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Formation of a complex pattern of sinapate esters in *Brassica napus* seeds, catalyzed by enzymes of a serine carboxypeptidase-like acyltransferase family?

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Abstract

Members of the Brassicaceae accumulate complex patterns of sinapate esters, as shown in this communication with seeds of oilseed rape (*Brassica napus*). Fifteen seed constituents were isolated and identified by a combination of high-field NMR spectroscopy and high resolution electrospray ionisation mass spectrometry. These include glucose, gentiobiose and kaempferol glycoside esters as well as sinapine (sinapoylcholine), sinapoylmalate and an unusual cyclic spermidine amide. One of the glucose esters (1,6-di-*O*-sinapoylglucose), two gentiobiose esters (1-*O*-caffeoylgentiobiose and 1,2,6′-tri-*O*-sinapoylgentiobiose) and two kaempferol conjugates [4′-(6-*O*-sinapoylglucoside)-3,7-di-*O*-glucoside and 3-*O*-sophoroside-7-*O*-(2-*O*-sinapoylglucoside)] seem to be new plant products. Serine carboxypeptidase-like (SCPL) acyltransferases catalyze the formation of sinapine and sinapoylmalate accepting 1-*O*-β-acetal esters (1-*O*-β-glucose esters) as acyl donors. To address the question whether the formation of other components of the complex pattern of the sinapate esters in *B. napus* seeds is catalyzed via 1-*O*-sinapoyl-β-glucose, we performed a seed-specific dsRNAi-based suppression of the sinapate glucosyltransferase gene (*BnSGT1*) expression. In seeds of *BnSGT1*-suppressing plants the amount of sinapoylglucose decreased below the HPLC detection limit resulting in turn in the disappearance or marked decrease of all the other sinapate esters, indicating that formation of the complex pattern of these esters in *B. napus* seeds is dependent on sinapoylglucose. This gives rise to the assumption that enzymes of an SCPL acyltransferase family catalyze the appropriate transfer reactions to synthesize the accumulating esters.

Keywords: *Brassica napus*; Brassicaceae; Oilseed rape; Sinapate esters; Molecular evolution; Serine carboxypeptidase-like acyltransferases

1. Introduction

Seeds of oilseed rape (*Brassica napus* L. var. *napus*) accumulate high amounts of sinapine (sinapoylcholine) among the various phenolics (Kozlowska et al., 1990; Bouchereau et al., 1991; Shahidi and Naczk, 1992; Bell, 1993) that are characteristic of the Brassicaceae family (Hegnauer, 1964). Sinapine is a member of the well-known pathway leading to sinapoylmalate in the seedlings (Linscheid et al., 1980; Bouchereau et al., 1992). A recent study of the molecular regulation of sinapine metabolism in *B. napus* (Milkowski et al., 2004) demonstrated transcriptional regulation of its biosynthesis via 1-*O*-sinapoyl-β-glucose. The role of such acylglucoses as acyl donors in plant secondary metabolism, as an alternative to CoA-dependent pathways, has been demonstrated with a remarkable number of other plants (Strack and Mock, 1993; Steffens, 2000).

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It has been shown (Li and Steffens, 2000; Lehfeldt et al., 2000; Shirley et al., 2001; Milkowski et al., 2004) that 1-*O*-β-acetal ester-dependent acyltransferases constitute a new class of serine carboxypeptidase-like (SCPL) proteins (SCPL acyltransferases). In Arabidopsis these enzymes form a distinct group within a large family of SCPL proteins (Milkowski and Strack, 2004). It is tempting to assume, that there is a corresponding group of SCPL acyltransferases catalyzing the formation of a vast array of structurally diverse sinapate and possibly other hydroxycinnamate esters in Arabidopsis.

The present work documents the formation of a complex pattern of hydroxycinnamate conjugates, mainly sinapate esters, in *B. napus* seeds, composed of glucose, gentiobiose and kaempferol glycoside esters as well as sinapine, sinapoylmalate and an unusual cyclic spermidine amide. We address the question whether the formation of these compounds is dependent on sinapoylglucose indicating that enzymes of a SCPL acyltransferase family would be involved, as hypothesized for Arabidopsis. For this reason we performed a seed-specific dsRNAi-based suppression of the sinapate glucosyltransferase gene (*BnSGT1*) expression. This approach is part of a study (Hüsken et al., 2005) focusing on reducing the amount of antinutritive sinapate esters (Ismail et al., 1981; Kozlowska et al., 1990; Naczk et al., 1998) that compromise the use of the valuable protein-rich seed meal, considered essential for possibly establishing rape as a protein crop.

Seeds of transgenic *B. napus* plants harbouring a dsRNAi construct for suppression of *BnSGT1* expression were analyzed (Hüsken et al., 2005). The *BnSGT1* gene codes for the enzyme that catalyzes the formation of an 1-*O*-β-acetal ester (Milkowski et al., 2000a), here 1-*O*-sinapoyl-β-glucose, the activated substrate of SCPL acyltransferases that transfer the sinapoyl moiety to various acceptors in the formation of the corresponding esters. As expected, the transformed seeds exhibit a significant decrease in the accumulation of 1-*O*-sinapoyl-β-glucose (barely detectable) that in turn results in the disappearance or marked decrease of all the other sinapate esters in *B. napus* seeds. Thus, these esters are synthesized via sinapoylglucose and therefore their formation is most likely catalyzed by a group of enzymes encoded by a SCPL acyltransferase gene family.

2. Results and discussion

2.1. Analysis of *B. napus* constituents

HPLC analyses of methanolic extracts from *B. napus* seeds showed complex patterns of UV-absorbing compounds. Fifteen out of at least 30 detectable compounds were isolated using a three-step protocol. Extracts were fractionated on polyamide SC6 followed by preparative HPLC and final purification on Sephadex LH-20 columns.

Purified compounds were identified using a combination of high-field NMR spectroscopy and high resolution electrospray ionisation mass spectrometry (HR ESI-MS). In all cases the characteristic ^{1}H NMR spin systems of the individual units in each molecule were identified from inspection of the 1D and 2D COSY data. In those cases where the chemical shift data were not sufficient to position substituents of the compounds, this information was determined from responses in 1D ^{1}H NOE difference spectra or from cross peaks in 2D ROESY spectra. HR ESI-MS afforded the molecular formulae and complemented the NMR data.

Three of the identified structures (Fig. 1), i.e. sinapoylglucose (**4**), sinapine (sinapoylcholine, **6**) and sinapoylmalate (**9**), are components of a well-known pathway that is characteristic for members of the Brassicaceae (Linscheid et al., 1980; Bouchereau et al., 1992; Milkowski et al., 2004). The spectroscopic data of these sinapate esters are in accordance with those from compounds isolated from *Raphanus sativus* (Linscheid et al., 1980) and their data are not included in this communication.

Summarizing the structure elucidation of the compounds isolated from *B. napus* seeds, it is noticeable that the molecules essentially fall into three groups, namely glucose, gentiobiose and kaempferol glycoside esters along with a spermidine amide. In most cases the aromatic acid substituent is the ubiquitous *E*-sinapoyl moiety that was recognized in the ^{1}H NMR spectra from its two proton singlet signal at ca. 7 ppm, the singlet of the two aromatic methoxyl groups at ca. 3.9 ppm and olefinic protons at 7.7 and 6.5 ppm with the characteristic *trans* vicinal coupling of ca. 15.8 Hz. Characteristic chemical shifts again identified the presence of the non-symmetrical *E*-4,5-dihydroxy-3-methoxycinnamoyl (5-hydroxyferuloyl) group in **2** and caffeoyl group in **1**. Similarly the universal sugar unit is the β-glucopyranosyl moiety detected from the presence in the ^{1}H spectra of its doublet anomeric proton (^3J ca. 7.8 Hz) and ring protons whose chemical shifts are determined by the individual substitution patterns.

The presence of a single acyl substituent in the glucose ring at the anomeric carbon, C-1 of compounds **1**, **2**, and **4**, was indicated by a doublet (7.8 Hz) for H-1 at ca. 5.6 ppm. Introduction of a second acyl unit at C-2 (**11**, **12**, **13**, **15**) caused a subtle shift to 5.8 ppm of H-1 and the low field shift of the double doublet of H-2 to ca. 5.1 ppm. A downfield shift of H-2 is also evident in the terminal sugar units of gentiobiose (**13**), sophorose (**5**) and glucose (**10**). Substitution at C-6 in **7**, **14** and **15** was evident from the downfield shifts of the two double doublets of H-6A and B to 4.6 and 4.3–4.5 ppm, respectively. Hence the number and simple assessment of chemical shifts provided unambiguous

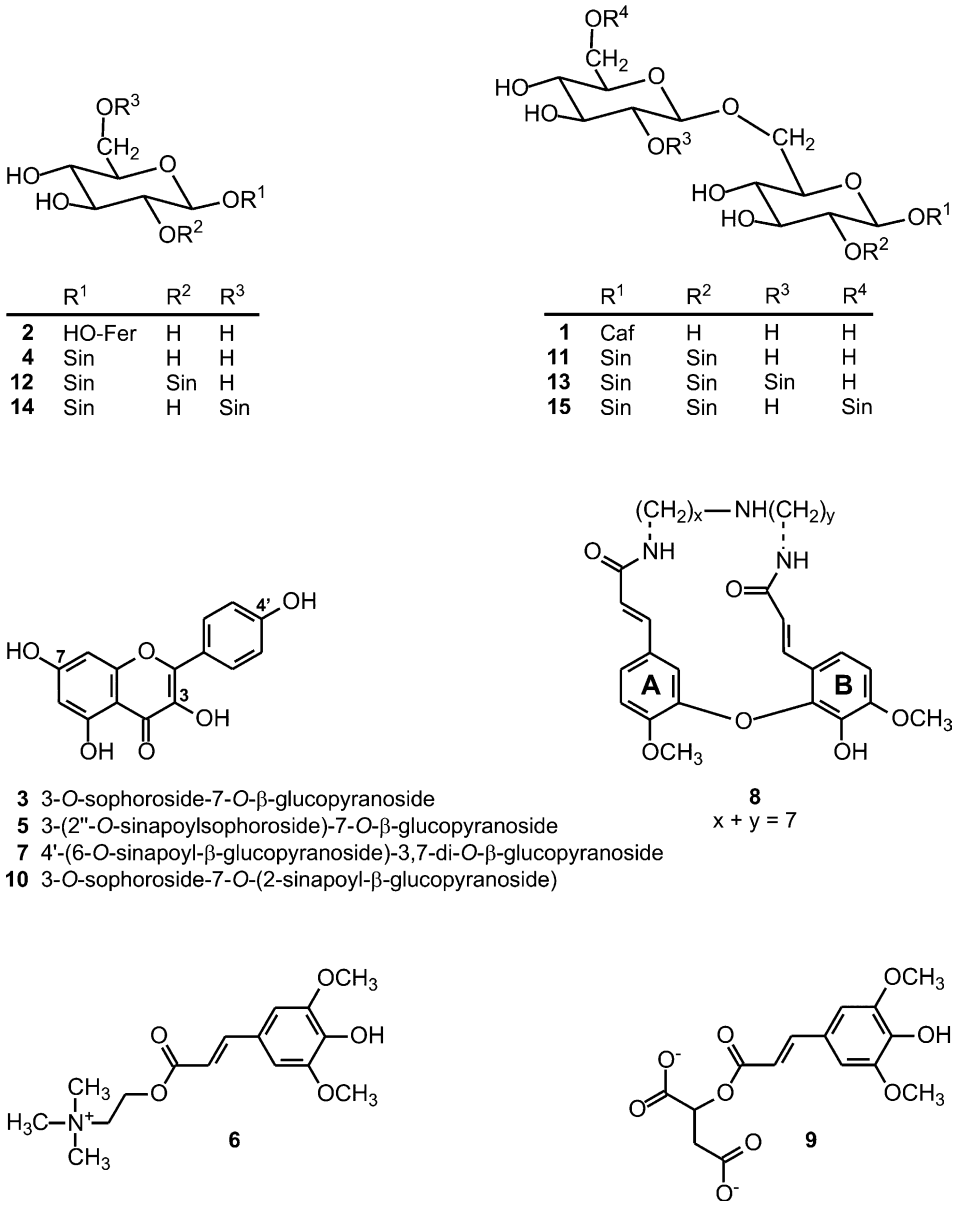

Fig. 1. Structures of compounds isolated from *Brassica napus* seeds; Sin = sinapoyl, HO-Fer = 5-hydroxyferuloyl, Caf = caffeoyl. Compound numbers are given according to the sequence in HPLC elution shown in Fig. 2.

evidence for the structure of the glucose, gentiobiose, and the sugar substituents in the kaempferol derivatives.

The C-7-substituted kaempferol derivatives were characterized by the low field substituent induced ^{1}H shifts of H-6 and H-8, and the AA″BB″ ^{1}H pattern of ring B. The assignments of the sugar units in these systems follow from the 2D COSY spectrum and the position of these units followed from correlations with the anomeric protons in the 1D NOE or 2D ROESY spectra.

The three units in the molecule of the unusual spermidine derivative (**8**), a cyclic spermidine alkaloid, were identified from the 2D COSY spectrum. The relative position of the substituents (methoxyl group and double bond side chains) in both aromatic systems was evident from the long range coupling in this spectrum and from cross peaks in the 2D ROESY spectrum. These substituents are the rare 3-hydroxy-4-methoxycinnamoyl (isoferuloyl) group (Greeaway et al., 1988), and the new 2,3-dihydroxy-4-methoxycinnamoyl (2-hydroxyisoferuloyl) group coupled via their *ortho* and *meta* hydroxyl groups forming a hydroxydiarylether. As two methylene groups of the spermidine suffer acylation and led to low field shifts relative to spermidine of ca. 0.5 ppm, there are two possible assignments; either the central nitrogen atom or the two terminal nitrogens are acylated. Consequently the HMQC spectrum (direct ^{13}C–^{1}H correlation) cannot distinguish between these two alternatives but does provide the two possible assignments. Fortunately, the signal to noise of the

HMBC spectrum (long-range ^{13}C–^{1}H correlation) was sufficient to detect the mutual three-bond correlations between the internal methylene groups H-4 and H-6 and the terminal methylene groups with the carbonyl groups. Thus, the data are only compatible with terminal group acylation. The HR ESI-MS data requires closure of a ring system through the aromatic residues in such a way as to provide a close proximity of H-2 of A with H-7 of B which is evident from their strong NOE interaction in the ROESY spectrum. This is only compatible with the isomeric structures (Fig. 1, compound **8**), arising from the two possible orientations of the spermidine moiety which can not be determined unambiguously.

The structure of this compound, the only amide found among the *B. napus* seed constituents, is described for the first time in a member of the Brassicaceae. It is similar to that of previously isolated compounds from the root bark of *Capparis decidua* (Ahmad et al., 1985, 1987). However, the proposed structures in these publications are questionable and Bienz et al. (2002) pointed to some inconsistencies in these studies and suggested alternative structures that are also different to compound **8**.

Most of the esters identified in the present study are known plant constituents, especially from members of the Brassicaceae. For example, compounds **4**, **6**, **9** and **12** have been found to accumulate in *Raphanus sativus* (Linscheid et al., 1980; Strack et al., 1984), *B. napus* (Bouchereau et al., 1992) and besides **4**, **6** and **9** additionally **2** in *Arabidopsis thaliana* (Lorenzen et al., 1996; Goujon et al., 2003). The gentiobiose di- and trisinapate esters (**11** and **13**) are known to accumulate in broccoli (Plumb et al., 1997; Price et al., 1997) together with gentiobioses esterified with sinapate as well as ferulate. Compound **13** was also found in fruits of *Boreava orientalis* (Sakushima et al., 1994). The kaempferol sinapoylglycoside **5** accumulates in leaves of *B. napus* (Olssen et al., 1998) and *B. oleracea* (Nielsen et al., 1993; Llorach et al., 2003) along with the non-acylated substance (**3**). Some other esters identified are to the best of our knowledge new plant products, i.e. one glucose ester (**14**), two gentiobiose esters (**1**, **15**) and two kaempferol sinapoylglucosides (**7**, **10**).

Changes in compound concentrations were followed during the first eight days of seedling development (Table 1). As shown in one of our previous publications on the sinapate ester metabolism in *B. napus* (Milkowski et al., 2004), the level of sinapine (**6**; 100 nmol seed^{-1}) decreases during seed germination resulting in the accumulation of sinapoylmalate (**9**; 86 nmol seed^{-1}) in the seedling cotyledons. Compounds **4**, **6** and **9** are members of the well-known pathway of sinapate esters in Brassicaceae seedlings (Linscheid et al., 1980; Bouchereau et al., 1992; Milkowski et al., 2004): sinapate is conjugated during seed development via 1-*O*-sinapoyl-β-glucose (**4**) with choline, resulting in the accumulation of sinapine (**6**). During seed germination, a sinapine esterase-catalyzed hydrolysis liberates sinapate that is conjugated a second time via sinapoylglucose with malate, resulting in the accumulation of sinapoylmalate (**9**).

Table 1
Content of *B. napus* compounds given as pmol seed^{-1} and developing seedlings (2-, 4- and 8-day-old), calculated as sinapate (**1**, **2**, **4**, **6**, **8**, **9**, **11**–**15**) or kaempferol equivalents (**3**, **5**, **7**, **10**)

Compound	Day 0 (Seed)	Day 2	Day 4	Day 8
1	320	480	n.d.[a]	n.d.
2	Trace	n.d.	n.d.	n.d.
3	692	130	150	115
4	14,500	39,100	13,000	5200
5	2680	1416	1850	1070
6	100,000	n.d.	n.d.	n.d.
7	560	443	292	n.d.
8	960	240	n.d.	n.d.
9	3600	37,800	73,300	86,000
10	281	n.d.	n.d.	n.d.
11	520	2560	2640	2200
12	3360	2480	400	n.d.
13	640	8900	13,800	10,000
14	100	240	n.d.	n.d.
15	100	80	n.d.	n.d.

[a] Not detected.

With regard to the minor compounds, it is interesting to note that along with sinapoylglucose (**4**) and traces of 5-hydroxyferuloylglucose (**2**), the disinapoylglucoses (**12**, **14**) also decrease in concentration during seedling development, whereas the amounts of two gentiobiose esters (**11**, **13**) slightly increase. From the kaempferol conjugates, the amount of **3** decreases slightly, that of **5** stays more or less constant, while **10** and **7** were not detected anymore in the seedlings after day 2 and 8, respectively. The spermidine alkaloid (**8**) also disappears during seedling development. The fate of these compounds is unknown.

2.2. Suppression of sinapate ester biosynthesis

The acyl donors in acyltransferase-catalyzed ester formation are mostly coenzyme A thioesters (Strack and Mock, 1993). It has been shown, however, with a number of plants that an alternative pathway is facilitated by acyltransferases that accept 1-*O*-β-acetal esters (1-*O*-β-glucose esters) (Strack and Mock, 1993; Milkowski and Strack, 2004). These acyltransferases apparently have been recruited from serine carboxypeptidases (SCPs) and adapted to take over acyltransfer functions (SCPL acyltransferase) (Li and Steffens, 2000; Lehfeldt et al., 2000; Shirley et al., 2001; Milkowski and Strack, 2004). According to the SwissProt database the proteome of Arabidopsis harbours 53 SCPL proteins, of which 21 form a distinct group including the known SCPL acyltransferases (Milkowski and Strack, 2004). In the light of the complex pattern of the *B. napus* sinapate esters and some other

hydroxycinnamate conjugates, we assumed that there is a corresponding SCPL acyltransferase family as in Arabidopsis. We addressed this problem by suppressing sinapoylglucose formation, the putative bottleneck reaction that is a prerequisite for the formation of all the *B. napus* sinapate esters.

Suppression of sinapate ester biosynthesis in seeds of *B. napus* was achieved by a dsRNAi approach designed to silence seed specifically the *BnSGT1* gene. In order to achieve this, *B. napus* was transformed with plasmid pLH–SGT–GUS, a dsRNAi construct that contains a part of the *BnSGT1*-encoding region as inverted repeat under the control of the seed-specific napin promoter (Hüsken et al., 2005). HPLC analysis of methanolic seed extracts revealed a strong reduction of sinapine content by 72% compared to the untransformed plants. For plants (T3 seeds from a homozygous T2 plant) with a single copy insertion it could be shown that the reduction of sinapine is a stable trait that seems not to interfere with other important seed characteristics. Currently this is under thorough investigation in our group using a metabolomics approach.

The loss of about 70% sinapine was accompanied by a strong decrease of the total sinapate ester content of almost 80%. The concentration of sinapoylglucose and most of the other sinapate esters seemed to be below the HPLC detection limit (Fig. 2). Thus suppression of the *BnSGT1* gene expression by the dsRNAi approach indicates that sinapoylglucose is indeed the central precursor not only for sinapine, sinapoylmalate and 1,2-disinapoylglucose biosyntheses (Fig. 3; SCT, SMT and SST activities) but also for transacylation reactions leading to the diverse pattern of the other sinapate esters in *B. napus* seeds (putative SCPL acyltransferase activities). We propose that the sinapate esters identified in this study are produced by distinct acyltransferases of the SCPL-type proteins giving rise to a large protein family of SCPL acyltransferases in *B. napus* as assumed for Arabidopsis (Milkowski and Strack, 2004).

Since we could not achieve stronger reduction of sinapine and some other minor components, it might be possible that *BnSGT1*-related genes are involved in sinapate ester biosynthesis. As was found for Arabidopsis (Milkowski et al., 2000b), there are related genes in *B. napus*

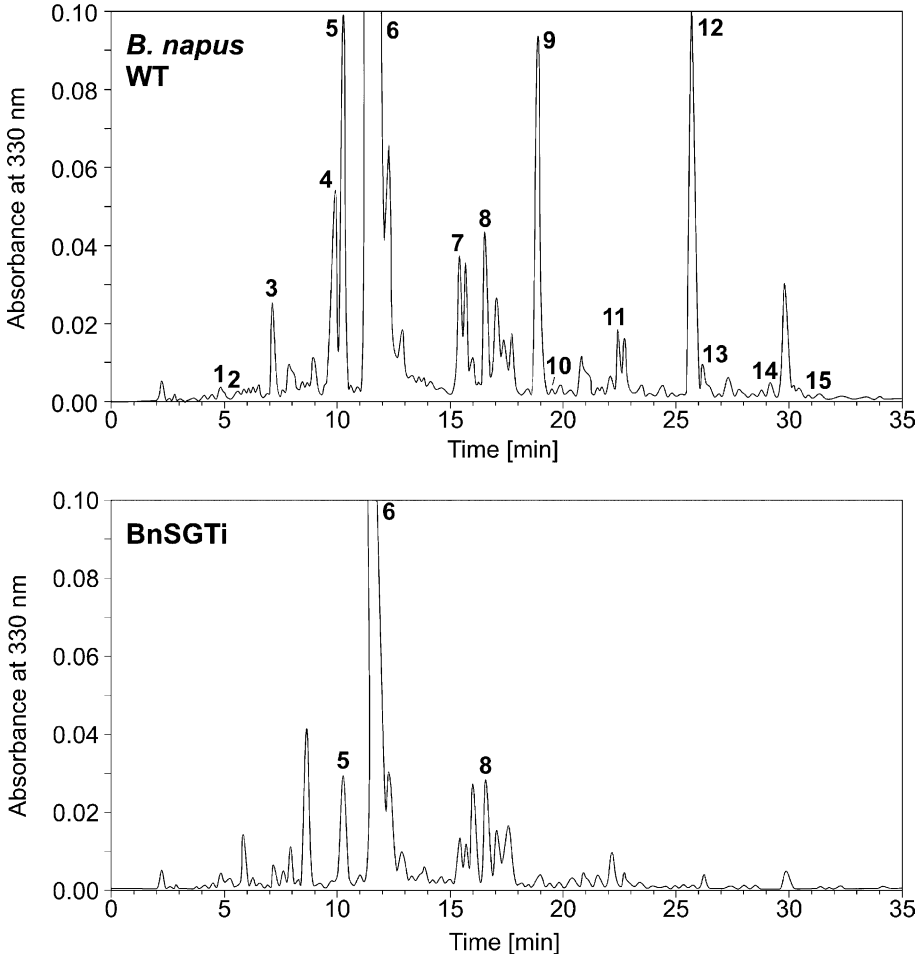

Fig. 2. HPLC traces of methanolic extracts of wild type (**WT**) *Brassica napus* seeds and those carrying the dsRNAi construct pLH–SGT–GUS (**BnSGTi**). Peak numbers correspond to compound numbers in Fig. 1.

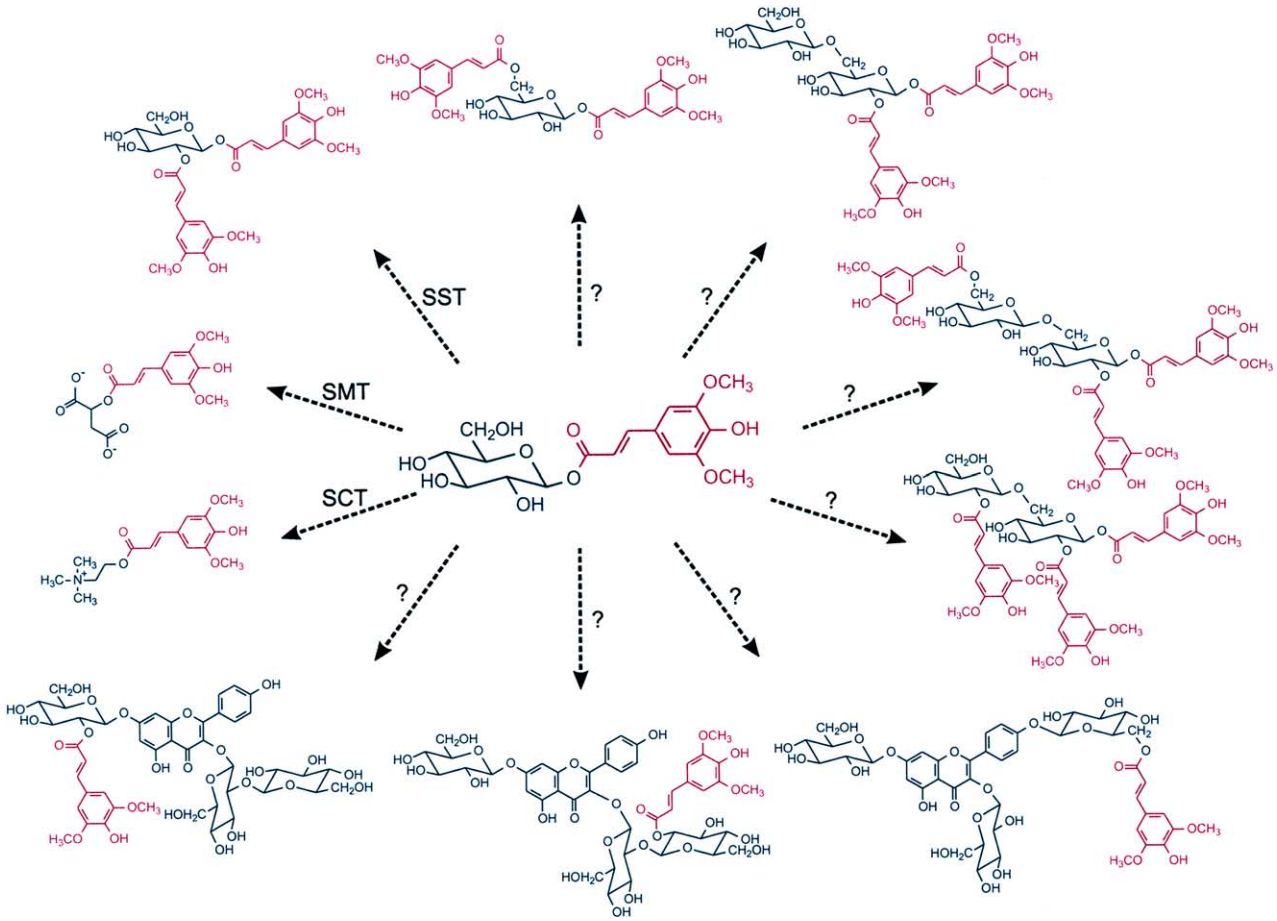

Fig. 3. Proposed *Brassica napus* SCPL acyltransferase family, catalyzing sinapoylglucose-dependent formation of sinapate esters; **SCT** and **SMT**, 1-*O*-sinapoylglucose:choline sinapoyltransferase and 1-*O*-sinapoylglucose:malate sinapoyltransferase from *Brassica napus*; **SST**, assumed to be homologous to the 1-*O*-sinapoylglucose:1-*O*-sinapoylglucose 2-*O*-sinapoyltransferase from *Raphanus sativus* (Dahlbender and Strack, 1986); ?, putative SCPL acyltransferase activities.

encoding enzymes that catalyze (in vitro) the formation of sinapoylglucose, along with other hydroxycinnamoylglucoses (J. Mittasch and C. Milkowski, unpublished). On the other hand, suppression of *BnSGT1* might not be complete. To test suppression strength, experiments are underway to quantify mRNA abundance in developing seeds of T4 plants.

In contrast to hydroxycinnamate esters, up to now the formation of amides seems to be exclusively dependent on coenzyme A activation, see e.g. amide formation with agmatine in barley seedlings (Bird and Smith, 1983) or tyramine and putrescine in tobacco (Negrel and Martin, 1984; Meurer-Grimes et al., 1989). In addition, a spermidine hydroxycinnamoyltransferase, dependent on hydroxycinnamoyl-CoA as acyl donor, has been characterized from tobacco (Negrel et al., 1991). It is most likely that the spermidine amide (**8**) in *B. napus* is also synthesized via coenzyme A thioesters. Thus, in comparison with the control plants, it is not surprising that the amount of this compound does not change significantly in the plants harbouring the dsRNAi construct for suppression of *BnSGT1* expression (Fig. 2).

2.3. Phenolic seed constituents of the progenitors of *B. napus*

We analyzed the phenolic seed constituents of *B. oleracea* and *B. rapa*, the progenitors of *B. napus*. It was shown in a previous publication (Milkowski et al., 2004) that Southern blot analyses of the genes that determine the formation of sinapine, i.e. *BnSGT1* and *BnSCT*, reflect the fact that the genome of the amphidiploid *B. napus* contains the genomes of *B. oleracea* and *B. rapa* (Schenck and Röbbelen, 1982). Thus, it can be assumed that the *B. napus* SCPL acyltransferase gene family most likely derives from the two progenitors. Since we do not know, however, anything about possible gene organization and control of expression of the respective genes, caution is advisable in assuming that the phenolic pattern of *B. napus* seeds is composed

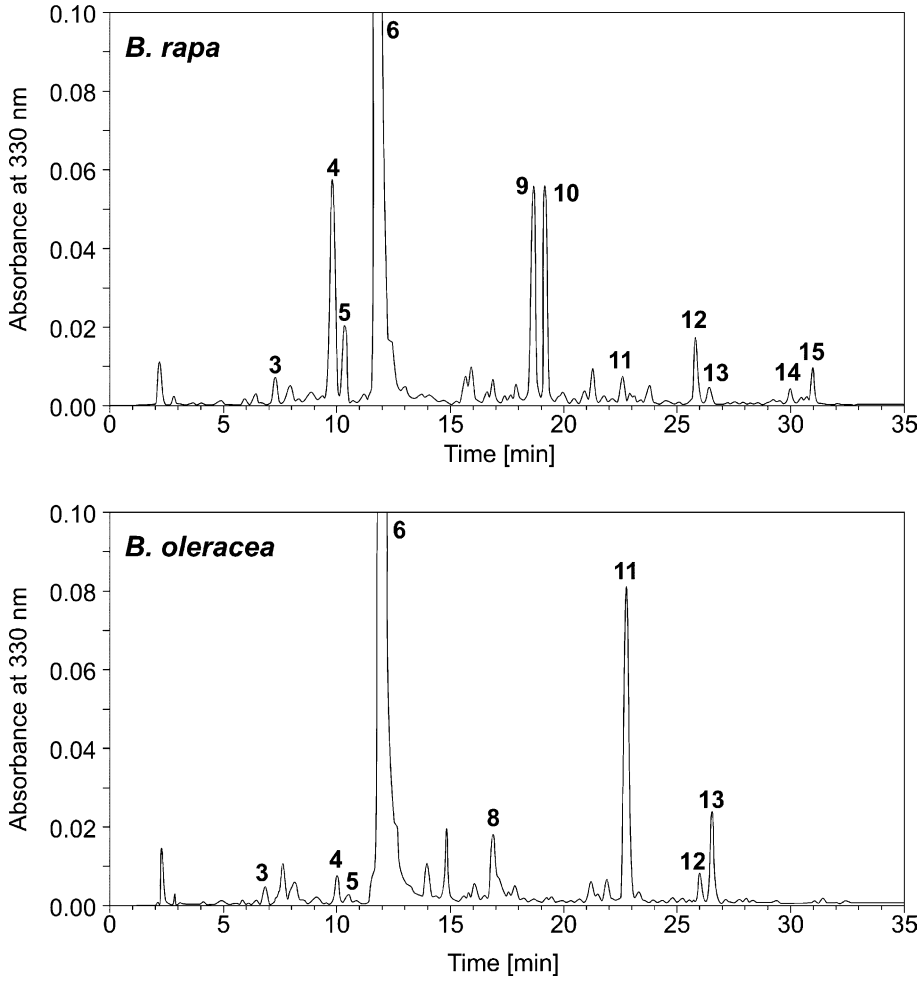

Fig. 4. HPLC traces of methanolic seed extracts from *Brassica oleracea* and *Brassica rapa*. Peak numbers correspond to compound numbers in Fig. 1.

of the compounds detected in the seeds of *B. oleracea* and *B. rapa* (Fig. 4) and the amount of some compounds might be below the HPLC detection limit. Nevertheless, it seems possible that compounds **9**, **10**, **14** and **15** of *B. rapa* add to those found in *B. oleracea* seeds (compare Fig. 4 with the HPLC trace of *B. napus* WT in Fig. 2). Genes involved in the formation of the spermidine amide (**8**) might be derived from *B. oleracea*. It is also interesting to note that formation of sinapoylmalate (**9**) in *B. napus* obviously results from expression of the *B. rapa* SMT gene. It was shown in an earlier publication that red cabbage (*B. oleracea* L. var. *oleracea*) seedlings lack accumulation of sinapoylmalate (Strack et al., 1978), indicating that this plant does not harbour or express the *SMT* gene.

On the genomic level, possible differences in gene organization between A and C genomes combined in the amphidiploid genome of *B. napus* (AACC $n = 19$) and those of the recent cultivars of *B. rapa* (AA, $n = 10$) and *B. oleracea* (CC, $n = 9$) used in this study should be considered (U, 1935). Regarding gene regulation, changes in gene expression patterns are likely to accompany amphidiploid formation to adjust the potential of the novel composite genome to the metabolic need of the plant.

3. Conclusion

In conclusion, we have shown in the present work that the complex pattern of sinapate esters in *B. napus* seeds is dependent on the formation of 1-*O*-sinapoyl-β-glucose. This gives rise to the assumption that enzymes of an SCPL acyltransferase family, known to accept this glucose ester as acyl donor, catalyze the formation of the accumulating esters. With these results we introduce another model system for studying evolution of plant secondary metabolism. As assumed for Arabidopsis (Milkowski and Strack, 2004) and discussed for *B. napus* herein, recruitment of SCPL acyltransferases from hydrolases of the serine carboxypeptidase-type, followed by diversification of the protein structures in plant evolution, leads to a distinct group of enzymes catalyzing an array of structurally diverse hydroxycinnamate esters.

The obvious demand of plant secondary product diversity drives evolution of genetic diversification leading to multigene families.

4. Experimental

4.1. Plant material

Seeds of spring oilseed rape (*Brassica napus* L. var. *napus* cv. Drakkar), forage kale (*B. oleracea* L. var. *medullosa* cv. Markola) and turnip (*B. rapa* L. var. *silvestris* cv. Rex) were purchased from the Norddeutsche Pflanzenzucht, Holtsee, Germany. Seedlings were grown under greenhouse conditions and cotyledons harvested after 2, 4, and 8 days of development. Construction of the dsRNAi suppression plasmid and *Agrobacterium tumefaciens*-mediated transformation and plant regeneration are described elsewhere (Hüsken et al., 2005). Seeds of T2 plants (T3 seeds) were analyzed to evaluate the effect of seed-specific suppression of *BnSGT1* gene expression.

4.2. Extraction and isolation

Seed material (400 g) was first extracted with hexane using an Ultra-Turrax homogenizer. After centrifugation, the defatted pellets were extracted three times with 80% aq. MeOH. The combined and concentrated extracts were chromatographed on Polyamide SC6 (Macherey and Nagel, Düren, Germany), stepwise eluted with H_2O, 50% aq. MeOH and 100% MeOH. Sinapine, the main product in the water fraction, was separated from other seed constituents by formation of the poorly soluble crystalline thiocyanate after addition of solid KSCN prior to further chromatographic steps.

The polyamide eluates were concentrated and further chromatographed by repeated preparative HPLC using a DELTAPAK 300/50 C18 column (Waters, USA) or VP 250/40 Nucleosil 100-10 C18 (Macherey-Nagel, Düren, Germany) using different gradients corresponding to the separation with 1% aq. HOAc (solvent A) and MeCN or MeOH (solvent B) with flow rates of 20 ml min^{-1}. The compounds were photometrically detected (HPLC-DAD, 330 nm). All compounds were finally purified on a Sephadex LH-20 column with 50% aq. MeOH as solvent.

4.3. Analytical HPLC

Fifty seeds or cotyledon pairs at different developmental stages were extracted three times with 80% aq. MeOH and the combined extracts were concentrated to dryness, redissolve in 2 ml 80% aq. MeOH, centrifuged and aliquots injected onto a Nucleosil C18 column (5 μm; 250 × 4 mm i.d.; Macherey-Nagel, Düren, Germany). Separation was achieved using a 40-min linear gradient at 1 ml min^{-1} from 10% to 50% MeCN in 1.5% aq. H_3PO_4. Compounds were photometrically detected (at 330 and 265 nm; maxplot between 210 and 500 nm) by a Waters 2996 photodiode array detector (DAD). Calibration data for quantification were obtained from sinapate for the hydroxycinnamate esters except from kaempferol as standard compound for the kaempferol conjugates.

4.4. NMR

All 1D and 2D [COSY, ROESY (mixing time 500 ms)] ^1H, and 2D ^1H detected ^{13}C–^1H [HMQC, HMBC] NMR spectra were recorded at 300 K on either a Bruker AVANCE DMX 600 or ARX 400 NMR spectrometers locked to the major deuterium signal of the solvent, CD_3OD. Chemical shifts are given in ppm relative to the residual solvent signal at 3.35 ppm and coupling constants in Hz.

4.5. HR ESI-FT-ICR-MS

The high resolution electrospray ionization (HR ESI) mass spectra were obtained from a Bruker Apex 70 e Fourier transform ion cyclotron resonance mass spectrometer (FT-ICR-MS) Bruker Daltonics, Billerica, USA) equipped with an Infinity^{TM} cell, a 7.0 T superconducting magnet (Bruker, Karlsruhe, Germany), an RF-only hexapole ion guide and an external electrospray ion source (Agilent, off axis spray). Nitrogen was used as drying gas at 150 °C. The sample solutions were introduced continuously via a syringe pump with a flow rate of 120 l h^{-1}. All data were acquired with 256k data points and zero filled to 1024k by averaging 32 scans.

4.6. ESI-MS/MS

The positive ion ESI-MS/MS data of the spermidine conjugate (**8**) were obtained from a Finnigan MAT TSQ 7000 instrument (electrospray voltage 4.5 kV; heated capillary temperature 220 °C; sheath gas: nitrogen) coupled with a Surveyor MicroLC system equipped with a RP18 column (5 μm, 1 × 100 mm, Ultrasep). For HPLC a gradient system was used starting from H_2O:MeCN 85:15 (each contained 0.3% HOAc) to 10:90 within 15 min followed by a 10-min isocratic period; flow rate 70 μl min^{-1}. The collision-induced dissociation (CID) mass spectrum of the $[M + H^+]$ of compound 8 (RT = 6.10 min) was performed during the HPLC run using a collision of 35 eV; collision gas: argon, collision pressure: 1.8×10^{-2} Torr.

4.7. 1-O-E-Caffeoyl-β-gentiobiose (1)

UV (HPLC-DAD): λ_{max} (nm) 217, 235, 300sh, 321. ^{1}H NMR: δ = 7.74 [*d*; H-7; *J*(7–8) 15.9], 7.58 [*d*; H-2; *J*(2–6) 2.0], 7.24 [*dd*; H-6; *J* (5–6) 8.4], 6.91 [*d*; H-5], 6.43 [*d*; H-8], 5.61 [*d*; H-1′, *J*(1′–2′) 7.9], 4.85 [*d*; H-1″, *J*(1″–2″) 7.4], 3.98 [*dd*; H-6′A; *J*(5′–6′A) 2.2, *J*(6′A–6′B) 12.1], 3.89 [*dd*; H-6″A; *J*(5″–6″A) 2.0, *J*(6″A-6″B) 12.2]; 3.76 [*dd*; H-6′B; *J*(5′–6′B) 6.1], 3.73 [*dd*; H-6″B; *J*(5″–6″B) 4.6], 3.57 [*dd*; H-2″; *J*(2″–3″) ca. 9], 3.52 [*m*; H-5′], 3.47 [*m*; H-2′, H-5″], 3.56-3.39 [*m*; rest sugar protons]. HR-(+)ESI-MS: 527.1372 $[M + Na]^+$ (calc. for $C_{21}H_{28}O_{14}Na$ 527.1377), HR-(−)ESI-MS: 503.1401 $[M - H]^-$ (calc. for $C_{21}H_{27}O_{14}$ 503.1406).

4.8. 1-O-E-(5-Hydroxyferuloyl)-β-glucopyranose (2)

UV (HPLC-DAD): λ_{max} (nm) 238, 331. ^{1}H NMR: δ = 7.69 [*d*; H-7; *J*(7–8) 15.9], 6.83, 6.80 [*d* × 2; H-2, H-6; *J*(2–6) 1.7], 6.38 [*d*; H-8], 5.61 [*d*; H-1′; *J*(1′–2′) 7.9], 3.92 [*s*; 3-OMe], 3.89 [*dd*; H-6′A; *J* (5′–6′A) 2.0, *J*(6′A–6′B) 12.1], 3.73 [*dd*; H-6′B; *J*(5′–6′B) 4.9], 3.50 [*dd*; H-2′; *J*(2′–3′) 9.4], 3.46 [*m*; H-5′], 3.47–3.40 [*m*; H-3′, H-4′]. HR-(+)ESI-MS: 395.0956 $[M + Na]^+$ (calc. for $C_{16}H_{20}O_{10}Na$ 395.0949), HR-(−)ESI-MS: 371.0986 $[M - H]^-$ (calc. for $C_{16}H_{19}O_{10}$ 371.0984).

4.9. Kaempferol 3-O-sophoroside-7-O-β-glucopyranoside (3)

UV (HPLC-DAD): λ_{max} (nm) 265, 331. ^{1}H NMR: δ = 8.11 ["*d*"; H-2′/6′; *J*(2′–3′) + (2′–5′) 8.9], 6.96 ["*d*"; H-3′/5′], 6.81 [*d*; H-8; *J*(6–8) 2.1], 6.54 [*d*; H-6], sophorose moiety: 5.54 [*d*; H-1; *J* (1–2) 7.4], 4.79 [*d*, H-1′ overlap with water signal], 3.82 [*dd*; H-6′A; *J*(5′–6′A) 2.5, *J* (6′A-6′B) 12.0], 3.78 [overlap; H-2], 3.75 [*dd*; H-6A], 3.73 [*dd*; H-6′B], 3.65 [*dd*; H-3; *J*(2–3) 8.9, *J*(3–4) 8.9], 3.53 [*dd*; H-6B], 3.45-3.40 [overlap; H-2′, H-3′, H-4′], 3.39 [*dd*; H-4], 3.33 [*ddd*; H-5′; *J*(5′–6′B) 6.5, *J*(4′–5′) 9.2], 3.25 [*ddd*; H-5; *J*(4–5) 9.8, *J*(5–6A) 2.2, *J*(5–6B) 5.5], glucose moiety: 5.10 [*d*; H-1; *J*(1–2) 7.5], 3.96 [*dd*; H-6A; *J*(5–6A) 2.2, *J* (6A–6B) 12.1], 3.75 [*dd*; H-6B], 3.57 [*ddd*; H-5; *J*(5–6B) 5.7, *J*(4–5) 9.6], 3.52 [*m*; H-2, H-3], 3.44 [*m*; H-4]. HR-(+)ESI-MS: 795.1976 $[M + Na]^+$ (calc. for $C_{33}H_{40}O_{21}Na$ 795.1954), HR-(−)ESI-MS: 771.1981 $[M - H]^-$ (calc. for $C_{33}H_{39}O_{21}$ 771.1989).

4.10. Kaempferol 3-(2″-O-E-sinapoylsophoroside)-7-O-β-glucopyranoside (5)

UV (HPLC-DAD): λ_{max} (nm) 226, 240sh, 268, 333. ^{1}H NMR: δ = 7.95 ["*d*"; H-2′/6′; *J*(2′–3′) + (2′–5′) 8.9], 6.94 ["*d*"; H-3′/5′], 6.46 [*d*; H-8; *J*(6–8) 2.1], 6.41 [*d*; H-6], sinapoyl moiety: 7.36 [*d*; H-7; *J*(7–8) 15.8], 6.33 [*s*; H-2/6], 6.14 [*d*; H-8], 3.67 [*s*; 3,5-OMe], sophorose moiety: 6.17 [*d*; H-1; *J*(1-2) 7.9], 5.25 [*d*; H-1′, *J*(1′–2′) 7.8], 4.96 [*dd*; H-2′; *J*(2′–3′) 9.5], 3.96 [*dd*; H-6′A; *J*(5′–6′A) small, *J*(6′A–6′B) ∼ 11.5], 3.81 [*dd*; H-6′B], 3.80 [*dd*; H-3′], 3.73 [*dd*; H-3; *J*(2-3) 9.0, *J*(3–4) 9.0], 3.70 [*dd*; H-6A; *J*(5–6A) 2.1, *J*(6A–6B) 12.2], 3.57 [*m*; H-5′], 3.60–3.55 [*m*; H-4′], 3.55 [*dd*; H-2], 3.52 [*dd*; H-6B], 3.35 [*dd*, H-4], 3.29 [*ddd*; H-5; *J*(5–6B) 5.4], glucose moiety: 5.13 [*d*; H-1; *J*(1-2) 7.69], 3.98 [*dd*; H-6A; *J*(5–6A) 2.3, *J*(6A–6B) 12.1], 3.77 [*dd*; H-6B; *J*(5–6B) 5.8], 3.61 [*dd*; H-3; *J*(2–3) 9.1, *J*(3–4) 9.1], 3.60 [*ddd*; H-5; *J*(4–5) 9.6], 3.53 [overlap; H-2], 3.45 [*dd*, H-4]. HR-(−)ESI-MS: 977.2538 $[M - H]^-$ (calc. for $C_{44}H_{49}O_{25}$ 977.2568).

4.11. Kaempferol 4′-(6-O-E-sinapoyl-β-glucopyranoside)-3,7-di-O-β-glucopyranoside (7)

UV (HPLC-DAD): λ_{max} (nm) 223, 240sh, 267, 321. ^{1}H NMR: δ = 8.06 ["*d*"; H-2′/6′; *J*(2′–3′) + (2′–5′) 9.0], 7.18 ["*d*"; H-3′/5′], 6.53, 6.53 [*d* × 2; H-8, H-6; *J*(6–8) 2.2], sinapoyl moiety: 7.57 [*d*; H-7; *J*(7–8) 15.9], 6.86 [*s*; H-2/6], 6.41 [*d*; H-8], 3.89 [*s*; 3,5-OMe], 4′-glucose moiety: 5.09 ["*d*"; H-1; *J*(1–2) 7.7], 4.57 [*dd*, H-6A, *J*(5–6A) 2.5, *J*(6A–6B) 11.9], 4.50 [*dd*; H-6B; *J*(5–6B) 7.3], 3.82 [*m*; H-5], 3.60–3.43 [*m*; H-4], 3.58 [*m*; H-2, H-3], 3-glucose moiety: 5.34 ["*d*"; H-1; *J*(1–2) 7.6], 3.73 [*m*; H-6A], 3.57 [*m*; H-6B], 3.45 [*m*; H-2, H-3], 3.35 [*m*; H-4], 3.25 [*ddd*; H-5; *J*(5–6A) 2.4, *J*(5–6B) 5.5, *J*(4–5) 9.6], 7-glucose moiety: 5.11 ["*d*"; H-1; *J*(1–2) 7.9], 3.94 [*dd*; H-6A; *J*(5–6A) 2.3, *J*(6A–6B) 12.2], 3.74 [*m*; H-6B], 3.60–3.43 [*m*; H-4], 3.56 [*m*; H-2, H-3], 3.55 [*m*; H-5]. HR-(+)ESI-MS: 1001.2516 $[M + Na]^+$ (calc. for $C_{44}H_{50}O_{25}Na$ 1001.2533), HR-(−)ESI-MS: 977.2559 $[M–H]^-$ (calc. for $C_{44}H_{49}O_{25}$ 977.2568).

4.12. Spermidine conjugate (8)

UV (HPLC-DAD): λ_{max} (nm) 236, 305, 310sh. ^{1}H NMR: δ = aromatic moiety A: 7.34 [*d*; H-7; *J*(7–8) 15.7], 7.19 [*dd*; H-6; *J*(2–6) 1.8, *J*(5–6) 8.4], 7.12 [*d*; H-5], 6.78 [*d*; H-2], 6.18 [*d*; H-8], 4.02 [*s*; 4-OMe], aromatic moiety B: 7.64 [*d*; H-7; *J*(7–8) 15.7], 7.31 [*d*; H-6; *J*(5–6) 8.7], 6.99 [*d*; H-5], 6.59 [*d*; H-8], spermidine methylene groups H-2 to H-4 and H-6 to H-9: 3.43 [*m*; H-2] 3.41 [H-9], 2.93 [*m*; H-6], 2.84 [*m*; H-4], 1.88 [*m*; H-3], 1.67 [*m*; H-7, H-8]. ^{13}C NMR (CD_3OD + trace CF_3CO_2H): δ = aromatic moiety A: 129.0 (C-1), 115.1 (C-2), 149.5 (C-3), 152.5 (C-4), 114.1(C-5) 124.9 (C-6), 141.6 (C-7), 119.8 (C-8), 168.8 (C-9), 56.8 (4-OMe), aromatic moiety B: 124.0 (C-1), 143.2 (C-2), 140.8 (C-3), 152.3 (C-4), 110.1 (C-5), 118.3 (C-6), 137.3 (C-7), 119.8 (C-8), 168.9 (C-9), 56.8 (4-OMe), spermidine: 36.8 (C-2), 27.6 (C-3), 45.8 (C-4), 48.5 (C-6), 26.9, 24.1 (C-7, C-8), 39.1 (C-9). HR-(+)ESI-MS: 496.2442 $[M + H]^+$ (calc. for $C_{27}H_{34}N_3O_6$ 496.2442), HR-(−)ESI-MS: 494.2288 $[M - H]^-$ (calc. for $C_{27}H_{32}N_3O_6$ 494.2297). ESI-CIDMS, *m/z* (rel. intensity, %): 496 ($[M + H]^+$, 14),

479 (5), 478 ([M + H − H_2O]$^+$, 5), 425 (7), 408 ([M + H-putrescine]$^+$, 100), 351 ([M + H-spermidine]$^+$, 18), 325 (12), 283 (5), 268 (6), 232 (8), 216 (5), 191 (6), 175 (18), 149 (12).

4.13. Kaempferol 3-O-sophoroside-7-O-(2-O-E-sinapoyl-β-glucopyranoside) (**10**)

UV (HPLC-DAD): λ_{max} (nm) 240, 265, 335. ^{1}H NMR: δ = 8.08 ["*d*"; H-2′/6′; *J*(2′–3′)+(2′–5′) 8.8], 6.92 ["*d*"; H-3′/5′], 6.74 [*d*; H-8; *J*(6–8) 2.1], 6.45 [*d*; H-6], sinapoyl moiety: 7.71 [*d*; H-7; *J*(7–8) 15.9], 6.93 [*s*; H-2/6], 6.46 [*d*; H-8], 3.89 [*s*; 3,5-OMe], sophorose moiety: 5.52 [*d*; H-1; *J*(1-2) 7.5], 4.78 [*d*, H-1′ overlap with water signal], 3.81 [*dd*; H-6′A; *J*(5′–6′A) 2.6, *J*(6′A–6′B) 11.9], 3.76 [overlap; H-2], 3.71 [*dd*; H-6A; *J*(5–6A) 2.1, *J*(6A–6B) 11.9], 3.71 [*dd*; H-6′B; *J*(5–6B) 5.0], 3.64 [*dd*; H-3; *J*(2–3) 9.0, *J*(3–4) 9.0], 3.50 [*dd*; H-6B; *J*(5–6B) 5.6], 3.45–3.40 [overlap; H-2′, H-3′, H-4′], 3.38 [*dd*; H-4], 3.32 [*ddd*; H-5′; *J*(4′–5′) 9.3], 3.23 [*ddd*; H-5; *J*(4–5) 9.7], glucose moiety: 5.38 [*d*; H-1; *J*(1–2) 8.0], 5.16 [*dd*; H-2; *J*(2-3) 9.5, 4.00 [*dd*; H-6A; *J*(5–6A) 2.1, *J*(6A–6B) 12.0], 3.80 [*dd*; H-6B; *J*(5–6B) 5.6], 3.79 [*dd*; H-3], 3.67 [*ddd*; H-5; *J*(4–5) 9.7], 3.57 [*dd*; H-4; *J*(3–4) 9.0]. HR-(+)ESI-MS: 1001.2516 [M + Na]$^+$ (calc. for $C_{44}H_{50}O_{25}Na$ 1001.2533), HR-(−)ESI-MS: 977.2548 [M − H]$^-$ (calc. for $C_{44}H_{49}O_{25}$ 977.2568).

4.14. 1,2-Di-O-E-sinapoyl-β-gentiobiose (**11**)

UV (HPLC-DAD): λ_{max} (nm) 240, 330. ^{1}H NMR: δ = 7.68, 7.67 [*d*×2; H-7^A, H-7^B; *J*(7^A–8^A)/*J*(7^B–8^B)15.8, 15.8], 6.93, 6.89 [*s*×2; H-2^A/6^A, H-2^B/6^B], 6.45, 6.37 [*d*×2; H-8^A, H-8^B], 5.82 [*d*; H-1′; *J*(1′–2′) 8.4], 5.13 [*dd*; H-2′; *J*(2′–3′) 9.5], 4.41 [*d*; H-1″, *J*(1″–2″) 7.8], 4.26 [*dd*; H-6′A; *J*(6′A–5′) 1.8, *J*(6′A–6′B) 11.4], 3.90 [*m*; H-6″A], 3.89 [*m*, H-6′B], 3.90, 3.88 [*s*×2; 3^A/5^A-OCH_3, 3^B/5^B-OCH_3], 3.77 [*dd*; H-3′; *J*(3′–2′) 9.1, *J*(3′-4′) 9.3], 3.73 [*m*; H-5′], 3.72 [*dd*; H-6″B; *J*(6″B–5″) 5.4, *J*(6″B–6″A) 11.8], 3.64 [*dd*; H-4′; *J*(4′–5′) 9.4], 3.41 [*dd*; H-3″; *J*(3″–2″) 9.1, *J*(3″–4″) 9.2], ~3.35 [*m*, H-4″], 3.31 [*m*; H-5″], 3.28 [*dd*, H-2″]. HR-(+)ESI-MS: 777.2187 [M + Na]$^+$ (calc. for $C_{34}H_{42}O_{19}Na$ 777.2213), HR-(−)ESI-MS: 753.2258 [M-H]$^-$ (calc. for $C_{34}H_{41}O_{19}$ 753.2248).

4.15. 1,2-Di-O-E-sinapoyl-β-glucopyranose (**12**)

UV (HPLC-DAD): λ_{max} (nm) 239, 330. ^{1}H NMR: δ = 7.68, 7.67 [*d*×2;H-7^A, H-7^B; *J* (7^A-8^A)/*J*(7^B-8^B) 15.9, 15.8], 6.92, 6.89 [*s*×2; H-2^A/6^A, H-2^B/6^B], 6.45, 6.37 [*d*×2; H-8^A, H-8^B], 5.84 [*d*; H-1′; *J*(1′–2′) 8.3], 5.11 [*dd*; H-2′; *J*(2′–3′) 9.6], 3.94 [*dd*; H-6′A; *J*(6′A–5′) 1.6, *J* (6′A-6′B) 12.3], 3.90, 3.88 [*s*×2; 3^A/5^A-OCH_3, 3^B/5^B-OCH_3], 3.79 [*dd*; H-6′B; *J*(6′B–5′) 4.8], 3.76 [*m*; H-3′], 3.58–3.53 [*m*; H-4′, H-5′]. HR-(+)ESI-MS: 615.1690 [M + Na]$^+$ (calc. for $C_{28}H_{32}O_{14}Na$ 615.1684), HR-(−)ESI-MS: 591.1723 [M − H]$^-$ (calc. for $C_{28}H_{31}O_{14}$ 591.1719).

4.16. 1,2,2′-Tri-O-E-sinapoyl-β-gentiobiose (**13**)

UV (HPLC-DAD): λ_{max} (nm) 239, 327. ^{1}H NMR: δ = 7.79, 7.63, 7.60 [*d*×3; H-7^A, H-7^B, H-7^C; *J*(7^A–8^A)/*J*(7^B–8^B)/*J*(7^C–8^C) 15.9, 15.8, 15.8], 7.02, 6.89, 6.87 [*s*×3; H-2^A/6^A, H-2^C/6^C, H-2^B/6^B], 6.61, 6.38, 6.27 [*d*×3; H-8^A, H-8^C, H-8^B], 5.78 [*d*; H-1′, *J*(1′–2′) 8.3], 5.04 [*dd*; H-2′, *J*(2′-3′) 9.5], 4.86 [*dd*; H-2″, *J*(1″–2″) 8.0, *J*(2″–3″) 9.4], 4.76 [*d*; H-1″], 4.19 [*dd*; H-6′A; *J*(5′–6′) ca. 1, *J*(6′A–6′B) 12.1], 3.93 [*dd*; H-6″A; *J*(5″–6″A) 2.2, *J*(6″A–6″B) 11.8], 3.89, 3.89, 3.86 [*s*×3; 3^B/5^B-OCH_3,3^C/5^C-OCH_3,3^A/5^A-OCH_3], 3.85 [*dd*; H-6′B; *J*(5′–6′B) 6.1] 3.74 [*dd*; H-6″B; *J*(5″–6″B] 5.8], 3.62 [*dd*; H-3″; *J*(3″–4″) 9.1], 3.62 [*ddd*; H-5′], 3.61 [*dd*; H-3′; *J*(3′–4′) 9.2], 3.44 [*dd*; H-4″; *J*(4″–5″) 9.5], 3.41 [*dd*; H-4′; *J*(4′–5′) 9.5], 3.34 [*ddd*; H-5″]. HR-(+)ESI-MS: 983.2794 [M + Na]$^+$ (calc. for $C_{45}H_{52}O_{23}Na$ 983.2792), HR-(−)ESI-MS: 959.2823 [M − H]$^-$ (calc. for $C_{45}H_{51}O_{23}$ 959.2827).

4.17. 1,6-Di-O-E-sinapoyl-β-glucopyranose (14)

UV (HPLC-DAD): λ_{max} (nm) 239, 329. ^{1}H NMR: δ = 7.74, 7.6 [*d*×2; H-7^A, H-7^B; *J*(7^A–8^A)/*J*(7^B–8^B)15.9, 15.9], 6.95, 6.94 [*s*×2; H-2^A/6^A, H-2^B/6^B], 6.45, 6.45 [*d*×2; H-8^A, H-8^B], 5.64 [*d*; H-1′; *J*(1′–2′) 7.5], 4.55 [*dd*; H-6′A; *J*(5′–6′A) 2.1, *J*(6′A–6′B) 12.1], 4.38 [*dd*; H-6′B; *J*(5′–6′B) 5.9], 3.91, 3.91 [*s*×2; 3^A/5^A-OCH_3,3^B/5^B-OCH_3], 3.73 [*ddd*; H-5′; *J*(4′–5′) 9.2], 3.55 [*dd*; H-2′; *J*(2′–3′) ca. 8.9], 3.53-3.45 [*m*; H-3′, H-4′]. HR-(+)ESI-MS: 615.1681 [M + Na]$^+$ (calc. for $C_{28}H_{32}O_{14}Na$ 615.1684), HR-(−)ESI-MS: 591.1719 [M − H]$^-$ (calc. for $C_{28}H_{31}O_{14}$ 591.1719).

4.18. 1,2,6′-Tri-O-E-sinapoylgentiobiose (**15**)

UV (HPLC-DAD): λ_{max} (nm) 239, 328. ^{1}H NMR: δ = 7.65, 7.64, 7.63 [*d*×3; H-7^A, H-7^B, H-7^C; *J*(7^A–8^A)/*J* (7^B–8^B)/*J*(7^C–8^C) 15.9, 15.9, 15.8], 6.93, 6.87, 6.86 [*s*×3; H-2^A/6^A, H-2^B/6^B, H-2^C/6^C], 6.47, 6.41, 6.32 [*d*×3; H-8^A, H-8^B, H-8^C], 5.84 [*d*; H-1′, *J*(1′–2′) 8.4], 5.12 [*dd*; H-2′, *J*(2′–3′) 9.5], 4.62 [*dd*; H-6″A; *J*(5″–6″A) 2.0, *J*(6″A–6″B) 11.9], 4.48 [*d*; H-1″; *J*(1″–2″) 7.8], 4.33 [*dd*; H-6″B; *J*(5″–6″B) 6.0], 4.21 [*dd*; H-6′A; *J*(5′–6′A) 1.6, *J*(6′A–6′B) 11.8], 3.94 [*dd*; H-6′B], 3.89, 3.87, 3.86 [*s*×3; 3^A/5^A-OCH_3, 3^B/5^B-OCH_3, 3^C/5^C-OCH_3], 3.80 [*m*; H-5′], 3.78 [*dd*; H-3′; *J*(3′–4′) 9.6], 3.57 [*dd*; H-4′; *J*(4′–5′) 9], 3.45 [*dd*; H-3″; *J*(2″–3″) 8.9, *J* (3″–4″) 8.9], 3.41 [*dd*; H-4″; *J* (4″–5″) 9.3], 3.30 [*dd*; H-2″]. HR-(+)ESI-MS: 983.2778 [M + Na]$^+$ (calc. for $C_{45}H_{52}O_{23}Na$ 983.2792), HR-(−)ESI- MS: 959.2804 [M − H]$^-$ (calc. for $C_{45}H_{51}O_{23}$ 959.2827).

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