Anti-inflammatory Acylphloroglucinol Derivatives from Hops (*Humulus lupulus*)

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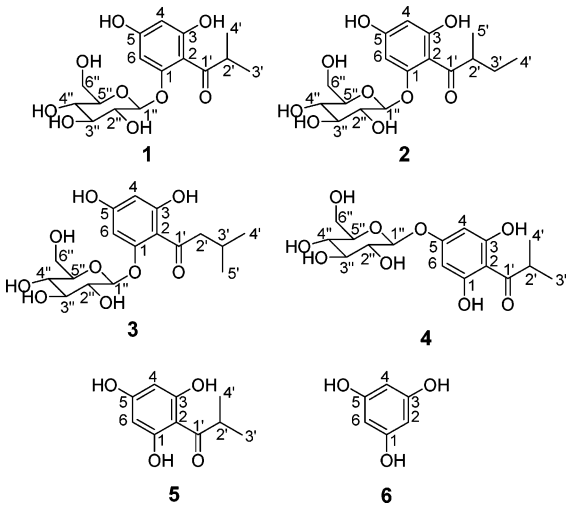
The polyphenol-enriched fraction of an ethanolic hops extract (*Humulus lupulus*) was separated to provide four acylphloroglucinol-glucopyranosides (**1**−**4**). 1-(2-Methylpropanoyl)phloroglucinol-glucopyranoside **1** has been isolated from hops before, whereas 1-(2-methylbutyryl)phloroglucinol-glucopyranoside **2**, known as multifidol glucoside, and 1-(3-methylbutyryl)phloroglucinol-glucopyranoside **3** were found in hops for the first time. 5-(2-Methylpropanoyl)phloroglucinol-glucopyranoside **4** was identified as a new natural product. The compounds were tested for inhibition of COX-1 activity. The aglycon **5**, obtained by acid hydrolysis of **1**, was equally effective as phloroglucinol, with an IC_{50} of 3.8 μM. The inhibitory potential of the glucosides was **1** > **2** > **3** and decreased with increasing length of the acyl side chain. Compound **4** was about 2.5-fold less active than **1** (IC_{50}: 23.7 and 58.7 μM, respectively).

Hops (*Humulus lupulus* L., Cannabinaceae) is an important source of phenolic constituents in beer. The dried hop cones contain 4−14% polyphenols, mostly phenolic acids, chalcones, prenylated flavonoids, catechins, and proanthocyanidins.[1−4] Hop products have been used over centuries almost exclusively in the brewing industry. They add aroma and bitterness to beer and provide antifungal and antibiotic properties.[5−7] These characteristics are mainly attributed to their content of α-acids such as humulone, which are converted during brewing to the bitter-tasting iso-α-acids. In recent years, hops have gained considerable interest because of the biological and potential cancer chemopreventive activities of some of its constituents (reviewed in refs 8, 9). As an example, the hop-derived prenylated chalcone, xanthohumol, was identified as a novel inhibitor of cyclooxygenase-1 (COX-1) by activity-guided fractionation of beer.[10] In continuation of these studies we here describe results of the phytochemical analysis and biological testing of a polyphenol-enriched hop extract, which led to the isolation and structure elucidation of four monoacylphloroglucinol-glucopyranosides (**1**−**4**) and one aglycon (**5**) with anti-inflammatory activity.

Fractionation of an ethanolic hop extract via size exclusion chromatography and preparative HPLC led to the isolation of four acylphloroglucinol-glucopyranosides (**1**−**4**). Additional phloroglucinol derivatives from hops have been described recently.[11]

Compound **1**, HRCIMS *m/z* 358.1278, was identified as 1-[(2-methylpropanoyl)phloroglucinyl]-β-D-glucopyranoside. Its ^{1}H NMR spectrum (Table 1) showed in the aliphatic region signals for a 2-methylpropanoyl moiety at δ 3.98, sept, 1.14 d (3H), and 1.13 d (3H) and a sugar moiety. In the aromatic region of the spectrum, two *meta*-coupled doublets appeared at δ 5.95 and 6.17, indicating an asymmetric substituted phloroglucinol moiety. Therefore, the sugar residue must be attached to C-1 of the phloroglucinol. The sugar moiety was identified by comparison of its ^{1}H and ^{13}C NMR data (Tables 1 and 2) with



those of known phloroglucinol-glucosides.[12] Compound **1** is a known constituent of hops,[13] but no NMR data have been published so far for this compound.

The NMR data of **2**, *m/z* 371.1421, were very similar to those of **1**, with only the values of the acyl moiety differing from **1**, indicating a 2-methylbutyryl moiety for compound **2** (Tables 1 and 2). The resulting 1-[(2-methylbutyryl)-phloroglucinyl]-β-D-glucopyranoside had been isolated initially as "multifidol glucoside" from the latex of *Jatropha multifida* L. (Euphorbiaceae) and shows interesting immunological activity.[12] Its ^{13}C NMR data fit very well with those of **2**, supporting the structure proposed. This is the first report of the occurrence of **2** in hops.

Compound **3** again differs from **1** only in the acyl moiety. In this case, NMR data were supportive of a 3-methylbutyryl moiety (Tables 1 and 2). The HREIMS with *m/z* 372.1412 supported the structure of 1-[(3-methylbutyryl)-phloroglucinyl]-β-D-glucopyranoside, which was described for the first time from strawberry fruit, *Fragaria ananassa* Duch.[14] Its aglycon, 2-(3-methylbutyryl)phloroglucinol, is a known ingredient of hops.[15]

The ^{1}H and ^{13}C NMR data of **4** (Tables 1 and 2) suggested a further acylphloroglucinol-glucoside. In contrast to compounds **1**−**3**, the chemical shifts of C-4 and C-6 (δ 96.6) as

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Table 1. ^{1}H NMR Data of Compounds **1**−**5** (in CD_3OD, 500 MHz)[a]

position	1	2	3	4	5
4	5.95 d (2.5)	5.94 d (2.5)	5.94 d (2.5)	6.08 s	5.80 s
6	6.17 d (2.5)	6.17 d (2.5)	6.17 d (2.5)	6.08 s	5.80 s
2′	3.98 sept (7.0)	3.90 m	3.17 dd (16.0/6.5)	3.97 sept (7.0)	3.96 sept (7.0)
			2.88 dd (16.0/7.5)		
3′	1.14 d (7.0, 3H)	1.80 m + 1.38 m	2.24 sept (7.0)	1.13 d (7.0, 3H)	1.12 d (7.0, 3H)
4′	1.13 d (7.0, 3H)	0.87 t (7.5, 3H)	0.96 d (7.0)	1.13d (7.0, 3H)	1.12 d (7.0, 3H)
5′		1.12 d (7.0, 3H)	0.93 d (7.0)		
1″	5.04 d (7.5)	5.03 d (7.5)	5.01 d (8.0)	4.91 d (7.5)	
2″	3.50 dd (9.0/7.5)	3.50 dd (9.0/7.5)	3.53 dd (9.0/7.5)	3.42 m	
3″	3.44 m	3.45 m	3.45 m	3.42 m	
4″	3.38 t (9.0)	3.38 t (9.0)	3.39 t (9.0)	3.38 m	
5″	3.44m	3.45 m	3.45 m	3.42 m	
6″ α	3.91 dd (12.3/2.0)	3.91 dd (12.3/2.0)	3.91 dd (12.3/2.0)	3.90 dd (12.3/2.0)	
6″ β	3.71 dd (12.3/5.5)	3.71 dd (12.3/5.5)	3.71 dd (12.3/5.5)	3.70 dd (12.3/5.5)	

[a] Chemical shifts δ_H (J in Hz).

Table 2. ^{13}C NMR Data of Compounds **1**−**5** (in CD_3OD, 125 MHz)[a]

position	1	2	3	4	5
1	161.6 (C)	161.8 (C)	162.2 (C)	165.2 (C)	165.7 (C)
2	106.2 (C)	106.8 (C)	107.0 (C)	106.3 (C)	104.6 (C)
3	167.5 (C)	167.4 (C)	167.6 (C)	165.2 (C)	165.7 (C)
4	98.3 (CH)	98.3 (CH)	98.3 (CH)	96.6 (CH)	95.9 (CH)
5	165.6 (C)	165.6 (C)	165.8 (C)	164.8 (C)	165.8 (C)
6	95.3 (CH)	95.3 (CH)	95.4 (CH)	96.6 (CH)	95.9 (CH)
1′	211.9 (C)	211.8 (C)	207.2 (C)	212.3 (C)	211.7 (C)
2′	40.4 (CH)	47.0 (CH)	54.2 (CH_2)	40.3 (CH)	39.9 (CH)
3′	20.2 (CH_3)	28.3 (CH_2)	26.2 (CH)	19.5 (CH_3)	19.6 (CH_3)
4′	19.5 (CH_3)	12.0 (CH_3)	23.4 (CH_3)	19.5 (CH_3)	19.6 (CH_3)
5′		16.8 (CH_3)	22.9 (CH_3)		
1″	101.5 (CH)	101.7 (CH)	101.9 (CH)	101.2 (CH)	
2″	74.8 (CH)	74.8 (CH)	74.8 (CH)	74.7 (CH)	
3″	78.7 (CH)	78.7 (CH)	78.6 (CH)	78.3 (CH)	
4″	71.2 (CH)	71.2 (CH)	71.2 (CH)	71.2 (CH)	
5″	78.4 (CH)	78.4 (CH)	78.4 (CH)	78.0 (CH)	
6″	62.5 (CH_2)	62.5 (CH_2)	62.5 (CH_2)	62.4 (CH_2)	

[a] Chemical shifts δ_C (mult.).

well as of H-4 and H-6 (δ 6.08) were identical. This was suggestive of symmetrical substitution of the aromatic ring moiety. The data for the acyl side chain were identical to those of **1**, revealing a 2-methylpropanoyl moiety. Therefore, **4** was assigned as 5-[(2-methyl-propanoyl)phloroglucinyl]-β-D-glucopyranoside. The HREIMS with *m/z* 358.1260 supports this structural assignment. Compound **4** is a new natural product.

Treatment of **1** with hydrochloric acid led to the loss of the sugar moiety and gave the expected aglycon **5**, HREIMS *m/z* 196.0736. Its NMR data (Tables 1 and 2) were in good agreement with the structure, 2-(2-methylpropanoyl)-phloroglucinol. The occurrence of compound **5** has been previously described for hops,[15] but spectroscopic data have not appeared in the literature.

The acyl side chains of compounds **1**−**5** are identical to those of the hops α-acids co-humulone, ad-humulone, and *n*-humulone. Therefore, by analogy with the findings of Zuurbier et al.,[16,17] we propose that **1**−**5** are formed from phloroisovalerophenone or phloroisobutyrophenone intermediates, which are generated by the enzyme phlorisovalerophenone synthase (VPS) during the biosynthesis of the hops bitter acids.[18] Accordingly, condensation of three malonyl-CoA units and one isobutyryl-CoA unit gives rise to phloroisobutyrophenone (**5**) as a precursor of co-humulone and compounds **1** and **4**, respectively. Condensation of three malonyl-CoA units and one isovaleryl-CoA unit generates phoroisovalerophenone as a precursor of *n*-humulone and compound **3**, and finally three malonyl-CoA units and one 2-methylbutyryl-CoA unit are condensed to the precursor of ad-humulone and compound **2**, respectively. The glycosylation of compounds **1**−**4** is mediated by glucosidases. We speculate that position C-1 is preferred to position C-5, but these observations require further biosynthetic studies. For compound **2**, the trivial names "multifidol" for the aglycon and "multifidol glucoside" are used.[12] According to the nomenclature of the hops bitter acids, we propose new trivial names for compounds **1**−**4**: "co-multifidol glucoside" for **1**, "ad-multifidol glucoside" for **2**, and "*n*-multifidol glucoside" for **3**. For compound **4**, we suggest "co-iso-multifidol glucoside" as a trivial name. In hops, the amount of ad-humulone is fairly constant (15%) and lower than that of co- and *n*-humulones (20−50% each in varying ratios).[5] In contrast, with the acylphloroglucinol derivates, we observed that the "co-" and the "ad-" forms were more abundant than the "*n*-" homologue (unpublished data).

The compounds were tested for inhibition of COX-1 activity using microsomes of ram seminal vesicles. COX-1 contains two catalytic sites, the cyclooxygenase site, which exists along a hydrophobic channel within the core of the protein, and a peroxidase site containing a heme moiety.[21,22] Using a Clark-style oxygen microelectrode, the oxygen consumption was measured to follow the bis-dioxygenation of arachidonic acid to yield prostaglandin G_2.[20] Dose−response curves for all acylphloroglucinol derivatives in comparison with phloroglucinol (**6**) are given in Figure 1. The aglycon **5** was as equally effective as phloroglucinol in inhibiting oxygen consumption, with a half-maximal inhibitory concentration (IC_{50}) of 3.8 μM. The insertion of a glucose moiety at position C-1 or C-5 to yield compounds **1** and **4**, respectively, reduced the COX-1 inhibitory potential about 6- and 15-fold, with IC_{50} values in the range of 23.7 and 58.7 μM. Elongation of the acyl

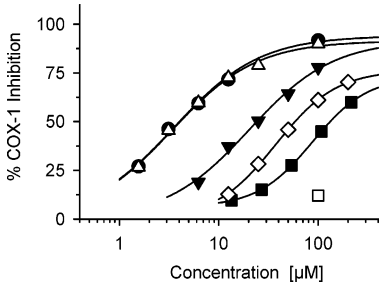

Figure 1. Inhibition of COX-1 activity by acylphloroglucinol derivatives, measured by inhibition of oxygen consumption during in vitro prostaglandin formation by COX-1. Dose-dependent inhibition by **1** (▼); **2** (■); **3** (□); **4** (◇); and **5** (●) in comparison with phloroglucinol (**6**) (△).

side chain with a methyl group in **2** further reduced the anti-inflammatory activity (IC_{50} = 131.3 μM), whereas the phloroisovalerophenone derivative **3** was basically inactive, with only 12% inhibition at a 100 μM concentration (IC_{50} > 100 μM). From these data, it was concluded that (i) phloroglucinol (**6**) is a good COX-1 inhibitor, (ii) substitution of compound **5** with the short 2-methylpropanoyl moiety at position C-2 does not reduce the inhibitory potential, (iii) further addition of a sugar moiety in position C-1 reduces the IC_{50} value of **5** about 6-fold, (iv) glycosylation at position C-5 reduces the inhibitory potential more than addition of a sugar moiety at position C-1, and (v) modifications of the acyl side chain severely lower the COX-1 inhibitory potential.

The anti-inflammatory potential of phloroglucinol derivatives has been reported before. For example, hyperforin, the major lipophilic constituent in *Hypericum perforatum* L. (St. John's wort), was described as a dual inhibitor of COX-1 and of lipoxygenase-5.[23] Szewczuk and Penning recently identified resorcinol as the minimum structure necessary for inactivation of COX-1.[24] The proposed mechanism involved inhibition of the peroxidase activity of COX-1, which is required to initiate the cyclooxygenase activity. Partial oxidation of the *m*-hydroquinone moiety within the compounds would then inactivate the cyclooxygenase activity.[25] Access to the cyclooxygenase site within the hydrophobic channel might discriminate the derivatives depending on their molecular structure and overall size. This could explain the lower activity of the glycosides tested in comparison with the aglycon **5** or with phloroglucinol (**6**).

In conclusion, we have isolated and structurally characterized acylphloroglucinol derivatives from hops, which were identified as novel inhibitors of COX-1. Our investigations provide some suggestions with respect to a structure−activity relationship; however, for a precise study, more derivatives should be tested.

Experimental Section

General Experimental Procedures. Optical rotations were measured with a Perkin-Elmer 241 polarimeter at 20 °C. IR spectra were recorded on a Bio Rad FTS3000 Excalibur S-Series infrared spectophotometer. NMR spectra were recorded on a Bruker Avance 500 and a Bruker Avance DRX 500 spectrometer in CD_3OD. Mass spectra were measured on a Finnigan MAT 90 mass spectrometer. Analytical HPLC was performed on a Waters Alliance 2690 separation module with a PDA-detector (Waters, Milford, MA) using an acetonitrile/water gradient. Column: RP-18ec (EC 250/4 Nucleosil 100-5 C_{18} Hop, Macherey-Nagel, Düren, Germany).

Plant Material. The (poly-)phenol-enriched fraction of an ethanolic hops extract was produced of hop variety "Hallertauer Perle" as described in ref 19 and supplied by Simon H. Steiner Hopfen GmbH, D-84048 Mainburg.

Extraction and Isolation. The aqueous extract (500 g) was partitioned with *n*-hexane (6 × 0.5 L) to remove residues of hop bitter acids, such as humulones and lupulones. Afterward, the aqueous layer was partitioned with ethyl acetate (EE) (6 × 0.5 L). The yield of the EE fraction (EE00) after evaporating the solvent was 4.42 g. EE00 (3.00 g) was separated by column chromatography using Sephadex LH-20 by isocratic elution with 100% methanol. Thereby, 12 fractions were obtained: EE01 (83 mg), EE02 (662 mg), EE03 (142 mg), EE04 (1303 mg), EE05 (102 mg), EE06 (129 mg), EE07 (88 mg), EE08 (179 mg), EE09 (50 mg), EE10 (17 mg), EE11 (48 mg), EE12 (50 mg). HPLC separation and UV spectra indicated the presence of acylphloroglucinols in fraction EE04. Therefore, this fraction (450 mg) was further separated by preparative HPLC.

HPLC was performed on an RP-18ec column (VP 250/10 Nucleodur 100-5 C18 ec, Macherey-Nagel, Düren, Germany) using acetonitrile/water (25:75) with 0.05% TFA. The solvent delivery system was a Waters M-45 (Waters, Milford, MA). Peaks were detected with an RI-Detector (RI-Detector 8110, Bischoff, Leonberg, Germany) and collected to yield 207 mg of compound **1**, 30 mg of **2**, 10 mg of **3**, and 7 mg of **4**.

1-[(2-Methylpropanoyl)phloroglucinyl]-*β*-D-glucopyranoside (1): white powder; $[\alpha]_D^{20}$ −55.6° (MeOH); IR (KBr) ν_{max} 3383 (OH), 1600 (C=O) cm^{-1}; ^1H and ^{13}C NMR data, see Tables 1 and 2; HRCIMS *m/z* 358.1278 (calcd for $C_{16}H_{22}O_9$, 358.1264).

1-[(2-Methylbutyryl)phloroglucinyl]-*β*-D-glucopyranoside (2): white powder; $[\alpha]_D^{20}$ −54.3° (MeOH); IR (KBr) ν_{max} 3386 (OH), 1601 (C=O) cm^{-1}; ^1H and ^{13}C NMR data, see Tables 1 and 2; HRCIMS *m/z* 372.1421 (calcd for $C_{17}H_{24}O_9$, 372.1420).

1-[(3-Methylbutyryl)phloroglucinyl]-*β*-D-glucopyranoside (3): white powder; $[\alpha]_D^{20}$ −58.8° (MeOH); IR (KBr) ν_{max} 3396 (OH), 1600 (C=O) cm^{-1}; ^1H and ^{13}C NMR data, see Tables 1 and 2; HREIMS *m/z* 372.1412 (calcd for $C_{17}H_{24}O_9$, 372.1420).

5-[(2-Methylbutyryl)phloroglucinyl]-*β*-D-glucopyranoside (4): beige powder; $[\alpha]_D^{20}$ −17.4° (MeOH); IR (KBr) ν_{max} 3391 (OH), 1608 (C=O) cm^{-1}; ^1H and ^{13}C NMR data, see Tables 1 and 2; HREIMS *m/z* 358.1260 (calcd for $C_{16}H_{22}O_9$, 358.1264).

Acid Hydrolysis of 1 to Generate 5. A 125 mg aliquot of compound **1** was dissolved in a mixture of 20 mL of water and 20 mL of hydrochloric acid (2 N) and heated under reflux for 30 min. On cooling, the mixture was partitioned with ethyl acetate (5 × 20 mL), dried over Na_2SO_4, and filtered over a paper filter that was wet with ethyl acetate. Evaporation of solvent yielded 60 mg of a yellowish oil, which was then purified using HPLC as described above. The yield of **5** was 28 mg.

2-(2-Methylpropanoyl)-1,3,5-benzenetriol (5): yellowish oil; $[\alpha]_D^{20}$ ±0.0° (MeOH); IR (KBr) ν_{max} 3319 (OH), 1601 (C=O) cm^{-1}; ^1H and ^{13}C NMR data, see Tables 1 and 2; HREIMS *m/z* 196.0736 (calcd for $C_{10}H_{12}O_4$, 196.0735).

Inhibition of Cyclooxygenase-1 Activity. Inhibition of cyclooxygenase-1 (COX-1) activity was measured by monitoring oxygen consumption during the conversion of arachidonic acid to prostaglandins using a Clark-type O_2-electrode (Hansatech Ltd., Kings Lynn, U.K.).[10,20] The reaction mixture contained approximately 0.5 U COX-1 in a 100 μL microsome fraction, prepared from ram seminal vesicles as a crude source of COX-1 (specific activity 0.2−1 U/mg protein). For calculation, the rate of O_2 consumption was compared to a DMSO control (100% activity). Phloroglucinol (**6**) was tested as a reference compound.

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