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Bacterial Scents

Biosynthesis of the Off-Flavor 2-Methylisoborneol by the Myxobacterium *Nannocystis exedens***

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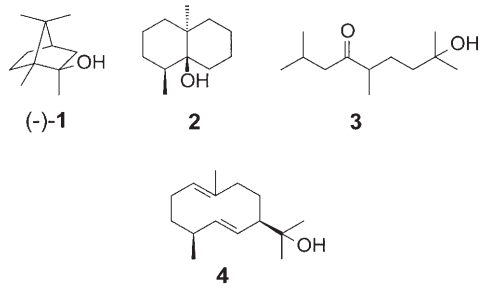
Bacteria are generally not regarded as organisms that contribute significantly to odors of our environment. Nevertheless, many bacteria produce volatiles of different substance classes, such as fatty acid derivatives, aromatic compounds, nitrogen and sulfur compounds, or terpenoids.[1] These odors can play important ecological roles, such as influencing the behavior of humans and other organisms. Highly complex profiles of volatiles are emitted by streptomycetes and myxobacteria,[2–7] and the frequently found musty or earthy smelling terpenoids 2-methylisoborneol (**1**) and geosmin (**2**)



(-)-**1** **2** **3** **4**

have extremely low odor thresholds. Compound **1**, a homomonoterpene, was first identified in *Streptomyces lavendulae*,[8] and later in several other actinomycetes,[6,9] cyanobacteria,[10] fungi,[11] and a liverwort, where it occurs as the (−)-enantiomer.[12]

Especially cyanobacteria can cause contaminations of freshwater with the undesirable flavor (off-flavor) **1**, that can result in significant economic losses in fishery.[13] Furthermore, low concentrations of **1** (10 ng L^{-1}) cause malodorous drinking water that is strongly rejected by humans.[14] Such concentrations are not toxic, but the offensive odor may lead to psychosomatic effects, such as headaches, stomach upsets, or stress.[15]

Interestingly, all known bacterial producers of the widespread terpenoids **1** and **2**, that is, myxobacteria, actinomycetes, and cyanobacteria, exhibit complex life cycles including the formation of multicellular complexes.[16] The inevitably coordinate behavior of single cells in the underlying processes to multicellular stages requires cell–cell communication, as has been demonstrated in the myxobacterium *Stigmatella aurantiaca* where fruiting body formation is initiated by the volatile pheromone stigmolone (**3**).[17] The terpenoids **1** and **2** might play a role in bacterial communication, but this remains speculative. The biosynthesis of the sesquiterpenoid **2** has recently been reported in myxobacteria[18] and streptomycetes,[19] whereas only little was known about the biosynthesis of **1** prior to this study. Early radiolabeling experiments suggested that **1** is a methylated monoterpene, the additional methyl-group being derived from *S*-adenosylmethionine (SAM).[20] Herein we report the unique biosynthesis of **1** in the myxobacterium *Nannocystis exedens* and the odor bouquet of this species.

Odor analysis of bacteria can be conveniently performed by headspace collection techniques from agar plate cultures, followed by GC/MS analysis.[3] Biosynthetic studies are thus possible with milligram amounts of labeled precursors added to the medium.[4] The volatiles of *N. exedens* (Na e485, Na eB37) and *N. exedens* subsp. *cinnabarina* (Na c29) were investigated using these techniques. Their complex bouquets are composed of up to 31 different compounds (Supporting Information). The main component of all strains is **1** accompanied by **2** and minor amounts of its biosynthetic precursor, (1(10)*E*,5*E*)-germacradien-11-ol (**4**). Further compounds can be classified as esters, lactones, aromatic compounds, terpenoids, fatty alcohols, or pyrazines; 2,5-diisopropylpyrazine being especially prominent in one strain. To clarify the absolute configuration of **1**, both enantiomers were synthesized from D-(+)- and L-(−)-camphor by $CeCl_3$-catalyzed addition of MeMgCl (Supporting Information).[21] GC on a chiral stationary phase established that only (−)-**1** is released by *N. exedens*. This is the same enantiomer as in the liverwort *Lophocolea heterophylla*,[12] whereas the absolute configuration of **1** produced by other bacteria is unknown.

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Supporting information for this article is available on the WWW under http://www.angewandte.org or from the author.

The biosynthesis of **1** by *N. exedens* was investigated in feeding experiments with the labeled precursors [*methyl*-^{13}C]methionine, and the isotopomers [4,4,6,6,6-$^{2}H_5$]- and [5,5,6,6,6-$^{2}H_5$]mevalolactone, that have been synthesized by known methods.[18,22] The methyl group from [*methyl*-^{13}C]methionine was incorporated into **1**, as indicated in the mass spectrum by an increase of the molecular ion by 1 amu (*m*/*z* 169, Figure 1B), although the major fragment ion is at *m*/*z* 95 for both unlabeled and ^{13}C-labeled **1** (for incorporation rates see Supporting Information). The mass spectrometric fragmentation of **1** follows the details elucidated by Weinberg and Djerassi for the related ketone camphor.[23] In particular, fragment **A** (*m*/*z* 95, Scheme 1) is formed by expelling the additional 2-methyl group by neutral loss of

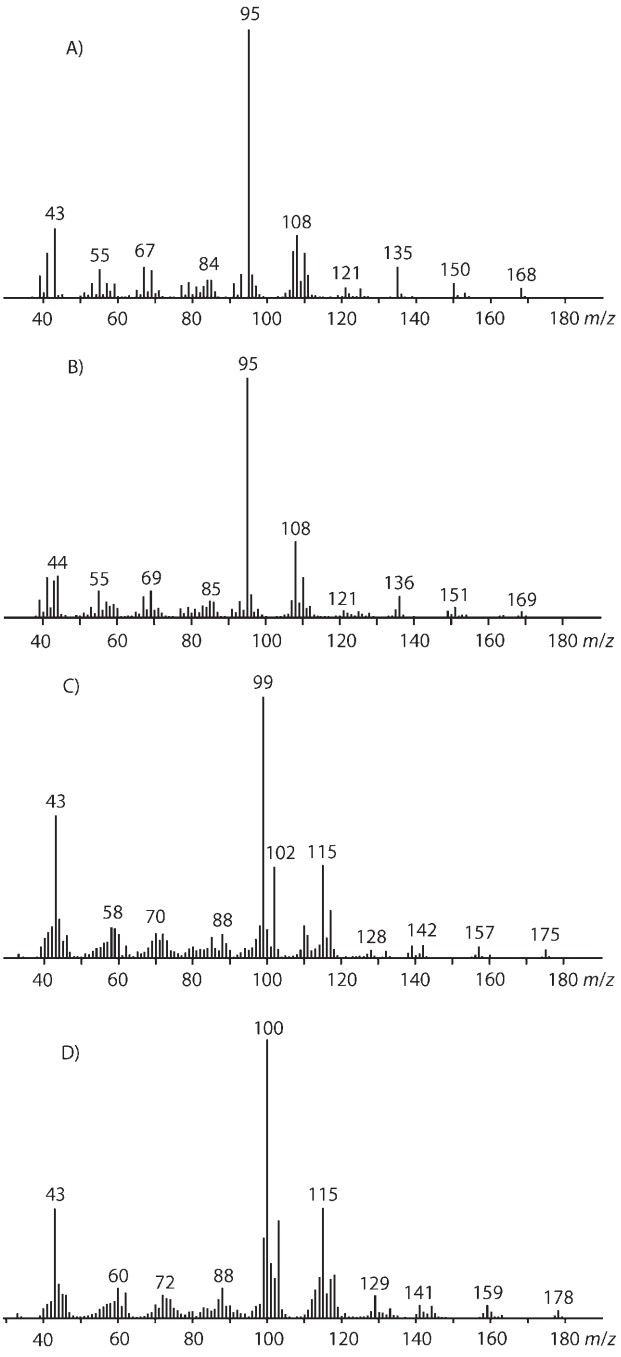
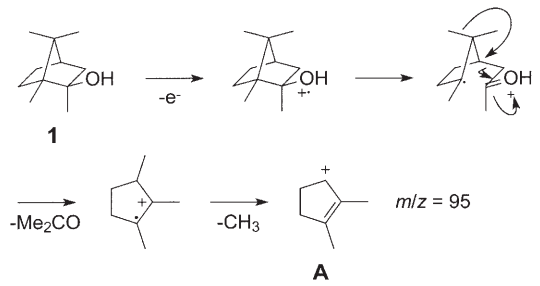
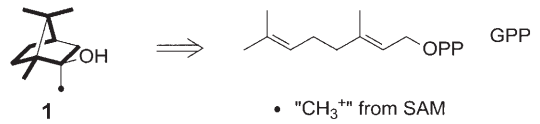


Figure 1. Mass spectra of A) **1**, B) [^{13}C]-**1** after feeding of [*methyl*-^{13}C]methionine, C) [$^{2}H_7$]-**1** after feeding of [4,4,6,6,6-$^{2}H_5$]mevalolactone, and D) [$^{2}H_{10}$]-**1** after feeding of [5,5,6,6,6-$^{2}H_5$]mevalolactone.



Scheme 1. Formation of the base peak ion **A** (*m*/*z* 95) in the mass spectrum of **1**, based on the reported fragmentation of camphor.[23]

acetone, followed by removal of one of the remaining methyl groups. Conclusively, the 2-methyl group originates from SAM, as proposed earlier.[20] The remaining ten carbon atoms were reasonably assumed to be derived from GPP (geranyl pyrophosphate, Scheme 2), the universal biosynthetic precursor of monoterpenes.

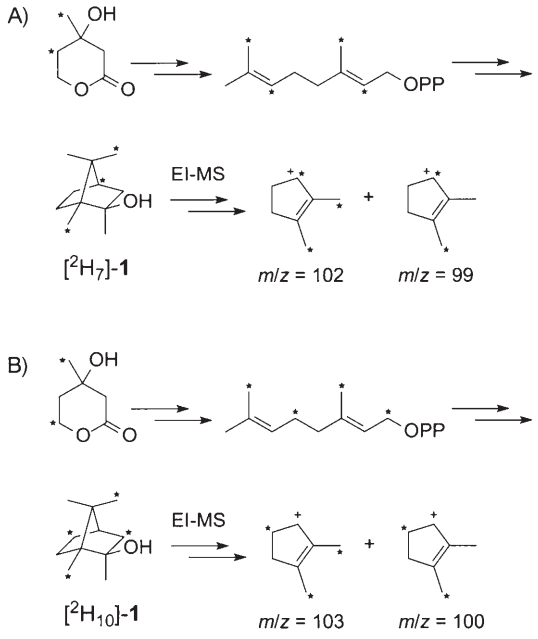


Scheme 2. Building blocks in the biosynthesis of **1**.

Feeding of [4,4,6,6,6-$^{2}H_5$]mevalolactone, and incorporation of two labeled isoprene units lead to a molecular ion at *m*/*z* 175, while the original base-peak ion was split into *m*/*z* 99 and 102 (Figure 1C), which is explainable by the loss of either CD_3 or CH_3. These data are in accordance with a labeling pattern and fragmentation of [$^{2}H_7$]-**1** depicted in Scheme 3A. Additionally, two coeluting isotopomers were found, each with only one labeled isoprene unit. Their molecular ions were at *m*/*z* 172 and 171 depending on whether the first or the second isoprene unit was labeled (Supporting Information).

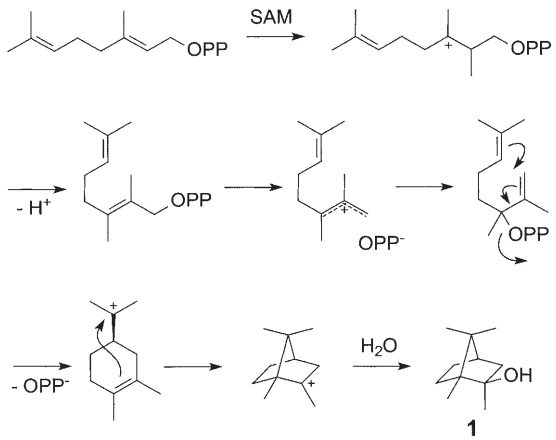
[5,5,6,6,6-$^{2}H_5$]mevalolactone was also fed to the bacteria. In this case the incorporation of two labeled isoprene units furnished a molecular ion at *m*/*z* 178, whereas the diagnostic fragment ions were found at *m*/*z* 100 and 103 (Figure 1D). The fragmentation of [$^{2}H_{10}$]-**1** and the labeling pattern shown in Scheme 3B are consistent with these data. Two isotopomers originating from only one labeled isoprene unit coeluted, both with molecular ions appearing at *m*/*z* 173 (Supporting Information).

Since SAM usually acts as an electrophile, it can attack double bonds, as for example, in cyclopropyl fatty acid biosynthesis.[24] The cyclization of terpene precursors such as GPP or farnesyl pyrophosphate proceeds via cationic intermediates,[25] that cannot be attacked by electrophiles, so that



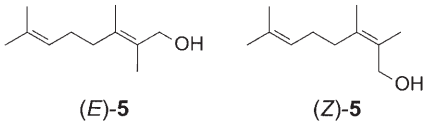
Scheme 3. Labeling pattern and fragmentation of A) [$^2H_7$]-**1** after feeding of [4,4,6,6,6-$^2H_5$]mevalolactone and of B) [$^2H_{10}$]-**1** after feeding of [5,5,6,6,6-$^2H_5$]mevalolactone.

methylation must occur either before or after cyclization. The alcohol function of **1** can be introduced by the final attack of water to a late cationic intermediate. However, once monoterpene alcohols, such as borneol or isoborneol, are generated, it is almost impossible for them to be methylated by SAM. Thus, the electrophilic double-bond attack most likely occurs prior to cyclization and by the methylation of GPP to β-methylgeranyl pyrophosphate (β-methyl-GPP, Scheme 4). Methylation of an earlier biosynthetic intermediate, for example, of dimethylallyl pyrophosphate, is unlikely, because methylation would yield a trimethylallyl pyrophosphate that cannot be used as a precursor for β-methyl-GPP. Other possible pathways to **1** can be excluded because of the observed labeling pattern.



Scheme 4. Biosynthesis of **1** in the myxobacterium *Nannocystis exedens.*

The biosynthesis of **1** through the methylation of GPP was further corroborated by the identification of β-methylgeraniol (**5**), the hydrolysis product of β-methyl-GPP, in the



(*E*)-**5** (*Z*)-**5**

volatile mixture emitted by *N. exedens.* Both (*E*)- and (*Z*)-**5** were synthesized by a Horner–Wittig-reaction of 6-methylhept-5-en-2-one with triethyl-2-phosphonopropionate (Supporting Information). Comparison of GC retention indices and mass spectra established that the natural volatile is (*E*)-**5**. It is most likely the hydrolysis product of (*E*)-β-methyl-GPP which in turn is formed intracellularly by enzymatic GPP methylation. This conclusion is further supported by the incorporation of [*methyl*-^{13}C]methionine into (*E*)-**5** (Supporting Information). Similar to the isomerization described for GPP when it is transformed into cyclic monoterpenes,[25] (*E*)-β-methyl-GPP may be isomerized to β-methyllinalyl pyrophosphate prior to cyclization to **1** (Scheme 4).

To our knowledge, the pathway described is unique because no methylation of the universal monoterpene building block GPP has been described to date. This deviation from text-book terpene biosynthesis points to a possible role of **1** in bacterial communication, since the methylation leads to a unique pathway which can be tightly regulated. This hypothesis is currently under investigation in our laboratories.

In summary, the volatiles released by different strains of the myxobacterium *N. exedens* have been investigated. The intriguing biosynthesis of the main volatile **1** has been elucidated by classical feeding studies with labeled precursors. The pathway involves the methylation of GPP to the novel biosynthetic intermediate β-methyl-GPP and its subsequent cyclization to **1**.

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