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Synthesis and characterization of anaerobic degradation biomarkers of *n*-alkanes via hydroxylation/carboxylation pathways

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Metabolite profiling is a powerful method in research on anaerobic biodegradation of hydrocarbons. Hydroxylation and carboxylation are proposed pathways in anaerobic degradation but very little direct evidence is available about metabolites and signature biomarkers. 2-Acetylalkanoic acid is a potential signature metabolite because of its unique and specific structure among possible intermediates. A procedure for the synthesis of four homologues with various carbon chain lengths was proposed and the characteristics of 2-acetyl–alkanoic acid esters were investigated using four derivatization processes, namely methyl, ethyl, *n*-butyl and trimethylsilyl esterification. Four intermediate fragments observed were at *m/z* 73 + 14*n*, 87 + 14*n*, 102 + 14*n* (*n* = 1, 2 and 4 for methyl, ethyl and *n*-butyl ester, respectively) and $[M - 42]^+$ for three of the derivatization methods. For silylation, characteristic ions were observed at *m/z* 73, 117, $[M - 42]^+$ and $[M - 55]^+$. These are basic and significant data for the future identification of potential intermediates of the hydroxylation and carboxylation pathways in hydrocarbon degradation.

Keywords: hydroxylation/carboxylation, potential biomarkers, 2-acetylalkanoic acid, synthesis of biomarkers, mass spectral characteristics

Introduction

It has been established since the 1990s that anaerobic degradation of alkanes plays an important role in nature.[1,2] To date, metabolite profiling has elucidated at least four kinds of initial activation steps in anaerobic alkane degradation.[3,4] A group of anaerobic microorganisms capable of degrading *n*-alkanes under nitrate-reducing,[5] sulfidogenic[6–8] and methanogenic conditions[9–12] has been reported.[13] Increasing evidence supports that fumarate addition plays a dominant role in the anaerobic activation of alkanes by those microorganisms.[14–16] However, isolated sulfate-reducing strain Hxd3, a sulfate reducer closely related to the genus *Desulfococcus*, is an exception.[2,17] This strain was elucidated for *n*-alkane activation by another mechanism compared to most other alkane-degrading strains, which showed an initial pathway of fumarate.[2,17] A putative biochemical pathway of degradation for alkanes, namely carboxylation, was proposed by So *et al.* based on $^{13}CO_2$-labeling experiments.[18] Strain Hxd3 would metabolize an alkane to form fatty acids via carboxylation with inorganic carbon (HCO_3^-/CO_2), most likely at C-3, with subsequent removal of two sub-terminal carbon atoms from the alkane chain.[18,19] Callaghan *et al.*[20] characterized an alkane-degrading and nitrate-reducing consortium, and

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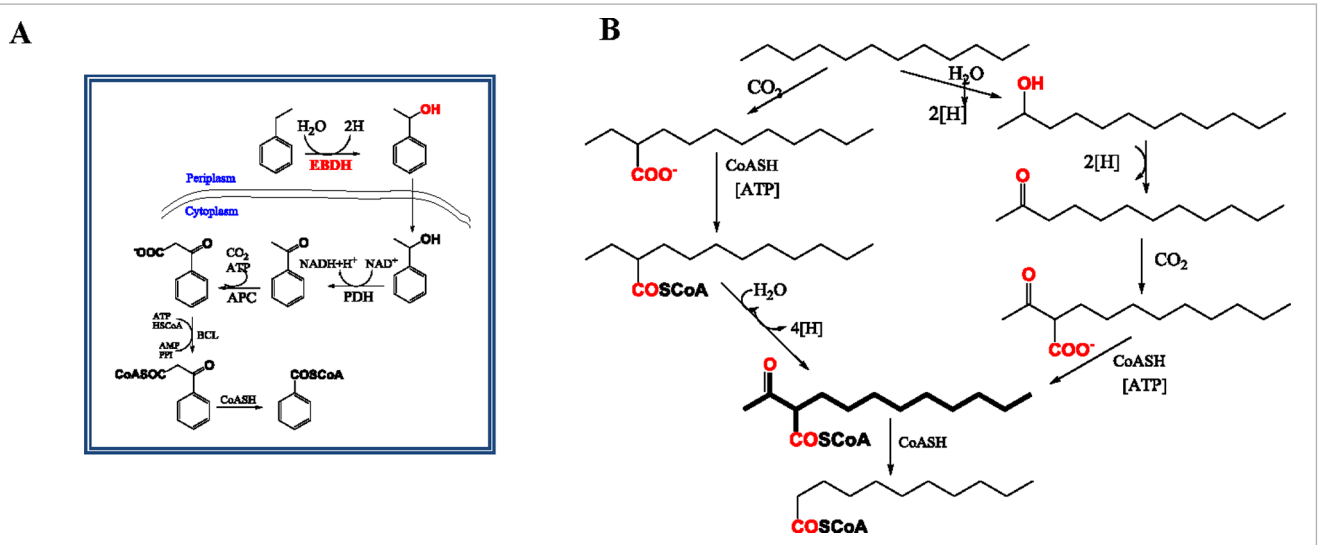


Figure 1. (A) Possible metabolic mechanisms initiated by carboxylation or hydroxylation. The common intermediates of these two pathways are highlighted in bold. (B) The pathway of anaerobic ethylbenzene degradation mechanism through an initial hydroxylation catalyzed by EBDH.

obtained evidence for a degradation mechanism analogous to the proposed carboxylation pathway under nitrate-reducing conditions.

However, recent omics-based analysis indicates that the genome of Hxd3 contains genes that encode an ethylbenzene dehydrogenase-like enzyme, which catalyzes the anaerobic hydroxylation of ethylbenzene [Figure 1(A)].[3,21,22] The new hypothetical activation strategy suggested an initial subterminal hydroxylation of the alkanes by an EBDH-like enzyme, followed by oxidation to a ketone and its subsequent carboxylation at C-3.[3,21] The latter process would be consistent with previous studies [Figure 1(B)].[14,19]

For metabolite analysis, deuterated long-chain fatty acids were detected while the proposed typical intermediates such as 2-ethylalkanoic acid and 2-acetylalkanoic acid were not identified in previous studies.[18,19] Notably, 2-acetylalkanoic acid, as a common intermediate, occurs in two similar pathways, namely hydroxylation and carboxylation.

Alkylsuccinate is a biomarker of fumarate addition. We had synthesized five biomarkers, specifically 2-ethylsuccinic acid, 2-(1-methyloctyl)succinic acid, 2-(1-methylpentadecyl) succinic acid, 2-cyclohexylsuccinic acid and 2-benzylsuccinic acid, and subsequently obtained their mass spectra characteristics in previous work which was concerned with the investigation of fumarate addition pathway in oilfield production water samples.[23,24] However, the corresponding mass spectrometry information of 2-acetylalkanoic acids is still not available in official libraries. In the research work reported here, we established a method for the chemical synthesis of 2-acetylalkanoic acids and obtained the characteristic fragments of them by GC-MS using four derivatization approaches, namely methyl, ethyl, *n*-butyl and trimethylsilyl (TMS) esterification. Our results provide the lacking data and greatly advance the detection of 2-acetylalkanoic acids in environmental and enrichment cultural samples.

Experimental

Chemical synthesis

All chemical reagents used in the syntheses were commercially available, of analytical grade and highest purity. *n*-Nonanol, ethyl acetoacetate, Na, ethanol, hexane, ethyl acetate, HBr, H_2SO_4, Na_2SO_4, $NaHCO_3$ and HCl were of analytical grade. *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) was purchased from Sigma-Aldrich (Shanghai Trading Co. Ltd, Shanghai, China). Where necessary, solvents were either de-watered using standard techniques or purchased as anhydrous form. In the following, 2-acetylundecanoic acid is used as an example to describe the synthetic route presented in Figure 2.

Preparation of bromononane. H_2SO_4 (0.035 mol, 5.04 g) was added dropwise into a mixture of *n*-nonanol (0.035 mol, 5.04 g) and HBr (0.0385 mol, 6.63 g) at 0 °C. The reaction was heated to reflux for 1 h at 120 °C. After cooling to room temperature, steam distillation was used to purify the target products. $NaHCO_3$ was added to adjust the pH to >7. The oil phase was separated and the water phase was extracted with hexane three times successively. Then, the organic phase was collected and dried with Na_2SO_4. The solvent was removed and the yield was approximately 90.0%.

Preparation of ethyl 2-acetylundecanoic acid ester. Sodium ethoxide was produced by Na (0.035 mol, 0.80 g) and ethanol (10 mL) at 0 °C.[23] Ethyl acetoacetate was added into the sodium ethoxide/ethanol solution dropwise and stirred for 30 min. Then, bromononane was added into the mixture. After

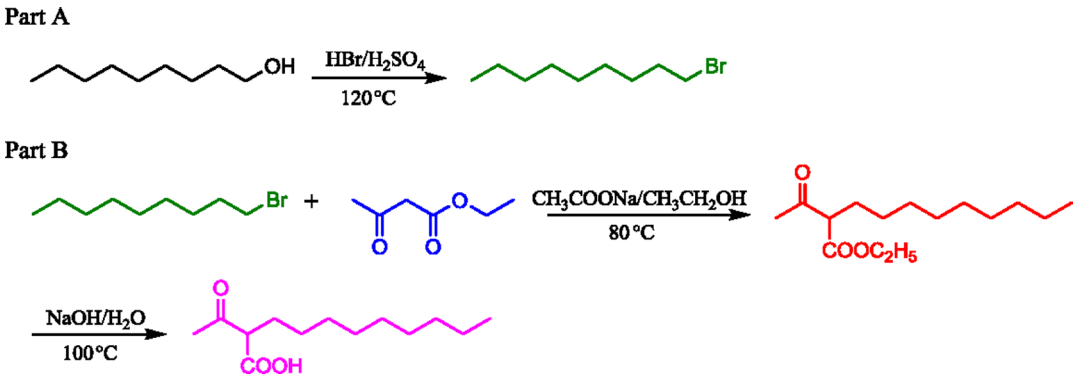


Figure 2. Synthesis of 2-acetylundecanoic acid.

further stirring for 1 h, the mixture was refluxed for 6 h at 80 °C. Ethanol was removed firstly by rotary evaporation. Deionized water and HCl were added into the product until the pH was <1. The oil phase was separated and the water phase was extracted with ethyl acetate three times successively. Then, the organic phase was collected and dried by passing through Na_2SO_4. The solvent was removed and the yield was approximately 68.5%.

Preparation of 2-acetylundecanoic acid. NaOH (0.035 mol, 1.40 g) and H_2O (20 mL) were added to ethyl 2-acetylundecanoate (0.035 mol, 8.96 g), and the mixture was refluxed for 4 h at 100 °C. After cooling to room temperature, hexane was used three times successively to extract the non-dissolved organic substances. Then, the water phase was acidified with HCl, and extracted three times with ethyl acetate and dried by passing through Na_2SO_4. The solvent was removed and the yield was about 92.5%.

2-Acetylpentanoic, 2-acetylhexanoic and 2-acetyldecanoic acids were also synthesized according to the method described above. Notably, the steps of Part B (Figure 2) should be kept water free. Thin-layer chromatography was used to monitor the progress of the whole reaction. The 2-acetylalkanoic acids were purified via column chromatography (petroleum ether–ethyl acetate, 8:1). The four compounds were derivatized to ethyl esters for GC-MS analysis via a method described elsewhere.[7,23,25]

NMR data

Ethyl 2-acetylpentanoic acid ester. ^{1}H NMR (400 MHz, $CDCl_3$, TMS; δ, ppm): 0.86 (t, *J* = 6.7 Hz, 3H), 1.26–1.31 (m, 5H), 1.81 (m, *J* = 14.8, 7.4 Hz, 2H), 2.06 (s, 3H), 3.39 (t, *J* = 7.3 Hz, 1H), 4.15 (q, *J* = 7.1 Hz, 2H).

Ethyl 2-acetylhexanoic acid ester. ^{1}H NMR (400 MHz, $CDCl_3$, TMS; δ, ppm): 0.86 (t, *J* = 6.7 Hz, 3H), 1.26–1.32 (m, 7H), 1.81 (m, *J* = 14.8, 7.4 Hz, 2H), 2.06 (s, 3H), 3.39 (t, *J* = 7.3 Hz, 1H), 4.16 (q, *J* = 7.1 Hz, 2H).

Ethyl 2-acetylundecanoic acid ester. ^{1}H NMR (400 MHz, $CDCl_3$, TMS; δ, ppm): 0.87 (t, *J* = 6.8 Hz, 3H), 1.17–1.37 (m, 15H), 1.83 (m, *J* = 14.8, 7.4 Hz, 2H), 2.22 (s, 3H), 3.42 (t, *J* = 7.4 Hz, 1H), 4.19 (q, *J* = 7.1 Hz, 2H).

Ethyl 2-acetyldodecanoic acid ester. ^{1}H NMR (400 MHz, $CDCl_3$, TMS; δ, ppm): 0.86 (t, *J* = 6.8 Hz, 3H), 1.14–1.39 (m, 17H), 1.83 (m, *J* = 17.5, 10.1 Hz, 2H), 2.22 (s, 3H), 3.43 (t, *J* = 7.4 Hz, 1H), 4.18 (q, *J* = 7.1 Hz, 2H).

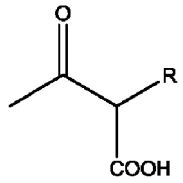
Derivatization

Four homologues with various carbon chain lengths were derivatized to methyl, ethyl, *n*-butyl and trimethylsilyl esters, respectively. Noteworthy, the mass spectra of ethyl ester products were obtained directly after synthesis. Briefly, the methods were as follows. A solution of 10% H_2SO_4–CH_3OH/H_2SO_4–butanol was added into each of the four compounds and heated at 90 °C for 1 h for methyl/butyl derivatizations.[26] For silylation, BSTFA and acetonitrile were rapidly mixed with each of the compounds and heated at 60 °C for 30 min.[27] Details of specific methods of derivatization were published previously by Bian *et al.*[23]

GC-MS analyses were carried out using an Agilent 7890A GC instrument equipped with an HP-5MS capillary column (30 m × 0.25 mm × 0.25 µm) and mass detector (MSD 5975). The injection port temperature was 280 °C and the oven temperature was initially held at 60 °C for 2 min, and then increased at 10 °C min^{-1} to 280 °C, held for 30 min. The MS detector acquired data in the scan mode, from 30 to 1000 mass units.

Results and discussion

Alkanes can be degraded anaerobically via a number of specific biochemical mechanisms, such as fumarate addition, carboxylation, hydroxylation etc. These proposed biochemical pathways can be observed in either enrichment cultures or pure cultures under laboratory conditions. However, it is difficult to identify them in hydrocarbon-rich subsurface environments, including oil reservoirs and contaminated environments. An efficient method to investigate the anaerobic degradation mechanisms in these environments is the direct detection and confirmation of the key degradation metabolites produced from these biochemical degradation pathways. For carboxylation and hydroxylation mechanisms, the



R= propyl, butyl, octyl, nonyl

Figure 3. Chemical structures of the four synthetized compounds.

intermediate 2-acetyl-alkanoic acids are the key compounds involved, but the mass spectral characteristics of these compounds are unavailable.

Four compounds, namely 2-acetylpentanoic, 2-acetylhexanoic, 2-acetyldecanoic and 2-acetylundecanoic acids, were synthesized in this research. Their structures are shown in Figure 3. They were characterized by both mass spectrometry and ^{1}H NMR spectroscopy. The synthesized compounds were derivatized by four methods respectively for higher volatility of the chemicals and enhanced detection sensitivity. The mass spectra of them are shown in Figure 4 and the supplementary data. There are certain apparent regularities in the mass spectra of these derivatization products.

Mass spectral characteristics of methyl, ethyl and *n*-butyl esterification products

Four intermediate fragments observed at *m/z* 73 + 14*n*, 87 + 14*n*, 102 + 14*n* (*n* = 1, 2 and 4 for methyl, ethyl and *n*-butyl ester, respectively) and $[M - 42]^+$ for the three derivatization methods are considered as mass spectral characteristics of 2-acetylalkanoic acid esters. We take ethyl esterification as an example to discuss the fragment ions in detail.

The mass spectra of the four ethyl esterification compounds are shown in Figure 4. The four most significant fragment ions at *m/z* 101, 115, 130 and $[M - 42]^+$ are supposed to be the characteristic fragments of ethyl 2-acetylalkanoic acid. They suggest a cleavage mechanism shown in Figure 5. These ions corresponding to different cleavage mechanisms are discussed in the following.

Fragment ions *m/z* 101, 115 and 130 (in red in Figure 4) remained unchanged in the compounds with increasing molecular weight. Ethyl esters of 2-acetylalkanoic acids undergo EI-induced fragmentation due to McLafferty rearrangement and γ-cleavage to form *m/z* 130. Note that alkyl of the carbon

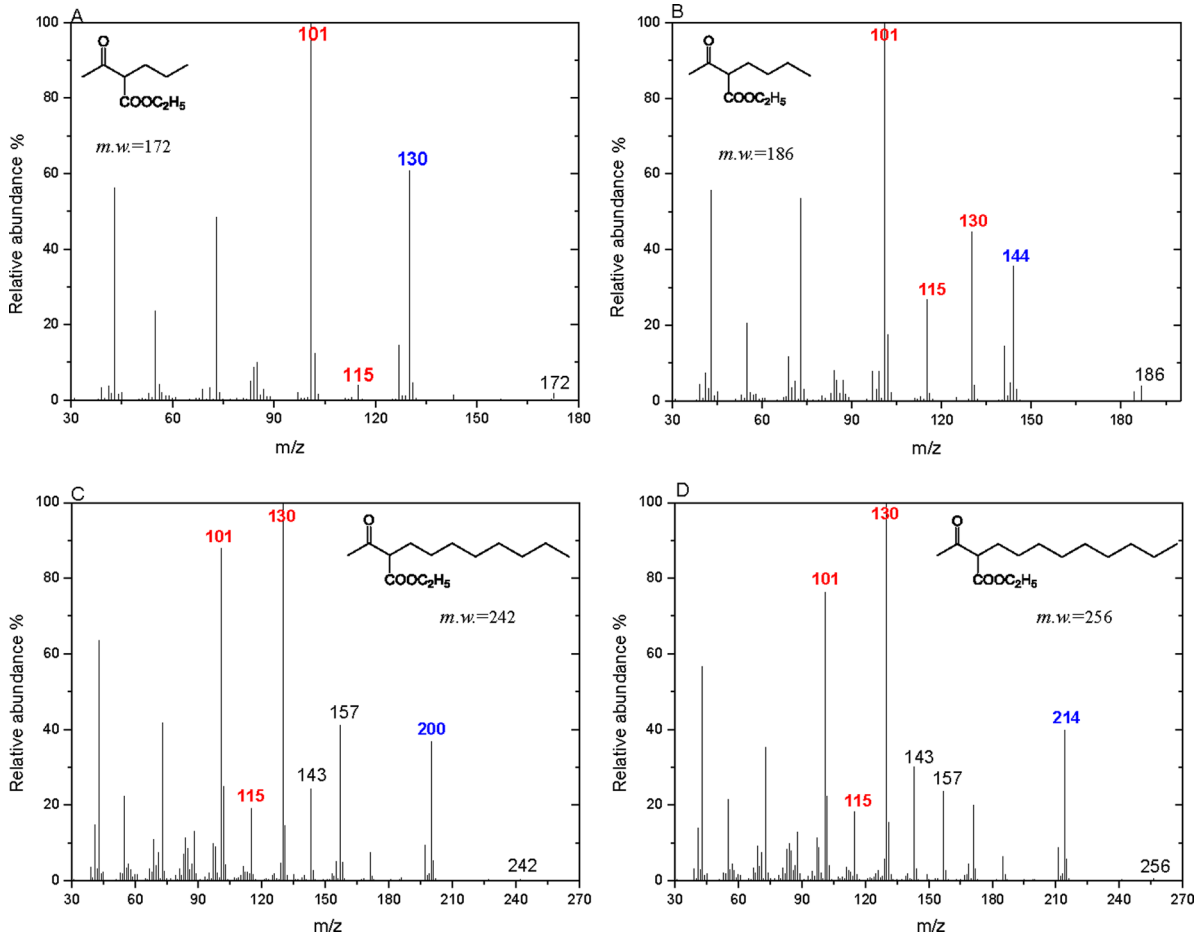


Figure 4. Mass spectra of ethyl 2-acetylalkanoic acids. (Values in red are constant for all chemical compounds. Values in blue are different for each compound and can reflect the length of the carbon chain.)

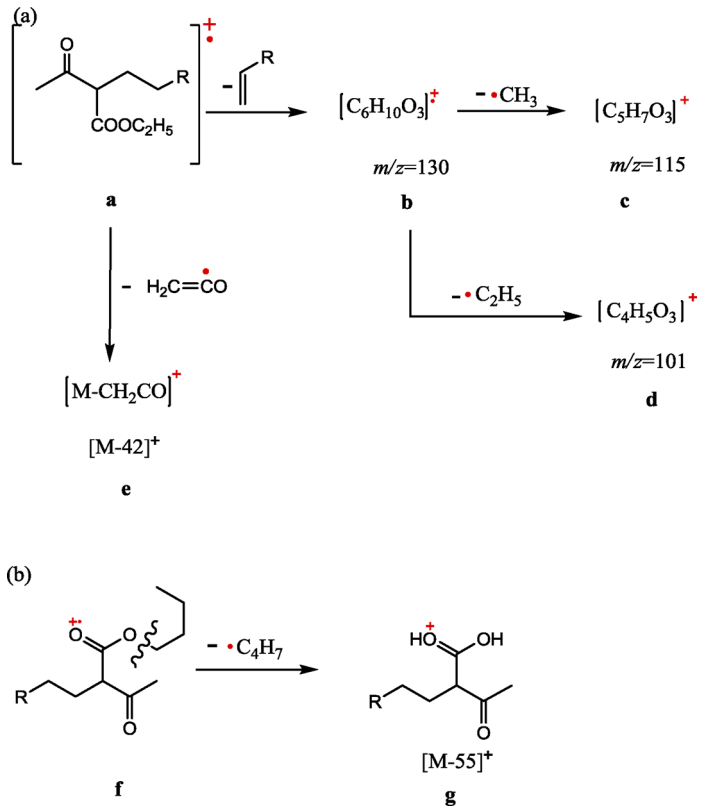


Figure 5. Proposed cleavage of ethyl 2-acetylalkanoic acid by mechanisms (a) and (b), and *n*-butyl 2-acetylalkanoic acid (c).

chain near the carbethoxy can provide a γ-H for this rearrangement (Figure 5, mechanism a).[28,29] Structure **b** loses a methyl radical to form **c** at *m/z* 115. Similarly, ethyl radical is eliminated from ion **b** to give fragment ion **d** at *m/z* 101. Ions *m/z* 130 and 101 have strong peaks, suggesting that they are relatively stable. Fragment ions *m/z* 143 and 157 are relatively abundant in mass spectra for long-chain compounds. They are simply generated by fracture of carbon chains.

Fragment ion *m/z* [M – 42]$^+$. EI mass spectra of the derivatives presented reveal a rather intense peak for the [M – 42]$^+$ ion (in blue in Figure 4) by elimination of a ketene [$^{\cdot}CH_2CO$] group due to McLafferty rearrangement (acetyl group can provide a γ-H for this rearrangement) (Figure 5).[28] Note that the elimination of a methyl or ethyl radical can also arise based on the rearrangement, but this process was not considered as characteristic fragmentation owing to either repeated *m/z* value or negligible ion peaks. The molecular mass can often be recognized by the presence of [M – 42]$^+$ ion which reflects indirectly the length of the carbon chain. Therefore, the general structure of compounds can be confirmed basically in terms of the key information. Fragment ion *m/z* [M – 42]$^+$ is constantly visible for the four derivatization methods (Figure 4 and supplementary data) since this cleavage mechanism of 2-acetylalkanoic acid esters is independent of the length of carbon chain and also the derivatization method.

Also, molecular ion peaks are found in the mass spectra. Consequently, the fragment ions *m/z* 101, 115, 130 and [M – 42]$^+$ could be regarded as the characteristic fragments of ethyl 2-acetylalkanoic acids.

For methyl and *n*-butyl esterification derivatives (supplementary data), except for common fragment ion *m/z* [M – 42]$^+$, the mass spectral characteristics have 14 or 28 mass units differences from ethyl esterification. The shifts of *m/z* are reasonable when methoxyl or butoxyl is substituted for ethoxyl. In addition, fragment [M – 55]$^+$ can be observed in mass spectrum of *n*-butyl esterification product representing a McLafferty rearrangement with double hydrogen transfer mechanism according to a previous study.[30]

Mass spectral characteristics of silylation

The fragment ions via mechanism (a) of Figure 5 are not regarded as the mass characteristics for silylation derivatization due to low abundance (supplementary data), except *m/z* [M – 42]$^+$. Instead, diagnostic fragmentations have been observed in the EI mass spectra of TMS esters with *m/z* 73 and 117. The EI mass spectra of TMS esters show rather abundant ions with *m/z* 73 suggesting the formation of trimethylsilyl monomers. Fragment ions *m/z* 117, corresponding to the formation of the Me_3SiOCO^+ ion by McLafferty rearrangement and γ-cleavage, also have a pronounced peak in each spectrum.[31,32] As for *m/z* [M – 42]$^+$, it has no strong correlation with the length of the carbon chain or the derivatization method. Another characteristic fragment ion *m/z* [M – 57]$^+$ is produced based on *m/z* [M – 42]$^+$ by eliminating a methyl radical which

Table 1. Characteristic fragment ions of 2-acetylalkanoic acid esters.

Derivation	Characteristic fragment ions
Methyl esterification	87, 101, 116, $[M - 42]^+$
Ethyl esterification	101, 115, 130, $[M - 42]^+$
n-Butyl esterification	129, 143, 158, $[M - 42]^+$, $[M - 55]^+$
Silylation	73, 117, $[M - 42]^+$, $[M - 57]^+$

belongs to TMS. Therefore, 73, 117, $[M - 42]^+$ and $[M - 57]^+$ are the mass spectral characteristics of silylation.

According to the discussion above, the mass spectral characteristics of hydrocarbon-derived acid esters are summarized in Table 1. Each derivatization method corresponds to different characteristic ions. Based on the information newly available, it is possible to choose one or several of the methods for detection of degradation intermediates to confirm carboxylation and hydroxylation mechanisms by direct detection of degradation intermediates.

Conclusions

Synthesis of chemical biomarkers in anaerobic degradation of alkanes via hydroxylation and carboxylation mechanisms was carried out and characteristic mass spectra were obtained. Four homologues with various carbon chain lengths were synthesized, and their characteristic mass spectra obtained by GC-MS include four characteristic fragments at m/z 73 + 14n, 87 + 14n, 102 + 14n (n = 1, 2 and 4 for methyl, ethyl and *n*-butyl ester) and $[M - 42]^+$ for three of the derivatization methods, and at m/z 73, 117, $[M - 42]^+$ and $[M - 55]^+$ for silylation. They can be used to identify potential biomarkers, 2-acetylalkanoic acids, in hydrocarbon degradation, namely carboxylation and hydroxylation pathways. Direct detection of biomarkers involved in carboxylation and hydroxylation pathways will advance our knowledge of anaerobic degradation processes.

Acknowledgements

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Supplementary data

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1255/ejms.1402.

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