Insecticidal Alkaloids from *Corydalis bulbosa* against *Drosophila melanogaster*

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A MeOH extract of tubers of *Corydalis bulbosa* had insecticidal activity against larvae of *Drosophila melanogaster* Meigen. Four protoberberine alkaloids, (−)-tetrahydroberberine (**1**), (−)-tetrahydrocopticine (**2**), (+)-corydaline (**3**), (±)-tetrahydropalmatine (**4**) and (±)-dehydrocorydaline (**5**) as its iodide were isolated from the extract. Compounds **5**, **1** and **2** exhibited LC_{50} values toward larvae of *D. melanogaster* of 0.23 μmol/mL, 0.91 μmol/mL and 1.70 μmol/mL diet concentration, respectively. Against adults, **1** showed the most potent activity with an LD_{50} value of 2.5 μg/adult. **1**, **2**, **3** and **4** inhibited acetylcholinesterase by 78.7, 71.8, 68.2 and 64.6%, respectively, at 1.0mM. Furthermore, **5** showed the most potent activity with inhibition of 61.3% at 0.40 mM. **5**, **1** and **2** were identified as insecticidal compounds from *C. bulbosa*. Investigation of the structure−activity relationship indicated the importance of the methylenedioxyl group and double bonds in the isoquinolizine moiety for enhanced activity of the protoberberine alkaloids.

Keywords: *Corydalis bulbosa; Papaveraceae; tubers; Drosophila melanogaster Meigen.; protoberberine alkaloids; (−)-tetrahydroberberine; (−)-tetrahydrocopticine; (+)-corydaline; (±)-tetrahydropalmatine; (±)-dehydrocorydaline iodide; 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. We found that a MeOH extract of tubers of *Corydalis bulbosa* had insecticidal activity against larvae of *Drosophila melanogaster*. (−)-Tetrahydroberberine from this plant was shown as one of the insecticidal compounds in our previous paper (Miyazawa et al., 1996a).

C. bulbosa is a perennial tuberous plant, widely distributed mainly in the moderate geographical regions of the northern hemisphere. It is mostly found in the forest and bush areas of the mountainous belts up to 2500 m above sea level. The tubers of *C. bulbosa* are called the Chinese crude drug "Engosaku" in Japanese. They have been used in Asian folk medicine as a febrifuge, antidote, or analgesic for a long time. They are well-known as rich sources of alkaloids, identified as types of protoberberine, aporphine, and protopine (Imaseki and Taguchi, 1961; Manske et al., 1978; Kiryakov et al., 1979, 1981; Fu et al., 1988; Slavík and Slavíková 1989; Ito et al., 1990; Sener and Temizer, 1991).

The alkaloids are structurally a very diverse class of secondary metabolites, and >5000 compounds are known, ranging from relatively simple structures such as coniine from hemlock to exceedingly complex ones such as the neurotoxin batrachtoxin, from the skin of a Colombian frog. They are most commonly encountered in the plant kingdom, but representatives also have been isolated from 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 (morphine and cocaine); and as medicines (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.

The biological activity of the protoberberine-type of alkaloids against insects has not been previously reported. The present paper deals with the isolation of active principles and their derivatives and their insecticidal activity against *D. melanogaster*.

EXPERIMENTAL PROCEDURES

Chemical Analysis. ^1H and ^{13}C NMR spectra were recorded on a JEOL GSX 270 NMR spectrometer with $CDCl_3$ or DMSO-d_6 as solvent. ^1H NMR was measured with TMS as internal standard. Electron impact mass spectra (EI-MS) were obtained at 70 eV by direct inlet on Shimadzu QP-1000A. 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 and powdered tubers of *C. bulbosa* were obtained from Takasago Yakugyou Co. (Osaka). *Drosophila melanogaster* Meigen. used in bioassay for insecticidal activity was obtained from Professor Ishikawa of the University of Tokyo. 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB) was purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo). Acetylcholinesterase iodide (ATC) was purchased from Kanto Chemical Co., Inc. (Tokyo).

Extraction and Isolation (Figure 1). Air-dried and powdered tubers of *C. bulbosa* (10 kg) were extracted with MeOH under reflux for 10 h. The solvent was removed under reduced pressure to give a crude extract. The MeOH extract

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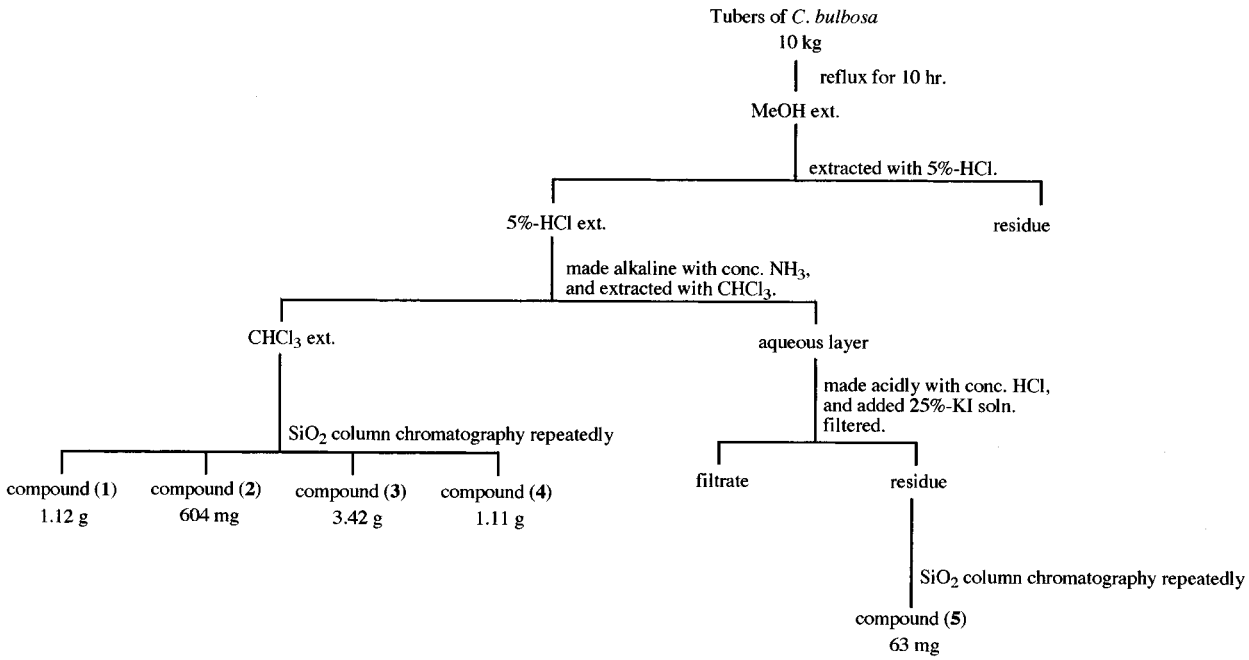

Figure 1. Scheme for isolation of insecticidal compounds from *C. bulbosa*.

(612 g) was dissolved in 1.5 L of 5% HCl and kept in the refrigerator for 24 h. Following filtration, the solution was washed with $CHCl_3$. The acidic aqueous layer was made alkaline with concentrated NH_4OH and extracted with 1 L of $CHCl_3$ three times. The $CHCl_3$ layer was washed with H_2O, dried over anhydrous Na_2SO_4, and removed under reduced pressure to give a mixture of tertiary alkaloids. The aqueous layer was again made acidic with concentrated HCl, and 200 mL of a 25% solution of potassium iodide was added. After one night, the mixture of iodides that crystallized out was collected by filtration.

The mixture (18.6 g) of tertiary alkaloids was fractionated by silica gel column chromatography with *n*-hexanes/EtOAc. The fraction eluted with *n*-hexanes/EtOAc (8:2, 2 L) was rechromatographed on silica gel with $CHCl_3$ (2 L) alone, and compounds **2** (604 mg) and **3** (3.42 g) were isolated. The fraction eluted with *n*-hexanes/EtOAc (75:15, 1 L) was recrystallized using $CHCl_3$/MeOH to obtained compound **1** (1.12 g). The fraction eluted with *n*-hexanes/EtOAc (6:4, 2 L) was purified by silica gel column chromatography with *n*-hexanes/EtOAc (7:3, 2 L) to obtain compound **4** (1.11 g).

The mixture of iodides was chromatographed on silica gel with $CHCl_3$/MeOH. Elution with $CHCl_3$ containing 2% MeOH (1 L) was repeated. Recrystallization of the substance in MeOH yielded compound **5** (63 mg) as its iodide salt.

Hydrochloride of (+)-Corydaline and (±)-Tetrahydropalmatine. Fifty milligrams each of (+)-corydaline and (±)-tetrahydropalmatine was dissolved in 50 mL of MeOH, and a mL of concentrated HCl was added at 0 °C. The solvent was removed under reduced pressure, and the residue was recrystallized from MeOH to afford hydrochloride salts.

Reduction of (±)-Dehydrocorydaline Iodide. To a stirred suspension of 40 mg of (±)-dehydrocorydaline iodide in MeOH was added 100 mg of $NaBH_4$. The reaction mixture was stirred at room temperature for 1 h. The product was purified by SiO_2 column chromatography.

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 (0.28, 0.84, 1.40, 1.96, and 2.80 μmol/mL of diet) of tertiary alkaloids and two hydrochlorides were used for determining LC_{50} values. Five concentrations (0.11, 0.22, 0.66, 1.10, and 2.20 μmol/mL of diet) of quaternary alkaloids 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, 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, and 10 new eggs were collected, transplanted onto each diet in 1 mL glass tubes, and reared at 25 °C and RH >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 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 were iced to stop their movement and treated on their abdomens with each of the test compounds at doses of 10, 7, 5, 3, and 1 μg in 0.5 μL of acetone with a 10 μL microsyringe. Controls were treated with 0.5 μL of acetone only. After 3 h, survival of the adults was recorded. Fifty adults were used in all assays. LD_{50} is the dose for 50% mortality and was determined using log-probit analysis (Litchfield and Wilcoxon, 1949).

Assay for Inhibition of Acetylcholinesterase from Heads of Adults of *D. melanogaster*. A crude extract containing acetylcholinesterase (AChE) was prepared from the heads of adults of *D. melanogaster* (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 the heads. Separation of the heads from bodies was then accomplished by sieving through mesh 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 source. 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 calorimetric method of Ellman (Ellman et al., 1961). The enzyme

solution (0.2 mL) and DTNB (0.1 mL) were added to 2.4 mL of 0.1 M phosphate buffer (pH 8.0) to be used for both the control and test solutions. The test solutions were added to each of the test compounds dissolved in 50 μL of EtOH or DMSO. Control was similarly prepared by the addition of 50 μL of EtOH or DMSO only. The 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 μL of ATC and further incubated at 25 °C for 20 min. After 20 min, the absorbance at 412 nm was measured spectrophotometrically and compared with that of control.

(−)-Tetrahydroberberine (1): white crystals; $[\alpha]_D$ −295.98 (*c* 0.3, $CHCl_3$); mp 132.8−134.5 °C; IR (υ_{max}, cm^{-1}) 2937, 2834, 2748, 1607, 1495, 1487, 1487, 1456, 1428, 1390, 1336, 1279, 1248, 1223, 1165, 1142, 1085, 1039, 992, 939, 861, 801, 753; ^{1}H NMR (270.1 MHz, $CDCl_3$) δ 6.81 (1H, *d*, *J* = 8.8 Hz, H-12), 6.78 (1H, *d*, *J* = 8.8 Hz, H-11), 6.73 (1H, *s*, H-1), 6.59 (1H, *s*, H-4), 5.91 (2H, *s*, 2,3-OCH_2O), 4.23 (1H, *d*, *J* = 15.0 Hz, H-8ax.), 3.84 (6H, *s*, 9, 10-OMe), 3.53 (1H, *d*, *J* = 15.0 Hz, H-8eq.), 3.52 (1H, *dd*, *J* = 11.8, 3.5 Hz, H-13a), 3.22 (1H, *dd*, *J* = 16.0, 11.8 Hz, H-13eq.), 3.05−3.22 (2H, *m*, H-5eq. and H-6eq.), 2.81 (1H, *dd*, *J* = 16.0, 3.5 Hz, H-13ax.), 2.56−2.71 (2H, *m*, H-5ax. and H-6ax.); ^{13}C NMR (67.8 MHz, $CDCl_3$) δ 29.5 (C-5), 36.4 (C-13), 51.3 (C-6), 53.9 (C-8), 55.8 (10-OMe), 59.6 (C-13a), 60.1 (9-OMe), 100.7 (2,3-OCH_2O), 105.5 (C-1), 108.3 (C-4), 111.0 (C-11), 123.8 (C-12), 127.7 (C-4a), 127.8 (C-8a), 128.6 (C-12a), 130.8 (C-14a), 145.0 (C-10), 145.9 (C-2), 146.1 (C-3), 150.2 (C-9); EIMS (70 eV, *m*/*z*) 339 ([M^+], 53.9%), 322 (4.7), 308 (11.1), 278 (2.5), 189 (2.6), 178 (13.0), 174 (29.9), 164 (100), 149 (99.1), 135 (11.1), 121 (29.4), 104 (18.6), 91 (21.2), 77 (24.3), 65 (9.3), 51 (8.5), 39 (7.2).

(−)-Tetrahydrocoptisine (2): white crystals; $[\alpha]_D$ −229.298 (*c* 0.7, $CHCl_3$); mp 192.6−194.7 °C; IR (υ_{max}, cm^{-1}) 2895, 2745, 1504, 1486, 1462, 1264, 1246, 1223, 1039; ^{1}H NMR (270.1 MHz, $CDCl_3$) δ 6.72 (1H, *s*, H-1), 6.68 (1H, *d*, *J* = 8.1 Hz, H-12), 6.62 (1H, *d*, *J* = 8.1 Hz, H-11), 6.59 (1H, *s*, H-4), 5.96 (1H, *d*, *J* = 1.6 Hz, 9,10-OCH_2O), 5.92 (1H, *d*, *J* = 1.6 Hz, 9,10-OCH_2O), 5.91 (2H, *s*, 2,3-OCH_2O), 4.09 (1H, *d*, *J* = 15.0 Hz, H-8ax.), 3.54 (1H, *d*, *J* = 15.0 Hz, H-8eq.), 3.57 (1H, *dd*, *J* = 3.8,11.2 Hz, H-13a), 3.23 (1H, *dd*, *J* = 11.2, 15.5 Hz, H-13eq.), 3.03−3.18 (2H, *m*, H-5eq. and H-6eq.), 2.80 (1H, *dd*, *J* = 3.8, 15.5 Hz, H-13ax.), 2.56−2.71 (2H, *m*, H-5ax. and H-6ax.); ^{13}C NMR (67.8 MHz, $CDCl_3$) δ 29.6 (C-5), 36.6 (C-13), 51.2 (C-6), 52.9 (C-8), 59.7 (C-13a), 100.7 (2,3-OCH_2O), 101.0 (9,10-OCH_2O), 105.5 (C-1), 106.7 (C-11), 108.4 (C-4), 116.8 (C-8a), 121.0 (C-12), 127.7 (C-4a), 128.5 (C-12a), 130.7 (C-14a), 143.2 (C-10), 144.9 (C-2), 146.0 (C-3), 146.2 (C-9); EIMS (70 eV, *m*/*z*) 323 ([M^+, 38.0%), 308 (1.5), 178 (4.4), 163 (10.8), 148 (100), 135 (4.4), 116 (5.7), 103 (5.6), 77 (6.9), 55 (2.3), 51 (5.5), 39 (5.1).

(+)-Corydaline (3): white crystals; $[\alpha]_D$ 255.108 (*c* 1.0, $CHCl_3$); mp 132.8−133.5 °C; IR (υ_{max}, cm^{-1}) 2933, 2834, 1611, 1516, 1494, 1456, 1279, 1257, 1230, 1056, 1016; ^{1}H NMR (270.1 MHz, $CDCl_3$) δ 6.91 (1H, *d*, *J* = 8.4 Hz, H-12), 6.78 (1H, *d*, *J* = 8.4 Hz, H-11), 6.69 (1H, *s*, H-1), 6.61 (1H, *s*, H-4), 4.25 (1H, *d*, *J* = 15.0 Hz, H-8ax.), 3.88 (6H, *s*, OMe), 3.87 (3H, *s*, OMe), 3.86 (3H, *s*, OMe), 3.51 (1H, *d*, *J* = 15.0 Hz, H-8eq.), 3.69 (1H, *dd*, *J* = 3.2 Hz, H-13a), 3.24 (1H, *dq*, *J* = 3.2, 6.8 Hz, H-13eq.), 2.95−3.19 (2H, *m*, H-5eq. and H-6eq.), 2.56−2.71 (2H, *m*, H-5ax. and H-6ax.), 0.94 (3H, *d*, *J* = 6.8 Hz, 13-Me); ^{13}C NMR (67.8 MHz, $CDCl_3$) δ 18.3 (13-Me), 29.3 (C-5), 38.3 (C-13), 51.3 (C-6), 54.4 (C-8), 55.8 (2,10-OMe), 56.1 (3-OMe), 60.0 (9-OMe), 63.0 (C-13a), 108.8 (C-1), 111.0 (C-11), 111.2 (C-4), 123.9 (C-12), 128.4 (C-4a), 128.5 (C-8a,14a), 128.6 (C-12a), 144.9 (C-10), 147.2 (C-2), 147.6 (C-3), 150.0 (C-9); EIMS (70 eV, *m*/*z*) 369 ([M^+, 37.0%), 354 (12.5), 338 (5.5), 323 (2.9), 190 (8.5), 178 (100), 163 (35.8), 148 (11.9), 135 (12.6), 117 (7.6), 103 (7.9), 91 (16.5), 77 (6.4), 65 (4.3), 55 (2.0), 51 (2.2), 39 (2.7).

(±)-Tetrahydropalmatine (4): white crystals; $[\alpha]_D$ ±08 (*c* 1.0, MeOH); mp 139.7−141.2 °C; IR (υ_{max}, cm^{-1}) 2937, 2834, 1612, 1515, 1495, 1456, 1279, 1258, 1231, 1085; ^{1}H NMR (270.1 MHz, $CDCl_3$) δ 6.89 (1H, *d*, *J* = 8.3 Hz, H-12), 6.79 (1H, *d*, *J* = 8.3 Hz, H-11), 6.74 (1H, *s*, H-1), 6.62 (1H, *s*, H-4), 4.25 (1H, *d*, *J* = 15.0 Hz, H-8ax.), 3.89 (3H, *s*, OMe), 3.87 (3H, *s*, OMe), 3.85 (3H, *s*, OMe), 3.84 (3H, *s*, OMe), 3.55 (1H, *d*, *J* = 15.0 Hz, H-8eq.), 3.69 (1H, *dd*, *J* = 3.2 Hz, H-13a), 3.55 (1H, *d*, *J* = 15.0 Hz, H-8eq.), 3.23 (1H, *dd*, *J* = 11.2, 15.5 Hz, H-13eq.), 3.09−3.23 (2H, *m*, H-5eq. and H-6eq.), 2.83 (1H, *dd*, *J* = 3.8, 11.5 Hz, H-13ax.), 2.59−2.72 (2H, *m*, H-5ax. and H-6ax.); ^{13}C NMR (67.8 MHz, $CDCl_3$) δ 29.1 (C-5), 36.3 (C-13), 51.5 (C-6), 54.0 (C-8), 55.8 (2,10-OMe), 56.0 (3-OMe), 59.3 (C-13a), 60.1 (9-OMe), 108.6 (C-1), 111.0 (C-11), 111.3 (C-4), 123.8 (C-12), 126.8 (C-8a), 127.7 (C-4a), 128.6 (C-12a), 129.7 (C-14a), 145.0 (C-10), 147.4 (C-2, 3), 150.2 (C-9); EIMS (70 eV, *m*/*z*) 355 ([M^+, 75.8%), 340 (9.0), 324 (16.4), 190 (33.9), 176 (9.2), 164 (96.5), 149 (100), 135 (11.0), 121 (29.4), 104 (20.5), 91 (19.0), 77 (24.3), 65 (6.7), 55 (3.8), 51 (5.4), 39 (4.2).

(±)-Dehydrocorydaline iodide (5): yellow crystals; $[\alpha]_D$ ±08 (*c* 0.2, MeOH); mp 243.8−245.2 °C; IR (υ_{max}, cm^{-1}) 3482, 3358, 3002, 2940, 2845, 1605, 1533, 1510, 1455, 1443, 1394, 1361, 1332, 1281, 1249, 1238, 1212, 1138, 1108, 1066, 1025; ^{1}H NMR (270.1 MHz, DMSO-d_6) δ 9.82 (1H, *s*, H-8), 8.17 (1H, *d*, *J* = 9.0 Hz, H-12), 8.05 (1H, *d*, *J* = 9.0 Hz, H-11), 7.56 (1H, *s*, H-1), 7.06 (1H, *s*, H-4), 4.94 (2H, *t*, *J* = 6.5 Hz, H-6), 4.12 (6H, *s*, OMe), 4.07 (3H, *s*, OMe), 3.91 (3H, *s*, OMe), 3.20 (2H, *m*, H-5), 2.50 (3H, *s*, 13-Me); ^{13}C NMR (67.8 MHz, DMSO-d_6) δ 26.0 (C-5), 39.8 (13-Me), 55.6 (C-6), 56.0 (2, 10-OMe), 57.2 (3-OMe), 61.9 (9-OMe), 111.6 (C-1), 112.4 (C-4), 119.1 (C-8a), 119.5 (C-13), 121.4 (C-14a), 123.4 (C-12), 127.0 (C-11), 128.5 (C-12a), 133.3 (C-4a), 137.9 (C-13a), 143.8 (C-10), 1145.2 (C-8), 146.5 (C-2), 150.1(C-3), 150.8 (C-9).

(+)-Corydaline Hydrochloride (3-HCl): white crystals; $[\alpha]_D$ 209.748 (*c* 0.5, $CHCl_3$); mp 155.3−157.8 °C; IR (υ_{max}, cm^{-1}) 3418, 2940, 2839, 1613, 1520, 1499, 1461, 1428, 1396, 1285, 1262, 1234, 1133, 1100, 109, 751; ^{1}H NMR (270.1 MHz, $CDCl_3$) δ 11.7 (1H, br *s*, HCl), 6.98 (1H, *d*, *J* = 8.5 Hz, H-12), 6.92 (1H, *d*, *J* = 8.5 Hz, H-11), 6.65 (2H, *s*, H-1, H-4), 4.98 (1H, *d*, *J* = 15.5 Hz, H-8ax.), 4.59 (1H, *d*, H-13a), 3.93 (3H, *s*, OMe), 3.89 (6H, *s*, OMe), 3.86 (3H, *s*, OMe), 3.8−4.13 (3H, *m*, H-6ax., H-6eq. and H-8eq.), 3.57−3.69 (1H, *m*, H-13eq.), 3.08−3.28 (1H, br, H-5eq.), 2.79 (1H, *dd*, H-5ax.), 1.36 (3H, *d*, *J* = 7.5 Hz, 13-Me); ^{13}C NMR (67.8 MHz, $CDCl_3$) δ 17.9 (13-Me), 25.8 (C-5), 36.0 (C-13), 52.5 (C-6), 53.4 (C-8), 55.9 (2,10-OMe), 56.2 (3-OMe), 60.5 (9-OMe), 63.6 (C-13a), 107.6 (C-1), 111.4 (C-11), 113.0 (C-4), 120.8 (C-14a), 121.1 (C-8a), 124.0 (C-12), 125.3 (C-4a), 130.2 (C-12a), 144.9 (C-10), 148.7 (C-2), 149.0(C-3), 150.8 (C-9).

(±)-Tetrahydropalmatine Hydrochloride (4-HCl): white crystals; $[\alpha]_D$ ±08 (*c* 1.0, MeOH); mp 178.7−179.2 °C; IR (υ_{max}, cm^{-1}) 3415, 2942, 2841, 1615, 1521, 1494, 1458, 1425, 1396, 1284, 1261, 1232, 1132, 1102, 1007, 751; ^{1}H NMR (270.1 MHz, $CDCl_3$) δ 13.1 (1H, br *s*, HCl), 6.94 (1H, *d*, *J* = 8.2 Hz, H-12), 6.89 (1H, *d*, *J* = 8.2 Hz, H-11), 6.65 (2H, *s*, H-1, H-4), 4.79 (1H, *d*, *J* = 15.8 Hz, H-8ax.), 4.59 (1H, br *s*, H-8eq.), 4.50−4.69 (1H, br, H-6eq.), 4.26−4.44 (1H, br, H-6ax.), 3.60−4.16 (2H, *m*, H-8eq. and H-13a), 3.91 (3H, *s*, OMe), 3.88 (3H, *s*, OMe), 3.87 (3H, *s*, OMe), 3.86 (3H, *s*, OMe), 3.52−3.70 (1H, *m*, H-13ax.), 3.47 (1H, *dd*, *J* = 17.0, 4.8 Hz, H-13eq.), 2.89−3.31 (1H, br, H-5eq.), 2.81 (1H, *d*, H-5ax.); ^{13}C NMR (67.8 MHz, $CDCl_3$) δ 25.7 (C-5), 32.9 (C-13), 51.8 (C-6), 52.8 (C-8), 55.9 (2,10-OMe), 56.2 (3-OMe), 60.1 (C-13a), 60.4 (9-OMe), 107.9 (C-1), 111.5 (C-4, 11), 122.6 (C-12), 121.5 (C-8a), 124.0 (C-4a), 124.1(C-12a), 124.4 (C-14a), 145.0 (C-10), 148.5 (C-2), 149.1 (C-3), 150.8 (C-9).

RESULTS AND DISCUSSION

Isolation of Active and Related Compounds. Air-dried and powdered tubers of *C. bulbosa* were extracted with MeOH under reflux for 6 h. Because tubers of *C. bulbosa* are known to be a rich source of alkaloids, the crude extract (612 g) was dissolved in 5% HCl and kept in the refrigerator for 24 h. Following filtration, the solution was washed with $CHCl_3$. The acidic aqueous layer was made alkaline with concentrated NH_4OH and extracted with $CHCl_3$. The $CHCl_3$ layer was washed with H_2O and dried over anhydrous Na_2SO_4, and the organic solvent was removed under

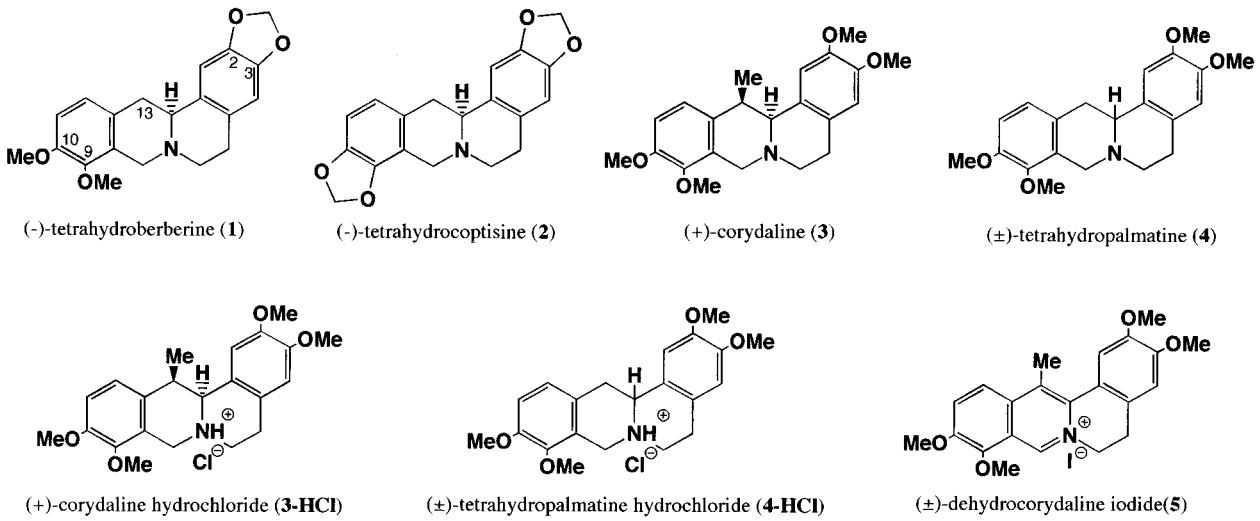

Figure 2. Structures of five alkaloids and two hydrochloride salts from *C. bulbosa*.

Table 1. Insecticidal Activities of Compounds 1−5 and Two Hydrochloride Salts 3-HCl and 4-HCl against Larvae of *D. melanogaster*

	no. of pupae[a] at concn[b] (μmol/mL of diet) of										
compd	control	2.80	2.20	1.96	1.40	1.10	0.84	0.66	0.28	0.22	0.11	LC50[a] (μmol/mL of diet)
1	10, 10, 10	0, 0, 0		0, 0, 0	0, 0, 1		7, 8, 8		7, 8, 9			0.91
2		2, 2, 3		1, 4, 6	5, 6, 8		7, 8, 8		7, 7, 8			1.70
3		5, 5, 6		6, 6, 7	7, 8, 8		8, 8, 8		6, 8, 10			>2.80
4		4, 6, 8		5, 5, 7	7, 8, 9		8, 9, 10		9, 9, 10			>2.80
3-HCl		7, 8, 10		10, 10, 10	9, 10, 10							>2.80
4-HCl		8, 8, 10		9, 10, 10	9, 10, 10							>2.80
5			0, 0, 0			0, 0, 1		3, 3, 4		5, 7, 9	7, 8, 9	

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

reduced pressure to give a mixture of tertiary alkaloids. The aqueous layer was again made acidic with concentrated HCl and a 25% solution of potassium iodide was able. After one night, the mixture of iodide salts, which crystallized out, was collected by filtration. Most of the insecticidal activity was recovered in each of mixtures. These mixtures were repeatedly fractionated by silica gel column chromatography as monitored by bioassay against larvae of *D. melanogaster*. Ultimately, four tertiary alkaloids and one quaternary alkaloid were isolated. These alkaloids (Figure 2) were identified as (−)-tetrahydroberberine (**1**), (−)-tetrahydrocoptisine (**2**), (+)-corydaline (**3**), (±)-tetrahydropalmatine (**4**), and (±)-dehydrocorydaline iodide (**5**) by spectral data compared with those reported previously (Chen and MacLean, 1968; Kamitani et al., 1975; Yu et al., 1976; Takao et al., 1977; Sugiura et al., 1978; Hussain et al., 1989; Janssen et al., 1990). **5** was reduced by $NaBH_4$ to yield **3**. Furthermore, **3** and **4** were treated with hydrochloric acid to obtain their hydrochloride salts (**3-HCl** and **4-HCl**).

Insecticidal Effect against Larvae. Bioassay for insecticidal activity against larvae was carried out according to a method by which larvae were fed with artificial diet containing test compounds for 7 days. The insecticidal activities of the five alkaloids and two hydrochlorides against larvae of *D. melanogaster* are shown in Table 1. In the course of the screening for novel naturally occurring insecticides from Chinese crude drugs, MeOH extract of tubers of *C. bulbosa* showed 80.0% of the larvae were killed at 2.0 mg/mL of diet. With the tertiary alkaloids, **1** showed the highest activity, with 99.7% of the larvae dead at 1.40 μmol/mL of diet and an LC_{50} value of 0.91 μmol/mL of diet (Miyazawa et al., 1996). **1** showed the same level of insecticidal activity as (*E*)-anethole (Miyazawa et al., 1993) and higher toxicity 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). **2** had the next highest activity, with 76.7% of the larvae dead at 2.80 μmol/mL of diet and an LC_{50} value of 1.70 μmol/mL of diet. However, at 2.80 μmol/mL of diet, **3** and **4** showed relatively low activities (LC_{50} values were 6.23 and 8.45 μmol/mL of diet, respectively). Two hydrochlorides, **3-HCl** and **4-HCl**, had no toxicity at 5.60 μmol/mL of diet. The most potent insecticidal activity against larvae was exhibited by **5**, which had an LC_{50} value of 0.23 μmol/mL of diet. All of these alkaloids were, however, less active than rotenone, a naturally occurring poison that killed all of the larvae at 0.13 μmol/mL.

Acute Toxicity against Adults. The acute toxicity of these alkaloids was determined by topical application on the abdomen of adults of *D. melanogaster* (Table 2). **1** killed 75% of adults at 5.0 μg/adult, and the LD_{50} value was 2.5 μg/adult (Miyazawa et al., 1996a). Similar to the results mentioned above, **1** was more active in this test when compared with **2**−**4**. Furthermore, **1** was more active than methyleugenol (LD_{50} = 6.2 μg/adult) (Miyazawa et al., 1992). **2** and **4** exhibited similar potency, and the LD_{50} values were 6.8 and 6.5 μg/adult, respectively. Furthermore, **1** was more active than rotenone (LD_{50} = 3.7 μg/adult). **3** had slight activity; at 20 μg/adult 70% of adults were killed. **3** was much

Table 2. Acute Toxicities of Compounds 1−5 and Two Hydrochloride Salts 3-HCl and 4-HCl against Adults of *D. melanogaster*

	survival[a] (in % relative to controls) at dose[b] (μg/adult) of					LD_{50}[c]
compd	10	7.0	5.0	3.0	1.0	(μg/adult)
1	0	20	25	40	100	2.5
2	0	40	90	100	100	6.8
3	75	80	100	100	100	>10.0
4	0	40	75	100	100	6.5
5	100	100	100	100	100	>10.0
3-HCl	100	100	100	100	100	>10.0
4-HCl	100	100	100	100	100	>10.0

[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 50 μL of acetone only. [c] LD_{50} is the lethal dose for 50% mortality determined by log-probit analysis.

Table 3. Insecticidal Activities of Compounds 1−5 and Two Hydrochloride Salts 3-HCl and 4-HCl against Acethylcholinesterase from Heads of Adults of *D. melanogaster*

	inhibition[a] (in % relative to controls) at concn[b] (mM) of						
compd	1.5	1.0	0.5	0.40	0.25	0.25	0.0625
1	81.5	78.7		75.6			
2		71.8	69.5		67.2		
3	69.2	68.2	67.9		66.9		
4	66.4	65.4	64.6		58.5		
3-HCl	66.7	65.5	64.5		62.1		
4-HCl	65.1	62.1	60.5		55.9		
5				63.9	61.7	60.7	58.6

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

less active (LD_{50} = 19.2 μg/adult). However, **5**, **3-HCl** and **4-HCl** were not toxic.

Inhibition of Acetylcholinesterase. Bioassay for acetylcholinesterase inhibition in vitro was carried out to investigate the mode of action of acute toxicity according to the colorimetric method of Ellman (Ellman et al., 1961). Inhibitions of acetylcholinesterase by the seven alkaloids are shown in Table 3. At 1.0 mM, **1** was the most inhibitory in four tertiary alkaloids. At this concentration, inhibitions for **1**, **2**, **3**, and **4** were 78.7, 71.8, 68.2, and 64.6%, respectively. **3-HCl** and **4-HCl** gave the same results as each of the tertiary alkaloids. Furthermore, the iodide salt **5** showed the most potent activity, with 61.3% inhibition at 0.40 mM. At a dose of 0.25 mmol/L, **1** caused an inhibition of 75.6%. At the same dose, inhibitions for the other six compounds were >55.0%. Furthermore, all of the alkaloids had strong activities comparable to that of (+)-pulegone, which had a 60.7% inhibition at 1.0 mmol/L (Grundy and Still, 1985; Miyazawa et al., 1997). Rotenone had no activity. Therefore, one possibility is that the acute toxicity of **1**, **2**, and **4** against adults is due to the inhibition of acetylcholinesterase.

According to these results, the insecticidally active compounds from tubers of *C. bulbosa* were **1**, **2**, and **5** in the test systems used in this investigation.

Although the potency of these protoberberine alkaloids was not improved by incorporation of a positively charged nitrogen, it was improved by the presence of double bonds in the isoquinolizine unit. Other structure−bioactivity relationship studies against microorganisms (Yamahara et al., 1972; Vennerstrom and Klayman, 1988) also indicated the importance of the presence of double bonds in the isoquinolizine unit for enhanced activity of the protoberberine alkaloids.

Against adults, **5**, **3-HCl**, and **4-HCl** were not toxic. However, they showed inhibition of acetylcholinesterase. Because these three alkaloids have a positive charge on the nitrogen atom, it is likely that they were little absorbed by the body of adults.

1 and **2** had the most potent activities of the four tertiary alkaloids in these assays. Furthermore, the alkyl substitution pattern at the 9,10-catechol function of both **1** and **2**, influences the toxicity. Although the dibenzoisoquinolizine skeleton has the potential for acute toxicity against adults, a methyl group at the 13-position in **3** reduced the activity. Other structure−bioactivity relationship studies on the effects on the intestine and uterus in mice (Imaseki et al., 1961) also indicated the importance of the methylenedioxyl group for enhanced activity of the tetrahydroprotoberberine alkaloids.

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