Three New Highly Acylated 3,4-*seco*-Grayanane Diterpenoids from the Fruits of *Pieris formosa*

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Phytochemical investigation on the fruits of *Pieris formosa* resulted in the isolation of three new highly acylated 3,4-*seco*-grayanane diterpenoids, pierisformotoxins E—G (1—3). Their structures were elucidated on the basis of extensive spectroscopic analysis, including 1D-, 2D-NMR, electrospray ionization-mass spectra (ESI-MS) and high resolution (HR)-MS.

Key words grayanane diterpenoid; *Pieris formosa*; Ericaceae

The genus *Pieris*, which includes only 7 species in the world, is a small genus of the big family Ericaceae.[1] In previous investigations, more than 40 new grayanane diterpenoids have been isolated from this genus, including asebotoxins I—X, pieristoxins A—K, and pierosides A—C from *P. japonica*[2—13]; pierisformosins A—D and pierisformosides A—I from *P. formosa*.[14—18] Recently, two new highly acylated 3,4-*seco*-grayanane diterpenoids, pierisoids A and B, were isolated from the flowers of *P. formosa*.[19] Some of these diterpenoids have shown significant physiological properties, including blood pressure and heart rate lowering, cardiotoxic, neurotoxic, growth inhibitory, and insecticidal activities.[20—27]

Our previous research on the flowers of *P. formosa* led to the discovery of a new grayanane diterpenoid, grayanotoxin XXII.[28] Since the secondary metabolites of the different parts of the medicinal plants often differ even growing in the same ecological environments, we further explored the fruits of this plant, in order to look for structural unique and bioactive diterpenoids. As a result, three new highly acylated 3,4-*seco*-grayanane diterpenoids, pierisformotoxins E—G (**1**—**3**, Fig. 1), were isolated. In addition, compounds **1**—**3** were tested for their acetylcholinesterase (AChE) inhibitory and nerve growth factor (NGF)-potentiating activities. Herein, we report the isolation, structure determination and biological activities of compounds **1**—**3**, and this is the first report of the chemical constituents of the fruits of *P. formosa*.

Results and Discussion

The 75% aqueous acetone extract of the fruits of *P. formosa* was concentrated under vacuum and then partitioned between EtOAc and H_2O (1 : 1). The EtOAc layer was subjected repeatedly to column chromatography over silica gel and Sephadex LH-20, and purified by semipreparative HPLC to yield compounds **1**—**3**.

Pierisformotoxin E (**1**), $[\alpha]_D^{27}$ −40.1 (c=0.14, $CHCl_3$), showed a pseudomolecular ion peak at m/z 655 $[M+Cl]^-$ in the negative electrospray ionization-mass spectra (ESI-MS), and the molecular formula, $C_{31}H_{40}O_{13}$, was established by high resolution (HR)-ESI-MS (m/z 655.2163, $[M+Cl]^-$), corresponding to twelve degrees of unsaturation. The IR spectrum showed absorptions at 3452 cm^{-1} due to the hydroxyl group, and the absorption bands at 1783 and 1737 cm^{-1} revealed the presence of ester carbonyl functional groups, while the absorptions at 1643 cm^{-1} were ascribed to the olefinic carbons.

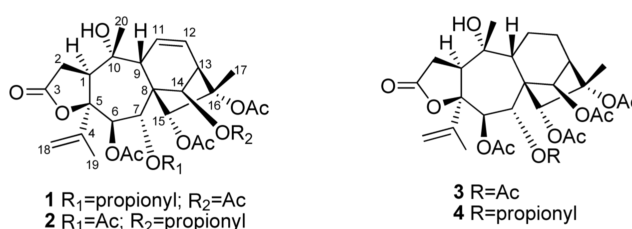

Fig. 1. Structures of Compounds **1**—**4**

The ^{1}H-NMR spectrum of **1** (Table 1) exhibited one propionyl unit at δ_H 1.30 (3H, t, J=7.5 Hz), 2.64 and 2.51 (each 1H, m), and four acetyl methyls at δ_H 2.00, 2.11, 2.16, and 2.18 (each 3H, s). Besides the signals above, three singlet methyls at δ_H 1.52, 1.60, and 2.00 (each 3H, s) were also observed in the high-field region. And in the low-field region, it displayed a terminal double bond at δ_H 5.45 and 4.88 (each 1H, br s), an endo-double bond at δ_H 5.73 (1H, dd, J=3.6, 9.9 Hz) and 5.92 (1H, br t, J=9.9 Hz), four singlet signals at δ_H 3.45 (br s), 5.13, 6.96 and 7.59, and a pair of AB doublet signals at δ_H 6.55 (1H, d, J=9.4 Hz) and 5.70 (1H, d, J=9.6 Hz) . Apart from carbon resonances of four *O*-acetyls and one *O*-propionyl unit, the ^{13}C-NMR and distortionless enhancement by polarization transfer (DEPT) spectra (Table 1) showed twenty carbon signals, including three methyls, two methylenes (one olefinic), nine methines (four oxygen-bearing, and two olefinic), and six quaternary carbons (one carboxyl, one olefinic and three oxygen-bearing). Signal at δ_H 7.59, showing no correlation with any carbons in the heteronuclear single quantum coherence (HSQC) spectrum, was assigned to the exchangeable proton of a hydroxyl group. The aforementioned facts suggested that compound **1** was probably a highly acylated grayanane diterpenoid.

By comparing the ^{1}H- and ^{13}C-NMR data of compound **1** with those of the polysterified grayanane diterpenoids previously reported, the planar structure of **1** was similar to that of

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Table 1. ^{1}H- and ^{13}C-NMR Data of Compounds **1**—**3** in C_5D_5N (500 MHz for ^{1}H and 125 MHz for ^{13}C)

Position	**1**		**2**		**3**	
	δ_H (J Hz)	δ_C	δ_H (J Hz)	δ_C	δ_H (J Hz)	δ_C
1	3.11 (overlapped)	54.1 d	3.11 (overlapped)	54.1 d	3.07 (dd, 11.7, 9.1)	54.6 d
2	3.08 (overlapped, H_α)	32.2 t	3.08 (overlapped, H_α)	32.2 t	2.92 (m)	32.3 t
	2.97 (dd, 10.0, 16.5, H_β)	—	2.97 (dd, 11.0 , 17.5, H_β)	—	—	—
3	—	174.4 s	—	174.5 s	—	174.6 s
4	—	148.0 s	—	148.0 s	—	147.9 s
5	—	91.0 s	—	91.0 s	—	90.9 s
6	6.55 (d, 9.6)	69.8 d	6.55 (d, 9.5)	69.8 d	6.54 (d, 9.2)	69.9 d
7	5.70 (d, 9.6)	67.2 d	5.68 (d, 9.5)	67.4 d	5.69 (d, 9.2)	68.2 d
8	—	54.9 s	—	55.0 s	—	55.8 s
9	3.45 (br s)	55.4 d	3.43 (br s)	55.4 d	2.73 (br d, 7.9)	47.9 d
10	—	75.8 s	—	75.8 s	—	76.8 s
10-OH	7.59 (s)	—	7.61 (s)	—	7.31 (s)	—
11	5.73 (dd, 3.6, 9.9)	129.6 d	5.71 (dd, 3.4, 8.6)	129.5 d	1.71 (m)	20.2 t
12	5.92 (br t, 9.9)	132.3 d	5.92 (br t, 8.6)	132.4 d	2.17 (overlapped, H_α)	25.3 t
	—	—	—	—	—	1.63 (m, H_β)
13	4.17 (d, 7.1)	45.5 d	4.14 (d, 7.2)	45.7 d	3.60 (br s)	45.0 d
14	6.96 (s)	76.7 d	7.02 (s)	76.5 d	6.86 (s)	79.0 d
15	5.13 (s)	88.5 d	5.14 (s)	88.4 d	5.44 (s)	87.0 d
16	—	89.7 s	—	89.7 s	—	88.1 s
17	1.60 (s)	19.2 q	1.60 (s)	19.2 q	1.69 (s)	18.9 q
18	5.45 (br s, H_α)	112.5 t	5.45 (br s, H_α)	112.5 t	5.41 (br s, H_α)	112.2 t
	4.88 (br s, H_β)	—	4.87 (br s, H_β)	—	4.87 (br s, H_β)	—
19	2.00 (s)	19.6 q	2.00 (s)	19.6 q	1.98 (s)	19.2 q
20	1.52 (s)	32.3 q	1.52 (s)	32.3 q	1.44 (s)	33.3 q
6-OAc	2.00 (s)	20.6 q	2.02 (s)	20.6 q	2.25 (s)	20.5 q
	—	169.1 s	—	169.1 s	—	169.1 s
7-OAc/-Opr	1.30 (t, 7.5)	9.4 q	2.28 (s)	21.5 q	2.03 (s)	21.5 q
	2.64 (m)	28.2 t	—	169.9 s	—	169.8 s
	2.51 (m)	173.2 s	—	—	—	—
14-OAc/-OPr	2.18 (s)	22.0 q	1.27 (t, 7.5)	9.3 q	2.17 (s)	21.9 q
	—	170.9 s	2.60 (m)	28.2 t	—	170.7 s
	—	—	2.34 (m)	174.4 s	—	—
15-OAc	2.11 (s)	21.1 q	2.14 (s)	21.1 q	2.15 (s)	20.9 q
	—	172.9 s	—	172.9 s	—	172.1 s
16-OAc	2.16 (s)	22.8 q	2.17 (s)	22.9 q	2.12 (s)	22.8 q
	—	170.5 s	—	170.5 s	—	170.1 s

secorhodomollolide A (**4**, Fig. 1),[22] and the only difference was that **1** had an additional endo-double bond [δ_H 5.73 (1H, dd, J=3.6, 9.9 Hz) and 5.92 (1H, br t, J=9.9 Hz), δ_C 129.6 d and 132.3 d]. In the heteronuclear multiple bond connectivity (HMBC) spectrum, signal at δ_H 5.73 was correlated to C-8 (δ_C 54.9) and C-13 (δ_C 45.5), and signal at δ_H 5.92 showed cross-peaks with C-9 (δ_C 55.4), C-13, and C-14 (δ_C 76.7) (Fig. 2), indicating that this double bond was located between C-11 and C-12.

The relative configuration of **1** was established on the basis of a rotating frame Overhauser enhancement spectroscopy (ROESY) experiment (Fig. 3). Biogenetically, Me-17 and Me-20 were assigned to be β-directed and H-13 to be α-oriented.[14—19] Accordingly, ROESY correlations of H-15/H-7, H-15/H-9, and H-15/Me-17, indicated that H-7 and H-15 were co-facial with H-9, and assigned to be β-oriented, while cross-peaks of H-1/H-6, H-6/H-13, and H-6/H-14 suggested that H-6 and H-14 were in the same α-orientations. Strong ROESY correlations of H-14/10-OH and Me-19/10-OH showed that 10-OH was α-directed. H-7 was assigned to be opposite to H-6 also based on a coupling constant of 9.6 Hz for the vicinal protons. Consequently, the structure of **1** was established and named as pierisformotoxin E.

Pierisformotoxin F (**2**) had the same molecular formula as

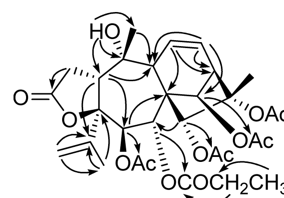

Fig. 2. Key HMBC Correlations of Compound **1**

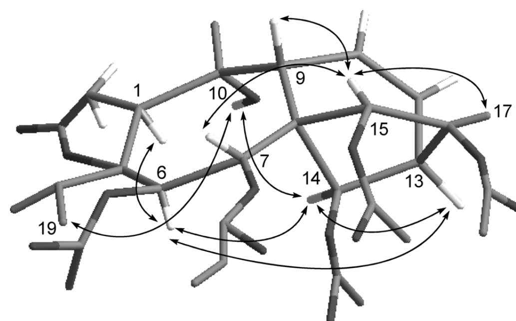

Fig. 3. Key ROESY Correlations of Compound **1**

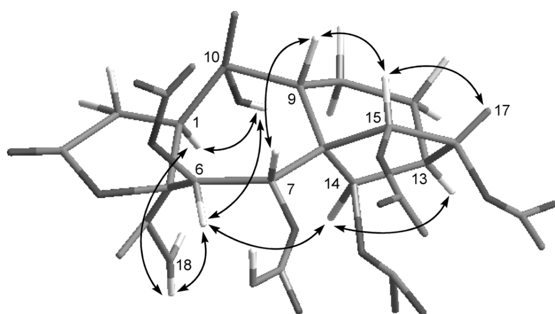

Fig. 4. Key ROESY Correlations of Compound **3**

1, as inferred from the HR-ESI-MS (*m/z* 655.2154, $[M+Cl]^-$). The ^{1}H- and ^{13}C-NMR data of **2** were very similar to those of **1** except for signals of H-7, H-14, C-7, C-14 and the propionyl carbonyl. The above signals of **2** were shifted by $\Delta\delta_H$ −0.02 and +0.06, and $\Delta\delta_C$ +0.2, −0.2, and +1.2 ppm, comparing with those of **1**. Moreover, the HMBC correlations of **2** from H-14 to the propionyl carbonyl (δ_C 174.4) and from H-7 to the acetyl carbonyl (δ_C 169.9) revealed that the propionyloxyl at C-7 and the acetyloxy at C-14 in **1** were exchanged in **2**. Therefore, the structure of compound **2** was determined and named as pierisformotoxin F.

The molecular formula of **3** was deduced as $C_{31}H_{40}O_{13}$, from its HR-ESI-MS (643.2151, $[M+Cl]^-$) and ^{13}C-NMR data. The ^{1}H-NMR spectral data (Table 1) indicated the presence of a terminal double bond at δ_H 5.41 (br s) and 4.87 (br s), four acylated oxymethines at δ_H 6.54 (d, J=9.2 Hz), 5.69 (d, J=9.2 Hz), 6.86 (s), and 5.44 (s), an exchangeable hydroxyl proton at δ_H 7.31 (s), five acetyl methys at δ_H 2.03, 2.12, 2.15, 2.17, and 2.25, as well as three singlet methyls at δ_H 1.44, 1.69, and 1.98. In addition, signals at δ_H 3.07 (dd, J=11.7, 9.1 Hz), 2.73 (br d, J=7.9 Hz) and 3.60 (br s), together with the resonances having complex coupling patterns or partially overlapped in the high-field region, were also observed. These spectroscopic data suggested that **3** was a highly acylated grayanane diterpenoid, and similar to secorhodomollide A (**4**). The main difference was that compound **3** contained an *O*-acetyl instead of the *O*-propionyl at C-7 of **4**. This was further confirmed by the HMBC correlation between H-7 and the acetyl carbonyl (δ_C 169.8).

In the ROESY spectrum of **3** (Fig. 4), correlations of Me-17/H-15, H-15/H-9 and H-9/H-7, indicated that H-7, H-9, H-15, and Me-17 were in the same β-orientations. Meanwhile, the cross-peaks observed between H-6/H-18α, H-18α/H-1, H-1/10-OH, 10-OH/H-6, H-6/H-14, and H-14/H-13 demonstrated that H-1, H-6, H-13, H-14 and 10-OH were all α-oriented. Thus, the structure of compound **3** was assigned and designated as pierisformotoxin G.

The AChE inhibitory activities of compounds **1**—**3** were assayed by using Ellman method.[29] However, none of the tested compounds showed obvious inhibitory activity at a concentration of 100 μM. The propensity of compounds **1**—**3** to enhance the effects of NGF to stimulate from PC 12 cells was also assessed, but they were inactive to enhance neurite outgrowth in NGF (5 ng/ml)-mediated PC 12 cells at 50 μM.

Experimental

General Experimental Procedures Optical rotations were made on a Jasco DIP-370 digital polarimeter (JASCO Corp., Tokyo, Japan). IR spectra were acquired using a Bio-Rad FTS-135 spectrophotometer with KBr pellets (Bio-Rad Corp., U.S.A.). NMR spectra, including HSQC, HMBC and ROESY, were recorded on a Bruker DRX-500 instrument with tetramethylsilane (TMS) as an internal standard (Bruker BioSpin group, German). ESI-MS and HR-ESI-MS data were obtained on an API Qstar Pulsar instrument (Applied Biosystem Corp., Canada). Semipreparative HPLC was carried on an Agilent 1200 liquid chromatography with a Zorbax SB-C_{18} (5 μm, 9.4×250 mm, Agilent, U.S.A.) column. Silica gel (200—300 or 100—200 mesh, Qingdao Marine Chemical Factory, P. R. China) and Sephadex LH-20 (Amersham Biosciences AB, Uppsala, Sweden) were used for column chromatography (CC). TLC was performed on silical gel GF_{254} plates (Qingdao Marine Chemical Factory, P. R. China), and spots were detected by spraying with 10% H_2SO_4–EtOH, followed by heating on a hot plate.

Plant Material The fruits of *P. formosa* were collected in Jindian, Kunming, China, in October 2009 and identified by Dr. Yong-Peng Ma of Kunming Institute of Botany, Chinese Academy of Sciences. The voucher specimen (KMUST 2009100901) was deposited at the Laboratory of Phytochemistry, the college of life science and technology, Kunming University of Science and Technology.

Extraction and Isolation The air-dried, powdered fruits of *P. formosa* (6 kg) were extracted with 75% Me_2CO/H_2O (3×18 l, 24 h each) at room temperature. The filtrate was concentrated *in vacuo* to give a crude extract, which was then partitioned between H_2O and EtOAc. The EtOAc fraction (350 g) was chromatographed over Sephadex LH-20 (MeOH–H_2O, 3 : 7, 6 : 4, 9 : 1, 1 : 0) to afford four fractions, and fraction 1 (MeOH–H_2O 3 : 7) was subjected to silica gel column ($CHCl_3$–MeOH 20 : 0, 19 : 1, 9 : 1, 8 : 2, 0 : 20) to give five main fractions (A—E). Compound **3** (7 mg) was crystallized from fraction B over silica gel ($CHCl_3$–MeOH 180 : 1), and the mother liquor was applied to Sephadex LH-20 ($CHCl_3$–MeOH, 1 : 1), then silica gel (petroleum ether–Me_2CO, 9 : 1, 8 : 2, 7 : 3, 6 : 4) to get four fractions (B1—B4). Subfraction B2 gave compounds **1** (5 mg) and **2** (6 mg), respectively, after being chromatographed over silica gel with petroleum ether–Me_2CO (10 : 1), and semipreparative HPLC with 30—50% MeOH–H_2O (3 ml/min).

Pierisformotoxin E (**1**): White amorphous powder, $[\alpha]_D^{27}$ −40.1 (c=0.14, $CHCl_3$); IR (KBr) ν_{max} 3452, 2984, 2941, 1783, 1737, 1643, 1371, 1232, 1064 cm^{-1}; ^{1}H- (500 MHz) and ^{13}C- (125 MHz) NMR data, see Table 1; ESI-MS (negative mode) *m/z*: 655 $[M+Cl]^-$; HR-ESI-MS (negative mode) *m/z*: 655.2163 $[M+Cl]^-$ (Calcd for $C_{31}H_{40}O_{13}Cl$, 655.2157).

Pierisformotoxin F (**2**): White amorphous powder, $[\alpha]_D^{27}$ −27.9 (c=0.05, $CHCl_3$); ^{1}H- (500 MHz) and ^{13}C- (125 MHz) NMR data, see Table 1; ESI-MS (negative mode) *m/z*: 655 $[M+Cl]^-$; HR-ESI-MS (negative mode) *m/z*: 655.2154 $[M+Cl]^-$ (Calcd for $C_{31}H_{40}O_{13}Cl$, 655.2157).

Pierisformotoxin G (**3**): White amorphous powder, $[\alpha]_D^{27}$ −11.6 (c=0.23, $CHCl_3$); ^{1}H- (500 MHz) and ^{13}C- (125 MHz) NMR data, see Table 1; ESI-MS (negative mode) *m/z*: 643 $[M+Cl]^-$; HR-ESI-MS (negative mode) *m/z*: 643.2151 $[M+Cl]^-$ (Calcd for $C_{30}H_{40}O_{13}Cl$, 643.2157).

Acetylcholinesterase Inhibitory Activity The acetylcholinesterase inhibitory activity was assayed by the spectrophotometric method developed by Ellman *et al.*,[29] and tacrine as positive control.

Neurite Outgrowth Assay For the bioassay of neurite outgrowth-promoting activity, PC 12 cells were seeded at a density of 2×10^4 cells/ml in 48-well plate coated with poly-L-lysine. After 24 h, the medium was changed to test medium containing various concentrations of NGF (50 ng/ml for positive control, 5 ng/ml for the negative control), 10% horse serum (HS), 5% fetal bovine serum (FBS), and 50 μM test compounds. After 72 h incubation, the neurite outgrowth was assessed under a phase-contrast microscope. The ratio of the neurite-bearing cells to total cells (with at least 100 cells examined/view area; 5 viewing area/well) was determined and expressed as a percentage.

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References

1) Editorial Committee of Flora of China of Chinese Academy of Sciences, "Flora Republicae Popularis Sinicae," Vol. 57, Science Press, Beijing, 2005, p. 22.
2) Eykman J. F., *Rec. Trav. Chim.*, **1**, 225 (1882).
3) Meguri H., *Yakugaku Zasshi*, **79**, 1052—1056 (1959).
4) Hikino H., Ito K., Takemoto T., *Chem. Pharm. Bull.*, **17**, 854—855 (1969).

5) Hikino H., Ito K., Ohta T., Takemoto T., *Chem. Pharm. Bull.*, **17**, 1078—1079 (1969).
6) Hikino H., Ogura M., Fuzita M., Ito K., Takemoto T., *Chem. Pharm. Bull.*, **19**, 856—857 (1971).
7) Hikino H., Ogura M., Takemoto T., *Chem. Pharm. Bull.*, **19**, 1980—1981 (1971).
8) Hikino H., Ogura M., Fushiya S., Konno C., Takemoto T., *Chem. Pharm. Bull.*, **25**, 523—524 (1977).
9) Sakakibara J., Kaiya T., Shirai N., *Yakugaku Zasshi*, **100**, 540—545 (1980).
10) Sakakibara J., Shirai N., Kaiya T., *Phytochemistry*, **20**, 1744—1745 (1981).
11) Kaiya T., Sakakibara J., *Chem. Pharm. Bull.*, **33**, 4637—4639 (1985).
12) Katai M., Matsushima T., Terai T., Meguri H., *Yakugaku Zasshi*, **95**, 778—783 (1975).
13) Katai M., Fujiwara M., Terai T., Meguri H., *Chem. Pharm. Bull.*, **28**, 3124—3126 (1980).
14) Wang L. Q., Ding B. Y., Zhao W. M., Qin G. W., *Chinese Chem. Lett.*, **9**, 465—467 (1998).
15) Wang L. Q., Ding B. Y., Zhao W. M., Qin G. W., Lin G., Cheng K. F., *Phytochemistry*, **49**, 2045—2048 (1998).
16) Wang L. Q., Chen S. N., Qin G. W., Cheng K. F., *J. Nat. Prod.*, **61**, 1473—1475 (1998).
17) Wang L. Q., Chen S. N., Cheng K. F., Li C. J., Qin G. W., *Phytochemistry*, **54**, 847—852 (2000).
18) Wang L. Q., Qin G. W., Chen S. N., Li C. J., *Fitoterapia*, **72**, 779—787 (2001).
19) Li C. H., Niu X. M., Luo Q., Xie M. J., Luo S. H., Zhou Y. Y., Li S. H., *Org. Lett.*, **12**, 2426—2429 (2010).
20) Codding P. W., *J. Am. Chem. Soc.*, **106**, 7905—7909 (1984).
21) Chen G., Gao Y., Mao H. Y., Li Y. S., *Chin. Med. J.*, **98**, 325—328 (1985).
22) Yakehiro M., Yamamoto S., Baba N., Nakajima S., Iwasa J., Seyama I., *J. Pharmacol. Exp. Ther.*, **265**, 1328—1332 (1993).
23) Yakehiro M., Yuki T., Yamaoka K., Furue T., Mori Y., Imoto K., Seyama I., *Mol. Pharmacol.*, **58**, 692—700 (2000).
24) Zhong G. H., Liu J. X., Weng Q. F., Hu M. Y., Luo J. J., *Pest Manage. Sci.*, **62**, 976—981 (2006).
25) Hikino H., Ohta T., Ogura M., Ohizumi Y., Konno C., Takemoto T., *Toxicol. Appl. Pharmacol.*, **35**, 303 (1976).
26) Masutani T., Seyama I., Narahashi T., Iwasa J., *J. Pharmacol. Exp. Ther.*, **217**, 812 (1981).
27) Klocke J. A., Hu M. Y., Chiu S. F., Kubo I., *Phytochemistry*, **30**, 1797 (1991).
28) Wang W. G., Li H. M., Li H. Z., Wu Z. Y., Li R. T., *J. Asian Nat. Prod. Res.*, **12**, 70—75 (2010).
29) Ellman G. L., Courtney K. D., Andres V. J., Featherstone R. M., *Biochem. Pharmacol.*, **7**, 88—95 (1961).