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Microbial synthesis of dihydrochalcones using *Rhodococcus* and *Gordonia* species

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ABSTRACT

Chalcones are important compounds in food and cosmetics industry, and in food chemistry research. We have developed a method of synthesis of dihydrochalcones from flavanone and α,β-unsaturated chalcones by microbial hydrogenation. It has been found that bacterial strains of *Rhodococcus* sp. and *Gordonia* sp. can be successfully used in the key step of dihydrochalcones synthesis. This kind of activity has not been previously examined.

Twelve microorganisms were initially screened for their ability to catalyze biotransformation reactions of selected flavonoid compounds. Of these, *Rhodococcus* sp. and *Gordonia* sp. transformed flavanone and chalcones to hydrogenation products in good isolated yield of 13–94%.

1. Introduction

Flavonoids are the group of natural polyphenolic compounds that are widespread in the plant kingdom [1–3]. Due to their health-promoting properties, in recent years there has been a growing interest in methods of synthesis of flavonoids and their application as food additives [4]. Among others, flavonoids have found application in food industry as a rich source of food colourings. Due to their antioxidant activity they play an important role as natural antioxidants and also they are used as protecting agents against microbial infections [5–7].

The use of microorganisms may be a highly efficient method of production of these compounds. This process is considered more effective than extraction from plants and than classic chemical synthesis. The major advantage of microbial biosynthesis of flavonoids is the possibility of cost reduction by using modified microbial strains or low-cost cultivation medium components.

The reactions involved in biotransformation of organic compounds by whole cells of various microorganisms include oxidation, reduction, hydroxylation, esterification, methylation, demethylation, isomerization, hydrolysis, glucosylation and hydrogenation [8–10]. In studies on intensification methods of obtaining natural compounds the mutagenesis techniques are also used [11].

The whole cells of various microorganisms have also been employed to catalyze asymmetric reduction of ketones, aldehydes, β-ketoesters and other α,β-unsaturated systems [12]. The application of whole growing or resting cells in the reduction reactions has clear advantages. Among them, the most important is production of chiral synthons and the ability to catalyze the reactions of high regio-, chemo- and enantioselectivity.

Many derivatives of flavone are known to have important biological functions. These natural products have been examined because of their diverse physiological and pharmaceutical properties such as estrogenic, antitumor, antimicrobial, antiallergic, and anti-inflammatory action [13–16]. In the literature there were also described studies on natural chalcone precursors and some other flavonoid derivatives, which aimed at determining the impact of structural modifications on pharmacological properties of the compounds (cytotoxic, antioxidant and anticancer) [17,18].

Satisfactory results of the original research concerning evaluation of pharmacological relevance of flavonoids isolated from natural sources, mainly from plants, inspired us to undertake the study on biotransformation of health-promoting polyphenols and their analogues. There are many reports in the literature on transformations 2-phenylchromane and its biosynthetic derivatives using plants, fungi and yeasts, leading to hydroxy-, methoxy- and glycosidic compounds [19,20].

Escherichia coli containing biphenyl dioxygenase from *Pseudomonas pseudoalcaligenes* transformed isoflavone into its 2′,3′-dihydro-2′,3′-*cis*-dihydroxy derivative [21]. When genes of naphthalene dioxygenase ware incorporated into *E. coli* cells,

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additionally analogous 5,6- and 7,8-dihydrodiol products were formed. In this case, flavone gave only 7,8-flavonedihydrodiol [22]. Above biphenyl dioxygenase produced 2′,3′-dihydroepoxy compounds from flavanone and *cis*-/*trans*-isoflavan-7-ols [23,24]. Another interesting biotransformation, occurred in the case of flavone and its derivatives, was C2-C3 double bond reduction leading to flavanone moiety [25]. Seo et al. reported also regio- and stereo-specific hydroxylation of flavanone and isoflavanone using sitedirected mutants of naphtalene dioxygenase which was impossible to carry out with the use of the native enzyme [26].

The big advantages of dihydrochalcones as potential food additives are their safety and stability. Nakamura et al. [5] reported that dihydrochalcones exhibited higher antioxidant activities than the corresponding flavanones. Lin et al. [27] characterized phloretin, dihydrochalcone of naringenin, and 12 other compounds isolated from apples for their hydroxyl radical scavenging activity and cellular tyrosinase inhibitory activity, suggesting their possible use as cosmetic agents. Phloretin was suggested to induce apoptosis in HL60 cells through the inhibition of protein kinase C activity [28]. Dihydrochalcones have also received considerable attention as food sweeteners, for instance neohesperidin dihydrochalcone, which is more than 300 times sweeter than sugar.

Chemical methods of hydrogenation with molecular hydrogen involve the use of catalysts, such as metal complexes and other compounds harmful to the environment. Selective hydrogenation of α,β-unsaturated carbonyl compounds has attracted great attention due to its wide application in synthesis of many fine chemicals and pharmaceutical compounds. This type reaction is a challenging task and many transition metals such as rhodium, ruthenium, palladium, iridium, and other metal complexes were used for these transformations [29,30]. Chemoselective hydrogenation of an olefinic fragment linked to an aromatic ring in α,β-unsaturated ketones has also been noted [31–33]. Kim et al. [34] reported about the method of reduction of flavone to dihydrochalcone using an excess of ammonium formate in the presence of Pd-C.

Due to the potential ability of bacterial cells to promote biotransformation reactions, we decided to study biotransformation of chalcone (**1**), 4-methoxychalcone (**2**), chalconaringenin (**3**), xanthohumol (**4**), flavanone (**5**) and naringenin (**6**). In this report, the whole cells of *Rhodococcus* sp. DSM 364 and *Gordonia* sp. DSM 44456 were used to obtain hydrogenation products of flavonoid compounds.

2. Experimental

2.1. Chemicals

All used chemicals were commercially available. Substrates for biotransformation were purchased from Sigma–Aldrich or obtained according to procedures described below.

2.1.1. Chalconaringenin (*3*)

Chalconaringenin (**3**) was prepared according to the general procedure described previously [35], which was modified. Thus, naringenin (1 g, 3.67 mmol) was dissolved in methanol (10 mL) and added to 200 mL of 20% KOH. The reaction mixture was stirred at 40 °C for 4 h, acidified with 2 M HCl to pH = 6 and extracted with diethyl ether (3 × 100 mL). The organic layer was separated, washed with water (3 × 100 mL). The combined organic extracts were dried over Na_2SO_4, filtered and concentrated in vacuo. The crude residue was purified by column chromatography on silica gel (chloroform/methanol 5/0.5, v/v) to afford 0.80 g (80%) of yellow crystals, mp. 256–258 °C. IR, ^{1}H NMR, ^{13}C NMR spectra and HR ESI-MS are given in supplementary data.

2.1.2. Xanthohumol (*4*)

Xanthohumol (**4**) was isolated from supercritical carbon dioxide extracted hops obtained from Production of Hop Extracts of Fertilizer Institute, Puławy, Poland. The method of isolation and purification was reported by Anioł et al. [36]. The product was obtained as yellow-orange crystals, mp. 170–172 °C. The spectroscopic data were in accordance with those previously reported [37].

2.2. General methods

The course of biotransformations was controlled by means of TLC and HPLC.

Thin layer chromatography was carried out using Kiesgel 60 F_{254} silica gel (0.2 mm, Merck) with various solvent systems as eluents. Compounds were visualized by spraying the plates with a solution of 10 g of $Ce(SO_4)_2$ and 20 g of phosphomolybdic acid in 1 L of 10% H_2SO_4 and heating.

Composition of reaction mixtures was established by high-performance liquid chromatography (HPLC), performed on a Waters 2690 instrument fitted with 2690 separations module and 996 photodiode array detector and Millennium 32 software. A reverse phase C-18 column (Marcherey Nagel, NUCLEODUR 100-5 C18 ec, 4.6 mm × 250 mm) was used. The mobile phase consisted of two solvents: A – 1% HCOOH in MeCN, B – 1% HCOOH in H_2O. A gradient elution from 40% solvent A to 60% solvent B over 28 min was used at the flow rate of 1 mL/min.

Products were separated by column chromatography using silica gel Kieselgel 60 (230–240 mesh, Merck) and hexane/acetone or hexane/dichloromethane/diethyl ether mixtures. Melting points were recorded on a Boetius apparatus.

Structures of isolated products were confirmed by spectroscopic methods. ^{1}H NMR and ^{13}C NMR spectra were recorded on NMR Bruker Avance II DRX 300 and Bruker Avance II DRX 600 MHz spectrometers with the tetramethylsilane (TMS) as an internal reference. The NMR spectra were measured in $CDCl_3$ or acetone-d_6. To confirm the presence of characteristic absorption bands, IR spectra were measured using a FT-IR Thermo-Nicolet IR 300 spectrometer. HRESI-MS spectra were taken on a Bruker micrOTOF-Q spectrometer. The structures of the known compounds were confirmed by comparison of their spectroscopic properties with data published in the literature.

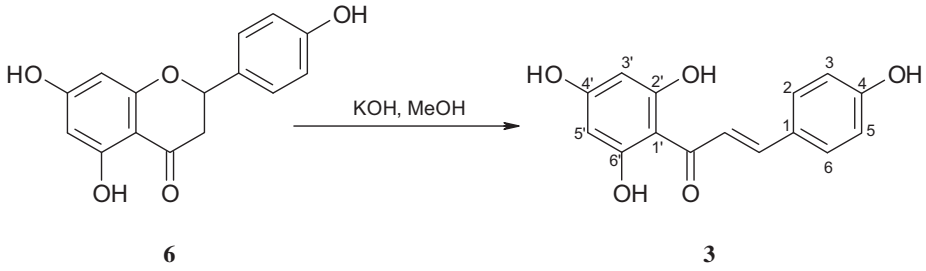
2.3. Microorganisms

Microorganisms (*Rhodococcus*, *Gordonia*, *Micrococcus*, *Streptomyces*, *Bacillus*, *Pseudomonas, Dietzia*) were purchased from the Polish Collection of Microorganisms (PCM) of the Institute of Immunology and Experimental Therapy Polish Academy of Sciences in Wroclaw and from German Microorganisms Culture Collection (DSMZ).

Biomass was determined by the dry weight method. Samples (10 mL) were filtered on Milipore HA filters, the residue was washed with distilled water (2 × 5 mL) and dried at 105 °C to a constant weight using the MAX 50/1/NH apparatus, RADWAG.

2.4. Screening procedure

25 mL of the medium in a 100 mL Erlenmeyer flask containing 1 g of glucose, 2.5 g of malt extract, and 1 g of yeast extract (pH 7.2) were inoculated with a suspension of a microorganism and then incubated for 48 h at 28 °C on a rotary shaker at 200 rpm. After that, 1 mg of a substrate dissolved in 1 mL of acetone was added. Every 24 h, portions of 5 mL of the transformation mixture were taken out and extracted with 10 mL of ethyl acetate and centrifuged at 4 °C for 10 min at 15,000 rpm. The extracts were dried over Na_2SO_4, concentrated in vacuum and analyzed by HPLC and TLC.



6 3

Fig. 1. Synthesis of chalconaringenin (**3**).

All experiments were done in duplicates and the results were reproducible. Verification of the stability of the substrates were performed by preparing and incubating control samples consisting of a tested flavonoid and the sterile cultivation medium. The culture controls consisted of the microbial cultures in fermentation media in which the microorganisms were cultivated, without substrate addition.

2.5. Preparative biotransformation

All transformations were performed on the preparative scale in 2000 mL flasks, each containing 250 mL of a cultivation medium. Precultures of *Gordonia sp. DSM 44456 and Rhodococcus sp. DSM 364* were prepared in two steps. At first, cells from the agar slants were transferred into 100 mL Erlenmeyer flask, each containing 25 mL of the medium (2.5% malt extract, 1% yeast extract, 4% glucose, pH 7.2), and incubated in a shaker at 28 °C and 200 rpm for 48 h. Then, 1 mL of this medium was added to a 250 mL Erlenmeyer flask containing 100 mL of the cultivation medium, and grown at 28 °C for 24 h. Portions of 1 mL of the pre-incubation culture suspension were used to inoculate four 2000 mL flasks, then 25 mg of substrate dissolved in 1 mL of acetone was added to the grown cultures under the same conditions as described above (24 h at 28 °C). After 72 h (biotransformations of chalcones **1–3**) and 7 days (biotransformations of flavanone **5**) of incubation the mixtures were extracted with ethyl acetate 3 × 200 mL and dried over Na_2SO_4. After the solvent evaporation, transformation products were separated by column chromatography and analyzed by chromatographic methods: TLC, HPLC, NMR and IR. The products were crystallized in hexane to obtain dihydrochalcones. IR, ^{1}H NMR, ^{13}C NMR spectra and HR ESI-MS are given in supplementary data.

2.6. Biotransformation products

2.6.1. Dihydrochalcone (*1a*)

Chalcone (**1**, 25.0 mg, 0.120 mmol) was used as a substrate. The crude product was purificated by column chromatography (hexane:acetone 5/1, v/v) to obtain white crystals of **1a** (DSM 364: 23.7 mg, yield 94%; DSM 44456: 16.4 mg, yield 65%), mp. 71–72 °C (hexane), lit. 71–73 °C [38]. The chemical synthesis of the compound and IR, ^{1}H–^{13}C NMR and MS spectra have been described previously [38,39].

2.6.2. 4-Methoxydihydrochalcone (*2a*)

4-Methoxychalcone (**2**, 25 mg, 0.105 mmol) was used as a substrate. The crude product was purificated by column chromatography (hexane:acetone 5/1, v/v) to obtain white crystals of **2a** (DSM 364: 20.2 mg, yield 80%; DSM 44456: 11.4 mg, yield 45%), mp. 58–59 °C (hexane), lit. 57.5–58.5 °C [38]. The chemical synthesis of the compound and IR, ^{1}H–^{13}C NMR and MS spectra have been described previously [38,39].

2.6.3. Dihydrochalconaringenin–phloretin (*3a*)

Chalconaringenin (**3**, 25 mg, 0.092 mmol) was used as a substrate. The crude product was purificated by column chromatography (hexane:acetone 5/1, v/v) to obtain pale yellow solid of **3a** (DSM 364: 21.2 mg, yield 84%), mp. 259–260 °C. Literature melting point (258–260 °C), chemical synthesis and IR, ^{1}H–^{13}C NMR spectra were reported previously [40].

2.6.4. 2′-Hydroxychalcone (*5a*) and 2′-hydroxydihydrochalcone (*5b*)

Flavanone (**5**, 25 mg, 0.111 mmol) was used as a substrate. The crude product mixture was purificated by column chromatography (hexane/diethyl ether/chloromethane 8/1.5/0.5, v/v/v) to obtain yellowish solid of **5a** (DSM 364: 7.5 mg, yield 30%), mp. 86–88 °C (hexane). Literature melting point (88–90 °C) and chemical synthesis were described by Rajput et al. [41]. Product (**5b**) was isolated as a white solid (DSM 364: 3.3 mg, yield 13%), mp. 127–129 °C (hexane). The chemical preparation and spectra data were described previously [20,42].

3. Results and discussion

The substrates containing 1,3-diphenyl-2-propen-1-one moiety: commercially available chalcone (**1**) and 4-methoxychalcone (**2**), obtained by chemical synthesis chalconaringenin (**3,** Fig. 1), isolated from spent hop xanthohumol (**4**), flavanone (**5**) and naringenin (**6**) were subjected to screening tests in order to select bacterial strains which were capable of enzymatic transformation. Among 12 microorganisms tested, *Rhodococcus* sp. DSM 364 and *Gordonia* sp. DSM 4456 were found to be the most effective in hydrogenation of some of these compounds.

The available literature evidence and our preliminary study indicate that it is difficult to select a biocatalyst capable of transforming flavonoids. For biotransformation of the compounds prepared by chemical synthesis we used the microorganisms selected on the basis of literature data. We expected to find the products of microbial aromatic ring hydroxylation, hydroxyl group acylation and glycosidation, carbonyl groups reduction and reduction of unsaturated bonds.

Rhodococcus and *Gordonia* strains are ubiquitous in the environment and have diverse biodegradative capabilities, breaking down a variety of alkanes, halogenated aliphatics and aromatics, and other xenobiotic pollutants [43–48]. To the best of our knowledge there are not many reports regarding biotransformation of chalcones with the help of bacteria belonging to *Gordonia* or *Rhodococcus*. In fact, known reports describing hydrogenation of chalcones by microorganisms to the corresponding saturated ketones concerned the reaction catalyzed by *Corynebacterium equi* IFO 3730, *Aspergillis flavus* isolated from *Paspalum maritimum* – in aqueous medium and *Saccharomyces cerevisiae* yeasts in biphasic system [31–33].

After the screening, we determined the biomass production at the chosen temperature of 28 °C by both the bacterial

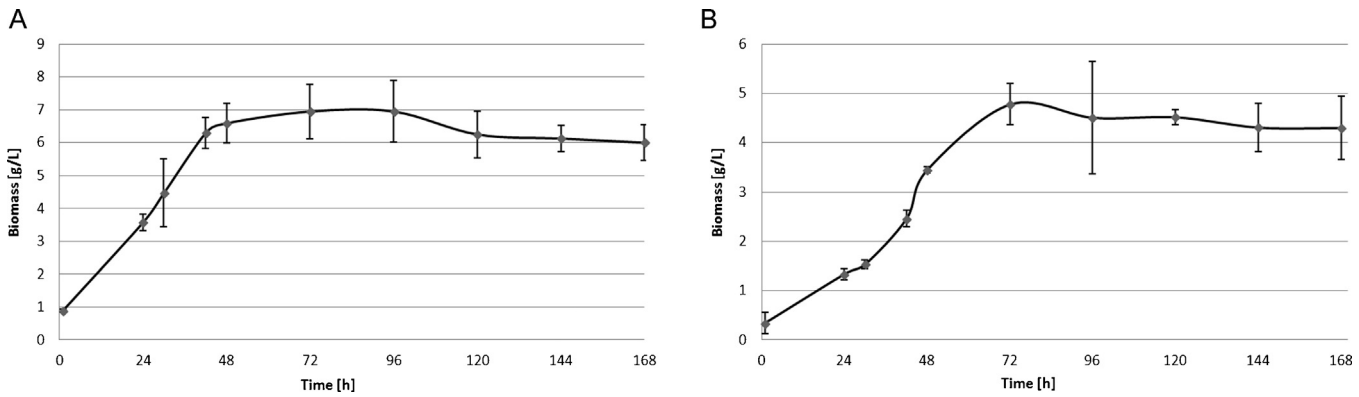


Fig. 2. Biomass production by (A) *Rhodococcus* sp. DSM 364, and (B) *Gordonia* sp. DSM.

strains – *Rhodococcus* sp. DSM 364 and *Gordonia* sp. DSM 4456 (Fig. 2). On the basis of the results obtained, in the preparative biotransformations the substrates were added after 72 h of incubation, when microorganisms reached the stationary phase of growth.

The biohydrogenation of chalcone (**1**), 4-methoxychalcone (**2**) and chalconaringenin (**3**) was completely chemoselective, producing only the corresponding saturated derivatives of 1,3-diphenyl-1-propanone (**1a**, **2a**, **3a** and **5a**) with a yield ranging from 13 to 94% after 72 h of the reaction. No other products were detected by HPLC and TLC. In the case of xanthohumol (**4**) we did not observe any product formation (Fig. 3 and Table 1), although in our previous study using the strain *Rhodotorula marina* this substrate was transformed into its dihydroderivative with 18% yield. This strain produced also compound (**2a**) from **2** [37].

Initially, we used two strains of bacteria. The experiments carried out on substrates **1** and **2** showed that better yields were obtained for *Rhodococcus* sp. DSM 364 – 94% and 80%, respectively. Thus, this strain was applied for the next biotransformations, using compounds **3**–**5**.

The structures of products were confirmed by means of IR, ^{1}H NMR, ^{13}C NMR, correlation spectroscopy and comparison with reported physical and spectroscopic data.

Biotransformation of chalcones **1** and **2** in the cultures of *Gordonia* sp. and *Rhodococcus* sp. led to the corresponding dihydrochalcones **1a** and **2a**. Comparison of the ^{1}H NMR, ^{13}C NMR and IR spectra of the products with the substrates clearly showed the lack of the double C=C bond and the presence of the unchanged carbonyl group C=O. Transformation of chalconaringenin (**3**) in the culture of *Rhodococcus* sp. led to phloretin (**3a**) in 84% yield. Compound **3a**, isolated as a white powder, exhibited a molecular ion at *m*/*z* 273.0749 in the high resolution electron ionization mass spectrum (HR-EIMS), corresponding to the molecular formula of $C_{15}H_{14}O_5$ (calcd. = 274.2686). The UV spectrum of **3a** showed maxima (λ_{max} = 284, 233 and 213 nm), that were comparable with a chalcone chromophore. The infrared spectrum exhibited absorption bands typical for hydroxyl (3285 cm^{-1}), methylene (2924 cm^{-1}), conjugated carbonyl (1605 cm^{-1}) and aromatic (1573 and 828 cm^{-1}) groups. In the ^{13}C NMR spectrum of **3a** 11 carbon signals, representing 15 carbon atoms, were observed. Comparison of the ^{1}H and ^{13}C NMR spectra of **3** and **3a** also revealed a structural similarity of these two compounds. The most significant difference observed in the ^{1}H NMR was the presence of protons of two methylene groups at α- and β positions in the spectrum of **3a**, and their absence in the spectrum of **3**. In addition, compound **3** exhibited a molecular ion at *m*/*z* 271.0608 in HR-EIMS, lower by 2.0141 compared to the product. Thus, compound **3a** was assigned as dihydrochalconarinenin (phloretin).

Phloretin (**3a**) was isolated from Formosan apple (*Malus doumeri* var. *formosana*) [27] and from tropic medicinal plant *Syzygium aqueum* and have potential as a cosmetic and antihyperglycaemic agent [49]. Furthermore, other research suggest that phloretin inhibits tumour cell growth by induction of apoptosis in the B16 mouse melanoma cells [28]. A literature search disclosed that phloretin (**3a**) has been synthesized by Siddaiah et al. [40] but to our knowledge, this paper is the first report of its production using bacterial biocatalysis.

Biotransformation of flavanone (**5**) catalyzed by the strain of *Rhodococcus* sp. DSM364 led to formation of two products. Thus, compound (**5**) was transformed into 2′-hydroxychalcone (**5a**) and next, like compounds (**1**–**3**), to 2′-hydroxydihydrochalcone (**5b**) by a bacterial enzyme system with 30 and 13% yield, respectively. Trihydroxy derivative of this compound: naringenin (**6**), was biotransformed neither by this strain nor by *Gordonia* sp. DSM 44456 (Fig. 4).

Compound **5a**, isolated as a yellowish powder, exhibited a molecular ion at *m*/*z*: [M−H$^+$] = 223.0764 corresponding to the molecular formula of $C_{15}H_{12}O_2$. The IR spectrum exhibited absorption bands typical for hydroxyl (3467 cm^{-1}), aromatic or alkenyl C—H (3058 cm^{-1}), conjugated carbonyl (1640 cm^{-1}) and aromatic (1573 cm^{-1}) groups. The ^{13}C NMR spectrum of **5a** showed 13 carbon signals representing 15 carbon atoms, all of them having chemical shifts of sp^2 carbon, in which two of the signals were assignable to a conjugated carbonyl (193.7 ppm) and an oxyaryl (163.5 ppm) carbon atom. The ^{1}H NMR spectrum showed a characteristic signal of a chalcone structure by the presence of a pair of doublets at 7.94 and 7.67 ppm with a *trans* coupling constant *J* = 15.3 Hz (*trans*-chalcone *J* = 15.6 Hz, *cis*-chalcone *J* = 13.0 Hz [50]). The spectroscopic data suggested that **5a** is a simple monohydroxylated chalcone derivative.

The ^{1}H NMR spectrum of **5b** showed presence of two methylene groups in the place of the chalcone double bond by the presence of a pair of triplets at 3.32 and 3.06 ppm with a coupling constant

Table 1
Synthesis of dihydrochalcones by *Rhodococcus* sp. DSM 364 (I) and *Gordonia* sp. DSM 44456 (II).[a]

Strain of bacteria	Substrate	Product	Time of biotransformation (days)	Yield (%)
I	**1**	**1a**	3	94
II	**1**	**1a**	3	65
I	**2**	**2a**	3	80
II	**2**	**2a**	3	45
I	**3**	**3a**	3	84
I or II	**4**	None	7	0
I	**5**	**5a**	7	30
		5b		13
I or II	**6**	None	7	0

[a] Culture conditions: 200 rpm, 28 °C. After 48 h of incubation 25 mg of substrate dissolved in 1 mL of acetone was added.

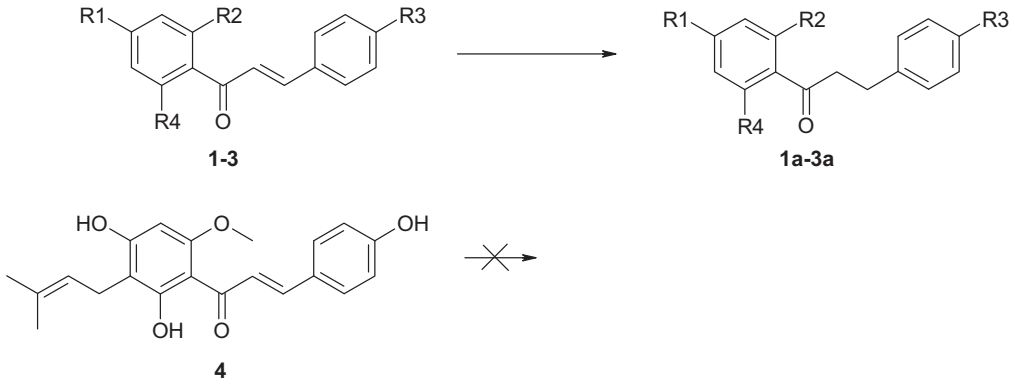


Fig. 3. Biotransformation of the chalcones (**1–4**) catalyzed by the selected bacteria strains.

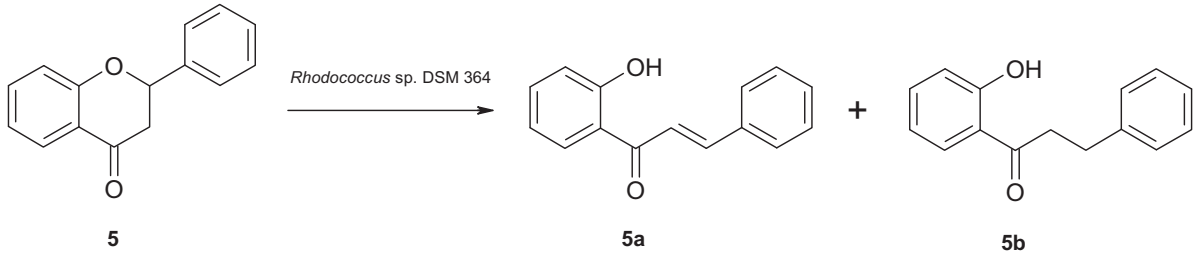


Fig. 4. Biotransformation of flavanones **5** catalyzed by *Rhodococcus sp*. DSM364.

J = 7.7 Hz. The phenolic group was determined to be at 12.28 ppm, whereas the multiplicities of four aromatic signals 7.73, 7.45, 6.96 and 6.86 ppm were typical for a 1,2-disubstituted benzene. Consequently, the ring B in **5b** was an unsubstituted phenyl group (7.18–7.33 ppm, 5H).

2′-Hydroxydihydrochalcone (**5b**) has been isolated from the *Pepermia obtusifolia* [51] and has been obtained in biotransformation of flavanone (**5**) by the culture of a UV mutant of *Aspergillus niger* MB [20]. It has been popular substrate in the solvent-free syntheses for generation of a variety of flavonoids [52]. Some of these compounds possess a wide range of antimicrobial activities [53].

4. Conclusion

In conclusion, the present study reports a development of highly efficient protocol for chemoselective conjugate reduction of α,β-unsaturated carbonyl compounds in aqueous media using bacterial cultures. The products obtained in the biotransformations of chalcones (**1**-**2**) using the strains of *Gordonia* sp. DSM 44456 and *Rhodococcus* sp. DSM 364 contained 1,3-diaryl-2-propan-1-one structure. The strain of *Rhodococcus* sp. DSM 364 was capable of hydrogenation of chalconaringenin (**3**) to phloretin (**3a**) and cleavage of the C-ring in flavanone (**5**), leading to 2′-hydroxychalcone (**5a**) and next to 2′-hydroxydihydrochalcone (**5b**). Xanthohumol (**4**) and naringenin (**6**) were resistant to biotransformation by these strains.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molcatb.2013.09.009.

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