Terpene Biosynthesis

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A Detailed View of 2-Methylisoborneol Biosynthesis**

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A displeasing earthy or musty odor of drinking water has frequently caused consumer complaints about water quality. This objectionable smell can usually be correlated with cyanobacterial blooms in reservoirs[1] and is mainly caused by the bacterial production of 2-methylisoborneol (2-MIB, **1**) and geosmin (**2**; Figure 1). Both compounds can be detected

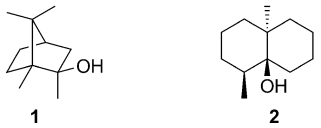

Figure 1. Structures of 2-MIB (**1**) and geosmin (**2**).

by humans in picomolar concentrations.[2,3] Although these molecules are non-toxic to humans in the low concentrations in which they may occur in drinking water, consumers often spuriously assume the contaminated water may not be safe to drink. Therefore, these contaminants must be removed by expensive treatments with UV light in combination with ozone[4,5] or with activated carbon.[6] The widespread occurrence of both terpenoids, together with their unpleasant properties, and also their mechanistically unusual biosynthetic pathways make both terpenoids interesting targets of biosynthetic studies.

2-MIB and geosmin both occur in various bacterial phyla.[7–12] 2-MIB is a methylated monoterpene, while geosmin (C_{12}) is a degraded sesquiterpene,[13] but details of their biosyntheses remained obscure and a subject of controversy for several decades. The biosynthesis of geosmin was elucidated by feeding experiments with labeled precursors,[14] followed by the identification of the geosmin synthase[15] and characterization of byproducts.[16,17] The identification of these side products was particularly important, giving indirect evidence for the proposed cationic intermediates along the cyclization cascade.

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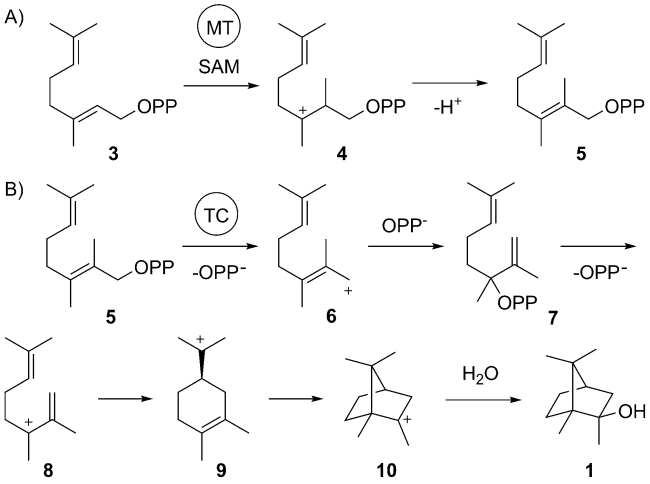
The biosynthesis of 2-MIB was also first established by feeding experiments with deuterated mevalonolactone isotopomers and [*methyl*-^{13}C]methionine in the myxobacterium *Nannocystis exedens*, resulting in a proposed pathway by *S*-adenosylmethionine (SAM) dependent methylation of geranyl diphosphate (GPP) followed by cyclization to 2-MIB (Scheme 1).[10] Subsequently, a GPP C-methyltransferase



Scheme 1. Biosynthesis of 2-MIB. A) Methylation of GPP by the SAM-dependent GPP C-methyltransferase (MT). B) Cyclization of (*E*)-2-methylgeranyl diphosphate (**5**) by the 2-MIB synthase (TC).

(MT) and a 2-MIB synthase, an unusual bacterial terpene cyclase (TC), were identified.[18,19] Herein we report on the discovery of a series of homomonoterpene byproducts, confirming the cationic intermediates in 2-MIB synthesis. Detailed mechanistic insights into the terpene cyclization were obtained in feeding studies with deuterated and ^{13}C-labeled compounds. A highly sensitive analytical method that avoids compound purification was developed for the feeding experiments with ^{13}C-labeled precursors.

In the biosynthesis of 2-MIB the product of GPP methylation, (*E*)-2-methylgeranyl diphosphate (**5**), undergoes a cyclization via a series of cationic intermediates (Scheme 1).[10] The sequence involves isomerization to 2-methyllinalyl diphosphate (**7**) via 2-methylgeranyl cation (**6**). The re-ionization of **7** to 2-methyllinalyl cation (**8**) enables a ring closure to the 2-methylterpinyl cation (**9**) followed by cyclization to the 2-methylbornyl cation (**10**) and capture of water to 2-MIB.

The volatiles emitted by bacteria can be analyzed using a closed-loop stripping apparatus (CLSA) coupled with GC-MS.[20] We have recently applied this technique for analysis of several genome-sequenced actinomycetes.[21,22] A series of

compounds with molecular ions at *m*/*z* 150 (homomonoterpenes) or *m*/*z* 168 (homomonoterpene alcohols) was detected in actinomycetes known to possess the biosynthetic genes for 2-MIB, suggesting that they arise from this biosynthetic machinery. One of the homomonoterpene alcohols, 2-methylgeraniol (**11**, Figure 2), has already been found in *N. exe-*

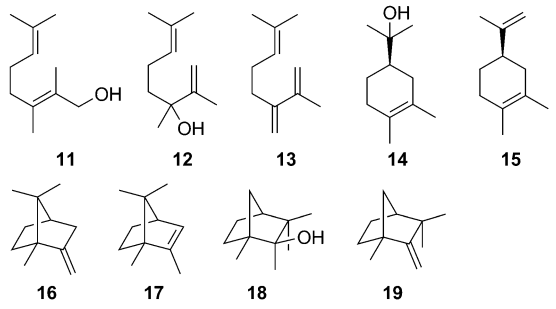


Figure 2. Side products of 2-MIB biosynthesis.

dens. Its identification was important for the understanding of 2-MIB biosynthesis, because it confirmed the methylation prior to cyclization and the existence of the cation **6** from which the alcohol is formed by water capture. The homomonoterpenes **11**, 2-methylenebornane (**16**), and 1-methylcamphene (**19**) are known products of a terpene cyclase with high homology to 2-MIB synthases in *Pseudomonas fluorescens* PfO-1.[23] The compounds **16**, **17**, and **19** have also been obtained chemically as dehydration products of 2-MIB.[24]

For a series of previously unidentified homomonoterpenes structural proposals were concluded from their mass spectra by comparisons to regular monoterpenes (Figure 3). The mass spectra of the unknown compounds showed characteristic fragment ions that are shifted by fourteen mass units (bold lines in Figure 3). Together with biosynthetic considerations this led to the suggested structures of 2-methyllinalool (**12**), 2-methylmyrcene (**13**), 2-methyl-α-terpineol (**14**), 2-methyllimonene (**15**), and 2-methyl-β-fenchol (**18**). In addition, the mass spectra of **14** and α-terpineol both contain a fragment ion at *m*/*z* 59 that indicates a 2-hydroxypropan-2-yl group. Particularly illuminative were the signal observed from a fragment ion arising by a retro-Diels–Alder fragmention in the mass spectrum of **15** at *m*/*z* 82 (limonene: *m*/*z* 68) and the dominating base peak ion in the mass spectra of both α-fenchol and **18** (*m*/*z* 81) resembling the left part of these molecules (as they are drawn in Scheme 2).

To provide evidence for these proposals a synthesis of each compound was carried out (Scheme S1 in the Supporting Information). Comparison of mass spectra and GC retention indices of the synthetic compounds with the natural products confirmed that the structures for the five compounds **12**–**15** and **18** are all new natural products. A reinvestigation of headspace extracts from fifteen actinomycetes[21,22] revealed that with the exception of *Streptomyces ambofaciens* all strains released **16** (Table S1). Compounds **17** and **19** were also produced by several actinomycetes, while the homomonoterpenes **12**–**15** and **18** occurred in strain-specific patterns. The identification of these volatiles in 2-MIB-producing

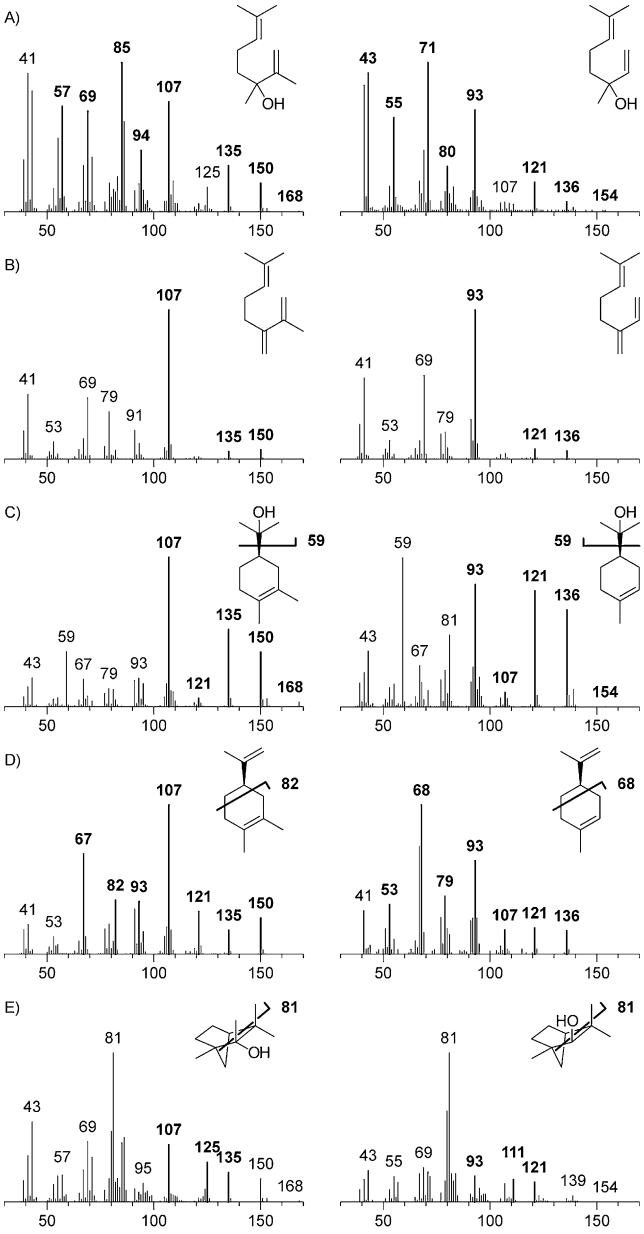
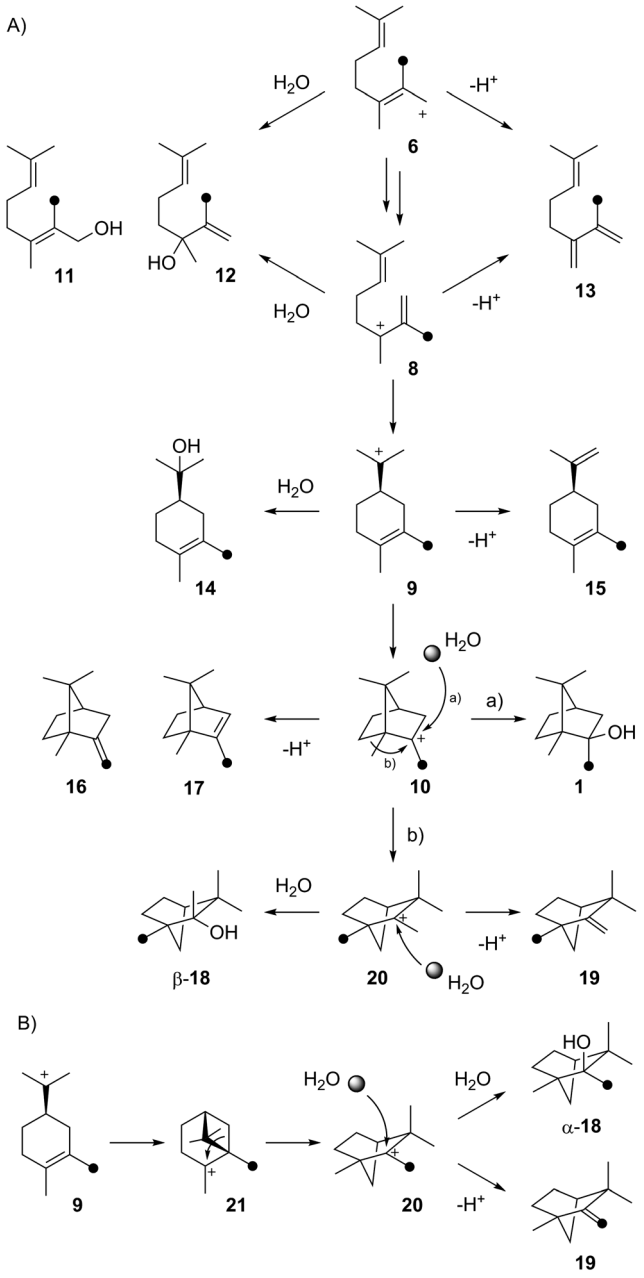


Figure 3. Mass spectra of homomonoterpenes compared to non-methylated analogues. A) **12** and linalool, B) **13** and myrcene, C) **14** and α-terpineol, D) **15** and limonene, E) **18** and α-fenchol.

bacteria is particularly interesting, because they arise from cationic intermediates en route to 2-MIB giving evidence for their existence as intermediates (Scheme 2 A).

The formation of **18** and **19** extends the 2-MIB pathway and requires an additional Wagner–Meerwein rearrangement. Two mechanisms for the formation of these compounds are possible. A rearrangement of **10** (pathway b in Scheme 2 A) would yield cation **20** that gives **18** if attacked by water or **19** by deprotonation. The attack of water at **10** to yield 2-MIB and at **20** to give **18** probably involves the same water molecule and should proceed from the same direction, therefore resulting in β-**18**. An alternative mechanism for the formation of **18** and **19** that is analogous to the cyclization of GPP to α-fenchol by the fenchol synthase from *Foenicum*

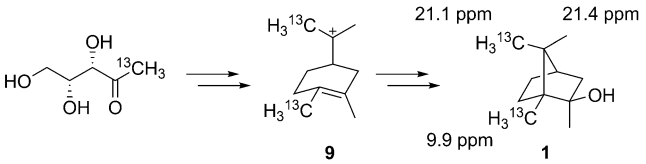


Scheme 2. Formation of homomonoterpenes by the 2-MIB synthase. Methyl groups originating from SAM are marked with black circles.

vulgare[25] starts from cation **9** that can be cyclized to the 1-methylpinyl cation **21** followed by rearrangement to **20** (Scheme 2B). In this case the attack of water should proceed from the *Si* face of **20** and result in α-**18** which, however, was not found. Although the structures of the cations **20** in Scheme 2A and B are the same, the carbon backbone in the two mechanisms has a different biosynthetic origin as can be followed for example, for the SAM-derived methyl groups (black circles in Scheme 2). For the mechanism in Scheme 2A, feeding of [*methyl*-$^2H_3$]methionine should result in a loss of a proton during formation of **19** from **20**, but of deuterium in case of the mechanism in Scheme 2B. The feeding experiment revealed formation of **19** by mechanism A (Figure S2).

A complementary experiment was performed to identify the relevant pathway for **18**. Feeding of [1-^{13}C]-1-deoxy-D-xylulose (synthesized according to Scheme S2)[26,27] resulted in [$^{13}C_2$]-**18** (Figure S3). The base peak ion of the labeled compound was observed at *m*/*z* 81, indicating that the 2-methyl group and one of the two geminal 3-methyl groups originate from C1 of 1-deoxy-D-xylulose, whereas the 1-methyl group must originate from SAM. This result also points to mechanism A, while mechanism B would require a base peak ion at *m*/*z* 82. Mechanism B may be used if GPP is converted in vitro by the 2-MIB synthase. It has been reported that such an incubation of GPP yields limonene, γ-terpinene, α- and β-pinene[19] that originate from the desmethyl analogues of **9** and **21** (missing SAM-derived methyl groups). Bornene, the deprotonation product of a desmethyl analogue of cation **10**, was not observed. Such an analogue of **10** is presumably not formed, because it is a less-stable secondary cation. The initial methylation of GPP may be important for an efficient formation of the 2-MIB skeleton via the tertiary cation **10**, making the 2-MIB pathway potentially more effective than the similar bornyl diphosphate pathway known from *Salvia officinalis*.[28]

Headspace extracts of *Micromonospora olivasterospora* contain large amounts of 2-MIB, but little of other materials. This strain was selected for investigation of the stereochemical course of the 2-MIB cyclization by feeding with [1-^{13}C]-1-deoxy-D-xylulose. This compound is converted into the doubly labeled cation **9** (Scheme 3). The final ring closure



Scheme 3. Stereochemical course of the 2-MIB biosynthesis and ^{13}C NMR chemical shifts of methyl groups in **1**.

could selectively take place from the *Re* face of the cationic center, or from both faces, if a rotation of the isopropyl group would be sterically possible in the enzyme's active site and faster than the further cyclization to **10**. The total amount of collected volatile material from one agar plate using the CLSA technique is insufficient for NMR spectroscopic analysis, but if a ^{13}C-labeled precursor is fed and efficiently incorporated into a natural product, analysis by ^{13}C NMR spectroscopy might give valuable insights. Investigation of the *M. olivasterospora* headspace extract after feeding of [1-^{13}C]-1-deoxy-D-xylulose by GC-MS showed the production of 2-MIB (64% of total volatile material by peak integration of total ion chromatogram), 2-methylenebornane (10%), geosmin (6%), and (8*S*,10*S*)-8,10-dimethyl-1-octalin (**22**, 1%) with high incorporation rates (ca. 75%) for all four compounds (Figure 4A). Analysis by ^{13}C NMR spectroscopy showed major ^{13}C signals at δ = 9.9 and 21.1 ppm (Figure 4B). Comparison with the completely assigned NMR spectroscop-

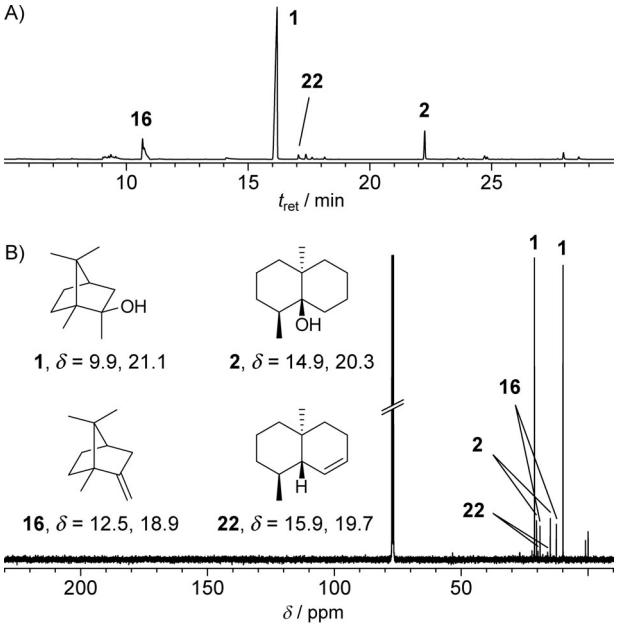


Figure 4. Analysis of headspace extracts from *M. olivasterospora* by A) GC-MS and B) ^{13}C NMR spectrum after feeding of [1-^{13}C]-1-deoxy-D-xylulose.

ic data for 2-MIB (see the Supporting Information) supports a cyclization of **9** to **10** with specific attack of the cationic center from the *Re* face. The signal at $\delta = 21.4$ ppm was below the limits of detection, establishing the high fidelity of the cyclization reaction. This tight control of the stereochemical course is a result of a cation–π interaction of **9** with a highly conserved phenylalanine residue (F309, Figure S4) that is located in the active site of 2-MIB synthase (Figure 5).[29]

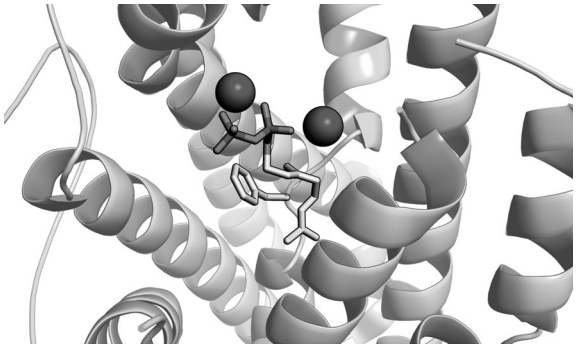


Figure 5. Active site of the 2-MIB synthase from *S. coelicolor* A2(3) complexed with Mg^{2+} ions and the substrate analogue geranyl-*S*-thiolo diphosphate (Protein data bank (PDB) code 3V1V).[29] The highly conserved phenylalanine residue (F309) suggested to be involved in cation–π interactions is also shown.

Further ^{13}C NMR signals were observed at $\delta = 12.5/19.0$, 14.9/20.3, and 15.9/19.7 ppm accounting for incorporations into 2-methylenebornane,[30] geosmin,[31] and (8*S*,10*S*)-8,10-dimethyl-1-octalin,[16] respectively.

In summary, 2-MIB biosynthesis proceeds via cationic intermediates that can be indirectly detected by the formation of byproducts. Feeding experiments allowed for a discrimination of two possible pathways to rearranged side products and demonstrated that the cations are conformationally fixed in the enzyme's active site by a cation–π interaction with a highly conserved phenylalanine residue. A new approach of combining the CLSA headspace technique with feeding of ^{13}C-labeled precursors and ^{13}C NMR spectroscopic investigation of the headspace extracts was developed. Its major advantage is the avoidance of culture extractions with solvents and laborious compound purifications.

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