ACS Medicinal Chemistry Letters

pubs.acs.org/acsmedchemlett

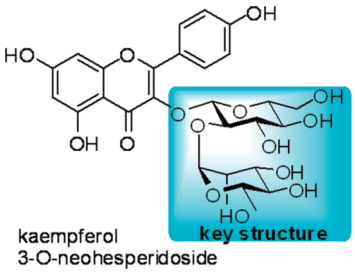
Study of Kaempferol Glycoside as an Insulin Mimic Reveals Glycon To Be the Key Active Structure

Kazuaki Yamasaki,[†] Ryogo Hishiki,[‡] Eisuke Kato,*[,†] and Jun Kawabata[†]

[†]Laboratory of Food Biochemistry, Division of Applied Bioscience, Graduate School of Agriculture and [‡]Laboratory of Food Biochemistry, Department of Bioscience and Chemistry, Faculty of Agriculture, Hokkaido University, Kita-ku, Sapporo, Hokkaido 060-8589, Japan

ABSTRACT Diabetes mellitus is increasing in prevalence with patient numbers rising throughout the world. Current treatments for diabetes mellitus focus on control of blood glucose levels. Certain kinds of flavonoids or their glycosides stimulate cells to improve glucose uptake and lower blood glucose levels. We synthesized kaempferol 3-*O*-neohesperidoside (**1**), a naturally occurring substance present in *Cyathea phalerata* Mart., reported to mimic the action of insulin. Synthetic **1** promoted glucose uptake in the cultured cell line, L6. Further studies to determine the core structure responsible for this activity using synthetic compounds revealed neohesperidose to be the primary pharmacophore. These findings support the use of certain saccharides as a potential novel treatment for diabetes mellitus by replacing or supporting insulin.

KEYWORDS Diabetes mellitus, insulin, flavonoid glycoside, glucose uptake



Insulin, a peptide secreted from β-cells located in the pancreatic islets of Langerhans, is the hormone responsible for reducing the blood glucose level to maintain homeostasis. Impairment in insulin secretion and resistance to the activity of insulin are both causally linked to the development of diabetes mellitus (DM), a disease becoming increasingly prevalent worldwide.[1] DM is termed as chronic hyperglycemia caused either by decreased availability of insulin due to deletion of β-cells (type 1 DM) or by decreased sensitivity to insulin, often accompanied by obesity (type 2 DM). Although the underlying cause of the disease can vary, the treatment of both types of DM is focused on the control of blood glucose levels. Current therapeutic strategies targeted at insulin action include the use of exogenous insulin to lower blood glucose levels; a sulfonylurea, a dipeptidylpeptidase-4 inhibitor, a glucagon-like peptide-1 analogue to improve secretion of insulin; and a peroxisomal proliferator-activated receptor (PPAR) agonist to tackle insulin resistance.[2−6] All of these drugs work to improve the symptoms of DM, but none of them except for insulin itself directly works on somatic cells to regulate the uptake of blood glucose. Thus, in the insulin-resistant type 2 DM, there is a continued need for pharmacological treatments to reduce the levels of blood glucose.

In an effort to overcome this deficit, there have been a number of recent reports describing substances that directly stimulate cells to enhance glucose uptake and decrease blood glucose levels. The essential trace elements, vanadium (or its complexes) and zinc, are both known to work in this way.[7−9] However, the risk of side effects such as metal intoxication precludes accurate estimation of an appropriate dosage and prevents their utilization as a novel therapy for DM. Other than metals, some natural organic compounds, notably flavonoids and their glycosides, are also known to enhance glucose uptake.[10−13] Although most of the reported flavonoids are PPAR agonists that enhance the activity of insulin, kaempferol 3-*O*-neohesperidoside (**1**) isolated from *Cyathea phalerata* Mart. was reported to exhibit an insulin-like activity and is thus a potential "insulin mimetic".[14] The flavonoid structure of **1** is similar to a flavonoid reported previously as a PPAR agonist. However, the insulin-mimetic activity is unique to **1** and has not been reported in any of the other flavonoids. In this paper, we investigate the molecular component responsible for this unique activity of **1**. We synthesized **1** and several related compounds containing substructures of **1** and tested their activity in cultured cells.

The synthesis of **1** has not been reported previously. Thus, we followed the synthetic procedure for related compounds that involves the reaction of neohesperidosyl bromide (**7**) with a flavonol (**14**).[15] Commercially available **2** was subjected to deacetylation and subsequent benzylation to give **3**. The double bond of **3** was then oxidized by dimethyldioxirane (DMDO) to form an α-epoxide, which was selectively opened by an addition of acetic acid to form **4**.[16,17] This selectively protected glucose (**4**) was then reacted with rhamnose imidate (**5**) in the presence of TMSOTf as the catalyst.[18] The resulting compound (**6**) was diastereoselectively brominated in HBr/acetic acid solution to give **7** (Scheme 1). The flavonol (**14**) was then synthesized by a previously

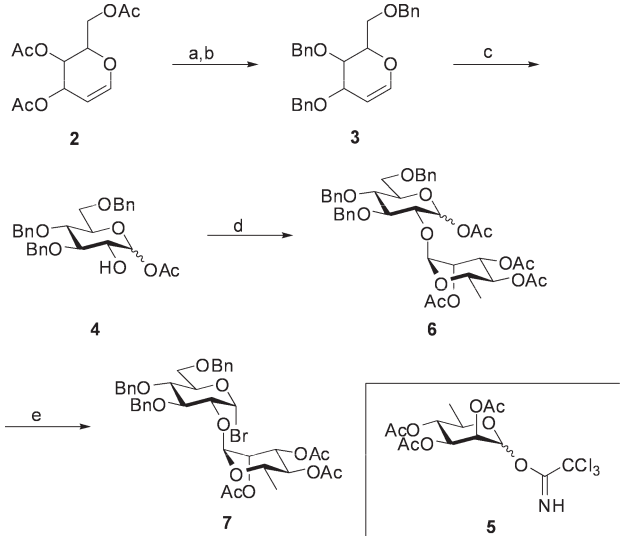
Received Date: July 15, 2010
Accepted Date: October 6, 2010
Published on Web Date: October 11, 2010

DOI: 10.1021/ml100171x | *ACS Med. Chem. Lett.* 2011, *2*, 17–21

used process starting from **8**.[19] After two hydroxyl groups of **8** were protected, the resulting **9** was subjected to the aldol condensation with **10**. Subsequent cyclization in the presence of a catalytic amount of iodine gave **12**. The flavone (**12**) was then oxidized using DMDO, which added a hydroxyl group at the 3-*C* position to give **13**. Although **13** had an amenable structure for the synthesis of **1**, a glycosylation reaction with **7** did not proceed. We suspect that the hydrogen bond between the 3-OH and the carbonyl group was interfering with the reaction. Thus, the benzyl-protecting group of 5-OH was selectively removed by heating in an aqueous acetic acid solution to give **14** (Scheme 2). The flavonol (**14**) was then coupled with **7** in $CHCl_3$-water biphasic solution with K_2CO_3 as a base and tetrabutylammonium bromide (TBAB) as a phase transfer catalyst to give **15** with 47% yield. Finally, the acetyl and benzyl groups of **15** were subsequently removed to yield **1** (7% overall yield) (Scheme 3).[20]

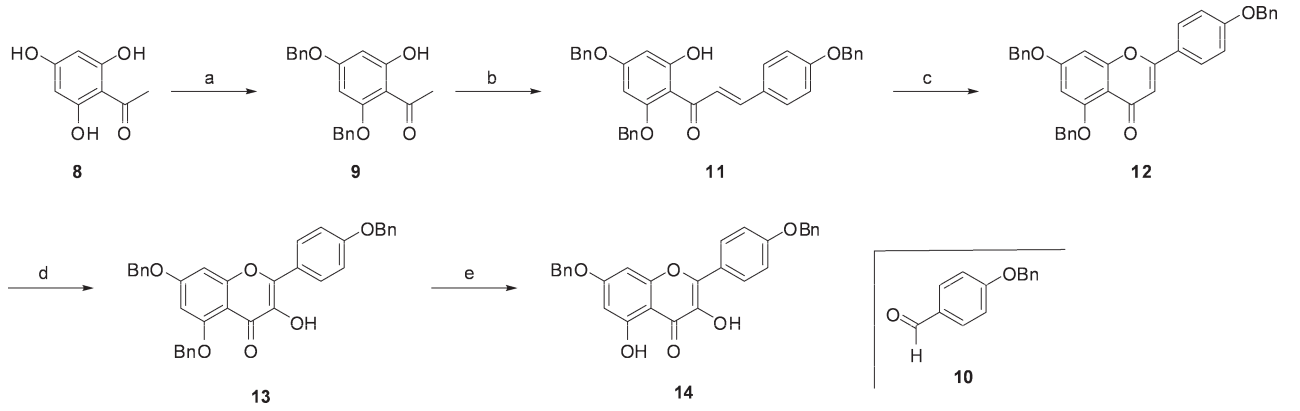
The previous report on the insulin-mimetic properties of **1** used rat muscle segments to test its activity.[14] To evaluate the activity of a large number of compounds, we required enhanced throughput and reproducibility than would be available with a native tissue assay. Cultured cell lines satisfy these requirements, and a muscle model cell line, L6, was selected to test the compounds generated in our study. The insulin-mimetic activity was measured by quantifying the glucose uptake promoted by each sample compound, using 2-deoxyglucose (2-DG) as a nonmetabolic glucose substitute. The amount of 2-DG transported was quantified

Scheme 1. Synthesis of Neohesperidosyl Bromide (**7**)[a]



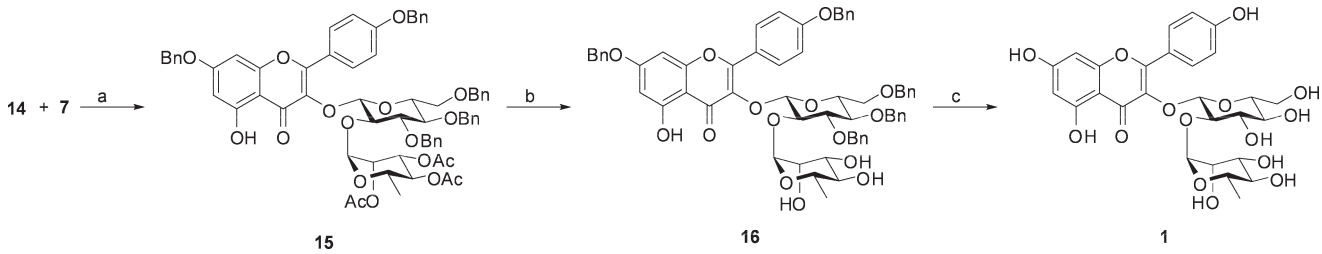
[a] Reagents and conditions: (a) NaOMe, MeOH. (b) BnBr, NaH, DMF (52% from **2**). (c) DMDO, acetone and then AcOH (68%). (d) Compound **5**, TMSOTf, CH_2Cl_2 (58%). (e) 33% HBr/AcOH, CH_2Cl_2 (90%).

Scheme 2. Synthesis of Flavonol (**14**)[a]



[a] Reagents and conditions: (a) BnBr, K_2CO_3, DMF (85%). (b) Compound **10**, NaH, DMF, 0 °C (80%). (c) I_2, DMSO, 120 °C (95%). (d) DMDO, acetone, CH_2Cl_2 (67%). (e) AcOH, H_2O, 110 °C (56%).

Scheme 3. Synthesis of Kaempferol 3-*O*-Neohesperidoside (**1**)[a]



[a] Reagents and conditions: (a) TBAB, K_2CO_3, $CHCl_3$, H_2O (47%). (b) NaOMe, MeOH, $CHCl_3$ (65%). (c) H_2, $Pd(OH)_2$, THF, MeOH (quant.).

DOI: 10.1021/ml100171x | *ACS Med. Chem. Lett.* 2011, *2*, 17–21

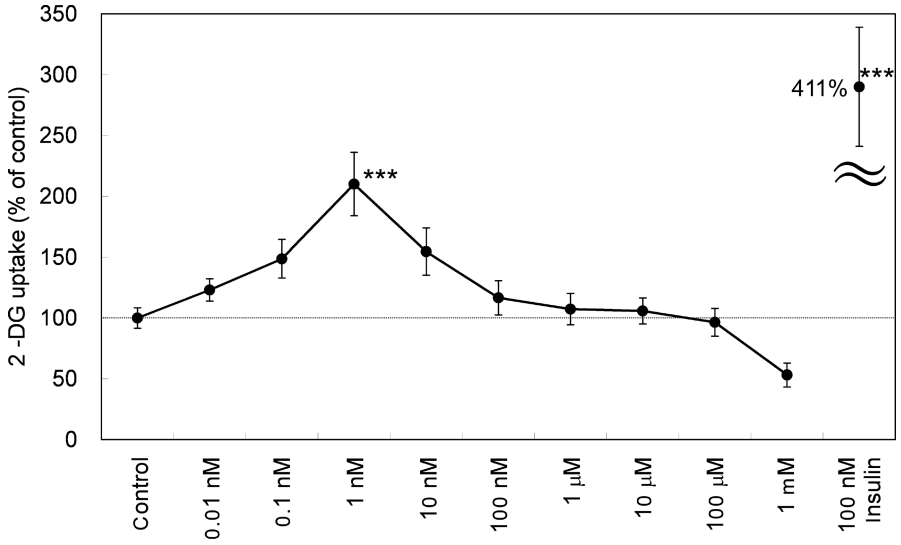


Figure 1. Insulin-mimetic activity of **1** at various concentrations. Differentiated L6 cells were treated with the indicated concentrations of **1** for 4 h. Control cells were incubated without sample; 100 nM insulin was used as a positive control. After incubation, cells were incubated with 2-DG for 20 min, and uptake was measured. Insulin-mimetic activity (i.e., 2-DG uptake) at each concentration of **1** is expressed as a percentage of 2-DG uptake in control cells. Data are means ± SEMs from five independent repeats of the experiment. Each data point was performed in quadruplicate in each experiment. *** denotes a statistically significant difference vs control ($p \leq 0.01$).

by the enzymatic method reported by Yamamoto et al.[21,22] Synthetic **1** exhibited insulin-mimetic activity and promoted the uptake of 2-DG, with peak uptake observed at 1 nM concentration (Figure 1). At this concentration, **1** showed over 200% enhancement of 2-DG uptake as compared with control cells. Higher and lower concentrations of **1** showed weaker activity, and uptake of 2-DG was even decreased as compared with control at 1 mM concentration. Some evidence suggests that the flavonoid moiety may be responsible for this decreased uptake. With chronic exposure (several days), some flavonoids enhance glucose uptake.[10−13] However, in the case of more acute exposure (typically a few hours), it is reported that certain types of flavonoids (including kaempferol) inhibit glucose uptake.[23] Thus, there may be intramolecular conflict in **1** where different moieties have opposite effects, and at high concentration, the effect of the inhibitory moiety may become predominant. Alternatively, it is possible that the inhibition of a glucose transporter may account for the observed decrease in 2-DG uptake, as **1** contains a glucoselike moiety in its structure. The concentration dependence of the activity of **1** follows the result of Zanatta et al., indicating validity of using cultured cell models to evaluate insulin-mimetic activity.[14]

To identify the structural unit responsible for the activity of **1**, we then synthesized and tested several compounds representing various substructures of **1**. Kaempferol (**17**), kaempferol 3-*O*-glucoside (**18**), and neohesperidose (**19**) are the core structural elements of **1** so were primarily selected for screening. Kaempferol (**17**) and neohesperidose (**19**) were easily obtained by deprotection of benzyl and acetyl groups in the synthetic intermediates **13** and **6**, respectively. Kaempferol 3-*O*-glucoside (**18**) was synthesized by attaching glucose to **14**, followed by removal of the protecting groups (Scheme 4). The insulin-mimetic activity of these synthetic compounds is summarized in Figure 2. As mentioned above, many flavonoids or flavonoid-containing plants are reported to show antidiabetic activity. We hypothesized that the aglycon compound **17** might be responsible for the activity of **1**. Unexpectedly, as shown in Figure 2, kaempferol (**17**) and its 3-glucoside (**18**) showed negligible activity. Unexpectedly, the major substructure responsible for the insulin-mimetic activity appeared to be neohesperidose (**19**). This compound induced 200−250% enhancement of 2-DG uptake as compared with control cells at both 0.1 and 1 nM concentrations. Additionally, **19** showed the highest activity at 0.1 nM, a 10-fold lower concentration than the most efficacious dose of **1** (Figure 2). A glycoside bond with enol is relatively weak and may be easily hydrolyzed in aqueous media or by endogenous intracellular glycosidase. The 10-fold lower potency of **1** might be explained by the hydrolysis of ∼10% of **1** to the active product, **19**. We currently have no information to confirm or refute that **1** requires hydrolysis to become active.

Irrespective of this, we believe that the disaccharide is the key structure for insulin-mimetic activity. The control of glucose uptake into the muscle cell by the disaccharide (i.e., sugar) is perhaps intuitive, but to the best of our knowledge, this is a new insight into the function of this disaccharide. Structurally analogous inositol derivatives are reported to mimic the activity of insulin and promote glucose uptake in L6 cells and rat muscle.[24,25] The apparent activity of these inositol derivatives is equivalent to insulin, but their mechanism is suggested to be divergent. Although **19** is dramatically more potent than the inositol derivatives (being active at subnanomolar concentrations, 10^6-fold lower than inositol derivatives), **19** bears structural similarities to inositol and may thus have a similar mechanism of action. Further structural, mechanistic, and pharmacokinetic studies on the active disaccharide may help to develop and optimize a novel treatment for DM.

DOI: 10.1021/ml100171x | *ACS Med. Chem. Lett.* **2011**, *2*, 17–21

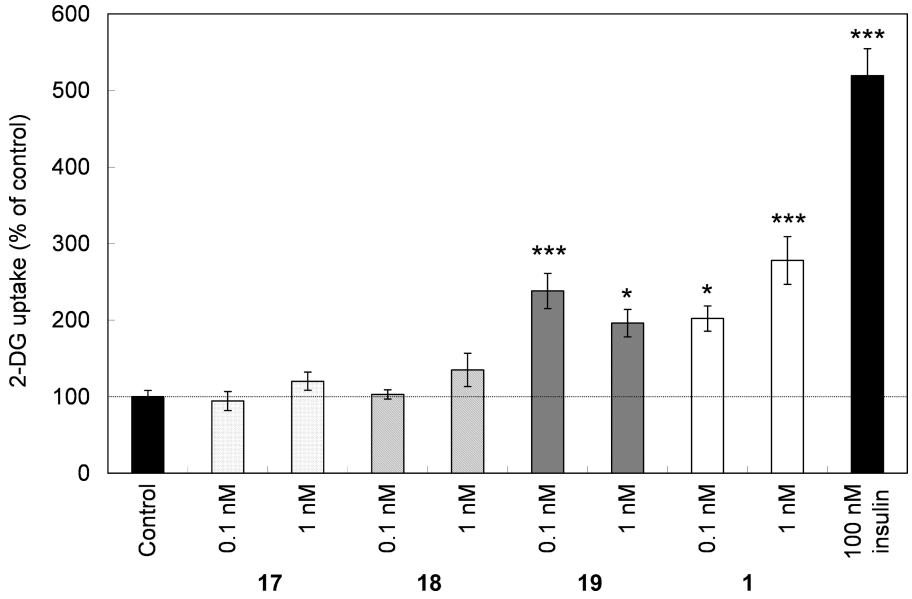
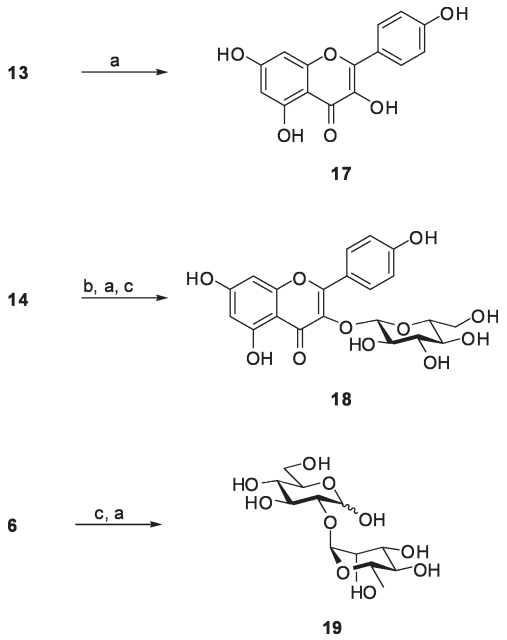


Figure 2. Insulin-mimetic activity of synthetic compounds. Differentiated L6 cells were treated for 4 h with either 0.1 or 1.0 nM concentrations of the indicated compounds. Control cells were incubated either without sample (negative control) or 100 nM insulin (positive control). After incubation, cells were incubated with 2-DG for 20 min, and uptake was measured. The insulin-mimetic activity is expressed relative to 2-DG uptake in control cells. Each data point was performed in quadruplicate. Data are means ± SEMs from five independent repeats of the experiment. *** and * denote statistically significant differences ($p \leq 0.01$ and $p \leq 0.05$, respectively) in relation to control.

Scheme 4. Synthesis of Compounds with Substructure of **1**[a]



[a] Reagents and conditions: (a) H_2, $Pd(OH)_2$, THF, MeOH. (b) 2,3,4,6-Tetra-*O*-acetylglucopyranosyl bromide, K_2CO_3, DMF. (c) NaOMe, MeOH, $CHCl_3$ (**17**, 58%; **18**, 13% in three steps; **19**, 45% in two steps).

In conclusion, we have synthesized **1** for the first time and developed a convenient assay model using cultured L6 cells to examine its insulin-mimetic activity. We have determined that the substructure responsible for this activity is neohesperidose (**19**). The key disaccharide structure responsible for this unique activity discriminates **1** from other flavonoids known to improve hyperglycemia. To our knowledge, this is the first evidence of a disaccharide enhancing cellular glucose uptake and may represent a novel strategy in the development of new treatments for DM. Questions remain with regard to the mechanism of action of **19** in muscle cells and to the possible existence of other bioactive disaccharides. We are currently studying disaccharide structure—activity relationships to address these questions.

SUPPORTING INFORMATION AVAILABLE

Detailed synthetic procedure and ^{1}H NMR spectrum data of new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author: *Tel/Fax: +81-11-706-2496. E-mail: eikato@chem.agr.hokudai.ac.jp.

REFERENCES

(1) WHO. Global burden of disease 2004 update; http://www.who.int/healthinfo/global_burden_disease/2004_report_update/en/index.html (accessed July, 2010).

(2) Müller, G.; Satoh, Y.; Geisen, K. Extrapancreatic effects of sulfonylureas—A comparison between glimepiride and conventional sulfonylureas. *Diabetes Res. Clin. Prac.* **1995,** *28* (Suppl.), S115–S137.

(3) Ye, J.; Doyle, P. J.; Iglesias, M. A.; Watson, D. G.; Cooney, G. J.; Kragen, E. W. Peroxisome proliferator-activated receptor (PPAR)-α activation lowers muscle lipids and improves insulin sensitivity in high fat-fed rats comparison with PPAR-γ activation. *Diabetes* **2001,** *50*, 411–417.

DOI: 10.1021/ml100171x | *ACS Med. Chem. Lett.* **2011,** *2,* 17–21

(4) Krentz, A. J.; Bailey, C. J. Oral Antidiabetic Agents Current Role in Type 2 Diabetes Mellitus. *Drugs* **2005,** *65*, 385–411.
(5) Zerilli, T.; Pyon, E. Y. Sitagliptin Phosphate: A DPP-4 Inhibitor for the Treatment of Type 2 Diabetes Mellitus. *New Drug* **2007,** *29*, 2614–2634.
(6) Knudsen, L. B.; Nielsen, P. F.; Huusfeldt, P. O.; Johansen, N. L.; Madsen, K.; Pedersen, F. Z.; Thøgersen, H.; Wilken, M.; Agersø, H. Potent Derivatives of Glucagon-like Peptide-1 with Pharmacokinetic Properties Suitable for Once Daily Administration. *J. Med. Chem.* **2000,** *43*, 1664–1669.
(7) Sakurai, H.; Shimomura, S.; Fukuzawa, K.; Ishizu, K. Detection of oxovanadium(IV) and characterization of its ligand environment in subcellular fractions of the liver of rats treated with pentavalent vanadium(V). *Biochem. Biophys. Res. Commun.* **1980,** *96*, 293–298.
(8) Cazarolli, L. H.; Zanatta, L.; Jorge, A. P.; Sousa, E.; Horst, H.; Woehl, V. M.; Pizzolatti, M. G.; Szpoganicz, B.; Silva, F. R. M. B. Follow-up studies on glycosylated flavonoids and their complexes with vanadium: Their anti-hyperglycemic potential role in diabetes. *Chem-Biol. Interact.* **2006,** *163*, 177–191.
(9) Sakurai, H.; Adachi, Y. The pharmacology of the insulinomimetic effect of zinc complexes. *BioMetals* **2005,** *18*, 319–323.
(10) Anton, S.; Melville, L.; Rena, G. Epigallocatechin gallate (EGCG) mimics insulin action on the transcription factor FOXO1a and elicits cellular responses in the presence and absence of insulin. *Cell. Signalling* **2007,** *19*, 378–383.
(11) Jorge, A. P.; Horst, H.; Sousa, E.; Pizzolatti, M. G.; Silva, F. R. M. B. Insulinomimetic effects of kaempferitrin on glycaemia and on ^{14}C-glucose uptake in rat soleus muscle. *Chem-Biol. Interact.* **2004,** *149*, 89–96.
(12) Hsu, F.; Liu, I.; Kuo, D.; Chen, W.; Su, H.; Cheng, J. Antihyperglycemic effect of puerarin in streptozotocin-induced diabetic rats. *J. Nat. Prod.* **2003,** *66*, 788–792.
(13) Sousa, E.; Zanatta, L.; Seifriz, I.; Creczynski-Pasa, T. B.; Pizzolatti, M. G.; Szpoganicz, B.; Silva, F. R. M. B. Hypoglycemic effect and antioxidant potential of kaempferol-3,7-*O*-(α)-dirhamnoside from *Bauhinia forficata* leaves. *J. Nat. Prod.* **2004,** *67*, 829–832.
(14) Zanatta, L.; Rosso, Â.; Folador, P.; Figueiredo, M. S. R. B.; Pizzolatti, M. G.; Leite, L. D.; Silva, F. R. M. B. Insulinomimetic effect of kaempferol 3-neohesperidoside on the rat soleus muscle. *J. Nat. Prod.* **2008,** *71*, 532–535.
(15) Du, Y.; Wei, G.; Linhardt, R. J. The first total synthesis of calabricoside A. *Tetrahedron Lett.* **2003,** *44*, 6887–6890.
(16) Murray, R. W.; Singh, M. Synthesis of epoxides using dimethyldioxirane: *trans*-Stilbene oxide. *Org. Synth. Coll. Vol.* **1998,** *9*, 288.
(17) Messaoudi, S.; Anizon, F.; Pfeiffer, B.; Golsteyn, R.; Prudhomme, M. Synthesis of a staurosporine analogue possessing a 7-azaindole unit instead of an indole moiety. *Tetrahedron Lett.* **2004,** *45*, 4643–4647.
(18) Francisco, C. G.; Herrera, A. J.; Kennedy, A. R.; Martín, A.; Melián, D.; Pérez-Martín, I.; Quintanal, L. M.; Suárez, E. Intramolecular 1,8-Hydrogen-Atom Transfer Reactions in (1→4)-*O*-Disaccharide Systems: Conformational and Stereochemical Requirements. *Chem.—Eur. J.* **2008,** *14*, 10369–10381.
(19) Smith, J. A.; Maloney, D. J.; Hecht, S. M.; Lannigan, D. A. Structural basis for the activity of the RSK-specific inhibitor, SL0101. *Bioorg. Med. Chem.* **2007,** *15*, 5018–5034.
(20) Spectral data of **1**: ^{1}H NMR (500 MHz, CD_3OD, room temeprature): 8.02 (2H, d, J = 8.8 Hz), 6.86 (2H, d, J = 8.8 Hz), 6.27 (1H, d, J = 1.2 Hz), 6.10 (1H, d, J = 1.2 Hz), 5.70 (1H, d, J = 7.5 Hz), 5.21 (1H, d, J = 1.2 Hz), 4.02 (1H, dq, J = 6.3, 9.6 Hz), 3.99 (1H, dd, J = 1.2, 3.4 Hz), 3.77 (1H, dd, J = 3.4, 9.6 Hz), 3.71 (1H, dd, J = 2.2, 12.3 Hz), 3.65—3.48 (5H, m), 3.22 (1H, ddd, J = 2.2, 5.8, 9.1 Hz), 0.95 (3H, d, J = 6.3 Hz) ppm. ^{13}C NMR (125 MHz, CD_3OD, room temeprature): 179.3, 170.0, 163.3, 161.7, 159.0, 158.2, 134.5, 132.3, 123.5, 116.5, 105.1, 102.9, 101.4, 100.6, 95.8, 80.4, 79.3, 78.7, 74.4, 72.7, 72.6, 72.1, 70.2, 62.9, 17.9 ppm. HR FAB MS (negative): $[M]^-$ found *m/z*, 593.1472; $C_{27}H_{30}O_{15}^-$ requires *m/z* 594.1585. $[\alpha]_D^{20}$ −80° (*c* 1.0, MeOH). Spectral data were identical to the reported data.[26]
(21) Yamamoto, N.; Sato, T.; Kawasaki, K.; Murosaki, S.; Yamamoto, Y. A nonradioisotope, enzymatic assay for 2-deoxyglucose uptake in L6 skeletal muscle cells cultured in a 96-well microplate. *Anal. Biochem.* **2006,** *351*, 139–145.
(22) Assay procedure of the insulin-mimetic activity. The tested compounds were dissolved in 2% fetal bovine serum (FBS)/Dulbecco's modified Eagle's medium (DMEM) and diluted to an appropriate concentration with the same media. L6 cells were plated in 24- or 48-well plates (5×10^4 and 2.5×10^4 cells per well, respectively) in DMEM media containing 10% FBS and grown until confluent. Cell differentiation was induced by replacing the growth medium with DMEM containing 2% FBS and growing them for an additional 5 days in these reduced-serum conditions, with culture media changed every other day. On the day of measurement, culture media were removed and replaced with either 2% FBS/DMEM (control cells) or sample solution or the same media containing 100 nM insulin [insulin (+) cells]. After 4 h of incubation, buffer was again removed and replaced with 1 mM 2-DG dissolved in 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). After 20 min of incubation, cells were rinsed three times with KRPH buffer and before addition of 0.1 M NaOH(aq). The cell lysate was frozen once and then heated at 85 °C for 40 min. The lysate was neutralized by 0.1 M HCl(aq), and triethanolamine buffer (150 mM, pH 8.1) was added. The amount of 2-DG contained in the lysate was then measured using an enzymatic method described previously.[27] The amount of 2-DG transported by the cells in each well was calculated in nmol/min. Uptake is normalized to the uptake value in control cells ("% control"). Each sample was tested in quadruplicate, and each experiment was repeated independently at least three times. Data are shown as means ± SEMs. Differences between control and insulin (+) groups were statistically analyzed using Tukey−Kramer's test, with p values < 0.05 being accepted as significant.
(23) Nomura, M.; Takahashi, T.; Nagata, N.; Tsutsumi, K.; Kobayashi, S.; Akiba, T.; Yokogawa, K.; Moritani, S.; Miyamoto, K. Inhibitory Mechanisms of Flavonoids on Insulin-Stimulated Glucose Uptake in MC3T3-G2/PA6 Adipose Cells. *Biol. Pharm. Bull.* **2008,** *31*, 1403–1409.
(24) Dang, N. D.; Mukai, R.; Yoshida, K.; Ashida, H. A nonradioisotope, enzymatic assay for 2-deoxyglucose uptake in L6 skeletal muscle cells cultured in a 96-well microplate. *Biosci., Biotechnol., Biochem.* **2010,** *74*, 1062–1067.
(25) Yap, A.; Nishiumi, S.; Yoshida, K.; Ashida, H. Rat L6 myotubes as an in vitro model system to study GLUT4-dependent glucose uptake stimulated by inositol derivatives. *Cytotechnology* **2007,** *55*, 103–108.
(26) Kazuma, K.; Noda, N.; Suzuki, M. Malonylated flavonol glycosides from the petals of Clitoria ternatea. *Phytochemistry* **2003,** *62*, 229–237.
(27) Kato, E.; Kawabata, J. Glucose uptake enhancing activity of puerarin and the role of C-glucoside suggested from activity of related compounds. *Bioorg. Med. Chem. Lett.* **2010,** *20*, 4333–4336.

DOI: 10.1021/ml100171x | *ACS Med. Chem. Lett.* **2011,** *2*, 17–21