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

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The hexane-soluble fr. was found to be as intense in antifeeding activity as the crude methanol extract of *Cryptomeria japonica* against *Acusta despesta*. This hexane soluble-fraction was used to isolate and identify two sesquiterpenols, (-)-cubebol and (+)-2,7(14),10-bisabolatrien-1-ol-4-one, as the active compounds. Both compounds strongly inhibited the feeding behavior of *A. despesta* at 120 μg/cm² and 80 μg/cm² concentrations, respectively.

Key words: Antifeedant; *Cryptomeria japonica*; *Acusta despesta*; (-)-cubebol; (+)-2,7(14),10-bisabolatrien-1-ol-4-one

*Acusta despesta*, a snail species, is a well-known pest of many vegetables and several other crops in the world covering more than 149 species of plants in over 28 families. It is especially a pest of Mikan, the Japanese orange in Japan.

*Cryptomeria japonica* is a popular indigenous cedar of Japan. The chemical components in this tree have been intensively studied and showed such bioactivities as termiticidal, antimite and plant growth regulation. In our studies, we fractionated the crude methanol extract of *C. japonica* into the hexane, AcOEt-soluble and aqueous frs., of which both the hexane and AcOEt-soluble frs. were isolated as highly active against *A. despesta* in antifeeding. The active components in the AcOEt-soluble fr. have recently been determined to be the two norlignanes, sequirin-C and agatharesinol. In this present paper, we report the isolation and identification of the antifeedants against *A. despesta* in the active hexane-soluble fr.

The active hexane-soluble fr. (94.5% ± 1.2, mean ± S.E.) was chromatographed on a silica gel column to separate it into the 100% hexane, 20% AcOEt/hexane, 50% AcOEt/hexane and 100% AcOEt frs. The bioassay results clearly confirmed that the active component(s) in the original hexane fr. were concentrated in the 20% AcOEt/hexane fr. (92.4% ± 1.6). The activities of the other frs. were as follows: 100% hexane fr. (0.0% ± 0.0), 50% AcOEt/hexane fr. (35.6% ± 1.3) and 100% AcOEt fr. (28.1% ± 1.5). The mean values for the antifeeding activity against *A. despesta* of the original hexane and 20% AcOEt/hexane frs. were not significantly different at p = 0.01 by ANOVA and the Duncan multiple-range test. Next, according to the retention times, the active 20% AcOEt/hexane fr. was separated into seven frs. (Frs. A-G) by HPLC. Of these seven frs., Frs. D (88.1% ± 1.1) and F (91.7% ± 1.3) both showed high activity. The other frs. (A, 21.2% ± 4.1; B, 50.8% ± 3.6; C, 19.6% ± 3.8; E, 46.0% ± 1.2; and G, 32.9% ± 1.6) showed much less or negligible activity against *A. despesta*. Preparative HPLC and several bioassays were applied to isolate active compounds 1 (fr. = 12.25 min) and 2 (fr. = 16.71 min) from Frs. D and F, respectively.

The molecular weight of compound 1 was determined to be 222, because the highest peak was found to be m/z 222 and demethylation and dehydration peaks were observed at m/z 207 and 204, respectively, in the mass spectrum. Since 15 sp²-carbons without any sp²-carbons nor carbonyl carbons were observed in the ¹³C-NMR spectrum, its molecular formula was determined as C₁₅H₂₆O. This molecular formula enabled the compound to be deduced as a tricyclic-sesquiterpenol. Of these three rings, one ring seems to have been three-membered, because two methine protons (0.87 ppm, H-5 and 0.83 ppm, H-6) which were coupled with each other (J = 3.4 Hz) were observed in a very high field of the ¹H-NMR spectrum. It is also reasonable that this compound contained one isopropyl moiety, as correlations between two methyl protons (0.97 ppm, H-12 and 0.91 ppm, H-13) and one methine proton (1.63 ppm, H-11) were observed in the H-H COSY spectrum. In addition to these data, one singlet methyl group was observed at 1.28 ppm. The chemical shift and the

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coupling pattern indicated that this methyl group was attached to the tertiary carbon (80.3 ppm) with a hydroxyl group. Judging from these data, this compound was deduced as a cubebol, which is one of the richest sesquiterpenoids in *C. japonica*.\textsuperscript{5,7} The \textsuperscript{13}C-NMR, \textsuperscript{1}H-NMR and MS data and the specific rotation value of this compound were, therefore, compared with the literature data\textsuperscript{8,9} for \((-\)-cubebol and completely matched, so compound 1 was identified as \((-\)-cubebol (Fig. 1).

\((-\)-Cubebol was first isolated and identified from *Piper cubebol*\textsuperscript{10} and its stereochemistry has been confirmed by synthetic methods.\textsuperscript{8,9}

The GC/MS analysis of compound 2 determined its molecular weight to be 234. Since 15 carbons were observed in the \textsuperscript{13}C-NMR spectrum and the absorptions of a hydroxyl group and a conjugated carbonyl carbon were observed in the IR spectrum, its molecular formula was determined as C\textsubscript{15}H\textsubscript{22}O\textsubscript{2}. These results, diagnostic the \textsuperscript{1}H- and \textsuperscript{13}C-NMR data, including two-dimensional NMR (H-H COSY and C-H COSY), the mass spectra and the specific rotation value enabled compound 2 to be identified as \((+\)-2,7(14),10-bisabolatrien-1-ol-4-one (Fig. 1). This compound was first isolated by Nagashima and Tazaki from *C. japonica* as a new sesquiterpenol in this tree,\textsuperscript{11} although the absolute configuration has not yet been confirmed. This is first report about the bioactivity of this unique sesquiterpenol, although there have been so many reports about the bioactivity of other sesquiterpenoids isolated from *C. japonica*.

\((-\)-Cubebol and \((+\)-2,7(14),10-bisabolatrien-1-ol-4-one that were isolated showed high antifeeding activities against *A. despesta* at 120 \(\mu\)g/cm\textsuperscript{2} (89.7% ± 1.8) and 80 \(\mu\)g/cm\textsuperscript{2} (91.8% ± 1.3), respectively, as shown in Fig. 2. No significant difference (\(p = 0.01\)) was apparent among the activities of \((-\)-cubebol, \((+\)-2,7(14),10-bisabolatrien-1-ol-4-one and the original hexane fr. throughout the range of concentrations examined, and no synergistic effect was observed when the two compounds were combined. These results seem to show that each compound individually inhibited the feeding behavior of *A. despesta*. The activities of these compounds were far lower than those of sequirin-C (91.1% ± 1.2 at 30 \(\mu\)g/cm\textsuperscript{2}) and agatharesinol (90.3% ± 1.7 at 40 \(\mu\)g/cm\textsuperscript{2}) in the AcOEt fr.,\textsuperscript{6} although these two sesquiterpenoids (\((-\)-cubebol; 460 \(\mu\)g/g weight of fresh wood (w. f. w.) and \((+\)-2,7(14),10-bisabolatrien-1-ol-4-one; 410 \(\mu\)g/g w. f. w.) had higher concentrations than those of the norligans (Sequirin-C; 110 \(\mu\)g/g w. f. w. and agatharesinol; 180 \(\mu\)g/g w. f. w.)\textsuperscript{6} in *C. japonica*.

Norligans, including sequirin-C and agatharesinol, have been observed in *C. japonica* wood after being subjected to physical injury or damage by insect feeding,\textsuperscript{11,12} so these compounds may play a role as defense substances induced by physical damage. \((-\)-Cubebol and \((+\)-2,7(14),10-bisabolatrien-1-ol-4-one are the richest among the sesquiterpenoids in *C. japonica*, so these compounds may play the role of resident defense substances in this tree.

**Experimental**

**Instruments.** GC/MS data for compounds 1 and 2 were measured with a JEOL MS600 mass spectrometer (GC system, Hewlett Packard HP6890; column, HP-5, 0.25 \(\mu\)m thickness, 30 m \(\times\) 0.32 mm i.d., cross-linked 5% PH Me siloxane) programmed from 100°C (2-min hold) to 250°C at a rate of 10°C/min. \textsuperscript{1}H- and \textsuperscript{13}C-NMR data for compounds 1 and 2 were
measured with a JEOL Lambda 400 spectrometer (400 MHz), using TMS as the internal standard. The letters br, s, d, t, q, and m represent a broad singlet, doublet, triplet, quartet, and multiplet, respectively, and coupling constants are given in Hz. IR spectra were recorded with a Shimadzu FT-IR-4300 instrument, using the liquid film method and specific rotation data were measured with a HORIBA SEPA-200 polarimeter.

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

Plant. The cedar wood used was obtained from the Reihoku area of Kochi Prefecture in Japan.

Bioassay. Two polypropylene ice cream cups (30 mm × 60 mm i. d.) were placed on top of one another, and a moistened paper filter was laid in the base of the lower cup for humidity maintenance, while 50–60 pinholes for ventilation were opened in the base of the upper cup. Two snails of 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 cm²) containing the plant extract with 20 ml of a 5% sucrose solution for 12 h under room conditions of 28°C in the dark. The percentage of the filter paper area which the snails did 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 and 1 cm thick) and extracted with 100% methanol (2L) 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 methanol extract was dissolved in 200 ml of water, and the solution was partitioned between hexane (250 ml × 4) and water, and then between AcOEt (250 ml × 4) and water. The fractions soluble in hexane (2.7 g), AcOEt (2.3 g) and water (1.2 g) were respectively obtained. The hexane-soluble fr. (0.9 g; 10 g weight of fresh wood equivalent) was chromatogrammed on a silica gel column (230 mm × 17 mm i. d., Wako gel C-300) that was eluted with an increasing concentration of AcOEt in hexane to obtain 100% hexane (56.6 mg), 20% AcOEt /hexane (726.7 mg), 50% AcOEt /hexane (76.7 mg), and 100% AcOEt (30.0 mg) frs. The 20% AcOEt /hexane eluate was then separated into seven frs., A (tR = 0–4.30 min), B (tR = 4.30–7.70 min), C (tR = 7.70–11.00 min), D (tR = 11.00–13.00 min), E (tR = 13.00–16.40 min), F (tR = 16.40–18.00 min) and G (tR = 18.00–25.00 min) by HPLC (YMC-Pack SIL column, S-5 μm, 300 mm × 10 mm i. d.) with a refractive index detector, eluting with 20% AcOEt in hexane at a flow rate of 4 ml/min. From D, compound 1 was isolated at tR = 12.25 min and compound 2 was isolated at tR = 16.71 min from Fr. F. The yields of compounds 1 and 2 were 460 μg/g w. f. w. and 410 μg/g w. f. w., respectively.

Compound 1. (−)-Cubebol, [α]D20 = −48.4° (c = 1.0, CHCl₃). GC/MS (tR = 8.44 min) m/z (%): 222(M⁺, 7.9), 207(95.3), 204(54.2), 189(9.1), 179(13), 161(100), 121(21.7), 119(29.4), 105(35.0), 91(20.8). IR ν max cm⁻¹ (liquid film): 3380(OH). 1H-NMR δH (CDCl₃): 1.84(1H, ddd, J = 11.5, 12.0, 8.6 H-2a), 1.66(1H, m, J = 6.5, H-10), 1.63(1H, m, J = 6.8, 6.8, H-11), 1.59(1H, m, J = 2.2, 12.9, H-8a), 1.54(1H, m, J = 8.1, 11.7, H-2b), 1.53(1H, m, J = 11.7, H-3a), 1.39(1H, m, J = 12.9, 2.2, H-9a), 1.37(1H, m, J = 8.6, H-3b), 1.28(3H, s, H-15), 0.99(1H, m, J = 3.4, H-7), 0.97(3H, d, J = 6.8, H-13), 0.94(3H, d, J = 6.5, H-14), 0.91(3H, d, J = 4.3, H-12), 0.87(1H, d, J = 3.4, H-5), 0.83(1H, d, J = 3.4, 3.4, H-6), 0.82(1H, m, J = 12.9, 2.2, H-8b), 0.52(1H, ddd, J = 12.9, 12.9, 2.2, H-9b). 13C-NMR δC (CDCl₃): 80.3(C-4), 44.1(d, C-7), 39.0(d, C-5), 36.3(t, C-3), 33.6(d, C-11), 33.4(s, C-1), 31.7(t, C-9), 30.8(d, C-10), 29.5(t, C-2), 27.9(q, C-15), 26.5(t, C-8), 22.5(d, C-6), 20.1(q, C-14), 19.6(q, C-13), 18.7(q, C-12).

Compound 2. (+)-2,7(14),10-Bisabolatrien-1-ol-l-4-one, [α]D20 +130° (c = 1.0, methanol). GC/MS (tR = 12.26 min) m/z (%): 234(M⁺, 24.4), 173(33.9), 124(15.9), 109(22.8), 98(60.9), 93(13.6), 70(19.8), 69(100), 41(63.4). IR ν max cm⁻¹ (Liquid film): 3400(OH), 1680(C=O). 1H-NMR δH (CDCl₃): 6.71(1H, dq, J = 1.5 and 1.3, H-2), 5.10(1H, tqq, J = 6.9, 1.3 and 1.2, H-10), 6.03(2H, br, s, H-14), 4.50(1H, br, dd, J = 9.7 and 1.3, H-1), 2.68(1H, ddd, J = 13.9, 9.7 and 3.8, H-6), 2.55(1H, dd, J = 16.4 and 3.8, H-5b), 2.36(1H, dd, J = 16.5 and 13.9, H-5a), 2.17(2H, m, H-9), 2.10(1H, br, s, OH), 2.09(2H, m, H-8), 1.80(3H, dd, J = 1.7 and 1.3, H-15), 1.69(3H, br, d, J = 1.3, H-12), 1.62(3H, br, d, J = 1.2, H-13). 13C-NMR δC (CDCl₃): 198.4(s, C-4), 147.5(s, C-7), 147.3(d, C-2), 135.1(s, C-3), 132.6(s, C-11), 123.4(d, C-10), 112.7(t, C-14), 69.3(d, C-1), 52.0(d, C-6), 41.6(t, C-5), 33.3(t, C-8), 26.3(t, C-9), 25.7(q, C-12), 17.8(q, C-13), 15.3(q, C-15).

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