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New dammarane-type glucosides as potential activators of AMP-activated protein kinase (AMPK) from *Gynostemma pentaphyllum*

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ABSTRACT

AMP-activated protein kinase (AMPK) is a key sensor and regulator of glucose, lipid, and energy metabolism throughout the body. Activation of AMPK improves metabolic abnormalities associated with metabolic diseases including obesity and type-2 diabetes. The oriental traditional medicinal herbal plant, *Gynostemma pentaphyllum*, has shown a wide range of beneficial effects on glucose and lipid metabolism. In this study, we found that *G. pentaphyllum* contains two novel dammarane-type saponins designated as damulin A (**1**), 2α,3β,12β–trihydroxydammar-20(22)-*E*,24-diene-3-*O*-[β-D-glucopyranosyl-(1→2)-β-D-glucopyranoside], and damulin B (**2**), 2α,3β,12β-trihydroxydammar-20,24-diene-3-*O*-[β-D-glucopyranosyl-(1→2)-β-D-glucopyranoside], that strongly activate AMPK in cultured L6 myotube cells. Damulins A and B also increased β-oxidation and glucose uptake with increasing GluT4 translocation to the plasma membrane in L6 myotube cells. Taken together our results indicate that activation of AMPK by damulins A and B may contribute to beneficial effect of *G. pentaphyllum* on glucose and lipid metabolism.

1. Introduction

AMP-activated protein kinase (AMPK) is an evolutionally conserved, intracellular energy sensor that increases ATP production and decreases energy consumption by responding to cellular stress that increases the intracellular AMP:ATP ratio.[1] Numerous studies have revealed that AMPK activation improves obesity, type-2 diabetes, and dyslipidemia by stimulating β-oxidation and glucose uptake in skeletal muscle tissues and inhibiting fat and cholesterol synthesis in the liver.[2–4]

AMPK is a serine/threonine kinase and is a heterotrimeric enzyme consisting of catalytic α and regulatory β and γ subunits.[4,5] AMPK activation occurs mainly via phosphorylation of Thr^{172} in the AMPK α subunit by the upstream kinase (LKB1).[6,7] AMP was originally reported to promote phosphorylation of Thr^{172} in AMPK α by LKB1. When ATP consumption increases the AMP concentration during intensive exercise or prolonged starvation, AMP is coupled to the γ subunit of AMPK and makes AMPK a better substrate for LKB1.[6,8] Conversely, inactivation of AMPK occurs when AMPK phosphatases, protein phosphatase 2Cα (PP2Cα), dephosphorylate Thr^{172}.[9,10] Moreover, AMP and ADP binding to the regulatory γ subunit were shown to protect AMPK from dephosphorylation catalyzed by AMPK phosphatases and to promote Thr^{172} phosphorylation by LKB1.[5,11,12]

Once activated, AMPK phosphorylates downstream substrates to switch off ATP-consuming pathways, including fatty acid and cholesterol synthesis, and to switch on ATP-generating pathways, including fatty acid oxidation and glycolysis.[4,13] Furthermore, activation of AMPK decreases blood glucose levels by stimulating translocation of glucose transporter 4 (GluT4) to the cell membrane as well as upregulating GluT4 expression independent of insulin signaling.[14,15] These beneficial effects of AMPK activation during glucose and lipid metabolism highlight AMPK as an emerging drug target for metabolic syndromes. Therefore, AMPK activators are considered promising candidates for the discovery of anti-obesity and anti-diabetes agents as well as drugs for treatment of other metabolic diseases.

Gynostemma pentaphyllum (Gp) has been widely used in Asian countries including Korea, China, Japan, and Vietnam as a traditional herbal medicine or tea. Total extract or total saponins from Gp have shown a wide range of beneficial effects such as cholesterol-lowering, hypoglycemic, and antitumor effects,[17–19] which likely overlap with the diverse downstream effects of AMPK activation.[3,16,20] We therefore investigated whether these beneficial effects of Gp-extract are due to AMPK activation. A phytochemical study on this Gp-extract was found that the activation of Gp-extract on AMPK is due to two new dammarane-type glycosides named damulins A and B.

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2. Results and discussion

Repeated column chromatography (HP-20, silica gel, RP-18, and semi-preparative HPLC) of the 50% EtOH-soluble extract of the leaves of *G. pentaphyllum* resulted in the isolation of two new dammarane-type glycosides named damulins A and B, respectively.

Compound **1** was obtained as white amorphous powder. Its HRFABMS showed a quasi-molecular ion peak at *m*/*z* 805.4717 $[M+Na]^+$, establishing a molecular formula of $C_{42}H_{70}O_{13}$. The IR spectrum suggested the presence of OH functional groups at 3400 cm^{-1}, olefinic C=C double bonds at 1658 to 1631 cm^{-1}, and C–O stretching vibrations at 1077–1045 cm^{-1}. The C–H bending vibration by *gem*-dimethyl groups was determined by the band at 1381 cm^{-1}. The ^{13}C NMR spectrum gave 42 signals, of which 12 were assigned to the sugar units and 30 to a triterpene moiety with typical resonances of two olefinic carbons (δ_C 124.7 and 124.4) and two quaternary carbons (δ_C 140.5 and 132.2). The ^1H NMR spectrum of compound **1** showed eight singlet peaks that were assigned to the aglycon tertiary-methyl groups at δ_H 0.92–1.67, three of which were diagnostic for methyls linked to an sp^2 carbon (δ_H 1.61, 1.63, and 1.67). Two signals for trisubstituted olefinic protons were also displayed at δ_H 5.08 (1H, br, *t*-like, *J* = 7.5 Hz) and δ_H 5.26 (1H, br, *t*-like, *J* = 7.0 Hz). Two oxymethines at δ_H 3.63 and 3.75 (each 1H, m), one axial oxygenated methine proton at δ_H 3.02 (1H, d, *J* = 9.3 Hz), and two anomeric protons at δ_H 4.45 (1H, d, *J* = 7.5 Hz) and 4.75 (1H, d, *J* = 7.5 Hz) assigned to the two sugar moieties were also determined from its ^1H NMR spectrum.

The comparison of the ^1H and ^{13}C NMR spectra of compound **1** with those of ginsenoside Rh_3[22] and ginsenoside F_4[23,24] suggested

Table 1
^1H (500 MHz) and ^{13}C (125 MHz) NMR Data for damulins A (**1**) and B (**2**) in CD_3OD

Position	Damulin A (**1**)		Damulin B (**2**)	
	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)
Aglycone				
1	48.1	0.92, m	48.1	0.94, m
		2.10, dd, 5.5, 12.5		2.11, m
2	68.3	3.75, m	68.3	3.75, m
3	96.8	3.02, d, 9.5	96.8	3.01, d, 9.5
4	42.0		42.0	
5	57.4	0.89, m	57.4	0.88, m
6	19.4	1.54, m 1.59, m	19.4	1.53, m 1.59, m
7	36.1	1.35, m 1.58, m	36.1	1.35, m
				1.58, m
8	41.3		41.3	
9	51.9	1.52, m	52.0	1.52, m
10	39.1		39.1	
11	32.7	1.28, m 1.82, m	33.1	1.28, m
				1.81, m
12	74.1	3.63, m	73.9	3.59, m
13	51.5	1.80, m	53.0	1.85, m
14	52.1		52.3	
15	33.5	1.11, m 1.69, m	33.3	1.11, m
				1.70, m
16	29.6	1.44, m 1.89, m	31.8	1.42, m
				2.00, m
17	51.4	2.59, m	49.6	2.58, m
18	16.3	1.05, s	16.3	1.06, s
19	18.1	0.99, s	18.1	0.99, s
20	140.5		156.3	
21	13.1	1.63, s	108.7	4.69, br, s 4.88, br, s
22	124.7	5.26, t, 7.0	35.1	2.02, m
				2.15, m
23	28.1	1.68, m	27.8	2.22, m
		2.67, m		2.14, m
24	124.4	5.08, t, 7.5	125.9	5.17, m
25	132.2		132.2	
26	26.0	1.67, s	26.0	1.69, s
27	17.9	1.61, s	17.9	1.63, s
28	17.3	0.93, s	17.3	0.93, s
29	28.8	1.14, s	28.8	1.13, s
30	18.1	0.92, s	18.0	0.91, s
Sugar moieties				
Glu′				
1′	105.0	4.45, d, 7.5	105.0	4.45, d, 8.0
2′	80.7	3.69, m	80.7	3.69, m
3′	78.8	3.59, m	78.8	3.60, m
4′	71.4	3.37, m	71.3	3.36, m
5′	78.2	3.20, m	78.2	3.22, m
6′	62.5	3.66, m 3.87, br, d, 12.5	62.5	3.62, m 3.84, br, d, 12.0
Glu″				
1″	104.5	4.75, d, 7.5	104.5	4.75, d, 8.0
2″	76.3	3.24, m	76.3	3.24, m
3″	78.4	3.25, m	78.4	3.26, m
4″	72.2	3.19, m	72.2	3.21, m
5″	78.1	3.36, m	78.0	3.36, m
6″	63.4	3.62, m 3.84, br, d, 10.5	63.3	3.66, m 3.87, dd, 2.5, 12.0

that its aglycon moiety is a similar with except for the absence of a hydroxy functional group at C-2. The comparison between compound **1** and the known compounds, gypenoside LXXVII[21] and gypenosides XLVI,[26] also showed that all three compounds have an identical skeleton with 2α,3β,12β-trihydroxy substitutions. The 2α,3β-dihydroxy substitutions of compound **1** were also confirmed from the carbon chemical shifts of both positions at δ_C 68.3 (C-2) and δ_C 96.8 (C-3), and from the *J* value of the proton, which was ascribable to H-3 at δ_H 3.02 (1H, d, J = 9.3 Hz). When the ^{13}C NMR data for compound **1** (Table 1) were compared with ginsenoside Rg_8,[27] ginsenoside F_4,[23] and ginsenoside Rh_5,[28] the stereochemistry of the double bond at C-20(22) of compound **1** was determined to be the *E*–configuration because the chemical shift at C-21 of compound **1** was observed at δ_C 13.1 ppm.[25,28,29]

From its hydrolysis and ^1H and ^{13}C NMR spectra data, compound **1** was suggested to contain two units of D-glucose. The chemical shifts, the multiplicity of the signals, the values of the coupling constants in the ^1H, and ^{13}C NMR data (See Table 1) indicated the presence of two β-configurations at the anomeric positions for the glucosyl units in compound **1**. One β-configuration for the first glucosyl unit with [δ_H 4.45 (1H, d, J = 7.5 Hz, H-1′); δ_C 105.0 (C-1′)], and the other for the second glucosyl unit [δ_H 4.75 (1H, d, J = 7.5 Hz, H–1″); δ_C 104.5 (C-1″)] were displayed. All ^1H and ^{13}C NMR signals of the two sugar moieties were also assigned using two-dimensional NMR spectra including ^1H–1H COSY, HMQC, and ^1H–13C HMBC correlations (see also Supplementary data and Fig. 1). The linkage sites and the sequences of the two saccharides and the aglycone were confirmed from an HMBC experiment. HMBC correlations were observed from H-1′ (δ_H 4.45) of the first glucose to C-3 (δ_C 96.8) of aglycon and from H-3 (δ_H 3.02) of aglycon to C-1′ (δ_C 105.0). Other correlations from H-1″ (δ_H 4.75) of the second glucose to C-2′ (δ_C 80.7) and from H-2′ (δ_H 3.69) to C-1″ (δ_C 104.5) suggested the connectivity of the sugar moiety (Fig. 1). Thus, the structure of compound **1** was established to be 2α,3β,12β–trihydroxydammar-20(22)-*E*,24-diene-3-*O*-[β-D-glucopyranosyl-(1→2)-β-D-glucopyranoside], and named damulin A.

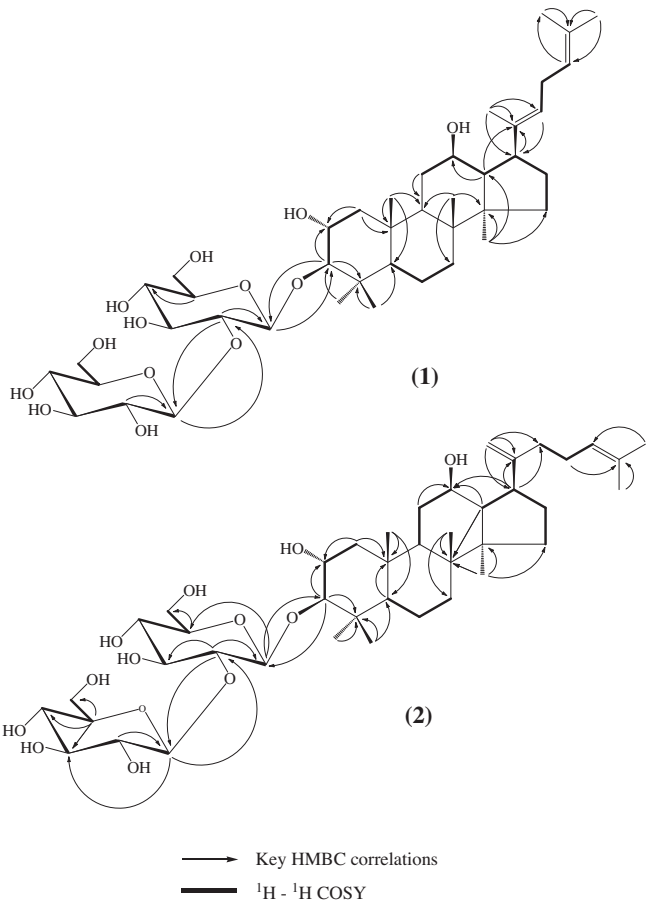
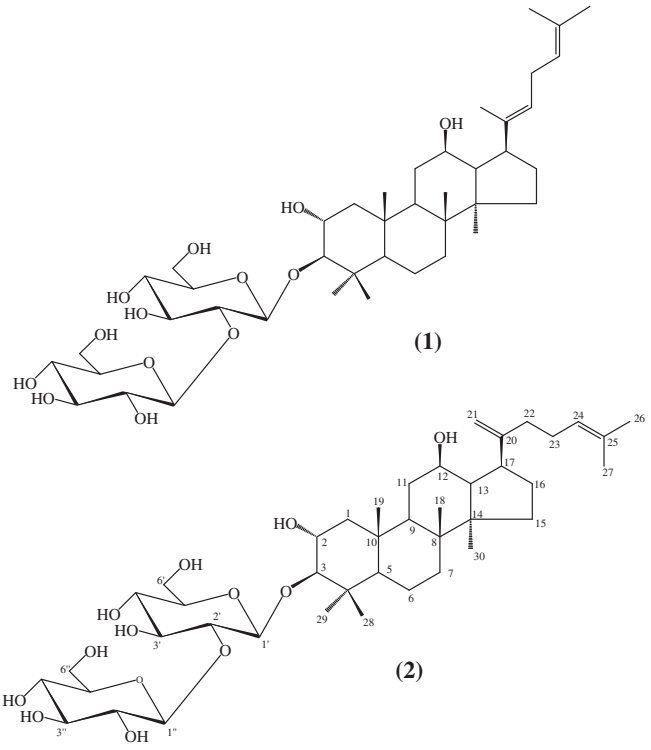


Figure 1. ^1H–^1H COSY and HMBC correlations of damulins A (**1**) and B (**2**).



Compound **2** was also purified as amorphous powder. The molecular formula of compound **2** was determined as $C_{42}H_{70}O_{13}$ from ^{13}C NMR and the quasi-molecular ion peak at *m*/*z* 783.4905 in a positive-mode HRESIMS ($[M+H]^+$; calcd. 783.4895). Comparison of the ^1H and ^{13}C NMR spectra of compounds **1** with **2** indicated that they had a similar aglycon moiety. However, compound **2** was a disappearance of a tertiary CH_3 group at C-20 and the double bond of the olefinic methine group at C-22 in the aglycon of compound **1**. The methyl proton signal at δ_H 1.63 (3H, s) in compound **1** was replaced by two broad singlet peaks at δ_H 4.69 and 4.88 (each 1H, br, s) in compound **2**, and one typical signal of the olefinic proton at δ_H 5.26 (1H, br, *t*-like, J = 7.0 Hz) in the ^1H NMR spectrum of compound **1** was disappeared. These results were also supported by the ^{13}C NMR spectrum of compound **2**. The olefinic carbon signal at δ_C 124.7 (C-22) and the methyl carbon signal at δ_C 13.1 (C-21) in compound **1** were replaced by a methylene peak at δ_C 35.1 and a terminal methylene peak at δ_C 108.7 in compound **2**, respectively.[30] In addition, the quaternary carbon at δ_C 140.5 (C-20) in the ^{13}C NMR spectrum of compound **1** was also shifted to downfield (δ_H 156.3) in compound **2**. ^1H and ^{13}C NMR data (Table 1), as well as the two-dimensional NMR experiments including ^1H–1H COSY, HMQC, and ^1H–13C HMBC spectroscopic data (see also Supplementary data and Fig. 1), supported that compound **2** (Table 1) showed two glucosyl units with β-configuration as in compound **1**. The linkage sites and the sequences of the two saccharides and the aglycon were also determined using HMBC experiments (Fig. 1). HMBC correlations from the anomeric proton

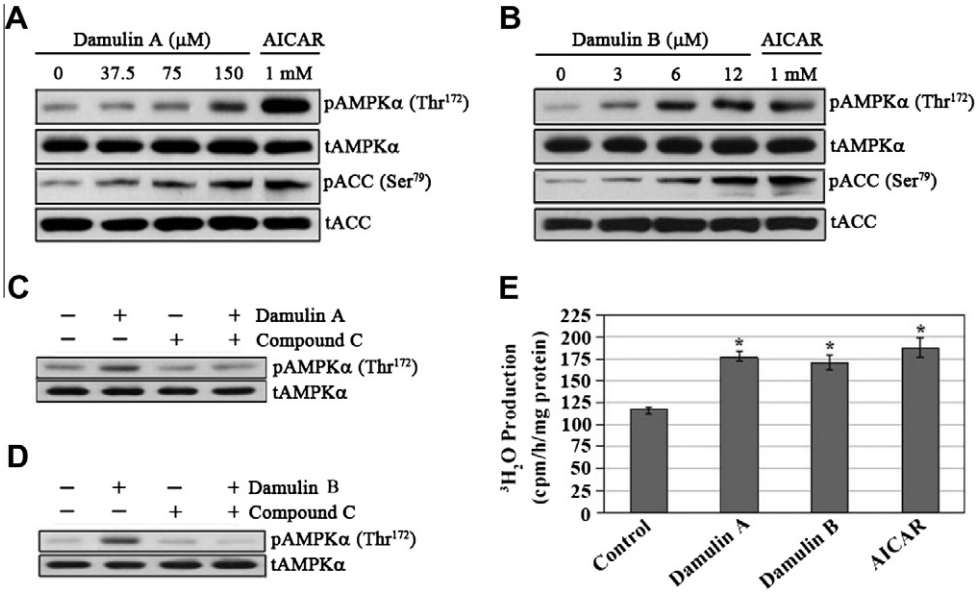


Figure 2. Effect of damulins A (**1**) and B (**2**) on AMPK activation and fatty acid oxidation in L6 myotube cells. L6 myotube cells were exposed to different doses of damulins A and B for 2 h or AICAR (1 mM) for 1 h, and phosphorylation of AMPK was analyzed with Western blotting. Damulins A (**A**) and B (**B**) increased phosphorylation of AMPK and ACC in a dose-dependent manner. Increased phosphorylation of AMPK induced by 150 μM damulin A (**C**) and 12 μM damulin B (**D**) was abrogated by pretreatment with compound C (10 μM) for 10 min. (**E**) Increased β-oxidation in cells by damulins A (150 μM) and B (12 μM). DMSO was used as a vehicle (control). AICAR (1 mM) was pretreated for 1 h. $^{*}P < 0.05$ compared with control.

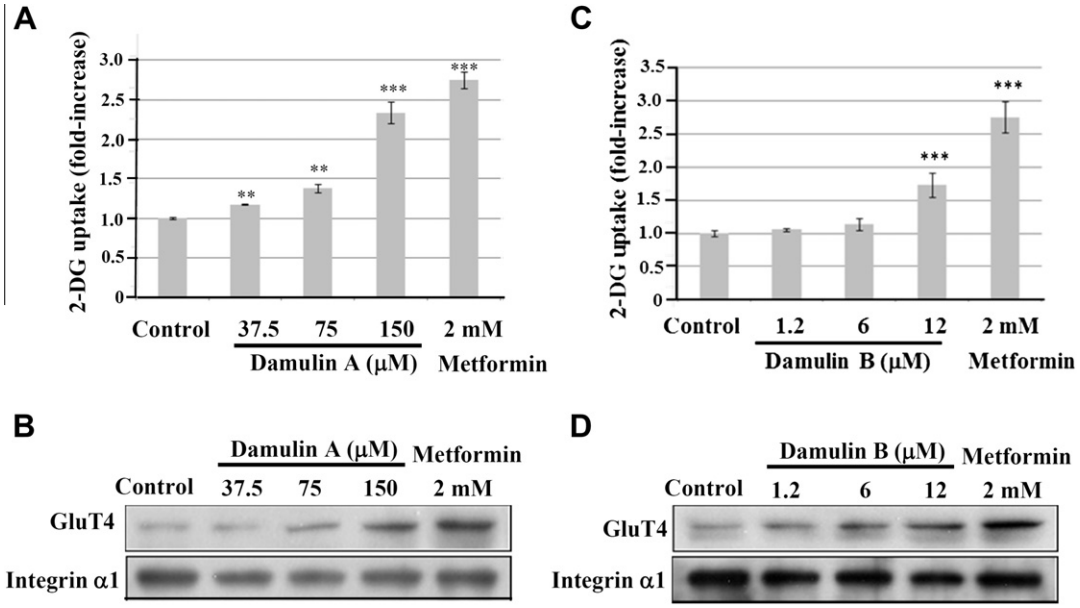


Figure 3. Dose-dependent increment of 2-DG uptake and GluT4 translocation to the cytoplasmic membrane in L6 myotube cells by damulins A and B. 2-DG uptake was increased by damulins A (**A**) and B (**C**). Stimulation of GluT4 translocation to the cytoplasmic membrane by damulins A (**B**) and B (**D**). Damulins A and B were treated to cells for 2 h and metformin was treated for 1 h. As a control DMSO was treated to cells. Cytoplasmic membrane faction of cell lysates (30 μg) was subjected to Western blot analyses. Data are the means ± S.D. of three experiments. $^{**}P < 0.01$; $^{***}P < 0.001$ versus control.

at δ_H 4.45 (1H, d, J = 8.0 Hz, H-1′) to δ_C 96.8 (C-3) and the other at δ_H 4.75 (1H, d, J = 8.0 Hz, H-1″) to δ_C 80.7 (C-2′) are a strong evidence for this conclusion. Based on the above results, the structure of compound **2**, damulin B, was elucidated as 2α,3β,12β-trihydroxydammar-20,24-diene-3-*O*-[β-D-glucopyranosyl-(1→2)-β-D-glucopyranoside].

In L6 myotube cells, the 50% ethanol extract of *G. pentaphyllum* increased Thr^{172} phosphorylation of the AMPKα subunit (data not shown). To investigate whether purified compounds are activators of AMPK, L6 myotube cells were treated with damulins A and B in different concentrations. As expected, treatment with damulins A and B led to a dose-dependent increase in phosphorylation of AMPK and acetyl-CoA carboxylase (Fig. 2A and B). To verify the specificity of damulins A and B for AMPK phosphorylation, we tested the effect of the AMPK inhibitor, compound C, on the phosphorylation of AMPK. As seen in Figure 2C and D, increased AMPK phosphorylation in the damulins A- and B-treated L6 myotube cells was almost completely abrogated by pretreating cells with 10 μM

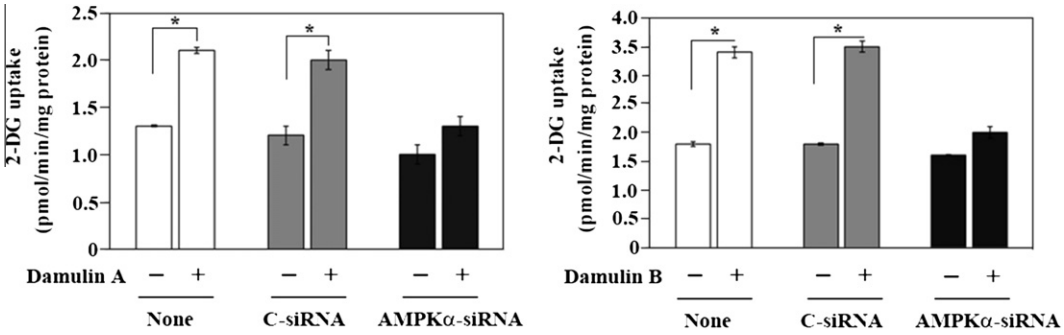


Figure 4. Effect of AMPKα2 knockdown on 2-DG uptake in L6 myotube cells. The increments of 2-DG uptake by damulins A and B were reduced by knockdown of AMPKα2 (AMPKsiRNA). Non-specific siRNA (C-siRNA) was transfected as a negative control. Data are the means ± S.D. of three independent experiments. $^{*}P < 0.05$ compared with control.

compound C. Accordingly, β-oxidation was also increased in L6 myotube cells by treatment of damulins A and B (Fig. 2E). Similarly, glucose uptake as determined by 2-deoxy-[^{3}H]D-glucose (2-DG) uptake was also stimulated by damulins A and B in a dose-dependent manner with increasing GluT4 translocation to the plasma membrane in L6 myotube cells (Fig. 3), and this increase of 2-DG uptake was abrogated by knockdown of AMPKα1/2 (Fig. 4). These results together indicate that damulins A and B stimulate glucose uptake via AMPK activation. *G. pentaphyllum* has been used in Asian counties as a folk herbal medicine with various therapeutic properties such as lowering blood cholesterol and glucose levels.[17,18] Furthermore, saponins from *G. pentaphyllum* also exert an apoptotic activity against various cultured cancer cells,[19,31,32] although its mechanism of action is poorly understood. However, these diverse biological effects of saponins from *G. pentaphyllum* overlap with well-documented downstream effects of AMPK activation,[3,16,20] prompting this research. In this study, we found that the 50% ethanol extract of *G. pentaphyllum* exerted AMPK activation and stimulated its downstream targets in vitro. We also characterized two novel dammarane-type saponins, damulins A and B, from *G. pentaphyllum* extract as active ingredients that increase phosphorylation of AMPK. As expected, both damulins A and B increased glucose uptake in L6 myotube cells via AMPK activation, explaining how the *G. pentaphyllum* extract lowered blood glucose levels in *db*/*db* diabetic mice, an effect that was seen previous report.[18]

Taken together, our results reveal a molecular mechanism underlying the beneficial effects of *G. pentaphyllum* on type-2 diabetes and obesity. Further investigation and optimization of these derivatives may enable the preparation of new AMPK activators with potential applications in the treatment of type-2 diabetes, obesity, and metabolic disorders.

3. Experimental section

3.1. General experimental procedures

IR spectra (KBr) were recorded on a Nicolet 6700 FT-IR spectrometer (Thermo electron Corp.), and the UV spectra in MeOH using a Shimadzu spectrometer. NMR spectra were obtained on Varian Unity Inova 500 MHz spectrometer at the Korea Basic Science Institute (KBSI, Gwangju Center, Korea). HRFABMS and HRESIMS data were recorded on a Micromass QTOF2 (Micromass, Wythenshawe, UK) mass spectrometer. Ion exchange resin (Diaion HP-20) was purchased from Mitsubishi Chemical Co. Silica gel (Merck, 63–200 μm particle size), RP-18 (Merck, 150 μm particle size) were used for column chromatography. TLC was carried out using Merck silica gel 60 F_{254} and RP-18 F_{254} plates. HPLC was carried out using a Gilson system with an UV detector and Optima Pak C_{18} column (10 × 250 mm, 10 μm particle size, RS Tech, Korea).

3.2. Chemicals and reagents

HPLC solvents were obtained from Burdick & Jackson, USA. AICAR (5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside), BAPTA/AM (1,2-bis(o-aminophenoxy) ethane-N,N,N′,N′-tetraacetic acid), and bovine serum albumin (BSA) were obtained from Sigma Chemical Co. Metformin, insulin, and protease inhibitor cocktail set 1 were purchased from Calbiochem. The active forms of recombinant AMPKα1,β1,γ1 protein and antibodies against phospho-AMPKα1/2 (Thr^{172}), total AMPK, phospho-ACC (Ser^{79}), and total ACC antibodies were purchased from Cell Signaling Technology (New England Biolabs). GLUT4 and β-actin antibodies were from Santa Cruz Biotechnology, Inc. Reagents for polyacrylamide gel electrophoresis were from Bio-Rad. 2-Deoxy-[^{3}H]D-glucose was from Perkin-Elmer Life Sciences. All other reagents were of the highest analytical grade.

3.3. Plant material

The cultivated whole plants of *G. pentaphyllum* were purchased from Wonkwang Food Co. in Geochang County, Gyeongsangnam-do, Republic of Korea, and authenticated by Professor Jae-Hong Pak, Department of Biology, Kyungpook National University. A voucher specimen (TLH 1001) was deposited in the herbarium (KNU) of Kyungpook National University, Republic of Korea.

3.4. Extraction and isolation of AMPK activators

After dried *G. pentaphyllum* leaves (5 kg) were extracted with 50% EtOH (8 L) twice for 6 h, the first and second supernatants were combined, filtered with gauze, and centrifuged (1500 *g*) for 30 min to remove insoluble matter. To isolate compounds that activate AMPK, the 50% EtOH extract of *G. pentaphyllum* was subjected to HP-20 ion exchange resin column chromatography (20 × 65 cm). Each fraction was collected by sequential elution with H_2O (10 L), 50% MeOH in H_2O (10 L), and acetone (10 L). Then, the acetone fraction was dried, suspended in H_2O (2 L), and successively partitioned with *n*-hexane (3 × 2 L), EtOAc (3 × 2 L), and *n*-BuOH (3 × 2 L). The partial BuOH fraction (50 g) was subjected to silica gel column (15 × 65 cm; 63–200 μm particle size) chromatography with a gradient solvent system of CH_2Cl_2/MeOH/H_2O (from 5/1/0.1 to 0/5/0.1) to yield five subfractions (B.1–B.5). The combined B.3 and B.4 fraction (B.34) was then subjected to reverse phase ODS–A column chromatography (6.5 × 65 cm; 150 μm particle size) with a gradient solvent system of MeOH/H_2O (from 2/1 to 2/0) to yield six subfractions (B.34–1 to B.34–6). Further purifi-

cation of sub-fraction B.34–5 (502 mg) by Gilson HPLC (Optima Pak C_{18} column, 10 × 250 mm, 10 μm particle size; UV detectors at 205 and 254 nm, flow rate 2 mL/min) with an isocratic solvent system (75% MeOH in 0.1% formic acid in H_2O for 45 min, then increased to 100% MeOH over 15 min) resulted in the isolation of compounds **1** (damulin A, 18 mg, t_R = 42 min) and **2** (damulin B, 14 mg, t_R = 31.5 min). The chemical structures of the pure compounds were elucidated with 1D (including ^1H and ^{13}C) and 2D (including gHSQC, COSY, HMBC, and NOESY) NMR spectra as well as mass data analyses.

3.5. Damulin A (1)

White amorphous powder; IR ν_{max} (KBr); 3400 (OH), 2943 (C–H), 1658, 1641, 1631 (C=C), 1381(C–H), 1077, 1045 (C–O) cm^{-1}; HRFABMS *m/z* 805.4717 $[M+Na]^+$, calcd. for $C_{42}H_{70}O_{13}Na$; 805.4714); ^1H (500 MHz) and ^{13}C (125 MHz) NMR data in MeOH–d_4 (see Table 1).

3.6. Damulin B (2)

Amorphous powder; IR ν_{max} (KBr): 3386 (OH), 2943 (C–H stretching), 1641, 1631 (C=C), 1453, 1379 (C–H bending), 1166, 1078, 1041(C–O), 887, 669 cm^{-1}; HRESIMS *m/z* 783.4905 $[M+H]^+$, calcd. for $C_{42}H_{70}O_{13}$; 783.4895); ^1H (500 MHz) and ^{13}C (125 MHz) NMR Data in MeOH–d_4 (see Table 1).

3.7. Cell culture

L6 cells were obtained from the American Type Culture Collection. L6 cells were differentiated into myotube cells and adipocyte cells, respectively, as previously described.[33,34]

3.8. Measurements of fatty acid oxidation and glucose uptake

Fatty acid oxidation was carried out, and ^3H_2O content was measured in a scintillation counter as previously described.[33] Glucose uptake was measured as the method described.[35] Cultured L6 myotube cells were suspended in glucose-free transport buffer containing 20 mM HEPES (pH 7.4), 140 mM NaCl, 5 mM KCl, 2.5 mM $MgSO_4$, 1 mM $CaCl_2$, and 1% BSA. Cells (~2×10^5) were transferred to plastic tubes and incubated at 37 °C in the presence or absence of damulins A and B and AICAR. Subsequently, 2-deoxy-[^{3}H]D-glucose was added to the cells for 10 min, and the incubations were terminated by washing three times with ice-cold 0.9% NaCl (w/v). Non-specific transport was measured in the same conditions, except that cytochalasin B (10 μM) was added to the medium before the addition of cells. Non-specific values were subtracted from all conditions. Cells were collected in 0.05 N NaOH by centrifugation for 30 s (6000 *g*), and cell-associated radioactivity was determined by scintillation counting. The results were expressed as pmol 2-DG/min/mg protein or fold increase compared to control.

3.9. Knock-down of AMPK in cultured cells

For targeting AMPKα2 isoforms in L6 cells, ON-TARGETplus SMARTpool small interfering RNA (siRNA) for rat AMPKα2 and negative control siRNA were purchased from Dharmacon. For gene knockdown experiments, differentiated L6 cells were transfected with siRNAs according to the manufacturer's instructions, cultured in α-Minimum Essential Media (MEM) without antibiotics and fetal bovine serum for 48 h, and then chemicals were added to the cells.

3.10. Western blot analysis

Cultured L6 myotube cells were lysed with cell lysis buffer A (50 mM Tris–HCl, 1% Nonidet P-40, 5 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 1 mM NaF and 1× protease inhibitor cocktail, pH 8.0). Isolated mouse soleus muscle tissues were homogenized with a glass homogenizer in HEPES buffer (20 mM HEPES, 250 mM sucrose, 1 mM EDTA, pH 7.4) containing protease inhibitor cocktail. Total membrane fracrions from L6 myotube cells were prepared as we previously described. Western blot analysis was carried out as previously described.[33]

3.11. Statistical analysis

All in vitro results are representative of at least three independent experiments, and data are the means ± S.D.

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Supplementary data

Supplementary data (1D and 2D (including COSY, HSQC, and HMBC) NMR spectra of the new compounds damulins A (**1**) and B (**2**)) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.09.013.

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