Antioxidant Principles from *Ephemerantha lonchophylla*

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One dihydrostilbene and three phenanthrene antioxidants were isolated from an ethanolic extract of the Chinese herbal *Ephemerantha lonchophylla*. One of these compounds, ephemeranthone (**4**) is a new natural product. Denbinobin (**1**) and 3-methylgigantol (**3**) have been previously isolated from this plant, and 3-ethoxy-5-hydroxy-7-methoxy-1,4-phenanthraquinone (**2**) is an artifact. The structures of these compounds were determined by spectroscopic analysis. The antioxidative activities for inhibiting human low density lipoprotein oxidation in vitro of compounds **1**−**4** were determined, and only **4** was active (5.3 times that of probucol).

Elevation of plasma low density lipoprotein (LDL) is strongly linked to atherosclerosis, the major cause of coronary heart disease. Recent studies have further demonstrated that atherogenesis involves endothelial dysfunction, monocyte infiltration and activation into macrophages, and smooth muscle cell proliferation.[1−3] Oxidative modification of LDL plays a key role in the molecular pathogenesis of atherosclerosis. Unlike intact LDL, which is metabolized through the receptor-mediated pathway, oxidized LDL (OxLDL) appears in the circulation and tends to infiltrate the aortic endothelium.[4] It is further oxidized in the intima until finally taken up by macrophages.[5] Antioxidants able to inhibit LDL oxidation may reduce early atherogenesis and slow its progress to the advanced stage.[6] Probucol is a cholesterol-lowering drug with antioxidant activity to inhibit LDL oxidation and reduce atherosclerosis in experimental animals.[7]

In recent years, we have extensively screened Chinese herbs for antioxidants that may inhibit LDL oxidation. Among these herbs a Chinese drug "Shi-Hu" exhibited significant inhibitory activity against Cu^{2+}/O_2-induced human LDL peroxidation in vitro.

The Chinese herb "Shi-Hu" is prepared from the dried stems of *Dendrobium* species (Orchidaceae). It is used as a tonic to nourish the stomach, promote the production of body fluid, and reduce fever.[8] The stems of *Ephemerantha lonchophylla* (Hook. f.) P. F. Hunt et Summerh (Orchidaceae) are also a source of "Shi-Hu" in Taiwan. Phenanthrenes and dihydrostilbenes have been isolated from the stem of *E. lonchophylla*.[9]

Results and Discussion

For the screening of antioxidants from the stems of *Ephemerantha lonchophylla*, the antioxidative effects of the fractions and extracts were tested for inhibition of human LDL peroxidation induced by $CuSO_4$.

The ethanolic extract of *E. lonchophylla* was dissolved into hexane-, CH_2Cl_2-, and EtOH-soluble parts. The hexane-soluble part was subjected to chromatography, and three isolated compounds were identified as denbinobin (**1**),[9] 3-ethoxy-5-hydroxy-7-methoxy-1,4-phenanthraquinone (**2**), and 3-methylgigantol (**3**).[9] The CH_2Cl_2 extract was also chromatographed on Si gel to obtain ephemeranthone (**4**). Among these four compounds isolated from *E. lonchophylla*, compound **4** is a new natural product. Compound **2** is an artifact. The structures of these compounds were identified by spectroscopic analysis.

Compound **1** was obtained as black prisms. Comparing the MS and ^1H, and ^{13}C NMR spectra of **1**, we confirmed this compound to be denbinobin.[9] Its HMBC spectrum revealed the following two- and three-bond couplings: H-2 (δ 6.14) to C-1 (δ 184.4), C-4 (δ 186.5), C-3 (δ 161.2), and C-10a (δ 132.4); H-10 (δ 8.12) to C-1, C-4a (δ 128.6), and C-8a (δ 139.9); H-9 (δ 8.06) to C-4b (δ 117.2), C-8 (δ 101.8), C-8a, and C-10a, which further supported the chemical assignment for C-4a and C-8a. This assignment hence revised the previously reported chemical shifts for C-6, C-8, C-4a, and C-8a.[9]

Compound **2** was obtained as black prisms. The ^1H and ^{13}C NMR spectrum of **2** were similar to those of **1** except for the absence of one MeO signal and the presence of an ethoxy group at δ 1.54 (3H, t, J = 7.0 Hz, $-CH_3$) and 4.10 (2H, q, J = 7.0 Hz, $-O-CH_2-$). Irradiation of the methoxy protons at δ 3.89 in **2** caused a NOE enhancement of H-8 (δ 6.76) and H-6 (δ 6.88), indicating the location of this methoxyl group to be at the C-7 position. In the HMBC spectrum (H-2 to C-1, C-3, and C-4; H-10 to C-1, C-4a, C-8a, and C-10a), the ethoxy moiety was confirmed to be located at C-3. Thus, the structure of **2** was established as 3-ethoxy-5-hydroxy-7-methoxy-1,4-phenanthraquinone. Compound **2** is an artifact, because it was not present when the stem of *E. lonchophylla* was extracted with acetone or methanol.

Ephemeranthone (**4**) was isolated as yellow crystals. The molecular formula for this compound was deduced as $C_{18}H_{16}O_5$ by a combination of HREIMS and ^1H and ^{13}C NMR analysis. Its IR spectrum revealed absorptions for hydroxy groups at 3246 cm^{-1}, carbonyl groups at 1688 and 1636 cm^{-1}, and phenyl groups at 1610 and 1556 cm^{-1}. The ^{13}C NMR and DEPT spectra indicated that **4** contained a skeleton based on 18 carbons: two methyl carbons (δ 30.9, 56.1), one methylene carbon (δ 51.3), six methine carbons (δ 98.0, 110.9, 121.6, 127.1, 128.1, 134.7), and nine quaternary carbons (δ 76.5, 120.1, 126.8, 135.0, 139.1, 155.7, 162.3, 200.9, 205.7). The ^1H NMR spectrum indicated the

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Table 1. Antioxidant Activities of Compounds and Probucol in Cu^{2+}-Induced LDL Peroxidation

compound	relative potency (%)[a]
probucol	1.0
2	0.2
4	5.3
resveratrol	0.7
1	0.4
3	0.9
moscatilin	7.8

[a] Compared with probucol.

presence of one methoxyl group at δ 3.77. It also displayed signals for an ABX (δ 7.17, d, J = 2.4 Hz, H-8; δ 7.21, dd, J = 2.4 and 8.1 Hz, H-6; δ 9.05, d, J = 8.1 Hz, H-5) system belonging to the aromatic protons. In addition, the lowfield doublet at δ 9.05 suggested that **4** belongs to the phenanthrene skeleton. Thus, the signals at δ 7.29 (d, J = 8.4 Hz) and 7.85 (d, J = 8.4 Hz) were assigned to H-10 and H-9, respectively. Because of the presence of NOEs between δ 3.77 (2-OCH_3) and 5.85, as well as between δ 7.29 (H-10) and 7.85 (H-9), 5.85, the singlet at δ 5.85 was determined to be H-1. The presence of a propanonyl moiety was indicated by signals at δ_H 2.12 (3H, s, H_3-13), 3.05 (1H, d, J = 15.3 Hz, H-11), 3.28 (1H, d, J = 15.3 Hz, H-11), and δ_C 205.7 (C-12). From the HMBC spectrum (H-1 to C-3; H_2-11 to C-3, C-4, and C-12; H_3-13 to C-12) the propanonyl moiety was confirmed to be located at C-3 (δ 76.5). Therefore, structure **4** was assigned to ephemeranthone.

It has been documented that Cu^{2+}-induced OxLDL exhibits biological and immunological properties similar to those in vivo.[10] Cu^{2+}-induced OxLDL is recognizable by scavenger receptors and causes cholesterol ester accumulation in macrophages. In screening for antioxidants to inhibit LDL oxidation, this method is commonly used.[11] The hypocholesterolemic drug probucol was used as a positive control in this study, because it is commonly used as a control to evaluate the antiatherogenic potential in animal study.

The antioxidative effects of these four compounds from *E. lonchophylla* and three other compounds moscatilin,[12] resveratrol, and probucol on in vitro lipid peroxidation of human LDL were studied. The antioxidative activities of compounds **1**−**3**, and resveratrol, an antioxidant,[13,14] were less potent than probucol (Table 1). Compound **4** and moscatilin exhibited much stronger antioxidative activities than probucol. The relative antioxidative activities of compounds **3**, **4**, and moscatilin were 0.9-, 5.3-, and 7.8-fold, respectively, that of probucol (Table 1).

Experimental Section

General Experimental Procedures. Melting points were determined on a Yanagimoto micromelting point apparatus and are uncorrected. IR spectra were recorded on a BIO-RAD FT−IR spectrometer. ^1H and ^{13}C NMR spectra were obtained on Varian Gemini 200 and Bruker AC-300 spectrometers. UV spectra were measured on a Hitachi U-3200 spectrophotometer. Mass spectra were obtained on JEOL SX-102A and JEOL JMS−HX100 spectrometers. Moscatilin was previously isolated from *Dendrobium loddigesii* Rolfe in our laboratory.[12] Resveratrol was purchased from Sigma Chemical Co., and probucol was obtained from Marion Merrell Dow.

Plant Materials. The stems of *Ephemerantha lonchophylla* (Hook. f.) P. F. Hunt et Summerh were obtained in a market in Taipei and identified by Professor Wang Z. T., Depatment of Pharmacognosy, China Pharmaceutical University, Nanjing, China. A voucher specimen is maintained in the herbarium of the National Research Institute of Chinese Medicine, Taipei, Taiwan.

Extraction and Isolation. The stems of *E. lonchophylla* (1.37 kg) were extracted with EtOH (10 L × 3). The extract was concentrated in vacuo to yield a dark brown mass, which was dissolved into hexane-, CH_2Cl_2-, and EtOH-soluble parts. The hexane, CH_2Cl_2, and EtOH extracts (0.4 mg/mL) exhibited 89.6, 62.5, and 66.3% inhibitory activities in the LDL peroxidation, respectively. The hexane extract was further subjected to Si gel chromatography using hexane, CH_2Cl_2, and EtOAc to yield 12 fractions. Fractions 11 and 12 showed the highest antioxidant activity. Fractions 11 and 12 were combined and rechromatographed on a Si gel column to obtain three compounds, **1** (23.6 mg), **2** (130.5 mg), and **3** (18.4 mg). The CH_2Cl_2 extract was chromatographed on Si gel column to yield compound **4** (44.6 mg).

Denbinobin (1): black prisms; mp 215−217 °C; ^1H NMR ($CDCl_3$) δ 3.91 (3H, s, 7-OCH_3), 3.94 (3H, 3-OCH_3), 6.14 (1H, s, H-2), 6.81 (1H, d, J = 2.5 Hz, H-8), 6.92 (1H, d, J = 2.5 Hz, H-6), 8.06 (1H, d, J = 8.5 Hz, H-9), 8.12 (1H, d, J = 8.5 Hz, H-10), 10.97 (1H, s, 5-OH); ^{13}C NMR ($CDCl_3$) δ 55.4 (7-OCH_3), 55.9 (3-OCH_3),101.8 (C-8), 107.3 (C-2), 108.6 (C-6), 117.1 (C-4b), 122.6 (C-10), 128.7 (C-4a), 132.4 (C-10a), 137.4 (C-9), 139.9 (C-8a), 156.4 (C-5), 160.8 (C-7), 161.2 (C-3), 184.4 (C-1), 186.5 (C-4); difference NOE (%) 7-OCH_3 [H-6 and H-8]; HMBC cross-peaks H-2/C-1, C-4, C-3, and C-10a; H-6/C-4b, C-5, C-7, and C-8; H-8/C-4b, C-6, C-7, and C-9; H-9/C-4b, C-8, C-8a, and C-10a; H-10/C-1, C-4a, and C-8a; 5-OH/C-4b, C-5, and C-6; EIMS m/z (%) 284 (M^+, 100), 269 (7), 213 (62), 185 (48).

3-Ethoxy-5-hydroxy-7-methoxy-1,4-phenanthraquinone (2): black prisms; mp 219−220 °C; ^1H NMR ($CDCl_3$) δ 1.54 (3H, t, J = 7.0 Hz, $-CH_3$), 3.89 (3H, s, 7-OCH_3), 4.10 (2H, q, J = 7.0 Hz, $-O-CH_2-$), 6.07 (1H, s, H-2), 6.76 (1H, d, J = 2.5 Hz, H-8), 6.88 (1H, d, J = 2.5 Hz, H-6), 7.99 (1H, d, J = 8.0 Hz, H-9), 8.04 (1H, d, J = 8.0 Hz, H-10), 11.00 (1H, s, 5-OH); ^{13}C NMR ($CDCl_3$) δ 13.9 ($-CH_3$), 55.4 ($-OCH_3$), 65.9 ($-OCH_2-$), 102.7 (C-8), 107.5 (C-2), 108.5 (C-6), 117.1 (C-4b), 122.5 (C-10), 128.7 (C-4a), 132.3 (C-10a), 137.2 (C-9), 139.8 (C-8a), 156.4 (C-5), 160.4 (C-7), 160.7 (C-3), 184.5 (C-1), 186.5 (C-4); difference NOE (%) H-2 [$-OCH_2$ (11)]; HMBC cross-peaks H-2/C-1, C-4, C-3, and C-10a; H-6/C-4b, C-5, and C-8; H-8/C-4b, C-6, C-7, and C-9; H-9/C-4b, C-8, C-8a, and C-10a; H-10/C-1, C-4a, C-8a, and C-10a; 5-OH/C-4b, C-5, and C-6; EIMS m/z (%) 298 (M^+, 100), 284 (75), 254 (26), 241 (23), 213 (48), 185 (42).

3-Methylgigantol (3): colorless prisms; mp 71−73 °C; UV λ_{max} nm 224, 266, 280; IR (KBr) ν_{max} (cm^{-1}) 3575 (br), 1600, 1516, 1465, 1155, 1061; ^1H NMR (Me_2CO-d_6) δ 2.78 (4H, br s, H-α and H-α'), 3.70 (3H, s, 3′-OCH_3), 3.81 (3H, s, 4-OCH_3), 3.82 (3H, s, 3-OCH_3), 6.25 (2H, d, J = 2.2 Hz, H-2′ and H-6′), 6.28 (1H, t, J = 2.0 Hz, H-4′), 6.64 (1H, d, J = 2.0 Hz, H-2), 6.69 (1H, dd, J = 2.0, 8.0 Hz, H-6), 6.77 (1H, d, J = 8.0 Hz, H-5); ^{13}C NMR (Me_2CO-d_6) δ 37.0 (t), 38.1 (t), 55.1 (q), 55.7 (q), 55.8 (q), 98.9 (d), 106.5 (d), 108.1 (d), 111.2 (d), 111.9 (d), 120.3 (d), 134.4 (s), 144.3 (s), 147.0 (s), 148.5 (s), 156.9 (s), 160.6 (s); EIMS m/z M^+ 288 (50), 185 (23), 151 (100).

Ephemeranthone (4): yellow crystals, mp 159−161 °C; $[\alpha]^{28}_D$ −11.76° (c 0.34, MeOH); UV λ_{max} (MeOH) (ϵ) 230 (26245), 263 (40230), 348 (5684), 430 (6360) nm; IR ν_{max} cm^{-1} 3246 (br), 1688, 1636, 1610, 1556, 1354, 1248, 1176, 1085; ^1H NMR (Me_2CO-d_6) δ 2.12 (3H, s, H-13), 3.05 (1H, d, J = 15.3 Hz, H-11), 3.28 (1H, d, J = 15.3 Hz, H-11), 3.77 (3H, s, 2-OCH_3), 5.85 (1H, s, H-1), 7.17 (1H, d, J = 2.4 Hz, H-8), 7.21 (1H, dd, J = 2.4, 9.0 Hz, H-6), 7.29 (1H, d, J = 8.4 Hz, H-10), 7.85 (1H, d, J = 8.5 Hz, H-9), 9.05 (1H, d, J = 9.0 Hz, H-5); ^{13}C NMR (Me_2CO-d_6) δ 30.9 (C-13), 51.3 (C-11), 56.1 (OCH_3), 76.5 (C-3), 98.0 (C-1), 110.9 (C-8), 120.1 (C-4a), 121.6 (C-6), 126.8 (C-4b), 127.1 (C-10), 128.1 (C-5), 134.7 (C-9), 135.0 (C-10a), 139.1 (C-8a), 155.7 (C-7), 162.3 (C-2), 200.9 (C-4), 205.7 (C-12); difference NOE (%) 2-OCH_3 [H-1 (19)], H-9 [H-10 (10), H-8 (11)]; HMBC cross-peaks H-1/C-2, C-3, C-4a, and C-10; H-9/C-4b, C-8, and C-8a; H-10/C-1, C-4a, and C-9; H_2-11/C-2, C-3, C-4, and C-12; H_3-13/C-12; EIMS m/z (%) 312 (M^+, 25), 280 (100), 252 (44), 223 (53), 184 (23), 155 (20); HREIMS m/z 312.1005, calcd for $C_{18}H_{16}O_5$ 312.0997.

LDL Lipid Peroxidation and Screening for Antioxidants. Blood samples were collected from healthy male adults

after a 12-hour overnight fast. Sera were fractionated by ultracentrifugation (Beckman L8−80M; R50 rotor) with the density adjusted by NaBr. LDL was obtained from the fractions corresponding to $\rho = 1.019-1.063$.[15,16] To remove H_2O-soluble antioxidants and NaBr, LDL-containing fractions (3−5 mL) were dialyzed extensively (at 4 °C/N_2) against phosphate buffer saline (PBS, 50 mM; pH 7.4) in darkness. Dialyzed LDL was used for assay as soon as possible.

After dialysis, LDL was diluted with PBS to 0.9 mg cholesterol/mL; 50-μL aliquots of LDL were placed into wells of a 96-well microtiter plate, and the plate was incubated with $CuSO_4$ (final concentrated 10 μM) at 37 °C to induce lipid peroxidation.[17] In a routine assay, incubation was carried out at 37 °C for 2 h (in a gyro-rotary incubator shaker at 120 rpm). For screening, LDL was preincubated with the test compounds at 37 °C for 1 h before adding Cu^{2+}. Probucol (10 μM) was used as a positive control.

Routinely, the time course of conjugated diene formation was also determined by following the increase of UV absorption at 232 nm.[17] Prolongation of the lag phase was used as index of antioxidative activity when an antioxidant was present in LDL oxidation with Cu^{2+}. The lag phase and rate of oxidation of LDL were dependent on the contents of lipophilic antioxidants, particularly α-tocopherol, and polyunsaturated fatty acids (PUFA) in LDL, which varied among donors. Their relative potency remained constant when probucol and lipophilic antioxidants were assayed using the same preparation of LDL.

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