Biosynthesis of Novel Shikonin Glucosides by Enzymatic Glycosylation

Bohan Li, Meilin Zhu, Hui Ma, Tao Ma, Yiqun Dai, Hongmei Li, Yu Li, and Cheng-Zhu Wu

School of Pharmacy, Bengbu Medical College; 2600 Donghai Road, Bengbu 233030, China;
and School of Pharmacy, Second Military Medical University; 325 Guohe Road, Shanghai 200433, China.

Received March 30, 2019; accepted June 27, 2019

Shikonin, a natural naphthoquinone, has attracted much attention due to its various biological activities. Two shikonin glucosides, shikonin-1,8-di-O-β-D-glucopyranoside (1) and shikonin-1′-O-β-D-glucopyranoside (2), were biosynthesized through in vitro enzymatic glycosylation and their structures were elucidated using spectroscopic techniques. The water-solubility and stability of compounds 1 and 2 were significantly higher than those of the parent compound. Furthermore, compound 2 showed moderate cytotoxicity against six cancer cell lines, with IC50 values ranging from 36.10 to 67.47 µM. This research indicated that in vitro enzymatic glycosylation of shikonin is an effective strategy to improve its water solubility and chemical stability.

Key words shikonin; enzymatic glycosylation; water-solubility; stability; cytotoxicity

Introduction

Natural products are an important source for the discovery and development of drugs. Shikonin is a naphthoquinone isolated from Lithospermum erythrorhizon Siebold & Zucarini that reportedly has a range of biological activities, including anticancer, antimicrobial, and anti-inflammatory. However, its poor water solubility and toxicity restrict its use as a drug. Many efforts have been made to chemically modify shikonin in order to synthesize derivatives that would yield more effective therapeutic agents. The glycosyltransferase GT YjiC has high substrate specificity for shikonin.

Identification of Novel Shikonin Glucosides

Compound 1 was obtained as a red powder and its molecular formula C28H36O15 was established by high resolution-electrospray ionization (HR-ESI)-MS (m/z 611.1962 [M–H]–) and NMR analysis. The NMR spectra of 1 were very similar to those of shikonin, except for the additional signals from two glucosyl moieties (Table 1). The two glucosyl moieties of 1 were both identified as being in the β-conformation, based on the coupling constant of their anomeric protons (J = 6.0 and 6.6 Hz, respectively). Additionally, the heteronuclear multiple bond correlation (HMBC) correlations from δH 4.55 (d, 1H, J = 6.0 Hz, H-1) to δC 75.8 (C-1) and δH 4.29 (d, 1H, J = 6.6 Hz, H-1′) to δC 169.8 (C-8) suggested that the glucosyl moieties were attached to C-1 and C-8, respectively (Fig. S2). Further, comprehensive HMBC analysis of 1 permitted the complete assignments of its carbons and protons. Therefore, the structure of 1 was identified as shikonin-1,8-di-O-β-D-glucopyranoside.

Compound 2 was obtained as a red powder and its molecular formula C28H36O15 was established by HR-ESI-MS (m/z 449.1447 [M–H]–). The 1H- and 13C-NMR data of compound

Results and Discussion

Effects of Reaction Conditions on the Glycosylation of Shikonin

Shikonin was subjected to in vitro glycosylation and the reaction mixture was analyzed by HPLC, which showed two product peaks (compounds 1 and 2). Optimization of the glycosylation reaction conditions showed that the most effective reaction occurred at pH 6.0 and 6 h incubation time with 3 mM of uridine-5′-diphosphate (UDP)-Glc, which yielded 0.32 mg/L and 1.09 mg/L of compounds 1 and 2, respectively (Fig. 2). These results indicate that GT YjiC has high substrate specificity for shikonin.

Fig. 1. Chemical Structures of Shikonin, Compounds 1 and 2
were consistent with those reported for shikonin-1-O-β-D-glucopyranoside.11) The relative positions of the glucose and alkyl chain were determined from HMBC correlations (Fig. S2). Therefore, the structure of 2 was identified as shikonin-1-O-β-D-glucopyranoside.

**Determination of Water-Solubility**

Commonly, modifying natural products by introducing glucose moieties improves their water solubility and chemical stability.18,19) The water solubilities of compounds 1 and 2 were found to be 15.20 mM and 10.05 mM, approximately 1520 and 1005 times higher than that of the parent compound, respectively (Table 2). These results showed that the enzymatic glucosylation of shikonin significantly enhances its water solubility.

**Determination of pH and Temperature Stability**

To clarify whether the glucosylation of shikonin enhanced its stability, the pH and temperature stabilities of compounds 1 and 2, and shikonin were determined using HPLC analysis. As expected, compounds 1 and 2 were more stable than shikonin at pH 6–10 and 50–100°C (Fig. 3). These results suggested that the enzymatic glucosylation of shikonin significantly enhances its stability under a range of different pH values and temperatures, which may lead to improvement in its bioavailability and biological activity.

**Cytotoxicity**

To understand the anti-tumor activity of the novel shikonin glucosides, we analyzed their anti-proliferative activity.

### Table 1. $^1$H- and $^{13}$C-NMR Data of Compounds 1 and 2 in CD$_3$OD (δ in ppm)

| No. | Compound 1 | | Compound 2 |
|-----|------------|------------|------------|
|     | δ$_C$      | δ$_H$ (J = Hz) | δ$_C$      | δ$_H$ (J = Hz) |
| 1   | 181.2      | —          | 183.3      | —          |
| 2   | 151.1      | —          | 152.0      | —          |
| 3   | 136.0      | 7.41 (s)   | 137.8      | 7.17 (s)   |
| 4   | 180.9      | —          | 183.2      | —          |
| 5   | 170.5      | —          | 174.5      | —          |
| 6   | 133.0      | 7.62 (s)   | 132.7      | 7.25 (s)   |
| 7   | 133.2      | 7.73 (s)   | 134.8      | 7.19 (s)   |
| 8   | 169.8      | —          | 174.0      | —          |
| 9   | 112.5      | —          | 113.3      | —          |
| 10  | 113.6      | —          | 114.3      | —          |
| 1’  | 75.8       | 5.28 (m)   | 75.7       | 5.10 (br s) |
| 2’  | 36.2       | 2.48–2.53 (m); 2.59–2.62 (m) | 34.3 | 2.48–2.53 (m); 2.63–2.65 (m) |
| 3’  | 120.4      | 5.25 (t, 15.6) | 120.6 | 5.31 (t, 14.8) |
| 4’  | 136.4      | —          | 135.7      | —          |
| 5’  | 25.6       | 1.65 (s)   | 26.0       | 1.66 (s)   |
| 6’  | 20.5       | 1.50 (s)   | 18.2       | 1.52 (s)   |
| 1’  | 105.8      | 4.55 (d, 6.0) | 104.2 | 4.47 (d, 7.7) |
| 2’  | 75.4       | 3.27 (m)   | 75.4       | 3.27 (m)   |
| 3’  | 78.1       | 3.38 (m)   | 78.1       | 3.28 (m)   |
| 4’  | 78.0       | 3.20 (m)   | 78.0       | 3.20 (m)   |
| 5’  | 71.7       | 3.26 (m)   | 71.7       | 3.26 (m)   |
| 6’  | 62.7       | 3.70 (m); 3.55 (m) | 62.7 | 3.70 (m); 3.55 (m) |
| 1’’ | 102.5      | 4.29 (d, 6.6) | —   | —          |
| 2’’ | 75.2       | 3.47 (m)   | —   | —          |
| 3’’ | 78.2       | 3.29 (m)   | —   | —          |
| 4’’ | 78.6       | 3.41 (m)   | —   | —          |
| 5’’ | 72.0       | 3.38 (m)   | —   | —          |
| 6’’ | 63.4       | 3.87 (m); 3.60 (m) | —   | —          |

### Table 2. Solubility of Shikonin and Its Glucosides 1 and 2 in Water

| Compounds | Water-solubility (mg/mL) | MW | Water-solubility (mM) | Relative solubility |
|-----------|--------------------------|----|-----------------------|---------------------|
| 1         | 9.303                    | 612.2 | 15.20                 | >1520               |
| 2         | 4.523                    | 450.2 | 10.05                 | >1005               |
| Shikonin  | <0.01                    | 288.3 | <0.01                 | 1                   |

Fig. 2. Production of Novel Shikonin Glucosides under Different Reaction Conditions

(A) Effect of buffer pH on in vitro glucosylation of shikonin. (B) Effect of incubation time on the in vitro glucosylation of shikonin. (C) Effect of UDP-Glc concentration on in vitro glucosylation of shikonin.

Fig. 3. Stability of Shikonin and Its Glucosides 1 and 2

(A) Effect of pH of Tris–HCl buffer on the stability of shikonin and its glucosides (1 and 2). (B) Effect of temperature on the stability of shikonin and its glucosides (1 and 2).
were incubated at 50, 60, 70, 80, 90, and 100°C in 500 µL of Tris–HCl buffer at pH 9 for 30 min. The pH and temperature stabilities were calculated as a percentage of the total peak area using HPLC analysis.

**MTT Assay** The six cancer (MCF-7, MDA-MB-231, H1975, HNE-1, SG7901, I-10) cell lines were grown in Dulbecco’s modified Eagle’s medium (DMEM) or RPMI 1640 medium containing 10% FBS (Hyclone, U.S.A.) and 1% penicillin/streptomycin (Gibco, U.S.A.). All cells were seeded in 96-well plates at a density of 3000 cells/well for overnight incubation. After adherence, the cells were treated with various concentrations of compounds for 72 h. The anti-proliferative activity was evaluated by standard MTT assay procedures.\(^{21}\)

**Acknowledgments** This research was financially supported by the Education Department of Anhui Natural Science Research Project China under Grant (KJ2018A0232, KJ2018A1009); the Science and Technology Development Fund Project of Bengbu Medical College (BYKF1718).

**Conflict of Interest** The authors declare no conflict of interest.

**Supplementary Materials** The online version of this article contains supplementary materials.

**References**

1. Nao L. T., Okogun J. I., Folk W. R., *Nat. Prod. Rep.*, 30, 584–592 (2013).
2. Newman D. J., Cragg G. M., *J. Nat. Prod.*, 79, 629–661 (2016).
3. Yoon Y., Kim Y. O., Lim N. Y., Jeon W. K., Sung H. J., *Planta Med.*, 65, 532–535 (1999).
4. Lu B., Gong X., Wang Z. Q., Ding Y., Wang C., Luo T. F., Piao M. H., Meng F. K., Chi G. F., Luo Y. N., Ge P. F., *Yao Hsueh Pao*, 38, 1543–1553 (2017).
5. Bhakuni D. S., Dhar M. L., Dhar M. M., Dhawan B. N., Mehrotra B. N., *Indian J. Exp. Biol.*, 7, 250–262 (1969).
6. Tanaka S., Tajima M., Tsukada M., Tabata M., *J. Nat. Prod.*, 49, 466–469 (1986).
7. Ahn B. Z., Baik K. U., Kweon G. R., Kim K., Hwang B. D., *J. Med. Chem.*, 38, 1044–1047 (1995).
8. Yang F., Chen Y., Duan W., Zhang C., Zhu H., Ding J., *Int. J. Cancer*, 119, 1184–1193 (2006).
9. Su Y., Xie J., Wang Y., Hu X., Lin X., *J. Med. Chem.*, 45, 2713–2718 (2010).
10. He H., Bai L. P., Jiang Z. H., *Bioorg. Med. Chem. Lett.*, 22, 1582–1586 (2012).
11. Hu X., Chen J., Su Y. H., Jiang Z., Wang Y. G., Patent CN102552296A (2012).
12. Garung R. B., Kim E. H., Oh T. J., Sohng J. K., *Mol. Cells*, 36, 355–361 (2013).
13. Pandey R. P., Li T. F., Kim E. H., Yamaguchi T., Park Y. I., Kim J. S., Sohng J. K., *Appl. Environ. Microbiol.*, 79, 3516–3521 (2013).
14. Dai Y., Ma T., Ge M., Li J., Hua Q., Li H. M., Zhang X., Liu H., Wu C. Z., *J. Chin. Chem. Soc.*, 63, 376–378 (2016).
15. Ghimire G. P., Koirala N., Pandey R. P., Jung H. J., Sohng J. K., *Chem. Pharm. Bull.*, 67, No. 10 (2019).
16) Pandey R. P., Parajuli P., Shin J. Y., Lee J., Lee S., Hong Y. S., Park Y. I., Kim J. S., Sohng J. K., *Appl. Environ. Microbiol.*, **80**, 7235–7243 (2014).
17) Choi O., Lee J. K., Kang S. Y., Pandey R. P., Sohng J. K., Ahn J. S., Hong Y. S., *J. Microbiol. Biot.*, **24**, 614–618 (2014).
18) Gantt R. W., Peltier-Pain P., Thorson J. S., *Nat. Prod. Rep.*, **28**, 1811–1853 (2011).
19) Mandai T., Okumoto H., Oshitari T., Nakanishi K., Mikuni K., Hara K. J., Hara K. Z., Iwantani W., Amano T., Nakamura K., Tsuchiya Y., *Heterocycles*, **54**, 561–566 (2001).
20) Cheng H., Cao X. H., Xian M., Fang L. Y., Cai T. B., Ji J. J., Tunac J. B., Sun D., Wang P. G., *J. Med. Chem.*, **48**, 645–652 (2005).
21) Yu C., Zhang Z., Zhang H., Zhen Z., Calway T., Wang Y., Yuan C. S., Wang C. Z., *Oncol. Rep.*, **30**, 2411–2418 (2013).
22) Dai Y., Zhang S., Liu D. C., Li H. M., Ma T., Huo Q., Wu C. Z., *Phytochemistry. Lett.*, **23**, 9–14 (2018).