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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.

Keywords

Liparis nervosa; Orchidaceae; Pyrrolizidine alkaloids; Cytotoxicity; Human colorectal cancer cell line; Autophagy; Apoptosis

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at <http://dx.doi.org/10.1016/j.phytochem.2018.06.001>. These data include MOL files and InChIKeys of the most important compounds described in this article.

InChIKey

MCJLHCBGPXQWLR-GKLPOLMESA-N

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.

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 $\times$ 2, 137.5 $\times$ 2 and 158.1] and a carboxyl group (δ_{C} 167.5) in the ¹H and ¹³C 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*-configured and C-1 was resonated in the range at δ_{C} 40.4–42.0 in ¹³C 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*-configured 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 C₈H₁₅NO (calcd. for [M + H]⁺, 142.1232) by HR-ESI-MS (Fig. S89), and the optical rotation [$\alpha_{\text{D}}^{20} + 62.1$ (c 0.213, EtOH)] was in good agreement with that of lindelofidine [$\alpha_{\text{D}}^{20} + 75.0$ (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 ¹³C 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 -configuration (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 $\rightarrow$ 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**, its molecular formula was deduced as $C_8H_{15}NO$, the retention time and R_f value were same as lindelofidine. The main differences between **1** and **2** were the presence of an additional acetyl group [δ_H 1.94 (3H, s); δ_C 20.7, 172.3] as indicated by the molecular formula and the connectivity of two glucose units. In HMBC (Fig. 2), H-6''' of Glc-I (δ_H 4.38) was correlated with the carbonyl carbon of the acetyl group at δ_C 172.3, indicating that the acetyl group was assigned to Glc-I C-6'''. Two D-glucose units were connected through a 1 $\rightarrow$ 4 linkage, which was deduced from the long-range correlations between Glc-II H-1''' (δ_H 4.33) and Glc-I C-4''' (δ_C 81.0) in HMBC spectrum. Accordingly, the structure of **2**, nervosine XI, was defined as 4-*O*-[β -D-glucopyranosyl-(1 $\rightarrow$ 4)-6-*O*-acetyl- β -D-glucopyranosyl] nervogenic acid ester of lindelofidine.

Compound **3** was also isolated as a white amorphous powder, and its molecular formula was determined to be $C_{37}H_{55}NO_{14}$ by HR-ESI-MS and ^{13}C NMR. Comparison of the spectral data of **3** (Table 2) with that of nervosine III (Huang et al., 2013a), 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 hydroxyl group and consisting in a 3-hydroxy-3-methyl-butenyl moiety based on the following analyses. The signals at δ_H 7.17 (1H, d, J = 16.2 Hz) and 6.34 (1H, d, J = 16.2 Hz) in the 1H NMR spectrum indicated the presence of a *trans*-double bonds. The two methyl groups of the prenyl moieties were shifted highfield compared to nervosine III, 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 quaternary carbon at δ_C 71.8 (C-3''') and the lack of an olefinic quaternary carbon in **3**, which indicated that there was an additional OH connected to C-3'''. The two methyl groups were connected to C-3''' and two *trans* olefinic carbons were located at C-1''' and C-2''' on the basis of the cross-peaks in the HMBC spectrum as shown in Fig. 2. The 1-hydroxymethyl pyrrolizidine unit of **3** was deduced as lindelofidine by its homologous NMR data with **1**, combined with TLC and UPLC-HR-ESI-MS analysis of the reaction mixture of alkaline hydrolysis of **3**. Consequently, **3** was established as 3-(3-methyl-2-butenyl)-4-*O*-[β -D-glucopyranosyl-(1 $\rightarrow$ 4)- β -D-glucopyranosyl]-5-(3-hydroxy-3-methyl-butenyl) benzoate ester of lindelofidine, and named nervosine XII.

Compound **4**, a white amorphous powder, had a molecular formula of $C_{25}H_{35}NO_4$ as deduced by HR-ESI-MS (m/z 414.2638 $[M + H]^+$, calcd for $C_{25}H_{36}NO_4$, 414.2644). Comparison of the 1D NMR chemical shifts of **4** (Table 3) with those of nervosine VII (Huang et al., 2016), a known compound isolated from this plant, implied that they shared the same planar structure except for the presence of an additional oxygen atom as indicated by the molecular formula. In the ^{13}C NMR spectrum (Table 3), the signals of C-3, C-5, and

C-8 were displaced downfield (δ_{C} 15.0, 14.4 and 19.6 ppm, respectively), in relation to those of nervosine VII (Huang et al., 2016). Therefore, the additional oxygen atom must be attached to the nitrogen atom with a coordination bond. The positive correlation was observed between H-1 and H-8 in NOESY spectrum, indicating a cis-configuration for these protons. The proposed structure was further supported by comparison of the NMR spectral data of **4** with liparis alkaloid A, a known compound described in the literature (Jiang et al., 2015), which also contained a unit of lindelofidine *N*-oxide. The above-mentioned conclusion was also confirmed through comparing the optical rotation $[\alpha]_{\text{D}}^{20} + 18.2$ (c 0.552, MeOH) with that of nervosine VII $[\alpha]_{\text{D}}^{20} + 23.0$ (c 0.860, CH₃Cl) (Huang et al., 2016). Furthermore, HMBC correlations from H-9 (δ_{H} 4.29 and 4.42) to C-7' (δ_{C} 168.0), C-1 (δ_{C} 39.3), C-2 (δ_{C} 27.5) and C-8 (δ_{C} 85.8) indicated that the nervogenic acid unit was attached to C-9 by an ester linkage (Fig. 2). Thus, structure **4** was defined as nervosine VII *N*-oxide.

Compound **5** was obtained as a white amorphous powder. The positive ion, C₂₅H₃₇ClNO₃⁺, was deduced from the quasimolecular ion peak at m/z 446.2468 [M]⁺ (calcd for C₂₅H₃₇ClNO₃⁺, 446.2462) and the isotopic peak at m/z 448.2448 [M + 2]⁺ in the HR-ESI-MS spectrum. The characteristic NMR spectra signals of compound **5** (Table 3) resembled those of nervosine VII, except for the presence of a *N*-chloromethyl group. This was supported by the appearance of a new methylene signal at δ_{H} 5.33 (2H, br.s) and δ_{C} 69.4 (Perez-Castorena et al., 1998; Shi et al., 2010; Bowman et al., 1994), and C-3, C-5, and C-8 appeared shifted downfield (δ_{C} 10.1, 9.8 and 14.6 ppm, respectively), in relation to those of nervosine VII (Huang et al., 2013a). The existence of an *N*-chloromethyl group at nitrogen atom was confirmed by the cross-peaks between H-10 (δ_{H} 5.33) with C-3 (δ_{C} 64.1), C-5 (δ_{C} 65.7) and C-8 (δ_{C} 80.8) in the HMBC spectrum (Fig. 2). From the above evidence, compound **5** was determined to be chloride-(*N*-chloromethyl nervosine VII). The tertiary amine was strongly nucleophilic and related chloromethyl derivatives were easily obtained from cyclized tertiary amines in the presence of methylene chloride (Beniddir et al., 2013; Bowman et al., 1994; Ndongo et al., 2017; Shi et al., 2010). These artifacts frequently occur during extraction and/or isolation process (Tan et al., 2013). Caution is therefore needed when quaternary ammonium derivatives are isolated. UPLC-HR-ESI-MS analysis of the crude extract from *L. nervosa* did not show any hint of chlorinated metabolites (see Supplementary Information, Fig. S54), and after purification with di-chloromethane in silica gel, strong chloromethyl derivatives signal were identified (see Supplementary Information, Fig. S55). Thus, compound **5** could be an artifact formed during the purification process with CH₂Cl₂.

The molecular formula of **6** (C₂₅H₃₅NO₅) was ascertained by HR-ESI-MS (m/z 430.2601 [M + H]⁺). Analyses of NMR data of **6** (Table 4) suggested that it also possessed a 1-hydroxymethyl pyrrolizidine moiety, which was estimated as lindelofidine following comparison of the pyrrolizidine part of **1** with NMR data, which was proved by alkaline hydrolysis of **6**, TLC and UPLC-HR-ESI-MS analysis of the reaction mixture. Furthermore, the NMR spectra exhibited characteristic signals for a tetra-substituted phenyl unit [δ_{H} 7.75 (1H, br.s), 7.89 (1H, br.s); δ_{C} 123.4, 125.3, 126.3, 130.9, 132.8, 164.0], a carboxyl group resonance at δ_{C} 166.6, and a prenyl group [δ_{H} 5.30 (1H, t, J = 7.2 Hz), 3.33 (2H, t, J = 7.2

Hz), 1.73 (3H, s) and 1.74 (3H, s); δ_C 134.3, 122.4, 28.8, 25.9, 17.9]. Based on the HMBC spectrum as shown in Fig. 2, the prenyl group and the carboxyl units were located at C-3' and C-1', respectively. In combination with the HMQC data, the remaining five aliphatic signals in ^{13}C NMR, two oxymethine at δ_C 73.3 and 98.9, one oxygen-bearing quaternary carbon at δ_C 71.8, and two methyl groups at δ_C 25.8 and 25.1 were observed. These NMR data hinted of a dihydrobenzofuran skeleton bearing a hydroxyl group at C-3''' and a hydroxyl-isopropyl residue C-2''' (Duan et al., 2008). In addition, correlation between H-9 and the carboxyl carbon (C-7') of the dihydrobenzofuran derivative unit in HMBC spectrum indicated that the dihydrobenzofuran derivative ester was located at C-9 of lindelofidine. The relative configuration of positions 2''' and 3''' could be deduced from the vicinal coupling constants, which should be 7.0 Hz in the case of a cis-configuration and 4.0–5.0 Hz in *trans*-derivatives (Friedrich et al., 2005). As a coupling constant of $^3J_{2''',3'''} = 4.2\text{Hz}$ was observed in **6**, the dihydrofuran ring had to be 2''', 3'''-*trans*-configured. The absolute configuration of C-2''' and C-3''' was identified as (2'''S, 3'''S)-conformer by CD experiment, indicating that it had the same curve pattern as that reported for the prenylated dihydrobenzofuran derivatives (Xu et al., 2016). Accordingly, the structure of compound **6** was determined as shown in Fig. 1, and accorded the trivial name nervosine XIII.

Compound **7**, a white amorphous powder, showed the molecular formula of $\text{C}_{25}\text{H}_{35}\text{NO}_4$, as established by the positive HR-ESI-MS ion at m/z 414.2644 $[\text{M} + \text{H}]^+$. The NMR data of compound **7** and nervosine III (Huang et al., 2013a) showed a close resemblance (Table 4). The 1-hydroxymethyl pyrrolizidine unit was identified as lindelofidine by TLC and UPLC-HR-ESI-MS analysis of the reaction mixture of its alkaline hydrolysis. The major differences were the absence of one oxygenbearing methine carbon at δ_C 73.3 and the presence of a methylene at δ_C 30.5, exhibiting that the hydroxyl group at C-3''' had disappeared. Complete structure assignments were obtained from exhaustive analysis of the HMQC, HMBC and COSY data. The circular dichroism curve of **7** showed the same pattern as that reported for acremine N (Arnone et al., 2009), and the absolute configuration of C-2''' was identified as (2'''S)-conformer, named nervosine XIV.

Compound **8** was obtained as a white amorphous powder. HR-ESI-MS data provided the molecular formula $\text{C}_{25}\text{H}_{35}\text{NO}_6$ (m/z 446.2544 $[\text{M} + \text{H}]^+$, calcd for $\text{C}_{25}\text{H}_{36}\text{NO}_6$, 446.2543). The NMR spectrum (Table 4) exhibited characteristic signals attributable to a carboxyl group, a lindelofidine moiety and a dihydrobenzofuran derivative bearing a hydroxyl-isopropyl residue at C-2''' and a hydroxyl group at C-3'''. The lindelofidine moiety was further supported by TLC and UPLC-HR-ESI-MS analysis of the reaction mixture of alkaline hydrolysis of **8**. Comparison of the NMR data of **8** with those of **6** indicated that the modification of one prenyl chain was the most notable difference, revealing the presence of a chain with a *trans* double bond, two methyl groups, and a tertiary hydroxyl group. This was confirmed by the presence of signals at δ_H 1.38 (6H, s, H-4''/5''), δ_H 6.65 (1H, d, $J = 16.2$ Hz, H-1''), and δ_H 6.72 (1H, d, $J = 16.2$ Hz, H-2'') in the ^1H NMR and at δ_C 30.0 (C-4''/5''), δ_C 71.6 (C-3''), δ_C 142.4 (C-2''), and δ_C 121.4 (C-1'') in the ^{13}C NMR (Flores et al., 2008). The dihydrobenzofuran derivative was attached to C-9 of lindelofidine moiety by the ester linkage base on the HMBC spectrum as shown in Fig. 2. The coupling constant (4.2 Hz) of H-2''' and H-3''' suggested a *trans*-configuration (Friedrich et al., 2005). Because the

circular dichroism curve of **8** showed the same pattern as that reported for the prenylated dihydrobenzofuran derivatives (Xu et al., 2016), the absolute configuration of C-2''' and C-3''' was identified as (2'''S, 3'''S)-conformer, and named nervosine XV.

2.2. Biological evaluation

2.2.1. Growth inhibition effects against HCT116 cells—To evaluate the effects of the isolated pyrrolizidine alkaloids on the growth of human cancer cells, the growth inhibitory potential of compounds **1–3** and **5–8** were determined by MTT assay. Compounds **1–3**, **7** and **8** did not show obvious growth inhibitory effects on HCT116 cell lines at concentrations up to 80 μ M. Compound **6** showed IC₅₀ values of $60.04 \pm 0.8 \mu$ M against HCT116 cells. Compound **5**, possessing a chloromethyl group connected to the N atom, exhibited cytotoxicity in a dose dependent manner against HCT116 cell lines with IC₅₀ values of $32.88 \pm 1.6 \mu$ M. Herein, adriamycin was used as the positive control, with IC₅₀ values of $3.5 \pm 6.7 \mu$ M against HCT116 cells.

2.2.2. Induction of apoptosis by compound 5 is associated with caspase-mediated pathway—In general, apoptosis occurs via either the mitochondrial (intrinsic) pathway with the activation of caspase-3 and -9 or the death receptor (extrinsic) pathway with the activation of caspase-8. To further explore the molecular mechanism involved in the compound **5**-mediated apoptosis of HCT116 cells, the effects of **5** on the activation of cleavage forms of caspase-3, -8, and -9 were observed after 24 h treatment with **5** (Fig. 3). A dose-dependent cleavage of procaspase-3 and -9 was observed, and the activated caspase-3 also cleaved PARP, which is required for repairing DNA damage. Meanwhile, cytochrome *c* expression was increased in a concentration-dependent manner. These findings suggested that the induction of apoptotic cell death by **5** might occur through a caspase-9 independent pathway.

2.2.3. Compound 5 induces autophagy in HCT116 cells—The conversion of LC3-I to LC3-II, as detected by western blotting, is commonly used as a marker to evaluate autophagy activity (Green and Levine, 2014). Western blot analyses showed that compound **5** induced accumulation of LC3-II in HCT116 cells (Fig. 4A), indicating that compound **5** might affect autophagy in HCT116 cells. To further confirm compound **5**-induced autophagy, the autophagy markers of LC3-II, p62 and Beclin 1 were detected by western blot analysis in HCT116 cells after 24 h treatment with **5** in a concentration-dependent manner. Treatment with compound **5** up-regulated the expression levels of LC3-II and Beclin 1, and down-regulated the expression of p62 in HCT116 cells (Fig. 4B), indicating that compound **5** could induce autophagy (Kim et al., 2016).

2.2.4. Effects of the MAPKs signaling pathways on 5-treated HCT116 cells—MAPKs signaling pathways consisting of ERK, p38 MAPK, and JNK have been considered as chemotherapeutic targets for sensitizing cancer cells to autophagy and apoptosis. To investigate the role of MAPK signaling pathways in **5**-induced autophagy and apoptosis, the activation of ERK1/2, p38 MAPK, and JNK was determined by western blot analysis. As shown in Fig. 5, compound **5** activated the phosphorylation of ERK1/2, p-p38 and p-JNK in

a concentration-dependent manner. These results support that **5**-induced autophagy and apoptosis in HCT116 cells is associated with activation of MAPKs signaling pathways.

2.2.5. Compound 5 induced the switch from autophagy to apoptosis in HCT116 cells—To elucidate the relationship between autophagy and apoptosis in HCT116 cells, the time-dependent expression of autophagy- and apoptosis-specific proteins after treatment with 40 μ M of compound **5** was inspected. As a result, the induction of autophagy by **5**, as indicated by the expression of LC3-II and Beclin 1, was gradually increased and reached a peak at 24 h. In contrast, the activation of apoptosis by **5**, as indicated by the levels of cleaved caspase-9 and cleaved PARP, continued increasing up to 48 h (Fig. 6). These data suggest that the induction of autophagy and apoptosis by **5** is not associated with a successive process, but rather a simultaneous approach in an early stage and the prolonged treatment with **5** inducing the switch from autophagy to apoptotic cell death.

3. Conclusions

Investigation on the whole plant of *L. nervosa* resulted in the isolation of seven previously undescribed pyrrolizidine alkaloids and a reaction artefact. Their structures were elucidated by extensive spectroscopic analyses and chemical reactions. Most of these compounds were investigated for their cytotoxicity *in vitro* against HCT116 human cancer cell line by MTT assay, and chloride-(*N*-chloromethyl nervosine VII) (**5**) was found to induce tumor cell death with IC_{50} 32.88 ± 1.6 μ M. The chloromethyl group connected to the N atom and hydroxy group on aromatic ring in compound **5** appear to be the important factors for potential antitumor activity. Furthermore, the study exploring possible mechanisms showed that compound **5** induced cell death by activating both apoptosis and autophagy in HCT116 cells. Compound **5** was able to induce apoptosis by activating the mitochondrial pathway, which was associated with mitochondrial release of cytochrome *c*, which functions to activate caspase-9, and then promoting the activation of caspase-3, leading to apoptosis. Also, we found that MAPK signaling pathways, ERK, p38 MAPK, and JNK, were the common upstream signals that simultaneously triggered apoptosis and autophagy. The time-dependent expression of autophagy- and apoptosis-specific proteins after treatment with compound **5** indicated that there was a switch from autophagy to apoptotic cell death. A schematic diagram summarizing these observed effects of compound **5** is shown in Fig. 7. Therefore, our findings highlight that compound **5** could be a suitable candidate for the treatment of human colorectal cancer cells.

4. Experimental

4.1. General experimental procedures

Optical rotations were measured on a Perkin-Elmer 341 polarimeter. 1D and 2D NMR spectra were recorded on a Bruker AV 600 NMR spectrometer with TMS, and IR spectra on a Thermo Fisher Nicolet 6700 spectrometer (KBr discs, cm^{-1}). UV spectra were determined with a Shimadzu UV-2450 spectrophotometer. Circular dichroism was obtained on a Jasco J-720 spectropolarimeter. Silica gel (Qingdao Haiyang Chemical Co., Ltd., China, 200–300 mesh), RP-18 silica gel (Merck, 40–60 μ m), D-101 macroporous resin (Rohm & Haas) and

alumina (Qingdao Haiyang Chemical Co., Ltd., China, 100–200 mesh) were used for column chromatography (CC). HPLC was carried out on a Waters SymmetryShield™ RP-18 column (5 μ m, 4.6 $\times$ 250 mm) with a Waters 600 controller and Waters 2487 detector. The TLC plates were precoated with silica gel GF₂₅₄ (Qingdao Haiyang Chemical Co., Ltd., China), and visualized under a UV lamp at 254 nm or by spraying Dragendorff's reagent or iodine. The HR-ESI-MS data were measured using a Q-TOF micro mass spectrometer (Waters). The antibodies for p-ERK1/2, ERK1/2, p-p38, p38, JNK, p-JNK, LC3-II, p62, Beclin 1, and the secondary antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA).

4.2. Plant material

The whole plants of *Liparis nervosa* (Thunb.) Lindl were collected in July 2014 from Kuankuo town, Suiyang county, Zunyi city, Guizhou Province, China (N28°14'35.48"E107°0'55.40"). The plant was identified by Professor Liang-Ke Song in School of Life Science and Engineering, Southwest Jiaotong University, Sichuan, China, where a voucher specimen is deposited (No. ZN361520140801).

4.3. Extraction and isolation

The air-dried and powdered whole plant of *L. nervosa* (14 kg) was extracted with 95% EtOH at room temperature (50 L $\times$ 3, every 7 days) to yield a dark extract (750 g). The extract was suspended in H₂O (2 L) and then partitioned with petroleum ether, EtOAc, and *n*-butanol, respectively. The *n*-butanol extract (180 g) was dissolved in 10% EtOH-H₂O, and subjected to a D101 macroporous resin column using the stepwise elution of H₂O-EtOH (90:10, 60:40, 40:60, and 5:95, *v/v*) to yield four fractions (Fr.A-Fr.D). Fr.B was separated by silica gel CC using a gradient of CH₂Cl₂-MeOH (90:10–5:95, *v/v*) to afford five sub-fractions (Fr.B1-Fr.B5). Fr.B1 was separated by silica gel CC and eluted with petroleum ether-Me₂CO-Et₂N (10:1:0.1–1:1:0.1, *v/v/v*) to produce compounds **2** (12 mg) and **3** (17 mg). Fr.B2 was subjected to silica gel CC with EtOAc-MeOH-Et₂N (20:1:0.2–1:1:0.2, *v/v/v*) to give three subfractions, Fr.B2–1-Fr.B2–3. Fr.B2–2 was subjected to ODS CC with CH₃CN-H₂O (2:8, *v/v*) to produce compounds **1** (14 mg) and **4** (2 mg). Fr.B4 was applied to silica gel CC and eluted with EtOA-MeOH-Et₂N (10:1:0.2, *v/v/v*), continually separated over an ODS column with MeOH-H₂O (5:95–90:10, *v/v*) to yield compound **6** (11 mg) and a mixture (15.5 mg), which was further isolated by preparative HPLC with CH₃CN-H₂O (3:7, *v/v*) to afford compound **7** (19 mg). Fr.C was subjected to neutral Al₂O₃ CC and silica gel CC in succession to yield three subfractions, Fr.C1-Fr.C3. Fr.C1 was submitted to silica gel CC and eluted with CH₂Cl₂-MeOH (40:1, *v/v*) to produce compound **5** (18 mg). Fr.C2 was subjected to ODS CC and silica gel CC in succession to yield compound **8** (28 mg).

4.3.1. Nervosine X (1)—White amorphous powder; $[\alpha]_D^{20} = +19.8$ (*c* 0.550, MeOH); UV (MeOH) $\lambda_{\max}$ (log ϵ), 244 (0.37), 208 (0.84) nm; IR (KBr) $\nu_{\max}$ 3369, 2958, 2920, 2879, 2851, 1716, 1631, 1600, 1455, 1384, 1350, 1317, 1220, 1182, 1077, 1042, 899, 772, 634 cm^{−1}. ¹H and ¹³C NMR data, see Table 1; HR-ESI-MS (*m/z*): 722.3754 [M + H]⁺ (calcd for C₃₇H₅₆NO₁₃, 722.3752).

4.3.2. Nervosine XI (2)—White amorphous powder; $[\alpha]_D^{20} = -1.47$ (*c* 0.950, MeOH); UV (MeOH) $\lambda_{\max}$ (log ϵ), 244 (0.31), 208 (0.80) nm; IR (KBr) $\nu_{\max}$ 3389, 2966, 2924, 2879, 2072, 1720, 1629, 1602, 1456, 1384, 1284, 1243, 1181, 1072, 1039, 901, 757, 637 cm^{-1} ; ^1H and ^{13}C NMR data, see Table 1; HR-ESI-MS (*m/z*): 764.3849 $[\text{M} + \text{H}]^+$ (calcd for $\text{C}_{39}\text{H}_{58}\text{NO}_{14}$, 764.3857).

4.3.3. Nervosine XII (3)—White amorphous powder; $[\alpha]_D^{20} = +1.0$ (*c* 0.300, MeOH); UV (MeOH) $\lambda_{\max}$ (log ϵ), 240 (0.86), 202 (0.68) nm; IR (KBr) $\nu_{\max}$ 3370, 2964, 2922, 2852, 1716, 1677, 1631, 1600, 1455, 1425, 1383, 1322, 1275, 1201, 1183, 1132, 1072, 902, 836, 800, 770, 721, 638 cm^{-1} ; ^1H and ^{13}C NMR data, see Table 2; HR-ESI-MS (*m/z*): 738.3707 $[\text{M} + \text{H}]^+$ (calcd for $\text{C}_{37}\text{H}_{56}\text{NO}_{14}$, 738.3701).

4.3.4. Nervosine VII N-oxide (4)—White amorphous powder; $[\alpha]_D^{20} = +18.2$ (*c* 0.550, MeOH); UV (MeOH) $\lambda_{\max}$ (log ϵ), 264 (1.63), 216 (2.22) nm; IR (KBr) $\nu_{\max}$ 3380, 2968, 2915, 2861, 1709, 1631, 1602, 1452, 1376, 1350, 1317, 1285, 1246, 1099, 983, 934, 906, 769, 628, 592 cm^{-1} ; ^1H and ^{13}C NMR data, see Table 3; HR-ESI-MS (*m/z*): 414.2638 $[\text{M} + \text{H}]^+$ (calcd for $\text{C}_{25}\text{H}_{36}\text{NO}_4$, 414.2644).

4.3.5. Chloride-(N-chloromethyl nervosine VII) (5)—White amorphous powder; $[\alpha]_D^{20} = -59.8$ (*c* 0.400, MeOH); UV (MeOH) $\lambda_{\max}$ (log ϵ), 264 (0.63), 215 (1.19) nm; IR (KBr) $\nu_{\max}$ 3365, 2969, 2926, 2877, 2858, 1711, 1631, 1604, 1464, 1382, 1351, 1313, 1284, 1243, 1184, 1098, 1007, 985, 769 cm^{-1} ; ^1H and ^{13}C NMR data, see Table 3; HR-ESI-MS (*m/z*): 446.2468 $[\text{M}]^+$ (calcd for $\text{C}_{25}\text{H}_{37}\text{ClNO}_3^+$, 446.2462), 448.2448 $[\text{M} + 2]^+$.

4.3.6. Nervosine XIII (6)—White amorphous powder; $[\alpha]_D^{20} = -4.3$ (*c* 1.400, MeOH); UV (MeOH) $\lambda_{\max}$ (log ϵ), 265 (0.69), 208 (1.48) nm; CD (MeOH) $\lambda_{\max}$ (ϵ): 209 (−1.38), 251 (+0.51) nm. IR (KBr) $\nu_{\max}$ 3368, 2972, 2927, 2796, 1711, 1631, 1605, 1465, 1440, 1377, 1345, 1311, 1284, 1224, 1182, 1084, 1008, 997, 913, 770 cm^{-1} ; ^1H and ^{13}C NMR data, see Table 4; HR-ESI-MS (*m/z*): 430.2601 $[\text{M} + \text{H}]^+$ (calcd for $\text{C}_{25}\text{H}_{36}\text{NO}_5$, 430.2593).

4.3.7. Nervosine XIV (7)—White amorphous powder; $[\alpha]_D^{20} = +20.4$ (*c* 0.450, CHCl_3); UV (MeOH) $\lambda_{\max}$ (log ϵ), 269 (0.29), 246 (0.76) nm; CD (MeOH) $\lambda_{\max}$ (ϵ): 248 (+1.51) nm. IR (KBr) $\nu_{\max}$ 2958, 2923, 2852, 1743, 1711, 1605, 1463, 1377, 1304, 1178, 1092, 962, 863, 770, 721 cm^{-1} ; ^1H and ^{13}C NMR data, see Table 4; HR-ESI-MS (*m/z*): 414.2644 $[\text{M} + \text{H}]^+$ (calcd for $\text{C}_{25}\text{H}_{36}\text{NO}_4$, 414.2644).

4.3.8. Nervosine XV (8)—White amorphous powder; $[\alpha]_D^{20} = +10.4$ (*c* 0.500, MeOH); UV (MeOH) $\lambda_{\max}$ (log ϵ), 279 (0.56), 206 (1.22) nm; CD (MeOH) $\lambda_{\max}$ (ϵ): 217 (−0.46) nm. IR (KBr) $\nu_{\max}$ 3403, 2973, 2927, 1706, 1655, 1599, 1513, 1451, 1376, 1353, 1283, 1208, 1177, 1061, 1025, 973, 914, 839, 811 cm^{-1} ; ^1H and ^{13}C NMR data, see Table 4; HR-ESI-MS (*m/z*): 446.2544 $[\text{M} + \text{H}]^+$ (calcd for $\text{C}_{25}\text{H}_{36}\text{NO}_6$, 446.2543).

4.4. Acid hydrolysis of 1–3

Compound **1** (about 2 mg) was heated in 1 N HCl-dioxane (1:1, 2 ml) at 85 °C for 3 h. The solution was diluted with H₂O (3 mL), neutralized with 1 N NaOH, and then extracted with CHCl₃. The H₂O layer was evaporated under a stream of nitrogen. The residue was dissolved in 1-(trimethylsilyl) imidazole (0.1 ml) and pyridine (0.2 ml), and the solution was stirred at 60 °C for 20 min. After drying, the residue was partitioned between H₂O and CHCl₃. The CHCl₃ layer was analyzed by GC using a L-CP-Chirasil-Val column (0.32 mm × 25 m). D-glucose was confirmed by comparison with the retention time of the authentic standards at 7.65 min. The same analysis was carried out on compounds **2–3** (Huang et al., 2013a).

4.5. Alkaline hydrolysis of 1

A solution of compound **1** (8 mg) in 0.5 mL of MeOH was refluxed with 1 mol/L NaOH (0.5 mL) for 1 h. The reaction mixture was acidified with 1 mol/L HCl and extracted with CHCl₃ (3 × 4 mL). The aqueous solution was made alkaline with 1 mol/L NaOH and extracted with CHCl₃ (3 × 4 mL), evaporation of the organic phase yielded lindelofidine (3.8 mg) (Huang et al., 2013a). The process was repeated for compounds **2**, **3**, and **6–8** (about 2 mg). 1-hydroxymethylpyrrolizidine was analyzed by TLC using CHCl₃:MeOH:diethylamine = 7:3:0.1 as developing solvent and iodine for detection, **1–3** and **6–8** gave lindelofidine (*R_f* 0.15) (Fig. S96). UPLC-HR-ESI-MS analysis was carried out on Waters ACQUITY UPLC/Xevo G2 QTOF mass spectrometer, flow rate: 0.3 ml/min, at 40 °C, eluent with CH₃OH/H₂O (2:8), its retention time was at 1.02 min (Fig. S90–Fig. S95).

4.6. Cell culture

HCT 116 human colon cancer cell line was obtained from ATCC. These cells were maintained at subconfluence in a 95% air and 5% CO₂ humidified atmosphere at 37 °C. RPMI medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin was used for routine subculturing.

4.7. Cytotoxicity assay

The cytotoxicity of the compounds against HCT116 human colon cell lines was evaluated by the MTT method as described in our previous paper (Huang et al., 2013a). Cells treated with DMSO (0.1% *v/v*) were used as negative controls, whereas adriamycin (98%; Sigma Chemical Co., Ltd., Shanghai, China) was used as the positive control.

4.8. Western blot analysis

After treatment with various concentrations of compounds, the cells were collected and lysed in lysis buffer. A 20 µg aliquot of total protein was separated by 10% SDS-PAGE gel, transferred to nitrocellulose membranes, blocked with 5% BSA, and probed with a primary antibody (1:1000) followed by the corresponding secondary antibody (1:3000). GAPDH (glyceraldehyde 3-phosphate dehydrogenase) was used as an internal standard. The signal was detected with Amersham Imager 600 (GE Healthcare, England) (Xu et al., 2014).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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

Fig. 2.
Key ^1H - ^1H COSY and HMBC correlations of compounds 1–8.

Fig. 3.

Effect of compound 5 on the expression of apoptosis-related proteins in HCT116 cells. HCT116 cells were cultured with indicated concentrations for 24 h. The activation of caspase-8, caspase-9 and caspase-3, PARP, and cytochrome *c* was determined by western blot analysis. GAPDH was used as an internal standard.

Fig. 4.

(A) Effect of compounds **1-3** and **5-8** on the induction of autophagy in HCT116 cells. HCT116 cells were cultured with 40 μ M of compounds **1-3** or **5-8** for 24 h, and the protein expression levels of LC3-II were determined by western blot analysis. (B) HCT116 cells were cultured with the indicated concentrations of compound **5** for 24 h. The protein expression levels of autophagy markers were determined by western blot analysis.

Fig. 5. Effect of compound 5 on the mitogen-activated protein kinase (MAPK) signaling in HCT116 cells. Cells were lysed after treatment with indicated concentrations of compound 5 for 24 h. The protein expression levels of p-ERK, ERK, p-p38, p38, p-JNK and JNK were determined by western blot analysis. GAPDH was used as an internal standard.

Fig. 6.

Effect of compound **5** on the modulation of crosstalk between autophagy and apoptosis. HCT116 cells were cultured with 40 μ M of compound **5** for 6, 12, 24, 36 and 48 h, and the protein expression levels of autophagy marker (LC3-II and Beclin 1) and apoptosis-related proteins (cleaved caspase 9, cleaved PARP) were determined by western blot analysis.

Fig. 7.

Schematic representing the proposed molecular mechanism underlying the action of compound **5** in induction of apoptosis and autophagy.

Table 1

NMR spectroscopic data of compounds 1 and 2.^a

No.	1		2		No.	1		2	
	δ_{H} (J in Hz)	δ_{C}	δ_{H} (J in Hz)	δ_{C}		δ_{H} (J in Hz)	δ_{C}	δ_{H} (J in Hz)	δ_{C}
1	2.91m	41.3 d	2.93m	41.1 d	Glc-I			Glc-I	
2	α 2.13m	27.1t	α 2.18m	27.0 t	1''	4.83 d (7.8)	104.2 d	4.74 d (6.0)	105.5 d
	β 2.00m		β 2.04m						
3	α 3.47m	55.0t	α 3.53m	54.9 t	2''	3.83m	82.8 d	3.61m	76.1 d
	β 3.28m		β 3.31m						
5	α 3.74m	57.1 t	α 3.82m	57.1 t	3''	3.63m	78.4 d	3.59m	75.3 d
	β 2.98m		β 3.02m						
6	α 2.18m	27.0t	α 2.18m	27.0 t	4''	3.42m	71.3d	3.62m	81.0d
	β 1.93m		β 1.94m						
7	α 2.09m	26.9t	α 2.12m	26.9 t	5''	3.15m	78.2 d	3.50 overlapped	74.0 d
	β 1.95m		β 1.98m						
8	4.20m	70.2 d	4.26m	70.4 d	6''	a 3.65 dd (5.4,12.0) b 3.78 dd (2.1,12.0)	62.6t	4.38 d (10.8)	63.8t
9	a 4.39 dd (8.4, 11.4) b 4.52 dd (6.6, 11.4)	64.2t	a 4.30 dd (8.4, 10.8) b 4.51 dd (6.0, 10.8)	64.1 t	Glc-II			Glc-II	
1'	-	127.1 s	-	127.1 s	1''	4.81 d (7.8)	105.4 d	4.33 d (7.8)	104.9 d
2'/6'	7.67 s	130.1 d	7.66s	130.0 d	2''	3.30m	76.1 d	3.21m	74.8 d
3'/5'	-	137.5s	-	137.4s	3''	3.49m	78.1 d	3.35m	77.8 d
4'	-	158.1 s	-	157.9s	4''	3.32m	71.7d	3.29m	71.4d
7'	-	167.5s	-	167.4s	5''	3.29m	78.1 d	3.35m	78.2 d
1''/1'''	3.59 d (7.2)	29.6t	3.50 overlapped	29.5 t	6''	a 3.67 dd (5.4,12.0) b 3.81 dd (2.4,12.0)	63.0t	a 3.66 br.d (10.8) b 3.90 dd (6.0, 10.8)	62.5t
2''/2'''	5.31 t (7.2)	124.0 d	5.25t (7.2)	123.8 d	OCOCH ₃	-	-	1.94s	20.7 q
3''/3'''	-	134.1 s	-	134.1 s	OCOCH ₃	-	-	-	172.3s
4''/4'''	1.76 s	18.3 q	1.73s	18.1 q					
5''/5'''	1.78 s	26.0 q	1.75s	25.9 q					

^1H NMR for 600 MHz, ^{13}C NMR for 150 MHz),
Measured in CD_3OD

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Table 2NMR spectroscopic data of compound **3**.^a

No.	3		No.		
	δ_{H} (J in Hz)	δ_{C}		δ_{H} (J in Hz)	δ_{C}
1	2.96m	41.2 d	1'''	7.17d (16.2)	123.0 d
2	α 2.19m β 2.04m	27.0 t	2'''	6.34 d (16.2)	140.8 d
3	α 3.52 overlapped β 3.32m	55.1 t	3'''	-	71.8 s
5	α 3.78 m β 3.03m	57.3 t	4'''	1.42 s	29.8 q
6	α 2.13m β 1.93m	27.0 t	5'''	1.42 s	29.9 q
7	α 2.17 m β 1.99m	26.9 t		Glc-I	
8	4.25m	70.6 d	1''''	4.72 d (7.8)	105.8 d
9	a 4.43 dd (6.0, 11.4) b 4.52 dd (6.0, 11.4)	64.2 t	2''''	3.57m	76.4 d
1'	-	127.2 s	3''''	3.60m	75.4 d
2'	8.01 d (1.8)	126.7 d	4''''	3.67m	80.6 d
3'	-	132.8 s	5''''	3.23m	76.7 d
4'	-	157.4 s	6''''	a 3.74 dd (2.4, 12.0) b 3.80 dd (3.6, 12.0)	61.8 t
5'	-	138.3 s		Glc-II	
6'	7.70 d (1.8)	130.7 d	1'''''	4.43 d (7.8)	104.6 d
7'	-	167.3 s	2'''''	3.21m	74.9 d
1''	a 3.64 overlapped b 3.52 overlapped	29.6 t	3'''''	3.23m	77.9 d
2''	5.30 t (7.2)	123.7 d	4'''''	3.30m	71.4 d
3''	-	134.2 s	5'''''	3.34m	78.2 d
4''	1.73s	18.1 q	6'''''	a 3.64 overlapped b 3.89 dd (1.8, 12.0)	62.5 t
5''	1.77s	25.9 q			

^aMeasured in CD₃OD (¹H NMR for 600 MHz, ¹³C NMR for 150 MHz).

Table 3NMR spectroscopic data of compounds **4** and **5**.^a

No.	4		5	
	δ_{H} (J in Hz)	δ_{C}	δ_{H} (J in Hz)	δ_{C}
1	3.23m	39.3 d	3.08m	41.3 d
2	α 2.38m	27.5 t	α 2.30m	27.7 t
	β 2.00m		β 2.18m	
3	α 3.90m	69.0 t	α 3.78m	64.1 t
	β 3.62m		β 3.78m	
5	α 3.78m	70.3 t	α 4.10m	65.7 t
	β 3.70m		β 3.51m	
6	α 2.30m	26.4 t	α 2.19m	27.2 t
	β 2.10m		β 2.19m	
7	α 2.26m	26.0 t	α 2.19m	25.4 t
	β 2.01m		β 2.19m	
8	4.13m	85.8 d	4.45m	80.8 d
9	a 4.29 dd (8.4, 10.8)	63.9 t	a 4.36 dd (6.4, 10.8)	63.1 t
	b 4.42 dd (6.0, 10.8)		b 4.47 dd (6.0, 10.8)	
10	-	-	5.33 br.s	69.4 t
1'	-	121.8 s	-	121.6 s
2'/6'	7.59 s	129.9 d	7.60 s	130.0 d
3'/5'	-	129.5 s	-	129.6 s
4'	-	158.8 s	-	158.9 s
7'	-	168.0 s	-	167.9 s
1''/1'''	3.33 d (7.2)	29.3 t	3.32 d (7.2)	29.3 t
2''/2'''	5.31 t (7.2)	123.0 d	5.30 t (7.2)	122.9 d
3''/3'''	-	134.3 d	-	134.3 d
4''/4'''	1.72 s	17.9 q	1.72 s	17.9 q
5''/5'''	1.77 s	26.0 q	1.77 s	26.0 q

^aMeasured in CD₃OD (¹H NMR for 600 MHz, ¹³C NMR for 150 MHz).

Table 4

NMR spectroscopic data of compounds 6–8.

No.	6^a		7^b		8^a	
	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C
1	2.92m	41.2 d	2.68m	40.8 d	2.93m	41.2 d
2	α 2.17m	27.0 t	α 1.84m	27.6 t	α 2.19m	27.0 t
	β 2.01m		β 1.62m		β 2.03m	
3	α 3.51m	54.9 t	α 3.07m	54.2 t	α 3.51m	55.0 t
	β 3.30m		β 2.66m		β 3.32m	
5	α 3.82m	57.1 t	α 3.16m	56.0 t	α 3.82m	57.2 t
	β 3.00m		β 2.49m		β 3.00m	
6	α 2.13m	26.9 t	α 1.89m	26.8 t	α 2.17m	26.9 t
	β 1.94m		β 1.76m		β 1.95m	
7	α 2.10m	26.8 t	α 1.70m	26.6 t	α 2.13m	26.8 t
	β 1.92m		β 1.42m		β 1.97m	
8	4.29m	70.3 d	3.62m	66.4 d	4.30m	70.4 d
9	a 4.36 br. d	64.0 t	a 4.28 dd	65.4 t	a 4.40 dd	64.1 t
	(8.4)		(7.8, 10.4)		(6.6, 10.8)	
	b 4.47 dd		b 4.40 dd		b 4.49 dd	
	(5.4, 10.2)		(7.2, 10.4)		(6.0, 10.8)	
1'	-	130.9 s	-	122.9 s	-	121.4 s
2'	7.75 br.s	132.8 d	7.67 br.s	130.8 d	7.97 br.s	131.5 d
3'	-	125.3 s	-	123.1 s	-	121.9 s
4'	-	164.0 s	-	161.9 s	-	163.1 s
5'	-	123.4 s	-	127.1 s	-	132.1 s
6'	7.89 br.s	126.3 d	7.67 br.s	124.4 d	7.90 br.s	126.7 d
7'	-	166.6 s	-	166.7 s	-	167.3 s
1''	3.33 (d, 7.2)	28.8 t	3.30 (d, 7.2)	28.4 t	6.65 (d, 16.2)	121.4 d
2''	5.30 (t, 7.2)	122.4 d	5.27 (t, 7.2)	121.5 d	6.72 (d, 16.2)	142.4 d
3''	-	134.3 s	-	133.3 s	-	71.6 s
4''	1.73 s	17.9 q	1.73 s	18.0 q	1.38 s	30.0 q
5''	1.74 s	25.9 q	1.74 s	25.9 q	1.38 s	30.0 q
2'''	4.34 (d, 4.2)	98.9 d	4.69 (d, 9.0)	90.2 d	4.38 (d, 4.2)	99.3 d
3'''	5.34 (d, 4.2)	73.3 d	3.19 (d, 9.0)	30.5 t	5.35 (d, 4.2)	72.9 d
4'''	-	71.8 s	-	72.0 s	-	71.7 s
5'''	1.22 s	25.8 q	1.21 s	24.2 q	1.28 s	25.4 q
6'''	1.29 s	25.1 q	1.33 s	25.9 q	1.30 s	25.6 q

^a Measured in CD₃OD (¹H NMR for 600 MHz, ¹³C NMR for 150 MHz).^b Measured in CDCl₃ (¹H NMR for 600 MHz, ¹³C NMR for 150 MHz).