ORIGINAL ARTICLE

Three new lignan glucoisides from the roots of *Scutellaria baicalensis*

Hailin Long, Haijing Zhang, Anjun Deng, Lin Ma, Lianqiu Wu*, Zhihong Li, Zhihui Zhang, Wenjie Wang, Jiandong Jiang, Hailin Qin*

State Key Laboratory of Bioactive Substance and Function of Natural Medicines, Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100050, China

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**KEY WORDS**

*Scutellaria baicalensis*; Roots; Labiatae; Lignan glucoside; Baicalensinosides A–C; 3,4-Dibenzyltetrahydrofuran; Osteoprotegerin-activating activity; Anti-osteoporotic activity

**Abstract**  Three new lignan glucosides, baicalensinosides A–C (1–3), were isolated from the roots of *Scutellaria baicalensis*. The structural elucidation was achieved by in-depth spectroscopic examinations and qualitative chemical test. Structurally, these compounds belong to the 3,4-dibenzyltetrahydrofuran-type lignan glycoside with a mono-hydroxyl substitution at the 7-position of benzylidene group on the numbering system of lignans being one of their shared critical features. The anti-osteoporotic activity of the isolated compounds was assessed in an *in vitro* osteoprotegerin (OPG) transcriptional activity assay using dual luciferase reporter detection. At 10 μmol/L, compounds 1–3 increased the relative activating ratio of OPG transcription to 1.83, 0.84 and 0.98 times that of the control group, respectively.

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1. Introduction

*Scutellaria baicalensis* Georgi from the Labiatae family is one of the most famous crude drugs in traditional Chinese medicine (TCM). In addition to the functions of clearing internal fire, removing damp-heat, and countering toxic pathogens according to the theory of TCM, it has also been reported to possess antiallergic, antipyretic, antibacterial, antiviral, anticancer, and anti-inflammatory properties in modern pharmacology. Phytomolecular profiling of *S. baicalensis* spectra. The hexose moiety of all the new compounds was determined as glucose, which, 2 new flavonoids isolated from the EtOAc-soluble fraction of the EtOH extract from the roots were recently reported by our group. As part of our ongoing effort to discover potential bioactive natural compounds from Chinese medicinal plants, the n-BuOH-soluble fraction of the EtOH extract from the roots was also investigated systematically and, in addition to known compounds, 3 new liganin glucosides were also obtained and identified. The isolated compounds were tested for their anti-osteoporotic activity in an *in vitro* osteoprotegerin (OPG) transcriptional activity assay using dual luciferase reporter detection. This article describes the isolation and structural determination of these new compounds, and their anti-osteoporotic activity.

2. Results and discussion

The neutral n-BuOH-soluble section of the EtOH extract from the roots of *S. baicalensis* was subjected to multiple column chromatographic purification steps and further purified by preparative HPLC, affording 3 new liganin glucosides (1–3) on top of some known compounds. These new compounds were all identified as liganin glucosides sharing 12 aromatic and 6 aliphatic carbons for the liganin skeletons and 6 aliphatic carbons for the hexose unit when removing the methoxy groups from the 

The molecular formula of compound 1 was determined as C_{29}H_{38}O_{13} by 

The IR spectrum displayed absorption bands of hydroxy (3396 cm\(^{-1}\)), aromatic (1598 and 1658 cm\(^{-1}\)), and etheric (1329 and 1120 cm\(^{-1}\)) functionalities. An in-depth analysis of the NMR data of 2, together with a comparison with those of 1, revealed that compound 2 was very similar to 1 structurally. In the H NMR spectrum, the main difference was that the set of resonances of the symmetrically 1,3,4,5-tetra substituted benzene ring at δ_H 6.46 (2H, s, H-2/6) in compound 1 was replaced by a set of resonances of a 1,3,4-trisubstituted benzene ring on the numbering system of lignans. Except for the signals from the glucose moiety which resonated at δ_H 4.86 (1H, d, J = 7.2 Hz, H-1’), 3.06-3.22 (4H, m, H-2’, 3’, 4’, 5’), 3.43 (1H, dd, J = 12.6, 5.4 Hz, H-6’a), and 3.59 (1H, dd, J = 12.6, 2.4 Hz, H-6’b), respectively, signals of a phenolic hydroxy group at δ_H 8.08 (1H, s), a methylene at δ_H 2.43 (1H, dd, J = 13.2, 11.4 Hz, H-7a) and 2.82 (1H, dd, J = 13.2, 4.8 Hz, H-7b), a methineoxy at δ_H 4.73 (1H, d, J = 6.0 Hz, H-7’), 2 methines at δ_H 2.59 (1H, m, H-8) and 2.26 (1H, dddd, J = 7.2, 7.2, 6.0, 6.0 Hz, H-8’), 2 methyleneoxys at δ_H 3.59 (1H, m, H-9a) and 3.92 (1H, dd, J = 7.8, 6.6 Hz, H-9b) and δ_H 3.52 (1H, dd, J = 10.8, 7.2 Hz, H-9’a) and 3.72 (1H, ov, H-9’b) were also evident in the H NMR spectrum (Table 1), which were confirmed by an HSQC experiment. The 13C NMR spectrum of 1 showed 28 carbons which were classified by the DEPT NMR experiment into 4 methyls, 4 methylenes, 12 methines, 6 oxygenated aromatic tertiary carbons, and 2 aromatic quaternary carbons, with the aromatic, aliphatic, and oxygenated carbons being categorized unequivocally by their chemical shifts. The HMBC spectrum unambiguously established the 2D structure of compound 1 as 4’-(β-D-glucopyranosyloxy)-3,3’,5,5’,5-tetramethoxy-9,9’-epoxylignane-4,7-diol by the correlations from H-2/6 to C-3/5, 4, and 7, from OH-4 to C-3/5 and 4, from H-2’/6’ to C-1’, 3’/5’, 4’, and 7’, from H-7’ to C-2’/6’, 8’, and 9’, from H-8’ to C-1’, 7’, 8, and 9’, and from H-9b (δ_C 3.92) to C-7 and 8’ (Fig. 2).

The molecular formula of compound 2 was established as C_{29}H_{38}O_{13} by 13C NMR analysis and by an HR-ESI-MS experiment in the positive ion mode with the sodium adduct ion at m/z 605.2107, indicating 10 indices of hydrogen deficiency. The IR spectrum displayed absorption bands of hydroxy (3396 cm\(^{-1}\)), aromatic (1598 and 1658 cm\(^{-1}\)), and etheric (1329 and 1120 cm\(^{-1}\)) functionalities. An in-depth analysis of the NMR data of 2, together with a comparison with those of 1, revealed that compound 2 was very similar to 1 structurally. In the H NMR spectrum, the main difference was that the set of resonances of the symmetrically 1,3,4,5-tetra substituted benzene ring at δ_H 6.46 (2H, s, H-2/6) in compound 1 was replaced by a set of resonances of a 1,3,4-trisubstituted benzene ring on the numbering system of lignans at δ_H 6.76 (1H, d, J = 1.8 Hz, H-2), 6.69 (1H, d, J = 7.8 Hz, H-5), and 6.60 (1H, dd, J = 7.8, 1.8 Hz, H-6), with 3 total methoxyls only, in compound 2. In the 13C and DEPT NMR spectra, the main difference was the appearance of an aromatic methine at δ_C 115.3 (C-5) in 2 which replaced the oxygenated tertiary aromatic carbon at δ_C 147.9 (C-5) in 1, with the downfield shifts of ∆δ +11.0 and +14.6 from C-4 and C-6, respectively, being observed (Table 1). Along with the HSQC spectrum, the correlations from H-2 to C-1, 3, 4, 6, and 7; from OH-4 to C-3, 4, and 5; from H-5 to C-1, 3, and 4; and from H-6 to C-2, 4, and 7 in the HMBC spectrum confirmed this assignment (Fig. 2). The HMBC correlations from H-2/6’ to C-1’, 3’/5’, 4’, and 7’, from H_2

![Figure 1](image-url) The structures of compounds 1–3.
Table 1 $^1$H and $^{13}$C NMR spectroscopic data for compounds 1–3.

| No. | $^1$H (mult. J, Hz) | $^1$C (DEPT) | $^1$H (mult. J, Hz) | $^1$C | $^1$H (mult. J in Hz) | $^1$C |
|-----|---------------------|--------------|---------------------|------|---------------------|------|
| 1   | 6.46 s              | 130.9 s      | 1.31 s              | 112.6 d | 6.74 d (2.4)       | 112.6 d |
| 2   | 105.9 d            | 6.76 d (1.8) | 1.31 s              | 115.3 d | 6.66 d (8.4)       | 115.3 d |
| 3   | 147.9 s            | 1.31 s       | 112.6 d            | 120.5 d | 6.57 d (8.4, 2.4)  | 120.5 d |
| 4   | 147.9 s            | 6.69 d (7.8) | 1.31 s              | 120.5 d | 6.57 d (8.4, 2.4)  | 120.5 d |
| 5   | 105.9 d            | 6.60 d (7.8, 1.8) | 1.31 s        | 120.5 d | 6.57 d (8.4, 2.4)  | 120.5 d |
| 7a  | 13.2, 11.4)        | 32.6 t       | 1.31 s              | 120.5 d | 6.57 d (8.4, 2.4)  | 120.5 d |
| 7b  | 32.6 t             | 2.80 dd (13.8, 4.8) | 1.31 s        | 120.5 d | 6.57 d (8.4, 2.4)  | 120.5 d |
| 8   | 2.59 m             | 1.31 s       | 112.6 d            | 120.5 d | 6.57 d (8.4, 2.4)  | 120.5 d |
| 9a  | 3.59 m             | 1.31 s       | 112.6 d            | 120.5 d | 6.57 d (8.4, 2.4)  | 120.5 d |
| 9b  | 3.92 dd (7.8, 6.6) | 1.31 s       | 112.6 d            | 120.5 d | 6.57 d (8.4, 2.4)  | 120.5 d |

Figure 2 Key HMBC correlations (H→C) of compounds 1–3.

-7 to C-1, 2, 6, 8, and 9, from H2-9 to C-7 and 8’, and from H-7’ to C-1’, 2’/6’, 8’, and 9’ confirmed unequivocally the 2D structure of compound 2 as 4’-(β-D-glucopyranosyloxy)-3,3’-trimethoxy-9,9’-epoxyilignane-4,7′-diol.

The molecular formula of compound 3 was established as C32H32O13 by $^{13}$C NMR data and by an HR-ESI-MS experiment in the positive ion mode with the sodium adduct ion at m/z 545.2002, indicating 10 indices of hydrogen deficiency. The IR spectrum displayed absorption bands of hydroxy (3395 cm⁻¹), aromatic (1598, 1515 and 1468 cm⁻¹), and etheric (1238, 1125 and 1070 cm⁻¹) functionalities. A detailed analysis of the NMR data of 3, along with a comparison with that of 2, found that compound 3 was very similar to 2. In the $^1$H NMR spectrum, the main difference was that the set of resonances of the symmetrically 1,3,3’,4’,5’-tetrasubstituted benzene ring at δH 6.604 (2H, s, H-2’/6’) in compound 2 was replaced by a set of resonances of a 1,3,4’-trisubstituted benzene ring at δH 6.88 (1H, d, J=1.8 Hz, H-2’), 7.02 (1H, d, J=8.4 Hz, H-5’), and 6.78 (1H, dd, J=8.4, 1.8 Hz, H-6’), with 2 methoxyls only being shown up in total, in compound 3. In the $^{13}$C and DEPT NMR spectra, the main difference was the appearance of an aromatic methine at δC 114.9 (C-5’) in 3, which replaced the oxygenated tertiary aromatic carbon at δC 152.4 (C-5’) in 2, with the downfield shifts of Δδ +12.0 and +13.8 from C-4’ and C-6’, respectively, being observed. Along with the HSQC spectrum, the correlations from H-2’ to C-4’, 6’, and 7’; from H-5’ to C-1’, 3’, and 4’; and from H-6’ to C-2’, 4’, and 7’ in the HMBC spectrum confirmed this assignment (Fig. 2). And the HMBC correlations from H-2 to C-4 and 6; from OH-4 to C-3, 4, and 5; from H-5 to C-1, 3, and 4; from H-6 to C-2 and 4; from H2-7 to C-1, 2, 6, 8, 8’, and 9; from H-7’ to C-1’, 2’, 6’, 8’, and 9’; from H8-8’ to C-1’; 7’, 8, 8’, and 9’; from H2-9 to C-7, 8, and 8’; and from H-glu-1 to C-4’ confirmed the 2D structure of compound 3 as 4’-(β-D-glucopyranosyloxy)-3,3’-dimethoxy-9,9’-epoxyilignane-4,7′-diol.

Even though it is well known that the establishment of the relative configurations of saturated hetero 5-membered rings is more complex than that for saturated hetero 6-membered rings, the
findings that the observed coupling constants of $J_{H_1-H_2}$, $J_{H_1-H_3}$, $J_{H_1-H_4}$, $J_{H_1-H_5}$, $J_{H_1-H_6}$, and $J_{H_1-H_7}$ of compounds 1–3 were of the same magnitude among the counterparts, respectively, of all the 3 molecules suggested that they have the same steric arrangements (Table 1). These $^1$H NMR coupling constants were also of the same magnitudes, respectively, as those of their counterparts in tripterygial, a known 3,4-dibenzyltetrahydrofuran-type lignan isolated previously from *Tripterygium wilfordii*, which possesses the same mono-hydroxyl substitution at the 7'-position of the benzylidine group as compounds 1–3. So, the relative configurations of the tetrahydrofuran moiety of compounds 1–3 were all elucidated to be the same as that of tripterygial, that is, H-8 and H-8' were in the trans-orientations. The absence of NOE correlation between H-8 and H-8' in the NOESY spectrum of compound 3 confirmed these assignments (also, the NOE correlation of H-8/H-8' was hardly observed in the 1D NOE difference experiment, see Supplementary Information Fig. S21), which is the same finding as that in the literature. The same value of $J_{H_1-H_7}$ = 6.0 Hz in all the 3 molecules confirmed that compounds 1–3 all possess the same relative configuration for the nonaromatic segments. Just because of the ambiguity involving the conformation of C(7) and C(7') ligands linked to C(8') is larger in bulk than another, the relative configuration of C(7') is not clear, notwithstanding the report for the configuration assignment of a similar structure only according to the $J_{H_1-H_7}$ value. In this study, baicalensinosides A–C were given as the trivial names of compounds 1–3, respectively.

To evaluate the potential bioactivity, the cytotoxicity in MG63 cells was first examined at 10 µmol/L using the MTT assay. Compounds 1–3 showed no obvious cytotoxicity when incubated with MG63 cells for 72 h (data not shown), suggesting that all the compounds are suitable for studying additional bioactivities, such as anti-osteoporotic activity. Compounds 1–3 were then evaluated for their anti-osteoporotic activity in an *in vitro* OPG transcriptional activity assay using dual luciferase reporter detection. This target has been proven to play a critical role in regulating bone remodeling. Although OPG was closely related to the potential treatment of osteoporosis, there has been no information as yet regarding the application of OPG agonists as anti-osteoporotic drug in clinic. Lack of a positive control compound led us to select the pGL3-basic-vector control in this study. As a result, compound 1 showed moderate activity at the concentration of 10 µmol/L with a relative activating ratio 1.83 times that of the control group. Compounds 2 and 3 showed weaker inhibitory activity with a relative activating ratio 0.84 and 0.98 times that of control group, respectively.

3. Conclusions

This study was conducted for the purpose of isolating and identifying new naturally-occurring compounds, especially bioactive compounds, from the roots of the titled plant. As a consequence, 3 new compounds (1–3) were obtained. The new compounds were identified as 3,4-dibenzyltetrahydrofuran-type lignan glucosides with a mono-hydroxy substitution at the 7'-position of the benzylidine group using the ligands numbering system being one of their shared critical features. Based on the report on the anti-inflammatory and immunomodulatory activity of the extract from the roots of the titled plant, the anti-osteoporotic activity of all 3 compounds was assessed in an *in vitro* OPG transcriptional activity assay using dual luciferase reporter detection. At 10 µmol/L, compounds 1–3 increased the relative activating ratio of OPG transcription to 1.83, 0.84 and 0.98 times that of the control group, respectively.

4. Experimental

4.1. General experimental procedures

Optical rotation was measured on a Perkin-Elmer 241 digital polarimeter at a temperature of 20 °C. UV spectra were recorded using a JASCO V-650 spectrophotometer, and IR (KBr) spectra were recorded using a Nicolet 5700 spectrometer. 1D and 2D NMR spectra were taken on a Varian Inova 600 MHz NMR spectrometer using DMSO-d$_6$ as a solvent and tetramethylsilane (TMS) as an internal standard. Both ESI-MS and HR-ESI-MS experiments were conducted on an Agilent 1100 series LC/MSD Trap SL mass spectrometer. Preparative HPLC procedure was performed on a Shimadzu LC-6AD instrument with a SPD-20A detector and a reversed-phase C18 column (BDS HYPERSIL, ODS-A, 250 mm x 21.2 mm, particle size 5 µm; Thermo, CA, USA). Silica gel (200–300 mesh; Qingdao Marine Chemical Inc., Qingdao, China), Diaion AB-8 (Chemical Plant of Nankai University, Tianjin, China), and Sephadex LH-20 (20–80 µm, Pharmacia Fine Chemicals, Uppsala, Sweden) were used for column chromatography (CC), and precoated GF$_2$54 silica gel plates (Qingdao Marine Chemical Inc., Qingdao, China) was used for TLC analyses. The spots were visualized by spraying with 10% H$_2$SO$_4$ in aqueous 95% EtOH and followed by heating using a hair dryer.

4.2. Plant material

The collection, identification, and deposit of the roots of *S. baicalensis* Georgi were described in our previous paper.

4.3. Extraction and isolation

After the pretreatment of dried and pulverized roots of *S. baicalensis* (18 kg) and the preliminary fractionation of the EtOH extract from the roots as described in our previous paper has served its intended purpose, the remaining water solution was extracted with n-BuOH for 3 times. Removal of the organic solvent from the n-BuOH extract under reduced pressure led to a brown semisolid residue (150 g). This residue was fractionated by CC on Diaion AB-8, eluting sequentially with H$_2$O, 20% aqueous EtOH, 40% aqueous EtOH, 60% aqueous EtOH, and 80% aqueous EtOH, to give 5 subfractions, Fr. 1 to Fr. 5, according to the eluents. Fr. 3 (28.0 g; 40% aqueous EtOH) was separated by CC on silica gel, using a gradient CHCl$_3$–MeOH (100:0–50:1–25:1–15:1–10:1–5:1) as an eluent, to yield 5 subfractions, Fr. 3-1 to Fr. 3-5, according to their TLC profiles. Fr. 3-2 (7.0 g; CHCl$_3$–MeOH, 50:1) was separated by CC on silica gel, using a gradient CHCl$_3$–MeOH (100:1–50:1–25:1–10:1) as an eluent, to yield 4 subfractions, Fr. 3-2-1 to Fr. 3-2-4, Fr. 3-2-3 (1.5 g; CHCl$_3$–MeOH, 25:1) was subjected to Sephadex LH-20 CC, using MeOH as an eluent, to give 3 subfractions, Fr. 3-2-3-1 to Fr. 3-2-3-3. Fr. 3-2-3-1 was purified by preparative RP-HPLC (mobile phase of CH$_3$CN–H$_2$O (30%, v/v); flow rate of 6 mL/min; and UV detection at 210 and 280 nm, simultaneously) to afford 1 (7 mg; $R_g$...
Three new lignan glucosides from *Scutellaria baicalensis* 233

=35.5 min). Fr. 3-2-3-3 was purified by preparative RP-HPLC (mobile phase of CH3CN–H2O (30%, v/v); flow rate of 6 mL/min; and UV detection at 210 and 280 nm, simultaneously) to afford 2 (5 mg; \( t_k = 33.2 \) min). Fr. 3-3 (9.0 g; CHCl3) was subjected to Sephadex LH-20 CC, using MeOH as an eluent, to give 3 fractions, Fr. 3-3-1 to Fr. 3-3-5. Fr. 3-3-1 (2.0 g; CHCl3–H2O, 280 nm, simultaneously) to afford 55.4 (2 positive ion mode ESI-MS \( m/z \) 545.1993). 55.5 (q, OMe-3), and see Table 1 for other data; positive ion mode HR-ESI-MS \( m/z \) 575.2099. White amorphous powder; \( [\alpha]_D^{20} + 10.3 \) (c 0.081, MeOH); UV (MeOH) \( \lambda_{max} \) (log e) 277 (3.24), 230 (3.90), 204 (4.53); IR (KBr) \( \nu_{\max} \) 3396, 3190, 2938, 2922, 2850, 1647, 1598, 1448, 1426, 1329, 1120, 830, 721, 648 cm\(^{-1}\); \(^1\)H NMR (DMSO-\( d_6 \), 600 MHz), see Table 1; \(^13\)C NMR (DMSO-\( d_6 \), 150 MHz) \( \delta \) 56.4 (q, OMe-3/5), 55.9 (q, OMe-3/5), and see Table 1 for other data; positive ion mode ESI-MS \( m/z \) 605.3 [M]+; positive ion mode HR-ESI-MS \( m/z \) 605.2202 [M]+[Na]\(^{+}\) (Calcd. for C\(_{28}\)H\(_{38}\)NaO\(_{13}\), 605.2202). Acid hydrolysates, derivatization reactions, and determination of the absolute configurations of sugars of compounds 1–3

Acid hydrolysates of compounds 1–3 and the derivatization reactions of the obtained sugars, and authentic glucose, were carried out according to the reported method\(^{10,11}\). Trimethylsilylated thiazolidine derivatives of the sugars were injected into a GC system. The sugars, as well as their absolute configurations, were determined by comparing the retention times with that of the trimethylsilylated thiazolidine derivative synthesized from the authentic glucose. GC conditions in the test was as follows: capillary column, HP-5 (60 m \( \times \) 0.25 mm, with a 0.25 \( \mu \)m film, 250 \({ }^\circ\)C; detection gas, N\(_2\); detection, FID; injection temperature, 260 \({ }^\circ\)C; detection temperature, 280 \({ }^\circ\)C; initial temperature, 16 \({ }^\circ\)C, raised up to 280 \({ }^\circ\)C at a rate of 5 \({ }^\circ\)C/min and the final temperature was maintained for 10 min. Along with the NMR examinations, \( \alpha \)-glucose was confirmed to be the only sugar for all 3 compounds. And under the above analytical conditions, the retention time of the authentic sugar was at 27.98 min.

4.5. Assay for anti-osteoporotic activity

MG63 cells in the growth phase were dispersed in a 48-well plate at a density of \( 5 \times 10^4 \) cells/well, and the plate was placed in a humidified incubator filled with 5% CO\(_2\) at 37 °C. Plasmid transfection (0.6 µg/well) was performed for 4 h when the cell confluence reached 70%–80%. Test compounds at a final concentration of 10 µmol/L were added into the wells (n = 3) after 4 h of transfection and were co-incubated for an additional 48 h. Luciferase activity was detected using a dual luciferase reporter gene detection kit (Promega, USA).

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.apsb.2016.03.007.

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