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Article

Chemical Constituents from Cultures of the Fungus *Marasmiellus ramealis* (Bull.) Singer

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A investigação química das culturas do fungo *Marasmiellus ramealis* resultou na primeira isolação de onze compostos incluindo dois novos sesquiterpenoides do tipo eudesmano 14(10→1)*abeo*-eudesmano-13-hidroxil-11-eno, 14(10→1)*abeo*-eudesmano-11,13-diol, e o novo derivado de meleína (*R*)-(–)-5-etoxicarbonil meleína. A atividade inibitória da aceticolinesterase (AChE) dos dois primeiros compostos foi avaliada, que mostraram ser ativos com uma porcentagem de inibição de 29 e 41%, respectivamente, em uma concentração de 100 μmol L^{-1}.

Chemical investigation on the cultures of the fungus *Marasmiellus ramealis* resulted in the first isolation of eleven compounds including two new eudesmane-type sesquiterpenoids 14(10→1)*abeo*-eudesmane-13-hydroxyl-11-ene, 14(10→1)*abeo*-eudesmane-11,13-diol, and the new mellein derivative (*R*)-(–)-5-ethoxycarbonyl mellein. The first two compounds were evaluated for their inhibitory activity against acetylcholinesterase (AChE), and proved to be active with a percentage inhibition of 29 and 41%, respectively, at a concentration of 100 μmol L^{-1}.

Keywords: *Marasmiellus ramealis* (Bull.) Singer, eudesmane, sesquiterpenoids, mellein, AChE inhibition

Introduction

As a key enzyme of biological neural conduction, acetylcholinesterase (AChE) can degrade the level of acetylcholine,[1] affecting the normal transmission of nerve signals *in vivo*. The search for AChE inhibitors, which reduce the degradation of acetylcholine, is an effective approach to drug discovery in this field. As a common neurodegenerative disease, Alzheimer's disease (AD) is caused by the absence of the brain neurotransmitter acetylcholine, so drug treatment for AD aims to improve the patient's level of acetylcholine by inhibiting AChE.[2,3] Many AChE inhibitors, such as tacrine, donepezil, rivastigmine and galantamine, are approved by the US Food and Drug Administration,[4] whereas huperzine A isolated from traditional Chinese medicine *Huperzia serrata* is the most successful AChE inhibitor developed in China.[5]

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The fungus *Marasmiellus ramealis* (Bull.) Singer belongs to the genus *Marasmiellus* in the family Marasmiaceae, and usually grows on deadwood. This fungus is a small, thin, white edible mushroom with a wide distribution in most parts of China, especially in Hainan, Hunan, Yunnan, and Tibet province.[6] Previous studies on chemical constituents of *M. ramealis* showed the presence of several natural products, such as marasin and isocoumarins.[7-9] In order to make full use of *M. ramealis*, a thorough chemical investigation was thus undertaken to find bioactive metabolites from cultures of this fungus, leading to the isolation of eleven compounds: 14(10→1)*abeo*-eudesmane-13-hydroxyl-11-ene (**1**), 14(10→1)*abeo*-eudesmane-11,13-diol (**2**), (*R*)-(–)-5-ethoxycarbonyl mellein (**3**), (*R*)-(–)-5-carboxylic acid mellein (**4**),[10] stachyline C (**5**),[11] 2-(4-hydroxyphenyl) acetate (**6**),[12] 5α,8α-epidioxy-ergosta-6,22-dien-3β-ol (**7**),[13] ergosta-7,22-dien-3β,5α,6β-triol (**8**),[14] cytochalasin D (**9**),[15] cytochalasin C (**10**),[16] and 13,14-epoxycytochalasin D (**11**).[17] Their structures were elucidated by mass spectrometry (MS),

nuclear magnetic resonance (NMR) and infrared (IR) spectroscopies, and comparison of spectroscopic data with those reported in literature. Compounds **1** and **2** are new eudesmane-type sesquiterpenoids and compound **3** is a new mellein derivative (Figure 1). Compounds **4**-**11** were isolated for the first time from *M. ramealis*. Two new eudesmane-type sesquiterpenoids (**1** and **2**) were assayed for AChE inhibitory activity and both showed moderate inhibition. Herein, the isolation and structural elucidation of these isolates, as well as their inhibitory activity against AChE, are described.

Experimental

General procedures

IR spectra were obtained on a Nicolet 380 FT-IR instrument, in KBr pellets (Thermo, Pittsburgh, PA, USA). Optical rotations were measured with a Rudolph Autopol III polarimeter (Rudolph Research Analytical, Hackettstown, NJ, USA). A Shimadzu UV-2550 spectrometer (Beckman, Brea, CA, USA) was used for scanning ultraviolet (UV) spectroscopy. High-resolution-electrospray ionization mass spectra (HRESIMS) were performed on an API QSTAR Pulsar mass spectrometer (Bruker, Bremen, Germany). 1D and 2D NMR spectra were recorded on AV-500 spectrophotometers (Bruker, Bremen, Germany) with tetramethylsilane (TMS) as the internal standard. Column chromatography (CC) was performed with Si gel (200-300 mesh; Qingdao Marine Chemical Inc., Qingdao, China) and Lichroprep RP-18 gel (40-63 μm; Merck, Darmastadt, Germany). The fractions were monitored by thin layer chromatography (TLC), and spots were visualized by heating Si gel plates sprayed with 5% H_2SO_4 in ethanol.

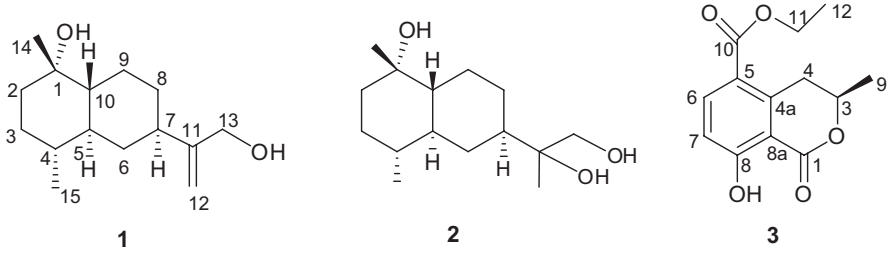
Fungus material

The fungus *M. ramealis* was collected in Jianfengling Mountain, Hainan province, China, in June 2012, and identified by Prof. Nian-kai Zeng, Hainan Medical College. The mycelium was isolated from the cap of *M. ramealis* and its strain was maintained on potato dextrose agar (PDA) slant at 4 °C. A voucher specimen (No. HUANG 201201) was deposited at the Institute of Tropical Bioscience and Biotechnology, Chinese Academy of Tropical Agricultural Sciences.

Fermentation, extraction and isolation

The fungus was cultured on PDA at room temperature for a week. Two pieces of mycelial agar plugs (0.5×0.5 cm^2) were inoculated into 1 L Erlenmeyer flasks containing 500 mL potato dextrose broth (PDB). The fermentation was carried out on a shaker at 25 °C and 150 rpm for 7 days, and then kept intact at room temperature for 23 days. The culture broth (90 L) was filtered to give the filtrate and mycelia. The filtrate was evaporated *in vacuo* to a small volume and then suspended in H_2O and partitioned successively with EtOAc and *n*-BuOH. The EtOAc solution was evaporated under reduced pressure to give a crude extract (15.6 g), which was separated into fractions 1-9 on silica gel CC using as gradient an eluent of petroleum ether-EtOAc (20:1-0:1, v/v, each 1 L). Fraction 2 (3.0 g) was separated by silica gel column using as gradient solvent petroleum ether-EtOAc (5:1-3:1, v/v, 500 mL) to afford compounds **5** (5.0 mg) and **6** (3.0 mg) according to their TLC pattern. Fraction 3 (2.0 g) was submitted to silica gel CC with petroleum ether-EtOAc (3:1, v/v, 600 mL) as eluent, and further purified by Sephadex LH-20 CC with $CHCl_3$/MeOH (1:1, v/v, 600 mL) as eluent, yielding compounds **9** (10.0 mg), **10** (15.0 mg) and **11** (10.0 mg). Compounds **3** (7.0 mg) and **4** (2.0 mg) were isolated from fraction 4 (3.5 g) by repeated silica gel CC eluted with petroleum ether-EtOAc (3:1, v/v, 800 mL). Compounds **1** (3.0 mg) and **2** (3.5 mg) were obtained from fraction 7 (2.0 g) by repeated silica gel CC with petroleum ether-EtOAc (2:1, v/v, 500 mL) and chromatographed over Sephadex LH-20 column, using $CHCl_3$-MeOH (1:1, v/v, 600 mL) as eluent. Fraction 8 (3.0 g) was purified by repeated silica gel CC eluted with petroleum ether-EtOAc (2:1, v/v, 600 mL) to yield compounds **7** (20.0 mg) and **8** (3.5 mg).

Compound **1**: colorless oil; $[\alpha]_D^{32}$ –3.7 (*c* 0.035, CH_3OH); UV (CH_3OH) λ_{max}/nm (log ε) 273 (3.36), 324 (3.30), 383 (3.24); IR (KBr) ν_{max}/cm^{-1} 3350, 2920, 1643, 1540, 1427,



1 **2** **3**

Figure 1. Structures of new compounds **1**-**3**.

Table 1 ^{1}H NMR and ^{13}C NMR data of **1** and **2** in $CDCl_3$ (δ in ppm)

No.	**1**		**2**	
	δ ^{1}H	δ ^{13}C	δ ^{1}H	δ ^{13}C
1	–	75.1	–	75.3
2	1.54 (β-H), 1.89 (α-H), m, 2H	36.9	1.23, 1.90, m, 2H	31.7
3	1.28, 1.68, m, 2H	31.7	1.23, 1.46, m, 2H	30.7
4	2.00, m, 1H	39.5	2.00, m, 1H	39.2
5	2.03, m, 1H	46.4	2.06, m, 1H	47.4
6	1.13, 1.45, m, 2H	30.1	1.45, 1.90, m, 2H	26.3
7	2.26, m, 1H	42.3	1.88, m, 1H	43.1
8	1.87, m, 2H	29.9	1.73 (β-H), 1.47 (α-H), m, 2H	24.5
9	1.53, 1.70, m, 2H	26.7	1.79, 1.73, m, 2H	21.1
10	2.11, m, 1H	55.4	2.08, m, 1H	55.8
11	–	156.9	–	76.2
12	4.93, dd, 1H, *J* 1.5, 2.1 Hz; 4.81, dd, 1H, *J* 1.5, 2.1 Hz	106.7	0.98, s, 3H	17.9
13	4.02, s, 2H	64.6	3.33, d, 1H, *J* 11.4 Hz; 3.58, d, 1H, *J* 11.4 Hz	68.4
14	1.16, s, 3H	29.2	1.15, s, 3H	32.2
15	0.86, s, 3H	16.4	0.95, s, 3H	16.5

1372, 1160, 1110, 1059, 611; HRESIMS *m/z* (%) 261.1826 (calcd. for $C_{15}H_{26}O_2Na$, 261.1830); ^{1}H NMR (500 MHz, $CDCl_3$) and ^{13}C NMR (125 MHz, $CDCl_3$) see Table 1.

Compound **2**: colorless oil; $[\alpha]_D^{32}$ –1.8 (*c* 0.05, CH_3OH); UV (CH_3OH) λ_{max}/nm (log ε) 196 (3.98), 202 (3.79), 273 (3.57), 306 (3.36), 322 (3.25), 365 (3.19); IR (KBr) ν_{max}/cm^{-1} 3419, 2925, 1628, 1451, 1031; HRESIMS *m/z* (%) 279.1938 $[M + Na]^+$ (calcd. for $C_{15}H_{28}O_3Na$, 279.1936); ^{1}H NMR (500 MHz, $CDCl_3$) and ^{13}C NMR (125 MHz, $CDCl_3$) see Table 1.

Compound **3**: white powder; $[\alpha]_D^{32}$ –6.4 (*c* 0.02, CH_3OH); UV (CH_3OH) λ_{max}/nm (log ε) 225 (1.56); IR (KBr) ν_{max}/cm^{-1} 3419, 2925, 1609, 1420, 1110; HREIMS *m/z* (%) 250.0847 $[M]^+$ (calcd. for $C_{13}H_{14}O_5$, 250.0841); ^{1}H NMR (500 MHz, $CDCl_3$) and ^{13}C NMR (125 MHz, $CDCl_3$) see Table 2.

Table 2 ^{1}H NMR and ^{13}C NMR data of **3** in $CDCl_3$ (δ in ppm)

No.	δ ^{1}H	δ ^{13}C
1	–	170.2
3	4.66, m, 1H	75.8
4	3.88, dd, 1H, *J* 3.1, 17.9 Hz; 3.04, dd, 1H, *J* 11.9, 17.9 Hz	32.7
4a	–	143.5
5	–	119.1
6	8.13, d, 1H, *J* 9.0 Hz	138.6
7	6.93, d, 1H, *J* 9.0 Hz	116.3
8	–	165.5
8a	–	109.0
9	1.55, d, 3H, *J* 6.4 Hz	20.9
10	–	165.9
11	4.32, q, 2H, *J* 7.1 Hz	61.1
12	1.38, t, 3H, *J* 7.1 Hz	14.4

Bioassay of AChE inhibitory activity

AChE inhibitory activity of these compounds was assayed by the spectrophotometric method developed by Ellman *et al.*.[18] Acetylthiocholine iodide (Sigma, St. Louis, MO, USA) was used as substrate in the assay. Compounds were dissolved in dimethyl sulfoxide (DMSO). The reaction mixture, consisting of 110 μL phosphate buffer (pH 8.0), 10 μL of tested compounds solution (2000 μmol L^{-1}), and 40 μL AChE solution (0.04 U *per* 100 μL), was mixed and incubated for 20 min (30 °C). The reaction was initiated by the addition of 20 μL 5,5-dithiobis-2-nitrobenzoic acid (6.25 mmol L^{-1}) and 20 μL acetylthiocholine. The hydrolysis of acetylthiocholine was monitored at 405 nm after 30 min. Tacrine (Sigma-Aldrich 99%) was used as positive control. All reactions were done in triplicate. The percentage inhibition was calculated as follows: %inhibition = $(E - S) / E \times 100$ (E is the activity of the enzyme without any test compound and S is the activity of enzyme with test compounds).

Results and Discussion

Compound **1** was isolated as a colorless oil, and its molecular formula was assigned as $C_{15}H_{26}O_2$ by the positive HRESIMS at *m/z* 261.1826 $[M + Na]^+$ (calcd. 261.1830) and NMR data (Table 1), indicating three degrees of unsaturation. The IR spectrum displayed the presence of hydroxyl groups (3350 cm^{-1}) and double bond (1643 cm^{-1}). Analysis of its ^{13}C NMR and distortionless enhancement by polarization transfer (DEPT) spectra (Table 1) showed the presence of 15 carbon resonances. These carbons were assigned to two methyl groups, seven methylenes (one olefinic and one oxygenated), four methines, and two quaternary carbons. Comparing the ^{13}C NMR data of **1** with those of (1*S*,4*S*,5*R*,7*R*,10*R*)-10-desmethyl-1-methyl-11-eudesmene showed that both compounds had the same carbon skeleton.[19] The main difference was that C-13 (δ_C 19.8) in (1*S*,4*S*,5*R*,7*R*,10*R*)-10-desmethyl-1-methyl-11-eudesmene was shifted downfield to δ_C 64.6 in compound **1**, which suggested the presence of a hydroxymethyl in the side chain of this compound. This was supported by its molecular formula and heteronuclear multiple-bond correlation (HMBC) spectrum correlations (Figure 2) from H-13 to C-11 [δ_C 156.9 (s)], C-12 [δ_C 106.7(t)], and C-7 [δ_C 42.3 (d)]. The other HMBC correlations indicated the atom connectivity in compound **1**. The relative configurations of the chiral centers (C-1, C-4, C-5, C-7, and C-10) in **1** were assigned from rotational frame nuclear Overhauser effect spectroscopy (ROESY) experiments (Figure 3), which indicated β-orientations of CH_3-14 and H-10, and α-orientations of H-5, H-7 and CH_3-15, as well as from the similarity of its NMR data with those of (1*S*,4*S*,5*R*,7*R*,10*R*)-10-desmethyl-1-methyl-11-eudesmene. Thus, the structure of compound **1** was assigned as shown and named as 14(10→1)*abeo*-eudesmane-13-hydroxyl-11-ene.

Compound **2** was obtained as a colorless oil and had the molecular formula $C_{15}H_{28}O_3$, based on the positive HRESIMS at *m/z* 279.1938 $[M + Na]^+$ (calcd. 279.1936), indicating two degrees of unsaturation. The IR spectrum of **2** showed absorption bands at 3419 cm^{-1} ascribable to hydroxyl groups. The ^{13}C NMR and DEPT spectra of **2** (Table 1) displayed a total of 15 carbon signals including three methyls, six methylenes, four methines and two oxygenated quaternary carbons, suggestive of a sesquiterpenoid skeleton. The ^{13}C NMR spectrum of **2** (Table 1) was similar to that of compound **1** except for the presence of one methyl (δ_C 17.9) and one oxygenated quaternary carbon (δ_C 76.2) in **2**, replacing olefinic carbons C-11 and C-12 in **1**. This feature indicated that compound **2** is the Δ^1 hydrolyzed analogue of **1**. This deduction was confirmed by the HMBC (Figure 2) correlations from H-12 [δ_H 0.98 (s)] to C-11, C-13 [δ_C 68.4 (t)], and C-7 [δ_C 43.1 (d)]. Compound **2** had the same relative configurations as those of **1** according to its ROESY spectrum (Figure 3) and their similar NMR data. Thus, the structure of compound **2** was assigned as shown and named as 14(10→1)*abeo*-eudesmane-11,13-diol.

The molecular formula of compound **3** was determined to be $C_{13}H_{14}O_5$ from its HREIMS at *m/z* 250.0847 $[M]^+$

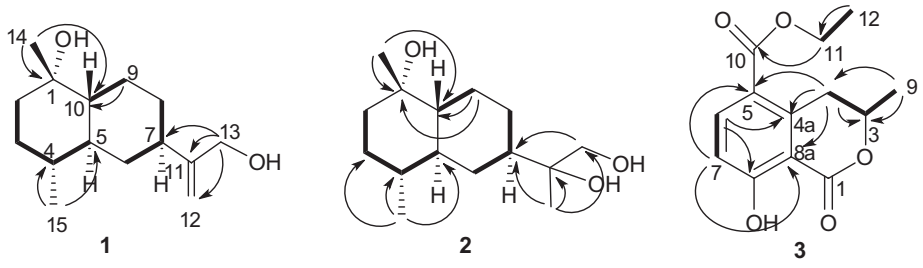


Figure 2. $^1H,^1H$-COSY (—) and key HMBC (⟶) correlations of compounds **1**-**3**.

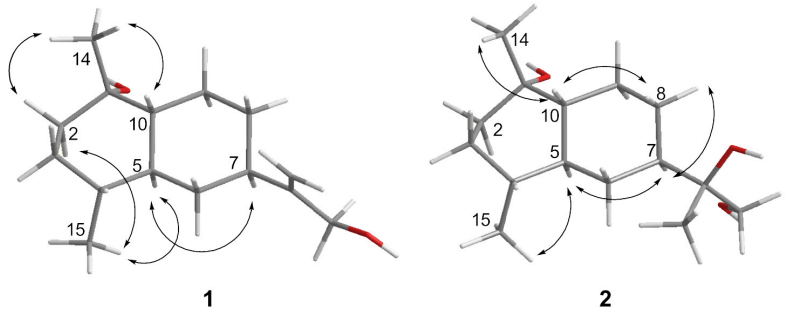


Figure 3. Key ROESY (⟷) correlations of compounds **1** and **2**.

(calcd. 250.0841). The ^{1}H and ^{13}C NMR spectra (Table 2) displayed 13 carbon resonances comprising of two methyl groups, two methylenes, three methines, six quaternary carbons (including four olefinic carbons and two carbonyls at δ_C 165.9 and 170.2). The NMR data of **3** were similar to those of (*R*)-(–)-5-methoxycarbonyl mellein except for the presence of ethoxyl signals (δ_C 61.1 and δ_C 14.4) attaching to the carbonyl at C-10 in **3**, replacing the methoxyl signal (δ_C 52.0) in (*R*)-(–)-5-methoxycarbonyl mellein,[20] which was confirmed by the HMBC (Figure 2) correlations from H-12 [δ_H 1.38 (t, 3H, *J* 7.1 Hz)] to C-11 (δ_C 61.1) and from H-11 [δ_H 4.32 (q, 2H, *J* 7.1 Hz)] to C-10 (δ_C 165.9). The $^{1}H,^{1}H$ correlation spectroscopy (COSY) and other HMBC correlations also supported the assignment of the mullein skeleton of **3**. The configuration of the stereocenter at C-3 in compound **3** was determined to be *R* based on a comparison of similar NMR data and negative optical rotation (–6.4) with those of (*R*)-(–)-5-methoxycarbonyl mellein.[20] Thus, compound **3** was identified as (*R*)-(–)-5-ethoxycarbonyl mellein.

The AChE inhibitory activity of compounds **1** and **2** were determined by a previously described method.[18] The known AChE inhibitor tacrine was used as positive control in this assay and showed a percentage inhibition of 57%. Compounds **1** and **2** both exhibited moderate inhibitory activity with a percentage inhibition of 29 and 41%, respectively, at a concentration of 100 μmol L^{-1}.

Conclusions

In this work, the new compounds 14(10→1)*abeo*-eudesmane-13-hydroxyl-11-ene (**1**), 14(10→1)*abeo*-eudesmane-11,13-diol (**2**), and (*R*)-(–)-5-ethoxycarbonyl mellein (**3**), together with eight known compounds were isolated from the cultures of the fungus *Marasmiellus ramealis* by column chromatography. The new eudesmane-type sesquiterpenoids **1** and **2** were evaluated for their inhibitory activity against acetylcholinesterase (AChE), and showed moderate inhibitory activity with a percentage inhibition of 29 and 41%, respectively, at a concentration of 100 μmol L^{-1}.

Supplementary Information

Supplementary data are available free of charge at http://jbcs.sbq.org.br as a PDF file.

Acknowledgements

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References

1. Kamal, M. A.; Greig, N. H.; Alhomida, A. S.; *Biochem. Pharmacol.* **2000**, *60*, 561.
2. Terry, R. D.; Masliah, E.; *Ann. Neurol.* **1991**, *30*, 572.
3. Rösler, M.; Anand, R.; Cicin-Sain, A.; Gauthier, S.; Agid, Y.; Dal-Bianco, P.; Stähelin, H. B.; Hartman, R.; Gharabawi, M.; *BMJ* **1999**, *318*, 633.
4. Giacobini, E.; *Pharmacol. Res.* **2004**, *50*, 433.
5. Wang, Y. E.; Yue, D. X.; Tang, X. C.; *Acta Pharmacol. Sin.* **1986**, *7*, 110.
6. Mao, X. L.; *Macromycetes of China*, 1st ed.; Science Press: Beijing, China, 2009.
7. Jarrah, M. Y.; Thaller, V.; *J. Chem. Soc., Perkin Trans. 1* **1983**, *1*, 1719.
8. Holroyde, J. K.; Orr, A. F.; Thaller, V.; *J. Chem. Soc., Chem. Commun.* **1976**, *7*, 242.
9. Holroyde, J. K.; Orr, A. F.; Thaller, V.; *J. Chem. Soc., Perkin Trans. 1* **1978**, *12*, 1490.
10. Huang, Z. J.; Shao, C. L.; Chen, Y. G.; She, Z. G.; Lin, Y. C.; Zhou, S. N.; *Chem. Nat. Compd.* **2007**, *43*, 655.
11. Almeida, C.; Part, N.; Bouhired, S.; Kehrans, S.; König, G. M.; *J. Nat. Prod.* **2011**, *74*, 21.
12. Que, D. M.; Dai, H. F.; Zeng, Y. B.; Wu, J.; Mei, W. L.; *Chin. J. Med. Chem.* **2009**, *19*, 200.
13. Cai, H. H.; Liu, X. M.; Chen, Z. Y.; Liao, S. T.; Zou, Y. X.; *Food Chem.* **2013**, *141*, 2873.
14. Tang, J. G.; Shao, H. J.; Liu, J. K.; *Chin. Tradit. Herb. Drugs* **2008**, *39*, 1776.
15. Liu, J. K.; Tian, J. W.; Dong, Z. J.; Ding, Z. H.; Wang, X. H.; Liu, P. G.; *Helv. Chim. Acta* **2002**, *85*, 439.
16. Zhan, Z. J.; Sun, H. D.; Wu, H. M.; Yue, J. M.; *Acta Bot. Sin.* **2003**, *45*, 248.
17. Chappuis, G.; Tamm, C.; *Helv. Chim. Acta* **1982**, *65*, 521.
18. Ellman, G. L.; Courtney, K. D.; Featherstone, R. M.; *Biochem. Pharmacol.* **1961**, *7*, 88.
19. Chavez, J. P.; Gottlieb, O. R.; Yoshida, M.; *Phytochemistry* **1995**, *39*, 849.
20. Klaiklay, S.; Rukachaisirikul, V.; Sukpondma, Y.; *Arch. Pharmacal Res.* **2012**, *35*, 1127.

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