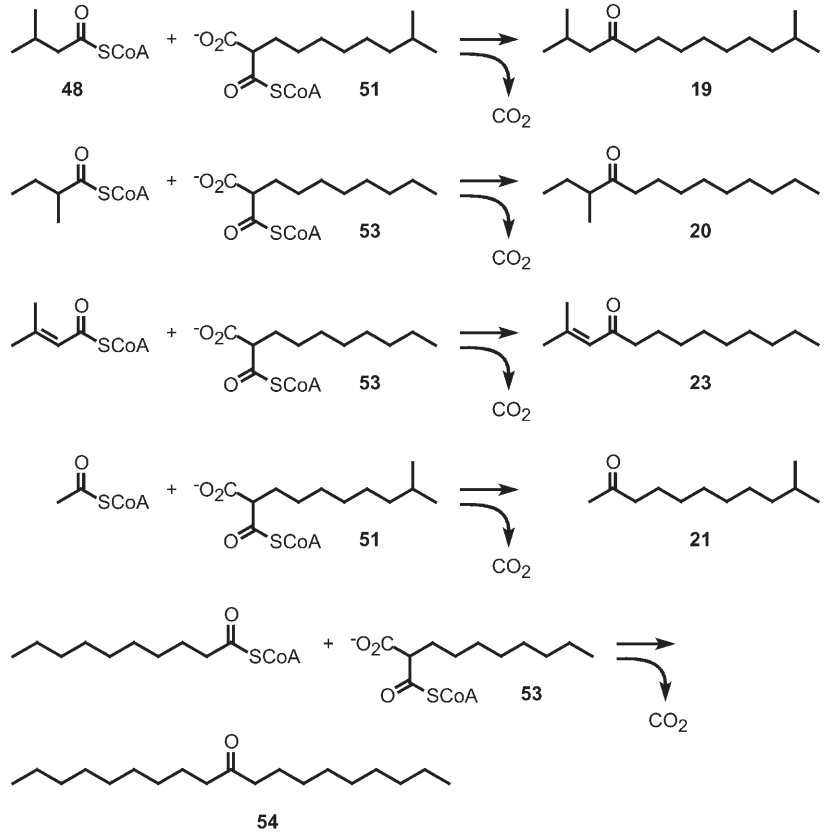
A biosynthetic pathway to the *S. aurantiaca* phenyl ketone **16**, based on the feeding experiments described above, is postulated (Scheme 6). The starting IV-CoA unit (**48**) is derived from leucine (**44**). The CoA-thioester **48** can be elongated by three malonyl-CoA units to form the key derivative 9-methyldecanoyl-CoA (**49**) directly. Alternatively, **49** may be derived from degradation of a fatty acid such as 15-methylhexadecanoic acid (**45**), itself derived from standard fatty acid biosynthesis. The latter route may be preferred, because incorporation rates of [$^2H_7$]-**45** are markedly higher than those of leucine. The $\omega-1$ methyl-branched fatty acid thioester **49** is then condensed with benzoyl-CoA (**50**), originating from phenylalanine (**47**) via cinnamic acid (**43**). It is most likely that **49** is activated by a carboxylase prior to the condensation, forming (7-methyloctyl)-malonyl-CoA (**51**), similarly to the cases of activation of fatty acyl-ACP by carboxylation in the biosynthesis of mycolic acids[24] or of acetyl-CoA to malonyl-CoA.[25] This activation by carboxylation at the α-position is corroborated by one feeding experiment with [$^2H_{31}$]-**46**, which is first degraded to [$^2H_{19}$]decanoyl-CoA according to the degradation of **45** to **49**. Carboxylation results in the formation of [$^2H_{18}$]octylmalonyl-CoA, which can lose another deuterium because of the C,H-acidity. Accordingly, the incorporation of 17 or 18 deuterium atoms is observed. The condensation step with **50** proceeds with decarboxylation, similarly to the elongation of a fatty acyl-CoA by malonyl-CoA, resulting in a β-keto acyl-CoA. Decarboxylation of the corresponding free β-keto acid **52** then gives **16**. In essence, the coupling step consists of the addition of an alkylmalonyl-CoA to an activated ester, followed by reductive loss of the carboxylic acid carbon of the free β-keto acid. In mycolic acid biosynthesis, the second decarboxylation step is omitted.[24] To the best of our knowledge, such a head-to-head condensation process has only rarely been described in the biosynthesis of secondary metabolites. A similar mechanism seems to be involved in the biosynthesis of some bacterial hydrocarbons.[26–28] Unbranched fatty acids are used in this head-to-head coupling process for the unbranched phenyl ketones **13**–**15**. Structural diversity in the biosynthesis of fatty acids is often achieved by use of different starter units, in this case IV-CoA. With the pathway described here further diversity can be achieved in the end group, in this case the coupling partner **50**.

This principle can explain the formation of several other ketones found in *S. aurantiaca* (Scheme 7). In the biosynthesis of the isobutyl ketones **17**–**19**, a fatty acid derivative such as **51** or **53** is again coupled with isovaleryl-CoA (**48**) instead of **50**. Other ketones could be formed similarly, by condensation of a fatty acyl-CoA precursor and isoleucine-derived 2-methylbutyryl-CoA (**20**), leucine-derived dimethylacryl-CoA (**23**), acetyl-CoA (**21**), or even decanoyl-CoA in the case of nonadecan-10-one (**54**). Interestingly, the formation of **21** by this pathway would be an alternative pathway to methyl ketones, normally believed to be produced by decarboxylation of the 3-oxoacyl intermediates of fatty acid biosynthesis.[29] Unfortunately, incorporation of [$^2H_{10}$]leucine or [$^2H_7$]-15-methylhexadecanoic acid into these ketones could not be observed, because only trace amounts were present in the headspace extracts.

Interestingly, the key fatty acid 9-methyldecanoic acid was found in the form of its methyl ester **8** in the analyses of strain DW4/3-1, while no other fatty acid methyl ester was detected. The methyl ester of the second building block in the head-to-head fusion process to phenyl ketones, methyl benzoate (**1**), was also produced by both strains of *Stigmatella aurantiaca*, although phenyl ketones such as **16** were only produced by strain Sg a15. Other acids or their corresponding CoA esters that can substitute benzoyl-CoA in the head-to-head coupling have been found in trace amounts. The acids 3-methylbutyric acid and 2-methylbutyric acid were identified in one sample of



Scheme 6. Biosynthetic pathway to 9-methyl-1-phenyldecan-1-one (**16**).



Scheme 7. Formation of several ketones by head-to-head coupling of fatty acid precursor and different end groups.

strain DW4/3-1. The related ketones **20** (derived from IV-CoA) and **17** (2-methylbutyryl-CoA) were released by both strains. Methyl 3-methylcrotonate was present in one sample of strain Sg a15, and the same sample of this strain contained trace amounts of **23**. Methyl 2-methylcrotonate was produced by both strains, whereas no derived ketone (e.g., 3-methyltridec-2-en-4-one) was found. In summary, the pattern of the building block methyl esters and fatty acids is correlated to the pattern of the constructed ketones.

The phenyl ketones **13** and **16** have not previously been reported in nature. Both **14** and **15** have been reported from the essential oils of the plants *Baccharis dracunculifolia*[30] and *Artabotrys odoratissimus*,[31] but the latter report at least seems to be doubtful with respect to the fact that several well known contaminants are declared as natural products in that article. Additionally, such phenyl ketones have been described as oxidation products of petroleum in sea air and water.[32]

The aliphatic ketones **17**–**20** and **23** and the alcohol **22** have not previously been reported from nature, whereas the ketone **21** occurs in the essential oil of *Ruta angustifolia*.[33] The amide **12** has been identified in wines[34] and is also known as an alarm pheromone of cockroaches[35] and wasps[36–38] and a as volatile constituent of *Nicotiana tabacum*, which also contains **9**.[39] The imine **10** is emitted by *Bacillus popillae*.[40] Both **10** and **11** are characteristic odor constituents of roasted seafood,[41,42] but **11** has not been reported from natural sources.

The bouquets emitted by the two *S. aurantiaca* strains are complex, with more than 80 components. They are different from those of *Myxococcus xanthus*[2] and *Chondromyces croca-*

tus,[1] which were investigated by identical methods. *M. xanthus* also produces ketones as characteristic components, but these are formed by a different biosynthetic pathway.[2] *S. aurantiaca* also forms imines and amides not present in the other bacteria. Common to all three species is the co-occurrence of the sesquiterpenoids (−)-geosmin (**4**) and (1(10)*E*,5*E*)-germacradien-11-ol (**5**), the biosyntheses of which have been addressed recently.[8]

In summary, the complex volatile bouquet emitted by the myxobacterium *Stigmatella aurantiaca* has been delineated, and several compound classes not previously known from myxobacteria were found. The biosynthesis of phenyl ketones was shown to proceed through an unusual "head-to-head" condensation process of a fatty acid precursor and benzoic acid. The biological function(s) of the volatiles and their relation to physiological changes in the life cycle of the bacteria are the subject of ongoing studies.

Experimental Section

Strains, culture conditions, and feeding experiments: The strains *Stigmatella aurantiaca* DW4/3–1 and Sg a15 and their cultivation were described previously.[43,44]

Feeding experiments were performed as described.[2] All compounds were dissolved in methanol or water and applied to TS-agar plates to a final concentration of 1 mM. After evaporation of the solvent under sterile conditions, the cell suspension (300 μL) was spread onto these plates as described previously[2] and also allowed to dry prior to incubation at 30 °C for four days.

Sampling: Volatile organic compounds emitted by cell cultures of *Stigmatella aurantiaca* were collected by the CLSA technique as described previously.[2]

GC-MS: GC-MS analyses were carried out on a HP 6890 Series GC system connected to a HP 5973 mass selective detector (Hewlett–Packard, Wilmington, USA) fitted with a BPX5 fused-silica capillary column (25 m × 0.22 mm i.d., 0.25 μm film, SGE, Melbourne, Australia). Conditions were as follows: inlet pressure: 77.1 kPa, He 23.3 mL min^{-1}; injection volume: 1 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 mL min^{-1}. Retention indices *I* were determined from a homologous series of *n*-alkanes (C_8–C_{38}). Identification of compounds was performed by comparison of mass spectra against the Wiley 7 Library and the Essential Oils Library (Massfinder) and by comparison with synthetic standards (see Table 1 for details).

Synthesis. General synthetic methods, synthetic procedures, and physical and spectroscopic data for the synthesized compounds can be found in the Supporting Information.

Acknowledgements

We thank W. A. König for samples of germacrene D and (1(10)E,5E)-germacradien-11-ol. J.S.D. thanks the Fonds der Chemischen Industrie and the BMBF for a stipend. Research in R.M.'s laboratory was funded by a grant from the Deutsche Forschungsgemeinschaft (DFG).

Keywords: biosynthesis · ketones · mass spectrometry · myxobacteria · volatile compounds

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Received: April 22, 2005
Published online on October 6, 2005

