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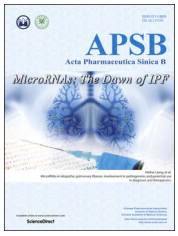

ORIGINAL ARTICLE

Aromatic compounds from an aqueous extract of "ban lan gen" and their antiviral activities

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KEY WORDS

Cruciferae;
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Abstract A pair of new diphenyl glycerol ether enantiomers (−)-**1** and (+)-**1** and two new methyl benzamidobenzoates **2** and **3**, named (−)-(*R*)- and (+)-(*S*)-isatindigotrioic acid [(−)-**1** and (+)-**1**] and isatindigoticamides A (**2**) and B (**3**), respectively, were isolated from an aqueous decoction of the roots of *Isatis indigotica* (ban lan gen). Their structures were elucidated by spectroscopic data analysis including 2D NMR experiments. The absolute configurations of (−)-**1** and (+)-**1** were assigned based on the CD exciton chirality method. Compounds **2** and **3** exhibited antiviral activities against HSV-1 with IC_{50} values of 4.87 and 25.87 μmol/L, respectively. Compound **2** was also found active against Coxsackie virus B3 and LPS-induced NO production.

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1. Introduction

"Ban lan gen", the dried roots of *Isatis indigotica* Fort. (Cruciferae), is an important traditional Chinese medicine mainly used for the treatment of influenza and infection diseases[1]. Previous studies showed that extracts of "ban lan gen" had diverse biological activities and contained various types of active constituents[2–9]. However, the previous studies were mainly focused on ethanol or methanol extracts, which is not in agreement with practical application of the herbal medicine by decocting with water. Therefore, an aqueous decoction of "ban lan gen" was investigated as part of our program to systematically study the chemical and biological diversity of several Chinese traditional medicines[10–22]. From the decoction, 43 new alkaloids and 54 known compounds, including a pair of indole alkaloid enantiomers containing dihydrothiopyran and 1,2,4-thiadiazole rings, a pair of bisindole alkaloid enantiomers, four stereoisomers of 3,5-bis(2-hydroxybut-3-en-1-yl)-1,2,4-thiadiazole, and twelve glycosidic indole alkaloids, as well as their antiviral and/or hepatocyte-protective activities, were characterized[23–30]. Continuous investigation on remaining fractions from the decoction resulted in the separation of a pair of unusual new diphenyl glycerol ether enantiomers (−)-**1** and (+)-**1** and two new methyl benzamidobenzoates (**2** and **3**) (Fig. 1). Herein, reported are details of the isolation, structure elucidation and biological activity of these compounds.

2. Results and discussion

Compound **1** was isolated as a white amorphous powder with $[\alpha]_D^{20} \approx 0$ (*c* 0.23, MeOH). The IR spectrum of **1** showed the presence of hydroxyl (3433 cm^{-1}), conjugated carbonyl (1705 and 1634 cm^{-1}), and aromatic ring (1604 and 1496 cm^{-1}) functional groups. The molecular formula of **1** was determined as $C_{19}H_{18}O_{10}$ by HR-ESI-MS at *m/z* 407.0986 $[M+H]^+$ (Calcd. for $C_{19}H_{19}O_{10}$ 407.0973) combined with the NMR data (Table 1). The ^1H NMR spectrum of **1** in DMSO-d_6 showed signals attributable to two 3,4-disubstituted phenyl moieties [δ_H 8.31 (d, J = 2.4 Hz, H-2), 6.60 (d, J = 9.0 Hz, H-5), and 7.66 (dd, J=9.0 and 2.4 Hz, H-6) and 7.48 (d, J = 1.8 Hz, H-2′), 6.97 (d, J = 8.4 Hz, H-5′), and 7.40 (brd, J=8.4 Hz, H-6′)], two oxygen-bearing methylenes [δ_H 4.43 (dd, J = 12.0 and 3.6 Hz, H-1″a), 4.33 (dd, J=6.0, 12.0 Hz, H-1″b), and 3.69 (brd, J=6.0 Hz, H_2-3'')], one oxygen-bearing methine [δ_H 4.50 (m, H-2″)], and a methoxy group [δ_H 3.70 (s)]. The ^{13}C NMR spectrum of **1** exhibited carbon signals corresponding to the above units and three additional carboxylic carbons resonated at δ_C 170.4 (C-8) and 169.8 and 169.7 (C-7 and C-7′), respectively. These spectroscopic data suggest that **1** is an unusual natural product consisting of two 3,4-disubstituted phenyl moieties, three carboxylic groups, and a glycerol unit. This was confirmed by 2D NMR data analysis of **1**. The ^1H–^1H COSY cross peak between H-5 and H-6 and HMBC correlations from H-2 to C-4, C-6, C-7, and C-8; from H-5 to C-1 and C-3; from H-6 to C-2, C-4, and C-7 (Fig. 2), together with their chemical shifts, indicated the presence of a 4-substituted isophthalic acid moiety in **1**. Meanwhile, the ^1H–^1H COSY correlation between H-5′ and H-6′ and the HMBC correlations from H-2′ to C-6′, C-4′, and C-7′; from H-5′ to C-1′ and C-3′; from H-6′ to C-4′, C-2′ and C-7′, and from OCH_3 to C-4′, in combination with the chemical shifts of these proton and carbon resonances, demonstrated that there was a 4′-substituted 3′-methoxybenzoic acid moiety. The existence of glycerol unit in **1** was verified by the ^1H–^1H COSY correlations H_2-1″/H-2″/H_2-3″ and their chemical shifts, as well as by the HMBC correlations from H-1″ to C-2″ and C-3″; from H-2″ to C-1″ and C-3″, and from H-3″ to C-1″ and C-2″. In addition, the HMBC correlations from H-1″ to C-4 and from H-2″ to C-4′, along with the molecular formula, revealed that C-1″ and C-2″ of the glycerol unit were linked *via* ether bonds with C-4 and C-4′ of the two aromatic acid moieties, respectively, providing the planar structure for **1**. The optical inactivity of **1** indicated that it was obtained as a racemate, which was proved by HPLC analysis on an analytical chiral column displayed two peaks with around 1:1 integration ratio. Subsequent HPLC separation of **1** using a semi-preparative chiral column yielded (−)-**1** {$[\alpha]_D^{20}$ −12.7 (*c* 0.06, MeOH)} and (+)-**1** {$[\alpha]_D^{20}$ +11.8 (*c* 0.05, MeOH)}, which had the consistent ^1H NMR spectroscopic data with those of **1** prior to separation. To determine the configurations of (−)-**1** and (+)−**1**, the electronic circular dichroism (ECD) spectra of (−)-**1** and (+)-**1** were calculated using the time-dependent density functional theory (TDDFT) method[31]. However, the calculated ECD spectra completely differed from the experimental circular dichroism (CD) spectra (Fig. 3). This indicates that comparison of the calculated ECD and the experimental CD spectra is not applicable for determination of the absolute configuration of (−)-**1** and (+)-**1**. An explanation is from flexibility of the structure, of which dynamic conformations under experimental conditions would not be consistent with the theoretically calculated conformers. The CD spectra of (−)-**1** and (+)-**1** displayed mirror curves with typical exciton-split Cotton effects at 207 and 225 nm, arising from interaction between the ^1L_a transition moments of two benzoate chromophores[32]. Based on the exciton chirality method, in the CD spectrum of (−)-**1**, the split Cotton effects with a positive sign (a positive first Cotton effect and a negative second Cotton effect) indicated that the exciton coupling of two benzoate chromophores had positive chirality (Fig. 3). This predicts the *R*-configuration for (−)-**1**. Whereas the split Cotton effects with a negative sign in the CD spectrum of (+)-**1** revealed negative chirality of the two

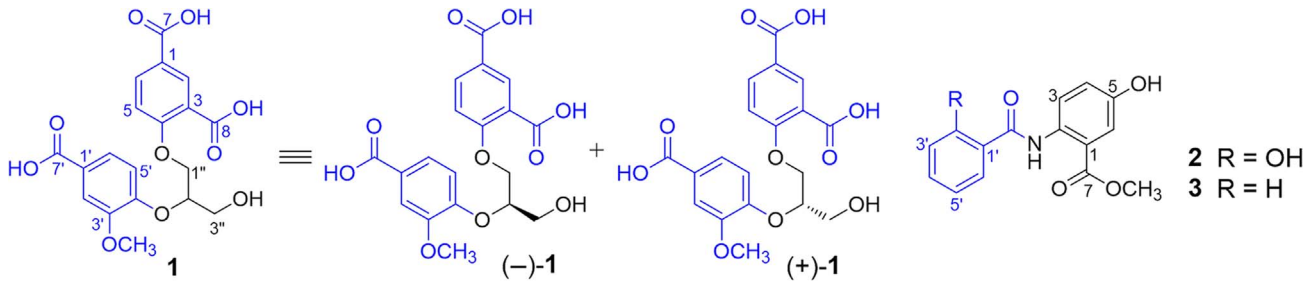

Figure 1 The structures of enantiomer mixture **1** and compounds (−)- and (+)-**1** and **2** and **3**.

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Table 1 NMR spectral data (δ)[a] for compounds (−)-/(+)-**1**, **2** and **3**.

	(−)-/(+)-**1**[b]		**2**[c]		**3**[d]	
No.	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1		119.1		118.6		117.2
2	8.31 d (2.4)	132.1		133.8		135.0
3		115.8	8.57 d (9.0)	123.5	8.76 d (9.0)	122.4
4		165.8	7.18 dd (9.0, 3.0)	122.4	7.16 dd (9.0, 2.5)	122.2
5	6.60 d (9.0)	116.9		154.0		153.0
6	7.66 dd (9.0, 2.4)	133.0	7.56 d (3.0)	117.5	7.55 d (2.5)	117.0
7		169.8[e]		169.4		169.1
8		170.4				
1′		134.8		116.1		135.8
2′	7.48 d (1.8)	113.5		162.8	8.01 d (7.0)	127.6
3′		148.8	6.97 dd (8.0, 1.0)	119.2	7.58 t (7.0)	129.4
4′		147.6	7.50 dt (1.0, 8.0)	135.4	7.62 t (7.0)	132.3
5′	6.97 d (8.4)	115.5	7.02 dt (1.0, 8.0)	120.0	7.58 t (7.0)	129.4
6′	7.40 brd (8.4)	121.7	7.83 dd (8.0, 1.0)	127.2	8.01 d (7.0)	127.6
7′		169.7[e]		169.2		164.9
1″a	4.43 dd (12.0, 3.6)	62.9				
1″b	4.33 dd (12.0, 6.0)					
2″	4.50 m	78.0				
3″	3.69 brd (6.0)	60.0				
OMe	3.70 s	55.3	3.99 s	53.2	3.96 s	52.7

[a]Data were measured in DMSO-d_6 for (−)-/(+)-**1** (600 MHz for ^{1}H NMR and 150 MHz for ^{13}C NMR) and in Me_2CO-d_6 for **2** and **3** (500 MHz for ^{1}H NMR and 125 MHz for ^{13}C NMR), respectively. The assignments were based on DEPT, ^{1}H–^{1}H COSY, HSQC, HMQC and HMBC experiments.

[b]Data for the hydroxyl group in (−)-/(+)-**1**: δ_H 4.98 (1H, brs, OH−3″).

[c]Data for the hydroxyl and amino groups in **2**: δ_H 12.22 (1H, brs, OH−5), 11.89 (1H, brs, OH−2′) and 8.65 (1H, brs, NH−2).

[d]Data for the hydroxyl and amino groups in **3**: δ_H 11.68 (1H, brs, OH−5) and 8.75 (1H, brs, NH−2).

[e]Data in the same column may be exchanged.

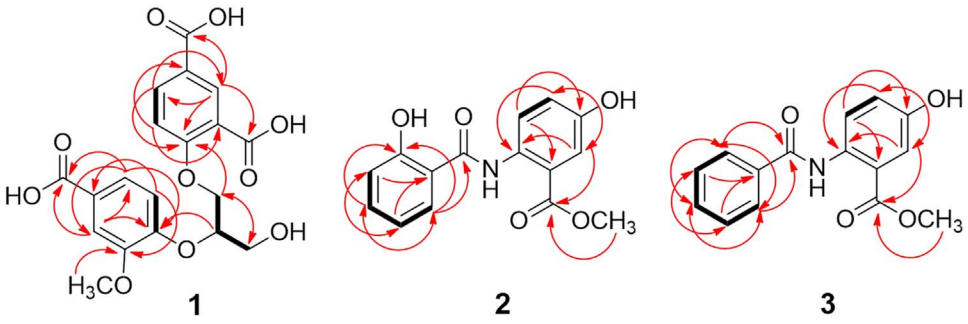

Figure 2 Main ^{1}H–^{1}H COSY (thick lines) and HMBC (arrows, from ^{1}H to ^{13}C) correlations of **1**−**3**.

chromophores, predicting the *S*-configuration. Therefore, the structures of compounds (−)-**1** and (+)-**1** were determined and named (−)-(*R*)- and (+)-(*S*)-isatindigotrioic acid, respectively.

Compound **2** was obtained as colorless needles (acetone). Its IR spectrum showed absorption bands for hydroxyl and amino (3356 and 3275 cm^{-1}), conjugated carbonyl (1691 and 1648 cm^{-1}), and aromatic ring (1611 and 1509 cm^{-1}) functionalities. The molecular formula of **2** was established as $C_{15}H_{13}NO_5$ by (+)-HR-ESI-MS at *m/z* 310.0688 $[M+Na]^+$. The NMR spectroscopic data (Table 1) revealed the presence of *ortho*-*meta*-trisubstituted and *ortho*-disubstituted benzene rings, a methoxy group, two carbonyls, and three exchangeable protons in **2**. This suggests that **2** is a natural product containing two benzoyl moieties with substitution of the hydroxyl, amino, and methoxy groups. Connections of the structural units in **2** were further elucidated by 2D NMR experiments. The ^{1}H–^{1}H COSY cross-peak between H-3 and H-4 and the HMBC correlations from H-3 to C-1 and C-5, from H-4 to C-2 and C-6, from H-6 to C-2, C-4, and C-7, and from OCH_3 to C-7, along with their chemical shifts, indicated the presence of a methyl *N*-substituted 2-amino-5-hydroxybenzoate moiety in **2**. The ^{1}H−^{1}H COSY spectrum also showed the vicinal coupling correlations of H-3′/H-4′/H-5′/H-6′. This, combined with the HMBC correlations from H-3′ to C-1′, C-5′, from H-4′ to C-2′ and C-6′, from H-5′ to C-1′ and C-3′, and from H-6′ to C-2′, C-4′ and C-7′, as well as with their chemical shifts, revealed the occurrence of a 2′-hydoxybenzoyl moiety in **2**. Although no information was obtained for a connection of two moieties, to satisfy the requirement of the molecular formula, the two moieties must be connected *via* an amide bond. This was supported by

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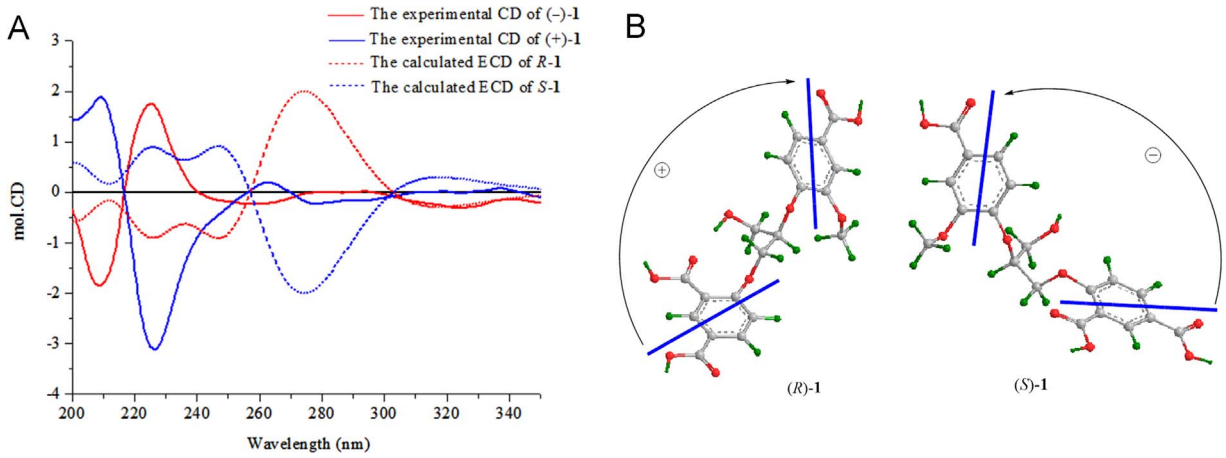

Figure 3 (A) The experimental CD (full lines) and calculated ECD (dash lines) spectra of (−)-**1** (blue) and (+)-**1** (red). (B) Illustration of the exciton chirality method predicting the absolute configurations of (−)-**1** and (+)-**1**.

comparison of the NMR data with the reported data for dianthramide B[33]. Therefore, compound **2** was determined as methyl 5-hydroxy-2-(2′-hydroxybenzamido)benzoate and named isatindigoticamide A.

Compound **3**, colorless needles (acetone), has the molecular formula of $C_{15}H_{13}NO_4$ as determined by HR-ESI-MS and NMR data. Comparison of the NMR spectroscopic data of **3** and **2** indicated that the 2′-hydroxybenzoyl moiety in **2** was replaced by a benzoyl unit in **3**. This suggests **3** is the analogue of **2** without the 2′-hydroxy group, which was confirmed by 2D NMR data analysis. Especially, the ^{1}H–^{1}H COSY cross-peaks of H-2′(H-6′)/H-3′(H-5′)/H-4′ and the HMBC correlations from H-2′(H-6′) to C-7′ verified the presence of an unsubstituted benzoyl moiety in **3**. Therefore, compound **3** was determined as methyl 2-(benzamido)-5-hydroxybenzoate and named isatindigoticamide B.

In the preliminary *in vitro* assays carried out in our studies, compounds **2** and **3** showed antiviral activity against herpes simplex virus1 (HSV-1)[24] with IC_{50} values of 4.87 and 25.87 μmol/L and SI values of 8.82 and 2.68, respectively, while the positive control acyclovir gave the IC_{50} and SI values of 0.71 μmol/L and 140.85. Compound **2** was also active against Coxsackie virus B3 with the IC_{50} and SI values of 8.62 μmol/L and 4.98, respectively (the positive control pleconaril gave IC_{50} 0.41 μmol/L and SI 243.9). In addition, compounds **2** and **3** exhibited inhibitory activity against the LPS-induced NO production in mouse peritoneal macrophage[25] with inhibitions of 86.9% and 39.6% at 10 μmol/L, and the positive control dexamethasone had 92.0% inhibition at the same concentration. Comparison of the structures between **2** and **3** suggests that the presence of hydroxyl group at C-2′ may enhance the activities. Due to limitation of the sample amounts, the enantiomers (−)-/(+)-**1** were not assayed.

3. Conclusions

Four new natural products, including a pair of new diphenyl glycerol ether enantiomers (−)-/(+)-**1** and two new methyl benzamidobenzoates **2** and **3**, were isolated and structurally determined from the aqueous decoction of Chinese traditional herbal medicine "ban lan gen". The enantiomers were separated by HPLC on a chiral semi-preparative column and their absolute configurations were determined by the CD exciton chirality method. Among the new isolates, compound **2** exhibited activities against replication of HSV-1 and Coxsackie virus B3 and against the LPS-induced NO production in mouse peritoneal macrophage. Compound **3** was only active against HSV-1. This result, together with our previous studies, continuously illustrate that the diverse chemical constituents with varied activities have contributions to pharmacological efficacy that supports the traditional usage of ban lan gen. Although the enantiomers (−)-/(+)-**1** were not assayed due to limitation of the sample amounts, these compounds provide new model structures for further synthesis and biological evaluation. In particular, as compared with **3**, an enhancement of activity by the C-2′ hydroxyl group in **2** provides an important clue for in-depth studies of structural modification and structure–activity relationship, as well as new drug development based on the drug-like methyl benzamidobenzoates.

4. Experimental

4.1. General experimental procedures

Optical rotations were measured on a P-2000 polarimeter (JASCO, Tokyo, Japan). UV spectra were obtained on a V-650 spectrometer (JASCO, Tokyo, Japan). IR spectra were acquired on a Nicolet 5700 FT-IR microscope instrument (FT-IR microscope transmission) (Thermo Electron Corporation, Madison, WI, USA). NMR spectra were measured at 500 MHz or 600 MHz for ^{1}H NMR, and 125 MHz or 150 MHz for ^{13}C NMR, respectively, on Inova 500 or VNS 600 (Varian Associates Inc., Palo Alto, CA, USA) in DMSO-d_6 or Me_2CO-d_6, using solvent peaks as references. ESI-MS and HR-ESI-MS data were collected on an AccuToFCS JMS-T100CS spectrometer (Agilent Technologies, Ltd., Santa Clara, CA, USA). Column chromatography (CC) was conducted 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., MD, USA). HPLC separation was carried out on an instrument equipped with an Agilent Chem Station 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 mm × 10 mm i.d.) packed with C18 reversed phase silica gel (5 μm) (W. R. Grace & Co., MD,

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USA) or a Chiralpak AD-H column (250 mm × 10 mm i.d.) packed with amylose tris(3,5-dimethylphenylcarbamate) coated on 5 μm silica gel (Daicel Chiral Technologies Co., Ltd., Shanghai, China). 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.

4.2. Plant material

The roots of *I. indigotica* were collected in December 2009 from Anhui Province, China. Plant identity was verified by Mr. Lin Ma (Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China). A voucher specimen (No. ID-S-2385) was deposited at the herbarium of Natural Medicinal Chemistry, Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College.

4.3. 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 (200 cm × 20 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 CC over MCI gel CHP 20 P (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 B3 (165 g) was chromatographed over silica gel, eluted by a gradient of increasing MeOH (0–100%) in EtOAc to yield B3-1–B3-16. Fraction B3-4 (11.0 g) was further fractionated by CC over Sephadex LH-20 (MeOH) to yield B3-4-1 − B3-4-9, of which B3-4-5 (2.5 g) was separated by reversed phase medium pressure liquid chromatography (RP-MPLC), eluted with a gradient of increasing MeOH (20%–100%) in H_2O, to give B3-4-5-1–B3-4-5-31. Fraction B3-4-5-15 (136.0 mg) was re-chromatographed over Sephadex LH-20 (MeOH) to yield B3-4-5-15-1–B3-4-5-15-6. Isolation of B3-4-5-15-3 (10.0 mg) by reversed phase semi-preparative HPLC (C18 column, 40% MeOH in H_2O, containing 0.3% AcOH, 1.5 mL/min) gave **1** (2.0 mg, t_R = 55 min). Subsequent separation of **1** by HPLC using the Chiralpak AD-H column (*n*-hexane-*i*PrOH, 4:1, containing 0.1% TFA, *v/v/v*, 1.5 mL/min) yielded (−)-**1** (0.74 mg, t_R = 35.3 min) and (+)-**1** (0.64 mg, t_R = 40.6 min). Fraction C (88.0 g) was fractionated by CC over silica gel, eluted with a gradient of increasing acetone concentration (0–100%) in petroleum ether, to yield C1–C11. Subfraction C7 (11.2 g) was subjected to CC over Sephadex LH-20 ($CHCl_3$-MeOH, 1:1, *v/v*) to give C7-1–C7-6, of which C7-4 (810 mg) was separated by reversed phase flash CC, eluted with a gradient of increasing MeOH (55% − 95%) in water to yield **2** (220.6 mg) and **3** (5.3 mg).

4.3.1. (−)-(*R*)- and (+)-(*S*)-isatindigotrioic acid [(−)- and (+)-**1**]

Mixture of (−)-**1** and (+)-**1** in around 1:1 ratio (**1**), white amorphous power; $[\alpha]_D^{20} \approx 0.0$ (*c* 0.23, MeOH); UV (MeOH) λ_{max} (logε) 209 (4.28), 258 (3.82), 292 (sh, 3.43) nm; IR ν_{max} 3433, 1705, 1634, 1604, 1562, 1496, 1384, 1278, 1218, 1188, 1117, 1049, 1027, 1002, 943, 887, 769, 703 cm^{-1}; ^1H NMR (DMSO-d_6, 600 MHz) spectral data, see Table 1; ^{13}C NMR (DMSO-d_6, 150 MHz) spectral data, see Table 1; ESI-MS *m/z* 405 $[M-H]^-$; HR-ESI-MS *m/z* 407.0986 $[M+H]^+$ (Calcd. for $C_{19}H_{19}O_{10}$ 407.0973), 429.0806 $[M+Na]^+$ (Calcd. for $C_{19}H_{18}O_{10}Na$ 429.0792). (−)-**1**: $[\alpha]_D^{20}$ −12.7 (*c* 0.06, MeOH); CD (MeOH) 208 ($\Delta\varepsilon$ −0.74), 225 ($\Delta\varepsilon$ +0.70); (+)-**1:** $[\alpha]_D^{20}$ +11.8 (*c* 0.05, MeOH); CD (MeOH) 207.5 ($\Delta\varepsilon$ +1.88), 225 ($\Delta\varepsilon$ −3.11).

4.3.2. Isatindigoticamide A (**2**)

Colorless needles, mp 156–158 °C; UV (MeOH) λ_{max} (logε) 208 (4.63), 239 (4.30), 309 (4.10), 338 (4.09) nm; IR (KBr) ν_{max} 3356, 3275, 1691, 1648, 1611, 1546, 1509, 1451, 1357, 1323, 1260, 1228, 1176, 1095, 1073, 1038, 984, 903, 845, 828, 786, 747, 685, 567 cm^{-1}; ^1H NMR (Me_2CO-d_6, 500 MHz) spectral data, see Table 1; ^{13}C NMR (Me_2CO-d_6, 125 MHz) spectral data, see Table 1; ESI-MS *m/z* 286 $[M-H]^-$; HR-ESI-MS *m/z* 310.0688 $[M+Na]^+$ (Calcd. for $C_{15}H_{13}NO_5$ 310.0686).

4.3.3. Isatindigoticamide B (**3**)

Colorless needles, mp 171–173 °C; UV (MeOH) λ_{max} (log ε) 203 (4.38), 221 (4.37), 281 (4.00), 339 (3.86) nm; IR (KBr) ν_{max} 3255, 3173, 2953, 1701, 1639, 1600, 1544, 1502, 1428, 1357, 1303, 1254, 1232, 1192, 1119, 1074, 985, 928, 835, 794, 700, 587, 556 cm^{-1}; ^1H NMR (Me_2CO-d_6, 500 MHz) spectral data, see Table 1; ^{13}C NMR (Me_2CO-d_6, 125 MHz) spectral data, see Table 1; ESI-MS *m/z* 294 $[M+Na]^+$, 565 $[2M+Na]^+$, 270 $[M-H]^-$, 541 $[2M-H]^-$; HR-ESI-MS *m/z* 294.0733 $[M+Na]^+$ (Calcd. for $C_{15}H_{13}NO_4Na$ 294.0737).

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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.2016.09.004.

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