Insecticidal Alkaloids against *Drosophila melanogaster* from *Nuphar japonicum* DC.

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In the course of screening for novel naturally occurring insecticides from Chinese crude drugs, MeOH extract of rhizomes of *Nuphar japonicum* DC. was found to give an insecticidal activity against larvae of *Drosophila melanogaster* Meigen. Four alkaloids, (−)-7-*epi*-deoxynupharidine (**1**), (−)-castoramine (**2**), (−)-nupharolutine (**3**), and (−)-nupharmine (**4**), were isolated by bioassy-guided fractionation from the extract. Insecticidal activity against larvae of *D. melanogaster* was demonstrated; **2** and **1** showed LC_{50} values of 1.00 and 4.33 μmol/mL of diet concentration, respectively. Acute toxicity against adults of *D. melanogaster* was also found; **1** had the most potent activity, with an LD_{50} value of 0.86 μg/adult. **1**, **2**, and **3** caused acetylcholinesterase inhibitions, at 0.5 mmol/L, of 99.6, 65.0, and 59.7%, respectively; however, **4** had only slight activity in this study. Therefore, **1** and **2** were identified as insecticidal compounds from rhizomes of *N. japonicum* DC. Structure−bioactivity relationship study indicated the importance of the position of the hydroxyl group and the piperizine skeleton for enhanced activity.

Keywords: *Nuphar japonicum DC.; Nymphaeaceae; rhizomes; Drosophila melanogaster Meigen; sesquiterpene alkaloids; (−)-7-epi-deoxynupharidine; (−)-castoramine; (−)-nupharolutine; (−)-nupharmine; 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. In the course of screening for novel naturally occurring insecticides from Chinese crude drugs, the MeOH extract of rhizomes of *Nuphar japonicum* DC. was found to exhibit an insecticidal activity against larvae of *Drosophila melanogaster* Meigen. (−)-7-*epi*-Deoxynupharidine from this plant was shown to be a potentially insecticidal compound in our previous paper (Miyazawa et al., 1996b).

N. japonicum DC. is a perennial rhizomatous herb with a wide distribution in the temperate zone of the northern hemisphere. From the Chinese crude drug the rhizomes of *N. japonicum* DC. are called "Senkotsu" in Japanese. In Asia they have been used in folk medicine as a diuretic and also as a stomach analgesic. The genus *Nuphar* contains many alkaloids (Arata and Ohashi, 1965; Cybulski et al., 1988; Iwanow et al., 1986; LaLonde et al., 1970, 1972; Maurer and Ohloff, 1976; Peura and Lounasamaa, 1977; Wong and LaLonde, 1970a,b; Wróbel, 1967) and tannins (Ishimatsu et al., 1989a,b; Nishizawa et al., 1982; Nonaka et al., 1987).

Over 5000 alkaloids are known, ranging from relatively simple structures such as coniine from hemlock to exceedingly complex ones such as the neurotoxin batrachtoxin, also found in the skin of a Colombian frog. They are most commonly encountered in the plant kingdom, but representatives have been isolated from most other orders of organisms ranging from fungi to mammals.

Their manifold pharmacological activities have always excited man's interest, and since early times selected plant products (many containing alkaloids) have been used as poisons for hunting, murder, and euthanasia; as euphoriants, psychedelics, and stimulants (i.e., morphine and cocaine), or as medicines (i.e. ephedrine). Many of our modern drugs now contain the same compounds or synthetic analogues, and the pharmacological and toxicological properties of these compounds are thus of immense interest and importance.

Biological activity of the genus *Nuphar* against insects had not been reported. In this paper, bioassay-guided isolation and insecticidal activities of compounds from rhizomes of *N. japonicum* DC. are described.

EXPERIMENTAL PROCEDURES

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 an internal. 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 rhizomes of *N. japonicum* DC. were obtained from Takasago Yakugyou Co. (Osaka). *Drosophila melanogaster* Meigen used in bioassay for insecticidal activity was distributed from Professor Ishikawa of the University of Tokyo. 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB) was purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo). Acetylthiocholine iodide (ATC) was purchased from

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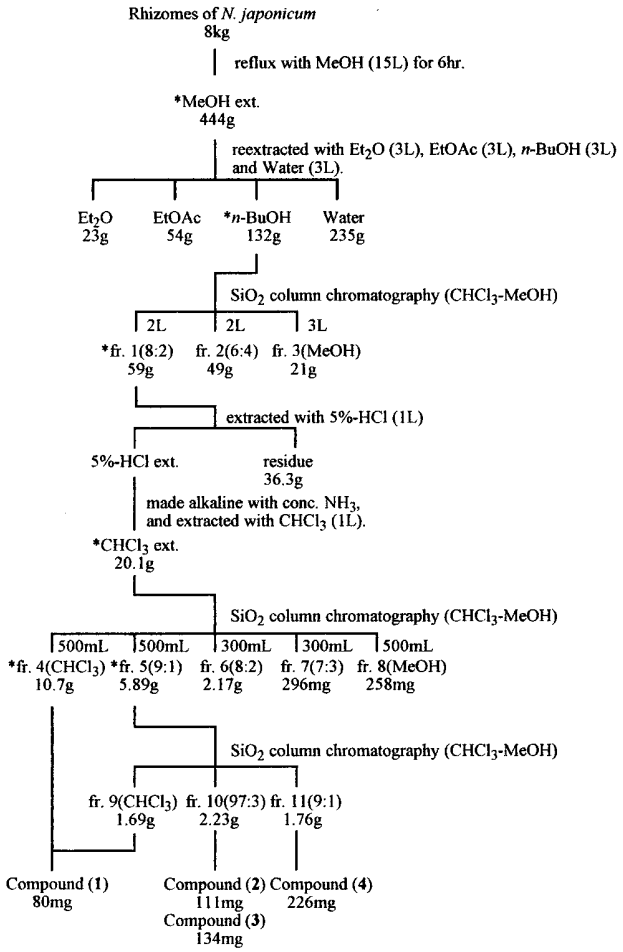

Figure 1. Isolation scheme for insecticidal compounds from rhizomes of *N. japonicum* DC.

Kanto Chemical Co., Inc. (Tokyo). Rotenone was purchased from Sigma Chemical Co. (St. Louis, MO). (+)-Pulegone was purchased from Taiyo Perfume Co., Ltd. (Osaka).

Extraction and Isolation. Air-dried rhizomes of *N. japonicum* (8 kg) were extracted with MeOH under reflux for 8 h (Figure 1). The solvent was removed in vacuo to give 444 g of the crude extract, which was successively reextracted with Et_2O, EtOAc, *n*-BuOH, and H_2O. The active *n*-BuOH extract after concentration (132 g) was fractionated by silica gel column chromatography with $CHCl_3$/MeOH. The active fraction 1 (59 g) eluted with $CHCl_3$/MeOH (8:1) was dissolved with $CHCl_3$ and treated with 5% HCl. The acidic aqueous layer was adjusted to pH 10 with concentrated NH_4OH, saturated with NaCl, and extracted with $CHCl_3$. The $CHCl_3$ layer was washed with H_2O, dried over anhydrous Na_2SO_4, and removed in vacuo to give 20.1 g of a mixture of alkaloids. The alkaloidal mixture was fractionated by silica gel column chromatography with $CHCl_3$/MeOH (Figure 1). The active fraction 4 (10.7 g) eluted with $CHCl_3$ and was rechromatographed on SiO_2 gel with *n*-hexanes/EtOAc. Eluate with *n*-hexanes/EtOAc (9:1) was purified on PTLC with *n*-hexanes/EtOAc/28% NH_3 (5:5:0.1) to yield compound (**1**) (35 mg). Further activity was obtained with fraction 5, which eluted with $CHCl_3$/MeOH (9:1); rechromatography of this fraction on SiO_2 gel eluted with $CHCl_3$, and the eluate was purified on PTLC with *n*-hexanes/EtOAc/28% NH_3 (5:5:0.1) to obtain **1** (26 mg). Fraction 10 eluted with $CHCl_3$/MeOH (97:3) was rechromatographed on SiO_2 gel with *n*-hexanes/EtOAc to yield 72 mg of compound **2** and 58 mg of compound **3**, respectively. Fraction 11 eluted with $CHCl_3$/MeOH (9:1) was rechromatographed on SiO_2 gel with *n*-hexanes/EtOAc to yield 137 mg of compound **4**.

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, 1994, 1996a,b): Five concentrations (0.42, 1.25, 2.08, 4.17, and 6.25 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 [brewer's 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.

The artificial diet was poured into Petri dishes and placed on the bottom of culture bottles. About 100 adults from colonies of *D. melanogaster* were introduced into the new culture bottle and allowed to oviposit for 3 h (25 °C and RH > 60%). 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 RH > 90% for 8 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). The adults of *D. melanogaster* from culture bottles were iced to stop their movement, and treated on their abdomens with each of the test compounds at doses of 50, 20, 10, 7, 5, 3, 1, and 0.75 μg in 0.5 μL of acetone with a 10 μL microsyringe. Controls were treated with 0.5 μL of acetone only. After 30 min, survival of the adults was recorded. Fifty adults were used for all assays containing control. LD_{50} is the lethal dose for 50% mortality and was determined from log-probit analysis (Litchfield and Wilcoxon, 1949).

Bioassay for Inhibition of Acetylcholinesterase from Heads of Adults of *D. melanogaster*. Enzyme mixture containing acetylcholinesterase (AChE) was extracted from heads of adults of *D. melanogaster* according to the method of Grundy and Still (1985). About 1000 adults were frozen at −80 °C for 7 days. The frozen adults were shaken for 1 min to detach heads. Separation of the heads from bodies was then accomplished by sieving through mesh (14 meshes/cm^2) so as to allow only the heads to pass. The heads were then homogenized in 10 mL of 0.1 M phosphate buffer at pH 8.0. The crude homogenate was centrifuged at 25000*g* for 30 min, and the supernatant was used as the enzyme. ATC was dissolved in 0.1 M phosphate buffer (pH 8.0). DTNB (39.6 mg) was dissolved in 10 mL of 0.1 M phosphate buffer at pH 7.0, and 15 mg of $NaHCO_3$ was added.

Inhibition of AChE was determined according to the colorimetric method of Ellman et al. (1961). Both the control and test solutions employed 0.2 mL of the enzyme solution and 0.1 mL of DTNB added to 2.4 mL of 0.1 M phosphate buffer (pH 8.0). The test solutions were added to each of the test compounds dissolved in 50 mL of EtOH. The control solution was similarly prepared by the addition of 50 mL of EtOH. Both control and each of the test solutions were preincubated at 25 °C for 5 min. After preincubation, the enzyme reaction was started by the addition of 40 mL of ATC followed by incubation at 25 °C for 20 min. After 20 min, the absorbance at 412 nm was measured spectrophotometrically and compared with that of the control.

(−)-7-*epi*-Deoxynupharidine (1): colorless oil; $[\alpha]_D$ −71.6° (*c* 1.0, $CHCl_3$); IR ($CHCl_3$, ν_{max} cm^{-1}) 2951, 2922, 2873, 2851, 2769, 1672, 1501, 1458, 1438, 1376, 1161, 1029, 874, 787, 772; ^{1}H NMR (270.1 MHz, $CDCl_3$) δ 7.35 (1H, m, α-furyl H), 7.31 (1H, br s, α-furyl H), 6.45 (1H, br s, β-furyl H), 2.78−2.95 (2H, m, H-4*ax*. and H-6*eq*.), 2.1−1.1 (12H), 0.91 (3H, d, J = 5.8 Hz, 1-Me), 0.73 (3H, d, J = 6.0 Hz, 7-Me); ^{13}C NMR (67.8 MHz, $CDCl_3$) δ 19.2 (1-Me), 19.8 (7-Me), 30.1 (C-9), 31.0 (C-7), 33.5 (C-8), 34.0 (C-2), 34.9 (C-3), 36.3 (C-1), 61.0 (C-4), 61.2 (C-6), 69.3 (C-10), 109.8 (C-15), 129.1 (C-13), 139.5 (C-14), 143.0 (C-16); EI-MS (70 eV) *m*/*z* 233 (M^+, 27.1%), 218 (5.4), 204 (12.6), 190 (18.1), 177 (8.4), 162 (12.2), 148 (16.1), 136 (31.4), 121 (9.9),

107 (12.9), 98 (100), 97 (22.6), 94 (85.4), 81 (22.6), 69 (9.9), 55 (29.5), 41 (31.7).

(−)-Castoramine (2): white crystal; $[\alpha]_D$ −79.4° (*c* 1.0, $CHCl_3$); mp 65.4−66.5 °C; IR (KBr, υ_{max} cm^{-1}) 3226, 2978, 2910, 2871, 2784, 1738, 1670, 1502, 1456, 1379, 1155, 1135, 1100, 1037, 874, 791, 764; ^{1}H NMR (270.1 MHz, $CDCl_3$) δ 7.35 (2H, m, α-furyl H), 6.45 (1H, br s, *β*-furyl H), 3.75 (2H, d, J = 2.4 Hz, 7-CH_2OH), 3.07 (1H, dd, J = 11.3, 2.1 Hz, H-6*eq*.), 2.95 (1H, dd, J = 6.4, 6.9 Hz, H-4*ax*.), 2.5−1.1 (12H), 0.92 (3H, d, J = 5.9 Hz, 1-Me); ^{13}C NMR (67.8 MHz, $CDCl_3$) δ 19.3 (1-Me), 65.1 (7-CH_2OH), 29.1 (C-9), 33.5 (C-8), 34.3 (C-2), 35.0 (C-3), 36.4 (C-1), 39.3 (C-7), 59.4 (C-4), 63.8 (C-6), 68.2 (C-10), 109.6 (C-15), 129.5 (C-13), 139.8 (C-14), 143.4 (C-16); EI-MS (70 eV) *m*/*z* 249 (M^+, 52.1%), 234 (14.3), 220 (19.0), 206 (23.2), 194 (9.0), 178 (38.5), 164 (12.8), 148 (15.0), 136 (53.8), 121 (14.4), 114 (73.8), 107 (29.5), 94 (100), 81 (25.9), 70 (23.6), 55 (18.8).

(−)-Nupharolutine (3): white crystal; $[\alpha]_D$ −104.3° (*c* 1.0, $CHCl_3$); mp 139.7−141.2 °C; IR (KBr, υ_{max} cm^{-1}) 3220, 2980, 2914, 2874, 2786, 1736, 1670, 1503, 1455, 1436, 1380, 1165, 1153, 1135, 1102, 1064, 1038, 1019, 874, 791, 764; ^{1}H NMR (270.1 MHz, $CDCl_3$) δ 7.33 (2H, m, α-furyl H), 6.35 (1H, br s, *β*-furyl H), 3.08−3.00 (1H, m, H-4*ax*.), 2.68 (1H, dd, J = 11.0, 2.3 Hz, H-6*eq*.), 2.0−1.0 (10H), 1.21 (3H, s, 7-Me), 0.90 (3H, d, J = 6.7 Hz, 1-Me); ^{13}C NMR (67.8 MHz, $CDCl_3$) δ 19.2 (1-Me), 25.4 (7-Me), 28.0 (C-9), 33.6 (C-2), 34.7 (C-3), 36.3 (C-1), 38.3 (C-8), 59.6 (C-4), 62.2 (C-6), 68.2 (C-7), 69.3 (C-10), 109.3 (C-15), 129.1 (C-13), 139.3 (C-14), 143.1 (C-16); EI-MS (70 eV) *m*/*z* 249 (M^+, 28.0%), 234 (8.7), 220 (11.6), 206 (15.5), 194 (6.0), 178 (28.3), 164 (9.5), 148 (11.2), 136 (46.4), 121 (12.9), 114 (72.8), 107 (28.3), 94 (100), 81 (25.6), 70 (26.0), 55 (18.5).

(−)-Nuphamine (4): colorless oil; $[\alpha]_D$ −47.8° (*c* 1.0, $CHCl_3$); IR ($CHCl_3$, υ_{max} cm^{-1}) 3297, 2928, 2852, 1736, 1670, 1502, 1438, 1378, 1160, 1105, 1064, 1025, 874, 792, 760; ^{1}H NMR (270.1 MHz, $CDCl_3$) δ 7.34 (2H, m, α-furyl H), 6.39 (1H, br s, *β*-furyl H), 5.43 (1H, br t, H-8), 3.99 (2H, s, H-11), 3.57 (1H, dd, J = 11.5, 2.6 Hz, H-2), 2.5−1.0 (8H), 1.69 (3H, s, 10-Me), 0.92 (3H, d, J = 6.4 Hz, 3-Me); ^{13}C NMR (67.8 MHz, $CDCl_3$) δ 14.0 (C-10), 18.4 (C-12), 31.6 (C-7), 33.8 (C-4), 34.3 (C-5), 35.5 (C-3), 53.5 (C-6), 63.6 (C-2), 61.0 (C-4), 68.4 (C-11), 109.1 (C-15), 122.2 (C-8), 129.2 (C-13), 137.3 (C-9), 138.4 (C-14), 142.8 (C-16); EI-MS (70 eV) *m*/*z* 248 (M^+, 0.5%), 216 (0.3), 200 (0.1), 188 (0.2), 175 (0.4), 164 (100), 147 (5.1), 135 (2.5), 120 (2.7), 107 (62.1), 94 (20.8), 79 (11.0), 67 (3.6), 53 (3.1).

RESULTS AND DISCUSSION

Isolation of Active Principles and Related Compounds. Insecticidal compounds were isolated from rhizomes of *N. japonicum* by bioassay-guided fractionation as outlined in Figure 1. Air-dried rhizomes of *N. japonicum* were extracted with MeOH under reflux for 6 h. The crude extract was concentrated and partitioned with Et_2O, EtOAc, *n*-BuOH, and H_2O successively. The *n*-BuOH fraction had the most potent activity against larvae of *D. melanogaster*. The extract with *n*-BuOH was fractionated by silica gel column chromatography with $CHCl_3$/MeOH. The active fraction 1 eluted with $CHCl_3$/MeOH (8:2) was dissolved with $CHCl_3$ and treated with 5% HCl. The acidic aqueous layer was brought to pH 10 with concentrated NH_4OH, saturated with NaCl, and extracted with $CHCl_3$. The $CHCl_3$ layer was washed with H_2O, dried over anhydrousNa_2SO_4, and removed in vacuo to give a mixture of alkaloids. The mixture of alkaloids was repeatedly chromatographed on silica gel and purified by PTLC, by which four alkaloids were isolated as the active constituents. The alkaloids were identified as (−)-7-*epi*-deoxynupharidine (**1**), (−)-castoramine (**2**), (−)-nupharolutine (**3**), and (−)-nuphamine (**4**) (Figure 2) by spectral data compared with previous paper (Itatani et al., 1976; Lalonde et al., 1975; Maurer and Ohloff, 1976; Wróbel et al., 1972).

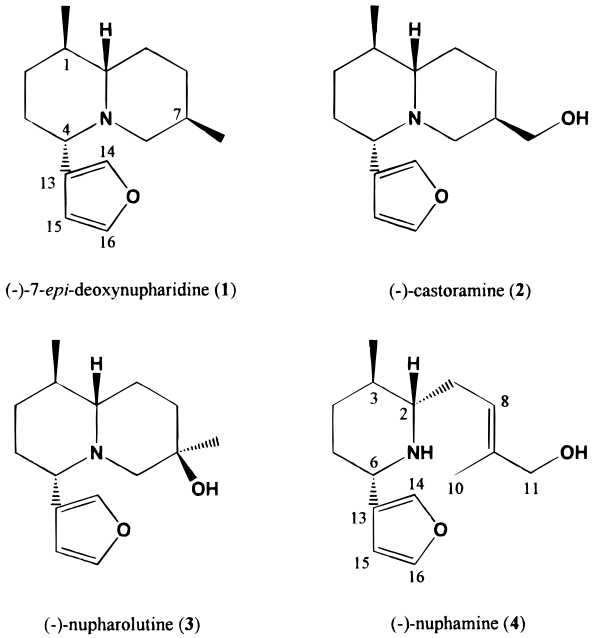

Figure 2. Structures of four alkaloids isolated from rhizomes of *N. japonicum* DC.

Insecticidal Activity against Larvae. The insecticidal effects of the four alkaloids against larvae of *D. melanogaster* are outlined in Table 1. The insecticidal effect against larvae of *D. melanogaster* was determined with an artificial diet containing test compounds. Of the four alkaloids, castoramine (**2**) had the greatest activity. When larvae were fed with the diet containing 2.08 μmol/mL of diet of **2**, all of larvae were killed; a 50% lethal concentration (LC_{50}) of larvae was 1.00 μmol/mL of diet concentration. Castoramine (**2**) showed the same level of insecticidal activity as (*E*)-anethole (Miyazawa et al., 1993) and (−)-tetrahydroberberine (Miyazawa et al., 1996a), but greater activity than safrole (Miyazawa et al., 1991), asaricin (Miyazawa et al., 1991), methyleugenol (Miyazawa et al., 1992), elemicine (Miyazawa et al., 1992), and *γ*-asarone (Miyazawa et al., 1992). (−)-7-*epi*-Deoxynupharidine (**1**) showed the second most potent activity; 48.3% of larvae were dead at 4.16 μmol/mL of diet, and LC_{50} value of larvae was 4.21 μmol/mL of diet (Miyazawa et al., 1996b). However, **1** showed less insecticidal activity than insecticidal compounds we had reported before (Miyazawa et al., 1991, 1992, 1993, and 1996a). Comparable to the above-mentioned two alkaloids, **3** and **4** were less active (LC_{50} values >4.16 μmol/mL of diet concentration, respectively). All of these alkaloids were, however, less active than rotenone, known as a naturally occurring poison, which killed all larvae at 0.13 μmol/mL.

Acute Toxicity against Adults. Acute toxicity against adults of *D. melanogaster* was determined by topical application on the abdomens of adults. Acute toxicities of these alkaloids are outlined in Table 2. In this test, **1** showed the most potent activity. Even at 1.0 μg/adult of **1** applied, 80% of adults were dead, and 50% lethal dose (LD_{50}) of adults was found to be 0.86 μg/adult (Miyazawa et al., 1996b). **1** was more active compared with (−)-tetrahydroberberine (LD_{50} =2.5 μg/adult; Miyazawa et al., 1996) and methyleugenol (LD_{50} = 6.2 μg/adult; Miyazawa et al., 1992). Furthermore, **1** was more active than rotenone (LD_{50} = 3.7 μg/adult). **3** had a slight activity; at 20 μg/adult 70% of adults were

Table 1. Insecticidal Activities (Expressed as Numbers of Pupae)[a] of Compounds 1−4 against Larvae of *D. melanogaster*

	concentration[a] (μmol/mL of diet)						
compd	control	4.17	2.92	2.08	1.25	0.42	LC_{50}[c] (μmol/mL of diet)
1	9, 10, 10	4, 4, 6	5, 7, 8	7, 9, 9	9, 10, 10	10, 10, 10	4.21
2		0, 0, 0	0, 0, 0	0, 0, 0	3, 4, 4	7, 7, 9	1.00
3		6, 8, 8	8, 9, 9	8, 9, 10	10, 10, 10	8, 10, 10	>4.17
4		7, 8, 9	8, 9, 9	8, 9, 10	7, 8,9	7, 9, 10	>4.17

	concentration[a] (μmol/mL of diet)			
compd	1.30	0.65	0.13	LC_{50}[c] (μmol/mL of diet)
rotenone	0, 0, 0	0, 0, 0	0, 0, 0	<0.13

[a] Eight days after transplantation (10 eggs newly laid, 3 replicates). [b] Test compounds of each concentration were dissolved in 50 μL of EtOH and mixed in 1 μL of artificial diet. [c] LC_{50} is the lethal concentration for 50% mortality, determined by log-probit analysis.

Table 2. Acute Toxicities (Expressed as Survival)[a] of Compounds 1−4 against Adults of *D. melanogaster*

	dose[b] (μg/adult)								LD_{50}[c]
compd	50	20	10	7.0	5.0	3.0	1.0	0.75	(μg/adult
1	0	0	0	0	0	0	20	70	0.86
2	30	70	80	100	100	100	100	100	35.3
3	10	30	90	100	100	100	100	100	16.3
4	60	100	100	100	100	100	100	100	>50.0

	dose[b] (μg/adult)						LD_{50}[c]
	10	7.0	5.0	3.0	1.0	0.5	(μg/adult)
rotenone	0	10	30	70	90	95	3.7

[a] After 30 min, survival of adults was recorded (percent relative to controls). [b] Test compounds of each dose were dissolved in 0.5 μL of acetone and applied on the abdomens of adults with a 10 μL microsyringe. Negative controls were treated with 50 μL of acetone only. [c] LD_{50} is the lethal dose for 50% mortality, determined by log-probit analysis.

Table 3. Inhibition[a] of Compounds 1−4 against Acetylcholinesterase from Heads of *D. melanogaster*

	concentration[b] (mmol/L)				
compd	1.5	1.0	0.5	0.25	0.125
1			99.6	74.9	
2		91.9	65.0		
3		67.8	59.7		
4	59.2	42.5			
(+)-pulegone			75.4	60.7	41.1
rotenone	0	0	0	0	

[a] After incubation for 20 min, changes in absorbance at 412 nm were recorded and compared with control. [b] Test compounds of each dose were dissolved in 50 μL of EtOH, added in each vial. Negative control was added 50 μL of EtOH only.

killed. The LD_{50} value of **3** was 16.3 μg/adult. Although **2** exhibited strong activity against larvae, it was much less active against adults (LD_{50}, = 35.3 μg/adult). Although **4** caused barely 40% mortality at a dose of 50 μg/adult, the LD_{50} was >50.0 μg/adult.

Inhibition of Acetylcholinesterase. Bioassay for acetylcholinesterase inhibition in vitro was carried out according to the colormetric method of Ellman et al. (1961) to investigate the mode of action of acute toxicity. Inhibitions of acetylcholinesterase of four alkaloids are outlined in Table 3. Similar to the results for acute toxicity against adult, **1** had the most potent activity of these alkaloids. At 0.5 and 0.25 mmol/L, inhibitions for **1** were 99.6 and 74.9%, respectively. At a dose of 1.0 mmol/L, inhibitions for **2** and **3** were 91.9 and 67.8%, respectively. At 0.5 mmol/L, **2** and **3** gave inhibitions of 65.0 and 59.7%, respectively. Furthermore, **1**−**3** had strong activities comparable to that of (+)-pulegone (i.e., 60.7% at 1.0 mmol/L; Grundy and Still, 1985; Miyazawa et al., 1997), whereas rotenone had no activity. However, **4** was the least active (42.5% at 1.0 mmol/L). Therefore, the acute toxicity of **1** against adults may be due to the inhibition of acetylcholinesterase.

In summary, the insecticidally active compounds from rhizomes of *N. japonicum* were **1** and **2** in the test systems used in this investigation. Comparison with the insecticidal activities toward larvae of these alkaloids shows that there was a great difference between the activities of **2** and **3**. Furthermore, the results suggest that for a strong activity against larvae a hydroxymethyl group at the C-7 position may be most important. Against adults, **1** was more potent compared to **2** and **3**. In addition, a great inanition in activity was observed among the results of acetylcholinesterase inhibitions of **1**−**3**. Therefore, the presence of a hydroxy substituent may be disadvantageous for acute toxicity against adults. Also, because **4** had a slight activity in this study, a piperizine skeleton, furthermore, had a potential of the revelation of an insecticidal activity.

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