PERGAMON

Phytochemistry 51 (1999) 771–780

PHYTOCHEMISTRY

Leaf surface flavonoids of *Chrysothamnus*

Jan F. Stevens[a,*], Eckhard Wollenweber[d], Monika Ivancic[b], Victor L. Hsu[b], Scott Sundberg[c], Max L. Deinzer[a]

[a]*Department of Chemistry, Oregon State University, 153 Gilbert Hall, Corvallis, OR 97331, USA*
[b]*Department of Biochemistry and Biophysics, Oregon State University, 2143 ALS, Corvallis, OR 97331, USA*
[c]*Department of Botany and Plant Pathology, Oregon State University, 1046 Cordley Hall, Corvallis, OR 97331, USA*
[d]*Institut für Botanik der Technischen Universität, Schnittspahnstrasse 3, D-64287 Darmstadt, Germany*

Received 2 July 1998; received in revised form 16 November 1998; accepted 16 November 1998

Abstract

Twenty-six flavonoid aglycones have been identified from eight plants covering three species of *Chrysothamnus* that were collected in eastern Oregon. The flavonoids were identified by NMR spectroscopy, tandem mass spectrometry and co-TLC with authentic markers. *Chrysothamnus nauseosus* yielded methyl ethers of apigenin, isoscutellarein, luteolin, kaempferol, herbacetin and quercetin. *O*-Methylated kaempferol and quercetin derivatives were isolated from the leaf exudate of *C. humilis*. The flavonoid chemistry of *C. viscidiflorus* was found different from the other two species by the presence of methyl ethers of quercetin, eriodictyol and taxifolin-3-acetate. Although the flavonoid profiles proved of diagnostic value at the species level, they provided little further evidence in favour of inclusion of *Chrysothamnus* into *Ericameria* as proposed earlier on the basis of morphological similarities.

Keywords: *Chrysothamnus nauseosus*; *C. viscidiflorus*; *C. humilis*, *Ericameria*, Asteraceae; Flavonoids; Dihydroflavonols; Taxifolin-3-*O*-acetate; Mass spectrometry; Chemotaxonomy

1. Introduction

Rabbitbrush (*Chrysothamnus* spp., Asteraceae) is a common shrub in xeric habitats in much of western North America. In the latest treatment of the genus (Anderson, 1986), five sections, 16 species and 41 subspecies are recognized. Recent authors have placed a portion or all of *Chrysothamnus* in *Ericameria*, a segregate of *Haplopappus* (Anderson, 1995; Lane, Morgan, Suh, Simpson, & Jansen, 1996; Nesom & Baird, 1993, 1995). In this paper we retain the genus *Chrysothamnus* in the broad sense, although a portion or all of it may more properly be placed in *Ericameria*. The geographical distribution of *C. nauseosus* (Pall. ex Pursh) Britt., *C. viscidiflorus* (Hook.) Nutt. and *C. humilis* Greene includes a portion of eastern Oregon, where the first two species are found as sub-dominants of the shrub vegetation. Both species become very conspicuous when they flower bright yellow in late summer.

The flavonoids of *C. nauseosus* and *C. viscidiflorus* have been investigated before. Bohlmann and coworkers (Bohlmann, Dutta, Robinson, & King, 1979) isolated three flavanones from *C. nauseosus* which they identified as pinocembrin, pinocembrin-5-methyl ether and 6-methoxypinocembrin-7-methyl ether. In a study by Wollenweber et al. (1997), the leaf exudate of *C. nauseosus* ssp. *albicaulis* (Nutt.) Hall and Clem. yielded five methyl ethers of kaempferol and quercetin together with apigenin and its 4′-methyl ether. Hydroxylation of the 6-position in flavonols appears to be a common feature in *C. viscidiflorus*, from which Urbatsch, Bacon and Mabry (1975) isolated six *O*-methylated flavonols derived from 6-hydroxykaempferol, quercetagetin (=6-hydroxyquercetin) and

* Corresponding author. Tel.: +1-541-737-1776; fax: +1-541-737-0497;.
E-mail address: stevensf@ucs.orst.edu (J.F. Stevens)

0031-9422/99/$ - see front matter
PII: S0031-9422(99)00110-7

8-methoxyquercetagetin. These significantly different flavonoid profiles suggest that both species are highly variable with regard to flavonoid chemistry. We now wish to report on a comparative study of the leaf exudate flavonoids of both species together with *C. humilis* from eastern Oregon, with the aim to provide more insight in the interspecific and intraspecific flavonoid variation in *Chrysothamnus*.

2. Results and discussion

Acetone leaf washings of eight plants covering three species of *Chrysothamnus* yielded 26 flavonoid aglycones by column chromatography on Sephadex LH-20 followed by preparative HPLC (Table 1). The flavonoids were identified as representatives (mostly methyl ethers) of flavones, flavonols, flavanones and dihydroflavonols by tandem mass spectrometry, NMR spectroscopy and co-TLC with markers.

2.1. Mass spectrometry

Atmospheric pressure chemical ionization (APCI) tandem mass spectrometry proved to be a powerful technique for characterization of *Chrysothamnus* flavonoids. APCI and other 'soft' ionization techniques, such as FAB (Ma, Li, Van de Heuvel, & Claeys, 1997) and electrospray ionization (Sägesser & Deinzer, 1996), generally produce strong molecular $[MH]^+$ ions without much fragmentation in the ion source. Structural information is obtained by fragmentation of selected $[MH]^+$ ions in collision-induced dissociation (CID) experiments performed in the tandem MS

Table 1
Flavonoid aglycone variation in *Chrysothamnus*

No.	Flavonoid	*C. nauseosus*[a]			Ref.[b]	*C. viscidiflorus*[a]				Ref.[b]	*C. humilis*[a]
		A	B	C		D	E	F	G		H
1	apigenin		+	+	Wollenweber et al., 1997						
2	4′-methyl ether isoscutellarein	+		+	Wollenweber et al., 1997						
3	8-methyl ether	+	+	+							
4	8,4-dimethyl ether	+		+							
5	luteolin							+			
6	3′,4′-dimethyl ether	+	+	+							
7	kaempferol	+			Wollenweber et al., 1997						
8	3-methyl ether	+	+		Wollenweber et al., 1997					Urbatsch et al., 1975	+
	4′-methyl ether				Wollenweber et al., 1997						
9	3,5-dimethyl ether										+
10	3,4′-dimethyl ether herbacetin	+			Wollenweber et al., 1997						
11	3,8-dimethyl ether			+							
12	quercetin	+			Wollenweber et al., 1997			+			
13	3-methyl ether					+	+	+	+	Urbatsch et al., 1975	+
	7-methyl ether				Wollenweber et al., 1997						
14	3′-methyl ether	+			Wollenweber et al., 1997			+		Urbatsch et al., 1975	
15	3,7-dimethyl ether						+	+	+		
16	3,3′-dimethyl ether					+	+	+	+	Urbatsch et al., 1975	+
17	3,4′-dimethyl ether						+	+	+		
18	3,7,3′-trimethyl ether						+	+			
19	3,3′,4′-trimethyl ether						+	+			
20	naringenin						+		+		+
21	eriodictyol						+	+	+		
22	3′-methyl ether					+		+			
23	4′-methyl ether taxifolin						+	+	+		
24	3-acetate					+	+	+	+		
25	3-acetate-7-methyl ether								+		
26	3-acetate-x′-methyl ether[c]						+		+		
	other flavonoids				Bohlmann et al., 1979					Urbatsch et al., 1975	

[a] Key to plants A through H examined: A through C = *C. nauseosus* ssp. *albicaulis*; D = *C. viscidiflorus* ssp. *lanceolatus*; E through G = *C. viscidiflorus* ssp. *viscidiflorus*; H = *C. humilis*.

[b] In Refs. (Bohlmann et al., 1979; Wollenweber et al., 1997; Urbatsch et al., 1975), plants were identified to species only.

[c] The position of the B-ring *O*-methyl (3′ or 4′) could not be established.

mode. We decided to follow Claeys' nomenclature for retro Diels–Alder (RDA) and other cleavage ions produced from protonated flavonoids under CID conditions (pers. comm., (Ma et al., 1997)). In this nomenclature system, adapted from CID mass fragmentation of carbohydrates (Domon & Costello, 1988), A and B ring fragments are labelled $^{i,j}A^+$ and $^{i,j}B^+$ where i and j indicate the cleavage positions in the C-ring. For instance, $^{1,3}A^+$ represents the A ring fragment produced by RDA cleavage of the 1–2 and 3–4 bonds (Fig. 1).

Each of the flavonoids examined yielded characteristic 'finger print' CID mass spectra, except for isomeric pairs **16**/**17** and **22**/**23** which could not be distinguished. In these cases, distinction was achieved by TLC comparison with markers (R_F and colour). RDA fission of the C-ring was most prominent in flavanones and dihydroflavonols. Both flavonoid classes yielded primarily $^{1,3}A^+$ daughter ions. Flavones and flavonols lacking *O*-methyl groups also gave strong RDA fragment ions on collisional dissociation. For instance, in the CID spectra of luteolin and kaempferol, the predominant $^{1,3}A^+$ (100%) fragments were accompanied by B-ring daughter ions, of which $^{1,3}B^+$ (luteolin, 40%) and $^{0,2}B^+$ (kaempferol, 70%) were most abundant.

Loss of methyl radicals (15 amu) from protonated flavonoids was prominent in many *O*-methylated flavones and flavonols, especially when carrying a methoxy function at C-8 as in compounds **3** and **4** (Fig. 1a–c). With herbacetin-3,8-dimethyl ether (**11**), the base peak at m/z 301 $[MH\text{-}30]^+$ may be taken as evidence for consecutive loss of two methyl radicals from the molecular ion with m/z 331 (Fig. 1n–p). This results in an unstable intermediate ion *o* with two odd electrons. The two unpaired electrons are likely to be rearranged so that a covalent O–H bond is formed whereby the charge is retained on the heterocyclic oxygen as shown in Fig. 1. The newly formed ion species *p* contains an α-diketo moiety which subsequently releases CO on collisional activation, giving rise to an ion with m/z 273 $[MH\text{-}30\text{–}28]^+$ (45%).

In many of the 3-methoxy flavonoids the $[MH\text{-}15]^{+\cdot}$ ion is accompanied by an $[MH\text{-}16]^+$ ion of equal or somewhat lesser intensity. Loss of 16 amu can be rationalized by formation of a furan ring involving C-2′ and the oxygen at C-3, upon which methane is released (see e.g. fragment ion *j* in Fig. 1). Loss of methane was also observed for luteolin-3′,4′-dimethyl ether (**6**) (Figs. 1 and 2, ion *g*) which formed a methylenedioxy structure *g* on collisional activation. Further ions were due to RDA ($^{1,3}A^+$, 5%) and expulsion of CO molecules. By contrast, electron impact mass fragmentation of **6** (reported in Audier, 1966) gave rise to RDA fragments ($^{1,3}A^{+\cdot}$, $^{1,3}AH^+$, $^{1,3}B^{+\cdot}$, $[^{1,3}B\text{-}15]^{+\cdot}$), but no loss of 16 amu from the $M^{+\cdot}$ ion was observed. This example demonstrates that EI and CID mass spectra of the same compounds can be very different.

2.2. Identification of flavonoids

Compounds **3** and **4** were isolated from *C. nauseosus* ssp. *albicaulis* and were identified as the 8- and 8,4′-methyl ethers of isoscutellarein (=8-methoxy apigenin) by ^1H NMR and co-TLC. With APCI–tandem mass spectrometry, both flavonoids yielded a fragment ion $[^{1,3}A\text{-}15]^{+\cdot}$ which was not observed for any of the other flavonoids. Moreover, this radical ion was much more intense in the CID spectra of scutellarein-6-methyl ether and its 4′-methyl ether (cf. Figs. 1 and 2, ions *d* and *e*). This indicates that CID experiments allow distinguishing between 6- and 8-methoxy apigenins. The striking difference in the intensity of the $[^{1,3}A\text{-}15]^{+\cdot}$ fragment can be explained by quinonoid stabilization. The 6-methoxy apigenins produce *para* quinonoid fragment ions which appear to be more stable than the *ortho* quinonoid fragments formed on CID of the 8-methoxy analogues. The higher stability of the *para* quinonoid fragment seems to render the 6-methoxy apigenins less resistent to RDA cleavage. Differentiation between 6- and 8-substituted flavonoids by APCI tandem mass spectrometry has been observed previously for 6/8-prenylnaringenins (Stevens, Ivancic, Hsu, & Deinzer, 1997) and vitexin/isovitexin (Rong, Stevens, Deinzer, De Cooman, & De Keukeleire, 1998).

The flavonoid chemistry of *C. humilis* has not been investigated before. Five flavonoids were identified in the leaf exudate of this species (Table 1). Compound **9** was absent from *C. nauseosus* and *C. viscidiflorus*. Its molecular weight (M_r 314) and its ^1H NMR spectrum pointed to a kaempferol dimethyl ether. With respect to mass fragmentation, **9** differed significantly from the 3,7- and 3,4′-dimethyl ethers of kaempferol. In the latter two flavonols, the loss of a methyl radical was much more abundant (>80%) than in **9**. Compound **9** yielded a major fragment ion with m/z 254 which was virtually absent from the fragmentation spectra of the 3,7- and 3,4′-dimethyl ethers. The possibility of the hydroxyls at C-7 and C-4′ being methylated in **9** was also excluded by ^1H NMR, because the H-8, H-2′/6′ and H-3′/5′ doublets did not shift in comparison with kaempferol itself. From the absence of a low field hydrogen bonded OH signal and a small downfield shift of H-6 it was concluded that the location of the two methyls had to be at OH-3 and OH-5. Compound **9** was thus identified as kaempferol-3,5-dimethyl ether. This is the second report of kaempferol-3,5-dimethyl ether from a natural source; it has previously been isolated from *Linaria dalmatica* (Scrophulariaceae) (Kapoor, Rishi, & Atal, 1985).

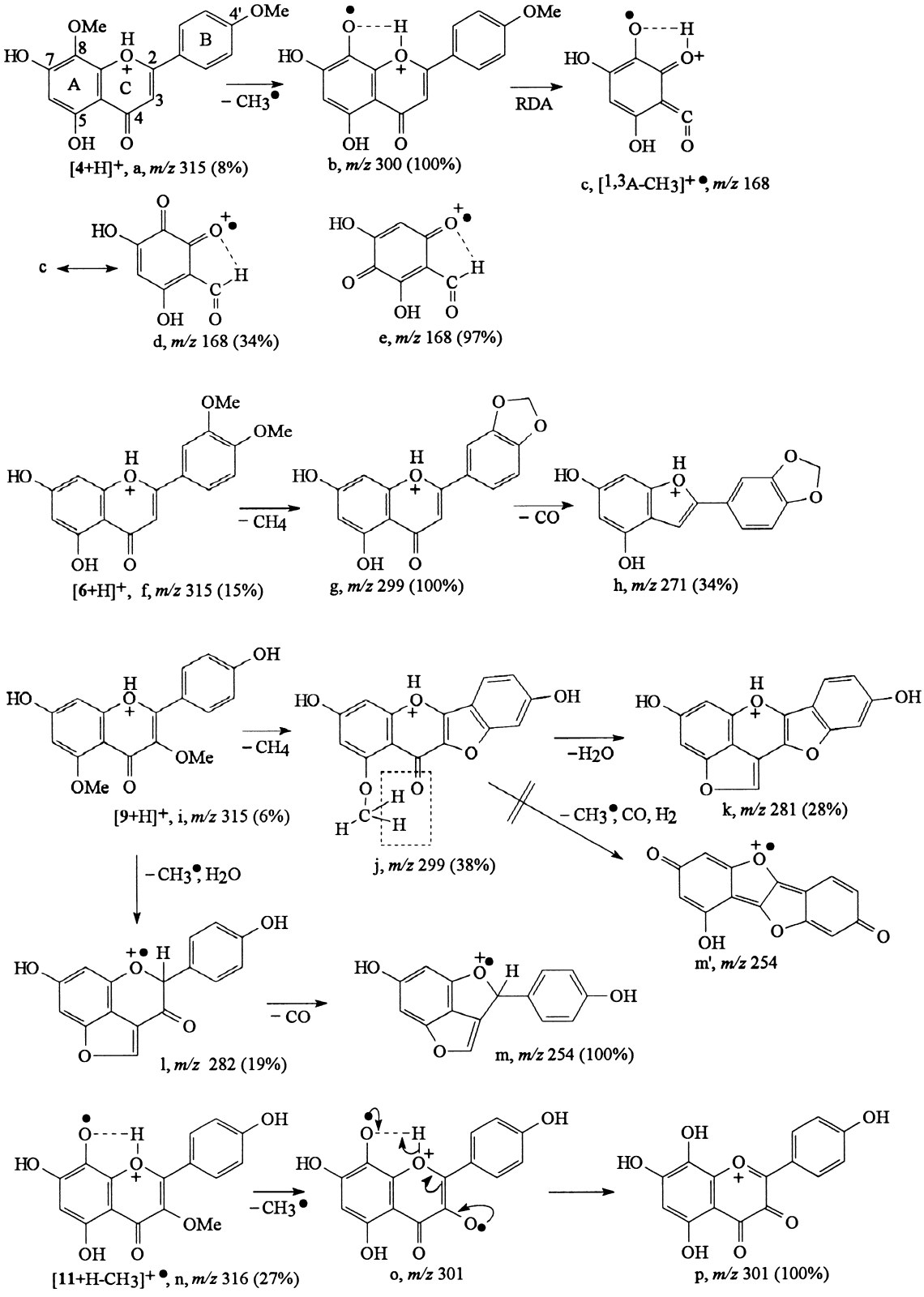


Fig. 1. APCI–CID mass fragmentation of selected flavonoids from *Chrysothamnus*.

The collision-induced fragmentation mass spectrum of **9** showed a base peak at *m/z* 254 (Fig. 2, ion *m*). The even mass of the ion indicates a radical cation. There are at least two ways to account for the loss of 61(=315–254) mass units. In the first route, ion *m'* arises from *j* by loss of a methyl radical, CO and two

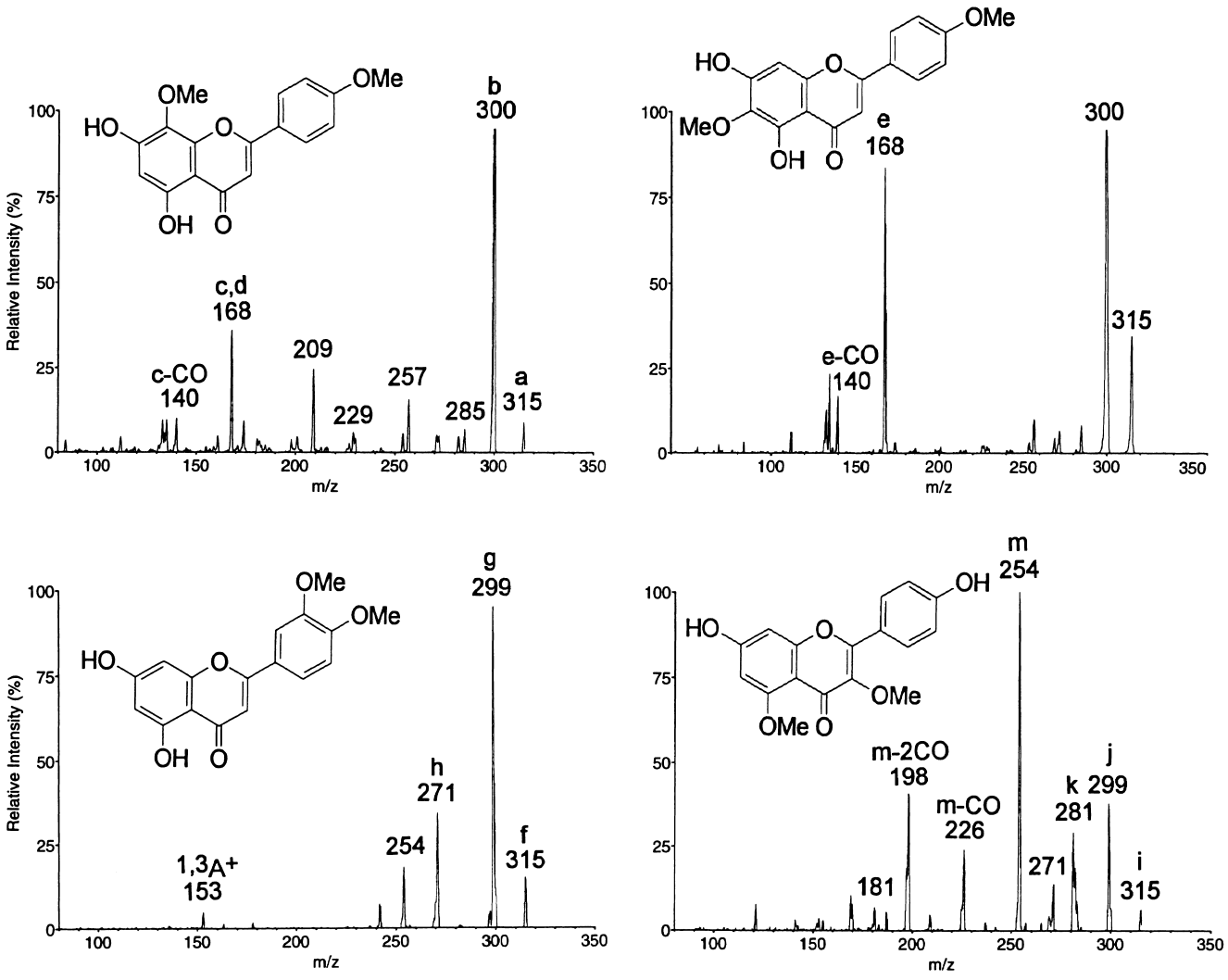


Fig. 2. APCI–CID mass spectra of $[MH]^+$ (m/z 315) ions of isoscutellarein-8,4′-diMe (upper left), scutellarein-6,4′-diMe (upper right), luteolin-3′,4′-diMe (lower left) and kaempferol-3,5-diMe (lower right). For structure of ions (a–m), compare Fig. 1.

hydrogens (16 + 15 + 28 + 2 = 61) (Fig. 1). Its highly conjugated nature would explain why the m/z 254 ion is fairly resistant to further decomposition. Alternatively, consecutive loss of a methyl radical, H_2O, and CO from the molecular ion *i* could lead to ion *m* in the second pathway (Fig. 1). The latter pathway gains support from an earlier study by Bowie and White (1969), who noted that $[M\text{-}H_2O]^{+\cdot}$ ions were formed from flavonol-5-*O*-methyl ethers by EIMS. The C-5 methoxyl and C-4 keto functions were shown to be involved in the release of a water molecule (Bowie & White, 1969). Since the phenolic hydroxyls do not participate in the alternative fragmentation pathway, deuterium labelling was used to investigate the origin of the m/z 254 ion. The hydroxyl hydrogens of compound **9** were allowed to exchange with deuterons by incubation with D_2O, and deuterium incorporation was monitored by electrospray MS (Fig. 3). Although there are two exchangeable hydroxyl protons in **9**, incorporation of the second deuteron was estimated to be ca. 6% as judged from the intensity of the peak at m/z 318; 63% of this peak was attributed to $[(d_2\text{-}\mathbf{9}) + D]^+$ and the remainder to $[(^{13}C, d_1\text{-}\mathbf{9}) + D]^+$ (the contribution of the second ^{13}C atom in an ion species was ignored in the calculations). For this reason, the peak at m/z 317 $[(d_1\text{-}\mathbf{9}) + D]^+$ with a much more favourable $D/^{13}C$ ratio was chosen for collision-induced fragmentation (Fig. 3). Each of the fragment ions *k*, *l*, and *m* shifted two mass units in the electrospray CID mass spectrum (that is, the deuterium labels were retained), indicating that at least one of the phenolic hydrogens is not involved in the loss of 61 amu from *i*. It is therefore hypothesized that the m/z 254 fragment ion arises from $[\mathbf{9} + H]^+$ as depicted in Fig. 1: $i \rightarrow l \rightarrow m$. The small peak at m/z 255 in Fig. 3 could be interpreted as ion m' with one deuteron, but it is far more likely that this ion arises from loss of $^{13}CH_3^{\cdot}$ or ^{13}CO from *i* and *l*, respectively.

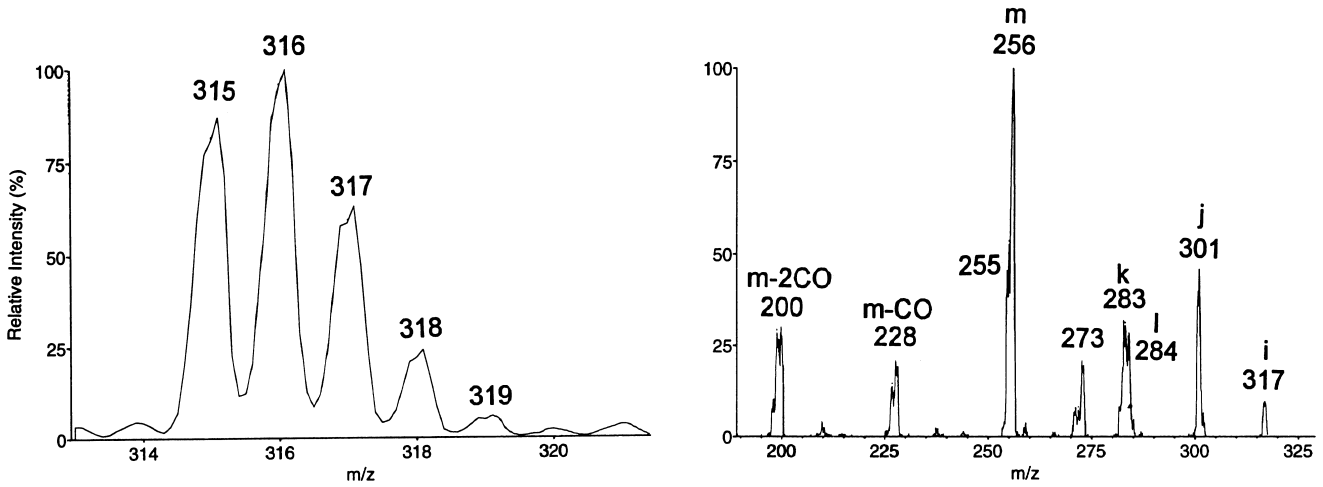


Fig. 3. Electrospray mass spectrometry of kaempferol-3,5-dimethyl ether (**9**) after incubation with deuterium oxide. Left panel: Isotopic pattern of the pseudo-molecular ion of **9** recorded in the single MS mode. Right panel: Collision-induced dissociation (CID) spectrum of the $[(d_1\text{-}\mathbf{9})+D]^+$ ion with m/z 317. The ESI-MS–MS spectrum of $[\mathbf{9}+H]^+$ with m/z 315 (not shown) was very similar to the APCI-MS–MS spectrum of the same ion, shown in Fig. 2. For key to peak labels, compare Fig. 1.

All four plants of *C. viscidiflorus* contained taxifolin-3-acetate (**24**) as a major leaf surface flavonoid. The presence of an acetic acid residue was apparent from a peak at m/z 287 ($[MH–HOAc]^+$, 100% int.) in the APCI mass spectrum recorded in the single MS mode (loss of 60 amu effected by collisions in the ion source at atmospheric pressure). On collisional activation of the molecular ion, fragment ions were formed as a result of loss of CO neutrals from $[MH\text{-}60]^+$ and fission of the C-ring. The predominant RDA fragment, $^{1,3}A$ (m/z 153, 100% int.), was accompanied by a peak at m/z 149 (79%) which was attributed to the cleavage fragment, $[^{0,2}A–HOAc]^+$. The identity was confirmed by ^1H and ^{13}C NMR (see Section 3). The ^{13}C signals were assigned by ^1H–^{13}C heteronuclear multiple quantum coherence (HMQC) and heteronuclear multiple bond correlation (HMBC) spectroscopy and were consistent with literature data (Gao, Wang, Mabry, & Kinghorn, 1990). Rather unexpectedly, H-8 appeared upfield relative to H-6 in the ^1H NMR spectrum recorded in DMSO-d_6. This was apparent from interaction between the A-ring proton at δ_H 5.95 and carbons 5, 7, 8 and 10 in the HMBC spectrum. Since no cross peak was observed with C-9, this signal had to be assigned to H-6. The other A-ring resonance (δ_H 5.91) interacted with all A-ring carbons except C-5 and was therefore attributed to H-8. Carbons 5, 6 and 10 were readily identified by their cross peaks with OH-5 at δ_H 11.5. These assignments were corroborated by HMQC. The positive optical rotation (+41°) together with the magnitude of $J_{2,3}$ (12 Hz) indicated the normal 2*R*:3*R* configuration (Markham, Webby, & Vilain, 1984).

In one plant of *C. viscidiflorus* ssp. *viscidiflorus* (plant G), taxifolin-3-acetate (**24**) was accompanied by minor quantities of compounds **25** and **26**. The latter flavonoid was also found in the leaf exudate of plant E of the same subspecies. Since both compounds showed APCI mass spectra with m/z 361 $[MH]^+$ and $[MH\text{-}60]^+$ ions, they were tentatively identified as mono methyl ethers of **24**. Mass fragmentation of **25** by CID gave two A-ring cleavage ions, i.e. $^{1,3}A^+$ ion with m/z 167 and $[^{0,2}A–HOAc]^+$ with m/z 163, indicating that the methyl is located at OH-7 or else at OH-5. The latter possibility was ruled out by TLC. Compound **25** proved to be identical to taxifolin-3-acetate-7-methyl ether by co-TLC on silica gel and polyamide (R_F and spot colour). Collisional activation of compound **26** yielded a strong RDA fragment ion, $^{1,3}A^+$, with m/z 153 (100%) and an $[^{0,2}A–HOAc]^+$ ion with m/z 149, thus leaving the methyl function to be located at one of the two B-ring hydroxyls. The presence of a B-ring *O*-methyl was also apparent from a peak at m/z 137 representing the $[^{1,3}B–C_2H_2O–H_2CO]^+$ fragment ion. This ion appeared at m/z 123 in the CID spectra of compounds **24** and **25**. The site of the *O*-methyl could not be established. Compounds **25** and **26** were obtained in insufficient amounts for structure confirmation by NMR spectroscopy.

2.3. Chemotaxonomic significance

The flavonoid profiles of *Chrysothamnus* (Table 1) indicate that the variation has diagnostic value at the species level. *Chrysothamnus nauseosus* is characterized by the combination of methyl ethers of apigenin, isoscutellarein, luteolin, and kaempferol. Except for the two isoscutellarein methyl ethers and luteolin-3,4-dimethyl ether, our findings are more or less consistent with an earlier survey of *C. nauseosus* from Arizona

(Wollenweber et al., 1997) (cf. Table 1). On the other hand, the presence of pinocembrin, pinocembrin-5-methyl ether and 6-methoxypinocembrin-7-methyl ether as reported from this species by Bohlmann et al. (1979) could not be confirmed. These authors did not mention the origin of their plant material nor did they include a statement as to whether additional flavonoids had been isolated along with the pinocembrin derivatives.

Kaempferol and its methyl ethers were found absent from the leaf exudate of *C. viscidiflorus*. The flavonoid chemistry of the latter species is mainly based on (methyl ethers of) quercetin, naringenin, eriodictyol and taxifolin-3-acetate. *Chrysothamnus viscidiflorus* ssp. *lanceolatus* (one plant examined) yielded only four flavonoids but they form a fair representation of the flavonols, flavanones and dihydroflavonols that characterize the three plants of *C. viscidiflorus* ssp. *viscidiflorus*. The differences between both subspecies with regard to the number of flavonoid scores are probably of a quantitative nature. Three quercetin methyl ethers were previously isolated from *C. viscidiflorus* by Urbatsch et al. (1975). However, our findings contrast sharply with their report of 6-hydroxyflavonols (jaceidin and quercetagetin methyl ethers) from plant material collected in Arizona. Furthermore, they made no mention of flavanones or taxifolin-3-acetate. The latter dihydroflavonols proved to be one of the major flavonoids in all four plants of *C. viscidiflorus* included in our survey. These differences seem to emphasize biogenetic divergence, i.e. presence/absence of (1) flavanone 3-hydroxylation and (2) flavonol 6-hydroxylation and may point to the existence of chemotypes.

In recent years several genera in the Astereae tribe of the Asteraceae have been realigned. The large genus, *Haplopappus*, has been segregated into sixteen, mainly North American genera (see review, Lane and Hartman (1996)), including *Ericameria*, a close relative of *Chrysothamnus*. In 1993, Nesom and Baird (1993) transferred a portion of *Chrysothamnus* (including *C. nauseosus*) to *Ericameria*, citing morphological, chloroplast DNA, hybridization, and other evidence. Anderson (1995) later transferred the rest of the genus, mainly on the basis of morphological similarity. Since then others have argued for retaining most species in *Chrysothamnus*, while transferring some to *Ericameria* (Lane et al., 1996; Nesom & Baird, 1995). Flavonoid reports are available for six species of *Ericameria*, viz. *E. bloomeri* (A. Gray) J.F. Macbr. (Wollenweber et al., 1997), *E. cooperi* (A. Gray) Hall (Urbatsch & Wussow, 1979), *E. diffusa* Benth. (Urbatsch, Mabry, Miyakado, Ohno, & Yoshioka, 1976), *E. laricifolia* (A. Gray) Shinners (Urbatsch & Wussow, 1979; Clark & Wollenweber, 1984), *E. linearifolia* (DC.) Urbatsch and Wussow (Wollenweber et al., 1997; Urbatsch & Wussow, 1979) and *E. parrasana* Blake (Urbatsch & Wussow, 1979). Their flavonoid profiles are mainly based on kaempferol and quercetin *O*-methyl ethers also found in *Chrysothamnus*. Furthermore, *C. nauseosus* and *E. laricifolia* share the presence of apigenin and its *O*-methyl ethers. Dihydroflavonols have been found in shoot surface exudates of *E. parassana* from Mexico; flavones and flavonols could not be detected in this species (Urbatsch & Wussow, 1979). Because *O*-methyl derivatives of common flavonols like kaempferol and quercetin are widely distributed in the Asteraceae (Wollenweber, 1994), the similarities between both genera with respect to flavonoid chemistry neither favour nor argue against inclusion of part or all of *Chrysothamnus* in *Ericameria* as suggested by earlier authors (Nesom & Baird, 1993, 1995; Anderson, 1995; Lane et al., 1996).

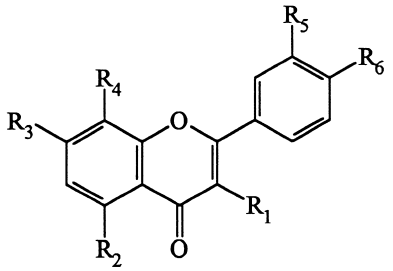
3. Experimental

3.1. Plant material

Overground parts with inflorescences were collected by J.F. Stevens in eastern Oregon in August 1996. Identifications were made by S. Sundberg. The collection sites were as follows:

Chrysothamnus nauseosus ssp. *albicaulis*
A: Oregon, Harney Co., 35 Mi west of Burns along US Hwy 20, *JF Stevens s.n.* 25 August 1996
B: Oregon, Harney Co., 34 Mi west of Burns along US Hwy 20, *JF Stevens s.n.* 25 August 1996
C: Oregon, Lake Co., 53 Mi west of Burns along US Hwy 20, *JF Stevens s.n.* 25 August 1996
Chrysothamnus viscidiflorus ssp. *lanceolatus*
D: Oregon, Harney Co., 16 Mi west of Burns along US Hwy 20, *JF Stevens s.n.* 18 August 1996
Chrysothamnus viscidiflorus ssp. *viscidiflorus*
E: Oregon, Harney Co., 34 Mi west of Burns along US Hwy 20, *JF Stevens s.n.* 25 August 1996
F: Oregon, Harney Co., 35 Mi west of Burns along US Hwy 20, *JF Stevens s.n.* 25 August 1996
G: Oregon, Lake Co., 53 Mi west of Burns on Buck Creek Road, north of US Hwy 20, *JF Stevens s.n.* 25 August 1996
Chrysothamnus humilis
H: Oregon, Lake Co., 53 Mi west of Burns along US Hwy 20, *JF Stevens s.n.* 25 August 1996.

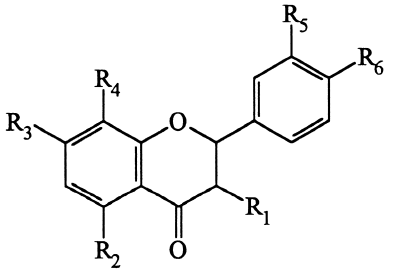
The samples were air-dried and stored at room temperature prior to extraction. Voucher specimens have been deposited at the Oregon State University Herbarium (OSC).



	R_1	R_2	R_3	R_4	R_5	R_6
1	H	OH	OH	H	H	OH
2	H	OH	OH	H	H	OMe
3	H	OH	OH	OMe	H	OH
4	H	OH	OH	OMe	H	OMe
5	H	OH	OH	H	OH	OH
6	H	OH	OH	H	OMe	OMe
7	OH	OH	OH	H	H	OH
8	OMe	OH	OH	H	H	OH
9	OMe	OMe	OH	H	H	OH
10	OMe	OH	OH	H	H	OMe
11	OMe	OH	OH	OMe	H	OH
12	OH	OH	OH	H	OH	OH
13	OMe	OH	OH	H	OH	OH
14	OH	OH	OH	H	OMe	OH
15	OMe	OH	OMe	H	OH	OH
16	OMe	OH	OH	H	OMe	OH
17	OMe	OH	OH	H	OH	OMe
18	OMe	OH	OMe	H	OMe	OH
19	OMe	OH	OH	H	OMe	OMe

3.2. Extraction and purification of flavonoids

Overground plant parts were air-dried and kept at room temp until analysis. After removal of the inflorescences, the vegetative parts (50–450 g) were immersed in Me_2CO for ca. 2 min. The Me_2CO washings were filtered and evapd in vacuo. The residues were extracted with 50–100 ml of boiling MeOH for ca. 5 min. The MeOH extracts were left standing overnight in a freezer (−18°C) and then centrifuged to separate the MeOH soluble flavonoids from precipitated lipophlic material. The supernatants were concentrated on a rotavapor and subsequently chromatographed on Sephadex LH-20 with MeOH as the eluting solvent. Fractions of ca. 10 ml were collected and monitored by TLC on silica gel (toluene–dioxane–HOAc, 18:5:1). Fractions of similar flavonoid composition were combined.



20	H	OH	OH	H	H	OH
21	H	OH	OH	H	OH	OH
22	H	OH	OH	H	OMe	OH
23	H	OH	OH	H	OH	OMe
24	OAc	OH	OH	H	OH	OH
25	OAc	OH	OMe	H	OH	OH
26	OAc	OH	OH	H	OH or OMe	

3.3. Semi-preparative HPLC separation

Individual flavonoids were isolated from pooled fractions by semi-prep. HPLC on a 10 μm Econosil RP-18 (250 × 10 mm) column using linear gradient elution, typically from 45% to 95% MeOH in 1% aq. HCOOH over 50 min at 5 ml min^{-1}. The UV trace was recorded at 280 nm. Peak fractions were collected manually, concentrated on a rotavapor and then lyophilized. Most peak fraction were of sufficient homogeneity to allow chromatographic and spectroscopic analysis. When necessary, mixed peak fractions were re-chromatographed on the same semi-preparative column using different solvent gradients, e.g. from 25 to 75% MeCN in 1% aq. HCOOH over 50 min at 5 ml min^{-1}.

3.4. Co-TLC analysis

Identities were confirmed by co-TLC with authentic markers available in E.W.'s laboratory in Darmstadt. TLCs were run on silica gel with toluene–dioxane–HOAc (18:5:1) and on polyamide DC-11 with toluene–petrol (100°–140°C)–MeCOEt–MeOH (12:6:2:1). Developed plates were examined under UV (350 nm) before and after spraying with diphenyl–boric acid–ethanolamine complex (1%) in MeOH.

3.5. NMR spectroscopy

Compounds **2**–**4**, **6**, **8**–**10**, **13**–**19** and **21**–**24** were examined by ^1H (600 MHz) NMR; selected data are presented below (a complete set is available from the first author on request). In addition, ^{13}C (150.9 MHz), ^1H–^{13}C HMQC and HMBC experiments were performed with compound **24** using standard pulse sequences. Spectral widths of 12 and 220 ppm were used in the H and C dimensions. All spectra were recorded in DMSO–d_6 at room temp and the DMSO resonance at δ_H 2.50 was used as internal shift reference.

3.6. Mass spectrometry

Atmospheric pressure chemical ionization (APCI) mass spectra were run on a PE Sciex API II Plus triple quadrupole instrument. Samples were introduced by

direct injection or by HPLC (flow rate 0.5–1.0 ml min^{-1}) via the heated nebulizer interface kept at 480°C which heats the column effluent to ca. 120°C. Ionization of the analyte vapour mixture was initiated by a corona discharge needle at ca. +6 kV and a discharge current of ca. 3 μA. The orifice plate voltage was set at 55 V in the positive ion mode. Collision-induced dissociation (CID) experiments were performed with $Ar–N_2$ (9:1) as target gas at a thickness of ca. 1.9×10^{14} atoms cm^{-2}. The collision energy was 30 V. Other operating conditions were standard.

3.7. Deuterium labelling experiments

Deuterium incorporation in kaempferol-3,5-dimethyl ether (**9**) was achieved by incubation of **9** with $MeCN–D_2O$ (1:2) for 1 day. The soln was then acidified with 0.1% AcOD and introduced into the mass spectrometer by loop injection (4 μl) at a flow rate of 5 μl min^{-1} (carrier solvent: $MeCN–D_2O$–AcOD, 500:500:1). The mass spectrometer was a PE–Sciex API III Plus triple–quadrupole instrument equipped with a nebulizer-assisted electrospray interface. The voltage on the spray capillary was +5.3 kV and the orifice plate voltage was +80 V. CID experiments were carried out as described above. The CID spectra of $[\mathbf{9}+H]^+$ obtained with the APCI source and the electrospray source were similar.

3.8. Isoscutellarein-8-methyl ether (*3*)

APCI–MS, *m/z* (rel. int.), 301 $[MH]^+$ (100); APCI–MS–MS, 286 $[MH–Me]^+$ (100), 195 (23), 168 $[^{1,3}A–Me]^+$ (29). ^1H NMR δ 6.82 (1H, s, H-3), 6.29 (1H, s, H-6), 7.94 (2H, d, $J=8.7$ Hz, H-2′/6′), 6.97 (2H, d, $J=8.7$ Hz, H-3′/5′), 12.64 (OH-5), 3.85 (OMe).

3.9. Isoscutellarein-8,4′-dimethyl ether (*4*)

APCI–MS, *m/z* (rel. int.), 315 $[MH]^+$ (100); APCI–MS–MS, see Figs. 1 and 2. ^1H NMR δ 6.88 (1H, s, H-3), 6.30 (1H, s, H-6), 8.03 (2H, d, $J=8.5$ Hz, H-2′/6′), 7.16 (2H, d, $J=8.5$ Hz, H-3′/5′), 12.59 (OH-5), 3.85 and 3.87 (2 × OMe).

3.10. Luteolin-3′,4′-dimethyl ether (*6*)

APCI–MS, *m/z* (rel. int.), 315 $[MH]^+$ (100); APCI–MS–MS, see Figs. 1 and 2. ^1H NMR δ 6.98 (1H, s, H-3), 6.21 (1H, d, $J=2$ Hz, H-6), 6.54 (1H, d, $J=2$ Hz, H-8), 7.58 (1H, d, $J=2$ Hz, H-2′), 7.14 (1H, d, $J=8.4$ Hz, H-5′), 7.69 (1H, dd, $J=2$, 8.4 Hz, H-6′), 12.93 (OH-5), 3.86 and 3.89 (2 × OMe).

3.11. Kaempferol-3,5-dimethyl ether (*9*)

APCI–MS, *m/z* (rel. int.), 315 $[MH]^+$ (100); APCI–MS–MS, see Figs. 1 and 2. ^1H NMR δ 6.35 (1H, d, $J=2.0$ Hz, H-6), 6.46 (1H, d, $J=2.0$ Hz, H-8), 7.88 (2H, d, $J=8.9$ Hz, H-2′/6′), 6.92 (2H, d, $J=8.9$ Hz, H-3′/5′), 3.70 and 3.80 (2 × OMe).

3.12. Eriodictyol-3′-methyl ether (*22*)

APCI–MS, *m/z* (rel. int.), 303 $[MH]^+$ (100); APCI–MS–MS, 177 $[^{1,4}B]^+$ (23), 153 $[^{1,3}A]^+$ (100), 145 $[^{1,4}B–MeOH]^+$ (56), 117 $[145\text{-}CO]^+$ (52). ^1H NMR δ 5.43 (1H, dd, $J=2.8$, 12.8, H-2), 3.33 (1H, dd, $J=12.8$, 17.2, H-3*ax*), 2.69 (1H, dd, $J=2.8$, 17.2, H-3*eq*), 5.89 (1H, d, $J=1.9$ Hz, H-6), 5.90 (1H, d, $J=1.9$ Hz, H-8), 7.10 (1H, d, $J=1.4$ Hz, H-2′), 6.80 (1H, d, $J=8.1$ Hz, H-5′), 6.91 (1H, dd, $J=1.5$, 8.1, H-6′), 12.16 (OH-5), 3.79 (OMe).

3.13. (2R,3R)-taxifolin-3-acetate (*24*)

$[\alpha]_D$ +41° (MeOH; *c* 0.42) (lit (Gao et al., 1990) +39°, MeOH, *c* 0.67). APCI–MS, *m/z* (rel. int.), 347 $[MH]^+$ (54), 287 $[MH–HOAc]^+$ (100); APCI–MS–MS, 287 (13), 259 $[287\text{-}CO]^+$ (17), 231 $[287\text{-}2CO]^+$ (60), 153 $[^{1,3}A]^+$ (100), 149 $[^{0,2}A–HOAc]^+$ (79), 123 $[^{1,3}B–C_2H_2O–H_2CO]^+$ (35). NMR δ_H 5.41 (1H, d, $J=11.7$ Hz, H-2), 5.81 (1H, d, $J=11.7$ Hz, H-3), 5.95 (1H, d, $J=2.0$ Hz, H-6), 5.91 (1H, d, $J=2.0$ Hz, H-8), 6.90 (1H, s, H-2′), 6.75 (2H, br s, H-5′/6′), 11.46 (OH-5), 1.97 (OAc); δ_C 80.15 (C-2), 72.00 (C-3), 191.35 (C-4), 163.22 (C-5), 96.38 (C-6), 167.43 (C-7), 95.41 (C-8), 162.37 (C-9), 100.54 (C-10), 126.24 (C-1′), 114.86 (C-2′), 145.13 (C-3′), 146.20 (C-4′), 115.29 (C-5′), 119.14 (C-6′), 168.74 (C-1″), 20.12 (C-2″).

3.14. Taxifolin-3-acetate-7-methyl ether (*25*)

APCI–MS, *m/z* (rel. int.), 361 $[MH]^+$ (100), 301 $[MH–HOAc]^+$ (34); APCI–MS–MS, 301 (11), 273 $[301\text{-}CO]^+$ (25), 245 $[301\text{-}2CO]^+$ (79), 167 $[^{1,3}A]^+$ (100), 163 $[^{0,2}A–HOAc]^+$ (79), 123 $[^{1,3}B–C_2H_2O–H_2CO]^+$ (25).

3.15. Taxifolin-3-acetate-x′-methyl ether (*26*)

APCI–MS, *m/z* (rel. int.), 361 $[MH]^+$ (35), 301 $[MH–HOAc]^+$ (100); APCI–MS–MS, 301 (7), 273 $[301\text{-}CO]^+$ (9), 245 $[301\text{-}2CO]^+$ (34), 153 $[^{1,3}A]^+$ (100), 149 $[^{0,2}A–HOAc]^+$ (50), 137 $[^{1,3}B–C_2H_2O–H_2CO]^+$ (17). The position of the *O*-methyl (3′ or 4′) could not be established.

Acknowledgements

We are grateful to Dr. C.S. Maier for help with collecting the plant material. The Sciex API III Plus mass spectrometer was purchased in part through grants from the National Science Foundation (BIR-9214371) and from the Anheuser–Busch Companies. The multinuclear Bruker DRX 600 NMR instrument was purchased in part through grants from the National Science Foundation (BIR-9413692) and the W.M. Keck Foundation.

References

Anderson, L. C. (1986). In E. D. McArthur, B. L. Welch, & Ogden, *Proceedings-symposium on the biology of Artemisia and Chrysothamnus; 1984 July 9–13; Provo, Utah* (pp. 29–45). U.S. Department of Agriculture, Forest Service, Intermountain Research Station General Technical Report INT-200.

Anderson, L. C. (1995). *Great Basin Naturalist*, *55*, 84.

Audier, H. (1966). *Bulletin de la Société chimique de France*, 2892.

Bohlmann, F., Dutta, L., Robinson, H., & King, R. M. (1889). *Phytochemistry*, *1979*, 18.

Bowie, J. H., & White, P. Y. (1969). *Journal of the Chemical Society (B)*, 89.

Clark, W. D., & Wollenweber, E. (1984). *Zeitschrift für Naturforschung*, *39c*, 1184.

Domon, B., & Costello, C. (1988). *Glycoconjugate Journal*, *5*, 397.

Gao, F., Wang, H., Mabry, T. J., & Kinghorn, A. D. (1990). *Phytochemistry*, *29*, 2865.

Kapoor, R., Rishi, A. K., & Atal, C. K. (1985). *Fitoterapia*, *56*, 296.

Lane, M. A., & Hartman, R. L. (1996). *American Journal of Botany*, *83*, 356.

Lane, M. A., Morgan, D. R., Suh, Y., Simpson, B. B., & Jansen, R. K. (1996). In D. J. N. Hind, & H. J. Beentje, *Compositae: systematics, Proceedings of the International Compositae Conference, Kew, 1994*, *vol. 1* (pp. 49–77). Kew: Royal Botanic Gardens.

Ma, Y. L., Li, Q. M., Van den Heuvel, H., & Claeys, M. (1997). *Rapid Communications in Mass Spectrometry*, *11*, 1357.

Markham, K. R., Webby, R. F., & Vilain, C. (1984). *Phytochemistry*, *23*, 2049.

Nesom, G. L., & Baird, G. I. (1993). *Phytologia*, *75*, 74.

Nesom, G. L., & Baird, G. I. (1995). *Phytologia*, *78*, 61.

Rong, H., Stevens, J. F., Deinzer, M. L., De Cooman, L., & De Keukeleire, D. (1998). *Planta Medica*, 620.

Sägesser, M., & Deinzer, M. (1996). *Journal of the American Society of Brewing Chemists*, *54*, 129.

Stevens, J. F., Ivancic, M., Hsu, V. L., & Deinzer, M. L. (1997). *Phytochemistry*, *44*, 1575.

Urbatsch, L. E., & Wussow, J. R. (1979). *Brittonia*, *31*, 265.

Urbatsch, L. E., Bacon, J. D., & Mabry, T. J. (1975). *Phytochemistry*, *14*, 2279.

Urbatsch, L. E., Mabry, T. J., Miyakado, M., Ohno, N., & Yoshioka, H. (1976). *Phytochemistry*, *15*, 440.

Wollenweber, E. (1994). In J. B. Harborne, *The Flavonoids: Advances in research since 1986* (p. 259). London: Chapman and Hall.

Wollenweber, E., Dörr, M., Fritz, H., Papendieck, S., Yatskievych, G., & Roitman, J. N. (1997). *Zeitschrift für Naturforschung*, *52c*, 301.