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Bio-assay guided isolation and identification of α-glucosidase inhibitors from the leaves of *Aquilaria sinensis*

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ABSTRACT

Eight α-glucosidase inhibitors including four new compounds were isolated from the 70% aqueous ethanolic extract of leaves of *Aquilaria sinensis* (Lour.) Gilg by activity-directed fractionation and purification processes. The ethanolic extract was first separated into petroleum ether, ethyl acetate, *n*-butanol and water soluble fractions and screened for inhibitory activity against α-glucosidase. Further activity-directed investigation lead to the isolation of four new compounds with moderate inhibitory activity, viz, aquilarisinin (**1**), aquilarisin (**2**), hypolaetin 5-*O*-β-D-glucuronopyranoside (**3**) and aquilarixanthone (**4**) from the *n*-butanol fraction, and four known compounds showing potent activity including mangiferin (**5**), iriflophenone 2-*O*-α-L-rhamnopyranoside (**6**), iriflophenone 3-*C*-β-D-glucoside (**7**) and iriflophenone 3,5-*C*-β-D-diglucopyranoside (**8**) from the most potent ethyl acetate fraction. The structures of these compounds were determined by extensive spectroscopic analyses, including IR, UV, ESIMS, HRESIMS, 1D and 2D NMR.

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1. Introduction

Diabetes mellitus is a chronic metabolic disorder which is characterized by hyperglycemia and accompanied by various chronic vascular complications; thus, the control of postprandial blood glucose surges is critical for the treatment of diabetes. One of the therapeutic approaches for postprandial hyperglycemia is to reduce carbohydrate uptake after meal. It is well-known that complex polysaccharides are hydrolyzed by amylases to dextrins which are further hydrolyzed to glucose by intestinal α-glucosidase before entering blood circulation through intestinal epithelium absorption. For this reason, postprandial hyperglycemia may be treated by amylase and α-glucosidase inhibitors via delaying glucose absorption. Synthetic amylase and α-glucosidase inhibitors, for example, acarbose (chemical structure shown in Fig. 1), are widely used for the treatment of patients with type II diabetes, but they are also reported to cause various side-effects; therefore, safer natural amylase and α-glucosidase inhibitors are desired and many compounds have been reported from plant sources (Hiroyuki et al., 2001; Matsui et al., 2001; Wansi et al., 2007; Kim et al., 2008; Lee et al., 2008). *Aquilaria sinensis* (Lour.) Gilg (Thymelaeaceae), distributed in South China such as Hainan, Guangxi, Guangdong, Fujian and Taiwan provinces, is the principal source of the expensive agarwood. The latter is a rich in resin which has been used as an incense as well as a traditional sedative, analgesic, and digestive medicine in East Asia. Phytochemical investigations of agarwood and its oil have led to isolation of sesquiterpenes and chromone derivatives (Nakanishi et al., 1984; Ishihara et al., 1991; Yagura et al., 2005; Liu et al., 2008). The leaves of *A. sinensis* have been used traditionally in China for treatments of inflammation and anaphylaxis and their extracts have exhibited notable analgesic, anti-inflammatory (Zhou et al., 2008) and mild cathartic (Iinuma et al., 2007) activities. As for the chemical constituents of the leaves, several flavonoids and benzophenone glycosides have been reported previously (Iinuma et al., 2007; Qi et al., 2009). In the course of our characterization studies on α-glucosidase inhibitors in natural medicines, the ethyl acetate (EtOAc) soluble fraction of the 70% aqueous ethanol extract from the leaves of *A. sinensis* was found to have inhibitory effects against α-glucosidase activity. Consequently, the chemical constituents of *Aquilaria* leaves were thoroughly investigated in this paper guided by bio-assay of α-glucosidase inhibitors.

2. Results and discussion

2.1. Chemistry

Dried leaves of *A. sinensis* were extracted with 70% aqueous ethanol, and the extract was concentrated *in vacuo* and kept overnight for precipitation. The resulting suspension was filtered and successively fractionated with petroleum ether (PE), EtOAc and *n*-butanol

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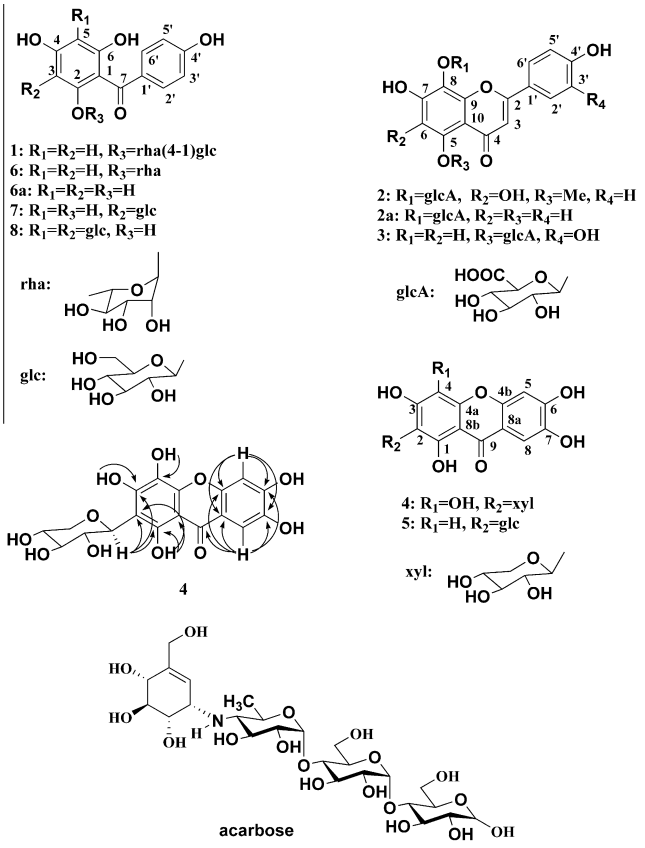

Fig. 1. Chemical structures of compounds **1**–**8**, **2a**, **6a**, acarbose and Key HMBC (H→C) correlations of **4**.

(*n*-BuOH). The EtOAc and *n*-BuOH extracts were successively subjected to silica gel, polyamide and Sephadex LH-20 column chromatography in combination with bioassays with the purpose of tracking α-glucosidase inhibitors. As a result, four new (**1**–**4**) and four known (**5**–**8**) compounds were obtained.

Compound **1** was obtained as a yellowish amorphous powder. Its molecular formula was determined as $C_{25}H_{30}O_{14}$ on the basis of ESIMS and HRESIMS data. In the ESIMS spectrum, two quasi-molecular ion peaks appeared at *m/z* 577 $[M+Na]^+$ and 555 $[M+H]^+$, suggesting a molecular weight of 554. The positive HRESIMS analysis also showed quasi-molecular ion peaks at *m/z* 555.1711 $[M+H]^+$ (calcd. for $C_{25}H_{31}O_{14}$, 555.1708) and 577.1516 $[M+Na]^+$ (calcd. for $C_{25}H_{30}NaO_{14}$, 577.1528), which confirmed the molecular formula mentioned above. Compound **1** was characterized as chalcone, this being partially supported by the stretching vibration signals at 3383 and 1725 cm^{-1} indicative of an associated hydroxyl and a carbonyl group, respectively, in its IR spectrum. The signals observed in ^1H and ^{13}C NMR (Table 1) spectra of **1** were similar to those of the known compound **6** (Iinuma et al., 2007), except for a set of signals representative of a β-D-glucopyranosyl group (Breitmayer and Voelter, 1989). This glucopyranosyl group was linked to C-4″ of the rhamnopyranosyl moiety through an 1 → 4 glycosidic bond as evidenced by the downfield chemical shift of this carbon (+10.5 ppm). The entire structure of **1**′ as shown in Fig. 1, was definitely determined based on the evidence below. Firstly, acid hydrolysis of **1** yielded an aglycone **6a** (iriflophenone), which was identical to that obtained by acidic hydrolysis of **6**, and two monosaccharides, i.e. glucose and rhamnose were identified by comparison with the respective authentic samples by paper chromatography (PC). Secondly, both ion peaks at *m/z* 247 $[M-162-146+H]^+$ in the positive mode and 245 $[M-162-146-H]^-$ in the negative mode ESIMS spectrum demonstrated the presence of a disaccharide chain consisting of a rhamnosyl–glycosyl moiety (Ding et al., 2008). Finally, the linkage pattern of the disaccharide chain was further confirmed by 2D NMR spectroscopy such as ^1H–^1H COSY, HSQC and HMBC analyses. The HMBC data showed correlations from H-1‴ [δ 4.32 (1H, d, *J* = 7.8 Hz, terminal H-1 of glucopyranosyl)] to C-4″ [δ 82.0 (inner C-4 of rhamnopyranosyl)] and from H-1″ [δ 5.14 (1H, br s, inner H-1 of rhamnopyranosyl)] to C-2 (δ 155.6), corroborating the linkage pattern of glucopyranosyl-(1→4)-rhamnopyranosyl and its attachment to C-2 of the aglycone. Thus, **1** was identified as iriflophenone 2-*O*-β-D-glucopyranosyl-(1→4)-*O*-α-L-rhamnopyranoside, named as aquilar- isinin.

Table 1
^1H and ^{13}C NMR spectroscopic data (DMSO-d_6) of **1**, **6** and **6a**.[a]

Position	**1**		**6**	**6a**
	δ_H	δ_C, mult.	δ_C, mult.	δ_C, mult.
1		109.7, qC	109.5, qC	106.6, qC
2		155.6, qC	155.5, qC	157.9, qC
3	6.14 d, 2.0	94.0, CH	93.9, CH	94.3, CH
4		159.5, qC	159.4, qC	160.4, qC
5	6.05 d, 2.0	96.8, CH	96.6, CH	94.3, CH
6		156.5, qC	156.4, qC	157.9, qC
7		193.1, qC	192.8, qC	194.4, qC
1′		130.4, qC	130.1, qC	130.6, qC
2′	7.56 d, 8.7	131.6, CH	131.4, CH	131.5, CH
3′	6.82 d, 8.7	115.3, CH	115.1, CH	114.7, CH
4′		161.9, qC	161.8, qC	161.5, qC
5′	6.82 d, 8.7	115.3, CH	115.1, CH	114.7, CH
6′	7.56 d, 8.7	131.6, CH	131.4, CH	131.5, CH
Rha-1″	5.14 br s	98.6, CH	98.9, CH	
Rha-2″	3.05 br d, 7.8	70.0, CH	70.1, CH	
Rha-3″	3.35 dd, 5.4, 7.8	70.0, CH	70.1, CH	
Rha-4″	3.34 dd, 5.4, 8.0	82.0, CH	71.5, CH	
Rha-5″	3.34 dd, 5.4, 8.0	68.1, CH	69.5, CH	
Rha-6″	1.14 d, 5.4	17.9, CH_3	17.9, CH_3	
Glc-1‴	4.32 d, 7.8	104.6, CH		
Glc-2‴	2.97, dd, 7.8, 8.4	74.5, CH		
Glc-3‴	3.11 t, 8.4	76.6, CH		
Glc-4‴	3.36 t, 8.4	69.7, CH		
Glc-5‴	3.06 t, 8.7	77.1, CH		
Glc-6‴	3.45 dd, 8.0, 12.0			
	3.62 d, 12.0	61.2, CH_2		

[a] C-multiplicities were established by a HSQC experiment.

Compound **2**, obtained as a yellowish amorphous powder, was a flavone (Fig. 1) as evidenced by comparison of its spectroscopic data with a known flavone, i.e. isoscutellarein 8-*O*-β-D-glucuronopyranoside (**2a**) (Billeter et al., 1991). In its IR spectrum, the strong IR bands at 3381 and 1726 cm^{-1} were closely similar to those of **1**, indicating an associated hydroxyl and a carbonyl group, respectively. This carbonyl group was confirmed by its ^{13}C NMR resonance signal at δ 182.0. The ^1H NMR spectroscopic data [δ 8.14 (2H, d, *J* = 8.4 Hz, H-2, 6); 6.88 (2H, d, *J* = 8.4 Hz, H-3, 5)] disclosed the characteristic of a 1,4-disubstituted B-ring, while six quaternary ^{13}C NMR carbon signals (δ 124.9, 129.2, 148.1, 149.6, 150.7 and 161.2) corresponding to *O*-linked aromatic carbons in combination with a ^1H NMR resonance at δ 6.68 (1H, s) that demonstrated the 5,6,7,8,4′-pentaoxygenated flavone skeleton of **2** (Agrawal, 1989; Breitmayer and Voelter, 1989; Ponce et al., 2004). A sharp three-proton singlet at δ_H 3.88 was assigned to a methoxyl group which was proven to be located at C-5, since this singlet showed a HMBC correlation with one of the oxygenated quaternary carbons at δ_C 149.6 (C-5). Compound **2** was thus established to be a monoglycosided flavone from the characteristic β-D-glucopyranosiduronyl signals of NMR [^1H and ^{13}C NMR spectroscopic data: see Table 2]. This β-D-glucopyranosiduronyl group connected the skeleton at C-8 via an oxygen atom, since the

Table 2
^{1}H and ^{13}C NMR spectroscopic data (DMSO-d_6) of **2**, **2a** and **3**.[a]

Position	**2**		**2a**[b]	**3**	
	δ_H	δ_C, mult.	δ_C	δ_H	δ_C, mult.
2		164.1, qC	163.8		163.5, qC
3	6.68 s	102.7, CH	102.3	6.43 s	102.4, CH
4		182.0, qC	181.7		180.5, qC
5		149.6, qC	157.2		157.5, qC
6		129.2, qC	99.0	8.36 s	99.0, CH
7		148.1, qC	157.2		163.5, qC
8		124.9, qC	125.2		128.3, qC
9		150.7, qC	149.2		149.8, qC
10		103.1, qC	103.3		102.0, qC
1′		121.4, qC	121.0		122.7, qC
2′	8.14 d, 8.4	129.2, CH	128.9	7.95 br s	114.3, CH
3′	6.88 d, 8.4	116.2, CH	116.0		146.5, qC
4′		161.2, qC	161.1		149.3, qC
5′	6.88 d, 8.4	116.2, CH	116.0	6.80 br d, 8.1	116.0, CH
6′	8.14 d, 8.4	129.2, CH	128.9	7.29 br d, 8.1	118.5, CH
GlcA-1″	4.66 d, 7.8	106.4, CH	106.3	4.37 d, 7.0	108.6, CH
GlcA-2″	3.52, m	73.7, CH	73.6	3.65–3.17 m	74.3, CH
GlcA-3″	3.57 t, 9.0	76.2, CH	76.1	3.65–3.17 m	76.2, CH
GlcA-4″	3.26, m	71.8, CH	71.5	3.65–3.17 m	71.9, CH
GlcA-5″	3.55 d, 9.0	75.5, CH	75.3	3.65–3.17 m	76.7, CH
GlcA-6″		170.5, qC	170.1		168.5, qC
5-OMe	3.88 s	56.1, CH_3			
OH				8.39 br s	

[a] C-multiplicities were established by a HSQC experiment.
[b] **2a** was isoscutellarein 8-*O*-β-D-glucuronopyranoside and the ^{13}C NMR spectroscopic data was reported in Billeter et al. (1991).

anomeric proton signal at δ_H 4.66 (H-1″) showed a HMBC correlation with another oxygenated quaternary carbon at δ_C 124.9 (C-8) (Yang et al., 2003). Acid hydrolysis of **2** confirmed the presence of this glucuronic acidyl group. At first, a quasi-molecular ion peak at *m/z* 217.0318 $[C_6H_{10}NaO_7]^+$ in the positive ESIMS spectrum of the hydrolysate established the presence of glucuronic acid. Secondly, comparison of the hydrolysate with an authentic glucuronic acid on PC (*n*-BuOH–H_2O–HOAc, 4:2:1, v/v/v) provided the same conclusion. The entire molecule of **2** was deduced to have a formula of $C_{22}H_{20}O_{13}$ from the quasi-molecular ion peaks at *m/z* 515 $[M+Na]^+$ and 493 $[M+H]^+$ in the positive mode, and at *m/z* 983 $[2M-H]^-$ and 491 $[M-H]^-$ in the negative mode ESIMS spectra. It was further confirmed by HRESIMS with this having an ion peak at *m/z* 493.0964 $[M+H]^+$ (calcd. for $C_{22}H_{21}O_{13}$, 493.0976). Hence, compound **2** was elucidated as 5-methoxy-6-hydroxy-isoscutellarein 8-*O*-β-D-glucuronopyranoside (Fig. 1), trivially named as aquilarisin.

Compound **3** was isolated as a yellowish powder. The strong IR bands at 3385 and 1725 cm^{-1} were indicative of an associated hydroxyl and a carbonyl group, respectively. The carbonyl group was confirmed by the resonance at δ_C 180.5 (C-4) in its ^{13}C NMR spectrum (Table 2) (Agrawal, 1989; Breitmayer and Voelter, 1989). Compound **3** was demonstrated to be a derivative of 5,7,8,3′,4′-pentahydroxyflavone (Fig. 1) by comparison of its NMR spectroscopic data with those published for hypolaetin 8-*O*-β-D-glucuronopyranoside (Billeter et al., 1991; Bilia et al., 1996). In the ^{1}H NMR spectra, the aromatic methine signals at δ 7.95 (1H, br s), 7.29 (1H, br d, *J* = 8.1 Hz) and 6.80 (1H, d, *J* = 8.1 Hz) were assigned to H-2′, H-6′ and H-5′ of the B ring, respectively, and the resonance at δ 6.43 (1H, s) was assigned to the H-3 of the C ring. Thus, another aromatic methine proton signal at δ 8.36 (1H, s) must be assignable to A ring, and its exact position was H-6 because of its HMBC correlation with a carbon signal at δ 102.0 (C-10) which was assigned based on its HMBC correlation with H-3. Similar to compound **2** it also possessed a glucuronosyl group as indicated from its ^{1}H and ^{13}C NMR spectroscopic data including δ_H 4.37 (1H, d, *J* = 7.0 Hz, H-1″), 3.65–3.17 (4H, m, H-2″ – H-5″), and δ_C 108.6 (C-1″), 74.3 (C-2″), 76.2 (C-3″), 71.9 (C-4″), 76.7 (C-5″) and 168.5 (C-6″) (Billeter et al., 1991). Acid hydrolysis (see Section 4) together with fragmentation ion peaks at *m/z* 325 $[M-176+Na]^+$ and 303 $[M-176+H]^+$ in the positive ESIMS spectrum established the existence of glucuronic acidyl group. This group connected C-5 (δ 157.5) via an oxygen atom, this being supported by a correlation noted between a proton signal at δ 4.37 (1H, d, *J* = 7.0 Hz, H-1″) and C-5 in the HMBC spectrum. This glucosidic bond was also β-oriented as judged from the coupling constant of H-1″ (*J* = 7.0 Hz) (Billeter et al., 1991). According to above description, the molecular formula of **3** was $C_{21}H_{18}O_{13}$, this also being confirmed by an quasi-molecular ion peak at *m/z* 479.0822 $[M+H]^+$ (calcd. for $C_{21}H_{19}O_{13}$, 479.0820). Finally, **3** was elucidated as hypolaetin 5-*O*-β-D-glucuronopyranoside (Fig. 1).

Compound **4** also was isolated in the form of a yellowish amorphous powder. Its IR spectrum differed slightly from the former 3 compounds in that the bands at 3394–3380 cm^{-1} were representative of aliphatic and aromatic hydroxyl groups, and bands at 1647 and 1587 cm^{-1} were characteristic signals of a chelated carbonyl group. Its ^{1}H and ^{13}C NMR signals, including five phenolic hydroxyl resonances at δ_H 13.59 (1H, s, 1-OH), 10.50 (1H, s, 6-OH), 9.75 (1H, s, 7-OH), 9.54 (1H, s, 3-OH) and 5.59 (1H, s, 4-OH) (Gómez-Zaleta et al., 2006), two shielded aromatic methine signals at δ_H 7.31 (1H, s, H-8) and 6.73 (1H, s, H-5), as well as a ^{13}C NMR resonance at δ_C 179.2 for a carbonyl group (Agrawal, 1989; Breitmayer and Voelter, 1989) (Table 3) closely resembled those of the known compound **5** (mangiferin), indicating that compound **4** also had a similar xanthone structure (Fig 1). Compared to mangiferin, compound **4** had an additional hydroxyl group at δ 5.59 (1H, s, 4-OH) and a set of NMR signals for a xylopyranosyl instead of a glucopyranosyl group. This additional hydroxyl group in **4** was located at C-4, inasmuch as the ^{13}C NMR signal of this carbon (δ 153.8) was shifted downfield about 60 ppm and the ^{1}H NMR signal for H-4 was absent in comparison to mangiferin. The pentosyl group was identified as β-D-xylopyranosyl on the basis of its ^{1}H and ^{13}C NMR spectroscopic data (Agrawal, 1989, 1992; Rancon et al., 1999). This xylopyranosyl group connected C-2 through the C–C

Table 3
^{1}H and ^{13}C NMR spectroscopic data (DMSO-d_6) of **4** and **5**.[a]

Position	**4**		**5**
	δ_H	δ_C, mult.	δ_C, mult.
1		158.3, qC	161.8, qC
2		105.4, qC	108.1, qC
3		160.7, qC	163.9, qC
4		153.8, qC	93.3, CH
4a		154.2, qC	156.3, qC
4b		150.9, qC	150.8, qC
5	6.73 s	102.4, CH	102.7, CH
6		154.2, qC	154.1, qC
7		143.6, qC	143.8, qC
8	7.31 s	107.7, CH	107.7, CH
8a		111.6, qC	111.8, qC
8b		101.4, qC	101.3, qC
C=O		179.2, qC	179.2, qC
1′	4.86 d, 9.5	74.3, CH	73.1, CH
2′	3.92 dd, 8.0, 9.5	71.9, CH	70.2, CH
3′	3.30 t, 8.0	81.0, CH	79.0, CH
4′	3.43 m	71.9, CH	70.7, CH
5′	3.30 d, 10.0 3.84 dd, 6.0, 10.0	69.2, CH_2	81.7, CH
6′			61.6, CH_2
1-OH	13.59 s		
3-OH	9.54 s		
4-OH	5.59 s		
6-OH	10.50 s		
7-OH	9.75 s		

[a] C-multiplicities were established by a HSQC experiment.

bond due to the HMBC correlations of H-1′ (δ 4.86) and C-1 (δ 158.3), C-2 (δ 105.4) and C-3 (δ 160.7) which not only established the linking position, but also showed the characteristic chemical shift pattern of a C-glycoside. The nature of the C-glycoside was confirmed by acid hydrolysis results of compound **4**, in which none of the usual fragmentation patterns for *O*-glycosides in the mass spectrum were observed (Rancon et al., 1999). The large coupling constant of H-1 (J = 9.5 Hz) indicated a β-orientation of this C–C glycosidic linkage. On the basis of both NMR spectroscopic data, in combination with the quasi-molecular ion peaks at *m/z* 855 $[2M+K]^+$ and 431 $[M+Na]^+$ detected in positive ESIMS spectrum, the molecular formula $C_{18}H_{16}O_{11}$ was established. Thus was further confirmed by the positive HRESIMS with an ion peak at *m/z* 431.0592 $[M+Na]^+$ (calcd. for $C_{18}H_{16}NaO_{11}$, 431.0590). Finally, the structure of **4** was elucidated as 2-*C*-β-D-xylopyranosyl-1,3,4,6,7-pentahydroxyxanthone (Fig. 1), which has been named as aquilarixanthone.

Known compounds were identified by various spectroscopic methods including MS, 1D and 2D NMR spectroscopic analysis and by comparing experimentally obtained data with those described in the literature. In this way, mangiferin (**5**) (Catalano et al., 1996; Mishra et al., 2006), iriflophenone 2-*O*-α-L-rhamnopyranoside (**6**) (Iinuma et al., 2007), iriflophenone 3-*C*-β-D-glucoside (**7**) (Murakami et al., 1986), and iriflophenone 3,5-*C*-β-D-diglucopyra-noside (**8**) (Iinuma et al., 2007) were identified.

2.2. α-Glucosidase inhibitory activity

The bio-assay of α-glucosidase inhibitor was employed in this investigation to direct the discovery of bioactive compounds. All of the chromatographic fractions, and compounds (**1**–**8**) isolated from leaves of *A. sinensis*, as well as an acidic hydrolysate (**6a**) of **6** were evaluated for their α-glucosidase inhibitory activity. Following preliminary screening, the EtOAc fraction showed very strong inhibitory effects against α-glucosidase, leading to the isolation of compounds **5**–**8**, whereas four new compounds **1**–**4** were obtained from the less potent *n*-BuOH fraction. Compounds **1**–**8** and **6a** were further studied for their concentration dependent activity in order to calculate IC_{50} values (Table 4). Compounds **5** and **6**, the major components in the leaves of *A. sinensis*, showed very strong inhibition with IC_{50} values of 126.5 ± 17.8 and 143.7 ± 10.6 μg/mL, respectively, when compared with that of the positive control acarbose with an IC_{50} value of 372.0 ± 37.8 μg/mL, which was comparable to the reported values (Li et al., 2005). In addition to **5** and **6**, **1**, **3**, **7** and **6a** also exhibited inhibitory effect against α-glucosidase which was about twofold of that of acarbose, while **2**, **4** and **8** showed inhibitory effect almost the same as that of acarbose (chemical structure shown in Fig. 1). Therefore, they may have potential as lead compounds for development of potent α-glucosidase inhibitors. This is the first report of α-glucosidase inhibitory activity of these compounds.

Table 4
Inhibitory effects of the fractions, compounds **1**–**8**, **6a**, and acarbose against α-glucosidase.

Samples	IC_{50} values[a]	
	μg/mL	μM
Total extract	1056.0 ± 28.6	
Petroleum ether fraction	1046.0 ± 42.1	
EtOAc fraction	366.0 ± 45.1	
n-BuOH fraction	990.1 ± 59.1	
H_2O-soluble fraction	993.2 ± 68.2	
1	151.6 ± 22.1	273.7 ± 39.8
2	312.3 ± 22.5	634.7 ± 45.7
3	142.9 ± 13.3	298.9 ± 27.9
4	276.7 ± 56.1	678.1 ± 137.4
5	126.5 ± 17.8	299.7 ± 42.3
6	143.7 ± 10.6	366.7 ± 27.0
7	165.1 ± 11.3	404.7 ± 27.7
8	273.6 ± 14.5	454.4 ± 24.0
6a	138.3 ± 7.3	562.1 ± 29.6
acarbose	372.0 ± 37.8	576.2 ± 58.5

[a] IC_{50}: concentration of inhibitor to inhibit 50% of its activity, which were obtained by interpolation of concentration–inhibition curves. All values were expressed as means ± standard deviation (n = 3).

3. Conclusion

In summary, compounds **5** and **6** are the main constituents of the leaves of *A. sinensis*, compounds **1–4** were new compounds, and compound **7** was isolated from the leaves of *A. sinensis* for the first time, whereas its pharmacokinetic profile had been reported by us previously (Feng et al., 2009). Compounds **1**–**8** and **6a** exhibited inhibitory activity *in vitro* against α-glucosidase, of which **5** was the most potent one. In addition, it is found that the chemical constituents of the leaves differ significantly from the resin-deposited part of the trunk of *A. sinensis* based on our results together with previous reports (Nakanishi et al., 1984; Ishihara et al., 1991; Yagura et al., 2005; Iinuma et al., 2007; Liu et al., 2008; Qi et al., 2009), and accordingly support their different folklore use for some diseases.

4. Experimental

4.1. General experimental procedures

Optical rotations were measured on an Autopol III polarimeter with MeOH as solvent at 22°. IR spectra were taken on a Thermo Nicolet Nexus 470 FT-IR spectrophotometer. UV spectra were obtained on a Varian Cary-300 ultraviolet–visible photometer in MeOH solution. Mass spectra were recorded on a MDS SCIEX API ASTAR spectrometer (for ESIMS) and a Bruker DALTONICS APEX IV Fourier transform ICR high-resolution mass spectrometer (for HRESIMS). 1D and 2D NMR spectra were recorded on a JEOL JNM 300 (300 MHz for ^{1}H NMR and 75 MHz for ^{13}C NMR) using DMSO-d_6 as solvents, with TMS as int. standard. Open column chromatography (CC) was carried out using silica gel (200–300 mesh, Qingdao Marine Chemical Co., Qingdao, PR China), polyamide C-100 (Wako Pure Chemical Industries Ltd., Osaka, Japan) and Sephadex LH-20 (Pharmacia, Fine Chemicals, Inc., Piscataway, NJ, USA) as stationary phase. TLC was conducted on silica gel GF_{254} plates (Merck) and reversed-phase C_{18} silica gel plates (Merck). *p*-Nitrophenyl-α-D-glucopyranoside (PNPG), *p*-nitrophenol (PNP), α-glucosidase, and authentic D-glucose, L-rhamnose, D-glucuronic acid were obtained from Sigma Chemical Co. (St. Louis, Mo, USA). Bovine serum albumin (BSA) and acarbose were ordered from Amersco Inc. (USA) and Bayer Healthcare Company Ltd. (Germany), respectively. All other chemicals were analytical-grade commercial preparations.

4.2. Plant material

Plant material was collected from "The National GAP Base of Chinese Materia Medica for *Aquilaria sinensis* (Lour.) Gilg" at Dianbai County, Guangdong Province of China in July 2005 and identified by Prof. Xiu-Wei Yang as the leaves of *A. sinensi*. A voucher specimen (No. 20050720) has been deposited in the State Key Laboratory of Natural and Biomimetic Drugs, School of Pharmaceutical Sciences, Peking University, China.

4.3. Exaction and isolation

Dried leaves of *A. sinensis* (10 kg) were powdered and extracted with EtOH–H_2O (7:3, v/v) (100 l × 3 times, 3 h for the first time, and 2 h for the 2nd and 3rd times) under conditions of reflux. The combined EtOH–H_2O solubles were evaporated under reduced pressure to afford a extract (2 kg, yield 20%, w/w). This was dissolved in H_2O (6 l) and partitioned successively with petroleum ether (18 l × 5 times), EtOAc (18 l × 5 times) and *n*-BuOH (18 l × 5 times) to yield the corresponding extracts of (150 g, 1.5%, w/w), (80 g, 0.8%, w/w), (165 g, 1.65%, w/w), and the residual H_2O layer of (705 g, 7.05%, w/w). All extracts were screened for inhibitory activity against α-glucosidase according to a slightly modified method described in previous papers (Krakenaite and Glemzha, 1983; Cremonesi et al., 2003; Li et al., 2005). As a result, only the EtOAc extract showed strong activity relative to the reference compound, acarbose.

The active EtOAc extract (70 g) was separated by silica gel CC and eluted with $CHCl_3$–MeOH mixtures of increasing polarity. A total of seven fractions (Fr.1: 1.1 g; Fr.2: 10.1 g; Fr.3: 1.7 g; Fr.4: 3.3 g; Fr.5: 4.5 g; Fr.6: 6.1 g, and Fr.7: 45 g) were collected and combined on the basis of TLC analysis. Fr. 7 (45 g) was fractionated using polyamide CC eluted with H_2O:MeOH gradient mixtures (55:45 → 0:100) to give four fractions (ca. 250 ml each) as fr. 7–1 ~ fr. 7–4. Fr. 7–1 was successively purified by CC on Sephadex LH-20 (MeOH–H_2O = 7:3, v/v) and RP-18 (MeOH–H_2O = 45:55, v/v) to afford compounds **5** (1.0 g), **6** (4.0 g), **7** (50.0 mg), and **8** (20.0 mg), respectively.

The *n*-BuOH extract (155 g) was dissolved in H_2O (465 l) and kept overnight at room temperature (for at least 8 h). The resulting precipitate was collected by filtration, and compound **5** (22.2 g) was obtained from it after repeated recrystallization with mixture of MeOH and $CHCl_3$. The filtrate was subjected to D-101 macroreticular resin CC eluted successively with H_2O and EtOH–H_2O (15:85, 30:70, 40:60, and 95:5, v/v) to give five fractions: B1 (43.5 g), B2 (30.8 g), B3 (30.0 g), B4 (33.0 g), and B5 (5.8 g), respectively. B3 (33.0 g) was fractionated by silica gel CC eluted with $CHCl_3$–MeOH gradient mixtures (9:1 → 6:1) to give five fractions (ca. 250 ml each) as fr. B3–1 – fr. B3–5. Fr. B3–2 was successively purified by CC on Sephadex LH-20 (MeOH:H_2O = 70:30, v/v) and RP-18 (MeOH:H_2O = 45:55, v/v), and preparation TLC ($CHCl_3$:MeOH:H_2O:HCOOH = 8:2:0.1:0.1, v/v/v/v), respectively, to provide compound **1** (20.5 mg). Fr. B3–3 was successively purified by Sephadex LH-20 CC (MeOH:H_2O = 70:30, v/v) and RP-18 (MeOH:H_2O = 45:55, v/v), respectively, to afford compounds **2** (8.3 mg), **3** (20.4 mg), and **4** (10.1 mg).

4.3.1. Aquilarisinin (iriflophenone 2-O-β-D-glucopyranosyl-(1→4)-O-α-L-rhamnopyranoside) (**1**)

Yellowish amorphous powder; $[\alpha]_D^{22}$ − 25.2 (*c* 1.02, MeOH); IR (KBr) ν_{max} 3383, 1725, 1626, 1597, 1509, 1458, 1383, 1322, 1280, 1170, 1073, 1032, 973, 925, and 821 cm^{-1}; UV (MeOH) λ_{max} 224, 290 nm; For ^1H and ^{13}C NMR spectroscopic data, see Table 1; positive ESITOFMS *m/z* 577 $[M+Na]^+$, 555 $[M+H]^+$, 247 $[M-162-146+H]^+$; negative ESITOFMS *m/z* 553 $[M-H]^-$, 245 $[M-162-146-H]^-$; positive HRESIMS *m/z* 555.1711 $[M+H]^+$ (calcd. for $C_{25}H_{31}O_{14}$, 555.1708); 577.1516 (calcd. for $C_{25}H_{30}NaO_{14}$, 577.1528).

4.3.2. Aquilarisin (5-methoxy-6-hydroxy-isoscutellarein 8-O-β-D-glucuronopyranoside) (**2**)

Yellowish amorphous powder; $[\alpha]_D^{22}$ − 62.1 (*c* 0.65, MeOH); IR (KBr) ν_{max} 3381, 2926, 1726, 1656, 1610, 1583, 1509, 1433, 1358, 1254, 1174, 1059, 1021, and 839 cm^{-1}; UV (MeOH) λ_{max} 225, 373, 344 nm; For ^1H and ^{13}C NMR spectroscopic data, see Table 2; positive ESITOFMS *m/z* 515 $[M+Na]^+$, 493 $[M+H]^+$; negative ESITOFMS *m/z* 983 $[2M-H]^-$, 491 $[M-H]^-$; positive HRESIMS *m/z* 493.0964 $[M+H]^+$ (calcd. for $C_{22}H_{21}O_{13}$, 493.0976).

4.3.3. Hypolaetin 5-O-β-D-glucuronopyranoside (**3**)

Yellowish powder; $[\alpha]_D^{22}$ − 53.5 (*c* 0.69, MeOH); IR (KBr) ν_{max} 3385, 1725, 1656, 1606, 1510, 1437, 1357, 1260, 1055, 1073, 1007, 945, 840, 715 cm^{-1}; UV (MeOH) λ_{max} 224, 259, 271, 355 nm; For ^1H and ^{13}C NMR spectroscopic data, see Table 2; positive ESITOFMS *m/z* 501 $[M+Na]^+$, 479 $[M+H]^+$, 325 $[M-176+Na]^+$, 303 $[M-176+H]^+$; negative ESITOFMS *m/z* 955 $[2M-H]^-$, 477 $[M-H]^-$, 301 $[M-176-H]^-$; positive HRESIMS $[M+H]^+$ *m/z* 479.0822 (calcd. for $C_{21}H_{19}O_{13}$, 479.0820).

4.3.4. Aquilarixanthone (2-C-β-D-xylopyranosyl-1,3,4,6,7-pentahydroxyxanthone) (**4**)

Yellowish amorphous powder; $[\alpha]_D^{22}$ − 10.3 (*c* 0.80, MeOH); IR (KBr) ν_{max} 3394–3380, 1647, 1621, 1587, 1478, 1430, 1357, 1291, 1173, 1077, 1036, 878, and 826 cm^{-1}; UV (MeOH) λ_{max} 241, 261, 317, 368 nm; For ^1H and ^{13}C NMR spectroscopic data, see Table 3; positive ESITOFMS *m/z* 855 $[2M+K]^+$, 431 $[M+Na]^+$, 331, 313, 301; positive HRESIMS *m/z* 431.0592 $[M+Na]^+$ (calcd. for $C_{18}H_{16}NaO_{11}$, 431.0590).

4.3.5. Acid hydrolysis of **6**

Compound **6** (200 mg) was hydrolyzed in 3 M HCl/MeOH (40 mL) at 70 °C for 3 h. After being neutralized with 4 M NaOH/H_2O and extracted with EtOAc (400 ml), the EtOAc solubles were evaporated *in vacuo* to give a residue. The latter was further purified by preparative silica gel TLC (CH_2Cl_2–EtOAc = 7:3, v/v) to yield iriflophenone (**6a**) (156 mg). Yellowish amorphous powder; ^1H NMR spectroscopic data (300 MHz, DMSO-d_6) δ 9.80 (4H, br s, OH × 4), 5.81 (2H, s, H-3, H-5), 7.53 (2H, d, *J* = 8.7 Hz, H-2′, H-6′), 6.76 (2H, d, *J* = 8.7 Hz, H-3′, H-5′); ^{13}C NMR spectral data (75 MHz, DMSO-d_6), see Table 1; EIMS *m/z* 246 $[M]^+$.

4.3.6. Acid hydrolysis of **1**, **2** and **3**

Compound **1** (1 mg) was dissolved in 10% H_2SO_4 (1 ml) and heated at 95 °C for 1 h. After cooling to room temperature, the reaction solution was neutralized with satd. $NaHCO_3$ and extracted with EtOAc (2 ml × 3). The combined EtOAc phase was evaporated, and iriflophenone was identified by direct comparison with **6a** on silica gel TLC. The aqueous layer was condensed and subjected to paper chromatography (PC) (*n*-BuOH–H_2O–HOAc, 4:2:1, v/v) together with authentic D-glucose and L-rhamnose. Consequently, the hydrolysates were coincident with authentic samples. Acid hydrolysis of compounds **2** and **3** were performed in the same manner as that of **1**. The aqueous layer was concentrated and subjected to PC (*n*-BuOH–H_2O–HOAc, 4:2:1, v/v) together with authentic D-glucuronic acid, as a result, the hydrolysate was coincident with authentic sample.

4.4. Biological assays

Inhibitory α-glucosidase activities were determined spectrophotometrically in a 96-well microtiter plate (Corning Costar, Cambridge, MA, USA) based on *p*-nitrophenyl-α-D-glucopyranoside (PNPG) as a substrate following the slightly modified method described in the papers (Krakenaite and Glemzha, 1983; Cremonesi et al., 2003; Li et al., 2005). In brief, 20 μl of enzyme solution [0.8 U/ml α-glucosidase in 0.01 M potassium phosphate buffer (pH 6.8) containing 0.2% of BSA] and 120 μl of the test compound or the extract in 0.5% DMSO of 0.01 M potassium phosphate buffer were mixed, and was preincubated at 37 °C prior to initiation of the reaction by adding the substrate. After 15 min of preincubation, PNPG solution (20 μl) [5.0 mM PNPG in 0.1 M potassium phosphate buffer (pH 6.8)] was added and then incubated together at

37 °C. After 15 min of incubation, 0.2 M Na_2CO_3 (80 μl) in 0.1 M potassium phosphate buffer was added to the test tube to stop the reaction. The amount of PNP released was quantified using a UV_{max} Kinetic Microplate Reader (Molecular Dynamics, Inc., Sunnyvale, California, CA, USA) at 405 nm.

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References

Agrawal, P.K., 1989. Carbon-13 NMR of Flavonoids. Elsevier, Amsterdam.
Agrawal, P.K., 1992. NMR spectroscopy in the structural elucidation of oligosaccharides and glycosides. Phytochemistry 31, 3307–3330.
Bilia, A.R., Ciampi, L., Mendez, J., Morelli, I., 1996. Phytochemical investigations of *Licania* genus. Flavonoids from *Licania pyrifolia*. Pharm. Acta Helv. 71, 199–204.
Billeter, M., Meier, B., Sticher, O., 1991. 8-Hydroxyflavonoid glucuronides from *Malva sylvestris*. Phytochemistry 30, 987–990.
Breitmayer, E., Voelter, W., 1989. Carbon-13 NMR Spectroscopy. VCH, Weinheim.
Catalano, S., Luschi, S., Flamini, G., Cioni, P.L., Nieri, E.M., Morelli, I., 1996. A xanthone from *Senecio mikanioides* leaves. Phytochemistry 42, 1605–1607.
Cremonesi, F., Torti, E., Pecile, A., Groppetti, D., Biondi, P., 2003. Evaluation of alpha-glucosidase activity in dog semen and its use in fertility diagnosis. Vet. Res. Commun. 27, 587–589.
Ding, S., Dudley, E., Plummer, S., Tang, J., Newton, R.P., Brenton, A.G., 2008. Fingerprint profile of *Ginkgo biloba* nutritional supplements by LC/ESI–MS/MS. Phytochemistry 69, 1555–1564.
Feng, J., Yang, X.W., Liu, T.H., 2009. RP-LC Quantification and pharmacokinetic study of iriflophenone 2-*O*-α-rhamnopyranoside in rat plasma. Chromatographia 70, 1227–1231.
Gómez-Zaleta, B., Ramírez-Silva, M.T., Gutiérrez, A., González-Vergara, E., Güizado-Rodríguez, M., Rojas-Hernández, A., 2006. UV/vis, ^1H, and ^{13}C NMR spectroscopic studies to determine mangiferin pK_a values. Spectrochim. Acta A 64, 1002–1009.
Hiroyuki, F., Tomohide, Y., Kazunori, O., 2001. Efficacy and safety of Touchi extract, an α-glucosidase inhibitor derived from fermented soybeans, in non-insulin-dependent diabetic mellitus. J. Nutr. Biochem. 12, 351–356.
Iinuma, M., Hara, H., Oyama, M., 2007. Laxative and food containing the same. PCT Int. Appl. WO2007083594, A1 20070726, p. 29.
Ishihara, M., Tsuneya, T., Uneyama, K., 1991. Guaiane sesquiterpenes from agarwood. Phytochemistry 30, 3343–3347.
Kim, K.Y., Nama, K.A., Kurihara, H., Kim, S.M., 2008. Potent α-glucosidase inhibitors purified from the red alga *Grateloupia elliptica*. Phytochemistry 69, 2820–2825.
Krakenaite, R.P., Glemzha, A.A., 1983. Some properties of two forms of alpha-glucosidase from *Saccharomyces cerevisiae*-II. Biokhimiia 48, 62–68.
Lee, S.S., Lin, H.C., Chen, C.K., 2008. Acylated flavonol monorhamnosides, α-glucosidase inhibitors, from *Machilus philippinensis*. Phytochemistry 69, 2347–2353.
Li, T., Zhang, X.D., Song, Y.W., Liu, J.W., 2005. A microplate-based screening method for alpha-glucosidase inhibitors. Chin. J. Clin. Pharmacol. Ther. 10, 1128–1134.
Liu, J., Wu, J., Zhao, Y.X., Deng, Y.Y., Mei, W.L., Dai, H.F., 2008. A new cytotoxic 2-(2-phenylethyl)chromone from Chinese eaglewood. Chin. Chem. Lett. 19, 934–936.
Matsui, T., Ueda, T., Oki, T., Sugita, K., Terhara, N., Matsumoto, K., 2001. α-Glucosidase inhibitory action of natural acylated anthocyanins: 1. Survey of natural pigments with potent inhibitory activity. J. Agric. Food Chem. 49, 1948–1951.
Mishra, B., Indira Priyadarshini, K., Sudheerkumar, M., Unnikrishhnan, M.K., Mohan, H., 2006. Pulse radiolysis studies of mangiferin: a *C*-glycosyl xanthone isolated from *Mangifera indica*. Radiat. Phys. Chem. 75, 70–77.
Murakami, T., Tanaka, N., Wada, H., Saiki, Y., Chen, C.M., 1986. Chemical and chemotaxonomical studies on Filices LXIII. Xanthone derivatives of *Hypodematium fauriei* Tagawa, *H. crenatum* Kuhn and *Gymnocarpium robertianum* Newm. (*G. jessoense* Koidz.). Yakugaku Zasshi 106, 378–382.
Nakanishi, T., Yamagata, E., Yoneda, K., Nagashima, T., Kawasaki, I., Yoshida, T., Mori, H., Miura, I., 1984. Three fragrant sesquiterpenes of agarwood. Phytochemistry 23, 2066–2067.
Ponce, M.A., Scervino, J.M., Erra-Balsells, R., Ocampo, J.A., Godeas, A.M., 2004. Flavonoids from shoots and roots of *Trifolium repens* (white clover) grown in presence or absence of the arbuscular mycorrhizal fungus *Glomus intraradices*. Phytochemistry 65, 1925–1930.
Qi, J., Lu, J.J., Liu, J.H., Yu, B.Y., 2009. Flavonoid and a rare benzophenone glycoside from the leaves of *Aquilaria sinensis*. Chem. Pharm. Bull. 57, 134–137.
Rancon, S., Chaboud, A., Darbour, N., Comte, G., Barron, D., Raynaud, J., Cabalion, P., 1999. A new *C*-glycosyl xanthone isolated from *Davallia solida*. Phytochemistry 52, 1677–1679.
Wansi, J.D., Lallemand, M.C., Chiozem, D.D., Toze, F.A.A., Mbaze, L.M., Naharkhan, S., Iqbal, M.C., Tillequin, F., Wandji, J., Fomum, Z.T., 2007. α-Glucosidase inhibitory constituents from stem bark of *Terminalia superba* (Combretaceae). Phytochemistry 68, 2096–2100.
Yagura, T., Shibayama, N., Ito, M., Kiuchi, F., Honda, G., 2005. Three novel diepoxy tetrahydrochromones from agarwood artificially produced by intentional wounding. Tetrahedron Lett. 46, 4395–4398.
Yang, H., Protiva, P., Cui, B.L., Ma, C.Y., Baggett, S., Hequet, V., Mori, S., Weinstein, I.B., Kennelly, E.J., 2003. New bioactive polyphenols from *Theobroma grandiflorum* (“Cupuacu”). J. Nat. Prod. 66, 1501–1504.
Zhou, M.H., Wang, H.G., Suolangjiba, Kou, J.P., Yu, B.Y., 2008. Antinociceptive and anti-inflammatory activities of *Aquilaria sinensis* (Lour.) Gilg. Leaves extract. J. Ethnopharmacol. 117, 345–350.