Discovery of New Lead Compounds as Inhibitors of Allene Oxide Synthase Based on Ozagrel Scaffold

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***Abstract*—Plant defense responses to herbivore attack and mechanical wounding are regulated by signal transduction pathways. Jasmonates (JAs) are important signal mediators that involve in plant defense signaling. To explore the regulation mechanism of JAs signaling, we have been working on JA biosynthesis inhibitors through design and synthesis small molecules targeting allene oxide synthase (AOS, CYP74A), a key enzyme in JA biosynthesis. In the present work, we report the synthesis of new imidazole-type AOS inhibitors based on ozagrel scaffold. The inhibitory activity of the synthesized compounds against AOS was determined by using purified recombinant protein expressed in E. Coli. Among the nine newly synthesized imidazole derivatives, we found that 6-[4-(2-imidazol-1-yl-ethyl) phenoxy] hexanoic acid ethyl ester (f) exhibits inhibitory activity against AOS with an IC_{50} value approximately 78±12 μM.**

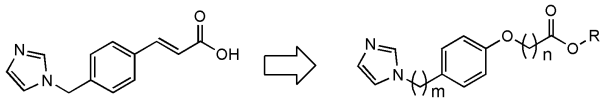
***Index Terms*—allene oxide synthase, Inhibitors, Jasmonic acid biosynthesis, Plant hormone, Plant defense response, Imidazole**

I. Introduction

The divergent oxidative metabolism of polyunsaturated fatty acids in most living organism leads to the production of a group of bioactive compounds, collectively called oxylipins. Jasmonates (JAs) are end products of octadecanoid pathway in phyto-oxylipin biosynthesis cascades [1], while the eicosanoids, which include prostaglandins, are biologically important oxylipins in mammals [2].

JAs serve as important signals that regulate gene expression in response to stress and developmental cues. JAs play key role in defense against herbivore attack and some infection of pathogens [3]. JAs are also implicated in the control of plant responses to abiotic stimuli such as mechanical wounding [3], salt stress and UV irradiation [4]. Exogenous JAs exert numerous inductive and inhibitory effects on plant developmental processes [4]. Because JAs are key signal mediators that regulate the gene expression in plant defense [3], [4], efforts have been made to manipulate JAs contents in plant tissue by using genetic approaches. Molecular functional analysis of enzymes in JA biosynthesis indicated that allene oxide synthase (AOS) is an important site in controlling overall of JA biosynthesis [5]. AOS expression is positively regulated by wounding [6], transgenic studies indicate that AOS is a rate-limiting step in JA biosynthesis [6]. Mutations that disrupt AOS in *Arabidopsis* have shown to cause male sterility in plant tissues and lost function in response to herbivior attack as well as response to mechanical wounding [7].

An alternative way to control JA biosynthesis is the use of specific inhibitors that targeting the important enzyme in JA biosynthesis. Since AOS is a key enzyme in JA biosynthesis, we have consequently worked on development of specific inhibitors targeting AOS [8]-[10].



Ozagrel Target compounds (**a-i**)

Figure 1. Using ozagrel as a molecular scaffold of AOS inhibitors

Molecular functional analysis of AOS indicated that it belongs to cytochrome P450s. Different from classic monoxygenase, it 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 [11]. Included in this class of P450 enzymes are thromboxane synthase (CYP5A1) and PGI2 synthase [12]. Thus, inhibitors targeting thromboxane synthase are possible candidate of AOS inhibitors scaffold (Figure 1). Since ozagrel is a known thromboxane inhibitor [13], we thus use ozagrel as a molecular scaffold to explore new AOS inhibitors. In the present work, we report the discovery of new imidazole lead compound of AOS inhibitors.

II. Material and Method

A. General

Chemicals for synthesis were purchased from Kanto Chemicals Co. Ltd. (Tokyo, Japan) and Tokyo Kasei Co.

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Ltd. (Tokyo, Japan). All the reagents were of the highest purity commercially available. ^{1}H-NMR spectra were recorded with a JEOL ECP-400 spectrometer, chemical shifts being expressed in ppm downfield from TMS as an internal standard. Electrospray ionization fourier transform ion cyclotron resonance mass spectra (ESI-FTICR) were recorded on an Exactive MS System (Thermo Fisher Scientific, USA).

B. Expression and Purification of Recombinant AtAOS

The coding region of *At*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 bacteria (2 mL) was incubated into 1 liter of fresh Turia-Bertani medium supplemented with ampicillin (100 μg/mL), placed in 2-liter culture flasks. Becateria were grown at 37 ^{o}C in a shaker at 150 rpm to an OD600 of 0.5. Cultures were cooled to 16 ^{o}C, and isopropyl-β-D-thiogalacto- pyranoside was added to a final concentration of 1 mM. Induced cultures were incubated for 24 h at 16 ^{o}C with gentle shaking (100 rmp). Cells were collected by centrifugation. Purification of recombinant *At*AOS was performed as described previously [9] with minor modification. Cells of E. coli from a 1-liter 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 sec. each time while on ice. The soluble fraction (supernatant) was prepared by centrifugation at 10000 x g for 1 hr at 4 ^{o}C. The supernatant fraction was then applied to a His-Tag affinity column, the *At*AOS being purified by use of His-Tag risin (Novagen). The purified *At*AOS was then eluted with a 20% elution buffer according to the Novagen protocol. Eluted sample was dialyzed for 24 hours at 4 oC by using an oscillatory dialysis system (Daiichi Pure Chemicals. CO., Ltd. Tokyo, Japan.) against 2 x 300 mL dialysis buffer (50 mM sodium phosphate buffer, pH 7.0). Protein measurements were performed using a Protein Assay Kit (Bio-Red, Herculs, CA, USA) and bovine serum albumin as a standard. The relative purity of recombinant AtAOS was estimated by SDS-polyacrylamide gel electrophoresis (12% polyacryl- amide) and staining of gels with Coomassie Brillant Blue R250.

C. In Vitro Assays for AtAOS

Enzyme reaction mixture contained 50 mM sodium phosphate buffer pH 7.0, enzyme (*At*AOS, (15 nM), and designed concentrations of substrate (13(*S*) HPOT) at 25 ^{o}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 44.5 μM-1cm-1 was used. The time course was followed for 400 sec to estimate the AOS activity. For kinetics studies of JM-8686 inhibition, AOS assay was carried out in triplicate in the presence of varying concentration of JM-8686. In case to determine the inhibitory activity of test compounds on AOS inhibition, enzyme reaction was started by adding *At*AOS to a final concentration of 15 nM.

D. Chemical Synthesis

1) Preparation of 5-(4-imidazol-1-ylmethyl- phenox)-pentanoic acid ethyl ester (a).

Synthesis of compound (**a**) was carried out by a method using 5-(4-hydroxymethyl- phenoxy)pentanoic acid ethyl ester as a starting material under a condition as discribed previously [14]. In a solution of anhydrate acetonitrle (5 ml) containing 252 mg (1 mmol) of 5-(4-hydroxymethylphenoxy)pentanoic acid ethyl ester, 162 mg (1 mmol) of diimidazol-1-yl-methanone desolved in acetonitrle (1 ml) was added and the reaction mixture was stirred for 5 hours at room temperature under nitrigen atmosphare. After the reaction has been monitored by TLC analysis, the sovent was removed under reduced pressure and the product was purified by silica column chromatography using ethylacetate and hexane (3:7) as elute. (89% yeild). ^{1}H NMR ($CDCl_3$) δ 1.25 (t, *J*=8.3 Hz, 3H), 1.81 (s, 4H), 2.38 (t, *J*=5.5 Hz, 2H), 3.96 (d, *J*=5.5 Hz, 2H), 4.13 (q, *J*=7.1 Hz, 2H), 5.07 (d, *J*=15.3 Hz, 2H), 6.89 (d, *J*=10.3 Hz, 2H), 7.07 (s, 1H), 7.28 (d, *J*=10.3 Hz, 2H), 7.42 (s, 1H), 8.13 (s, 1H). MS-ESI m/z calculated for $C_{17}H_{22}N_2O_3$ $[M]^+$ 303, found 303. Other compounds (**b–i**) were prepared in a similar method as described above by reacting diimidazol-1-yl-methanone with the corresponding alcohols.

2) Preparation of 6-(4-Imidazol-1-ylmethylphenoxy) hexanoic acid ethyl ester (b).

(92% yeild) ^{1}H NMR ($CDCl_3$) δ 1.26 (t, *J*=7.2 Hz, 3H), 1.44-1.85 (m, 6H), 2.34 (t, *J*=7.5 Hz, 2H), 3.97 (t, *J*=6.3 Hz, 2H), 4.13 (q, *J*=7.2 Hz, 2H), 5.35 (s, 2H), 6.91 (d, *J*=8.4 Hz, 2H), 7.05 (s, 1H), 7.37 (d, *J*=8.4 Hz, 2H), 7.42 (s, 1H), 8.13 (s, 1H). $C_{18}H_{24}N_2O_3$ $[M]^+$ 317, found 317.

3) Preparation of 11-(4-Imidazol-1-ylmethyl phenoxy)undecanoic acid ethyl ester (c).

(78% yeild) ^{1}H NMR ($CDCl_3$) δ 1.25 (t, *J*=7.2 Hz, 3H), 1.41-1.44 (m, 2H), 1.57-1.63 (m, 2H), 1.74-1.81 (m, 2H), 2.28 (t, *J*=7.4 Hz, 2H), 3.96 (t, *J*=6.6 Hz, 2H), 4.12 (q, *J*=7.1 Hz, 2H), 5.35 (s, 2H),6.91 (d, *J*=8.3 Hz, 2H), 7.05 (s, 1H), 7.26 (s, 5H), 7.37 (d, *J*=8.3 Hz, 2H), 7.41 (s, 1H), 8.13 (s, 1H). $C_{22}H_{32}N_2O_3$ $[M]^+$ 373, found 373.

4) Preparation of 4-[4-(2-Imidazol-1-yl-ethyl) phenoxy]butyric acid ethyl ester (d).

(88% yeild) ^{1}H NMR ($CDCl_3$) δ 1.25 (t, *J*=7.1 Hz, 3H), 2.01 (t, *J*=6.6 Hz, 2H), 2.51 (t, *J*=7.3 Hz, 2H),3.02 (t, *J*=4.2 Hz, 2H), 4.13 (q, *J*=7.1 Hz, 2H), 4.58 (t, *J*=4.2 Hz, 2H), 6.85 (d, *J*=9.4 Hz, 2H), 7.06 (s, 1H), 7.15 (d, *J*=9.4 Hz, 2H), 7.39 (s, 1H), 8.10 (s, 1H). $C_{17}H_{22}N_2O_3$ $[M]^+$, found 303.

5) Preparation of 5-[4-(2-Imidazol-1-yl-ethyl) phenoxy]pentanoic acid ethyl ester (e).

(95% yeild) ^{1}H NMR ($CDCl_3$) δ 1.26 (t, *J*=7.1 Hz, 3H), 1.80-1.83 (m 4H), 2.38 (t, *J*=7.0 Hz, 2H), 3.03 (t, *J*=7.0 Hz, 2H), 3.95 (t, *J*=5.5 Hz, 2H), 4.13 (q, *J*=7.3 Hz, 2H), 4.57 (t, *J*=7.0 Hz, 2H), 6.85 (d, *J*=8.8 Hz, 2H), 7.06 (s, 1H), 7.14

(d, *J*=8.8 Hz, 2H), 7.39 (s, 1H), 8.10 (s, 1H). $C_{18}H_{24}N_2O_3$ $[M]^+$ 317, found 317.

6) Preparation of 6-[4-(2-Imidazol-1-yl-ethyl) phenoxy]hexanoic acid ethyl ester (f).

(92% yeild) ^{1}H NMR ($CDCl_3$) δ 1.25 (t, *J*=7.2 Hz, 3H), 1.47-1.54 (m, 2H), 1.66-1.84 (m, 4H), 2.33 (t, *J*=7.5 Hz, 2H), 3.03 (t, *J*=7.0 Hz, 2H), 3.94 (t, *J*=6.4 Hz, 2H), 4.13 (m 2H), 4.57 (t, *J*=7.0 Hz, 2H), 6.85 (d, *J*=8.4 Hz, 2H), 7.05 (s, 1H), 7.15 (d, *J*=8.4 Hz, 2H), 7.39 (s, 1H), 8.10 (s, 1H). $C_{19}H_{26}N_2O_3$ $[M]^+$ 331, found 331.

7) Preparation of 4-[4-(2-Imidazol-1-yl-ethyl) phenoxy]butyric acid (g).

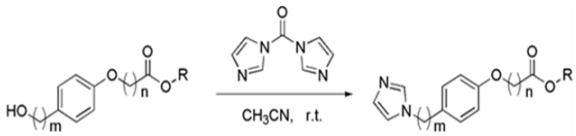
(68% yeild) ^{1}H NMR ($CDCl_3$) δ 1.25 (s, 1H), 2.11 (tt, J_1=J_2=6.6 Hz, 2H), 2.58 (t, *J*=7.3 Hz, 2H), 3.03(t, *J*=6.8 Hz, 2H), 4.02 (t, *J*=6.1 Hz, 2H), 4.57 (t, *J*=6.8 Hz, 2H), 6.86 (d, *J*=8.4 Hz, 2H), 7.07 (s, 1H), 7.14 (d, *J*=8.4 Hz, 2H), 7.26(s, 2H), 7.39 (s, 1H), 8.09 (s, 1H). $C_{15}H_{18}N_2O_3$ $[M]^+$ 275, found 275.

8) Preparation of 5-[4-(2-Imidazol-1-yl-ethyl) phenoxy]pentanoic acid (h).

(60% yeild) ^{1}H NMR ($CDCl_3$) δ 1.82-1.85 (m, 4H), 2.44 (t, *J*=6.8 Hz, 2H), 3.03 (t, *J*=6.8 Hz, 2H), 4.00 (t, *J*=5.8 Hz, 2H), 4.58 (t, *J*=6.8 Hz, 2H), 6.85 (d, *J*=8.4 Hz, 2H), 7.07 (s, 1H), 7.14 (d, *J*=8.4 Hz, 2H), 7.40 (s, 1H), 8.10 (s, 1H). $C_{16}H_{20}N_2O_3$ $[M]^+$ 289, found 289.

9) Preparation of 6-[4-(2-Imidazol-1-yl-ethyl) phenoxy]hexanoic acid (i).

(55% yeild) ^{1}H NMR ($CDCl_3$) δ 1.53 (tt, J_1=3.5, J_2=4.07 Hz, 2H), 1.72 (tt, J_1=J_2=7.59 Hz, 2H), 1.80 (tt, J_1=7.04, J_2=7.14 Hz, 2H), 2.39 (t, *J*=7.1 Hz, 2H), 3.02 (t, *J*=6.8 Hz, 2H), 3.96 (t, *J*=6.4 Hz, 2H), 4.58 (t, *J*=7.0 Hz, 2H), 6.85 (d, *J*=7.0 Hz, 2H), 7.06 (s, 1H), 7.14 (d, *J*=7.0 Hz, 2H), 7.39 (s, 1H), 8.08 (s, 1H). $C_{17}H_{22}N_2O_3$ $[M]^+$ 303, found 303.



Scheme 1. General method for synthesis the target compound

III. Results and Discussion

The imidazole derivatives reported in this work were prepared by a method shown in Scheme 1 [14]. The biological activity of test compounds on AOS inhibition was carried out by using spectrophotometric method [10]. JM-8686 [9], a specific inhibitor of AOS developed in our laboratory was used as a positive control for structure-activity relationship studies. (4-Imidazol-1-ylmethylphenoxy) alkylcarboxic acid ethyl ester derivatives (**a-c**) are analogues which share a common structure of 4-imidazol-1-ylmethylphenyl moiety with ozagrel. At a concentration of 100 μM, these compounds exhibited inhibitory activity against AOS from 12 to 36% (Table 1). This result indicates that these analogues did not show potent inhibitory activity against AOS. Introduction of 4-(2-Imidazol-1-yl-ethyl) phenyl instead of 4-imidazol- 1-yl-methylphenyl to the compounds (**d-f**) promoted the inhibitory activity against AOS (Table I). The degrees of AOS inhibition are found from 23 to 68%. Among these compounds, compound **f** is the most potent inhibitor with 68±6% inhibitory activity against AOS at 100 μM (Table 1). This result also indicates that the carbon number between imidazole moiety and the phenyl moiety is sensitive to AOS inhibition in this synthetic series.

To further explore the structure-activity relationships of this newly synthesized imidazole derivatives, three carboxylic acid analogues (**g-i**) were prepared and their inhibitory activity against AOS was investigated (Table 1). We found the inhibitory activity of carboxylic acid analogues was significantly decreased (Table I). The inhibitory activity of compound **i** which share a common structure with compound f, except the ethyl ester moiety, significantly reduced the inhibitory activity against AOS from 68±6 to 12±6%, indicating that a carboxylic acid ester moiety is essential for AOS inhibition. Among the synthesized compounds, we found that compound **f** is the most potent inhibitor. Does-dependent inhibition analysis of compound **f** revealed that the IC_{50} of this compound is approximately 78±12 μM (Fig. 2).

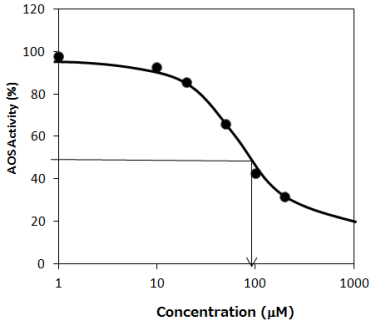
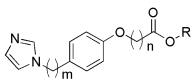


Figure 2. Does dependent inhibition of AOS by compound f.

Although there are only nine analogues were synthesized and used for biological studies, data obtained in this work indicated that compound **f** is a new lead of AOS inhibitor. We expect further chemical optimization of this synthetic series will lead to discover novel agents for JA biosynthesis inhibitors as well as new agents for dissertation the functions of JA biosynthesis.

TABLE I: Inhibitory Activity of Imidazole Compounds Against AOS



Compound ID.	m	n	R	Inhibition (%)
a	1	4	ethyl	12±5
b	1	5	ethyl	15±6
c	1	10	ethyl	36±7
d	2	3	ethyl	23±3
e	2	4	ethyl	42±4
f	2	5	ethyl	68±6
g	2	3	hydroxy	10±5
h	2	4	hydroxy	8±2
i	2	5	hydroxy	12±6
JM-8686(1 μM)				95±3

*the final concentration of test compounds (**a-i**) were 100 μM, all the experiments were done at least three times to establish the repeatability.

IV. Conclusion

To explore novel chemicals for regulating JA biosynthesis, ozagrel, a potent inhibitor of thromboxane synthase was used as a molecular scaffold.

Enzyme reaction mixture was carried out as indicated in the experimental section. The concentrations of compound **f** were 0, 10, 20, 50, 100, and 200 μM. All the experiments were triplicated to establish the repeatability.

We used purified recombinant AOS as an enzyme system to evaluate the inhibitory activity of synthesized compounds against AOS. Among the nine analogues of ozagrel, we found that 6-[4-(2-Imidazol-1-yl-ethyl)phenoxy] hexanoic acid ethyl ester (**f**) exhibits inhibitory activity against AOS with an IC_{50} value approximately 78±12 μM. Structure-activity relationship studies of newly synthesized compounds provided information about the structure requirements for AOS inhibition.

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