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Microbial Synthesis of Linoleate 9*S*-Lipoxygenase derived Plant C18 Oxylipins from C18 Polyunsaturated Fatty Acids

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ABSTRACT: Plant oxylipins, including hydroxy fatty acids, epoxy hydroxy fatty acids, and trihydroxy fatty acids, biosynthesized from C18 polyunsaturated fatty acids (PUFAs), are involved in pathogen-specific defense mechanisms against fungal infections. However, the quantitative biotransformation by plant enzymes has not been reported. A few bacteria produce C18 trihydroxy fatty acids, but the enzymes and pathways related to the biosynthesis of plant oxylipins in bacteria have not been reported. In this study, we first report the biotransformation of C18 PUFAs into plant C18 oxylipins by expressing linoleate 9*S*-lipoxygenase with or without epoxide hydrolase from the proteobacterium *Myxococcus xanthus* in recombinant *Escherichia coli*. Among nine types of plant oxylipins, 12,13-epoxy-14-hydroxy-*cis,cis*-9,15-octadecadienoic acid was identified as a new compound by NMR analysis, and 9,10,11-hydroxy-*cis,cis*-6,12-octadecadienoic acid and 12,13,14-trihydroxy-*cis,cis*-9,15-octadecadienoic were suggested as new compounds by LC-MS/MS analysis. This study shows that bioactive plant oxylipins can be produced by microbial enzymes.

KEYWORDS: *Biotransformation; Epoxy hydroxy fatty acid; Trihydroxy fatty acid; Microbial enzyme; Enzyme catalysis*

INTRODUCTION

Oxylipins, oxygenated unsaturated fatty acids, have important biological roles in various organisms. They are divided into fatty acid aldehydes, divinyl ethers, hydroxy fatty acids (HFAs), keto fatty acids, epoxy hydroxy fatty acids, epoxy fatty acids, polyhydroxy fatty acids, diepoxides, tetrahydrofurandiols, and prostanoids.[1-5] Plant oxylipins are usually biosynthesized from C18 polyunsaturated fatty acids (PUFAs), including linoleic acid, α-linolenic acid, and γ-linolenic acid, as starting materials.

Among oxylipins, HFAs, epoxy hydroxy fatty acids (EHFAs), and trihydroxy fatty acids (THFAs) are extremely important bioactive molecules in plants. The plant C18 oxylipins, 9*S*- and 13*S*-hydroxyoctadecadienoic acids have antifungal activity,[6] 9,10-epoxy-18-hydroxy-octadecanoic acid and 9,10,18-trihydroxy-octadecanoic acid have been described as potential messengers in plant-pathogen interactions,[7] and 9*S*,12*S*,13*S*-trihydroxy-octadecenoic acid shows strong inhibition activity toward the germination and elongation of rice blast fungus in plants, and is also used as an adjuvant for vaccines.[8,9] The 7,8,9-, 9*S*,10*S*,11*R*-, and 12,13,17-trihydroxy-octadecenoic acids in plants inhibit the growth of the plant pathogenic fungi related to wheat powdery mildew, potato late blight, and cucumber botrytis.[10-12] These oxylipins can also be used in the industry as starting materials for resins, waxes, nylons, plastics, and cosmetics because the hydroxyl group gives fatty acids special properties such as higher viscosity and reactivity compared to non-hydroxylated fatty acids.[6,13]

Lipoxygenase (LOX) converts PUFAs having a *Z*,*Z*-1,4-pentadiene structure into different regiospecific hydroperoxy fatty acids (HpFAs), which are readily reduced to HFAs under physiological conditions. In animals, EHFAs are converted from HpFAs by LOX, undergoing bi-functional catalytic reactions,[14,15] whereas, in plants, this is achieved by different enzymes, i.e., peroxygenase or epoxy alcohol synthase.[11,16] THFAs are converted from EHFAs by the

hydrolysis of epoxide rings by epoxide hydrolase (EH) in both plants and animals.[11,17] Especially, human EH divided into EH1 (microsomal EH), EH2 (soluble EH), EH3, EH4, cholesterol hydrolase, leukotriene A_4 hydrolase, and hepoxilin EH.[18] Among them, soluble EH and hepoxilin EH had activity on the conversion of trioxilin (THFA) from hepoxilin (EHFA).[17,19] The study of the biosynthesis of oxylipins in plants is challenging because oxylipins exist in trace amounts *in vivo* and rapidly degrade. Moreover, the quantitative biotransformation by plant enzymes has not been reported.

Plant oxylipins have also been found in corals, fungi, and bacteria.[20] A few types of bacteria have been found to produce plant THFAs,[21,22] however, the biosynthetic pathways and related enzymes have not been reported to date. Recently, we have discovered that arachidonate 11*S*- and 12*S*-LOXs and EH from the proteobacterium *Myxococcus xanthus* DK1622 convert C20 and C22 PUFAs into the EHFAs, hepoxilins, and the THFAs, trioxilins.[23] Arachidonate 11*S*- and 12*S*-LOXs from *M. xanthus*, corresponding linoleate 9*S*- and 13*S*-LOX, respectively, catalyze first a two-step reaction of peroxidation and epoxidation to date among bacterial LOXs. The two-step reactions enable the conversion of C20 and C22 PUFAs into EHFAs. EHFAs are converted into THFAs by *M. xanthus* EH because its activity is similar to human soluble EH (EH2). In this study, we attempted the biotransformation for the production of plant C18 oxylipins (e.g., HFAs, EHFAs, and THFAs) from C18 PUFAs (e.g., linoleic acid, *α*-linolenic acid, and *γ*-linolenic acid) by expressing only linoleate 9*S*-LOX or both linoleate 9*S*-LOX and EH from *M. xanthus* in *Escherichia coli* ER2566 (Figure 1).

MATERIALS AND METHODS

Preparation of Standards. The PUFA standards linoleic acid **(1)**, α-linolenic acid **(6)**,

and γ-linolenic acid **(11)** were purchased from Sigma-Aldrich. The HFA standards 9*S*-hydroxy-*trans*,*cis*-10,12-octadecadienoic acid **(5)** and 9*R*-hydroxy-*trans*,*cis*-10,12-octadecadienoic acid were purchased from Cayman Chemical. The other HFA, EHFA, and THFA standards were prepared as follows: The reactions were performed for 3 h at 35 °C in 50 mM 3-[4-(2-hydroxyethyl)piperazin-1-yl]propane-1-sulfonic acid (HEPPS, pH 8.5) buffer containing 100 mg L^{-1} PUFA as a substrate and 20 g L^{-1} recombinant *E. coli* expressing only linoleate 9*S*-LOX or both linoleate 9*S*-LOX and EH from *M. xanthus*. The reaction solution was extracted with an equal volume of ethyl acetate, the solvent layer was harvested, and the solvent in the layer was removed using a rotary evaporator. The solvent-free extract was applied to a preparative HPLC (Agilent 1260, Santa Clara, CA, USA) equipped with a Nucleosil C18 column (10×250 mm, 5-μm particle size; Phenomenex). The column was eluted at 30 °C at a flow rate of 6 mL min^{-1}, and the product fractions were collected by detecting the absorbance at 202 nm. The adsorbent resin SP825 (Ion Technology, Sungnam, Republic of Korea), which bound specifically hydrophilic compounds like fatty acid derivatives, at 10 g L^{-1} was added to 10 mM of the collected products. The absorption of products to the resin was performed in shaking at 200 rpm at 40 °C during 3 h. The absorbed resin was recovered by filtration through a 45-μm pore-size filter and the purified products were obtained by extraction of the resin with ethyl acetate. To investigation of purity of reaction products, the purified 9-HODE as an example was estimated to be 98% purity by comparing with the standard 9-HODE, which was purchased from Cayman Chemical (See the Supporting Information, Figure S1). All the collected samples were purified to ≥93% purity (See Table 2 and Supporting Information, Figures S2−S4 for product purification), and were used as standards after identification by LC-MS/MS.

Microbial Strains, Plasmids, and Gene Cloning. *M. xanthus* DK1622 (KCCM

44251, Seoul, Republic of Korea) was used as the source of the genomic DNA for cloning linoleate 9*S*-LOX (Genbank accession number ABF88826.1) and EH (Genbank accession number ABF91519.1). *E. coli* ER2566 and pET-28a (Takara) and pACYC duet vectors (Novagen) were used as host cells and expression vectors, respectively (Supporting Information Table S1). The genes encoding linoleate 9*S*-LOX and EH of *M. xanthus* were amplified by polymerase chain reaction (PCR) with the primers as shown in the Supporting Information Table S2. The DNA fragments were cloned into pET-28a or the pACYC duet vector. The plasmid was transformed into *E. coli* ER2566.

Culture Conditions. *M. xanthus* DK1622 was cultivated in a 500-mL flask containing 100 mL of Casitone medium at 30 °C with shaking at 200 rpm for 24 h. Recombinant *E. coli* ER2566 was cultivated in a 2-L flask containing 500 mL Luria–Bertani medium supplemented with 0.1 mM kanamycin for pET-28a vector or chloramphenicol for the pACYC duet vector at 37 °C and shaken at 200 rpm. When the optical density of the bacterial culture at 600 nm reached 0.6, 0.1 mM isopropyl-*β*-D-thiogalactopyranoside (IPTG) was added, and incubation was continued at 16 °C with shaking at 150 rpm for 18 h to induce enzyme expression.

Enzyme Purification. After cultivation, recombinant *E. coli* ER2566 cells were harvested from culture broth by centrifugation at 6000×*g* for 30 min at 4 °C and washed with 0.85% NaCl by centrifugation at 6,000 *g* for 30 min at 4 °C. The washed cells were suspended in 50 mM phosphate buffer (pH 7.0) containing 10 mM imidazole, 300 mM NaCl, and 0.1 mM phenylmethylsulphonyl fluoride as a protease inhibitor and the suspended cells were disrupted by sonication and kept on ice for 30 min. Cell debris was removed by centrifugation at 13000×*g* for 20 min at 4 °C, and the supernatant was applied to an

immobilized metal-ion affinity chromatography cartridge (GE Healthcare) equilibrated with 50 mM phosphate buffer (pH 8.0) containing 300 mM NaCl. The bound protein in the cartridge was eluted by the same buffer with a linear gradient of 10−250 mM imidazole. The active fractions were collected and dialyzed against 50 mM HEPPS (pH 8.5) buffer. After dialysis, the resulting solution was used as the purified enzyme. The purification was performed in a cold chamber at 4 °C using a fast protein liquid chromatography system (Bio-Rad).

Specific Activity and Kinetic Parameters. The specific activity of the purified linoleate 9*S*-LOX from *M. xanthus* for C18 PUFA substrates was determined by measuring the increase in absorbance at 234 nm using a Beckman Coulter DU-700 spectrophotometer after incubation at 35 °C in 50 mM HEPPS (pH 8.5) buffer containing 0.1 mM substrate and 3.0–6.0 μg mL^{-1} enzyme for 2 min. Only the part of each reaction showing a linear correlation between product concentration and time was used, and the enzyme activity was calculated with the extinction coefficient of 25000 M^{-1} cm^{-1} for conjugated fatty acid products. The enzyme activity (U mg^{-1}) was defined as the amount of product concentration (μmol) per amount of protein (mg) per reaction time (min). To determine the kinetic parameters, the reactions were conducted at 35 °C in 50 mM HEPPS (pH 8.5) buffer by varying the concentration of C18 PUFA from 10 to 900 μM for 1 min, and the enzyme activity was determined by measuring the absorbance. The kinetic parameters K_m (mM) and k_{cat} (min^{-1}) were determined by non-linear regression method using a GraphPad software (See the Supporting Information, Figure S5). To calculate k_{cat}, the amount of protein was divided by the total molecular mass.

Biotransformation of C18 PUFAs into Plant C18 Oxylipins. The concentrations of

cells and substrate for the maximal production of 9,10,11-trihydroxy-*cis*-12-octadecenoic acid **(4)** were determined with 3 mM linoleic acid **(1)** by varying the cell concentration from 0.5 to 6 g L^{-1} and with 3 g L^{-1} cell by varying substrate concentration from 0.5 to 3.5 mM, respectively. The cell concentration was measured from the optical density at 600 nm of the cell suspension and converted into the dry cell weight using a linear calibration curve of the optical density at 600 nm *versus* dry cell weight. The reactions for the biotransformation of C18 PUFAs into plant C18 oxylipins by recombinant *E. coli* expressing only LOX or both LOX and EH from *M. xanthus* were performed at 35 °C in 50 mM HEPPS (pH 8.5) buffer containing 1−3 mM substrate and 3−15 g L cells for 60 and 90 min, respectively. The reactions were conducted in 100-mL baffled flasks containing 10 mL reaction solution in an incubator shaken at 200 rpm.

Quantification of the biotransformation products by HPLC analysis. The reaction products were extracted using an equal volume of ethyl acetate, the solvent was then removed with a rotary evaporator, and methanol was added to the dried extract. All fatty acids and their derivatives were quantitatively analyzed using an HPLC system (Agilent) with a UV detector at a wavelength of 202 nm and a reverse-phase Nucleosil C18 column (3.2×150 mm, 5-μm particle size; Phenomenex). The column was eluted at 35 °C with a gradient of solvent A (acetonitrile/water/acetic acid, 50:50:0.1, v/v/v) and solvent B (acetonitrile/acetic acid, 100:0.1, v/v). CP HPLC was run using a Chiralcel OD-H column (2.1 × 150 mm, 5-μm particle size; Daicel) with solvent system of *n*-hexane/2-propanol/acetic acid (98: 2:0.1, v/v/v).

The concentrations of PUFAs, HFAs, EHFAs, and THFAs were calculated by calibrating the peak areas to the concentrations of standards (Supporting Information Table S3 for calibration curves). For an example, the calibration curve of linoleic acid using determination

methods was provided in Supporting Information Figure S6. The concentrations of PUFAs, including linoleic acid **(1)**, α-linolenic acid **(6)**, and *γ*-linolenic acid **(11)**, and the HFA 9*S*-hydroxy-*trans*,*cis*-10,12-octadecadienoic acid **(5)** were determined by commercially available standards. The calibration curves of HFAs, including 12-hydroxy-*cis*,*trans*,*cis*-9,13,15-octadecatrienoic acid **(10)** and 9-hydroxy-*cis*,*trans*,*cis*-6,10,12-octadecatrienoic acid **(15)**, were made after linoleate 9*S*-LOX from *M. xanthus* converted completely **6** and **11** into **10** and **15**, without any byproducts, respectively. The reactions were performed in 50 mM HEPES buffer (pH 7.5) containing 400 mM cysteine as a reducing agent and 1 mg mL^{-1} enzyme at 35 °C by varying the substrate concentration from 0.2 to 0.8 mM with 0.2 mM increase for 90 min. The calibration curves of EHFAs were made after linoleate 9*S*-LOX from *M. xanthus* converted completely PUFAs, including **1**, **6**, and **11**, into HFAs, including **5**, **10**, and **15**, and EHFAs, including 9,10-epoxy-11-hydroxy-*cis*-12-octadecenoic acid **(3)**, 12,13-epoxy-14-hydroxy-*cis*,*cis*-9,15-octadecadienoic acid **(8)**, and 9,10-epoxy-11-hydroxy-*cis*,*cis*-6,12-octadecadienoic acid **(13)**, without other byproducts, respectively. The concentrations of EHFAs were calculated by subtracting the concentrations of HFAs from the concentrations of PUFAs, which were known from the already made calibration curves. The calibration curves of THFAs were made after linoleate 9*S*-LOX from *M. xanthus* converted completely PUFAs, including **1**, **6**, and **11**, into HFAs, including **5**, **10**, and **15**, and THFAs, including 9,10,11-trihydroxy-*cis*-12-octadecadienoic acid **(4)**, 12,13,14-trihydroxy-*cis*,*cis*-9,15-octadecadienoic acid **(9)**, and 9,10,11-trihydroxy-*cis*,*cis*-6,12-octadecadienoic acid **(14)**, without other byproducts, respectively. The concentrations of THFAs were calculated by subtracting the concentrations of HFAs from the concentrations of PUFAs because the concentrations of HFAs and PUFAs were known from the already made calibration curves. The concentrations of HpFAs were the same as those of HFAs. The reactions for EHFAs and THFAs were performed in 50 mM HEPES buffer (pH 7.5) containing 1 mg mL^{-1} enzyme at

35 °C by varying the substrate concentration from 0.2 to 0.8 mM with 0.2 mM increase for 120 min and 150 min, respectively. The purity of all products was estimated by the calibration curves.

LC-MS/MS Analysis for the Identification of Products. LC-MS/MS analysis of nine types of the biotransformation products plant C18 oxylipins, including HFAs, EHFAs, and THFAs, was performed using a Thermo-Finnigan LCQ Deca XP Plus ion trap mass spectrometer (Thermo Scientific). The instrument consisted of an LC pump, an auto sampler, and a photodiode array detector. Ionization of the samples was carried out through electrospray ionization at 15 V fragmentor voltage in negative mode, 46 V capillary voltage in positive ionization mode, 0.01 min average scan time, 0.02 min average time to change polarity, 5kV ion source voltage, nebulizer gas of 30 psi, 275 °C capillary temperature, and 35 % abundant precursor ions at collision energy.

NMR Analysis for the Identification of Product. The structure of compound **8** was identified by recording 1D (^{1}H and ^{13}C) and 2D (COSY, correlation spectroscopy; TOCSY, total correlation spectroscopy; ROESY, rotating frame nuclear over-hauser effect spectroscopy; HSQC, hetero-nuclear single-quantum correlation spectroscopy; HMBC, hetero-nuclear multiple-bond correlation spectroscopy) NMR spectra on a Bruker Avance HD (850 MHz) equipped with TCI cryoprobe (NCIRF, Seoul National University). $CDCl_3$ was used as a solvent and TMS was used as an internal standard. All chemical shifts are quoted in δ (ppm).

RESULTS AND DISCUSSION

Gene Cloning, Expression, and Biochemical Properties of LOX and EH from *M. xanthus*. Linoleate 9*S*-LOX, the same as arachidonate 11*S*-LOX, and EH from *M. xanthus* DK1622, which had been already used in our previous study, were applied in the biotransformation of C18 PUFAs into plant oxylipins. The genes encoding LOX (2,028 bp) and EH (957 bp) from *M. xanthus* DK1622 were cloned and expressed in *E. coli* ER2566. The recombinant LOX and EH, which were expressed from the pACYC duet vector, showed two distinct bands in crude extract, having molecular masses of approximately 76 and 35 kDa, respectively (Supporting Information Figure S7). LOX, which was expressed from the pET-28a vector, was purified as a soluble protein from crude extract through His-Trap affinity chromatography, and showed a single band in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) at a molecular mass of approximately 76 kDa,[23] which is almost consistent with the molecular mass calculated from 675 amino acids with the hexa-histidine tag.

The amino acid sequence of *M. xanthus* LOX exhibited 6%, 25%, and 34% identities with the reported LOXs from *Cyanothece* sp.,[24] *Pseudomonas aeruginosa*,[25] and humans,[26] respectively. Interestingly, *M. xanthus* LOX exhibited the highest identity to human LOX but a low identity to bacterial LOXs. Thus, *M. xanthus* LOX probably converts PUFAs to EHFAs by a two-step reaction in a similar way to human LOX. The amino acid sequence of *M. xanthus* EH showed 21% identity to that of human soluble EH (EH2).[27]

Catalytic Activity of LOX from *M. xanthus*. To identify the reaction products of *M. xanthus* LOX, the purified enzyme was reacted with C18 PUFAs. The total molecular masses of the reaction products obtained from the conversion of linoleic acid (*cis*,*cis*-9,12-octadecadienoic acid) **(1)**, α-linolenic acid (*cis*,*cis*,*cis* -9,12,15-octadecatrienoic acid) **(6)**, and γ-linolenic acid (*cis*,*cis*,*cis*-6,9,12-octadecatrienoic acid) **(11)** are represented by peaks at

mass-to-charge ratios (*m/z*) of 295.3, 293.2, and 293.2, respectively, in the liquid chromatography (LC)-tandem mass spectrometry (MS/MS) spectra. A peak at *m/z* 171.2 for the product obtained from the conversion of **1** resulted from the cleavage of the hydroxyl group at the C9 position (Figure 2A). The peaks at *m/z* 183.1 and 211.0 for the product of **6** resulted from the cleavage of the hydroxyl group at the C12 position (Figure 2B). The peaks at *m/z* 141.1 and 169.0 for the product of **11** resulted from the cleavage of the hydroxyl group at the C9 position (Figure 2C). Based on the LC-MS/MS data, the reaction products were suggested as 9-hydroxy-*trans*,*cis*-10,12-octadecadienoic acid **(5)**, 12-hydroxy-*cis*,*trans*,*cis*-9,13,15-octadecatrienoic acid **(10)**, and 9-hydroxy-*cis*,*trans*,*cis*-6,10,12-octadecatrienoic acid **(15)**, respectively.

The chirality of **5**, which was obtained from the conversion of **1** by *M. xanthus* LOX, was identified as the *S*-form by chiral-phase (CP) high-performance liquid chromatography (HPLC) analysis with the 9*S*- and 9*R*- standards because it showed the same retention time as the 9*S*-standard (Supporting Information Figure S8). The specific activity and catalytic efficiency (k_{cat}/K_m) of LOX from *M. xanthus* were the highest for **1**, followed by **6** and **11** (Table 1). These results indicated that LOX from *M. xanthus* is a linoleate 9*S*-LOX. 9*S*-LOX from *M. xanthus* showed high catalytic efficiency for C20 and C22 PUFAs, including arachidonic acid, EPA and DHA, as arachidonate 11*S*-LOX.[28] The proteobacterium *M. xanthus* had two LOXs, and the other LOX, known as arachidonate 12*S*-LOX,[29] was identified as linoleate 13*S*-LOX. Most of bacterial LOXs were found only several gram-negative bacteria. LOXs from cyanobacteria including *Nostoc* sp.,[30] *Anabaena* sp.,[31] and *Acaryochloris marina*,[32] are linoleate 9*R*-LOXs, whereas LOXs of two strains in proteobacteria, *P. aeruginosa*[33] and *Burkholderia thailandensis*,[34] are linoleate 13*S*-LOX. Therefore, this is the first 9*S*-form of bacterial linoleate LOXs.

Unfortunately, *M. xanthus* linoleate 9*S*-LOX has lower turnover number as 7.2 s^{-1} for

linoleic acid than those of the previously reported bacterial LOXs. Linoleate 13*S*-LOX, which are reported as arachidonic 12S-LOX form *M. xanthus* LOX, showed 9.2 s^{-1} turnover number, showed 1.3-fold higher turnover number[29] and linoleate 13*S*-LOXs from *P. aeruginosa*[35] and *B. tailandensis*[34] belonging to the same proteobacteria showed 2.8- and 13.0-fold higher turnover number than that of *M. xanthus* linoleate 9*S*-LOX. Also, linoleate 9*R*-LOX, other stereochemical LOX from cyanobacterium *A. marina* shown 13.4 s^{-1}, showing 1.9-folds higher.[32] However, this is the first 9*S*-form of bacterial linoleate LOXs although the activity was lower than those of the reported LOXs.

Biotransformation of PUFAs (1, 6, 11) into HFAs (5, 10, 15). The specific productivity of recombinant *E. coli* to produce HFAs using PUFAs during the production of EHFAs was higher than that during the production of THFAs (Table 2). Thus, only the biotransformation activity of PUFAs to HFAs during the production of EHFAs is described in this sections.

Linoleic acid **(1)** was transformed into 9*S*-hydroxy-*trans,cis*-10,12-octadecenoic acid **(5)** *via* 9*S*-hydroperoxy-*trans,cis*-10,12-octadecadienoic acid **(2)** by linoleate 9*S*-LOX from *M. xanthus*, which had been expressed in *E. coli* ER2566, and **2** was readily reduced to **5** without reductant (Figure 1A). The **5** was produced to a concentration of 0.28 mM in the reaction medium from 1 mM linoleic acid with a specific and volumetric productivity of 123 μmol h^{-1} g-cells^{-1} and a 370 μM h^{-1}, respectively (Figure 3A). The isolated yield of **5** reached 28% with a purity of 99% (Table 2). Although the conversion of **1** to **5** by LOXs of plants and fungi has been reported, the compound **5** could be prepared by using the bacterial 9*S*-LOX.

The biosynthesis of 12-hydroxyoctadecatrienoic acid was very rare in nature. Among bacterial LOXs, linoleate 9*R*-LOX from *A. marina* converts α-linolenic acid **(6)** into 12*R*-hydroxy-*cis*,*trans*,*cis*-9,13,15-octadecatrienoic acid as a stereoisomer,[32] whereas *M. xanthus*

linoleate 9*S*-LOX converted **6** into 12*S*-hydroxy-*cis,trans,cis*-9,13,15-octadecatrienoic acid **(10)** (Figure 1B). Recombinant *E. coli* at 9 g L^{-1} produced 0.35 mM **10** from 1 mM **6** *via* 12*S*-hydroperoxy-*cis,trans,cis*-9,13,15-octadecatrienoic acid **(7)** in 60 min (Figure 3B). Recombinant *E. coli* at 15 g L^{-1} converted 1 mM *γ*-linolenic acid **(11)** into 0.60 mM 9*S*-hydroxy-*cis,trans,cis*-6,10,12-octadecatrienoic acid **(15)** *via* 9*S*-hydroperoxy-*cis,trans,cis*-6,10,12-octadecatrienoic acid **(12)** in 45 min (Figure 1C and 3C). CYP443D1 (CYP74 family) from *Nematostella vectensis*, a sea anemone, has been shown to convert **11** into **15**. However, no quantitative data was shown.[36] The yield and specific productivity of the recombinant *E. coli* expressing LOX for the production of HFAs followed the orders **11** > **6** > **1** and **1** > **11** > **6**, respectively (Table 2).

Biotransformation of PUFAs (1, 6, 11) into EHFAs (3, 8, 13). EHFAs were qualitatively identified in mostly plants and marine organisms and were converted from C18 PUFAs by epoxy alcohol synthase, kind of CYP74 family. However, the biotransformation of PUFAs into EHFAs with two-step reaction by bacterial LOX has not been reported to date. Thus, linoleate 9*S*-LOX from *M. xanthus* was used for the production of EHFAs from C18 PUFAs.

9,10-Epoxy-11-hydroxy-*cis*-12-octadecenoic acid **(3)** has been qualitatively identified from the reaction product of linoleic acid (**1**) by epoxy alcohol synthase (CYP74-related enzyme) from the brown algae *Ectocarpus siliculosus*, However, the quantitative production of **3** has not been reported. Thus, the quantitative production of **3** by recombinant *E. coli* expressing *M. xanthus* 9*S*-LOX was attempted. The cells converted 1 mM **1** into 0.72 mM **3** (Figure 4A) *via* 9-hydroperoxy-*trans,cis*-10,12-octadecadienoic acid **(2)** in 45 min with a specific productivity of 321 μmol h^{-1} g-cells^{-1}, a productivity of 960 μM h^{-1}, and a purity of 97% (Figure 3A and Table 2). α-Linolenic acid **(6)** (1 mM) was converted into 0.65 mM 12,13-

epoxy-14-hydroxy-*cis*,*cis*-9,15-octadecadienoic acid **(8)** (Figure 4B) *via* **7** in 60 min by recombinant *E. coli* (Figure 3B). Compound **8** is a new compound, and thus the structure was additionally identified through NMR analysis (Supporting Information Figure S9−S11). γ-Linolenic acid **(11)** (1 mM) was converted into 0.40 mM 9,10-epoxy-11-hydroxy-*cis*,*cis*-6,12-octadecadienoic acid **(13)** (Figure 4C) by recombinant *E. coli via* 9-hydroperoxy-*cis*,*trans*,*cis*-6,10,12-octadecatrienoic acid **(12)** in 45 min (Figure 3C). The CYP74 family from *N. vectensis* also converts **11** into **13**.[36] The yield and specific productivity of recombinant *E. coli* for EHFAs described in Table 2.

Biotransformation of PUFAs (1, 6, 11) into THFAs (4, 9, 14). *P. aeruginosa* converts 35.6 mM linoleic acid into total 13.3 mM of 9,10,13- and 9,12,13-trihydroxy-*cis*-11-octadecenoic acid from after 72 h fermentation with a productivity of 184 μmol h^{-1} and a yield of 37%.[37] *Clavibacter* sp. converts 25.3 mM linoleic acid into 6.1 mM 12,13,17-trihydroxy-trans-9-octadecenoic acid in 140 h fermentation with a productivity of 43 μmol h^{-1} and a yield of 24%.[21,22] However, the enzymes involved in the production of these THFAs were not determined. The bacteria produced 9,10,13-, 9,12,13-, and 12,13,17-THFAs, whereas recombinant *E. coli* expressing linoleate 9*S*-LOX and EH from *M. xanthus* produced 9,10,11- and 12,13,14-THFAs.

The time course reactions for the biotransformation of C18 PUFAs into THFAs were performed using recombinant *E. coli* expressing linoleate 9*S*-LOX and EH from *M. xanthus*. The conversion of THFA from EHFA by EH was confirmed by the reactions of recombinant *E. coli* expressing linoleate 9*S*-LOX with and without EH under the same conditions (Supporting Information Figure S12). Recombinant *E. coli* at 3 g L^{-1} converted 1 mM linoleic acid **(1)** into 0.75 mM 9,10,11-trihydroxy-*cis*-12-octadecadienoic acid **(4)** (Figure 5A) *via* 9-hydroperoxy-*trans*,*cis*-10,12-octadecadienoic acid **(2)** and 9,10-epoxy-11-hydroxy-*cis*-

12-octadecenoic acid **(3)** sequentially in 60 min with a specific productivity of 250 μmol h^{-1} g-cells^{-1} and a purity of 99% (Figure 6A and Table 2). Although **4** has been isolated from *Boehmeria nivea* root and potato leaf,[11,12] this is the first quantitative biotransformation. The concentrations of cells and substrate for the production of **4** were determined to be 3 g L^{-1} cells and 3 mM **1** (Supporting Information Figure S13). Under these conditions, 3 mM **1** was converted into 2.11 mM **4** in 90 min by 3 g L^{-1} recombinant *E. coli* with a specific productivity of 469 μmol h^{-1} g-cells^{-1}, a productivity of 1,410 μM h^{-1}, and a yield of 70% (Figure 7 and Table 2), which were 2.0- and 1.9-fold increases and 5% decrease, compared to those of 1 mM **1**. The productivity and yield of recombinant *E. coli* for 9,10,11-trihydroxy-*cis*-12-octadecadienoic acid **(4)** were 7.7-times and 33% higher than those of *P. aeruginosa* for 9,10,13- and 9,12,13-trihydroxy-*cis*-11-octadecenoic acid, respectively, which showed the previously highest productivity and yield. The recombinant *E. coli* at 9 g L^{-1} converted 1 mM α-linolenic acid **(6)** into 0.63 mM 12,13,14-trihydroxy-*cis*,*cis*-9,15-octadecadienoic acid **(9)** (Figure 5B) *via* 12-hydroperoxy-*cis*,*trans*,*cis*-9,13,15-octadecatrienoic acid **(7)** and 12,13-epoxy-14-hydroxy-*cis*,*cis*-9,15-octadecadienoic acid **(8)** sequentially in 90 min (Figure 6B). Recombinant *E. coli* at 15 g L^{-1} converted 1 mM γ-linolenic acid **(11)** into 0.29 mM 9,10,11-trihydroxy-*cis*,*cis*-6,12-octadecadienoic acid **(14)** (Figure 5C) in 90 min *via* 9-hydroperoxy-*cis,trans,cis*-6,10,12-octadecatrienoic acid **(12)** and 9,10-epoxy-11-hydroxy-*cis*,*cis*-6,12-octadecadienoic acid **(13)** sequentially (Figure 6C and Table 2). The yield and specific productivity of recombinant *E. coli* for THFAs followed the order **1** > **6** > **11**. Compounds **9** and **14** have not been reported to date and, thus, are newly synthesized in this study. However, unfortunately, two of new THFA compounds were not analyzed by NMR because a sufficient amount with high purity was not secured for NMR analysis.

In conclusion, plant C18 oxylipins with antifungal activity have not been commercialized because studies of their quantitative biotransformation and function have little been carried

out. In the present study, *E. coli* expressing linoleate 9*S*-LOX and EH from *M. xanthus* were used to synthesize plant C18 oxylipins, including HFAs, EHFAs, and THFAs, from C18 PUFAs. This is the first 9*S*-form of bacterial linoleate LOXs although the activity was lower than those of the reported LOXs. The biosynthesis of plant EHFAs and THFAs by microbial enzymes has been demonstrated for the first time. Moreover, the productivity and yields of the recombinant *E. coli* expressing enzymes for the production of THFAs were significantly higher than those of other bacteria. Our results will contribute to the industrial production of plant oxylipins from C18 PUFAs.

ASSOCIATED CONTENT

Supporting Information

Tables S1: Strains and plasmids used in this study. Table S2: Primers designed for PCR analysis. Table S3: Calibration curves of all fatty acids and their derivatives in this study. Figure S1: HPLC profile of the standard and purified compounds of 9-HODE. Figure S2: (A) HPLC profiles of products obtained from the conversion of linoleic acid (LA). (B) HPLC profile of purified 9-hydroxy-*trans*,*cis*-10,12-octadecadienoic acid (9-HODE). (C) HPLC profile of purified 9,10-epoxy-11-hydroxy-*cis*-12-octadecenoic acid (9,10-EP-11-HOD). (D) HPLC profile of purified 9,10,11-trihydroxy-*cis*-12-octadecenoic acid (9.10.11-THOD). Figure S3: HPLC profiles of the purified products obtained from the conversion of α-linolenic acid. (A) HPLC profile of products obtained from the conversion of α-linolenic acid (ALA). (B) HPLC profile of purified 12-hydroxy-*cis*,*trans*,*cis*-9,13,15-octadecatrienoic acid (12-HOTrE). (C) HPLC profile of purified 12,13-epoxy-14-hydroxy-*cis*,*cis*-9,15-octadecadienoic acid (12,13-EP-14-HODE). (D) HPLC profile of purified 12,13,14-trihydroxy-*cis*,*cis*-9,15-octadecadienoic acid (12,13,14-THODE). Figure S4: (A) HPLC profiles of products obtained from the conversion of γ-linolenic acid (GLA). (B) HPLC

406 profiles of purified 9-hydroxy-*cis*,*trans*,*cis*-6,10,12-octadecatrienoic acid (9-HOTrEγ). (C)
407 HPLC profiles of purified 9,10-epoxy-11-hydroxy-*cis*,*cis*-6,12-octadecadienoic acid (9,10-
408 EP-11-HODE). (D) HPLC profiles of purified 9,10,11-trihydroxy-*cis*,*cis*-6,12-
409 octadecadienoic acid (9,10,11-THODE). Figure S5: Determination for kinetics parameter of
410 *M. xanthus* LA 9S-LOX by non-linear regression method using a GraphPad software. (A)
411 LA (B) ALA (C) GLA. Figure S6: Determination for the concentration of LA by HPLC. (A)
412 HPLC profile of LA standard at each concentration ranging from 0.2 to 0.8 mM. (B)
413 Calibration curve of LA standard between peak area and concentration. Figure S7: SDS-
414 PAGE analysis of recombinant proteins, linoleate 9*S*-LOX and EH, expressed in
415 recombinant *E. coli* ER2566. Figure S8: CP HPLC analysis of the reaction product obtained
416 from the conversion of linoleic acid. Figure S9: The structure of 12,13-EP-14-HODE. (A)
417 Chemical structure of (*Z*)-11-((2*S*,3*R*)-3-((*R*,*Z*)-1-hydroxypent-2-en-1-yl)oxiran-2-yl)undec-
418 9-enoic acid. (B) Chemical structure of (*Z*)-11-((2*S*,3*R*)-3-((*S*,*Z*)-1-hydroxypent-2-en-1-
419 yl)oxiran-2-yl)undec-9-enoic acid. Figure S10: NMR data of 12,13-EP-14-HODE. (**A**) ^{1}H
420 NMR peak of compound **8**. (B) ^{13}C NMR peak of compound **8**. Figure S11: 2D NMR data
421 of 12,13-EP-14-HODE. (A) COSY spectrum of compound **8**. (B) TOCSY spectrum of
422 compound **8**. (C) ROESY spectrum of compound **8**. (D) HSQC spectrum of compound **8**.
423 (E) HMBC spectrum of compound **8**. Figure S12: HPLC profiles of the reaction of PUFA
424 (linoleic acid) into THFA (9,10,11-THOD, 9,10,11-trihydroxy-*cis*-12-octadecenoic acid) by
425 *E. coli* expressing 9S-LOX with and without EH. Figure S13: Determination of the
426 concentrations of cells and substrate for the biotransformation of 9,10,11-trihydroxy-*cis*-12-
427 octadecenoic acid from linoleic acid by recombinant *E. coli* expressing linoleate 9*S*-LOX
428 and EH from *M. xanthus*. (A) Determination of cell concentration. (B) Determination of
429 substrate concentration.
430

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Notes

The authors declare no competing financial interest.

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544

Figure captions

Figure 1. Biotransformation pathways of C18 PUFAs into plant C18 oxylipins. (A) Linoleic acid **(1)** is converted into 9-hydroperoxy-*trans,cis*-10,12-octadecadienoic acid **(2)** by linoleate 9*S*-LOX, which is sequentially converted into 9,10-epoxy-11-hydroxy-*cis*-12-octadecenoic acid **(3)** and 9,10,11-trihydroxy-*cis*-12-octadecenoic acid **(4)** by linoleate 9*S*-LOX and EH. Compound **2** is transformed into 9-hydroxy-*trans*,*cis*-10,12-octadecadienoic acid **(5)** *via* auto reduction. (B) *α*-Linolenic acid **(6)** is converted into 12-hydroperoxy-*cis,trans,cis*-9,13,15-octadecatrienoic acid **(7)** by linoleate 9*S*-LOX, which is sequentially converted into 12,13-epoxy-14-hydroxy-*cis*,*cis*-9,15-octadecadienoic acid **(8)** and 12,13,14-trihydroxy-*cis*,*cis*-9,15-octadecadienoic acid **(9)** by linoleate 9*S*-LOX and EH, respectively. Compound **7** is transformed into 12-hydroxy-*cis*,*trans*,*cis*-9,13,15-octadecatrienoic acid **(10)** *via* auto reduction. (C) *γ*-Linolenic acid **(11)** is converted into 9-hydroperoxy-*cis*,*trans*,*cis*-6,10,12-octadecatrienoic acid **(12)** by linoleate 9*S*-LOX, which is sequentially converted into 9,10-epoxy-11-hydroxy-*cis*,*cis*-6,12-octadecadienoic acid **(13)** and 9,10,11-trihydroxy-*cis*,*cis*-6,12-octadecadienoic acid **(14)** by linoleate 9*S*-LOX and EH. Compound 12 is transformed into 9-hydroxy-*cis*,*trans*,*cis*-6,10,12-octadecatrienoic acid **(15)** *via* auto reduction. Compounds **8**, **9**, and **14** are newly discovered in this study.

Figure 2. LC-MS/MS analysis of the HFA products obtained from the conversion of C18 PUFAs. The reactions were carried out using recombinant *E. coli* ER2566 containing linoleate 9*S*-LOX from *M. xanthus*. (A) LC-MS/MS fragments of the HFA obtained from the conversion of linoleic acid **(1)**. The HFA was identified as 9-hydroxy-*trans,cis*-10,12-octadecadienoic acid **(5)**. (B) LC-MS/MS fragments of the HFA obtained from the conversion of *α*-linolenic acid **(6)**. The HFA was identified as 12-hydroxy-*cis,trans,cis*-

9,13,15-octadecatrienoic acid **(10)**. (C) LC-MS/MS fragments of the HFA obtained from the conversion of *γ*-linolenic acid **(11)**. The HFA was identified as 9-hydroxy-*cis,trans,cis*-6,10,12-octadecatrienoic acid **(15)**. The asterisks (*) and numbers underlined with red indicate the molecular masses of key fragments and total molecular mass of the compound, respectively, used for the identification of the structure.

Figure 3. Time-course reactions for the biotransformation of C18 PUFAs into EHFAs by recombinant *E. coli* ER2566:pET28a-MXLOX. (A) Biotransformation of linoleic acid **(1)** into 9,10-epoxy-11-hydroxy-*cis*-12-octadecenoic acid **(3)**. (B) Biotransformation of *α*-linolenic acid **(6)** into 12,13-epoxy-14-hydroxy-*cis*,*cis*-9,15-octadecadienoic acid **(8)**. (C) Biotransformation of *γ*-linolenic acid **(11)** into 9,10-epoxy-11-hydroxy-*cis*,*cis*-6,12-octadecadienoic acid **(13)**. The biotransformation was performed in 50 mM HEPPS buffer (pH 8.5) containing 3−15 g L^{-1} cells and 1 mM PUFA at 35 °C for 60 min. The data represent the mean of three experiments, and the error bars represent the standard deviations. The symbols indicate the concentrations of PUFAs (**1**, **6**, and **11**) (●), HpFAs (**2**, **7**, and **12**) (■), HFAs (**5**, **10**, and **15**) (▲), and EHFAs (**3**, **8**, and **13**) (♦).

Figure 4. LC-MS/MS analysis of EHFA products obtained from the conversion of C18 PUFAs. The reactions were carried out using recombinant *E. coli* ER2566 containing linoleate 9*S*-LOX from *M. xanthus*. (A) LC-MS/MS fragments of the EHFA obtained from the conversion of linoleic acid **(1)**. The EHFA was identified as 9,10-epoxy-11-hydroxy-*cis*-12-octadecenoic acid **(3)**. (B) LC-MS/MS fragments of the epoxy hydroxy fatty acid obtained from the conversion of *α*-linolenic acid **(6)**. The EHFA was identified as 12,13-epoxy-14-hydroxy-*cis,cis*-9,15-octadecadienoic acid **(10)**. (C) LC-MS/MS fragments of the EHFA obtained from the conversion of *γ*-linolenic acid **(11)**. The EHFA was identified as 9,10-

epoxy-11-hydroxy-*cis,cis*-6,12-octadecadienoic acid **(15)**. The asterisks (*) and numbers underlined in red indicate the molecular masses of key fragments and the total molecular mass of the compound, respectively, used for structure identification.

Figure 5. LC-MS/MS analysis of THFA products obtained from the conversion of C18 PUFAs. The reactions were carried out using recombinant *E. coli* ER2566 containing linoleate 9*S*-LOX and EH from *M. xanthus*. (A) LC-MS/MS fragments of THFA obtained from the conversion of linoleic acid **(1)**. The THFAwas identified as 9,10,11-trihydroxy-*cis*-12-octadecenoic acid **(4)**. (B) LC-MS/MS fragments of THFA obtained from the conversion of α-linolenic acid **(6)**. The THFA was identified as 12,13,14-trihydroxy-*cis,cis*-9,15-octadecadienoic acid **(10)**. (C) LC-MS/MS fragments of THFA obtained from the conversion of γ-linolenic acid **(11)**. The THFA was identified as 9,10,11-hydroxy-*cis,cis*-6,12-octadecadienoic acid **(15)**. The asterisks (*) and numbers underlined in red indicate the molecular masses of key fragments and the total molecular mass of the compound, respectively, used for structure identification.

Figure 6. Time-course reactions for the biotransformation of C18 PUFAs into THFAs by recombinant *E. coli* ER2566:pACYC-MXLOX-MXEH. (A) Biotransformation of linoleic acid **(1)** into 9,10,11-trihydroxy-*cis*-12-octadecenoic acid **(4)**. (B) Biotransformation of α-linolenic acid **(6)** into 12,13,14-trihydroxy-*cis*,*cis*-9,15-octadecadienoic acid **(9)**. (C) Biotransformation of γ-linolenic acid **(11)** was converted into 9,10,11-trihydroxy-*cis*,*cis*-6,12-octadecadienoic acid **(14)**. The biotransformation was performed in 50 mM HEPPS buffer (pH 8.5) containing 3−15 g L^{-1} cells and 1 mM PUFA at 35 °C for 90 min. The data represent the mean of three experiments, and the error bars represent the standard deviations. The symbols indicate the concentrations of PUFAs (**1**, **6**, and **10**) (●), HpFAs (**2**, **7**, and **11**)

621 (■), HFAs (**5**, **10**, and **15**) (▲), EHFAs (**3**, **8**, and **13**) (♦), and THFAs (**4**, **9**, and **14**) (▼).

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623 **Figure 7.** Time-course reactions for the biotransformation of linoleic acid **(1)** into 9,10,11-
624 trihydroxy-*cis*-12-octadecenoic acid **(4)** by recombinant *E. coli* ER2566:pACYC-MXLOX-
625 MXEH. The biotransformation was performed in 50 mM HEPPS buffer (pH 8.5) containing
626 3 g L^{-1} cells and 3 mM linoleic acid at 35 °C for 90 min. The data represent the mean of three
627 experiments, and error bars represent the standard deviations. The symbols indicate the
628 concentrations of linoleic acid **(1)** (●), 9-hydroperoxy-*trans*,*cis*-10,12-octadecadienoic acid **(2)**
629 (■), 9-hydroxy-*trans*,*cis*-10,12-octadecadienoic acid **(5)** (▲), 9,10-epoxy-11-hydroxy-*cis*-12-
630 octadecenoic acid **(3)** (♦), and 9,10,11-trihydroxy-*cis*-12-octadecenoic acid **(4)** (▼).

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Table 1. Specific activity and kinetic parameters of *M. xanthus* LOX for C18 PUFAs

Substrate	Product[a]	Specific activity[b] (U mg^{-1})	K_m (mM)	k_{cat}[c] (min^{-1})	k_{cat}/K_m (min^{-1} mM^{-1})
Linoleic acid (C18:2$^{\Delta 9,12/\omega-6}$)	9-Hydroperoxy-*trans*,*cis*-10,12-octadecadienoic acid	11.4 ± 0.2	0.330	477.1	1,446
α-Linolenic acid (C18:3$^{\Delta 9,12,15/\omega-6}$)	12-Hydroperoxy-*cis*,*trans*,*cis*-9,13,15-octadecatrienoic acid	3.4 ± 0.1	0.081	76.1	940
γ-Linolenic acid (C18:3$^{\Delta 6,9,12/\omega-3}$)	9-Hydroperoxy-*cis*,*trans*,*cis*-6,10,12-octadecatrienoic acid	1.9 ± 0.3	0.073	48.4	663

[a]These products were the first-step reaction products of *M. xanthus* linoleate 9*S*-LOX.

[b]Unit is micromoles per minute (μmol min^{-1}).

[c]The kinetics parameters were calculated by nonlinear regression analysis.

Data represent the mean of three experiments, and the error bars represent the standard deviations.

Table 2. Plant C18 oxylipins accessible through biocatalysis and the biocatalytic performance

Starting material	Intermediate product	Yield (%)[a]	Specific productivity (μmol h^{-1} g-cells^{-1})	Final product	Yield (%)[a]	Specific productivity (μmol h^{-1} g-cells^{-1})
Linoleic acid **(1)**	9,10-Epoxy-11-hydroxy-12-octadecenoic acid **(3)**	72.2 ± 1.3 (97%[c])	321 ± 4.8	9,10,11-Trihydroxy-12-octadecenoic acid **(4)**	75.1 ± 3.8 70.3 ± 1.9[b] (99%)	205 ± 6.2 469 ± 8.1[b]
				9-Hydroxy-10,12-octadecadienoic acid **(5)**	24.7 ± 1.1[d] (94%) 27.7 ± 2.3[e]	54.8 ± 2.5[d] 123 ± 3.2[e]
α-Linolenic acid **(6)**	12,13-Epoxy-14-hydroxy-9,15-octadecadienoic acid **(8)**	65.2 ± 2.3 (96%)	72.5 ± 2.9	12,13,14-Trihydroxy-9,15-octadecadienoic acid **(9)**	62.6 ± 4.1 (98%)	45.9 ± 3.6
				12-Hydroxy-9,13,15-octadecatrienoic acid **(10)**	37.8 ± 1.9[d] (93%) 34.7 ± 0.9[e]	28.0 ± 1.1[d] 38.5 ± 2.5[e]
γ-Linolenic acid **(11)**	9,10-Epoxy-11-hydroxy-6,12-octadecadienoic acid **(13)**	40.1 ± 2.1 (99%)	26.7 ± 0.6	9,10,11-Trihydroxy-6,12-octadecadienoic acid **(14)**	29.3 ± 1.3 (99%)	19.3 ± 1.1
				9-Hydroxy-6,10,12-octadecatrienoic acid **(15)**	70.1 ± 3.3[d] (99%) 59.8 ± 1.8[e]	31.1 ± 0.7[d] 39.9 ± 1.3[e]

[a]The product yield is the ratio of the number of moles of product formed to those of substrate consumed. The mole concentrations of substrates and products were determined by HPLC analysis.

[b]The product yield and specific productivity of 9,10,11-trihydroxy-12-octadecenoic acid (**4**) from linoleic acid (**1**) by recombinant *E. coli*

containing pACYC-MXLOX-MXEH were measured at t = 90 min from Figure 3.

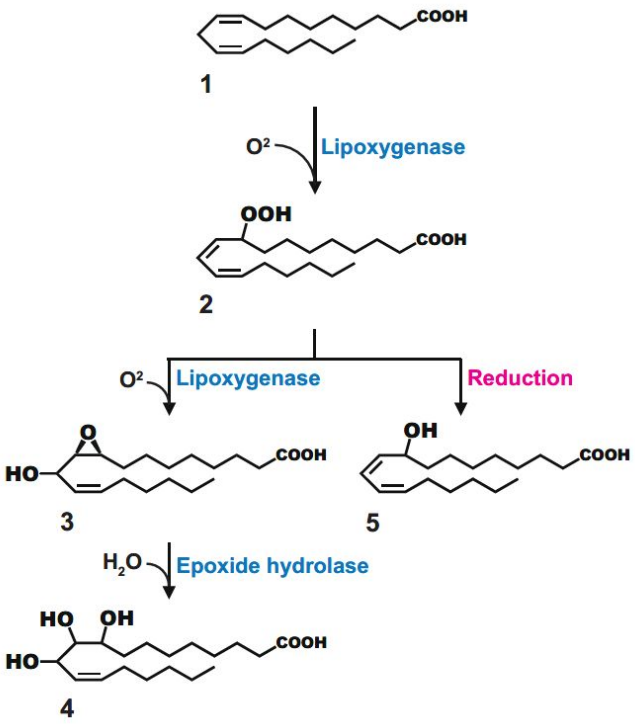
[c]The numbers in parenthesis indicate the purity of the reaction products.

[d]The product yields and specific productivities for HFAs represent those from the biotransformation of PUFAs into THFAs by recombinant *E. coli* containing pACYC-MXLOX-MXEH.

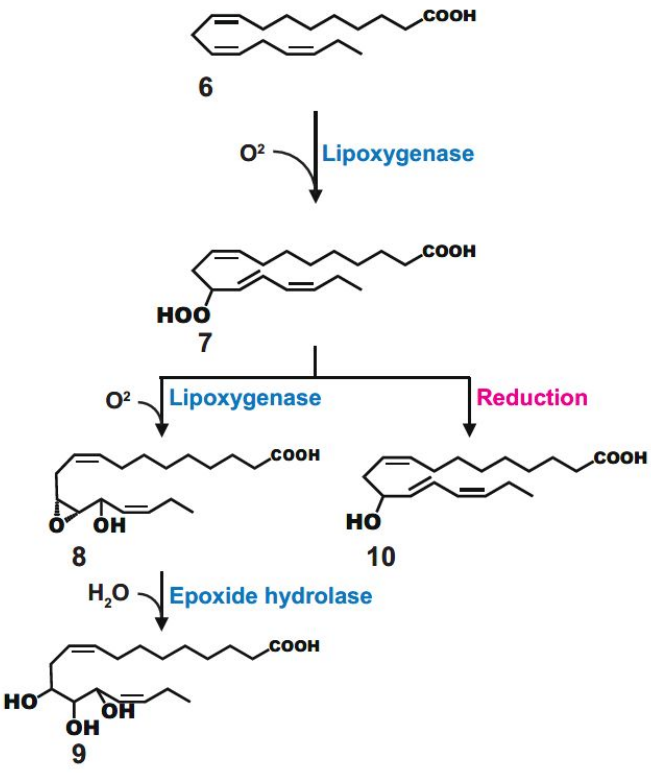
[e]The product yields and specific productivities for HFAs represent those from the biotransformation of PUFAs into EHFAs by recombinant *E. coli* containing pET28a-MXLOX.

All data represent the means of three experiments, and ± represents the standard deviations.

A


COOH
1
O2
Lipoxygenase
OOH
COOH
2
O2
Lipoxygenase
Reduction
O
HO
COOH
3
OH
COOH
5
H2O
Epoxide hydrolase
HO
OH
HO
COOH
4

B


COOH
6
O2
Lipoxygenase
COOH
HOO
7
O2
Lipoxygenase
Reduction
COOH
O
OH
8
COOH
HO
10
H2O
Epoxide hydrolase
COOH
HO
OH
OH
9

C

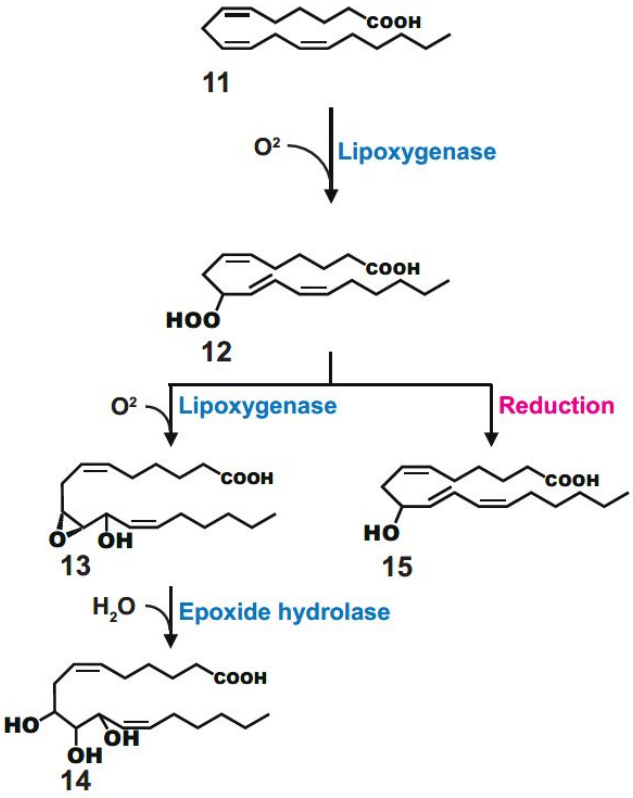
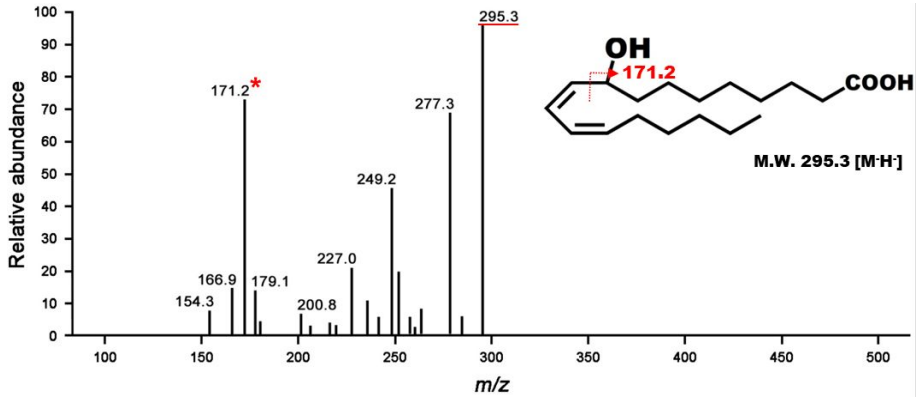
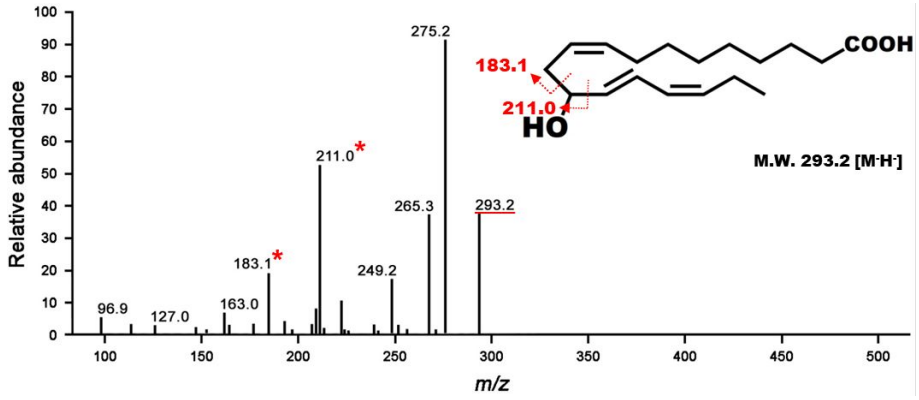

COOH
11
O2
Lipoxygenase
COOH
HOO
12
O2
Lipoxygenase
Reduction
COOH
O
OH
13
COOH
HO
15
H2O
Epoxide hydrolase
COOH
HO
OH
OH
14

Figure 1

A


Relative abundance
m/z
295.3
171.2*
277.3
249.2
227.0
166.9
179.1
154.3
200.8
OH
171.2
COOH
M.W. 295.3 [M-H-]

B


Relative abundance
m/z
275.2
183.1
211.0
HO
COOH
M.W. 293.2 [M-H-]
211.0*
265.3
293.2
183.1*
249.2
96.9
127.0
163.0

C

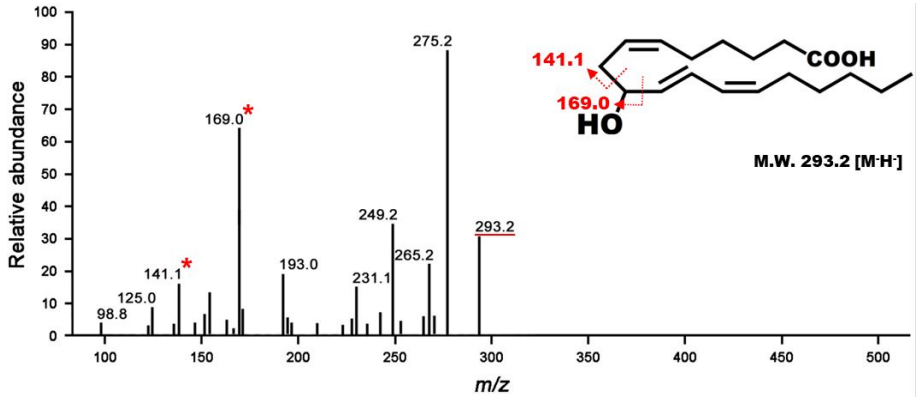
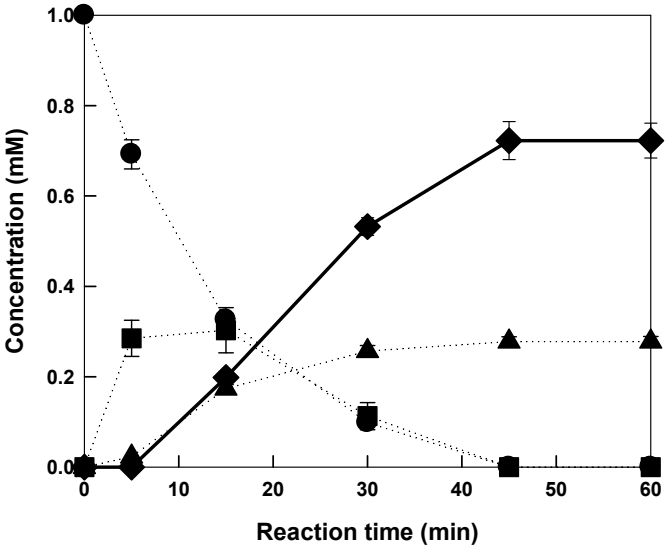
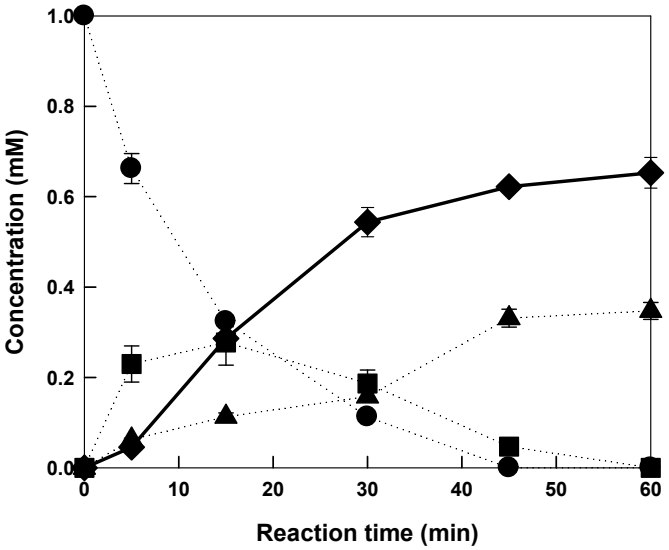

Relative abundance
m/z
275.2
141.1
169.0
HO
COOH
M.W. 293.2 [M-H-]
169.0*
249.2
293.2
265.2
193.0
231.1
141.1*
125.0
98.8

Figure 2

A


Concentration (mM)
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
40
50
60
Reaction time (min)

B


Concentration (mM)
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
40
50
60
Reaction time (min)

C

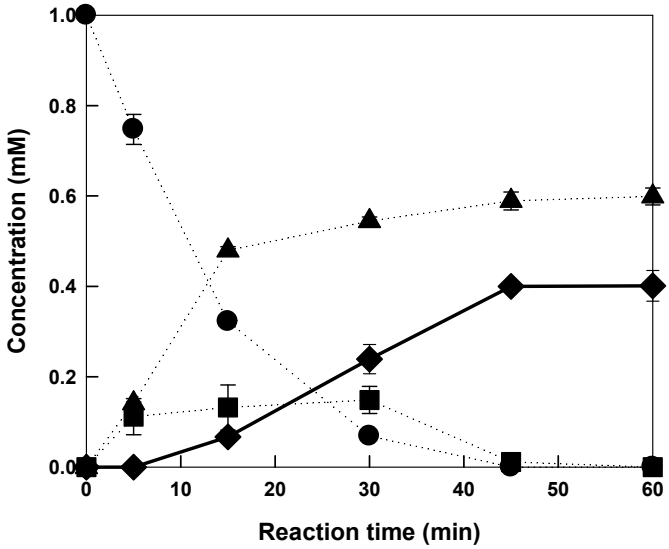
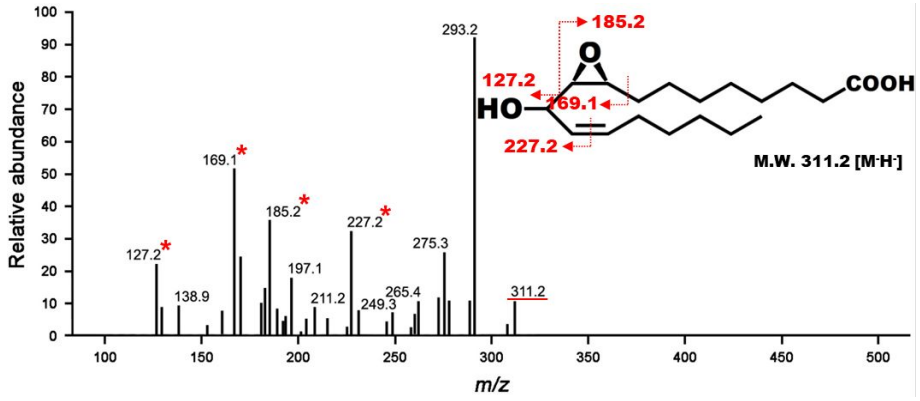
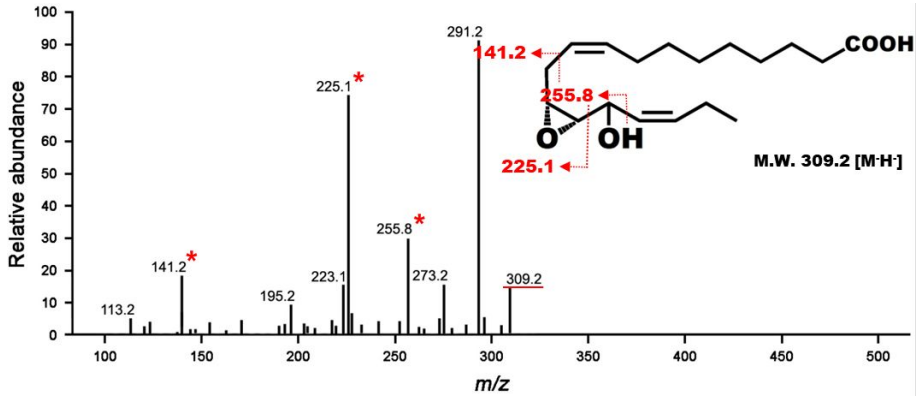

1.0
0.8
0.6
0.4
0.2
0.0
Concentration (mM)
0
10
20
30
40
50
60
Reaction time (min)

Figure 3

A


Relative abundance
m/z
293.2
185.2
127.2
169.1
227.2
COOH
HO
O
M.W. 311.2 [M-H-]
169.1
185.2
227.2
127.2
275.3
197.1
138.9
211.2
249.3
265.4
311.2

B


Relative abundance
m/z
291.2
141.2
255.8
225.1
COOH
O
OH
M.W. 309.2 [M-H-]
225.1
255.8
141.2
223.1
273.2
309.2
195.2
113.2

C

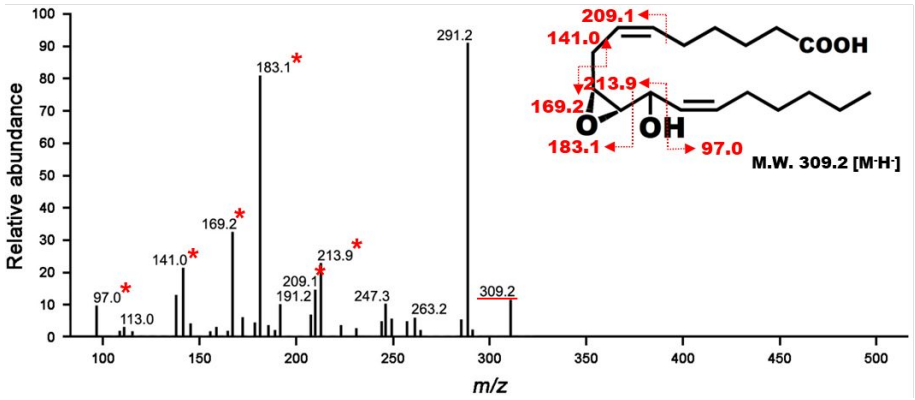
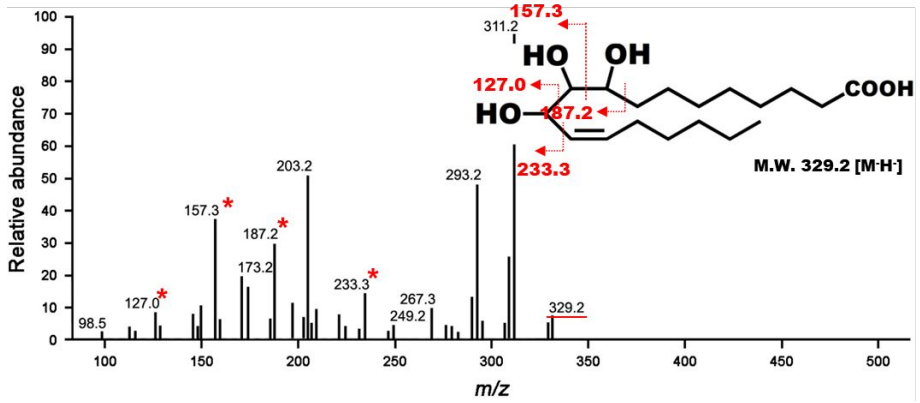
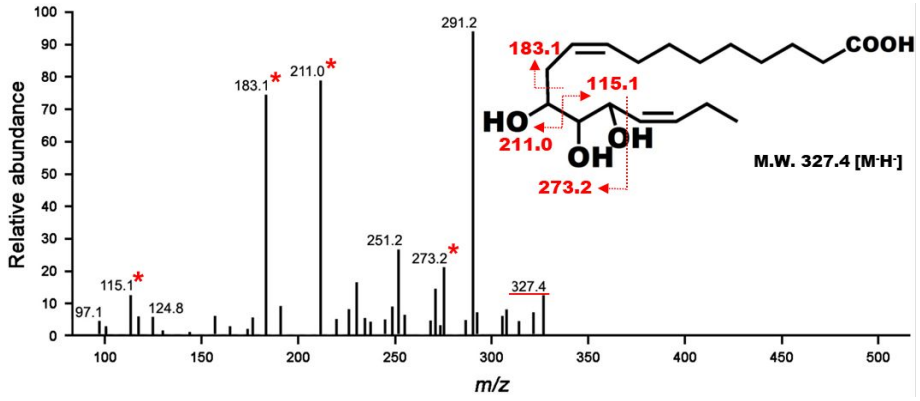

Relative abundance
m/z
291.2
209.1
141.0
213.9
169.2
183.1
97.0
COOH
O
OH
M.W. 309.2 [M-H-]
183.1
169.2
141.0
213.9
209.1
191.2
97.0
113.0
247.3
263.2
309.2

Figure 4

A


Relative abundance
m/z
157.3
311.2
HO
OH
127.0
HO
187.2
COOH
233.3
M.W. 329.2 [M-H-]
203.2
293.2
157.3
187.2
173.2
233.3
267.3
249.2
127.0
98.5
329.2

B


Relative abundance
m/z
291.2
183.1
COOH
115.1
HO
211.0
OH
OH
273.2
M.W. 327.4 [M-H-]
183.1
211.0
251.2
273.2
115.1
97.1
124.8
327.4

C

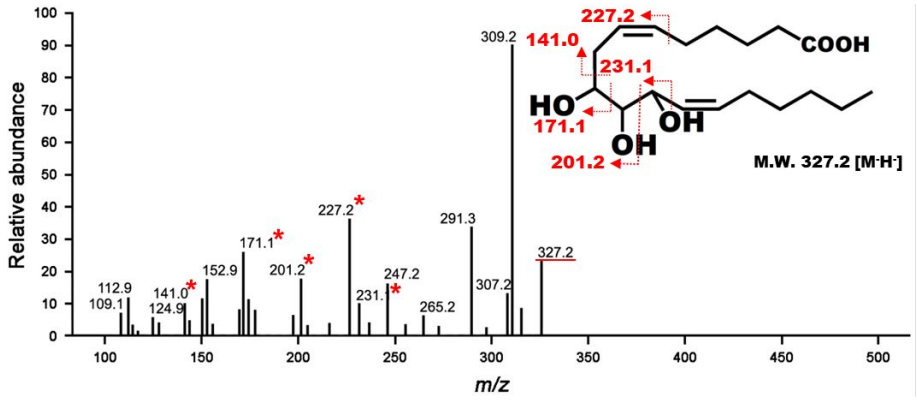
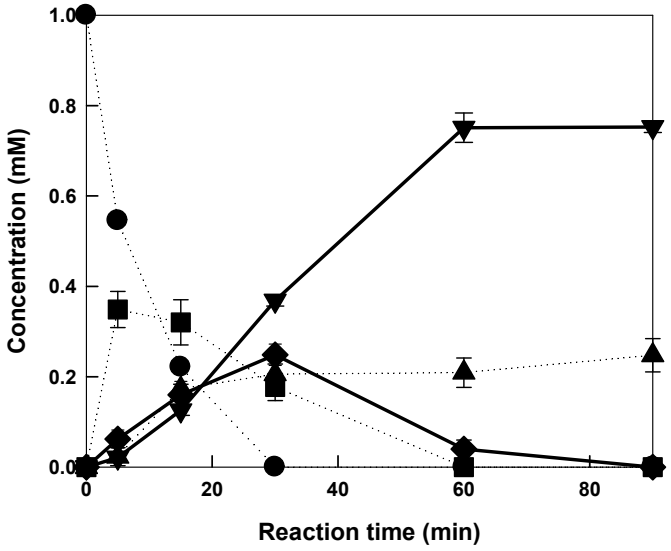
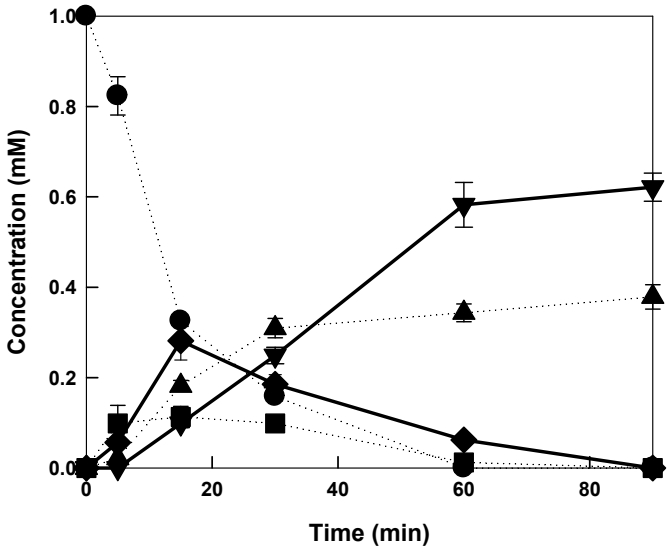

Relative abundance
m/z
227.2
309.2
141.0
COOH
231.1
HO
171.1
OH
OH
201.2
M.W. 327.2 [M-H-]
227.2
291.3
171.1
327.2
152.9
201.2
247.2
112.9
109.1
141.0
124.9
231.1
307.2
265.2

Figure 5

A


Concentration (mM)
1.0
0.8
0.6
0.4
0.2
0.0
0
20
40
60
80
Reaction time (min)

B


Concentration (mM)
1.0
0.8
0.6
0.4
0.2
0.0
0
20
40
60
80
Time (min)

C

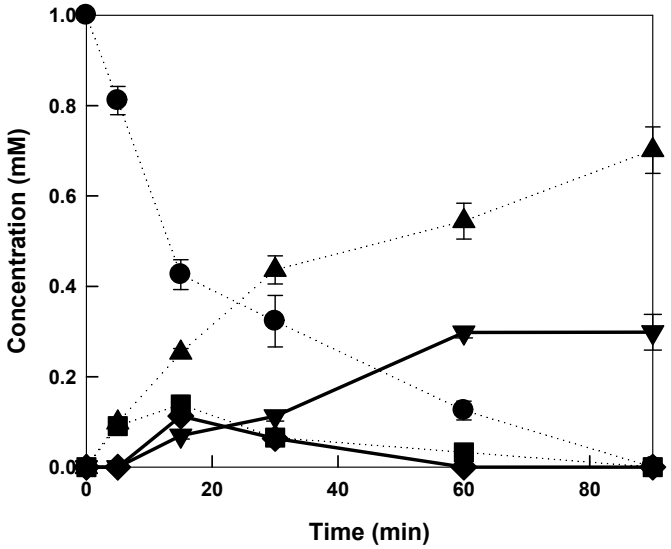


Figure 6

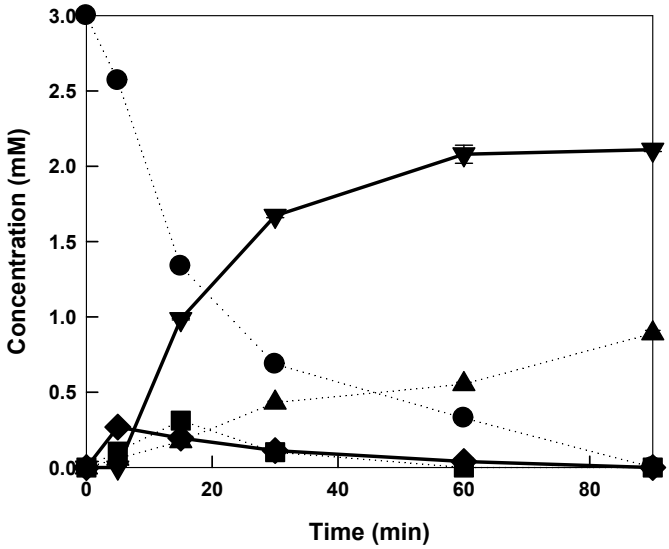


Figure 7

For Table of Contents Only