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Microbial transformation of xanthohumol

Wimal H.M.W. Herath[a], Daneel Ferreira[a], Ikhlas A. Khan[a,b,*]

[a]*National Center for Natural Products Research, Research Institute of Pharmaceutical Sciences, The University of Mississippi, University, MS 38677, USA*

[b]*Department of Pharmacognosy, School of Pharmacy, The University of Mississippi, University, MS 38677, USA*

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Abstract

Microbial transformation of xanthohumol using the culture broth of *Pichia membranifaciens* afforded three metabolites, (*E*)-2″-(2‴-hydroxyisopropyl)-dihydrofurano[2″,3″:4′,3′]-2′, 4-dihydroxy-6′-methoxychalcone, (2*S*)-2″-(2‴-hydroxyisopropyl)-dihydrofurano[2″,3″:7,8]-4′-hydroxy-5-methoxyflavanone and (*E*)-2″-(2‴-hydroxyisopropyl)-dihydrofurano[2″,3″:2′,3′]-4′-hydroxy-5-methoxychalcone.

Keywords: Xanthohumol; Microbial metabolites; Chalcone; Flavanone; Prenyl; NMR

1. Introduction

Xanthohumol **1**, a chalcone type prenylflavanoid, is a constituent of beer and some dietary supplements. It is the major component of the glandular exudates of the female inflorescences (hops) of *Humulus lupulus* L. (Cannabinaceae). Hops are used in the brewing industry to provide aroma and bitterness to beer. The α-acids of hops undergo thermal isomerization in the brew kettle to give the iso-acids, the bitter principles of beer. The brewing process also facilitates the conversion of xanthohumol **1** and other minor prenylchalones in hops into the corresponding isomeric prenylflavanones (Stevens et al., 1999). Studies have revealed that **1** is an antiproliferative agent in ovarian, colon and breast cancer cells (Miranda et al., 1999). Its ability to inhibit CYP enzymes (Henderson et al., 2000) and to induce quinone reductase has suggested that **1** may have cancer-chemopreventive properties (Miranda et al., 2000). Xanthohumol has also been patented as a drug for osteoporosis treatment (US Patent, 1997). Investigation of the metabolism of **1** by rat liver microsomes (Yilmazer et al., 2001a), has resulted in the isolation of three polar metabolites. Two of these have been tentatively identified as hydroxylated isopropyldihydrofurano derivatives of **1** and the third as 2- or 3-hydroxyxanthohumol. In addition to the three polar metabolites, the non-polar dehydrocycloxanthohumol has also been obtained by treating **1** with liver microsomes from isosafrole- and β-naphthoflavone-pretreated rats. In another study it has been demonstrated that **1** produces glucuronides by liver microsomes from human or rat (Yilmazer et al., 2001b).

The use of microbial models to mimic mammalian metabolism is well known (Rosazza and Duffel, 1986; Clark et al., 1985). The present work was an attempt to generate mammalian metabolites of xanthohumol **1** with fungal models.

2. Results and discussion

Twenty microorganisms were used in a standard two-stage procedure (Abourashed and Khan, 2000) to identify organisms capable of metabolizing xanthohumol (**1**). TLC indicated that several organisms were capable of transforming **1**. The fungus, *Pichia membranifaciens* (ATCC 2254) was selected for scale-up studies as it showed higher transformational efficiency. The metabolites formed were, (*E*)-2″-(2‴-hydroxyisopropyl)-dihydrofurano[2″,3″:4′,3′]-2′, 4-dihydroxy-6′-methoxychalcone (**2**), (2*S*)-2″-(2‴-hydroxyisopropyl)-dihydrofurano[2″,3″:7,8]-4′-hydroxy-5-methoxyflavanone (**3**), and (*E*)-2″-(2‴-hydroxyisopropyl)-dihydrofurano [2″,3″:2′,3′]-4′-hydroxy-6′-

* Corresponding author. Tel.: +1-91-662-915-7821; fax: +1-91-662-915-7989.

E-mail address: rikhan@olemiss.edu (I.A. Khan).

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methoxychalcone (**4**) (Fig. 1). High-resolution electrospray ionization mass spectrometric data (HR–ESI–MS) of the metabolites suggested a molecular formula of $C_{21}H_{22}O_6$ for each indicating that they were monoxygenated derivatives of **1**. The UV spectra of **2** and **4** were similar to that of xanthohumol **1**, showing maximum absorption at 368 and 360 nm, respectively (Markham, 1982).

The ^1H and ^{13}C NMR spectral data of **2** and **4** (Table 1) showed close resemblance to those of **1**. The aromatic A ring proton H-5′ of **2** appeared as a singlet at δ 6.01 and those of the B ring, H-2/H-6 and H-3/H-5 were observed as doublets at δ 7.49 and δ 6.86 ($J = 8.4$ Hz). The two olefinic protons, H-α and H-β resonated as a second order singlet at δ 7.75 with the OMe singlet at δ 3.91. These data suggested oxygenation at the prenyl substituent of xanthohumol. The presence of ^1H–^1H COSY couplings between the signals at δ 4.77 (H-2″) and δ 3.13 (H-3″) in the spectrum of **2**, indicated a–CH(O)–CH_2– spin system fixed in a dihydrofurano ring system (Yilmazer et al., 2001a). The long-range (COSY and HMBC) ^1H and ^{13}C NMR correlations were in complete agreement with structure **2**. The hydrogen bonded C-2′ hydroxylic proton resonated at δ 14.42. Compound **2** was isolated from the mycelium of *P. membranifaciens* and it was comparable to one of the four metabolites formed by incubating xanthohumol **1** with rat liver microsomes (Yilmazer et al., 2001a).

The NMR data of the metabolite, (*E*)-2″-(2‴-hydroxyisopropyl)-dihydrofurano[2″,3″:2′,3′]-4′-hydroxy-5-methoxychalcone **4** showed no low-field hydrogen-bonded hydroxylic proton and spin systems and chemical shifts of the dihydrofurano moiety very similar to those of compound **2**. This suggested spontaneous nucleophilic attack at C-2″ by the C-2′ phenolic group (Fig. 2, route b) (Yilmazer et al., 2001a) to form the [2″,3″:2′,3′]-bicyclic ring system. Such a preference for cyclization at C-2″ presumably stems from the considerable degree of steric hindrance at C-3″. Conspicuously, both compounds **2** and **4** exhibit optical activity, hence indicating at least a degree of stereoselectivity in the cyclization process which leads to the creation of the C-2″ stereocenter with unidentified absolute configuration.

The metabolite, (2*S*)-2″-(2‴-hydroxyisopropyl)-dihydrofurano-[2″,3″:7,8]-4′-hydroxy-5-methoxyflavanone (**3**) recorded a maximum absorption at 290 nm and an inflection at 320 nm suggesting it to be a flavanone (Markham, 1982). In the ^1H NMR spectrum the B ring protons, H-2′/H-6′ and H-3′/H-5′ appeared as doublets at δ 7.21 and δ 6.84 ($J = 8.4$Hz). The presence of a –CH(O)–CH_2– spin system in a dihydrofurano ring system in **3** was also evident by the ^1H–^1H COSY couplings between signals at δ 4.68 (H-2″, *dd*, $J = 8.4$, 14.0 Hz) and δ 3.00 (H-3″, *m*, overlapped by H-3) in the

Table 1
^1H and ^{13}CNMR spectroscopic data of compounds **2**, **4**

Position	**2**		**4**	
	δ_H (J Hz)	δ_C	δ_H (J Hz)	δ_C
α	7.75 *s*	125.43	7.05 (*d*, 15.7)	125.56
β	7.75 *s*	142.79	7.52 (*d*, 15.7)	143.68
1	–	128.59	–	127.23
2,6	7.49 (*d*, 8.4)	130.66	7.40 (*d*, 8.4)	130.60
3,5	6.86 (*d*, 8.4)	116.34	6.79 (*d*, 8.4)	116.20
4	–	158.33	–	159.63
1′	–	107.08	–	106.51
2′	–	162.70	–	156.51
3′	–	106.05	–	106.51
4′	–	167.01	–	161.73
5′	6.01 *s*	86.35	5.93 *s*	92.57
6′	–	164.22	–	160.30
3″	3.05 (*dd*, 15.1, 8.1) 3.13 (dd, 15.1, 8.1)	27.59	3.00 *m*	27.22
2″	4.77 (*t*, 8.5)	92.18	4.59 (*t*, 8.9)	91.66
1‴	1.37 *s*	26.23	1.30 *s*	26.42
2‴	–	72.66	–	71.66
3‴	1.26 *s*	24.22	1.12 *s*	24.13
6′-OMe	3.91 *s*	56.43	3.72 *s*	56.32
2′-OH	14.42	–	–	–

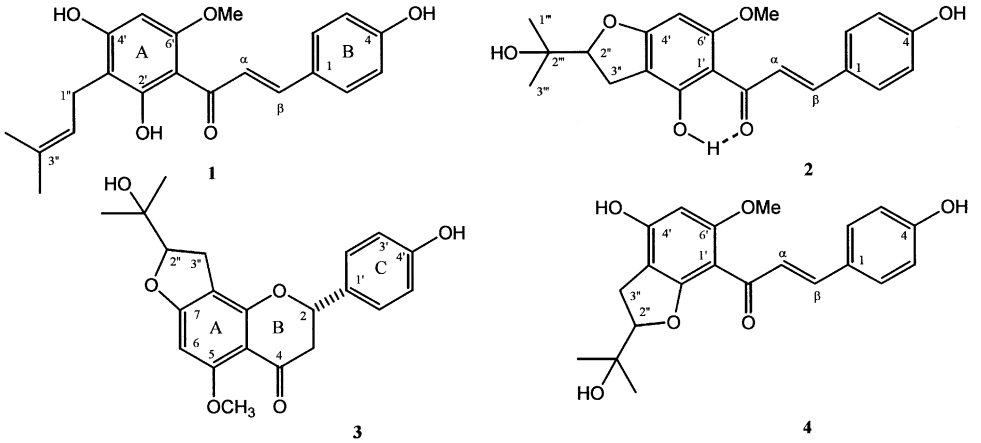

Fig. 1. Microbial metabolites from xanthohumol, **1**.

NMR spectrum (Yilmazer et al., 2001a). The major differences in the NMR spectra of **3** as compared to **1**, were the disappearance of signals due to the olefinic H-α and H-β protons and the appearance of signals at δ 5.26 (H-2, *dd*, $J=1.8$, 8.2 Hz), 2.70 (H-3, *br. d*, $J=16.2$Hz) and 2.93 (H-3, m, overlapped by H-3″), typical of the C-ring protons of flavanones. The high amplitude negative Cotton effect in the 290–300 nm region and the weak positive Cotton effect in the 324–328 region allowed the assignment of (*S*)-configuration at the C-2 stereocenter (Gaffield, 1970), indicating the stereospecificity of the C-ring cyclization process. Differentiation between the five-membered dihydrofurano ring system and the alternative of the six-membered 2*H*-dihydrochromeno arrangement in compounds **2**–**4** were based on comparison of ^{13}C chemical shifts with literature values (Yilmazer et al., 2001a; Tabata et al., 1997).

The present study was an attempt to generate mammalian metabolites of xanthohumol **1** which may contribute to the understanding of its biological activities, especially the cancer chemo-preventive effects. These compounds could also be used as analytical standards for the detection of the metabolites of **1** in biological fluids. In vitro studies of **1** with rat and human liver microsomes have yielded several oxygenated derivatives and glucuronides whose biological activities are yet to be determined (Yilmazer et al., 2001a,b). Glucuronidation could also play a role in reducing the biological half-life of **1** (Vermeulen, 1996). Of the oxygenated derivatives obtained in the present study, only (*E*)-2″-(2‴-hydroxyisopropyl)-dihydrofuran [2″,3″:4′,3′]-2′, 4-dihydroxy-6′-methoxychalcone (**2**) was identical to that obtained using rat liver microsomes. A detailed study of the biological activities of the bio-transformed products (Yilmazer et al., 2001a,b and present study) obtained so far would contribute to the understanding of the mechanisms of action of **1** on mammalian systems.

3. Experimental

3.1. General

The IR spectra were run in $CHCl_3$ using an ATI Mattson Genesis Series FTIR Spectrophotometer. UV spectra were run on a Hewlett Packard 8452A diode array spectrometer. Optical rotations were measured with a Jasco DIP-370 digital polarimeter and CD measurements on a Jasco J-710 instrument in MeOH. ^{1}H and ^{13}C NMR were recorded in $CDCl_3$ on a Bruker Avance DRX-500 FT spectrometer. HR-ESI-MS data were obtained using a Brüker GioApex 3.0.

3.2. Substrate

Xanthohumol was from Hops Research Council, St. Paul, Oregon.

3.3. Organisms and metabolism

The 20 microorganisms used for screening were obtained from the National Center for Natural Products Research, University of Mississippi. Fermentation experiments were carried out in medium α, consisting of dextrose, 20 g; NaCl, 5 g; K_2HPO_4, 5 g; bacto-peptone (Difco Labs), 5 g and yeast extract (Difco Labs, Detroit, MI), 5 g per liter of distilled water. Initial fermentations were conducted in 125 ml Erlenmeyer flasks containing 25 ml medium α. A two-stage fermentation procedure was adopted in all experiments (Abourashed and Khan, 2000). Xanthohumol **1** (940 mg) was added in DMF (0.5 mg/ml) to 24 h old stage II cultures. These were incubated at room temperature on a rotary shaker (New Brunswick Model G10-21) at 100 rpm for a period of 14 days. Sampling and TLC monitoring were carried out at 7-day intervals. Precoated Si gel 60 F_{254} plates

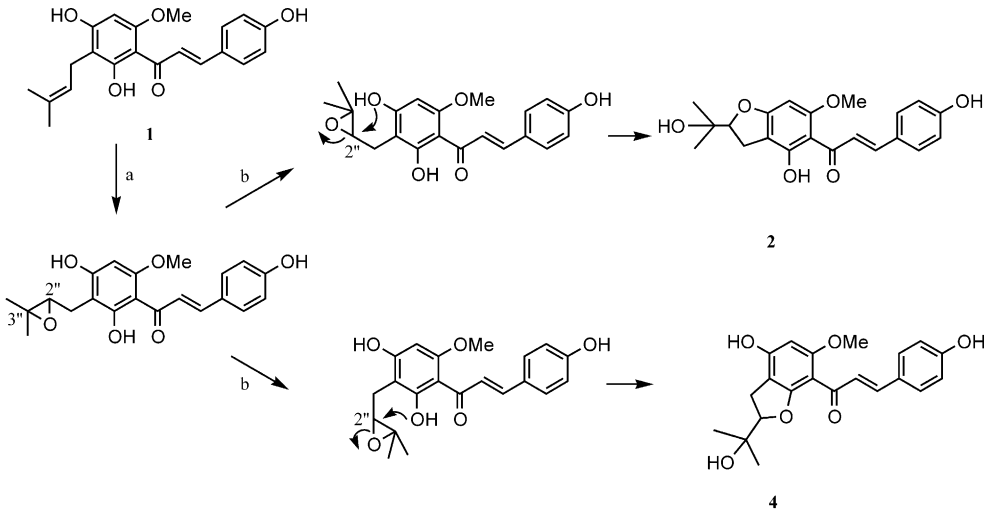

Fig. 2. Suggested pathway (Yilmazer et al., 2001a) for the formation of **2** and **4**. a, Epoxidation; b, nucleophilic cyclization.

(E. Merck) were used with $CHCl_3$:CH_3OH, (9:1) as the solvent system. UV light (254 nm) and *p*-anisaldehyde spray reagent were used to visualize the spots. Scale-up fermentations were performed under the same conditions with six 2-l flasks, containing 500 ml of medium and 150 mg of substrate, each. Extractions of the culture filtrates and residues were carried out with EtOAc. The solvent was evaporated in vacuo at 40 °C to obtain the residues. The isolations of metabolites were by column (Si gel 230–400 mesh: E. Merck) and preparative layer (Si gel 60 F_{254}) chromatography. Culture and substrate controls were run simultaneously with the above experiments (Orabi et al., 1999).

3.4. Microbial transformation of xanthohumol (*1*) by *P. membranifaciens*

The combined fermentation broth was filtered and the filtrate was exhaustively extracted with EtOAc. The yellowish brown solid obtained (260 mg) on evaporation of the solvent was subjected to column chromatography ($CHCl_3$ gradually enriched with MeOH), followed by preparative layer chromatography ($CHCl_3$:CH_3OH, 9:1). (2S)- 2″-(2‴-hydroxyisopropyl)-dihydrofurano [2″,3″:7,8]-4″5-hydroxy-5-methoxyflavanone (**3**) was isolated as a light yellow solid (30 mg, 3.3% yield); $[\alpha]_D^{26}$ −96.25 (*c* 0.0016, MeOH); R_f 0.50; UV (MeOH) λ_{max} (log ε): 216 (4.09), 242 (3.86), 290 (3.87), 320 (3.47) nm; CD (MeOH); $[\theta]_{291} = -10.13$; IR ν_{max} ($CHCl_3$) cm^{-1}: 3322, 2971, 2927, 1659, 1614, 1518, 1454, 1373, 1258, 1140, 1098; ^1H NMR (500 MHz, $CDCl_3$): δ 7.21 (2H, *d*, *J* = 8.1Hz, H-2′/H-6′), 6.84 (2H, *d*, *J* = 8.1Hz, H-3′/H-5′), 6.04 (1H, *s*, H-8), 5.26 (1H, *dd*, *J* = 1.8, 8.2 Hz, H-2), 4.68 (1H, *dd*, *J* = 8.4, 14.0 Hz, H-2″), 3.80 (3H, *s*,−OMe), 3.00 (2H, *m*, H-3″), 2.93 (1H, *m*, H-3), 2.70 (1H, *br*. *d*, *J* = 16.2Hz H-3), 1.29 (3H, *s*, Me-2‴), 1.19 (3H, *s*, Me-2‴) and ^{13}CNMR: ($CDCl_3$) δ 190.63 (C-4), 167.38 (C-7), 163.72 (C-5), 159.80 (C-8a), 157.60 (C-4′), 130.11 (C-2′/6′), 128.06 (C-1′), 116.01 (C-3′/5′), 106.02 (C-6), 92.03 (C-2″), 88.17 (C-8), 79.13 (C-2), 71.30 (C-2‴), 56.48 (5-OMe), 45.61 (C-3), 27.74 (C-3″), 25.98 (C-3‴), 24.60 (C-3‴); HR-ESI-MS *m*/*z* [M + Na]: 393.1314 (calc. for $C_{21}H_{22}O_6$ Na: 393.1314).

(*E*)-2″-(2‴-hydroxyisopropyl)-dihydrofurano [2″,3″:2′,3′]-4′-hydroxy-5-methoxychalcone (**4**) was purified as a yellow solid (5 mg, 0.55% yield), $[\alpha]_D^{26}$ −96.25 (*c* 0.0027, MeOH); R_f 0.42; ^1H and ^{13}C NMR: see Table 1. UV (MeOH) λ_{max} (log ε): 214 (3.51), 236 (3.60), 284 (3.31), 338 (3.56), 360 (3.49) nm; IR ν_{max} ($CHCl_3$) cm^{-1}: 3430, 2957, 2928, 1728, 1644, 1602, 14‘62, 1275, 1136, 1073, 742; ^1H and ^{13}C NMR: see Table 1; HR–ESI–MS *m*/*z* [M + H]: 371.1514 (calc. for $C_{21}H_{22}O_6$ H: 371.1495). $CHCl_3$ extract (112mg) of mycelium when chromatographed over silica yielded (*E*)-2″-(2‴hydroxyisopropyl)-dihydrofurano [2″,3″:4′,3′]-2′,4-dihydroxy-6′-methoxychalcone, **2** as a yellow solid (5.2 mg, 0.58% yield), $[\alpha]_D^{26}$ −96.25 (*c* 0.0035, MeOH); R_f 0.62; UV (MeOH) λ_{max} (log ϵ): 212 (4.19), 242 (3.88), 368 (4.25) nm; IR ν_{max} ($CHCl_3$) cm^{-1}: 3309, 2925, 1632, 1605, 1553, 1523, 1433, 1339, 1236, 1130; ^1H and ^{13}C NMR: see Table 1; HR-ESI-MS *m*/*z* [M + H] : 371.1380 (calc. for $C_{21}H_{22}O_6$ H : 371.1495).

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