**Note**

**Antifeedants against Locusta migratoria from the Japanese Cedar, Cryptomeria japonica II**

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A methanol extract of Cryptomeria japonica completely inhibited feeding by Locusta migratoria. Based on bioassay-guided fractionation, two active terpenols, (+)-ferruginol and (-)-cubebol, were isolated and identified as antifeedants against this insect species. Each compound separately showed weak activity, but they showed intense activity against this insect species when they were combined.

**Key words:** Locusta migratoria; Cryptomeria japonica; antifeedant; (+)-ferruginol; (-)-cubebol

The Japanese cedar, Cryptomeria japonica (Taxodiaceae), is a popular indigenous cedar of Japan. The chemical components in this tree have been intensively studied for a long time, and such bioactivity as termiticidal,1,2) anti-mite,3,4) anti-fungal,3) antifeeding4-6) and inhibiting germination8) have been reported. In addition to these, we have recently found that a crude methanol extract of C. japonica strongly inhibited feeding of the migratory locust, Locusta migratoria L.9) which is a well-known and serious pest to cereals throughout the world and sometimes causes massive damage to crops in every continent except Antarctica.10,11)

The active components were extracted by hexane from a crude methanol extract of C. japonica. The fractions from 10% percent diethyl ether in hexane and 30% diethyl ether in hexane that were separated from the hexane layer by silica-gel column chromatography each showed high activity. (1S,6R)-2,7(14),10-Bisabolatrien-1-ol-4-one12) and (++)-7(14),10-bisaboladien-1-ol-4-one were isolated and identified as the active compounds from the active 30% diethyl ether in hexane fraction.9) Each compound alone did not show any activity, but together they showed intense activity against this insect species.9)

We report in this paper the isolation and identification of the antifeedants against L. migratoria in the active 10% diethyl ether in hexane fraction.

The active 10% diethyl ether in hexane fraction (the proportion remaining on the filter paper was 72.9%±21.4, mean ± S.E.)13) was separated into three fractions by HPLC according to the retention times (A, 0–6.0 min; B, 6.0–9.0 min; C, 9.0–12.0 min). Although each fraction showed low antifeeding activity against the locust (A, 33.3%±11.1; B, 29.2%±11.7; C, 58.2%±10.1), the solution showed strong antifeeding activity against L. migratoria as the original methanol extract did when all three fractions were combined and presented for the bioassay (A + B + C, 97.7%±1.67). The combination of fractions A and C showed high activity (A + C, 98.9%±0.6), but the combinations of fractions A and B and of fractions B and C only had low activity (A + B, 12.8%±2.3; B + C, 66.3%±5.5). These results indicate that the activity was not due to a single component, but to multiple components.

Fraction A was further separated into three fractions (A1 fr., 0–9.5 min; A2 fr., 9.5–10.5 min; A3 fr., 10.5–12.5 min) by HPLC, and each of these fractions combined with fraction C was submitted to the bioassay. Of the three fractions obtained from fraction A (A1 + C, 46.3±4.1; A2 + C, 87.9±6.0; A3 + C, 10.4±2.6), only fraction A2 had equally high activity to that of the original methanol extract of C. japonica when combined with fraction C. Likewise, fraction C was further separated into three fractions (C1 fr., 0–9.5 min; C2 fr., 9.5–11.1 min; C3 fr., 11.1–12.5 min) by HPLC according to the retention time, and each fraction combined with fraction A was submitted to the bioassay. Fraction C2 showed the highest activity when combined with fraction A (A + C1, 30.0±9.3; A + C2, 99.3±0.4; A + C3, 43.7±5.6). Fractions A2 and C2 each consisted only of a single component, i.e., compounds 1 and 2, respectively, on all HPLC traces.

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Identification of compounds 1 and 2. Twenty carbon signals were observed in the $^{13}$C-NMR spectrum of compound 1. The diagnostic $^{1}$H- and $^{13}$C-NMR data, including two-dimensional NMR (H-H COSY and C-H COSY), mass spectra and specific rotation value, enabled compound 1 to be identified as a (+)-ferruginol (Fig. 1). The $^{13}$C-NMR, $^{1}$H-NMR and MS data and the specific rotation value for this compound were, therefore, compared with the literature data.13,14

Judging from the $^{1}$H- and $^{13}$C-NMR data, including two-dimensional NMR (H-H COSY and C-H COSY) and mass spectra, compound 2 was deduced to be cubebol. A comparison of the $^{1}$H- and $^{13}$C-NMR, GC-MS data and the specific rotation value for compound 2 with the literature data5,16 showed close agreement, confirming compound 2 to be (−)-cubebol which is one of the richest sesquiterpenols in C. japonica (Fig. 1).4,17

Compounds 1 and 2 were respectively present as 46.1 and 3.49 µg/cm² on the filter paper in the bioassay. As shown in Fig. 2, these two isolated compounds inhibited feeding of L. migratoria as strongly as the original methanol extract did when they were combined, although each compound alone only showed weak activity (1, 43.7% ± 8.4; 2, 18.4% ± 5.2; 1 + 2, 84.7% ± 10.8).

(+)-Ferruginol was first isolated and identified from a miro tree (Podocarpus ferrugineus).18 This compound is known to have the antifungal activity14 and inhibit mycelial growth against Lentinus edodes.19 (−)-Cubebol was first isolated and identified from Piper cubeba and has antifeeding activity against a snail species (Acusta despesta).9

(15S,6R)-2,7(14),10-Bisabolatrien-1-ol-4-one and (+)-7(14),10-bisaboladien-1-ol-4-one, which were the active compounds in the 30% diethyl ether in hexane fraction, showed high antifeeding activity against L. migratoria only when they were combined.9 In this case, (+)-7(14),10-bisaboladien-1-ol-4-one was the main active compound and (15S,6R)-2,7(14),10-bisabolatrien-1-ol-4-one was a support component in the antifeeding activity of C. japonica against L. migratoria. Both compounds showed a highly synergistic effect. In contrast, (+)-ferruginol and (−)-cubebol each showed activity when used alone against this insect species, so there seems not to be a synergistic effect with (+)-ferruginol and (−)-cubebol. These results may indicate the presence of different active sites in this insect species for each compound.

Our next target is to find the optimum concentration of each compound and to examine the antifeeding activity with a combination of the four active compounds.

These compounds completely inhibited this serious pest’s feeding, and the results of this work might contribute to the development of integrated pest control methods.

**Experimental**

*Instruments.* GC-MS data for compounds 1 and 2 were measured with a Jeol MS600 mass spectrometer. GC analyses were conducted with a Shimadzu GC-14A instrument 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. $^{1}$H- and $^{13}$C-NMR data for compounds 1 and 2 were measured with a Jeol Lambda 400 spectrometer at 400 MHz, using TMS as an internal standard. Letters (br.)s, d, t, q, and m represent (broad) singlet, doublet, triplet, quartet, and multiplet, respectively, and coupling constants are given in Hz.

*Insects and plants.* L. migratoria was collected from a field in Nankoku City, Kochi Prefecture and kept at 28 ± 1°C with 16 h lighting and 8 h darkness, gramineous weed being provided as a feed. Adult locusts were used for the bioassay. Heartwood of C. japonica from a 35-year-old tree in Rei-hoku, Kochi Prefecture, was used as the material for extraction.

*Bioassay.* A semicircular filter paper (7 cm in diameter) was made three cuts of 2.5 cm from the circumference toward the center. The methanol extract of 3 g of fresh wood equivalent of C. japonica was applied to this filter paper. After drying, 0.3 ml of a 15% aqueous sucrose solution was added to the filter paper. The filter paper containing both the extract and the 15% sucrose solution and a filter paper containing only 0.3 ml of the
15% sucrose solution were put at the center of a rectangular polypropylene tray (24 cm long, 32 cm wide, 11 cm high). Ten adults of *L. migratoria* were allowed to feed on the two filter papers for 24 h at 27 °C with 16:8 (L:D) illumination. The percentage of the filter paper area which the locusts did not feed on represented as the antifeedant rate. Each sample was tested at least three times.

**Preparation of the plant extract.** Fresh *C. japonica* heartwood (3.15 kg) was cut into pieces (5 cm long, 1 cm thickness) and extracted with methanol (16-liter) for 3 d in darkness at room temperature. This procedure was conducted twice. After evaporating the solvent under reduced pressure at 40 °C, a methanol extract (56.9 g) was obtained. The residue of this methanol extract (48.8 g, 3.0 kg of fresh wood equivalent) was dissolved in 1400 ml of water, and the solution was next partitioned between hexane (1000 ml × 4) and, water and then between ethyl acetate (1000 ml × 4) and water. The hexane (17.6 g), ethyl acetate (16.3 g) and water (4.7 g) fractions were respectively obtained. The hexane fraction (5.86 g, 1.0 kg of fresh wood equivalent) was chromatographed in a silica gel column (400 mm × 30 mm i. d., Wako gel C-300) that was eluted with an increasing concentration of diethyl ether in hexane to obtain hexane (0.40 g), 10% diethyl ether in hexane (3.12 g), 30% diethyl ether in hexane (1.33 g), 70% diethyl ether in hexane (0.51 g), diethyl ether (0.32 g) and methanol eluates (0.17 g). The eluate of 50 g of fresh wood equivalent from 10% diethyl ether in hexane was separated into three fractions, fraction A (tR = 0–6.0 min; 28.7 mg), fraction B (tR = 6.0–9.0 min; 14.0 mg) and fraction C (tR = 9.0–12.0 min; 7.8 mg) by using normal-phase HPLC (YMC Pack SIL 06A-024-5 column, 300 × 10 mm i.d.) eluted with 30% diethyl ether in hexane containing 1% ethanol at a flow rate of 4 ml/min and linked to a refractive index detector. The 50 g of fresh wood equivalent of fraction A was divided three fractions, fraction A1 (tR = 0–9.5 min; 0.9 mg), fraction A2 (tR = 9.5–10.5 min; 29.4 mg) and fraction A3 (tR = 10.5–12.5 min; 0.2 mg) by using normal-phase HPLC (YMC Pack SIL 06A-024-5 column, 300 × 10 mm i.d.) eluted with 1% diethyl ether in hexane containing 1% ethanol at a flow rate of 4 ml/min. The 50 g of fresh wood equivalent of fraction C was divided three fractions, fraction C1 (tR = 0–9.8 min; 0.9 mg), fraction C2 (tR = 9.8–11.1 min; 3.7 mg) and fraction C3 (tR = 11.1–12.5 min; 4.5 mg) by HPLC performed in the same column at a 4 ml/min flow rate, eluting with 5% ethyl acetate in hexane containing 1% ethanol. Compound 1 was isolated at tR = 10.0 min from fraction A2, and compound 2 was isolated at tR = 11.5 min from fraction C. The yield of each compound from 1.0 g of fresh wood equivalent of the methanol extract was 296 μg (1) and 22.4 μg (2).

**Compound 1.** (+)-ferruginol, [α]D20 +56.2° (c = 1.0 CHCl3). GC-MS m/z (%): 286 (M+, 100), 271 (79), 201 (40), 189 (41), 175 (50), 69 (62). 1H-NMR δH (CDCl3): 0.85 (3H, s, H-18), 0.90 (3H, s, H-19), 1.15 (3H, s, H-20), 1.18 (1H, m, H-3), 1.20 (3H, d, J = 6.8, H-16), 1.22 (3H, d, J = 6.8, H-17), 1.32 (1H, dd, J = 12.4, 2.4, H-5), 1.39 (1H, m, H-1), 1.41 (1H, m, H-3), 1.57 (1H, m, H-2), 1.65 (1H, m, H-6), 1.73 (1H, m, H-2'), 1.79 (1H, m, H-7), 1.84 (1H, m, H-6'), 2.17 (1H, d, J = 12.6, H-1'), 2.83 (1H, m, H-7'), 3.11 (1H, s, J = 6.8, H-15), 6.62 (1H, s, H-11), 6.83 (1H, s, H-14). 13C-NMR δC (CDCl3): 150.6 (s, C-12), 148.6 (s, C-9), 131.5 (s, C-13), 127.3 (s, C-8), 126.5 (d, C-14), 111.0 (d, C-11), 50.3 (d, C-5), 41.7 (t, C-3), 38.8 (t, C-1), 37.4 (s, C-10), 33.4 (q, C-18), 33.3 (s, C-4), 29.7 (t, C-7), 26.7 (d, C-15), 24.7 (q, C-20), 22.8 (q, C-17), 22.6 (q, C-16), 21.6 (q, C-19), 19.3 (t, C-2), 19.2 (t, C-6).

**Compound 2.** (−)-cubebol, [α]D20 −48.4° (c = 1.0, CHCl3). GC-MS m/z (%): 222 (M+, 7.9), 207 (95.3), 204 (54.2), 189 (91.9), 179 (13), 161 (100), 121 (21.7), 119 (29.4), 105 (35.0), 91 (20.8). IR νmax cm−1 (Liquid film): 3380 (OH). 1H-NMR δH (CDCl3): 1.84 (1H, ddd, J = 12.0, 11.7, 8.6 H-2a), 1.66 (1H, m, H-10), 1.63 (1H, m, H-11), 1.59 (1H, m, H-8a), 1.54 (1H, m, H-2b), 1.53 (1H, m, H-3a), 1.39 (1H, m, H-9a), 1.37 (1H, m, H-3b), 1.28 (3H, s, H-15), 0.99 (1H, m, 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 = 6.8, H-12), 0.87 (1H, d, J = 3.4, H-5), 0.83 (1H, dd, J = 3.4, 3.4, H-6), 0.82 (1H, ddd, J = 12.9, 12.9, 12.9, 2.4, H-8b), 0.52 (1H, ddd, J = 13.9, 12.9, 12.9, 2.4, H-9b). 13C-NMR δC (CDCl3): 80.3 (s, 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).

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