1,4-Dimethoxyglucobrassicin in *Barbarea* and 4-Hydroxyglucobrassicin in *Arabidopsis* and *Brassica*

Niels Agerbirk,*,† Bent Larsen Petersen,‡ Carl Erik Olsen,† Barbara Ann Halkier,‡ and Jens Kvist Nielsen†

Chemistry Department and Department of Plant Biology, Center of Molecular Plant Physiology (PlaCe), Royal Veterinary and Agricultural University, Thorvaldsensvej 40, DK-1871 Frederiksberg C, Denmark

A novel indole glucosinolate, 1,4-dimethoxyglucobrassicin (1,4-dimethoxyindol-3-ylmethylglucosinolate), was isolated as the desulfo derivative from roots of the P-type of *Barbarea vulgaris* ssp. *arcuata*, and its structure was determined by spectroscopy including 2D NMR spectroscopy. 4-Hydroxyglucobrassicin (4-hydroxyindol-3-ylmethylglucosinolate) was isolated as the desulfo derivative from green siliques (fruits) of *Arabidopsis thaliana* and identified by comparison of its ^{1}H NMR spectrum with the spectrum of the known desulfoglucosinolate from *Brassica napus*. The delayed elution of desulfo indole glucosinolates from the DEAE Sephadex column used in sample preparation was examined, and the diode-array UV spectra of desulfo indole glucosinolates were measured, to ensure a reliable determination of 1,4-dimethoxyglucobrassicin and 4-hydroxyglucobrassicin with the existing analysis method based on the HPLC of desulfoglucosinolates. 1,4-Dimethoxyglucobrassicin was not detected in 10 other *Arabidopsis*, *Brassica*, and *Barbarea* species, indicating an evolutionarily recent mutation in the indole glucosinolate biosynthesis in *B. vulgaris* ssp. *arcuata* type P.

Keywords: *Brassicaceae; Barbarea; Arabidopsis; Brassica; 1,4-dimethoxyindol-3-ylmethylglucosinolate; 1,4-dimethoxyglucobrassicin; 4-hydroxyindol-3-ylmethylglucosinolate; 4-hydroxyglucobrassicin; desulfoglucosinolate; indole phytoalexin; NMR; MS; UV; HPLC*

INTRODUCTION

Indole glucosinolates (indol-3-ylmethylglucosinolates, glucobrassicins) are secondary metabolites that are commonly accumulated in cruciferous plants (*1*). Upon hydrolysis by the endogenous plant enzyme myrosinase (thioglucosidase, EC 3.2.3.1), they are converted into isothiocyanates (*2*) or nitriles (*3*, *4*). This reaction occurs after physical disruption of the plant tissue, e.g., by insect feeding or fungal infection. The isothiocyanates formed from indole glucosinolates are unstable and quickly react with ascorbic acid to form thiocyanate ion and ascorbigens as the main aglucone hydrolysis products (*4*−*7*). Other products are formed as well, especially if ascorbic acid is not present (*4*, *8* and references therein). Although the nitriles are chemically stable, they can be hydrolyzed to carboxylic acids by the endogenous plant enzyme nitrilase (nitrile aminohydrolase, EC 3.5.5.1) (*9*).

Several lines of evidence have been reported for an in vivo conversion of indole glucosinolates via isothiocyanates to fungitoxic and phytotoxic indole phytoalexins. The conversion seems to be induced by either pathogens, UV light, or heavy metal salts and thus to be independent of any physical damage to the plant tissue (*10*). This offers a new physiological function for the indole glucosinolates as nonphytotoxic intermediates in the biosynthesis of the phytoalexins. The evidence for such a biochemical pathway is still incomplete (*10*).

In addition to the parent indole glucosinolate glucobrassicin (**1**), the substituted indole glucosinolates 1-methoxyglucobrassicin (**2**), 4-methoxyglucobrassicin (**3**), and 4-hydroxyglucobrassicin (**5**) (Figure 1) are commonly accumulated in cruciferous plants (*11*−*13*). Substituted indole phytoalexins corresponding to the substituted indole glucosinolates **1**, **2**, and **3** are known (*10*), which supports a biosynthetic connection. The *N*-sulfo derivative of glucobrassicin is also known (*14*), but it cannot be detected by the common analysis method based on desulfoglucosinolates (*15*, *16*) and has consequently seldom been studied.

The nutritional, agronomic and ecological properties of indole glucosinolates are of interest (*1*, *13*, *17*−*19*). The phenolic indole glucosinolate **5** has strong antinutritional properties in animal fodder (*20*), and the discovery of **5** as the dominating glucosinolate in seeds of modern cultivars of oilseed rape (*Brassica napus* L.) (*21*) catalyzed the development and validation of HPLC-based methods for analysis of individual intact or desulfated glucosinolates (*22* and references therein).

Arabidopsis thaliana (L.) Heynh. is used extensively as a molecular genetic model for crop plants. Many *Brassica* species are important crops. Species of the genus *Barbarea* are being tested as possible new oil crops (*23*, *24*) or as gene donors for *Brassica* crop plants using somatic hybridization (*25*). *Arabidopsis* and *Barbarea* are evolutionary closely related, whereas *Brassica* is of more distant origin (*26*).

During ongoing investigations of the influence of glucosinolates on insect herbivory in *Barbarea* species and *A. thaliana*, a novel indole glucosinolate was discovered in the P-type (*27*) of *Barbarea vulgaris* R.

* Author to whom correspondence should be addressed [telephone (+45) 35 28 24 38; fax (+45) 35 28 23 98; e-mail nia@kvl.dk].

† Chemistry Department.

‡ Department of Plant Biology.

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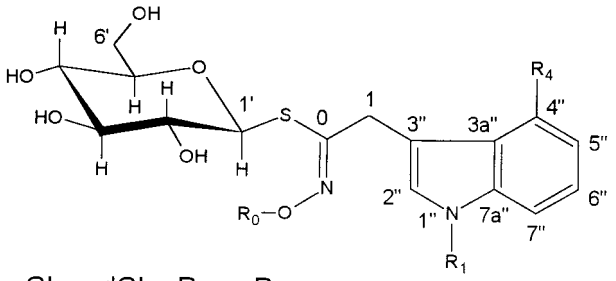

Glu	dGlu	R_1	R_4
1	**d1**	H	H
2	**d2**	OMe	H
3	**d3**	H	OMe
4	**d4**	OMe	OMe
5	**d5**	H	OH

Figure 1. Indole glucosinolates (Glu, $R_0 = SO_3^-$) and desulfo indole glucosinolates (dGlu, $R_0 = H$).

Br. ssp. *arcuata* (Opiz.) Simkovics, and the previously known indole glucosinolate **5** was identified in *A. thaliana* (*28*), in which it had previously escaped detection (*19*, *29*). The present study reports on the identification of these two secondary metabolites using 1D and 2D NMR techniques and MS. Several analytical parameters were measured to ensure a reliable analysis of indole glucosinolates. The occurrence of indole glucosinolates in *A. thaliana* and several *Barbarea* and *Brassica* species was reevaluated, as previous methods might have failed to detect some of these compounds.

MATERIALS AND METHODS

Plants. Plants were grown from seeds in artificial soil (Cornell mix + osmocote), with addition of fertilizer every week, in a greenhouse with supplemental light, as described elsewhere (*30*). Roots were harvested from the bottom of the pots, or sometimes from within the soil, and gently rinsed with water if necessary. The seeds were obtained as follows. Seeds of wild *Barbarea* populations were collected by J. K. Nielsen at various localities on the Danish island Zealand; names of localities, collection years, and accession numbers are in parentheses after each species: G-type of *B. vulgaris* R. Br. ssp. *arcuata* (Opiz.) Simkovics (Herlev, 1994, B1), P-type of *B. vulgaris* R. Br. ssp. *arcuata* (Opiz.) Simkovics (Trundholm Mose, 1997, B4), *B. vulgaris* ssp. *vulgaris* R. Br. (Risby, 1996, B5), *B. intermedia* Boreau (Klampenborg, 1998, B7), and *B. stricta* Andrz. (Tissø, 1997, B8). Seeds of *Barbarea verna* (Miller) Ascherson were from the Botanical Garden, University of Copenhagen, 1997 (B16). Seeds of *Brassica napus* L. cv. Polo were a gift from Danisco Seed, Holeby, Denmark. Seeds of *Brassica oleracea* L. cv. Golden Acre were from Agway Inc., Syracuse, NY. Seeds of *Brassica rapa* L., Crucifer Genetics Cooperative rapid cycling line 1−1, and of *B. juncea* (L.) Czern. et Coss, Crucifer Genetics Cooperative rapid cycling line 4−1, were a gift from D. E. Costich, Boyce Thomson Institute at Cornell University. Seeds of *Brassica nigra* (L.) Koch and of *B. carinata* A. Br. were a gift from B. Traw, Department of Entomology, Cornell University. *Arabidopsis thaliana*, ecotype Colombia for purification of desulfo-4-hydroxyglucobrassicin, was grown as described elsewhere (*28*). The analytical results in Table 2 were obtained with independent material of *A. thaliana*, ecotype Colombia, grown and kindly supplied by P. Conklin, Boyce Thompson Institute at Cornell University. Voucher specimens of the *Barbarea* accessions will be deposited at the Botanical Museum, University of Copenhagen, after flowering in spring 2001.

Chemicals and Enzymes. Benzylglucosinolate, used as internal standard, was from Merck (Darmstadt, Germany). The concentration of the internal standard solution was determined by UV spectroscopy, using $\epsilon_{227.5} = 8870\ M^{-1}\ cm^{-1}$ (*31*). *Helix pomatia* type H-1 sulfatase (aryl sulfate sulfatase, EC 3.1.6.1.) was from Sigma (S-9626) and was used without further purification. DEAE Sephadex A25 was from Pharmacia (Uppsala, Sweden).

NMR Spectroscopy and MS. NMR spectra were recorded on a Bruker Avance 400 instrument in D_2O at 400.1 MHz (^1H) and 100.6 MHz (^{13}C). ^1H and ^{13}C chemical shifts are based on dioxane as the internal reference, with δ_H(dioxane) = 3.75 and δ_C(dioxane) = 67.4. Mass spectra were obtained on a Bruker Esquire-LC ion trap mass spectrometer in positive electrospray ionization mode. Samples were dissolved in 50 μM aqueous sodium acetate−methanol (1:1) and introduced by flow injection.

Extraction of Glucosinolates and Enzymatic Hydrolysis to Desulfoglucosinolates. Analytical-scale DEAE columns were prepared in Pasteur pipets (short type, dead volume ca. 100 μL) with glass wool stoppers, as follows: DEAE Sephadex A25 (0.10 g dry) was incubated overnight in 0.5 M aqueous acetic acid−NaOH, pH 5. The swelled material was transferred to the pipet, and excess acetate was washed out by 5 × 2 mL water. Dried or freeze-dried plant tissue (40 mg) was extracted three times, each for 1 min, in 3 mL of boiling MeOH−water (7:3). During the first round of extraction, 100 μL of 5 mM benzylglucosinolate was added to the boiling extract as an internal standard. The crude extract was briefly centrifuged and loaded directly on a DEAE column. The column was washed with 5 × 2 mL water, followed by 2 × 2 mL of 0.02 M aqueous acetic acid−NaOH, pH 5. When the column was drained, 0.4 mL of *Helix pomatia* sulfatase, 2.5 mg/mL dissolved in 0.02 M aqueous acetic acid−NaOH, pH 5, was applied to the column, and collection of the eluate was started. After incubation overnight, the resulting desulfoglucosinolates were eluted by 5 × 1 mL of water. The desulfoglucosinolate eluates were kept frozen until analysis by HPLC.

Differential Elution from DEAE Columns. The elution step, in which desulfoglucosinolates were eluted after the overnight sulfatase incubation, was controlled by collection of 0.5 mL fractions and subsequent analysis of each fraction separately. For the measurement of elution order, a DEAE column twice the size of the analytical column was used (i.e., 0.2 g DW of DEAE Sephadex, as a column 5 mm wide and 53 mm high packed in a short-type Pasteur pipet), and 0.25 mL fractions were collected. Crude extracts of *Brassica napus* seeds, green parts of broccoli, *B. oleracea* (*4*), and roots of *Barbarea vulgaris* ssp. *arcuata* type P were mixed to provide a sample containing five different indole glucosinolates, as well as several non-indole glucosinolates.

HPLC Analysis of Desulfoglucosinolates. For analysis of desulfoglucosinolates, a Hewlett-Packard series 1100 HPLC with a diode-array detector was equipped with a Luna C18 column, 150 × 4.6 mm, 3 μ (Phenomenex, Torrance, CA). The column temperature was 40 °C, and the flow rate was 0.75 mL/min. A sample (40 μL) was injected onto the column. After 5 min of elution with pure water, a linear gradient from 0 to 35% acetonitrile in 30 min was found to separate the desulfoglucosinolates. Peaks were identified by diode-array UV spectroscopy (200−400 nm) and quantified by the peak area at 229 nm (bandwidth 4 nm) relative to the area of the internal standard peak. Peak areas of desulfoglucosinolates were corrected for differences in molar absorption, using the response factors measured by Buchner (*32*). The response factors used were 0.95 for desulfobenzylglucosinolate, 0.29 for desulfoglucobrassicin (**d1**), 0.20 for desulfo-1-methoxyglucobrassicin (**d2**), 0.25 for desulfo-4-methoxyglucobrassicin (**d3**), and 0.28 for desulfo-4-hydroxyglucobrassicin (**d5**). For desulfo-1,4-dimethoxyglucobrassicin (**d4**), the correction factor of 0.20 for **d2** was assumed to be valid.

Purification of Desulfoglucosinolates. d1, **d3**, and **d4** for NMR experiments were purified from freeze-dried roots of *B. vulgaris* ssp. *arcuata* type P, accession B4. **d5** for ^1H NMR experiments (Figure 3) was purified from freeze-dried stems

Table 1. Comparison of the Measured ^{1}H and ^{13}C NMR Chemical Shifts and Molecular Weights Measured by MS for the Desulfoglucosinolates d1, d3, d4, and d5[a]

	d1	d3	d4	d5	d1	d3	d4	d5
thioglucoside moiety	δH	δH	δH	δH	δC	δC	δC	δC[b]
1′	4.82 d[c]	4.87 d (10)	4.87 d (10)	4.89 d (9)	81.9	82.4	82.4	82.3
2′	3.29 t (9)	3.31 t (9)	3.32 t (9)	3.30 t (9)	72.8	72.8	72.7	72.8
3′	3.21 t (9)	3.20 t (9)	3.21 t (9)	3.21 t (9)	77.9	78.1	78.0	78.1
4′	3.35 t (9)	3.38 t (10)	3.39 t (9)	3.40 t (9)	69.7	69.3	69.4	69.4
5′	2.97 m	2.86 m	2.91 m	2.99 ddd (10, 4, 3)	80.6	80.4	80.4	80.2
6′a	3.58 m	3.51 d (13)	3.48 d (12)	3.55 dd (13, 2)	61.1	60.8	60.9	60.9
6′b	do.	3.58 dd (13,4)	3.57 dd (12, 4)	3.59 dd (13, 4)	–	–	–	–
C0 and side chain								
0	–	–	–	–	155.9	156.8	156.6	157.0
1a	4.09 d (16)	4.25 d (17)	4.22 d (17)	4.24 d (17)	29.8	30.6	30.5	30.4
1b	4.18 d (16)	4.33 d (17)	4.29 d (17)	4.39 d (17)	–	–	–	–
2″	7.29 s	7.09 s	7.26 s	7.08 s	124.5	123.6	122.1	123.4
3″	–	–	–	–	109.8	109.8	107.2	109.8
3a″	–	–	–	–	127.0	116.7	113.4	116.4
4″	7.69 d (8)	–	–	–	119.3	154.7	155.1	151.2
5″	7.19 t (7)	6.67 d (7)	6.70 d (8)	6.54 m	120.2	100.9	101.8	104.8
6″	7.26 t (8)	7.18 t (8)	7.26 t (8)	7.04−7.09 m	123.0	123.9	125.0	124.0
7″	7.53 d (8)	7.15 d (8)	7.17 d (8)	7.04−7.09 m	112.8	106.3	103.1	105.4
7a″	–	–	–	–	137.0	138.6	134.8	139.3
1″-OCH_3	–	–	4.07 s	–	–	–	66.8	–
4″-OCH_3	–	3.94 s	3.94 s	–	–	56.1	56.3	–
m/z for $[M+Na]^+$	391	421	451	407				

[a] Assignments were based on 2D NMR methods (see text). δH is followed by the multiplicity of the signal and the coupling constant in Hz. [b] Extracted from HSQC and HMBC spectra. [c] Overlap with water signal at 4.79 ppm, revealed by COSY.

and siliques (fruits) of *A. thaliana*. **d5** for ^{1}H and ^{13}C NMR experiments (Table 1, Figure 3) was purified from seeds of *B. napus* cv. Polo. The desulfo indole glucosinolates were purified by scaling up the analytical group separation procedure, followed by preparative HPLC. For **d5**, a LC-10AT HPLC from Shimadzu (Kyoto, Japan), equipped with a Supelcosil LC-ABZ column, 25 cm × 4.6 mm, 5 μm, from Supelco, Bellefonte, PA, was used. For **d1**, **d3**, and **d4**, an LKB HPLC system (Pharmacia, Uppsala, Sweden) equipped with a semipreparative Nucleosil C18 column, 25 cm × 10 mm (Mikrolab, Højbjerg, Denmark), was used with a MeOH gradient in water as eluent. Solvents were evaporated by a rotary evaporation at ca. 40 °C or by lyophilization (**d5**). Purified **d5** was stable in aqueous solution for several weeks at 5 °C.

RESULTS AND DISCUSSION

1,4-Dimethoxyglucobrassicin from *Barbarea*. After sulfatase treatment of anionic metabolites from roots of *B. vulgaris* ssp. *arcuata* type P, three desulfo indole glucosinolates, **d1**, **d3**, and the novel hydrophobic compound **d4**, were detected and purified. The ^{1}H NMR spectra of **d1** and **d3** were in agreement with partially assigned spectra of natural **d1** and **d3** (*11*) and fully assigned spectra of synthetic **d1** and **d3** (*33*).

The UV spectrum of **d4** suggested a substituted indole derivative. ^{1}H NMR, ^{13}C NMR, and MS results all suggested a desulfo dimethoxyglucobrassicin. The methoxy groups were found to be positioned at 1″ and 4″ by NOESY and COSY as follows: H1a and H1b were easily identified as two widely separated doublets ($J = 17$ Hz), as is often seen when the free rotation around the methylene bridge in the indol-3-ylmethyl group is hindered by attachment to a bulky structure. H1a and H1b showed NOE to H2″, which showed NOE to the 1″-methoxy group. NOE between the 1″-methoxygroup and H7″ defined the position of H7″. COSY confirmed the relative positions of H7″, H6″, and H5″. Finally, H5″ showed NOE to the 4-methoxy group (Figure 2). In this way, all side-chain protons were assigned. A ^{1}H−^{13}C correlated spectrum (HSQC) identified the signals of the proton-bearing side-chain carbon atoms; the remaining signals were then identified by HMBC. In the thioglucose moiety, COSY easily allowed assignments of all protons, and a HSQC spectrum identified the corresponding carbon signals (Table 1).

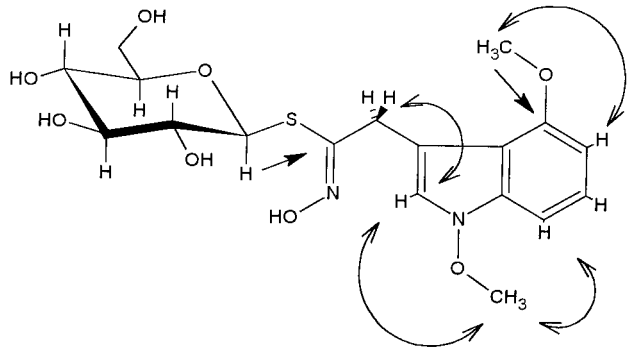

Figure 2. Selected NOE (curved arrows) and HMBC (straight arrows) long-range interactions that confirm the substitution pattern and connectivity of **d4**.

The expected characteristics for a 4″-methoxy substitution were identified as follows: (1) Signals from only three coupled aromatic protons (5″, 6″, and 7″) were seen. The aromatic 5″ proton was shifted by 0.49 ppm upfield in **d4** and by 0.52 ppm upfield in **d3** relative to that in **d1**. Similar effects were seen for the 5″, 3a″, and 7″ carbons, all as expected for a 4″-methoxy derivative. (2) The 4″-methoxy proton and carbon signals were identical and similar, respectively, to the corresponding signals from **d3**. (3) HMBC detected long-range (three-bond) coupling between the 4″-MeO group and C4″, confirming the position of the ether linkage.

The expected characteristics for a 1″-methoxy substitution were also identified as follows: (1) The aromatic singlet (2″, exactly overlapping the 6″ triplet, but resolved by HSQC) of **d4** was shifted by 0.17 ppm downfield compared to that of **d3**, a feature also seen for other *N*-methoxy derivatives compared to the corresponding compounds without an *N*-methoxy group (*4*, *7*, *11*). (2) The 1″-methoxy proton and carbon chemical

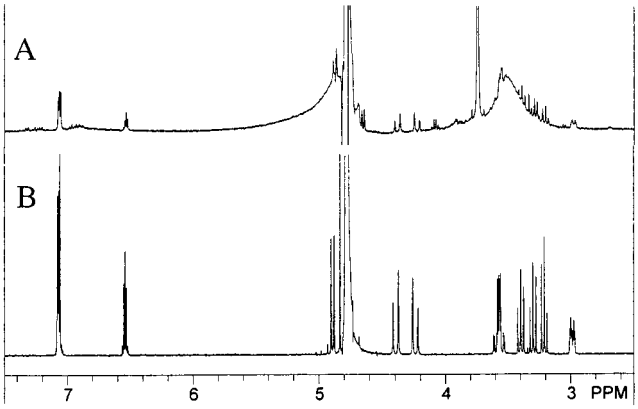

Figure 3. Comparison of **d5**, isolated from *A. thaliana* after sulfatase treatment, with authentic **d5** by ^{1}H NMR spectroscopy. (A) **d5** from *A. thaliana*. (B) Authentic **d5**.

shifts were close to the chemical shifts of the *N*-methoxy group in other *N*-methoxyindole derivatives (*4*, *7*, *11*). (3) The 1″-methoxy protons did not show HMBC long-range (three-bond) coupling with any carbon, as expected if the hetero atom was indeed the site of the substitution.

The structure of the thiohydroximate part of **d4** was inferred from the known general structure of glucosinolates, which is based on studies of allylglucosinolate (*34*, *35*). The expected molecular weight for a desulfoglucosinolate and the similarity of the NMR chemical shifts of H1′ to the chemical shift reported for synthetic desulfo indole glucosinolates (*33*) support this part of the proposed structure. HMBC established the position of the thioether bond by a long-range interaction between C0 and H1′.

From the identification of the desulfoglucosinolates **d1**, **d3**, and **d4**, after binding of the native metabolites to an anion exchange column and subsequent release by treatment with sulfatase (*36*), the existence of the intact glucosinolates **1**, **3**, and **4** in the extracted plant material was deduced. Apart from being derivatives obtained by sulfatase treatment, desulfoglucosinolates also occur in plants as intermediates in glucosinolate biosynthesis (*13*), but whether **d1**, **d3**, and **d4** occur as biosynthetic intermediates in the investigated plant species cannot be concluded from this study.

4-Hydroxyglucobrassicin from *Arabidopsis*. HPLC showed a hydrophilic desulfo indole glucosinolate from green siliques of *A. thaliana* after on-column conversion of the native glucosinolates to desulfoglucosinolates by sulfatase. By diode-array HPLC, the compound was found to be indistinguishable from the previously known **d5** from seeds of *Brassica napus*. The *A. thaliana* desulfoglucosinolate and **d5** from *B. napus* were isolated and analyzed by ^{1}H NMR spectroscopy (Figure 3). It was concluded that the indole glucosinolate **5**, previously known from *B. napus*, was present in *A. thaliana*. Whereas **5** is the dominating glucosinolate in seeds of *B. napus* cv. Polo, it was only present as a minor glucosinolate in *A. thaliana* (*28*). Accordingly, 15000 scans and water suppression were used to obtain the shown spectrum of **d5** from *A. thaliana*.

The glucosinolate **5** was detected in stems, flower buds, and green siliques of *A. thaliana,* whereas only a trace was detected in mature seeds (*28*). The identification of the economically important glucosinolate **5** in siliques of *A. thaliana* should facilitate further biochemical studies of this compound.

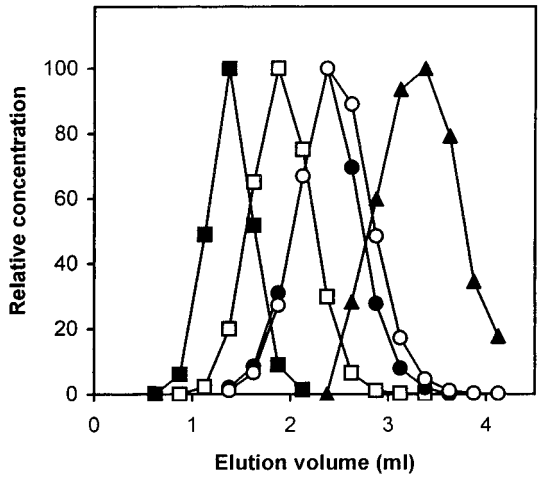

Figure 4. Side-chain-dependent elution of desulfoglucosinolates from a 200-mg DEAE Sephadex column after incubation with sulfatase. (■) desulfo-2-phenylethylglucosinolate, (□) **d2** and **d4** (coeluting), (●) **d3**, (○) **d1**, (▲) **d5**.

4-Hydroxyglucobrassicin from *B. napus*. ^{1}H and ^{13}C NMR spectra and an electrospray ionization mass spectrum of the hydroxyglucobrassicin from *B. napus* seeds were recorded to ensure the identity of the reference compound. Hanley et al. (*12*) have shown that the hydroxyascorbigen derived from the *B. napus* hydroxyglucobrassicin is converted into the 4-methoxy derivative by methylation. This and other results indicate that **5** is the correct structure of the hydroxyglucobrassicin in *Brassica* (*12*, *21*). To serve the dual purpose of confirming the substitution pattern and obtaining ^{13}C NMR data, which have not been reported for **d5**, we recorded the HSQC and HMBC spectra, which together with COSY fully confirmed that the hydroxyindole glucosinolate from *B. napus* is 4-hydroxyglucobrassicin (Table 1).

Analytical Aspects. Several analytically relevant characteristics were measured to ensure a reliable determination of **4** and **5** with the existing analytical method based on HPLC of desulfoglucosinolates (*22*). All of the desulfo indole glucosinolates were retained on the DEAE Sephadex column used in the group separation relative to nonindole desulfoglucosinolates (Figure 4). The order of elution from the DEAE Sephadex column was non-indoles (first), **d2**/**d4**, **d3**, **d1**, and **d5** (last), so elution volumes optimized to include **d5** (*32*) will also include **d4**. With a 100-mg column, at least 3 mL was necessary for the elution of **d5**. The delayed elution of desulfo indole glucosinolates from DEAE Sephadex columns was reported by Buchner and investigators involved in the discovery of **5** (*22*). The commonly used internal standard desulfobenzylglucosinolate coeluted with desulfo-2-phenylethylglucosinolate (result not shown). It can be concluded that the use of elution volumes that are too low in the desulfoglucosinolate method will lead to serious underestimation of indole glucosinolates, especially **1**, **3**, and **5**.

In the HPLC determination, the retention time and diode-array UV spectrum allowed for identification of each desulfo indole glucosinolate (Figure 1). Absorption bands (λ_{max} in nm) and typical retention times (RTs) were as follows. **d1**: 220, 273 (shoulder), 280, 289, RT = 21.8 min. **d2**: 223, 278 (shoulder), 290, 298 (shoulder), RT = 28.2 min. **d3**: 221, 265, 282 (shoulder), 292, RT = 23.9 min. **d4**: 224, 262, 272 (shoulder), 293, 301, RT = 29.7 min. **d5**: 221, 267, 283, 293, RT = 16.8 min. The

Table 2. Taxonomic Distribution of the Indole Glucosinolates 1−5 in Roots of Some *Barbarea* and *Brassica* Species and in Roots and Siliques of *A. thaliana*

species	N^a	indole glucosinolates (μmol/g dry weight)				
		1	**2**	**3**	**4**	**5**
Barbarea vulgaris ssp. *vulgaris*	1	6.2	2.9	0.8	ND[b]	ND[b]
Barbarea vulgaris ssp. *arcuata* type G	4	0.9−1.3	0.3−2.1	0.1−1.8	ND[b]	ND[b]
Barbarea vulgaris ssp. *arcuata* type P	5	2.3−3.6	0−0.3	0.7−1.0	0.5−3.1	ND[b]
Barbarea intermedia	3	1.9−2.6	ND[b]	0−0.2	ND[b]	ND[b]
Barbarea stricta	3	1.2−9.3	0.4−3.1	0.1−0.4	ND[b]	ND[b]
Barbarea verna	1	4.9	1.3	0.6	ND[b]	ND[b]
Brassica oleracea	1	0.6	1.1	0.7	ND[b]	0.06
Brassica napus	3	0.6−1.1	0.8−1.6	0.7−1.8	ND[b]	0.07−0.13
Brassica rapa	1	0.5	1.8	0.3	ND[b]	ND[b]
Brassica juncea	1	0.1	0.4	0.04	ND[b]	0.05
Brassica nigra	1	0.2	0.2	0.2	ND[b]	0.08
Brassica carinata	1	0.5	0.9	0.4	ND[b]	0.4
Arabidopsis thaliana, roots	1	0.2	3.4	0.1	ND[b]	ND[b]
A. thaliana, siliques	3	2.4−2.9	ND[b]	ND[b]	ND[b]	0.07−0.11

[a] *N* is the number of independent samples analyzed, the observed range of concentrations is reported. [b] ND = not detected; the limit of detection was around 0.02 mmol/gram dry weight

UV spectrum of **d4** was characteristic, especially the distinct band at 301 nm and the local minimum at 280 nm. The combination of the UV spectrum and the longer retention time on C18 HPLC columns than **d2** allows for a high degree of certainty in the routine identification of **4** by sulfatase treatment of anionic metabolites followed by diode-array HPLC. HPLC response factors at 229 nm (*37*) of the four previously tested desulfo indole glucosinolates seem to depend mainly on the position of the UV band at ca. 220 nm. Based on the similarity of **d2** and **d4** in this respect, we assumed that an approximately similar HPLC response factor at 229 nm was valid for **d2** and **d4**.

Occurrence. Hypothetically, **4** could occur as a side product in plant tissues in which both **2** and **3** are biosynthesized. To test this hypothesis, the content of indole glucosinolates was determined in roots and siliques of *A. thaliana*, in roots of several *Barbarea* species, and in roots of the six *Brassica* species known as "U's triangle". We concentrated our efforts on roots as **4** was not found in foliage or seeds of *B. vulgaris* ssp. *arcuata* type P and as *Brassica* roots were known to contain both **2** and **3** (*11*, *15*).

Whereas **1**, **2**, and **3** were of widespread occurrence in *Barbarea* and **1**, **2**, **3**, and **5** in *A. thaliana* and *Brassica*, **4** was only detected in *Barbarea vulgaris* ssp. *arcuata* type P (Table 2). The new indole glucosinolate seemed to be of restricted taxonomic occurrence and is possibly accumulated in the P-type because of an evolutionarily recent mutation in a gene responsible for the biosynthesis of **2** or **3** in related species. In support of this hypothesis, the content of **2** was relatively low in the plants in which **4** occurred. If accumulation of indole phytoalexins can be induced in roots of *Barbarea vulgaris* ssp. *arcuata*, it would be interesting to test whether the difference in indole glucosinolate substitution between the P- and G-type is correlated with a difference in the substitution pattern of the phytoalexins. This might shed light on the possible involvement of substituted indole glucosinolates in the biosynthesis of substituted indole phytoalexins.

Regarding indole glucosinolates in *A. thaliana* and *Brassica* roots, our results are in general agreement with those of other authors (*11*, *15*, *19*, *38*). Our identification of **5** in *A. thaliana* and of **4** in *B. vulgaris* ssp. *arcuata* type P can be attributed to the investigation of new genotypes and plant organs.

ABBREVIATIONS USED

NOE, nuclear overhauser effect; COSY, correlated spectroscopy (2D $^1H-^1H$ chemical shift correlation); HSQC, heteronuclear single quantum coherence (one-bond 2D $^{13}C-^1H$ chemical shift correlation); HMBC, heteronuclear multiple bond correlation (multiple-bond 2D $^{13}C-^1H$ chemical shift correlation); NOESY, nuclear Overhauser effect spectroscop**y** (2D $^1H-^1H$ correlation via NOE).

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