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A Novel Type of Geosmin Biosynthesis in Myxobacteria

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The biosynthesis of geosmin (**1**) and (1(10)*E*,5*E*)-germacradien-11-ol (**2**), two volatile terpenoid compounds emitted by the myxobacteria *Myxococcus xanthus* and *Stigmatella aurantiaca*, was investigated in feeding experiments with different labeled precursors. In these experiments, the volatiles released by the cell cultures grown on agar plates were collected with a closed-loop stripping apparatus (CLSA) and analyzed by GC−MS. [$^2H_{10}$]Leucine and [4,4,4,5,5,5-$^2H_6$]dimethylacrylate were fed to wild-type strains and *bkd* mutant strains, which are impaired in the degradation of leucine to isovaleryl-CoA. [$^2H_{10}$]Leucine was incorporated into **1** and **2** only by the wild-type strains via the biosynthetic pathway that involves leucine degradation and branching into the mevalonate pathway. Dimethylacrylyl-CoA (DMA-CoA) is an intermediate in the leucine degradation and in the recently discovered pathway from HMG-CoA to isovaleryl-CoA. The corresponding free acid, [4,4,4,5,5,5-$^2H_6$]dimethylacrylic acid, was incorporated into **1** and **2** only by the mutants impaired in leucine degradation. [4,4,6,6,6-$^2H_5$]Mevalonic acid lactone (**12**) was synthesized and fed to *M. xanthus* and *S. aurantiaca* wild-type strains and a double mutant strain of *M. xanthus*. This strain does not degrade leucine and is impaired in the reduction of 3-hydroxy-3-methylglutaryl-CoA to mevalonic acid. The mass spectral analysis of labeled **1** and **2** obtained in these feeding experiments led to a biosynthetic scheme to **1** with intermediate **2**. This pathway differs from that observed in the liverwort *Fossombronia pusilla* and thus suggests microbial geosmin biosynthesis following a route different from that in liverworts. Our results are supported by a 1,2-hydride shift of the tertiary hydrogen atom at C-4a into the ring opposite to that in *F. pusilla*.

Introduction

Geosmin (**1**) is a degraded sesquiterpene with low odor threshold that is produced by several microorganisms and responsible for the characteristic odor of freshly plowed earth.[1] It was first isolated from the actinomycete *Streptomyces griseus*[2] and subsequently also found in fungi,[3] cyanobacteria,[4] plants,[5] mosses,[6] protozoans,[7] and insects.[8] The occurrence of **1** is of considerable importance because it can be accumulated by fish and other foodstuff to produce an off-flavor and resists conventional water treatment.[9] Several biological functions such as growth inhibition have been described, but this seems to be the case in high concentration only.[9] Geosmin is known as a signal to the glass eel *Anguilla anguilla*. The eels are attracted to the compound in a low salinity environment, which leads to the speculation that this combination acts as a landmass marker.[10] More importantly, humans avoid water inhabited by toxic cyanobacteria that release **1**.[11]

Recently, we have investigated the profile of volatile compounds emitted by the myxobacterium *Myxococcus xanthus* (strain DK1622) by use of a modified closed-loop stripping apparatus (CLSA).[12] Several terpenoid compounds are produced by this strain. (−)-Geosmin (**1**) is

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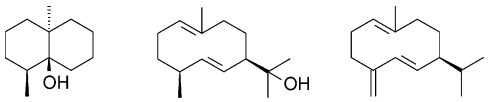
one of the major compounds and is responsible for the earthy smell of the cultures. It is also produced by other myxobacteria like *Nannocystis* exedens.[13] Myxobacteria, in general, are known for the production of several secondary metabolites that often show interesting biological activities[14] and biosyntheses.[15,16] Previously unknown pathways for the biosynthesis of *iso*-fatty acids and its precursor isovaleryl-CoA have been identified.[17−19] However, volatile secondary metabolites were only investigated recently.[12] Other terpenoids released by *M. xanthus* are (1(10)*E*,5*E*)-germacradien-11-ol (**2**), often produced in large amounts, and the trace component (−)-germacrene D (**3**). Biosynthetic studies can be carried out by feeding labeled precursors to agar plate cultures and subsequent analysis with the CLSA technique.[12] This method was used in the present study to investigate the biosynthesis of terpenes in *M. xanthus*.



1 **2** **3**

Results and Discussion

During investigations on the biosynthesis of 9-methyldecan-3-one and (*S*)-9-methyldecan-3-ol,[12] the two main components in the headspace profile of *M. xanthus*, labeled [$^2H_{10}$]leucine was fed to the wild-type strain DK1622 and to a mutant strain JD300. The latter strain is impaired in the degradation of leucine to isovaleryl-CoA due to a mutation in the *bkd* gene (branched-chain keto acid dehydrogenase). Headspace extracts were sampled from agar plate cultures by CLSA and analyzed by GC−MS.[12] Unexpectedly, the incorporation of [$^2H_{10}$]-leucine into **1** and **2** by the wild-type strain was observed, while the mutant strain showed no incorporation (for incorporation rates, see Table 1). Identical feeding experiments were carried out with the myxobacterium *Stigmatella aurantiaca* (wild-type strain DW 4/3-1 and *bkd* mutant strain) whose results matched those obtained with *M. xanthus*.

The retention time of a deuterated compound is always shortened as compared to that of its unlabeled counterpart.[12] Thus, pure mass spectra of labeled compounds arising from deuterated precursors can be obtained, even in the presence of unlabeled material. The incorporation of [$^2H_{10}$]leucine into **1** is indicated by a shift of the molecular ion from *m/z* = 182 to 188 and 187, respectively (compare Figure 1a and b). Conclusively, labeled **1** contained up to six deuterium atoms, accompanied by minor amounts of isotopomers with five deuterium atoms. The same pattern is found for **2** by an increase of the molecular ion from *m/z* = 222 to 228 and 227 (compare Figure 1f and g). Leucine can enter the mevalonate pathway by the leucine-dependent isoprenoid pathway, giving rise to dimethylallyl pyrophosphate (DMAPP) and isopentenyl pyrophosphate (IPP),[18,20] the universal building blocks in the terpene biosynthesis. Herein, [$^2H_{10}$]-leucine is degraded by transamination and subsequent oxidative decarboxylation to [$^2H_9$]isovaleryl-CoA (IV-CoA), and then further to [$^2H_7$]dimethylacrylyl-CoA (DMA-CoA) as outlined in Scheme 1. This pathway is continued by the carboxylation to [$^2H_6$]-3-methylglutaconyl-CoA (MG-CoA) and the addition of water to result in [$^2H_6$]-3-hydroxy-3-methylglutaryl-CoA (HMG-CoA), a central intermediate in the mevalonate pathway to terpenes.[21] Reduction furnishes [$^2H_6$]mevalonic acid (MVA), and several phosphorylation and decarboxylation steps lead to [$^2H_6$]IPP and [$^2H_6$]DMAPP. The *pro-R* proton at C-2 of IPP is removed in the isomerization process from IPP to DMAPP. The stereochemistry at C-2 of labeled IPP and therefore the number of deuterium atoms retained in DMAPP is thus determined by the stereochemical course of the MG-CoA hydration. This step is catalyzed by the methylglutaconyl-CoA hydratase and proceeds by the *syn*-addition of water from the *Si*-side.[22] The enzyme is part of the crotonase superfamily, an enzyme superfamily catalyzing biotransformations in which enolate anion intermediates derived from acyl-CoA substrates need to be stabilized.[23] A transformation similar to the hydration of MG-CoA is the addition of water to enoyl-CoA intermediates in the fatty acid *β*-oxidation. The enoyl-CoA hydratase (E.C. 4.2.1.17) is involved in the stereospecific hydration of α,*β*-unsaturated acyl-CoA thioesters that proceeds also via the *syn*-

TABLE 1. Incorporation Rates in Feeding Experiments

experiment[a]	**1**[b]	**2**[b]
Myxococcus xanthus		
DK1622 + [$^2H_{10}$]leucine	6	8
JD300 + [$^2H_{10}$]leucine	0	0
DK1622 + [$^2H_6$]DMAA	0	0
JD300 + [$^2H_6$]DMAA	49	
DK1622 + **12**	44	46
Stigmatella aurantiaca		
DW 4/3-1 + [$^2H_{10}$]leucine	3	4
DW 4/3-1 *bkd*$^-$ + [$^2H_{10}$]leucine	0	0
DW 4/3-1 + [$^2H_6$]DMAA	0	0
DW 4/3-1 *bkd*$^-$ + [$^2H_6$]DMAA	15	19
DW 4/3-1 + **12**	22	25

[a] Feeding experiments with *Myxococcus xanthus* (wild-type strain DK1622 and *bkd* mutant strain JD300) and *Stigmatella aurantiaca* (wild-type strain DW 4/3-1 and *bkd* mutant strain) supplying different labeled precursors. [b] Incorporation rates in % into volatiles **1** and **2**. The rates are given as ratio of labeled to unlabeled isoprene units used for the formation of the sesquiterpenoids. This ratio was calculated from the ion chromatograms of molecular ions of the different isotopomers.

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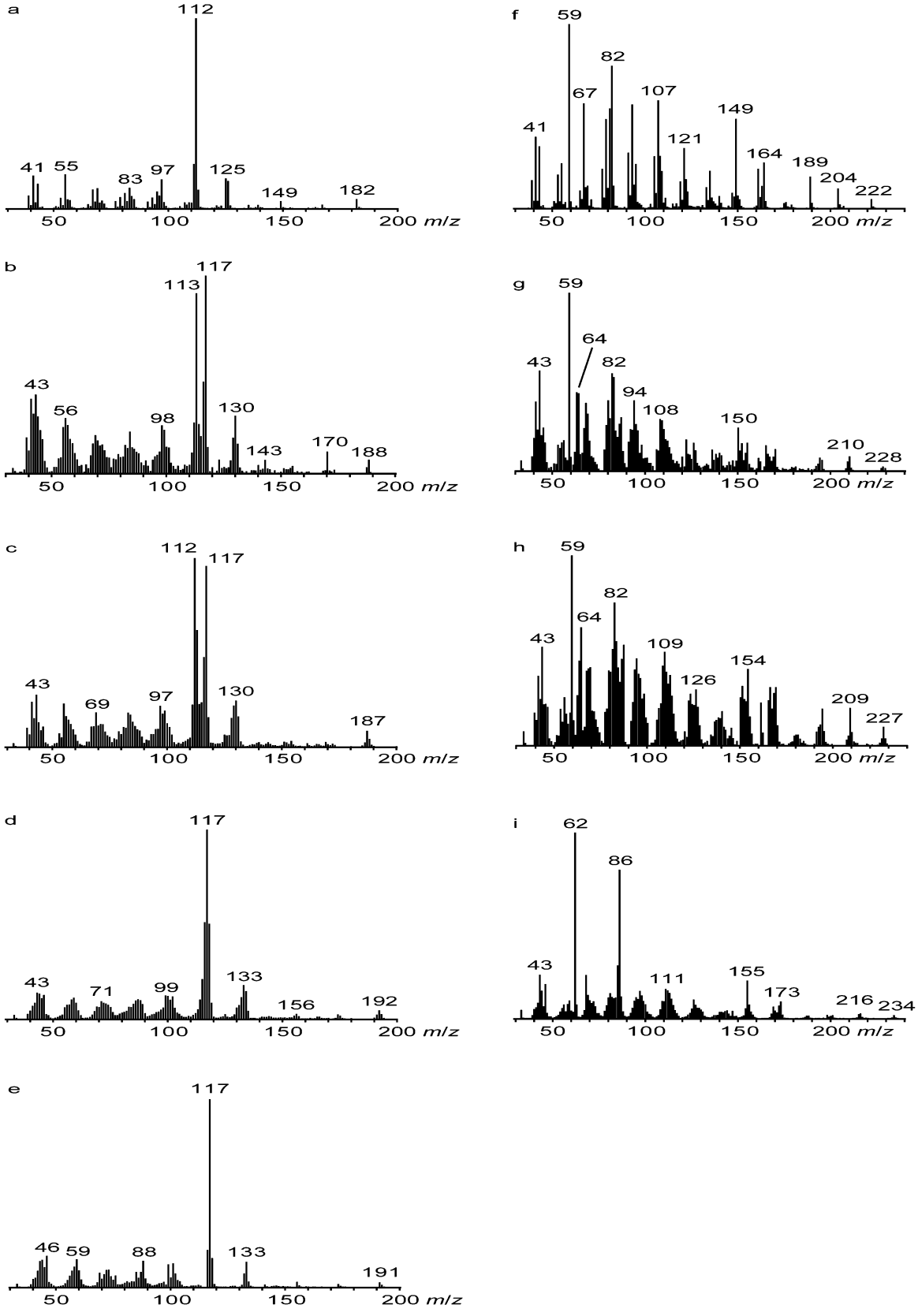
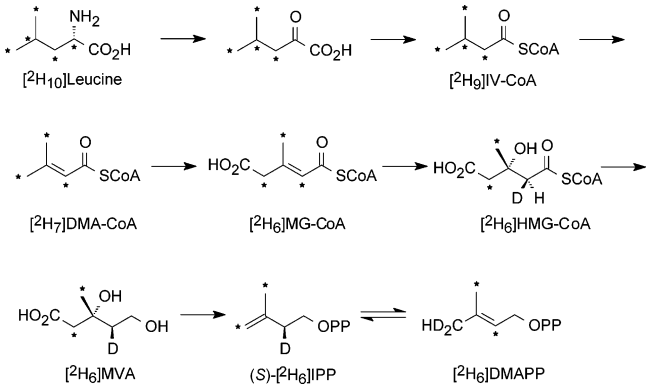


FIGURE 1. Mass spectra of terpenoid compounds present in the headspace extracts of *Myxococcus xanthus* and *Stigmatella aurantiaca*. Mass spectra of **1** (a), [$^2H_6$]-**1** after feeding of [$^2H_{10}$]leucine (b), [$^2H_5$]-**1** after feeding of [$^2H_6$]DMAA and incorporation of one labeled IPP unit (c), [$^2H_{10}$]-**1** after feeding of [$^2H_6$]DMAA and incorporation of two labeled IPP units (d), **1g** after feeding of **12** (e), **2** (f), [$^2H_6$]-**2** after feeding of [$^2H_{10}$]leucine (g), [$^2H_5$]-**2** after feeding of [$^2H_6$]DMAA (h), and **2g** after feeding of **12** (i).

SCHEME 1. Biosynthesis of the Terpene Building Blocks [$^2H_6$]IPP and [$^2H_6$]DMAPP from [$^2H_{10}$]Leucine[a]

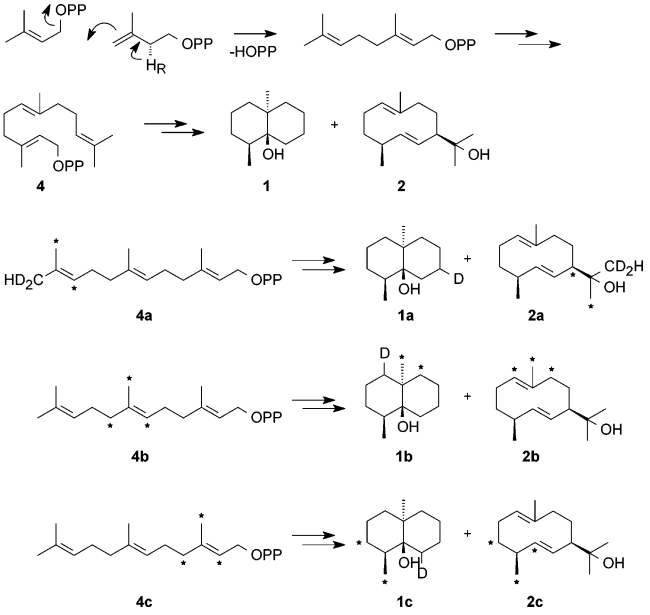


[a] Asterisks indicate fully deuterated carbons.

addition of water.[24] Furthermore, in recent investigations on *Pseudomonas putida*, an operon encoding the leucine catabolic pathway was identified.[25] The enzyme methylglutaconyl-CoA hydratase (E.C. 4.2.1.18) is encoded by one gene of this operon. Because of the presence of the same catalytic residues as found in other enzymes of this superfamily, it is supposed to catalyze the *syn*-addition of water. Therefore, the 1-fold deuterated carbon in [$^2H_6$]-HMG-CoA (C-2) generated on the leucine pathway is suggested to be *S*-configured. This stereochemistry is retained in the next steps to [$^2H_6$]IPP. The following reversible isomerization to DMAPP is catalyzed by type-I isomerases in animals, plants, and fungi, whereas archaea and many eubacteria use a structurally unrelated type-II isomerase.[26] The isomerization process is associated with the loss of the *pro-R* proton for both types of isomerases.[26] Thus, the isomerization of (*S*)-[$^2H_6$]IPP furnishes [$^2H_6$]DMAPP. Hypothetically, (*R*)-[$^2H_6$]IPP would lead to [$^2H_5$]DMAPP.

The universal sesquiterpene precursor farnesyl pyrophosphate (FPP, **4**) is formed from one unit of DMAPP and two units of IPP. This process is associated with the loss of the *pro-R* proton at C-2 of IPP (Scheme 2).[27] The statistical incorporation of the [$^2H_{10}$]leucine-derived isoprene units [$^2H_6$]DMAPP and (*S*)-[$^2H_6$]IPP leads to the formation of three isotopomers of [$^2H_6$]FPP (**4a**, **4b**, and **4c**). In contrast, *R*-configured [$^2H_6$]IPP would yield three isotopomers of [$^2H_5$]FPP. Therefore, the observed formation of [$^2H_6$]-**1** (**1b** and **1c**) and [$^2H_6$]-**2** (**2a**, **2b**, and **2c**), which is indicated by the molecular ions at *m/z* = 188 and 228, is only explainable by the formation of (*S*)-[$^2H_6$]-IPP. The occurrence of six deuteriums in labeled **1** indicates by inversion of the arguments that *syn*-addition of water from the *Si*-side to MG-CoA must take place in the leucine-dependent isoprenoid pathway of the myxobacteria. The generation of [$^2H_5$]-**1** and [$^2H_5$]-**2** that are found in low amounts can be explained by the reisomerization of [$^2H_6$]DMAPP to (*S*)-[$^2H_5$]IPP and back

SCHEME 2. Biosynthesis of Terpenoids via FPP from DMAPP and IPP[a]



[a] Labeling patterns in the sesquiterpene precursor FPP, **1**, and **2** after feeding of [$^2H_{10}$]leucine and incorporation of one labeled isoprene unit. Completely deuterium-labeled carbons are indicated by asterisks.

to [$^2H_5$]DMAPP. In this process, the terminal (*E*)-methyl group can either lose an H or D, thus giving access to (*S*)-[$^2H_5$]IPP.

The isopropylidene group of [$^2H_6$]DMAPP incorporated into **4a** is lost during the biosynthesis of **1** as outlined below, forming **1a**. This compound coelutes with unlabeled **1** because of its low deuterium content and is not detectable due to the low incorporation rates.

The molecular ions and diagnostic fragment ions expected in the mass spectra of labeled **1** and **2** after feeding of different deuterium-labeled precursors are summarized in Figure 2. The mass spectrum of labeled [$^2H_6$]-**2** shows fragment ions at *m/z* = 59 and 64, respectively, in a ratio of about 2:1 after feeding of [$^2H_{10}$]leucine (Figure 1g). These fragment ions result from α-cleavage next to the alcohol function, giving *m/z* = 64 for **2a**. This ion points to a content of five deuterium atoms according to the composition $[C_3H_2D_5O]^+$ (Figure 2a). Thus, the isopropylidene group in labeled **4a** must contain up to five deuteriums as is expected for the degradation of [$^2H_{10}$]leucine to [$^2H_6$]DMAPP via the leucine-dependent isoprenoid biosynthesis.

Further insight into the biosynthesis of **1** was possible by the following observations: The chromatographic peak of [$^2H_6$]-**1** obtained after feeding of [$^2H_{10}$]leucine originates from the two isotopomers **4b** and **4c** (Scheme 2), thus containing the two isotopomers **1b** and **1c**. This is indicated by a shift of the highly diagnostic base peak of natural **1** from *m/z* = 112 to *m/z* = 113 and 117, respectively, in the expected 1:1 ratio (Figures 1b and 2a). Other diagnostic ions found at *m/z* = 125 and 126 in unlabeled **1** move to *m/z* = 130 and 131. These major ions arise from a rearrangement process according to Scheme 3 and can be used to localize the deuterium atoms.[5] The formation of **1b** (Figure 2a) results in five deuterium

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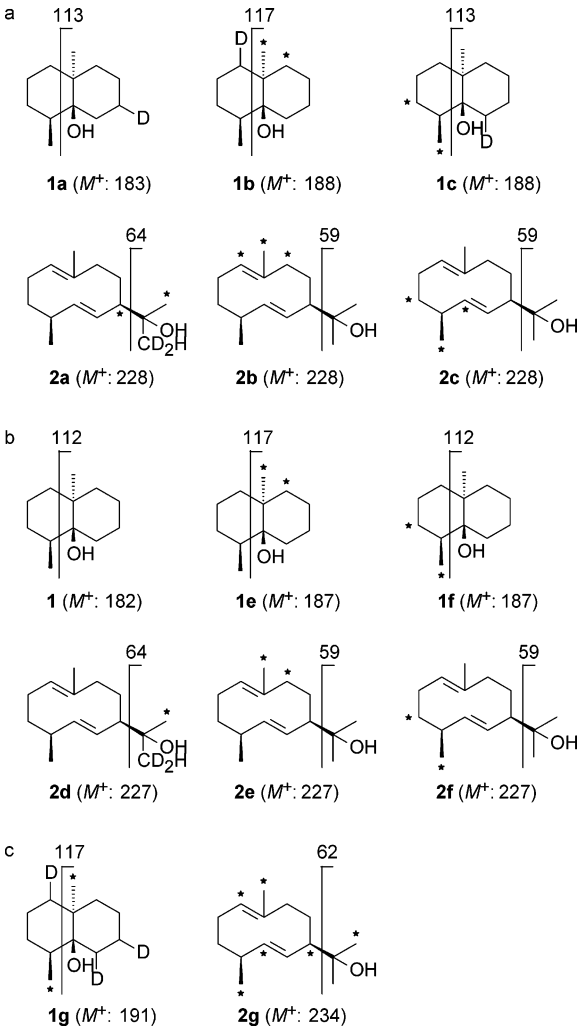
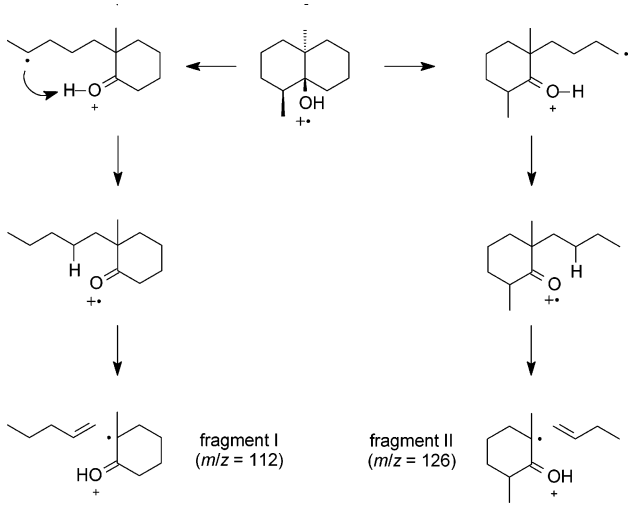


FIGURE 2. Labeling patterns in **1** and **2** after feeding of [$^2H_{10}$]-leucine and incorporation of one labeled isoprene unit (a). Labeling patterns in **1** and **2** after feeding of [$^2H_6$]DMAA and incorporation of one labeled isoprene unit (b). Labeling patterns in **1** and **2** after application of **12** and incorporation of three labeled isoprene units (c). Expected molecular ions and diagnostic fragment ions are shown. Asterisks indicate fully deuterated carbons.

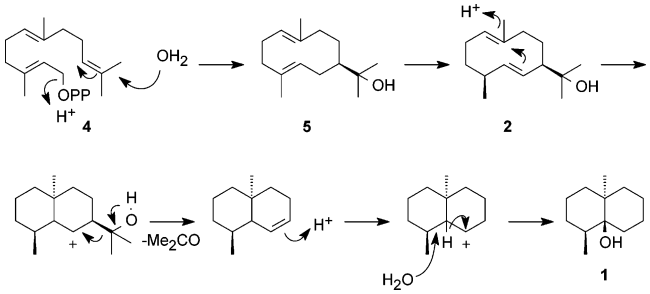
atoms in fragment I (m/z = 117) and four deuterium atoms in fragment II (m/z = 130). One deuterium is present in fragment I (m/z = 113) and five deuterium atoms in fragment II (m/z = 131) of isotopomer **1c**. These data are not in agreement with the biosynthetic pathways to **1** suggested by Boland et al. for *Streptomyces*[5] and Cane and Watt for *Streptomyces coelicolor*.[28]

The biosynthesis of **1** is controversially discussed in the literature. Recently, the gene encoding the enzyme responsible for the production of **1** and **2** in *Streptomyces coelicolor* was identified.[28,29] This protein has two domains: The N-terminal domain is used for the formation of **2**,[28] while the C-terminal domain is not involved in the biosynthesis of either **1** or **2** and shows no sesquiterpene synthase activity in vitro. Furthermore, the production

SCHEME 3. MS Fragmentation Pathways to the Diagnostic Fragment Ions of 1



SCHEME 4. Biosynthesis of 1 in the Myxobacteria *M. xanthus* and *S. aurantiaca*



of **1** in *S. coelicolor* is lost after the deletion of the N-terminal domain, but not after the removal of the C-terminal domain.[29] Conclusively, Cane and Watt postulated **2** to be an intermediate in the biosynthesis of **1**. A geosmin-overproducing strain of *Streptomyces citreus* also emits considerable amounts of **2**, thus suggesting this compound to be the sesquiterpene precursor of **1**.[30] Pollak and Berger postulated a route for the biosynthesis of **1** relying on volatile side products they identified in cultures of *S. citreus*.[30] Based on the mass spectral data obtained after feeding of [$^2H_{10}$]leucine, we proposed a biosynthetic pathway to **1** with intermediate **2** similar in its early steps to the biosynthetic scheme postulated by Pollak and Berger (Scheme 4). Herein, FPP is cyclized to hedycaryol (**5**) and further isomerized to **2**. Protonation initiates the formation of the bicyclic carbon skeleton. The isopropyl moiety is then removed by the loss of acetone. Protonation of the double bond facilitates the attack of water in combination with a 1,2-hydride shift from the tertiary bridgehead carbon into the right ring. The formation of **1** with the correct relative configuration is in coherence with this mechanism. The hydride shift leading to isotopomer **1c** from **4c** results in the formation of an ion at m/z = 113.

The last step in the pathway postulated by Boland et al. (Scheme 5a) is characterized by a hydrogen shift of the same hydrogen, but into the left ring of **1**.[5] Thus, the

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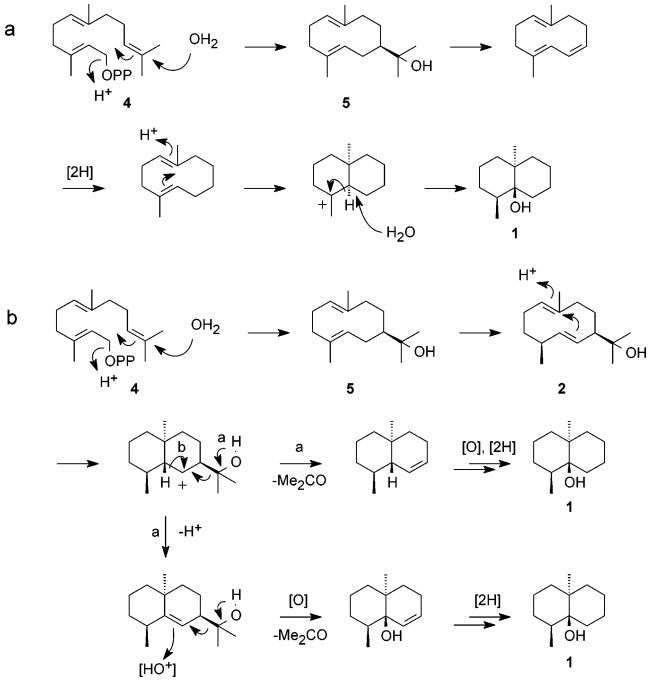
SCHEME 5. Biosynthesis of 1 in the Liverwort *Fossombronia pusilla* (a) and Two Suggested Pathways for the Biosynthesis of 1 in *Streptomyces* sp. (b)



incorporation of **4c** following this pathway would necessarily lead to the detection of the diagnostic fragment ion at *m/z* = 112. Boland et al. used different labeled precursors as [5,5-$^2H_2$]DOX ([5,5-$^2H_2$]deoxyxylulose), [4,4,6,6,6-$^2H_5$]MVA, and [2,2-$^2H_2$]MVA, respectively. [5,5-$^2H_2$]DOX was used in investigations on *Streptomyces* sp. (sesquiterpenes can arise in streptomycetes through the deoxyxylulose phosphate pathway as well as the mevalonate pathway depending on the growth phase[31,32]), whereas [4,4,6,6,6-$^2H_5$]MVA and [2,2-$^2H_2$]MVA were used in investigations on the liverwort *Fossombronia pusilla* (sesquiterpenes are formed in this species via the mevalonate pathway only[33,34]). While the results of feeding experiments with *F. pusilla* employing [4,4,6,6,6-$^2H_5$]-MVA clearly indicated the hydrogen shift into the left ring of **1** and therefore gave evidence for the pathway outlined in Scheme 5a, the feeding of [5,5-$^2H_2$]DOX to *Streptomyces* sp. led to an ambiguous result. The H-2 in FPP is not labeled, and thus the fate of this diagnostic hydrogen atom cannot be followed. Both pathways shown in Schemes 4 and 5a are in accordance with the fragmentation pattern of labeled **1** obtained by Boland et al. after feeding of [5,5-$^2H_2$]DOX to *Streptomyces* sp. It should be noticed, however, that different organisms might use different pathways for the biosynthesis of the same compound.

Two alternative pathways suggested by Cane and Watt[28] are presented in Scheme 5b (pathways a and b). Both comprise the formation of **2** by cyclization of FPP to **5** and subsequent isomerization. The proton-mediated cyclization leads to a bicyclic cation that is suggested to be an intermediate in both pathways. The methine hydrogen attached to the tertiary bridgehead carbon is lost in the following steps of both pathways under discussion. Consequently, both pathways would require the diagnostic fragment ion at *m/z* = 112 after the incorporation of **4c**. It can therefore be ruled out that either of them is used by *M. xanthus*.

Furthermore, the sesquiterpene **3** is emitted by *M. xanthus* as a trace component, but no incorporation of [$^2H_{10}$]leucine is observed because of the low amounts present in the extract. In a recent study on *Streptomyces coelicolor*, the cyclization of FPP to **2** and **3** is shown to be catalyzed by the same enzyme.[35] An alternative pathway to **2** via nerolidyl pyrophosphate and a common cationic intermediate in the biosynthesis of **2** and **3** (helminthogermacradienyl cation) is presented. This pathway is also consistent with the data obtained after feeding [$^2H_{10}$]leucine.

In a subsequent experiment, [4,4,4,5,5,5-$^2H_6$]dimethylacrylate ([$^2H_6$]DMAA), one of the proposed intermediates on the catabolic route from leucine to HMG-CoA,[18,19] was fed to the wild-type strains and the *bkd* mutant strains of *M. xanthus* and *S. aurantiaca*. While the wild-type strains did not incorporate [$^2H_6$]DMAA into the isoprenoids, the mutant strains readily incorporated this labeled intermediate into **1** and **2** (Figure 1c, d, and h). One feeding experiment (feeding of [$^2H_6$]DMAA to the mutant strain JD300 of *M. xanthus*) did not yield **2**, and therefore no incorporation into this compound was observed. The incorporation rates for [$^2H_6$]DMAA in the case of the wild-type strains seem to be low. Accordingly, the incorporation is beyond the limits of detection in the GC−MS system. In contrast, the mutant strains impaired in the production of dimethylacrylyl-CoA from leucine show significant incorporation (Table 1). The incorporation of one and two labeled isoprene units into **1** was indicated by a shift of the molecular ion from *m/z* = 182 to 187 and 192, respectively. Thus each [4,4,4,5,5,5-$^2H_6$]-DMAA is responsible for the occurrence of five deuterium atoms in **1**. The incorporation of one unit of [$^2H_6$]DMAA leads to three FPPs with different labeling patterns similar to **4a**, **4b**, and **4c**, only missing the olefinic deuterium. While the incorporation of [$^2H_5$]FPP leads to the complete loss of deuterium in **1** (similar to **4a**), labeling in the other two respective isoprene units affords the two different isotopomers **1e** and **1f** (Figure 2b), which cannot be separated by GC. The incorporation of [$^2H_6$]DMAA into the second isoprene unit of FPP (similar to **4b**) results in a base peak shift from *m/z* = 112 to 117, while the incorporation into the third isoprene unit of FPP (similar to **4c**) gives rise to a fragment with *m/z* = 112 (Figure 1c). Furthermore, the incorporation of two units [$^2H_6$]DMAA into **1** is accompanied by a base peak at *m/z* = 117 (Figure 1d). In the case of **2**, the incorporation of only one unit [$^2H_6$]DMAA is observed furnishing the coeluting isotopomers **2d**, **2e**, and **2f** (Figure 1h, Figure 2b). This is indicated by a common molecular ion at *m/z* = 227 and fragment ions at *m/z* = 59 and 64, respectively, in the expected ratio of about 2:1.

Deuterium-labeled mevalonic acid lactone [4,4,6,6,6-$^2H_5$]MVA (**12**) was synthesized to further investigate the

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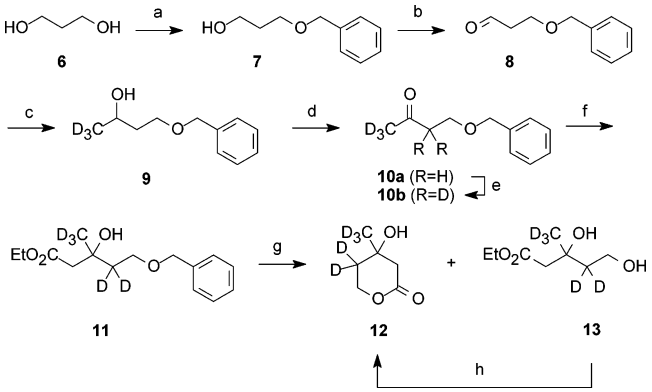
SCHEME 6. Synthesis of 12[a]



[a] (a) NaH, THF, DMF, BnBr, 58%; (b) PCC, CH_2Cl_2, 78%; (c) D_3CMgI, Et_2O, 90%; (d) PCC, CH_2Cl_2, 93%; (e) K_2CO_3, D_2O, 94%; (f) Rieke-Zn, ethyl bromoacetate, THF, 79%; (g) H_2, Pd/C, Et_2O; (h) *p*-TsOH, CH_2Cl_2, 85% over two steps.

biosynthetic pathway to **1** (Scheme 6). One of the alcohol groups of propane-1,3-diol (**6**) was protected as benzyl ether to give **7**. Oxidation with PCC provided the aldehyde **8** that was treated with the Grignard reagent of [$^2H_3$]methyl iodide. PCC oxidation furnished **10a**. The required additional deuterium labeling was introduced into **10a**, resulting in **10b** by H/D exchange. While strong bases (NaOMe in MeOD) led immediately to the elimination of benzyl alcohol, K_2CO_3 in D_2O required prolonged reaction times (7 d), but resulted in the formation of **10b** in high yield (94%). NaOD in D_2O caused a fast H/D exchange (2 h), but some elimination occurred. A Reformatsky reaction[36] with Rieke zink[37,38] and ethyl bromoacetate then furnished **11**. Cleavage of the benzyl ether moiety with Pd/C in a hydrogen atmosphere gave **13** and **12** arising by spontaneous cyclization. Another portion of **12** was obtained from **13** by cyclization with *p*-toluenesulfonic acid. This procedure allows the convenient synthesis of **12** in a gram scale.

In subsequent experiments, **12** was fed to *M. xanthus* and *S. aurantiaca* wild-type strains and a double mutant strain of *M. xanthus* (DK5624). The latter is impaired in the transformation of HMG-CoA to MVA in addition to the *bkd* negative genotype[39] and does not produce **1** and **2** (compare Figure 3a and 3b). Total ion chromatograms obtained after the application of **12** are depicted in Figure 3c (DK1622) and d (DK5624). The wild-type strain used **12** and natural MVA for the production of **1** and **2** with different degrees of labeling. In contrast, the mutant strain only emitted **1g** and **2g** derived from **12** with the maximum deuterium content of 9 or 12 deuterium atoms, respectively. The isotopomers of **1** and **2** with different deuterium content obtained from the wild-type strains were separable by gas chromatography, but the isotopomers with identical deuterium content coeluted. This is shown for **1** by the ion chromatograms for the expected molecular ions above *m/z* = 182 (Figure 4a). Similarly, the incorporation of one to three labeled isoprene units into **2** can be observed (Figure 4b). The incorporation rates were higher in the experiment with *M. xanthus* than with *S. aurantiaca* (Table 1). The mass spectrum of **1g** (Figure 1e) showed a molecular ion at *m/z* = 191, and the diagnostic fragment ions shifted from *m/z* = 112 to 117 and *m/z* = 126 to 133. These data further corroborate the suggested biosynthetic pathway to **1** (Scheme 4), whereas the pathway described by Boland et al. (Scheme 5a) would require *m/z* = 116 and *m/z* = 134 due to a 1,2-hydride shift into the left instead of the right ring of **1**. The pathways suggested by Cane and Watt (Scheme 5b) would require the detection of *m/z* = 116 and *m/z* = 133, because the respective deuterium atom is lost in both pathways. The mass spectrum of **2g** (Figure 1i) showed a molecular ion at *m/z* = 234 and a base peak at *m/z* = 62, indicating the presence of three deuterium atoms in the isopropyl group (Figure 2c).

In conclusion, it was shown that the leucine-dependent isoprenoid biosynthesis can also operate for the production of sesquiterpenes.[19] In myxobacteria, this novel pathway seems to be sort of a back-up system, because wild-type strains only poorly incorporate leucine. The biosynthetic pathway to **1** was clarified by feeding small amounts of labeled leucine, DMAA, and MVA to *M. xanthus* and *S. aurantiaca* that were cultivated on agar plates. Leucine is transformed via the leucine-dependent isoprenoid biosynthetic pathway to HMG-CoA, further supported by the incorporation of the intermediate DMAA. Cyclization and isomerization of FPP leads to **2**. The data are consistent with, but do not prove the intermediacy of **2** in the formation of **1**. The subsequent steps, cyclization to the bicyclic system, loss of acetone, and proton-mediated addition of water in combination with a 1,2-hydride shift, are in accordance with the fragmentation pattern observed after feeding of the precursors. In particular, the biosynthetic pathway to **1** is different from that described for the liverwort *Fossombronia pusilla*. Obviously, two independent pathways to **1** exist in nature. Whether the pathway described here also operates in *Streptomyces* remains to be determined (as well as its dependence on the MVA or DOX pathways), because the data in the literature do not allow a discrimination.[5]

Experimental Section

Strains, Culture Conditions, Feeding Experiments. *Myxococcus xanthus* (strains DK1622[40] and the *bkd* mutant strain JD300[41]) and *Stigmatella aurantiaca* (strain DW4/3-1 with the corresponding *bkd* mutant[18]) have been described previously. Cultivation and feeding experiments using these strains were performed as described.[12] Labeled compounds were dissolved in MeOH and spread on top of freshly prepared agar plates with 40 μg/mL kanamycin if required (for DK5624) to reach a final concentration of 1 mM. Plates were inoculated with 500 μL of a log-phase liquid culture of the required strain and incubated 1−3 d until significant growth was visible.

Sampling. Volatile organic compounds emitted by cell cultures of *Myxococcus xanthus* and *Stigmatella aurantiaca* were collected using the CLSA technique as described previously.[12]

Preparation of [1,1,1-$^2H_3$]-4-Benzyloxy-2-butanol (9). The synthesis of the known compounds[42] **7** and **8** is described

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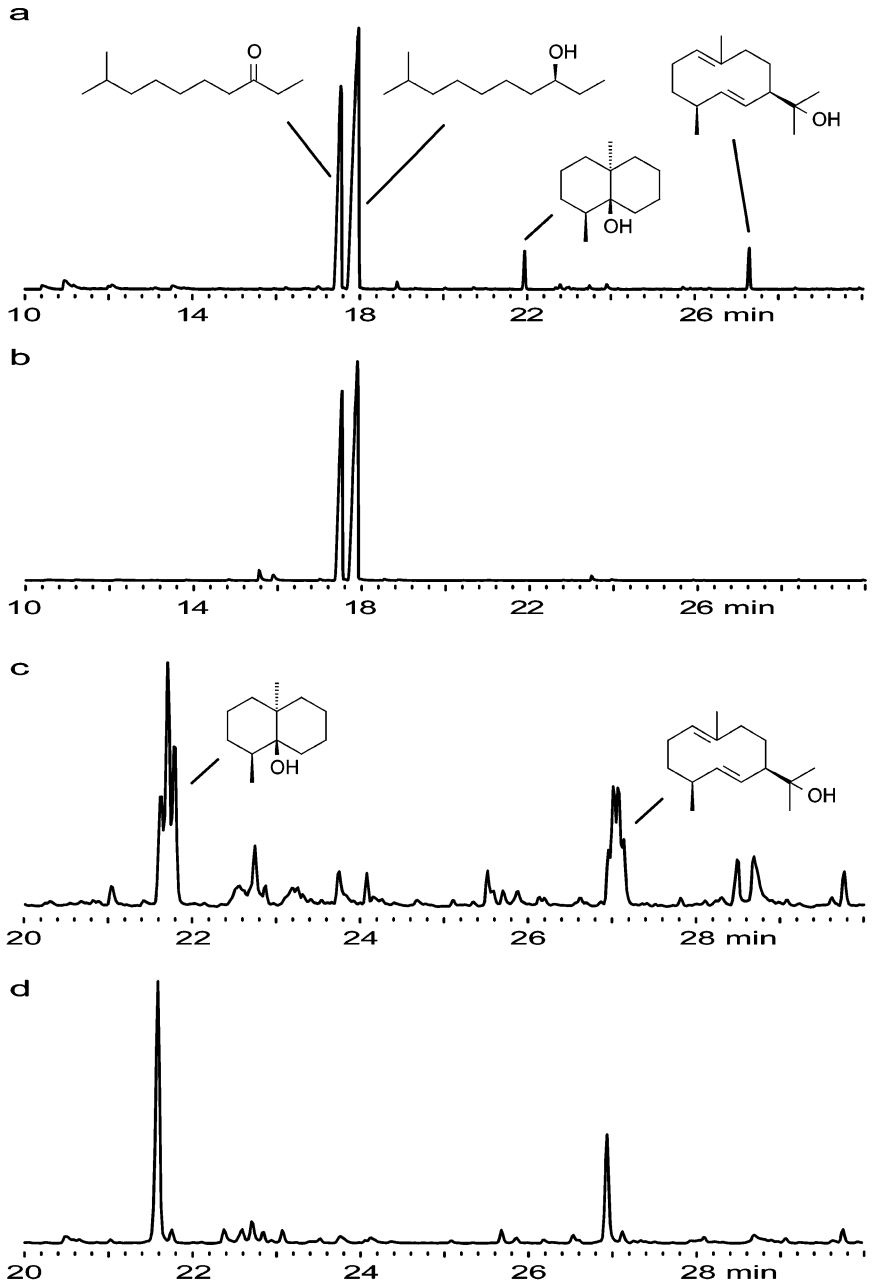

FIGURE 3. Total ion chromatograms of headspace extracts of *Myxococcus xanthus*. Wild-type strain DK1622 (a), a mutant strain (DK5624) that releases no more **1** and **2** (b), DK1622 after feeding of **12** (c), and a mutant strain (DK5624) after feeding of **12** (d).

in the Supporting Information. A solution of **8** (14.8 g, 90.5 mmol, in dry diethyl ether (50 mL)) was added dropwise to an ice-cooled solution of [$^2H_3$]methylmagnesium iodide prepared from [$^2H_3$]methyl iodide (14.5 g, 100 mmol, 99% ^2H) and Mg (2.43 g, 100 mmol) in dry diethyl ether (250 mL). The reaction mixture was stirred overnight at room temperature and then quenched by the addition of 2 N HCl. The aqueous layer was extracted three times with diethyl ether. The combined organic layers were dried with $MgSO_4$ and concentrated. The residue was purified by column chromatography on silica gel (eluent: pentane/diethyl ether, 1:1) to give **9** (14.9 g, 90%) as a colorless liquid. The deuterium content was determined to be >98% by ^1H NMR.

9. R_f = 0.25; I = 1466. ^1H NMR (400 MHz, $CDCl_3$): δ = 7.25−7.36 (m, 5H), 4.51 (s, 2H), 3.99 (dd, J = 8.1, 2.9 Hz, 1H), 3.60−3.72 (m, 2H), 2.91 (br s, 1H), 1.67−1.81 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 137.9 (C), 128.4 (2 × CH), 127.7 (CH), 127.6 (2 × CH), 73.2 (CH_2), 69.0 (CH_2), 67.3 (CH), 38.1 (CH_2). MS (EI) *m/z* (%) = 164 (15), 147 (1), 120 (16), 107 (40), 91 (100), 79 (19), 65 (19), 59 (15), 48 (15).

Preparation of [1,1,1-$^2H_3$]-4-Benzyloxy-2-butanone (10a). A solution of **9** (14.9 g, 81.5 mmol) in dry CH_2Cl_2 (50 mL) was added to a suspension of PCC (26.3 g, 122.3 mmol) in dry CH_2Cl_2 (300 mL). The reaction mixture was stirred overnight. Workup and purification as described for **8** yielded **10a** (13.7 g, 93%) as a colorless liquid. The deuterium content was determined to be 91% by ^1H NMR.

10a. R_f = 0.25; I = 1463. ^1H NMR (400 MHz, $CDCl_3$): δ = 7.25−7.36 (m, 5H), 4.50 (s, 2H), 3.73 (t, J = 6.3 Hz, 2H), 2.70 (t, J = 6.3 Hz, 2H). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 207.2 (CO), 138.0 (C), 128.3 (2 × CH), 127.7 (2 × CH), 127.6 (CH), 73.1 (CH_2), 65.2 (CH_2), 43.7 (CH_2). MS (EI) *m/z* (%) = 120 (21),

(42) Cleater, E.; Harter, J.; Ley, S. V. *Heterocycles* **2004**, *62*, 619–634.

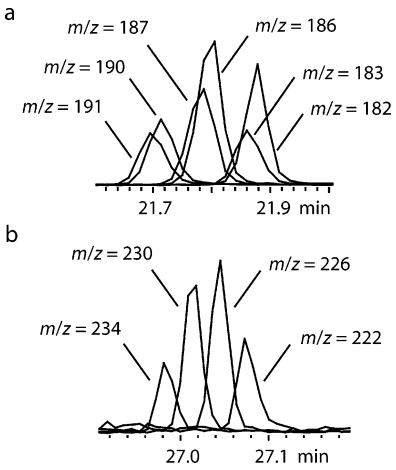

FIGURE 4. (a) Ion chromatograms showing the molecular ions of **1** after feeding DK1622 with **12**: $m/z = 182$ (unlabeled **1**, arising from three units MVA), $m/z = 183$ ([$^2H_1$]-**1**, first unit **12**, second and third unit MVA), $m/z = 186$ ([$^2H_4$]-**1**, two units MVA, second or third unit **12**), $m/z = 187$ ([$^2H_5$]-**1**, two units **12**, second or third unit MVA), $m/z = 190$ ([$^2H_8$]-**1**, first unit MVA, second and third unit **12**), and $m/z = 191$ ([$^2H_9$]-**1**, three units **12**). The ion chromatograms at $m/z = 184$, 185, 188, and 189 showed no significant peak intensities. (b) Ion chromatograms showing the molecular ions of **2** after feeding DK1622 with **12**: $m/z = 222$ (unlabeled **2**, 3 × MVA), $m/z = 226$ ([$^2H_4$]-**2**, 2 × MVA, 1 × **12**), $m/z = 230$ ([$^2H_8$]-**2**, 1 × MVA, 2 × **12**), and $m/z = 234$ ([$^2H_{12}$]-**2**, 3 × **12**). The ion chromatograms at $m/z = 223$, 224, 225, 227, 228, 229, 231, 232, and 233 showed no significant peak intensities.

107 (59), 91 (100), 79 (26), 65 (30), 57 (14), 51 (15), 46 (85), 39 (12).

Preparation of [1,1,1,3,3-$^2H_5$]-4-Benzyloxy-2-butanone (10b) by H/D Exchange. A mixture of **10a** (13.7 g, 75.8 mmol), K_2CO_3 (2.09 g, 15.2 mmol), and D_2O (50 mL) was stirred at room temperature for 7 d. The reaction mixture was extracted three times with diethyl ether. The combined extracts were dried ($MgSO_4$) and concentrated. Purification by column chromatography on silica gel (eluent: pentane/diethyl ether, 2:1) gave **10b** (13.3 g, 94%) as a colorless liquid. The deuterium content was determined to be >95% by ^{1}H NMR.

10b. $R_f = 0.25$; $I = 1458$. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.25−7.36 (m, 5H), 4.51 (s, 2H), 3.72 (s, 2H). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 207.4 (CO), 138.0 (C), 128.4 (2 × CH), 127.7 (2 × CH), 127.6 (CH), 73.2 (CH_2), 65.2 (CH_2). MS (EI) m/z (%) = 120 (12), 107 (65), 91 (96), 77 (35), 65 (27), 59 (16), 51 (13), 46 (100), 39 (11).

Preparation of Ethyl [4,4,6,6,6-$^2H_5$]-5-Benzyloxy-3-hydroxy-3-methylpentanoate (11). As described by Nakamura,[43] freshly cut Li (0.66 g, 94.8 mmol) was added to a solution of naphthalene (12.1 g, 94.8 mmol) in dry THF (50 mL). The reaction mixture immediately turned to dark green and was stirred until the Li was totally consumed. $ZnCl_2$ (12.9 g, 94.8 mmol) was dried at 120 °C in vacuo for 2 h and then dissolved in dry THF (50 mL). The resulting solution was added dropwise to the solution of lithium naphthalenide to give a fine slurry of highly reactive Rieke zinc.[37,38] Caution! Rieke zinc might spontaneously ignite on air and should always be handled in an inert gas atmosphere. Subsequently, a solution of ethyl bromoacetate (15.2 g, 91.0 mmol) and **10b** (13.2 g, 71.2 mmol) in dry THF (50 mL) was added dropwise. The Zn was consumed; the resulting reaction mixture was stirred overnight, and then quenched by the addition of 2 N HCl. The aqueous layer was extracted three times with diethyl ether. The combined extracts were dried with $MgSO_4$. The solvents were removed under reduced pressure. Column chromatography on silica gel (eluent: pentane/diethyl ether, 1:1) furnished **11** (15.2 g, 79%) as a colorless oil. The deuterium content was determined to be >95% by ^{1}H NMR.

11. $R_f = 0.43$; $I = 1915$. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.25−7.37 (m, 5H), 4.49 (s, 2H, CH_2), 4.13 (dq, $J = 7.2$, 1.8 Hz, 2H), 3.95 (br s, 1H), 3.67 (s, 2H), 2.57 (d, $J = 15.2$ Hz, 1H), 2.49 (d, $J = 15.3$ Hz, 1H), 1.24 (t, $J = 7.2$ Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 172.1 (CO), 137.6 (C), 128.0 (2 × CH), 127.3 (CH), 127.3 (2 × CH), 72.9 (CH_2), 70.2 (C), 66.5 (CH_2), 60.1 (CH_2), 45.1 (CH_2), 13.8 (CH_3). MS (EI) m/z (%) = 184 (1), 165 (21), 147 (12), 134 (8), 115 (5), 107 (6), 91 (100), 74 (10), 65 (11), 46 (19).

Preparation of [4,4,6,6,6-$^2H_5$]Mevalonic Acid Lactone (12). Benzyl ether **11** (2.71 g, 10.0 mmol) was added to a suspension of Pd/C (532 mg, 0.50 mmol, 10% Pd) in diethyl ether (20 mL). The mixture was stirred in a H_2 atmosphere at room temperature for 2 h, filtered, and concentrated. Column chromatography on silica gel (eluent: diethyl ether) gave **12** (0.83 g, 46%) and ethyl [$^2H_5$]mevalonate (**13**, 0.57 g) as colorless liquids. For GC−MS analysis, both products were transformed into TMS derivatives with MSTFA. Compound **13** (0.57 g, 3.15 mmol) was dissolved in dry CH_2Cl_2 (50 mL). A catalytic amount of *p*-toluenesulfonic acid (2 mg, 0.01 mmol) was added. The mixture was stirred for 48 h and concentrated. The residue was purified by column chromatography on silica gel (eluent: diethyl ether) to give another portion of **12** (315 mg, 74% from **13**; total yield of **12**: 1.15 g, 85%). The deuterium content was determined to be >95% by ^{1}H NMR.

12. $R_f = 0.10$; $I = 1385$. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 4.60 (d, $J = 11.3$ Hz, 1H), 4.34 (d, $J = 11.3$ Hz, 1H), 2.71 (br s, 1H), 2.66 (d, $J = 17.4$ Hz, 1H), 2.52 (d, $J = 17.3$ Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 170.5 (CO), 68.0 (C), 65.9 (CH_2), 44.6 (CH_2). MS (EI) m/z (%) = 192 (12), 162 (11), 150 (100), 133 (5), 118 (44), 103 (11), 73 (39), 59 (6), 46 (15).

13. $R_f = 0.25$; $I = 1500$. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 4.21 (br s, 1H), 4.18 (q, $J = 7.1$ Hz, 2H), 3.88 (dd, $J = 11.1$, 2.0 Hz, 1H), 3.81 (dd, $J = 11.2$, 4.1 Hz, 1H), 3.37 (br s, 1H), 2.62 (d, $J = 15.4$ Hz, 1H), 2.48 (d, $J = 15.4$ Hz, 1H), 1.29 (t, $J = 7.1$ Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 172.6 (CO), 71.6 (C), 60.6 (CH_2), 59.1 (CH_2), 45.1 (CH_2), 14.0 (CH_3). MS (EI) m/z (%) = 310 (18), 280 (6), 261 (12), 250 (7), 238 (28), 221 (10), 206 (100), 190 (20), 177 (10), 160 (25), 147 (58), 133 (13), 119 (16), 103 (42), 88 (17), 73 (59), 59 (4), 45 (5).

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Supporting Information Available: General methods; preparation of **7** and **8**; ^{1}H and ^{13}C NMR spectra of compounds **7**−**12**. This material is available free of charge via the Internet at http://pubs.acs.org.

JO050449G

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