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Higenamine 4'-*O*-ß-D-glucoside in the lotus plumule induces glucose uptake of L6 cells through ß2-adrenergic receptor

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25 **Abstract**

26 Hypoglycemic effect is an efficient means to modulate elevated blood glucose levels in
27 patients with diabetes. We found that the extract of lotus plumule (the germ of *Nelumbo*
28 *nucifera* Gaertn. seed) showed potent glucose uptake enhancement activity against L6
29 myotubes, which results in a hypoglycemic effect. This activity was further investigated,
30 and an active constituent was identified as a single bioactive compound, higenamine
31 4'-*O*-ß-D-glucoside. Mechanistic studies employing phosphatidylinositol 3-kinase (PI3K)
32 inhibitor, AMP-activated protein kinase (AMPK) inhibitor, or adrenergic receptor
33 antagonist showed that the compound induced its activity through ß2-adrenergic
34 receptor. Patients with type II diabetes mellitus frequently develop insulin resistance.
35 Owing to the differences between the mechanism of action of insulin and of the isolated
36 compound, the compound or lotus plumule itself may have the possibility of modulating
37 blood glucose levels in insulin-resistant patients effectively.

38

39 **Keywords**

40 *Nelumbo nucifera* Gaertn.; hypoglycemic effect; glucose uptake; ß2-adrenergic receptor
41 agonist; Diabetes mellitus.

42

1. Introduction

Hyperglycemia is a characteristic symptom of diabetes mellitus, which is currently considered an urgent problem worldwide. Since hyperglycemia results in various disorders,[1–3] control of blood glucose level is the major goal of diabetes treatments. Insulin formulation is one of an accepted treatment for patients with diabetes mellitus. However, for insulin-resistant patients with type II diabetes, decreased insulin sensitivity limits its use, and an alternative method to modulate blood glucose levels is often required. Therefore, exploring hypoglycemic agents that can improve glucose utilization and decrease blood glucose level is an efficient target of research.

Sacred lotus (*Nelumbo nucifera* Gaertn.), a large aquatic herb native to India, is widely distributed in India, Japan, and China. The plant is known for its potential health benefits and is employed in traditional medications. Rhizome, leaf, flower, seed, and all other parts are used to treat vomiting, leprosy, inflammation, skin diseases, neuronal disorders, diarrhea, poisoning, and various other symptoms.[4,5]

Many scientific studies have shown the beneficial effects of this plant, including anti-ischaemic, antioxidant, hepatoprotective, antiproliferative, anti-inflammatory, anti-fertility, anti-arrhythmic, anti-fibrosis, antiviral, and other activities.[5,6] Among them, hypoglycemic activity of the plant extract has been reported.[4,7–9]

During our search for hypoglycemic agents using glucose uptake assay against muscle model cells, the extract of the lotus plumule showed high activity. Lotus plumule is the germ inside the lotus seed, located between two cotyledons. Although the hypoglycemic activity of this plant has been reported, its active component was not fully investigated and the mechanism of action was not revealed. In this research, we isolated the component with glucose uptake enhancing activity from the extract and

67 investigated the target signaling pathway that induces its activity.

68

69 **2. Material and methods**

70 2.1.General

71 All commercially available chemicals were used without further purification.
72 Chemicals and enzymes were purchased from Wako Pure Chemical Industries, unless
73 otherwise specified. Fluorescence was measured by a Synergy™ MX microplate reader
74 (BioTek Instruments, Inc.). A Bruker AMX 500 (Bruker BioSpin K.K.) was used to
75 obtain nuclear magnetic resonance (NMR) spectra and *tert*-butanol was used as an
76 internal standard (^{1}H NMR: 1.24 ppm, ^{13}C NMR: 30.29 ppm). A Waters LCT Premier
77 spectrometer (Waters Co.) was used to obtain electrospray ionisation mass spectra
78 (ESI-MS). Lotus plumule (germ of *Nelumbo nucifera* Gaertn. seed) was purchased
79 commercially from the Kampo market.

80

81 2.2.Isolation procedure of higenamine 4-*O*-β-D-glucoside (**1**)

82 Dried lotus plumule (50 g) was crushed using a blender and extracted twice with
83 300 mL of 50% aqueous (aq) methanol over 3 days at room temperature (rt). The extract
84 was filtered and evaporated under reduced pressure to remove methanol and then
85 purified using the following activity guided fractionation. The obtained aqueous
86 solution was extracted with ethyl acetate and then with 1-butanol. The water layer was
87 concentrated under reduced pressure to remove the organic solvents and adsorbed onto
88 the column filled with DIAION HP-20 (Mitsubishi Chemical Co., ϕ40×160 mm). The
89 column was washed with water, 50% aq methanol, and methanol (600 mL each). The
90 50% aq methanol eluate was concentrated and separated using Cosmosil 75C_{18}-OPN

column chromatography (Nacalai Tesque, Inc., ϕ30×230 mm) by stepwise elution with water, 10% aq methanol, 20% aq methanol, 30% aq methanol, 50% aq methanol, and methanol (300 mL each). The 10% aq methanol fraction was separated again using Cosmosil 75C_{18}-OPN column chromatography (ϕ20×220 mm) by stepwise elution with water (200 mL, Fr. 1–3), 5% aq methanol (300 mL, Fr. 4–7), 10% aq methanol (200 mL, Fr. 8–10), and 50% aq methanol (200 mL, Fr. 11). Fr. 6 and 7 were combined and separated thrice using high performance liquid chromatography (HPLC) [1) Inertsil ODS-3 (GL Science Co., ϕ20×250 mm), gradient elution from 5% aq methanol to 50% aq methanol in 60 min; 2) Inertsil ODS-3 (ϕ20×250 mm), 0.1% trifluoroacetic acid (TFA), 15% aq methanol; 3) InertSustain C18 (GL Science Co., ϕ20×250 mm), 0.1% TFA, 10% aq methanol] to obtain 7.4 mg of TFA salt of higenamine 4-*O*-β-D-glucoside (**1**), a slightly brown oil. The structure was confirmed by comparing MS (HR-ESI-MS (positive): $[M + H]^+$ found *m*/*z* 434.1806, $C_{22}H_{28}NO_8^+$ requires *m*/*z* 434.1809) and ^{1}H-NMR data (CD_3OD, see Supplementary Fig. 1) with reference values.[10,11] The data in Table 1 was obtained in D_2O because it was easier to distinguish the signals. The specific rotation was $[\alpha]_D^{25}$ = -17.8 (*c* = 0.8, MeOH).

2.3.Synthesis

2.3.1. 6,7-diacetoxy-1-(4-hydroxybenzyl)-1,2,3,4-tetrahydroisoquinoline (**3**)

Aldehyde (**2**) (1.02 g, 4.08 mmol) and dopamine hydrochloride (515.3 mg, 2.72 mmol) were dissolved in acetonitrile (20 mL) and 0.1 M potassium phosphate buffer (pH 6.0, 5 mL) and stirred for 16 h at 50°C. Saturated (sat) aq sodium bicarbonate was added to the reaction mixture and extracted with ethyl acetate. The organic layer was washed with brine, dried over sodium sulfate, and evaporated. The residue was

115 partially purified using silica gel column chromatography (Chloroform/methanol = 9/1
116 to 4/1). Since the remaining dopamine was difficult to separate, the obtained crude
117 mixture was directly subjected to the following reactions.
118 Crude product (186.4 mg) was dissolved in 1,4-dioxane (15 mL), water (5 mL),
119 and benzyl chloroformate (120 μL, 0.84 mmol). To the mixture, sat aq sodium
120 bicarbonate was added and the mixture was adjusted to pH 8. After stirring for 10 min,
121 the reaction mixture was diluted with water and extracted with ethyl acetate. The
122 organic layer was washed with brine, dried over sodium sulfate, and evaporated. The
123 residue was dissolved in dichloromethane (10 mL), and trimethylamine (0.7 mL, 5.00
124 mmol) was added. Acetyl chloride (0.2 mL, 2.81 mmol) was added to the mixture and
125 stirred for 15 min in an argon atmosphere. Then, 1 M tetrabutylammonium fluoride
126 solution in tetrahydrofuran (0.5 mL) was added and stirred for 5 min. The reaction
127 mixture was diluted with water and extracted with chloroform. The organic layer was
128 washed with brine, dried over sodium sulfate, and evaporated. The residue was purified
129 using silica gel column chromatography (Hexane/acetone = 3/1 to 2/1) to yield **3** (140.5
130 mg, 11% from dopamine), an enantiomer mixture that showed *m*/*z* 512 [ESI-MS
131 (positive), $[M + Na]^+$] in the mass spectrum. Since ^{1}H-NMR data of the obtained **3**
132 showed complex signals, probably owing to isomers from benzyl group rotation
133 (rotamer) and chiral carbon (enantiomer), the current and following intermediate
134 products (**3**–**5**) were confirmed only using the mass spectra.
135
136 2.3.2. 6,7-diacetoxy-2-carbobenzyloxy-1-(4-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosylox
137 y)benzyl)-1,2,3,4-tetrahydroisoquinoline (**4**)
138 Compound **3** (50.2 mg, 0.103 mmol) and 2,3,4,6-tetra-*O*-acetyl-D-glucopyranosyl

trichloroacetimidate (80.8 mg, 0.164 mmol) were dissolved in dry dichloromethane (2.0 mL), and activated molecular sieves 4A (powder, 219 mg, Sigma-Aldrich Co.) was added and stirred for 10 min in an argon atmosphere. To this mixture, boron trifluoride diethyl etherate (10% v/v in dichloromethane, 150 μL) was added at 0°C and stirred for 10 min in an argon atmosphere. The reaction mixture was passed through Celite® pad, sat aq sodium bicarbonate was added, and extracted with chloroform. The organic layer was washed with brine, dried over sodium sulfate, and evaporated. The residue was purified by preparative thin layer chromatography (Hexane/acetone = 2/1) to produce **4** (84.2 mg, quant.), which showed *m*/*z* 842 (ESI-MS (positive), $[M + Na]^+$) in the mass spectra.

2.3.3. 6,7-dihydroxy-2-carbobenzyloxy-1-(4-(β-D-glucopyranosyloxy)benzyl)-1,2,3,4-tetrahydroisoquinoline (**5**)

Compound **4** (84.2 mg, 0.103 mmol) was dissolved in methanol (2 mL), and 28% sodium methoxide in methanol (0.1 mL) was added and stirred for 5 min. The reaction mixture was neutralized by the addition of Dowex® 50W-X8, filtered, and evaporated. The residue was purified by preparative thin layer chromatography (chloroform/methanol = 7/1) to yield **5** (34.1 mg, 58%), which showed *m*/*z* 590 (ESI-MS (positive), $[M + Na]^+$) in the mass spectra.

2.3.4. Higenamine 4'-*O*-β-D-glucoside (**1**)

Compound **5** (17.1 mg, 0.03 mmol) was dissolved in methanol (1 mL), and palladium hydroxide was added and stirred for 1 h in a hydrogen atmosphere. The reaction mixture was passed through a Celite® pad and evaporated. The residue containing the mixture of diastereomers was purified using HPLC equipped with InertSustain® C18

and 0.1% TFA, 10% methanol aq as an eluent to yield TFA salt of **1** (5.3 mg, 32%), a slightly brown oil. The spectra data matched the reference data, and the data of isolated 1 (Supplementary Fig. 2). [10,11] The specific rotation was $[\alpha]_D^{25}$ = -20.0 (c = 0.5, MeOH).

2.4. Cell culture

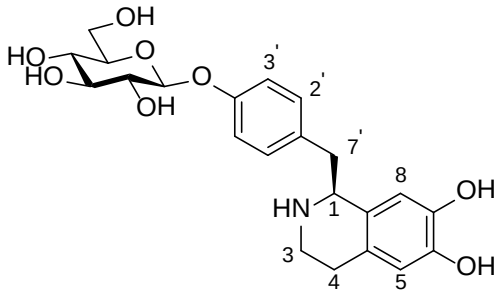
L6 cells (JCRB9081, Lot10102000) were provided by the JCRB Cell Bank through the National Institute of Biomedical Innovation, Japan. L6 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum at 37°C in a humidified atmosphere with 10% CO_2. Cells were reseeded in 48-well plates for glucose uptake assay. After the cells reached confluence, the medium was switched to DMEM containing 2% horse serum for differentiation. The medium was replaced every second day, and experiments were performed 6–8 days after initiating differentiation.

2.5. Glucose uptake enhancement assay

A glucose uptake assay was performed according to the reported method with modifications.[12] The medium was switched to serum-free DMEM and the cells were serum starved for 15 h. The cells were then incubated with the corresponding concentration of a sample diluted in serum-free DMEM for 4 h. After treatment, the cells were rinsed twice with Krebs-Ringer phosphate-HEPES (KRPH) buffer (20 mM HEPES, 136 mM NaCl, 4.7 mM KCl, 1 mM NaH_2PO_4, 1 mM $MgSO_4$, and 1 mM $CaCl_2$, pH 7.4) and incubated in KRPH buffer containing 1 mM 2-deoxyglucose for 20 min. After washing the cells thrice with KRPH buffer, 0.05 M NaOH aq was added to lyse the

187 cells. The lysate was frozen, thawed, and heated at 85°C for 60 min. A mixture of 0.1 M
188 HCl aq and 50 mM triethanolamine buffer (pH 8.1, with 0.5 mM $MgCl_2$ and 50 mM KCl)
189 was added and adjusted to pH 8.1. For quantitation of 2-deoxyglucose, 50 µL of the cell
190 lysate, or standards containing various concentrations of 2-deoxyglucose, and 50 µL of
191 the assay cocktail [1.3 mM ATP, 20 µM $NADP^+$, 50 µM resazurin sodium salt, 12
192 units/mL hexokinase (Sigma-Aldrich Co.), 32 units/mL glucose 6-phosphate
193 dehydrogenase (from *Leuconostoc mesenteroides*), and 4 units/mL diaphorase dissolved
194 in triethanolamine buffer] were mixed in 96-well plates. After 1 h of incubation at 37°C,
195 the fluorescence was measured (λ_{ex} = 530 nm, λ_{em} = 590 nm). Background fluorescence
196 (performed without 2-deoxyglucose incubation) was subtracted, and the values are
197 represented relative to the control. For the experiments using inhibitors, cells were
198 pre-incubated in the presence or absence of inhibitors for 60 min prior to sample
199 treatment and were performed as written in section 2.5.
200
201 2.6.Statistical analysis
202 Glucose uptake enhancement assays were performed independently; each
203 experiment was repeated thrice, with n = 4 for each, and the representative values are
204 shown on the graphs. Student's *t*-test was used to assess the significant differences
205 between the two mean values. $p < 0.05$ was considered statistically significant.
206
207 **3. Results and discussions**
208 3.1.Isolation and the activity of higenamine 4'-*O*-β-D-glucoside (**1**)
209 The 50% aq methanol extract from dried lotus plumule showed a glucose uptake
210 enhancement activity approximately half of that of the positive control (100 nM insulin)

211 at 0.001*n*. The concentration term *n* was employed in the following activity guided
212 fractionations, which 1*n* represents the concentration of a sample when the extractable
213 constituent obtained from 1 g of plant material is dissolved in 10 mL of the medium.
214 The crude extract was separated following activity-guided fractionations. The
215 solvent partition between the water, ethyl acetate, and 1-butanol established the water
216 layer as the active layer. The water layer was fractionated using open column
217 chromatography with a DIAION HP-20 (Mitsubishi Chemical Co.) and Cosmosil
218 75C_{18}-OPN (Nacalai Tesque, Inc.). The obtained active fraction was further purified
219 using HPLC with Inertsil ODS-3 (GL Science Co.) and InertSustain C18 (GL Science
220 Co.) as the column to afford 7.4 mg of higenamine 4'-*O*-β-D-glucoside (**1**) as the active
221 compound (Table 1).
222



223 Higenamine 4'-*O*-β-D-glucoside (**1**)
224
225 There is only one previous study reporting the isolation of **1**, which is from the fruit
226 of *Phoebe chekiangensis*; [11] this is the first study to report its isolation from lotus
227 plumule. The aglycon moiety of **1**, higenamine also called norcoclaurine, is a component
228 of lotus and both its enantiomers have been isolated previously from the plant.[13,14] The
229 stereochemistry of the higenamine moiety of the current isolated compound was easily
230 determined as *S*-enantiomer by comparison with reported NMR data.[10] Specific rotation

231 of the isolated **1** ($[\alpha]_D^{25}$ = -17.8) also showed similar value to the reported data of

232 *S*-enantiomer ($[\alpha]_D$ = -2.3) compared to the reported value of its epimer ($[\alpha]_D$ = +42.6).

233

234 Table 1. NMR data of higenamine 4'-*O*-β-D-glucoside (**1**) in D_2O at rt.

Position	δ_C (ppm)	δ_H (ppm)	Mult.	*J* (Hz)
4'	156.8			
6 or 7	144.8			
6 or 7	143.5			
2'	131.6	7.28	d	8.5
1'	130.3			
9 or 10	124.5			
9 or 10	123.8			
3'	117.7	7.14	d	8.5
5	116.5	6.78	s	
8	114.5	6.68	s	
Glc1	100.8	5.14	d	7.3
Glc5	76.8	3.64	ddd	1.9, 5.7, 9.8
Glc3	76.2	3.62	dd	8.8, 9.1
Glc2	73.6	3.58	dd	7.3, 9.1
Glc4	70.1	3.50	dd	9.8, 8.8
Glc6a	61.2	3.94	dd	1.9, 12.5
Glc6b		3.76	dd	5.7, 12.5
1	56.8	4.69	dd	6.0, 9.0
3a	39.9	3.54-3.48	m	
3b		3.31	ddd	6.3, 6.6, 12.9
7'a	39.1	3.47	dd	6.0, 14.5
7'b		3.08	dd	9.0, 14.5
4a	24.7	2.99	ddd	6.3, 6.3, 17.3
4b		2.96	ddd	6.6, 6.6, 17.3

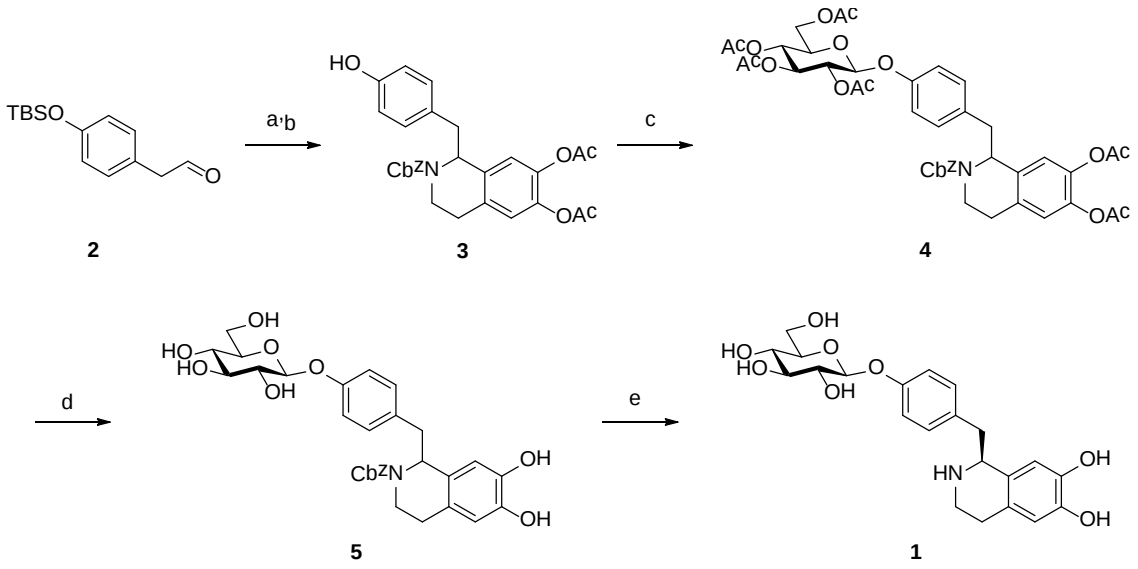
235

236 3.2. Synthesis of higenamine 4'-*O*-β-D-glucoside (**1**)

237 To confirm the structure and activity of the isolated compound, **1** was synthesized.

238 According to the literature,[15] aldehyde (**2**)[16] was reacted with dopamine, and protective

groups were modified to afford higenamine (**3**) as the mixture of enantiomers. After a glycosylation reaction of **3** with glucosyl imidate in the presence of boron trifluoride, the obtained **4** was deprotected and the desired diastereomer **1** was selectively purified using HPLC. The ^{1}H-NMR spectra of synthetic **1** matched the reported data,[10] and the data of isolated **1**. The specific rotation of the synthetic **1** ($[\alpha]_D^{25}$ = -20.0) also matched with the isolate ($[\alpha]_D^{25}$ = -17.8) thus confirming the structure.



Scheme 1. Synthesis of **1**. a) dopamine hydrochloride, MeCN, 0.1 M potassium phosphate buffer (pH 6.0), 50°C. b) [1. CbzCl, $NaHCO_3$, 1,4-dioxane, H_2O; 2. AcCl, triethylamine, CH_2Cl_2 then 1 M tetrabutylammonium fluoride in tetrahydrofuran], 11% from dopamine. c) 2,3,4,6-tetra-*O*-acetyl-D-glucopyranosyl trichloroacetimidate, BF_3-Et_2O, CH_2Cl_2, 0°C, quant. d) NaOMe, MeOH, 58%. e) $Pd(OH)_2$, H_2, MeOH, 32%.

3.3. Glucose uptake enhancement activity

Glucose uptake enhancement activity of the synthetic **1** was measured, and a 2.1-fold increase compared to the control, or equivalent to 100 nM insulin at 10 μM was

observed (Fig. 1). In addition, a concentration-dependent increase in glucose uptake enhancement activity was observed between 1 and 100 μM.

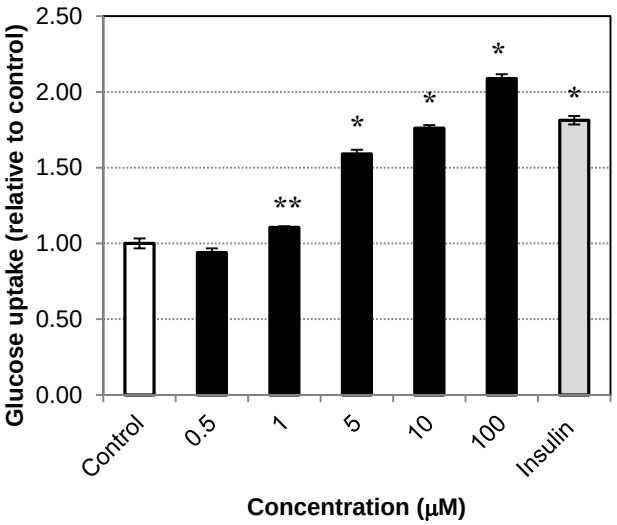


Fig. 1. Glucose uptake enhancement activity of **1**. Insulin: 100 nM; *$p < 0.01$, **$p < 0.05$ to control.

Higenamine 4'-*O*-β-D-glucoside (1), also named SCH 71450, is known as a D_2/D_4 dopamine receptor antagonist.[11] Other activities of the compound are currently not reported and the glucose uptake enhancement activity, which is related to the hypoglycemic effect, will increase its utility.

3.4.Mechanistic study

To study the mechanism by which **1** enhances glucose uptake in muscle cells, several inhibitors were co-incubated with **1** and its activity was measured. Neither the PI3K inhibitor, LY294002, nor the AMPK inhibitor, dorsomorphin, inhibited the activity of **1**, indicating that the insulin signaling pathway or the AMPK pathway, which are

involved in the effect of various anti-diabetic medications, are not involved (Fig. 2, 3). In contrast, the addition of a non-selective ß-adrenergic receptor antagonist, propranolol, or a ß2 selective adrenergic receptor antagonist, ICI118,551, eliminated the activity of **1**, showing that the ß2-adrenergic receptor is the target of this compound that induces enhanced glucose uptake (Fig. 4).

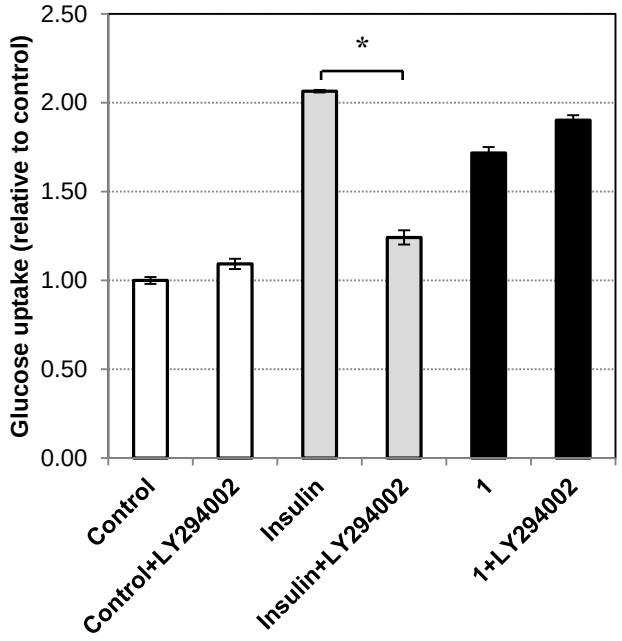


Fig. 2. Co-incubation of **1** with PI3K inhibitor LY294002. Insulin (positive control): 100 nM; Compound **1**: 10 μM; LY294002: 25 μM; *$p < 0.01$ between the indicated two bars.

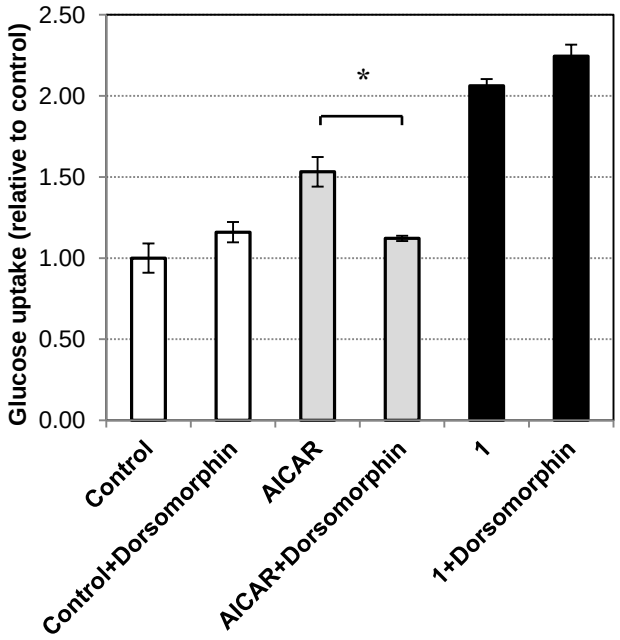
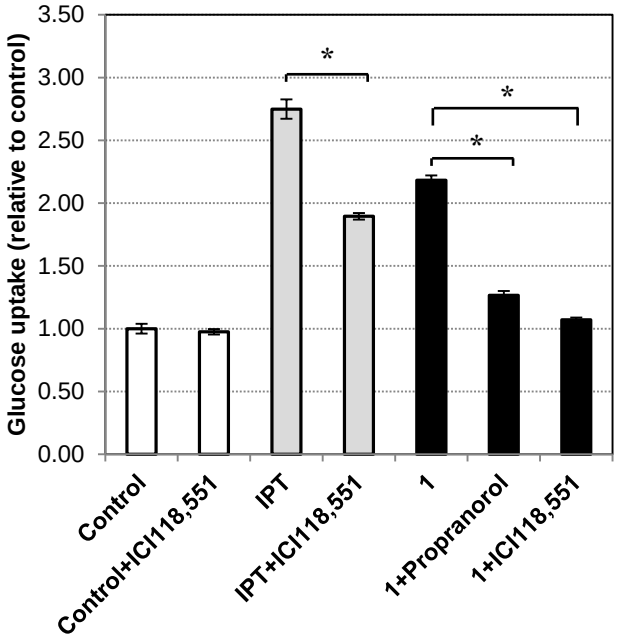


Fig. 3. Co-incubation of **1** with AMPK inhibitor dorsomorphin.
5-amino-1-ß-D-ribofuranosyl-imidazole-4-carboxamide (AICAR, positive control): 1 mM;
Compound **1**: 10 μM; Dorsomorphin: 10 μM; *$p < 0.01$ between the indicated two bars.

As previously reported, the ß2-adrenergic receptor agonist mediates glucose uptake in the muscle cells.[17–19] The mechanistic study revealed that two distinctive pathways are involved in the glucose uptake enhancement of ß2-adrenergic receptor agonist. First is via the accumulation of cyclic AMP (cAMP), which activates cAMP-dependent protein kinase, phosphorylates the mammalian target of the rapamycin complex 2, and translocates the glucose transporter 4. Second is via PI3K, the protein involved in the insulin signaling pathway. In the case of **1**, involvement of PI3K in enhancing glucose uptake was not observed as shown in Fig. 2, and thus accumulation of cAMP might be the only pathway.



298

299 Fig. 4. Co-incubation of **1** with ß-adrenergic receptor antagonists. IPT (isoproterenol,
300 positive control): 1 μM; Compound **1**: 10 μM; Propranorol: 1 μM; ICI118,551: 100 nM; *p
301 < 0.01 between the indicated two bars.

302

303 **4. Conclusions**

304 We have identified higenamine 4'-*O*-ß-D-glucoside (**1**) from lotus plumule as the
305 bioactive molecule that enhances glucose uptake of L6 cells and is one of the molecules
306 responsible for the hypoglycemic effect of the plant. By the use of specific inhibitors, the
307 pathway involved in the activity of **1** was identified as ß2-adrenergic receptor mediated
308 signaling (Fig. 5). Since ß2-adrenergic receptor agonist is shown to be effective in
309 mediating glucose uptake of skeletal muscle in type 2 diabetes model rats, [19] the
310 compound may be effective for the treatment of insulin-resistant patients with type II
311 diabetes.

312

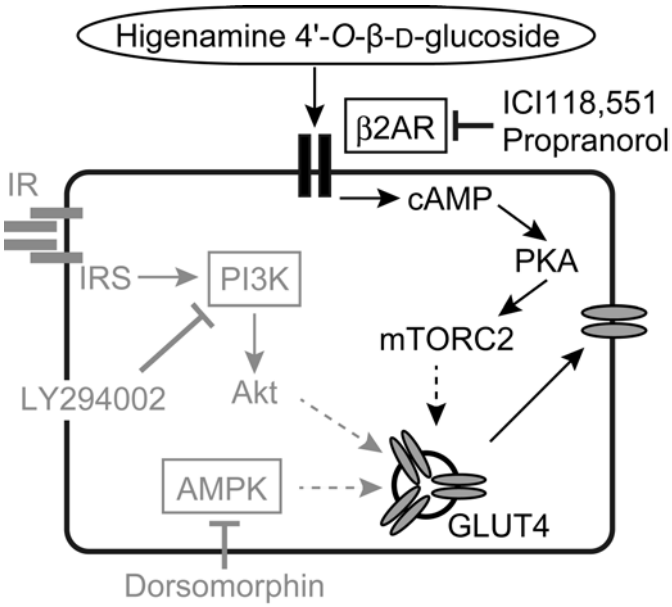


Fig. 5. Signaling pathways of glucose uptake. Black words and arrows are the pathway involved in the activity of higenamine 4'-*O*-ß-D-glucoside (**1**). Gray words and arrows are the denied pathways. ß2AR: ß2 adrenergic receptor, PKA: cAMP-dependent protein kinase, mTORC2: mammalian target of the rapamycin complex 2, GLUT4: glucose transporter 4, IR: insulin receptor, IRS: insulin receptor substrate.

5. Acknowledgements

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