Biological Activity of 4-Acetyltropolone, the Minor Component of *Thujopsis dolabrata* Sieb. et Zucc. *hondai* Mak.

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4-Acetyltropolone, a minor component of *Thujopsis dolabrata* Sieb. et Zucc. *hondai* Makino, showed antimicrobial activity against various microorganisms including wood-rotting fungi, a phytogrowth-inhibitory effect with chlorophyll biosynthesis inhibition, cytotoxic effect and inhibitory activity on metalloproteases. This compound had strong antifungal activity on *Daedalea dickinsii* IFO-4979 [minimum inhibitory concentration (MIC): 0.2 μg/ml] and *Coriolus versicolor* IFO-4940 (MIC: 0.39 μg/ml). Its cytotoxic effect at 20.0 μg/ml on human stomach cancer KATO-III and Ehrich's ascites carcinoma was stronger than those of podophyllotoxin, vincristine and vinblastine, the anticancer agents isolated from higher plants and used clinically. This compound also had potent antibacterial activity against *Staphylococcus epidermidis* IFO-12993, its MIC being 1.56 μg/ml. However, other biological activities of 4-acetyltropolone were lower than those of hinokitiol which is the main component of this plant, suggesting that the contribution of the acetyl group at C-4 to biological activity is smaller than that of the isopropyl group at that position. The acute toxicity of 4-acetyltropolone (LD_{50}: 335.2 mg/kg) to mice was much lower than that of hinokitiol (LD_{50}: 191 mg/kg).

Key words 4-acetyltropolone; antimicrobial activity; metalloprotease inhibition; phytogrowth-inhibitory effect; cytotoxic activity; hinokitiol

4-Acetyltropolone, like hinokitiol (β-thujaplicin), β-dolabrin, γ-thujaplicin and α-thujaplicin, is known to be a component of *Thujopsis dolabrata* Sieb. et Zucc. *hondai* Makino (Chart 1).[1] This compound is nothing more than a compound with the acetyl group, instead of the isopropyl group, at the 4-position of hinokitiol. Hinokitiol, the major component (1.0%) of this plant, has already been found to show the antimicrobial activity,[2—4] inhibitory activity on metalloproteases[4] or catechol-*O*-methyltransferase,[5] insecticidal activity,[6,7] cytotoxic effect on mammalian tumor cells[8,9] and phytogrowth-inhibitory activity.[10] We previously reported that β-dolabrin and γ-thujaplicin, like hinokitiol, had a phytogrowth-inhibitory effect,[11] inhibited metalloproteases,[4] and had an antimicrobial activity[4] and a cytotoxic effect on mammalian tumor cell lines.[12] It has been reported that three compounds showed insecticidal activity against *Tyrophagus putrescentiae* and *Coptotermes formosanus* and antifungal activity on wood-rotting fungi.[13] α-Thujaplicin, another minor component of the plant, exhibited the antibacterial activity, phytogrowth-inhibitory effect, metalloprotease inhibition and cytotoxic effect on mammalian tumor cell lines.[14] However, as 4-acetyltropolone is a minor component, no work has yet been done on the above-mentioned biological activities of its other hinokitiol-related compounds, While γ-thujaplicin and α-thujaplicin, other minor components understood, have previously been reported to show potent and broad biological activities.studies on these biological activities of 4-acetyltropolone which was also minor component are needed.

In this work, to determine the range of biological activities of hinokitiol-related compounds, the synthesis of 4-acetyltropolone and its antimicrobial activity against various microorganisms including the wood-rotting fungi, phytogrowth-inhibitory effect, inhibitory activity on metalloproteases and cytotoxic effect on two kinds of mammalian tumor cell lines were compared with that of hinokitiol. Its acute toxicity following intraperitoneal administration was investigated as a basic study on its biological activity in mice.

MATERIALS AND METHODS

4-Acetyltropolone was synthesized according to the following methods. The ^{1}H-NMR spectra were measured with a JEOL GSX-270 spectrometer using tetramethylsilane as an internal standard. High resolution (HR)-MS spectrum was measured with JEOL JMS-HX-100.

Synthesis of 4-Acetyltropolone A solution of 50 g of acid oil obtained by distillation of the wood of *T. dolabrata.* var. *hondai* dissolved in 175 g of 85% formic acid, to which was added 40 g of 35% hydrogen peroxide, was stirred at 40 °C for 2 h and formic acid was evaporated under reduced pressure. Four hundred sixty grams of 4.8% sodium hydroxide was then added and the mixture was stirred at 40 °C for another 3 h. This was acidified with 10% hydrochloric acid and extracted with toluene. The aqueous layer was cooled with ice, an aqueous solution of 14.6 g periodic acid dihydrate was added dropwise with stirring and 3.98 g of yellow

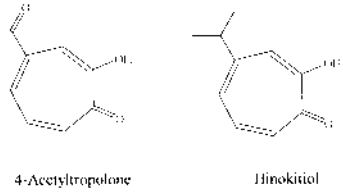

Chart 1

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powder was obtained from the aqueous layer. The powders were recrystallized from ethanol to give 2.0 g of 4-acetyltropolone as yellow plates. ^{1}H-NMR (300 MHz, $CDCl_3$: 2.67 (s, 3H, 4-$COCH_3$), 7.44—7.90 (m, 4H, 3,5,6,7-CH). HR-MS *m*/*z*: 164.0461 (M^+ Calcd for $C_9H_8O_3$: 164.0473).

Chemicals 4-Acetyltropolone, synthesized according to the above mentioned method, was used for various biological activity tests. Hinokitiol[4] isolated from *T. dolabrata.* var. *hondai* was also used for these activity tests. 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 metalloproteases) was purchased from Nakalai Tesque, Inc. Kyoto, Japan. Gentamicin (a positive control for the antibacterial activity test) was purchased from Schering-Plough Co., Ltd. Amphotericin B (a positive control for the antifungal activity test) was purchased from Bristol-Myers Squibb Co., Ltd. Sodium 2,4-dichlorophenoxyacetate (2,4-D, a positive control for the phytogrowth-inhibitory activity test) was purchased from Tokyo Kasei Kogyo Co., Ltd. Podophyllotoxin, vincristine and vinblastine (a positive control for the cytotoxic activity test) were purchased from Sigma Chemical Co.

Organisms Bacteria: Bacteria used for the antibacterial activity test were as follows: *Staphylococcus aureus* IFO-12732, methicillin-resistant *S. aureus* (MRSA, minimum inhibitory concentration (MIC) against methicillin: 1600 μg/ml), methicillin-resistant *S. aureus* (MIC against 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. Fungi: Fungi used for the antifungal activity test on wood-rotting fungi were: *Daedalea dickinsii* IFO-4979, *Pycnoporus coccineus* IFO-4924, *Schizophyllum commune* IFO-4928, *Coriolus versicolor* IFO-4940, and *Lenzites betulina* IFO-4964. Plants: Plants used for the phytogrowth-inhibitory activity test were: *Brassica campestris* L. subsp. *rapa* Hook. f. et Anders and *Echinochloae utilis* Ohwi et Yabuno.

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

Methods The agar dilution method was used for the antibacterial and antifungal activity tests. Inhibition testing of the metalloproteases carboxypeptidase A, collagenase and thermolysin was performed according to the method reported previously.[4] The phytogrowth-inhibitory activity test was administered according to the method of Inamori *et al.*[10] The cytotoxic effect was also determined by the method reported previously.[11] The acute toxicity of 4-acetyltropolone was examined using the following method. Mice were divided into groups of 5 animals each. The compound was suspended in 5% gum arabic saline solution and intraperitoneally administered. The LD_{50} was calculated by the Van der Waerden method.[15]

RESULTS AND DISCUSSION

Antifungal Activity of 4-Acetyltropolone on Wood-rotting Fungi Antifungal activity of 4-acetyltropolone on the wood-rotting fungi was examined by the agar dilution method and compared with that of hinokitiol. As shown in Table 1, 4-acetyltropolone showed the antifungal activity toward all of these fungi examined. The antifungal activity of this compound on *D. dickinsii* IFO-4979 was particularly strong, its MIC being 0.2 μg/ml; this was as high as that of amphotericin B used as a positive control. The antifungal activity of 4-acetyltropolone on this fungus was almost equal to that of hinokitiol. However, unlike hinokitiol, this compound exhibited potent antifungal activity against *Coriolus versicolor* IFO-4940, its MIC being 0.39 μg/ml. In this respect, strong antifungal activity of the compound on *C. versicolor* is of considerable interest. The antifungal activity of hinokitiol in the present work was almost equal to that of this compound in previous papers.[12] The antifungal activity of 4-acetyltropolone on wood-rotting fungi except for *D. dickinsii* and *C. versicolor* was lower than that of hinokitiol. Considering that, in addition to 4-acetyltropolone, hinokitiol, γ-thujaplicin and β-dolabrin also showed antifungal activity on the wood-rotting fungi, this seems to be a common biological activity of hinokitiol-related compounds.[13] Such a strong antifungal activity of these four hinokitiol-related compounds on wood rotting fungi seems to be closely related to our paper[13] that Konjiki-do, the well-known national treasure, known in English as Chusonji-Temple, Iwate-ken, Japan, which was built of *T. dolabrata.* var. *hondai* remained free of harm from these fungi for a long eight hundred year period.

Antibacterial Activity of 4-Acetyltropolone As reported previously, hinokitiol,[4] β-dolabrin,[4] γ-thujaplicin[4] and α-thujaplicin[14] have particularly been reported to show potent antibacterial activity on *Staphylococcus* sp., including MRSA and *Enterococcus faecalis* IFO-12993. Therefore, our attention has also been focused on the antibacterial activity of 4-acetyltropolone on these bacteria. This activity was investigated using the agar dilution method and compared with that of hinokitiol. The results are summarized in Table 2. 4-

Table 1. Antifungal Activity of 4-Acetyltropolone on Wood-Rotting Fungi

Fungus	Antifungal activity MIC (μg/ml)[a]		
	4-Acetyltropolone	Hinokitiol	Amphotericin B
Coriolus versicolor IFO-4940	0.4	12.5	0.2
Daedalea dickinsii IFO-4979	0.2	0.2	0.2
Lenzites betulina IFO-4964	6.3	12.5	0.2
Pycnoporus coccineus IFO-4924	50.0	12.5	0.2
Schizophyllum commune IFO-4928	50.0	12.5	0.2

a) Minimum inhibitory concentration. Assay: agar dilution method. Medium: The mixture of potato dextrose agar "Nissui" (A) and *Staphylococcus* medium "Eiken" (B) was used. Ratio of A and B was adjusted to 4 : 1. Culture condition: 25 °C for 7 d.

Table 2. Antibacterial Activity of 4-Acetyltropolone

Pathogenic bacteria	MIC (μg/ml)[a]		
	4-Acetyltropolone	Hinokitiol	Gentamicin
Staphylococcus epidermidis IFO-12993	1.56	0.2	1.56
Staphylococcus aureus IFO-12732	25.00	12.5	1.56
Methicillin-resistant *S. aureus* (MIC against methicillin: 1600 μg/ml)	6.25	6.25	100.0
Methicillin-resistant *S. aureus* (MIC against methicillin: 12.5 μg/ml)	6.25	6.25	12.5

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

Acetyltropolone showed the antibacterial activity against the *Staphylococcus* sp. examined, with that on *S. epidermidis* IFO-12993 especially strong, its MIC being 1.56 μg/ml. This antibacterial activity, however, was lower than that of hinokitiol, while it was as high as that of gentamicin used as a standard.

Judging from papers that 4-aetyltropolone, hinokitiol,[4] β-dolabrin[4] and α-thujaplicin[14] showed rather strong antibacterial activities against *S. epidermidis* IFO-12993, a nosocomial infectious bacterium, the activity of hinokitiol-related compounds on this bacterium is of considerable interest. Hinokitiol has already been reported to be a useful agent for skin.[16—18] Since *S. epidermidis* IFO-12993 is well known to be present on skin, the application of other hinokitiol-related compounds to the skin should also be investigated. 4-Acetyltropolone also exhibited antibacterial activity against MRSA with activity equal to that of hinokitiol. This activity on MRSA was also found in other hinokitiol-related compounds.[4,14] There are few effective antibacterial agents on MRSA currently known, making the synthesis of various derivatives of hinokitiol-related compounds and their antibacterial activities on these bacteria of interest.

In contrast, contrary to our expectation and unlike other hinokitiol-related compounds, the antibacterial activity of 4-acetyltropolone on *E. faecalis* IFO-1296 was low (MIC: >50 μg/ml). Nor did it also have any antibacterial activity on any of the gram-negative bacteria examined even at high concentration of 50 μg/ml (data not shown).

The Inhibitory Activity of 4-Acetyltropolone on Metalloproteases The inhibitory activity of 4-acetyltropolone on the metalloproteases carboxypeptidase A, collagenase and thermolysin was investigated using the method previously reported[4] and is shown in Table 3. The inhibitory activity was as high as that of 1,10-phenanthroline used as a positive control, and was slightly lower but almost equal to that of hinokitiol.[4] Hinokitiol,[4] γ-thujaplicin,[4] β-dolabrin[4] and α-thujaplicin[14] have previously been reported to show the same activity by the authors.

These metalloproteases have already been reported to be found in mast cells,[19] rheumatoid arthritis[20] and corneal ulcers[20] during the inflammatory phase. Since metalloprotease inhibition is found not only in 4-acetyltropolone, but also in hinokitiol, γ-thujaplicin, β-dolabrin[4] and α-thujaplicin,[14] knowledge of the antiinflammatory effect of hinokitiol-related compounds would be valuable.

The Phytogrowth-Inhibitory Activity of 4-Acetyltropolone The phytogrowth-inhibitory activity of 4-acetyltropolone was examined as reported previously[10] and compared with that of hinokitiol. The results are summarized in Table 4. 4-Acetyltropolone showed weak inhibitory activity against the seeds of *Brassica campestris* L. subsp. *rapa* Hook. f. et Anders, while its growth-inhibitory effect on *E. utilis* Ohwi et Yabuno was strong and it inhibited germination of the seeds of this plant even at the low concentration of 30 ppm. However, the phytogrowth-inhibitory activity of 4-acetyltropolone was much lower than that of hinokitiol and sodium 2,4-dichlorophenoxyacetate, which was used as a positive control. The growth effect of hinokitiol in the present work was as strong as that reported earlier.[10] Other hinokitiol-related compounds have also been reported to ex-

Table 3. Inhibitory Activity of 4-Acetyltropolone on Carboxypeptidase A, Collagenase and Thermolysin

Compound	IC_{50} (M)[a]		
	Carboxypeptidase A[b]	Collagenase[c]	Thermolysin[d]
4-Acetyltropolone	3.50×10^{-4}	8.50×10^{-5}	2.10×10^{-4}
Hinokitiol	2.75×10^{-6}	2.40×10^{-5}	6.10×10^{-5}
1,10-Phenanthroline	4.22×10^{-4}	1.85×10^{-4}	3.60×10^{-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.* Collagenase 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 4. Inhibitory Activity of 4-Acetyltropolone on Plant Growth

Compound	Concentration (ppm)	Growth (ratio)[a]	
		Plant	
		Brassica campestris L.	*Echinochloa utilis* Ohwi et Yabuno
4-Acetyltropolone	50	0.34	0
	30	0.50	0
	10	0.84	0.53
Hinokitiol	50	0	0
	30	0.06	0.03
	10	0.44	0.20
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.

Table 5. Chlorophyll Contents of *Brassica campestris* Treated with 4-Acetyltropolone

Compound	Concentration (ppm)	Total chlorophyll (%)[b)]	Chlorophyll a (%)	Chlorophyll b (%)
4-Acetyltropolone	10	13.20	9.20	4.10
	20	10.48	6.80	3.68
	30	8.36	5.20	3.16
Hinokitiol	10	3.70	2.67	1.03
	20	2.80	1.91	0.89
	30	0.35	0.27	0.08
2,4-D[a)]	10	5.70	4.51	1.19
	20	5.53	4.47	1.06
	30	3.00	2.07	0.93
Control[c)]		18.36	12.41	5.95

a) Sodium 2,4-dichlorophenoxyacetate. *b*) Total chlorophyll: % (wet/wt). Analytical method, A.O.A.C. method; observation time, 7 d; temperature, 27 °C; quality of ligtht, 9000 m^2. cd. sr. *c*) 1% DMSO alone.[10)]

hibit the same inhibitory activity.[10,11,14)]

The surface of *B. campestris* leaves treated with 4-acetyltropolone turned yellow-white in every treated group, and the amount of chlorophyll 7 d after germination was determined in leaves treated with 10, 20 and 30 ppm of this compound. As shown in Table 5, the amounts decreased more in the leaves treated with 4-acetyltropolone than in the control group. However, the decrease in chlorophyll concentration in cotyledons treated with this compound was lower than in those treated with hinokitiol and 2,4-D, which was used as a positive control, suggesting weaker inhibition of chlorophyll biosynthesis by the former. The decrease in *B. campestris* cotyledons treated with hinokitiol in the present work was similar to that reported previously.[10)]

The reduction of chlorophyll concentration in cotyledons treated with other hinokitiol-related compounds[10,11,14)] has already been reported.

The Cytotoxic Activity of 4-Acetyltropolone The cytotoxic activity of 4-acetyltropolone was examined with two cell lines of human stomach cancer KATO-III and Ehrlich's ascites carcinom. Podophyllotoxin, vinblastine and vincristine, antitumor agents isolated from higher plants were used as comparative agents. 4-Acetyltropolone was dissolved in dimethyl sulfoxide (DMSO) and 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).

As shown in Figs. 1 and 2, 4-acetyltropolone showed weak cytotoxic activity on the two of cell lines. Unlike hinokitiol, the growth-inhibitory activity was not found at the low concentrations of 0.32 and 1.25 μg/ml, but 4-acetyltropolone at 5 μg/ml inhibited cell growth of the cancer by 25%, and Ehrlich's ascites by 10%. This compound at 20 μg/ml also inhibited cell growth of the former by 88%, and the latter by 75%. The cytotoxic activity of 4-acetyltropolone at every concentrations was much lower than that of hinokitiol. However, the cytotoxic activity of 4-acetyltropolone at 20 μg/ml was much higher than those of podophyllotoxin, vincrystine and vinblastine which were used as positive controls. The cytotoxic activity of other hinokitiol-related compounds has been reported by the authors.[8,12,14)]

Toxicity Profile of Mice and Mortality Following the Administration of 4-Acetyltropolone The toxicity profiles of mice and mortality following the intraperitoneal administration of 4-acetyltropolone showed that control mice gained in body weight from 27.4±0.57 g (mean±S.E.) to 33.1±0.86 g by administration of 5% gum arabic saline solution at 9 d after administration. The results are summarized in Table 6. Administration of 400 mg/kg, convulsive attack developed at approximately 30 min later, and 4 out of 5 mice died within 3 h. In a group given 380 mg/kg of this compound, reduction in voluntary movement began about 1 h after administration and crouching together with eye-closing; the ani-

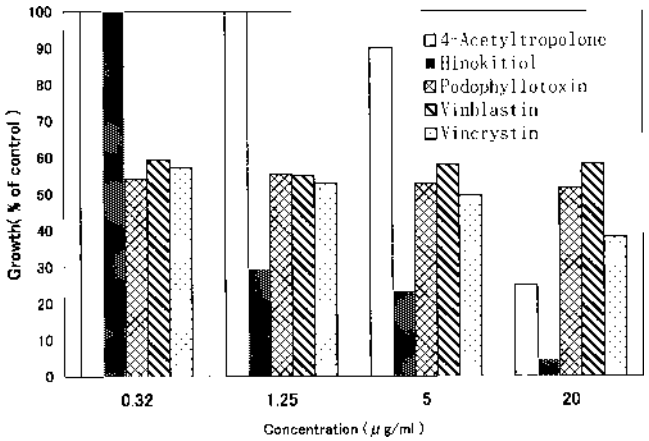

Fig. 1. Inhibitory Activity of 4-Acetyltropolone on Growth of Cell Lines of Ehrlich's Ascites Carcinoma in Comparison with Several Compounds

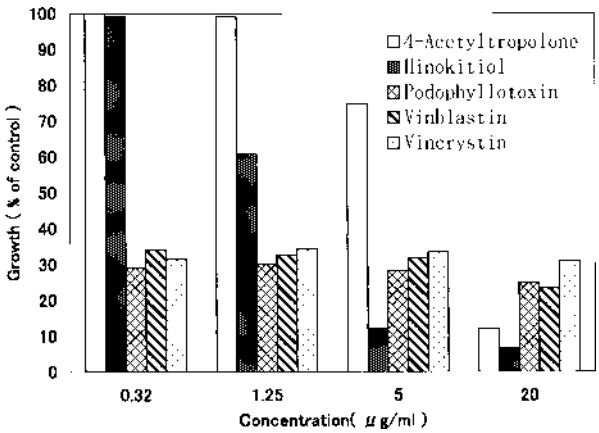

Fig. 2. Inhibitory Activity of 4-Acetyltropolone on Growth of Cell Lines of Human Stomach Cancer KATO-III in Comparison with Several Compounds

Table 6. Toxicity Profile of Mice Injected with 4-Acetyltropolone

Dose (mg/kg)	Mortality (%)								
	day								
	1	2	3	4	5	6	7	8	9
400	80	100	100	100	100	100	100	100	100
380	60	80	80	80	80	80	80	80	80
360	20	40	60	60	60	60	60	60	60
340	20	20	40	40	40	40	40	40	40
320	20	20	40	40	40	40	40	40	40
300	20	20	20	20	20	20	20	20	20
280	0	20	20	20	20	20	20	20	20
260	0	0	0	0	0	0	0	0	0

Conditions were as described in this paper. LD_{50}=335.2 mg/kg.

Table 7. Body Weight of Mice Injected with 4-Acetyltropolone

Dose (mg/kg)	*n*	Body weight (g)
400	0	—
380	1	30
360	2	31±1.41
340	3	31.7±0.58
320	3	32.7±0.58
300	4	32.3±0.96
280	4	31.5±1.30
260	5	33.2±1.92

n: number of surviving mice 9 d after body weight are the mean±S.E. Other conditions are as described in this paper.

mals began to die approximately 2 h later. Although the body weight gain of surviving mice administered each concentration of this compound was somewhat lower than control mice, there was no significant difference in body weight change between them (Table 7). The acute toxicity of 4-acetyltropolone (335.2 mg/kg) to mice was weaker than those of hinokitiol (191 mg/kg),[14] β-dolabrin (232 mg/kg),[14] γ-thujaplicin (277 mg/kg)[14] and α-thujaplicin (256 mg/kg).[14]

The lower biological activity of 4-acetyltropolone than of hinokitiol suggested that at least the isopropyl group was more contributory to enhancement of the activity of the latter than the acetyl group of the former. However, it is not clear whether the difference in the two biological activities between 4-acetyltropolone and hinokitiol is due to (1) the difference of their molecular conformations caused by introduction of carbonyl group or (2) the difference of their physicochemical characteristics resulting from difference of their functional groups. Considering that above-stated activities were found not only in 4-acetyltropolone, but also in hinokitiol,[2—4,6—10,12,13] β-dolabrin,[4,9,11—13] γ-thujaplicin[4,9,11—13] and α-thujaplicin[14] these effects seem to be common biological activities of hinokitiol-related compounds.

The biological activities of hinokitiol-acetate lack by acetylation of the hydroxyl group at C-2 in molecules (data not shown), indicating that cause of the activities of hinokitiol-related compounds is due to metal chelation between the carbonyl group at C-1 and the hydroxyl group at C-2 in molecules of the compounds.

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