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Full Length Research Paper

Biotransformation of 3,4-cyclocondensed coumarins by transgenic hairy roots of *Polygonum multiflorum*

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Two new coumarin glycosides (5 and 6), together with one known compound (4), were biosynthesized by transgenic hairy roots of *Polygonum multiflorum* with three 3,4-cyclocondensed coumarins as substrates (1 to 3). Their structures were elucidated by physicochemical and extensive spectroscopic analyses. The results of this study demonstrated that the hairy roots of *P. multiflorum* have the abilities to glycosylate 3,4-cyclocondensed coumarins in a regio- and stereo-selective manner. Furthermore, the effects of three elicitors (salicylic acid, yeast extract and $HgCl_2$) on the accumulation of the products were evaluated.

Key words: *Polygonum multiflorum*, transgenic hairy roots, biotransformation, 3,4-cyclocondensed coumarins.

INTRODUCTION

Coumarin and its derivatives are distributed throughout the nature and show broad spectra of biological activities like anticoagulation (Chen et al., 2003), anti-inflammation (Chan et al., 2010), antioxidation (Manojkumar et al., 2009), anticancer (Musa et al., 2008), etc. They can also be used for the treatment of psoriasis (Chan et al., 2010). Besides, their anti-HIV activity has especially attracted scientists' interest (Yang et al., 2001). However, the water solubility of the compounds is inadequate, which limits their applications, especially for usage in pharmaceuticals. Compared with 3,4-cyclocondensed coumarins, their glycosides are more soluble in water with strong bioactivities. It has been proven that glycocoumarin could be used as inhibitors of viral adsorption, such as the case of their anti-HIV actions (Yu et al., 2003). Recently, some studies also showed that 3,4-cyclocondensed coumarin O-glycosides exhibited anticoagulant activity (Garazd et al., 2005). Their glycosides could also be used in the treatment of skin fungal infection, preventing the skin fungal infections or suspending its development (Musa et al., 2008).

The methods of coumarins glycosylation include chemical synthesis, enzyme synthesis and biological synthesis. There are some disadvantages in the method of chemical synthesis, like high cost, very complex reactions (Garazd et al., 2005), and the yields are always very low. The insufficiency of the method of enzyme synthesis showed strict conditions and relative high cost. Comparing the aforementioned methods, biological synthesis has a lot of advantages. For instance, many reactions might occur in the cells according to the variety of enzymes, such as reductions, oxidations, and particularly glycosylation (Li et al., 2003). The glycosy- lation reaction of phenylpropanoids could be observed as the biotransformation of cinnamic, caffeic, and ferulic acids occur in plant cell cultures of *Eucalyptus perriniana* (Katsuragi et al., 2010). In our previous work, the transgenic hairy roots of *Polygonum multiflorum* have been demonstrated to have the abilitilies of glycosylating simple hydroxycoumarins and some phenolic compounds (Yan et al., 2008; Yu et al., 2008). The hairy roots have the characters of fast growth, genetic stability, cultivation without growth regulators, etc (Wang et al., 2002). There

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are lots of studies on the biotransformation of coumarin compounds by hairy roots. The root cultures of *Panax ginseng* have been demonstrated to have high glycosylation ability to the 7-OH coumarin (Li et al., 2002). The glycosylation reactions also have substrate specificity and the position selectivity. For example, phenolic compounds could be easily glycosylated by *Pharbitis nil* hairy roots. The 7-OH group of coumarins might be particularly vulnerable to glycosylation (Kanho et al., 2004). Therefore, in order to produce the coumarin glycosides with the method of biosynthesis and investigate the regio- and stereo-selectivity of transgenic hairy roots of *P. multiflorum* to 3,4-cyclocondensed coumarins, three 3,4-cyclocondensed coumarins (1, 2 and 3) were chosen as substrates in this paper. Furthermore, the effects of three elicitors (salicylic acid (SA), yeast extract and $HgCl_2$) on the yields of biosynthesis products were also investigated primarily.

MATERIALS AND METHODS

General procedures

Three substrates were synthesized by Pechmann condensation. The structures of the substrates were determined using ^1H NMR, and those of the biosynthesis products were elucidated using ^1H NMR, ^{13}C NMR and heteronuclear multiple bond correlation (HMBC). The spectra were recorded by a Bruker Advance 400 MHz spectrometer in DMSO-d_6 solution and chemical shifts were expressed in δ (ppm) referring to tetramethylsilane (TMS). Electrospray ionization-mass spectra (ESI-MS) were measured by a DSQ spectrometer (Thermo Electron Co.). High performance Liquid chromatography (HPLC) analyses were carried out by Agilent 1200 series with a diode array detection and Phenomenex C_{18} column (5 µm, 4.6 × 250 mm). Separation was performed on a C_{18} column by isocratic elution with water (A) and MeOH (B) (45:55, v/v), and the products were detected at 320 nm. Detection temperature was 30°C. The products were eluted with mobile phase of a mixture of water-MeOH (45:55, v/v) in 20 min.

Preparation of substrates

7-Hydroxy-2,3-dihydrocyclopenta[c]chromen-4-one (1), 7,9-dihydroxy-2,3-dihydro-cyclop- enta[c]chromen-4-one (2), 6,7-dihydroxy-2,3-dihydrocyclopenta[c]chromen-4-one (3) were prepared by Pechmann condensation. Ethyl-2-oxocyclopentane-carboxylate (30 ml) with resorcinol (phloroglucinol or pyrogallol) (22 g) in absolute ethanol (50 ml) was cooled and vigorously stirred. The reactions were treated dropwise with concentrated sulfuric acid, respectively at 0°C until congealed, left overnight and poured into 600 ml of icewater (Garazd et al, 2002).

The three compounds (1 to 3) were finally obtained by filtration and recrystallization from methanol. All the reactions were monitored by TLC (developing agent: ethyl acetate/petroleum ether, 1/1), and ultraviolet (UV) detection at 365 nm. The structures were finally determined by ^1H NMR, and the purity was given to be over 95% by HPLC analysis.

Culture conditions

Hairy roots of *P. multiflorum* were induced in our laboratory. The cultures (7.5 g, fresh weight) have been sub-cultured 10 days at intervals on Murashige and Skoog medium (MS: 200 ml in a 500 ml conical flask, pH 5.75). The incubation was cultured on a rotary shaker (110 rpm) for 8 days at 25°C in the dark. Prior to use, the cultures were sub-cultured three times with dropculture method. All groups were cultured in the same conditions throughout the experiments.

Biotransformation of substrates 1, 2 and 3

Each substrate (1, 2 and 3, each at 600 mg) was administered to 20 flasks of suspension cultures of *P. multiflorum* hairy roots (30 mg/flask). The hairy roots and media were separated by filtration with suction after cultures were incubated for 7 days. The medium was extracted with EtOAc for three times, and then was further extracted with *n*-BuOH for three times. The cultures were dried at 55°C and extracted with MeOH for 2.5 h (0.5 h × 5, 50°C). All the extracts were condensed and passed through silica gel column using a gradient elution of petroleum ether-EtOAc-MeOH system. First, the substrate and other impurities were eluted using the petroleum ether. When the solvents were EtOAc-MeOH (1:9 v/v) and the amount were five column volumes, the products could be completely separated. Subsequently, products were further purified by recrystallization in methanol.

Optimization of the biotransformation

In order to determine the biotransformation capacity of the hairy roots on the three 3,4-cyclocondensed coumarins and evaluate the optimization condition of compound 1, the effects of DMSO to the yields of products were investigated. Furthermore, the time course of compounds 1, 2 and 3 were also established. All experiments were repeated 3 times, and the data were the mean values of three experiments.

The earlier experiment estimated the effect of DMSO to the hairy roots. Every concentration was administered to three flasks cultures, which dissolved 10 mg of compound 1. Final concentrations of the DMSO in the medium were 0.25, 1.00, 2.50, 5.00 and 7.50% (v/v), respectively. After incubation, the hairy roots and media were separated by suction filtration. The medium was condensed to 100 ml and the cultures were dried at 55°C. The yield of the product was determined on the basis of the peak area from HPLC and expressed as a relative percentage to the total amount of the whole reaction products. The extraction and analytic procedures of the following experiments were the same as described earlier.

Meanwhile, to test the relationship of substrate concentration and product yield, compound 1 was administrated to three flasks cultures. Final concentrations of compound 1 in the medium were 0.05, 0.10, 0.25, 0.50 and 1.0 mg/L.

The relationship between the time of substrate supply and glucoside formation was examined as the following procedure. Compound 1 (10 mg) was administered to each flask; then, at 0.5, 1, 2, 3, 4, 5, 6, and 7 days intervals, three flasks were harvested. The time courses of compounds 2 and 3 were investigated with the same procedure as mentioned earlier.

Preparation of the elicitor solutions and administration

SA, yeast extract and $HgCl_2$ were dissolved in DMSO separately and prepared in three concentrations. The elicitor solutions were added into three suspension cultures. Final concentrations of each elicitor in the medium were 0.1, 1.0 and 10.0 mg/L. And each flask was added as the same concentration of compound 1 (20 mg/flask).

Analysis of the sample

All samples were detected under the following conditions: the samples obtained from media were detected after being filtrated by the 0.45 μm microporous membrane. The samples from cultures (0.2 g) were extracted with 10 ml methanol, cooled to room temperature, filtrated by the 0.45 μm microporous membrane, and then determined. The yield of the products was determined on the basis of the peak area from HPLC and expressed as a relative percentage to the total amount of whole reaction products.

RESULTS

Structures of the biotransformation products

Structures of compounds 1, 2, and 3 were verified by comparing their NMR spectral data with literature (Garazd et al., 2002). Two new compounds (5 and 6) along with one known compound (4) were biosynthesized by hairy roots of *P. multiflorum* when compounds 1, 2 and 3 were administrated as substrates. The spectral data of compound 4 and two new compounds (5 and 6) are as follows.

7-O-*β*-D-glucopyranosyl-2,3-dihydrocyclopenta[c]chromen-4-one (4). Colorless needle crystals. ^{1}H NMR (400 MHz, DMSO-d_6, δ ppm, *J*/Hz): δ 2.12 (2H, m, CH_2-2), 2.75 (2H, m, CH_2-1), 3.07 (2H, m, CH_2-3), 3.14-3.73 (6H, m, H of glucopyranosyl), 4.60 (t, *J* = 6 Hz, OH of glucopyranosyl), 5.03 (1H, d, *J* = 7.2 Hz, H'), 5.31 (d, *J* = 4.8 Hz, OH of glucopyranosyl), 5.07 (d, *J* = 5.6 Hz, OH of glucopyranosyl), 5.13 (d, *J* = 4.4 Hz, OH of glucopyranosyl), 5.31 (d, *J* = 4.8 Hz, OH of glucopyranosyl), 7.02 (1H, dd, *J* = 8.8 Hz, 1.6, H-8), 7.09 (1H, d, *J* = 1.6 Hz, H-6), 7.56 (1H, d, *J* = 8.8 Hz, H-9). ^{13}C NMR (100 MHz, DMSO-d_6, δ ppm): 160.09 (C-7), 159.69 (C-4), 156.91 (C-6a), 155.37 (C-1a), 126.73 (C-3a), 124.53 (C-9), 113.92 (C-6), 113.25 (C-9a), 103.71 (C-8), 100.66 (C-1'), 77.62 (C-5'), 76.98 (C-3'), 73.65 (C-2'), 70.17 (C-4'), 61.16 (C-6'), 32.13 (CH_2-3), 30.535 (CH_2-1), 22.48 (CH_2-2). Each signal in the ^{1}H NMR, ^{13}C NMR spectra of compound 4 was compared with substrate in the literature (Garazd et al., 2002), and the data were practically consistent with the reference. Therefore, the structure of compound 4 was determined to be 7-O-*β*-D-glucopyranosyl-2,3-dihydrocyclopenta[c]chromen-4-one.

9-O-*β*-D-glucopyranosyl-7-hydroxy-2,3-dihydrocyclopenta[c]chromen-4-one (5). Needle crystals. ESI-MS: *m/z* 381.0 $[M+H]^+$, *m/z* 379.6 $[M-H]^+$, *m/z* 217.4 $[M-162-H]^{+1}$. ^{1}H NMR (400 MHz, DMSO-d_6, δ ppm): δ 2.21 (2H, m, CH_2-2), 2.63 (2H, m, CH_2-1), 3.23 (2H, m, CH_2-3), 3.30-3.73 (6H, m, H of glucopyranosyl), 4.59 (1H, d, *J* = 5.2 Hz, OH of glucopyranosyl), 4.94 (1H, d, *J* = 7.6 Hz, H-1'), 5.05 (1H, d, *J* = 5.2 Hz, OH of glucopyranosyl), 5.12 (1H, d, *J* = 2.4 Hz, OH of glucopyranosyl), 5.31 (1H, d, *J* = 4.0 Hz, OH of glucopyranosyl), 6.41 (1H, d, *J* = 2 Hz, H-6), 6.50 (1H, d, *J* = 2.0 Hz, H-8), 10.45 (1H, s, 7-OH). ^{13}C NMR (100 MHz, DMSO-d_6, δ ppm): δ 161.24 (C-7), 159.62 (C-4), 156.44 (C-9), 156.28 (C-6a), 156.08 (C-1a), 122.95 (C-3a), 102,94 (C-9a), 101.06 (C-1'), 98.87 (C-8), 96.70 (C-6), 77.57 (C-5'), 77.20 (C-3'), 73.77 (C-2'), 69.92 (C-4'), 61.97 (C-6'), 36.14 (CH_2-3), 29.64 (CH_2-1), 22.55 (CH_2-2). Compound 5 was obtained as colorless needle crystals (in MeOH). The ESI-MS demonstrated a quasimolecular ion peak $[M-H]^-$ at *m/z* 379.6, $[M+H]^+$ at *m/z* 381.0, $[M-162-H]^+$ at *m/z* 217.4 and the molecule weight were 162 more than the substrate 2, which showed the molecular formula $C_{18}H_{20}O_9$, indicating that compound 5 might be a glucosylation product of substrate 2. ^{1}H NMR spectrum displayed anomeric proton signal (δ 4.94, H, d, *J* = 7.6 Hz). The coupling constant (7.6 Hz) indicated that the configuration of sugar was *β* type. The ^{13}C NMR spectrum showed anomeric carbon at δ 101.1. The sugar generated by acid hydrolysis was compared with the standard of *β*-D-glucose by paper chromatography, and found that they are both R_f value were the same. In the HMBC spectrum H-1 (4.60) of the terminal glucose and C-6 (61.0) of the inner glucose gave the linkage of the sugar units to be D-glucose. Thus, the structure of the product was proposed to be *β*-D-glucose. The signals of δ 2.21 (2H, m), 2.63 (2H, m), 3.23 (2H, m) 6.41 (1H, d, *J* = 2 Hz), 6.50 (1H, d, *J* = 2.0 Hz), 10.45 (1H, s) showed that the positions of 2, 3, 8, and 9 were not replaced, while sugar group was connected in one of the phenolic hydroxyl group. HMBC spectrum showed that the signal of C-9 (δ 156.4) was related to the anomeric proton and the δ 6.50 (1H, d, *J* = 2.0 Hz) signal, indicating that the sugar group was attached to the 9-OH. The signal δ 6.50 (1H, d, *J* = 2.0 Hz) was H-8. However, the δ 6.41 (1H, d, *J* = 2 Hz) was the H-6. δ 156.44 was related with the δ H 6.41, 6.50, indicating that the signal is the C-7 signal. δ 156.2 was connected with δ 6.50, it is for the C-6a signal. There were connection between δ 156.1 and δ H 2.21 (2H, m), 2.63 (2H, m), 3.23 (2H, m), indicating the signal is C-1a. δ 121.95 and δ H 2.21 (2H, m), 2.63 (2H, m), 3.23 (2H, m) were related, δ 121.95 is C-3a signal. Correlations were observed between δ 102.94 and H-8, suggesting δ 102.94 is C-9a signal. δ 101.06 is sugar carbon. Thus, compound 5 was elucidated to be 9-O-*β*-D-glucopyranosyl-7-hydroxyl-2,3-dihydrocyclopenta[c]chromen- 4-one, which is a new compound.

6-O-*β*-D-glucopyranosyl-7-hydroxy-2,3-dihydrocyclopenta[c]chromen-4-one (6). White needle crystal. ESI-MS: m/z 403.3 $[M+Na]^+$, m/z 783.5 $[2M+Na]^+$, m/z 379.6 $[M-H]^+$. ^{1}H NMR (400 MHz, DMSO-d_6, δ ppm): δ 2.10 (2H, m, CH_2-2), 2.73 (2H, m, CH_2-1), 3.03 (2H, m, CH_2-3), 3.18-3.64 (6H, m, H of glucopyranosyl), 4.20 (1H, d, *J* = 5.2 Hz, OH of glucopyranosyl), 4.95 (1H, d, *J* = 7.6 Hz, H-1'), 4.96 (d, *J* = 4.4 Hz, OH of glucopyranosyl), 5.09 (d, *J* = 4.4 Hz, OH of glucopyranosyl), 6.89 (1H, d, *J* = 8.4 Hz, H-8), 7.20 (1H, d, *J* = 8.4 Hz, H-9). ^{13}C NMR (100 MHz, DMSO-d_6, *δ* ppm): *δ* 158.93 (C-4), 156.87 (C-1a), 152.44 (C-7), 147.66 (C-6a), 131.25 (C-6), 122.67 (C-3a), 120.84 (C-9), 113.14 (C-8), 111.61 (C-9a), 103.92 (C-1'), 77.29 (C-5'),

76.26 (C-3'), 73.88 (C-2'), 69.69 (C-4'), 60.82 (C-6'), 31.74 (CH_2-3), 9.97 (CH_2-1), 22.04 (CH_2-2). The ESI-MS demonstrated a quasimolecular ion peak $[M–H]^-$ at *m/z* 379.6, $[M+Na]^+$ at m/z 403.3 and the molecule weight were 162 more than substrate 3, which showed the molecular formula $C_{18}H_{20}O_9$, indicating that compound 6 might be a glucosylation product of substrate 2. In the ^1H NMR spectra of compound 6, an anomeric proton signal was observed at δ 4.95 (1H, d, J = 7.6 Hz), indicating that the configuration of sugar was *β* -type. The sugar generated by acid hydrolysis was compared with the standard of *β*-D-glucose by paper chromatography (PC), and it found that the R_f value of both were the same. In addition, the component sugar in compound 6 was indicated to be *β*-D-glucose on the basis of the patterns of the carbon and proton signals caused by the sugar moiety in NMR spectra. In the HMBC spectrum, the signal of δ 131.3 (C-6) was related to the anomeric proton and indicated that the sugar group was connected to C_6-OH. The ^{13}C NMR spectrum showed anomeric carbon at δ 101. Other signals were confirmed the same as compound 5. Thus, compound 6 was elucidated to be 6-O-*β*-D-glucopyranosyl -7-hydroxyl-2,3-dihydrocyclopenta[c]chromen- 4-one, which is a new compound. The structures of compounds 1, 2, 3, 4, 5 and 6 are as shown in Figure 1.

Optimization of the biotransformation condition

The yields of compound 4 displayed a declining tendency as the addition of DMSO, with the highest yield (78.42%) at the concentration of 0.25%. It was also found that the product was only stored in the hairy roots and not detected in the medium. The product secretion rate was 36.96% at the highest concentration of DMSO (7.50%), while the yield of compound 4 was only 14.81%. The 0.25% of DMSO was chosen to dissolve the substrates as the optimal concentration.

The yields of compound 4 did not display a significant difference when the concentrations of compound 1 were between 0.05 and 0.25 mg/ml. The range of the yield was among 78 and 82%, but the yield was only 24.20% when the concentration was 0.5 mg/ml. Therefore, the concentrations were chosen among 0.05 and 0.25 mg/ml in the following experiments.

As shown in Figure 2, the concentration of compound 1 decreased rapidly in one day (10 to 3.44 mg). The yield of compound 4 increased sharply at the first day, indicating that biotransformation happened on the first day, and compound 4 reached the maximum transformation rate at the fourth day (81.35%).

The time course of compound 5 (Figure 3) showed that compound 5 was rapidly synthesized in a half-day. The accumulation of compound 5 reached the highest at the third day, and the yield was 93.40%. As shown in Figure 4, the biosynthesis reaction of compound 6 occurred at the first day. The highest yield was 18.14% at the second day. After that, the total of the substrate and product tends to a steady state.

Effects of the elicitor administration

The yield of compound 4 was 82.15% (the concentration of 1 was 0.1 mg/ml) without any elicitor and increased about 11% as the addition of SA in the concentration of 1 mg/L (Table 1). The yield was very low when the concentration of SA was 10 mg/L, and compound 4 are only 5.78 mg/flask. The yield was 94.24% which increased 12% as the yeast extract were added in the concentration of 1.0 mg/L, respectively. There was little inducing effect with $HgCl_2$ addition, and the accumulation of the product was lower than those of when the concentration was 1.0 and 10 mg/L.

The yield of compound 4 declined obviously when the concentration of the three elicitors reached the maximum (10 mg/L). Over dosage of elicitors failed to increase the product accumulation.

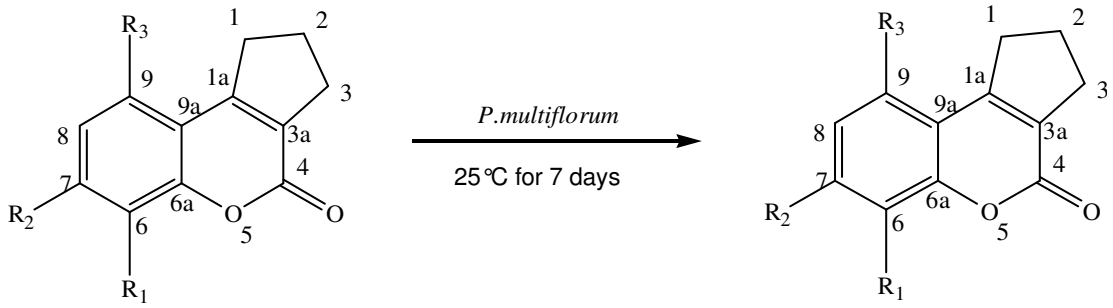
Water solubility of the substrates and their products

The comparison of the water solubility of the substrates and their products was shown in Table 2. The water solubility of three biosynthesis products increased obviously, especially for compound 5, which has increased 75.6 folds. The result showed that conjugation of glucose residue to 3,4-cyclocondensed coumarins could increase the water solubility. Therefore, it is a useful way to improve the water solubility of the 3,4-cyclocondensed coumarins by glycosylation method.

DISCUSSION

The transgenic hairy roots of *P. multiflorum* showed strong ability to transform the 3,4-cyclocondensed coumarins, and the yield was much higher when compared with the chemical reaction (42.84%) (Garazd et al., 2005). The lowest conversion rate of 6,7-dihydroxy-2,3-dihydrocyclopenta[c]chromen-4 coumarins (3) may be due to steric hindrance of the *o*-hydroxy. The sugar group connection positions indicated that the hairy roots were superior to glycosylate 9-OH or 6-OH when 7,9-dihydroxy or 6,7-dihydroxy 2,3-dihydrocyclopenta[c]chromen-4 coumarins were administrated. It shows that the hairy roots of *P. multiflorum* have the position selectivity. This result certifies that the biotransformation is indeed a useful way for the production of 3,4-cyclocondensed coumarin glycosides and new compounds.

The biotransformation was so rapid that it was an effective way to produce the 3,4-cyclocondensed coumarin glycosides. The major influence factors of



1. R_2=OH; R_1=R_3=H
2. R_2=R_3=OH; R_1=H
3. R_1=R_2=OH; R_3=H
4. R_2=OGlc; R_1=R_3=H
5. R_3=OGlc; R_2=OH; R_1=H
6. R_1=OGlc; R_3=H; R_2=OH

Figure 1. Biotransformation of the three 3,4-cyclocondensed coumarins by hairy roots of *P. multiflorum* showing the structures of the substrates (**1**, **2** and **3**) and their products (**5**, **6** and **7**) were as above. The structures of the products are all glycosides.

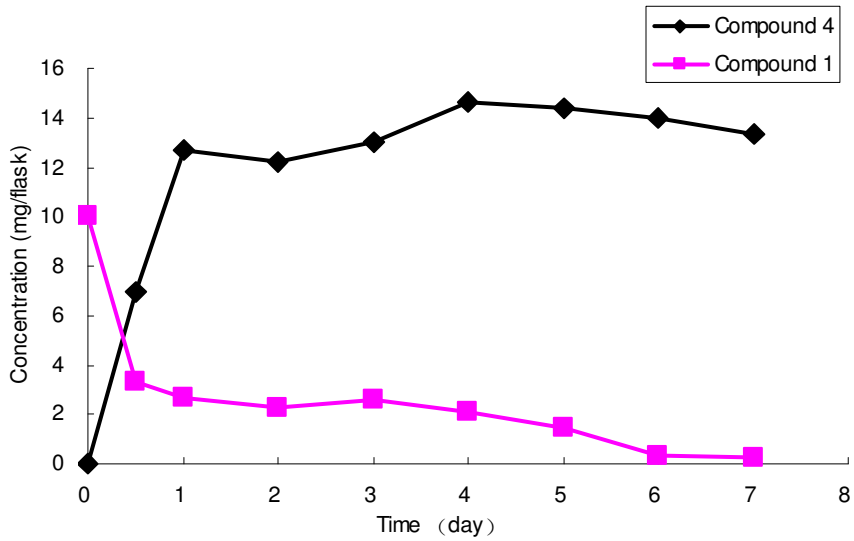


Figure 2. Time-course of 7-hydroxy-2,3-dihydrocyclopenta[c]chromen-4-one (**1**) by hairy roots of *P. multiflorum*. At one day interval, three flask cultures were harvested and the yields of the substrate and product were analyzed. The result showed that the product yield reached the highest (81.35%) on the fourth day. The substrate was the lowest on the seventh day. Total quantity of compound **1** (■); fields of the compound **4** (product of the compound **1**) (◆).

biotransformation occurring and the conversion rate (cell, organ, etc.) are enzyme system as well as the substrate structural features, which include co-culturing time, substrate concentration, etc (Michiko et al., 1995). The substrate concentration increasing within a certain range could effectively improve the yield of the product, but the appropriate maximum concentration should be studied. Yields and conversion rates will decline, because the high concentration will cause harm to the culture (Tripathi et al., 2002). The effects of the co-culturing time, concentrations of the DMSO and substrates were investigated, and the results have shown that the over dosage failed to increase the product accumulation. Therefore, the optimization conditions of the biotransformation were 0.25% of DMSO, concentrations of substrates among 0.05 and 0.25 mg/ml. The appropriate co-culturing time of the compound 4, 5, and 6 were four, three, two days, respectively.

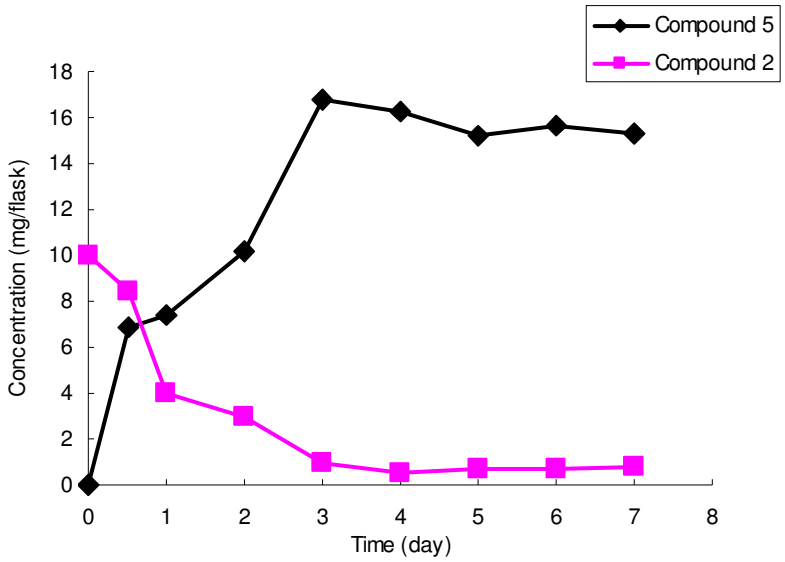


Figure 3. Time-course of 7,9-dihydroxy-2,3-dihydrocyclop-enta[c]chromen-4-one (**2**), by hairy roots of *P. multiflorum*. At one day interval, three flask cultures were harvested and the yields of the substrate and product were analyzed. The result showed that the product yield reached the highest (93.40%) on the third day. The substrate was the lowest on the fourth day. Total quantity of compound **2** (■); fields of the compound **5** (product of the compound **2**) (◆).

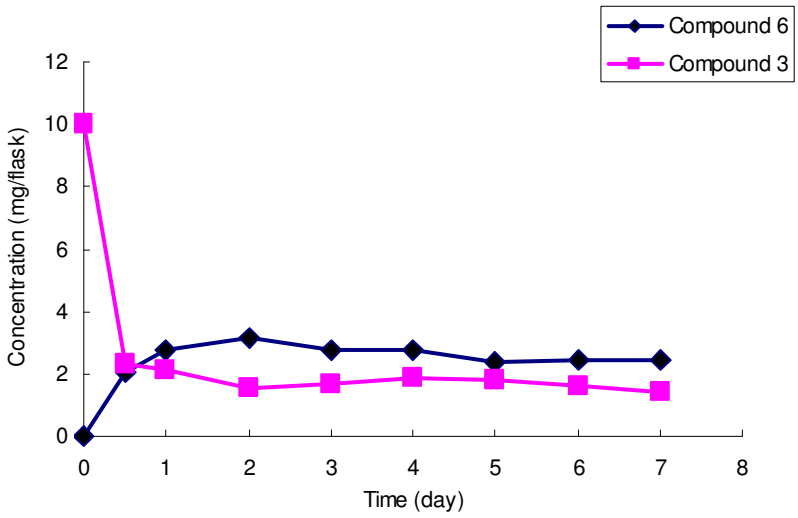


Figure 4. Time-course of 6,7-dihydroxy-2,3-dihydrocyclopenta[c]chromen-4-one (**3**) by hairy roots of *P. multiflorum*. At one day interval, three flask cultures were harvested and the yields of the substrate and product were analyzed. The result showed that the product yield reached the highest (18.14%) on the second day. The substrate was the lowest on the seventh day. Total quantity of compound **3** (■); fields of the compound **6** (product of the compound **3**) (◆).

According to the literatures, the plasma membrane could be stronger with the increase of elicitor concentration. Some studies showed umbelliferone could be stimulated in suspension cultures of plant tissues using 1.0 mg/L $HgCl_2$ (Smith et al., 2001). Over dosage of elicitors failed to increase the product accumulation. The yield of taxol increased 4 times as the concentration of SA was 0.10 mg/L, but when the concentrations of SA

Table 1. Yields of the compound **4** with different concentration elicitors.

Elicitor	Concentration of the elicitor (mg/L)	Compound 4 (mg/flask)	Yield (%)
Blank	0	14.80	82.15
SA	0.1	14.10	78.25
	1.0	16.79	93.15
	10	5.78	32.10
$HgCl_2$	0.1	14.66	81.34
	1.0	10.85	60.21
	10	10.22	56.71
YE	0.1	15.24	84.56
	1.0	16.98	94.24
	10.	12.88	71.49

The accumulations of the product were increasing when the concentrations of the SA and YE were 1.0 mg/L.

Table 2. Water solubility of 3,4-cyclocondensed coumarins and their biosynthesis products.

Substrate	W1 (mol/ml)	W2 (mol/ml)	Fold
1	0.080	1.92	24.00
2	0.025	1.89	75.60
3	0.14	0.79	5.64

W1 were mean water solubility of substrate; W2 were mean water solubility of product.

were more than 0.1 mg/L, the yield of the taxol decreased, because of the decrease of the plant cell activity (Miao et al., 2000). The release of puerarin increased gradually with increase in SA concentrations (Zhang and Li, 2003). The accumulation of glycyrrhizic acid could also be improved with effective concentration ranges for SA and yeast extract (Bian et al., 2008). These mentioned elicitor experiments demonstrate that it is important to choose the effective concentration of elicitor to improve product accumulation. When the concentrations of the elicitors were over dosage, the yield of the product decreased because of the decrease of the hairy roots activity. Therefore, the optimum concentration of SA and yeast extract was 1.0 mg/ml.

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REFERENCES

Bian AH, Gao WY, WJ (2008). The effect of glycyrrhizic acid accumulation of licorice son suspension with different elicitors. China. Pharm. J. 22:1690–1693.

Chan YY, Li CH, Shen YC, Wu TS (2010). Anti-inflammatory principles from the stem and root barks of *Citrus medic*. Chem. Pharm. Bull. 58(1):61-65.

Chen KS, Wu CC, Chang FR, Chin YC, Chiang MY, Wang WY, Wu YC (2003). Bioactive coumarins from the leaves of *Murraya Omphalocarpa*. Planta. Med. 69(7):654-657.

Garazd MM, Garazd YaL, Shilin SV, Panteleimonova TN, Khilya VP (2002). Modified coumarins. 4. synthesis and biological properties of cyclopentaneannelated furocoumarins. Chem. Nat. Compd. 38:230–242.

Garazd YL, Kornienko EM, Maloshtan LN, Garazd MM, Khilya VP (2005). Modified coumarins synthesis and anticoagulant activity of 3,4-cycloannelated coumarin D-glycopyranosieds. Chem. Nat. Comp. 41:508–512.

Kanho H, Yaoya S, Itani T, Nakane T, Kawahara N, Takase Y, Masuda K, Kuroyanagi M (2004). Glucosylation of phenolic compounds by *Pharbitis nil* hairy roots: I. Glucosylation of coumarin and flavone derivatives. Biosci. Biotechnol. Biochem. 68:2032–2039.

Katsuragi H, Shimoda K, Kubota N, Nakajima N, Hamada H, Hamada H (2010). Biotransformation of cinnamic acid, *p*-coumaric acid, caffeic acid, and ferulic acid by plant cell cultures of *Eucalyptus perriniana*. Biosci. Biotechnol. Biochem. 74:1920–1924.

Li W, Koike K, Asada Y, Yoshikawa T, Nikaida T (2002). Biotransformation of umbelliferone by *Panax ginseng* root cultures. Tetrahedron. Lett. 43:5633–5635.

Li W, Koike K, Asada Y, Yoshikawa T, Nikaido T (2003). Biotransformation of low-molecular-weight alcohols by *Coleus forskohlii* hairy root cultures. Carbohydr. Res. 338:729–731.

Manojkumar P. Ravi TK, Gopalakrishnan S (2010). Antioxidant and antibacterial studies of arylazopyrazoles and arylhydrazonopyrazolones containing coumarin moiety. Eur. J. Med. Chem. 44:4690–4694,

Miao ZQ, Wei ZJ, Yuan YJ (2000). The effect of salicylic acid in taxol biosynthesis. Chin. J. Biotechnol. 16:509–513.
Michiko Y, Koichiro S, Kazuo S, Kunitoshi Y, Kanjil (1995). Glucosylation of phenolics by hairy root cultures of *Lobelia sessitifolia*. Phytochemistry 39:1149–50.
Musa MA, Cooperwood JS, Khan MOF (2008). A review of coumarin derivatives in pharmacotherapy of breast cancer. Curr. Med. Chem.15:2664-2679,
Smith RA, Drummond S, Haines A, Porter JR, Hock RS (2001). Induction of umbelliferone in sweet potato cell suspension culture using mercuric chloride. Biotechnol. Lett. 23:1397–1400.
Tripathi U, Rao SR, Ravishankar GA (2002). Biotransformation of phenylpropanoid compounds to vanilla flavor metabolites in cultures of *Haematococcus pluvialis*. Process. Biochem. 38:419-426.,
Wang L, Yu RM, Zhang H, Cheng KL (2002). The cultivating and active ingredients producing of the *Polygonum multiflorum* hairy roots. Chin. J. Biotechnol. 18:69–73.
Yan CY, Yu RM, Zhang Z, Kong, LY (2007). Biotransformation of arbutin by transgenic hairy roots of *Polygonum multiflorum*. J. Integer. Plant Biol. 49:207–212.
Yang JS (2001). Research progress of anti-HIV activity of coumarins. West Chin. J. Pharm. Sci. 16(4):285-288.
Yu DL, Suzuki M, Xie L, Morris-Natschke SL, Lee KH (2003). Recent progress in the development of coumarin derivatives as potent anti-HIV agents. Med. Res. Rev. 23:322-345.
Yu RM, Zhou LB, Yan CY, Duan GY, Zhao Y (2008). Two new coumarin lucosides biosynthesized by transgenic hairy roots of *Polygonum multiflorum*. Chin. Chem. Lett. 19:76-78.
Zhang CR, Li L (2003). Salicylic acid, jasmonic acid methyl ester and ethylene poison ivy cells of the influence of puerarin suspension culture production. J. Plant Res. Environ. 12:56–57.