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Polymorphism for Novel Tetraglycosylated Flavonols in an Eco-model Crucifer, *Barbarea vulgaris*

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ABSTRACT: Nineteen apparent flavonoids were determined by HPLC-DAD in foliage of a chemotype (G-type) of *Barbarea vulgaris*, and four were isolated. Two were novel tetraglycosylated flavonols with identical glycosylation patterns, kaempferol 3-*O*-(2,6-di-*O*-*β*-D-glucopyranosyl)-*β*-D-glucopyranoside-7-*O*-α-L-rhamnopyranoside (**1**) and quercetin 3-*O*-(2,6-di-*O*-*β*-D-glucopyranosyl)-*β*-D-glucopyranoside-7-*O*-α-L-rhamnopyranoside (**2**). The identification of D/L configuration was tentatively based on susceptibility to α-L-rhamnosidase and *β*-D-glucosidases. A characteristic feature of **1** and **2** was appreciable water solubility, an expected consequence of the extensive glycosylation. A less complex pair of flavonols comprised 3-*O*-*β*-D-glucopyranoside-7-*O*-α-L-rhamnopyranosides of kaempferol and quercetin. Two natural chemotypes of *B. vulgaris* differed in levels of **1** and **2**, with the P-type deficient in **1** and **2** and the insect-resistant G-type rich in **1** (ca. 3−4 *μ*mol/g dry wt) and with moderate levels of **2** (ca. 0.3−0.8 *μ*mol/g dry wt). However, there was only modest seasonal variation in flavonols **1** and **2**, in contrast to a strong seasonal variation in insect resistance.

KEYWORDS: flavonoid, glycosylation, structural elucidation, *β*-D-glucosidase, α-L-rhamnosidase, NMR, quantitative analysis, polymorphism

INTRODUCTION

The wild plant *Barbarea vulgaris* R. Br. [Brassicales: Brassicaceae] has acquired the status of model organism for investigation of plant−insect interactions due to its complex but remarkable interactions with plant pests and other herbivorous insects. Pioneer ecological entomologists noticed a high oviposition rate by a number of Brassicales-adapted insects combined with low larval survival.[1] Such apparently maladaptive behavior can be termed an "oviposition mistake". A search for the oviposition stimulants demonstrated oviposition-stimulating glucosinolates in the leaves,[2] recently demonstrated to be present at the leaf surface as well as in the leaf interior.[3] Searches for larval deterrents revealed saponins with variable deterrent or perhaps toxic effects to specialist insect larvae.[3−8] The combination of oviposition-stimulating glucosinolates and deterrent saponins explained the oviposition mistake and inspired in agronomists the concept of "dead-end" trap cropping as a general method of biological pest control.[3,9,10] Additional genetic−ecological research documented major, dominant flea beetle R-genes conferring the ability to accept and survive on *B. vulgaris* plants.[11] In combination with a natural polymorphism of resistance in *B. vulgaris*, as described below, this system seems to provide a unique opportunity for investigations of plant−insect coevolution in a natural setting.[12−14]

Traditional subspecific taxonomy of *B. vulgaris* is based on morphology, but there is no general agreement of the value of the various characters used, or whether it is at all fruitful to subdivide this highly variable species into subspecific taxa.[15] Chemical analysis and specific resistance tests of accessions of traditional taxa showed a pattern that did not follow the traditional taxonomy.[15] For practical purposes the traditional taxonomy was supplemented with additional "type" designations. One type of var. *arcuata* (Opiz.) Fries was named the G-type (for glabrous leaves), and this type showed the same glucosinolate profile, saponin content, and insect resistance phenotype as ssp. *vulgaris*.[5,10,15] A deviating type, morphologically also belonging to var. *arcuata*, was named the P-type (for pubescent leaves). The P-type has a highly distinct glucosinolate profile, is susceptible to the above-mentioned specialist insects, and is devoid of the resistance-correlated saponins.[5,10,15,16] The P-type is cytologically similar to the G-type, and the two types occasionally hybridize in nature and after artificial crossing.[17−19]

In Dutch populations of *B. vulgaris*, a different, glucosinolate-based polymorphism in insect resistance has been discovered.[20] Recently, additional polymorphism in resistance to the oomycete *Albugo candida* (Pers. ex Fr.) O. Kuntze has been discovered.[21] In this case, the pattern is opposite the pattern of insect resistance, with the P-type being resistant and the G-type susceptible. With the growing complexity of the ecological interactions of the species, it has become relevant to construct a genetic map of *B. vulgaris* with a focus on genes of ecological interest. Current *B. vulgaris* gene mapping is based on segregation in the F2 generation from hybrids (F1) between the G- and P-types of *B. vulgaris*, and genetic loci for a number of unidentified polar and apolar metabolites have been reported, in addition to loci for saponins and glucosinolates.[22] For this reason, investigations of flavonoids and all other classes of secondary metabolites in *B. vulgaris* have become a priority,[16] with particular focus on genetically based polymorphism.

The flavonoids are structurally diverse phenolic metabolites in higher plants containing an aromatic bicyclic or tricyclic

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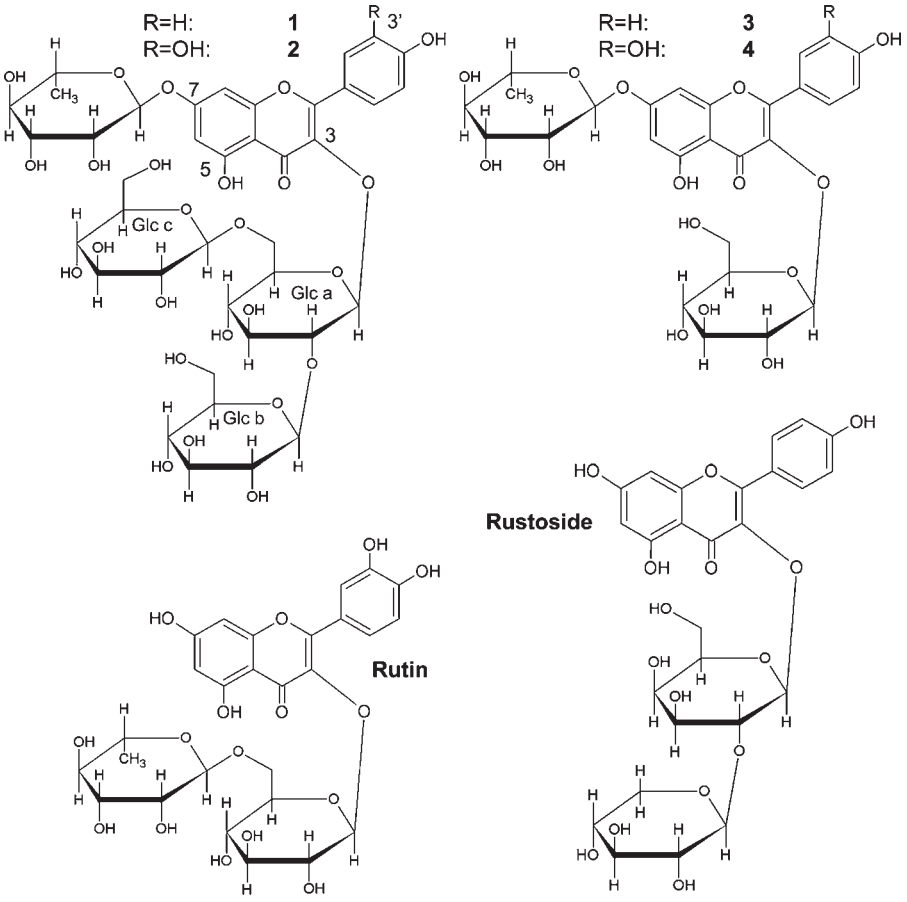

Figure 1. Structures and numbering systems of the isolated flavonoids (**1**−**4**) and reference flavonoids. "Glc a", "Glc b", and "Glc c" at **1**/**2** refer to individual glucose residues to simplify discussion in the text.

C6−C3−C6 core structure with a number of phenol groups, some of which are usually glycosylated and/or conjugated with other types of structures. In agreement with their extreme structural diversity, these metabolites have diverse physiological and ecological activities including specialist insect resistance.[23,24] Flavonoids include several structural subclasses, of which flavones are tricyclic structures carrying a ketone functional group in the central ring, and flavonols are characterized by an additional hydroxy group at C3 next to the ketone group of flavones (Figure 1). Information on the flavonoids in *B. vulgaris* has so far relied on investigations by Senatore et al.,[25] who elucidated the structures of seven flavonoids in *B. vulgaris* collected in southern Italy, where the plant is consumed and used in popular medicine. The identified flavonoids were methyl, mono- and diglycosyl derivatives of the flavone luteolin and the flavonols quercetin and kaempferol. These structures are referred to as **S1**−**S7** in this paper ("S" for Senatore), with conservation of the individual compound numbers assigned by Senatore et al.[25]

On the basis of our experience of the variability of the species, including a seasonal decline in insect resistance from summer to fall,[17] the aims of the present work were to (i) develop a method for the separation and quantification of flavonoids in the insect-resistant G-type of *B. vulgaris*, (ii) elucidate the structures of detected flavonoids, (iii) probe the natural seasonal variation in flavonoid profiles of insect-resistant G-type *B. vulgaris*, and (iv) search for genetic polymorphisms in flavonoid content between the G- and P-types of *B. vulgaris*.

MATERIALS AND METHODS

Chemicals. Rustoside (kaempferol 3-*O*-(2-*O*-*β*-D-xylopyranosyl)-*β*-D-galactopyranoside) was isolated from horseradish.[26] Other fine chemicals were from Sigma-Aldrich (St. Louis, MO): rutin (R9000) (quercetin 3-*O*-(6-*O*-α-L-rhamnopyranosyl)-*β*-D-glucopyranoside), kaempferol (K0125), quercetin (Q0125), *β*-glucosidase from almonds (G4511), and hesperidinase from *Aspergillus niger* (H8137).

Plants. Foliage and seeds of the two types of *B. vulgaris* var. *arcuata* were from three localities on the Danish island Zealand described previously.[17] G-Type foliage and seeds were for logistic reasons from either locality 1 (old grass field near the town of Herlev), locality 2 (waste area near the village of Gl. Svebølle), or locality 4 (wet meadow near the town of Kværkeby). P-Type foliage and seeds were from locality 5 (wet meadow at Lake Tissø). Foliage (G-type) for isolation of flavonoids was from locality 2 and collected by J.K.N. in November 1997. For optimization of the SPE-HPLC analysis method in 2000−2001 we used G-type leaves collected by J.K.N. in August 1998 from locality 4, and for investigation of seasonal variation of flavonoids we used previously described[17] samples (collected in 1998 by J.K.N.) from the same locality. Foliage from naturally grown P-type plants at locality 5 were also from the same study[17] and collected in August. To obtain P- and G-type plants exposed to identical environmental conditions, we grew plants from seeds collected at locality 1 (G-type) and locality 5 (P-type) in parallel (from seeds to ca. 1.5-month-old rosette plants) at the previously reported growth chamber conditions.[11] Each sample was prepared from a total of five fully developed rosette leaves selected at random from five different plants

harvested in March 2010, lyophilized directly, and homogenized before a 50 mg sample was taken.

Flavonoid Isolation. Dry foliage (50 g dry wt) of *B. vulgaris* var. *arcuata* G-type, collected and lyophilized ca. 3 months earlier and stored frozen (−20 °C), was repeatedly extracted in 70% aqueous EtOH (first extraction hot) with homogenization. The volume was reduced to 250 mL (rotary evaporation), and the extract was defatted three times with 250 mL of $CHCl_3$ (organic phase discarded). The volume of the aqueous phase was reduced to 25 mL to remove traces of $CHCl_3$. The aqueous phase was then diluted to 125 mL with H_2O and defatted three times with 125 mL of EtAc (organic phase discarded). The aqueous phase was reduced to 50 mL, centrifuged, and applied to an Amberlite XAD7 (Sigma) column (83 cm × 2.6 cm). The column was eluted with 3 L of water, 1.2 L of 5% EtOH, 1.5 L of 10% EtOH, 3.5 L of 15% EtOH, 7.5 L of 20% EtOH, 3.5 L of 40% EtOH, and 0.7 L of 50% EtOH, collecting 25 mL fractions. UV spectroscopy of selected fractions suggested the fractions eluted in 20% EtOH (fractions 488−577) and 40% EtOH (fractions 728−747) to be rich in flavonoids. Each of these groups of fractions was concentrated and subjected to preparative paper chromatography (descending PC for 48 h in isoPrOH/HOAc/H_2O 12:3:5 on 8 sheets (fractions 488−577) or 16 sheets (fractions 728−747) of Whatman no. 1 paper), yielding **1** and **2** as separate bands from fractions 488−577 and a band with a mix of **3** and **4**, among other bands, from fractions 728−747. **3** and **4** were separated by chromatography on an LH 20 (Pharmacia) column (72 cm × 2.6 cm), with **3** (4 mg) eluted with water and **4** (3.5 mg) eluted with 20% EtOH. The already separated **1** and **2** were (separately) subjected to additional chromatography on LH 20 columns (eluted with water), yielding pure **1** (21.5 mg) and **2** (7 mg).

NMR, MS, and Optical Rotation. NMR, FAB-MS (positive and negative modes), high-resolution MS, and LC-ESI-MS were carried out with the previously reported instrumentation and conditions.[27] For one- and two-dimensional ^1H NMR at 400.1 MHz and ^{13}C NMR at 100.6 MHz, we used hexadeuterated DMSO with 10% D_2O as solvent and based chemical shifts on the signal from DMSO (δ_H 2.49, δ_C 39.4). 2D NMR experiments included COSY, HSQC, HMBC, TOCSY, and JRES. Specific optical rotations of small amounts (3−10 mg weighed with a five-decimal balance) were measured in 2 dm cells at 20 °C and 589.3 nm (D line) with an AA-1000 polarimeter (Optical Activity Ltd., Ramsey, UK), using sucrose ($[\alpha]^{20}_D$ = 65 (H_2O, c = 1 g/100 mL)) and a solvent blank as controls.

Extraction and Solid Phase Extraction (SPE). Dry G-type foliage (100 mg), collected and lyophilized 2 years earlier and stored frozen (−20 °C), was extracted for 2 min in 5 mL of boiling EtOH/H_2O (7:3), keeping the solvent boiling by immersion in a boiling water bath while homogenizing with an Ultra Turrax homogenizer (Janke & Kunkel, Staufen, Germany). After centrifugation (2 min), the supernatant was collected and the residue extracted again for 2 min in 5 mL of the same solvent at room temperature with homogenization; this second extraction was repeated, and the three extracts were pooled to give the crude extract (ca. 15 mL). The majority or all of the solvent was evaporated under a gentle stream of pressurized atmospheric air and the residue brought to 5.00 mL with H_2O in a volumetric flask. A C_{18} type SPE cartridge, 'Isolute', 500 mg, pore size = 54 Å, particle diameter = 60 μm (International Sorbent Technology, Mid Glamorgan, UK), was activated with EtOH (20 mL) and gradually adjusted to 100% H_2O over 35 mL. Group separation to obtain a flavonoid fraction was carried out by applying 2.5 mL (50%) of the redissolved crude extract to the SPE cartridge, allowing the extract to slowly penetrate the cartridge driven by gravity only (accelerated loading was found to be reduce capacity significantly). The cartridge was subsequently washed with 25 mL of H_2O (discarded) and eluted with 5 × 2 mL of 40% EtOH to give the flavonoid fraction, of which 2 mL was taken to dryness under a gentle stream of pressurized atmospheric air and redissolved in 1 mL of water, of which 50 μL was injected to the HPLC-DAD.

The above validated conditions were used for all reported quantitative analyses of G-type foliage, carried out in the years 2000−2001. For a qualitative comparison of P- and G-type leaves carried out in 2010, the previously used SPE columns were not available. A modified method for the detection of **1** and **2** was devised with currently available SPE cartridges, taking into account the optimized conditions above and the more robust characteristics of present day SPE cartridges. Dry foliage (50 mg) collected and lyophilized ca. 3 months earlier and stored frozen (−20 °C) was extracted (4 mL, 1 min) for three consecutive rounds in boiling MeOH/H_2O (7:3) without homogenization, decanting the three extracts to a beaker. The solvent was evaporated under a gentle stream of pressurized atmospheric air. A C_{18}-type SPE cartridge, Strata C18-E, 200 mg, pore size = 55 Å, particle diameter = 70 μm (Phenomenex, Torrance, CA), was activated in 2 mL of 96% EtOH and directly equilibrated in 2 mL of H_2O. The crude extract residue was dissolved in 3.00 mL of H_2O, the solution was centrifuged, and 2.00 mL was applied to the SPE cartridge (forced by gravity only), which was rinsed with a further 2 × 1 mL of H_2O. The run-through was collected as a control. The flavonoid fraction was eluted with 3 × 1 mL of 40% aqueous EtOH (96% EtOH/H_2O (42:58)). From both the run-through and the flavonoid fraction, the solvent was evaporated under a gentle stream of pressurized atmospheric air, and the residue was redissolved in 1 mL of water, of which 50 μL was injected to the HPLC-DAD.

High-Pressure Liquid Chromatography (HPLC-DAD). For the optimized method, we used the previously reported HPLC equipment with diode array UV detection (DAD),[17] fitted with a 250 mm × 4.6 mm i.d., 5 μm, Luna phenylhexyl column with a Security Guard filter (4 mm × 3 mm i.d.) with a phenylpropyl stationary phase from Phenomenex. Both column and filter were fitted with a homemade column oven kept at 30 (± 0.2) °C. The flow rate was constant at 0.8 mL/min, the A-eluent was 0.1% aqueous trifluoroacetic acid (TFA), and the B-eluent was pure MeOH. The gradient program consisted of 5 min at 30% B and a linear gradient (55 min) to 50% B, followed by a brief wash (linear 5 min to 80% B, hold for 5 min at 80% B, linear 5 min to 30% B) and equilibration (10 min at 30% B). For routine UV detection we used peak areas at 260 and 330 nm (bandwidth = 1 nm), often supplemented with automated peak identification using a UV spectrum library constructed with the software native to the HPLC (Shimadzu Class-VP 5.032).

Optimization and Validation of the Analytical Method. A preliminary flavonoid fraction (40% EtOH eluate from SPE) of G-type *B. vulgaris* was used for optimization of column type and gradient. After various MeOH and MeCN gradients had been tested on various column types, the phenylhexyl column with MeOH as eluent was chosen on the basis of a high number of separated peaks and favorable peak characteristics. Additional optimization of temperature and gradient was carried out, aiming at maximizing peak number and peak shapes. An acidic A-eluent, 0.1% aqueous TFA, was selected as it improved peak shape and separation.

Reproducibility, linearity, detection limit and recovery of the method, lack of effect of the extraction solvent (70% aqueous MeOH or 70% aqueous EtOH), lack of difference of flavonoid profiles after solvent evaporation by rotary evaporation versus evaporation under a gentle stream of pressurized atmospheric air, lack of flavonoids in the initial H_2O eluate from SPE cartridges, and flavonoid stability during short-term storage in aqueous solution and long-term storage in frozen solutions or as dry preparations were evaluated by HPLC analysis using the optimized conditions. Recovery, linearity, and detection limit were tested with the flavonol diglycosides rutin (ε = 19500 M^{-1} cm^{-1}) and rustoside (ε = 14000 M^{-1} cm^{-1}). The limit of detection was defined as the concentration corresponding to 3 times the standard deviation (SD) of the area of the lowest level standard (4 μM). Quantification of individual flavonoids was based on peak areas measured at 330 nm and compared with a standard curve of rustoside.

Table 1. UV Spectroscopic and Mass Spectrometric Data of Isolated *B. vulgaris* Flavonoids

informative major ions observed in the mass spectrum (FAB negative mode) (m/z)					
1	**2**	**3**	**4**	suggested fragment ion	interpretation
917	933	593	609	$[M - H]^-$	
771	787	447	463	$[M - H - 146]^-$	loss of Rha
755	771	431	447	$[M - H - 162]^-$	loss of Glc
431	447			$[M - H - 3 \times 162]^-$	loss of Glc_3
UV absorption maxima (nm) measured by on-column DAD in ca. 30−45% aq EtOH[a]					
266	256	264	254		
346	354	346	354		

[a] Identical or similar (within ±2 nm) values obtained for isolated compounds in H_2O (**1**, **2**) or 10% aqueous EtOH (**3**, **4**).

Flavonoid Hydrolysis. Solutions in 0.1 M sodium acetate (pH 5) of **1** (220 μg) and β-D-glucosidase from almonds (2 U = 0.1 mg) were incubated at 20 °C for 3 or 6 days with appropriate blanks (no **1** or no enzyme). The reaction was stopped by boiling for 2 min, denatured protein was removed by freezing plus centrifugation, and the flavonoid contents were analyzed by HPLC-DAD and LC-MS. The pellet obtained upon centrifugation was redissolved in aqueous 50% MeOH and also analyzed by HPLC-DAD and LC-MS with quercetin and kaempferol as references. HPLC-DAD peaks were assigned by comparison with LC-MS analyses. A number of additional experiments with variation of buffer pH (pH 6 and 7 obtained with sodium phosphate) and concentration of **1** and enzyme were also carried out. In a further experiment, hesperidinase from *Aspergillus niger* (either 0.03 or 0.003 U) and **1** (110 μg) dissolved in 0.4 M sodium acetate (pH 4.2) were incubated at 20 °C for 1 or 3 h or overnight. Appropriate blanks (no **1** or no enzyme) were included. As specificity controls, rutin (saturated, ca. 75 μg) and rustoside (110 μg) were incubated with the same two amounts of hesperidinase and the same buffer except addition of EtOH to 5% (which was found not to affect hesperidinase activity when tested with **1**). Reactions were terminated and products analyzed as above. Hesperidinase treatment of **2**, **3**, and **4** was carried out similarly, at various flavonoid concentrations and EtOH concentrations (up to 7.5%).

RESULTS AND DISCUSSION

Isolation and Structural Elucidation of Flavonoids. By a combination of column chromatography and paper chromatography, four flavonoids (**1**, **2**, **3**, **4**) were isolated from foliage of the G-type of *B. vulgaris* (Figure 1). Spectroscopic analysis by NMR and MS and susceptibility to hesperidinase as explained below for **1** identified **4** as quercetin 3-*O*-β-D-glucopyranoside-7-*O*-α-L-rhamnopyranoside, which had previously been reported (**S2**) from *B. vulgaris* and other plant species,[25] and **3** as the corresponding kaempferol derivative (kaempferol 3-*O*-β-D-glucopyranoside-7-*O*-α-L-rhamnopyranoside), known from *Arabidopsis thaliana* and other plant species.[28] As expected, compound **4** was optically active with $[\alpha]_D = -1.5 \times 10^2$ (MeOH, *c* 0.009 g/100 mL), whereas remaining amounts of **3** (in 2011) did not allow determination of specific optical activity. A literature search suggested that the quite common flavonol diglycoside **4** had apparently never before been investigated with respect to either optical activity or the D/L configuration of the monosaccharide moieties. In *A. thaliana*, **3** was shown to be based on D-glucose and L-rhamnose,[28] but specific optical activity was not reported. Hence, this aspect of the structures of **3** and **4** remained tentative, relying solely on susceptibility to hesperidinase and the general dominance of D-glucosides and L-rhamnosides in plants. Comparison of HPLC retention times of **1**−**4** with those of authentic *B. vulgaris* flavonoid standards, kindly supplied by F. Senatore, confirmed that **4** was identical to **S2**, whereas **1**−**3** were different from the flavone and flavonol glycosides previously reported from *B. vulgaris* collected in Italy.[25]

The UV spectrum of **1** resembled the spectrum of **3**, suggesting that they were based on the same aglycone (Table 1). High-resolution MS of the Na^+ adduct of **1** gave the mass (m/z) 941.2494, compatible with the calculated value 941.2533 for $[M + Na]^+$ of $C_{39}H_{50}O_{25}$. The aromatic region of the ^1H NMR spectrum showed signals from a *para*-substituted phenyl group and from two additional H with long-range coupling, compatible with kaempferol being the aglycone, and this identity of the aglycone was confirmed by comparison of ^{13}C NMR signals with reference values.[28,29]

The negative mode FAB mass spectrum of **1** showed major fragment ions due to loss of fragments corresponding to (m/z) 146 and 162. This fragmentation was similar to the fragmentation of **3**, suggesting that both contained a rhamnoside group and a group with the same mass as a glucoside (i.e., either glucose or one of its epimers, referred to in general as a "hexoside" in the following) (Table 1). The difference of **1** relative to **3** appeared to be a more polar nature and a higher molecular mass, corresponding to the presence of two additional hexoside groups. Major fragment ions corresponding to loss of three hexosides were detected (Table 1), in contrast to the near absence of signals for ions corresponding to loss of two hexosides. This fragmentation pattern suggested that the three deduced hexosides in **1** were connected and present as a branched structure. As expected for a glycoside of biological origin, compound **1** was optically active, with $[\alpha]_D = -86$ (MeOH, *c* 0.05 g/100 mL). A relatively high water solubility (at least 5 mg/mL at 20 °C for **1**) was also noted for **1**, compatible with a high degree of glycosylation.

The presence of a rhamnoside group in **1** was supported by the presence of a well-separated doublet (3H) in the ^1H NMR high-field region due to the characteristic rhamnoside methyl group (Table 3). Signals from four anomeric hydrogens, of which three had coupling constants between 7 and 8 Hz and one had a coupling constant of 1.5 Hz, were also obvious, supporting the extent of glycosylation suggested by MS. However, the remaining carbohydrate region in the 400 MHz ^1H NMR spectrum was extremely complex.

The elucidation by 2D NMR of the structure of the glycoside parts of the molecule began with the above-mentioned methyl group tentatively assigned to a rhamnoside residue. Coupling

Table 2. ^{13}C NMR Spectroscopic Data of Four Isolated *B. vulgaris* Flavonoids

	δ_C			
position	1	2	3	4
aglycone				
2	156.7	156.6	157.1	157.0
3	133.4	133.5	133.8	133.6
4	177.7	177.7	177.8	177.6
5	160.8	160.8	160.8	160.7
6	99.6	99.6	99.7	99.5
7	161.8	161.9	161.8	161.6
8	94.9	94.8	94.9	94.6
9	156.2	156.1	156.3	156.1
10	105.9	105.9	105.9	105.7
1′	120.9	121.0	121.1	120.6
2′	131.4	116.3	131.3	115.3
3′	115.5	144.9	115.4	145.0
4′	160.2	149.0	160.1	149.4
5′	115.5	115.6	115.4	116.0
6′	131.4	122.4	131.3	122.1
triglucoside at C3(Glc a, Glc b, Glc c)				
a1	98.4	98.5	101.0	100.9
a2	81.7	82.2	74.3	74.1
a3	76.4	76.4	76.4	76.4
a4	69.4	69.4	70.0	69.9
a5	76.5	76.6	77.6	77.6
a6	67.9	68.0	60.9	61.0
b1	103.8	104.0		
b2	74.3	74.4		
b3	76.5	76.6		
b4	69.8	69.8		
b5	77.0	76.9		
b6	60.8	60.9		
c1	103.3	103.3		
c2	73.3	73.4		
c3	76.5	76.4		
c4	69.8	69.8		
c5	76.5	76.6		
c6	60.8	60.9		
rhamnoside at C-7				
1	98.7	98.7	98.5	98.5
2	69.8	69.9	69.9	69.8
3	70.2	70.3	70.3	70.2
4	71.7	71.8	71.7	71.6
5	70.2	70.3	70.3	70.2
6	18.1	18.1	18.1	18.0

analysis by COSY revealed the connectivity of this methyl group, via a sequence of four separated hydrogens, to an anomeric hydrogen at 5.50 ppm. This spin system was also revealed by TOCSY. An HSQC spectrum identified the corresponding carbon signals. These signals could be attributed to an α-rhamnopyranoside residue by comparison with chemical shift reference values of common flavonoid carbohydrate moieties.[28,29] The position of the rhamnoside residue was identified as C7 of the aglycone by an HMBC interaction between the anomeric proton at 5.50 ppm and C7 at 161.8 ppm.

The three anomeric hydrogens with large coupling constants (mentioned above) were part of three hexoside-like spin systems (eventually named "Glc a", "Glc b", and "Glc c", see Figure 1) as revealed by TOCSY. The sequence of individual hydrogens and corresponding carbon atoms in each spin system was determined by a combination of COSY, HSQC, and HMBC, allowing assignment of all remaining carbon signals and determination of all hydrogen chemical shifts (Figure 1; Tables 2 and 3). Furthermore, HMBC revealed that one of the spin systems (Glc a) (with a rather deshielded anomeric hydrogen at 5.54 ppm) was connected to the aglycone at C3 (with an HMBC interaction from the anomeric H to C3 at 133.4 ppm). HMBC also revealed that the two remaining hexoside-like spin systems (Glc b and Glc c) were connected in a branched manner to the Glc a spin system (with HMBC interactions from the anomeric H of Glc b at 4.61 ppm to C2 of Glc a at 81.7 ppm and from the anomeric H of Glc c at 3.95 ppm to C6 of Glc a at 67.9 ppm). At this point, it was relevant to compare with the spectrum of **3**, as all evidence suggested **1** to be a dihexoside derivative of **3**. Indeed, the signals of the Glc a spin system were compatible with a disubstituted β-glucopyranose residue, taking into account the expected effect of the substitutions (+7.4 ppm at C2 of Glc a and +7.0 ppm at C6 of Glc a when compared with the corresponding chemical shifts in **3**). The remaining two hexose residues were finally identified as β-glucopyranose residues on the basis of coupling constants and comparison of carbon chemical shifts with reference values of common flavonoid carbohydrate moieties.[28,29]

We assumed, along with previous authors,[25] that the sugars of *B. vulgaris* flavonoids belong to the usual D/L series (D-glucose and L-rhamnose).[28,29] However, the NMR spectroscopic data would also be compatible with one or more of the glucoside moieties being L-glucosides and/or the rhamnoside moiety being D-rhamnoside, although L-glucose and D-rhamnose are considered to be extremely rare in higher organisms. To resolve this final uncertainty, the suitability of **1** as substrate of commercial enzyme preparations was tested.

The first tested enzyme, β-D-glucosidase (EC 3.2.1.21) from almond, catalyzed very slow hydrolysis of **1** into a product with a 6 min higher retention time and containing one less glucose residue according to LC-MS (ca. 38% conversion in 6 days at pH 5, no conversion in blank with enzyme omitted) (Figure 2A). This was the only observed product of **1** at a range of pH values (pH 5−7) and substrate concentrations (up to 4 mM). The observation of specific hydrolysis by almond β-D-glucosidase suggested that at least one terminal glucose residue was a β-D-glucoside.

An additional enzyme preparation was tested, a commercial preparation of β-D-glucosidase and α-L-rhamnosidase (EC 3.2.1.40) from *Aspergillus niger* with the trade name "Hesperidinase". Hesperidinase has been separated into one α-L-rhamnosidase and two β-D-glucosidase components with quantitatively similar activities.[30] After incubation with Hesperidinase for 1 h, essentially all substrate (99.9%) was hydrolyzed into kaempferol (4.2%) and three expected intermediates (Figure 2B). Loss of one of the terminal glucose residues appeared to represent a minor kinetic bottleneck, but after prolonged incubation, kaempferol was the sole major product (98%). This was considered to be in agreement with all glycosylation being based on the conventional enantiomers, D-glucose and L-rhamnose. The following control experiments were also in agreement with this

Table 3. ^{1}H NMR Spectroscopic Data of the Novel *B. vulgaris* Flavonoids 1 and 2

	δ_H[a] (multiplicity; *J* in Hz)	
position	**1**	**2**
aglycone		
6	6.41 (d; 2)	6.40 (d; 2)
8	6.75 (d; 2)	6.73 (d; 2)
2′	8.05 (d; 9)	7.57 (d; 2)
3′	6.90 (d; 9)	
5′	6.90 (d; 9)	6.85 (d; 9)
6′	8.05 (d; 9)	7.60 (dd; 2, 9)
triglucoside at C3		
(Glc a, Glc b, Glc c)		
a1	5.54 (d; 7)	5.56 (d; 7)
a2	3.50	3.55 (t; 8)
a3	3.48	3.46 (t; 9)
a4	3.18 (t; 9)	3.22 (t; 9)
a5	3.30	3.30 (m)
a6	H_a = 3.76; H_b = 3.39	H_a = 3.76; H_b = 3.42 (dd; 7, 14)
b1	4.61 (d; 8)	4.59 (d; 8)
b2	3.05 (t; 8)	3.05 (t; 8)
b3	3.18 (t; 9)	3.18 (t; 8)
b4	3.12	3.12
b5	3.12	3.13
b6	H_a = 3.58 (br d; 11); H_b = 3.45	H_a = 3.53 (d; 10); H_b = 3.45 (dd; 7, 10)
c1	3.95 (d; 8)	3.96 (d; 8)
c2	2.76 (t; 8)	2.77 (t; 8)
c3	2.87 (t; 9)	2.87 (t; 9)
c4	2.94 (t; 9)	2.95 (t; 9)
c5	2.76	2.77 (m)
c6	H_a = 3.48; H_b = 3.32	H_a = 3.48 (d; 11); H_b = 3.33 (dd; 5, 11)
rhamnoside at C7		
1	5.50 (d; 1.5)	5.50 (d; 1.1)
2	3.83 (dd; 1.5, 3)	3.83 (dd; x, 3)
3	3.62 (dd; 3, 9)	3.62 (dd; 3, 9)
4	3.28 (t, 9)	3.28 (t; 9)
5	3.44 (m)	3.44 (dd; 6, 9)
6	1.11 (d; 6)	1.11 (d; 6)

[a] Signals without indicated coupling constants were extracted from 2D spectra.

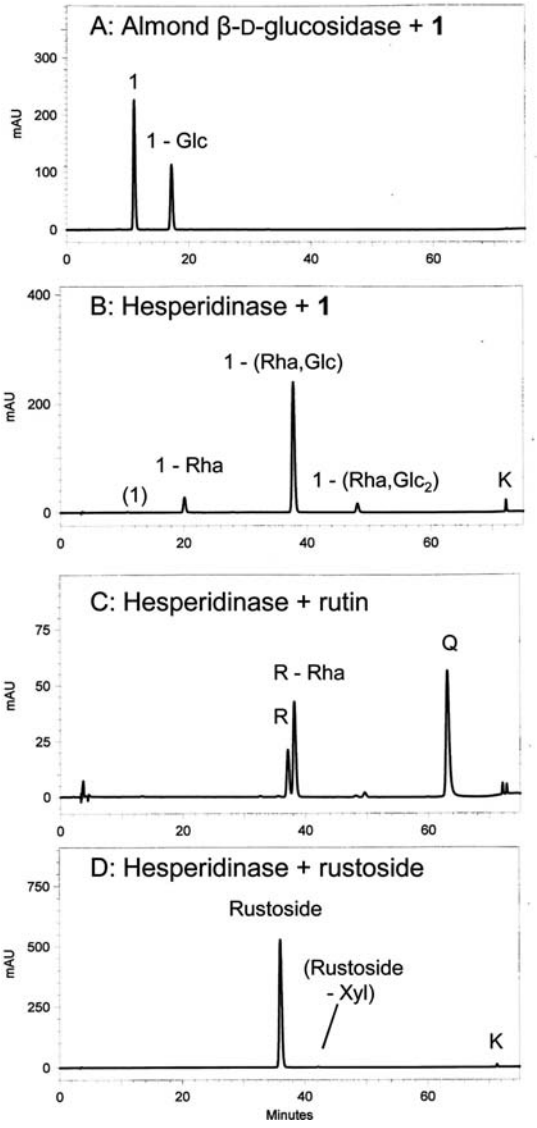

Figure 2. HPLC chromatograms (UV detection at 330 nm) of products of enzymatic hydrolysis of flavonoids performed to test D/L configuration of glycoside linkages: (A) remaining substrate and one exclusive product after incubation of **1** for 6 days with almond *β*-D-glucosidase; (B) water-soluble products after incubation of **1** for 1 h with hesperidinase, with both *β*-D-glucosidase and α-L-rhamnosidase activity; (C) remaining substrate and soluble products after incubation of rutin with hesperidinase for 1 h; (D) rustoside (98.5% of initial concentration) and product traces after incubation with hesperidinase for 1 h. K, kaempferol; Q, quercetin; R, rutin.

interpretation. Incubation for 1 h with 10-fold less Hesperidinase (0.003 U) resulted in 56% of **1** remaining after hydrolysis for 1 h, demonstrating that the above used amount of Hesperidinase was appropriate for removal of **1** in 1 h. Similar dependency of added Hesperidinase activity was found for hydrolysis of rutin (Figures 1 and 2C), a known substrate of Hesperidinase.[30] Hence, the observed hydrolysis of **1** could be attributed to the main *β*-D-glucosidase and α-L-rhamnosidase activities of Hesperidinase,[30] but lack of published specificity data for these enzymes prevented a firm conclusion concerning the D/L configuration of carbohydrate monomers of **1**. As a preliminary specificity test, rustoside (Figure 1) was incubated at the same conditions, and negligible hydrolysis (1.5%) was observed after 1 h (Figure 2D), demonstrating that the Hesperidinase enzyme components were sensitive to the lack of a C6 in the terminal *β*-D-xylopyranoside moiety of rustoside. However, even with rustoside, significant hydrolysis was observed after incubation overnight, meaning that the Hesperidinase components were not absolutely specific. On the basis of the combined evidence, the structure of **1** was concluded to be kaempferol 3-*O*-(2,6-di-*O*-*β*-D-glucopyranosyl)-*β*-D-glucopyranoside-7-*O*-α-L-rhamnopyranoside, with the D/L configurations remaining tentative.

The aglycone of **2** was unequivocally identified as quercetin, on the basis of NMR chemical shifts, ^{1}H NMR coupling patterns, and UV and MS data in the same way as discussed for **1** above (Tables 1−3). *Meta*-coupling between H2′ and H6′ was detectable in this spectrum because of the different chemical shifts of these signals induced by the 3′ OH substituent (Table 3). The MS fragmentation pattern was similar to the fragmentation pattern of **1**, suggesting that the glycosylation of **2** could be identical to that of **1**. This hypothesis was confirmed by sensitivity to Hesperidinase and elucidation of the NMR spectra, including 2D spectra, using an equivalent approach as explained above in the case of **1** but with additional acquisition of a *J*-resolved ^{1}H NMR spectrum (JRES) to reveal coupling constants of overlapping carbohydrate signals (Tables 2 and 3). From these results, the structure of **2** was concluded to be quercetin 3-*O*-(2,6-di-*O*-*β*-D-glucopyranosyl)-*β*-D-glucopyranoside-7-*O*-α-L-rhamnopyranoside. The D/L configurations of the individual carbohydrate moieties were tentatively based on conversion to quercetin by Hesperidinase.

Comprehensive Flavonoid Profile of *B. vulgaris* var. *arcuata* G-Type Foliage. To be able to test hypotheses of flavonoid involvement in insect resistance or other biological characteristics, we optimized and validated an HPLC-based analytical method. Foliage of G-type plants collected in August, when the level of insect resistance is high, was used to include the expected resistance factor in the developed method. Traditional natural product chemistry procedures were taken as the starting point, and the optimization was carried out one step at a time in the reverse direction of the procedure, that is, starting with HPLC and ending with extraction.

Applying the optimized HPLC gradient to pure compounds and simple mixtures, we obtained excellent separation of the four isolated flavonol glycosides and hydrolysates of **1** (Figures 2 and 3A). The entire analytical method was linear and recovery of internal standard close to 100%. We concluded that the method was satisfactory for both qualitative and quantitative investigations of *B. vulgaris* var. *arcuata* G-type foliage with the purpose of searching for flavonoids related to insect resistance or other biological properties.

With the complete *B. vulgaris* var. *arcuata* G-type flavonoid fraction, we observed a complex mixture of 260 + 330 nm absorbing metabolites in the model sample (foliage collected in late summer), including 17 almost baseline separated major peaks (with >1% of the total 330 nm peak area) and two overlapping major peaks (Figure 2B). The UV spectra of the individual major peaks typically had two maxima, at 256−268 nm and at 318−352 nm, with either the former or the latter being the principal, as is typical for many flavones and flavonols. Separation of peaks labeled a, b, and c (respectively) in Figure 2 was difficult. Baseline separation of peaks b and c was obtained during the quantitative analyses performed in 2001, but was unfortunately not reproducible in 2010 when this work was finished. Comparison of our material with standards kindly provided by F. Senatore did not allow identification of additional major peaks.

Seasonal Flavonoid Variation. The natural variation in flavonoids in rosette leaves collected from summer to late fall was probed for G-type leaves from locality 4. Leaves from this locality had a similar seasonal decline in flea beetle resistance (August/September, 0% survival; October, 48% survival; November, 73% survival) as observed at several other localities.[17] For this reason, duplicate analysis of pooled leaves from the same investigation should allow us to test whether any flavonoid constituent was correlated to resistance or susceptibility. The structurally elucidated flavonoids **1**−**4** constituted around half of the total flavonoid level throughout the period, with the kaempferol derivatives being dominant (Figure 4). The summed levels of the tetraglycosylated **1** + **2** and the diglycosylated **3** + **4**

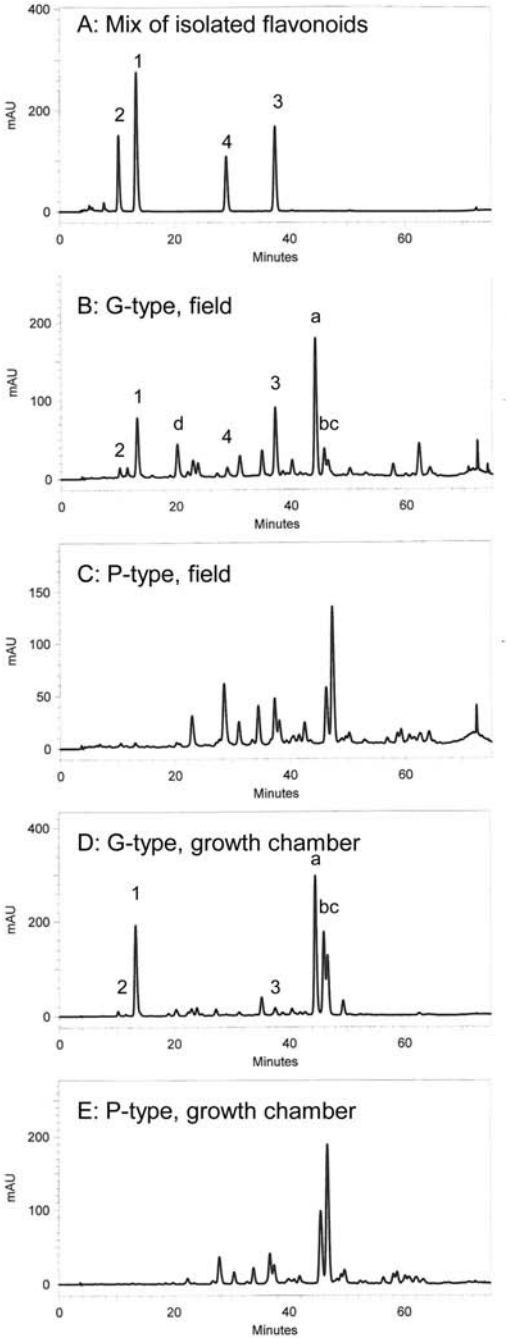

Figure 3. HPLC chromatograms (UV detection at 330 nm) of *B. vulgaris* flavonoid fractions and isolated flavonoids: (A) mixture of isolated flavonoids; (B, C) flavonoid profiles of leaves of G-type (B) or P-type (C) *B. vulgaris* plants sampled at natural growth sites; (D, E) flavonoid profiles of leaves of G-type (D) or P-type (E) *B. vulgaris* plants grown at identical conditions in a growth chamber. Plant chemotype (G-type or P-type) and growth condition (field collected in August or grown in growth chamber) were as indicated in individual panels. Peaks 1−4 correspond to flavonoid structures in Figure 1, whereas letters a−d indicate unidentified peaks with flavonoid-like UV spectra.

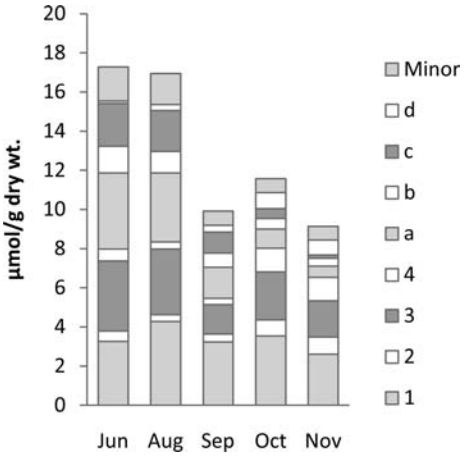

Figure 4. Seasonal variation of flavonoids **1**−**4** and apparent flavonoids in the G-type of *B. vulgaris* ssp. *arcuata* at a natural locality in Denmark (means of two similar replicates from each month). The vertical order of the flavonoids is identical in the legend and all five columns. **1**−**4** signify the four flavonoids illustrated in Figure 1. Letters a−d refer to individual unidentified flavonoid-like peaks in the chromatogram in Figure 3B,D. "Minor" signifies additional, unidentified flavonoid-like peaks.

exhibited only modest variation during the period, but the relative amounts of the quercetin derivatives **2** and **4** showed an increasing tendency compared to the kaempferol derivatives **1** and **3**.

Overall, there was a tendency for lower flavonoid levels in the fall than in summer, with levels in the fall between half and two-thirds of levels in June and August, and this seasonal decline was mainly due to the three unidentified, putative flavonoids a−c (Figure 3) that were major constituents in the summer season and minor constituents in the fall. These putative flavonoids were hence potential flea beetle deterrents, although their relatively early decline between August and September did not exactly match the gradual decline in resistance from September to November. Among additional, more minor flavonoid-like peaks, one showed a significant increasing tendency during the period (labeled "d" in Figure 3), whereas the remaining showed decreasing tendencies or were rather independent of the season. As original chromatograms of field-collected samples (obtained in 1999) were not printed in a style suitable for publication, chromatograms B and C illustrated here (Figure 3) were produced in 2010 with 10-year-old flavonoid preparations, in which peaks b and c had apparently become less prominent and peak d more prominent during storage.

Flavonoids and Insect Resistance. The original impetus for investigating the seasonal variation of *B. vulgaris* flavonoids reported here was an attempt to identify metabolites responsible for the seasonal variation in specialist insect resistance.[17] Flavonoids were secondary metabolites known to exhibit specialist insect deterrence[23] and seasonal variation.[34] The patterns of seasonal variation revealed here were to some extent compatible with several of our hypotheses, for example, that resistance in the summer could be due to deterrent effects of compounds such as peak a or peak c that declined during the fall. However, subsequent tests of deterrent effects of *B. vulgaris* extracts[5] showed that the flavonoids were present in fractions devoid of detectable deterrence toward the test insect (the diamondback moth), whereas saponins were deterrent and exhibited a seasonal decline.[4,5] For this reason we subsequently concentrated on investigations of saponins, and the role of flavonoids, if any, in the interaction between *B. vulgaris* and insects has not yet been elucidated. The available information is compatible with saponins being the sole *B. vulgaris* deterrents to specialist insects tested so far,[5−8,22] but does not rule out that flavonol glycosides may influence host plant selection in flea beetles and other insects. Indeed, flavonol glycosides have in some cases been found to act synergistically with other secondary compounds.[24,26,33]

Polymorphism in Tetraglycosylated Flavonoids. The novel, tetraglycosylated flavonoids **1** and **2** were not detected in a sample of field-collected P-type leaves (Figure 3C) (minor peaks visible in Figure 3C did not match the exact retention time of **1**). To test whether there was a genetic difference between the plant types, the two types were grown under identical conditions in a growth chamber. Flavonoid analysis of the resulting leaves demonstrated that contents of **1** and **2** in leaves of growth chamber grown *B. vulgaris* G-type plants ($N = 3$) were similar to profiles of plants exposed to natural growth conditions and confirmed that P-type leaves ($N = 4$) were deficient in the tetraglycosylated flavonoids **1** and **2** (Figure 3D,E). When both retention times and UV spectra were taken into account, **4** could not be demonstrated in the P-type and was certainly not a major constituent, whereas the presence or absence of **3** in a group of overlapping peaks in P-type chromatograms could not be concluded with certainty. The complete optimization of the analytical method was carried out with the 2001 procedure, but the analysis of growth chamber plants had to be done with SPE cartridges available in 2010. Lack of **1**, **2**, or any major flavonoid-like peak was hence tested and confirmed for the SPE run obtained with the modified protocol and cartridges used for qualitative analyses in 2010. This test confirmed that the currently available SPE cartridges used in 2010 were also capable of binding all *Barbarea* flavonoids from 50 mg of dry leaf material, including the rather hydrophilic **1** and **2**. Hence, the lack of detection of **1** and **2** from P-type plants grown in the growth chamber was reliable.

The comparison of the two plant types also suggested that the two types differ in one or more dominating, late peaks crowded at 44−47 min in the G-type chromatogram (a−c in Figure 3) and 45−47 min in the P-type chromatogram, but we have not so far investigated these additional peaks. Finally, we noted that **3** and **4** were not major constituents in foliage from G-type plants grown in the growth chamber and that peaks a−c were prominent in plants from the growth chamber, as they were in field-collected plants from the summer season (Figure 4).

Flavonoid Biochemistry and Genetics in P-Type and G-Type *B. vulgaris*. With the current expansion of *B. vulgaris* investigations to include molecular genetics and interactions with other insects and pathogens, attention to the flavonoids of the species has returned.[22] For future investigations including additional chemotypes and hybrids, HPLC with both MS and DAD detection will be the optimal choice.[31,32] Fortunately, the conditions developed here should be easily adaptable to HPLC-MS as well, although the TFA used for acidification should perhaps be substituted with, for example, formic acid for optimal ionization. The method may also have preparative potential, but TFA should be omitted[25] or carefully removed after peak collection[27] to avoid damage to sensitive flavonoids. Limited enzymatic degradation of **1** and **2** was found to be a realistic, complementary strategy for preparation of potential biosynthetic intermediates (Figure 2).

Differential flavonol content in types of *B. vulgaris* and their hybrids may help to elucidate the biological roles of these compounds. A simple, yet potentially physiologically relevant characteristic of tetraglycosylated **1** and **2** is their appreciable water solubility, in contrast to the sparingly water-soluble flavonol diglycosides. More subtle effects could be due to specific effects on binding to, for example, insect taste receptors.[33] A QTL in linkage group 3 for major genes for relatively polar metabolites with m/z values for $[M + Na]^+$ ions of 957 and 941[22] would seem to correspond to one or more major genes controlling levels of flavonols **2** and **1** identified here. When the genetics of flavonol production and insect/disease resistance in *B. vulgaris* is known in more detail, it may be possible to produce near-isogenic *Barbarea* plants with different flavonol glycosylation patterns and investigate whether such variation would influence interactions with pests, diseases, and environment.

The flavonoid content of *B. vulgaris* shares some similarities with that of the closely related general purpose model plant *Arabidopsis thaliana*. A major flavonol glycoside in leaves of *A. thaliana* is kaempferol 3-*O*-(6-*O*-β-D-glucopyranosyl)-β-D-glucopyranoside-7-*O*-α-L-rhamnopyranoside.[28] This flavonol glycoside would correspond to **1** after removal of the Glc b moiety (Figure 1) and is a potential intermediate in the biosynthesis of **1** in *B. vulgaris*. Although we found identical glycosylation patterns of quercetin and kaempferol in *B. vulgaris*, it is likely that the glycosylation patterns are due to glycosyl transferases with considerable substrate specificities, as other known glycosides in these plants have glycoside parts with a different branching pattern (4′-glycosylation in saponins) or without known glycoside branching but rather glycoside acylation (6′-acylation of glucosinolates[16]). Glycosylation of natural products can be crucial for biological activity,[8] and mutants in flavonoid glycosylation in *A. thaliana* have been useful for the identification of the responsible genes and proteins.[35] Extensive synteny between *A. thaliana* and *B. vulgaris*[22] may enable the characterization of similar genes and enzymes in *B. vulgaris*.

Continued investigation of the chemical ecology of *B. vulgaris* appears to be attractive because the plant offers agronomical applications against a major crucifer pest, is increasingly well investigated chemically and genetically, is involved in an apparent natural coevolutionary process with a model herbivorous insect, and is currently being investigated by a considerable number of independent but cooperating research groups.[3,6,8,14,16,18−20,22] The polymorphism for highly glycosylated flavonols reported here should allow investigations of relationships between flavonoid biochemistry and ecology in this model system.

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REFERENCES

(1) Courtney, S. P. Coevolution of Pierid butterflies and their cruciferous host plants III. *Anthocharis cardamines* (L.) Survival, development and oviposition on different host plants. *Oecologia* **1981**, *51*, 91–96.

(2) Huang, X.; Renwick, J. A. A.; Sachdev-Gupta, K. Oviposition stimulants in *Barbarea vulgaris* for *Pieris rapae* and *P. napi oleraceae*: isolation, identification and differential activity. *J. Chem. Ecol.* **1994**, *20*, 423–438.

(3) Badenes-Pérez, F. R.; Reichelt, M.; Gershenzon, J.; Heckel, D. G. Phylloplane location of glucosinolates in *Barbarea* spp. (Brassicaceae) and misleading assessment of host suitability by a specialist herbivore. *New Phytol.* **2011**, *189*, 549–556.

(4) Shinoda, T.; Nagao, T.; Nakayama, M.; Serizawa, H.; Koshioka, M.; Okabe, H.; Kawai, A. Identification of a triterpenoid saponin from a crucifer, *Barbarea vulgaris*, as a feeding deterrent to the diamondback moth, *Plutella xylostella*. *J. Chem. Ecol.* **2002**, *28*, 587–599.

(5) Agerbirk, N.; Olsen, C. E.; Bibby, B. M.; Frandsen, H. O.; Brown, L. D.; Nielsen, J. K.; Renwick, J. A. A. A saponin correlated with variable resistance of *Barbarea vulgaris* to the diamondback moth *Plutella xylostella*. *J. Chem. Ecol.* **2003**, *29*, 1417–1433.

(6) Nielsen, N. J.; Nielsen, J.; Staerk, D. New resistance-correlated saponins from the insect-resistant crucifer *Barbarea vulgaris*. *J. Agric. Food Chem.* **2010**, *58*, 5509–5514.

(7) Kuzina, V.; Ekstrøm, C. T.; Andersen, S. B.; Nielsen, N. J.; Olsen, C. E.; Bak, S. Identification of defence compounds in *Barbarea vulgaris* against the herbivore *Phyllotreta nemorum* by an ecometabolomics approach. *Plant Physiol.* **2009**, *151*, 1977–1990.

(8) Nielsen, J. K.; Nagao, T.; Okabe, H.; Shinoda, T. Resistance in the plant, *Barbarea vulgaris*, and counter-adaptations in flea beetles mediated by saponins. *J. Chem. Ecol.* **2010**, *36*, 277–285.

(9) Shelton, A. M.; Nault, B. A. Dead-end trap cropping: a technique to improve management of the diamondback moth, *Plutella xylostella* (Lepidoptera: Plutellidae). *Crop Prot.* **2004**, *23*, 497–503.

(10) Badenes-Pérez, F. R.; Reichelt, M.; Heckel, D. G. Can sulfur fertilisation improve the effectiveness of trap crops for diamondback moth, *Plutella xylostella* (L.) (Lepidoptera: Plutellidae)? *Pest Manag. Sci.* **2010**, *66*, 832–838.

(11) Nielsen, J. K. Specificity of a Y-linked gene in the flea beetle *Phyllotreta nemorum* for defences in *Barbarea vulgaris*. *Entomol. Exp. Appl.* **1999**, *115*, 265–270.

(12) Thompson, J. N. *The Geographic Mosaic of Coevolution*; University of Chicago Press: Chicago, IL, 2005.

(13) Breuker, C. J.; De Jong, P. W.; Victoir, K.; Vrieling, K.; Brakefield, P. M. Pleiotropic effects associated with an allele enabling the flea beetle *Phyllotreta nemorum* to use *Barbarea vulgaris* as a host plant. *Evol. Ecol.* **2007**, *21*, 13–26.

(14) De Jong, P. W.; Breuker, C. J.; De Vos, H.; Vermeer, K. M. C. A.; Oku, K.; Verbaarschot, P.; Nielsen, J. K.; Brakefield, P. M. Genetic differentiation between resistance phenotypes in the phytophagous flea beetle, *Phyllotreta nemorum*. *J. Insect Sci.* **2009**, *9*, 1–8.

(15) Agerbirk, N.; Ørgaard, M.; Nielsen, J. K. Glucosinolates, flea beetle resistance, and leaf pubescence as taxonomic markers in the genus *Barbarea* (Brasicceae). *Phytochemistry* **2003**, *63*, 69–80.

(16) Agerbirk, N.; Olsen, C. E. Isoferuloyl derivatives of five seed glucosinolates in the crucifer genus *Barbarea*. *Phytochemistry* **2011**, *72*, 610–623.

(17) Agerbirk, N.; Olsen, C. E.; Nielsen, J. K. Seasonal variation in leaf glucosinolates and insect resistance in two types of *Barbarea vulgaris* ssp. *arcuata*. *Phytochemistry* **2001**, *58*, 91–100.

(18) Ørgaard, M.; Linde-Laursen, I. Meiotic analysis of Danish species of *Barbarea* (Brassicaceae) using FISH: chromosome numbers and rDNA sites. *Hereditas* **2008**, *145*, 215–219.

(19) Toneatto, F.; Nielsen, J. K.; Ørgaard, M.; Hauser, T. P. Genetic and sexual separation between insect resistant and susceptiple *Barbarea vulgaris* plants in Denmark. *Mol. Ecol.* **2010**, *19*, 3456–3465.

(20) van Leur, H.; Vet, L. E. M.; van der Putten, W. H.; van Dam, N. M. *Barbarea vulgaris* glucosinolate phenotypes differentially affect performance and preference of two different species of lepidopteran herbivores. *J. Chem. Ecol.* **2008**, *34*, 121–131.

(21) Toneatto, F. Genetics and distribution of insect resistance polymorphism in *Barbarea vulgaris*. Ph.D. thesis, University of Copenhagen, 2009.

(22) Kuzina, V.; Nielsen, J. K.; Augustin, J. F.; Torp, A. M.; Bak, S.; Andersen, S. B. *Barbarea vulgaris* linkage map and quantitative trait loci for saponins, glucosinolates, hairiness and resistance to the herbivore *Phyllotreta nemorum*. *Phytochemistry* **2011**, *72*, 188–198.

(23) Haribal, M.; Renwick, J. A. A. Dual chemical barriers protect a plant against different larval stages of an insect. *J. Chem. Ecol.* **2001**, *27*, 1575–1583.

(24) Simmonds, M. S. J. Flavonoid−insect interactions: recent advances in our knowledge. *Phytochemistry* **2003**, *64*, 21–30.

(25) Senatore, F.; D'Agostini, M.; Dini, I. Flavonoid glycosides of *Barbarea vulgaris* L. (Brassicaceae. *J. Agric. Food Chem.* **2000**, *48*, 2659–2662.

(26) Nielsen, J. K.; Larsen, L. M.; Sørensen, H. Host plant selection of the horseradish flea beetle *Phyllotreta armoraciae* (Coleoptera: Chrysomelidae). Identification of two flavonol glycosides stimulating feeding in combination with glucosinolates. *Entomol. Exp. Appl.* **1979**, *26*, 40–48.

(27) Agerbirk, N.; Olsen, C. E.; Poulsen, E.; Jakobsen, N.; Hansen, P. R. Complex metabolism of aromatic glucosinolates in *Pieris rapae* caterpillars involving nitrile formation, hydroxylation, demethylation, sulfation, and host plant dependent carboxylic acid formation. *Insect Biochem. Mol. Biol.* **2010**, *40*, 126–137.

(28) Veit, M.; Pauli, G. F. Major flavonoids from *Arabidopsis thaliana* leaves. *J. Nat. Prod.* **1999**, *62*, 1301–1303.

(29) Markham, K. R.; Chari, V. M.; Mabry, T. J. Carbon-13 NMR spectroscopy of flavonoids. In *The Flavonoids: Advances in Research*; Harborne, J. B., Mabry, T. J., Eds.; Chapman and Hall: London, U.K., 1982; pp 19−133.

(30) Manzanares, P.; de Graaf, L. H.; Visser, J. Purification and characterization of an α-L-rhamnosidase from *Aspergillus niger*. *FEMS Microbiol. Lett.* **1997**, *157*, 279–283.

(31) Truchado, P.; Tourn, E.; Gallez, L. M.; Moreno, D. A.; Ferreres, F.; Tomás-Barberán, F. A. Identification of botanical biomarkers in Argentinean *Diplotaxis* honeys: flavonoids and glucosinolates. *J. Agric. Food Chem.* **2010**, *58*, 12678–12685.

(32) Olsen, H.; Aaby, K.; Borge, G. I. A. Characterization, quantification, and yearly variation of the naturally occurring polyphenols in a common red variety of curly cale (*Brassica oleracea* L. convar. *acephala* var. *sabellica* cv. 'Redbor'. *J. Agric. Food Chem.* **2010**, *58*, 11346–11354.

(33) van Loon, J. J. A.; Wang, C. Z.; Nielsen, J. K.; Gols, R.; Qiu, Y. T. Flavonoids from cabbage are feeding stimulants for diamondback moth larvae additional to glucosinolates: chemoreception and behaviour. *Entomol. Exp. Appl.* **2002**, *104*, 27–34.

(34) Haribal, M.; Renwick, J. A. A. Seasonal and population variation in flavonoid and alliarinoside content of *Alliaria petiolata*. *J. Chem. Ecol.* **2001**, *27*, 1585–1594.

(35) Yonekura-Sakakibara, K.; Takayuki, T.; Niida, R.; Saito, K. Identification of a flavonol 7-*O*-rhamnosyltransferase gene determining flavonoid pattern in *Arabidopsis* by transcriptome coexpression analysis and reverse genetics. *J. Biol. Chem.* **2007**, *282*, 14932–14941.