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Microbial transformations of flavanone by *Aspergillus niger* and *Penicillium chermesinum* cultures

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Abstract

As a result of biotransformation of flavanone (**1**) by the strain *Aspergillus niger MB* (being the UV mutant) and by the wild strain *Penicillium chermesinum 113* the products of hydroxylation at C-6 (**2**) and C-4′ (**5**) were obtained. Additionally, three dihydrochalcones with hydroxyl groups at C-2′ (**4**), C-2′ and C-5′ (**3**) and C-2′ and C-4 (**6**) were formed.

Keywords: Biotransformations; Flavanones; Dihydrochalcones; *Aspergillus niger*; *Penicillium chermesinum*

1. Introduction

Flavonoids, which are the object of our investigation, are naturally occurring, biologically active compounds which are present in plants [1]. Widespread studies in several research centres proved their antiallergic, antiatherogenic, antidiabetic, anti-flammatory and anticancerogenic properties [2,3].

The chemical structure of flavonoids, especially the presence and location of the hydroxyl groups, have strong impact on the scope of their biological activity [4,5].

Microbial transformations may be the way of obtaining optically active products from racemic mixtures, via modification of a substrate structure. These products, which are often difficult to obtain by chemical synthesis, may have different biological properties. Additionally, biotransformations may bring information about possible metabolic pathways of flavonoids in mammals [6].

The research presented in this report is a continuation of our earlier work concerning biotransformations of flavanone [7]. In this case, except for *Aspergillus niger MB* strain (being the UV mutant), wild strain *Penicillium chermesinum 113* has been used.

2. Materials and methods

2.1. Analysis

The course of microbial transformation was monitored by TLC (SiO_2, DC—Alufolien Kieselgel 60 F_{254}, Merck). Chromatograms were developed using the following developing systems: hexane:ethyl acetate 7:3, dichloromethatne:ethyl acetate 1:1, toluene:diethyl ether 4:1. Column chromatography (SiO_2, Kieselgel 60, 230–400 mesh, 40–63 μm, Merck, Darmstadt) was performed using the same eluents.

^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker Avance DRX 300 spectrometer. IR spectra were determined on a Mattson IR 300 Thermo Nicolet spectrometer. Optical rotations were measured on an Autopol IV automatic polarymeter (Rudolph). HPLC analysis was performed on a Waters 2690 instrument with a Waters 996 Photodiode Array Detector, equipped with a ODS 2 column (4.6 mm × 250 mm, Waters) and a Guard-Pak Inserts μBondapak C18 precolumn. Separation conditions: gradient elution, using 80% of acetonitrile in 4.5% formic acid solution (eluent A) and 4.5% formic acid (eluent B); flow 1 ml/min; detection at 280 nm; program: 0–7 min 10% A: 90% B, 7–10 min 50% A: 50% B, 10–13 min 60% A: 40% B, 13–15 min 70% A: 30% B, 15–20 min 80% A: 20% B, 20–30 min 90% A: 10% B, 30–40 min 100% A. The enantiomeric excess was determined using a Chiralpak AD-H HPLC column, 4.6 mm × 250 mm

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Table 1
Yield of biotransformation products determined by HPLC

Microorganism	Time of incubation (days)	Biotransformation products (%)					Unreacted substrate (%)
		2	**3**	**4**	**5**	**6**	
Aspergillus	1	3.7	–	2.4	–	–	88.0
niger	3	26.7	2.6	5.3	–	–	46.4
MB	6	23.7	5.5	7.6	–	–	31.1
	9	18.9	7.2	8.1	–	–	21.0
Penicillium	1	–	–	–	38.2	–	56.8
cher-	3	–	–	–	49.3	1.0	31.7
mesinum	6	–	–	–	36.1	13.9	18.2
113	9	–	–	–	12.1	37.8	6.2

(Diacel), with hexane:isopropanol (9:1) as eluent (isocratic resolution).

Crystallographic measurements were performed at 100 K using an Oxford Cryosystem device on a Kuma KM4CCD κ-axis diffractometer with a graphite-monochromated Mo Kα radiation ($\lambda = 0.71073$ Å). The data were corrected for Lorentz and polarization effects. No absorption correction was applied. Data reduction and analysis were carried out with the CrysAlis CCD and CrysAlis Red programs [8]. Structures were solved by direct methods (program SHELXS97) and refined by the full matrix least-squares method on all F^2 data using the SHELXL97 programs [9] (for details of refinement and the description of the crystal structures of 6-hydroxyflavanone (**2**) and 4′-hydroxyflavanone (**5**) see [11] and [15]).

2.2. Materials

The reacemic substrate for biotransformation—flavanone (**1**) was purchased from Aldrich.

2.2.1. Flavanone (*1*)

Melting point 76–77 °C; ^{1}H NMR see Table 2; ^{13}C NMR see Table 3; IR (KBr, ν_{max}, cm^{-1}): 1690,1606,1574.

2.3. Microorganisms

The UV mutant—*A. niger MB* was obtained from Wrocław University of Economics (Poland). The microorganism was maintained on potato slants at 5 °C.

The wild strain *P. chermesinum 113* was obtained from the culture collection of the Department of Chemistry of Wrocław University of Environmental and Life Sciences (Poland). The microorganism was maintained on agar slants at 5 °C.

3. Biotransformations

3.1. 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 acetone, was added to the grown culture. Control cultivation with no substrate was also performed. After 1, 3, 6 and 9 days of incubation under the above conditions, portions of 5 ml of the transformation mixture were taken out and extracted with ethyl acetate (3× 3 ml). The extracts were dried over $MgSO_4$, concentrated in vacuo and analyzed by TLC. Quantitative analyses of the mixtures were performed by means of HPLC using modified internal standard procedure, which means that the isolated and purified biotransformation products were used as standards for preparation of calibration curves.

3.2. 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 acetone was added to each flask (100 mg of the substrate per 1 l of the cultivation mixture). After 9 days of incubation the mixtures were extracted with ethyl acetate (3× 200 ml), dried ($MgSO_4$) and concentrated in vacuo. The transformation products were separated by column chromatography. Pure products were identified by means of spectral analyses (TLC, ^{1}H NMR, ^{13}C NMR, IR) and optical rotation measurements.

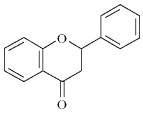
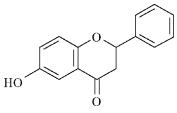
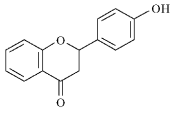
Physical and spectral data of the products obtained are presented below.

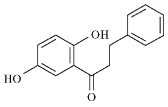
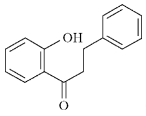
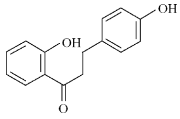
3.2.1. 6-Hydroxyflavanone (*2*)

Melting point 233–234 °C; $[\alpha]_{546}^{20} = +3.61$ ($c = 0.94$, CH_3OH); ee = 19.6%; ^{1}H NMR see Table 2; ^{13}C NMR see Table 3; IR (KBr, ν_{max}, cm^{-1}): 3420, 1680, 1587.

Crystal data for (**2**): $C_{15}H_{12}O_3$, $M_w = 240.25$, colourless block, crystal size 0.20 mm × 0.20 mm × 0.20 mm, monoclinic, space group $P2_1/c$, $a = 5.251(2)$ Å, $b = 21.809(4)$ Å, $c = 10.025(3)$ Å, $\beta = 93.34(3)°$, $V = 1146.1(6)$ Å^3, $Z = 4$, $D_x = 1.392$ Mg m^{-3}, $T = 100(2)$ K, $R = 0.0405$, w$R = 0.0946$ (for 1456 with $I > 2\sigma(I)$) for 180 variables [11].

Table 2
^{1}H NMR chemical shifts (δ) of compounds **1–6**

Proton	Flavanones		
	 **1**[a]	 **2**[a]	 **5**[b]
H-2	5.50(dd) (J = 13.2; 3.0)	5.50(dd) (J = 13.0; 2.9)	5.42(dd) (J = 13.2; 2.8)
H-3	H-3$_{ax}$ 3.11(dd) (J = 16.9; 13.2) H-3$_{eq}$ 2.91(dd) (J = 16.9; 3.0)	H-3$_{ax}$ 3.12(dd) (J = 17.0; 13.0) H-3$_{eq}$ 2.73(dd) (J = 16.9; 3.0)	H-3$_{ax}$ 3.15(dd) (J = 16.9; 13.2) H-3$_{eq}$ 2.80(dd) (J = 17.0; 2.8)
H-5	7.95(dd) (J = 8.1; 1.8)	7.10(d) (J = 2.9)	7.75(dd) (J = 7.8; 1.4)
H-6	7.47(m)		7.25(m)
H-7	7.47(m)	7.00(dd) (J = 8.9; 2.9)	7.55(td) (J = 7.8; 1.8)
H-8	7.47(m)	6.91(d) (J = 8.9)	7.25(m)
H-2′	7.07(m)	7.50(d) (J = 7.5)	7.33(d) (J = 8.5)
H-3′	7.47(m)	7.37(m)	6.82(d) (J = 8.6)
H-4′	7.47(m)	7.37(m)	
H-5′	7.47(m)	7.37(m)	6.82(d) (J = 8.6)
H-6′	7.07(m)	7.50(d) (J = 7.5)	7.33(d) (J = 8.5)

Proton	Dihydrochalcones		
	 **3**[a]	 **4**[a]	 **6**[a]
H-α	3.25(t) (J = 7.6)	3.32(t) (J = 7.7)	3.25(t) (J = 7.6)
H-β	3.04(t) (J = 7.6)	3.05(t) (J = 7.6)	2.95(t) (J = 7.6)
H-3′	6.88(d) (J = 8.9)	6.95(d) (J = 8.4)	6.96(d) (J = 8.6)
H-4′	7.00(d) (J = 8.9)	7.46(t) (J = 8.6)	7.45(t) (J = 8.5)
H-5′		6.85(t) (J = 8.1)	6.85(t) (J = 8.1)
H-6′	7.17(d) (J = 3.0)	7.73(d) (J = 8.1; 1.5)	7.75(d) (J = 8.0)
H-2	7.25(m)	7.24(m)	7.10(d) (J = 8.3)
H-3	7.25(m)	7.24(m)	6.75(d) (J = 8.4)
H-4	7.25(m)	7.24(m)	
H-5	7.25(m)	7.24(m)	6.75(d) (J = 8.4)
H-6	7.25(m)	7.24(m)	7.10(d) (J = 8.3)
2′-OH	11.85(s)	12.50(s)	12.40(s)
4-OH			5.83(s)

[a] Solvent $CDCl_3$.
[b] Solvent CD_3OD.

*3.2.2. 2′,5′-Dihydroxydihydrochalcone (**3**)*

Melting point 110–112 °C, lit. mp 110 °C [10]; ^{1}H NMR see Table 2; ^{13}C NMR see Table 3; IR (KBr, ν_{max}, cm^{-1}): 3585, 2987, 1733, 1648, 1488.

*3.2.3. 2′-Hydroxydihydrochalcone (**4**)*

Oily liquid; ^{1}H NMR see Table 2; ^{13}C NMR see Table 3; IR (KBr, ν_{max}, cm^{-1}): 3055, 2929, 1731,1640, 1423.

*3.2.4. 4′-Hydroxyflavanone (**5**)*

Melting point 192–194 °C; $[\alpha]_{546}^{20} = -85.18$ (c = 0.88, CH_3OH); ee = 35.3%; ^{1}H NMR see Table 2; ^{13}C NMR see Table 3; IR (KBr, ν_{max}, cm^{-1}): 3163, 2971, 1730, 1670, 1465.

Crystal data for (**5**): $C_{15}H_{12}O_3$, M_w = 240.25, colourless needle, crystal size 0.35 mm × 0.15 mm × 0.15 mm, monoclinic, space group $P2_1/n$, a = 5.247(2) Å, b = 15.374(3) Å, c = 14.648(3) Å, β = 97.84(3)°, V = 1170.6(6) Å^3, Z = 4, D_x = 1.363 Mg m^{-3}, T = 100(2) K, R = 0.0495, wR = 0.0892 (for 1921 with $I > 2\sigma(I)$) for 188 variables [15].

*3.2.5. 2′,4-Dihydroxydihydrochalcone (**6**)*

Melting point 105–107 °C, lit. mp 106 °C [10]; ^{1}H NMR see Table 2; ^{13}C NMR see Table 3; IR (KBr, ν_{max}, cm^{-1}): 3585, 1699, 1650, 1485.

4. Results and discussion

In our earlier paper [7] microbial transformations of flavanone by a wild strain *A. niger* KB and three of its UV mutants (*A. niger* 13/5, *A. niger* IBR 6/2, *A. niger* SBP) were described. Those biotransformations gave several different products, including dehydrogenation, reduction and hydroxylation ones. The present paper focuses on biotransformations by means of another UV mutant of *A. niger* KB (*A. niger* MB) and a wild

Table 3
^{13}C NMR chemical shifts (δ) of compounds **1–6**.

Carbon	Flavanones		
	1[a]	**2**[c]	**5**[b]
C-2	79.6	79.1	80.8
C-3	44.7	44.4	45.2
C-4	191.9	192.0	194.6
C-5	127.1	110.2	129.1
C-6	121.6	152.0	119.2
C-7	136.2	124.9	137.5
C-8	118.2	119.3	116.3
C-9	161.6	155.0	163.4
C-10	121.0	121.3	122.0
C-1′	138.8	139.5	131.2
C-2′	126.2	127.0	129.0
C-3′	128.9	129.1	116.3
C-4′	128.8	129.0	159.0
C-5′	128.9	129.1	116.3
C-6′	126.2	127.0	129.0
Carbon	Dihydrochalcones		
	3[c]	**4**[a]	**6**[c]
C-α	40.1	40.0	40.3
C-β	29.9	29.9	29.2
C-1′	118.9	119.2	119.2
C-2′	156.6	162.4	162.2
C-3′	119.3	118.6	118.4
C-4′	124.8	136.3	136.3
C-5′	147.5	118.9	118.9
C-6′	114.6	129.8	129.8
C-1	140.6	140.7	132.4
C-2	128.4	128.6	129.4
C-3	128.6	128.4	115.4
C-4	126.2	126.3	154.2
C-5	128.6	128.4	115.4
C-6	128.4	128.6	129.4
C═O	204.8	205.4	205.8

[a] Solvent $CDCl_3$.
[b] Solvent CD_3OD.
[c] Solvent DMSO-d_6.

strain *P. chermesinum 113*. Except for hydroxylation, both of the strains employed are capable of cleaving of the C-ring in flavonoids.

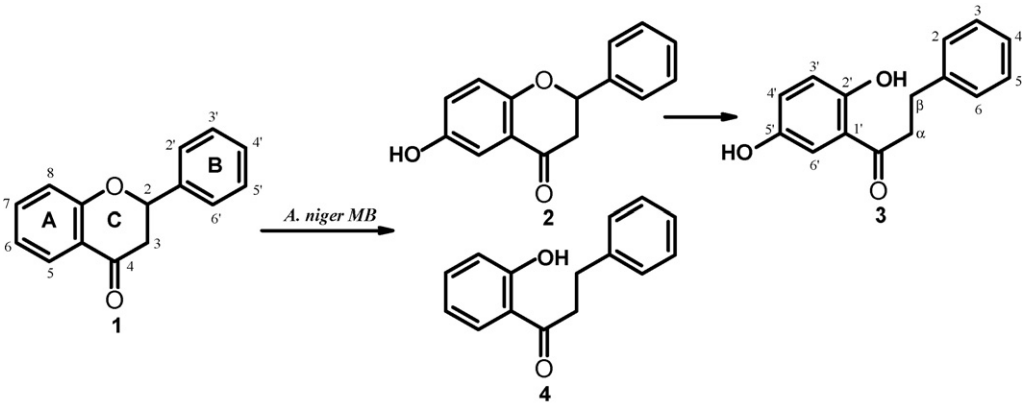
Biotransformation of flavanone (**1**) by means of *A. niger MB* culture led to formation of three products: 6-hydroxyflavanone (**2**), 2′,5′-dihydroxydihydrochalcone (**3**) and 2′-hydroxydihydrochalcone (**4**), which after 9 days of biotransformation were isolated in 18% (27 mg), 6% (9 mg) and 7.5% (11.25 mg) yields, respectively (Scheme 1).

The reactions were monitored by means of TLC and HPLC, what gave information about changes in quantitative ratio of products during the course of the process. In the first 24 h only two products were formed: 6-hydroxyflavanone (**2**) and 2′-hydroxydihydrochalcone (**4**). In the third day 2′,5′-dihydroxydihydrochalcone (**3**) appeared, and since then the amount of 6-hydroxyflavanone (**2**) gradually decreased, whereas the amount of 2′,5′-dihydroxydihydrochalcone (**3**) increased, proportionally (Table 1). This led to the conclusion that product **3** had possibly been formed in the second step of flavanone biotransformation, due to the C-ring cleavage of 6-hydroxyflavanone (**2**), formed in the first day of the biotransformation. 2′-Hydroxydihydrochalcone (**4**), which is produced independently, is probably the product of the C-ring cleavage of flavanone (**1**).

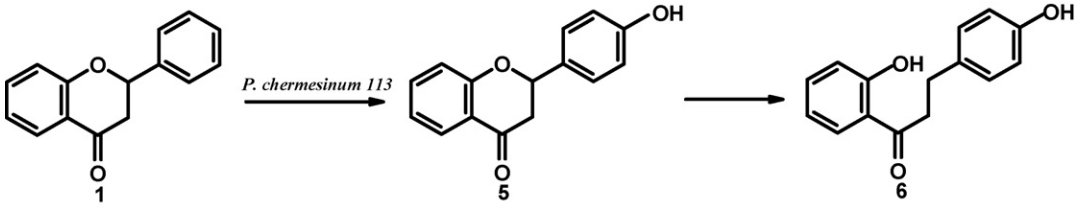
In the ninth day of the biotransformation there was still 21% of the unreacted substrate (**1**) present in the reaction mixture, however, continuing the reaction up to 10 days resulted in complete degradation of both the substrate and the products.

Biotransformation of flavanone (**1**) in *P. chermesinum 113* culture led to two products: 4′-hydroxyflavanone (**5**) and 2′,4-dihydroxydihydrochalcone (**6**), which were isolated after 9 days of the biotransformation in 11.8% (17.7 mg) and 36.5% (54.7 mg) yields, respectively (Scheme 2).

When monitored the progress of the reaction with time by means of TLC and HPLC, we observed a certain analogy to the biotransformation conducted in *A. niger MB* culture. The product of C-4′ hydroxylation of the B-ring (**5**) was formed in the first 24 h of the reaction and its amount increased up to the



Scheme 1.



Scheme 2.

third day. In the third day of the reaction product **6** appeared, and since then an amount of 4′-hydroxyflavanone (**5**) gradually decreased. The changes in quantity of 4′-hydroxyflavanone (**5**) were proportional to the increase in amount of 2′,4-dihydroxydihydrochalcone (**6**) (Table 1). This suggests that in flavanone (**1**) the hydroxylation occurs prior to the C-ring cleavage, leading to the respective chalcone (**6**).

The longer biotransformation time, the smaller total amount of the extracted compounds, which was observed for both of the tested cultures—*A. niger MB* and *P. chermesinum 113*.

The products of biotransformation of flavanone (**1**) were identified by means of ^{1}H NMR and ^{13}C NMR, as well as X-ray analysis.

The only product of microbial transformation of flavanone (**1**) catalysed by *A. niger MB* that retained the benzo-γ-pyrone structure was 6-hydroxyflavanone (**2**).

The site of hydroxylation was established by signals of the A-ring protons, which indicate substitution at C-6. We observed three signals, each of one proton: a doublet at $\delta = 7.10$ ppm, $J_{5,7} = 2.9$ Hz, the signal of H-5, a doublet at $\delta = 6.91$ ppm, $J_{8,7} = 8.9$ Hz, the signal of H-8, and a doublet of doublets at $\delta = 7.00$ ppm, $J_{7,8} = 8.9$ Hz and $J_{7,5} = 2.9$ Hz, being the signal of H-7. There is no H-6 proton signal in the NMR spectrum of **2** (Table 2). The hydroxylation at C-6 was also confirmed by the ^{13}C NMR. The signal of C-6 has been shifted from $\delta = 121.6$ ppm for the substrate (**1**) to $\delta = 152.0$ ppm for 6-hydroxyflavanone (**2**). The measured specific rotation of product **2** was $[\alpha]_{546}^{20} = +3.61$ ($c = 0.94$, CH_3OH), whereas enantiomeric excess established by means of HPLC (chiral column) was ee = 19.6%.

X-ray analysis of product **2** confirms the structure ascribed on the basis of the NMR spectra. The molecular structure of **2**, together with the numbering scheme employed, is presented in Fig. 1. The present structure of 6-hydroxyflavanone (**2**) displays both enantiomers somewhat randomly, not systematically, arranged in the unit cell. In the present analysis, atoms at position 2 in the pyrone ring [C2 and H2 (major component) and C2A and H2A (minor component)] and phenyl ring [C1′-C6′ (major component) and C1A′-C6A′ (minor component)] are clearly resolved. Thus, two enantiomers occupy equivalent sites in the unit cell, but not in a systematic way. The ratio of the two enantiomers (R:S) in the asymmetric unit is 0.75:0.25, which gives a 3:1/1:3 ratio in the crystal structure overall [11].

This type of disorder appears in the crystal structures of 5-hydroxy-7,4′-dimethoxyflavanone [12], 7-hydroxy-4′-methoxyflavanone [13], and naringenin [14]. Cox et al. suggest that the integrity of the crystal structures is maintained by the close overlap of equivalent atom positions in the two enantiomers, which can easily substitute for each other [14].

The product of biotransformation of flavanone (**1**) by the strain of *P. chermesinum 113* that retained the benzo-γ-pyrone structure was 4′-hydroxyflavanone (**5**). Hydroxylation at C-4′ of the B-ring was established by ^{1}H NMR analysis. In the spec-

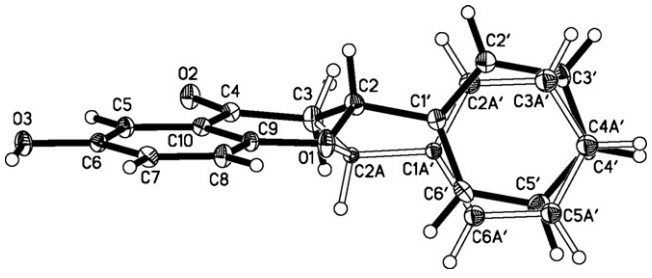


Fig. 1. The molecular structure of the overlapping enantiomers of 6-hydroxyflavanone (**2**).

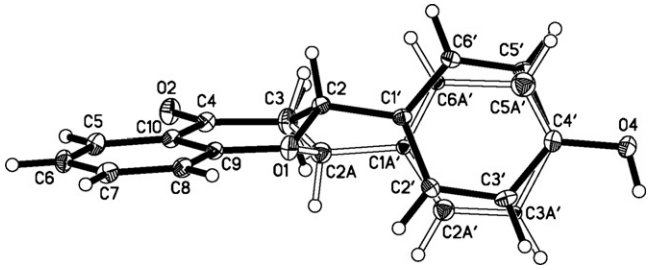


Fig. 2. The molecular structure of the overlapping enantiomers of 4′-hydroxyflavanone (**5**).

trum of **5** there are two doublets visible, of two protons each, at $\delta = 6.82$ ppm ($J = 8.6$ Hz) and $\delta = 7.33$ ppm ($J = 8.5$ Hz), of the similar coupling constants, which come from H-3′, H-5′ and H-2′, H-6′ protons (Table 2). In the ^{13}C NMR spectrum there is a shift of C-4′ signal observed from $\delta = 128.8$ ppm in the spectrum of substrate **1** to $\delta = 159$ ppm in the spectrum of product **5** (Table 3). The specific rotation of 4′-hydroxyflavanone (**5**) was $[\alpha]_{546}^{20} = -85.18$ ($c = 0.88$, CH_3OH) and the enantiomeric excess ee = 35.3%. The absolute configuration of products **2** and **5** was not established.

The structure of 4′-hydroxyflavanone (**5**) was confirmed by X-ray analysis. The molecular structure of **5**, together with the numbering scheme employed, is presented in Fig. 2. In the present analysis, many atoms of R and S enantiomers of 4′-hydroxyflavanone (**5**) overlap directly (Fig. 2) and some overlap partially, but atoms at position 2 in the pyrone ring [C2 and H2 (higher occupancy factor) and C2A and H2A (lower occupancy factor)] are clearly resolved. Two enantiomers occupy equivalent sites in the crystal latice in not systematical way. The ratio of the two enantiomers (R:S) in the asymmetric unit in the crystallographic analysis is 0.80:0.20, which gives an 4:1/1:4 ratio in the centrosymmetric crystal structure overall [15].

Three of the flavanone biotransformation products contained the structure of dihydrochalcone. In the ^{1}H NMR spectrum of product **3** there are two triplets observed at $\delta = 3.25$ ppm and $\delta = 3.04$ ppm of H-α and H-β protons, and a singlet at $\delta = 11.85$ ppm of 2′-OH (Table 2). These signals are typical for the dihydrochalcone structure. In the ^{13}C NMR spectrum there are signals at $\delta = 40.1$ and 29.9 ppm present, which come from of C-α and C-β, respectively. Similar signals were observed in the ^{1}H NMR and ^{13}C NMR spectra of compounds **4** and **6**, being the next two biotransformation products of dihydrochalcone structures (Tables 2 and 3).

In the ^{13}C NMR spectra a shift of C-5′ signal from 118.9 ppm in the spectrum of 2′-hydroxydihydrochalcone (**4**) to 147.5 ppm in the spectrum of 2′,5′-dihydroxydihydrochalcone (**3**) was observed, which confirmed the presence of OH group at C-5′ in product **3**. In the ^{1}H NMR spectrum of 2′,4-dihydroxydihydrochalcone (**6**) there are two doublets, of two protons each, visible at $\delta = 6.75$ ppm (H-3, H-5) and 7.1 ppm (H-2, H-6), of the similar coupling constants. These suggest substitution at C-4.

The presence of a singlet of one proton at $\delta = 5.83$ ppm in the ^{1}H NMR and a shift of the C-4 signal from $\delta = 126.3$ ppm for product **4** to $\delta = 154.2$ ppm for product **6** in the ^{13}C NMR confirms the presence of hydroxyl group at C-4 in 2′,4-dihydroxydihydrochalcone (**6**).

5. Conclusions

1. The products obtained in the biotransformations of flavanone (**1**) using the strains of *A. niger MB* and *P. chermesinum 113* contain benzo-γ-pyrone and dihydrochalcone structures.
2. The strains of *A. niger MB* and *P. chermesinum 113* are capable of hydroxylation of flavanone (**1**).
3. Hydroxylation in either C-6 or C-4′ position occurred prior to the C-ring cleavage of flavanone (**1**).

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