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Prenylflavonoid variation in *Humulus lupulus*: distribution and taxonomic significance of xanthogalenol and 4′-*O*-methylxanthohumol

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Abstract

The resins produced by either lupulin or leaf glands of over 120 plants of *Humulus lupulus* and one plant of *H. japonicus* (Cannabinaceae) were analyzed for the presence of prenylated flavonoids. The *H. lupulus* taxa investigated were *H. lupulus* var. *lupulus* from Europe, *H. lupulus* var. *cordifolius* from Japan, and *H. lupulus* from North America. Fifty-two of the plants examined were cultivars of European, American, and Japanese origin. Twenty-two flavonoids were detected in the glandular exudates of *H. lupulus* by HPLC-MS–MS. Xanthohumol (3′-prenyl-6′-*O*-methylchalconaringenin) was the principal prenylflavonoid in all *H. lupulus* plants and was accompanied by 11 structurally similar chalcones. Ten flavonoids were identified as the flavanone isomers of these chalcones. Three other prenylchalcones were isolated from *H. lupulus* cv. 'Galena', one of which was identified as 3′-prenyl-4′-*O*-methylchalconaringenin (named 'xanthogalenol'). The distribution of three 4′-O-methylchalcones, i.e. xanthogalenol, 4′-*O*-methylxanthohumol, and 4′,6′-di-*O*-methylchalconaringenin, was found to be limited to wild American plants from the Missouri–Mississippi river basin, *H. lupulus* var. *cordifolius*, and most of their descendents. These 4′-*O*-methylchalcones were absent from cultivars of European origin, and from wild hops from Europe and southwestern USA. The flavonoid dichotomy (presence versus absence of 4′-*O*-methylchalcones) indicates that there are at least two evolutionary lineages within *H. lupulus* (European and Japanese–American), which is in agreement with morphological, molecular, and phytogeographical evidence. Leaf glands of *H. japonicus* from eastern Asia did not produce the *H. lupulus* prenylflavonoids.

Keywords: *Humulus lupulus*; *H. japonicus*; Cannabinaceae; Hops; Prenylated flavonoids; Xanthohumol; Xanthogalenol; Mass spectrometry; Chemotaxonomy

1. Introduction

The hop plant (*Humulus lupulus* L., Cannabinaceae) is cultivated in the temperate zones of the world for its female inflorescences (commonly referred to as 'hop cones' or 'hops'), which are used in the brewing industry to add bitterness and aroma to beer. The female flower clusters are partly covered with lupulin glands while male flowers have only a few glands in the crease of their anthers and on their sepals. The resin secreted by these glands contains bitter acids, essential oils and prenylated flavonoids. In a limited prenylflavonoid survey of hops, Stevens and co-workers (Stevens, Ivancic, Hsu & Deinzer, 1997) found no significant difference between nine female hop cultivars of European origin.

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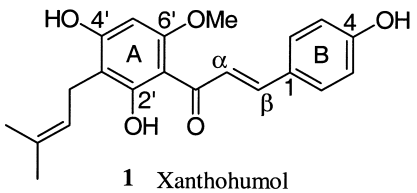
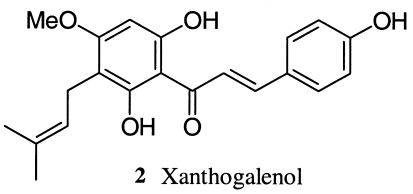
Xanthohumol (**1**, see Fig. 1 and Table 1 for structures) was the principal flavonoid in all samples examined, while eight other prenylated flavonoids were found only in small quantities. Following the isolation and identification of the novel prenylated chalcone, xanthogalenol (**2**), and the known chalcones, 4′-*O*-methylxanthohumol (**3**) and flavokawin (**4**), from the American cultivar Galena, we conducted a more comprehensive flavonoid survey with three wild taxa and 52 cultivars of European, American and Japanese origin. The distribution of **1**–**4**, as well as 18 other flavonoids (**5**–**22**) is compared with the hybridization patterns and morphological and molecular (rDNA) variation in *H. lupulus*.

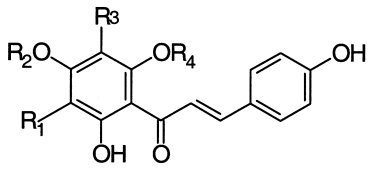
Although some authors have argued against the splitting of *H. lupulus* into varieties, subspecies, or even separate species (Burgess, 1964; Neve, 1991; and refs. cited), we decided to follow largely the infraspeci-



1 Xanthohumol

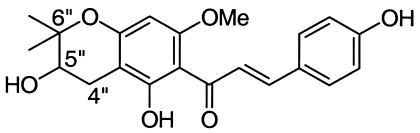


2 Xanthogalenol

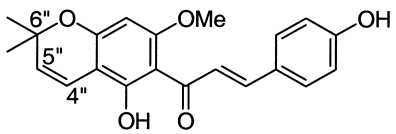


	R_1*	R_2	R_3*	R_4
3	Pn	Me	H	Me
4	H	Me	H	Me
5	Pn	H	H	H
6	Gn	H	H	H
7	Pn	H	Pn	H
8	Pn	H	Pn	Me

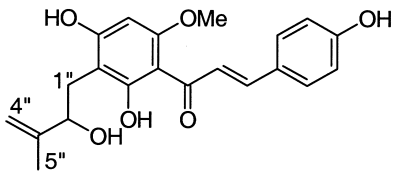
*Pn = Prenyl, Gn = Geranyl



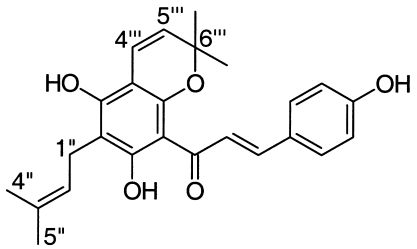
9 Xanthohumol B



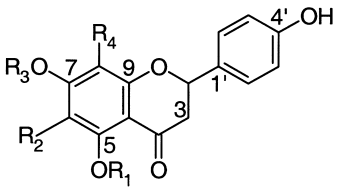
10 Xanthohumol C



11 Xanthohumol D



12 Xanthohumol E



	R_1	R_2*	R_3	R_4*
13	Me	H	H	Pn
14	H	Pn	Me	H
15	H	H	Me	Pn
16	Me	H	Me	Pn
17	Me	H	Me	H
18	H	Pn	H	H
19	H	H	H	Pn
20	H	Pn	H	Pn
21	H	Gn	H	H
22	H	H	H	Gn

*Pn = Prenyl, Gn = Geranyl

Fig. 1. Prenylflavonoids from *Humulus lupulus*. For compound names, see Table 1.

fic classification according to Small (1978, 1980), which is primarily based on geographic distribution and leaf morphology. Wild hops of Europe are relatively uniform and therefore grouped in one taxon, *H. lupulus* var. *lupulus*. Small (1978) recognized three varieties endemic to North America: *H. lupulus* var. *lupuloides* E. Small (central and eastern N. America), *H. lupulus* var. *pubescens* E. Small (midwestern USA), and *H. lupulus* var. *neomexicanus* Nelson and Cockerell (western N. America). Due to sympatry and morphological intergradation of these groups in most parts of central and midwestern USA, and for reasons discussed below, they are here referred to as wild American hops. The indigenous wild hops of Japan and perhaps eastern mainland Asia are known as *H. lupulus* var. *cordifolius* (Miquel) Maximowicz.

2. Results

Twenty-two flavonoids (Table 1) were detected in methanolic extracts of hops by HPLC-MS comparison with authentic flavonoids obtained in the present study and during previous work (Stevens et al., 1997; Stevens, Taylor & Deinzer, 1999a; Stevens, Taylor, Clawson & Deinzer, 1999b). Of the five newly isolated chalcones from *H. lupulus* cv. 'Galena', compounds **2**, **11**, and **12** are new natural products. Mass fragmentation of prenylated chalcones and flavanones by collision-induced dissociation (CID; also referred to as collision-activated decomposition, CAD) has been discussed in previous work (Stevens et al., 1997). As pointed out in that report, chalcones cannot be distinguished from their isomeric flavanones by mass spectrometry due to thermal isomerization in the ion source. Distinction between both flavonoid types was made by UV spectroscopy (Markham, 1982): chalcones have UV maxima near 370 nm while those of the flavanones are near 290 nm (Table 1).

2.1. Identification of flavonoids

The acetone extract of 'Galena' hops was fractionated on Sephadex LH-20 with methanol as the eluent.

Table 1
Chromatographic and spectral data of prenylflavonoids from *Humulus lupulus*

No.	Trivial name	R_t (min)[a]	UV λ_{max} (nm) in MeOH (log ε)	APCI-MS, m/z (rel. int.)[b]	
				$[MH]^+$	Daughter ions of $[MH]^+$
	Chalcones				
1	Xanthohumol	15.1	368 (4.56)	355	179 (100)
2	Xanthogalenol	15.9	365 (4.49)	355	179 (100)
3	4′-*O*-Methylxanthohumol	18.6	367 (4.55)	369	193 (100), 163 (13)
4	4′,6′-Di-*O*-methylchalconaringenin	14.0	367 (4.51)	301	181 (100), 166 (11), 147 (10)
5	Desmethylxanthohumol	13.2	366	341	183 (58), 165 (100)
6	3′-Geranylchalconaringenin	16.9	367, 310 *sh*	409	285 (7), 183 (22), 165 (100)
7	3′,5′-Diprenylchalconaringenin	17.0	372	409	297 (14), 233 (49), 195 (47), 177 (100), 121 (15)
8	5′-Prenylxanthohumol	19.2	371	423	247 (19), 191 (100)
9	Xanthohumol B	12.7	370 (4.35), 275*sh* (3.92), 243 (4.09)	371	251 (45), 233 (100), 191 (24), 179 (93), 147 (30), 109 (10)
10	Xanthohumol C	18.0	371, 298*sh*, 285	353	233 (100), 191 (19), 109 (10)
11	Xanthohumol D	12.7	369 (4.42)	371	251 (6), 233 (89), 191 (26), 179 (100), 109 (13)
12	Xanthohumol E	17.9	369	407	231 (100), 189 (22), 135 (7)
	Flavanones				
13	Isoxanthohumol	9.6	287, 325*sh*	355	179 (100)
14	7-*O*-Methyl-6-Prenylnaringenin	17.3	291, 339*sh*	355	179 (100), 149 (12), 121 (6)
15	7-*O*-Methyl-8-Prenylnaringenin	16.3	291, 339*sh*	355	179 (100), 149 (10), 121 (5)
16	5,7-Di-*O*-methyl-8-Prenylnaringenin	13.2	287, 322*sh*	369	193 (100), 163 (21)
17	5,7-Di-*O*-methylnaringenin	8.2	283 (4.27)	301	181 (100), 166 (8), 147 (12), 119 (8)
18	6-Prenylnaringenin	14.1	291, 334*sh*	341	183 (18), 165 (100)
19	8-Prenylnaringenin	12.4	291, 334*sh*	341	183 (21), 165 (100)
20	6,8-Diprenylnaringenin	17.4	295, 347*sh*	409	297 (19), 233 (44), 177 (100), 121 (18)
21	6-Geranylnaringenin	17.9	294, 336*sh*	409	285 (20), 183 (17), 165 (100)
22	8-Geranylnaringenin	16.0	294, 338*sh*	409	285 (10), 183 (19), 165 (100)

[a] HPLC separations were achieved on a 5 μm LiChrosphere (4 × 250 mm) column using a linear solvent gradient starting from 40% MeCN (B) in 1% aq. HCOOH (A) to 100% B over 15 min, then 100% B for 7 min, at 0.8 ml min^{-1}.

[b] Atmospheric pressure chemical ionization-mass spectrometry. Daughter ions were obtained in the tandem MS mode with the collision energy set at 30 V (Table 1), 15 V (Section 2), or 11 V (Fig. 2).

Table 2
^{1}H-NMR spectroscopic data for compounds **2**, **3**, **9**, **11**, and **12** [δ ppm, *mult.* (*J* in Hz)]

H	2	3	9	11	12
α	7.98 *d* (15.5)	7.75 *d* (15.5)	7.77 *d* (15.5)	7.75 *d* (15.5)	7.90 *d* (15.5)
β	7.69 *d* (15.5)	7.69 *d* (15.5)	7.70 *d* (15.5)	7.66 *d* (15.5)	7.68 *d* (15.5)
2,6	7.54 *d* (8.4)	7.59 *d* (8.3)	7.58 *d* (8.7)	7.57 *d* (8.6)	7.54 *d* (8.6)
3,5	6.84 *d* (8.4)	6.84 *d* (8.3)	6.84 *d* (8.7)	6.84 *d* (8.6)	6.86 *d* (8.6)
5′	6.11 *s*	6.27 *s*	6.01 *s*	6.06 *s*	
1″	3.13 *d* (7.0)	3.16 *d* (7.0)		2.75 *dd* (6.7, 13.4) 2.64 *dd* (6.9, 13.4)	3.21 *d* (7.0)
2″	5.09 *t* (7.0)	5.09 *t* (7.0)		4.22 *t* (6.7)	5.12 *t* (7.0)
4″	1.69 *s*	1.70 *s*	2.71 *dd* (5.2, 16.5) 2.38 *dd* (7.0, 16.5)	4.64 *s*, 4.60 *s*	1.71 *s*
5″	1.60 *s*	1.60 *s*	3.66 *dd* (5.3, 6.9)	1.72 *s*	1.62 *s*
6″-Me*s*			1.29 *s*, 1.22 *s*		
4‴					6.66 *d* (9.9)
5‴					5.60 *d* (9.9)
6‴-Me*s*					1.49 *s*
4-OH	10.07 *s*	10.09 *s*	10.09 *br s*	10.06 *s*	9.95 *s*
2′-OH	14.17 *s*	14.20 *s*	14.78 *s*	14.68 *s*	14.38 *s*
4′-OH				10.55 *s*	10.11 *s*
6′-OH	10.93 *s*				
4′-OMe	3.80 *s*	3.91 *s*			
6′-OMe		3.98 *s*	3.88 *s*	3.87 *s*	

In the fractions richest in xanthohumol (**1**), compound **2** was detected, which eluted slightly later than xanthohumol (**1**) on a reversed-phase C_{18} column, and which showed an MS–MS fragmentation pattern identical to that of xanthohumol (**1**). Both compounds yielded fragment ions with *m*/*z* 299 $[MH–C_4H_8]^+$ and the retro Diels–Alder fragments, *m*/*z* 235 $[^{1,3}A]^+$ and *m*/*z* 179 $[^{1,3}A–C_4H_8]^+$, indicating that **2**, like **1**, contains a phloroglucinol A-ring with one C-prenyl and one *O*-methyl substituent. The ^{1}H- and ^{13}C-NMR spectra of **1** (Stevens et al., 1997) and **2** (Tables 2 and 3) were also very similar. Because **2** showed a low-field hydrogen-bonded hydroxyl (OH-2′ at δ_H 14.17) in the ^{1}H spectrum, two structures seemed possible: (i) 3′-prenyl-4′-*O*-methylchalconaringenin and (ii) 5′-prenyl-6′-*O*-methylchalconaringenin. Possibility (ii) was excluded by ^{1}H–^{13}C HMBC spectroscopy as follows. The identities of C-1′, C-2′ and C-3′ followed from their cross peaks with the OH-2′ resonance at δ_H 14.17. Since the H-1″ protons of the prenyl substituent showed interactions with C-3″, C-2″, C-2′, C-3′ and C-4′, the prenyl group was located at C-3′. The methoxy protons interacted with only one carbon atom, which was identified as C-4′ by its other cross peaks with H-1″ and H-5′.

For the other A-ring hydroxyl, interactions were observed with C-1′, C-5′ and C-6′: this hydroxyl was therefore located at C-6′. Chalcone **2** was thus identified as 3′-prenyl-4′-*O*-methylchalconaringenin. The trivial name, ‘xanthogalenol’, is proposed for this compound to express its structural resemblance with

Table 3
^{13}C-NMR spectroscopic data for compounds **2**, **3**, **9**, **11**, and **12** (δ ppm)

C	2	3	9	11	12
C═O	192.4	192.3	191.8	191.7	191.9[a]
α	123.8	123.7	123.5	123.8	123.6
β	142.6	143.0	143.0	142.5	142.7
1	126.0	125.9	126.0	126.0	126.0
2,6	130.5	130.6	130.6	130.5	130.3
3,5	116.0	116.0	116.0	116.0	116.1
4	159.9	160.0	159.7	159.9	160.1
1′	104.8	105.5	104.9	104.5	105.1[a]
2′	162.6	162.8	164.7	165.2	163.4
3′	106.9	108.4	100.4	105.2	108.4
4′	163.0	163.1	160.0	163.3	157.4[a]
5′	90.7	87.7	91.7	91.1	102.5
6′	160.3	161.1	160.5	160.6	153.7
1″	20.9	21.0		28.8	21.3
2″	123.0	122.7		73.7	122.9
3″	129.9	130.2		148.0	130.2
4″	17.6	17.6	25.1	109.8	17.8
5″	25.5	25.4	67.3	17.3	25.5
6″			78.8		
4‴					117.0
5‴					125.0
6‴					77.2
6″-Me			25.3		
			21.0		
6‴-Me					27.3
4′-OMe	55.5	55.9			
6′-OMe		56.2	56.0	55.7	

[a] These ^{13}C shift values were obtained from the HMQC and HMBC spectra because the corresponding signals did not exceed the noise level in the ^{13}C 1D spectrum.

xanthohumol and its first isolation from *H. lupulus* cv. 'Galena'.

Xanthogalenol (**2**) was converted into two flavanone isomers, **14** and **15**, upon heating in aqueous solution. This may be taken as additional proof for the position of the *O*-methyl at C-4′, leaving two free hydroxyls *ortho* to C-1′ available for cyclization.

Xanthohumol (**1**) forms only one flavanone, isoxanthohumol (**13**), because the second *ortho* hydroxyl is methylated and not available for ring closure. The position of the C-prenyl function in the two isoxanthogalenols was determined by the intensity of the $[MH-C_4H_8]^+$ ion with *m/z* 299 in the daughter ion spectra of **14** and **15**. Cleavage of the prenyl group is much more prominent in the 6-prenylflavanone **14**, presumably as a result of *para* quinonoid stabilization (Fig. 2). In the *ortho* quinone-methide fragment ion of the 8-prenylflavanone **15**, quinonoid stabilization is less extended, and prenyl cleavage is therefore intuitively less favorable. Distinction between 6- and 8-substituted flavanoids by APCI tandem mass spectrometry has previously been observed for 6/8-prenylnaringenins (Stevens et al., 1997), 6/8-geranylnaringenins (Stevens et al., 1999b), vitexin/isovitexin (Rong, Stevens, Deinzer, de Cooman & de Keukeleire, 1998), and for 6/8-methoxy apigenins (Stevens, Wollenweber, Ivancic, Hsu, Sundberg & Deinzer, 1999c).

Compound **3** was also characterized as a chalcone on the basis of its UV maximum at 367 nm and its ^{1}H spectrum with two mutually coupled bridge protons (J = 15.5 Hz, H-α and H-β). Collision-induced dissociation of its molecular ion, *m/z* 369 $[C_{22}H_{25}O_5]^+$, yielded the following fragment ions in its MS–MS spectrum: *m/z* 313 $[MH-C_4H_8]^+$, *m/z* 249 $[^{1,3}A]^+$ and *m/z* 193 $[^{1,3}A-C_4H_8]^+$. This fragmentation pattern is consistent with chalconaringenin bearing one C-prenyl and two *O*-methyls on the A-ring. ^{1}H-NMR spectroscopic analysis revealed the presence of a low-field res-

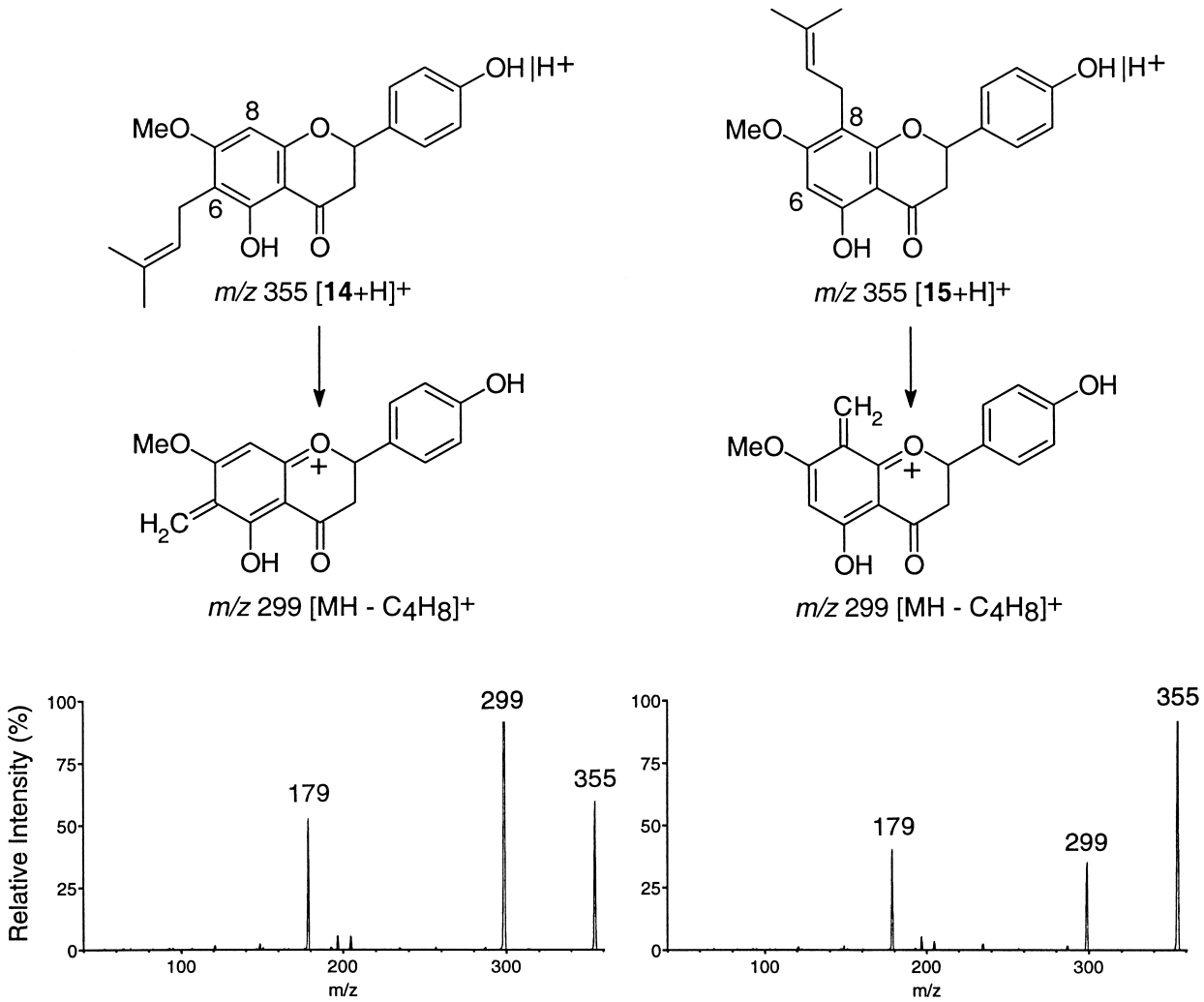


Fig. 2. Distinction between isoxanthogalenols (**14** and **15**) by APCI-CID-MS (collision energy, 11V).

onance which was attributed to OH-2′ (Table 2). As a consequence, the two methoxy substituents had to be at C-4′ and C-6′. In the HMBC spectrum of **3**, the identity of C-1′, C-2′ and C-3′ readily followed from their cross peaks with the OH-2′ signal. The aromatic A-ring proton (δ_H 6.27) interacted with all A-ring carbons but C-2′, and was therefore identified as H-5′, leaving the prenyl substituent to be located at C-3′. This was confirmed by interactions between H-1″ of the prenyl group and carbons 2′, 3′ and 4′. The structure of **3** was now determined to be 4′-*O*-methylxanthohumol. Compound **3** has previously been isolated from hops collected in northern Japan by Sun and co-workers (Sun, Watanabe & Saito, 1989). These authors did not report ^{13}C shift data, and therefore compound **3** is included in Table 3. Chalcone **3** produced one isomeric flavanone (**16**) when heated in a 1:1 mixture of methanol and water.

Compound **4** was identified as 2′,4-dihydroxy-4′,6′-dimethoxychalcone (=4′,6′-di-*O*-methylchalconaringenin) on the basis of UV, MS and ^1H-NMR spectroscopic data. MS–MS fragmentation of the $[MH]^+$ ion (*m*/*z* 301) yielded two diagnostic RDA fragment ions, *m*/*z* 181 $[^{1,3}A]^+$ and *m*/*z* 147 $[^{1,4}B]^+$, indicating that two of chalconaringenin's A-ring hydroxyls were methylated. A free OH-2′, apparent from a low-field resonance at δ_H 13.65, left the methoxy substituents to be located at carbons 4′ and 6′ (^1H-NMR spectral data listed under Section 4.7). Isomerization of chalcone **4** yielded 5,7-di-*O*-methylnaringenin (**17**), which was identical to an authentic sample of **17** by LC-MS. Chalconaringenin-4′,6′-dimethyl ether (**4**) has been reported from the fern *Notholaena dealbata* (Wollenweber & Roitman, 1991). Sun et al. (1989) also isolated a dimethyl ether of chalconaringenin from *H. lupulus* cv. 'Shinshuwase'. They placed the *O*-methyls at positions 2′ and 6′ on the basis of EI MS data, that is, they stated that absence of retro Diels–Alder fragmentation indicated the absence of a free hydroxyl group at C-2′ and C-6′ in the chalcone. However, the authors did observe a base peak at *m*/*z* 181 (100%) which one could identify as the RDA fragment, $[^{1,3}A]^+$, according to Claeys' proposed nomenclature for RDA fragments of flavonoids (Ma, Li, van den Heuvel & Claeys, 1997). This implies that Sun's chalconaringenin-dimethyl ether and compound **4** could be identical. In the present survey, only one chalcone with M_r 300 was detected in Shinshuwase hops, which was identified as 2′,4-dihydroxy-4′,6′-dimethoxychalcone, by retention time and MS comparison with compound **4**.

The resin fraction of Galena hops yielded two chalcones with M_r 370. The first isomer, compound **9**, was earlier obtained from 'Saazer hops' in small amounts and identified as the hydrate of compound **10** by CID mass fragmentation (Stevens et al., 1997). The proposed structure has been confirmed by NMR (^1H, ^{13}C, ^1H–^1H COSY, ^1H–^{13}C HMBC and HMQC, Tables 2 and 3). It was later isolated again from hops by Tabata and co-workers (Tabata, Ito, Tomoda & Omura, 1997), who named it 'xanthohumol B'. We decided to adopt Tabata's name for compound **9** to avoid confusion, and now propose the name 'xanthohumol C' for compound **10** which was earlier named dehydrocycloxanthohumol (Stevens et al., 1997).

The second isomer (M_r 370, $C_{21}H_{22}O_6$), xanthohumol D (**11**), is a new natural product. The CID mass spectrum resembled that of xanthohumol B (**9**). Both chalcones had the following peaks in common: *m*/*z* 353 $[MH-H_2O]^+$, *m*/*z* 299 $[MH-C_4H_{10}O]^+$, *m*/*z* 251 $[^{1,3}A]^+$, *m*/*z* 233 $[^{1,3}A-H_2O]^+$, and *m*/*z* 179 $[^{1,3}A-C_4H_{10}O]^+$. From these diagnostic fragment ions it was deduced that **11**, like **9**, is a derivative of xanthohumol with one oxygen in the prenyl group. Since chalcone **11** showed three phenolic hydroxyls (OH-2′, OH-4′ and OH-4) in its ^1H-NMR spectrum, the possibility was excluded that the prenyl substituent was fixed in a pyrano or furano ring with OH-4′ delivering the hetero atom. The side-chain resonances were attributed to two olefinic protons (=CH_2, δ_H 4.64 *s* and 4.60 *s*), two methylene protons (δ_H 2.75 *dd* and 2.64 *dd*, J = 13, 7 Hz), one –CH< proton (δ_H 4.22, apparent *t*, coupled with the methylene protons, J = 7 Hz), and three methyl protons (δ_H 1.72 *s*). These signals are consistent with a 2-hydroxy-3-methyl-but-3-enyl substituent (Seo et al., 1997). Structure **11** was fully supported by 2D NMR spectroscopy (^1H–^1H COSY, ^{13}C, ^1H–^{13}C HMQC and HMBC, Tables 2 and 3).

Compound **12** (M_r 406, $C_{25}H_{26}O_5$) showed a UV maximum at 369 nm, and was therefore also characterized as a chalcone. Collisional activation of the $[MH]^+$ ion yielded daughter ions with *m*/*z* 351 $[MH-C_4H_8]^+$, *m*/*z* 287 $[^{1,3}A]^+$, *m*/*z* 231 $[^{1,3}A-C_4H_8]^+$, and *m*/*z* 189 $[231\text{-}C_3H_6]^+$. This fragmentation pattern pointed to a chalconaringenin derivative with two C-prenyl substituents on the A-ring, one of which is cyclized with an adjacent phenolic hydroxyl to form a dimethyl chromeno ring (Fig. 3). These assumptions gained support from ^1H-NMR spectroscopic analysis (Table 2), but the ^1H spectrum offered no clue as to which of the two possible hydroxyls (4′ or 6′) provided the hetero atom. It showed three phenolic hydroxyls, two of which were identified as OH-2′ (δ_H 14.38) and OH-4 (δ_H 9.95) by HMBC spectroscopy. For OH-2′, interactions were observed with carbons 2′ (δ_C 163.4), 1′ and 3′ (δ_C 105.1 and 108.4) in the HMBC spectrum. Since H-1″ of the prenyl substituent also interacted with the carbon resonating at δ_C 108.4, this carbon signal was assigned to C-3′ and the other (δ_C 105.1) to C-1′. The third hydroxyl resonance (δ_H 10.11) showed cross peaks with δ_C 102.5 (C-5′) and δ_C 108.4 (C-3′), but

not with C-1′, and was thus identified as OH-4′. From these assignments it became clear that the oxygen atom attached to C-6′ was part of the dimethyl chromeno ring. Structure **12**, as depicted in Fig. 1, was consistent with other interactions observed in the HMBC spectrum. The methine proton H-4‴ of the dimethyl chromeno moiety interacted with C-6‴ (δ_C 77.2) and C-6′ (δ_C 153.7), while the other methine signal (H-5‴) showed cross peaks with C-6‴ and C-5′. Assignments of all proton and carbon signals are listed in Tables 2 and 3. Although not directly a derivative of xanthohumol, compound **12** was named 'xanthohumol E'.

2.2. Flavonoid survey

The LC-MS–MS analyses of the plant materials yielded a data matrix listing the amount of 19 flavonoids as per cent of xanthohumol (**1**) for each of 121 samples covering three wild taxa and 52 cultivars. The leaf gland flavonoids and lupulin gland flavonoids showed similar profiles (by principal components analysis, see below) in a wild hop plant from Montana, despite the fact that the leaves (early Summer 1999) and hop cones (late Summer 1998) were collected in different seasons and years. Based on this observation, we decided we could include leaf gland samples from other hop plants in this study. These plants had not yet developed lupulin glands at the time the analyses were performed, and therefore leaf gland sampling offered an alternative way to investigate their prenylflavonoid chemistry. Similarity between both types of secretory structures has also been observed with humulones and lupulones (Nickerson, unpubl. results).

The most conspicuous finding was the dichotomous distribution (presence versus absence) of the 4′-*O*-methylchalcones **2**–**4** (wild hops and cultivars are listed accordingly in Tables 4 and 5). A sample of the data set is given in Table 6 to illustrate the differences between the flavonoid profiles observed for plants of different geographic origins. Fig. 4 shows a typical LC-MS–MS chromatogram.

Principal components analysis (PCA) of the whole

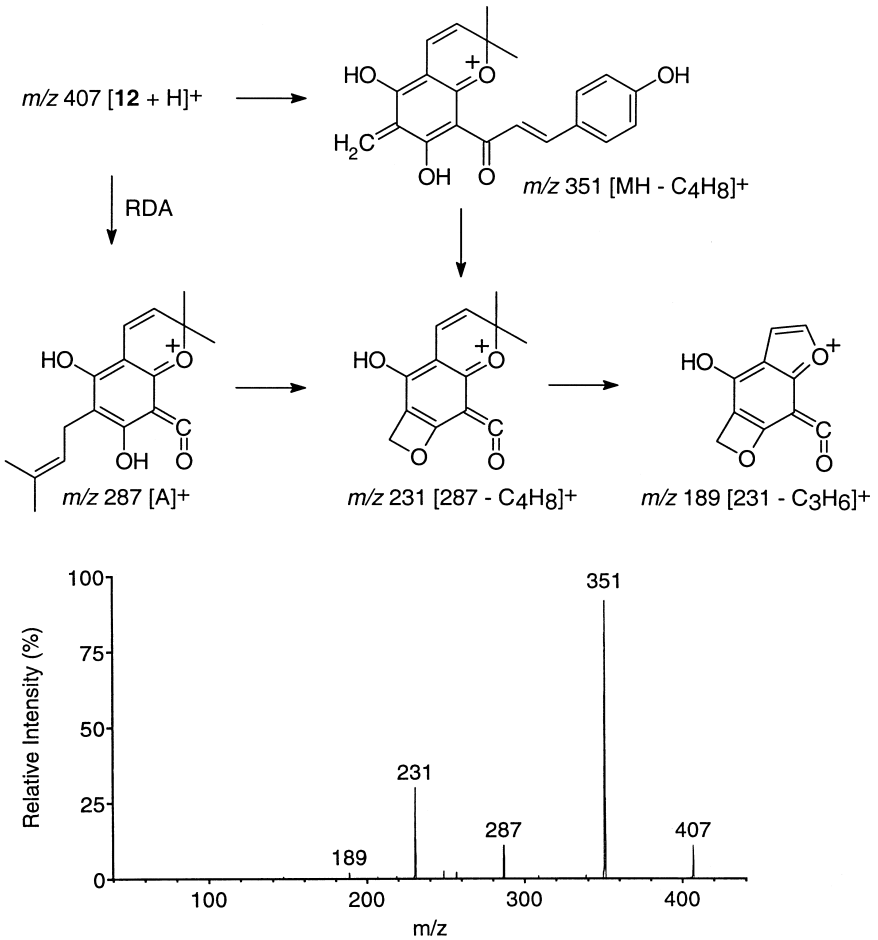


Fig. 3. Proposed APCI-CID (15V) mass fragmentation of xanthohumol E.

data set revealed three groups of hop plants: (i) cultivars and wild hops of European origin and southwestern US wild hops, (ii) hybrids between European cultivars and central US or Japanese wild hops, and (iii) central US and Japanese wild hops (Fig. 5). In PCA, a set of independent linear combinations of the original data vectors (e.g., the flavonoid composition) are calculated in such a way

Table 4
Hop varieties containing xanthogalenol (**2**), 4′-*O*-methylxanthohumol (**3**), and 4′,6′-di-*O*-methylchalconaringenin (**4**)

Sample code	Variety	USDA acc. no.[a]	Parentage (female × male), remarks[b]	Collection site and year[c]
	H. lupulus			
Cr	var. *cordifolius*		Originally from Japan	Leiden Univ., 1999 (leaf glands)
Co2	wild American var.	60026M	Originally from Colorado	OSU,[e]1999 (leaf glands)
Co3	wild American var.	60028M	Originally from Colorado	OSU, 1999 (leaf glands)
Co4	wild American var.	60031M	Originally from Colorado	OSU, 1999 (leaf glands)
Io	wild American var.	21565M	Originally from Iowa	OSU, 1999 (leaf glands)
Mi1	wild American var.	21605	Originally from Minnesota	OSU, 1998
Ms	wild American var.	21558M	Originally from Missouri	OSU, 1999 (leaf glands)
M1	wild American var.	21585M	Originally from Montana	OSU, 1998
M2	wild American var.	21586	Originally from Montana	OSU, 1998
M3	wild American var.	21586	Originally from Montana	OSU, 1999 (leaf glands)
M4	wild American var.	21595M	Originally from Montana	OSU, 1998
M5	wild American var.	21601M	Originally from Montana	OSU, 1992
M6	wild American var.	21602	Originally from Montana	OSU, 1998
M7	wild American var.	21580	Originally from Montana	OSU, 1999 (leaf glands)
M8	wild American var.	21580	Originally from Montana	OSU, 1998
N2	wild American var.	21550M	Originally from Nebraska	OSU, 1999 (leaf glands)
N7	wild American var.	21574M	Originally from North Dakota	OSU, 1999 (leaf glands)
No	wild American var.	9032-028M[d]	Originally from North Dakota	OSU, 1998
Wn	wild American var.	21117M	Originally from Wisconsin	OSU, 1999 (leaf glands)
	Cultivars			
Aq	Aquila	21222	Brewer's Gold (USDA 19001) seedling × OP	WSU[f], 1998
Ba	Banner	21287	Brewer's Gold (USDA 19001) seedling × OP	WSU, 1998
Bg	Brewer's Gold	19001	Wild Manitoba BB1 × OP	1997
Bv	Brewer's Gold, v.f.	21116	virus free clone of Brewer's Gold (19001)	OSU, 1998
Bu	Bullion	64100	Wild Manitoba BB1 × OP	1998
Ca	Calicross	66054	California Cluster × Fuggle seedling	OSU, 1998
Ch	Chinook	21226	Petham Golding × (Brewer's Gold × Wild Utah hop)	OSU, 1998
Cl1	Cluster	65102	Probably derived from an American Wild hop	Idaho, 1997
Cl2	Cluster	65102	Probably derived from an American Wild hop	Washington, 1997
Cl3	Cluster	65102	Probably derived from an American Wild hop	Washington, 1998
Ga1	Galena	21182	Brewer's Gold (USDA 19001) × OP	Idaho, 1997
Ga2	Galena	21182	Brewer's Gold (USDA 19001) × OP	Washington, 1998
Ga3	Galena	21182	Brewer's Gold (USDA 19001) × OP	OSU, 1998
Ga4	Galena	21182	Brewer's Gold (USDA 19001) × OP	Washington, 1997
Gv	Galena, v.f.	21699	Virus free clone of Galena (21182)	OSU, 1998
Gs	Golden Star	21039	Mutant of Shinshuwase	OSU, 1999 (leaf glands)
Kc	Kirin C-601	21709	1/2 Toyomidori, 1/8 Kirin II, 1/8 OP (Japan)	OSU, 1998
Ki	Kirin II	21286	Clonal selection from Shinshuwase hops	OSU, 1998
O	Olympic	21225	3/4 Brewer's Gold	OSU, 1999 (leaf glands)
Pt	Pocket Talisman	21115	Selected from Talisman (Late Cluster × OP)	WSU, 1998
S1, S2	Shinshuwase	60042	Saazer × wild Japanese male (OP)	OSU, 1999 (leaf glands)
Sk	Sticklebract	21403	Tetraploid First Choice (USDA 66055, Californa Cluster × OP) × OP (New Zealand)	OSU, 1998
Ta	Talisman	65101	Late Cluster × OP	OSU, 1998

[a] USDA accession nos. ending with 'M' are male plants.
[b] OP: open pollination.
[c] Collection sites of commercial samples are not given. Lupulin glands of the flowers were used for analysis (unless otherwise mentioned).
[d] USDA nursery number.
[e] Oregon State University.
[f] Washington State University.

Table 5
Hop varieties lacking xanthogalenol and other 4′-*O*-methylchalcones

Sample code	Variety	USDA acc. no.[a]	Parentage (female × male), remarks[b]	Collection site and year[c]
	H. lupulus			
E1	var. *lupulus*		Seeds from Humboldt Univ., Berlin	Leiden University, 1997
E2	var. *lupulus*		Originally from Reeuwijk, The Netherlands	Leiden University, 1998
E3	var. *lupulus*		Originally from Boskoop, The Netherlands	Leiden University, 1997
E4	var. *lupulus*		Plant raised from seeds of E1	Leiden University, 1997
Az	Wild American var.	60013M	Originally from Arizona	OSU, 1999 (leaf glands)
Co1	Wild American var.	60023M	Originally from San Juan Riv.Valley, Colorado	OSU, 1999 (leaf glands)
Nm1	Wild American var.	60016	Originally from New Mexico	OSU, 1999 (leaf glands)
Nm2	Wild American var.	60020	Originally from New Mexico	OSU, 1999 (leaf glands)
Nm3	Wild American var.	60021	Originally from New Mexico	OSU, 1999 (leaf glands)
	Cultivars			
Ap	Apolon	21051	Brewer's Gold × Yugoslavian wild male	OSU, 1999 (leaf glands)
At	Atlas	21052	Brewer's Gold × Yugoslavian wild male	OSU, 1999 (leaf glands)
Cc	Cascade	56013	[Fuggle × (Serebrianka-Fuggle Seedling)] × OP	Washington, 1998
Cb	Columbia	21040	Tetraploid Fuggle (USDA 21003) × Fuggle seedling	OSU, 1998
Eg	Eastern Gold	21678	Japanese cv × Wye OB79 (USDA 64103M)	WSU, 1998
Er	Eroica	21183	Brewer's Gold (USDA 19001) × OP	OSU, 1998
Fh	Fuggle H	48209	Selected from Fuggle (USDA 19209). Pedigree of Fuggle unknown, originally from Kent, UK	OSU, 1998
Fu	Fuggle N	21016	Clonal selection of Fuggle material	OSU, 1998
Ha	Hallertauer	56001	Probably derived from an old German land race	Idaho, 1997
Hm	Hallertauer mittelfrüh	21014	Selected from an old German land race	OSU, 1998
Ht	Hallertauer Tradition	21672	Hallertau Gold × a German male	1997
He	Hersbrucker	21516	Probably derived from an old German land race	OSU, 1998
Ke	Keyworth's Early	21278	OP seedlings of New Mexican wild female	OSU, 1999 (leaf glands)
Ks	Keyworth's Midseason	21279	OP seedlings of New Mexican wild female	OSU, 1999 (leaf glands)
Km	Kitamidori	21677	Japanese cultivar	WSU, 1998
Lc	Late Cluster seedling	19005M	Late Cluster × OP	OSU, 1992
Ma1	Magnum	21670	Galena × a German male	1997
Ma2	Magnum	21670	Galena × a German male	Washington, 1998
Nb	Northern Brewer	64107	Canterbury Golding (old English hop) × OB21 (seedling of Brewer's Gold × Wild American)	Germany, 1997
Nu1	Nugget	21193	USDA 65009 × 63015M; 5/8 Brewer's Gold	OSU, 1998 (stored −15°C)
Nu2	Nugget	21193	USDA 65009 × 63015M; 5/8 Brewer's Gold	OSU, 1998 (stored +20°C)
Nu3	Nugget	21193	USDA 65009 × 63015M; 5/8 Brewer's Gold	Oregon, 1997
Nu4	Nugget	21193	USDA 65009 × 63015M; 5/8 Brewer's Gold	Washington, 1997
Nu5	Nugget	21193	USDA 65009 × 63015M; 5/8 Brewer's Gold	Idaho, 1997
Pe1	Perle	21227	Northern Brewer (64107) × a German male	Germany, 1997
Pe2	Perle	21227	Northern Brewer (64107) × a German male	Oregon, 1997
Pe3	Perle	21227	Northern Brewer (64107) × a German male	Washington, 1997
Pw	Pride of Ringwood	66052	Pride of Kent (Brewer's Gold × OP) × OP (seedling raised and comm. grown in Australia)	WSU, 1998
Sa	Saazer	21077	Selected from an old Czechoslovakian land race	1997
Sb1	Saazer-36	21521	Virus free clone of Saazer (USDA 21077)	OSU, 1998 (stored −15°C)
Sb2	Saazer-36	21521	Virus free clone of Saazer (USDA 21077)	OSU, 1998 (stored +20°C)
Sb3	Saazer-36	21521	Virus free clone of Saazer (USDA 21077)	Idaho, 1997
Sc	Saazer-38	21522	Virus free clone of Saazer (USDA 21077)	Idaho, 1997
Sd	Saazer-72	21532	Virus free clone of Saazer (USDA 21077)	Idaho, 1997
Sn1	Santiam	21664	Swiss Tettnanger × (Tetraploid Hallertauer m.f. × USDA 21381M) = 61021 × 21618M	Washington, 1997
Sn2	Santiam	21664	Swiss Tettnanger × (Tetraploid Hallertauer m.f. × USDA 21381M) = 61021 × 21618M	Oregon, 1997
Sm	Smooth Cone	66056	California cluster × OP	OSU, 1999 (leaf glands)
Ss	Spalter	21186	Selected in the Spalt area of Germany from an old land race	Germany, 1997
Sp1	Spalter Select	21674	Cross between two German breeding lines	1997
Sp4	Spalter Select	21674	Cross between two German breeding lines	OSU, 1998
St	Sterling	21689	1/2 Saazer-38, 1/4 Cascade, 1/8 Brewer's Gold (19001), 1/8 Zattler seedling	Oregon, 1997
Te1	Tettnanger	21015	Selected in the Tettnang area of Germany from an old land race	1997

(continued on next page)

Table 6 (*continued*)

Sample code	Variety	USDA acc. no.[a]	Parentage (female × male), remarks[b]	Collection site and year[c]
Te2	Tettnanger	21015	Selected in the Tettnang area of Germany from an old land race	Oregon, 1997
Ty	Toyomidori	21676	Northern Brewer (64107) × Wye OB79 (USDA 64103M); Japanese cultivar	WSU, 1998
Us	USDA 21120	21120	Has Fuggle and Late Cluster in ancestral line	WSU, 1998
Wi1,3	Willamette	21041	Tetraploid Fuggle (21003) × Fuggle seedling	Idaho, 1997
Wi2	Willamette	21041	Tetraploid Fuggle (21003) × Fuggle seedling	Oregon, 1997
Wi4	Willamette	21041	Tetraploid Fuggle (21003) × Fuggle seedling	Washington, 1997
Wi5	Willamette	21041	Tetraploid Fuggle (21003) × Fuggle seedling	Washington, 1998

[a,b,c]See footnotes in Table 4.

that the first linear combination, or principal component, accounts for as much of the variation in the original data as possible. The second PC accounts for as much of the remaining variability as possible, then a third PC is calculated, and so on. In effect, a new set of axes is created on which the data can be plotted that might better reveal patterns. In this case, the separation of the three groups along the axis of the first principal component, accounting for 68% of the variance in the original data, is largely due to the amounts of the 4′-*O*-methylchalcones **2**–**4**. The dispersion of the observations along the second PC axis, which accounts for 17% of the variance, is associated with the relative amounts of desmethylxanthohumol (**5**) and, to a lesser extent, the prenylnaringenins **18** and **19**. For instance, the sample of Cascade hops (Cc, in top region of the European cluster) contained a significantly higher percentage of **5** (44%) than the samples of Willamette hops, which were placed near the bottom of that cluster (range 1.7–6.1%, average 3.8%).

As a group, the flavanones had little impact on the separation of the three clusters due to their low abundance. However, the progressive isomerization of chalcones into flavanones during storage did influence separation within the clusters. For example, a 1997 sample of a given hop cultivar will be located lower on the second PC axis than its corresponding 1998 sample. The levels of the prenylnaringenins **18** and **19**, the geranylnaringenins **21** and **22**, and 6,8-diprenylnaringenin (**20**) were elevated in the 1997 samples. The increased levels were also seen in 1998 samples stored at room temperature versus those stored in the freezer immediately after harvest (Table 6). These changes lend support to

Table 6
Prenylflavonoid concentration as percentage of xanthohumol

Compound number	Japanese wild var. *cordifolius*	Japanese cultivar Kirin II	American wild Montana 8	American cultivar Cluster	European wild Reeuwijk	European cultivar Saaz-36, Stored −15°C	European cultivar Saaz-36, Stored 20°C
1	100	100	100	100	100	100	100
2	36.94	4.06	16.77	3.64	0.00	0.00	0.00
3	75.36	8.28	27.25	8.27	0.00	0.00	0.00
4	6.36	1.16	5.13	0.69	0.00	0.00	0.00
5	7.71	4.95	16.76	6.49	7.99	29.98	17.61
6	10.11	0.69	2.63	1.44	0.21	2.10	0.84
7	2.16	0.12	2.46	0.74	0.53	3.85	1.79
8	0.04	0.05	0.45	0.11	0.07	0.06	0.17
9	0.28	0.00	0.18	0.00	0.92	0.06	0.24
10	0.19	1.74	0.10	0.21	0.48	0.22	0.63
12	0.00	0.03	0.13	0.09	0.06	0.20	0.19
13	0.56	1.74	1.41	1.65	1.96	0.81	3.48
14	1.35	0.76	2.31	0.45	0.00	0.00	0.00
16	0.40	0.10	0.34	0.10	0.00	0.00	0.00
17	0.34	0.16	0.73	0.11	0.00	0.00	0.00
18	0.31	0.63	1.60	0.71	0.69	1.45	2.87
19	0.08	0.18	0.46	0.21	0.26	0.49	1.06
20	0.24	0.18	0.59	0.10	0.28	0.37	1.12
21	0.27	0.12	0.29	0.18	0.03	0.13	0.32
22	0.08	0.00	0.09	0.05	0.00	0.13	0.09

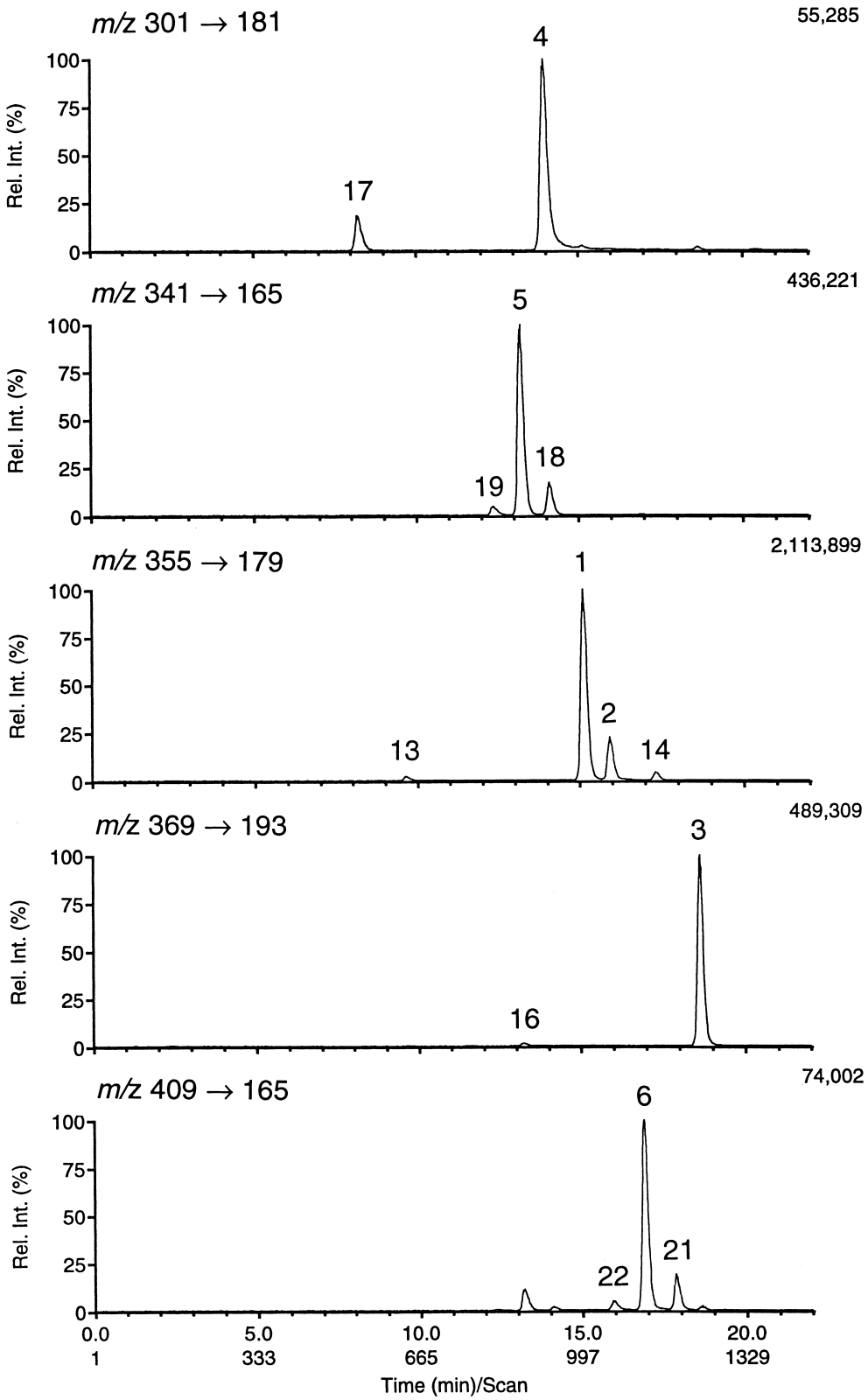


Fig. 4. LC-MS–MS chromatogram of the lupulin resin of a wild *H. lupulus* plant from Montana. For key to peak identities, see Fig. 1 and Table 1.

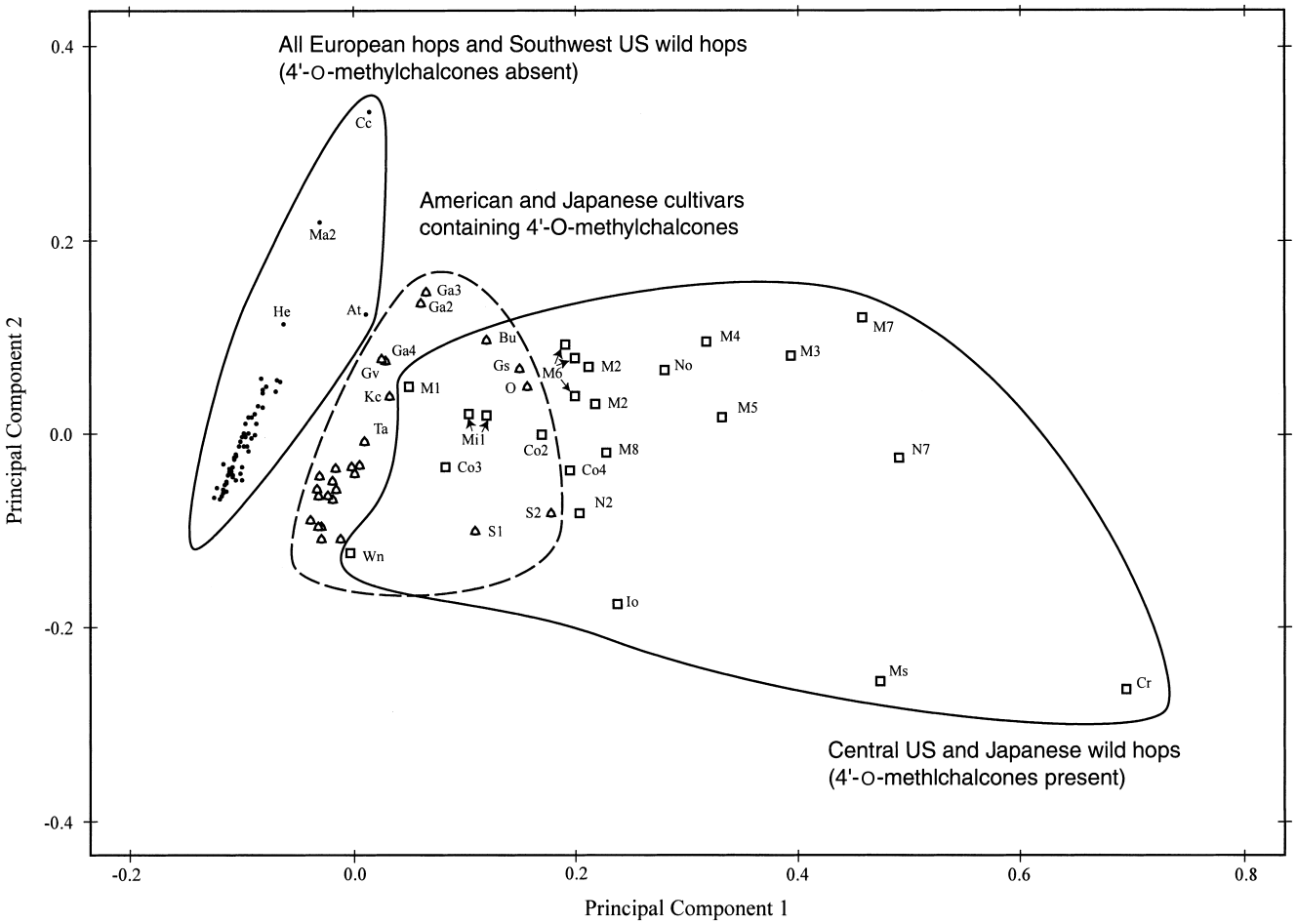


Fig. 5. Principal components analysis of the flavonoid data. The plants are plotted on the first two PC axes; see Section 3. For sample abbreviations, see Tables 4 and 5.

our earlier hypothesis that the flavanones of hops are formed chemically from their isomeric chalcones during drying and storage, and not enzymatically in the lupulin glands (Stevens et al., 1997). The results obtained with the leaf glands provide additional evidence for absence of chalcone isomerase in these secretory structures. Prenylflavanones were virtually absent in leaf glands from young leaves which were collected in Oregon State University's hop yard on the same day the analyses were performed.

The leaf glands of *H. japonicus* Sieb. & Zucc. did not contain the prenylated flavonoids found in the glandular exudates of *H. lupulus*. Only one plant of *H. japonicus* was examined, and clearly more material (more plants, lupulin) needs to be analyzed before the exudate chemistry of this species can be established. The data collected for this plant (with the absence of all 22 flavonoids) were not included in the PCA.

3. Discussion

The dichotomous distribution of the 4′-*O*-methylchalcones **2**–**4** correlates well with leaf morphology and hybridization patterns in *H. lupulus*. It is generally accepted that the early European cultivars originated by local domestication of wild hops (*H. lupulus* var. *lupulus*). The American and Japanese cultivars are considered to be hybrids between introduced European cultivars and local wild (male) plants (Small, 1980; Neve, 1991). (Note: some American-developed cultivars, for example, Santiam, Sterling, and Willamette, contain only European germplasm and are here considered "European"). The origin of the three groups of cultivars is in good agreement with their flavonoid patterns. Both wild hops and cultivars of European origin lack xanthogalenol (**2**), and both groups were otherwise also very similar with regard to flavonoid chemistry. The hybrid origin of the American cultivars is

reflected by the placement of these varieties in the PCA between the European cultivars and the American wild plants from the Missouri and Mississippi river basins (Fig. 5). The latter group and one plant of *H. lupulus* var. *cordifolius* contained the highest amounts of xanthogalenol, 4′-*O*-methylxanthohumol, and 4′,6′-di-*O*-methylchalconaringenin, while intermediate levels of these 4′-*O*-methylchalcones were found in the hybrid cultivars. The plants from Arizona, New Mexico and southern Colorado lacked the 4′-*O*-methylchalcones, and were consequently grouped together with the European hops in the PCA scatter plot. The cultivars, Keyworth's Midseason and Keyworth's Early, are the 'granddaughter' and 'great-granddaughter' of a wild female plant from New Mexico. Each generation was raised from open pollinated flowers at Wye College, England. The absence of 4′-*O*-methylchalcones in these cultivars reflects the absence of biosynthetic capability in the ancestral lines.

Small's classification of wild North American hops was based on morphological characters and geographic distribution, and he recognized the complications of sympatry and intergradation (Small, 1978). Our study did not include morphological examination, nor did we know if our selection of material coincided with Small's. Except for one sample originally collected in Wisconsin, all wild American hops in this study were from west of the Mississippi River. Excepting those from Arizona and New Mexico, they could, by geography, be any of Small's varieties. Many specimens were originally collected west of the 100th meridian, a region in which Small considered *H. lupulus* var. *neomexicanus* to be largely allopatric. In contrast, the presence or absence of the 4′-*O*-methyl chalcones divides the wild hops in this study into one group from the Missouri–Mississippi drainage, and a second one south and west of that basin, including Arizona, New Mexico, and southwestern Colorado. Thus, we refer to these hops as wild American hops with two geographic/chemical groups.

The phylogenetic relationship between *H. lupulus* from the southwestern USA, most likely of var. *neomexicanus*, and the other taxa of *H. lupulus* is not clear. It could have been derived from *H. lupulus* of central or midwestern USA, or perhaps directly from an east Asian taxon (either capable or incapable of accumulating 4′-*O*-methylchalcones), if one considers the Rocky Mountains as a physical barrier between the two American taxa. The Rocky Mountains continued to be uplifted since early Tertiary times, and it became an increasingly effective east–west barrier for plants growing at lower elevations (Morain, 1984). It is therefore not clear whether absence of 4′-*O*-methylchalcones in *H. lupulus* of the American southwest is the result of loss of the trait, or if this taxon is a more direct descendent from a lineage that never possessed the character.

In a study of genetic variation using restriction fragment length polymorphisms of the ribosomal RNA genes, Pillay and Kenny (1996) also recognized two major groups within *H. lupulus*, (1) wild and cultivated European hops and wild hops from western China (Xinjiang province), and (2) wild American hops. In contrast to our flavonoid variation data, Pillay & Kenny's analysis of rDNA variation did not reveal differences between wild hops from the Missouri–Mississippi river basins and those from Arizona and New Mexico (Pillay & Kenny, 1996). Wild *H. lupulus* plants from eastern China and Japan were not included in their studies.

4′-*O*-methylchalcones are quantitatively well represented in the leaf gland exudate of *H. lupulus* var. *cordifolius* of Japanese origin. Many of the Japanese cultivars presumably received gene material from male plants of this taxon by wind pollination. The intermediate position of cv. 'Golden Star' and the Kirin cultivars from Japan is apparently the result of inheritance of the 4′-*O*-methylchalcones from *H. lupulus* var. *cordifolius* through the classic Japanese cultivar, 'Shinshuwase'. The lupulin resin of the latter cultivar is known as a source of 4′-*O*-methylxanthohumol and xanthohumol (Sun et al., 1989), and the leaf gland exudates of both the Shinshuwase hop plants examined contained the three 4′-*O*-methylchalcones and xanthohumol. Although Shinshuwase has been recorded as a hybrid between the European cultivars, 'Saazer' and 'White Vine', these ancestors would not account for the presence of the 4′-*O*-methylchalcones. In fact, morphological evidence indicates that the female Saazer plant was open pollinated by a wild Japanese plant, a hypothesis supported by the flavonoid composition. Ono (Ono, 1959; cited by Neve, 1991) noted that Shinshuwase resembles the American hop more than the European one from a morphological point of view. Golden Star has been reported as a mutant form of Shinshuwase, while 'Kirin II' is a clonal selection from Shinshuwase, and as such is virtually identical to Shinshuwase (Neve, 1991). The other Japanese cultivar containing the three 4′-*O*-methylchalcones, Kirin C-601, received 1/8 gene material from Kirin II and 1/8 from an unknown male plant, both of which could have passed on the capability to accumulate 4′-*O*-methylchalcones.

The classic American cultivars are the Clusters. Although the origin of the Clusters is not entirely clear, hybridization between introduced European cultivars and American male plants most likely occurred during the settlement of the eastern seaboard in the 17th century. It is therefore assumed that the American Clusters inherited the 4′-*O*-methylchalcones from local wild males. The 'Early Cluster' (Oregon) and

'California Cluster' arose on the west coast as clonal selections of the 'Late Cluster' that the settlers brought with them in the mid to late 1800s (Neve, 1991).

Many of the xanthogalenol-containing cultivars listed in Table 4 are derived from *H. lupulus* cv. 'Brewers Gold', for example, the American cultivars 'Aquila' and 'Galena'. The varieties 'Brewer's Gold' and 'Bullion' are selections from seedlings of a hybrid between a wild female plant from Manitoba and an unknown European male plant. This hybrid resulted from an open-pollination event at Wye College, England, in 1918 (Neve, 1991). There is little doubt that the 4′-*O*-methylchalcones were passed on to 'Brewer's Gold', 'Bullion' and their descendents, by this Manitoba female plant, which is from the same geographic region as the wild hops from Montana, North Dakota and Minnesota examined in this study. However, some cultivars contain wild American gene material, notably Apolon, Atlas, Hallertauer Magnum, Nugget, and Smooth Cone, yet they lack the capability to produce 4′-*O*-methylchalcones. A possible explanation is loss of the trait by genetic drift due to selection for other characters. This is presumably also true for the cultivars, Northern Brewer, Pride of Ringwood, and Toyomidori, all of which contain less than 1/16th wild American gene material.

The distribution of xanthogalenol and 4′-*O*-methylxanthohumol leads us to suggest that 4′-*O*-methylation is a primitive character in *H. lupulus*. The most parsimonious explanation for the observed dichotomy is that 4′-*O*-methylation of chalcones has been retained (or arisen during the early stages of infraspecific differentiation) in the Japanese and American lineages, and was lost in the European lineage (var. *lupulus*). The present-day disjunct distribution of *H. lupulus* is considered to be the result of its eastward migration to Japan and America and westward to Europe. Geographic separation of the species has earlier been associated with the development of two genetically different populations, and may explain why wild hops of American origin are more similar to wild Japanese hops than to wild European plants, with regard to pubescence and shape of the leaves (Neve, 1991; Small, 1980).

These assumptions are in line with phytogeographical and palaeobotanical data. Three species of *Humulus* are known as endemics of China (*H. lupulus* var. *cordifolius*, *H. japonicus* [syn. *H. scandens* (Lour.) Merr] and *H. yunnanensis* Hu), which led Neve (1991) to suggest that this part of Asia is the center of origin of the genus. Most of the fossil fruits of *Humulus* (*H. irtyshensis*, *H. rotundatus*, *H. scandens*) and the presumably closely related *Humularia* (now believed to be extinct), were found in China and Russia. These records all date back to the Oligocene, Miocene, and Pliocene periods (35 million to 2 million years ago) (Collinson, 1989), indicating that speciation was most vigorous during the mid to late Tertiary. In this period, the temperate zone of the northern hemisphere was extended towards the pole (Takhtajan, 1969), and, although the sea level was rising, the Bering Strait consisted of a dry land bridge (Pielou, 1979) or a chain of islands and archipelagos (Morain, 1984), for extended periods of time. These conditions allowed some species to use the Bering Strait as a land route for migration between Eurasia and North America (Pielou, 1979; Morain, 1984). Moreover, Raven and Axelrod (1974) argued that the Bering land bridge has been more important as a land route for migrating species than the North Atlantic passage via Greenland and Iceland, since the middle of the Eocene. Examples of genera that presumably owe their present disjunct distribution (eastern Asia and N. America) to the Bering land bridge include *Carya* (hickory), *Acer* (maple), *Epigaea* (trailing arbutus), and *Stewartia* (shrubs of the tea family) (Pielou, 1979).

4. Experimental

4.1. Plant material

Hop cones and male lupulin materials were obtained from various sources; their origin, collection site and year of harvest are listed in Tables 4 and 5. The hop cones were dried at ca. 50°C to final moisture contents of ca. 12% and stored at −15°C, until analysis (unless otherwise stated in Tables 4 and 5).

Leaves were collected at the experimental hop yards of Oregon State University (OSU) and the National Clonal Germplasm Repository, Corvallis, in early Summer 1999. Leaf samples were processed fresh shortly after collection of the leaves. *Humulus japonicus* (originally from China) was grown in the greenhouses of the NCGR at Corvallis.

4.2. Reference flavonoids

Flavonoids **1**, **5**, **8**, **9**, **10**, and **13** were obtained during earlier work or isolated as described by Stevens et al. (1997). Flavanones **18** and **19** were prepared by prenylation of naringenin (Stevens et al., 1999a); the geranylflavanones **21** and **22** were synthesized from naringenin and linalool (Stevens et al., 1999b). The remaining flavonoids were obtained as described in Section 4.7.

4.3. Sample preparation for LC-MS

Female plant material (one hop cone or ground hops, typically 0.1 g) was immersed in 100 ml of MeOH–HCOOH (99:1 by vol.). The extraction mix-

ture was gently swirled several times over 2 h and then allowed to settle for 2 h. A portion of the cleared extract was carefully removed by pipetting and directly analyzed by LC-MS. Male lupulin samples were prepared as follows: ca. 0.1 mg resin was immersed in MeOH–HCOOH (99:1, 1 ml) at room temperature for 2 h with occasional agitation. A portion of the supernatant was analyzed by LC-MS.

Leaf gland extracts of female or male plants were prepared as follows: leaf glands (ca. 30) were removed from the bottom side of young leaves with a solid needle at 7× (stereomicroscope), transferred to a 0.25 ml sample vial, and extracted with MeOH–HCOOH (99:1, 0.1 ml) at room temp for 2 h with occasional agitation.

4.4. LC-tandem MS analysis

Hop extracts were analyzed for prenylflavonoids by LC-MS as described previously (Stevens et al., 1999a). In short, hop extracts were separated on a 5 μm C_{18} column (250 × 4 mm) using a linear solvent gradient from 40 to 100% B (MeCN) in A (1% aq. HCOOH) over 15 min, followed by 100% B for 7 min. The flow rate was 0.8 ml min^{-1}. The HPLC instrument was connected to a PE Sciex API III Plus triple-quadrupole mass spectrometer, equipped with an atmospheric pressure chemical ionization (APCI) source. The APCI source was operated in the positive ion mode. The HPLC effluent was introduced into the mass spectrometer via a heated nebulizer interface at 500°C.

Ionization of the analyte vapor mixture was initiated by a corona discharge needle at ca. 8 kV and a discharge current of ca. 6 μA. For MS–MS, argon–nitrogen (9:1) was used as target gas in the collision cell (ca. 1.8×10^{14} atoms cm^{-2}). The collision energy was set at 15 V to obtain diagnostic daughter ions for structure determination (Section 2), and increased to 30 V to enhance the yield of the $[^{1,3}A-C_4H_8]^+$ type ions for semi-quantitation by multiple reaction monitoring (MRM, see below).

The 22 flavonoids (Table 1) were selectively detected by MRM in the tandem MS mode. In MRM, specific daughter ions were detected that were produced by collision-induced dissociation of selected $[MH]^+$ ions. That is, for each $[MH]^+$ ion, the most intense ion in the full-scan daughter ion spectrum was chosen for detection (cf. Table 1): m/z 355 → 179 for compounds **1**, **2**, **13**, **14** and **15**; m/z 369 → 193 for **3** and **16**; m/z 301 → 181 for **4** and **17**; m/z 341 → 165 for **5**, **18** and **19**; m/z 409 → 165 for **6**, **21** and **22**; m/z 409 → 177 for **7** and **20**; m/z 423 → 191 for **8**; m/z 371 → 233 for **9** + **11** (indistinguishable from each other); m/z 353 → 233 for **10**; and m/z 407 → 231 for chalcone **12**. Although the MRM software allows rapid sequential scanning of up to 10 ion pairs in a single HPLC run, the number of ion pairs scanned in a single HPLC run was limited to six in order to retain sufficient sensitivity for detection of each daughter ion. The remaining ion pairs were scanned in a second HPLC–MRM run for each sample. Scanning of the ion pair, m/z 355 → 179, was included in both runs in order to be able to use xanthohumol (**1**), xanthogalenol (**2**), and isoxanthohumol (**13**) as internal calibrants.

4.5. Data analysis

The chromatographic peak areas of the flavonoids analyzed by HPLC–MRM were determined using PE Sciex's MacQuan 1.5 software. Compounds **9** and **11** coeluted and were treated as a single amount. Compound **15** was not detected in the survey of hop varieties. If the peak areas of xanthohumol (**1**), xanthogalenol (**2**), and isoxanthohumol (**13**) from the two chromatographic runs for each hop sample were comparable, the data were pooled and used to calculate the amount of 19 flavonoids relative to xanthohumol (**1**) for that sample.

The SAS System 6.12 statistics software was used to calculate a covariance matrix from these relative amounts and then to perform a principal component analysis. Xanthohumol (**1**), serving as an internal standard, had a relative amount of 100%, and was not used in the PCA calculation. The scatter plot (Fig. 5) was generated from the first two PCs.

4.6. NMR spectroscopy

Nuclear magnetic resonance spectra were recorded on a multinuclear Bruker DRX 600 instrument at 600 MHz for ^1H and at 150.9 MHz for ^{13}C. Samples were dissolved in DMSO-d_6 and analyzed at room temperature. DMSO resonances (δ_H 2.50 and δ_C 39.51) were used as internal chemical shift references. ^1H–^{13}C HMQC and HMBC experiments were performed using standard pulse sequences.

4.7. Extraction and purification of flavonoids

Whole hop cones (cv. 'Galena', 301 g) were immersed in Me_2CO for a few minutes, and then the extraction solvent was decanted and filtered. After a second extraction with Me_2CO, the filtrates were combined and concentrated on a rotary evaporator at 45°C. The oily residue was fractionated on a Sephadex LH-20 column with MeOH as eluent. Fractions of ca. 10 ml were collected and monitored with TLC (silica gel plates run in toluene–dioxane–HOAc, 18:5:1). Chalcone-containing fractions were combined and fractionated again by column chromatography on Sephadex LH-20. Fractions of similar composition, monitored by HPLC-UV (370 nm), were combined to

give three main fractions: (i) containing compounds **1**, **3**, **4**, **9**, **10**, **11**, and **12**, (ii) containing **1**, **2**, and **6**, and fraction (iii) with mainly **1** and **5**. These compound mixtures were separated by preparative HPLC on a 10 μm Econosil RP-18 column (22 × 250 mm) using a linear gradient starting from 40 to 100% MeCN in 1% aq. HCOOH over 40 min at a flow rate of 11.2 ml min^{-1}. The UV trace was recorded at 370 nm. Peak fractions were collected manually and dried by rotary evaporation and lyophilization. Mixed peak fractions were re-chromatographed on the same column using different solvent gradients or isocratic elution (MeCN–1% aq. HCOOH).

4′,6′-di-*O*-methylchalconaringenin (**4**). Yellow powder after lyophilization. UV and MS data, Table 1; ^{1}H-NMR spectral data δ_H: 13.65 (OH-2′), 10.08 (OH-4), 7.62 (2H, *s*, H-α and H-β), 7.58 (2H, *d*, *J* = 8.6 Hz, H-2/6), 6.83 (2H, *d*, *J* = 8.6 Hz, H-3/5), 6.15 (1H, *d*, *J* = 2.3 Hz, H-5′), 6.11 (1H, *d*, *J* = 2.3 Hz, H-3′), 3.90 and 3.82 (each 3H, *s*, 2 × OMe).

4.8. Conversion of chalcones into flavanones

Chalcones **2**, **3**, and **4** (ca. 0.2 mg) were dissolved separately in a mixture of MeOH–H_2O (1:1, 3 ml) and kept overnight at 60°C. This treatment resulted in complete conversion of the chalcones, monitored by HPLC-MS. The flavanone products (**14/15** from **2**; **16** from **3**; and **17** from **4**) were isolated from the reaction mixtures by preparative HPLC and analyzed by UV and MS (Table 1). Compound **17** was identical with a sample of 4′-hydroxy-5,7-dimethoxyflavanone (═5,7-di-*O*-methylnaringenin), purchased from Indofine (Somerville, NJ), by UV and HPLC-MS.

4.9. Preparation of 6,8-diprenylnaringenin and 3′,5′-diprenylchalconaringenin

Prenylated naringenins were obtained by treatment of naringenin with 2-hydroxy-2-methyl-but-3-ene in dioxane/BF_3 etherate as described by Jain, Gupta and Sarpal (1978). A portion of the reaction products was separated on a 10 m Econosil RP-18 column (10 × 250 mm) using a linear gradient starting from 40 to 100% MeCN in 1% aq. HCOOH over 60 min at a flow rate of 5.0 ml min^{-1}. The UV trace was recorded at 290 nm. Peak fractions were collected manually and dried by rotary evaporation and lyophilization. 8-Prenylnaringenin (13 mg), 6-prenylnaringenin (5 mg), and 6,8-diprenylnaringenin (4 mg) were obtained as white powders.

6,8-Diprenylnaringenin (**20**). ^{1}H-NMR spectral data; δ_H 12.39 (OH-2), 9.61 and 9.54 (OH-4 and OH-4′), 7.30 (2H, *d*, *J* = 8.5 Hz, H-2′/6′), 6.78 (2H, *d*, *J* = 8.5 Hz, H-3′/5′), 5.39 (1H, *dd*, *J* = 12.6, 2.9 Hz, H-2), 5.08 and 5.05 (2H, 2*t*, *J* = 7.2 Hz, H-2″ and H-2‴), 3.22–3.15 (5H, H-3ax, H-1″ and H-1‴), 2.72 (1H, *dd*, *J* = 17.0, 3.1 Hz, H-3*eq*), 1.70, 1.61, 1.59, and 1.52 (each 3H, 4*s*, H-4″, H-5″, H-4‴ and H-5‴); UV and MS data are listed in Table 1.

3′,5′-Diprenylchalconaringenin (**7**) was prepared by treatment of flavanone **20** (4 mg) with 5% NaOH in MeOH (4 ml) under reflux conditions for 30 min. The reaction mixture was poured into 25 ml of 2N HCl. The aqueous layer was extracted with Et_2O (25 ml) and discarded. The Et_2O layer was washed with water (3 × 25 ml) and evaporated to near-dryness. A small amount of 3′,5′-diprenylchalconaringenin (ca. 0.1 mg) was obtained from the residue by preparative HPLC (UV 370 nm). UV and MS data are listed in Table 1.

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