Biotransformation of C20- and C22-polyunsaturated fatty acids and fish oil hydrolyzates to *R*,*R*-dihydroxy fatty acids as lipid mediators by double-oxygenating 15*R*-lipoxygenase

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Table of Contents

Supporting Tables

Table S1.	Primer design for amplification of specific DNA sequences	5
Table S2.	Regression equations for calibration curves of PUFAs, MonoHFAs, and DiHFAs	7
Table S3.	Docking energy and distance of ARA (1) from 10 LOXs existed in S. cellulosum	8
Table S4.	Specific activities of double-oxygenating LOXs from mouse, Endozoicomonas numazuensis, Archangium violaceum, and S. cellulosum	9
Table S5.	1D NMR data of 5 <i>R</i> ,15 <i>R</i> -DiHETE (4) in MeOD (850 MHz NMR)	10
Table S6.	1D NMR data of 5 <i>R</i> ,15 <i>R</i> -DiHEPA (8) in MeOD (850 MHz NMR).	11
Table S7.	1D NMR data of 7 <i>R</i> ,17 <i>R</i> -DiHDHA (12) in MeOD (850 MHz NMR).	12

Supporting Figures

Fig. S1. Alignment of the amino acid sequences of 10 putative LOXs in S. cellulosum	3
Fig. S2. Reverse-phase HPLC profiles of MonoHFAs obtained from the conversion of ARA (1) by the selected 6 LOXs derived from S. cellulosum with star	ndard.
	4
Fig. S3. 15 <i>R</i> -LOX sequence derived from <i>S. cellulosum</i> so ce1871	5
Fig. S4. Normal-phase HPLC profiles of MonoHFAs obtained from the conversion of ARA (1) by the selected 3 LOXs derived from S. cellulosum with H	IETE
standards1	7
Fig. S5. Chiral-phase HPLC profiles of MonoHFAs obtained from the conversion of ARA (1) by the selected 3 putative LOXs derived from S. cellulo	osum
(UniProtKB protein numbers, A0A2LESS6, A0A2L0ESU2, and S4XZS0) with 9S-, 9R-, 12S-, 12R-, 15S-, and 15R-HETE (3) standards.	8
Fig. S6. Reverse- and chiral-phase HPLC profiles of the reaction products obtained from the conversion of ARA (1) by the putative LOX cloned with the products obtained from the conversion of ARA (1) by the putative LOX cloned with the products obtained from the conversion of ARA (1) by the putative LOX cloned with the products obtained from the conversion of ARA (1) by the putative LOX cloned with the products obtained from the conversion of ARA (1) by the putative LOX cloned with the products obtained from the conversion of ARA (1) by the putative LOX cloned with the products obtained from the conversion of ARA (1) by the putative LOX cloned with the products obtained from the conversion of ARA (1) by the putative LOX cloned with the products obtained from the conversion of ARA (1) by the putative LOX cloned with the products obtained from the conversion of ARA (1) by the putative LOX cloned with the products obtained from the conversion of ARA (1) by the putative LOX cloned with the products obtained from the conversion of ARA (1) by the putative LOX cloned with the products obtained from the conversion of ARA (1) by the putative LOX cloned with the products obtained from the conversion of ARA (1) by the putative LOX cloned with the products obtained from the conversion of ARA (1) by the putative LOX cloned with the products obtained from the conversion of ARA (1) by the putative LOX cloned with the products obtained from the conversion of ARA (1) by the putative LOX cloned with the products obtained from the conversion of ARA (1) by the putative LOX cloned with the products obtained from the conversion of ARA (1) by the putative LOX cloned with the products obtained from the conversion of ARA (1) by the putative LOX cloned with the products obtained from the conversion of ARA (1) by the putative LOX cloned with the products obtained from the conversion of ARA (1) by the putative LOX (1) by	rimer
of S4XZS0 with standards	0
Fig. S7. SDS-PAGE analysis and gel filtration chromatography of S. cellulosum 15R-LOX expressed in E. coli C2566	2

Fig. S8. Effects of temperature and pH on the production of 15 <i>R</i> -HETE (3) from ARA (1) by 15 <i>R</i> -LOX from <i>S. cellulosum</i>	23
Fig. S9. LC-MS/MS profiles of MonoHFA and DiHFA obtained from the conversion of ARA (1) by 15 <i>R</i> -LOX from <i>S. cellulosum</i>	24
Fig. S10. LC-MS/MS profiles of MonoHFA and DiHFA obtained from the conversion of EPA (5) by 15 <i>R</i> -LOX from <i>S. cellulosum</i> .	25
Fig. S11. LC-MS/MS profiles of MonoHFA and DiHFA obtained from the conversion of DHA (9) by 15 <i>R</i> -LOX from <i>S. cellulosum</i> .	26
Fig. S12. Normal-phase HPLC profiles of DiHFA obtained from the conversion of ARA (1) by S. cellulosum 15R-LOX with 5R,15S-DiHETE, 5A	S,15 <i>R</i> -DiHETE,
5S,15S-DiHETE, and 5R,15R-DiHETE (4) standards.	27
Fig. S13. ¹ H NMR and ¹³ C NMR peaks of 5 <i>R</i> ,15 <i>R</i> -DiHETE (isomer of leukotriene B4; 4) in deuterated methanol (MeOD) (850 MHz NMR).	28
Fig. S14. 2D NMR data of 5 <i>R</i> ,15 <i>R</i> -DiHETE (4) in MeOD (850 MHz NMR)	29
Fig. S15. ¹ H NMR and ¹³ C NMR peaks of 5 <i>R</i> ,15 <i>R</i> -DiHEPA (enantiomer of resolvin E4; 8) in MeOD (850 MHz NMR).	30
Fig. S16. 2D NMR data of 5 <i>R</i> ,15 <i>R</i> -DiHEPA (8) in MeOD (850 MHz NMR)	31
Fig. S17. ¹ H NMR and ¹³ C NMR peaks of 7 <i>R</i> ,17 <i>R</i> -DiHDHA (enantiomer of resolvin D5; 12) in MeOD (850 MHz NMR).	32
Fig. S18. 2D NMR data of 7 <i>R</i> ,17 <i>R</i> -DiHDHA (12) in MeOD (850 MHz NMR).	
Fig. S19. Effects of temperature and pH on the biotransformation of ARA (1) into 5 <i>R</i> ,15 <i>R</i> -DiHETE (4) by <i>E. coli</i> expressing 15 <i>R</i> -LOX from the biotransformation of ARA (1) into 5 <i>R</i> ,15 <i>R</i> -DiHETE (4) by <i>E. coli</i> expressing 15 <i>R</i> -LOX from the biotransformation of ARA (1) into 5 <i>R</i> ,15 <i>R</i> -DiHETE (4) by <i>E. coli</i> expressing 15 <i>R</i> -LOX from the biotransformation of ARA (1) into 5 <i>R</i> ,15 <i>R</i> -DiHETE (4) by <i>E. coli</i> expressing 15 <i>R</i> -LOX from the biotransformation of ARA (1) into 5 <i>R</i> ,15 <i>R</i> -DiHETE (4) by <i>E. coli</i> expressing 15 <i>R</i> -LOX from the biotransformation of ARA (1) into 5 <i>R</i> ,15 <i>R</i> -DiHETE (4) by <i>E. coli</i> expressing 15 <i>R</i> -LOX from the biotransformation of ARA (1) into 5 <i>R</i> ,15 <i>R</i> -DiHETE (4) by <i>E. coli</i> expressing 15 <i>R</i> -LOX from the biotransformation of ARA (1) into 5 <i>R</i> ,15 <i>R</i> -DiHETE (4) by <i>E. coli</i> expressing 15 <i>R</i> -LOX from the biotransformation of ARA (1) into 5 <i>R</i> ,15 <i>R</i> -DiHETE (4) by <i>E. coli</i> expressing 15 <i>R</i> -LOX from the biotransformation of ARA (1) into 5 <i>R</i> ,15 <i>R</i> -DiHETE (1) by <i>E. coli</i> expressing 15 <i>R</i> -LOX from the biotransformation of ARA (1) into 5 <i>R</i> ,15 <i>R</i> -DiHETE (1) by <i>E. coli</i> expressing 15 <i>R</i> -LOX from the biotransformation of ARA (1) into 5 <i>R</i> ,15 <i>R</i> -DiHETE (1) by <i>E. coli</i> expressing 15 <i>R</i> -LOX from the biotransformation of ARA (1) into 5 <i>R</i> ,15 <i>R</i> -DiHETE (1) by <i>E. coli</i> expressing 15 <i>R</i> -LOX from the biotransformation of ARA (1) into 5 <i>R</i> ,15 <i>R</i> -DiHETE (1) by <i>E. coli</i> expressing 15 <i>R</i> -LOX from the biotransformation of ARA (1) into 5 <i>R</i> ,15 <i>R</i> -DiHETE (1) by <i>E. coli</i> expressing 15 <i>R</i> -LOX from the biotransformation of ARA (1) into 5 <i>R</i> ,15 <i>R</i> -DiHETE (1) by <i>E. coli</i> expressing 15 <i>R</i> -LOX from the biotransformation of ARA (1) into 5 <i>R</i> ,15 <i>R</i> -DiHETE (1) by <i>E. coli</i> expressing 15 <i>R</i> -LOX from the biotransformation of ARA (1) into 5 <i>R</i> ,15 <i>R</i> -DiHETE (1) by <i>E. coli</i> expressing 15 <i>R</i> -LOX from the biotransformation of ARA (1) into 5 <i>R</i> ,15 <i>R</i> -LOX from the biotransformation of ARA (1) by the biotransformati	m S. cellulosum
	34
Fig. S20. Optimization of cell and substrate concentrations for the biotransformation of ARA (1) into 5 <i>R</i> ,15 <i>R</i> -DiHETE (4) by <i>E. coli</i> expre	ssing 15 <i>R</i> -LOX
from S. cellulosum	35
Fig. S21. Optimization of cell and substrate concentrations for the biotransformation of ARA (1) into 5 <i>R</i> ,15 <i>R</i> -DiHETE (4) by <i>E. coli</i> expre	ssing 15 <i>R</i> -LOX
from S. cellulosum at the optimal ratio of cells to substrate.	
Fig. S22. Binding of absorbent resins to ARA (1) as a substrate.	
Fig. S23. Effect of resin SP825 concentration on the biotransformation of ARA (1) into 5 <i>R</i> ,15 <i>R</i> -DiHETE (4) by 15 <i>R</i> -LOX from <i>S. cellulosum</i>	with varying the
concentrations of cells and ARA (1).	
Fig. S24. Biotransformation of PUFAs into DiHFAs with the addition of resin by E. coli expressing 15R-LOX from S. cellulosum in filtrate	
Fig. S25. Biotransformation of PUFAs into DiHFAs with the addition of resin by <i>E. coli</i> expressing 15 <i>R</i> -LOX from <i>S. cellulosum</i> in resin	41
Fig. S26. Reliability of the models of 15 <i>R</i> -LOX from <i>S. cellulosum</i> built by Alphafold and Discovery studio (DS)	43

Fig. S27. Confirmation of the active site in 15 <i>R</i> -LOX from <i>S. cellulosum</i> in DS model by Alphafold model	45
Fig. S28. Reverse-phase HPLC profiles of the products obtained from the conversion of ARA (1) by the wild-type and variant LOXs at position 3	54 from <i>S</i> .
cellulosum	46
Fig. S29. Reverse-phase HPLC profiles of the products obtained from the conversions of ARA (1) and 15 <i>R</i> -HETE (3) by the wild-type and varian	it LOXs at
position 606 from S. cellulosum	47
Fig. S30. Effects of temperature and pH on the biotransformation of ARA (1) into 15 <i>R</i> -HETE (3) by <i>E. coli</i> expressing 15 <i>R</i> -LOX from <i>S. cellulosu</i>	<i>m</i> 48
Fig. S31. Effects of cell and substrate concentration on the biotransformation of ARA (1) into 15 <i>R</i> - HETE (3) by <i>E. coli</i> expressing engineered	15 <i>R</i> -LOX
(L606F variant)	51
Fig. S32. Optimization of cell and substrate concentration on the biotransformation of ARA (1) into 15 <i>R</i> -HETE (3) by <i>E. coli</i> expressing engineered	15 <i>R</i> -LOX
(L606F variant)	52

Supporting Tables

UniprotKB	Name	Restriction enzyme	Sequence $(5' \rightarrow 3')$
S4XZS0	pET28a-SCLOX- F (LOX)	Nde I	AGCGGCCTGGTGCCGCGCGGCAGC <u>CATATG</u> ATGAGTAACCCGTCCCTGCCTCAAAACGAC
	pET28a-SCLOX- R (LOX)	Nde I	GTGGTGGTGCTCGAGTGCGGCCGCAAGCTT TCAGATGTTGATGCTCTGCGGGGATCTGCGA
	pET28a-SCLOX- F (Vector)	Hind III	TCGCAGATCCCGCAGAGCATCAACATCTGA <u>AAGCTT</u> GCGGCCGCACTCGAGCACCACCAC
	pET28a-SCLOX- R (Vector)	Hind III	GTCGTTTTGAGGCAGGGACGGGTTACTCATC ATATGGCTGCCGCGCGGCACCAGGCCGCT
A0A150S935	pET28a-F (LOX)	Nde I	AGCGGCCTGGTGCCGCGCGGCAGC <u>CATATG</u> ATGAGCCTGTTCGACGTTCCGGTCCTGCCG
	pET28a-R (LOX)	Nde I	ATCTCAGTGGTGGTGGTGGTGGTGCTCGAGT CAGATATTGATGCTCTGCGGGATGGCCGA
	pET28a-F (Vector)	Xho I	TCGGCCATCCCGCAGAGCATCAATATCTGA CTCGAGCACCACCACCACCACTGAGAT
	pET28a-R (Vector)	Xho I	CGGCAGGACCGGAACGTCGAACAGGCTCAT CATATGGCTGCCGCGCGGCACCAGGCCGCT
A0A150QTF 6	pET28a-F (LOX)	Nde I	AGCGGCCTGGTGCCGCGCGGCAGC <u>CATATG</u> [[] ^{a]} ATGAGCCTGTTCGACGTTCCGGTCCTGCCG
	pET28a-R (LOX)	Nde I	GTGGTGGTGCTCGAGTGCGGCCGCAAGCTT TCAGATATTGATGCTCTGCGGGATGGCCGA
	pET28a-F (Vector)	Hind III	TCGGCCATCCCGCAGAGCATCAATATCTGA <u>AAGCTT</u> ^[a] GCGGCCGCACTCGAGCACCACCA C
	pET28a-R (Vector)	Hind III	CGGCAGGACCGGAACGTCGAACAGGCTCAT CATATGGCTGCCGCGCGGCACCAGGCCGCT
A0A4P2Q3W 5	pET28a-F (LOX)	Nde I	AGCGGCCTGGTGCCGCGCGGGCAGC <u>CATATG</u> ATGAGGTACCCCTCTCTGCCGCAGAAGGAC
	pET28a-R (LOX)	Nde I	GTGGTGGTGCTCGAGTGCGGCCGCAAGCTT TCAGATGTTGATGCTCTGGGGGGATCTGGGA
	pET28a-F	Hind III	TCCCAGATCCCCCAGAGCATCAACATCTGA

Table S1 Primer design for amplification of specific DNA sequences^a

	(Vector)		AAGCTTGCGGCCGCACTCGAGCACCACCAC
	pET28a-R (Vector)	Hind III	GTCCTTCTGCGGCAGAGAGGGGGTACCTCAT CATATGGCTGCCGCGCGGGCACCAGGCCGCT
A0A2L0ESS 6	pET28a-F (LOX)	Nde I	AGCGGCCTGGTGCCGCGCGGCAGCC <u>ATATG</u> ATGGGGGCATTGATGACAGTCGACTACAAG
	pET28a-R (LOX)	Nde I	GTGGTGGTGCTCGAGTGCGGCCGCAAGCTT TCAGATGGTGATTCCGCAGGGGGATCTTGTC
	pET28a-F (Vector)	Hind III	GACAAGATCCCCTGCGGAATCACCATCTGA <u>AAGCTT</u> GCGGCCGCACTCGAGCACCACCAC
	pET28a-R (Vector)	Hind III	CTTGTAGTCGACTGTCATCAATGCCCCCATC ATATGGCTGCCGCGCGCGCACCAGGCCGCT
A0A2L0ESU 2	pET28a-F (LOX)	Nde I	AGCGGCCTGGTGCCGCGCGGCAGC <u>CATATG</u> ATGCTCGCCGCGCTGCGCCGCCTGTTATCG
	pET28a-R (LOX)	Nde I	GTGGTGGTGCTCGAGTGCGGCCGCAAGCTT TCAGATGTTGATTCGGGACTGCACCGTCCT
	pET28a-F (Vector)	Hind III	AGGACGGTGCAGTCCCGAATCAACATCTGA <u>AAGCTT</u> GCGGCCGCACTCGAGCACCACCAC
	pET28a-R (Vector)	Hind III	CGATAACAGGCGGCGCGCGCGCGGCGAGCAT CATATGGCTGCCGCGCGGCACCAGGCCGCT

^{*a*} The restriction sites are underlined.

Туре	Compound	Regression equation ^{<i>a</i>}	r^2
PUFAs	1	y = 0.0000358x - 0.0001	0.9912
	5	y = 0.0000301x + 0.0007	0.9904
	9	y = 0.0000277x - 0.0008	0.9812
MonoHpFAs	2 and 3	y = 0.00009061x - 0.0008	0.9812
and MonoHFAs ^b	6 and 7	y = 0.000192x + 0.0015	0.9761
	10 and 11	y = 0.0000688x - 0.0091	0.9914
DiHFAs	4	y = 0.0000751x - 0.0002	0.9880
	8	y = 0.00006181x + 0.0007	0.9970
	12	y = 0.00004814x + 0.00108	0.9671

Table S2 Regression equations for calibration curves of PUFAs, MonoHFAs, and DiHFAs^{a,b}

PUFAs, polyunsaturated fatty acid; MonoHFAs, hydroxy fatty acids; MonoHpFAs, Monohydroperoxy fatty acids; DiHFAs, dihydroxy fatty acids; ARA (1), arachidonic acid; EPA (5), eicosapentaenoic acid; DHA (9), docosahexaenoic acid; 15R-HpETE (2), 15R-hydroperoxy-5Z,8Z,11Z,13E-eicosatetraenoic acid; 15R-HpEPA (6), 15R-hydroperoxy-5Z,8Z,11Z,13E,17Z-eicosapentaenoic acid; 17R-HpDHA (10), 17R-hydroperoxy-4Z,7Z,10Z,13Z,15E,19Z-docosahexaenoic acid; 15*R*-HETE (3), 15R-hydroxy-5Z,8Z,11Z,13Eeicosatetraenoic acid; 15R-HEPA (7), 15R-hydroxy-5Z,8Z,11Z,13E,17Z-eicosapentaenoic acid; 17R-HDHA (11), 17R-hydroxy-4Z,7Z,10Z,13Z,15E,19Z-docosahexaenoic acid; 5R,15R-DiHETE (isomer of LTB4; 4), 5R,15R-dihydroxy-6E,8Z,11Z,13E-eicosatetraenoic acid; 5R,15R-DiHEPA (enantiomer of RvE4; 8), 5R,15Rdihydroxy-6E,8Z,11Z,13E,17Z-eicosapentaenoic acid; 7R,17R-DiHDHA (enantiomer of RvD5; 12), 7R,17Rdihydroxy-4Z,8E,10Z,13Z,15E,19Z-docosahexaenoic acid. a x, peak area in the HPLC profile; y, molar concentration of standard (mM). ^b Data represent the mean \pm SD (n = 3) values. MonoHFAs or MonoHFAs were obtained from the conversion of PUFAs by E. coli expressing ARA15R-LOX from S. cellulosum with or without cysteine as a reducing agent, respectively. The concentrations of HFAs calibrated by the peak areas were the same as those of HpFAs.¹

UniprotKB	Length	Docking energy (kcal/mol), <i>E</i>	Distance (Å, δ)	Ε/ δ	Regio- and stereoselectivity
A0A150S935	703	-76.1	10.9	-6.9	ND ^a
A0A150QTF6	704	-104.4	11.2	-9.3	ND
S4XZS0	673	-157.6	7.5	-21.0	15 <i>R</i>
A0A4P2Q3W5	673	-105.3	12.4	-8.5	ND
A0A4P2QUL1	673	+13.5	6.7	+2.0	ND
A0A2L0ESU2	687	-50.1	8.4	-5.9	12 <i>S</i>
A0A150TEU6	673	+20.4	10.5	+1.9	ND
A0A150T884	673	+18.5	9.7	+1.9	ND
A0A150PUW0	673	+5.0	4.9	+1.0	ND
A0A2L0ESS6	680	-40.5	6.9	-5.8	9 <i>S</i>

Table S3 Docking energy and distance of ARA (1) from 10 LOXs existed in S. cellulosum

^a ND, not detected. The blue-colored letters indicate negative docking energy values.

Table S4 Specific activities of double-oxygenating LOXs from mouse, Endozoicomonas numazuensis, Archangium violaceum, and S. cellulosum^{a,b}

	Substrate	Specific activity (µmol/min/mg)				
Oxygenation		S. cellulosum 15R-LOX	A. violaceum 15S-LOX ²	<i>E. numazuensis</i> 12 <i>S</i> -LOX ³	Mouse 8S-LOX ⁴	
First	1	505 ± 1.5	282 ± 1.5	286 ± 2.2	14.9 ± 0.01	
Second	HETE	80.1 ± 2.2	67.6 ± 2.2	54.3 ± 1.4	0.098 ± 0.003	

ARA (1), arachidonic acid. ^{*a*} Data were detected by measuring reaction solution from 1 as substrate using HPLC in absorbance at 234 nm or reaction

solution from HFAs as substrates in absorbance at 270 nm.

^b Data represent the means of three experiments, and error bars represent the standard deviation.

C No.	1Η (δ)	Multiplet	$J(\mathrm{Hz})$	Protons	¹³ C (δ)
1					177.7
2	2.32	t	7.36	2H	34.97
3	1.75-1.61	m		2H	22.33
4	1.59-1.51	m		2H	37.83
5	4.13	td	7.23, 6.93	2H	73.01
6	5.68	dd	15.09, 6.93	1H	137.93
7	6.583	dd	14.71, 10.95	1H	126.43
8	6.01	dd	10.96, 10.72	1H	129.7
9	5.39	dt	9.95, 7.54	1H	130.46
10	3.1	t	7.44	2H	27.5
11	5.38	dt	10.65, 7.42	1H	130.23
12	6.01	dd	10.96, 10.72	1H	129.77
13	6.56	dd	15.22, 10.91	$1\mathrm{H}$	126.22
14	5.67	dd	15.22, 5.73	1H	138.31
15	4.10	td	6.46, 5.73	1H	73.4
16	1.56-1.45	m		2H	38.54
17	1.42-1.29	m		2H	26.43
18	1.36-1.26	m		2H	33.12
19	1.37-1.29	m		2H	23.85
20	0.91	t	6.71	3H	14.57

 Table S5 1D NMR data of 5R,15R-DiHETE (4) in MeOD (850 MHz NMR)

C No.	1Η (δ)	Multiplet	$J(\mathrm{Hz})$	Protons	¹³ C (δ)
1					178.1
2	2.31	t	7.27	2H	35.2
3	1.75-	m		2H	22.4
4	1.61			211	27.0
4	1.59-	m		2H	37.9
5	4.15-	m		1H	73.3
-	4.11				
6	5.68	dd	14.87, 6.64	IH	138
7	6.57	dd	14.87,	1H	126.4
			11.04		
8	6	dd	12.03,	1H	129.7
9	5 41-	m	11.04	1H	130.4
)	5.35	111		111	150.1
10	3.1	t	7.5	2Н	27.5
11	5.41-	m		1H	130.4
12	5.35	44	12.24	111	120.7
12	0.01	uu	12.24	111	129.1
13	6.58	dd	14.99,	1H	126.4
1.4	5 (0)	11	12.24	111	1077
14	5.69	dd	14.99, 6 80	IH	137.7
15	4.15-	m	0.00	1H	73.3
1.6	4.11			ATT	264
16	2.34-	m		2H	36.4
17	5.38-	m		1H	125.6
	5.34				
18	5.49	dt	10.73,	1H	134.8
19	2.06	qd	7.51,	2H	21.8
20	0.96	t	7.17 7.51	3Н	14.7

Table S6 1D NMR data of 5*R*,15*R*-DiHEPA (8) in MeOD (850 MHz NMR)

C No.	1Η (δ)	Multiplet	$J(\mathrm{Hz})$	Protons	¹³ C (δ)
1					177.3
2	2.35-	m		2H	35.1
3	2.31 2.35-	m		2Н	24.2
4	2.31 5.50-	m		1H	131.2
E	5.45			111	107 (
5	5.30- 5.45	III		ΙП	127.0
6	2.35- 2.29	m	6.63	2H	36.4
7	4.16	td	6.63, 6.46	1H	73.2
8	5.7	dd	15.04, 6.46	1H	137.6
9	6.57	dd	15.04, 10.54	1H	126.4
10	6.01	t	10.54	1H	129.7
11	5.39- 5.35	m		1H	130.4
12	3.09	t	7.59	2H	27.5
13	5.39- 5.35	m		1H	130.4
14	6	dd	10.37	1H	129.7
15	6.58	dd	15.14, 10.37	1H	126.4
16	5.69	dd	15.14, 6.38	1H	137.6
17	4.14	td	6.63, 6.38	1H	73.3
18	2.32-	m	6.63	2H	36.4
19	5.39-	m		1H	125.6
20	5.55 5.50-	m		1H	134.8
21	5.45 2.06	qd	7.55,	2H	21.8
22	0.96	t	7.21 7.55	3Н	14.7

 Table S7 1D NMR data of 7R,17R-DiHDHA (12) in MeOD (850 MHz NMR)

Supporting Figures

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A0A150QTF6	A0A150QTF6_SORCE	387	IADGNYHELISHLGLTHLLTEPFVLATARQLDPTHPLNLLLTPHFAGTLLINYAAQTSLI	446
S4XZS0	S4XZS0 SORCE	355	VADGNFHELISHLGOTHLVLEAFAMATPROLAPEHPVNVLLTPHFOGTLAINNAAEADLI	414
A0A4P203W5	A0A4P203W5 SORCE	355	VADGNYHEMISHLGOTHLVVEAFAMATPROLAPEHPVNVLLTPHLOGTLAINDAAFADLV	414
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AOAZLOESUZ	A0A2L0ESU2 SORCE	366	VSATLDAELGNHLAOCHLNLEOYAIAAHRNLR-RSPLRWLLMPHLREVVLINHSANEFLL	424
A0A150TEU6	A0A150TEU6 SORCE	355	VADGNFHELISHLGOTHLVLEAFAMATPROLAPEHPVNVLLTPHFOGTLAINNAAEADLI	414
A0A150T884	A0A150T884 SORCE	355	VADGNEHELTSHLGOTHLVLEAFAMATPROLAPEHPVNVLLTPHFOGTLAINNAAFADLT	414
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AUAISUPUWU	AUAISUPUWU_SORCE	355	VADGNEHELISHLGQIHLVLEAFAMAIPRQLAPEHEVNVLLIPHEQGILAINNAAEADLI	414
A0A2L0ESS6	A0A2L0ESS6 SORCE	360	CSEGNAHOMVAHAIRTHEVTE PFVMATMRNLPDKHPIYKLLRRHFRYTLAINEGARVTLL	419
	-			
A0A150S935	A0A150S935 SORCE	446	TPGGPVDLLLTGTSASLNALAASAVOEVRYNOTFLPOALAARGVD-SKEA	494
AGA1 SOOTES	AGAISOOTES SORCE	447	TREEPUDITISCIES	405
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54XZS0	S4XZS0_SORCE	415	APGGPVDNLLAGTIASSIQLAVDAVLSYSVNQQFLPVALAQRGVD-DPSI	463
A0A4P2Q3W5	A0A4P2Q3W5 SORCE	415	APGGPVDSLLAGTIASSVQLAVDSVLGYRVNQQFLPAALASRGVD-DPGA	463
A0A4P2OUL1	A0A4P2OUL1 SORCE	415	APGGPVDNLLAGTIASSTRLAVDAVLSYSVNOOFLPVALABRGVD-DPST	463
BABAT APPRITS	LALAT APPRILATEADAP	405		450
RUNZLULJUZ	AURZEUESUZ SUNCE	120	GFGGIIIRASALIDA - AIQARLANLUG	105
AGAISOTEUS	ADAISUTEU6_SORCE	415	APGGPVDNLLAGIIASSIQLAVDAVLSYSVNQQFLPVALAQRGVD-DPSI	463
A0A150T884	A0A150T884 SORCE	415	APGGPVDNLLAGTIASSTQLAVDAVLSYSVNQQFLPVALAHRGVD-DPSS	463
202150PUW0	AGA1 SOPUWO SORCE	415	APGGPUDNLLAGTIA-SSTOLAUDAVLSYSUNOOFLPUALAORGUD-DPST	463
BABAT APPER	ACALOUT OF CODOR	100		170
RURZLUESSE	AUAZLULSS6_SURCE	420	REGGVEDDE INIGGEDRGRVELGKRGERRWREIDNRERPDEERRGVE-DEWV	470
A0A1505935	AOA1505935 SORCE	495	LPDYPYRDDALTVWNAIHDWVSDYVGIYYESDGDVAGD-VELOXWVOELUTAGHTO	540
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S4XZS0	SAXZSO SORCE	464	LFSYFYRDDATLLWGAIHTWVSRYLAVYYTSDADVLGD-YELQHWIAELSSPS	515
A0A4P203W5	A0A4P203W5 SORCE	464	LPSYPYRDDALLLWGAIHPWVSRYLALYYTSDADVOGD-YELOGWFAELSSPS	515
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A0A150TEU6	A0A150TEU6 SORCE	464	LPSYPYRDDAMLLWGAIHTWVSRYLAVYYTSDADVLGD-YELOHWIAELSSPS	515
3031507994	BOBISOTREA SODOF	464	I DEVENDENTI I NGATHTWUSEVI A TVYTEDA UT CD_VELONNTAET SEDS	616
AUAIOUICCA	AUAISUISSA SURCE	101	LESTETRUDANLLWGATHIWVSRILATITISDAAVLGD-TLLGMMIALLSES	010
AGAISOPOWO	A0A150PUW0_SORCE	404	LPSYPYRDDAILLWGAIHIWVSRYLAVYYISDADVLGD-YELQHWIAELSSPS	515
A0A2L0ESS6	A0A2LOESS6 SORCE	471	LPYYPYRDDALALWDAIEEYVGGVLDHFYRSDADLAED-AEMOAWWADLTARGLPA	525
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A0A150S935	A0A150S935 SORCE	550	QIKTVGYLSTLLTQVIF	577
3031500TE6	2021 SOOTES SODCE	5.5.1	DIGEGNE-GNA	570
AVALOUX11	AUNTOS CODET			
348230	SAA2SU_SORCE	210	AIRIVAILADLLINVIP	543
A0A4P2Q3W5	A0A4P2Q3W5 SORCE	516	ALRTVGYLATLLTHVIF	543
A0A4P2OUL1	A0A4P2OUL1 SORCE	516	AIRTVAYLANLITHVIF	543
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A0A150TEU6	A0A150TEU6 SORCE	516	AIRTVAYLADLLTHVIF	543
A0A150T884	A0A150T884 SORCE	516	AIRTVAYLADLLTHVIF	543
AAA1 SADITUA	AAAA SODEWA SODEF	616		549
AUAISUPUWU	AUAISUPUWU SURCE	210	RIRIVALLADLLINVIC	543
AOA2LOESSE	A0A2LOESS6_SORCE	526	==========EK====EK====LPCA======ELSRVADLVDILSTVLF	548
2021505935	ADA1505935 SORCE	578	TASACHAAUNEPORTIMSYTPALPLAGEAPEDAP-AGAPATOVLDVLAPLGESTLOO	633
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S4XZS0	S4XZS0 SORCE	544	TASACHAAVNFPQSSLMSFTPALPLAAYAPAPSITAGLPPSEIFOHLPPLQQALLQI	600
A0A4P2O3W5	A0A4P203W5 SORCE	544	TASACHAAVNFPORSIMSFTPAYPLAAYAPAPSVAEGLPPSEIFRHLPPLOOALLOI	600
AOA4P2OUT 1	A0A4P2OUL1 SOPCE	544	TASACHAANNEPOSSIMSETPALPLAAVAPAPAPSTTACI DESETFORT PDIO	600
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AUAZLULSU2	RUNZLULSUZ SUKCE	201	EATERDWARKLOWEDGEVEIGELWGRG-NVLVAEDDPDVAPPPDEATEMLWI-	030
A0A150TEU6	A0A150TEU6 SORCE	544	TASACHAAVNFPOSSLMSFTFALPLAAYAPAPSITAGLPPSEIFOHLPPLOOALLOI	600
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ADAZLOESSE	AUAZLUESS6_SORCE	549	IVSVOMBALNYLQYEMYAFVFNAFLCMRMAPPREKGKIGPTELAAMLPTRSQTLWQI	605
	-			
A0A1505935	A0A150S935 SORCE	634	AVLGGLGAVYHTVLGOYGGHFSDLRARAALASFOKRLOATEOOINLANGL	68
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SAXZSO	SAXZSO_SORCE	601	NVMMLLGGVYYSRLGDYDRNIPGAYFTDARVREPLEAFQRELMEIEATIGKRN	65:
A0A4P2Q3W5	A0A4P2Q3W5 SORCE	601	NVMMLLGGVYYSRLGDYDRNIPGAYFTDPRVREPLEAFORALIEIEATIGKRN	65:
A0A4P2OUT.1	A0A4P2OUL1 SOPCE	601	NVMMLLGGVYYSRLGDYDRNIPGAYFTDARIREPLEAFORELMDIEATTGKRN	65
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AUAISUQTE	AUAISUQIF6_SORCE	005	GERTAIDILLEDAIFQSINI	704
S4XZS0	S4XZS0 SORCE	654	LQRFPYVVLLPSQIPQSINI	673
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A0A150T884 A0A150PUW0	AOA150TEU6_SORCE AOA150T884_SORCE AOA150PUW0_SORCE	654 654 654	LQRFYYVLLSQIFQSII LQRFYYVLLSQIFQSII LQRFYYVLLSQIFQSII	673

Fig. S1 Alignment of the amino acid sequences of 10 putative LOXs in *S. cellulosum*. The UniProtKB protein numbers of the LOXs are A0A150S935, A0A150QTF6, S4XZS0, A0A4P2Q3W5, A0A4P2QUL1, A0A2L0ESU2, A0A150TEU6, A0A150T884, A0A150PUW0, and A0A2L0ESS6. Red boxes indicate metal-binding residues (catalytic residues).



Fig. S2 Reverse-phase HPLC profiles of MonoHFAs obtained from the conversion of ARA (1) by the selected 6 LOXs derived from *S. cellulosum* with standard. The reactions were performed at 30 °C in 50 mM EPPS buffer (pH 8.0) containing 1.0 mM 1, 0.1 g/L LOX, and 100 mM cysteine as reducing agent for 10 min. The peak area of 1 as substrate was not decreased by A0A150S935, A0A15QTF6, and A0A4P2Q3W5 proteins. However, A0A2LESS6, A0A2L0ESU2 and S4XZS0 proteins converted 1 into monoHFA.

А

New_15 <i>R</i> LOX	1	MSNPSLPQNDSPAEQQQRKSELARAQQTYYYNHDTRLSPLGVAQSVPNGQGFAPILVGSA	60
Template_S4XZS0	1	MSNPSLPQNDSPAEQQQRKSELARAQQTYYYDHDTRLSPLGYAQSYPNGQGFAPILYGSA	60
New_15 <i>R</i> LOX	61	ALVILELVGNVVAKASKLVGSDGSLRSPKGAPLDEATSARIQGVLRDLKQRHRELTLALT	12(
Template_S4XZS0	61	ALVILTLYGNYYAKASKLYGSDGSLKSPKGAPLDTAISAKIGGYL DLKGKHRELTL LI ALVILDLYGNYYAKASKLYGSDGSLRSPKGAPLDDATSARIGGYLHDLKQRHRELTLYLT	120
New_15RLOX	121	PLHGEPERAPAPPPRPAAALVEGFAOKFVATAENRVMESVLGAPNLSTKNVGDIIKSLL	180
Template_S4XZS0	121	PLHGEPERAPAPPPR PA ALVEG AURFY TAENK MESYLGA NLSTKNYGDTTNSLL PLHGEPERAPAPPPRAPAVALVEGVAOKFYTTAENRAMESYLGATNLSTKNYGDTTNSLL	18(
New_15RLOX	181	DTLLSAAGKLLLDLYGMYGQATSYYDYYSEFOTFRLPEIASAYQDDAIFAWMRYAGPNPL	240
Template_S4XZS0	181	DTLLSAAGKLLLDLYGMYGGATSYYDYYSEFUTFKLPETAS YGDDATFAWMKYGGPNPL DTLLSAAGKLLLDLYGMYGGATSYYDYYSEFUTFKLPETASYYQDDATFAWMKYAGPNPL	240
New_15RLOX	241	VLKRVASPGASFPYTDAQVRSVMGEGDSLASAGKEGRLVLADVEVLSQLVPGTAPDQQKV	300
Template_S4XZS0	241	VLKRYASPGASFFYTDAUTHSYMGEGDSLASAGREGRLYLADY+ LSULYFGTAPDQUAT VLKRYASPGASFPYTDAQYRSYMGEGDSLASAGREGRLYLADYKALSQLYPGTAPDQQKY	300
New_15RLOX	301	VEAPLALFAVPAAGAPSRLLRPVAIQCAQAPGAASPIFTPSDGVAWEVAKLHVQVADGNF	360
Template_S4XZS0	301	VEAPLALFAYPAAGAPSILLIKPYATQCAQAPGAASPTFTSJGYAWEYAKLHYQYADGN VEAPLALFAYPAAGAPSILLIKPYATQCAQAPGAASPTFTSJGYAWEYAKLHYQYADGNF	360
New_15RLOX	361	HELISHLGOTHLYMEAFAMATPROLAPEHPYNYLLTPHFOGTLAINNAAEADLIAPGGPY	42(
Template_S4XZS0	361	HELISHLGGTHLVLEAFAMATPRQLAPEHPVNVLLTPHFQGTLAINNAAEADLIAPGGPV	42(
New_15 <i>R</i> LOX	421	DNLLAGTIASSTQLAVDAVLSYSVNQQFLPVALAHRGVDDSALPSVPVRDDAVLLWGVI	480
Template_S4XZS0	421	DNLLAGTIASSIGLAVDAYLSISYNQUFLYYLA HQYDDFS LFSTYNDDA LLHG I DNLLAGTIASSTQLAYDAYLSYSYNQOFLPYALAQRGYDDPSTLPSYPYRDDATLL\@AI	48(
New_15RLOX	481	HTWYSRYLATYYTSDADYLGDYELQSWIAELSSPSECGLKDIGEDGATRTYAYLTDLLTH	540
Template_S4XZS0	481	HTWYSRYLAYYYTSDADYLGDYELQHWIAELSSPS CGEKDIGEDGAIRTYAYL DLLTH	540
New_15RLOX	541	VYFTASAQHAAVNFPQSSLMSFTPALPLAAVAPAPSITAGLPSSEIFQHLPPLQQALLQL	600
Template_S4XZS0	541	VIFTASAGHAAVNFPGSSLMSFIFALPLAAVAPAPSITAGLF SEIFGHLFPLQGALLGT	600
New_15RLOX	601	NYMMLLGGYYYSRLGDYDRN I PGAYFTDARYREPLEAFORELME I EAT I GKRNLORFPYL	660
Template_S4XZS0	601	NYMMLLGGYYYSRLGDYDRNIFGATFIDARYREFLEAFGRELMEIEATIGKRNLUKFYY NYMMLLGGYYYSRLGDYDRNIFGAYFTDARYREPLEAFGRELMEIEATIGKRNLQRFPYY	660
New_15RLOX	661	VLLPS0IP0SINI 673	
Template S4X7S0	661	VLLPSQIPQSINI 673	

B

>S. cellulosum_15R_LOX

MSNPSLPQNDSPAEQQQRKSELARAQQTYVYNHDTRLSPLGVAQSVPNGQGFAPILVGSAALVILELVGNVVAKASKLVG SDGSLRSPKGAPLDEATSARIQGVLRDLKQRHRELTLALTPLHGEPERAPAPPPPRPPAAALVEGFAQKFVATAENRVMES VLGAPNLSTKNVGDIIKSLLDTLLSAAGKLLLDLVGMYGQATSVYDYVSEFQTFRLPEIASAYQDDAIFAWMRVAGPNPL VLKRVASPGASFPVTDAQYRSVMGEGDSLASAGKEGRLYLADYEVLSQLVPGTAPDQQKYVEAPLALFAVPAAGAPSRLL RPVAIQCAQAPGAASPIFTPSDGVAWEVAKLHVQVADGNFHELISHLGQTHLVMEAFAMATPRQLAPEHPVNVLLTPHFQ GTLAINNAAEADLIAPGGPVDNLLAGTIASSTQLAVDAVLSYSVNQQFLPVALAHRGVDDPSALPSYPYRDDAVLLWGVI HTWVSRYLAIYYTSDADVLGDYELQSWIAELSSPSECGLKDIGEDGAIRTVAYLTDLLTHVVFTASAQHAAVNFPQSSLM SFTPALPLAAYAPAPSITAGLPSSEIFQHLPPLQQALLQLNVMMLLGGVYYSRLGDYDRNIPGAYFTDARVREPLEAFQR ELMEIEATIGKRNLQRFPYLVLLPSQIPQSINI **Fig. S3** 15*R*-LOX sequence derived from *S. cellulosum* so ce1871. The sequence of this gene was not the same as that of the gene encoding S4XZS0, however, similar to the sequence. (A) Alignment of 15*R*-LOX from *S. cellulosum* so ce1871 with the target protein sequence as S4XZS0 from *S. cellulosum* so ce56. (B) 15*R*-LOX sequence derived from *S. cellulosum* so ce1871.



Fig. S4 Normal-phase HPLC profiles of MonoHFAs obtained from the conversion of ARA (1) by the selected 3 LOXs derived from *S. cellulosum* with HETE standards. HPLC profiles of the 15-, 12-, 9-, 5-HETE standards. HPLC profiles of the reaction products obtained from the conversion of ARA (1) by the selected 3 LOXs derived from *S. cellulosum*. The reaction products were analyzed with MonoHFA standards, including 15-, 12-, 9-, and 5-HETEs. The reactions were performed at 30 °C in 50 mM EPPS buffer (pH 8.0) containing 1.0 mM 1, 0.1 g/L LOX, and 100 mM cysteine as reducing agent for 10 min. The reaction products of A0A2LESS6, A0A2L0ESU2, and S4XZS0 proteins were suggested as 9-, 12-, and 15-HETE, respectively.



Fig. S5 Chiral-phase HPLC profiles of MonoHFAs obtained from the conversion of ARA (1) by the selected 3 putative LOXs derived from *S. cellulosum* (UniProtKB protein numbers, A0A2LESS6, A0A2L0ESU2, and S4XZS0) with 9*S*-, 9*R*-, 12*S*-, 12*R*-, 15*S*-, and 15*R*-HETE (**3**) standards. The MonoHFAs were analyzed using the Amylose-1 column. (A) Chiral-phase HPLC analysis of the product obtained from the conversion of **1** by the putative LOX cloned with the primer of A0A2LESS6 with 9*S*- and 9*R*-HETE standards. (B) Chiral-phase HPLC analysis of the product obtained from the conversion of **1** by LOX cloned with the primer of A0A2L0ESU2 with 12*S*- and 12*R*-HETE standards. (C) Chiral-phase HPLC analysis of the product obtained from the conversion of **1** by LOX analysis of the product obtained from the conversion of **1** by LOX cloned with the primer of A0A2L0ESU2 with 12*S*- and 12*R*-HETE standards. (C) Chiral-phase HPLC analysis of the product obtained from the conversion of **1** by LOX analysis of the product obtained from the conversion of ARA by the putative LOX cloned with the primer of S4XZS0 with **3** and 15*S*-HETE standards. The reaction products of A0A2LESS6, A0A2L0ESU2, and S4XZS0 proteins were suggested as **3**, 12*S*-, and 9*S*-HETE, respectively.



Fig. S6 Reverse- and chiral-phase HPLC profiles of the reaction products obtained from the conversion of ARA (1) by the putative LOX cloned with the primer of S4XZS0 with standards. (A) Reverse-phase HPLC profiles of the reaction products obtained from the conversion of 1 by the putative LOX with 15*S*-HETE (**3**) and 5*S*,15*S*-DiHETE (**4**) standards. The MonoHFAs were analyzed using the Nucleosil C18 column. (B) Chiral-

phase HPLC profiles of MonoHFA obtained from the biotransformation of **1** by the putative LOX with **3** and 15*S*-HETE standards. The MonoHFAs were analyzed using the Amylose-1 column. (C) Reverse-phase HPLC profiles of DiHFA obtained from the bioconversion of **1** by the putative LOX with 8*S*,15*S*-DiHETE and 5*S*,15*S*-DiHETE standards. The MonoHFAs were analyzed using the Nucleosil C18 column. The reactions were performed at 30 °C in 50 mM EPPS buffer (pH 8.0) containing 1.0 mM **1**, 0.1 g/L enzyme, and 100 mM cysteine as reducing agent for 10 min.

А

B



Fig. S7 SDS-PAGE analysis and gel filtration chromatography of *S. cellulosum* 15*R*-LOX expressed in *E. coli* C2566. (A) SDS-PAGE of *S. cellulosum* 15*R*-LOX. M, the protein marker; P, pellet; C, crude; PF, purified enzyme (*S. cellulosum* 15*R*-LOX). (B) Determination of the molecular mass of the purified native *S. cellulosum* 15*R*-LOX by using Sephacryl S-300 HR column with reference proteins. The reference proteins were carbonic (29 kDa), ovalbumin (44 kDa), conalbumin (75 kDa), aldolase (158 kDa), and ferritin (440 kDa).



Fig. S8 Effects of temperature and pH on the production of 15R-HETE (**3**) from ARA (**1**) by 15R-LOX from *S. cellulosum*. (A) Effect of temperature on the biotransformation of ARA into 15R-HETE. The reactions were performed in 50 mM HEPES (pH 8.0) buffer containing 0.01 g/L enzyme and 0.5 mM ARA by varying the temperature from 20 to 40 °C in the presence of 100 mM cysteine for 10 min. (B) Effect of pH on the biotransformation of ARA into 15R-HETE. The reactions were performed using 50 mM HEPES, 50 mM EPPS, and 50 mM CHES buffers containing 0.01 g/L enzyme, 0.5 mM ARA, and 100 mM cysteine by varying the pH from 7.5 to 9.5 at 30 °C for 10 min. Data represent the mean \pm SD (n = 3) values.



5,15-DiHETE

Fig. S9 LC-MS/MS profiles of MonoHFA and DiHFA obtained from the conversion of ARA (1) by 15*R*-LOX from *S. cellulosum*. The red arrows indicate critical fragments around the hydroxyl group.
(A) 15-HETE. (B) 5,15-DiHETE.



Fig. S10 LC-MS/MS profiles of MonoHFA and DiHFA obtained from the conversion of EPA (5) by 15*R*-LOX from *S. cellulosum*. The red arrows indicate critical fragments around the hydroxyl group.
(A) 15-HEPA. (B) 5,15-DiHEPA.



Fig. S11 LC-MS/MS profiles of MonoHFA and DiHFA obtained from the conversion of DHA (9) by 15*R*-LOX from *S. cellulosum*. The red arrows indicate critical fragments around the hydroxyl group. (A) 17-HDHA. (B) 7,17-DiHDHA.



Fig. S12 Normal-phase HPLC profiles of DiHFA obtained from the conversion of ARA (1) by *S. cellulosum* 15*R*-LOX with 5*R*,15*S*-DiHETE, 5*S*,15*R*-DiHETE, 5*S*,15*S*-DiHETE, and 5*R*,15*R*-DiHETE (4) standards. The DiHFAs were analyzed using a Zorbax Rx-Sil column. The reactions were performed at 30 °C in 50 mM EPPS (pH 8.0) containing 1.0 mM 1, 1.0 g/L enzyme, and 100 mM cysteine for 10 min. The compound is suggested as 5*R*,15*R*-DiHETE or 5*S*,15*S*-DiHETE. However, the chirality of the DiHFAs produced by double-oxygenating LOXs did not change from that of the MonoHFAs,⁵⁻⁷ indicating that the product obtained from the conversion of 15*R*-HETE (3) by *S. cellulosum* 15*R*-LOX was 4, but not 5*S*,15*S*-DiHETE. Furthermore, the NMR data for 5,15-DiHETE obtained from the conversion of 3 by *S. cellulosum* 15*R*-LOX were different from those of 5*S*,15*S*-DiHETE. The product obtained was thus 5*R*,15*R*-DiHETE.



Fig. S13 ¹H NMR and ¹³C NMR peaks of 5*R*,15*R*-DiHETE (isomer of leukotriene B4; **4**) in deuterated methanol (MeOD) (850 MHz NMR).



Fig. S14 2D NMR data of 5*R*,15*R*-DiHETE (4) in MeOD (850 MHz NMR). (A) Correlation spectroscopy (COSY). (B) Heteronuclear single quantum coherence (HSQC). (C) Rotating frame Overhauser enhancement spectroscopy (ROSEY). (D) Heteronuclear multiple bond coherence (HMBC).



Fig. S15 ¹H NMR and ¹³C NMR peaks of 5*R*,15*R*-DiHEPA (enantiomer of resolvin E4; **8**) in MeOD (850 MHz NMR).



Fig. S16 2D NMR data of *5R*,1*5R*-DiHEPA (**8**) in MeOD (850 MHz NMR). (A) COSY. (B) HSQC. (C) ROSEY. (D) HMBC.



Fig. S17 ¹H NMR and ¹³C NMR peaks of 7*R*,17*R*-DiHDHA (enantiomer of resolvin D5; **12**) in MeOD (850 MHz NMR).

S32



Fig. S18 2D NMR data of 7*R*,17*R*-DiHDHA (**12**) in MeOD (850 MHz NMR). (A) COSY. (B) HSQC. (C) ROSEY. (D) HMBC.



Fig. S19 Effects of temperature and pH on the biotransformation of ARA (1) into 5R, 15R-DiHETE (4) by *E. coli* expressing 15*R*-LOX from *S. cellulosum*. (A) Effect of temperature on the biotransformation of **1** into **4**. The reactions were performed in 50 mM HEPES (pH 8.0) buffer containing 1.0 g/L cells, 1.0 mM **1**, and 100 mM cysteine by varying the temperature from 20 to 40 °C with 100 mM cysteine for 60 min. (B) Effect of pH on the biotransformation of **1** into **4**. The reactions were performed in 50 mM CHES buffers by varying the performed in 50 mM HEPES, 50 mM EPPS, and 50 mM CHES buffers by varying the pH from 7.5 to 9.5 containing 1.0 g/L cells, 1.0 mM **1**, and 100 mM cysteine at 25 °C for 60 min. Data represent the mean \pm SD (n = 3) values.





Fig. S20 Optimization of cell and substrate concentrations for the biotransformation of ARA (1) into 5R, 15R-DiHETE (4) by *E. coli* expressing 15R-LOX from *S. cellulosum*. (A) Optimization of cell concentration. The reactions were performed in HEPES (pH 8.0) buffer containing 1.0 mM 1 and 100 mM cysteine by varying the cell concentration from 0.1 to 6.0 g/L cells for 60 min. (B) Optimization of substrate concentration. The reactions were performed in HEPES (pH 8.0) buffer containing 1.0 g/L cells and 100 mM cysteine by varying the concentration of 1 as substrate from 0.5 to 10 mM for 60 min. Data represent the mean \pm SD (n = 3) values.

А



Fig. S21 Optimization of cell and substrate concentrations for the biotransformation of ARA (1) into 5R, 15R-DiHETE (4) by *E. coli* expressing 15R-LOX from *S. cellulosum* at the optimal ratio of cells to substrate. The reactions were performed in a 100-mL flask containing 10 mL HEPES (pH 8.0) buffer supplemented with resin SP825 at the optimal ratio of 5:4 (g/L per mM) by varying the concentrations of cells and 1 from 0.5 g/L and 2.0 mM to 2.5 g/L and 10.0 mM at the optimal ratio of 1.0 g/L to 4.0 mM for 60 min. The symbols indicate the concentration (•) and conversion yield (%) (\circ) of 4. Data represent the mean \pm SD (n = 3) values.



Fig. S22 Binding of absorbent resins to ARA (1) as a substrate. (A) Selection of absorbent resins for 1 as substrate. Adsorbent resins were used to test binding to 1. (B) Effect of SP825 resin concentration on 1 at 4.0 mM. The reaction was performed in a 100-mL flask containing 10 mL 50 mM HEPES (pH 8.0) buffer supplemented 4.0 mM 1, 1.0 g/L cells, and 100 mM cysteine with 5.0 g/L resin at 25 °C for 60 min. The resin and buffer were separated by filtration through a 45- μ m pore-size filter. Data represent the mean \pm SD (n = 3) values.

А



Fig. S23 Effect of resin SP825 concentration on the biotransformation of ARA (1) into 5R, 15R-DiHETE (4) by 15R-LOX from *S. cellulosum* with varying the concentrations of cells and ARA (1). The reactions were performed at 25 °C in a 100-mL flask containing 10 mL reaction HEPES buffer (pH 8.0) with 100 mM cysteine by varying the concentrations of cells and 1 from 0.5 g/L and 2.0 mM to 3.5 g/L and 20 mM for 60 min. Dark, grey, and dark grey bars indicate the concentrations of 4 after the reactions without resin, with the optimal ratio of cells to substrate (1:4, g/L per mM) at a constant concentration of 5.0 g/L resin, and with the optimal ratio of cells, substrate, and resin resin (1:4:5, g/L per mM per g/L), respectively. Data represent the mean \pm SD (n = 3) values.



Conventration (mM) *R*,15*R*-DiHEPA (Enantiomer of RvE4) 15*R*-HEPA - EPA Time (min)



С

B

A

Fig. S24 Biotransformation of PUFAs into DiHFAs with the addition of resin by *E. coli* expressing 15*R*-LOX from *S. cellulosum* in filtrate. (A) Biotransformation of ARA (1) to 5*R*,15*R*-DiHETE (isomer of LTB4; **4**). (B) Biotransformation of EPA (**5**) to 5*R*,15*R*-DiHEPA (enantiomer of RvE4; **8**). (C) Biotransformation of DHA (**9**) to 7*R*,17*R*-DiHDHA (enantiomer of RvD5; **12**). The reactions were performed at 25 °C in a 100-mL flask containing 50 mM HEPES (pH 8.0) buffer supplemented with 7.5 g/L absorbent resin SP825, 6.0 mM PUFAs, 1.5 g/L cells, and 100 mM cysteine for 90 and 120 min. The resin and buffer were separated by filtration through a 45-µm pore-size filter. Data represent the mean \pm SD (n = 3) values.







A



Fig. S25 Biotransformation of PUFAs into DiHFAs with the addition of resin by *E. coli* expressing 15*R*-LOX from *S. cellulosum* in resin. (A) Biotransformation of ARA (1) into 5*R*,15*R*-DiHETE (isomer of LTB4; **4**). (B) Biotransformation of EPA (**5**) into 5*R*,15*R*-DiHEPA (enantiomer of RvE4; **8**). (C) Biotransformation of DHA (**9**) into 7*R*,17*R*-DiHDHA (enantiomer of RvD5; **12**). The reactions were performed at 25 °C in a 100-mL flask containing 50 mM HEPES (pH 8.0) buffer supplemented with 7.5 g/L absorbent resin SP825, 6.0 mM PUFA, 1.5 g/L cells, and 100 mM cysteine for 90 and 120 min. The resin and buffer were separated by filtration through a 45-µm pore-size filter. Data represent the mean \pm SD (n = 3) values.





Fig. S26 Reliability of the models of 15*R*-LOX from *S. cellulosum* built by Alphafold and Discovery studio (DS). (A) Alphafold model. Blue color represents high reliability, whereas the remaining colors represent low reliability. (B) DS model. (C) Ramachandran plot for demonstrating reliability in DS model. The Ramachandran plot was shown with the verify score, and the residues displayed represents low reliability.



Fig. S27 Confirmation of the active site in 15*R*-LOX from *S. cellulosum* in DS model by Alphafold model. Superimposition of the catalytic residues built by Alphafold and DS. The catalytic residues His366/His371/His549/Asn553/Ile673 in the model built by Alphafold is shown in cyan whereas the those in the model built by DS is shown in gray. When the substrates were docked, interaction residues (Gln354, Leu413, Val602, Met603, and Leu606) are located at the hydrophobicity region of the substrate-binding pocket.



Fig. S28 Reverse-phase HPLC profiles of the products obtained from the conversion of ARA (1) by the wild-type and variant LOXs at position 354 from *S. cellulosum*. The MonoHFAs and DiHFAs were analyzed using the Nucleosil C18 column. The reactions were performed at 25 °C in 50 mM HEPES (pH 8.0) containing 1.0 mM 1, 0.5 g/L cells, and 100 mM cysteine for 120 min.







Fig. S29 Reverse-phase HPLC profiles of the products obtained from the conversions of ARA (1) and 15*R*-HETE (**3**) by the wild-type and variant LOXs at position 606 from *S. cellulosum*. The MonoHFAs and DiHFAs were analyzed using the Nucleosil C18 column. (A) HPLC profiles for reaction products obtained from the conversion of **1** by the wild-type and variant 15*R*-LOXs with **1** standard. The reactions were performed at 25 °C in 50 mM HEPES (pH 8.0) containing 1.0 mM **1**, 0.1 g/L cells, and 100 mM cysteine for 120 min. (B) HPLC profiles for the products obtained from the conversion 606. The reactions were performed at 25 °C in 50 mM the cysteine for 120 min. (C) HPLC profiles for the products obtained from the conversion of 15*R*-HETE by the variants at position 606. The reactions were performed at 30 °C in 50 mM HEPES (pH 7.5) containing 0.5 mM **3**, 0.01 g/L of purified enzyme, and 100 mM cysteine for 30 min.



Fig. S30 Effects of temperature and pH on the biotransformation of ARA (1) into 15*R*-HETE (**3**) by *E. coli* expressing 15*R*-LOX from *S. cellulosum*. (A) Effect of temperature on the biotransformation of **1** into **3**. The reactions were performed in 50 mM HEPES (pH 8.0) buffer containing 1.0 g/L cells, 1.0 mM **1**, and 100 mM cysteine for 10 min by varying the temperature from 20 to 40 °C. (B) Effect of pH on the biotransformation of **1** into **3**. The reactions were performed in 50 mM HEPES, 50 mM EPPS, and 50 mM CHES buffers containing 1.0 g/L cells, 1.0 mM **1**, and 100 mM cysteine at 25 °C for 10 min by varying the pH from 7.5 to 9.5. Data represent the mean \pm SD (n = 3) values.

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Fig. S31 Effects of cell and substrate concentration on the biotransformation of ARA (1) into 15*R*-HETE (**3**) by *E. coli* expressing engineered 15*R*-LOX (L606F variant). (A) Optimization on cell concentration for the biotransformation of **1** into **3**. The reactions were performed in HEPES (pH 8.0) buffer containing 1.0 mM **1**, 5.0 g/L SP825, and 100 mM cysteine for 30 min by varying the cell concentration from 0.5 to 6.0 g/L cells. (B) Optimization on substrate concentration for the biotransformations were performed in HEPES (pH 8.0) buffer with cysteine concentration of **1** into **3**. The reactions were performed in HEPES (pH 8.0) buffer with cysteine for 30 min by varying the cell biotransformation of **1** into **3**. The reactions were performed in HEPES (pH 8.0) buffer with cysteine containing 1.0 g/L cells, 5.0 g/L SP825, and 100 mM cysteine for 30 min by varying the substrate concentration from 0.5 to 6.0 mM. The optimal ratio of cells to substrate was 1:2 (g/L per mM). Data represent the mean \pm SD (n = 3) values.

A



Fig. S32 Optimization of cell and substrate concentration on the biotransformation of ARA (1) into 15R- HETE (3) by *E. coli* expressing engineered 15R-LOX (L606F variant). The reactions were performed in a 100-mL flask containing 10 mL HEPES (pH 8.0) buffer supplemented with cells, substrate, and resin at the optimal ratio of 2:4:5 (g/L per mM per g/L) for 60 min by varying the concentrations of cells and 1 from 0.5 g/L and 1.0 mM to 2.5 g/L and 5.0 mM at the optimal ratio of 2.0 g/L to 4.0 mM. Data represent the mean \pm SD (n = 3) values.

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