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Microbial Transformation of Isoxanthohumol, a Hop Prenylflavonoid

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Abstract – Microbial transformation of isoxanthohumol (**1**), a prenylated flavanone from hops, has resulted in the production of a pair of glucosylated derivatives. The structures of these compounds were elucidated to be (2*S*)-5-methoxy-8-prenylnaringenin 7-*O*-β-D-glucopyranoside (**2**) and (2*R*)-5-methoxy-8-prenylnaringenin 7-*O*-β-D-glucopyranoside (**3**) based on the spectroscopic analyses.
Keywords – Microbial transformation, hop prenylflavonoid, isoxanthohumol, 5-methoxy-8-prenylnaringenin 7-*O*-β-D-glucopyranoside

Introduction

Isoxanthohumol (**1**) (5-methoxy-8-prenylnaringenin) is a well known prenylated flavanone, together with a major prenylated chalcone xanthohumol, contained in the female inflorescences of *Humulus lupulus* L. (hops) (Cannabaceae), which are added to brewing process of beer (Stevens, *et al*., 1997; Stevens and Page, 2004). This flavanone has been regarded as the main cyclized product of xanthohumol in hop preparation and brewing process (Stevens, *et al*., 1999a; Stevens, *et al*., 1999b). It has been described that isoxanthohumol exhibited moderate estrogenic activity (Milligan, *et al*., 1999), antiproliferative activity in human cancer cells (Miranda, *et al*., 1999; Delmulle, *et al*., 2006) and cancer chemoprevention properties (Gerhäuser, *et al*., 2002). Although isoxanthohumol has been one of the major prenylated flavonoids in hops and beer, few metabolism studies have been performed to identify the metabolic fate of isoxanthohumol in mammalian metabolism. Also, some metabolism studies have focused on the conversion of isoxanthohumol to 8-prenylnaringenin, the potent phytoestrogen (Possemiers, *et al*., 2005; Possemiers, *et al*., 2006). The oxidative metabolism study *in vitro* using human liver microsomes provided several metabolites, which were identified as *cis*- and *trans*-hydroxyisopentenylated alcohols or oxidized aldehyde at the methyl groups of isoxanthohumol, *O*-demethylated isoxanthohumol, hydroxymethylbutenyl derivatives at isopentenyl group and hydroxylated or oxidative derivatives in the B-ring by liquid chromatography-tandem mass spectrometry (Nikolic, *et al*., 2005). Microbial transformation studies are also known as useful tools to mimic and predict mammalian metabolism for the better understanding of xenobiotic metabolism (Clark, *et al*., 1985; Venisetty and Cidii, 2003). We previously reported several metabolites of xanthohumol and 8-prenylnaringenin from hops, which were produced by microbial transformation method (Kim and Lee, 2006; Kim, *et al*., 2008). In an ongoing metabolism study of prenylflavonoids from hops, a preparative-scale transformation of isoxanthohumol (**1**) by fungus *Mucor hiemalis* KCTC 6165 gave a pair of glucosylated metabolites including a novel compound. We describe production and structure elucidation of these metabolites (**2-3**) by microbial transformation herein.

Experimental

General experimental Procedures – Optical rotations were recorded with a Jasco DIP 1000 digital polarimeter, and CD spectra at 20 ºC were measured on a Jasco J-810 spectrometer. UV spectra were recorded on a Jasco V-530 spectrophotometer, and IR spectra were obtained on a Jasco FT/IR 300-E spectrometer. NMR experiments were recorded using a Varian Unity INOVA 500. ESIMS and HRESIMS were determined on a Micromass QTQF2 and Waters Synapt HDMS LC-MS spectrometers, respectively.

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TLC was carried out on Merck silica gel F_{254}-precoated glass plates and RP-18 F_{254S} plates. Medium pressure liquid chromatography (MPLC) was carried out using silica gel (40 - 63 μm, Merck). HPLC was performed on a Hewlett-Packard 1100 series composed of a degasser, a binary mixing pump, a column oven and a DAD detector using Agilent Zorbax Eclipse XDB-C18 column (4.6 × 250 mm, 5 μm) with acetonitrile (solvent A) and water containing 0.1% formic acid (solvent B) at the flow rate of 1.0 mL/min.

Chemicals and Ingredients – Isoxanthohumol was prepared by chemical cyclization in aqueous NaOH solution at 0 °C as described by Stevens and colleagues (Stevens, *et al*., 1999b), and microbial transformation method by the fungus *R. oryzae* KCTC 6946 as previous reported by Kim and Lee (Kim and Lee, 2006). Produced isoxanthohumol by both was extracted with EtOAc and then purified with chromatographic method including silica gel and reversed C_{18} MPLC. The spectroscopic data of isoxanthohumol were in good agreement with data in the literature (Stevens, *et al*., 1997) and its structure was also confirmed by 2D NMR experiments. Optical rotation and CD spectrum exhibited the substrate isoxanthohumol was a racemic mixture of (2*S*)- and (2*R*)-isoxanthohumol. Ingredients for media including D-glucose, peptone, malt extract, yeast extract, and potato dextrose medium were purchased from Becton, Dickinson and Co. and sucrose was purchased from Sigma-Aldrich Co.

Microorganisms and Fermentation – All of the microorganisms were obtained from the Korean Collection for Type Cultures (KCTC). The cultures used for preliminary screening were as follows (Kim, *et al*., 2008): *Absidia spinosa* KCTC 6588, *Alternaria alternata* 6005, *Aspergillus fumigatus* 6145, *Aspergillus niger* 6910, *Benisingtonia intermedia* 7207, *Candida albicans* 7965, *Candida famata* 7000, *Candida solani 7689, Cunninghamella elegans* var. *elegans* 6992, *Curvularia lunata* var. *lunata* 6919, *Debaryomyces hansenii* var. *hansenii* 7645, *Debaryomyces occidentalis* var. *occidentalis* 7194, *Debaryomyces robertsiae* 7299, *Filobasidium capsuligenum* 7102, *Filobasidium neoformans* 7902, *Fusarium oxysporum* f.sp. *lini* 16325, *Gliocladium deliquescens* 6173, *Hormoconis resinae* 6966, *Kluyveromyces marxianus* 7155, *Metarhizium flavoviride* var. *minus* 6310, *Metschnikowia pulcherrima* 7605, *Microbacterium lacticum* 9230, *Mortierella ramanniana* var. *angulispora* 6137, *Monascus rubber* 6122, *Mucor hiemalis* 6165, *Mycobacterium phlei* 3037, *Penicillium chrysogenum* 6933, *Pichia membranifaciens* 7006, *Pichia pastoris* 7190, *Polyporus arcularius* 6341, *Rhizopus oryzae* 6399, *Rhizopus oryzae* 6946, *Rhodotorula rubra* 7909, *Saccharomyces cerevisiae* 7904, *Saccharomycodes ludwigii* 7126, *Torulaspora delbrueckii* 7116, *Tremella mesenterica* 7131, *Trichoderma koningii* 6042, *Trichophyton mentagrophytes* 6085, *Trigonopsis variabilis* 7263, *Zygosaccharomyces rouxii* 7191. Fermentation experiments were performed in three types of media; *F. oxysporum* f.sp. *lini*, *C. lunata* var. *lunata*, *R. oryzae* (KCTC 6399) and *C. elegans* var. *elegans* were cultured on potato dextrose medium (24 g/L). *A. niger* was cultured on malt medium (Blekeslee's formula; malt 20 g/L, D-glucose 20 g/L, peptone 1 g/L). *M. hiemalis* was incubated on potato sucrose medium (potato dextrose 24 g/L and sucrose 20 g/L). *A. alternata* and *P. membranifaciens* were incubated on malt medium (malt extract 20 g/L and peptone 5 g/L). Other microorganisms were cultured on yeast-malt medium (D-glucose 10 g/L, peptone 5 g/L, malt extract 3 g/L, and yeast extract 3 g/L).

Metabolism Screening Procedure – Microbial cultures for microbial transformation studies were grown according to the two-stage procedure (Clark, *et al*., 1985). In the screening studies, the actively growing microbial cultures were inoculated in 100 mL flasks containing 20 mL of media, and incubated with gentle agitation (200 rpm) at 25 °C in a temperature-controlled shaking incubator. The ethanolic solution (2 mg/0.1 mL) of **1** was added to each flask 24 h after inoculation, and further incubated at the same condition for 3 days. Sampling and TLC monitoring were generally performed on RP-18 TLC_{254S} on 60% MeOH at 24 h intervals. UV light (254 and 365 nm) and anisaldehyde-sulfuric acid reagent was used for identification of metabolites on TLC. Substrate controls consisted of **1** and sterile medium incubated without microorganisms, and culture controls consisted of fermentation cultures in which the microorganisms were grown without addition of substrate **1**.

Microbial transformation of 1 by *Mucor hiemalis* KCTC 6165 – Preparative-scale fermentations were performed with 1 L flasks each containing 250 mL medium and 20 mg isoxanthohumol (**1**) for 10 d under the same conditions. The cultures were extracted with 1 L of EtOAc two times and the organic layers were combined and concentrated at reduced pressure. The EtOAc extract (420 mg) was subjected to silica gel (40 - 63 μm) column chromatography with a $CHCl_3$-MeOH (8 : 1) solvent system to give a pair of metabolites **2** and **3** (68.3 mg, 58.5 % yield). An aliquot of compounds (3.4 mg) was further chromatographed by HPLC with a gradient solvent system of 20% A to 30% A for 25 min to afford two isomers **2** (1.1 mg, t_R 20.51 min) and **3** (1.0

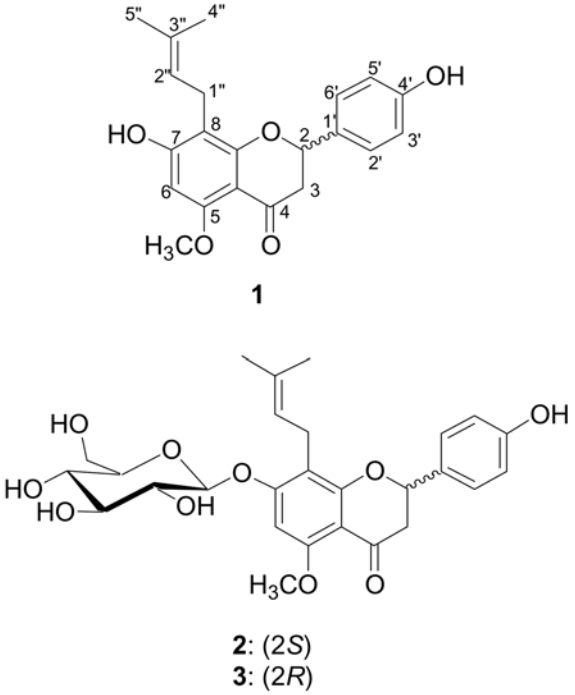

Fig. 1. Chemical structures of isoxanthohumol (**1**) and its metabolites (**2** - **3**).

mg, t_R 21.16 min).

(2*S*)-5-methoxy-8-prenylnaringenin 7-*O*-β-D-glucopyranoside (2): white amorphous powder; $[\alpha]_D$ –41.6 (*c* 0.2, MeOH); UV λ_{max} (MeOH) 222, 282, 327 nm; CD (*c* 0.00039, MeOH) 286 (–8.6), 324 (0.0), 335 (+2.2); IR (KBr) ν_{max} 3421, 2922, 1652, 1601, 1520, 1345, 1279, 1099 cm^{-1}; ^1H NMR (DMSO-d_6, 500 MHz) δ 9.84 (1H, s, 4'-O*H*), 7.29 (2H, d, *J* = 8.5 Hz, H-2',6'), 6.78 (2H, d, *J* = 8.5 Hz, H-3',5'), 6.47 (1H, s, H-6), 5.35 (1H, dd, *J* = 12.5, 3.0 Hz, H-2), 5.13 (1H, brt, *J* = 7.3 Hz, H-2"), 4.94 (1H, d, *J* = 8.0 Hz, H-1'"), 3.77 (3H, s, 5-OC*H*$_3$), 3.73 (1H, m, H-6'"a), 3.42 (1H, m, H-6'"b), 3.41 (1H, m, H-5'"), 3.33 (1H, m, H-1"a), 3.30 (1H, m, C-3'"), 3.30 (1H, m, C-2'"), 3.10 (1H, m, C-4'"), 3.09 (1H, dd, *J* = 13.5, 6.5 Hz, H-1"b), 2.99 (1H, dd, *J* = 16.5, 12.3 Hz, H-3a), 2.62 (1H, dd, *J* = 16.5, 3.0 Hz, H-3b), 1.58 (3H, s, H-5"), 1.55 (3H, s, H-4"); ^{13}C NMR (DMSO-d_6, 125 MHz) δ 188.7 (C-4), 160.7 (C-7), 160.5 (C-8a), 159.6 (C-5), 157.4 (C-4'), 130.1 (C-3"), 129.4 (C-1'), 127.8 (C-2',6'), 122.7 (C-2") 115.1 (C-3',5'), 109.8 (C-8), 106.1 (C-4a), 100.3 (C-1'"), 92.6 (C-6), 78.0 (C-2), 77.5 (C-5'"), 76.9 (C-3'"), 73.3 (C-2'"), 70.1 (C-4'"), 60.9 (C-6'"), 55.5 (5-OCH_3), 44.8 (C-3), 25.6 (C-5"), 21.7 (C-1"), 17.6 (C-4"); ESIMS *m/z* 517 $[M + H]^+$.

(2*R*)-5-methoxy-8-prenylnaringenin 7-*O*-β-D-glucopyranoside (3): yellow amorphous powder; $[\alpha]_D$ +11.0 (*c* 0.2, MeOH); UV λ_{max} (MeOH) (log ε) 223 (4.46), 282 (4.21), 324 (3.70) nm; CD (*c* 0.00039, MeOH) 287 (+9.8), 317 (0.0), 338 (–6.8); IR (KBr) ν_{max} 3422, 2920, 1652, 1599, 1350, 1277, 1094 cm^{-1}; ^1H NMR (CD_3OD, 500 MHz) δ 7.31 (2H, d, *J* = 8.8 Hz, H-2',6'), 6.81 (2H, d, *J* = 8.8 Hz, H-3',5'), 6.55 (1H, s, H-6), 5.32 (1H, dd, *J* = 12.5, 3.0 Hz, H-2), 5.16 (1H, brt, *J* = 7.0 Hz, H-2"), 5.03 (1H, d, *J* = 7.5 Hz, H-1'"), 3.93 (1H, dd, *J* = 12.0, 2.0 Hz, H-6'"a), 3.87 (3H, s, 5-OC*H*$_3$), 3.66 (1H, *J* = 12.0, 7.0 Hz, H-6'"b), 3.59 (1H, brd, *J* = 8.0 Hz, C-3'"), 3.53 (1H, m, H-5'"), 3.53 (1H, brd, *J* = 8.0 Hz, C-2'"), 3.48 (1H, brt, *J* = 9.0 Hz, C-4'"), 3.37 (1H, m, H-1"a), 3.26 (1H, dd, dd, *J* = 13.8, 7.0 Hz, H-1"b), 3.03 (1H, dd, *J* = 16.5, 12.5 Hz, H-3a), 2.72 (1H, dd, *J* = 16.5, 3.0 Hz, H-3b), 1.60 (3H, s, H-5"), 1.56 (3H, s, H-4"); ^{13}C NMR (CD_3OD, 125 MHz) δ 193.3 (C-4), 163.3 (C-7), 163.2 (C-8a), 161.9 (C-5), 159.0 (C-4'), 132.0 (C-3"), 131.4 (C-1'), 129.1 (C-2',6'), 124.0 (C-2") 116.4 (C-3',5'), 112.6 (C-8), 107.8 (C-4a), 102.1 (C-1'"), 93.8 (C-6), 80.3 (C-2), 78.9 (C-5'"), 78.6 (C-3'"), 75.1 (C-2'"), 71.8 (C-4'"), 62.9 (C-6'"), 56.4 (5-OCH_3), 46.3 (C-3), 26.1 (C-5"), 23.0 (C-1"), 18.2 (C-4"); HRESIMS *m/z* 517.2074 $[M + H]^+$ (calcd for $C_{27}H_{33}O_{10}$, 517.2074).

Acid hydrolysis of metabolites 2 and 3 – Solutions of compounds **2** and **3** (ca. 5 mg) in 2N HCl were heated for 2 h. After cooling, each mixture was neutralized and partitioned between EtOAc and H_2O, respectively. The aqueous layer was concentrated and developed by cellulose TLC (BuOH-C_6H_6-C_6H_5N-H_2O = 5 : 1 : 3 : 3, R_f 0.17) in comparison with authentic D-glucose.

Results and Discussion

Preparation of isoxanthohumol ($C_{21}H_{22}O_5$, MW 354) (**1**) used as the substrate was preceded by both chemical cyclization in aqueous alkali solution (Stevens, *et al.*, 1999b) and enzymatic cyclization using microbial transformation method (Kim and Lee, 2006). The produced isoxanthohumol did not exhibit optical isomerism at C-2, which was confirmed by no absorption in its CD spectrum and no optical rotation. It was therefore indicated that this isoxanthohumol is a racemic mixture of (2*S*)- and (2*R*)-flavanones.

Of forty one microbial cultures screened, the fungus *Mucor hiemalis* KCTC 6165 was selected for further scale-up fermentation studies since its culture displayed metabolizing ability to transform isoxanthohumol (**1**) based on TLC analyses. The R_f value of the metabolites (a mixture of **2** and **3**: R_f 0.46) were larger on reverse phase C_{18} plates, in comparison with that of the substrate **1** (R_f 0.15), which indicated that metabolites with the

higher polarity were produced by the fungus. Separate control studies showed that these metabolites were produced as a result of enzymatic activity in fungus, not as a consequence of chemical or non-metabolic conversion.

Metabolites **2** and **3** were obtained as white and yellow amorphous powder, respectively, by HPLC separation. UV spectra of both compounds displayed maximal absorptions at ~ 223, 282, and 325 nm, which indicated that they were flavanone derivatives. In addition, their identical spectral data of UV, IR, and ESIMS ($[M + H]^+$ peak at *m/z* 517) suggested that two diastereomeric metabolites were produced by microbial transformation method. ^1H and ^{13}C NMR data of these metabolites showed aglycone signals corresponding to an isopentenyl and a flavanone moiety which are almost identical with those of **1**, except for the six typical signals of a sugar moiety in **2** and **3**. The sugar was assigned a glucopyranose based on NMR data and the R_f value comparison with authentic sample after acidic hydrolysis of **2** and **3**. The *J* values (8.0 Hz in **2** and 7.5 Hz in **3**) of the anomeric protons (H-1) at δ ~ 5.00 indicated that this sugar had a β-configuration. The glucosylated position was assigned to be C-7 by observation of HMBC correlations between H-1 (δ 4.94 in **2** and δ 5.03 in **3**) to C-7 (δ 160.7 in **2** and δ 163.3 in **3**). From these results, the structure of a pair of metabolites was established to be 5-methoxy-prenylnaringenin 7-*O*-β-D-glucopyranoside. The absolute configuration at the C-2 stereocenter was established on the basis of circular dichroism (CD) spectra. Metabolite **2** showed the positive and negative Cotton effects at 335 nm (n → π* transition) and 286 nm (π → π* transition) corresponding to 2*S*. In contrast, the C-2 stereocenter in metabolite **3** was assigned 2*R* configuration from the negative and positive Cotton effects at 338 and 287 nm (Gaffield, 1970; Slade, *et al*., 2005). Based on these results, structures of metabolites **2** and **3** were unambiguously assigned to be (2*S*)-5-methoxy-8-prenylnaringenin 7-*O*-β-D-glucopyranoside and (2*R*)-5-methoxy-8-prenylnaringenin 7-*O*-β-D-glucopyranoside, respectively.

Previous microbial metabolism studies revealed that metabolite **2** was also produced by cyclization procedure of the prenylated chalcone xanthohumol with the fungus *Cunninghamella elegans* var. *elegans* KCTC 6992 (Kim and Lee, 2006). However, the presence of compound **3** has not been reported yet in the literatures.

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