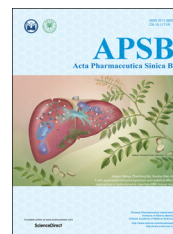




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ORIGINAL ARTICLE

Indole alkaloid sulfonic acids from an aqueous extract of *Isatis indigotica* roots and their antiviral activity



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Isatindosulfonic acids;
Antiviral activity

Abstract Six new indole alkaloid sulfonic acids (**1–6**), together with two analogues (**7** and **8**) that were previously reported as synthetic products, were isolated from an aqueous extract of the *Isatis indigotica* root. Their structures including the absolute configurations were determined by spectroscopic data analysis, combined with enzyme hydrolysis and comparison of experimental circular dichroism and calculated electronic circular dichroism spectra. In the preliminary assay, compounds **2** and **4** showed antiviral activity against Cocksackie virus B3 and influenza virus A/Hanfang/359/95 (H3N2), respectively.

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1. Introduction

“Ban lan gen”, the dried roots of *Isatis indigotica* Fort. (Cruciferae), is the most popular traditional Chinese herbal medicine for the treatment of influenza during pandemics in China¹. Previous investigations showed that extracts of “ban lan gen” had extensive pharmacological effects including antiviral, antibiotic, and anti-inflammatory activities^{2–6}. Around 100 chemical constituents with different structural types (sulfur-containing alkaloids, lignans, and flavonoids, etc.) and various biological activities were isolated from the extracts^{7–15}. However, chemical studies were dominated by extracting “ban lan gen” with ethanol or methanol, differing from practically decocting with water. Accordingly, we investigated an aqueous decoction of “ban lan gen” as part of a program to assess the chemical diversity of traditional Chinese medicines and their biological activities^{16–36}. In our previous papers, more than 70 constituents including 40 new alkaloids in free and glycosidic forms and some with antiviral and cell-damage protective activities^{37–45} were reported from *I. indigotica* for the first time. Herein, reported are isolation, structural characterization, and bioactivity assay of six new indole alkaloid sulfonic acids (**1–6**), together with two analogues previously prepared as synthetic products (**7** and **8**, Fig. 1).

2. Results and discussion

Compound **1** was isolated as a white amorphous powder with $[\alpha]_D^{20} -12.3$ (c 0.07, MeOH). Its IR spectrum showed absorption bands for hydroxyl (3396 cm^{-1}) and aromatic ring (1600 and 1512 cm^{-1}) functional groups. The molecular formula of **1** was determined as $\text{C}_{23}\text{H}_{24}\text{N}_2\text{O}_9\text{S}$ by $(-)\text{-HR-ESI-MS}$ at m/z 503.1148 $[\text{M-H}]^-$ (Calcd. for $\text{C}_{23}\text{H}_{23}\text{N}_2\text{O}_9\text{S}$, 503.1130) and NMR spectroscopic data. As compared with those of the previously isolated chemical constituents from the same extract^{37–45}, the NMR spectroscopic data of **1** in CD_3OD (Table 1) indicated the presence of one 2,3-disubstituted 1*H*-indole ring, one 1'*H*-indol-3'-yl, a β -glucopyranosyl, and an isolated methine. This, together with the molecular formula, indicated that **1** was an uncommon sulfur-containing bis(indolyl)methane β -glucopyranoside^{37,40,41}, of which the planar structure was further elucidated by interpretation of 2D NMR spectroscopic data.

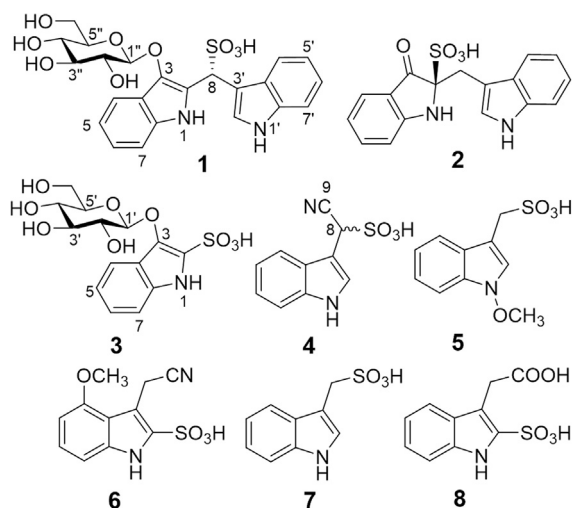


Figure 1 The structures of compounds **1–8**.

The proton-bearing carbon and corresponding proton signals in the NMR spectra of **1** were assigned by analysis of the ^1H - ^1H COSY and HSQC spectra. The HMBC spectrum of **1** exhibited two- and three-bond heteronuclear correlations (Fig. 2) from H-4 to C-3, C-6, and C-7a; from H-5 to C-3a and C-7; from H-6 to C-4 and C-7a; from H-7 to C-3a and C-5; from H-8 to C-2 and C-3;

Table 1 The NMR spectroscopic data (δ) of compounds **1** and **2**^a.

No.	1 ^b		2	
	δ_{H}	δ_{C}	δ_{H}	δ_{C}
2		126.9		85.6
3		134.5		200.0
3a		122.3		122.0
4	7.63 brd (8.4)	118.5	7.25 brd (7.2)	125.0
5	6.90 dt (1.2, 8.4)	119.8	6.52 brt (7.2)	118.8
6	6.97 dt (1.2, 8.4)	122.2	7.23 dt (1.2, 7.2)	138.3
7	7.22 brd (8.4)	112.4	6.74 brd (7.2)	113.1
7a		134.8		163.6
8a	6.25 s	54.9	3.99 d (15.0)	29.3
8b			3.56 d (15.0)	
2'	7.54 s	125.3	6.96 s	125.0
3'		112.3		108.9
3'a		128.7		129.4
4'	7.73 brd (8.4)	120.4	7.66 brd (7.2)	119.9
5'	6.92 dt (1.2, 8.4)	119.8	6.94 dt (1.2, 7.2)	119.6
6'	7.00 dt (1.2, 8.4)	122.2	6.97 dt (1.2, 7.2)	122.0
7'	7.27 brd (8.4)	122.6	7.16 brd (7.2)	111.9
7'a		137.3		137.4

^a ^1H and ^{13}C NMR data (δ) were measured at 600 and 150 MHz in CD_3OD for **1** and **2**, respectively. Proton coupling constants (J) in Hz are given in parentheses. The assignments were based on ^1H - ^1H COSY, HSQC, and HMBC experiments.

^bData for glucopyranosyl in **1**: δ_{H} 4.70 (1 H, d, $J = 7.8$ Hz, H-1'), 3.53 (1 H, dd, $J = 7.8, 9.0$ Hz, H-2'), 3.40 (1 H, t, $J = 9.0$ Hz, H-3'), 3.31 (1 H, t, $J = 9.0$ Hz, H-4'), 3.18 (1 H, m, H-5'), 3.82 (1 H, dd, $J = 12.0, 2.4$ Hz, H-6'a), 3.71 (1 H, dd, $J = 12.0, 6.6$ Hz, H-6'b); δ_{C} 106.7 (C-1'), 75.5 (C-2'), 78.0 (C-3'), 71.9 (C-4'), 78.8 (C-5'), 63.2 (C-6').

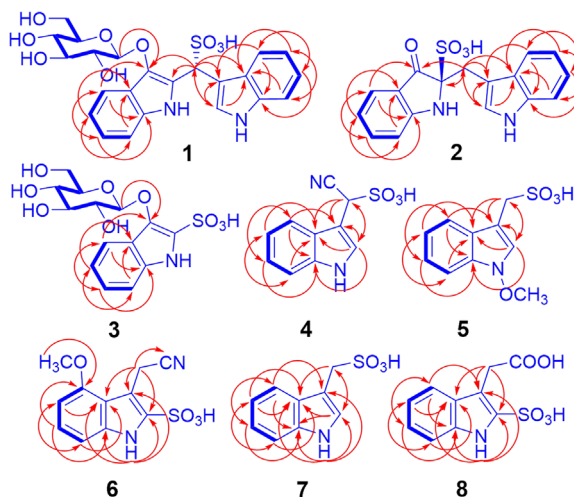


Figure 2 Main ^1H - ^1H COSY (thick lines) and HMBC (arrows, from ^1H to ^{13}C) correlations of compounds **1–8**.

and from H-1'' to C-3. These correlations, together with the ^1H - ^1H COSY cross-peaks of H-4/H-5/H-6/H-7 and H-1''/H-2''/H-3''/H-4''/H-5''/H-6'' and the chemical shifts of the proton and carbon resonances, revealed that there was a 3- β -glucopyranosyloxy-1*H*-indol-2-yl-methine moiety in **1**. The HMBC correlations of H-2'/C-3', C-3'a, C-7'a, and C-8; H-4'/C-3', C-6', and C-7'a; H-5'/C-3'a and C-7'; H-6'/C-4' and C-7'a; H-7'/C-5' and C-3'a; and H-8/C-2', C-3', and C-3'a; in combination with their chemical shifts and the ^1H - ^1H COSY cross-peaks of H-4'/H-5'/H-6'/H-7', proved that the 1*H*-indol-3'-yl was substituted at the methine (CH-8) to a (3- β -glucopyranosyloxy-1*H*-indol-2-yl)(1*H*-indol-3'-yl)methine parent structure for **1**. To match requirements of the molecular formula and the substituted aliphatic methine (CH-8), a sulfonic acid unit must be placed at C-8, which is supported by the chemical shifts of the CH-8 resonances (δ_{H} 6.25 and δ_{C} 54.9). Thus, the planar structure of **1** was established as shown in Fig. 2.

The stereochemistry of **1** was assigned by hydrolysis with snailase, combined with explanation of circular dichroism (CD) spectroscopic data. From the hydrolysate, D-glucose was isolated and identified by comparison of retention factor (R_f) on TLC, specific rotation $[\alpha]_{\text{D}}^{20}$, and ^1H NMR spectroscopic data with those of an authentic sugar sample (see in Experimental Section and Figs. S24 and S111 in Supplementary Information), while the aglycone was decomposed into a complex mixture that failed to be separated. The CD spectrum of **1** displayed typical split Cotton effects at λ_{max} 214 ($\Delta\epsilon$ +6.13) and 233 ($\Delta\epsilon$ -9.66) nm, arising from coupling between $\pi \rightarrow \pi^*$ transitions of the two indole chromophores and corresponding to an absorption band at λ_{max} 223 nm in the UV spectrum. Application of the CD exciton chirality method⁴⁶, the negative CD exciton chirality indicates the *R* configuration for **1**. This was supported by calculations of electronic CD (ECD) spectra of **1**, its 8'*S* diastereoisomer (**1'**), and the aglycone (**1a**) (see Figs. S5-9 in Supplementary Information) based on the time-dependent density functional theory (TDDFT)⁴⁷. The main Cotton effects in the experimental CD spectrum of **1** were in agreement with that in the calculated ECD spectrum of **1**, but almost completely opposite in the calculated ECD spectrum of **1'** (Fig. 3). Therefore, the structure of compound **1** was determined and named as isatibisindosulfonic acid A 3-*O*- β -D-glucopyranoside.

Compound **2**, a white amorphous powder with $[\alpha]_{\text{D}}^{20}$ +10.7 (*c* 0.05, MeOH), has the molecular formula $\text{C}_{17}\text{H}_{14}\text{N}_2\text{O}_4\text{S}$ as determined by HR-ESI-MS at m/z 341.0607 [$\text{M}-\text{H}$]⁻ (Calcd. for $\text{C}_{17}\text{H}_{13}\text{N}_2\text{O}_4\text{S}$, 341.0602) and NMR spectroscopic data.

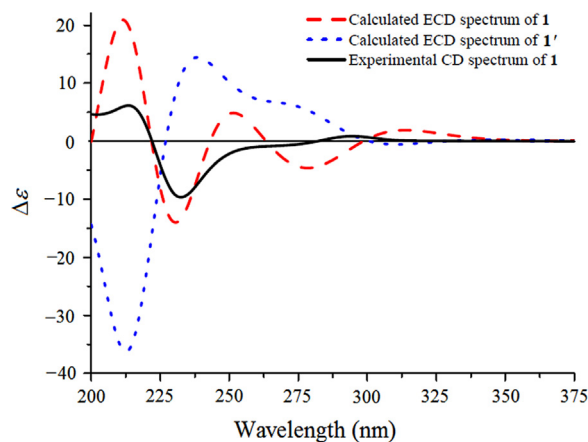


Figure 3 The experimental CD spectrum of **1** (black) and the calculated ECD spectra of **1** (red dash) and **1'** (blue dot).

Comparison of the NMR spectroscopic data of **2** with those of **1** indicated that the 3- β -glucopyranosyloxy-1*H*-indol-2-yl and the aliphatic methine in **1** were replaced by a 2-substituted 3-oxoindoline-2-yl and an aliphatic methylene (CH_2 -8) in **2**, respectively. This was verified by 2D NMR spectroscopic data of **2**. Especially, besides the correlations confirming the 1*H*-indol-3'-ylmethylene moiety (Fig. 2), the HMBC correlations of H-4/C-3, C-6, and C-7a; H-5/C-3a, and C-7; H-6/C-4 and C-7a; H-7/C-5 and C-3a; and H₂-8/C-2 and C-3; together with the ^1H - ^1H COSY cross-peaks of H-4/H-5/H-6/H-7 as well as the chemical shifts of these proton and carbon resonances, proved the presence of 2-substituted 3-oxo-indolin-2-yl at C-8. The sulfonic acid unit must be located at the remaining substitution site (C-2) to satisfy the molecular composition of **2**. This was supported by the deshielded chemical shift of C-2 (δ_{C} 85.6) induced by three electronic withdraw substituents. The *R* configuration of **2** was assigned by comparison of the experimental CD and calculated ECD spectra of **2** and its enantiomer (Fig. 4). Therefore, the structure of compound **2** was determined and designated as isatibisindosulfonic acid B.

Compound **3** was isolated as a white amorphous powder with $[\alpha]_{\text{D}}^{20}$ -8.4 (*c* 0.10, MeOH). Its molecular formula was deduced as $\text{C}_{14}\text{H}_{17}\text{NO}_9\text{S}$ from (+)-HR-ESI-MS at m/z 398.0509 [$\text{M}+\text{Na}$]⁺ (Calcd. for $\text{C}_{14}\text{H}_{17}\text{NO}_9\text{SNa}$, 398.0516). The NMR spectrum of **3** in CD_3OD showed signals assignable to a 2,3-disubstituted indole ring and a β -glucopyranosyloxy. The presence of these two units was supported by cross-peaks of H-4/H-5/H-6/H-7 and H-1'/H-2'/H-3'/H-4'/H-5'/H-6' in the ^1H - ^1H COSY spectrum of **3**, as well as by their chemical shifts and coupling constants (Table 2). In the HMBC spectrum of **3**, besides the correlations confirming the indole ring and β -glucopyranosyloxy moieties (Fig. 2), the correlation from H-1' to C-3 located the β -glucopyranosyloxy group at C-3 of the indole ring. Considering the molecular formula and quaternary nature of C-2, a sulfonic acid unit must be located at C-2. Using the same protocols as described for **1**, D-glucose was isolated and identified from the enzymatic hydrolysate of **3**. Therefore, the structure of compound **3** was determined and named isatindosulfonic acid A 3-*O*- β -D-glucopyranoside.

Compound **4** was obtained as a white amorphous powder with $[\alpha]_{\text{D}}^{20}$ \approx 0 (*c* 0.07, MeOH). The (-)-HR-ESI-MS at m/z 235.0182 [$\text{M}-\text{H}$]⁻ (Calcd. for $\text{C}_{10}\text{H}_7\text{N}_2\text{O}_3\text{S}$ 235.0183) indicated the molecular formula $\text{C}_{10}\text{H}_8\text{N}_2\text{O}_3\text{S}$ for **4**. The NMR spectroscopic data showed that **4** contained structural units of a methine, a 1*H*-indol-3-yl, and a cyano group. The presence of cyano group

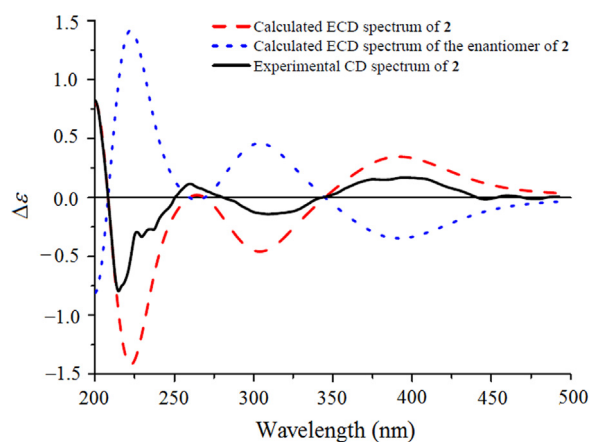


Figure 4 The experimental CD spectrum of **2** (black) and the calculated ECD spectra of **2** (red dash) and its enantiomer (blue dash).

Table 2 The NMR spectral data (δ) for compounds **3–8**^a.

No	3^b		4		5^c		6^d		7		8	
	δ_{H}	δ_{C}	δ_{H}	δ_{C}	δ_{H}	δ_{C}	δ_{H}	δ_{C}	δ_{H}	δ_{C}	δ_{H}	δ_{C}
1							11.29		10.82 s		10.92 s	
2		127.9	7.39 s	126.8	7.45 s	124.7		139.0	7.20 s	124.6		137.5
3		135.5		105.8		104.5		99.8		108.3		109.1
3a		121.5		127.3		125.5		116.1		127.8		127.9
4	7.91 brd (7.8)	120.8	7.75 brd (8.4)	120.5	7.69 brd (7.8)	120.8		154.3	7.59 brd (7.8)	119.5	7.52 brd (7.8)	120.1
5	7.03 dt (1.2, 7.8)	120.7	7.00 dt (1.2, 8.4)	120.5	7.06 dt (1.2, 7.8)	120.7	6.48 d (8.0)	99.7	6.92 brt (7.8)	117.9	6.91 dt (1.2, 7.8)	118.2
6	7.17 dt (1.2, 7.8)	124.8	7.07 dt (1.2, 8.4)	122.8	7.17 dt (1.2, 7.8)	123.2	7.00 t (8.0)	123.3	7.01 brt (7.8)	120.4	7.04 dt (1.2, 7.8)	121.8
7	7.33 brd (7.8)	113.0	7.31 brd (8.4)	112.4	7.37 brd (7.8)	108.9	6.92 d (8.0)	105.4	7.30 brd (7.8)	110.9	7.29 brd (7.8)	111.6
7a		133.9		137.9		133.4		135.6		135.8		133.8
8			5.39 s	50.9	4.21 s	49.5	4.20 s	14.0	3.86 s	48.4	3.66 s	34.4
9				118.4				119.8				

^aNMR data (δ) were measured in CD₃OD for **3–5** and in DMSO-*d*₆ for **6–8**, at 600 MHz for ¹H and 150 MHz for ¹³C of **3–5**, **7**, and **8** and at 500 MHz for ¹H and 125 MHz for ¹³C of **6**, respectively. Proton coupling constants (*J*) in Hz are given in parentheses. The assignments were based on ¹H–¹H COSY, HSQC, and HMBC experiments.

^bData for glucopyranosyl in **3**: δ_{H} 4.81 (1 H, d, *J* = 7.8 Hz, H-1''), 3.58 (1 H, dd, *J* = 9.6, 7.8 Hz, H-2''), 3.44 (1 H, t, *J* = 9.6 Hz, H-3''), 3.45 (1 H, t, *J* = 9.6 Hz, H-4''), 3.28 (1 H, m, H-5''), 3.91 (1 H, dd, *J* = 12.0, 2.4 Hz, H-6'a), 3.79 (1 H, dd, *J* = 12.0, 4.8 Hz, H-6'b); δ_{C} 107.4 (C-1''), 75.3 (C-2''), 77.7 (C-3''), 71.2 (C-4''), 78.3 (C-5''), 62.5 (C-6'').

^cData for methoxy in **5**: δ_{H} 4.07 (3 H, s); δ_{C} 66.2.

^dData for methoxy in **6**: δ_{H} 3.85 (3 H, s); δ_{C} 55.3.

was supported by a characteristic absorption band at 2254 cm^{−1} in the IR spectrum, while the 1*H*-indol-3-yl was confirmed by 2D NMR spectroscopic data (Fig. 2). Especially the HMBC correlations from H-2 to C-3, C-3a, C-7a, and C-8 and from H-8 to C-2, C-3, and C-3a revealed that there was a main structural moiety of 1*H*-indol-3-ylmethine in **4**. The molecular formula required that the cyano group and an additional sulfonic acid unit must be located at the methine. This was supported by an exchangeable property of the methine proton (H-8) which was disappeared in the 2D NMR spectra of the same sample after the 1D NMR spectra of **4** in CD₃OD were acquired and the sample was kept at 4 °C for several months (see Figs. S60–62 in Supplementary Information). For this exchange, an appropriate explanation is due to a combination of electron-withdraw effects from the three substituents at the methine. This, together with the optical inactivity, indicated that **4** was isolated as a racemate. Thus, the structure of compound **4** was determined as (±)-cyano(1*H*-indol-3-yl)methanesulfonic acid and named isatindosulfonic acid B.

Compound **5**, a white amorphous powder, has the molecular formula C₁₀H₁₁NO₄S as indicated from (+)-HR-ESI-MS at *m/z* 242.0481 [M+H]⁺ (Calcd. for C₁₀H₁₂NO₄S, 242.0482) and the NMR spectroscopic data. Comparison of the NMR spectroscopic data between **5** and **4** (Table 2) demonstrated replacement of the cyano group and the aliphatic methine in **4** by a methoxy group and an aliphatic methylene in **5**, respectively. Analysis of 2D NMR spectroscopic data (Fig. 2) proved that **5** had the parent structure of 1*H*-indol-3-ylmethylene. The methoxy group was positioned at *N*-1 on the basis of the deshielded chemical shifts of the methoxy proton and carbon resonances (δ_{H} 4.07 and δ_{C} 66.2)^{41,42}. Meanwhile, a sulfonic acid unit must be put at the methylene unit to fulfill the molecular formula and the remaining substitution position in **5**. Therefore, the structure of compound **5**

was determined as (1-methoxy-1*H*-indol-3-yl)methanesulfonic acid and named isatindosulfonic acid C.

Compound **6** was isolated as a white amorphous powder. Its molecular formula was determined as C₁₁H₁₀N₂O₄S by (+)-HR-ESI-MS *m/z* 267.0434 [M+H]⁺ (Calcd. for C₁₁H₁₁N₂O₄S, 267.0434) and 289.0253 [M+Na]⁺ (Calcd. for C₁₁H₁₀N₂O₄SNa, 289.0253). Comparison of the NMR spectroscopic data between **6** and **4** indicated that the 1*H*-indol-3-ylmethine in **4** was replaced by a 2-substituted 4-methoxy-1*H*-indol-3-ylmethylene in **6**, while the sulfonic acid group at the methine in **4** was migrated to C-2 of the indole ring in **6**. This was confirmed by a typical cyano absorption band at 2257 cm^{−1} in the IR spectrum of **6**, especially by the HMBC correlations from H-1 to C-2, C-3, C-3a, and C-7a; from H₂-8 to C-2, C-3, C-3a, and C-9, and from OCH₃ to C-4. In addition, as compared with those of the previously isolated analogues from this extract^{37–45}, the chemical shifts of C-2 (δ_{C} 139.0, deshielded) and C-3 (δ_{C} 99.8, shielded) supported location of the sulfonic acid unit. Therefore, the structure of compound **6** was determined as 3-(cyanomethyl)-4-methoxy-1*H*-indole-2-sulfonic acid and named isatindosulfonic acid D.

Compound **7**, a white amorphous powder, showed NMR spectroscopic data similar to those of **5**, except that a nitrogen-bearing hydrogen (δ_{H} 10.82) in **7** substituted the nitrogen-bearing methoxy group in **5**. Thus, the structure of compound **7** was determined as (1*H*-indol-3-yl)methanesulfonic acid and named isatindosulfonic acid E, which was proved by (−)-HR-ESI-MS and 2D NMR spectroscopic data (Fig. 2).

Compound **8**, a white amorphous powder, has the molecular formula C₁₀H₉NO₅S as indicated from (−)-HR-ESI-MS at *m/z* 254.0126 [M−H][−] (Calcd. for C₁₀H₈NO₅S 254.0129). Comparison of the NMR spectroscopic data between **8** and **6** demonstrated replacement of the cyano group in **6** by a carboxylic acid group in

8, in addition to the absence of the methoxy group. Accordingly, the structure of compound **8** was determined as shown and named isatindosulfonic acid **F**, which was also verified by 2D NMR spectroscopic data (Fig. 2).

Although compound **7** was previously synthesized by treatment of gramine with a sodium sulfite solution^{48,49} or the sulfomethylation of indole with formaldehyde and sodium sulfite⁵⁰ and **8** was obtained from aerobic oxidation of indole-3-acetic acid with bisulfite^{51–53}, the two compounds have never been isolated from a natural source. Thus, they are new natural products. The detailed spectroscopic data of **7** and **8** are included in this paper since these data are absent in the literatures^{48–53}.

Theoretical ECD spectral calculations are powerful methods that increasingly applied for the determination of absolute configurations of various natural products⁴⁷. Our previous^{26,41,42,45} and present ECD spectra calculations demonstrate that the presence of β -D-glucopyranosyloxy on the chromophore(s) of natural products has significant influences on intensities, wavelengths, and signs of the Cotton effects in the calculated ECD spectra. Especially, in several cases^{26,41} the calculated ECD spectra of β -D-glucopyranosides are incomparable with those of the aglycones, for instance, **1** and **1'** (see Figs. S6 and S7 Supplementary Information). This suggests that comprehensive comparison and analysis of the experimental CD and calculated ECD spectra of the aglycone and diastereoisomers for a β -D-glucopyranoside are essential to make correct assignment of the absolute configuration. Direct and simple comparison of the experimental CD and calculated ECD spectra of the β -D-glucopyranoside or between β -D-glucopyranoside and aglycone may causes an ambiguous conclusion.

In the preliminary *in vitro* assays^{37–45}, compound **2** exhibited antiviral activity against Coxsackie virus B3 with an IC_{50} value of 33.3 μ mol/L and $SI > 3.0$ (the positive controls pleconaril and ribavirin gave $IC_{50} = 0.0009$ and 517.38 μ mol/L and $SI = 17122.2$ and 3.0, respectively), while compound **4** were active against influenza virus A/Hanfang/359/95 (H3N2) with $IC_{50} = 33.3$ μ mol/L and $SI > 3.0$ (the positive controls oseltamivir and ribavirin had $IC_{50} = 1.63$ and 1.06 μ mol/L and $SI = 773.0$ and 1098.2, respectively). The other isolates were inactive at a concentration of 50 μ mol/L. In addition, these compounds were also assessed for their inhibitory activity against HIV-1 replication, and several human cancer cell lines, but all were inactive at a concentration of 10 μ mol/L.

3. Conclusions

Eight indole alkaloid sulfonic acids (**1–8**), having diverse structure features, were isolated from the aqueous extract of “ban lan gen”. Six (**1–6**) are new compounds that have never been obtained as natural or synthetic products and two (**7** and **8**) are new natural products that were previously synthesized, while two (**2** and **4**) were active against Coxsackie virus B3 and influenza virus A/Hanfang/359/95 (H3N2), respectively. Compounds **1** and **2** are the first examples of bis(indolyl)methane sulfuration metabolites, and **1** and **3** are the first glycosidic forms of indole sulfuration products. Characterization of **1–8** indicates that the abnormal sulfonic acid forms of the indole alkaloids abundantly exist in the aqueous extract of “ban lan gen”, which have never been known before. This, together with previous results^{37–45}, indicates the occurrence of a variety of previously unknown components in the aqueous extract of “ban lan gen”. As components of the medicinal extract, more detailed chemical and biological investigations of the abnormal forms of the plant metabolites are required to find out

their contributions to pharmacological efficacy that support the traditional application of the herbal medicine, and to search for hits of new drug development.

4. Experimental

4.1. General experimental procedures

Optical rotations were measured on a P-2000 polarimeter (JASCO, Tokyo, Japan). UV spectra were acquired on a V-650 spectrometer (JASCO, Tokyo, Japan). CD spectra were measured on a JASCO J-815 CD spectrometer (JASCO, Tokyo, Japan). IR spectra were obtained on a Nicolet 5700 FT-IR microscope instrument (FT-IR microscope transmission) (Thermo Electron Corporation, Madison, WI, USA). NMR spectra were recorded at 600 or 500 MHz for 1H NMR and 150 or 125 MHz for ^{13}C NMR, respectively, on a Bruker AVIIIHD 600 (Bruker Corp., Karlsruhe, Germany) or a SYS 600 or an Inova 500 instrument (Varian Associates Inc., Palo Alto, CA, USA) in CD_3OD , $DMSO-d_6$, or D_2O with solvent peaks used as references. ESI-MS and HR-ESI-MS data were taken on an Agilent 1100 Series LC-MSD-Trap-SL and an Agilent 6520 Accurate-Mass Q-TOFL CMS spectrometers (Agilent Technologies, Ltd., Santa Clara, CA, USA), respectively. Column chromatography (CC) was carried out on macroporous adsorbent resin (HPD-110, Cangzhou Bon Absorber Technology, Co., Ltd., Cangzhou, China), CHP 20 P (Mitsubishi Chemical Inc., Tokyo, Japan), silica gel (200–300 mesh, Qingdao Marine Chemical Inc. Qingdao, China), Sephadex LH-20 (Pharmacia Biotech AB, Uppsala, Sweden), HW-40C (Mitsubishi Chemical Inc.), or reversed phase C18 silica gel (W. R. Grace & Co., MD, USA). HPLC separation was performed on an instrument equipped with an Agilent ChemStation for LC system, an Agilent 1200 pump, and an Agilent 1100 single-wavelength absorbance detector (Agilent Technologies, Ltd.) using a Grace semipreparative column (250 mm \times 10 mm, i.d.) packed with C18 reversed phase silica gel (5 μ m) (W. R. Grace & Co., MD, USA). TLC was carried out on glass precoated silica gel GF₂₅₄ plates (Qingdao Marine Chemical Inc.). Spots were visualized under UV light or by spraying with 7% H_2SO_4 in 95% EtOH followed by heating. Unless otherwise noted, all chemicals were purchased from commercially available sources and were used without further purification.

4.2. Plant material

The *Isatis indigotica* roots “ban lan gen” were purchased in Anhui province, China, in December 2009. The plant was identified by Mr. Lin Ma (Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China). A voucher specimen (No. ID-S-2385) was deposited at the Herbarium of the Department of Medicinal Plants, Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China.

4.3. Extraction and isolation

For the extraction and preliminary fractionation of the extract see Ref. 37. Fraction B2 (547 g) was subjected to CC over silica gel and eluted by a gradient of increasing MeOH (0–100%) in EtOAc to yield B2-1–B2-5. Subfraction B2-4 (120 g) was separated

by CC over Sephadex LH-20 (CHCl_3 -MeOH, v/v, 1:1) to yield B2-4-1-B2-4-3, of which B2-4-1 (40 g) was further separated by CC over Sephadex LH-20 (H_2O) to yield B2-4-1-1-B2-4-1-13. Separation of B2-4-1-7 (6 g) by CC over Sephadex LH-20 (MeOH- H_2O , v/v, 4:6) obtained B2-4-1-7-1-B2-4-1-7-4, of which B2-4-1-7-2 (2 g) was separated again by CC over Sephadex LH-20 (MeOH- H_2O , v/v, 4:6) to afford B2-4-1-7-2-1-B2-4-1-7-2-3. Isolation of B2-4-1-7-2-1 (130 mg) by CC over HW-40C (H_2O) yielded **8** (8 mg), while separation of B2-4-1-7-2-2 (1.5 g) by CC over HW-40C (H_2O) afforded B2-4-1-7-2-2-1-B2-4-1-7-2-2-5. Purification of B2-4-1-7-2-2-2 (60 mg) by RP-HPLC (3% MeCN in H_2O containing 0.2% acetic acid, v/v/v, 1.5 mL/min) gave **3** (6.0 mg, t_R = 13 min). Subfraction B2-4-1-8 (2 g) was separated by CC over silica gel and eluted by a gradient of increasing MeOH (0–100%) in CHCl_3 to yield B2-4-1-8-1-B2-4-1-8-9, of which B2-4-1-8-2 (200 mg) was chromatographed over Sephadex LH-20 (MeOH) to afford B2-4-1-8-2-1-B2-4-1-8-2-5. Isolation of B2-4-1-8-2-5 (100 mg) by RP-HPLC (2% MeCN in H_2O containing 0.2% acetic acid, 1 mL/min) obtained **4** (6.0 mg, t_R = 11 min).

Fraction B3 (165 g) was separated by CC over silica gel, eluting with a gradient of increasing MeOH (0–100%) in EtOAc, to yield B3-1-B3-16, of which B3-4 (11 g) was further fractionated by CC over Sephadex LH-20 (MeOH) to give B3-4-1-B3-4-6. Isolation of B3-4-5 (1.8 g) by RP-MPLC eluting with a gradient of increasing MeOH (20%–100%, v/v) in H_2O yielded B3-4-5-1-B3-4-5-12. Preparative TLC separation of B3-4-5-2 (127.0 mg), using the mobile phase of EtOAc-MeOH- H_2O (8:2:1, v/v/v), obtained B3-4-5-2-1-B3-4-5-2-3. Subsequent purification of B3-4-5-2-1 (37 mg) by RP-HPLC (15% MeOH in H_2O , v/v, 1.5 mL/min) afforded **6** (6.0 mg, t_R = 45 min). Fractionation of B3-11 (1.8 g) by CC over Sephadex LH-20 (H_2O) yielded B3-11-1-B3-11-3, of which B3-11-2 (120 mg) was fractionated again by CC over Sephadex LH-20 (H_2O) to give B3-11-2-1-B3-11-2-4. Isolation of B3-11-2-1 (18 mg) by CC over Sephadex LH-20 (MeOH) gave **5** (4 mg) and of B3-11-2-4 (30 mg) by Sephadex LH-20 (H_2O) obtained **2** (5 mg), while B3-11-3-1-B3-11-3-8 was afforded from B3-11-3 (300 mg) by CC over Sephadex LH-20 (MeOH). Subsequent separation of B3-11-3-2 (45 mg) by RP-HPLC (2% MeCN in H_2O containing 0.2% acetic acid, v/v/v, 1.8 mL/min) obtained **1** (4.0 mg, t_R = 35 min), and of B3-11-3-8 (30 mg) by RP-HPLC (2% MeCN in H_2O containing 0.3% acetic acid, v/v/v, 1.5 mL/min) yielded **7** (10.0 mg, t_R = 17 min).

4.3.1. *Isatibisindosulfonic acid A 3-O-β-D-glucopyranoside (1)*

White amorphous solid; $[\alpha]_D^{20}$ –12.3 (c 0.07, MeOH). UV (MeOH) λ_{max} (log ϵ) 203 (3.32), 223 (3.05), 281 (2.74) nm; CD (MeOH) 214 ($\Delta\epsilon$ +6.13), 233 ($\Delta\epsilon$ –9.66), 295 ($\Delta\epsilon$ +0.84); IR ν_{max} 3396, 3188, 3011, 2922, 2850, 2500, 2361, 1645, 1512, 1468, 1420, 1343, 1325, 1301, 1246, 1215, 1188, 1119, 817, 722, 648, 547, 521 cm^{-1} . ^1H NMR (CD_3OD , 600 MHz) data, see Table 1; ^{13}C NMR (CD_3OD , 150 MHz) data, see Table 1; (–)-ESI-MS: m/z 503 $[\text{M}-\text{H}]^-$; (–)-HR-ESI-MS: m/z 503.1148 $[\text{M}-\text{H}]^-$ (Calcd. for $\text{C}_{23}\text{H}_{23}\text{N}_2\text{O}_9\text{S}$, 503.1130).

4.3.2. *Isatibisindosulfonic acid B (2)*

White amorphous solid; $[\alpha]_D^{20}$ +10.7 (c 0.05, MeOH). UV (MeOH) λ_{max} (log ϵ) 202 (3.79), 221 (3.44), 288 (3.09) nm; CD (MeOH) 215 ($\Delta\epsilon$ –0.80), 260 ($\Delta\epsilon$ +0.11), 310 ($\Delta\epsilon$ –0.14), 399 ($\Delta\epsilon$ +0.17); IR ν_{max} 3396, 3186, 3011, 2922, 2850, 1646, 1511, 1469, 1420, 1343, 1325, 1301, 1245, 1215, 1119, 1043, 817, 722, 647, 548 cm^{-1} . ^1H NMR (CD_3OD , 600 MHz) data, see Table 1; ^{13}C NMR (CD_3OD ,

150 MHz) data, see Table 1; (–)-ESI-MS: m/z 341 $[\text{M}-\text{H}]^-$; (–)-HR-ESI-MS: m/z 341.0607 $[\text{M}-\text{H}]^-$ (Calcd. for $\text{C}_{17}\text{H}_{13}\text{N}_2\text{O}_4\text{S}$, 341.0602).

4.3.3. *Isatindosulfonic acid A 3-O-β-D-glucopyranoside (3)*

White amorphous solid; $[\alpha]_D^{20}$ –8.4 (c 0.10, MeOH). UV (MeOH) λ_{max} (log ϵ) 203 (2.70), 223 (2.53), 281 (1.80) nm; IR ν_{max} 3391, 2922, 2850, 1686, 1575, 1418, 1343, 1301, 1248, 1208, 1137, 1106, 1046, 928, 835, 802, 748, 722, 663, 624, 579, 534 cm^{-1} . ^1H NMR (CD_3OD , 600 MHz) data, see Table 2; ^{13}C NMR (CD_3OD , 150 MHz) data, see Table 2; (–)-ESI-MS: m/z 374 $[\text{M}-\text{H}]^-$; (+)-HR-ESI-MS: m/z 398.0509 $[\text{M}+\text{Na}]^+$ (Calcd. for $\text{C}_{14}\text{H}_{17}\text{NO}_9\text{SNa}$, 398.0516).

4.3.4. *Isatindosulfonic acid B (4)*

White amorphous solid; UV (MeOH) λ_{max} (log ϵ) 204 (3.47), 217 (3.52), 287 (2.85) nm; IR ν_{max} 3395, 3185, 3011, 2922, 2850, 2500, 2254, 1645, 1512, 1468, 1420, 1343, 1300, 1246, 1216, 1119, 1047, 817, 744, 722, 647, 546, 522 cm^{-1} . ^1H NMR (CD_3OD , 600 MHz) data, see Table 2; ^{13}C NMR (CD_3OD , 150 MHz) data, see Table 2; (–)-ESI-MS: m/z 235 $[\text{M}-\text{H}]^-$; (–)-HR-ESI-MS: m/z 235.0182 $[\text{M}-\text{H}]^-$ (Calcd. for $\text{C}_{10}\text{H}_7\text{N}_2\text{O}_3\text{S}$, 235.0183).

4.3.5. *Isatindosulfonic acid C (5)*

White amorphous solid; UV (MeOH) λ_{max} (log ϵ) 220 (2.40), 273 (1.80), 291 (1.79) nm; IR ν_{max} 3395, 3186, 3011, 2921, 2850, 1646, 1468, 1420, 1343, 1324, 1301, 1245, 1215, 1188, 1119, 1049, 816, 722, 647 cm^{-1} ; ^1H NMR (CD_3OD , 600 MHz) data, see Table 2; ^{13}C NMR (CD_3OD , 150 MHz) data, see Table 2; (–)-ESI-MS: m/z 240 $[\text{M}-\text{H}]^-$; (+)-HR-ESI-MS: m/z 242.0481 $[\text{M}+\text{H}]^+$ (Calcd. for $\text{C}_{10}\text{H}_{12}\text{NO}_4\text{S}$, 242.0482).

4.3.6. *Isatindosulfonic acid D (6)*

White amorphous solid; UV (MeOH) λ_{max} (log ϵ) 205 (4.07), 222 (4.25), 271 (3.62), 287 (3.35), 297 (3.25) nm; IR ν_{max} 3460, 2257, 1643, 1619, 1588, 1514, 1396, 1369, 1350, 1258, 1204, 1117, 1039, 999, 829, 787, 768, 740, 714, 648 cm^{-1} ; ^1H NMR ($\text{DMSO}-d_6$, 500 MHz) data see Table 2; ^{13}C NMR ($\text{DMSO}-d_6$, 125 MHz) data see Table 2; (–)-ESI-MS: m/z 265 $[\text{M}-\text{H}]^-$; (+)-HR-ESI-MS: m/z 267.0434 $[\text{M}+\text{H}]^+$ (Calcd. for $\text{C}_{11}\text{H}_{11}\text{N}_2\text{O}_4\text{S}$, 267.0434), 289.0253 $[\text{M}+\text{Na}]^+$ (Calcd. for $\text{C}_{11}\text{H}_{10}\text{N}_2\text{O}_4\text{SNa}$, 289.0253).

4.3.7. *Isatindosulfonic acid E (7)*

White amorphous solid; UV (MeOH) λ_{max} (log ϵ) 220 (3.33), 271 (2.77), 289 (2.74) nm; IR ν_{max} 3399, 3114, 3052, 2957, 2927, 2852, 2531, 2304, 1926, 1887, 1771, 1696, 1616, 1543, 1487, 1457, 1415, 1351, 1281, 1222, 1190, 1162, 1123, 1097, 1067, 1008, 927, 897, 878, 851, 792, 774, 742, 646, 609, 583, 535, 520 cm^{-1} ; ^1H NMR ($\text{DMSO}-d_6$, 600 MHz) data, see Table 2; ^{13}C NMR ($\text{DMSO}-d_6$, 150 MHz) data, see Table 2; (–)-ESI-MS: m/z 210 $[\text{M}-\text{H}]^-$; (–)-HR-ESI-MS: m/z 210.0235 $[\text{M}-\text{H}]^-$ (Calcd. for $\text{C}_9\text{H}_8\text{NO}_3\text{S}$, 210.0230).

4.3.8. *Isatindosulfonic acid F (8)*

White amorphous solid; UV (MeOH) λ_{max} (log ϵ) 204 (2.08), 277 (0.92) nm; IR ν_{max} 3395, 3187, 3011, 2921, 2850, 2256, 1731, 1646, 1512, 1469, 1420, 1343, 1325, 1301, 1246, 1215, 1119, 1027, 1005, 818, 763, 722, 648, 547 cm^{-1} ; ^1H NMR ($\text{DMSO}-d_6$, 600 MHz) data, see Table 2; ^{13}C NMR ($\text{DMSO}-d_6$, 150 MHz) data, see Table 2; (–)-ESI-MS: m/z 254 $[\text{M}-\text{H}]^-$; (–)-HR-ESI-MS: m/z 254.0126 $[\text{M}-\text{H}]^-$ (Calcd. for $\text{C}_{10}\text{H}_8\text{NO}_5\text{S}$, 254.0129).

4.4. Enzymatic hydrolysis of **1** and **3**

Compounds **1** and **3** (~1.5 mg) were separately hydrolyzed in H₂O (3 mL) with snailase (3.0 mg, CODE S0100, Beijing Biodee Biotech Co., Ltd., Beijing, China) at 37 °C for 24 h. The hydrolysate was concentrated under reduced pressure and the residue was isolated by CC over silica gel eluting with CH₃CN–H₂O (8:1, v/v) to afford sugar and decomposed mixture of aglycone. The sugar (0.3–0.4 mg) showed a retention factor ($R_f \approx 0.38$) on TLC (EtOAc–MeOH–AcOH–H₂O, 12:3:3:2), $[\alpha]_D^{20} +44.1$ –44.5 (c 0.03–0.04, H₂O), and ¹H NMR (D₂O) data in agreement with those of an authentic D-glucose (see Supporting Information).

4.5. ECD Calculation of **1** and **2**

For details, see Supporting Information. Briefly, conformational analysis was carried out by using the MMFF94 molecular mechanics force field via the MOE software package for **1** and **2**. The lowest-energy conformers with relative energies lower than 2 kcal/mol were re-optimized using the density functional theory (DFT) at the B3LYP/6–31+G (d,p) level via the Gaussian 09 program. The conductor-like polarizable continuum model (CPCM) was employed to consider the solvent effects using the dielectric constant of MeOH ($\epsilon=32.6$). The energies, oscillator strengths, and rotational strengths of excitations were calculated using the TDDFT methodology at the B3LYP/6–31+G (d,p) level in vacuum. The re-optimized conformers having relative Gibbs free energies (ΔG) within 2 kcal/mol were used to simulate the ECD spectra with the Gaussian function ($\sigma=0.28$ eV). To obtain the final spectrum, the simulated spectra of the lowest energy conformers were averaged based on the Boltzmann distribution theory and their ΔG . All quantum computations were conducted using Gaussian 09 program package.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.apsb.2017.04.003>.

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