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Biotransformation of 5,7-Methoxyflavones by Selected Entomopathogenic Filamentous Fungi

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ABSTRACT: 5,7-Dimethoxyflavone, a chrysin derivative, occurs in many plants and shows very low toxicity, even at high doses. On the basis of this phenomenon, we biotransformed a series of methoxy-derivatives of chrysin, apigenin, and tricetin obtained by chemical synthesis. We used entomopathogenic fungal strains with the confirmed ability of simultaneous hydroxylation/demethylation and glycosylation of flavonoid compounds. Both the amount and the place of attachment of the methoxy group influenced the biotransformation rate and the product's amount nascent. Based on product and semi-product structures, it can be concluded that they are the result of cascading transformations. Only in the case of 5,7,3′,4′,5′-pentamethoxyflavone, the strains were able to attach a sugar molecule in place of the methoxy substituent to give 3′-*O*-*β*-D-(4″-*O*-methylglucopyranosyl)-5,7,4′,5′-tetramethoxyflavone. However, we observed the tested strains' ability to selectively demethylate/hydroxylate the carbon C-3′ and C-4′ of ring B of the substrates used. The structures of four hydroxyl-derivatives were determined: 4′-hydroxy-5,7-dimethoxyflavone, 3′-hydroxy-5,7-dimethoxyflavone, 3′-hydroxy-5,7,4′,5′-tetramethoxyflavone, and 5,7-dimethoxy-3′,4′-dihydroxyflavone (5,7-dimethoxy-luteolin).

KEYWORDS: *biotransformation, Beauveria sp., Isaria sp., methoxyflavones, 5,7-dimethoxyflavone, 4-O-methyloglycosylation*

1. INTRODUCTION

Nowadays, it is estimated that there are over 9000 flavonoid compounds present in the plant world.[1] These secondary metabolites fulfill a number of functions, including regulating the overall development of plants, pigmentation, or protecting against ultraviolet (UV) radiation.[2] In addition, new biological properties of flavonoids, such as a positive effect on model organisms (including the human body) after their consumption, are constantly investigated. Studies show that flavonoid compounds can have a wide range of applications from frequently described antioxidant, anticancer, anti-inflammatory,[2,3] antifungal, antibacterial, or antiviral ones[3,4] up to the use in the treatment of Alzheimer's disease[5] or reduction of blood glucose levels (tested on rat models).[6] It is highly likely that we do not know all their properties, and discovering new possibilities of using these substances will take a long time.

Flavonoids show positive effects when tested on cell lines, but usually at a concentration that, when scaled up to the human body, may exceed the maximum daily dose of the drug.[7] It is mainly related to the poor bioavailability and low absorption of these compounds due to their low water solubility.[8] So far, the most frequently described flavonoids have been naturally occurring derivatives containing the hydroxy substituent(s) located at carbon C-5 and C-7. A model example of such a compound (besides quercetin, apigenin, luteolin, and diosmetin) is chrysin (5,7-dihydroxyflavone). It has been determined that chrysin (apart from its antibacterial, anti-inflammatory, antiallergic, anticancer, and antioxidative properties) has an antityrosinase-inhibiting effect and a moderate aromatase-inhibiting effect, improves the development of cognitive functions, reduces brain damage, and has antianxiety and antiestrogenic activities.[9] Chrysin can be extracted, among other sources, from *Passiflora caerulea* L. (blue passion flower) or bee honey,[10] while for laboratory and industrial use, it is synthesized from intermediate compounds[11] and now more and more often from other flavonoids.[9]

Flavonoids with hydroxyl groups are usually characterized by much higher biological activities than counterparts with other substituents (*e.g.*, methoxy).[12] However, the substitution of the hydroxy group, for example, with a methyl or methoxy group, can change their biological activity while increasing the compound's absorbability due to their better lipophilic properties.[13] What is more, introducing a sugar moiety into the flavonoid structure increases its water solubility and, thus, often its biological bioavailability.[8] The lower biological response of such compounds may be compensated by their ability to penetrate biological membranes, including the blood–brain barrier, and a higher level of accumulation in the body, compared to the starting substances.[14]

The methoxy derivative of chrysin is 5,7-dimethoxyflavone (5,7-DMF), a natural compound that can be found, among other sources, in the rhizome of *Boesenbergia pandurata* (Roxb.), that is, a plant which has been used for a long time in traditional Thai medicine,[15] *Piper caninum*,[16] or in

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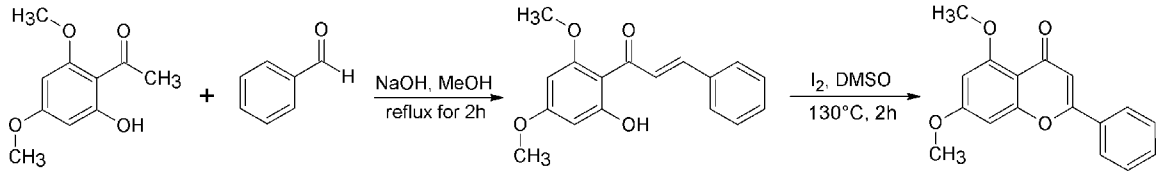
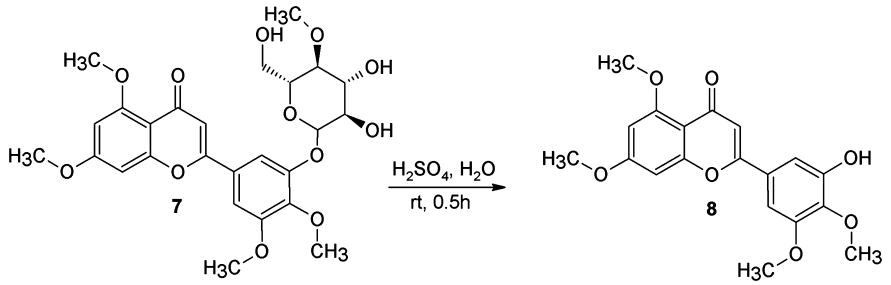
Scheme 1. Example of Chalcone Synthesis by Claisen−Schmidt Condensation Reaction and Flavone Synthesis



Scheme 2. Glycoside Hydrolysis with Sulfuric Acid



Kaempferia parviflora.[17] 5,7-DMF showed very low toxicity, with practically no side effects, even at doses up to 3 g/kg body weight, tested in rats.[15] The authors compared the anti-inflammatory properties of 5,7-DMF with aspirin and found that 5,7-DMF simultaneously inhibits prostaglandin production (anti-inflammatory effect) and lowers the rat's temperature. It was also reported that 5,7-DMF acts as an inhibitor of sarcopenia and, at the same time, causes the development of muscle mass and volume in a mouse model,[18] whose properties are very similar to chrysin.

It was demonstrated that the intracellular transport of 5,7-dimethoxyflavone was approximately 10-fold higher than that of chrysin (5,7-dihydroxyflavone).[19] Moreover, chrysin was rapidly metabolized by the human liver (S9 fraction), with no parent compound remaining after a 20 min incubation. In contrast, 5,7-DMF was metabolically stable over the whole 60 min time-course studied.[20] Interestingly, it was found that the concentration of 5,7-DMF after oral administration was significantly higher in the tissues of the animal than in the plasma, where its half-life was 3.4 ± 2.8 h, and its complete removal from the body took about 17 h.[5] In addition, it has also been shown that the metabolism of 5,7-DMF in comparison to chrysin is significantly reduced, which means that it lasts longer in the body, making it a promising chemopreventive substance.[14]

For this reason, we decided to perform biotransformations of three methoxyflavones obtained by chemical synthesis, containing methoxy substituents at major positions, that is, at the C-5 and C-7 carbon: 5,7-dimethoxyflavone (dimethylchrysin), 5,7,4′-trimethoxyflavone (trimethylapigenin), and 5,7,3′,4′,5′-pentamethoxyflavone (pentamethyltricetin). These compounds were previously identified in medicinal plants: 5,7-DMF, for example, in *K. parviflora*; 5,7,4′-trimethoxyflavone, for example, in *P. caninum*;[16] and 5,7,3′,4′,5′-pentamethoxyflavone in *Murraya paniculata*,[21] whereas the biocatalysts used include strains of entomopathogenic filamentous fungi belonging to the species *Beauveria bassiana* (KCh J1.5, KCh J2.1, KCh J3.2, KCh J1, and KCh BBT), *B. caledonica* (KCh J3.3, KCh J3.4),[22] *Isaria fumosorosea* KCh J2,[8] and *I. farinosa* KCh KW 1.1.[22] Strains from these species have a unique capacity for 4-*O*-methylglycosylation of flavonoids. This reaction is most often described for flavonoid compounds containing a free hydroxyl group in their structure.[3,23−28] The ability of entomopathogenic strains to hydroxylate and 4-*O*-methylglycosylate flavonoid compounds was also described.[8,29,31] In our previous work, we also observed that they perform demethylation and then 4-*O*-methylglycosylation of methoxyflavones.[30,31]

The present research aimed to evaluate the catalytic possibilities of entomopathogenic fungi toward flavones containing methoxy groups located at carbon C-5 and C-7.

2. MATERIALS AND METHODS

2.1. Substrates. The substrates 2-hydroxy-4,6-dimethoxyacetophenone, benzaldehyde, 4-methoxybenzaldehyde, and 3,4,5-trimethoxybenzaldehyde were purchased from Sigma-Aldrich (St. Louis, MO, USA). Flavones used in biotransformations were synthesized from those substrates, and the reactions are described below. The resulting chalcones were used as substrates for the flavone synthesis, and their NMR spectral data are identical to those previously published.[32]

2.2. Synthesis. All used substrates were synthesized in the laboratory by carrying out two kinds of reactions. First, five different methoxychalcones were synthesized in the Claisen−Schmidt reaction of 2-hydroxy-4,6-dimethoxyacetophenone with suitable benzaldehyde. The reaction was described previously[32,33] and is shown in Scheme 1. The resulting flavones (**1**−**3**) were used as substrates for the biotransformation. Their NMR spectral data are identical to those previously published.[32]

After 2 h of reflux, the product of the Claisen−Schmidt reaction was transferred into an acid environment and filtered using a Buchner funnel. The obtained product (appropriate methoxychalcone) was confirmed by NMR analysis. All other methoxychalcones were synthesized analogously. Methoxyflavones were synthesized from methoxychalcones by reaction with iodine in DMSO with 2−3 h incubation (until the substrate had reacted completely) at 130 °C,[34] as presented in the example above (Scheme 1). All substrates for biotransformations were synthesized in the same way, as described in our recent publication.[31] Detailed information on the synthesis is included in the Supporting Information. The course of biotransformation was monitored using thin-layer chromatography (TLC). The composition of product mixtures was established by HPLC. The obtained compounds were confirmed by NMR [^{1}H NMR, ^{13}C NMR, correlation spectroscopy (COSY), HMBC and HSQC] analysis and their mass was checked by liquid chromatography (LC)−mass spectrometry (MS) analysis (Supporting Information).

2.2.1. Hydrolysis of 3′-O-β-D-(4″O-Methylglucopyranosyl)-5,7,4′,5′-tetramethoxyflavone (7). The reaction for glycoside

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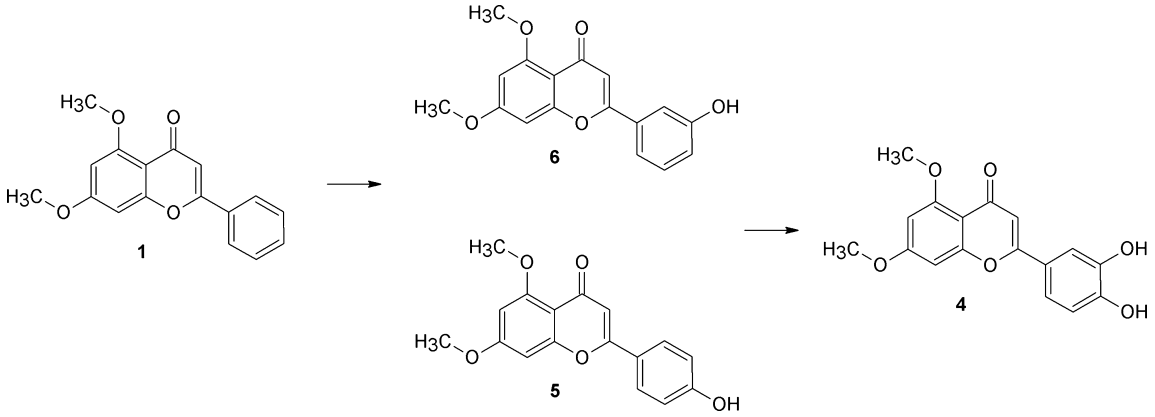

Figure 1. Biotransformation of 5,7-dimethoxyflavone (**1**) by *I. farinosa* KCh KW 1.1.

hydrolysis with sulfuric acid was carried out on the basis of the study by Zhang *et al.*[35] with solvent modification. In this case, the sample was dissolved in dimethyl sulfoxide (DMSO). The reaction was carried out in a round bottom flask on a magnetic stirrer. 15 mg of compound (**7**) was dissolved in 800 μL of DMSO. 500 μL of concentrated sulfuric acid (96% v/v) was added dropwise to the sample; then 500 μL of water was added very carefully (Scheme 2). The progress of the reaction was controlled by TLC plates every 30 min. After two hours of reaction, when the starting substrate was no longer observed, 5 mL of H_2O was added to the reaction. Then, the reaction mixture was extracted three times with ethyl acetate. Organic fractions were collected together, and the remains of water were removed with anhydrous $MgSO_4$. Then, the sample was evaporated using a vacuum evaporator. The sample was then analyzed by NMR and LC−MS spectroscopy to confirm the structure of the product obtained.

2.3. Microorganisms. The microorganisms *B. bassiana* KCh J1.5, KCh J2.1, KCh J1, KCh J3.2, and KCh BBT; *B. caledonica* KCh J3.3 and KCh J3.4; *I. farinosa* KCh KW 1.1; and *I. fumosorosea* KCh J2 were obtained from the public culture collection of the Department of Chemistry, Wrocław University of Environmental and Life Sciences (Wrocław, Poland). Isolation and identification procedures of all strains were described in our previous papers.[8,22]

2.4. Screening Procedure. Erlenmeyer flasks (300 mL), each containing 100 mL of the sterile cultivation medium (3% glucose, 1% aminobac-bacteriological peptone—enzymatic hydrolysate of selected animal tissue high in free amino acids and low molecular mass peptides), were inoculated with a suspension of each entomopathogenic strain and then incubated for 3 days at 24 °C on a rotary shaker. After this time, 10 mg of a substrate dissolved in 1 mL of dimethyl sulfoxide (DMSO) was added. Samples were collected on the 1st, 3rd, 7th, and 10th day of the process. Then, all products were extracted using ethyl acetate, and extracts were dried using $MgSO_4$, concentrated *in vacuo*, and analyzed using TLC and HPLC methods.

2.4.1. Scale-up Biotransformation. For the scale-up process, Erlenmeyer flasks (2000 mL) were used, each containing 500 mL of the same cultivation medium (3% glucose, 1% aminobac), which was inoculated in the same way as described above. Three days after inoculation, 100 mg of a substrate dissolved in 2 mL of DMSO was added. Processes of substrate conversion were performed individually depending on substrates and previously obtained HPLC results. Products were extracted three times using ethyl acetate and then analyzed using TLC, HPLC, and NMR spectroscopy (^{1}H NMR, ^{13}C NMR, COSY, HMBC, and HSQC) analysis.

2.5. Analysis. Basic analyses were carried out using TLC plates (SiO_2, DC Alufolien Kieselgel 60 F_{254} (0.2 mm thick), Merck, Darmstadt, Germany). The mobile phase contained a mixture of chloroform and methanol in a 9:1 (v/v) ratio. The plates were observed using a UV lamp (254 and 365 nm). The scale-up biotransformation products were separated using 1000 μm preparative TLC silica gel plates (Anatech, Gehrden, Germany). The mobile phase contained a mixture of chloroform and methanol in a 9:1 (v/v) ratio. The products were isolated by scraping out successive bands and extracted twice with ethyl acetate.

2.5.1. HPLC. A Waters 2690 instrument equipped with a Waters 996 photodiode array detector, using an ODS 2 column (4.6 × 250 mm, Waters, Milford, MA, USA) and a Guard-Pak Inserts μBondapak C18 pre-column, was used to perform HPLC analyses. The mobile phase consisted of eluent A (80% acetonitrile in 4.5% acetic acid solution) and eluent B (4.5% acetic acid) with gradient elution: 0−7 min, 10% A/90% B; 7−10 min, 50% A/50% B; 10−13 min. 60% A/40% B; 15−20 min 80% A/20% B; 20−30 min, 90% A/10% B; 30−40 min, 100% A. The flow rate was 1.0 mL/min, injection volume was 10 μL, and detection wavelength was 323 nm.

2.5.2. NMR Spectroscopy. The NMR analysis was performed with a DRX 600 MHz Bruker spectrometer (Bruker, Billerica, MA, USA) with an UltraShield Plus magnet and measured in DMSO-d_6 or $CDCl_3$. All NMR data is available in the Supporting Information.

2.5.3. LC−MS. Molecular formulas of products were confirmed by LC−MS 8045 SHIMADZU analysis. The mobile phase was a mixture of 0.1% aqueous formic acid v/v (A) and acetonitrile (B). The program was as follows: 80% B and 20% A in 5 min. The flow rate was 0.3 mL/min, and the injection volume was 2 μL. The column (Kinetex 2.6 μm C18 100 Å, 100 mm × 3 mm, Phenomenex, Torrance, CA, USA) was operated at 30 °C. The major operating parameters were as follows: nebulizing gas flow: 3 L/min, heating gas flow: 10 L/min, interface temperature: 300 °C, drying gas flow: 10 L/min, data acquisition range m/z 100−1000 Da; and ionization mode-positive. Data were collected with LabSolutions (Shimadzu, Kyoto, Japan) software.

3. RESULTS AND DISCUSSION

The 5,7-methoxyflavones obtained by chemical synthesis were biotransformed in the cultures of nine entomopathogenic filamentous fungal strains. The cultures of five *B. bassiana* strains (KCh J1.5, KCh J2.1, KCh J3.2, KCh J1, and KCh BBT), two *B. caledonica* strains (KCh J3.3 and KCh J3.4), and two Isaria strains (*I. fumosorosea* KCh J2 and *I. farinosa* KCh KW 1.1.) were used as biocatalysts. All mentioned strains were used in our recent work,[31] in which we described the catalytic abilities of these fungi toward flavones containing a methoxy group/groups within the B ring.

All the used substrates, containing one to three methoxy groups in the structure, were obtained by a two-step chemical synthesis. Three chalcones were synthesized from 2′-hydroxy-4,6-dimethoxyacetophenone and the appropriate benzaldehyde in a basic medium in the first stage. Then, they were transformed into the appropriate methoxyflavones by reaction with I_2 in DMSO. As a result of these reactions, 5,7-

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dimethoxyflavone, 5,7,4′-trimethoxyflavone, and 5,7,3′,4′,5′-pentamethoxyflavone were obtained.

3.1. Biotransformation of 5,7-Dimethoxyflavone (1). Biotransformation of 5,7-dimethoxyflavone (**1**) in the cultures of most of the tested strains resulted in three products (**4**–**6**) (Figure 1). None of the isolated products contained a sugar molecule in its structure. Such a result of the biotransformation of this substrate is surprising because, in our previous research, we mainly observed 4-*O*-methylglycosylation products preceded by hydroxylation/demethylation.[30,31] After 7-day incubation of 5,7-dimethoxyflavone (**1**) in the culture of the *B. bassiana* KCh J1.5 strain, the main product constituted almost 80% of the reaction mixture, with a retention time of 11.4 min according to HPLC (Table S1—Supporting Information.), and was identified as 5,7-dimethoxy-3′,4′-dihydroxyflavone (**4**).

The ^{1}H NMR spectrum of this product shows signals confirming that the structure of the flavone A and C rings has been preserved. The presence and multiplicity of the signals indicate that the modification has been made in the B ring of the flavone skeleton. The presence and chemical shifts of signals in both the ^{1}H and ^{13}C NMR spectra and the correlation spectra (COSY, HMQC, and HMBC) indicate that 5,7-dimethoxyflavone (**1**) underwent hydroxylation at the carbon C-3′ and C-4′ (shifted signals visible in the ^{13}C NMR spectrum from these carbons are in the field of 145.70 and 148.96 ppm, respectively).

The NMR spectra characterized the other two products (**5** and **6**) (Figure 1.) as 5,7-dimethoxy-4′-hydroxyflavone (**5**) and 5,7-dimethoxy-3′-hydroxyflavone (**6**) (Supporting Information). The molecular mass of these compounds was confirmed by LC−MS analysis. As a result of three-day preparative biotransformation of 5,7-dimethoxyflavone (**1**) in the culture of the *B. bassiana* KCh J1.5 strain, 5,7-dimethoxy-4′-hydroxyflavone (**5**) was isolated with 10% yield. The multiplicities and positions of the signals visible in both the ^{1}H and ^{13}C NMR spectra and the correlation spectra (COSY, HMQC, and HMBC) indicate that this product has one hydroxyl group in its structure. The ^{1}H NMR spectrum shows two multiplets (each derived from two protons) characteristic for a *para*-substituted aromatic ring. In the HMBC spectrum, the signal from the hydrogen of the hydroxyl group (δ = 10.22 ppm) is coupled with the signal from the C-3′ and C-5′ carbon, which confirms the structure of the isolated product **5**.

The main product of this three-day biotransformation by *B. bassiana* KCh J1.5 was 5,7-dimethoxy-3′-hydroxyflavone (**6**), which was isolated with a yield of 40%. The NMR and MS analysis indicated that this compound is a product of hydroxylation. The shape and positions of the signals present in both the ^{1}H and ^{13}C NMR spectra and the correlation spectra (COSY, HMQC, and HMBC) indicate that the 5,7-dimethoxyflavone skeleton was preserved, and the B ring was hydroxylated. In the HMBC spectrum, the signal from the hydroxyl group (δ = 9.87 ppm) is coupled with the signals from the C-2′, C-3′, and C-4′ carbon, which confirms the structure of 5,7-dimethoxy-3′-hydroxyflavone (**6**). This compound was the main product (observed between 1 and 3 days of the substrate incubation process) in the tested strains' cultures. The concentration of compound **6** was significantly higher than that of 5,7-dimethoxy-4′-hydroxyflavone (**5**) (Tables 1 and S1—Supporting Information). The difference in the amounts of obtained compounds **5** and **6** proves the preference of enzymes in the cells of the tested entomopathogenic strains to put the hydroxyl group in 5,7-dimethoxyflavone (**1**) at carbon C-3′. Previous studies described the entomopathogenic strains' preference for the hydroxylation of flavonoid substrates into the C-4′ position. This tendency was observed during the biotransformation of flavone, 5-hydroxyflavone, 6-methylflavone, and 7-aminoflavone in *I. fumosorosea* KCh J2 culture.[8,24] 6-Methoxyflavanone in the culture of *I. fumosorosea* KCh J2 was hydroxylated to 4′-hydroxy and 3′,4′-dihydroxy derivatives, while 6-methoxyflavone was hydroxylated to 3′-hydroxy, 4′-hydroxy, and 3′,4′-dihydroxy derivatives.[30] All products isolated in the mentioned studies were corresponding β-D-(4-*O*-methyl)-glucopyranosides.[8,24,30]

Table 1. Product Yields at 7th Day of Biotransformation of 5,7-Dimethoxyflavone (1) and 5,7,4′-Trimethoxyflavone (2), According to HPLC

		composition of products [%]		
strain no.	substrate	(**6**)	(**5**)	(**4**)
B. bassiana KCh J1.5	**1**	17.0	2.5	79.5
	2	0	64.5	0.4
B. bassiana KCh J2.1	**1**	1.5	0	1.7
	2	0	62.6	0.3
I. farinosa KCh KW 1.1.	**1**	1	0	1.2
	2	0	17.2	0.4
B. bassiana KCh J1	**1**	21.4	1.7	9.8
	2	0	46.1	2.4
I. fumosorosea KCh J2	**1**	44.4	0.4	54.8
	2	0	8.6	0.4
B. caledonica KCh J34	**1**	18.8	1.7	6.4
	2	0	7.7	0
B. bassiana KCh J3.2	**1**	30.7	3	4.5
	2	0	34.8	1.9
B. caledonica KCh J3.3	**1**	10.2	0.7	4
	2	0	6.8	0
B. bassiana KCh BBT	**1**	30.9	3.9	7.5
	2	0	60.2	3.6

3.2. Biotransformation of 5,7,4′-Trimethoxyflavone (2). Two products were isolated (**4** and **5**) (Figure 2) after incubation of 5,7,4′-trimethoxyflavone (**2**) in the cultures of the tested entomopathogenic strains. These compounds were also observed in the biotransformation of 5,7-dimethoxyflavone (**1**). In this case, the main product was 5,7-dimethoxy-4′-hydroxyflavone (**5**), which is the result of the *O*-demethylation of the substrate used. An analogous effective regioselective demethylation of 5,7,4′-trimethoxyisoflavone and sinensetin was previously described using a strain of the species *Aspergillus niger*.[36,37]

Product **5** was produced by the enzymes of all the microorganisms used in the studies. In the cultures of three of them (*B. bassiana* KCh J1.5, KCh J2.1, and KCh BBT), this product was observed with over 60% conversion (after seven days of substrate incubation according to HPLC (Table 1). Extending the biotransformation process to 10 days resulted in increased production of 5,7-dimethoxy-4′-hydroxyflavone (**5**) up to 60 and 75% for two other strains, *I. fumosorosea* KCh J2 and *I. farinosa* KCh KW 1.1., respectively (Scheme 3). Of equal importance, the concentration of 5,7-dimethoxy-3′,4′-dihydroxyflavone (**4**) in the reaction mixture increased after a prolonged time. In the cultures of *B. bassiana* KCh J2.1 and KCh BBT and *I. farinosa* KCh KW 1.1. strains, a concentration exceeding 10% was reached, while in the *B. bassiana* KCh J1 strain culture, the concentration of this product was recorded

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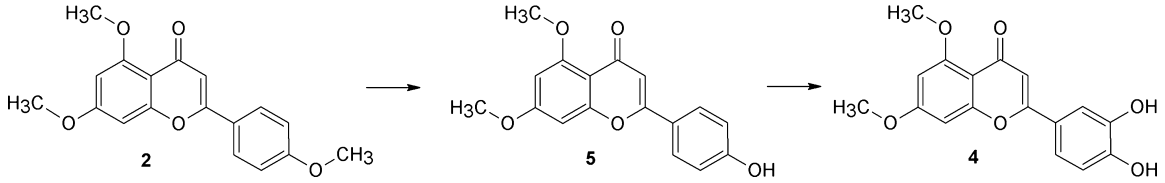
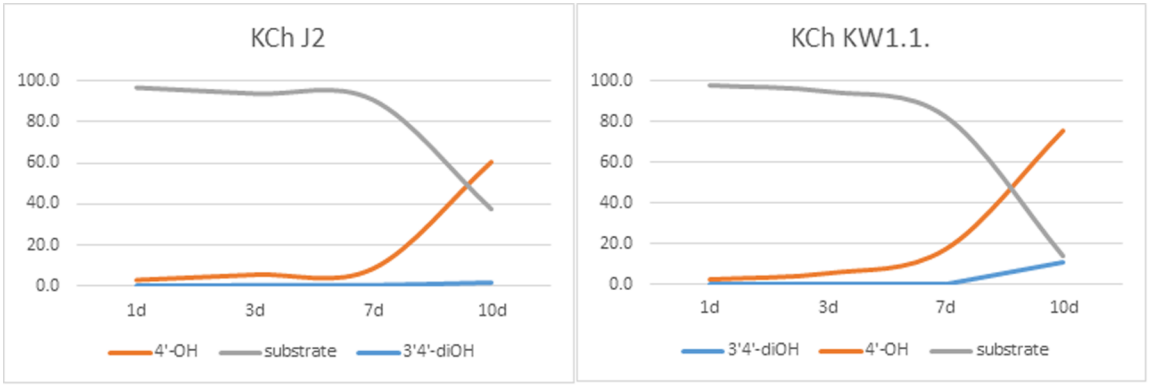


Figure 2. Biotransformation of 5,7,4′-trimethoxyflavone (**2**) by *I. farinosa* KCh KW 1.1.

Scheme 3. Microbial Transformation of 5,7,4′-Trimethoxyflavone (2) in *I. fumosorosea* KCh J2 and *I. farinosa* KCh KW 1.1 in Time



at the level of 26% after ten days of transformation (Tables 1 and S2—Supporting Information). Hydroxylation of C-3′ carbon and 4′-hydroxyl flavonoids is often observed and well-characterized in plant cells.[38,39] The hydroxylation pattern of the B ring of flavonoids is determined by the flavonoid 3′-hydroxylase (F3′H). The phylogenetic tree results showed that F3′H belongs to CYP75B.[38,40]

The application of the *Bacillus megaterium* cytochrome P450 BM3 (CYP450 BM3) and its mutants for the hydroxylation of naringenin to eriodictyol was described by Chu *et al.*[41] As a result of conducting *in vitro* experiments with human liver microsomes and recombinant enzymes, aromatic hydroxylation was also observed at position 3′ of the B ring of naringenin and sakuranetin.[42] The dominant enzyme responsible for this hydroxylation is CYP1A2, whereas other human liver cytochromes P450 are CYP2C19, CYP2D6, CYP2E1, and CYP3A4.[42]

3.3. Biotransformation of 5,7,3′,4′,5′-Pentamethoxyflavone (PMF) (3). The main transformation product of 5,7,3′,4′,5′-pentamethoxyflavone (**3**) in the culture of the *B. bassiana* KCh J1.5 strain nascent with a conversion of >99% after just three days was 3′-*O*-*β*-D-(4″-*O*-methylglucopyranosyl)-5,7,4′,5′-tetramethoxyflavone (**7**). This product was also observed in the other tested strains' cultures but with much lower efficiency (max 20−24% conversion after 7 days) (Table 2). Moreover, the *B. bassiana* KCh J1 strain was the only one of the tested strains unable to attach the sugar molecule into the flavonoid. In this culture, the highest concentration of the regioselective *O*-demethylation product of the methoxy group bound to the C-3′ carbon was observed. The product of this process was 5,7,4′,5′-tetramethoxy-3′-hydroxyflavone (**8**) (Figure 3). Such regioselective demethylation is consistent with the observations previously presented by Nielsen *et al.*[43] *In vitro* biotransformation of tangeretin and nobiletin (the typical polymethoxy flavonoids in plants[44,45]) by rat liver microsomes indicated that the demethylation only occurs at the C-3′ and C-4′ of the B ring.[43] On the other hand, successive *O*-demethylation and 4-*O*-methylglucosylation were previously described during the biotransformation of methoxyflavones and flavanones in cultures of entomopathogenic filamentous fungi of the genera *Beauveria* and *Isaria*.[30,31]

Structure (**8**) was characterized on the basis of data obtained by NMR analyses, and its molecular mass was confirmed by LC−MS analysis. Demethylation of only one group is confirmed by the presence on the ^{1}H NMR spectrum in the position 3.79−3.90 ppm, where only 3 singlets originate from the protons of four methoxyl groups. Additionally, different chemical shifts of signals coming from H-2′ and H-6′ protons (7.48 and 7.34 ppm, respectively) inform that the symmetrical structure of the substrate B ring has changed. On the ^{1}H NMR spectrum, signals coming from the sugar substituent are visible. On the basis of the analysis of multiplicity and chemical shifts of signals coming from this substituent, it was identified as glucose and was visible in the HMBC spectrum of the coupling between the signal coming from the protons of the group $-OCH_3$ visible in the position 3.47 ppm with C-4 carbon of the sugar unit clearly indicating that this substituent is 4-*O*-glucopyranoside. In comparison, on the HMBC spectrum of compound **7**, there is also visible coupling of the doublet coming from the hemiacetal proton H-1″ with the signal from C-3′ carbon which unambiguously confirms the structure of 3′-*O*-*β*-D-(4″-*O*-methylglucopyranosyl)-5,7,4′,5′-tetramthoxyflavone (**7**).

The second product observed during the biotransformation of compound **3** in the studied strains' cultures was a result of regioselective demethylation—3′-hydroxy-5,7,4′,5′-tetramethoxyflavone (**8**), an intermediate product, essential to obtaining compound **7**. The structure of 3′-hydroxy-5,7,4′,5′-tetramethoxyflavone (**8**) was confirmed by comparing its experimental data with the standard obtained as a result of acid deglucosylation of compound **7**. The molecular mass of product **8** was confirmed by LC−MS analysis, and the structure was confirmed by NMR analysis. The shape and position of the signals visible in both the ^{1}H and ^{13}C NMR

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Table 2. Microbial Transformation of 5,7,3′,4′,5′-Pentamethoxyflavone (3), According to HPLC

strain no.	number of compound	retention time [min]	composition of products [%] after 1, 3, and 7 days		
			1	3	7
B. bassiana KCh J1.5	7	10.7	27.9	99.5	99.9
	8	13.0	11.9	0.5	0.1
	3	15.1	60.2	0.0	0.0
B. bassiana KCh J2.1	7	10.7	0.0	0.6	5.3
	8	13.0	1.8	6.2	12.0
	3	15.1	98.2	93.2	82.8
I. farinosa KCh KW 1.1.	7	10.7	0.7	4.1	15.4
	8	13.0	8.5	22.7	29.0
	3	15.1	90.8	73.2	55.6
B. bassiana KCh J1	7	10.7	0.0	0.0	0.0
	8	13.0	2.7	38.7	56.9
	3	15.1	97.4	61.3	43.1
I. fumosorosea KCh J2	7	10.7	0.0	3.0	3.9
	8	13.0	0.0	0.0	0.0
	3	15.1	100.0	97.0	96.1
B. caledonica KCh J34	7	10.7	1.2	18.6	19.8
	8	13.0	0.4	1.9	3.5
	3	15.1	98.4	79.6	76.7
B. bassiana KCH J3.2	7	10.7	0.0	3.1	24.0
	8	13.0	0.3	3.9	16.7
	3	15.1	99.7	93.0	59.2
B. caledonica KCh J3.3	7	10.7	0.9	4.8	7.0
	8	13.0	1.4	0.2	0.4
	3	15.1	97.7	95.1	92.6
B. bassiana KCh BBT	7	10.7	1.0	2.1	5.5
	8	13.0	2.7	7.3	17.2
	3	15.1	96.3	90.6	77.3

spectra and correlation spectra (COSY, HMQC, HMBC) indicate that this product has one hydroxyl group in its structure (singlet in position 9.55 ppm). The ^{1}H NMR spectrum of this product shows four singlets derived from the protons of the methoxy groups. In the HMBC spectrum, the signal from the hydroxyl group (δ = 9.55 ppm) is coupled with the signals from the C-2′, C-3′, and C-4′ carbon, which confirms the isolated product's structure.

The hydroxyl analogues of the 5,7-dimethoxyflavone substrates described in this work (dihydroxyflavone with substituents in the 5 and 7 positions) have already been transformed by the filamentous fungi (including strains of the *B. bassiana* species) to appropriate glycosidic derivatives, where the sugar molecule was mainly attached to carbon C-7′ and C-3′.[28]

In our study, none of the tested microorganisms were able to *O*-demethylate the methoxy groups present in the A ring. Demethylation was observed only in the B ring of the tested substrates. Surprisingly, in the light of the previously described effective 4-*O*-methylglucosylation of hydroxy flavonoids in the cultures of entomopathogenic fungi,[3,23,24,46] only one glucopyranoside was isolated during the biotransformation of 5,7-dimethoxyflavones—3′-*O*-*β*-D-(4″-*O*-methylglucopyranosyl)-5,7,4′,5′-tetramethoxyflavone (**7**). Two other substrates described in this work underwent ortho-hydroxylation leading to 5,7-dimethoxy-3′,4′-dihydroxyflavone (**4**) (dimethoxy-luteolin). Flavonoid compounds containing hydroxyl groups in the 3′ and 4′ positions in their structure show the desired positive effect on the human body.[47] Luteolin is highly pharmacologically effective in inflammatory and neurodegenerative diseases.[48] In addition, luteolin has been reported as a potential anticancer agent that could inhibit a wide range of human cancers.[47,49,50]

The entomopathogenic fungal strains used in the study showed the capacity for selective demethylation/hydroxylation in the B ring of the substrates used at C-3′ and C-4′ carbon. Three monohydroxy products were obtained: 4′-hydroxy-5,7-dimethoxyflavone (**5**), 3′-hydroxy-5,7-dimethoxyflavone (**6**), and 3′-hydroxy-5,7,4′,5′-tetramethoxyflavone (**8**). It was also demonstrated that the tested strains have produced enzymes capable of orthohydroxylation, resulting in 5,7-dimethoxy-3′,4′-dihydroxyflavone (**4**) (dimethoxy-luteolin). In our previous study, 4-*O*-methylglucosides were the major biotransformation products of methoxyflavones. In this case, where the A ring is substituted in two positions (5,7), the tested microorganisms mainly performed the demethylation and hydroxylation reaction. The location of the methoxyflavones in the A ring—characteristic of many natural flavones—makes them modify only in the B ring.[31]

In summary, the manuscript describes a biotransformation study of 5,7-methoxyflavones by selected entomopathogenic filamentous fungi. Moreover, efficient methods of obtaining bioactive flavonoids are sought continuously. So far, the best and most frequently described flavonoids are naturally occurring derivatives containing the hydroxy or methoxy substituents located at carbon C-5 and C-7. A model example of such a compound (besides quercetin, apigenin, luteolin, and diosmetin) is chrysin (5,7-dihydroxyflavone). The methoxy

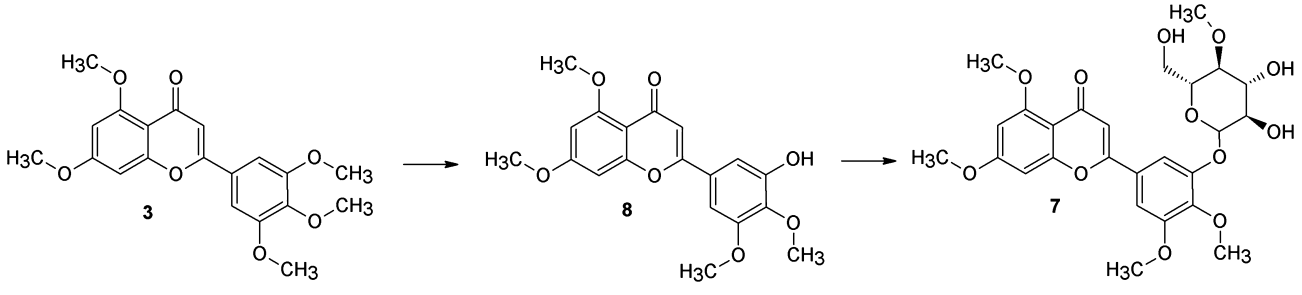

Figure 3. Biotransformation of 5,7,3′,4′,5′-pentamethoxyflavone (**3**) by *B. bassiana* KCh J1.5 and *B. bassiana* KCh J1.

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derivative of chrysin is 5,7-dimethoxyflavone, which also occurs in many plants and shows very low toxicity, with practically no side effects, even at high doses (even up to 3 g/kg body weight, tested in rats).[15] Based on this phenomenon, in this study, we biotransformed a series of methoxy derivatives of chrysin, apigenin, and tricetin obtained by chemical synthesis. We used entomopathogenic fungal strains with the confirmed ability of simultaneous hydroxylation/demethylation and glycosylation of flavonoid compounds, like *B. bassiana*, *B. caledonica*, *I. fumosorosea*, and *I. farinosa*, as biocatalysts. Both the amount and the place of attachment of the methoxy group influenced the biotransformation rate and the product's amount nascent. All obtained products were characterized by spectroscopic methods. On the basis of products and semi-products structures, it can be concluded that they are the result of cascading transformations. Five compounds were obtained; among them, four are hydroxyl derivatives of substrates and one 4-*O*-methylglucoside. Each of the obtained products was determined by HPLC, LC−MS, and NMR analysis (Supporting Information). The only glycosylation product was identified as 3′-*O*-*β*-D-(4″-*O*-methylglucopyranosyl)-5,7,4′,5′-tetramethoxyflavone (**7**), which was obtained during the 5,7,3′,4′,5′-pentamethoxyflavone biotransformation. These results clearly indicate that entomopathogenic filamentous fungi produce numerous active enzymes capable of effectively converting flavonoid compounds of varying structures. High yield and short time of conversion of substrates are crucial for increasing the described process into an industrial scale. Moreover, the structure of the obtained products promotes their usage in the food and pharmaceutical industries as active substances in health-promoting preparations.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.1c00136.

NMR data of all compounds described in the publication; MS analysis, ^{1}H NMR, ^{13}C NMR, COSY, HMQC, and HMBC of all substrate and biotransformation products; efficiencies of chalcone synthesis during the Claisen−Schmidt reaction; and flavone synthesis efficiencies by cyclization reactions (PDF)

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Notes

The authors declare no competing financial interest.

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