Original

In Vitro Antifungal and Antiviral Activities of γ - and δ -Lactone Analogs Utilized as Food Flavoring

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Massoialactone, γ -, and δ -decalactones inhibited the growth of *Aspergillus niger*, *Candida albicans*, and *Staphylococcus aureus* at 31.5-125, 62.5-500, and 62.5-250 μ g/ml, respectively, as seen using the agar dilution method and 96-well microbioassay system with a liquid culture system. These lactones fungicidally inhibited their growth because their minimum fungicidal concentrations were only 2 to 4 times that of their minimum inhibitory concentrations. Results obtained from their structure-fungicidal activity against two species of fungi were as follows: γ -lactones exhibit higher antifungal activity than δ -lactones; the antimicrobial effects of γ -lactones are intensified as the side chain length increases; massoialactone with an unsaturated δ -lactone ring gives a smaller minimum inhibitory concentration against the two types of fungi compared with γ - and δ -decalactones; and the (*R*)-forms of γ -undecalactone and γ -dodecalactone inhibit the growth of *A. niger* at smaller concentrations compared with the (*S*)-forms. Vaporized massoialactone inhibited the growth of the two fungi at 1.2 μ g/ml in a sealed vial, whereas the γ - and δ -decalactones exhibited no inhibition against the growth of the two fungi. Massoialactone arrested the oxygen consumption by *C. albicans* at 240 μ M, suggesting that the antimicrobial mechanism responsible for massoialactone activity against *C. albicans* may be the inhibition of the respiratory system. The reproduction of influenza virus A was inhibited at 23 and 57 % by massoialactone and δ -decalactone at 0.25 and 2.5 μ g/ml, respectively.

Key words : γ -Lactone/ δ -Lactone/Antifungal activity/Antiinfluenza viral activity.

INTRODUCTION

Essential oils, which are concentrated herbal extracts that contain volatile aromatic chemicals, possess a variety of pharmacological activities such as antimicrobial activity, sedation, and anti-inflammatory effects (Arakawa et al. 1992; Carle and Gomaa, 1992; Santos and Rao, 2000). These oils inhibit the

growth of a wide variety of microorganisms and have great potential as antimicrobial agents. They exhibit their strongest activity against fungi, and then against gram-positive and acid-fast bacteria, and finally yeast (Farag et al. 1989a; Hammer et al. 1998), whereas their activities against gram-negative bacteria are weak because the outer membrane of such bacteria is composed of a hydrophilic side chain which inhibits the passage of oil into the cytoplasm (Chao et al. 2000; Hinou et al. 1989; Mann et al. 2000).

γ - and δ -Lactones are also frequently used as

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FIG. 1. Chemical structures of the tested lactones. A, γ -decalactone; B, δ -decalactone; C, massoialactone.

fragrance or flavor compounds: γ -decalactone has an oily peach-like tenacious odor, δ -decalactone is sweet and creamy and has a nut-like odor, while massoialactone is creamy and has a butter-like odor (Fig. 1). Lactones have been actively investigated with regard to their chemical synthesis (Corma et al., 2004; Kula et al., 1996; Gupta et al., 2004), but few studies have been conducted on their physiological activities other than flavor. Sakurai et al. (1968) reported that the antifungal activity of six γ -lactones among seventy-one chemosynthetic γ -lactones were as effective as dehydroacetic acid and ethyl *p*-hydroxybenzoate. Alfa-methylene- γ -lactones with an alkyl group at the C-4 position, which adds a roast-like odor, exhibited potent inhibition of the growth of three types of bacteria and two types of fungi compared to butyl *p*-hydroxybenzoate as a standard antibiotic (Miyazawa et al., 2000). Therefore, we hypothesized that the principal γ - and δ -lactones utilized as flavoring in food and drink products may also inhibit the growth of bacteria and fungi. The objectives of this study were to test the antimicrobial and antiviral activities of C_{10} - C_{14} γ - and δ -lactones against six types of bacteria, two types of fungi, and an influenza virus, and examine their structure-activity relationships.

MATERIALS AND METHODS

Materials

Lactones were obtained from Soda Aromatic Co., Ltd. (Tokyo, Japan) and Ogawa & Co., Ltd. (Tokyo, Japan) and other chemicals of analytical grade were obtained from Wako Pure Chemicals Ltd. (Osaka, Japan) and Nacalai Tesque Inc. (Kyoto, Japan).

Microorganisms

The lactones were tested for antimicrobial activity against the gram-positive bacteria *Staphylococcus aureus* NBRC 12732, *Bacillus subtilis* NBRC 3009, *Lactobacillus casei* JCM 1134, and *Streptococcus salivarius* subsp. *thermophilus* IAM 10064, and the gram-negative bacteria *Escherichia coli* NBRC 3301, *Pseudomonas aeruginosa* NBRC 3080, and the fungi

Candida albicans NBRC 1594 and *Aspergillus niger* NBRC 4414.

Antimicrobial activity

The antimicrobial activities of the lactones were determined by the agar dilution method and 96-well microbioassay system using a liquid broth. Muller-Hinton broth (Difco Lab., USA) and Lactobacilli MRS broth (Difco Lab.) media were used for the determination of the antibacterial activity against four non-lactic acid bacteria species and two lactic acid bacteria species, respectively, while Sabouraud medium (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) was used to examine the antifungal activity against the two species of fungi. For both methods, DMSO without lactones was used as a negative control, and streptomycin for bacteria and butyl *p*-hydroxybenzoate for fungi were used as a positive control. The minimum inhibitory concentration (MIC) and minimum bactericidal and fungicidal concentrations (MBC and MFC) were determined by two independent experiments ($n=4$).

The agar dilution method used was as follows. Test microorganisms adjusted to 10^6 cfu/ml were streaked on an agar plate containing two-fold stepwise diluted lactones dissolved in DMSO at a maximum concentration of 1,000 μ g/ml. After incubation at 37°C for 18 h (bacteria other than the lactic acid bacteria) or 48 h (lactic acid bacteria), and at 28°C for 24 h (*C. albicans*) or 48 h (*A. niger*), the plates were visually examined for growth.

The micro-liquid dilution method used was as follows. Approximately 180 μ l of the suspension of the test microorganism in which the density was adjusted to 10^4 cfu/ml of the medium was dispensed into 96-well microplates, and 36 μ l of the lactones dissolved in DMSO (maximum concentration of 10,000 μ g/ml) and 144 μ l of the microorganism suspension were added to the wells in the first column of the microplate. The solution in each well in the first column was then serially 2-fold diluted with a micropipette. After incubation, the wells were visually examined for growth. The bactericidal and fungicidal activities were examined as follows. The test microorganisms on the agar plate using the agar dilution method were collected with a sterile cotton bud, and the washed cells were inoculated into new medium. After incubation, the tubes were visually examined for growth.

The antimicrobial activity of the vaporized lactone was determined as follows. The suspensions (50 μ l) of the two species of fungi adjusted to 10^6 cfu/ml were inoculated on the dried surface of Sabouraud agar medium in a 50 ml vial, and 8 mm diameter

paper disks impregnated with DMSO solutions of the test lactones, were inserted into the rubber caps with a setting pin. After sealing with vial-cap, the bottle was incubated at 28°C for 48 h. The inner gas (20 μ l) collected through the rubber cap of the vial by a microsyringe was analyzed by gas chromatography (Shimadzu Co., Kyoto, Japan) using a capillary column (DB-5, 15 m x 0.25 mm i.d., 0.25 μ m film; Agilent, Palo Alto, CA). Nitrogen gas was used as a carrier gas at 48 ml/min, and the eluted compounds were detected by FID. The column, injector, and detector temperatures were maintained at 100 to 120 (2°C/min), 120, and 120°C, respectively.

Inhibition of the respiratory system

The oxygen consumption rate of *C. albicans* was determined using a Respire 1 (Kyokko Trading Co., Tokyo, Japan) at 28°C. Dissolved oxygen in the cell suspension (10⁷ cfu/ml) was decreased at a constant rate for 2 min of preincubation and then 20 μ l of 12 mM massoialactone or 10 mM KCN (as a positive control) solutions were added into 1 ml of the cell suspension.

Inhibition of germ tube formation from conidiospores

The conidiospores of *A. niger* were cultivated in a manner similar to the micro-liquid dilution method. After incubation at 28°C for 48 h, the spores in each well were observed with a stereoscopic microscope.

Antiviral activity

The antiviral activities of the lactones were determined by a plaque assay method using Influenza virus A and Madin Darby Canine Kidney (MDCK) cells. Influenza virus AH 1N1 (A/USSR/92/77) and MDCK cells were obtained from the National Institute of Infectious Diseases (Tokyo, Japan) and the National Institute of Public Health (Tokyo, Japan), respectively. The MDCK cells were cultured in Eagle's Minimal Essential Medium (MEM; Nissui Pharmaceutical Co. Ltd.) supplemented with 10% calf serum (Flow Laboratories, Irvine, U.K.), 2.92 μ g of L-glutamine/ml, 100 units of penicillin/ml, 60 μ g of kanamycin/ml, and 100 μ g of streptomycin/ml. The cells were cultured at 37°C in a humidified atmosphere in the presence of 5% CO₂, and cells in the log phase (48 h after culturing) were used for the determination of antiviral activity. The virus (20 pfu/ml) adsorbed on the cells formed single layer in the plate at 35°C for 60 min. After washing the cells with phosphate-buffered saline (Sigma-Aldrich Co., St. Louis, MO), 2.0 ml of maintenance medium, prepared from Dulbecco's Modified Eagle's Medium (Gibco

Laboratories, NY) containing 10 IU of trypsin/ml, 3 mg of glucose/ml, and the test lactones adjusted to each concentration with 1 % (v/v) ethanol solution, were added to the cells, which were then further incubated at 35°C for 72 h. After two cycles of the freezing and thawing of the plate, the virus concentration in the supernatant was determined by the plaque assay method. The cells were plated at 2x10⁵ cells in tissue culture plates (35 mm i.d.) and incubated at 37°C for 4 days to form a single cell layer. After the cells were washed, 0.2 ml of ten-fold stepwise diluents of the supernatant were added to the cells in the plate, and they were then incubated at 35 °C for 60 min to permit adsorption of the virus onto the cells. After the cells were washed, 2 ml of the maintenance medium containing 1.15 % agar (Gibco Lab.) were layered over the cells in the plate and then the plate was incubated at 35°C for 3 days. Maintenance medium containing neutral red was layered over the agar and the number of plaques was then counted. Antiviral activity was calculated using the following formula: antiviral activity (%) = [(plaque number without the sample) - (plaque number with the sample)] / (plaque number without the sample) x 100.

RESULTS

Antimicrobial activities of lactones

γ - and δ -Decalactones and massoialactone inhibited the growth of the two fungi and *S. aureus* among the bacteria and fungi tested. Their MICs against *A. niger*, *C. albicans*, and *S. aureus* were 31.5-125, 62.5-500, and 62.5-250 μ g/ml, respectively (Table 1), and they exerted fungicidal actions against the fungi and *S. aureus*, because their MFCs were only 2-4 fold higher than their MICs. The MICs of the lactones with different C₁₀-C₁₄ structures against the fungi are summarized in Table 2. The test lactones exhibited potent inhibition of *A. niger* growth compared to *C. albicans*, and γ -lactones with a carbon number the same as the δ -lactones showed higher antifungal activity than the δ -lactones. The antimicrobial effects of the γ -lactones were intensified as the number of carbons in the side chain increased from 5 to 9. The massoialactone with an unsaturated δ -lactone ring had a smaller MIC against the fungi compared to the γ -, and δ -decalactones with a saturated lactone ring. The (*R*)-forms of γ -undecalactone and γ -dodecalactone inhibited the growth of *A. niger* at smaller concentrations compared with their (*S*)-forms. γ -Dodecalactone showed an MIC similar to cis- γ -dodec-6-enolactone with a double bond in the side chain.

TABLE 1. Antimicrobial activities of lactones.

Microorganism	γ -Decalactone		δ -Decalactone		Massoialactone	
	MIC	MBC/MFC	MIC	MBC/MFC	MIC	MBC/MFC
<i>S. aureus</i>	250	500	250	500	62.5	250
<i>B. subtilis</i>	500	1000	>1000	>1000	1000	1000
<i>E. coli</i>	>1000	>1000	>1000	>1000	1000	>1000
<i>P. aeruginosa</i>	500	1000	500	1000	500	1000
<i>L. casei</i>	>1000	>1000	>1000	>1000	>1000	>1000
<i>S. salivarius</i> subsp. <i>thermophilus</i>	>1000	>1000	>1000	>1000	>1000	>1000
<i>C. albicans</i>	250	500	500	1000	62.5	125
<i>A. niger</i>	125	250	125	250	31.5	62.5

Units, $\mu\text{g/ml}$ **TABLE 2.** Antifungal activities of lactones.

Lactone	MIC ($\mu\text{g/ml}$)	
	<i>C. albicans</i>	<i>A. niger</i>
γ -Decalactone (C_{10})	250	125
γ -Undecalactone (C_{11})	202	101
(<i>S</i>)- γ -Undecalactone (C_{11})	102	102
(<i>R</i>)- γ -Undecalactone (C_{11})	102	51.2
γ -Dodecalactone (C_{12})	51.7	51.7
(<i>S</i>)- γ -Dodecalactone (C_{12})	60.7	60.7
(<i>R</i>)- γ -Dodecalactone (C_{12})	52.6	26.3
<i>cis</i> - γ -Dodec-6-enolactone (C_{12})	49.5	99
δ -7-Decenolactone (C_{10})	>108	108
δ -Decalactone (C_{10})	500	125
Massoialactone (C_{10})	62.5	31.5
δ -Undecalactone (C_{11})	>103	103
δ -Dodecalactone (C_{12})	>121	121
ε -Decalactone (C_{12})	>105	>105
δ -Tetradecalactone (C_{14})	108	>108

The vaporized massoialactone exhibited growth inhibition against *A. niger* and *C. albicans* at 1.2 $\mu\text{g/ml}$ in the sealed vial, but this was not true for γ -, and the δ -decalactones, and their MICs fell to one fifty-second against *C. albicans* and one twenty-sixth against *A. niger* compared to the values obtained by the solid dilution method.

Lactones antiinfluenza viral activities

Inhibition of the reproduction of the influenza virus A at 23 and 57 % was observed for massoialactone and δ -decalactone at 0.25 and 2.5 $\mu\text{g/ml}$, respectively, whereas γ -decalactone presented a weak inhibition of 6.7 % at 25 $\mu\text{g/ml}$ (Table 3).

Inhibition of germ tube formation from *A. niger* conidiospores by lactones

Massoialactone inhibited the formation of germ tubes from conidiospores at 31.5 $\mu\text{g/ml}$. Although the conidiospores were elongated at 15.8 $\mu\text{g/ml}$, their extended length was shorter than that of the

TABLE 3. Antiviral activities of lactones.

Lactone	Inhibition ratio of virus reproduction (%)		
	25 $\mu\text{g/ml}$	2.5 $\mu\text{g/ml}$	0.25 $\mu\text{g/ml}$
Massoialactone	>90	90	23
δ -Decalactone	67	57	0
γ -Decalactone	6.7	0	0

control. The volume of conidium increased almost six-fold due to water absorption at 31.5 $\mu\text{g/ml}$, whereas swelling of the conidium was inhibited at 125 $\mu\text{g/ml}$.

Inhibition of the respiratory system of *C. albicans* by massoialactone

Massoialactone inhibited the oxygen consumption by *C. albicans* at 240 μM similar to 200 μM KCN as a positive control (Fig. 2). The lactone caused no leakage of intracellular substances with an absorbance at 280 nm, because this absorbance for the supernatant and optical density at 660 nm of the cell suspension incubated with massoialactone (31.5-1000 $\mu\text{g/ml}$) at 28°C for 20 min were similar to the values shown by the control without lactone.

DISCUSSION

Lactones tested in this study presented the strongest antifungal activity against *A. niger* among the six types of bacteria and two types of fungi, and massoialactone exhibited potent inhibition against the fungi compared with the γ - and δ -decalactones for the vapor and solution conditions. Specifically, the vaporized massoialactone inhibited their growth at a smaller MIC (1.2 $\mu\text{g/ml}$) compared with the MICs (31.5-62.5 $\mu\text{g/ml}$) given by the agar dilution method and microbioassay system with a liquid broth culture. Vaporized lactones are able to actively move with high energy because there is no interaction with water, and therefore, are more efficiently adsorbed onto

FIG. 2. Inhibition of oxygen consumption by massoialactone. The arrows A and B indicate the addition of 12 mM massoialactone and 10 mM KCN, respectively. The curves of dissolved oxygen concentration in the cell-suspended solution are recorded with dissolved oxygen concentration on the vertical axis and time horizontally.

lipophilic mycelia and spores exposed to air. Conversion of the δ -lactone ring into an unsaturated ring may result in an increase in antifungal activity, because three test lactones had almost the same boiling points at 267–287°C and the concentrations of vaporized γ - and δ -decalactones in the sealed vials were the same as that of massoialactone, whereas for the γ -lactone ring the unsaturated ring had no effect on their fungicidal activity (Sakurai et al. 1968).

According to the MIC values, the antimicrobial effects of the γ -lactones were intensified as the number of carbons in the side chain increased from 5 to 9. Sakurai et al. (1968) also reported that γ -lactones with a side chain length of C₄–C₆ show the strongest activity against fungi, and a similar result was reported for their primary alcohols and saturated carboxylic acids with normal chains (Kourai et al., 1994). The increase in the antimicrobial effects with increasing side chain length may reflect the ability of γ -lactones to distribute into the cell membrane as a result of the increasing hydrophobicity. However, these results were not observed for the C₁₀–C₁₄ δ -lactones (Table 2). Delta-lactones also exhibited antifungal activity weaker than that of the γ -lactones. These results show that the chemical reactivity of the lactone ring of γ -lactone has higher chemical reactivity than that of the δ -lactone ring.

Regarding the antimicrobial activity of γ -undecalactone and γ -dodecalactone, the (*R*)-form exhibited potent inhibition of fungal growth compared with the (*S*)-form in the same manner as that of limonene (Aggarwal et al., 2002; Lis-Balchin et al., 1996). These results suggest that the chiral lactones directly

and stereospecifically combine with receptors or enzymes in the cell membrane and cause their inactivation.

Massoialactone and δ -decalactone effectively inhibited the reproduction of human A-type influenza virus, as was also true for 3-carbethoxy-4,5-dimethyl-5,6-dihydro-2-pyrone with an unsaturated δ -lactone ring (Avetisyan et al., 1982). Thus, anti-influenza agents with higher activity occur among the unsaturated δ -lactones.

The mechanism responsible for the antimicrobial activities of essential oils was reported to be due to oil adsorbed on the cell membrane causing an increase in their membrane fluidity, the acceleration of ion permeation such as the influx of Na⁺ and Ca²⁺, the loss of the pH gradient and membrane potential formed across the cell membrane, inactivation of membrane enzymes, and the leakage of cellular materials resulting from disintegration of the cell membrane (Cox et al., 1998 and 2000; Lambert et al., 2001; Seeman, 1972; Sikkema et al., 1994). Massoialactone causes the inhibition of the respiratory system in *C. albicans* but not the disintegration of the cell membrane, because it arrests oxygen consumption by *C. albicans* at 240 μ M. In addition, no leakage of intercellular substances with an absorbance at 280 nm was caused by the lactone at 1,000 μ g/ml. Further studies are needed to clarify the structure-activity relationship and the antimicrobial mechanism of γ - and δ -lactones.

Our findings suggest that representative γ - and δ -lactones utilized as flavoring in food and drink products, especially vaporized massoialactones, exhibit potent inhibition of the growth of *A. niger*, *C. albicans*, and influenza virus A, and therefore might be useful as protective agents against fungi and viruses in the air in the same manner as that of essential oils.

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