Biologically Active Components against *Drosophila melanogaster* from *Podophyllum hexandrum*

Mitsuo Miyazawa,*,† Mami Fukuyama,† Kimio Yoshio,† Takenori Kato,† and Yukio Ishikawa‡

Department of Applied Chemistry, Faculty of Science and Engineering, Kinki University, Kowakae, Higashiosaka-shi, Osaka 577-8502, Japan, and Laboratory of Applied Entomology, Faculty of Agriculture, University of Tokyo, Bunkyo-ku, Tokyo 113-8657, Japan

In the course of screening for novel naturally occurring insecticides from Chinese crude drugs, a dichloromethane extract of *Podophyllum hexandrum* was found to give an insecticidal activity against larvae of *Drosophila melanogaster* Meigen. From the extract, an insecticidal compound was isolated by bioassay-guided fractionation. The compound was identified as podophyllotoxin (**1**) by comparison of its spectroscopic characteristics with literature data. In bioassays for insecticidal activity, **1** showed a LC_{50} value of 0.24 μmol/mL diet against larvae of *D. melanogaster* and a LD_{50} value of 22 μg/adult against adults. Acetylpodophyllotoxin (**1A**), however showed slight insecticidal activity in both assays, indicating that the 4-hydroxyl group was an important function for enhanced activity of **1**.

Keywords: *Podophyllum hexandrum; Podophyllaceae; lignan; Drosophila melanogaster Meigen; podophyllotoxin; 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 medicine (Miyazawa et al., 1991, 1992, 1993, 1996a,b). A CH_2Cl_2 extract of *Podophyllum hexandrum* was found to exhibit insecticidal activity against larvae of *Drosophila melanogaster* Meigen.

The genus *Podophyllum* has four well-known species, one in the Himalayas, one in America, and two of Chinese origin. Several other less-known Chinese species have also been identified and chemically investigated. The genus was the subject of vigorous phytochemical investigations in the early 1960s after the isolation of several lignans (Atta-ur-Rahman et al., 1995).

Among the plant polyphenols, of which over 8000 are known, the flavonoids such as quercetin form the largest group. However, phenolic quinones, lignans, xanthones, coumarins, and other classes exist in considerable numbers. In addition to monomeric and dimeric structures, there are three important groups of phenolic polymers—the lignins of the plant cell wall, the black melanin pigments of plants, and the tannins of woody plants.

Plant polyphenols are economically important because they make major contributions to the taste and color of our food and drinks. The flavor and taste of tea is related to the fact that the tea leaf contains up to 30% of its dry weight as polyphenol. Likewise, the bitterness of beer is due to the content of the phloroglucinol derivative, humulone, while the color of red wine is imparted by anthocyanins, such as the pigment malvin. In nature, phenolics have a significant role in protecting plants from being overeaten by herbivores. They also act as chemical signals in the flowering and pollination of plants and in the processes of plant symbioses and plant parasitism.

A number of lignans isolated from *Podophyllum* species have shown a wide range of biological activities, such as antitumor, antimitotic, and antiviral activities. Some of them have also shown toxicity to fungi, insects, and vertebrates (Macrea et al., 1984). In a previous paper, the successful chemical conversion of the podophyllotoxin into the clinically useful anticancer drugs Etoposide and Teniposide has also triggered further research in this area (Issel et al., 1984). The insecticidal activity of podophyllotoxins and congeners against *Blattella germanica*, *Epilachna sparsa orientalis*, and *Plutella xylostella* has been reported (Inamori et al., 1986). However, there is no detailed report about the insecticidal activity of podophyllotoxin against insects of *Diptera* order. In this paper, we describe the isolation and identification of the insecticidal compounds against *D. melanogaster* Meigen from *P. hexandrum*.

EXPERIMENTAL PROCEDURES

Chemical Analysis. ^1H and ^{13}C NMR spectra were recorded on a JEOL GSX 270 NMR spectrometer with $CDCl_3$ as the solvent. ^1H NMR spectra were measured with TMS as the internal standard. Electron impact mass spectra (EI-MS) were obtained at 70 eV by GC-MS on a Hewlett-Packard 5890. 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. A commercially available air-dried root of *P. hexandrum* was obtained from Takasago Yakugyou Co. (Osaka). *D. melanogaster* Meigen., used in the bioassay for insecticidal activity, was provided by Professor Ishikawa of University of Tokyo.

Extraction and Isolation. Air-dried rhizomes of *P. hexandrum* (800 g) were extracted with CH_2Cl_2 under reflux for

* To whom correspondence should be addressed (telephone +81-6-6721-2332; fax +81-6-6727-4301).
† Kinki University.
‡ University of Tokyo.

10.1021/jf9903509 CCC: $18.00
Published on Web 11/18/1999

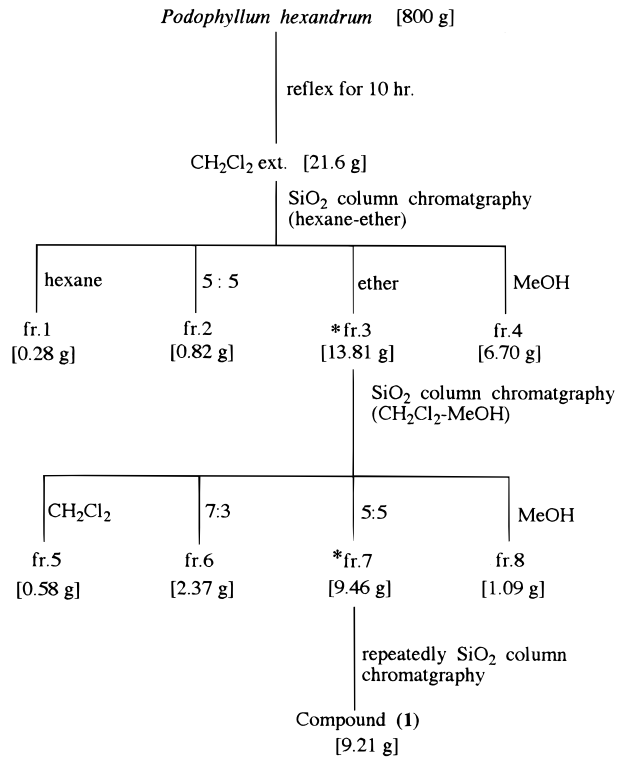


Figure 1. Isolation scheme of compound **1** from *Podophyllum hexandrum*.

10 h. The solvent was removed under reduced pressure to give a crude extract. The crude extract with CH_2Cl_2 (21.6 g) was fractionated by silica gel column chromatography with a hexane−ether mixture. Furthermore, fraction 3 eluated with ether fractionated by silica gel column chromatography with CH_2Cl_2−MeOH. Rechromatography of fraction 7 eluting with CH_2Cl_2−MeOH (5:5) gave 9.21 g of compound **1**.

Acetylation of 1. The acetate (compound **1A**) of **1** was obtained by reaction with acetic anhydride and pyridine.

Bioassay for Insecticidal Activity against Larvae of *D. melanogaster*. The bioassay for insecticidal activity against larvae of *D. melanogaster* was carried out as described previously (Miyazawa et al., 1991, 1992, 1993, 1996a,b). Five concentrations (0, 0.1, 0.5, 1, and 2 μmol/mL diet) were used for determining the LC_{50} value. 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.

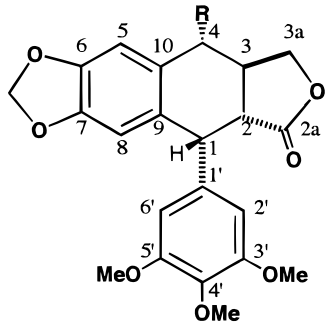
About 100 adults from the colonies of *D. melanogaster* were introduced into a new culture bottle; in which artificial diet poured into the bottom of a Petri dish was placed, and allowed to oviposit at 25 °C and RH >60% for 3 h. The diet was taken out of the bottle; 10 newly laid eggs were collected and transplanted onto each diet in a 1 mL glass tube and reared at 25 °C and RH >90% for 8 days. The developmental stage was observed, and the number of pupae was recorded and compared with those of a control. Ten newly laid eggs were used in each of the three replicates. LC_{50} is the lethal concentration for 50% mortality, as 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 a culture bottle were frozen to stop their movement, and treated with each of the test compounds at doses of 100, 50, 30, 20, 15, 10, and 5 μg in 0.5 μL of acetone 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. LD_{50} is the lethal dose for 50% mortality, as was determined from log-probit analysis (Litchfield and Wilcoxon, 1949).

Podophyllotoxin (1): $[\alpha]_D^{23}$ −96.4°; mp 103−109 °C; IR γ_{max}, cm^{-1} 1774, 1593, 1481, 1230, 940, 1428, 1381, 1126; ^1H NMR (270, 1 MHz, $CDCl_3$) δ 2.04 (1H, s, OH), 2.80 (2H, m, H-2,3), 3.74 (6H, s, OCH_3-3′,5′), 3.80 (3H, s, OCH_3-4′), 4.02 (H, t, J = 9.5 Hz, H-3aβ), 4.58 (2H, m, H-1,3aα), 4.73 (1H, d, J = 8.6 Hz, H-4b), 5.95 (1H, d, J = 1.6 Hz, OCH_2O), 5.97 (1H, d, J = 1.6 Hz, OCH_2O), 6.37 (2H, s, H-2′,6′), 6.49 (1H, s, H-8), 7.11 (1H, s, H-5).

RESULTS AND DISCUSSION

Isolation of Active Principle. The insecticidal compounds were isolated from *P. hexandrum* by bioassay-guided fractionation. *P. hexandrum* was extracted with CH_2Cl_2 under reflex for 10 h. The CH_2Cl_2 extract was fractionated by silica gel column chromatography with hexane−ether. The isolation scheme for **1** by bioassay-guide is shown in Figure 1. Fraction 3 had the most potent activity against larvae of *D. melanogaster*. Furthermore, the fraction 3 was fractionated by silica gel column chromatography with CH_2Cl_2−MeOH. The fraction 7 was repeatedly chromatgraphied on silica gel by which **1** was isolated as the active principle. Compound **1** was identified as podophyllotoxin by spectral data compared with previous reports (Sibata et al., 1961; Jackson et al., 1984; Ayres and Lim, 1972).



1: R=OH
1A: R=OAc

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 larvae fed with artificial diet containing test compound. When larvae were fed with the diet containing 2.0 μmol/mL diet, **1** and **1A** killed 100% and 93.3% of the larvae. At 0.5 μmol/mL diet, **1** killed 76.6% of larvae whereas **1A** caused only 40.0% mortality. Therefore, the concentration of compounds **1** and **1A** at 50% lethal concentration (LC_{50}) of larvae as 0.24 and 0.64 μg/mL diet, respectively. Furthermore, compounds **1** and **1A** had a more powerful insecticidal activity than (*E*)-anethole (Miyazawa et al., 1993), safrole (Miyazawa et al., 1991), (−)-tetrahydroberberine (Miyazawa et al., 1996a), asaricin (Miyazawa et al., 1991), methyleugenol (Miyazawa et al., 1992), elemicine (Miyazawa et al., 1992), and γ-asarone (Miyazawa et al., 1992).

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. Acute toxicities of these lignans were shown in Table 2. Application at 30 μg/adult of **1** per adult resulted in 75% mortality. At 15 μg/adult, **1** killed 30%

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

		concentration (μmol/mL diet)[a]				
compound	control	2	1	0.5	0.1	LC_{50} (μmol/mL diet)[c]
1	10, 10, 10[b]	1, 0, 0	1, 1, 1	2, 3, 2	6, 4, 6	0.24
1A		1, 1, 0	2, 3, 2	7, 5, 6	8, 7, 9	0.64

	concentration (μmol/mL diet)[a]						LC_{50} (μmol/mL diet)[c]
	1.30	0.65	0.13	0.05	0.01	0.005	
Rotenone	0, 0, 0	0, 0, 0	0, 0, 0	2, 3, 2	7, 8, 6	9, 9, 9	0.022

[a] Test compounds of each concentrations 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 newly 10 eggs laid 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 Adult of *D. melanogaster*

	survival[a] (in % to controls) at dose[b] (μg/adult)						LD_{50} (μg/adult)[c]
compound	100	50	30	20	15	5	
1	0	0	25	50	70	100	22
1A	45	60	63	70	85	100	80

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

[a] Test compounds of each doses were dissolved in 0.5 μL of acetone and treated on the abdomen of an adult with a 10 μL-microsyringe. Controls were treated with 50 μL of acetone only. [b] After a set time interval, survival of the adults were recovered and compared with controls. [c] LD_{50} (the lethal dose for 50% mortality) determined by log-probit analysis.

of adults. The LD_{50} of adults was found to be 22 μg/adult. At 100 and 30 μg/adult, **1A** killed 55% and 37% of adults, respectively. Thus, **1A** had a lower activity and the LD_{50} value was 80 μg/adult.

In this research, only **1** was detected as an insecticidal component from *P. hexandrum*. In previous paper, this species was shown to contain at least 10 aryltetralin: 4′-demethylpodophyllotoxin, β-peltatin, α-peltatin, desoxypodophyllotoxin, isopicropodophyllone, podophyllotoxone, 4′-demethylpodophyllotoxone, 4′-demethyldesoxypodophyllotoxin and 4′-demethylisopicropodophyllone (Jackson and Dewick, 1985). These compounds were useful to cathartics in America. Also, they had a were reported antitumor action but cannot adapt to the whole body because of their high toxicity (Karime, 1967). With regard to insecticidal activity, it was previously reported that against *Blattella germanica*, *Epilachna sparsa orientalis*, *Plutella xylostella*, and *Culex pipiens molestus*, **1** and deoxypodophyllotoxin interestingly gave contrasting results in that the insecticidal activity of **1** was weaker than that of deoxypodophyllotoxin except for this action on *Epilachna sparsa orientalis* (Inamori et al., 1986). Although **1** and **1A** demonstrated an insecticidal activity against *D. melanogaster* similar to the recent paper, the activity of **1A** was weaker than that of **1**. Furthermore, investigation of structure-activity relationship suggested that the 4-hydroxyl group was an important function for enhanced insecticidal activity of **1**. The difference between our results and a previous report is considered to be due to the difference in the metabolic pathways of test insects.

LITERATURE CITED

Ayres, D. C.; Lim, C. K. Lignans and related phenols. Part XIII. Halogenated derivatives of podophyllotoxin. *J. Chem. Soc. Perkin 1* **1972**, 1350−1355.

Inamori, Y.; Kubo, M.; Tsujibo, H.; Ogawa, M.; Baba, K.; Kozawa, M.; Fujita, E. The Biological activities of Podophyllotoxin compounds. *Chem. Pharm. Bull.* **1986**, *34*, 3928−3932.

Issel, B. F.; Muggia, F. M.; Carter, S. K. *Etoposide (VP-16), Current status and new development*; Academic Press: Orlando, FL, 1984.

Jackson, D. E.; Dewick, P. M.; Aryltetralin lignans from *Podophyllum hexandrum* and *Podophyllum peltatum*. *Phytochemistry* **1984**, *23*, 1147−1152.

Jackson, D. E.; Dewick, P. M. Tumour-inhibitory aryltetralin lignans from *Podophyllium pleianthum*. *Phytochemistry* **1985**, *24*, 2407−2409.

Karime, T. *Saishin Syokubutukagaku*; Hirokawa Press: Tokyo, Japan, p 80.

Litchfield, J. T., Jr.; Wilcoxon, F. A simplified method of evaluating dose−effect experiments. *J. Pharmacol. Exp. Ther.* **1949**, *98*, 99−113.

Macrae, W. D.; Towers, G. H. N. Review article number 2 biological activities of lignans. *Phytochemistry* **1984**, *23*, 1207−1220.

Miyazawa, M.; Ishikawa, Y.; Toshikura, M.; Kameoka, H.; Insecticidal compounds from *Asiasarum heterotropoides* Maek. var mandshuricum Maek. *Chem. Express* **1991**, *6*, 703−706.

Miyazawa, M.; Ishikawa, Y.; Toshikura, M.; Kameoka, H.; Insecticidal allylbenzenes from *Asiasarum heterotropoides* Maek. var mandshuricum Maek. *Chem. Express* **1992**, *7*, 69−72.

Miyazawa, M.; Ota, H.; Ishikawa, Y.; Kameoka, H. An insecticidal compound from *Illicium verum*. *Chem. Express* **1993,** *8*, 437−440.

Miyazawa, M.; Yoshio, K.; Ishikawa, Y.; Kameoka, H. Insecticidal alkaloid against *Drosophila Melanogaster* from tubers of *Corydalis bulbosa*. *Nat. Prod. Lett.* **1996a**, *8*, 299−302.

Miyazawa, M.; Yoshio, K.; Ishikawa, Y.; Kameoka, H. An insecticidal alkaloid against *Drosophila Melanogaster* form rhizomes of *Nuphar japonicum* DC. *Nat. Prod. Lett.* **1996b**, *8*, 307−310.

Rahman, A.; Ashraf, M.; Choudhary, M. I.; Rehman, H.; Kazmi, M. H. Antifungal aryltetralin from leaves of *Podophyllum hexandrum*. *Phytochemistry* **1995**, *40*, 427−431.

Sibata, S.; Murata, T.; Fufita, M. Studies on the Constituents of Japanese and Chinese Drugs. On the Constituents of Rhizome and Root of *Podophyllum pleianthum* Hance. *Yakugaku Zasshi* **1961**, *82*, 777−779.

Received for review April 9, 1999. Revised manuscript received September 1, 1999. Accepted September 14, 1999.

JF9903509