A Biosynthetic Pathway to Isovaleryl-CoA in Myxobacteria: The Involvement of the Mevalonate Pathway

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Dedicated to Professor Heinz G. Floss on the occasion of his 70th birthday.

A biosynthetic shunt pathway branching from the mevalonate pathway and providing starter units for branched-chain fatty acid and secondary metabolite biosynthesis has been identified in strains of the myxobacterium Stigmatella aurantiaca. *This pathway is upregulated when the branched-chain α-keto acid dehydrogenase gene (*bkd*) is inactivated, thus impairing the normal branched-chain amino acid degradation process. We previously proposed that, in this pathway, isovaleryl-CoA is derived from 3,3-dimethylacrylyl-CoA (DMA-CoA). Here we show that DMA-CoA is an isomerization product of 3-methylbut-3-enoyl-CoA (3MB-CoA). This compound is directly derived from 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) by a decarboxylation/dehydration reaction resembling the conversion of mevalonate 5-diphosphate to isopentenyl diphosphate. Incubation of cell-free extracts of a* bkd *mutant with HMG-CoA gave product(s) with the molecular mass of 3MB-CoA or DMA-CoA. The shunt pathway most likely also operates reversibly and provides an alternative source for the monomers of isoprenoid biosynthesis in myxobacteria that utilize L-leucine as precursor.*

Introduction

Branched-chain fatty acids (BCFAs) constitute more than half of the cellular fatty acids in myxobacteria[1] and some Gram-positive bacteria, for example, Bacillus, Micrococcus, and Sarcina. They cover about 65% of total fatty acids of *Myxococcus xanthus* and about 90% of those in *Bacillus anthracis* and *Bacillus subtilis*.[2,3] In general, BCFAs are important for the control of membrane fluidity of the cells, but in myxobacteria the BCFAs are also involved in cell–cell communication and signaling during development.[4] One of the essential signals in these processes is known as the E-signal and is required for the expression of numerous developmental genes. This signal has been proposed to be particularly mediated by the isovalerate-derived BCFA species *iso*-15:0, *iso*-13:0, and *iso*-17:0.[4]

Isovaleryl-CoA (IV-CoA), 2-methylbutyryl-CoA (2MB-CoA), and isobutyryl-CoA (IB-CoA) serve as primers of branched-chain fatty acids and are derived from the branched-chain amino acids leucine, isoleucine, and valine, respectively. They also function as starter units during the biosynthesis of many microbial secondary metabolites, for example, avermectin,[5] myxothiazol,[6] and myxalamid.[7] An aminotransferase is involved in the conversion of branched-chain amino acids to α-keto acids, and an evolutionarily conserved multienzyme assembly, the branched-chain α-keto acid dehydrogenase complex (BCKAD), is responsible for decarboxylation of the keto acids. Downard and co-workers reported that *bkd* transposon-insertion mutants of *M. xanthus* still produce reduced levels of BCFA and exhibit reduced BCKAD enzyme activity.[8] These facts indicate the existence of a *bkd*-independent pathway for the production of these fatty acid species or the presence of a *bkd* isogene. A similar phenomenon has also been observed during studies on the biosynthesis of the antifungal agent myxothiazol produced by the myxobacterium *Stigmatella aurantiaca* DW4/3–1.[9] Here, a *bkd* mutant retains the production of its total branched-chain fatty acids and of the secondary metabolite myxothiazol, albeit at a reduced level.[9] Incorporation experiments with the wild-type strain showed that IV-CoA, the starter unit of myxothiazol, is derived from leucine.[10] However, an analogous experiment with the *bkd* mutant revealed that leucine was no longer the immediate precursor of the starter unit, which is instead derived from acetate through a novel branch of the mevalonate pathway.[9] 3,3-Dimethylacrylate (DMAA) or its coenzyme A-activated form has been found as intermediate in this pathway; this further suggested a direct link between the mevalonate pathway and branched-chain fatty acid biosynthesis in myxobacteria.[9]

Besides branched-chain fatty acid biosynthesis, the utilization of leucine as the carbon source for sterol biosynthesis has been observed in mammals, plants, and fungi.[11–13] However,

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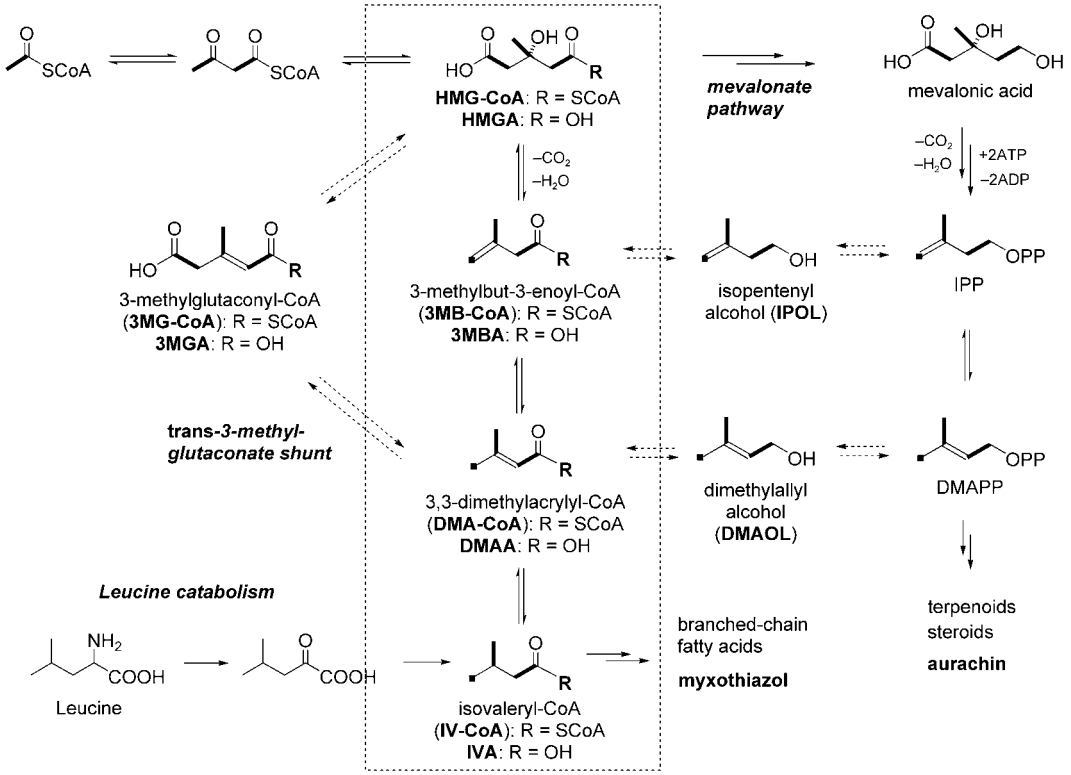
this phenomenon is mainly due to a complete degradation of leucine to acetyl-CoA in the mitochondrion before this compound is transferred and used further in the cytosol as the precursor for the biosynthesis of a number of metabolites, for example, fatty acids and steroids. However, Goad and co-workers reported that the trypanosome *Leishmania mexicana* efficiently utilizes the amino acid leucine for steroid production, and found that the leucine skeleton is incorporated, after degradation to IV-CoA, into the isoprenoid pathway leading to sterol.[14] It was suggested that the integration of leucine into the mevalonate pathway might take place through the leucine degradation pathway, in which leucine is converted to IV-CoA, then 3,3-dimethylacrylyl-CoA (DMA-CoA), 3-methylglutaconyl-CoA (3MG-CoA), and HMG-CoA (Scheme 1). The last compound is then directly reduced to mevalonic acid, the precursor of steroids. Alternatively, leucine might be incorporated into isoprenoids through the hypothetical *trans*-3-methylglutaconate pathway, in which one of the intermediates in leucine degradation (e.g., 3MB-CoA or DMA-CoA) is directly reduced to the corresponding alcohol (isopentenyl alcohol (IPOL) or dimethylallyl alcohol (DMAOL)) followed by a phosphorylation to enter the mevalonate pathway.

To investigate the metabolic route that links the mevalonate pathway and IV-CoA biosynthesis in myxobacteria, we have therefore undertaken the studies described here using wild-type and *bkd* mutants of *S. aurantiaca* Sg a15 (producer of the isoprenoid aurachin) and *S. aurantiaca* DW 4/3–1.[9] The characterization of this alternative pathway on the molecular level is critical for our understanding of its regulation and function in myxobacteria, and it could also be a model for the investigation of similar pathways in various other microorganisms.

Results

Involvement of *trans*-3-methylglutaconate in the alternative pathway to IV-CoA

To evaluate whether 3MG-CoA is involved in the alternative pathway from acetyl-CoA to IV-CoA, deuterium-labeled 3MGA was synthesized and fed to the *bkd* mutants of the myxothiazol producer. The synthesis was initiated from methyl acetoacetate by employing a modification of the method reported by Kuchkova et al.[15] and using D_2SO_4/D_2O and KOD/D_2O as deuterium sources. The product was identified based on direct comparisons of its TLC and mass spectrometry with those of an unlabeled analogue. Incorporation experiments with [D_6]3MGA and the *bkd* mutants of *S. aurantiaca* DW 4/3–1 showed that the compound was not incorporated into myxothiazol and branched-chain fatty acids. 3-Hydroxy-3-[D_3]methylglutaric acid ([D_3]HMGA) was then fed to the wild-type and the *bkd* mutant of myxothiazol producers. HMGA is a free acid analogue of HMG-CoA and known as an antilipemic agent that acts by interfering with the enzymatic steps involved in the conversion of acetyl-CoA to HMG-CoA, as well as inhibiting the activity of mammalian HMG-CoA reductase. However, it was also expected that HMGA would be activated to HMG-CoA and utilized further for the biosynthesis of steroids and IV-CoA in *S. aurantia*ca. In fact, in the presence of this compound, both



Scheme 1. *Possible pathways connecting mevalonate and IV-CoA. The dashed box shows the most likely intermediates involved in the shunt pathway. The solid arrows show reactions that are likely to occur in the new shunt pathway and the dashed arrows show those that are not likely to occur.*

the wild-type and the *bkd* mutant strains of *S. aurantiaca* DW 4/3–1 grew normally and produced the antibiotic. However, no incorporation of [D_3]HMGA into myxothiazol could be detected in the LC-MS analysis.

Synthesis and evaluation of [D_5]3MBA, [D_7]IPOL, and [D_8]DMAOL as possible intermediates in IV-CoA biosynthesis

3MB-CoA, IPP and its free alcohol (IPOL), or DMAPP and DMAOL (3-methylbut-2-en-1-ol) are possible intermediates that might link acetyl-CoA with IV-CoA through the mevalonate pathway (Scheme 1). While DMAOL was proposed to be one of the intermediates in the *trans*-3-methylglutaconate shunt,[14,16] 3MB-CoA has never been associated with any related biosyntheses. To determine whether any of these compounds is involved in IV-CoA biosynthesis, the isotopically labeled corresponding free acids, [D_5]3MBA, [D_7]IPOL, and [D_8]DMAOL were synthesized and fed to the myxothiazol producer (strain EBS7). Thus, [D_5]3MBA was prepared from [D_6]DMAA[9] by treating the latter with lithium diisopropylamide (LDA) at −78 °C for 1 h. The reaction was quenched with 10% hydrochloric acid solution to give a mixture of [D_5]DMAA and [D_5]3MBA in a ratio of about 4:6. By using silica gel chromatography, [D_5]3MBA could be isolated as yellowish syrup (% atom D > 95% as judged by GC-MS and ^{1}H NMR data). In contrast to [D_5]DMAA, the product [D_5]3MBA lacks UV absorption at 254 nm, but its spot on TLC can be easily visualized by potassium permanganate exposure. Reduction of [D_5]3MBA with lithium aluminum deuteride ($LiAlD_4$) at room temperature gave [D_7]IPOL (% atom D > 85%), whereas the reaction of ethyl [D_6]3,3-dimethylacrylate with the same reducing agent gave [D_8]DMAOL (% atom D > 90%), the fragmentation pattern of which in the GC-MS analysis is consistent with that of the authentic compound, 3-methylbut-2-en-1-ol (Aldrich). Ethyl [D_6]3,3-dimethylacrylate was prepared from [D_6]acetone and triethyl phosphonoacetate employing the Wittig/Wadsworth–Emmons reaction.[9]

Mass spectral analysis of the biosynthetic products revealed that [D_5]3MBA was the only compound that was incorporated into myxothiazol (45%; Table 1). No deuterium incorporation into myxothiazol was observed in the feeding experiments with [D_7]IPOL and [D_8]DMAOL. Parallel incorporation experiments with [D_6]DMAA and [D_9]IVA were also performed for comparisons, in which both compounds were incorporated into myxothiazol with incorporations of 40% and 55%, respectively. Similar results were obtained for the incorporation of [D_5]3MBA into BCFA (Table 2).

Incorporation of mevalonolactone into aurachin, but not into myxothiazol

The nonincorporation of [D_8]DMAOL and [D_7]IPOL into myxothiazol suggests that the shunt pathway is not bridged by either IPP or DMAPP. To further prove this assumption, feeding experiments with [2-^{13}C] mevalonolactone to the *bkd* mutants of both the myxothiazol and the aurachin producers were carried out. The products were isolated and analyzed by ESI-MS and the results showed that mevalonolactone was incorporated into aurachin (~20%) but not into myxothiazol in *S. aurantiaca* DW4/3–1/EBS7.

Table 1. *Incorporation of isotopically labeled intermediates into myxothiazol and aurachin.*[a]

Compounds	Amount Fed [mM]	myxothiazol DW4/3–1	myxothiazol DWEBS7	aurachin Sg a15	aurachin Sg aEBS7
[D_6]3MGA	1	–	–	–	–
[D_3]HMGA	1	–	–	NT	–
[D_5]3MBA	1	–	++++	+	+++
[D_6]DMAA	1	–	++++	+–	++
[D_9]IVA	1	NT	++++ +	NT	+++
[D_7]IPOL	1	–	–	–	–
[D_8]DMAOL	1	–	–	–	–
[D_{10}]leucine	3.8	++++ +	–	+	NT
[2-^{13}C]mevalonolactone	1	NT	–	NT	+

[a] –: 0%, +–: 1–5%, +: 5–15%, ++: 16–25%, +++: 26–35%, +++ +: 36–45%, +++++: 46–55%; NT: not tested.

Table 2. *Incorporation of [D_6]DMAA and [D_5]3MBA into fatty acids.*

Fatty acid	Sg a15 [D_6]DMAA	Sg a15 [D_5]3MBA	Sg aEBS7 [D_6]DMAA	Sg aEBS7 [D_5]3MBA
iso-15:0	0	0	33	74
iso-16:0	0	0	51	75
iso-17:0	0	0	36	80
iso-17:0 2OH	0	0	39	77
iso-17:0 3OH	0	0	35	60

Enzymatic decarboxylation/dehydration of HMG-CoA

In further attempts to prove a direct involvement of HMG-CoA in the shunt pathway, in vitro enzymatic reactions were carried out with cell-free extracts of the *bkd* mutant and the wild-type strain DW4/3–1. The reactions were performed by using HMG-CoA as substrate in the presence of adenosine triphosphate (ATP), dithiothreitol, EDTA, PMSF, and the HMG-CoA-reductase inhibitor mevinolin. The last compound was added to block HMG-CoA reductase, which might exhaust the substrate supply. ESI-MS (negative-ion mode) analysis of the reaction products revealed a significant enzyme activity in the cell-free extract of the *bkd* mutant that converts HMG-CoA to a product with pseudomolecular ions at $m/z = 848$ $[M-H]^-$ and 424 (the doubly charged ion species; Figure 1B). ESI-MS analysis of commercially available standard DMA-CoA (Sigma) and synthetic 3MB-CoA, which was synthesized from 3MBA and coenzyme A by using the procedure reported by Kawaguchi et al.,[17] both gave an identical set of pseudomolecular ions. Tandem mass spectrometry experiments with these compounds did not provide any information to differentiate the two CoA esters. While approximately 10% of the enzyme activity was also observed in the cell-free extract of the wild-type

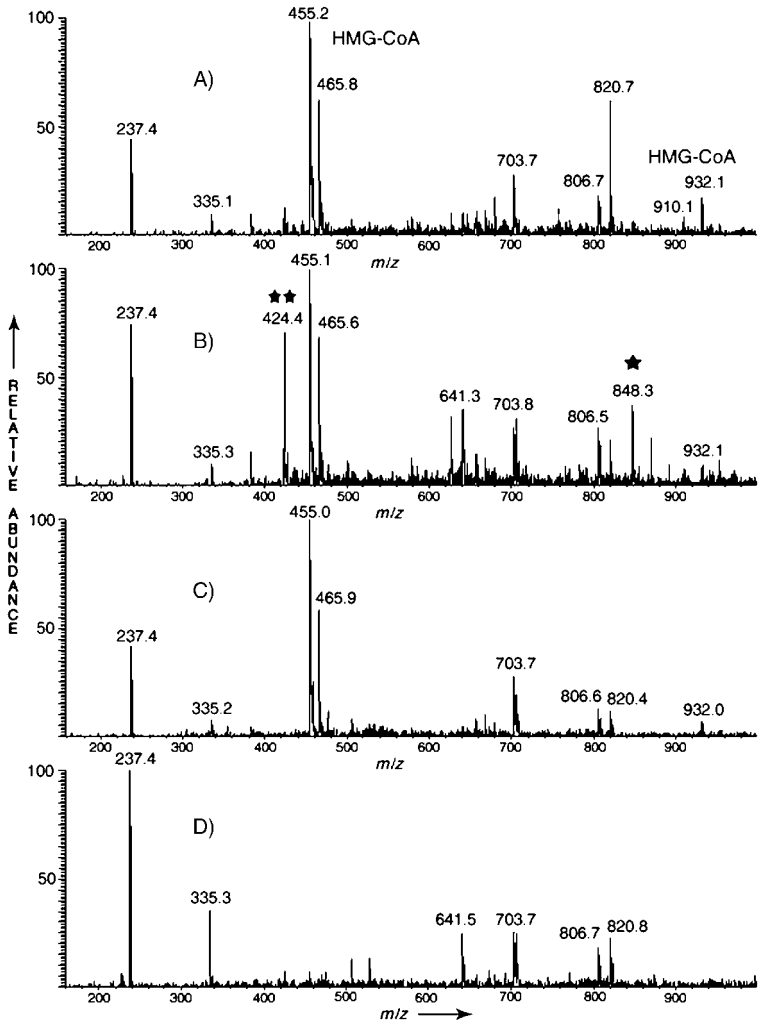
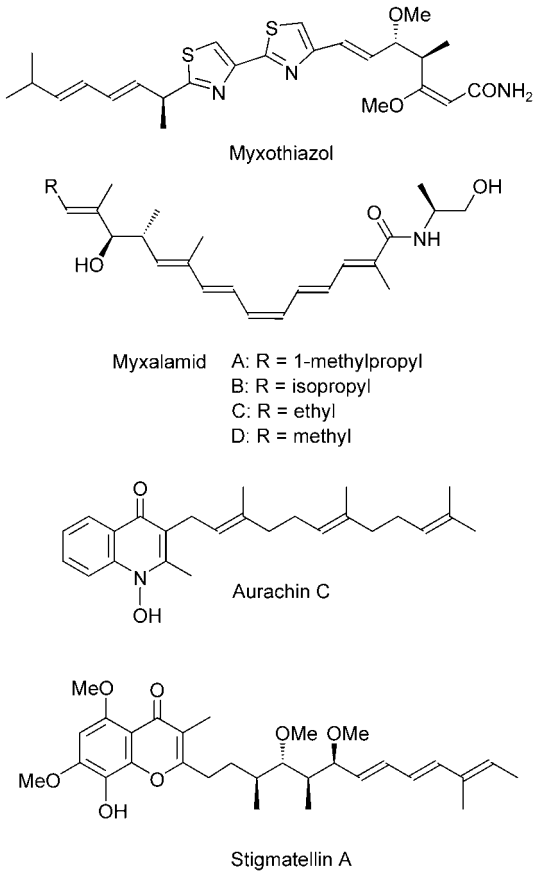


Figure 1. *Negative-ion-mode mass spectral analysis of samples from enzymatic experiments with HMG-CoA and cell-free extracts of* S. aurantiaca *DW4/3–1 (wild-type) and the* bkd *mutant. A) cell-free extract of the wild-type incubated with HMG-CoA; B) cell-free extract of* bkd *mutant incubated with HMG-CoA; C) boiled cell-free extract of* bkd *mutant and HMG-CoA; D) cell-free extract of bkd mutant only. Peaks at 910 and 932 (singly charged) and 455 and 466 (doubly charged) represent pseudomolecular ion species of HMG-CoA and its sodium salt, respectively. Peaks at 848 and 424 (marked as single and double stars, respectively) represent pseudomolecular ion species of the product.*

strain (Figure 1 A), no products could be detected in the reaction with boiled cell-free extract (Figure 1 C). On the other hand, incubation of cell-free extract of the *bkd* mutant and HMG-CoA in the absence of ATP resulted in only trace amounts of the expected product (data not shown).

The fate of leucine in *S. aurantiaca* Sg a15

In many organisms leucine is catabolized to acetyl-CoA before entering the isoprenoid (mevalonate) pathway. However, in some species of *Leishmania*, leucine was found to be efficiently used and incorporated as IVA backbone into isoprenoids. Although a number of possible pathways have been proposed for this direct conversion, no experimental data are available to prove or disprove any of these notions. Because in myxobacteria, IV-CoA is derived from HMG-CoA via 3MB-CoA and DMA-CoA, it is intriguing to explore whether isoprenoids can be derived from L-leucine through the same pathway, albeit in the opposite direction. To investigate this possibility, incorporation experiments with [D_{10}]leucine (3.8 mM) were carried out with the wild-type strain of *S. aurantiaca* Sg a15 (aurachin producer). This strain has been described as producing three structurally unrelated antibiotics, stigmatellin,[18] the myxalamids,[7] and the aurachins (Scheme 2).[19] The aurachins are a complex mixture of quinoline alkaloids containing sesquiterpene side chains that are normally derived from the mevalonate pathway.[20]



Scheme 2. *Chemical structures of secondary metabolites isolated from* S. aurantiaca *DW4/3–1 (myxothiazol) and* S. aurantiaca *Sg a15 (myxalamid, aurachin, and stigmatellin).*

As leucine is utilized as substrate in protein biosynthesis and is thus involved in primary metabolism, the incorporation experiments require a high concentration of labeled leucine in order to observe a significant incorporation of the isotope-label into the secondary metabolite. Nevertheless, leucine was found to be incorporated intact $[M+H+5]^+$ (from incorporation of leucine derived DMAPP) and $[M+H+6]^+$ (from incorporation of leucine derived IPP; ~10% for both isotopomers)

into the isoprene units of the aurachins. This result suggests that the shunt pathway might operate reversibly in myxobacteria. Very similar results were observed for the incorporation of label into cycloartenol[21] with approximately 15% incorporation into the $[M-HOSiMe_3+5]^+$ and the $[M-HOSiMe_3+6]^+$ isotopomers.

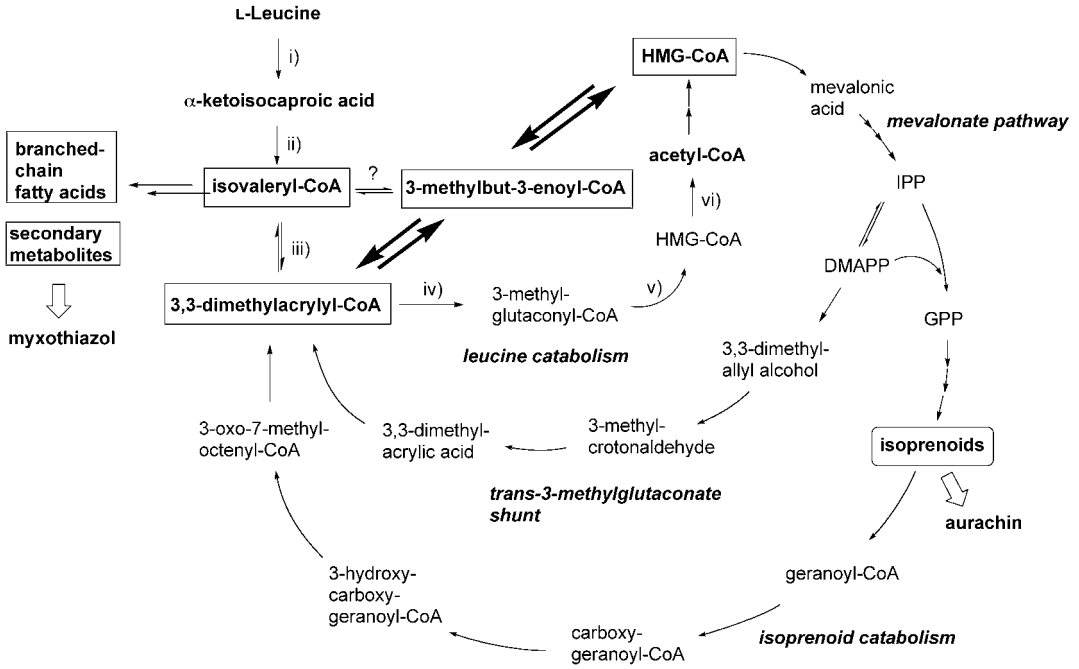
Cloning and inactivation of the *bkd* locus from *S. aurantiaca* Sg a15 and further evaluation of the involvement of the shunt pathway in isoprenoid biosynthesis

To study further the involvement of the shunt pathway in the biosynthesis of isoprenoids in myxobacteria, a *bkd* mutant of the aurachin producer (strain Sg a15/EBS7) was prepared. The mutant was constructed by using a strategy similar to that reported for the myxothiazol producer (strain DW4/3–1/EBS7).[9] Starting from the genomic sequence of *bkd* E1β from *M. xanthus* DK1622, oligonucleotides esgup and esgdown were deduced. By using this set of primers and genomic DNA of strain Sg a15 as template, a PCR product of the expected size (600 bp) could be amplified from the heterologous primer set. This product was cloned into pCR2.1/TOPO, resulting in plasmid pCBS6. The insert of the plasmid was sequenced, revealing an insert size of 592 bp. The *bkd* fragment from strain Sg a15 shows 92.7% identity with the fragment reported from strain DW4/3–1, the deduced proteins show 93.7% identity. The size of the insert from pCBS6 seemed too large to be used for a gene-inactivation experiment by homologous recombination, because the resulting expected 3′ deletion in one of the two copies of the β-subunit after integration is relatively small and might not be of significance for the function of the gene. Therefore, plasmid pCBS6 was used as a template for a PCR reaction with primers esgup and esg2. The corresponding 476 bp PCR product was again cloned and resulted in plasmid pCBS8, which was resequenced to verify the correctness of the clone. Strain Sg a15 was electroporated with pCBS8 and pEBS7 (fragment from DW4/3–1[9]) according to published procedures.[22] Chromosomal DNA from resulting kanamycin-resistant mutants was prepared and analyzed by Southern hybridization with the labeled insert of pEBS7 as a probe. Different strains showing genotypes consistent with site-specific integration into the target gene were named Sg a15/EBS7. These mutants showed a hybridization pattern almost identical to strain DW4/3–1/EBS7.

As expected from the previous experiments, incorporation of $[D_5]$3MBA and $[D_6]$DMAA into branched-chain fatty acids of *S. aurantiaca* Sg a15 and its descendants was observed exclusively in the *bkd* mutants, and no incorporation of label was observed in the wild-type (Table 2). $[D_5]$3MBA, $[D_6]$DMAA, and $[D_9]$IVA were also found to be incorporated into aurachin, while $[D_6]$3MGA, $[D_8]$DMAOL, and $[D_7]$IPOL were not; this provides evidence for the participation of the proposed intermediates in the biosynthesis of isoprenoids. Feeding with $[D_5]$3MBA and $[D_6]$DMAA both gave $[M+H+4]^+$ and $[M+H+5]^+$ as the predominant pseudo-ion species (~35% incorporation for 3MBA and ~15% for DMAA) together with other pseudo-ions that have different levels of deuterium label (isotopomers with various percentage incorporations). On the other hand, $[D_9]$IVA predominantly gave $[M+H+5]^+$ and $[M+H+6]^+$ pseudo-ion species (~25% incorporation); this is consistent with the loss of three deuterium atoms due to dehydrogenation and isomerization reactions to 3MB-CoA. The same phenomenon was also observed in the experiments with L-$[D_{10}]$leucine, in which at least four deuterium atoms are lost during transamination, dehydrogenation, and isomerization processes. An unspecific wash out of one or more deuteriums also appeared to occur during the conversion of 3MB-CoA to the isoprene moieties of aurachin, as pseudomolecular ions with lower levels of deuterium labeling were also observed.

Discussion

A shunt pathway from acetyl-CoA to IV-CoA via DMA-CoA in myxobacteria could occur by various routes including a number of possible intermediates (Scheme 1). First, it may branch from the mevalonate pathway at HMG-CoA via either 3MB-CoA or 3MG-CoA. The involvement of the former compound requires a dehydration/decarboxylation of HMG-CoA, whereas the intermediacy of the latter compound would require stepwise reactions. Alternatively, the shunt pathway may branch at a point further downstream in the mevalonate pathway, for example, via isopentenyl diphosphate (IPP) or dimethylallyl diphosphate (DMAPP). If the latter is true, a dephosphorylation step is required prior to the oxidation of the alcohol to the carboxylic acid by the action of an alcohol dehydrogenase and an aldehyde dehydrogenase, as has been proposed by Edmond and Popják.[16] The carboxylic acid is then converted to the CoA ester and reduced to IV-CoA. 3MG-CoA appeared to be one of the likely intermediates due to its involvement in the leucine catabolism pathway in mammals and plants (Scheme 3). It was also proposed to be involved in the transfer of carbon atoms from mevalonate to *n*-fatty acids in mammals and insects (known as the *trans*-3-methylglutaconate shunt).[16,23] In addition, the free acid is present in the organic acid excretion in humans with HMG-CoA lyase deficiency.[24] While it is not clear whether the mammalian leucine catabolism processes operate fully in myxobacteria, it was intriguing to investigate the existence of such a pathway in *S. aurantiaca* in connection with the possibility of its involvement in the alternative shunt pathway from HMG-CoA to IV-CoA. We assumed that the conversion of HMG-CoA to IV-CoA takes place through a reverse direction of leucine catabolism. However, this turned out to be an unlikely scenario, as feeding experiments with a number of possible intermediates did not support this hypothesis. The nonincorporation of 3MGA into myxothiazol suggests that the *trans*-3-methylglutaconate shunt pathway is not involved in the alternative biosynthetic pathway to IV-CoA in myxobacteria. However, this notion suffers from the classical possibility of cellular uptake problems, in which the dicarboxylate compound might not be able to freely penetrate the cell membranes or might not be activated to its CoA ester for further utilization in the cell. This is underlined by the results from feeding experiments with another dicarboxylic acid, $[D_3]$HMGA, which resulted in no effect on the



Scheme 3. *The shunt pathway from HMG-CoA to IV-CoA identified in this study together with other possible pathways present in other organisms. The question mark indicates a possibility of 3MB-CoA directly reduced to IV-CoA and vice versa. The enzymes of leucine catabolism are: i) BCAA aminotransferase, ii) BCKAD complex, iii) isovaleryl-CoA dehydrogenase, iv) 3-methylcrotonyl-CoA carboxylase, v) 3-methylglutaconyl-CoA hydratase, vi) HMG-CoA lyase.*

growth of the bacteria. Neither did it result in the incorporation of the compound into myxothiazol. Even if 3MGA and HMGA can make their way into the cells, they may not be further processed if the appropriate activating enzyme that converts the free acid to its CoA ester is not present. On the other hand, the free monocarboxylic acids appear to be fairly well taken up by the cells and are converted to their activated forms. In the form of their *N*-acetylcysteamine (SNAC) derivatives, which resemble coenzyme A esters, they were also able to penetrate the cell membranes. However, the amounts of incorporation into myxothiazol are lower than those of the free acids (T.M., M. Xu, unpublished data). It is assumed that the SNAC esters are not directly utilized by the enzymes, but are rather hydrolyzed and activated to their coenzyme A esters. However, the activation process might not be equally effective for all compounds. Data from a number of experiments consistently indicate that percent incorporations of $[D_6]$DMAA into myxothiazol and aurachin were lower than those of $[D_5]$3MBA and $[D_9]$IVA. Whether or not the substrate specificity of cellular coenzyme A ligases is responsible for the observed discrepancy remains to be biochemically elucidated. Alternatively, DMA-CoA might not be directly involved in the pathway, and its incorporation might take place exclusively via 3MB-CoA after isomerization. The latter compound can be reduced directly to IV-CoA or carboxylated and hydrated to HMG-CoA.

Nevertheless, the incorporation of $[D_5]$3MBA, $[D_6]$DMAA, and $[D_9]$IVA into myxothiazol, BCFA, and aurachin provided new insight into an alternative pathway connecting the mevalonate pathway and the BCFA biosynthesis in myxobacteria. This is strongly supported by the results of in vitro experiments, in which cell-free extracts of *bkd* mutants in the presence of ATP efficiently convert HMG-CoA to a product with a pseudomolecular ion at $m/z = 848$ $[M-H]^-$, which could be rationally interpreted as a decarboxylation/dehydration product of HMG-CoA ($M_W = 911$). This decarboxylation/dehydration reaction of HMG-CoA resembles the reaction from mevalonate 5-diphosphate to IPP. A dedicated enzyme, which is upregulated in the mutant strain, is believed to be responsible for this conversion, as the cell-free extract of the wild-type strain did not show a comparable activity. The enzyme presumably requires ATP for its activity, because only trace amounts of the expected product could be observed in the absence of exogenous ATP. In addition, no molecular ion peak correlating to 3MG-CoA ($M_W = 893$) was observed; this further suggests that the involvement of the normal leucine catabolism is unlikely. A direct conversion of HMG-CoA to 3MB-CoA also indicates that HMG-CoA is the branching point that connects the mevalonate pathway to BCFA biosynthesis. Participation of the mevalonate pathway further downstream is less likely, as none of mevalonolactone, IPOL, or DMAOL was incorporated into myxothiazol. We believe that some of the HMG-CoA decarboxylation/dehydration product, 3MB-CoA, in the reaction mixture might have been converted further to DMA-CoA, as an enzyme capable of such conversion may be present in the cell-free extract. However, attempts to distinguish the two isomers by means of HPLC and mass spectrometry have been unsuccessful.

The involvement of the shunt pathway in isoprenoid biosynthesis is also revealed by the ability of *S. aurantiaca* to efficiently synthesize steroids from leucine.[21] Presently, this phenomenon has only been clearly demonstrated in the trypanosome *Leishmania mexicana* and related species.[14] However, it was proposed that the integration of leucine into the mevalo-

nate pathway in *Leishmania* takes place through the normal leucine-degradation pathway to give HMG-CoA, which is then directly reduced to mevalonic acid, or through the hypothetical *trans*-3-methylglutaconate pathway. While both proposed pathways are equally attractive, it will be intriguing to determine if the alternative shunt pathway identified in this work is also present in *Leishmania* and other species. A complete understanding of the biosynthetic pathways to IV-CoA and isoprenoids in pathogenic bacteria and other infectious agents will potentially lead to new ways of combating diseases caused by these organisms.

Experimental Section

Instruments and chemicals: The ^{1}H and ^{13}C NMR spectra were recorded on Bruker AF-300 or AM-500 NMR spectrometers with *Mac*NMR 5.5 PCI as the instrument controller and data processor. Low-resolution mass spectra were recorded on a Bruker–Esquire (electrospray ionization) and a ThermoFinnigan LCQ Advantage (electrospray and atmospheric pressure chemical ionizations) liquid chromatograph–ion trap mass spectrometer. A Bruker APEX III 47e Fourier Transform (Ion Cyclotron Resonance) mass spectrometer was used for the high-resolution mass spectrometry. A quadrupole mass spectrometer (Hewlett Packard model 5971A) with gas chromatograph (model 5890) inlet, was used for the GC-MS experiment. A ISF-4V shaker, Adolf Kuhner AG, was used for the fermentation. All synthetic reactions were carried out under an atmosphere of dry argon at room temperature in oven-dried glassware, unless otherwise noted. Reactions were monitored by TLC (silica gel 60 F_{254}, Merck) with detection by UV light or by alkaline permanganate or $Ce(SO_4)_2/H_2SO_4$ solutions. Column chromatography was performed on 230–400 mesh silica gel (Aldrich). For HPLC, a Beckman System Gold Programmable Solvent Module was used with a Beckman System Gold Diode Array Detector Module. All non-isotope-labeled chemicals were purchased from Aldrich or Sigma and used without further purification, unless otherwise noted. [D_2]Sulfuric acid was purchased from Isotec, Inc. [D_6]Acetone, [D_4]methanol, $CDCl_3$, and D_2O were purchased from Cambridge Isotope Laboratories, Inc. [D_9]Isovaleric and 3-hydroxy-3-[D_3]methylglutaric acids were purchased from CDN Isotopes, Inc. DL-[D_{10}]leucine was purchased from Campro Scientific.

DNA manipulations, analysis, sequencing, and PCR: Routine genetic procedures, such as genomic and plasmid DNA isolations, restriction endonuclease digestions, DNA ligations, and other DNA manipulations, were performed according to standard protocols.[25] PCR was carried out by using *Taq* DNA polymerase (Invitrogen) according to the manufacturer's protocol. The conditions for amplification with the Eppendorf Mastercycler gradient were denaturation: 30 s at 95 °C, annealing: 30 s at 55 °C, and extension: 45 s at 72 °C for 30 cycles and a final extension at 72 °C for 10 min. Sequencing was performed by using the Big Dye RR terminator cycle sequencing kit (PerkinElmer Biosystems), and the gels were run on ABI-377 sequencers. Southern analysis of the genomic DNA was performed by using the standard protocol for homologous probes of the DIG High Prime DNA labeling and detection starter kit II (Roche Molecular Biochemicals). Amino acid and DNA alignments were performed by using the programs of the Lasergene software package (DNASTAR Inc.) and Clustal W.[26]

Cloning and inactivation of the *bkd* locus of β *aurantiaca* Sg a15: Based on the genomic sequence of *bkd* E1b from *M. xanthus* DK1622, a set of oligonucleotides, esgup (5′-ggcatccgcgggtccatct-3′) and esgdown (5′-gcggggccagcagcgactag-3′), were deduced and used to amplify a *bkd* gene fragment from genomic DNA of *S. aurantiaca* Sg a15 by PCR. The product (592 bp) was cloned into pCR2.1/TOPO, resulting in plasmid pCBS6. This plasmid was then used as a template for a PCR reaction with primers esgup and esg2 (5′-aggccttgatgagctcccagtcg-3′) to give a 476 bp PCR product, which was cloned into vector pCR2.1/TOPO (harboring a kanamycin-resistance gene), resulting in plasmid pCBS8. Plasmids pCBS8 and pEBS7 (fragment from DW4/3–1[9]) were electroporated into strain Sg a15 according to published procedures.[22] The correctness of the integration of the plasmid into the chromosome of kanamycin-resistant colonies was verified by Southern hybridization (data not shown).

Incorporation experiments with isotopically labeled compounds: Incorporation experiments with [D_5]3MBA, [D_6]DMAA, [D_9]IVA, [D_6]3MGA, [D_7]IPOL, [D_8]DMAOL, [D_3]HMGA, and DL-[D_{10}]leucine were carried out in tryptone–starch medium (100 mL)[6] after inoculation with 1×10^7 cells. The cells were grown at 28 °C in a gyratory shaker (200 rpm) for 24 h, and the labeled compounds were pulse-fed in equal amounts at 24, 26, 28, 44, 46, 52, 68, and 74 h to give final concentrations of 1 mM. Amberlite XAD-1180 (2 g) were added to each flask after 80 h. The cells and the resin were harvested after 96 h.

Analysis of precursor incorporation into fatty acids, myxothiazol, aurachin, and cycloartenol: Fatty acid analysis was carried out according to published procedures.[9] Myxothiazol was isolated from *S. aurantiaca* DW4/3–1 and the *bkd* mutant by repeated acetone extraction of cell pellets and resins, and the extracts were dried over anhydrous sodium sulfate. The organic solvent was evaporated to dryness, and the residues were dissolved in a small amount of methanol, filtered through an Acrodisc 0.2 μm PVDF membrane (Pall), and subjected to LC-MS analysis (analytical column: Agilent Zorbax SB-C_8; solvent gradient: acetonitrile/0.2% aqueous acetic acid 6:4→100% acetonitrile). Aurachin was isolated from *S. aurantiaca* Sg a15 and its *bkd* mutant (strain EBS7) by using the same procedure described for myxothiazol. Cycloartenol was isolated as described previously.[21] The incorporation rates were obtained by comparisons of the selected ion monitoring mass spectra of the isotope peaks with the parent peaks of the samples.

Cell disruption and enzyme assay: Cells from culture of *S. aurantiaca* DW 4/3–1 and strain EBS7 (50 mL each) were disrupted by sonication in buffer A (sodium phosphate buffer pH 7.4 (50 mM), EDTA (1 mM), dithiothreitol (1 mM), and phenylmethyl sulfonyl fluoride (PMSF) (1 mM)), and the resulting cell-free extracts were clarified by centrifugation (10000 *g*, 20 min, 4 °C). Incubations of HMG-CoA with the cell-free extracts were carried out with substrate (5 mM), ATP (25 mM), mevinolin (1 mM), cell-free extract (50 μL), and buffer A to a final incubation volume of 100 μL. The reaction mixtures were incubated at 30 °C for 3 h and then lyophilized. MeOH (200 μL) was added to the residues, and the mixtures were agitated in a Vortex mixer and allowed to stand for 30 min. The suspensions were centrifuged to remove the precipitates, and the supernatants were subjected to mass spectrometry analysis.

Synthesis of 3MGA: Methyl acetoacetate (9.29 mL) was added dropwise to concentrated H_2SO_4 (16.2 mL) at 0 °C. The mixture was stirred at room temperature for four days, ice (40 g) was added, and the mixture was stirred for another 5 min. The products were extracted with diethyl ether (3×50 mL), and the organic layer was pooled and washed with water and brine. The solution was dried over anhydrous sodium sulfate and concentrated in vacuo to give crude crystals, which were dissolved in MeOH (10 mL) and cooled

to 0 °C. A solution of KOH (20 g) in H_2O (20 mL) was added dropwise over 30 min, and the mixture was stirred at room temperature for 1 h. Water (100 mL) was added, and the mixture was acidified to pH 1 with concentrated hydrochloric acid. The products were isolated with ethyl acetate, and the organic layer was washed with brine and dried over anhydrous sodium sulfate. The organic solvent was evaporated under reduced pressure, and the residue was separated by chromatography over a silica gel column (50 g silica gel, *n*-hexane/EtOAc 2:1→1:1→1:2→EtOAc) to give white crystals of *trans*-3MGA and its *cis*-isomer in a 1:1 ratio. The geometrical configurations of the products were determined by NOE experiments. Repeated recrystallization (in *n*-hexane/Et_2O 1:1) of the mixture increased the *trans*/*cis*-isomer ratio to approximately 95:5. ^{1}H NMR (300 MHz, $CDCl_3$): for the *trans*-isomer $\delta = 2.21$ (d, $J =$ 1.75 Hz, 3H; 6-CH_3), 3.20 (d, $J = 1.0$ Hz, 2H; 4-H_2), 5.82 (m, 2-H, 1H); for the *cis*-isomer $\delta = 1.98$ (d, $J = 1.56$ Hz, 3H; 6-CH_3), 3.76 (s, 4-H_2, 2H), 5.86 (m, 2-H, 1H); Electrospray-MS: *m/z*: 167.0 $[M+Na]^+$; Electrospray-HRMS: *m/z*: 167.0323 $[M+Na]^+$; calcd for $C_6H_8O_4Na$: 167.0320.

Synthesis of [D_6]3MGA: [D_6]3MGA was synthesized according to the procedure developed for the unlabeled analogue. However, D_2SO_4 (96% in D_2O, 99% D), CD_3OD, and a solution of KOD (24 g) in D_2O (24 mL) were used in the synthesis to give [D_6]3MGA as amorphous crystals. $R_f = 0.2$ (*n*-hexane/EtOAc 1:1); Electrospray-MS *m/z*: 173.1 $[M+Na]^+$; electrospray-HRMS: *m/z*: 173.0698 $[M+Na]^+$; calcd for $C_6H_2D_6O_4Na$: 173.0697.

Synthesis of [D_5]3MBA: Butyl lithium solution (5.89 mL, 9.4 mmol) in hexane (1.6 M) was added to a solution of diisopropylamine (1.32 mL, 9.4 mmol) in anhydrous THF (10 mL) at −20 °C under an argon atmosphere. The solution was stirred at room temperature for 15 min, then cooled to −78 °C, and a solution of [D_6]DMAA (500 mg, 4.7 mmol) in THF (7 mL) was added. The reaction mixture was stirred at −78 °C for another 1 h, then quenched by stirring with 10% HCl (10 mL) at −78 °C, then allowed to warm to room temperature. The product was extracted with diethyl ether (2× 15 mL), and the extract was washed with brine, dried over anhydrous sodium sulfate and concentrated in vacuo. The resulting residue was purified by silica gel column chromatography (*n*-hexane/ethyl ether 5:1→4:1→3:1, *v/v*). Concentration of the appropriate fractions afforded [D_5]3MBA (192 mg, 1.83 mmol, 38.9%) as a yellowish liquid: $R_f = 0.35$ (*n*-hexane/Et_2O 2:1, UV negative) and recovered [D_5]DMAA (279 mg, 2.63 mmol, 56%): $R_f = 0.40$ (UV positive). ^{1}H NMR (300 MHz, $CDCl_3$) $\delta = 3.08$ (s, 2-H_2, 2H); for unlabeled analogue: $\delta = 1.84$ (d, $J = 0.7$ Hz, 3H; 3-CH_3), 3.09 (s, 2-H_2, 2H), 4.90 (m, 4-H_a, 1H), 4.96 (m, 4-H_b, 1H); GC-MS for [D_5]3MBA (Agilent J&W Scientific GC DB-5MS column, initial oven $T = 50$ °C (run for 5 min), gradient: 10 °C min^{-1}, $T_{final} = 220$ °C), $R_t = 5.1$ min, EI-MS (%): 105 $[M]^+$, 87, 77, 60 (100), 52; GC-MS for [D_6]DMAA: $R_t = 5.3$ min; EI-MS: 106 $[M]^+$, 88, 60, 52.

Synthesis of [D_8]DMAOL: A solution of $LiAlD_4$ in THF (1.0 M, 7.0 mL) was added to a solution of ethyl [D_6]3,3-dimethylacryloate (500 mg, 3.4 mmol) in THF (12 mL) at −78 °C. The mixture was stirred for 1.5 h at room temperature and quenched by an addition of hydrochloric acid solution (2 N, 10 mL), diethyl ether (50 mL), and water (40 mL). The product was extracted with diethyl ether (3×), and the ether portion was washed with brine and dried over anhydrous sodium sulfate. The product was separated by chromatography over a silica gel column (*n*-hexane/Et_2O 5:1) to give [D_8]DMAOL (160 mg, 50%) as a colorless liquid. $R_f = 0.25$ (*n*-hexane/ethyl acetate 2:1, *v/v*); electrospray-MS *m/z*: 211.2 $[2M+Na]^+$; electrospray-HRMS: *m/z*: 211.2264 $[2M+Na]^+$; calcd for $C_{10}H_4D_{16}O_2Na$: 211.2365; GC-MS for [D_8]DMAOL (Agilent J&W Scientific GC DB-5MS column; oven initial $T = 50$ °C (run for 5 min), gradient: 10 °C min^{-1}, $T_{final} = 220$ °C), $R_t = 3.87$ min; EI-MS (%): 94 $[M]^+$, 76 (100), 57; GC-MS for the authentic compound (Aldrich), $R_t = 4.00$ min; EI-MS (%): 86 $[M]^+$, 71 (100), 53.

Synthesis of [D_7]IPOL: A solution of $LiAlD_4$ in THF (1.0 M, 4.0 mL) was added to a solution of [D_5]3MBA (200 mg, 1.9 mmol) in THF (6.4 mL) at −78 °C. The mixture was stirred at room temperature for 1 h. The reaction was quenched by addition of hydrochloric acid solution (2 N, 6 mL), diethyl ether (30 mL), and water (24 mL). The product was extracted with diethyl ether (3×), and the ether portion was washed with brine and dried over anhydrous sodium sulfate. The product was separated by chromatography over a silica gel column (*n*-hexane/Et_2O 5:1) to give [D_7]IPOL (80 mg, 45%) as a colorless liquid. $R_f = 0.25$ (*n*-hexane/ethyl acetate 2:1, *v/v*); GC-MS for [D_7]IPOL (Agilent J&W Scientific GC DB-5MS column, initial oven $T = 50$ °C (run for 5 min), gradient: 10 °C min^{-1}, $T_{final} = 220$ °C), $R_t = 4.05$ min; EI-MS (%): 93 $[M]^+$, 73 (100), 61, 56.

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