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Microbial transformation of baicalin and baicalein

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Abstract

As a result of microbial transformation of baicalin and baicalein the products of 4′-hydroxylation of the B ring, *O*-methylation at C-6, and both *O*-methylation at C-6 and hydroxylation at C-4′ were obtained. Transformations of baicalin were accompanied by the reaction of hydrolysis.

Keywords: Biotransformations; Baicalin; Baicalein; *Coryneum betulinum*; *Chaetomium* sp.; *Penicillium chrysogenum*; *Cryptosporiopsis radicicola*

1. Introduction

Baicalin (baicalein-7-*O*-glucuronide) (**1**) and baicalein (5,6,7-trihydroxyflavone) (**2**) are the most important biologically active components of *Radix Scutellariae* [1–4]. Both the raw material and the flavones isolated from it – baicalin (**1**) and baicalein (**2**) – exhibit wide range of pharmacological activities due to their antiallergic, anti-inflammatory, antiatherogenic, antithrombotic, antibacterial, antiviral and anticancerogenic properties [5–8]. These properties arise mainly from antioxidant activity of flavonoids [9] and may be changed or improved by modification of compound structures. Biotransformations may be a convenient way of achieving this goal [10,11].

Antioxidant activity of the compounds may be enhanced by increasing the number of hydroxyl groups in the flavone system, especially in the B ring [12]. The presence of methoxyl groups increases solubility in lipid phases and changes affinity for active centers in biological membranes, which influence biological activity of chemical compounds [13].

The metabolism of baicalin and baicalein in mammals, as well as in the case of other flavonoids, is not fully known. Microbial transformations may be helpful in studies on this subject due to similarities in transformations of several compounds by mammals and by microorganisms [14–16].

2. Experimental

2.1. General

The course of microbial transformations was monitored by HPLC, performed on Waters 2690 instrument fitted with Waters 996 Photodiode Array Detector and Millennium 32 software. Resolution was achieved using an ODS 2 analytical column from Waters (4.6 mm × 250 mm) with a Guard-Pak Inserts μBondapak C18 pre-column (Waters) and a preparative column LiChrosorb RP-18 (7 μm), 25 mm × 250 mm (Merck). Gradient elution was applied using acetonitrile/4.5% formic acid solution (80:20) as eluent A and 4.5% formic acid solution as eluent B; detection at 280 nm; flow for analytical resolution 1 ml/min; flow for preparative resolution 3 ml/min. The following gradient conditions have been used to resolution and purification of the compounds: 0–7 min (100% eluent B), 7–15 min (85% eluent B, 15% eluent A), 15–20 min (50% eluent B, 50% eluent A), 20–30 min (20% eluent B, 80% eluent A), 30–40 min (10% eluent B, 90% eluent A). ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker Avance^{TM} 600 spectrometer. The ^{1}H NMR spectra were recorded in DMSO-d_6, whereas ^{13}C NMR in DMSO-d_6 and Me_2CO-d_6.

2.2. Microorganisms and substrates

The strains *Coryneum betulinum* (6534), *Chaetomium* sp. (6665), *Cryptosporiopsis radicicola* (6671) came from collection of the Department of Forest Pathology of the Agricultural

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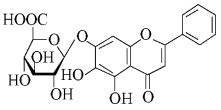
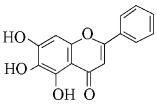
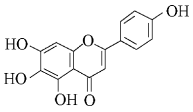
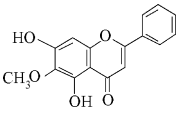
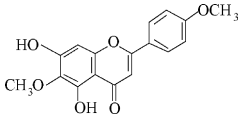
Table 1
^{1}H NMR chemical shifts (δ) of compounds 1–5 (solvent: DMSO-d_6)

Proton	Compound				
					
H-3	6.99, s	6.92, s	6.71, s	6.90, s	6.86, s
H-8	7.04, s	6.63, s	6.80, s	6.98, s	6.64, s
H-2′	8.07, m	8.05, m	7.80, d, J = 8 Hz	8.12, m	8.02, d, J = 9 Hz
H-3′	7.59, m	7.58, m	6.94, d, J = 8Hz	7.70, m	7.12, d, J = 9 Hz
H-4′	7.59, m	7.58, m		7.70, m	
H-5′	7.59, m	7.58, m	6.94, d, J = 8Hz	7.70, m	7.12, d, J = 9 Hz
H-6′	8.07, m	8.05, m	7.80, d, J = 8 Hz	8.12, m	8.02, d, J = 9 Hz
5-OH	12.58, s	12.66, s	12.89, br s	12.97, br s	13.01, s
6-OH	8.65, s	8,83, br s	8,78, br s		
7-OH		10,52 br s	10,43, br s	10.27, br s	10.30, br s
6-OMe				3.98, s	3.87, s
4′-OMe					3.78, s

University of Kraków. The strain *Penicillium chrysogenum* (112) came from the collection of Department of Chemistry of the Wrocław University of Environmental and Life Sciences. The microorganisms were stored on agar slants with Sabouraud, at 8 °C. Substrates for the biotransformation: baicalin (**1**) and baicalein (**2**) were purchased from Aldrich.

2.3. Screening procedure

Cultivation media consisted of 3% glucose and 1% peptobac in water. The microorganisms were transferred from the slants to 250 ml Erlenmayer flasks, each containing 100 ml of the medium. Pre-incubation was performed at 25 °C for 24–48 h, until the proper growth of the microorganisms was achieved. Then portions of 1 ml of the culture solution were transferred to inoculate 250 ml flasks, each containing 100 ml of the medium. After cultivation at 25 °C for 48 h on a rotary shaker, 10 mg of a substrate, dissolved in 0.5 ml of THF (tetrahydrofuran), was added to the grown culture. Control cultivation with no substrate was also performed. After 3, 6 and 12 days of incubation under the above conditions, portions of 5 ml of the transformation mixture were taken out and extracted with ethyl acetate (3 ml × 3 ml). The extracts were dried over $MgSO_4$, concentrated in vacuo and analyzed by HPLC.

2.4. Preparative biotransformation

Portions of 1 ml of the pre-incubation culture solution were used to inoculate three 2000 ml flasks, each containing 500 ml of the cultivation medium. The cultures were incubated at 25 °C for 48 h on a rotary shaker. Then 50 mg of a substrate dissolved in 2.5 ml of THF was added to each flask (100 mg of the substrate per 1 l of the cultivation mixture). After 6 days of incubation the mixtures were extracted with ethyl acetate (3 ml × 200 ml), dried ($MgSO_4$) and concentrated in vacuo. The transformation products were separated by HPLC. Pure products were identified by means of spectral analyses (^{1}H NMR, ^{13}C NMR).

Table 2
^{13}C NMR chemical shifts (δ) of compounds 1–5

Carbon	Compound				
	1[a]	**2**[a]	**3**[a]	**4**[b]	**5**[a]
C-2	103.4	163.4	166.8	163.7	163.0
C-3	106.1	105.0	103.4	104.9	102.7
C-4	182.3	182.6	184.4	182.5	181.2
C-5	146.8	147.4	147.9	152.2	153.3
C-6	130.6	131.4	131.9	132.8	131.2
C-7	151.2	154.1	152.8	154.2	157.0
C-8	93.8	94.4	95.8	94.2	94.0
C-9	149.4	150.3	151.3	152.4	152.1
C-10	104.7	104.7	107.5	106.1	104.0
C-1′	130.8	131.5	123.2	130.6	122.7
C-2′	126.4	126.8	129.6	126.3	127.8
C-3′	129.1	129.7	117.0	129.1	114.2
C-4′	132.0	132.3	162.9	132.1	162.1
C-5′	129.1	129.7	117.0	129.1	114.2
C-6′	126.4	126.8	129.6	126.3	127.8
C-1″	100.0				
C-2″	72.7				
C-3″	75.3				
C-4″	71.2				
C-5″	75.5				
C-6″	170.1				
6-OMe				60.2	59.6
4′-OMe					55.4

[a] Solvent: DMSO-d_6.
[b] Solvent: Me2CO-d_6.

Spectral data of the products obtained are presented in Tables 1 and 2.

3. Results and discussion

Biotransformations were performed on two flavone substrates: baicalin (**1**) and its aglycone—baicalein (**2**). We started from screening tests that comprised the strains of species: *Aspergillus*, *Coryneum*, *Chaetomium*, *Cryptosporiopsis* and

Penicillium. This preliminary study led to selection of 4 out of 16 microorganisms which were capable of transformation of the selected substrates: *Coryneum betulinum*, *Chaetomium* sp., *Cryptosporiopsis radicicola* oraz *Penicillium chrysogenum*. The transformations observed included: glycoside bond hydrolysis, hydroxylation in the B ring, *O*-methylation and methoxylation. Microbial transformations on both substrates were continued for the time sufficient for the full consumption of the starting material (6 days).

Biotransformation of glucuronide—baicalin (**1**) in the culture of *C. betulinum* led to glycoside bond hydrolysis along with hydroxylation at 4′ position in the B ring (Fig. 1). The resulting product – 4′,5,6,7-tetrahydroxyflavone (**3**) – was isolated in 40% yield (60 mg/150 mg of the substrate). There was no baicalein (the product of baicaline hydrolysis) observed in the post-reaction mixture, therefore we conclude that the hydroxylation of baicalin (**1**) occurs prior to the hydrolysis, in which the glucuronic acid molecule is detached. Additional confirmation of this reaction course is the observation that baicalein (**2**) does not undergo biotransformation by the same strain.

Product **3** was identified by means of ^{1}H NMR and ^{13}C NMR. In the ^{1}H NMR spectrum there are two 2H doublets at $\delta = 6.94$ ppm and $\delta = 7.80$ ppm of the same coupling constants $J = 8$ Hz observed, which come from 3′,5′ and 2′,6′ protons. These indicate substitution at C-4′ in the B ring (Table 1). In the ^{13}C NMR spectrum the presence of hydroxyl group at C-4′ in product **3** is confirmed by the change of chemical shift of C-4′ signal from 132.0 ppm in the spectrum of baicalin (**1**) to 162.9 ppm in the spectrum of 4′,5,6,7-tetrahydroxyflavone (**3**). Hydrolysis of baicalin (**1**) results in disappearing the signals of glucuronic acid carbons in the ^{13}C NMR spectrum of 4′,5,6,7-tetrahydroxyflavone (**3**) (Table 2).

Biotransformation of baicalin catalyzed by the strain of *Chaetomium* sp. led to formation of product of *O*-methylation at C-6 in the A ring. The product, 5,7-dihydroxy-6-methoxyflavone (**4**), was isolated in 35% yield (52.5 mg/150 mg of the substrate). Probably the reaction of methylation was preceded by hydrolysis of the glycoside bond, because biotransformation of baicalein (**2**) (aglycone) by means of *Chaetomium* sp. gave *O*-methylation product (**4**) in much higher yield (79.2%) and all the starting material was consumed. The structure of 5,7-dihydroxy-6-methoxyflavone (**4**) was determined by means of ^{1}H NMR. The signal of 6H at $\delta = 8.65$ ppm in the spectrum of baicalin (**1**) was replaced by the singlet of 3H at $\delta = 3.98$ ppm of the 6-methoxyl group of product **4**. Additionally, in the ^{1}H NMR spectrum of **4** two broad singlets at $\delta = 12.97$ ppm and $\delta = 10.27$ ppm are observed, which are ascribed to 5-OH and 7-OH hydroxyl protons (Table 1). Therefore, the only possible position of *O*-methylation is C-6. In the ^{13}C NMR *O*-methylation along with hydrolysis resulted in appearance of a new signal at $\delta = 60.2$ ppm, characteristic for methoxyl carbon atom, and disappearance of glucuronic acid signals in the spectrum of **4** (Table 2).

5,7-Dihydroxy-6-methoxyflavone (**4**) was also the product of the microbial transformation of the aglycone—baicalein (**2**). The catalysts suitable for this reaction (apart from the mentioned before *Chaetomium* sp.) were the strains of *C. radicicola* and *P. chrysogenum* (Fig. 2). In the first case the product of methylation (**4**) was isolated in 11.7% yield (17.6 mg), in the second one in 12% (18 mg).

In the presence of enzymatic system of *P. chrysogenum* 5,7-dihydroxy-4′,6-dimethoxyflavone (**5**) was obtained as the second product of transformation of baicalein (**2**) (along with **4**) and isolated in 5.2% yield (7.8 mg) (Fig. 2). Its structure was confirmed by the ^{1}H NMR spectrum in which, unlike in the spectrum of substrate **2**, two 2H doublets at $\delta = 8.02$ ppm and $\delta = 7.12$ ppm ($J = 9$ Hz) are visible. These are the signals of 2′,6′ and 3′,5′ protons, respectively, which indicate substitution at 4′ in the B ring. The presence of methoxyl group at 4′ is confirmed by 3H singlet at $\delta = 3.78$ ppm. The presence of the second methoxyl group is indicated by the singlet of 3H at $\delta = 3.87$ ppm, whereas its location at C-6 is confirmed by two signals at $\delta = 13.01$ ppm and $\delta = 10.30$ ppm, which are ascribed to 5-OH and 7-OH, respectively, and also lack of the 6-OH proton signal, when compared

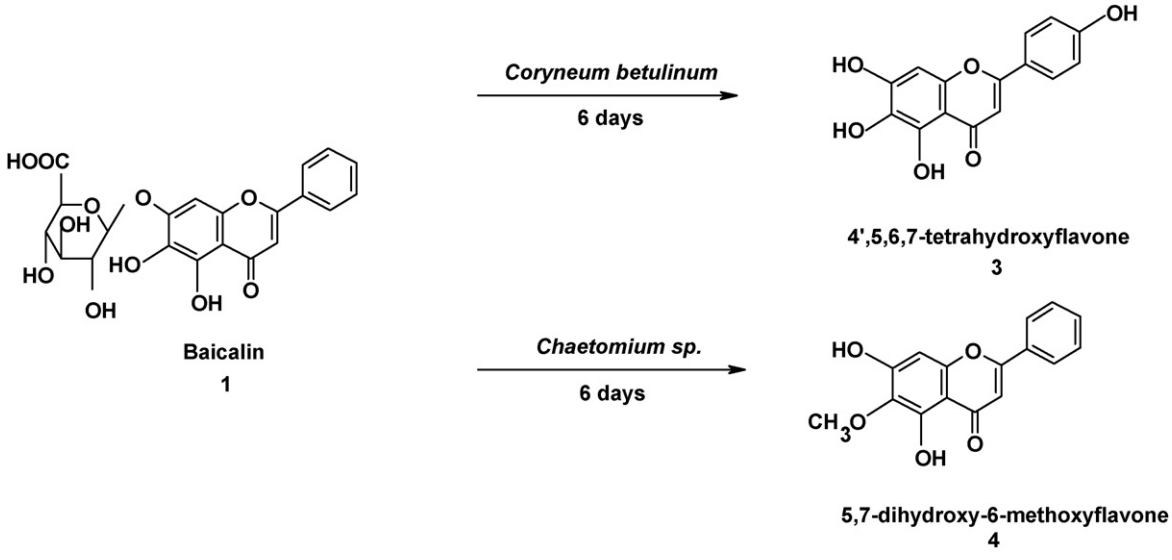


Fig. 1. Products of microbial transformation of baicalin.

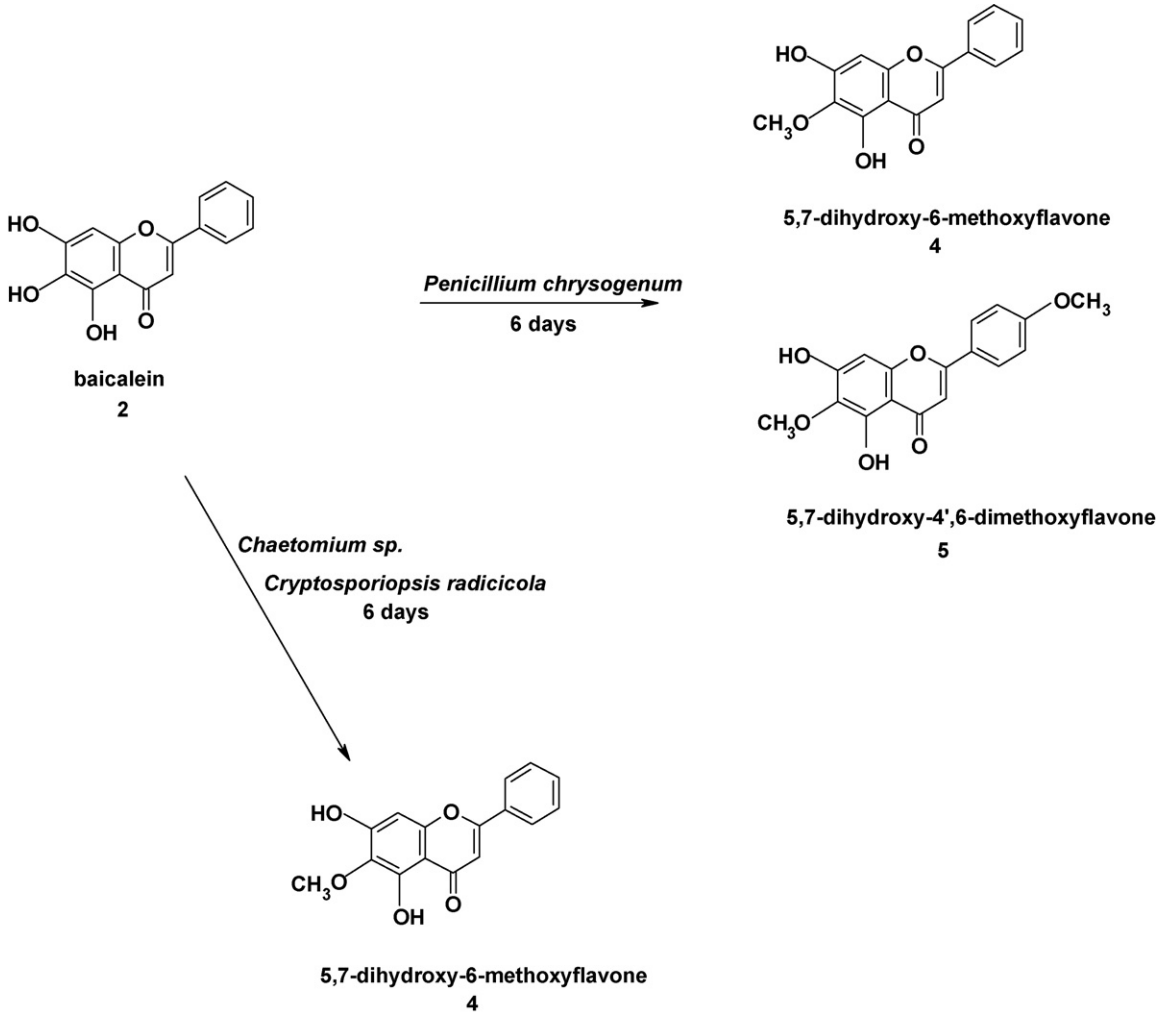


Fig. 2. Products of microbial transformation of baicalein.

to the spectrum of substrate **2** (Table 1). In the ^{13}C NMR of **5** two new signals compared to the substrate baicalein (**2**) were observed: the first one at $\delta = 59.6$ ppm, which comes from the carbon of a methoxyl group at C-6 in the A ring, and the second one at $\delta = 55.4$ ppm, being the signal of the methoxyl carbon at C-4′ in the B ring (Table 2). The chemical shift of C-4′ signal changes from $\delta = 132.3$ ppm in the case of baicalein (**2**) to $\delta = 162.1$ ppm in the case of 5,7-dihydroxy-4′,6-dimetoxyflavone (**5**) (Table 2).

The strain of *P. chrysogenum* did not transform baicalin (**1**) at all.

The results of our experiments indicate high regiospecifity of both methylation and hydroxylation. Only one out of three hydroxyl groups undergoes methylation—the one at C-6 in the A ring, whereas the favored position of B ring functionalization is C-4′.

O-Methylation of flavonoids changes chemical reactivity of the phenolic hydroxyl groups and increases lipophilic properties of the compounds. This may be important for retaining optimal hydrophilic–lipophilic properties of newly formed flavones. Biological activity of compounds depends on their hydrophilic–lipophilic properties, which determine transport across membranes and affinity for an active site. Therefore, retaining optimal hydrophilic–lipophilic properties may be essential for designing new compounds of desired activity, which could find potential application as drugs.Two products obtained in the microbial transformations: 5,7-dihydroxy-6-methoxyflavone (**4**) and 4′,5,6,7-tetrahydroxyflavone (**3**) were found in flavonoid fraction isolated from *Radix Scutellariae* [4,17]. 5,7-Dihydroxy-6-methoxyflavone (oroxylin A) and 4′,5,6,7-tetrahydroxyflavone (scutellarein) as well as about 40 other flavonoid compounds were identified in *Radix Scutellariae* baicalensis. Total content in the raw material from China exceeds 20 % and 12–17 % of this is baicalin. Therefore, looking at the chemical composition of the *Radix Scutellariae baicalensis* and the biotransformation products one may draw a conclusion that oroxylin A and scutellarein are formed from baicalin due to enzymatic reactions. The microorganisms that catalyze hydroxylation (*C. betulinum*) and methylation (*Chaetomium* sp.) of baicalin probably use the enzymatic systems similar to those present in the plant. *O*-methyltransferases that take part in the microbial methylation of baicalin and baicalein are similar to the plant *O*-methyltransferases involved in secondary metabolism

in *Radix Scutellariae baicalensis*. Our observations suggest that fungal *O*-methyltransferases, as well as the plant ones, act highly regioselectively [16].

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