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Title: Polyunsaturated fatty acid saturation by gut lactic acid bacteria affecting host lipid composition

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Abstract

Microorganisms in the gastrointestinal tract interact with their host in many ways and contribute significantly to the maintenance of host health (*1*). Lipid metabolism by gastrointestinal microbes generates multiple fatty-acid species, such as conjugated fatty acids and *trans*-fatty acids, that can affect host lipid metabolism (*2*). In the representative gut bacterium *Lactobacillus plantarum*, we identified genes encoding the enzymes involved in a novel saturation metabolism of polyunsaturated fatty acids, and revealed in detail the metabolic pathway that generates hydroxy fatty acids, oxo fatty acids, conjugated fatty acids, and partially saturated *trans*-fatty acids as intermediates. Furthermore, we observed these intermediates, especially hydroxy fatty acids, in host organs. Levels of hydroxy fatty acids were much higher in specific pathogen–free mice than in germ-free mice, indicating that these fatty acids are generated through polyunsaturated fatty acids metabolism of gastrointestinal microorganisms. These findings suggested that lipid metabolism by gastrointestinal microbes affect the health of the host by modifying fatty-acid composition.

1. Round JL, Mazmanian SK (2009) The gut microbiota shapes intestinal immune responses during health and disease. *Nat. Rev. Immunol.* 9(5): 313-323.
2. Griinari JM, Bauman DE (1999) in *Advances in conjugated linoleic acid research.*, eds Yurawecz MP *et al*. (AOCS Press, Champaign), pp 180-199.

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Introduction

Dietary fats are metabolized not only by humans, but also by microbes in our gastrointestinal tracts. However, lipid metabolism by gastrointestinal microbes has not been explored in detail. Saturation metabolism of polyunsaturated fatty acids, a representative mode of lipid metabolism by gastrointestinal microbes, is a detoxifying metabolism of anaerobic bacteria, such as lactic-acid bacteria, that reside in colon and intestine. This process transforms growth-inhibiting free polyunsaturated fatty acids into less toxic free saturated fatty acids (*3*). This saturation metabolism generates characteristic fatty acids, e.g., conjugated fatty acids and *trans*-fatty acids, which are well known to present in ruminant-derived foods and exert various physiological activities.

'Conjugated fatty acid' is a collective term for positional and geometric isomers of fatty acids with conjugated double bonds. In particular, conjugated linoleic acids (CLAs) such as *cis*-9,*trans*-11-CLA and *trans*-10,*cis*-12-CLA reduce carcinogenesis (*4*), atherosclerosis (*5*), and body fat (*6*). In regard to lipid metabolism, CLA is a potent peroxisome proliferator-activated receptor α (PPARα) agonist (*7*), and treatment with CLA increases the catabolism of lipids in the liver of rodents (*8*). Based on these findings, CLA is now commercialized as a functional food for control of body weight, especially in the USA and European countries.

On the other hand, consumption of *trans*-fatty acids increases the risk of coronary heart disease by increasing LDL and reducing HDL cholesterol levels (*9*). Consequently, *trans*-fatty acids are considered to be harmful for health, and nutritional authorities have recommended that consumption of *trans*-fatty acids be reduced to trace amounts (*10*). Therefore, it is important to control fatty acid saturation processes that generate these fatty acids (*11*); however, the precise metabolic pathway and enzymes involved have not been clearly identified.

Our analyses on conjugated fatty acid synthesis in representative gut bacteria, the lactic-acid bacteria (*12-15*), demonstrated that *Lactobacillus plantarum* AKU 1009a can transform the *cis*-9,*cis*-12 diene structure of C18 fatty acids such as linoleic acid, α-linolenic acid, and γ-linolenic acid into the conjugated diene structures *cis*-9,*trans*-11 and *trans*-9,*trans*-11 (*16-21*). In addition, this strain can saturate these conjugated dienes into the *trans*-10 monoene. Our subsequent metabolic analysis indicated that 10-hydroxy-12-octadecenoic acid is an intermediate of CLA synthesis, and further investigations of hydroxy fatty-acid metabolism by lactic-acid bacteria revealed that CLA is produced from hydroxy fatty acids such as ricinoleic acid in castor oil (*22-25*). In cell-free extracts from this strain, we identified the enzymes involved in CLA synthesis (*26*). Three enzymes, CLA-HY, CLA-DH, and CLA-DC, are necessary for synthesis of conjugated fatty acids such as CLA. Only the combined action of these three enzymes can generate CLA from linoleic acid, with 10-hydroxy-*cis*-12-octadecenoic acid arising as an intermediate (Figure 1B-3). The reactions catalyzed by each enzyme, however, were not revealed in those studies. Through genomic analysis in *L. plantarum* WCFS1, we found that *cla-dh* (GeneID: 1061221) and *cla-dc* (GeneID: 1061220) are located in a cluster with another gene, *cla-er* (GeneID: 1061219) (Figure 1A). In light of this, we tried to identify the function of the gene product (CLA-ER) together with those of CLA-HY, CLA-DH, and CLA-DC.

Results and Discussion

Based on the sequence of the *L. plantarum* WCFS1 genome, we designed primers to amplify the CLA-ER gene from *L. plantarum* AKU 1009a genomic DNA. The PCR-amplified product, *cla-er* (GenBank ID: AB812091)*,* consisted of 654 bp and encoded a protein whose amino-acid sequence is 99.8% identical to the homologous sequence from *L. plantarum* WCFS1. We transformed the resulting plasmid containing *cla-er* from *L. plantarum* AKU 1009a, pCLA-ER, into *E. coli* Rosetta™2 (DE3) to generate *E. coli* Rosetta/pCLA-ER. The gene product, CLA-ER, had a molecular weight of ~25 kDa (including a His tag), and could be detected in soluble cell-free extracts of *E. coli* Rosetta/pCLA-ER. We examined the function of CLA-ER in fatty-acid metabolism using purified CLA-ER in combination with other purified enzymes (CLA-HY, CLA-DH, and CLA-DC) from the corresponding transformants (*E. coli* Rosetta/pCLA-HY, *E. coli* Rosetta/pCLA-DH, and *E. coli* Rosetta/pCLA-DC).

In a reaction with these four enzymes (CLA-HY, CLA-DH, CLA-DC, and CLA-ER) as catalysts, *cis*-9,*trans*-11-CLA (CLA1), *trans*-9,*trans*-11-CLA (CLA2), 10-hydroxy-*cis*-12-octadecenoic acid, *trans*-10-octadecenoic acid, and *cis*-9-octadecenoic acid (oleic acid) were generated from linoleic acid (Figure 1B-4). Thus, the combined action of these four enzymes generated saturated products of oleic acid and *trans*-10-octadecenoic acid from linoleic acid, i.e., these four enzymes catalyzed the saturation of an polyunsaturated fatty acid.

In our previous studies, we revealed that linoleic acid could be converted into 10-hydroxy-*cis*-12-octadecenoic acid by CLA-HY (Figure 1B-2), as well as into CLA1 and CLA2 along with 10-hydroxy-*cis*-12-octadecenoic acid by CLA-HY, CLA-DH, and CLA-DC (Figure 1B-3). We purified the resulting 10-hydroxy-*cis*-12-octadecenoic acid by high-performance liquid chromatography (HPLC) and used it as a substrate for reactions containing each enzyme together with the oxidoreduction cofactors, i.e., FAD, NADH, or NADPH, that enhanced CLA synthesis by CLA-HY, CLA-DH, and CLA-DC (*14*). 10-Hydroxy-*cis*-12-octadecenoic acid was converted into linoleic acid and *trans*-10,*cis*-12-octadecadienoic acid by CLA-HY in the presence of FAD and NADH (Figure S1). The same substrate was converted into 10-oxo-*cis*-12-octadecenoic acid by CLA-DH in the presence of NAD$^+$ (Figure S2). As before, we purified the resulting *trans*-10,*cis*-12-octadecadienoic acid and 10-oxo-*cis*-12-octadecenoic acid by HPLC and used them as substrates in subsequent reactions.

None of the enzymes converted *trans*-10,*cis*-12-octadecadienoic acid under any conditions tested. By contrast, 10-oxo-*cis*-12-octadecenoic acid was converted into 10-oxo-*trans*-11-octadecenoic acid by CLA-DC in the absence of cofactors (Figure S3). HPLC-purified 10-oxo-*trans*-11-octadecenoic acid was converted into 10-oxo-octadecanoic acid by CLA-ER in the presence of FAD/FMN and NADH (Figure S4), and was converted into 10-hydroxy-*trans*-11-octadecenoic acid by CLA-DH in the presence of NADH (Figure S5). Purified 10-oxo-octadecanoic acid was converted into 10-hydroxy-octadecanoic acid by CLA-DH in the presence of NADH (Figure S6), and this product was in turn converted into *cis*-9-octadecenoic acid and *trans*-10-octadecenoic acid by CLA-HY in the presence of FAD and NADH (Figure S7). The 10-hydroxy-*trans*-11-octadecenoic acid could also be converted into *cis*-9,*trans*-11-CLA and *trans*-9,*trans*-11-CLA by CLA-HY in the presence of FAD and NADH (Figure S8).

These results demonstrate that the linoleic-acid saturation metabolism of *Lactobacillus plantarum* consists of multiple reactions. The first reaction of linoleic-acid metabolism is hydration of the carbon-carbon double bond at the delta-9 position, catalyzed by CLA-HY, to generate 10-hydroxy fatty acid. The second reaction is dehydrogenation of the hydroxy group at C10, catalyzed by CLA-DH, to generate 10-oxo fatty acid. The third reaction is isomerization of the carbon-carbon double bond at delta-12, catalyzed by CLA-

DC, to generate the conjugated enone structure, 10-oxo-*trans*-11-fatty acid. The fourth reaction is hydrogenation of the carbon-carbon double bond at delta-11, catalyzed by CLA-ER, to generate the carbon-carbon single bond. The fifth reaction is hydrogenation of the oxo group at C10, catalyzed by CLA-DH, to generate 10-hydroxy fatty acid. The last reaction is dehydration of hydroxy group at C10, catalyzed by CLA-HY, to generate *cis*-9 and *trans*-10 monoenoic fatty acids. Through a branch of the saturation-metabolism pathway, conjugated fatty acids are generated by the combined actions of three of the enzymes, CLA-HY, CLA-DH, and CLA-DC. The branched pathway starts with hydrogenation of the oxo group at C10 in 10-oxo-*trans*-11-fatty acid, catalyzed by CLA-DH, to generate 10-hydroxy-*trans*-11-fatty acid; the final reaction is dehydration of hydroxy group at C10 in 10-hydroxy-*trans*-11-fatty acid, catalyzed by CLA-HY, to generate *cis*-9,*trans*-11 and *trans*-9,*trans*-11 conjugated fatty acids (Figure 2). As we reported previously, C18 fatty acids with Δ9 and Δ12 diene systems such as α-linolenic acid, γ-linolenic acid, and stearidonic acid undergo the same transformations in *L. plantarum* AKU 1009a (*20*), indicating that the corresponding intermediates (hydroxy, oxo, conjugated, and partially saturated *trans*-fatty acids) are produced by the combined actions of these enzymes. The revealed fatty acid saturation metabolism consists of similar reactions in known fatty acid biosynthesis and degradation pathway, however, it is completely new pathway using only free fatty acids as substrates but not CoA- or acyl carrier protein-activated fatty acids.

In the experiments described above, we revealed the pathway of unsaturated fatty-acid metabolism in *L. plantarum* and the enzymes involved in this metabolism. These enzymes function as catalysts of hydration/dehydration (CLA-HY), oxidation of hydroxy groups/reduction of oxo groups (CLA-DH), migration of carbon-carbon double bonds (CLA-DC), and saturation of carbon-carbon double bonds (CLA-ER). The genes that encode CLA-DH, CLA-DC, and CLA-ER form a gene cluster in *L. plantarum.* When we searched for gene clusters containing *cla-dh*, *cla-dc,* and *cla-er* in other microorganisms using the KEGG database (Kyoto Encyclopedia of Genes and Genomes, http://www.genome.jp/kegg/), we found that *Lactobacillus casei* and *Lactobacillus rhamnosus* have the same gene cluster as well as the *cla-hy* gene. Furthermore, many species of lactic-acid bacteria have one or more of these four genes. For example, *Lactobacillus salivarius* has *cla-dc*, *cla-er,* and *cla-hy*; and *Lactobacillus amylovorus*, *Lactobacillus johnsonii*, *Lactobacillus helveticus*, and *Lactobacillus crispatus* have *cla-dh*, *cla-er*, and *cla-hy*. Therefore, acting in concert, these species may mediate the polyunsaturated fatty acid saturation metabolism in gastrointestinal tract. The *in vivo* distributions of these strains in relation with the fatty acid profiles and the health conditions of host organisms are further interests.

The reactions and enzymes we identified will be useful for modifying the properties of fatty acids in foods. The apparent isomerization reaction catalyzed by the combined activities of CLA-HY, CLA-DH, and CLA-DC will be useful for production of *cis*-9,*trans*-11- and *trans*-9,*trans*-11-conjugated fatty acids. The other isomerization reaction catalyzed by CLA-HY will be useful for production of *trans*-10,*cis*-12-conjugated fatty acids. Furthermore, the dehydration reaction catalyzed by CLA-HY might determine the ratio of *trans* to *cis*-fatty acids. In other words, enhancing *cis*-dehydration by CLA-HY could be useful for reducing the amounts of *trans*-fatty acid in foods. This might be possible as we reported previously that the CLA1/CLA2 ratio (*cis*/*trans* ratio) can be controlled by optimizing the reaction conditions (*14, 16*). Furthermore, not only in food industry, but also in chemical industry, the reactions found in the fatty acid saturation metabolism are useful, e.g. enzymatic production of hydroxy fatty acids for polymer industry.

To evaluate the effects of gastrointestinal bacteria on the profile of fatty acids in host tissues, we monitored endogenous formation of the fatty-acid intermediates of polyunsaturated fatty acid saturation metabolism, i.e., hydroxy and oxo fatty acids, in mice

bred in either germ-free or specific pathogen–free (SPF) conditions. In the colon, small intestine, and plasma of both groups of mice, we detected 10-hydroxy-*cis*-12-octadecenoic acid, 10-hydroxyoctadecanoic acid, and 10-oxooctadecanoic acid. By contrast, we did not detect 10-oxo-*cis*-12-octadecenoic acid or 10-oxo-*trans*-11-octadecenoic acid derived from linoleic acid, or metabolites derived from α-linolenic acid and γ-linolenic acid. There were significant differences in the levels of hydroxy fatty acids between SPF and germ-free mice: in particular, we observed higher levels of 10-hydroxy fatty acids derived from linoleic acid, oleic acid, or both in SPF mice than in germ-free mice (Figure 3). We also detected a structurally related hydroxy fatty acid, 13-hydroxy-9-*cis*-octadecenoic acid, which is produced by lactic-acid bacteria (*27*), at higher levels in SPF mice than in germ-free mice (Figure 3). These differences in the levels of hydroxy fatty acids could be clearly observed in the small intestine, the primary site of fatty acid absorption. These results indicate that gastrointestinal microbes play roles in modifying the fatty-acid profiles of their host mice, in particular by increasing the levels of hydroxy fatty acids that are initial intermediates of polyunsaturated fatty acid saturation metabolism. The investigations using mono-colonize germ-free mice with *L. plantarum* or a strain deficient in one or more of the fatty acid saturating genes to see whether the host accumulates intermediates are also future interests.

The intermediates found in the novel polyunsaturated fatty acid saturation metabolism are predicted to exert specific physiological functions. For example, hydroxy and oxo fatty acids act as ligands for PPARγ (*28*); oxo-fatty acids found in tomato juice are potent PPARα activators and decrease the amount of triacylglycerol in obese diabetic mice (*29*). Therefore, functional investigations of these fatty-acid intermediates of the polyunsaturated fatty acid saturation metabolism will provide new methods for improving our health by altering lipid metabolism related to the onset of metabolic syndrome. Exploration of the lipid metabolism of gastrointestinal microorganisms at the enzymatic and genetic levels, and integration of these findings with metagenomic information, might enable us to promote health by controlling intestinal lipid metabolism.

Materials and Methods
Chemicals
Standard samples of *cis*-9,*trans*-11-CLA, *trans*-9,*trans*-11-CLA, and 10-hydroxy-*cis*-12-octadecenoic acid were prepared as previously described (*11, 13*). Linoleic acid and fatty acid–free (<0.02%) bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO, USA). All other chemicals were analytical grade and obtained commercially.

Cloning and expression of recombinant CLA-ER proteins in *E. coli*
Primers were designed to amplify the *cla-er* sequence from *L. plantarum* AKU 1009a genomic DNA. The PCR-amplified product was ligated into the pET101/D-TOPO expression vector (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. The resulting plasmid, pCLA-ER, was transformed into *E. coli* Rosetta[TM]2 (DE3) (Novagen, Madison, WI, USA) to generate *E. coli* Rosetta/pCLA-ER. The integrity of the cloned gene was verified by DNA sequencing using a Beckman-Coulter CEQ8000 (Beckman Coulter, Fullerton, CA, USA).

Expression of recombinant proteins in *E. coli*
Transformants (*E. coli* Rosetta/pCLA-HY, *E. coli* Rosetta/pCLA-DH, *E. coli* Rosetta/pCLA-DC, and *E. coli* Rosetta/pCLA-ER) were cultured in Luria-Bertani (LB) medium at 37°C for 1 h with shaking at 300 rpm, and then isopropyl-β-thiogalactopyranoside (IPTG) was added to a final concentration of 1.0 mM. After addition of IPTG, the transformed cells were cultivated at 20°C for 6 h with shaking at 300 rpm. The cells were

harvested by centrifugation (12,000 × g, 10 min), washed twice with 0.85% NaCl, and stored at −20°C until further use.

Purification of enzymes from transformants

To determine the approximate concentration of protein eluted in chromatography, effluents were monitored by UV detection at 280 nm. Enzymes were purified using a fast protein liquid chromatography (FPLC) system (Amersham Pharmacia Biotech Co., Uppsala, Sweden) equilibrated with binding buffer (20 mM potassium phosphate buffer (KPB), 50 mM imidazole, pH 7.4) or standard buffer (20 mM KPB, 1 mM DTT, 10% ethylene glycol, pH 6.5). Fractions with enzymatic activity were collected and concentrated using an Amicon Ultra YM-10 (Millipore, Billerica, MA, USA). All procedures were carried out at 4°C.

CLA-HY and CLA-ER were purified as His-tagged proteins. *E. coli* Rosetta/pCLA-HY and *E. coli* Rosetta/pCLA-ER cells (8 g) in 1.5 L of culture broth were suspended in binding buffer and disrupted with an Insonator 201M ultrasonic oscillator (Kubota, Osaka, Japan). After ultracentrifugation (100,000 × g, 60 min) of the cell lysate, the resulting supernatant containing His-tagged CLA-HY or His-tagged CLA-ER was loaded onto a Ni-Sepharose column (His Trap HP; GE Healthcare, Buckinghamshire, UK) pre-equilibrated with binding buffer. After washing, the bound proteins were eluted with elution buffer (20 mM KPB, 250 mM imidazole, pH 7.4). Active fractions were collected and concentrated with a Centriprep YM-3 (Millipore) and applied to a Hi-load 26/60 Superdex 200 prep-grade column (GE Healthcare) equilibrated with 50 mM KPB (pH 6.5). Active fractions were collected, dialyzed with 50 mM KPB (pH 6.5) including 50% (v/v) glycerol, and stored at −20°C until use.

For purification of CLA-DH, *E. coli* Rosetta/pCLA-DH cells (8 g) in 1.5 L of culture broth were suspended in BugBuster Master Mix (Merck, Darmstadt, Germany) (30 ml) and incubated for 20 min at room temperature. After ultracentrifugation (100,000 × g, 60 min) of the cell lysate, the resulting supernatant was concentrated with a Centriprep YM-3 and applied to a HiLoad 26/60 Superdex 200 prep-grade column that had been equilibrated with standard buffer and eluted. CLA-DH was purified further using a Mono Q 10/100 GL column and a Superdex 200 10/300 GL column (GE Healthcare). The purified CLA-DH was dialyzed with 50 mM KPB (pH 6.5) including 50% (v/v) glycerol and stored at −20°C until use.

For purification of CLA-DC, *E. coli* Rosetta/pCLA-DC cells (8 g) in 1.5 L of culture broth were suspended in standard buffer and disrupted with an Insonator 201M ultrasonic oscillator. After centrifugation (20,000 × g, 30 min) of the cell lysate, solid sulfate was added to the resulting supernatant to 50–80% saturation. The precipitate was recovered by centrifugation, dissolved in 10 ml standard buffer, and then dialyzed three times against 2 L standard buffer for 8 h. CLA-DC was purified further using a Phenyl Superose HR 10/10 column and a Mono Q 5/50 GL column (GE Healthcare). Purified CLA-DC was dialyzed with 50 mM KPB (pH 6.5) including 50% (v/v) glycerol and stored at −20°C until use.

Reaction conditions

Reactions were performed in test tubes (16.5 × 125 mm) that contained 1 mL of reaction mixture (20 mM KPB, pH 6.5) with 0.1% (w/v) fatty acid complexed with BSA (0.02% [w/v]) as the substrate, and purified enzymes (CLA-HY, CLA-DH, CLA-DC, and CLA-ER) in various combinations. The reactions were performed with 5 mM NADH, 5 mM NAD$^+$, 0.1 mM FMN, or 0.1 mM FAD under microaerobic conditions in a sealed chamber with an O_2 absorber (Anaeropack "Kenki", Mitsubishi Gas Chemical Co., Ltd., Tokyo, Japan), and gently shaken (120 strokes/min) at 37°C for 12 h. The oxygen concentration under microaerobic conditions was maintained below 0.1% (<1000 ppm) and monitored with

an oxygen indicator (Mitsubishi Gas Chemical Co., Ltd.). All experiments were performed in triplicate, and the averages of three separate experiments that were reproducible within ±10% are presented in the figures.

Lipid analyses

Before lipid extraction, *n*-heptadecanoic acid was added to the reaction mixture as an internal standard. Lipids were extracted from 1 mL of the reaction mixture with 5 mL of chloroform/methanol/1.5% KCl in H_2O (2:2:1, by vol.), according to the procedure of Bligh-Dyer, and concentrated by evaporation under reduced pressure (*31*). The resulting lipids were dissolved in 2 mL of methanol and 3 mL of benzene, and then methylated with 300 μL of 1% trimethylsilyldiazomethane at 28°C for 30 min. After methylation, the resulting fatty-acid methyl esters were concentrated by evaporation under reduced pressure. The resulting fatty-acid methyl esters were analyzed by gas-liquid chromatography (GC) using a Shimadzu (Kyoto, Japan) GC-1700 gas chromatograph equipped with a flame-ionization detector and split-injection system, fitted with a capillary column (SPB-1, 30 m × 0.25 mm I.D., Supelco, Bellefonte, PA, USA). The initial column temperature was 180°C for 30 min, but was subsequently increased to 210°C at a rate of 60°C/min, and then maintained at that temperature for 29.5 min. The injector and detector were operated at 250°C. Helium was used as a carrier gas at a flow rate of 1.4 mL/min. The fatty-acid peaks were identified by comparing retention times to known standards.

Isolation and identification of reaction products

Reaction products were separated by reverse-phase HPLC using a Shimadzu LC-10A system equipped with a Cosmosil column ($5C_{18}$-AR, 20 × 250 mm, Nacalai Tesque, Kyoto, Japan). The mobile phase was acetonitrile-H_2O (8:2, by vol.) at a flow rate of 3.0 ml/min, and the effluent was monitored by ultraviolet detection (205 nm and 233 nm). The methyl esters of purified fatty acids were transformed to the pyrrolidide and trimethylsilyl (TMS) derivatives. Pyrrolidide derivatives were prepared by direct treatment of the purified fatty-acid methyl esters with pyrrolidine-acetic acid (10:1, vol/vol) in screw-cap tubes for 1 h at 115°C, followed by extraction with dichloromethane. The organic extract was washed with water and dried over anhydrous Na_2SO_4, and then the solvent was removed under vacuum in a rotary evaporator. The TMS derivatives were prepared by direct treatment of the purified fatty-acid methyl esters with a mixture of TMS agent (pyridine/hexamethyldisilazane/trimethylchlorosilane, 9:3:1, by vol.) in screw-cap tubes for 30 min at 60°C followed by extraction with chloroform. The chemical structures of purified fatty-acid methyl esters, pyrrolidide derivatives, and TMS derivatives were determined by mass spectroscopy (MS), and the chemical structures of purified free fatty acids were determined by two-dimensional proton nuclear magnetic resonance (^{1}H-NMR) techniques including ^{1}H-^{1}H double–quantum-filtered chemical-shift correlation spectroscopy (DQF-COSY), and two-dimensional nuclear Overhauser effect spectroscopy (NOESY), as described previously (*20*).

Mice

SPF and germ-free BALB/c mice (9 weeks, female) were obtained from CLEA Japan (Tokyo, Japan) and maintained under SPF and germ-free conditions with a sterile diet (CL-2, CLEA Japan), respectively, at the Experimental Animal Facility, Institute of Medical Science, The University of Tokyo. Isolated tissues were immediately frozen by liquid nitrogen and always kept at −80°C prior to fatty-acid analysis. All experiments were approved by the

Animal Care and Use Committee of The University of Tokyo and conducted in accordance with their guidelines.

Fatty-acid analysis in mice

LC-MS/MS-based lipidomics was performed as described (*30*). Briefly, samples were subjected to solid-phase extraction using a Sep-Pak C_{18} cartridge (Waters) with a deuterium-labeled internal standard (AA-d8, LTB_4-d4, 15-HETE-d8, PGE2-d4). Lipidomic analyses were performed using an HPLC system (Waters UPLC) with a linear ion-trap quadrupole mass spectrometer (QTRAP 5500; AB SCIEX) equipped with an Acquity UPLC BEH C_{18} column (1.0 mm × 150 mm × 1.7 μm; Waters). Samples were eluted with a mobile phase consisting of of water/acetate (100:0.1, vol/vol) and acetonitrile/methanol (4:1, vol/vol) (73:27) for 5 min; ramped to 30:70 after 15 min; ramped to 20:80 after 25 min, and held for 8 min; and then ramped to 0:100 after 35 min, and held for 10 min with flow rates of 70 μL/min (0–30 min), 80 μL/min (30-33 min), and 100 μL/min (33-45 min). MS/MS analyses were conducted in negative-ion mode, and fatty-acid metabolites were identified and quantified by multiple-reaction monitoring (MRM). Quantitation was perfomed using calibration curves constructed for each compound, and recoveries were monitored using added deuterated internal standards.

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Figure legends
Figure 1   Gene clusters for polyunsaturated fatty-acid metabolism and GC chromatograms.
A) Gene clusters for fatty-acid metabolic enzymes in *L. plantarum.* B) GC chromatograms of 1) substrate; 2) reaction with CLA-HY; 3) reaction with CLA-HY, CLA-DH, and CLA-DC together with FAD and NADH; and 4) reaction with CLA-HY, CLA-DH, CLA-DC, and CLA-ER together with FAD and NADH. I.S., internal standard.

Figure 2 Polyunsaturated fatty-acid metabolism pathway.

Figure 3 Detection and quantitative analyses of polyunsaturated fatty acid saturation meatabolism intermediates in mice.
Lipids extracted from colon (100 mg), intestine (100 mg), or plasma (100 μL) of specific pathogen–free (SPF) or germ-free (GF) mice were analyzed by LC-MS/MS-based lipidomics as described in Methods. Data are presented as means ± SEM (n = 8). The statistical significance between mean values was determined by unpaired t-test with Welch's correction.
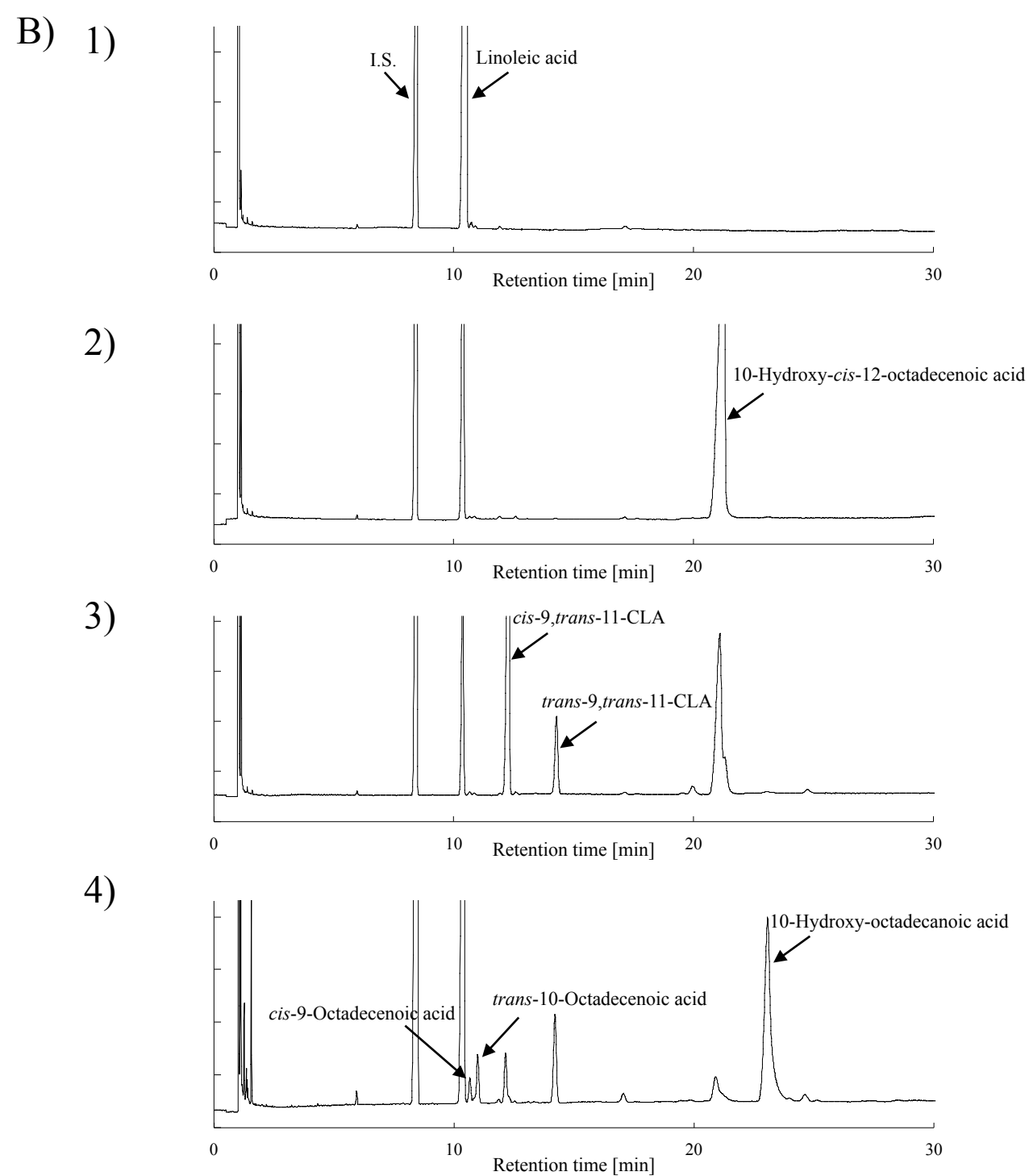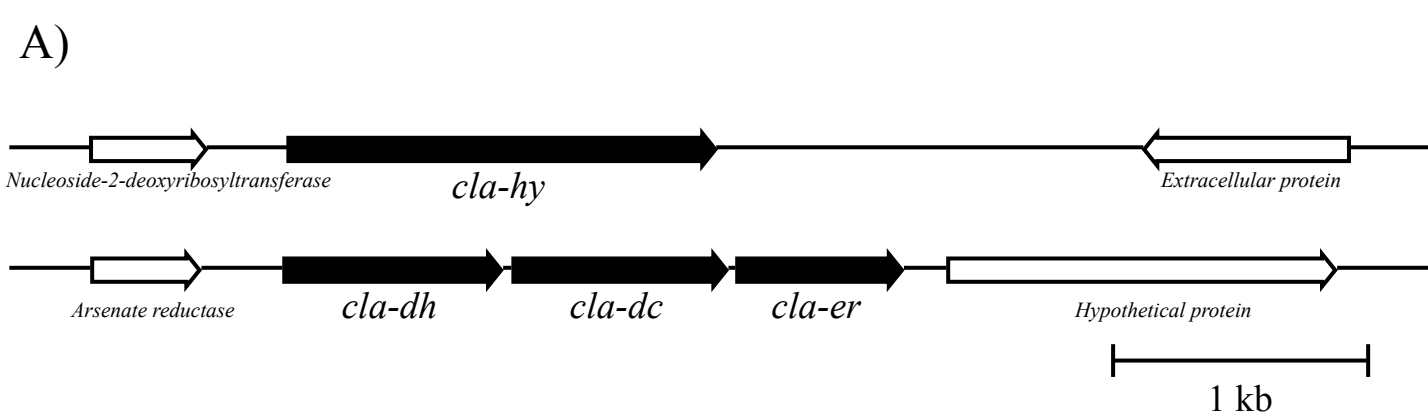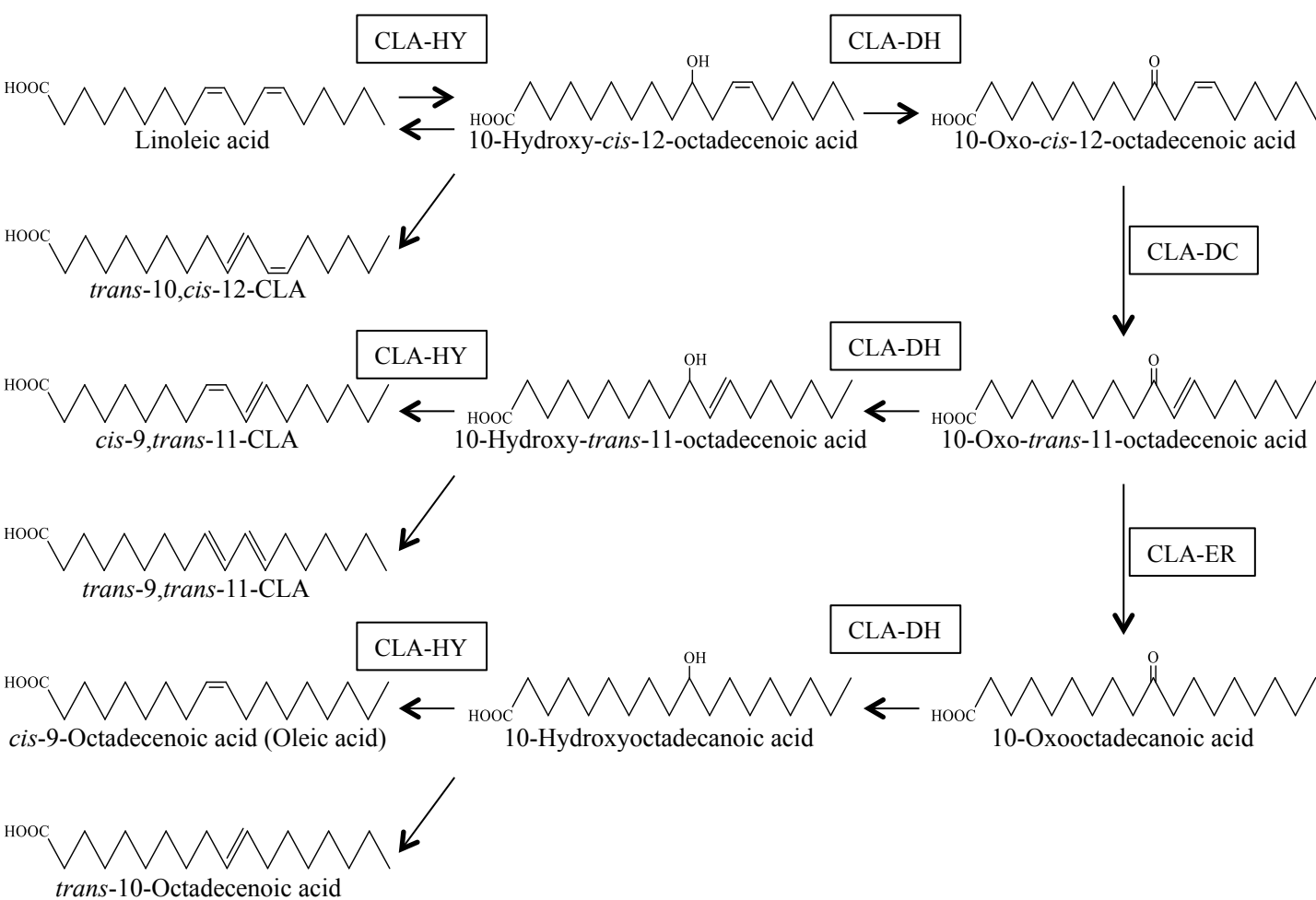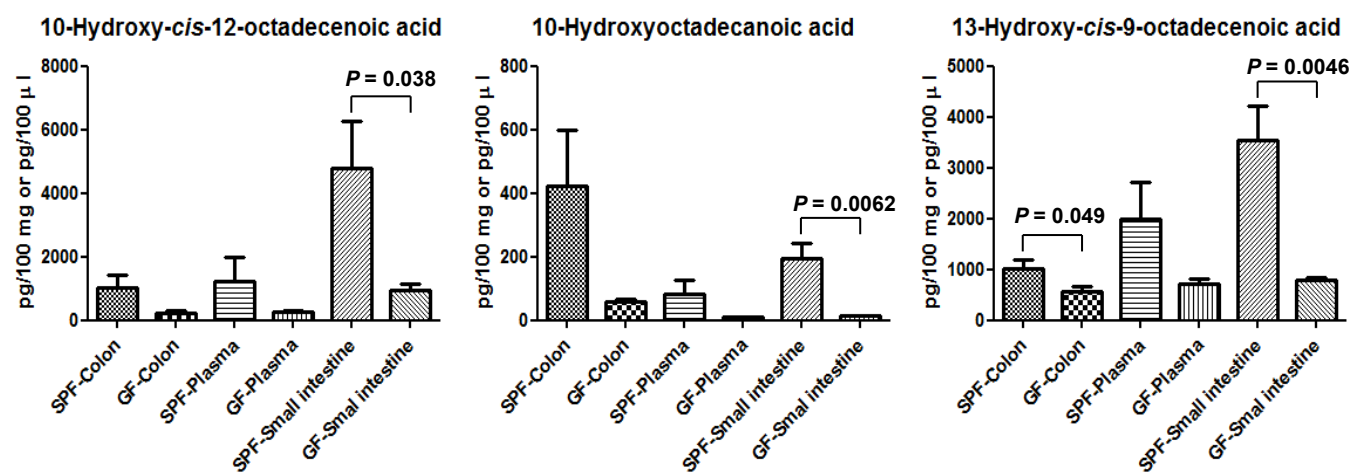
A)

Nucleoside-2-deoxyribosyltransferase   *cla-hy*   Extracellular protein

Arsenate reductase   *cla-dh*   *cla-dc*   *cla-er*   Hypothetical protein

1 kb

B)

1)

I.S.   Linoleic acid

0   10   Retention time [min]   20   30

2)

10-Hydroxy-*cis*-12-octadecenoic acid

0   10   Retention time [min]   20   30

3)

cis-9,*trans*-11-CLA

trans-9,*trans*-11-CLA

0   10   Retention time [min]   20   30

4)

10-Hydroxy-octadecanoic acid

cis-9-Octadecenoic acid   *trans*-10-Octadecenoic acid

0   10   Retention time [min]   20   30

Figure 1

Figure 2

Figure 3

Structural analysis of *trans*-10,*cis*-12-CLA

The structure of *trans*-10,*cis*-12-CLA was confirmed by GC-MS and NMR analyses.   Mass spectra of pyrrolidide derivatives of the isolated fatty-acid methyl ester showed the same mass fragments pattern of pyrrolidide derivatives as commercially available *trans*-10,*cis*-12-CLA methyl ester (Figure S9).   Furthermore, ^{1}H-NMR and DQF-COSY also suggested one partial structure as $-CH_2-CH=CH-CH=CH-CH_2-$ (Figure S9).   Coupling constants between H-10 and H-11 and between H-12 and H-13 were $J = \sim15$ Hz and $J = \sim11$ Hz, respectively, indicated that the $\Delta10$ double bond was in the *trans* configuration and the $\Delta12$ double bond was in the *cis* configuration [^{1}H NMR d_H (CDCl$_3$): 6.29 (1H, *dd*, $J = 11.6, 15.7$ Hz, -CH=C*H*-CH=, H-11), 5.94 (1H, *dd*, $J = 11.0, 11.0$ Hz, =CH-C*H*=CH-, H-12), 5.65 (1H, *dt*, *J*= 15.1, 7.3 Hz, $-CH_2$-C*H*=CH-, H-10), 5.30 (1H, *dt*, $J = 10.8, 7.6$ Hz, -CH=C*H*-CH$_2$-, H-13), 3.67 (3H, *s*, -OC*H*$_3$), 2.30 (2H, *t*, $J = 7.6$ Hz, -C(=O)-C*H*$_2$-CH$_2$-, H-2), 2.15 (2H, *dt*, $J = 6.3, 7.2$ Hz, =CH-C*H*$_2$-CH$_2$-, H-14), 2.10 (2H, *dt*, $J = 7.1, 6.5$ Hz, -CH$_2$-C*H*$_2$-CH=, H-9), 1.26-1.63 (18H, *m*, -CH$_2$-C*H*$_2$-CH$_2$-, H-3, H-4, H-5, H-6, H-7, H-8, H-15, H-16, and -CH$_2$-C*H*$_2$-CH$_3$, H-17), 0.89 (3H, *t*, $J = 7.0$, -CH$_2$-C*H*$_3$, H-18)].

Based on the results of these spectral analyses, the isolated fatty acid was identified as *trans*-10,*cis*-12-octadecadienoic acid (*trans*-10,*cis*-12-CLA) (Figure S9).

Structural analysis of 10-oxo-*cis*-12-octadecenoic acid

The structure of 10-oxo-*cis*-12-octadecenoic acid was confirmed by GC-MS and NMR analyses.   Mass spectra of the isolated fatty-acid methyl ester identified the isolated fatty acid as 10-oxo-12-octadecenoic acid.   ^{1}H-NMR and DQF-COSY also suggested one partial structure as $-CH_2-C(=O)-CH_2-CH=CH-CH_2-$ (Figure S10).   The coupling constant between H-12 and H-13 was $J = 10.8$ Hz, indicated that the $\Delta12$ double bond was in the *cis* configuration [^{1}H NMR d_H (CDCl$_3$): 5.59 (1H, *dt*, $J = 10.8, 7.1$ Hz, -CH=C*H*-CH$_2$-, H-13), 5.53 (1H, *dt*, $J = 10.8, 7.0$ Hz, -CH$_2$-C*H*=CH-, H-12), 3.15 (2H, *d*, $J = 6.8$ Hz, -C(=O)-C*H*$_2$-CH=, H-11), 2.43 (2H, *t*, $J = 7.5$ Hz, -CH$_2$-C*H*$_2$-C(=O)-, H-9), 2.35 (2H, *t*, *J*= 7.5 Hz, HOOC-C*H*$_2$-CH$_2$-, H-2), 2.03 (2H, *dt*, $J = 7.3, 7.1$ Hz, =CH-C*H*$_2$-CH$_2$-, H-14), 1.63 (2H, *tt*, $J = 7.4, 7.4$ Hz, -CH$_2$-C*H*$_2$-CH$_2$-, H-3), 1.56 (2H, *tt*, $J = 7.3, 7.3$ Hz, -CH$_2$-C*H*$_2$-CH$_2$-, H-8), 1.24–1.39 (14H, *m*, -CH$_2$-C*H*$_2$-CH$_2$-, H-4, H-5, H-6, H-7, H-15, H-16, and -CH$_2$-C*H*$_2$-CH$_3$, H-17), 0.89 (3H, *t*, $J = 7.0$ Hz, -CH$_2$-C*H*$_3$, H-18)].

Based on the results of these spectral analyses, the isolated fatty acid was identified as 10-oxo-*cis*-12-octadecenoic acid (Figure S10).

Structural analysis of 10-oxo-*trans*-11-octadecenoic acid

The structure of 10-oxo-*trans*-11-octadecenoic acid was confirmed by GC-MS and NMR analyses.   Mass spectra of the isolated fatty-acid methyl ester identified the isolated fatty acid as 10-oxo-11-octadecenoic acid.   ^{1}H-NMR and DQF-COSY also suggested one partial structure as -CH_2-C(=O)-CH=CH-CH_2- (Figure S11).   The coupling constant between H-11 and H-12 was J = 15.9 Hz, indicated that the Δ11 double bond was in the *trans* configuration [^{1}H NMR d_H ($CDCl_3$): 6.83 (1H, *dt*, J = 15.9, 6.9 Hz, -CH=C*H*-CH_2-, H-12), 6.09 (1H, *d*, J = 15.9 Hz, -C(=O)-C*H*=CH-, H-11), 2.52 (2H, *t*, J = 7.5 Hz, -CH_2-C*H$_2$*-C(=O)-, H-9), 2.34 (2H, *t*, J = 7.5 Hz, HOOC-C*H$_2$*-CH_2-, H-2), 2.21 (2H, *dt*, J = 6.8, 6.8 Hz, =CH-C*H$_2$*-CH_2-, H-13), 1.61 (4H, *m*, -CH_2-C*H$_2$*-CH_2-, H-3, H-8), 1.46 (2H, *tt*, J = 7.4 Hz, -CH_2-C*H$_2$*-CH_2-, H-14), 1.23–1.38 (14H, *m*, -CH_2-C*H$_2$*-CH_2-, H-4, H-5, H-6, H-7, H-15, H-16, and -CH_2-C*H$_2$*-CH_3, H-17), 0.89 (3H, *t*, J = 6.9 Hz, -CH_2-C*H$_3$*, H-18)].

Based on the results of these spectral analyses, the isolated fatty acid was identified as 10-oxo-*trans*-11-octadecenoic acid (Figure S11).

Structural analysis of 10-hydroxy-*trans*-11-octadecenoic acid

The structure of 10-hydroxy-*trans*-11-octadecenoic acid was confirmed by GC-MS and NMR analyses.   Mass spectra of TMS derivatives of the isolated fatty-acid methyl ester identified the isolated fatty acid as 10-hydroxy-11-octadecenoic acid.   ^{1}H-NMR and DQF-COSY also suggested one partial structure as -CH_2-CH(OH)-CH=CH-CH_2- (Figure S12).   The coupling constant between H-11 and H-12 was J = 15.4 Hz, indicated that the Δ11 double bond was in the *trans* configuration [^{1}H NMR d_H ($CDCl_3$): NMR d_H ($CDCl_3$): 5.63 (1H, *dt*, J = 15.4, 6.7 Hz, -CH=C*H*-CH_2-, H-12), 5.44 (1H, *dd*, J = 7.2, 15.4 Hz, -CH(OH)-C*H*=CH-, H-11), 4.12 (1H, *dt*, J = 7.2, 7.1 Hz, -CH_2-C*H*(OH)-CH=, H-10), 2.33 (2H, *t*, J = 7.5 Hz, HOOC-C*H$_2$*-CH_2-, H-2), 2.02 (2H, *dt*, J = 7.0, 7.2 Hz, =CH-C*H$_2$*-CH_2-, H-13), 1.63 (2H, *tt*, J = 7.5, 7.3 Hz, -CH_2-C*H$_2$*-CH_2-, H-3), 1.50 (2H, *m*, -CH_2-C*H$_2$*-CH(OH)-, H-9), 1.22–1.39 (18H, *m*, -CH_2-C*H$_2$*-CH_2-, H-4, H-5, H-6, H-7, H-8, H-14, H-15, H-16, and -CH_2-C*H$_2$*-CH_3, H-17), 0.87 (3H, *t*, J = 13.9 Hz, -CH_2-C*H$_3$*, H-18)].

Based on the results of these spectral analyses, the isolated fatty acid was identified as 10-hydroxy-*trans*-11-octadecenoic acid (Figure S12).

Structural analysis of 10-oxo-octadecanoic acid

The structure of 10-oxo-octadecanoic acid was confirmed by GC-MS analysis. Mass spectra of the isolated fatty-acid methyl ester identified the isolated fatty acid as 10-oxo-octadecanoic acid (Figure S13).

Structural analysis of 10-hydroxy-octadecanoic acid

The structure of 10-hydroxy-octadecanoic acid was confirmed by GC-MS analysis. Mass spectra of TMS derivatives of the isolated fatty-acid methyl ester identified the isolated fatty acid as 10-hydroxy-octadecanoic acid (Figure S14).

Structural analysis of oleic acid and *trans*-10-octadecenoic acid

For isolation of octadecanoic acids, extracted fatty acids were separated by thin-layer chromatography (TLC) using TLC Glass Plates (Silica Gel 60, Merck), using a solvent system of hexane/diethyl ether/acetic acid (60/40/1, by vol.). Fatty acids were detected under ultraviolet light (366 nm) after being sprayed with 0.01% primulin in 80% acetone. The extracted free fatty acids were methylated, and the resultant fatty-acid methyl esters were purified with a Shimadzu LC-VP system fitted with a Silver column Kanto (4.6 × 250 mm, Kanto Chemical Co., Inc., Tokyo, Japan). The mobile phase was hexane-acetonitrile (99.9:0.1, by vol.) at a flow rate of 1.0 ml/min, and the effluent was monitored by ultraviolet detection (200 nm and 206 nm). Normal fatty acids were divided into *trans*-fatty acids, whose retention time was ~12 min, and *cis*-fatty acids, whose retention time was ~29 min. The methyl esters of purified fatty acids were transformed to pyrrolidide derivatives as described previously[20]. The structure of *trans*-10-octadecenoic acid was confirmed by GC-MS analysis. Mass spectra of pyrrolidide derivatives of the purified *trans*–fatty-acid methyl ester identified the purified *trans*–fatty acid as *trans*-10-octadecenoic acid (Figure S15). The structure of *cis*-9-octadecenoic acid was confirmed by GC-MS analysis. Mass spectra of pyrrolidide derivatives of the purified *cis*–fatty-acid methyl ester showed the same mass fragments pattern of pyrrolidide derivatives as commercially available *cis*-9-octadecenoic acid methyl ester. Based on the results of these analyses, the purified fatty acids were identified as *trans*-10- and *cis*-9-octadecenoic acids.
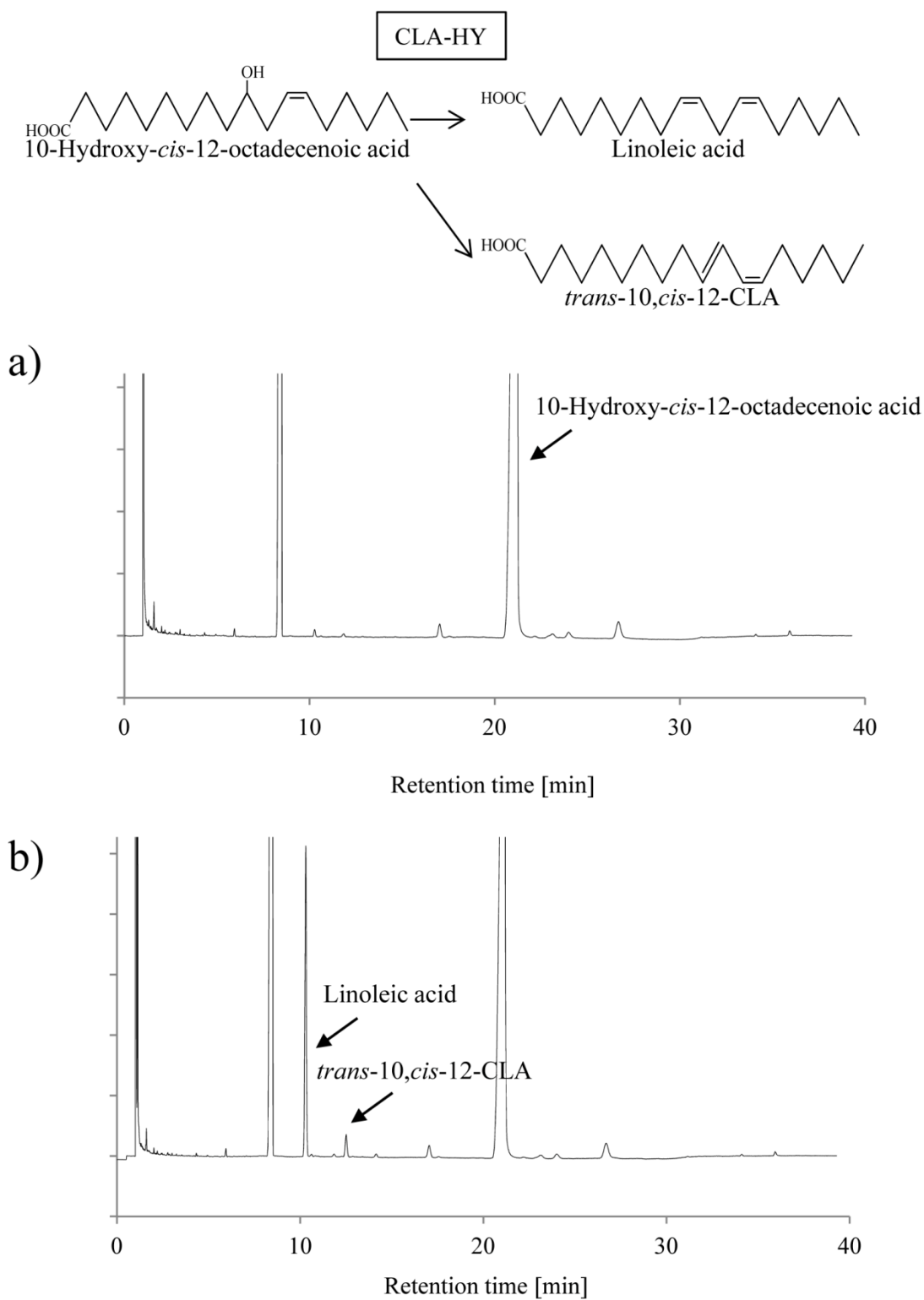
Figure S1 **Pathway for conversion of 10-hydroxy-*cis*-12-octadecenoic acid by CLA-HY, and GC chromatograms of a) before and b) after the reaction.**
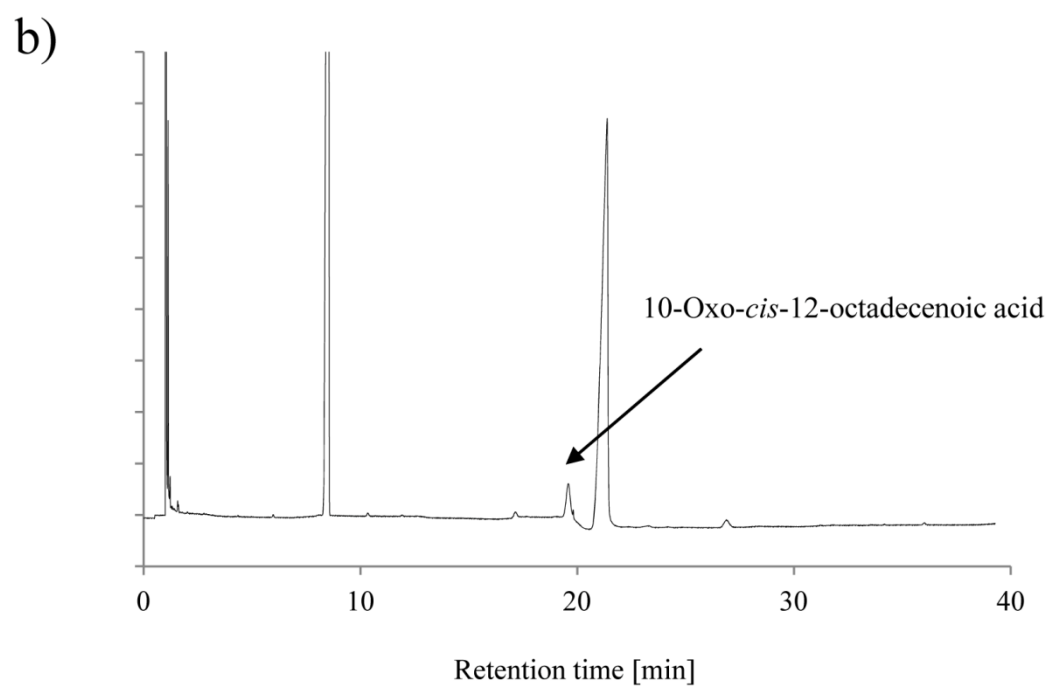
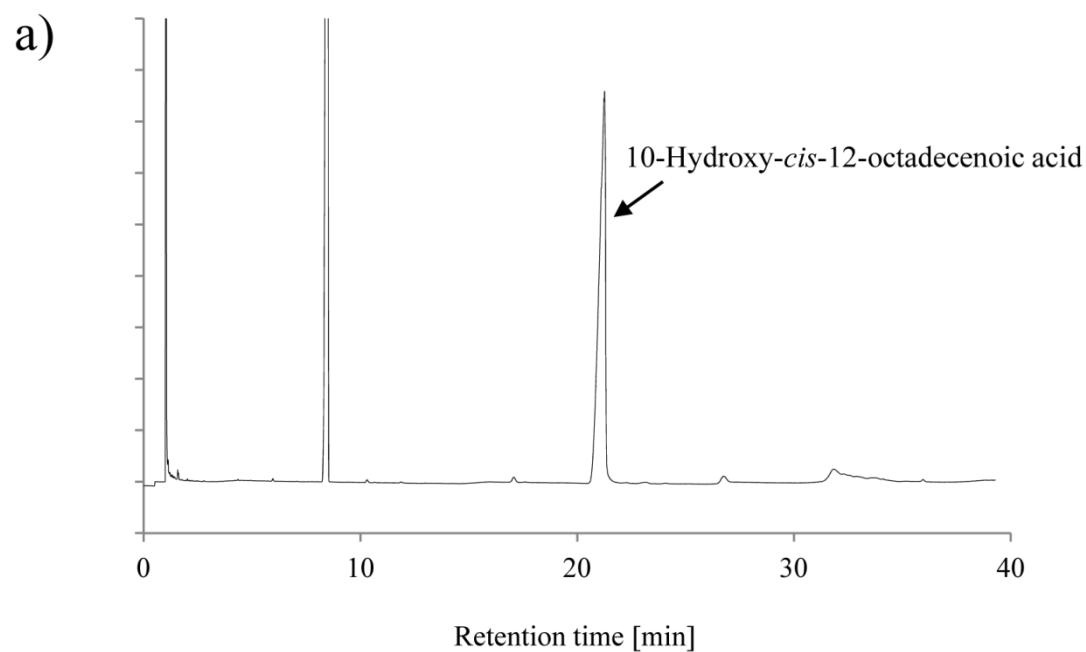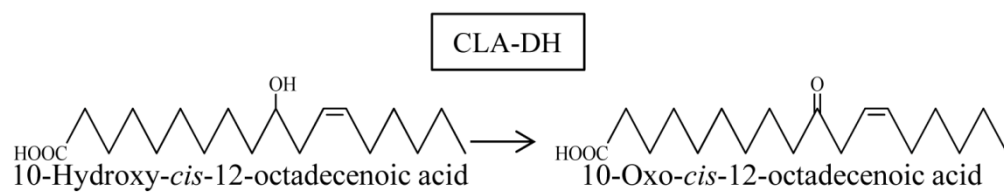**Figure S2 Pathway for conversion of 10-hydroxy-*cis*-12-octadecenoic acid by CLA-DH, and GC chromatograms of a) before and b) after the reaction.**

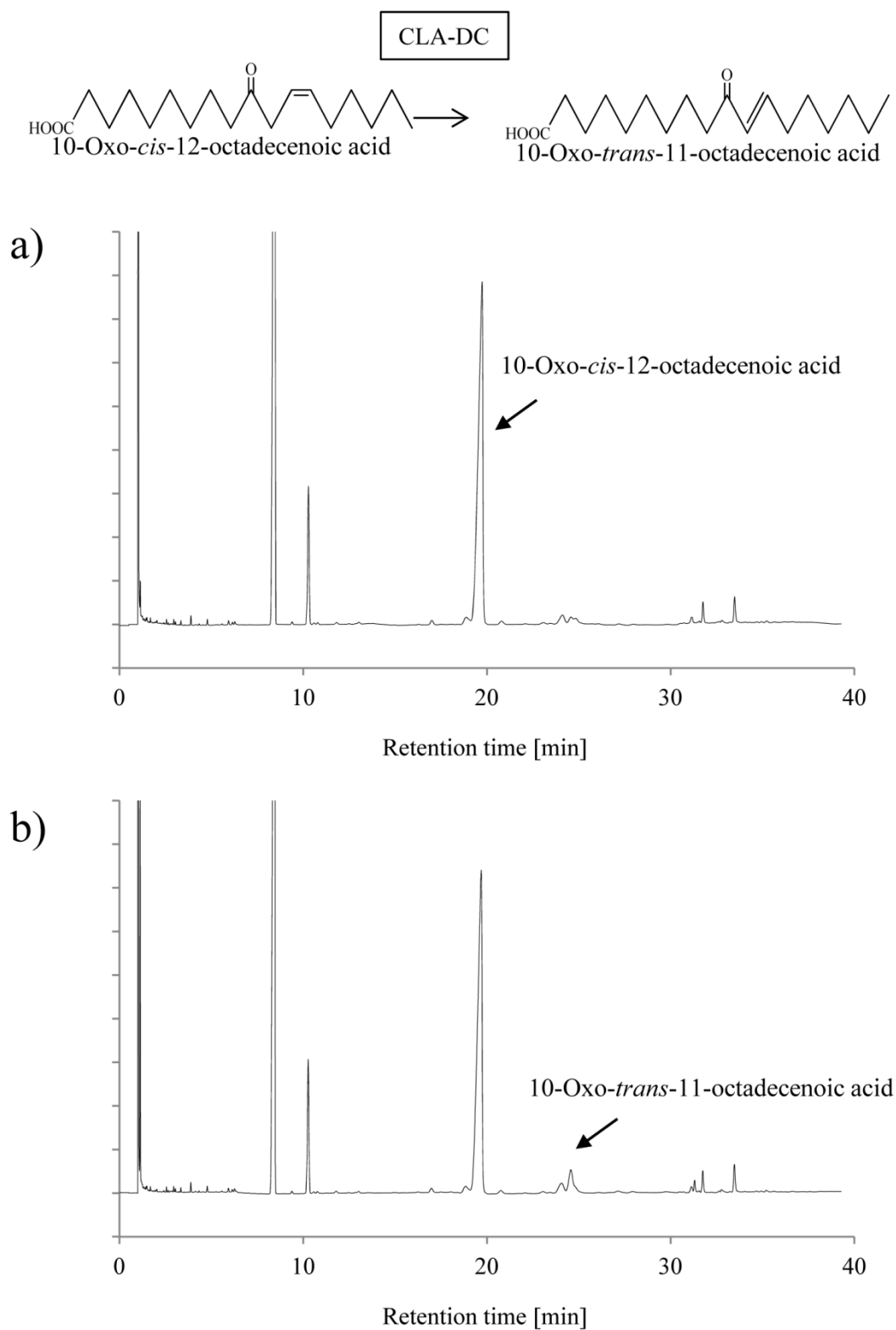**Figure S3** **Pathway for conversion of 10-oxo-*cis*-12-octadecenoic acid by CLA-DC, and GC chromatograms of a) before and b) after the reaction.**
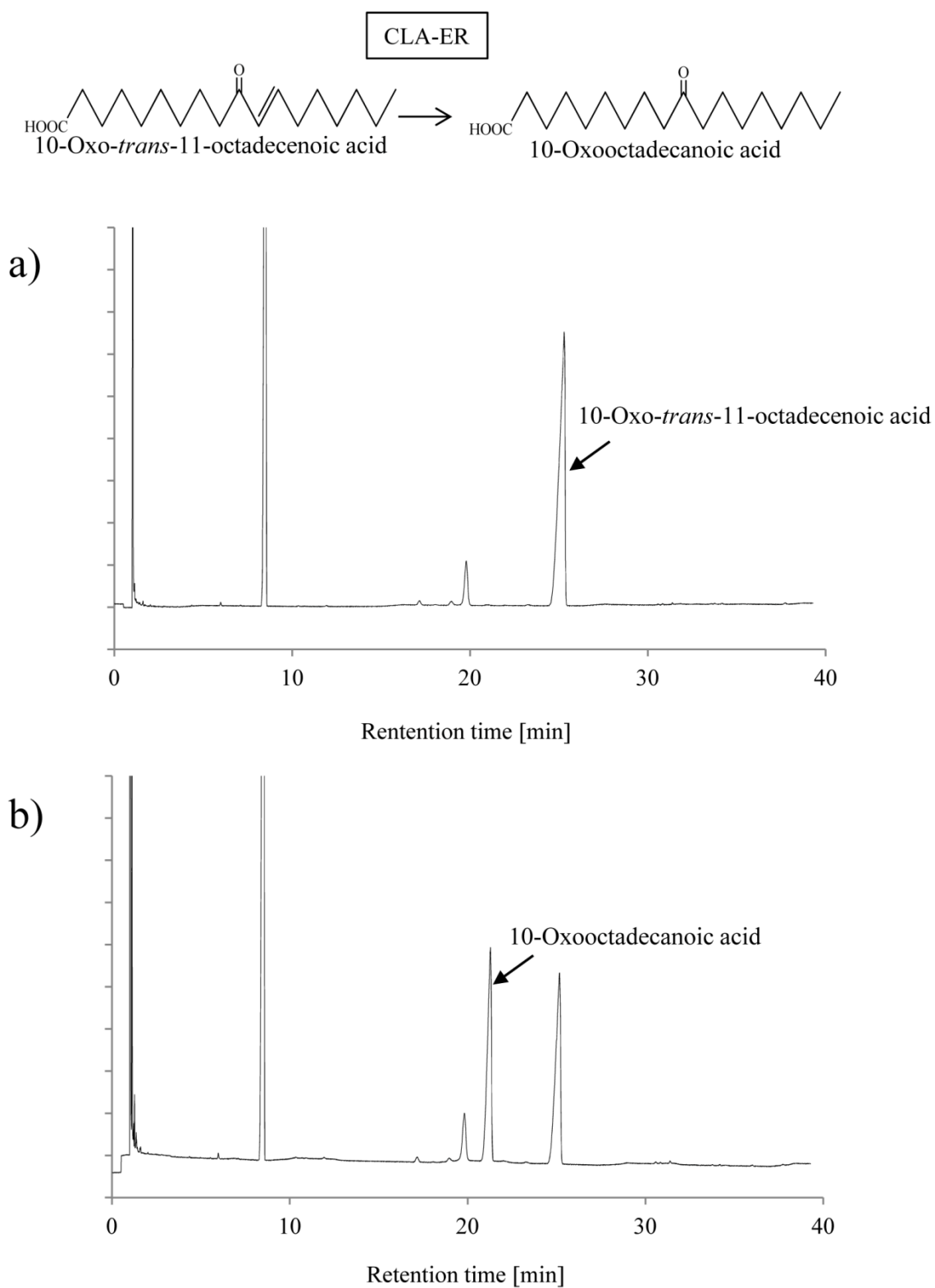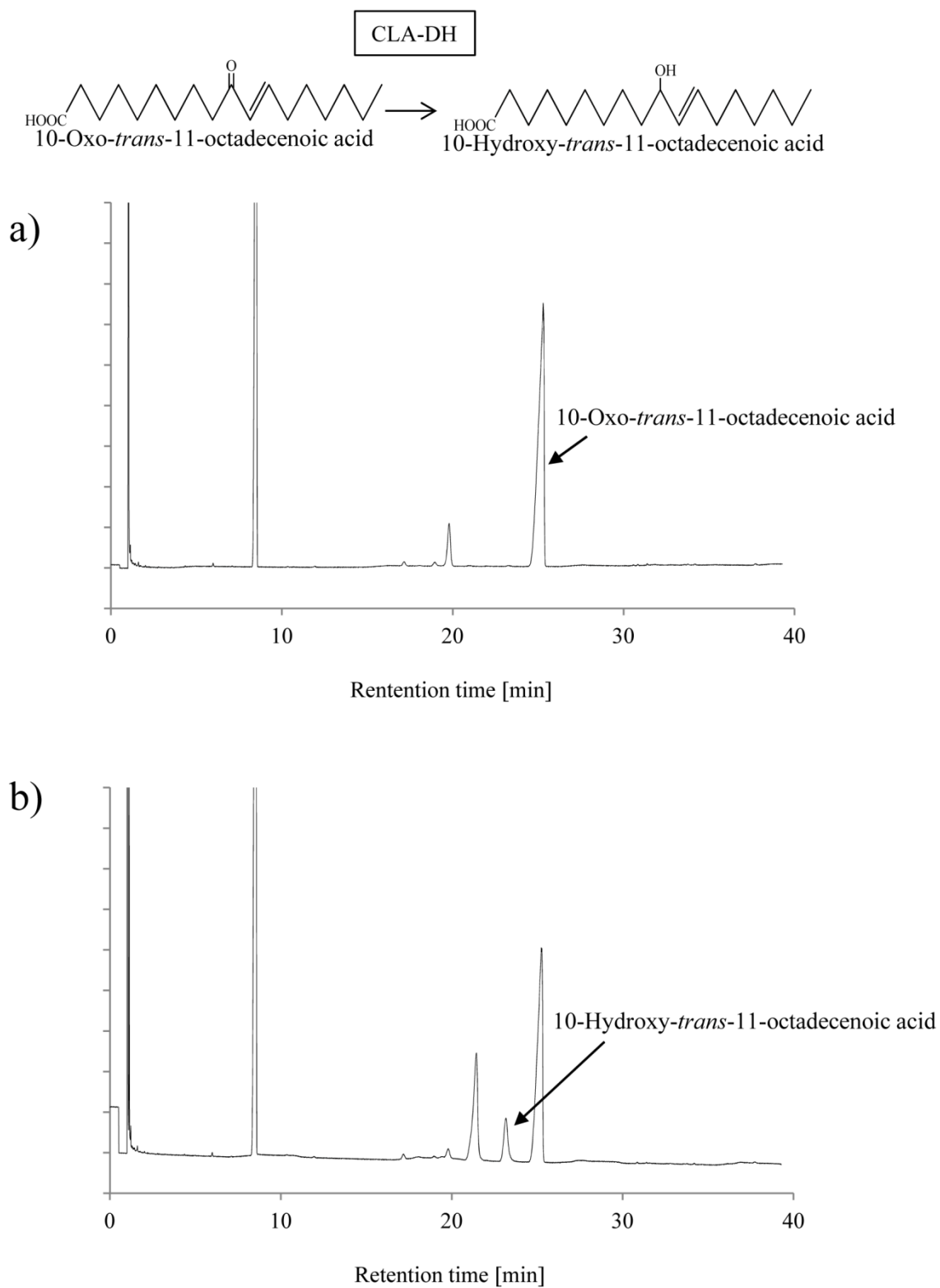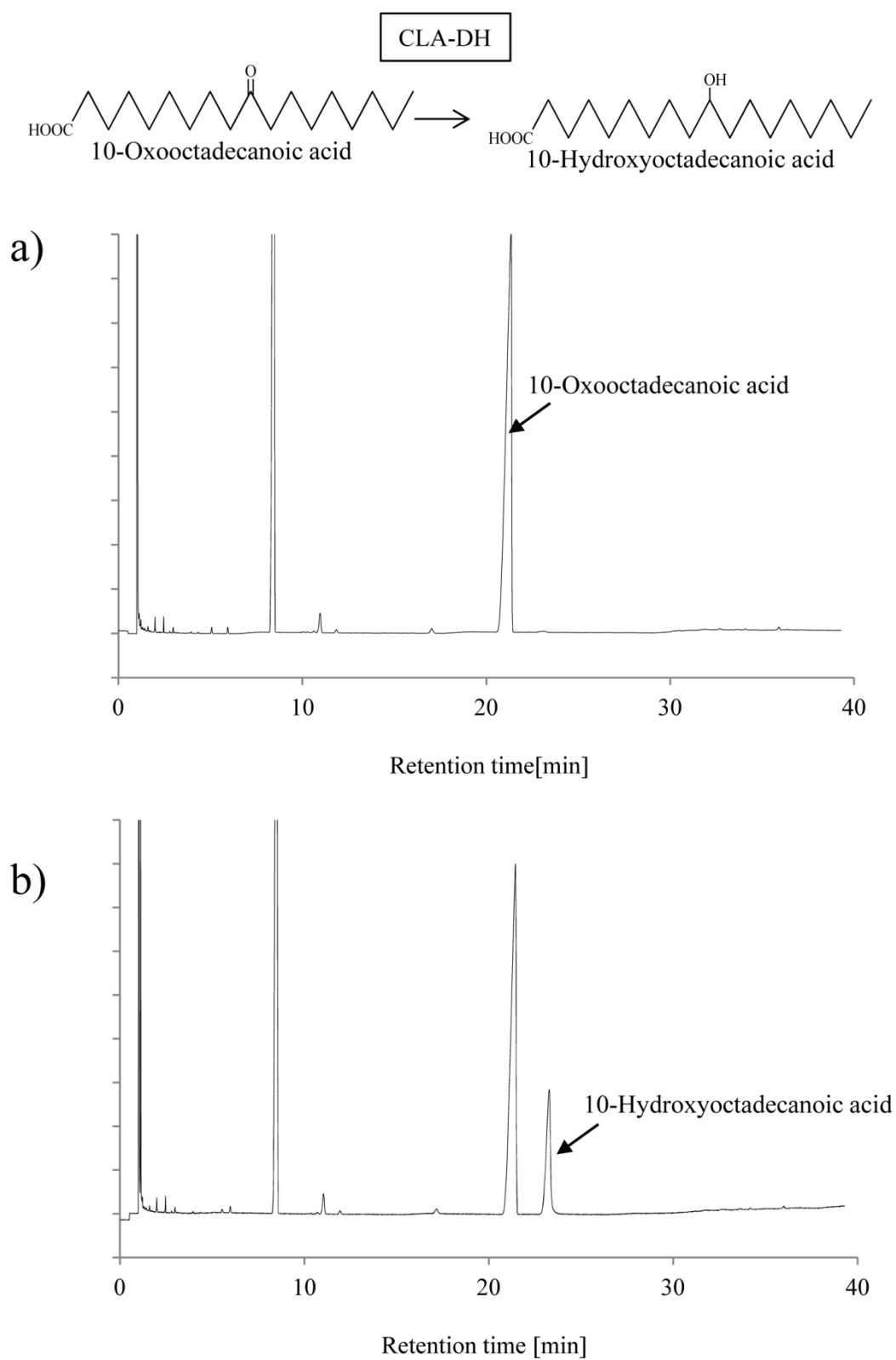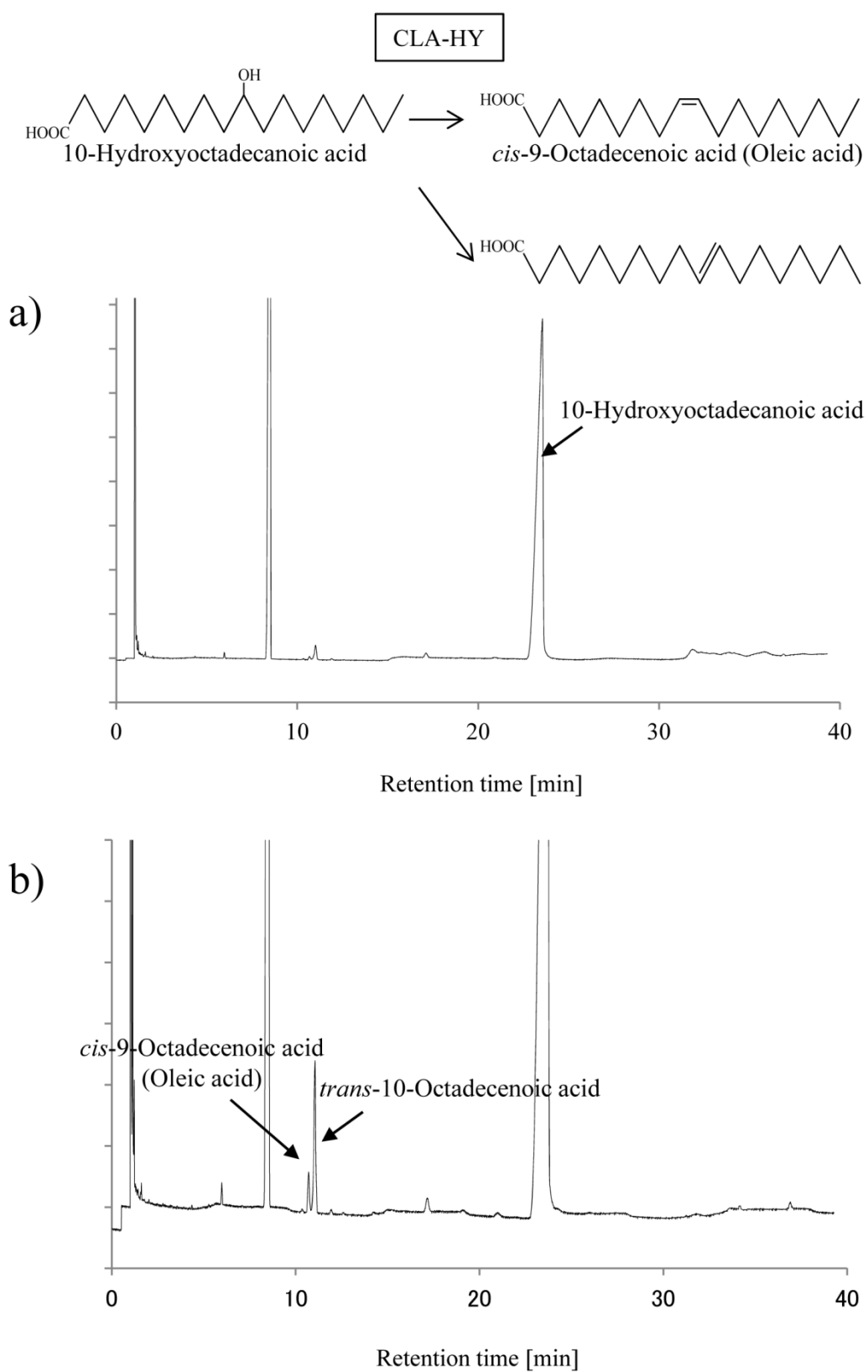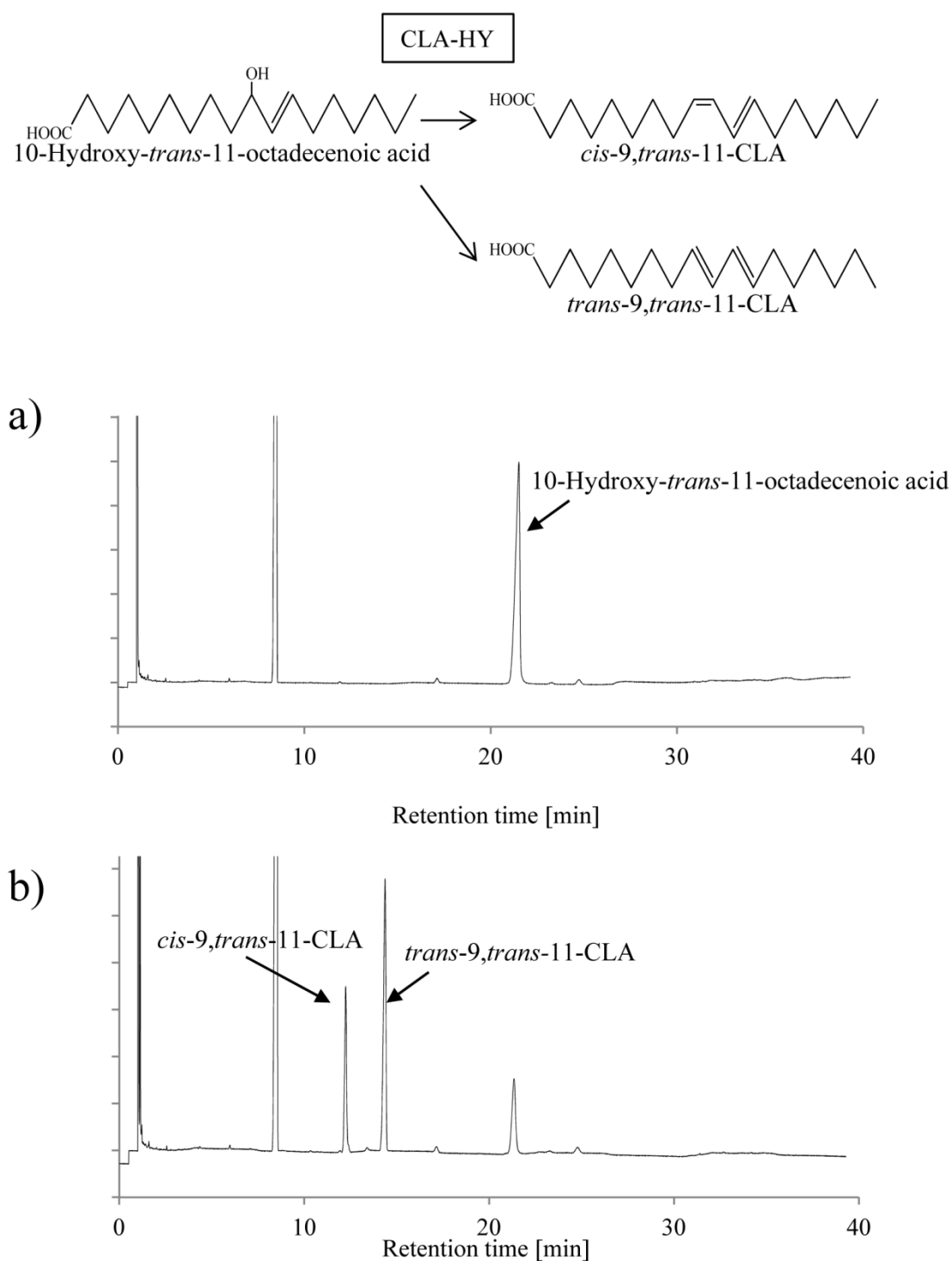
Figure S4   Pathway for conversion of 10-oxo-*trans*-11-octadecenoic acid by CLA-ER, and GC chromatograms of a) before and b) after the reaction.

CLA-DH

10-Oxo-*trans*-11-octadecenoic acid → 10-Hydroxy-*trans*-11-octadecenoic acid

a)

10-Oxo-*trans*-11-octadecenoic acid

Rentention time [min]

b)

10-Hydroxy-*trans*-11-octadecenoic acid

Retention time [min]

Figure S5   Pathway for conversion of 10-oxo-*trans*-11-octadecenoic acid by CLA-DH, and GC chromatograms of a) before and b) after the reaction.

Figure S6　Pathway for conversion of 10-oxooctadecanoic acid by CLA-DH, and GC chromatograms of a) before and b) after the reaction.

Figure S7 **Pathway for conversion of 10-hydroxyoctadecanoic acid by CLA-HY, and GC chromatograms of a) before and b) after the reaction.**

Figure S8   Pathway for conversion of 10-hydroxy-*trans*-11-octadecenoic acid by CLA-HY, and GC chromatograms of a) before and b) after the reaction.
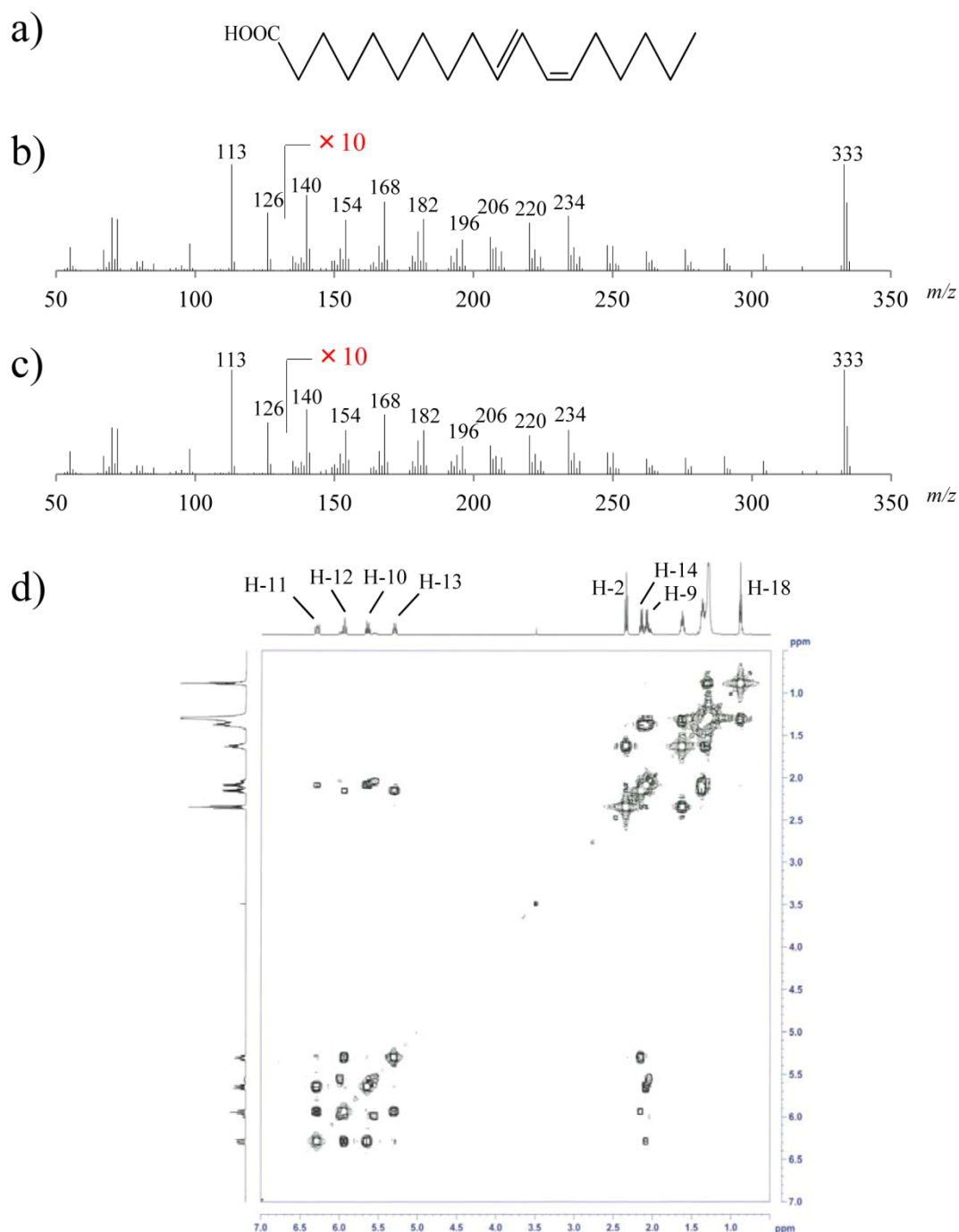
Figure S9   Structural identification of *trans*-10,*cis*-12-octadecadienoic acid.

a) The structure of *trans*-10,*cis*-12-octadecadienoic acid. b) GC-MS analysis of pyrrolidide derivative of commercially available *trans*-10,*cis*-12-octadecadienoic acid methyl ester. c) GC-MS analysis of pyrrolidide derivative of isolated *trans*-10,*cis*-12-octadecadienoic acid methyl ester. d) DQF-COSY of isolated *trans*-10,*cis*-12-octadecadienoic acid.

a)

b)

c)
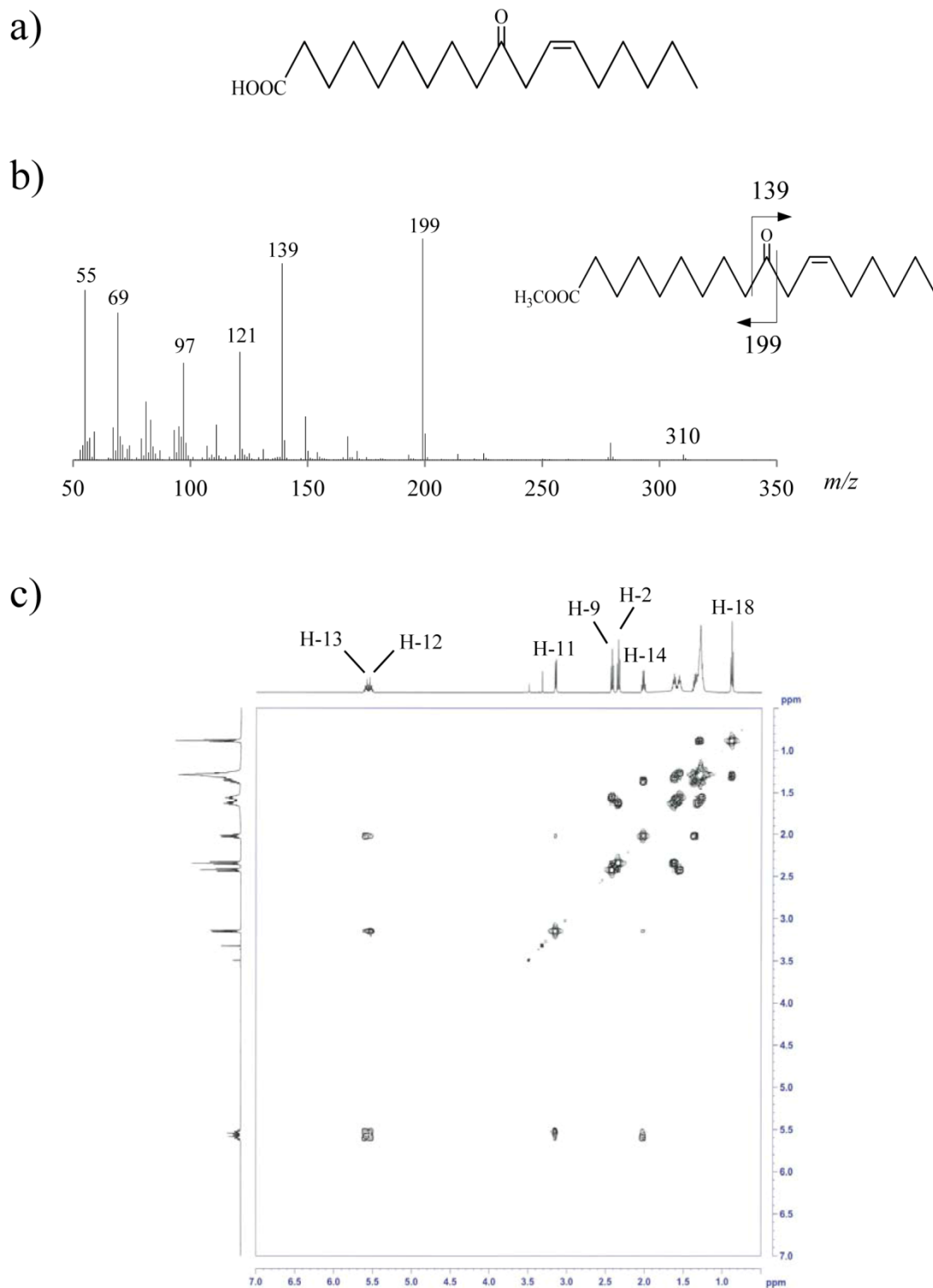
Figure S10 Structural identification of 10-oxo-*cis*-12-octadecenoic acid.
a) The structure of 10-oxo-*cis*-12-octadecenoic acid. b) GC-MS analysis of 10-oxo-*cis*-12-octadecenoic acid methyl ester. c) DQF-COSY of 10-oxo-*cis*-12-octadecenoic acid.
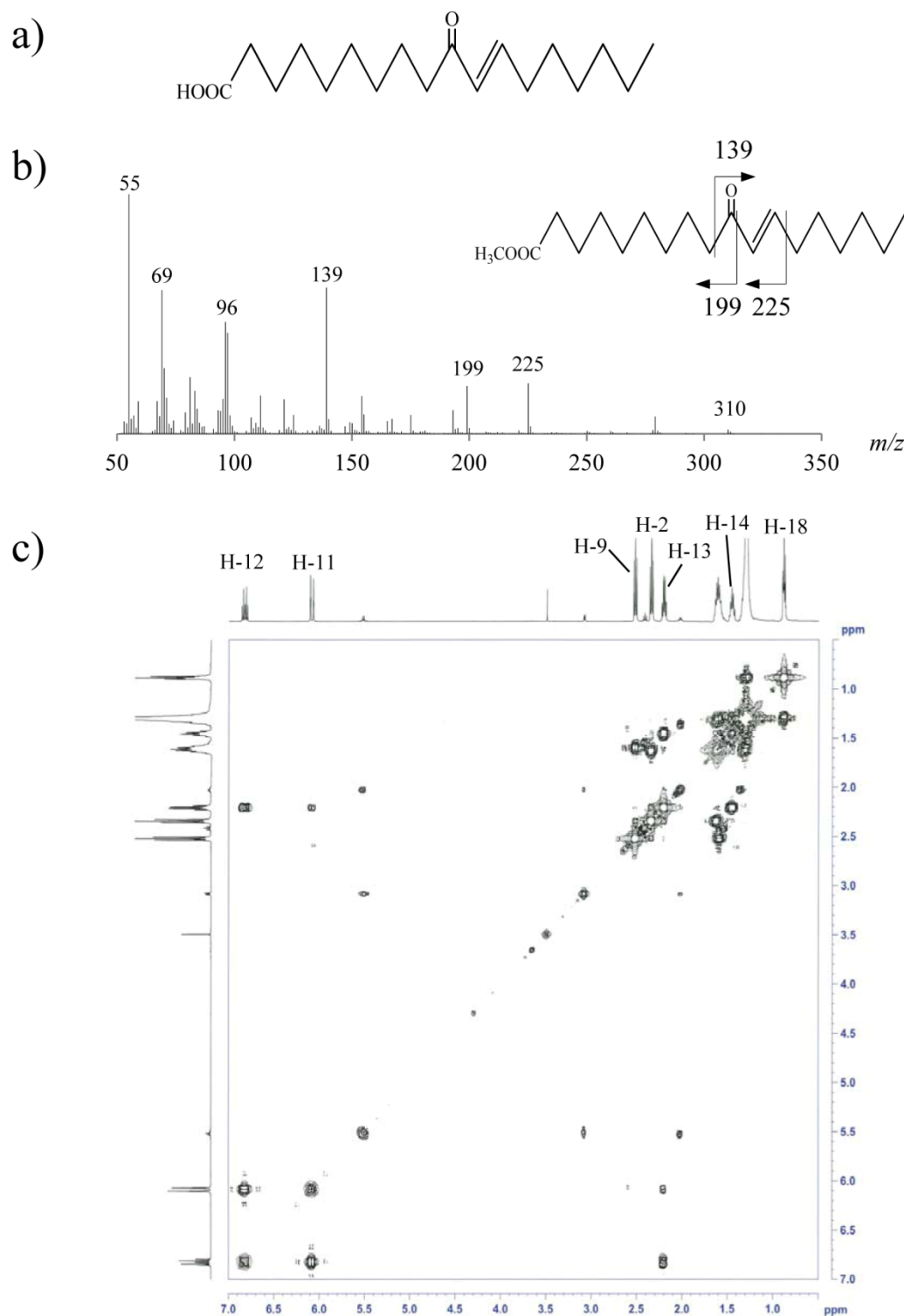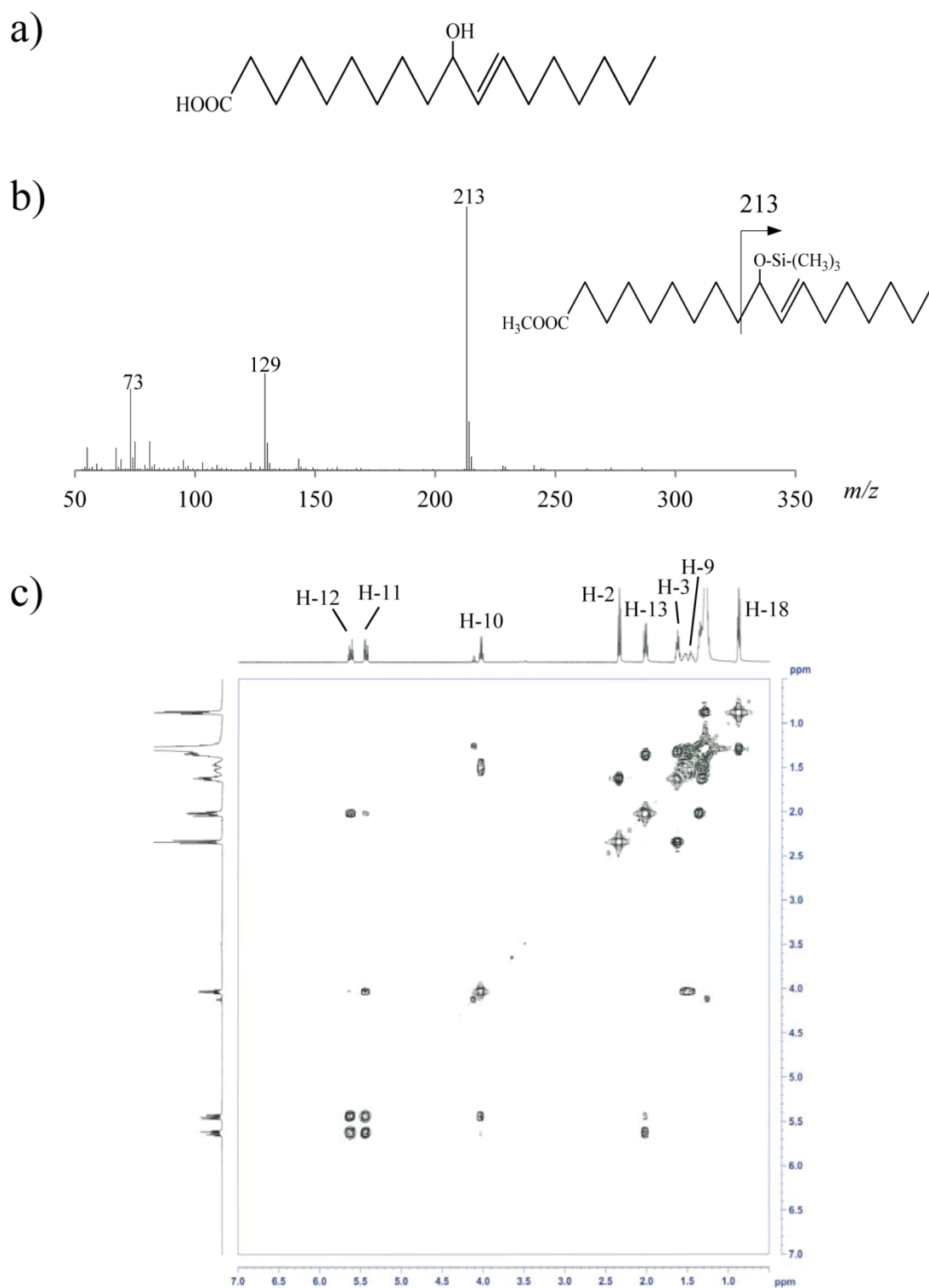
Figure S11 Structural identification of 10-oxo-*trans*-11-octadecenoic acid.
a) The structure of 10-oxo-*trans*-11-octadecenoic acid. b) GC-MS analysis of 10-oxo-*trans*-11-octadecenoic acid methyl ester. c) DQF-COSY of 10-oxo-*trans*-11-octadecenoic acid.

Figure S12   Structural identification of 10-hydroxy-*trans*-11-octadecenoic acid.
a) The structure of 10-hydroxy-*trans*-11-octadecenoic acid. b) GC-MS analysis of TMS derivative of 10-hydroxy-*trans*-11-octadecenoic acid methyl ester. c) DQF-COSY of 10-hydroxy-*trans*-11-octadecenoic acid.
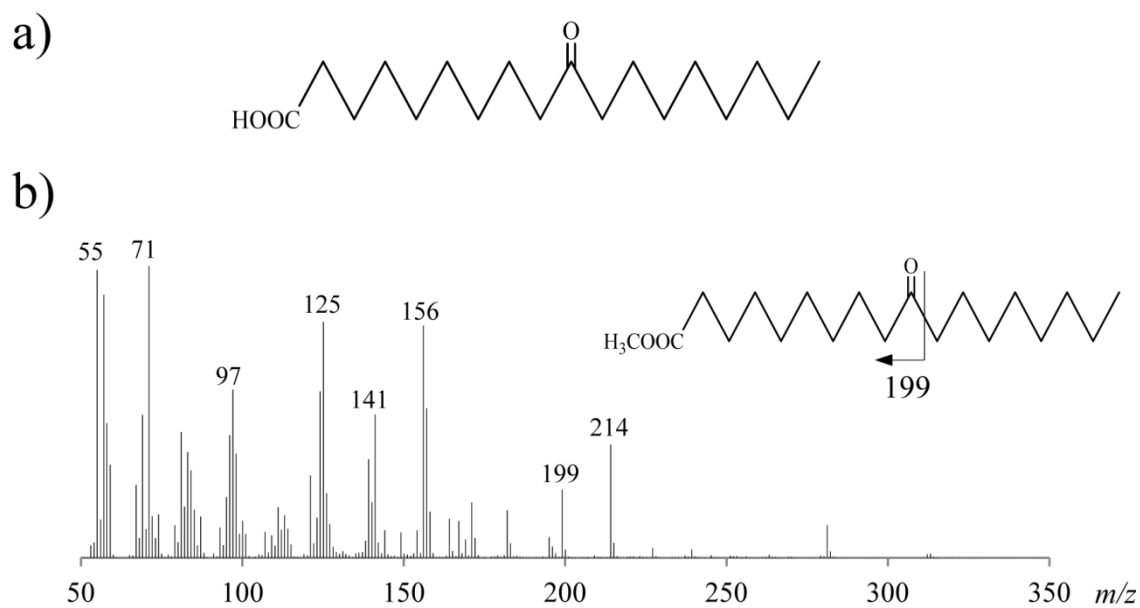
Figure S13   Structural identification of 10-oxooctadecanoic acid.

a) The structure of 10-oxooctadecanoic acid. b) GC-MS analysis of 10-oxooctadecanoic acid methyl ester.
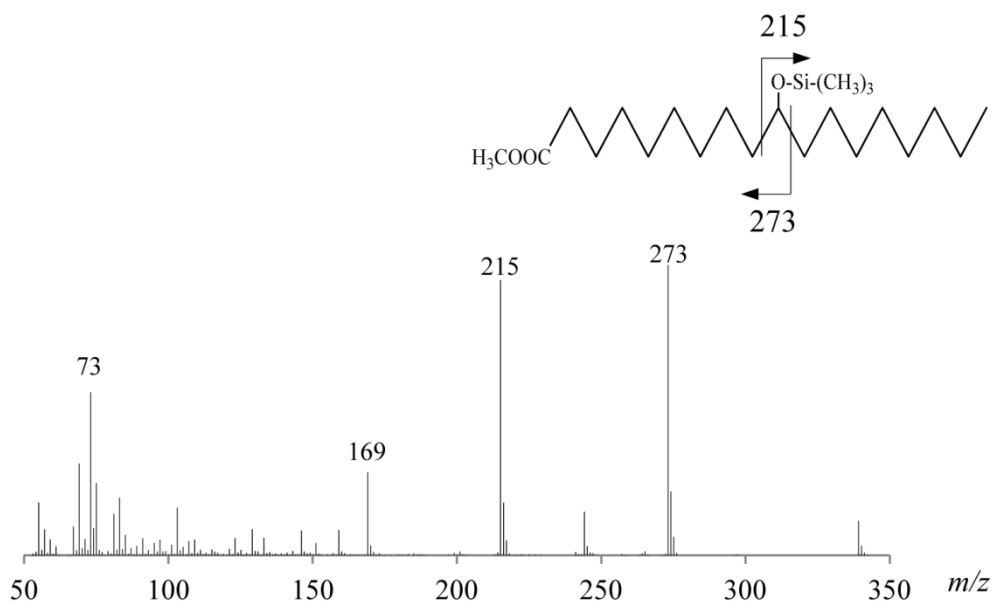
Figure S14   Structural identification of 10-hydroxyoctadecanoic acid.
a) The structure of 10-hydroxyoctadecanoic acid. b) GC-MS analysis of TMS derivative of 10-hydroxyoctadecanoic acid methyl ester.
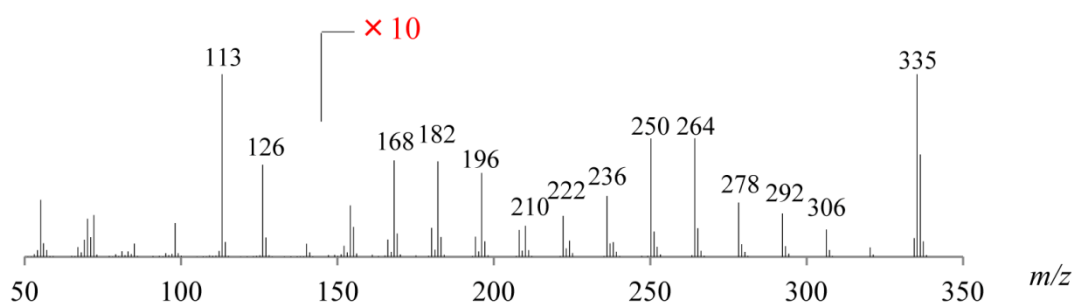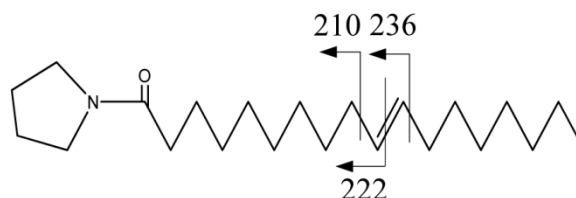
a)

b)

Figure S15   Structural identification of *trans*-10-octadecenoic acid.

a) The structure of *trans*-10-octadecenoic acid. b) GC-MS analysis of pyrrolidide derivative of *trans*-10-octadecenoic acid methyl ester.