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α-Glucosidase inhibitory triterpenoids from the stem barks of *Uncaria laevigata*

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ABSTRACT

A phytochemical study on the ethanolic extract of the stem barks of *Uncaria laevigata* leads to the isolation and characterization of 18 triterpenoid compounds. Ten of these are new, including one novel heptanortriterpenoid (**1**), four ursane triterpenes (**2–4, 10**), and five oleanane triterpenes (**5–9**). Their structures were established by spectroscopic methods, especially by 2D-NMR and MS analyses. All these isolates were evaluated for their inhibitory effects on α-glucosidase: ursolic acid and **3** showed potent activities with IC_{50} values of 16 ± 2.2 and 49 ± 3.7 μM, respectively.

1. Introduction

Diabetes mellitus is a complex chronic disease characterized by hyperglycemia resulting from the metabolic disorders of insulin resistance and abnormal insulin secretion [1]. The persistent hyperglycemic condition in diabetes plays a predisposing role for dysfunction and failure of various organs, such as kidneys, heart, blood vessels and eyes. Thus, sustained control of hyperglycemia is considered to be important to the effective treatment of diabetes. During the last few decades, there have been widespread interest in finding α-glucosidase inhibitors in modern natural product medicines and medicinal chemistry, because of their promising therapeutic potential in the treatment of diabetes [2,3]. Located in the brush-border surface membrane of intestinal cells, α-glucosidase plays a crucial role in dietary carbohydrates digestion and post-translational processing of glycoprotein [3]. By inhibiting the function of α-glucosidase, its inhibitors can delay the digestion of oligosaccharide and disaccharide to monosaccharide, thus reducing the rate of glucose absorption [4]. Currently available α-glucosidase inhibitors include sugar derivatives (iminosugars, carbasugars, thiosugars, etc.) and other non-sugar derivatives [5], however, most of them are associated with undesirable side effects, drug interactions and the treatment is expensive. Therefore, safer natural α-glucosidase inhibitors are urgently desired. Natural products and their derivatives have been a successfully source of bioactive molecules in medicines for thousands of years with advantages of effectiveness, low incidence of side effects.

Uncaria laevigata is a commonly used traditional Chinese medicine to treat diseases of the cardiovascular and central nervous systems, e.g. hypertension, lightheadedness, numbness, convulsions [6]. The plant genus *Uncaria* is widely distributed in tropical countries of Asia, such as India and southern mainland China. Previous phytochemical and pharmacological studies on *Uncaria* genus mainly focus on oxindole and indole alkaloids. In contrast with earlier investigations, research interests have increasingly shifted towards to triterpenoids of *Uncaria* genus due to their pharmacological effects as anti-diabetic, anti-inflammatory, and antioxidant agents [7]. Recently, Domingues and his co-workers reported that the extract of another species of *Uncaria* was effective to prevent the progression of immune-mediated diabetes [8]. Besides, studies also indicated that triterpenoids possess strong inhibitory activities against

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α-glucosidase [9,10]. Thus, the stem barks of *U. laevigata* were investigated as a part of our ongoing effort in the isolation and structure characterization of anti-α-glucosidase constituents from natural sources [11,12]. Eighteen triterpenoid compounds (**1**-**18**) were isolated as a result of our efforts, and their inhibitory activities against yeast α-glucosidase were evaluated.

2. Experimental

2.1. General methods

NMR spectra were recorded on Bruker AVANCE III 500 NMR instruments (^{1}H: 500 MHZ, ^{13}C: 125 MHZ), with TMS as internal standard. Mass spectra were obtained on a MS Agilent 1100 series LC/MSD ion-trap mass spectrometer (ESIMS) and an Agilent UPLC-Q-TOF (6520B), respectively. IR (KBr disks) spectra were recorded by a Bruker Tensor 27 spectrometer. UV spectra were measured on a Shimadzu UV-2501 PC spectrophotometer. Optical rotations were conducted on a JASCO P-1020 polarimeter. Melting points were measured using an X-4 digital display micromelting apparatus, uncorrected. RP-C_{18} (40–63 μm, Fuji) was used for column chromatography. Preparative HPLC was carried out using an Agilent 1100 Series instrument with a shim-park RP-C_{18} column (20 × 200 mm) and a 1100 Series multiple wavelength detector.

2.2. Plant material

The air-dried stem barks of *U. laevigata* were collected from Xishuangbanna, Yunnan Province, China, in April 2011, and were authenticated by Professor Min-Jian Qin of Research Department of Pharmacognosy, China Pharmaceutical University. The voucher specimen (No. UL-201104) was deposited at the Department of Natural Medicinal Chemistry, China Pharmaceutical University.

2.3. Extraction and isolation

The air-dried stem barks (15 kg) were extracted by refluxing with 95% ethanol four times. The EtOH extract was concentrated under reduced pressure and then was suspended in H_2O and extracted with CH_2Cl_2. The CH_2Cl_2 extract (400 g) was chromatographed on a column of silica gel eluted with a gradient of petroleum ether–EtOAc (30:1, 15:1, 7:1, 3:1, 1:1), to afford five fractions (Fr. A–E). Fr. D (81 g) was fractioned to 9 subfractions (Fr. D1–D9) using silica gel CC eluted with $CHCl_3$–MeOH in a gradient (100:1, 50:1, 25:1, 10:1, 5:1). An aliquot of Fr. D4 (3 g) was subjected to reversed-phase C_{18} silica gel, eluted with MeOH–H_2O (4:6 to 7:3) to give three fractions (Fr. D4A–D4C). Fr. D4C was purified by preparative HPLC using MeOH–H_2O (65:35, 10 ml/min) as the mobile phase to give **1** (9.5 mg). Fr. D5 (4.8 g) was chromatographed on a column of reversed-phase C_{18} silica gel, eluted with MeOH–H_2O (4:6 to 8:2), to give four subfractions (Fr. D5A–D5D). Fr. D5B was chromatographed on Sephadex LH 20 (MeOH), and then purified by preparative HPLC using MeOH–H_2O (65:35, 10 ml/min) as the mobile phase to give **3** (7 mg), **6** (37 mg), **8** (30 mg), and **11** (28 mg). Fr. D5C was subjected to silica gel column eluted with petroleum ether–EtOAc (20:1, 10:1, 5:1, 2:1) to give four subfractions (Fr. D5C1-D5C4). Fr. D5C1 was separated by preparative HPLC eluted with MeOH–H_2O (72:28, 10 ml/min) to yield **5** (11 mg) and **12** (7 mg). Fr. D5C2 was purified by preparative HPLC eluted with MeOH–H_2O (72:28, 10 ml/min) to give **4** (4 mg), **7** (6 mg), and **13** (7 mg). Fr. D5C3 was separated by preparative HPLC eluted with MeOH–H_2O (72:28, 10 ml/min) to give **14** (4 mg), **15** (6 mg), and **16** (18 mg). Fr. D5C4 was separated by preparative HPLC eluted with MeOH–H_2O (72:28, 10 ml/min) to give **2** (6 mg), **9** (11 mg), and **10** (4 mg). Fr. D5D was subjected to silica gel column eluted with petroleum ether–EtOAc (25:1, 10:1, 5:1, 2:1) to give three subfractions (Fr. D5D1–D5D3). Fr. D5D1 was purified by preparative HPLC eluted with MeOH–H_2O (90:10, 10 ml/min) to yield **17** (27 mg). Fr. D5D3 was purified by preparative HPLC eluted with MeOH–H_2O (80:10, 10 ml/min) to yield **18** (21 mg).

2.4. Laevigatone A (**1**)

White powder; mp 213–214 °C; $[\alpha]_D^{25}$ + 35.0 (*c* 0.032, MeOH); IR (KBr) ν_{max} 3442, 2958, 2927, 1654, 1459, 1380 cm^{-1}; ^{1}H (500 MHz, $CDCl_3$) and ^{13}C NMR (125 MHz, $CDCl_3$) data, see Tables 1 and 3; HRESIMS *m/z*: 359.2593 [M-H]$^-$ (Calcd for $C_{23}H_{35}O_3$: 359.2592).

2.5. 3β, 19α-Dihydroxy-6β, 7β-epoxyl-urs-12-en-28-oic acid (**2**)

White powder; mp 244–245 °C; $[\alpha]_D^{25}$ + 2.8 (*c* 0.162, MeOH); IR (KBr) ν_{max} 3443, 2974, 2927, 1684, 1401, 1206, 1140 cm^{-1}; ^{1}H (500 MHz, methanol-d_4) and ^{13}C NMR (125 MHz, methanol-d_4) data, see Tables 1 and 3; HRESIMS *m/z*: 485.3271 [M-H]$^-$ (Calcd for $C_{30}H_{45}O_5$: 485.3272).

2.6. 3β-Hydroxy-30-methoxy-6-oxo-urs-12, 19 (20)-dien-28-oic acid (**3**)

White powder; mp 237–238 °C; $[\alpha]_D^{25}$ + 20.0 (*c* 0.044, MeOH); IR (KBr) ν_{max} 3416, 2958, 2925, 1689, 1456, 1380, 1206, 1138 cm^{-1}; ^{1}H (500 MHz, pyridine-d_5) and ^{13}C NMR (125 MHz, pyridine-d_5) data, see Tables 1 and 3; HRESIMS *m/z*: 497.3263 [M-H]$^-$ (Calcd for $C_{31}H_{45}O_5$: 497.3272).

2.7. 19α-Hydroxy-3, 6-dioxo-urs-1 (2), 12-dien-28-oic acid (**4**)

White powder; mp 273–274 °C; $[\alpha]_D^{25}$ + 21.3 (*c* 0.048, MeOH); IR (KBr) ν_{max} 3451, 2975, 2932, 1686, 1460, 1399 cm^{-1}; ^{1}H (500 MHz, methanol-d_4) and ^{13}C NMR (125 MHz, methanol-d_4) data, see Tables 1 and 3; HRESIMS *m/z*: 481.2957 [M-H]$^-$ (Calcd for $C_{30}H_{41}O_5$: 481.2959).

2.8. 6α, 19α-Dihydroxy-3-oxo-olean-12-en-28-oic acid (**5**)

White powder; mp 258–260 °C; $[\alpha]_D^{25}$ + 88.8 (*c* 0.057, MeOH); IR (KBr) ν_{max} 3443, 2949, 1688, 1454, 1385, 1206, 1143 cm^{-1}; ^{1}H (500 MHz, methanol-d_4) and ^{13}C NMR (125 MHz, methanol-d_4) data, see Tables 2 and 3; HRESIMS *m/z*: 485.3281 [M-H]$^-$ (Calcd for $C_{30}H_{45}O_5$: 485.3272).

Table 1
^{1}H NMR Data of compounds **1–5** (500 MHz).

Position	**1**[a]	**2**[b]	**3**[c]	**4**[b]	**5**[b]
1	1.76, dt (3.5, 13.0) 1.04, m	1.49, m 0.98, m[d]	1.70, m[d] 1.32, dd (4.0, 13.0)	7.15, d (10.0)	1.75, m[d]
2	1.69, m[d]	1.67, m[d]	1.90, m[d]	5.78, d (10.0)	2.76, ddd (6.0, 11.0, 16.4) 2.23, ddd (6.0, 11.0, 16.4)
3	3.18, dd (6.0, 10.0)	3.22, dd (5.0, 11.5)	3.48, dd (5.0, 11.0)		
4					
5	0.77, d (1.3)	1.30, s	2.48, s	3.09, s	1.71, m[d]
6	4.60, brs	3.47, d (4.0)			3.84, dt (7.5, 10.5)
7	1.69, m[d]	3.05, d (4.0)	2.76, d (12.0) 2.12, m[d]	2.73, d (13.0) 1.89, d (13.0)	1.60, m[d]
9	1.59, m[d]	1.77, m[d]	2.16, m[d]	2.57, m[d]	1.89, t (9.0)
11	1.69, m[d] 1.59, m[d]	1.89, m	1.95, m[d]	2.44, m 2.25, ddd (3.0, 11.0, 18.3)	2.00, dd (3.5, 7.3)
12	2.40, m[d]	5.45, brs	5.76, brs	5.40, brs	5.35, brs
15	2.20, td (5.5, 13.5) 1.57, m[d]	1.99, m 1.42, m[d]	2.20, m[d] 1.14, m	1.76, m[d] 0.94, m[d]	1.70, m[d] 1.09, m
16	2.40, m[d]	2.61, td (4.0, 13.5) 1.62, m[d]	2.07, m[d]	2.63, dd (4.0, 13.5) 1.54, m	2.31, td (3.5, 14.5) 1.64, m[d]
18	5.81, brs	2.55, brs	3.73, brs	2.55, brs	3.08, brs
20		1.37, m[d]		1.35, m	3.26, d (3.5)
21		1.77, m[d] 1.26, m	2.46, m[d] 2.26, m	1.73, m[d] 1.25, m[d]	1.75, m[d] 1.03, m
22		1.77, m[d] 1.62, m[d]	2.12, m[d] 2.02, m[d]	1.73, m[d] 1.63, dd (3.5, 12.3)	1.77, m[d] 1.64, m[d]
23	1.09, s	1.16, s	1.44, s	1.18, s	1.32, s
24	1.16, s	1.02, s	1.69, s	1.39, s	1.32, s
25	1.29, s	1.16, s	1.00, s	1.25, s	0.84, s
26	1.20, s	1.01, s	1.11, s	0.90, s	0.84, s
27	1.27, s	1.46, s	1.25, s	1.48, s	1.38, s
29		1.19, s	1.87, s	1.21, s	0.94, s
30		0.95, d (7.0)	4.04 (11.5)[e] 4.01 (11.5)[e]	0.93, d (6.5)	0.97, s
OCH_3-30			3.33, s		

[a] Measured in $CDCl_3$.
[b] Measured in methanol-d_4.
[c] Measured in pyridine-d_5.
[d] Signal pattern unclear due to overlapping.
[e] AB system.

2.9. 3β, 19α-Dihydroxy-6-oxo-olean-12-en-28-oic acid (**6**)

White powder; mp 285–286 °C; $[\alpha]_D^{25}$ + 30.9 (*c* 0.070, MeOH); IR (KBr) ν_{max} 3491, 2935, 1697, 1457, 1394, 1206 cm^{-1}; ^{1}H (500 MHz, DMSO-d_6) and ^{13}C NMR (125 MHz, DMSO-d_6) data, see Tables 2 and 3; HRESIMS *m/z*: 485.3266 [M-H]$^-$ (Calcd for $C_{30}H_{45}O_5$: 485.3272).

2.10. 19α-Hydroxy-3, 6-dioxo-olean-12-en-28-oic acid (**7**)

White powder; mp 277–278 °C; $[\alpha]_D^{25}$ + 16.7 (*c* 0.042, MeOH); IR (KBr) ν_{max} 3475, 2975, 2926, 1699, 1457, 1384, 1215 cm^{-1}; ^{1}H (500 MHz, methanol-d_4) and ^{13}C NMR (125 MHz, methanol-d_4) data, see Tables 2 and 3; HRESIMS *m/z*: 483.3110 [M-H]$^-$ (Calcd for $C_{30}H_{43}O_5$: 483.3116).

2.11. 19α-Hydroxy-3, 6-dioxo-24-norolean-12-en-28-oic acid (**8**)

White powder; mp 278–279 °C; $[\alpha]_D^{25}$ + 17.7 (*c* 0.077, MeOH); IR (KBr) ν_{max} 3451, 2950, 2933, 2866, 1698, 1451, 1398, 1205 cm^{-1}; ^{1}H (500 MHz, DMSO-d_6) and ^{13}C NMR (125 MHz, DMSO-d_6) data, see Tables 2 and 3; HRESIMS *m/z*: 469.2955 [M-H]$^-$ (Calcd for $C_{29}H_{41}O_5$: 469.2959).

2.12. 6β, 19α-Dihydroxy-3-oxo-24-norolean-12-en-28-oic acid (**9**)

White powder; mp 234–235 °C; $[\alpha]_D^{25}$ + 35.4 (*c* 0.048, MeOH); IR (KBr) ν_{max} 3442, 2934, 1689, 1451, 1400, 1385, 1206, 1142 cm^{-1}; ^{1}H (500 MHz, methanol-d_4) and ^{13}C NMR (125 MHz, methanol-d_4) data, see Tables 2 and 3; HRESIMS *m/z*: 471.3118 [M-H]$^-$ (Calcd for $C_{29}H_{43}O_5$: 471.3116).

2.13. 6β, 19α-Dihydroxy-3-oxo-24-norurs-12-en-28-oic acid (**10**)

White powder; mp 230–232 °C; $[\alpha]_D^{25}$ + 27.8 (*c* 0.074, MeOH); IR (KBr) ν_{max} 3442, 2931, 1701, 1635, 1454, 1400 cm^{-1}; ^{1}H (500 MHz, methanol-d_4) and ^{13}C NMR (125 MHz, methanol-d_4) data, see Tables 1 and 3; HRESIMS *m/z*: 471.3125 [M-H]$^-$ (Calcd for $C_{29}H_{43}O_5$: 471.3116).

2.14. α-Glucosidase inhibitory assay

The inhibitory activity of all isolated compounds against yeast α-glucosidase (Sigma) was determined by modification of the procedure previously reported [3]. Briefly, 10 μL of the enzyme (1 U/mL) and 10 μL of the samples were successively

Table 2
^{1}H NMR Data of compounds **6–10** (500 MHz).

Position	**6**[a]	**7**[b]	**8**[a]	**9**[b]	**10**[b]
1	1.54, m[c]	2.03, dd (4.0, 13.0)	1.92, dd (5.5, 12.5)	1.93, m	1.95, m[c]
	1.25, m	1.62, m[c]	1.63, m[c]	1.29, m[c]	1.29, m
2	1.47, m[c]	2.85, td (6.0, 14.5)	2.50, m[c]	2.58, td (6.5, 14.5)	2.58, m[c]
		2.12, m[c]	2.13, dd (3.0, 15.5)	2.23, m[c]	2.24, m
3	2.98, dd (5.0, 10.5)				
4			2.56, m		2.85, m
5	2.26, s	2.67, s	2.71, d (11.5)	1.12, m[c]	1.12, m[c]
6				4.16, brs	4.16, brs
7	2.56, d (12.0)	2.62, m[c]	2.65, d (11.5)	1.71, m[c]	1.74, m[c]
	1.67, m[c]	1.83, d (12.0)	1.74, d (11.5)	1.64, m[c]	1.64, m[c]
9	2.26, m[c]	2.43, dd (7.5, 10.5)	2.36, dd (7.5, 10.5)	1.87, dd (7.5, 11.0)	1.83, dd (7.0, 11.0)
11	1.93, m	2.12, m[c]	2.02, m	2.10, m	2.16, m
12	5.27, brs	5.37, brs	5.29, brs	5.37, brs	5.33, brs
15	1.44, m[c]	1.76, m[c]	1.49, m[c]	1.74, m[c]	1.92, m[c]
	0.85, m[c]	1.01, m	0.85, m[c]	1.07, m[c]	1.04, m[c]
16	2.22, m[c]	2.33, td (3.0, 14.0)	2.22, td (4.5, 12.5)	2.30, m[c]	2.58, m[c]
	1.47, m[c]	1.62, m[c]	1.50, m[c]	1.61, m[c]	1.56, m[c]
18	2.93, brs	3.07, brs	2.95, brs	3.09, brs	2.54, brs
19	3.13, brs	3.26, d (3.5)	3.14, brs	3.27, d (3.5)	1.38, m
21	1.67, m[c]	1.62, m[c]	1.63, m[c]	1.74, m[c]	1.74, m[c]
	0.92, m[c]		0.91, m[c]	1.03, m[c]	1.64, m[c]
22	1.67, m[c]	1.76, m[c]	1.63, m[c]	1.77, m[c]	1.74, m[c]
	1.51, m[c]	1.62, m[c]	1.51, m[c]	1.64, m[c]	1.64, m[c]
23	0.92, s	1.06, s	0.85, d (5.0)	1.03, d (6.5)	1.04, d (6.0)
24	1.07, s	1.47, s			
25	0.82, s	1.21, s	0.96, s	1.43, s	1.44, s
26	0.65, s	0.82, s	0.74, s	1.14, s	1.16, s
27	1.38, s	1.43, s	1.41, s	1.29, s	1.33, s
29	0.85, s	0.93, s	0.85, s	0.94, s	1.20, s
30	0.88, s	0.96, s	0.88, s	0.97, s	0.93, d (6.5)

[a] Measured in DMSO-d_6.
[b] Measured in methanol-d_4.
[c] Signal pattern unclear due to overlapping.

added to 96-well plates containing 150 μL of 50 mM potassium phosphate buffer (PH 6.8), and the inhibitors were incubated with the enzyme at 37 °C for 60 min. Then, 30 μL of the substrate (*p*-nitrophenyl α-D-glucoside, 1 mM) was added and the enzymatic reaction was carried out at 37 °C for 30 min monitoring spectrophotometrically by measuring the absorbance at 405 nm. Genistein was used as a positive control. The inhibition type of new compound **3** was evaluated using the Lineweaver–Burk double-reciprocal plot method. Two different concentrations of **3** around the IC_{50} value were chosen. Under each concentration, a-glucosidase activity was assayed by varying the concentration of PNP glucoside.

3. Results and discussion

The EtOH extract of the stem barks of *U. laevigata* was suspended in water and partitioned with CH_2Cl_2. The CH_2Cl_2 soluble fraction was repeatedly subjected to column chromatography on silica gel, reversed-phase C18 silica gel, and preparative HPLC to afford ten new triterpenoids (**1-10**, Fig. 1), along with eight known compounds (**11–18**).

Compound **1** was isolated as a colorless amorphous powder with a molecular formula of $C_{23}H_{36}O_3$ as deduced from the high resolution-electrospray ionization-mass spectra (HRESIMS) pseudo-molecular ion at *m/z* 359.2593 $[M\text{-}H]^-$ (Calcd for $C_{23}H_{35}O_3$: 359.2592). The IR spectrum of **1** showed absorption bands at 3442 and 1654 cm^{-1} due to hydroxyl and carbonyl groups, respectively. The ^{1}H NMR spectrum of **1** (Table 1) showed five tertiary methyls, an olefinic proton at δ_H 5.81 (1H, brs), and two hydroxylated methine protons at δ_H 3.18 (1H, dd, J = 6.0, 10.0 Hz) and 4.60 (1H, brs). The ^{13}C NMR spectrum of **1** (Table 3) combined with HSQC experiment revealed 23 carbon signals, including five methyl, seven methylene, five methine, and six quaternary carbons. The HMBC correlations (Fig. 2) from the two gem-dimethyl protons at δ 1.09 and 1.16 (each 3H, s) to the oxymethine carbon resonance at δ 79.2 suggested the presence of a hydroxyl group at C-3 position. The configuration of the hydroxyl group was inferred as a β-orientation on the basis of the multiplicity of H-3 (δ 3.18, dd, J = 6.0, 10.0 Hz) [13] and the observation of ROESY correlations between H-3 with H-5/Me-23. On the other hand, the HMBC cross-peaks of Me-25 with C-5, of Me-26 with C-7, and of H-5/H-7 with the oxygen-bearing carbon resonance at δ 68.7 confirmed the existence of one hydroxyl group at C-6 position. The ROESY cross-peaks between H-6 and H-5/Me-23 indicated that the configuration of the hydroxyl group at C-6 was a β-orientation. The olefinic proton at δ_H 5.81 (1H, brs) and corresponding carbon resonances at δ_C 126.0 and 170.4, the strongly deshielded chemical shift for the quaternary carbon suggested that it may be placed β to a carbonyl (δ_C 199.4). The HMBC spectrum showed correlations of Me-27 with an olefinic carbon at δ_C 170.4, and of H-12 with the other one at δ_C 126.0, which confirmed their attribution to C-13 and C-18,

Table 3
^{13}C NMR Data of compounds **1–10** (125 MHz).

Position	**1**[a]	**2**[b]	**3**[c]	**4**[b]	**5**[b]	**6**[d]	**7**[b]	**8**[d]	**9**[b]	**10**[b]
1	41.2	39.8	40.3	157.4	39.6	38.2	41.1	38.2	42.9	43.2
2	27.7	28.2	28.3	125.0	34.2	26.5	34.8	36.1	38.5	38.1[e]
3	79.2	79.3	78.2	205.6	223.0	76.3	216.9	211.7	217.4	217.5
4	39.8	40.2	38.7	44.5	[f]	37.0	48.2	[f]	43.8	43.8
5	55.6	54.2	65.6	63.9	60.0	64.1	66.3	62.8	56.4	56.2
6	68.7	56.5	212.5	213.4	68.5	212.0	214.5	210.4	68.8	68.8
7	41.8	57.9	51.9	52.0	43.9	50.5	52.0	49.0	39.6	39.7
8	40.2	41.5	47.1	48.3	41.6	45.8	47.8	46.5	40.0	40.3
9	51.2	47.4	48.9	44.2	47.4	46.9	[f]	44.7	47.1	46.4
10	37.4	37.4	43.7	46.2	39.7	42.5	44.3	42.4	37.4	37.2
11	21.6	24.0	24.4	25.3	24.9	23.4	25.2	23.9	25.3	25.3
12	33.0	131.8	127.9	128.6	124.9	122.0	124.5	122.1	125.1	129.7
13	170.4	139.8	138.3	140.2	144.3	142.9	144.4	143.3	144.4	139.8
14	45.3	43.9	44.5	43.6	43.1	41.5	43.2	42.0	43.5	43.5
15	28.7	29.7	29.1	29.6	29.7	27.7	29.6	27.8	29.8	29.8
16	34.5	26.8	24.5	26.5	28.8	26.9	28.5	27.0	28.8	26.8
17	199.4	[f]	47.2	[f]	46.9	44.6	46.8	[f]	46.9	[f]
18	126.0	56.8	51.4	55.2	45.4	43.1	45.3	43.3	45.4	55.4
19		73.4	134.5	73.7	82.6	80.0	82.5	80.0	82.7	73.7
20		43.2	127.3	43.2	36.2	34.8	36.2	34.8	36.2	43.3
21		27.4	25.5	27.4	29.8	28.3	29.5	28.3	29.6	27.4
22		38.7	33.7	38.9	34.1	32.1	34.0	32.1	34.2	39.1
23	28.1	28.9	28.9	24.8	32.4	27.4	24.7	12.1	11.5	11.5
24	17.2	18.0	16.8	23.0	20.6	15.6	22.6			
25	18.0	17.7	17.5	18.4	17.0	15.9	16.2	12.8	15.4	15.7
26	20.6	15.7	18.7	18.9	18.0	17.0	18.3	17.3	19.0	19.0
27	19.5	26.3	22.7	25.7	24.8	24.7	25.8	24.8	25.3	25.1
28		182.1	180.0	182.0	182.3	178.9	182.1	179.0	182.4	182.4
29		27.2	17.6	27.1	28.7	28.0	28.7	28.0	28.8	27.2
30		16.6	74.0	16.6	25.4	24.3	25.2	24.4	25.4	16.7
OCH_3-30			58.1							

[a] Measured in $CDCl_3$.
[b] Measured in methanol-d_4.
[c] Measured in pyridine-d_5.
[d] Measured in DMSO-d_6.
[e] Signal is weak.
[f] Overlapped with residual solution signal.

respectively. Furthermore, the HMBC correlations of Me-27 with C-15, of H-15 with C-16, and of H-16 with the carbon resonance at δ_C 199.4 suggested the carbonyl group located at C-17. The relative configurations of the methyl groups and other protons in **1** were determined by ROESY experiment. Thus, the structure of **1** was unambiguously established and named as laevigatone A.

Compound **2** was shown to have the molecular formula $C_{30}H_{46}O_5$ on the basis of the pseudo-molecular ion at *m/z* 485.3271 $[M-H]^-$ (Calcd for $C_{30}H_{45}O_5$: 485.3272) in the HRESIMS, corresponding to eight degrees of unsaturation. The IR spectrum revealed the presence of hydroxyl group (3443 cm^{-1}) and carboxylic acid group (1684 cm^{-1}). The ^{1}H NMR spectrum of **2** (Table 1) exhibited characteristic signals for seven methyls including six singlets at δ_H 1.01, 1.02, 1.16, 1.16, 1.19, 1.46 and one doublet at δ_H 0.95 (3H, d, J = 7.0 Hz), three oxygenated methine protons at δ_H 3.05 (1H, d, J = 4.0 Hz), 3.22 (1H, dd, J = 5.0, 11.5 Hz), and 3.47 (1H, d, J = 4.0 Hz), and one olefinic proton at δ_H 5.45 (1H, brs). Analysis of its ^{13}C NMR (Table 3) and HSQC spectroscopic data revealed **2** contained 30 carbon resonances, including seven methyl, seven methylene, eight methine, and eight quaternary carbons. Of these, the signals at δ_C 73.4, and 79.3 revealed the presence of two oxygen-bearing carbons. In the HMBC spectrum, compound **2** exhibited significant cross-peaks from Me-27/H-18 to one olefinic carbon at δ_C 139.8, from H-18 to the other one at δ_C 131.8 (corresponding olefinic proton δ_H 5.45, 1H, brs) and to the carboxylic acid group signal at δ_C 182.1, which are characteristic of a $\Delta^{12\ (13)}$-18-COOH-ursane-type structure. The HMBC correlations of Me-23/Me-24/H-5 with one oxymethine carbon signal at δ_C 79.3 (δ_H 3.22, 1H, dd, J = 5.0, 11.5 Hz), and of H-18/Me-29/Me-30 with the other one at δ_C 73.4 (quaternary carbon) indicated the hydroxyl groups located at C-3 and C-19, respectively. In the ROESY spectrum, the correlations of H-3 with H-5/Me-23, of H-12 with Me-29, and of H-18 with Me-29/H-20 suggested that the hydroxyl groups at C-3 and C-19 were β- and α-configuration, respectively. In full consideration of the degrees of unsaturation, the mutually coupled protons at δ_H 3.05 and 3.47 (each 1 H, d, J = 4.0 Hz) and the corresponding oxygenated carbons at δ_C 56.5 and 57.9 indicated the presence of an epoxide ring, which was ascertained on the basis of HMBC cross-peaks from Me-23/Me-24/Me-25 to C-5, from H-5 to one oxymethine carbon at δ_C 56.5 (δ_H 3.47, d, J = 4.0 Hz), and from the proton signal at δ_H 3.47 and Me-26 to the carbon resonance at δ_C 57.9 (δ_H 3.05, d, J = 4.0 Hz). Meanwhile, the 6,7-epoxide ring can be confirmed from the above analysis. The ROESY correlations of H-6 with Me-23, and of H-7 with

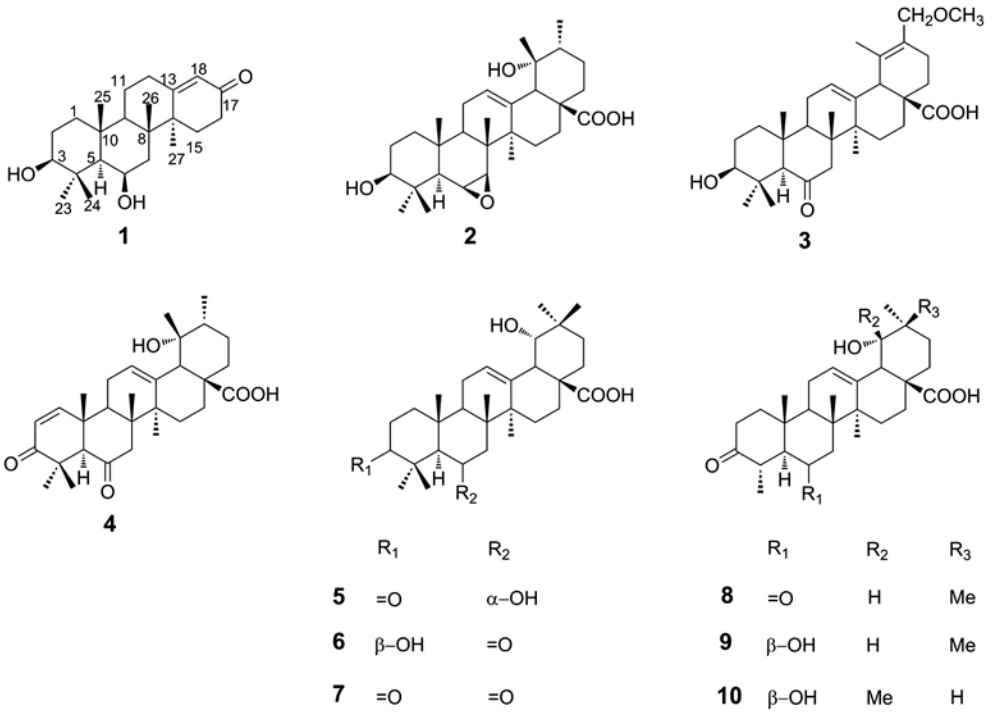

Fig. 1. Chemical structures of new compounds **1–10**.

Me-27 indicated a β-orientation for the 6,7-epoxide ring. Therefore, the structure of **2** was determined to be 3β, 19α-dihydroxy-6β, 7β-epoxyl-urs-12-en-28-oic acid.

Compound **3** was isolated as white powder, with a molecular formula of $C_{31}H_{46}O_5$ as deduced from the HRESIMS pseudo-molecular ion at *m/z* 497.3263 $[M\text{-}H]^-$ (Calcd for $C_{31}H_{45}O_5$: 497.3272). The IR spectrum showed the absorptions of hydroxyl group (3416 cm^{-1}) and carboxylic acid group (1689 cm^{-1}). The ^1H NMR spectrum of **3** (Table 1) displayed the presence of six singlet signals for methyl at δ_H 1.00, 1.11, 1.25, 1.44, 1.69, and 1.87, one methoxyl group at δ_H 3.33, one oxygenated methine proton at δ_H 3.48 (1H, dd, $J = 5.0$, 11.0 Hz), and one olefinic proton at δ_H 5.76 (1H, brs). In the ^{13}C NMR spectrum (Table 3), three oxygenated carbons at δ_C 58.1, 74.0, 78.2, signals at δ_C 180.0 and 212.5 for carboxylic acid and ketone, respectively, and four olefinic carbons at δ_C 127.3, 127.9, 134.5 and 138.3 were observed. The NMR spectroscopic data of **3** showed a close similarity to those of 3β, 19α-dihydroxy-6-oxo-urs-12-en-28-oic acid (**11**), especially on the responses associated with rings A–D but were different in ring E. In the HMBC spectrum, the correlations from H-18/Me-29/H-30 to the olefinic carbon signals at δ_C 134.5 and 127.3 indicated the presence of a double bond between C-19 and C-20. Besides, the correlation between the signal at δ_H 3.33 (3H, s) and δ_C 74.0 (C-30) located the methoxyl group at C-30. Furthermore, the β-configuration of hydroxyl group at C-3 was confirmed on the basis of the correlations from H-3 to H-5/Me-23 in the ROESY spectrum of **3**. Consequently, the structure of **3** was established as 3β-hydroxy-30-methoxy-6-oxo-urs-12, 19 (20)-dien-28-oic acid.

Compound **4** was obtained as white powder and showed a pseudo-molecular ion at *m/z* 481.2957 $[M\text{-}H]^-$ (Calcd for $C_{30}H_{41}O_5$: 481.2959), corresponding to the molecular formula $C_{30}H_{42}O_5$. The IR spectrum exhibited absorptions of hydroxyl group (3451 cm^{-1}) and carboxylic acid group (1686 cm^{-1}).

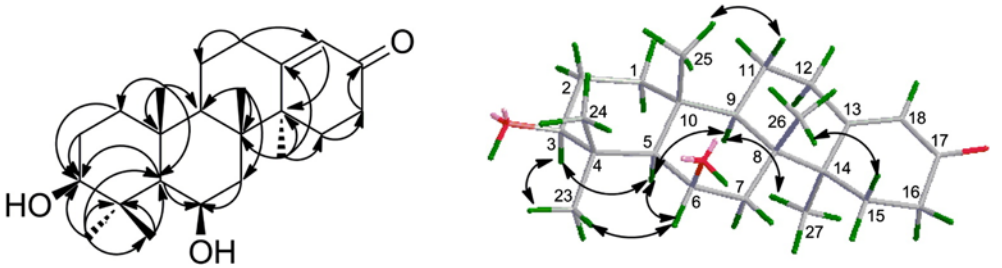

Fig. 2. Key HMBC (→) and ROESY (↔) correlations of **1**.

Careful inspection of the NMR spectra of **4** (Tables 1 and 3) and 19α-hydroxy-3, 6-dioxo-urs-12-en-28-oic acid (**13**, both recorded in methanol-d_4) indicated closely related structures of them. The major differences were the presence of additional olefinic proton signals at δ_H 5.78 (1H, d, J = 10.0 Hz) and 7.15 (1H, d, J = 10.0 Hz), and the corresponding carbon resonances at δ_C 125.0 and 157.4 in **4**, which suggested that one more double bond existed in **4**. The olefinic signals at δ_C 157.4 and 125.0 were further assigned to C-1 and C-2, respectively, deduced from the correlations of H-5/Me-25 with C-1, and of H-2 with C-10 in the HMBC spectrum of **4**. Thus, the structure of **4** was determined to be 19α-hydroxy-3, 6-dioxo-urs-1 (2), 12-dien-28-oic acid.

Compound **5** was obtained as white powder and showed a pseudo-molecular ion at m/z 485.3281 $[M-H]^-$ (Calcd for $C_{30}H_{45}O_5$: 485.3272), corresponding to the molecular formula $C_{30}H_{46}O_5$, two hydrogen atom less than that of 3β, 6β, 19α-trihydroxy-olean-12-en-28-oic acid (**14**). The IR spectrum indicated the presence of hydroxyl group (3443 cm^{-1}) and carboxylic acid group (1688 cm^{-1}). Compound **5** resembles **14** in their NMR data (Tables 1 and 3) with some major differences: the disappearance of the oxygenated carbon signal at δ_C 80.3, and instead, the presence of one more ketone carbon signal at δ_C 223.0 in **5**. The HMBC correlations of Me-23/Me-24 with the carbon signal at δ_C 223.0 located the ketone group at C-3. Besides, the proton signal at δ_H 3.84 (1H, dt, J = 7.5, 10.5 Hz) for H-6 in **5** was different from the corresponding signals at δ_H 4.48 (1H, brs) in **14**, indicating the relative configuration of hydroxyl group located at C-6 maybe α, which was ascertained by the ROESY correlations of H-6 with Me-25/Me-26. Therefore, the structure of **5** was determined to be 6α, 19α-dihydroxy-3-oxo-olean-12-en-28-oic acid.

Compound **6** gave a molecular formula of $C_{30}H_{46}O_5$, the same as that of **5**, as deduced from the HRESIMS pseudo-molecular ion at m/z 485.3266 $[M-H]^-$ (Calcd for $C_{30}H_{45}O_5$: 485.3272). The IR spectrum exhibited the absorption bands at 3491 cm^{-1} (hydroxyl group) and 1697 cm^{-1} (carboxylic acid group). Analyses of the 1D and 2D NMR data of **6** suggested that **6** possessed the same skeleton and functional group as **5**. The locations of two hydroxyl and one ketone groups were assigned to C-3, C-19 and C-6 on the basis of the HMBC correlations from Me-23/H-5/H-2 to one oxymethine carbon at δ_C 76.3, from Me-29/Me-30/H-18 to the other one at δ_C 80.0, and from H-5/H-7 to the signal at δ_C 212.0, respectively. The relative configuration of hydroxyl groups connected to C-3 and C-19 was further determined to be β and α, deducing from the correlations of H-3 with H-5/Me-23, and of H-12 with H-19, respectively. Accordingly, compound **6** was established as 3β, 19α-dihydroxy-6-oxo-olean-12-en-28-oic acid.

Compound **7** showed a pseudo-molecular ion at m/z 483.3110 $[M-H]^-$ (Calcd for $C_{30}H_{43}O_5$: 483.3116), corresponding to the molecular formula $C_{30}H_{44}O_5$, which possessed two less hydrogen atoms than that of **5**. The IR spectrum exhibited the absorption bands at 3475 cm^{-1} (hydroxyl group) and 1699 cm^{-1} (carboxylic acid group). The similarity of the NMR data (Table 2 and 3) of **7** to those of **5** indicated that **7** possessed the same 19α-hydroxyoleanane-type skeleton. The major difference between **5** and **7** lies in the functionality at C-6: the oxygenated methine group was absent in **5**, but instead, an additional ketone carbon signal at δ_C 214.5 was observed. The HMBC correlations of H-5/H-7 with the carbon signal at δ_C 214.5 assigned the ketone group to C-6. Based on the above analysis and further confirmed by the HSQC, HMBC and ROESY experiments, the structure of **7** was established as 19α-hydroxy-3, 6-dioxo-olean-12-en-28-oic acid.

Compound **8** was obtained as a white powder. The molecular formula $C_{29}H_{42}O_5$ was determined from the HR-ESI-MS pseudo-molecular ion at m/z 469.2955 $[M-H]^-$ (Calcd for $C_{29}H_{41}O_5$: 469.2959). The absorption bands in the IR spectrum suggested the presence of hydroxyl group (3451 cm^{-1}) and carboxylic acid group (1698 cm^{-1}). In the ^1H NMR spectrum of **8** (Table 2), five singlet signals at δ_H 0.74, 0.85, 0.88, 0.96, and 1.41, and one doublet signal at δ_H 0.85 (3H, d, J = 5.0 Hz) for six methyl groups were observed. The ^{13}C NMR spectrum of **8** (Table 3) exhibited total 29 carbon signals, including the characteristic olefinic carbon signals at δ_C 122.1 and 143.3, which indicated that **8** possessed a noroleanane-type skeleton. Comparing the ^{13}C NMR spectra of **8** and **6**, one oxygenated carbon signal for C-3 (δ_C 76.3) in **6** was replaced by a ketone signal at δ_C 211.7 in **8**. The HMBC correlations from one methyl group at δ_H 0.85 (3H, d, J = 5.0 Hz) to C-3/C-4/C-5 and the ROESY correlations from the same methyl group to H-5 further suggested that **8** had a 24-noroleanane-type skeleton. The locations and relative configurations of other functional groups were determined with the help of HSQC, HMBC, and ROESY experiments. Based on the above evidences, the structure of **8** was established as 19α-hydroxy-3, 6-dioxo-24-norolean-12-en-28-oic acid.

Compound **9** was isolated as white powder and showed a pseudo-molecular ion at m/z 471.3118 $[M-H]^-$ (Calcd for $C_{29}H_{43}O_5$: 471.3116), corresponding to the molecular formula $C_{29}H_{44}O_5$, which possessed two more hydrogen atoms than that of **8**. The IR spectrum indicated the presence of hydroxyl group (3442 cm^{-1}) and carboxylic acid group (1689 cm^{-1}). Comparison of the NMR (Tables 2 and 3) data of **9** and **8** revealed that the significant differences were the disappearance of a ketone carbon signal at δ_C 210.4, and the additional chemical shifts of δ_H 4.16 (1H, brs) and δ_C 68.8 in **9**, which indicated that the ketone group at C-6 in **8** was replaced by a hydroxyl group in **9**. Other NMR data of **9** corresponded well to those of **8**. Based on further analysis of HSQC, HMBC, and ROESY spectra of **9**, its structure was determined to be 6β, 19α-dihydroxy-3-oxo-24-norolean-12-en-28-oic acid.

Compound **10** was obtained as a white powder and showed a pseudo-molecular ion at m/z 471.3125 $[M-H]^-$ (Calcd for $C_{29}H_{43}O_5$: 471.3116), corresponding to the molecular formula $C_{29}H_{46}O_4$. The IR spectrum of **10** indicated the presence of hydroxyl group (3442 cm^{-1}), carboxylic acid group (1701 cm^{-1}) and double bond (1635 cm^{-1}). The ^1H NMR spectrum of **10** (Table 2) displayed four singlet signals of methyl groups at δ_H 1.16, 1.20, 1.33, and 1.44, two doublet signals of methyl groups at δ_H 0.93 (3H, d, J = 6.5 Hz) and 1.04 (3H, d, J = 6.0 Hz), one oxygenated methine proton at δ_H 4.16 (1H, brs), and one olefinic proton at δ_H 5.33 (1H, brs). Based on the analysis of its ^{13}C NMR (Table 3) and HSQC spectral data, a total of 29 carbon signals were observed, including one oxygenated carbon signal at δ_C 73.7 and the signals at δ_C 129.7 and 139.8, which were characteristic for the 19α-hydroxy-$\Delta^{12\ (13)}$-ursane skeleton [13]. Comparing

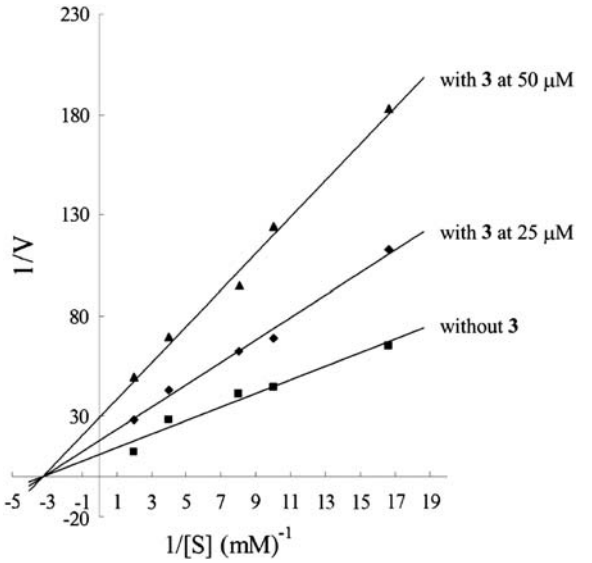

Fig. 3. Double-reciprocal plots of the inhibition kinetic of yeast α-glucosidase by compound **3**.

the NMR data of **10** and **9**, the signals of rings A and B in **10** were almost the same as those in **9**, which indicated that one ketone group and one hydroxyl group were located at C-3 and C-6 in **10**, respectively, and that **10** also possessed a 24-norursane-type skeleton. Based on further analysis of HSQC, HMBC, and ROESY spectra of **10**, the structure of **10** was determined to be 6β, 19α-dihydroxy-24-norurs-12-en-28-oic acid.

The other isolated known compounds **11**–**18** were identified as 3β, 19α-dihydroxy-6-oxo-urs-12-en-28-oic acid (**11**) [14], 2α, 19α-dihydroxy-3-oxo-urs-12-en-28-oic acid (**12**) [13], 19α-hydroxy-3, 6-dioxo-urs-12-en-28-oic acid (**13**) [14], 3β, 6β, 19α-trihydroxy-olean-12-en-28-oic acid (**14**) [15], 3β, 6β, 19α-trihydroxy-urs-12-en-28-oic acid (**15**) [16], 6β, 19α-dihydroxy-3-oxo-urs-12-en-28-oic acid (**16**) [17], ursolic acid (**17**) [18], and 3β, 6β-dihydroxy-urs-12, 18 (19)-dien-28-oic acid (**18**) [6] through comparison of their physical data (ESIMS and NMR) with published information.

Compounds **1**–**18** were tested for their inhibitory effect on yeast α-glucosidase activity. Among them, ursolic acid and new compound **3** possessed high potency with the IC_{50} value of 16 ± 2.2 and 49 ± 3.7 μM, respectively, as compared with that of genistein (IC_{50} = 36 ± 1.5 μM) which was used as a positive control. Other sixteen tested compounds exhibited only weak activity with inhibition below 20% at the concentration of 100 μM.

The inhibition type of new compound **3** was determined from Lineweaver–Burk plots with the purpose to know how it interacts with yeast α-glucosidase. The double-reciprocal plots (Fig. 3) showed straight lines with the same Michaelis–Menten constant K_m, indicating that compound **3** was noncompetitive α-glucosidase inhibitor (K_i = 28 μM).

Conflict of interest

The authors have declared that there is no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.fitote.2013.07.005.

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