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Identification of Metabolites of the Anaerobic Degradation of *n*-Hexane by Denitrifying Betaproteobacterium Strain HxN1

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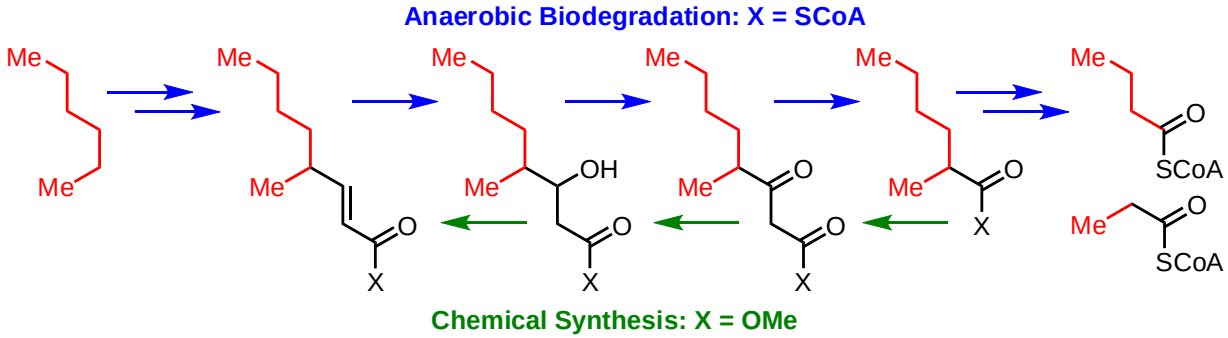
Abstract: The constitutions of seven metabolites formed during anaerobic degradation of *n*-hexane by the denitrifying betaproteobacterium strain HxN1 were elucidated by comparison of gas chromatographic and mass spectrometric data with synthetic reference standards. Synthesis of 4-methyloctanoic acid derivatives was accomplished by conversion of 2-methylhexanoyl chloride with Meldrum's acid. The β-oxoester was reduced with $NaBH_4$, the hydroxy group eliminated and the double bond displaced to yield the methyl esters of 4-methyl-3-oxooctanoate, 3-hydroxy-4-methyloctanoate, (*E*)-4-methyl-2-octenoate and (*E*)- and (*Z*)-4-methyl-3-octenoate, respectively. The methyl esters of 2-methyl-3-oxohexanoate and 3-hydroxy-2-methylhexanoate were similarly prepared from butanoyl chloride and Meldrum's acid. However, methyl (*E*)-2-methyl-2-hexenoate was prepared by HWE-reaction, followed by isomerization to methyl (*E*)-2-methyl-3-hexenoate. This investigation, with the exception of 4-methyl-3-oxooctanoate which was not detectable in the cultures, completes the unambiguous identification of all intermediates of anaerobic biodegradation of *n*-hexane to 2-methyl-3-oxohexanoyl-CoA which is then thiolytically cleaved to butanoyl-CoA and propionyl-CoA the two latter being further transformed

according to established pathways.

Key Topic: Anaerobic Biodegradation

Graphical Abstract: Metabolites formed during anaerobic degradation of *n*-hexane by the denitrifying betaproteobacterium strain HxN1 were identified by comparison with newly synthesized reference standards using GC-MS.



Keywords: alkanes, anaerobic degradation, "*Aromatoleum*" sp. HxN1, C–H-bond activation, oxoester

Introduction

The key challenge in the biodegradation of saturated hydrocarbons is C–H-bond activation. The initial activation step requires the cleavage of a C–H-bond, being associated with a high energy barrier, which has to be overcome. In oxic environments (presence of O_2) this is accomplished by the well-studied oxygenase enzymes employing O_2-derived highly reactive oxygen species.[1] Investigations on the biodegradation of hydrocarbons under anoxic conditions (absence of O_2) led to the discovery of a large diversity of novel microorganisms and biochemical transformations (for overviews see[2]).

The betaproteobacterium strain HxN1, which affiliates with the newly described genus *Aromatoleum*,[3] was originally isolated from ditch sediments in Bremen (Germany) and has been shown to completely oxidize *n*-hexane (**1**) to CO_2 under strictly anoxic conditions coupled to denitrification.[4] Based on metabolite and EPR studies, anaerobic degradation of *n*-hexane (**1**) by strain HxN1 has been proposed to proceed *via* an 1-methylpentyl radical, which adds to fumarate yielding (1-methylpentyl)succinate (MPS, **2**, Scheme 1, step a) in a reaction catalyzed by a glycyl radical enzyme.[5]

Subsequent investigations with stereoisomers of (2,5-$^2H_2$)hexane revealed inversion of configuration at C-2 of *n*-hexane (**1**) during formation of dicarboxylic acid **2**. Based on this evidence it has been suggested that C–H-bond cleavage and C–C-bond formation may proceed in a concerted manner which thus would avoid a highly reactive 1-methylpentyl radical as a free intermediate.[6] Further degradation of MPS (**2**) was inferred from metabolite analysis to proceed by the following reaction sequence (Scheme 1):[7] Thioesterification to (1-methylpentyl)succinyl-CoA (**3**, step b) is followed by a vitamin B_{12}-dependent mutase-catalyzed rearrangement of the C-skeleton in the succinyl-CoA moiety by an 1,2-acyl shift (step c) forming (2-methylhexyl)malonyl-CoA (**4**) to then furnish 4-methyloctanoyl-CoA (**5a**) by decarboxylation (step d). The β-oxidation sequence of the latter *via* 4-methyl-2-octenoyl-CoA (**6a**, step e) and 3-hydroxy-4-methyloctanoyl-CoA (**7a**, step f) leads to 4-methyl-3-oxooctanoyl-CoA (**8a**, step g), which undergoes thiolytic cleavage (step h) forming acetyl-CoA (**9**) and 2-methylhexanoyl-CoA (**10a**). A second round of β-oxidation starting with 2-methylhexanoyl-CoA (**10a**) would involve sequential formation of 2-methyl-2-hexenoyl-CoA (**11a**, step i), 3-hydroxy-2-methylhexanoyl-CoA (**12a**, step j) and 2-methyl-3-oxohexanoyl-CoA (**13a**, step k), with the latter being thiolytically cleaved into butanoyl-CoA (**16**) and propionyl-CoA (**17**). A third round of β-oxidation would then transform C_4-compound **16** into two acetyl-CoA (**9**). While the three acetyl-CoA (**9**) formed are terminally oxidized to CO_2 in the TCA cycle, the propionyl-CoA (**17**) could be converted to fumarate by the methylmalonyl-CoA pathway formally utilizing one equivalent of CO_2, and thus recycling the co-substrate of the initial *n*-hexane activation reaction.

The aim of the present study was to complete our metabolite-based understanding of the anaerobic degradation of *n*-hexane (**1**) downstream of MPS (**2**), by unambiguously elucidating the constitutions of intermediates **6a**, **7a**, and **11a**–**15a** which to date have only tentatively been assigned based on mass spectrometric data. This task was accomplished by chemical synthesis of the respective compounds (as their methyl esters **6b**, **7b**, and **11b**–**15b**) and their application as reference standards for unambiguous metabolite identification by GC-MS. Compounds **14a** and **15a** with isomerized C–C-double bond are actually not included in the metabolic pathway shown in Scheme 1, anyhow, the respective methyl esters **14b** and **15b** are present in the methylated culture extract of strain HxN1.

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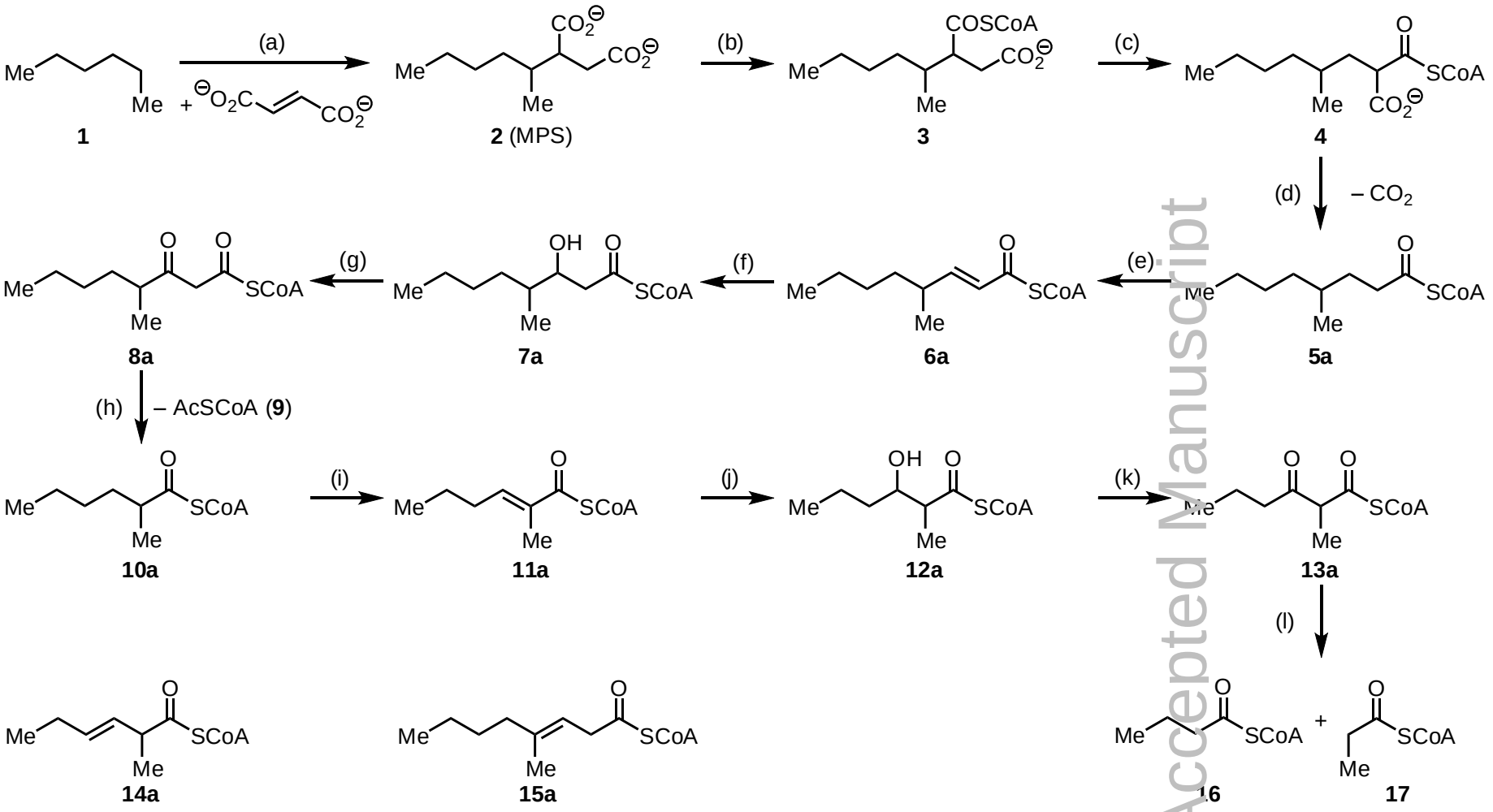

Scheme 1. Proposed pathway for the anaerobic degradation of *n*-hexane (**1**) by strain HxN1, for corresponding transformations (a) to (l) see text.

Results and Discussion

Organic Synthesis. The synthesis of the 4-methyloctanoic acid series started from commercially available 2-methylhexanoic acid **18**. The latter was first converted to its acid chloride, which was isolated and purified by distillation (Scheme 2).[8] The conversion with Meldrum's acid and subsequent solvolysis with methanol under standard conditions (with pyridine and catalytic amounts of DMAP)[9] furnished however complex reaction mixtures containing only small amounts of β-oxoester **8b**.[10] After some experimentation, the use of an overstoichiometric amount of DMAP (1.8 equiv.) turned out to furnish compound **8b** in preparatively useful quantities (70% yield). Treatment of compound **8b** with $NaBH_4$ gave both diastereoisomers of the β-hydroxyester **7b**[11] (78% yield) without any stereoselectivity (*dr* 53:47). The isomers were not separated, but its mixture submitted to elimination *via* the methanesulfonate to furnish α,β-unsaturated ester **6b**[12] in 77% yield and exclusively as the (*E*)-isomer. The C–C-double bond was shifted to furnish the β,γ-unsaturated isomer **15b**[13] by a two-step strategy originally introduced by Orsini *et al.*[14] The first step is the allylic bromination with NBS–AIBN, yielding intermediate product **19** (79%), which was then submitted to reduction with Zn–AcOH with displacement of the C–C-double bond. Compound **15b** (63%) was obtained as a mixture of diastereoisomers (*E*/*Z* 2:1), which were not separated. The configuration was assigned by the following NOE-experiments: Irradiation of the 4-CH_3 group at 1.62 ppm lead to almost no NOE effect at the olefinic signal at 5.31 ppm; thus, this signal belongs to the (*E*)-isomer. If the 4-CH_3 group at 1.73 ppm is irradiated, a pronounced NOE of the olefinic signal is observed, hence, this is the (*Z*)-isomer.

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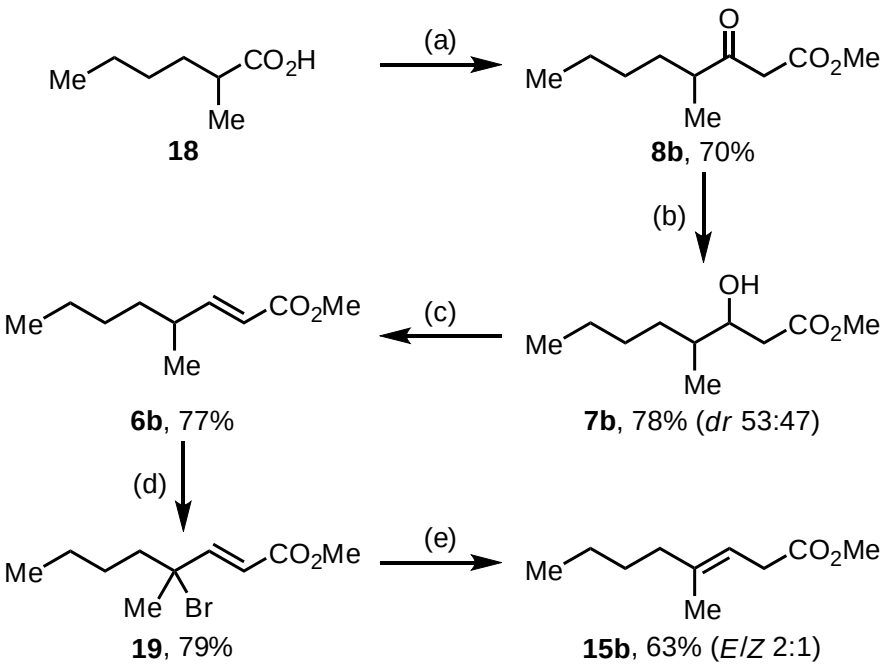

Scheme 2. Synthesis of members of the 4-methyloctanoic acid series. Reagents and conditions: (a) 1. 4.0 equiv. $SOCl_2$, 76°C, 3 h; 2. distillation; 3. 0.9 equiv. Meldrum's acid, 1.8 equiv. DMAP, CH_2Cl_2, 23°C, 15 h; 4. MeOH, 65°C, 24 h; (b) 1.2 equiv. $NaBH_4$, MeOH, 23°C, 17 h; (c) 1.4 equiv. $MeSO_2Cl$, 9.0 equiv. NEt_3, CH_2Cl_2, 23°C, 28 h; (d) 1.0 equiv. NBS, 6 mol% AIBN, $CHCl_3$, 75°C, 19 h; (e) 8.2 equiv. Zn, AcOH, 23°C, 1 h.

The synthesis of the 2-methylhexanoic acid series also started with the acylation of Meldrum's acid,[9] in this case with butanoyl chloride (**20**) furnishing oxoester **21**[9,15] in moderate yield (38%); anyhow, pyridine and only catalytic amounts of DMAP could be applied here (Scheme 3). The methylation with MeI occurred with K_2CO_3 in acetone with surprisingly high selectivity towards the mono-alkylated product **13b**[16] (55%). Reduction with $NaBH_4$ gave the alcohol **12b**[17] (57%) as an inseparable mixture of two diastereoisomers (*dr* 3:2). Upon attempts of elimination using the same protocol as for compound **6b** (Scheme 2) the α,β-unsaturated compound **11b** (see Scheme 4) was obtained, however, together with unspecified impurities, which could not be separated by column chromatography. For this reason, compound **11b** was accessed as outlined below.

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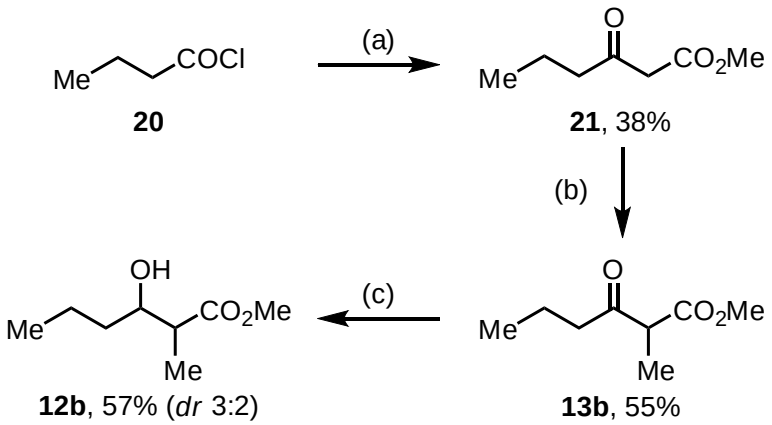
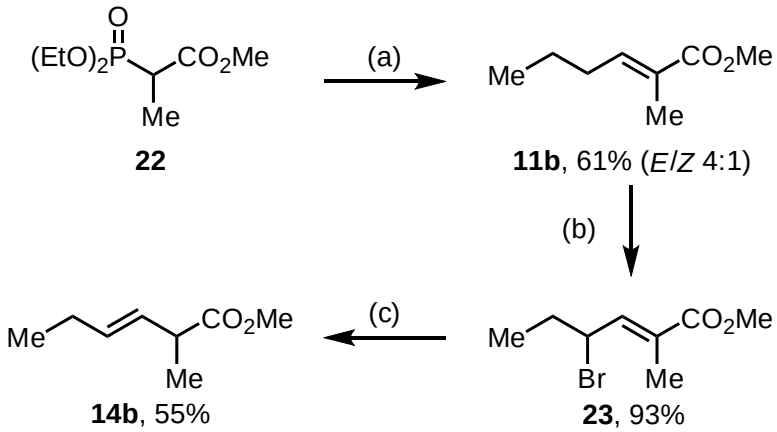


Scheme 3. Synthesis of members of the 2-methylhexanoic acid series. Reagents and conditions: (a) 1. 0.9 equiv. Meldrum's acid, 0.2 equiv. DMAP, 1.8 equiv. pyridine, CH_2Cl_2, 23°C, 19 h; 2. MeOH, 65°C, 4 h; (b) 1.5 equiv. MeI, 1.0 equiv. K_2CO_3, acetone, 23°C, 3.5 h; (c) 1.2 equiv. $NaBH_4$, MeOH, 23°C, 3 h.

HWE-reaction of phosphorylpropionate **22**[18] with butanal gave α,β-unsaturated ester **11b**[19] as mixture of diastereoisomers (61%, *E*/*Z* 4:1, Scheme 4), which could be separated by column chromatography. The (*E*)-selectivity of the HWE-reaction in the formation of α-methyl-α,β-unsaturated esters has been investigated before in the literature.[20] Furthermore, the NMR spectra of both diastereoisomers of ester **11b** were previously reported.[19] The (*E*)-isomer was submitted to two-step double bond displacement as performed above to furnish the β,γ-unsaturated isomer **14b**[21] (55%, only *trans*-configurated) *via* the allylic bromide **23** (93%).



Scheme 4. Synthesis of unsaturated derivatives of 2-methylhexanoic acid. Reagents and conditions: (a) 1.0 equiv. butanal, 1.1 equiv. KO*t*Bu, THF, 23°C, 1 h; (b) 1.0 equiv. NBS, 6 mol% AIBN, CCl_4, 80°C, 19 h; (c) 2.0 equiv. Zn, AcOH, 0°C, 1 h, then 23°C, 1 h.

Identification of Metabolites. Methylated culture extracts of strain HxN1 after anaerobic growth with *n*-hexane were analyzed by gas chromatography-mass spectrometry (GC-MS). Accordingly, all reference standards needed for unambiguous identification of metabolites have been synthesized as the respective methyl esters (see above). Target structures of the so far unidentified metabolites were proposed based on their mass spectrometric fragmentation patterns. Conclusive identification of the detected metabolites was accomplished by GC co-injection experiments and comparison of mass spectra (Figures 1 and 2, Figures S1–S16 in the Supporting Information). Oxoester **8b** could not be detected which may indicate that the steady-state concentration of transient metabolite **8a** is below detection limit in growing cultures of strain HxN1. The underlying β-thiolase reaction is actually known to be far on the side of the oxoester cleavage products.[22] Interestingly, the two diastereoisomers of methyl 3-hydroxy-2-methylhexanoate (**12b**) were present in the culture extracts of strain HxN1 in a similar proportion as furnished by the synthetic procedure. The two diastereoisomers of hydroxyester **7b** were not separable under the GC conditions applied.

The comparison of synthetic esters **14b** and **15b** with the methylated extract from cultures of strain HxN1 by GC-MS have doubtlessly confirmed, that these compounds with isomerized β,γ-C–C-double bonds are present in the extract. It might be assumed, however, that compounds have been formed artificially during heat deactivation and acid treatment of the culture broth, e.g. by isomerization of compounds **6a** or **11a** or by elimination of alcohols **7a** or **12a**, respectively. For this reason, we have performed the following control experiments: The β-hydroxyesters **7b** or **12b** as well as the free carboxylic acid corresponding to ester **6b** were treated under respective conditions (85°C at pH 1.5, hydrochloric acid). These mixtures were then analyzed by GC-MS and ^{1}H NMR, and in none of the cases, compounds with β,γ-double bonds could be detected. Therefore we conclude, that compounds **14a** and **15a** are native metabolites of strain HxN1, though their possible roles remain unclear at this point.

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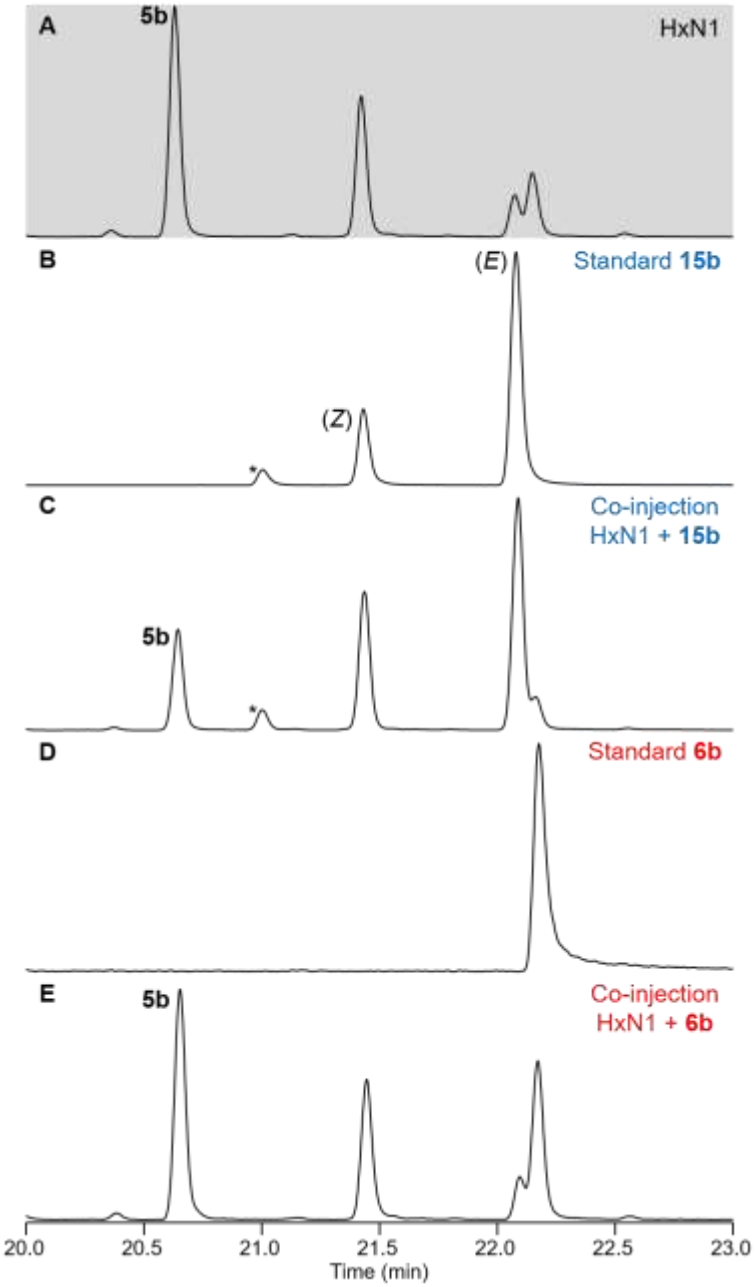

Figure 1. Gas chromatographic separation of isomers of methyl 4-methyloctenoates [**6b** and (*E*)-, (*Z*)-**15b**] and co-injections with methylated culture extracts of strain HxN1 after anaerobic growth with *n*-hexane (**1**). Depicted are the total ion chromatograms. The metabolite methyl 4-methyloctanoate (**5b**) had been identified and structurally elucidated before.[7] The relative abundance of component **5b** is used as an internal reference to assess the increase of peaks upon co-injection. (A) Methylated culture extract of strain HxN1; (B) synthetic standard methyl (*Z*)- and (*E*)-4-methyl-3-octenoate (**15b**) (* = impurity); (C) co-injection of extract and standard **15b**; (D) synthetic standard methyl (*E*)-4-methyl-2-octenoate (**6b**); (E) co-injection of extract and standard **6b**.

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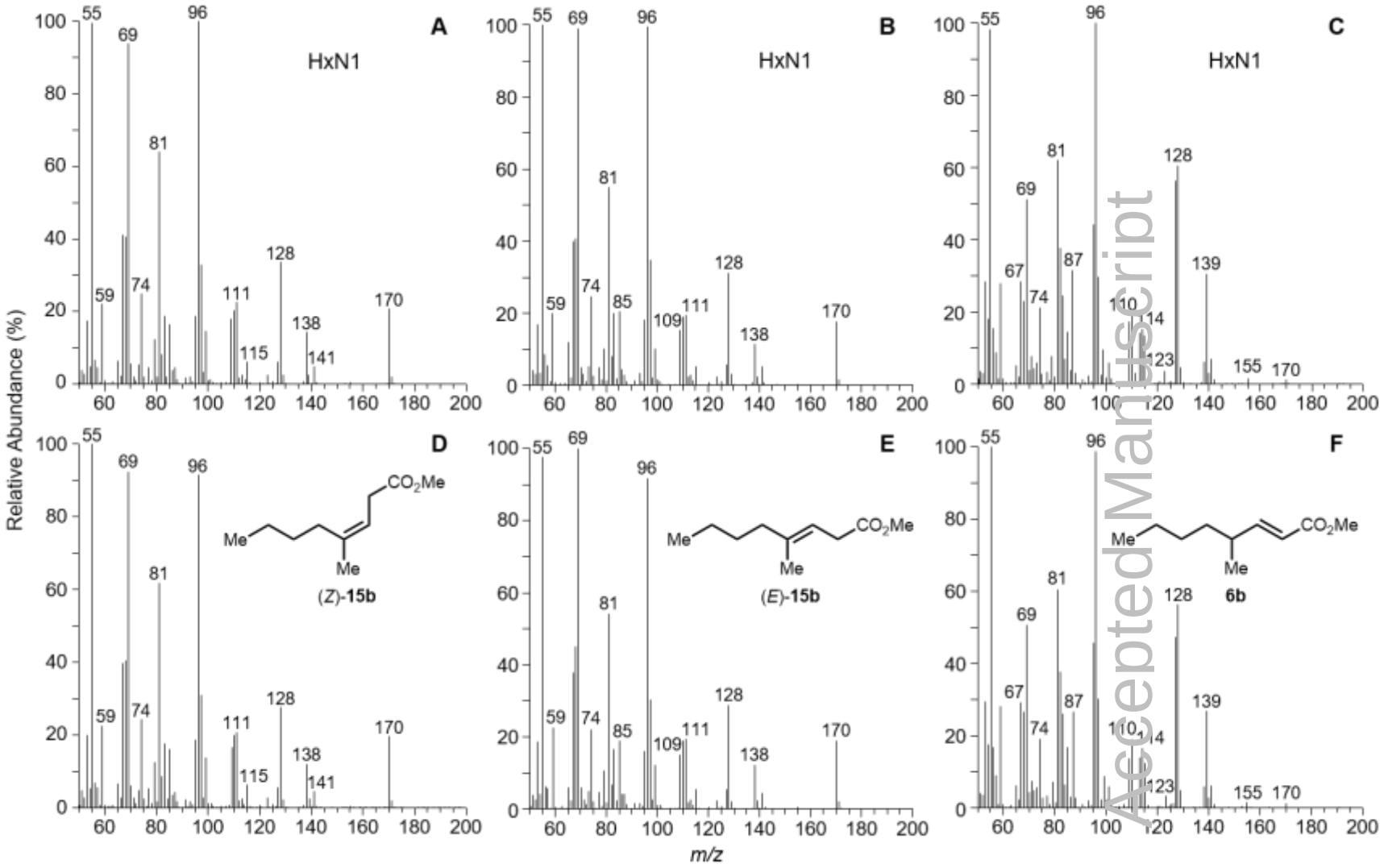

Figure 2. Mass spectra of compounds eluting at 21.42 (A), 22.07 (B) and 22.15 (C) min from methylated culture extracts of HxN1 (cf. Figure 1A) compared with the mass spectra of synthetic standards (*Z*)-**15b** (D), (*E*)-**15b** (E) and **6b** (F).

Conclusion

The betaproteobacterium strain HxN1 degrades *n*-hexane (**1**) under anoxic conditions into three equivalents of acetyl-CoA (**9**), which are further oxidized to CO_2 in the TCA cycle. A metabolic pathway has been proposed earlier to proceed from (2-methylhexyl)malonyl-CoA (**4**) through two rounds of β-oxidation *via* 4-methyloctanoate derivatives **5a**–**8a** and 2-methylhexanoate derivatives **10a**–**13a** (Scheme 1). Extracts of a strain HxN1 culture anaerobically grown with *n*-hexane (**1**) were actually submitted to thioester hydrolysis with hydrochloric acid and methylation with diazomethane to furnish the respective methyl esters **5b**–**13b** for analysis by GC-MS. While the structures of compounds **5b** and **10b**, the methyl esters related to thioesters **5a** and **10a**, have been elucidated earlier, the constitutions of methylated metabolites **6b**–**13b** were so far proposed based on mass spectrometric data. Furthermore, two β,γ-unsaturated congeners **14b** and **15b** were also proposed to be present in the culture extracts, although their role in the metabolic pathway remains so far unclear.

In this study, we have prepared synthetic compounds **6b**–**8b** and **11b**–**15b** and compared them with the constituents of the methylated culture extract by gas chromatography including co-injection experiments and mass spectrometry. This enabled us to unequivocally establish the molecular identities of seven metabolites which so far were only tentatively identified. The only exception was the methyl ester of 4-methyl-3-oxooctanoate (**8b**) which could not be detected in the extract, presumably because the steady-state concentration of this transient metabolite is below the detection limit.

In both, the 4-methyloctanoate (**6b**–**8b** and **15b**) and the 2-methylhexanoate (**10a**–**14a**) series, the organic synthesis started with the acylation of Meldrum's acid with the appropriate acid chlorides to furnish the β-oxoesters **8b** and **21**; the latter was α-methylated to give target compound **13b**. The β-oxoesters **8b** and **13b** were submitted to reduction with $NaBH_4$ to give the respective β-hydroxyesters **7b** and **12b** (both as mixtures of two racemic diastereoisomers). While compound **7b** could be eliminated to compound **6b** after activation of the hydroxy group as methyl sulfonate, this transformation was rather sluggish in the 2-methylhexanoate series. Therefore, we have prepared compound **11b** by HWE reaction of butanal with phosphorylpropionate **22**.

Isomerization of the α,β-double bonds in compounds **6b** and **11b** was accomplished in two steps: allylic bromination with NBS–AIBN furnished the γ-bromo congeners **19** and **23**. The C–C-double bond was then shifted to the β,γ-positon by reduction with Zn in AcOH to give compounds **14b** and **15b**.

The synthetic routes to obtain the reference standards, in particular for those of 4-methyloctanoate series, to a certain extent mimic the reversed pathway of *n*-hexane degradation in strain HxN1. This study furthers our metabolite-based understanding of the anaerobic degradation of *n*-hexane (**1**) by "*Aromatoleum*" sp. HxN1. Since this biodegradation pathway is archetypical for *n*-alkanes of a very broad chain length range and diverse anaerobic microorganisms including nitrate-, sulfate-, and arsenate-reducing bacteria and even a sulfate-reducing archaeon, the present findings will serve as valuable reference for pathway-oriented studies with pure cultures as well as environmental samples (e.g. from hydrocarbon-containing soils and sediments). It can be envisaged that the synthetic procedures described may also provide access to homologues with shorter and longer carbon chains and thus enable comprehensive metabolite-based investigations of anaerobic biodegradation of *n*-alkanes in laboratory-based as well as field studies.

Experimental Section

General. Preparative column chromatography was carried out using Merck SiO_2 (35–70 µm, type 60 A) with hexanes (mixture of isomers, bp. 64–71°C), *tert*-butylmethylether (MTBE), and CH_2Cl_2 as eluents. TLC was performed on aluminum plates coated with SiO_2 F_{254}. ^1H and ^{13}C NMR spectra were recorded on Bruker Avance DRX 500 and 300 instruments. Multiplicities of carbon signals were determined with DEPT experiments. HRMS spectra of products were obtained with Waters Q-TOF Premier (ESI) or Thermo Scientific DFS (EI) spectrometers. IR spectra were recorded on a Bruker Tensor 27 spectrometer equipped with a diamond ATR unit. All starting materials were commercially available.

2-Methylhexanoyl chloride. A mixture of 2-methylhexanoic acid (**18**) (8.7 mL, 8.0 g, 61 mmol, 1.0 equiv.) and $SOCl_2$ (17.8 mL, 29.2 g, 246 mmol, 4.0 equiv.) was heated to reflux for 3 h (gas evolution). Subsequently, the mixture was submitted to vacuum distillation through a 10 cm-Vigreux column yielding 2-methylhexanoyl chloride (8.51

g, 57.3 mmol, 93%) at 53°C (19 mbar) as a colorless liquid. ^{1}H-NMR (500 MHz, $CDCl_3$): δ = 0.91 (t, *J* = 6.9 Hz, 3H), 1.28 (d, *J* = 6.9 Hz, 3H), 1.31–1.36 (m, 4H), 1.48–1.57 (m, 1H), 1.76–1.85 (m, 1H), 2.86 (sex, *J* = 6.9 Hz, 1H) ppm. ^{13}C{^{1}H}-NMR (75 MHz, $CDCl_3$): δ = 13.8 (CH_3), 16.9 (CH_3), 22.4 (CH_2), 28.8 (CH_2), 33.1 (CH_2), 51.4 (CH), 177.8 (C) ppm. IR (ATR): nu(tilde) 2960 (s), 2935 (s), 2863 (m), 1790 (vs), 1459 (s), 1381 (m), 1144 (m), 934 (vs), 892 (m), 861 (s), 801 (w), 733 (w), 705 (s), 680 (m), 646 (w) cm^{-1}. $C_7H_{13}ClO$ (148.63).

Methyl 4-Methyl-3-oxooctanoate (8b). DMAP (5.86 g, 48.0 mmol, 1.8 equiv.) was added to a solution of Meldrum's acid (3.46 g, 24.0 mmol, 0.9 equiv.) in CH_2Cl_2. After stirring the mixture for 15 min at ambient temperature, 2-methylhexanoyl chloride (4.1 mL, 3.9 g, 26 mmol, 1.0 equiv, prepared as given above) was dropwise added over a period of 15 min. After stirring the mixture for further 15 h at ambient temperature, hydrochloric acid (2 mol L^{-1}, 100 mL) was added and the resulting suspension vigorously stirred for 5 min. The layers were separated and the organic layer was washed with water (100 mL). Both combined aqueous layers were extracted with CH_2Cl_2 (2 x 75 mL, 1 x 50 mL). All four organic layers were combined, dried ($MgSO_4$) and evaporated after filtration. The residue was dissolved in MeOH (100 mL) and the solution heated to reflux for 1 d. After evaporation, the residue was submitted to chromatography (SiO_2, hexanes/MTBE 6:1, R_f = 0.38) to furnish the title compound **8b** (3.13 g, 16.8 mmol, 70%) as a colorless liquid. According to ^{1}H NMR, the compound exists as two tautomers (keto/enol 85:15). ^{1}H-NMR (500 MHz, $CDCl_3$), keto tautomer: δ = 0.90 (t, *J* = 7.0 Hz, 3H), 1.11 (d, *J* = 7.0 Hz, 3H), 1.23–1.42 (m, 5H), 1.64–1.72 (m, 1H), 2.62 (sex, *J* = 6.9 Hz, 1H), 3.49 (s, 2H), 3.74 (s, 3H) ppm; enol tautomer: δ = 0.89 (t, *J* = 7.0 Hz, 3H), 1.13 (d, *J* = 7.0 Hz, 3H), 1.23–1.42 (m, 5H), 1.55–1.64 (m, 1H), 2.22 (sex, *J* = 6.9 Hz, 1H), 3.73 (s, 3H), 4.98 (s, 1H), 12.03 (s, 1H) ppm. ^{13}C{^{1}H}-NMR (125 MHz, $CDCl_3$), keto tautomer: δ = 14.1 (CH_3), 16.1 (CH_3), 22.8 (CH_2), 29.4 (CH_2), 32.5 (CH_2), 46.8 (CH), 47.6 (CH_2), 52.4 (CH_3), 167.9 (C), 206.6 (C) ppm; signals for the enol tautomer could not be identified with certainty. IR (ATR): nu(tilde) 2957 (s), 2933 (s), 2875 (m), 2861 (m), 1749 (vs), 1714 (vs), 1652 (s), 1626 (s), 1451 (s), 1437 (s), 1404 (m), 1377 (m), 1311 (s), 1232 (vs), 1155 (s), 1003 (s), 840 (m), 805 (m) cm^{-1}. MS (EI, 70 eV): *m/z* (%) 186 (0.5) [M^{+}], 155 (1), 143 (9), 130 (100), 101 (47), 98 (86), 85 (65), 74 (26), 69 (68), 59 (36), 57 (54). HRMS (ESI): calcd. 193.1416 (for $C_{10}H_{18}LiO_3^{+}$), found 193.1409 [M + Li^{+}]. $C_{10}H_{18}O_3$ (186.25).

Methyl 3-Hydroxy-4-methyloctanoate (7b). At 0°C (ice–water bath), $NaBH_4$ (62 mg, 1.6 mmol, 1.2 equiv.) was added to a solution of compound **8b** (254 mg, 1.36 mmol, 1.0 equiv.) in MeOH (5 mL). After stirring the mixture for 17 h at ambient temperature, AcOH (1 mol L^{-1}, 10 mL) was added. It was extracted with MTBE (3 x 15 mL) and the organic layers were combined, dried ($MgSO_4$) and evaporated after filtration. The residue was submitted to chromatography (SiO_2, hexanes/MTBE 1:1, R_f = 0.40) to furnish the title compound **7b** (200 mg, 1.06 mmol, 78%) as a colorless liquid. According to ^1H NMR, the compound exists as two diastereoisomers (*dr* 53:47). ^1H-NMR (500 MHz, $CDCl_3$), major isomer: δ = 0.89 (t, *J* = 6.6 Hz, 3H), 0.91 (d, *J* = 6.7 Hz, 3H), 1.08–1.41 (m, 5H), 1.42–1.55 (m, 1H), 1.55–1.64 (m, 1H), 2.47 (dd, *J* = 16.3 Hz, *J* = 9.7 Hz, 1H), 2.49 (dd, *J* = 16.3 Hz, *J* = 2.6 Hz, 1H), 2.83 (d, *J* = 3.8 Hz, 1H), 3.71 (s, 3H), 3.87 (dddd, *J* = 9.7 Hz, *J* = 6.4 Hz, *J* = 3.8 Hz, *J* = 2.6 Hz, 1H) ppm; minor isomer: δ = 0.89 (t, *J* = 6.6 Hz, 3H), 0.91 (d, *J* = 6.7 Hz, 3H), 1.08–1.41 (m, 5H), 1.42–1.55 (m, 2H), 2.45–2.48 (m, 2H), 2.68 (d, *J* = 3.7 Hz, 1H), 3.71 (s, 3H), 3.91–3.96 (m, 1H) ppm. $^{13}C\{^1H\}$-NMR (125 MHz, $CDCl_3$), major isomer: δ = 14.2 (CH_3), 15.0 (CH_3), 23.09 (CH_2), 29.5 (CH_2), 32.1 (CH_2), 37.8 (CH_2), 38.3 (CH), 51.9 (CH_3), 72.0 (CH), 174.1 (C) ppm; minor isomer: δ = 14.2 (CH_3), 14.4 (CH_3), 23.07 (CH_2), 29.6 (CH_2), 32.6 (CH_2), 38.2 (CH), 38.8 (CH_2), 51.9 (CH_3), 71.4 (CH), 174.0 (C) ppm. IR (ATR): nu(tilde) 3480 (s, br), 2957 (s), 2928 (s), 2873 (m), 2860 (m), 1725 (vs), 1460 (m), 1438 (m), 1379 (w), 1338 (w), 1338 (w), 1277 (m), 1194 (m), 1170 (vs), 1048 (s), 1013 (m), 989 (s) cm^{-1}. MS (EI, 70 eV): *m/z* (%) 170 (0.5), 139 (4), 128 (4), 103 (100), 74 (12), 71 (42). HRMS (ESI): calcd. 195.1572 (for $C_{10}H_{20}LiO_3^+$), found 195.1570 [M + Li^+]. $C_{10}H_{20}O_3$ (188.26).

Methyl (*E*)-4-Methyl-2-octenoate (6b). At 0°C (ice–water bath), $MeSO_2Cl$ (626 mg, 5.47 mmol, 1.4 equiv.) and NEt_3 (3.56 g, 35.0 mmol, 9.0 equiv.) were added to a solution of compound **7b** (735 mg, 3.90 mmol, 1.0 equiv.) in CH_2Cl_2 (20 mL). After stirring the mixture for 28 h at ambient temperature, saturated aqueous NH_4Cl solution (40 mL) was added. The mixture was extracted with CH_2Cl_2 (3 x 40 mL) and the organic layers were combined, dried ($MgSO_4$) and evaporated after filtration. The residue was submitted to chromatography (SiO_2, hexanes/MTBE 1:1, R_f = 0.62) to furnish the title compound **6b** (513 mg, 3.01 mmol, 77%) as a colorless liquid. ^1H-NMR (500 MHz, $CDCl_3$): δ = 0.88 (t, *J* = 7.0 Hz, 3H), 1.04 (d, *J* = 6.7 Hz, 3H), 1.21–1.41

(m, 6H), 2.29 (qtdd, *J* = 7.9 Hz, *J* = 6.8 Hz, *J* = 6.4 Hz, *J* = 1.2 Hz, 1H), 3.73 (s, 3H), 5.77 (dd, *J* = 15.7 Hz, *J* = 1.2 Hz, 1H), 6.87 (dd, *J* = 15.7 Hz, *J* = 7.9 Hz, 1H) ppm. $^{13}C\{^1H\}$-NMR (125 MHz, $CDCl_3$): δ = 14.2 (CH_3), 19.6 (CH_3), 22.9 (CH_2), 29.5 (CH_2), 35.9 (CH_2), 36.7 (CH), 51.5 (CH_3), 119.3 (CH), 155.3 (CH), 167.5 (C) ppm. IR (ATR): nu(tilde) 2958 (s), 2929 (s), 2873 (m), 2859 (m), 1724 (vs), 1656 (s), 1458 (m), 1435 (s), 1379 (w), 1352 (m), 1310 (m), 1268 (s), 1213 (m), 1175 (s), 1151 (m), 1138 (m), 1036 (w), 1012 (w), 983 (m), 941 (w), 916 (w), 861 (w), 725 (w), 715 (w), 646 (w) cm^{-1}. MS (EI, 70 eV): *m/z* (%) 170 (1.5) [M^+], 155 (2), 141 (5), 139 (29), 128 (56), 127 (54), 96 (100). HRMS (ESI): calcd. 177.1467 (for $C_{10}H_{18}LiO_2^+$), found 177.1465 [M + Li^+]. $C_{10}H_{18}O_2$ (170.25).

Methyl (*E*)-4-Bromo-4-methyl-2-octenoate (19). NBS (0.53 g, 3.0 mmol, 1.0 equiv.) and AIBN (30 mg, 0.18 mmol, 6 mol%) were added to a solution of compound **6b** (0.51 g, 3.0 mmol, 1.0 equiv.) in $CHCl_3$ (5 mL). After stirring the mixture for further 19 h at 75°C, it was cooled (ice–water bath) and filtered, the residue rinsed with CH_2Cl_2 (5 mL) and the filtrate was washed with water (3 x 20 mL). The organic layer was dried ($MgSO_4$) and evaporated after filtration. The residue was submitted to Kugelrohr distillation (175°C, 0.9 mbar) to furnish the title compound **19** (0.59 g, 2.4 mmol, 79%) as a colorless liquid. ^1H-NMR (300 MHz, $CDCl_3$): δ = 0.92 (t, *J* = 7.0 Hz, 3H), 1.30–1.46 (m, 4H), 1.87 (s, 3H), 1.91–2.03 (m, 2H), 3.77 (s, 3H), 5.91 (d, *J* = 15.7 Hz, 1H), 7.09 (d, *J* = 15.7 Hz, 1H) ppm. $^{13}C\{^1H\}$-NMR (75 MHz, $CDCl_3$): δ = 13.9 (CH_3), 22.6 (CH_2), 28.1 (CH_2), 29.4 (CH_3), 45.0 (CH_2), 51.8 (CH_3), 65.4 (C), 118.5 (CH), 151.8 (CH), 166.7 (C) ppm. IR (ATR): nu(tilde) 2956 (m), 2938 (m), 2920 (w), 2877 (m), 1727 (vs), 1653 (w), 1437 (s), 1382 (w), 1313 (m), 1283 (s), 1199 (m),1177 (m), 1158 (m), 1045 (m), 1015 (m), 985 (w), 868 (w), 723 (w), 634 (w), 621 (w) cm^{-1}. HRMS (ESI): calcd. 249.0485 (for $C_{10}H_{18}BrO_2^+$), found 249.0484 [M + H^+]. $C_{10}H_{17}BrO_2$ (249.15).

Methyl 4-Methyl-3-octenoate (15b). Zinc powder (1.00 g, 15.3 mmol, 1.0 equiv.) was added to a solution of compound **19** (0.46 g, 1.86 mmol, 8.2 equiv.) in AcOH (4 mL) and the solution was stirred at ambient temperature for 1 h. It was diluted with water (20 mL), filtered and the filtrate was extracted with MTBE (3 x 20 mL). The combined organic layers were dried ($MgSO_4$) and evaporated after filtration. The residue was submitted to chromatography (SiO_2, hexanes/MTBE/CH_2Cl_2 100:5:1, R_f =

0.43) to furnish the title compound **15b** (0.20 g, 1.2 mmol, 63%) as a mixture of two stereoisomers (*E*/*Z* = 2:1) and as a colorless oil. ^{1}H-NMR (300 MHz, $CDCl_3$): δ = 0.89 (t, *J* = 7.1 Hz, 3H), 1.22–1.44 (m, 4H), 1.62 [s, 2/3 x 3H; (*E*)-isomer], 1.73 [s, 1/3 x 3H; (*Z*)-isomer], 2.02 (t, *J* = 7.0 Hz, 2H), 3.05 (d, *J* = 7.1 Hz, 2H), 3.68 (s, 3H), 5.31 (tq, *J* = 7.1 Hz, *J* = 1.1 Hz, 1H), ppm. ^{13}C{^{1}H}-NMR (75 MHz, $CDCl_3$), (*E*)-isomer: δ = 13.97 (CH_3), 16.2 (CH_3), 22.3 (CH_2), 29.9 (CH_2), 33.5 (CH_2), 39.2 (CH), 51.7 (CH_3), 115.3 (CH), 139.5 (C), 173.0 (C) ppm; (*Z*)-isomer: δ = 14.01 (CH_3), 22.6 (CH_2), 23.4 (CH_3), 30.0 (CH_2), 31.7 (CH_2), 33.3 (CH_2), 51.7 (CH_3), 115.9 (CH), 139.7 (C), 173.0 (C) ppm. IR (ATR): nu(tilde) 2961 (s), 2930 (s), 2872 (m), 2859 (m), 1741 (vs), 1627 (vw), 1435 (s), 1379 (w), 1312 (m), 1261 (s), 1193 (m), 1164 (s), 1015 (m), 983 (m), 836 (w), 729 (w), 613 (w) cm^{-1}. GC-MS (EI, 70 eV), (*E*)-isomer: *m/z* (%) 170 (19) [M^+], 138 (12), 128 (29), 127 (6), 111 (20), 109 (15), 96 (93), 85 (19), 81 (55), 74 (23), 69, (100), 68 (45), 67 (43), 65 (7), 59 (23), 55 (100); (*Z*)-isomer: *m/z* (%) 170 (19) [M^+], 138 (12), 128 (29), 127 (6), 111 (20), 109 (17), 97 (30), 96 (90), 81 (63), 74 (24), 69, (91), 67 (40), 65 (7), 59 (22), 55 (100). HRMS (ESI): calcd. 177.1461 (for $C_{10}H_{18}LiO_2^+$), found 177.1463 [M + Li^+]. $C_{10}H_{18}O_2$ (170.25).

Methyl 3-Oxohexanoate (21). DMAP (0.68 g, 5.6 mmol, 0.2 equiv.) and pyridine (4.4 g, 56 mmol, 1.8 equiv.) were added to a solution of Meldrum's acid (4.00 g, 28.0 mmol, 0.9 equiv.) in CH_2Cl_2 (35 mL). After stirring the mixture for 15 min at ambient temperature, butanoyl chloride (**20**) (3.3 g, 31 mmol, 1.0 eq.) was added dropwise at 0°C (ice–water bath) over a period of 15 min. After stirring the mixture for further 19 h at ambient temperature, hydrochloric acid (2 mol L^{-1}, 100 mL) was added and the resulting suspension vigorously stirred for 5 min. The layers were separated and the organic layer was washed with water (100 mL). Both combined aqueous layers were extracted with CH_2Cl_2 (3 x 50 mL). All four organic layers were combined, washed with hydrochloric acid (2 mol L^{-1}, 2 x 50 mL) and brine (50 mL), dried ($MgSO_4$) and evaporated after filtration. The residue was dissolved in MeOH (35 mL) and the solution heated to reflux for 4 h. After evaporation, the residue was submitted to chromatography (SiO_2, hexanes/MTBE 3:1, R_f = 0.40) to furnish the title compound **21** (1.52 g, 10.5 mmol, 38%) as a colorless liquid. According to ^{1}H NMR, the compound exists as two tautomers (keto/enol 9:1). ^{1}H-NMR (300 MHz, $CDCl_3$), keto-tautomer: δ = 0.92 (t, *J* = 7.4 Hz, 3H), 1.63 (sex, *J* = 7.3 Hz, 2H), 2.51 (t, *J* = 7.3 Hz, 2H), 3.44 (s, 2H), 3.74 (s, 3H) ppm; enol-tautomer: δ = 0.97 (t, *J* = 7.5 Hz, 3H), 1.63 (sex, *J* = 7.3 Hz,

2H), 2.17 (t, *J* = 7.5 Hz, 2H), 3.72 (s, 3H), 4.99 (s, 1H), 12.01 (s, 1H) ppm. $^{13}C\{^1H\}$-NMR (75 MHz, $CDCl_3$), keto-tautomer: δ = 13.5 (CH_3), 16.9 (CH_2), 44.9 (CH_2), 49.0 (CH_2), 52.3 (CH_3), 167.7 (C), 202.7 (C) ppm; signals for the enol tautomer could not be identified with certainty. IR (ATR): nu(tilde) 2963 (m), 2937 (w), 2878 (w), 1744 (vs), 1714 (vs), 1630 (w), 1437 (s), 1408 (m), 1319 (s), 1259 (s), 1230 (s), 1155 (s), 1124 (m), 1072 (m), 1006 (s), 853 (w) cm^{-1}. MS (EI, 70 eV): *m/z* (%) 144 (9) [M^+], 129 (2), 116 (4), 101 (27), 84 (7), 71 (100), 59 (33), 57 (13). HRMS (ESI): calcd. 151.0941 (for $C_7H_{12}LiO_3^+$), found 151.0943 [M + Li^+]. $C_7H_{12}O_3$ (144.17).

Methyl 2-Methyl-3-oxohexanoate (13b). Compound **21** (1.45 g, 10.1 mmol, 1.0 equiv.) was added to a solution of K_2CO_3 (1.39 g, 10.1 mmol, 1.0 equiv.) in acetone (10 mL). After stirring the mixture for 30 min at ambient temperature, methyl iodide (2.14 g, 15.1 mmol, 1.5 equiv.) was added dropwise at 0°C (ice–water bath) over a period of 15 min. After stirring the mixture for further 3 h at ambient temperature, water (20 mL) was added. It was extracted with MTBE (3 x 20 mL) and the organic layers were combined, dried ($MgSO_4$) and evaporated after filtration. The residue was submitted to chromatography (SiO_2, hexanes/MTBE 5:1, R_f = 0.37) to furnish the title compound **13b** (0.87 g, 5.5 mmol, 55%) as a colorless liquid. According to ^1H NMR, the compound exists as two tautomers (keto/enol 9:1). ^1H-NMR (500 MHz, $CDCl_3$), keto-tautomer: δ = 0.91 (t, *J* = 7.4 Hz, 3H), 1.34 (d, *J* = 7.1 Hz, 3H), 1.62 (sex, *J* = 7.4 Hz, 2H), 2.47 (dt, *J* = 17.3 Hz, *J* = 7.1 Hz, 1H), 2.55 (dt, *J* = 17.4 Hz, *J* = 7.3 Hz, 1H), 3.53 (q, *J* = 7.2 Hz, 1H), 3.73 (s, 3H) ppm; enol-tautomer: δ = 0.96 (t, *J* = 7.6 Hz, 3H), 1.62 (sex, *J* = 7.4 Hz, 2H), 1.75 (s, 3H), 2.28 (t, *J* = 7.6 Hz, 2H), 3.76 (s, 3H), 12.66 (s, 1H) ppm. $^{13}C\{^1H\}$-NMR (125 MHz, $CDCl_3$), keto-tautomer: δ = 12.8 (CH_3), 13.5 (CH_3), 17.0 (CH_2), 43.2 (CH_2), 52.3 (CH_3), 52.7 (CH), 171.1 (C), 205.8 (C) ppm; signals for the enol tautomer could not be identified with certainty. IR (ATR): nu(tilde) 2961 (m), 2877 (w), 1745 (vs), 1714 (vs), 1455 (s), 1436 (m), 1377 (m), 1327 (m), 1248 (s), 1202 (s), 1177 (m), 1122 (m), 1072 (m), 1018 (m), 967 (w), 898 (w), 860 (m) cm^{-1}. MS (EI, 70 eV): *m/z* (%) 158 (2) [M^+], 129 (3), 127 (5), 115 (6), 88 (13), 87 (7), 83 (2), 71 (100), 59 (16). HRMS (ESI): calcd. 165.1103 (for $C_8H_{14}LiO_3^+$), found 165.1108 [M + Li^+]. $C_8H_{14}O_3$ (158.19).

Methyl 3-Hydroxy-2-methylhexanoate (12b). $NaBH_4$ (0.29 g, 7.6 mmol, 1.2 equiv.) was added to a solution of compound **13b** (1.00 g, 6.32 mmol, 1.0 equiv.) in MeOH

(20 mL). After stirring the mixture for 3 h at ambient temperature, water (20 mL) was added. The mixture was extracted with MTBE (3 x 30 mL) and the organic layers were combined, dried ($MgSO_4$) and evaporated after filtration. The residue was submitted to chromatography (SiO_2, hexanes/MTBE 1:1, R_f = 0.36) to furnish the title compound **12b** (0.58 g, 2.6 mmol, 57%) as a colorless liquid. According to ^1H NMR, the compound exists as two diastereoisomers (*dr* 3:2). ^1H-NMR (500 MHz, $CDCl_3$), major isomer: δ = 0.93 (t, *J* = 7.2 Hz, 3H), 1.21 (d, *J* = 7.3 Hz, 3H), 1.29–1.59 (m, 4H), 2.49 (d, *J* = 6.9 Hz, 1H), 2.51–2.57 (m, 1H), 3.64–3.70 (m, 1H), 3.71 (s, 3H) ppm, the signal at 2.49 ppm was identified as OH-signal by H-D-exchange (D_2O); minor isomer: δ = 0.94 (t, *J* = 7.0 Hz, 3H), 1.18 (d, *J* = 7.3 Hz, 3H), 1.29–1.59 (m, 4H), 2.43 (d, *J* = 4.7 Hz, 1H), 2.51–2.57 (m, 1H), 3.71 (s, 3H), 3.89–3.93 (m, 1H) ppm, the signal at 2.43 ppm was identified as OH-signal by H-D-exchange (D_2O). $^{13}C\{^1H\}$-NMR (125 MHz, $CDCl_3$), major isomer: δ = 10.8 (CH_3), 14.2 (CH_3), 18.9 (CH_2), 37.1 (CH_2), 45.4 (CH), 51.9 (CH_3), 73.3 (CH), 176.7 (C) ppm; minor isomer: δ = 10.8 (CH_3), 14.5 (CH_3), 19.3 (CH_2), 36.1 (CH_2), 44.4 (CH), 52.0 (CH_3), 71.6 (CH), 176.7 (C) ppm. IR (ATR): nu(tilde) 3457 (s, br), 2957 (s), 2874 (m), 1720 (vs), 1459 (m), 1436 (m), 1255 (m), 1197 (s), 1170 (s), 1120 (m), 1055 (m), 1026 (m), 1013 (m), 985 (m), 851 (m) cm^{-1}. GC-MS (EI, 70 eV), major isomer: *m/z* (%) 145 (0.5), 129 (2), 117 (40), 111 (6), 88 (100), 85 (34), 83 (9); minor isomer: *m/z* (%) 145 (2), 129 (1), 117 (16), 111 (5), 88 (100), 85 (23), 83 (7). HRMS (ESI): calcd. 167.1259 (for $C_8H_{16}LiO_3^+$), found 167.1260 [M + Li^+]. $C_8H_{16}O_3$ (160.21).

Methyl (*E*)- and (*Z*)-2-Methyl-2-hexenoate (11b). At 0°C (ice–water bath), KO*t*Bu (6.10 g, 54.3 mmol, 1.1 equiv.) was added to a solution of methyl 2-(diethoxyphosphoryl)propanoate (**22**) (11.1 g, 49.4 mmol, 1.0 equiv.) in THF (100 mL). After stirring the mixture for 15 min at ambient temperature, butanal (3.57 g, 49.4 mmol, 1.0 equiv.) was added at 0°C (ice–water bath). After stirring the mixture for further 1 h at ambient temperature, saturated aqueous NH_4Cl solution (40 mL) was added. The mixture was extracted with MTBE (3 x 40 mL) and the organic layers were combined, dried ($MgSO_4$) and evaporated after filtration. The residue was submitted to chromatography (SiO_2, hexanes/MTBE/CH_2Cl_2 20:1:0.1) to yield the title compound (*Z*)-**11b** (930 mg, 6.54 mmol, 13%) as the first fraction (R_f = 0.53) as a colorless liquid. Secondly, compound (*E*)-**11b** (3.36 g, 23.6 mmol, 48%) was eluted (R_f = 0.35), also a colorless liquid; ratio (*E*/*Z* 3.6:1).

(*E*)-Isomer: ^{1}H-NMR (300 MHz, $CDCl_3$): δ = 0.92 (t, *J* = 7.4 Hz, 3H), 1.45 (sex, *J* = 7.4 Hz, 2H), 1.81 (s, 3H), 2.13 (q, *J* = 7.4 Hz, 2H), 3.71 (s, 3H), 6.74 (t, *J* = 7.4 Hz, 1H) ppm. ^{13}C{^{1}H}-NMR (75 MHz, $CDCl_3$): δ = 12.4 (CH_3), 13.9 (CH_3), 21.8 (CH_2), 30.7 (CH_2), 51.7 (CH_3), 127.6 (C), 142.6 (CH), 168.8 (C) ppm. IR (ATR): nu(tilde) 2958 (m), 2931 (w), 2876 (w), 1714 (vs), 1650 (m), 1434 (m), 1280 (m), 1220 (m), 1143 (s), 1094 (m), 742 (m) cm^{-1}. MS (EI, 70 eV): *m/z* (%) 142 (33) [M^+], 127 (17), 111 (33), 101 (42), 95 (25), 88 (31), 83 (26), 82 (24), 81 (17), 73 (23), 69 (19), 67 (22), 59 (19), 55 (100). HRMS (ESI): calcd. 149.1154 (for $C_8H_{14}LiO_2^+$), found 149.1155 [M + Li^+]. $C_8H_{14}O_2$ (142.20).

(*Z*)-Isomer: ^{1}H-NMR (300 MHz, $CDCl_3$): δ = 0.91 (t, *J* = 7.4 Hz, 3H), 1.41 (sex, *J* = 7.4 Hz, 2H), 1.88 (d, *J* = 1.2 Hz, 3H), 2.42 (q, *J* = 7.4 Hz, 2H), 3.72 (s, 3H), 5.93 (tq, *J* = 7.4 Hz, *J* = 1.2 Hz, 1H) ppm. ^{13}C{^{1}H}-NMR (75 MHz, $CDCl_3$): δ = 13.8 (CH_3), 20.6 (CH_3), 22.6 (CH_2), 31.6 (CH_2), 51.1 (CH_3), 126.8 (C), 143.5 (CH), 168.5 (C) ppm. IR (ATR): nu(tilde) 2952 (m), 2930 (w), 2866 (w), 1715 (vs), 1647 (m), 1434 (m), 1274 (m), 1218 (m), 1148 (s), 1070 (m), 768 (m) cm^{-1}. MS (EI, 70 eV): *m/z* (%) 142 (45), 127 (28), 111 (32), 101 (35), 95 (38), 81 (24), 67 (35), 55 (100). HRMS (ESI): calcd. 143.1067 (for $C_8H_{14}LiO_2^+$), found 143.1070 [M + H^+]. $C_8H_{14}O_2$ (142.20).

Methyl (*E*)-4-Bromo-2-methyl-2-hexenoate (23). NBS (4.62 g, 26.0 mmol, 1.0 equiv.) and AIBN (0.26 g, 1.6 mmol, 6 mol%) were added to a solution of compound (*E*)-**11b** (3.69 g, 26.0 mmol, 1.0 equiv.) in CCl_4 (40 mL). After stirring the mixture for further 19 h at 80°C, it was cooled (ice–water bath) and filtered, the residue rinsed with CH_2Cl_2 (5 mL) and the filtrate was washed with water (3 x 20 mL). The organic layer was dried ($MgSO_4$) and evaporated after filtration. The residue was submitted to chromatography (SiO_2, hexanes/MTBE 7:1, R_f = 0.56) to furnish the title compound **23** (5.37 g, 24.3 mmol, 93%) as a colorless liquid. ^{1}H-NMR (300 MHz, $CDCl_3$): δ = 1.01 (t, *J* = 7.3 Hz, 3H), 1.90 (d, *J* = 1.4 Hz, 3H), 1.86–2.07 (m, 2H), 3.76 (s, 3H), 4.68 (dt, *J* = 10.7 Hz, *J* = 7.0 Hz, 1H), 6.81 (dq, *J* = 10.7 Hz, *J* = 1.4 Hz, 1H) ppm. ^{13}C{^{1}H}-NMR (75 MHz, $CDCl_3$): δ = 12.2 (CH_3), 12.6 (CH_3), 32.0 (CH_2), 50.3 (CH_3), 52.1 (CH), 129.1 (C), 140.3 (CH), 167.9 (C) ppm. IR (ATR): nu(tilde) 2972 (m), 2956 (w), 2938 (w), 2876 (w), 1715 (vs), 1646 (m), 1435 (m), 1276 (s), 1234 (s), 1191 (m), 1119 (m), 748 (s) cm^{-1}. HRMS (ESI): calcd. 242.9991 (for $C_8H_{13}BrNaO_2^+$), found 242.9997 [M + Na^+]. $C_8H_{13}BrO_2$ (221.09).

Methyl 2-Methyl-3-hexenoate (14b). Zinc powder (3.17 g, 48.5 mmol, 2.0 equiv.) was added to a solution of compound **23** (5.37 g, 24.3 mmol, 1.0 equiv.) in AcOH (100 mL) and the solution was stirred at 0°C for 1 h, then at ambient temperature for 1 h. It was diluted with water (50 mL), filtered and the filtrate was extracted with MTBE (3 x 80 mL). The combined organic layers were washed successively with hydrochloric acid (2 mol L^{-1}, 50 mL), saturated aqueous $NaHCO_3$ solution (150 mL), and brine (80 mL), then dried ($MgSO_4$), filtered and evaporated after filtration. The residue was submitted to chromatography (SiO_2, hexanes/MTBE 10:1, R_f = 0.33) to furnish the title compound **14b** (1.91 g, 13.4 mmol, 55%) as a colorless liquid. ^{1}H-NMR (300 MHz, $CDCl_3$): δ = 0.96 (t, *J* = 7.5 Hz, 3H), 1.22 (d, *J* = 7.1 Hz, 3H), 2.02 (pent, *J* = 7.1 Hz, 2H), 3.09 (pent, *J* = 7.1 Hz, 1H), 3.66 (s, 3H), 5.46 (dd, *J* = 15.5 Hz, *J* = 7.3 Hz, 1H), 5.57 (dt, *J* = 15.4 Hz, *J* = 5.7 Hz, 1H) ppm. ^{13}C{^{1}H}-NMR (75 MHz, $CDCl_3$): δ = 13.4 (CH_3), 17.5 (CH_3), 25.4 (CH_2), 42.7 (CH), 51.7 (CH_3), 127.7 (CH), 133.8 (CH), 175.6 (C) ppm. IR (ATR): nu(tilde) 2968 (m), 2935 (w), 2876 (w), 1737 (vs), 1457 (m), 1434 (m), 1280 (m), 1249 (s), 1193 (s), 1165 (s), 1051 (m), 967 (m) cm^{-1}. MS (EI, 70 eV): *m/z* (%) 142 (7) [M^+], 127 (5), 88 (32), 83 (48), 67 (13), 55 (100). HRMS (ESI): calcd. 149.1148 (for $C_8H_{14}LiO_2^+$), found 143.1151 [M + Li^+]. $C_8H_{14}O_2$ (142.20).

Cultivation. The betaproteobacterium "*Aromatoleum*" sp. HxN1 has been subcultured in our laboratory since its isolation.[4] Cultivation was carried out in defined, bicarbonate-buffered medium, essentially as described earlier.[5] Cultures were grown in stopper-sealed flat glass bottles (500 mL) containing 400 mL medium under an anoxic atmosphere (N_2/CO_2 90:10, v/v). *n*-Hexane (**1**) was provided as dilution (5%, v/v) in 2,2,4,4,6,8,8-heptamethylnonane serving as inert carrier phase. Sodium *n*-hexanoate from a sterile stock solution was added to control cultures at a final concentration of 3 mM.

Preparation of culture extracts. Extracts for metabolite analysis were obtained from cultures of "*Aromatoleum*" sp. HxN1 as previously described.[5] Essentially, cultures were inactivated by heat (85°C in a water bath for 15 min). Overlying carrier phase (cultures with *n*-hexane) was removed by means of a separatory funnel, the obtained culture broth acidified (pH 1.5 with hydrochloric acid) and thereafter extracted three times with Et_2O. Finally, the combined organic layers were dried (Na_2SO_4) and after filtration, stored in teflon-sealed glass bottles until further analyses. In addition, co-in-

jection experiments for some compounds were performed using samples obtained by solid phase extraction of cell-free supernatants of strain HxN1 grown with *n*-hexane (**1**). Waters Oasis MAX (30 µm) was used as the solid phase. The adsorbed metabolites were eluted with 5% formic acid in methanol. The identity of the metabolite pattern with that of the original extracts was confirmed by GC-MS.

Derivatization and analysis of metabolites. Dried extracts were evaporated to dryness, solubilized in CH_2Cl_2 and methylated using freshly prepared diazomethane essentially as described before.[7] Methylated extracts were then analyzed by GC-MS using a Trace GC Ultra gas chromatograph coupled to an ISQ OD mass spectrometer (both Thermo Scientific). The GC was equipped with an Agilent DB5 fused silica capillary (30 m length, 0.25 mm internal diameter, 0.25 µm film thickness). Helium was used as carrier gas. The GC oven temperature was programmed from 40°C (2 min hold time) to 200°C at a rate of 3 K min^{-1} and further to 320°C (2 min hold time) at a rate of 20 K min^{-1}. The MS was operated in electron impact ionization mode (70 eV) at a source temperature of 220°C and a transfer line temperature of 280°C. The mass range was 50–650 Dalton at scan cycle time of 0.2 s. The analyses of reference standards and the co-injection experiments were performed on the same system using identical conditions.

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