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

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The constitutions of seven metabolites formed during anaerobic degradation of *n*-hexane by the denitrifying betaproteobacterium strain HxN1 were elucidated by comparison of their GC and MS data with those of synthetic reference standards. The synthesis of 4-methyloctanoic acid derivatives was accomplished by the conversion of 2-methylhexanoyl chloride with Meldrum's acid. The β-oxoester was reduced with $NaBH_4$, the hydroxy group was eliminated, and the double bond was 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. 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 Horner–Wadsworth–Emmons 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 the anaerobic biodegradation of *n*-hexane to 2-methyl-3-oxohexanoyl coenzyme A (CoA), which is then thiolytically cleaved to butanoyl-CoA and propionyl-CoA; these two metabolites are further transformed according to established pathways.

Introduction

The key challenge in the biodegradation of saturated hydrocarbons is C–H activation. The initial activation step requires cleavage of a C–H bond, which is associated with a high energy barrier that has to be overcome. In oxic environments (presence of O_2), this is accomplished by the well-studied oxygenase enzymes, which employ O_2-derived, highly reactive oxygen species.[1] Investigations into 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 is affiliated 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, the anaerobic degradation of **1** by strain HxN1 has been proposed to proceed via a 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 **1** during the 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 **2** was inferred from metabolite analysis to proceed through 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 carbon skeleton in the succinyl-CoA moiety by a 1,2- shift of the thioester moiety (step c), forming (2-methylhexyl)malonyl-CoA (**4**) to then give 4-methyloctanoyl-CoA (**5a**) by decarboxylation (step d). The β-oxidation sequence of **5a** 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) to form acetyl-CoA (**9**) and 2-methylhexanoyl-CoA (**10a**). A second round of β-oxidation, starting with **10a**, would involve the 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); the last of these is thiolytically cleaved into butanoyl-CoA (**16**) and propionyl-CoA (**17**). A third round of β-oxidation would then transform C_4-

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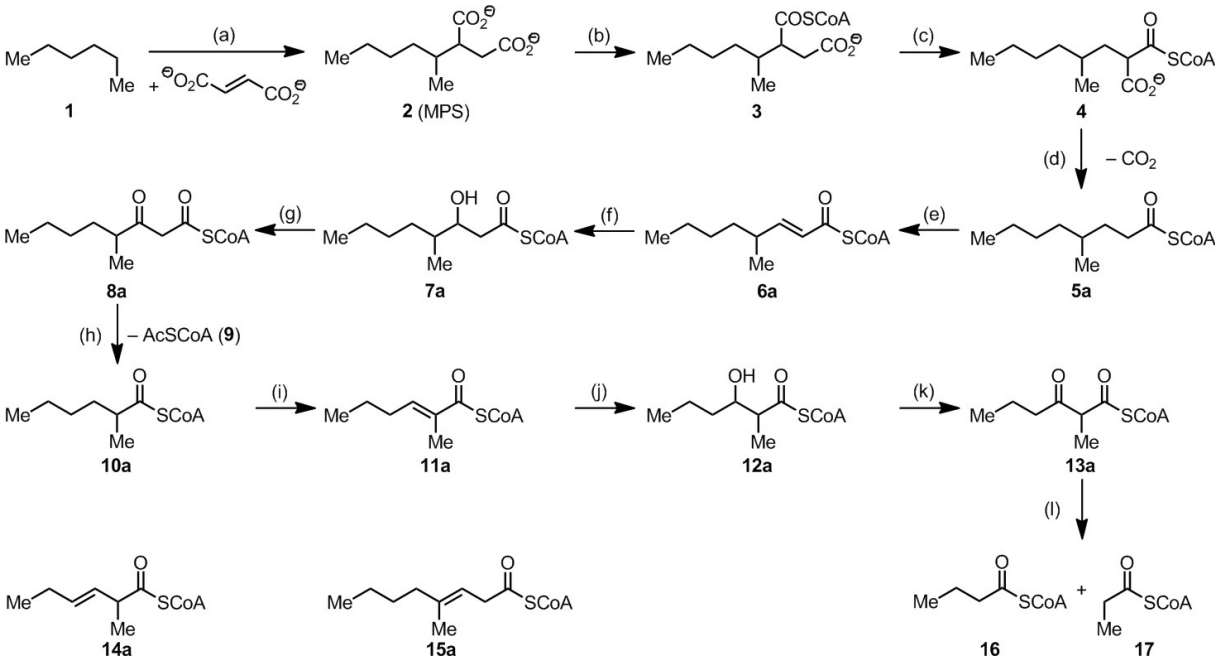
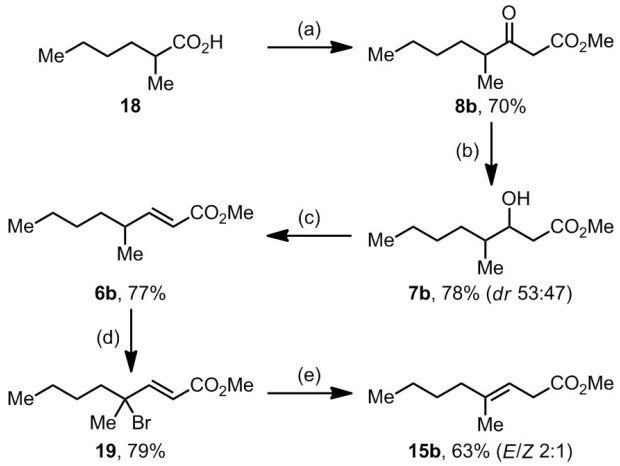


Scheme 1. Proposed pathway for the anaerobic degradation of **1** by strain HxN1; for corresponding transformations (a) to (l), see the main text. CoA: coenzyme A.

compound **16** into two molecules of **9**. Whereas the three molecules of **9** formed are terminally oxidized to CO_2 in the tricarboxylic acid (TCA) cycle, compound **17** could be converted into fumarate by the methylmalonyl-CoA pathway, which formally utilizes one equivalent of CO_2, and thus, recycles the co-substrate of the initial activation reaction of **1**.

Herein, we aimed to complete our metabolite-based understanding of the anaerobic degradation of **1** downstream of **2** by unambiguously elucidating the constitutions of intermediates **6a**, **7a**, and **11a**–**15a**, which, to date, have only tentatively been assigned based on MS data. This task was accomplished by the 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 means of GC-MS. Compounds **14a** and **15a**, with isomerized C=C bonds, are actually not included in the metabolic pathway shown in Scheme 1, but the respective methyl esters **14b** and **15b** are present in the methylated culture extract of strain HxN1.



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

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 into its acid chloride, which was isolated, and purified by distillation (Scheme 2).[8] Conversion with Meldrum's acid and subsequent solvolysis with methanol under standard conditions (with pyridine and catalytic amounts of DMAP),[9] however, gave complex reaction mixtures that contained only small amounts of β-oxoester **8b**.[10] After some experimentation, the use of an overstoichiometric amount of DMAP (1.8 equiv) gave 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 the mixture was submitted to elimination via the methanesulfonate to give α,β-unsaturated ester

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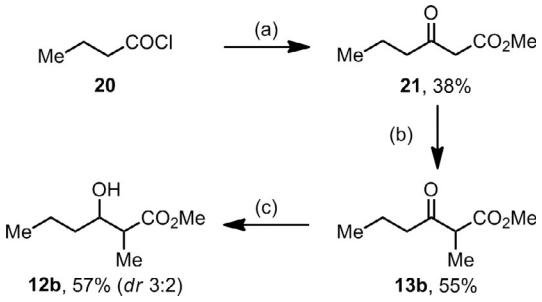
6b[12] in 77% yield and exclusively as the (*E*)-isomer. The C=C bond was shifted to give the β,γ-unsaturated isomer **15b**[13] through a two-step strategy, originally introduced by Orsini et al.[14] The first step was allylic bromination with NBS–AIBN, yielding intermediate product **19** (79%), which was then submitted to reduction with Zn–AcOH, with displacement of the C=C bond. Compound **15b** (63%) was obtained as a mixture of diastereoisomers (*E*/*Z* 2:1), which were not separated. The configurations were assigned by subsequent NOE experiments: Irradiation of the 4-CH_3 group at $\delta = 1.62$ ppm led to almost no NOE effect at the olefinic signal at $\delta = 5.31$ ppm; thus, this signal belonged to the (*E*)-isomer. If the 4-CH_3 group at $\delta =$ 1.73 ppm was irradiated, a pronounced NOE effect of the olefinic signal was observed; hence, this was the (*Z*)-isomer.

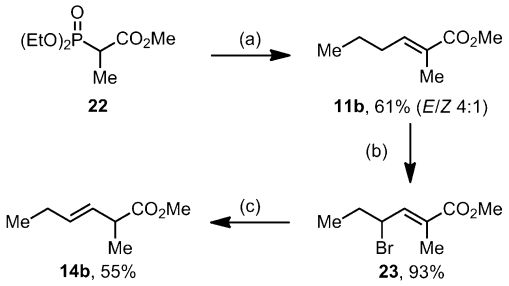
The synthesis of the 2-methylhexanoic acid series also started with the acylation of Meldrum's acid,[9] in this case with butanoyl chloride (**20**) to give oxoester **21**[9,15] in moderate yield (38%). Pyridine and only catalytic amounts of DMAP could be applied here (Scheme 3). Methylation with MeI occurred with



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

K_2CO_3 in acetone, with surprisingly high selectivity towards monoalkylated product **13b**[16] (55%). Reduction with $NaBH_4$ gave alcohol **12b**[17] (57%) as an inseparable mixture of two diastereoisomers (*dr* 3:2). Attempts at elimination by using the same protocol as that for compound **6b** (Scheme 2) yielded α,β-unsaturated compound **11b** (Scheme 4), together with unspecified impurities that could not be separated by column chromatography. For this reason, compound **11b** was accessed as outlined below.

The Horner–Wadsworth–Emmons (HWE) reaction of phosphoryl propionate **22**[18] with butanal gave α,β-unsaturated ester **11b**[19] as a mixture of diastereoisomers (61%, *E*/*Z* 4:1; Scheme 4), which could be separated by column chromatography. The (*E*)-selectivity of the HWE reaction for the formation of α-methyl-α,β-unsaturated esters has been reported previously 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 give β,γ-unsaturated isomer **14b**[21] (55%, only *trans*-isomer) via allylic bromide **23** (93%).



Scheme 4. Synthesis of unsaturated derivatives of **18**. Reagents and conditions: (a) butanal (1.0 equiv), KO*t*Bu (1.1 equiv), THF, 23 °C, 1 h; (b) NBS (1.0 equiv), 6 mol% AIBN, CCl_4, 80 °C, 19 h; (c) Zn (2.0 equiv), AcOH, 0 °C, 1 h, then 23 °C, 1 h.

Identification of metabolites

Methylated culture extracts of strain HxN1 after anaerobic growth with **1** were analyzed by means of GC-MS. Accordingly, all reference standards needed for unambiguous identification of metabolites were synthesized as the respective methyl esters (see above). Target structures of the as-yet unidentified metabolites were proposed based on their MS fragmentation patterns. Conclusive identification of the detected metabolites was accomplished by GC coinjection experiments and a comparison of mass spectra (Figures 1 and 2 and Figures S1–S16 in the Supporting Information). Oxoester **8b** could not be detected; this may indicate that the steady-state concentration of transient metabolite **8a** is below the limit of detection 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 to that obtained through the synthetic procedure. The two diastereoisomers of hydroxyester **7b** were not separable under the GC conditions applied.

A comparison of synthetic esters **14b** and **15b** with the methylated extract from cultures of strain HxN1 by GC-MS have confirmed that these compounds with isomerized β,γ-C=C 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, for example, 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 the respective conditions (85 °C at pH 1.5, hydrochloric acid). These mixtures were then analyzed by means of GC-MS and ^{1}H NMR spectroscopy, and in none of the cases could compounds with β,γ-double bonds be detected. Therefore, we conclude that compounds **14a** and **15a** are native metabolites of strain HxN1, although their possible roles remain unclear at this point.

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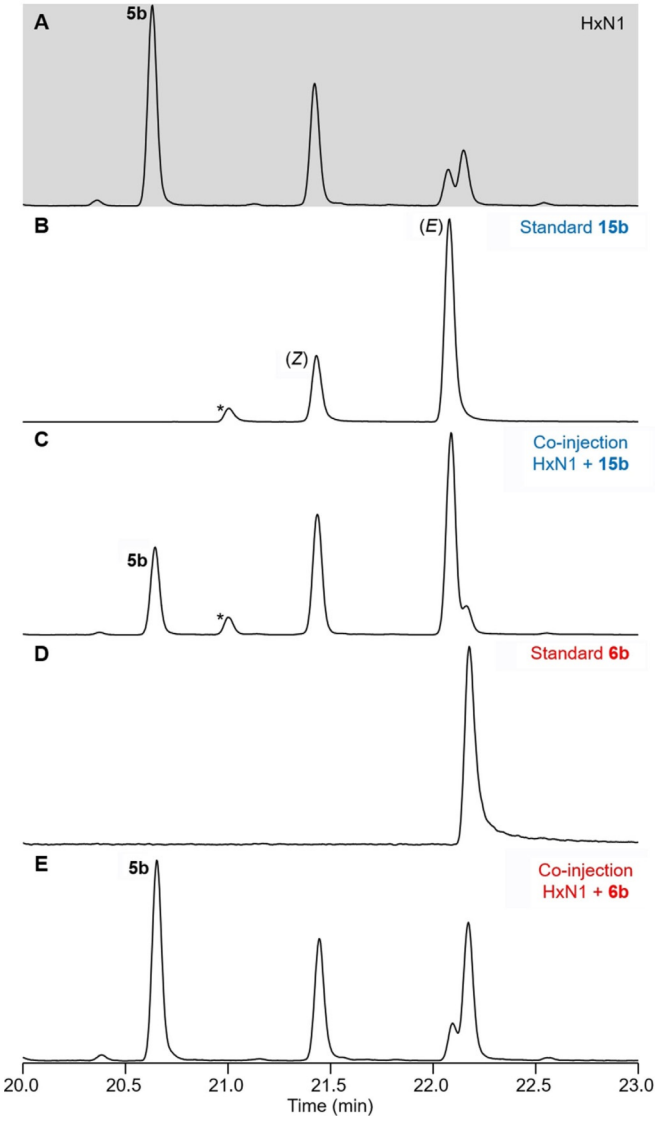

Figure 1. GC separation of isomers of **6b** and (*E*)- and (*Z*)-**15b** and coinjection with methylated culture extracts of strain HxN1 after anaerobic growth with **1**. The total ion chromatograms are depicted. The metabolite **5b** had been identified and structurally elucidated before.[7] The relative abundance of **5b** was used as an internal reference to assess the increase of peaks upon coinjection. A) Methylated culture extract of strain HxN1; B) synthetic standard (*Z*)- and (*E*)-**15b** (* = impurity); C) coinjection of extract and standard **15b**; D) synthetic standard **6b**; and E) coinjection of extract and standard **6b**.

Conclusion

The betaproteobacterium strain HxN1 degrades **1** under anoxic conditions into three equivalents of **9**, which are further oxidized to CO_2 in the TCA cycle. A metabolic pathway was previously proposed to proceed from **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 **1** were submitted to thioester hydrolysis with hydrochloric acid and methylation with diazomethane to give the respective methyl esters **5b**–**13b** for analysis by means of GC-MS. While the structures of compounds **5b** and **10b**, that is, the methyl esters related to thioesters **5a** and **10a**, were previously elucidated, the constitutions of methylated metabolites **6b**–**13b** have so far been proposed based on MS 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 unclear, to date.

We prepared synthetic compounds **6b**–**8b** and **11b**–**15b** and compared them with the constituents of the methylated culture extract by GC, including coinjection experiments, and MS. This enabled us to unequivocally establish the molecular identities of seven metabolites, which previously 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 was below the limit of detection.

In both, the 4-methyloctanoate (**6b**–**8b** and **15b**) and 2-methylhexanoate (**10b**–**14b**) series, organic synthesis started with the acylation of Meldrum's acid with the appropriate acid chlorides to give the β-oxoesters **8b** and **21**; the last of which 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). Although 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 prepared compound **11b** by the HWE reaction of butanal with phosphoryl propionate **22**.

Isomerization of the α,β-double bonds in compounds **6b** and **11b** was accomplished in two steps: allylic bromination with NBS–AIBN gave the γ-bromo congeners **19** and **23**. The C=C bond was then shifted to the β,γ-position 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 the 4-methyloctanoate series, to a certain extent, mimic the reverse degradation pathway of *n*-hexane (**1**) in strain HxN1. This study furthers our metabolite-based understanding of the anaerobic degradation of **1** by *Aromatoleum* sp. HxN1. Because 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 a valuable reference for pathway-oriented studies with pure cultures and 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 and field studies.

Experimental Section

General: Preparative column chromatography was performed by using Merck SiO_2 (35–70 μm, type 60 A) with hexanes (mixture of isomers, b.p. 64–71 °C), *tert*-butyl methyl ether (MTBE), and CH_2Cl_2

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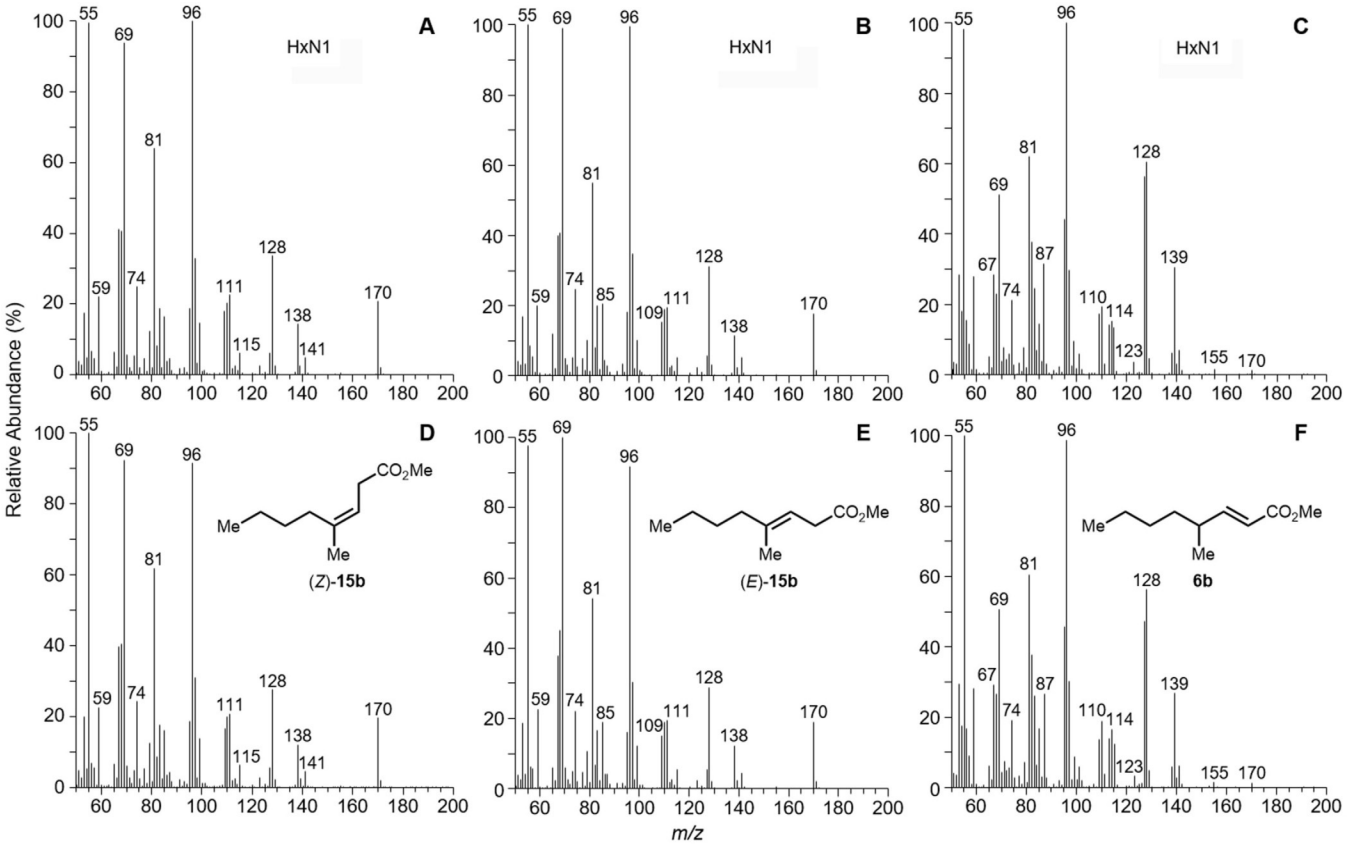

Figure 2. Mass spectra of compounds eluting at A) 21.42, B) 22.07, and C) 22.15 min from methylated culture extracts of strain HxN1 (cf. Figure 1 A) compared with the mass spectra of synthetic standards D) (*Z*)-**15 b**, E) (*E*)-**15 b**, and F) **6 b**.

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 MHz instruments. Multiplicities of carbon signals were determined through 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 attenuated total reflectance (ATR) unit. All starting materials were commercially available.

2-Methylhexanoyl chloride: A mixture of **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 to yield 2-methylhexanoyl chloride (8.51 g, 57.3 mmol, 93%) at 53 °C (19 mbar) as a colorless liquid. ^1H NMR (500 MHz, $CDCl_3$): $\delta = 0.91$ (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; $^{13}C\{^1H\}$ NMR (75 MHz, $CDCl_3$): $\delta = 13.8$ (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): $\tilde{\nu} = 2960$ (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 (8 b): 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 added dropwise over a period of 15 min. After stirring the mixture for a further 15 h at ambient temperature, hydrochloric acid (2 mol L^{-1}, 100 mL) was added and the resulting suspension was 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 (2×75 mL, 1×50 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 was 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 give **8 b** (3.13 g, 16.8 mmol, 70%) as a colorless liquid. According to ^1H NMR spectroscopy, the compound existed as two tautomers (keto/enol 85:15). ^1H NMR (500 MHz, $CDCl_3$), keto tautomer: $\delta = 0.90$ (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: $\delta = 0.89$ (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; $^{13}C\{^1H\}$ NMR (125 MHz, $CDCl_3$), keto tautomer: $\delta = 14.1$ (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): $\tilde{\nu} = 2957$ (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): *m/z* calcd for $C_{10}H_{18}LiO_3^+$ 193.1416 [M+Li^+]; found 193.1409.

Methyl 3-hydroxy-4-methyloctanoate (7 b): At 0 °C (ice–water bath), $NaBH_4$ (62 mg, 1.6 mmol, 1.2 equiv) was added to a solution

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of **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. The solution was extracted with MTBE (3× 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 give **7b** (200 mg, 1.06 mmol, 78%) as a colorless liquid. According to ^{1}H NMR spectroscopy, the compound existed as two diastereoisomers (*dr* 53:47). ^{1}H 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, 9.7 Hz, 1H), 2.49 (dd, J = 16.3, 2.6 Hz, 1H), 2.83 (d, J = 3.8 Hz, 1H), 3.71 (s, 3H), 3.87 (dddd, J = 9.7, 6.4, 3.8, 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{^{1}H} 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): $\tilde{\nu}$ = 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): *m/z* calcd for $C_{10}H_{20}LiO_3^+$ 195.1572 [*M*+Li^+]; found 195.1570.

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 **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, a saturated aqueous solution of NH_4Cl (40 mL) was added. The mixture was extracted with CH_2Cl_2 (3×40 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 give **6b** (513 mg, 3.01 mmol, 77%) as a colorless liquid. ^{1}H 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, 6.8, 6.4, 1.2 Hz, 1H), 3.73 (s, 3H), 5.77 (dd, J = 15.7, 1.2 Hz, 1H), 6.87 (dd, J = 15.7, 7.9 Hz, 1H) ppm; ^{13}C{^{1}H} 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): $\tilde{\nu}$ = 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): *m/z* calcd for $C_{10}H_{18}LiO_2^+$ 177.1467 [*M*+Li^+]; found 177.1465.

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 **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 (3×20 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 give **19** (0.59 g, 2.4 mmol, 79%) as a colorless liquid. ^{1}H 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{^{1}H} 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): $\tilde{\nu}$ = 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): *m/z* calcd for $C_{10}H_{18}BrO_2^+$ 249.0485 [*M*+H^+]; found 249.0484.

Methyl 4-methyl-3-octenoate (15b): Zinc powder (1.00 g, 15.3 mmol, 1.0 equiv) was added to a solution of **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. The reaction mixture was diluted with water (20 mL), filtered, and the filtrate was extracted with MTBE (3×20 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 give **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, 2/3×3H; (*E*)-isomer), 1.73 (s, 1/3×3H; (*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, 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): $\tilde{\nu}$ = 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): *m/z* calcd for $C_{10}H_{18}LiO_2^+$ 177.1461 [*M*+Li^+]; found 177.1463.

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, compound **20** (3.3 g, 31 mmol, 1.0 equiv) 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 was 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 (3×50 mL). All four organic layers were combined, washed with hydrochloric acid (2 mol L^{-1}, 2×50 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 give **21** (1.52 g, 10.5 mmol, 38%) as a colorless liquid. According to ^{1}H NMR spectroscopy, the compound existed 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{^{1}H} 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): $\tilde{\nu}$ = 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): *m/z* calcd for $C_7H_{12}LiO_3^+$ 151.0941 [*M*+Li^+]; found 151.0943.

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Methyl 2-methyl-3-oxohexanoate (13 b): 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 a further 3 h at ambient temperature, water (20 mL) was added. The solution was extracted with MTBE (3×20 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 give **13 b** (0.87 g, 5.5 mmol, 55 %) as a colorless liquid. According to ^{1}H NMR spectroscopy, the compound existed as two tautomers (keto/enol 9:1). ^{1}H NMR (500 MHz, $CDCl_3$), keto tautomer: δ = 0.91 (t, J = 7.4 Hz, 3 H), 1.34 (d, J = 7.1 Hz, 3 H), 1.62 (sex, J = 7.4 Hz, 2 H), 2.47 (dt, J = 17.3, 7.1 Hz, 1 H), 2.55 (dt, J = 17.4, 7.3 Hz, 1 H), 3.53 (q, J = 7.2 Hz, 1 H), 3.73 (s, 3 H) ppm; enol tautomer: δ = 0.96 (t, J = 7.6 Hz, 3 H), 1.62 (sex, J = 7.4 Hz, 2 H), 1.75 (s, 3 H), 2.28 (t, J = 7.6 Hz, 2 H), 3.76 (s, 3 H), 12.66 (s, 1 H) ppm; ^{13}C{^{1}H} 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): $\tilde{\nu}$ = 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): *m/z* calcd for $C_8H_{14}LiO_3^+$ 165.1103 [M+Li$^+$]; found 165.1108.

Methyl 3-hydroxy-2-methylhexanoate (12 b): $NaBH_4$ (0.29 g, 7.6 mmol, 1.2 equiv) was added to a solution of **13 b** (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 (3×30 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 give **12 b** (0.58 g, 2.6 mmol, 57 %) as a colorless liquid. According to ^{1}H NMR spectroscopy, the compound existed as two diastereoisomers (*dr* 3:2). ^{1}H NMR (500 MHz, $CDCl_3$), major isomer: δ = 0.93 (t, J = 7.2 Hz, 3 H), 1.21 (d, J = 7.3 Hz, 3 H), 1.29–1.59 (m, 4 H), 2.49 (d, J = 6.9 Hz, 1 H), 2.51–2.57 (m, 1 H), 3.64–3.70 (m, 1 H), 3.71 (s, 3 H) ppm; the signal at 2.49 ppm was identified as the OH signal by H–D exchange (D_2O); minor isomer: δ = 0.94 (t, J = 7.0 Hz, 3 H), 1.18 (d, J = 7.3 Hz, 3 H), 1.29–1.59 (m, 4 H), 2.43 (d, J = 4.7 Hz, 1 H), 2.51–2.57 (m, 1 H), 3.71 (s, 3 H), 3.89–3.93 (m, 1 H) ppm; the signal at 2.43 ppm was identified as the OH signal by H–D exchange (D_2O); ^{13}C{^{1}H} 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): $\tilde{\nu}$ = 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): *m/z* calcd for $C_8H_{16}LiO_3^+$ 167.1259 [M+Li$^+$]; found 167.1260.

Methyl (*E*)- and (*Z*)-2-methyl-2-hexenoate (11 b): At 0 °C (ice–water bath), KO*t*Bu (6.10 g, 54.3 mmol, 1.1 equiv) was added to a solution of **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 a further 1 h at ambient temperature, a saturated aqueous solution of NH_4Cl (40 mL) was added. The mixture was extracted with MTBE (3×40 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 give (*Z*)-**11 b** (930 mg, 6.54 mmol, 13 %) as the first fraction (R_f = 0.53) as a colorless liquid. Second, compound (*E*)-**11 b** (3.36 g, 23.6 mmol, 48 %) was eluted (R_f = 0.35), also a colorless liquid (*E*/*Z* 3.6:1).

(*E*)-Isomer: ^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.92 (t, J = 7.4 Hz, 3 H), 1.45 (sex, J = 7.4 Hz, 2 H), 1.81 (s, 3 H), 2.13 (q, J = 7.4 Hz, 2 H), 3.71 (s, 3 H), 6.74 (t, J = 7.4 Hz, 1 H) 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): $\tilde{\nu}$ = 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): *m/z* calcd for $C_8H_{14}LiO_2^+$ 149.1154 [M+Li$^+$]; found 149.1155.

(*Z*)-Isomer: ^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.91 (t, J = 7.4 Hz, 3 H), 1.41 (sex, J = 7.4 Hz, 2 H), 1.88 (d, J = 1.2 Hz, 3 H), 2.42 (q, J = 7.4 Hz, 2 H), 3.72 (s, 3 H), 5.93 (tq, J = 7.4, 1.2 Hz, 1 H) 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): $\tilde{\nu}$ = 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): *m/z* calcd for $C_8H_{14}LiO_2^+$ 143.1067 [M+H$^+$]; found 143.1070.

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 (*E*)-**11 b** (3.69 g, 26.0 mmol, 1.0 equiv) in CCl_4 (40 mL). After stirring the mixture for a 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 (3×20 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 give **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, 3 H), 1.90 (d, J = 1.4 Hz, 3 H), 1.86–2.07 (m, 2 H), 3.76 (s, 3 H), 4.68 (dt, J = 10.7, 7.0 Hz, 1 H), 6.81 (dq, J = 10.7, 1.4 Hz, 1 H) 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): $\tilde{\nu}$ = 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): *m/z* calcd for $C_8H_{13}BrNaO_2^+$ 242.9991 [M+Na$^+$]; found 242.9997.

Methyl 2-methyl-3-hexenoate (14 b): Zinc powder (3.17 g, 48.5 mmol, 2.0 equiv) was added to a solution of **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. The mixture was diluted with water (50 mL), filtered, and the filtrate was extracted with MTBE (3×80 mL). The combined organic layers were washed successively with hydrochloric acid (2 mol L^{-1}, 50 mL), a saturated aqueous solution of $NaHCO_3$ (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 give **14 b** (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, 3 H), 1.22 (d, J = 7.1 Hz, 3 H), 2.02 (pent, J = 7.1 Hz, 2 H), 3.09 (pent, J = 7.1 Hz, 1 H), 3.66 (s, 3 H), 5.46 (dd, J = 15.5, 7.3 Hz, 1 H), 5.57 (dt, J = 15.4, 5.7 Hz, 1 H) 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): $\tilde{\nu}$ = 2968 (m), 2935 (w), 2876 (w),

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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): *m/z* calcd for $C_8H_{14}LiO_2^+$ 149.1148 [*M*+Li^+]; found 143.1151.

Cultivation: The betaproteobacterium *Aromatoleum* sp. HxN1 has been subcultured in our laboratory since its isolation.[4] Cultivation was performed in defined, bicarbonate-buffered medium, essentially as described previously.[5] Cultures were grown in stopper-sealed flat glass bottles (500 mL) containing medium (400 mL) under an anoxic atmosphere (N_2/CO_2 90:10, *v/v*). *n*-Hexane (**1**) was provided as a dilution (5%, *v/v*) in 2,2,4,4,6,8,8-heptamethylnonane, which served as an 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 **1**) was removed by means of a separatory funnel, the obtained culture broth was 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-injection experiments for some compounds were performed by using samples obtained by means of solid-phase extraction of cell-free supernatants of strain HxN1 grown with **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 means of GC-MS.

Derivatization and analysis of metabolites: Dried extracts were evaporated to dryness, solubilized in CH_2Cl_2, and methylated by using freshly prepared diazomethane, essentially as described previously.[7] Methylated extracts were then analyzed by GC-MS on a Trace GC Ultra gas chromatograph coupled to an ISQ OD mass spectrometer (both Thermo Scientific). The gas chromatograph 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 the carrier gas. The GC oven temperature was programmed from 40 (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}. MS was performed 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 Da at a scan cycle time of 0.2 s. Analyses of reference standards and coinjection experiments were performed on the same system under identical conditions.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: alkanes · anaerobic degradation · C–H activation · metabolism · structure elucidation

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