A New Type of Antimicrobial Phenolics Produced by Plant Peroxidase and Its Possible Role in the Chemical Defense Systems against Plant Pathogens

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Syringaldehyde readily reacted in the horse-radish peroxidase (HRPOD) system. The ethyl acetate extract of the reaction mixture showed a marked antimicrobial activity against bacteria and fungi. After repeated column chromatography three potential antimicrobial compounds were obtained from the extract. The structural elucidation of active compounds was achieved by a combination of spectroscopic techniques and chemical modification.

Introduction

Plants induce varied resistance response toward microbial challenge. In order to establish an infection, pathogenic fungi must break such defense barriers as lignin, phytoalexins, and hydrolytic enzymes (Kobayashi *et al.*, 1993). At the early stage of infection, peroxidase activity in the plant tissue is enhanced dramatically (Hammerschmidt *et al.*, 1982). POD activation and the resulting lignin accumulation at the infected site are known to play an important part in the defense system (Ride, 1975), and several phenolics have been reported to be converted into oxidized or polymerized compounds by the activated POD (Patzlaff *et al.*, 1978).

The accumulation of POD products at the infection site might be of disadvantage to invaders such as pathogens and insects. This had prompted us to examine what type of simple phenolics were converted into antimicrobial compounds by HRPOD. In our previous research, guaiacol was found to be converted into pronounced antimicrobial compounds in *in vitro* HRPOD system (Kobayashi *et al.*, 1994). As a continuation of our studies, we also found syringaldehyde to be converted into marked antimicrobial compounds. This paper reports the chemical structures of these compounds, together with antimicrobial activities.

Materials and Methods

^{1}H NMR spectra were recorded with a Varian VXR-500 instrument, using deuterized solvent as an internal standard. Mass spectra were recorded with a JEOL JMS-D 300 instrument, and UV spectra were recorded with a Shimadzu UV-3000 spectrophotometer. Peroxidase was kindly presented by Toyobo Co., and syringaldehyde was purchased from Tokyo Kasei Co.

POD reaction with syringaldehyde

The reaction system consisted of 4 mmol of syringaldehyde, 5 ml of methanol, 20 mmol of hydrogen peroxide, and 600 units of POD in 500 ml of a 0.1 M Na–Pi buffer at pH 5.8, and was stirred at 25 °C. After 1, 3, 10, 30, 90 min incubation, an aliquot (1 ml) was taken from the reaction mixture, and extracted with EtOAc. The extracts were subjected to HPLC analysis and antimicrobial tests. The reaction was stopped after 90 min incubation by adding EtOAc. Commercially available POD was used in this experiment. This enzyme was reported to be prepared from horse-radish and to contain both acidic and basic isoenzyme (Toyobo Catalog). One unit of POD was defined as that to decompose 1 μmol of hydrogen peroxide per min at 25 °C at pH 7.0 in the guaiacol system.

Extraction and isolation of POD reaction products

The reaction mixture (500 ml) was successively extracted twice with EtOAc and *n*-butanol. The resulting organic phases showed no reaction to the

KI–I$_2$ starch solution but faint blue color was seen in the aqueous phase. The organic phases and the aqueous phase were concentrated *in vacuo*, and then tested for antimicrobial activities. The EtOAc extract (650 mg) with a pronounced antimicrobial activity was chromatographed on a silica gel column (Wakogel C-100) eluted stepwise with 500 ml each of 20, 40, 70% EtOAc in *n*-hexane and EtOAc. The active fractions (40% EtOAc eluate and 100% EtOAc eluate) were further purified by silica gel column chromatography (Wakogel C-300) to give active compounds, **1** (30 mg), **2** (20 mg), and **3** (2.5 mg), using linear gradient elution by *n*-hexane to EtOAc.

Properties of 1

EI-MS *m/z*: 168 (M$^+$); UV λ$_{max}$ (MeOH) nm (log ε): 265 (4.1); ^{1}H NMR δ (500 MHz, CDCl$_3$): 3.80 (6 H, s), 5.83 (2 H, s).

Properties of 2

EI-MS *m/z*: 320.0877 ([M+2H]$^+$, calcd. for C$_{16}$H$_{16}$O$_7$; 320.0897); CI-MS *m/z* (rel. int.): 319 (M+H$^+$, 1.6), 183 (100.0); UV λ$_{max}$ (MeOH) nm (log ε): 273 (4.5); ^{1}H NMR δ (500 MHz, CDCl$_3$): 3.87 (3 H, s), 3.91 (6 H, s), 5.15 (1 H, d, *J* = 2.4 Hz), 6.10 (1 H, d, *J* = 2.4 Hz), 7.17 (2 H, s), 9.93 (1 H, s).

Preparation of 2 a and 2 b

Compound **2** (10 mg) was dissolved in MeOH (0.5 ml) containing pyrogallol (10 mg), and subjected to preparative TLC (silica gel) to afford **2a** (2 mg). ^{1}H NMR δ (500 MHz, CDCl$_3$): 3.86 (6 H, s), 3.97 (3 H, s), 5.92 (1 H, d, *J* = 2.8 Hz), 6.27 (1 H, d, *J* = 2.8 Hz), 6.75 (1 H, s), 7.14 (1 H, s), 7.18 (2 H, s), 9.93 (1 H, s).

Compound **2a** was treated with ethereal diazomethane in a sealed vial at 25 °C for 12 h, and then the reaction mixture was subjected to preparative HPLC to give **2b** (0.5 mg). EI-MS *m/z* (rel. int.): 362 (M$^+$, 72.5), 347 (100.0), 319 (11.6); ^{1}H NMR δ (500 MHz, CDCl$_3$): 3.47 (1 H, d, *J* = 5.4 Hz), 3.73 (6 H, s), 3.73 (1 H, hidden), 3.77 (3 H, s), 3.77 (1 H, hidden), 3.85 (6 H, s), 6.08 (2 H, s), 7.27 (2 H, s).

Properties of 3

EI-MS *m/z*: 334 (M$^+$); UV λ$_{max}$ (MeOH) nm (log ε): 278 (4.2); ^{1}H NMR δ (500 MHz, CDCl$_3$): 3.71 (3 H, s), 3.81 (3 H, s) 3.89 (6 H, s), 6.82 (1 H, s), 7.12 (2 H, s), 9.91 (1 H, s).

Antimicrobial assay

The antimicrobial assay was previously described (Kobayashi *et al.*, 1994). In this assay, tetracycline inhibited the growth of *Bacillus subtilis* and *Escherichia coli* at the minimum inhibitory concentrations (MICs) of 0.8 μg/ml and 1.6 μg/ml, respectively. Cycloheximide suppressed the spore germination of *Aspergillus candidus* and *Cladosporium herbarum* at the MICs of 6.3 μg/ml and 12.5 μg/ml, respectively.

Results and Discussion

Commercially available POD from horse-radish was used for this experiment. Syringaldehyde in aqueous methanolic solution was added to Na–Pi buffer at pH 5.8 containing POD and hydrogen peroxide, and the mixture was stirred at 25 °C for 90 min. Syringaldehyde was not consumed when either POD or hydrogen peroxide was removed from the reaction system. After 1, 3, 10, 30, 90 min incubation an aliquot (1 ml) was taken from the reaction mixture for HPLC analysis and antimicrobial tests. The antibacterial activities increased as the reaction proceeded (Table I). After 90 min incubation the reaction mixture was successively extracted with ethyl acetate and *n*-butanol, then the organic layers and aqueous layer were concentrated *in vacuo* and tested for antimicrobial activities separately. The ethyl acetate layer showed a strong antibacterial activity (Table II). Three active compounds, **1**, **2** and **3**, were purified as single compounds from the ethyl acetate layer by repeated column chromatography under the guidance of an antibacterial assay. These compounds exhibited strong antibacterial or moderate antifungal activities (Table II).

Table I. The increase in antibacterial activities as the reaction progressed.

| | MIC [μg/ml] | | | | | |
	0 min	1 min	3 min	10 min	30 min	90 min
Bacillus subtilis	>1000	1000	500	125	125	125
Escherichia coli	>1000	1000	500	125	125	125

MIC, minimum inhibitory concentration.

Table II. Antimicrobial activity of syringaldehyde and its POD reaction products.

| | Syring-aldehyde | MIC [μg/ml] | | | |
		Extract[a]	1	2	3
Bacillus subtilis	>1000	125	63	250	>500
Escherichia coli	>1000	125	63	500	500
Aspergillus candidus	1000	500	250	250	250
Cladosporium herbarum	1000	1000	500	500	>500

[a] Ethyl acetate extract of reaction mixture.
MIC, minimum inhibitory concentration.

Compounds, **1** and **2,** showed a yellow and a red spot respectively on siliga gel TLC (R_f 0.6, 0.5; benzene–EtOAc, 7:3) and the compounds rapidly oxidized a reduced methylene green reagent, indicating the presence of a quinone chromophore. The MS spectra [**1**, EI-MS *m/z*: 168 (M⁺); **2,** CI-MS *m/z*: 319 (M+H⁺)] suggested that **1** and **2** were a monomeric and a dimeric compound, respectively.

Compound **1** was identified as 1,3-dimethoxy-*p*-benzoquinone by analyzing the ¹H NMR spectrum [δ 3.80 (6H, s), 5.83 (2H, s)], and by comparison with the chemical shift values reported for this compound (Achenbach and Wörth, 1977).

The molecular formula of **2** was $C_{16}H_{14}O_7$, by the high resolution mass spectrometry of its re-

duced form. The ¹H NMR spectrum indicated the presence of a 1,2,3,5-tetra-substituted benzene (a symmetric structure) and a di-substituted quinone. A 6H singlet at δ 3.91, a 2H singlet at δ 7.17, and a 1H singlet at δ 9.93 were assignable to two aromatic methoxyls, two aromatic protons, and an aromatic formyl respectively, and a 3H singlet at δ 3.87 was attributed to a methoxyl on the quinone. Six oxygen atoms out of 7 were rationally assigned to three methoxyls, a formyl, and two carbonyls. The remainder must be used as an ether linkage connecting a benzene and a quinone. These observations were supported by a significant ion peak at *m/z* 183 afforded by cleavage occurring at the β to the benzene ring. The color due to the quinone chromophore and the chemical shift values in the ¹H NMR suggested the presence of an *o*-benzoquinone. In order to confirm this, phenazine formation was carried out using *o*-phenylenediamine, but the attempt failed due to the unstability of this compound. Therefore, another approach was performed in the NMR experiment, and the presence of *o*-benzoquinone system was verified by analyzing the ¹H NMR spectrum of a derivative **2b** which was obtained by methylation of **2a** (hydroxyquinone form of **2**). The ¹H NMR spectrum of **2b** indicated that the benzene ring originating from the quinone moiety had a symmetrical substitution pattern, suggesting the pres-
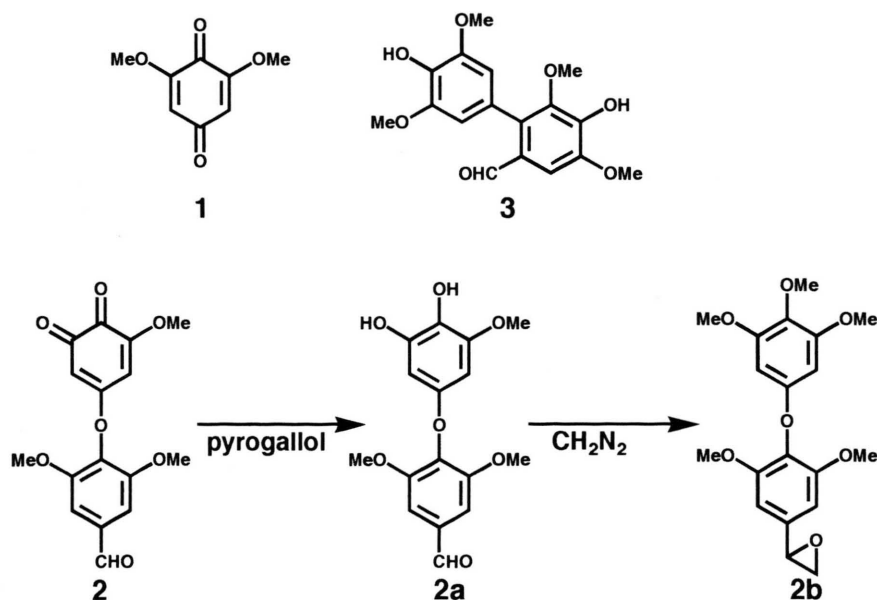

Fig. 1. Structures of the antimicrobial POD products and their derivatives.

ence of 3,5-di-substituted *o*-quinone. The substitution pattern of a methoxyl and a formyl on the benzene and a methoxyl on the quinone was rationally assigned based on that of the original building block, *i.e.*, syringaldehyde. The novel structure of **2** was thus established as shown in Fig. 1.

Compound **3** was characterized by a strong UV-absorbing spot on silica gel TLC (R_f 0.3; benzene – EtOAc, 7:3). The EI-MS spectrum, *m/z* 334 (M⁺), suggested that it was a dimeric compound. Bathochromic shift was observed in the UV spectrum upon addition of NaOH, indicating the presence of phenolic hydroxyls. The ¹H NMR spectrum showed that this compound consisted of a penta-substituted benzene and a tetra-substituted benzene (a symmetric structure). A 6 H singlet at δ 3.89 and a 2 H singlet at δ 7.12 were attributed to two aromatic methoxyls and two aromatic protons respectively on the tetra-substituted benzene ring, and a singlet at δ 9.91, a singlet at δ 6.82, and two 3 H singlets at δ 3.71 and 3.81 were ascribed to a formyl, an aromatic proton, and two methoxyls on the penta-substituted benzene ring. The substitution pattern of methoxyls, hydroxyls, and a formyl on the benzene rings was determined based on that of the starting material, syringaldehyde. Thus, the novel structure of **3** was elucidated as shown in Fig. 1.

Among the products, **1** was a major product and showed a marked antibacterial activity toward *Bacillus subtilis*. Compounds, **1** and **2**, had a moderate antifungal activity.

These facts encouraged us to investigate further the plant defense mechanism where POD is involved and to examine the POD products originated from prevalent phenolics for antimicrobial or insecticidal activities. Screening efforts to investigate new HRPOD products are now in progress using phenol-containing fractions from several plant species.

Compound **1** was not produced from syringic acid in the same reaction condition. This type of compound was known to be afforded by alkaline hydrogen peroxide. This suggests that decarboxylation could not be involved in the formation of **1**, and a Baeyer-Villiger type of reaction could account for its formation. The detailed mechanism is now investigated.

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