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Biotransformation of Paeonol and Emodin by Transgenic Crown Galls of *Panax quinquefolium*

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Abstract Two aromatic substrates, paeonol (**1**) and emodin (**2**), were biotransformed by using transgenic crown galls of *Panax quinquefolium*. Four biotransformed products (**3**–**6**) were isolated and identified by physicochemical and spectral methods. A β-glucoside (**3**, 73.2% of biotransformation yield) and a 1-(2,4-dimethoxyphenyl)- ethanone (**4**, 8.03%) were isolated from the suspension cultures after 7-day incubation of substrate **1**. Upon administration of substrate **2**, another β-glucoside [emodin-6-O-β-D-glucopyranoside (**5**), 19.2%] and a hydroxylated derivative, citreorosein (**6**, 54.6%), were also obtained. The results demonstrate that transgenic crown galls of *P. quinquefolium* have the capacities to catalyze glycosylation, hydroxylation, and methylation reactions in the plant cells on those aromatic compounds.

Keywords Transgenic crown galls · *Panax quinquefolium* · Biotransformation · Paeonol · Emodin

Abbreviations

UDP	Uridine bisphosphate
MS Medium	Murashige and Skoog Medium (1962)
TLC	Thin-layer chromatography
Varian Inova NMR	Varian Inova nuclear magnetic resonance
TMS	Tetramethylsilane
HPLC	High-performance liquid chromatography

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Introduction

Biotransformation by cultured plant cells is considered to be an important method to convert low-cost and plentiful organic compounds into highly valuable and useful constituents [1]. The reactions involved in the biotransformation of organic compounds by cultured plant cells include oxidation, reduction, hydroxylation, esterification, methylation, isomerization, hydrolysis, and glycosylation [1, 2].

Glycosylation occurs readily in plant cells, i.e., many kinds of secondary metabolites such as saponins and anthocyanins can be produced in the form of glycosides in higher plants, and most of them accumulate in the vacuole of plant cells [3]. It is of great use to study one-step enzymatic glycosylation by cultured plant cells in contrast to chemical glycosylation, which requires tedious steps and high cost [4, 5]. Biotransformation is also considered economical, as the reaction is carried out without the need for expensive purified enzymes or UDP-sugars. As a result, biotransformation by plant cells has been the subject of increasing scientific attention and has been carried out for exogenous compounds in many cell culture systems, such as *Panax ginseng*, *Eucalyptus perriniana*, *Nicotiana tabacum*, *Gardenia jasminoides*, etc., in which the relevant compounds were converted into their corresponding glycosides [2, 6].

The plant suspension culture system plays an important role in glycosylation, as chemical synthesis and microbial systems are difficult to perform for this reaction [7]. The crown galls is the expressing product of transgenic *Panax quinquefolium*, in which the plant has been infected by *Agrabacterium tumefaciens*, and the Ti plasmid in *A. tumefaciens* has been introduced into the plants' nuclear genome [8, 9]. *Agrobacterium*-Ti plasmids are natural gene vectors and have been extensively employed in a number of studies in genetic engineering of secondary metabolism in many important plants within the last several decades. One recent important progress in transgenic technology is the application of transgenic organ cultures such as crown galls and hairy roots to the production and biotransformation of secondary metabolites [10].

Two bioactive and medicinal compounds, paeonol (**1**) and emodin (**2**), were used as substrates in this experiment. Paeonol has various biological activities such as anti-inflammatory and cardiovascular-protective activities, inhibition against anaphylactic reaction, antidiabetic effects, and inhibition against platelet aggregation [11]. Emodin also possesses a number of biological activities, such as inhibitory activity against inflammation, anti-viral and anti-bacterial activities, vasorelaxation, immunosuppression, and hepatoprotection [12]. Both paeonol and emodin are water-insoluble aromatic compounds, which limits their potentially wide application as nutritional and medicinal materials.

In this paper, we report the transgenic crown galls of *P. quinquefolium* as a novel biocatalyst to convert substrate **1** and **2** into their corresponding glycosides [paeonol-2-O-β-D-glucopyranoside (**3**) and emodin-6-O-β-D-glucopyranoside (**5**)]. Besides glycoside products, two other transformed products, one from methylation [1-(2,4-dimethoxyphenyl)-ethanone, **4**] and another from hydroxylation [citreorosein (**6**)], were also obtained.

Materials and Methods

Cultivation

The crown galls of *P. quinquefolium* L., which have been established as described in our previous report [9], were subcultured on MS liquid medium (Murashige and Skoog's

medium containing 30 g/L sucrose without agar) at 25 °C in the dark at 110 rpm on a rotary shaker at 25-day intervals.

Materials and Instruments

Silica gel (100–200 and 200–300 mesh) was used for column chromatography (CC), and silica GF_{254} (10–40 μm) for TLC were supplied by the Qingdao Marine Chemical Factory, People's Republic of China. TLC visualization was accomplished with a UV lamp at 254 nm, I_2 evaporation, and heating after spraying with 5% H_2SO_4 in ethanol (*v*/*v*). Macroporous Resin AB-8 (the Chemical Plant of NanKai University, Tianjin, People's Republic of China) and Sephadex LH-20 (Pharmacia) were also used for separation. Melting points were measured by SGW X-4 (Shanghai Precise Scientific Instrument). Optical rotations were measured on a Polax-2L polarimeter. Infrared spectra were obtained with a Bruker Vector-22 (by a KBr disk method) spectrometer. ^{1}H-NMR and ^{13}C-NMR spectra were recorded on an Varian Inova NMR spectrometer with TMS as internal standard.

Paeonol (**1**) was purchased from Xuancheng Baicao Plants Industry and Trade, People's Republic of China. Emodin (**2**) was purchased from Nanjing Medical Technology Development, People's Republic of China. The purity (>98%) of both two substrates was determined by HPLC analysis.

Biotransformation and Purification of Products

One hundred milligrams of substrate (10 mg for each flask and 250 ml liquid medium in each flask) were administered to ten flasks containing the suspension cells of *P. quinquefolium*, and the cultures were incubated at 25°C for 7 days on a rotary shaker (120 rpm) in the dark. After incubation, the cells and medium from each experiment were separated by filtration with suction and two components, the medium portion and the cells portion, were obtained. Glucosides in the former was first extracted with EtOAc and then with n-BuOH. Two fractions were analyzed by TLC and HPLC. The transformed products in the latter were extracted with MeOH for 12 h and sonicated for 20 min. The yields of the products were calculated on the basis of the peak area using calibration curves prepared by HPLC. The MeOH fraction was concentrated and partitioned between H_2O and EtOAc. The EtOAc fractions were combined and analyzed by TLC and HPLC. The products were further purified by silica gel (200–300 mesh) and Sephadex LH-20 column chromatography.

Identification of Transformed Products

Paeonol-2-O-β-D-glucopyranoside (**3**): Colorless amorphous solid, mp: 81–83 °C, Molish reaction is positive, $[\alpha]_D$ −62° (*c* 0.22, MeOH, 20°C). FAB-MS: *m*/*z* = 327.1$[M–H]^+$, 165.1 $[M–162–H]^+$. IR (KBr)γ_{max} (cm^{-1}): 1,073, 1,174, 1,203, 1,261, 1,426, 1,501, 1,602, 1,656, 2,924, 3,407. ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 3.26–3.71 (m, 6H), 4.97 (d, 1 H, *J*=7.6), 6.61 (dd, 1 H, *J*=2.4; 8.8), 6.77 (d, 1 H, *J*=2.0), 7.61 (d, 1 H, *J*=8.8). ^{13}C-NMR (100 MHz, DMSO-d_6) δ: 197.0 ($COCH_3$), 32.4 ($COCH_3$), 55.8 (OCH_3), 120.9 (C-1), 159.2 (C-2), 101.2 (C-3), 164.3 (C-4), 108.2 (C-5), 131.2 (C-6), 100.9 (C-1′), 73.5 (C-2′), 77.6 (C-3′), 70.1 (C-4′), 77.1 (C-5′), 61.0 (C-6′).

1-(2,4-dimethoxyphenyl)-ethanone (**4**): Colorless needle crystal (MeOH), mp: 44-45°C, FAB-MS: *m*/*z*=180.7 $[M+H]^+$. ^{1}H-NMR (400 MHz, CD_3OD) δ: 2.22 (s, 3 H), 3.76 (s, 3 H), 3.77 (s, 3 H), 7.13 (d, 1 H, *J*=8.4), 7.21 (d, 1 H, *J*=6.8), 7.56 (brs, 1 H). ^{13}C-NMR

(100 MHz, $CDCl_3$) δ: 197.7 ($COCH_3$), 164.5 (C-4), 161.0 (C-2), 132.6 (C-6), 121.2 (C-1), 105.0 (C-5), 98.3 (C-3), 55.5 (OCH_3), 55.4 (OCH_3), 31.8 ($COCH_3$).

Emodin-6-O-β-D-glucopyranoside (**5**): orange amorphous solid, mp: 209–211 °C, the reaction of Molish is positive, $[\alpha]_D$ −74°(*c* 0.18, MeOH, 20 °C); FAB-MS: *m/z*=455.2 $[M + Na]^+$, 431.1 $[M - H]^+$, 269.2 $[M–162–H]^+$; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 2.43 (3 H, s, CH_3), 3.17–3.73 (6 H, m, H of sugar), 5.12 (1 H, d, *J*=6.8, H-1′), 6.96 (1 H, d, *J*= 2.4, H-2), 7.21 (1 H, s, H-7), 7.28 (1 H, d, *J*=2.0, H-4), 7.54 (1 H, s, H-5), 11.93 (1 H, brs, OH-8), 12.12 (1 H, brs, OH-1); ^{13}C-NMR (100 MHz, DMSO-d_6) δ: 190.4 (C=O, C-9), 181.4 (C=O, C-10), 164.3 (C-8), 164.1 (C-6), 161.7 (C-1), 148.9 (C-3), 135.1 (C-10a), 133.1 (C-4a), 124.5 (C-2), 120.9 (C-4), 113.8 (C-8a), 111.0 (C-9a), 109.2 (C-5,C-7), 100.2 (C-1'), 77.5–69.7 (C-2', C-3', C-4', C-5'), 60.8 (C-6'), 30.9 (CH_3).

Citreorosein (**6**): Yellow needle crystal (MeOH), mp: 281–283 °C, FAB-MS: *m/z*=284.9 $[M–H]^+$; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 4.60 (2 H, s, CH_2OH), 5.583 (1 H, brs, CH_2OH), 6.61 (1H, d, *J*=2.4, H-2), 7.14 (1 H, d, *J*=2.4, H-4), 7.25 (1 H, s, H-7), 7.65 (1 H, s, H-5), 11.40 (1 H, brs, OH-6), 12.05 (1 H, s, OH-8), 12.08 (1 H, s, OH-1); ^{13}C-NMR (100 MHz, DMSO-d_6) δ: 190.1 (C=O, C-9), 181.9 (C=O, C-10), 166.0 (C-6), 164.9.(C-8), 161.8 (C-1), .153.2 (C-3), 135.6 (C-10a), 133.4 (C-4a), 121.2 (C-4), 117.5 (C-2), 114.5 (C-9a), 109.3 (C-5), 108.4 (C-8a, C-7), 62.4 (CH_2OH).

Establishment of Time Course

Substrate (10 mg) was administered to each of the flasks and the mixtures were incubated on a rotary shaker at 25 °C. At 1-day intervals, one of the flasks was taken out from the rotary shaker, and the cells and medium were separated by filtration. The extraction and analysis procedures were the same as described in the section of "biotransformation and purification of products." The yield of the products was determined on the basis of the peak area from HPLC.

Results and Discussions

Biotransformation of Paeonol (**1**)

The crown galls of *P. quinquefolium* were incubated in the substrate containing 40 mg/L of phaenol. After 7 days' incubation, paeonol-2-*O*-β-D-glucopyranoside (**3**) and 1-(2,4-dimethoxyphenyl)-ethanone (**4**) were isolated from the cells and the corresponding medium.

Paeonol-2-*O*-β-D-glucopyranoside (**3**) was obtained as oil at the beginning, but through freeze-drying, turned into a colorless amorphous solid. It revealed an $[M–H]^+$ ion peak at *m/z* 327.1 in the FAB-MS spectrum, suggesting that **3** is larger than **1** by a hexose unit. In the ^{1}H-NMR spectra of **3**, besides the signals for a paeonol moiety, it showed a set of β-glucopyranosyl signals, with the resonance for the anomeric proton at δ5.03 (1 H, d, *J*= 7.6 Hz). Its ^{13}C-NMR spectra exhibited 15 carbon signals including one anomeric carbon signal at δ100.9, and suggesting **3** is larger than **1** by a sugar moiety. Further comparison of the NMR and $[\alpha]_D$ data of **3** with those in the reference [13, 14] showed that it is paeonol 2-*O*-β-D-glucopyranoside.

1-(2,4-Dimethoxyphenyl)-ethanone (**4**) was obtained as a colorless needle crystal (MeOH). It revealed an $[M+H]^+$ ion peak at *m/z* 180.7 in FAB-MS spectrum, suggesting that **4** is larger than **1** by a methyl unit, which was further confirmed by the ^{1}H-NMR

spectrum. Comparing with the data in the reference [15], the structure of **4** was identified as 1-(2,4-dimethoxyphenyl)-ethanone.

To investigate the biotransformation pathway, a time course in the conversion of **1** was followed. As shown in Fig. 3a, both products of **3** and **4** were detected after 48 h administration of **1**. But the content of **3** was evidently more than that of **4**. Paeonol-2-*O*-β-D-glucopyranoside (**3**) as the main product reached its maximum concentrations on the eighth day and then decreased sharply. The maximum biotransformation rate of **3** was determined by HPLC to be 73.2%. The content of 1-(2,4-dimethoxyphenyl)-ethanone (**4**) increased slowly within the time-course experiment, and its transformed rate was 8.03% on the 12th day.

Based on these results, the biosynthesis pathways of paeonol-2-*O*-β-D-glucopyranoside (**3**) and 1-(2,4-dimethoxyphenyl)-ethanone (**4**) were hypothesized as shown in Fig. 1, and the time course was established as demonstrated in Fig. 3a.

Biotransformation of Emodin (**2**)

Emodin (**2**) was subjected to the same biotransformation system. After a 5-day incubation period, transformed products **5** and **6** were obtained from the MeOH extracts of the cells.

Emodin-6-O-β-D-glucopyranoside (**5**) was obtained as orange amorphous solid. The FAB-MS spectrum of **5** show a $[M + Na]^+$ ion peak at *m/z* 455.2, suggesting that **5** is larger than **1** by a hexose unit. In the ^{1}H-NMR spectra of **5**, besides the signals for an emodin moiety, a set of β-glucopyranosyl signals appeared, with the resonance for the anomeric proton at δ5.12 (1 H, d, *J*=6.8 Hz). Its ^{13}C-NMR spectra exhibited 21 carbon signals including one anomeric carbon signal at δ100.2, suggesting **5** is larger than **2** by a sugar moiety. Further comparison of the NMR of **5** with those in the reference [16] showed that it is emodin-6-*O*-β-D-glucopyranoside.

Citreorosein (**6**) was obtained as yellow needle crystal (MeOH). The FAB-MS spectrum of **6** show a $[M–H]^+$ ion peak at *m/z* 284.9. In the ^{1}H-NMR spectra of **6**, besides the signals for an emodin moiety, they showed additional 2H (s, δ 4.6) and OH (brs, δ 5.6) signals and loss of methyl signal, with the resonance for the methyl changing into corresponding hydroxyl-methyl. Its ^{13}C-NMR spectra exhibited 15 carbon signals including the carbon signal at δ 62.4, which is evidently different from the emodin moiety. Further comparison of the NMR spectral data of **6** with those in the reference [17] showed that it is citreorosein.

A time course was made to investigate the biotransformation pathway of **2** by the culture cells of *P. quinquefolium*, as shown in Fig.3b. The maximum concentration appeared on the fourth day for Emodin-6-*O*-β-D-glucopyranoside (**5**) and the fifth day for citreorosein (**6**);

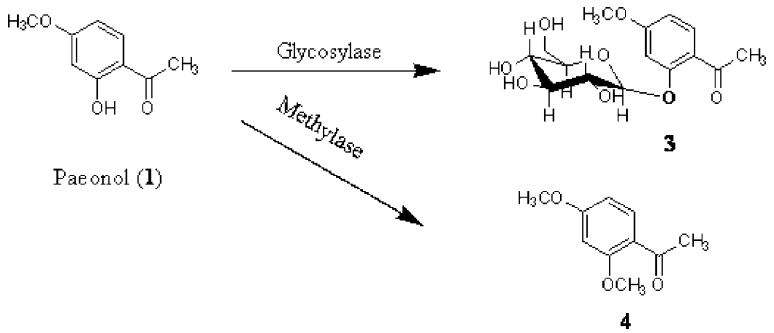

Fig. 1 Glycosylation and methylation of paeonol (**1**) by the crown galls of *P. quinquefolium*

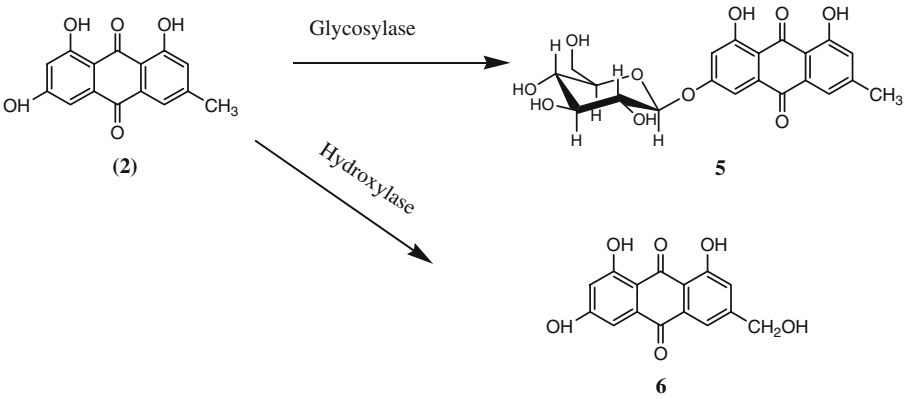

Fig. 2 Glycosylation and hydroxylation of emodin (**2**) by the crown galls of *P. quinquefolium*

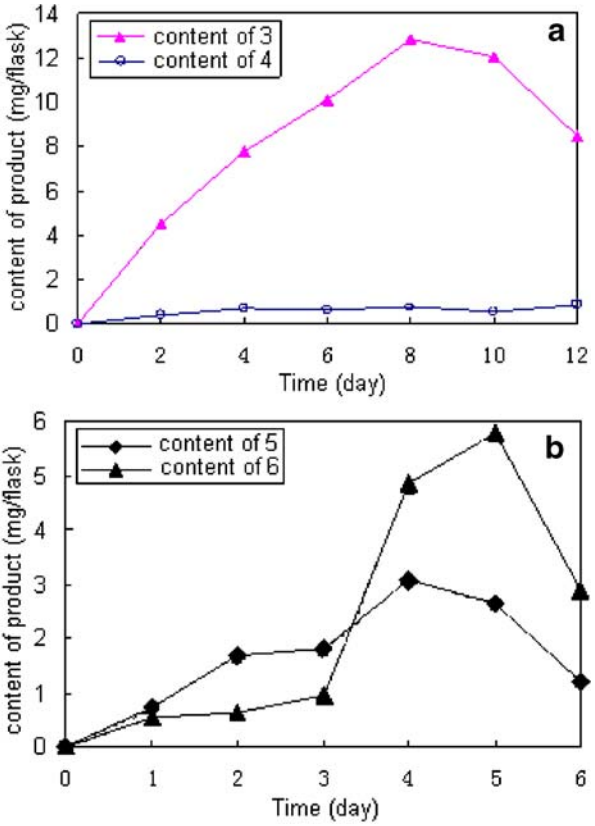

Fig. 3 Time courses in the biotransformations of paeonol (**1**, **a**) and emodin (**2**, **b**) by the crown galls of *P. quinquefolium*

the maximum biotransformation rate was determined by HPLC to be 19.2% for **5** and 54.6% for **6**, respectively. Then, the concentration decreased gradually, potentially indicating the formation of other secondary products.

Based on these results, the biosynthesis pathways of transformed products **5** and **6** were hypothesized as shown in Fig. 2, and the time course was established seen in Fig. 3b.

Conclusions

The results of this experiment revealed that transgenic crown galls of *P. quinquefolium* have the capacity to convert certain aromatic compounds such as paeonol (**1**) and emodin (**2**) into their corresponding β-glucosides. It is well known that glycosylation by plant cells serves as the detoxification step for toxic phenolic compounds, which could arise either from normal plant metabolism or from the environment [18, 19]. The results above also suggested that exogenous aromatic compounds such as paeonol (**1**) and emodin (**2**) could act as chemical stress against the culture cells of *P. quinquefolium*. As shown in Fig. 3, the maximum yield of glycosides of paeonol (**1**) was about 3.8 times higher than that of emodin (**2**). This demonstrated that glucosyltransferases in the cultured cells of *P. quinquefolium* may have high specificity for helical compounds among those aromatic constituents. This may have relationship with the many factors, such as molecular weight and structural styles of substrates. Its exact mechanistic details call for further investigations.

In addition to glycosylation, products of hydroxylation and methylation were also obtained by the transformation of the crown gall cells of *P. quinquefolium*, demonstrating the capacity for culture cells of *P. quinquefolium* as a catalyst to transform exogenous compounds in a wide array of useful and potentially harvestable ways.

Finally, authors measured the levels of paeonol-2-*O*-β-D-glucopyranoside (**3**) and 1-(2,4-dimethoxyphenyl)-ethanone (**4**) [or emodin-6-*O*-β-D-glucopyranoside (**5**) and citreorosein (**6**)] from the mixtures of cells and media. We have done a lot of experiments using several plant cell systems in our lab. Some transformed products may exist mainly in the medium [20], and others may appear predominantly in culture cells [21]. In this experiment of using the crown galls of *P. quinquefolium*, most products were found to exist in the culture cells.

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References

1. Suga, T., & Hirata, T. (1990). *Phytochemistry, 29*, 2393–2406. doi:10.1016/0031-9422(90)85155-9.
2. Ishihara, K., Hamada, H., Hirata, T., & Nakajima, N. (2003). *J Mol Catal, B Enzym, 23*, 145–170. doi:10.1016/S1381-1177(03)00080-8.
3. Furuya, T., Ushiyama, M., Ashida, Y., & Yoshikawa, T. (1989). *Phytochemistry, 28*, 483–487. doi:10.1016/0031-9422(89)80036-6.
4. Mastelic, J., Jerkovic, I., Vinkovic, M., Dzolic, Z., & Vikic-Topic, D. (2004). *Croat Chem Acta, 77*, 491–500.
5. Namme, R., Mitsugi, T., Takahashi, H., & Ikegami, S. (2005). *Tetrahedron Lett, 46*, 3033–3036. doi:10.1016/j.tetlet.2005.03.016.
6. Matkowski, A. (2008). *Biotechnol Adv, 26*, 548–560. doi:10.1016/j.biotechadv.2008.07.001.

7. Banthorpe, D. V., & Osborne, M. J. (1984). *Phytochemistry, 23*, 905–907. doi:10.1016/S0031-9422(00)85057-8.
8. Song, Y. B., & Yu, R. M. (2001). *J Senyang Pharmaceut Univ, 18*(2), 152–156.
9. Yu, R. M., Song, Y. B., Zhang, H., Ye, W. C., Zhang, Y. L., & Yao, X. S. (2003). *Chin J Biotechnol, 19*, 372–375.
10. Saito, K., Yamazaki, M., & Murakoshi, I. (1992). *J Nat Products, 55*, 149–162. doi:10.1021/np50080a001.
11. Sun, Y. S., Liu, Z. B., Wang, J. H., Tian, W., Zhou, H. Y., Zhu, L. X., et al. (2008). *Sep Purif Technol, 64*, 221–226.
12. Ding, Y., Zhao, L., Mei, H., Zhang, S. L., Huang, Z. H., Duan, Y. Y., et al. (2008). *Eur J Pharmacol, 590*, 377–386. doi:10.1016/j.ejphar.2008.06.044.
13. Li, W., Koike, K., Asada, Y., Yoshikawa, T., & Nikaido, T. (2005). *J Mol Catal, B Enzym, 35*(4–6), 117–121. doi:10.1016/j.molcatb.2005.06.006.
14. Kuwajima, H., Shibano, N., Baba, T., Takaishi, K., Inoue, K., & Shingu, T. (1996). *Phytochemistry, 41*, 289–292. doi:10.1016/0031-9422(95)00525-0.
15. Li, G. L., & Zhao, G. (2003). Chinese. *J Org Chem, 23*, 375–379.
16. Demirezer, L. Ö., Kuruüzüm-Uz, A., Bergere, I., Schiewe, H. J., & Zeeck, A. (2001). *Phytochemistry, 58*, 1213–1217. doi:10.1016/S0031-9422(01)00337-5.
17. Xiang, L., Liu, X. H., Fan, G. Q., Cui, Y. X., Du, L. J., & Guo, D. A. (2005). *Chinese Tradit Herbal Drugs, 36*, 1306–1309.
18. Kamel, S., Brazier, M., Desmet, G., Fliniaux, M. A., & Jacquin-Dubreuil, A. (1992). *Phytochemistry, 31*, 1581–1583. doi:10.1016/0031-9422(92)83111-B.
19. Zaalishvili, G. V., Khatisashvili, G. A., Ugrkhelidze, D. S., Gordeziani, M. S., & Kvesitadze, G. I. (2002). *Applied Biochemistry and Microbiology, 6*, 515–524.
20. Yan, C. Y., Zhang, Z., Yu, R. M., & Kong, L. Y. (2007). China J. *Chinese Materia Medica, 32*(3), 192–195.
21. Yan, C. Y., Yu, R. M., Zhang, Z., & Kong, L. Y. (2007). *J Integr Plant Biol, 49*, 207–212. doi:10.1111/j.1744-7909.2007.00400.x.