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Synthesis and mass spectra of rearrangement bio-signature metabolites of anaerobic alkane degradation via fumarate addition

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ABSTRACT

Metabolite profiling in anaerobic alkane biodegradation plays an important role in revealing activation mechanisms. Apart from alkylsuccinates, which are considered to be the usual biomarkers via fumarate addition, the downstream metabolites of C-skeleton rearrangement can also be regarded as biomarkers. However, it is difficult to detect intermediate metabolites in both environmental samples and enrichment cultures, resulting in lacking direct evidence to prove the occurrence of fumarate addition pathway. In this work, a synthetic method of rearrangement metabolites was established. Four compounds, namely, propylmalonic acid, 2-(2-methylbutyl) malonic acid, 2-(2-methylpentyl)malonic acid and 2-(2-methyloctyl)malonic acid, were synthesized and determined by four derivatization approaches. Besides, their mass spectra were obtained. Four characteristic ions were observed at m/z 133 + 14n, 160 + 28n, 173 + 28n and [M - (45 + 14n)]$^+$ (n = 0 and 2 for ethyl and n-butyl esters, respectively). For methyl esterification, mass spectral features were m/z 132, 145 and [M - 31]$^+$, while for silylation, fragments were m/z 73, 147, 217, 248, 261 and [M - 15]$^+$. These data provide basis on identification of potential rearrangement metabolites in anaerobic alkane biodegradation via fumarate addition.

1. Introduction

Hydrocarbons, the critical components of crude oil, were reported to be biodegraded anaerobically under sulfate-reducing, nitrate-reducing and methanogenic conditions [1–4]. Research on biochemical mechanisms involved in the anaerobic biodegradation of hydrocarbons deepens our understandings on how the microorganisms activate alkanes in complex anoxic subsurface environments [5–7]. Based on pure cultures and enrichment cultures from laboratory studies, several initial activation pathways of alkane degradation were proposed, such as fumarate addition, hydroxylation/carboxylation and alkyl-CoM pathways [8–12]. Typically, pathway exploration of anaerobic hydrocarbon degradation is mainly divided into two main means. One is through metaomics approaches, but there still exist some limitations [13]. The other is a combination of key-metabolite detection and related functional gene analysis [14–16]. Functional genes can be relatively easy to acquire by molecular techniques, but intermediate metabolites are hard to detect, which eventually causes a lack of strong evidence to confirm the presence of activation mechanisms. Therefore, investigations on bio-signature metabolites contribute to the exploration of degradation pathways.

In most cases, alkanes are anaerobically activated via addition to fumarate, resulting in the generation of 1-methylalkyl succinic acids, which is followed by the C-skeleton rearrangement reaction to form 2-(2-methylalkyl)malonic acids and, subsequently, oxidization to fatty acids and further conversion to methane and carbon dioxide [17–19]. Alkylsuccinates are commonly recognized as biomarkers indicating the presence of fumarate addition [17,19]. In previous studies, alkylsuccinates with different carbon chain lengths were synthesized, and the mass spectral characteristics of these derivatives were also obtained and

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summarized [20–22], which provides substantial convenience for the identification of fumarate addition to alkanes occurred in environmental samples and enrichment cultures [15,23]. But due to the short duration and low concentration of alkylsuccinates, and the relatively low sensitivity of the instruments [4,18], the knowledge on the fumarate addition pathway of *in situ* petroleum reservoirs and enrichment cultures is not enough [17,24]. Besides, little was known about the detection of downstream metabolites, especially C-skeleton rearrangement metabolites. Since the biological source of rearrangement product alkylmalonate is unique, it can also be considered as an indicator for the fumarate addition pathway. However, a lack of standard substances and insufficient commercial sources of rearrangement bio-signature metabolites lead to unavailable mass spectrometry information in official libraries. Thus, a general synthesis approach and mass spectral features of these bio-signature metabolites with different derivatization methods are necessary.

In the current study, a synthetic method for 2-(2-methylalkyl) malonic acids was established. Four synthesized compounds that differ in terms of carbon chain lengths were treated via four different derivatization methods (namely methyl, ethyl, *n*-butyl and trimethylsilyl esterifications) to acquire the informative fragments of various molecular masses by gas chromatography-mass spectrometer (GC-MS). The obtained mass spectral characteristics provide fundamental basis on identification of alkylmalonates as an alternative to be indicative of intermediates in anaerobic alkane degradation via the fumarate addition pathway.

2. Experimental

2.1. Materials

All reagents that were used (HBr, H_2SO_4, HCl, NaOH, Na, $LiAlH_4$, *n*-hexane, Na_2SO_4, *n*-hexyl alcohol, *n*-propanol, ethyl acetate, ethanol, methanol, LiCl, DMSO, THF, $NaHCO_3$, diethyl methylmalonate and petroleum ether) in the chemical synthesis were of analytical grade and of the highest purity and purchased from Shanghai Lingfeng Chemical Reagent Co. Ltd (Shanghai, China). Sodium sulfate and ethanol were pretreated to remove water prior to use or were purchased in anhydrous form. N, O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) was purchased from Sigma-Aldrich (Shanghai Trading Co. Ltd, Shanghai, China).

2.2. Synthesis of 2-(2-methylalkyl)malonic acid

2.2.1. Preparation of 1-bromoalkane

Alkylalcohol (0.035 mol) and HBr (0.039 mol) were mixed into a 100-mL flask. At 0 °C, H_2SO_4 was cautiously added, dropwise, into the mixture above and stirred intensely [25]. After returning to room temperature, the mixture was transferred to an oil bath and heated to reflux at 120 °C for 1 h. After cooling to room temperature, steam distillation was conducted to initially purify the target product. $NaHCO_3$ was added into the mixture to adjust the pH to > 7. The mixture was extracted with *n*-hexane three times and organic phase was obtained. Then, anhydrous Na_2SO_4 was used to dry the solvent. The yield was approximately 90%.

2.2.2. Preparation of diethyl 2-alkyl-2-methylmalonate

To a 100-mL flask equipped with a drying tube, 10 mL absolute ethanol was added. Na (0.035 mol, 0.80 g) was added in an ice water bath, and the solution was stirred untill Na was completely dissolved. The reaction apparatus was transferred to room temperature. Diethyl methylmalonate was added dropwise, which was followed by stirring for 30 min. Then, the prepared 1-bromoalkane was added into the above mixed system, stirred for 1 h, and placed in an oil bath at 80 °C for 6 h. The anhydrosity of the entire reaction was maintained. After the reaction cooled to room temperature, the ethanol was removed via rotary evaporation, and distilled water was added to the flask. Hydrochloric acid was added to adjust the pH to < 1. The obtained intermediate product was extracted three times with ethyl acetate. The combined oil layers were dried over anhydrous sodium sulfate and evaporated [21]. In this step, the brominated product replaced the active hydrogen from diethyl methylmalonate. The yield was approximately 68.5%.

2.2.3. Preparation of ethyl 2-methylalkylcarboxylate

This step was conducted under Krapcho decarboxylation condition [26]. The above product, namely, diethyl 2-alkyl-2-methylmalonate (0.035 mol), was added to a 100-mL flask and dissolved with 20 mL DMSO. LiCl (0.07 mol, 2.96 g) and distilled water (0.07 mol, 1.26 g) were added into the mixture, which was transferred to an oil bath at 150 °C and heated to reflux for 10 h. Following cooling to room temperature, distilled water was added, and extraction was conducted with ethyl acetate three times. The organic phase was combined, and the solvent was dried over anhydrous Na_2SO_4 and evaporated. The yield was approximately 60%.

2.2.4. Preparation of 2-methylalkyl alcohol

$LiAlH_4$ powder (0.035 mol, 1.33 g) was added into a dry 100-mL flask. In an ice water bath, 20 mL of the prepared re-distilled anhydrous THF was added dropwise with a constant pressure liquid funnel and stirred until the $LiAlH_4$ powder was completely dissolved. The drying tube was used throughout the process to prevent moisture in the air from entering the reaction system. The intermediate product, namely, ethyl 2-methylalkylcarboxylate, that was synthesized in the previous step was dissolved in 10 mL of re-distilled THF. Then, the mixture above was added dropwise to a solution of $LiAlH_4$ in THF by adjusting a constant pressure liquid funnel with a drying tube. The mixture was stirred vigorously at room temperature for 6 h until the whole reaction mixture turned into a gray turbid liquid [27].

The following procedure was conducted to quench the reaction. When quenching of the reaction, a constant-pressure liquid funnel was used to strictly control the rate of addition. When adding water at the beginning, a drop was added and stirred for a specified duration prior to continuing. The solution gradually changed from gray to white turbid. At the same time, precipitation could also occur in the reaction, and if the stirrer is unable to stir, a volume of anhydrous THF could be added to dissolve the precipitation. Water was added until no hydrogen was released from the solution. At this time, a large amount of white precipitation appeared in the flask, and dilute hydrochloric acid was added dropwise under an ice water bath. The mixture was stirred vigorously until the precipitation dissolved; then, the solution was clarified. The obtained clarification liquid was extracted with ethyl acetate three times. Then, the organic phase was combined and the solvent was removed from via the addition of anhydrous sodium sulfate and evaporation. The yield was approximately 43%.

2.2.5. Preparation of 1-bromo-2-methylalkane

This synthetic method was the same as that for the preparation 1-bromoalkane in step 2.2.1. The yield was approximately 82%.

2.2.6. Preparation of diethyl 2-(2-methylalkyl)malonate

In this step, the 1-bromo-2-methylalkane that was obtained in the above step was reacted with diethyl malonate. The method was as described in step 2.2.2, and the yield was approximately 60%.

2.2.7. Preparation of 2-(2-methylalkyl)malonic acid

2-(2-Methylalkyl)malonic acid was acquired via an ester hydrolysis reaction. The product that was acquired in the above step, namely, diethyl 2-(2-methylalkyl)malonate, was added with NaOH (0.07 mol, 2.80 g) and H_2O (20 mL) into a 100-mL flask and heated to reflux at 100 °C for 4 h. After cooling to room temperature, the mixture was extracted with *n*-hexane 3 times to remove undissolved organic matter

[20]. Then, the aqueous phase was collected and acidified with hydrochloric acid to pH < 1 and extracted with ethyl acetate three times. Finally, the oil phase was combined and dried over anhydrous sodium sulfate and evaporated. The yield was approximately 90%.

The final product, namely, 2-(2-methylalkyl)malonic acid, was purified via column chromatography (petroleum ether: ethyl acetate = 15:1, *v/v*).

2.3. NMR analysis

Diethyl 2-(2-methylalkyl)malonates were analyzed via ^{1}H NMR. The spectra were recorded on a Bruker AM 400-MHz spectrometer, and tetramethylsilane (TMS) was used as an internal standard.

2.4. Derivatization

Four homologous compounds that differed in terms of carbon chain length were derivatized to methyl, ethyl, *n*-butyl and trimethylsilyl esters. The mass spectral characteristics of the diethyl ester products could be acquired directly during chemical synthesis. Other derivatizations were conducted as described below.

In test tubes, 0.5 mL of 10% (*v/v*) H_2SO_4-methanol/H_2SO_4-butanol solution was added into 100 mg of each product for complete mixing. Then, the tubes were sealed and placed in an oil bath at 90 °C for 60 min for methyl/*n*-butyl esterifications [28]. Regarding the silylation products, BSTFA and acetonitrile were quickly well-mixed with each of the synthesized compounds and reacted in an oven at 60 °C for 30 min [29].

All derivatives above were identified directly via GC-MS for mass spectrometry characterization. All GC-MS analyses were performed on an Agilent 6890 GC instrument equipped with an HP-5MS capillary column (30 m × 0.25 mm × 0.25 μm) and mass detector (MSD 5975). The experimental details regarding various methods of derivatization and the conditions for the GC-MS program were studied previously [20].

3. Results and discussion

Four 2-(2-methylalkyl)malonic acids that differed in terms of carbon chain length, namely, propylmalonic acid, 2-(2-methylbutyl)malonic acid, 2-(2-methylpentyl)malonic acid and 2-(2-methyloctyl)malonic acid, were synthesized in this study (**1–4**, Fig. 1).

The complete synthetic route for 2-(2-methylalkyl)malonic acid is illustrated in Fig. 2. Four synthesized compounds were derivatized via four methods for GC-MS detection (Fig. 3, S1, S2 and S3). The diethyl esterification compounds were identified via both mass spectrometry (Fig. 3) and ^{1}H NMR spectroscopy (Fig. S4).

3.1. NMR data

Diethyl propylmalonate. ^{1}H NMR (400 MHz, $CDCl_3$, TMS; δ, ppm): δ = 4.19 (q, J = 8.0 Hz, 4H), 3.33 (t, J = 7.6 Hz, 1H), 1.87 (q, J = 7.6 Hz, 2H), 1.38-1.33 (m, 2H), 1.27 (t, J = 7.2 Hz, 6H), 0.94 (t, J = 7.3 Hz, 3H).

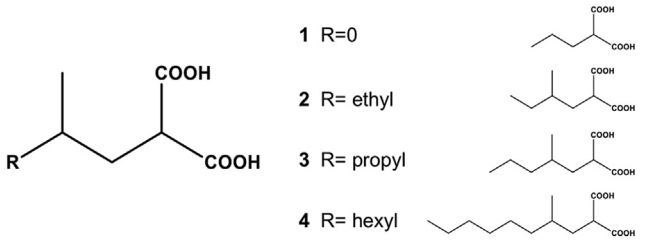

Fig. 1. Chemical structures of the four 2-(2-methylalkyl)malonic acids.

Diethyl 2-(2-methylbutyl)malonate. ^{1}H NMR (400 MHz, $CDCl_3$, TMS; δ, ppm) δ = 4.24-4.17 (m, 4H), 3.47-3.41 (m, 1H), 2.01-1.93 (m, 1H), 1.72-1.65 (m, 1H), 1.42-1.34 (m, 2H), 1.31-1.20 (m, 6H), 1.23-1.17 (m, 1H), 0.91-0.86 (m, 6H).

Diethyl 2-(2-methylpentyl)malonate. ^{1}H NMR (400 MHz, $CDCl_3$, TMS; δ, ppm): δ = 4.19-4.10 (m, 4H), 3.39-3.33 (m, 1H), 1.91-1.83 (m, 1H), 1.64-1.54 (m, 1H), 1.43-1.26 (m, 2H), 1.22-1.17 (m, 8H), 1.12-0.99 (m, 1H), 0.87-0.75 (m, 6H).

Diethyl 2-(2-methyloctyl)malonate. ^{1}H NMR (400 MHz, $CDCl_3$, TMS; δ, ppm): δ = 4.21-4.16 (m, 4H), 3.45-3.41 (m, 1H), 1.98-1.91 (m, 1H), 1.70-1.63 (m, 1H), 1.42-1.38 (m, 1H), 1.28-1.25 (m, 16H), 0.89-0.86 (m, 6H).

3.2. Mass spectrometry characterization of methyl, ethyl and n-butyl esterification products

The mass spectral characteristics are presented in Fig. 3 and Figs. S1, S2 and S3. Regularities were observed in the mass spectra of all the derivatization products. Since the diethyl esterification product can be obtained according to the designed synthetic route, it can be directly analyzed via GC-MS. The fragment ions were examined in detail by considering the diethyl ester as an example.

3.2.1. Mass spectrometry characterization of ethyl esterification products

The mass spectral characterizations of the four diethyl esterification compounds are presented in Fig. 3. The four most significant intermediate fragments, which are observed at *m/z* 133, 160, 173 and $[M - 45]^+$, are speculated to be the characteristic fragment ions of diethyl 2-(2-methylalkyl)malonate. The cleavage mechanism is proposed (Fig. 4) for further discussion.

The strong peaks at *m/z* 133, 160 and 173 (in red in Fig. 3) remained unchanged with changes in the relative molecular mass. It is posited that diethyl 2-(2-methylalkyl)malonates (structure **a**) loses the alkyl moiety (the R group) and forms a carbon cation of diethyl methylenemalonate (structure **b**) at *m/z* 173. This fragment ion presents the unique molecular structure of diethyl malonate from diethyl 2-(2-methylalkyl)malonates and is indicative of the structural characteristics of such substances. Among all of the peaks, the fragment ion *m/z* 160 shows the highest relative abundance in each mass spectrum. Since the alkyl group of the carbon chain can provide a γ-H for this rearrangement reaction, it is speculated that structure **d** undergoes EI-induced fragmentation via McLafferty rearrangement and γ-cleavage to form structure **f** at *m/z* 160 [30,31]. At the same time, structure **g** can further proceed a McLafferty rearrangement with double hydrogen transfer, leading to an alkenyl loss and yielding structure **h**, *m/z* 133 [31,32]. The fragment ions *m/z* 133, 160 and 173 are all relatively abundant in the four mass spectra and can be used as the primary determinants of compounds with structures of this type.

The relative abundance of the fragment ion *m/z* $[M - 45]^+$ (in blue in Fig. 3), which corresponds to structure **c**, is also high in all the EI mass spectra. As presented in Fig. 4, it is presumed that the loss of an ethyoxyl moiety ($[\cdot OC_2H_5]$, *m/z* 45) from the ethyl ester group in structure **a** results in the formation of fragment **c** [33–36]. The relative molecular mass of the compound can be inferred from *m/z* $[M - 45]^+$ to determine the length of the alkyl chain.

Therefore, the four fragment ion peaks at *m/z* 133, 160, 173 and $[M - 45]^+$ can be regarded as the characteristic fragments that indicate the presence of diethyl 2-(2-methylalkyl)malonates.

3.2.2. Mass spectrometry characterization of methyl esterification products

Three relatively intense peaks at *m/z* 132, 145 (in red) and $[M - 31]^+$ (in blue) are visible in all the spectra of the methyl esterification derivatives (Figure S1). Fragment ions *m/z* 132 and 145 are similar to those of ethyl esters, which differ by 28 mass units, suggesting the presence of two methylene groups, analogous to structures **b** and **f**. The shifts of *m/z* are rational when methoxyl is substituted for ethoxyl.

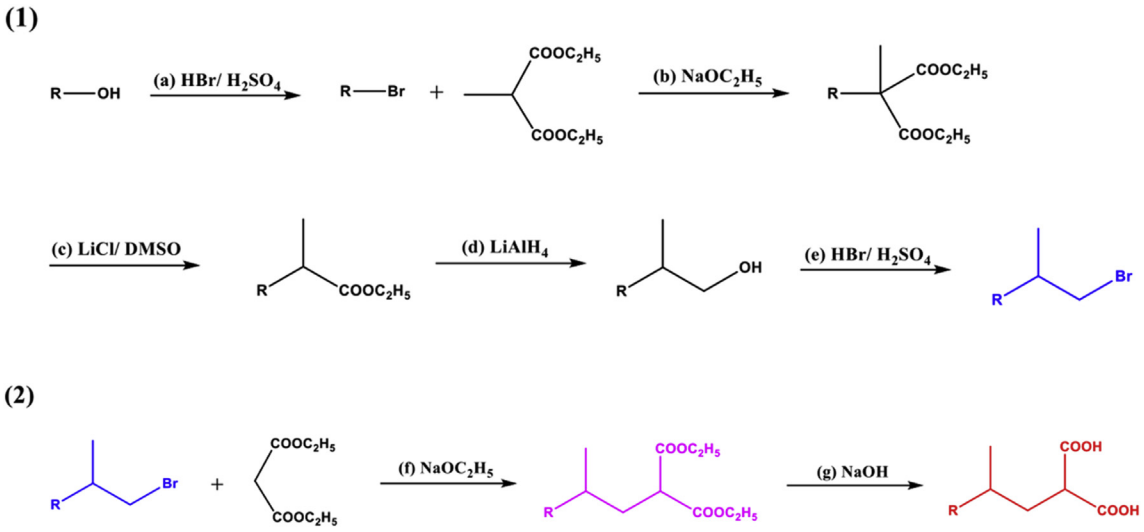

Fig. 2. Chemical synthetic procedure for 2-(2-methylalkyl)malonic acid.

Additionally, a fragment ion *m/z* [M - 31]$^+$ can be observed in all mass spectra, indicating the elimination of a methyl group. According to mechanism (b), the fragment at *m/z* 119 that corresponds to structure **h** is missing from all EI mass spectra of methyl esters. However, this is reasonable because when the methyl moiety replaces the ethyl moiety, McLafferty rearrangement with double hydrogen transfer (steps **g** to **h**) will not occur, resulting in the absence of the fragment at *m/z* 119 [31]. Therefore, the mass spectral characteristics of dimethyl 2-(2-methylalkyl)malonates are *m/z* 132, 145 and [M - 31]$^+$.

3.2.3. Mass spectrometry characterization of n-butyl esterification products

Likewise, the mass spectral characteristics of *n*-butyl esters differ by 28 or 56 mass units from those of ethyl esterification. As displayed in Fig. S2, the mass spectral characteristics *m/z* 161, 216, 229 (in red) and [M - 73]$^+$ (in blue) can be identified in each mass spectrum of *n*-butyl esterification products, which coincide with mechanisms (a) and (b) in Fig. 4. In addition, two fragment ions, namely, *m/z* [M - 55]$^+$ and [M - 129]$^+$ (in green), are observed in all mass spectra of *n*-butyl esters. According to Fig. 4(c), due to the presence of γ-H on the butyl group of fragment **i**, the McLafferty rearrangement with double hydrogen transfer reaction occurs to yield fragment **j** at *m/z* [M - 55]$^+$, and a butenyl moiety is lost [31,37]. The butanol group of fragment **j** is further removed to generate structure **k** at *m/z* [M - 129]$^+$, which is in agreement with previous observations [20]. Hence, the fragment ions *m/z* 161, 216, 229, [M - 73]$^+$, [M - 55]$^+$ and [M - 129]$^+$ are regarded as characteristic peaks of dibutyl 2-(2-methylalkyl)malonates.

3.3. Mass spectrometry characterization of silylation products

As shown in Fig. S1 and S2, in contrast to alkyl esterification,

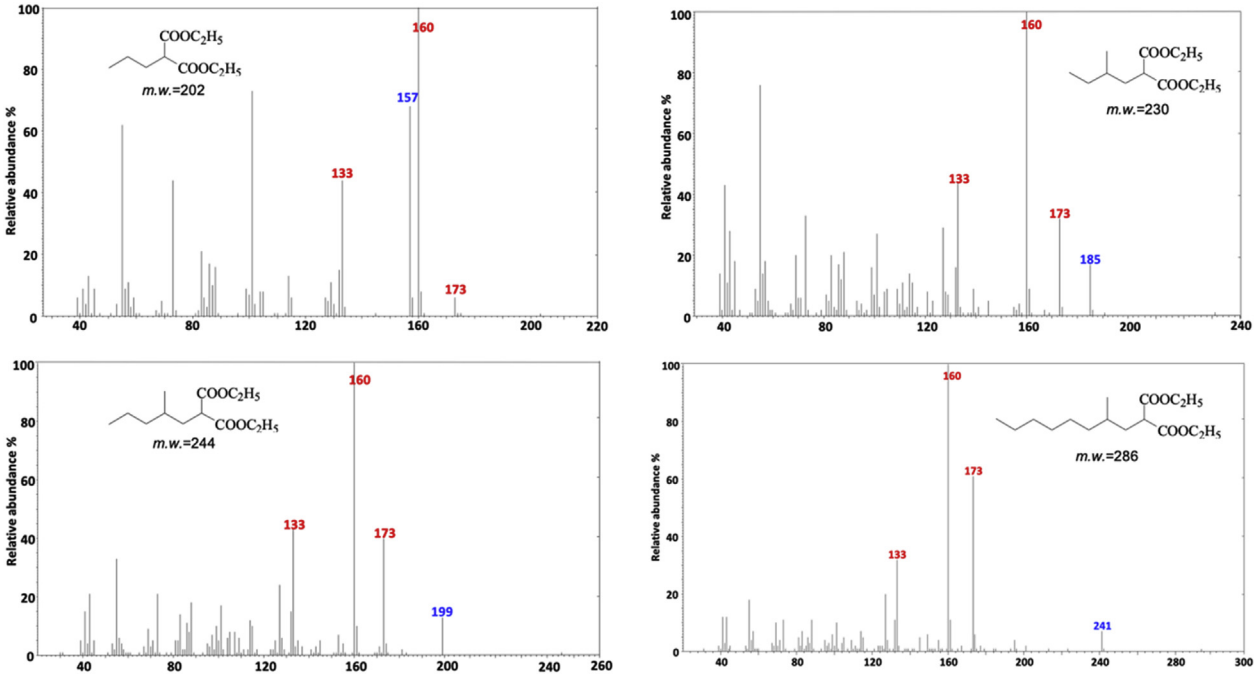

Fig. 3. Mass spectral characteristics of the synthesized diethyl 2-(2-methylalkyl)malonates as fumarate addition pathway rearrangement derivatives. The values in red are constant for all chemical compounds. The values in blue differ among the compounds and can reflect the length of the carbon chain. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

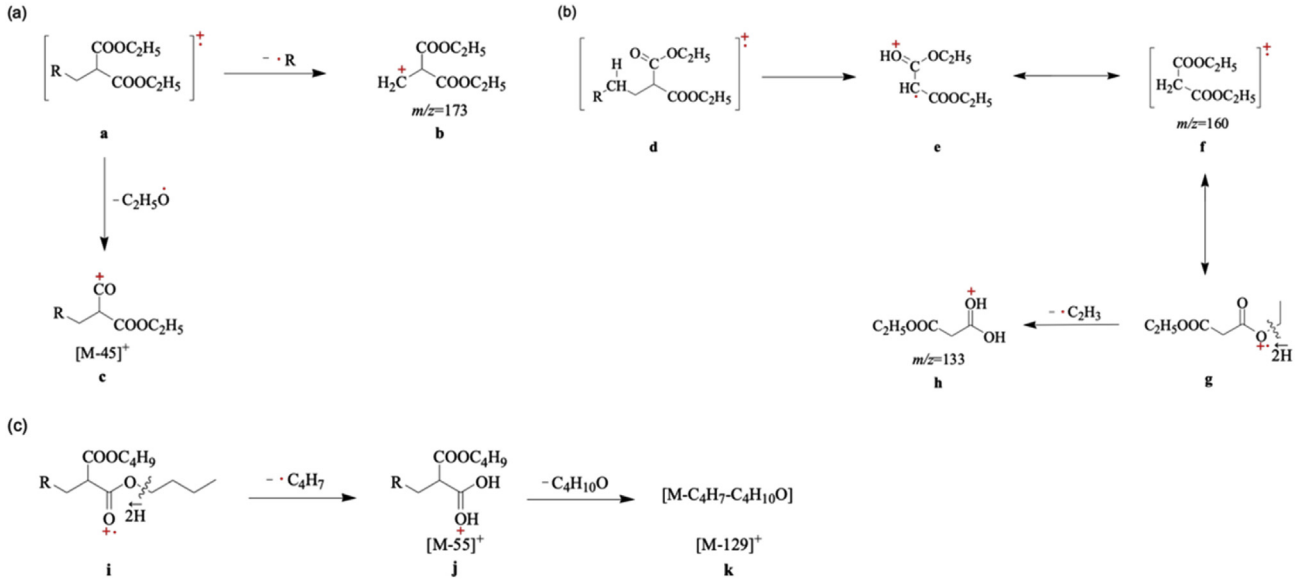

Fig. 4. Proposed cleavage of diethyl 2-(2-methylalkyl)malonate via mechanisms (a) and (b) and of dibutyl 2-(2-methylalkyl)malonate via mechanism (c).

Table 1
Characteristic fragment ions of diester 2-(2-methylalkyl)malonates.

Derivation	Characteristic fragment ions
Methyl esterification	132, 145, $[M - 31]^+$
Ethyl esterification	133, 160, 173, $[M - 45]^+$
n-Butyl esterification	161, 216, 229, $[M - 73]^+$, $[M - 55]^+$, $[M - 129]^+$
Silylation	73, 147, 217, 248, 261, $[M - 15]^+$

fragment ions at *m/z* 261 and 248 via mechanisms (a) and (b) in Fig. 4 are identified in each spectrum, but no ion peaks that corresponded to the process of converting structure **a** to structure **c** via mechanism (a) were observed. Similar to methyl esterification, the correspondence of the missing fragment at *m/z* 175 to structure **h** is rational because McLafferty rearrangement with double hydrogen transfer (steps **g** to **h**) will not occur [31], whereas the four fragment ions at *m/z* 73, 147, 217 and $[M - 15]^+$ (Fig. S3) are presented with relatively high abundance. It is suggested that *m/z* $[M - 15]^+$ corresponds to the loss of the methyl group from the bis-(trimethylsilyl) esters. The fragment at *m/z* 217 is presumed to be a product of the migration of the trimethylsilyl group on the carboxyl monomer, and the ions at *m/z* 73 and 147 correspond to the trimethylsilyl monomer and dimer [38–41]. Consequently, the fragment ions at *m/z* 73, 147, 217, 248, 261 and $[M - 15]^+$ can be regarded as the mass spectral characteristics of trimethylsilyl esters.

In conclusion, the mass spectral characteristics of diester 2-(2-methylalkyl)malonates are summarized in Table 1. Each of the derivatization methods corresponds to a distinct sets of characteristics. According to the newly obtained information, it is possible to choose one or several suitable methods for proving fumarate addition via the direct detection of rearrangememnt bio-signature metabolites.

4. Conclusions

A synthetic procedure for 2-(2-methylalkyl)malonic acids as rearrangement bio-signature metabolites of anaerobic alkane activation via fumarate addition was conducted. Four model products with various carbon chain lengths were derivatized via four approaches, and their mass spectra information was determined via GC-MS. The characteristic fragments were observed at *m/z* 133 + 14*n*, 160 + 28*n*, 173 + 28*n* and $[M - (45 + 14n)]^+$ (n = 0 and 2 for ethyl and *n*-butyl esters, respectively). Additionally, mass spectra features at *m/z* 132, 145, and $[M - 31]^+$ and ions at *m/z* 73, 147, 217, 248, 261 and $[M - 15]^+$ can be used for the identification of methyl and trimethylsilyl esters, respectively. These results provide an alternative method for detecting downstream biomarkers of the fumarate addition pathway in both *in situ* and in the laboratory, which deepens the exploration of anaerobic hydrocarbon degradation.

CRediT authorship contribution statement

Jing Chen: Conceptualization, Methodology, Writing - original draft. **Lei Zhou:** Software, Visualization, Writing - original draft. **Yi-Fan Liu:** Data curation, Writing - original draft, Writing - review & editing. **Zhao-Wei Hou:** Writing - original draft, Writing - review & editing. **Wei Li:** Writing - original draft, Writing - review & editing. **Serge Maurice Mbadinga:** Writing - original draft, Formal analysis. **Jing Zhou:** Writing - original draft, Methodology. **Tao Yang:** Writing - original draft, Methodology. **Jin-Feng Liu:** Writing - original draft, Project administration. **Shi-Zhong Yang:** Writing - original draft, Project administration. **Xiao-Lin Wu:** Writing - original draft, Supervision. **Ji-Dong Gu:** Writing - original draft, Conceptualization, Supervision. **Bo-Zhong Mu:** Writing - original draft, Project administration, Funding acquisition, Supervision.

Declaration of competing interest

The authors declare that there is no conflict of interest.

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Appendix A. Supplementary data

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