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The Scent of Bacteria: Headspace Analysis for the Discovery of Natural Products

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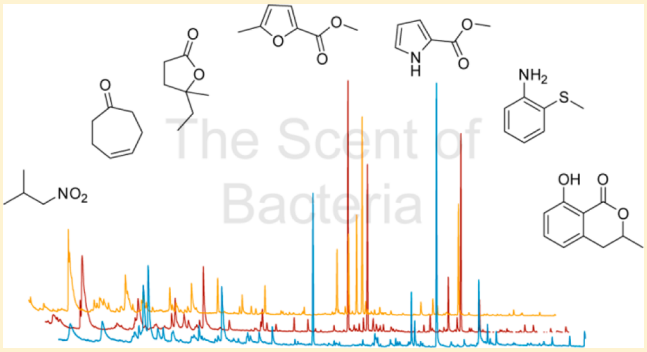
Supporting Information

ABSTRACT: Volatile compounds released by 50 bacterial strains, 45 of them actinobacteria in addition to three chloroflexi and two myxobacteria, have been collected by use of a closed-loop stripping apparatus, and the obtained headspace extracts have been analyzed by GC-MS. Excluding terpenes that have recently been published elsewhere, 254 compounds from all kinds of compound classes have been identified. For unambiguous compound identification several reference compounds have been synthesized. Among the detected volatiles 12 new natural products have been found, in addition to mellein, which was released by *Saccharopolyspora erythraea*. The iterative PKS for this compound has recently been identified by in vitro experiments, but mellein production in *S. erythraea* has never been reported before. These examples demonstrate that headspace analysis is an important tool for the discovery of natural products that may be overlooked using conventional techniques. The method is also useful for feeding experiments with isotopically labeled precursors and was applied to investigate the biosynthesis of the unusual nitrogen compound 1-nitro-2-methylpropane, which arises from valine. Furthermore, several streptomycetes emitted compounds that were previously recognized as insect pheromones, thus questioning if bacterial symbionts are involved in insect communication.



Bacteria, especially actinomycetes, are prolific producers of various secondary metabolites including polyketides, nonribosomal peptides, terpenoids, alkaloids, and lipids. The traditional method of screening for new natural products starts with a fermentation followed by culture extraction and compound purification. During concentration steps volatile secondary metabolites are easily lost and, therefore, have frequently been overlooked.

An approach for the discovery of volatile secondary metabolites requires specialized headspace techniques such as the closed-loop stripping apparatus (CLSA).[1,2] Using this method we have recently identified a large number of terpenes emitted by bacteria, most of them actinomycetes, known to encode terpene cyclases.[3] Only a few other reports exist on volatiles from actinomycetes,[4−13] and a general overview summarizing the accumulated knowledge about bacterial volatiles was published in 2007.[14] Following the survey on terpenoid bacterial volatiles,[3] here we report on the volatiles from other compound classes. A discussion of the significance of the analytical results with respect to the potential of finding new natural products or known natural products from previously unrecognized sources is given.

RESULTS AND DISCUSSION

The volatiles released by 50 bacterial strains, mainly actinomycetes together with three chloroflexi and two myxobacteria, were collected on charcoal using the CLSA headspace technique.[1,2] After a collection time of about one day the adsorbed volatiles were eluted with dichloromethane, and the extracts were analyzed by GC-MS. Each microorganism was investigated in at least four replicates to test for reproducibility. The species included in this study are summarized in Table 1 of the Supporting Information (SI), the identified compounds are listed in Table 2 of the SI, and one representative chromatogram of each investigated species is shown in Figure 1 of the SI. Compound identification was performed by the following approaches: (a) comparison of the mass spectrum to electronic mass spectral libraries,[15] (b) comparison of the retention index to tabulated literature data,[15] (c) comparison with a commercially available authentic sample, (d) chemical synthesis of a reference compound.

A total of 254 volatile compounds from various classes were identified. Some of these compounds were almost ubiquitously present, whereas others occurred in only one or a few species. Each compound class will be discussed separately in the following sections. A detailed description of the terpenoids that make up the largest and struturally most diverse class of volatiles from actinomycetes with more than 150 different compounds identified in 35 strains was recently published elsewhere.[3]

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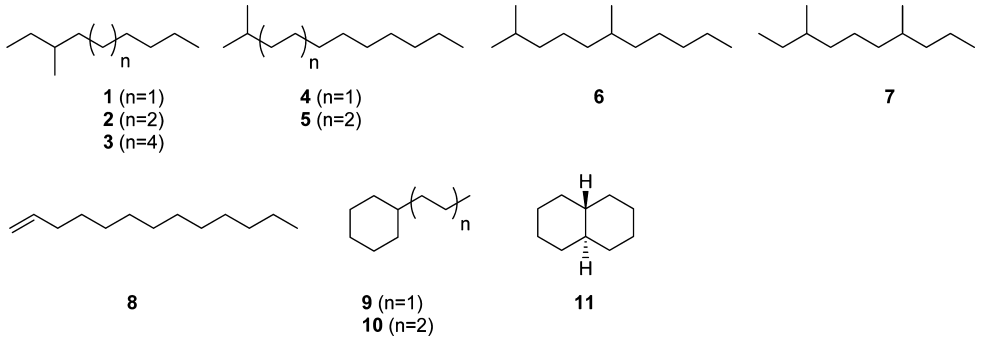

Figure 1. Hydrocarbons.

Hydrocarbons. The hydrocarbons identified in this study include unbranched and methyl-branched alkanes, alkenes, and cycloalkanes (Figure 1). The methyl-branched compounds 3-methylnonane (**1**), 3-methyldecane (**2**), 2,6-dimethylundecane (**6**), and 3,7-dimethyldecane (**7**) were released by *Chitinophaga pinensis*, while 3-methyldodecane (**3**), 2-methylundecane (**4**), and 2-methyltridecane (**5**) were found in *Streptomyces griseoflavus* and tridec-1-ene (**8**) was identified as the main compound from *Streptomyces auratus*. This olefin has not been described previously as a volatile from a particular bacterium, but its shorter homologue undec-1-ene is well known from *Pseudomonas*.[16] The cyclic compounds propylcyclohexane (**9**), pentylcyclohexane (**10**), and *trans*-decalin (**11**) were produced by *C. pinensis*.

Alcohols. Primary alcohols predominated, but a few secondary alcohols, one cycloalkanol, and one diol were also found (Figure 2). Several species emitted unbranched alcohols

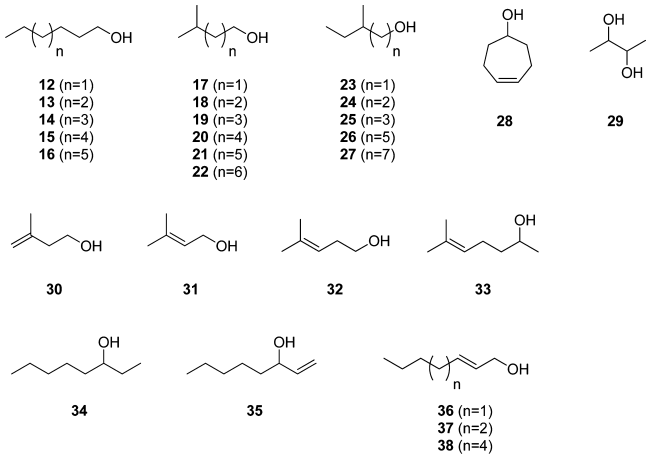

Figure 2. Alcohols.

ranging from hexanol (**12**) to decanol (**16**). The complete series was found in *Streptomyces parvulus*, while particularly large amounts of **12** were released by *Kitasatospora setae* and *Streptomyces avermitilis*. Furthermore, various ω-1 methyl-branched alcohols (**17**−**22**) and a series of odd ω-2 methyl-branched alcohols (**23**−**27**) were identified. This pattern is indicative of their formation from the corresponding branched amino acids valine, leucine, and isoleucine by transamination and oxidative decarboxylation, fatty acid chain elongation of the thus obtained starter units, and final reduction to the alcohols. This mechanism yields the complete series of ω-1 methyl-branched alcohols, but only the odd ω-2 methyl-branched compounds, whereas compound **24** may require one α-oxidation step for its biosynthesis.

Cyclohept-4-enol (**28**) was emitted by *Streptomyces sviceus*, while 2,3-butanediol (**29**) was found in several strains. This diol was previously described as a volatile from *Bacillus* and shown to promote growth and induce systemic resistance in *Arabidopsis thaliana*.[17,18] Several unsaturated methyl-branched alcohols (**30**−**33**) were detected. The compounds 3-methylbut-3-enol (**30**) and 3-methylbut-2-enol (**31**) represent the formal hydrolysis products of the terpene precursors isopentenyl diphosphate and dimethylallyl diphosphate, respectively, but alternative biosynthetic origins, e.g., from leucine, may also be possible. Octan-3-ol (**34**) and oct-1-en-3-ol (**35**) are known as fungal volatiles comprising the typical fungal smell.[19] The isomer (*E*)-oct-2-en-1-ol (**36**) and its higher homologues **37** and **38** were also occasionally found.

Aldehydes and Ketones. Several aldehydes and ketones were identified, in addition to hydroxyketones, diketones, and a few cyclic ketones (Figure 3). The aldehydes ranging from hexanal (**39**) to tetradecanal (**47**) were widespread, with nearly the complete series present in *C. pinensis* and *Herpetosiphon aurantiacus*. The α,β-unsaturated analogues **48**−**50**, prenal (**51**), and the bis-unsaturated (*E*,*E*)-nona-2,4-dienal (**52**) occurred less often. Furthermore, methyl ketones (**53**−**62**) and ethyl ketones (**63**−**66**) were identified that can arise via fatty acid biosynthesis from β-keto acids or, in the case of incorporation of a terminal methylmalonyl-CoA unit (CoA = coenzyme A), from α-methyl-β-keto acids, respectively, by decarboxylation.[2,20] Interestingly, the compound 3-ethylpentan-2-one (**61**) derived from an internal ethylmalonyl-CoA elongation unit was emitted by *Streptomyces bottropensis*. We have recently also identified a series of blastmycinone derivatives with internally ethyl branched *O*-acyl moieties,[4] thus reflecting the biosynthetic potential of this particular microorganism.

In *K. setae* the typical fungal metabolites[19] octan-3-one (**64**) and oct-1-en-3-one (**67**) occur together with the related alcohols **34** and **37**. In addition, a variety of α-hydroxyketones (**68**−**75**) together with the diones **76** and 77 were found. The hydroxyketones were widespread and are known as precursors of alkylated pyrazines in *Corynebacterium glutamicum*.[5] Among cyclic ketones cyclopentanone and α,β-unsaturated cyclopentenone derivatives **78**−**82** were identified. The trisubstituted 2,3,5-trimethylcyclopent-2-enone (**82**) is released in large quantities by *Streptomyces coelicolor* and is a new natural product. The structurally related 2,3-dimethyl-5-methylenecyclopent-2-enone has previously been reported from *S. coelicolor* as a decarboxylation product of methylenomycin C.[21] Similarly, **82** can also be regarded as a shunt product of methylenomycin

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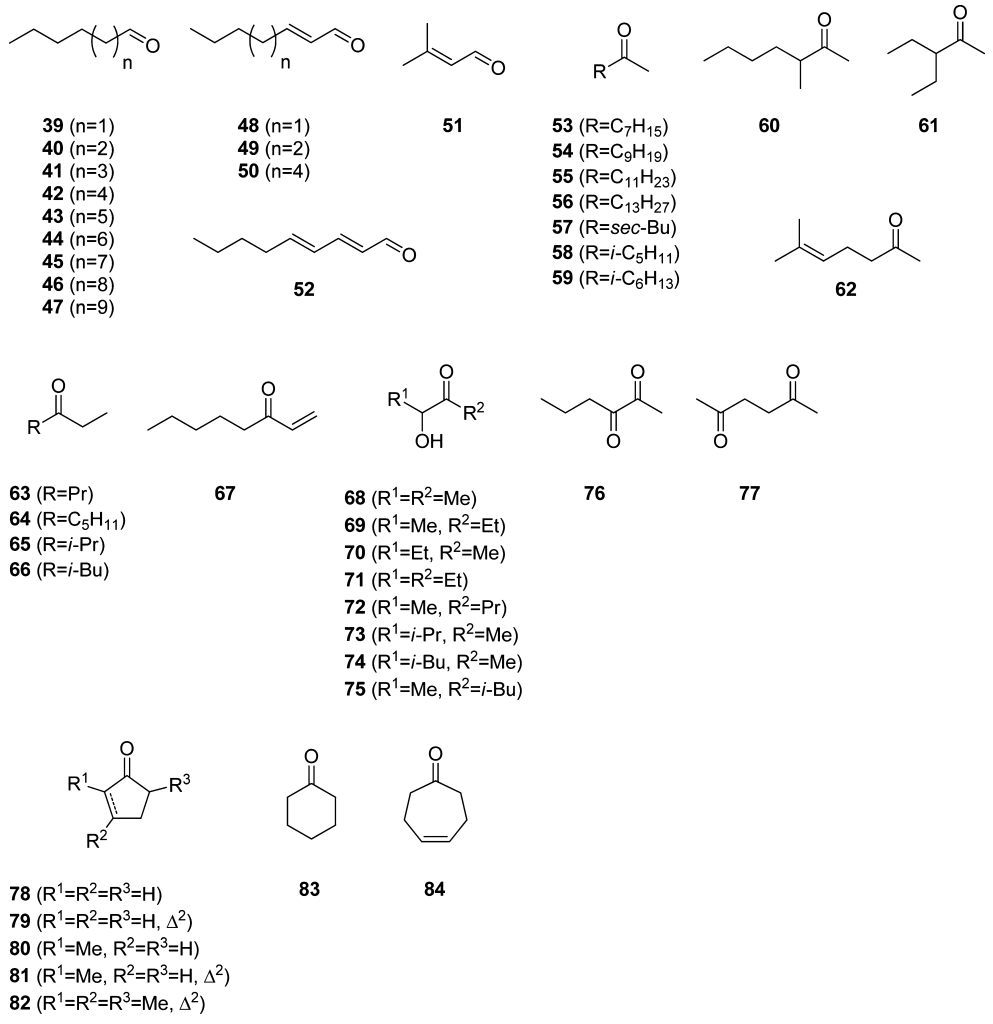

Figure 3. Aldehydes and ketones.

biosynthesis. Cyclohexanone (**83**) and cyclohept-4-enone (**84**) were also occasionally found. Compound **84** is related to the alcohol **28**, and both compounds may originate via the phenylacetate catabolon as previously suggested for tropone.[22] Neither the alcohol **28** nor the accompanying ketone **84** has been reported from any organism before.

Carboxylic Acids and Esters. Two previous studies on the volatiles released by *Micromonospora aurantiaca* and *Chitinophaga* have revealed that bacteria can produce a large diversity of volatile fatty acids and fatty acid methyl esters.[8,23] During the course of this study the carboxylic acids 3-methylbutyric acid (**85**) and 2-methylbutyric acid (**86**) were identified in *C. pinensis* and *H. aurantiacus*, whereas various methyl esters such as the branched compounds **87**−**97** were found within the whole set of strains (Figure 4). Unsaturated esters included the compounds **98**−**102** and methyl 2,4-dimethylhexa-2,4-dienoate (**103**), which has previously been described from the fungus *Fusarium fujikuroi*.[24] The unsaturated esters **98** and **99** likely originate from leucine and isoleucine by transamination, oxidative decarboxylation, FAD-dependent introduction of the olefinic double bond, and methylation of the corresponding free acid. In addition, the α- and β-hydroxy esters **104**−**109** occurred. Compounds **105**, **107**, and **108** also exhibit the leucine- and isoleucine-derived carbon backbones, respectively, and can be regarded as alternative precursors for **98** and **99**. Ethyl esters occurred less often and were represented by the compounds **110**−**112**. The identification of the methyl-branched compounds was based on the retention indices of these compounds, which were 40 units lower than for the unbranched isomers that were commercially available. This difference of retention indices is evident for a methyl branch in the ω-1 position, which can further be rationalized by the biosynthesis of these compounds from valine- and leucine-derived starter units, respectively.[8] Finally, the acetate esters **113**−**115** were identified. In most strains only one or a few esters were found, whereas a potpourri of several esters occurred in *Actinosynnema mirum*, *Stackebrandtia nassauensis*, and *Streptomyces violaceusniger*. While most esters were present only in traces, **87** was emitted as one of the main compounds by *S. auratus*, as was its isomer **90** by *Streptomyces lividans*. The volatiles **93** and **109** from *A. mirum* and **97** from *S. nassauensis* are new natural products.

Lactones. Lactones are well known cell-to-cell signaling molecules in *Streptomyces*, and as such, they control various processes including sporulation and antibiotic production.[25,26] The A-factor from *Streptomyces griseus* was discovered in the 1960s by Khokhlov and is a butanolide with a 2-acyl side chain and a 3-hydroxymethyl function (Figure 5).[27] The structurally related factor I from *Streptomyces viridochromogenes* and virginiae butanolide A (VB-A) from *Streptomyces virginiae* exhibit a hydroxylated 2-alkyl group with different stereochemistry at the hydroxy function.[28,29] The factor NFX-4 from

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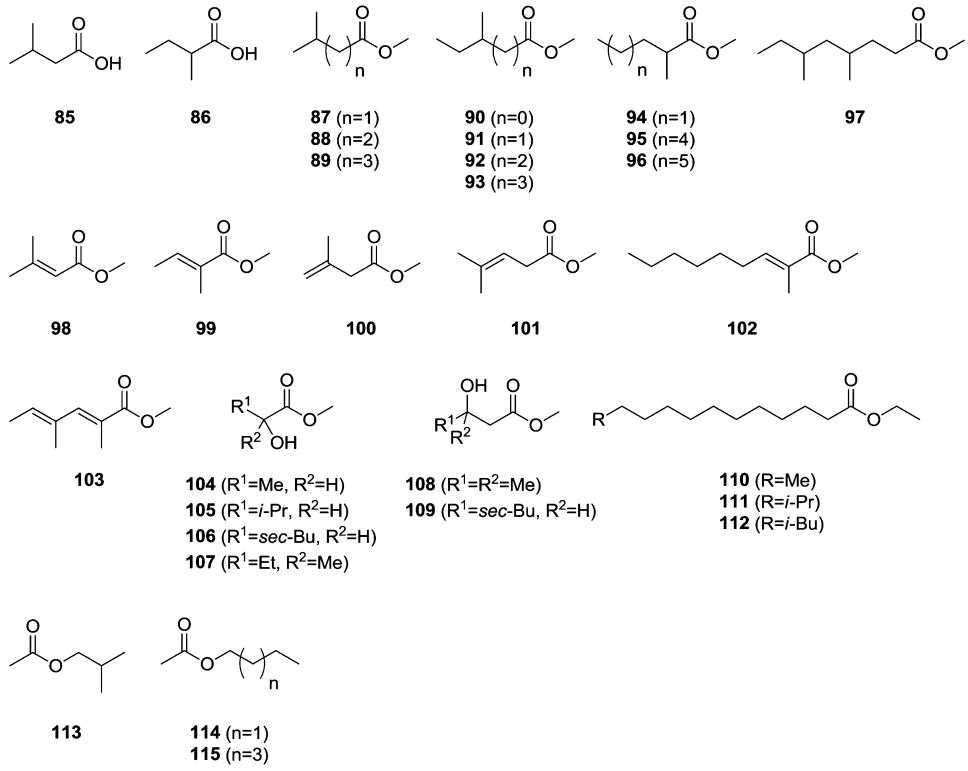

Figure 4. Carboxylic acids and esters.

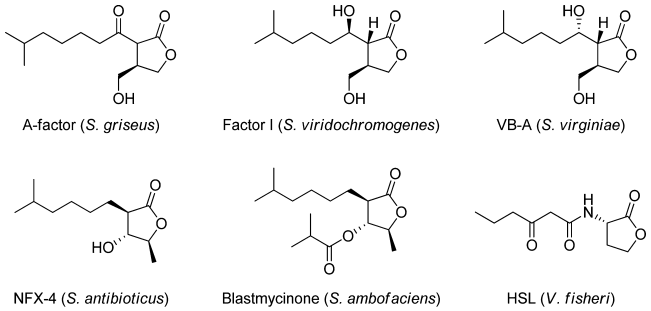

Figure 5. Lactone signaling compounds from bacteria.

Streptomyces antibioticus is a butanolide substituted with a 2-alkyl group and, in contrast to the other butanolides, has a 3-hydroxy function and a 4-methyl group.[30] This substitution pattern is also found in the blastmycinones, a large class of butanolides produced by *Streptomyces ambofaciens* and various other streptomycetes known as degradation products of the fungicidal antimycins.[4,31] Gram-negative bacteria use a wide range of species-specific *N*-acylhomoserine lactones, such as *N*-(3-oxohexanoyl)-L-homoserine lactone from *Vibrio fisheri*, for this purpose.[32,33] Although these molecules are biosynthetically different from the signaling factors in streptomycetes, the

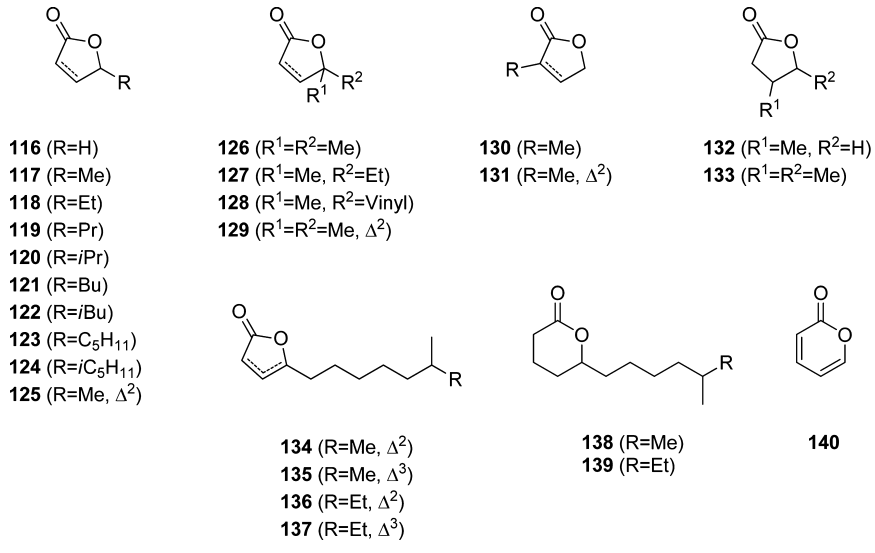

Figure 6. Lactones.

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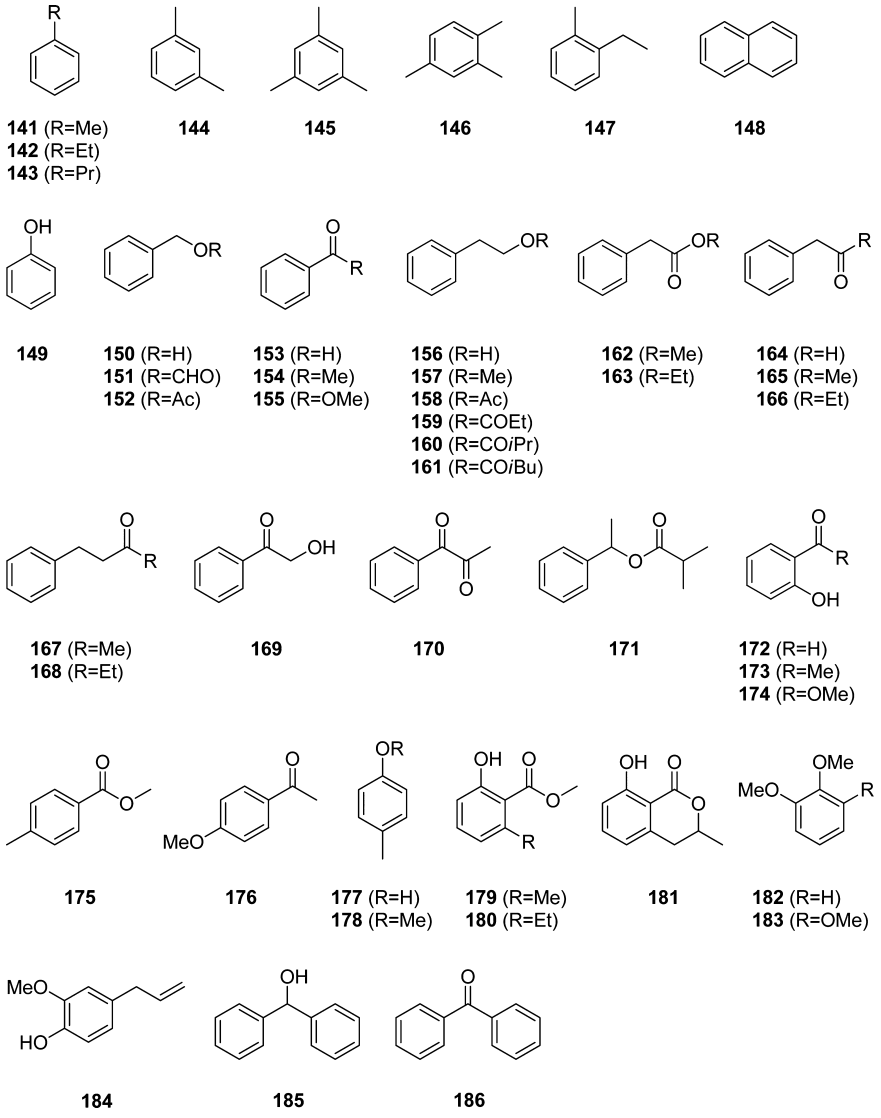

Figure 7. Aromatic compounds.

structural resemblence between these two compound classes is intriguing.

Volatile butenolides have previously been reported from streptomycetes by Gerber.[9] The headspace extracts of various actinomycetes included in this study also contained a large variety of butanolides and butenolides, while strains from other taxa did not release any lactones, suggesting that the substance class is characteristic for actinomycetes. Besides the unsubstituted pentan-4-olide (**117**) and pent-2-en-4-olide (**125**) and a series of 4-alkylated lactones, a few butanolides and butenolides with alkyl substitutents in the 2-position or with two alkyl substitutents were found (Figure 6). The compound 4-methylhex-5-en-4-olide (**128**) is also known as lavender lactone and contains a vinyl substituent. The α,β-unsaturated butenolides **134** and **136**, the corresponding β,γ-unsaturated butenolides **135** and **137**, and the pentanolides **138** and **139** have previously been reported from a marine streptomycete.[11] During the course of this study these lactones were detected in various streptomycetes (*S. albus*, *S. citreus*, *S. ghanaensis*, *S. griseus*, and *S. parvulus*). A retrobiosynthetic analysis of these compounds suggests that three are derived from valine (**134**, **135**, **138**) and three originate from isoleucine (**136**, **137**, **139**). Interestingly, neither in the present nor in our previous study[11] were the leucine-derived congeners found. Finally, 2*H*-pyran-2-one (**140**) occurred in *Thermomonospora curvata*. Some strains included in this study released only one or a few lactones. Particularly rich in lactones were the headspace extracts from *Saccharopolyspora erythraea*, *T. curvata*, and the streptomycetes *S. albus*, *S. ambofaciens*, *S. citreus*, *S. clavuligerus*, *S. griseus*, and *S. odorifer*. Since several butanolides are known to act as signaling compounds in streptomycetes, a similar role of the lactones identified here cannot be ruled out.

Aromatic Compounds. Aromatic compounds made up by far the largest class of volatiles. A series of aromatic hydrocarbons (**141**−**148**) was found (Figure 7). Particularly interesting is the occurrence of propylbenzene (**143**) together with its saturated counterpart propylcyclohexane (**9**) in *C. pinensis*. Compound **143** may either be biosynthetically derived from **9** or, more likely, be its biosynthetic precursor, because the biosynthesis of both compounds from phenylalanine is the easiest explanation. However, pentylbenzene, which would similarly be the precursor of (or derived from) pentylcyclohexane (**10**), was not detected. Mesitylene (**145**) has previously been described as a natural product that originates in the light-

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induced polyene splicing of the polyketide neoaureothin to orinocin.[34]

Besides these hydrocarbons various oxygenated aromatic compounds were found. Phenol (**149**) was emitted only by *S. violaceusniger*, whereas benzyl alcohol (**150**) occurred in several strains. Its formate ester **151** was only present in *Streptomyces* sp. Tü 6071, and benzyl acetate (**152**) was released by *S. erythraea* and *Streptomyces* sp. Tü 6071. Benzaldehyde (**153**), acetophenone (**154**), and methyl benzoate (**155**) were also found in various species, with **153** and **154** being the main constituents of the headspace extracts from *C. pinensis* and *S. nassauensis*. One of the most widespread bacterial volatiles is 2-phenylethanol (**156**),[14] which was identified in 42 of the 50 strains investigated in this study and has been previously reported from several actinomycetes,[6,8,11−13] myxobacteria,[2,33,35] and enterobacteria.[36,37] In contrast, its derivatives such as the methyl ether **157** and the esters **158**−**161** occurred less frequently. The biosynthesis of **156** can be rationalized from phenylalanine by transamination to phenylpyruvate and subsequent decarboxylation to phenylacetaldehyde (**164**), which is found in *S. erythraea* and *S. ambofaciens*. A final reduction of **164** yields **156**. Oxidation of **164** may yield phenylacetic acid as precursor of the esters **162** and **163**, which were both identified in a few strains. Phenylacetone (**165**), present in headspace extracts of *S. erythraea* and both strains of *Streptomyces filamentosus*, may be generated by a two-carbon elongation of phenylacetyl-CoA with malonyl-CoA and subsequent decarboxylation of the free β-keto acid. A similar reaction sequence with methylmalonyl-CoA furnishes 1-phenylbutan-2-one (**166**), which is released by *S. nassauensis*. The related compounds 4-phenylbutan-2-one (**167**) and 1-phenylpentan-3-one (**168**) were detected in *Rubrobacter xylanophilus*. Their formation can be rationalized from phenylpropanoyl-CoA by elongation with either malonyl-CoA or methylmalonyl-CoA, respectively, and decarboxylation of the resulting free β-keto acid. The ketone **167** is known from the myxobacterium *Chondromyces crocatus*,[35] while **168** has not been reported from bacteria before. Compounds with a rather unusual oxidation pattern were 2-hydroxy-1-phenylethanone (**169**) from *T. curvata*, 1-phenylpropane-1,2-dione (**170**) from *S. erythraea*, and 1-phenylethyl isobutanoate (**171**) from *S. ambofaciens*.

Some strains produced derivatives of salicylic acid that can arise from the shikimate pathway intermediate chorismic acid. Salicylaldehyde (**172**) was present in *H. aurantiacus* and *T. curvata*, *o*-hydroxyacetophenone (**173**) was found in *R. xylanophilus*, and methyl salicylate (**174**) was produced by five strains. Several further compounds identified in this study occurred only in one or very few strains. These are methyl *p*-toluate (**175**), 4-methoxyacetophenone (**176**), *p*-cresol (**177**), *p*-methylanisol (**178**), methyl 6-methylsalicylate (**179**), methyl 6-ethylsalicylate (**180**), mellein (**181**), veratrole (**182**), 1,2,3-trimethoxybenzene (**183**), eugenol (**184**), diphenylmethanol (**185**), and benzophenone (**187**).

Particularly interesting is the common occurrence of mellein and the two structurally related methyl esters **179** and **180** in *S. erythraea*. Mellein was first recognized in the fungus *Aspergillus melleus* in 1950 by Burton.[38] In ants mellein and methyl 6-methylsalicylate function as alarm or trail pheromones, and in some species both metabolites occur as mixtures.[39−42] In addition, mellein is known from the giant danaine butterfly *Idea leuconoe*, where it acts as a male sex pheromone,[43] and methyl 6-methylsalicylate is a female sex pheromone from the wasp *Spalangia endius*.[44] Both methyl esters **179** and **180** are also known as constituents of the defensive secretions from the beetle *Chrysopeplus expolitus*.[45] The occasional co-occurrence of mellein and methyl 6-methylsalicylate or of methyl 6-methylsalicylate and methyl 6-ethylsalicylate in one species supports their common biosynthesis. Results from feeding experiments with isotopically labeled acetate were in agreement with a polyketide origin of **181** and the free carboxylic acid of **179**, 6-methylsalicylic acid.[46] Recently the iterative polyketide synthase (PKS) for mellein in *S. erythraea* encoded by SACE5532 (or *pks8*) has been identified by in vitro experiments with the recombinant enzyme,[47] but so far mellein production by *S. erythraea* has never been reported. Therefore, our observations confirm that mellein is indeed produced by *S. erythraea*. The high sensitivity of our analytical method furthermore enables the detection of potential side products of the mellein PKS. The biosynthesis of mellein, **179**, and **180** by one and the same PKS would be explainable by slight modifications of a common mechanism: the incorporation of one acetyl-CoA starter and three malonyl-CoA extension units leads to **179**, **180** is made using a propionyl-CoA starter unit and three malonyl-CoA units, and **181** requires an acetyl-CoA starter and four malonyl-CoA elongation units, but formation of the aromatic ring is mechanistically the same for all three compounds. A gene knockout experiment is currently underway to investigate whether production of **179** and **180** also depends on the mellein PKS.

Furans. Furans were detected in several of the investigated strains. Furfuryl formate (**187**) was detected only in *S. clavuligerus*, whereas 2-acetylfuran (**188**) is one of the most widespread compounds of this class, which was emitted by 20 of the 50 strains and is the main volatile from *Haliangium ochraceum* (Figure 8). The compound 2-hexanoylfuran (**189**)

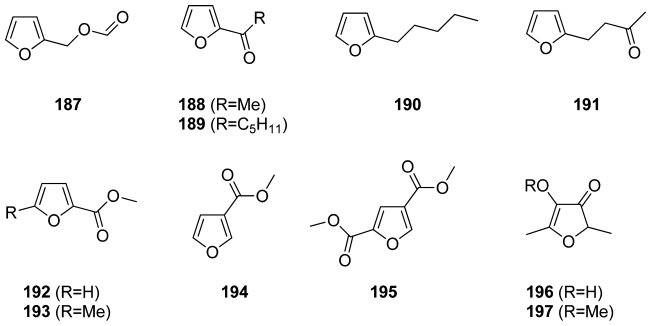

Figure 8. Furans.

was released by *S. bottropensis*, 2-pentylfuran (**190**) was identified in *S. avermitilis*, and the ketone 1-(2-furyl)butan-3-one (**191**) occurred in *S. filamentosus*. A series of methyl furancarboxylates was found including methyl 2-furoate (**192**), which was produced by several strains, whereas its isomer methyl 3-furoate (**194**) was limited to *S. griseoflavus*. Methyl 5-methyl-2-furoate (**193**) was detected only in *Streptomyces cattleya* and *S. griseoflavus*, and dimethyl furan-2,4-dioate (**195**) was present in the extracts from *S. griseoflavus* and *S. parvulus*. The compounds **192** and **195** have also been reported from other streptomycetes,[13] but compound identification was based only on their mass spectra and not on comparison to authentic samples. Since the mass spectra of isomers such as **192** and **194**, or **195** and the 2,3-, 2,5-, and 3,4-disubstituted analogues, are very similar, compound identification in this earlier report

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remained tentative. Therefore, for unambiguous compound identification all possible regioisomers of these furan derivatives were obtained by synthesis (cf. below) or from commercial suppliers. The furanone **196** and the corresponding methylated derivative **197** were identified in *S. clavuligerus*. The furan **193** has not been reported as a natural product before.

Sulfur Compounds. A structurally diverse array of sulfur volatiles was identified (Figure 9), with the headspace extracts

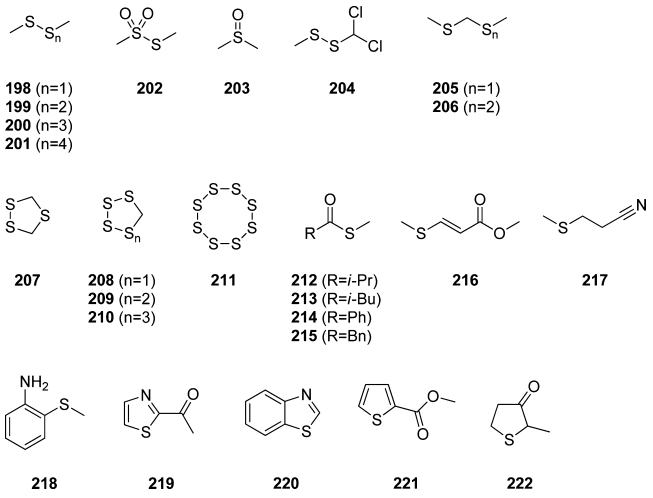

Figure 9. Sulfur compounds.

from *R. xylanophilus* and *S. bottropensis* being particularly rich in these compounds. The most widespread sulfur compounds were the dimethyl polysulfides ranging from dimethyl disulfide (**198**) to dimethyl pentasulfide (**201**). The oxidation product of **198**, *S*-methyl methanethiosulfonate (**202**), was also present in various samples, while the formal oxidation product of dimethyl sulfide, dimethyl sulfoxide (**203**), was detected only in *S. auratus*. The chlorinated sulfur volatile dichloromethyl methyl disulfide (**204**) was tentatively identified from its mass spectrum and was found in *R. xylanophilus*, *S. griseus*, and *Streptomyces pristinaespiralis*. This compound may arise by chlorination of **198** and represents a new natural product. Further dialkyl (poly)sulfides were 2,4-dithiapentane (**205**) from *S. bottropensis* and *S. griseoflavus* and methyl methylthiomethyl disulfide (**206**).

Cyclic polysulfides included 1,2,4-trithiolane (**207**) from *S. filamentosus* and tetrathiolane (**208**), pentathiane (**209**), and hexathiepan (**210**) from *S. bottropensis*. Several sulfur heterocycles including **207** are known from Shiitake mushrooms (*Lentinus edodes*),[48] while **208** and **209** have not been reported from natural sources before. Cyclooctasulfur (**211**) was also present in a few headspace extracts.

Several methyl alkanethioates (**212**–**215**) were occasionally found. The two volatiles methyl 3-(methylsulfanyl)prop-2-enoate (**216**) emitted by *S. nassauensis* and *S. griseoflavus* and 3-(methylsulfanyl)propionitrile (**217**) released by *S. clavuligerus* are probably methionine-derived. The compound **218** from *S. bottropensis* was unambiguously identified as 2-(methylsulfanyl)aniline by comparison to all three possible regioisomers and is a new natural product. This metabolite has been discussed as a degradation product of benzothiazole (**220**),[49] which was, however, not emitted by *S. bottropensis*, but only by a few other strains. The thiazole derivative acetylthiazole (**219**) also occurred in some bacterial bouquets. The new natural product thiophene-2-carboxylate (**221**) is the sulfur analogue of the furan derivative **192** and was found in *S. nassauensis*. The compound 2-methyltetrahydrothiophen-3-one (**222**) is derived from homocysteine and pyruvate[50] and was detected in *Saccharopolyspora spinosa*.

Nitrogen Compounds. Several alkylated pyrazines (**223**–**235**) were identified (Figure 10). Some of these pyrazines such as 2,5-dimethylpyrazine (**226**) were particularly widespread, whereas others occurred in only one or a few samples, e.g., 2-ethyl-3,5-dimethylpyrazine (**233**) from *Streptomyces* sp. Tü 6071. The biosynthesis of alkylated pyrazines has recently been investigated in our laboratories and proceeds via acetoin and its derivatives.[5] For this reason acetoins (cf. the discussion of aldehydes and ketones above) and pyrazines frequently co-

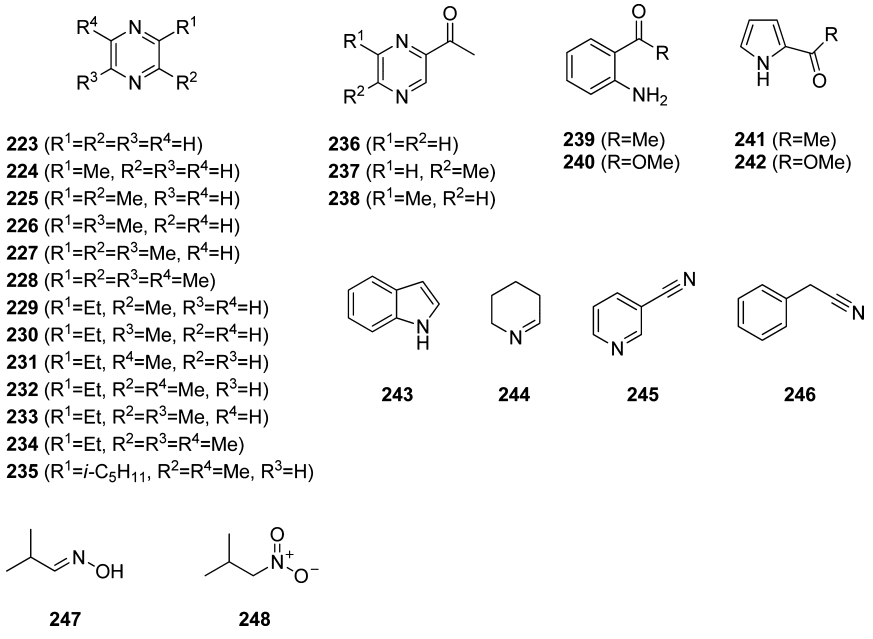

Figure 10. Nitrogen compounds.

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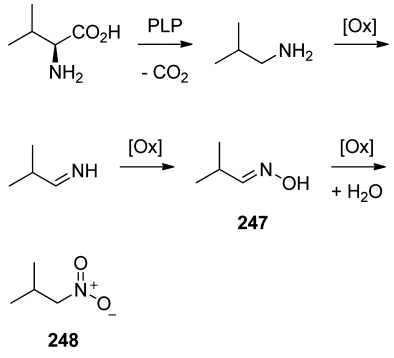
occur in one species. Furthermore, a few acetylpyrazines were identified including the stem compound acetylpyrazine (**236**), 2-acetyl-5-methylpyrazine (**237**), and 2-acetyl-6-methylpyrazine (**238**), which are all produced by *T. curvata*.

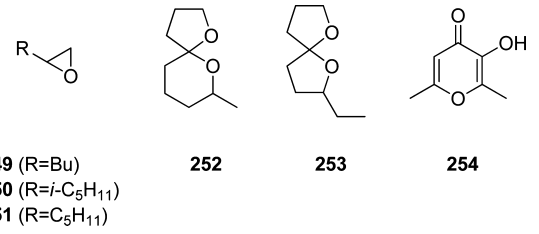
Two derivatives of anthranilic acid were released by a few species. These were identified as 2-aminoacetophenone (**239**) and methyl anthranilate (**240**), the latter being one of the main components from *Haliangium ochraceum*. The pyrrole derivative 2-acetylpyrrole (**241**) was found in *S. erythraea*, *S. pristinaespiralis*, and *T. curvata*, while methyl pyrrole-2-carboxylate (**242**) completed the series of structurally related five-membered heterocycles in *S. nassauensis*, in which the respective furan and thiophene derivatives (**194** and **221**) were also found (cf. above). The compound **242** is known as a trail pheromone of the ant *Metapone madagascaria*.[51] Indole (**243**) was present in the extracts of three species, while tetrahydropyridine (**244**) was a headspace constituent of *T. curvata* and is known to be generated from lysine via cadaverine. It serves as a reactive building block in the biosynthesis of various piperidine alkaloids such as isopelletierine and anabasine from *Nicotiana*.[52] However, no piperidine alkaloid is known from *T. curvata* that could rationalize the occurrence of **244** in this species. The cyanides nicotinonitrile (3-cyanopyridine, **245**) and phenylacetonitrile (**246**) were found in *T. curvata* and *A. mirum*, respectively. Finally, *S. viridochromogenes* released isobutylaldoxime (**247**) and 1-nitro-2-methylpropane (**248**), which are structurally and biosynthetically related. The biosynthesis of the unusual volatiles **247** and **248** was investigated in a feeding experiment with isotopically labeled [$^2H_8$]valine in which a maximum incorporation of seven to eight deuterium atoms with a ratio of ca. 1:1 into both compounds was observed by GC-MS (Figure 2 of the SI). The delineated biosynthetic pathway (Scheme 1) starts with the

Scheme 1



pyridoxal phosphate-dependent decarboxylation of valine to isobutylamine followed by oxidations via the imine and the oxime **247** to the nitrite **248**. The oxidation of isobutylamine to the imine may proceed with low stereospecificity, thus resulting in the loss of either a proton or a deuterium in this step. An alternative pathway that cannot be distinguished on the basis of our feeding experiment is the oxidation of isobutylamine via *N*-isobutylhydroxylamine to the oxime **247**.

Other Compounds. A set of epoxides (**249**–**251**) were emitted by *S. spinosa* (Figure 11). Most interestingly, in several actinomycetes the spiroketals conophtorin (**252**) and chalcogran (**253**) were detected. In contrast to the two diastereoisomers of **252**, the diastereoisomers of **253** could be chromatographically separated, but without reference material



249 (R=Bu)
250 (R=*i*-C_5H_{11})
251 (R=C_5H_{11})
252 **253** **254**

Figure 11. Other compounds.

it was impossible to assign the structures of a distinct diastereoisomer. The spiroketal **252** is a repellant or antiaggregative pheromone of *Paravespula vulgaris*,[53] while **253** is an aggregation pheromone of the beetle *Pityogenes chalcographus*.[54] Finally, 6-methylmaltol (**254**) was identified in *S. clavuligerus*.

Synthesis of Reference Compounds. For unambiguous identification several reference compounds were synthesized. All synthetic compounds were identical to the natural products as discussed above and listed in Table 2 of the SI.

For the synthesis of cyclohept-4-enol (**28**) and the corresponding ketone **84** homoallylmagnesium bromide was reacted with ethyl formate (**255**) to yield the alcohol **256** (Scheme 2). Ring-closing metathesis with Grubbs' catalyst of the second generation resulted in **28**, while **84** was available from **256** by a sequence of oxidation with PCC followed by olefine metathesis.[55] The β-hydroxy esters **108** and **109** were prepared by the addition of the ester enolate of methyl acetate to acetone or 2-methylbutanal, respectively. In contrast, the α-hydroxy esters **105** and **107** were obtained by transformation of the commercially available carboxylic acids into the methyl esters using diazomethane. The lactones **126**–**128** were synthesized by the addition of the required Grignard reagent to ethyl levulinate (**263**),[56] while the addition to the ester function was avoided by careful control of the reaction conditions. The adducts cyclized spontaneously to the lactones during moderately acidic workup with ammonium chloride. Treatment of lactone **127** with phosphoric acid and P_2O_5 gave the ketone **262**,[57] which was methylated using LDA and MeI to yield **82**.

Mellein (**181**) and the biosynthetically related compounds methyl 6-methylsalicylate (**179**) and methyl 6-ethylsalicylate (**180**) were prepared from 2,6-dihydroxybenzoic acid (**263**, Scheme 3). The acid **263** was protected as acetonide **264**,[58] followed by conversion of the free phenol into the triflate **265**.[59] A Stille coupling with allyltributylstannane yielded **266**, which was deprotected with potassium hydroxide to give 6-allylsalicylic acid (**270**).[60,61] A metal-catalyzed cyclization[62] resulted in the target compound mellein. The copper-catalyzed methylation[63] of **265** provided **267**, which upon reaction with potassium carbonate in methanol yielded in **179**. Since a direct ethylation of **265** using ethyllithium and ethyl iodide failed, the synthesis of **180** was carried out by radical bromination[63] of **267** to the benzyl bromide **268** and subsequent cross coupling with methylmagnesium bromide using Kochi's catalyst.[64]

For the identification of the dimethyl furandioate **195** released by *S. griseoflavus* and *S. parvulus* three possible regioisomers were prepared, while the fourth regioisomer dimethyl furan-3,4-dioate was commercially available. These compounds show very similar mass spectra, and therefore the natural product could be unambiguously identified only based on its retention index in comparison to all four standards. The

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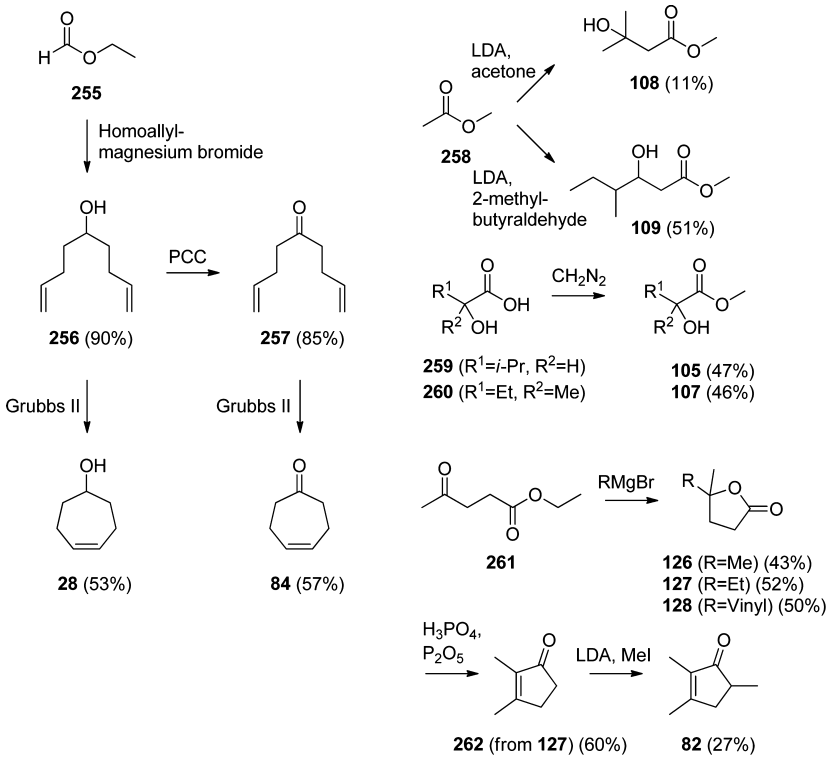
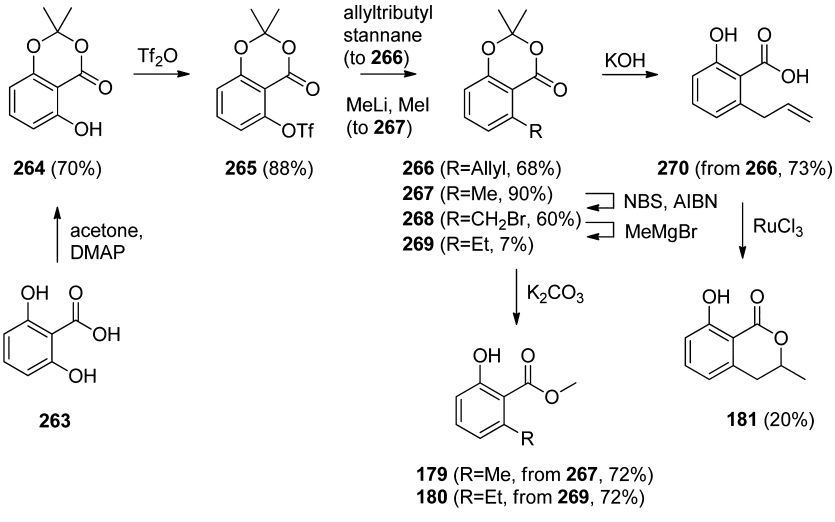
Scheme 2



Scheme 3



2,4-regioisomer was synthesized by methylation of the monomethyl ester **273** with diazomethane (Scheme 4). The preparation of **273** from furan-3-carbaldehyde (**271**) via carboxymethylation with methyl chloroformate to **272** and oxidation was published by Süßmuth.[65] The 2,5-regioisomer **275** was easily obtained from the commercially available diacid **274** by esterification with diazomethane, while the 2,3-regioisomer (**278**) was synthesized from furan-2-carboxylic acid (**276**) by carboxymethylation with LDA and methyl cyanoformate to the monomethylester **277**. Subsequent esterification with diazomethane furnished **278**. Comparison of all three synthetic regioisomers and the commercially available fourth regioisomer to the natural volatile established its structure as dimethyl furan-2,4-dioate (**195**). The esters **193** and **194** were obtained by methylation of the corresponding carboxylic acids.

The synthesis of 2-pentylfuran (**190**) was performed by deprotonation of furan with *n*-BuLi followed by treatment with pentyl iodide (Scheme 5).[11] The heterocyclic compounds methyl thiophene-2-carboxylate (**221**) and methyl pyrrole-2-carboxylate (**242**) from *S. nassauensis* were prepared with diazomethane from the corresponding carboxylic acids. The unusual nitrite **248** was obtained from isobutyl bromide (**284**) by nucleophilic substitution with sodium nitrite. Finally, the epoxides **250** and **251** were synthesized from the respective alkenes by oxidation with *m*-CPBA.

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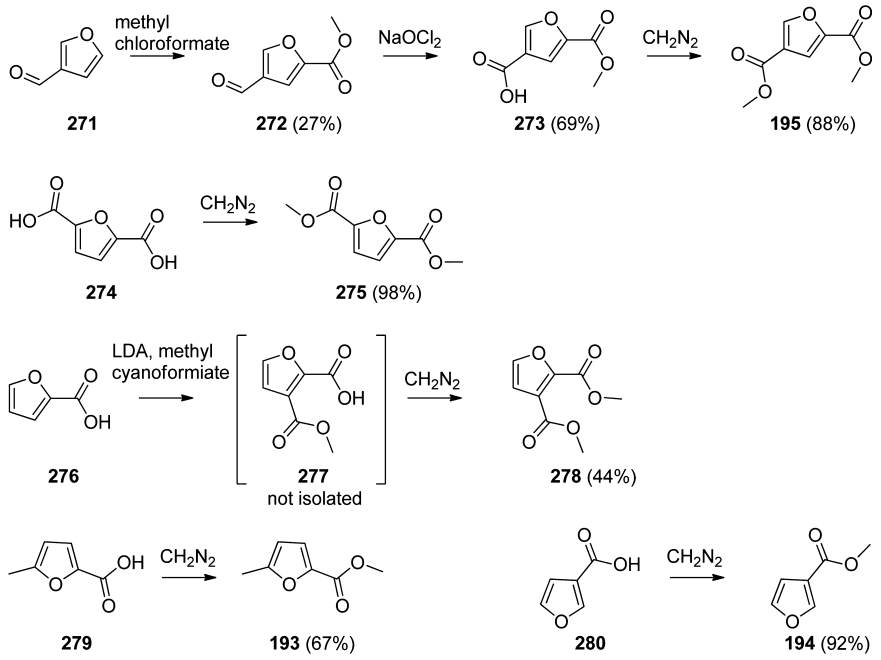
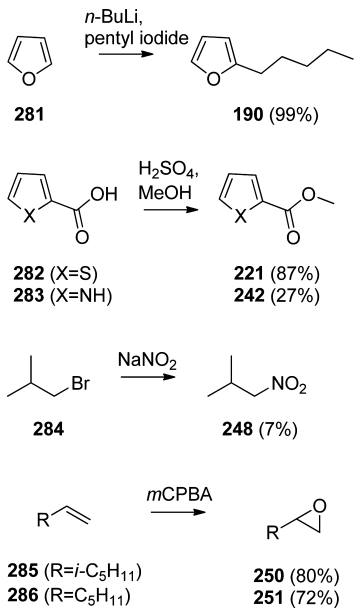
Scheme 4



Scheme 5



CONCLUSIONS

Within the headspace extracts of 50 bacterial strains investigated in this study, 254 compounds from various classes were identified, excluding terpenes, which have been reported separately.[3] Although a large number of bacterial volatiles are already known,[13,14] our analyses demonstrate that new compounds can still be discovered. In particular, the compounds cyclohept-4-enol (**28**), cyclohept-4-enone (**84**), 2,3,5-cyclopent-2-enone (**82**), methyl 5-methylheptanoate (**93**), methyl 3-hydroxy-4-methylhexanoate (**109**), methyl 4,6-dimethyloctanoate (**97**), methyl 4-methylfuran-2-carboxylate (**193**), dichloromethyl methyl disulfide (**204**), tetrathiolane (**208**), pentathiane (**209**), 2-(methylsulfanyl)aniline (**218**), and methyl thiophene-2-carboxylate (**221**) have not been previously reported from any organism, thus demonstrating that bacterial volatiles can be a rich source of new natural products. Furthermore, known natural products can be identified in previously unrecognized sources, as shown for mellein from *S. erythraea*, for which the iterative PKS has recently been reported based on in vitro experiments.[47] The large structural diversity of bacterial volatiles also reflects the fascinating biosynthetic potential of microorganisms. The biosynthetic capacity was highlighted by the occurrence of 1-nitro-2-methylpropane (**248**) in *S. viridochromogenes*. Feeding experiments with isotopically labeled precursors can easily be performed with agar plate cultures using the CLSA headspace technique and GC-MS analysis. This approach demonstrated that the nitro compound **248** is formed from valine by decarboxylation and successive oxidation steps via the corresponding aldoxime. A prominent example for a similar formation of a nitro group has been reported for the biosynthesis of the *p*-nitrobenzoate starter unit for the polyketide aureothin in *Streptomyces thioluteus* from *p*-aminobenzoate.[66]

In some cases bacterial volatiles were recognized as biosynthetic shunt products of other known metabolites; for example, 2,3,5-cyclopent-2-enone (**82**) found in *S. coelicolor* is obviously related to the methylenomycin biosynthesis,[21] and the ethyl branched ketone **61** coincides nicely with the occurrence of similarly ethyl branched blastmycinones in *S. bottropensis*.[4] Several of the other volatiles may potentially be shunt products of biosynthetic pathways to yet unidentified larger secondary metabolites. Another important finding of this study is the bacterial origin of several compounds that are known as insect pheromones including methyl 6-methylsalicylate (**179**), methyl 6-ethylsalicylate (**180**), mellein (**181**), pyrrole-2-carboxylate (**242**), conophthorin (**252**), and chalcogran (**253**). Together with the accumulating knowledge about symbiotic relationships between actinomycetes and insects[67] this questions the origin of these compounds in the arthropods. Further research will be required to clarify this interesting point. The odor marks set by many highly productive bacteria

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such as actinomycetes, myxobacteria, and cyanobacteria can easily be recognized by the earthy smell due to geosmin and 2-methylisoborneol production, but we have demonstrated that there is still more to be discovered within the scent of bacteria.

EXPERIMENTAL SECTION

General Experimental Procedures. Chemicals were purchased from Acros Organics (Geel, Belgium) or Sigma Aldrich Chemie GmbH (Steinheim, Germany) and used without further purification. All nonaqueous reactions were performed under an inert atmosphere (N_2) in flame-dried flasks. Solvents were purified by distillation and dried according to standard methods. For all general procedures, the relative amounts of the reagents are given as equivalents (equiv) referring to the molar ratios of the compounds, and the relative amounts of the solvents are given as final concentrations of the transformed starting material (set to 1.0 equiv). Melting points were measured on a Büchi 530, UV spectra were obtained using a Varian Cary 100 Bio, and IR spectra were recorded with a Bruker Tensor 27 ATR (attenuated total reflectance). NMR spectra were recorded on Bruker DRX-400 (400 MHz) or AV III-400 (400 MHz) spectrometers and were referenced against TMS (δ = 0.00 ppm) for ^{1}H NMR and $CHCl_3$ (δ = 77.16 ppm) for ^{13}C NMR. GC-MS analyses of synthetic products were carried out with an Agilent 7890A gas chromatograph connected to an Agilent 5975C inert mass detector fitted with a BPX-5 (25 m, 0.25 mm i.d., 0.25 μm film) and, in the case of natural products, also on a HP-5MS fused silica capillary column (25 m, 0.25 mm i.d., 0.25 μm film). Instrumental parameters were (1) inlet pressure, 77.1 kPa, He 23.3 mL min^{-1}, (2) injection volume, 2 μL, (3) transfer line, 300 °C, and (4) electron energy 70 eV. The GC was programmed as follows: 5 min at 50 °C increasing at 10 °C min^{-1} to 320 °C, and operated in split mode (20:1, 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_{32}). Thin-layer chromatography was performed with 0.2 mm precoated plastic sheets (Polygram Sil G/UV254, Machery-Nagel). Column chromatography was carried out using Merck silica gel 60 (70–200 mesh).

Strains, Culture Conditions, and Feeding Experiments. All bacterial strains (Table 1 of the SI) were precultured in the appropriate liquid medium (for medium compositions cf. SI) until full growth (ca. 3–7 days). Subsequently, 1 mL of liquid culture was transferred to an agar plate containing the same medium, and the plates were allowed to dry, incubated for 2–3 days, and subjected to CLSA headspace analysis. In the case of *Roseiflexus castenholzii*, which did not grow on agar plates, the liquid cultures were directly used for sampling of volatiles by CLSA. For each medium a control experiment with medium alone was performed. The compounds identified in these control experiments are not listed in Table 2. For the feeding experiment on the biosynthesis of compound **248** by *S. viridochromogenes* SFM agar was amended with [$^2H_8$]valine (1 mM) followed by collection of the volatiles by CLSA.

Collection of Volatiles by CLSA. The volatiles released by agar plate cultures, or in the case of *R. castenholzii* by liquid cultures, were collected by use of a CLSA.[1,2] The agar plates were placed in a small chamber as part of a closed apparatus with a circulating air stream that was passed over the agar plates and then through a charcoal filter (Chromtech GmbH, Idstein, Precision charcoal filter 5 mg). Alternatively, the liquid cultures were directly attached, and the airstream was directed over the liquid culture that was stirred by a magnetic stirrer to cast out the volatiles and maintain culture aeration. After a collection time of 18–24 h the absorbed volatiles were eluted from the charcoal filter with analytically pure dichloromethane (40–50 μL), and the extract was immediately analyzed by GC-MS and stored at −80 °C. After analysis of a particular strain a blank run was performed prior to analysis of the next strain.

GC-MS Analyses of Headspace Extracts. GC-MS analyses of headspace extracts were carried out on an Agilent 7890A gas chromatograph connected with an Agilent 5975C inert mass detector fitted with an HP-5MS fused silica capillary column (30 m, 0.25 mm i.d., 0.25 μm film, Agilent). GC conditions were as follows: inlet pressure 77.1 kPa, He 23.3 mL min^{-1}, injection volume 1.5 μL, transfer line 300 °C, electron energy 70 eV. The operation mode was splitless (60 s valve time), and the carrier gas was He at 1.2 mL min^{-1}. The GC was programmed as follows: 5 min at 50 °C increasing at 5 °C min^{-1} to 320 °C. Retention indices (I) were determined from a homologous series of n-alkanes (C_8–C_{32}).

ASSOCIATED CONTENT

Supporting Information

List of investigated strains and culture conditions, medium compositions, tabulated results of analyses, chromatograms of headspace extracts, mass spectra obtained in feeding experiments, synthetic procedures, and spectroscopic data including ^{1}H and ^{13}C NMR spectra of synthetic compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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