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Biological and chemical guided isolation of 3,4-secograyanane diterpenoids from the roots of *Pieris formosa*†

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Seventeen new 3,4-secograyanoids (1–17), together with seven known compounds (18–24), were isolated from the roots of *Pieris formosa*. Their structures with absolute configurations were characterized by a series of spectroscopic methods and X-ray diffraction. Compounds 1, 2, 4–8, 10–13, and 16–24 exhibited significant analgesic activity at 5.0 mg kg^{−1} (ip) compared to vehicle-injected mice ($p < 0.05$). In particular, compounds 16 and 17 showed highly potent activities with inhibition rates of 63.5% and 69.9%, respectively.

Introduction

Grayanoids, a special type of diterpenoid, exist exclusively in the Ericaceae family with diverse structures and wide bioactivities.¹ To date, almost two hundred grayanoids with eleven carbon skeletons, including grayanane,^{2,3} 1,5-secograyanane,⁴ 3,4-secograyanane,⁵ 9,10-secograyanane,⁶ 1,10:2,3-disecograyanane,⁷ kalmane,⁸ 1,5-secokalmane,⁹ leucothane,¹⁰ micranthane,¹¹ mollane,¹² and rhodomollane,¹³ have been reported. Notably, 3,4-secograyanane is a rare type of grayanoid, and only seventeen 3,4-secograyanoids have been isolated and characterized. Several grayanoids display analgesic and insect antifeedant activities.

Pieris formosa (Wall) D. Don, a traditional Chinese medicine, has been used to treat tinea and scabies.¹⁴ In previous phytochemical investigations of *P. formosa*, fifteen grayananes and three diterpenes with an unusual carbon skeleton were isolated and characterized, and most compounds were found to exhibit an analgesic effect.^{15,16} In our on-going efforts, biological and chemical guided fractionation led to the isolation of seventeen new 3,4-secograyananes (1–17), together with seven known 3,4-secograyanoids (18–24) (see Fig. 1). Herein, we report their isolation, structural elucidation, and bioactivity. The main text of the article should appear here with headings as appropriate.

Results and discussion

Biological and chemical guided fractionation and isolation of the roots of *P. formosa*

The fractionation and isolation procedures were guided by acetic acid-induced writhing test and online HPLC/UV/ESIMS² analyses. The roots of *P. formosa* were extracted with 95% EtOH and the evaporation of the solvent under reduced pressure subsequently yielded a black residue. The residue was mixed with diatomite (1 : 2) and Soxhlet was successively extracted thereafter using a gradient of petroleum ether, CH₂Cl₂, EtOAc and CH₃OH. Acetic acid-induced writhing test showed analgesic activity for the CH₂Cl₂ portion (D) and the EtOAc portion (E), with 20% and 25% writhing inhibitions (100 mg kg^{−1}, op), respectively. Subsequently, the CH₂Cl₂ portion (D) and the EtOAc portion (E) were applied to a macroporous resin D101 column and eluted with a gradient of 30% EtOH–H₂O, 50% EtOH–H₂O and 70% EtOH–H₂O, to yield three fractions, respectively (D1–D3, and E1–E3). Fractions D1, D2, E1, and E2 showed analgesic activity with 20%, 33%, 73%, and 41% writhing inhibition (100 mg kg^{−1}, op), respectively. Fractions D1 and D2 were separated through a polyamide column and eluted with H₂O–EtOH (100 : 0, 30 : 70, 50 : 50, 70 : 30, v/v), to yield eight fractions (D1a–D1d and D2a–D2d). Fractions E1 and E2 were separated through a polyamide column and eluted with a gradient of H₂O–EtOH (100 : 0, 30 : 70, 60 : 40, v/v), to yield six fractions (E1a–E1c and E2a–E2c). Online HPLC/UV/ESIMS² analyses showed that there were many constituents that exhibited a maximum absorption band at λ 210–220 nm in the UV spectra and [M – 60]⁺, [M – 2 × 60]⁺, and [M – 3 × 60]⁺ fragment ions in the positive MS² mode in fractions D1a, D2a, and D2b (see Fig. S1–S3†), characteristic of the highly acylated 3,4-secograyanane diterpenoids.⁵ Similarly, there were many constituents that exhibited a maximum absorption band at λ

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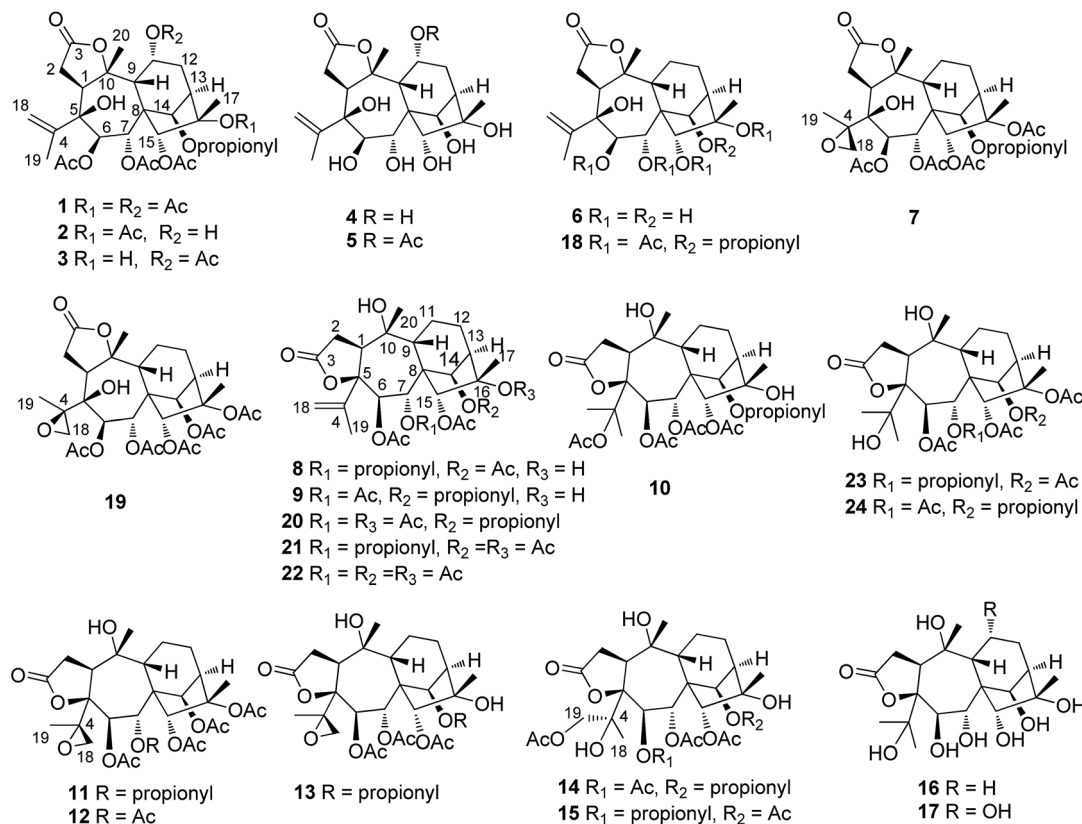


Fig. 1 Chemical structures of the isolated compounds 1–24.

210–220 nm in the UV spectra and $[M - 18]^+$, $[M - 2 \times 18]^+$, and $[M - 3 \times 18]^+$ fragment ions in the positive MS^2 mode in fractions E1a, E1b, and E2a (see Fig. S4–S6[†]), characteristic of the highly hydroxylated grayanane diterpenoids. Finally, seventeen new 3,4-secograyanoids (1–17), together with seven known 3,4-secograyanoids (18–24), were isolated from the fractions D1a, D2a, D2b, E1a, E1b, and E2a by a combination of different chromatographic methods. Acetic acid-induced writhing test showed that compounds 1, 2, 4–8, 10–13, and 16–24 exhibited significant analgesic activity at 5.0 mg kg^{-1} (ip).

Characterization of compounds 1–17

Pieristoxinlactone A (1) was obtained as a colorless crystal, and its molecular formula was determined to be $\text{C}_{33}\text{H}_{44}\text{O}_{15}$ based on the HRESIMS $[M + \text{Na}]^+$ ion peak m/z 703.2568 (calcd 703.2572), indicating twelve indices of hydrogen deficiency. Its ^1H NMR spectrum (see Table 1) showed one triplet methyl (δ_{H} 1.35, t, $J = 7.0$ Hz) and eight singlet methyls (δ_{H} 2.25, 2.24, 2.19, 2.18, 2.12, 2.00, 1.73, 1.70). In the low-field region, a pair of AB doublets (δ_{H} 6.41, 5.64, d, $J = 10.0$ Hz) and an exchangeable hydroxy proton (δ_{H} 7.26) were also observed. The ^{13}C NMR (see Table 2) and DEPT spectra displayed 33 carbon resonances, ascribed to nine methyls, four methylenes (one olefinic at δ_{C} 113.9), eight methines (five oxygenated at δ_{C} 92.2, 75.6, 74.0, 68.6, 67.5), and twelve quaternary carbons (seven ester carbonyls at δ_{C} 174.8, 174.1, 171.9, 170.7, 170.3, 169.1, 168.9; one olefinic at δ_{C} 147.9; three oxygenated at δ_{C} 88.3, 88.2, 76.3). The ^1H – ^1H COSY (see

Fig. 3) and HSQC spectra revealed the presence of four spin systems: C(1)H–C(2)H₂, C(6)H–C(7)H, C(9)H–C(11)H–C(12)H₂–C(13)H–C(14)H, and a propionyl unit. These structural features suggested that 1 was a highly acylated 3,4-secograyanane. Further HSQC and HMBC experiments (see Fig. 3) enabled the full assignments of the ^1H and ^{13}C NMR spectra of 1. Compound 1 showed the same planar structure as secorhomololidine D⁵. NOESY correlations (see Fig. 3) from H-14 to H-1 and H-12a and from H-6 to H-1, H-14, and H₃-19 revealed that these protons were α -oriented. In contrast, the NOESY correlations from H-7 to H-9 and H₃-20, from H-15 to H-9 and H-12b, from H₃-17 to H-12b, suggested that these protons were β -oriented. Furthermore, the small coupling constant between H-9 and H-11 ($^3J_{\text{H9,H11}} = 5.0$ Hz) in the ^1H NMR spectrum, with the aid of NOESY correlations from H-11 to H-9 and H-12b, indicated that H-11 was β -orientated. To further corroborate the structural assignment and establish the absolute configuration, a single X-ray diffraction [Cu K α radiation; Flack parameter: 0.00(8)] of 1 (see Fig. 2; CCDC: 1548366[†]) was successfully carried out, allowing an explicit assignment of the absolute configuration of 1 as 1*S*, 5*S*, 6*R*, 7*R*, 8*S*, 9*R*, 10*R*, 11*R*, 13*R*, 14*R*, 15*R*, 16*S*. Thus, the structure of 1 was defined, and it was named pieristoxinlactone A.

Pieristoxinlactone B (2) was assigned the molecular formula $\text{C}_{31}\text{H}_{42}\text{O}_{14}$ based on the HRESIMS ion at m/z 661.2477 $[M + \text{Na}]^+$, indicative of eleven degrees of unsaturation. The ^1H and ^{13}C NMR data (see Tables 1 and 2) of 2 closely resembled those of 1,



Table 1 ^1H NMR data of compounds 1–5 in pyridine- d_5

No.	1 ^a	2 ^a	3 ^b	4 ^a	5 ^b
1	3.74, dd (13.5, 8.0)	4.45, dd (13.5, 7.0)	3.65, dd (10.2, 6.5)	4.49, dd (13.5, 7.0)	3.52, dd (13.2, 7.2)
2	a 3.12, dd (17.5, 13.5), b 2.51, dd (17.5, 8.0)	a 3.13, dd (17.5, 13.5), b 2.36, dd (17.5, 7.0)	a 3.12, dd (18.6, 10.2), b 2.50, overlap	a 3.16, dd (17.0, 13.5), b 2.41, dd (17.0, 7.0)	a 3.12, dd (17.4, 13.2), b 2.47, dd (17.4, 7.2)
6	5.64, d (10.0)	6.03, d (9.0)	5.74, overlap	4.88, d (9.5)	4.78, d (9.0)
7	6.41, d (10.0)	6.84, d (9.0)	6.63, d (10.8)	5.52, d (9.5)	5.45, d (9.0)
9	3.58, d (5.0)	2.48, overlap	3.21, brs	1.98, d (2.5)	2.40, brs
11	5.70, t (5.0)	4.71, dd (7.0, 5.0)	5.76, overlap	4.70, d (5.5, 2.5)	5.71, dd (4.8, 3.6)
12	a 2.46, brd (11.0), b 2.15, m	a 2.40, overlap, b 2.11, dd (16.0, 5.5)	a 2.45, overlap, b 2.18, overlap	a 2.15, dd (15.5, 9.5), b 2.03, dd (15.5, 5.5)	a 2.22, dd (16.8, 9.6), b 2.11, dd (16.8, 5.4)
13	3.62, d (10.0)	3.45, d (9.5)	3.48, overlap	2.59, d (9.5)	2.52, d (9.6)
14	7.04, brs	7.40, brs	6.92, brs	6.25, brs	5.57, brs
15	5.36, s	5.66, s	5.45, s	4.40, s	4.39, s
17	1.73, s	1.94, s	1.57, s	1.56, s	1.53, s
18	a 5.68, brs, b 5.12, brs	a 5.64, brs, b 5.05, brs	a 5.68, brs, b 5.14, brs	a 5.56, brs, b 5.04, brs	a 5.58, brs, b 5.12, brs
19	2.00, s	1.83, s	1.96, s	2.07, s	2.08, s
20	2.12, s	2.17, s	2.10, s	1.92, s	1.88, s
6-OAc	1.70, s	1.75, s	1.75, s		
7-OAc	2.25, s	2.15, s	2.20, s		
11-OAc	2.19, s		2.15, s		2.04, s
14-OPr	a 2.78, m, b 2.41, overlap, 1.35, t (7.0)	a 2.60, m, b 2.47, overlap, 1.30, t (7.5)	a 2.67, m, b 2.57, m, 1.27, t (7.0)		
15-OAc	2.24, s	2.23, s	2.24, s		
16-OAc	2.18, s	2.06, s			
5-OH	7.26, s	7.18, s			6.30, s
11-OH		7.61, d (3.5)			

^a Recorded at 500 MHz. ^b Recorded at 600 MHz.

with the only difference between the two compounds was a hydroxy group replacing the acetoxy group at C-11 of **2**. The observation of upfield shift of H-11 ($\Delta\delta_{\text{H}}$ 1.69) and C-11 ($\Delta\delta_{\text{C}}$ 5.8) of **2** compared to those of **1** and the key HMBC correlation from 11-OH to C-11 confirmed the above deduction. The NOESY correlations from H-14 to H-1, H-6, and H-12a, and from H-1 to H₃-19 and 11-OH, suggested these protons are α -oriented. The NOESY correlations from H-9 to H-7, H-11, H-15, and H₃-17, and from H₃-20 to H-7 and 5-OH indicated that these protons are β -oriented. Thus, the structure of compound **2** was determined and it was given the trivial name pieristoxinlactone B.

Pieristoxinlactone C (**3**) exhibited the molecular formula of C₃₁H₄₂O₁₄, similar to **2**, as deduced from the HRESIMS ion (m/z 661.2467 [M + Na]⁺). Analysis of the NMR data (see Tables 1 and 2) clearly indicated that **3** is closely related to **1**. The major differences are that the signals due to an acetyl group was absent, and the resonances for C-13, C-15, C-16, and C-17 were shifted by $\Delta\delta_{\text{C}}$ + 6.3, −1.6, −7.1, and +4.9 ppm, respectively, compared to those of **1**. This finding indicated that the acyloxy group at C-16 in **1** was replaced by a hydroxy group in **3**. The NOESY correlations from H-14 to H-1, H-6, from H-6 to H₃-19 and 11-OH, suggested that these protons are α -orientated. The NOESY correlations from H-9 to H-15 and H₃-17, and from H₃-20 to H-7 indicated that these protons were β -orientated. Furthermore, NOESY correlations from H-9 to H-11, together with the small coupling constant of H-9 (brs), indicated that H-

11 was also β -orientated. Thus, the structure of **3** was established as assigned, and it was named pieristoxinlactone C.

Pieristoxinlactone D (**4**) had the molecular formula C₂₀H₃₀O₉ based on its HRESIMS ion at m/z 415.1984 [M + H]⁺, indicative of six degrees of unsaturation. The ^1H NMR (see Table 1), together with the HSQC showed three tertiary methyls (δ_{H} 2.07, 1.92, 1.56), a terminal double bond (δ_{H} 5.56, 5.04, brs) and a pair of AB doublets (δ_{H} 5.52, 4.88, d, J = 9.5 Hz) in the low-field region. The ^{13}C NMR (see Table 2) and DEPT spectra displayed 20 carbon resonances (one olefinic quaternary carbon resonance should appear at δ_{C} 151.4) ascribed to three methyls, three methylenes (one olefinic at δ_{C} 111.7), eight methines (five oxygenated at δ_{C} 91.2, 79.6, 73.4, 72.5, 63.2), and six quaternary carbons (one ester carbonyl at δ_{C} 175.4; one olefinic at δ_{C} 151.4; three oxygenated at δ_{C} 89.8, 82.3, 77.0). The ^1H – ^1H COSY (see Fig. 3) and HSQC spectra established the presence of three spin systems: C(1)H–C(2)H₂, C(6)H–C(7)H and C(9)H–C(11)H–C(12)H₂–C(13)H–C(14)H. Analysis of the ^1H and ^{13}C NMR data of **4** showed that its structure was closely related to **1** except for the absent of six acyl groups. A more detailed analysis of the 2D NMR data (see Fig. 3) revealed that **4** is based on the same carbon skeleton as **1**. The NOESY correlations (see Fig. 3) from H-14 to H-1, H-6, and H-12a and from H-6 to H-19 established these protons were spatially and were α -orientated. On the other hand, the NOESY correlations from H-7 to H-9 and H₃-20, from H-9 to H-15 and H₃-17, and from H-12b to H-11 and H₃-17



Table 2 ¹³C NMR data of compounds 1–15 in pyridine-*d*₅

No.	1 ^a	2 ^a	3 ^b	4 ^a	5 ^b	6 ^b	7 ^a	8 ^b	9 ^b	10 ^b	11 ^a	12 ^b	13 ^b	14 ^b	15 ^b
1	42.6	42.7	43.3	42.4	43.7	42.9	41.9	54.7	54.7	51.5	52.6	52.5	52.7	49.6	49.6
2	30.5	31.0	30.7	31.5	31.2	32.3	31.3	32.3	32.3	33.6	32.7	32.7	32.7	33.7	33.8
3	174.1	174.8	174.0	175.4	174.5	174.9	174.5	174.6	174.7	175.0	174.4	174.3	174.3	175.4	175.4
4	147.9	148.5	147.6	151.4	150.3	150.2	60.2	147.9	147.8	89.7	62.0	62.0	62.0	76.9	76.9
5	76.3	76.6	76.4	77.0	76.7	76.0	73.0	90.8	90.8	92.4	87.8	87.7	87.6	92.3	92.3
6	74.0	74.1	73.9	73.4	73.8	75.2	74.0	70.0	70.0	68.9	69.8	69.8	69.9	70.1	69.9
7	67.5	66.7	67.4	72.5	72.4	74.4	67.9	68.5	68.8	68.5	68.4	68.6	69.1	68.7	68.7
8	53.3	53.1	53.4	54.4	53.9	53.8	52.9	56.1	56.1	56.0	55.7	55.7	56.1	55.9	55.9
9	60.8	57.6	58.8	57.0	57.0	53.6	56.9	47.6	47.5	47.4	47.3	47.2	46.8	48.1	48.1
10	88.3	89.2	88.1	89.8	88.6	90.2	89.3	76.9	76.9	76.8	76.4	76.4	76.5	76.4	76.5
11	68.6	62.8	68.4	63.2	68.1	19.0	18.8	20.5	20.5	20.6	20.6	20.6	21.0	20.3	20.3
12	30.6	35.1	31.4	35.8	31.6	23.7	22.6	25.4	25.4	25.3	25.5	25.4	25.5	25.1	25.1
13	41.5	44.9	47.8	50.9	49.7	50.4	42.5	50.4	50.6	50.4	45.6	45.7	51.3	50.2	49.6
14	75.6	78.1	77.8	79.6	79.3	80.4	76.9	80.1	80.1	80.3	79.1	79.1	80.1	80.0	80.2
15	92.2	88.9	90.6	91.2	92.0	90.6	91.1	86.9	86.7	86.7	86.5	86.3	86.2	87.3	87.3
16	88.2	89.9	81.1	82.3	81.8	81.5	88.4	80.0	80.2	80.0	88.2	88.2	80.2	80.1	80.0
17	18.9	22.7	23.8	26.3	25.5	24.1	19.1	21.9	21.7	21.8	19.0	19.0	21.9	21.6	21.7
18	113.9	113.0	113.9	111.7	112.3	112.3	49.5	112.1	112.0	22.8	52.7	52.7	52.7	23.3	23.3
19	19.2	18.8	19.1	19.7	20.0	19.9	18.3	19.2	19.1	20.8	17.6	17.5	17.5	68.4	68.4
20	30.2	27.4	28.8	26.9	27.8	27.5	29.6	33.3	33.4	34.0	33.1	33.0	33.1	33.7	33.7
4-OAc										169.0, 22.5					
6-OAc/OPr	169.1, 19.9	169.1, 20.0	169.2, 19.9				169.6, 20.4	169.2, 20.6	169.2, 20.5	169.2, 21.1	169.2, 20.6	169.2, 20.5	169.3, 20.9	168.8, 21.3	172.2, 27.9, 9.0
7-OAc/OPr	170.7, 21.5	170.1, 21.6	170.2, 21.5				170.5, 21.6	172.6, 28.1, 9.1	169.5, 21.4	169.4, 21.6	173.0, 28.4, 9.4	169.8, 21.5	169.6, 21.5	169.5, 21.6	169.4, 21.6
11-OAc	168.9, 21.3		169.0, 21.3		169.2, 21.6										
14-OAc/OPr	174.8, 28.3, 9.2	173.6, 28.5, 9.5	173.2, 28.4, 9.1				174.8, 28.4, 9.3	170.0, 21.7	173.1, 28.5, 9.1	173.1, 28.6, 9.1	170.7, 22.0	170.6, 21.9	173.1, 28.4, 9.1	173.2, 28.6, 9.1	170.0, 22.1
15-OAc	171.9, 20.8	171.0, 21.0	171.5, 20.9				171.7, 20.8	171.9, 21.0	171.8, 20.9	171.7, 20.9	171.9, 21.0	171.8, 20.9	171.4, 20.5	171.9, 20.9	171.9, 20.9
16-OAc	170.3, 22.9	169.8, 22.5					170.3, 22.9				170.1, 22.8	170.0, 22.7			
19-OAc														170.9, 20.8	170.9, 20.8

^a Recorded at 125 MHz. ^b Recorded at 150 MHz.

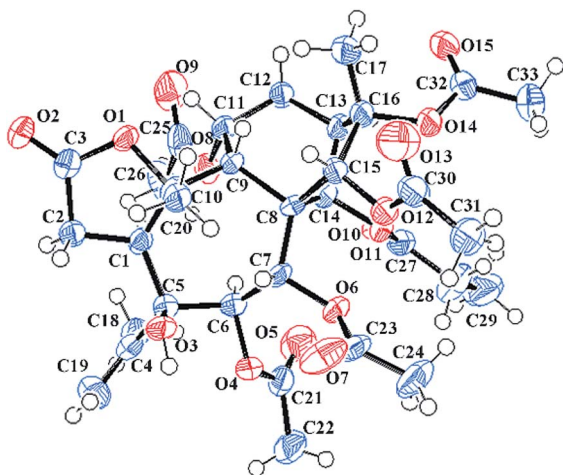


Fig. 2 X-ray crystal structure of 1.

suggested the β -orientation of H-7, H-9, H-11, H-12b, H-15, and H₃-17. The small coupling constant between H-11 and H-12b ($J_{\text{H11,H12b}} = 5.5$ Hz) in the ^1H NMR spectrum also proved that H-11 was β -orientated. Therefore, the structure of 4 was assigned as shown, and it was named pieristoxinlactone D.

Pieristoxinlactone E (5) possessed a molecular formula of $\text{C}_{22}\text{H}_{32}\text{O}_{10}$ as assigned by the HRESIMS data (m/z 457.2075 $[\text{M} + \text{H}]^+$). Comparison of the NMR data of 5 (see Tables 1 and 2) with those of 4 revealed that they are structural analogues. The only difference between the two compounds was the presence of an acetoxy group at C-11 of 5 instead of a hydroxy group in 4, which was further confirmed by the HMBC correlation from H-11 to the acetate carbonyl carbon (δ_{C} 169.2). Therefore, the structure of 5 was assigned as shown, and it was named pieristoxinlactone E.

The molecular formula of 6 was assigned as $\text{C}_{20}\text{H}_{30}\text{O}_8$, by the HRESIMS ion at m/z 421.1831 $[\text{M} + \text{Na}]^+$ and ^{13}C NMR data. Analysis of the NMR data (see Tables 2 and 3) indicated that the structure of 6 closely resembled that of 4 with the only

difference was the absence of the hydroxy group at C-11 in 6, which was confirmed by the spin system C(9)H–C(11)H₂–C(12)H₂–C(13)H–C(14)H verified by ^1H – ^1H COSY and HSQC spectra. Further 2D NMR experiments enabled the full assignments of the ^1H and ^{13}C NMR data of 6. Therefore, 6 was assigned as shown, and was named pieristoxinlactone F.

The HRESIMS ion at m/z 661.2478 $[\text{M} + \text{Na}]^+$ and the ^{13}C NMR data of 7 suggested a molecular formula of $\text{C}_{31}\text{H}_{42}\text{O}_{14}$. The ^1H and ^{13}C NMR data of 7 (see Tables 2 and 3) closely resembled those of pierisoid A¹⁷ except for the location of the propionyloxy. Key HMBC correlations (see Fig. 3) from H-7 to one acetyl carbonyl (δ_{C} 170.5) and from H-14 to the propionyl carbonyl (δ_{C} 174.8) suggested that one acetyloxy and the propionyloxy were connected to C-7 and C-14, respectively. Further HSQC and HMBC experiments (see Fig. 3) enabled the full assignments of the ^1H and ^{13}C NMR spectra of 7 to be carried out. The NOESY correlations (see Fig. 3) from H-14 to H-1, H-6, and H-12a established that these protons were located on the same face of the ring, while the NOESY correlations (see Fig. 3) from H-7 to H-9 and H₃-20, from H-9 to H-15 and H₃-17, and from 5-OH to H-9 and H₃-20 suggested these protons were on the other face of the ring. However, the chiral quaternary C-4 was in the side chain and its relative configuration couldn't be assigned. Subsequently, an X-ray single-crystal structure of 7 (see Fig. 4; CCDC: 1548369[†]), obtained using Cu K α radiation [Flack parameter: 0.04(7)], not only permitted the unambiguous assignment of the propionyloxy but also defined its absolute configuration as 1*S*, 4*R*, 5*S*, 6*R*, 7*R*, 8*S*, 9*R*, 10*R*, 13*R*, 14*R*, 15*R*, 16*S*. Thus, the structure of 7, named pieristoxinlactone G, was finally determined.

The molecular formula $\text{C}_{29}\text{H}_{40}\text{O}_{12}$ of pieristoxinlactone H (8) was assigned according to the HRESIMS ion at m/z 603.2398 $[\text{M} + \text{Na}]^+$. The ^1H and ^{13}C NMR data of 8 (see Tables 2 and 3) revealed a close resemblance to those of secorhodomollolide A,⁵ and the only difference between the two compounds involved a hydroxy group at C-16 of 8 replacing an acetoxy group of secorhodomollolide A. Compared to secorhodomollolide A, the ^1H NMR resonances for H-13 and H₃-17 of 8 were shifted by $\Delta\delta_{\text{H}}$ –1.15 and –0.25, while the ^{13}C NMR resonances for C-13, C-16,

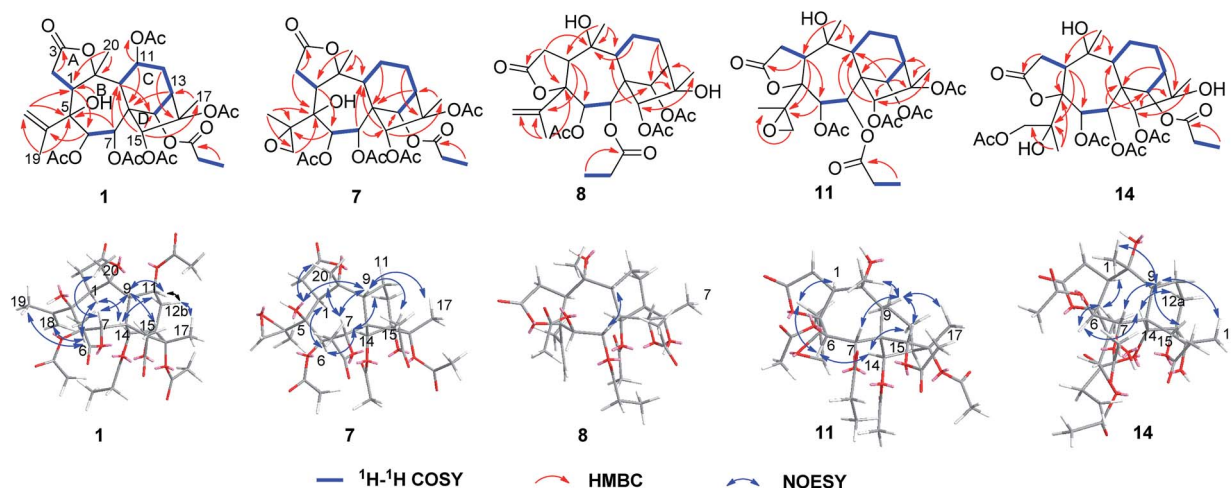


Fig. 3 Key ^1H – ^1H COSY, HMBC and NOESY correlations of compounds 1, 7, 8, 11, and 14.



Table 3 ^1H NMR data of compounds **6–10** in pyridine- d_5

No.	6 ^b	7 ^a	8 ^b	9 ^b	10 ^b
1	3.20, dd (13.2, 6.6)	3.43, dd (13.0, 7.0)	3.09, t (10.2)	3.09, t (10.2)	3.62, dd (12.0, 7.8)
2	a 3.13, dd (16.2, 13.2), b 2.55, dd (16.2, 6.6)	a 3.55, overlap, b 3.11, dd (17.5, 7.0)	a 2.94, overlap, b 2.91, overlap	a 2.94, overlap, b 2.91, overlap	a 3.11, dd (19.2, 12.0), b 2.94, dd (19.2, 7.8)
6	4.65, d (8.4)	5.60, d (10.0)	6.59, d (9.0)	6.60, d (9.0)	6.78, d (10.2)
7	5.27, d (8.4)	5.35, d (10.0)	5.78, d (9.0)	5.77, d (9.0)	5.77, d (10.2)
9	2.37, t (6.0)	3.23, dd (11.5, 7.0)	2.71, brd (7.2)	2.66, brd (7.8)	2.66, brd (7.8)
11	a 1.63, overlap, b 1.61, overlap	a 1.78, m, b 1.71, overlap	a 2.12, m, b 1.78, m	a 2.12, m, b 1.77, m	a 2.13, overlap, b 1.81, overlap
12	a 1.93, dd (15.0, 7.8), b 1.57, overlap	a 2.06, overlap, b 1.57, m	a 2.15, m, b 1.67, m	a 2.15, m, b 1.67, m	a 2.16, overlap, b 1.65, overlap
13	2.41, brd (7.8)	3.53, brd (7.0)	2.49, brs	2.47, brs	2.49, overlap
14	4.93, brs	6.15, brs	6.94, brs	6.99, brs	6.86, brs
15	4.26, s	5.35, s	5.44, s	5.46, s	5.48, s
17	1.53, s	1.73, s	1.45, s	1.46, s	1.46, s
18	a 5.54, s, b 5.08, s	a 3.29, d (5.5), b 2.48, d (5.5)	a 5.42, s, b 4.89, s	a 5.41, s, b 4.88, s	1.91, s
19	1.99, s	1.61, s	1.99, s	1.99, s	2.01, s
20	1.84, s	2.05, s	1.47, s	1.47, s	1.57, s
4-OAc					1.90, s
6-OAc		2.01, s	2.02, s	2.03, s	2.15, s
7-OAc/OPr		2.29, s	a 2.58, m, b 2.47, m, 1.23, t (6.0)	2.22, s	2.27, s
14-OAc/OPr		a 2.79, m, b 2.47, overlap, 1.36, t (7.5)	2.23, s	a 2.58, m, b 2.51, m, 1.26, t (6.0)	a 2.55, m, b 2.50, overlap, 1.27, t (7.8)
15-OAc		2.24, s	2.10, s	2.13, s	2.14, s
16-OAc		2.17, s			
5-OH		5.65, s			
10-OH			7.29, s	7.30, s	7.32, s
16-OH			3.64, s	3.94, s	

^a Recorded at 500 MHz. ^b Recorded at 600 MHz.

and C-17 of **8** were shifted by $\Delta\delta_{\text{C}}$ +5.5, −8.1, +3.0 ppm, respectively, suggesting the presence of a hydroxy group at C-16 in **8**. The NOESY correlations (see Fig. 3) from H-9 to H-7 and H-15, from H-15 to H-11b and H₃-17, from H₃-17 to H-12b suggested the β -orientation of these protons. The large coupling constant between H-6 and H-7 ($^3J_{\text{H6,H7}} = 9.0$ Hz) in the ^1H NMR

spectrum indicated that H-6 and H-7 were in the *trans* configuration. Therefore, H-6 was α -orientated. Moreover, the NOESY correlations from H-14 to H-12a, from H₃-19 to H-1 and H-6, and from H-1 to 10-OH established these protons to be α -orientated. Thus, the structure of pieristoxinlactone **H** (**8**) was assigned as drawn.

Compound **9** had the same molecular formula $\text{C}_{29}\text{H}_{40}\text{O}_{12}$ as **8**, as assigned by the HRESIMS (m/z 603.2417 [$\text{M} + \text{Na}$]⁺) and ^{13}C NMR data. Its NMR data were highly similar to those of **8** except for the slight variations in the chemical shifts of H-14 ($\Delta\delta_{\text{H}}$ +0.05), C-7 ($\Delta\delta_{\text{C}}$ +0.3), and the propionyl carbonyl ($\Delta\delta_{\text{C}}$ +0.5), suggesting that the propionyloxy group may connect to C-14 in **9**. The key HMBC correlations from H-7 to one acetyl carbonyl (δ_{C} 169.5) and from H-14 to the propionyl carbonyl (δ_{C} 173.1) confirmed the above assignment. The relative configuration of **9** was assigned as the same as **8** by their similar NMR and NOESY data. Therefore, the structure of **9** was assigned, and it was named pieristoxinlactone **I**.

The molecular formula $\text{C}_{31}\text{H}_{44}\text{O}_{14}$ of **10** was identical to **24**, as determined by ^{13}C NMR data and the HRESIMS ion at m/z 663.2618 [$\text{M} + \text{Na}$]⁺. The general features of the NMR data of **10** (see Tables 2 and 3) resembled those of **24**, except for the substituents at C-4 and C-16. The observation of the upfield shift of H-13 ($\Delta\delta_{\text{H}}$ 1.11) and H₃-17 ($\Delta\delta_{\text{H}}$ −1.11), and downfield

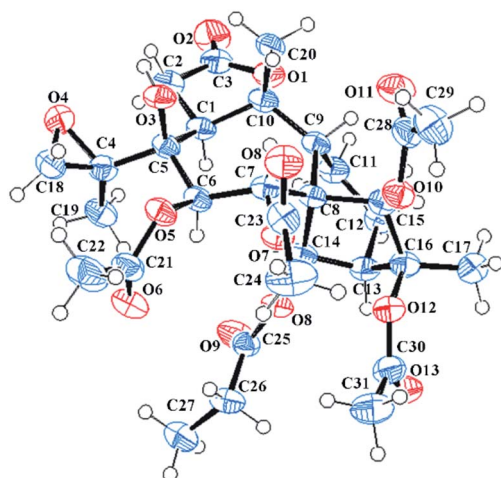
Fig. 4 X-ray crystal structure of **7**.

Table 4 ^1H NMR data of compounds **11**–**15** in pyridine- d_5

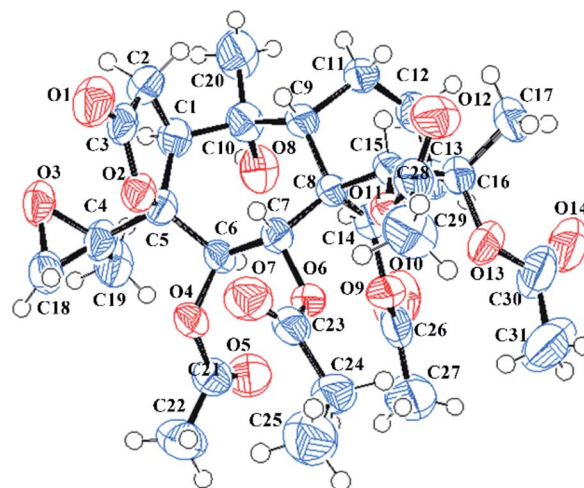
No.	11 ^a	12 ^b	13 ^b	14 ^b	15 ^b
1	3.42, brd (11.5)	3.41, dd (11.4, 7.8)	3.41, dd (11.4, 8.4)	4.09, t (10.2)	4.09, t (10.2)
2	a 3.00, dd (19.0, 11.5), b 2.81, dd (19.0, 8.0)	a 2.99, dd (18.6, 11.4), b 2.79, dd (18.6, 7.8)	a 3.01, dd (18.6, 11.4), b 2.80, dd (18.6, 8.4)	a 3.03, m, b 2.98, m	a 3.03, m, b 2.98, m
6	6.55, d (9.5)	6.55, d (9.6)	6.60, d (9.0)	6.62, d (8.4)	6.68, d (9.0)
7	5.80, d (9.5)	5.76, d (9.6)	5.84, d (9.0)	5.73, d (8.4)	5.73, d (9.0)
9	2.64, brd (8.0)	2.61, d (7.8)	2.52, overlap	2.74, brd (8.4)	2.74, brd (7.8)
11	a 2.09, overlap, b 1.78, m	a 2.11, m, b 1.75, m	a 2.08, m, b 1.79, m	a 2.12, overlap, b 1.79, m	a 2.11, overlap, b 1.79, m
12	a 2.18, overlap, b 1.66, overlap	a 2.17, m, b 1.65, m	a 2.14, m, b 1.65, m	a 2.18, overlap, b 1.67, m	a 2.17, overlap, b 1.67, m
13	3.61, t (4.0)	3.58, t (3.6)	2.46, t (3.0)	2.44, m	2.48, overlap
14	7.04, brs	7.03, brs	7.14, brs	6.78, brs	6.75, brs
15	5.53, s	5.52, s	5.51, s	5.45, s	5.45, s
17	1.71, s	1.71, s	1.47, s	1.46, s	1.47, s
18	a 3.35, d (5.5), b 2.59, d (5.5)	a 3.34, d (5.4), b 2.59, d (5.4)	a 3.35, d (4.8), b 2.60, d (4.8)	1.74, s	1.75, s
19	1.62, s	1.61, s	1.61, s	a 4.76, d (11.4), b 4.70, d (11.4)	a 4.78, d (11.4), b 4.69, d (11.4)
20	1.49, s	1.49, s	1.50, s	1.47, s	1.47, s
6-OAc	2.14, s	2.14, s	2.13, s	2.17, s	a 2.52, m, b 2.45, overlap, 1.16, t (7.2)
7-OAc/-OPr	a 2.68, m, b 2.56, overlap, 1.34, t (7.5)	2.29, s	2.25, s	2.26, s	2.24, s
14-OAc/-OPr	2.24, s	2.22, s	a 2.65, m, b 2.53, overlap, 1.28, t (7.2)	a 2.60, m, b 2.50, m, 1.26, t (7.2)	2.23, s
15-OAc	2.13, s	2.13, s	2.12, s	2.14, s	2.14, s
16-OAc	2.15, s	2.15, s			
19-OAc				2.06, s	2.05, s
10-OH	7.55, s	7.51, s	7.50, s	7.33, s	7.32, s

^a Recorded at 500 MHz. ^b Recorded at 600 MHz.

shift of H₃-18 ($\Delta\delta_{\text{H}}$ 0.24) and H₃-19 ($\Delta\delta_{\text{H}}$ 0.36) in the ^1H NMR spectrum of **10**, as well as the downfield shift of C-4 ($\Delta\delta_{\text{C}}$ 13.8), C-13 ($\Delta\delta_{\text{C}}$ 5.3), and C-17 ($\Delta\delta_{\text{C}}$ 2.9) and an upfield shift of C-4 ($\Delta\delta_{\text{C}}$ 8.2) in the ^{13}C NMR spectrum of **10** compared with those of **24** suggested that an acetyloxy group and a hydroxy group were connected to C-4 and C-16, respectively. The HMBC cross-peaks from H-1 and H₃-19 to C-4 (δ_{C} 89.7) and from H-14, H-13 and H₃-17 to C-16 (δ_{C} 80.0) reconfirmed the above assignment. Compound **10**, pieristoxinlactone J, was thereby structurally characterized.

The molecular formula C₃₁H₄₂O₁₄ was assigned to **11** with eleven indices of hydrogen deficiency by the HRESIMS ion at m/z 661.2469 [M + Na]⁺ and ^{13}C NMR data. The NMR data of **11** (see Tables 2 and 4) showed many similarities to those of **23**, revealing that these two compounds are homologues, except for the presence of a 4,18-oxirane group in **11**. Compared to **23**, the C-4 carbon resonance was shielded ($\Delta\delta_{\text{C}}$ 13.9) and the C-18 carbon resonance was deshielded ($\Delta\delta_{\text{C}}$ 26.9), suggesting that an oxirane group was present between C-4 and C-18. Moreover, the key HMBC correlations (see Fig. 3) from H₃-19 to C-4 (δ_{C} 62.0) and C-18 (δ_{C} 52.7) further confirmed this deduction. The NOESY correlations (see Fig. 3) from H-14 to H-6 and H-12a and from H₃-19 to H-1 and H-6 suggested that these protons are spatially close and α -orientated. In contrast, the NOESY

correlations from H-9 to H-7, H-15, H₃-17, and H₃-20 demonstrated the β -orientation of H-7, H-9, H-15, H₃-17, and H₃-20. However, the relative configuration of C-4 still remains uncertain because C-4 was on the side chain. Finally, an X-ray diffraction study using the anomalous dispersion radiation of

Fig. 5 X-ray crystal structures of **11**.

Cu $K\alpha$ [Flack parameter: $-0.07(15)$] of **11** (see Fig. 5; CCDC: 1548367†) defined its absolute configuration as 1*S*, 4*R*, 5*S*, 6*R*, 7*R*, 8*S*, 9*R*, 10*R*, 13*R*, 14*R*, 15*R*, 16*S*. Thus, the structure of **11** was determined, and it was named pieristoxinlactone K.

The molecular formula of **12**, $C_{30}H_{40}O_{14}$, was determined by the HRESIMS ion at m/z 647.2307 $[M + Na]^+$. A careful analysis of the NMR data of **12** revealed **12** to be based on a 3,4-seco-geranyne skeleton in a similar manner to **11**, except for an acetoxy group substituent at C-7 in **12** instead of a propionyloxy group in **11**. The key HMBC cross-peak from H-7 to an acetyl carbonyl carbon (δ_C 169.8) confirmed the assignment. The structure of compound **12**, pieristoxinlactone L, was thus defined as depicted.

Compound **13** had a molecular formula of $C_{29}H_{40}O_{13}$, as assigned by HRESIMS (m/z 619.2355 $[M + Na]^+$). The 1H and ^{13}C NMR spectra of compound **13** (see Tables 2 and 4) were similar to those of **11**, suggesting that these two compounds were homologues. Compound **13** differs from **11** by substitutions at C-7, C-14, and C-16. The HMBC correlations from H-7 to one acetyl carbonyl (δ_C 169.6) and from H-14 to the propionyl carbonyl (δ_C 173.1) suggested that C-7 and C-14 were connected to an acetoxy and propionyloxy, respectively. In addition, compared to **11**, the resonances of H-13 and H₃-17 were shifted by $\Delta\delta_H$ -1.15 and -0.24 , and the resonances of C-13, C-16, and C-17 were shifted by $\Delta\delta_C$ $+5.7$, -8.0 , and $+2.9$ ppm, respectively, suggesting a hydroxy group substituent at C-16 in **13**. Thus, the structure of **13** was eventually determined, and it was named pieristoxinlactone M.

Compound **14** exhibited a molecular formula of $C_{31}H_{44}O_{15}$ based on the HRESIMS ion at m/z 679.2572 $[M + Na]^+$ and with the aid of ^{13}C NMR data. The 1H and ^{13}C NMR spectra of **14** (see Tables 2 and 4) were similar to those of **10**, differing only in the presence of a hydroxy group and an oxygenated methylene group (δ_C 68.4) and the absence of a methyl group in **14**. Compared to **10**, the C-4 carbon resonance of **14** was shielded ($\Delta\delta_C$ 12.8), revealing that the hydroxy group substituent was at C-4. Moreover, the HMBC correlations (see Fig. 3) from H₂-19 to one acetyl carbonyl (δ_C 170.9) and from H₃-18 to C-4 (δ_C 76.9)

and C-19 (δ_C 68.4) indicated that the hydroxy group and one acetyl group were placed at C-4 and C-19, respectively. The NOESY correlations (see Fig. 3) from H-14 to H-12a and H-6 and from H₃-18 to H-1 and H-6 established the α -orientations of H-1, H-6, H-12a, H-14, and H₃-18, while correlations from H-9 to H-7, H-15, H₃-17, and H₃-20 confirmed the β -orientations of these protons. The relative configuration of C-4 remains uncertain because C-4 is located on the side chain. Finally, the X-ray diffraction [Cu $K\alpha$; Flack parameter: $0.04(16)$] of **14** (see Fig. 6; CCDC: 1548368†) was successfully performed, which not only confirmed the assigned structure but also established the absolute configuration as 1*S*, 4*S*, 5*S*, 6*R*, 7*R*, 8*S*, 9*R*, 10*R*, 13*R*, 14*R*, 15*R*, 16*S*. Thus, the structure of pieristoxinlactone N (**14**) was characterized as shown.

The molecular formula of **15** was deduced to be $C_{31}H_{44}O_{15}$, identical to that of **14**, as indicated by the HRESIMS ion at m/z 679.2572 $[M + Na]^+$. NMR data analysis showed that its structure was closely related to that of **14**, differing only in the location of the propionyloxy group (at C-6 in **15**; at C-14 in **14**). The key HMBC correlations from H-6 to the propionyl carbonyl (δ_C 172.2) and from H-14 to one acetyl carbonyl (δ_C 170.0) confirmed that the propionyloxy group was connected to C-6. Thus, the structure of compound **15** was determined as drawn, and it was named pieristoxinlactone O.

The HRESIMS ion peak at m/z 417.2123 $[M + H]^+$ and the ^{13}C NMR data suggested a molecular formula of $C_{20}H_{32}O_9$ for compound **16**. Apart from the signals for the five acyl groups, the 1H and ^{13}C NMR data of **16** (see Table 5) and **10** were similar, suggesting that these two compounds were homologues. Further 1H - 1H COSY, HSQC, and HMBC experiments allowed for the full assignments of the 1H and ^{13}C NMR spectra of **16**. Compound **16** shared the same carbon skeleton as **10**. The NOESY correlations from H-14 to H-12a, H₃-19 and from H-1 to H-2a, H-6, and H₃-19 demonstrated that the α -orientation of these protons, while the NOESY correlations from H-9 to H-7 and H-15, H₃-20 to H-2b, and from H-15 to H₃-17 confirmed the β -orientation of these protons. Compound **16**, named pieristoxinlactone P, was thereby structurally characterized.

Compound **17** possessed the molecular formula of $C_{20}H_{32}O_{10}$, as assigned by the HRESIMS ion at m/z 433.2065 $[M + H]^+$ and ^{13}C NMR data. The NMR data of **17** resembled those of **16**, except for the only difference of the presence of an oxygenated methine carbon and the absence of a methylene carbon in **17**. The spin system C(9)H-C(11)H-C(12)H₂-C(13)H-C(14)H established by 1H - 1H COSY and HSQC spectra, suggested that a hydroxy group was connected to C-11 (δ_C 68.1). The α -orientation of 11-OH was confirmed by the NOESY correlations from H-11 to H-9 and H₃-20. The structure of pieristoxinlactone Q (**17**) was thus defined as shown.

The seven known diterpenoids, namely, secorhodomollolide C (**18**),⁵ pierisoid B (**19**),¹⁷ secorhodomollolide B (**20**),⁵ secorhodomollolide A (**21**),⁵ pierisformotoxin G (**22**),¹⁸ pierisformotoxin B (**23**),¹⁹ and pierisformotoxin A (**24**),¹⁹ were identified by the comparison of the experimental and previously reported spectroscopic data.

The antinociceptive activities of compounds **1**, **2**, **4**–**8**, **10**–**13**, and **16**–**24** were evaluated using an acetic acid-induced writhing

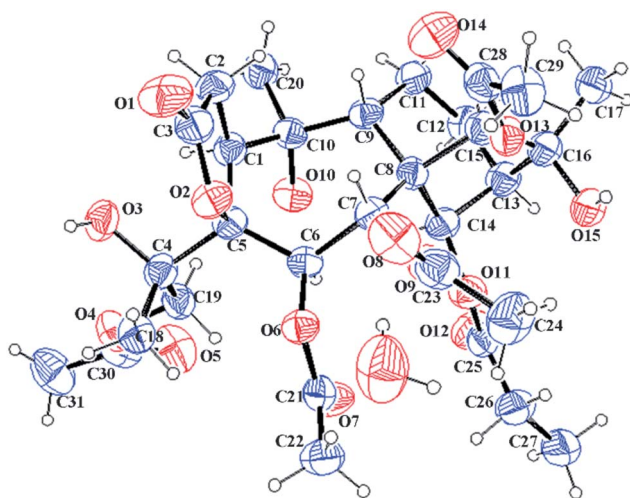
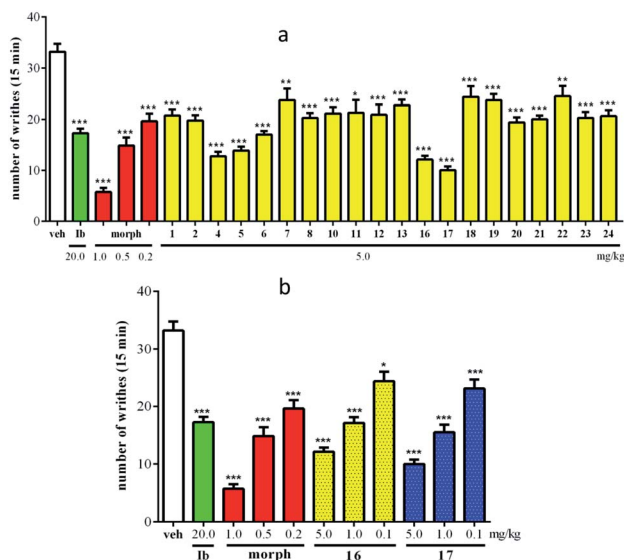


Fig. 6 X-ray crystal structures of **14**.



Table 5 ^1H and ^{13}C NMR data (600 Hz) of compounds **16** and **17** in pyridine- d_5

No.	16		17	
	δ_{H} (J in Hz)	δ_{C}	δ_{H} (J in Hz)	δ_{C}
1	3.80, dd (13.2, 5.4)	51.5	3.71, dd (11.4, 10.8)	51.1
2	a 3.10, dd (18.0, 13.2), b 2.72, dd (18.0, 5.4)	35.0	a 3.04, dd (18.0, 10.8), b 2.98, dd (18.0, 11.4)	34.7
3		176.6		176.2
4		76.7		75.6
5		95.2		95.1
6	5.60, brs	70.4	5.61, d (7.2)	71.6
7	4.65, brs	73.6	5.08, d (7.2)	70.9
8		57.6		57.6
9	2.02, overlap	47.2	2.41, d (4.2)	54.9
10		77.6		78.2
11	a 1.96, overlap, b 1.56, m	21.0	4.54, t (4.8)	68.1
12	a 1.91, overlap, b 1.60, m	26.0	a 2.39, overlap, b 2.02, dd (15.6, 4.2)	34.7
13	2.42, t (3.0)	51.2	2.58, brd (10.2)	46.8
14	5.73, brs	81.1	5.89, brs	77.8
15	4.20, s	88.2	4.04, s	90.7
16		81.2		82.2
17	1.50, s	22.5	1.61, s	23.3
18	2.05, s	28.1	1.86, s	27.9
19	1.82, s	25.9	1.76, s	26.8
20	1.47, s	34.7	1.67, s	33.6
11-OH	6.53, s			

**Fig. 7** (a) Antinociceptive activities of the purified compounds from the roots of *P. formosa* in an acetic acid-induced writhing test. (b) Antinociceptive activities of various concentrations of **16** and **17** in an acetic acid-induced writhing test. Ibuprofen (lb) and morphine (morph) were used as control drugs. Each bar and vertical line represents the mean $\pm$ SEM of the values obtained from 8 mice. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, significant differences between the test compound or control drug groups and vehicle group (veh) (one-way ANOVA followed by the Bonferroni test).

test. As shown in Fig. 7a, all evaluated compounds exhibited significant analgesic activity at 5.0 mg kg $^{-1}$ (ip) compared to vehicle-injected mice ($p < 0.05$). In particular, compounds **16** and **17** showed highly potent activities, with writhes inhibition rates of 63.5% and 69.9%, respectively. To further understand the action of the antinociceptive activities of compounds **16** and **17**, three doses (5.0, 1.0, and 0.1 mg kg $^{-1}$) were employed in an acetic acid-induced writhing test. The results revealed that **16** and **17** were able to reduce acetic acid-induced writhes in a dose-dependent manner (Fig. 7b), with ID $_{50}$ values of 1.24 and 0.72 mg kg $^{-1}$, respectively.

Experimental

General experimental procedures

Optical rotations were measured on an AUTOPOL V polarimeter. IR spectra were recorded using a Nicolet 5700 FT-IR microscope (FT-IR microscope transmission). 1D and 2D NMR spectra were obtained using an INOVA-500 or a Bruker-600 spectrometer. Online HPLC/UV/ESIMS 2 analyses were recorded on an Agilent 1100 Series liquid chromatography system chromatograph. HRESIMS data were recorded on an Agilent 6520 Accurate-Mass Q-TOF LC/MS spectrometer. Preparative HPLC was performed using a Shimadzu LC-6AD instrument equipped with RID-20A and SPD-20A detectors, using a YMC-Pack RP-C18 column (250 $\times$ 10 mm, 5 μ m) or a COSMOSIL packed column (250 $\times$ 10 mm, 5 μ m). Column chromatography was performed using polyamide (30–60 mesh, Changzhou Changfeng Chemical Factory, China), macroporous resin D101 (Qingdao Marine Chemical Factory, China), MCI (Mitsubishi Chemical Corporation), and ODS (50 μ m, Merck, Germany). TLC was carried out with glass precoated Si gel GF254 plates (Qingdao Marine Chemical Factory, China). Spots were visualized by spraying with 10% H $_2$ SO $_4$ in EtOH–H $_2$ O (95 : 5, v/v) followed by heating.

Plant material

Roots of *Pieris formosa* (120 kg) were collected in Chuxiong Yi Autonomous Prefecture in Yunnan Province, People's Republic of China, in June 2014 and were identified by Prof. Lin Ma (Department of Natural Products Chemistry, Institute of Materia Medica, Chinese Academy of Medical Sciences). A voucher specimen (ID-s-2747) was deposited in the herbarium at the Department of Medicinal Plants, Institute of Materia Medica, Chinese Academy of Medical Sciences.

Extraction and isolation

The isolation procedures were guided by acetic acid-induced writhing test and online HPLC/UV/ESIMS 2 analyses as described above. The roots of *Pieris formosa* (120 kg) were extracted with 95% EtOH (150 l $\times$ 3 h $\times$ 3). The evaporation of the solvent under reduced pressure yielded a black residue (5.0 kg). The residue was mixed with diatomite (1 : 2) and then successively Soxhlet extracted using a gradient of petroleum ether, CH $_2$ Cl $_2$, EtOAc and CH $_3$ OH. The CH $_2$ Cl $_2$ portion (D) and the EtOAc portion (E) were then applied to a macroporous resin



D101 column (10 kg) and eluted with a gradient of 30% EtOH–H₂O, 50% EtOH–H₂O and 70% EtOH–H₂O, to yield six fractions (D1–D3, and E1–E3). Fractions D1 (60 g) and D2 (57 g) were separated through a polyamide column and eluted with H₂O–EtOH (100 : 0, 30 : 70, 50 : 50, 70 : 30, v/v), to yield eight fractions (D1a–D1d and D2a–D2d). Fractions E1 (20 g) and E2 (35 g) were separated through a polyamide column and eluted with a gradient of H₂O–EtOH (100 : 0, 30 : 70, 60 : 40, v/v), to yield six fractions (E1a–E1c and E2a–E2c). Fraction D1a (4 g) was separated *via* an MCI column and eluted with a gradient of H₂O–MeOH (1 : 9–9 : 1, v/v), to obtain four subfractions D1a1–D1a4. Subfraction D1a1 (0.9 g) was purified by semipreparative HPLC with MeCN–H₂O (23 : 77, v/v, 4.5 ml min^{−1}), to yield **13** (4.5 mg, *t_R* = 37.5 min), **14** (1.0 mg, *t_R* = 34.5 min) and **15** (0.6 mg, *t_R* = 32.5 min). Fraction D2a (4 g) was separated through a MCI column and eluted with a gradient of H₂O–MeOH (1 : 9–9 : 1, v/v), to obtain four subfractions D2a1–D2a4. Subfraction D2a1 (1.5 g) was purified further using semipreparative HPLC with MeCN–H₂O (30 : 70, v/v, 4.0 ml min^{−1}), to yield **1** (26.0 mg, *t_R* = 70.0 min), **18** (11.0 mg, *t_R* = 72.5 min), **20** (83.0 mg, *t_R* = 57.5 min), and **21** (180.0 mg, *t_R* = 55.5 min). Subfraction D2a2 (0.5 g) was purified further using semipreparative HPLC with MeCN–H₂O (22.5 : 77.5, v/v, 4.0 ml min^{−1}), to yield **3** (1.2 mg, *t_R* = 75.0 min), **8** (2.5 mg, *t_R* = 79.0 min), **10** (3.5 mg, *t_R* = 80.0 min), and **19** (14.0 mg, *t_R* = 91.0 min). Subfraction D2a3 (1.3 g) was purified further using semipreparative HPLC with MeCN–H₂O (24 : 76, v/v, 4.0 ml min^{−1}), to yield **11** (36.0 mg, *t_R* = 103.0 min), **12** (2.5 mg, *t_R* = 70.0 min), **23** (16.0 mg, *t_R* = 61.0 min), and **24** (71.0 mg, *t_R* = 67.0 min). Fraction D2b (8 g) was separated through an ODS column and eluted with 10% CH₃OH–H₂O, 30% CH₃OH–H₂O, 50% CH₃OH–H₂O, and 70% CH₃OH–H₂O, successively, to yield four fractions (D2b1–D2b4). Subfraction D2b3 (1.5 g) was purified by reversed-phase semipreparative HPLC with MeCN–H₂O (23 : 77, v/v, 4.0 ml min^{−1}), to yield **2** (5.7 mg, *t_R* = 115.5 min), **7** (8.0 mg, *t_R* = 151.5 min), **9** (1.0 mg, *t_R* = 104.5 min), and **22** (7.6 mg, *t_R* = 114.5 min). Fraction E1a (3 g) was chromatographed on a YMC RP-C18 column (17% CH₃CN–H₂O, v/v, 5 ml min^{−1}) to yield seven fractions (E1a1–E1a7). Subfraction E1a2 (0.6 g) was purified further using semipreparative HPLC with MeCN–H₂O (8 : 92, v/v, 4.5 ml min^{−1}), to yield **4** (6.5 mg, *t_R* = 21.5 min) and **6** (5.0 mg, *t_R* = 38.0 min). Fraction E1b (5 g) was separated through an ODS column and eluted with 10% CH₃OH–H₂O, 30% CH₃OH–H₂O, 50% CH₃OH–H₂O, and 70% CH₃OH–H₂O, successively, to yield four fractions (E1b1–E1b4). Subfraction E1b1 (2 g) was chromatographed on a YMC RP-C18 column (14% CH₃CN–H₂O, v/v, 5 ml min^{−1}) to yield four fractions (E1b1a–E1b1d). Subfraction E1b1a (0.3 g) was purified by semipreparative HPLC with MeCN–H₂O (12 : 88, v/v, 5.0 ml min^{−1}), to yield **5** (5.0 mg, *t_R* = 25.5 min). Fraction E2a (15 g) was separated *via* an MCI column and eluted with a gradient of H₂O–MeOH (1 : 9–9 : 1, v/v), to obtain five subfractions E2a1–E2a5. Subfraction E2a1 (1 g) was purified further using a semipreparative HPLC with MeCN–H₂O (6 : 94, v/v, 5.0 ml min^{−1}), to yield **16** (6.0 mg, *t_R* = 32.0 min) and **17** (6.0 mg, *t_R* = 18.5 min).

Pieristoxinlactone A (1). Colorless crystals; mp 268–269 °C; [α]_D²⁰ −9.9 (*c* 0.29, MeOH); IR (KBr) $\nu_{\max}$ 3472, 2986, 2941, 1790,

1741, 1368, 1223, 1087 cm^{−1}; ¹H and ¹³C NMR data, see Tables 1 and 2; HRESIMS *m/z* 703.2568 [M + Na]⁺ (calcd for C₃₃H₄₄O₁₅Na, 703.2572).

Pieristoxinlactone B (2). White, amorphous powder; [α]_D²⁰ −5.2 (*c* 0.23, MeOH); IR (KBr) $\nu_{\max}$ 3505, 2921, 2850, 1744, 1646, 1374, 1233, 1053 cm^{−1}; ¹H and ¹³C NMR data, see Tables 1 and 2; HRESIMS *m/z* 661.2477 [M + Na]⁺ (calcd for C₃₁H₄₂O₁₄Na, 661.2467).

Pieristoxinlactone C (3). White, amorphous powder; [α]_D²⁰ −46.2 (*c* 0.03, MeOH); IR (KBr) $\nu_{\max}$ 3500, 2924, 2853, 1785, 1745, 1376, 1231, 1067, 925 cm^{−1}; ¹H and ¹³C NMR data, see Tables 1 and 2; HRESIMS *m/z* 661.2467 [M + Na]⁺ (calcd for C₃₁H₄₂O₁₄Na, 661.2467).

Pieristoxinlactone D (4). White, amorphous powder; [α]_D²⁰ −11.9 (*c* 0.07, MeOH); IR (KBr) $\nu_{\max}$ 3395, 2976, 2936, 1759, 1676, 1202, 1139, 1076, 928 cm^{−1}; ¹H and ¹³C NMR data, see Tables 1 and 2; HRESIMS *m/z* 415.1984 [M + H]⁺ (calcd for C₂₀H₃₁O₉, 415.1963).

Pieristoxinlactone E (5). White, amorphous powder; [α]_D²⁰ −25.3 (*c* 0.17, MeOH); IR (KBr) $\nu_{\max}$ 3390, 2965, 2944, 1777, 1741, 1232, 1075, 923 cm^{−1}; ¹H and ¹³C NMR data, see Tables 1 and 2; HRESIMS *m/z* 457.2075 [M + H]⁺ (calcd for C₂₂H₃₃O₁₀, 457.2068).

Pieristoxinlactone F (6). White, amorphous powder; [α]_D²⁰ −7.9 (*c* 0.19, MeOH); IR (KBr) $\nu_{\max}$ 3377, 2965, 2939, 1766, 1677, 1202, 1140, 911 cm^{−1}; ¹H and ¹³C NMR data, see Tables 3 and 2; HRESIMS *m/z* 421.1831 [M + Na]⁺ (calcd for C₂₀H₃₀O₈Na, 421.1833).

Pieristoxinlactone G (7). Colorless crystals; mp 270–272 °C; [α]_D²⁰ +57.0 (*c* 0.26, MeOH); IR (KBr) $\nu_{\max}$ 3505, 2982, 2920, 1773, 1731, 1373, 1238, 1059, 929 cm^{−1}; ¹H and ¹³C NMR data, see Tables 3 and 2; HRESIMS *m/z* 661.2478 [M + Na]⁺ (calcd for C₃₁H₄₂O₁₄Na, 661.2467).

Pieristoxinlactone H (8). White, amorphous powder; [α]_D²⁰ +49.5 (*c* 0.10, MeOH); IR (KBr) $\nu_{\max}$ 3536, 2937, 1747, 1370, 1235, 1045 cm^{−1}; ¹H and ¹³C NMR data, see Tables 3 and 2; HRESIMS *m/z* 603.2398 [M + Na]⁺ (calcd for C₂₉H₄₀O₁₂Na, 603.2412).

Pieristoxinlactone I (9). White, amorphous powder; [α]_D²⁰ +7.5 (*c* 0.04, MeOH); IR (KBr) $\nu_{\max}$ 3536, 2939, 1746, 1372, 1233, 1044 cm^{−1}; ¹H and ¹³C NMR data, see Tables 3 and 2; HRESIMS *m/z* 603.2417 [M + Na]⁺ (calcd for C₂₉H₄₀O₁₂Na, 603.2412).

Pieristoxinlactone J (10). White, amorphous powder; [α]_D²⁰ +23.2 (*c* 0.19, MeOH); IR (KBr) $\nu_{\max}$ 3440, 2978, 2942, 1741, 1373, 1235, 1049 cm^{−1}; ¹H and ¹³C NMR data, see Tables 3 and 2; HRESIMS *m/z* 663.2618 [M + Na]⁺ (calcd for C₃₁H₄₄O₁₄Na, 663.2623).

Pieristoxinlactone K (11). Colorless crystals; mp 231–233 °C; [α]_D²⁰ +67.3 (*c* 0.10, MeOH); IR (KBr) $\nu_{\max}$ 3469, 2942, 1786, 1731, 1371, 1245, 1054 cm^{−1}; ¹H and ¹³C NMR data, see Tables 4 and 2; HRESIMS *m/z* 661.2469 [M + Na]⁺ (calcd for C₃₁H₄₂O₁₄Na, 661.2467).

Pieristoxinlactone L (12). White, amorphous powder; [α]_D²⁰ +52.1 (*c* 0.07, MeOH); IR (KBr) $\nu_{\max}$ 3472, 2983, 2942, 1732, 1372, 1222, 1053 cm^{−1}; ¹H and ¹³C NMR data, see Tables 4 and



2; HRESIMS m/z 647.2307 $[M + Na]^+$ (calcd for $C_{30}H_{40}O_{14}Na$, 647.231).

Pieristoxinlactone M (13). White, amorphous powder; $[\alpha]_D^{20} +25.0$ (c 0.10, MeOH); IR (KBr) ν_{max} 3423, 2978, 2941, 1785, 1730, 1375, 1265, 1239, 1214, 1048, 913 cm^{-1} ; 1H and ^{13}C NMR data, see Tables 4 and 2; HRESIMS m/z 619.2355 $[M + Na]^+$ (calcd for $C_{29}H_{40}O_{13}Na$, 619.2361).

Pieristoxinlactone N (14). Colorless crystals; mp 267–268 °C; $[\alpha]_D^{20} -62.0$ (c 0.05, MeOH); IR (KBr) ν_{max} 3368, 2919, 1738, 1374, 1242, 1049 cm^{-1} ; 1H and ^{13}C NMR data, see Tables 4 and 2; HRESIMS m/z 679.2572 $[M + Na]^+$ (calcd for $C_{31}H_{44}O_{15}Na$, 679.2572).

Pieristoxinlactone O (15). White, amorphous powder; $[\alpha]_D^{20} -53.2$ (c 0.04, MeOH); IR (KBr) ν_{max} 3490, 2986, 2941, 1739, 1373, 1239, 1061 cm^{-1} ; 1H and ^{13}C NMR data, see Tables 4 and 2; HRESIMS m/z 679.2572 $[M + Na]^+$ (calcd for $C_{31}H_{44}O_{15}Na$, 679.2572).

Pieristoxinlactone P (16). White, amorphous powder; $[\alpha]_D^{20} -17.3$ (c 0.30, MeOH); IR (KBr) ν_{max} 3480, 3391, 2975, 2945, 1761, 1445, 1232, 1117, 1077 cm^{-1} ; 1H and ^{13}C NMR data, see Table 5; HRESIMS m/z 417.2123 $[M + H]^+$ (calcd for $C_{20}H_{33}O_9$, 417.2119).

Pieristoxinlactone Q (17). White, amorphous powder; $[\alpha]_D^{20} -21.6$ (c 0.19, MeOH); IR (KBr) ν_{max} 3355, 2977, 2937, 1761, 1676, 1201, 1184, 1140 cm^{-1} ; 1H and ^{13}C NMR data, see Table 5; HRESIMS m/z 433.2065 $[M + H]^+$ (calcd for $C_{20}H_{33}O_{10}$, 433.2068).

X-ray crystallographic analysis

The crystals of compounds **1**, **7**, **11**, and **14** were obtained from recrystallization in a mixture of solvents (MeOH/H₂O, 10 : 1) at room temperature. The X-ray crystallography studies of the four compounds were conducted according to the usual procedure (for details, see Tables S1–S4 in the ESI†). The crystallographic data for compounds **1**, **7**, **11**, and **14** reported in this paper have been deposited at the Cambridge Crystallographic Data Centre (deposition No. CCDC 1548366, 1548369, 1548367, and 1548368† for **1**, **7**, **11**, and **14**, respectively).

Animals

All animal care and experimental procedures were in accordance with the current guidelines of the National Institutes of Health (NIH). Animal experiments were approved by the Ethics Committee of Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College.

Acetic acid-induced writhing test

Groups of 8 Kunming mice were used as control and test mice. The mice were given an intraperitoneal injection of 1% v/v acetic acid solution 15 minutes after the administration of the compounds (blank control received the vehicle, 0.9% NaCl, 5 ml kg^{-1} , ip; positive control received the morphine, 0.5 mg kg^{-1} , ip). The mice were placed individually in glass boxes. The numbers of writhes produced in these mice were counted for 20 minutes. For scoring purposes, a writhe was indicated by a stretching of the abdomen with a simultaneous stretching of at least one hind limb.

Conclusions

In summary, herein we have reported seventeen new 3,4-secograyanoids (**1–17**), together with seven known compounds (**18–24**), which were isolated from the roots of *Pieris formosa*. Their structures with absolute configurations were characterized by a series of spectroscopic methods and X-ray diffraction. Compounds **1**, **2**, **4–8**, **10–13**, and **16–24** exhibited significant analgesic activity at 5.0 mg kg^{-1} (ip) compared to vehicle-injected mice ($p < 0.05$). In particular, compounds **16** and **17** showed highly potent activities with writhe inhibition rates of 63.5% and 69.9%, respectively.

Conflicts of interest

There are no conflicts to declare.

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References

- Y. Li, Y. B. Liu and S. S. Yu, *Phytochem. Rev.*, 2013, **12**, 305–325.
- W. H. Tallent, M. L. Riethof and E. C. Horning, *J. Am. Chem. Soc.*, 1954, **79**, 4548–4554.
- P. Narayanan, M. Rohrl, K. Zechmeister and W. Hoppe, *Tetrahedron Lett.*, 1970, **11**, 3943–3944.
- S. Fushiya, H. Hikino and T. Takemoto, *Tetrahedron Lett.*, 1974, **15**, 183–186.
- S. J. Wang, S. Lin, C. G. Zhu, Y. C. Yang, S. Li, J. J. Zhang, X. G. Chen and J. G. Shi, *Org. Lett.*, 2010, **12**, 1560–1563.
- Z. Y. Wu, H. Z. Li and W. G. Wang, *Phytochem. Lett.*, 2012, **5**, 87–90.
- Y. Li, Y. B. Liu, J. J. Zhang, Y. H. Li, J. D. Jiang, S. S. Yu, S. G. Ma, J. Qu and H. N. Lv, *Org. Lett.*, 2013, **15**, 3074–3077.
- J. W. Burke, R. W. Doskotch, C. Z. Ni and J. Clardy, *J. Am. Chem. Soc.*, 1989, **111**, 5831–5833.
- S. Z. Zhou, S. Yao, C. P. Tang, C. Q. Ke, L. Li, G. Lin and Y. Ye, *J. Nat. Prod.*, 2014, **77**, 1185–1192.
- A. Furusaki, N. Hamanaka and H. Miyakoshi, *Chem. Lett.*, 1972, **1**, 783–786.
- M. K. Zhang, Y. Zhu, G. Q. Zhan, P. H. Shu, R. J. Sa, L. Lei, M. Xiang, Y. B. Xue, Z. W. Luo, Q. Wan, G. M. Yao and Y. H. Zhang, *Org. Lett.*, 2013, **15**, 3094–3097.
- Y. Li, Y. B. Liu, Y. L. Liu, C. Wang, L. Q. Wu, L. Li, S. G. Ma, J. Qu and S. S. Yu, *Org. Lett.*, 2014, **16**, 4320–4323.
- Y. Li, Y. B. Liu, H. M. Yan, Y. L. Liu, Y. H. Li, H. N. Lv, S. G. Ma, J. Qu and S. S. Yu, *Sci. Rep.*, 2016, **6**, 36572.
- J. S. Chen and S. Zheng, *Chinese Poisonous Plants; Science*, Beijing, 1987, pp. 226–227.



- 15 C. S. Niu, Y. Li, Y. B. Liu, S. G. Ma, F. Liu, L. Li, S. Xu, X. J. Wang, R. B. Wang, J. Qu and S. S. Yu, *Org. Lett.*, 2017, **19**, 906–909.
- 16 C. S. Niu, Y. Li, Y. B. Liu, S. G. Ma, L. Li, J. Qu and S. S. Yu, *Tetrahedron*, 2016, **72**, 44–49.
- 17 C. H. Li, X. M. Niu and Q. Luo, *Org. Lett.*, 2010, **12**, 2426–2429.
- 18 Z. Y. Wu, Y. D. Li and G. S. Wu, *Chem. Pharm. Bull.*, 2011, **59**, 492–495.
- 19 W. G. Wang, Z. Y. Wu, R. Chen, H. Z. Li, H. M. Li, Y. D. Li, R. T. Li and H. R. Luo, *Chem. Biodiversity*, 2013, **10**, 1061–1071.

