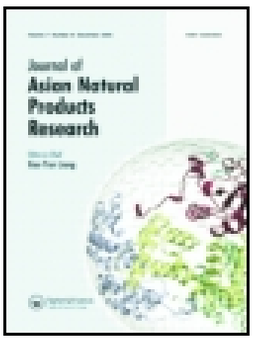

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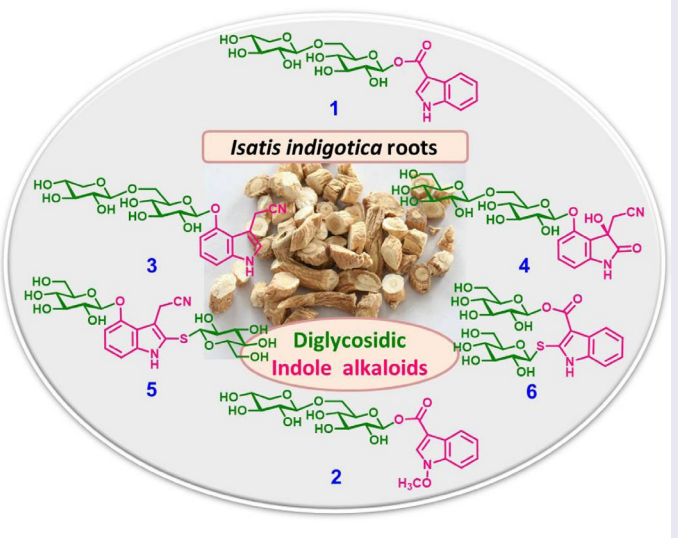
ABSTRACT

Six new indole alkaloid diglycosides named isatigotindolediosides A-F (**1–6**), along with three known analogs (**7–9**), were isolated from an aqueous extract of the *Isatis indigotica* roots (ban lan gen). Their structures including the absolute configurations were determined by comprehensive spectroscopic data analysis, combined with enzyme or acid hydrolysis, and comparison of experimental circular dichroism (CD) and calculated electronic circular dichroism (ECD) spectra. In the preliminary assays, compounds **3**, **5**, and **8** showed antiviral activity against Coxsackie virus B3.



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1. Introduction

The dried roots of *Isatis indigotica* Fort. (Cruciferae), Chinese name "ban lan gen", is a popular traditional medicine for the treatment of influenza and infection diseases. Considerable studies have revealed that extracts of this herbal medicine had various pharmacological effects including antiviral and anti-inflammatory activities [1]. Although around 100 chemical constituents were isolated from the extracts, only a few showed marginal bioactivities [2,3]. Literature shows that the previous chemical studies were mainly performed with the ethanol and methanol extracts, whereas the herbal medicine is practically utilized by decocting with water. Therefore, an aqueous extract of "ban lan gen" was investigated as part of our program to assess the chemical diversity of traditional Chinese medicines and their biological activities, focusing on water-soluble and/or minor components [3–15]. Previously we reported more than 70 constituents from *I. indigotica* for the first time, including 39 new alkaloids in free and monoglycosidic forms, some of them showed potent antiviral and cell-damage protective activities [16–22]. With a continuation of our study on the same extract, six new diglycosidic indole alkaloid derivatives named isatigotindolediosides A-F (**1–6**, Figure 1), together with three known analogs (**7–9**), have been obtained. This paper describes details of the isolation, structure elucidation, and bioactivity assay of the new isolates.

2. Results and discussion

Compound **1** was isolated as a white amorphous powder with $[\alpha]_D^{20}$ – 27.2 (*c* 0.09, MeOH). Its IR spectrum showed absorption bands for hydroxyl (3396 cm^{-1}), conjugated carbonyl (1706 and 1645 cm^{-1}), and aromatic ring [1600 (sh) and 1516 cm^{-1}] functionalities. The molecular formula of **1** was determined as $C_{21}H_{27}NO_{12}$ by (+)-HR-ESI-MS at *m/z* 508.1434 $[M + Na]^+$ and NMR spectroscopic data (Tables 1 and 2). The ^1H NMR spectrum of **1** in MeOH-d_4 showed diagnostic signals for a 3′-substituted 1′*H*-indole ring [21] at δ_H 8.28 (s, H-2′), 8.14 (d, *J* = 7.8 Hz, H-4′), 7.27 (t, *J* = 7.8 Hz, H-5′), 7.33 (t, *J* = 7.8 Hz, H-6′) and

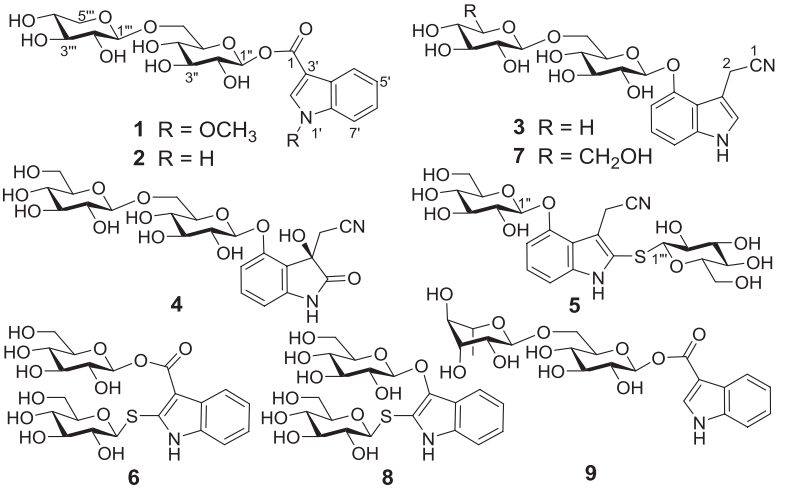

Figure 1. The structures of compounds **1–9**.

Table 1. ^{1}H NMR spectral data (δ) for compounds **1–6**.[a]

No	**1**[b]	**2**	**3**	**4**	**5**[c]	**6**
2a			4.20 dd (18.6, 0.6)	3.38 d (16.2)	4.33 d (16.8)	
2b			4.16 dd (18.6, 0.6)	3.16 d (16.2)	4.10 d (16.8)	
1′					11.23 s	
2′	8.28 s	8.07 s	7.13 brs			
4′	8.14 d (7.8)	8.10 dd (7.2, 1.8)				7.98 dd (7.2, 1.8)
5′	7.27 t (7.8)	7.20 dt (7.2, 1.2)	6.82 dd (7.8, 0.6)	6.94 brd (8.4)	6.72 d (7.8)	7.10 dt (1.8, 7.2)
6′	7.33 t (7.8)	7.22 dt (7.2, 1.8)	7.08 t (7.8)	7.34 t (8.4)	7.05 t (7.8)	7.12 dt (1.8, 7.2)
7′	7.53 d (7.8)	7.45 dd (7.2, 1.2)	7.05 dd (7.8, 0.6)	6.65 brd (8.4)	6.99 d (7.8)	7.31 dd (7.2, 1.8)
1″	5.69 d (7.8)	5.70 d (7.8)	5.05 d (7.8)	5.00 d (7.8)	4.91 d (7.8)	5.71 d (8.4)
2″	3.53 dd (9.0, 7.8)	3.54 dd (9.0, 7.8)	3.60 dd (9.0, 7.8)	3.57 dd (9.0, 7.8)	3.40 dd (9.0, 7.8)	3.56 dd (9.0, 8.4)
3″	3.50 t (9.0)	3.51 t (9.0)	3.49 t (9.0)	3.47 t (9.0)	3.29 brt (9.0)	3.45 t (9.0)
4″	3.46 t (9.0)	3.46 t (9.0)	3.44 t (9.0)	3.52 t (9.0)	3.21 brt (9.0)	3.38 t (9.0)
5″	3.63 m	3.64 m	3.65 m	3.63 m	3.32 m	3.41 m
6″a	4.13 dd (11.4, 1.8)	4.13 dd (11.4, 1.8)	4.09 dd (11.4, 1.8)	3.96 dd (10.8, 4.8)	3.73 dd (11.4, 2.4)	3.82 dd (12.0, 1.8)
6″b	3.75 dd (11.4, 5.4)	3.75 dd (11.4, 5.4)	3.79 (11.4, 6.0)	3.78 brd (10.8)	3.49 dd (11.4, 5.4)	3.63 dd (12.0, 5.4)
1‴	4.30 d (6.6)	4.30 d (7.2)	4.31 d (7.8)	4.88 d (7.8)	4.36 d (9.6)	4.84 d (9.6)
2‴	3.57 dd (9.0, 6.6)	3.57 dd (9.0, 7.2)	3.19 dd (9.0, 7.8)	3.39 dd (9.0, 7.8)	2.76 dd (9.6, 9.0)	3.25 dd (9.6, 9.0)
3‴	3.53 t (9.0)	3.52 t (9.0)	3.26 t (9.0)	3.67 t (9.0)	3.15 t (9.0)	3.38 t (9.0)
4‴	3.76 m	3.76 m	3.47 m	3.30 t (9.0)	2.97 t (9.0)	3.27 t (9.0)
5‴a	3.81 dd (12.6, 3.0)	3.81 dd (12.6, 3.0)	3.80 dd (11.4, 5.4)	3.64 m	3.13 m	3.49 m
5‴b	3.48 dd (12.6, 10.2)	3.48 dd (12.6, 10.2)	3.06 dd (11.4, 10.2)			
6‴a				3.75 brd (10.2)	3.65 brd (10.4)	3.92 dd (12.0, 2.4)
6‴b				3.62 dd (10.8, 5.4)	3.48 dd (10.4, 5.4)	3.66 dd (12.0, 5.4)

[a]^{1}H NMR data (δ) were measured at 600 MHz in CD_3OD for **1–4** and **6** and in DMSO-d_6 for **5**, respectively. Proton coupling constants (J) in Hz are given in parentheses. The assignments were based on ^{1}H-^{1}H COSY, HSQC, and HMBC experiments.
[b]Data for the methoxy group in **1**: δ_H 4.18 (3H, s).
[c]Data for hydroxyl protons of **5** in DMSO-d_6: δ_H 5.32 (1H, d, J = 3.6 Hz, O*H*-2″), 5.29 (1H, brs, O*H*-2‴), 5.15 (1H, brs, O*H*-4″), 5.06 (1H, brs, O*H*-3″), 5.06 (1H, brs, O*H*-3‴), 4.97 (1H, brs, O*H*-4‴), 4.61 (1H, brs, O*H*-6″), 4.54 (1H, brs, O*H*-6‴).

Table 2. ^{13}C NMR spectral data (δ) for compounds **1–6**.[a]

No	**1**[b]	**2**	**3**	**4**	**5**	**6**
1	164.5	165.5	121.3	117.1	120.0	165.6
2			16.2	25.5	14.6	
2′	131.0	134.3	123.6	178.4	122.4	140.5
3′	103.2	107.5	105.3	74.7	110.7	107.3
3′a	124.4	127.5	118.6	117.9	116.9	127.8
4′	122.5	122.0	152.9	156.6	151.3	121.9
5′	123.7	122.8	105.4	112.5	103.6	122.9
6′	124.8	123.9	124.1	133.6	123.7	123.9
7′	109.9	113.1	107.6	106.6	105.7	112.2
7′a	133.6	138.2	140.0	144.4	138.0	138.3
1″	95.5	95.3	102.7	103.2	101.2	95.6
2″	74.1	74.1	75.2	75.0	73.6	74.2
3″	78.0	78.0	78.1	77.8	76.8	78.3
4″	71.3	71.3	71.4	71.3	69.8	71.2
5″	77.7	77.7	77.4	76.8	77.2	78.9
6″	69.2	69.2	69.6	67.4	60.9	62.5
1‴	104.7	104.7	105.2	100.1	87.2	86.7
2‴	72.4	72.4	75.0	73.6	72.1	74.3
3‴	74.2	74.2	77.6	75.2	77.9	79.4
4‴	69.5	69.5	71.2	71.7	69.5	71.4
5‴	66.7	66.7	66.8	73.7	81.1	82.3
6‴				62.6	60.8	62.5

[a]^{13}C NMR data (δ) were measured at 150 MHz in CD_3OD for **1–4** and **6** and in DMSO-d_6 for **5**, respectively. The assignments were based on DEPT, ^{1}H-^{1}H COSY, HSQC, and HMBC experiments.
[b]Data for the methoxy group in **1**: δ_C 67.4.

7.53 (d, J = 7.8 Hz, H-7′), a methoxy group at δ_H 4.18 (s, OCH_3-N), and two sugar units, of which anomeric protons resonated at δ_H 5.69 (d, J = 7.8 Hz, H-1″) and 4.30 (d, J = 6.6 Hz, H-1‴), respectively, and partially overlapped hydroxyl-geminating protons between δ_H 4.13 and 3.46 (Table 1). The presence of the structural units was supported by the ^{13}C NMR and DEPT spectra of **1**, which displayed 21 carbon resonances (Table 2) corresponding to the 3′-substituted 1′*H*-indole ring and methoxy units, along with resonances due to a pentosyl, a hexosyl, and an additional ester carbonyl carbon at δ_C 164.5 (C-1). These spectroscopic data suggest that **1** is an uncommon diglycosidic 1′*H*-indole derivative containing an ester carbonyl unit, of which the structure was further elucidated by 2D NMR data analysis.

The proton-bearing carbon and corresponding proton signals in the NMR spectra of **1** were assigned by analysis of the ^{1}H-^{1}H COSY and HSQC spectra. The HMBC spectrum of **1** exhibited two- and three-bond long range heteronuclear correlations (Figure 2) from H-2′ to C-1, C-3′, C-3′a, and C-7′a; from H-4′ to C-3′, C-6′, and C-7′a; from H-5′ to C-3′a and C-7′; from H-6′ to C-4′ and C-7′a, and from H-7′ to C-3′a and C-5′. These correlations, together with their chemical shifts and the ^{1}H-^{1}H COSY cross-peaks of H-4′/H-5′/H-6′/H-7′, revealed the presence of a 1′*H*-indole-3′-carboxyl moiety in **1**. The HMBC correlation from H-1″ to C-1, in combination with the ^{1}H-^{1}H COSY cross-peaks of H-1″/H-2″/H-3″/H-4″/H-5″/H_2-6″ and the coupling constant values between the vicinal protons ($J_{1'',2''}$ = 7.8 Hz and $J_{2'',3''}$ = $J_{3'',4''}$ = $J_{4'',5''}$ = 9.0 Hz), demonstrated that the 1′*H*-indole-3′-carboxyl connected via an ester bond to the anomeric carbon (C-1″) of *β*-glucopyranosyl. In addition, the HMBC correlations of H-1‴/C-5‴ and H_2-5‴/C-1‴, along with the cross-peaks of H-1‴/H-2‴/H-3‴/H-4‴/H_2-5‴ and the coupling constant values ($J_{1''',2'''}$ = 6.6 Hz and $J_{2''',3'''}$ = $J_{3''',4'''}$ = 9.0 Hz), indicated that there was a *β*-xylopyranosyl in **1**. Meanwhile, the HMBC correlations of H-1‴/C-6″, H_2-6″/C-1‴ revealed a 1‴→6″ connection between *β*-xylopyranosyl and *β*-glucopyranosyl in **1**. The remaining methoxy group was located at the nitrogen atom to satisfy the requirement of the molecular formula of **1**. This was supported by the chemical shift of the methoxy carbon resonance (δ_c 67.4) [18,20]. From the enzymatic hydrolysate of **1**, a sugar mixture

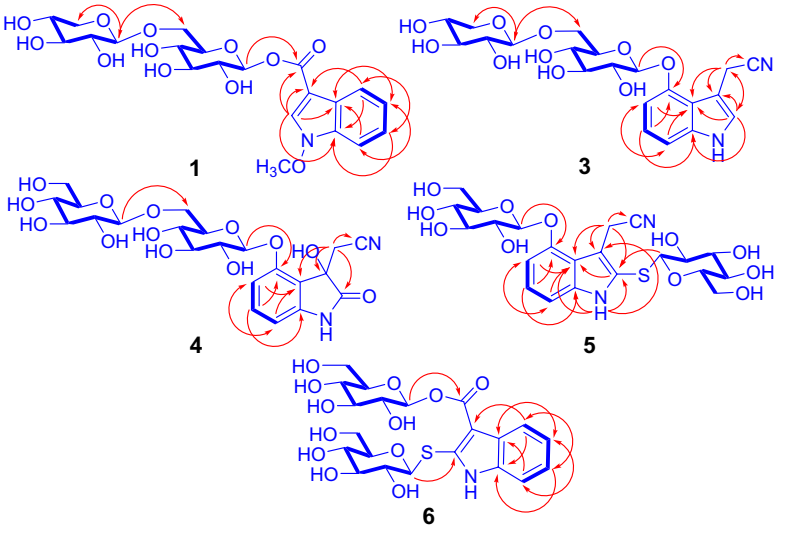

Figure 2. Main ^{1}H-^{1}H COSY (thick lines) and HMBC (arrows, from ^{1}H to ^{13}C) correlations of compounds **1** and **3–6**.

was isolated. Subsequent chiral derivatization and GC analysis of the mixture (Section 3), as compared with derivatives of authentic sugar samples, proved liberation of D-xylose and D-glucose by enzyme hydrolysis of **1**. Thus, the structure of compound **1** was determined as *β*-D-xylopyranosyl-(1‴→6″)-*β*-D-glucopyranosyl 1′-methoxy-1′*H*-indole-3′-carboxylate, and given a trivial name isatigotindoledioside A.

Compound **2**, a white amorphous powder with $[\alpha]_D^{20}$ − 25.7 (*c* 0.05, MeOH), showed similar spectroscopic features as those of **1** and has the molecular formula $C_{20}H_{25}NO_{11}$ as determined by HR-ESIMS at *m/z* 478.1326 $[M + Na]^+$ and NMR spectroscopic data. Comparison of the NMR spectroscopic data of **2** and **1** (Tables 1 and 2) indicated the absence of the methoxy group in **2**, suggesting that **2** is a demethoxy derivative of **1**. The suggestion was supported by shielded shifts of H-4′, H-5′, H-6′, and H-7′ ($\Delta\delta_H$ −0.04 to −0.21) and deshielded shifts of C-2′, C-3′, C-3′a, C-7′, and C-7′a ($\Delta\delta_C$ +3.1 to +4.6) for 1′*H*-indole-3′-carboxyl in **2**, as compared with those in **1**. This was further verified by 2D NMR data analysis and enzyme hydrolysis of **2**. Especially using the same protocols as described for **1**, D-glucose and D-xylose were identified from the hydrolysate of **2**. Therefore, the structure of compound **2** was determined as *β*-D-xylopyranosyl-(1‴→6″)-*β*-D-glucopyranosyl 1′*H*-indole-3′-carboxylate and named isatigotindoledioside B.

Compound **3** was obtained as a white amorphous powder with $[\alpha]_D^{20}$ − 55.2 (*c* 0.12, MeOH). The molecular formula of **3** was determined as $C_{21}H_{26}N_2O_{10}$ by HR-ESIMS at *m/z* 489.1491 $[M + Na]^+$ and NMR spectroscopic data. Comparison of the NMR data between **3** and **2** (Tables 1 and 2) indicated replacement of the aglycone in **2** by a 4′-substituted 2-(1′*H*-indol-3′-yl)-acetonitrile in **3**, while the sugar moiety was same in the two compounds. Particularly the presence of the 2-substituted acetonitrile unit was indicated by a characteristic absorption band (2254 cm^{-1}) for the cyano group in the IR spectrum of **3**, in combination with the resonances due to an isolated methylene [δ_H 4.20 (1H, dd, *J* = 18.6, 0.6 Hz, H-2a) and 4.16 (1H, dd, *J* = 18.6, 0.6 Hz, H-2b) and δ_C 16.2 (C-2)] and a quaternary cyano carbon at δ_C 121.3 (C-1) in the NMR spectra. This suggests that **3** is 2-(4′-hydroxy-1′*H*-indol-3′-yl)-acetonitrile 4′-*O*-*β*-D-xylopyranosyl-(1‴→6″)-*β*-D-glucopyranoside, which was further confirmed by 2D NMR spectroscopic data analysis and enzyme hydrolysis of **3**. Especially, the HMBC correlations of H_2-2/C-1, C-2′, C-3′, C-3′a; H-2′/C-2, C-3′, C-3′a, and C-7′a; H-5′/C-3′a, C-4′, and C-7′; H-6′/C-4′ and C-7′a, H-7′/C-3′a and C-5′ confirmed the aglycone while the correlations of H-1″/C-4′, H-1‴/C-6″, and H_2-6″/C-1‴ proved the linkage among the sugar and aglycone units. Enzyme hydrolysis of **3** also liberated D-glucose and D-xylose as identified by application of the aforementioned method. Therefore, the structure of compound **3** was determined and named isatigotindoledioside C.

Compound **4**, a white amorphous powder with $[\alpha]_D^{20}$ − 26.8 (*c* 0.02, MeOH), has the molecular formula $C_{22}H_{28}N_2O_{13}$ as indicated by HR-ESIMS at *m/z* 551.1482 $[M + Na]^+$. The IR and NMR spectroscopic data showed that this compound is another diglycosidic indole alkaloid differing from **1–3** in both the aglycone and sugar moieties. As compared with those of **3**, the IR absorption bands (2259 and 1728 cm^{-1}) and NMR spectroscopic data (Tables 1 and 2) demonstrated that **4** had an aglycone of 2-(3′,4′-dihydroxy-2′-oxoindolin-3′-yl)acetonitrile. This was confirmed by the ^1H-^1H COSY cross-peaks of H-2a/H-2b and H-5′/H-6′/H-7′ and the HMBC correlations of H-2/C-1, C-2′, C-3′, and C-3′a; H-5′/C-3′a, and C-7′; H-6′/C-4′ and C-7′a; and H-7′/C-3′a, and C-5′, together with the chemical shifts of these proton and carbon resonances. Meanwhile, the presence of two *β*-glucopyranosyls in **4** were indicated by the ^1H-^1H COSY cross-peaks of H-1″/H-2″/H-3″/H-4″/H-5″/

H_2-6″ and H-1‴/H-2‴/H-3‴/H-4‴/H-5‴/H_2-6‴, as well as by their chemical shifts and coupling constants (Table 1). In addition, in the HMBC spectrum of **4**, the correlations from H-1″ to C-4′, from H_2-6″ to C-1‴, and from H-1‴ to C-6″ revealed substitution of a (1‴→6″)-di-*β*-glucopyranosyloxy at C-4′ of the aglycone.

Enzyme hydrolysis of **4** afforded the aglycone **4a** and sugar. The ^{1}H NMR spectrum of **4a** was completely consistent with that of (+)-(*S*)-2-(3,4-dihydroxy-2-oxoindolin-3-yl) acetonitrile, which was previously isolated from the same extract and structurally proved by single-crystal X-ray crystallographic analysis [16]. However, **4a** gave a negative specific rotation, while the circular dichroism (CD) spectra of **4** and **4a** displayed positive Cotton effects around 217 and 265 nm and a negative Cotton effect around 240 nm. These Cotton effects mirrored to those of (+)-(*S*)-2-(3,4-dihydroxy-2-oxoindolin-3-yl)acetonitrile [16], suggesting the *R* configuration for **4** and **4a**. The suggestion was further supported by comparison of the experimental CD and calculated electronic circular dichroism (ECD) spectra of **4a** as shown in Figure 3. The sugar was identified as D-glucose by comparison of the ^{1}H NMR spectrum and specific rotation data ($[\alpha]_D^{20}$ + 44.0 (*c* 0.02, H_2O)) with those of the authentic sugar sample. Therefore, the structure of compound **4** was determined as (–)-(*R*)-2-(3′,4′-dihydroxy-2′-oxoindolin-3′-yl)-acetonitrile 4′-*O*-*β*-D-glucopyranosyl-(1‴→6″)-*β*-D-glucopyranoside and named isatigotindoledioside D.

Compound **5**, a white amorphous powder with $[\alpha]_D^{20}$ – 43.5 (*c* 0.07, MeOH), showed IR and NMR spectroscopic data similar to those of **3**. The molecular formula $C_{22}H_{28}N_2O_{11}S$ of **5** was assigned by HR-ESIMS at *m/z* 551.1305 $[M + Na]^+$, suggesting that this compound is a sulfur-containing derivative of **3**. Comparison of the NMR spectroscopic data between **5** and **3** indicated that the sp^2 hybridized methine of the aglycone in **3** was replaced by a sp^2 hybridized quaternary carbon (δ_C 122.4, C-2′) in **5**, and that the terminal *β*-xylopyranosyl of **3** was replaced by a *β*-thioglucopyranosyl of **5** with characteristic anomeric proton and carbon resonated at δ_H 4.36 (d, *J* = 9.6 Hz, H-1‴) and δ_C 87.2 (C-1‴) [18,20], respectively. This suggests that **5** is 2-(4′-hydroxy-2′-mercapto-1′*H*-indol-3′-yl)-acetonitrile

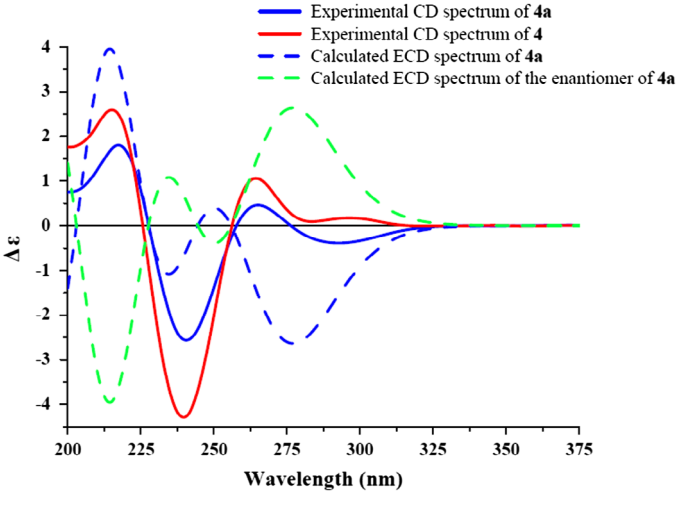

Figure 3. The experimental CD spectra of **4a** (blue) and **4** (red) and the calculated ECD spectra of **4a** (blue, dash line) and the enantiomer of **4a** (green, dash line). The calculated ECD curves were blue-shifted by 10.5 nm.

4′-*O*-*β*-D-glucopyranosyl-2′-*S*-*β*-D-glucopyranoside, which was confirmed by 2D NMR data analysis (Figure 2 and Supporting Information) and acid hydrolysis. The aglycone was verified by the ^{1}H-^{1}H COSY cross peaks of H-5′/H-6′/H-7′ and the HMBC correlations of H-1′/C-2′, C-3′, C-3′a, and C-7′a; H-2/C-1, C-2′, C-3′, and C-3′a; H-5′/C-3′a, C-4′, and C-7′; H-6′/C-4′ and C-7′a; and H-7′/C-5′ and C-3′a. The occurrence and location of *β*-glucopyranosyl and *β*-thioglucopyranosyl were proved by the ^{1}H-^{1}H COSY cross-peaks of H-1″/H-2″/H-3″/H-4″/H-5″/H_2-6″ and H-1‴/H-2‴/H-3‴/H-4‴/H-5‴/H_2-6‴ and their chemical shifts and coupling constants (Table 1), along with the HMBC correlations from H-1″ to C-4′ and from H-1‴ to C-2′. Acid hydrolysis of **5** produced a sole sugar, which was identified as D-glucose by comparison of the ^{1}H NMR spectrum and specific rotation data ($[\alpha]_D^{20}$ + 47.5 (*c* 0.08, H_2O)) with those of the authentic sugar sample. Therefore, the structure of compound **5** was determined as shown and named isatigotindoledioside E.

Compound **6**, a white amorphous powder with $[\alpha]_D^{20}$ – 23.3 (*c* 0.07, MeOH), is another sulfur-containing 1′*H*-indole diglucopyranoside having the molecular formula $C_{21}H_{27}N_2O_{12}S$ that was established by HR-ESIMS at *m/z* 540.1152 $[M + Na]^+$ and the NMR spectroscopic data. As comparison of the NMR spectroscopic data (Tables 1 and 2), the difference between **6** and **2** was similar to that between **5** and **3**. Accordingly, compound **6** was deduced to be *β*-D-glucopyranosyl 2′-*β*-D-thioglucopyranosyl-1′*H*-indole-3′-carboxylate. The deduction was proved by subsequent 2D NMR data analysis and acid hydrolysis. The aglycone was confirmed by the ^{1}H-^{1}H COSY cross peaks of H-4′/H-5′/H-6′/H-7′ and the HMBC correlations of H-4′/C-3′, C-3′a, C-6′, and C-7′a; H-5′/C-3′a, C-4′, C-6′, and C-7′; H-6′/C-4′, C-5′, C-7′, and C-7′a; and H-7′/C-3′a, C-5′, and C-7′a (Figure 2). Meanwhile, the occurrence of *β*-glucopyranosyl and *β*-thioglucopyranosyl, as well as their locations, was demonstrated by the ^{1}H-^{1}H COSY cross-peaks of H-1″/H-2″/H-3″/H-4″/H-5″/H_2-6″ and H-1‴/H-2‴/H-3‴/H-4‴/H-5‴/H_2-6‴ and the HMBC correlations of H-1″/C-1 and H-1‴/C-2′. From the acid hydrolysate of **6**, D-glucose was isolated and identified by measurements of the ^{1}H NMR spectrum and specific rotation data ($[\alpha]_D^{20}$ + 45.3 (*c* 0.03, H_2O)). Thus, the structure of compound **6** was determined and named isatigotindoledioside F.

By comparing the spectroscopic data with the reported data in the corresponding literature, the known compounds were identified as cappariloside B (**7**) [23], calanthoside (**8**) [24], and *α*-L-rhamnopyranosyl-(1→6)-*β*-D-glucopyranosyl 3-indolecarbonate (**9**) [25], respectively. In the preliminary *in vitro* assays [16–22], compounds **3, 5**, and **8** showed equally inhibitory activity against Coxsackie virus B3 with an IC_{50} value of 33.3 μM and SI >3.0 (the positive control ribavirin gave an IC_{50} value of 0.0009 μM and a SI value of 17,122.2). However, all the new isolates were inactive against influenza virus A/Hanfang/359/95 (H3N2) at a concentration of 10 μM.

In summary, six new indole alkaloid diglycosides, having diverse structure features, were isolated from the aqueous extract of "ban lan gen". Among them, three showed antiviral activity against Coxsackie virus B3 but were inactive against influenza virus A/Hanfang/359/95 (H3N2). This, together with our previous results [16–22], shows that diverse indole alkaloid components have varied contributions to pharmacological efficacy that supports the traditional applications of the herbal medicine. Especially, the diverse structures provide a clue for in-depth investigation of structure-activity relationships of the indole-derived analogs.

3. Experimental

3.1. 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, U.S.A.). NMR spectra were recorded at 600 MHz for ^{1}H and 150 MHz for ^{13}C, respectively, on a SYS 600 instrument (Varian Associates Inc., Palo Alto, CA, U.S.A.) in CD_3OD, DMSO-d_6, or D_2O with solvent peaks used as references. ESIMS and HRESIMS data were taken on an Agilent 1100 Series LC-MSD-Trap-SL and Agilent 6520 Accurate-Mass Q-TOFL CMS spectrometers (Agilent Technologies, Ltd., Santa Clara, CA, U.S.A.), respectively. Column chromatography (CC) was carried out on macroporous adsorbent resin (HPD-110, Cangzhou Bon Absorber Technology Co. Ltd, Cangzhou, China), silica gel (200–300 mesh, Qingdao Marine Chemical Inc., Qingdao, China), Sephadex LH-20 (Pharmacia Biotech AB, Uppsala, Sweden), CHP 20P (Mitsubishi Chemical Inc., Tokyo, Japan), or reversed phase C-18 silica gel (W. R. Grace & Co., Maryland, U.S.A.). 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 semi-preparative column (250 × 10 mm i.d.) packed with C_{18} reversed phase silica gel (5 μm) (W. R. Grace & Co., Maryland, U.S.A.). TLC was carried out on glass precoated silica gel GF_{254} 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.

3.2. Plant material

The roots of *Isatis indigotica* were collected in December 2009 from Bozhou, Anhui Province, China. Plant identity was verified by Mr. Lin Ma (Institute of Materia Medica, Beijing 100,050, China). A voucher specimen (No. ID-S-2385) was deposited at the herbarium of Natural Medicinal Chemistry, Institute of Materia Medica.

3.3. Extraction and isolation

The air-dried and pulvarized plant material (50 kg) was decocted with H_2O (150 L; 3 × 1 h). The aqueous extracts were combined and evaporated under reduced pressure to yield a dark-brown residue (32 kg). The residue was dissolved in H_2O (122 L), loaded on a macroporous adsorbent resin (HPD-110, 19 kg) column (20 × 200 cm), and eluted successively with H_2O (50 L), 50% EtOH (125 L), and 95% EtOH (100 L) to yield three corresponding fractions A, B, and C. After removing the solvent under reduced pressure, fraction B (0.9 kg) was separated by column chromatography (CC) over MCI gel CHP 20P (5 L), with successive elution using H_2O (10 L), 30% EtOH (30 L), 50% EtOH (20 L), 95% EtOH (10 L), and Me_2CO (8 L), to give fractions B1-B5. Fraction B2 (547 g) was subjected to silica gel CC eluting with 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, 1:1) to give B2-4-1–B2-4-3, of which B2-4-1 (40 g) was further fractionated by CC over Sephadex LH-20 (H_2O) to afford B2-4-1-1–B2-4-1-13. Subfraction B2-4-1-6 (20 g) was separated by CC over Sephadex LH-20 (H_2O) to yield B2-4-1-6-1–B2-4-1-6-5. Further fractionation of B2-4-1-6-1 (8 g) by flash CC over C-18 silica gel (0–40% MeOH in H_2O) gave B2-4-1-6-1-1–B2-4-1-6-1-9, of which B2-4-1-6-1–4 (5 mg) was purified by RP-HPLC (6% MeOH in H_2O containing 0.2% acetic acid, *v*/*v*/*v*, 1.8 ml/min) to afford **4** (1.5 mg, t_R = 40 min). Subfraction B2-4-1-8 (2 g) was further isolated by silica gel CC ($CHCl_3$–MeOH, 15:1~0:1) to yield B2-4-1-8-1–B2-4-1-8-6, of which B2-4-1-8-2 (200 mg) was further fractionated by CC over Sephadex LH-20 (MeOH) to afford B2-4-1-8-2-1–B2-4-1-8-2-5. Purification of B2-4-1-8-2-4 (50 mg) by RP-HPLC (8% MeCN in H_2O, 2.0 ml/min) to yield **3** (10.0 mg, t_R = 50 min). Subfraction B2-4-1-8-3 (550 mg) was fractionated by CC over Sephadex LH-20 (MeOH) to yield B2-4-1-8-3-1–B2-4-1-8-3-4, of which B2-4-1-8-3-1 (220 mg) was separated by HW-40C (H_2O) to yield B2-4-1-8-3-1-1–B2-4-1-8-3-1-4. Subsequent isolation of B2-4-1-8-3-1-4 (50 mg) by RP-HPLC (6% MeCN in H_2O, 2.0 ml/min) obtained **5** (9.0 mg, t_R = 62 min) and **7** (7.0 mg, t_R = 52 min). Separation of B2-4-1-8-3-2 (90 mg) by CC over HW-40C (MeOH–H_2O, 4:6) afforded B2-4-1-8-3-2-1 and B2-4-1-8-3-2-2, of which B2-4-1-8-3-2-1 (30 mg) was purified by RP-HPLC (10% MeCN in H_2O, *v*/*v*, 2.0 ml/min) to afford **8** (7.0 mg, t_R = 19 min). Isolation of B2-4-1-8-3-2-2 (28 mg) by preparative TLC (EtOAc:MeOH:H_2O, 8:2:1, *v*/*v*/*v*) yielded B2-4-1-8-3-2-2-1 (6 mg) and B2-4-1-8-3-2-2-2 (10 mg), and further purification by RP-HPLC (20% MeOH in H_2O, 2.0 ml/min) obtained **1** (3.0 mg, t_R = 38 min) from the former and by RP-HPLC (12% MeCN in H_2O, 2.0 ml/min) yielded **2** (5.0 mg, t_R = 37 min) from the latter. Subfraction B2-4-1-9 (3 g) was chromatographed over silica gel and eluted by a gradient of increasing MeOH (0–100%) in $CHCl_3$ to yield B2-4-1-9-1–B2-4-1-9-4, of which B2-4-1-9-4 (1.8 g) was further separated by HW-40C (H_2O) to yield B2-4-1-9-4-1–B2-4-1-9-4-6. Separation of B2-4-1-9-4-6 (200 mg) by CC over Sephadex LH-20 (H_2O) yielded B2-4-1-9-4-6-1–B2-4-1-9-4-6-4. Successive purification of B2-4-1-9-4-6-2 (8 mg) and B2-4-1-9-4-6-3 (10 mg) by RP-HPLC (10% MeCN in H_2O containing 0.2% acetic acid, *v*/*v*/*v*, 2.0 ml/min) afforded **6** (2.0 mg, t_R = 32 min) and **9** (1 mg, t_R = 58 min), respectively.

3.3.1. Isatigotindoledioside A (1)

White amorphous powder; $[\alpha]_D^{20}$ – 27.2 (*c* 0.09, MeOH); UV (MeOH) λ_{max} (log ε) 204 (2.66), 214 (sh, 2.53), 231 (sh, 2.26), 291 (1.99) nm; IR ν_{max} 3396, 3186, 3011, 2922, 2849, 1706, 1645, 1600 (sh), 1516, 1468, 1419, 1372, 1326, 1300, 1247, 1207, 1118, 1072, 1011, 816, 774, 745, 722, 646 cm^{-1}; ^1H NMR (CD_3OD, 600 MHz) spectral data, see Table 1; ^{13}C NMR (CD_3OD, 150 MHz) spectral data, see Table 2; (+)-ESI-MS: *m*/*z* 508 $[M + Na]^+$; (–)-ESI-MS: *m*/*z* 484 $[M - H]^-$, 520 $[M + Cl]^-$; (+)-HR-ESI-MS: *m*/*z* 508.1434 $[M + Na]^+$ (calcd for $C_{21}H_{27}NO_{12}Na$, 508.1425).

3.3.2. Isatigotindoledioside B (2)

White amorphous powder; $[\alpha]_D^{20}$ – 25.7 (*c* 0.05, MeOH); UV (MeOH) λ_{max} (log ε) 204 (3.01), 214 (sh, 2.75), 229 (sh, 2.59), 285 (2.37) nm; IR ν_{max} 3395, 3187, 3011, 2923, 2850, 1691, 1646, 1530, 1468, 1420, 1370, 1343, 1325, 1301, 1246, 1215, 1173, 1119, 1073, 1012, 817, 776, 753, 722, 648 cm^{-1}; ^1H NMR (CD_3OD, 600 MHz) spectral data, see Table 1; ^{13}C NMR (CD_3OD, 150 MHz) spectral data, see Table 2; (+)-ESI-MS: *m*/*z* 478 $[M + Na]^+$; (–)-ESI-MS:

m/z 454 [M − H]$^-$, 490 [M + Cl]$^-$; (+)-HR-ESI-MS: *m/z* 478.1326 [M + Na]$^+$ (calcd for $C_{20}H_{25}NO_{11}Na$, 478.1320).

3.3.3. Isatigotindoledioside C (3)

White amorphous powder; $[\alpha]_D^{20}$ − 55.2 (*c* 0.12, MeOH); UV (MeOH) λ_{max} (log ε) 219 (2.70), 267 (1.78) nm; IR ν_{max} 3395, 3011, 2922, 2850, 2254, 1646, 1588, 1509, 1468, 1420, 1358, 1253, 1170, 1077, 1046, 783, 751, 651 cm^{-1}; ^{1}H NMR (CD_3OD, 600 MHz) spectral data, see Table 1; ^{13}C NMR (CD_3OD, 150 MHz) spectral data, see Table 2; (+)-ESI-MS: *m/z* 489 [M + Na]$^+$, 505 [M + K]$^+$; (−)-ESI-MS: *m/z* 465 [M − H]$^-$, 501 [M + Cl]$^-$; (+)-HR-ESI-MS: *m/z* 489.1491 [M + Na]$^+$ (calcd for $C_{21}H_{26}N_2O_{10}Na$, 489.1480).

3.3.4. Isatigotindoledioside D (4)

White amorphous powder; $[\alpha]_D^{20}$ − 26.8 (*c* 0.02, MeOH); UV (MeOH) λ_{max} (log ε) 202 (3.65), 216 (sh, 3.39), 297 (2.66) nm; CD (MeOH) $\Delta\varepsilon_{215\ nm}$ + 2.59, $\Delta\varepsilon_{240\ nm}$ − 4.29, $\Delta\varepsilon_{265\ nm}$ + 1.06, $\Delta\varepsilon_{298\ nm}$ + 0.17; IR ν_{max} 3394, 2922, 2849, 2259, 1728, 1627, 1497, 1467, 1418, 1270, 1247, 1203, 1149, 1080, 1031, 923, 863, 818, 774, 708, 651 cm^{-1}; ^{1}H NMR (CD_3OD, 600 MHz) spectral data, see Table 1; ^{13}C NMR (CD_3OD, 150 MHz) spectral data, see Table 2; (+)-ESI-MS: *m/z* 551 [M + Na]$^+$, 567 [M + K]$^+$; (−)-ESI-MS: *m/z* 527 [M − H]$^-$, 563 [M + Cl]$^-$; (+)-HR-ESI-MS: *m/z* 551.1482 [M + Na]$^+$ (calcd for $C_{22}H_{28}N_2O_{13}Na$, 551.1484).

3.3.5. Isatigotindoledioside E (5)

White amorphous powder; $[\alpha]_D^{20}$ − 43.5 (*c* 0.07, MeOH); UV (MeOH) λ_{max} (log ε) 202 (2.98), 223 (2.52), 278 (2.01) nm; IR ν_{max} 3329, 2917, 2893, 2247, 1623, 1582, 1511, 1427, 1400, 1347, 1296, 1247, 1173, 1099, 1069, 1031, 921, 884, 855, 810, 774, 746, 582 cm^{-1}; ^{1}H NMR (DMSO-d_6, 600 MHz) spectral data, see Table 1; ^{13}C NMR (DMSO-d_6, 150 MHz) spectral data, see Table 2; (+)-ESI-MS: *m/z* 551 [M + Na]$^+$, 567 [M + K]$^+$; (−)-ESI-MS: *m/z* 527 [M − H]$^-$, 563 [M + Cl]$^-$; (+)-HR-ESI-MS: *m/z* 551.1305 [M + Na]$^+$ (calcd for $C_{22}H_{28}N_2O_{11}SNa$, 551.1306).

3.3.6. Isatigotindoledioside F (6)

White amorphous powder; $[\alpha]_D^{20}$ − 23.3 (*c* 0.07, MeOH); UV (MeOH) λ_{max} (log ε) 203 (3.00), 217 (sh, 2.70), 241 (sh, 2.53), 3.05 (2.35) nm; IR ν_{max} 3396, 3185, 3011, 2922, 2850, 1646, 1469, 1420, 1344, 1325, 1301, 1246, 1216, 1187, 1118, 1064, 1037, 817, 783, 752, 722, 648 cm^{-1}; ^{1}H NMR (CD_3OD, 600 MHz) spectral data, see Table 1; ^{13}C NMR (CD_3OD, 150 MHz) spectral data, see Table 2; (+)-ESI-MS: *m/z* 540 [M + Na]$^+$; (−)-ESI-MS: *m/z* 516 [M − H]$^-$; (+)-HR-ESI-MS: *m/z* 540.1152 [M + Na]$^+$ (calcd for $C_{21}H_{27}NO_{12}SNa$, 540.1146).

3.3.7. Enzymatic hydrolysis of 1–4 and acidic hydrolysis of 5 and 6

Compounds **1–4** (1.0–3.0 mg) were separately hydrolyzed in H_2O (3 ml) with snailase (3.0 mg, CODE S0100, Beijing Biodee Biotech Co., Ltd., Beijing, China) at 37 °C for 24 h. Compounds **5** and **6** (~1.5 mg) were separately hydrolyzed with 2 N HCl (3 ml) at 80 °C for 6 h. The hydrolysate was concentrated under reduced pressure and the residue was isolated by CC over silica gel eluting with CH_3CN–H_2O (8:1), to afford two mixtures, respectively, containing sugar and aglycone. The sugar mixtures from hydrolysis of **1–3**, as well as the authentic D- and L-glucose and D- and L-xylose (1 mg, each), were individually reacted with L-cysteine methyl ester hydrochloride (2.5 mg) in pyridine (1.3 ml) at 60 °C for 2 h,

then N-trimethylsilylimidazole (0.3 ml) was added and reacted at 60 °C for another 2 h. The reaction mixtures were separately analyzed by GC under the following conditions: injection temperature, 300 °C; detector temperature (FID), 300 °C; capillary column, HP-5 (60 m × 0.32 mm, Dikma); the carrier gas, N_2; start temperature 200 °C raised to 260 °C at a rate of 10 °C/min, and the final temperature maintained for 38.5 min. Retention times (t_R) for the derivatives of D-glucose, L-glucose, D-xylose, L-xylose were determined to be 29.82, 30.55, 18.90, and 20.03 min, respectively. For the derivatives of the sugar mixture from the hydrolysate of **1**, GC analysis displayed two peaks with the t_R values of 29.44 min and 19.01 min, which were consistent with those of the derivatives of D-glucose and D-xylose, respectively, and similar peaks with the t_R values of 29.46 and 19.01 min and 29.47 and 19.02 min were observed in the sugar mixtures from the hydrolysis of **2** and **3** (see in Supporting information). The sugar (0.2–0.8 mg) from **4–6** showed retention factor ($R_f \approx$ 0.38) on TLC (EtOAc-MeOH-AcOH-H_2O, 12:3:3:2), with $[\alpha]_D^{20}$ values of +44.0 to +47.5 (*c* 0.02–0.08, H_2O), and ^{1}H NMR (D_2O) data in agreement with those of an authentic D-glucose. Purification of the mixture containing the aglycone of **4** by Sephadex LH-20 (MeOH) obtained **4a**, $[\alpha]_D^{20}$ − 48.3 (*c* 0.02, MeOH). The ^{1}H NMR spectrum of **4a** was the same as that of (+)-(*S*)-2-(3,4-dihydroxy-2-oxoindolin-3-yl)acetonitrile [16].

3.3.8. ECD Calculation of 4a

Briefly, conformational analysis was performed by Monte Carlo searching with the MMFF94 molecular mechanics force field using the Spartan 10 software **4a**. 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 ($\varepsilon = 32.6$). The energies, oscillator strengths, and rotational strengths of excitations were calculated using the TDDFT methodology at the B3LYP/6-311++G (2d, 2p) 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 relative Gibbs free energy (ΔG). All quantum computations were conducted using Gaussian 09 program package on an IBM cluster machine located at the High Performance Computing Center of Peking Union Medical College.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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