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Pyrrolizidine alkaloids from *Liparis nervosa* with antitumor activity by modulation of autophagy and apoptosis

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ABSTRACT

Seven pyrrolizidine alkaloids, nervosine X–XV and nervosine VII *N*-oxide, together with a reaction product, namely chloride-(*N*-chloromethyl nervosine VII), were isolated from *Liparis nervosa*. Their structures were elucidated by extensive spectroscopic analyses. Most of these compounds were investigated for their cytotoxicity *in vitro* against HCT116 human cancer cell line, and the results showed that chloride-(*N*-chloromethyl nervosine VII) induced tumor cell death in a dose-dependent manner. Furthermore, the mechanisms underlying its cytotoxicity were investigated, including apoptosis and autophagy. Apoptosis in HCT116 cells was associated with up-regulation of caspase-3 and -9 expressions by activation of the mitochondrial pathway. The autophagy inducing effect was associated with the regulation of autophagic markers, including LC3-II, p62, and Beclin 1. Mechanistic studies showed that JNK, ERK1/2, and p38 MAPKs signaling cascades play an important role in chloride-(*N*-chloromethyl nervosine VII) induced autophagy and apoptosis.

1. Introduction

The genus *Liparis*, belonging to the Orchidaceae family that is comprised of more than 250 species, is mainly distributed in the tropic and subtropic region (Editorial committee of Flora of China, 1999). *Liparis nervosa* (Thunb.) Lindl, is a herbaceous plant, widely distributed in China, and used for detoxicating and hemostatic functions (Hua et al., 1999). Research on phytochemical constituents has been conducted since decades and a series of pyrrolizidine alkaloids (PAs) has been isolated (Nishikawa and Hirata, 1967, 1968). The PAs represent a class of typical metabolites, and it is well known that the majority of PAs cause serious diseases in domestic animals and humans through liver bioactivation (Bush et al., 1993). However, saturated PAs are not hepatotoxic and must be distinguished from the 1, 2-unsaturated pyrrolizidine alkaloids (Bush et al., 1993; Castells et al., 2014). Further evidence showed that PAs are biologically active molecules with antitumor, antibiosis, and antifeedant activities (Hartmann, 1999; Siciliano et al., 2005; Liu et al., 2017). Previous investigations on chemical constituents of *L. nervosa* in our group have reported eleven pyrrolizidine alkaloids and sixteen nervogenic acid derivatives (Huang et al., 2013a, 2013b, 2013c, 2016). Our continuing study on the ethanol extract of the same specimen led to the isolation of seven previously undescribed saturated PAs, nervosine X–XV (**1–3, 6–8**) and nervosine VII *N*-oxide (**4**), together with a reaction product of nervosine VII (**5**) (Fig. 1). Compounds **1–3** and **5–8** were investigated in this study for their cytotoxicity *in vitro* against HCT116 human cancer cells, and we found that compound **5** induced tumor cell death in a dose-dependent manner.

Programmed cell death (PCD), a major cytotoxic mechanism of antitumor agents including two classical forms, apoptosis (type I PCD) and autophagy (type II PCD), along with the regulation of PCD is an important target in cancer chemotherapy (Kim et al., 2016). Accordingly, the underlying molecular mechanism of the antitumor activity of compound **5**, and how it modulates the crosstalk between autophagy and apoptosis were investigated in HCT116 human colon cancer cells. Here, we report the isolation and structural elucidation of eight previously undescribed PAs from the whole plant of *L. nervosa*, as well as the investigation about the mechanisms by which compound **5** affects apoptosis and autophagy in HCT116 cells.

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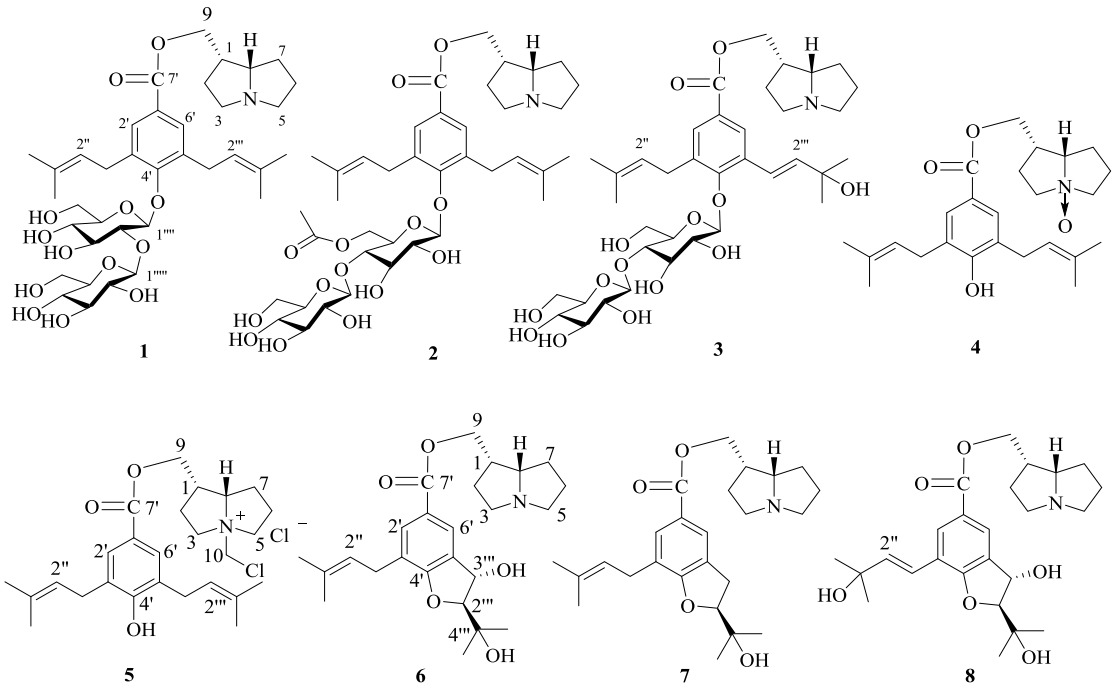

Fig. 1. Structures of compounds **1–8** isolated from *Liparis nervosa*.

2. Results and discussion

2.1. Structure elucidation and identification

Compound **1** was isolated as a white, amorphous powder, and assigned a molecular formula of $C_{37}H_{55}NO_{13}$ based on the protonated molecule at *m/z* 722.3754 $[M + H]^+$ ($C_{37}H_{56}NO_{13}$, calcd for 722.3752), as revealed by HR-ESI-MS (positive mode) and ^{13}C NMR spectra. IR spectrum of **1** gave a broad peak at 3369 cm^{-1} indicating the presence of hydroxy groups, a peak at 1716 cm^{-1}, which was attributed to a carbonyl group, and two peaks at 1600 and 772 cm^{-1} confirming the presence of an aromatic ring. In the ^1H NMR of **1** (Table 1), resonances for four vinylic methyl groups at δ_H 1.76 (6H, s) and 1.78 (6H, s), two olefinic methine protons at δ_H 5.31 (2H, t, J = 7.2 Hz) and two vinylic methylene groups at δ_H 3.59 (4H, d, J = 7.2 Hz) were readily distinguished, which indicated the existence of two prenyl moieties in the molecule (Nishikawa and Hirata, 1967, 1968; Huang et al., 2013a, 2016). Moreover, resonances reminiscent of a tetra-substituted aromatic ring [δ_H 7.67 (2H, s); δ_C 127.1, 130.1 × 2, 137.5 × 2 and 158.1] and a carboxyl group (δ_C 167.5) in the ^{1}H and ^{13}C NMR spectra, along with the key HMBC correlations (Fig. 2), δ_H H-2′/6′ (δ_H 7.67) to C-1″/1‴ (δ_C 29.6), and C-7′ (δ_C 167.5), suggested the presence of a 4-hydroxy-3,5-bis-(3-methyl- 2-butenyl)-benzoic acid moiety, previously described as a nervogenic acid unit (Nishikawa and Hirata, 1967, 1968; Huang et al., 2013a, 2016).

Additionally, the substructure 1-hydroxymethyl pyrrolizidine was deduced from the NMR data, which displayed a nitrogen-bearing methine (δ_H 4.20; δ_C 70.2), one oxymethylene (δ_H 4.39, 4.52; δ_C 64.2), two nitrogen-bearing methylene [(δ_H 3.28, 3.47; δ_C 55.0) and (δ_H 2.98, 3.74; δ_C 57.1)], three methylene [(δ_H 2.00, 2.13; δ_C 27.1), (δ_H 1.93, 2.18; δ_C 27.0) and (δ_H 1.95, 2.09; δ_C 26.9)] and one methine (δ_H 2.91; δ_C 41.3), which were connected by COSY and confirmed by HMBC correlations. Eleven pyrrolizidine alkaloids have been isolated from *L. nervosa*, and 1-hydroxymethyl pyrrolizidine moiety of those alkaloids was defined as lindelofidine and laburnine (Nishikawa and Hirata, 1967, 1968; Huang et al., 2013a, 2016). The main differences between lindelofidine and laburnine were that, H-1 and H-8 of lindelofidine were *cis*-configurated and C-1 was resonated in the range at δ_C 40.4–42.0 in ^{13}C NMR, while in laburnine, H-1 and H-8 were *trans*-configuration, C-1 was resonated at δ_C 45.0–46.3, respectively (Huang et al., 2013a, 2016). 1-hydroxymethyl pyrrolizidine moiety of **1** presented similar properties [δ_C (41.3, C-1, d), H-1 and H-8 were a *cis*-configurated according to the NOESY correlations] with lindelofidine based on the NMR spectra. Moreover, 1-hydroxymethyl pyrrolizidine was obtained by alkaline hydrolysis of **1**, whose molecular formula was deduced as $C_8H_{15}NO$ (calcd. for $[M+H]^+$, 142.1232) by HR-ESI-MS (Fig. S89), and the optical rotation $[\alpha]_D^{20}$ +62.1 (*c* 0.213, EtOH) was in good agreement with that of lindelofidine $[\alpha]_D^{20}$ +75.0 (*c* 3.2, EtOH) (Nishikawa and Hirata, 1967). From the above-mentioned evidence, the substructure in **1** was estimated as lindelofidine. HMBC correlations from H-9 (δ_H 4.39 and 4.52) to C-7′ (δ_C 167.5), the carboxyl carbon of the nervogenic acid unit, indicating that an ester group is attached at C-9.

The remaining signals in the ^1H and ^{13}C NMR spectra of **1** could be assigned to two D-glucose units, which was supported by acid hydrolysis of **1**, and GC analysis of 1-(trimethylsilyl) imidazole derivatives. According to the coupling constants of the hydrogens attached to the anomeric center at δ 4.81 (1H, d, J = 7.8 Hz) and 4.83 (1H, d, J = 7.8 Hz), the two glucose units (Glc-I and Glc-II) were determined as β-configuraion (Huang et al., 2013a). In the HMBC, the proton signals Glc-I H-1⁗ (δ_H 4.83) and Glc-II H-1⁗⁗ (δ_H 4.81) showed long-range correlations with carbons C-4′ (δ_C 158.1) and Glc-I C-2⁗ (δ_C 82.8), respectively. Based on the above data and analysis, the structure of compound **1** was elucidated as 4-*O*-[β-D-glucopyranosyl-(1 → 2)-β-D-glucopyranosyl] nervogenic acid ester of lindelofidine, named as nervosine X.

Compound **2** was purified as a white, amorphous powder with the molecular formula $C_{39}H_{57}NO_{14}$ based on its HR-ESI-MS *m/z* 764.3849 ($[M + H]^+$, calcd for $C_{39}H_{58}NO_{14}$, 764.3857). Comparison of the spectral data of **2** with that of **1** indicated that it also possessed one nervogenic acid, one 1-hydroxymethyl pyrrolizidine moiety, and two β-D-glucose units. The 1-hydroxymethyl pyrrolizidine moiety of **2** in the 1D NMR spectroscopic data (Table 1) were similar to those of **1**, suggesting that this compound also possessed a lindelofidine group, which was further supported by UPLC-HR-ESI-MS and TLC analysis of the reaction mixture of alkaline hydrolysis of **2**, it's molecular formula was