Alkane Activation

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Stereochemical Investigations Reveal the Mechanism of the Bacterial Activation of *n*-Alkanes without Oxygen**

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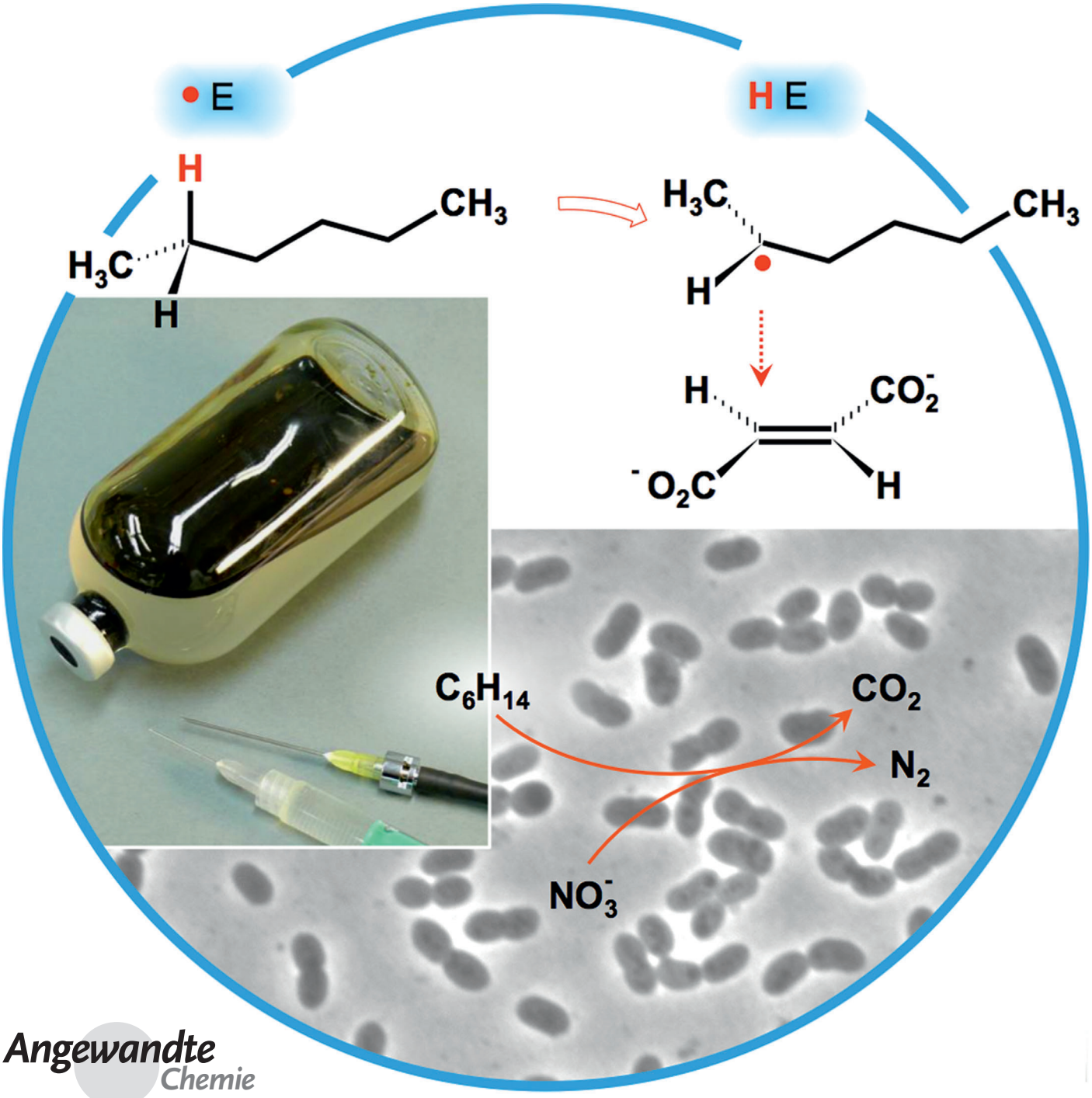
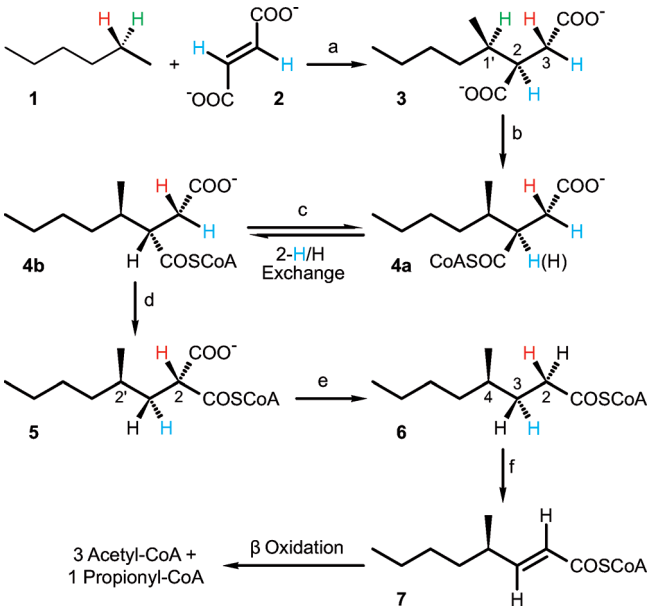
Dedicated to Professor Wittko Francke



Certain heterotrophic bacteria conserve energy by coupling the complete oxidation of *n*-alkanes to carbon dioxide with the reduction of different electron acceptors. Aerobic *n*-alkane-utilizing bacteria, which were first recognized at the beginning of the last century, use dioxygen not only as the terminal electron acceptor, but also as the co-substrate for enzymatic reactions that transform these inert substrates to oxygen-containing metabolites suitable for further degradation.[1] Owing to the specific function of dioxygen in the activation of *n*-alkanes in aerobic bacteria, utilization of these substrates by anaerobic bacteria under strictly anoxic conditions has long been considered impossible. However, in the last two decades numerous denitrifying, iron(III)-reducing, and sulfate-reducing bacteria have been described that oxidize *n*-alkanes and other hydrocarbons to CO_2 in the absence of molecular oxygen.[2] *n*-Alkanes contain exclusively apolar C–H σ bonds, making homolytic mechanisms of enzymatic activation most likely.[3] Accordingly, anaerobic bacteria frequently employ glycyl radical enzymes to achieve selective removal of a hydrogen atom from C2 of *n*-alkanes, resulting in addition of an alk-2-yl radical to the double bond of fumarate (**2**; Scheme 1).[4,5]

Mechanistically, this reaction appears to be similar to the formation of benzylsuccinate from toluene catalyzed by the glycyl radical enzyme benzylsuccinate synthase.[6] Indeed, EPR spectroscopy provided strong evidence for the presence of a glycyl radical enzyme in cells of the denitrifying betaproteobacterium "*Aromatoleum*" strain HxN1 anaerobically grown with *n*-hexane.[4] A tentative (1-methylalkyl)succinate synthase similar to benzylsuccinate synthase has been identified.[7] However, abstraction of a hydrogen atom from any C–H bond of an *n*-alkane is intrinsically more difficult than such a process at the methyl group of toluene.[8] To better understand the mechanism of *n*-alkane functionalization under anoxic conditions, we have studied the stereochemical features of this defining example of C–H activation.



Scheme 1. Initial steps of the anaerobic oxidation of *n*-hexane in the denitrifying strain HxN1[9] including the proposed stereochemistry of the reactions involved as elucidated in this study. a) (1-Methylalkyl)-succinate synthase; b) (1-methylalkyl)succinate-CoA ligase; c) (1-methylalkyl)succinyl-CoA epimerase; d) (2-methylalkyl)malonyl-CoA mutase; e) (2-methylalkyl)malonyl-CoA decarboxylase; f) 4-methylalkanoyl-CoA dehydrogenase.

Analysis of metabolites present in cells of strain HxN1 anaerobically grown with *n*-hexane (**1**) had shown that the formed (1-methylpentyl)succinate (**3**) consists of two diastereoisomers,[4] indicating an apparent imperfect stereoselectivity of the enzymatic reaction. The analogous formation of benzylsuccinate from toluene by benzylsuccinate synthase yields exclusively the *R* enantiomer.[10] Anaerobic incubation of strain HxN1 with perdeuterated *n*-hexane revealed that the hydrogen atom abstracted from C2 of the *n*-alkane is transferred to C3 of the succinate moiety.[4] It has been suggested that subsequent degradation of **3** by means of activation as a coenzyme A thioester, intramolecular rearrangement to (2-methylhexyl)malonyl-CoA (**5**), and decarboxylation leads to 4-methyloctanoyl-CoA (**6**), which is then further degraded by dehydrogenation and β-oxidation (Scheme 1).[9]

To elucidate the configuration at the newly formed stereocenters we synthesized all four stereoisomers of **3** as standards for comparison. This was achieved from racemic and pure (*R*)- and (*S*)-hexan-2-ol through activation of the hydroxy group and displacement with diethyl malonate, followed by alkylation with ethyl bromoacetate, hydrolysis of the ester groups, and decarboxylation (see the Supporting Information). The mixtures of **3** resulting from (*S*)-hexan-2-ol [(2*R*,1′*R*) and (2*S*,1′*R*) isomers **3a** and **3d**] and (*R*)-hexan-2-ol [(2*S*,1′*S*) and (2*R*,1′*S*) isomers **3b** and **3c**] were used directly for analysis, whereas the mixture from racemic hexan-2-ol was separated into the (2*R*,1′*R*)/(2*S*,1′*S*) isomers (**3a** and **3b**) and (2*R*,1′*S*)/(2*S*,1′*R*) isomers (**3c** and **3d**) by fractional recrystal-

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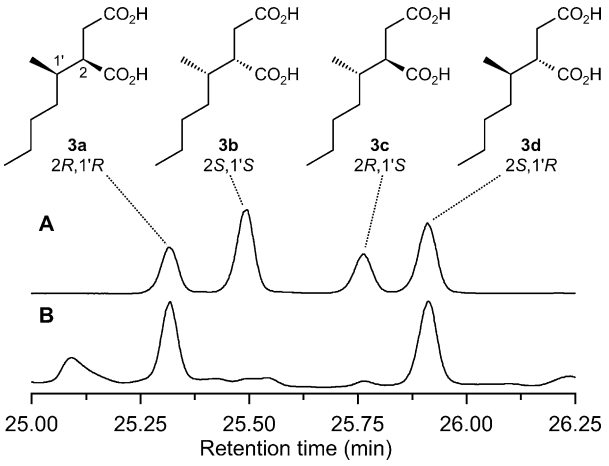
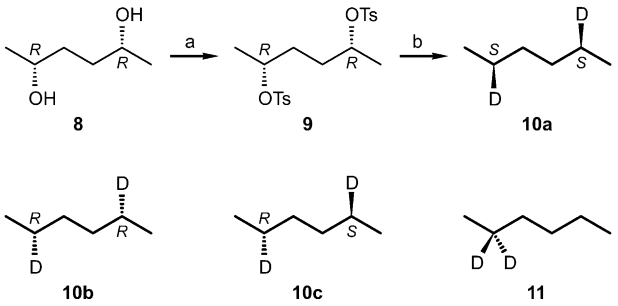


Figure 1. Gas chromatograms showing the specific formation of the (2*R*,1′*R*) and (2*S*,1′*R*) isomers of **3** during anaerobic growth of strain HxN1 with **1**. A) Synthetic mixture of all four stereoisomers; B) products formed by strain HxN1. For GC separation, the stereoisomers of **3** were derivatized with (*R*)-1-phenylethanamine (see the Supporting Information).



Scheme 2. Synthesis of (2*S*,5*S*)-*n*-(2,5-$^2H_2$)hexane (**10a**). a) *p*-Toluenesulfonyl chloride, pyridine in dichloromethane (*p*-toluenesulfonyl = Ts); b) $LiAl^2H_4$ in tetraglyme (see the Supporting Information for full experimental details). Also shown are the structures of other dideuterated *n*-hexanes used in this study.

lization. As shown in Figure 1, strain HxN1 specifically forms equal amounts of **3a** and **3d** during anaerobic growth with **1**. This demonstrates that both configurations at C2 in the succinate moiety are present. However, the stereocenter at C1′ in the products (C2 of the hydrocarbon substrate) has exclusively the *R* configuration. Consequently, solely (*R*)-4-methyloctanoyl-CoA (**6**) is formed as the product of subsequent steps in the degradation pathway as revealed by comparison with synthetic authentic standards of (*R*)- and (*S*)-4-methyloctanoic acid (see the Supporting Information).

The formation of a stereocenter at a secondary alkyl moiety with complete stereocontrol suggested that the abstraction of the hydrogen atom from the *n*-alkane must also proceed in a stereoselective manner. To investigate this point, we traced the fate of deuterium atoms in stereospecifically labeled *n*-(2,5-$^2H_2$)hexane isomers **10a–c** and in *n*-(2,2-$^2H_2$)hexane (**11**) during anaerobic growth of strain HxN1. For this purpose, a strategy for the synthesis of (*R*,*R*)-, (*S*,*S*)-, and (*meso*)-*n*-(2,5-$^2H_2$)hexane from (*S*,*S*)-, (*R*,*R*)-, and (*meso*)-hexane-2,5-diol, respectively, was developed; the synthesis of **10a** is representative of the sequence employed (Scheme 2). Diol **8** was converted into di-*p*-toluenesulfonate **9**, which was reduced by lithium aluminum deuteride in tetraethyleneglycol dimethyl ether (tetraglyme) with inversion of configuration (S_N2) at each stereocenter.[11] We used the high-boiling solvent tetraglyme (bp = 276 °C) so that the pure deuterated *n*-hexane (bp ≈ 69 °C) could be removed from the reaction mixture directly by distillation. The stereochemical assignments for the *n*-(2,5-$^2H_2$)hexane isomers were based on the known sense of chirality of the precursor diols and confirmed by X-ray analysis of the derived di-*p*-toluenesulfonates (see the Supporting Information). The deuterated *n*-hexanes were characterized by ^1H, ^2H, and ^{13}C NMR spectroscopy and by electron ionization mass spectrometry (EIMS; see the Supporting Information). The estimated ^2H content at each isotopically labeled position was greater than 98%. Previously, stereoisomeric *n*-(2,3-$^2H_2$)butanes of relatively low isotopic purity were used to study the aerobic oxidation of *n*-butane by the particulate methane monooxygenase (p-MMO) from *Methylococcus capsulatus* (Bath).[12]

Incubation experiments were performed with cultures of strain HxN1 that had been adapted to *n*-heptane using a mixture of *n*-heptane and a dideuterated *n*-hexane (8:1), to avoid the formation of unlabeled **3**. Rotational symmetry of the *n*-(2,5-$^2H_2$)hexane isomers forces the enzyme to attack a configurationally defined CHD center and simplifies the product analysis. Complete transfer of a deuterium atom to the succinate moiety of **3** was observed upon addition of **10a**, while essentially no deuterium transfer occurred with **10b** (Figure 2). This proves that in the initial step of the catalytic cycle of the (1-methylpentyl)succinate-forming enzyme exclusively the pro-*S* hydrogen atom is abstracted from C2 of **1**. Subsequently, the secondary alkyl species binds with a fumarate molecule on the face opposite to that from which the hydrogen atom was abstracted, resulting in the 1′*R* configuration of **3**. Thus, an overall inversion of configuration at C2 of **1** takes place. The formation of **3** is completed by back transfer of the originally abstracted hydrogen atom, which regenerates the cysteinyl radical. The hydrogen abstraction and addition to fumarate may even occur in a concerted manner, similar to an S_N2 reaction (Scheme 3). Such a mechanism could explain how the high difference in bond dissociation energy (BDE) between a thiol (RS–H) and a C–H bond of a methylene group in an alkane (ΔBDE ≈ 40 kJ mol^{-1})[8] could be overcome. Hence, the highly energetic hex-2-yl radical may not exist as a single enzyme-bound species. The only detectable radical in this reaction would be that at C3 of the succinate moiety stabilized by the adjacent carboxylate, similar to the radical detected in the carbon-skeleton rearrangement catalyzed by glutamate mutase.[13] With **10c** and **11**, mixtures of dideuterated isotopologues of **3** were formed in which the main product was formed by the transfer of the pro-*S* hydrogen atom from C2 and C5, respectively (see the Supporting Information). This indicates a significant primary kinetic isotope effect (≥ 3) for the abstraction of the hydrogen atom.

Considering the proven stereoselectivity of benzylsuccinate synthase,[10] the formation of equal amounts of diaste-

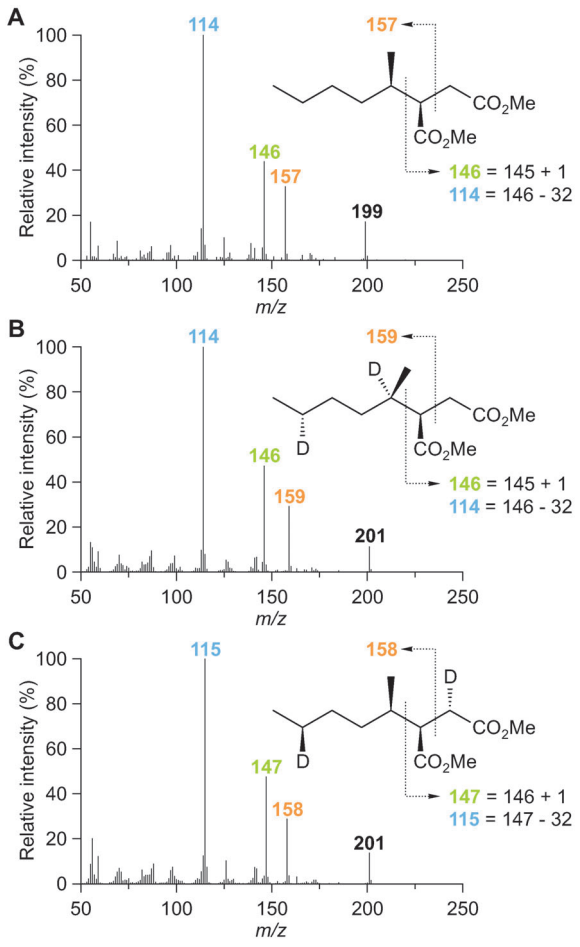
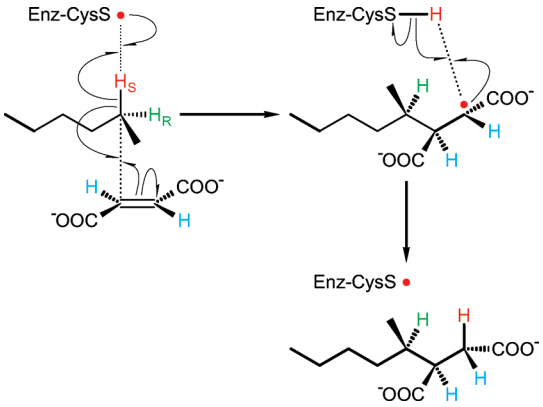


Figure 2. Mass spectra showing the labeling patterns of deuterated isotopologues of **3** (as dimethyl esters after derivatization with diazomethane) formed during anaerobic growth of strain HxN1 in the presence of stereospecifically deuterated *n*-hexanes. A) **3** formed from **1**. B) (1′,4′-$^2H_2$)-**3** formed from **10b**; C) (3,4′-$^2H_2$)-**3** formed from **10a**. Depicted are the mass spectra of the (2*R*,1′*R*) isomers; essentially identical mass spectra were obtained for the corresponding (2*S*,1′*R*) isomers. The fragment ion at *m*/*z* 199 ($[M-31]^+$ resulting from loss of $-OCH_3$ from one of the methyl ester moieties) in (A) is shifted to *m*/*z* 201 in (B) and (C), indicating the presence of two deuterium atoms. The fragment ion at *m*/*z* 157 in (A) is shifted to *m*/*z* 159 and *m*/*z* 158 in (B) and (C), respectively. The fragment ions at *m*/*z* 114 and *m*/*z* 146 in (A) are shifted to *m*/*z* 115 and *m*/*z* 147, respectively, in (C), while no shift is observed in (B). Thus exclusively the pro-*S* hydrogen atom is abstracted from the *n*-alkane.

reoisomers **3a** and **3d** is unlikely to be the result of a non-stereoselective addition of the hex-2-yl radical to fumarate. Rather, the complete exchange with external hydrogen of the deuterium atom at C2 of **3** derived from (2,3-$^2H_2$)fumarate[4] points to an epimerization. A precedent for such a reaction is that catalyzed by α-methylacyl-CoA racemase,[14] an enzyme required for the metabolism of certain 2-methyl fatty acids with *R* configuration, as only the *S* isomers are substrates for β-oxidation. We therefore suggest that the initially formed isomer of **3**, as the CoA-thioester **4**, is epimerized to generate the diastereoisomer required by the putative mutase. Notably, the homologue of α-methylacyl-CoA racemase from *Mycobacterium tuberculosis* belongs to the family III CoA-transferases, representing the only known example of a CoA racemase of this relatively new family.[15] Other members of this superfamily are (*E*)-cinnamoyl-CoA:(*R*)-phenyllactate CoA-transferase and succinyl-CoA:(*R*)-benzylsuccinate CoA-transferase.[16] We thus speculate that in strain HxN1 activation of **3** and epimerization of **4** might be catalyzed by one and the same enzyme.



Scheme 3. Proposed mechanism of the (1-methylpentyl)succinate-forming enzyme reaction. Please note that the active form of the enzyme is generated by abstraction of a hydrogen atom from an adjacent cysteine residue by the glycyl storage radical.[3]

It is notable that exclusively the fumarate- rather than the *n*-hexane-derived hydrogen atom at C3 of **3** migrates during the carbon-skeleton rearrangement of **4** to **5** (Scheme 1).[9] Further, the *n*-hexane-derived hydrogen atom at C2 and the fumarate-derived hydrogen atom at C3 of **6** are exclusively removed during the dehydrogenation to 4-methyloct-2-enoyl-CoA (**7**). This observation is in accord with the expected stereochemistry of hydrogen removal during the first step of β-oxidation catalyzed by acyl-CoA dehydrogenase.[17] Considering that the analogous decarboxylation/transcarboxylation of methylmalonyl-CoA and the intramolecular rearrangement catalyzed by methylmalonyl-CoA mutase proceed with retention of configuration,[18] we propose the overall stereochemical course of the (1-methylpentyl)succinate-forming reaction and subsequent steps leading to **7** shown in Scheme 1. This requires a *syn* addition of **1** to **2**, which is in agreement with the proposed mechanism of benzylsuccinate synthase.[19] The only difference from the conversion of succinyl-CoA via (*R*)- and (*S*)-methylmalonyl-CoA to propionyl-CoA is the postulated decarboxylation/transcarboxylation of (2*R*,2′*R*)-(2-methylhexyl)malonyl-CoA (**5**) rather than the (2*S*,2′*R*) isomer (for details see the Supporting Information). This would require a second epimerization, which is not consistent with retention of the *n*-hexane-derived hydrogen at C2 of **6**.

Overall, our results document a highly stereospecific anaerobic C–H activation of an *n*-alkane with stereochemical features in complete contrast to the aerobic oxidations of methylene groups by cytochrome P450 monooxygenases and other monooxygenases for which a retention pathway is preferred.[12,20] Further investigations to confirm the proposed epimerization of an initially formed diastereoisomer of **3** with defined configuration at C2 are underway.

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