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IDENTIFICATION OF 3-*O*-[2-*O*-(β-D-XYLOPYRANOSYL)-β-D-GALACTOPYRANOSYL] FLAVONOIDS IN HORSERADISH LEAVES ACTING AS FEEDING STIMULANTS FOR A FLEA BEETLE

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Key Word Index—*Armoracia rusticana*; Cruciferae; flavonoids; 3-*O*-[2-*O*-(β-D-xylopyranosyl)-β-D-galactopyranosyl]-kaempferol; 3-*O*-[2-*O*-(β-D-xylopyranosyl)-β-D-galactopyranosyl]-quercetin; *Phyllotreta armoraciae*; Coleoptera; Chrysomelidae; flea beetle; feeding stimulants.

Abstract—The concentration of flavonol glycosides in leaves of *Armoracia rusticana* harvested at various times during the growing season has been determined. Quantitatively dominating were 3 - *O* - [2 - *O* - (β - D - xylopyranosyl) - β - D - galactopyranosyl] - quercetin and 3 - *O* - [2 - *O* - (β - D - xylopyranosyl) - β - D - galactopyranosyl] - kaempferol. The latter was present in highest concentration in leaves throughout the growing season, the highest concentration being found in spring. This compound had the highest feeding stimulatory effect towards the flea beetle *Phyllotreta armoraciae*. The glycosides are new natural products which have been identified by use of enzymatic and spectroscopic methods, including ^{13}C NMR.

INTRODUCTION

Most flea beetles of the genus *Phyllotreta* feed in nature solely on plants belonging to the order Capparales, but not all species of Capparales are equally acceptable. Some species prefer Cruciferae, others Capparaceae or Resedaceae [1–4]. The only natural host plant of *Phyllotreta armoraciae* is horseradish, *Armoracia rusticana* G., M. and Sch. (Cruciferae), but in the laboratory a few more crucifers are acceptable [5, 6].

Members of Capparales are known to contain glucosinolates which are important feeding stimulants for *Phyllotreta* spp. as well as for other insects [5]. However, *P. armoraciae* is not able to recognize horseradish solely by its content of glucosinolates [5]. We have isolated and partially identified two flavonol glycosides from horseradish which are feeding stimulants to *P. armoraciae* [6]. Flavonol glycosides are widely distributed in Cruciferae [7, 8]. However, *P. armoraciae* is able to discriminate between flavonol glycosides with different carbohydrate moieties (unpublished results). If *P. armoraciae* is able to discriminate between the flavonol glycosides of horseradish and those of other crucifers, horseradish might be recognized by these beetles by its content of glucosinolates and particular flavonol glycosides. In order to further investigate these aspects of host plant selection of *P. armoraciae*, exact identification of the flavonol glycosides in leaves of various crucifers is essential. We report here on the quantitatively dominant flavonol glycosides in leaves of horseradish.

RESULTS AND DISCUSSION

The flavonol glycosides in the methanol extracts of *A. rusticana* leaves harvested at various times during the growing season were purified and separated by CC and investigated by PC, TLC and HVE (see Experimental). The results are presented in Table 1. The glycoside **1** was found to be quantitatively dominating in the leaves throughout the growing season. This compound was the most potent feeding stimulant for *P. armoraciae* [6]. Minor amounts of **2** and a flavonoid (**3**) with a 2 - *O* - (β - D - xylopyranosyl) - β - D - galactopyranosyl moiety like **1** and **2** and a B-ring system as in **1**, but diverging from **1** in some part of the A- and C-ring systems, as revealed from the ^{13}C NMR spectrum, were also found. Compounds **1** and **3** were separated by use of a Polyclar-AT column but otherwise they were almost identical. Very small amounts of 3 - *O* - (β - D - galactopyranosyl) - kaempferol (**4**) and 3 - *O* - (β - D-galactopyranosyl) - quercetin (**5**) were also detected by use of PC and UV spectroscopy, but they were considered to be artifacts produced during the purification and isolation procedure from **1** and **2**, respectively. Compounds **1–5** showed no electrophoretic mobility, in contrast to other flavonoids isolated from crucifers (see below).

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Table 1. Flavonoid concentration in leaves of *A. rusticana* harvested at various times during the growing season

Compound	Flavonoid concentration (μmol/g freeze-dried leaves)			
	May*	June	July	September
3-*O*-[2-*O*-(β-D-xylopyranosyl)-β-D-galactopyranosyl]-kaempferol (1)	40	40	35	19
3-*O*-[2-*O*-(β-D-xylopyranosyl)-β-D-galactopyranosyl]-quercetin (2)	1	1	1	4
3-*O*-[2-*O*-(β-D-xylopyranosyl)-β-D-galactopyranosyl]-flavonoid (3)	20	20	10	6

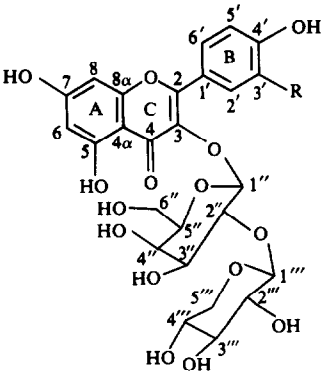
*Young leaves.

Hydrolysis of **1** and **2** in 1 M HCl resulted in a 1:1:1 ratio of D-galactose, xylose, and the aglycone kaempferol or quercetin, respectively, as determined by UV spectroscopy, quantitative determination of D-galactose using β-D-galactose dehydrogenase, and GC of the trimethylsilyl derivatives of the carbohydrates. Gentle hydrolysis of **1** and **2** in 1 M acetic acid at room temperature resulted in minor amounts of the xylosyl-galactose disaccharide. The compounds **1**, **2**, and the hydrolysis products thereof were identified by PC and TLC in several solvent systems using authentic reference compounds. The position of the glycosylated phenol group in **1** and **2** was revealed from UV spectroscopy. Table 2 shows R_f values and spectroscopic data obtained for **1–3**.

In Table 3 the chemical shift values obtained from ^{13}C NMR spectra of **1–3** are presented. These data confirm the kaempferol and quercetin structures of **1** and **2**, respectively. By comparison with previously reported data on the effects of glycosylation on the aglycone spectrum [9] it is also confirmed that only the hydroxyl at C-3 is glycosylated in both **1** and **2**. The β-D-xylopyranosyl structure is revealed by comparison with reported values [10, 11] as are the β-D-galactopyranosyl structure [9–13]. The above-mentioned data including the downfield shift observed for C-2 in the galactose part of **1–3** show that all three compounds have the disaccharide 2 - *O* - (β - D - xylopyranosyl) - β - D - galactopyranosyloxy connected to the C-3 position of the aglycone part as shown in the formula.

Compound **1** is possibly identical with rustoside which has previously been isolated from *A. rusticana* but assigned the structure kaempferol 3 - (*O* - β - D - glucopyranosyl - β - D - xylofuranoside) [14]. The presence of xylogalactosides of both kaempferol and quercetin has been proposed in *A. rusticana*, but no information concerning the interconnection and structure of the carbohydrates is given [8].

The results from semi-quantitative determinations of flavonoids in leaves of selected crucifers gave the following results: plant species (μmol flavonoids/g freeze-dried leaf); *Armoracia rusticana* G., M. and Sch. (60); *Brassica nigra* (L.) Koch (3); *Sisymbrium officinale* (L.) Scop. (55); *Nasturtium microphyllum* (Boenn.) Rchb. (2); *Sinapis alba* L. (19); *Cardamine amara* L. (44); *Raphanus sativus* L. (2). The identification was based on chromatographic and UV-spectroscopic methods (see Experimental). The results revealed the presence of some major and several minor flavonoid glycosides. The major flavonoids in leaves of *R. sativus* are kaempferol glycosides with carbohydrates different from those of horseradish glycosides. This is also the case for the major flavonoids in leaves of the other crucifers investigated but, furthermore, some of these compounds have acidic properties corresponding to a carboxylic acid function, e.g. the compounds in *S. alba* and *B. nigra*. Acidic properties corresponding to sulphate groups have recently been reported for other flavonoids [15, 16], but they are easily distinguished from carboxylate groups by high-voltage electrophoresis at pH 1.9, 3.6 and 6.5 [17, 18].



1 R = H

2 R = OH

The concentration of flavonol glycosides in leaves of selected crucifers is higher than the concentration of **1** necessary to stimulate feeding in *P. armoraciae* [6]. Glucosinolates extracted from the above-mentioned species stimulate feeding in *P. armoraciae*, and leaves of all except *C. amara* and *R. sativus* are accepted by the beetles in laboratory assays [5]. Evidently, the acceptability of plants to *P. armoraciae* is neither correlated to the glucosinolate content nor to the total concentrations of flavonol glycosides in leaves. Exact identification of isolated

Table 2. R_f values and UV spectral data for *A. rusticana* flavonoids

	R_f values in solvent*								UV λ_{max} in nm and corresponding (E) from UV spectra in solvent†					
Compound	1	2	3	4	5	6	7	8	I	II	III	IV	V	VI
1	0.53	0.59	0.07	0.31	0.62	0.60	0.90	0.37	267(1.4) 300(0.8) 356(1.1)	275(1.5) 327(0.9) 400(1.5)	273(1.5) 306(0.7) 356(1.0) 410(1.1)	275(1.4) 304(0.8) 348(1.0) 408(0.9)	276(1.5) 315(0.7) 395(1.0)	269(1.2) 306(0.6) 360(1.0)
2	0.45	0.41	0.03	0.35	0.63	0.60	0.88	0.26	263(0.8) 365(0.6)	274(0.9) 324(0.7) ‡	277(0.9) 306(0.3) 445(0.8)	274(0.8) 302(0.3) 374(0.4) 412(0.5)	275(0.7) 406(0.5)	265(0.8) 380(0.5)
3	0.52	0.60	0.07	0.28	0.63	0.60	0.90	0.39	268(1.0) 303(0.6) 354(0.8)	276(1.1) 329(0.6) 402(1.2)	276(1.0) 302(0.5) 353(0.7) 406(0.7)	277(0.9) 306(0.5) 348(0.7) 406(0.6)	276(1.0) 313(0.5) 392(0.7)	263(0.9) 305(0.5) 358(0.7)

*For solvent systems, see Experimental.

†I, MeOH; II, MeOH + MeONa; III, MeOH + $AlCl_3$; IV, MeOH + $AlCl_3$ + HCl; V, MeOH + NaOAc; VI, MeOH + NaOAc + H_3BO_3.

‡A peak at *ca* 420 nm disappears after a short time.

flavonol glycosides from some of these plants is in progress. Feeding responses of *P. armoraciae* to the compounds are being evaluated in order to determine whether plant acceptability is correlated with the presence of particular flavonoid glycosides.

EXPERIMENTAL

Plant material. The plants used in this work were from the previously described collection [5].

Insects and bioassay. The phyllotreta flea beetle *P. armoraciae* and the applied assay are described elsewhere [5, 19].

General methods and instrumentation. Methods and equipment used for GC, ^{13}C NMR, ^{1}H NMR, PC, and high-voltage electrophoresis (HVE) have been described previously [17, 18]. The applied PC solvents and HVE buffers were: solvent 1, *n*-BuOH–HOAc–H_2O (12:3:5); solvent 2, PhOH–H_2O–13 M NH_3 (120:30:1) (w/v/v); solvent 3, *iso*-PrOH–H_2O–13 M NH_3 (8:1:1); solvent 4, H_2O; solvent 5, 15% HOAc; solvent 6, HOAc–conc HCl–H_2O (10:3:30); buffer pH 1.9, HOAc–HCO_2H–H_2O (4:1:45); buffer pH 3.6, Pyridine–HOAc–H_2O (1:10:200); buffer pH 6.5, Pyridine–HOAc–H_2O (25:1:500). TLC was performed on DC-Alufolie Cellulose F-254, 20 × 20 cm, Merck in solvent 7, Pyridine–EtOAc–HOAc–H_2O (9:9:2:5) and solvent 8, EtOAc–MeCOEt–HCO_2H–H_2O (5:3:1:1). Flavonoids were detected by long-wave UV light, and the isolated flavonoids were investigated by UV spectral analysis in different solvents and compared with authentic samples as described elsewhere [20].

Table 3. ^{13}C NMR chemical shifts (δ) for the different atoms in *A. rusticana* flavonoids*

	Atom no.	1	2	3
C-ring	2	156.9	157.1	157.6
	3	134.3	134.6	134.0
	4	178.8	178.8	178.5
	4a	105.2	105.3	103.5
A-ring	5	160.7	160.7	158.1
	6	99.5	99.6	101.7
	7	163.3	163.7	160.5
	8	95.1	95.2	94.6
	8a	157.9	157.8	135.5
B-ring	1′	122.0	123.5	122.0
	2′	131.8	116.2	131.8
	3′	115.8	144.3	116.3
	4′	159.3	148.2	160.1
	5′	115.8	117.3	116.3
	6′	131.8	122.5	131.8
Galactose moiety	1″	100.8	100.8	100.8
	2″	78.9	78.7	78.8
	3″	74.0	74.0	74.0
	4″	69.4	69.5	69.5
	5″	74.4	74.3	74.4
	6″	61.1	61.2	61.2
Xylose moiety	1‴	104.2	104.1	104.2
	2‴	75.8	76.0	75.9
	3‴	76.4	76.3	76.4
	4‴	70.1	70.1	70.1
	5‴	66.0	65.9	66.0

*For comparison with corresponding values for some reference glycosides, see ref. [13]; for quercetin, robinin and rutin, see ref. [12]; for kaempferol and quercetin 3-*O*-galactopyranoside (5), see ref. [9].

Isolation and semi-quantitative determination of flavonoids from different crucifers. Freeze-dried leaves (1 g) were homogenized with an Ultra-Turrax homogenizer in 100 ml boiling MeOH × 3, 3 min each time. The homogenates were cooled, filtered, concd to dryness and redissolved in 10 ml H_2O before extraction with 3 × 10 ml $CHCl_3$. Part of the H_2O extract (0.1 ml) was purified by prep. 2D–PC in solvents 4 and 1, after which spots containing flavonoids were eluted from the paper with MeOH and investigated by UV [20]. The semi-quantitative determinations were based on $\epsilon_{max} = 1.4 \times 10^7$ cm^2/mol for the peak corresponding to λ 350–370 nm. Another part of the H_2O extract (9 ml) was chromatographed on a Sephadex G-10 (1.5 × 80 cm) column. H_2O was used as eluant, flow rate 250 ml/hr, 20 ml/fraction, using continuous UV (260 nm) monitoring. Flavonoid-containing fractions were pooled, concd, investigated by PC in solvents 1–6 and UV (as for the eluates from 2D–PC). Results from both of these procedures are used for the semi-quantitative values given in Table 1.

Isolation of 1–3. Freeze-dried leaves of *A. rusticana* (10 g portions) harvested at various times during the growing season were homogenized (Ultra-Turrax) and extracted with boiling MeOH–H_2O (3:1) (250 ml portions) ×3, 5 min each time. The homogenates were cooled, filtered, concd, diluted with H_2O (15 ml), and extracted with $CHCl_3$ (3 × 20 ml). The H_2O extract was chromatographed on a Sephadex G-10 (2.5 × 90 cm) column using H_2O as eluant and continuous UV monitoring. Fractions (20 ml) were collected at 250 ml/hr and investigated by PC. The flavonoid-containing fractions (200–800) were investigated by TLC (solvent 7), PC (solvents 1–6), and UV which revealed **1** as the quantitatively dominating compound in fractions 200–500, accompanied by minor amounts of **3** and followed by **2**. Further purification and separation were obtained by a Polyclar-AT (2.5 × 45 cm) column, MeOH–H_2O (3:1) as eluant (20 ml fractions), and the above mentioned collection and detection system. Compound **3** appeared in fractions 16–45, **1** in fractions 60–120, and **2** in fractions 130–170. Final purification of the isolated **1–3** was obtained on a Sephadex G-25 (fine) (2.5 × 45 cm) column using H_2O as eluant and the collection and detection system described above. In contrast to the Sephadex G-10 column, the Sephadex G-25 column had only weak adsorption of **1–3** and they were eluted in fractions nearly corresponding to their MWs. For UV, PC, and TLC behaviour, see Table 2; for ^{13}C NMR, see Table 3.

Hydrolysis of 1–3. A MeOH soln containing *ca* 10 μmol of the isolated compound (determined from UV) was evaporated, re-dissolved in 1 ml 1 M HCl, and hydrolysed by heating in a boiling water bath for 4 hr. The reaction mixture was evaporated, re-dissolved in H_2O (1 ml), and evaporated (× 3), redissolved in H_2O (3 ml), and extracted with EtOAc (5 ml) × 3. The EtOAc extract (15 ml) was extracted with H_2O (5 ml) × 3 and the H_2O and EtOAc solns evaporated to dryness. Both residues were investigated by PC (solvents 1–3) and TLC (solvent 7) using authentic kaempferol, quercetin, glucose, galactose, rhamnose, xylose, and arabinose as references. The residue from the H_2O soln was furthermore investigated by GC as previously described [18], and by enzymatic methods. Corresponding samples of the isolated compounds were hydrolysed in 10% HOAc for 15 and

90 min, respectively, followed by investigation as described for the HCl reaction mixture.

Enzymatic determination of D-*galactose.* The residue from the H_2O soln obtained after HCl-catalysed hydrolysis was purified by prep. PC in solvent 1. The isolated hexose was then investigated and quantitatively determined by use of β-D-galactose dehydrogenase in an assay as described in the information sheet from Boehringer–Mannheim. No reaction was obtained by D-glucose oxidase. Authentic D-glucose and D-galactose, respectively, were used for preparation of the standard curves.

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