Pycnalin, a New α-Glucosidase Inhibitor from *Acer pycnanthum*

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A new compound, pycnalin (1), together with four known compounds, ginnalins A (2), B (3), C (4), and 3,6-di-*O*-galloyl-1,5-anhydro-D-glucitol (3,6-di-GAG) (5), were isolated from *Acer pycnanthum*. The structure of 1 was determined on the basis of 2D-NMR spectral data and synthesis of 1. Pycnalin (1) is the first 1,5-anhydro-D-mannitol linked to a gallic acid, while compounds 2—5 were 1,5-anhydro-D-glucitol linked to gallic acids. All compounds were tested *in vitro* for α-glucosidase inhibitory and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activities. Pycnalin (1) exhibited moderate α-glucosidase inhibitory activity as well as free radical scavenging activity. Ginnalin A (2) and 3,6-di-GAG (5), which have two galloyl groups, exhibited potent α-glucosidase inhibition, compared to those of other compounds 1, 3, and 4 containing a galloyl group. These results suggest that α-glucosidase inhibition is influenced by the number of galloyl groups.

Key words *Acer pycnanthum*; pycnalin; ginnalin; α-glucosidase inhibition; 1,1-diphenyl-2-picrylhydrazyl radical scavenging activity

Postprandial hyperglycemic excursion has been implicated as an independent risk factor for cardiovascular disease, not only among diabetes patients, but also among other pre-diabetic subjects in the general population with pre-diabetic states.[1,2] Therefore, sustained postprandial hyperglycemia (PPHG) also results in postprandial oxidative stress (PPOS). PPOS is characterized by an increased susceptibility of the organism toward oxidative damage after consumption of a meal rich in lipids and/or carbohydrates, and is associated with a higher risk for atherosclerosis, diabetes and obesity.[3] Hence, combinations of agents that reduce PPHG and PPOS are becoming therapeutics of interest to combat diabetes and its related complications. α-Glucosidase inhibitors are used in the management of non-insulin-dependent diabetes mellitus, acting by reversible inhibition of the gastrointestinal sucrases, glucoamylase, dextrinase, maltase, and isomaltase. These enzymes normally catalyze the conversion of dietary starch and sucrose into absorbable monosaccharides; therefore, enzyme inhibition delays and reduces the peak of postprandial blood glucose.[4] In a recent study, it was reported that oral administration of the *Acer* extract to mice caused their blood glucose level to be lower than control at 30 min.[5,6] Additionally, it was suggested that the effect is also involved in α-glucosidase inhibitory activity, and ginnalins B (**3**) and C (**4**) from *A. pycnanthum* were found to be a portion of the active compounds.[7] In this study, we report the isolation and structure determination of a new compound, pycnalin (**1**), and investigated of its α-glucosidase inhibitory and 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activities.

Results and Discussion

Isolation and Structure Elucidation The leaves of *A. pycnanthum* were extracted with hot water. The hot water extract was partitioned among EtOAc, BuOH, and H_2O and ginnalin A (**2**, 4.4%) was obtained from the EtOAc-insoluble portion. The BuOH-soluble portion was separated by octadecylsilyl (ODS) column chromatography [MeOH–H_2O (5 : 95 →100 : 0)] and ODS HPLC [MeOH–H_2O (10 : 90→14 : 86)] to give pycnalin (**1**, 0.008%), together with ginnalins B (**3**, 0.1%), C (**4**, 0.033%), and 3,6-di-*O*-galloyl-1,5-anhydro-D-glucitol (3,6-di-GAG) (**5**, 0.012%).

The molecular formula of pycnalin (**1**), $C_{13}H_{16}O_9$, was established by high resolution-electrospray ionization-mass spectra (HR-ESI-MS) [*m/z* 339.0949 $(M+Na)^+$, Δ+0.7 mmu]. IR spectrum implied the presence of hydroxyl (3423 cm^{-1}) and ester carbonyl (1699, 1237 cm^{-1}) groups. The gross structure of **1** was deduced from detailed analyses of the ^{1}H- and ^{13}C-NMR data (Table 1) aided by 2D-NMR (^{1}H–^{1}H correlation spectroscopy (COSY), ^{1}H-detected heteronuclear multiple quantum coherence (HMQC), and heteronuclear multiple bond connectivity (HMBC)). The ^{1}H-NMR spectral data showed nine proton signals. A singlet at δ_H 7.13, integrating for two protons, was attributed to the two aromatic protons of a galloyl group. Eight proton signals at δ_H 3.40—4.52 suggested the presence of a structure similar to that of a sugar moiety. The ^{13}C-NMR and distortionless enhancement by polarization transfer (DEPT) spectra of **1** indicated the presence of two oxymethylene carbons, four oxymethine carbons, two aromatic methine carbons, three

Table 1. ^{1}H- and ^{13}C-NMR Spectral Data of Pycnalin (**1**)

Position	^{13}C, δ_C	^{1}H, δ (mult, *J*, Hz)
1_{ax}	71.2, CH_2	3.57 (1H, dd, 12.4, 1.3)
1_{eq}		3.88 (1H, dd, 12.4, 2.0)
2	70.6, CH	3.84 (1H, m)
3	75.8, CH	3.54 (1H, dd, 9.1, 3.5)
4	69.3, CH	3.70 (1H, t, 9.1)
5	80.0, CH	3.40 (1H, m)
6	65.5, CH_2	4.52 (1H, dd, 11.8, 2.0)
		4.32 (1H, dd, 11.8, 6.1)
1′	122.2, qC	—
2′, 6′	110.3, CH	7.13 (2H, s)
3′, 5′	146.4, qC	—
4′	139.3, qC	—
7′	167.4, qC	—

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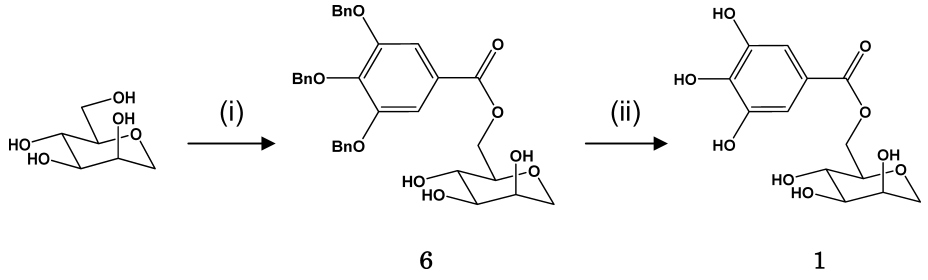

(i) 3,4,5-Tribenzylgallic acid, EDCI·HCl, DMAP, Pyr, rt, 1 week, 54%; (ii) 10% Pd–C, EtOAc–EtOH (1 : 3), rt, 30 min, 93%.

Chart 1

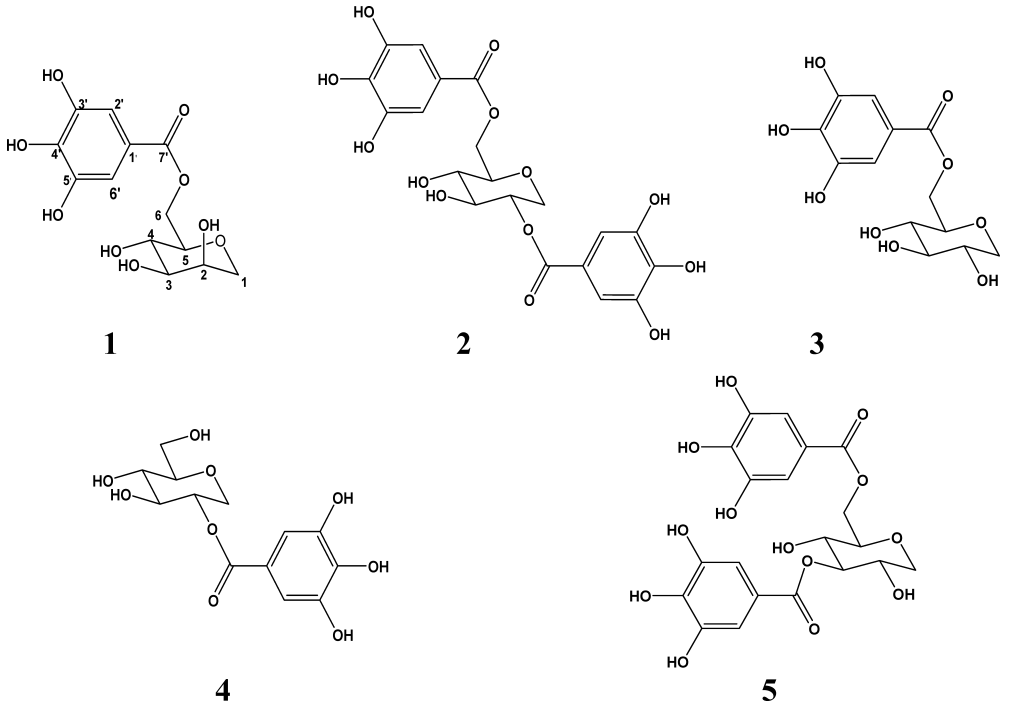

Fig. 1. Structures of Compounds **1**—**5** from *A. pycnanthum*

phenolic carbons, and two quaternary carbons including an ester carbonyl carbon at δ_C 167.4. The ^{1}H–^{1}H COSY connectivities of C-1 to C-6 indicated the presence of a 1-deoxysugar moiety in **1**. The stereochemistry of the 1-deoxysugar moiety was assigned by ^{1}H–^{1}H coupling constants and nuclear Overhauser effect spectroscopy (NOESY) correlations. The ^{1}H–^{1}H coupling constants ($J_{1ax,2}$=1.3 Hz, $J_{1eq,2}$=2.0 Hz, and $J_{2,3}$=3.5 Hz) revealed that the hydroxyl group at C-2 is in the β-orientation. The ^{1}H–^{1}H coupling constants ($J_{3,4}$=9.1 Hz and $J_{4,5}$=9.1 Hz) and NOESY correlations of H-1ax to H-3 and H-5 suggested the presence of 1,5-anhydro-mannitol in **1**. The HMBC correlation between H-6 and the ester carbonyl carbon at δ_C 167.4 in a galloyl group identified the structure of **1** to be 6-*O*-galloyl-1,5-anhydro-mannitol. Pycnalin (**1**) is the first 1,5-anhydro-mannitol linked to a galloyl group.

Additionally, four known compounds **2**—**5** were identified to be ginnalins A (**2**),[8] B (**3**),[9] and (**4**),[9] and 3,6-di-GAG (**5**)[8] by comparison of their physicochemical and spectroscopic data with the literature.

Synthesis of Pycnalin (1) The absolute stereochemistry of pycnalin (**1**) was achieved through its synthesis (Chart 1). First, 1,5-anhydro-D-mannitol was treated with *N*,*N*-dimethyl-4-aminopyridine (DMAP), EDCI·HCl, and 3,4,5-tribenzylgallic acid in dry pyridine at room temperature under N_2 for 1 week to afford 6-*O*-(3′,4′,5′-tribenzylgalloyl)-1,5-anhydro-D-mannitol (**6**) in 54% yield.[10,11] Then palladium-activated carbon was added to a solution of **6** in EtOAc–EtOH (1 : 3) at room temperature under H_2 for 30 min to give **1** in 93% yield.[10] This product corresponded to the natural product and was confirmed by $[\alpha]_D$, ^{1}H- and ^{13}C-NMR spectral data, which matched those of natural **1**.

***α*-Glucosidase Inhibitory Activity of 1—5** Recently, the α-glucosidase inhibitory activity of ginnalins B (**3**) and C (**4**) from *A. pycnanthum* has been reported in our group.[7] In this α-glucosidase inhibitory activity, we investigated the structure–activity relationship of pycnalin (**1**), as well as ginnalins A (**2**), B (**3**), and C (**4**), and 3,6-di-GAG (**5**). Compounds **1**—**5** were found to be inhibitors of α-glucosidase, showing promising inhibitory activities in a dose-dependent manner. Ginnalin A (**2**) and 3,6-di-GAG (**5**) showed similar potency as the clinically used standard drug acarbose that is widely prescribed to type II diabetic patients (Fig. 2). The IC_{50} values of pycnalin (**1**), ginnalins A (**2**), B (**3**), C (**4**), 3,6-di-GAG (**5**), and acarbose were 395.6, 45.1, 784.2, 257.6, 25.6, and 6.7 μM, respectively.

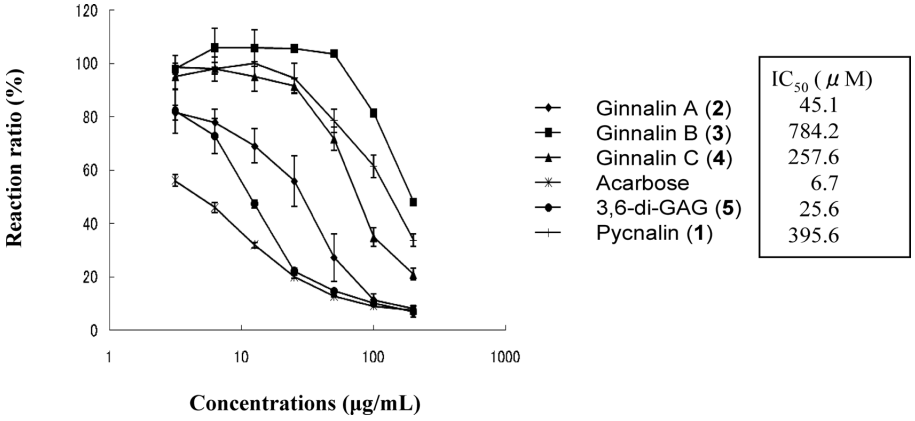

Fig. 2. α-Glucosidase Inhibitory Activity of Compounds **1**—**5**

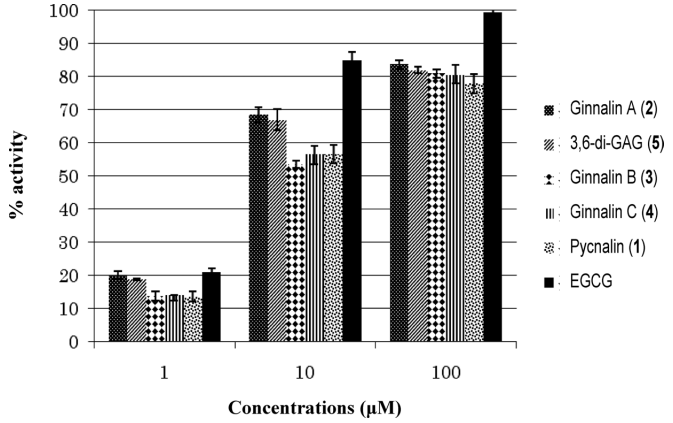

Fig. 3. DPPH Free Radical Scavenging Activity of Compounds **1**—**5**

These results suggest that α-glucosidase inhibition is influenced by the number of galloyl groups.

DPPH Scavenging Activity of 1—5 Although the antioxidative capacity of ginnalin A (**2**) has already known,[12] differences in the number of bound molecules or binding position of the galloyl group have not been examined. Figure 3 showed the concentration-dependent DPPH free radical scavenging activities of pycnalin (**1**), ginnalins A (**2**), B (**3**), C (**4**), and 3,6-di-GAG (**5**). Ginnalin A (**2**) and 3,6-di-GAG (**5**), containing two galloyl groups, at 1 and 10 μM, exhibited better activities than the other compounds **1**, **3**, and **4**, containing one gallyol group, while there were no differences in all compounds at 100 μM.

These results may suggest that the antioxidation is affected by the number of galloyl groups at low concentrations, but is hardly affected at high concentrations.

In conclusion, postprandial hyperglycemia (PPHG) is an exaggerated rise in blood sugar following a meal that is rich in carbohydrates and/or lipids. A rich meal also causes increased susceptibility of the organism toward oxidative damage, known as postprandial oxidative stress (PPOS). PPOS is characterized by the overt generation of free radicals and increased susceptibility of the organism toward oxidative damage. Furthermore, PPOS is associated with a higher risk for atherosclerosis, diabetes, and obesity. Therefore, a combination of agents that reduce PPHG and PPOS may become therapeutics of interest for these multiple disorders.[13] Hence, pycnalin (**1**) and the other compounds **2**—**5** that possess α-glucosidase inhibitory activity rapidly inhibit the increase of blood glucose and also exhibit antioxidative activity, and thus have a great potential in food and medicine.

Experimental

General Experimental Procedure Optical rotation was recorded on a Jasco DIP-370. UV spectrum was recorded on a HITACHI U-2000A spectrometer. IR spectrum was recorded on a JASCO FT/IR-300 spectrometer. ^{1}H- and ^{13}C-NMR spectra were measured and recorded on a Bruker Avance 500 spectrometer in acetone-d_6 and CD_3OD. The resonances of acetone-d_6 and CD_3OD at δ_H 2.00, δ_C 30.3, and δ_H 3.35, δ_C 49.8 were used as internal references for the NMR spectra. HR-ESI-MS was recorded on a Waters Xevo Q-Tof mass spectrometer.

Plant Material Leaves of *A. pycnanthum* were collected from Komaki, Aichi, Japan in August, 2008. A voucher specimen has been deposited at the Graduate School of Marine Science and Technology, Tokyo University of Marine Science and Technology, Tokyo, Japan. The leaves were expeditiously dried at 80 °C and were then crushed.

Extraction and Isolation Procedures The dried and crushed leaves (52.4 g) of *A. pycnanthum* were extracted with a 200-fold volume of hot water for 1 h and left at room temperature for 1 d. After filtration, the extract was evaporated in a rotary evaporator. The residue was partitioned among EtOAc (200 ml×3), BuOH (200 ml×3), and H_2O (200 ml). Ginnalin A (**2**, 2.33 g) was obtained from the EtOAc-insoluble portion. The BuOH-soluble portion (320 mg) was chromatographed on Cosmosil 75C_{18}-OPN (ϕ 2.2×35 cm, Nacalai Tesque, Inc., Japan) with MeOH–H_2O (5 : 95→100 : 0) yielding eight fractions 1—8. Fraction-3 (104 mg) eluted with MeOH–H_2O (5 : 95), was purified by ODS HPLC [Develosil ODS HG-5 (ϕ 1.0×25 cm, Nomura Chemical Co., Ltd., Japan), flow rate 1.6 ml/min; MeOH–H_2O (10 : 90→14 : 86); detection UV (217, 275 nm)] to give pycnalin (**1**, 4.2 mg, t_R 37 min), ginnalin B (**3**, 52.4 mg, t_R 48 min), and ginnalin C (**4**, 17.2 mg, t_R 52 min), respectively. Fraction-4 (27.2 mg) eluted with MeOH–H_2O (15 : 85) was purified by ODS HPLC [Develosil ODS HG-5, flow rate 1.5 ml/min; MeCN : 0.1% AcOH in H_2O (14 : 86); detection UV (217, 275 nm)] to give 3,6-di-GAG (**5**, 6.3 mg, t_R 30 min).

Pycnalin (1) $[\alpha]_D^{24}$ +21.0° (c=1.00, MeOH); IR (KBr) ν_{max} 3423, 1699, 1237 cm^{-1}; UV λ_{max} (MeOH) nm (ε) 217 (14020), 276 (6480); ^{1}H-NMR (acetone-d_6, 500 MHz) and ^{13}C-NMR (acetone-d_6, 125 MHz) data (Table 1). ESI-MS (positive ion) m/z: 339 $(M+Na)^+$, HR-ESI-MS m/z: 339.0949 (Calcd for $C_{13}H_{16}O_9Na$: 339.0942).

Synthesis of Pycnalin (1) The 1,5-anhydro-D-mannitol (10.0 mg, 0.061 mmol) was treated with DMAP (1.0 mg, 0.072 mmol), EDCI·HCl (18.0 mg, 0.091 mmol), and 3,4,5-tribenzylgallic acid (40.0 mg, 0.091 mmol) in dry pyridine (3.5 ml) at room temperature under N_2 for 1 week. The reaction mixture was partitioned with EtOAc (10 ml) and H_2O (10 ml). The organic layer was dried with $MgSO_4$, and evaporated. The residue was purified by silica gel, column chromatography ($CHCl_3$: MeOH=14 : 1) to afford 6-*O*-(3′,4′,5′-tribenzylgalloyl)-1,5-anhydro-D-mannitol (**6**, 19.4 mg) in 54% yield; ^{1}H-NMR (CD_3OD, 500 MHz) δ_H 7.22—7.43 (15H, m), 7.38 (2H, s), 5.09—5.14 (6H, m), 4.74 (1H, d, J=2.1 Hz), 4.47 (1H, d, J=8.3 Hz), 4.06 (1H, d, J=12.3 Hz), 3.98 (1H, m), 3.66 (1H, m), 3.63 (1H, m), 3.58 (1H, d, J=12.3 Hz), 3.40 (1H, m); ESI-MS (positive ion) m/z 609 $(M+Na)^+$.

Palladium-activated carbon (2.5 mg) was added to a solution of **6**

(15.8 mg, 0.028 mmol) in EtOAc–EtOH (1 : 3, 4 ml) at room temperature under H_2 for 30 min. The reaction mixture was filtered through celite and the filtrate was evaporated to give pycnalin (**1**, 8.2 mg) in 93% yield.

Assay for the Inhibitory Activity against α-Glucosidase Intestinal α-glucosidase inhibitory activity was determined as described in the literature with some modification.[14] Fasted ddY male mice were sacrificed and the small intestines were immediately removed. The contents of the small intestines which included brush border enzymes were squeezed out and used to prepare samples at 25 mg/ml. Ice-cold phosphate buffer (pH 6.9) containing 0.2% bovine serum albumin was used as a solvent. The samples were centrifuged at 4500×***g*** for 5 min at 4 °C. The supernatant was used as a crude enzyme solution in the experiment.

The activity of α-glucosidase was assayed with 27.7 mM maltose or 55.3 mM sucrose (final concentrations) with or without *A. pycnanthum* at different concentrations. Acarbose was used as a positive control. After the samples and the substrate were pre-incubated for 5 min at 36.5 °C, the enzyme solution was added. The samples were then incubated at the same temperature for 40 min for sucrose. The reaction was terminated by heating with boiling water for 5 min, and the quantity of glucose produced was measured with a commercial GOD-POD kit (Glucose CII-test, WAKO Pure Chem, Osaka, Japan).[7]

DPPH Free Radical Scavenging Activity In a 96-well micro plate, a 10 μM of test sample was dissolved in EtOH (1, 10, 100 μM) and 190 μl of a mixed solution, and a 3 : 1 : 4 solution of MilliQ: 400 mM MES buffer: 0.4 mM DPPH solution dissolved in EtOH were added. The reaction solution was measured at a wavelength 490 nm using a spectrophotometer. The percentage of DPPH scavenging was calculated as $(1-B/A)\times100$, where A represents the absorbance of the control without the test samples and B represents the absorbance in the presence of test samples.[15]

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