Alkaloids with Nitric Oxide Inhibitory Activities from the Roots of *Isatis tinctoria*

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**Abstract:** As our ongoing research project on Ban Lan Gen (*Isatis tinctoria* roots), a total of 23 alkaloids were obtained. Compounds 1 and 2 contain an unusual C–C bond between the 2(1H)-quinolinone moiety and the phenol moiety and between the 2(1H)-quinolinone moiety and the 1H-indole moiety, respectively. Compound 3 possesses an unusual carbon skeleton and its putative biosynthetic pathway was discussed, and Compound 23 was deduced as a new indole alkaloid glycoside. Compounds 4–7 were identified as four new natural products by extensive spectroscopic experiments. Additionally, the anti-inflammatory activity was assessed based on nitric oxide (NO) production using Lipopolysaccharide-stimulated RAW264.7 macrophages. Compounds 9, 15, and 17 showed inhibitory effects with IC50 values of 1.2, 5.0, and 74.4 µM.

**Keywords:** *Isatis tinctoria* roots; alkaloids; structure identification; anti-inflammatory activity

1. Introduction

*Isatis tinctoria* L. (synonym, *Isatis indigotica* Fort.), named Ban Lan Gen in the Chinese Pharmacopoeia, belongs to the gene *Isatis* (Brassicaceae family), which is widely distributed and cultivated in the North of the Yangtze River, China [1–4]. Alkaloids were considered as one of the characteristic constituents of this plant, which possess diverse bioactivities such as anti-inflammatory, antiviral, antibacterial, antitumor, and antioxidant activities [5–7]. Up to now, more than 100 alkaloids have been isolated from *I. tinctoria*, such as indole alkaloids, quinazolone alkaloids, quinoline alkaloids, and so on [1–5]. As our ongoing phytochemical and pharmacological research project on this plant [8–12], four new alkaloids and four new natural products, along with 15 known analogues, were obtained, and their structures and absolute configurations were determined by extensive spectroscopic data analysis, including one-dimensional and two-dimensional-NMR, HRESIMS, and IR, specific rotation data, and electronic circular dichroism (ECD) experiments. The known compounds (4–22, Figure 1) were identified by comparison of their spectroscopic and optical rotation data with those in the reported literature as 4-p-hydroxyphenyl-2(1H)-quinolinone (4) [13], 2-(1H-indol-2-yl)-6-methoxy-4(3H)-quinazolinone (5) [14], 2-(2-hydroxyphenyl)-4(3H)-quinazolinone (6) [15], 2-(but-3-en-1-yl)-4(3H)-quinazolinone (7) [16], 2-(1H-indol-2-yl)-4(3H)-quinolinone (8) [17], tryptanthrin (9) [18], 3-(2,4-dioxo-1,2- dihydroquinazolin-3(4H)-yl)propanoic acid (10) [19], indiforine [20,21], etc.
C (11) [3], 4-(2,4-dioxo-1,2-dihydroquinazolin-3(4H)-yl)butanoic acid (12) [20], methyl 4-(2,4-dioxo-1,2-dihydroquinazolin-3(4H)-yl)butanoate (13) [21], 3-(2-hydroxyphenyl)-4(3H)-quinazolinone (14) [22], 3-(2-carboxyphenyl)-4(3H)-quinazolinone (15) [23], 4-methyl-1,2-dihydro-2-oxoquinazoline (16) [24], 2-methyl-4(3H)-quinazolinone (17) [25], 4-hydroxy-3-methyl-2(1H)-quinolinone (18) [26], 2-amino-4-quinolinecarboxylic acid (19) [27], 4(1H)-quinolinone (20) [28], 4(1H)-quinolone-3-carboxylic acid (21) [29], and 1,2,3,4-tetrahydro-4-hydroxy-quinolinecarboxylic acid (22) [30]. The NO inhibitory activities of the isolates (1–23) were also evaluated against the LPS-stimulated RAW264.7 macrophages. In the present paper, we report the isolation and structure determination, putative biosynthetic pathway, and the NO inhibitory activities of these alkaloids.

2. Results and Discussion

Isatisindigoticanine E (1) was obtained as a yellow amorphous powder. The molecular formula was assigned as C_{15}H_{11}NO_{3} on the basis of the negative ion HREIMS peak at m/z 252.0666 [M−H]− (calculated 252.0666 [M−H]−), together with its one-dimensional-NMR data (Table 1). The 1H-NMR spectrum displayed signals of a 1,2,4-trisubstituted benzene ring [31] at δ_{H} 7.20 (1H, d, J = 2.2 Hz, H-5), 6.63 (1H, dd, J = 8.3, 2.2 Hz, H-7), and 6.66 (1H, d, J = 8.3 Hz, H-8]), a 1,4 disubstituted benzene ring at [δ_{H} 7.57 (2H, d, J = 8.5 Hz, H-2',6')] and 6.90 (2H, d, J = 8.5 Hz, H-3',5') and also showed a trisubstituted double bond [9] at δ_{H} 7.48 (1H, s, H-3) and three exchangeable protons at δ_{H} 10.19 (1H, brs, NH-1), 10.12 (1H, brs, OH-6), and 8.96 (1H, brs, OH-4'). The 13C-NMR spectrum showed 15 carbon signals, among which 7 × C carbons at δ_{C} (169.5, 159.6, 152.2, 135.4, 126.1, 125.4, 122.5) and 8 × CH carbons at δ_{C} (136.7, 132.1, 132.1, 116.5, 116.1, 116.1, 110.7, 110.1) were found based on the DEPT 135 experiment. The two-dimensional-NMR spectroscopic features confirmed the inference above. The proton and protonated carbon resonances in the NMR spectra of 1 were unambiguously assigned by the HSQC

Figure 1. Structures of Compounds 1–23.

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Isatisindigocanine F (2) was obtained as a yellow amorphous powder. The molecular formula was assigned as C_{18}H_{14}N_{2}O_{2} by the one-dimensional-NMR data and the HRESIMS positive ion peak at m/z 291.1125 [M + H]^+ (calculated 291.1128 [M + H]^+). The 1H-NMR spectrum (Table 1) of 2 showed signals of a 1,2,3-trisubstituted benzene ring at [δH 6.67 (1H, d, J = 7.5 Hz, H-6), 7.15 (1H, overlap, H-7)
and 6.85 (1H, d, J = 7.3 Hz, H-8)], an ortho-disubstituted benzene ring at [δ_H 7.50 (1H, d, J = 7.5 Hz, H-4'), 7.15 (1H, overlap, H-5'), 7.00 (1H, dd, J = 7.5, 7.4 Hz, H-6') and 7.14 (1H, overlap, H-7')] [35]; two trisubstituted double bonds at δ_H 8.63 (1H, s, H-3) and 9.54 (1H, s, H-3'), as well as two exchangeable protons at δ_H 12.06 (1H, brs, NH-1) and 10.51 (1H, brs, NH-1') and a methoxy group at δ_H 4.04 (3H, s, 5-OMe). After analysis of the 13C-NMR, DEPT 135 and HSQC data (Table 1), a 1H-indol-2-yl moiety (112.5, C; 133.5, CH; 126.3, C; 117.9, CH; 121.1, CH; 127.0, CH; 109.5, CH; 139.4, C) [8,10] and a 5-methoxy-2(1H)-quinoline moiety (168.3, C; 130.6, CH; 118.8, C; 116.8, C; 155.0, C; 102.6, CH; 123.9, CH; 106.2, CH; 138.0, C; 55.9, CH3) were observed [34]. HMBCs of H-3/C-2' and H-3'/C-4 indicated the 1H-indol-2-yl moiety connected with the 5-methoxy-2(1H)-quinoline moiety via a C-4-C-2' bond. These inferences were confirmed by detailed analysis of the two-dimensional-NMR data including HSQC, HMBC (Figure 2), and 1H-1H COSY (Figure 2) experiments. The structure of 2 was thus deduced, as depicted in Figure 1.

Isatisindigoticanine G (3), a yellow amorphous powder, possessed the molecular formula of C20H15N3O based on the positive HRESIMS ion at m/z 314.1297 [M + H]+ (calculated 314.1288 [M + H]+) and one-dimensional-NMR data. The 1H-NMR spectrum (Table 1) of 3 showed signals of two ortho-disubstituted benzene rings at [δ_H 8.13 (1H, d, J = 8.0 Hz, H-5), 7.47 (1H, dd, J = 8.0, 7.2 Hz, H-6), 7.82 (1H, dd, J = 8.1, 7.2 Hz, H-7) and 7.72 (1H, d, J = 8.1 Hz, H-8)] and [δ_H 7.87 (1H, d, J = 7.5 Hz, H-4'),7.22 (1H, dd, J = 8.1, 7.5 Hz, H-5'), 7.25 (1H, dd, J = 8.1, 7.5 Hz, H-6') and 7.50 (1H, d, J = 7.5 Hz, H-7')] and 8.13 (1H, s, H-1’), as well as an exchangeable proton at δ_H 11.98 (1H, brs, NH-1') [35]. The 13C-NMR and the DEPT 135 spectra (Table 1) displayed 8 × C carbons at δ_C (160.5, 156.8, 148.8, 136.4, 127.4, 125.6, 120.3, 112.5), 10 × CH carbons at δ_C (134.9, 128.3, 126.3, 126.2, 126.0, 123.0, 122.6, 120.9, 118.5, 112.6), and 2 × CH2 carbons at δ_C (44.7, 26.2). The two-dimensional-NMR spectra (Figure 2) of 3 showed the 1H-1H COSY correlations of H-5/H-6/H-7/H-8, H-3'/H-4” and HMBCs from H-5/C-4 from H-1”/C-2 and C-3” and from H-4”/C-2 and C-4, which indicated a 8H-pyrido[2,1-b]-11(9H)-quinazolinone moiety in 3 [36]; 1H-1H COSY correlations of H-4'/H-5'/H-6'/H-7' and the HMBCs from NH-1/C-2’, C-3’, C-3’a, and C-7’a indicated a 1H-indol-3-yl moiety in 3 [10]. HMBCs from NH-1/C-9 and C-6, and from H-2’/C-2” and H-1”/C-3’ determined the 8H-pyrido[2,1-b]-11(9H)-quinazolinone moiety connected with the 1H-indol-3-yl moiety via a C-2”-C-3’ bond. The structure of 3 was thus determined, as depicted in Figure 1.

Isatindigoside D (23) was isolated as a red amorphous powder with [α]20D + 12.1° (c 0.19, MeOH). Its molecular formula of C23H22N2O7 (14 IHD) was deduced from the NMR data and the HRESIMS positive ion peak at m/z 490.1592 [M + Na]+, (calculated 490.1585 [M + Na]+). When comparing the one-dimensional (Table 2) and two-dimensional-NMR data (Figure 2) with the reported bisindoloside of isatindigobisindoloside C [35], they showed almost identical NMR spectroscopic features except for the differences around C-2 (downfield of C-2’, C-3’, C-3”’, upfield of C-2”). These differences, along with the optical rotation data ([α]20D + 12.1, c 0.19 in MeOH) supported Compound 23, would be the C2-epimer of isatindigobisindoloside C ([α]20D ~ 33.9, c 0.11 in MeOH) [35]. The experimental and calculated ECD curves of (2S)-23 matched well (Figure 3), which confirmed the S absolute configuration of 23 [35,37], and the calculation details are listed in the Supporting Information (Figures S33 and S34). Acid hydrolysis of 23 resulted in the product of D-glucose, which was confirmed by GC analysis of the acetylation derivative of the hydrolysate of 23 and the authentic sugars (D-glucose 45.23 min, L-xylose 45.38 min) [8,9]. The large coupling constant of Glc-H1 (J = 7.8 Hz) revealed the β-glucopyranosyl linkage in 23 [38,39]. Accordingly, the structure of isatindigoside D (23) was elucidated as depicted (Figure 1).
Table 2. $^1$H-NMR (600 MHz in DMSO-$d_6$) and $^{13}$C-NMR data (150 MHz in DMSO-$d_6$) of 23.

<table>
<thead>
<tr>
<th>No.</th>
<th>$\delta_H$ (J in Hz)</th>
<th>$\delta_C$</th>
<th>No.</th>
<th>$\delta_H$ (J in Hz)</th>
<th>$\delta_C$</th>
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<td>5.68, s</td>
<td>38.7</td>
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<td>7.56, d (8.0)</td>
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<td>10.35, brs</td>
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<td>6.93, dd (8.0, 7.1)</td>
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<tr>
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<td>133.0</td>
<td>7&quot;</td>
<td>7.32, d (8.1)</td>
<td>111.4</td>
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<tr>
<td>3'a</td>
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<td>121.2</td>
<td>7&quot;a</td>
<td></td>
<td>136.0</td>
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<td>6b</td>
<td>3.67, dd (10.8, 1.8)</td>
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</table>

Figure 3. Experimental and calculated ECD spectra of 23.

NO is a messenger molecule that is widespread in cells and can affect a variety of physiological and pathological processes. The production of NO causes tissue damage and can trigger a variety of inflammatory diseases. LPS induces the release of NO from RAW264.7 cells by detecting the release of NO widely used to investigate the anti-inflammatory effects of the compounds [2,10,40]. As our ongoing phytochemical and pharmacological research project on I. tinctoria [8–12], Compounds 1–23 were obtained and were evaluated for their anti-inflammatory activity based on NO inhibitory effects in the LPS-activated RAW 264.7 cells [40]. The cytotoxicity of Compounds 1–23 were tested at three different concentrations (25, 50, and 100 μM), and the results showed that only Compound 9 showed cytotoxicity above 25 μM, while the other compounds were above 100 μM. The results of NO production showed that Compounds 9, 15, and 17 exhibited inhibitory activities with IC$_{50}$ values of 1.2, 5.0, and 74.4 μM (Table 3).
Table 3. NO inhibitory activities of Compounds 1–23 in RAW 264.7 cell line.

<table>
<thead>
<tr>
<th>Compounds</th>
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<th>Cytotoxicity</th>
<th>Compounds</th>
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<td>15</td>
<td>5.0 ± 1.3</td>
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<tr>
<td>5</td>
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<td>&gt;100</td>
<td>17</td>
<td>74.4 ± 3.8</td>
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<td>18</td>
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<td>20</td>
<td>&gt;100</td>
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<tr>
<td>9</td>
<td>1.2 ± 0.9</td>
<td>&gt;25</td>
<td>21</td>
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<tr>
<td>10</td>
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<td>&gt;100</td>
<td>&gt;100</td>
<td>AG b</td>
<td>22.7 ± 0.4</td>
<td>&gt;100</td>
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</table>

a IC<sub>50</sub> values were expressed as mean ± SD (n = 3). b AG = aminoguanidine hydrochloride was used as the positive control.

Isatisindigoticanine G (3) is the first example of a 8H-pyrido[2,1-b]-11(9H)-quinazolinone moiety connected with a 1H-indol-3-yl moiety via a C–C bond of C-2″–C-3′. For its unusual structural features, a plausible biosynthetic pathway is discussed in Figure 4. First, myrosinase catalyzed hydrolysis of progoitrin and epiprogoitrin to give 3a [1]. 3a was connected with 2-aminobenzoic acid moiety by steps of dehydration to give 3b [10], and then 3c was obtained via a cyclization reaction of 3b [2,11]. 3c was connected with 1H-indole moiety by enzyme-catalyzed reaction to give 3d [5] and was then changed via a dehydration reaction to give 3 [9–11].

Figure 4. Putative biosynthetic pathway of 3.

3. Experimental Section

The General Experimental Procedures, Extraction and Isolation, Plant Materials, Inhibitory Assay of NO Production and ECD Calculation sections are listed in the Supporting Information.

3.1. Physical and Spectroscopic Data of Isatisindigoticanines E–G and Isatindigoside D

Isatisindigoticanine E (1), a yellow amorphous powder; IR (KBr) ν<sub>max</sub>: 3406, 2923, 1647, 1609, 1556, 1517, 1466, 1383, 1273, 1093, 745 cm<sup>-1</sup>; m/z 356.1398 [M + H]<sup>+</sup> (calculated 356.1394 [M + H]<sup>+</sup>); <sup>1</sup>H-NMR (DMSO-<sup>d</sup>6, 600 MHz) and <sup>13</sup>C-NMR (DMSO-<sup>d</sup>6, 150 MHz); see Table 1.
Isatisindigoticanine F (2), a yellow amorphous powder; IR (KBr) $v_{\text{max}}$: 3456, 1679, 1621, 1516, 1461, 1319, 1206, 1135, 1021, 859, 813 cm$^{-1}$; $m/z$ 291.1125 [M – H]$^-$ (calculated 291.1128 [M + H]$^+$); $^1$H-NMR (DMSO-$d_6$, 600 MHz) and $^{13}$C-NMR (DMSO-$d_6$, 150 MHz); see Table 1.

Isatisindigoticanine G (3), a yellow amorphous powder; IR (KBr) $v_{\text{max}}$: 3404, 2919, 1708, 1601, 1468, 1400, 1384, 1092, 745 cm$^{-1}$; $m/z$ 314.1297 [M + H]$^+$ (calculated 314.1288 [M + H]$^+$); $^1$H-NMR (DMSO-$d_6$, 600 MHz) and $^{13}$C-NMR (DMSO-$d_6$, 150 MHz); see Table 1.

Isatindigoside D (23), a red amorphous powder; $[\alpha]_D^{20} + 12.1$ (c 0.19, MeOH); IR (KBr) $v_{\text{max}}$: 3420, 2939, 1722, 1598, 1514, 1461, 1261, 1069, 1025, 859, 813 cm$^{-1}$; HRESIMS: $m/z$ 490.1592 [M + Na]$^+$, (calculated 490.1585 [M + Na]$^+$); $^1$H and $^{13}$C-NMR (600 and 150 MHz in DMSO-$d_6$); see Table 2.

3.2. Absolute Configuration Determination of Sugar

Compound 23 (2 mg) was hydrolyzed in 2 M hydrochloric acid (4 mL) at 80 ºC for 2 h. After cooling, the solution was concentrated under vacuum, dissolved with water, and extracted twice with dichloromethane (CH$_2$Cl$_2$). The residue was dissolved in distilled water and reduced with NaBH$_4$ for 3 h at room temperature. After neutralization with AcOH and evaporation to dryness, the residue was acetylated with Ac$_2$O for 1 h at 100 ºC. The resulting alditol acetate was subjected to GC analysis under the following conditions: capillary column, HP-5ms (60 m × 0.25 mm × 0.25 µm); detector, FID; detector temperature, 280 ºC; injection temperature, 280 ºC; initial temperature 140 ºC, subsequently increased to 240 ºC at a rate of 5 ºC/min, and then 1 min to increase to 260 ºC, finally, subsequent increase to 280 ºC at a rate of 2 ºC/min; carrier, N$_2$ gas [8,9]. The D glucose moiety in 23 was confirmed by the comparison of their retention times ($t_R$) with those of authentic sugars ($t_R$ D-glucose 45.23 min, $t_R$ 1-glucose 45.38 min).

4. Conclusions

In this paper, a total of 23 alkaloids were reported, including four new ones: isatisindigoticanines E–G (1–3) and isatindigoside D (23). Four new natural products and 15 known analogues were isolated from Ban Lan Gen. Isatisindigoticanine G possesses an unusual carbon skeleton of an 8H-pyrido[2,1-b]-11(9H)-quinazolinone moiety connected with a 1H-indole moiety via a C–C bond of C-2”–C-3’.

Supplementary Materials: The following are available online http://www.mdpi.com/1420-3049/24/22/4033/s1, Copies of IR, HREIMS, $^1$H-NMR, $^{13}$C-NMR, DEPT 135, HSQC, HMBC, and $^1$H-$^1$H COSY of 1–3 and 23. Experimental and calculated ECD spectra of 23.

Author Contributions: R.W., Y.L., and K.C. conducted the experiments; D.R. and J.L. carried out the anti-inflammatory activity experiments; Y.S., Q.J., and W.Z. analyzed the MS, ECD, and NMR data; D.Z. did the isolation, confirmed the structures, and wrote the paper; R.W. oversaw the research project and drafted the paper.

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Conflicts of Interest: No competing financial interests were declared by the authors.

References


Sample Availability: Samples of the Compounds 1–23 are available from the authors.

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