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THREE NEW ISOINDOLIN-1-ONES FROM THE LEAVES OF YUNNAN LOCAL SUN CURED TOBACCO AND THEIR BIOACTIVITIES

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Abstract – Three new (**1-3**) and one known (**4**) isoindolin-1-ones were isolated from the leaves of Yunnan local sun cured tobacco. Their structures were determined by means of HRESIMS and extensive 1D and 2D NMR spectroscopic studies. Compounds **1-3** were tested for their anti-tobacco mosaic virus (anti-TMV) activities and compounds **1-4** were tested for their cytotoxicity activities. The results showed that compounds **1-3** showed high anti-TMV activity with inhibition rates of 43.8, 45.6 and 52.7%. These rates are higher than that of positive control. Compounds **1-4** also showed moderate-to-weak inhibitory activities against some tested human tumor cell lines with IC_{50} values in the range of 2.8–8.2 μM.

Nicotiana tabacum, tobacco, is an annually-grown herbaceous plant in the Solanaceae (nightshade family). It is found only in cultivation, where it is the most commonly grown of all plants in the *Nicotiana* genus, and its leaves are commercially grown in many countries to be processed into tobacco.[1] *N. tabacum* is also a kind of plant containing most complex secondary metabolites in nature.[2,3] The recent phytochemical investigations revealed that many new bioactive compounds, such as, sesquiterpenes,[4-6] alkaloids,[7,8] lignans,[9,10] flavonoids,[11-14] phenylpropanoids,[15,16] chromanones,[17,18] biphenyls,[19] phenolic amides,[20] isocoumarins,[21] and furan-2-carboxylic acids,[22] were isolated from the genus of this plants. Some of which showed various bioactivities, such as anti-HIV-1, anti-TMV, and cytotoxicity. Therefore, the multipurpose utilization of this plant is an interesting topical and attracts more and more attentions. As part of an ongoing effort aimed at elucidating the bioactive natural products from *Nicotiana* genus, the phytochemistry investigation of the leaves of Yunnan local sun cured tobacco (a variant of *N. tabacum*)

was carried out, and led to the isolation of three new (**1**-**3**) and one known (**4**) isoindolin-1-ones. Herein we present the isolation, structural elucidation, the anti-tobacco mosaic virus (anti-TMV) activities, and the cytotoxicity activities of these compounds.

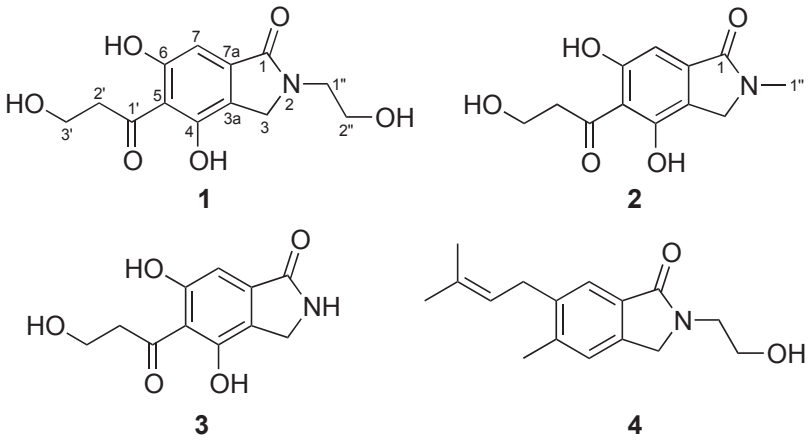

Figure 1. Isoindolin-1-ones from the leaves of Yunnan local sun cured tobacco

A 70% aq. acetone extract prepared from the leaves of tobacco was subjected repeatedly to column chromatography and preparative HPLC to afford three new isoindolin-1-ones, 4,6-dihydroxy-2-(2-hydroxyethyl)-5-(3-hydroxypropan)isoindolin-1-one (**1**), 4,6-dihydroxy-5-(3-hydroxypropan)-2-methyl-isoindolin-1-one (**2**), and 4,6-dihydroxy-5-(3-hydroxypropan)isoindolin-1-one (**3**), and one known isoindolin-1-one, 2-(2-hydroxyethyl)-5-methyl-6-3-prenylisoindolin-1-one (**4**).[7] The structures of the compounds **1**-**4** were shown in Figure 1, and the ^{1}H and ^{13}C NMR data of **1**-**3** were listed in Table 1.

Compound **1** was isolated as a pale yellow gum. The molecular formula of **1** was determined to be $C_{13}H_{15}NNaO_6$ by the pseudomolecular ion peak at *m/z* 304.0790 $[M+Na]^+$ in its HRESIMS, suggesting 7 degrees of unsaturation. The UV spectrum showed absorption maxima at 210, 268 and 306 nm, and the IR spectrum showed absorption bands at 3386, 1688, 1652, 1612, 1576, and 1452 cm^{-1}, indicating the presence of hydroxy group, carbonyl group, and aromatic ring. The ^{1}H, ^{13}C NMR data (Table 1), and HSQC correlations of **1** showed resonances due to a isoindolin-1-one nucleus[23] (C-1 to C-7a; H_2-3 and H-7), a 3-hydroxypropane group[24] (C-1' to C-3'; H_2-2' and H_2-3'), a *N*-2-hydroxyethyl group[25] (C-1" and C-2"; H_2-1" and H_2-2"), and two phenolic hydroxy groups (δ_H 11.05 s, 10.84 s). The HMBC correlations (Figure 2) of H_2-3 with C-1, C-3a, C-4, C-7a, and C-1", of H-7 with C-1, C-3a, and C-7a, and of H_2-1" with C-1 and C-3 also suggested that compound **1** should be an isoindolin-1-one. The HMBC correlations of H_2-2′ (δ_H 3.25) with C-5 (δ_C 120.6) indicated that the 3-hydroxypropane group was attached to C-5.

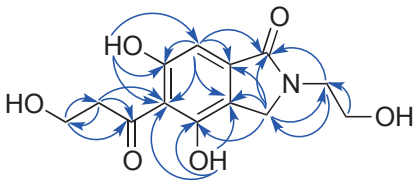

Figure 2. Key HMBC (↷) correlations of **1**

Two phenolic hydroxy groups were assigned to C-4 and C-6 on the basis of HMBC correlations between the hydroxy proton (δ_H 11.05) and C-4 (δ_C 153.1), C-5 (δ_C 120.6), and C-3a (δ_C 136.3), as well as those between the other hydroxy proton (δ_H 10.84) and C-5 (δ_C 120.6), C-6 (δ_C 158.3), and C-7 (δ_C 101.2). Finaly, the 2-hydroxyethyl group linked to nitrogen-atom (N-2) was confirmed by the HMBC correlation of H-1″ (δ_H 3.56) with C-1 (δ_C 166.3) and C-3 (δ_C 44.1). Thus, the structure of **1** was established as 4,6-dihydroxy-2-(2-hydroxyethyl)-5-(3-hydroxypropan)isoindolin-1-one.

Table 1. ^{1}H and ^{13}C NMR data of compounds **1-3** ($CDCl_3$, δ, ppm, J/Hz)

No.	Compound **1**		Compound **2**		Compound **3**	
	δ_C	δ_H (m, J, Hz)	δ_C	δ_H (m, J, Hz)	δ_C	δ_H (m, J, Hz)
1	166.3 s		167.2 s		169.3 s	
3	44.1 t	4.20 s	46.5 t	4.12 s	44.3 t	4.12 s
3a	136.3 s		136.5 s		136.8 s	
4	153.1 s		153.7 s		153.7 s	
5	120.6 s		122.8 s		120.0 s	
6	158.3 s		158.7 s		158.4 s	
7	101.2 d	6.63 s	101.3 d	6.60 s	102.2 d	6.66 s
7a	116.8 s		116.5 s		119.4 s	
1′	198.8 s		1998 s		199.6 s	
2′	42.1 t	3.25 (t) 6.8	42.3 t	3.25 (t) 6.8	43.5 t	3.24 (t) 6.8
3′	58.1 t	4.33 (t) 6.8	58.4 t	4.31 (t) 6.8	58.8 t	4.30 (t) 6.8
1″	46.6 t	3.56 (t) 5.6	33.5 s	2.82 s		
2″	59.6 t	3.86 (t) 5.6				
-NH						9.02 s
Ar-OH-4		11.05 s		11.12 s		10.80 s
Ar-OH-6		10.84 s		10.86 s		10.67 s

Compound **2** was also obtained as yellow gum. A molecular formula $C_{12}H_{13}NO_5$ was assigned from HRESIMS (*m/z*: 276.0734 $[M+Na]^+$, calcd 276.0734). The ^{1}H and ^{13}C NMR data of **2** (Table 1) displayed 12 carbon and 13 proton signals, corresponding to a isoindolin-1-one nucleus, a 3-hydroxypropane group, a methyl group linked to nitrogen-atom (N-2 position), and two phenolic hydroxy groups. The ^{1}H and ^{13}C NMR spectral data of **2** were similar to those of **1**. The obvious differences resulted from the replacement of the *N*-2-hydroxyethyl group in **1** by the *N*-methyl group in **2**. The HMBC correlations of the *N*-methyl proton (δ_H 2.82) with C-1 (δ_C 167.2) and C-3 (δ_C 46.5) also indicated that the location of *N*-methyl group. Accordingly, the structure of 4,6-dihydroxy-5-(3-hydroxypropan)-2-methylisoindolin-1-one (**2**) was established.

The ^{1}H and ^{13}C NMR spectra of **3** were also similar to those of **1**. The chemical shift differences resulted from the disappearance of an *N*-2-hydroxyethyl group and appearance of an amino proton resonance (δ_H

9.02 s) in **2**. This indicated that the *N*-2-hydroxyethyl group in **1** was converted into an amino proton in **2**. The HMBC correlations of amino proton to C-1, C-3, C-3a, and C-7a also indicated this structure change. Thus, the structure of **3** was established as 4,6-dihydroxy-5-(3-hydroxypropan)isoindolin-1-one.

Compounds **1-3** were tested for their anti-TMV activity at the concentration of 20 μM. The anti-TMV activity were tested using the half-leaf method.[26,27] Ningnanmycin (a commercial product for plant disease in China, with inhibition rate of 33.6% at the concentration of 20 μM), was used as a positive control. The results showed that compounds **1-3** showed high anti-TMV activity with inhibition rate of 43.8, 45.6, and 52.7%, respectively. These rates are higher than that of positive control.

The cytotoxicities of compounds **1-4** were also tested using a previously reported procedure.[28,29] The cytotoxic abilities against five human tumor cell lines (NB4, A549, SHSY5Y, PC3, and MCF7) by MTT-assay were summarized in Table 2. The results revealed that compounds **1-6** showed moderate-to-weak inhibitory activities against some tested human tumor cell lines with IC_{50} values in the range of 2.8–8.2 μM.

Table 2. Cytotoxic activity of compounds **1-4**

Compounds	Cell lines and IC_{50} (μM)				
	NB4	A549	SHSY5Y	PC3	MCF7
1	2.8	>10	4.2	6.3	5.1
2	3.6	>10	5.8	>10	5.4
3	>10	8.2	6.7	4.8	7.0
4	>10	3.8	4.2	5.9	>10
Taxol	0.03	0.02	0.05	0.05	0.05

NB4, human leukemia cell; A549, carcinomic human alveolar basal epithelial cell; SHSY5Y, human neuroblastoma cell; PC3, human prostate cancer cell; MCF7, human breast adenocarcinoma cell.

EXPERIMENTAL

General. UV spectra were obtained using a Shimadzu UV-2401A spectrophotometer. A Tenor 27 spectrophotometer was used for scanning IR spectroscopy with KBr pellets. 1D and 2D NMR spectra were recorded on DRX-500 spectrometers with TMS as internal standard, and the chemical shifts (δ) were expressed in ppm. HRESIMS was performed on an API QSTAR time-of-flight spectrometer and a VG Autospec-3000 spectrometer, respectively. Preparative HPLC was performed on a Shimadzu LC-8A preparative liquid chromatograph with a ZORBAX PrepHT GF (21.2 mm × 25 cm, 7 μm) column or a Venusil MP C_{18} (20 mm × 25 cm, 5 μm) column. Column chromatography was performed with Si gel (200–300 mesh, Qing-dao Marine Chemical, Inc., Qingdao, China). The fractions were monitored by TLC, and spots were visualized by heating Si gel plates sprayed with 5% H_2SO_4 in EtOH.

Plant Material. The sun cured tobacco leaves (Weishan red sun cured tobacco, a variety of *Nicotiana tabacum*) were collected in Dali Prefecture, Yunnan Province, People's Republic of China, in September 2015. The identification of the plant material was verified by Prof. H. W. Yang (School of Tobacco, Yunnan Agriculture University).

Extraction and Isolation. The air-dried and powdered tobacco leaves (4.3 kg) were extracted three times

with 70% aqueous acetone (3 × 6.0 L) at room temperature and filtered to yield a filtrate, and the extract was partitioned between EtOAc and 3% tartaric acid. The aqueous layer was adjusted to pH 9 with saturated sodium carbonate aq. and extracted with EtOAc. The EtOAc-soluble alkaloidal materials (53.6 g) were applied to silica gel (200–300 mesh) column chromatography, eluting with $CHCl_3$/MeOH gradient system (10:0, 9:1, 8:2, 7:3, 6:4, and 5:5) to give six fractions A-F. The further separation of fraction B (9:1, 8.26 g) was subjected to silica gel column chromatography eluting with acetone and then run on preparative HPLC (40% MeOH/H_2O, flow rate 12 mL/min) to yield compound **4** (16.3 mg). Further separation of fraction C (8:2, 15.4 g) by silica gel column chromatography, eluted with $CHCl_3$/acetone (8:2, 7:3, 6:4, 5:5, 4:6, 3:7, and 2:8), yielded mixtures C1–C7. Fraction C4 (5:5, 0.87 g) was subjected to silica gel column chromatography using petroleum ether/acetone, and then semi-preparative HPLC (30% MeOH/H_2O, flow rate 12 mL/min) to give **1** (10.8 mg) and **2** (11.4 mg). Fraction C-5 (4:6, 0.47 g) was subjected to silica gel column chromatography using petroleum ether/acetone, and then semi-preparative HPLC (26% MeOH/H_2O, flow rate 12 mL/min) to give **3** (12.0 mg).

Anti-TMV Assays. TMV (U1 strain) was obtained from the Key Laboratory of Tobacco Chemistry of Yunnan Province, China Tobacco Yunnan Industrial Co., Ltd. The virus was multiplied in *Nicotiana tabacum* cv. K326 and purified as described.[30] The concentration of TMV was determined as 20 mg/mL with a UV spectrophotometer [virus concentration = (A_{260} × dilution ratio) / $E_{1\,cm}^{0.1\%,\,260\,nm}$]. The purified virus was kept at -20 °C and was diluted to 32 μg/mL with 0.01 M PBS before use.

Nicotiana glutinosa plants were cultivated in an insect-free greenhouse. *N. glutinosa* was used as a local lesion host. The experiments were conducted when the plants grew to the 5-6-leaf stage. The tested compounds were dissolved in DMSO and diluted with distilled H_2O to the required concentrations. A solution of equal concentration of DMSO was used as negative control. The commercial antiviral agent ningnanmycin (purity > 98%) was used as a positive control.

For Half-Leaf Method,[26,27] the virus was inhibited by mixing with the solution of compound. After 30 min, the mixture was inoculated on the left side of the leaves of *N. glutinosa*, whereas the right side of the leaves was inoculated with the mixture of DMSO solution and the virus as control. The local lesion numbers were recorded 3–4 days after inoculation. Three repetitions were conducted for each compound. The inhibition rates were calculated according to the formula:

$$\text{inhibition rate (\%)} = [\,(C\text{-}T) / C\,] \times 100\%$$

where C is the average number of local lesions of the control and T is the average number of local lesions of the treatment. Ningnanmycin, a commercial virucide for plant disease in China, was used as a positive control.

Cytotoxicity Assay. Colorimetric assays were performed to evaluate each compound's activity. NB4 (human acute promyelocytic leukemia cells), A549 (human lung adenocarcinoma epithelial cells), SHSY5Y (human neuroblastoma cells), PC3 (human prostate cancer cell), and MCF7 (human breast adenocarcinoma cells) tumor cellcells were purchased from the American Type Culture Collection (ATCC). All cells were cultured in RPMI-1640 or DMEM medium (Hyclone, Logan, UT) supplemented with 10% fetal bovine serum (Hyclone) at 37 °C in a humidified atmosphere with 5% CO_2. Cell viability was assessed by conducting colorimetric measurements of the amount of insoluble formazan formed in living cells based on the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma, St. Louis, MO). Briefly, 100 μL of suspended adherent cells were seeded into each well of a 96-well cell culture plate and allowed to adhere for 12 h before drug addition. In addition, suspended cells were seeded just before drug addition, with an initial density of 1×10^5 cells/mL in 100 μL of medium. Each tumor cell line was exposed to each test compound at various concentrations in triplicate for 48 h; paclitaxel (Sigma, purity > 95%) was used as a positive control. After the incubation, MTT (100 μg) was added to each well, and the incubation was continued for 4 h at 37 °C. The cells were lysed with 100 μL of 20% SDS-50% DMF after removal of 100 μL of the medium. The optical density of the lysate was measured at 595 nm in a 96-well microtiter plate reader (Bio-Rad 680). The IC_{50} value of each compound was calculated by Reed and Muench's method.

4,6-Dihydroxy-2-(2-hydroxyethyl)-5-(3-hydroxypropan)isoindolin-1-one (1): Obtained as yellow gum; UV (MeOH) λ_{max} nm (log ε) 210 (4.18), 268 (3.52), and 306 (3.08); IR (KBr) ν_{max} 3386, 2938, 1688, 1652, 1612, 1576, 1452, 1358, 1246, 1167, 1062, 973, and 852 cm^{-1}; positive ESIMS *m/z* 304 $[M+Na]^+$, positive HRESIMS *m/z* 304.0790 $[M+Na]^+$ (calcd for $C_{13}H_{15}NNaO_6$, 304.0797).

4,6-Dihydroxy-5-(3-hydroxypropan)-2-methylisoindolin-1-one (2): Obtained as yellow gum; UV (MeOH) λ_{max} nm (log ε) 210 (4.06), 265 (3.75), and 304 (3.12); IR (KBr) ν_{max} 3390, 2935, 1685, 1657, 1612, 1569, 1460, 1352, 1255, 1160, 1054, 968, and 846 cm^{-1}; positive ESIMS *m/z* 276 $[M+Na]^+$, positive HRESIMS *m/z* 276.0734 $[M+Na]^+$ (calcd for $C_{12}H_{13}NNaO_5$, 276.0734).

4,6-Dihydroxy-5-(3-hydroxypropan)isoindolin-1-one (3): Obtained as yellow gum; UV (MeOH) λ_{max} nm (log ε) 210 (4.02), 265 (3.47), and 302 (3.03); IR (KBr) ν_{max} 3390, 2930, 1686, 1659, 1615, 1558, 1464, 1352, 1253, 1170, 1069, 958, and 847 cm^{-1}; positive ESIMS *m/z* 260 $[M+Na]^+$, positive HRESIMS *m/z* 260.0535 $[M+Na]^+$ (calcd for $C_{11}H_{11}NNaO_5$, 260.0535).

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REFERENCES

1. The Editorial Committee of the Administration Bureau of Flora of China, 'Flora of China,' 67 vols., Beijing Science and Technology Press, Beijing, 2005.
2. M. F. Dube and C. R. Green, *Rec. Adv. Tob. Sci*., 1982, **8**, 42.
3. A. Rodgman and T. A. Perfetti, 'The Chemical Components of Tobacco and Tobacco Smoke,' CRC Press, Taylor and Francis Group, Boca Raton, Florida, 2008.
4. S. Z. Shang, W. Zhao, J. G. Tang, X. M. Xu, H. D. Sun, J. X. Pu, Z. H. Liu, M. M. Miao, Y. K. Chen, and G. Y. Yang, *Fitoterapia*, 2016, **108**, 1.
5. X. Feng, Xin, J. S. Wang, J. Luo, and L. Y. Kong, *J. Asian Nat. Prod. Res.*, 2010, **12**, 252.
6. G. Y. Yang, W. Zhao, Y.-K. Chen, Z.-Y. Chen, Q.-F. Hu, and M.-M. Miao, *Asian J. Chem.*, 2013, **25**, 4932.
7. G.-H. Kong, Y.-P. Wu, W. Li, Z.-Y. Xia, Q. Liu, K.-M. Wang, P. He, R.-Z. Zhu, X.-X. Si, and G.-Y. Yang, *Heterocycles*, 2016, **92**, 331.
8. X. C. Wei, S. C. Sumithran, A. G. Deaciuc, H. R. Burton, L. P. Bush, L. P. Dwoskin, and P. A. Crooks, *Life Sci.*, 2005, **78**, 495.
9. Y. K. Chen, X. S. Li, G. Y. Yang, Z. Y. Chen, Q. F. Hu, and M. M. Miao, *J. Asian Nat. Prod. Res*., 2012, **14**, 450.
10. X.-M. Gao, X. Li, X. Yang, H. Mu, Y. Chen, G. Yang, and Q.-F. Hu, *Heterocycles*, 2012, **85**, 147.
11. M. M. Miao, L. Li, Q. P. Shen, C. B. Liu, Y. K. Li, T. Zhang, F. M. Zhang, P. He, K. M. Wang, R. Z. Zhu, Y. K. Chen, and G. Y. Yang, *Fitoterapia*, 2015, **103**, 260.
12. Z. Y. Chen, J. L. Tan, G. Y. Yang, M. M. Miao, Z. Y. Chen, and T. F. Li, *Phytochem. Lett*., 2012, **5**, 233.
13. Y. Li, Y. Zhao, N. Xiang, L. Yang, F. Wang, G. Yang, and Z. Wang, *Heterocycles*, 2014, **89**, 2771.
14. Y. Wang, C.-B. Liu, Q.-P. Shen, F.-M. Zhang, P. He, Z.-H. Liu, H.-B. Zhang, X.-D. Yang, M.-M. Miao, and G.-Y. Yang, *Heterocycles*, 2015, **91**, 1198.
15. H. Q. Leng, J. X. Chen, Y. Hang, Y. X. Duan, G. Y. Yang, Y. K. Chen, Y. D. Guo, Q. F. Hu, and M. M. Miao, *Chem. Nat. Compd.*, 2014, **49**, 1028.
16. J. Tan, Z. Chen, G. Yang, M. Miao, Y. Chen, and T. Li, *Heterocycles*, 2011, **83**, 2381.
17. D. Mou, W. Zhao, T. Zhang, L. Wan, G. Yang, Y. Chen, Q. Hu, and M. Miao, *Heterocycles*, 2012, **85**, 2485.
18. G. Yang, W. Zhao, T. Zhang, Y. Duan, Z. Liu, M. Miao, and Y. Chen, *Heterocycles*, 2014, **89**, 183.
19. S. Z. Shang, W. X. Xu, P. Lei, W. Zhao, J. G. Tang, M. M. Miao, H. D. Sun, J. X. Pu, Y. K. Chen, and G. Y. Yang, *Fitoterapia*, 2014, **99**, 35.
20. S. Z. Shang, Y. X. Duan, X. Zhang, J. X. Pu, H. D. Sun, Z. Y. Chen, M. M. Miao, G. Y. Yang, and

Y. K. Chen, *Phytochem. Lett.*, 2014, **7**, 413.

21. S. Z. Shang, W. X. Xu, L. Li, J. G. Tang, W. Zhao, P. Lei, M. M. Miao, H. D. Sun, J. X. Pu, Y. K. Chen, and G. Y. Yang, *Phytochem. Lett.*, 2015, **11**, 53.
22. C.-B. Liu, X.-M. Xu, Q.-P. Shen, W. Zhang, X.-F. Shen, Y.-K. Yang, F.-M. Zhang, P. He, X.-X. Si, Y.-D. Guo, J.-J. Xia, and G.-Y. Yang, *Heterocycles*, 2016, **92**, 1095.
23. K. Wang, L. Bao, Q. Y. Qi, F. Zhao, K. Ma, Y. F. Pei, and H. W. Liu, *J. Nat. Prod*., 2015, **78**, 146.
24. Q. F. Hu, B. Zhou, X. M. Gao, L. Y. Yang, L. D. Shu, Y. Q. Shen, G. P. Li, C. T. Che, and G. Y. Yang, *J. Nat. Prod.*, 2012, **75**, 1909.
25. J. M. Wojnar, K. O. Dowle, and P. T. Northcote, *J. Nat. Prod*., 2014, **77**, 2288.
26. M. Zhou, K. Zhou, X. M. Gao, Z. Y. Jiang, J. J. Lv, Z. H. Liu, G. Y. Yang, M. M. Miao, C. T. Che, and Q. F. Hu, *Org. Lett*., 2015, **17**, 2638.
27. M. Zhou, M. M. Miao, G. Du, S. Z. Shang, W. Zhao, Z. H. Liu, G. Y. Yang, C. T. Che, Q. F. Hu, and X. M. Gao, *Org. Lett*., 2014, **16**, 5016.
28. Q. F. Hu, B. Zhou, J. M. Huang, Z. Y. Jiang, X. Z. Huang, L. Y. Yang, X. M. Gao, G. Y. Yang, and C. T. Che, *J. Nat. Prod*., 2013, **76**, 1866.
29. Q. F. Hu, B. Zhou, Y. Q. Ye, Z. Y. Jiang, X. Z. Huang, Y. K. Li, G. Du, G. Y. Yang, and X. M. Gao, *J. Nat. Prod*., 2013, **76**, 1854.
30. L. J. Reed and H. Muench, *Am. J. Epidemiol*., 1938, **27**, 493.