INTRODUCTION

Leaf beetles (Chrysomelidae) are known to protect themselves against enemies by various defensive mechanisms, including behavioral, mechanical, and chemical (Deroe and Pasteels, 1982). Adult Criocerinae and Chrysomelinae, and some Galerucinae and Alticinae possess pronotal and elytoral glands from which defensive substances are secreted (Deroe and Pasteels, 1982; Pasteels et al., 1988, 1989). Of these subfamilies, the secretions of Chrysomelinae have been well studied and good correlations have been established between the secretion components and species classification at the subtribal level, although a few exceptions exist (Pasteels et al., 1982; Pasteels, 1993). Pasteels et al. (1982, 1994) reported that the adults of the subtribe Chrysomelina secrete isoxazolinone glucosides (compounds 1 and 6, Table 1) and dinitropropanoyl isoxazolinone glucosides (compounds 3 and 4, Table 1). However, no chemical studies have been undertaken on Japanese species.

In the present study, we report the identification of the secreted components from four Japanese Chrysomelinae (subtribe Chrysomelina), *Gastrophysa atrocyanea*, *Plagiodera versicolora distincta*, *Chrysomela vigintipunctata costella*, and *Gastrolina depressa* (Coleoptera: Chrysomelidae), and their deterrent effects against ants (*Tetramorium caespitum*).

MATERIALS AND METHODS

**Insects.** *Gastrophysa atrocyanea* and *Plagiodera versicolora distincta* were selected from a laboratory culture maintained on *Rumex obtusifolius* (Polygonaceae) and willow (genus *Salix* (Salicaceae)) leaves, respectively. *Chrysomela vigintipunctata costella* and *Gastrolina depressa* were collected at egg or larval stages and reared on willow (genus *Salix* (Salicaceae)) and *Juglans mandshurica* Maxim. var. *Siebeldiana* (Juglandaceae) leaves, respectively.

**Collection of secretions.** The adults of each species were fed the leaves for at least a week after eclosion. These beetles were disturbed using forceps and the resulting secretions were collected on bits of filter paper under a stereomicroscope. The secretions were extracted in acetone. After freeze-drying, hexane was added to the extraction, the sample was mixed and centrifuged. The nonpolar components were removed from the sample using hexane. The remaining sample was stored at −18°C.
Chemical analysis. Thin Layer Chromatography (TLC) was performed using Silica gel 60 F254 plates (5×10 cm, Merck) developed with CHCl3 : CH3OH 5:8 : 2. After development, spots on the plate were observed under UV -254 nm and visualized with 10% aqueous sulfuric acid (heated for 30 min at 120°C) or diazotized p-nitroaniline reagent (Majak and Bose, 1974).

The *G. atrocyanea* secretions (58.2 mg dry weight) were obtained from about 2,000 adults and isolated by preparative TLC (Silica gel 60 F254, 10×20 cm, Merck) using the above-mentioned solvent. After development, the bands were observed under UV-254 nm and visualized by spraying with diazotized p-nitroaniline reagent to confirm the position of compounds 1–5. The band from each compound was scraped off the plate, and the silica gel was extracted with acetone. The isolated compounds were analyzed by Nuclear Magnetic Resonance (NMR) and Fast Atom Bombardment Mass Spectrometry (FAB-MS). NMR spectra were recorded on a Varian Unity INOVA 500 (500 MHz) using D2O for compound 1 or acetone-d6 (a drop of D2O was added to remove OH coupling) for the other compounds together with tetramethylsilane as an internal standard. FAB mass spectra were measured by a JEOL JMS-700 using glycerol (positive ion) or triethanolamine (negative ion) as a matrix.

High Performance Liquid Chromatography (HPLC) analysis was performed using a reverse phase column (YMC-Pack ODS-AM, 250×4.6 mm I.D., YMC Co., Ltd.). A gradient was created using acetonitrile in water (0–5 min, 5–20%, linear→5–15 min, 20%→15–25 min, 20–50%, linear) at a flow rate of 1 ml/min and the eluate was detected by UV-absorbance at 210 nm. A CCCMP pump and a UV-8000 detector (TOSO Co., Ltd.) were used in the HPLC system.

All samples were quantitated by HPLC. Isolated compounds 1–5 and secreted extracts from the four species were adjusted to 1 mg/ml and one adult secretion equivalent (ASE)/12 μl with acetone and 1 μl of each was injected. The peak area was measured and the concentrations of these compounds in the secretions were calculated using the calibration curve obtained from the peak area of isolated compounds 1–5.

Bioassay. The deterrent effects of the secretions on the ant *Tetramorium caespitum* were examined. Ants were starved for 24 h in a petri dish lined with damp filter paper prior to bioassay. Each sample was dissolved in acetone (2 ASEs or 15 μg of each compound) or acetone control was applied to a square piece of filter paper (5×5 mm) and the solvent allowed to air-dry. Each piece of treated and control filter paper was placed symmetrically on a petri dish (60 mm diam.). Sucrose solution (1M, 5 μl) was added to each filter paper immediately before the test. Ten ants were released into the petri dish. After 3 min, the number of ants feeding on each filter paper was counted. Each test was replicated 10 times and the results were statistically different when evaluated by Wilcoxon’s signed-ranks test (Sokal and Rohlf, 1995).

### RESULTS

**Identification of the secretion components of *G. atrocyanea***

Five major spots, compounds 1, 2, 3, 4, and 5 (Rf 0.27, 0.39, 0.43, 0.54, and 0.62, respectively) were detected in the secretions of *G. atrocyanea* after treatment with 10% H2SO4 by TLC. Compounds 1, 3, and 4 were also detected under UV-254 nm and compounds 1–5 showed positive reaction to diazotized p-nitroaniline reagent. This reac-
Table 2. $^1$H-NMR chemical shifts* of secretion components from Chrysomelinae and references

<table>
<thead>
<tr>
<th>Compound</th>
<th>3-Isoxazolin-5-one</th>
<th>Glucopyranose unit</th>
<th>3-Nitropropanoyl group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H-3</td>
<td>H-4</td>
<td>H-1</td>
</tr>
<tr>
<td>1</td>
<td>8.48, d</td>
<td>5.51, d</td>
<td>5.15, d</td>
</tr>
<tr>
<td>J</td>
<td>3.9</td>
<td>3.6</td>
<td>(4.9, d)</td>
</tr>
<tr>
<td>(published data&lt;sup&gt;d&lt;/sup&gt;)</td>
<td>(8.40, d)</td>
<td>(5.35, d)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>8.45, d</td>
<td>5.30, d</td>
<td>5.13, d</td>
</tr>
<tr>
<td>J</td>
<td>3.9</td>
<td>3.7</td>
<td>9.0</td>
</tr>
<tr>
<td>(published data&lt;sup&gt;e&lt;/sup&gt;)</td>
<td>(8.3)</td>
<td>(8.5, 9.5)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>8.45, d</td>
<td>5.30, d</td>
<td>5.13, d</td>
</tr>
<tr>
<td>J</td>
<td>3.9</td>
<td>3.7</td>
<td>9.0</td>
</tr>
<tr>
<td>(published data&lt;sup&gt;f&lt;/sup&gt;)</td>
<td>(5.74)</td>
<td>(4.92)</td>
<td></td>
</tr>
</tbody>
</table>

*a Chemical shifts are in δ, and J values are in Hz.

*b Compounds 2 and 5 are replaced by H-1, H-2, H-3, H-4, H-5, H-6a, b, H-2' and H-3', respectively.

c d, t, dd, ddd and m stand for doublet, triplet, double doublet, two set-double doublet and multiplet, respectively.

d Pasteels et al., 1982; * Stermitz et al., 1972; † Majak et al., 1992.
tion is restricted to primary and secondary aliphatic nitro compounds with an $\alpha$ hydrogen(s), and is useful for detection of nitropropanoyl glucosides (Majak and Bose, 1974). Compounds 1–5 were clearly separated by HPLC, but were isolated by preparative TLC.

The $^1$H- and $^{13}$C-NMR spectra of compounds 1, 2, and 5 (Tables 2 and 3) corresponded well with previously published data (Stermitz et al., 1972; Pfeffer et al., 1979; Pasteels et al., 1982; Majak et al., 1992). Therefore, we concluded that compounds 1–5 were replaced by C-1, C-2, C-3, C-4, C-5, C-6, C-1', C-2', and C-3', respectively.

The $^1$H-NMR spectrum of compound 1 was similar to that of compound 3, and the $^1$H-$^1$H COSY spectrum revealed a downfield shift in the H'-3 signal indicating that C'-3 was esterified. The FAB mass spectrum of compound 3 exhibited fragments at $m/z$ 450 [M+H]$^+$. Therefore, we concluded that compound 3 was 2-[2',6'-di-(3'-nitropropanoyl)-$\beta$-d-glucopyranosyl]-3-isoxazolin-5-one.

The $^1$H-NMR spectrum of compound 4 (Table 2) was similar to that of compound 3, but the $^1$H-$^1$H COSY spectrum revealed a downfield shift in the H'-3 signal indicating that C'-3 was esterified. The FAB mass spectrum of compound 4 exhibited fragments at $m/z$ 450 [M+H]$^+$. Therefore, compound 4 was identified as 2-[3',6'-di-(3'-nitropropanoyl)-$\beta$-d-glucopyranosyl]-3-isoxazolin-5-one.

**Comparison of secretions from the four species**

TLC and HPLC revealed that *P. versicolora distincta*, *C. vigintipunctata costella*, and *G. depressa* secrete the same compounds as *G. atrocyanea*, although *G. depressa* secreted only trace amounts of compounds 2 and 5 (Table 4).

**Deterrent effects of the secretions**

Ants were observed to immediately move away from the filter papers treated with secretions from the four species. The secretions were significant deterrents ($p \leq 0.01$) to *T. caespitum* (Fig. 1). Compounds 3 and 5 were also significant deterrents ($p \leq 0.05$) at the tested concentrations (Fig. 2). Compounds 2 and 4 exhibited no significant activity, although several ants avoided the filter paper.

**DISCUSSION**

Pasteels et al. (1982, 1984) reported that secre-
Adult Secretions of Japanese Chrysomelinae

Table 4. Components of secretions from four Japanese Chrysomelinae

<table>
<thead>
<tr>
<th>Component</th>
<th>G. atrocyanea</th>
<th>P. versicolora distincta</th>
<th>C. vigintipunctata costella</th>
<th>G. depressa</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>24.0</td>
<td>15.8</td>
<td>9.2</td>
<td>11.3</td>
</tr>
<tr>
<td>2</td>
<td>3.4</td>
<td>6.0</td>
<td>17.5</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>29.7</td>
<td>11.4</td>
<td>4.6</td>
<td>3.3</td>
</tr>
<tr>
<td>4</td>
<td>3.4</td>
<td>11.0</td>
<td>9.4</td>
<td>1.1</td>
</tr>
<tr>
<td>5</td>
<td>11.6</td>
<td>7.6</td>
<td>12.5</td>
<td>+</td>
</tr>
</tbody>
</table>

*a Each value was calculated from the HPLC peak area for the secretions from G. atrocyanea (N=700), P. versicolora distincta (N=100), C. vigintipunctata costella (N=191), G. depressa (N=788) dissolved in 8.4, 1.2, 2.3 and 9.5 ml acetone, respectively.

b Trace amount.

Fig. 1. Deterrent activity of the secretions of four Japanese Chrysomelinae against the ant, Tetramorium caespitum. Solid bar: the mean number (±SD) of ants feeding on the control solution (5 µl 1 M sucrose). Open bar: the mean number (±SD) of ants feeding on the treated solution (5 µl 1 M sucrose plus 2 ASEs). Wilcoxon’s signed-ranks test: **p≤0.01 (N=10).

Fig. 2. Deterrent activity of the components of the secretion from Chrysomelinae against the ant, Tetramorium caespitum. Solid bar: the mean number (±SD) of ants feeding on the control solution (5 µl 1 M sucrose). Open bar: the mean number (±SD) of ants feeding on the treated solution (5 µl 1 M sucrose plus 15 µg of each compound). Compound names (1-5): see text. Wilcoxon’s signed-ranks test: *p≤0.05 (N=10).
subsequent encounters, the ants actively avoided
the secretions. The bioassay results revealed that
the secretions from each beetle were significantly
deterrent to T. caespitum immediately after con-
tacting the treated filter paper with antennae or
mouth parts. These responses suggest that the
secretions act as contact deterrents as non-volatile
compound(s). Pasteels et al. (1986) reported that
compound 1 was a deterrent against the ant, Myr-
mica rubra. However, in the present study, com-
 pound 1 showed no activity. The conflicting data
are likely due to the use of different ant species and
test methods. Compounds 3 and 5 were significant
deterrents, whereas compounds 2 and 4 exhibited
slight, but not significant deterrency. The differ-
ence in chemical structure likely influences the ef-
ficacy of the compounds. Although the secretions
from the four species were highly potent deter-
r ents, each compound individually was substan-
tially less effective. This difference in efficacy was
likely a result of the concentrations tested. The
beetle secretions (Fig. 1) were applied to the filter
paper at 2 ASEs, which is equivalent to at least 30
µg (30 µg was twice the total amount of G. de-
pressa secretion, Table 4), whereas only 15 µg of
each individual compound (Fig. 2) was tested.

Compounds 2, 3, 4, and 5 deterred T. caespitum
in this study, and Pasteels et al. (1986) reported
that compounds 1 and 6 were protective against M.
rubra. Nitropropanoyl glucosides exhibit toxicity
and deter feeding in insects (Byers et al., 1977;
Hutchins et al., 1984), and nitropropanoic acid, its
aglycone, is toxic to insects, birds, and nonrumin-
nants (Williams et al., 1969; Bell, 1974; Janzen et
al., 1977; Gustine, 1979). Therefore, these isolated
compounds are likely to participate in the defense
of Chrysomelinae against predators.

REFERENCES

Bell, M. E. (1974) Toxicology of karaka kernel, karakin, and

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