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Coupled biosynthesis of volatiles and salinosporamide A in *Salinispora tropica*

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Abstract

Terrestrial bacteria, especially actinomycetes, are known to be prolific producers of volatile compounds. We show here that bacteria from ocean sediments can also release complex bouquets of volatiles. The actinomycete *Salinispora tropica* produces novel cyclohexenyl compounds, such as methyl cyclohex-2-ene-1-carboxylate (**9**), methyl 2-(cyclohex-2-en-1-yl)acetate (**10**), methyl (*E/Z*)-2-(cyclohex-2-en-1-ylidene)acetate (**11**, **12**), and related alcohols (**8**, **13**). These compounds were identified by GC/MS and confirmed by synthesis. In addition, rare spiroacetals, aromatic compounds, short chain acids and esters, alcohols, and various cyclic compounds were produced by the bacteria. The biosynthesis of the cyclohexenyl compounds is closely coupled to that of cyclohexenylalanine (**4**), a building block of salinosporamide A, a proteasome inhibitor produced by *S. tropica*. Analysis of *S. tropica* strains that harbor knockouts of the salinosporamide biosynthetic genes *salX* and *salD*, coupled with feeding experiments, revealed that 3-(cyclohex-2-en-1-yl)-2-oxopropanoic acid (**60**) and 3-(cyclohex-2-en-1-ylidene)-2-oxopropanoic acid (**61**, **62**) are important intermediates in the biosynthesis of salinosporamide A, **4**, and **8–13**.

Graphical Abstract

The marine sediment bacterium *Salinispora tropica* does not only produce the proteasome inhibitor salinosporamide A, but at the same time a plethora of volatile compounds. Mutant feeding experiments showed that biosynthesis of the major volatile cyclohexenyl-derivatives is closely connected to the biosynthesis of cyclohexenylalanine, a building block of salinosporamide.

Keywords

actinomycetes; marine bacteria; amino acids; spiro acetals; feeding experiments

Introduction

Terrestrial actinomycete bacteria are potent producers of volatile compounds.^[1–5] These volatiles can be classified into five major groups, including fatty acid derivatives, sulfur-, nitrogen-, and aromatic compounds, as well as terpenes.^[1] The latter comprise the largest and structurally most diverse class of actinomycete volatiles, containing mostly sesquiterpenes,^[6,7] but also monoterpenes and diterpenes. Two prominent such terpenes are the earthy-odor trisnorsesquiterpene geosmin (**1**)^[3–5,8] and the musty-odor homomonoterpene 2-methylisoborneol (**2**).^[3,5,8] Geosmin was the first volatile compound identified from bacteria in 1965.^[9]

We became interested in comparing volatiles from deep-sea marine actinomycetes to those released by their terrestrial relatives. Volatiles released by marine bacteria have so far been investigated preferentially in bacteria of the roseobacter clade, which emit sulfur compounds, lactones and tropone.^[10,11] Other studies included arctic flavobacteria that were isolated from ice or surface waters, which produce long chain ketones in strain specific mixtures.^[14] *Streptomyces* GWS-BW-H5, closely related to *S. caviscabies* and isolated from the German Wadden Sea, produces terpenes such as geosmin (**1**) and several sesquiterpenes as well as long chain aliphatic lactones.^[8] These compounds are similar or identical to those produced by terrestrial streptomycetes,^[7] and no specific difference in bouquet composition compared to terrestrial relatives was observed. Most of the bacteria investigated so far were isolated near the ocean surface. We therefore became interested whether bacteria from marine sediments, sometimes living far away from surface waters, are able to produce volatiles. Actinobacteria seemed good candidates because of their diverse biosynthetic

machinery for secondary metabolites. The following questions we like to answer with our work are therefore: (i) Do marine sediment actinobacteria produce volatiles? (ii) Are these volatiles similar to those found in terrestrial actinomycetes? (iii) Is their biosynthesis related to those of known natural products?

The latter question arose because recently we could show that in some cases the volatiles released by antibiotic producing strains likely originate from shunt metabolites of antibiotic biosynthesis. *Streptomyces* FORM5 emits streptopyridines as strain specific volatiles, which biosynthesis seems tightly connected to that of the also produced antibiotic streptazoline.^[12] *Phaeobacter inhibens* from the marine *Roseobacter* clade emits the volatile tropone that is biosynthetically related to the antibiotic tropodithietic acid (TDA).^[11,13] *Streptomyces ambofaciens* produces volatile blastmycinones and the corresponding antibiotic antimycins, which share genes in their biosynthetic gene cluster.^[15] In contrast, there are also bacteria where no correlation was found between antibiotic and volatile biosynthesis. *Chitinophaga* Fx7914 is known for the production of the antibiotic elansolide.^[16] Analysis of the volatiles revealed no related compounds, but a plethora of fatty acid esters, sulfur compounds, amino acid derivatives, and terpenes.^[17]

Salinispora tropica is an obligate marine actinomycete isolated from ocean sediments. This bacterium is best known for the production of the anticancer compound salinosporamide A (3),^[18,19] a γ -lactam- β -lactone proteasome inhibitor, which is presently in clinical trials. Salinosporamide A (3) is biosynthesized from three building blocks: an acetate, the *S*-adenosyl-methionine (SAM)-derived chlorinated precursor 2-chloroethylmalonyl-CoA (50), and the non-proteinogenic amino acid L-3-cyclohex-2'-enylalanine (4).^[20] We report here on the identification and synthesis of new, strain specific volatile cyclohexene derivatives produced by *S. tropica* and their correlation to the salinosporamide A biosynthetic gene cluster. Knockout mutants and feeding experiments confirmed the biosynthetic relationship of the volatiles to this non-proteinogenic amino acid.

Results and Discussion

Analysis of volatiles

The volatiles released by liquid cultures of *S. tropica* CNB-440 were collected using closed-loop stripping analysis (CLSA)^[21] and analyzed by GC/MS. The analysis revealed a mixture of approximately 40 different compounds, eight of which were consistently higher in abundance across different experiments (Figure 1). Three of the major compounds were readily identified as the widespread aromatic volatiles 2-phenylethanol (6), methyl phenylacetate (7), and methyl benzoate (5),^[1,2] whereas the other five compounds (a-e) were unknown.

Amongst the minor volatiles, we identified a plethora of methyl esters, acids, ketones, lactones, and alcohols in the headspace extract (Scheme 1, Table 1). Most of the volatiles were already known from previous analyses of other actino- and other bacteria. The α -hydroxyketones **28** and **29** are widespread and were identified as precursors in the biosynthesis of alkylated pyrazines in *Corynebacterium glutamicum*.^[22] The ketone 4-hydroxy-4-methyl-2-pentanone (diacetone alcohol, **27**) indicates the release of acetone, which itself is not detectable by the method used. Another interesting ketone is cyclohept-4-enone **37**. Recently, this compound was found in several actinomycetes, e.g. *Streptomyces* FORM5^[12] and *Streptomyces ambofaciens*.^[2] Compound **37** may derive from the phenylacetate biosynthetic pathway as previously suggested for tropone.^[2,23] The dioxaspiro-acetals **35** and **36** have so far not been found to be produced by bacteria. Spiroacetal **36** is the sex pheromone of the fruit fly *Bactrocera oleae*,^[24] while **35** is an aroma compound of green Mexican coffee.^[25] In general, actinomycetes are potent terpene producers, but such compounds were rare in the headspace extract of *S. tropica* CNB-440. Only methyl geranate (**23**) was identified. To the best of our knowledge, diethyl ester **40** has not yet been reported from nature.

The five unknown compounds **a–e** shared similar ions in their mass spectra (Figure 2). A fragment m/z 77 is characteristic in the mass spectra of benzenoid compounds such as **5**. In the spectra of the unknown compounds **a**, **b**, and **c**, a base peak of m/z 80/81 is present, which led us to propose a cyclohexene-ring as structural motif. The mass spectrum of compound **a** (Figure 2a) showed high similarity with that of cyclohex-2-enylmethanol; a compound that is not known to be produced in bacteria, but has been identified from the plant *Foeniculum vulgare*.^[27] The molecular ion at m/z 112 is present in very low abundance. Ion m/z 94 is formed by the loss of water, and the loss of the CH_2OH results in the base peak m/z 81. The structure of the natural compound **a** was confirmed by synthesis via cyclohexenyl anion addition to formaldehyde^[28] (Scheme 2a).

Compound **b** has a clearly visible molecular ion at m/z 140. Fragment ions at m/z 108 and 81 point to the loss of methanol (M-32) and a methyl ester group (M-59), resulting in the proposal of methyl cyclohex-2-ene-1-carboxylate as the structure. The position of the double bond was assumed to be identical to that of the cyclohexenyl ring of salinosporamide A (**3**). The structure of compound **b** was proven by synthesis (Scheme 2b); commercially available 3-bromocyclohex-1-ene (**45**) was transformed into the corresponding nitrile **46** by substitution with cyanide.^[29] A Pinner reaction under mild conditions with HCl and methanol led to compound **9**, which was identical to the natural compound **b** and confirmed the proposed structure.

The next putative cyclohexene-derivative **c** had a mass spectrum similar to that of **b** with a molecular ion of m/z 154, indicating one additional methylene group. The ions at m/z 94 and m/z 122 arise from the loss of methanol and the methyl ester group, respectively, while the m/z 80 suggests a loss of CH_2COOMe . These data led to the assignment of methyl 2-(cyclohex-2-en-1-yl)acetate as the structure for compound **c**. To confirm this proposal, the compound was synthesized starting from the alcohol **8** (Scheme 2a). Transformation of **8** with *N*-bromosuccinimide and triphenylphosphine yielded bromide **43**.^[30] A Grignard

reaction with dry ice led to acid **44** that was esterified with methanol to arrive at the desired methyl ester, **10**, which was identical to the natural compound **c**. The reduction product of **44**, alcohol **13**, occurred as minor component of the odor bouquet.

Compounds **d** and **e** have identical mass spectra (Figure 2d) and are probably diastereomers. The molecular ion of **d/e** is 2 amu lower than that of **c**, indicating an additional double bond. The position of the second double bond is likely not in the ring, as no diastereomers would be possible, and thus no identical mass spectra would be expected. Therefore, we proposed the isomers **d/e** to be methyl (*E*)- and (*Z*)-2-(cyclohex-2-en-1-ylidene)acetate (**11**, **12**). The two isomers were synthesized by a Horner-Wadsworth-Emmons reaction^[31] with cyclohexanone (**47**) and the phosphonoester **15**, leading to a 4:1 *Z/E* ratio of the products (Scheme 2c). In contrast, Peterson olefination^[32] of **47** with methyl 2-(trimethylsilyl)acetate (**49**) preferentially furnished the *E*-isomer in a 2:1 *E/Z* ratio. Comparison of the mass spectra and GC retention times confirmed our structural proposal for the isomers **d/e**. NOESY-experiments allowed assignments of the geometrical isomers.

The identified 2-cyclohexenyl-derivatives **a–e** are new bacterial compounds and to the best of our knowledge, except for the alcohol **a**,^[27] are new natural compounds. Examples for other 2-cyclohexenyl-derivatives occurring in nature are quite rare. The aldehyde 2-cyclohexene-carbaldehyde was detected in the peel of citrus fruits.^[33] Naturally occurring derivatives of **c**, **d** and **e**, such as the corresponding acids or aldehydes have not been reported. These compounds are therefore a new class of compounds likely derived from the biosynthetic pathway of the rare amino acid cyclohexenylalanine (**4**).

Biosynthesis of cyclohexenyl-compounds

The biosynthesis of salinosporamide A was previously investigated and is accomplished by incorporation of three metabolic building blocks (Figure 3b).^{[19] [20] [34]} The genes and the corresponding enzymes for the formation and incorporation of acetyl-CoA (**51**) and the SAM-derived PKS extender unit 2-chloroethylmalonyl-CoA (**51**) are well established.^[35] In contrast, the biosynthesis of the amino acid building block β -hydroxy-L-3-cyclohex-2'-enylalanine (**52**) is less well understood.^[36–38] Isotopic feeding experiments, mutagenesis, and mechanistic enzymology support a new branch point in the typical aromatic amino acid biosynthetic pathway that leads to the formation of cyclohexenylalanine (**4**). Two genes have been confirmed and two others have been proposed to be in the cyclohexenylalanine pathway (Figure 3a, blue colored genes).^[19]

The gene *salU* encodes a 3-deoxy-D-arabinoheptulosonate-7-phosphate (DAHP) synthase, which catalyzes the first step of the shikimate pathway and synthesizes DAHP from phosphoenolpyruvate and D-erythrose 4-phosphate. The putative SalU product would then proceed via primary metabolism to prephenic acid. We previously characterized the purified recombinant SalX enzyme, which performs the decarboxylative dehydration of prephenic acid (**54**).^[37] The prephenate decarboxylase SalX is part of a newly described prephenate-utilizing enzyme family that avoids the typical aromatization of the cyclohexadienol ring by protonating C-6' to yield the endocyclic diene dihydro-4-hydroxyphenylpyruvate (**55**).^[37,39] SalX is a homolog to the enzyme BacA in bacilysin biosynthesis. Walsh *et al.* showed that

intermediate **55** is transformed by olefin isomerization into the isomers **56** and **57**, of which the 3*E*-olefin **56** was the main product. This reaction can take place spontaneously or be enzyme catalyzed.^[39] In bacilysin biosynthesis, BacB accelerates the conversion of the endocyclic diene **55** into the exocyclic diene **56**. While the *sal* gene cluster does not harbor a *bacB* homolog, we suspect that *salZ*, which is homologous to *phzF* in phenazine biosynthesis, might serve a similar function. While the remainder of the cyclohexenylalanine pathway has not yet been characterized, we suspect that *salW*, which encodes a PLP-dependent aminotransferase, may catalyze the installation of the amino group in **4**. Finally, the gene *salD* codes for a cytochrome P450 hydroxylase, which installs the β -hydroxyl group in hydroxycyclohexenylalanine **58**.^[38]

The close structural similarity between **4** and **10** pointed to a common biosynthetic pathway for these compounds. Therefore, we tested the production of volatiles **8–12** in *salX* and *salD* *S. tropica* knockout mutants, as SalX catalyzes the first committed step in cyclohexenylalanine biosynthesis and SalD catalyzes a post cyclohexenylalanine assembly modifying hydroxylation reaction.^[38] Consistent with our proposal, we did not observe any volatile cyclohexenyl compounds in the headspace of the *salX* mutant (Figure 4), thereby correlating the cyclohexenyl analogues with cyclohexenylalanine biosynthesis. In contrast, the *salD* mutant maintained, and in fact, had increased production of compounds **8–12** (Figure 4). This observation was consistent with prior measurements of decreased overall salinosporamide production levels of the deoxy analogue salinosporamide J,^[38] suggesting that the absence of the SalD hydroxylase results in the accumulation of cyclohexenylalanine-derived volatiles.

From these results we postulate the biosynthetic pathway shown in Scheme 3. The volatile compounds **11/12** may arise from the diene acids **61/62** by enzymatic oxidative decarboxylation and SAM methylation. The next step towards the biosynthesis of **4** would be the regioselective reduction of **61/62** to the monoene-acid **60**. This acid can serve as a precursor of the volatile shunt product **10**, formed by similar steps as mentioned for **11/12**: oxidative decarboxylation to **44** and SAM methylation. Reduction of acid **44** leads to the minor alcohol **13**. The compounds **8** and **9** are products of oxidative degradation of the side chain of **44** via **59** and **63**, followed by methylation or reduction (Scheme 3). The intermediate **60** is also the substrate for transamination to form **4**.

As the acids **60–62** may be important biosynthetic intermediates in the proposed cyclohexenylalanine pathway, we next sought to explore their intermediacy. To do so, we synthesized and supplemented them to the *salX* mutant, which blocks the first committed step in cyclohexenylalanine biosynthesis, to restore the production of both volatiles **8–12** and salinosporamide A.

The syntheses of all three compounds started from bromide **43**, which was converted in a Grignard reaction with diethyl oxalate into the α -keto ester **66** with 95% yield.^[40] Saponification with aqueous potassium hydroxide furnished acid **60**. According to Miller *et al.*,^[41] reaction of α -ketoester **66** with copper(II)bromide should result in the direct formation of the desired double bond in **68/69**. However, only bromide **67** was formed in our hands. Elimination with 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) yielded the isomers

68/69 in a 1:1 *E/Z* ratio. Saponification of the ethyl esters led to isomers **64** and **65**. The free acids proved to be unstable, although analysis of their more stable sodium salts by HPLC/MS confirmed their formation. To circumvent problems occurring during feeding experiments with the unstable acids, the ethyl esters **68/69** were evaluated as ethyl esters are readily incorporated and hydrolyzed *in vivo*.

Synthetic **66** and **68/69** were then fed to liquid cultures of *salX S. tropica* in a concentration of 1 mmol/L (Figure 5). We assumed that the production of methyl phenylacetate (**7**) would remain approximately constant during the feeding experiments, because the previous experiment had shown no influence on its formation. In addition, feeding of ²H₅-phenylalanine furnished ²H₅-labeled 2-phenylethanol (**6**), methyl benzoate (**5**) and **7**, but unlabeled **8–12**, confirming that there is no biosynthetic link between the cyclohexenyl compounds and phenylalanine metabolism.^[20] Therefore, the chromatograms were normalized to compound **7**.

Gratifyingly, the production of the volatiles **8–12** could be restored by feeding of **66** or **68/69** to the *salX* mutant. Both the diene acids and the monoene acid produce mixtures of diene esters **11** and **12** and the monoene ester **10**. This result confirmed the acids as intermediates in the biosynthesis of the volatiles **10–12**. Moreover, we analyzed the cell extract and confirmed the restoration of salinosporamide A production, thereby establishing that **66** is indeed a biosynthetic intermediate to **52** (SI). While the administration of **66** to *salX* resulted mainly in the production of **10**, **68/69** conversely furnished an equal amount of **10** and the *E/Z* isomers **11** and **12**, supporting the reduction of isomers **61/62** to **60** (Scheme 3). The later step may also be reversible, because small amounts of **11/12** are also formed after administration of **60**. Lastly, feeding of [²H₃]-methionine produced [²H₃]-methyl esters **5**, **7**, and **10–12** labeled at the methyl group, proving the origin of the methyl group.

Conclusion

We show here for the first time that marine actinomycete bacteria occurring in the deep ocean sediments can be prolific producers of volatiles. In addition to a range of volatiles common amongst terrestrial actinomycetes, we showed that *Salinispora tropica* produces rare compounds such as spiroacetals and a series of novel cyclohexenyl compounds. The biosynthesis of compounds **8–12** is closely coupled to that of salinosporamide A's distinct nonproteinogenic amino acid **4**. Analysis of knockout mutants of salinosporamide A biosynthetic genes revealed that the biosynthesis of **4** and the volatile compounds **8–12** are in fact coupled, and the α-oxo-acids **60–62** are important biosynthetic intermediates. We are further investigating the biosynthesis of **4** by establishing the suspected roles of the genes *salV*, *salW*, *salY*, and *salZ* and plan to use the volatile cyclohexenyl chemistry as a diagnostic tool in establishing the complete metabolic pathway to this unique nonproteinogenic amino acid.

Experimental Section

See the Supporting Information.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Total ion chromatogram of a headspace extract of a five day liquid culture of *S. tropica* CNB-440.

Figure 2.

EI-mass spectra of A) cyclohex-2-enylmethanol (**a**, **8**), B) methyl cyclohex-2-ene-1-carboxylate (**b**, **9**), C) methyl 2-(cyclohex-2-en-1-yl)acetate (**c**, **10**), and D) methyl (*E*)- and methyl (*Z*)-2-(cyclohex-2-en-1-ylidene)acetate (**d/e**, **11/12**).

Figure 3.

A) Organization of the salinosporamide A (**3**) gene cluster. B) Proposed mechanism of the biosynthesis of **3**. Corresponding genes for the biosynthesis of the building blocks have the same color. Not assigned genes are shown in black.^[35]

Figure 4.

Total ion chromatograms of the wild type of *S. tropica* and its knockout mutants *salX* and *salD*; cyclohexenyl compounds are marked by an asterisk. The TIC traces are normalized to compound 7. The *salD* trace is reduced by a factor of five to show accumulation of target compounds.

Figure 5.

Total ion chromatograms of the volatiles of the knockout mutants *salX*, after feeding 1mM of A) monoene ester **66**, and B) diene acids **68/69**. Cyclohexenyl compounds are noted by an asterisk. Chromatograms were normalized to methyl phenylacetate (**7**). Chromatograms is zoomed by a factor of four for better visibility.

Scheme 1. Structures of volatiles identified in *S. tropica* CNB-440.

Scheme 2.

a) Synthesis of cyclohex-2-enylmethanol (**a**, **8**), and methyl 2-(cyclohex-2-en-1-yl)acetate (**c**, **10**), b) synthesis of methyl cyclohex-2-ene-1-carboxylate (**b**, **9**), and c) synthesis of methyl (*E*)- and methyl (*Z*)-2-(cyclohex-2-en-1-ylidene)acetate (**d/e**, **11/12**).

Scheme 3.

Proposed biosynthetic pathway to PCP-bound β -hydroxycyclohexenylanine **52** and the volatile cyclohexenyl compounds **8–13**.

Scheme 4.
Synthesis of biosynthetic intermediates.

Table 1

Volatile compounds identified in the headspace of *S. tropica* CNB-440. The amounts of the compounds are given as 0–1% (o), 1–10% (x), 10–30% (xx), 30–100% (xxx) relative to the largest peak area in the total ion chromatogram. The identification (Ident.) of the compound based on comparison of the mass spectrum to databases (ms), comparison of retention index to a published retention index on the same or similar GC phase (ri),^[26] and/or based on comparison to a synthetic or commercially available reference compound (std). I_{exp} : experimental gas chromatographic retention index; I_{lit} : literature values of the gas chromatographic retention index.

	compound ^a	I_{exp}	I_{lit}^c	Ident.	<i>S. tropica</i>
1	3-methyl-2-butene-1-ol (14)	n.d. ^b		ms	o
2	methyl 3-methylbutanoate (17)	n.d.		ms	x
3	cyclopentanone (37)	802	808	ms, ri, std	x
4	2-hydroxypentan-3-one (28)	805	803	ms, ri, std	x
5	3-hydroxypentan-2-one (29)	811	818	ms, ri, std	x
6	isobutyric acid (15)	818	795	ms, ri	x
7	4-hydroxy-4-methyl-2-pentanone (27)	846	851	ms, ri	o
8	hexan-1-ol (20)	868	867	ms, ri, std	o
9	methyl 4-methylpentanoate (18)	893		ms	x
10	3-methylbutanoic acid (16)	896	894	ms, ri	o
11	4-butanolide (30)	915	915	ms, ri, std	x
12	(<i>E</i>)-methyl 4-methylpent-2-enoate (19)	933		ms	o
13	(<i>E</i>)-methyl 4-methylpent-3-enoate (20)	943		ms	o
14	4-pentanolide (31)	955	956	ms, ri, std	o
15	3-methyl-4-butanolide (32)	960	974	ms, ri, std	o
16	dimethyl trisulfide (34)	966	970	ms, ri, std	o
17	methyl 2-furoate (25)	979	983	ms, ri, std	o
18	4-methylpent-3-enoic acid (21)	985	980	ms, ri, std	x
19	methyl 4-methylhexanoate (22)	999	1001	ms, ri, std	o
20	cyclohept-4-enone (37)	1006	1004	ms, ri, std	o
21	cyclohex-2-enylmethanol (a, 8)	1011	1011	ms, std	xx
22	methyl 3-hydroxypentanoate (24)	1026		ms	o
23	1,6-dioxaspiro[4.5]decane (35)	1040		ms	o
24	methyl cyclohex-2-ene-1-carboxylate (b, 9)	1073	1073	ms, std	xx

compound ^a	<i>I</i> _{exp}	<i>I</i> _{lit} ^c	Ident.	<i>S. tropica</i>
25 unknown 3-ketomethyl ester	1080			o
26 methyl benzoate (5)	1090	1091	ms, ri, std	xx
27 2-phenylethanol (6)	1108	1109	ms, ri, std	xx
28 2-cyclohexen-1-ethanol (13)	1120		ms, std	o
29 1,7-dioxaspiro[5.5]undecane (36)	1124		ms	o
30 unknown methyl 3-hydroxyalkanoate	1127			o
31 methyl 2-(cyclohex-2-en-1-yl)acetate (c , 10)	1160	1160	ms, std	xxx
32 methyl 2-phenylacetate (7)	1173	1177	ms, ri, std	xxx
33 benzothiazole (39)	1215	1218	ms, ri, std	o
34 methyl (<i>E</i>)-2-(cyclohex-2-en-1-yliden)acetate (d , 11)	1253	1253	ms, std	xxx
35 methyl (<i>Z</i>)-2-(cyclohex-2-en-1-yliden)acetate (e , 12)	1274	1274	ms, std	xx
36 (<i>E</i>)-methyl 3,7-dimethylocta-2,6-dienoate (23)	1323	1323	ms, ri	o
37 diethyl 2-(but-3-enyl)malonate (40)	1334	1339	ms, ri, std	o
38 benzophenone (41)	1623	1625	ms, ri, std	o

^a artifacts found in both control and inoculated samples are not mentioned,

^b not determined,

^c data from NIST Chemistry WebBook[26] or our own compound database