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Five Pairs of Enantiomer as Rearrangement Products from Secoiridoids in *Gentiana macrophylla* Pall.

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ABSTRACT: Five racemates (**1**−**5**) were isolated from *Gentiana macrophylla*, in which **2**−**5** were successfully separated into four pairs of enantiomers (**2a** and **2b**, **3a** and **3b**, **4a** and **4b**, and **5a** and **5b**), whereas the resolution of **1** failed due to the hemiacetal functionality at the stereogenic center. Using electronic circular dichrosim calculation, the relationship of the molecular rotation direction and the carbon *R/S* chirality was revealed, and each pair of enantiomer was identified as (−)-(*S*)-gentianmacrol B (**2a**) and (+)-(*R*)-gentianmacrol B (**2b**), (−)-(*S*)-8-methoxy-gentianol (**3a**) and (+)-(*R*)-8-methoxy-gentianol (**3b**), (+)-(*S*)-8-methyl-gentianadine (**4a**) and (−)-(*R*)-8-methyl-gentianadine (**4b**), and (−)-(*S*)-gentianol (**5a**) and (+)-(*R*)-gentianol (**5b**). Besides, these compounds could be divided into two series, **1**−**2** containing the benzene ring moiety and **3**−**5** containing the pyridine ring moiety. Considering that their molecular skeleton could not be generated from the classical biosynthesis pathway in plants, the plausible biosynthesis pathways of **1**−**5** were deduced to be transformed from secoiridoids in *G. macrophylla*. Due to the significant difference in the pharmacological effect for the optical factor, our research provided new diverse molecules for further optical activity studies in drug research.
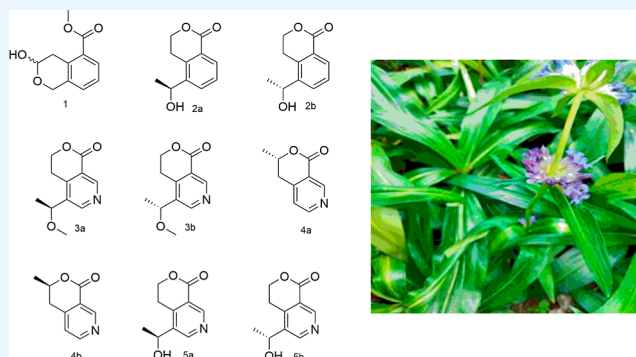
INTRODUCTION

Gentiana macrophylla is a perennial herb in the genus *Gentiana* of Gentianaceae, distributed widely in almost all regions of northern China.[1] Its root (Gentianae Macrophyllae Radix), named as "Qing-Jiao" in Chinese according to Chinese Medicine Principles, has always been used as one of the primary ingredients in many famous prescriptions for the treatment of rheumatic pain, stroke hemiplegia, muscle and vein spasms, soreness in the joints, damp heat jaundice, hot flashes in the bones, and fever in children,[2] while its flower, named as "Jie-Ji-Na-Bao" in Tibetan according to the Four Medical Classics (one of the traditional Tibetan medicine books),[3] has always been used alone for the treatment of rheumatoid arthritis and jaundice hepatitis.[4,5] Moreover, earlier studies have revealed the presence of secoiridoids as its main chemical components.[6] However, considering a high activity of the hemiacetal in the structure, various unpredictable rearrangement reactions might occur, and some secoiridoids were transformed into alkaloids, such as gentianine A, gentianidine, and gentianol under a nitrogenous environment,[7,8] and some secoiridoids were transformed into rare furan derivatives, such as genfurtate, swertianglide, and anglelone.[9]

In this paper, two series of natural products were isolated from *G. macrophylla*, one containing a pyridine ring moiety and the other having a benzene ring moiety. According to their plausible biosynthetic pathways (Scheme 1), all compounds were deduced to be transformed from secoiridoids before the formation or after the breakage of the glycoside bond. During the rearrangement reactions, molecular chirality might suffer great changes; thus, enantiomers were present. Furthermore, it should be stressed that no acids or bases were employed during our procedure; thus, the alkaloids of **3**−**5** were obtained as natural products. The biosynthesis mechanism in *G. macrophylla* for the pyridine ring moiety was deduced to acquire a nitrogen source from the plant growing environment. Particularly, since severe birth defects were caused by thalidomide in 1960s, the optical factor in drug applications has attracted a lot of attention from national governments around the world, for D/L-thalidomide presents significantly different pharmacological effects, a sedative effect due to the dextroisomer, and a teratogenesis effect due to the levoisomer.[10] Nowadays, more pure enantiomers are being developed for clinical drugs with high activity and low adverse effect, such as levodopa, levofloxacin, dextro-penicillamine,
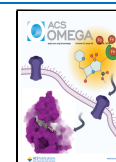
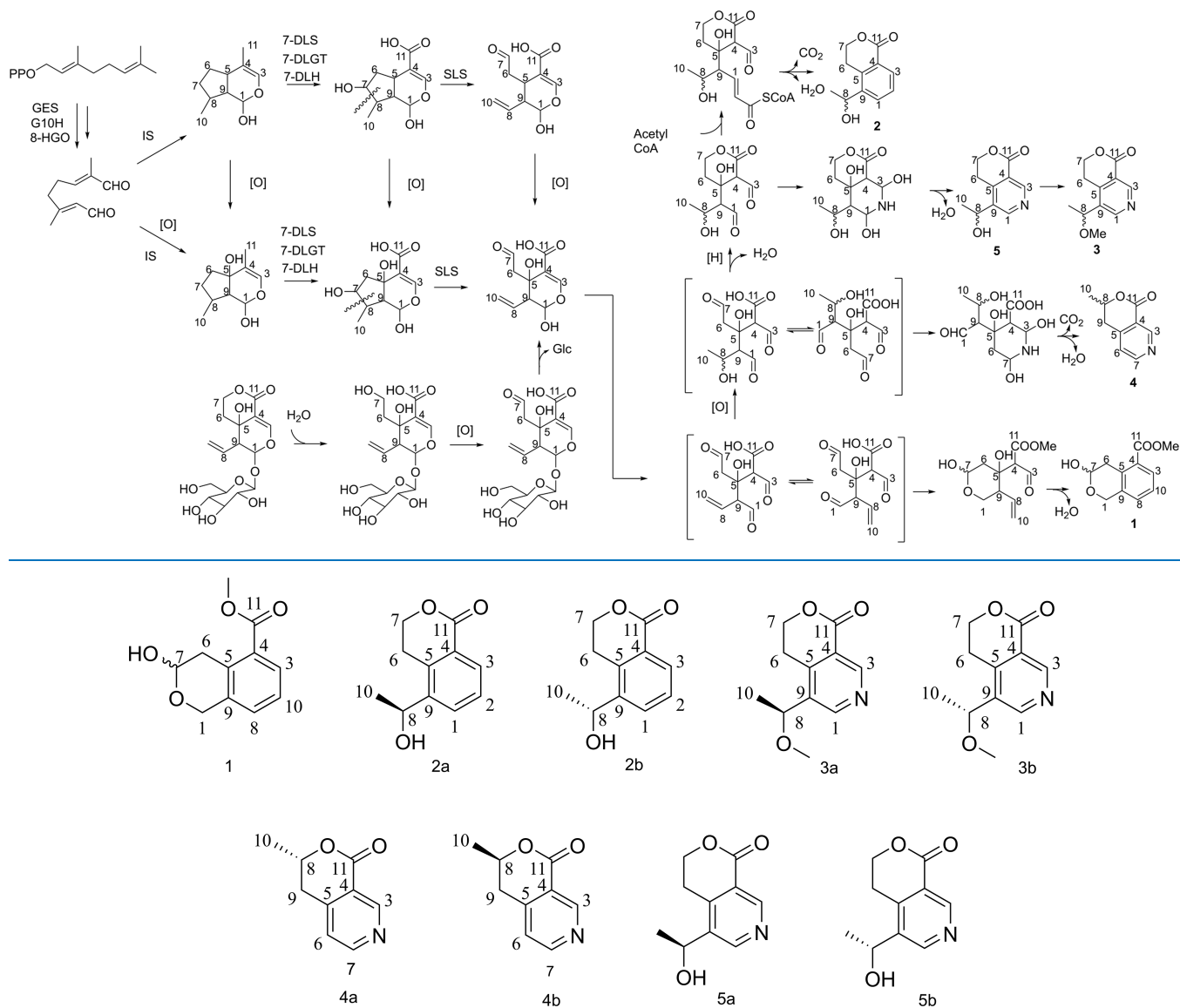## Scheme 1. Plausible Biosynthesis Pathway of Compounds 1−5

Figure 1. Structures of compounds 1−5.

etc.[11] Thus, these enantiomers in *G. macrophylla* provided new, diverse precursors for drug research.

RESULTS AND DISCUSSION

Structure Elucidation. Five pairs of enantiomers were identified: four new compounds, (±) gentianmacrol A (**1**), (±) gentianmacrol B (**2**), (±) 8-methoxy-gentianol (**3**), and (±) 8-methyl-gentianadine (**4**), and one known compound, (±)-gentianol (**5**); they were isolated from the radix of *G. macrophylla* Pall. Moreover, these five pairs of enantiomer were performed with chiral separation, and individual enantiomer of **2**−**5** were obtained unless the interconversion feature of **1**. Besides, the relationship of the molecular rotation direction and the carbon *R*/*S* chirality was revealed using electronic circular dichrosim (ECD) calculation; thus, each pair of enantiomer was identified as (−)-(*S*)-gentianmacrol B (**2a**) and (+)-(*R*)-gentianmacrol B (**2b**), (−)-(*S*)-8-methoxy-gentianol (**3a**) and (+)-(*R*)-8-methoxy-gentianol (**3b**), (+)-(*S*)-8-methyl-gentianadine (**4a**) and (−)-(*R*)-8-methyl-

gentianadine (**4b**), and (−)-(*S*)-gentianol (**5a**) and (+)-(*R*)-gentianol (**5b**)[8] (Figure 1).

Compound **1** displayed pseudo molecular ion peaks at *m/z* 209.0806 [M + H]$^+$ (calcd for $C_{11}H_{13}O_4$, 209.0814) in the HR-ESI-MS spectrum, corresponding to the molecular formula $C_{11}H_{12}O_4$ (Figure S1). The ^1H NMR spectrum displayed signals of one hemiacetal proton at δ_H 5.25 (dd, *J* = 5.47, 3.83 Hz, H-7), one methoxy signal at δ 3.87 (s, H-11), ortho-trisubstituted benzene ring moiety at δ_H 7.27 (m, H-10), δ_H 7.26 (m, H-8), and δ_H 7.80 (dd, *J* = 6.23, 2.96 Hz, H-3), one oxygenated methylene proton at δ_H 4.97 (d, *J* = 15.00 Hz, H-1a) and δ_H 4.78 (d, *J* = 15.00 Hz, H-1b), and one methylene proton at δ_H 3.34 (dd, *J* = 17.76, 3.83 Hz, H-6a) and δ_H 3.05 (dd, *J* = 17.76, 5.47 Hz, H-6b). The ^{13}C NMR spectrum displayed 11 carbon signals, which were attributed to one carbonyl group at δ_C 168.9 (C-11), six sp^2 carbons of one benzene ring moiety at δ_C 136.7 (C-9), δ_C 130.9 (C-4), δ_C 134.7 (C-5), δ_C 126.8 (C-10), δ_C 129.3 (C-8), and δ_C 130.2 (C-3), one hemiacetal at δ_C 93.2 (C-7), one oxygenated methylene at δ_C 64.5 (C-1), and one methylene at δ_C 34.8 (C-

Table 1. ^{1}H NMR (600 MHz) and ^{13}C NMR (150 MHz) Data^a of Compounds 1 and 2 (in CD$_3$OD) (δ_H in ppm, J in Hz)

no.	1		2	
	δ_C	δ_H	δ_C	δ_H
1	64.5	4.97 (1H, d, J = 15.00 Hz, H-1a)	131.9	7.80 (1H, dd, J = 7.79, 1.40 Hz, H-1)
		4.78 (1H, d, J = 15.00 Hz, H-1b)		
2			128.4	7.46 (1H, t, J = 7.71 Hz, H-2)
3	130.2	7.80 (1H, dd, J = 6.23, 2.96 Hz, H-3)	130.0	7.98 (1H, dd, J = 7.77, 1.40 Hz, H-3)
4	130.9		126.4	
5	134.7		138.5	
6	34.8	3.34 (1H, dd, J = 17.76, 3.83 Hz, H-6a)	25.2	3.16 (2H, m, H-6)
		3.05 (1H, dd, J = 17.76, 5.47 Hz, H-6b)		
7	93.2	5.25 (1H, dd, J = 5.47, 3.83 Hz, H-7)	68.3	4.54 (2H, m, H-7)
8	129.3	7.26 (1H, m, H-10)	66.8	5.11 (1H, q, J = 6.53 Hz, H-8)
9	136.7		144.7	
10	126.8	7.27 (1H, m, H-8)	24.2	1.47 (3H, d, J = 6.53 Hz, H-10)
11	168.9		167.8	
OMe	52.3	3.87 (3H, s, 11-OCH$_3$)		

^{a}^{1}H NMR and ^{13}C NMR were measured at 600 and 150 MHz; the assignments were based on HSQC, HMBC, and ROESY experiments.

6) and one methoxy at δ_C 52.3 (C-11). The correlative signals of the ^{1}H NMR and ^{13}C NMR were assigned by HSQC experiments (Table 1). In the HMBC spectrum (Figure 2), the
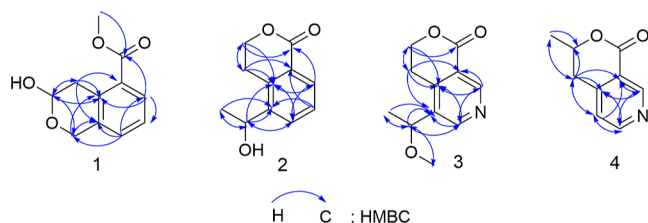

Figure 2. Selected HMBC correlations of compounds 1−4.

correlations of H-7 to C-1, 5 and 6, H-6 to C-4, 5, 7 and 9, and H-1 to C-5, 7 and 9 suggested the presence of an [4,5-*c*]-endocyclic ring owing to a pyran ring moiety attached with the benzene ring moiety; the correlations of OMe to C-11 suggested that the carbonyl was methyl esterifed. Thus, the structure of **1** was determined as gentianmacrol A. Considering the lability of the hemiacetal group, the stereochemistry configuration at C-7 might be unstable. This deduction was verified by the optical rotation test and an interconversion feature during the chiral separation. Thus, **1** was present as racemate as (±)-gentianmacrol A (Figures 1, S3−S7).

Compound **2**, **3**, **4**, and **5** were also present as a pair of racemate in *G. macrophylla*. Each enantiomer was separated by HPLC using CHIRALPAK AD-H (250 × 4.6 mm, 5 μm), UV detector 240 nm, and flow rate 0.8 mL/min (Figure 3). Racemates of **2**, **3**, and **5** were eluted with *n*-hexane/ isopropanol (89:11) as the molie phase to yield **2a** (t_R = 15.08 min) and **2b** (t_R = 16.83 min), **3a** (t_R = 21.75 min) and **3b** (t_R = 22.64 min), and **5a** (t_R = 23.45 min) and **5b** (t_R = 25.24 min), and the racemates of **4** were eluted with *n*-hexane/ isopropanol (85:15) to yield **4a** (t_R = 18.87 min) and **4b** (t_R = 17.08 min).

Compound **2** displayed pseudo molecular ion peaks at m/z 193.0861 [M + H]$^+$ (calcd for C$_{11}$H$_{13}$O$_3$, 193.0865) in the HR-ESI-MS spectrum, corresponding to the molecular formula C$_{11}$H$_{12}$O$_3$ (Figure S9). The ^{1}H NMR spectrum displayed signals of ortho-trisubstituted benzene ring protons at δ_H 7.98 (dd, J = 7.78, 1.40 Hz, H-3), δ_H 7.46 (t, J = 7.78 Hz, H-2), and δ_H 7.80 (dd, J = 7.78, 1.40 Hz, H-1), one ethoxy group at δ_H

5.11 (q, J = 6.53 Hz, H-8) and δ_H 1.47 (d, J = 6.53 Hz, H-10), and two methylene protons at δ_H 4.54 (m, H-7) and δ_H 3.16 (m, H-6). The ^{13}C NMR spectrum displayed 11 carbon signals, which are attributed to one lactone carbonyl group at δ_C 167.8 (C-11), one benzene ring moiety at δ_C 138.5 (C-5), δ_C 126.4 (C-4), δ_C 130.0 (C-3), δ_C 128.4 (C-2), δ_C 131.9 (C-1), and δ_C 144.7 (C-9), one oxygenated methylene at δ_C 68.3 (C-7), one methylene at δ_C 25.2 (C-6), and one methyl at δ_C 24.2 (C-10). The correlative signals of the ^{1}H NMR and ^{13}C NMR were assigned by HSQC experiments (Table 1). In the HMBC spectrum (Figure 2), the correlations of H-7 to C-5, 6, and 11, H-6 to C-4, 5, 7, and 9, and H-3 to C-11 suggested the presence of an [4,5-*c*]-endocyclic ring owing to a δ-lactone pyran ring moiety attached with the benzene ring moiety; the correlations of H-8 to C-1, 5, 9, and 10, and H-10 to C-8 and 9 suggested that the ethoxy group was attached to the benzene ring at C-9. Thus, the structure of **2** was indicated as gentianmacrol B. In the molecule, the stereochemistry at C-8 generated a molecular chirality; however, **2** presented racemization during the optical rotation test. Thus, chiral separation was performed as referred to by the above chromatographic condition. A pair of racemate **2a** and **2b** was present as (−)-gentianmacrol B with [α]$_D^{20}$ −67.0° and (+)-gentianmacrol B with [α]$_D^{20}$ + 67.0° (Figures 1 and S11−S15).

Compound **3** displayed pseudo molecular ion peaks at m/z 208.0968 [M + H]$^+$ (calcd for C$_{11}$H$_{14}$NO$_3$, 208.0974), corresponding to the molecular formula C$_{11}$H$_{13}$NO$_3$ (Figure S16). The proton signals at δ_H 8.72 (s, H-1) and δ_H 9.05 (s, H-3) in the ^{1}H NMR spectrum and the carbon signals at δ_C 148.3 (C-5), δ_C 137.3 (C-9), δ_C 152.2 (C-1), δ_C 150.7 (C-3), and δ_C 123.1 (C-4) in ^{13}C NMR indicated the presence of a characteristic β,γ,δ-trisubstituted pyridine ring moiety. The signals of two methylene at [δ_H 4.61 (t, J = 5.80 Hz, H-7) and δ_C 67.9 (C-7)] and [δ_H 3.24 (td, J = 5.80, 2.1 Hz, H-6) and δ_C 24.8 (C-6)] and a lactone carbonyl group at δ_C 165.6 (C-11) showed the correlated signals of H-7 to C-5, 6, and 11, H-6 to C-4, 5, 7, and 9, and H-3 to C-11 in the HMBC spectrum, indicating the presence of an [4,5-*c*]-endocyclic ring owing to a δ-lactone pyran ring moiety attached with the pyridine ring moiety. Besides, the signals of an ethoxy group at [δ_H 4.70 (q, J = 6.53 Hz, H-8) and δ_C 76.2 (C-8)] and [δ_H 1.49 (d, J = 6.53 Hz, H-10) and δ_C 22.1 (C-10)] and a methoxy group at [δ_H
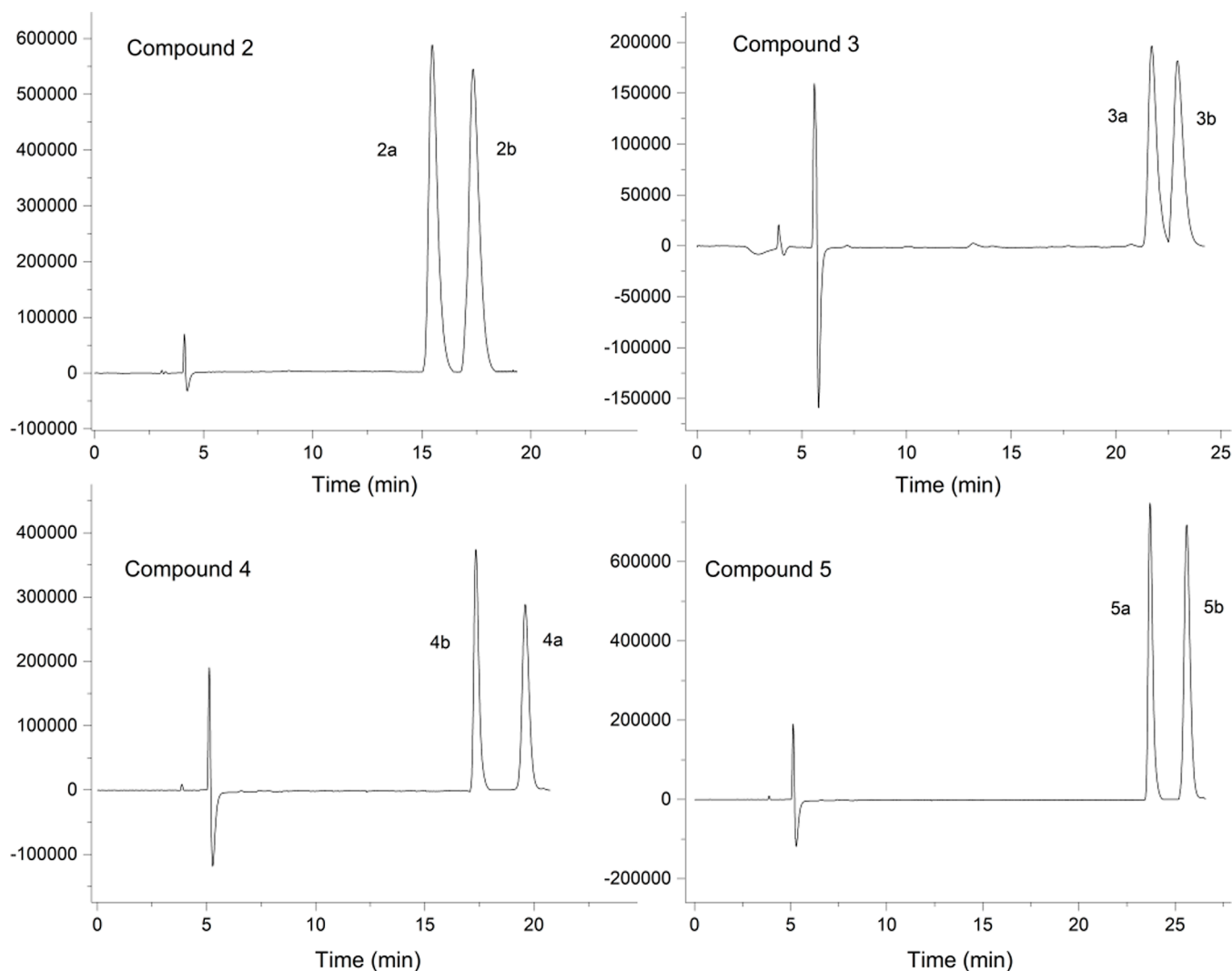
Figure 3. Chiral separation chromatography of compounds **2**−**5**.

3.32 (s, OMe) and δ_C (57.0)] showed correlated signals of H-8 to C-1, 5, 9, and OMe, H-10 to C-9, and OMe to C-8, indicating that the exthoxy group was attached with the methoxy and the pyridine ring moiety at C-9. Thus, the structure of **3** was determined as an analogue of **5**,[8] named 8-methoxy-gentianol. Due to the same stereochemistry at C-8, racemates of **3** and **5** showed similar optical rotation properties with **2**. Thus, **3a** and **3b** were present as (−)-8-methoxy-gentianol with $[\alpha]_D^{20}$ −20.0° and (+)-8-methoxy-gentianol with $[\alpha]_D^{20}$ +20.0°, while **5a** and **5b** were present as (−)-gentianol with $[\alpha]_D^{20}$ −64.0° and (+)-gentianol with $[\alpha]_D^{20}$ +64.0° (Figures 1 and S18−S22).

Compound **4** was indicated as alkaloid derivatives as **3** and **5**, according to pseudo molecular ion peaks at m/z 194.0815 [M + H]$^+$ (calcd for $C_{10}H_{12}NO_3$, 194.0817) corresponding to the molecular formula $C_{10}H_{11}NO_3$ (Figure S23). Similar characteristic spectra were present for a [4,5-c]-endocyclic ring owing to a δ-lactone pyran ring moiety attached with the pyridine ring moiety. However, the proton signals at δ_H 7.45 (d, J = 5.14 Hz, H-6), δ_H 8.69 (d, J = 5.14 Hz, H-7), and δ_H 9.08(s, H-3) in the ^1H NMR spectrum and the carbon signals at δ_C 150.9 (C-5), δ_C 124.1 (C-6), δ_C 154.1 (C-7), δ_C 151.3 (C-3), and δ_C 122.9 (C-4) in the ^{13}C NMR spectrum indicated the presence of a β,γ-disubstituted pyridine ring moiety and

the absence of the ethoxy group. In addition, a methyl at δ_H 1.53 (d, J = 6.34 Hz) was correlated with C-8 and 9 in the HMBC spectrum, indicating that the methyl was attached with the δ-lactone pyran ring moiety at C-8. Thus, the structure of **4** was determined as 8-methly gentian. In addition, C-8 was a chiral carbon atom in the structure, and continuous separation of racemate obtained **4a** as (+)-8-methly gentian with $[\alpha]_D^{20}$ +20.0° and **4b** as (−)-8-methly gentian with $[\alpha]_D^{20}$ −20.0° (Figures 1 and S25−S29).

Electronic Circular Dichrosim Calculation and Experimental CD Measurements. ECD has been proven to be a powerful and reliable method in elucidating the absolute configuration of natural products.[12] To get the relationship between the absolute configuration of the chiral carbon atom and optical rotation properties of enantiomers, ECD calculations using TDDFT (time-dependent density functional theory) and experimental CD measurements were performed. According to the data of **2**, **3**, and **5**, one of the enantiomers with a negative value of optical rotation had 8S configuration, while the positive one had 8R configuration. According to the data of **4**, one of the enantiomers with a negative value of optical rotation had 8R configuration, while the positive one had 8S configuration. The result was consistent with the reported deduction in the literature.[8,13] Therefore, the pair of
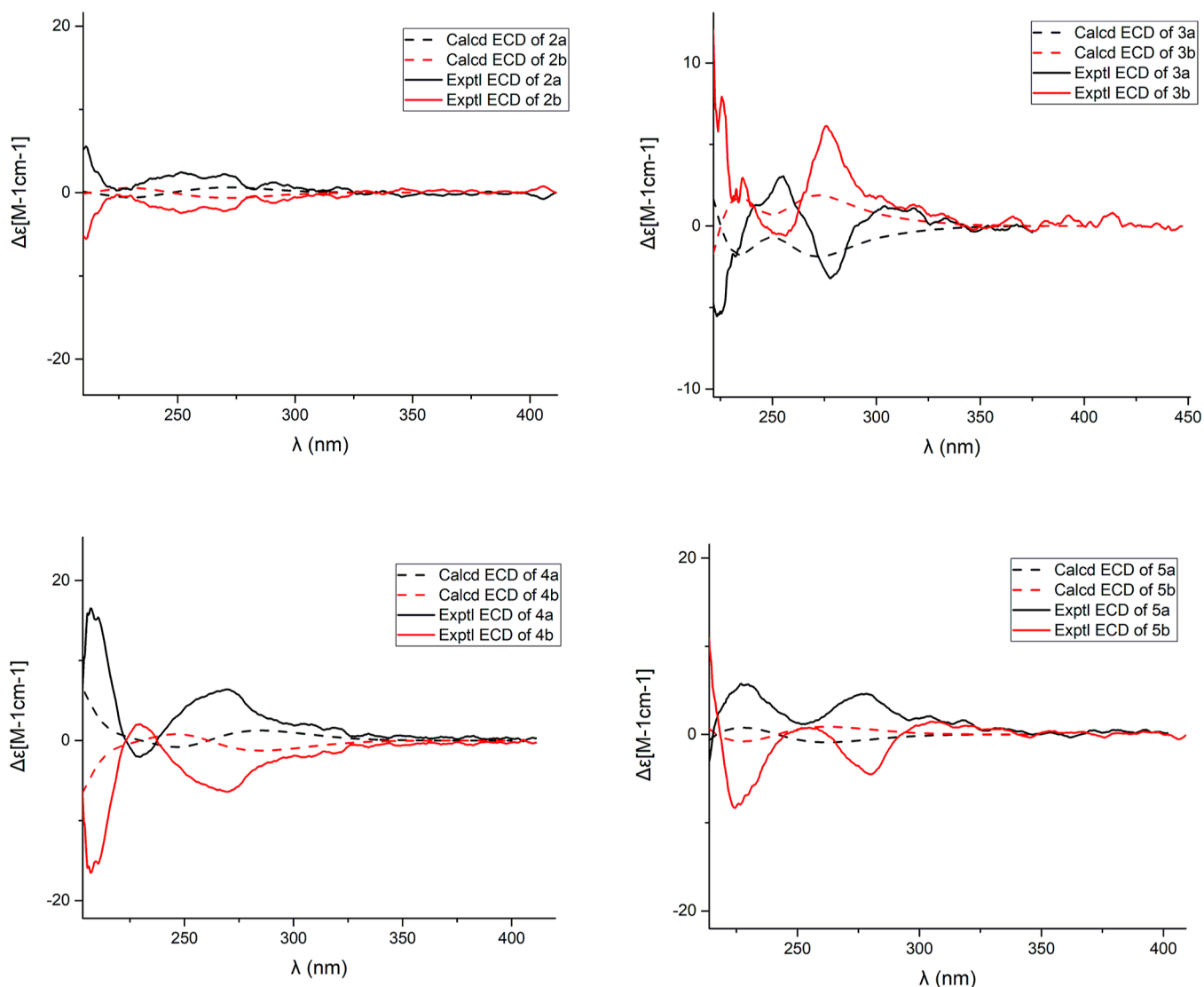
Figure 4. Calculated and experimental ECD spectra of compounds **2**−**5**.

racemate was identified to be **2a** as (−)-(*S*)-gentianmacrol B and **2b** as (+)-(*R*)-gentianmacrol B, **3a** as (−)-(*S*)-8-methoxy-gentianol and **3b** as (+)-(*R*)-8-methoxy-gentianol, **4a** as (+)-(*S*)-8-methly gentian and **4b** as (−)-(*R*)-8-methly gentian, and **5a** as (−)-(*S*)-gentianol and **5b** as (+)-(*R*)-gentianol (Figure 4).

Plausible Biosynthesis Pathway Deduction. Considering that the structure of these products could not be generated from the classical biosynthesis pathway (acetate−malonate pathway, mevalonic acid pathway, cinnamic acid pathway, and amino acid pathway) in plants, the plausible biosynthesis pathway of these compounds was proposed. As shown in Scheme 1, these compounds were produced as allosteric products transformed from secoiridoids. 5-Hydroxy-deglyco-sylsecologanate, which could be biosynthesized from three pathways, was deduced as the key intermediates. Although this key intermediate had not been identified from the plant, its analogue 5-hydroxysecologanolic acid with a labile feature was detected and isolated under an excess solvent of MeOH−CH$_2$N$_2$·Et$_2$O (1:1) from *Fontanesia phillyreoides*.[14] It should be stressed that hydroxy protonation at C-5 was vital to initiate the subsequent reactions, thus resulting in electron rearrange-

ment to form the benzene ring moiety or pyridine ring moiety. Moreover, the presence of hemiacetal at C-1 was another key factor. All subsequent reactions were conducted after the ring-opening of the hemiacetal. For compound **1**, the ring-opening reaction resulted in the formation of a new hemiacetal at C-7 and the formation of a benzene ring due to the reaction of the terminal olefinic bond (C-8 and C-10) and the aldehyde group (C-3) as well as the electron rearrangement after the leaving of 5-OH. For compound **2**, the ring-opening reaction, the hydroxylation of the olefinic bond at C-8, the reduction of the aldehyde group at C-7 to the hydroxyl group, following esterification with the acyl group, and the lengthening reaction of the carbon chain due to acetyl CoA successively occurred; finally, **2** was biosynthesized after the formation of a benzene ring due to the reaction of the newly generated terminal olefinic bond and the aldehyde group (C-3) and the electron rearrangement due to the leaving of COOH and OH. For compounds **3**, **4**, and **5**, besides the similar abovementioned reactions, a pyridine ring moiety was generated under NO$_3^-$ or NH$_4^+$ from a soil fertilizer providing a nitrogen source.[15] Finally, owing to the hemiacetal variability in **1** and the nonselective reaction property of the olefinic bond (C-8 and

C-10) during the biosynthesis of **2**−**5**, their enantiomers were generated simultaneously. Accordingly, the number of carbon atoms in the structure and the nomenclature of compounds were employed on the basis of their precursor secoiridoids.

■ EXPERIMENTAL PROCEDURES

General Experimental Procedures. The IR spectra were recorded on a Bruker VERTEX 70 instrument. 1D and 2D NMR spectra were recorded on a Bruker AVANCE III-600 instrument. The HR-ESI-MS spectra were taken on an AB SCIEX Triple TOF™ 5600+ HRESIMS. Silica gel (200−300 mesh) was purchased from Qingdao Meigao Group Corporation. Column chromatography (CC) was performed with Sephadex LH-20 gel, which was purchased from Pharmacia Biotech, Sweden. D101-macroporous absorption resin was purchased from Sinopharm Chemical Reagent Corporation (Shanghai, China) and used for CC. The analytical HPLC was performed on a Waters 2695 Separations Module coupled with a 2996 photodiode array detector and an Ultimate XB-C18 (4.6 × 250 mm, 5 μm particles). Semipreparative HPLC was performed using Jiangsu Hanbang Technology Corporation's Ultimate XB-C18 (10 × 250 mm, 5 μm particles) and YMC-Pack ODS-A (10 μm, 20 × 250 mm) (YMC). TLC was carried out with GF254 plates by Qingdao Marine Chemical Corporation. ECD spectra were obtained on a JASCO J-815 spectrophotometer (Jasco, Tokyo, Japan). The optical rotation was obtained on a Anton Paar.

Plant Material. The roots of *G. macrophylla* were purchased from the Fengxian country in Shaanxi Province of China in April 2019. The plant was identified by Prof. Wei Wang (Shaanxi University of Chinese Medicine). A voucher specimen (no. GM-20191020) was deposited in the Herbarium of Shaanxi University of Chinese Medicine.

Extraction and Isolation. The roots of *G. macrophylla* (40 kg) were crushed and extracted with MeOH (80 L × 2) at 50 °C for 3 h. The MeOH extracts were concentrated under reduced pressure to extracts, which was separated by macroporous resin CC (methanol−water gradient elution) to four fractions, the water part (Fr. A), the 30% methanol part (Fr. B), the 60% methanol part (Fr. C), and the methanol part (Fr. D). The 30% methanol part (Fr. B) (340 g) was further separated by HPLC (DAC-50 C₁₈ 75 μm, H$_2$O−MeOH 1:0−0:1, flow rate: 30 mL/min) to afford five fractions (Fr. B-1 to Fr. B-5). Fr. B-4 (120 g) was subjected to CC on silica gel, eluting with CH$_2$Cl$_2$−MeOH (20:1−0:1) to give six fractions (Fr. B-4-1 to Fr. B-4-6). Fr. B-4-4 (25 g) was subjected to CC on silica gel, eluting with CH$_2$Cl$_2$−MeOH (10:1−0:1) to give five fractions (Fr. B-4-4-1 to Fr. B-4-4-5). Fr. B-4-4-3 (856 mg) was purified by HPLC (Ultimate XB-C18, 10 mm × 250 mm, 5 μm particles, flow rate: 3.0 mL/min) with MeCN−H$_2$O (20:80) as the mobile phase to afford racemates **1** (4 mg; t_R = 13.4 min). Fr. B-4-4-4 (67 mg) was purified by HPLC (Ultimate XB-C18, 10 mm × 250 mm, 5 μm particles, flow rate: 3.0 mL/min) with MeCN−H$_2$O (10:90) as the mobile phase to afford racemates **4** (2 mg; t_R = 41.8 min). **1** was further isolated by HPLC with an AD-H column with *n*-hexane−isopropanol (85:15, 0.8 mL/min) to afford two peaks **1a** (t_R = 16.19 min) and **1b** (t_R = 23.67 min). **4** was further isolated by HPLC-ECD with an AD-H column with *n*-hexane−isopropanol (85:15, 0.8 mL/min) to afford two peaks **4a** (1 mg t_R = 18.87 min) and **4b** (1 mg t_R = 17.08 min). The 60% methanol part (Fr. C) (350 g) was further separated by macroporous resin CC (methanol−water gradient elution) to

four fractions (Fr. C-1 to Fr. C-4). Fr. C-3 (30 g) underwent silica gel column eluting with CH$_2$Cl$_2$−MeOH (1:0−0:1) to afford seven fractions (Fr. C-3-1 to Fr. C-3-7). Fr. C-3-4 (240 mg) was purified by HPLC (Ultimate XB-C18, 10 × 250 mm, 5 μm particles, flow rate: 3.0 mL/min) with MeCN−H$_2$O (15:85) as the mobile phase to afford racemates **2** (3 mg; t_R = 29.8 min). **2** was further isolated by HPLC with an AD-H column with *n*-hexane−isopropanol (89:11, 0.8 mL/min) to afford two peaks **2a** (1.5 mg t_R = 15.08 min) and **2b** (1.5 mg t_R = 16.83 min). Fr. C-3-1-5 (157 mg) was purified by HPLC (Ultimate XB-C18, 10 mm × 250 mm, 5 μm particles, flow rate: 3.0 mL/min) with MeCN−H$_2$O (20:80) as the mobile phase to afford racemates **5** (5 mg; t_R = 20.5 min) and **3** (3 mg; t_R = 33.4 min). **3** was further isolated by HPLC-ECD with an AD-H column with *n*-hexane−isopropanol (89:11, 0.8 mL/min) to afford two peaks **3a** (1.5 mg t_R = 21.75 min) and **3b** (1.5 mg t_R = 22.64 min). **5** was further isolated by HPLC-ECD with an AD-H column with *n*-hexane−isopropanol (89:11, 0.8 mL/min) to afford two peaks **5a** (2.5 mg t_R = 23.45 min) and **5b** (2.5 mg t_R = 25.24 min).

ECD Spectra Calculations. The structure of compounds **2**−**5** was built with ChemDraw Professional 15.0, and all trial structures were first minimized (MM2 method in Chem3D 15.0) based on molecular mechanics calculations. To sample the conformational space, a conformational search was performed (GMMX method of MMFF94) based on molecular mechanics calculations within 10 kcal/mol, via the GaussView 6.0 software package. Thus, **2**-(*S*) and **2**-(*R*) gave 12 numbers of conformations; **3**-(*S*) and **3**-(*R*) gave 15 numbers of conformations; **4**-(*S*) and **4**-(*R*) gave 2 numbers of conformations; **5**-(*S*) and **5**-(*R*) gave 11 numbers of conformations. Then, all the conformations for geometry optimization and energy calculation used the job type of Opt Freq and method of DFT with the B3LYP functional and the 6-31G(d,p) basis set with the Gaussian 16W program. All optimized conformations were performed with Boltzmann distributions of SCF energies (sum of electronic and thermal free energies) (more than 1%).[16] Thus, **2**-(*S*) and **2**-(*R*) obtained 12 numbers of optimized conformations; **3**-(*S*) and **3**-(*R*) obtained 8 numbers of optimized conformations; **4**-(*S*) and **4**-(*R*) obtained 2 numbers of optimized conformations; **5**-(*S*) and **5**-(*R*) obtained 11 numbers of optimized conformations, respectively. Next, the job type of energy and method of TD-DFT with the CAM-B3LYP/6-31G(d,p) basis set was used to calculate the spin-allowed excitation energies and rotatory (R_n) and oscillator strengths (f_n) of the lowest 30 excited states. All ECD calculations were performed using the method of no solution. Finally, the ECD spectra were combined after Boltzmann weighting according to their population contribution.

(±)-Gentianmacrol A (1). It was obtained as colorless oil; IR (KBr) ν_{max}: 3362, 2056, 1710, 1666, and 1146 cm^{-1} (Figure S2); ^{1}H NMR (CDOD$_3$, 600 MHz) and ^{13}C NMR (CDOD$_3$, 150 MHz) spectral data, see Table 1; *m/z* 209.0806 [M + H]$^+$ (calcd for C$_{11}$H$_{13}$O$_4$, 209.0814) (Figure S1).

(±)-Gentianmacrol B (2). It was obtained as colorless oil; IR (KBr) ν_{max}: 3358, 2920, 2060, 1705, 1665, and 1599 cm^{-1} (Figure S10); ^{1}H NMR (CDOD$_3$, 600 MHz) and ^{13}C NMR (CDOD$_3$, 150 MHz) spectral data, see Table 1; *m/z* 193.0861 [M + H]$^+$ (calcd for C$_{11}$H$_{13}$O$_3$, 193.0865) (Figure S9).

(−)-(S)-Gentianmacrol B (2a). $[\alpha]_D^{20}$ = −67.0° (*c* 0.01, MeOH); experimental ECD (MeOH, *c* 2.62 × 10^{-3} M) λ_{max} ($\Delta\varepsilon$) 235 (+1.1) nm.

Table 2. ^{1}H NMR (600 MHz) and ^{13}C NMR (150 MHz) Data^a of Compounds 3 and 4 (in CD$_3$OD) (δ_H in ppm, J in Hz)

no.	3		4	
	δ_C	δ_H	δ_C	δ_H
1	152.2	8.72 (1H, s, H-1)		
2				
3	150.7	9.05 (1H, s, H-3)	151.3	9.08 (1H, s, H-3)
4	123.1		122.9	
5	148.9		150.9	
6	24.8	3.24 (2H, td, J = 5.80, 2.11 Hz, H-6)	124.1	7.45 (1H, d, J = 5.14 Hz, H-6)
7	67.9	4.61 (2H, t, J = 5.80 Hz, H-7)	154.1	8.69 (1H, d, J = 5.14 Hz, H-7)
8	76.2	4.70 (1H, q, J = 6.53 Hz, H-8)	76.7	4.79 (1H, m, H-3)
9	137.3		34.6	3.12 (1H, dd, J = 17.01, 3.35 Hz, H-9a)
				3.04 (1H, dd, J = 17.01, 11.15 Hz, H-9b)
10	22.1	1.49 (3H, d, J = 6.53 Hz, H-10)	20.9	1.53 (3H, d, J = 6.34 Hz, H-10)
11	165.6		165.8	
OMe	57.0	3.32 (3H, s, −OCH$_3$)		

^{a} ^{1}H NMR and ^{13}C NMR were measured at 600 and 150 MHz; the assignments were based on HSQC, HMBC, and ROESY experiments.

(+)-(R)-Gentianmacrol B (2b). $[\alpha]_D^{20}$ = +67.0° (c 0.01, MeOH); experimental ECD (MeOH, c 2.62 × 10^{-3} M) λ_{max} ($\Delta\varepsilon$) 235 (−1.0) nm.

(±)-9-Methoxy-gentianol (3). It was obtained as colorless oil; IR (KBr) ν_{max}: 2955, 2052, 1726, 1663, 1591, and 1113 cm^{-1} (Figure S17); ^{1}H NMR (CDOD$_3$, 600 MHz) and ^{13}C NMR (CDOD$_3$, 150 MHz) spectral data, see Table 2; m/z 208.0968 [M + H]$^+$ (calcd for C$_{11}$H$_{14}$NO$_3$, 208.0974) (Figure S16).

(−)-(S)-8-Methoxy-gentianol (3a). $[\alpha]_D^{20}$ = −20.0° (c 0.01, MeOH); experimental ECD (MeOH, c 2.41 × 10^{-3} M) λ_{max} ($\Delta\varepsilon$) 228 (−2.9) nm, 277 (−3.2) nm, 255 (+3.0) nm.

(+)-(R)-8-Methoxy-gentianol (3b). $[\alpha]_D^{20}$ = +20.0° (c 0.01, MeOH); experimental ECD (MeOH, c 2.41 × 10^{-3} M) λ_{max} ($\Delta\varepsilon$) 228 (+3.6) nm, 277 (+5.8) nm, 255 (−0.5) nm.

(±)-8-Methly Gentian (4). It was obtained as colorless oil; IR (KBr) ν_{max}: 2920, 2361, and 1720 cm^{-1} (Figure S24); ^{1}H NMR (CDOD$_3$, 600 MHz) and ^{13}C NMR (CDOD$_3$, 150 MHz) spectral data, see Table 2; m/z 164.0702 [M + H]$^+$ (calcd for C$_9$H$_{10}$NO$_2$, 164.0712) (Figure S23).

(+)-(S)-8-Methly Gentian (4a). $[\alpha]_D^{20}$ = +20.0° (c 0.01, MeOH); experimental ECD (MeOH, c 3.07 × 10^{-3} M) λ_{max} ($\Delta\varepsilon$) 230 (−2.0) nm, 278 (+4.4) nm.

(−)-(R)-8-Methly Gentian (4b). $[\alpha]_D^{20}$ = −20.0° (c 0.01, MeOH); experimental ECD (MeOH, c 3.07 × 10^{-3} M) λ_{max} ($\Delta\varepsilon$) 230 (+2.1) nm, 278 (−4.4) nm.

(±)-Gentianol (5). It was obtained as colorless oil; ^{1}H NMR (CDOD$_3$, 600 MHz) and ^{13}C NMR (CDOD$_3$, 150 MHz) spectral data (Figures S31 and 32); m/z 194.0815 [M + H]$^+$ (calcd for C$_9$H$_{10}$NO$_2$, 194.0817) (Figure S30).

(−)-(S)-Gentianol (5a). $[\alpha]_D^{20}$ = −64.0° (c 0.01, MeOH); experimental ECD (MeOH, c 2.59 × 10^{-3} M) λ_{max} ($\Delta\varepsilon$) 225 (+4.2) nm, 261 (+1.1) nm, 275 (+3.9) nm.

(+)-(R)-Gentianol (5b). $[\alpha]_D^{20}$ = −64.0° (c 0.01, MeOH); experimental ECD (MeOH, c 2.59 × 10^{-3} M) λ_{max} ($\Delta\varepsilon$) 225 (−3.0) nm, 253 (+1.5) nm, 280 (−1.4) nm.

CONCLUSIONS

In this investigation, five pairs of enantiomers such as (±) gentianmacrol A (1), (±) gentianmacrol B (2), (±) 8-methoxy-gentianol (3), (±) 8-methyl-gentianadine (4), and (±)-gentianol (5) were isolated from the radix of *G. macrophylla*, in which 1−4 were identified as new compounds. Further, chiral separation was performed, and each enantiomer

of 2−5 was obtained. Besides, the relationship of the molecular rotation direction and the carbon R/S chirality was revealed using an ECD calculation. Considering that the structure of these products could not be generated from the classical biosynthesis pathway in plants, the plausible biosynthesis pathways of 1−5 as rearrangement products from secoiridoids in *G. macrophylla* were deduced reasonably.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.4c02476.

HR-ESI-MS spectra of compounds 1−5; IR spectra of compounds 1−4; 1D and 2D NMR of compounds 1−4; 1D NMR of compound 5; chiral separation chromatography of compound 1; and the calculated ECD related data of compounds 2−5 (PDF)

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