Chemistry of Natural Compounds, Vol. 46, No. 5, 2010

INDOLE ALKALOIDS FROM THE ROOTS OF *Isatis ingigotica* AND THEIR ANTIHERPES SIMPLEX VIRUS TYPE 2 (HSV-2) ACTIVITY *IN VITRO*

Dong-Dong Sun,[1*] Wei-wei Dong,[2] Xiang Li,[1] and Han-Qing Zhang[3]

UDC 547.944/945

*A new indole alkaloid **1**, together with four known indole alkaloids **2–5**, was isolated from the 95% EtOH extract of the traditional Chinese medicinal plant* Isatis indigotica. *Alkaloids **2–5** were found in the plant for the first time. Their structures were elucidated as 3-hydroxy-3-acetonitrile-4-hydroxy-2-indolone (**1**), 1-methoxy-3-indoleacetonitrile (**2**), 3-indoleacetic acid (**3**), 3-indolealdehyde (**4**), and 1-methoxy-3-indolecarbaldehyde (**5**) on the basis of spectroscopic data. Their anti-HSV effects* in vitro *were evaluated by plaque reduction assay.*

Keywords: *Isatis indigotica*, indole alkaloids, antiherpes simplex virus type 2 (HSV-2) activity.

The root of *Isatis indigotica* Fort. (Brassicaceae), a traditional Chinese medicine, has been used to treat fever, phlogosis, headache, rheumatism, and viruses in China since ancient times [1]. Nowadays, it is routinely applied for the treatment of diseases such as catarrhal rhinitis, influenza, bacterial infection, sickness with the symptom of hypoimmunity, etc. that are caused by bacteria and virus infection [2, 3]. In the literature, many biological activities, including antiviral, have been reported for *Isatis indigotica* [4]. In previous studies, the constituents isolated from the plant are acids [5], alkaloids [6, 7], benzenoids [8, 9], flavonoids [10], lignans [11], amino acids [4], sterols [5], nucleosides [9], and sulfur-containing compounds [12].

In this investigation, a new indole derivative (**1**), together with four indole compounds **2–5**, which were separated for the first time, was isolated from the 95% EtOH extract of the traditional Chinese medicinal plant *Isatis indigotica.* Their structures were elucidated as 3-hydroxy-3-acetonitrile-4-hydroxyl-2-indolone (**1**), 1-methoxy-3-indoleacetonitrile (**2**), 3-indoleacetic acid (**3**), 3-indolealdehyde (**4**), and 1-methoxy-3-indolecarbaldehyde (**5**) on the basis of spectroscopic data. Their anti-HSV effects *in vitro* were evaluated by plaque reduction assay.

Purification of the EtOAC fraction of the root of *Isatis indigotica* by several column chromatographic methods has afforded a new indole derivative **1**. The structure elucidation of the indole compound was performed by HR-ESI-MS and 1D and 2D NMR experiments.

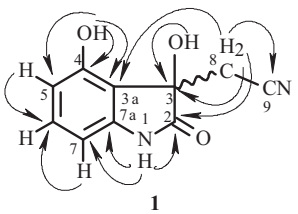

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Fig. 1. Partial HMBC correlations of **1**.

1) Nanjing University of Chinese Medicine, 210046, Jiangsu, People′s Republic of China, fax: +86 25 85811006, e-mail: sun_21373@163.com; 2) Nanjing University of Technology, 210009, Jiangsu, People′s Republic of China; 3) Institute of Botany, Jiangsu Province and Chinese Academy of Sciences, 210014, Jiangsu, People′s Republic of China. Published in Khimiya Prirodnykh Soedinenii, No. 5, pp. 642–644, September–October, 2010. Original article submitted May 22, 2009.

0009-3130/10/4605-0763

TABLE 1. ^{1}H and ^{13}C NMR Data of **1** (300 and 75 MHz, DMSO-d_6, δ, ppm, J/Hz)

Position	δ_H	δ_C	Position	δ_H	δ_C
NH	11.92(1H, s)		8 (-CH_2-)	3.84 (2H, d, J = 16.1)	32.1
2(C=O)		178.7	9 (-CN)		117.6
3		74.5	3a		121.1
4		157.1	7a		132.6
5	6.85 (1H, d, J = 8.4)	122.3	3-OH	4.02 (1H, s)	
6	7.21 (1H, dd, J = 8.4, 7.8)	145.0	4-OH	5.17 (1H, s)	
7	6.62 (1H, d, J = 7.8)	114.5			

TABLE 2. Cytotoxic Effect and Antiviral Activities of Compounds **1–5** *in vitro*

Compound	Cytotoxic effect, μM Vero cells CC_{50}	Antiviral activity, μM				
		HSV-2			HSV-1	
		IC_{50}	IC_{90}	TI	IC_{50}	TI
1	111.3 ± 9.1	34.4 ± 3.2[a,b]	89.1 ± 6.2	3.2	> 80.0	–
2	117.3 ± 10.9	39.1 ± 4.1	95.7 ± 7.1	3.0	> 90.0	–
3	–	> 90.0	–	–	> 90.0	–
4	–	> 90.0	–	–	> 90.0	–
5	–	> 90.0	–	–	> 90.0	–
ACV	75.3 ± 8.1	17.2 ± 3.6	41.7 ± 5.9	4.4	16.1 ± 2.8	4.9
Solvents	> 200.0	–	–	–	–	–

Data were mean ± S.D. of three independent experiments, [a]$p < 0.05$ (compared between reference and test compound), [b]$p < 0.05$ (compared between 1 and 2); ACV was used as a reference compound, ACV: Acyclovir (acycloguanosine), 2-amino-9-((2-hydroxyethoxy)methyl)-1H-purin-6(9H)-one.

The molecular formula of **1** was assigned as $C_{10}H_8N_2O_3$ by HR-ESI-MS, which suggests eight degrees of unsaturation. A positive Dragendorff test was obtained, which showed that **1** was an alkaloid. The IR spectrum displayed absorption bands at 3285, 3160, 2250, and 1685 cm^{-1}, indicating the presence of imino, hydroxyl, nitrile, and carbonyl groups. The ^{1}H NMR spectrum of this metabolite (Table 1) showed three aromatic protons δ 6.85 (1H, d, J = 8.4 Hz), δ 7.21 (1H, dd, J = 8.4, 7.8 Hz), and δ 6.62 (1H, d, J = 7.8 Hz). The spin systems of the aromatic protons showed that the proton at δ 7.21 (dd, J = 8.4, 7.8 Hz, H-6) was coupled to two protons at δ 6.85 (d, J = 8.4 Hz, H-5) and δ 6.62 (d, J = 7.8 Hz, H-7), the coupling constants further suggesting that the orientation of H-6 to H-5 and H-7 is *ortho* [13]. An exchangeable broad proton at δ 11.92 (NH) suggested that the aromatic protons are part of an indole ring substituted at either position 4 or 7. Analysis of the HMBC spectra showed correlations of the downfield carbon at δ 157.1 (C-4) with the hydroxyl proton (4-OH), the methylene protons at δ 3.84 (H-8, d, J = 16.1 Hz) with carbons at δ 117.6 (CN), δ 178.7 (C-2), δ 74.5 (C-3), and δ 121.1 (C-3a), and the proton at δ 11.92 (-NH-) with carbons at δ 178.7 (C-2), δ 132.6 (C-7a), and δ 114.5 (C-7). IR data showed a weak absorption at 2250 cm^{-1}, which was attributed to a nitrile group, also consistent with the ^{13}C NMR chemical shift at δ 117.6. Analysis of the HMQC, HMBC (Fig. 1), and ^{1}H–^{1}H COSY spectra led to the complete assignment of proton and carbon signals for **1**. The positions of the substituting group were further confirmed by the 2D NMR spectra described above. Accordingly, the planar structure of **1** was determined to be 3-hydroxy-3-acetonitrile-4-hydroxyl-2-indolone (**1**).

The known compounds **2–5** were identified by comparison of their IR, MS, and ^{1}H and ^{13}C NMR spectral data with those in the literature [14–17].

Table 2 shows the anti-HSV-1 and HSV-2 activity of **1–5**; none of them significantly suppressed HSV-1 infection, but compounds **1** and **2** have a certain effect on inhibiting HSV-2 reproduction ($p < 0.05$). The IC_{50} of **1** was 34.4 ± 3.2 μM, and that of **2** was 39.1 ± 4.1 μM. Both compounds inhibited 90% of HSV-2 multiplication (IC_{90}) at concentration of 89.1 ± 6.2 μM for **1**, and of 95.7 ± 7.1 μM for **2**. Both **1** and **2** were not toxic towards Vero cells at the concentrations that inhibited HSV infection. The CC_{50} of **1** was 111.3 ± 9.1 μM, and that of **2** was 117.3 ± 10.9 μM. The therapeutic index (TI) was calculated as the ratio of CC_{50} to IC_{50}. For anti-HSV-2 assay, **1** had the highest TI (3.2), followed by **2** (3.0).

EXPERIMENTAL

General. Melting points: Gallenkamp apparatus. Optical rotations: Jasco P-1020. IR: Hitachi 260–30. NMR: Bruker AMX or Varian UNITY INOVA 300 MHz in DMSO-d_6. ESI-MS^n: API 3000. HR-ESI-MS: PE LC/MS spectrometer. Open column chromatography: silica gel (70–230 mesh).

Plant Material. *I. indigotica* Fort. (Brassicaceae) roots, supplied by a traditional Chinese medicine company (Anguo Co.) in Hebei in 2007, was identified by Prof. Jian-Wei Chen (Nanjing University of Chinese Medicine, Jiangsu, China). The samples were authenticated and deposited in the Pharmaceutical College of the Nanjing University of Chinese Medicine, Jiangsu (SDD-BLG-005).

Extraction and Isolation. The air-dried roots (50 kg) of *Isatis indigotica* were extracted with 95% EtOH at room temperature. The resulting extract (2000 g) was suspended in water and successively partitioned to provide EtOAC (500 g) and *n*-BuOH (250 g) fractions. The EtOAC extract (500 g) was subjected to Si-gel CC and eluted with mixtures of $CHCl_3$–MeOH (100:1–3:1) to provide ten fractions (P1–P10). The P3 fraction (100 g) was subjected to silica gel (200–300 mesh) column chromatography using $CHCl_3$–Me_2CO (100:1–1:1) as eluent to afford seven other fractions (P11–P17); the P13 fraction (20 g) was further subjected to Sephadex LH-20 CC ($CHCl_3$–MeOH 8:2) and purified using a Lobar A RP-18 column (80% MeOH) to afford **3** (20 mg), **4** (50 mg), and **5** (15 mg). The P15 fraction (15 g) was subjected to Sephadex LH-20 CC ($CHCl_3$–MeOH 5:1) and purified using a Lobar A RP-18 column (80% MeOH) to afford **1** (18 mg) and **2** (800 mg).

3-Hydroxy-3-acetonitrile-4-hydroxy-2-indolone (1), white amorphous powder; mp 250–252°C; $[\alpha]_D^{20}$ –91.1° (*c* 0.2, pyridine). IR (KBr, ν, cm^{-1}): 3285, 3160, 2250, and 1685. ESI-MS: *m/z* 205, 204, 164, 136, 108, 90. HR-ESI-MS: *m/z* 227.0431 $[M + Na]^+$ (calcd for $C_{10}H_8N_2O_3Na$, 227.0433). ^1H and ^{13}C NMR data, see Table 1.

1-Methoxy-3-indoleacetonitrile (2), yellow liquid. IR (KBr, ν, cm^{-1}): 2935, 2250, 1615, 1455, 1415, 742. ESI-MS: *m/z* 186, 171, 155, 146, 128, 101, 77. ^1H NMR (300 MHz, $CDCl_3$, δ, ppm, J/Hz): 7.62 (1H, d, J = 7.8, H-4), 7.46 (1H, d, J = 8.2, H-7), 7.36 (1H, t, J = 7.8, H-6), 7.22 (1H, t, J = 7.8, H-5), 7.24 (1H, s, H-2), 3.93 (3H, s, -OCH_3), 3.63 (2H, s, H-8). ^{13}C NMR (75 MHz, $CDCl_3$, δ, ppm): 122.5 (C-2), 121.7 (C-3), 100.1 (C-3a), 119.6 (C-4), 117.9 (C-5), 121.2 (C-6), 107.9 (C-7), 131.5 (C-7a), 33.6 (C-8), 117.9 (C-9), 65.5 (OCH_3).

3-Indoleacetic acid (3), white amorphous powder. IR (KBr, ν, cm^{-1}): 3385, 1645, 1616, 740. ESI-MS: *m/z* 175, 174, 130, 103, 77. ^1H NMR (300 MHz, CD_3OD, δ, ppm, J/Hz): 7.53 (1H, d, J = 7.9, H-4), 7.35 (1H, d, J = 8.0, H-7), 7.17 (1H, s, H-2), 7.11 (1H, t, J = 7.5, H-6), 7.02 (1H, t, J = 7.5, H-5), 3.65 (2H, s, H-8). ^{13}C NMR (75 MHz, CD_3OD, δ, ppm): 119.4 (C-2), 128.3 (C-3), 108.9 (C-3a), 121.1 (C-4), 122.6 (C-5), 124.7 (C-6), 112.3 (C-7), 138.1 (C-7a), 33.9 (C-8), 177.7 (C-9).

3-Indolealdehyde (4), white needles. IR (KBr, ν, cm^{-1}): 3166, 1638, 1619, 1575, 760. ESI-MS: *m/z* 145, 144, 116, 89. ^1H NMR (300 MHz, CD_3OD, δ, ppm, J/Hz): 9.88 (1H, s, H-8), 8.15 (1H, d, J = 7.5, H-4), 8.04 (1H, s, H-2), 7.46 (1H, d, J = 7.9, H-7), 7.27 (1H, t, J = 7.3, H-6), 7.22 (1H, t, J = 7.2, H-5). ^{13}C NMR (75 MHz, CD_3OD, δ, ppm): 22.7 (C-2), 119.7 (C-3), 125.5 (C-3a), 123.8 (C-4), 125.2 (C-5), 140.1 (C-6), 113.4 (C-7), 138.7 (C-7a), 187.8 (C-8).

1-Methoxy-3-indolecarbaldehyde (5), colorless solid. IR (KBr, ν, cm^{-1}): 2925, 1660, 1617, 1520, 745. ESI-MS: *m/z* 175, 144, 132, 116, 85, 71, 57. ^1H NMR (300 MHz, CD_3OD, δ, ppm, J/Hz): 9.85 (1H, s, H-8), 8.21 (1H, d, J = 7.8, H-4), 7.77 (1H, s, H-2), 7.40 (1H, d, J = 8.0, H-7), 7.28 (1H, t, J = 7.5, H-6), 7.23 (1H, t, J = 7.5, H-5), 4.07 (3H, s, -OCH_3). ^{13}C NMR (75 MHz, CD_3OD, δ, ppm): 122.0 (C-2), 114.3 (C-3), 121.6 (C-3a), 123.9 (C-4), 124.2 (C-5), 131.9 (C-6), 108.8 (C-7), 132.4 (C-7a), 184.1 (C-8), 66.6 (OCH_3).

Cytotoxic Assay. The cytotoxic effect of monomers **1–5** on Vero cells was determined by XTT (sodium 3′-[1-(phenylaminecarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene sulfonic acid) (Sigma, USA) assay using the method of Scudiero D. A. [18]. Briefly, Vero cell suspensions were seeded onto 96-well tissue culture plate at densities of 5000–10 000 cells per well for 4 h incubation. Pure compounds **1–5** in DMSO-d_6 were serially diluted to various concentrations in MEM and each dilution was added to quadruplicate wells of a 96-well tissue culture plate. The plate was then incubated for another 72 h. Later, the XTT reagent was added and the plate was reincubated at 37°C for an additional 2 h. Acyclovir (ACV) dilution and DMSO-d_6 dilution were used as positive and negative controls, respectively. The optical densities (OD) were then determined with an enzyme immunoassay (EIA) reader (Lab System, MTX Labs, VA, USA) at a test wavelength of 492 nm and a reference wavelength of 690 nm. The toxicity effects of the pure compounds **1–5** were calculated, and the 50% cytotoxic concentration (CC_{50}) was evaluated by regression analysis of the dose-response curve generated from data [19].

Antiviral Assay. According to the method described previously [20], assessment of anti-HSV activity of **1–5** and acyclovir (ACV) was performed in 24-well culture plates. Vero cells were seeded and incubated for 48 h in a CO_2 incubator at 37°C after plating 500 μL of cell suspension, which contained 1×10^6 cells/mL. Approximately 100 pfu of HSV-1 or HSV-2

isolates and standard strains were added to each well of the plate and allowed to adsorb for 1 h at room temperature. Then, unadsorbed viruses were removed and 200 μL of media, containing compounds **1**–**5** at nontoxic concentrations and ACV, were applied into duplicate test wells, whereas media were added into control wells. After that, 400 μL of growth media, containing 2% sodium carboxymethylcellulose, was added to the infected cells. After incubation for an additional 72 h in a CO_2 incubator, the overlay media were discarded and the cells were stained with 0.1% crystal violet in 1% ethanol for 10 min. Plaques were counted and the concentration of pure compounds **1**–**5** required to suppress the formation of virus plaque by 50% (IC_{50}) was calculated [21].

ACKNOWLEDGMENT

The authors would like to acknowledge the assistance of Prof. Han-Qing Zhang, Institute of Botany, Jiangsu Province and the Chinese Academy of Sciences. This work was cofinanced by grants from the Jiangsu Province administration of traditional chinese medicine basic research projects (HZ0704407KJD).

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