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Xanthohumol metabolites in faeces of rats

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Dedicated to the memory of Professor J.B. Harborne

Abstract

Xanthohumol (**1**), isolated from hop, was fed to rats in a dose of 1000 mg kg^{-1} body weight. The faeces of the animals were collected after 24 and 48 h and analysed for metabolites of **1**. Approximately 89% of the recovered flavonoid-compounds consisted of unchanged **1**. Sixteen metabolites and six previously known metabolites were isolated and characterized by coupling techniques (HPLC–NMR, HPLC–MS and HPLC–DAD). Their structures were elucidated by spectroscopic methods, especially using NMR spectroscopy. Twenty metabolites had a modified chalcone structure and two metabolites were flavanone derivatives.
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1. Introduction

Xanthohumol (1), a prenylated chalcone, is the most abundant flavonoid in hops (*Humulus lupulus* L.). Recently it has attracted some interest because of its various biological activities. It has been shown to be an effective anti-proliferative agent in human cancer cell lines (Gerhäuser et al., 2002b). Recent studies have suggested that it may have potential cancer-chemopreventive activity (Henderson et al., 2000; Gerhäuser et al., 2002a,b). In addition, it exhibited antimutagenic and antioxidative effects (Miranda et al., 2000) in vitro and protected against arteriosclerosis and osteoporosis (Tobe et al., 1997; Tabata et al., 1997). The in vitro biotransformation of **1** by rat and human liver microsomes has been investigated and glucuronides, hydroxylated and cyclic dehydro-metabolites have been identified (Yilmazer et al., 2001a,b). Until now no investigations have been carried out on in vivo transformation of **1**. The present study deals with the in vivo metabolites of **1** found in faeces of rats.

2. Results and discussion

2.1. General aspects

Repeated chromatography of the extract from faeces of rats yielded 22 metabolites of **1** (see Fig. 1). Untransformed **1** was isolated in mg quantities and its metabolites in μg amounts only. The extract was composed of approximately 89% xanthohumol (**1**) and 11% metabolites, based on integrated HPLC-peak areas. The high amount of unchanged compound **1** complicated the identification of the metabolites. Therefore **1** was removed prior to isolation of metabolites. The metabolites were characterized by conventional NMR spectroscopy and/or HPLC–^{1}H NMR coupling in the stopped-flow mode, HPLC–MS, and HPLC–DAD. UV spectra resembling those of **1**, molecular masses in the order of magnitude of **1**, and ^{1}H NMR fingerprint signals of the *p*-substituted phenyl ring, the singlets of H-5′ and 6′-OCH$_3$ were considered to discriminate

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Fig. 1. Structures of xanthohumol metabolites.

between metabolites related to **1** and matrix compounds. Variations in the chemical shifts of these fingerprint signals, and especially those of the prenyl side chain, and the appearance of additional signals in the spectrum were used to deduce the structures of the individual metabolites as discussed in the following paragraphs.

The parent compound, xanthohumol (**1**) was identified by means of the retention time and spectroscopic data (Table 1), which were in agreement with published data (Tabata et al., 1997). In some cases the combination of liquid chromatography with spectroscopic methods (HPLC–NMR, HPLC–MS) enabled the identification of microgram quantities of low-abundant metabolites without isolation. The results of HPLC–^{1}H NMR were verified exemplary for xanthohumol (**1**) and some isolated metabolites by means of conventional 1D and 2D NMR spectroscopic techniques.

Products of the following transformations were observed: hydroxylation, *O*-methylation, *O*-acetylation, epoxidation, cyclisation involving the prenyl side chain, and glucuronidation at the hydroxyl in position 4′. Two metabolites showed a flavanone skeleton. The other metabolites had a chalcone skeleton with unchanged or modified prenyl moieties. Modification of the prenyl substituent almost covered hydroxylations and/or cyclisation to give a pyrano or furano ring with one of the adjacent 2′- or 4′-hydroxyl groups.

2.2. Metabolites with altered non-cyclic prenyl side chain

HPLC–MS analysis of **2** created a quasi molecular ion $[M-H]^-$ at m/z 371, with a mass difference of 18 in comparison to **1**, which is consistent with hydration of the parent compound. In the HPLC–^{1}H NMR spectrum the proton signals of the core structure, 6′-OCH_3 (δ 3.87), H-α (δ 7.80) and H-β (δ 7.67) of **2** were almost identical with those found in the proton NMR spectrum of **1** (Table 1). This indicated that the chalcone skeleton of **2** was the same as for **1** and the structural difference must be located in the prenyl side chain at C-3′. However, the characteristic signal of H-2″ was not found in the HPLC–^{1}H NMR spectrum, but the signal of an additional methylene group at δ 1.56 was present instead. Together with the singlet of two equivalent methyl groups at δ 1.20, these data indicated the presence of a 3-hydroxy-3-methylbutyl group. Comparison with published data of the modified prenyl chain of a coumaronochromone (Lo et al., 2002) further confirmed the structure of the side chain. Thus, metabolite 2 has been established as 3′-(3″methyl-3″-hydroxy-butyl)-2′,4,4′,-trihydroxy-6′-methoxychalcone, which is reported here for the first time.

HPLC–MS analysis of compounds **3** and **4** gave the same molecular mass $M_R = 372$, suggesting hydroxylated metabolites of **1**. With the exception of side chain

Table 1
^{1}H NMR spectroscopic data (500 MHz) of compounds **1**–**4** δ, mult. (J in Hz). The metabolites were measured in the HPLC–NMR mode in MeCN–D_2O. Compounds **1** and **4** were additionally measured under conventional NMR conditions in MeOH-d_4

Position	**1**		**2**	**3**	**4**	
	MeCN–D_2O	MeOH-d_4	MeCN–D_2O	MeCN–D_2O	MeCN–D_2O	MeOH-d_4
α	7.80, 1H (*d*, 16.0)	7.79, 1H (*d*, 15.7)	7.80, 1H (*d*, 15.5)	7.81, 1H (*d*, 15.9)	7.80, 1H (*d*, 16.0)	7.79, 1H (*d*, 15.5)
β	7.68, 1H (*d*, 16.0)	7.67, 1H (*d*, 15.7)	7.67, 1H (*d*, 15.5)	7.68, 1H (*d*, 15.9)	7.68, 1H (*d*, 16.0)	7.68, 1H (*d*, 15.5)
2/6	7.56, 2H (*d*, 8.4)	7.50, 2H (*d*, 8.6)	7.56, 2H (*d*, 8.5)	7.58, 2H (*d*, 8.5)	7.57, 2H (*d*, 8.4)	7.50, 2H (*d*, 8.6)
3/5	6.87, 2H (*d*, 8.4)	6.87, 2H (*d*, 8.6)	6.88, 2H (*d*, 8.5)	6.89, 2H (*d*, 8.5)	6.88, 2H (*d*, 8.4)	6.82, 2H (*d*, 8.6)
5′	6.09, 1H, *s*	6.02, 1H, *s*	6.10, 1H, *s*	6.11, 1H, *s*	6.09, 1H, *s*	6.04, 1H, *s*
1″	3.20, 2H (*d*, 7.0)	3.23, 2H (*d*, 7.2)	2.55, 2H, *m*	3.26, 2H (*d*, 7.3)	2.87, 1H (*dd*, 14.0, 5.0)	2.96, 1H (*dd*, 13.8, 5.4)
					2.77, 1H (*dd*, 14.0, 7.5)	2.79, 1H (*dd*, 13.8, 7.3)
2″	5.15, 1H (*t*, 7.0)	5.20, 1H (*t*, 7.2)	1.56, 2H, *m*	5.39, 1H (*t*, 7.3)	4.30, 1H (*dd*, 7.5, 5.0)	4.33, 1H (*dd*, 7.3, 5.4)
4″	1.73, 3H, *s*	1.75, 1H, *s* }	1.20, 6H, *s*	3.86, 2H, *s*	4.79, 1H, *s*	4.80, 1H, *s*
		1.63, 3H, *s* }			4.72, 1H, *s*	4.71, 1H, *s*
5″	1.64, 3H, *s*			1.74, 3H, *s*	1.75, 3H, *s*	1.81, 3H, *s*
6′-OCH_3	3.88, 3H, *s*	3.90, 3H, *s*	3.87, 3H, *s*	3.88, 3H, *s*	3.88, 3H, *s*	3.91, 3H, *s*

signals, the ^{1}H NMR resonances were similar to those of **1**, indicating that the hydroxylation must have occurred in the side chain. The positions of the new hydroxyl groups and the double bond were established by the coupling pattern and chemical shifts of the side chain signals. In the HPLC–^{1}H NMR spectrum of compound **3** a signal due to one of the methyl groups of the prenyl chain was not observed. Instead, a methylene group (δ 3.86) appeared as a singlet at relatively low field, which is consistent with an electronegative substituent in this position. Due to the mass spectral data this substituent must be a hydroxyl group and the structure of metabolite 3 was attributed to 3′-(3″-hydroxymethyl-but-2″-enyl)-2′,4,4′-trihydroxy-6′-methoxychalcone, which is a new compound.

The side-chain signals in the ^{1}H NMR spectrum of compound 4 measured in MeOH-d_4 (Table 1) showed a low-field doublet of doublets of a hydroxylated methine at δ 4.33 adjacent to two methylene protons at δ 2.96 and 2.79 attributable to H-1″a and H-1″b. The signal lines of an olefinic methylene group at δ 4.80 and 4.71 and a methyl group at δ 1.81 completed the set of resonances of the hydroxylated prenyl chain consistent with a 2-hydroxy-3-methyl-but-3-enyl moiety. From these data, metabolite **4** was assigned to 3′-(2″-hydroxy-3″-methyl-but-3″-enyl)-2′,4,4′-trihydroxy-6′-methoxychalcone, xanthohumol D (Stevens et al., 2000). The structure determined by HPLC–^{1}H NMR spectroscopy was further confirmed by conventional ^{1}H (Table 1) and 2D NMR spectral analysis (data not shown). The shift differences between ^{1}H NMR spectra of **4** measured in the HPLC–NMR mode (MeCN–D_2O) and by conventional NMR (MeOH-d_4) did not exceed 0.1 ppm, indicating the suitability of HPLC–NMR spectroscopic data for structure elucidation of xanthohumol metabolites.

2.3. Metabolites with substituted or altered chalcone moiety

The proton NMR spectra of compounds **5**, **6**, and **7** exhibited the complete set of signals of the parent compound, xanthohumol (**1**). Additional NMR signals (Table 2) and mass spectral data readily indicated substituents such as a glucuronide unit in compound **5** with $[M-H]^-$at m/z 529, and a methyl or acetyl group in **6**, and **7**, respectively. The substitution positions were assigned by chemical shift differences in comparison with compound **1**. Compared with the H-5′ resonance of 1 (δ 6.09) in the HPLC–^{1}H NMR spectrum the low-field shift of the corresponding signal of **5** at δ 6.30 pointed to a substitution of the neighbouring phenolic OH group at C-4′. The almost unchanged resonances of the phenyl ring excluded substitution of the hydroxyl group at C-4 and substitution of the chelated 2′-OH would result in shift changes of the proton in α-position, which is not the case. These data resembled those obtained previously, although in a different solvent, from the same compound (Yilmazer et al., 2001a). Thus, compound **5** was elucidated as 4′-*O*-glucuronyl-2′,4-dihydroxy-6′-methoxy-3′-prenylchalcone.

The molecular mass of compound **6** ($M_R = 368$) suggested an additional methyl group in this compound. The corresponding signal was found in the ^{1}H NMR spectrum at δ 3.84 (Table 2) next to the 6′-OCH_3 (δ 3.90) singlet. The signal of H-3/5 (δ 6.96) in MeOH-d_4 appeared at 0.09 ppm, and that of H-2/6 (δ 7.57) 0.07 ppm at lower field, which is indicative of the methyl group at 4-OH, because all the other signals did not show any significant changes. Based on these data, compound **6** was characterized as 2′,4′-dihydroxy-4,6′-dimethoxy-3′-prenylchalcone.

Table 2
^{1}H NMR spectroscopic data (500 MHz) of compounds **5**–**8** δ, mult. (J in Hz). Metabolite **5** was measured in the HPLC–NMR mode in MeCN–D_2O, metabolites **6** and **8** were measured under conventional NMR conditions in MeOH-d_4 and metabolite **7** was measured under both conditions

Position	5	6	7		8
	MeCN–D_2O	MeOH-d_4	MeCN–D_2O	MeOH-d_4	MeOH-d_4
α	7.77, 1H (*d*, 15.5)	7.83, 1H (*d*, 15.5)	7.88, 1H (*d*, 15.8)	7.88, 1H (*d*, 15.5)	3.83, 2H (*d*, 8.0)
β	7.71, 1H (*d*, 15.5)	7.67, 1H (*d*, 15.5)	7.66, 1H (*d*, 15.8)	7.68, 1H (*d*, 15.5)	
2/6	7.58, 2H (*d*, 8.5)	7.57, 2H (*d*, 8.6)	7.67, 2H (*d*, 8.5)	7.62, 2H (*d*, 8.7)	7.20, 2H (*d*, 8.5)
3/5	6.88, 2H (*d*, 8.5)	6.96, 2H (*d*, 8.6)	7.31, 2H, (*d*, 8.5)	7.35, 2H, (*d*, 8.7)	6.73, 2H (*d*, 8.5)
5′	6.30, 1H *s*	6.01, 1H, *s*	6.09, 1H, *s*	6.03, 1H, *s*	6.07, 1H, *s*
1″	3.32, 1H (*dd*, 14.2, 7.4)	3.23, 2H (*d*, 6.4)	3.20, 2H (*d*, 7.2)	3.23, 2H (*d*, 7.1)	3.11, 2H (*dd*, 6.7)
	3.23, 1H (*dd*, 14.2, 6.3)				
2″	5.16, 1H (*dd* (7.4, 6.3)	5.19, 1H (*t*, 6.4)	5.14, 1H (*t*, 7.2)	5.20, 1H (*t*, 7.1)	5.06, 1H (*t*, 6.7)
4″	1.74, 3H, *s*	1.75, 3H, *s*	1.72, 3H, *s*	1.76, 3H, *s*	1.67, 3H, *s*
5″	1.62, 3H, *s*	1.64, 3H, *s*	1.63, 3H, *s*	1.65, 3H, *s*	1.60, 3H, *s*
6′-OCH_3	3.91, 3H, *s*	3.90, 3H, *s*	3.88, 3H, *s*	3.91, 3H, *s*	3.89, 3H, *s*
1‴	5.22, 1H (*d*, 7.1)				
2‴-4‴	3.54-3.65, 3H, *m*				
5‴	4.09, 1H (*d*, 9.7)				
4-OCH_3		3.84, 3H, *s*			
4-$COCH_3$			n.d.	2.03, 3H, *s*	

Although HPLC-MS analysis of compound **7** yielded a quasi molecular ion $[M-H]^-$at m/z 395, suggesting an acetylated metabolite of **1**, the methyl signal of the acetyl group was not visible in the HPLC–^{1}H NMR spectrum because of overlay by the large solvent signal (MeCN). Measurement in MeOH-d_4 (Table 2) revealed this signal at δ 2.03. The position of the acetyl group was assigned by inspection of the chemical shift differences between compound **1** and metabolite **7**. The signals of H-3/5 (δ 7.31) and H-2/6 (δ 7.67) appeared at substantially lower fields compared to the corresponding signals of **1** (Table 1). Similar shifts and shift differences were found by HPLC–NMR in MeCN–D_2O (Tables 1 and 2). Thus, the acetyl group must be attached to 4-OH and metabolite **7** was elucidated as 4-acetoxy-2′,4′-dihydroxy-6′-methoxy-3′-prenylchalcone.

Owing to the molecular mass information $M_R = 370$, metabolite **8** was supposed to be an oxygenated derivative of **1**. The prenyl side chain and the A and B ring protons showed almost the same chemical shift as those of the parent compound. However, typical *trans*-olefinic protons (H-α and H-β) were missing. Instead, a doublet ($J = 8.0$ Hz) integrating for two protons appeared at δ 3.83 in the HPLC–^{1}H NMR spectrum (Table 2). The chemical shift and multiplicity of this signal was consistent with an epoxide structure with the two protons (H-α and H-β) possessing the same chemical shifts. According to these data, the structure of the metabolite **8** most probably is 1-[(2′,4′-dihydroxy-3′-isoprenyl-6′-methoxy)-phenyl]-[3-(4-hydroxyphenyl)]-2,3-epoxypropan-1-on. For comparison purpose, chalcone numbering was retained for compound **8** in Fig. 1 and Table 2 of this paper.

2.4. Metabolites of the flavanone type

The ^{1}H NMR spectrum of **9** exhibited typical signals of a flavanone and a prenyl side chain (Table 3). The singlet of the methoxyl group appeared at δ 3.76. The values were in agreement with published data (Hänsel and Schulz, 1988) for isoxanthohumol (**9**). Mass spectral data like $M_R = 354$ obtained from HPLC–MS confirmed the suggested structure.

The molecular mass of **10**, which was determined on the basis of HPLC-MS measurement to be at $M_R = 372$, showed a mass difference of 18 to isoxanthohumol **9**. The ^{1}H NMR spectrum exhibited signals of the flavanone moiety (Table 3), which closely resembled those of compound **9**. The signals at δ 1.61 and δ 2.60 and the singlet of two equivalent methyl groups at δ 1.15 were attributed to a 3-hydroxy-3-methylbutyl side chain. Comparison of the ^{1}H NMR spectral data of the 3″-hydroxylated prenyl moiety of **10** with corresponding signals of compound **2** and with published data of the modified prenyl chain of a coumaronochromone (Lo et al., 2002) showed similar chemical shift values and thus confirmed the proposed structure of the side chain at C-8 of the flavanone skeleton. This is the first report of 8-(3″-methyl-3″-hydroxybutyl)-4′,6-dihydroxy-5′-methoxy-flavanone (**10**) as a metabolite of xanthohumol.

Table 3
^{1}H NMR data (500 MHz) of compounds 9 and 10 δ, mult., J in Hz. The spectrum of 9 was measured in the HPLC-NMR mode in MeCN-D_2O and the data of 10 were obtained from conventional ^{1}H NMR in MeOH-d_4

Position	**9**	**10**
	MeCN–D_2O	MeOH-d_4
2	5.34, 1H (*dd*, 12.8, 3.1)	5.30, 1H (*dd*, 12.7, 2.9)
3 (ax)	2.98, 1H (*dd*, 17.1, 12.8)	2.95, 1H (*dd*, 17.8, 12.7)
3 (eq)	2.70, 1H (*dd*, 17.1, 3.1)	2.67, 1H (*dd*, 17.8, 2.9)
6	6.16, 1H, *s*	6.10, 1H, *s*
2′/6′	7.31, 2H (*d*, 8.5)	7.33, 2H (*d*, 8.6)
3′/5′	6.85, 2H (*d*, 8.5)	6.80, 2H (*d*, 8.6)
1″	3.17, 2H (*d*, 6.9)	2.60, 2H, *m*
2″	5.10, 1H (*t*, 6.9)	1.61, 2H, *m*
4″/5″	1.60, 3H, *s*	1.15, 6H, *s*
	1.56, 3H, *s*	
5-OCH_3	3.76, 3H, *s*	3.79, 3H, *s*

2.5. Metabolites of the benzodihydrofurane type

While the metabolites which have been discussed above contain an open prenyl chain, the following compounds are characterized by a cyclic prenyl moiety. Since ring closure by nucleophilic attack of either of the two *ortho* hydroxyl groups of xanthohumol (**1**), 2′-OH or 4′-OH, to C-2″ or C-3″ may occur, two five and two six-membered isomeric types of structures are possible. The benzofurane-type metabolites such as **11** and **12** will be considered first. These compounds were first identified in metabolic studies on xanthohumol (**1**) in rat liver microsomes (Yilmazer et al., 2001b) and *Pichia membranifaciens* (Herath et al., 2003), respectively, and the structure elucidated by ^{1}H–^{13}C heterocorrelated NMR spectroscopy. Both metabolites were found in the present study as well, but the amounts were insufficient for 2D NMR experiments and therefore had to be characterized by ^{1}H NMR and MS only. Despite the same number and type of spin systems occurring in both isomers, discrimination was possible by inspection of chemical shifts of H-α, H-β, and in part 6′-OCH_3. In the spectra of chalcones with chelating 2′-hydroxy group, H-α is deshielded and appears at relatively low field, almost between δ 7.4 to 7.7 (Pistelli et al, 1996; Abegaz et al., 1998). Compounds without hydrogen bond formation due to missing or substituted 2′-OH exhibit δ values for H-α at significantly higher field. The chemical shift of H-β is influenced as well, but differences are less significant. Moreover, the 6′-OCH_3 singlet appears at approximately δ 3.9 in compounds with furane ring formation via 4′-OH and at δ 3.7–3.8 in compounds

with ring closure via 2′-OH. According to the chemical shifts characteristics of H-α, H-β, and 6′-OCH_3, further ^{1}H NMR (Table 4) and mass spectra showing the same quasi molecular ion $[M–H]^-$ at *m/z* 369, the structures of metabolites **11** and **12** were determined as 2″-(2‴ - hydroxyisopropyl) - dihydrofurano[4″,5″:3′,4′]2′,4-dihydroxy-6′-methoxy chalcone and 2″-(2‴-hydroxyisopropyl)-2″,3″-dihydrofurano[4″,5″:2′,3′]4,4′-dihydroxy-6′-methoxychalcone, respectively.

^{1}H NMR spectrum of **13** (Table 4) exhibited two olefinic protons H-α/H-β at δ 7.78 and 7.72 which are similar to those of **11**. This indicated that compound 13 was fixed in a furano ring with 2′-OH. The other ^{1}H NMR data (Table 4) resembled those of **11** except the signal of H-3″ (δ 3.74) which in comparison to the corresponding signal of **11** was shifted to lower field and integrated for one proton only, indicating substitution by an electronegative substituent, which was found to be a methoxyl group resonating at δ 3.65. The HPLC-MS analysis gave the molecular mass information $M_R = 400$ and suggested a methoxylated derivative of **11** and therefore structure of **13** was assigned as 2″-(2‴-hydroxyisopropyl)-3″-methoxy-2′,3′-dihydrofurano[4″,5″:3′,4′]2′,4-dihydroxy-6′-methoxychalcone.

The ^{1}H NMR spectrum of **14** displayed signals at δ 6.75 and δ 7.23 for H-α and H-β at relatively high field and 6′-OCH_3 at δ 3.71. These data are consistent with a furano ring structure fixed with 2′-OH, which is comparable with metabolite **12**. The spectrum of the prenyl unit of **14** showed two singlets at δ 1.31 and δ 1.16 due to geminal methyl groups and two doublets at δ 3.59 and δ 4.71 which were attributed to H-2″ and the hydroxylated H-3″, respectively. Together with the signals of ring **B** this led to the determination of metabolite 14 as 2″-(2‴-hydroxyisopropyl)-3″-hydroxy-2″,3″-dihydrofurano[4″,5″:2′,3′]4,4′-dihydroxy-6′-methoxychalcone.

2.6. Metabolites of the benzofurane type

The HPLC–NMR spectrum of metabolite **15** resembled that of compound **11** but signals due to H-2″ were missing. The presence of a low-field proton at H-3″(δ 6.75), and the quasi molecular ion $[M-H]^-$ at *m/z* 367 suggested a dehydro analogue of **11**. The two methyl groups of the hydroxy isopropyl moiety appeared as a singlet at relatively low field (δ 1.57) supporting the allylic structural feature. These data led to the identification of metabolite **15** as 2″-(2‴-hydroxyisopropyl)-furano[4″,5″:3′,4′]4,2′-dihydroxy-6′-methoxychalcone.

The doublet of the geminal methyl groups at δ 1.32 and the multiplet integrating for one proton of H-2‴ (δ 3.03) indicated an isopropyl moiety attached to metabolite **16** while the multiplicities and chemical shifts of the signals (Table 4) of the remaining part of the molecule are similar to those of **15**. The singlet at δ 6.43 was assigned to H-3″ because of its broadened shape, which

Table 4
^{1}H NMR data (500 MHz) of metabolites **11–18** δ, mult. (*J* in Hz). The spectra of compounds **11** and **15** were measured in the HPLC-NMR mode in MeCN–D_2O and the data of metabolites **12–14** and **16–18** were obtained from conventional ^{1}H NMR in MeOH-d_4

Position	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**
	MeCN–D_2O	MeOH-d_4	MeOH-d_4	MeOH-d_4	MeCN–D_2O	MeOH-d_4	MeOH-d_4	MeOH-d_4
α	7.76, 1H (d, 15.3)	7.12, 1H (d, 16.0)	7.78, 1H (d, 15.5)	6.75, 1H (d, 16.0)	7.73, 2H, s	7.73, 1H, s 7.74, 1H, s	7.75, 2H, s	7.29, 1H (d, 16.0)
β	7.68, 1H (d, 15.3)	7.49, 1H (d, 16.0)	7.72, 1H (d, 15.5)	7.23, 1H (d, 16.0)				7.53, 1H (d, 16.0)
2/6	7.57, 2H (d, 8.5)	7.47, 2H (d, 8.5)	7.51, 2H (d, 8.5)	7.42, 2H (d, 8.5)	7.59, 2H (d, 8.5)	7.52, 2H (d, 8.5)	7.54, 2H (d, 8.5)	7.49 2H (d, 8.5)
3/5	6.88, 2H (d, 8.5)	6.79, 2H (d, 8.5)	6.82, 2H (d, 8.5)	6.79, 2H (d, 8.5)	6.89, 2H (d, 8.5)	6.83, 2H (d, 8.5)	6.84, 2H (d, 8.5)	6.81 2H (d, 8.5)
5′	6.15, 1H, s	6.01, 1H, s	5.96, 1H, s	6.16, 1H, s	6.67, 1H, s	6.68, 1H, s	6.77, 1H, s	6.41, 1H, s
2″	4.75, 1H (dd, 9.4, 8.0)	4.65, 1H (dd, 9.4, 8.5)	4.47, 1H (d, 4.4)	4.71, 1H (d, 7.1)			7.58, 1H (d, 2.2)	7.57, 1H (d, 2.2)
3″	2.93, 1H (dd, 15.1, 8.0) 3.06, 1H (dd, 15.1, 9.4)	3.00, 1H (dd,16.0, 8.5) 3.03, 1H (dd, 16.0, 9.4)	3.74, 1H (d, 4.4)	3.59, 1H (d, 7.1)	6.75, 1H, s	6.43, 1H, s	6.87, 1H (d, 2.2)	6.85, 1H (d, 2.2)
1‴/3‴	1.21, 3H, s 1.16, 3H, s	1.26, 1H, s 1.18, 1H, s	1.42, 3H, s 1.39, 3H, s	1.31, 3H, s 1.16, 3H, s	1.57, 6H, s	1.32, 6H (d, 7.3)		
2‴						3.03, 1H, m		
6′-OCH_3	3.90, 3H, s	3.76, 3H, s	3.91, 3H, s	3.71, 3H, s	3.95, 3H, s	3.97, 3H, s	4.00, 3H, s	3.88, 3H, s
3″-OCH_3			3.65, 3H, s					

presumably was due to long-range ^{1}H, ^{1}H-coupling with H-2‴ and thus allowed discrimination from the singlet attributed to H-5′ (δ 6.68). A quasi molecular ion $[M-H]^-$ at *m/z* 351 was found with HPLC–MS and confirmed the suggested structure. Thus, metabolite **16** was established as 2″-isopropyl-furano[4″,5″:3′,4′]4,2′-dihydroxy-6-methoxychalcone.

The substantially lower molecular mass of $M_R = 310$ of metabolites **17** and **18**, in comparison with xanthohumol (**1**) and the other metabolites, suggested loss of a partial structure. The ^{1}H NMR spectra exhibited signals of A and B rings, the *trans* double bond, the 6′-OCH_3 and two doublets of a furanyl moiety (Table 4) but signals of the isopropyl side chain were missing. From these ^{1}H NMR and MS data, both compounds **17** and **18** were established as isomeric furano-dihydroxy-6′-methoxychalcones. Discrimination of both isomers was possible by means of the different chemical shifts of the H-α and methoxyl signals. The low-field signals of H-α (δ 7.75) and 6′-OCH_3 (δ 4.00) of metabolite **17** suggested that in this compound the furan ring has been formed with 4′-OH. In contrast, H-α (δ 7.29) of **18** appeared at substantially higher field and the singlet of 6′-OCH_3 is shifted slightly but significantly to δ 3.88. These shift differences are in agreement with those discussed above for metabolites **11**–**14**. Thus, compounds **17** and **18** are furano-[4″,5″:3′,4′]4,2′-dihydroxy-6′-methoxychalcone and furano-[4″,5″:2′,3′]4,4′-dihydroxy-6′-methoxychalcone, respectively.

2.7. Metabolites of the benzoisochromane and benzoisochromene type

The HPLC–MS analysis of compound **19** showed a quasi molecular ion $[M-H]^-$ at *m/z* 351 indicating an isomer of the benzofurane metabolite **16**. However, the ^{1}H NMR signals of the prenyl group of **19** provided evidence for a cyclization as a dimethylpyrano moiety. Two doublets at δ 6.60 and 5.58 with a 10 Hz coupling constant were attributed to the *cis* double bond, and the singlet at δ 1.42 integrating for six protons to the *gem*-dimethyl group. These data were consistent with those of 2″,2″-dimethyl-(2*H*)-pyrano[5″,6″:3′,4′]2′,4-dihydroxy-6′-methoxychalcone, a natural product, which was first isolated from hop (Stevens et al., 1997) and later named xanthohumol C (Stevens et al., 2000).

The ^{1}H NMR spectrum of metabolite **20** did not show any *cis* double bond signals but, instead singlets of a double bound proton at δ 6.74 and an additional methoxy group at δ 3.10 were detected. By contributing a mass information of $M_R = 382$, HPLC–MS also suggested an additional methoxy group. The position of this substituent was deduced from the proton signal at δ 6.74, which appeared broadened due to the long-range ^{1}H,^{1}H coupling with the geminal methyl groups at C-2″ and therefore was attributed to H-3″. Thus, only C-4″ remained for the position of the methoxy group. From these data the structure of metabolite **20** was established as 4″-methoxy-2″,2″-dimethyl-(2*H*)-pyrano[5″,6″:3′,4′]2′,4-dihydroxy-6′-methoxychalcone.

The structure of metabolite **21** was established from comparison of ^{1}H NMR (Table 5) and mass spectral data with those of compound **19**. Two triplets (δ 2.60 and 1.81), which in the ^{1}H NMR spectrum of **21** appeared instead of the *cis* double bond signals in the spectrum of compound **19**, and the two units higher molecular mass information ($M_R = 354$) of **21** clearly proved this compound as 2″,2″-dimethyl-3″,4″-dihydro-(2*H*)-pyrano[2″,3″:3′,4′]2′,4-dihydroxy-6′-methoxychalcone.

The molecular mass $M_{R=}354$ of metabolite **22**, which was indicated by the HPLC–MS spectrum, was identical with that of compound **21**, suggesting isomeric structures. Moreover, the signals in the ^{1}H NMR spectrum of **22** (Table 5) closely resembled those of **21**, except the chemical shifts of the two *trans* olefinic protons and the singlet of the 6′-OCH_3 group, which appeared at relatively high field at δ 6.76 and δ 7.23 (H-α and H-β, respectively) and δ 3.68 (6′-OCH_3). This phenomenon

Table 5
^{1}H NMR spectroscopic data (500 MHz) of metabolites **19**–**23** δ ppm, mult. (*J* in Hz). The spectrum of compound **19** was measured in the HPLC–NMR mode in MeCN–D_2O and the data of metabolites **20–23** were obtained from conventional ^{1}H NMR in MeOH-d_4

Position	**19**	**20**	**21**	**22**	**23**
	MeCN–D_2O	MeOH-d_4	MeOH-d_4	MeOH-d_4	MeOH-d_4
α	7.79, 1H (*d*, 15.3)	7.74, 1H, s	7.82, 1H (*d*, 15.5)	6.76, 1H (*d*, 16.0)	6.74, 1H (*d*, 15.3)
β	7.72, 1H (*d*, 15.3)	7.74, 1H, s	7.70, 1H (*d*, 15.5)	7.23, 1H (*d*, 16.0)	7.22, 1H (*d*, 15.3)
2/6	7.58, 2H (*d*, 8.6)	7.53, 2H (*d*, 8.9)	7.51, 2H (*d*, 8.5)	7.40, 2H (*d*, 9.7)	7.38, 2H (*d*, 8.6)
3/5	6.88, 2H (*d*, 8.6)	6.83, 2H (*d*, 8.9)	6.83, 2H (*d*, 8.5)	6.79, 2H (*d*, 9.7)	6.77, 2H (*d*, 8.6)
5′	6.02, 1H, *s*	6.79, 1H, *s*	5.96, 1H, *s*	6.10, 1H, *s*	6.09, 1H, *s*
3″	5.58, 1H (*d*, 10.4)	6.74, 1H, *s*	1.81, 2H (*t*, 7.0)	1.75, 2H (*t*, 7.0)	3.67, 1H (*dd*, 5.7, 7.5)
4″	6.60, 1H (*d*, 10.4)		2.60, 2H (*t*, 7.0)	2.61, 2H (*t*, 7.0)	2.84, 1H (*dd*, 16.7, 5.7) 2.47, 1H (*dd*, 16.7, 7.5)
2″-$(CH_3)_2$	1.42, 6H, *s*	1.59, 6H, *s*	1.34, 6H, *s*	1.21, 6H, *s*	1.20, 3H, *s*1.13, 3H, *s*
6′-OCH_3	3.92, 3H, *s*	3.99, 3H, *s*	3.90, 3H, *s*	3.68, 3H, *s*	3.65, 3H, *s*
4″-OCH_3		3.10, 3H, *s*			

was discussed already for compound **11** and **12** as a means to discriminate between benzofuran-type compounds which were cyclized via 2′-OH and 4′-OH and is even more pronounced in the compounds of the benzochromane series, e.g. metabolites **22** and **21**. From these analytical data and comparative considerations, metabolite 22 has been established as 2″,2″-dimethyl-3″,4″-dihydro-(2*H*)-pyrano[5″,6″:2′,3′]4,4′-dihydroxy-6′-methoxychalcone.

Finally, the ^{1}H NMR spectrum of metabolite **23** also exhibited signals of a dihydropyrano unit fixed with the 2′-OH group which delivered the oxygen (Table 5). Again, this was deduced from the high-field chemical shifts of H-α (δ 6.74), H-β (δ 7.22), and 6′-OCH_3 (δ 3.65). The HPLC-MS spectrum displayed a quasi molecular ion $[M-H]^-$ at 369, thus indicating an oxygenated metabolite. This finding was supported by a signal of an oxygenated methine resonating at δ 3.67, which appeared as a doublet of doublets in the ^{1}H NMR spectrum, owing to an adjacent methylene (δ 2.84/2.47). Due to the hypothetical biotransformation pathway of xanthohumol (**1**) (Yilmazer et al., 2001b) and in analogy with the chemical shifts and coupling patterns of corresponding signals of xanthohumol B (Tabata et al., 1997; Stevens et al., 2000) the hydroxyl functionality very likely is attached to C-3″. Thus the structure of metabolite **23** has been assigned as 2″,2″-dimethyl-3″-hydroxy-4″,5″-dihydro-(2*H*)-pyrano[5″,6″:2′,3′]4,4′-dihydroxy-6′-methoxychalcone.

Glucuronation and methylation of phenolic OH-groups are common metabolic features and can thus explain metabolites **5** and **6**. In vitro studies of XN with rat an human liver microsomes yielded several oxygenated derivatives and glucuronides (Ylmazer et al., 2001a,b). Isomerisation of the chalcon to flavanone is observed under alkaline conditions in vitro (compounds **9** and **10**). Oxidation of the prenyl side chain, followed by cyclization to either pyran or furan ring systems could be due to *P*450 enzymatic reactions. Oxidation and cyclisation was also observed as microbial transformation of **1** (Herath et al., 2003). Experiments in collaboration with Braune/Blaut (Bergholz-Rehbrücke, Germany) are under way to test the metabolic activity of gut bacteria. The metabolites of **1** in various body compartments of rats will be further characterized over a period of 48 h (Nookandeh et al., unpublished results).

3. Experimental

3.1. Chemicals

Xanthohumol (**1**) was isolated from an ethanolic extract of hops (Nookandeh, unpublished results) obtained from Hallertauer Hopfenveredlungsgesellschaft mbH (Mainburg, Germany). MeCN, formic acid, *n*-hexane, EtOAc, TFA were purchased from Riedel-de-Haen Germany.

3.2. Analytical methods

3.2.1. HPLC of metabolites

HPLC was performed on a Waters 2690 HPLC system with a 996 diode-array detector. LiChrospher 100 RP-18 (5 μm) was used as the stationary phase, in a 4×250 mm column (Knauer).

The flow rate was 1 ml/min and 0.1% formic acid was added to both the MeCN and water component of the eluent. Gradient elution with MeCN/water 9/91 v/v over 35 min to MeCN/water 100/0 v/v over the next 5 min; after 45 min the MeCN/water was altered to 9/91 v/v within 5 min. For detection, UV spectra were recorded from 254 to 400 nm.

The retention times of the described compounds were as follows: **1** (26.88 min), **2** (20.42 min), **3** (19.99 min), **4** (22.99 min), **5** (19.53 min), **6** (31.72 min), **7** (20.25 min), **8** (13.82 min), **9** (19.00 min), **10** (12.83 in), **11** (23.29 min), **12** (14.13 min), **13** (24.19 min), **14** (12.33 min), **15** (22.00 min), **16** (32.21 min), **17** (26.36 min), **18** (15.91 min), **19** (30.91 min), **20** (28.68 min), **21** (30.44 min), **22** (18.71 min), **23** (13.29 min).

3.2.2. HPLC for isolation

A Bischoff HPLC pump model No. 2200 (Bischoff, Leonberg) with a UV-variable wavelength monitor detector and a 5 μm LiChrospher-100, RP 18-column (4×250 mm, Knauer) were used to separate metabolites. Metabolites were eluted with MeCN/water at a flow rate of 1.5 ml/min.

3.2.3. HPLC–^{1}H NMR (stopped-flow mode)

Reversed-phase HPLC was performed on a HP 1100 chromatography system (quarternary pump G1311A, autosampler G1313A) using a LiChrospher 100 RP-18 (5 μm) column (250×4 mm); gradient MeCN-D_2O (0.1% TFA): 0 min 9% MeCN, 35 min 91%, 40 min 100%, 45 min 9%; diode array detection from 254 to 400 nm, monitoring wavelength UV 284 nm; flow rate at 1 ml min^{-1}. The HPLC was connected to an Bruker Avance DRX 500 spectrometer. HPLC–^{1}H NMR spectra were measured in the stopped-flow mode at 500.13 MHz using a 4 mm inverse-detection LC probe (detection volume 120 μl). Double solvent suppression of MeCN and HDO of the MeCN–D_2O gradient was performed by presaturation applying standard Bruker pulse sequences. The suppressed signal of MeCN was set to δ 2.0 for calibration.

3.2.4. Conventional NMR

1D and 2D NMR spectra of isolated compounds were measured on a Bruker Avance DRX 500 spectrometer

at 500.13 MHz (^{1}H), using a 2.5 mm inverse-detection microprobe (*z*-gradient).

3.2.5. LC–MS

3.2.5.1. HPLC. Chromatographic separations were performed on a Hewlett-Packard HPLC-System (HP 1100) with a LiChrospher 100 RP-18, 5 μm, 2×150 mm column at a flow rate of 0.2 ml min^{-1}. Xanthohumol metabolites were separated with a linear solvent gradient starting from MeCN/water 17/83 v/v to MeCN/water 90/10 v/v over 35 min and then to MeCN/water 100/0 v/v during the next 5 min. At 45 min the MeCN/water was changed to 17/83 v/v within 5 min, and the column was equilibrated for 15 min prior to the next injection.

A UV-Variable wavelength monitor VWDG1314A detector (Agilent Technologies) was connected in line between the column and the electrospray MS.

3.2.5.2. MS detection. The mass selective detection was carried out on an Esquire LC ion trap (Bruker Daltonics GmbH). Electrospray ionisation was run in negative mode with a voltage of 4000 V, nebulizing gas of 42 psi, and drying gas of 10 l/min at 220 °C. MS spectra were recorded with a scan range from $m/z = 200$ to $m/z = 600$.

3.2.6. CC and TLC

CC was performed using silica gel (230–400 mesh). TLC was carried out with Merck precoated silica gel (60 F_{254}) and EtOAc/hexane (80/20) as solvent. The TLC plates were sprayed with anisaldehyde/sulfuric acid reagent and heated. Xanthohumol and its metabolites appeared as orange-yellow spots.

3.3. Animal experiments and extraction of the tests

Twelve female SD-rats (144–175 g body weight) were purchased from Charles River Wiga, Sulzfeld, Germany. The animals received 1000 mg/kg **1** suspended in 4% starch solution by gavage. Faeces were collected on ice for 24 h and from 6 rats additionally for the period from 24 to 48 h after the gavage. The samples were frozen and stored at −20 °C until extraction.

3.4. Extraction and Isolation

The total amount of faeces was 3.6 g dry weight (9.6 g wet weight), and were extracted with EtOAc/CH_2Cl_2/isopropanol 3:1:1 mixtures at room temp. The extract was concentrated under reduced pressure of 35 °C to give 0.45 g and chromatographed (solid phase extraction) SiO_2 gel chromatography (5 g, 2.5 cm diameter) with a gradient of *n*-hexane/EtOAc to give five fractions: KE1 (51.3 mg) [*n*-hexane/EtOAc (85/15, v/v)], KE2 (62.9 mg) [*n*-hexane/EtOAc (83/17, v/v)], KE3 (6.8 mg) [*n*-hexane/EtOAc (20/80, v/v)], KE4 (10.4 mg) [*n*-hexane/EtOAc (80/20, v/v)] and KE5 (28.6 mg) [*n*-hexane/EtOAc(20/80, v/v) followed by MeOH].

Further solid phase extraction and HPLC-analysis of five series yielded compounds **1**–**23**.

Fractionation of KE1 after solid phase extraction with *n*-hexane/EtOAc 15% and 18% was subjected to HPLC RP-18 using MeCN/H_2O (55/45, 65/35, v/v) as solvent system and afforded **20**, **21**, **19** and **6**.

Fractionation of KE2 eluted with *n*-hexane EtOAc **18** and 50% and HPLC RP-18 using MeCN/H_2O (50/50, v/v), afforded **17** and **1**. Fractionation of KE3 eluted with *n*-hexane EtOAc 18% and 80% and HPLC RP-18 using MeCN/H_2O (50/50, v/v), afforded **4**, **15** and **13**. Fractionation of KE4 eluted on HPLC RP-18 using MeCN/H_2O (37/63, v/v), afforded of **18**, **22**, **9**, **5**, **3**, **7** and **11**.

Fractionation of KE5 eluted on HPLC RP-18 using MeCN/H_2O (23/77, 35/65, v/v), afforded of **14**, **10**, **23**, **8** and **12**.

3.4.1. Chalcone *2*

UV(MeCN/H_2O) λ_{max} 371.5 nm; HPLC/MS (electrospray) $[M-H]^-$ m/z 371; ^{1}H NMR data see Table 1.

3.4.2. Chalcone *3*

UV (MeCN/H_2O) λ_{max} 371.5 nm; HPLC/MS (electrospray) $[M-H]^-$ m/z 369: ^{1}H NMR data see Table 1.

3.4.3. Chalcone *6*

UV (MeCN/H_2O) λ_{max} 364.3 nm; HPLC/MS (electrospray) $[M-H]^-$ m/z 367; ^{1}H NMR data see Table 2.

3.4.4. Chalcone *7*

UV (MeCN/H_2O) λ_{max} 355.1 nm; HPLC/MS (electrospray) $[M-H]^-$ m/z 395; ^{1}H NMR data see Table 2.

3.4.5. Chalcone *8*

UV (MeCN/H_2O) λ_{max} 315.9 nm; 255.6 nm *sh*; HPLC/MS (electrospray) $[M-H]^-$ m/z 369; ^{1}H NMR data see Table 2.

3.4.6. Flavonone *10*

UV (MeCN/H_2O) λ_{max} 291.0 nm; HPLC/MS (electrospray) $[M-H]^-$ m/z 371; ^{1}H NMR data see Table 3.

3.4.7. Chalcone *11*

UV (MeCN/H_2O) λ_{max} 364.3 nm; 252.1 nm *sh*; HPLC/MS (electrospray) $[M-H]^-$ m/z 367; ^{1}H NMR data see Table 4.

3.4.8. Chalcone *12*

UV (MeCN/H_2O) λ_{max} 344.4 nm; HPLC/MS (electrospray) $[M-H]^-$ m/z 369; ^{1}H NMR data see Table 4.

3.4.9. Chalcone ***13***

UV ($MeCN/H_2O$) λ_{max} 371.5 nm; HPLC/MS (electrospray) $[M-H]^-$ m/z 399; ^1H NMR data see Table 4.

3.4.10. Chalcone ***14***

UV ($MeCN/H_2O$) λ_{max} 330.1 nm; HPLC/MS (electrospray) $[M-H]^-$ m/z 385; ^1H NMR data see Table 4.

3.4.11. Chalcone ***15***

UV ($MeCN/H_2O$) λ_{max} 371.5 nm; HPLC/MS (electrospray) $[M-H]^-$ m/z 367; ^1H NMR data see Table 4.

3.4.12. Chalcone ***16***

UV ($MeCN/H_2O$) λ_{max} 364.3 nm, 252.1 *sh*; HPLC/MS (electrospray) $[M-H]^-$ m/z 351; ^1H NMR data see Table 4.

3.4.13. Chalcone ***17***

UV ($MeCN/H_2O$) λ_{max} 364.1 nm, 252.1 *sh*; HPLC/MS (electrospray) $[M-H]^-$ m/z 309; ^1H NMR data see Table 4.

3.4.14. Chalcone ***18***

UV ($MeCN/H_2O$) λ_{max} 337.2 nm; HPLC/MS (electrospray) $[M-H]^{btl}$ m/z 309; ^1H NMR data see Table 4.

3.4.15. Chalcone ***20***

UV ($MeCN/H_2O$) λ_{max} 364.3 nm, 255.6 *sh*; HPLC/MS (electrospray) $[M-H]^-$ m/z 381; ^1H NMR data see Table 5.

3.4.16. Chalcone ***21***

UV ($MeCN/H_2O$) λ_{max} 371.5 nm; HPLC/MS (electrospray) $[M-H]^-$ m/z 353; ^1H NMR data see Table 5.

3.4.17. Chalcone ***22***

UV ($MeCN/H_2O$) λ_{max} 330.1 nm; HPLC/MS (electrospray) $[M-H]^-$ m/z 353; ^1H NMR data see Table 5.

3.4.18. Chalcone 23

UV ($MeCN/H_2O$) λ_{max} 330.1 nm; HPLC/MS (electrospray) $[M-H]^-$ m/z 369; ^1H NMR data see Table 5.

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