Insecticidal Sesquiterpene from *Alpinia oxyphylla* against *Drosophila melanogaster*

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In the course of screening for novel naturally occurring insecticides from Chinese crude drugs, an MeOH extract of *Alpinia oxyphylla* was found to possess insecticidal activity against larvae of *Drosophila melanogaster* Meigen. From the extract, an insecticidal compound was isolated by bioassay-guided fractionation and identified as nootkatone (**1**) by GC, GC-MS, and ^{1}H and ^{13}C NMR spectroscopy. In bioassays for insecticidal activity, **1** showed an LC_{50} value of 11.5 μmol/mL of diet against larvae of *D. melanogaster* and an LD_{50} value of 96 μg/adult against adults. Epinootkatol (**1A**), however, showed slight insecticidal activity in both assays, indicating that the carbonyl group at the 2-position in **1** was the important function for enhanced activity of **1**.

Keywords: *Alpinia oxyphylla; Drosophila melanogaster Meigen.; sesquiterpene; nootkatone; epinootkatol; insecticidal activity; structure−activity relationship*

INTRODUCTION

In our search for new naturally occurring insecticidal compounds, we have used Chinese crude drugs having a history of safe use as medicines (Miyazawa et al., 1991, 1992, 1993, 1996a,b).

The crude drug "yakuchi" in Japan, prepared from the fruits of *Alpinia oxyphylla*, originated in southern China. This plant has been used for gastrointestinal disturbances as a folk remedy in Japan and other Oriental countries. As for its chemical components, monoterpenes (pinene, camphor, 1,8-cineole), sesquiterpenes (zingiberene, zingiberol, nootkatone, nootkatol, oxyphyllenonic acid A and B, oxyphyllenone A and B, and oxyphyllenodiol), and vanillyl compounds (yakuchinone A and B) have been reported (Namba, 1980; Itokawa et al., 1981, 1982; Shoji et al., 1984; Muraoka et al., 1998).

In screening for the development of new drugs from natural products, an MeOH extract of *A. oxyphylla* was found to have insecticidal activity against *Drosophila melanogaster* Meigen. Further analysis of the extract indicated strong activity in the fraction containing nootkatone, which was confirmed to be the active constituent.

The sesquiterpene nootkatone, the principal flavoring constituent in grapefruit and Japanese summer orange, was reported to be an antiulcer compound (Yamahara et al., 1990). Insecticidal and antifeeding effects of sesquiterpenes against *Heliothis armigera*, *Aphis gossypii*, *Pieris rapae*, *Plutella xylostella*, *Brevicoryne brassicae*, *Spodoptera littoralis*, *Ostrina furnacalis*, and *Tribollum castaneum* have been reported (Tu et al., 1990; Liu et al., 1990; Wang et al., 1991; González et al., 1993; Dadang et al., 1996).

Biological activity of *A. oxyphylla* against *D. melanogaster* has not been reported.

The present paper deals with the isolation of the active principles, their derivatives, and their insecticidal activity against *D. melanogaster*.

MATERIALS AND METHODS

Chemical Analysis. ^{1}H and ^{13}C NMR spectra were recorded on a JEOL JNM GSX 270 NMR spectrometer with $CDCl_3$ as solvent. ^{1}H NMR was measured with TMS as internal standard. Electron impact mass spectra (EI-MS) were obtained at 70 eV by GC-MS on a Hewlett-Packard 5972 series mass spectrometer interfaced with a Hewlett-Packard 5890 gas chromatograph fitted with a column (HP-5MS, 30 m × 0.25 mm i.d., temperature = 140 °C, 4 °C/min). IR spectra were determined with a Perkin-Elmer 1760-X infrared Fourier transform spectrometer with an ordinated scale for the region 4000−450 cm^{-1}. Specific rotation was determined with a Jasco DIP-140 digital polarimeter.

Materials. Commercially available air-dried fruits of *A. oxyphylla* MIQ. were obtained from Takasago Yakugyou Co. (Osaka, Japan). *D. melanogaster* Meigen., used in bioassays for insecticidal activity, was provided by Professor Ishikawa of the University of Tokyo. Rotenone was purchased from Sigma Chemical Co. (St. Louis, MO).

Extraction and Isolation (Figure 1). Insecticidal compound was isolated from fruit of *A. oxyphylla* by bioassay-guided fractionation as outlined in Figure 1. Air-dried fruits of *A. oxyphylla* (5 kg) were extracted under reflux with MeOH for 12 h. The solvent was removed in vacuo to give 333.6 g of the crude extract, which was successively reextracted with CH_2Cl_2, EtOAc, BuOH, and water. The active CH_2Cl_2 extract after concentration (202.9 g) was fractionated by SiO_2 column chromatography with hexane−EtOAc. Fraction 3 (18.7 g) had the most potent activity against larvae of *D. melanogaster*. Furthermore, fraction 3 eluted with hexane−ether (4:1) was fractionated by SiO_2 column chromatography with hexane−ether.

Rechromatography of fraction 7 (1.7 g) eluted with hexane−ether (9:1) was rechromatographed on an SiO_2 column with hexane−ether to yield 298 mg of compound **1**.

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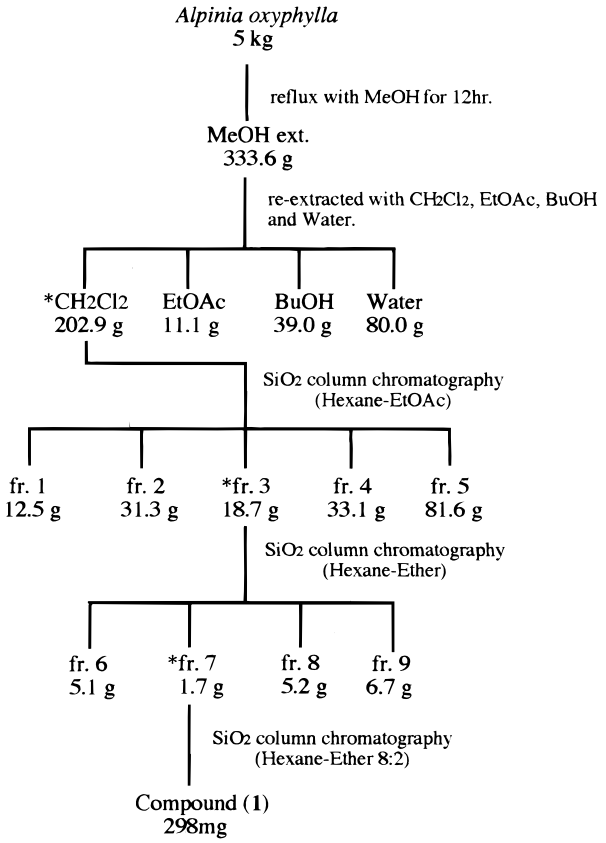
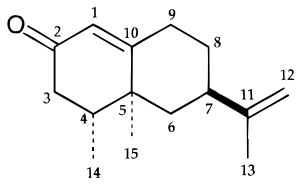
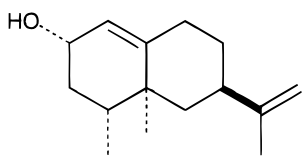


Figure 1. Scheme for the isolation of the insecticidal compound from *A. oxyphylla*.

Reduction of Nootkatone. To the ether solution (6 mL) of nootkatone (**1**) (200 mg) was added 25 mg of $LiAlH_4$, and the mixture was stirred overnight at room temperature. The



nootkatone (**1**)



epinootkatol (**1A**)

suspension was extracted with ether. The organic phase was dried over anhydrous Na_2SO_4, and the solvent was removed under reduced pressure. The residue was chromatographed on silica gel [hexane−acetone (97:3)]. Nootkatol (7 mg) was eluted, followed by epinootkatol (**1A**) (oily, 185 mg).

Bioassay for Insecticidal Activity against Larvae of *D. melanogaster*. The bioassay for insecticidal activity against larvae of *D. melanogaster* was carried out as follows (Miyazawa et al., 1991, 1992, 1993, 1996a,b). Four concentrations (4.6, 9.2, 18.3, and 36.7 μmol/mL of diet) were used for determining LC_{50} values. Test compounds were dissolved in 50 μL of EtOH and mixed in 1 mL of artificial diet [brewers' yeast (60 g), glucose (80 g), agar (12 g), and propionic acid (8 mL) in water (1000 mL)]. A control diet was treated with 50 μL of EtOH only.

About 100 adults from the colonies of *D. melanogaster* were introduced into a new culture bottle, into which artificial diet had been poured into a Petri dish, and allowed to oviposit at 25 °C and relative humidity >60% for 3 h. The diet was taken out of the bottle, and 10 new eggs were collected and transplanted onto each diet in 1 mL glass tubes and reared at 25 °C and relative humidity >90% for 8 days. One day after the transplantation, larvae were hatched and fed each test compound with the artificial diet. At 25 °C, larvae generally change to pupae for 7 days. The developmental stage was observed, and the numbers of pupae were recorded and compared with those of a control. Ten new eggs were used in each of the three replicates. LC_{50} is the lethal concentration for 50% mortality and was determined by log-probit analysis (Litchfield and Wilcoxon, 1949).

Bioassay for Acute Toxicity against Adults of *D. melanogaster*. Acute toxicity was determined by topical application to adults of *D. melanogaster* (Miyazawa et al., 1996a,b). Adults of *D. melanogaster* from culture bottles were iced to stop their mobility and treated with each of the test compounds at doses of 300, 200, 100, and 50 μg in 0.5 μL of acetone placed on their abdomens with a 10 μL microsyringe. Controls were treated with 0.5 μL of acetone only. After a set time interval, survival of the adults was recorded. Fifty adults were used in all assays. The LD_{50} was determined using log-plobit analysis (Litchfield and Wilcoxon, 1949).

Nootkatone (1) was obtained as bright yellow needles: mp 36−39 °C; $[\alpha]_D$ +181° (*c* 1.0, $CHCl_3$); MS (70 eV), *m/z* 218 ([M^+], 19%), 147 (100), 121 (68), 91 (66), 41 (64); IR υ_{max}, 2935, 1671, 1456, 1456, 1286, 888 cm^{-1}; ^1H NMR (270.1 MHz, $CDCl_3$) δ 5.77 (1H, s, H-1), 4.74 (2H, d, H-12), 1.74 (3H, s, H-13), 1.13 (3H, s, H-15), 0.97 (3H, d, H-14); ^{13}C NMR (67.8 MHz, $CDCl_3$) δ 14.87 (C-14), 16.82 (C-15), 20.80 (C-13), 31.60 (C-8), 33.00 (C-6), 39.29 (C-5), 40.30 (C-4), 40.44 (C-7), 42.05 (C-3), 43.91 (C-9), 109.22 (C-12), 124.65 (C-1), 149.05 (C-11), 170.47 (C-10), 199.59 (C-2).

Epinootkatol (1A) was obtained as a colorless oil: $[\alpha]_D$ +90° (*c* 1.0, $CHCl_3$); MS (70 eV), *m/z* 220 ([M^+], 75%), 177 (100), 121 (60), 41 (59); IR υ_{max}, 3317, 2858, 1645, 1456, 1373, 1024, 888, 757 cm^{-1}; ^1H NMR (270.1 MHz, $CDCl_3$) δ 5.33 (1H, bd, H-1), 4.68 (2H, m, H-12), 4.25 (1H, m, H-2), 1.00 (3H, s, H-15), 0.89 (3H, d, H-14), 1.72 (3H, m, H-13); ^{13}C NMR (67.8 MHz, $CDCl_3$) δ 15.36 (C-14), 18.20 (C-15), 20.80 (C-4), 32.32 (C-6), 32.86 (C-8), 37.20 (C-3), 38.14 (C-5), 39.23 (C-13), 40.73 (C-7), 44.56 (C-9), 67.99 (C-2), 108.52 (C-12), 124.24 (C-1), 146.01 (C-11), 150.22 (C-10).

RESULTS AND DISCUSSION

Isolation of Active Principle. An insecticidal compound was isolated from fruit of *A. oxyphylla* by bioassay-guided fractionation as outlined in Figure 1. Air-dried fruit of *A. oxyphylla* were extracted with MeOH under reflux for 12 h. The crude extract was concentrated and partitioned with CH_2Cl_2, EtOAc, BuOH, and water successively. The extract with CH_2Cl_2 was fractionated by SiO_2 column chromatography with hexane−EtOAc. Fraction 3 had the most potent activity against larvae of *D. melanogaster*. Furthermore, fraction 3 was fractionated by SiO_2 column chromatography with hexane−ether. Fraction 7 was repeatedly chromatographed on an SiO_2 column, by which **1** was isolated as the active principle. Compound **1** was identified as nootkatone by GC, GC-MS, and ^1H and ^{13}C NMR spectroscopy, respectively.

Insecticidal Effects of 1 and 1A against Larvae. The insecticidal effect of **1** and **1A** against larvae of *D. melanogaster* is shown in Table 1. The insecticidal effect against larvae of *D. melanogaster* was determined by our own system of feeing larvae with artificial diet containing test compound. When larvae were fed with the diet containing 36.7 μmol of **1** or **1A**/mL of diet, **1** and **1A** killed 100 and 73.3% of larvae, respectively. At 4.6 μmol/mL of diet, **1** killed 26.7% of larvae. At the same concentration **1A** did not kill larvae. Therefore, 50% lethal concentrations (LC_{50}) of larvae were 11.5 and 25.6 μmol/mL of diet for **1** and **1A**, respectively. All of these compounds were, however, less active than ro-

Table 1. Insecticidal Activities of Compounds 1 and 1A against Larvae of *D. melanogaster*

	concentration (μmol/mL of diet)[a]					LC_{50}[c] (μmol/mL of diet)
compd	control	36.7	18.3	9.2	4.6	
1	10, 10, 10[b]	0, 0, 0	1, 2, 2	6, 5, 5	8, 7, 7	11.5
1A		3, 3, 2	7, 7, 6	8, 8, 9	10, 10, 10	25.6

	concentration (μmol/mL of diet)[a]					LC_{50}[c] (μmol/mL of diet)
compd	0.65	0.13	0.05	0.01	0.005	
rotenone	0, 0, 0	0, 0, 0	3, 2, 2	7, 8, 7	9, 9 ,9	0.022

[a] Test compounds of each concentration were dissolved in 50 μL of EtOH and mixed in 1 mL of artificial diet. [b] Numbers of pupae: After 8 days from transplantation, the 10 newly laid eggs on the diet, and three replicates. [c] LC_{50} (the lethal concentration for 50% mortality) determined by log-probit analysis.

Table 2. Acute Toxicities of Compounds 1 and 1A against Adults of *D. melanogaster*

	survival[a] (% relative to controls) at dose[b] (μg/adult)				LD_{50}[c] (μg/adult)
compd	300	200	100	50	
1	0	17	47	73	96
1A	27	57	87	100	222

	survival[a] (% relative to controls) at dose[b] (μg/adult)						LD_{50}[c] (μg/adult)
compd	10	7.0	5.0	3.0	1.0	0.5	
rotenone	0	10	30	70	90	95	3.7

[a] After 3 h, survival of the adults was recorded and compared with controls. [b] Test compounds of each dose were dissolved in 0.5 μL of acetone and treated on the abdomen of adult with a 10 μL microsyringe. Controls were treated with 0.5 μL of acetone only. [c] LD_{50} is the lethal dose for 50% mortality determined by log-probit analysis.

tenone, a naturally occurring poison that killed all of the larvae at 0.13 μmol/mL of diet.

Acute Toxicities of 1 and 1A against Adults. Acute toxicity against adults of *D. melanogaster* was determined by topical application on the abdomen of adults (Table 2).

Acute toxicities of these sesquiterpenes are shown in Table 2. At a dose of 100 μg/adult of **1**, 53% of adults were dead. At 50 μg/adult, **1** killed 27% of adults. The 50% lethal dose (LD_{50}) of adults was found to be 96 μg/adult. At 300 and 200 μg/adult, **1A** killed 73 and 43% of adults, respectively. However, **1A** had slight activity, and the LD_{50} value was 222 μg/adult.

In this research, **1** was only detected as an insecticidal component from *A. oxyphylla*. However, it was previously reported to contain at least nine sesquiterpenoids: zingiberene, zingiberol, nootkatone, nootkatol, oxyphyllenonic acid A and B, oxyphyllenone A and B, and oxyphyllenodiol (Shoji et al., 1984; Muraoka et al., 1998). However, the insecticidal activity was not reported. Although **1** and **1A** demonstrated insecticidal activity against *D. melanogaster* in this study, the activity of **1A** was weaker than that of **1**. Furthermore, investigation of the structure−activity relationship suggested that the carbonyl group at the 2-position was an important structural feature for the enhanced insecticidal activity of **1**.

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