Sulfur-enriched alkaloids from the root of *Isatis indigotica*

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**Abstract** Five new sulfur-enriched alkaloids isatithioetherins A–E (1–5), and two pairs of scalemic enantiomers (+) and (−)-isatithiopyrin B (6a and 6b) and isoepegoitrin and isogoitrin (7a and 7b), along with the known scalemic enantiomers epigoitrin and goitrin (8a and 8b), were isolated and characterized from an aqueous extract of the *Isatis indigotica* roots. Their structures were determined by extensive spectroscopic data analysis, including 2D NMR and theoretical calculations of electronic circular dichroism (ECD) spectra based on the quantum-mechanical time-dependent density functional theory (TDDFT). Compounds 1–5 represent a novel group of sulfur-enriched alkaloids, biogenetically originating from stereoselective assemblies of epigoitrin-derived units. Isolation and structure characterization of 6a and 6b support the postulated biosynthetic pathways for the diastereomers 9a and 9b via a rare thio-Diels–Alder reaction. Compounds 2 and 4 showed antiviral activity against the influenza virus A/Hanfang/359/95 (H3N2, IC\(_{50}\) 0.60 and 1.92 μmol/L) and the herpes simplex virus 1 (HSV-1, IC\(_{50}\) 3.70 and 2.87 μmol/L), and 2 also inhibited Coxsackie virus B3 (IC\(_{50}\) 0.71 μmol/L).

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1. Introduction

The dried roots and leaves of *Isatis indigotica* Fort. (Cruciferae), having names of "ban lan gen" and "da qing ye", respectively, are used in traditional Chinese medicine for the treatment of various diseases. They are among the most common ingredients of formulations used for treating influenza, cold, and fever. Chemical and pharmacological studies demonstrated that extracts of these drug materials contained diverse chemical constituents with various biological activities. Although the drug materials are practically utilized by decocting with water, only few chemical studies on the water decoctions were previously reported. Because the constituents of extracts are highly dependent upon extraction methods, we consider that there must be unknown bioactive chemical constituents in the decoctions. Therefore, the water decoction of the *I. indigotica* roots was investigated as part of a program to assess the chemical and biological diversity of traditional Chinese medicines. This has led to discovery of many new chemical constituents with diverse structural types and biological activities from "ban lan gen". A continuation on the same decoction has resulted in structure characterization of nine new sulfur-containing natural products via a thioether bond between C-1 and C-2. The proton and carbon resonances in the NMR spectra were assigned by the HSQC experiment of 1. In the 1H–1H COSY spectrum, the vicinal coupling cross-peaks of H2-4/H-5/H-6/H2-7 and the HMBC correlations from H2-4 to C-2, C-5, and C-6 (H2-4 to C-2, C-5, and C-6) in combination with comparison of the chemical shifts for most of the pairing signals were less than Δδc ± 0.1. As compared with those of the reported compounds from this plant species, these spectroscopic data suggested that 1 was an unusual asymmetric dimer of alkaidcontaining three sulfur atoms, of which the structure was further elucidated by 2D NMR data analysis.

The proton and carbon-bearing carbon resonances in the NMR spectra were assigned by the HSQC experiment of 1. In the 1H–1H COSY spectrum, the vicinal coupling cross-peaks of H2-4/H-5/H-6/H2-7 and the HMBC correlations from H2-4 to C-2, C-5, and C-6 (H2-4 to C-2, C-5, and C-6) in combination with comparison of the chemical shifts of these proton and carbon signals with those of the co-occurring epigotrin (8a), revealed the presence of a pair of N- and N'-substituted epigotirim units in 1. In addition, the 1H–1H COSY cross-peaks of H2-1'/H-2'/H-3'/H-4''/N'H and H2-1'/H-2'/H-3'/H-4''/N''H, together with the HMBC correlations from H2-1' to C-1'' and from H2-1'' to C-1' as well as their chemical shifts, indicated that there were a pair of N'- and N''-substituted 4'-amino-but-2'-enyl and 4''-amino-but-2''-enyl units, connecting each other via a thioether bond between C-1' and C-1'''. Moreover, the HMBC spectrum of 1 exhibited the correlations from both H2-4' and H2-4'' to the carbon resonance at δC 152.0 (C-5') and C-5''. This demonstrated that the amino groups of the sulfur-bridged bis-butenamine moiety must connect via C-5' and C-5'' with the two epigotirim units to match requirement of the molecular structure.

![Figure 1 The structures of compounds 1–9.](image)
formula and \( \text{N} \) and \( \text{N}^0 \)-substitution, though no three-bond correlations from \( \text{H}_2-4 \) and/or \( \text{H}_2-4^0 \) to \( \text{C}-5 \) and \( \text{C}-5^0 \) were observed in the HMBC spectrum. Accordingly, the planar structure of 1 was determined as shown.

The ROESY spectrum of 1 displayed the NOE correlations between \( \text{H}_2-1^0 \) and \( \text{H}_2-4^0 \) as well as between \( \text{H}_2-1^0 \) and \( \text{H}-3^0 \) and between \( \text{H}-2^0 \) and \( \text{H}_2-4^0 \) (Fig. 3), suggesting a 2\(^\text{trans}\)-2\(^\text{cis}\) geometric configuration for 1. The suggestion was supported by the chemical shift rule for the \( \alpha \)-alkyl carbons connecting to the \( \text{trans} \) and \( \text{cis} \)-double bonds (\( \delta_{\text{trans}} \gt \delta_{\text{cis}} \)), because the chemical shift values of \( \text{C}-1^0 \) and \( \text{C}-4^0 \) (\( \delta_{\text{C}} 32.9 \) and 37.9) were larger than those of \( \text{C}-1^\text{tt} \) and \( \text{C}-4^\text{tt} \) (\( \delta_{\text{C}} 27.5 \) and 37.9) in 1. The specific rotation value of 1 was almost doubled as compared with that of 8a, \( [\alpha]_{D}^{20} +21.6 \) (c 2.4, CHCl\(_3\)), suggesting that the absolute configuration at \( \text{C-5} \) and \( \text{C-5}^0 \) in 1 are identical to that in 8a. This was further supported by comparison of the experimental CD and calculated ECD spectra of 1 (Fig. 4). Therefore, the structure of compound 1 was determined and named isatithioetherin A.

Compound 2, a colorless gum with \( [\alpha]_{D}^{20} +32.0 \) (c 1.7, MeCN), showed similar spectroscopic data to those of 1, except that the NMR spectra of 2 displayed only half the number of resonances corresponding to the proton and carbon atoms expected from the molecular formula. This suggested that 2 was an isomer of 1 with the symmetric structure, which was supported by EI-MS data of 2 at \( m/z \) (%) 129 (100) and 225 (22) arising from cleavage of the carbamide and thioether bonds, respectively (Supplementary Information Fig. S30). Comparison of the spectroscopic NMR data between 2 and 1 (Table 1) demonstrated that the 2\(^\text{trans}\)-2\(^\text{cis} \) double bond in 1 was absent in 2. Thus, 2 was assigned as the 2\(^\text{trans}\)-2\(^\text{trans} \) isomer of 1, which was proved by 2D NMR data analysis, especially by the HMBC correlation from \( \text{H}_2-1^0 \) to \( \text{C}-1^0 \) and the NOESY correlations between \( \text{H}_2-1^0 \) and \( \text{H}-3^0 \) (\( \delta_{\text{C}} 32.9 \) and 42.2) as well as chemical shifts of \( \text{C}-1^0 \) and \( \text{C}-4^0 \) (\( \delta_{\text{C}} 27.5 \) and 37.9) in 1. The similarity of specific rotation and CD data between 2 and 1 indicated that the two compounds had the same absolute configuration, which was supported by comparison of the experimental CD and calculated ECD spectra of 2 (Fig. 4). Thus, the structure of compound 2 was determined and named isatithioetherin B.

Compound 3 was obtained as a colorless gum with \( [\alpha]_{D}^{20} +30.4 \) (c 0.2, MeCN). Its molecular formula \( \text{C}_{20}\text{H}_{20}\text{N}_{4}\text{O}_{4}\text{S}_{4} \) with one more sulfur atom than 1 and 2 was determined by HR-ESI-MS and NMR spectroscopic data. The UV, IR, and NMR spectroscopic
| No. | $\delta_H$ | $\delta_C$ | $\delta_H$ | $\delta_C$ | $\delta_H$ | $\delta_C$ | $\delta_H$ | $\delta_C$ | $\delta_H$ | $\delta_C$ |
|-----|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|
| 2   | 4.47 t (10.0) | 186.4 | 4.47 dd (11.0, 9.0) | 52.5 | 4.47 dd (11.0, 9.0) | 52.5 | 4.47 dd (11.0, 9.0) | 52.5 | 4.47 dd (11.0, 9.0) | 52.5 |
| 4a  | 4.02 dd (10.0, 8.0) | 52.5 | 4.00 dd (11.0, 8.0) | 52.5 | 4.00 dd (11.0, 8.0) | 52.5 | 4.00 dd (11.0, 8.0) | 52.5 | 4.00 dd (11.0, 8.0) | 52.5 |
| 5   | 5.34 dt (10.0, 8.0) | 80.3 | 5.35 dt (9.0, 8.0) | 80.3 | 5.35 dt (9.0, 8.0) | 80.3 | 5.35 dt (9.0, 8.0) | 80.2 | 5.35 dt (9.0, 8.0) | 80.3 |
| 6   | 6.10 ddd (17.5, 10.0, 8.0) | 134.5 | 6.09 ddd (17.5, 10.5, 8.0) | 134.5 | 6.09 ddd (17.5, 10.5, 8.0) | 134.5 | 6.08 ddd (17.0, 10.5, 8.0) | 134.5 | 6.08 ddd (17.0, 10.5, 8.0) | 134.5 |
| 7a  | 5.53 d (17.5) | 121.0 | 5.53 d (17.5) | 121.0 | 5.53 d (17.5) | 121.0 | 5.53 d (17.0) | 121.0 | 5.53 d (17.0) | 121.0 |
| 7b  | 5.42 d (10.0) | 32.9 | 5.42 d (10.5) | 32.9 | 5.42 d (10.5) | 32.9 | 5.42 d (10.5) | 32.9 | 5.42 d (10.5) | 32.9 |
| 1’a | 3.14 d (5.0) | 186.4 | 3.14 d (5.0) | 186.4 | 3.14 d (5.0) | 186.4 | 3.14 d (5.0) | 186.4 | 3.14 d (5.0) | 186.4 |
| 1’b | 3.14 d (5.0) | 186.4 | 3.14 d (5.0) | 186.4 | 3.14 d (5.0) | 186.4 | 3.14 d (5.0) | 186.4 | 3.14 d (5.0) | 186.4 |
| 2’  | 5.70 t (5.0) | 129.6 | 5.70 t (5.0) | 129.6 | 5.70 t (5.0) | 129.6 | 5.70 t (5.0) | 129.6 | 5.70 t (5.0) | 129.6 |
| 3’  | 5.71 t (5.0) | 129.5 | 5.71 t (5.0) | 129.5 | 5.71 t (5.0) | 129.5 | 5.71 t (5.0) | 129.5 | 5.71 t (5.0) | 129.5 |
| 4’a | 3.94 t (5.0) | 42.2 | 3.94 t (5.0) | 42.2 | 3.94 t (5.0) | 42.2 | 3.94 t (5.0) | 42.2 | 3.94 t (5.0) | 42.2 |
| 4’b | 3.94 t (5.0) | 42.2 | 3.94 t (5.0) | 42.2 | 3.94 t (5.0) | 42.2 | 3.94 t (5.0) | 42.2 | 3.94 t (5.0) | 42.2 |
| 5’  | 152.0 | 152.0 | 152.0 | 152.0 | 152.0 | 152.0 | 152.0 | 152.0 | 152.0 | 152.0 |
| 4’a | 4.47 t (10.0) | 186.4 | 4.47 t (10.0) | 186.4 | 4.47 t (10.0) | 186.4 | 4.47 t (10.0) | 186.4 | 4.47 t (10.0) | 186.4 |
| 4’b | 4.01 dd (10.0, 8.0) | 186.4 | 4.01 dd (10.0, 8.0) | 186.4 | 4.01 dd (10.0, 8.0) | 186.4 | 4.01 dd (10.0, 8.0) | 186.4 | 4.01 dd (10.0, 8.0) | 186.4 |
| 5’  | 5.34 dt (10.0, 8.0) | 186.4 | 5.34 dt (10.0, 8.0) | 186.4 | 5.34 dt (10.0, 8.0) | 186.4 | 5.34 dt (10.0, 8.0) | 186.4 | 5.34 dt (10.0, 8.0) | 186.4 |
| 6’  | 6.09 ddd (17.5, 10.0, 8.0) | 134.5 | 6.09 ddd (17.5, 10.0, 8.0) | 134.5 | 6.09 ddd (17.5, 10.0, 8.0) | 134.5 | 6.09 ddd (17.5, 10.0, 8.0) | 134.5 | 6.09 ddd (17.5, 10.0, 8.0) | 134.5 |
| 7’a | 5.53 d (17.5) | 121.0 | 5.53 d (17.5) | 121.0 | 5.53 d (17.5) | 121.0 | 5.53 d (17.5) | 121.0 | 5.53 d (17.5) | 121.0 |
| 7’b | 5.42 d (10.0) | 186.4 | 5.42 d (10.0) | 186.4 | 5.42 d (10.0) | 186.4 | 5.42 d (10.0) | 186.4 | 5.42 d (10.0) | 186.4 |
| 1’n | 3.23 d (6.0) | 32.3 | 3.23 d (6.0) | 32.3 | 3.23 d (6.0) | 32.3 | 3.23 d (6.0) | 32.3 | 3.23 d (6.0) | 32.3 |
| 2’n | 5.58 t (6.0) | 129.6 | 5.58 t (6.0) | 129.6 | 5.58 t (6.0) | 129.6 | 5.58 t (6.0) | 129.6 | 5.58 t (6.0) | 129.6 |
| 3’n | 5.59 t (6.0) | 130.2 | 5.59 t (6.0) | 130.2 | 5.59 t (6.0) | 130.2 | 5.59 t (6.0) | 130.2 | 5.59 t (6.0) | 130.2 |
| 4’n | 3.98 t (6.0) | 42.2 | 3.98 t (6.0) | 42.2 | 3.98 t (6.0) | 42.2 | 3.98 t (6.0) | 42.2 | 3.98 t (6.0) | 42.2 |
| 5’n | 152.0 | 152.0 | 152.0 | 152.0 | 152.0 | 152.0 | 152.0 | 152.0 | 152.0 | 152.0 |
| N’H | 9.57 brs | 9.62 brs | 9.62 brs | 9.62 brs | 9.62 brs | 9.62 brs | 9.62 brs | 9.62 brs | 9.62 brs | 9.62 brs |

aData ($\delta$) were measured in acetone-$d_6$ for 1-5 at 500 MHz for $^1$H NMR and 125 MHz for $^{13}$C NMR. Coupling constants ($J$) in Hz are given in parentheses. The assignments were based on DEPT, $^1$H–$^1$H COSY, HSQC and HMBC experiments.

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features of 3 resembled those of 2. However, as compared the NMR spectroscopic data between 3 and 2 (Table 1), the chemical shifts of H₂-1'(H₂-1'') and C-1'(C-1'') in 3 were significantly deshielded by ΔδH +0.26 and ΔδC +9.0, respectively. This suggested replacement of the sulfide bond in 2 by a disulfide bond in 3, which was verified by the 2D NMR data analysis (Figs. 2 and 3), particularly by the absence of the correlation from H₂-1' to C-1''', (H₂-1'' to C-1') in the HMBC spectrum of 3. The presence of the disulfide bond was further proved by EI-MS data of 3 at m/z 320 (6), 288 (23), and 256 (29) due to breakdown of the carbamide, carbon-sulfur, and disulfide bonds, respectively (Supplementary Information Fig. S51). The 5 R,S,R'-configuration of 3 was supported by the specific rotation and CD data as well as by comparison of the experimental CD and calculated ECD spectra of 3 (Fig. 4). Therefore, the structure of compound 3 was determined and named isatithioetherin C.

Compound 4 was obtained as a colorless gum with [α]D 20 +39.2 (c 0.21, MeCN), an isomer of 3 as indicated by spectroscopic data (Experimental Section 4.3 and Table 1). However, the NMR spectroscopic data showed that 5 had an asymmetric structure. Comparison of the NMR spectroscopic data between 5 and 3 (Table 1) demonstrated that a terminal double bond [δH 5.76 (1H, ddd, J = 17.0, 10.5, and 7.0 Hz, H-3'), 5.30 (1H, d, J = 17.0 Hz, H-4'a), and 5.23 (1H, d, J = 10.5 Hz, H-4'b); and δC 136.2 (C-3') and 119.3 (C-4')] and a sulfur-bearing methine [δH 3.68 (1H, q, J = 7.0 Hz, H-2’)] and δC 53.5 (C-2')] in 5 replaced one trans-distributed double bond and one sulfur-bearing methane in 3. In addition, the resonances for one nitrogen-bearing methylene changed from δH 3.96 (2H, t, J = 6.0 Hz, H₂-4) and δC 42.2 (C-4') in 3 to δH 3.74 and 3.57 (1H each, d, J = 12.0 and 7.0 Hz, H-1'a and H-1'b) and δC 43.6 (C-1') in 5. This demonstrated replacement of the 4'-amino-but-2'-enyI unit in 3 by an 1'-amino-but-3'-en-2'-yI unit in 5. The deduction was proved by the 1'H COSY cross peaks of NH/H₂-1'/H-2'/H-3'/H-4', the HMBC correlations from H₂-1' to C-5', and the ROESY correlations between H₂-1''' and H-3''' and between H-2'''' and H₂-4'''' (Figs. 2 and 3). The chemical shifts of H₂-2', H₂-2'' and C-2''', together with the molecular composition, demonstrated that a 1''',2'''-disulfide bond must be formed in 5, which was further supported by EI-MS data (Supplementary Information Fig. S82). Similarity of the specific rotation and CD data between 5 and 3 suggested that they had the same 5 R,S,R'-configuration.

### Table 2 NMR spectroscopic data for compounds 6 and 7.

| No. | δH (DMSO-d_6) | δC (Acetone-d_6) | δH (MeOH-d_4) | δC (Acetone-d_6) |
|-----|---------------|------------------|---------------|------------------|
| 2   | 176.7         | 128.9            | 177.6         | 130.3            |
| 3   | 48.4          | 128.6            | 49.5          | 130.3            |
| 3a  | 128.9         | 128.6            | 130.3         | 130.3            |
| 4a  | 7.04 brd (7.2)| 124.3            | 7.07 dd (7.2, 0.6)| 125.3          |
| 4a  | 7.04 brd (7.2)| 124.3            | 7.07 dd (7.2, 0.6)| 125.3          |
| 5   | 6.91 ddd (7.8, 7.2, 0.6)| 122.0| 6.91 ddd (7.8, 7.2, 0.6)| 122.9|
| 6   | 7.27 ddd (7.8, 7.8, 0.6)| 129.8| 7.26 ddd (7.8, 7.2, 0.6)| 130.5|
| 7a  | 6.93 brd (7.8)| 110.1            | 7.01 brd (7.2)| 110.9          |
| 7b  | 142.7         | 143.7            | 143.7         | 118.0            |
| 3'  | 126.6         | 128.5            | 128.5         | 118.0            |
| 4'  | 7.42 dd (5.4, 3.0)| 141.3| 7.42 dd (4.8, 4.2)| 142.0|
| 5'a | 2.79 m        | 266.6            | 2.83 m        | 27.8             |
| 5'b | 2.79 m        | 26.6             | 2.83 m        | 27.8             |
| 6'a | 3.56 ddd (15.6, 10.8, 4.8)| 21.1| 3.75 ddd (13.2, 8.4, 6.6)| 22.2|
| 6'b | 2.77 m        | 21.1             | 2.74 ddd (13.2, 4.2, 3.6)| 22.2|
| 3'' | 173.0         | 174.2            | 174.2         | 187.2            |
| 5'' | 185.4         | 187.2            | 187.2         | 187.2            |
| 1''a| 2.81 dd (13.8, 7.2)| 40.5| 2.85 dd (14.4, 5.4)| 41.4|
| 1''b| 2.75 dd (13.8, 6.6)| 40.5| 2.82 dd (14.4, 7.2)| 41.4|
| 2'' | 4.23 ddd (7.2, 6.6, 5.4)| 70.0| 4.37 dt (7.2, 5.4)| 71.3|
| 3'' | 5.63 ddd (16.8, 10.8, 5.4)| 140.9| 5.70 ddd (16.8, 10.8, 5.4)| 141.5|
| 4''a| 5.02 dt (16.8, 1.8)| 113.7| 5.10 dt (16.8, 1.8)| 114.1|
| 4''b| 4.89 dt (10.8, 1.8)| 113.7| 4.90 dt (10.8, 1.8)| 114.1|
| 1-NH| 10.74 s       | 9.69 s           | 9.69 s        | 118.0            |
| 2''-OH| 4.83 d (5.4)| 3.68 d (5.4)| 3.68 d (5.4)| 3.68 d (5.4)|

Data (δ) were measured at 600 MHz for 1H NMR and 150 MHz for 13C NMR. Coupling constants (J) in Hz are given in parentheses. The assignments were based on DEPT, 1H-1H COSY, HSQC and HMBC experiments.
Especially, the experimental CD spectrum of 5 matched well with the calculated ECD spectrum (Fig. 5), but significantly differed from that of the 2’-epimer of 5 (Supplementary Information Figs. S10 and S12). This supported that 5 had the 2$S,5R'$R'-configuration. Therefore, the structure of compound 5 was determined and named isatithioetherin E.

Compound 6 was obtained as a colorless gum with [α]$_D^{20}$ +15.2 (c 0.24, MeOH). Its spectroscopic data were similar to those of the scamicle mixture of 9a and 9b in a 2:1 ratio from the same decocion$_{49}$ [herein, given trivial names (−)-isatithiopyrin A for 9a and (+)-isatithiopyrin A for 9b, respectively]. However, TLC and reversed-phase HPLC analysis indicated that 6 was different from 9. Comparison of the NMR spectroscopic data between 6 (Table 2) and 9 in the same solvent DMSO-$_d_6$$_{10}$ demonstrated that H-1’”a and 2’’’-OH in 6 were shielded by Δδ$_H$ -0.05 and -0.10, respectively, whereas H-1’’’b, H-2’’’, H-3’’’, H-4’’’a, and H-4’’’b were deshielded by Δδ$_H$ +0.05, +0.06, +0.16, +0.23, and +0.12, while differences of the chemical shifts for the other proton resonances and all the carbon resonances were less than Δδ$_C$ ±0.03 and Δδ$_C$ ±0.3. Based on these changes and the optical activity, 6 was deduced as either a diastereomer of 9a or 9b or a scamicle mixture of the diastereomers of 9a and 9b, which was further confirmed by 2D NMR data analysis of 6 in both the solvents of acetone-$_d_6$ and DMSO-$_d_6$ (Fig. 2). Because the specific rotation data of 6 was opposite to that of 9 with almost an equal magnitude and because the later was proved as the scamicle mixture of 9a and 9b in a 2:1 ratio$_{49}$, 6 must be a mixture containing two enantiomers in the same 2:1 ratio. Although subsequent chiral HPLC separation proved the presence of two partially resolved peaks with an integration of about 2:1 ratio in the chromatogram of 6 (Supplementary Information Figs. S113 and S114), further isolation of the two components failed due to decomposition of the sample in solid state storing at 10°C for 6 months. Since two chiral centers exist in the structures, there are only four stereoisomers including two pairs of enantiomers. With the previous chiral separation and structural assignment of 9a and 9b as the (−)-(2’’’S,3’’’S)- and (+)-(2’’’R,3’’’R)-enantiomers$_{49,62}$, respectively, 6 must be a mixture consisting of (2’’’S,3’’’R)- and (2’’’R,3’’’S)-enantiomers in the approximate 2:1 or 1:2 ratio and the optical properties of 6 must be from the exceed enantiomer. The experimental spectrum of 6 was in good agreement with the theoretically calculated ECD spectrum of the (2’’’S,3’’’R)-enantiomer (6a, Fig. 6), whereas mirrored to that of the (2’’’R,3’’’S)-enantiomer (6b) (Supplementary Information Fig. S14). This supported that 6 consisted of 6a and 6b in the approximate 2:1 ratio and that the positive specific rotation of 6 was from the exceed 6a. Accordingly, 6b must have the negative specific rotation. Therefore, the structures of compounds 6a and 6b were assigned and named as (+)- and (−)-isatithiopyrin B, respectively. It is worth noting that the four stereoisomers were very recently synthesized by a biomimetic thio-Diels–Alder reaction, a very rare reaction in nature, and that the presence of 6a and 6b in the 2:1 ratio was also predicted by density functional theory (DFT) calculations$_{62}$. Therefore, compounds 6a and 6b are new natural products, which were chemically synthesized and theoretically predicted.

Compound 7 was obtained as a colorless gum with [α]$_D^{20}$ +26.3 (c 0.1, MeOH). Its spectroscopic features was similar to those of 8 (the scamicle mixture of 8a and 8b in the 2:1 ratio$_{64}$), indicating that 7 was either an isomer of 8a and 8b or a scamicle mixture of the isomers of 8a and 8b. As compared with those of 8, the H-5 and C-5 resonances in the NMR spectra of 7 were significantly shielded by Δδ$_H$ -0.90 and Δδ$_C$ -30.0, respectively, while the C-2 resonance was shielded by Δδ$_C$ – 10.0. The differences indicated isomerization of the oxazolidine-2-thione ring in 8 into a thiazolidin-2-one ring in 7, which was proved by 2D NMR data analysis of 7 (Fig. 2). HPLC analysis using a chiral column (CD-ph) confirmed that 7 was a scamicle mixture of the enantiomers 7a and 7b in the 2:1 ratio (Supplementary Information Fig. S126). Although further preparative separation of the enantiomers failed due to decomposition of the sample in solid state during storage, similarity of the specific rotation and CD curve between 7 with 8 and 8a indicated that the exceed enantiomer in 7 had the same configuration as 8a. This was further supported by comparing the experimental CD spectrum of 7 with the theoretically calculated spectra of 7a and 7b (Fig. 6). Therefore, the structure of 7a and 7b were determined and named as isoepigroitrin and isogroitrin, respectively.

Compounds 1 – 5 are the first example of natural products with dimeric structure features likely deriving from epigoitrin (8a). Based on our previous speculations, these sulfur-containing metabolites are biosynthesized (Scheme 1) from the precursors glucosinolates$_{49,55}$ including epiprogroitrin (10) and/or progoitrin (11) also, which are abundant in the 2:1 ratio of 10:11 in _L. indigotica_$.^{65}$ Myrosinases catalyzed hydrolysis of epiprogroitrin (10) and progoitrin (11)
liberates intermediate 12, which undergoes the Lossen rearrangement, either via a direct process or via imidothioate 13, to yield isothiocyanate 14. An intramolecular nucleophilic addition of 14 produces 8\textsuperscript{65}. Isolation of 7 indicates the possible presence of enzyme-catalyzed hydrolysis and dehydration processes of 14 via an unstable intermediate 15 to generate both 7 and 8, because the proportion and configuration of the exceed enantiomers in the scalemic mixtures are sustained. In a stereoselective manner, an enzyme-catalyzed nucleophilic intermolecular addition between the R-isomers of 8 and 14 would give the optically active intermediate 16. A further intramolecular addition of 16 generates an intermediate 17, which undergoes migration of the thiol group to the terminal double-bond with simultaneous double bond rearrangement and breakdown of the oxygen-bridge to afford the thiol carbamides 18 and 19 (geometric isomers). The thiol group in 17 would also be migrated to the oxygen-bridged methine carbon to afford the thiol carbamides 20, accompanying with reversion of the C-2\textsuperscript{1} configuration from 2'R in 17 to 2'S in 20. Condensation between 18 and 19 and between two molecules of 19 produces 1 and 2, respectively. Meanwhile, a molecule of H\textsubscript{2}S would be simultaneously liberated as a sulfur donor to form the disulfide 3 and trisulfide 4 from 19 as well as to form disulfide 5 from 19 and 20. Moreover, together with 9\texttextsubscript{a} and 9\texttextsubscript{b}\textsuperscript{95}, the isolation and structure determination of 6\texttextsubscript{a} and 6\texttextsubscript{b} confirmed biosynthetic formation of the stereoisomers via a rare thio-Diels–Alder reaction in nature\textsuperscript{92}. The experimental data demonstrate that the configuration at the spiro carbon (C-3) plays a decisive role in the specific rotations and the CD spectroscopic features of 6\texttextsubscript{a}, 6\texttextsubscript{b}, 9\texttextsubscript{a}, and 9\texttextsubscript{b}. The biogenetic speculations fully support the structural assignments of 1–9.

Although the postulated biosynthetic precursors occur as the stereoisomers epiprogoitrin (10) and progoitrin (11) in the inequivalent amounts (2:1), 1–5 were obtained as the optically pure forms and their diastereomers were not founded in the decoction. The fact indicates that 1–5 are biosynthesized in a stereoselective manner, implying the presence of specific enzyme(s) to control the stereoselectivity. Additionally, the precursors glucosinolates can thermally be decomposed into diverse bioactive breakdown products\textsuperscript{65}. Therefore, influences of the decocting process on the bioactive components as well as the pharmacological effects of the ban lan gen decoction deserves further investigation in future studies.

In the preliminary in vitro assays, compounds 2 and 4 showed antiviral activity against influenza virus A/Hanfang/35995 (H3N2)\textsuperscript{66}, with IC\textsubscript{50} values of 0.60 and 1.92 μmol/L and SI values of 9.62 and 3.61, respectively (the positive control RBV, IC\textsubscript{50} = 0.97 μmol/L and SI = 1200). These two compounds also exhibited activity against the herpes simplex virus 1 (HSV-1)\textsuperscript{66} with IC\textsubscript{50} values of 3.70 and 2.87 μmol/L and SI values of 5.20 and 2.68, respectively (the positive control acyclovir, IC\textsubscript{50} = 0.71 μmol/L and SI = 140.9). In addition, 2 inhibited Coxackie virus B3 replication\textsuperscript{66}, with IC\textsubscript{50} and SI values of 0.71 μmol/L and 9.04 (the positive control Pleconaril, IC\textsubscript{50} = 0.41 μmol/L and SI = 243.9; RBV, IC\textsubscript{50} = 222.22 μmol/L and SI = 9.0). Moreover, 2 and 4 reduced d-L-galactosamine (GAlN)-induced hepatocyte (WB-F344 cell) damage\textsuperscript{67} with 70% and 73% inhibition at 10 μmol/L, respectively, while the positive control bicyclocl gave 66% inhibition.

3. Conclusions

From the aqueous extract of the *I. indigotica* root, five novel sulfur-enriched alkaloids isatithioethers A–E (1–5), together
with two pairs of scalenic enantiomers (+)- and (−)-isatisthiopyrin B (6a and 6b) and isoepigiotrin and isoepogitrin (7a and 7b) were isolated and structurally determined. Compounds 1−5 represent the first examples of sulfur-enriched natural products biogenetically assembled by four epigoitrin-derived units, while 6a and 6b having the unique structural feature are the scalenic mixture (2:1), which were biomimetically synthesized by the rare thio-Diels−Alder reaction and theoretically predicted by the DFT calculations.3 The relatively broad antiviral spectra of 2 and 4 demonstrate that the sulfur-enriched metabolites are potentially active constituents responsible for the treatment of influenza and other diseases in clinic application of the ban lan gen decoction, though other compounds were not assayed due to limitation of the sample amounts and/or decomposition of the compounds during storage. The labile properties of the novel sulfur-enriched compounds indicate that the decocting procedure must be an important factor to significantly influence on content and composition of the chemical constituents in the ban lan gen extracts. Therefore, the extracting process must be taken into consideration in research and evaluation of ban lan gen and da qing ye. This consideration would also be valid for some of the herbal medicines. Additionally, our previous and present results48−61 reveal that, in the ban lan gen decoction there are diverse active components against different types of viruses and continuously provide novel candidate for further studies of synthetic/biosynthetic and medicinal chemistry as well as pharmacology.

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 recorded on a Nicolet 5700 FT-IR microscope instrument (FT-IR microspectroscopy, Thermo Electron Corporation, Madison, WI, USA). NMR spectra were recorded at 600 or 500 MHz for 1H NMR and 150 or 125 MHz for 13C NMR, respectively, on a SY500 instrument (Varian Associates Inc., Palo Alto, CA, USA) or Bruker 500 NMR (Bruker Corp. Karlsruhe, Germany) spectrometer in DMSO-d6, acetone-d6 or MeOH-d4 with TMS or solvent peaks used as references. EI-MS data were measured on an AutoSpec Ultima-TOF spectrometer (Micromass, UK). 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-TOF LCMS spectrometers (Agilent Technologies, Ltd., Santa Clara, CA, USA), respectively. Column chromatography (CC) was carried out on macroporous adsorbent resin (HPD-110, 19 kg) column (200 cm × 20 cm), and eluted successively with H2O (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 subjected to column chromatography (CC) over MCI gel CHP 20P (5 L), with successive elution using H2O (10 L), 30% EtOH (30 L), 50% EtOH (20 L), 95% EtOH (10 L), and Me2CO (8 L), to give fractions B1–B5. Fraction B2 (547 g) was subjected to CC over silica gel with elution by a gradient of increasing MeOH concentration (0−100%) in EtOAc and then with 30% EtOH, to yield fractions B2-1−B2-5 based on TLC analysis. Fraction B2-1 (16.3 g) was chromatographed over Sephadex LH-20 with elution by a petroleum ether/chloroform/methanol (5:5:1) mixture to yield B2-1-1−B2-1-10. Fraction B2-1-1 (2.5 g) was separated by silica gel CC (CHCl3/Mc2CO, 100:1) to give B2-1-1-1−B2-1-1-6. Subsequent separation of B2-1-1-3 (54.3 mg) by reversed-phase (RP) HPLC (63% CH3CN in H2O) gave 4 (15.2 mg) and 5 (2.3 mg). Fraction B2-1-1-4 (120.7 mg) was chromatographed over Sephadex LH-20 (CHCl3/McOH, 1:1) to give fractions B2-1-1-4-1 and B2-1-1-4-2, of which B2-1-1-4-1 (34.2 mg) was purified by preparative TLC (mobile phase: CHCl3/Mc2CO, 15:1) to yield I (2.6 mg), and B2-1-1-4-2 by RF HPLC (63% CH3CN in H2O) to afford 2 (18.7 mg). Fraction B2-1-1-6 (122.7 mg) was separated by RF flash CC (0−100% MeOH in H2O) to give 3 (8.2 mg), B2-1-2 (600 mg) was fractionated by RF flash CC with a gradient of increasing MeOH concentration (0−100%) in H2O to yield B2-1-2-1−B2-1-2-4. Separation of B2-1-2-4 (10.7 mg) by RF HPLC (60% MeOH in H2O) afforded 6 (1.9 mg) and 9 (2.1 mg). Chiral HPLC analysis of 6 using AD-H column (250 mm × 10 mm) and mobile phase iPrOH−n-hexane (1:4, 2.0 mL/min) showed two peaks (6a and 6b) with an approximate integration ratio of 2:1. B2-1-3 (7.6 g) was fractionated by silica gel CC (CHCl3/McOH, 50:1) to give B2-1-3-1−B2-1-3-3, of which B2-1-3-1 (5 g) was chromatographed over silica gel CC (petroleum ether/Mc2CO, 10:1) to yield 8 (3.5 g). Subsequent separation of 8 (20 mg) by HPLC using Chiralpak IC column (250 mm × 10 mm) and mobile phase H. 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.

4.2. Plant material

The H. 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

The air-dried and pulverized plant material (50 kg) was decocted with H2O (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 H2O (122 L), loaded on a macroporous adsorbent resin (HPD-110, 19 kg) column (200 cm × 20 cm), and eluted successively with H2O (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 H2O (10 L), 30% EtOH (30 L), 50% EtOH (20 L), 95% EtOH (10 L), and Me2CO (8 L), to give fractions B1–B5. Fraction B2 (547 g) was subjected to CC over silica gel, with elution by a gradient of increasing MeOH concentration (0−100%) in EtOAc and then with 30% EtOH, to yield fractions B2-1−B2-5 based on TLC analysis. Fraction B2-1 (16.3 g) was chromatographed over Sephadex LH-20 with elution by a petroleum ether/chloroform/methanol (5:5:1) mixture to yield B2-1-1−B2-1-10. Fraction B2-1-1 (2.5 g) was separated by silica gel CC (CHCl3/Mc2CO, 100:1) to give B2-1-1-1−B2-1-1-6. Subsequent separation of B2-1-1-3 (54.3 mg) by reversed-phase (RP) HPLC (63% CH3CN in H2O) gave 4 (15.2 mg) and 5 (2.3 mg). Fraction B2-1-1-4 (120.7 mg) was chromatographed over Sephadex LH-20 (CHCl3/McOH, 1:1) to give fractions B2-1-1-4-1 and B2-1-1-4-2, of which B2-1-1-4-1 (34.2 mg) was purified by preparative TLC (mobile phase: CHCl3/Mc2CO, 15:1) to yield I (2.6 mg), and B2-1-1-4-2 by RF HPLC (63% CH3CN in H2O) to afford 2 (18.7 mg). Fraction B2-1-1-6 (122.7 mg) was separated by RF flash CC (0−100% MeOH in H2O) to give 3 (8.2 mg), B2-1-2 (600 mg) was fractionated by RF flash CC with a gradient of increasing MeOH concentration (0−100%) in H2O to yield B2-1-2-1−B2-1-2-4. Separation of B2-1-2-4 (10.7 mg) by RF HPLC (60% MeOH in H2O) afforded 6 (1.9 mg) and 9 (2.1 mg). Chiral HPLC analysis of 6 using AD-H column (250 mm × 10 mm) and mobile phase iPrOH−n-hexane (1:4, 2.0 mL/min) showed two peaks (6a and 6b) with an approximate integration ratio of 2:1. B2-1-3 (7.6 g) was fractionated by silica gel CC (CHCl3/McOH, 50:1) to give B2-1-3-1−B2-1-3-3, of which B2-1-3-1 (5 g) was chromatographed over silica gel CC (petroleum ether/Mc2CO, 10:1) to yield 8 (3.5 g). Subsequent separation of 8 (20 mg) by HPLC using Chiralpak IC column (250 mm × 10 mm) and mobile phase
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4.3.5. Isatisitioetherin E (5)

Colorless gum; [α]_D^20 +39.2 (c 0.21, MeCN); UV (MeOH) \( \lambda_{\max} \) (log e) 258 (4.56) nm; CD (MeCN) 208 (\( \Delta \epsilon +0.37 \), 261 (\( \Delta \epsilon +0.38 \)), 292 (\( \Delta \epsilon -0.78 \)), 314 (\( \Delta \epsilon -0.83 \)) nm; IR \( \nu_{\max} \) 3204, 3050, 2921, 2855, 1702, 1541, 1477, 1402, 1350, 1233, 1094, 1047, 966, 859, 803, 753, 721, 651, 595 cm\(^{-1}\); \(^1\)H NMR (acetone-d\(_6\), 500 MHz) data Table 1; \(^13\)C NMR (acetone-d\(_6\), 125 MHz) data Table 1; EI-MS m/z (\%) 320 (3), 286 (28), 256 (6), 233 (5), 129 (100), 118 (12), 96 (26), 85 (34), 68 (54); (+)-ESI-MS m/z 537 [M + Na]\(^+\), 553 [M + K]\(^+\); (-)-ESI-MS m/z 549 [M + Na]\(^+\); (+)-HR-ESI-MS m/z 519.0916 [M + H]\(^+\) (Calcd. for C\(_{20}\)H\(_{27}\)N\(_4\)O\(_4\)S\(_3\)Na, 519.0910); 537.0732 [M + Na]\(^+\) (Calcd. for C\(_{20}\)H\(_{27}\)N\(_4\)O\(_4\)S\(_3\)Na, 537.0729).

4.3.6. (+)- and (-)-isatisipyrin B (6a and 6b) in a 2:1 ratio

Colorless gum; [α]_D^20 +15.2 (c 0.24, MeOH); UV (MeOH) \( \lambda_{\max} \) (log e) 209 (4.37), 250 (sh, 3.56) nm; CD (MeCN) 231 (\( \Delta \epsilon -3.61 \)), 259 (\( \Delta \epsilon +2.87 \)), 284 (\( \Delta \epsilon +1.10 \)) nm; IR \( \nu_{\max} \) 3255, 3089, 3026, 2924, 2852, 1716, 1619, 1474, 1413, 1321, 1243, 1184, 1135, 1077, 1027, 997, 929, 835, 753, 721, 690, 633, 564, 492 cm\(^{-1}\); \(^1\)H NMR (acetone-d\(_6\), 600 MHz) data Table 2, \(^13\)C NMR (acetone-d\(_6\), 150 MHz) data Table 2, \(^13\)C NMR (DMSO-d\(_6\), 150 MHz) data Table 2; (+)-ESI-MS m/z 372 [M + H]\(^+\), 394 [M + Na]\(^+\), 410 [M + K]\(^+\); (-)-ESI-MS m/z 406 [M + Cl]\(^-\); (+)-HR-ESI-MS m/z 372.0849 [M + H]\(^+\) (Calcd. 372.0835 for C\(_{18}\)H\(_{22}\)N\(_2\)O\(_3\)S\(_2\)); 394.0667 [M + Na]\(^+\) (Calcd. 394.0654 for C\(_{18}\)H\(_{22}\)N\(_2\)O\(_3\)S\(_2\)).

4.3.7. Isoeipogitrin and Isoisogrist (7a and 7b) in a 2:1 ratio

Colorless gum; [α]_D^20 +26.3 (c 0.1, MeOH); UV (MeOH) \( \lambda_{\max} \) (log e) 207 (4.12), 250 (sh, 1.22) nm; CD (MeCN) 233 (\( \Delta \epsilon +0.08 \)), 287 (\( \Delta \epsilon -0.04 \)), 339 (\( \Delta \epsilon -0.02 \)) nm; IR (KBr) \( \nu_{\max} \) 3254, 3078, 2879, 1679, 1539, 1473, 1420, 1355, 1296, 1245, 1214, 1138, 1070, 989, 969, 930, 799, 723, 677, 614 cm\(^{-1}\); \(^1\)H NMR (MeOH-d\(_6\), 600 MHz) data Table 2; \(^13\)C NMR (MeOH-d\(_6\), 150 MHz) data Table 2; EI-MS m/z (\%) 129 [M + H]\(^+\), 63 (97), 85 (64), 73 (100), 71 (61), 69 (90), 57 (40); (+)-HR-ESI-MS m/z 130.0320 [M + H]\(^+\) (Calcd. for C\(_3\)H\(_5\)NO\(_2\)S, 130.0321), 152.0136 [M + H]\(^+\) (Calcd. for C\(_3\)H\(_5\)NO\(_2\)S, 152.0141).

4.3.8. ECD calculations of 1–5, 6a/6b, and 7a/7b

For details, see Supplementary Information. Briefly, conformational analysis and quantum computations were performed using Gaussian 16 program package. The lowest energy conformers whose relative energy within 4 kcal/mol were further optimized at the B3LYP/6–31 g(d,p) level. The energies, oscillator strengths, and rotational strengths were calculated using the TDDFT methodology at the CAM-B3LYP/6–311+G (d,p) level. Conductor-like polarizable continuum model (CPCM) was adopted to consider solvent effects using the dielectric constant of MeCN (\( \varepsilon = 35.7 \)) for 1–5 and MeOH (\( \varepsilon = 32.6 \)) for 6.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at https://doi.org/10.1016/j.apsb.2018.08.005.

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