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Transformation of isoxanthohumol by fungi

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ABSTRACT

Isoxanthohumol is the most abundant and an important prenylated flavonoid present in hopped beers. In order to select microorganisms capable of transforming isoxanthohumol screening tests on 44 fungi cultures were performed. This kind of activity has not been previously examined. *Beauveria bassiana* AM278 and *Absidia glauca* AM177 converted isoxanthohumol into glucoside derivatives, whereas *Fusarium equiseti* AM15 transformed it into (2*R*)-2″-(2‴-hydroxyisopropyl)-dihydrofurano[2″,3″:7,8]-4′-hydroxy-5-methoxyflavanone with high efficiency. Isoxanthohumol 7-*O*-β-D-4‴-methoxyglucopyranoside is a new compound.

1. Introduction

Hops, the female inflorescences of *Humulus lupulus* L. (Cannabaceae) have found many applications [1]. The most important is in brewing industry to provide aroma, bitterness, and flavour to beer. Beer is the main dietary source of hop prenylflavonoids, with an estimated daily intake of about 0.14 mg [2]. Xanthohumol, the most important prenylated chalcone of hop (up to 1% in dry hop cones) is converted to isoxanthohumol by thermal treatment and increased pH value during the brewing process [3]. Content of isoxanthohumol depends on a type of beer and ranges from 40 (European lager) to 3440 μg/l (strong ale) [2]. It was first isolated (named humulol) and later identified as a flavanone by Verzele et al. in 1957 [4].

Isoxanthohumol has received much attention as a proestrogen [5], an antiviral agent [6], an antioxidant [7], and in recent years as a cancer chemopreventive agent [8,9].

Metabolism of isoxanthohumol has been poorly documented, so far. Nikolic et al. [10] described 10 human liver microsomal metabolites of isoxanthohumol. One of them was 8-prenylnaringenin, which is considered to be the most potent phytoestrogen to-date, present in hop and other plants e.g. *Sophora flavescens* [11]. Human intestinal microbiota also demethylated isoxanthohumol to 8-prenylnaringenin with a high efficiency, up to 80% conversion [12]. *Eubacterium limosum* isolated from this community was found to be capable of 90% conversion [13].

Microbial transformations allow selective conversion of compounds into derivatives which are difficult to obtain in chemical synthesis. Biotransformations are also used to mimic mammalian metabolism [14]. The aim of such studies is to generate mammalian metabolites in an easy way, for testing their biological potential, and to contribute to the knowledge of mechanisms of mammalian system.

The purpose of the present work is to isolate and characterize the fugal metabolites of isoxanthohumol, since no reports are available to-date on the biotransformation of this compound.

The valuable initial substance – xanthohumol – was obtained from the by-product of the brewing industry.

2. Experimental

2.1. Microorganisms

Microorganisms used in the study were purchased from Institute of Biology and Botany of the Medical Academy of Wrocław (indexed AM), Department of Forest Pathology of the Agricultural University of Kraków (indexed ARK), Department of Biotechnology and Food Microbiology of Wrocław University of Environmental and Life Sciences (indexed AR), Department of Plant Protection of Wrocław University of Environmental and Life Sciences (indexed UPF). The cultures used for preliminary screening were as follows: *Absidia glauca* AM177, *Absidia coerulea* AM93, *Absidia cylindrospora* AM336, *Aspergillus fumigatus* UPF703, *Aspergillus glaucus* AM211, *Aspergillus nidulans* AM243, *Aspergillus niger* UPF702, *Aspergillus ochraceus* AM370, *A. ochraceus* AM456, *Beauveria bassiana* AM278, *B. bassiana* AM446, *Botrytis cinerea* AM235, *Chaetomium* sp. ARK16651, *Chaetomium* sp. ARK16665, *Chaetomium* sp. AM432, *Chaetomium*

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indicum AM32, *C. indicum* AM158, *Cunnighamella japonica* AM472, *Epithyrium resinae* ARK16051, *Fusarium equiseti* AM15, *Inonotus radiatus* ARK15970, *Laetiporus sulphurens* AM525, *Mortierella isabellina* AM212, *Mortierella mutabilis* AM404, *Mortierella vinaceace* AM149, *Penicillium chermesinum* AM113, *Penicillium chrysogenum* AM112, *Penicillium purpurogenum* AM49, *Penicillium vermiculatum* AM50, *Penicillium camembertii* AM51, *Pezicula cinnamomea* ARK15753, *Piptoporus betulinus* ARK5213, *P. betulinus* ARK15980, *P. betulinus* ARK20129, *Poria placenta* ARK2213, *Pycnidiella resinae* ARK16050, *Rhizopus nigricans* UPF701, *Sclerophoma pythiophila* ARK3215/6, *Spicaria divaricata* AM423, *Spicaria fusispora* AM136, *Spicaria violacea* AM439, *Trametes versicolor* AM536, *Trichothecium roseum* UPF700, *Yarrowia lipolytica* AR71.

2.2. Cultivation of fungi

The fungi were maintained on Sabouraud or Czapek or potato agar slants (depending on genus) and grown on a Sabouraud medium consisting of 3% glucose and 1% peptone. The cultures were shaken at 25 °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.

2.3. Microbial transformation of isoxanthohumol (1)

2.3.1. Screening tests

A solution of **1** (10 mg in 0.5 ml of methanol) was added to the fungi cultures. After 7-day incubation on a rotary shaker (25 °C, in the darkness) pH was measured and the metabolites and any remaining substrate were extracted.

All the experiments were performed in duplicate. Substrate control consisted of isoxanthohumol (**1**) and sterile growth medium incubated without fungi.

2.3.2. Preparative transformation

70 mg isoxanthohumol (**1**) dissolved in 8 ml of methanol was distributed among four flasks with fungi cultures (2 ml each): *A. glauca* AM177 – 4-day culture, pH 4.1, *B. bassiana* AM278 – 2-day culture, pH 4.3; *Fusarium equiseti* AM15 – 2-day culture, pH 6.6. The reactions were carried out under the same conditions as in screening tests and continued until the substrate **1** was metabolized (progress of conversion was monitored by HPLC).

2.3.3. Reaction work-up and product analysis

In the screening experiments the products were extracted from the medium with 15 ml of ethyl acetate. In the preparative biotransformations the fermentation broth was extracted three times with 20 ml of ethyl acetate. Then the extracts were dried over magnesium sulfate and the solvent was evaporated off. The residues were separated by column chromatography on silica gel (0.05–0.2, Merck), using chloroform:methanol (from 9:1 to 3:1, v/v) as eluent.

TLC was carried out using silica gel 60, F_{254}, 0.2 mm thick plastic plates (Merck) with chloroform:methanol (from 9:1 to 6:1, v/v) as developing solvent. HPLC was performed on a Waters 2690 Aliance chromatograph with a photodiode array detector Waters 996 (detection at 290 and 370 nm wavelength) using an analytical HPLC column Waters Spherisorb 5 μm ODS2 (4.6 mm × 250 mm) at the flow rate 1 ml/min. A linear solvent gradient from 40 to 60% aq. MeCN containing 1% HCOOH over 40 min was used. The NMR spectra (^{1}H NMR, ^{13}C NMR, DEPT 135°, COSY, HMQC, HMBC) were recorded at 600 MHz on a DRX 600 Bruker Avance instrument in DMSO-d_6. Optical rotation was measured on an Autopol IV Rudolph polarimeter, in methanol solutions. UV spectra were recorded on a Visible Spectrophotometer Cintra 20, GBC, in methanol solutions. CD spectrum was obtained on a Jasco J-715 CD/ORD spectropolarimeter in methanol. Positive-ion ESI-MS spectra were taken on a Bruker micrOTOF-Q spectrometer.

2.4. Chemicals

2.4.1. Xanthohumol (3′-(3,3-dimethylallyl)-2′,4′,4-trihydroxy-6′-methoxychalcone)

Xanthohumol was isolated from supercritical carbon dioxide extracted hops ("Marynka", crop 2005), obtained from Production of Hop Extracts of Fertilizer Institute, Puławy, Poland. Spent hops were immersed in ethyl acetate, sonificated and extracted. The extract was filtered, the solvent evaporated off and the residue chromatographed over Sephadex LH-20 using methanol as eluent. The fractions containing xanthohumol were collected, evaporated in vacuo and purified by repeated column chromatography on silica gel using chloroform:methanol (9:1, v/v) as eluent to give pure xanthohumol as yellow-orange crystals.

The method of isolation was reported by Stevens et al. [15]. Xanthohumol obtained by us was identical with the standard (Alexis Biochemicals, Switzerland).

2.4.2. Isoxanthohumol (1) (5-methoxy-8-prenylnaringenin)

Isoxanthohumol (**1**) was obtained by chemical conversion of xanthohumol.

Xanthohumol was dissolved in 1% aqueous sodium hydroxide (ca. 15 mg/ml) and stirred at 0 °C until the substrate was consumed. Then formic acid was added (50 μl per ml of sodium hydroxide). The reaction mixture was extracted with ethyl acetate and washed with water. The extract was dried, the solvent evaporated off, and the residue was separated by column chromatography on silica gel (0.05–0.2, Merck), using chloroform:methanol (9:1, v/v) as eluent.

The spectral characteristic of the obtained substance was in agreement with literature date for isoxanthohumol [15].

3. Results and discussion

Although prenylated flavonoids occur in a variety of plant species, little is known about the metabolism of this class of molecules. Isoxanthohumol is the major prenylflavonoid in beer, which is the most important dietary source of this compound.

Our study focused on checking whether isoxanthohumol (**1**) may be transformed by fungi and on identification of the metabolites of these processes. 44 fungi cultures were screened for potential isoxanthohumol (**1**) transformation activity. Several microorganisms showed the capability of transforming isoxanthohumol. *A. glauca* AM177, *B. bassiana* AM278, and *F. equiseti* AM15 were selected for scale-up studies, because they gave the products with the highest efficiency compared to the other fungi.

3.1. Transformation of isoxanthohumol (1) by A. glauca AM177

Incubation of the microorganism with isoxanthohumol (**1**) for 7 days (pH of the culture 3.7) led to product **2** (62.9 mg isolated, 61.6% yield). UV spectrum of **2** showed maximum absorption bands at 236 and 283 nm, similar to that of **1** (237 and 289 nm, respectively), which indicated that it was a flavanone derivative. The ^{1}H NMR spectrum of product **2** showed seven new characteristic signals typical for a sugar moiety, with chemical shifts between 4.95 and 3.10 ppm. The ^{13}C NMR spectrum exhibited six carbon signals from 100.8 to 61.3 ppm, which were not observed in substrate (**1**). The location of glucose was confirmed by the absence of the signal of 7-hydroxyl proton present in the ^{1}H NMR spectrum of **2**, and by HMBC correlation between H-1‴ and C-7. The low-field shift of H-6 in the ^{1}H NMR spectrum of product **2** (δ 6.46)

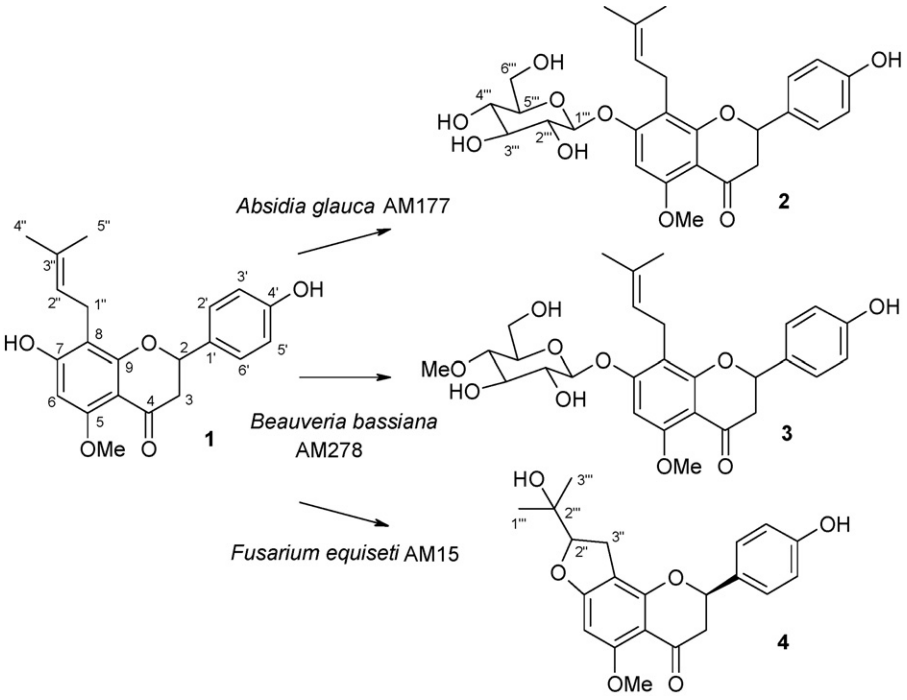

Fig. 1. Transformation of isoxanthohumol (**1**) by the selected fungi.

compared to isoxanthohumol **1** (δ 6.14) additionally confirmed substitution of the 7-hydroxyl group. On the basis of the above spectral analysis metabolite **2** was identified as isoxanthohumol 7-*O*-β-D-glucopyranoside (Fig. 1). The spectral data are in accordance with the literature [16].

^{1}H NMR δ: 9.53 (1H, br. s, 4′-OH), 7.28 (2H, d, J = 8.6 Hz, H-2′,6′), 6.77 (2H, d, J = 8.5 Hz, H-3′,5′), 6.46 (1H, s, H-6), 5.35 (1H, m, H-2), 5.12 (1H, t, J = 7.0 Hz, H-2″), 4.95 (1H, d, J = 7.6 Hz, H-1‴), 3.73 (1H, m, H-6‴), 3.76 (3H, s, 5-OMe), 3.45 (1H, m, H-5‴), 3.44 (1H, m, H-6‴), 3.37 (1H, m, H-1″), 3.36 (1H, m, H-3‴), 3.32 (1H, m, H-2‴), 3.10 (1H, m, H-4‴), 3.10 (1H, m, H-1″), 2.97 (1H, m, H-3ax), 2.62 (1H, m, H-3eq), 1.57 (3H, s, H-4″), 1.54 (3H, s, H-5″);

^{13}C NMR δ: 189.8 (C-4), 161.1 (C-7), 160.1 (C-9), 159.0 (C-5), 157.9 (C-4′), 130.7 (C-3″), 129.9 (C-1′), 128.3 (C-2′,6′), 123.2 (C-2″), 115.5 (C-3′,5′), 110.7 (C-8), 106.7 (C-10), 100.8 (C-1‴), 93.0 (C-6), 78.5 (C-2), 78.0 (C-5‴), 77.4 (C-3‴), 73.8 (C-2‴), 70.6 (C-4‴), 61.3 (C-6‴), 56.0 (5-OMe), 45.0 (C-3), 26.0 (C-5″), 22.1 (C-1″), 18.0 (C-4″).

3.2. Transformation of isoxanthohumol (1) by B. bassiana AM278

Fermentation was carried out for 12 days (pH of the culture 8.4) and 52.6 mg of product **3** was obtained (50.2% yield). The UV spectrum of **3** showed maximum absorption bands at 236 and 283 nm and an inflection at 328 nm, which suggested that it was a flavanone derivative, like metabolite **2**. Seven proton signals and six carbon ones corresponding to a sugar were observed in the regions from 4.97 to 2.98 ppm in the ^{1}HNMR and from 100.5 to 60.9 ppm in the ^{13}CNMR spectra (details below). HMQC, HMBC and COSY experiments allowed establishing unambiguously the sugar moiety structure. The methyl carbon (C-4‴-O-*C*H$_3$) resonating at δ 60.2 showed a one-bond correlation with three protons at δ 3.45 on HMQC spectrum. These protons showed also three-bond correlation with methine carbon at δ 76.4 (C4‴) on HMBC spectrum. Compound **3** showed an $[M+H]^+$ peak at m/z 531 in ESI-MS, which is in agreement with formula $C_{28}O_{10}H_{34}$. These spectral data allow identifying metabolite **3** as isoxanthohumol 7-*O*-β-D-4‴-methoxyglucopyranoside (Fig. 1). To the best of our knowledge, this compound has never been reported before.

^{1}H NMR δ: 9.55 (1H, br. s, 4′-OH), 7.29 (2H, d, J = 8.5 Hz, H-2′,6′), 6.77 (2H, d, J = 8.4 Hz, H-3′,5′), 6.43 (1H, s, H-6), 5.35 (1H, dd, J = 12.2, 2.9 Hz, H-2), 5.12 (1H, t, J = 7.2 Hz, H-2″), 4.97 (1H, d, J = 7.8 Hz, H-1‴), 3.76 (3H, s, 5-OMe), 3.66 (1H, m, H-6‴), 3.49 (1H, m, H-6‴), 3.48 (1H, m, H-4‴), 3.45 (3H, s, 4‴-OMe), 3.43 (1H, m, H-3‴), 3.30 (1H, m, H-2‴), 3.09 (1H, m, H-1″), 2.98 (1H, m, H-3ax), 2.98 (1H, m, H-5‴), 2.63 (1H, m, H-3eq), 1.58 (3H, s, H-4″), 1.55 (3H, s, H-5″);
^{13}C NMR δ: 189.3 (C-4), 161.1 (C-7), 160.1 (C-9), 160.0 (C-5), 157.9 (C-4′), 130.6 (C-3″), 129.9 (C-1′), 128.3 (C-2′,6′), 123.2 (C-2″), 115.5 (C-3′,5′), 110.3 (C-8), 106.6 (C-10), 100.5 (C-1‴), 93.0 (C-6), 79.9 (C-5‴), 78.5 (C-2), 77.0 (C-3‴), 76.4 (C-4‴), 74.0 (C-2‴), 60.9 (C-6‴), 60.2 (4‴-OMe), 56.0 (5-OMe), 45.2 (C-3), 26.0 (C-5″), 22.2 (C-1″), 18.1 (C-4″).

ESI-MS m/z: 531 $[M+H]^+$.

Glucuronidation and methylation of phenolic groups are the main pathways of the Phase II detoxification processes for most xenobiotics in mammals. Glucuronidation of a prenylated flavonoid by rat and human liver microsomes was first described by Yilmazer et al. [17]. However, glycosylation of flavonoids is rare in microbial systems and the reports are limited. Glucopiranosides with methoxyl group at C-4 in glucose moiety are typical products of flavonoids transformation for the species *B. bassiana* [18–20]. It is noteworthy that all these transformations in *B. bassiana* AM278 culture proceeded regioselectively.

3.3. Transformation of isoxanthohumol (1) by F. equiseti AM15

Incubation of the strain with substrate (**1**) was performed for 5 days (pH of the culture 5.9) and 29.2 mg of product **4** was isolated (41.7% yield). The UV spectrum of **4** showed maximum absorption bands at 240 and 292 nm, suggesting flavanone structure.

The major differences in the ^{1}H NMR spectra of metabolite **4** and substrate **1** include disappearance of signal of 7-hydroxyl group proton and the up-field shift of H-2″ signal (from δ 5.10 for **1** to δ 4.67 for **4**). The presence of a –CH(O)CH$_2$– spin system in the dihydrofurano ring system in product **4** was confirmed by COSY couplings between signals at δ 4.67 (H-2″) and δ 3.05 (H-3″).

Formation of product **4** can be rationalized by an initial epoxidation of the prenyl group, followed by an intramolecular attack of the neighboring hydroxyl group. In this case ring closure leads to a five-member ring. This metabolic pathway was found to be the major route for prenyl group metabolism in xanthohumol by human liver microsomes [21]. The cyclization process leads to the formation of a new chiral center at C-2″, with unidentified absolute configuration. The absolute configuration of C-2 was established by CD analysis. The CD spectrum showed a high amplitude positive Cotton effect in the 280–300 nm region ($[\theta]_{289}$ 14.2) and a weak negative Cotton effect near 330 nm ($[\theta]_{332}$ − 0.3), which allowed an assignment of *R*-configuration at the C-2 stereocenter [22]. On the basis of the spectral data product **4** was identified as (2*R*)-2″-(2‴-hydroxyisopropyl)-dihydrofurano[2″,3″:7,8]-4′-hydroxy-5-methoxyflavanone (**4**) (Fig. 1).

^{1}H NMR δ: 9.58 (1H, s, 4′-OH), 7.30 (2H, d, J = 8.5 Hz, H-2′,6′), 6.78 (2H, d, J = 8.4 Hz, H-3′,5′), 6.20 (1H, s, H-6), 5.40 (1H, dd, J = 12.0, 1.6 Hz, H-2), 4.67 (1H, m, H-2″), 4.66 (1H, *s*, 2‴-OH), 4.65 (1H, s, 2‴-OH), 3.74 (3H, s, 5-OMe), 3.05 (1H, m, H-3″), 3.00 (1H, m, H-3ax), 2.58 (1H, m, H-3eq), 1.13 (3H, s, H-1‴), 1.10 (3H, s, H-3‴); ^{13}C NMR δ: 188.0 (C-4), 166.8 (C-7), 162.9 (C-5), 158.9 (C-9), 158.0 (C-4′), 129.7 (C-1′), 128.5 (C-2′,6′), 115.6 (C-3′,5′), 105.7 (C-8), 105.6 (C-10), 91.6 (C-2″), 88.3 (C-6), 78.5 (C-2), 70.4 (C-2‴), 56.4 (5-OMe), 45.1 (C-3), 27.0 (C-3″), 26.2 (C-1‴), 25.2 (C-3‴). ESI-MS m/z: 371 $[M+H]^+$. $[\alpha]_D$: 38.0.

The data obtained for **4** correspond closely (with exclusion of the specific rotation) to those described in the literature for the metabolite that was obtained from xanthohumol in *Pichia membranifaciens* culture [23]. The product was identified as (2*S*)-2″-(2‴-hydroxyisopropyl)-dihydrofurano[2″,3″:7,8]-4′-hydroxy-5-methoxyflavanone and it showed antimalarial activity against *Plasmodium falciparum* [24].

4. Conclusion

In the present study we found that many of the tested fungi cultures are capable of effective transformation of isoxanthohumol (**1**). A novel methoxyglucosylated metabolite of isoxanthohumol (**1**) was described. To the best of our knowledge, biotransformation of **1** by fungi has never been reported before, so these results are a noteworthy contribution to research in this area.

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Ewa Huszcza was born in 1966 in Wrocław, Poland, studied biotechnology at the Technical University of Wrocław and obtained her PhD in 1996. Since 1996 she has been at Department of the Wrocław University of Environmental and Life Sciences. Her research is focused on the biotransformation of various biological active compounds.

Tomasz Tronina was born in 1983 in Oleśnica, Poland. He studied biotechnology at the Wrocław University of Environmental and Life Sciences and received his Diploma in 2007. His main research interest includes the biotransformation of active compounds from natural sources.