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- Fungal metabolism of twelve naphthoflavone compounds was investigated
- Effect of different substituent in B-ring was evaluated
- Products of 4'-hydroxylation and 4'-*O* demethylation have been obtained
- *Aspergillus* and *Verticilium* strains were most effective biocatalyst
- Fungal metabolism do not coincides with human or rodent metabolism

Fungal metabolism of naphthoflavones. Effect of substitution in the B-ring.

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Abstract

Naphthoflavones (benzoflavones) are synthetic flavonoids commonly used in drug metabolism studies as selective activators or inhibitors of cytochrome P-450 enzymes. Nowadays they are also used as a component of food supplements for body builders. There is no data regarding naphthoflavone microbial metabolism. In the present studies sixty-three fungal strains have been screened for their ability to transform α-naphthoflavone (7,8-benzoflavone) or β-naphthoflavone (5,6-benzoflavone). Five strains belonging to the genera *Penicillium*, *Cladosporium, Aspergillus and Verticillium* transformed α-naphthoflavone and β-naphthoflavone to the corresponding 4'-hydroxy derivatives. These selected fungi have been used in a further study on biotransformation of naphthoflavones with a differently substituted B-ring. Only 4'-methoxy derivatives have been transformed to the related 4'-hydroxy products. Selected strains are good biocatalysts to obtain 4'-hydroxy naphthoflavones in the one step reaction.

keywords: naphthoflavones, benzoflavones, biotransformation, hydroxylation, demethylation, fungi

1. Introduction

Naphthoflavones are synthetic derivatives of flavones, a group of naturally-occurring flavonoids widely distributed in the plant world. At the beginning of the 1970s α-naphthoflavone (α-NF, 7,8-benzoflavone, **1**) was found to be in the spotlight of researchers because of its effect on the metabolism of carcinogens [1]. The subsequent studies showed that this compound inhibits the activation of procancerogenic polycyclic hydrocarbons and activates aflatoxin B1 to mutagenic metabolites *in vitro* [2,3]. Interestingly, β-naphthoflavone (β-NF, 5,6-benzoflavone, **2**), an isomer of α-NF (**1**), also affects the activity of microsomal polycyclic hydrocarbon hydroxylases, however, it acts as an inducer of these enzymes activities in vitro [4, 5]. A few years later Kellis and coworkers discovered that α-NF (**1**) is a potent inhibitor of aromatase, the enzyme responsible for transformations of androgens into estrogens [6]. They also showed that β-NF (**2**) has no inhibitory activity. Nowadays the properties of these two naphthoflavones, that rely on the selective modulation of the activity of particular cytochrome P-450 monooxigenases, are widely applied in drug metabolism studies [7, 8].

The biological activity of naphtoflavones was tested in parallel with their metabolism (Table 1). The much greater interest in α-NF (**1**) compared to β-NF (**2**) may be associated with the fact that some metabolites of α-NF (**1**) have a higher inhibitory potency [9, 10]. Further studies on the metabolism of naphtoflavones seem to be mandatory especially due to the action of aromatase-inhibiting α-NF (**1**) that is used as a component of dietary supplements sold for bodybuilding. In a consequence humans may be exposed to gut microbial metabolites of α-NF (**1**) and such may differ from those already identified.

For years whole cell microorganism biotransformations have been used to mimic mammalian metabolism or to obtain human particular metabolites at reasonable quantities using simple methods [11]. To our best knowledge there is no data concerning naphthoflavones microbial metabolism, therefore we decided to study this phenomenon.

Table 1. Comparative data from different studies on α-NF (**1**) and β-NF (**2**) metabolism.

	Metabolites of	
	α-NF (**1**)	β-NF (**2**)
Long-Evans rat liver microsomes and reconstituted Cytochrome P-450 System [12]	5,6-oxide, 5,6-dihydrodiol, 7,8-dihydrodiol, 6-hydroxy,7-hydroxy, 9-hydroxy and one unknown compound	5,6-dihydrodiol, 7,8-dihydrodiol, 8-hydroxy, 5-hydroxy and five unknown compounds
rat liver microsomes and reconstituted selection of Cytochrome P-450 System [13]	5,6-oxide, 5,6-dihydrodiol, 7,8-dihydrodiol, 6-hydroxy and one unknown metabolite	
Charles River CD rat liver microsomes [14]	5,6-oxide, 5,6-dihydrodiol,6-hydroxy, 9-hydroxy	
Charles River CD rat, mouse, rabbit and Syrian golden hamster liver microsomes [15]	5,6-oxide, 5,6-dihydrodiol, 7,8-dihydrodiol, 6-hydroxy,7-hydroxy, 9-hydroxy	
Sprague Dawley rat liver microsomes [16]	5,6-oxide, 5,6-dihydrodiol, 9,10-dihydrodiol, 6-hydroxy	
hepatic microsomes from the marine fish *Stenotomus versicolor* [17]	unidentyified dihydrodiol	
liver microsomes [18]	5,6-oxide, 5,6-dihydrodiol,9,10-dihydrodiol, 5-hydroxy	
Syrian golden hamster liver microsomes [19]	6-hydroxy,7-hydroxy and six other metabolites	
Syrian golden hamster hepatocytes [20]	5,6-dihydrodiol, 7,8-dihydrodiol, 6-hydroxy,7-hydroxy	
Syrian golden hamster liver and kidney microsomes [21]	5,6- dihydrodiol, two isomeric hydroxyderivatives	
human liver microsomes [22]	5,6-oxide, 5,6-dihydrodiol, 7,8-dihydrodiol, 6-hydroxy,7-hydroxy	

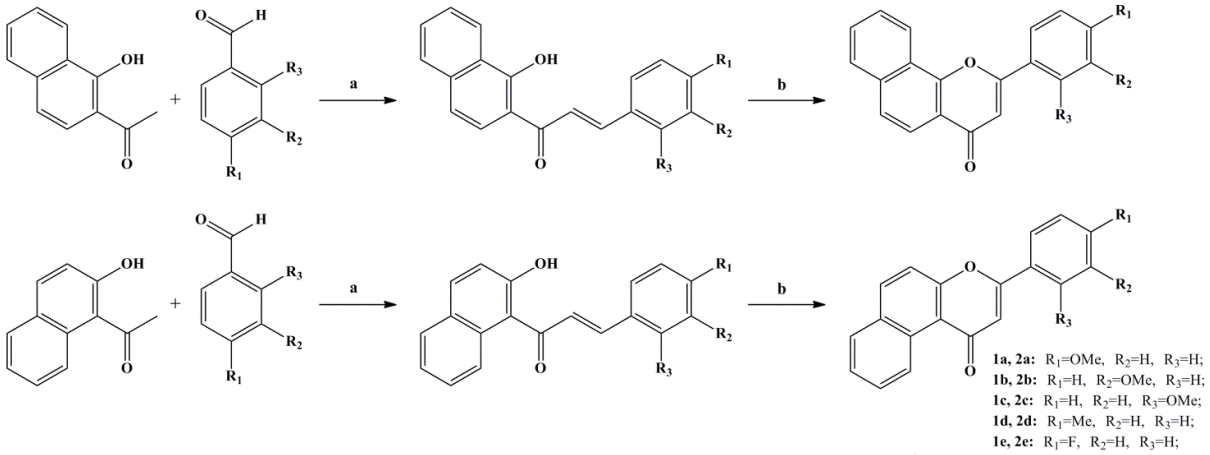
Table 1. Comparative data from different studies on α-NF (**1**) and β-NF (**2**) metabolism.

	Metabolites of	
recombinant human CYP3A4 and NADPH:CYP reductase with cytochrome b5 [23]	5,6-oxide, 7,8-dihydrodiol	

2. Experimental

2.1 Materials

α-NF (**1**), β-NF (**2**), 1-hydroxy-2-acetonaphthone, 2-hydroxy-1-acetonaphthone, 2-methoxybenzaldehyde, 3-methoxybenzaldehyde, 4-methoxybenzaldehyde, 4-methylbenzaldehyde and 4-fluorbenzaldehyde were purchased from Sigma Aldrich. Ten naphthoflavone derivatives were obtained from corresponding hydroxy-acetonaphthones and benzaldehydes as a starting materials. Two stage synthesis was used in which first step was base aldol condensation of hydroxy-acetonaphthones and benzaldehydes to afford chalcones. Second step was oxidative-cyclisation of chalcones with iodine in DMSO to afford flavones (Scheme 1) [24].



Scheme 1: Synthesis of naphthoflavone derivatives ([a)]KOH, EtOH; [b)]I_2, DMSO)

The structures of the obtained compounds were confirmed by NMR spectra analysis. Spectroscopic data of methoxy-derivatives (**1a-c**, **2a-c**) are with agreement with literature data [24]. Data of other compounds (**1d-e**, **2d-e**) are as follows:

4'-methyl-α-naphthoflavone (**1d**). ^{1}H NMR (600 MHz, DMSO-d_6) δ: 2.39 (3H, s, H-Me), 7.11 (1H, s, H-3), 7.40 (2H, m, H-3', H-5'), 7.80 (2H, m, H-8, H-9), 7.97 (1H, m, H-5), 7.90 (1H, m, H-6), 8.08 (2H, m, H-2', H-6'), 8.10 (1H, m, H-7), 8.63 (1H, m, H-10). ^{13}C NMR (150 MHz, DMSO-d_6) δ: 21.05 (C-Me), 107.45 (C-3), 119.59 (C-13), 119.97 (C-5), 122.23 (C-10), 123.47 (C-11), 125.29 (C-6), 126.24 (C-2', C-6'), 127.66 (C-9), 128.22 (C-7), 128.36 (C-1'), 129.48 (C-8), 129.79 (C-3', C-5'), 141.91 (C-4'), 135.40 (C-14), 152.65 (C-12), 162.04 (C-2), 176.76 (C-4).

4'-fluoro-α-naphthoflavone (**1e**). ^{1}H NMR (600 MHz, DMSO-d_6) δ: 7.21 (1H, s, H-3), 7.47 (2H, m, H-3', H-5'), 7.83 (2H, m, H-8, H-9), 7.95 (1H, m, H-6), 8.00 (1H, m, H-5), 8.12 (1H, m, H-7), 8.33 (2H, m, H-2', H-6'), 8.69 (1H, m, H-10); ^{13}C NMR (150 MHz, DMSO-d_6) δ: 108.07 (C-3), 116.29 (d, J = 22.00 Hz, C-3', C-5'), 119.53 (C-13), 119.93 (C-5), 122.29 (C-10), 123.43 (C-11), 125.41 (C-6), 127.67 (C-9), 127.77 (d, J = 2.90 Hz, C-1'), 128.22 (C-7), 129.07 (d, J = 9.00 Hz, C-2', C-6'), 129.55 (C-8), 135.44 (C-14), 152.74 (C-12), 161.05 (C-2), 164.09 (d, J = 250.3 Hz, C-4'), 176.74 (C-4).

4'-methyl-β-naphthoflavone (**2d**). ^{1}H NMR (600 MHz, DMSO-d_6) δ: 2.41 (3H, s, H-Me), 7.16 (1H, s, H-3), 7.42 (2H, m, H-3', H-5'), 7.69 (1H, m, H-7), 7.78 (1H, m, H-6), 7.90 (1H, d, J = 9.0 Hz, H-10), 7.97 (1H, m, H-8), 8.06 (2H, m, H-2', H-6'), 8.36 (1H, d, J = 9.0 Hz, H-9), 9.97 (1H, m, H-5). ^{13}C NMR (150 MHz, DMSO-d_6) δ: 21.05 (C-Me), 109.12 (C-3), 116.18 (C-12), 118.16 (C-10), 126.08 (C-5), 126.11 (C-2', C-6'), 126.55 (C-7), 127.51 (C-1'), 128.57 (C-8), 129.01 (C-6), 130.36 (C-13), 129.69 (C-14), 129.75 (C-3', C-5'), 135.69 (C-9), 141.85 (C-4'), 156.94 (C-11), 160.39 (C-2), 179.18 (C-4).

4'-fluoro-β-naphthoflavone (**2e**). ^{1}H NMR (600 MHz, DMSO-d_6) δ: 7.20 (1H, s, H-3), 7.45 (2H, m, H-3', H-5'), 7.68 (1H, m, H-7), 7.78 (1H, m, H-6), 7.89 (1H, m, H-10), 8.10 (1H, m, H-6"), 8.23 (2H, m, H-2', H-6'), 8.36 (1H, m, H-9), 9.95 (1H, m, H-5). ^{13}C NMR (150 MHz, DMSO-d_6) δ: 109.65 (C-3), 116.11 (C-12), 116.25 (d, J = 22.0, C3', C-5'), 118.13 (C-10), 126.04 (C-5), 126.60 (C-7), 127.30 (d, J = 2.9 Hz, C-1'), 128.58 (C-8), 128.85 (d, J = 9.0 Hz, C-2', C-6'), 129.06 (C-6), 129.63 (C-14), 130.37 (C-13), 135.78 (C-9), 156.90 (C-11), 160.35 (C-2), 164.07 (d, J = 250.4, C-4'), 179.15 (C-4).

2.2. Analytic procedures

TLC was carried out on Merck silica gel 60, F_{254} (0.2 mm thick) plates using chloroform/methanol (9:1 v/v) or hexane/acetone/2-propanol (8:1:1 v/v) as developing solvents. After drying, spots were visualized under short-and long-wavelength UV light, the plates were then sprayed with methanol-sulfuric acid (1:1 v/v) solution. HPLC was performed on a Waters 2695 Alliance instrument with a photodiode array detector Waters 2996 (detection from 220 to 500 nm wavelength) using the analytical HPLC column Cosmosil Cholester 5 μm (4.6 x 250 mm) at the flow rate of 1 ml/min. A linear solvent gradient from 45% to 95% *aq* MeOH containing 0.05% HCOOH of over 39 min was used. ^{1}H NMR, ^{13}C NMR, DEPT 135°, ^{1}H–^{1}H NMR (COSY), ^{1}H– ^{13}C NMR (HMQC and HMBC)) were recorded on a DRX Bruker Avance TM 600 (600 MHz) instrument in DMSO-d_6, UV spectra were run on a Spectrofotometer Cintra 303, GBC, in methanol. Positive and negative-ion ESI-MS spectra were taken on a Bruker microTOF-Q spectrometer.

2.3. Microorganisms

Sixty-three strains of fungal cultures from the culture collection of the Department of Chemistry at the Wrocław University of Environmental and Life Sciences (Poland) have been used in the screening procedure. The strains used came from the Department of Pharmaceutical Biology and Botany of the Wrocław Medical University, Poland (indexed AM), Department of Forest Pathology of the Agricultural University of Kraków, Poland (indexed ARK), Department of Chemistry of the Wrocław University of Environmental and Life Sciences, Poland (indexed KCh), Department of Plant Protection of the Wrocław University of Environmental and Life Sciences, Poland (indexed UPF). The strains used in the screening procedure are as follows: *Absidia coerulea* AM 93, *A. glauca* AM 177, *Acremoniella atra* AM 12, *Armillaria mellea* AM 296, *A. mellea* AM 477, *Aspergillus* sp. AM 14, *A. glaucus* AM 211, *A. nidulans* AM 243, *A. ochraceus* AM 370, *A. ochraceus* AM 456, *A. candidus* AM 386, *A. niger* UPF 702, *A. niger* UPF 709, *A. niger* UPF 724, *A. fumigatus* UPF 703, *A. versicolor* UPF 703, *Beauveria bassiana* AM 278, *Botrytis cinarea* AM 235, *Chalara* sp. ARK 16664, *Cladosporium avellaneum* AM 135, *Coryneum betulinum* ARK 16534, *Crumenulopsis sororia* ARK 15940, *Cunninghamella japonica* AM 472, *Disculina betulina* ARK 16538, *Fusarium culmorum* AM 10, *F. culmorum* AM 196, *F. culmorum* AM 282, *F. scirpi* AM 199A, *F. oxysporum* AM 2, *F. oxysporum* AM 145, *F. oxysporum* UPF 727, *F. tricinctum* AM 395, *Laetiporus sulphurens* AM 525, *Morteriella vinacea* AM 149, *M. isabellina* AM 212, *Mucor hiemalis* UPF 729, *Penicillium albidum* AM 47, *P. purpurogenum* AM 49, *P. vermiculatum A*M 13, *P. vermiculatum* AM 50, *P. camembertii* AM 51, *P. urticae* AM 54, *P. thomi* AM 91, *P. vinaceum* AM 110, *P. lilacinum* AM 111, *P. chrysogenum* AM 112, *P. chermesinum* AM 113, *P. spinulosum* AM 114, *P. frequentas* AM 351, *P. frequentas* AM 359, *P. diversum* AM 388, *P. notatum* KCh 904, *P. notatum* UPF 725, *Piptoporus betulinus* AM 475, *Pleurotus ostreatus* AM 482, *P. ostreatus* UPF 721, *Rhizopus nigricans* UPF 701, *Spicaria divaricata* AM 423, *Stemphylium botryosum* AM

279, *Syncephalastrum racemosum* AM 105, *Trametes versicolor* AM 536, *Trichothecium roseum* UPF 700, *Verticillium* sp. AM 424. Microorganisms were maintained on Sabouraud medium (3% glucose and 1% peptone) agar slants at 5°C.

2.4. Conditions for biotransformations

The cultures were shaken on rotary shakers (130 speed, 6.5 amplitude) at 28°C in 100-ml Erlenmeyer flasks with 30 ml of the medium in the screening studies and in 300-ml Erlenmeyer flasks with 100 ml of the medium in the preparative scale transformation. In all experiments fungi were cultivated on Sabouraud medium (3% glucose and 1% peptone). Agar slant cultures were used to obtain the preculture, and then 3-day precultures were transferred to the main cultures media (1 ml to the 30 ml and 3 ml to the 1500 ml). Substrates were added after 5 days of culturing. Substrate control for all naphthoflavones consisted of the substrate and a sterile growth medium incubated without microorganisms.

In the screening studies 20 mg of substrate (**1, 1a-e, 2, 2a-e**) dissolved in 2 ml of DMSO were equally distributed among 4 flasks (0,5 ml each). Biotransformations were carried out for 7 and 14 days in duplicate, acidified with 1M HCl to pH around 5 (if necessary) and then reaction mixtures were extracted with ethyl acetate (2 x 10 ml).

In the preparative biotransformations, 60 mg of substrate (**1**, **1a**, **2** or **2a**) dissolved in 4 ml of DMSO was equally distributed among four flasks with 5-day fungal cultures (1 ml each). Reactions were carried out for 21 days, acidified with 1M HCl to pH around 5 (if necessary), and then reaction mixtures were extracted with ethyl acetate (3 x 40 ml).

Extracts were dried over anhydrous magnesium sulphate and the solvent was filtered and evaporated under vacuum. Residues were dissolved in methanol and analysed by TLC and HPLC.

2.5. Products isolation and analysis

The products of naphtoflavones biotransformation were separated by column chromatography on silica gel 60 (230 – 400 mesh, Merck) using chloroform/methanol (9:1 v/v) as an eluent. Products structures were elucidated by NMR and MS spectroscopy methods. Spectral data of products are as follows:

4'-hydroxy-α-naphthoflavone (**3**).^{1}H NMR (600 MHz, DMSO-d_6) δ: 7.00 (2H, m, H-3', H-5'), 7.04 (1H, s, H-3), 7.83 (2H, m, H-8, H-9), 8.01 (1H, s, H-5), 7.95 (1H, m, H-6), 8.12 (2H, m, H-2', H-6'), 8.13 (1H, m, H-7), 8.71 (1H, m, H-10), 10.33 (1H, s, C4'-OH); ^{13}C NMR (150 MHz, DMSO-d_6) δ: 105.98 (C-3), 116.07 (C-3', C-5'), 119.51 (C-13), 120.04 (C-5), 121.70 (C-1'), 122.24 (C-10), 123.50 (C-11), 125.14 (C-6), 127.64 (C-9), 128.23 (C-7), 128.32 (C-2', C-6'), 129.50 (C-8), 135.37 (C-14), 152.58 (C-12), 160.88 (C-4'), 162.49 (C-2), 176.64 (C-4); HRESI-MS [M-H$^+$] (calculated/found) (m/z 257.0990/257.0979).

4'-hydroxy-β-naphthoflavone (**4**).^{1}H NMR (600 MHz, DMSO-d_6) δ: 6.96 (2H, m, H-3', H-5'), 7.02 (1H, s, H-3), 7.67 (1H, m, H-7), 7.77 (1H, m, H-6), 7.86 (1H, m, J = 9.0 Hz, H-10), 8.01 (2H, m, H-2', H-6'), 8.09 (1H, m, H-8), 8.34 (1H, m, J = 9.0 Hz, H-9), 9.97 (1H, m, H-5), 10.33 (1H, s, C4'-OH).^{13}C NMR (150 MHz, DMSO-d_6) δ: 107.67 (C-3), 115.98 (C-3', C-5'), 116.06 (C-12), 118.14 (C-10), 121.19 (C-1'), 126.14 (C-5), 126.45 (C-7), 128.14 (C-2', C-6'), 128.54 (C-8), 129.78 (C-14), 128.91 (C-6), 130.34 (C-13), 135.47 (C-9), 156.81 (C-11), 160.78 (C-2), 160.80 (C-4'), 179.10 (C-4); HRESI-MS [M-H$^+$] (calculated/found) (m/z 257.0990/257.0983).

3. Results

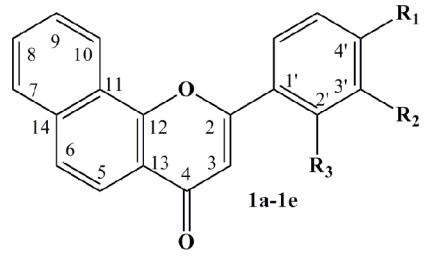
3.1. Screening studies

Sixty-three fungal strains were screened for their ability to transform α-NF (**1**) and β-NF (**2**). The selection of some species was done based on literature data concerning flavone biotransformations [25]. Progress of the biotransformation was determined by TLC and HPLC

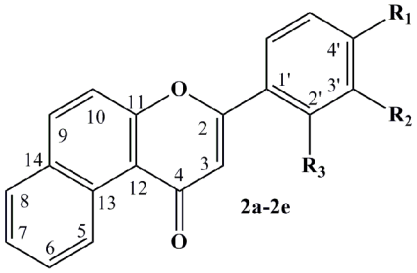
analysis of culture extracts. Five fungal strains: *Penicillium thomi* AM 91, *Cladosporium avellaneum* AM 135, *Verticillium* sp. AM 424, *Aspergillus glaucus* AM 211 and *A. niger* UPF 724 were capable of transforming α-NF (**1**) and/or β-NF (2). These microorganisms were chosen for screening studies for their metabolic potential to transform derivatives of **1** and **2**, and for further transformations in a preparative scale.

3.2. Preparative transformations of naphtoflavones

Scale-up biotransformations of naphtoflavones: α-NF (**1**), β-NF (**2**), and ten derivatives of these compounds (Scheme 2) afforded two products: 4'-hydroxy-α-naphthoflavone (**3**), 4'-hydroxy-β-naphthoflavone (**4**) (Scheme 3). To obtain the highest transformation rates, we prolonged the reaction time up to 21 days. However, the conversion rates measured by HPLC in comparison with the results obtained in the screening tests slightly differed towards an increased yield. Also, if any products were formed, they were observed after only one day of substrate exposition.



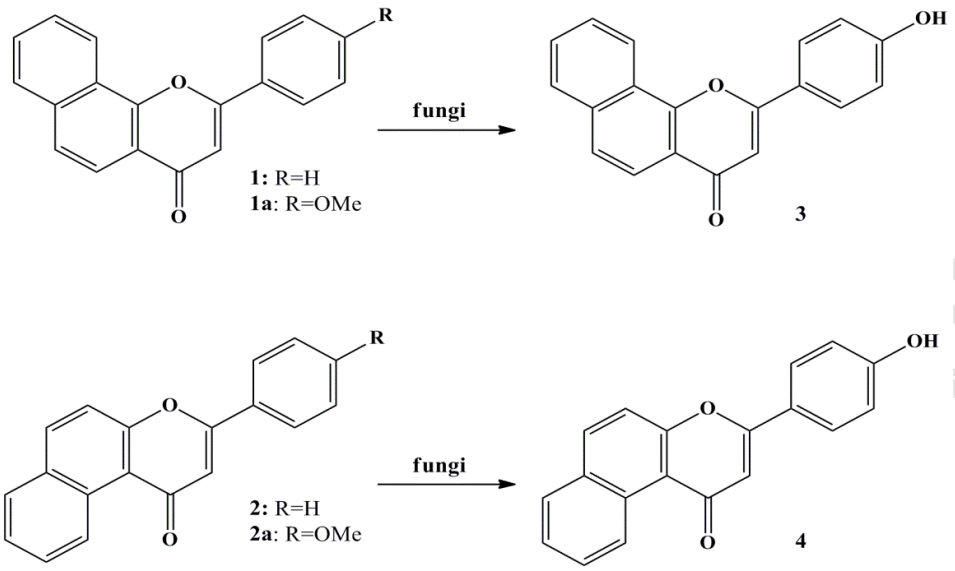
1a R_1=OMe R_2=H R_3=H
1b R_1=H R_2=OMe R_3=H
1c R_1=H R_2=H R_3=OMe
1d R_1=Me R_2=H R_3=H
1e R_1=F R_2=H R_3=H



2a R_1=OMe R_2=H R_3=H
2b R_1=H R_2=OMe R_3=H
2c R_1=H R_2=H R_3=OMe
2d R_1=Me R_2=H R_3=H
2e R_1=F R_2=H R_3=H

Scheme 2: Synthetized naphthoflavone derivatives used in the metabolism studies.

All five selected strains were able to metabolize **1** while only three were able to transform **2** (Table 2). The most effective biocatalysts were *Aspergillus glaucus* AM 211 and *Verticillium* sp. AM 424 which transformed **1** to **3** with an isolated yield of 43% and 63%, respectively. These strains were also the most efficient in transforming **2** to **4** with a 24% and 17% isolated yield. Out of the ten tested derivatives of naphtoflavones **1** and **2**, only two have been efficiently converted. These were the 4'-methoxy-α-NF (**1a**) and 4'-methoxy-β-NF (**2a**).



Scheme 3. Transformations of naphthoflavones by fungi.

One strain, *Penicillium thomi* AM 91, exhibited some substrate specificity because it was able to transform only flavones with 7,8-benzo moiety, whereas *Aspergillus niger* UPF 724 was the only strain capable of the *O*-demethylation of both **1a** and **2a** with isolated yields of 71% and 34% exceeding the hydroxylation of naphthoflavones. Also the *O*-demethylation of **1a** by *Penicillium thomi* AM 91 resulted in compound **3** with a higher isolated yield (43%) then hydroxylation of **1** (24%).

Table 2. The yield of naphthoflavones metabolites obtained by selected fungal strains.

Strain	**3** [%]* from		**4** [%]* from	
	α-NF (**1**)	4'-OMe-α-NF (**1a**)	β-NF (**2**)	4'-OMe-β-NF (**2a**)
Aspergillus glaucus AM 211	43	traces**	24	traces**
Aspergillus niger UPF 724	17	71	traces**	34
Cladosporium avellaneum AM 135	36	nd	9	nd
Penicillium thomi AM 91	24	43	traces**	nd
Verticillium sp. AM 424	63	nd	17	nd

*all yields are of pure isolated compounds
** traces refer to values under 2% of the conversion rate detected by HPLC
nd – not determined

4. Discussion

Screening studies performed on sixty-three fungal strains resulted in the selection of five strains and afforded two products. Biotransformations in which the conversion rate exceeded 2% (HPLC, λ = 290 nm) were transferred to a larger scale. All the other microorganisms that produced traces of products 3 and 4 (< 2%) belonged to the Aspergillus, Fusarium and Penicillium genus. It is possible that optimization of the biotransformation conditions could increase its efficiency. Comparing the data concerning the metabolism of naphtoflavones in animals, summarized in Table 1, with the results of our studies on microbial transformations, it can be seen that the enzyme systems of mammals and fish modify naphthalene moiety, while microbial transformation products were changed in the B-ring. Although the hydroxylation of the B-ring of unsubstituted flavones, particularly 4'-hydroxylation, have been reported previously [25, 26], we found it interesting to test the substrate specificity of the selected strains, especially since flavone microbial transformations in only *Aspergillus* and *Penicillium* strains yielded positive transformation results in our preliminary studies [27].

Further reactions with differently substituted derivatives were carried to check the impact of additional steric hinderance in the B-ring and altered groups at position 4' on the reaction results. Reactions with 4' substituted methoxy naphthoflavones **1a** and **2a** provided two parallel products: **3** and **4**. Demethylation reactions for the flavonoid compounds with flavone or flavanone structure have already been described, but not for compounds with additional benzyl moiety like methoxynaphthoflavones. There are many literature examples of microbial *O*-demethylation of flavones among which 4'-*O*-demethylation being the most frequent, most often using the fungi of the genus *Aspergillus* [28]. *Aspergillus niger* UPF 724 was the only strain capable of the *O*-demethylation of both **1a** and **2a** with the high isolated yields 71% and 34%, respectively (Table 2, Scheme 3), exceeding the hydroxylation of naphthoflavones, which corresponds with literature where the majority of the demethylation tends to occur at 3' or 4' of flavon or flavanone derivatives and exceeds the related hydroxylation yields [28].

The obtained results indicate that different enzymes may be associated with hydroxylation and demethylation in the selected strains. The lack of O-demethylation or 4'-hydroxylation products for other methoxy derivatives may suggests different substrate specificity, but without further research it will remain as an assumption. Moreover, taking into account α-NF (1) and β-NF (2) activities regarding cytochrome enzymes, it is possible that its naphthoflavones derivatives inhibit or do not induce certain enzymes required for transformations.

There are no data about the biological activities of 4'-hydroxynaphthoflavones, therefore another step in our research will be the determination of these compounds' cytotoxicity. Moreover, the products obtained are more related to *prokaryote* flavonoid transformations and such transformations seem to be expected, also for gut microflora exposed for α-NF (**1**) during supplementation with dietary supplements for body builders containing α-NF (**1**) [26].

5. Conclusions

Among sixty-three fungal strains screened for ability to transform naphtoflavones only five were efficient biocatalysts. Preparative scale experiments yielded the 4'-hydroxy derivatives of both α-NF (**1**), β-NF (**2**) and its differently substituted analogues. *Verticillium* sp. and *Aspergillus glaucus* proved to be the best biocatalyst to obtain 4'-hydroxynaphtoflavones through direct hydroxylation, while *Aspergillus niger* through demethylation of 4'-methoxy substrates. The process of demethylation was the most efficient. Our results indicate that fungi are useful biocatalysts to obtain a 4'-hydroxy derivatives of naphthoflavones in the one step reaction. Fungal metabolism of naphthoflavones do not coincides with mammalian metabolism.

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*Graphical Abstract (for review)