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New Resistance-Correlated Saponins from the Insect-Resistant Crucifer *Barbarea vulgaris*

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Isolation and characterization of plant constituents responsible for insect resistance are of the utmost importance for better understanding of insect−host plant interactions, for selection and breeding of resistant plant varieties, and for development of natural insecticides to be used in future sustainable agriculture and food production. In this study, 3-*O*-cellobiosyl-cochalic acid (**1**), 3-*O*-cellobiosyl-gypsogenin (**3**), and 3-*O*-cellobiosyl-4-epihederagenin (**4**) were isolated from the glabrous type of *Barbarea vulgaris* var. *arcuata* exhibiting resistance to the flea beetle *Phyllotreta nemorum*. In addition to the new constituents, 3-*O*-cellobiosyl-hederagenin (**2**), a known insect repellant, was identified. The structures were established by one- and/or two-dimensional homo- and heteronuclear NMR experiments acquired at 800 MHz and by fragmentation and high-resolution mass spectrometric analysis. Compounds **1**, **3**, and **4** are glycosides of cochalic acid, gypsogenin, and 4-epihederagenin, respectively, none of which have previously been identified in Brassicaceae. Compounds **3** and **4** have both recently been targeted as unidentified constituents exhibiting correlation with *P. nemorum* resistance, but this is the first report of their structures.

KEYWORDS: ***Barbarea vulgaris*; cochalic acid; hederagenin; 4-epihederagenin; gypsogenin; wintercress**

INTRODUCTION

Modern agriculture and food production have become successes in terms of providing a surplus of food and feed in developed countries. However, this relies partly on the development and extensive use of synthetic chemicals for pest management, and there are increasing concerns about the impact of these pesticides on human health and the environment (*1*). This has led to restrictions in the use of conventional organophosphate and carbamate insecticides, for example, in the United States (Food Quality Protection Act of 1996), and there is an increasing awareness of the potential of natural pesticides or biopesticides for use in organic as well as conventional agriculture (*2*). Natural insecticides encompass plant-derived natural products used as insect repellants, antifeedants, growth inhibitors, mating disruptors, etc., many of which are both nontoxic to mammals and nonpersistent in aqueous and soil environments (*3*). Another approach is the biotechnological engineering of plants to express genes involved in the biosynthesis of the constituents causing insect resistance (*4*). Whatever the objective, a deeper understanding of the constituents responsible for and the mechanisms involved in insect resistance is desirable.

Barbarea vulgaris R. Br. (Brassicaceae) is a small perennial herb commonly known as bitter wintercress or yellow rocket (*5*). It prefers moist habitats, is natively distributed in Europe, and has been naturalized in North America, Africa, Australia, and Japan (*6*). The plant has traditionally been used as a medicine against scurvy and cough and as a diuretic and an edible plant. As with other Brassicaceae, *B. vulgaris* is bitter due to the glucosinolates present, namely, 2-hydroxy-2-(4-hydroxyphenyl)ethyl glucosinolate, 2-hydroxy-2-phenylethyl glucosinolate, 2-phenylethyl glucosinolate (*5*), and 1,4-dimethoxyglucobrassicin (*7*). *B. vulgaris* var. *arcuata* can be divided into two genetically, morphologically, and chemically different types: the pubescent type (P-type) with more than 20 hairs along the basal fourth of the leaf margin, starting from the petiole, and pubescent leaf surface and the glabrous type (G-type) with less than 10 hairs along the basal fourth of the leaf margin and glabrous to glabrate leaf surface (*8*, *9*). The P-type is susceptible to herbivores like the flea beetle, *Phyllotreta nemorum*, and the diamondback moth, *Plutella xylostella*, the latter being a significant pest on Brassicaceous crops (*10*), while the G-type is resistant to herbivore attack from *P. xylostella* and most common genotypes of *P. nemorum* (*5*). It has previously been shown that there is no correlation between the content of the above-mentioned glucosinolates and the resistance against *P. nemorum* (*5*). In contrast, two triterpenoid β-amyrin saponins, that is, 3-*O*-cellobiosyl-hederagenin (**2**) (*11*) and 3-*O*-cellobiosyl-oleanoic acid (**5**) (*8*), have been isolated from *B. vulgaris* and found to be, at least partly, responsible for the resistance against *P. xylostella*. Saponins are a diverse group of steroid or triterpene glycosides, and they are widely distributed in the plant kingdom. Numerous pharmacological and biological activities have been reported, including hemolytic, antifungal, antibacterial, antiviral, piscicidal, molluscicidal, insecticidal, and antifeedant activities (*12*). In a recent study, an ecometabolomic approach was used for global metabolite analysis of the P- and G-type of *B. vulgaris*, as well as their F1 and F2 offspring (*6*). Methanolic extracts were analyzed by liquid chromatography

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coupled with mass spectrometry (LC-MS), and principal component analysis of the data showed that **2** and **5** correlated with resistance against *P. nemorum*. However, two unidentified compounds showed higher linear correlation with the insect resistance than these constituents. LC-MS data suggested these constituents to be triterpenoid saponins based on the sodiated adducts of mass-to-charge ratios (*m*/*z*) 817 and 819, respectively, as well as fragment patterns. In this study, we performed a targeted isolation of these insect repellants and elucidated their structures.

MATERIALS AND METHODS

General Experimental Procedures. Pressurized liquid extraction (PLE) was performed with a Dionex ASE-200 Accelerated Solvent Extractor (Dionex, Sunnyvale, CA). All LC-MS analyses for monitoring extraction efficiency and isolation progress were performed on a system consisting of a Waters 2795 separation module (Waters, Milford, MA) hyphenated with a Micromass LCT time-of-flight (TOF) mass spectrometer (MS) (Micromass, Manchester, United Kingdom). The system was equipped with an electrospray ionization (ESI) interface, and analyses were performed in positive ion mode. Exact mass measurements and collision-induced fragmentation were performed on an Ultima Global quadrupole/orthogonal acceleration time-of-flight mass spectrometer (QTOF-MS-MS) (Waters Micromass). The instrument was equipped with an ESI source operated in both negative and positive ion mode, and analyses were performed by syringe infusion of purified compounds. ^{1}H, ^{13}C, and two-dimensional (2D) NMR spectra of isolated compounds were recorded using a Bruker Avance III NMR spectrometer (^{1}H and ^{13}C resonance frequencies 799.96 and 201.12 MHz, respectively) equipped with a 5 mm ^{1}H observe TCI cryoprobe operated at 298.15 K (Bruker, Fällanden, Switzerland). ^{1}H spectra and homonuclear experiments were calibrated using tetramethylsilane as the internal standard, whereas heteronuclear experiments were referenced to methanol-d_4 at δ_H 3.31 and δ_C 49.0, respectively. Preparative-scale isolation was performed with a Waters high-performance liquid chromatography (HPLC) system consisting of a 2525 binary gradient module equipped with a column fluidic organizer, a 2996 PDA detector, a UV fraction manager, and a 2767 sample manager (Waters). Analytical-scale isolation was performed with an Agilent 1200 HPLC system consisting of a quaternary pump, an autosampler, a sample collector, and a photodiode array detector (Agilent, Santa Clara, CA).

LC-MS and High Resolution−Mass Spectrometry (HR-MS) Measurements. LC-MS was performed on the above-mentioned system, and the column used was a 100 mm × 2.1 mm i.d., 4 μm, Synergi Fusion-RP (Phenomenex, Torrance, CA) thermostated at 30 °C. Separations were performed with positive ion mode monitoring using 0.1% formic acid in 50 μM aqueous sodium chloride (eluent A) and 0.1% formic acid in 80% aqueous acetonitrile (v/v) (eluent B) for the following linear gradient profile: 0 min, 18% B; 3 min, 18% B; 60 min, 80% B; 65 min, 100% B; 70 min, 100% B; 71 min, 18% B; and 85 min, 18% B. The MS was operated at default settings, and external calibration was performed with 2 mM sodium hydroxide:0.02% formic acid (1:1, v/v). The calibration was performed in the range of *m*/*z* 200−1000 units with a fifth order polynomial fit using 12 sodium formate clusters. HR-MS and fragmentations were performed on the above-mentioned QTOF-MS-MS using default settings. The mass spectrometer was operated in TOF scan mode (*m*/*z* 100−1000) for exact mass measurement, where the quadropole served as an ion beam focusing device (RF only) and in MS-MS mode for fragmentation. The collision gas was argon, and reported collisions were performed at 50 eV. External calibration was performed as described above.

Plant Material. Seeds from the G-type of *B. vulgaris* R. Br. var. *arcuata* (Opiz.) Simkovics (Brassicaceae) were collected by Dr. Jens Kvist Nielsen in Herlev, Zealand, Denmark, in 1999. Leaves from three batches grown in climate chamber or indoor between January and June 2007 were pooled, yielding 332 g of fresh plant material. The leaves were freeze-dried, and petioles were removed. The plant material was powdered and subsequently stored at −80 °C. A voucher specimen (accession number B44) was deposited in Herbarium C (Botanical Museum, University of Copenhagen, Copenhagen, Denmark) (*8*).

Extraction and Sample Prepurification. Pilot-scale PLE experiments were performed at 40, 70, and 100 °C, and maximum extraction efficiency with unaltered analyte pattern, as evaluated by visual inspection of LC-MS chromatograms, was obtained at 70 °C. Parallel extractions of a total of 60 g of plant material mixed with 0.2 L of SpeedMatrix were performed with 70% aqueous methanol in 12 33 mL extraction cells plugged with glassfiber (pressure, 1500 psi; temperature, 70 °C; preheat, 0 min; static, 5 min; flush volume, 50%; purge time, 120 s; and static cycles, 2). Extracts were pooled, concentrated in vacuo at 40 °C, and freeze-dried to give 11.7 g of raw extract, which was redissolved in 960 mL of 30% aqueous methanol. Pilot-scale solid-phase extraction (SPE) was performed by applying redissolved extract equivalent to ∼4 mg of dry matter on StrataX (0.2 g), Oasis HLB (1 g), BondElut C8 (0.3 g), and BondElut C18 (0.3 g) cartridges, eluting with 40, 50, 60, 70, 80, 90, and 100% methanol and monitoring the eluate by LC-MS. The saponins were eluted in the narrowest band and at the lowest solvent strength with BondElut C18 cartridges, and the remainder of the extract was prepurified on SPE-cartridges containing 10 g of MegaBond Elut C18 material. Thus, portions of 60 mL were applied to conditioned (50 mL of methanol, 50 mL of Milli-Q water, and 50 mL of 30% methanol) cartridges, washed with 50 mL of 40% methanol, and eluted with 50 mL of 80% methanol. The eluent was concentrated in vacuo at 40−60 °C and freeze-dried to obtain 1.9 g of saponin-enriched fraction.

Isolation. The saponin-enriched fraction was dissolved in 10 mL of dimethylsulfoxide for semipreparative HPLC, and the column used was a 100 mm × 21.2 mm i.d., 4 μm, Synergi Fusion-RP (Phenomenex). The separation was performed with 0.1% aqueous formic acid (eluent A) and 80% aqueous acetonitrile acidified with 0.1% formic acid (eluent B) using the following linear gradient elution profile: 0 min, 15% B; 55 min, 55% B; 56 min, 100% B; 59.5 min, 100% B; 60.5 min, 15% B; and 65 min, 15% B. The flow rate was 30 mL/min, and 18 separations (injection of 500 μL each) were collected in 30 s time slots from 22 to 59 min. All fractions were monitored by LC-MS, and selection of fractions based on positive-mode signals at *m*/*z* 817 and *m*/*z* 819 afforded 5.7 mg of **1** (fraction 40) and 9.6, 31.2, and 29.6 mg of **2** (fraction 46, 47, and 48, respectively). Fraction 53 (3 mg) was subjected to analytical-scale HPLC. The column used was a 150 mm × 4.6 mm i.d., 3 μm, Luna C_{18}(2) (Phenomenex) using isocratic elution with 63.5% methanol acidified with 0.1% formic acid at a flow rate of 0.8 mL/min. This afforded submilligram quantities of **3** (apex at 15.8 min) and **4** (22.4 min).

Compound ***1***. 3-*O*-Cellobiosyl-cochalic acid (synonym: 3-*β*-[*O*-*β*-D-glucopyranosyl-(1→4)-*β*-D-glucopyranosyloxy]-16-*β*-hydroxyolean-12-en-28-oic acid or 3-*O*-[*O*-*β*-D-glucopyranosyl-(1→4)-*β*-D-glucopyranosyl]-cochalic acid). ^{1}H NMR (methanol-d_4, 800 MHz): See **Table 1**. ^{13}C NMR (methanol-d_4, 200 MHz): See **Table 1**. ESI-QTOF-MS-MS (positive mode) *m*/*z* (%): 819 $[M + Na]^+$ (100), 801 $[M - H_2O + Na]^+$ (45), 775 $[M - CO_2 + Na]^+$ (5), 757 $[M - CO_2 - H_2O + Na]^+$ (50), 657 $[M - C_6H_{10}O_5 + Na]^+$ (< 5), 639 $[M - C_6H_{12}O_6 + Na]^+$ (< 5), 595 $[M - C_6H_{12}O_6 - CO_2 + Na]^+$ (< 5), 365 $[C_{12}H_{22}O_{11} + Na]^+$ (10), 347 $[C_{12}H_{20}O_{10} + Na]^+$ (5), 203 $[C_6H_{12}O_6 + Na]^+$ (< 5), 185 $[C_6H_{10}O_5 + Na]^+$ (< 5), (negative mode) 795 $[M - H]^-$ (100), 633 $[M - C_6H_{10}O_5 - H]^-$ (85), 615 $[M - C_6H_{12}O_6 - H]^-$ (40), 471 $[M - C_{12}H_{20}O_{10} - H]^-$ (35), 245 $[C_{16}H_{23}O_3 - H_2O]^-$ (< 5). HR-ESI-QTOF-MS *m*/*z* 795.4504 $[M - H]^-$ (calcd for $[C_{42}H_{67}O_{14}]^-$, 795.4536), 819.4532 $[M + Na]^+$ (calcd for $[C_{42}H_{68}O_{14}Na]^+$, 819.4501).

Compound ***2***. 3-*O*-Cellobiosyl-hederagenin (synonym: 3-*β*-[*O*-*β*-D-glucopyranosyl-(1→4)-*β*-D-glucopyranosyloxy]-23-hydroxyolean-12-en-228-oic acid or 3-*O*-[*O*-*β*-D-glucopyranosyl-(1→4)-*β*-D-glucopyranosyl]-hederagenin). ^{1}H NMR (methanol-d_4, 800 MHz): See the Supporting Information. ^{13}C NMR (methanol-d_4, 200 MHz): See the Supporting Information. ESI-QTOF-MS-MS (positive mode) *m*/*z* (%): 819 $[M + Na]^+$ (100), 801 $[M - H_2O + Na]^+$ (< 5), 775 $[M - CO_2 + Na]^+$ (10), 757 $[M - CO_2 - H_2O + Na]^+$ (< 5), 657 $[M - C_6H_{10}O_5 + Na]^+$ (< 5), 639 $[M - C_6H_{12}O_6 + Na]^+$ (< 5), 365 $[C_{12}H_{22}O_{11} + Na]^+$ (5), 347 $[C_{12}H_{20}O_{10} + Na]^+$ (5), 203 $[C_6H_{12}O_6 + Na]^+$ (< 5), 185 $[C_6H_{10}O_5 + Na]^+$ (< 5), (negative mode): 795 $[M - H]^-$ (15), 633 $[M - C_6H_{10}O_5 - H]^-$ (45), 615 $[M - C_6H_{12}O_6 - H]^-$ (35), 471 $[M - C_{12}H_{20}O_{10} - H]^-$ (100). HR-ESI-QTOF-MS *m*/*z* 795.4496 $[M - H]^-$ (calcd for $[C_{42}H_{67}O_{14}]^-$, 795.4536), 819.4532 $[M + Na]^+$ (calcd for $[C_{42}H_{68}O_{14}Na]^+$, 819.4501).

Compound ***3***. 3-*O*-Cellobiosyl-gypsogenin (synonym: 3-*β*-[*O*-*β*-D-glucopyranosyl-(1→4)-*β*-D-glucopyranosyloxy]-23-oxo-olean-12-en-28-oic acid or 3-*O*-[*O*-*β*-D-glucopyranosyl-(1→4)-*β*-D-glucopyranosyl]-gypsogenin). ^{1}H NMR (methanol-d_4, 800 MHz) *δ*: 0.87 (3H, s, H-26), 0.88 (3H, s, H-29), 0.95 (3H, s, H-30), 1.00 (3H, s, H-25), 1.11

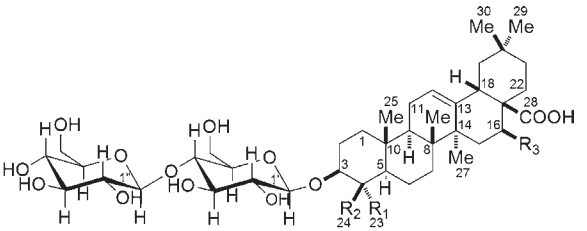
Table 1. NMR Spectroscopic Data of **1**

position	$^{13}C^{a}$	$^{1}H^{a,b}$	NOESY^{a,c,d}	HMBC^{a,c,e}
1	39.5	ax: 0.98 (m)	1eq, 2eq, 3, 9, 11α	9, 10
		eq: 1.62 (dt, $J_{1eq,1ax}$ = 13.2, $J_{1eq,2ax}$ = $J_{1eq,2eq}$ = 3.5)	1ax, 2ax, 2eq, 11α, 25	3, 5, 10, 25
2	26.8	ax: 1.69 (qd, $J_{2ax,1ax} \approx J_{2ax,2eq} \approx J_{2ax,3} \approx$ 12.0, $J_{2ax,1eq}$ = 3.5)	1eq, 2eq, 24, 25	3
		eq: 1.92 (m)	1ax, 1eq, 2ax, 3, 1′	3
3	90.5	3.16 (dd, $J_{3,2ax}$ = 11.9, $J_{3,2eq}$ = 4.4)	1ax, 2eq, 5, 1′	4, 23, 24, 1′
4	39.8			
5	56.8	0.78 (dd, $J_{5,6ax}$ = 11.9, $J_{5,6eq}$ = 1.2)	3, 7ax, 9, 23, 27	3, 4, 6, 9, 10, 23, 24, 25
6	19.1	ax: 1.41 (m)	6eq, 26	
		eq: 1.56 (m)	6ax, 7eq, 23	
7	34.0	ax: 1.52 (m)	5, 7eq, 27	6, 8
		eq: 1.34 (m)	6eq, 7ax	5, 6
8	40.2			
9	48.1	ax: 1.53 (m)	1ax, 5, 11α, 27	5, 8, 10, 11, 26
10	37.5			
11	24.3	α: 1.85 (dm, $J_{11\alpha,11\beta}$ = 18.5)	1ax, 1eq, 9, 12	9, 12, 13
		β: 1.91 (m)	12, 25, 26	9, 12, 13
12	122.6	5.26 (t, $J_{12,11\alpha}$ = $J_{12,11\beta}$ = 3.5)	11α, 11β, 18, 19eq, 30	9, 11, 18
13	144.7			
14	44.7			
15	38.7	ax: 1.91 (m)	15eq, 26	16, 27
		eq: 1.36 (dd, $J_{15eq,15ax}$ = 13.6, $J_{15eq,16}$ = 4.3)	15ax, 16	13, 14, 16, 27
16	66.0	4.07 (dd, $J_{16,15ax}$ = 11.6, $J_{16,15eq}$ = 4.3)	15eq, 19ax, 21ax, 22eq, 27	
17	50.9			
18	44.6	3.03 (dd, $J_{18,19ax}$ = 14.0, $J_{18,19eq}$ = 4.5)	12, 19eq, 22ax, 30	12, 13, 16, 19
19	47.3	ax: 1.65 (t, $J_{19ax,19eq}$ = $J_{19ax,18}$ = 14.0)	16, 19eq, 21ax, 27	13, 18, 20, 29, 30
		eq: 1.08 (m)	12, 18, 19ax, 29, 30	
20	31.3			
21	34.7	ax: 1.43 (m)	16, 19ax, 21eq, 22eq, 29	22
		eq: 1.17 (m)	21ax, 29, 30	
22	28.3	ax: 1.53 (m)	18, 22eq	18
		eq: 2.19 (br dt, $J_{22eq,22ax}$ = 13.9, $J_{22eq,21ax} \approx J_{22eq,21eq} \approx$ 3.0)	16, 22ax	28
23	28.2	1.05 (s)	5, 6eq, 1′	5, 24
24	16.8	0.85 (s)	2ax, 25	3, 4, 5, 23
25	15.7	0.95 (s)	1eq, 2ax, 11β, 24, 26	1, 5, 9, 10
26	17.9	0.89 (s)	6ax, 11β,15ax, 25	8
27	26.9	1.19 (s)	5, 7ax, 9, 16, 19ax	8, 13, 14, 15
28	179.3			
29	33.5	0.89 (s)	19eq, 21ax, 21eq	19, 20, 21, 30
30	24.1	0.97 (s)	12, 18, 19eq, 21eq	19, 20, 21, 29
1′	106.1	4.34 (d, $J_{1',2'}$ = 7.9)	2eq, 3, 23, 3′, 5′	3′
2′	75.0	3.25 (dd, $J_{2',3'}$ = 9.1, $J_{2',1'}$ = 7.9)	4′	1′, 3′
3′	76.3	3.49 (t, $J_{3',4'}$ = $J_{3',2'}$ = 9.1)	1′, 5′, 1″	2′, 4′
4′	80.3	3.56 (t, $J_{4',3'}$ = $J_{4',5'}$ = 9.1)	2′, 6′, 1″, 2″	3′, 5′, 6′, 1″
5′	75.9	3.37 (m)	1′, 3′, 6′	
6′	61.6	3.85 (m)	4′, 5′, 1″, 2″	4′, 5′
1″	104.2	4.41 (d, $J_{1',2'}$ = 7.9)	3′, 4′, 6′, 3″, 5″	4′, 3″
2″	74.6	3.21 (dd, $J_{2'',3''}$ = 9.1, $J_{2'',1''}$ = 7.9)	4′, 6′	1″, 3″, 4″
3″	77.5	3.36 (t, $J_{3'',4''}$ = $J_{3'',2''}$ = 9.1)	1″	2″, 4″
4″	71.0	3.29 (t, $J_{4'',3''}$ = $J_{4'',5''}$ = 9.1)[f]		3″, 5″, 6″
5″	77.8	3.33 (m)	1″, 6″A, 6″B	4″
6″	62.1	A: 3.65 (dd, $J_{6''A,6''B}$ = 11.9, $J_{6''A,5''}$ = 5.8)	6″B	4″, 5″
		B: 3.87 (dd, $J_{6''A,6''B}$ = 11.9, $J_{6''B,5''}$ = 2.2)	5″, 6″A	4″, 5″

[a] ^{1}H (800 MHz) and ^{13}C (200 MHz) NMR spectroscopic data were measured in methanol-d_4, δ values relative to internal TMS. [b] Multiplicity of signals is given in parentheses: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; and br, broad; coupling constants (apparent splittings) are reported as numerical values in Hz. [c] Signal correlating with ^{1}H resonance. [d] Mixing time, 600 ms. [e] Optimized for $^{n}J_{C,H}$ = 7.7 Hz. [f] Identified from the HSQC spectrum.

(3H, s, H-24), 1.16 (3H, s, H-27), 0.85−2.0 (m, H-1-H-2, H-5-H-22), 2.91 (1H, br d, $J_{18,19ax} \approx$ 13.8 Hz, H-18), 3.12−3.65 (m, H-2′-H-5′, H-2″-H-5″), 3.65 (1H, dd, $J_{6''A,6''B}$ = 11.9 Hz, $J_{6''A,5''}$ = 5.7 Hz, H-6″A), 3.85 (2H, m, H-6′), 3.86 (1H, dd, $J_{6''A,6''B}$ = 11.9 Hz, $J_{6''B,5''}$ = 2.0 Hz, H-6″B), 3.89 (1H, dd, $J_{3ax,2ax}$ = 12.0 Hz, $J_{3ax,2eq}$ = 4.6 Hz, H-3ax), 4.21 (1H, d, $J_{1',2'}$ = 7.9 Hz, H-1′), 4.39 (1H, d, $J_{1'',2''}$ = 7.9 Hz, H-1″), 5.22 (1H, t, $J_{12,11\alpha}$ = $J_{12,11\beta}$ = 3.5 Hz, H-12), 9.41 (1H, s, H-23). ESI-QTOF-MS-MS (positive mode) *m*/*z* (%): 817 $[M + Na]^+$ (100), 773 $[M - CO_2 + Na]^+$ (5), 757 $[M - CO_2 - H_2O + Na]^+$ (< 5), 655 $[M - C_6H_{10}O_5 + Na]^+$ (< 5), 637 $[M - C_6H_{12}O_6 + Na]^+$ (< 5), 365 $[C_{12}H_{22}O_{11} + Na]^+$ (5), 347 $[C_{12}H_{20}O_{10} + Na]^+$ (5), 203 $[C_6H_{12}O_6 + Na]^+$ (< 5), 185 $[C_6H_{10}O_5 + Na]^+$ (< 5), (negative mode) 795 $[M - H]^-$ (< 5), 631 $[M - C_6H_{10}O_5 - H]^-$ (100), 613 $[M - C_6H_{12}O_6 - H]^-$ (50), 469 $[M - C_{12}H_{20}O_{10} - H]^-$ (90). HR-ESI-QTOF-MS *m*/*z* 793.4377 $[M - H]^-$ (calcd for $[C_{42}H_{65}O_{14}]^-$, 793.4380), 817.4327 $[M + Na]^+$ (calcd for $[C_{42}H_{66}O_{14}Na]^+$, 817.4345).

Compound ***4***. 3-*O*-Cellobiosyl-4-epihederagenin (synonym: 3-*β*-[*O*-*β*-D-glucopyranosyl-(1→4)-*β*-D-glucopyranosyloxy]-24-hydroxyolean-12-en-28-oic acid or 3-*O*-[*O*-*β*-D-glucopyranosyl-(1→4)-*β*-D-glucopyranosyl]-4-epihederagenin). ^{1}H NMR (methanol-d_4, 800 MHz) δ: 0.85 (3H, s, H-26),



1: $R_1 = R_2 = CH_3$ $R_3 = OH$
2: $R_1 = CH_2OH$ $R_2 = CH_3$ $R_3 = H$
3: $R_1 = CHO$ $R_2 = CH_3$ $R_3 = H$
4: $R_1 = CH_3$ $R_2 = CH_2OH$ $R_3 = H$
5: $R_1 = R_2 = CH_3$ $R_3 = H$

Figure 1. Structures of triterpenoid saponins **1**−**4** from *B. vulgaris* var. *arcuata*.

0.87 (3H, s, H-25), 0.88 (3H, s, H-29), 0.93 (1H, br d, $J_{5ax,6ax}$ = 12.0 Hz, H-5ax), 0.95 (1H, s, H-30), 0.96 (1H, m, H-1eq), 1.20 (3H, s, H-23), 1.14 (3H, s, H-27), 1.36 (1H, m, H-6ax), 1.57 (1H, m, H-9), 1.63 (1H, m, H-1ax), 1.65 (1H, m, H-19ax), 1.80−1.92 (2H, m, H-11α, H11-β), 1.94 (1H, m, H-16ax), 2.90 (1H, br d, $J_{18,19ax}$ = 13.6 Hz, H-18), 3.20 (1H, m, H-3ax), 3.21 (1H, m, H-2′), 3.22 (1H, m, H-2″), 3.30 (1H, m, H-24A), 3.30 (1H, m, H-4″), 3.31 (1H, m, H-5″), 3.35 (1H, m, H-3″), 3.36 (1H, br t, $J_{4',5'}$ = 9.6 Hz, H-5′), 3.50 (1H, m, H-3′), 3.57 (1H, m, H-4′), 3.65 (1H, dd, $J_{6''A,6''B}$ = 11.2 Hz, $J_{5'',6''A}$ = 5.6 Hz, H-6″A), 3.86 (2H, m, H-6′), 3.88 (1H, dd, $J_{5'',6''B}$ = 2.4 Hz, $J_{6''A,6''B}$ = 11.2 Hz, H-6″B), 4.02 (1H, d, $J_{24A,24X}$ = 12.0 Hz, H-24X), 4.41 (1H, $J_{1'',2''}$ = 8.0 Hz, H-1′), 4.43 (1H, d, $J_{1',2'}$ = 8.0 Hz, H-1′), 5.21 (1H, t, $J_{12,11\alpha} = J_{12,11\beta}$ = 3.5 Hz, H-12). ^{13}C NMR (methanol-d_4, 200 MHz) δ: 15.5 (C-25), 17.8 (C-28), 23.1 (C-23), 23.9 (C-30), 26.1 (C-27), 28.9 (C-15), 31.4 (C-20), 33.7 (C-29), 35.1 (C-21), 37.4 (C-10), 39.4 (C-1), 40.2 (C-8), 42.4 (C-14), 44.6 (C-4), 47.9 (C-19), 48.7 (C-9), 57.2 (C-5), 61.7 (C-6′), 63.8 (C-24), 90.3 (C-3), 146.1 (C-13). ESI-QTOF-MS-MS (positive mode) *m*/*z* (%): 819 $[M + Na]^+$ (100), 775 $[M - CO_2 + Na]^+$ (5), 759 $[M - CO_2 - H_2O + Na]^+$ (< 5), 657 $[M - C_6H_{10}O_5 + Na]^+$ (< 5), 365 $[C_{12}H_{22}O_{11} + Na]^+$ (10), 347 $[C_{12}H_{20}O_{10} + Na]^+$ (5), 203 $[C_6H_{12}O_6 + Na]^+$ (< 5), 185 $[C_6H_{10}O_5 + Na]^+$ (< 5), (negative mode) 795 $[M - H]^-$ (< 5), 633 $[M - C_6H_{10}O_5 - H]^-$ (50), 615 $[M - C_6H_{12}O_6 - H]^-$ (30), 471 $[M - C_{12}H_{20}O_{10} - H]^-$ (100). HR-ESI-QTOF-MS *m*/*z* 795.4496 $[M - H]^-$ (calcd for $[C_{42}H_{67}O_{14}]^-$, 795.4536), 819.4509 $[M + Na]^+$ (calcd for $[C_{42}H_{68}O_{14}Na]^+$, 819.4501).

RESULTS AND DISCUSSION

Dried and pulverized leaves of *B. vulgaris* were subjected to PLE. On the basis of pilot PLE experiments monitored by LC-MS, 70% methanol extractions of 60 g of plant material were performed at 70 °C, yielding 11.7 g of raw extract. The extract was redissolved in 30% methanol and subjected to C_{18} SPE. This yielded 1.9 g of a saponin-enriched sample, which after fractionation by preparative-scale HPLC and targeted isolation of fractions with *m*/*z* of 817 or 819 gave 5.7 mg of **1**, 70.4 mg of **2**, and 3 mg of a mixture. The latter was separated by analytical-scale HPLC to give submilligram amounts of **3** and **4** (**Figure 1**). Retention times and mass spectra from LC-MS analyses of **3** and **4** were compared with those of extract of the G-type of *B. vulgaris* var. *arcuata*, and this rendered **3** and **4** identical with unknown 2 and 1, respectively, possessing the highest linear correlation to resistance against *P. nemorum* (*6*). Thus, retention time differences between **3** and **4** as pure compounds and as constituents in the crude extract were less than 0.1 min, and similarly, the mass spectra of **3** (base peak *m*/*z* 817 and aglycone *m*/*z* 453) and **4** (base peak *m*/*z* 819 and aglycone *m*/*z* 455) were practically identical for isolated material and in crude extract.

Compound **1** was assigned the molecular formula $C_{42}H_{68}O_{14}$ based on results from HR-MS. Two β-D-glucopyranosyl units were identified by correlations in the correlation spectroscopy (COSY) spectrum, starting from the characteristic resonances of the anomeric hydrogens H1′ (δ_H 4.34, d, $J_{H1',H2'}$ = 7.9 Hz) and H1″ (δ_H 4.41, d, $J_{H1'',H2''}$ = 7.9 Hz), respectively, and throughout the two glucose moieties. The β(1″→4′) glucosidic linkage was identified from the downfield shift of C-4′ (δ_C 80.3) in addition to correlations in the heteronuclear multiple bond correlation (HMBC) spectrum from H-1″ and H-4′ to C-4′ and C-1″, respectively. The remainder of the resonances originated from the aglycone, and seven methyl singlets were observed in addition to resonances for two oxygenated methines (δ_H 3.16, dd, $J_{H3,H2ax}$ = 11.9 Hz, $J_{H3,H2eq}$ = 4.4 Hz, H-3, δ_C 90.5, C-3 and δ_H 4.07, dd, $J_{H16,H15ax}$ = 11.6 Hz, $J_{H16,H15eq}$ = 4.3 Hz, H-16, δ_C 66.0, C-16), an alkenic resonance (δ_H 5.26, t, $J_{H12,H11\alpha} = J_{H12,H11\beta}$ = 3.5 Hz, H-12, δ_C 122.6, C-12) and a resonance for a carboxylic acid (δ_C 179.2, C-28). These observations were in agreement with an oleanane type triterpene, and full assignment of all ^{1}H and ^{13}C NMR resonances based on COSY, nuclear Overhauser effect spectroscopy (NOESY), heteronuclear single quantum coherence (HSQC), and HMBC experiments acquired at 800 MHz established the aglycone as cochalic acid. **Table 1** summarizes all observed correlations. Selected HMBC and NOESY correlations used for assignment of the structure are presented in **Figure 2**. Thus, the equatorial positions of *O*-3 and *O*-16 were determined based on 1,2-trans diaxial couplings between H-3 and H-2ax ($J_{H3,H2ax}$ = 11.9 Hz) and H-16 and H-15ax ($J_{H16,H15ax}$ = 11.6 Hz), respectively. The equatorial position of *O*-16 was further supported by the resonance position of H-27 (δ_H 1.19), which was not downfield shifted due to 1,3-diaxial interaction with *O*-16 as observed with echinocystic acid ($\delta_H \sim 1.81$) (*13*). Using H-16 as an anchoring point, the nuclear Overhauser effect (NOE) correlation path H-16→H-27→H-9→H-5→H-23 showed the α-position of these groups (**Figure 2**), whereas the NOE correlation path H-24→H-25→H-11β→H-26→H-15ax demonstrated the β-position of these groups. Both the downfield shift of C-3 (δ_C 90.5) and the HMBC correlations from H-3 and H-1′ to C-1′ and C-3, respectively, demonstrated the glucosidic linkage at C-3. Furthermore, the large glucosidation shift of C-3 [$\Delta\delta_A = \delta_{\mathbf{1}} - \delta_{oleanoicacid}$ = 90.5 − 79.8 (*8*) = 10.7] is in agreement with the β-D-configuration of the glucose unit attached to the aglycone (*14*) and consequently also of the second glucose unit of cellobioside. Compound **1**, that is, 3-*O*-cellobiosyl-cochalic acid, is a new compound containing the sapogenin cochalic acid. Cochalic acid was first isolated from *Myrtillocactus cochal* (*15*) but has also been identified in hydrolysate of *Myrtillocactus geometrizans* and *Myrtillocactus eichlamii* (*16*), *Brenania brieyi* (*17*), and *Pachycereus weberi* (*18*). Cochalic acid is the 16-epimer of echinocystic acid of which the latter exhibited a downfield shift of the equatorial positioned H-16 (δ_H 5.25, br s) (*13*) due to diamagnetic anisotropy effects of the C-28 carboxylic group. The slightly broadened signal does not reveal any clear splittings due to the relatively small equatorial−axial couplings. This is contrasted by the axially positioned H-16 (δ_H 4.07, dd, $J_{16,15ax}$ = 11.6 Hz, $J_{16,15eq}$ = 4.3 Hz) of cochalic acid, for which typical axial−axial and axial−equatorial coupling constants are observed. Thus, the unambiguous structure elucidation of **1** performed by full assignment of 1D as well as 2D homo- and

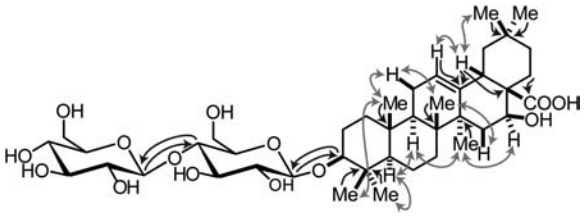


Figure 2. Selected correlations of **1** observed in the HMBC spectrum (black single-headed arrows, H→C) and the NOESY spectrum (gray double-headed arrows).

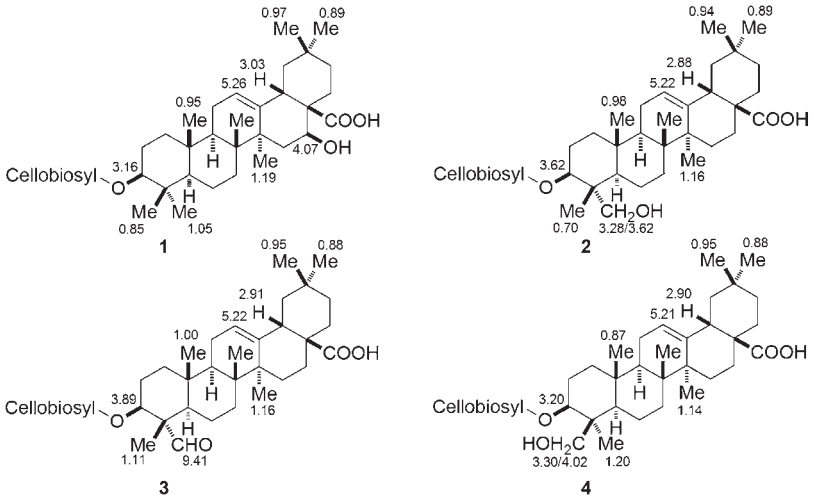


Figure 3. Selected diagnostic ^{1}H NMR chemical shift values of **1**−**4**.

heteronuclear NMR experiments at 800 MHz proves the equatorial β-position of the hydroxyl group at C-16, which previously has been falsely assigned as axial (*19*, *20*). This is the first report of a cochalic acid derivative from Brassicaceae.

Compound **2** was assigned the molecular formula $C_{42}H_{68}O_{14}$ based on results from HR-MS and identified as 3-*O*-cellobiosyl-hederagenin by full assignment of all ^{1}H and ^{13}C NMR resonances from COSY, NOESY, HSQC, and HMBC experiments acquired at 800 MHz. 3-*O*-Cellobiosyl-hederagenin has previously been isolated from *B. vulgaris* by Shinoda et al (*11*), but because fully assigned ^{1}H and ^{13}C NMR data at ultrahigh field strength were not included, these are now reported in this paper (Supporting Information).

Compound **3** was assigned the molecular formula $C_{42}H_{66}O_{14}$ based on results from HR-MS. Collision-induced fragmentation showed sequential loss of two hexose units in negative ion mode {m/z 631 $[M - C_6H_{10}O_5 - H]^-$ (100%) and 469 $[M - C_{12}H_{20}O_{10} - H]^-$ (90%)} as well as a disaccharide moiety in positive ion mode (m/z 363 $[C_{12}H_{22}O_{11} + Na]^+$), which shows that **4** is a monodesmosidic saponin. The ^{1}H spectrum acquired at 800 MHz showed close resemblance with that of **2** for all signals originating from hydrogens on rings B−E. However, the resonance signals observed for H-23A and H-23B in **2** were absent in the spectrum of **3**, and instead, a singlet resonance was observed at δ_H 9.41, which indicated an aldehyde group at the C-23 position. This was further supported by the downfield shifted signal for H-3 (δ_H 3.89, dd, $J_{H3,H2ax}$ = 11.8 Hz, $J_{H3,H2eq}$ = 4.5 Hz), as also observed by a related saponin reported by Nord and Kenne (*21*). The ^{1}H resonance signals of the disaccharide unit were almost identical to those observed for **1** and **2**, and thus, **3** is a new compound containing the gypsogenin skeleton, that is, 3-*O*-cellobiosyl-gypsogenin. A closely related analogue containing a β-D-glucopyranosyl-(1→2)-β-D-glucopyranosyloxy glycoside moiety has previously been isolated from *Fatsia japonica* (Araliaceae) (*22*), but this is the first report of gypsogenin in Brassicaceae.

Compound **4** was assigned the molecular formula $C_{42}H_{68}O_{14}$ based on results from HR-MS, and **4** was identified as a monodesmosidic saponin due to sequential loss of two hexose units (negative ion mode: m/z 633 $[M - C_6H_{10}O_5 - H]^-$ and 471 $[M - C_{12}H_{20}O_{10} - H]^-$) and the observation of a disaccharide moiety (positive ion mode: m/z 365 $[C_{12}H_{22}O_{11} + Na]^+$). The ^{1}H NMR spectrum of **4** acquired at 800 MHz showed high resemblance with that of **2**, suggesting two closely related analogues. Thus, the upfield-shifted ^{1}H resonance singlet observed for H-24 in **2** was absent in the ^{1}H spectrum of **4**. Instead, an upfield shift of H-25 and a downfield shift of H-23 (δ 0.87 and 1.20, respectively) were observed for **4** as compared to **1**. The two diastereotopic hydrogen atoms of H-24 appeared as an AX spin system (δ 3.30/4.02, H-24A/B; J = 12.0 Hz). The relatively large $\Delta\delta$ is in agreement with previously reported ^{1}H NMR data of a β-hydroxymethyl attached to the chiral C-4 center (*23*, *24*). In addition, selected ^{13}C NMR data of the sapogenin of **4** are in agreement with those previously reported for a 4-epihederagenin diglycoside (*24*) and for the 28-methyl ester of 4-epihederagenin (*23*). This was further supported by correlations observed in COSY, NOESY, and HMBC experiments acquired at 800 MHz. The ^{1}H resonance signals of the disaccharide unit were almost identical to those observed with **1** and **2**, and thus, **4** was identified as 3-*O*-cellobiosyl-4-epihederagenin. Compound **4** is a new glycoside of 4-epihederagenin. **Figure 3** shows selected diagnostic ^{1}H NMR chemical shift values used to identify the closely related analogues **1**−**4**. 4-Epihederagenin has previously been isolated from *Lantana indica* (*23*), and a 4-epihederagenin diglycoside was isolated from *Clematis chinensis* (*24*). Zhong and co-workers (*25*) isolated a series of hederagenin glycosides from *Clematis tangutica* but erroneously depicted the structure of 4-epihederagenin. Wu and co-workers (*26*) therefore subsequently claimed the identification of 4-epihederagenin from *Gentiana aristata* incorrectly using data from Zhong et al. (*25*). This is the first report of a 4-epihederagenin derivative from Brassicaceae.

The current work identified the triterpene saponines 3-*O*-cellobiosyl-gypsogenin (**3**) and 3-*O*-cellobiosyl-4-epihederagenin (**4**), which together with 3-*O*-cellobiosyl-hederagenin (**2**) are partly responsible for *P. nemorum* and *P. xylostella* resistance observed for the G-type of *B. vulgaris* var. *arcuata*. This finding helps us understand the mechanism underlying the potential of *B. vulgaris* var. *arcuata* as a "dead-end trap crop" (*27*, *28*), that is, a crop that is highly attractive for oviposition by an insect plant but on which larvae cannot survive. The results presented in this work together with previously published results (*6*, *8*, *11*) show that a series of pentacyclic triterpene saponines with a cellobiosyl glycosidic moiety are among the constituents responsible for the resistance of the plant. Triterpene saponines constitute a wide range of structurally diverse compounds commonly occurring in plants, and a wide range of biological activities have been reported within this diverse group (*12*, *29*, *30*). It is interesting that the reported insect resistance seems to be specifically restricted to oleanoic acid analogues with a 3-*O*-β-cellobiosyl moiety and different degrees of oxidation at C-16, C-23, and C-24. Further studies aiming at delineating the relationship between

P. nemorum and/or *P. xylostella* resistance and a chemically diverse array of saponins related to **1**−**4** are therefore needed, and this might lead to discovery of natural insecticides with potential in organic agriculture.

ABBREVIATIONS USED

P-type, pubescent type; G-type, glabrous type; PLE, pressurized liquid extraction; TOF, time-of-flight; ESI, electrospray ionization; QTOF-MS-MS, quadropole/orthogonal acceleration time-of-flight mass spectrometer; HPLC, high-performance liquid chromatography; SPE, solid-phase extraction; COSY, correlation spectroscopy; NOESY, nuclear Overhauser effect spectroscopy; HSQC, heteronuclear single quantum coherence; HMBC, heteronuclear multiple bond correlation; NOE, nuclear Overhauser effect.

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Supporting Information Available: Table containing ^{1}H and ^{13}C NMR spectroscopic data of **2** acquired at 800 MHz. This material is available free of charge via the Internet at http://pubs.acs.org.

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