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Design and Synthesis of Novel Imidazole Derivatives as Potent Inhibitors of Allene Oxide Synthase(CYP74)

Keimei Oh*,† and Noboru Murofushi

Department of Biotechnology, Akita Prefectural University, Shimoshinjo-Nakano, Akita-shi, Akita 010-0195, Japan

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Abstract—Allene oxide synthase (AOS) is a key enzyme in the oxylipin pathway in plants leading to jasmonic acid and other jasmonates (JAs), important signal mediators of defense signal networks in plants. AOS uses hydroperoxylinolenic acid as an oxygen donor as well as the substrate, thus the biochemical conversion of 13(*S*)-hydroperoxylinolenic acid to allene oxide can proceed in the absence of oxygen and NADPH. We have designed the synthesized of a series of novel imidazole derivatives and tested them in a bioassay as AOS inhibitors using a purified recombinant AOS enzyme isolated from *Arabidopsis* and expressed in *E. coli*. Among the derivatives prepared, heptyl 8-[1-(2,4-dichlorophenyl)-2-imidazolylethoxy]octanoate (k) was found to be the most potent inhibitor, with an IC_{50} of 10 ± 5 nM, which is 250,000-fold and 1,000,000-fold more potent than the known AOS inhibitors, acetylsalicyclic acid (2.5 mM) and ketoconazole (10 mM), respectively.

The oxylipin biosynthetic pathway in plants leads to pentacyclic fatty acids, generically called jasmonates (JAs). JAs are considered to be essential signal mediators in the plant's defense system against pest attack, mechanical injury and also in several developmental processes.[1–5] Use of a JAs-deficient mutant[3] and other JA-related mutants have further demonstrated that JAs are key compounds in controlling the plant's defense system.[6,7] It was with this background that we began a systematic search for specific inhibitors of the biosynthesis of JAs for use in the analysis of plant defense signal networks.

The cloning and function analysis of three enzymes in jasmonic acid (JA) biosynthesis, 13-lipoxygenase (LOX), allene oxide synthase (AOS) and 12-oxo-10, 15(*Z*)-phytodienoic acid (OPDA) reductase, has provided an understanding of not only the regulation of the biosynthesis of JAs, but also of their integration into signalling networks.[8] AOS is of particular importance in the biosynthesis of JAs because it catalyzes the first specific reaction in the pathway. Additionally, recent evidence indicates that AOS is a focal point in controlling overall biosynthesis of JAs.[9] Thus, AOS is an important site to emphasize in the search for potent inhibitors of biosynthesis of JAs.

Molecular functional analysis of AOS indicates that it is a cytochrome P450 enzyme[10,11] and its catalytic action in biosynthesis of epoxyalcohols has been well characterized.[12] The hydroperoxide substrate is thought to interact with the ferric state of AOS and one-electron transfers from AOS Fe(III) to the O–O bond of 13(*S*)-hydroperoxylinolenic acid, resulting in the formation of AOS Fe(IV)–OH and a substrate alkoxy radical. The subsequent free radical oxidation and loss of a β-proton result in the formation of an unstable oxide, the product of AOS. This observation, which is practically useful for the design of inhibitors, implies that AOS recognizes its substrate through a binding of the lipophilic fatty acid moiety with the protein. Simultaneously, the hydroperoxy group interacts with the prosthetic heme iron.

The inhibition mechanism of the cytochrome P450 enzyme has been studied in considerable detail.[13] It is known that many imidazole derivatives exhibit inhibitory activities against cytochrome P450s,[14] apparently due to the intrinsic affinity of the nitrogen electron pair in heterocyclic molecules for the prosthetic heme iron.[15] The imidazoles thus bind not only to lipophilic regions of the protein, they also simultaneously bind to the prosthetic heme iron.[16] We have thus designed new inhibitors by choosing a highly effective compound among the known imidazole derivatives and introducing

*Corresponding author. Tel.: +81–18-872–1590; fax: +81-872–1670; e-mail: jmwang@akita-pu.ac.jp
†This author has previously published under his Chinese name Jing-Ming Wang.

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an alkylcarboxynate moiety on the possibility that this moiety might mimic the partial structure of 13(*S*)-hydroperoxylinolenic acid, thereby enhancing its binding to the protein. Another structure feature of AOS substrate, 13(*S*)-hydroperoxylinolenic acid, is that there is a (3*Z*) hex-3-enyl moiety. Thus, we introduce a 2,4-dichlorophenyl substitute into newly designed inhibitors to mimic the partial structure of this moiety. Consequently, we have synthesized new derivatives of 2-(1*H*-imidazole-1-yl)-1-(2,4-dichlorophenyl) ethanol.

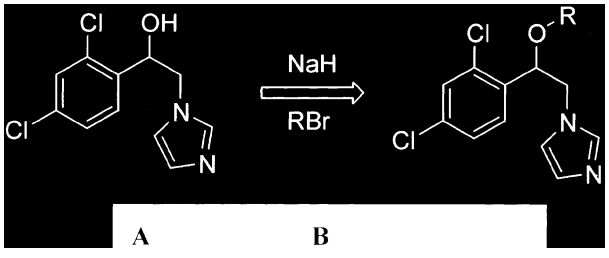
Biologically active recombinant enzymes are useful for evaluation of the inhibitory activity of inhibitors. Hence, we constructed an assay system by using a recombinant *Arabidopsis* AOS expressed in *E. coli*. In the present work, we report on the design and synthesis of a novel series of imidazole derivatives that exhibit potent inhibitory activity against AOS.

Chemistry

The imidazole derivatives reported in this work were prepared by a method shown in Scheme 1. The preparation of target compounds, [1-(1*H*-imidazole-1-yl)-2-(2,4-dichlorophenyl) ethoxy] alkylcaboxynate derivatives (**B**), was carried out by allowing (**A**) to react with alkyl halids or bromoalkylcarbonates under a condition described in the literature.[17] The enzyme substrate, 13(*S*)-hydroperoxylinolenic acid, was prepared from linolenic acid by oxidation with soybean LOX 1 and purified to homogeneity.[18]

Results and Discussions

We tested our synthesized compounds as a racemic mixture. The enzyme activity of AOS was measured using a reverse phase HPLC system to separate the product(s) from the reaction mixture. Typical chromatograms of the reaction mixture are shown in Figure 1. Here, a heated enzyme was used as a negative control (Fig. 1A). We assigned peak I (t_R 8.7 min) to 13(*S*)-hydroperoxylinolenic acid by comparison of its t_R and UV spectrum with that of the authentic specimen. In the presence of AOS (1 pmol protein, Fig. 1B), the amount of 13(*S*)-hydroperoxylinolenic acid was significantly decreased relative to the heated enzyme treatment (Fig. 1A), indicating the biochemical conversion of 13(*S*)-hydroperoxylinolenic acid to 12, 13(*S*)-epoxy-9 (*Z*), 11, 15(*Z*)-octadecatrienoic acid by AOS. Thus, the recombinant assay system can be used effectively to estimate the activity of AOS inhibitors.



Scheme 1. Synthesis of compounds of general structure.

It has been reported that acetylsalicyclic acid (ASA) inhibits AOS at high concentrations[19] and it is known that ketoconazole exhibits inhibitory activity against many types of cytochrome P450s.[20] We thus used these two compounds as baseline references in the estimation of AOS inhibitory activity by our synthesized compounds.

As indicated in Table 1, the IC_{50} (see note of Table 1) value of compounds with double-bond (**a, b**) is about 10 times that of compounds without the double-bond in this moiety (**c, d**). Thus, placing a double-bond appears to have an appreciable negative effect on the potential inhibitory activity of this synthetic series.

The methyl octanate derivative (**g**) exhibits potent inhibitory activity against AOS, with the IC_{50} value of 0.85 ± 0.3 (μM), Suggesting that a carboxylic acid ester substituent is important for increasing inhibitory activity.

An increase in the carbon number in the alcolol of the seter moiety enhanced the inhibitory activity, with compound **k** exhibiting the highest inhibitory activity against AOS, that is, an IC_{50} value of 10 ± 5 nM. Compound **k** is thus the most potent inhibitor of AOS activity reported to date.

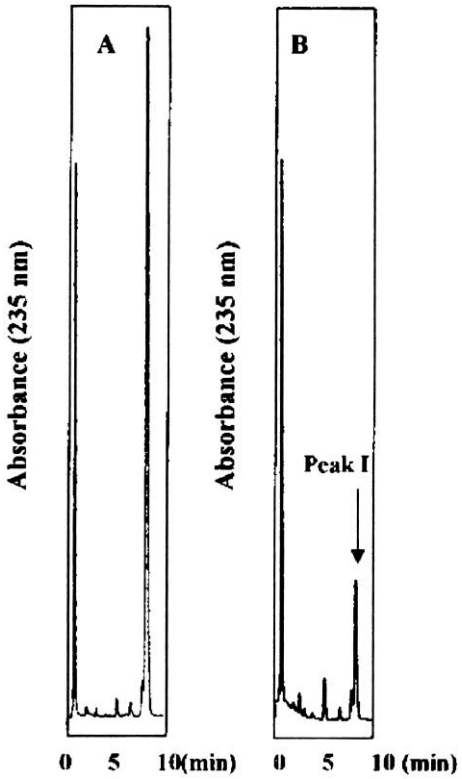
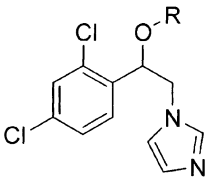


Figure 1. An example of the HPLC analysis of purified recombinant Arabidopsis AOS activity. The enzyme reaction was carried out by incubation of purified AOS (1 pmol, either heated or fresh enzyme) with 13(*S*)-hydroperoxylinolenic acid (2 ng) as substrate at 25 °C for 30 min. The HPLC procedure is described in the Experimental. Chromatograms shown are: **A**, a heated sample of AOS; **B**, a fresh sample of AOS. The example shown was replicated three times to establish repeatability. This system was then used to establish AOS inhibitory activity (IC_{50}) of novel compounds **a**–**k**, ketoconazole and ASA.

Table 1. Inhibition of AOS by synthesized compounds



Compd	R	IC_{50} (μM)[a]
a	CH_2 CH=CH_2	8.8±2.2
b	CH_2 CH=C $(CH_3)_2$	5.9±3.2
c	CH_2 $(CH_2)_7CH_3$	0.61±0.2
d	CH_2 $(CH_2)_2CH_3$	0.90±0.3
e	CH_2 $(CH_2)_3COOCH_2$ CH_3	1.0±0.6
f	CH_2 $(CH_2)_4COOCH_2$ CH_3	0.67±0.2
g	CH_2 $(CH_2)_6COOCH_3$	0.85±0.3
h	CH_2 $(CH_2)_6COO(CH_2)_2$ CH_3	0.21±0.6
i	CH_2 $(CH_2)_6COO(CH_2)_3$ CH_3	0.12±0.02
j	CH_2 $(CH_2)_6COOCH_2$ $CH_2CH(CH_3)_2$	0.08±0.022
k	CH_2 $(CH_2)_6COO(CH_2)_6CH_3$	0.01±0.005
Acetylsalicyclic acid		2500±50[19]
Ketoconazole		10,000±150[20]

[a]IC_{50} values are calculated from the inhibition curve using 5–7 different concentrations (from 1 nM to 1 mM) of inhibitors. Data represent the mean (±SEM) from two to four independent determinations.

Finally, all of the novel derivatives were more potent than either ketoconazole or ASA. The most active, compound **k** with an IC_{50} value of 10 nM, is 250,000-fold and 1,000,000-fold more potent than acetylsalicyclic acid (2.5 mM) and ketoconazole (10 mM), respectively.

AOS belongs to a unique class of cytochrome P450s, 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.[12,21] The catalytic mechanism of AOS and similar cytochrome P450s is still not fully understood. Included in this class of P450 enzymes are thromboxane synthase and PGI_2 synthase.[22] Since our novel compounds exhibit potent inhibitory activity against 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.

Conclusions

We have synthesized highly potent inhibitor for AOS. Structure–activity relationship analysis of these novel compounds leads to the following conclusion: (i) introduction of a double-bond into the alkyl moiety (**a**–**d**) affects adversely; (ii) introduction of a lipophilic environment in the alkyl carboxynate moiety significantly increases AOS-inhibitory activity.

Experimental

^{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. Solvents are either reagent or HPLC grade. Reagents used were of the highest grade commercially available.

Preparation of methyl 8-[1-(2,4-dichlorophenyl)-2-imidazolylethoxy]octanoate (g). To a stirred solution of 1-(1H-imidazole-1yl)-2-(2,4-dichlorophy) ethanol (1.0 g 3.9 mmol) (ACROS ORGANICS) in DMF (3 mL) was gradually added sodium hydride (0.156 g, 3.9 mmol) at room temperature. The reaction mixture was stirred at 40 °C for 30 min. After cooling to −15 °C, to the reaction mixture was added 8-bromooctanonic acid methyl ester (0.92 g, 3.9 mmol) in DMF with continuous stirring for further 30 min at room temperature. After evaporation of solvent under reduced pressure, the residue was diluted with chloroform. The chloroform layer was washed with water and dried over anhydrous sodium sulfate. Separation of the concentrate by column chromatography on sillica gel (chloroform/methanol = 10:1; v:v) gave 0.48 g (29.8%) of **g**. Light yellow oil. ^{1}H NMR ($CDCl_3$) δ 1.28 (br, 6H), 1.50–1.69 (m, 4H), 2.30 (t, J = 7.5 Hz, 2H), 3.18–3.22 (m, 1H), 3.29–3.34 (m, 1H), 3.67 (s, 3H), 3.98 (dd, J = 7.3, 14.3 Hz, 1H), 4.16 (dd, J = 2.8, 14.5 Hz, 1H), 4.83 (dd, J = 2.6, 7.3 Hz, 1H), 6.92 (s, 1H), 7.02 (s, 1H), 7.25 (s, 2H), 7.41 (s, 1H), 7.45 (s, 1H). ESI-MS m/e 413 (M + H)+. Anal. calcd for $C_{20}H_{26}Cl_2N_2O_3$: C, 58.12; H, 6.34; N, 6.78; Cl, 17.15. Found: C, 58.19; H, 6.39; N, 6.90; Cl, 17.25. Other compounds (**a**–**f**, **h**–**k**) were prepared in a similar way by reaction **A** with corresponding halides.

1-[2-Allyloxy-2-(2,4-dichlorophenyl)-ethyl]-1*H*-imidazole (a). Light yellow oil. ^{1}H NMR ($CDCl_3$) δ 3.74 (dd, J = 5.9, 12.8 Hz, 1H), 3.93 (dd, J = 5.1, 12.8 Hz, 1H), 4.02 (dd, J = 7.3, 14.2 Hz, 1H), 4.19 (dd, J = 2.6, 14.3 Hz, 1H), 4.93 (dd, J = 2.6, 7.3 Hz, 1H), 5.15–5.20 (m, 2H), 5.70–5.80 (m, 1H), 6.92 (s, 1H), 7.02 (s, 1H), 7.27 (s, 2H), 7.42 (s, 1H), 7.45 (s, 1H). ESI-MS m/e 297 (M + H). Anal. calcd for $C_{14}H_{14}Cl_2N_2O$: C, 56.58; H, 4.75; N, 9.43; Cl, 23.86. Found: C, 56.48; H, 4.80; N, 9.49; Cl, 23.96.

1-[2-(2,4-Dichlorophenyl)-2-(3-methylbut-2-enyloxy)-ethyl]-1*H*-imidazole (b). Light yellow oil. ^{1}H NMR ($CDCl_3$) δ 1.50 (s, 3H), 1.71 (s, 3H), 3.75 (dd, J = 7.9, 10.9 Hz, 1H), 3.87 (dd, J = 6.4, 10.9 Hz, 1H), 3.98 (dd, J = 7.3, 14.3 Hz, 1H), 4.15 (dd, J = 2.6, 14.3 Hz, 1H), 4.91 (dd, J = 2.5, 7.3 Hz, 1H), 5.19 (br, 1H), 6.92 (s, 1H), 7.02 (s, 1H), 7.27 (s, 2H), 7.41 (s, 1H), 7.45 (s, 1H). ESI-MS m/e 326 (M + H). Anal. calcd for $C_{16}H_{18}Cl_2N_2O$: C, 59.09; H, 5.58; N, 8.61; Cl, 21.80. Found: C, 58.96; H, 5.45; N, 8.72; Cl, 21.88.

1-[2-(2,4-Dichlorophenyl)-2-nonyloxy-ethyl]-1*H*-imidazole (c). Light yellow oil. ^{1}H NMR ($CDCl_3$) δ 0.88 (t, J = 7.0 Hz 3H), 1.25 (br, 16H), 1.49–1.56 (m, 2H), 3.19–3.25 (m, 1H), 3.32–3.35 (m, 1H), 3.98 (dd, J = 7.4, 14.6 Hz, 1H), 4.17 (dd, J = 2.7, 14.5 Hz, 1H), 4.83 (dd, J = 2.7, 7.4 Hz, 1H), 6.92 (s, 1H), 7.01 (s, 1H), 7.25 (s,

2H), 7.41 (s, 1H), 7.45 (s, 1H). ESI-MS *m/e* 412 (M + H) + . Anal. calcd for $C_{20}H_{28}Cl_2N_2O$: C, 62.66; H, 7.36; N, 7.30; Cl, 18.50. Found: C, 62.58; H, 7.29; N, 7.25; Cl, 18.55.

1-[2-Butoxy-2-(2,4-Dichlorophenyl)-ethyl]-1*H*-imidazole (d). Light yellow oil. ^{1}H NMR ($CDCl_3$) δ 0.88 (t, J = 7.5 Hz 3H), 1.25–1.37 (m, 2H), 1.48–1.55 (m, 2H), 3.20–3.26 (m, 1H), 3.31–3.36 (m, 1H), 3.97 (dd, J = 7.5, 14.6 Hz, 1H), 4.17 (dd, J = 2.6, 14.3 Hz, 1H), 4.83 (dd, J = 2.7, 7.3 Hz, 1H), 6.92 (t, J = 1.3 Hz,1H), 7.01 (d, J = 1.1 Hz, 1H), 7.25 (s, 2H), 7.41 (s, 1H), 7.45 (s, 1H). ESI-MS *m/e* 314 (M + H). Anal. calcd for $C_{15}H_{18}Cl_2N_2O$: C, 57.52; H, 5.79; N, 8.94; Cl, 22.64. Found: C, 57.53; H, 5.70; N, 8.96; Cl, 22.69.

Ethyl 5-[1-(2,4-dichlorophenyl)-2-imidazolylethoxy] pentanoate (e). Light yellow oil. ^{1}H NMR ($CDCl_3$) δ 1.26 (t, J = 7.1 Hz, 3H), 1.55–1.66 (m, 2H), 2.28 (t, J = 7.1 Hz, 2H), 3.21–3.26 (m, 1H), 3.32–3.37 (m, 1H), 3.98 (dd, J = 7.3, 14.6 Hz, 1H), 4.11–4.20 (m, 3H), 4.84 (dd, J = 2.6, 7.3 Hz, 1H), 6.92 (s, 1H), 7.02 (s, 1H), 7.25 (s, 2H), 7.41 (s, 1H), 7.45 (s, 1H). ESI-MS *m/e* 385 (M + H). Anal. calcd for $C_{18}H_{22}Cl_2N_2O_3$: C, 56.11; H, 5.76; N, 7.27; Cl, 18.40. Found: C, 56.11; H, 5.70; N, 7.30; Cl, 18.56.

Ethyl 6-[1-(2,4-dichlorophenyl)-2-imidazolylethoxy]-hexanoate (f). Light yellow oil. ^{1}H NMR ($CDCl_3$) δ 1.25 (t, J = 7.2 Hz, 3H), 1.55–1.66 (m, 4H), 2.27 (t, J = 7.2 Hz, 2H), 3.19–3.24 (m, 1H), 3.31–3.37 (m, 1H), 3.97 (dd, J = 7.3, 14.6 Hz, 1H), 4.10–4.29 (m, 3H), 4.83 (dd, J = 2.6, 7.3 Hz, 1H), 6.92 (s, 1H), 7.02 (s, 1H), 7.25 (s, 2H), 7.41 (s, 1H), 7.44 (s, 1H). ESI-MS *m/e* 400 (M + H). Anal. calcd for $C_{19}H_{24}Cl_2N_2O_3$: C, 57.15; H, 6.06; N, 7.02; Cl, 17.76. Found: C, 57.11; H, 6.15; N, 7.10; Cl, 17.80.

Propyl 8-[1-(2,4-dichlorophenyl)-2-imidazolylethoxy]-octanoate (h). Light yellow oil. ^{1}H NMR ($CDCl_3$), δ 0.94 (t, J = 7.3 Hz, 3H), 1.24–1.29 (m, 2H), 1.50–1.69 (m, 10H), 2.30 (t, J = 7.3 Hz, 2H), 3.19–3.24 (m, 1H), 3.29–3.35 (m, 1H), 3.98 (dd, J = 7.5, 14.7 Hz, 1H), 4.03–4.19 (m, 3H), 4.82 (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/e* 442 (M + H). Anal. calcd for $C_{22}H_{30}Cl_2N_2O_3$: C, 59.86; H, 6.85; N, 6.35; Cl, 16.04. Found: C, 59.99; H, 6.82; N, 6.39; Cl, 16.06.

Butyl 8-[1-(2,4-dichlorophenyl)-2-imidazolylethoxy]-octanoate (i). Light yellow oil. ^{1}H NMR ($CDCl_3$) δ 0.93 (t, J = 7.3 Hz, 3H), 1.24–1.30 (m, 4H), 1.51–1.71 (m, 10H), 2.29 (t, J = 7.2 Hz, 2H), 3.20–3.24 (m, 1H), 3.29–3.35 (m, 1H), 3.98 (dd, J = 7.4, 14.6 Hz, 1H), 4.03–4.19 (m, 3H), 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/e* 456 (M + H) + . Anal. calcd for $C_{23}H_{32}Cl_2N_2O_3$: C, 60.66; H, 7.08; N, 6.15; Cl, 15.57. Found: C, 60.59; H, 6.90; N, 6.22; Cl, 15.69.

3-Methylbutyl 8-[1-(2,4-dichlorophenyl)-2-imidazolylethoxy]octanoate (j). Light yellow oil. ^{1}H NMR ($CDCl_3$) δ 0.87 (s, 3H), 0.89 (s, 3H), 1.24–1.27 (m, 8H), 1.48–1.73 (m, 6H), 2.30 (t, J = 7.2 Hz, 2H), 3.20–3.24 (m, 1H), 3.29–3.35 (m, 1H), 3.98 (dd, J = 7.5, 14.6 Hz, 1H), 4.08–4.19 (m, 3H), 4.82 (dd, J = 2.7, 7.2 Hz, 1H), 6.92 (s, 1H), 7.02 (s, 1H), 7.27 (s, 2H), 7.41 (s, 1H), 7.45 (s, 1H). ESI-MS *m/e* 470 (M + H). Anal. calcd for $C_{24}H_{34}Cl_2N_2O_3$: C, 61.41; H, 7.30; N, 5.97; Cl, 15.10. Found: C, 61.52; H, 7.35; N, 6.02; Cl, 15.14.

Heptyl 8-[1-(2,4-dichlorophenyl)-2-imidazolylethoxy]-octanoate (k). Light yellow oil. ^{1}H NMR ($CDCl_3$) δ 0.88 (t, J = 3.3 Hz, 3H), 1.27–1.40 (m, 10H), 1.51–1.71 (m, 10H), 2.29 (t, J = 7.2 Hz, 2H), 3.20–3.24 (m, 1H), 3.29–3.35 (m, 1H), 3.98 (dd, J = 7.4, 14.6 Hz, 1H), 4.03–4.19 (m, 3H), 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/e* 498 (M + H). Anal. calcd for $C_{26}H_{38}Cl_2N_2O_3$: C, 62.77; H, 7.70; N, 5.63; Cl, 14.25. Found: C, 62.85; H, 7.85; N, 5.83; Cl, 14.34.

Preparation of 13(*S*)-hydroperxy-9(*Z*), 11(*E*), 15(*Z*)-octadecatrienoic acid. Linolenic acid (925 mg, 3.3 mmol) was dissolved in a sodium borate buffer solution (470 mL; 0.1 M, pH = 9) under nitrogen atmosphere and cooled to 0 °C in a ice bath. Soybean lipoxygenase I (89 mg) was added, a gentle stream of oxygen was bubbled through the solution via a Pasteur pipet, and the mixture was stirred for 30 min. Progress of the reaction was monitored by UV spectroscopy at 234 nm. After the reaction, the mixture was adjusted to pH 3 with 0.1 M hydrochloric acid solution and extracted twice with 300 mL of chloroform/methanol (2:1 v/v). The chloroform layer was collected and concentrated under reduced pressure. The samples were resuspended in 1 mL *n*-hexane and loaded onto a silica gel column (1–20 cm) prewashed with *n*-hexane. The loaded column was washed with 30 mL of *n*-hexane/diethyl ether/acetic acid (90:10:1 v/v/v), and 13(*S*)-HPOT was eluted with 30 mL of hexane/diethyl ether/acetic acid (75:25:1 v/v/v). After evaporation of the solvent under N_2, 13 (*S*)-HPOT was dissolved in methanol and stored at −80 °C.

Expression and purification of recombinant AOS in *E. coli*. The coding region of AOS cDNA that was restricted by the enzymes BamHI and KpnI, was inserted into an E. 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. An overnight culture of M15 *E. coli* (15 mL) was added to 1 L of fresh Turia-Bertani medium with amphicillin (100 μg/mL), placed in 2-L culture flasks and shaken at 100 rpm and at 37 °C. After 3 h of incubation, isopropyl-D-thiogalacto-pyranoside (IPTG) was added to the culture to a final concentration of 1 mM to induce expression of the recombinant Arabidopsis AOS protein. The culture was shaken continuously at 100 rpm for 8 h at 16 °C.

Cells of *E. coli* from a 1-L culture were pelleted and resuspended in 50 mL of phosphate buffer (50 mM, pH = 8.0) plus 0.1% Triton X-100. The cell suspension was sonicated 15 times for 30 s each time while on ice. The soluble fraction (supernatant) was prepared by centrifugation at 10,000*g* for 1 h at 4 °C. This supernatant fraction was then applied to a His-Tag affinity

column, the AOS being purified by use of His-Tag risin (Novagen). The purified AOS was then eluted with a 20% elution buffer according to the Novagen protocol.

HPLC analysis of AOS activity. A HPLC system equipped with a UV detector (Hitachi model L7400, Hitachi Co., Ltd., Tokyo, Japan) and a flow system (Hitachi, model L7100) were used. Sample injection was controlled by using an autosampler (Hitachi, model L7200). The recombinant Arabidopsis AOS reaction mixture was separated on an octadecylsilane column (C-18, UG120, 4.6 mm inner diameter ×150 mm, Shiseido Co., Ltd., Tokyo, Japan), keeping the column temperature at 25 °C.

Elution of the HPLC column was carried out with 50% aqueous acetonitrile (containing 0.1% phosphoric acid) at a flow-rate of 2 mL/min. The initial enzyme reaction, prior to HPLC, was carried out using purified AOS enzyme, as above, that is 1 pmol of either heated or fresh enzyme incubated with 13(*S*)-hydroperoxylinolenic acid (2 ng/mL) as substrate at 25 °C for 30 min. The reaction was stopped by acidifying to pH 3 with HCl.

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