Lycopodium Alkaloids from *Huperzia serrata*

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Three new lycopodium alkaloids, huperserramines A–C (**1**–**3**, resp.), along with 15 known ones, lycopodine-6α,11α-diol (**4**), lycoposerramine H (**5**), lycoposerramine I (**6**), lycopodine-6α-ol (**7**), lycoposerramine M (**8**), diphaladine A (**9**), lycoposerramine K (**10**), lycoposerramine W (**11**), huperzine M (**12**), luciduline (**13**), phlegmariuine N (**14**), huperzine A (**15**), huperzine B (**16**), lycodine (**17**), and lycoposerramine R (**18**), were isolated from the whole plant of *Huperzia serrata*. Their structures were established by spectroscopic methods, including 2D-NMR and MS analyses. All the isolates were evaluated for their inhibitory effects on acetylcholinesterase (AChE) and α-glucosidase. As a result, lycopodine-6α,11α-diol (**4**) exhibited more potent α-glucosidase inhibitory activity (IC_{50} 148 ± 5.5 μM) than the positive control acarbose (IC_{50} 376.3 ± 2.7 μM).

Introduction. – The whole plant of *Huperzia serrata* (THUNB. ex MURRAY) TREVIS., with a trivial name *Qian Ceng Ta*, has earned its worldwide fame since the discovery of huperzine A, a potent, highly specific, and reversible inhibitor of acetylcholinesterase (AChE) [1]. Subsequent chemical and pharmaceutical investigation revealed that lycopodium alkaloids represent the main bioactive constituents of this plant [2]. The lycopodium alkaloids, featuring unique skeletons with varying levels of oxidation [3], have attracted the attention of both synthetic organic chemists and pharmacologist due to their complex structures and bioactivities [4–6]. In the present work, 18 lycopodium alkaloids **1**–**18** (*Fig. 1*), including three new ones, huperserramines A–C (**1**–**3**, resp.), were isolated from the whole plant of *Huperzia serrata* and subjected to *in vitro* AChE and α-glucosidase inhibitory assays. Herein, we report the isolation, identification, and structure elucidation of these compounds, along with their AChE and α-glucosidase inhibitory activities.

Results and Discussion. – The crude alkaloids of *Huperzia serrata* were subjected to extensive column chromatography to afford three new compounds **1**–**3**, as well as the 15 known ones **4**–**18** (*Fig. 1*).

Huperserramine A (**1**) was obtained as colorless oil with a molecular formula of $C_{17}H_{26}N_2O_2$ according to the $[M+H]^+$ peak at m/z 291.2070 (calc. 291.2073) in the HR-ESI mass spectrum, corresponding to six degrees of unsaturation. The IR spectrum displayed the absorption band for OH groups (3364 cm^{-1}). The ^{1}H-NMR spectrum (*Table*) displayed signals for one O-bearing CH group (δ(H) 4.18–4.25 (*m*, H–C(3))), one N-bearing CH_2 group at (δ(H) 4.11 (*d*, *J* = 13.0, H_a–C(1)) and 3.80 (*d*, *J* = 11.0, H_b–C(1))), one N-bearing CH group at (δ(H) 4.04–4.13 (*m*, H–C(5))), one N-bearing Me group (δ(H) 3.59 (*s*, Me(17))), and an additional Me group (δ(H) 1.23 (*d*, *J* = 6.5,

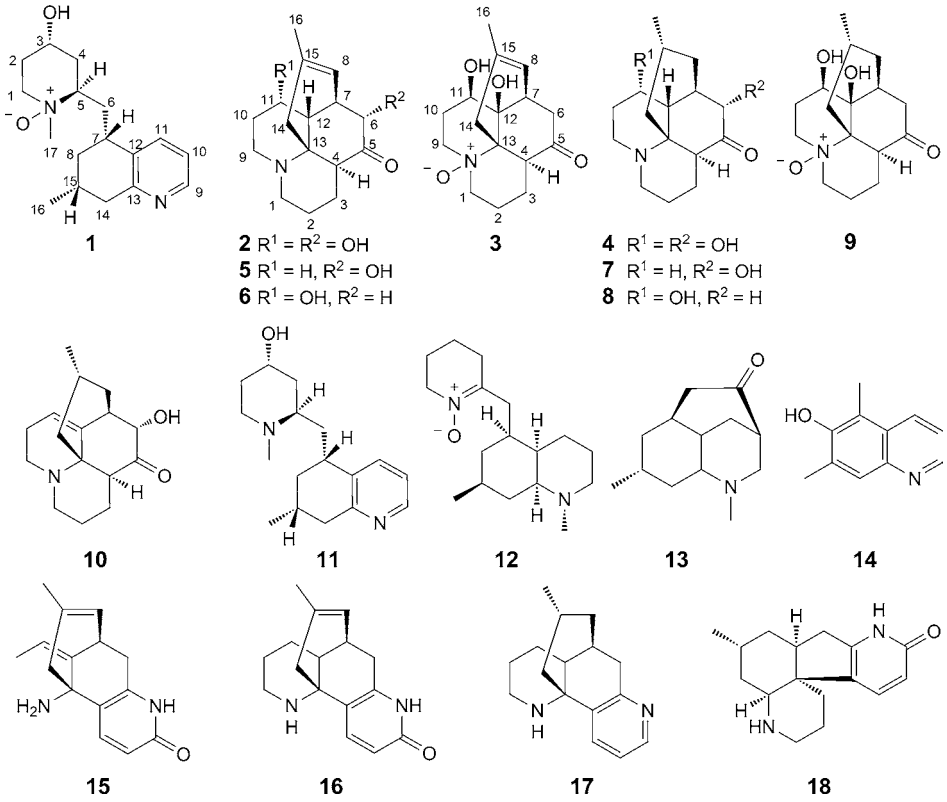

Fig. 1. *The structures of compounds* **1**–**18**

Me(16))). Further, signals of three aromatic H-atoms (δ(H) 8.46 (*d*, $J = 4.5$, H–C(9)), 7.80 (*d*, $J = 8.0$, H–C(11)), and 7.20 (*dd*, $J = 8.0$, 5.0, H–C(10)) composed the characteristic resonances for a 2,3-*ortho*-substituted pyridine ring, which corresponded to the three sp^2 CH groups (δ(C) 147.1, 134.8, and 121.3) and two sp^2 quaternary C-atom resonances (δ(C) 157.5 and 133.9) observed in the ^{13}C-NMR spectrum. The remaining twelve ^{13}C resonances including two Me, six CH_2, and five CH groups, were all positioned at δ(C) < 70 ppm. The above mentioned data, combined with a comparison with those of the known compound lycoposerramine W (**11**) [7], indicated that **1** possessed a phlegmarine skeleton with the 5,6,7,8-tetrahydroquinoline moiety, which could be established and confirmed by the 2D-NMR data (*Fig. 2*). The NMR spectra of **1** (*Table*) resembled those of lycoposerramine W (**11**), except that the resonances of C(1), C(5), and C(17) were downfield-shifted by 12.2–14.5 ppm. These unusual shifts, combined with the molecular formula, suggested that **1** should be an *N*-oxide of **11**. These shifts were similar to those observed for galanthamine and its corresponding *N*-oxide [8]. According to biogenetic considerations, the relative configurations at C(3), C(4), C(7), and C(15) of **1** should be in agreement with those in **11**. The structure of **1** was thus established and was given the trivial name huperserramine A.

Table. *^{1}H- and ^{13}C-NMR Data (500 and 125 MHz, resp.) of Compounds* **1**–**3**. δ in ppm, J in Hz. The atom numbering as indicated in *Fig. 1*.

Position	**1**[a])		**2**[b])		**3**[a])	
	δ(H)	δ(C)	δ(H)	δ(C)	δ(H)	δ(C)
1	4.11 (*d*, *J* = 13.0), 3.80 (*d*, *J* = 11.0)	62.9	3.16 (*ddd*, *J* = 13.0, 13.0, 3.2), 2.59–2.69 (*m*)	48.5	3.36–3.48 (*m*), 2.90–3.00 (*m*)	63.8
2	2.45–2.53 (*m*), 2.00–2.02 (*m*)	27.7	1.88–1.98 (*m*), 1.32–1.44 (*m*)	19.3	1.81–1.91 (*m*)	21.0
3	4.18–4.25 (*m*)	61.3	1.72–1.79 (*m*), 1.62–1.71 (*m*)	20.3	1.80–1.89 (*m*)	17.3
4	2.18–2.26 (*m*), 1.88–1.94 (*m*)	35.9	3.31–3.33 (*m*)	49.1	3.95 (br. *d*, *J* = 12.0)	48.6
5	4.04–4.13 (*m*)	67.9	–	213.6		207.9
6	2.28 (*ddd*, *J* = 15.0, 8.0, 3.5), 1.30 (*ddd*, *J* = 15.0, 9.0, 6.0)	35.8	3.56 (*d*, *J* = 2.8)	75.9	3.66 (*dd*, *J* = 14.0, 5.0), 2.23 (*dd*, *J* = 14.0, 2.0)	45.3
7	3.11–3.19 (*m*)	36.7	2.66 (br. *s*)	43.9	2.55–2.63 (*m*)	42.6
8	2.18–2.31 (*m*), 1.15–1.30 (*m*)	38.5	5.52 (*d*, *J* = 5.2)	124.2	5.35 (*d*, *J* = 4.5)	123.8
9	8.46 (*d*, *J* = 4.5)	147.1	3.67 (*dt*, *J* = 12.5, 2.8), 2.46 (*dt*, *J* = 12.2, 3.5)	42.2	4.41 (*dt*, *J* = 12.0, 2.0), 2.90–3.01 (*m*)	59.4
10	7.20 (*dd*, *J* = 8.0, 5.0)	121.3	1.97–2.08 (*m*), 1.89–2.02 (*m*)	34.0	3.34–3.48 (*m*), 1.65–1.75 (*m*)	26.9
11	7.80 (*d*, *J* = 8.0)	134.8	4.15 (*ddd*, *J* = 2.8, 2.8, 2.8)	67.7	4.18 (br. *s*)	72.5
12	–	133.9	2.02 (br. *s*)	47.0	–	71.8
13	–	157.5	–	61.0	–	72.3
14	3.01–3.09 (*m*), 2.66 (*dd*, *J* = 17.0, 11.5)	41.7	2.67 (*d*, *J* = 18.7), 1.89–1.98 (*m*)	41.8	3.58 (*d*, *J* = 17.0), 2.16 (*d*, *J* = 17.0)	30.2
15	1.96–2.08 (*m*)	29.1	–	136.2	–	130.6
16	1.23 (*d*, *J* = 6.5)	22.2	1.58 (*s*)	23.0	1.67 (*s*)	22.5
Me(17)	3.59 (*s*)	55.7	–	–	–	–

[a]) Recorded in $CDCl_3$. [b]) Recorded in $CD_3OD/CDCl_3$ 1 : 1.

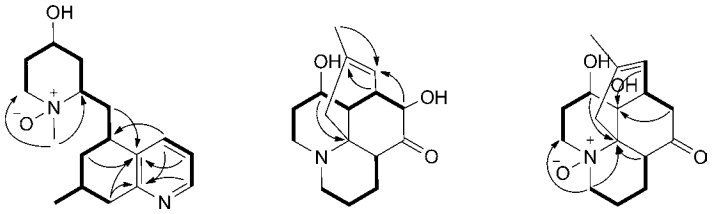

Fig. 2. *Selected ^{1}H,^{1}H-COSY* (—) *and HMBC* (H → C) *features of compounds* **1**–**3**

Huperserramine B (**2**) was obtained as colorless prisms and assigned the molecular formula $C_{16}H_{23}NO_3$, as deduced from its HR-EI-MS (*m*/*z* 278.1763 ($[M+H]^+$; calc. 278.1756)) and NMR data. The IR spectrum displayed absorption bands for ketone

(1712 cm^{-1}) and OH groups (3313 cm^{-1}). In the ^{1}H-NMR spectrum, signals assignable to one olefinic H-atom at δ(H) 5.52 (*d*, $J = 5.2$, H–C(8)), and two O-bearing CH groups at 4.15 (*ddd*, $J = 2.8$, 2.8, 2.8, H–C(11)) and 3.56 (*d*, $J = 2.8$, H–C(6)) were discernable. The ^{13}C-NMR and DEPT spectra revealed the presence of 16 C-atoms, *i.e.*, one ketone C=O group, two quaternary C-atoms, six CH and six CH_2 groups, and one Me group. Among them, the sp^2 quaternary C-atom (δ(C) 136.2) and sp^2 CH group (δ(C) 124.2) were ascribed to the trisubstituted C=C bond. The quaternary C-atom at (δ(C) 61.0) and two CH_2 groups (δ(C) 48.5 and 42.2) were determined to be N-bearing, whereas the resonances at δ(C) 75.9 and 67.7 were assigned to two O-bearing CH groups. The NMR data of **2** exhibited similarities with those of lycoposerramine H (**5**) [9], a lycopodine-type alkaloid with a $C_{16}N$ skeleton. The key discrepancy was the replacement of a CH_2 group in **5** (δ(H) 2.45, 1.69; δ(C) 25.8) by an O-bearing CH group in **2** (δ(H) 4.15; δ(C) 67.7), which, in combination with the molecular formula, indicated that **2** should be a hydroxylated derivative of **5**. The ^{1}H,^{1}H-COSY correlations CH_2(9)/CH_2(10)/δ(H) 4.15/H–C(12)/H–C(7)/H–C(6) suggested that the second OH group was located at C(11), which was confirmed by the HMBC from H–C(11) to the N-bearing quaternary C-atom C(13), and was also in accordance with the downfield shifts of C(10) ($\Delta\delta + 7.4$ ppm), C(11) ($\Delta\delta + 41.9$ ppm), and C(12) ($\Delta\delta +$ 4.9 ppm). The configuration of the OH group at C(11) was deduced as α-axial based on the coupling constants (*ddd*, $J = 2.8$, 2.8, 2.8) of H–C(11).

Huperserramine C (**3**) had the molecular formula of $C_{16}H_{23}NO_4$ as deduced from the HR-ESI-MS (*m/z* 294.1698 ($[M+H]^+$, $C_{16}H_{24}NO_4^+$; calc. 294.1705)). The IR absorptions at 1707 and 3367 cm^{-1} indicated the presence of ketone and OH groups, respectively. In the ^{1}H-NMR spectrum, resonances assignable to one olefinic H-atom (δ(H) 5.35 (*d*, $J = 4.5$, H–C(8))) and one O-bearing CH group (δ(H) 4.18 (br. *s*, H–C(11))) were easily discernable. The ^{13}C-NMR and DEPT spectra exhibited resonances of 16 C-atoms; *i.e.*, one ketone C=O group, three quaternary C-atoms, four CH and seven CH_2 groups, and one Me group. The NMR data exhibited features of a lycopodine-type alkaloid. Two CH_2 groups at δ(C) 59.4 and 63.8), as well as a quaternary C-atom with a signal at δ(C) 72.3 were identified as bound to a nitrogen oxide function [10]. Comparison with the known lycopodine-type skeletons revealed diphaladine A (**9**) as a lead. Comparative analysis of the NMR data of the two compounds indicated that **3** should be an analog of **9** [11] with a C(8)=C(15) bond. This assumption was verified by the ^{1}H,^{1}H-COSY plots CH_2(6)/H–C(7) and H–C(7)/ H–C(8), as well as the HMBC from H–C(8) to C(12). The relative configurations at C(4), C(7), C(11), C(12), and C(13) of **3** were elucidated to be identical with those of **9** by pertinent NOESY correlations. The structure of huperserramine C (**3**) was thus established.

Compounds **4**–**18** were identified by comparison with the corresponding literature data to be lycopodine-6α,11α-diol (**4**) [12], lycoposerramine H (**5**) [9], lycoposerramine I (**6**) [9], lycopodine-6α-ol (**7**) [9], lycoposerramine M (**8**) [9], diphaladine A (**9**) [11], lycoposerramine K (**10**) [9], lycoposerramine W (**11**) [7], huperzine M (**12**) [13], luciduline (**13**) [14], phlegmariuine N (**14**) [15], huperzine A (**15**) [1][16], huperzine B (**16**) [1], lycodine [17][18] (**17**), and lycoposerramine R (**18**) [19], respectively.

Acetylcholinesterase (AChE) and α-glucosidase inhibitory activities of compounds **1**–**18** were evaluated. Unfortunately, except the two well-known AChE inhibitors,

huperzine A (**15**) and B (**16**), none of them exhibited apparent AChE inhibitory activity at a concentration of 20 μM. However, it was surprising that compound **4**, the alkaloid we reported in our previous work as a new compound [15], showed more potent α-glucosidase inhibitory activity (IC_{50} 148.1 ± 5.5 μM) than acarbose (IC_{50} 376.3 ± 2.7 μM). Compound **4** belongs to the class of lycopodine-type alkaloids. This class comprised the largest group of lycopodium alkaloids and appeared to be the most widely distributed [2], though they exhibited extremely lower acetylcholinesterase or butyrylcholinesterase inhibitory activities compared to huperzine A and B (**15** and **16**, resp.) that possess the lycodine skeleton [20]. It seemed that the hydroxylation at C(6) and C(11), and the reduction of C(8)=C(15) bond may contribute to the α-glucosidase inhibitory activity. To the best of our knowledge, there has been no report concerning the α-glucosidase inhibitory activities of lycopodium alkaloids. Our results provided a preliminary indication that the lycopodine-type alkaloids may represent an untapped source of α-glucosidase inhibitors and warrant further studies.

Experimental Part

General. All solvent used were of anal. grade and obtained from commercially available sources. Column chromatography (CC): silica gel (SiO_2, 200–300 mesh; *Qingdao Marine Chemical Inc.*, Qingdao, China), GH amino-functionalized silica gel (NH_2–SiO_2; *Greenherbs Science & Technology Development Co., Ltd.*, Beijing, China), *MCI CHP20P* gel (75–150 μm; *Mitsubishi Chemical Industries Ltd.,* Japan), *ODS C-18* gel (50 μm; *YMC Co. Ltd.*, Kyoto, Japan), and *Toyopearl HW-40C* gel (50–100 μm; *Tosoh Corporation*, Japan). TLC: Precoated silica gel GF_{254} plates (*Qingdao Marine Chemical Inc.*, Qingdao, China); visualized with UV light and *Dragendorff*'s reagent (soln. of potassium bismuth iodide). M.p.: *X-4* melting-point apparatus; uncorrected (*Beijing Tech Instrument Co., Ltd.*, Beijing, China). Optical rotations: *Rudolph Research Autopol III* automatic polarimeter. UV Spectra: *Shimadzu-UV-2450* spectrometer; λ_{max} (log ε) in nm. IR Spectra: *Thermo-Nicolet-6700 FT-IR* microscope instrument (FT-IR microscope transmission); $\tilde{\nu}$ in cm^{-1}. ^1H- and ^{13}C-NMR spectra: *Bruker AM-500* apparatus at 500 and 125 MHz, resp.; δ in ppm rel. to Me_4Si as internal standard, J in Hz. ESI-MS and HR-ESI-MS: *Agilent-6210-LC/TOF* mass spectrometer; in *m/z* (rel. %).

Plant Material. The whole plant of *Huperzia serrata* was collected from Anhui Province in P. R. China, in October 2008, and identified by Prof. *Hai-Bo Bai* of Zhejiang University City College. A voucher specimen (ZJUT HS-08-10) was deposited with the Zhejiang University of Technology, P. R. China.

Extraction and Isolation. The air-dried whole plant of *Huperzia serrata* (5.8 kg) was extracted three times with 95% EtOH at r.t. (3 × 30 l). The resulting residue (1.7 kg) was dissolved in H_2O (5 l) to form a suspension, and then pH was adjusted with 0.5N H_2SO_4 to *ca.* 5. The acidic mixture was extracted with AcOEt (6 × 1.5 l) to remove the non-alkaloid components. The aq. phase was brought to pH of *ca.* 10 by addition of 1N Na_2CO_3 and partitioned with $CHCl_3$ (6 × 1.5 l) to give the crude alkaloids (28.7 g). CC of the crude alkaloids was performed on *MCI* gel (MeOH/H_2O 20:80 → 100:0) to yield five fractions *Frs. A–F. Fr. A* was purified by CC (SiO_2; $CHCl_3$/MeOH 20:1 → 12:1) to afford **17** (37.6 mg). *Fr. B* was first subjected to CC (SiO_2; $CHCl_3$/MeOH 15:1 → 5:1) and then separated by a further CC (*ODS C-18*; MeOH/H_2O 30:70 → 60:40) to yield **9** (12 mg), **1** (3.2 mg), **12** (5.4 mg), **18** (12.3 mg). *Fr. C* was successively subjected to CC (*ODS C-18*; MeOH/H_2O 50:50 → 55:45; and *HW 40C*; MeOH) to give **10** (8 mg) and **5** (10 mg). *Fr. D* was subjected to CC (*ODS C-18*; MeOH/H_2O 40:60 → 70:30; and then SiO_2; $CHCl_3$/MeOH 20:1 → 12:1), and finally purified by CC (*HW 40C*, MeOH) to furnish **8** (10 mg), **14** (301 mg), **6** (98 mg), **13** (7.8 mg), **4** (4.0 mg), and **2** (11.7 mg). *Fr. E* was purified by CC (SiO_2; $CHCl_3$/MeOH/Et_2NH 20:1:0.05; and then *ODS C-18*; MeOH/H_2O 55:45 → 75:25) to yield **15** (50 mg), **11** (20 mg), and **7** (3.6 mg). *Fr. F* was separated by CC (NH_2–SiO_2; $CHCl_3$/MeOH 50:1 → 15:1) to yield **3** (16.5 mg) and **16** (14.7 mg).

Huperserramine A (=(*1S,4S*)*-1-Methyl-2-{[(5R,7R)-5,6,7,8-tetrahydro-7-methylquinolin-5-yl]methyl}piperidin-4-ol 1-Oxide*; **1**). Colorless oil. $[\alpha]_D^{20} = +43.2$ ($c = 0.07$, $CHCl_3$). UV (CH_3OH): 264 (3.16), 236 (3.18). IR (neat): 3364, 2928, 2873, 2727, 2485, 1642, 1577, 1447, 1377, 1289, 1132, 1035, 964, 802. ^{1}H- and ^{13}C-NMR: see the *Table*. HR-ESI-MS: 291.2070 ($[M + H]^+$, $C_{17}H_{27}N_2O_2^+$; calc. 291.2073).

Huperserramine B (=(*6α,11α*)*-6,11-Dihydroxy-15-methyllycopod-8-en-5-one*; **2**). Colorless prisms. M.p. > 300°. $[\alpha]_D^{20} = +25.7$ ($c = 0.35$, MeOH). IR (KBr): 3313, 2929, 2894, 2829, 1712, 1468, 1338, 1316, 1277, 1184, 949, 872, 787. ^{1}H- and ^{13}C-NMR: see the *Table*. HR-ESI-MS: 278.1763 ($[M + H]^+$, $C_{16}H_{24}NO_3^+$; calc. 278.1756).

Huperserramine C (=(*1S,8R,8aS,9R,12aS*)*-1,2,3,4,6,7,8,8a,9,12-Decahydro-8,8a-dihydroxy-11-methyl-1,9-ethanopyrido[2,1-j]quinolin-14-one 5-Oxide*; **3**). Colorless oil. $[\alpha]_D^{20} = -3.1$ ($c = 0.07$, $CHCl_3$). IR (neat): 3367, 2922, 2854, 1707, 1453, 1357, 1311, 1191, 1122, 1057, 995. ^{1}H- and ^{13}C-NMR: see the *Table*. HR-ESI-MS: 294.1698 ($[M + H]^+$, $C_{16}H_{24}NO_4^+$; calc. 294.1705).

AChE Inhibitory Assay. The AChE inhibitory activities of **1**–**18** were assayed according to the spectroscopic *Ellmann*'s method [21], employing huperzine A as a positive control.

α-Glucosidase Inhibitory Assay. The inhibitory activities of **1**–**18** against yeast α-glucosidase (from *Saccharomyces cerevisiae*; *Sigma-Aldrich*, St. Louis, MO, USA) were evaluated as described in [22][23] with slight modifications [22][23]. The assay was performed in 96-well microplates. Briefly, 20 µl of enzyme soln. (0.2 U/ml α-glucosidase in 0.01M potassium phosphate buffer (pH 6.8)) and 130 µl of the compound in 0.2% DMSO of 0.01M potassium phosphate buffer were mixed, and the mixture was pre-incubated at 37° prior to initiation of the reaction by adding the substrate. After 15 min of pre-incubation, PNPG (*p*-nitrophenyl-α-D-glucopyranoside) soln. (20 µl; 5.0 mM PNPG in 0.01M potassium phosphate buffer (pH 6.8)) was added, and then incubated together at 37°. After 30 min of incubation, 1.0M Na_2CO_3 (100 µl) in 0.01M potassium phosphate buffer was added to the test tube to stop the reaction. The increment in absorption at 405 nm due to the hydrolysis of PNPG by α-glucosidase was monitored continuously with an auto-multifunctional microplate reader. The percentage of enzyme inhibition was calculated as $(1 - B/A) \times 100$, where A represents the absorbance of the control without test samples, and B represents the absorbance in the presence of test samples. All the tests were run in triplicate. Acarbose was used as a positive control in this study. The 50% inhibitory concentration (IC_{50}) values were expressed as mean ± SE ($n = 3$).

This work was supported by the *National Natural Science Foundation of China* (No. 21402174); the *Scientific Foundation of Zhejiang Province* (Y2111243), and the *Scientific Foundation of Zhejiang University of Technology* (2013XY010).

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Received January 12, 2014