Biological Activity of β-Dolabrin, γ-Thujaplicin, and 4-Acetyltropolone, Hinokitiol-Related Compounds

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β-Dolabrin, γ-thujaplicin, and 4-acetyltropolone, the components of Aomori Hiba (*Thujopsis dolabrata* SIEB. *et* ZUCC. var. *hondai* MAKINO), showed antifungal activity on seven kinds of plant-pathogenic fungi, antibacterial activity against two kinds of *Legionella* sp., and *in vitro* cytotoxic effect on murine P388 lymphocytic leukemia cell line. Firstly, β-dolabrin, γ-thujaplicin and 4-acetyltropolone had clear antifungal activity against seven kinds of plant-pathogenic fungi tested. In particular, β-dolabrin and 4-acetyltropolone showed strong antifungal activity against *Pythium aphanidermatum* IFO 32440, with minimum inhibitory concentration (MIC) values of 6.0 μg/ml. Secondly, β-dolabrin, γ-thujaplicin and 4-acetyltropolone had obvious growth-inhibitory effect on two kinds of *Legionella* sp. 4-Acetyltropolone especially had strong antibacterial activity toward *Legionella pneumophila* SG 1, and its MIC value was 3.1 μg/ml. These three compounds showed cytotoxic effects against murine P388 lymphocytic leukemia cell line *in vitro*. The cytotoxic effect of three compounds in the murine P388 lymphocytic leukemia cell line were clear when cell growth was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. At 48 h after treatment, γ-thujaplicin and 4-acetyltropolone at 0.63 μg/ml inhibited cell growth of murine P388 lymphocytic leukemia by 85% and 65%, respectively. At the same time after treatment, the growth of the murine P388 lymphocytic leukemia cell line was completely suppressed by the three compounds at concentrations higher than 5.0 μg/ml. Among these three compounds, γ-thujaplicin had the strongest cytotoxic activity on the growth of this tumor cell line *in vitro*.

Key words β-dolabrin; γ-thujaplicin; 4-acetyltropolone; antimicrobial activity; cytotoxic activity; murine P388 lymphocytic leukemia cell line

We have been investigating the biological activity of hinokitiol-related compounds (Chart) such as hinokitiol (β-thujaplicin), β-dolabrin, γ-thujaplicin, α-thujaplicin and 4-acetyltropolone isolated from Aomori Hiba (*Thujopsis dolabrata* SIEB. *et* ZUCC. var. *hondai* MAKINO). Of these hinokitiol-related compounds, hinokitiol and β-dolabrin are the major components isolated from this plant by our group. On the other hand, γ-thujaplicin, α-thujaplicin, and 4-acetyltropolone, the minor components, were chemically synthesized[1—3] for various biological activity tests. It has been found by authors that these five compounds showed antimicrobial activity,[1—4] metalloprotease inhibition,[1—4] phytogrowth-inhibitory effects,[1,2,5] insecticidal activity,[4,6,7] and *in vitro* cytotoxic effect on human and murine tumor cell lines.[1,2,8,9] Among these five compounds, hinokitiol has also been used as a preservative for vegetabls,[10] flowers,[11] and mushrooms[12] as well as a plant growth stimulator[13] because of its strong antibacterial activity. Hinokitiol has been widely used as a preservative, a toothpaste, a cosmetic, and a hair tonic. On the other hand, unlike hinokitiol, the other four hinokitiol-related compounds have not been used practically, because no basic toxicologic and histopathologic studies of these four compounds have been done. We have recently reported that in addition to the above-mentioned activities, α-thujaplicin,[7] hinokitiol,[4] and tropolone[4] showed antifungal activity on plant-pathogenic fungi and antibacterial effect against *Legionella* sp., and *in vitro* cytotoxic effect on murine P388 lymphocytic leukemia cell line. However, no study has been done on the similar biological activities of β-dolabrin, γ-thujaplicin, and 4-acetyltropolone.

Therefore in the series of our basic studies on the biological activity of hinokitiol-related compounds isolated from *T. dolabrata* and their clinical application, antifungal activity on plant-pathogenic fungi and antibacterial activity against *Legionella* sp. of β-dolabrin, γ-thujaplicin and 4-acetyltropolone were investigated. Cytotoxic effect on murine P388 leukemia lymphocytic cell line of β-dolabrin, γ-thujaplicin and 4-acetyltropolone, the components of this plant was also examined *in vitro* and compared with that of hinokitiol.

MATERIALS AND METHODS

Chemicals β-Dolabrin was isolated from acid oil obtained by distillation of the wood of *T. dolabrata* SIEB. *et* ZUCC. var *hondai* MAKINO according to the method of Nozoe *et al.*[14] and used for various biological activity assays. γ-Thujaplicin[3] and 4-acetyltropolone,[2] the minor components, were chemically synthesized and used for various biological activity assays. Vinblastine and vincristine (a positive control for *in vitro* cytotoxic activity) were obtained from Sigma Chemical Co. (U.S.A.).

Microorganisms Plant-pathogenic fungi used for the antifungal activity assays were as follows: *Pythium aphanidermatum* IFO 32440, *Thanatephorus cucumeris* IFO 30455, *Fusarium solani* IFO 9955, *Botryotinia fuckeliana* IFO 30915, *Phomopsis obscurans* MAFF 744018 and *Colletotrichum orbiculare* MAFF 306518. *Colletotrichum lagenarium* was a wild strain kindly supplied by the National

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Table 1. Antifungal Activity of β-Dolabrin, γ-Thujaplicin and 4-Acetyltropolone on Plant-Pathogenic Fungi

Plant-pathogenic fungi	MIC (μg/ml)[a]			
	β-Dolabrin	γ-Thujaplicin	4-Acetyltropolone	Hinokitiol
Pythium aphanidermatum IFO 32440	6.0	12.0	6.0	12.0
Thanatephorus cucumeris IFO 30455	12.0	12.0	50.0	12.0
Fusarium solani IFO 9955	50.0	50.0	100.0	50.0
Botryotinia fuckeliana IFO 30915	25.0	25.0	200.0	50.0
Phomopsis obscurans MAFF 744018	25.0	12.0	50.0	12.0
Colletotrichum orbiculare MAFF 306518	6.0	12.0	12.0	25.0
Colletotrichum lagenarium	25.0	12.0	50.0	25.0

Potato dextrose agar medium, incubated at 24 °C for 15 d. *a*) Minimum inhibitory concentration was determined using the agar dilution method.

Institute of Vegetable and Tea Science. Bacteria used for antibacterial activity assays were *Legionella pneumophila* SG 1 and *L. pneumophila* SG 3. Both bacteria, which originated in the environment, were isolated by the Byotai-Seiri Laboratory.

Cells The tumor cell line used for *in vitro* cytotoxic activity assays was the murine P388 lymphocytic leukemia cell line $P388D_1$ (ATCC TIB63).

Methods The antifungal activity of the three compounds on plant-pathogenic fungi was investigated using the agar dilution method,[4] 1) incubation temperature: 24 °C, 2) incubation time: 15 d, 3) medium: potato dextrose agar (pH 6.0). The antibacterial activity of the three compounds against *Leginonella* sp. was also examined by the agar dilution method.[4] 1) incubation temperature: 35 °C, 2) incubation time: 7 d, 3) medium: BCYE α-agar. The cytotoxic assay was performed according to the method in the previous paper.[9] Cell line of murine P388 lymphocytic leukemia in the exponential growth phase were plated in 96-well flat bottom microplates at a density of 3×10^3 cells per 100 μl in each well and grown for 24 h in each medium and then 100 μl of fresh medium with various concentrations of test compounds was added. After 24 and 48 h of culture, cell growth was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method.[15] Test compounds were dissolved in DMSO and diluted in complete medium at 0.32—20 μg/ml. The final concentrations (0.0012—0.08% in complete medium) of DMSO did not influence the cell growth of the cell line of murine P388 lymphocytic leukemia (data not shown).

RESULTS AND DISCUSSION

Antifungal Activity of β-Dolabrin, γ-Thujaplicin and 4-Acetyltropolone on Plant-Pathogenic Fungi As previously reported, hinokitiol,[4] tropolone[4] and α-thujaplicin[7] were found to show strong growth-inhibitory activity on plant-pathogenic fungi, so antifungal activity of β-dolabrin, γ-thujaplicin and 4-acetyltropolone on plant-pathogenic fungi were investigated in comparison with that of hinokitiol. As shown in Table 1, the three compounds had clear antifungal activity against seven kinds of plant-pathogenic fungi and their minimum inhibitory concentration (MIC) values were in the range of 6.0—200.0 μg/ml. In particular, the antifungal effect of β-dolabrin on *Pythium aphanidermatum* IFO 32440 and *Colletotrichum orbiculare* MAFF 306518 was strong, and its MIC value was 6.0 μg/ml. The antifungal activity of β-dolabrin was higher than that of hinokitiol, the comparative agent. The antifungal effect of hinokitiol in the present work was as strong as that of this compound in the previous paper.[3] 4-Acetyltropolone also showed strong antifungal activity against *P. aphanidermatum* IFO 32440, its MIC value being 6.0 μg/ml, but its antifungal activity against other fungi except for *P. aphanidermatum* IFO 32440 were weak in comparison with those of the other three compounds. The antifungal activity of γ-thujaplicin was slightly higher than that of hinokitiol, while that of 4-acetyltropolone on other fungi except for *P. aphanidermatum* IFO 32440 and *C. orbiculare* MAFF 306518 was weaker than that of hinokitiol. We reported that β-dolabrin, γ-thujaplicin, and hinokitiol showed antifungal activity on pathogenic fungi.[3] We also found that the four compounds except for α-thujaplicin strongly inhibited the growth of wood-rotting fungi.[2,6] It has previously been reported in two patents that hinokitiol has strong antifungal activity on *Helicobasidum mompa*[16] and *Rosellinia necatrix*.[17] In the present work and the previous paper,[4,7] five hinokitiol-related compounds and tropolone were found to have clear antifungal activity against plant-pathogenic fungi. These facts suggest that antifungal activity on pathogenic fungi including plant-pathogenic fungi seems to be common biological activity of hinokitiol-related compounds. We are now performing pot testing of these five hinokitiol-related compounds against plant-pathogenic fungi.

Table 2. Antibacterial Activity of β-Dolabrin, γ-Thujaplicin and 4-Acetyltropolone on *Legionella* Spp.

Compound	MIC (μg/ml)[a]	
	L. pneumophila SG 1	*L. pneumophila* SG 3
β-Dolabrin	6.25	25.0
γ-Thujaplicin	50.0	25.0
4-Acetyltropolone	3.1	12.5
Hinokitiol	6.25	25.0

BCYE α-agar medium, incubated at 35 °C for 7 d. *a*) Minimum inhibitory concentration was determined using the agar dilution method.

Antibacterial Activity of β-Dolabrin, γ-Thujaplicin, and 4-Acetyltropolone on *Legionella* sp. In a previous paper,[7] hinokitiol and α-thujaplicin were reported to have antibacterial activity on *Legionella* sp., so we investigated the antibacterial activity of β-dolabrin, γ-thujaplicin and 4-acetyltropolone on *Legionella pneumophila* SG 1 and *L. pneumophila* SG 3. As shown in Table 2, three compounds showed obvious growth-inhibitory activity against the two

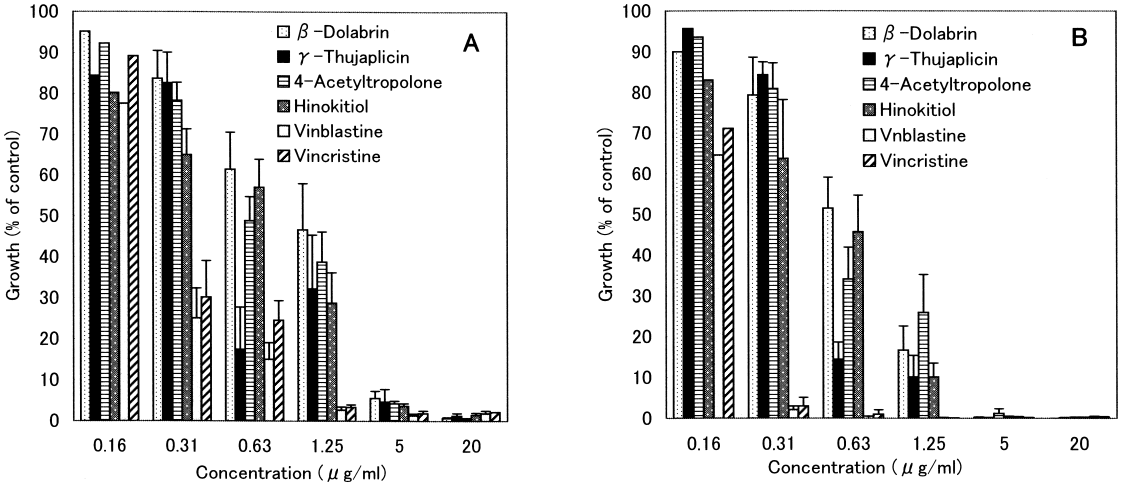

Fig. 1. Inhibitory Activity of β-Dolabrin, γ-Thujaplicin and 4-Acetyltropolone on Growth of Cell Line of Murine P388 Lymphocytic Leukemia at 24 h after Treatment (A) and at 48 h after Treatment (B)

Values are expressed as % of control and represent the mean±S.E. ($n=5$).

Legionella sp. In particular, 4-acetyltropolone had the strongest antibacterial activity against both *Legionella* sp., and its MIC value on *L. pneumophila* SG 1 was 3.1 μg/ml, while that on *L. pneumophila* SG 3 was 12.5 μg/ml. The antibacterial activity of 4-acetyltropolone on both bacteria was higher than those of other hinokitiol-related compounds. The antibacterial activity of hinokitiol on both bacteria in this work was as high as that in the previous paper.[7] Next, β-dolabrin also showed strong antibacterial activity against *L. pneumophila* SG 1, its MIC value being 6.25 μg/ml. The antibacterial activity of β-dolabrin on this bacterium was as high as that of hinokitiol. On the other hand, among the five compounds, γ-thujaplicin showed the weakest antibacterial activity on this bacterium. Since all hinokitiol-related compounds tested had antibacterial activity toward both *Legionella* sp., antibacterial activity on these bacteria seems to be a common physiological activity of these five compounds. Because rather strong antibacterial effect against *Legionella* sp. was found in hinokitiol-related compounds that have low toxicity in mice, the synthesis of many derivatives of these compounds and studies of their antibacterial activities are in progress.

Cytotoxic Activity of β-Dolabrin, γ-Thujaplicin, and 4-Acetyltropolone on Cell Line of Murine P388 Lymphocytic Leukemia *in Vitro* The cytotoxic effect of hinokitiol[7] and α-thujaplicin[7] on murine P388 lymphocytic leukemia has previously been investigated *in vitro*. However, no work has been done on the cytotoxic activity of β-dolabrin, γ-thujaplicin, and 4-acetyltropolone in the murine P388 lymphocytic leukemia cell line, so the same activity of three compounds on this tumor cell line was examined *in vitro*. These compounds showed strong cytotoxic activity against the murine P388 cell line. As shown in Fig. 1A (24 h after treatment), the growth of murine P388 cell line by test compounds, except for γ-thujaplicin, was suppressed in a concentration-dependent fashion. At 24 h after treatment, γ-thujaplicin, 4-acetyltropolone, and β-dolabrin at 0.63 μg/ml inhibited cell growth of murine P388 by 83, 51 and 39%, respectively. Among the three compounds, the inhibitory activity of γ-thujaplicin was higher than that of vincristine (growth inhibitory activity: 75%), used as a positive control. The inhibitory activity of γ-thujaplicin and 4-acetyltropolone was higher than that of hinokitiol (growth inhibitory activity: 43%). The cytotoxic effect at 48 h after treatment with the three compounds at 0.63 μg/ml are shown in Fig. 1B. γ-Thujaplicin, 4-acetyltropolone and β-dolabrin also inhibited cell growth of murine P388 by 85, 65 and 48%, respectively. Like 24 h after treatment, the inhibitory activity of γ-thujaplicin and 4-acetyltropolone was higher than that of hinokitiol (growth inhibitory activity: 52%). On the other hand, unlike 24 h after treatment, the inhibitory activity of γ-thujaplicin, the strongest growth inhibitor, was much lower than that of vincristine (growth inhibitory activity: 99%), used as a positive control. However, the growth of murine P388 lymphocytic leukemia cell line was completely suppressed by the three compounds at concentrations higher than 5.0 μg/ml.

In addition to these three compounds, α-thujaplicin has been recently reported to show strong cytotoxic activity against murine P388 lymphocytic leukemia cell line *in vitro*.[7] Five hinokitiol-related compounds have previously been found to show strong cytotoxic activity on cell lines of human stomach cancer KATO-III and Ehrlich's ascites carcinoma *in vitro*.[1,2,9] Of these five compounds, hinokitiol has already been reported to show strong cytotoxic effects in established tumor cell lines such as colon 26, RK, and MDCK cells and the minimal cytopathogenic concentration was reported to be around 10 μg/ml.[18] We previously reported that hinokitiol and tropolone showed strong cytotoxic effects *in vitro* on the growth of murine and human tumor cell lines including RL-♂, MH124, HL60, K526, and KATO-III, and the inhibitory concentration (IC_{50}) values for all cell lines were 0.3—0.6 μg/ml.[8] The cumulative evidence suggests that *in vitro* cytotoxicity is a common pharmacological activity of hinokitiol-related compounds. Because these five hinokitiol-related compounds was previously found to show low toxicity in mice,[1,2,9] The activity of these compounds against various human tumor cell lines *in vitro* and antitumor activity of the five compounds in mice should further be investigated, together with the mechanism of their cytotoxic effects.

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