

# Antifeedants against *Acusta despesta* from the Japanese Cedar, *Cryptomeria japonica*

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Antifeedant, *Cryptomeria japonica*, *Acusta despesta*

During our studies on the components in Japanese cedar, *Cryptomeria japonica*, we found that the crude methanol extract of *C. japonica* showed intense antifeeding activity against one snail species, *Acusta despesta*, which is well-known as a pest of many vegetables and crops.

The active components in the extract were separated into the hexane and ethyl acetate soluble fractions. From the active ethyl acetate soluble fraction, two norlignans, sequirin-C and agatharesinol, were isolated and identified as the active compounds. Both compounds inhibited feeding behavior of *A. despesta* at 30 µg/cm<sup>2</sup> and 40 µg/cm<sup>2</sup> concentrations, respectively, when applied by an eggplant leaf or filter paper containing 20 µl of 5% sucrose solution.

## Introduction

*Acusta despesta*, a snail species, is well-known as a pest to many vegetables and crops. Its host range is very wide and it can attack more than 149 species plants in over 28 families (Kegazawa, 1985). Thus they are typical phytophagous pests. Though a meta-aldehyde is used for pest control against *A. despesta*, the development of a new pest control method against this pest species is necessary because the efficacy of this pesticide is dependent on weather (Takeda, 1985).

*Cryptomeria japonica* is a popular indigenous tree of Japan. The chemical components of this tree have been studied intensively. These components show various bioactivities such as termiticidal (Sogabe *et al.*, 2000), antimite and plant growth regulation activities (Yatagai *et al.*, 1991; Morita *et al.*, 1991; Morita and Yatagai, 1994). In addition to these, we recently found that the crude methanol extract of *C. japonica* showed intense antifeeding activity against *A. despesta*. In this paper we report the isolation and identification of antifeedants against *A. despesta* from *C. japonica*.

**Abbreviations:** fr, fraction; ODS, octa-decanoyl-silicon; UV, ultraviolet radiation; Rt, retention time; GCMS, gas chromatography mass spectrometry; LCMS, liquid chromatography mass spectrometry.

## Materials and Methods

### Instruments

GCMS and LCMS data of compounds **1** and **2** were measured with a JEOL MS600 mass spectrometer. GC analyses were done with a Shimadzu GC-14A fitted with a fused silica column (HR1701, 0.25 µm thickness, 25 m × 0.2 mm i.d.) and programmed from 200 °C (2-min hold) to 250 °C at a rate of 10 °C/min. <sup>1</sup>H- and <sup>13</sup>C-NMR data for compounds **1** and **2** were measured with a JEOL Lambda 400 spectrometer (400 MHz), using TMS as the internal standard. Letters br, s, d, t, q, and m represent broad singlet, doublet, triplet, quartet, and multiplet, respectively, and coupling constants given in Hz.

### Snail

Stock colonies of *A. despesta* were reared on lettuce at 25–28 °C, relative humidity of 60–70% and with a 16:8 h (L:D) illumination.

### Plant

The cedar wood used was from the Reihoku area of Kochi Prefecture, Japan.



### Bioassay

Two polypropylene ice cream cups (30 mm × 60 mm i.d.) were piled up and a moistened paper filter was laid in the base of the lower cup for humidity maintenance and 50~60 pinholes for the ventilation were opened in the base of the upper cup. Two snails with over 1.5 cm shell diameter were introduced into the upper cup and allowed to feed on a plant leaf or paper disc (Toyo Advantec, #2, 1 cm<sup>2</sup>) containing the plant extract with 20 µl of a 5% sucrose solution for 12 hrs under conditions of 28 °C in the dark. The percentage of the filter paper areas which the snails could not feed was computed as the antifeedant rate. Five cups were prepared for each bioassay.

### Isolation of compounds **1** and **2**

Fresh *C. japonica* wood (300 g) was cut into pieces (5 cm long, 1 cm thickness) and extracted with 100% methanol (2 l) for 3 days in darkness at room temperature. This procedure was conducted twice. After evaporating the solvent under reduced pressure at 40 °C, a methanol extract (6.4 g) was obtained. This residue of the methanol extract was dissolved in 200 ml of water, and the solution was then partitioned between n-hexane (250 ml × 4) and water and subsequently, ethyl acetate (250 ml × 4) and water. Fractions soluble in n-hexane (2.7 g), ethyl acetate (2.3 g) and water (1.2 g) were obtained respectively. The ethyl acetate-soluble fr was chromatographed on an ODS resin (235 mm × 17 mm i. d., Chromatorex DM1020T, Fuji Silysia Chemical) eluted with increasing concentrations of methanol in water to obtain 20% methanol in water (122.9 mg), 50% methanol in water (239.6 mg), and 100% methanol eluates (238.6 mg). The 50% methanol in water eluate was separated into four frs, Fraction A (Rt = 0–10.0 min), Fraction B (Rt = 10.0–11.0 min) Fraction C (Rt = 11.0–13.0 min), and Fraction D (Rt = 13.0–20.0 min) by using reversed-phase HPLC (column: CAPCELLPAK C18 AG120Å, 250 mm × 10 mm i.d.) eluting with 20% acetonitrile in water at a flow rate of 4 ml/min with a refractive index detector. From Fraction A, compound **1** was isolated at Rt = 6.95 min and compound **2** was isolated at Rt = 10.04 min from Fraction B. The yield of compounds **1** and **2** was 110 µg/g weight fresh

wood and 180 µg/g weight fresh wood, respectively.

### Ozonolysis of compound **1**

Compound **1** (6.5 mg) was dissolved in 5 ml of dry methanol after displacing the air of the 25 ml-flask by N<sub>2</sub> gas. Ozone was introduced into the flask during stirring for 10 min at -78 °C. Then, dimethylsulfoxide was added to stop the reaction. The product was extracted under acid condition by diethyl ether after methanol was removed, and then GC and GCMS analyses were carried out.

### Compound **1** (Sequirin-C)

[α]<sub>D</sub><sup>25</sup> -90.0°(c 1.0, methanol). LCMS (FRIT-FAB<sup>+</sup>, flow injection) *m/z* (%): 303(M<sup>+</sup>+H, 19.0), 285(36.6), 267(20.2), 241(100), 209(46.5), 123(81.0), 107(92.5). <sup>1</sup>H-NMR δ<sub>H</sub> (acetone-*d*<sub>6</sub>): 7.22(2H, d, *J* = 8.8, H-2', 6'), 6.81(1H, d, *J* = 1.9, H-2''), 6.74(1H, d, *J* = 8.3, H-3''), 6.69(2H, d, *J* = 8.8, H-3', 5'), 6.65(1H, d, *J* = 8.3 and 1.9, H-6''), 6.35(1H, d, *J* = 15.6, H-1), 6.23(1H, d, *J* = 8.8 and 15.6, H-2), 3.92(1H, ddd, *J* = 3.6, 6.8 and 7.8, H-4), 3.63(1H, dd, *J* = 3.6 and 11.2, H-5a), 3.47(1H, dd, *J* = 6.8 and 11.2, H-5b), 3.38(1H, dd, *J* = 7.8 and 8.8, H-3). <sup>13</sup>C-NMR δ<sub>C</sub> (acetone-*d*<sub>6</sub>): 157.8(s, C-4'), 146.1(s, C-5''), 144.8(s, C-4''), 134.6(s, C-1''), 131.6(d, C-1), 130.7(d, C-2', 6'), 129.1(d, C-2), 128.4(s, C-1'), 121.1(d, C-2''), 116.9(d, C-3', 5'), 116.3(d, C-3'', 6''), 76.3(d, C-4), 65.8(t, C-5), 53.0(d, C-3).

### Compound **2** (Agatharesinol)

[α]<sub>D</sub><sup>25</sup> -37.6°(c 1.7, acetone). LCMS (FRIT-FAB<sup>+</sup>, flow injection) *m/z* (%): 287((M<sup>+</sup>+H, 2.1), 277(6.8), 225(18.3), 185(2G+H. 100)167(10.1), 149(15), 131(11). <sup>1</sup>H-NMR δ<sub>H</sub> (acetone-*d*<sub>6</sub>): 7.21(2H, d, *J* = 8.8, H-2', 6'), 7.19(2H, δ *J* = 8.8, H-2'', 6''), 6.73(4H, d, *J* = 8.8, H-3', 5' and H-3'', 5''), 6.34(1H, br. s, H-2), 6.33(1H, br. s, H-1), 3.92(1H, ddd, *J* = 7.0, 6.8 and 4.0, H-4), 3.57(1H, dd, *J* = 10.8 and 4.0, H-5b), 3.47(1H, ddd, *J* = 8.0, 7.0 and 2.3, H-3), 3.42(H, dd, *J* = 10.8 and 6.8, H-5a). <sup>13</sup>C-NMR δ<sub>C</sub> (acetone-*d*<sub>6</sub>): 157.8(s, C-4''), 156.8(s, C-4'), 133.8(s, C-1''), 130.9(d, C-1), 130.8(d, C-2'', 6''), 130.4(s, C-1'), 129.7(d, C-2), 128.3(d, C-2', 6'), 116.3(d, C-3', 5'), 115.9(d, C-3'', 5''), 75.9(d, C-4), 65.6(t, C-5), 53.4(d, C-3).

*p*-Hydroxybenzaldehyde

Rt = 2.26 min (by GC). GCMS (EI<sup>+</sup>) *m/z* (%): 122 (M<sup>+</sup>, 86.7), 121(100), 93(45.5), 65(65.6), 44(60.0), 39(76.1).

**Results and Discussion**

When *A. despesta* were given 1 cm<sup>2</sup> of eggplant leaf (*Solanum melongena* L., Solanaceae), their host plant, or a filter paper containing a 5% sucrose solution they fed on all of them within 12 hrs. When 1 cm<sup>2</sup> of eggplant leaf with a methanol extract (200 mg weight of fresh wood equivalent) of *C. japonica* was given or filter paper with the methanol extract containing a 5% sucrose solution, they did not feed on them (the eggplant leaf: 100% ± 0.0, the filter paper, 96.4% ± 0.8, mean ± S.E.). The snails which were used in the bioassay normally fed on an eggplant leaf or a filter paper with a 5% sucrose solution after the bioassay. These results clearly indicate that the methanol extract of *C. japonica* strongly inhibited the feeding behavior of *A. despesta*, that is, the extract contained antifeedant(s) against the polyphagous *A. despesta*. Next, when the hexane, ethyl acetate, and water frs derived from the active methanol extract were submitted to bioassay, the results indicated that both the hexane (94.5% ± 1.2) and ethyl acetate (92.1% ± 1.6) frs had high antifeeding activities against *A. despesta* but the activity in the water fr (28.1% ± 2.0) was low. The means of antifeeding activity against *A. despesta* for the methanol extract and hexane and ethyl acetate frs were not significantly different at *P* = 0.01 by an *Anova*, *Duncan multiple range test*. Of these two active frs, the ethyl acetate fr was chromatographed on an ODS open column to separate it into 20% methanol, 50% methanol, and 100% methanol frs. Based on bioassay results of these frs, the 50% methanol fr was isolated as the most active fr (20% methanol fr, 38.0% ± 1.6, 50% methanol fr, 92.1% ± 1.8 and 100% methanol fr, 30.1% ± 2.0). According to the retention times, the active 50% methanol fr was separated into four frs by using reversed-phase HPLC. Of the four frs, Frs. A (88.1% ± 1.4) and B (90.1% ± 1.5) showed high activities. The other frs (Fr. C, 41.5% ± 1.8, Fr. D, 24.4% ± 2.0) did not show antifeeding activity against *A. despesta*. Through preparative HPLC and several bioassays, active com-

pounds **1** (Rt = 6.95 min) and **2** (Rt = 10.04 min) were isolated from Frs. A and B, respectively.

By ozonolysis of compound **1**, an equimolar amount of *p*-hydroxybenzaldehyde was generated. In addition to this results, judging from the results of specific rotation and LCMS and <sup>1</sup>H- and <sup>13</sup>C NMR analyses, compound **1** could be identified as a sequirin-C (Fig. 1) (Hatam and Whiting, 1969; Takahashi, 1981 and Nishida *et al.*, 1995). Similarly, compound **2** was identified as an agatharesinol (Enzell and Thomas, 1966; Enzell *et al.*, 1967a; Enzell *et al.*, 1967b and Takahashi, 1981) through the spectroscopic analyses (Fig. 1) and the specific rotation value.

Sequirin-C and agatharesinol inhibited the feeding behavior of *A. despesta* at 30 µg/cm<sup>2</sup> (91.1% ± 1.2) and 40 µg/cm<sup>2</sup> (90.3% ± 1.7) concentrations, respectively, when applied on an eggplant leaf or a filter paper containing 20 µl of a 5% sucrose solution as shown in Fig. 2. As shown, the activity of agatharesinol was weaker than that of sequirin-C but its concentration (180 µg/g weight fresh wood) was apparently more than that of sequirin-C (110 µg/g weight fresh wood) in *C. japonica*. The

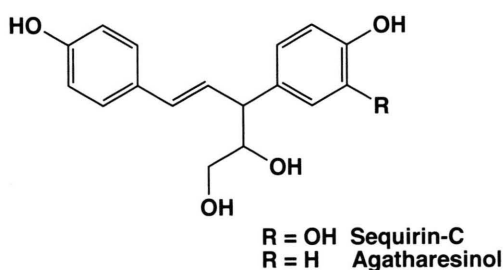


Fig. 1. Structures of sequirin-C and agatharesinol.

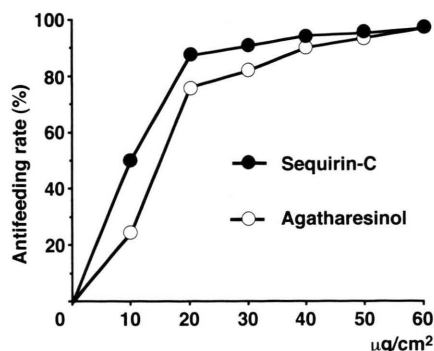


Fig. 2. Antifeeding activities of sequirin-C and agatharesinol against *A. despesta*.

activity of the ethyl acetate fr was considered to be based on these two compounds, although a synergistic effect was not observed when combined. However, the fact that these compounds strongly inhibited the feeding behavior suggests that they may have the potential to effectively control it as a pest.

There are some reports that these norlignans were observed in *C. japonica* wood when *C. japonica* received physical injury or damages by insect feeding (Shimada *et al.*, 1987; Takahashi, 1996; Ta-

kahashi and Ogiyama, 1985a, 1985b). Apparently these compounds may play a role as defense substances.

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