2,6-Dimethoxy-*p*-benzoquinone as an Antibacterial Substance in the Bark of *Phyllostachys heterocycla* var. Pubescens, a Species of Thick-Stemmed Bamboo

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Active antibacterial substances contained in the bark of *Phyllostachys heterocycla* var. Pubescens were isolated by HPLC. As a result of the identification using high-resolution MS, ^{1}H NMR, ^{13}C NMR, etc., 2,6-dimethoxy-*p*-benzoquinone was found to be an antibacterial compound in this species of bamboo. Comparison of the antibacterial spectra of benzoquinone derivatives suggested that the antibacterial activity of benzoquinones was influenced by the type of substituted group.

INTRODUCTION

Bamboo is a very popular plant in Asia, and a variety of implements, household goods, etc. have been manufactured by utilizing the characteristic physical properties of bamboo. Both bamboo grass and bamboo have been widely used plants in the diet of Asians and utilized not only as a wrapping material for foods, e.g., meat, sushi, and candy, but also as tableware. It has also been believed that foods can be preserved better by wrapping them with bamboo bark or bamboo grass. In spite of the fact that bamboo has been used extensively as a foodstuff in Asia, little investigation has been carried out on bamboo from a food chemistry point of view.

Antibacterial substances and medical materials used as antiinflammation or antitumor treatments in bamboo grass, a plant closely related to bamboo, have been reported (Chuyen et al., 1982; Shibata et al., 1975; Okabe et al., 1975). However, we have previously found that an extract from bamboo possesses a stronger antibacterial activity than that from bamboo grass.

In the present study, the antibacterial activity of an extract from the bark of *Phyllochys heterocycla* var. Pubescens was measured and an antibacterial compound was isolated and identified. The antibacterial compound thus identified was confirmed to be 2,6-dimethoxy-*p*-benzoquinone. The antibacterial potencies of some *p*-benzoquinone derivatives were also compared to the potency of 2,6-dimethoxybenzoquinone.

MATERIALS AND METHODS

Materials. The bark of *P. heterocycla* var. Pubescens collected in Hioki-Gun, Kagoshima Prefecture, Japan, was pulverized by using a grinder.

Reagents. The reagents used for the comparison of the antibacterial potencies were potassium sorbate (Wako Pure Chemicals Industry Co., Ltd.), propionic acid, benzoic acid, *p*-benzoquinone, 2,3-dimethoxy-5-methyl-*p*-benzoquinone, tetramethyl-*p*-benzoquinone, 2,5-dihydroxy-*p*-benzoquinone, tetrahydroxy-*p*-benzoquinone, 2,5-diphenyl-*p*-benzoquinone (these eight were from Tokyo Kasei Industry Co., Ltd.), and capric acid monoglyceride (Riken Vitamin Co., Ltd.). All reagents were used without further purification.

Extraction Method. The bamboo powder, undried (400 g), was suspended in 2000 mL of a chloroform/methanol mixture (2:1). The suspension was ultrasonicated for 2 h and then filtered. The filtrate was condensed by a rotary evaporator to obtain the whole extract.

Crude Fractions. The whole extract was fractionated into four fractions—basic, acidic, phenolic, and neutral—by the method illustrated in Figure 1 (Chuyen, 1982).

HPLC. A Gilson Model 303 (100SC pump head) was used. The columns used were (a) silica gel 60 (50 cm × 50 mm, Toyo Soda Co., Ltd.) and (b) GC-310 (50 cm × 7.6 mm, Asahi Kasei Kogyo Co., Ltd.). Chloroform was used as the mobile phase for both columns. A Model HM UV detector from Gilson was used at 286 nm for both columns. The flow rate was 50 mL/min for column a and 1 mL/min for column b.

Instruments. Instruments used were as follows: UV absorption spectrophotometer, Model UV-260 (Shimadzu Co., Ltd.); infrared absorption spectrophotometers, Model JIR-200 [Japan Electron and Optics Laboratory (JEOL) Co., Ltd.] NMR, Model JNM-FX200 (JEOL); mass spectrometer, Model JX-303 (JEOL); and high-resolution mass spectrometer, Model JMS-OISG (JEOL).

Bacterial Stocks and Culture Media. The bacterial stocks used for antibacterial activity assays were *Bacillus subtilis* (IFO-13719), *Staphylococcus aureus* (IFO-13276), *Sarcina lutea* (ATCC-1001), *Escherichia coli* (IFO-3301), *Salmonella typhimurium* (IFO-13245), and *Pseudomonus aeruginosa* (IFO-3080). Ordinary bouillon and Tryptosoy agar medium (Eiken Kagaku Co., Ltd.) were used as culture media.

Assay of Antibacterial Activity. (*1*) *Paper Disk Method.* One platinum loop of bacteria was incubated in 10 mL of ordinary bouillon solution overnight at 37 °C to prepare the seeded solution. Then, 0.1 mL of the seeded solution was diluted with 10 mL of Tryptosoy agar medium (× 100 dilution). After cooling, the disk (8 M/M DIA, Toyo Seisakusho Co. Ltd.), onto which 1 mg of a test substance had been adsorbed, was placed to the diluted seeded medium and incubated for 24 h at 37 °C. The diameter of an inhibited growth circle was then measured.

(*2*) *Measurement of Minimum Inhibitory Concentration (MIC).* The MIC of each sample was measured by using the twofold serial dilution method as previously reported (Sakanaka, 1989).

RESULTS AND DISCUSSION

Comparison of the Antibacterial Activities of the Whole Extract from *P. heterocycla* var. Pubescens and Commercial Antibacterial Agents for Foods. To first examine the practical usefulness of the bamboo extract, its antibacterial activity was compared with the

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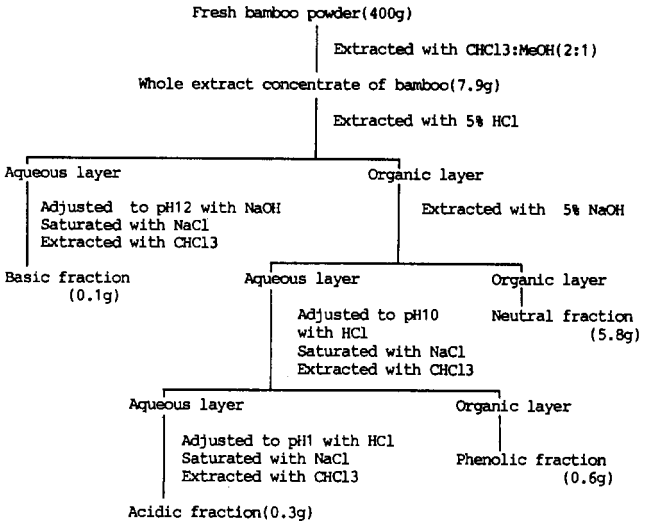

Figure 1. Fractionation of whole extract concentrate of bamboo.

Fraction No.	1	2	3	4	5	6	7	8	9	10
Antibacterial activity	-	-	-	-	-	-	+	+	-	+

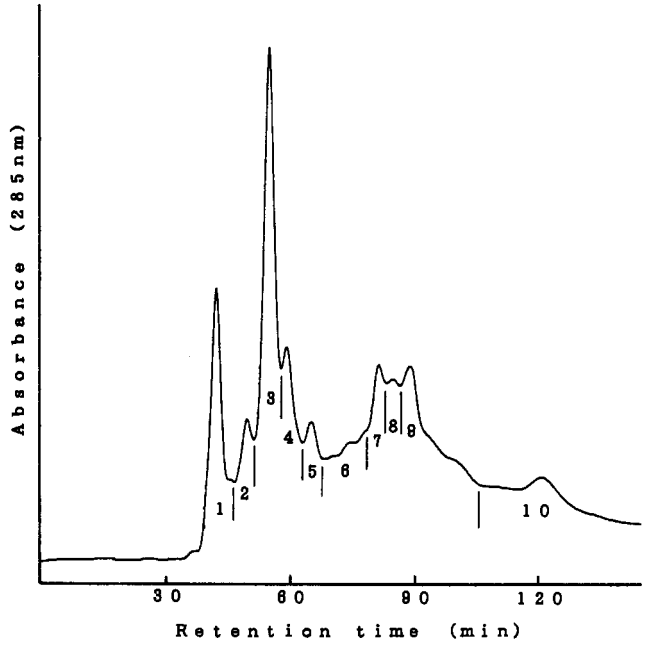

Figure 2. HPLC chromatogram and antimicrobial activities of various fractions of the neutral fraction. Antimicrobial activities were determined by disc assay procedure (*B. subutilis*, bouillon–agar medium incubated at 37 °C for 24 h). ++, Very strong; +, strong; –, no activity.

antibacterial activities of four presently popular antibacterial agents, potassium sorbate, propionic acid, benzoic acid, and capric acid monoglyceride. As shown in Table I, the whole extract from bamboo showed a fairly strong antibacterial activity against Gram-positive bacteria with a potency similar to that of benzoic acid or capric acid monoglyceride. Against Gram-negative bacteria, however, the extract showed no growth inhibition.

From these results, the whole extract was judged to have a practical antibacterial activity, though objective bacterial species are limited.

Antibacterial Activity of Crude Fractions. Table II shows the antibacterial activities of the four crude fractions prepared by the method illustrated in Figure 1. The neutral fraction was found to have the strongest

Fraction No.	A	B	C
Antibacterial activity	-	+	+ -

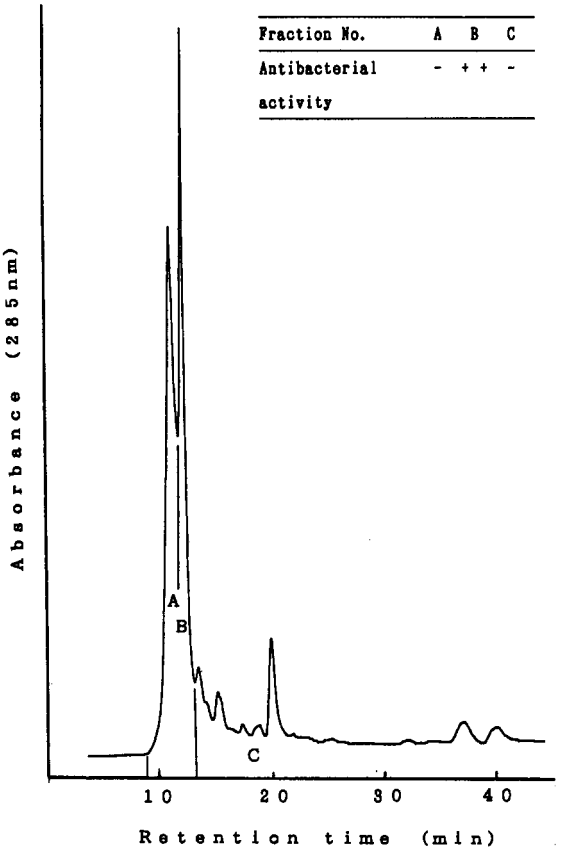

Figure 3. HPLC chromatogram and antimicrobial activities of various fractions of fraction 7. Antimicrobial activities were determined by disc assay procedure (*B. subutilis*, bouillon–agar medium incubated at 37 °C for 24 h). ++, Very strong; –, no activity.

Table I. Comparison of Antimicrobial Activities of Whole Extract with That of Food Preservatives[a]

	microorganism			
reagent	*B. subtilis*	*S. aureus*	*E. coli*	*S. typhimurium*
whole extract	+	+	–	–
potassium sorbate	–	–	–	–
propionic acid	–	–	++	++
benzoic acid	++	++	+	+
monocaprylin	+	–	++	+

[a] Antimicrobial activities were determined by disc assay procedure (bouillon–agar medium incubated at 37 °C for 24 h). ++, Very strong; +, strong; –, no activity.

Table II. Antimicrobial Activities of Various Fractions of Whole Bamboo Extract[a]

	microorganism			
fraction	*B. subtilis*	*S. aureus*	*E. coli*	*S. typhimurium*
acidic	–	+	–	–
basic	–	+	–	–
neutral	+	++	–	–
phenolic	–	+	–	–

[a] Antimicrobial activities were determined by disc assay procedure (bouillon–agar medium incubated at 37 °C for 24 h). ++, Very strong; +, strong; –, no activity.

activity. All crude fractions, however, showed no antibacterial activity against Gram-negative bacteria.

Thus, the neutral fraction was selected for subsequent isolation of antibacterial substances.

Isolation of Antibacterial Substances from the Neutral Fraction. The chromatogram obtained by further fractionation of the neutral fraction by preparative

Table III. Comparison of Antibacterial Activity of Whole Extract Concentrate and *p*-Benzoquinone Derivatives

sample	MIC (ppm)					
	S. aureus	*B. subtilis*	*S. lutea*	*E. coli*	*S. typhimurium*	*P. aeruginosa*
whole extract concentrate	5×10^3	5×10^3	10^4	10^4	10^4	5×10^4
2,6-dimethoxy-*p*-benzoquinone	200	200	400	400	400	800
p-benzoquinone	>800	800	>800	200	200	400
tetramethyl-*p*-benzoquinone	800	400	800	800	>800	>800
2,5-dihydroxy-*p*-benzoquinone	800	>800	>800	800	800	>800
tetrahydroxy-*p*-benzoquinone	>800	>800	>800	>800	>800	400
2,3-dimethoxy-5-methyl-*p*-benzoquinone	100	100	100	100	100	>800
2,5-diphenyl-*p*-benzoquinone	>800	>800	>800	>800	>800	>800

[a] MIC was determined by twofold serial broth dilution method.

HPLC is shown in Figure 2. The antibacterial activities of 10 fractions in the chromatogram were then measured by using *B. subtilis*. As shown in Figure 2, fraction 7 showed the strongest antibacterial activity, and this fraction was further purified by GPC. The chromatogram obtained on GPC fractionation and the antibacterial activities of three fractions against *B. subtilis* are shown in Figure 3. It was found from Figure 3 that fraction B corresponded to the substance primarily exhibiting the antibacterial activity of the extract from the bamboo.

Structure Analysis of Fraction B. The molecular formula of the compound isolated as the fraction B was analyzed by using a high-resolution mass spectrometer, and it was estimated to be $C_8H_8O_4$ from the m/z value of 168.0417. ^1H NMR showed two single peaks, δ 3.9 (2, $-OCH_3$) and δ 5.8 (2, $=CH-$). ^{13}C NMR presented the following single peaks: δ 56.33 ($-OCH_3$), δ 107.21 ($-CH=$), δ 157.07 [$-(=C)O-$], δ 176.25 [$-HC(O=C)CH-$] and δ 186.24 [$-(H_3CO)(O=C)(OCH_3)-$]. The IR (KBr) spectrum showed absorption peaks at 1592, 1623, 1645, and 1695 cm^{-1}. UV absorption peaks, $\lambda(\epsilon)$, were at 283.0 (14500) and 375.6 (560) nm in $CHCl_3$.

From these results of analyses, the antibacterial substance contained in the extract from *P. heterocycla* var. Pubescens was identified as 2,6-dimethoxy-*p*-benzoquinone. This was supported by preliminary data (Handa et al., 1983).

MIC Values of the Whole Extract Concentrate and *p*-Benzoquinone Derivatives. The MIC values of the whole extract concentrate and *p*-benzoquinone derivatives against bacteria are shown in Table III. It was immediately found from Table III that the antibacterial spectrum of the whole extract concentrate of *P. heterocycla* Pubescens resembles that of 2,6-dimethoxy-*p*-benzoquinone, one of the constituents of the extract, having an exceptionally stronger antibacterial activity than the other constituents. Therefore, if 2,6-dimethoxy-*p*-benzoquinone was assumed to be the only antibacterial compound in the extract, the content of this compound was estimated to be 0.8–4% of the whole extract concentrate. However, the content of 2,6-dimethoxy-*p*-benzoquinone assayed by HPLC was 0.0532%, which is $^1/_{15}$–$^1/_{75}$ of the estimated content. This difference is thought to be due to the existence of 2,6-dimethoxy-*p*-benzoquinone synergists in *P. heterocycla* Pubescens.

The following findings were also obtained from Table III as to the correlation between the structures of *p*-benzoquinone derivatives and antibacterial activities: (1) The molecule that has both phenyl and hydroxyl groups showed either no or a very weak antibacterial activity. (2) The molecule having a methoxy group had the strongest antibacterial activity and was more effective against Gram-positive bacteria. (3) The molecule that has an isopropyl group showed a strong antibacterial action particularly against Gram-positive bacteria, while the molecule having four methyl groups had a very weak antibacterial activity. (4) Unlike other derivatives, *p*-benzoquinone had a strong antibacterial activity against Gram-negative bacteria.

CONCLUSIONS

Phenolic substances such as flavonoids (Nakatani, 1988), organic acids (Yamamoto et al., 1984), and essential oils (Kurita et al., 1981) are known as antibacterial substances derived from plants. As for quinones, the antibacterial activities of naphthoquinone and anthraquinone were reported by many investigators (Shcherbonovskii et al., 1975; Brewer et al., 1984; Tabata et al., 1982; Sharma et al., 1985; Matsueda et al., 1980). 2,6-Dimethoxy-*p*-benzoquinone has been reported in higher plants (Handa et al., 1983) and the antibacterial activity of other benzoquinones was also reported, but only by a few investigators (Haraguchi et al., 1986).

On the other hand, Nikaido et al. (1984) isolated 2,5-dimethoxy-*p*-benzoquinone as an inhibitor of AMP phosphodiesterase from some Gramineae. However, so far as we are aware, there is no study describing 2,6-dimethoxy-*p*-benzoquinone isolated from bamboo. Moreover, the finding that the antibacterial activity of *p*-benzoquinone derivatives is influenced by the type of substituted group is interesting from the viewpoint of the structure–activity relationship for antibacterial substances.

It is known that plants have various biological protective systems to accommodate environmental conditions (Ramarathnam et al., 1988; Osawa et al., 1985). The use of the antibacterial substances contained in spices for the preservation of meat is one good example of the utilization of biological protective systems in plants. The antibacterial compound in the bamboo extract discovered in the present study is thought to be useful as an antibacterial agent not only for foods but also for cosmetics, etc.

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Registry No. 2,6-Dimethoxy-*p*-benzoquinone, 530-55-2; potassium sorbate, 24634-61-5; propionic acid, 79-09-4; benzoic acid, 65-85-0; monocaprylin, 26402-26-6; *p*-benzoquinone, 106-51-4; tetramethyl-*p*-benzoquinone, 527-17-3; 2,5-dihydroxy-*p*-benzoquinone, 615-94-1; tetrahydroxy-*p*-benzoquinone, 319-89-1; 2,3-dimethoxy-5-methyl-*p*-benzoquinone, 605-94-7; 2,5-diphenyl-*p*-benzoquinone, 844-51-9.