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Asymmetric synthesis and stereochemical structure–activity relationship of (*R*)- and (*S*)-8-[1-(2,4-dichlorophenyl)-2-imidazol-1-yl-ethoxy] octanoic acid heptyl ester, a potent inhibitor of allene oxide synthase

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Abstract—The preparation of both enantiomers of 8-[1-(2,4-dichlorophenyl)-2-imidazol-1-yl-ethoxy] octanoic acid heptyl ester (JM-8686), a potent inhibitor of allene oxide synthase, has been achieved using 2,4-dichlorophenacyl bromide as a starting material. The key step was the asymmetric reduction of 1-(2,4-dichlorophenyl)-2-imidazol-1-yl-ethanone with chiral BINAL-H. The products were purified by chiral high-performance liquid chromatography (HPLC) to afford pure (*R*)-JM-8686 and (*S*)-JM-8686. The inhibitory activities and binding affinities of these enantiomers toward allene oxide synthase were determined. We found that the inhibition potency of (*R*)-JM-8686 is approximately 200 times greater than that of (*S*)-JM-8686, with IC_{50} values of approximately 5 ± 0.2 nM and 950 ± 18 nM, respectively. The dissociation constants of (*R*)-JM-8686 and (*S*)-JM-8686 with respect to the recombinant allene oxide synthase were approximately 1.4 ± 0.3 μM and 4.8 ± 0.6 μM, respectively.

1. Introduction

The oxylipin biosynthetic pathway in plants leads to the production of a group of bioactive compounds.[1] The initial step of most phyto-oxylipin biosyntheses is catalyzed by lipoxygenase (LOX), which adds molecular oxygen to either the C-9 or C-13 position of linolenic or linoleic acid.[2] The resulting hydroperoxides are further metabolized by several enzymes including three closely related members of the CYP74 family of cytochrome P-450: allene oxide synthase (AOS), hydroperoxide lyase (HPL), and divinyl ether synthase (DES).[1] AOS catalyzes the first reaction in the specific biosynthetic pathway of jasmonic acid related compounds, collectively called Jasmonates (JAs).[3] JAs are essential signal mediators in the defense system of plants against pest attack, in mechanical injury, and also in several developmental processes.[4–7] Correlations between endogenous JA levels in specific tissues and the effects of the applied JAs have provided evidence that JAs have a role in fruit ripening, embryo development,[5] senescence,[8] and the accumulation of storage proteins.[9] Analysis of JA-deficient mutants of *Arabidopsis* has provided evidence that JAs play a key role in flower development. The critical requirement of JAs in male fertility was established by the characterization of an *Arabidopsis* mutant that fails to produce linolenic acid, the fatty acid precursor of JAs.[10] Analysis of JA-deficient mutants of tomato has indicated that JAs are key regulators involved in the defense response to herbivore attack and mechanical wounding.[11,12]

The importance of JAs in the plant life cycle has promoted increasing interest in understanding the mechanisms by which JAs biosynthesis is regulated.[7]

Abbreviations: AOS, allene oxide synthase; BINAL-H, 2,2′-dihydroxy-1,1′-binaphthyl-lithium aluminum hydride; DES, divinyl ether synthase; HPL, hydroperoxide lyase; 13(*S*) HPOT, 13(*S*)-hydroperoxy-9(*Z*),11(*E*),15(*Z*)-octadecatrienoic acid; JAs, jasmonates; JM-8686, 8-[1-(2,4-dichlorophenyl)-2-imidazol-1-yl-ethoxy] octanoic acid heptyl ester; LOX, lipoxygenase.

Keywords: Allene oxide synthase inhibitor; Imidazole; Jasmonic acid biosynthesis.

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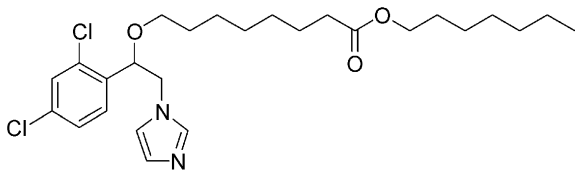


Figure 1. Chemical structure of JM-8686.

Available evidence indicates that AOS is an important site of regulation. Herbivore attack and mechanical wounding positively regulate AOS expression.[13] Mutations that disrupt AOS function in *Arabidopsis* caused male sterility and attenuated responses of leaves to mechanical wounding.[14] Thus, specific inhibitors interfering with AOS functions may be useful for dissecting the functions associated with JA biosynthesis.

We have previously reported the first JA biosynthesis inhibitor, JM-8686 (the chemical structure is shown in Fig. 1), that exhibits potent inhibitory activity against AOS.[15] Kinetic analysis of AOS inhibition by JM-8686 indicated that it binds to the active site of AOS. In addition, JM-8686 exhibits high selectivity between AOS and HPL, a closely related member of the CYP74 family of cytochrome P-450.[16] Thus, using the stereoisomers of JM-8686 to investigate the properties of the inhibitor–enzyme interaction may provide stereochemical information about the binding site of AOS, which is useful for designing new AOS inhibitors as well as for understanding the structural functions of AOS. Here, we report the asymmetric synthesis of (*R*)-(−)-JM-8686 (**4a**) and (*S*)-(+)-JM-8686 (**4b**), and their biological activities against AOS.

2. Results and discussion

2.1. Chemistry

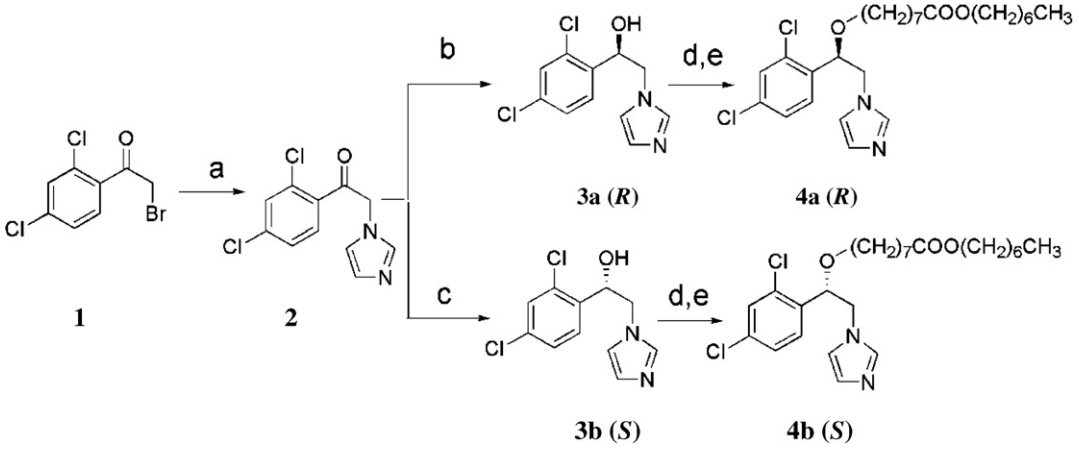
As shown in Scheme 1, for the asymmetric syntheses of (*R*)-JM-8686 {(*R*)-8-[1-(2,4-dichlorophenyl)-2-imidazol-1-yl-ethoxy] octanoic acid heptyl ester (**4a**)} and (*S*)-JM-8686 {(*S*)-8-[1-(2,4-dichlorophenyl)-2-imidazol-1-yl-ethoxy] octanoic acid heptyl ester (**4b**)}, the starting material 2,4-dichlorophenacyl bromide (**1**) was used. Compound **1** was coupled with imidazole according to the previously described method[17] to give **2**. (*R*)-1-(2,4-Dichlorophenyl)-2-imidazol-1-yl-ethanol (**3a**) and (*S*)-1-(2,4-dichlorophenyl)-2-imidazol-1-yl-ethanol (**3b**) were prepared by asymmetric reduction of **2** using chiral BINAL-H.[18] The target compounds **4a** and **4b** were prepared from their corresponding (*R*) and (*S*) precursors (**3a** and **3b**) by the method we previously described.[15] The optical purity of the synthesized compounds was examined by chiral HPLC (described in Section 4). Data from the chiral HPLC and ^{1}H NMR analyses indicate that the yield of the asymmetric reduction of **2** by (*R*)-BINAL-H is approximately 56%, and the enantiomeric excess (ee) of **3a** is approximately 48.6%. The yield and optical purity of the product for the reaction to prepare **3b** are approximately 62% and 56.3%, respectively (Table 1). The relationships of the absolute configurations and specific rotations of the individual stereoisomers (**3a** and **3b**) were confirmed by comparison with the data reported previously.[19]

The target compounds, **4a** and **4b**, were prepared from **3a** and **3b** and purified by chiral HPLC. The optical purities are greater than 99% (ee) as determined from the chiral HPLC peak areas (Table 1). The absolute stereo configurations of **4a** and **4b** were determined by comparison of their CD spectra with those of the corre-

Table 1. Specific rotations and retention times on chiral HPLC of **3a**, **3b**, **4a**, and **4b**

Compound	Specific rotation	Retention time[a] (min)
3a	$[\alpha]_D^{24}$ +992 ± 24 (C = 1, MeOH)	8.81 ± 0.05
3b	$[\alpha]_D^{24}$ −998 ± 42 (C = 1, MeOH)	10.95 ± 0.07
4a	$[\alpha]_D^{24}$ +416 ± 25 (C = 0.01, MeOH)	8.29 ± 0.08
4b	$[\alpha]_D^{24}$ −435 ± 29 (C = 0.01, MeOH)	17.42 ± 0.3

[a] To separate **3a** and **3b**, a flow rate of 4 mL/min was used on a chiral HPLC column.



Scheme 1. Reagents and conditions: (a) imidazole (5 equiv), DMF; (b) (*R*)-BINAL-H, THF, −100 °C; (c) (*S*)-BINAL-H, THF, −100 °C; (d) NaH, DMF; (e) $Br(CH_2)_7COO(CH_2)_6CH_3$.

sponding precursors, **3a** and **3b**. As shown in Figure 2, the CD spectral pattern of **3a** is the same as **4a**, and that of **3b** is the same as **4b**, indicating that **4a** is (*R*)-(−)-JM-8686 and **4b** is (*S*)-(+)-JM-8686.

2.2. Inhibitory activity of the enantiomers against allene oxide synthase

The inhibitory activity of the two isomers against purified recombinant AOS was investigated by continuously observing the dehydration process of the substrate. As shown in Figure 3, without inhibitor (*squares*), the absorbance of the substrate was decreased by approximately 0.1 within 120 s, which is associated with the AOS activity. At concentrations of 1 (μM) for the two isomers, (*R*)-(−)-JM-8686 (*triangles*) exhibits approximately 93% inhibition while (*S*)-(+)-JM-8686 (*circles*) shows approximately 50% inhibition, indicating that (*R*)-(−)-JM-8686 is more potent than (*S*)-(+)-JM-8686. The IC_{50} values of (*R*)-(−)-JM-8686 and (*S*)-(+)-JM-8686 calculated from the dose-dependent inhibition curves of AOS were approximately 5 ± 0.2 nM and 950 ± 18 nM, respectively (Table 2).

2.3. Binding affinity of the stereoisomers to allene oxide synthase

Binding of JM-8686 to AOS was determined by measuring the optical difference spectra upon addition of JM-8686 to recombinant AOS. AOS exhibited a soret absorption peak at 419 nm, which is characteristic of low-spin P450s (Fig. 4A, solid line). The addition of (*R*)-(−)-JM-8686 to AOS induced an absorbance shift of the heme soret band from 419 to 429 nm (Fig. 4A, broken line, data for (*S*)-(+)-JM-8686 are not shown). The difference spectrum patterns induced by (*R*)-(−)-JM-8686 and (*S*)-(+)-JM-8686 are shown in Figures 4B and C. Both stereoisomers induced typical type II binding spectra, which are characteristic of a change from a low to a high spin state of the ferric iron. This behavior is usually associated with the direct coordination of the imidazole group of JM-8686 to the heme iron of AOS.[20] The dissociation constant (K_d) was determined by titrating the observed spectral absorbance difference ΔA (429–391 nm) versus the concentration of inhibitor (Fig. 4D). The K_d values for (*R*)-(−)-JM-8686 and (*S*)-(+)-JM-8686 were approximately 1.4 ± 0.3 μM and 4.8 ± 0.6 μM, respectively (Table 2). These results clearly indicate a positive correlation between inhibitory activity and binding affinity of the two stereoisomers.

AOS (CYP74A) belongs to a unique class of P450s. The catalytic action of AOS in the biosynthesis of epoxyalcohols has been well characterized.[21] The hydroperoxide substrate is thought to interact with the ferric ion of AOS. One-electron transfer from AOS Fe (III) to the O–O bond of 13(*S*)-hydroperoxy-9(*Z*),11(*E*),15(*Z*)-octadecatrienoic acid [13(*S*) HPOT] results in the formation of AOS Fe (IV)–OH and a substrate alkoxy radical. The

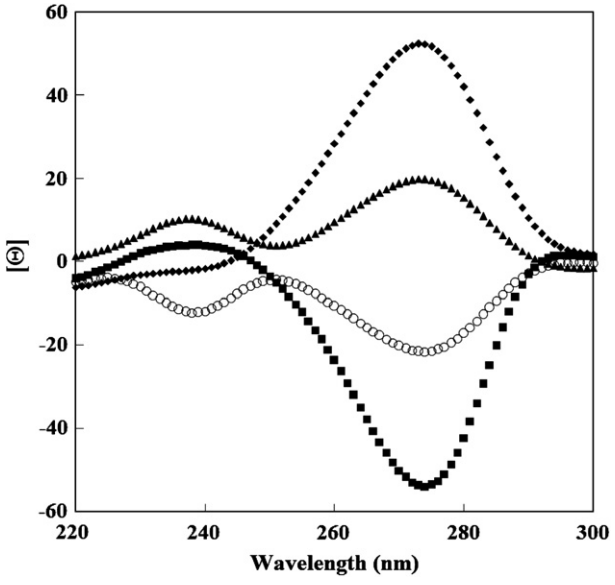


Figure 2. Induced circular dichroism spectra of **3a** (*triangles*), **3b** (*circles*), **4a** (*diamonds*), and **4b** (*squares*) in a methanol solution (10^{-4} M).

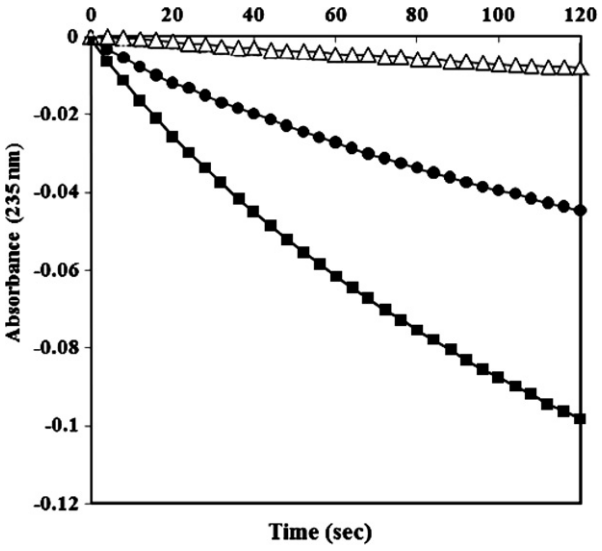


Figure 3. Inhibitory activity of (*R*)- and (*S*)-JM-8686 against AOS. The experiments were carried out by incubation of 13(*S*) HPOT (20 μM) with 1 μM (*R*)-(−)-JM-8686 (*triangles*), 1 μM (*S*)-(+)-JM-8686 (*circles*), or without adding inhibitor (*diamonds*). The reaction was initiated by adding the enzyme. The progress curve was recorded for 120 s. Data are shown as plots of the average of three independent experiments.

Table 2. I_{50} values of the stereoisomers of JM-8686 upon AOS inhibition and binding affinities

Compound	I_{50}[a] (nM)	K_d[b] (μM)
(*S*)-(+)-JM-8686	950 ± 18	4.8 ± 0.6
(*R*)-(−)-JM-8686	5 ± 0.2	1.4 ± 0.3

[a] The concentration that produces 50% inhibition of AOS activity when compared to the control (untreated) condition.

[b] The dissociation constant K_d was determined by titrating the observed spectral absorbance difference ΔA (429–391 nm) versus the inhibitor concentration (Fig. 4).

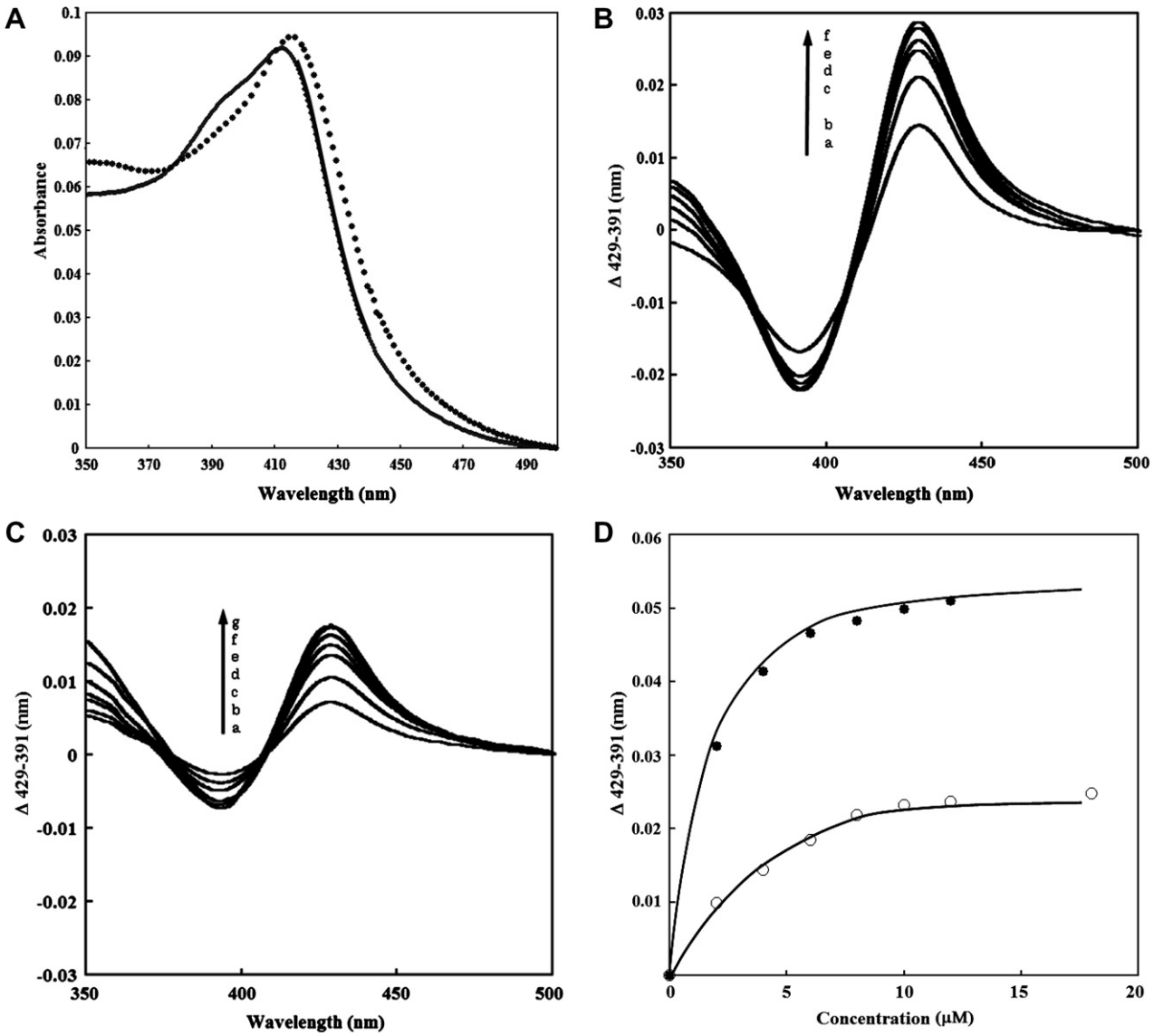


Figure 4. Binding of JM-8686 to AOS. (A) Absorption spectra of oxidized AOS (solid line) and its (*R*)-(−)-JM-8686 complex (broken line). Recombinant AOS (2.5 μM) was dissolved in 50 mM NaH_2PO_4 (pH = 7.0) with 0.1% Tween 20 containing 20% glycerol, and (*R*)-(−)-JM-8686 was added to AOS at a final concentration of 12 μM. (B) Spectrophotometric titration of AOS with (*R*)-(−)-JM-8686. (*R*)-(−)-JM-8686 was added to AOS (2.5 μM) at various final concentrations (a, 2; b, 4; c, 6; d, 8; e, 10; f, 12 μM). (C) Spectrophotometric titration of AOS with (*S*)-(+)-JM-8686 induced spectral changes in AOS. The amount of (*S*)-(+)-JM-8686 added was: a, 2; b, 4; c, 6; d, 8; e, 10; f, 12; g, 18 μM. (D) A plot of absorbance differences ΔA (429–391 nm) against (*R*)-(−)-JM-8686 (*circles*) and (*S*)-(+)-JM-8686 (*open circles*) concentrations. Data are shown as plots of the average of two independent experiments.

subsequent free radical oxidation and loss of a β-proton result in the formation of an unstable allene oxide, which is the product of the AOS reaction. These observations suggest the asymmetric carbon of the substrate plays a key role in the catalytic action of AOS. Data taken from binding assays of the two stereoisomers of JM-8686 to AOS clearly indicate that (*R*)-(−)-JM-8686 exhibits a high binding affinity toward AOS, which suggests there is a stereochemical recognition mechanism for inhibitor–enzyme interaction. In addition, AOS has no absolute requirement for molecular oxygen and NADPH in catalyzing the biochemical conversion of 13(*S*)-hydroperoxylinolenic acid. Instead, it uses hydroperoxylinolenic acid both as an oxygen donor (using the peroxy moiety) and as a substrate.[21,22] Included in this class of P450 enzymes are thromboxane synthase and PGI_2 synthase.[23] Since JM-8686 exhibits potent inhibition of AOS activity, we expect that experimental use of this synthetic series will provide important information about the catalytic mechanism of this class of cytochrome P450s. The structure of crystallized plant AOS has not been investigated. Oldham et al. recently reported the crystal structure of coral allene oxide synthase,[24] the enzyme that uses 8-(*R*)-hydroperoxyeicosatetraenoic acid as a substrate. With the aid of a structure-based sequence alignment, we found the sequence identity between *Arabidopsis* AOS and coral AOS is only about 10% (data not shown), so that the 3D structure of *Arabidopsis* AOS may be different from that of coral AOS. Data obtained from this study

indicate that the AOS–JM-8686 interaction is highly restricted by the stereochemical configuration of the inhibitor. This observation implies that JM-8686 is a useful tool for determining the structure of AOS. Further studies of the in vivo actions of this synthetic inhibitor are now in progress.

3. Conclusion

We synthesized two enantiomers of JM-8686 by asymmetric reduction of 1-(2,4-dichlorophenyl)-2-imidazol-1-yl-ethanone using chiral BINAL-H derivatives coupled with chiral HPLC. Analyses of the binding affinity and inhibitory activity of both enantiomers indicate that the potency of the (*R*)-isomer is greater than that of the (*S*)-isomer.

4. Experimental

4.1. General

Melting points (mp) were determined with a Yanako melting point apparatus. ^{1}H NMR spectra were recorded with a JOEL ECP-400 spectrometer, chemical shifts being expressed in ppm downfield from TMS as an internal standard. Electrospray ionization mass spectra (ESI-MS) were recorded on a PE Sciex API-2000 LC/MS System. Optical rotations were measured on a JASCO P-1020 polarimeter using methanol solutions at concentrations given in g/100 cm^3. Solvents were either dehydrated reagent or of HPLC grade. Ampicillin and isopropyl-D-thiogalactopyranoside were purchased from Wako Chemical Inc. (Tokyo, Japan). Linolenic acid and soybean lipoxygenase were obtained from Sigma (Tokyo, Japan). Other reagents of the highest quality were purchased from Tokyo Kasei Co. (Tokyo, Japan).

4.2. Chemistry

4.2.1. Preparation of 13(*S*)-hydroperoxy-9(*Z*),11(*E*),15(*Z*)-octadecatrienoic acid. 13(*S*) HPOT was prepared according to the method described previously.[15] The stock solution of 13(*S*) HPOT was prepared by dissolving 13(*S*) HPOT in methanol (10 mM) and was stored at −20 °C.

4.2.2. Preparation of 1-(2,4-dichlorophenyl)-2-imidazol-1-yl-ethanone (2). Compound **2** was prepared by using 2,4-dichlorophenacyl bromide as described previously.[17] White needle crystal: mp 78.3–79.3 °C. ^{1}H NMR ($CDCl_3$) δ 5.33 (s, 2H), 6.92 (s, 1H), 7.02 (s, 1H), 7.38 (d, J = 9.1 Hz, 1H), 7.51 (s, 2H), 7.57 (d, J = 9.1 Hz, 1H). ESI-MS *m*/*z* 255.1 (M+H), Calcd 255.0 (M+H).

4.2.3. Preparation of (*R*)-1-(2,4-dichlorophenyl)-2-imidazol-1-yl-ethanol (3a). Preparation of **3a** was carried out by the method using (*R*)-BINAL-H as described previously.[18] White rod crystal: mp 115.6–117.1 °C. ^{1}H NMR ($CDCl_3$) δ 3.87 (dd, J = 8.4, 4.3 Hz, 1H), 4.22 (dd, J = 2.2, 14.3 Hz, 1H), 5.24 (dd, J = 2.0, 8.4 Hz, 1H), 6.87 (s, 1H), 6.91 (s, 1H), 7.31 (dd, J = 8.4, 2.2 Hz, 1H), 7.41 (d, J = 4.8 Hz, 1H), 7.42 (s, 1H), 7.59 (d, J = 8.4 Hz, 1H). ESI-MS *m*/*z* 257.2 (M+H), Calcd 257.1 (M+H).

4.2.4. Preparation of (*S*)-1-(2,4-dichlorophenyl)-2-imidazol-1-yl-ethanol (3b). The **3b** isomer was prepared in a similar way using (*S*)-BINAL-H. White rod crystal: mp 111.7–112.7. ^{1}H NMR ($CDCl_3$) δ 3.86 (dd, J = 8.3, 14.2 Hz, 1H), 4.22 (dd, J = 2.2, 13.9 Hz, 1H), 5.23 (dd, J = 1.8, 8.4 Hz, 1H), 6.87 (s, 1H), 6.92 (s, 1H), 7.31 (dd, J = 8.4, 2.2 Hz, 2H), 7.41 (d, J = 2.2 Hz, 2H), 7.59 (d, J = 8.4 Hz, 1H). ESI-MS *m*/*z* 257.2 (M+H), Calcd 257.2 (M+H).

4.2.5. Preparation of (*R*)-8-[1-(2,4-dichlorophenyl)-2-imidazol-1-yl-ethoxy] octanoic acid heptyl ester (4a). Compound **4a** was prepared using (*R*)-1-(2,4-dichlorophenyl)-2-imidazol-1-yl-ethanol (**3a**) by the method described previously.[15] Colorless oil. ^{1}H NMR ($CDCl_3$) δ 0.88 (t, J = 6.6 Hz, 3H), 1.24–1.32 (m, 16H), 1.45–1.65 (m, 4H), 2.29 (t, J = 7.7 Hz, 2H), 3.18–3.23 (m, 1H), 3.29–3.35 (m, 1H), 3.98 (dd, J = 7.4, 14.4 Hz, 1H), 4.05 (t, J = 6.6 Hz, 2H), 4.17 (dd, J = 2.5, 14.4 Hz, 1H), 4.83 (dd, J = 2.5, 7.4 Hz, 1H), 6.92 (s, 1H), 7.01 (s, 1H), 7.26 (s, 2H), 7.41 (s, 1H), 7.45 (s, 1H). ESI-MS *m*/*z* 498.3 (M+H), Calcd 498.2 (M+H).

4.2.6. Preparation of (*S*)-8-[1-(2,4-dichlorophenyl)-2-imidazol-1-yl-ethoxy] octanoic acid heptyl ester (4b). Compound **4b** was prepared in a similar way by using **3b**. Colorless oil. ^{1}H NMR ($CDCl_3$) δ 0.88 (t, J = 6.6 Hz, 3H), 1.24–1.32 (m, 16H), 1.49–1.69 (m, 4H), 2.29 (t, J = 7.7 Hz, 2H), 3.18–3.24 (m, 1H), 3.29–3.35 (m, 1H), 3.98 (dd, J = 7.3, 14.3 Hz, 1H), 4.05 (t, J = 6.6 Hz, 2H), 4.17 (dd, J = 2.6, 14.3 Hz, 1H), 4.81 (dd, J = 2.6, 7.3 Hz, 1H), 6.92 (s, 1H), 7.02 (s, 1H), 7.26 (s, 2H), 7.41 (s, 1H), 7.45 (s, 1H). ESI-MS *m*/*z* 498.2 (M+H), Calcd 498.2 (M+H).

4.2.7. Circular dichroism (CD). CD measurements were performed by using a J-720 spectropolarimeter (Jasco) as described previously.[25] Methanol was used as a blank. Samples were dissolved in methanol at a concentration of 0.1 mg/mL and placed in a cuvette with a 1 cm path length. Spectra were scanned at 25 °C in the region 220–300 nm. The observed specific ellipticity (difference between sample and blank) was converted to the mean residue ellipticity θ (deg/cm^2/dmol).

4.2.8. HPLC separation of stereoisomers. The optical purity of the synthesized compounds was examined by HPLC with a chiral stationary phase column (Daicel Chem. Ltd, CHIRALPAK OJ, 4.6 mm 250 mm), using *n*-hexane/2-propanol (15:1) as the eluent, at a flow rate of 2 mL/min with a detection wavelength of 254 nm.

4.3. Biology

4.3.1. Expression and purification of recombinant AOS. The coding region of AOS cDNA that was restricted by the enzymes BamHI and KpnI was inserted into an

Escherichia coli expression vector pQE30 (Qiagen). *E. coli* M15, transformed with this construct, was kindly provided by Prof. E.W. Weiler, Lehrstuhl für Pflanzenphysiologie, Fakultät für Biologie Ruhr-Universität, Germany. Expression and purification of recombinant AOS were performed as described previously.[16]

4.3.2. In vitro assays for AOS. The enzyme reaction mixture contained 50 mM sodium phosphate buffer (pH 7.0, 0.1% Tween 20), enzyme (AOS, 5 nM), and designated concentrations of substrate (13(*S*) HPOT) at 25 °C. Activity was measured by following the decrease in absorption at 235 nm using a Shimadzu UV3100 spectrophotometer (Shimadzu, Kyoto, Japan). An absorption coefficient of 22.8 mM^{-1}/cm^{-1} was used.

4.3.3. Binding assay of JM-8686 to recombinant AOS. Binding of JM-8686 to AOS was measured by optical difference spectroscopy of the purified recombinant AOS using a Shimadzu UV3100 spectrophotometer as described previously.[16]

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