# Synthesis and Biological Evaluation of Two Salidroside Analogues in the PC12 Cell Model Exposed to Hypoglycemia and Serum Limitation

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**Salidroside is a phenylpropanoid glycoside isolated from *Rhodiola rosea* L., a traditional Chinese medicinal plant, and has displayed a broad spectrum of pharmacological properties. In this paper, two analogues were prepared with the glucosamine and *N*-acetylglucosamine as glycosyl donor, 2-(4-hydroxyphenyl)ethanol as glycosyl acceptor. The effects of them over PC12 cell model exposed to hypoglycemia and serum limitation were assessed with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, Flow Cytometry and Western blot analysis.**

**Key words** salidroside; analogues; *N*-acetylglucosamine; hypoglycemia; PC12 cell

Salidroside (Fig. 1), a traditional Chinese medicine, which may be synthesized by glucosylation of 2-(4-hydroxyphenyl)ethanol, showed a range of pharmacological properties, such as resisting anoxia,[1)] anti-radiation and anti-fatigue,[2,3)] postponing ageing,[4)] preventing cardiovascular disease[5)] and anti-tumor.[6)] It has been reported that the species of glycosyl donor could affect activities of glycosides, for example, the antioxidant activity increases in the order glucose$<$mannose$\leq$fructose.[7)]

*N*-Acetylglucosamine (GlcNAc) can be obtained by either chemical or enzymatic hydrolysis of chitin and chitosan.[8)] Both natural and synthetic glucosamine-containing compounds have been reported to have a range of biological activities.[9—12)] *N*-Acetylglucosamine has been shown, therefore, to be an available starting material for physiologically or pharmacologically important products.

The purpose of this study was to synthesize two salidroside analogues with the glucosamine and *N*-acetylglucosamine as glycosyl donor, 2-(4-hydroxyphenyl)ethanol as glycosyl acceptor, and to investigate their activities in the PC12 cell model exposed to hypoglycemia and serum limitation, as compared with salidroside.

The synthesis of the compound **3** was completed, starting from 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-$\alpha$-D-glucopyranosyl chloride **1** and 2-(4-(benzyloxy)phenyl)ethanol **2**. The reaction was conducted in dry dichloromethane for 3 h with zinc chloride and 4,4′-dimethoxytriphenylmethyl chloride as catalysts. Compound **1** can readily be prepared in a single step from *N*-acetylglucosamine by reaction with acetyl chloride.[13)] The designed target compound **5** was obtained from **3** by direct deacetylation with $CH_3ONa/CH_3OH$ and catalytic hydrogenation by refluxing with 5% Pd/C and $HCOONH_4$ in anhydrous methanol. Compound **6** was obtained from **5** by refluxing with 30% KOH/ethanol (m/v) (Chart 1). Compounds **5** and **6** were beta configuration identified from $^1$H-NMR studies.

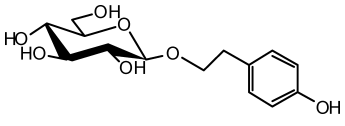
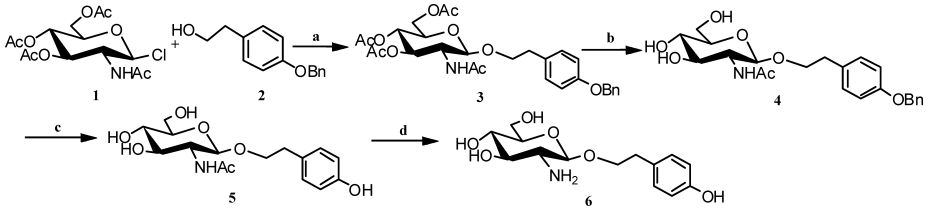


Fig. 1. The Chemical Structure of Salidroside

According to 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, after the PC12 cells were exposed to hypoglycemia and serum limitation for 24 h, cell viabilities were significantly decreases, as compared to control group. And further showed that pretreatment with compounds **5**, **6** and salidroside at different concentrations (80, 160, 320 $\mu$g/ml) significantly attenuated the cell viability loss evoked by hypoglycemia and serum limitation, and the attenuating effect on the survival of cultured PC12 cells displayed a dose dependent pattern (Fig. 2).

The change in the percentage of apoptotic cells were also analyzed by Hoechst 33342 staining (Fig. 3). In comparison to control group, exposure to hypoglycemia and serum limitation alone produced significantly apoptosis. Pre-incubation with 80, 160, and 320 $\mu$g/ml of compounds **5**, **6** and salidroside, the apoptotic percentages were significantly lower than that produced by exposure to hypoglycemia and serum limitation alone.

The quantitative comparison indicated that the percentage of early apoptotic PC12 cells induced by exposure to hypoglycemia and serum limitation alone was significantly larger than in control group, and also significantly larger than that



Reagents: a) $ZnCl_2$, DMT·Cl, $CH_2Cl_2$; b) NaOMe, MeOH, rt, 3 h; c) $HCOONH_4$, 5% Pd/C, $CH_3OH$, reflux; d) 30% KOH, EtOH.

Chart 1. Synthetic Route for Compounds **5** and **6**

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induced by pretreatment with 320 $\mu$g/ml compounds **5**, **6** and salidroside followed by exposure to hypoglycemia and serum limitation. The percentage of necrotic cells, however, displayed no significant difference among the different groups (Fig. 4).

Western blot analysis showed that compounds **5** and **6** against hypoglycemia and serum limitation-induced cell apoptosis in the PC12 cells partly attributed to their evoked modulation of apoptosis-related gene expression, which was in accordance with salidroside[14] (Fig. 5).

## Experimental

**General** Commercial reagents were used without further purification unless otherwise stated. Melting points were uncorrected. $^1$H-NMR spectra were recorded on a Bruker AC 400 instrument at 300 MHz, with tetramethylsilane (TMS, $\delta$ 0.00) as the internal standard and were run in $D_2O$, $J$ values were expressed in Hz. Elemental analysis (C, N and H) were performed on ElementarVarioEL III analyzer (German). Flash chromatography was performed on silica gel (200—300 mesh). Primary monoclonal anti-B-cell lymphoma-2 (Bcl-2) antibody, primary monoclonal anti-Bax antibody, and primary monoclonal anti-$\beta$-actin antibody were purchased from Sigma. AnnexinV-fluorescein isothiocyanate (FITC) ApoptosisDetection Kit was purchased from BenderMedsystens. IRDye 800 Conjugated Affinity Purified Goat Anti-Mouse immunoglobulin G (IgG) was purchased from KPL (USA). Slidroside was purchased from Aladdin.

**General Procedure for Compounds 5 and 6** 2-Acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-$\alpha$-D-glucopyranosyl chloride **1** (1.0 g, 2.7 mmol) and 2-(4-(benzyloxy)phenyl)ethanol **2** (500 mg, 2.3 mmol) were added to a suspension of zinc chloride (280 mg, 2 mmol) and 4,4′-dimethoxytriphenylmethyl (710 mg, 2 mmol) in a dry dichloromethane. The mixture was stirred at room temperature for 5 h until reaction was complete as shown by TLC, then 20 ml dichloromethane was added, and the mixture was washed with saturated sodium bicarbonate. Solvent was removed *in vacuo* and the residue was purified by column chromatography to yield the glycoside **3** 0.91 g (72.2%).

To a solution of compound **3** (1.6 mmol) in MeOH (25 ml) was added NaOMe (40 mg), and the reaction mixture was stirred at room temperature for 3 h. After that diluted with MeOH and acidified with Amberlite IRA-120 (H+) resin to pH=7. The reaction mixture was filtered and evaporated to dryness under reduced pressure afford **4** (0.69 g, 86.9%) as white solid.

A mixture of **4** (600 mg, 1.4 mmol), 5% Pd/C (210 mg) and ammonium formate (441 mg, 7 mmol) in MeOH (10 ml) was stirred at refluxing for 6 h. Then filtered, concentrated and purified by column chromatography using 8 : 1 $CHCl_3$–MeOH to give white solid 430 mg (90%) of **5**, mp 176—178 °C, $Rf$=0.22 (MeOH : $CHCl_3$=1 : 5). $^1$H-NMR ($D_2O$) $\delta$: 7.17 (2H, d, $J$=8.07 Hz), 6.87 (2H, d, $J$=7.86 Hz), 4.46 (1H, d, $J$=8.07 Hz), 4.12—4.17 (1H, m), 3.94 (1H, d, $J$=12.3 Hz), 3,73—3.76 (2H, m), 3.61 (1H, t, $J$=9.8, 8.58 Hz), 3.43—3.48 (3H, m), 2.77—2.82 (2H, m), 1.81 (3H, s). IR (KBr) cm$^{-1}$: 3345, 2928, 2842, 1655, 1520, 1451, 1382, 1274, 1128, 1082, 1006, 825. *Anal.* Calcd for $C_{16}H_{23}NO_7$: C, 56.30; H, 6.79; N, 4.10. Found: C, 56.36; H, 6.83; N, 4.03.

Compound **5** (375 mg, 1.1 mmol) was added to 15 ml 30% KOH/EtOH (m/v) in 50 ml flask, refluxing for 10 h, extracted with dichloromethane, concentrated and purified by column chromatography using 1 : 5 MeOH–$CHCl_3$ to give 290 mg (88%) of **6**, mp 151—153 °C, $Rf$=0.34 (MeOH : $CHCl_3$=

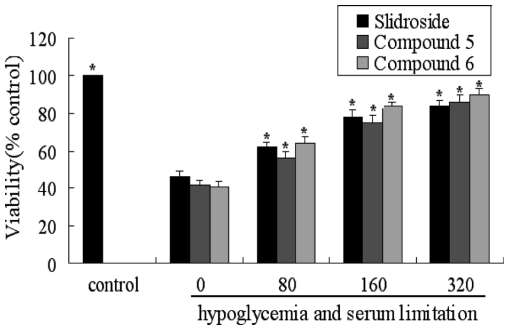


Fig. 2. Protective Effect of Compounds **5**, **6** and Salidroside on Hypoglycemia and Serum Limitation Induced Cytotoxicity in PC12 Cells

The incubation in the high-glucose medium during the whole treatment period served as control group, and the treatment only with hypoglycemia and serum limitation for 24 h served as hypoglycemia and serum limitation alone group. The cell viabilities were expressed as the percent (%) of the control value by using MTT assay. The data were expressed as means±S.D. of three independent experiments ($n$=3). $*p<0.01$ *vs.* hypoglycemia and serum limitation alone group.

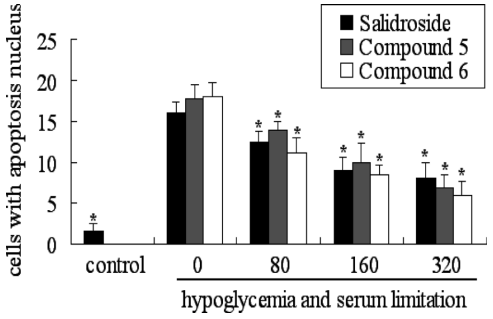


Fig. 3. The Percentage of Apoptotic Cells in the Total Cell Population Is Shown for Different Treatments of PC12 Cell the Same as in Fig. 2

$*p<0.01$ *vs.* hypoglycemia and serum limitation alone group.

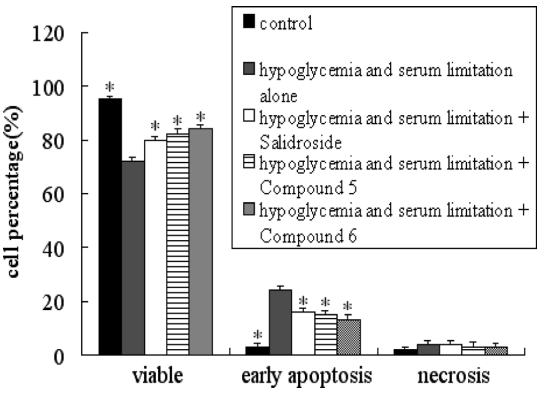


Fig. 4. Flow Cytometry with AnnexinV/PI Staining for Cultured PC12 Cells in Control Group, Hypoglycemia and Serum Limitation Alone Group, Pretreatment with 320 $\mu$g/ml Compounds **5**, **6** and Salidroside Plus Exposure to Hypoglycemia and Serum Limitation

The percentage of viable, early apoptotic, and necrotic cells in the total cell population was shown for the above five different treatments. The data were expressed as means±S.D. of three independent experiments ($n$=3). $*p<0.01$ *vs.* hypoglycemia and serum limitation alone group.

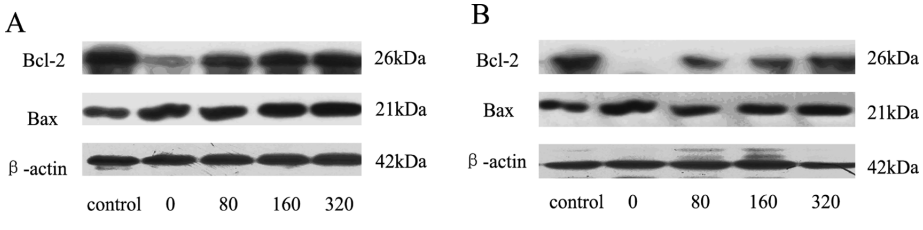


Fig. 5. Western Blotting Image for (a) Compound **5**, (b) Compound **6**

1 : 3), $^1$H-NMR ($D_2O$) $\delta$: 8.46 (1H, s), 7.24 (2H, d, $J$=8.07 Hz), 6.90 (2H, d, $J$=8.07 Hz), 4.70 (1H, d, $J$=8.9 Hz), 4.12—4.17 (1H, m), 3.90—3.94 (2H, m), 3.72—3.76 (1H, m), 3.61—3.67 (1H, m), 3.45—3.47 (2H, m), 3.01 (1H, d, $J$=9.27 Hz), 2.89—2.95 (2H, m). IR (KBr) cm$^{-1}$: 3404, 2917, 2840, 1639, 1517, 1460, 1095, 1066, 713, 627. *Anal.* Calcd for $C_{14}H_{21}NO_6$: C, 56.18; H, 7.07; N, 4.68. Found: C, 56.22; H, 7.12; N, 4.60.

**Cell Culture** Rat PC-12 cells, obtained from the American Type Culture Collection (Manassas, VA, U.S.A.), were plated and maintained in high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% horse serum, 5% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 U/ml streptomycin in 5% $CO_2$/air at 37 °C. After being pretreated with 80, 160, or 320 $\mu$g/ml **5**, **6** and salidroside for 24 h, the cells were subjected to hypoglycemia and serum limitation by replacing the culture medium with the glucose-free DMEM supplement with 1% horse serum and 1% FBS, 100 U/ml penicillin, and 100 U/ml streptomycin, and in the presence of **5**, **6** and salidroside at original concentrations for another 24 h incubation.[15] At the end of incubation, the MTT solution was added and further incubated for 4 h at 37 °C followed by the addition of dimethyl sulfoxide (DMSO) to dissolve the resulting formazan. The absorbance (OD) values were measured at 570 nm with Thermo Multiskan MK3 (U.S.A.).

**Detection of Apoptosis** On the detection of apoptosis, PC12 cells were fixed in 4.0% paraformaldehyde, then stained with Hoechst 33342 at 37 °C, followed by observation under a fluorescence microscope (Olympus, Japan). In order to quantify the apoptotic process, cells with fragmented or condensed DNA and normal DNA were counted, data were expressed as the ratio of apoptotic cells to total cells.

**Flow Cytometry**[14] The PC12 cells were resuspended in buffer at a concentration of $1\times10^6$ cell/ml. FITC-conjugated annexin V and propidium iodide (PI) were added, after incubation in the dark at room temperature, cells were analyzed with a flow cytometer (BD FACSAriaII, U.S.A.).

**Western Blot** The PC12 cells were subjected to Western blot analysis for Bcl-2 and Bax protein expression. Cell proteins were extracted and quantified by a bicinchoninic acid (BCA) kit, followed by electrophoretic separation on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After transferring to polyvinylidene difuoride (PVDF) membranes, samples were allowed to react with primary mouse monoclonal antibodies against Bcl-2 (1 : 400) and primary mouse monoclonal antibodies against Bax (1 : 200) respectively, and subsequently with IRDye 800-Conjugated Affinity Purified Goat Anti-Mouse IgG (1 : 5000). The images were scanned with GS800 Densitometer Scanner (Bio-Rad). $\beta$-Actin served as an internal control.

**Statistical Analysis** All measurements were repeated 3 times, data were expressed as means±S.D. Statistical differences among groups were analyzed by one-way analysis of variance (ANOVA) tests, and differences were considered significant if $p<0.05$.

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**References**

1) Chen Y. M., Chen J., Mao G. M., Fu J. Y., *Zhejiang Prev. Med.*, **19**, 92—93 (2007).
2) Tong G. H., Tan Z. S., Yang Q., *J. Health Toxicol.*, **18**, 183—184 (2004).
3) Luo C. H., Shu R., *Modern Appl. Med.*, **13**, 5—7 (1996).
4) Saratikov A. S., *Pharmazie*, **69**, 394—398 (1968).
5) Bonanome A., Pagnan A., Caruso D., Toia A., Xamin A., Fedeli E., Berra B., Zamburlini A., Ursini F., Galli G., *Nutr. Metab. Cardiovasc. Dis.*, **10**, 111—120 (2000).
6) Udintsev S. N., Schakhov V. P., *Eur. J. Cancer*, **27**, 1182 (1991).
7) Grankvist K., *Biochem. J.*, **200**, 685—690 (1981).
8) Akiyama K., Kawazu K., Kobayashi A., *Carbohydr. Res.*, **279**, 151—160 (1995).
9) Petitou M., van Boeckel C. A. A., *Pure Appl. Chem.*, **69**, 1839—1846 (1997).
10) Solini A., Vergnani L., Ricci F., Crepaldi G., *Diabetes Care*, **20**, 819—823 (1997).
11) Hyers T. M., Agnelli G., Hull R. D., Weg J. G., Morris T. A., Samama M., Tapson V., *Chest*, **114**(Suppl.), 561S—578S (1998).
12) Sears P., Wong C. H., *Science*, **291**, 2344—2350 (2001).
13) Frankfurt W. S., Eschborn G. K., U.S. Patent 5696246 (1997).
14) Yu S., Liu M., Gu X. S., Ding F., *Cell. Mol. Neurobiol.*, **28**, 1067—1078 (2008).
15) Guo Y. B., Zhao Y. H., Zheng C., Meng Y., Yang Y. M., *Chem. Pharm. Bull.*, **58**, 1627—1629 (2010).