Antimicrobial Components, Trachrysone and 2-Methoxystypandrone, in *Rumex japonicus* Houtt.

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Active antimicrobial substances contained in *Rumex japonicus* Houtt. were isolated by HPLC. As a result of the identification using MS, ^{1}H NMR, and ^{13}C NMR, trachrysone and 2-methoxystypandrone were found to be antimicrobial compounds. Comparison of the antimicrobial spectra of trachrysone and 2-methoxystypandrone analogs suggested that the antimicrobial activity of trachrysone and 2-methoxystypandrone analogs was influenced by the types of functional groups and positions of the hydroxyl groups.

Antimicrobial agents for improving the shelf stability of foods and cosmetics are indispensable. Although conventional synthetic antimicrobial agents have excellent activities, their safety in the human body has been considered doubtful (Inui *et al.*, 1978). Since it is known that antimicrobial agents are contained in natural materials (Nakatani and Inatani, 1984; Chuyen *et al.*, 1982; Swaminathan and Koehler, 1976; Kimura *et al.*, 1981; Sawai *et al.*, 1981; Kodama *et al.*, 1985; Nagata *et al.*, 1985; Mizobuchi and Sato, 1984; Sankawa *et al.*, 1989), many research studies have been carried out to improve the shelf stability of foods and cosmetics using natural antimicrobial agents (Hara and Watanabe, 1989; Ueda *et al.*, 1982; Ali and Hasnain, 1986). No satisfactory natural antimicrobial material has yet been discovered.

Accordingly, in this investigation a study of the natural antimicrobial constituent of *Rumex japonicus* Houtt., a crude drug (Tanaka, 1976), has been conducted.

It is known that some crude drugs have antimicrobial action. In particular, they have been used as a remedy for cutaneous diseases. For instance, components in *R. japonicus* Houtt. have been used for athlete's foot in folk remedies. Odani *et al.* (1977) reported that the effective antifungal contained in *R. japonicus* Houtt. is 2-acetyl-1,8-dihydroxy-3-methylnaphthalene [musizin (MUS)]. The authors have reinvestigated this species using preparative high-performance liquid chromatography (HPLC), which has been widely used in recent years, and have found that *R. japonicus* Houtt. also contains 6-acetyl-5-hydroxy-2-methoxy-7-methyl-1,4-naphthoquinone [2-methoxystypandrone (MSD)] and 2-acetyl-1,8-dihydroxy-3-methyl-6-methoxynaphthalene [trachrysone (TRA)] as two further effective antimicrobial components.

EXPERIMENTAL MATERIALS AND METHODS

Experimental Materials. The root of *R. japonicus* Houtt., collected in Wakayama Prefecture, Japan, was dried in a vacuum freeze-dryer. The dried root of *R. japonicus* Houtt. was pulverized in a household mixer and the pulverized sample (55.4 g) extracted, in turn, with *n*-hexane, ethyl acetate, chloroform, 1-butanol, and water. Yields of each fraction were 0.198, 0.500, 0.053, 0.428, and 3.87 g, respectively.

Test Compounds. The following test compounds were used for the experiments: benzoic acid (BA), sorbic acid (SA), butyl hydroxybenzoate (BH), 1,4-naphthoquinone (NQ), 2-methyl-1,4-naphthoquinone (MNQ), and 2-hydroxy-1,4-naphthoquinone (2HNQ) manufactured by Wako Pure Chemical Industries Ltd., Tokyo; hydroquinone (HQ) and 5-hydroxy-1,4-naphthoquinone (5HNQ) manufactured by Tokyo Kasei Kogyo Co., Ltd., Tokyo; 1-naphthol (NT) manufactured by Koso Chemicals Co., Ltd., Tokyo; 1-naphthoic acid (NA), 1-hydroxy-2-naphthoic acid (HNA), 2,3-dihydroxynaphthalene (2,3-DN), 1,4-dihydroxynaphthalene (1,4-DN), 1,5-dihydroxynaphthalene (1,5-DN), and 1,6-dihydroxynaphthalene (1,6-DN) manufactured by Tokyo Kasei Kogyo; 1,3-dihydroxynaphthalene (1,3-DN) and 2,7-dihydroxynaphthalene (2,7-DN) manufactured by Wako Pure Chemical Industries; and 2,6-dihydroxynaphthalene (2,6-DN) manufactured by Aldrich Chemical Co., Milwaukee, WI.

High-Performance Liquid Chromatography (HPLC). A Gilson Model 303 chromatograph with 10SC pump head, columns of (a) Develosil 60-10 (25 cm long and 20 mm in diameter), (b) Develosil ODS10 (25 cm long and 20 mm in diameter), and (c) Develosil ODS5 (25 cm long and 5 mm in diameter) from Nomura Chemical Co., Ltd., Aichi, Japan, and chloroform for column a and a mixture of acetonitrile and water (mixing ratio by volume 80/20) for columns b and c was used. An SPD-M6A detector from Shimadzu Works Co., Ltd., Tokyo, was used at a wavelength of 273 nm. The flow rate in columns a and b was 6 mL/min and in column c was 1 mL/min.

Analytical Instruments. The analytical instruments used were an ultraviolet spectrometer (UV-260) from Shimadzu Works, an infrared spectrophotometer (FIR-5500) from Japan Electron and Optics Laboratory (JEOL), Tokyo, a nuclear magnetic resonance spectrometer (GSX-270) from JEOL, and a mass spectrometer (JMS-DX300) from JEOL.

Determination of Antimicrobial Activity. *Strains.* Three kinds of Gram-positive bacteria, *Staphylococcus aureus* FDA-209P, *Bacillus subtilis* PCI-219, and *Micrococcus lutea* ATCC-1001, three kinds of Gram-negative bacteria, *Escherichia coli* 0-80, *Salmonella typhimurium* H-901 W, and *Pseudomonas aeruginosa* IFO-3080, and two kinds of fungi, *Saccharomyces cerevisiae* KF-25 and *Aspergillus niger* ATCC-6275, were used for determining the antimicrobial activity.

Paper Disk Method. The antimicrobial activity of each fraction was determined by the paper disk method (Nishina *et al.*, 1991). A seeded solution was prepared by culturing *B. subtilis* in 10 mL of an ordinary nutrient broth at 37 °C for 24 h. Then 0.1 mL of the seeded solution was diluted 100 times with 10 mL of a trypto-soy agar medium kept at 50 °C. The diluted solution was put into a sterilized basin. After cooling, a disk 8 mm thick (Toyo Seisakusho Co., Ltd., Tokyo) soaked with 1 mg of the sample was put on the medium and cultured at 37 °C for 24 h. After that, the diameter of an inhibited growth circle was measured.

Determination of Minimum Inhibitor Concentration (MIC). Trypto-soy agar and *sabouraud* dextrose agar media (manufactured by Eiken Chemical Co., Ltd., Tokyo) were used

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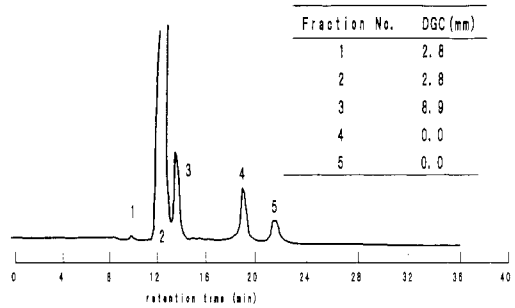

Figure 1. HPLC chromatogram and antimicrobial activity of fractions of the *n*-hexane extract of *R. japonicus* Houtt. roots. The diameter of an inhibited growth circle [DGC(mm)] was measured by a paper disk method (*B. subtilis*, bouillon–agar medium incubated at 37 °C for 24 h).

for the test media for bacteria and fungi, respectively. For less water-soluble samples, a series of media was prepared by diluting an ethanol solution containing 400 μg of each sample per milliliter with water to a specified concentration and adding 10 mL each of the diluted solution to the test medium. A series of media thus prepared was seeded with 10 μL each of the bacterial solution containing 10^6–10^8 cells precultured in an ordinary nutrient broth at 37 °C for 24 h. The media seeded with the bacteria and fungi were cultured at 37 °C for 48 h and at 30 °C for 7 days, respectively, to determine MIC by observing in what media the microorganisms grew (Sankawa *et al.*, 1989).

RESULTS AND DISCUSSION

Antimicrobial Activities of Crude Fractions. The diameters of inhibited growth circles of crude fractions, extracted with *n*-hexane, ethyl acetate, chloroform, 1-butanol, and water, were 8.2, 4.8, 3.8, 0.0, and 0.0 mm, respectively. The water and 1-butanol fractions had no antimicrobial activity, while the *n*-hexane fraction had the highest activity. The activities of the ethyl acetate and chloroform fractions were lower than that of the *n*-hexane fraction.

Fractionation of *n*-Hexane Fraction by HPLC. The *n*-hexane extract was fractioned into five fractions (1–5) by reversed-phase HPLC as shown in Figure 1. The antimicrobial activities of fractions 1–5 are also illustrated in Figure 1. Fractions 1 and 2 have low antimicrobial activity, while fraction 3 has high antimicrobial activity. Fractions 4 and 5 have no activity. Fractions 1–3 with antimicrobial activity or relatively high content were almost perfecty repurified by normal-phase HPLC and submitted for structural analyses.

Identification of Compounds 1–3. The molecular formula of compound 1 was determined as $C_{12}H_{14}O_5$ from M^+ = 260.0686 in the mass spectrometric results. The results of ^{1}H NMR ($CDCl_3$) indicate that signals at δ 2.35 (3H, s, CH_3), 2.59 (3H, s, CH_3), 3.93 (3H, s, O—CH_3), 6.11 (1H, s, Ar H), 7.52 (1H, s, Ar H), and 12.51 (1H, s, OH) are observed. ^{13}C NMR presented the following single peaks: δ 20.0 (CH_3), 31.8 (CH_3), 56.7 (O—CH_3), 109.6 (C_3), 112.4 (C_{10}), 121.7 (C_8), 130.5 (C_6), 135.7 (C_9), 143.5 (C_7), 158.2 (C_5), 161.1 (C_2), 179.1 (C_1), 190.3 (C_4), and 202.9 (C=O). The results of heteronuclear multiple-bond correlation spectroscopy (HMBC) indicated connections of naphthoquinone carbons. The results of IR ν_{KBr} indicate that absorption bands are observed at 1697, 1631, 1592, 1192, and 877 cm^{-1}. The results of UV spectrometry (MeOH) show that absorption bands are observed at wavelengths of 204, 288, and 407 nm. Fraction 1, therefore, is identified as 6-acetyl-5-hydroxy-2-methoxy-7-methyl-1,4-naphthoquinone [2-methoxystypandrone (MSD)] from the results mentioned above. The analytical results of mass spectrometry and ^{1}H and ^{13}C NMR agree with the data obtained by Kimura *et al.* (1983).

The result of the mass spectrometry indicates that the molecular weight of fraction 2 is 216. The results of ^{1}H NMR ($CDCl_3$) indicate that signals at δ 2.45 (3H, s, CH_3), 2.55 (3H, s CH_3), 6.84 (1H, dd, Ar H), 6.89 (1H, s, Ar H), 7.05 (1H, dd, Ar H), 7.23 (1H, t, Ar H), 10.22 (1H, s, OH), and 17.33 (1H, s, OH) are observed. Infrared spectrophotometric analysis showed absorption bands at IR ν_{KBR} 3449 (OH), 1586 (C=O), 1168 (CO), and 857 (aromatic CH), and ultraviolet absorption bands at wavelengths of 224, 261, 320, 336, and 394 nm (MeOH) were observed. Since those data agree with the measurements of Ogweno and Rukunga (1985), fraction 2 is identified as 2-acetyl-1,8-dihydroxy-3-methylnaphthalene [musizin (MUS)].

The molecular weight of fraction 3 is 246. The results of ^{1}H NMR ($CDCl_3$) indicate that signals are observed at δ 2.62 (3H, s, CH_3), 2.72 (3H, s, CH_3), 3.88 (3H, s, OCH_3), 6.47 (1H, s, Ar H), 6.49 (1H, s, Ar H), 6.83 (1H, s, Ar H), 10.42 (1H, s, OH), and 17.73 (1H, s, OH). IR ν_{KBR} results showed absorption bands at 1585, 1201, 1162, and 858. Since those results agree with the result obtained by Tsuboi *et al.* (1977), fraction 3 is identified as 2-acetyl-1,8-dihydroxy-3-methyl-6-methoxynaphthalene [trachrysone (TRA)].

Antimicrobial Activity of MSD and TRA Analogs. The antimicrobial activities of standard antimicrobial agents, MSD and TRA, are shown in Table I; those of MUS are also shown for comparison. Comparison of the MICs with those of standard antimicrobial agents shows that MSD has an especially high inhibitory action on Gram-positive bacteria. Although TRA has well-balanced inhibitory action on both bacteria and fungi, the inhibitory action of TRA on Gram-positive bacteria is lower than that of MSD. Table I reveals that the antimicrobial activity of MUS is low, and, particularly, that on fungi is zero. These results indicate that the antimicrobial activities of MSD and TRA are remarkably higher than that of MUS.

The antimicrobial spectra of 1,4-naphthoquinones, which are MSD analogs, are also shown in Table I. Although the antimicrobial activities of NQ, MNQ, and HQ on most Gram-positive and -negative bacteria, expressed in MIC, are 5–50 times higher than that of MSD, that of MSD on *B. subtilis* is higher. The antimicrobial activity of 2HNQ is nearly equal to that of MSD, while that of 5HNQ is lower than that of MSD. Those results indicate that the antimicrobial activity of NQ, which is the basic skeleton of MSD analogs, is the highest, and the activity decreases with an increase in the number of functional groups connected to the basic structure. Particularly, the antimicrobial activity is unexpectedly decreased by the presence of a hydroxyl group at the 5-position.

^{1}H NMR spectra were determined for 2HNQ and 5HNQ in an effort to elucidate why the antimicrobial activity of the former is higher than that of the latter. A broad signal (s, 1H) derived from the hydroxyl group is observed at 11.79 ppm in the spectrum of 2HNQ, while a sharp signal (s, 1H) is observed at 11.67 ppm in that of 5HNQ. From the fact that the signal derived from the hydroxyl group in the spectrum of 5HNQ is sharp, it is inferred that there is hydrogen bonding between an oxygen atom at the 4-position of a carbon atom and a hydrogen atom at the 5-position. The difference in antimicrobial activities between 2HNQ and 5HNQ may relate to the existence of the internal hydrogen bonding of 5HNQ.

Although MSD also has a hydroxyl group at the

Table I. Comparison of Antimicrobial Activities of 2-Methoxystypandrone, Trachrysone, and Related Compounds [MIC (ppm)][a]

sample	S. aureus	B. subtilis	S. lutea	E. coli	S. typhimurium	P. aeruginosa	S. cerevisiae	A. niger
MSD	40	<10	40	1000	>1000	>1000	40	1000
TRA	100	100	200	100	100	>1000	40	1000
MUS	400	400	1000	>1000	>1000	>1000	400	>1000
BA	>1000	>1000	>1000	>1000	>1000	>1000	100	100
SA	>1000	>1000	>1000	>1000	>1000	>1000	200	1000
BE	200	200	200	100	1000	>1000	200	1000
NQ	10	10	10	40	100	200	40	1000
MNQ	10	10	10	20	40	>1000	40	1000
2HNQ	40	40	40	200	200	>1000	400	>1000
5HNQ	400	1000	1000	1000	1000	>1000	400	>1000
NT	400	400	400	400	400	400	400	400
NA	400	400	1000	1000	1000	>1000	1000	1000
HNA	400	400	400	1000	1000	1000	400	1000
HQ	10	10	10	20	20	>1000	40	>1000
2,3-DN	200	100	100	100	100	400	400	200
1,3-DN	40	100	100	100	100	>1000	100	>1000
1,4-DN	10	10	10	40	40	400	10	100
1,5-DN	40	40	20	200	200	1000	100	1000
1,6-DN	200	100	100	200	200	1000	200	1000
2,6-DN	200	200	40	200	200	1000	200	200
2,7-DN	200	200	200	200	400	>1000	200	1000

[a] MIC was determined by the 2-fold serial broth dilution method.

5-position, its antimicrobial activity is higher than that of 5HNQ. The other functional groups, particularly the methoxy group at the 2-position, may prevent the activity from decreasing. The authors have previously compared the antimicrobial activity of *p*-benzoquinone analogs and found that molecules having a methoxy functional group have high antimicrobial activity (Nishina and Uchibori, 1991). The mechanism by which the antimicrobial activity is improved by a methoxy group must be further elucidated.

The antimicrobial spectra of TRA analogs, including the isomeric naphthalene diols, are also shown in Table I. The antimicrobial activities follow the general order (highest to lowest) (1) HQ and 1,4-DN, (2) 1,5- and 1,3-DN, (3) TRA, (4) 1,6-, 2,6-, and 2,7-DN, (5) MUS, NT, NA, and HNA. It is inferred from those results that dihydroxynaphthalenes with hydroxyl groups at the para position have the highest antimicrobial activity and those with hydroxyl groups at positions further and nearer than the para position have lower activities. NT with a single hydroxyl group has lower antimicrobial activity than any dihydroxynaphthalene. Therefore, the hydroxyl group itself may take such an extensive part in the antimicrobial activity that the activity is synergistically increased by positioning two hydroxyl groups at the para position.

The difference in antimicrobial activities between TRA and MUS cannot be explained by the positions of the hydroxyl groups, since the only structural difference between the two compounds is the presence of a methoxy group at the 6-position of the former. Although the antimicrobial activity of TRA may be increased by the methoxy group in the same way as in *p*-benzoquinones (Nishina and Uchibori, 1991), further studies are required to elucidate the mechanism.

It is known at present that MSD is contained in *Ventilago calyculata* (Hanumaiah *et al.*, 1985), *Polygonum cuspidatum* (Kimura *et al.*, 1983), and *Rhamnus fallax* Boiss (Rauwald and Meithing, 1982, 1985). Hughes and Sargent (1989) synthesized MSD from trimethoxybenzaldehyde. Neither a paper reporting that MSD is contained in *R. japonicus* nor a paper describing the physiological activity of MSD has been found so far. The chemical structure of 1,4-naphthoquinones and the correlation with antimicrobial activity are presented only in Yamazoe *et al.*'s (1968) paper on the study of the halogenated molecules.

It is known that TRA is also contained in the seed of *Cassia obtusifolua* L. and *Cassia tura* L. (Shibata *et al.*, 1969) and that the glucoside of TRA is contained in rhubarb (Tsuboi *et al.*, 1977). Nikaido *et al.*'s (1984) paper describes the inhibitory activities of TRA and a material related to it, adenosine 3′,5′-cyclic monophosphate phosphodiesterase. The antimicrobial activity and mechanism, however, have not been yet elucidated. No paper has reported that TRA is contained in plants in the genus *Rumex*. There are a few papers (Moussa *et al.*, 1981; Misra and Tewari 1971a,b; Misra, 1978; Drobnica *et al.*, 1968) on the antimicrobial activity of naphthalene derivatives and only two papers (Odani *et al.*, 1977; Zaki *et al.*, 1971) on the antimicrobial activity of naphthalenediols.

In conclusion, this paper demonstrates that MSD and TRA have been isolated from *R. japonicus* Houtt. and have high antimicrobial activity. The types of functional groups in 1,4-naphthoquinone analogs and the possible correlation between the existence of hydrogen bonding and the antimicrobial activity, as well as the correlation between the positions of the hydroxyl groups in naphthalenediol analogs and the antimicrobial activity, must be clarified in the future. The toxicity and safety of MSD and TRA will continue to be of interest.

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