PERGAMON

Phytochemistry 50 (1999) 219–225

PHYTOCHEMISTRY

HPLC–ESI–MS/MS analysis of sulfated flavor compounds in plants

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Revised 1 July 1998

Abstract

Synthesis of six new flavor sulfates (benzyl sulfate, 2-phenylethyl sulfate, 2,5-dimethyl-4-hydroxy-3(2H)-furanone sulfate, α-ionol sulfate, vomifoliol sulfate, linalyl sulfate) was performed in order to screen for these compounds in plants. Structural elucidation was performed by NMR spectroscopy and a screening method developed by using high performance liquid chromatography–electrospray ionization tandem mass spectrometry (HPLC–ESI–MS/MS). The results obtained with various plant tissues indicate that sulfation of flavor compounds is a common pathway in plant metabolism. © 1998 Elsevier Science Ltd. All rights reserved.

Keywords: Sulfate esters; Flavor; Benzyl sulfate; 2-Phenylethyl sulfate; 2,5-Dimethyl-4-hydroxy-3(2H)-furanone sulfate; α-Ionol sulfate; Vomifoliol sulfate; Linalyl sulfate; HPLC–ESI–MS/MS; Electrospray ionization (ESI)

1. Introduction

In 1937, Kawaguchi and Kim (1937) reported for the first time a sulfate ester of a flavonol, namely isorhamnetin 3-sulfate, as naturally occurring in *Persicaria hydropiper*. Up to the seventies, when Harborne (1975) reviewed this new class of sulfur compounds in plants, little attention has been paid to these polar flavonoid derivatives. Later there has been an increasing number of reports dealing with these conjugates, summarized by Barron, Varin, Ibrahim, Harborne, and Williams (1988). The data show the common occurrence of sulfate esters, mostly derivatives of hydroxyflavones and -flavonols or their methyl ethers and, less widely distributed, the corresponding glycosylated derivatives in a variety of plants. Besides, other reports describe the occurrence of sulfated derivatives of hydroxycinnamic acids (Imperato, 1982), coumarins (Lemmich & Shabana, 1984), anthraquinones (Harborne & Mokhtari, 1977), cyanogenic glycosides (Spencer & Seigler, 1985) and anthocyanins (Toki et al., 1994).

In contrast to the sulfation mechanism in animal tissues leading to changes of solubility and metabolic activity of phenols, steroids, xenobiotics, or flavor compounds (Mulder, 1981), the physiological role of these plant constituents has not been clarified to date. Nothing is known about the sulfation of flavor compounds in plants leading to the question about a possible co-occurrence of sulfates besides well-known flavor precursors like glycosides (Winterhalter & Schreier, 1994) or phosphates (Ney, Jäger, Herderich, Schreier, & Schwab, 1996). Previously, for flavonoids a co-occurrence of sulfates and glycosides has been described (Varin, Barron, & Ibrahim, 1986).

In order to screen for sulfated flavor precursors, synthesis of sulfate esters **1**–**6** of different chemical classes (benzyl alcohol, 2-phenylethanol, 2,5-dimethyl-4-hydroxy-3(2H)-furanone, α-ionol, vomifoliol, and linalol) was performed and a screening method developed by HPLC–ESI–MS/MS. The present paper describes the results obtained by screening a widespread variation of plant tissues for these compounds.

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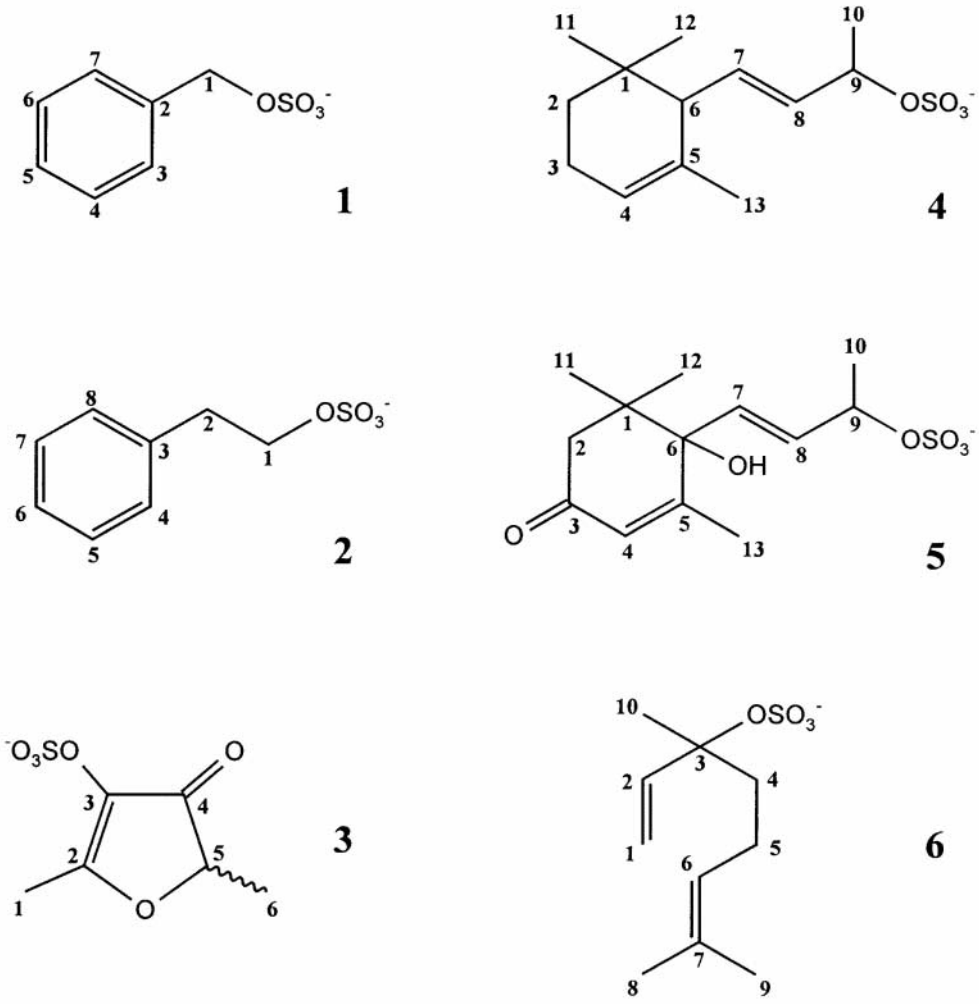
0031-9422/98/$ - see front matter
PII: S0031-9422(98)00526-3



2. Results and discussion

For the screening of sulfated flavor compounds, synthesis of a representative selection of such conjugates was performed by standard methods (Lieberman, Hariton, & Fukushima, 1948; Piasecki, 1992). The purified compounds were obtained in overall yields ranging from 20% to 90% and characterized by ^{1}H and ^{13}C NMR spectroscopy (cf. Tables 1 and 2 and Section 3). Distinct downfield shifts (1–4 ppm) of the α-carbons and slightly upfield shifts of the β-carbons observed in comparison with their corresponding alcohols were consistent with data reported for coumarin sulfates (Lemmich & Shabana, 1984) as well as carbohydrate and steroid sulfates (Lillard & Seib, 1978; Goto, Kato, Hasegawa, & Nambara, 1979). In addition, the ^{1}H NMR data confirmed the observations in the ^{13}C NMR spectra by slightly downfield shifts of the hydrogens at the α-carbons.

Using HPLC–ESI–MS analysis in negative mode, an abundant pseudomolecular ion $[M]^-$ corresponding to the molecular mass of the anion was observed for each sulfated flavor compound under study. MS/MS experiments of these precursor ions produced characteristic product ion spectra. Sulfate ester **3** was the only compound under study which showed an abundant fragment *m/z* 127 corresponding to the aglycone moiety $[\text{aglycone}-H]^-$. This observation is in good agreement with MS/MS studies using 2,5-dimethyl-4-hydroxy-3(2H)-furanone derivatives in positive mode, where the aglycone fragment was the main fragment in the product ion spectrum (Roscher, Herderich, Steffen, Schrier, & Schwab, 1996). The other compounds produced base peaks with *m/z* 97 (for **2**, **4**, and **5**) corresponding to $[HSO_4]^-$ or *m/z* 96 (for **1** and **6**). In accordance to data recorded for steroid sulfates (Weidolf, Lee, & Henion, 1988) the fragment *m/z* 96 corresponds to a lack of the aglycone moiety leading possibly to $[SO_4]^{-\cdot}$. By increasing the collision induced dissociation (CID) offset voltage the fragmentation pattern could be shifted to the fragment *m/z* 80 corresponding to $[SO_3]^-$ (cf. Fig. 1). The detection of *m/z* 80 was less sensitive in comparison to *m/z* 96/97/127, as it was not possible to shift the fragmentation pattern completely to *m/z* 80. To distinguish between phosphates and sulfates, it is imperative to have a

Table 1
^{1}H NMR data of **4** and the corresponding alcohol (400 MHz; δ ppm relative to solvent signal, coupling constants in Hz)

	4 (DMSO-d_6)		Alcohol[a] ($CDCl_3$)	
H-2α/3α	1.11–1.14	2H, m	1.13–1.19	2H, m
H-2β	1.31–1.45	1H, m	1.38–1.45	1H, m
H-3β	1.98	1H, br s	1.98	1H, br s
H-4	5.28–5.49	m[b]	5.39–5.53	m[b]
H-6	1.89	1H, d, 8.4	2.07	1H, d, 9.2
H-7/8	5.28–5.49	m[b]	5.39–5.53	m[b]
H-9	4.25–4.30	1H, m	4.22	1H, quin, 6.2
H-10	1.19	3H, d, 6.2	1.26	3H, d, 6.4
H-11[c]	1.01/1.03	3H, 2 × s	0.88	3H, s
H-12[c]	0.90/0.96	3H, 2 × s	0.80/0.82	3H, 2 × s
H-13	1.71	3H, 2 × s	1.57	3H, 2 × d, 1.5

[a]See Pabst, Barron, Sémon, & Schreier (1992). [b]Signals overlapped (3H). [c]Interchangeable values.

Table 2
^{13}C NMR data of **4** and the corresponding alcohol (100 MHz, δ ppm relative to solvent signal)

	4 (DMSO-d_6)	Alcohol[a] ($CDCl_3$)
C-1	32.3	32.0
C-2	31.3/31.4	31.6
C-3	23.7	23.6
C-4	119.6/119.7	121.0
C-5	136.4	134.0
C-6	48.9	54.0
C-7	132.0	131.2
C-8	132.5	136.0
C-9	72.0/72.7	68.8/68.9
C-10	20.0	23.1
C-11[b]	28.8	27.4/27.5
C-12[b]	27.6	27.0
C-13	22.6	22.8

[a]See Pabst et al. (1992). [b]Assignments may be reversed.

second detectable product ion specific for sulfates, because both conjugates exhibit as abundant fragment in the ESI negative mode m/z 97 corresponding to $[H_2PO_4]^-$ and $[HSO_4]^-$, respectively (Ney et al., 1996; Feurle, Jomaa, Wilhelm, Gutsche, & Herderich, 1998). In contrast, the second fragment for sulfates m/z 80 corresponding to $[SO_3]^-$ is absent in the spectra of phosphates exhibiting m/z 79 corresponding to $[PO_3]^-$ (Ney et al., 1996; Feurle et al., 1998). With these data in hand an unambiguous assignment of the sulfates is possible.

With a test-mixture of **1**–**6**, a screening method for these new conjugates in plants was developed. To separate the reference compounds from each other, RP-18 HPLC separation using a gradient with ammonium acetate in water and methanol, respectively, was chosen. In order to increase the sensitivity of the analysis and to avoid co-elutions of compounds **2** and **5**, separate runs for compounds **1**–**3** (group 1) and **4**–**6** (group 2) were performed. For each group two different time-dependent selected reaction monitoring (SRM) experiments, corresponding to the different fragmentation pattern at various CID offset voltages, were selected to screen plant extracts: (i) SRM 1 and 3 yielding the product ions m/z 96/97/127, respectively; and (ii) SRM 2 and 4 yielding the product ion m/z 80 for all compounds under study. SRM increased the sensitivity and was highly selective because of excluding matrix effects by filtration of the precursor ion $[M]^-$ and its specific product ion.

With this method in hand a wide variety of plant tissues was screened for compounds **1**–**6**. Extracts from fresh and dried leaves and fruits were obtained by XAD-2 solid phase extraction. In order to further purify the extracts a second XAD-2 column was eluted with 30% methanol sufficient for elution of these polar conjugates. A representative example from our study is shown in Fig. 2. The results are summarized in Table 3. In leaves, the aromatic compounds were detectable in most of the samples, while the norisoprenoid derivatives were less widely distributed. The terpene derivative could not be detected in any of the samples, probably due to the observed instability of the compound under extraction and storage conditions. The furanone conjugate was also not detectable in any of the samples. This finding is in accordance to literature data; the alcohol and its derivatives occur only in fruits but not in leaves (Schwab & Roscher, 1998).

In fruits (blackcurrant, guava and passionfruit), detection of the compounds could not be achieved, probably due to too low concentrations of the conjugates. This observation is in good accordance with Harborne's previously published data, showing the occurrence of flavonoid sulfates mainly in leaves (Harborne, 1975). For this reason, further attempts with fruits were not performed.

In summary, we identified for the first time sulfated flavor conjugates in plant tissues. HPLC–ESI–MS/MS offers the possibility to detect selectively these polar minor compounds. In order to distinguish between phosphates and sulfates exhibiting the same precursor and product ions, an additional SRM experiment specific for sulfates was developed to confirm their occurrence. These new flavor conjugates seem to be common constituents of secondary plant metabolism being overlooked in the last years.

3. Experimental

3.1. Plant material

Guava, blackcurrant, passionfruit, cress and dried leaves were available from a local market. Vine leaves cv. muscat and shiraz originated from INRA,

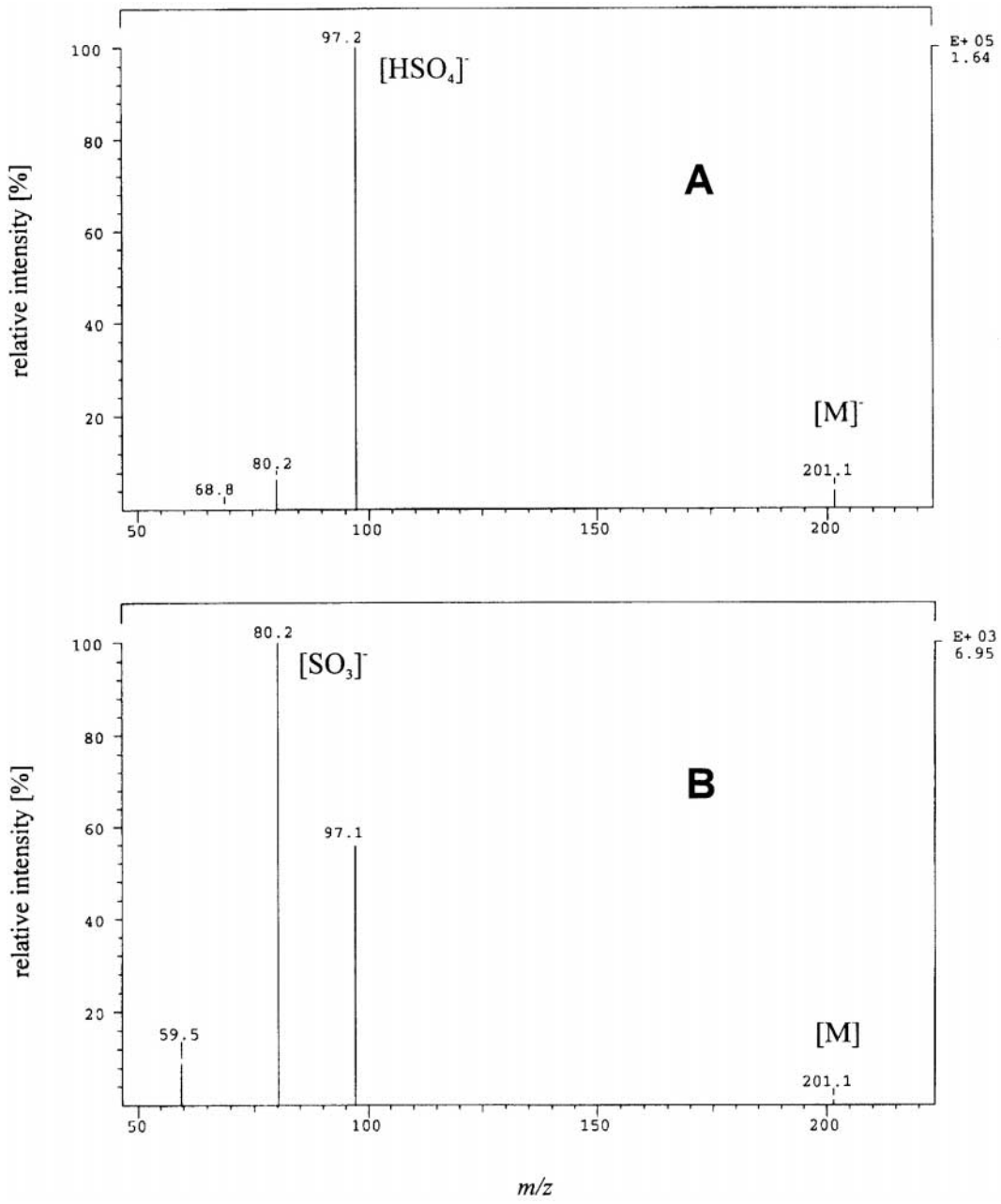


Fig. 1. Product ion mass spectra of **2** obtained by collision induced dissociation (CID) with different offset voltages (presursor ion: *m/z* 201.1 $[M]^-$): (A) offset voltage 21 eV; (B) offset voltage 57 eV.

Montpellier. All other fresh leaves were collected in summer 1997 on the campus of the University of Würzburg.

3.2. Plant extracts

The first step corresponded to the method described for the isolation of a glycosidic extract (Gunata, Bayonove, Baumes, & Cordonnier, 1985). Thus, after mixing of 1000 g of fruits with 1000 ml of 0.2 M citrate–phosphate buffer (pH 7.0), a clear extract was obtained by centrifugation (3000*g*, 20 min). To extract leaves the method was modified as follows: after mixing of 200 g of leaves with 1000 ml MeOH and macerizing the mixture at ambient temp. overnight, a clear extract was obtained by filtration. MeOH was removed under reduced pressure. The aq. residue was extracted three times with 100 ml of pentane to remove chlorophyll. The clear extract was then applied to an Amberlite XAD-2-column (4×35 cm). After a rinse with 2000 ml of distilled H_2O and 500 ml of pentane–Et_2O (1 + 1), the extract was obtained by eluting with

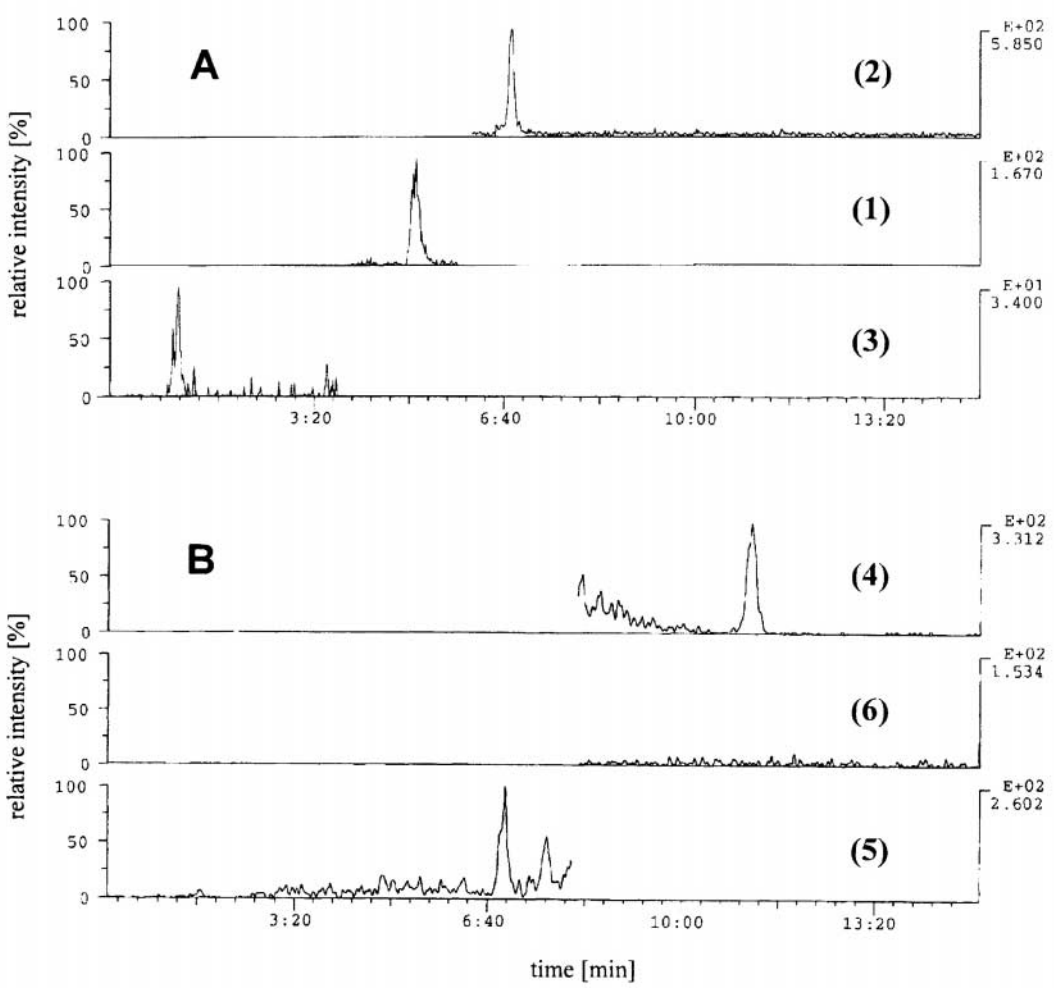


Fig. 2. Time-dependent SRM experiments for compounds **1**–**6** in strawberry leaf extract. Chromatogram A shows the product ion traces of group 1: **1**–**3** (SRM 1). The characteristic ion pairs are as follows: *m*/*z* 207.0/127.1 (**3**), 187.1/96.1 (**1**), 201.1/97.2 (**2**). Compound **3** (RR_t 2.5 min) was not detectable in this sample. Chromatogram B shows the product ion traces of group 2: **4**–**6** (SRM 3). The characteristic ion pairs are as follows: *m*/*z* 303.3/97.2 (**4**), 273.3/97.2 (**5**), 233.2/96.2 (**6**). Compound **6** (RR_t 10.2 min) was not detectable in this sample (ESI, negative mode, RP-18; gradient: 10 mM NH_4Ac in H_2O–10 mM NH_4Ac in 90% MeOH).

1000 ml of MeOH. The MeOH eluate was conc. under reduced pressure to dryness (yields ranging from 0.5 g to 2.0 g).

A part of the extract (corresponding to 25 g of dried leaves, 50 g of fresh leaves or 250 g of fruits) was redissolved in 10 ml 0.2 M citrate–phosphate buffer (pH 3.5) and applied to a second Amberlite XAD-2-column (1.5×15 cm). After a rinse with 50 ml H_2O, elution was performed with 50 ml 30% MeOH in H_2O to yield the desired fraction. The solution was concentrated under reduced pressure to dryness and redissolved in 1 ml distilled H_2O for the analysis of sulfated conjugates by HPLC–ESI–MS/MS.

3.3. Reference compounds

The synthesis of **1** and **2** was performed by treatment of the commercially available alcohols (3 mmol, **1**: 320 mg; **2**: 370 mg) with chlorosulfonic acid (3 mmol, 350 mg) in 30 ml Et_2O (Lieberman et al., 1948). After stirring of the mixture for one hour at room temp., Et_2O was removed by distillation under reduced pressure. Neutralization with 2 N NaOH yielded a crystalline precipitate which was dried and used without further purification for structural elucidation by NMR and HPLC–ESI–MS/MS. The sodium salts **1** and **2** were obtained in overall yields ranging from 83% to 90%, respectively (**1**: 2.5 mmol, 520 mg; **2**: 2.7 mmol, 605 mg). In case of **3** the procedure was modified as follows: before treatment of the alcohol (3 mmol, 385 mg) with chlorosulfonic acid (3 mmol, 350 mg) diethylamine (400 μl) had to be added to ensure milder conditions. After one hour at room temp. the solution was neutralized as described before. The residue was dissolved in 5 ml H_2O with 0.05% HCO_2H and purified on Lichrospher 100-C18 material (3×10 cm). Elution was performed with increasing amounts of CH_3CN in H_2O with 0.05% HCO_2H. Pure **3** was

Table 3
Screening results for compounds **1**–**6** in various plant tissues

Leaves	1	2	3	4	5	6
Blackberry[a]	✓	✓				
Blackcurrant	✓					
Blueberry[a]	✓	✓		✓	✓	
Cress		✓			✓	
Elder				✓		
Plum						
Raspberry[a]	✓	✓		✓		
Redcurrant	✓			✓		
Rosehip	✓	✓		✓		
Strawberry[a]	✓	✓		✓	✓	
Sweet cherry		✓			✓	
Vine cv. muscat	✓					
Vine cv. shiraz	✓			✓	✓	

[a]Dried leaves.

obtained with an overall yield of 50% (1.5 mmol, 340 mg). Synthesis of **4**–**6** was performed by the method of Piasecki (1992). To a well stirred and cooled solution of 10 mmol of the appropriate alcohol (**4**: 1.94 g; **5**: 2.24 g; **6**: 1.54 g) in 15 ml dry CCl_4 and 500 μl dry pyridine 11 mmol (1.75 g) of sulfur trioxide·pyridine complex was added slowly in small portions. After 2–3 h of stirring in an ice bath the mixture was left at room temp. to the next day. Then, the solution was evaporated under reduced pressure and a suspension of 12 mmol Na_2CO_3 ($Na_2CO_3 \times 12\ H_2O$: 3.43 g) in 20 ml EtOH and 7.5 ml H_2O was added slowly under vigorous stirring in an ice bath. After four hours the reaction mixture was evaporated, the residue redissolved in 5 ml H_2O with 0.05% HCO_2H and purified on Lichrospher 100-C18 material as described before. Yields ranged from 20% to 30% (**4**: 660 mg; **5**: 945 mg; **6**: 560 mg).

3.4. NMR data

1: ^{1}H NMR cf. White, Li, and Lu (1992); ^{13}C NMR (100 MHz, DMSO-d_6): δ 67.6 (C-1), 127.7 (C-3, C-7), 128.3 (C-5), 128.8 (C-4, C-6), 138.1 (C-2).

2: ^{1}H NMR cf. White et al. (1992); ^{13}C NMR (100 MHz, DMSO-d_6): δ 35.5 (C-2), 66.4 (C-1), 126.1 (C-6), 128.3 (C-5, C-7), 128.9 (C-4, C-8), 139.0 (C-3).

3: ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.36 (3H, d, $J = 7.0$ Hz, H-6), 2.28 (3H, s, H-1), 4.66 (1H, q, $J = 7.0$ Hz, H-5); ^{13}C NMR (100 MHz, DMSO-d_6): δ 14.4 (C-1), 16.4 (C-6), 80.0 (C-5), 130.6 (C-3), 181.0 (C-2), 196.8 (C-4).

4: cf. Tables 1 and 2.

5: ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.98 (3H, s, H-11*), 1.00 (3H, s, H-12*), 1.19 (3H, m, H-10), 1.87 (3H, m, H-13), 2.17–2.32 (2H, m, H-2), 4.52 (1H, m, H-9), 5.67 (1H, m, H-4), 5.77 (1H, m, H-7), 5.86 (1H, m, H-8) (*signals interchangeable); ^{13}C NMR (100 MHz, DMSO-d_6): δ 19.0 (C-13), 22.3 (C-10), 23.1 (C-11*), 24.1 (C-12*), 40.3 (C-1), 49.5 (C-2), 71.5/72.3 (C-9), 77.8 (C-6), 125.7 (C-4), 131.5 (C-7), 133.1 (C-8), 163.8 (C-5), 197.0 (C-3) (*signals interchangeable).

6: ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.55 (3H, s, H-8*), 1.64 (3H, s, H-9*), 1.93 (3H, s, H-10), 2.26 (4H, m, H-4, H-5), 5.04 (1H, t, $J = 7.0$ Hz, H-6), 5.60 (1H, d, $J = 17.6$ Hz, H-1a), 5.68 (1H, d, $J = 10.6$ Hz, H-1b), 6.35 (1H, dd, $J = 10.7$ Hz, 17.5 Hz, H-2) (*signals interchangeable); ^{13}C NMR (100 MHz, DMSO-d_6): δ 17.5 (C-9), 22.2 (C-5), 23.9 (C-10), 25.4 (C-8), 38.7 (C-4), 74.7 (C-3), 119.5 (C-1), 122.4 (C-6), 132.2 (C-7), 138.9 (C-2).

3.5. HPLC–ESI–MS/MS analysis

Analysis of sulfated conjugates **1**–**6** was performed on a triple stage quadrupole TSQ 7000 LC–MS/MS system (Finnigan MAT, Bremen). Data acquisition and data evaluation were carried out on a Personal DEC station 5000/33 (Digital Equipment, Unterföhring) and ICIS 8.1 software (Finnigan MAT, Bremen). For HPLC an Applied Biosystems dual syringe pump model 140B (bai, Bensheim) was used. HPLC separation was carried out on an Eurospher 100-C18 (2×100 mm, 5 μm, Knauer, Berlin) using a linear gradient at a flow rate of 200 μl/min. The HPLC gradient was as follows: solvent A (10 mM NH_4Ac in H_2O), solvent B (10 mM NH_4Ac in 90% MeOH); 0–10 min 10–90% B, 10–11 min 90–100% B, 11–15 min 100% B. For injection a Spark Holland Triathlon autosampler (SunChrom, Friedrichsdorf) was used, the injection volume was 10 μl using the μl pick-up mode. Electrospray ionization (ESI) in negative mode was used. The temp. of the heated capillary was set to 230°C and the capillary voltage to 3.4 kV. Nitrogen served both as sheath (60 psi) and auxiliary gas (10 l/min).

The product ion spectra were available by collision induced dissociation (CID) (2.5 mTorr argon; 20–60 eV). From the characteristic fragmentation pattern the most abundant product ion was selected for selected reaction monitoring (SRM) experiments.

3.6. HPLC–ESI–MS/MS data

1: product ions of m/z 187 $[M]^-$: m/z 96 $[M-C_7H_7]^-$, 80 $[SO_3]^-$; **2**: cf. Fig. 1; **3**: product ions of m/z 207 $[M]^-$: m/z 127 $[\text{aglycone}-H]^-$, 80 $[SO_3]^-$; **4**: product ions of m/z 273 $[M]^-$: m/z 97 $[HSO_4]^-$, 80 $[SO_3]^-$; **5**: product ions of m/z 303 $[M]^-$: m/z 97 $[HSO_4]^-$, 80 $[SO_3]^-$; **6**: product ions of m/z 233 $[M]^-$: m/z 96 $[M-C_{10}H_{17}]^-$, 80 $[SO_3]^-$. Depending on the offset voltage of the CID the intensity of the product ions is shifted to smaller fragments.

For improved sensitivity the six compounds were analyzed in two groups (group 1: **1**–**3**; group 2: **4**–**6**) and two different MS/MS experiments were performed for the screening for each group in plants: Group 1: **1**–**3**: (i) SRM 1, time-dependent: 0–4 min m/z 207.0/127.1 (20 eV) for **3**; 4–6 min m/z 187.1/96.1 (30 eV) for **1**; 6–15 min m/z 201.1/97.2 (21 eV) for **2**; scan-time 1 s; (ii) SRM 2, time-dependent: 0–4 min m/z 207.0/80.2 (23 eV) for **3**; 4–6 min m/z 187.1/80.2 (57 eV) for **1**; 6–15 min m/z 201.1/80.2 (57 eV) for **2**; scan-time 1 s. Group 2: **4**–**6**: (iii) SRM 3, time-dependent: 0–8 min m/z 303.3/97.2 (35 eV) for **5**; 8–15 min m/z 273.3/97.2 (31 eV) for **4**, 233.2/96.2 (20 eV) for **6**; scan-time 1 s; (iv) SRM 4, time-dependent: 0–8 min m/z 303.3/80.2 (60 eV) for **5**; 8–15 min m/z 273.3/80.2 (60 eV) for **4**, 233.2/80.2 (57 eV) for **6**; scan-time 1 s.

3.7. Nuclear magnetic resonance (NMR) spectroscopy

NMR spectra were recorded on a Bruker WM 400 spectrometer. The spectra were measured in DMSO-d_6 as solvent and referenced to the solvent signal, respectively.

Acknowledgements

We thank the NMR crew (B. Pink, M. Roth, Z. Lukacs, M. Wein) for recording the NMR spectra and S. Sauer for performing a part of the HPLC–ESI–MS/MS analyses. We also thank Y. Gunata (INRA, Montpellier, France) for providing vine leaves and the European Community for financial support (AIR3-CT94-2193).

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