Identification and Biological Activity of Microbial Metabolites of Xanthohumol

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Microbial transformation of xanthohumol using the culture broth of *Cunninghamella echinulata* NRRL 3655 afforded (2*S*)-8-[4″-hydroxy-3″-methyl-(2″-*Z*)-butenyl]-4′,7-dihydroxy-5-methoxyflavanone (5) and (2*S*)-8-[5″-hydroxy-3″-methyl-(2″-*E*)-butenyl]-4′,7-dihydroxy-5-methoxyflavanone (6). Xanthohumol (1) and flavanone 6 as well as (*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) obtained with *Pichia membranifaciens* showed antimalarial activity against *Plasmodium falciparum*.

Key words xanthohumol; microbial metabolite; chalcone; NMR; antimalarial

The prenylflavonoid, xanthohumol (**1**) is the major constituent of the female inflorescences (hops) of *Humulus lupulus* L. (Cannabinaceae).[1)] The use of hops in the brewing industry makes **1** an important constituent of beer. **1** and other minor prenylchalcones are converted into the corresponding isomeric prenylflavanones during the brewing process.[2)] Xanthohumol is also a component of some dietary supplements and acts as an antiproliferative agent in ovarian, colon and breast cancer cells.[3)] In addition, it may have cancer-chemopreventive properties[4,5)] and has been patented as a drug for osteoporosis treatment.[6)] *In vitro* studies of **1** with rat and human liver microsomes have yielded several oxygenated derivatives and glucuronides.[7,8)] The biological activities of those metabolites are yet to be determined.

The aim of the microbial transformation studies of **1** was to generate mammalian metabolites which may contribute to the understanding of its biological activities and to be used as analytical standards for the detection of these metabolites in biological fluids.[9—11)] Previous work on the microbial transformation of **1** with cultures of the fungus, *Pichia membranifaciens* (ATCC 2254), resulted in the formation of (*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-5-methoxychalcone (**4**) (Fig. 1).[12)] (*E*)-2″-(2‴-Hydroxyisopropyl)-dihydrofuran[2″,3″:4′,3′]-2′,4-dihydroxy-6′-methoxychalcone (**2**) was identical to that obtained using rat liver microsomes.[7)]

We now report on the microbial transformation of **1** with *Cunninghamella echinulata* NRRL 3655 and the screening of these products for cytotoxicity towards mammalian cell lines and for their potential antimicrobial and antimalarial properties.

Results and Discussion

Twenty microorganisms were used to identify organisms capable of metabolizing xanthohumol (**1**). The standard two-stage procedure[9)] was adopted to screen the organisms. TLC indicated that several organisms were capable of transforming **1**. In the previous investigations with the fungus, *Pichia membranifaciens* (ATCC 2254) three metabolites, (**2**), (**3**), and (**4**) (Fig. 1) were isolated and characterized.[12)]

We selected *Cunninghamella echinulata* NRRL 3655 for scale-up studies as it gave different metabolites than those obtained with the former organism. This transformation afforded the new (2*R*)-8-[4″-hydroxy-3″-methyl-(2″-*Z*)-butenyl]-4′,7-dihydroxy-5-methoxyflavanone (**5**) (2.2% yield) and (2*R*)-8-[5″-hydroxy-3″-methyl-(2″-*E*)-butenyl]-4′,7-dihydroxy-5-methoxyflavanone (**6**) (1.6% yield) (Table 1). Three chalcone related products were also isolated. However, these were formed in concentrations (less than 1 mg, each) that did not permit their structure elucidation.

High-resolution electrospray ionization mass spectrometric data (HR-ESI-MS) of **5** and **6** suggested a molecular formula of $C_{21}H_{22}O_6$ for each indicating that they were monooxygenated derivatives of **1**. Both compounds recorded a maximum absorption at 290 nm and an inflection at 320 nm suggesting a flavanone skeleton.[13)] In the ^{1}H-NMR spectrum of **5**, the B-ring protons, H-2′/H-6′ and H-3′/H-5′ appeared as doublets at δ 7.29 (J=8.5 Hz) and δ 6.78 (J=8.5 Hz). The major differences in the NMR spectrum of **5** as compared to **1**, were the disappearance of signals due to the olefinic H-α, H-β protons and the appearance of signals at δ 5.33 (H-2, dd, J=3.0, 12.0 Hz) and double-doublets at δ 2.91 and δ 2.59, typical of the C-ring protons of flavanones. These data, together with observation of a singlet at δ 1.63 due to a single allylic methyl group and a two-proton singlet at δ 3.96 emanating from a hydroxymethylene functionality, suggested oxygenation at one of the methyl groups of the isoprenyl moiety. The long-range ^{1}H- and ^{13}C-NMR correlations were in complete agreement with structure **5**. The nuclear Overhauser effect spectroscopy (NOESY) spectrum of **5** showed spatial proximities between the methine proton (δ 5.15, H-2″) and the methyl protons (δ 1.63, H-2″). A similar dipolar interaction was observed between the methylene protons (δ 3.96, H-4″) and the benzylic methylene protons (δ 3.17, H-1″) confirming the *Z*-configuration of the double bond. In the CD spectrum of **5**, the high amplitude positive Cotton effect in the 290—300 nm region and the negative Cotton effect in the 330—340 nm region allowed the assignment of (*R*)-configuration at the C-2 stereocenter[14)] indicating the stereospecificity of the C-ring cyclization process. This contrasts with the formation of the 2*S*-flavanone **3** using *P. membrani-*

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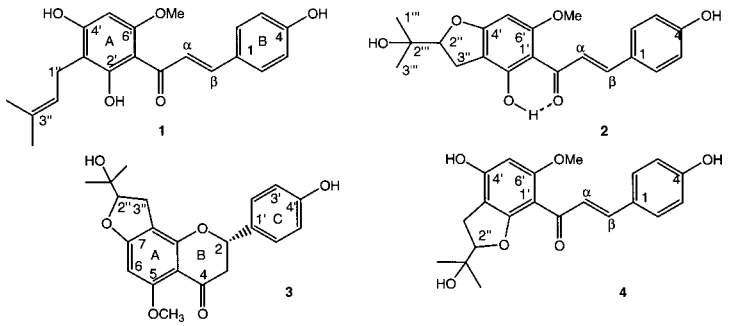

Fig. 1. Structures of Xanthohumol (**1**) and Its Metabolites Obtained with *P. membranifaciens*

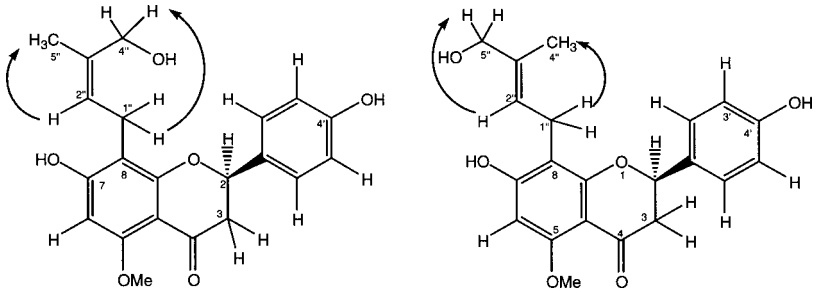

Fig. 2. Structures of the Flavanones **5** and **6** Showing Key NOESY Correlations

Table 1. ^{1}H- and ^{13}C-NMR Spectroscopic Data of Compounds **5**, **6**

Position	**5**		**6**	
	δ_H (J Hz)	δ_C	δ_H (J Hz)	δ_C
1′	—	129.9	—	130.0
2′,6′	7.29 d (8.5)	128.1	7.29 d (8.5)	128.2
3′,5′	6.78 d (8.5)	115.9	6.78 d (8.5)	115.5
4′	—	157.5	—	157.5
2	5.33 dd (12.0, 3.0)	78.4	5.32 dd (12.5, 3.0)	78.4
3	2.91 dd (16.0, 12.0, H_{ax})	45.3	2.92 dd (16.5, 12.5, H_{ax})	45.3
	2.59 dd (16.0, 3.0, H_{eq})		2.58 dd (16.0, 3.0, H_{eq})	
4	—	188.4	—	188.4
4a	—	104.9	—	104.9
5	—	159.9	—	161.6
6	6.13 s	93.3	6.15 s	93.3
7	—	162.0	—	159.9
8	—	107.6	—	107.7
8a	—	161.5	—	162.1
1″	3.17 d (7)	21.8	3.18 d (7.5)	21.8
2″	5.15 t (7)	124.4	5.34 t (7.5)	122.2
3″	—	135.0	—	134.9
4″	3.96 s	22.1	1.52 s	67.0
5″	1.63 s	60.2	3.72 s	14.3
5-OMe	3.69 s	56.0	3.70 s	56.0

facians and represents a rare example of the preferential generation of 2*R*-flavanones.

The reasoning for structure elucidation of flavanone **6** was the same as that for metabolite **5**. Dipolar interactions of the methine proton (δ 5.34, H-2″) with the methylene protons (δ 3.72, H-5″) and similar interactions between the benzylic methylene protons (δ 3.18, H-1″) and the methyl protons (δ 1.52, H-4″) in the NOESY spectrum indicated an *E*-configuration of the double bond in **6**. The CD spectrum of **6** showed similar Cotton effects in the 330—340 and 290—300 nm regions but with lower amplitudes compared to those of **5**. This presumably reflects a lower degree of optical purity as is also reflected by comparison of $[\alpha]_D$ values of flavanones **5** and **6**.

The ^{1}H and ^{13}C data collated in Table 1 for flavanones **5** and **6** were fully corroborated by appropriate 2D NMR experiments, *e.g.* correlation spectroscopy (COSY), NOESY, ^{1}H-detected heteronuclear multiple quantum coherence (HMQC) and heteronuclear multiple bond connectivity (HMBC).

Xanthohumol (**1**) and its products of microbial transformation **2**—**6** (see ref. 12 for **2**—**4**) together with the three undefined polar compounds, showed no cytotoxic activity at 2.78, 8.33 and 25 μg/ml (data not shown) against human cancer cell lines, SK-MEL (malignant, melanoma), KB (epidermal carcinoma, oral), BT-549 (ductal carcinoma, breast) and SK-OV-3 (ovary carcinoma) as well as non-cancerous Vero cells (monkey kidney fibroblasts). Such absence of cytotoxicity towards mammalian cells may contribute towards the safety of xanthohumol containing products for human consumption. No cell proliferation activity was detected. None of the metabolites showed antibacterial or antifungal activity at 50, 10, and 2 μg/ml (data not shown). However, antimalarial activity was exhibited by compounds **1**—**3** and **6** against D6 (chloroquine sensitive) and W2 (chloroquine resistant) strains of the malaria parasite, *Plasmodium falciparium* (Table 2). Both **1** and **2** were active against D6 and W2 strains with IC_{50} values of 3.3 and 1.0 μg/ml and 4.1 and 1.8 μg/ml, respectively. Flavanone **3** was active against D6 strain (IC_{50}, 2 μg/ml) and **6** was active against W2 strain (IC_{50}, 3.2 μg/ml). The selectivity index of antimalarial activity of these compounds ranged from $>$6.1 for compound **2** to $>$25.0 for compound **1**.

Table 2. Antimalarial Activity of Compounds **1**—**6**

Compound	*P. falciparum* (D6)[a]		*P. falciparum* (W2)[b]		Cytotoxicity to Vero cells[d]
	IC_{50} (μg/ml)	SI[c]	IC_{50} (μg/ml)	SI[c]	
1	3.3	>7.6	1.0	>25.0	NC
2	4.1	>6.1	1.8	>13.9	NC
3	2.0	>12.5	NA	—	NC
4	NA	—	NA	—	NC
5	NA	—	NA	—	NC
6	NA	—	3.2	>7.8	NC

a) Chloroquine-sensitive clone. *b*) Chloroquine-resistant clone. *c*) Selectivity index=IC_{50} (Vero cells)/IC_{50} *P. falciparum*. *d*) The highest concentration=25.0 μg/ml. NA=not active. NC=no cytotoxicity.

Experimental

General Experimental Procedures IR spectra were run in $CHCl_3$ using an ATI Mattson Genesis Series FTIR Spectrophotometer. UV spectra were recorded 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 Bruker GioApex 3.0.

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

Organisms and Metabolism The twenty 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, U.S.A.), 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.[15] Xanthohumol **1** was added in dimethylformamide (0.5 mg/ml) to 24 h old stage II cultures. They were incubated at room temperature on a rotary shaker (New Brunswick Model G10-21) at 100 rpm for a period of 14 d. Sampling and TLC monitoring were carried out at 7-d intervals. Precoated Si gel 60 F_{254} plates (E. Merck) were used with $CHCl_3$–MeOH (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 α 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 isolation of metabolites was 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.[16]

Microbial Transformation of Xanthohumol (1) by *C. echinulata* The filtrate of the combined fermentation broth was exhaustively extracted with EtOAc. On evaporation of the solvent a yellowish solid was obtained (500 mg). It was column chromatographed (Si gel 230—400 mesh: E. Merck, 30 g, column diameter: 20 mm) using $CHCl_3$ gradually enriched with MeOH.

The combined fractions were purified by preparative layer chromatography ($CHCl_3$–MeOH, 9 : 1). (2*S*)-8-[5″-Hydroxy-methyl-(2″-*Z*)-butenyl]-4′,7-dihydroxy-5-methoxyflavanone (**5**) was isolated as a light yellow solid (15 mg, 1.7% yield). $[\alpha]_D^{26}$ +1.081° (*c*=0.74, MeOH). *Rf* 0.46; ^{1}H- and ^{13}C-NMR: see Table 1. UV λ_{max} (MeOH) nm (log ε): 214 (4.45), 228 (4.44), 288 (4.23), 326 (3.77) nm. CD (MeOH) $[\theta]_{335}=-3.1$, $[\theta]_{289}=+9.1$; IR ν_{max} ($CHCl_3$) cm^{-1}: 3202, 2922, 1658, 1598, 1518, 1415, 1351, 1275, 1147, 1095. HR-ESI-MS *m*/*z*. $[M+Na]^+$: 393.1321 (Calcd for $C_{21}H_{22}O_6$+Na: 393.13086).

(2*S*)-8-[4″-Hydroxy-3″-methyl-(2″-*E*)-butenyl]-4′,7-dihydroxy-5-methoxyflavanone (**6**) was purified as a light yellow solid (14.5 mg, 1.6% yield). $[\alpha]_D^{26}$ +0.625° (*c*=0.320, MeOH). *Rf* 0.42; ^{1}H- and ^{13}C-NMR: see Table 1. UV λ_{max} (MeOH) nm (log ε): 216 (3.82), 236 (3.91), 288 (3.87), 326 (3.45). CD (MeOH) $[\theta]_{334}=-1.0$, $[\theta]_{291}=+4.7$. IR ν_{max} ($CHCl_3$) cm^{-1}: 3214, 2917, 1650, 1596, 1518, 1414, 1274, 1094, 834. HR-ESI-MS *m*/*z*. $[M+H]^+$: 371.1534 (Calcd for $C_{21}H_{22}O_6$+H : 371.1495).

Assays for Biological Activity Cytotoxicity assays on xanthohumol and the microbial metabolites were carried out against four types of human cancer cell lines, SK-MEL (malignant, melanoma), KB (epidermal carcinoma, oral), BT-549 (ductal carcinoma, breast) and SK-OV-3 (ovary carcinoma) as well as non-cancerous Vero cells (monkey kidney fibroblasts). Samples were added at 2.78, 8.33 and 25 μg/ml. The assay was performed in 96-well tissue culture-treated microplates and utilized the neutral red staining procedure for cytotoxicity determination.[17] Antimicrobial activity of compounds were evaluated using standard protocols of NCCLS standards,[18,19] against a panel of microorganisms including *Candida albicans*, *Cryptococcus neoformans*, *Staphylococcus aureus* and Methicillin resistant *S. aureus*, *Mycobacterium intercellulare* and *Pseudomonas aeruginosa. In vitro* antimalarial activity was tested against two strains (D6, chloroquine sensitive and W2, chloroquine resistant) of the malarial parasite, according to a modification of the method described by Makler *et al.*[20] The activity of plasmodial LDH is determined as an indicator of the number of parasites remaining in infected RBCs, following incubation with samples. IC_{50}, the concentration that causes 50% growth inhibition of the parasite, was calculated from the dose curves generated by plotting concentrations against percent growth inhibition.

Acknowledgements The authors thank Dr. Chuck Dunbar for conducting HR-ESI-MS analysis and Mr. Frank Wiggers for assistance in obtaining 2D NMR spectra. This work was supported, in part, by the United States Department of Agriculture, Agricultural Research Specific Cooperative Agreement No. 58-6408-2-00009 and the Hop Research Council, St. Paul, Oregon.

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