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Biosynthesis of iso-fatty acids in myxobacteria†

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The fatty acid (FA) profiles of the myxobacteria *Stigmatella aurantiaca* and *Myxococcus xanthus* were investigated by acidic methanolysis of total cell extracts and GC or GC-MS analysis. The main components were 13-methyltetradecanoic acid (iso-15:0) and (*Z*)-hexadec-11-enoic acid (16:1, *ω*-5 *cis*). The biosynthesis of iso-FAs was investigated in several feeding experiments. Feeding of isovaleric acid (IVA) to a mutant impaired in the degradation of leucine to isovaleryl-CoA (IV–CoA) (*bkd* mutant) of *M. xanthus* only increased the amount of iso-odd FAs, whereas feeding of isobutyric acid (IBA) gave increased amounts only of iso-even FAs. In contrast, a *bkd* mutant of *S. aurantiaca* gave increased amounts of iso-odd and iso-even fatty acids in both experiments. We assumed that in *S. aurantiaca* α-oxidation takes place. [D_7]-15-Methylhexadecanoic acid (**8**) was synthesised and fed to *S. aurantiaca* as well as [D_{10}]leucine and [D_8]valine to elucidate this pathway in more detail. The iso-fatty acid **8** was degraded by α- and β-oxidation steps. [D_{10}]Leucine was strongly incorporated into iso-odd and iso-even fatty acids, whereas the incorporation rates for [D_8]valine into both types of fatty acids were low. Thus α-oxidation plays an important role in the biosynthesis of iso-fatty acids in *S. aurantiaca*. The incorporation rates observed after feeding of [D_{10}]leucine and [D_8]valine are the highest for iso-17:0 compared to the other acids. This indicates the central role of iso-17:0 in the biosynthesis of iso-FAs. The shorter homologues seem to be formed mainly by α-oxidation and β-oxidation of this acid. After feeding of **8** traces of unsaturated counterparts of this labelled FA occurred in the extracts indicating that desaturases are active in the biosynthesis of unsaturated fatty acids in *S. aurantiaca*.

Introduction

Myxobacteria are of considerable interest because of their complex life cycle, unusual physiology, and broad metabolic potential.[1,2] During the last decades several compounds with pharmacological activity have been isolated from different species such as *Myxococcus fulvus*,[3] *Myxococcus xanthus*,[4] *Stigmatella aurantiaca*,[5] and *Sorangium cellulosum*.[6] The biosynthesis of their metabolites often comprises unusual biosynthetic mechanisms.[7,8] One example is the unexpected effect of *bkd* (*b*ranched chain *k*eto acid *d*ehydrogenase) mutagenesis in *M. xanthus* and *S. aurantiaca*; *bkd* mutants are impaired in the degradation of the branched chain amino acids leucine, valine, and isoleucine to isovaleryl-CoA (IV–CoA), isobutyryl-CoA (IB–CoA), and 2-methylbutyryl-CoA (2MB–CoA), respectively. In streptomycetes, a disruption of the *bkd* locus leads to a total depletion of the cells of any of these thioesters (and therefore also of iso-fatty acids and secondary metabolites that require these thioesters as starting units).[9,10] However, in the two myxobacteria only a reduction of branched chain keto acid dependent secondary metabolite formation and iso-fatty acid biosynthesis is observed. The reason for this is an alternative pathway to synthesise at least IV–CoA and presumably also IB–CoA from acetyl-CoA. This novel pathway branches from hydroxymethylglutaryl-CoA, a central intermediate of the mevalonate dependent isoprenoid biosynthesis.[11,12] In the course of further studies regarding this pathway and its connection to fatty acid biosynthesis in general, we became interested in whether the fatty acid biosynthesis in myxobacteria follows established pathways or shows differences to other bacteria. The investigations were performed on the two species *Stigmatella aurantiaca* and *Myxococcus xanthus*, the fatty acid pattern of which had previously been investigated briefly by Ware and Dworkin and Schröder and Reichenbach, respectively.[13,14] Furthermore, the fatty acid pattern of *M. xanthus* cells is highly dependent on the growth medium.[15] Recently, the bound fatty acids in phosphatidylethanolamine (PE) purified from *M. xanthus* cell membranes have been identified.[16]

In a previous communication we reported on the biosynthesis of iso-fatty acids (iso-FA) in *M. xanthus* and *S. aurantiaca*, including results from genetically modified strains.[17] The iso-FAs with an odd number of carbon atoms (iso-odd FAs) were shown to be generated from the leucine-derived starter IV–CoA, while the iso-even FAs were derived from iso-odd FAs by *α*-oxidation, a new pathway to methyl-branched FAs. The valine-derived starter isobutyryl-CoA (IB–CoA) was not involved in the biosynthesis of these FAs. In the present article we will present the synthesis of the labelled key component **8**. The biosynthesis of iso-even FAs by α-oxidation of iso-odd FAs in *S. aurantiaca* was clearly shown by the degradation of [D_7]-15-methylhexadecanoic acid (**8**) to iso-even FAs and iso-odd FAs with shorter chain length. Furthermore, full details on the fatty acid composition of the investigated strains used in feeding experiments and further conclusions on the fatty acid biosynthesis pathways that operate in these myxobacteria are presented.

Results and discussion

Extracts of liquid cultures of *S. aurantiaca* and *M. xanthus* (wild-type strains and *bkd* mutants) were methanolysed under acidic conditions and analysed by GC–MS. Identifications of the resulting methyl esters of free and bound fatty acids were

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based upon mass spectra[18,19] and gas chromatographic retention indices (see ESI†).[20,21]

The fatty acid profiles of both species are summarised in Tables S1 and S2 of the ESI, respectively†. *S. aurantiaca* cells contained a number of saturated and unsaturated unbranched FAs (C_{10}–C_{18}). The main component of this class was (*Z*)-hexadec-11-enoic acid (16:1, *ω*-5 *cis*, 24.0% of total extract). Some saturated and unsaturated iso-FAs were also present (C_{11}–C_{17}), predominated by 13-methyltetradecanoic acid (iso-15:0, 22.3%) and 15-methylhexadecanoic acid (iso-17:0, 11.9%). In addition, 2-hydroxy and 3-hydroxy FAs (iso-17:0 3OH, iso-15:0 3OH, and 16:0 2OH) were present in minor amounts. Fautz *et al.* already reported on these hydroxy fatty acids as characteristic lipid constituents of *S. aurantiaca* (strain Sg a1) and other gliding bacteria.[22] The fatty acid profile of other myxobacteria also comprises hydroxy fatty acids.[23] In contrast, the *bkd* mutant which is impaired in the generation of methyl-branched starting units from branched amino acids produced significantly reduced relative amounts of branched FAs, whereas the relative amounts of unbranched FAs increased, *e.g.* iso-15:0 (11.9%) and 16:1 (*ω*-5 *cis*, 32.4%).

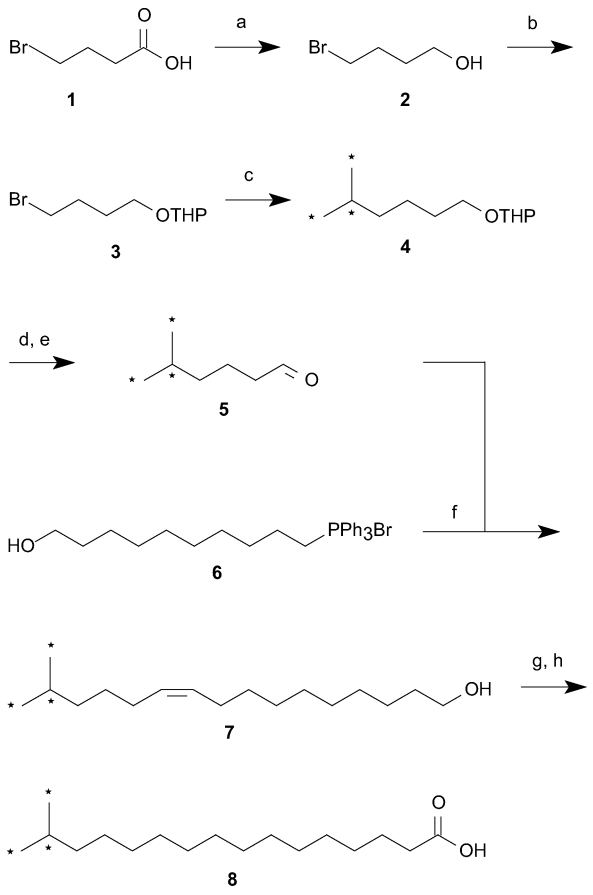
This pattern was repeated in *M. xanthus*. The fatty acid profile consisted of saturated and unsaturated unbranched (C_{10}–C_{17}) and methyl-branched (C_{13}–C_{17}) FAs with 16:1 (*ω*-5 *cis*, 13.8%) and iso-15:0 (37.4%) as major components. 2-Hydroxy and 3-hydroxy FAs (iso-17:0 3OH, iso-17:0 2OH, iso-15:0 3OH, 8:0 3OH, and 16:0 3OH) were also found in low amounts. In the *bkd* mutant the relative amounts of iso-FAs decreased compared to unbranched FAs, reflected by 16:1 (*ω*-5 *cis*) (27.6%) and iso-15:0 (14.6%). This decrease of iso-FAs in combination with an increase of unbranched unsaturated FAs in the *bkd* mutant of *M. xanthus* was reported earlier.[12,15,16,24]

In earlier investigations on *M. xanthus* and *S. aurantiaca* among the unbranched FAs only C_{11}–C_{17} (*M. xanthus*) and C_{12}–C_{17} (*S. aurantiaca*) have been detected, whereas iso-FAs ranged from C_{14}–C_{17} (*M. xanthus*) and C_{13}–C_{17} (*S. aurantiaca*), respectively, with iso-15:0, iso-17:0, and 16:1 as main components in the fatty acid extracts of both species.[13,14] These FAs also predominate in other myxobacteria.[23,25]

In contrast to a total depletion of the cells of any iso-branched fatty acids after disruption of the *bkd* locus as observed in other bacteria (*e.g. Streptomyces*),[9,10] myxobacteria have an alternative pathway to IV–CoA that branches from the mevalonate dependent isoprenoid biosynthesis.[11,12] This pathway is induced in the *bkd* mutants and gives rise to residual iso-FAs.

In order to elucidate the biosynthetic pathways to branched FAs we fed the *bkd* mutants with the methyl-branched acids isobutyric acid (IBA) and isovaleric acid (IVA) known to be formed by the degradation of valine and leucine, respectively (Tables S1 and S2 in the ESI†). The biosynthesis of iso-even FAs normally takes place by chain elongation of IB–CoA with acetate-derived malonyl-CoA, while iso-odd FAs arise from elongation of IV–CoA. We obtained significantly increased amounts only for the expected FAs after feeding the *bkd* mutant of *M. xanthus* with IBA or IVA (*e.g.* increased amounts of 14-methylpentadecanoic acid, iso-16:0, after feeding of IBA, and increased amounts of 13-methyltetradecanoic acid, iso-15:0, after feeding of IVA, respectively), whereas the addition of either IBA or IVA increased the amounts of both iso-odd and iso-even FAs in the *bkd* mutant of *S. aurantiaca*. Therefore we concluded that fatty acid biosynthesis in *S. aurantiaca* proceeds with the involvement of α-oxidation of long chain fatty acids or transformation of IBA to IVA and *vice versa* (see below), whereas the respective pathway should not be active in *M. xanthus*.

The labelled key compound [D_7]-15-methylhexadecanoic acid **8** ([D_7]-iso-17:0) was fed to *S. aurantiaca* to further investigate the participation of α-oxidation in the biosynthesis of iso-FAs. This experiment is particularly important because it gives the unambiguous proof for the formation of iso-even FAs from iso-odd FAs by α-oxidation. As will be shown below, this acid furthermore plays a central role in the FA metabolism of *S. aurantiaca*, because all other iso-FAs with shorter chain length are at least partially generated from this acid *via* α- and/or β-oxidation steps. Compound **8** was prepared starting with 4-bromobutyric acid **1** that was reduced to the corresponding alcohol **2** using borane-dimethyl sulfide (Scheme 1).[26,27] The deuterium label and the methyl branch were introduced in a cross coupling reaction with [D_7]isopropylmagnesium bromide catalysed by $Li_2[CuCl_4]$,[28] after protection of the alcohol with dihydropyran.[29] Deprotection furnished [D_7]-6-methylhexan-1-ol which was transformed into the corresponding aldehyde **5** by PCC oxidation.[30,31] This aldehyde was immediately used in a Wittig reaction with 10-hydroxydecyltriphenylphosphonium ylide prepared from 2-(10-bromodecyloxy)oxane.[32] Unexpectedly, the protecting group was lost during the formation of the phosphonium salt with triphenylphosphane. The obtained unsaturated alcohol [D_7]-(*Z*)-15-methylhexadec-10-en-1-ol **7** was hydrogenated using Pd/C to give [D_7]-15-methylhexadecan-1-ol, and then oxidised with PDC in DMF to yield **8**.[33] The results of the feeding experiments with *S. aurantiaca* are summarised in Table 1 and the total ion chromatogram is presented in Fig. 1.



Scheme 1 Synthesis of [D_7]-15-methylhexadecanoic acid (**8**). a) BH_3–SMe_2, THF, 74%; b) 3,4-dihydro-2*H*-pyran, *p*-TsOH, Et_2O, 91%; c) [D_7]-iPrMgBr, $Li_2[CuCl_4]$, THF, 52%; d) *p*-TsOH, MeOH, 64%; e) PCC, CH_2Cl_2, 53%; f) BuLi, THF, 45%; g) H_2, Pd/C, Et_2O, 78%; h) PDC, DMF, 67%.

The iso-odd FAs occurred in significantly higher amounts than the iso-even FAs. Incorporation of the deuterium label originating from **8** (for the mass spectrum of its methyl ester see Fig. 2A) is shown for all iso-FAMEs obtained after acidic methanolysis by the occurrence of peaks that elute directly before the unlabelled FAMEs in GC. These labelled methyl esters are readily identified by their mass spectra. Molecular ions are increased by seven units, *e.g.* from $m/z = 270$ to 277 in the case of methyl 14-methylpentadecanoate derived from iso-16:0 (compare Figs. 2B and C). Furthermore, some high

Table 1 Fatty acid profile of *Stigmatella aurantiaca* after feeding of **8**. Fatty acids were identified as methyl esters in a methylated fatty acid extract. Peak: peak marker in Fig. 1, trace compounds are not marked (—), *I*: retention index, Int.: percent of total area in GC, Inc.: incorporation rate of deuterium labelling in labelled compounds

Peak	Compound	*I*	Int.	Inc. (%)
—	10:0	1333	0.04	
—	[D_7]iso-11:0	1390	0.04	36
—	iso-11:0	1395	0.05	
a	11:0	1432	0.18	
b	12:0	1526	0.91	
c	[D_7]iso-13:0	1583	0.18	54
d	iso-13:0	1588	0.14	
e	13:0	1626	0.15	
f	[D_7]iso-14:0	1682	0.09	33
g	iso-14:0	1689	0.17	
h	14:1 (*ω*-5 *cis*)	1715	0.12	
i	14:0	1726	0.67	
j	[D_7]iso-15:1	1765	0.10	44
k	iso-15:1	1770	0.13	
l	[D_7]iso-15:0	1783	5.42	58
m	iso-15:0	1789	5.00	
—	15:1	1820	0.07	
—	15:0	1824	0.15	
n	[D_7]iso-16:0	1882	2.32	58
o	iso-16:0	1887	1.68	
p	16:1 (*ω*-5 *cis*)	1914	3.64	
q	16:0	1925	2.29	
—	[D_7]iso-15:0 3OH	1935	0.08	10
r	iso-15:0 3OH	1940	0.85	
s	[D_7]iso-17:1[a]	1963	1.31	
t	[D_7]iso-17:1[a]	1971	1.12	
u	[D_7]iso-17:0	1985	49.3	
—	iso-17:0	1990	2.34	
—	17:0	2026	0.08	
—	16:0 2OH	2048	0.25	
v	16:0 3OH	2080	0.26	
—	18:2	2091	0.07	
—	18:1	2098	0.07	
—	[D_7]iso-17:0 2OH	2103	0.25	13
w	iso-17:0 2OH	2109	1.67	
—	18:0	2124	0.20	
—	[D_7]iso-17:0 3OH	2136	0.10	13
x	iso-17:0 3OH	2142	0.68	

[a] Partially coeluting [D_7]iso-17:1 (*ω*-4 *cis*, *ω*-5 *cis*, *ω*-6 *cis*, and *ω*-7 *cis*) as determined from also partially coeluting DMDS derivatives.

mass fragment ions shift from $m/z = 213$, 227, 239, and 241 to $m/z = 220$, 234, 246, and 248, respectively, all indicating the incorporation of seven deuterium atoms. Notably, fragment ions characteristic of methyl esters ($m/z = 74$ and 87) do not shift because the deuterium label is not located in the functional group moiety of the FAMEs. Furthermore, mass spectra of deuterated compounds show a decreased fragment ion at $m/z = 43$ in combination with an increased signal at $m/z = 50$. Both effects are in agreement with the [D_7]isopropyl group present in iso-FAMEs. The same changes in mass spectra from unlabelled to labelled compounds can be observed for methyl 13-methyltetradecanoate (obtained from iso-15:0, Figs. 2D and E), methyl 12-methyltridecanoate (iso-14:0), and methyl 11-methyldodecanoate (iso-13:0), respectively (data not shown). The incorporation of deuterium label into methyl 14-methylpentadecanoate (iso-16:0) proves that iso-17:0 must be degraded by α-oxidation in *S. aurantiaca*. The occurrence of the deuterium label in methyl 13-methyltetradecanoate (iso-15:0) shows that β-oxidation also takes place, or, less likely, double α-oxidation. The chain lengths of methyl-branched FAMEs vary from methyl 9-methyldecanoate (iso-11:0) to methyl 14-methylpentadecanoate (iso-16:0, with missing methyl 10-methylundecanoate, iso-12:0), and incorporation rates rise from 33% for methyl 12-methyltridecanoate (iso-14:0) to 58% in case of methyl 14-methylpentadecanoate (iso-16:0). An increasing incorporation rate depending on the chain length in both series of iso-even and iso-odd FAs can be observed (Table 1).

Furthermore, incorporation into 2-hydroxy and 3-hydroxy FAs is shown by the presence of the deuterated analogs of methyl 2-hydroxy-15-methylhexadecanoate (iso-17:0 2OH) and methyl 3-hydroxy-15-methylhexadecanoate (iso-17:0 3OH), respectively. The mass spectra of labelled and unlabelled methyl 2-hydroxy-15-methylhexadecanoate (iso-17:0 2OH) are shown in Figs. 2F and G. The molecular ion indicates an incorporation of seven deuterium atoms by an increase from $m/z = 300$ to 307. Other fragment ions shift from $m/z = 222$, 241, and 268 to $m/z = 229$, 248, and 275. The occurrence of a base peak at $m/z = 50$ in the spectrum of the deuterated compound clearly indicates the position of the deuterium atoms in the isopropyl moiety. This pattern is repeated in the mass spectra of labelled and unlabelled methyl 3-hydroxy-15-methylhexadecanoate (iso-17:0 3OH, Figs. 2H and I). The fragment ion at $m/z = 103$ resulting from 3,4-cleavage next to the alcohol function, characteristic of 3-hydroxy fatty acid methyl esters,[34] does not shift, and this is in agreement with the location of labelling.

Besides the branched and unbranched saturated compounds some unsaturated FAMEs were readily identified from their mass spectra in the extracts obtained after feeding of **8**. For the determination of the double bond positions derivatisation with dimethyl disulfide (DMDS) was performed.[35] The mass spectra of DMDS derivatives show strong fragmentation between the methylthio groups, and the fragment ion containing the methyl ester function is characterised by the loss of methanol (Figs. S1 and S2 and Table S3 in the ESI†). Interestingly, the unbranched unsaturated fatty acids produced by *S. aurantiaca* are 16:1 (*ω*-5 *cis*) and 14:1 (*ω*-5 *cis*), respectively. The latter one can arise from

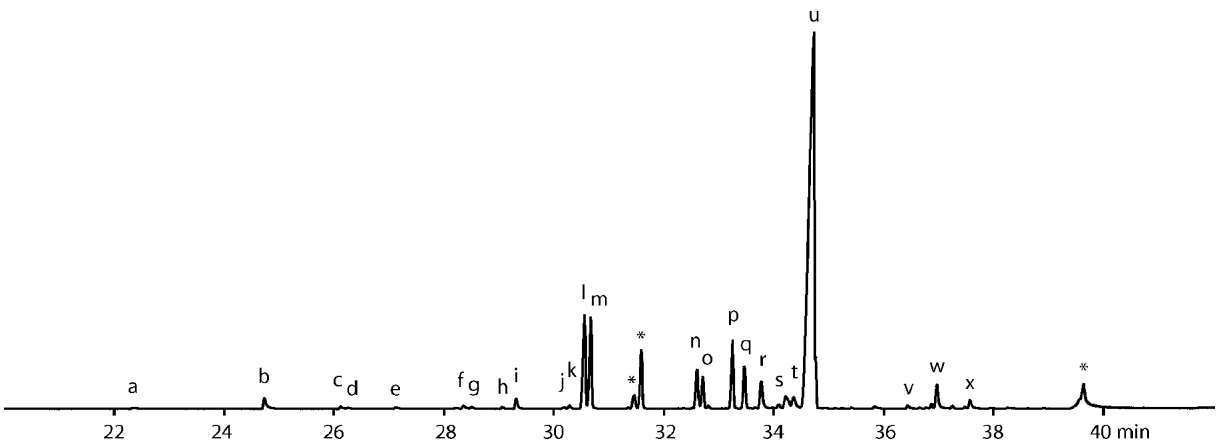


Fig. 1 Total ion chromatogram of an methylated fatty acid extract of *Stigmatella aurantiaca* after feeding of **8**. Letters refer to compounds in Table 1. Non-fatty acids are indicated by asterisks.

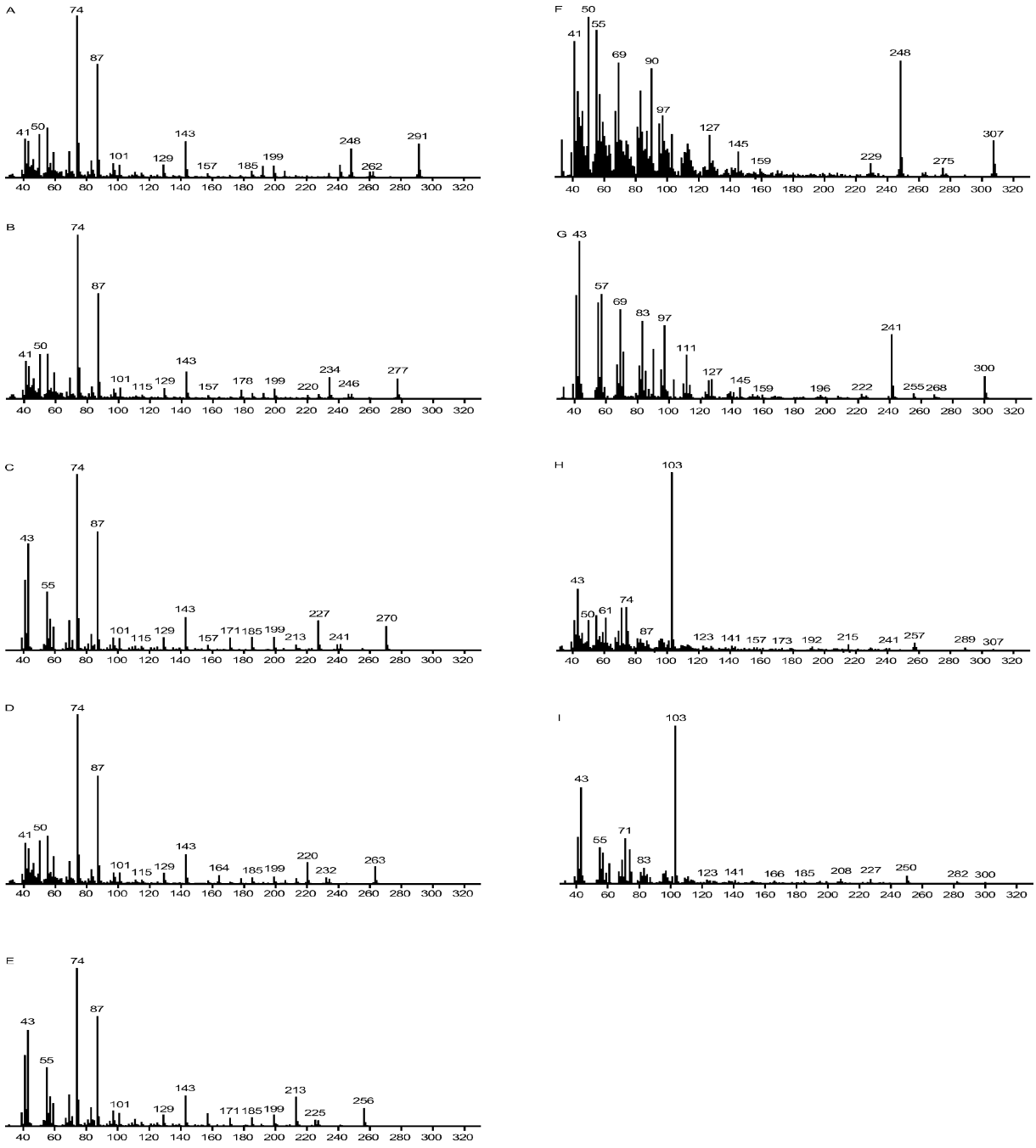
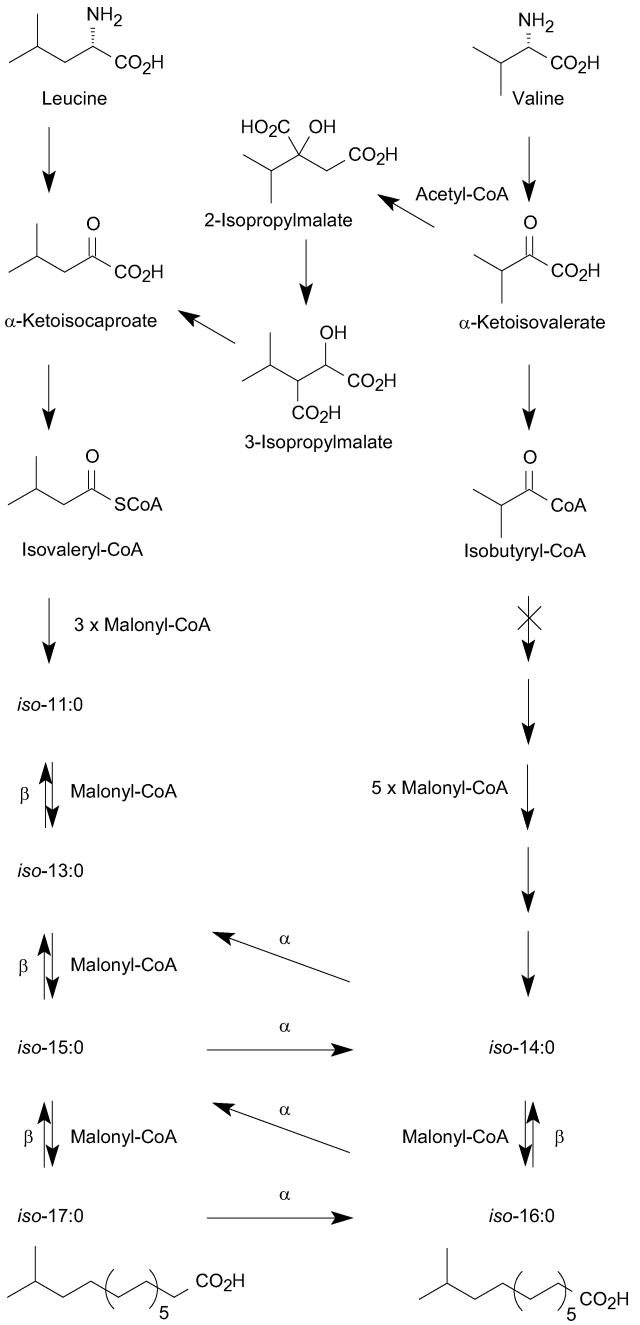


Fig. 2 Mass spectra of labelled and unlabelled fatty acid methyl esters occurring in the methylated fatty acid extract of *Stigmatella aurantiaca* after feeding with [D_7]-15-methylhexadecanoic acid (**8**). [D_7]-Methyl 15-methylhexadecanoate, [D_7]-iso-17:0, peak u in Fig. 1 (A), [D_7]-methyl 14-methylpentadecanoate, [D_7]-iso-16:0, peak n (B), methyl 14-methylpentadecanoate, iso-16:0, peak o (C), [D_7]-methyl 13-methyltetradecanoate, [D_7]-iso-15:0, peak l (D), methyl 13-methyltetradecanoate, iso-15:0, peak m (E), [D_7]-methyl 2-hydroxy-15-methylhexadecanoate, [D_7]-iso-17:0 2OH (F), methyl 2-hydroxy-15-methylhexadecanoate, iso-17:0 2OH, peak w (G), [D_7]methyl 3-hydroxy-15-methylhexadecanoate, [D_7]-iso-17:0 3OH (H), methyl 3-hydroxy-15-methylhexadecanoate, iso-17:0 3OH, peak x (I).

16:1 (*ω*-5 *cis*) through one β-oxidation step. Double α-oxidation is unlikely, because 15:1 is only present in trace amounts. The double bond position and configuration of 16:1 (*ω*-5 *cis*) was previously not identified in investigations on *S. aurantiaca*. Fautz *et al.* reported on the presence of a hexadecenoic acid in this species which they assumed to be 16:1 (*ω*-5 *cis*) because of its retention time relative to 16:1 (*ω*-7 *cis*).[22] Unsaturated 16:1 (*ω*-5 *cis*) has also been identified in *M. xanthus*.[15,16]

Methyl-branched unsaturated FAs were only produced in minute amounts and they all showed the labelling pattern of **8**. The position of the double bonds in the labelled methyl esters obtained from iso-17:1 varies from *ω*-7 to *ω*-4, also determined from DMDS derivatives (Table S3 in the ESI†). The corresponding free acids were only found in extracts of cultures fed with **8**. The incorporation of labelling into the unsaturated iso-FAs indicates that they arise from **8** by the action of a desaturase, and this might also be the case for the major unsaturated acids 16:1 (*ω*-5 *cis*) and 14:1 (*ω*-5 *cis*), respectively. Bacteria can synthesise unsaturated fatty acids by introduction of the double bond during chain assembly (anaerobic pathway), while eukaryotes generally introduce double bonds after chain assembly into the saturated hydrocarbon chain requiring NAD(P)H and O_2 (aerobic pathway) by action of a desaturase.[36] Nevertheless, such desaturases are also known from prokaryotes as *Bacillus subtilis*.[37–41]

In subsequent experiments we tested whether the degradation of long chain iso-odd FAs by α-oxidation is the only pathway to iso-even FAs or the alternative route, transformation of valine



Scheme 2 Biosynthetic pathways from leucine and valine to iso-FAs. α: α-oxidation, β: β-oxidation.

to the starter IB–CoA followed by elongation with malonyl-CoA, is also active (Scheme 2). [D_8]Valine and [D_{10}]leucine, respectively, were fed to *S. aurantiaca* and the incorporation rates into the fatty acids were compared. The results are summarised in Table 2. Addition of [D_{10}]leucine to the bacterial cultures led to the formation of iso-FAs carrying nine deuterium atoms due to the loss of one deuterium in the transamination reaction to [D_9]-α-ketoisocaproic acid. Oxidative decarboxylation then forms [D_9]IV–CoA, the methyl-branched starting unit usually used for the biosynthesis of iso-odd FAs. The fatty acid pattern of *S. aurantiaca* obtained after feeding of [D_{10}]leucine was characterised by the presence of [D_9]iso-17:0 that showed the highest incorporation rate (40%) among the iso-FAs (Table 2). All other labelled iso-FAs had a shorter chain length and lower incorporation rates. Therefore these fatty acids seem to be generated by the degradation of [D_9]iso-17:0. The degradation pathways can be reconstructed based on the incorporation rates, because it is reasonable that each additional biosynthetic trans-

Table 2 Fatty acid profile of *Stigmatella aurantiaca* after feeding of [D_8]valine and [D_{10}]leucine, respectively. Fatty acids were identified as methyl esters in a methylated fatty acid extract. *I*: retention index, Int.: percent of total area in GC after feeding of [D_8]valine and [D_{10}]leucine, respectively. Incorporation rates are given in percent in parentheses

Compound	*I*	Int. [D_8]Val	Int. [D_{10}]Leu
10:0	1333	0.42	0.82
[D_9]iso-11:0	1388		0.04 (29)
[D_7]iso-11:0	1390	0.00 (0)	
iso-11:0	1395	0.10	0.10
12:0	1526	0.15	0.11
[D_9]iso-13:0	1582		0.10 (33)
[D_7]iso-13:0	1583	0.00 (0)	
iso-13:0	1588	0.43	0.20
[D_9]iso-14:0	1682		0.21 (32)
[D_7]iso-14:0	1683	0.00 (0)	
iso-14:0	1689	0.89	0.44
14:1 (ω-5 *cis*)	1715	0.55	0.19
14:0	1726	1.63	1.59
[D_9]iso-15:0	1781		7.63 (35)
[D_7]iso-15:0	1783	0.40 (2)	
iso-15:0	1789	20.0	14.1
15:1	1820	0.40	
15:0	1824	1.81	
[D_9]iso-16:0			2.64 (32)
[D_7]iso-16:0	1882	0.44 (3)	
iso-16:0	1887	13.5	5.50
16:1 (ω-5 *cis*)	1914	16.6	23.4
16:0	1925	16.8	7.35
[D_9]iso-17:0	1983		1.71 (40)
[D_7]iso-17:0	1985	0.34 (4)	
iso-17:0	1990	8.81	2.59
17:0	2026	0.62	0.00

formation can dilute the original label. Two different pathways are therefore important in the degradation of iso-FAs. The first one starts with iso-17:0 (40% incorporation) and proceeds through subsequent β-oxidation steps, affording iso-15:0 (35%), iso-13:0 (33%), and iso-11:0 (29%), respectively (Scheme 2). The second degradation pathway from iso-17:0 starts with an initial α-oxidation step forming iso-16:0 (32%) and successive β-oxidation to iso-14:0 (32%). Thus the normal biogenesis of iso-odd FAs from leucine as well as the unusual formation of iso-even FAs from this amino acid through one α-oxidation and subsequent β-oxidation steps is demonstrated. Transfer between these pathways might still be possible by α-oxidation of acids other than iso-17:0. This acid is the key component for the biosynthesis of iso-FAs. The lower homologues iso-15:0, iso-13:0, and iso-11:0 are preferentially liberated in the catabolic process, but not at all or only in lower amounts during the anabolic process leading to iso-17:0. Liberation during the latter process would result in a decrease of the incorporation rate with longer chain length.

If the other possibility for the formation of iso-even FAs, the use of the starter IB–CoA arising from valine, was active, high incorporation after feeding *S. aurantiaca* with [D_8]valine would be expected. However, only low incorporation rates were observed, the highest being 4% in the case of [D_7]iso-17:0. Two other fatty acids contained the deuterium label, iso-16:0 (3%) and iso-15:0 (2%), whereas incorporation into shorter ω-1 methyl-branched FAs could not be found, probably due to the low amounts of these acids in the extract. The reduced labelling in iso-16:0 and iso-15:0 compared to iso-17:0 indicates that the latter one might be their precursor, formed through one α-oxidation or one β-oxidation step, respectively. An anabolic pathway may also contribute to these acids.

The strongly reduced incorporation rates after feeding of [D_8]valine compared to [D_{10}]leucine clearly indicate that the valine-derived possible starter unit IB–CoA has not the same if any relevance as leucine derived IV–CoA. Furthermore, the highest incorporation after application of [D_8]valine was found for iso-17:0 that should arise from leucine degradation and chain

elongation of the respective starter IV–CoA with six malonyl-CoA units. Therefore, two pathways for the biosynthesis of iso-FAs from valine can be discussed. The first is initiated by the formation of an IB–CoA starter from valine, chain elongation with seven malonyl-CoA units to yield iso-18:0, and final degradation through α- and β-oxidation steps to furnish the shortened iso-FAs. This pathway is unlikely because of the low incorporation rates compared to feeding of [D_{10}]leucine. Furthermore, the required valine derived precursor for iso-17:0, iso-18:0, is not present in the fatty acid extract. It seems more likely that valine is funnelled into the leucine metabolism by transamination to α-ketoisovalerate and the well-known subsequent transformation to the leucine precursor α-ketoisocaproate[42] that is widely distributed among diverse bacteria[43] (Scheme 2). This α-keto acid then serves as a precursor of IV–CoA as a starter in branched chain fatty acid biosynthesis as discussed above. The clearly reduced incorporation rates for [D_8]valine compared to [D_{10}]leucine as well as the highest incorporation rate observed for typically leucine derived iso-17:0 are in accordance with this pathway.

Even-numbered unbranched FAs are normally built up through an acetate starter unit and chain elongation with malonyl-CoA units, while the use of propionate starters furnishes odd-numbered FAs, normally a disfavoured process.[36,44] In both investigated myxobacteria we obtained a typical pattern that showed significantly higher amounts of unbranched even-numbered FAs than odd-numbered ones. Whether α-oxidation is involved in the formation of unbranched odd from even FAs in myxobacteria and a fatty acid as *e.g.* 16:0 has a similar role as iso-17:0 as precursor for shorter as well as unsaturated FAs remains to be investigated. Fatty acid degradation by α-oxidation has been described already in 1959 by Martin and Stumpf in peanuts (palmitic acid),[45] in higher plants,[46] in humans,[47] and in the bacterium *Sphingomonas paucimobilis*.[48] The α-oxidation has also been found in the phytanic acid degradation pathway.[49] The methyl group at C-3 of phytanic acid blocks its catabolism by β-oxidation. Instead an α-oxidation step leads to pristanic acid that can be readily degraded by subsequent β-oxidation steps furnishing propionate and acetate units, respectively.

Conclusions

The data obtained by feeding of labelled valine and leucine as well as **8** support the described mechanisms, especially the uncommon α-oxidation mechanism used by *S. aurantiaca*. Both organisms, *S. aurantiaca* and *M. xanthus*, lack the capability to produce IB–CoA from valine under the conditions tested. In *M. xanthus* this is demonstrated by the fact that the wild-type produces no iso-even FAs, whereas feeding the *bkd* mutant with IBA leads to an increased formation of this type of fatty acids. It remains to be elucidated on which pathways the *bkd* mutant of *M. xanthus* is able to produce minute amounts of iso-even FAs. Probably a second *bkd* complex enabling IB–CoA production is induced in the *bkd* mutant. The low incorporation rates for [D_8]valine into iso-FAs indicate that *S. aurantiaca* is not able to produce fatty acids *via* IB–CoA. In contrast to *M. xanthus* the diversity of FAs in this species is broadened by α-oxidation giving access to iso-even FAs that are thus derived from IV–CoA and not from IB–CoA. Further studies will show whether the α-oxidation is more common in the biosynthesis of fatty acids in nature.

Experimental

Synthesis

General methods. Chemicals were purchased from Fluka Chemie GmbH (Buchs, Switzerland) or Sigma-Aldrich Chemie GmbH (Steinheim, Germany) and used without further purification. Solvents were purified by distillation and dried according to standard methods. All reactions were carried out in oven-dried glassware in a N_2 atmosphere. Thin layer chromatography was carried out using 0.2 mm pre-coated plastic sheets Polygram Sil G/UV_{254} (Marcherey-Nagel GmbH & Co. KG, Düren, Germany). ^{1}H NMR spectra were recorded on a Bruker AC200 (200 MHz) spectrometer with TMS as an internal standard. *J* values are given in Hz. ^{13}C NMR spectra were obtained using a Bruker AC200 (50 MHz) with TMS as internal standard. Column chromatography was carried out using Merck Kieselgel 60.

Feeding experiments

The strains *Stigmatella aurantiaca* DW4/3-1 and EBS7-11 (*bkd* mutant), and *Myxococcus xanthus* DK1622 and JD300 (*bkd* mutant), their cultivation and generation of fatty acid methyl esters have previously been described.[12,20,24]

Feeding experiments were performed as previously described.[11,12] IVA, IBA, [D_{10}]leucine, or [D_8]valine (1 mM each) were added to 50 ml cultures of the strains in 250 ml Erlenmeyer flasks. All strains were cultivated on a rotary shaker at 30 °C and cells were harvested by centrifugation. The labelled fatty acid **8** was dissolved in $CHCl_3$ (0.2 M) and 100 µl were added on a CTT-agar plate to a final concentration of 6.5 mM. After evaporation of the solvent under sterile conditions, 300 µl of cells were added on this spot and also allowed to evaporate prior to incubation at 30 °C for four days.

Derivatisation with DMDS

As described by Leonhardt and de Vilbiss,[35] freshly distilled dimethyl disulfide (DMDS, 0.1 cm^3) and a solution of iodine (0.05 cm^3, 5% in diethyl ether) were added to the methylated fatty acid extract of *Stigmatella aurantiaca* (0.05 cm^3). The reaction mixture was kept for 12 h at 50 °C. Then a saturated solution of Na_2SO_3 (0.02 cm^3) was added to remove the iodine. The mixture was diluted with diethyl ether (1 cm^3) and dried with Na_2SO_4. The volatiles were removed at 40 °C in a gentle stream of N_2 and the residue was diluted with 0.05 cm^3 of diethyl ether. The obtained solution was immediately used for GC–MS analysis.

GC–MS

GC–MS analyses were carried out on a HP 6890 Series GC System connected to a HP 5973 Mass Selective Detector (Hewlett-Packard Company, Wilmington, USA) fitted with a BPX-5 fused-silica capillary column (25 m × 0.22 mm id, 0.25 µm film, SGE Inc., Melbourne, Australia). Conditions were as follows: inlet pressure: 77.1 kPa, He 23.3 mL min^{-1}; injection volume: 1 µL; transfer line: 300 °C; electron energy: 70 eV. The GC was programmed as follows: 5 min at 50 °C increasing at 5 °C min^{-1} to 300 °C, and operated in splittless mode (60 s valve time). The carrier gas was He at 1 cm^3 min^{-1}. Retention indices *I* were determined from a homologous series of *n*-alkanes (C_7–C_{25}).

Preparation of 4-bromobutan-1-ol 2[26]

A solution of **1** (4.18 g, 25 mmol) in THF (10 cm^3) was added dropwise at 0 °C to a solution of borane dimethylsulfide complex (2.28 g, 30 mmol, 3.0 cm^3) in dry THF (20 cm^3). The reaction mixture was stirred overnight at room temperature and then quenched by the addition of ethanol (5 cm^3) and water (20 cm^3). The crude product was obtained by extraction with pentane (3 × 50 cm^3), drying with $MgSO_4$, and removing of the solvents *in vacuo*. Purification of the residue by column chromatography on silica gel with pentane–diethyl ether (3 : 1) afforded **2** (2.81 g, 74%) as a colourless liquid. TLC (pentane–diethyl ether = 3 : 1): R_F 0.18; GC: *I* 1033; δ_H(400 MHz; $CDCl_3$; Me_4Si) 1.65–1.81 (2 H, m, CH_2), 1.83–2.04 (2 H, m, CH_2), 3.46 (2 H, t, *J* 6.6, CH_2) and 3.70 (2 H, t, *J* 6.3, CH_2); δ_C(100 MHz; $CDCl_3$; Me_4Si) 29.1 (CH_2), 30.9 (CH_2), 33.6 (CH_2) and 61.8 (CH_2); *m/z* (EI) 136 (36%), 134 (36), 108 (10), 106 (10), 95 (5), 93 (5), 73 (14), 55 (100) and 41 (38).

Preparation of THP ethers

Similar to the method of Parham and Anderson,[29] a solution of **2** or 10-bromodecan-1-ol, respectively, (1.0 equiv.), 3,4-dihydro-2*H*-pyran (1.1 equiv.) and a catalytic amount of *p*-toluenesulfonic acid (5 mol%) in dry diethyl ether (final concentration of the alcohol was approximately 5 mol dm^{-3}) was stirred at room temperature for 3 h. The reaction mixture was stirred for 30 min after the addition of K_2CO_3 (50 mg) and then washed with saturated $NaHCO_3$ solution. The organic layer was separated and dried over $MgSO_4$. The organic solvent was removed under reduced pressure. The crude product was purified by column chromatography on silica gel with pentane–diethyl ether (5 : 1) to obtain the THP ethers as colourless liquids.

2-(4-Bromobutyloxy)oxane 3. Yield: 3.18 g (91%); TLC (pentane–diethyl ether = 5 : 1): R_F 0.62; GC: *I* 1479; δ_H(200 MHz; $CDCl_3$; Me_4Si) 1.52–1.63 (2 H, m, CH_2), 1.65–1.85 (2 H, m, CH_2), 1.91–2.05 (2 H, m, CH_2), 3.37–3.56 (2 H, m, CH_2) and 4.56–4.60 (1 H, m, CH); δ_C(50 MHz; $CDCl_3$; Me_4Si) 19.6 (CH_2), 25.4 (CH_2), 28.3 (CH_2), 29.8 (CH_2), 30.7 (CH_2), 33.7 (CH_2), 62.3 (CH_2), 66.4 (CH_2) and 98.8 (CH); *m*/*z* (EI) 237 (8), 235 (8), 137 (62), 135 (64), 101 (2), 85 (100), 67 (10), 55 (70) and 41 (35).

2-(10-Bromodecyloxy)oxane. Yield: 1.08 g (80%); TLC (pentane–diethyl ether = 20 : 1): R_F 0.15; GC: *I* 2135; δ_H(200 MHz; $CDCl_3$; Me_4Si) 1.20–1.92 (22 H, m, 11 × CH_2), 3.32–3.55 (2 H, m, CH_2), 3.41 (2 H, t, *J* 6.8, CH_2), 3.67–3.93 (2 H, m, CH_2) and 4.56–4.59 (1 H, m, CH); δ_C(50 MHz; $CDCl_3$; Me_4Si) 19.7 (CH_2), 25.5 (CH_2), 26.2 (CH_2), 28.1 (CH_2), 28.7 (CH_2), 29.3 (CH_2), 29.4 (CH_2), 29.4 (CH_2), 29.7 (CH_2), 30.7 (CH_2), 32.8 (CH_2), 33.9 (CH_2), 62.3 (CH_2), 67.6 (CH_2) and 98.8 (CH); *m*/*z* (EI) 321 (4%), 319 (4), 249 (1), 247 (1), 150 (2), 148 (2), 137 (2), 135 (2), 115 (2), 101 (9), 85 (100), 69 (10), 55 (22) and 41 (22).

Preparation of [D_7]-2-(5-methylhexyloxy)oxane 4

According to the method of Tamura and Kochi,[28] a flame dried reaction vessel was charged with Mg (158 mg, 6.50 mmol) and dry THF (0.5 cm^3) in a nitrogen atmosphere. A solution of [D_7]isopropyl bromide (845 mg, 6.50 mmol) in THF (2.5 cm^3) was added dropwise during 15 min. The reaction mixture was stirred for an additional 30 min. After formation of the Grignard reagent the mixture was cooled down to −78 °C, a solution of **3** (1.66 g, 7.00 mmol) in dry THF (25 cm^3) and a solution of $Li_2[CuCl_4]$ in THF (0.7 cm^3, 0.1 mol dm^{-3}, 0.7 mmol) were added dropwise. The reaction mixture was stirred overnight, allowed to warm up slowly to room temperature and then quenched by the addition of 2 N HCl (20 cm^3). The aqueous layer was extracted with diethyl ether (3 × 50 cm^3). The combined organic layers were dried with $MgSO_4$ and concentrated *in vacuo*. The residue was purified by column chromatography on silica gel with pentane–diethyl ether (20 : 1), and yielded **4** (700 mg, 52%) as a colourless liquid. TLC (pentane–diethyl ether = 2 : 1): R_F 0.37; GC: *I* 1373; δ_H(200 MHz; $CDCl_3$; Me_4Si) 1.15–1.44 (4 H, m, 2 × CH_2), 1.51–1.85 (8 H, m, 4 × CH_2), 3.33–3.55 (2 H, m, CH_2), 3.68–3.93 (2 H, m, CH_2) and 4.56–4.60 (1 H, m, CH); δ_C(50 MHz; $CDCl_3$; Me_4Si) 19.6 (CH_2), 23.9 (CH_2), 25.5 (CH_2), 29.9 (CH_2), 30.7 (CH_2), 38.5 (CH_2), 62.3 (CH_2), 67.6 (CH_2) and 98.8 (CH); *m*/*z* (EI) 207 (1%), 206 (3), 134 (2), 115 (3), 101 (6), 85 (100), 67 (7), 56 (15) and 41 (15).

Preparation of [D_7]-5-methylhexan-1-ol

A solution of **4** (623 mg, 3.01 mmol) and *p*-toluenesulfonic acid (58 mg, 0.30 mmol) in dry methanol (20 cm^3) was stirred for 30 min at room temperature, and then the solvent was removed under reduced pressure. The residue was diluted with water and extracted with diethyl ether (3 × 50 cm^3). The combined organic layers were dried with $MgSO_4$ and concentrated. Purification by column chromatography with pentane–diethyl ether (5 : 1) yielded [D_7]-5-methylhexan-1-ol (238 mg, 64%) as a colourless liquid. TLC (pentane–diethyl ether = 5 : 1): R_F 0.15; GC: *I* 942; δ_H(200 MHz; $CDCl_3$; Me_4Si) 1.16–1.37 (4 H, m, 2 × CH_2), 1.48–1.64 (2 H, m, CH_2), 1.81 (1 H, br s, OH) and 3.63 (2 H, t, *J* 6.6, CH_2); δ_C(50 MHz; $CDCl_3$; Me_4Si) 23.5 (CH_2), 33.0 (CH_2), 38.7 (CH_2) and 62.9 (CH_2); *m*/*z* (EI) 104 (5%), 87 (38), 77 (100), 62 (92), 50 (81) and 46 (89).

Preparation of [D_7]-5-methylhexanal 5

Similar to the method described by Corey and Suggs[30] a solution of [D_7]-5-methylhexan-1-ol (238 mg, 1.93 mmol) in dry dichloromethane (5.0 cm^3) was added to a suspension of PCC (619 mg, 2.88 mmol) in dry dichloromethane (5.0 cm^3). The reaction mixture was stirred at room temperature for 3 h and then quenched by the addition of water (20 cm^3). The aqueous layer was separated and extracted with diethyl ether (3 × 20 cm^3). The combined extracts were dried with $MgSO_4$ and the organic solvents were removed under reduced pressure. The residue was purified by column chromatography on silica gel with pentane–diethyl ether (5 : 1) to give **5** (124 mg, 53%) as a colourless liquid. Due to its instability against oxidation this aldehyde was immediately used in the next step. TLC (pentane–diethyl ether = 5 : 1): R_F 0.79; GC: *I* 861; *m*/*z* (EI) 102 (43%), 93 (25), 85 (32), 78 (71), 71 (33), 59 (82), 50 (73) and 46 (100).

Preparation of (10-hydroxydecyl)triphenylphosphonium bromide 6

A solution of 2-(10-bromodecyloxy)oxane (1.09 g, 3.38 mmol) and triphenylphosphane (885 mg, 3.38 mmol) in acetonitrile (20 cm^3) was heated under reflux for 48 h. The reaction mixture was concentrated *in vacuo* and the residue was purified by column chromatography on silica gel with dichloromethane–methanol (10 : 1) to yield **6** (1.20 g, 71%) as a colourless solid. TLC (dichloromethane–methanol = 10 : 1): R_F 0.38; δ_H(200 MHz; $CDCl_3$; Me_4Si) 1.10–1.40 (10 H, m, 5 × CH_2), 1.49–1.61 (6 H, m, 3 × CH_2), 2.53 (1 H, br s, OH), 3.55–3.75 (2 H, m, CH_2), 3.59 (2 H, t, *J* 6.6, CH_2) and 7.73–7.87 (15 H, m, 15 × CH); δ_C(50 MHz; $CDCl_3$; Me_4Si) 22.5 (d, $^3J_{P,C}$ 4.4, CH_2), 22.7 (d, $^1J_{P,C}$ 49.9, CH_2), 25.6 (CH_2), 28.9 (CH_2), 28.9 (CH_2), 29.1 (CH_2), 29.1 (CH_2), 30.3 (d, $^2J_{P,C}$ 15.5, CH_2), 32.6 (CH_2), 62.5 (CH_2), 118.2 (d, $^1J_{P,C}$ 85.8, C), 130.5 (d, $^2J_{P,C}$ 12.3, CH), 133.5 (d, $^3J_{P,C}$ 9.9, CH) and 135.1 (d, $^4J_{P,C}$ 2.9, CH).

Preparation of [D_7]-(*Z*)-15-methylhexadec-10-en-1-ol 7

As described by Martinez and Ruiz,[32] a solution of BuLi in hexane (3.0 cm^3, 1.6 mol dm^{-3}, 4.8 mmol) was added dropwise to a suspension of **6** (1.20 g, 2.4 mmol) in dry THF (20 cm^3). The reaction mixture was stirred for 30 min. During this time a dark red ylide was formed. A solution of **5** (97 mg, 0.80 mmol) in dry THF (3.0 cm^3) was added. The reaction mixture was stirred at room temperature for 2 h and then quenched by the addition of 2 N HCl. The aqueous layer was separated and extracted with diethyl ether (3 × 50 cm^3). The combined extracts were dried with $MgSO_4$ and concentrated under reduced pressure. Compound **7** (94 mg, 45%) was obtained by column chromatography on silica gel with pentane–diethyl ether (5 : 1) as a colourless liquid. Only minor amounts (<5%) of the *E*-isomer were detected by ^{1}H NMR.

TLC (pentane–diethyl ether = 5 : 1): R_F 0.15; GC: *I* 1933; δ_H(200 MHz; $CDCl_3$; Me_4Si) 1.13–1.41 (14 H, m, 7 × CH_2), 1.50–1.61 (4 H, m, 2 × CH_2), 1.86 (1 H, br s, OH), 1.94–1.99 (4 H, m, 2 × CH_2), 3.62 (2 H, t, *J* 6.6, CH_2) and 5.27–5.43 (2 H, m, 2 × CH); δ_C(50 MHz; $CDCl_3$; Me_4Si) 25.7 (CH_2), 27.2 (CH_2), 27.4 (CH_2), 27.5 (CH_2), 29.2 (CH_2), 29.4 (CH_2), 29.4 (CH_2), 29.4 (CH_2), 29.5 (CH_2), 29.7 (CH_2), 32.7 (CH_2), 62.9 (CH_2), 129.8 (CH) and 129.9 (CH); *m*/*z* (EI) 261 (3), 243 (16), 215 (5),

187 (3), 152 (3), 138 (9), 124 (18), 110 (31), 96 (74), 82 (100), 75 (34), 67 (86), 55 (95) and 41 (72).

Preparation of [D_7]-15-methylhexadecan-1-ol

Pd/C (21 mg, 10% Pd, 0.02 mmol) was added to a solution of **7** (94 mg, 0.36 mmol) in diethyl ether (5.0 cm^3). The reaction mixture was stirred in an H_2 atmosphere (10 N cm^{-2}) for 20 min. Pd/C was filtered of and the resulting solution was concentrated. The product was purified by column chromatography on silica gel with pentane–diethyl ether (3 : 1) to obtain [D_7]-15-methylhexadecan-1-ol (74 mg, 78%) as a colourless solid.

TLC (pentane–diethyl ether = 3 : 1): R_F 0.32; GC: *I* 1951; δ_H(200 MHz; $CDCl_3$; Me_4Si) 1.15–1.40 (24 H, m, 12 × CH_2), 1.50–1.64 (2 H, m, CH_2), 1.66 (1 H, br s, OH) and 3.63 (2 H, t, *J* 6.5, CH_2); δ_C(50 MHz; $CDCl_3$; Me_4Si) 25.7 (CH_2), 27.3 (CH_2), 29.4 (2 × CH_2), 29.6 (3 × CH_2), 29.7 (4 × CH_2), 29.9 (CH_2), 32.8 (CH_2) and 63.0 (CH_2); *m*/*z* (EI) 245 (8%), 217 (14), 189 (5), 157 (4), 140 (4), 125 (8), 111 (20), 97 (52), 83 (82), 69 (79), 55 (100) and 41 (63).

Preparation of [D_7]-15-methylhexadecanoic acid 8

According to the method of Corey and Schmidt[33] PDC (368 mg, 0.98 mmol) was suspended in dry DMF (1.0 cm^3). A solution of [D_7]-15-methylhexadecan-1-ol (74 mg, 0.28 mmol) in dry DMF (3.0 cm^3) was added to this suspension. The reaction mixture was stirred for 12 h at room temperature and then water (20 cm^3) was added. The aqueous layer was separated and extracted with diethyl ether (3 × 50 cm^3). The combined organic layers were dried with $MgSO_4$ and concentrated to dryness. The crude product was purified by column chromatography on silica gel with pentane–diethyl ether (3 : 1) to yield **8** (52 mg, 67%) as a colourless solid. For GC–MS analysis a small sample was transformed into its trimethylsilyl ester with MSTFA.[50] TLC (pentane–diethyl ether = 3 : 1): R_F 0.51; δ_H(200 MHz; $CDCl_3$; Me_4Si) 1.10–1.40 (20 H, m, 10 × CH_2), 1.55–1.72 (4 H, m, 2 × CH_2) and 2.35 (2 H, t, *J* 7.5, CH_2); δ_C(50 MHz; $CDCl_3$; Me_4Si) 24.7 (CH_2), 27.4 (CH_2), 29.0 (CH_2), 29.2 (CH_2), 29.4 (CH_2), 29.6 (CH_2), 29.6 (2 × CH_2), 29.9 (CH_2), 34.0 (CH_2), 38.8 (CH_2) and 179.9 (CO); *m*/*z* (EI, MSTFA) 349 (14%), 334 (100), 320 (1), 306 (5), 290 (4), 201 (6), 185 (3), 145 (20), 132 (27), 129 (27), 117 (50), 75 (33), 73 (38) and 50 (9).

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