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ANALYSIS OF PROPOSED AROMATIC PRECURSORS OF HOP BITTER ACIDS

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ABSTRACT.—Four proposed precursors of hop bitter acids, 2-acyl-1,3,5-benzenetriol and 2-acyl-4-(3-methyl-2-butenyl)-1,3,5-benzenetriol (acyl = 2-methylpropanoyl or 3-methylbutanoyl), were synthesized (compounds **7–10**). Tlc, hplc and gc systems were developed for the analysis of these compounds in the presence of the hop bitter acids **1–6**. All four precursors were found to be present in cone extracts of several hop cultivars.

A characteristic of the hop plant, *Humulus lupulus* L. (Cannabinaceae), is the accumulation of bitter acids in its ripe cones. These compounds are derivatives of 1,3,5-benzenetriol (=phloroglucinol), with an acyl group and two or three 3-methyl-2-butenyl (=prenyl) side-chains as substituents in the aromatic nucleus; bitter acids consist of α-acids, mainly cohumulone **1**, humulone **2**, and adhumulone **3**, and β-acids, chiefly colupulone **4**, lupulone **5**, and adlupulone **6**. According to Drawert and Beier (1), 2-(2-methylpropanoyl)-4-(3-methyl-2-butenyl)-1,3,5-benzenetriol [**9**] and 2-(3-methylbutanoyl)-4-(3-methyl-2-butenyl)-1,3,5-benzenetriol [**10**] are the first aromatic intermediates formed in the biosynthesis of the bitter acids **1** and **4**, and **2** and **5**, respectively. However, the isolation of a glycoside of 2-(2-methylpropanoyl)-1,3,5-benzenetriol from hop cones (2) indicates that the aromatic ring is probably formed first during biosynthesis, which is then prenylated to yield the various bitter acids. Aromatic rings in flavonoids are formed through the action of the enzyme chalcone synthase from the substrates 4-coumaroyl-CoA and malonyl-CoA (3). Because it has been shown that chalcone synthase also catalyzes the formation of acylphloroglucinols from aliphatic starter molecules (4), we think that this enzyme is also involved in the biosynthesis of hop bitter acids.

For the study of the aromatic precursors of the main α- and β-acids, suitable methods to analyze small amounts of these metabolites in extracts are needed. First we synthesized 2-(2-methylpropanoyl)-1,3,5-benzenetriol [**7**], 2-(3-methylbutanoyl)-1,3,5-benzenetriol [**8**] and their prenylated derivatives **9** and **10**. We then developed chromatographic systems for the analysis of these compounds by modifying assays which are employed in the study of hop bitter acids. Subsequently, the analytical methods were used for the screening of extracts of several hop cultivars for compounds **7–10**.

RESULTS AND DISCUSSION

The synthesis of 2-acyl-1,3,5-benzenetriol and its prenylated derivatives has been studied before (5–9). We obtained **7** and **8** in satisfactory yields (35–38%) by a Friedel-Crafts reaction between phloroglucinol and the acid chlorides of 2-methylpropanoic acid and 3-methylbutanoic acid in a carbon disulphide-nitrobenzene mixture in the presence of aluminum trichloride (6). We found that crystallization of the products from H_2O (pH <2) was improved by removing interfering impurities by means of activated carbon. Different solvents can be used for the prenylation of **8** by 3-methyl-2-butenyl bromide

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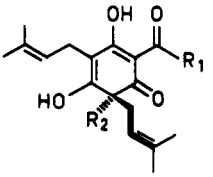
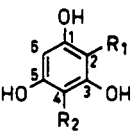


	R_1	R_2
1	$-CH(CH_3)_2$	$-OH$
2	$-CH_2-CH(CH_3)_2$	$-OH$
3	$-CH(CH_3)-CH_2-CH_3$	$-OH$
4	$-CH(CH_3)_2$	$-CH_2-CH=C(CH_3)_2$
5	$-CH_2-CH(CH_3)_2$	$-CH_2-CH=C(CH_3)_2$
6	$-CH(CH_3)-CH_2-CH_3$	$-CH_2-CH=C(CH_3)_2$



	R_1	R_2
7	$-C(=O)-CH(CH_3)_2$	$-H$
8	$-C(=O)-CH_2-CH(CH_3)_2$	$-H$
9	$-C(=O)-CH(CH_3)_2$	$-CH_2-CH=C(CH_3)_2$
10	$-C(=O)-CH_2-CH(CH_3)_2$	$-CH_2-CH=C(CH_3)_2$

under alkaline conditions (5,7). When we carried out this reaction using toluene, a complex mixture of **10** and several polyprenylated products was obtained. Prenylation of **7** and **8** in H_2O led to a better result. During the reaction a precipitate was formed, which consisted mainly of **9** or **10**, respectively. The precipitate obtained after acidification also contained the original 2-acyl-1,3,5-benzenetriol. Purification of **10**, collected without acidification, was less laborious than the workup of **9**, that was obtained in a crude mixture after acidification.

Compounds **7–10** were identified by uv, ^{1}H-nmr, and mass spectrometry. We found that, due to keto-enol tautomerism and exchange of the aromatic protons with deuterium from deuterated protic solvents, the signals of the aromatic protons in the nmr spectra were only partially recorded. These signals were fully observed when an aprotic solvent like DMSO was used. Mass spectra of the four synthesized phloroglucinol derivatives have not been reported before. They were studied by gc-ms and lc-ms. In gc-ms, the M^+ peak and a base peak at *m/z* 153, probably corresponding to a fragment resulting from cleavage in the acyl chain with retention of the carbonyl group, are observed for both **7** and **8**. The M^+ peak, an ion at *m/z* 221, probably resulting from a similar reaction as mentioned above for **7** and **8**, and a base peak at *m/z* 165, are characteristic of the mass spectra of **9** and **10**. In lc-ms employing a thermospray interface, only $[M+H]^+$ peaks are recorded for the phenolics **7–10**. Fragmentation of these compounds is induced by lc-ms/ms. For the fragmentation of **7** and **8** more energy (20 V) is required than for their prenylated analogues **9** and **10** (10 V). Loss of H_2O, rearrangement, and further degradation of **7** and **8** yield a fragment ion at *m/z* 151 for both compounds. In the fragmentation of **9** and **10**, a cleavage in the prenyl side-chain is probably involved, resulting in the formation of ions at *m/z* 209 and 223, respectively. The formation of these specific fragments from the compounds **7–10** in ms/ms experiments was recorded by selective-reaction monitoring (srm).

The four proposed precursors **7–10** were analyzed with tlc and hplc systems (10–12) routinely employed at our laboratory for the analysis of hop bitter acids. In tlc, group separation of **7–10** from the bitter acids is observed. When analyzed as a mixture, the four compounds are observed as two zones, **7** plus **8**, and **9** plus **10**, respectively. The same is true for the bitter acids, where no distinction can be made between the individual components of the α- and β-acids (10). The results in hplc were more satisfactory. By employing a reversed-phase C_{18} column and a gradient mixture of MeOH-H_2O-85% H_3PO_4 (700:300:2.5) to 100% MeOH, all four compounds **7–10** are well separated from the bitter acids **1–6**. Additional information on the identity of the chromophores from the bitter acids and their precursors was obtained by using a photodiode array detector (pda). In our work on the precursors of the hop bitter acids, analysis by lc-ms proved to be useful for the identification of small amounts of the potential intermediates.

Hop bitter acids are known as labile compounds that are not suitable for gc analysis (13), although gc of the bitter acids and their precursors **9** and **10** seems possible on packed columns after silylation (14). We decided to examine the behavior of hop bitter acids and their possible precursors in a fused-silica capillary column which is more inert towards unstable compounds. In our experiments, decomposition of α-acids took place during gc, leading to the formation of products which masked the peaks for **9** and **10**. However, the compounds **7–10** could be analyzed directly without derivatization, although the peaks for **7** and **8** were broad and showed tailing, probably due to adsorption of these polar compounds. Gc-ms also was shown to be suitable for the analysis of the four possible precursors in hop extracts.

Cones of several hop cultivars were extracted with MeOH. After some clean-up steps to remove neutral and alkaline fatty substances, the residues of these extracts were

resuspended in MeOH and analyzed by hplc. These samples consisted mainly of the α- and β-acids **1–6** and contained also possibly small amounts of compounds **7–10** (Figure 1). The extracts were further purified by solid-phase extraction, using a C_{18} column, to

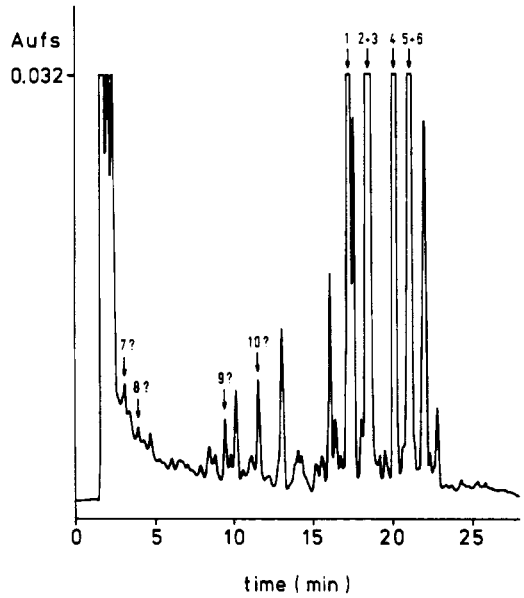


FIGURE 1. Hplc of a cone extract of hop cultivar "Hallertau mittelfrüh." Sample: 10 µl. Detection: 290 nm. For other conditions, see Experimental.

remove the bitter acids which interfere with the gc analysis of intermediates **9** and **10**. The more polar compounds **7–10** in the extracts were separated from the apolar bitter acids, as they were eluted from the column by a 80% MeOH mixture while the bitter acids remained adsorbed to the C_{18} column. Although a loss of the potential precursors took place during the purification, extracts were obtained which were suitable for further studies by hplc and gc. The recoveries of 0.2 mg of precursor added to a 30 ml MeOH cone extract were for compound **7**: 49±5% (n=4), for compound **8**: 37±8% (n=5), for compound **9**: 21±8% (n=5), and for compound **10**: 25±9% (n=5). By means of hplc-pda, gc-ms, lc-ms and lc-ms/ms all four precursors were found to be present in hop cone extracts (Figures 2 and 3).

In total, cone extracts of eight hop cultivars were screened for compounds **7–10**. The results of the analyses by hplc-pda, gc-ms, lc-ms and/or lc-ms/ms are summarized in Table 1. Compounds **9** and **10** were found in the extracts of six cultivars. The acylphloroglucinols **7** and **8** occurred in trace amounts, but were detected with certainty in extracts of four cultivars. The aqueous phase of the extracts of the cultivars "Hallertau mittelfrüh" and "Yeoman" were subjected to acid hydrolysis to test whether the precursors were also present as glycosides, but **7–10** were not detected in the organic extract of the H_2O layer. Probably due to their occurrence in small amounts, the metabolites **7** and **8** were not detected in hop cones in a previous study on the biosynthesis of hop bitter acids (15). We conclude from our screening that the acylphloroglucinols **7** and **8** can indeed be the first aromatic intermediates formed in the biosynthesis of hop bitter acids. In further studies we will examine the enzyme(s)

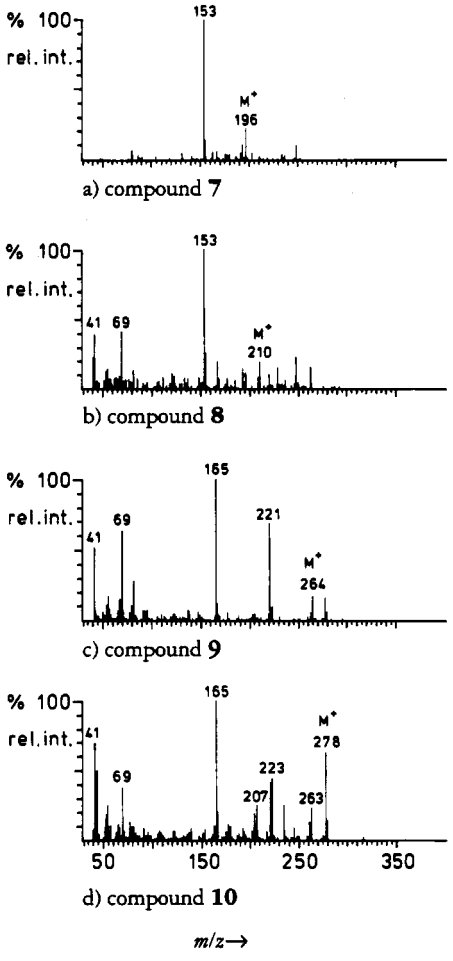


FIGURE 2. Mass spectra obtained by gc-ms of a purified cone extract of hop cultivar "Hallertau mittelfrüh." For conditions, see Experimental.

involved in this biosynthesis in order to test whether the aromatic precursors are formed through catalysis by chalcone synthase.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—A supercritical CO_2 extract of cones of *Humulus lupulus*, provided by L.C. Verhagen, was used as a reference mixture for hop α- and β-acids. Mps are uncorrected. Uv spectra were recorded on a DMS 80 Varian spectrophotometer. ^{1}H-Nmr spectra were recorded at 200 MHz with TMS as internal reference on a JEOL FX-200. Tlc was carried out according to (10) on Si gel plates using a mixture of cyclohexane-EtOAc-propionic acid (60:38:2) as eluent; phenolic compounds were detected at uv 254 nm and after spraying with vanillin/H_2SO_4. The hplc system consisted of a Pharmacia LKB LCC 2252 controller, a Pharmacia LKB low-pressure mixer, an LKB 2150 pump, a Rheodyne 7125 loop (20 μl), a Hypersil ODS column from Shandon (250 mm×4.6 mm i.d., 5 μm) with an Upchurch pre-column (C_{18}; 30 μm), an LKB 2151 uv detector (set at 290 nm), and a Shimadzu CR 501 chromatographic data processor. Gradient elution was performed with MeOH-H_2O-85% H_3PO_4 (700:300:2.5) to 100% MeOH in 25 min, and a flow rate of 1.1 ml·min^{-1} was used. Hplc-pda was carried out with a Waters 712 WISP, a Waters 600E system controller and a Waters 991 pda. The hplc system described above was also used for lc-ms, except for the following modifications: high-pressure mixing was applied by employing two LKB 2150 pumps, an LKB 2152 LC controller was used and no pre-column was employed. Gradient elution

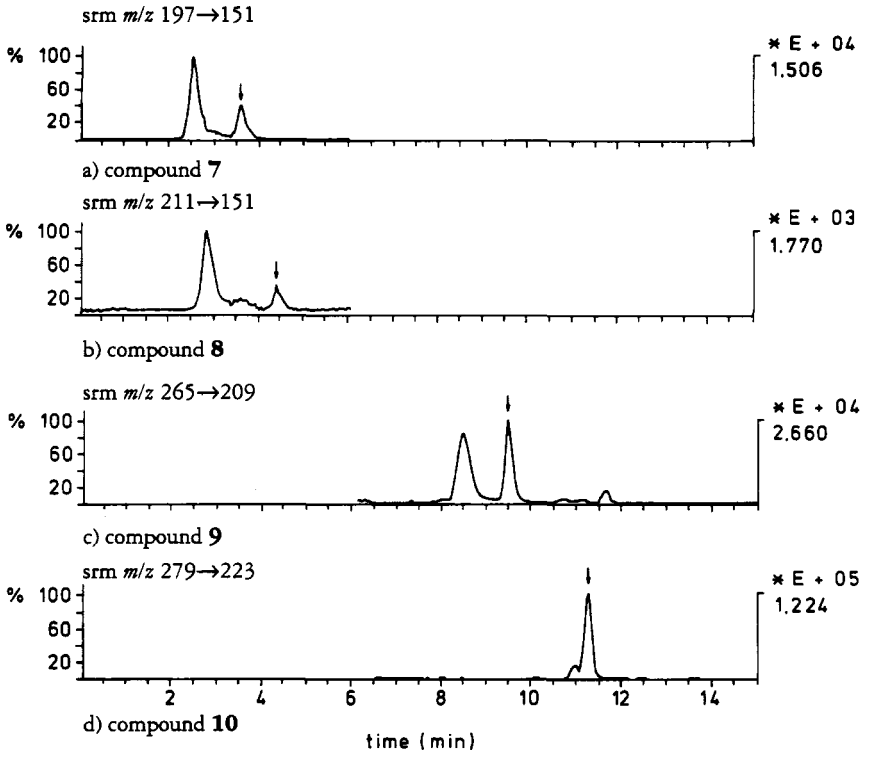


FIGURE 3. Lc-ms/ms by selective reaction monitoring (srm) of a purified cone extract of hop cultivar "Hallertau mittelfrüh." For conditions, see Experimental.

was performed using mixtures of MeOH-0.05 M NH_4Ac (pH 3.5) (7:3) and MeOH. The flow rate was 1.2 $ml \cdot min^{-1}$. Lc-ms was carried out on a Finnigan MAT TSQ-70 triple quadrupole mass spectrometer equipped with a Finnigan MAT thermospray interface in discharge-on mode (1 kV). Vaporizer and source temperatures were 75° and 250°, respectively. Repeller voltage was 70 V. In the ms/ms experiments air was used as collision gas at 0.5 Pa. The optimized collision energy for the different compounds was 10 or 20 V. Product-ion spectra were acquired under these conditions using pure samples of **7–10**. In the analysis of hop extracts an srm procedure was applied: during the first 6 min of elution the reactions *m/z* 197→*m/z* 151 (for **7**) and *m/z* 211→*m/z* 151 (for **8**) (selective precursor-scan mode with product-ion *m/z* 151) were monitored, while thereafter the reactions *m/z* 265→*m/z* 209 (for **9**) and *m/z* 279→*m/z* 223 (for **10**) (selective neutral loss-scan mode with a constant neutral loss of 56 daltons) were observed. Gc was carried out on a Chrompack CP 9000 gas chromatograph equipped with a Chrompack WCOT CP-Sil 5 cb fused-silica capillary column (10 m×0.25 mm i.d., film thickness 0.13 μm) and connected to a Shimadzu C-R3A

TABLE 1. Presence of Compounds **7–10** in Extracts From Hop Cones.

Cultivar	Compound				
	Voucher No.	**7**	**8**	**9**	**10**
Humulus lupulus "Hallertau mittelfrüh" ...	880500 E	+	+	+	+
H. lupulus "Yeoman"	880500 L	+	+	+	+
H. lupulus "Eroica"	880500 C	−	−	+	+
H. lupulus "Hersbrücker spät".....	880500 F	+	+	+	+
H. lupulus "Cobbs Goldings"	910044 II	?	?	+	+
H. lupulus "Brewers Gold"	910044 A	?	−	?	?
H. lupulus "Olympic"	910044 J	+	+	+	+
H. lupulus "Fuggle"	910044 EE	?	−	+	?

chromatographic data processor. N_2 at 0.9 ml·min^{-1} (50 kPa) was used as carrier gas, the split ratio was 1:30, the injector temperature 220°, and the fid was set at 240°. An oven temperature program was employed: 100°–5°·min^{-1}–230° (15 min). Gc-ms was carried out on a Packard 438 gas chromatograph coupled to a Finnigan MAT ion trap detector. He (50 or 100 k Pa) was used as carrier gas, the split ratio was 1:40. The temperature of the transfer line was 250°. The scan range was from *m/z* 40 to 435, the scan time 1 sec.

2-(-2-Methylpropanoyl)-1,3,5-benzenetriol [**7**].—$C_{10}H_{12}O_4$. Synthesized according to (6). Yellow needles. Yield: 38% (2×recrystallized; hplc: 98% pure). Mp 138°–139° [lit. 138° (4)]; uv λ max (MeOH) 209, 228, 288 (log ε 4.1) nm; tlc R_f 0.32, orange after spraying; hplc R_t 3.2 min; gc R_t 1954; gc-ms *m/z* 196 $[M]^+$ (17), 153 $[M-C_3H_7]^+$ (100), 69 (18), 41 (23); ms/ms *m/z* 197 $[M+H]^+$ (100), 179 $[M-H_2O+H]^+$, 151 $[M-H_2O-CO+H]^+$; ^{1}H nmr (DMSO) δ 1.07 (6H, d, *J*=7.0 Hz, H-3′ and H-4′), 3.87 (1H, m, H-2′), 5.81 (2H, s, H-4 and H-6).

2-(3-Methylbutanoyl)-1,3,5-benzenetriol [**8**].—$C_{11}H_{14}O_4$. Synthesized according to (6). Yellow crystals. Yield: 35% (2×recrystallized; hplc: 97% pure). Mp 144°–145° [lit. 145° (4)]; uv λ max (MeOH) 211, 227, 288 (log ε 4.2) nm; tlc R_f 0.34, orange after spraying; hplc R_t 4.0 min; gc R_t 2052; gc-ms *m/z* 210 $[M]^+$ (12), 195 $[M-CH_3]^+$ (12), 153 $[M-C_4H_9]^+$ (100), 69 (23), 41 (28); ms/ms *m/z* 211 $[M+H]^+$, 193 $[M-H_2O+H]^+$, 155 (100), 151 $[M-H_2O-COCH_2+H]^+$; ^{1}H nmr (DMSO) δ 0.91 (6H, d, *J*=6.6 Hz, H-4′ and H-5′), 2.14 (1H, m, H-3′), 2.86 (2H, d, *J*=6.6 Hz, H-2′), 5.79 (2H, s, H-4 and H-6).

2-(2-Methylpropanoyl)-4-(3-methyl-2-butenyl)-1,3,5-benzenetriol [**9**].—$C_{15}H_{20}O_4$. KOH (0.95 g; 15 mmol) was dissolved in 40 ml H_2O under stirring, while Ar was passed through the solution. Then, 2.2 g of **7** (10 mmol) was dissolved in this solution, which was then cooled to 0° and shielded from light. Next, 1.5 g of 3-methyl-2-butenyl bromide (10 mmol) was added slowly. A yellow precipitate gradually formed. The mixture, cooled to 0°, protected from light and kept under Ar, was left stirred for 22 h. The suspension was then acidified with concentrated HCl to pH<2, whereupon more precipitate was formed. The precipitate was filtered and dissolved in 50 ml Et_2O. The organic layer was washed with H_2O (pH<2), dried over $MgSO_4$ and concentrated. The resulting red oil was purified by column chromatography over Si gel (twice). The column was eluted with petroleum ether 40°–60°, Et_2O (8:2), (7:3), and (6:4). Compound **9** was mainly eluted in the (6:4) fractions. Fractions containing **9** as main component were combined and concentrated. Compound **9** was recrystallized from $CHCl_3$/pentane. Yellow crystals. Yield: 7% (hplc: 99% pure). Mp 159°–160°; uv λ max (MeOH) 211, 227, 292 (log ε 4.1), 340 (sh) nm; tlc R_f 0.37, orange-red after spraying; hplc R_t 9.6 min; gc R_t 2199; gc-ms *m/z* 264 $[M]^+$ (33), 221 $[M-C_3H_7]^+$ (63), 165 (100), 69 $[C_5H_9]^+$ (23), 55 $[C_4H_7]^+$ (15), 41 (38); ms/ms *m/z* 265 $[M+H]^+$, 209 $[M-CH_2C(CH_3)_2+H]^+$ (100), 191 $[M-CH_2C(CH_3)_2-H_2O+H]^+$; ^{1}H nmr (DMSO) δ 1.07 (6H, d, *J*=6.8 Hz, H-3′ and H-4′), 1.60 and 1.68 (2×3H, 2×s, H-4″ and H-5″), 3.07 (2H, d, *J*=7.0 Hz, H-1″), 3.90 (1H, m, H-2′), 5.11 (1H, t, *J*=7.0 Hz, H-2″), 5.99 (1H, s, H-6).

2-(3-Methylbutanoyl)-4-(3-methyl-2-butenyl)-1,3,5-benzenetriol [**10**].—$C_{16}H_{22}O_4$. See under **9** with following modifications: 2.1 g of **8** (10 mmol) were dissolved in 80 ml H_2O. At the end of the reaction, the precipitate was collected without acidification. The oil with **10** as main product was purified once by column chromatography. Yellow crystals. Yield: 13% (hplc: 99% pure). Mp 138.5°–139.5° [lit. 139°–140° (7)]; uv λ max (MeOH) 208, 226, 292 (log ε 4.2), 340 (sh) nm; tlc R_f 0.38, orange-red after spraying; hplc R_t 11.7 min; gc R_t 2294; gc-ms *m/z* 278 $[M]^+$ (66), 263 $[M-CH_3]^+$ (22), 223 (42), 221 $[M-C_4H_9]^+$ (46), 165 (100), 69 $[C_5H_9]^+$ (33), 55 $[C_4H_7]^+$ (24), 41 (68); ms/ms *m/z* 279 $[M+H]^+$, 223 $[M-CH_2C(CH_3)_2+H]^+$ (100), 205 $[M-CH_2C(CH_3)_2-H_2O+H]^+$; ^{1}H nmr (DMSO) δ 0.88 (6H, d, *J*=6.4 Hz, H-4′ and H-5′), 1.57 and 1.65 (2×3H, 2×s, H-4″ and H-5″), 2.11 (1H, m, H-3′), 2.83 (2H, d, *J*=6.6 Hz, H-2′), 3.04 (2H, d, *J*=7.0 Hz, H-1″), 5.08 (1H, t, *J*=7.0 Hz, H-2″), 5.95 (1H, s, H-6).

EXTRACTION AND PURIFICATION OF PLANT EXTRACTS.—Hop plants were grown in the experimental garden of the Division of Pharmacognosy. Voucher specimens are deposited at the Rijksherbarium, Leiden University. After harvest, hop cones were stored at −80° until use. Quantities (1–10 g) of hop cones were extracted with 50 ml MeOH by grinding with an ultra-turrax and subsequent sonification of the suspension for 5 min. After filtration, the filtrate was concentrated *in vacuo* to ca. 5 ml, after which 10 ml of H_2O was added and the aqueous phase was made alkaline by 1 N NaOH to pH 11. The H_2O layer was extracted with 20 ml petroleum ether 40°–60°, acidified with 6 N HCl to pH 1 and extracted twice with 20 ml EtOAc. The EtOAc layers were combined, dried over anhydrous Na_2SO_4, and concentrated *in vacuo*. The residue was redissolved in 2.0 ml of MeOH. A (1:10) dilution of a part of this solution was used for analysis by hplc. To obtain purified extracts without bitter acids, 1.6 ml of the MeOH solution was mixed with 0.1 ml H_2O, 0.1 ml 6 N HCl, and 0.2 ml Na_2EDTA (1 mg·ml^{-1}) and applied onto a 3 ml disposable C_{18} column (J.T. Baker), conditioned by washing with 3 ml H_2O and 3 ml 80% MeOH containing 0.1 mg·ml^{-1} Na_2EDTA and 0.3 N HCl. A teflon liner was used. The column was sucked dry and washed twice with 0.5 ml 80% MeOH containing 0.1 mg·ml^{-1} Na_2EDTA and 0.3 N HCl. The eluates were combined and concentrated to a small volume. The H_2O layer was then extracted 3 times with an equal volume of Et_2O. The organic

layers were combined and concentrated *in vacuo*. The residue was resuspended in 1.0 ml of MeOH and analyzed by hplc and gc.

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