Biological Activity of α-Thujaplicin, the Minor Component of *Thujopsis dolabrata* Sieb. *et* Zucc. var. *hondai* Makino

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α-Thujaplicin, a minor component of *Thujopsis dolabrata* Sieb. *et* Zucc. var. *hondai* Makino, which was synthesized, showed the antibacterial activity, phytogrowth-inhibitory effect, inhibition of carboxypeptidase A and cytotoxic effect. Antibacterial activity of α-thujaplicin on *Enterococcus faecalis* IFO-12965 [minimum inhibitory concentration (MIC): 1.56 μg/ml] was higher than that of gentamicin (MIC: 6.25 μg/ml) used as a positive control. Inhibitory activity of α-thujaplicin on carboxypeptidase A [50% inhibitory concentration (IC_{50}): 3.24×10^{-5} M] was higher than that of 1,10-phenanthroline used as a positive control. α-Thujaplicin showed germination inhibition toward the seed of *Echinochloa utilis* Ohwi et Yabuno even at the low concentration of 10 ppm and its growth inhibitory effect was stronger than that of sodium 2,4-dichlorophenoxyacetate used as a standard. α-Thujaplicin at 1.25 μg/ml inhibited cell growth of human stomach cancer KATO-III by 86%, and Ehrlich's ascites carcinoma by 87%, respectively. This compound even at the low concentration of 0.32 μg/ml also inhibited cell growth of the former by 66%, and the latter by 75%, respectively. The acute toxicity of α-thujaplicin [50% lethal dose (LD_{50}) value: 256 mg/kg] in mice was as strong as those of β-dolabrin (LD_{50} value: 232 mg/kg) and γ-thujaplicin (LD_{50} value: 277 mg/kg).

Key words α-thujaplicin; antibacterial activity; phytogrowth-inhibitory effect; carboxypeptidase A; inhibition; cytotoxic activity

It has already been reported that the hinokitiol[1]-related compounds hinokitiol (β-thujaplicin), β-dolabrin and γ-thujaplicin are among the constituents of *Thujopsis dolabrata* Sieb. *et* Zucc. var. *hondai* Makino (Chart 1).[2] Among them, hinokitiol, the major component of this plant has been found to show a broad spectrum of biological activities, including antimicrobial activity,[3—5] insecticidal effect,[6] phytogrowth-inhibitory activity,[7] cytotoxic effect on mammalian tumor cells[8] and inhibitory activity on catechol-*O*-methyltransferase,[9] and metalloproteases such as carboxypeptidase A,[10] collagenase[10] and thermolysin.[10] At the same time, we have reported that β-dolabrin and γ-thujaplicin, like hinokitiol,[7] showed a phytogrowth-inhibitory effect,[11] and inhibitory activities on metalloproteases.[10] We recently reported that hinokitiol, β-dolabrin and γ-thujaplicin had antifungal activities against wood-rotting fungi and insecticidal effects on *Tyrophagus putrescentiae* and *Coptotermes formosanus*.[12] Strong cytotoxic effects on two kinds of tumor cell lines were also found in β-dolabrin and γ-thujaplicin[13] as well as hinokitiol.[8] On the other hand, because α-thujaplicin is a minor component (0.2%) of the wood of *T. dolabrata* Sieb. *et* Zucc. var. *hondai* Makino, no work has yet been done on its above-mentioned biological activities.

In this work, to expand our knowledge of the biological activity of hinokitiol-related compounds, the synthesis of α-thujaplicin (Chart 1) and its antibacterial activity, phytogrowth-inhibitory effect and inhibitory activity on metalloproteases were investigated in comparison with those of other hinokitiol-related compounds. As basic research on the cytotoxic effect of hinokitiol-related compounds, this effect of α-thujaplicin on two kinds of tumor cell lines of human stomach cancer, KATO-III and Ehrlich's ascites carcinoma, was also examined.

MATERIALS AND METHODS

Synthesis of α-thujaplicin was performed according to the following method. Measurement: The ^{1}H-NMR spectra were measured with a JEOL GSX-270 spectrometer using tetramethylsilane as an internal standard. HR-MS spectrum was measured with JEOL JMS-HX-100.

Synthesis of 7,7-Dichloro-4-isopropylidene-bicyclo[3.2.0]-hept-2-en-6-ones To a stirred solution of freshly distilled 6,6-dimethylfilvene (70.0 g, 74.9%, 0.49 mol)[14] and dichloroacetylchloride (106.6 g, 0.72 mol) in *n*-hexane (500 ml) was added dropwise Et_3N (72.8 g, 0.72 mol) over a period of

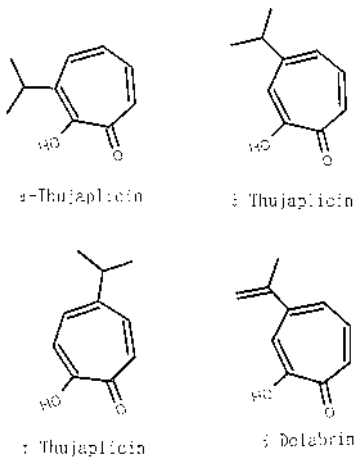

Chart 1

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1 h. The mixture was stirred for an additional 6 h and allowed to stand overnight. The reaction mixture was washed with H_2O (3×300 g). After removal of the solvent, the residue was distilled under vacuum to give 53.6 g of cycloadduct.

Synthesis of 3-Isopropenyl tropolone (α-Dolabrin)[15] To a solution of 50% NaOH aq (88 g) in AcOH (160 g) was added the cycloadduct (50.0 g, 0.23 mol). The mixture was refluxed 18.5 h. After cooling to room temperature, toluene (150 g) was added. The combined solution was washed with H_2O (50 g) and dried over sodium sulfate. After removal of the solvent, the residue was distilled under reduced pressure to give 18.7 g of α-dolabrin.

Synthesis of α-Thujaplicin A solution of α-dolabrin (18.7 g, 0.115 mol) in EtOH (100 g) was stirred for 11 h in the presence of 5% Pd–C (4.0 g) under hydrogen atmosphere. After filtration of the catalyst, the residue from evaporation of the filtrate was distilled under vacuum, and recrystallized from iso-hexane to give α-thujaplicin (2.0 g). ^{1}H-NMR (300 MHz, $CDCl_3$) 1.26 [d, J=4.3 Hz, 6 H, 3-CH$(CH_3)_2$], 3.74 [seven lines, J=4.3 Hz, 1H, 3-CH$(CH_3)_2$], 7.00—7.51 (m, 4 H, 4, 5, 6, 7-CH), 9.81 (br s, 1H, OH). HR-MS *m/z*: 164.0840 (M^+ Calcd for $C_{10}H_{12}O_2$: 164.0837).

GC-MS of acid oil obtained by distillation of the wood of *T. dolabrata* Sieb. *et* Zucc. var. *hondai* Makino was measured. Analytical conditions of GC-MS (Shimadzu GC-MS QP-5000) were as follows. Column: DB 1701, 15 m×I.D. 0.25 mm; oven temperature: 60 to 250 °C at 8 °C/min; injection temperature: 250 °C; detector temperature: 250 °C. Retention time (t_R) of α-thujaplicin, hinokitiol and β-dolabrin were 9.703, 11.197 and 11.377 min, respectively. The contents of the three compounds were 7.28, 63.59 and 26.3%, respectively.

Chemicals α-Thujaplicin, which was synthesized according to the above method, was used for various biological activities. Hinokitiol,[10] β-dolabrin[10] and γ-thujaplicin[10] isolated from *T. dolabrata* Sieb. *et* Zucc. var. *hondai* Makino were also used for these biological activities. The metalloproteases carboxypeptidase A, collagenase and thermolysin were purchased from Sigma Chemical Co. (U.S.A.). 1,10-Phenanthroline (a positive control for inhibition of metalloprotease) was purchased from Nakalai Tesque, Inc., Kyoto, Japan. Gentamicin (a positive control for the antibacterial activity test) was purchased from Schering-Plough Co., Ltd. Sodium 2,4-dichlorophenoxyacetate (2,4-D, a positive control for a phytogrowth-inhibitory activity test) was purchased from Tokyo Kasei Kogyo Co., Ltd.

Organisms Bacteria: Bacteria used for the antibacterial activity test were as follows: *Staphylococcus aureus* IFO-12732, methicillin-resistant *S. aureus* (MIC against methicillin: 1600 μg/ml), methicillin-resistant *S. aureus* (MIC against methicillin: 12.5 μg/ml), *S. epidermidis* IFO-12993, *Enterococcus faecalis* IFO-12965, *Escherichia coli* IFO-3806, *Proteus vulgaris* IFO-3988, *Serratia marcescens* IFO-12648, *Enterobacter cloacae* IFO-13535, Enterohaemorrhagic *E. coli*, *Klebsiella planticola* IFO-3317, and *Pseudomonas aeruginosa* IFO-13275. Plants: Plants used for the phytogrowth-inhibitory activity test were *Brassica campestris* L. and *Echinochloae utilis* Ohwi et Yabuno.

Cells Tumor cell lines used for the cytotoxic activity test were human stomach cancer KATO-III and Ehrlich's ascites carcinoma.

Animals Male Slc:ddY mice aged 4 weeks were purchased from Japan SLC (Shizuoka, Japan). The mice were fed an autoclaved commercial diet (NMF: Oriental Yeast, Tokyo, Japan) and given drinking water *ad libitum*. After prefeeding for 1 week, mice were used for the acute toxicity test.

Methods The antibacterial activity test was evaluated by the agar dilution method. The phytogrowth–inhibitory activity test was evaluated according to the method of Inamori *et al*.[7] Inhibition testing of the metalloproteases carboxypeptidase A, collagenase and thermolysin was performed according to the method reported previously.[16] The cytotoxic effect was also determined by the method reported previously.[13] The acute toxicity of α-thujaplicin was examined according to the following method. Mice were divided into groups of 5 animals each. α-Thujaplicin was suspended in 5% gum arabic saline solution and intraperitoneally administered. The condition of mice and mortality were followed for 8 d after administration. The LD_{50} was calculated according to the Van der Waerden method.[17]

RESULTS AND DISCUSSION

Antibacterial Activity of α-Thujaplicin The antibacterial activity of α-thujaplicin was investigated by the agar dilution method and compared with those of other hinokitiol-related compounds (Table 1). α-Thujaplicin, like the other compounds, showed clear antibacterial activity against all of the bacteria examined. The activity on *E. faecalis* IFO-12965 was particularly strong, its minimum inhibitory concentration (MIC) being 1.56 μg/ml. The antibacterial activity of α-thujaplicin on *E. faecalis* IFO-12965 was higher than those of other hinokitiol-related compounds and gentamicin used as a positive control. Although on *S. epidermidis* IFO-12993 the activity was lower than those of hinokitiol and β-dolabrin, its growth-inhibitory activity on this bacterium was higher than that of gentamicin used as a positive control. The antibacterial activities of hinokitiol, β-dolabrin and γ-thujaplicin in the present work were almost equal to those reported in the previous paper.[10] In addition to these three compounds,[10] the strong antibacterial effect of α-thujaplicin on *E. faecalis* IFO-12965 and *S. epidermidis* IFO-12993 seems to be a common biological activity of most hinokitiol-related compounds. Such a strong antibacterial activity of α-thujaplicin, hinokitiol, β-dolabrin and γ-thujaplicin on *E. faecalis* IFO-12965, which is a nosocomial infectious bacterium, is of considerable interest. Therefore, their antibacterial activities on vancomycin-resistant *Enterococci* (VRE) should be investigated. Considering that many application of hinokitiol to skin[18—22] have already been reported and α-thujaplicin, hinokitiol, β-dolabrin and γ-thujaplicin showed strong antibacterial activities against *S. epidermidis* IFO-12993, one of the residents of skin, basic dermatological research on hinokitiol-related compounds seems desirable.

Inhibitory Activity of α-Thujaplicin on Metalloproteases The inhibitory activity of α-thujaplicin on carboxypeptidase A was examined according to the method of Petra (Table 2).[16] The compound showed the inhibitory activity toward this enzyme, although this activity was slightly lower than those of other hinokitiol-related compounds[10]; it was higher, however, than that of 1,10-phenanthroline used

Table 1. Antibacterial Activity of α-Thujaplicin, Hinokitiol, γ-Thujaplicin and β-Dolabrin

Pathogenic bacteria	MIC (μg/ml)[a]				
	α-Thujaplicin	Hinokitiol	γ-Thujaplicin	β-Dolabrin	Gentamicin
Gram-positive bacteria					
Staphylococcus aureus IFO-12732	12.5	25	25	25	3.13
MRSA IID-1677	12.5	25	25	25	12.5
Methicillin-resistant *S. aureus* (MIC against methicillin: 1600 μg/ml)	12.5	12.5	25	25	50
Methicillin-resistant *S. aureus* (MIC against methicillin: 12.5 μg/ml)	12.5	12.5	12.5	12.5	12.5
Staphylococcus epidermidis IFO-12993	0.78	0.39	12.5	0.39	1.56
Enterococcus faecalis IFO-1296	1.56	6.25	3.13	3.13	6.25
Gram-negative bacteria					
Escherichia coli IFO-3806	12.5	12.5	12.5	12.5	1.56
Proteus vulgaris IFO-3988	12.5	12.5	12.5	12.5	1.56
Serratia marcescens IFO-12648	12.5	12.5	12.5	12.5	1.56
Enterobacter cloacae IFO-13535	12.5	12.5	12.5	12.5	1.56
Enterohaemorrhagic *Escherichia coli*	12.5	12.5	12.5	12.5	1.56
Klebsiella planticola IFO-3317	12.5	12.5	12.5	12.5	1.56
Pseudomonas aeruginosa IFO-13275	100	100	100	100	6.25

a) Minimum inhibitory concentration value was determined by the agar dilution method on a Muller–Hinton medium, which was incubated at 37 °C for 24 h.

Table 2. Inhibitory Activity of α-Thujaplicin on Carboxypeptidase A, Collagenase and Thermolysin

Compound	IC_{50} (M)[a]		
	Metalloprotease		
	Carboxypeptidase A[b]	Collagenase[c]	Thermolysin[d]
α-Thujaplicin	3.24×10^{-5}	$>1.00\times10^{-3}$	$>1.00\times10^{-3}$
Hinokitiol	2.76×10^{-6}	2.40×10^{-5}	6.10×10^{-5}
γ-Thujaplicin	1.95×10^{-5}	8.90×10^{-5}	$>1.00\times10^{-3}$
β-Dolabrin	1.06×10^{-5}	1.89×10^{-5}	6.85×10^{-5}
1,10-Phenanthroline	4.21×10^{-4}	1.83×10^{-4}	3.40×10^{-4}

a) 50% Inhibitory concentration. *b*) Carboxypeptidase A was isolated from bovine pancrease(Sigma Chemical Co.). Carboxypeptidase A solution was incubated at 37 °C and pH 7.5 for 15 min. Each value represents the mean of duplicate assays. *c*) Collagenase was isolated from *Clostridium histolyticum* and was incubated at 37 °C and pH 7.5 for 15 min. Each value represents the mean of duplicate assays. *d*) Thermolysin was isolated from *Bacillus thermoproteolucum*. Thermolysin solution was incubated at 37 °C and pH 7.5 for 15 min. Each value represents the mean of duplicate assays.

Table 3. Inhibitory Activity of α-Thujaplicin on Plant Growth

Compound	Concentration (ppm)	Growth (ratio)[a]	
		Plant	
		Brassica campestris L.	*Echinochloa utilis* Ohwi *et* Yabuno
α-Thujaplicin	50	0[b]	0
	30	0.01	0
	10	0.36	0
Hinokitiol	50	0	0
	30	0.06	0.03
	10	0.44	0.20
γ-Thujaplicin	50	0	0
	30	0.04	0.02
	10	0.42	0.19
β-Dolabrin	50	0	0
	30	0	0.05
	10	0.27	0.32
2,4-D[c]	50	0.05	0
	30	0.07	0
	10	0.09	0.02

a) Growth (length of radicle) in control experiments after 7 d was taken as 1.00. Experimental size: quality of light, 9000 m^2. cd. sr; 20 seeds/group, 2 groups; observation time, 7 d; temperature, 27 °C; illumination time, 12 h/d. *b*) No germination. *c*) Sodium 2,4-dichlorophenoxyacetate.

as a positive control. Unlike other hinokitiol-related compounds, it did not show the inhibitory activity toward collagenase or thermolysin.[10] However, it is not clear whether the difference in inhibitory activity between α-thujaplicin and other hinokitiol-related compounds is due to 1) the difference in enzyme specificity or 2) the difference in steric effect caused by the difference in position of the isopropyl group in their molecules. Considering that α-thujaplicin did not show the inhibitory activity toward collagenase or thermolysin, the cause of metalloprotease inhibition seems not always due to metal chelation between the carbonyl group at C-1 and the hydroxyl group at C-2 in molecules of hinokitiol-related compounds. However, the reason for this is not clear at present.

Phytogrowth-Inhibitory Activity of α-Thujaplicin

The phytogrowth-inhibitory activity of α-thujaplicin was investigated according to the method of Inamori *et al.*[7] and, as shown in Table 3, was seen to be strong. Its growth-inhibitory activity on *B. campestris* L. was as high as those of other hinokitiol-related compounds, while this activity on *E. utilis* Ohwi *et* Yabuno it was higher than that of these other compounds. It completely inhibited the germination of the seeds of *E. utilis* Ohwi *et* Yabuno even at the low concentration of 10 ppm. Considering that carboxypeptidase A has already been found to play an important role in the early stage of germination in the seeds of plants,[23] this activity of α-thujaplicin is supported from the above inhibition on carboxypeptidase A. These findings suggest that phytogrowth-inhibitory action is a common biological activity of hinokitiol-related compounds.

As the color of cotyledons of *B. campestris* L. treated with α-thujaplicin changed to yellowish white, its chlorophyll content was measured by the A.O.A.C. method[24]; the results are summarized in Table 4. Like other hinokitiol-related

Table 4. Chlorophyll Contents of *Brassica campestris* Treated with α-Thujaplicin and Other Hinokitiol-Related Compounds

Compound	Concentration (ppm)	Total chlorophyll (%)[a]	Chlorophyll a (%)	Chlorophyll b (%)
α-Thujaplicin	10	8.50	6.73	1.77
	20	0	0	0
	30	0	0	0
Hinokitiol	10	3.81	2.64	1.17
	20	2.71	1.91	0.80
	30	0.38	0.25	0.13
γ-Thujaplicin	10	5.38	4.25	1.13
	20	4.23	3.92	0.31
	30	0	0	0
β-Dolabrin	10	6.80	5.66	1.14
	20	5.28	4.26	1.02
	30	4.29	3.40	0.89
2,4-D[b]	10	5.86	4.51	1.35
	20	4.98	3.11	1.87
	30	3.18	2.87	0.31
Control		19.81	13.32	6.49

a) % (wet/wt). Analytical method, A.O.A.C. method; observation time, 7 d; temperature, 27 °C; illumination, 9000 lux. *b*) Sodium 2,4-dichlorophenoxyacetate.

compounds, the chlorophyll content in cotyledons of this plant treated with α-thujaplicin was decreased as compared with the control. However, this decrease by α-thujaplicin treatment was weaker than those of other hinokitiol-related compounds. Based on the findings that the chlorophyll content in the cotyledons of *B. campestris* L. treated with all of the hinokitiol-related compounds examined was greatly decreased compared with the control, at least part of the phytogrowth-inhibitory actions of these compounds also seems to cause this decrease.

Cytotoxic Activity of α-Thujaplicin on Cell Lines of Human Stomach Cancer KATO-III and Ehrlich's Ascites Carcinoma The cytotoxic activity of α-thujaplicin was examined with two kinds of cell lines of human stomach cancer KATO-III and Ehrlich's ascites carcinoma and compared with those of other hinokitiol-related compounds. α-Thujaplicin was dissolved in DMSO and was diluted in complete medium at 0.32—20 μg/ml. Final concentration (0.0012—0.08% in complete medium) of DMSO at this time did not influence the cell growth of the two cell lines (data not shown), while α-thujaplicin showed strong cytotoxic effect on both cell lines *in vitro*. As shown in Figs. 1 and 2, the growth of both tumor cell lines by α-thujaplicin was suppressed in concentration-dependent fashion. α-Thujaplicin at 1.25 μg/ml inhibited cell growth of human stomach cancer KATO-III by 86%, and Ehrlich's ascites carcinoma by 87%. This compound even at the low concentration of 0.32 μg/ml also inhibited cell growth of the former by 66%, and the latter by 75%. Cytotoxic activity of α-thujaplicin was almost equal to that of β-dolabrin.[13] Judging from the results of cytotoxic activity of α-thujaplicin in the present work and other hinokitiol-related compounds in the previous paper,[8,13] these effects seem to be common biological activities of hinokitiol-related compounds.

Toxicity Profile of Mice and Mortality Following the Administration of α-Thujaplicin The toxicity profile of mice and mortality following the intraperitoneal administration of α-thujaplicin was investigated, and the mortality is compared with other hinokitiol-related compounds in Table 5. When 320 mg/kg of α-thujaplicin was administered, convulsion developed at approximately 30 min after administration and all the mice died within 2 d.

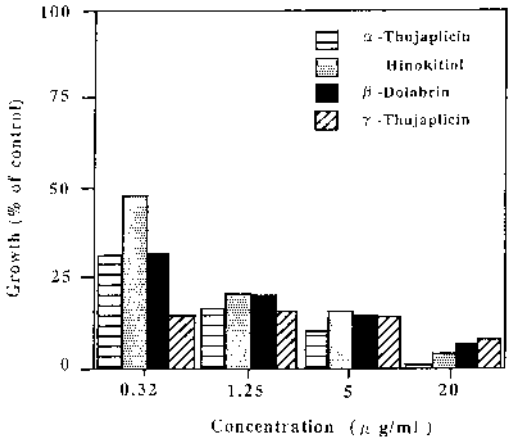

Fig. 1. Inhibitory Activity of α-Thujaplicin on Growth of Cell Lines of Human Stomach Cancer KATO-III in Comparison with Other Hinokitiol-Related Compounds

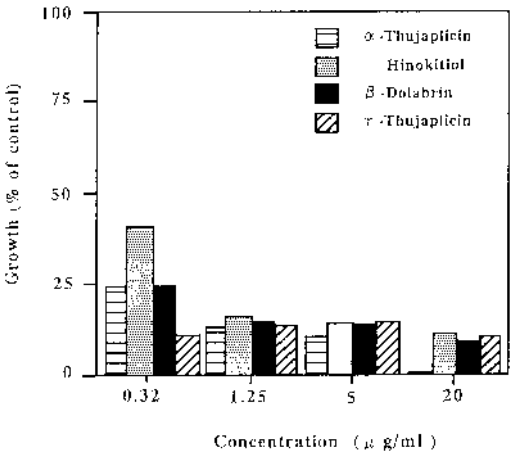

Fig. 2. Inhibitory Activity of α-Thujaplicin on Growth of Cell Lines of Ehrlich's Ascites Carcinoma in Comparison with Other Hinokitiol-Related Compounds

In each group given 300 mg/kg of α-thujaplicin, the reduction in voluntary movement began about 18 h after administration and crouching accompanied with eye-closing developed. The animals began to die approximately 2 d later.

Table 5. Toxicity Profile of Mice Injected with α-Thujaplicin, Hinokitiol, β-Dolabrin and γ-Thujaplicin

	Dose (mg/kg)	Mortality (%) day 1	2	3	4	5	6	7	8
α-Thujaplicin	320	0	100	100	100	100	100	100	100
	300	0	20	40	60	60	60	60	80
	280	0	0	20	60	60	60	60	60
	260	0	0	20	40	40	40	40	40
	240	0	40	40	40	40	40	40	40
	220	0	0	0	20	20	20	20	20
	200	0	0	0	0	0	0	0	0
									LD_{50}: 256 mg/kg
Hinokitiol	240	80	100	100	100	100	100	100	100
	225	60	80	80	80	80	80	80	80
	210	60	60	60	60	60	60	60	60
	195	60	60	60	60	80	60	60	60
	180	20	20	20	40	40	40	40	40
	165	0	0	20	20	20	20	20	20
	150	0	0	0	0	0	0	0	0
									LD_{50}: 191 mg/kg
β-Dolabrin	310	100	100	100	100	100	100	100	100
	295	80	80	80	80	80	80	80	80
	280	80	80	80	80	80	80	80	80
	265	60	60	60	60	60	60	60	60
	250	20	60	80	80	80	80	80	80
	235	20	40	40	40	40	40	40	40
	220	0	40	40	40	40	40	40	40
	205	0	20	20	40	40	40	40	40
	190	0	20	20	20	20	20	20	20
	175	0	0	0	0	0	0	0	0
									LD_{50}: 232 mg/kg
γ-Thujaplicin	325	100	100	100	100	100	100	100	100
	310	80	80	80	80	80	80	80	80
	295	60	60	60	60	60	60	60	60
	280	60	60	60	60	60	60	60	60
	265	40	40	40	40	40	40	40	40
	250	20	20	20	20	20	20	20	20
	235	0	0	0	0	0	0	0	0
									LD_{50}: 277 mg/kg

Animals: ddY strain mice (male) 26—29 g (body weight). Route: intraperitoneal injection. Calculation of LD_{50}: Van der Waerden method.

Comparison of the 50% lethal dose (LD_{50}) value of α-thujaplicin to that of other hinokitiol-related compounds in mice is also summarized in Table 5. The LD_{50} value of α-thujaplicin was found to be 256 mg/kg. Its acute toxicity was lower than that of hinokitiol, but was almost equal to those of β-dolabrin and γ-thujaplicin. Though the acute toxicity of hinokitiol, β-dolabrin and γ-thujaplicin was reported earlier,[13] this toxicity of α-thujaplicin is reported for the first time here.

Considering that similar activities were found not only in α-thujaplicin, but also in other hinokitiol-related compounds,[7,8,10—13] other biological activities of these compounds should be further investigated.

Studies on the synthesis of 4-acetyltropolone, one of the minor components of *T. dolabrata* Sieb. *et* Zucc. var. *hondai* Makino and its biological activities are in progress.

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