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8,4′-Oxyneolignane glucosides from an aqueous extract of "ban lan gen" (*Isatis indigotica* root) and their absolute configurations

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Abstract   Three pairs of glycosidic 8,4′-oxyneolignane diastereoisomers, named isatioxyneolignosides A−F (**1–6**), were isolated from an aqueous extract of *Isatis indigotica* roots. Their structures and absolute configurations were elucidated by comprehensive spectroscopic data analysis and enzyme hydrolysis. The validity of $\Delta\delta_{\text{C8-C7}}$ values to distinguish *threo* and *erythro* aryl glycerol units and Cotton effects at 235 ± 5 nm to determine absolute configurations at C-8 in **1–6** and their aglycones (**1a–6a**) are discussed.

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1. Introduction

"Ban lan gen", the root of *Isatis indigotica* Fort. (Cruciferae), is an important traditional Chinese medicine for the treatment of fever and influenza[1]. This herbal medicine has been pharmacologically and chemically investigated for more than half century[1–16]. However, previous studies were predominated by extracting "ban lan gen" with ethanol or methanol[7,8,10,12], which alters a practical application by decocting with water. As part of a program to assess the chemical diversity and biological activity of traditional Chinese medicines[17–37], our investigation on an aqueous extract of "ban lan gen" led to discovery of various new constituents with different biological activities[38–49]. Herein, we report isolation and structure characterization of six new 8,4′-oxyneolignane β-D-glucopyranosides (1–6, Fig. 1) from the same decoction. In addition, determination of *threo* and *erythro* as well as absolute configurations of 8,4′-oxyneolignane derivatives is discussed by analysis of NMR spectroscopic and circular dichroism (CD) data in combination with electronic CD (ECD) calculations.

2. Results and discussion

Compound 1, a white amorphous powder with $[\alpha]_D^{20}$ –5.2 (*c* 0.12, MeOH), showed IR absorption bands for hydroxyl (3390 cm^{-1}), conjugated carbonyl (1644 cm^{-1}), and aromatic ring (1600 and 1512 cm^{-1}) functionalities. The molecular formula $C_{24}H_{30}O_{13}$ of 1 was determined by (+)-HR-ESI-MS at *m/z* 549.1594 [M+Na]$^+$. The NMR spectroscopic data of 1 in DMSO-d_6 (Tables 1 and 2) indicated the presence of two *meta-para*-disubstituted phenyls, two aromatic methoxy groups, an oxymethylene, and two oxy-methines, as well as a β-glucopyranosyl. However, in the NMR spectra, resonances for one of the two phenyls were significantly broadened (See Supplementary Information Figs. S46 and S47). Especially an expected coupling between two vicinal aromatic protons ($J_{5',6'}$) was unresolved while the ^{13}C NMR spectrum displayed two fewer carbon resonances (C-1′ and C-7′) than those expected from the molecular formula. Subsequently, the structure of 1 was further elucidated by acquirement and analysis of 2D NMR spectroscopic data.

The proton and proton-bearing carbon resonances in the NMR spectra of 1 were assigned unambiguously by interpretation of ^1H–^1H COSY and HSQC spectroscopic data. The HMBC spectrum of 1 showed two- and three-bond correlations from H-1″ to C-4; from H-2 to C-1, C-3, C-4, and C-6; from H-5 to C-1, C-3, C-4, and C-6; from H-6 to C-2 and C-4; and from OCH₃-3 to C-3 (Fig. 2). These correlations, together with the ^1H–^1H COSY cross-peaks of H-5/H-6/H-2 and H-1″/H-2″/H-3″/H-4″/H-5″/H₂-6″ and chemical shifts of these proton and carbon resonances as well as

the coupling constant values (7.8 Hz for $J_{1'',2''}$ and $J_{5,6}$), indicated the presence of a 4-β-glucopyranosyloxy-3-methoxyphenyl in 1. The HMBC correlations from both H-2 and H-6 to C-7; from H-7 to C-1, C-2, C-6, C-8, and C-9; from H-8 to C-1, C-7, and C-9; and from H₂-9 to C-7 and C-8; along with their chemical shifts and the ^1H–^1H COSY cross-peaks of H-7/H-8/H₂-9, demonstrated that the 4-β-glucopyranosyloxy-3-methoxyphenyl connected to a glycerol unit to form a 4-β-glucopyranosyloxy-3-methoxyphenylglycerol moiety. In addition, the HMBC correlations from H-2′ to C-4′ and C-6′; from H-5′ to C-3′; from H-6′ to C-2′ and C-4′; and from OCH₃-3′ to C-3′; in combination with their chemical shifts and the ^1H–^1H COSY cross-peaks of H-5′/H-6′, revealed that there was a 3′-methoxy-4′-oxyphenyl though these correlations had much less intensities and the proton and carbon resonances were broad in the spectra, along with the unobserved C-1′ resonance and unresolved coupling ($J_{5',6'}$). Meanwhile, the intensive HMBC correlation from H-8 to C-4′ revealed that the 4-β-glucopyranosyloxy-3-methoxyphenylglycerol and 3′-methoxy-4′-oxyphenyl connected *via* an ether bond between C-8 and C-4′. The above deduction suggests that there must be a COOH unit at C-1′ in 1 to match requirement of the molecular formula and to explain the unobserved and broadened NMR resonances due to conjugation and/or dissociation of the COOH unit. Therefore, the gross structure of 1 was elucidated as guaiancylglycerol-8-vanillic acid 4-O-β-D-glucopyranoside, of which the aglycone with the *threo* or *erythro* configuration was reported[50,51] and obtained also in this study. However, their absolute configurations were not determined in the literatures (according to nomenclature of lignans and neolignans recommended by IUPC[52], herein numberings 8 and 4 are used instead of 8′ and 4′ in the literatures).

Enzymatic hydrolysis of 1 with snailase afforded sugar and aglycone (1a). The sugar exhibited retention factor (Rf) on TLC, specific rotation $[\alpha]_D^{20}$, and ^1H NMR spectroscopic data identical to those of an authentic D-glucose (see Experimental Section 4.3.7). The NMR spectroscopic data of 1a in CD₃OD were different from those of *threo* guaiancylglycerol-8-vanillic acid in the same solvent[50], but highly consistent with those of the *erythro* isomer in acetone-d_6[51] or CD₃OD. This suggested that 1 possessed the *erythro* configuration. The CD spectra of 1 and 1a displayed negative Cotton effects at 229 and 236 nm (Fig. 3), respectively. Because the sign of Cotton effect at 235 ± 5 nm are validated for assignment of the C-8 configuration of the arylglycerol units in various 8,4′-oxyneolignans (positive for 8S and negative for 8R)[53–55], the 8R configuration was assigned for 1 and 1a. Therefore, the structure of compound 1 was determined as shown in Fig. 1 and designated as isatioxyneolignoside A.

Compound 2 was isolated as a white amorphous powder with $[\alpha]_D^{20}$ –31.4 (*c* 0.07, MeOH). The spectra of 2 were almost identical to those of 1, except that the CD spectrum of 2 displayed a mirrored curve to that of 1 (Fig. 3). This suggested that the aglycone (2a) in 2
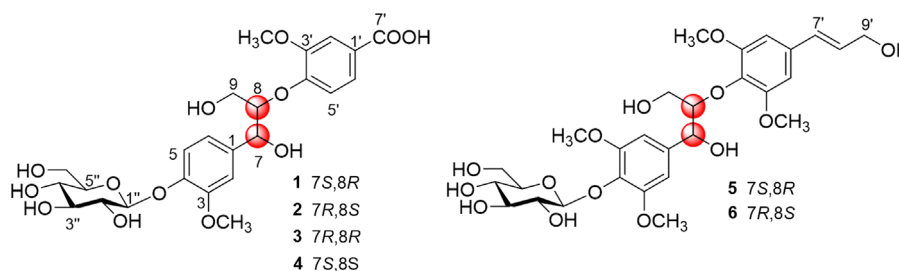

Figure 1 The structures of compounds 1–6.

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

No.	1[b]	2[b]	3[b]	4[b]	5[b]	6[b]
2	7.05 brs	7.07 brs	7.02 brs	7.04 brs	6.68 s	6.69 s
5	6.99 d (7.8)	6.99 d (8.4)	6.99 d (7.8)	6.99 d (7.8)		
6	6.88 d (7.8)	6.88 d (8.4)	6.85 d (7.8)	6.86 d (7.8)	6.68 s	6.69 s
7	4.76 d (4.2)	4.77 d (4.8)	4.76 brs	4.78 brs	4.87 d (5.4)	4.86 d (6.0)
8	4.43 m	4.45 m	4.38 m	4.37 m	4.21 m	4.21 m
9a	3.59 m	3.60 m	3.59 brd (8.4)	3.61 brd (9.6)	3.85 dd (12.0, 4.8)	3.85 dd (12.0, 5.4)
9b	3.58 m	3.58 m	3.23 m	3.24 m	3.55 dd (12.0, 3.6)	3.55 dd (12.0, 2.4)
2′	7.42 brs	7.42 brs	7.47 brs	7.51 brs	6.66 s	6.66 s
5′	7.01 brs	7.04 brd (7.8)	7.00 brs	7.00 brs		
6′	7.42 brs	7.45 brd (7.8)	7.42 brs	7.44 brs	6.66 s	6.66 s
7′					6.48 d (15.6)	6.48 d (16.2)
8′					6.26 m	6.26 m
9′					4.17 dd (5.4, 1.2)	4.17 dd (5.4, 1.2)
1″	4.84 d (7.8)	4.86 d (7.2)	4.84 d (7.2)	4.86 d (7.2)	4.75 d (7.2)	4.73 d (7.8)
2″	3.21 brdd (8.4, 7.8)	3.22 brdd (9.0, 7.2)	3.22 brdd (9.0, 7.2)	3.24 brdd (9.0, 7.2)	3.40 dd (9.6, 7.8)	3.41 m
3″	3.22 brt (8.4)	3.26 brt (9.0)	3.24 brt (9.0)	3.26 brt (9.0)	3.35 m	3.38 m
4″	3.14 brt (8.4)	3.16 brt (9.0)	3.14 brt (9.0)	3.16 brt (9.0)	3.35 m	3.38 m
5″	3.26 m	3.26 m	3.26 m	3.28 m	3.14 m	3.14 m
6″	3.64 brd (12.0)	3.64 brd (11.4)	3.64 brd (11.4)	3.65 brd (11.4)	3.71 dd (12.0, 2.4)	3.71 dd (12.0, 2.4)
6‴b	3.43 dd (12.0, 4.8)	3.45 dd (11.4, 4.8)	3.44 dd (11.4, 4.8)	3.45 dd (11.4, 4.8)	3.61 dd (12.0, 5.4)	3.61 dd (12.0, 4.8)

[a]^{1}H NMR data (δ) were measured at 600 MHz in DMSO-d_6 for **1–4** and in CD$_3$OD for **5** and **6**, 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 groups, **1**: δ_H 3.72 (6H, s, OCH_3-3/3′); **2**: δ_H 3.73 (6H, s, OCH_3-3/3′); **3**: δ_H 3.71 (3H, s, OCH_3-3), 3.77 (3H, s, OCH_3-3′); **4**: δ_H 3.73 (3H, s, OCH_3-3), 3.78 (3H, s, OCH_3-3′); **5**: δ_H 3.78 (6H, s, OCH_3-3/5), 3.75 (6H, s, OCH_3-3′/5′); **5**: δ_H 3.78 (6H, s, OCH_3-3/5), 3.75 (6H, s, OCH_3-3′/5′).

was the enantiomer of **1a** since the specific rotation of **2** had the same negative sign as that of **1** and since L-glucoside is rare in natural products. The suggestion was confirmed by 2D NMR spectroscopic data analysis and enzyme hydrolysis of **2**. Particularly, from the hydrolysate of **2**, D-glucose was isolated and identified by using the aforementioned protocol, while the ^{1}H NMR spectroscopic data of the aglycone (**2a**) were identical to those of **1a**, whereas the specific rotation and Cotton effect curve were reversed (Fig. 3). Therefore, the structure of compound **2** was determined and assigned as isatioxyneolignoside B.

Compound **3**, a white amorphous powder with $[\alpha]_D^{20}$ –16.0 (c 0.12, MeOH), displayed spectroscopic data similar to those of **1** and **2**. The significant differences were two resonances of methoxy groups at δ_H 3.71 (OCH_3-3) and 3.77 (OCH_3-3′) (separated by $\Delta\delta_H$ 0.06) in the ^{1}H NMR spectrum of **3** replaced the overlapped resonances of the methoxy groups around δ_H 3.72 (OCH_3-3/3′) in the spectra of **1** and **2**, while the H-9b resonance in **3** was significantly shielded by $\Delta\delta_H$ –0.35. The differences indicated that **3** was a *threo* stereoisomer of **1** and **2**[50,51]. This was further proved by 2D NMR spectroscopic data analysis and enzyme hydrolysis of **3** that liberated D-glucose and an aglycone (**3a**). Especially the differences of the ^{1}H NMR spectroscopic data between **3a** and **1a** (or **2a**) were in good agreement with those between **3** and **1** (or **2**). The CD spectra of **3** and **3a** displayed the negative Cotton effects at 235 and 236 nm (Fig. 4), respectively, which were in accordance with those of **1** and **1a**, but opposite to **2** and **2a**. Application of the Cotton effect rule[53–55], the 7R,8R configuration was assigned for **3** and **3a**. Consequently, the structure of compound **3** was determined and named trivially isatioxyneolignoside C.

Compound **4**, a white amorphous powder with $[\alpha]_D^{20}$ –15.0 (c 0.07, MeOH), is another stereoisomer of **1–3** as indicated from spectroscopic data (see Experimental Section 4.3 and Tables 1 and 2)

and confirmed by 2D NMR spectroscopic data analysis (Fig. 2) and enzyme hydrolysis. The spectroscopic differences between **4** and **3** were consistent with those between **2** and **1**, suggesting that **4** is the 7S,8S diastereoisomer of **3**. Especially, the Cotton effect curves of **4** and the aglycone **4a** almost mirrored to those of **3** and **3a** (Fig. 4), respectively. Thus, the structure of compound **4** was determined and designated as isatioxyneolignoside D.

Compound **5** was isolated as a white amorphous powder with $[\alpha]_D^{20}$ +13.3 (c 0.03, MeOH). The molecular formula of **5** was elucidated as $C_{28}H_{38}O_{14}$ by (+)-HR-ESI-MS and NMR spectroscopic data. The NMR spectroscopic data of **5** in CD$_3$OD showed signals assignable to syringylglycerol, sinapyl alcohol, and β-glucopyranosyl moieties. Analysis of 2D NMR spectroscopic data confirmed the presence of the three structural moieties and established connections among them. Especially, the HMBC correlation from H-8 to C-4′, together with their chemical shifts, revealed a typical structure of 8,4′-oxyneolignane for the aglycone (**5a**) as shown in Fig. 2, while the HMBC correlation from H-1″ to C-4 located the β-glucopyranosyloxy at C-4 of the aglycone. Hydrolysis of **5** with snailase afforded sugar and **5a**. The sugar was identified as D-glucose by using the same methods as described for **1–4**, while **5a** was identified as *erythro* syringylglycerol-8-*O*-4′-(sinapyl alcohol) ether (*erythro*-SGSE) by comparison of the measured ^{1}H NMR spectroscopic data with that reported in the literature[56]. The 8R configuration was assigned by the negative Cotton effects at 236 and 241 nm[53–55] in the CD spectra of **5** and **5a** (Fig. 5). Therefore, the structure of compound **5** was determined and trivially named as isatioxyneolignoside E. A compound from an ethanol extract of *Indocalamus latifolius* leaves[57] was recently reported to have the same structure as **5**. However, the reported data does not support the claimed structure and the structure drawing is incorrect in the literature.

Compound **6** exhibited spectroscopic data almost identical to those of **5** (see Experimental section and Tables 1 and 2). However, the specific rotation value $\{[\alpha]_D^{20}$ –11.5 (*c* 0.05, MeOH)$\}$ and CD curve of **6** were reversed (Fig. 5). This demonstrated that **6** was the 7*R*,8*S* stereoisomer of **5**, which was confirmed by enzymatic hydrolysis of **6**. From the hydrolysate, the sugar was isolated and identified as D-glucose, while the aglycone (**6a**) gave the same ^{1}H NMR spectroscopic data as **5a** (See Supplementary Information Figs. S117 and S130), but opposite specific rotation and CD data (Fig. 5). Thus, the structure of compound **6** was determined and named as isatioxyneolignoside F.

Our previous investigation demonstrated that in various 8,4′-oxyneolignane derivatives the difference of chemical shift values ($\Delta\delta_{C8-C7}$) for an *erythro* arylglycerol unit was consistently smaller than that for the *threo* diastereoisomer[53,54] as compared the data acquired in a same solvent. Accordingly, the $\Delta\delta_{C8-C7}$ values are validated to distinguish *threo* and *erythro* arylglycerol units in 8,4′-oxyneolignane derivatives. The validity of this rule for the *threo* and *erythro* guaiancylglycerol-8-vanillic acids was confirmed by the reported ^{13}C NMR spectroscopic data of **1a/2a** and/or **3a/4a** in CD$_3$OD[50] or acetone-d_6[51] and those of the related analogues in different solvents[55,56,58]. Application of the rule for

Table 2 ^{13}C NMR spectroscopic data (δ) for compounds **1–6**.

No.	1	2	3	4	5	6
1	135.8	135.8	135.7	135.6	139.5	139.5
2	111.7	111.8	111.2	111.2	106.0	106.0
3	148.3	148.3	148.3	148.3	153.8	153.8
4	145.5	145.5	145.5	145.5	135.5	135.5
5	114.5	114.5	114.6	114.5	153.8	153.8
6	119.2	119.2	118.6	118.7	106.0	106.0
7	71.3	71.3	70.7	70.6	74.0	74.0
8	83.2	83.2	83.7	83.6	87.1	87.1
9	59.9	59.9	60.0	59.9	61.6	61.6
1′					134.7	134.7
2′	113.0	112.9	113.0	113.2	104.9	104.9
3′	148.6	148.6	148.4	148.4	154.5	154.5
4′	150.5	150.5	150.0	149.7	136.4	136.4
5′	113.7	113.8	113.7	113.7	154.5	154.5
6′	122.5	122.5	122.2	122.2	104.9	104.9
7′					131.3	131.3
8′					129.9	129.9
9′					63.6	63.6
1″	100.2	100.1	100.1	100.1	105.6	105.6
2″	73.2	73.2	73.2	73.2	75.7	75.7
3″	76.8	76.8	76.8	76.8	77.8	77.8
4″	69.6	69.6	69.6	69.6	71.3	71.3
5″	77.0	77.0	77.0	77.0	78.4	78.4
6″	60.6	60.6	60.6	60.6	62.6	62.6
OCH$_3$-3	55.5	55.5	55.4	55.4	57.0	57.0
OCH$_3$-5					57.0	57.0
OCH$_3$-3′	55.5	55.5	55.4	55.4	56.7	56.7
OCH$_3$-5′					56.7	56.7

^{13}C NMR data (δ) were measured at 150 MHz in DMSO-d_6 for **1–4** and in CD$_3$OD for **5–6**, respectively. The assignments were based on ^{1}H–^{1}H COSY, HSQC, and HMBC experiment.
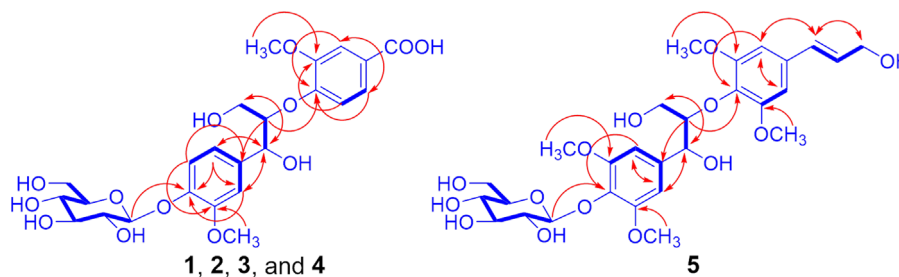
Figure 3 (A) The overlaid experimental CD spectra of **1** (red) and **2** (blue) and the calculated ECD spectra of **1** (red dash) and **2** (blue dash). (B) The overlaid experimental CD spectra of **1a** (red) and **2a** (blue) and the calculated ECD spectra of **1a** (red dot) and **2a** (blue dot).

Figure 2 Main ^{1}H–^{1}H COSY (thick lines) and three-bond HMBC (arrows, from ^{1}H to ^{13}C) correlations of compounds **1–5**.
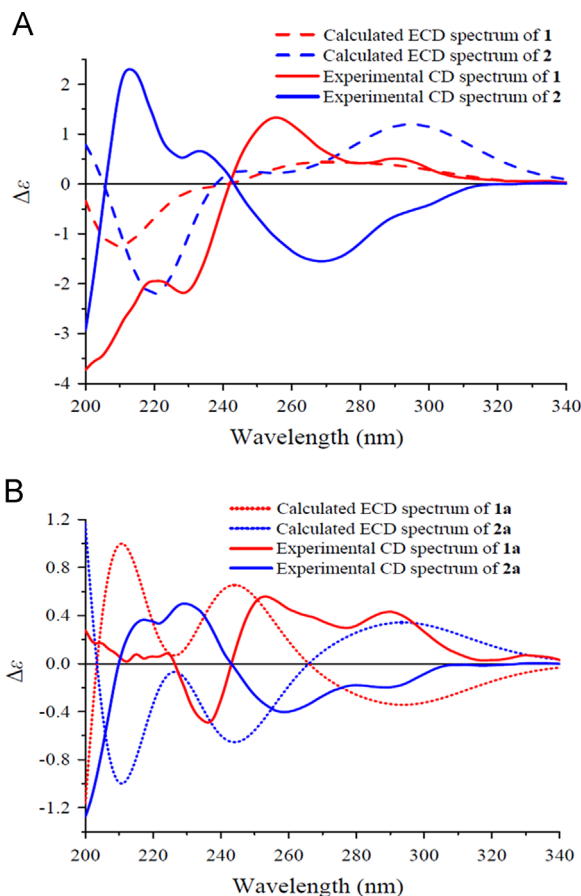
Figure 4 (A) The overlaid experimental CD spectra of **3** (red) and **4** (blue) and the calculated ECD spectra of **3** (red dash) and **4** (blue dash). (B) The overlaid experimental CD spectra of **3a** (red) and **4a** (blue) and the calculated ECD spectra of **3a** (red dot) and **4a** (blue dot).

Figure 5 (A) The overlaid experimental CD spectra of **5** (red) and **6** (blue) and the calculated ECD spectra of **5** (red dash) and **6** (blue dash). (B) The overlaid experimental CD spectra of **5a** (red) and **6a** (blue) and the calculated ECD spectra of **5a** (red dot) and **6a** (blue dot).
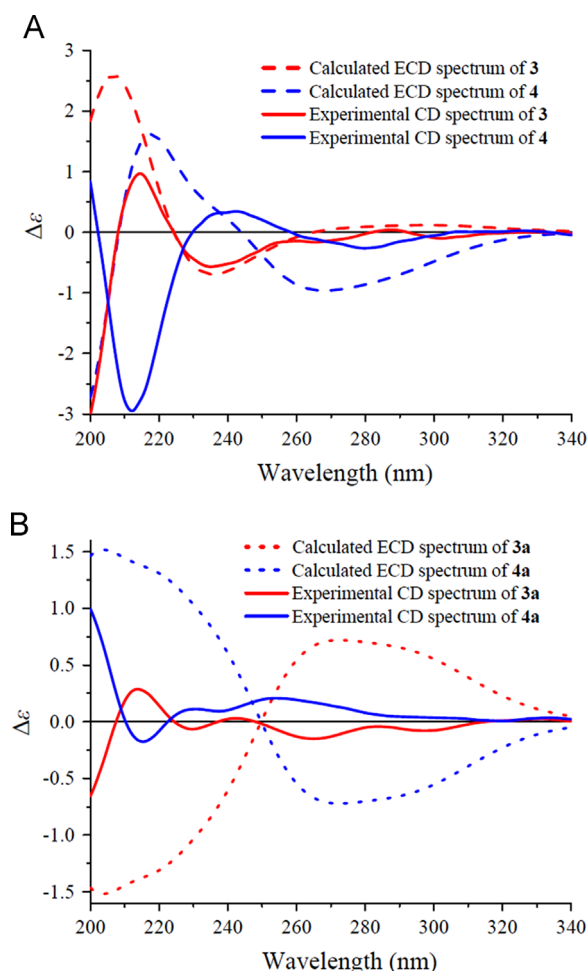
the ^{13}C NMR data of **1–4** in DMSO-d_6 and **5** and **6** in CD$_3$OD (Table 2) supported the assignment of the relative configurations for **1–6**.

Theoretical calculations of the ECD spectra are increasingly applied for determination of absolute configurations of natural products[59]. However, our previous ECD spectra calculations of β-D-glucosidic natural products showed that intensities, wavelengths, and signs of the Cotton effects in the calculated ECD spectra were varied significantly by the β-D-glucopyranosyl unit on the chromophores and that the β-D-glucopyranosyloxy possibly played a decisive role for the signs of the partial or whole Cotton effects[27,42]. With the experimental CD spectra of **1–6** and enantiomeric pairs (**1a/2a**, **3a/4a**, and **5a/6a**) in hand, the theoretical ECD spectra were calculated to evaluate applicability of the calculated ECDs for determination of the absolute configurations of the arylglycerol units in the 8,4′-oxyneolignane derivatives. Comparison of the calculated ECD spectra between **1** and **2**, **3** and **4**, and **5** and **6** indicated that the β-D-glucopyranosyl indeed had significant influences on the intensities, wavelengths, and signs of the Cotton effects (See Supplementary Information Figs. S4, S16 and S28). This was confirmed by comparing the ECD spectra between the glucoside
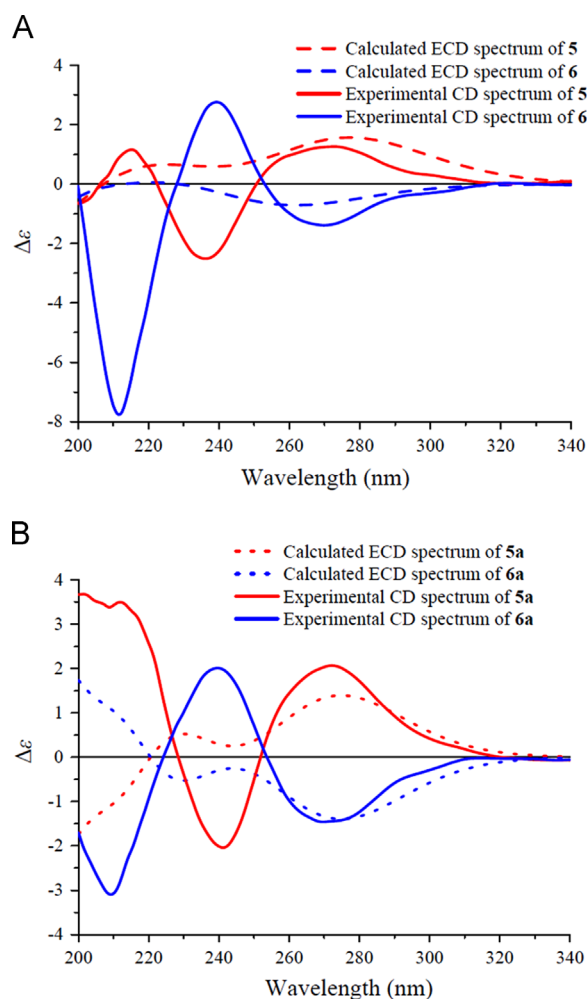
and aglycone pairs (**1/1a**, **2/2a**, **3/3a**, **4/4a**, **5/5a**, and **6/6a**, See Supplementary Information Figs. S5, S6, S17, S18, S29 and S30). Furthermore, the experimental CD spectra of **1–6** and **1a–6a** significantly differed the corresponding calculated ECD spectra in part or whole wavelength regions (See Supplementary Information Figs. 3–5, S7, S8, S10, S11, S19, S20, S22, S23, S31, S32, S34 and S35) except for those of **1** and **3**. As compared with the experimental CD spectra, in short or/and long wavelength region(s) of the calculated ECD spectra of **2**, **4–6**, and **1a–6a** the sign(s) of Cotton effect(s) was reversed. Especially, each experimental CD spectrum displayed a characteristic Cotton effect around 235 ± 5 nm, whereas this Cotton effect was indistinguishable in the calculated spectra of **1a–4a**, **2**, **5**, or **6**. This indicated that, under the experimental conditions relatively stable conformers of these compounds differed from that predicted by the theoretical calculations. Therefore, the theoretical ECD calculation is generally invalid to determine the absolute configuration of 8,4′-oxyneolignane derivatives. This result, together with our previous investigations[53,54], supports that the Cotton effect around 235 ± 5 nm in the experimental CD

spectrum remains to be characteristic and specific to assign the absolute configuration at C-8 of the aryl glycerol units in the aryl glycerols and neolignans (positive for 8S and negative for 8R).

3. Conclusions

Six new β-D-glucopyranosides having three pairs of enantiomeric 8,4′-oxyneolignane aglycones (1–6) were isolated from an aqueous extract of *Isatis indigotica* roots (ban lan gen). Analysis of the ^{13}C NMR data of the diastereoisomers confirmed the rule using the $\Delta\delta_{C8\text{-}C7}$ values to distinguish *threo* and *erythro* aryl glycerol units in various 8,4′-oxyneolignanes. Comparison of the experimental CD and calculated ECD spectra of these compounds indicates that the ECD calculation is generally invalid to determine their absolute configurations. However, the experimental CD spectra of 1–6 substantiated relationship of the signs of the Cotton effects around 235 ± 5 nm with the C-8 configurations of the aryl glycerol units in the aryl glycerols and neolignans. Although biological assay was not performed due to limitation of sample amounts, characterization of these compounds enriched diversity of chemical constituents of "ban lan gen" while their potential bioactivities are deserved in future investigation[15].

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 MHz for ^{1}H NMR and 150 MHz for ^{13}C NMR, respectively, on a SYS 600 instrument (Varian Associates Inc., Palo Alto, CA, USA) in CD$_3$OD, DMSO-d_6, or D$_2$O 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 C-18 silica gel (W. R. Grace & Co., Maryland, 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 × 10 mm i.d.) packed with C18 reversed phase silica gel (5 μm, W. R. Grace & Co., Maryland, USA). 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 5% H$_2$SO$_4$ 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 collected in December 2009 from Bozhou, Anhui Province, China. Plant identity was verified by Mr. Lin Ma (Institute of Materia Medica, Beijing, China). A voucher specimen (No. ID-S-2385) was deposited at the herbarium of Natural Medicinal Chemistry, Institute of Materia Medica.

4.3. Extraction and isolation

For the extraction and preliminary fractionation of the extract see Ref. 42. Subfraction B2-4 (120 g) was separated by CC over Sephadex LH-20, eluting with CHCl$_3$–MeOH (1:1) to yield subfractions B2-4-1–B2-4-3, of which B2-4-1 (40 g) was rechromatographed over Sephadex LH-20 eluting with H$_2$O to yield B2-4-1-1–B2-4-1-13. Subfraction B2-4-1-6 (20 g) was separated by CC over Sephadex LH-20 (H$_2$O) 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 reversed phase C18 silica gel (0–40% MeOH in H$_2$O, v/v) gave B2-4-1-6-1-1–B2-4-1-6-1-9, of which B2-4-1-6-1-3 (800 mg) was separated again by CC over Sephadex LH-20 (H$_2$O) to yield B2-4-1-6-1-3-1 and B2-4-1-6-1-3-2. Isolation of B2-4-1-6-1-3-2 (100 mg) by RP-HPLC (22% MeOH in H$_2$O with 0.2% acetic acid, v/v, 2.0 mL/min) afforded mixtures of 1 and 3 (4.0 mg, $t_R = 45$ min) and 2 and 4 (3.5 mg, $t_R = 50$ min). The two mixtures were separated by HPLC on a Chiral CD-PH column (8% MeCN in H$_2$O with 0.2% acetic acid, v/v, 2.0 mL/min) to obtain 1 (1.5 mg, $t_R = 45$ min) and 3 (1.1 mg, $t_R = 52$ min) from the former, and 2 (1.3 mg, $t_R = 65$ min) and 4 (1.2 mg, $t_R = 85$ min) from the latter.

Subfraction B2-4-2 (10 g) was subjected to MPLC over reversed phase C18 silica gel, eluting with a gradient of increasing MeOH (0%–60%) in H$_2$O, to yield B2-4-2-1–B2-4-2-8. Separation of B2-4-2-3 (800 mg) by CC over HW-40C (H$_2$O) gave B2-4-2-3-1–B2-4-2-3-10. Further isolation of B2-4-2-3-3 (60 mg) by flash CC over reversed phase C$_{18}$ silica gel, eluted by a gradient of increasing MeOH (0%–100%) in H$_2$O, afforded B2-4-2-3-3-1-B2-4-2-3-3-3, of which B2-4-2-3-3-2 (7 mg) was purified by RP-HPLC (25% MeOH in H$_2$O, 2.0 mL/min) to obtain 6 (1.5 mg, $t_R = 120$ min), and B2-4-2-3-3-3 (4 mg) by RP-HPLC (35% MeOH in H$_2$O, 2.0 mL/min) to yield 5 (1.5 mg, $t_R = 28$ min).

4.3.1. Isatioxyneolignoside A (1)
White amorphous powder; $[\alpha]_D^{20}$ −5.2 (c 0.12, MeOH); UV (MeOH) $\lambda_{\max}$ (logε) 203 (3.62), 255 (3.09), 284 (2.93, sh) nm; CD (MeOH) 229 (Δε −2.18), 256 (Δε +1.33), 291 (Δε +0.51) nm; IR $\nu_{\max}$ 3390, 3010, 2921, 2850, 1644, 1600, 1554, 1512, 1468, 1417, 1382, 1268, 1222, 1185, 1118, 1076, 1030, 884, 782, 723, 647 cm^{-1}; ^{1}H NMR (DMSO-d_6, 600 MHz) data, see Table 1; ^{13}C NMR (DMSO-d_6, 150 MHz) data, see Table 2; (+)-ESI-MS: m/z 549 [M+Na]$^+$; (−)-ESI-MS: m/z 525 [M−H]$^-$; (+)-HR-ESI-MS: m/z 549.1594 [M+Na]$^+$ (Calcd. for C$_{24}$H$_{30}$O$_{13}$Na, 549.1579).

4.3.2. Isatioxyneolignoside B (2)
White amorphous powder; $[\alpha]_D^{20}$ −31.4 (c 0.07, MeOH); UV (MeOH) $\lambda_{\max}$ (logε) 203 (3.58), 256 (3.06), 283 (2.93, sh) nm; CD (MeOH) 213 (Δε +2.30), 233 (Δε +0.66), 269 (Δε −1.55) nm; IR $\nu_{\max}$ 3395, 3186, 3011, 2922, 2850, 1646, 1601, 1548, 1512, 1469, 1419, 1381, 1325, 1300, 1269, 1218, 1187, 1119,

1076, 1030, 889, 816, 722, 647 cm^{-1}; ^{1}H NMR (DMSO-d_6, 600 MHz) data, see Table 1; ^{13}C NMR (DMSO-d_6, 150 MHz) data, see Table 2; (+)-ESI-MS: m/z 549 [M+Na]$^+$; (−)-ESI-MS: m/z 525 [M−H]$^-$; (+)-HR-ESI-MS: m/z 549.1575 [M+Na]$^+$ (Calcd. for $C_{24}H_{30}O_{13}Na$, 549.1579).

4.3.3. Isatioxyneolignoside C (3)

White amorphous powder (MeOH); $[\alpha]_D^{20}$ −16.0 (c 0.12, MeOH); UV (MeOH) λ_{max} (logε) 203 (3.07), 254 (2.65), 282 (2.51, sh) nm; CD (MeOH) 215 ($\Delta\varepsilon$ +0.97), 235 ($\Delta\varepsilon$ −0.57) nm; IR ν_{max} 3394, 3188, 3011, 2921, 2850, 1645, 1601, 1548, 1512, 1468, 1419, 1383, 1301, 1265, 1219, 1188, 1118, 1075, 1030, 933, 861, 784, 722, 647 cm^{-1}; ^{1}H NMR (DMSO-d_6, 600 MHz) data, see Table 1; ^{13}C NMR (DMSO-d_6, 150 MHz) data, see Table 2; (+)-ESI-MS: m/z 549 [M+Na]$^+$; (−)-ESI-MS: m/z 525 [M–H]$^-$; (+)-HR-ESIMS: m/z 549.1577 [M+Na]$^+$ (Calcd. for $C_{24}H_{30}O_{13}Na$, 549.1579).

4.3.4. Isatioxyneolignoside D (4)

White amorphous powder; $[\alpha]_D^{20}$ −15.0 (c 0.07, MeOH); UV (MeOH) λ_{max} (logε) 203 (3.60), 254 (2.94), 284 (2.72, sh) nm; CD (MeOH) 212 ($\Delta\varepsilon$ −2.95), 243 ($\Delta\varepsilon$ +0.34), 280 ($\Delta\varepsilon$ −0.26) nm; IR ν_{max} 3395, 3190, 3011, 2922, 2850, 1646, 1601, 1553, 1512, 1468, 1419, 1383, 1267, 1220, 1187, 1118, 1075, 1030, 890, 860, 784, 722, 647 cm^{-1}; ^{1}H NMR (DMSO-d_6, 600 MHz) data, see Table 1; ^{13}C NMR (DMSO-d_6, 150 MHz) data, see Table 2; (+)-ESI-MS: m/z 549 [M+Na]$^+$; (−)-ESI-MS: m/z 525 [M–H]$^-$; (+)-HR-ESIMS: m/z 549.1581 [M+Na]$^+$ (Calcd. for $C_{24}H_{30}O_{13}Na$, 549.1579).

4.3.5. Isatioxyneolignoside E (5)

White amorphous powder; $[\alpha]_D^{20}$ +13.3 (c 0.03, MeOH); UV (MeOH) λ_{max} (logε) 202 (3.25), 223 (2.69, sh), 271 (2.23) nm; CD (MeOH) 216 ($\Delta\varepsilon$ +1.16), 236 ($\Delta\varepsilon$ −2.51), 273 ($\Delta\varepsilon$ +1.26) nm; IR ν_{max} 3391, 3011, 2921, 2849, 1639, 1596, 1503, 1465, 1421, 1333, 1229, 1125, 1073, 921, 847, 803, 722, 628 cm^{-1}; ^{1}H NMR (CD$_3$OD, 600 MHz) data, see Table 1; ^{13}C NMR (CD$_3$OD, 150 MHz) data, see Table 2; (+)-ESI-MS: m/z 621 [M+Na]$^+$; (−)-ESI-MS: m/z 633 [M+Cl]$^-$; (−)-HR-ESI-MS: m/z 643.2268 [M+HCOO]$^-$ (Calcd. for $C_{29}H_{39}O_{16}$, 643.2244).

4.3.6. Isatioxyneolignoside F (6)

White amorphous powder; $[\alpha]_D^{20}$ −11.5 (c 0.05, MeOH); UV (MeOH) λ_{max} (logε) 202 (3.19), 222 (2.71, sh), 268 (2.27) nm; CD (MeOH) 212 ($\Delta\varepsilon$ −7.76), 240 ($\Delta\varepsilon$ +2.76), 270 ($\Delta\varepsilon$ −1.39) nm; IR ν_{max} 3374, 2918, 2850, 1731, 1639, 1596, 1503, 1462, 1421, 1333, 1228, 1126, 1074, 919, 846, 803, 619 cm^{-1}; ^{1}H NMR (CD$_3$OD, 600 MHz) data, see Table 1; ^{13}C NMR (CD$_3$OD, 150 MHz) data, see Table 2; (+)-ESI-MS: m/z 621 [M+Na]$^+$; (−)-ESI-MS: m/z 633 [M+Cl]$^-$, 643 [M+HCOO]$^-$; (−)-HR-ESI-MS: m/z 643.2235 [M+HCOO]$^-$ (Calcd. for $C_{29}H_{39}O_{16}$, 643.2244).

4.3.7. Enzymatic hydrolysis of 1–6

Compounds 1–6 (1.0–1.5 mg) were separately hydrolyzed in H$_2$O (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 CH$_3$CN–H$_2$O (8:1) to afford sugar and aglycone. The sugar (0.3–0.6 mg) showed a retention factor (^{n}Rf ≈ 0.38) on TLC (EtOAc–MeOH–AcOH–H$_2$O, 12:3:3:2), with $[\alpha]_D^{20}$ values of

+43.9–+48.0 (c 0.03–0.06, H$_2$O), and ^{1}H NMR (D$_2$O) data in agreement with those of an authentic D-glucose (See Supplementary Information Figs. S53, S70, S86, S102, S118, S131, and S132). The aglycones, 1a: $[\alpha]_D^{20}$ −8.6 (c 0.04, MeOH); CD (MeOH) 236 ($\Delta\varepsilon$ −0.49), 253 ($\Delta\varepsilon$ +0.56), 290 ($\Delta\varepsilon$ +0.43) nm; 2a: $[\alpha]_D^{20}$ +9.8 (c 0.03, MeOH); CD (MeOH) 217 ($\Delta\varepsilon$ +0.36), 229 ($\Delta\varepsilon$ +0.50), 259 ($\Delta\varepsilon$ −0.40), 290 ($\Delta\varepsilon$ −0.20) nm; 3a: $[\alpha]_D^{20}$ +8.5 (c 0.03, MeOH); CD (MeOH) 214 ($\Delta\varepsilon$ +0.29), 229 ($\Delta\varepsilon$ −0.07), 265 ($\Delta\varepsilon$ −0.15); 4a: $[\alpha]_D^{20}$ −10.7 (c 0.05, MeOH); CD (MeOH) 216 ($\Delta\varepsilon$ −0.18), 229 ($\Delta\varepsilon$ +0.11), 254 ($\Delta\varepsilon$ +0.21) nm; 5a $[\alpha]_D^{20}$ +12.5 (c 0.05, MeOH); CD (MeOH) 212 ($\Delta\varepsilon$ +3.50), 241 ($\Delta\varepsilon$ −2.04), 272 ($\Delta\varepsilon$ +2.07) nm; 6a: $[\alpha]_D^{20}$ −8.5 (c 0.03, MeOH); CD (MeOH) 209 ($\Delta\varepsilon$ −3.09), 240 ($\Delta\varepsilon$ +2.01), 270 ($\Delta\varepsilon$ −1.46) nm. The ^{1}H NMR spectroscopic data (See Supplementary Information Figs. S52, S69, S85, S101, S117 and S130) of enantiomeric pairs of 1a and 2a, 3a and 4a, and 5a and 6a were identical, which are consistent with the reported data for *erythro*-guaiancylglycerol-8-vanillic acid[51], *threo*-guaiancylglycerol-8-vanillic acid[50], and *erythro*-SGSE[56], respectively.

4.3.8. ECD Calculation of 1/1a, 2/2a, 3/3a, 4/4a, 5/5a, and 6/6a

For details, see Supporting Information. Briefly, conformational analysis was performed by using the MMFF94 molecular mechanics force field *via* the MOE software package. The lowest energy conformers whose relative energy within 2 kcal/mol were further optimized at the B3LYP/6–31 G(d,p) level in methanol. The energies, oscillator strengths, and rotational strengths of the first electronic excitations were calculated using the TDDFT methodology at the B3LYP/6–311+G(d,p) level. The ECD spectrum of the conformer was simulated using a Gaussian function (0.28 eV for 1/1a, 2/2a, 5/5a, and 6/6a, and 0.30 eV for 3/3a, 4/4a).

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.apsb.2017.09.006.

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