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THREE NEW NERVOGENIC ACID DERIVATIVES FROM *LIPARIS NERVOSA*

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Abstract – In the present study, three new nervogenic acid derivatives (**1**–**3**) were isolated from the whole plant of *Liparis nervosa* (Thunb. ex A. Murray). Their structures were elucidated by extensive spectroscopic analyses including 1D, 2D NMR and HR-ESI-MS. The cytotoxicity of all the compounds was investigated against HCT116 human cancer cell line.

Liparis nervosa (Thunb. ex A. Murray) Lindl., a member of the Orchidaceae family, is an herbaceous plant widely distributed in China.[1] *L. nervosa* exhibits various biological effects on inflammation, detoxication and hemostatic. Previous chemical investigations of *L. nervosa* led to the isolation of a series of nervogenic acid derivatives and pyrrolizidine alkaloids.[2] Pharmacological studies demonstrated that those nervogenic acid derivatives and pyrrolizidine alkaloids were the effective components of *L. nervosa*. Chloride-(N-chloromethylnervosine VII) and nervosine VII were found to induce apoptosis and autophagy in HCT116 human colorectal cancer cells by activation the ERK1/2, JNK and p38 pathway, and suppressing the p53 signaling pathway.[3] Nervosine V has been reported[4] to exert the inhibition of LPS-induced nitric oxide (NO) production in RAW264.7 macrophages with IC_{50} value 2.16 μmol·L^{-1}. To find more biologically active substances, our continued studies on the whole plant extract of *L. nervosa* have resulted in the isolation and structure determination of three new nervogenic acid derivatives (**1**–**3**) (Figure 1). Herein, we report the isolation and structural elucidation of these compounds, as well as their cytotoxicity against HCT116 cells.

Compound **1** was obtained as an amorphous solid. It had a molecular formula of $C_{23}H_{32}O_8$ from the quasi-molecular ion peak in positive HR-ESI-MS at *m/z* 459.2093 $[M + Na]^+$ (Calcd. for $C_{23}H_{32}O_8Na$, 459.1995). Resonance in ^1H NMR spectrum (Table 1) for an anomeric proton at δ_H 4.65 (1H, d, J = 7.2

Hz), which gave correlations in the HMQC spectrum (Figure 2), with the anomeric carbon signal at δ_C 107.1. After acid hydrolysis of **1**, GC analysis of 1-(trimethylsilyl)imidazole derivatives showed that the arabinose was determined to be L-configuration. According to the coupling constant of the anomeric hydrogens and GC analysis, it was confirmed that there is a α-L-arabinopyranosyl unit in compound **1**. The signals at δ_H 8.09 (1H, d, J = 1.8 Hz) and δ_H 7.78 (1H, d, J = 1.8 Hz) in ^{1}H NMR and δ_C 128.2 (s), 127.0 (d), 131.2 (d), 131.9 (s), 138.2 (s), 157.3 (s) in ^{13}C NMR revealed the presence of a tetrasubstituted aromatic ring.[4] In addition, signals of a prenyl group [δ_C 18.0 (q), 25.9 (q), 123.7 (d), 29.7 (t), 134.0 (s)], and a carboxyl group resonance at δ_C 169.8, were also readily distinguished.

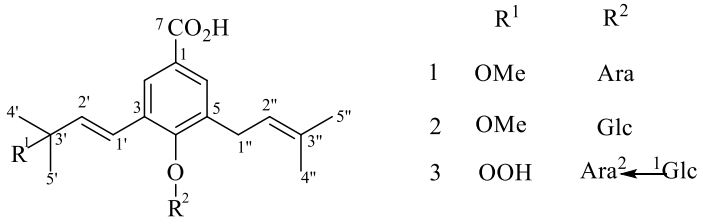

Figure 1. Structures of compounds **1**–**3**

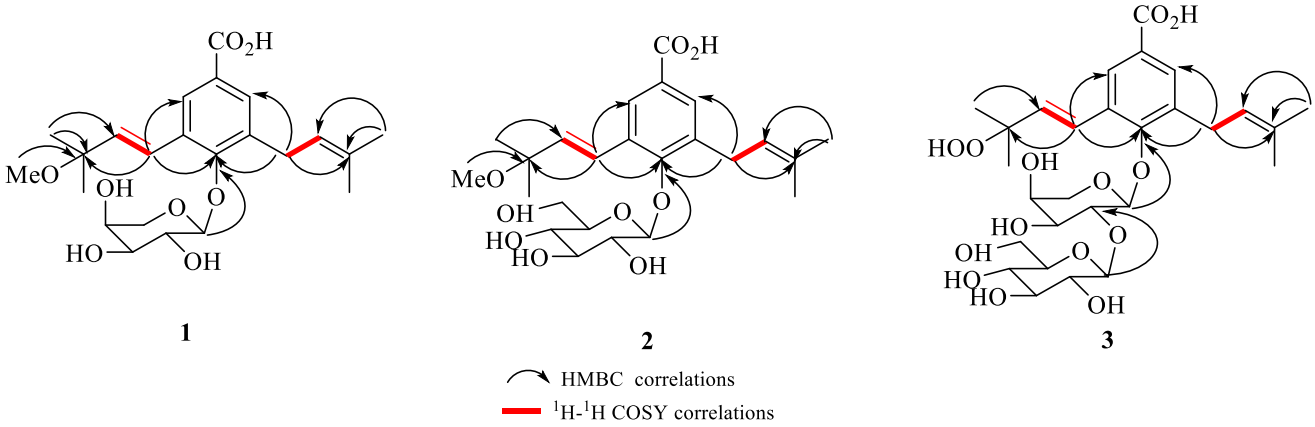

Figure 2. Key HMBC and ^{1}H-^{1}H COSY correlations for compounds **1**–**3**

Comparison of the spectral data of **1** (Table 1) with that of nervogenic acid derivatives,[1] previously isolated from this plant, indicated that they were similar in structure, with the main difference being that one isoprene chain was substituted by a methoxy group and consisting in a 3-methoxy-3-methyl-butenyl moiety based on the following analyses. The two methyl groups of the prenyl moieties were shifted highfield, indicating that the double bond was shifted from the 2,3-position to the 1,2-position. Moreover, the ^{13}C NMR spectral data showed the presence of an oxygenated tertiary carbon at δ_C 77.1 (C-3′) and the lack of an olefinic quaternary carbon, indicated that there was an additional methoxy connected to C-3′, which was confirmed by HMBC correlation from the methoxy group (δ_H 3.29) to C-3′ (δ_C 77.1). The signals at δ_H 7.26 (1H, d, J = 16.8 Hz) and δ_H 6.25 (1H, d, J = 16.8 Hz) in the ^{1}H NMR spectrum indicated the presence of a *trans*-double bond. Moreover, the 3-methoxy-3-methyl-1-butenyl moiety was

confirmed by the presence of signals in the ^{13}C NMR spectrum at δ_C 26.0 (q), 26.4 (q), 77.1(s), 126.6 (d),

Table 1. ^{1}H NMR data [a] and ^{13}C NMR data [b] of compounds **1**–**3**

No.	**1** [c]		**2** [c]		**3** [c]	
	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C
1	—	128.2 s	—	128.4 s	—	127.2 s
2	8.09 d (1.8)	127.0 d	8.09 d (1.8)	127.0 d	8.04 s	127.2d
3	—	131.9 s	—	131.8 s	—	131.7 s
4	—	157.3 s	—	157.1 s	—	156.5 s
5	—	138.2 s	—	138.2 s	—	137.7 s
6	7.78 d (1.8)	131.2 d	7.81 d (1.8)	131.3d	7.73 s	131.1 d
7	—	169.8 s	—	169.9 s	—	171.2 s
1′	7.26 d (16.8)	126.6 d	7.28 d (16.8)	126.5 d	7.27 d (16.2)	126.5 d
2′	6.25 d (16.8)	137.3 d	6.27 d (16.8)	137.7 d	6.35 d (16.8)	137.1d
3′	—	77.1 s	—	78.3 s	—	83.4 s
4′	1.47 s	26.0 q	1.48 s	25.9 q	1.48 s	25.1 q
5′	1.48 s	26.4 q	1.49 s	26.5 q	1.44 s	25.4 q
3′-OMe	3.29 s	51.0 q	3.31 s	51.0 q	—	—
1″	3.68 d (7.2) 3.54 d (7.2)	29.7 t	3.70 d (7.2) 3.55 d (7.2)	29.8 t	3.59 d (7.2) 3.48 d (7.2)	29.8 t
2″	5.36 t (7.2)	123.7 d	5.39 t (7.2)	123.8 d	5.31 t (7.2)	123.9 d
3″	—	134.0 s	—	133.9 s	—	133.7 s
4″	1.80 s	18.0 q	1.81 s	18.1 q	1.74 s	18.1 q
5″	1.82 s	25.9 q	1.83 s	25.8 q	1.75 s	26.0 q
	Ara		Glc		Ara	
1‴	4.65 d (7.2)	107.1 d	4.79 d (7.2)	106.2 d	4.71 overlapped	106.2 d
2‴	3.98 dd (7.2, 9.6)	72.9 d	3.63 dd (7.2, 9.6)	75.6 d	4.12 dd (7.2, 9.0)	82.0 d
3‴	3.59 dd (3,6, 9.6)	74.5 d	3.44 dd (3,6, 9.6)	78.1 d	3.77 overlapped	74.2 d
4‴	3.86 m	69.8 d	3.45 m	71.6 d	3.85 m	69.4 d
5‴	3.90 dd (2.4, 12.6) 3.43 dd (1.2, 12.6)	67.9 t	3.18 m	78.3 d	3.39 overlapped 3.85 overlapped	67.3 t
6‴	—	—	3.83 dd (2.4, 12.6) 3.71 dd (1.2, 12.6)	62.8 t	—	—
					Glc	
1⁗	—	—	—	—	4.72 d (7.8)	104.8 d
2⁗	—	—	—	—	3.39 m	76.0 d
3⁗	—	—	—	—	3.30 m	78.1 d
4⁗	—	—	—	—	3.31 m	71.6 d
5⁗	—	—	—	—	3.40 m	78.1 d
6⁗	—	—	—	—	3.77 overlapped 3.65 dd (5.4, 12)	62.9 t

[a] ^{1}H NMR (600 MHz, δ, *J* in Hz).

[b] ^{13}C NMR (600 MHz, δ).

[c] Data in CD_3OD.

137.3 (d), 51.0 (q). In the HMBC experiment, correlations between the resonance of H-2′/C-3 and H-1′/C-2, C-3, C-4, C-2′, C-3′ were observed, indicating the linkage of the 3-methoxy-3-methyl-1-butenyl moiety at C-3 of the aromatic ring (Figure 2). Further signals in the ^{1}H and ^{13}C NMR spectra of **1** could be assigned to a sugar unit, which was identified as α-L-arabinose by gas chromatography of the hydrolyzed product, and its anomeric proton resonance at δ 4.65 (H-1‴, 1H, d, J = 7.2 Hz) and carbon signals at δ 107.1 (d), 74.5 (d), 72.9 (d), 69.8 (d) and 67.9 (t). These data supported the structure of **1** as 3-((*E*)-3-methoxy-3-methyl-1-butenyl)-5-(3-methyl-2-butenyl)-4-*O*-(*α*-L-arabinopyranosyl)benzoic acid.

Compound **2** was obtained as an amorphous solid. It had a molecular formula of $C_{24}H_{34}O_9$ from the quasi-molecular ion peak in positive HR-ESI-MS at *m/z* 489.2173 $[M + Na]^+$ (Calcd. for $C_{24}H_{34}O_9Na$, 489.2101). The NMR spectroscopic data (Table 1) of **2** were comparable to those of **1**, indicating that **2** was also a nervogenic acid derivative. The sugar residue of **2** was identified as *β*-D-glucopyranosy unit by gas chromatography of the hydrolyzed product, and its anomeric proton resonance at Glc H-1‴ (δ_H 4.79, d, J = 7.2 Hz). In the HMBC experiment, long-range correlation was observed from Glc H-1‴ with C-4 (δ_C 157.1). Thus, the structure of compound **2** was determined as 3-((*E*)-3-methoxy-3-methyl-1-butenyl)-5-(3-methyl-2-butenyl)-4-*O*-(*β*-D-glucopyranosyl)benzoic acid.

Compound **3** was obtained as an amorphous solid. It had a molecular formula of $C_{28}H_{40}O_{14}$ from the quasi-molecular ion peak in positive HR-ESI-MS at *m/z* 623.2245 $[M + Na]^+$ (Calcd. for $C_{28}H_{40}O_{14}Na$, 623.2316). It was found to have a similar structure to compound **1** by comparison of their NMR data (Table 1), the main difference observed was that the methoxy group at C-3′ was replaced by a hydroperoxy group, and one more *β*-D-glucopyranoside unit than compound **1**. It was confirmed by gas chromatography of the hydrolyzed product and by the coupling constant of their anomeric protons Ara H-1‴ (δ_H 4.71, d, J = 7.2 Hz) and Glc H-1⁗ (δ_H 4.72, d, J = 7.8 Hz). In the HMBC experiment, long-range correlations were observed from Ara H-1‴ with C-4 (δ_C 156.5), Glc H-1⁗ with Ara C-2‴ (δ_C 82.0). Moreover, the linkage of the sugar units was as same as in nervosine IV.[5] Thus, the structure of compound **3** was determined as 3-((*E*)-3-hydroperoxy-3-methyl-1-butenyl)-5-(3-methyl-2-butenyl)-4-*O*-[*β*-D-glucopyranosyl-(1→2)-*α*-L-arabinopyranosyl]benzoic acid.

Compounds **1**–**3** were evaluated for their cytotoxicity against HCT116 cells. However, all compounds had no obvious inhibitory activity against the tumor cells used (IC50 > 50 μM, n = 3).

EXPERIMENTAL

General procedures.

Optical rotations were recorded on a Perkin–Elmer 341 polarimeter. 1D and 2D NMR spectra were measured by a Bruker AV 600 NMR spectrometer. HR–ESIMS were carried out on a Q–TOF micro mass spectrometer (Waters, USA). Silica gel (Qingdao Haiyang Chemical Co., Ltd., China, 200–300 mesh)

were used for column chromatography (CC). The TLC plates were precoated with silica gel GF_{254} (Qingdao Haiyang Chemical Co., Ltd., China).

Plant material.

L. nervosa were collected in Zunyi, Guizhou Province (N28° 14′ 35.48″ E107° 0′ 55.40″), China in July 2014. A voucher specimen (No. ZN361520140801) is deposited in School of Life Science and Engineering, Southwest Jiaotong University, Sichuan, China.

Extraction and Isolation.

The whole plants of *L. nervosa* (10 kg) were extracted with 95% EtOH at room temperature to obtain the EtOH extract (750 g). The EtOH extract (750 g) was suspended in H_2O (7 L) at 50 ℃ and adjusted to pH 2.8 with 10% HCl, and extracted with petroleum ether to obtain the petroleum ether extract (18 g). The pH of the aqueous layer was adjusted to 9.4 with aqueous ammonia solution and extracted with CH_2Cl_2, EtOAc, and *n*-butanol to obtain the CH_2Cl_2 extract (75 g), EtOAc extract (16 g) and *n*-butanol extract (44 g) successively.

The CH_2Cl_2 extract (75 g) was subjected to a silica gel column eluted in a step gradient manner with CH_2Cl_2: MeOH (50:1 - 0:1) to afford fractions (A-E) based on TLC analysis. Fraction A (12 g) was separated by RP-18 silica gel column CC using 50% MeOH in H_2O to afford A_1, A_2 and A_3. Compounds **1** (13 mg) and **2** (8 mg) were obtained from subfraction A_1 after CC (silica gel, light petroleum−CH_2Cl_2−MeOH, 9:2:0.3). Subfraction A_2 was further purified by CC (silica gel, light petroleum−CH_2Cl_2−MeOH, 8:2:0.2) to give compound **3** (6 mg).

3-((*E*)-3-Methoxy-3-methyl-1-butenyl)-5-(3-methyl-2-butenyl)-4-*O*-(*α*-L-arabinopyranosyl)benzoic acid (1)

White powder. $[\alpha]_D^{25}$ + 11.3 (c 0.280，MeOH); HR – ESI – MS (*m/z* 459.2093 $[M + Na]^+$) (Calcd. for $C_{23}H_{32}O_8Na$, 459.1995); ^1H NMR (600 MHz, CD_3OD) and ^{13}C NMR (150 MHz, CD_3OD) data, see Table 1.

3-((*E*)-3-Methoxy-3-methyl-1-butenyl)-5-(3-methyl-2-butenyl)-4-*O*-(*β*-D-glucopyranosyl)benzoic acid (2)

White powder. $[\alpha]_D^{25}$ + 9.2 (c 0.230，MeOH); HR – ESI – MS (*m/z* 480.2173 $[M + Na]^+$) (Calcd. for $C_{24}H_{34}O_9Na$, 480. 2101); ^1H NMR (600 MHz, CD_3OD) and ^{13}C NMR (150 MHz, CD_3OD) data, see Table 1.

3-((*E*)-3-Hydroperoxy-3-methyl-1-butenyl)-5-(3-methyl-2-butenyl)-4-*O*-[*β*-D-glucopyranosyl-(1→2)-*α*-L-arabinopyranosyl]benzoic acid (3)

White powder. $[\alpha]_D^{25}$ + 7.5 (c 0.190，MeOH);HR – ESI – MS (*m/z* 623.2245 $[M + Na]^+$) (Calcd. for $C_{28}H_{40}O_{14}Na$, 623.2316); ^1H NMR (600 MHz, CD_3OD) and ^{13}C NMR (150 MHz, CD_3OD) data, see

Table 1.

Acid hydrolysis

A solution of each compound (about 2 mg) in 1 mol/L HCl–dioxane (1:1, 5 mL) was heated at 80 ℃ for 4 h，respectively. After cooling, the solution was neutralized with 1 mol/L NaOH and evaporated to dryness. H_2O (5 mL) was added and the solution was extracted with $CHCl_3$ (3×3 mL). The aqueous layer was concentrated under a stream of nitrogen. The residue was silylated with HMDS-TMCS-pyridine (hexamethyldisilazane-trimethylchlorosilane-pyridine, 3:1: 9). The thiazolidine derivatives were analyzed by GC for sugar identification. D-Glucose and L-arabinose were confirmed by comparison with the retention time of the authentic standards at 11.81 min and 9.04 min, respectively. The same analysis was carried out on compounds **2**–**3**.[6]

Cell culture and cytotoxicity assay

HCT116 human colon cancer cell line were obtained from the American Type Culture Collection (ATCC). The cytotoxicity of the compounds against HCT116 human colon cell line was evaluated by MTT assay as described in our previous paper.[5] Cells treated with dimethyl sulfoxide (DMSO) (0.1% v/v) were used as negative controls, whereas adriamycin (≥98%; Sigma Chemical Co., Ltd., Shanghai, China) was used as positive control.

SUPPORTING INFORMATION

Supplementary (synthesis of the starting azides, HPLC chromatograms, IR, ^{1}H and ^{13}C NMR, MS spectra, etc.) data associated with this article can be found, in the online version, at URL: https://www.heterocycles.jp/newlibrary/downloads/PDFsi/27587/104/6

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DISCLOSURE STATEMENT

No potential conflict of interest was reported by the authors.

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