Repellents in the Japanese Cedar, Cryptomeria japonica, against the Pill-bug, Armadillidium vulgare

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Sandaracopimarinel and (1S,6R)-2,7(14),10-bisabolatrien-1-ol-4-one were isolated and identified from Cryptomeria japonica as repellents against Armadillidium vulgare which is well known as an unpleasant pest in the house and as vegetable pest in Japan. These compounds strongly repelled A. vulgare when they were combined, although each compound alone did not show any activity.

Key words: Armadillidium vulgare; Cryptomeria japonica; repellent; sandaracopimarinel; (1S,6R)-2,7(14),10-bisabolatrien-1-ol-4-one

Armadillidium vulgare (Oniscidae, Isopoda), which is a species of terrestrial isopods and called the pill-bug, is widely distributed in the world and is well known as an invasive and unpleasant pest in the house and as a phytophagous pest feeding on the growing point of plants such as young buds of fruits, vegetables, and flowers in Japan.1,2)

The Japanese cedar, Cryptomeria japonica (Taxodiaceae), a plant peculiar to Japan, has been vigorously studied for the bioactivities of its components: for example, termiticidal,3,4) anti-mite,5,6) antifungal7) and antifeeding against snail species8) activities, and growth-regulation effect.9) In addition to these, we have recently found that a crude methanol extract of C. japonica strongly repelled A. vulgare. We report here the identification of the repellents in C. japonica against A. vulgare in a multi-component system requiring plural components to exhibit its activity.

The MeOH extract (3 g of fresh wood equivalent) of C. japonica was applied to a quarter of a filter paper and then submitted to a bioassay. When fifty specimens of A. vulgare were allowed to move freely at the center of a plastic tray and randomly seek moisture, the isopods which reached at the control filter paper containing only water remained on it. On the contrary, they went back, cleaned their antennas and then dispersed from the filter paper containing the MeOH extract as soon as they touched it. After 30 min, almost 100% of the isopods (2.0 ± 0.06, av. of arresting rate ± S.E.) had settled on the control filter paper. These results and observations made clear that the MeOH extract of C. japonica strongly disturbed the A. vulgare isopods and made them move to the filter paper containing only water, indicating that the extract contained a repellent(s) against the isopod species.

The crude MeOH extract suspended in water was successively extracted with hexane, diethyl ether, ethyl acetate, and butanol. As shown in Fig. 1, only the diethyl ether-soluble fraction showed repellent activity against the isopod as strongly as the original MeOH extract (hexane fr., 41.4 ± 1.67; diethyl ether fr., 2.6 ± 1.56; ethyl acetate fr., 31.5 ± 6.59; butanol fr., 49.0 ± 5.61; aqueous fr., 44.9 ± 5.69). The active diethyl ether-soluble fraction was then chromatographed in a silica gel column, eluting in sequence with a mixture of increasing concentrations of diethyl ether in hexane. Of the obtained fractions (hexane fr., 35.8 ± 4.15; 10% EtOH fr., 47.4 ± 5.29; 30% EtOH fr., 4.7 ± 1.01; 100% EtOH fr., 28.7 ± 1.55; diethyl ether fr., 47.2 ± 5.14), the fraction of 30% diethyl ether in hexane was the most active.

The active fraction of 30% diethyl ether in hexane was further separated into three fractions (A fr., 0-31 min; B fr., 31-56 min; C fr., 56-55 min) by HPLC according to the retention time. When these three fractions were randomly combined and submitted to the bioassay, any combination lacking either the A or B fraction in the resulting mixture had low activity, while the combinations containing both A and B fractions showed high activity (A + B + C fr., 2.6 ± 1.48; A fr., 46.4 ± 0.99; B fr., 31.9 ± 8.75; C fr., 35.2 ± 9.04; A + B fr., 0.9 ± 0.93; A + C fr., 53.8 ± 0.75; B + C fr., 36.6 ± 2.48) as shown in Fig. 2. These results indicated that the activity was not due to a single component, but to multiple components.

Fraction A was further separated into the four

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Fig. 1. Activities of the MeOH Extract and of the Separated Fractions.

Fig. 2. Activities of the Fractions Separated by HPLC.

fractions (A₁ fr., 0–22 min; A₂ fr., 22–23 min; A₃ fr., 23–24 min; A₄ fr., 24–31 min) by HPLC, and each was submitted to the bioassay. Of the four fractions obtained from fraction A (A₁–A₄ + B fr., 6.7 ± 2.25; A₁ + B fr., 49.8 ± 4.74; A₂ + B fr., 10.4 ± 2.63; A₃ + B fr., 42.0 ± 2.63; A₄ + B fr., 37.8 ± 3.30; A₁ + A₂ + B fr., 43.5 ± 2.87)
Fig. 3. Activities of the Four Fractions Separated from Active Fraction A When Combined with Fraction B.

fr., 11.5 ± 3.29; A3 + A2 + B fr., 11.4 ± 3.70; A4 + A2 + B fr., 10.7 ± 0.41), only fraction A3 had similar activity to that of the original MeOH extract of C. japonica when combined with fraction B (Fig. 3). Fractions A2 and B each consisted only of a single component, i.e., compounds 1 and 2, respectively, on any HPLC chromatogram.

These two isolated compounds strongly repelled A. vulgare as strongly as the original MeOH extract only when they were combined, although each compound alone did not show any activity (I, 52.7 ± 7.72; 2, 30.9 ± 6.49; 1 + 2, 3.3 ± 1.92).

Identification of compound 1. The 13C-NMR, 1H-NMR and IR spectra of compound 1 indicate 20 different kinds of carbon, 32 hydrogens, and an absorption at 3400 cm⁻¹ based on a hydroxyl group. In addition to these data, the largest mass fragment peak was observed at 288 (m/z) in the GC-MS spectrum, so the molecular formula of compound 1 was estimated to be C20H32O (Δ5). As this compound also had two double bonds (C-8, 137.0 ppm; C-14, 128.7 ppm; H-14, 5.22 ppm), including one vinyl group (C-15, 149.1 ppm; C-16, 110.0 ppm; H-15, 5.78 ppm; H-16, 4.91 and 4.88 ppm) and three methyl groups (C-17, 25.9 ppm; C-19, 17.9 ppm; C-20, 15.6 ppm; H-17, 1.04 ppm; H-19, 0.84 ppm; H-20, 0.81 ppm) with a singlet in the 1H-NMR spectrum, it is reasonable to assume that three rings formed a pimaran skeleton in this compound. H-H COSY, C-H COSY and COLOC spectral data also imply the pimaran skeleton. Since the specific rotation value ([α]D20° = -20°, c = 0.1, MeOH) and 13C-NMR data were the same as the literature data,9 compound 1 was identified as sandaracopimarrol (Fig. 4).

Identification of compound 2. The IR spectrum indicated absorptions at 3400 cm⁻¹ and 1680 cm⁻¹ which were respectively based on a hydroxyl group and α,β-unsaturated ketone moiety. Since 15 different kinds of carbon and 22 hydrogens were respectively observed in the 13C- and 1H-NMR spectra, and a largest mass fragment peaks appeared at 234 (M⁺) and 257 (M + Na⁺) in the GC-MS and LC-MS spectra, respectively, the molecular formula of compound 2 was estimated to be C19H23O2 (Δ5). One enone (C-4, 198.4 ppm; C-2, 147.3 ppm; C-3, 135.1 ppm; H-2, 6.71 ppm), one exomethylene (C-7, 147.5 ppm; C-14, 112.7 ppm; H-14, 5.03 ppm), and one double bond (C-11, 132.6 ppm; C-10, 123.4 ppm; H-10, 5.10 ppm) were shown by both the 13C- and 1H-NMR spectra, indicating that compound 2 had one ring system. All of three methyl groups (C-12, 25.7 ppm; C-13, 17.8 ppm; C-15, 15.3 ppm; H-15, 1.80 ppm; H-12, 1.69 ppm; H-13, 1.62 ppm) were attached to double bonds from their chemical shift value. Two methyl groups were coupled with the H-10 proton and another with the H-2 proton at long range. These results and their chemical shift values indicate that one 2-methylpropenyl and one 2-
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Experimental

**Instruments.** GC-MS data for compounds 1 and 2 were recorded with a Shimadzu QP-2000 instrument and measured at 20 eV. GC analyses were done with a Shimadzu GC-14A instrument with a fused silica column (HR1701, 0.25 mm thickness, 25 m × 0.2 mm i.d.), the temperature being programmed from 200°C (2 min hold) to 250°C at a rate of 10°C/min. LC-MS data were recorded with a VG Quatro II instrument by ESI (positive) with injection at a flow rate at 10 µl/min. 1H-NMR and 13C-NMR spectra, including two-dimensional correlation spectra, were measured with a JEOL JNM-LA400 spectrometer (400 MHz), TMS being used as an internal standard. Letters s, (d) t, q, and m represent singlet, (double) doublet, triplet, quartet and multiplet, respectively, and coupling constants are given in Hz. IR spectra were recorded with a Shimadzu FT-IR-4300 instrument by the liquid film method, and specific rotation values were recorded with a Horiba High Sensitive Polarimeter SEPA-200.

**Insects and plants.** A. vulgare was collected from a field in Nankoku City, Kochi Prefecture and kept at 28 ± 1°C with 16 h lighting–8 h darkness, vegetables and humus being provided as a feed. Mature isopods over about 8 mm in length were used for the bioassay.

Heartwood of Cryptomeria japonica from a 35-year-old tree in Rei-hoku, Kochi Prefecture was used as the sample extract.

**Bioassay.** The MeOH extract of 3 g of fresh wood equivalent of C. japonica was applied to a quadrant of circular filter paper (9 cm in diameter). After drying, 0.3 ml of water was added to the filter paper. Two filter papers containing only 0.3 ml of water and two with both the extract and water were alternately put at the four corners of a rectangular plastic tray (23 cm × 30 cm). Fifty adults of A. vulgare were introduced at the center on the tray and allowed to move freely. After 30 min, the numbers of isopods on the filter papers containing the sample and those containing only water were respectively counted. The repellent activity was estimated from arresting rate of the isopods by the following equation:

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\text{Arresting rate} (\%) = \frac{\text{number of isopods on the filter papers containing the sample}}{\text{number of isopods on the filter papers containing only water} + \text{number of isopods on the filter papers containing the sample}} \times 100.
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Each sample was tested at least three times.

**Isolation of compounds 1 and 2.** The MeOH extract (500 g of fresh wood equivalent) of C. japonica was dissolved in water (300 ml) and successively...
extracted with hexane (250 ml × 3), diethyl ether (250 ml × 3), ethyl acetate (250 ml × 3), and water-saturated butanol (250 ml × 3). The diethyl ether-soluble fraction (1.2 g) was then chromatographed on silica gel (Wako-gel C-300, 40 g), eluting successively with hexane, 10% diethyl ether in hexane, 30% diethyl ether in hexane, 50% diethyl ether in hexane, and diethyl ether (500 ml each). The 30% diethyl ether-in-hexane fraction yielded compounds 1 and 2 which were purified by preparative HPLC (Cosmosil 55L, 250 mm × 10 mm i.d.), eluting with 20% ethyl acetate in hexane containing 1% ethanol (2 ml/min). Compounds 1 and 2 were isolated at tR=22.0 and 33.3 min, respectively. The yield of each compound from 1.0 g of fresh wood equivalent of the MeOH extract was 0.35 mg (1) and 2.1 mg (2).

**Compound 1** (sandaracopimarinal). [α]D +20° (c = 0.1, MeOH). GC-MS m/z (%): 288 (M+, 17.3), 273 (14.3), 258 (23.0), 257 (100), 161 (20.5), 147 (26.0), 135 (32.0), 133 (29.7), 121 (48.3), 109 (40.1), 107 (44.9), 95 (38.5), 93 (52.3), 91 (54.4), 81 (57.4), 79 (48.9), 67 (39.0), 55 (70.1), 43 (48.7), 41 (75.4), 29 (34.2). IR νmax (liquid film) cm⁻¹: 3400 (OH). 1H-NMR (CDCl₃) δ: 5.78 (IH, dd, J=17.1 and 9.9, H-15), 5.22 (1H, s, H-14), 4.91 (1H, d, J=17.1, H-16a), 4.88 (1H, d, J=9.9, H-16b), 3.40 (1H, d, J=10.8, H-18a), 3.12 (1H, d, J=10.8, H-18b), 2.24 (1H, dd, J=13.9 and 4.1, H-7a), 2.08 (1H, m, H-7b), 1.78–1.71 (2H, m, H-1a and H-9), 1.63 (1H, m, H-11a), 1.57 (1H, m, H-3a), 1.55–1.45 (6H, m, H-2, H-6a, H-11b, H-12a, and OH), 1.40 (1H, dd, J=12.4 and 4.9, H-3b), 1.35–1.21 (3H, m, H-5, H-6b, and H-12b), 1.04 (3H, s, H-17), 1.00 (1H, m, H-1b), 0.84 (3H, s, H-19), 0.81 (3H, s, H-20). 13C-NMR (CDCl₃) δ: 149.1 (d, C-15), 137.0 (s, C-8), 128.7 (d, C-14), 110.0 (t, C-16), 72.2 (t, C-18), 50.5 (d, C-9), 47.8 (d, C-5), 38.9 (t, C-1), 38.1 (s, C-10), 37.8 (s, C-13), 37.4 (s, C-4), 35.7 (t, C-5), 35.4 (t, C-7), 34.5 (t, C-12), 25.9 (q, C-17), 22.4 (t, C-6), 18.8 (t, C-11), 18.3 (t, C-2), 17.9 (q, C-19), 15.6 (q, C-20).

**Compound 2** (1S,6R)-2,7(14),10-bisabolatrien-1-ol-4-one. [α]D +130° (c=1.0, MeOH). LC-MS (ESI+) m/z (%): 257 (M+Na+, 100). GC-MS m/z (%): 234 (M+, 1.7), 98 (34.3), 70 (21.4), 69 (96.1), 41 (100). IR νmax (liquid film) cm⁻¹: 3400 (OH), 1680 (C=O). 1H-NMR (CDCl₃) δ: 6.71 (1H, dq, J=1.6 and 1.3, H-2), 5.10 (1H, qq, J=6.9, 1.3, and 1.2, H-10), 5.03 (2H, s, H-14), 4.50 (1H, dd, J=9.7 and 1.3, H-1), 2.68 (1H, dd, J=13.9, 9.7, and 3.8, H-6), 2.55 (1H, dd, J=16.5 and 3.8, H-5a), 2.36 (1H, dd, J=16.5 and 13.9, H-5b), 2.17 (2H, m, H-9), 2.10 (1H, s, OH), 2.09 (2H, m, H-8), 1.80 (3H, dd, J=1.6 and 1.3, H-15), 1.69 (3H, d, J=1.3, H-12), 1.62 (3H, d, J=1.2, H-13). 13C-NMR (CDCl₃) δ: 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-15), 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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**References**