Bioconversion of n-3 and n-6 PUFA by *Clavibacter* sp. ALA2

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ABSTRACT: *Clavibacter* sp. ALA2 oxidized n-3 and n-6 PUFA into a variety of oxylipins. Structures of products converted from EPA and DHA were determined as 15,18-dihydroxy-14,17-epoxy-5(*Z*),8(*Z*),11(*Z*)-eicosatrienoic acid and 17,20-dihydroxy-16,19-epoxy-4(*Z*),7(*Z*),10(*Z*),13(*Z*)-docosatetraenoic acid by GC–MS and NMR analyses. In contrast, γ-linolenic acid and arachidonic acid were converted to diepoxy bicyclic FA, tetrahydrofuranyl monohydroxy FA, and trihydroxy FA. Thus, the structures of bioconversion products were different between n-3 and n-6 PUFA. Furthermore, strain ALA2 placed hydroxy groups and cyclic structures at the same position from the ω-terminal despite the number of carbons in the chain and the double bonds in the PUFA.

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KEY WORDS: Bioconversion, *Clavibacter* sp. ALA2, cyclic fatty acids, oxygenated polyunsaturated fatty acids, trihydroxy fatty acids.

Hydroxy FA are common in nature (1,2). For example, in mammals, PUFA are metabolized to prostaglandins, leukotrienes, lipoxins, and other hydroxy and epoxy FA (3). These FA metabolites are important chemical mediators of biological responses in mammals. In contrast, plant systems produce hydroxy FA, such as castor oil, which are important industrial materials. They are used widely in products including resins, waxes, nylons, plastics, cosmetics, and coatings (4).

Microbial systems reportedly convert unsaturated FA to monohydroxy-, dihydroxy-, and trihydroxy-FA (5–8). Various value-added hydroxy FA and their derivatives for industrial applications can be produced by using the unique reaction specificities of microbial enzymes. In our previous work, *Clavibacter* sp. ALA2 converted linoleic acid to 12,13,17-trihydroxy-9(*Z*)-octadecenoic acid (12,13,17-THOA) and 12,13,16-THOA (9,10). Furthermore, we also obtained and identified novel diepoxy bicyclic FA and tetrahydrofuranyl FA as metabolites of THOA from the bioconversion of linoleic acid (11–14). These cyclic FA have unique chemical structures and are expected to have some biological functions.

To clarify these biological functions and to develop an industrial production process for these cyclic FA, one must understand the substrate- and positional-specificity of bioconversion by strain ALA2. In this paper, we identify eight bioconversion products from n-3 and n-6 PUFA by strain ALA2. Furthermore, we discuss the positional- and substrate-specificity of PUFA bioconversion by strain ALA2.

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MATERIALS AND METHODS

Microorganism cultivation. *Clavibacter* sp. ALA2 (NRRL B-21660) was isolated from a dry soil sample collected from McCalla, Alabama (9). Strain ALA2 was cultivated aerobically in 125-mL Erlenmeyer flasks containing 50 mL of medium with shaking at 200 rpm, 30°C. The medium had the following composition (per liter): 5 g dextrose, 15 g yeast extract, 10 g tryptone, 5 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.01 g $FeSO_4 \cdot 7H_2O$, 0.014 g $ZnSO_4$, 0.08 g $MnSO_4 \cdot H_2O$, and 0.01 g nicotinic acid. The medium was adjusted to pH 6.8 with dilute phosphoric acid.

Chemicals. Linoleic acid, α-linolenic acid, γ-linolenic acid, arachidonic acid, and DHA were purchased from Nu-Chek-Prep Inc. (Elysian, MN). EPA was purchased from Cayman Chemical Co. (Denver, CO). The purities of all substrate FA were over 95%. Silica Gel 60 (70–230 mesh) and preparative Silica Gel 60 F_{254} TLC plates were purchased from EM Science (Cherry Hill, NJ). All other chemicals were reagent grade and obtained from commercial sources.

Bioconversion. Bioconversions were carried out by adding 125 μL PUFA to a 1-d-old culture and shaking the flasks at 200 rpm at 30°C for an additional 7 d. After incubation, the culture was acidified to pH 2 with 6 N hydrochloric acid. A crude lipid fraction was extracted with 100 mL of ethyl acetate and then with 100 mL of diethyl ether using a separation funnel. The solvent was evaporated to obtain the crude lipid extract.

Products analyses. The crude lipid extracts were methylated with diazomethane for GC analyses. Methyl ester derivatives were injected into a Hewlett-Packard 5890 gas chromatograph equipped with an FID, a Supelco (Bellefonte, PA) SPB-1 capillary column (15 m × 0.32 mm i.d.; 0.25 μm thickness), and a Hewlett-Packard 6890 integrator. The column temperature was raised from 170 to 210°C at a rate of 3°C/min and then kept at 210°C. The injection and detector temperatures were 240 and 250°C, respectively. Palmitic acid was added as an internal standard prior to solvent extraction. The relative activity was calculated as the ratio of a product peak vs. the methyl palmitate peak.

Purification of products. The crude lipid extracts were fractionated by column chromatography (2.5 cm i.d. × 35 cm length) packed with silica gel 60. Elutions were carried out using 500 mL of methylene chloride, 500–1000 mL methylene

chloride/methanol (97:3, vol/vol), and 500–1000 mL methylene chloride/methanol (95:5, vol/vol). Products **1**–**4**, product **6**, and product **7** were obtained from the methylene chloride/methanol (97:3, vol/vol) fraction. Product **5** and product **8** were eluted by methylene chloride/methanol (95:5, vol/vol). Fractionated products were methylated with diazomethane and further purified on preparative Silica Gel 60 F_{254} TLC plates. Product **1** (converted from EPA): (developing solvent) *n*-hexane/ethyl ether (1:9, vol/vol), R_f = 0.44, purity (by GC analysis) 96%; product **2** (converted from DHA): *n*-hexane/ethyl ether (1:9, vol/vol), R_f = 0.43, purity 92%; product **3** (converted from γ-linolenic acid): methylene chloride, R_f = 0.22, purity 95%; product **4** (converted from γ-linolenic acid): *n*-hexane/acetone (7:3, vol/vol), R_f = 0.52, purity 87%; product **5** (converted from γ-linolenic acid): ethyl ether/acetone (4:1, vol/vol), R_f = 0.52, purity 97%; product **6** (converted from arachidonic acid): methylene chloride/methanol (99:1, vol/vol), R_f = 0.70, purity 99%; product **7** (converted from arachidonic acid): *n*-hexane/ethyl acetate (7:3, vol/vol), R_f = 0.53, purity 96%; product **8** (converted from arachidonic acid): methylene chloride/methanol (9:1, vol/vol), R_f = 0.43, purity by GC–MS 95%.

Identification of products. The structures of bioconversion products were analyzed with GC–MS and proton and ^{13}C NMR. For GC–MS analyses, silylation of methyl-esterified extracts was achieved with the mixture of 1-(trimethylsilyl)imidazole + pyridine (1:4, vol/vol) for 30 min at room temperature. GC–MS analyses were performed with a Hewlett-Packard Model 5890 gas chromatograph interfaced with a Model 5971 mass-selective detector operating at 70eV. The capillary column used was a Hewlett-Packard HP-5-MS cross-linked 5% phenyl methyl silicone, 30 m × 0.25 mm i.d., film thickness 0.25 µm. The carrier gas was helium, and its flow rate was 0.65 mL/min. The GC column was programmed from 65 to 260°C at a rate of 20°C/min and then kept at 260°C for 20 min. NMR spectra were obtained with a Bruker model ARX-400 spectrometer (Billerica, MA) equipped with a 5 mm $^{13}C/^{1}H$ dual probe (^{13}C NMR, 100 MHz; ^{1}H NMR, 400 MHz). NMR spectra were recorded with $CDCl_3$ as internal standard and solvent.

RESULTS AND DISCUSSION

Bioconversion of n-3 PUFA by Clavibacter *sp. ALA2*. The crude lipid extract was obtained from incubation of EPA and *Clavibacter* sp. ALA2 in culture media for 7 d. GC analysis of the methyl ester of the crude lipid extract showed new product peaks (Fig. 1). The major product (product **1**), with a GC retention time of 24 min, was purified by silica gel column chromatography followed by preparative silica gel TLC as described in the Method and Materials section for analyses with GC–MS and NMR.

The EI-MS of the methyl ester/trimethylsilyloxy ethers (OTMSi) of the isolated product **1** gave a molecular ion at (relative intensity) *m/z* 510 $[M]^+$ (1). Typical fragment ions were interpreted as follows: 379 $[M - CH_3CH_2CHOTMSi]^+$ (0.7), 289 $[M - (CH_2CH{=}CH)_3(CH2)_3COOCH_3]^+$ (4), 271 (2), 239 (2), 199 $[289 - TMSiOH]^+$ (29), 181 (6), 157 (20), 145 (21), 131 $[CH_3CH_2CHOTMSi]^+$ (59), and 73 $[TMSi]^+$ (100). The structure of product **1** (methyl ester derivative) was further confirmed by ^{13}C and ^{1}H NMR analyses (Table 1). The ^{13}C NMR signals at 71.2 and 74.2 ppm corresponded to a hydroxy group in the C-15 and C-18 positions in the molecule, respectively. The ^{13}C NMR signals of 129.8 (C-5), 128.9 (C-6), 128.2 (C-8), 128.1 (C-9), 128.8 (C-11), and 125.9 ppm (C-12) indicated the presence of three double bonds. The carbon peaks for C-7 (25.7 ppm), C-10 (25.6 ppm), and C-13 (27.1 ppm) indicated that the three double bonds remained in the *cis* configuration. An epoxy structure was found at C-14 (83.6 ppm) and C-17 (79.1 ppm). The positions of the hydroxy group and the double bonds also were supported by ^{1}H NMR signals (Table 1). From these data, the structure of product **1** converted from EPA was identified to be 15,18-dihydroxy-14,17-epoxy-5(*Z*),8(*Z*),11(*Z*) eicosatrienoic acid (Fig. 2). Strain ALA2 hydroxylated the two double bonds between C-14 and C-15, C-17 and C-18, but did not alter the double bonds at the C-5, C-8, and C-11 positions.

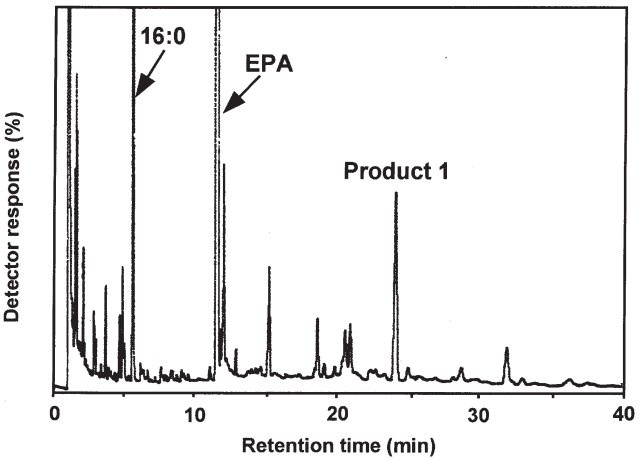

FIG. 1. GC chromatogram of the crude lipid extract obtained from incubation of EPA and *Clavibacter* sp. ALA2. The 1-d-old culture (125 mL) was incubated with 125 µL of EPA at 30°C for 7 d. The crude lipid extract was methylated for GC analysis.

Strain ALA2 also converted DHA to several products. A major product (product **2**) with GC retention time at 37 min and three minor products with GC retention times at 21, 27, and 30 min were detected on GC chromatogram of the methyl esters of the crude lipid extract. The EI-MS of the methyl ester/OTMSi of the isolated product **2** was interpreted as follows: EI-MS *m/z* (relative intensity): 536 $[M]^+$ (0.8), 405 $[M - CH_3CH_2CHOTMSi]^+$ (0.3), 289 $[M - (CH_2CH{=}CH)_4(CH_2)_2{-}COOCH_3]^+$ (2), 271 (1), 199 $[289 - TMSiOH]^+$ (24), 181 (6), 157 (17), 145 (17), 131 $[CH_3CH_2CHOTMSi]^+$ (52), 73 $[TMSi]^+$ (100). Therefore, the chemical structure of product 2 was estimated to be 17,19-dihydroxy-16,18-epoxy-4,7,10,13-docosatetraenoic acid because typical fragmentations such as *m/z* 289, 199, and 131 were similar to those of product **1**. Proton and ^{13}C NMR analyses (Table 1) confirmed this structure for product **2**. ^{13}C NMR signals of 129.1 (C-4), 129.8 (C-5), 128.0 (C-7), 127.8 (C-8), 128.2 (C-10), 128.2 (C-11), 129.3

TABLE 1
Proton and ^{13}C NMR Signals for Products Converted from EPA and DHA by *Clavibacter* sp. ALA2

	Resonance signals (ppm)			
	Product **1**		Product **2**	
Carbon number	Proton	^{13}C	Proton	^{13}C
1	—	174.3	—	174.0
2	2.34 *t* (*J* = 7.5 Hz)	33.4	2.30*m*	33.9
3	1.70 *m*	24.8	2.30 *m*	22.7
4	2.12 *m*	26.5	5.40 *m*	128.1
5	5.40 *m*	129.8	5.40 *m*	129.8
6	5.40 *m*	128.9	2.85*m*	26.7
7	2.87 *m*	25.7	5.40 *m*	128.0
8	5.40 *m*	128.2	5.40 *m*	127.8
9	5.40 *m*	128.1	2.85 *m*	25.7
10	2.87 *m*	25.6	5.40 *m*	128.2
11	5.40 *m*	128.8	5.40 *m*	128.2
12	5.40 *m*	125.9	2.85 *m*	25.4
13	2.45 *m*	27.1	5.40 *m*	129.3
14	3.64 *m*	83.6	5.40 *m*	125.9
15	4.03 *m*	71.2	2.30 *m*	27.0
16	1.84, 2.15 *m*	38.1	3.63 *m*	83.6
17	4.01 *m*	79.1	4.03 *m*	71.1
18	3.39 *m*	74.2	1.83, 2.30 *m*	37.9
19	1.60 *m*	26.9	4.00 *m*	79.2
20	0.99 *t* (*J* = 7.5 Hz)	10.2	3.37 *m*	74.7
21	—	—	1.59 *m*	25.6
22	—	—	0.98 *t* (*J* = 7.5 Hz)	10.1
OCH_3[a]	3.67 *s*	51.4	3.66 *s*	51.5

[a]Products were analyzed as methyl ester derivatives by ^1H and ^{13}C NMR.

(C-13), and 125.9 ppm (C-14) indicated the presence of four double bonds. The ^{13}C NMR signals of 26.7 (C-6), 25.7 (C-9), 25.4 (C-12), and 27.0 ppm (C-15) indicated the four double bonds remained in *cis* configuration. Therefore, the chemical structure of product **2** is 17,19-dihydroxy-16,18-epoxy 4(*Z*), 7(*Z*),10(*Z*),13(*Z*)-docosatetraenoic acid (Fig. 2). When EPA or DHA was incubated with an autoclaved 3-d-old strain ALA2, none of the products were detected. Additionally, none of the products were observed when strain ALA2 was incubated alone in culture media for 7 d without substrate. The products were therefore likely to be formed from EPA and DHA enzymatically by strain ALA2 and not by autoxidation.

The identified products **1** and **2** had two hydroxy groups and a tetrahydrofuranyl (THF) ring in their molecules (Fig. 2). We

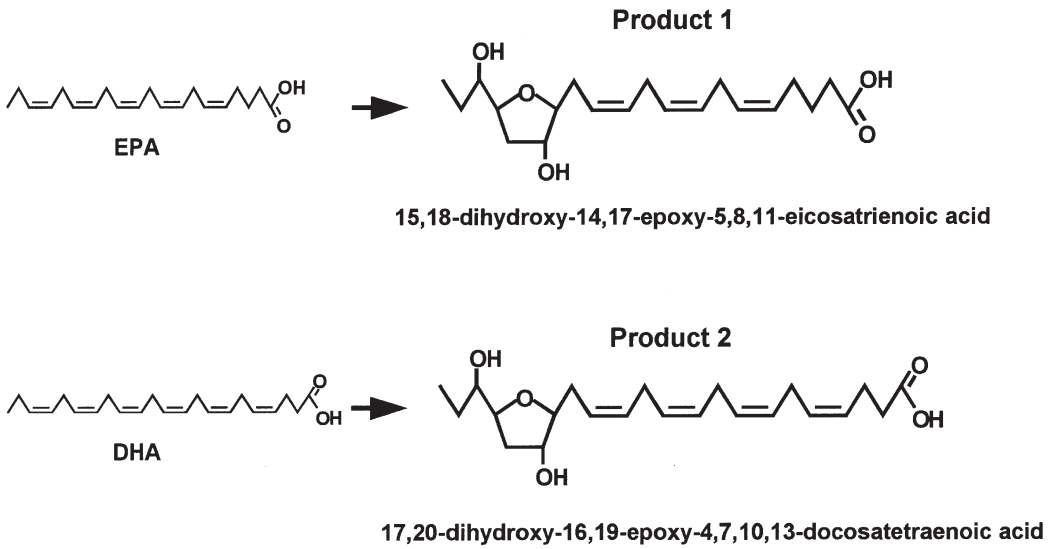

FIG. 2. Bioconversion products from n-3 PUFA by *Clavibacter* sp. ALA2.

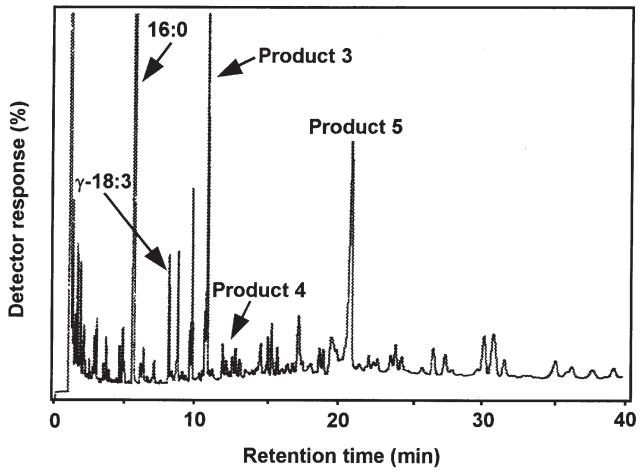

FIG. 3. GC chromatogram of the crude lipid extract obtained from incubation of γ-linolenic acid and *Clavibacter* sp. ALA2. The 1-d-old culture (125 mL) was incubated with 125 μL of γ-linolenic acid at 30°C for 7 d. The crude lipid extract was methylated for GC analysis.

reported earlier that strain ALA2 converted α-linolenic acid to 13,16-dihydroxy-12,15-epoxy-9(*Z*)-octadecenoic acid and 7,13,16-trihydroxy-12,15-epoxy-9(*Z*)-octadecenoic acid (15). From product structures obtained from these n-3 PUFA, it seems that strain ALA2 places hydroxyl groups at the same positions from the omega (ω)-terminal and cyclizes them to THF ring despite their varying degrees in carbon chain numbers and double bonds.

Bioconversion of n-6 PUFA by Clavibacter *sp. ALA2*. The crude lipid extract obtained from incubation of γ-linolenic acid with strain ALA2 for 7 d was methylated with diazomethane. GC analysis of the methyl esters of the crude lipid extract showed many product peaks and a remarkable decrease of substrate γ-linolenic acid (Fig. 3). Two major products with GC retention time at 11 min (product **3**) and 22 min (product **5**) were purified as described in the Materials and Methods section.

The EI-MS of the methyl ester/OTMSi derivative of product **3** gave a molecular ion at *m/z* (relative intensity) 322 $[M]^+$ (0.2). Typical fragment ions were interpreted as follows: 291 $[M - CH_3O]^+$ (1), 280 $[M - CH_2CO]^+$ (0.6), 262 (0.6), 194 (3), 180 (2), 127 $[M - (CH_2CH{=}CH)_2(CH_2)_4COOCH_3]^+$ (100), 99 (11), 81 (19), 67 (14), 55 (10). A fragment ion of *m/z* 127 was a characteristic ion of a diepoxy bicyclic structure as reported previously (12). Therefore, the chemical structure of product **3** is possibly 12,17;13,17-diepoxy-6,9-octadecadienoic acid. This structure was confirmed by proton and ^{13}C NMR analyses. The NMR data were similar to those of 12,17;13,17-diepoxy-9(*Z*)-octadecenoic acid obtained from linoleic acid by strain ALA2 (12). The singlet multiplicity of terminal methyl showed that there was no coupling with C-17 (Table 2), and the chemical shift of C-17 by ^{13}C NMR was consistent with diethyl functionality (Table 3). The dihedral angle for H-13 and either of the H-14 protons is about 60 degrees, resulting in small, unresolved splitting of the H-13 signal. ^{13}C NMR chemical shifts in a low field corresponding to C-6, C-7, C-9, and C-10 indicated there were two double bonds in the molecule.

The GC–MS analysis of the methyl ester/OTMSi ether derivative of the isolated product **5** gave the following characteristic signal fragments: EI-MS, *m/z* (relative intensity) 468 $[M - TMSiOH]^+$(0.1), 453 $[M - CH_3 - TMSiOH]^+$(0.1), 437 $[M - OCH_3 - TMSiOH]^+$ (0.2), 363 $[M - CH_3 - 2TMSiOH$ or $M - (CH_2CH{=}CH)_2(CH_2)_4COOCH_3]^+$ (3), 297 $[CHOTMSi(CH_2{-}CH{=}CH)_2(CH_2)_4COOCH_3]^+$ (3), 268 (9), 261 $[M - CHOTMSi{-}(CH_2CH{=}CH)_2(CH_2)_4COOCH_3]^+$ (13), 191 (12),

TABLE 2
^1H NMR Data for Bioconversion Products from γ-Linolenic Acid and Arachidonic Acid by *Clavibacter* sp. ALA2

Carbon	Product **3**			Product **4**			Product **5**			Product **6**			Product **7**			Product **8**		
no.	δ	mult.	Hz	δ	mult.	Hz	δ	mult.	Hz	δ	mult.	Hz	δ	mult.	Hz	δ	mult.	Hz
	2.33	*t*	7.5	2.33	*t*	7.5	2.33	*t*	7.5	2.34	*t*	7.6	2.34	*t*	7.5	2.34	*t*	7.5
3	?	?		1.67	*m*		1.50	*m*		1.70	*m*		1.70	*m*		1.70	*m*	
4	1.39	*m*		1.42	*m*		1.50	*m*		2.12	*m*		2.12	*m*		2.11	*m*	
5	2.09	*m*		2.12	*m*		2.10	*m*		5.40	*m*		5.39	*m*		5.40	?	
6	5.40	*m*		5.40	*m*		5.40	*m*		5.40	*m*		5.39	*m*		5.40	?	
7	5.40	*m*		5.40	*m*		5.40	*m*		2.85	*m*		2.84	*m*		2.85	?	
8	2.82	*m*		2.82	*m*		2.84	*m*		5.40	*m*		5.39	*m*		5.40	?	
9	5.40	*m*		5.40	*m*		5.40	*m*		5.40	*m*		5.39	*m*		5.40	?	
10	5.40	*m*		5.40	*m*		5.50	*m*		2.85	*m*		2.84	*m*		2.85	*m*	
11	2.28	*m*		2.25	*m*		2.30	*m*		5.40	*m*		5.39	*m*		5.40	*m*	
12	4.04	*t*	6.7	3.44	*m*		3.00	*m*		5.40	*m*		5.39	*m*		5.40	*m*	
13	4.14	*b,s*		3.83	*m*		3.40	*b,s*		2.28	*m*		2.25	*m*		2.30	*m*	
14	1.40,1.79	?		1.60	*m*		1.50	*m*		4.04	*t*	6.7	3.45	*m*		3.45	*m*	
15	1.61	*m*		1.60	*m*		1.50	*m*		4.14	*b,s*		3.85	*m*		3.45	*m*	
16	1.65,1.79	?		3.83	*m*		1.50	*m*		1.50,1.80	*m*		1.54	*m*		1.50	*m*	
17	—	—		1.60	*m*		3.75	*m*		1.60,1.80	*m*		2.05	*m*		1.60	*m*	
18	1.41	*s*		0.94	*t*	7.5	1.18	?	6.2	1.60	*m*		3.85	*m*		1.45	*m*	
19	—			—			—			—	—		1.47,1.63	*m*		3.79	*m*	
20	—			—			—			1.41	*s*		0.93	*t*	7.4	1.17	?	6.2
OCH_3[a]	3.66	*s*		3.67	*s*		3.66	*s*		3.67	*s*		3.66	*s*		3.67	*s*	

[a]Products were analyzed as methyl ester derivatives by ^1H NMR.

TABLE 3
^{13}C NMR Chemical Shifts (ppm) for Bioconversion Products from γ-Linolenic Acid and Arachidonic Acid by *Clavibacter* sp. ALA2

Carbon no.	Product **3**	Product **4**	Product **5**	Product **6**	Product **7**	Product **8**
1	174.6	174.2	174.4	174.3	174.4	174.5
2	33.9	33.9	33.9	33.6	33.3	33.4
3	24.5	24.6	25.8	24.8	24.8	24.8
4	29.0	29.1	29.0	26.5	26.5	26.5
5	26.8	26.9	26.9	130.2	128.9	128.9
6	130.5	129.7	129.7	129.0	128.8	128.8
7	127.9	128.0	128.0	25.8	25.7	25.7
8	25.7	25.7	24.6	128.3	128.2	128.3
9	129.7	130.0	130.3	128.0	128.0	128.0
10	125.0	125.8	125.8	25.6	25.6	25.6
11	33.6	31.4	31.5	128.8	129.8	130.0
12	79.4	73.8	73.6	125.3	125.8	125.9
13	78.3	81.5	73.8	33.4	31.3	31.4
14	27.7	28.4	33.4	78.3	73.8	73.5
15	17.0	31.9	21.8	79.4	81.5	73.8
16	34.8	80.8	39.0	27.8	28.4	33.3
17	108.1	28.5	67.5	17.1	31.8	21.8
18	24.7	10.1	23.1	34.9	80.9	40.0
19	—	—	—	108.1	28.5	67.5
20	—	—	—	24.8	10.0	22.9
OCH_3[a]	51.3	54.0	51.4	51.4	51.4	51.4

[a]Products were analyzed as methyl ester derivatives by ^{13}C NMR.

171 (14), 147 (25), 129 (45), 117 $[CH_3CHOTMSi]^+$(17), 73 $[TMSi]^+$ (100), 55 (7). All of these data indicated that product **5** is 12,13,17-trihydroxy-6,9-octadienoic acid. Proton and ^{13}C NMR data confirmed this structure (Tables 2, 3). Double bonds were seen at both C-6–C-7 and C-9–C-10. Three hydroxyl groups were seen at C-12, C-13, and C-17. Therefore, product **5** was identified as 12,13,17-trihydroxy-6(*Z*),9(*Z*)-octadienoic acid.

We also isolated a minor product (product **4**) with a GC retention time of 13 min. The MS of the methyl ester/OTMSi derivative of this product was as follows: EI-MS *m/z* (relative intensity): 396 $[M]^+$ (4), 306 $[M - TMSiOH]^+$ (1), 297 [M – ethyltetrahydrofuranyl]$^+$ (6), 268 [rearrangement TMSi + $(CH_2CH{=}CH)_2(CH_2)_4COOCH_3]^+$ (18), 201 [M –$(CH_2CH{=}CH)_2(CH_2)_4COOCH_3]^+$ (52), 175 (17), 129 (21), 99 [ethyltetrahydrofuranyl]$^+$ (28), 81 (39), 73 $[TMSi]^+$ (100), 55 (31). The peak at *m/z* 99 indicated a terminal ethyl tetrahydrofuranyl group in its molecule, as reported previously (11). All of these data indicated a possible structure of 12-hydroxy-13,16-epoxy-6,9-octadecadienoic acid. This structure was confirmed by ^{1}H and ^{13}C NMR (Tables 2, 3). Double bonds were seen at both C-6–C-7 and C-9–C-10. One hydroxyl group was seen at C-12. And the epoxy structure was seen at C-13 and C-16. ^{1}H NMR of the terminal methyl group showed a triplet multiplicity, indicating the structure to have a terminal THF ring. Other features of NMR data were also in agreement with the structure of 12-hydroxy-13,16-epoxy-6(*Z*),9(*Z*)-octadecadienoic acid.

Strain ALA2 also converted arachidonic acid to cyclic FA and trihydroxyl FA as reported previously (16). In the present study, we further purified three products by preparative TLC and confirmed the structures by ^{1}H and ^{13}C NMR analyses (Tables 2, 3) as 14,19;15,19-diepoxy-5(*Z*),8(*Z*),11(*Z*)-eicosatrienoic acid (product **6**, GC retention time of 15 min), 14-hydroxy-15,18-epoxy-5(*Z*),8(*Z*),11(*Z*)-eicosatrienoic acid (product **7**, GC retention time of 18 min), and 14, 15, 19-trihydroxy-5(*Z*),8(*Z*),11(*Z*)-eicosatrienoic acid (product **8**, GC–MS retention time of 18 min) (Fig. 4). Thus, the products obtained from γ-linolenic acid and arachidonic acid by strain ALA2 consist of multiple compounds with diepoxy bicyclic structure, tetrahydrofuranyl ring, or trihydroxy group in their molecules. These products had a very similar structure to those products obtained from linoleic acid by strain ALA2 (12).

Bioconversion products identified in the present study are summarized in Figures 2 and 4. The structures of these products are remarkably different from those obtained from n-3 and from n-6 PUFA. It seems that the presence of a double bond at the ω-3 position in substrate PUFA affects the outcome of the bioconversion products by ALA2 enzymes. In addition, both the hydroxyl group and cyclic structure are placed at the same position from the ω-carbon terminal, despite differences in the number of carbons and double bonds of the substrate PUFA. Strain ALA2 enzymes may be able to recognize the ω-carbon terminal of PUFA and proceed with hydroxylation and cyclization accordingly. It is known that hydroxylation at the ω-1, ω-2, and ω-3 positions are catalyzed by cytochrome P-450. Their enzymes have been characterized in bacteria, fungi, and mammals (17–19). Furthermore, Moghaddam *et al.* (20) reported that P-450 isolated from the mouse-catalyzed epoxidation at C-12 and C-13 position of linoleic acid, or at the C-14 and C-15 position of arachidonic acid, corresponded to the ω-5 and ω-6 position of their PUFA, followed by conversion to diols. Therefore, the bioconversion by strain ALA2, investigated in the present study, may be related to P-450 enzymes.

Substrate specificity. The relative activities were measured by the formation of products detectable by GC. Strain ALA2

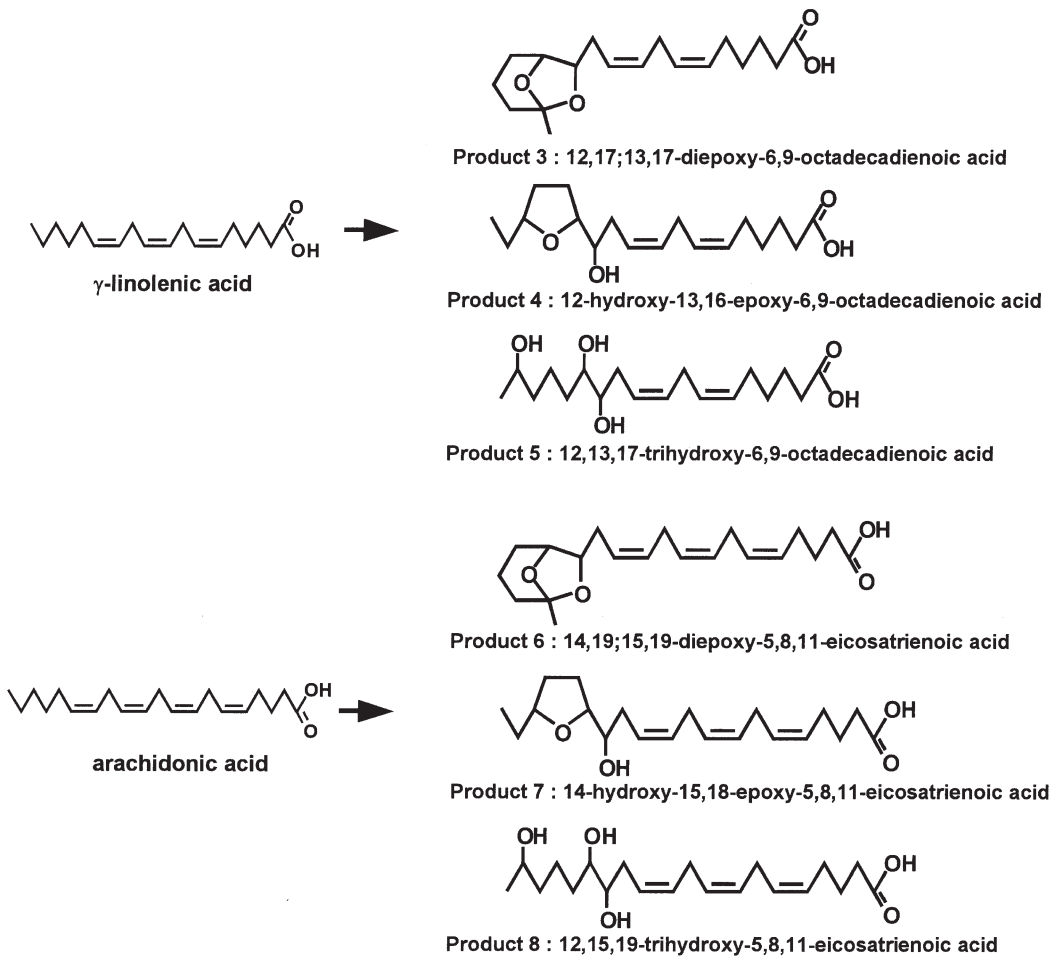

FIG. 4. Bioconversion products from n-6 PUFA by *Clavibacter* sp. ALA2.

showed the highest relative activity for the bioconversion to trihydroxy-9(*Z*)-octadecenoic acid from linoleic acid. The relative activities for α- and γ-linolenic acids were lower than that of linoleic acid. Increases of carbon chain number and of the number of double bonds led to a remarkable reduction in relative activity (Table 4).

Some hydroxyl FA are known to have antifungal and cytotoxic activity (21–23). However, little is known about biological

TABLE 4
Relative Activity for Bioconversion of n-3 and n-6 PUFA by *Clavibacter* sp. ALA2

Substrate	Product (product no.)	Relative activity (%)
n-3 PUFA		
α-Linolenic acid[a]	7,13,16-Trihydroxy-12,15-epoxy-9(*Z*)-octadecadienoic acid	100
	13,16-Dihydroxy-12,15-epoxy-10(*Z*)-octadecadienoic acid	19
EPA[a]	15,18-Dihydroxy-14,17-epoxy-5,8,11 eicosatrienoic acid (**1**)	16
DHA[a]	17,20-Dihydroxy-16,19-epoxy-4,7,10,13 docosatetraenoic acid (**2**)	14
n-6 PUFA		
Linoleic acid[b]	Trihydroxy-9(*Z*)-octadecenoic acid	130
	12,17;13,17-Diepoxy-9(*Z*)-octadecenoic acid	19
	12-Hydroxy-13,16-epoxy-9(*Z*)-octadecenoic acid	2
γ-Linolenic acid[a]	Trihydroxy-6,9-octadecadienoic acid (**5**)	76
	12,17;12,17-Diepoxy-6,9-octadecadienoic acid (**3**)	27
	12-Hydroxy-13,16-epoxy-6,9-octadecadienoic acid (**4**)	9
Arachidonic acid[a]	Trihydroxy-5,8,11-eicosatrienoic acid (**8**)	—
	14,19;15,19-Diepoxy-5,8,11-eicosatrienoic acid (**6**)	11
	14-Hydroxy-15,18-epoxy-5,8,11-eicosatrienoic acid (**7**)	7

[a]PUFA 125 μL, 30°C, 7 d incubation.
[b]Linoleic acid 125 μL, 30°C, 2 d incubation.

functions and chemical properties of THF FA and diepoxy bicyclic FA (24). Therefore, it will be interesting to explore the biological functions of cyclic FA identified in the present study.

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