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Higenamine 4'-*O*-β-D-glucoside in the lotus plumule induces glucose uptake of L6 cells
through β2-adrenergic receptor

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Abstract

Hypoglycemic effect is an efficient means to modulate elevated blood glucose levels in patients with diabetes. We found that the extract of lotus plumule (the germ of *Nelumbo nucifera* Gaertn. seed) showed potent glucose uptake enhancement activity against L6 myotubes, which results in a hypoglycemic effect. This activity was further investigated, and an active constituent was identified as a single bioactive compound, higenamine 4'-*O*- β -D-glucoside. Mechanistic studies employing phosphatidylinositol 3-kinase (PI3K) inhibitor, AMP-activated protein kinase (AMPK) inhibitor, or adrenergic receptor antagonist showed that the compound induced its activity through β 2-adrenergic receptor. Patients with type II diabetes mellitus frequently develop insulin resistance. Owing to the differences between the mechanism of action of insulin and of the isolated compound, the compound or lotus plumule itself may have the possibility of modulating blood glucose levels in insulin-resistant patients effectively.

Keywords

Nelumbo nucifera Gaertn.; hypoglycemic effect; glucose uptake; β 2-adrenergic receptor agonist; Diabetes mellitus.

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

investigated the target signaling pathway that induces its activity.

2. Material and methods

2.1. General

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

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

Dried lotus plumule (50 g) was crushed using a blender and extracted twice with 300 mL of 50% aqueous (aq) methanol over 3 days at room temperature (rt). The extract was filtered and evaporated under reduced pressure to remove methanol and then purified using the following activity guided fractionation. The obtained aqueous solution was extracted with ethyl acetate and then with 1-butanol. The water layer was concentrated under reduced pressure to remove the organic solvents and adsorbed onto the column filled with DIAION HP-20 (Mitsubishi Chemical Co., φ40×160 mm). The column was washed with water, 50% aq methanol, and methanol (600 mL each). The 50% aq methanol eluate was concentrated and separated using Cosmosil 75C₁₈-OPN

column chromatography (Nacalai Tesque, Inc., $\phi 30 \times 230$ 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 75C₁₈-OPN column chromatography ($\phi 20 \times 220$ 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., $\phi 20 \times 250$ mm), gradient elution from 5% aq methanol to 50% aq methanol in 60 min; 2) Inertsil ODS-3 ($\phi 20 \times 250$ mm), 0.1% trifluoroacetic acid (TFA), 15% aq methanol; 3) InertSustain C18 (GL Science Co., $\phi 20 \times 250$ 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, C₂₂H₂₈NO₈⁺ requires m/z 434.1809) and ¹H-NMR data (CD₃OD, see Supplementary Fig. 1) with reference values.^{10,11} The data in Table 1 was obtained in D₂O 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

partially purified using silica gel column chromatography (Chloroform/methanol = 9/1 to 4/1). Since the remaining dopamine was difficult to separate, the obtained crude mixture was directly subjected to the following reactions.

Crude product (186.4 mg) was dissolved in 1,4-dioxane (15 mL), water (5 mL), and benzyl chloroformate (120 μ L, 0.84 mmol). To the mixture, sat aq sodium bicarbonate was added and the mixture was adjusted to pH 8. After stirring for 10 min, the reaction mixture was diluted with water and extracted with ethyl acetate. The organic layer was washed with brine, dried over sodium sulfate, and evaporated. The residue was dissolved in dichloromethane (10 mL), and trimethylamine (0.7 mL, 5.00 mmol) was added. Acetyl chloride (0.2 mL, 2.81 mmol) was added to the mixture and stirred for 15 min in an argon atmosphere. Then, 1 M tetrabutylammonium fluoride solution in tetrahydrofuran (0.5 mL) was added and stirred for 5 min. The reaction mixture was diluted with water and extracted with chloroform. The organic layer was washed with brine, dried over sodium sulfate, and evaporated. The residue was purified using silica gel column chromatography (Hexane/acetone = 3/1 to 2/1) to yield **3** (140.5 mg, 11% from dopamine), an enantiomer mixture that showed m/z 512 [ESI-MS (positive), $[M + Na]^+$] in the mass spectrum. Since 1H -NMR data of the obtained **3** showed complex signals, probably owing to isomers from benzyl group rotation (rotamer) and chiral carbon (enantiomer), the current and following intermediate products (**3–5**) were confirmed only using the mass spectra.

2.3.2. 6,7-diacetoxy-2-carbobenzyloxy-1-(4-(2,3,4,6-tetra-*O*-acetyl- β -D-glucopyranosyloxy)benzyl)-1,2,3,4-tetrahydroisoquinoline (**4**)

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 μ L) 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 $[\alpha]_{\text{D}}^{25} = -20.0$ ($c = 0.5$, 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% CO₂. 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 (KRPB) buffer (20 mM HEPES, 136 mM NaCl, 4.7 mM KCl, 1 mM NaH₂PO₄, 1 mM MgSO₄, and 1 mM CaCl₂, pH 7.4) and incubated in KRPB buffer containing 1 mM 2-deoxyglucose for 20 min. After washing the cells thrice with KRPB buffer, 0.05 M NaOH aq was added to lyse the

cells. The lysate was frozen, thawed, and heated at 85°C for 60 min. A mixture of 0.1 M HCl aq and 50 mM triethanolamine buffer (pH 8.1, with 0.5 mM MgCl₂ and 50 mM KCl) was added and adjusted to pH 8.1. For quantitation of 2-deoxyglucose, 50 µL of the cell lysate, or standards containing various concentrations of 2-deoxyglucose, and 50 µL of the assay cocktail [1.3 mM ATP, 20 µM NADP⁺, 50 µM resazurin sodium salt, 12 units/mL hexokinase (Sigma-Aldrich Co.), 32 units/mL glucose 6-phosphate dehydrogenase (from *Leuconostoc mesenteroides*), and 4 units/mL diaphorase dissolved in triethanolamine buffer] were mixed in 96-well plates. After 1 h of incubation at 37°C, the fluorescence was measured ($\lambda_{\text{ex}} = 530 \text{ nm}$, $\lambda_{\text{em}} = 590 \text{ nm}$). Background fluorescence (performed without 2-deoxyglucose incubation) was subtracted, and the values are represented relative to the control. For the experiments using inhibitors, cells were pre-incubated in the presence or absence of inhibitors for 60 min prior to sample treatment and were performed as written in section 2.5.

2.6. Statistical analysis

Glucose uptake enhancement assays were performed independently; each experiment was repeated thrice, with $n = 4$ for each, and the representative values are shown on the graphs. Student's t -test was used to assess the significant differences between the two mean values. $p < 0.05$ was considered statistically significant.

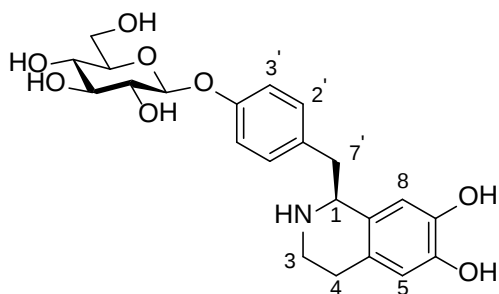
3. Results and discussions

3.1. Isolation and the activity of higenamine 4'-*O*- β -D-glucoside (**1**)

The 50% aq methanol extract from dried lotus plumule showed a glucose uptake enhancement activity approximately half of that of the positive control (100 nM insulin)

at 0.001*n*. The concentration term *n* was employed in the following activity guided fractionations, which 1*n* represents the concentration of a sample when the extractable constituent obtained from 1 g of plant material is dissolved in 10 mL of the medium.

The crude extract was separated following activity-guided fractionations. The solvent partition between the water, ethyl acetate, and 1-butanol established the water layer as the active layer. The water layer was fractionated using open column chromatography with a DIAION HP-20 (Mitsubishi Chemical Co.) and Cosmosil 75C₁₈-OPN (Nacalai Tesque, Inc.). The obtained active fraction was further purified using HPLC with Inertsil ODS-3 (GL Science Co.) and InertSustain C18 (GL Science Co.) as the column to afford 7.4 mg of higenamine 4'-*O*-β-D-glucoside (**1**) as the active compound (Table 1).



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

There is only one previous study reporting the isolation of **1**, which is from the fruit of *Phoebe chekiangensis*; ¹¹ this is the first study to report its isolation from lotus plumule. The aglycon moiety of **1**, higenamine also called norcoclaurine, is a component of lotus and both its enantiomers have been isolated previously from the plant.^{13,14} The stereochemistry of the higenamine moiety of the current isolated compound was easily determined as *S*-enantiomer by comparison with reported NMR data.¹⁰ Specific rotation

of the isolated **1** ($[\alpha]_{\text{D}}^{25} = -17.8$) also showed similar value to the reported data of *S*-enantiomer ($[\alpha]_{\text{D}} = -2.3$) compared to the reported value of its epimer ($[\alpha]_{\text{D}} = +42.6$).

Table 1. NMR data of higenamine 4'-*O*-β-D-glucoside (**1**) in D₂O at rt.

Position	δ _C (ppm)	δ _H (ppm)	Mult.	<i>J</i> (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

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

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

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

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

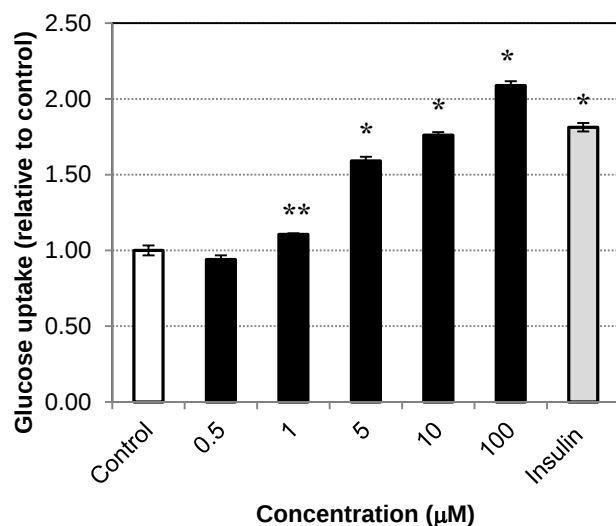


Fig. 1. Glucose uptake enhancement activity of **1**. Insulin: 100 nM; * $p < 0.01$, ** $p < 0.05$ to control.

Higenamine 4'-*O*- β -D-glucoside (**1**), also named SCH 71450, is known as a D₂/D₄ 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 β -adrenergic receptor antagonist, propranolol, or a β_2 selective adrenergic receptor antagonist, ICI118,551, eliminated the activity of **1**, showing that the β_2 -adrenergic receptor is the target of this compound that induces enhanced glucose uptake (Fig. 4).

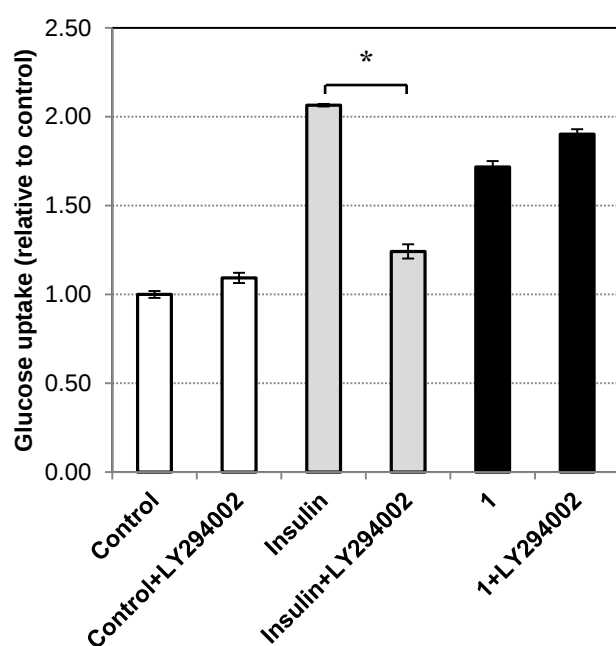


Fig. 2. Co-incubation of **1** with PI3K inhibitor LY294002. Insulin (positive control): 100 nM; Compound **1**: 10 μ M; LY294002: 25 μ M; * p < 0.01 between the indicated two bars.

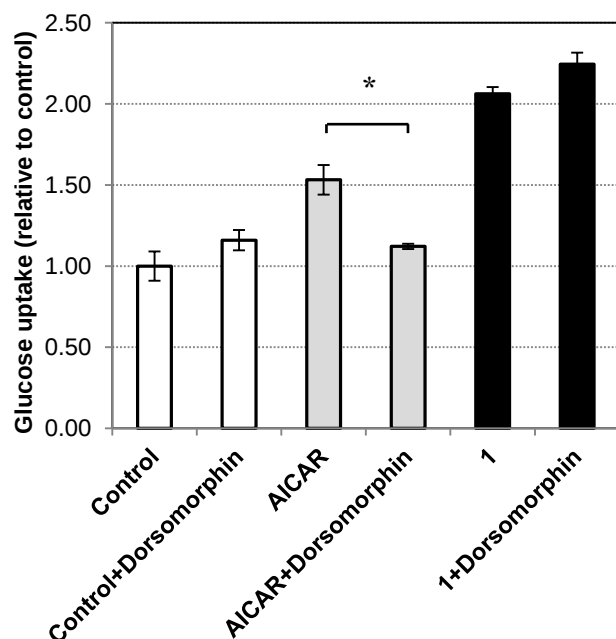


Fig. 3. Co-incubation of **1** with AMPK inhibitor dorsomorphin.

5-amino-1- β -D-ribofuranosyl-imidazole-4-carboxamide (AICAR, positive control): 1 mM; Compound **1**: 10 μ M; Dorsomorphin: 10 μ M; * p < 0.01 between the indicated two bars.

As previously reported, the β 2-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 β 2-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.

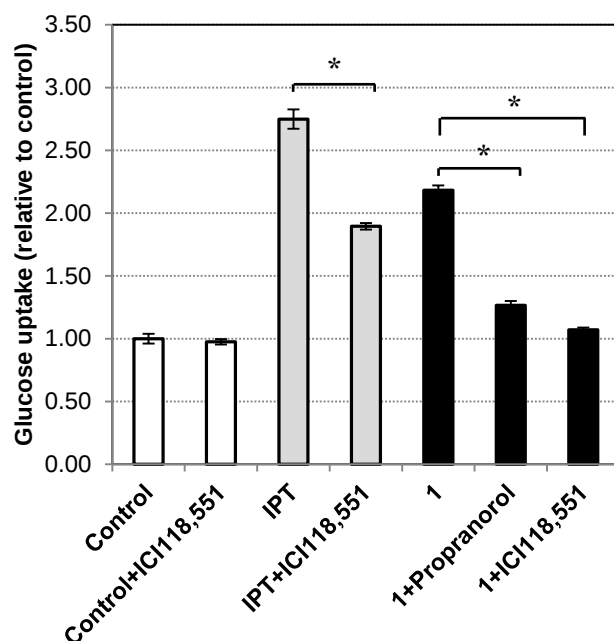


Fig. 4. Co-incubation of **1** with β -adrenergic receptor antagonists. IPT (isoproterenol, positive control): 1 μ M; Compound **1**: 10 μ M; Propranolol: 1 μ M; ICI118,551: 100 nM; * p < 0.01 between the indicated two bars.

4. Conclusions

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

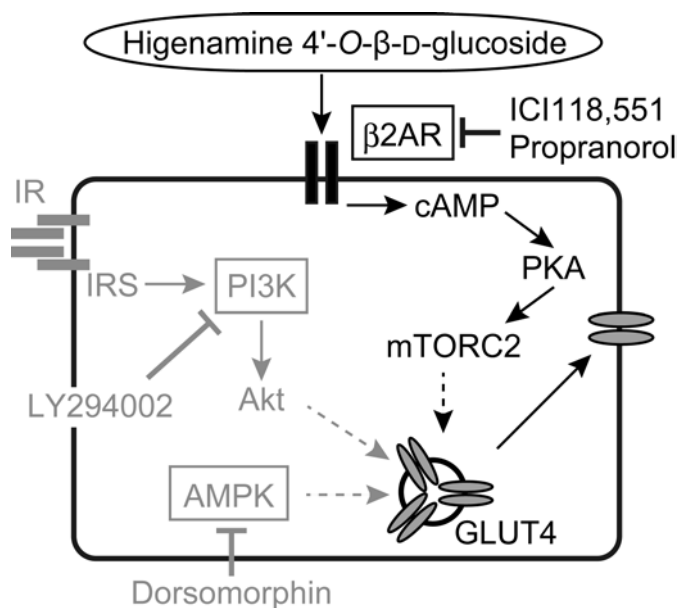


Fig. 5. Signaling pathways of glucose uptake. Black words and arrows are the pathway involved in the activity of higenamine 4'-O-β-D-glucoside (1). Gray words and arrows are the denied pathways. β2AR: β2 adrenergic receptor, PKA: cAMP-dependent protein kinase, mTORC2: mammalian target of the rapamycin complex 2, GLUT4: glucose transporter 4, IR: insulin receptor, IRS: insulin receptor substrate.

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