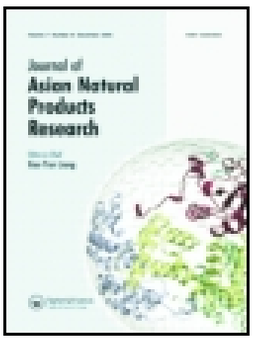

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Phenylpropanoids from the stems of *Ephedra sinica*

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Two new compounds of phenylpropanoids, (*S*)-*N*-((1*R*,2*S*)-1-hydroxy-1-phenylpropan-2-yl)-5-oxopyrrolidine-2-carboxamide (**1**) and (3*R*)-3-*O*-*β*-D-glucopyranosyl-3-phenylpropanoic acid (**2**), were isolated from the stems of *Ephedra sinica*. Their structures were elucidated by in-depth examination of spectroscopic data, mainly including those from the 1D and 2D NMR and HRESIMS techniques, and chemical method. The absolute configurations of the two compounds were also corroborated through CD procedure.

Keywords: *Ephedra sinica*; Ephedraceae; amphetamine-type alkaloid; glycoside of phenylpropanoic acid

1. Introduction

Ephedra sinica Stapf. (Ephedraceae) is one of the original plants of Ephedrae Herba that was recorded in the Chinese Pharmacopoeia as "Ma Huang." Just like the other two original plants, *E. intermedia* Schrenk et C. A. Mey. and *E. equisetina* Bunge, the stem of *E. sinica* has been widely used to treat rheums, asthma, and cough with dyspnea, *inter alia*, in traditional Chinese medicine (TCM) for thousands of years [1,2]. It has been reported that *E. sinica* contains amphetamine-type alkaloids and flavonoids as the most important and accepted constituents in pharmaceutics and chemotaxonomy. In general, the amphetamine-type alkaloids were illustrated to bear some interesting bioactivities. For instance, the most famous *l*-ephedrine and its analogs isolated from the title plant have been applied to treat hypotension, obesity, asthma, and allergic rhinitis [3–6]. and so on, clinically. However, it was also found, through the investigation on literatures, that there were only limited amphetamine-type alkaloids from the *Ephedra* species [7,8], of which, (±)-2-imino-1-phenylpropan-1-ol was reported by our laboratory for the first time [9]. On the other hand, there were not many literatures about the NMR studies on the isolated amphetamine-type alkaloids yet. In our ongoing search for new amphetamine-type alkaloids, including amphetamine-type compounds with alkalescence, from *Ephedra* species, a new amide of amphetamine-type was isolated from the neural *n*-BuOH-soluble fraction of the ethanol extract from the stem of *E. sinica*. In the meantime, one new glycoside of phenylpropionic acid was also isolated from the same neural fraction. In an exact sense, both new compounds belong in phenylpropanoids because of their shared propylbenzene core. This paper deals mainly with the isolation and structural elucidation of these new compounds.

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2. Results and discussion

The neutral *n*-BuOH-soluble section of the ethanol extract from the stems of *E. sinica* was subjected to multiple column chromatography (CC) and further purified by preparative HPLC procedure, affording two new compounds (**1** and **2**) reported herein (Figure 1).

Compound **1** was obtained as a white amorphous powder, with $[\alpha]_D^{20}$ − 29.6 (*c* 0.61, CH_3OH). Its molecular formula, $C_{14}H_{18}N_2O_3$, was assigned by ^{13}C NMR data and protonated ion at *m/z* 263.1392 $[M + H]^+$ in the HRESIMS measurement of positive-ion mode, indicative of seven degrees of unsaturation. The IR absorptions at 3309, 3091, 1662, 1639, 1554, and 1452 cm^{-1}, in conjunction with the ^{13}C NMR spectrum, indicated the existence of hydroxyl, amide, and aryl groups. The ^1H NMR spectrum revealed the diagnostic signals of the alkaloid of 2-amino-1-hydroxy-1-phenylamphetamine-type, with a monosubstituted benzene ring and a 2-amino-1-hydroxy-1-phenylsubstituted propane being exhibited by the signals at δ 7.28–7.32 (4 H, m, H-2′, 3′, 5′, 6′), 7.20–7.23 (1 H, m, H-4′), 5.43 (1 H, br s, OH), 4.50 (1 H, d, $J = 5.5$ Hz, H-1), 3.86–3.92 (1 H, m, H-2), and 0.98 (3 H, d, $J = 7.0$ Hz, H-3), which is very consistent with those of the known alkaloids of this kind [10]. In addition to the hydroxyl group, two other labile protons at δ 7.83 (1 H, d, $J = 8.5$ Hz, NH-2) and 7.70 (1 H, br s, NH-1″) were also exhibited in the ^1H NMR spectrum. All three labile protons were confirmed by HSQC experiment which showed that no ^{13}C NMR signals correlated to the ^1H NMR signals at δ_H 7.83, 7.70, and 5.43. Also, an AA'BB'X-type coupling system of —CH—CH_2—CH_2— was determined, in combination with the ^1H, ^1HCOSY experiment, by a set of ^1H NMR signals at δ 1.48–1.50 and 2.04–2.06 (1 H each, 2 × m, H_2-4″), 1.98 (2 H, dd, $J = 10.5$, 6.5 Hz, H_2-3″), and 3.92–3.96 (1 H, m, H-5″). The ^{13}C NMR spectrum of **1** exhibited 14 carbon signals, which were classified by DEPT experiment into one methyl, two methylenes, eight methines including two pairs of aromatic carbons showing the uniform resonance frequencies at δ_C 126.3 (d) and 127.7 (d), respectively, one quaternary carbon, and two carbonyls at δ_C 171.2 and 177.3 (Table 1). Considering the molecular formula of seven unsaturation degrees, and in association with the HSQC spectrum, the HMBC experiment unambiguously established the AA'BB'X-type coupling system as a 5-oxopyrrolidine-2-carboxamide moiety by the correlations from NH-1″ to C-2″, C-3″, C-4″, and C-5″, from H_2-3″ to C-2″, C-4″, and C-5″, from H_2-4″ to C-2″ and C-6″, and from NH-2 to C-6″ (Figure 2), which also suggested that the 2D structure of **1** was *N*-(1-hydroxy-1-phenylpropan-2-yl)-5-oxopyrrolidine-2-carboxamide. With this constitution established by critical NMR examination, the next consideration was to elucidate the absolute configurations of C-1 and C-2 of the 1-hydroxy-1-phenylpropan-2-yl and C-5″ of the 5-oxopyrrolidine-2-carboxamide moieties. The CD spectrum of

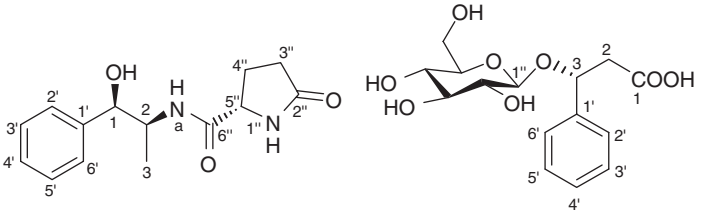


Figure 1. Structures of compounds **1** and **2**.

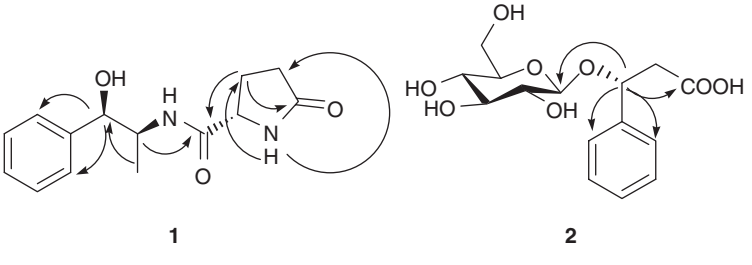
Table 1. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR spectroscopic data for **1** and **2** (in DMSO-d_6).

No.	1		2	
	δ_H (mult[a], *J* in Hz)	δ_C (dept[b])	δ_H (mult, *J* in Hz)	δ_C (dept)
1	4.50 d (5.5)	74.9 d		172.4 s
2	3.86–3.92 m	50.0 d	a 2.93 dd (16.0, 7.5) b 2.66 dd (16.0, 7.5)	41.2 t
3	0.98 d (7.0)	14.9 q	5.06 t (7.5)	76.2 d
OH-1	5.43 br s			
NH-2	7.83 d (8.5)			
1′		143.3 s		141.4 s
2′	7.28–7.32 m	126.3 d	7.38 d (7.5)	126.9 d
3′	7.28–7.32 m	127.7 d	7.32 t (7.5)	127.9 d
4′	7.20–7.23 m	126.7 d	7.26 t (7.5)	127.4 d
5′	7.28–7.32 m	127.7 d	7.32 t (7.5)	127.9 d
6′	7.28–7.32 m	126.3 d	7.38 d (7.5)	126.9 d
1″	7.70 br s		4.34 d (8.0)	101.6 d
2″		177.3 s	2.94–2.97 m	73.7 d
3″	1.98 dd (10.5, 6.5)	29.1 t	3.01–3.15 m	76.6 d
4″	a 2.04–2.06 m b1.48–1.50 m	25.1 t	3.01–3.15 m	70.0 d
5″	3.92–3.96 m	55.4 d	3.01–3.15 m	76.9 d
6″		171.2 s	3.60 dd (12.5, 4.5) 3.36–3.40 m	61.0 t

Notes: [a] multiplicity; [b] DEPT NMR.

1 displayed a positive Cotton effect at 218 nm (log $\Delta\varepsilon + 5.03$) (Figure 3), demonstrating the 5″*S* configuration [11–13]. The coupling constant of $J_{1,2} = 5.5$ Hz suggested that **1** bore the 1,2-*erythro* configuration, which was consistent with the determination of 1*R*, 2*S* configuration through another positive Cotton effect at 258 nm (log $\Delta\varepsilon + 3.68$) in the CD spectrum [10,14,15]. Accordingly, **1** was determined as (*S*)-*N*-((1*R*,2*S*)-1-hydroxy-1-phenylpropan-2-yl)-5-oxopyrrolidine-2-carboxamide, and was given the trivial name sinicine.

Compound **2** was obtained as a white amorphous powder, and it has $[\alpha]_D^{20}$ − 19.4 (*c* 0.112, MeOH). The ^{13}C NMR data and the deprotonated ion at *m/z* 327.1087 $[M - H]^-$ in the HRESIMS experiment of negative-ion mode indicated the molecular formula of $C_{15}H_{20}O_8$. The IR spectrum suggested the existence of hydroxyl (3452 cm^{-1}), carbonyl (1705 cm^{-1}), and aromatic groups (1636 cm^{-1}). Especially, the broad peak between 2400 cm^{-1} and 3400 cm^{-1} in the IR spectrum indicated the existence of carboxy. The ^{1}H and ^{13}C NMR spectra, in



1 **2**

Figure 2. Key HMBC (→) correlations of **1** and **2**.

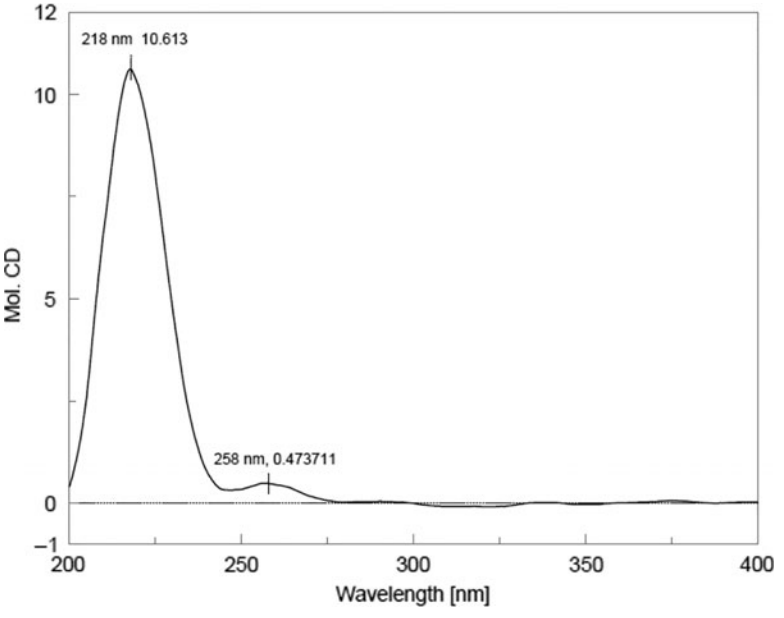


Figure 3. CD spectra of **1.**

conjunction with the DEPT and HSQC experiments, showed up a monosubstituted benzene ring by the resonances at δ_H/δ_C 7.26 (1 H, t, $J = 7.5$ Hz, H-4′)/127.4 (d, C-4′), 7.32 (2 H, t, $J = 7.5$ Hz, H-3′, 5′)/127.9 (d, C-3′, 5′), 7.38 (2 H, d, $J = 7.5$ Hz, H-2′, 6′)/126.9 (d, C-2′, 6′), and δ_C 141.4 (s, C-1′). An aliphatic ABX coupling system was also determined by a set of resonances at δ_H/δ_C 2.66 (1 H, dd, $J = 16.0$, 7.5 Hz, H-2a) and 2.93 (1 H, dd, $J = 16.0$, 7.5 Hz, H-2b)/41.2 (t, C-2) and 5.06 (1 H, t, $J = 7.5$ Hz, H-3)/76.2 (d, C-3) (Table 1). One hexopyranse moiety was detected by the resonances for one anomeric oxymethine at δ_H/δ_C 4.34 (1 H, d, $J = 8.0$ Hz, H-1″)/101.6 (d, C-1″), one oxymethylene at δ_H/δ_C 3.60 (1 H, dd, $J = 12.5$, 4.5 Hz, H-6″a) and 3.36–3.40 (1 H, m, H-6″b)/61.0 (t, C-6″), and four other oxymethines at δ_H 2.94–3.15 (4 H, m, H-2″, 3″, 4″, 5″) as multiplets and at δ_C 73.7 (d, C-2″), 76.6 (d, C-3″), 70.0 (d, C-4″), and 76.9 (d, C-5″). Acid hydrolysis of **2** afforded D-glucose, which was affirmed by derivatization and GC analysis, as described in section "*3.5*″ [16]. In addition, the ^{13}C NMR signal at δ_C 172.4 (s, C-1) matched the determination of carboxylic acid. With the elucidation of the structural moieties accomplished, the 2D structure of **2** was established as 3-O-β-D-glucopyranosyl-3-phenylpropanoic acid by the HMBC experiment which exhibited the correlations from the oxymethine at δ_H 5.06 (H-3) to C-1, C-2, C-1′, C-2′/6′, and C-1″, from H-1″ to C-3, from H-2a and H-2b to C-1, C-3, and C-1′, and the like (Figure 2). Now that the NMR technique, as well as acid hydrolysis, had served its intended purpose, we sought to address the issue of elucidating the absolute configuration of C-3. Calculation of electronic circular dichroism (ECD) using time-dependent density functional theory (TDDFT) [17], in conjunction with the experimental ECD data, has been successfully applied to determine the absolute configuration of compounds in recent years. In the case of **2**, the calculated ECD value for the 3*R* configuration is log $\Delta\varepsilon + 8.13$ at 212.5 nm, a value closely comparable to the log $\Delta\varepsilon + 2.68$ at 212.5 nm of experimental ECD value, as

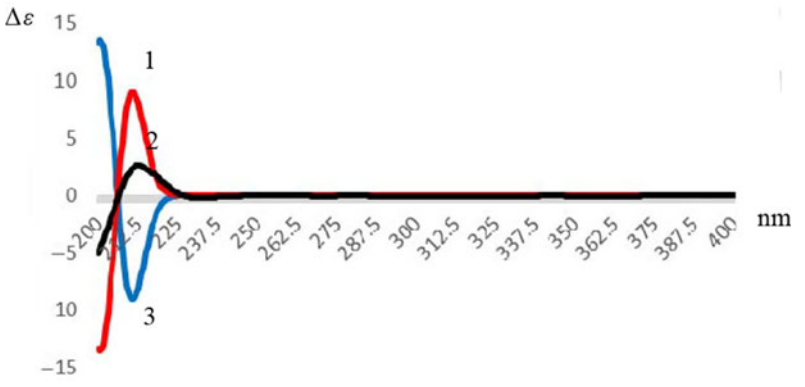


Figure 4. Calculated and experimental ECD spectra of **2**((1) calculated in methanol; (2) experimental in methanol; (3) calculated in methanol for the *S* configuration).

shown in Figure 4. Thus, the structure of **2** was characterized as (3*R*)-3-O-*β*-D-glucopyranosyl-3-phenylpropanoic acid.

3. Experimental

3.1 General experimental procedures

Optical rotations were measured on a Perkin − Elmer 241 digital polarimeter (JASCO, Tokyo, Japan) at a temperature of 20°C. UV spectra were obtained on a JASCO V-650 spectrophotometer (JASCO, Tokyo, Japan), and IR (KBr) spectra on a Nicolet 5700 infrared spectrometer (Thermo Nicolet, Waltham, MA, USA). CD spectra were taken using a JASCO J-815 CD spectrometer (JASCO, Tokyo, Japan). 1D and 2D NMR spectra were recorded on a Bruker Avance-III 500 NMR (Bruker Instruments Inc., Fällanden, Switzerland) spectrometer, using dimethyl sulfoxide-d_6 (DMSO-d_6) as solvents and tetramethylsilane (TMS) as internal standard. Both ESIMS and HRESIMS experiments in negative or positive modes were performed using an Agilent 1100 series LC/MSD Trap SL mass spectrometer (Agilent, Santa Clara, CA, USA). Preparative HPLC was carried out on a Shimadzu LC-6AD system (Shimadzu, Kyoto, Japan) equipped with a SPD-10A detector, and a reversed-phase C_{18} column (YMC-Pack ODS-A 20 × 250 mm, 5 μm, YMC CO., Kyoto, Japan) was employed. GC analysis was undertaken on an Agilent 7890A instrument (Agilent, Santa Clara, CA, USA). CC was conducted over silica gel (200−300 mesh, Qingdao Marine Chemical Inc. Qingdao, China) or Sephadex LH-20 (Pharmacia Fine Chemicals, Uppsala, Sweden), and thin layer chromatography (TLC) over glass plate precoated silica gel GF_{254}. Spots were visualized under UV light of 254 nm and 360 nm, simultaneously, and by spraying with 10% H_2SO_4 in 95% EtOH, followed by heating. All solvents were of analytical grade. L-Cysteine methyl ester hydrochloride, *N*-trimethylsilylimidazole, and authentic D-glucose were purchased from Sigma-Aldrich Chemical Co. (St Louis, MO, USA).

3.2 Plant material

The stems of *Ephedra sinica* were obtained from Bozhou crude drug market, Anhui Province, China, in December 2011, and was identified by Associate Prof. Lin Ma (a savant in plant systematics with Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College) through careful examination of the morphological features

and according to the description for *E. sinica* in the state Pharmacopoeia of China [1]. All the experimental material was carefully examined to ensure the purity and a voucher specimen (ID-S-2555) was deposited in the Herbarium of Institute of Materia Medica, Chinese Academy of Medical Sciences, Beijing, China.

3.3 Extraction and isolation

Dried and cut stems of *E. sinica* (19 kg) were extracted for three times with 85% aqueous (aq.) EtOH (2 h, 1 h, 1 h) under reflux condition. After evaporation of the aq. EtOH solvent under vacuum, a dark-brown residue of 3.0 kg was obtained. The residue was suspended in 80% EtOH in water (8 L) and extracted in a separatory funnel with petroleum ether (8 L each) for several times until the upper fraction was very transparent. The 80% aq. ethanol-soluble section was evaporated under vacuum condition to afford another dark-brown residue, which was re-dissolved in water (7 L), and then extracted in a separatory funnel with EtOAc for three times (7 L each). Next, the water layer was further extracted in a separatory funnel with *n*-BuOH (7 L each) for three times. The combined *n*-BuOH solution was extracted in a separatory funnel with the solution of 5% $NaHCO_3$ in water for three times (3 × 1.5 L) and then pure H_2O (2 × 1 L), respectively, to pH = 7.0. The neutral *n*-BuOH solution was evaporated under vacuum condition to afford the corresponding brown residue (220 g) of *n*-BuOH extract.

The *n*-BuOH extract was separated, by CC over silica gel using a gradient of $CHCl_3$/MeOH (100:1–1:1) as eluent, into seven fractions (designated as F1 to F7) according to their TLC profiles. F3 (21 g, $CHCl_3$/MeOH = 10:1) was subjected to silica gel CC and eluted with a gradient of $CHCl_3$/MeOH (50:1 to 20:1), yielding seven subfractions (F3-1 to F3-7). F3-2 (5 g, $CHCl_3$/MeOH = 50:1) was separated by CC over silica gel using an isocratic elution of $CHCl_3$/MeOH = 50:1 into three subfractions (F3-2-1 to F3-2-3) according to their TLC profiles. F3-2-2 (3 g) was separated into three subfractions (F3-2-2-1 to F3-2-2-3) by silica gel CC using an isocratic elution of EtOAc/Me_2CO = 15:1 and TLC detection. F3-2-2-2 (100 mg) was purified by preparative RP-HPLC [mobile phase: CH_3OH/H_2O (23%, v/v); flow rate: 6 ml min^{-1}; UV detection at 280 nm] to afford **1** (2 mg). F3-7 (1 g, $CHCl_3$/MeOH = 20:1) was submitted to Sephadex LH-20 CC with MeOH as eluent to yield four subfractions (F3-7-1 to F3-7-4) according to their TLC profiles. **2** (10 mg) was obtained from F3-7-2 by using preparative RP-HPLC [mobile phase: 25% MeOH in H_2O, containing 0.1% formic acid; flow rate: 6 mL min^{-1}; UV detection at 280 nm].

3.3.1 (S)-N-((1R,2S)-1-Hydroxy-1-phenylpropan-2-yl)-5-oxopyrrolidine-2-carboxamide (1)

$[\alpha]_D^{20}$ − 29.6 (*c* 0.61, MeOH); CD (MeOH) $\Delta\varepsilon_{218\,nm}$ + 5.03, $\Delta\varepsilon_{258\,nm}$ + 3.68; UV (MeOH) λ_{max} (log ε) 204.8 (4.31), 229.2 (3.09), and 274.8 (2.13) nm; IR (KBr) ν_{max}3309, 3091, 2978, 1662, 1639, 1554, 1452, 1250, 1124, 1084, 1054, 968, 909, 760, and 700 cm^{-1}; ^1H and ^{13}C NMR spectral data see Table 1; ESIMS (negative-ion mode): *m/z* 261.5 $[M - H]^-$; HRESIMS (positive-ion mode): *m/z* 263.1392 $[M + H]^+$ (calcd for $C_{14}H_{19}N_2O_3$, 263.1390).

3.3.2 (3R)-3-O-β-D-Glucopyranosyl-3-phenylpropanoic acid (2)

$[\alpha]_D^{20}$ − 19.4 (*c* 0.112, MeOH); CD (MeOH) $\Delta\varepsilon_{212.5\,nm}$ + 2.68; UV (MeOH) λ_{max} (log ε) 206.6 (3.75) and 258.8 (2.46) nm; IR (KBr) ν_{max} 3452, 3400–2400, 2951, 2921, 2877, 2665, 2598, 1705, 1636, 1452, 1277, 1208, 1086, 1018, 905,

754, and 701 cm^{-1}; ^{1}H and ^{13}C NMR spectral data see Table 1; ESIMS (negative-ion mode): *m/z* 327.1 $[M - H]^-$; HRESIMS (negative-ion mode): *m/z* 327.1087 $[M - H]^-$ (calcd for $C_{15}H_{19}O_8$ 327.1085).

3.4 Calculation procedure for CD spectrum of 2

The elucidation of the absolute configuration of **2** was carried out via Monte Carlo searching in the MMFF94 molecular mechanics force field using the SPARTAN 08 [18]. Geometry re-optimizations at B3LYP/6-31G(d) in vacuo, with the PCM solvent model for MeOH followed by TDDFT calculations using various functionals (B3LYP, BH&HLYP, PBE0) and the TZVP basis set were performed by the Gaussian 09 [19] package. Boltzmann distributions were estimated from the ZPVE-corrected B3LYP/6-31G(d) energies. ECD curves were obtained on the basis of rotator strengths with a half-band of 0.25 eV using SpecDis v1.61 [20]. The Microsoft Excel software was used for visualization of the results.

3.5 Acid hydrolysis of 2 and determination of the absolute configuration of sugar

The acid hydrolysis of **2** and the determination of the absolute configuration of sugar were performed using reported method [16]. About 2 mg of **2** was dissolved in 2 M HCl (2 ml) and heated at 85°C for 10 h in oil bath. The mixture was concentrated under reduced pressure to dryness. The resulting residue was suspended in H_2O and extracted in a separatory funnel with $CHCl_3$ for three times. The aqueous layer was evaporated under vacuum, then diluted with H_2O, and then re-evaporated under vacuum, the re-dilution and re-evaporation procedure being repeated for several times, to produce a neutral residue. The residue was dissolved in anhydrous pyridine (1 ml). L-Cysteine methyl ester hydrochloride (2 mg) was then added, and the reaction mixture was incubated at 60°C for 2 h. After the mixture was concentrated to dryness under reduced pressure, 0.2 ml of *N*-trimethylsilylimidazole was added, and the mixture was further incubated at 60°C for 2 h. Then H_2O (2 ml) was added, and the solution was extracted with hexane for three times (2 ml each). The hexane extract was subjected to GC system under the following conditions to monitor: capillary column: HP-5 (30 m × 0.25 mm, with a 0.25 μm film, Dikma); detection: FID; detector temperature: 280°C; injection temperature: 260°C; initial temperature 16°C, which was raised to 280°C at the rate of 5°C/min and the final temperature was maintained for 10 min; Carrier: N_2 gas. The authentic D-glucose was also treated by the same procedure as that for **2**. From the acidic hydrolysate of **2**, D-glucose was confirmed by comparing the retention time of the derivative with that of authentic sugar, with the retention time of D-glucose being 27.96 min.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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