Morphinane Alkaloids with Cell Protective Effects from *Sinomenium acutum*

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One new morphinane alkaloid, sinomenine *N*-oxide (**1**), and one new natural occurring morphinane alkaloid, *N*-demethylsinomenine (**2**), together with six known alkaloids, 7,8-didehydro-4-hydroxy-3,7-dimethoxymorphinan-6-ol (**3**), sinomenine (**4**), sinoacutine (**5**), *N*-norsinoacutine, acutumine, and acutumidine, were isolated from the stems of *Sinomenium acutum*. Their structures were elucidated on the basis of spectroscopic analysis and chemical methods. Compounds **2**, **3**, and **5** have protective effects against hydrogen peroxide-induced cell injury.

Sinomenium acutum (Thumb.) Rehd. et Wils. (Menispermaceae) has long been used in traditional Chinese medicine to treat various rheumatic diseases.[1,2] Sinomenine is the main alkaloid isolated from the stems and roots of this plant. Previous reports have demonstrated that the pharmacological profile of sinomenine includes immunosuppression,[3] arthritis amelioration,[4] anti-inflammation,[2] and protection against hepatitis induced by lipopolysaccharide.[5] In addition, using intramuscular injection and multiple dosing, a combination of sinomenine and cyclosporin A showed immunomodulatory effects in a cardiac transplant model.[6] Our recent studies showed that sinomenines may be used for the treatment of cognitive disorders,[7] which led to our continuing research on constituents of the roots and stems of this species. One new morphinane alkaloid, sinomenine *N*-oxide (**1**), and one new natural occurring morphinane alkaloid, *N*-demethylsinomenine (**2**), together with six known morphinane alkaloids, 7,8-didehydro-4-hydroxy-3,7-dimethoxymorphinan-6-ol (**3**),[8] sinomenine (**4**),[9] sinoacutine (**5**),[10] *N*-norsinoacutine,[11] acutumine,[12] and acutumidine,[13] were isolated. In this Note, we describe the isolation and structural determination of **1** and **2** and the protective effects against free radical-induced cell injury of **2**, **3**, and **5**.

Sinomenine *N*-oxide (**1**) was obtained as an amorphous powder. The molecular formula $C_{19}H_{23}NO_5$ was established by HREIMS as 16 mass units greater than that of sinomenine. Its IR spectrum showed characteristic absorptions for hydroxyl (3400 cm^{-1}), an α,β-unsaturated carbonyl moiety (1685, 1629 cm^{-1}), and a substituted phenyl group (1600, 1486 cm^{-1}). The ^1H NMR spectrum indicated the presence of two *O*-methyl groups (δ 3.82, 3.74, each 3H, s), two *o*-coupled aromatic protons (δ 6.52, 6.70, each 1H, d, $J = 8.2$ Hz), two methylene protons (δ 2.57, 4.43, each 1H, d, $J = 15.5$ Hz), and one methyl group (δ 3.32, s, 3 H) at the oxygenated N atom suggested by the deshielded *N*-methyl singlet, compared with that of sinomenine (δ 2.42, s, 3 H). The ^1H and ^{13}C NMR data of **1** were similar to those of sinomenine, except for the presence of the oxygenated *N*-methyl (δ_C 57.81) instead of the *N*-methyl (δ_C 42.81) in sinomenine (**4**) (Table 1). The CD spectrum of **1** showed negative (Δε, −25.7) and positive (Δε, +20.2) Cotton effects at 269 and 232 nm, respectively, which was similar to those of sinomenine,[9] suggesting the absolute configuration of **1** was identical with that of sinomenine. On the basis of these data, the structure of **1** was established as the *N*-oxide derivative of sinomenine. Oxidation of sinomenine by H_2O_2 indeed afforded **1**, hence confirming the structure.

N-Demethylsinomenine (**2**) was obtained as white crystals. The molecular formula $C_{18}H_{21}NO_4$ was established by HREIMS as 14 mass units less than that of sinomenine. Its IR spectra showed characteristic absorptions for hydroxyl (3318 cm^{-1}), an α,β-unsaturated carbonyl moiety (1679, 1625 cm^{-1}), and a substituted phenyl group (1580, 1483 cm^{-1}). The ^1H NMR data of **2** were similar to those of sinomenine, except for the lack of the *N*-methyl group (δ_H 2.42, 3H, s) in sinomenine (**4**) (Table 1), suggesting that compound **2** was *N*-demethylsinomenine. The absolute configuration of **2** was detemined by CD, which showed negative (Δε, −46.4) and positive (Δε, +20.2) Cotton effects at 269 and 233 nm, respectively, similar to those of sinomenine.[9] *N*-Demethylsinomenine is a new naturally occurring morphinane alkaloid, although it was reported as a microtransformed derivative.[14]

Cell protective activities were assessed by measuring the ability of the compounds to protect against H_2O_2 injury of the cells pretreated with compounds **1**, **2**, **3**, and **5**. This in vitro bioassay evaluation indicated that **2**, **3**, and **5** had protective effects against free radical-induced cell injury (Figure 2). A number of independent lines of studies have now converged to suggest that increased oxidative stress and disturbed defensive mechanisms occur in the brain of AD (Alzheimer's disease), which might result in a self-propagating cascade of neurodegenerative events.[15,16] As the neurotoxic effects of β-amyloid (Aβ) peptides are at least in part mediated by free radicals and some antioxidants have been proved to rescue cells from Aβ toxicity, therapeutic efforts aimed at removal of free radicals or prevention of their formation may be beneficial in AD.[15,17] Protective effects against free radical-induced cell injury of **2**, **3**, and **5** suggested that these morphinane alkaloids may be a potential type of lead compounds beneficial in AD.[15,17,18]

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Table 1. ^{1}H and ^{13}C NMR Data (δ in ppm, J in Hz) of **1**, **2**, and **4** in $CDCl_3$[a]

	1		2	4	
position	proton	carbon	proton	proton	carbon
1	6.52 (d, 8.2)	118.29 (d)	6.53 (d, 8.4)	6.56 (d, 8.2)	119.23 (d)
2	6.70 (d, 8.2)	110.16 (d)	6.63 (d, 8.4)	6.68 (d, 8.2)	108.94 (d)
3		146.29 (s)			144.95 (s)
4		145.68 (s)			144.70 (s)
5α	2.57 (d, 15.5)	47.85 (t)	2.47 (d, 15.6)	2.54 (d, 15.6)	49.23 (t)
5β	4.43 (d, 15.5)		4.32 (d, 15.6)	4.32 (d, 15.6)	
6		193.15 (s)			194.03 (s)
7		152.72 (s)			152.35 (s)
8	5.51 (d, 2.1)	113.41 (d)	5.44 (d, 2.2)	5.37 (s)	115.15 (d)
9	3.85 (t, 3.8)	73.04 (d)	3.44 (dd, 4.4, 4.0)	3.18 (dd, 5.5, 3.8)	56.70 (d)
10α	3.06 (br d, 19.6)	29.10 (t)	3.16 (dd, 18.0, 1.5)	3.03 (d, 18.3)	24.23 (t)
10β	3.35 (br d, 19.6)		2.75 (dd, 18.0, 1.5)	2.69 (dd, 18.3, 5.5)	
11		125.50 (s)			130.45 (s)
12		121.29 (s)			122.63 (s)
13		39.42 (s)			40.50 (s)
14	4.36 (br s)	39.06 (d)	2.94 (t, 1.9)	2.98 (dd, 3.8, 2.1)	45.97 (d)
15a	2.63 (br d, 13.2)	31.60 (t)	2.59 (ddd, 12.5, 12.2, 3.8)	1.92 (ddd, 12.5, 3.7, 2.2)	36.05 (t)
15β	1.90 (br d, 13.2)		1.72 (ddd, 12.5, 12.2, 3.8)	1.87 (ddd, 12.5, 12.3, 4.5)	
16α	3.16 (br d, 11.6)	61.27 (t)	1.90 (dd, 11.4, 1.8)	2.06 (ddd, 12.3, 11.8, 3.7)	47.14 (t)
16β	2.90 (br d, 11.6)		2.76 (dd, 11.4, 1.8)	2.53 (ddd, 11.8, 4.5, 2.2)	
NMe	3.32 (s)	57.81 (q)		2.42 (s)	
3-OMe	3.82 (s)	56.13 (q)	3.80 (s)	3.79 (s)	56.05 (q)
7-OMe	3.74 (s)	55.03 (q)	3.47 (s)	3.45 (s)	54.77 (q)

[a] ^{1}H NMR recorded at 400 MHz and ^{13}C NMR at 100 MHz.

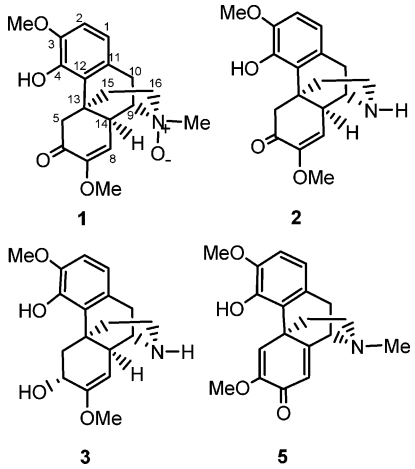

Figure 1. Structure of compounds **1**, **2**, **3**, and **5**.

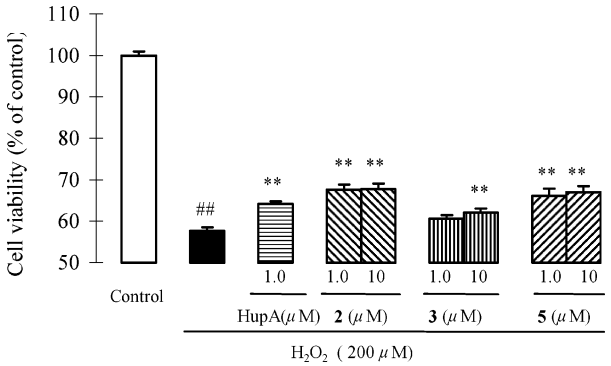

Figure 2. Protective effects of Hup A (positive reference), **2**, **3**, and **5** on cell injury induced by hydrogen peroxide (200 μM) in PC12 cells. $^{**}p < 0.01$ vs H_2O_2 group (##).

Experimental Section

General Experimental Procedures. $[\alpha]_D$ values were obtained on a JASCO DIP-181 polarimeter. IR spectra (KBr) were obtained on a Nicolet Magna FTIR-750. All NMR spectra were recorded on a Bruker DRX-500 instrument in $CDCl_3$. Chemical shifts are reported in ppm with TMS as internal standard. MS spectra were recorded on a MAT-95 spectrometer. Alkaloids were detected by Dragendorff's reagent; CD spectra were measured on a J-810 spectrometer in MeOH, and data are given as $\Delta\epsilon$ (nm).

Plant Material. The stems of *S. acutum* were collected in Tonglin County of Anhui Province in February 2000 and identified by one of the authors (G.-H.B.). A voucher specimen (No. SIMM000212) was deposited in the herbarium of Shanghai Institute of Materia Medica. Huperzine A was provided as a positive reference by the State Key Laboratory of Drug Research, Shanghai Institute of Materia Medica. Bismuth potassium iodide (analytical standard) was bought from Shanghai Chemical Co.; H_2O_2, MTT, and DMSO were from Sigma Chemical Co; RPMI 1640 medium was from Gibco-BRL. All other reagents were of the highest available quality.

Extraction and Isolation. The stems of *S. acutum* (40 kg) were air-dried, ground, and extracted with 95% EtOH (2 × 100 L). After removal of the solvent by evaporation under vacuum, the residue was dissolved in tartaric acid and filtered. The acid aqueous layer was adjusted to pH 10 with NH_4OH and extracted with petroleum ether (3 × 2.5 L), $CHCl_3$ (4 × 2.5 L), and *n*-BuOH (3 × 2.5 L), successively. The $CHCl_3$ extract was evaporated under vacuum to give a red mass (485 g). A portion (350 g) of the red mass was subjected to column chromatography on silica gel (200−300 mesh, 4.5 × 70 cm) and eluted stepwise with $CHCl_3$/CH_3OH [20:1 (5.0 L), 9:1 (3.0 L), 4:1 (3.0 L), and 0:1 (5.0 L)] to give four fractions. The fraction $CHCl_3$/CH_3OH (20:1) was further subjected to column chromatography on silica gel (200−300 mesh, 2.5 × 40 cm) and stepwise eluted with petroleum ether/$CHCl_3$/CH_3OH [3:1:0 (5.0 L), 0:1:0 (4.0 L), 0:20:1 (3.0 L), 0:9:1 (3.0 L)] to give compounds **4** (3 g) and **5** (300 mg). The fraction $CHCl_3$/CH_3OH (9:1) was further subjected to column chromatography on silica gel (200−300 mesh, 2.5 × 40 cm) and stepwise eluted with $CHCl_3$/CH_3OH [1:0 (5.0 L), 20:1 (5.0 L), 9:1 (3.0 L)] to give compounds **1** (12 mg), **2** (109 mg), **3** (18.8 mg), **4** (2 g), *N*-norsinoacutine (7 mg), acutumine (2 g), and acutumidine (1.5 g).

Sinomenine *N*-oxide (1): white powder (CH_3OH); mp 155−156 °C; $[\alpha]^{20}_D$ −79° (*c* 0.50, CH_3OH); IR (KBr) ν_{max} 3399, 2929, 1685, 1629 1600, 1487, 1486, 1432, 1280, 1145, 750 cm^{-1}; CD −25.7 (269), +20.2 (232) (*c* 0.50, CH_3OH); NMR data ($CDCl_3$), see Table 1; EIMS *m/z* 345 $[M]^+$, 329, 328, 314, 296, 270 (base peak), 255, 239, 227; HREIMS *m/z* 345.1568 (calcd for $C_{19}H_{23}NO_5$ 345.1560).

***N*-Demethylsinomenine (2):** white crystals ($CHCl_3$); mp 222−223 °C; $[\alpha]^{20}_D$ −108° (*c* 0.50, CH_3OH); IR (KBr) ν_{max} 3318,

2921, 1679, 1625, 1483, 1436, 1278, 1199, 1064, 869, 783, 756 cm^{-1}; CD −46.4 (269), +20.2 (233) (*c* 0.80, CH_3OH); NMR data ($CDCl_3$), see Table 1; EIMS *m/z* 315 $[M]^+$ (base peak), 314, 300, 287, 178, 164, 132; HREIMS *m/z* 315.1470 $[M]^+$ (calcd for $C_{18}H_{21}NO_4$ 315.1470).

Oxidation of Sinomenine.[19] Sinomenine was soaked in H_2O_2 and stirred for about 48 h and then extracted by CH_2Cl_2. The CH_2Cl_2 extracts were crystallized to give just one compound, which was identical with the natural sinomenine *N*-oxide (**1**) by co-TLC detection. The ^1H NMR and ESIMS data of the product agreed with those of **1**.

Cell Protection Test.[18] PC12 cells (rat pheochromocytoma line) were high passages from ATCC (American Type Culture Collection) and maintained at 37 °C in a humidified atmosphere containing 5% CO_2. Cells were seeded into multiwell plates (Greiner) at a density of 5×10^4 cells per mL in RPMI1640 medium (Gibco), supplemented with 10% heat-inactivated bovine calf serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. Experiments were carried out 24 h after cells were seeded. Hydrogen peroxide (H_2O_2, 8.8 M solution) was stored at 4 °C until 100 mM stock solutions were prepared in phosphate buffer saline (PBS) on the day of application to cultures. The 100 mM H_2O_2 was further diluted for addition to the cultures. Compounds **1**, **2**, **3**, and **5** were dissolved in 2% DMSO first, then diluted with PBS. Pretreatment of the cells with huperzine A [18] (1 μM) and compounds **1**, **2**, **3**, and **5** (1 and 10 μM) was conducted 2 h prior to H_2O_2 exposure. Medium was replaced with fresh RPMI1640 after 30 min treatment with H_2O_2 (200 μM). Cell survival was evaluated by MTT reduction. Briefly, 6 h after H_2O_2 exposure, MTT solution in PBS was added with a final concentration of 0.5 mg/mL, and the incubation was continued for 4 h. Finally, 100 μL of solution containing 50% DMF and 20% SDS, pH 4.8, was added. The mixtures were kept overnight, and then the amount of MTT formazan was quantified by determining the absorbance at 570 and 630 nm using a universal microplate reader (Bio-Tek).[20] Statistical analysis: Data are expressed as means ± SD and evaluated for statistical significance with one-way ANOVA followed by Duncan's multiple range test.

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Supporting Information Available: 2D NMR spectra for compound **1** and spectroscopic data for known compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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