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Note

Inhibition of *trans*-Cinnamate 4-Hydroxylase by 4-Amino-5-aryl-2,3-dihydro-3*H*-1,2,4-triazole-3-thiones

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A number of 4-amino-5-aryl-2,3-dihydro-3*H*-1,2,4-triazole-3-thiones were synthesized and tested for inhibitory activity against *trans*-cinnamate 4-hydroxylase (C4H) from *Populus kitakamiensis*, which was expressed in yeast. Of the compounds tested, 4-amino-5-phenyl-2,4-dihydro-3*H*-1,2,4-triazole-3-thione (**1**) was the most effective, having an IC_{50} value of 0.32 μM. Replacement of the phenyl group with heterocycles or substituted phenyl groups drastically reduced the activity. Compound **1** behaved as a mixed-inhibitor of C4H. When potato tuber disks were treated with **1** (0.2–20 ng/disk) in the presence of laminarin, a β-1,3-glucooligosaccharide elicitor, *trans*-cinnamic acid was accumulated at levels 3 times higher than in disks treated with laminarin alone.

Keywords: *trans*-cinnamate 4-hydroxylase, phenylpropanoid, 4-amino-5-phenyl-2,4-dihydro-3*H*-1,2,4-triazole-3-thione, *Populus kitakamiensis*.

INTRODUCTION

trans-Cinnamate 4-hydroxylase (C4H) is a member of the P450 superfamily (CYP73) which catalyzes the first oxygenation in the general phenylpropanoid pathway from *trans*-cinnamate to *p*-coumarate in higher plants.[1] Together with phenylalanine ammonia-lyase and 4-coumaryl CoA ligase, C4H is involved in the core reactions of the phenylpropanoid metabolism which provides lignins, flower pigments, signal molecules, UV protectants, phytoalexins and so on. Since C4H is highly inducible in response to stress, including wounding, pathogen infection and chemical treatment, it is accepted that this enzyme plays a central role in the regulation of various biological responses related to the phenylpropanoid pathway.[2] C4H has not been found in any invertebrate or vertebrate animals. Therefore, specific C4H inhibitors would be useful not only as a biochemical probe in elucidating the role of C4H, but also as a bio-rational lead compound for the development of a novel herbicide.

We have previously reported that a series of 5-aryl-1,3,4-oxadiazole-2-thiols inhibited C4H from *Populus kitakamiensis*, which was expressed in yeast.[3] We further examined several heterocycles, and found that 4-amino-5-phenyl-2,4-dihydro-3*H*-1,2,4-triazole-3-thione (**1**) showed strong inhibitory activity against C4H expressed in yeast. Although some 5-aryl-2,4-dialkyl-2,4-dihydro-3*H*-1,2,4-triazole-3-thiones have been reported to show antidepressant activity,[4] there has been no report on the inhibitory activity of **1** toward C4H. This report describes the structure-activity relationships concerning the inhibition of C4H activity by 4-amino-5-aryl-2,4-dihydro-3*H*-1,2,4-triazole-3-thiones and the accumulation of *trans*-cinnamic acid (*t*-CA) in potato tubers treated with compound **1**.

MATERIALS AND METHODS

1. Chemicals

Laminarin was purchased from Sigma-Aldrich Co. The ^{1}H-NMR spectra were recorded with a JEOL EX-400 (400 MHz) spectrometer. 4-Amino-5-aryl-2,4-dihydro-3*H*-1,2,4-triazole-3-thiones were prepared according to the methods described by Sung and Lee (Fig. 1).[5] The following procedure for the preparation of 4-amino-5-phenyl-2,4-dihydro-3*H*-1,2,4-triazole-3-thione (**1**) is typical.

To a solution of benzohydrazide (2 g, 15 mmol) in 20 ml of ethanol was added pulverized KOH (1 g, 18 mmol). After the solution became clear, carbon disulfide (2.7 ml, 45 mmol) was added. After stirring for 15 hr at room temperature, 100 ml of diethyl ether was added to the mixture, and the precipitate was collected by filtration, washed with diethyl ether, and dried to afford 2.7 g (74%) of crude potassium 3-benzoyldithiocarbazate. To a solution of the dithiocarbazate (1 g, 4 mmol) in 1 ml of water was added 80% hydrazine hydrate (0.5 ml, 8 mmol). The solution was refluxed for 6 hr until it became clear green. After the solution had cooled to room temperature, 5 ml of water was added and the solution was neutralized with 3 N HCI to form a precipitate. The precipitate was collected by filtration and recrystallized from ethanol to give 0.49 g (64%) of **1**, mp 180–182°C (decomp.). ^{1}H-NMR δ ($CDCl_3$): 4.85 (2H, s, NH_2), 7.48–7.56 (3H, m, phenyl), 7.95–8.10 (2H, m, phenyl), 10.48 (1H, s, NH) Anal. Found: C, 50.09; H, 4.18; N, 28.86%. Calcd. for $C_8H_8N_4S$: C, 49.98; H, 4.19; N; 29.14%.

Compounds **2**–**13** were prepared in the same manner as compound **1** with use of the corresponding hydrazide instead of benzohydrazide. The structures of the compounds were confirmed with ^{1}H-NMR spectra.

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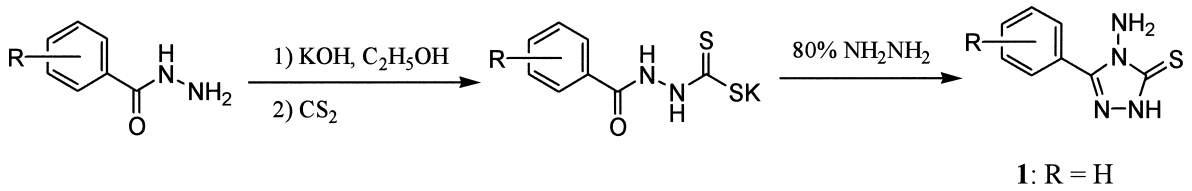


Fig. 1. Scheme for the synthesis of 4-amino-5-aryl-2,4-dihydro-3*H*-1,2,4-triazole-3-thiones.

2. Yeast Microsomes and Enzyme Assays

Yeast microsomes expressing C4H (CYP73a) from *Populus kitakamiensis* were prepared as described previously.[3] The NADPH-dependent hydroxylation of *t*-CA using microsomes was monitored by reverse-phase HPLC as reported.[3] 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.

3. Treatment of Potato Tubers and Determination of t-CA

Tubers of potato (*Solanum tuberosum*) were used as described by Matsuda *et al*.[6] The tuber was cut into disks (8 mm in diameter and 2 mm thick), and washed with deionized water. Five disks were treated with each amount of compound **1** dissolved in acetone (40 μl). After evaporation of the solvent at room temperature, 1 ml of a laminarin solution (0.2 mg/ml) was applied to the tuber disks in a Petri dish. The disks were incubated at 25°C under wet and dark conditions for 24 hr. Five disks were combined and homogenized with 5 ml of methanol. After filtration of the homogenate, the filtrate was concentrated under reduced pressure. *t*-CA was extracted with ethyl acetate in acidic condition (pH 2.0) and its amount was determined by HPLC according to the method used in enzyme assays.

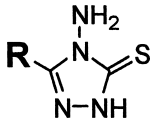
RESULTS AND DISCUSSION

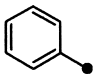
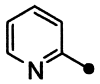
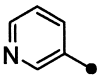
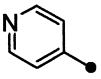
Table 1 shows the inhibitory activity of 4-amino-5-aryl-2,4-dihydro-3*H*-1,2,4-triazole-3-thiones against C4H from *P. kitakamiensis*. Compound **1** was more active than 5-phenyl-1,3,4-oxadiazole-2-thiol reported previously, the IC_{50} value of which was 0.80 μM.[3] The inhibitory activity of the pyridyl (**2**–**4**) and 2-thienyl (**6**) analogs was decreased in comparison with that of the phenyl analog **1**. Notably, the furyl analog **5** did not inhibit C4H even at 100 μM. The introduction of a substituent such as a chloro, fluoro or methyl group into the benzene ring (**7**–**12**) decreased or eliminated the activity. The 4-hydroxyphenyl analog **13** was quite inactive. Thus, in the 1,2,4-triazole-3-thione series, the unsubstituted phenyl analog **1** was the most active of the compounds tested on C4H from *P. kitakamiensis*.

The Lineweaver-Burk plots for the inhibition of C4H showed that compound **1** was a mixed-type inhibitor (Fig. 2), suggesting that it binds to the active site of C4H and at a separate site as well.

The expected impact of the inhibition of C4H *in vivo* is a decrease in the formation of downstream metabolites in the phenylpropanoid pathway and the accumulation of *t*-CA. To see whether **1** caused the accumulation of *t*-CA, we conducted experiments

Table 1. Inhibitory activity of 4-amino-5-aryl-2,4-dihydro-3*H*-1,2,4-triazole-3-thiones against C4H from *P. kitakamiensis*



No.	R	IC_{50} (μM)
1		0.32
2		45
3		51
4		13
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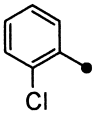
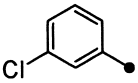
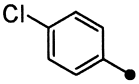
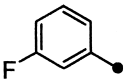
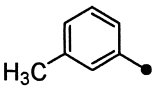
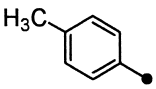
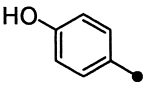
7		>100
8		5.0
9		>100
10		3.9
11		36
12		>100
13		>100

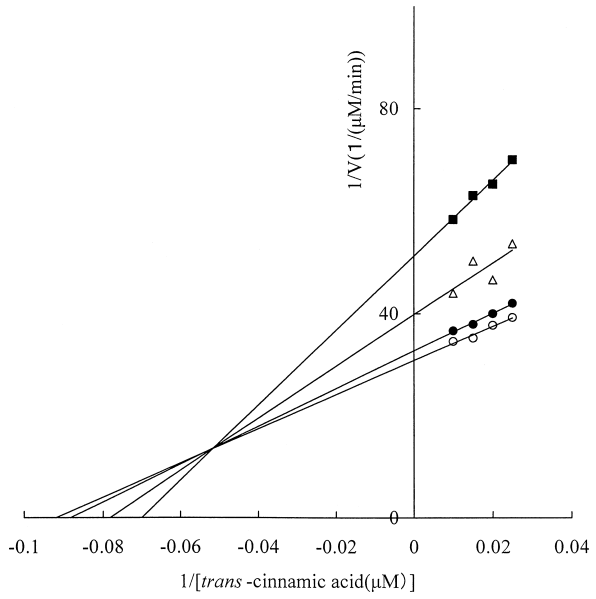


Fig. 2. Lineweaver-Burk plots of the inhibition by compound **1** of the hydroxylation of *trans*-cinnamic acid. The substrate concentrations were 40, 50, 67, and 100 μM. The concentrations of inhibitor were 0 (○), 5 (●), 10 (△), and 15 μM (■). Each plot is expressed as the mean of triplicate experiments.

using potato tubers, in which the phenylpropanoid pathway is known to be activated by an oligosaccharide elicitor.[6] The basal level of *t*-CA in the acetone-treated control was 0.03 mg per potato tuber disk (Fig. 3). *p*-Coumaric acid in the disks could not be quantified because of the overlapping of several peaks. When the disks were treated with laminarin, a β-1,3-glucooligosaccharide elicitor, the amount of *t*-CA increased by about 4-fold from the control level 24 hr after treatment, indicating that this elicitor caused a significant activation of upstream enzymes of the phenylpropanoid pathway as reported by Matsuda *et al*.[6] When **1** was applied to the disks at a wide range of doses (0.02–20 ng/disk) in the absence of the elicitor, a slight increase in *t*-CA content was observed. In the presence of the elicitor, treatment with 0.2 ng of **1** induced the accumulation of *t*-CA to a level 3 times higher than that in disks treated with laminarin alone. At higher doses of **1** (2 and 20 ng), the *t*-CA content was almost the same as that on treatment with 0.2 ng. Compound **1** seems to be a potent and selective inhibitor of C4H.

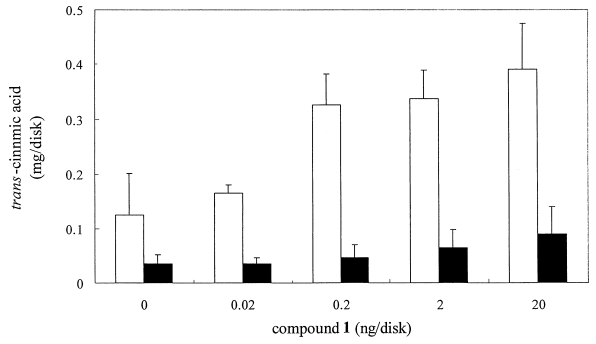


Fig. 3. Accumulation of *trans*-cinnamic acid caused by compound **1** in potato tuber disks. Compound **1** was applied in the presence (white bars) or absence (black bars) of 0.2 mg of laminarin. *trans*-Cinnamic acid was quantified using HPLC 24 hr after treatment. Data are the mean±SE from at least three independent experiments.

Salicylic acid (SA) is a key endogenous signal involved in the activation of numerous plant defense responses.[7] Chong *et al.* have reported that the accumulation of SA in tobacco plants requires *de novo* synthesis of benzoic acid from *t*-CA.[8] Specific C4H inhibitors might be useful for elucidating the role of C4H in the regulation of the SA and general phenylpropanoid pathways.

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