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Neopierisoids A and B, Two New Chlorinated 3,4-*seco*-Grayanane Diterpenoids with Antifeedant Activity from Flowers of *Pieris japonica*

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Supporting Information

ABSTRACT: Two new chlorinated multiacylated 3,4-*seco*-grayanane diterpenoids, neopierisoids A and B (**1** and **2**), were isolated from flowers of the poisonous plant *Pieris japonica* and were identified from spectroscopic analysis and X-ray diffraction data. Both compounds showed obvious antifeedant activity against *Pieris brassicae* with an EC_{50} of 10.07 $\mu g/cm^2$ for **1** and 5.33 $\mu g/cm^2$ for **2**, indications of toxic properties. Chlorinated 3,4-*seco*-grayanane diterpenoids in *P. japonica* may play a defensive role against herbivores.

KEYWORDS: *chlorinated 3,4-seco-grayanane diterpenoids, Pieris japonica, antifeedant, defensive role*

INTRODUCTION

Plant secondary chemicals play a defensive role by discouraging insect herbivory, either by deterring feeding and oviposition or by impairing larval growth.[1] Antifeedants are found among all of the major classes of secondary metabolites: alkaloids, phenolics, and terpenoids.[2] Many diterpenoids, an important subclass of terpenoids, show antiviral,[3] antibacterial,[4] and allelopathic effects,[5] and are obvious phytoalexins with a defensive function against generalist and specialist insect and mammalian herbivores.[6−8] Grayanane diterpenoids, the main poisonous constituents of toxic species in the family Ericaceae, exhibit remarkable antifeedant and insecticidal activities.[9−11]

Among plants of southwestern China, *Pieris japonica*, a well-known poisonous plant belonging to the family Ericaceae, is widely distributed in hill and valley regions.[12,13] It has been reported that livestock fall into a coma after accidentally ingesting this plant, and even the traditional Chinese name "Ma-Zui-Mu" describes enebria in horses after consumption.[14] In folk medicine, the juice of fresh leaves can be used as an insecticide and as a lotion for the treatment of ring worm and scabies.[15]

Accordingly, we were interested in investigating the possible relationship between poisonous secondary metabolites of *P. japonica* and its plant defenses effect. In order to find new secondary metabolites with antifeedant and insecticidal activities, we studied the poisonous constituents grayanane diterpenoids in the flowers of *P. japonica*.

MATERIALS AND METHODS

General Experimental Procedures. Optical rotations were measured with a JASCO DIP-370 digital polarimeter (JASCO Corporation, Tokyo, Japan). UV data were obtained on a Shimadzu UV-2401A spectrophotometer (Shimadzu, Kyoto, Japan). A BioRad FtS-135 spectrophotometer was used for scanning IR spectroscopy with KBr pellets (Bio-Rad Corporation, Hercules, CA). One-dimensional (1D) and two-dimensional (2D) NMR spectra were recorded on DRX-400 spectrometers (Bruker BioSpin Group, German). Unless otherwise specified, chemical shifts (δ) were expressed in parts per million with reference to the solvent signals. Column chromatography was performed with silica gel (100−200 mesh) (Qingdao Marine Chemical, Inc., Qingdao, People's Republic of China) and Sephadex LH-20 (Amersham Biosciences AB, Uppsala, Sweden). EI-MS, HREIMS (70 eV) were measured on a VG Auto Spec-3000 spectrometer (VG PRIMA, Birmingham, England). Semipreparative HPLC was performed on an Agilent 1200 liquid chromatograph with a Zorbax SB-C_{18}, 250 × 9.4 mm i.d. column (Agilent, USA) at 198 nm. Fractions were monitored by TLC (Si gel GF_{254}), (Qingdao Marine Chemical Factory, Qingdao, China), and spots were visualized by heating silica gel plates sprayed with 10% H_2SO_4 in EtOH. All solvents, including petroleum ether (60−90 °C), were distilled prior to use.

Plant Material. The flowers of *P. japonica* were collected from Xundian County, Yunnan province, People's Republic of China, in April 2012. Voucher specimens (KM20120405) were deposited at the Faculty of Life Science and Technology, Kunming University of Science and Technology, and were identified by one of the authors, Haizhou Li.

Extraction and Isolation. The air-dried, powdered flowers of *P. japonica* (3.9 kg) were extracted with 75% Me_2CO/H_2O (3 × 15 L, 48 h each) at room temperature. The crude extract was then partitioned between H_2O and EtOAc, and the EtOAc fraction (580 g) was chromatographed over a 2000 × 150 mm i.d., (100−200 mesh) silical gel column (1:0, 9:1, 8:2, 7:3, and 6:4 $CHCl_3$:Me_2CO, each 10 L) to afford five fractions (fractions 1−5). Fraction 3 (8:2 $CHCl_3$:Me_2CO, 20 g) was subjected to Sephadex LH-20 chromatography on a 800 × 60 mm i.d. column (3:7, 6:4, 9:1, 1:0 MeOH:H_2O, each 3 L) to give five main subfractions (A−E). Subfraction A (3:7 MeOH:H_2O, 10.5 g) was repeatedly chromatographed on a 600 × 50 mm i.d. (10:1 $CHCl_3$:Me_2CO, 2.5 L) and a 300 × 24 mm i.d. (4:1 petroleum

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ether:Me_2CO, 1.7 L) silica gel column, respectively, to get a diterpenoids mixture of 480 mg. Then, the diterpenoids mixture was separated by semipreparative HPLC on a 250 × 9.4 mm i.d., 5 μm ZORBAX SB-C18 (Agilent, USA) column with 32% MeCN:H_2O (3 mL/min, 198 nm), to yield neopierisoids A (38 mg) and B (44 mg).

Neopierisoid A (**1**), colorless needles; $[\alpha]_D^{24}$ + 16.19 (*c* 0.20, MeOH); UV (MeOH) λ_{max} (log ε): 202.8 (2.79) nm, 274.4 (1.63) nm; IR (KBr) ν_{max}: 3443, 2986, 2943, 1783, 1732, 1640, 1550, 1462, 1429, 1370, 1242, 1223, 1103, 1082, 1056, 990, 946, 891, 844, 825, 804, 751, 719, 690, 662, 645, 608, 536, 474, 414 cm^{-1}; positive ESIMS: *m/z* 697/698 (3:1) $[M + Na]^+$; positive HREIMS *m/z* 674.2363 (calcd for $C_{31}H_{42}O_{13}Cl$, 674.2341).

Neopierisoid B (**2**), white amorphous powder; $[\alpha]_D^{24}$ + 8.09 (*c* 0.50, MeOH); UV (MeOH) λ_{max} (log ε): 203.2 (2.12) nm; IR (KBr) ν_{max}: 3444, 2987, 2944, 2885, 1998, 1782, 1732, 1640, 1549, 1463, 1429, 1373, 1263, 1222, 1169, 1104, 1082, 1054, 1022, 991, 959, 945, 903, 846, 826, 805, 750, 738, 719, 688, 642, 606, 536, 468, 414 cm^{-1}; positive ESIMS: *m/z* 697/698 (3:1) $[M + Na]^+$; positive HREIMS *m/z* 674.2331 (calcd for $C_{31}H_{42}O_{13}Cl$, 674.2341).

Antifeedant Assay. A dual-choice bioassay modified from previous methods was performed for antifeedant tests.[16] The *Pieris brassicae* larvae were reared on an artificial diet in our laboratory under a controlled photoperiod (12:8 h light:dark) and temperature (25 ± 2 °C). *P. brassicae* larvae (third instar) were starved 4−5 h prior to each bioassay. Fresh leaf discs were cut from *Brassica chinensis*, using a cork borer (1.8 cm in diameter). Treated leaf discs were painted with 20 μL of acetone solution containing the test compound (concentration 2000, 1500, 1000, 800, and 500 μg/mL) and control leaf discs with the same amount of acetone. After air drying, two tested leaf discs and two control ones were set in alternating position in the same Petri dish (150 mm in diameter) with moistened filter paper at the bottom. One third instar larvae was placed at the center of the Petri dish. Ten replicates were run for each treatment. After feeding for 24 h, areas of leaf discs consumed were measured. The relative amounts (recorded in percentages from 0 to 100) of the treated and untreated substrate area eaten in each feeding choice test were estimated visually by dividing the food area in imaginary quarters. The measurements were always done by the same operator. The antifeedant index (AFI) was calculated according to the formula AFI = $[(C - T)/(C + T)] \times 100$, where *C* and *T* represent the control and treated leaf areas consumed by the insect. The insect antifeedant potency of the test compound was evaluated in terms of the EC_{50} value (the effective dosage for 50% feeding reduction), which was determined by Probit analysis for each insect species.

HPLC Analysis of the Neopierisoids A and B and the Crude Extract. A treated EtOAc extract was prepared by using the crude EtOAc extract subjected to Sephadex LH-20 chromatography and eluted with CH_3OH to get rid of most flavonoids and phenolic compounds and enrich diterpenoids. The neopierisoids A and B (Figure 5A) and treated EtOAc extract (Figure 5B) were analyzed by HPLC to identify neopierisoids A and B (**1** and **2**) (see Figure 1). HPLC analysis was performed on an Agilent 1200 liquid chromatograph with 28% MeCN:H_2O (1 mL/min, 198 nm) on a 250 × 4.6 mm i.d., 5 μm, ZORBAX SB-C18 (Agilent).

X-ray Crystallographic Analysis of Neopierisoid A (1). Neopierisoid A (**1**): $C_{31}H_{42}O_{13}Cl$, *M* = 674.23, colorless needles, size 0.52 × 0.48 × 0.13 mm^3. Crystal data for neopierisoid A (**1**): $2(C_{31}H_{43}ClO_{14})\cdot C_2H_6O\cdot 2(H_2O)$, *M* = 1432.31, monoclinic, *a* = 13.1973(18) Å, *b* = 10.9585(15) Å, *c* = 24.226(3) Å, α = 90.00°, β = 100.216(2)°, γ = 90.00°, *V* = 3448.1(8) $Å^3$, *T* = 100(2) K, space group *P*21, *Z* = 2, μ(Mo Kα) = 0.183 mm^{-1}, 34543 reflections measured, 17211 independent reflections (R_{int} = 0.0446). The final R_1 values were 0.0627 [$I > 2\sigma(I)$]. The final $wR(F^2)$ values were 0.1628 [$I > 2\sigma(I)$]. The final R_1 values were 0.0865 (all data). The final $wR(F^2)$ values were 0.1804 (all data). The goodness of fit on F^2 was 1.037. Flack parameter = 0.02(7). The Hooft parameter is 0.07(3) for 7195 Bijvoet pairs.

Crystallographic data for the structure of neopierisoid A (**1**) has been deposited in the Cambridge Crystallographic Data Centre (deposition number CCDC 917847). Copies of the data can be obtained free of charge from the CCDC via www.ccdc.cam.ac.uk.

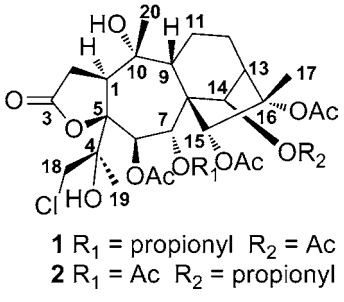

Figure 1. Chemical structures of compounds **1** and **2**.

RESULT AND DISCUSSION

Compound **1** was obtained as colorless needles from methanol. Its positive ESIMS showed an $[M + Na]^+$ peak at *m/z* 697/698 (rel. 3:1), indicating the presence of a chlorine atom in **1**. The molecular formula ($C_{31}H_{43}O_{14}Cl$, with ten degrees of unsaturation) of **1** was confirmed from HRESIMS. The IR spectrum showed absorption bands for hydroxy (3444 cm^{-1}), lactone (1784 cm^{-1}),[17] and ester carbonyl (1733 cm^{-1}) functional groups.[18−20]

The ^1H NMR of **1** in C_5D_5N showed resonances attributed to a propionyl unit at δ_H 1.34 (3H, t, *J* = 7.5 Hz), 2.58 (1H, m), 2.68 (1H, m), as well as four acetyl methyls at δ_H 2.12, 2.13, 2.16, and 2.18 (s, each 3H) (Table 1). In addition, three tertiary methyls at δ_H 1.47, 1.68, and 1.77 (s, each 3H) were observed in the high-field region. Resonances due to an AB group at [δ_H 4.18 (d, *J* = 11.5 Hz), 4.36 (d, *J* = 11.5 Hz)], together with an ABX group at δ_H [2.90 (dd, *J* = 18.0, 8.0 Hz), 3.10 (dd, *J* = 18.0, 11.9 Hz), and 4.04 (dd, *J* = 11.9, 8.0 Hz)] were also present. The low-field region displayed four acetoxy methines at δ_H 5.44 (br s), 5.72 (d, *J* = 8.4 Hz), 6.73 (br s), and 6.75 (d, *J* = 2.6 Hz).

Analyses of the ^{13}C NMR and DEPT spectra of **1** revealed 31 carbon resonances (Table 1), including eight methyls (one propionyl methyl and four acetyl methyls), five methylenes, seven methines (four oxygen-bearing), and eleven quaternary carbons (one lactone, one propionyl ester carbonyl groups, four acetyl ester carbonyl groups, and three oxygen-bearing). These data suggested that compound **1** is a highly acylated pentacyclic diterpenoid with a C_{20} nucleus different from the normal grayanane or kalmane skeletons reported previously.[15]

The HMBC spectrum of **1** showed obvious correlations from Me-19 (δ_H 1.77, s) to C-4, C-5, and C-18, and from the AB methylene H-18a to C-4, C-5, and C-19 and H-18b to C-19. Furthermore, the ABX system H-2a and H-2b displayed HMBC correlations with C-1, C-3, and C-10, as well as H-2a to C-5 and H-1 to C-2, C-3, C-4, C-5, C-6, C-9, and C-10. Other HMBC correlations were noted from H-6 to an acetyl carbonyl (δ_C 169.1 s) and Me-20 to C-1, C-5, C-9, and C-10. The lactone group was located between C-3 (δ_C 175.2, s) and C-5 (δ_C 94.0, s) rather than between C-3 and C-10 based on the fact that C-5 was shifted by $\Delta\delta_H$ −21.0 ppm in **1** compared with pierisoid A[22] (C-5, δ_C 73.0, s) and the observed HMBC correlations from 10-OH (δ_H 7.36, s) to C-1, C-9, C-10, and C-20. The chlorine atom was assigned at C-18, because of the extraordinary high-field shift of C-18 (δ_C 54.0, t) compared with the oxygenated methylenes (δ_C 60−70).[18,21] The partial structure **1a** (Figure 2) was deduced from the above evidence, coupled with a H-1/H_2-2 proton spin system established from $^1H-^1H$ COSY correlations.

Detailed analyses of the $^1H-^1H$ COSY and HSQC spectra starting from the proton H-9 (δ_H 2.73, d, *J* = 8.0 Hz) revealed the

Table 1. ^{13}C and ^{1}H NMR Spectroscopic Data of Neopierisoids A (1) and B (2) (pyridine-d_5, δ in ppm)[a]

position	1		2	
	^{13}C	^{1}H	^{13}C	^{1}H
1	51.2 d	4.04 (dd, 11.9, 8.0)	51.2 d	4.04 (dd, 11.9, 8.0)
2a	33.6 t	3.10 (dd, 18.0, 11.9)	33.6 t	3.10 (dd, 18.0, 11.9)
2b		2.90 (dd, 18.0, 8.0)		2.88 (dd, 18.0, 8.0)
3	175.2 s		175.3 s	
4	78.6 s		78.6 s	
5	94.0 s		94.1 s	
6	69.7 d	6.73 (br s)	69.6 d	6.74 (d, 8.5)
7	68.0 d	5.72 (d, 8.4)	68.2 d	5.69 (d, 8.4)
8	55.7 s		55.7 s	
9	48.2 d	2.73 (d, 8.0)	48.1 d	2.70 (d, 8.0)
10	76.7 s		76.6 s	
11a	20.2 t	1.74 (m)	20.3 t	1.71 (m)
11b		1.64 (m)		1.64 (m)
12a	25.2 t	1.72 (m)	25.3 t	1.67 (m)
12b		1.62 (m)		1.62 (m)
13	45.0 d	3.61 (m)	45.3 d	3.56 (m)
14	79.3 d	6.75 (d, 2.6)	79.1 d	6.81 (br s)
15	87.2 d	5.44 (br s)	87.0 d	5.46 (br s)
16	88.2 s		88.3 s	
17	19.0 q	1.68 (s)	19.0 q	1.69 (s)
18a	54.0 t	4.36 (d, 11.5)	54.0 t	4.36 (d, 11.5)
18b		4.18 (d, 11.5)		4.18 (d, 11.5)
19	20.5 q	1.77 (s)	20.5 q	1.77 (s)
20	34.0 s	1.47 (s)	34.0 s	1.47 (s)
6-OAc	21.0 q	2.16 (s)	21.8 q	2.15 (s)
	169.1 s		169.1 s	
7-OPr/OAc	9.3 q	1.34 (t, 7.5)	21.1 q	2.33 (s)
	28.4 t	2.68 (m)	169.9 s	
	173.0 s	2.58 (m)		
14-OAc/OP r	22.1 q	2.12 (s)	9.3 q	1.30 (t, 7.4)
	170.1 s		28.4 t	2.56 (m)
			174.1 s	2.36 (m)
15-OAc	22.8 q	2.13 (s)	20.9 q	2.16 (s)
	172.1 s		172.0 s	
16-OAc	21.2 q	2.18 (s)	22.9 q	2.13 (s)
	170.7 s		170.1 s	
10-OH		7.36 (s)		7.36 (s)

[a] ^{1}H NMR spectra were recorded at 500 MHz and ^{13}C NMR spectra at 125 MHz, and the assignments were based on DEPT, HSQC, COSY, HMBC, and ROESY experiments.

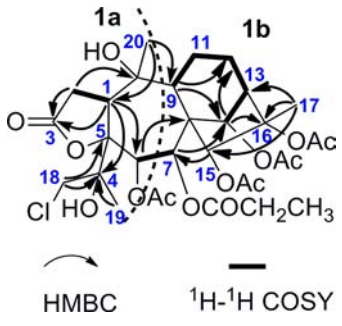

Figure 2. Structural fragments and key HMBC and $^{1}H-^{1}H$ COSY correlations of **1**.

presence of the following spin system: $CHCH_2CH_2CHCH$, H-9/H_2-11/H_2-12/H-13/H-14. This information was confirmed by key HMBC correlations from H-11b (δ_H 1.64, m) to C-8 and C-13, H-13 (δ_H 3.61, m) to C-8, C-11, C-14, C-15, and C-16, Me-17 (δ_H 1.68, s) to C-13, C-15, and C-16, and H-15 (δ_H 5.44 br s) to C-7, C-8, C-9, C-14, C-16, and C-17. Meanwhile, the HMBC spectrum showed correlations of H-14 and H-15 with two acetyl carbonyls, respectively, and of H-7 with the propionyl carbonyl. These correlations located the two acetoxy groups at C-14 and C-15 and the propionyloxyl at C-7. The remaining acetoxy moiety was located unequivocally at C-16 on the basis of the oxygenated nature of C-16 and the molecular formula of 1. These data further corroborated the structure of fragment **1b** (Figure 2).

Moreover, HMBC correlations from H-9 to C-8, C-10, C-11, C-15, and C-20 and from H-7 to C-5, C-6, C-8, C-9, and C-14, along with the proton spin system of H-6 and H-7, established by $^{1}H-^{1}H$ COSY, permitted **1a** and **1b** to be jointed through carbon−carbon connections between C-9 and C-10 and between C-6 and C-7. Accordingly, the planar structure of compound **1** could be established.

In the ROESY spectrum of **1**, cross-peaks observed between H-1α/H-6/Me-19, H-6/H-14, and H-13/H-14 demonstrated that H-1, H-6, H-13, H-14, and possibly Me-19, have α orientations (Figure 3). Meanwhile, H-7, H-9, H-15, Me-17,

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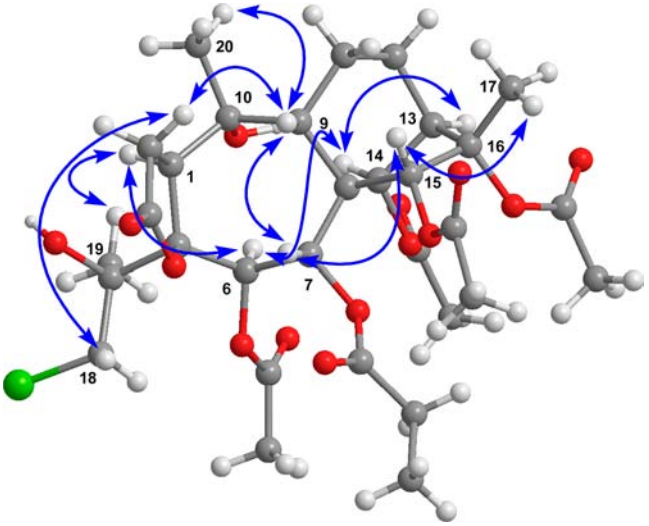

Figure 3. Key ROESY correlations of **1**.

C-18, and Me-20 were established as having β orientations based on NOE interactions of H-2β with H-7, H-9, and H_2-18, H-7 with H-9 and H-15, H-9 with Me-20, and H-15 with Me-17. However, the relative configuration of C-4 could not be determined from the ROESY experiment due to the free rotation of the σ bond between C-4 and C-5. Fortunately, a single-crystal X-ray [anomalous scattering of Mo Kα radiation with a Flack parameter of 0.02 (7) and a Hooft parameter of 0.07 (3) for 7195 Bijvoet pairs (CCDC917847)] was obtained, and thus, the stereochemistry of C-4 could be fully determined. The absolute configurations of **1** were confirmed as 1*S*, 4*S*, 5*S*, 6*R*, 7*S*, 8*S*, 9*R*, 10*R*, 13*R*, 14*R*, 15*R*, and 16*S*, and its chemical structure was fully elucidated. The new compound has been named neopierisoid A (Figure 4).

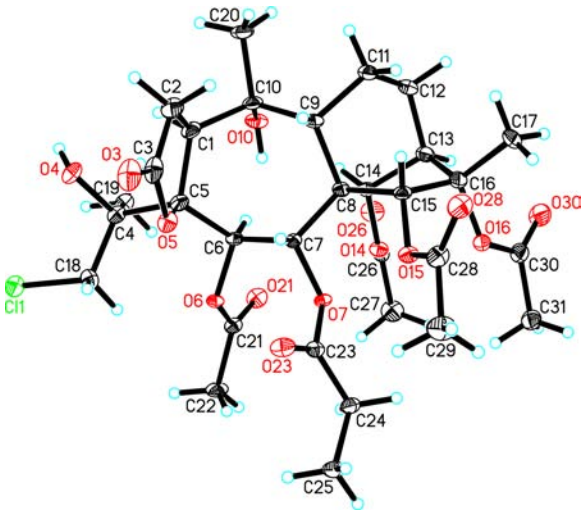

Figure 4. X-ray crystallographic structure of **1**.

Neopierisoid B (**2**) was obtained as white amorphous powder. Its molecular formula was assigned as $C_{31}H_{42}O_{13}Cl$ by HRESIMS. Comparison of the IR, MS, 1D (^{1}H, ^{13}C, and DEPT) (Table 1), and 2D (^{1}H−^{1}H COSY, HSQC, HMBC, and ROESY) NMR spectroscopic data of **2** with those of **1** clearly revealed that **2** was also a 18-chlorinated-3,4-*seco*-grayanane diterpenoid. The only difference between the two compounds was that the propionyloxy group at C-7 in **1** was replaced by an acetoxy group in **2**, and the acetoxy group at C-14 in **1** was replaced by a propionyloxy group in **2**. Accordingly, the struture of neopierisoid B was determined, as shown in **2**.

Since neopierisoids A and B were the first examples of organohalogenated compounds from the family Ericaceae, it is not definite that these compounds originally occurred in the plant. Experiments should be carried out to verify that the compounds **1** and **2** came from the extract of the plant. Since no chlorinated solvent was used for extraction, a treated EtOAc extract was prepared by using the crude EtOAc extract subjected to Sephadex LH-20 (eluted with CH_3OH) to get rid of most flavonoids and phenolic compounds and enrich diterpenoids. Then, the treated EtOAc extract was analyzed by HPLC to identify neopierisoids A and B (**1** and **2**). The retention times of **1** and **2** (R_t = 26.6 and 28.8 min, respectively) (Figure 5, panels **A** and **B**) were in good agreement with those in the treated EtOAc extract (Figure 5**C**), which indicated that the compounds were not artifacts. Other evidence came from the fact that no chlorinated diterpenes were obtained from reactions of possible precursors such as secorhodomollolide B[15] and pierisoid A[22] in chloroform at room temperature and 60 °C overnight. Furthermore, although a chlorinated solvent was also used in the extraction and isolation in previous experiments,[15,22] no chlorinated compounds were found. Actually, it is very difficult for halogenation to occur on unactivated methyl carbon atoms, so the hypothesis that neopierisoids A and B are artifacts is unlikely. Large numbers of organohalogenated compounds have been found in marine plants and animals; however, only very small numbers of halogenated compounds have been isolated from terrestrial plants.[23−25] Halogenation at aliphatic, unactivated carbon centers is usually more difficult than at aromatic, electron-rich substrates.[26] Thus, neopierisoids A and B represent a new class of chlorinated diterpenes. In accordance with Vaillancourt,[27] natural products halogenated on aliphatic carbons could be generated enzymatically. Iron enzymes and generated high-valent oxoiron species could act as powerful oxidants to catalyze the addition of a radical Cl• species on an aliphatic carbon.[28] The chlorination mechanism of compounds **1** and **2** might be related to catalysis by such enzymes, requiring a thorough biochemical study of the plant.

Compounds **1** and **2** were tested for their antifeedant effects against *P. brassicae*, a plant-feeding generalist insect herbivore, using an established method as described previously.[29] Both compounds showed obvious antifeedant activities, with an EC_{50} of 10.07 μg/cm^2 for **1** and 5.33 μg/cm^2 for **2**. In accordance with a previous report, [30] neem oil containing 1% azadirachtin as a commericial antifeedant showed antifeedant activities against two generalist insect herbivores, beet armyworm (*Spodoptera exigua*) and cotton bollworm (*Helicoverpa armigera*) with EC_{50} = 3.71 μg/cm^2 and 2.62 μg/cm^2, respectively. Although neopierisoids A and B are less active than neem oil containing 1% azadirachtin, they also displayed good antifeedant activity. These toxic properties of **1** and **2** might suggest a defensive role of 18-chlorinated-3,4-*seco*-grayanane diterpenoids in *P. japonica* against herbivores.

The interest in *P. japonica* is mainly because of its toxicity to mammals. It is reported that 36 samples of ericaceous toxins and their congeners showed acute toxicity on mice.[31] The poisonous mechanism of grayanotoxin is probably that grayanotoxin exerts selective effects on voltage-dependent sodium channels by eliminating fast sodium inactivation and causing a hyperpolarizing shift in voltage dependence of channel activation.[32]

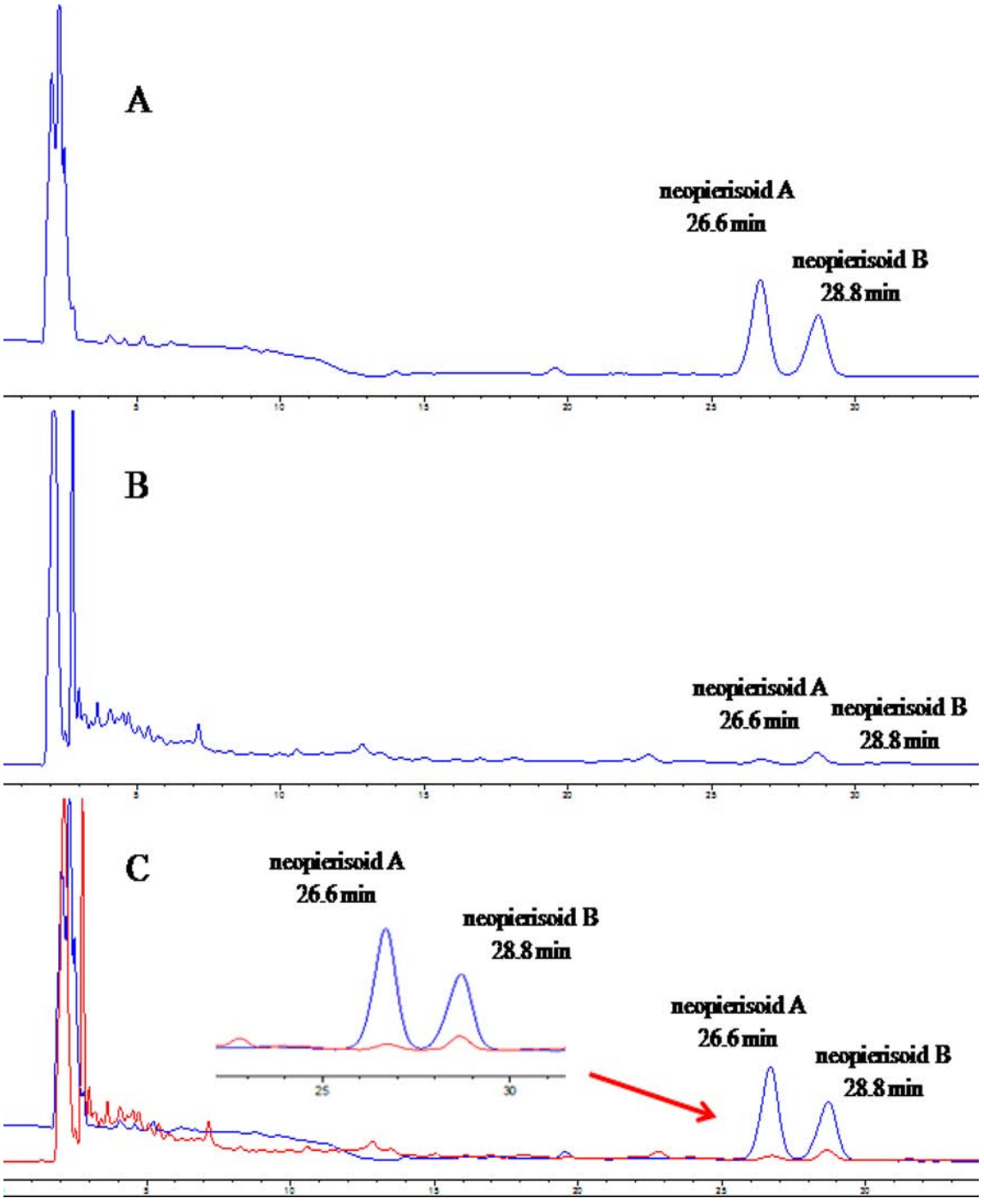

Figure 5. (A) HPLC analysis of neopierisoids A and B. Neopierisoid A with the retention time R_t = 26.6 min and neopierisoid B with the retention time R_t = 28.8 min. λ_{max} = 198 nm. (B) HPLC analysis of the treated EtOAc extract. Neopierisoid A, R_t = 26.6 min and neopierisoid B, R_t = 28.8 min. λ_{max} = 198 nm. (C) The overlapped and amplified picture of panels A and B .

Compared with the toxicity to mammals of grayanotoxin, its antifeedant and insecticidal activities were actually not well-studied. However, Klocke and colleagues showed that the most active constituents with antifeedant activity in the flowers of *Rhododendron molle* were identified as rhodojaponin-III, grayanotoxin III, and kalmanol.[33] Furthermore, *R. molle*, which is rich in grayanane diterpenoids, had been developed into a commercial botanical insecticide.[34] The results of antifeedant assay of neopierisoids A and B might provide some reference for the developing of *P. japonica* or other grayanane diterpenoid abundant plants to a commercial botanical insecticide.

Neopierisoids A and B are two unusual highly acylated chlorinated 3,4-*seco*-grayanane diterpenoids with a 5,7,6,5 ring system and 12 chiral centers obtained from the family Ericaceae. In addition, the antifeedant effects of these two grayananes suggest that the poisonous constituents of *P. japonica* might relate with its defensive function.

■ ASSOCIATED CONTENT

Supporting Information

Figures include spectra and overlapped and amplified picture of Figures 22 and 23. Tables include crystal data and structure

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refinement for neopierisoid A, atomic coordinates, bond lengths, anisotropic displacement parameters, hydrogen coordinates and isotropic displacement parameters, torsion angles, and hydrogen bonds. Detailed experimental procedures, method for antifeedant activity testing, physicochemical properties, 1D and 2D NMR, MS, UV, IR, ORD data of compounds **1** and **2**, and X-ray crystal structure of **1**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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REFERENCES

(1) Isman, M. Insect antifeedants. *Pestic. Outlook* **2002**, *13*, 152−157.
(2) Akhtar, Y.; Isman, M. B.; Niehaus, L. A.; Lee, C.-H.; Lee, H.-S. Antifeedant and toxic effects of naturally occurring and synthetic quinones to the cabbage looper, Trichoplusia ni. *Crop Prot.* **2012**, *31*, 8−14.
(3) Kittakoop, P.; Wanasith, S.; Watts, P.; Kramyu, J.; Tanticharoen, M.; Thebtaranonth, Y. Potent antiviral potamogetonyde and potamogetonol, new furanoid labdane diterpenes from *Potamogeton malaianus*. *J. Nat. Prod.* **2001**, *64*, 385−388.
(4) Jaki, B.; Heilmann, J.; Sticher, O. New antibacterial metabolites from the cyanobacterium *Nostoc commune* (EAWAG 122b). *J. Nat. Prod.* **2000**, *63*, 1283−1285.
(5) Kong, C.; Liang, W.; Xu, X.; Hu, F.; Wang, P.; Jiang, Y. Release and activity of allelochemicals from allelopathic rice seedlings. *J. Agric. Food. Chem.* **2004**, *52*, 2861−2865.
(6) Zhang, H. P.; Wang, L. Q.; Qin, G. W. Grayanane diterpenoids from the leaves of *Craiobiodendron yunnanense*. *Bioorg. Med. Chem.* **2005**, *13*, 5289−5298.
(7) Langenheim, J. H. Higher plant terpenoids: A phytocentric overview of their ecological roles. *J. Chem. Ecol.* **1994**, *20*, 1223−1280.
(8) Raccuglia, R. A.; Bellone, G.; Loziene, K.; Piozzi, F.; Rosselli, S.; Maggio, A.; Bruno, M.; Simmonds, M. S. J.; Hastifolins, A.-G. Antifeedant *neo*-clerodane diterpenoids from *Scutellaria hastifolia*. *Phytochemistry* **2010**, *71*, 2087−2091.
(9) Sakakibara, J.; Shirai, N.; Kaiya, T. Diterpene glycosides from *Pieris japonica*. *Phytochemistry* **1981**, *20*, 1744−1745.
(10) Zhong, G.; Hu, M.; Wei, X.; Weng, Q.; Xie, J.; Liu, J.; Wang, W. Grayanane diterpenoids from the flowers of *Rhododendron molle* with cytotoxic activity against a *spodoptera frugiperda* cell line. *J. Nat. Prod.* **2005**, *68*, 924−926.
(11) Wu, Z. Y.; Li, H. Z.; Wang, W. G.; Li, H. M.; Chen, R.; Li, R. T.; Luo, H. R. Lyonin A, a new 9,10-secograyanotoxin from *Lyonia ovalifolia*. *Chem. Biodiversity* **2011**, *8*, 1182−1187.
(12) Yao, G. M.; Ding, Y.; Zuo, J. P.; Wang, H. B.; Wang, Y. B.; Ding, B. Y.; Chiu, P.; Qin, G. W. Dihydrochalcones from the leaves of *Pieris japonica*. *J. Nat. Prod.* **2005**, *68*, 392−396.
(13) Shirai, N.; Sakakibara, J.; Kaiya, T.; Kobayashi, S.; Hotta, Y.; Takeya, K. Quantitative structure-inotropy relationship applied to substituted grayanotoxins. *J. Med. Chem.* **1983**, *26*, 851−855.
(14) Chow, S.; Kress, C.; Albaek, N.; Jessen, C.; Williams, C. M. Determining a synthetic approach to pierisformaside C. *Org. Lett.* **2011**, *13*, 5286−5289.
(15) Wang, S.; Lin, S.; Zhu, C.; Yang, Y.; Li, S.; Zhang, J.; Chen, X.; Shi, J. Highly acylated diterpenoids with a new 3,4-secograyanane skeleton from the flower buds of *Rhododendron molle*. *Org. Lett.* **2010**, *12*, 1560−1563.
(16) Isman, M. B; Koul, O.; Luczynski, A.; Kaminski, J. Insecticidal and antifeedant bioactivities of neem oils and their relationship to azadirachtin content. *J. Agric. Food Chem.* **1990**, *38*, 1406−1411.
(17) Li, S. H.; Wang, J.; Niu, X. M.; Shen, Y. H.; Zhang, H. J.; Sun, H. D.; Li, M. L.; Tian, Q. E.; Lu, Y.; Cao, P.; Zheng, Q. T. Maoecrystal V cytotoxic diterpenoid with a novel C19 skeleton from *Isodon eriocalyx* (Dunn.) hara. *Org. Lett.* **2004**, *6*, 4327−4330.
(18) Wang, W. G.; Du, X.; Li, X. N.; Wu, H. Y.; Liu, X.; Shang, S. Z.; Zhan, R.; Liang, C. Q.; Kong, L. M.; Li, Y.; Pu, J. X.; Sun, H. D. New bicyclo[3.1.0]hexane unit *ent*-kaurane diterpene and its *seco*-derivative from *Isodon eriocalyx* var. *laxiflora*. *Org. Lett.* **2012**, *14*, 302−305.
(19) Wang, W. G.; Li, X. N.; Du, X.; Wu, H. Y.; Liu, X.; Su, J.; Li, Y.; Pu, J. X.; Sun, H. D. Laxiflorolides A and B, epimeric bishomoditerpene lactones from *Isodon eriocalyx*. *J. Nat. Prod.* **2012**, *75*, 1102−1107.
(20) Nakanishi, K.; Solomon, P. H. *Infrared Absorption Spectroscopy*, 2nd ed.; Holden Day: Oakland, 1977; pp 17−44.
(21) Breitmaier, E.; Voelter, W. *Carbon-13 NMR Spectroscopy*, VCH: Weinheim, 1987; pp 183−280.
(22) Li, C.-H.; Niu, X.-M.; Luo, Q.; Xie, M.-J.; Luo, S.-H.; Zhou, Y.-Y.; Li, S.-H. Novel polyesterified 3,4-*seco*-grayanane diterpenoids as antifeedants from *Pieris formosa*. *Org. Lett.* **2010**, *12*, 2426−2429.
(23) Gribble, G. W. The diversity of naturally produced organohalogens. *Chemosphere* **2003**, *52*, 289−297.
(24) Blasiak, L. C.; Drennan, C. L. Structural perspective on enzymatic halogenation. *Acc. Chem. Res.* **2009**, *42*, 147−155.
(25) Runguphan, W.; Qu, X.; O'Connor, S. E. Integrating carbon-halogen bond formation into medicinal plant metabolism. *Nature* **2010**, *468*, 461−464.
(26) Vaillancourt, F. H.; Yeh, E.; Vosburg, D. A.; O'Connor, S. E.; Walsh, C. T. Cryptic chlorination by a non-haem iron enzyme during cyclopropyl amino acid biosynthesis. *Nature* **2005**, *436*, 1191−1194.
(27) Vaillancourt, F. H.; Yeh, E.; Vosburg, D. A.; Garneau-Tsodikova, S.; Walsh, C. T. Nature's inventory of halogenation catalysts: Oxidative strategies predominate. *Chem. Rev.* **2006**, *106*, 3364−3378.
(28) Allard, P.-M.; Martin, M.-T.; Dau, M.-E. T. H.; Leyssen, P.; Gueritte, F.; Litaudon, M. Trigocherrin A, the first natural chlorinated daphnane diterpene orthoester from *Trigonostemon cherrieri*. *Org. Lett.* **2012**, *14*, 342−345.
(29) Luo, S. H.; Luo, Q.; Niu, X. M.; Xie, M. J.; Zhao, X.; Schneider, B.; Gershenzon, J.; Li, S. H. Glandular trichomes of *Leucosceptrum canum* harbor defensive sesterterpenoids. *Angew. Chem., Int. Ed.* **2010**, *49*, 4471−4475.
(30) Li, C. H.; Jing, S. X.; Luo, S. H.; Shi, W.; Hua, J.; Liu, Y.; Li, X. N.; Schneider, B.; Gershenzon, J.; Li, S. H. Peltate glandular trichomes of *Colquhounia coccinea* var. *mollis* harbor a new class of defensive sesterterpenoids. *Org. Lett.* **2013**, *15*, 1694−1697.
(31) Hikino, H.; Ohta, T.; Ogura, M.; Ohizumi, Y.; Konno, C.; Takemoto, T. Structure-activity relationship of ericaceous toxins on acute toxicity in mice. *Toxicol. Appl. Pharmacol.* **1976**, *35*, 303−310.
(32) Maejima, H.; Kinoshita, E.; Seyama, I.; Yamaoka, K. Distinct sites regulating grayanotoxin binding and unbinding to D4S6 of Na(v)1.4 sodium channel as revealed by improved estimation of toxin sensitivity. *J. Biol. Chem.* **2003**, *278*, 9464−9471.
(33) Klocke, J. A.; Mei-Ying, H.; Shin-Foon, C.; Kubo, I. Grayanoid diterpene insect antifeedants and insecticides from *Rhododendron molle*. *Phytochemistry* **1991**, *30*, 1797−1800.
(34) Popescu, R.; Kopp, B. The genus *Rhododendron*: An ethnopharmacological and toxicological review. *J. Ethnopharmacol.* **2013**, *147*, 42−62.