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Novel indole *C*-glycosides from *Isatis indigotica* and their potential cytotoxic activity

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ABSTRACT

Two novel indole *C*-glycosides, which were the first reported alkaloids *C*-glycosides from *Isatis indigotica*, together with five known alkaloids were isolated. The novel alkaloids were elucidated to be indole-3-acetonitrile-4-methoxy-2-*C*-β-D-glucopyranoside (**1**) and *N*-methoxy-indole-3-acetonitrile-2-*C*-β-D-glucopyranoside (**2**) on the basis of spectroscopic analysis. **1** exhibited significant cytotoxic activities against human myeloid leukemia HL-60 and human liver cancer HepG2 cells with the IC_{50} of 1.3 ± 0.1 and 2.1 ± 0.3 μM, respectively. **2** showed potential cytotoxic activities against HL-60 and human myeloid leukemia Mata cells with the IC_{50} of 5.1 ± 0.4 and 12.1 ± 0.8 μM, respectively.

1. Introduction

Radix isatidis (*Ban-Lan-Gen* in Chinese) is the root of the plant *Isatis indigotica*, which widely distributes in China. It has been used as a medicinal plant for more than 2000 years in traditional Chinese medicine, usually used for seasonal febrile diseases, pestilence, eruptive diseases, and inflammatory diseases with redness of sore throat [1]. Previously studies indicated that *R. isatidis* has wide pharmacological bioactivities, including antivirus, anti-bacterial, anti-endotoxic, anti-tumor, anti-inflammatory, immune regulatory effects, etc. [2,3]. The aqueous extract of *R. isatidis* has been used for curing flu and hepatitis in China for decade of years, which has achieved prominent clinical effects.

Previous chemical research indicated that there were many reports of alkaloids in the genus *Isatis*. The major alkaloids were indoles, such as indigotin, isatin, indirubin, hydroxyl indoles, and their glucosides [4–8]. The blue dye indigo (indigotin) is one of the oldest natural dyestuffs known to human beings [9]. Also, indole alkaloids in *Isatis* showed many bioactivities, such as antivirus and anticancer activities [10–13]. Beside indole alkaloids, quinoline and quinazolone were also identified from *Isatis* [14,15]. There are also some sphingolipids [16] and lignans [17] in the plant of *I. indigotica*.

In our continuous investigation of bioactive natural products derived from traditional Chinese medicines, a systematic phytochemical investigation on *I. indigotica* resulted in the isolation of 7 alkaloids, including two novels and five known. In this paper, the isolation, structure elucidation and cytotoxic activity of those alkaloids were reported herein.

2. Experimental

2.1. General

NMR spectra were recorded on a Bruker DPX-400 spectrometer using standard Bruker pulse programs (Bruker BioSpin GmbH, Rheinstetten, Germany). Chemical shifts were expressed as the δ-value with reference to tetramethylsilane (TMS) as an internal standard. ESI-MS data were obtained on an Agilent 1200 HPLC/6410B TripleQuad mass spectrometer (Agilent Technologies, Inc., Santa Clara, USA), and HRESIMS

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were measured on a Bruker APEX II mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany). Sephadex LH-20 (Pharmacia Biotech AB, Uppsala, Sweden), silica gel (Qingdao Ocean Chemical Co. Ltd, Qingdao, China), and ODS (40–63 μm, Merck KGaA, Darmstadt, Germany) were used for column chromatography. TLC was carried out on preparative Silica gel 60 F_{254} and RP-18 F_{254} plates (Merck KGaA, Darmstadt, Germany), and spots were visualized by spraying with 15% H_2SO_4 followed by heating at 105 °C for 10 min. Preparative HPLC was performed using a semipreparative ODS column (Amethyst C18, 10 mm × 250 mm, 5 μm, Sepax Technologies Inc., Newark, USA).

2.2. Plant material

R. isatidis was purchased from Wuhan Longtai Pharmaceutical Company, Wuhan, China, in March 2009, and was identified as the roots of *I. indigotica* by Prof. Xiangjiu He, College of Pharmacy, Wuhan University. A voucher specimen (No. 20090301) is available at the College of Pharmacy, Wuhan University, Wuhan (430071), China.

2.3. Extraction and isolation

R. isatidis (2.0 kg) was crushed into 40 meshes and extracted with 70% ethanol (8 L × 2). The solvent was removed under vacuum at 55 °C to yield a crude extract (303.0 g). The extract was resuspended in water and partitioned with ethyl acetate (2 L × 3) to afford an ethyl acetate extract (43.0 g). The ethyl acetate extract was fractionated through a D101 macroporous resin column (6 × 100 cm) eluted with a gradually increasing amount of ethanol in water and got 5 fractions. Fraction II (10% EtOH elution, 1.86 g) was further applied to a silica gel column (200–300 mesh, 25 × 340 mm), eluted with a gradually increasing amount of MeOH in chloroform, and compound **6** (10.3 mg) was obtained from $CHCl_3$/MeOH 2:1 elution and recrystallized with acetone. Fraction III (30% EtOH elution of the macroporous resin, 2.05 g) was applied to a silica gel column (200–300 mesh, 30 × 220 mm), eluted with $CHCl_3$/MeOH system, and got 9 subfractions. The subfraction 2 ($CHCl_3$/MeOH 100:1 elution, 0.36 g) was purified by semipreparative RP-HPLC (Amethyst C18, 10 mm × 250 mm, 5 μm, Sepax Technologies Inc., Newark, DE) using 22% methanol as mobile phase to give **3** (7.1 mg). **4** (12.0 mg) and **7** (8.6 mg) were from the subfraction 3 ($CHCl_3$/MeOH 50:1 elution, 0.16 g) purified by the semipreparative RP-HPLC using 20% methanol as mobile phase. Fraction IV (50% EtOH elution of the macroporous resin, 1.25 g) was further applied to a silica gel column (200–300 mesh, 25 × 340 mm), eluted with $CHCl_3$/MeOH system, and got 15 subfractions. The subfraction 3 ($CHCl_3$/MeOH 50:1 elution, 0.20 g) was purified by the semipreparative HPLC using 15% methanol as mobile phase and got **1** (6.3 mg) and **2** (10.2 mg). **5** (12.1 mg) was purified from subfraction 12 with repeated open ODS column chromatography (Fig. 1).

Compound **1:** indole-3-acetonitrile-4-methoxy-2-*C*-β-D-glucopyranoside, yellow amorphous powder (acetone). HRESIMS (positive-ion mode) *m/z* 371.1213 (calcd. for $C_{17}H_{20}N_2O_6Na$, 371.1219). ^{1}H NMR (Acetone-d_6, 400 MHz) and ^{13}C NMR (Acetone-d_6, 100 MHz), see Table 1.

Compound **2**: *N*-methoxy-indole-3-acetonitrile-2-*C*-β-D-glucopyranoside, colorless amorphous powder. HRESIMS (positive-ion mode) *m/z* 371.1215 (calcd. for $C_{17}H_{20}N_2O_6Na$, 371.1219). ^{1}H NMR (Acetone-d_6, 400 MHz) and ^{13}C NMR (Acetone-d_6, 100 MHz), see Table 1.

2.4. Cytotoxic activity assay

The cytotoxic activity against four human tumor cell lines, human myeloid leukemia HL-60, human myeloid leukemia Mata, and human liver cancer HepG2 were performed according to the protocol reported by Tao Feng et al. [18]. All cells were cultured in RPMI-1640 medium (Hyclone, Logan, UT), supplemented with 10% fetal bovine serum (Hyclone) and antibiotic (100 units/mL penicillin and 100 μg/mL streptomycin) in 5% CO_2 at 37 °C. The cytotoxic activity assay was performed according to the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] method in 96-well microplates. Briefly, 200 μL adherent cells were seeded into each well of 96-well microplates and allowed to adhere for 12 h before drug addition, while suspended cells were seeded just before drug addition with initial density of 5×10^4 cells/mL. Each tumor cell line was exposed to the test compound at concentrations of 100, 50, 25, 12.5, and 6.25 μg/mL in quadruples for 48 h.

3. Results and discussion

Two new indole alkaloids named indole-3-acetonitrile-4-methoxy-2-*C*-β-D- glucopyranoside (**1**) and *N*-methoxy-indole-3-acetonitrile-2-*C*-β-D-glucopyranoside (**2**), which were the first reported alkaloids *C*-glycosides from *Isatis*, together with 5 known compounds, were isolated and identified. The known compounds were identified as 2,3-dihydro-4-hydroxy-2-oxo-1H-indole-3-acetonitrile (**3**) [19], 1H-indole-3-carboxylic acid (**4**) [20], indole-3-methyl acetate (**5**) [21], adenosine (**6**) [22], and epigoitrin (**7**) [23], respectively, by compared their spectroscopic data with the reported values.

Compound **1** was isolated as yellow amorphous powder. The positive electrospray ionization mass spectrometry (ESI-MS) gave an ion $[M+H]^+$ at *m/z* 349 and the molecular formula of $C_{17}H_{20}N_2O_6$ was drawn combined with ^{1}H and ^{13}C NMR. Its molecular formula was confirmed by HRESIMS, which showed an ion $[M+Na]^+$ at *m/z* 371.1213 (cald. for $C_{17}H_{20}N_2O_6Na$: 371.1219). The IR absorptions of **1** indicated the presence of nitrile group (2256 cm^{-1}). The ^{1}H NMR spectrum showed signals of an active proton (δ_H 11.26), three aryl protons (δ_H 7.10, 1H, dd, J = 7.8, 8.2 Hz; 6.95, 1H, d, J = 8.2 Hz; 6.54, 1H, d, J = 7.8 Hz), two methylene protons (δ_H 4.10, 2H, s), three methoxyl protons (δ_H 2.88, 3H, s), and some protons of the sugar linkage (Table 1). The ^{13}C NMR and DEPT spectra exhibited 17 carbon signals, consisting of a methyl, two methylenes, eight methines, and six quaternary carbons. These NMR data suggested that **1** is a glucopyranoside, possessing an aryl skeleton. The HMBC showed the signal of H-5 (δ_H 6.54, 1H, d, J = 7.8 Hz) correlated with C-4α ((δ_C 116.0) and C-7 (δ_C 104.7), the signal of H-6 (δ 7.10, 1H, dd, J = 7.8, 8.2 Hz) correlated with C-4 (δ_C 153.2) and C-7α (δ_C 139.7), and the signal of H-7 (δ 6.95, 1H, d, J = 8.2 Hz)

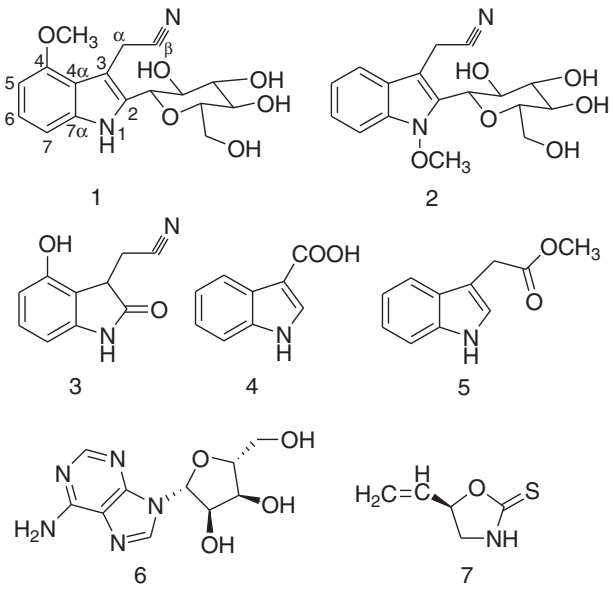

Fig. 1. Structure of compounds **1**–**7** from *Isatis indigotica*.

correlated with C-5 (δ_C 99.7) and C-4α (δ_C 116.0), respectively. The correlation between –OCH_3 (δ 2.88, 3H, s) and C-4 (δ_C 153.2) in HMBC indicated the connection site of the methoxyl group with the benzene. Analysis of its ^{1}H–^{1}H COSY spectra indicated the correlations of the glucose protons from H-1′ (δ 4.33, 1H, d) to H-2′ (δ 2.76, 1H, m), H-2′ to H-3′ (δ 3.13, 1H, m), H-3′ to H-4′ (δ 2.96, 1H, m), H-4′ to H-5′ (δ 3.13, 1H, m), and from H-5′ to H-6′ (δ 3.67, 3.46, 2H, m). The correlation in HMBC between the anomeric proton (δ 4.33, 1H, d, H-1′) and C-2 (δ 121.8) indicated the connection site of the glucose unit. The large coupling constant of anomeric proton at δ 4.33 (H-1′, d, J = 9.1 Hz) suggested β-configuration of the glucose. Comparing the ^{1}H and ^{13}C NMR data [24], the moiety was identified as β-D-glucopyranoside.

Table 1
NMR spectroscopic data (400 MHz, Acetone-d_6) for compounds **1** and **2.**

	1		2	
Position	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)
1		11.26 (s)		
2	121.8		124.1	
3	110.8		109.4	
4	153.2		119.0	7.72 (d, 8.0)
4α	116.0		121.6	
5	99.7	6.54 (d, 7.8)	120.6	7.19 (dd, 8.0, 7.2)
6	123.9	7.09 (dd, 8.2, 7.8)	124.3	7.34 (dd, 8.3, 7.2)
7	104.7	6.95 (d, 8.2)	108.9	7.52 (d, 8.3)
7α	137.9		132.7	
α	14.9	4.10 (s)	13.5	4.22 (m)
β	119.5		119.0	
1′	87.5	4.33 (d, 9.5)	89.3	4.42 (d, 9.7)
2′	72.2	2.76 (m)	72.8	3.01 (m)
3′	77.8	3.13 (m)	77.9	3.16 (m)
4′	69.5	2.96 (m)	69.5	3.08 (m)
5′	81.0	3.13 (m)	81.0	3.08 (m)
6′	61.0	3.67 (m)	61.0	3.66 (m)
		3.46 (m)		3.43 (m)
OCH_3	69.6	3.88 (s)	66.4	4.16 (s)

Apart from benzene and glucose, there were a carbon–carbon double bond and an acetonitrile unit in the structure of **1**. In HMBC spectrum, correlations were observed from the proton signal of H-α (δ_H 4.10 2H, s, CH_2CN) to C-4α (δ_C 116.0), C-2 (δ_C 121.8), C-3 (δ_C 110.8), C-β (δ_C 119.5, $CH_2\underline{C}N$). Based on above analysis, compound **1** was elucidated to be indole-3-acetonitrile-4-methoxy-2-*C*-β-D-glucopyranoside.

Compound **2** was isolated as colorless amorphous powder. The ESI-MS exhibited an ion $[M+H]^+$ at m/z 349 with the positive-ion mode indicating a mass 348 compatible with the molecular formula of $C_{17}H_{20}N_2O_5$. Its molecular formula was confirmed by HRESIMS, which showed an ion $[M+Na]^+$ at m/z 371.1215 (cald. for $C_{17}H_{20}N_2O_6Na$: 371.1219). Comparing the NMR data of **2** to that of **1**, it indicated that they had similar structures, and the difference between them existed in the substituent position of methoxyl group. Analysis of its ^{1}H–^{1}H COSY spectra indicated the correlations of the protons of sugar from H-1′ (δ 4.42, H, d) to H-6′ (δ 3.66, 3.43, 2 H, m). The correlation in HMBC between the anomeric proton (δ 4.42, 1H, d, H-1′) and C-2 (δ 124.1) indicated the connection site of the glucose unit. The large coupling constant of anomeric proton at δ 4.42 (H-1′, J = 9.7 Hz) indicated the β-configuration of the glucose. Comparing the

Table 2
Cytotoxic activities of compounds **1**–**7** against HL-60, Mata, and HepG-2 cells.

Compound	IC_{50} (μM)		
	HL-60	Mata	HepG-2
1	1.3 ± 0.1	92.4 ± 7.1	2.1 ± 0.3
2	5.1 ± 0.4	12.1 ± 0.8	55.8 ± 4.5
3	80.4 ± 8.1	46.3 ± 5.1	21.0 ± 3.1
4	81.2 ± 6.2	78.0 ± 8.2	>100
5	>100	>100	76.0 ± 6.3
6	35.1 ± 2.2	62.3 ± 5.1	>100
7	>100	>100	87.1 ± 7.3
Cisplatin	1.5 ± 0.2	5.1 ± 0.4	4.9 ± 0.3

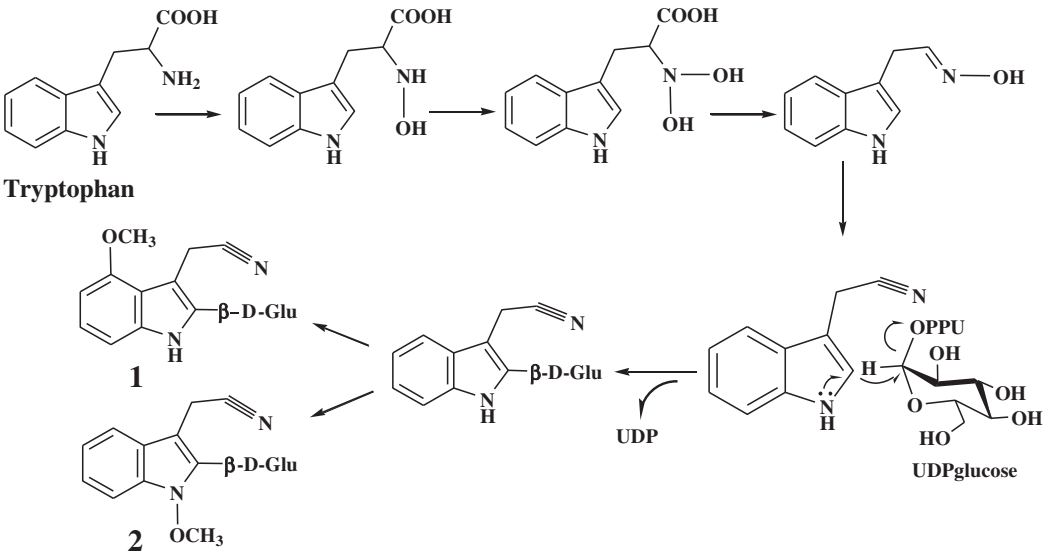

Fig. 2. The proposed biosynthesis pathway of compounds **1** and **2.**

^{1}H and ^{13}C NMR data [24], the moiety was identified as β-D-glucopyranoside. In HMBC spectrum, correlations between H-4 (δ_H 7.72, 1H, d, $J = 8.0$ Hz) and C-3 (δ_C 109.4), H-5 (δ_H 7.19, dd, $J = 8.0$, 7.2 Hz) and C-4α (δ_C 121.6) showed that **2** had a consecutive disubstituted benzene. Therefore, **2** was elucidated to be *N*-methoxy-indole-3-acetonitrile-2-*C*-β-D-glucopyranoside.

The alkaloids were evaluated for cytotoxic activity against three human tumor cell lines, HL-60 (human myeloid leukemia), Mata (human myeloid leukemia), and HepG2 (human liver cancer) using a MTT assay and the results were shown in Table 2. Two novel alkaloids showed potential cytotoxic activity. **1** had significant cytotoxic activity against HL-60 and HepG2 with the IC_{50} of 1.3 ± 0.1 and 2.1 ± 0.3 μM, respectively. **2** had significant cytotoxic activity against HL-60 and Mata with the IC_{50} of 5.1 ± 0.4 and 12.1 ± 0.8 μM, respectively.

Indole alkaloids were the main type of alkaloids in the genus *Isatis*, which exhibited antivirus and anticancer activities [4–8,10–13]. In this research, compounds **1** and **2** exhibited potential cytotoxic activities against human liver cancer and leukemia cell lines, which gave some scientific providence for the traditional use as folk medicine of *Isatis*.

Nitriles are relative rare in natural products. They were found in the genus *Isatis* previously [25,26]. Compounds **1** and **2** were the first reported alkaloids *C*-glycosides from *Isatis*. Their biosynthesis pathways were proposed in Fig. 2 [27–31]. Nitriles were biosynthesized from the precursor of tryptophan. Then *C*-glycosides were possibly formed through a S_N2 nucleophilic replacement reaction with UDPglucose. Finally, compounds **1** and **2** were the oxidation products at different skeleton sites of the nitrile glycosides.

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