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Note

5-Aryl-1,3,4-oxadiazole-2-thiols as a New Series of *trans*-Cinnamate 4-Hydroxylase Inhibitors

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A series of 5-aryl-1,3,4-oxadiazole-2-thiols was found to inhibit *trans*-cinnamate 4-hydroxylase (C4H) from *Populus kitakamiensis*, which was expressed in yeast. 5-Phenyl-1,3,4-oxadiazole-2-thiol showed inhibitory activity comparable to 2-hydroxy-1-naphthoic acid, a known C4H inhibitor. Studies on the structure-activity relationship indicated that the presence of a thiol group was significant for stronger activity. Of the compounds tested, 5-(3-fluorophenyl)-1,3,4-oxadiazole-2-thiol was the most active. © Pesticide Science Society of Japan

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INTRODUCTION

The *trans*-cinnamate 4-hydroxylase (C4H) is a cytochrome P450 which catalyzes the hydroxylation of *trans*-cinnamic acid into *p*-coumaric acid, the first oxygenation step in the metabolism of phenylpropanoids in plants.[1] Phenylpropanoid metabolism is a plant-specific pathway leading to a variety of compounds such as lignins, lignans, flavonoids, isoflavonoids and coumarins, which are essential for plant development and defense against ultraviolet light, predators and pathogens. C4H, which is highly inducible by wounding and pathogen infection, is indicated to be the major enzyme in the regulation of lignification and defense-related reactions.[2] Therefore, specific C4H inhibitors would offer an attractive means of studying the important phenylpropanoid pathway of plants. Since C4H has not been found in any invertebrate or vertebrate animals, it might be an unexploited target for development of a new herbicide.

Schalk *et al*. described 2-hydroxy-1-naphthoic acid (**1**)[3] and piperonylic acid (**2**)[4] as a competitive inhibitor and a mechanism-based inactivator of C4H, respectively. There is no report in the literature of C4H inhibitors other than these structural analogs of cinnamic acid. By random screening, we have recently found that 5-phenyl-1,3,4-oxadiazole-2-thiol strongly inhibits C4H, CYP73a from *Populus kitakamiensis*, which is expressed in yeast. In the present paper we report the structure-activity relationships of a series of 5-aryl-1,3,4-oxadiazole-2-thiols.

MATERIALS AND METHODS

1. Chemicals

2-Hydroxy-1-naphthoic acid and piperonylic acid were purchased from Tokyo Kasei Co. Compound **3** was purchased from Aldrich Co.

2-Methylthio-5-phenyl-1,3,4-oxadiazole (**4**) was prepared by methylation of **3** with methyl iodide.[5] 2-Phenyl-1,3,4-oxadiazole (**5**) was prepared according to the reported method.[6] 5-Substituted-1,3,4-oxadiazole-2-thiols (**7–30**) were synthesized from the reaction of the corresponding hydrazides and carbon disulfide in the presence of potassium hydroxide according to procedures reported previously.[6–8] The structures of the compounds were confirmed using ^{1}H-NMR spectra which were recorded on a JEOL EX-400 (400 MHz) spectrometer. The following procedure for the preparation of 5-(3-fluorophenyl)-1,3,4-oxadiazole-2-thiol (**23**) is typical.

To a solution of 2.6 g of 3-fluorobenzohydrazide and 1.2 g of potassium hydroxide in 20 ml of ethanol was added 3.2 g of carbon disulfide. The mixture was refluxed for 9 hr and then the solvent was evaporated under reduced pressure. The residue was dissolved in 5 ml of water and the solution was acidified to pH 5–6 with 1 M HCl solution. The resulting precipitate was collected by filtration and recrystallized from ethanol to give 1.6 g (40%) of **23**, mp 134–135.5°C. ^{1}H-NMR ($CDCl_3$) δ: 7.48–7.79 (4H, m, phenyl), 11.6 (1H, s, SH). Anal. Found: C, 49.08; H, 2.58; N; 14.34%. Calcd. for $C_8H_5N_2OFS$: C, 48.97; H, 2.54; N; 14.28%.

2-Hydroxy-5-phenyl-1,3,4-oxadiazole (**6**). To a solution of 2.2 g of bis(trichloromethyl)carbonate in 50 ml of *tert*-butyl methyl ether at 0–5°C was added a solution of 1 g of benzohydrazide in 10 ml of tetrahydrofuran. After stirring for 1 day at room temperature, 50 ml of 5% Na_2CO_3 solution was added to the mixture, and the organic layer was washed with 5% $NaHCO_3$ solution and brine, and dried over Na_2SO_4. After removal of the solvent, the residue was recrystallized from diisopropyl ether to give 1.0 g (84%) of **6**, mp 141–142°C, ^{1}H-NMR ($CDCl_3$) δ: 7.44–7.55 (3H, m, phenyl), 7.84–7.88 (2H, m, phenyl), 9.59 (1H, broad s, OH). Anal. Found: C, 59.37; H, 3.69; N, 17.33%. Calcd. for $C_8H_6N_2O_2$: C, 59.26; H, 3.73; N; 17.28%.

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2. Construction of C4H Expression Vector in Yeast

The cDNA of *cyp73a* (DNA database accession number: D82815) from a hybrid aspen, *Populus kitakamiensis*, has been isolated.[9] For heterogeneous gene expression in yeast, the forward primer was synthesized to include a start codon (in bold) with addition of a *Bam*HI site (*italic*) to aid in cloning in the yeast expression vector described below, (C4H-sense: 5′-CC*G-GATCC*AAGATA**ATG**GATCTCCTCCTC-3′) and the reverse primer was also designed with addition of a *Bam*HI site, (C4H-antisense: 5′-GGGATCCGCATCCAAAACCATCCCG-3′). All *Escherichia coli* manipulations were carried out in strain MV1190. The amplified fragment was digested with *Bam*HI and purified with agarose gel electrophoresis. It was inserted into the *Bam*HI site of pUC119, and the resultant products were sequenced to confirm PCR fidelity. The fragment was subcloned into the *Bam*HI site downstream between of *GAL1* promoter and *CYC1* terminator of pYES2 (Invitrogen, Carlsbad, CA), yielding the expression construct pYES73a.

3. Yeast Transformation and Heterologous Gene Expression in Yeast Cells

Introduction of the plasmid into *Saccharomyces cerevisiae* BJ2168, selection of the transformant, and induction of the P450 protein were carried out as described previously.[10] The transformed yeast cells grown in expression medium were periodically collected, and microsomes from them were prepared. The yeast cells transformed with pYES2 were used as controls in the enzyme assay.

4. Preparation of Microsomes from Yeast

Yeast spheroplasts were prepared by enzymatic digestion of yeast cell walls with zymolyase as reported previously[11] and disrupted at 0–4°C with glass beads in 0.1 M potassium phosphate buffer (pH 7.5) containing 0.25 M sucrose, 15 mM 2-mercaptoethanol, 1 mM EDTA, 40 mM sodium ascorbate, 10 mM $MgCl_2$, 3 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride. The lysate was centrifuged at 3,800 *g* for 10 min, then at 10,000 *g* for 10 min at 0°C, and the resulting supernatant was centrifuged at 100,000 *g* for 60 min at 4°C. Pellets (microsomal fraction) were resuspended (35 mg protein/ml) in 0.1 M potassium phosphate buffer (pH 7.5) containing 30% glycerol and 1.5 mM 2-mercaptoethanol, and kept frozen at −80°C for one month without any detectable activity change.

5. Enzymatic Assays and HPLC Analysis

trans-Cinnamic acid hydroxylation was assayed by a procedure modified from that described in the literature.[12] In a final volume of 500 μl, the reaction mixture contained 1 mM NADPH, 0.1 M potassium phosphate (pH 7.5), 15 mM 2-mercaptoethanol, 100 μM *trans*-cinnamic acid, 150 μl of microsomal fraction and 0.1–500 μM test compound. After 60 min incubation at 30°C, the reaction was stopped by adding 30 μl of 2 M HCl. Precipitated proteins were removed by filtration and 20 μl of the filtrate was taken for analysis by reverse-phase HPLC on a Shimadzu LC-10A equipped with a Shimadzu UV-VIS diode array. Separations were performed on a 4.6×250 mm Shimadzu ODS-II (5 μm) column at 40°C. The elution program consisted of a linear gradient of methanol and a mixture of phosphoric acid solution (pH 3.0)/methanol/2-propanol (75 : 20 : 5, v/v/v) delivered at a flow rate of 1 ml/min. The C4H oxidation products were monitored at 310 nm. Under these conditions, *p*-coumaric acid and *trans*-cinnamic acid eluted at approximately 9.5 and 14.2 min, respectively (Fig. 1). Authentic *p*-coumaric acid was compared

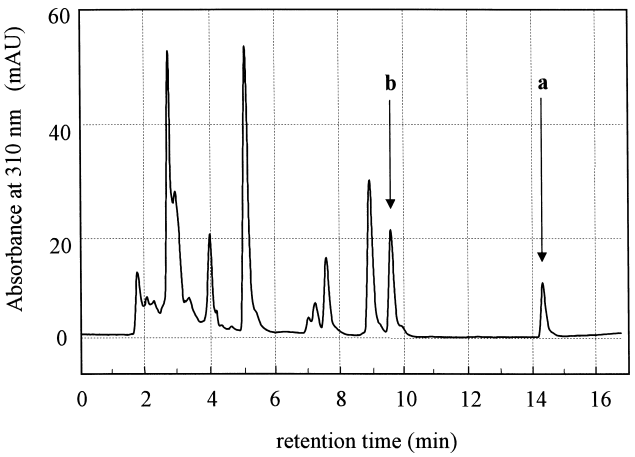
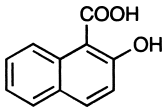
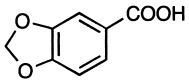
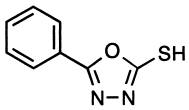
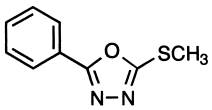
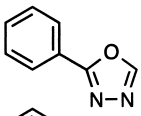
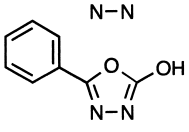


Fig. 1. HPLC analysis of the hydroxylation of *trans*-cinnamic acid catalyzed by C4H expressed in yeast. a, *trans*-cinnamic acid; b, *p*-coumaric acid.

Table 1. Inhibitory activity of various compounds toward cinnamate 4-hydoxylase from *P. kitakamiensis*

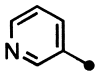
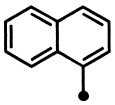
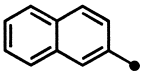
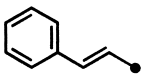
No	Compound	IC_{50} (μM)
1		0.88
2		>100
3		0.80
4		>100
5		>100
6		6.7

with the product of C4H oxidation in both retention time and the UV spectra of the peak. *para*-Coumaric acid was not detectable in the incubation with control yeast microsomes. The potency of inhibitory activity was represented by the IC_{50} value, which was defined as the concentration of the test compound that resulted in 50% inhibition of the peak areas of *p*-coumaric acid produced by C4H oxidation.

RESULTS AND DISCUSSION

The inhibitory activity of several compounds against C4H, CYP73a from *P. kitakamiensis*, is given in Table 1. 2-Hydroxy-1-naphthoic acid (**1**) significantly inhibited this C4H with an IC_{50} value of 0.88 μM. Its activity was very similar to that (IC_{50}: 0.89 μM) found against C4H, CYP73A1 from *Helianthus tuberosus*.[3] Piperonylic acid (**2**), which has previously been reported as a mechanism-based inactivator of C4H, did not show strong inhibitory activity. Schalk *et al*. have demonstrated that compound **2** inhibited the activity of CYP73A1 by 58% at a high concentration of 100 μM.[4]

Table 2. Inhibitory activity of 5-substitued-1,3,4-oxadiazole-2-thiols toward cinnamate 4-hydroxylase from *P. kitakamiensis*

No	R	IC_{50} (μM)
7		0.58
8		0.65
9		15
10		5.9
11		6.2
12		>100
13		>100
14		>100
15		>100

5-Phenyl-1,3,4-oxadiazole-2-thiol (**3**) showed activity comparable to **1**. Conversion of the thiol group to a methylthio group (**4**) eliminated the activity. 5-Phenyl-1,3,4-oxadiazole (**5**), which lacks the thiol group, had no activity. The 2-hydroxy analog **6** was 8-fold less active than **3**. These results indicate that the presence of a thiol group is essential for inhibitory activity.

A modification was made by replacing the phenyl group of **3** with other substituents (Table 2). The 2- and 3-pyridyl analogs (**7** and **8**) had slightly higher level of activity than the phenyl analog **3**, while the 4-pyridyl analog **9** showed much weaker activity. The 2-furyl (**12**) and 2-thienyl (**13**) analogs were about 8-fold less active than **3**. The 1-naphthyl (**12**), 2-naphthyl (**13**), styryl (**14**) and cyclohexyl (**15**) analogs were almost inactive at 100 μM.

Since the phenyl analog **3** was found to show considerably strong activity, several substituents were introduced on the benzene ring (Table 3). The introduction of a chloro (**17**), fluoro (**23**) or bromo (**26**) substituent at the *meta* position on the benzene ring increased the activity in comparison with that of **3**, while the 3-methylphenyl (**20**) and 3-methoxyphenyl (**27**) analogs had lower level of activity and the trifluoromethyl analog (**28**) did not significantly inhibit even at 100 μM. Introducing a chloro (**16**), methyl (**19**) or fluoro (**22**) substituent at the *ortho* position also improved the inhibitory activity. On the other hand, substitution at the *para* position on the benzene ring (**18**, **21** and **24**) caused a drastic decrease in activity in comparison with the phenyl analog

Table 3. Inhibitory activity of 5-aryl-1,3,4-oxadiazole-2-thiols toward cinnamate 4-hydroxylase from *P. kitakamiensis*

No	R	IC_{50} (μM)
16	2-Cl	0.68
17	3-Cl	0.21
18	4-Cl	23
19	2-CH_3	0.50
20	3-CH_3	1.1
21	4-CH_3	50
22	2-F	0.19
23	3-F	0.13
24	4-F	3.3
25	2-Br	0.98
26	3-Br	0.42
27	3-OCH_3	6.1
28	3-CF_3	>100
29	2,5-Cl	2.0
30	3,5-Cl	>100

3. Although the 2- and 3-chlorophenyl analogs (**16** and **17**) had increased activity, the additional introduction of a chlorine atom on the benzene ring (**29** and **30**) led to a decrease in inhibitory activity. Consequently, the 3-fluorophenyl analog **23** was the most active of the compounds tested on C4H from *P. kitakamiensis*. The mode of action and the *in vivo* inhibitory activity of these 5-aryl-1,3,4-oxadiazole-2-thiols are under investigation.

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