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Gentisides A and B, two new neuritogenic compounds from the traditional Chinese medicine *Gentiana rigescens* Franch

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ABSTRACT

Two new alkyl 2,3-dihydroxybenzoates, gentisides A and B, were isolated from the traditional Chinese medicine *Gentiana rigescens* Franch. Their structures and stereochemistry were elucidated by spectroscopic methods and chemical derivatization. These compounds showed a significant neuritogenic activity at 30 μM against PC12 cells that was comparable to that seen for the best nerve growth factor (NGF) concentration of 40 ng/mL. Gentisides A and B showed parallel activity, indicating that the observed structural difference at the end of their alkyl chain did not affect neuritogenic activity.

1. Introduction

Neurotrophic factors such as nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), and glial cell line derived neurotrophic factor (GDNF) play critical roles in neuronal development, survival, and the functional maintenance of neurons.[1–3] Neurotrophic factors have been targeted as potential therapeutic drugs for treatment of neurodegenerative disorders. At this time, NGF has been the most intensively studied and has been subjected to animal experiments.[4] However, because it is a high molecular weight polypeptide, NGF is unstable and is also unable to cross the blood–brain barrier.[5] Therefore, its application as a medicine for the treatment of neurodegenerative disorders is assumed to be difficult. To address this issue, considerable efforts have been made to find small molecules that have neurotrophic properties similar to those of NGF and/or that can enhance the activity of endogenous neurotrophic factors.[6]

PC12 cells, a line derived from a rat pheochromocytoma, respond to NGF by switching from an immature chromaffin-cell-like phenotype to a sympathetic-neuron-like one, complete with the outgrowth of long branching neurites. This cell line is therefore useful as a bioassay system for the investigation of potential drugs for neurodegenerative diseases.[7] Our previous search for compounds that mimic and/or enhance the neuritogenic activity of NGF using the PC12 cell line system resulted in the isolation of novel cerebrosides, the termitomycesphins, from the edible Chinese mushroom *Termitomyces albuminosus*[8,9] and a series of steroid glycosides named linckosides from the Okinawan blue starfish *Linckia laevigata*.[10] In our present study, gentisides A (**1**) and B (**2**) (Fig. 1), two new neuritogenic compounds, were isolated from a methanol extract of the traditional Chinese medicine *Gentiana rigescens* Franch. In this paper, we report the isolation, structures, and biological activity of these substances.

2. Results and discussion

2.1. Isolation

A methanol extract of dried roots of *G. rigescens* was partitioned between 80% aqueous MeOH and *n*-hexane. The active *n*-hexane

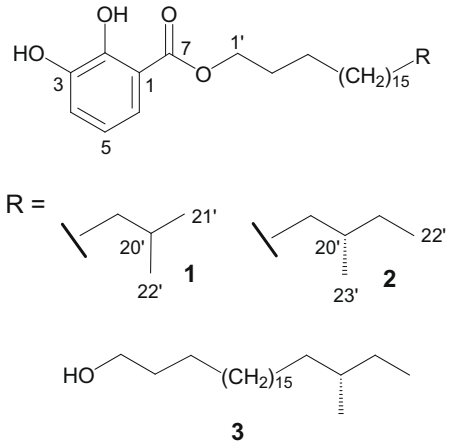

Figure 1. Structures of gentisides A (**1**) and B (**2**), and 20*S*-methyl docosanol (**3**).

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fraction was chromatographed on silica gel, then on ODS to yield an active fraction. This was further purified by repeated reversed phase HPLC to yield gentisides A (**1**, 0.00075% of dry wt.) and B (**2**, 0.0012%).

2.2. Structure elucidation

Gentiside A (**1**) was obtained as a colorless powder with the molecular formula $C_{29}H_{50}O_4$ as determined by HR FT-ICR MS measurement. The IR absorption band at 3469 cm^{-1} suggested the presence of a hydroxyl group. The proton nuclear magnetic resonance (^{1}H NMR) spectrum indicated the presence of two methyl (CH_3) groups (δ_H 0.86), many aliphatic methylene (CH_2) and/or methine (CH) groups (δ_H 1.14–1.77), a benzene ring (δ_H 6.80–7.38), one oxygen functionality (CH_n–O, δ_H 4.34), and two singlet hydroxyl groups (δ_H 5.65 and 11.00). The sharp signal of a hydroxyl group at δ_H 11.00 is due to the formation of hydrogen-bond with a carboxyl group. The signals at δ_H 0.86 (d) and many aliphatic methylenes δ_H 1.14–1.77, δ_C 26.0–39.1 suggested is the presence of a long alkyl in this molecule. Two fully overlapped methyl doubleted signals (δ_H 0.86) revealed the presence of an isopropyl group at the end of the long alkyl chain. The signal at δ_C 170.5 supported the presence of one carboxyl group. The ^{13}C and DEPT NMR data combined with the 2D NMR as well as HR-MS data, confirmed the presence of two CH_3, eighteen CH_2, one CH, one CH_2–O, one benzene ring with three substituted positions, and one carboxyl group. Taking the molecular formula into consideration, two of the three substituted positions of benzene ring turned out to be two hydroxyl groups. The analysis of COSY and HOHAHA spectra led to the determination of the partial structures (C-4 to C-6, C-1′ to C-3′, C-19′ to C-21′ and 22′, and a long alkyl chain) depicted with the bold bonds in Figure 2. These partial structures were connected by the long-range H–C correlations obtained by an HMBC experiment to give a gross structure of **1**. The HMBC correlations establishing the gross structure of **1** were as follows: a proton of hydroxyl group at C-2 to C-1, C-2, and C-3; a proton of hydroxyl group at C-3 to C-2, C-3, and C-4; H-6 to C-7; H-1′ to C-7. Important HMBC correlations are summarized in Figure 2 with arrows. Thus, the structure of **1** was elucidated as shown in Figure 1.

Gentiside B (**2**) was obtained as a colorless powder with the molecular formula $C_{30}H_{52}O_4$ as determined by an HR FT-ICR MS measurement. The ^{1}H and ^{13}C NMR of **2** were superimposable on those of **1** (Table 1) except for the signals at the end of the long alkyl chain. The **2** possesses an isobutyl group at C-19′, as determined by analysis of 2D NMR. Subsequently, **2** was hydrolyzed under weakly basic conditions to afford a 20*S*-methyl docosanol (**3**) molecule, which was confirmed by the comparison of ^{1}H NMR, MS as well as specific rotation, with those reported.[11,12] The structure of **2** was thus determined as shown in Figure 1.

2.3. Biological activity

The neuritogenic activity of gentisides A (**1**) and B (**2**) was evaluated using PC12 cells and compared to the activity of 40 ng/mL NGF. Figure 3 shows the dose–dependent increase in the percentage of PC12 cells with long neurite outgrowths. The gentiside concentrations were varied from 1 to 30 μM, which corresponded to the minimum concentration with detectable activity and the maximum concentration without cytotoxicity observed 48 h after treatment. The gentisides showed a dose-dependent increasing activity ranging across the test range. At the best concentration without obvious cytotoxicity, gentisides exhibited neurite outgrowth equivalent to that seen with the positive NGF control at its best concentration (40 ng/mL). The solvent control also induced few neurite outgrowths. Gentisides A (**1**) and B (**2**), differing only in the isopropyl group and isobutyl group at the end of side chain, respectively, showed significant neuritogenic activities of 82% (Fig. 3a), and 87% (Fig. 3b) at a concentration of 30 μM on day 2 after the treatment. Moreover, gentisides A (**1**) and B (**2**) showed quite similar activity at all concentrations tested. At the concentration of 100 μM, although cytotoxicity was clearly observed, almost all of the living cells possessed long neurite outgrowths in the presence of either compound. Thus, the small structural difference at the end of the alkyl chain did not affect the biological activity of the gentisides on PC12 cells.

Subsequently, possible synergistic effects of gentisides were examined on the neurite outgrowth of PC12 cells induced by low concentration of NGF (1 ng/mL). No significant synergistic activity was observed, which is probably due to the high activity of gentisides alone (data not shown).

Interestingly, 2,3-dihydroxybenzoic acid obtained from alkaline hydrolysis of gentiside B did not induce obvious neurite outgrowth in PC12 cells (data not shown).

Figure 4 shows the morphological changes of PC12 cells after treatment with gentisides A (**1**, Fig. 4c) and B (**2**, Fig. 4d) in comparison with the solvent control (1% DMSO, Fig. 4a) and the positive NGF control (40 ng/mL, Fig. 4b). When treated with gentisides at 30 μM, the cells extended long multipolar and bipolar neurite out-

Table 1
^{1}H and ^{13}C NMR data for gentisides A (**1**) and B (**2**) in $CDCl_3$

Carbon No.	1		2	
	^{1}H[a]	^{13}C[b]	^{1}H[a]	^{13}C[b]
1	—	112.7	—	112.7
2	—	148.9	—	149.0
3	—	145.1	—	145.1
4	7.10 dd (8.0, 1.5)	119.7	7.10 dd (8.0, 1.5)	119.7
5	6.80 t (8.0)	119.1	6.80 t (8.0)	119.1
6	7.38 dd (8.0, 1.5)	120.6	7.37 dd (8.0, 1.5)	120.6
7	—	170.5	—	170.5
1′	4.34 t (6.5)	65.8	4.34 t (6.5)	65.8
2′	1.77 m	28.6	1.78 m	28.6
3′	1.43 m	26.0	1.44 m	26.0
4′-18′	1.22–1.38 m	27.4–30.0[c]	1.10–1.38 m	27.1–30.1[d]
19′	1.14 m	39.1	1.10–1.38 m	27.1–30.1[d]
20′	1.51 m	28.0	1.33 m	34.5
21′	0.86 d (7.0)	22.7	1.09, 1.30 m	36.7
22′	0.86 d (7.0)	22.7	0.85 t (7.0)	11.4
23′	—	—	0.84 d (6.0)	19.3
2-OH	11.00 s	—	11.00 s	—
3-OH	5.65 s	—	5.68 s	—

[a] 500 MHz, coupling constants (*J* in Hz) are in parentheses.
[b] 125 MHz.
[c] δ: 27.4, 29.2, 29.5, 29.6, 29.7, 30.0.
[d] δ: 27.1, 29.2, 29.5, 29.6, 29.7, 30.1.

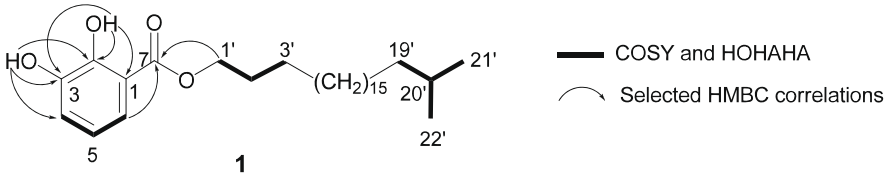

Figure 2. Gross structure of gentiside A (**1**) with selected HMBC correlations.

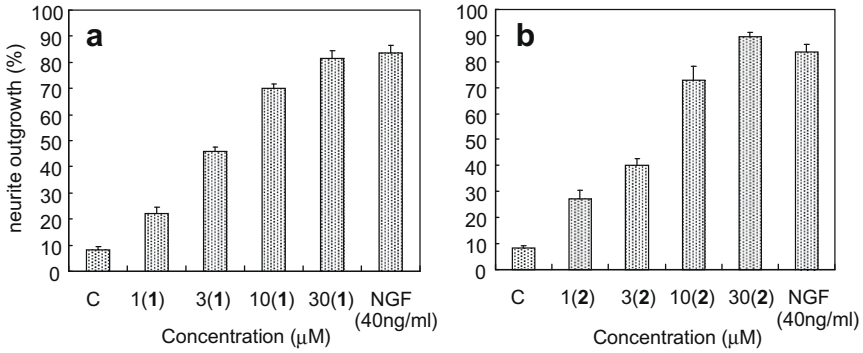

Figure 3. Dose-dependent response of the NGF-mimicking activity of gentisides A (**1**, a) and B (**2**, b) 48 h after treatment. Activity is represented by percentages of PC12 cells with neurite outgrowths longer than the diameter of the cell body. C: control (1% DMSO); NGF (40 ng/mL): positive control.

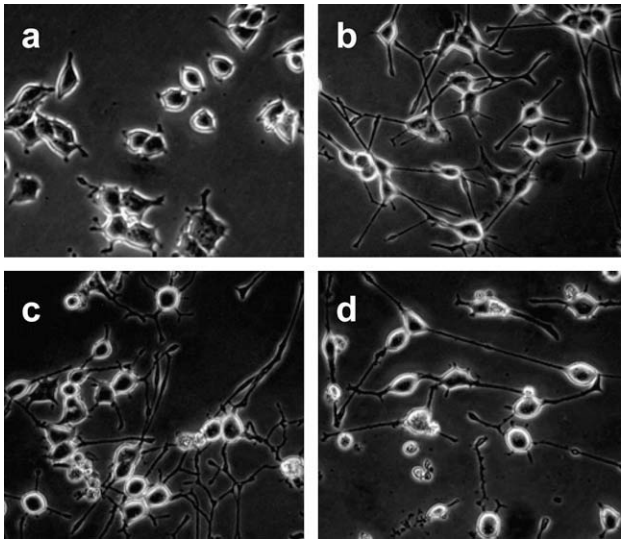

Figure 4. Photomicrographs of PC12 cells under a phase-contrast microscope 48 h after treatment. (a) Solvent control (1% DMSO), (b) NGF (40 ng/mL), (c) gentiside A (**1**, 30 μM), and (d) gentiside B (**2**, 30 μM).

growths 48 h after treatment, which were similar to those produced following treatment with NGF at 40 ng/mL. Control cells cultured without added compounds induced few short neurite outgrowths.

3. Experimental

3.1. General procedures

Preparative HPLC was performed using ELITE P-230 pumps, and the optical rotations were measured on a JASCO P-1030 digital polarimeter. IR spectra were recorded on a JASCO FT/IR-4100 and the UV spectra were recorded on a PGENERAL TU-1901. High-resolution (HR) MS measurements were performed on a Apex III (7.0 Tesla) FT-ICR mass spectrometer (Bruker, Billerica, MA, USA) using CF_3COONa as an external calibration standard. The NMR spectra were recorded on a Bruker AV III-500 spectrometer, and the NMR chemical shifts in δ (ppm) were referenced to the solvent peaks of δ_C 77.0 and δ_H 7.26 for $CDCl_3$.

3.2. Extraction and isolation

A commercial preparation of dried roots of *G. rigescens* (1980 g, dried wt.) was purchased from HuQingYuTang in Hanzhou, Zhejiang province, China. The sample was powdered and soaked in MeOH (10 L) for five days at room temperature with occasional stirring. The supernatant was separated by filtration and concentrated to give 353.6 g of a crude extract, which was divided into two portions and subjected to solvent partition. In a typical case, a portion (176.8 g) of extract was dissolved in 80% aqueous MeOH (1.5 L) and then washed three times with *n*-hexane (500 mL for each). The *n*-hexane fraction from the two portions were combined and concentrated to give a light green viscous oil (14.6 g), which was chromatographed on silica gel (200–300 mesh, Yantai Chemical Industry Research Institute) and eluted with a *n*-hexane/EtOAc step gradient (100:0, 90:10, 85:15, 80:20, 70:30, 50:50) to afford 24 fractions. The active sample, eluted with *n*-hexane/EtOAc (90:10, 85:15), was separated through ODS (Cosmosil 75 C18-OPN, Nacalai Tesque) and eluted with a MeOH/H_2O step gradient (80:20, 85:15, 90:10, 95:5, 100:0), and MeOH/$CHCl_3$ (1:1), successively, to afford 42 fractions. The active sample (203.4 mg) eluted with MeOH/H_2O (90:10, 95:5) was subjected to HPLC [Develosil ODS-HG-5 (φ 10/250 mm), Nomura chemical, flow rate: 3 mL/min, MeOH/H_2O (98:2)] to give two active fractions. The active fraction with shorter retention time was purified by HPLC [Develosil ODS-HG-5 (φ 10/250 mm), Nomura chemical, flow rate: 3 mL/min, 85–100% aqueous EtOH in 90 min linear gradient] to give pure gentiside A (**1**) (14.8 mg, t_R = 43.0 min). Another active fraction was also purified by HPLC [Develosil ODS-HG-5 (φ 10/250 mm), Nomura chemical, flow rate: 3 mL/min, 90–100% aqueous EtOH in 90 min linear gradient] to yield gentiside B (**2**) (24.2 mg, t_R = 31.9 min) as the active component.

3.2.1. Gentiside A (1)

Colorless powder, IR (KBr): 3469, 2920, 1671, 1469, 1311, 1155, 1071 cm^{-1}; UV (MeCN) λ_{max} (log ε): 314 nm (5.82); HR FT-ICR MS *m*/*z* 485.3610 $(M+Na)^+$, calcd for $C_{29}H_{50}O_4Na$ 485.3601; for ^1H and ^{13}C NMR see Table 1.

3.2.2. Gentiside B (2)

Colorless powder, $[\alpha]_D^{22}$ +2.4 (*c* 2.04, $CHCl_3$); IR (KBr): 3476, 2920, 1674, 1468, 1309, 1153, 1073 cm^{-1}; UV (MeCN) λ_{max} (log ε): 321 nm (6.01); HR FT-ICR MS *m*/*z* 499.3775 $(M+Na)^+$, calcd for $C_{30}H_{52}O_4Na$ 499.3758; for ^1H and ^{13}C NMR see Table 1.

3.3. Degradation of 2

Gentiside B (**2**) (8 mg) in methanol (4 mL) was treated with potassium carbonate (20 mg) at room temperature for 27 h. The crude product was dissolved in H_2O (10 mL) and extracted three times with *n*-hexane (10 mL). The *n*-hexane layers were combined, concentrated, and purified by silica gel open column eluted with $CHCl_3$ to afford the **3** (4.4 mg): colorless powder, $[\alpha]_D^{22}$ +3.7 (*c*

2.04, $CHCl_3$) [lit.[11] $[\alpha]_D^{23}$ +4.0], ^1H NMR ($CDCl_3$, 500 MHz) δ: 3.64 (2H, t, J = 6.5 Hz), 1.57 (2H, m), 1.20–1.38 (35H, m), 1.07–1.14 (2H, m), 0.85 (3H, t, J = 7.0 Hz), 0.84 (3H, d, J = 6.0 Hz).[12]

3.4. Bioassay methods

Biological activity was evaluated according to the methods described in our previous paper.[8,9] Briefly, 20,000 of PC12 cells in DMEM medium (1 mL) were placed in each well of a 24-well microplate and precultured under a humidified atmosphere of 5% CO_2 at 37 °C. Twenty-four hours later, the medium was replaced by 1 mL of serum-free DMEM medium containing a test sample and DMSO (1%). In the case for evaluation of the NGF-enhancement effects, the medium was replaced by 1 mL of serum-free DMEM medium containing 1.0 ng of NGF (Recombinant Human β-NGF, SIGMA) in addition to a test sample. The morphological changes of the cells were monitored under a phase-contrast microscope at every 24 h. About one hundred cells were counted from a randomly chosen sight and this was repeated three times.

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