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Metabolic Profiling of Lignans and Other Secondary Metabolites from Rapeseed (*Brassica napus* L.)

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ABSTRACT: A metabolic profiling study was carried out on rapeseed (*Brassica napus* L.). Eleven glucosinolates were identified by high-performance liquid chromatography (HPLC) coupled with diode array detection (DAD) and mass spectrometry (MS). Additionally, 18 phenolic compounds were profiled from an ethanol extract of rapeseed. Besides two major phenols, sinapine and methyl sinapate, 16 minor phenolic compounds were isolated and identified. Seven of them are new lignans including three (±)-thomasidioic acid derivatives and four (*E*,*E*)-dienolignan derivatives. The structures of novel phenolic compounds were elucidated by 1D and 2D nuclear magnetic resonance (NMR) spectroscopy and MS. The analytical data of secondary metabolites in rapeseed winter cultivar "Emerald" and information about purification on a microscale are useful for upcoming studies on tissue-specific localization of these compounds.

KEYWORDS: *Brassica napus, dienolignan derivatives, glucosinolates, lignans, rapeseed, sinapates, thomasidioic acid derivatives*

INTRODUCTION

Rapeseed (*Brassica napus* L.) is an important oilseed in temperate regions and contributes up to 15% of the global oleiferous production.[1] Rapeseed is categorized into two forms, on the basis of different seed seasons, as winter cultivars and spring cultivars. Winter cultivars of *B. napus* usually lead to higher seed and oil yields than spring varieties.[2,3] Although mainly used as an oil crop, rapeseed has high protein and essential amino acid contents, which make it both animal feed and a potential human food additive. However, high levels of glucosinolates in rapeseed meal of conventional cultivars limit its nutritional value,[4] because the decomposition products of glucosinolates are goitrogenic and hepatotoxic.[5] Therefore, tremendous efforts were put into breeding low-glucosinolate cultivars,[2,4] which contain only 0.5−1.0% glucosinolates.[5] Phenolic choline esters, mainly sinapate esters, are the other major class of antinutritional compounds in rapeseed, because they are responsible for the bitterness and astringency[6] and can form complexes with amino acids and proteins that cause digestion and absorption problems to mammals.[2,7,8] The predominant sinapate is sinapine, **4** (Figure 1), which is present in 1−2% (w/w) of the rapeseed meal.[5] Due to the economic importance of rapeseed and in order to investigate the consequences of genetic modifications, several studies were performed on profiling phenolic choline esters in rapeseed.[1,7,9−11] The sinapine content in rapeseed has been successfully reduced by genetically modifying the biosynthetic pathway.[12]

High levels of glucosinolates and sinapates are unwanted in rapeseed-derived food and feed because of their antinutritive properties. However, they may have positive physiological effects on plant development and protect seeds against pathogens and herbivores. For example, glucosinolates, which are very important for plants as defense compounds, have been thoroughly explored during the past decade.[13] Around 132 glucosinolates had been identified by 2011.[14] Sinapine, which is the choline ester of sinapic acid, was suggested to be involved in supplying choline for phosphatidylcholine in young *Raphanus sativus* seedlings.[15] Sinapate esters, especially with phenylpropanoids, play an important role in UV protection.[16] Although the ecological effects of secondary metabolites of cruciferous plants have been extensively studied, information about their specific role in rapeseed is limited. The cell- and tissue-specific distribution of metabolites is one of the clues to understand their physiological and ecological importance in plant seeds. Hence, a thorough phytochemical screening of the seeds of *B. napus* (winter cultivar 'Emerald') was undertaken to assemble analytical data for subsequent localization studies of secondary metabolites in rapeseed. Because the analytical methods of glucosinolate identification based on high-performance liquid chromatography (HPLC) have been well established and the glucosinolate structures were highly explored,[17] HPLC−diode array detection/mass spectrometry (HPLC-DAD/MS) was used to investigate the glucosinolate profile in the 'Emerald' cultivar. Due to the high complexity of other secondary metabolite profiles previously identified from rapeseed,[1,7,9−11,18] non-glucosinolate secondary metabolites were purified from seed extract as well, and their structures were unambiguously elucidated by nuclear magnetic resonance (NMR) spectroscopy and MS.

The aim of this study was to identify from a selected rapeseed cultivar as many as possible secondary metabolites of different structural classes, which presumably are of physiological relevance or may be involved in ecological interactions. The information and analytical data gained from this study are intended to be used in ongoing investigations on the spatial distributions of these metabolites in various organs of the seed. Here we report the identification of 11 glucosinolates by HPLC-DAD/MS and the purification and structure elucidation

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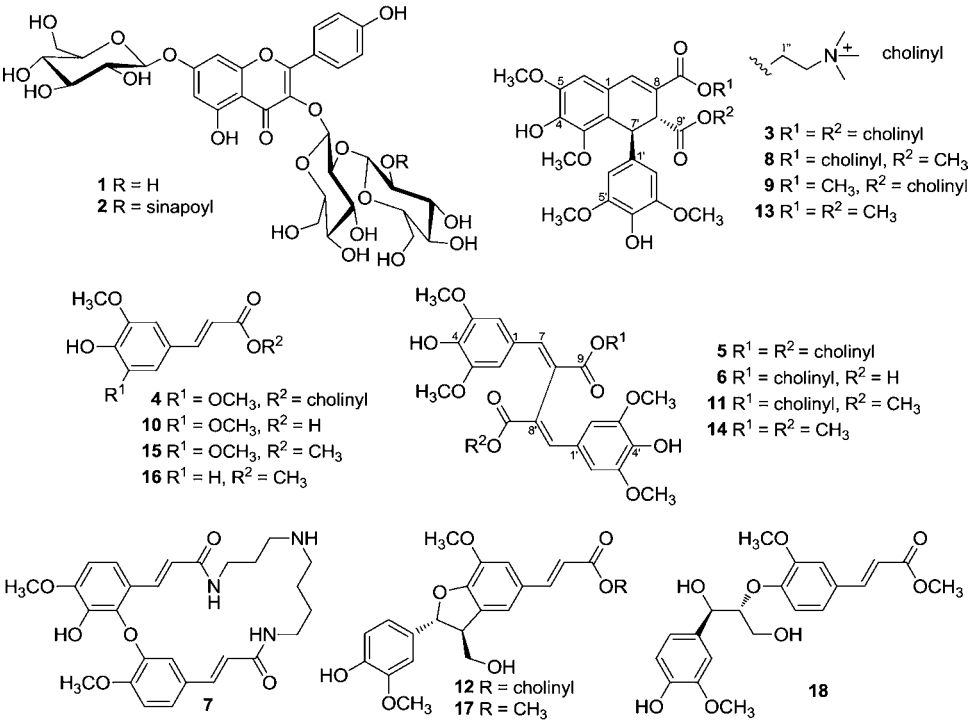


Figure 1. Structures of non-glucosinolate compounds isolated from rapeseed in this study.

of 18 phenolic compounds from rapeseed. Among them, seven new lignans were determined by means of NMR and MS.

MATERIALS AND METHODS

General Experimental Procedures. Glucosinolates were identified in their desulfated form with HPLC-DAD/MS by comparing the retention times and the mass data with those of references. HPLC was conducted on an Agilent series HP1100 consisting of a G1312A binary pump, a G1367A autosampler, and a G1315A diode array detector (Agilent Technologies, Waldbronn, Germany). The column used was a 250 × 4.6 mm i.d., 5 μm, LiChrospher 100 RP18, with a 4 × 4 mm i.d. guard column of the same material (Merck KGaA, Darmstadt, Germany). A stepwise binary gradient of H_2O (solvent A) containing 0.2% (v/v) formic acid and MeCN (solvent B), at a flow rate of 1.0 mL/min at 25 °C, was used as follows: 0 min, 1.5% B; 1 min, 1.5% B; 6 min, 5% B; 8 min, 7% B; 18 min, 21% B; 23 min, 29% B; 23.1 min, 100% B; 24 min, 100% B; 24.1 min, 1.5% B; and 28 min, 1.5% B. The injection volume was 50 μL. The HPLC eluate was monitored by DAD at 229 nm. An Esquire 6000 ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany) was coupled to the same HPLC system for recording electrospray ionization mass spectra (ESIMS). Positive ionization mode was used in the range m/z 60−1000.

Semipreparative HPLC was performed on a Prominence system with an LC-20AT gradient pump and an SPD-20A UV−vis detector (Shimadzu Corp., Tokyo, Japan). The column used in the first chromatographic step was a 250 × 21 mm i.d., 7 μm, Nucleosil 100 C_{18}, with a 50 × 21 mm i.d. guard column of the same material (Macherey-Nagel GmbH & Co. KG, Düren, Germany). The column used in the second chromatographic step was a 250 × 10 mm i.d., 5 μm, Purospher STAR RP18e with a 10 × 10 mm i.d. guard column of the same material (Merck KGaA).

NMR spectra of isolated compounds were recorded on a Bruker AV 500 NMR spectrometer (Bruker Biospin, Karlsruhe, Germany), operating at 500.13 MHz for ^1H and at 125.75 MHz for ^{13}C. The NMR spectrometer was equipped with a TCI cryoprobe (5 mm). Standard Bruker pulse sequences were used for measuring ^1H, ^{13}C, $^1H-^1H$ correlation spectroscopy (COSY), heteronuclear single-quantum correlation (HSQC), and heteronuclear multiple-bond correlation (HMBC) spectra at 298 K. Tetramethylsilane (TMS) was used as internal chemical shift reference.

LC-ESIMS of isolated phenolic compounds were recorded in positive ionization mode on a LC-ESIMS/MS system, which consists of an Agilent 1100 chromatography including a G1311A quaternary solvent delivery pump and a G1313A autosampler (Agilent Technologies), and a Bruker Esquire 3000 ion trap mass spectrometer (Bruker Daltonics). The column used was a 250 × 4.6 mm i.d., 5 μm, Purospher STAR RP18e with a 4 × 4 mm i.d. guard column of the same material (Merck KGaA). A stepwise binary gradient of H_2O (solvent A) and MeOH (solvent B), both containing 0.1% (v/v) formic acid, at a constant flow rate of 1.0 mL/min, was used as follows: 0 min, 20% B; 15 min, 100% B; 20 min, 100% B; 21 min, 20% B; 25 min, 20%. UV spectra were recorded during the LC-ESIMS runs by a DAD detector, detection 200−700 nm (J&M Analytik AG, Aalen, Germany), which was integrated in the LC-ESIMS system.

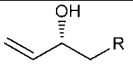
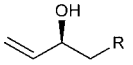
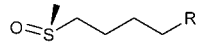
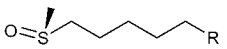
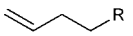
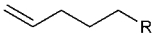
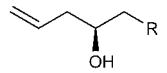
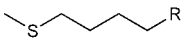
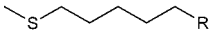
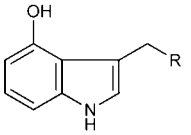
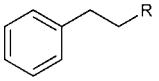
LC−high resolution ESIMS (LC-HRESIMS) data of new compounds were recorded on a LC-MS/MS system consisting of an Ultimate 3000 series RSLC system (Dionex, Sunnyvale, CA, USA), and an Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). The column used was a 150 × 2.1 mm i.d., 2.2 μm, Acclaim C18 (Dionex). A stepwise binary gradient of H_2O (solvent A) and MeCN (solvent B), both containing 0.1% (v/v) formic acid was used: 0 min, 0.5% B; 10 min, 10% B; 14 min, 80% B; 19 min, 80% B; 19.1 min, 0.5% B; 25 min, 0.5% B; flow rate, 300 μL/min. HRESIMS spectra were measured in positive ionization mode on the Orbitrap mass analyzer.

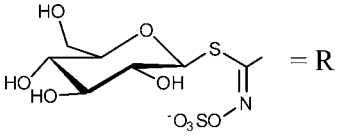
Optical rotation was recorded on a P-1030 automatic digital polarimeter (Jasco, Tokyo, Japan) at 25 °C.

Plant Material. Rapeseed winter cultivar 'Emerald' used in this experiment was purchased from Raps GbR (Langballig, Germany).

Metabolic Profiling of Glucosinolates. Methods for extracting and desulfating glucosinolates were modified from the literature[19] to cope with the single-seed samples. One rapeseed (4.9 mg) was put into a 2 mL Eppendorf tube. MeOH (1 mL, 80%, v/v) and four metal balls (3 mm) were added to the tube; the tube was then put into a paint-shaker, Skandex SO-10m (Fluid Management, Sassenheim, The Netherlands), for 10 min. After centrifugation at 13000 rpm for 10 min in a 5415R centrifuge (Eppendorf, Hamburg, Germany), 800 μL of supernatant was taken and added to a weak anion exchange DEAE

Table 1. Glucosinolate Structures Identified from Rapeseed in This Study

Common name	Systematic name	Structure
progoitrin	(*2R*)-2-hydroxy-3-butenyl glucosinolate	
epiprogoitrin	(*2S*)-2-hydroxy-3-butenyl glucosinolate	
glucoraphanin	4-methylsulfinylbutyl glucosinolate	
glucoalyssin	5-methylsulfinylpentyl glucosinolate	
gluconapin	3-butenyl glucosinolate	
glucobrassicanapin	4-pentenyl glucosinolate	
gluconapoleiferin	(*2R*)-2-hydroxy-4-pentenyl glucosinolate	
glucoerucin	4-(methylsulfanyl)butyl glucosinolate	
glucoberteroin	5-(methylsufanyl)pentyl glucosinolate	
4-hydroxyglucobrassicin	4-hydroxyindol-3-ylmethyl glucosinolate	
gluconasturtiin	2-phenylethyl glucosinolate	



Sephadex A25 (Sigma, Steinheim, Germany) cartridge, which was made as follows: DEAE Sephadex A25 (30 mg) was added into each well of a 1 mL Nunc 96 fritted (filter) deepwell plate (Thermo Fisher Scientific, Rochester NY, USA) and compressed to form a cartridge, which was conditioned with 800 μL of H_2O and equilibrated with 500 μL of 80% (v/v) MeOH before use. The cartridge was eluted with 500 μL of 80% (v/v) MeOH, 1 mL of H_2O twice, and 500 μL of 0.02 M MES buffer (pH 5.2), successively, after which 30 μL of sulfatase (Sigma, Steinheim, Germany) solution prepared as described was added.[20] The cartridge was capped and incubated at ambient temperature overnight. Afterward, the cartridge was eluted with 500 μL of H_2O for desulfated glucosinolate analysis.

Extraction and Purification of Phenolic Compounds. Rapeseed (25 g) was ground in liquid N_2 and extracted with 100 mL of 80% ethanol (24 h × 3). The combined extract was filtered and evaporated in a vacuum (<40 °C) to yield 3.24 g of residue, which was dissolved in 20 mL of H_2O. The aqueous solution was loaded on a Discovery DSC-18 SPE cartridge, 10 g, 60 mL (Supelco, Bellafonte, PA, USA), which was conditioned with 20 mL of MeCN and then equilibrated with 20 mL of H_2O before use. After the eluate of 60 mL of H_2O was discarded, the eluate of 60 mL of 80% MeCN aqueous solution was collected and dried at <40 °C in a vacuum to give 760 mg of residue. The residue was fractionated on a 250 × 21 mm i.d., 7 μm, Nucleosil 100 C_{18} column, with a 50 × 21 mm i.d. guard column of the same material (Macherey-Nagel GmbH & Co. KG). The flow rate was 10 mL/min; UV detection was at 240 and 330 nm. The following stepwise gradient of H_2O (solvent A) containing 0.1% (v/v) trifluoroacetic acid and MeOH (solvent B) was applied: 0 min, 35% B; 30 min, 80% B; 32 min, 100% B; 42 min, 100% B; 45 min, 35% B; 50 min, 35%. Two fractions were collected as F1 (18.5−28.1 min) and F2 (28.1−38.0 min). Further separation of fractions F1 and F2 was performed on a 250 × 10 mm i.d., 5 μm, Purospher STAR RP18e column with a 10 × 10 mm i.d. guard column of the same material (Merck KGaA) at a flow rate of 4 mL/min. H_2O (A) containing 0.1% (v/v) trifluoroacetic acid and MeCN (B) was used to elute compounds **1**−**12** from fraction F1 as follows: 0 min, 15% B; 30 min, 25% B; 32 min, 100% B; 37 min, 100% B; 39 min, 15% B; 42 min, 15% B. H_2O (A) containing 0.1% (v/v) trifluoroacetic acid and MeOH (B) was used to elute compounds **13**−**18** from F2 as follows: 0 min, 55% B; 15 min, 65% B; 16 min, 100% B; 21 min, 100% B; 22 min, 55% B; 25 min, 55% B.

(±)-Dicholinyl thomasidioate [dicholinyl (±)-4,4′-dihydroxy-3,3′,5,5′-tetramethoxy-2,7′-cyclolign-7-en-9,9′-dioate], ***3:*** 2.3 mg; UV (MeOH/H_2O) λ_{max} 247.9, 340.2 nm; $[\alpha]^{25}_D$ 0.0 (*c* 0.1, MeOH); ^{1}H and ^{13}C NMR data, see Table 2; ESIMS *m*/*z* 309.1 $[M]^{2+}$; HRESIMS *m*/*z* 309.1568 $[M]^{2+}$ (calcd for $C_{16}H_{23}O_5N$, 309.1576).

Dicholinyl (E,E)-4,4′-dihydroxy-3,3′,5,5′-tetramethoxylign-7,7′-dien-9,9′-dioate, ***5:*** 0.5 mg; UV (MeOH/H_2O) λ_{max} 243.4, 338.7 nm; ^{1}H and ^{13}C NMR data, see Table 3; ESIMS *m*/*z* 309.1 $[M]^{2+}$; HRESIMS *m*/*z* 309.1566 $[M]^{2+}$ (calcd for $C_{16}H_{23}O_5N$, 309.1576).

Cholinyl hydrogen (E,E)-4,4′-dihydroxy-3,3′,5,5′-tetramethoxy-lign-7,7′-dien-9,9′-dioate, ***6:*** 3.5 mg; UV (MeOH/H_2O) λ_{max} 240.9, 330.7 nm; ^{1}H and ^{13}C NMR data, see Table 3; ESIMS *m*/*z* 532.4 $[M]^+$; HRESIMS *m*/*z* 532.2169 $[M]^+$ (calcd for $C_{27}H_{34}O_{10}N$, 532.2183).

9-Cholinyl-9′-methyl (±)-thomasidioate [9-cholinyl-9′-methyl (±)-4,4′-dihydroxy-3,3′,5,5′-tetramethoxy-2,7′-cyclolign-7-en-9,9′-dioate], ***8****, and 9-methyl-9′-cholinyl (±)-thomasidioate [9-methyl-9′-cholinyl (±)-4,4′-dihydroxy-3,3′,5,5′-tetramethoxy-2,7′-cyclolign-7-en-9,9′-dioate],* ***9:*** 0.6 mg (ratio **8**/**9** = 8:7, calculated from integrals of ^{1}H NMR signals in the isolated mixture of **8** and **9**); UV (MeOH/H_2O) λ_{max} 248.4, 337.7 nm; $[\alpha]^{25}_D$ 0.0 (*c* 0.05, MeOH); ^{1}H and ^{13}C

Table 2. ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) Data of Compounds 3, 8, 9, and 13 (CD_3OD)

	3		8		9		13	
position	δ_H (J, Hz)	δ_C	δ_H (J, Hz)	δ_C	δ_H (J, Hz)	δ_C	δ_H (J, Hz)	δ_C
1		123.93		124.01		124.04		124.24
2		125.00		125.05		125.10		124.92
3		146.74		146.92		146.82		146.85
4		144.31		144.14		143.91		143.61
5		149.71		149.59		149.56		149.42
6	6.98 (s)	109.60	6.94 (s)	109.56	6.92 (s)	109.42	6.89 (s)	109.38
7	7.81 (s)	141.05	7.78 (s)	140.89	7.73 (s)	139.86	7.70 (s)	139.62
8		133.91		134.39		134.17		134.64
9		167.20		167.28		168.85		168.93
3-OCH_3	3.52 (s)	60.83	3.58 (s)	60.81	3.55 (s)	60.79	3.58 (s)	60.80
5-OCH_3	3.93 (s)	56.83	3.924 (s)	56.80	3.919 (s)	56.82	3.91 (s)	56.78
9-$COOCH_3$					3.75 (s)	52.56	3.73 (s)	52.44
1″	4.62 (br t)	59.37	4.62 (br s)	59.24				
2″	3.74 (t, 4.8)	66.08	3.73 (t, 4.7)	66.14				
N″-CH_3	3.18 (s)	54.34	3.18 (s)	54.34				
1′		121.81		122.22		122.61		123.16
2′/6′	6.23 (s)	105.96	6.26 (s)	105.87	6.29 (s)	105.87	6.28 (s)	105.77
3′/5′		149.20		149.16		149.12		149.06
4′		135.76		135.55		135.63		135.37
7′	5.06 (br s)	40.47	5.01 (br s)	40.78	5.02 (br s)	40.66	4.97 (br s)	40.88
8′	4.07 (d, 1.3)	48.39	3.99 (d, 1.4)	48.33	4.05 (d, 1.4)	48.08	3.97 (d, 1.4)	48.11
9′		172.55		174.02		172.67		174.19
3′/5′-OCH_3	3.69 (s)	56.76	3.69 (s)	56.69	3.70 (s)	56.71	3.69 (s)	56.64
9′-$COOCH_3$			3.64 (s)	53.01			3.62 (s)	52.92
1‴	4.52 (br m)	59.95			4.51 (br m)	59.80		
2‴	3.67 (m)	65.96			3.64 (t, 4.6)	65.99		
N‴-CH_3	3.12 (s)	54.34			3.11 (s)	54.34		

NMR data, see Table 2; ESIMS m/z 546.5 $[M]^+$; HRESIMS m/z 546.2314 $[M]^+$ (calcd for $C_{28}H_{36}O_{10}N$, 546.2339).

Cholinyl methyl (E,E)-4,4′-dihydroxy-3,3′,5,5′-tetramethoxylign-7,7′-dien-9,9′-dioate, ***11:*** 3.5 mg; UV (MeOH/H_2O) λ_{max} 241.9, 337.7 nm; ^{1}H and ^{13}C NMR data, see Table 3; ESIMS m/z 546.5 $[M]^+$; HRESIMS m/z 546.2333 $[M]^+$ (calcd for $C_{28}H_{36}O_{10}N$, 546.2339).

Dimethyl (E,E)-4,4′-dihydroxy-3,3′,5,5′-tetramethoxylign-7,7′-dien-9,9′-dioate, ***14:*** 1.7 mg; UV (MeOH/H_2O) λ_{max} 240.4, 329.7 nm; ^{1}H and ^{13}C NMR data, see Table 3; ESIMS m/z 497.2 $[M + Na]^+$, 475.1 $[M + H]^+$; HRESIMS m/z 475.1600 $[M + H]^+$ (calcd for $C_{24}H_{27}O_{10}$, 475.1604).

RESULTS AND DISCUSSION

Glucosinolate Profiling. Glucosinolates were determined in the desulfated form by comparing their MS data and retention times with those of references. A total of 11 glucosinolates were identified. The majority of them, except 4-hydroxyglucobrassicin and gluconasturtiin, are aliphatic glucosinolates (progoitrin, epiprogoitrin, glucoraphanin, gluconapoleiferin, glucoalyssin, gluconapin, glucobrassicanapin, glucoerucin, glucoberteroin) (Table 1). All of the glucosinolates detected in this study have previously been found in rapeseed.[21,22] The successful identification of glucosinolates in a single rapeseed described here suggested that the current protocol could be applied to study the distribution of glucosinolates in different tissues of the rapeseed and other microscopic pieces of the rape plant. The homemade anion exchange cartridges in a 96-well plate allow high-throughout profiling of glucosinolates in a small amount of plant material.

Isolation of Phenolic Compounds. From the ethanol extract of 25 g of rapeseed, 18 phenolic compounds (Figure 1) were isolated and identified, including three new (±)-thomasidioic acid derivatives, **3**, **8**, and **9**, and four new (*E*,*E*)-dienolignan derivatives, **5**, **6**, **11**, and **14**. In addition, for the first time compound **18** was isolated as a natural product.

(±)-Thomasidioic Acid Derivatives. Four (±)-thomasidioic acid derivatives, namely, compounds **3**, **8**, **9**, and **13**, were isolated from rapeseed extract and identified by NMR, MS, and optical rotation data. Compound **13** (NMR data in Table 2 for comparison with data of **3**, **8**, and **9**) was elucidated as dimethyl (±)-thomasidioate by comparing the analytical data with those reported for the synthetic product.[23]

The singlets at δ 7.81 (H-7), 6.98 (H-6), and 6.23 (2H, H-2′/6′) in the aromatic range of the ^{1}H NMR spectrum of compound **3** (Table 2), a broad singlet at δ 5.06 (H-7′), a doublet at δ 4.07 (H-8′) with a coupling constant of J = 1.3 Hz, and three *O*-methyl signals at δ 3.93 (5-OCH_3), 3.69 (3′/5′-OCH_3), and 3.52 (3-OCH_3) resemble the ^{1}H NMR spectrum of **13** and therefore were attributed to the lignan part of the molecule. In addition, the spectrum displays two broad signals at δ 4.62 (H_2-1″) and 4.52 (H_2-1‴), a triplet at δ 3.74 (H_2-2″), a multiplet at δ 3.67 (H_2-2‴), and two singlets, each integrating for nine protons at δ 3.18 and 3.12 assignable to N−CH_3 groups. These ^{1}H NMR data suggest two choline moieties in compound **3**. The ^{13}C NMR spectrum displays signals of two choline ester moieties with identical (N″/N‴−CH_3, δ 54.34) or <1 ppm different (C-1″ and C-1‴; C-2″ and C-2‴) chemical shifts (Table 2) and signals of two phenylpropanoids (two C_9 units), as well as four phenolic *O*-methyl groups (two of them, C-3′/5′, δ 56.76, are equivalent). HSQC and HMBC data were used to assign all proton and carbon signals (Table 2). The connection positions of two phenylpropanoid moieties were

determined by the key correlations of H-7 (δ 7.81) with C-2 (δ 125.00) and C-8′ (δ 48.39); H-8′ (δ 4.07) with C-9 (δ 167.20), C-7 (δ 141.05), C-2 (δ 125.00), and C-1′ (δ 121.81); and H-7′ (δ 5.06) with C-8 (δ 133.91), C-1 (δ 123.93), C-3 (δ 146.74), C-9′ (δ 172.55), and C-2′/6′ (δ 105.96) in the HMBC spectrum (Figure 2A), confirming the constitution of

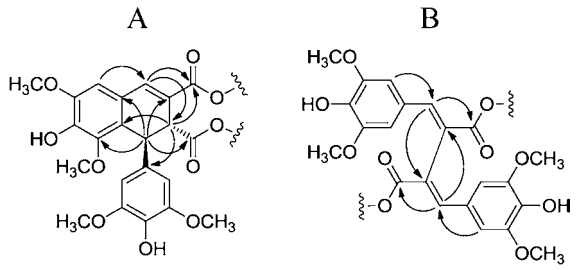


Figure 2. Selected HMBC correlations of (±)-thomasidioic acid derivatives **3**, **8**, **9**, **13** (A) and (*E*,*E*)-dienolignan derivatives **5**, **6**, **11**, **14** (B).

compound **3** as an arylnaphthalene lignan derivative. The small coupling constant of $J_{H\text{-}7'-H\text{-}8'}$ = 1.3 Hz was consistent with a *trans* (diaxial) configuration of the syringyl ring at C-7′ and the choline ester substituent at C-8′ as proposed by Wallis.[24] No optical activity was observed, indicating that compound **3** is racemic. Hence, the structure was elucidated as dicholinyl (±)-thomasidioate [dicholinyl (±)-4,4′-dihydroxy-3,3′,5,5′-tetramethoxy-2,7′-cyclolign-7-en-9,9′-dioate] (Figure 1), which is further confirmed by ESIMS and HRESIMS.

Despite many efforts, compounds **8** and **9** could not be separated by HPLC, because they coeluted on several reversed phase sorbent columns. Therefore, the structures of the two compounds were elucidated from the NMR spectra of the mixture. The signals in the ^{1}H NMR spectrum showed two sets of signals (Table 2), which were readily distinguished by means of their integral values (ratio **8**/**9** = 8:7). Furthermore, NMR spectra of the two compounds are highly similar to those of compounds **3** and **13**. 2D NMR data (^{1}H−^{1}H COSY, HSQC, HMBC) suggest the two compounds are also (±)-thomasidioate derivatives (Figure 2A). In addition to the thomasidioate signals, ^{1}H and ^{13}C NMR spectra from each of the two compounds display signals of one *O*-methyl group and one cholinyl moiety. The HMBC correlations of H_2-1″ (δ 4.62) with C-9 (δ 167.28) and of COOC$\underline{H}_3$ (δ 3.64) with C-9′ (δ 174.02) assign the choline ester to position 9 and the methyl ester to position 9′ in **8**. In contrast, in compound **9**, the choline ester moiety is attached to C-9′ (δ 172.67) and the methyl ester to position 9 (δ 168.85), as inferred from their HMBC correlations with H_2-1‴ (δ 4.51) and COOC$\underline{H}_3$ (δ 3.75), respectively. Thus, compounds **8** and **9** are isomers, a fact that was confirmed by their ESIMS and HRESIMS data, and their structures were elucidated as 9-cholinyl-9′-methyl (±)-thomasidioate [9-cholinyl-9′-methyl (±)-4,4′-dihydroxy-3,3′,5,5′-tetramethoxy-2,7′-cyclolign-7-en-9,9′-dioate] (**8**) and 9-methyl-9′-cholinyl (±)-thomasidioate [9-methyl-9′-cholinyl (±)-4,4′-dihydroxy-3,3′,5,5′-tetramethoxy-2,7′-cyclolign-7-en-9,9′-dioate] (**9**).

(*E*,*E*)-Dienolignan Derivatives. The ^{1}H NMR spectrum of compound **5** (Table 3) shows an *O*-methyl singlet at δ 3.76

Table 3. ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) Data of Compounds 5, 6, 11, and 14 (CD_3OD)

	5		**6**		**11**		**14**	
position	δ_H (*J*, Hz)	δ_C	δ_H (*J*, Hz)	δ_C	δ_H (*J*, Hz)	δ_C	δ_H (*J*, Hz)	δ_C
1		126.2		126.6		126.3[a]		126.7
2/6	6.99 (s)	109.1	6.97 (s)	109.1	6.94 (s)	109.0	6.88 (s)	108.7
3/5		149.4		149.3		149.3		149.1
4		140.2		139.6		139.6		139.1
7	8.00 (s)	145.9	7.95 (s)	145.8	7.95 (s)	146.0	7.85 (s)	144.2
8		124.0		124.7		124.2		125.6
9		167.9		168.2		168.0		169.5
3/5-OCH_3	3.76 (s)	56.8	3.75 (s)	56.7	3.75 (s)	56.7	3.74 (s)	56.6
9-$COOCH_3$							3.69 (s)	52.9
1″	4.57 (m)	59.8	4.57 (m)	59.7	4.56 (m)	59.7		
2″a	3.63 (m)	66.0	3.64 (m)	66.0	3.62 (dd, 6.7, 3.2)	66.0		
2″b	3.54 (m)		3.58 (m)		3.56 (dd, 6.2, 3.2)			
N″-CH_3	3.04 (s)	54.3	3.08 (s)	54.3	3.06 (s)	54.3		
1′		126.2		126.5		126.4[a]		126.7
2′/6′	6.99 (s)	109.1	6.92 (s)	108.8	6.93 (s)	108.9	6.88 (s)	108.7
3′/5′		149.4		149.2		149.2		149.1
4′		140.2		139.4		139.8		139.1
7′	8.00 (s)	145.9	7.90 (s)	143.9	7.89 (s)	144.2	7.85 (s)	144.2
8′		124.0		125.8		125.2		125.6
9′		167.9		170.5		169.4		169.5
3′/5′-OCH_3	3.76 (s)	56.8	3.76 (s)	56.7	3.75 (s)	56.7	3.74 (s)	56.6
9′-$COOCH_3$					3.71 (s)	53.0	3.69 (s)	52.9
1‴	4.57 (m)	59.8						
2‴a	3.63 (m)	66.0						
2‴b	3.54 (m)							
N‴-CH_3	3.04 (s)	54.3						

[a]Values in the same column may be interchanged.

(12H, H-3/5/3′/5′-OCH_3), and singlets at δ 8.00 (2H, H-7/7′) and 6.99 (4H, H-2/6/2′/6′), assignable to the protons attached to the double bond and in the aromatic ring, respectively. Another series of signals, representing choline moieties, appears at δ 4.57 (4H, H-1″/1‴), 3.63 (2H, H-2″a/2‴a), 3.54 (2H, H-2″b/2‴b), and 3.04 (18H, N″/N‴−CH_3). The signals in the ^{1}H NMR spectrum of **5** resemble those of sinapine, except the missing signal of the olefinic H-8 and decoupling of the other olefinic proton, H-7. The lack of H-8, together with ESIMS and HRESIMS data, suggested a dimer, consisting of two sinapine units connected through a C-8/8′ carbon−carbon bond. The downfield shift of C-8 from δ 114.7 in the ^{13}C NMR spectrum of sinapine to δ 124.0 in the spectrum of compound **5** also supported the connection through C-8/8′. The *E* configuration of the two double bonds was established by comparing the chemical shifts of the olefinic protons with those of reported compounds.[25,26] Finally, **5** was elucidated as dicholinyl (*E*,*E*)-4,4′-dihydroxy-3,3′,5,5′-tetramethoxylign-7,7′-dien-9,9′-dioate.

The ^{1}H NMR spectrum of compound **6** (Table 3) displays four singlets at δ 7.95 (H-7), 7.90 (H-7′), 6.97 (H-2/6), and 6.92 (H-2′/6′), two *O*-methyl group signals at δ 3.75 (3/5-OCH_3) and 3.76 (3′/5′-OCH_3), and a series of choline moiety signals at δ 4.57 (H_2-1″), 3.64 (H-2″a), 3.58 (H-2″b), and 3.08 (N″−CH_3). The proton and carbon signals (Table 3) were assigned by analyzing connections in the HSQC and HMBC spectra, from which a sinapine moiety and a sinapic acid moiety were deduced. Mutual correlations between H-7 and C-8′ and between H-7′ and C-8 in the HMBC spectrum (Figure 2B) indicated a connection between the two sinapoyl units through a carbon−carbon bond between C-8 and C-8′. Therefore, the structure of compound **6** was elucidated as cholinyl hydrogen (*E*,*E*)-4,4′-dihydroxy-3,3′,5,5′-tetramethoxylign-7,7′-dien-9,9′-dioate, which was confirmed by ESIMS and HRESIMS.

The ^{1}H NMR spectrum of compound **11** (Table 3) highly resembles that of **6**, except there is an additional *O*-methyl signal at δ 3.71 (3H, 9′-COOC$\underline{H}_3$) in the spectrum of **11**. Compound **11** was deduced as a dimer of sinapine and methyl sinapate, which are connected through a carbon−carbon bond between C-8 and C-8′. The ^{1}H and ^{13}C NMR signals (Table 3) and the ESIMS and HRESIMS data confirmed the structure of **11** as cholinyl methyl (*E*,*E*)-4,4′-dihydroxy-3,3′,5,5′-tetramethoxylign-7,7′-dien-9,9′-dioate.

The ^{1}H NMR spectrum of the symmetric compound, **14** (Table 3), displays only four singlets, which were assigned to H-7/7′ (2H, δ 7.85), H-2/6/2′/6′ (4H, δ 6.88), and the *O*-methyl signals at δ 3.74 (12H, H-3/5/3′/5′-OCH_3) and 3.69 (6H, 9/9′-COOC$\underline{H}_3$). The ^{13}C NMR spectrum shows nine signals, all of which were readily assigned by the analyses of HSQC and HMBC (Figure 2B) spectra. The HRESIMS data suggest a formula of $C_{24}H_{26}O_{10}$. Thus, the structure was determined as a dimer of two methyl sinapate units, which are connected to each other through positions 8/8′, named dimethyl (*E*,*E*)-4,4′-dihydroxy-3,3′,5,5′-tetramethoxylign-7,7′-dien-9,9′-dioate.

Known Phenolic Compounds. Known compounds were determined by comparing their 1D and 2D NMR spectra and MS data with those of corresponding compounds in the literature as 3-*O*-(*β*-D-glucopyranosyl-(1→2)-*β*-D-glucopyranosyl)-7-*O*-*β*-D-glucopyranosylkaempferol (**1**),[27] 3-*O*-[(2-*O*-sinapoyl-*β*-D-glucopyranosyl)-(1→2)-*β*-D-glucopyranosyl]-7-*O*-*β*-D-glucopyranosylkaempferol (**2**),[28] sinapine (**4**),[29] a cyclic spermidine conjugate (**7**),[9] sinapic acid (**10**),[30] cholinyl 3-[2,3-dihydro-2-(4-hydroxy-3-methoxyphenyl)-3-(hydroxymethyl)-7-methoxy-5-benzofuranyl]-(2*Z*)-acrylate (**12**),[10,31] dimethyl (±)-thomasidioate (**13**),[23] methyl sinapate (**15**) and methyl ferulate (**16**),[32] methyl 3-[2,3-dihydro-2-(4-hydroxy-3-methoxyphenyl)-3-(hydroxymethyl)-7-methoxy-5-benzofuranyl]-(2*Z*)-acrylate (**17**),[33] and methyl 4-[2-hydroxy-2-(4-hydroxy-3-methoxyphenyl)-1-(hydroxymethyl)ethyl]-ferulate (**18**),[34] a synthetic model compound.

In total, 11 glucosinolates and 18 phenolic compounds were identified from rapeseed. All of the glucosinolates detected in this study have previously been found from rapeseed. In addition to eight new natural products (**3**, **5**, **6**, **8**, **9**, **11**, **14**, and **18**), compounds **13** and **17** were reported from rapeseed for the first time. The acquired information about purification and structures of secondary metabolites in rapeseed and their analytical data are useful for upcoming studies on tissue-specific localization of these compounds.

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Notes

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NOTE ADDED AFTER ASAP PUBLICATION

This paper published October 15, 2012 with errors in the analytical data for compounds **3**, **5**, **6**, **8**, **9**, and **11**. The correct version published October 24, 2012.