The Structures of Sinapic Acid Esters and Their Metabolism in Cotyledons of *Raphanus sativus*

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Five sinapic acid esters were isolated from cotyledons of *Raphanus sativus* and have been identified with the aid of electron impact (EI)-, field desorption (FD)-mass spectrometry and NMR spectroscopy: 6,3′-disinapoylsucrose, sinapoylcholine (sinapine), 6-sinapoylglucoraphenine, 1-sinapoylglucose, and sinapoylmalate. Three of these derivatives are metabolically related in the sequence sinapoylcholine → 1-sinapoylglucose → sinapoylmalate.

Introduction

Cotyledons of *Raphanus sativus* exhibit a complex pattern of sinapic acid derivatives which undergo marked quantitative changes [1]. Besides sinapoylcholine (sinapine), which is widespread in Brassicaceae seeds [2], another ester was found in growing cotyledons to be 1-sinapoylglucose [3].

At present, in cotyledons of *Raphanus* three new major sinapic acid esters have been found [1] and this report presents their structural elucidation. In addition the known sinapoylcholine and 1-sinapoylglucose were reinvestigated and their structures verified.

Detailed studies on the quantitative changes of the five sinapic acid esters in *Raphanus* and the enzymatic studies in ref. [4–6] lead to the proposal that sinapoylcholine is interconverted to sinapoylmalate *via* the intermediate 1-sinapoylglucose. The compounds are designated with the symbols B-1 through B-5, according to the first study on this subject [1].

Materials and Methods

Plant material and culture conditions

Plant source and culture conditions for analytical work are described in ref. [1]. Plants used for preparative isolation of sinapoyl derivatives were grown in a greenhouse.

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Isolation of sinapoyl derivatives

Extraction and chromatography of sinapoyl derivatives were carried out as described in ref. [1]. Purification of these isolated compounds was achieved on Sephadex LH-20 (Pharmacia, Uppsala, Sweden) (90×2 cm) eluted with CH_3OH. B-1, B-3, and B-4 were isolated from dry seeds. B-2 came form 3-day and B-5 from 8-day old seedlings. Elution on polyamide CC 6: water, B-1 and B-2; 60% CH_3OH in water, B-3; 0.035% NH_4OH in CH_3OH, B-4 and B-5.

Instrumentation

High performance liquid chromatography (HPLC) for studying the kinetics of sinapoyl derivatives from *Raphanus* germination is described in ref. [4, 7]. The HPLC columns were LiChrosorb RP-8 and LiChrosorb Si-60 (both 5 µm, 250×4 mm) (Merck, Darmstadt).

Mass spectrometry was carried out with a MAT 731 spectrometer (Varian MAT, Bremen), equipped with a FD/FI ion source and a total ion current controlled emitter heating device; spectra were recorded with a strip chart recorder. Accurate masses were evaluated by peak matching at a minimum resolu-

tion of 10000 (10% valley definition). For ^1H NMR spectroscopy, a Varian EM 390 CW spectrometer (90 MHz) and a Bruker WH-360 FT spectrometer (360 MHz) were used. ^{13}C NMR spectroscopy was carried out with a Bruker WH-360 FT spectrometer (90.52 MHz).

Derivatization

Silylation was achieved with BSTFA/CH_3CN at 80 °C. The samples were evaporated to dryness and redissolved in $CHCl_3$ for mass spectrometry.

For acetylation, samples were dissolved in $(CH_3CO)_2O$/pyridine (1:3) and kept at room temperature for 36 h. Products were analyzed by TLC on silicagel SI F (Riedel de Haën, Seelze) with $CHCl_3/CH_3OH$ in various ratios. For mass spectrometry, samples were dissolved in $CHCl_3$. For NMR spectroscopy, the acylated products were purified on silicagel columns eluted with $CHCl_3/CH_3OH$ (gravity or high pressure flow).

For methylation, samples were treated in CH_3OH with diazomethane in ether, evaporated to dryness and dissolved in $CHCl_3$.

Accurate mass measurements

B-3. *m/e* 574: 574.1681 (0.51 mmu err.), $C_{28}H_{30}O_{13}$.

B-4. To find the values for *m/e* 402 and 386, the series of fragmentations indicated in Table I was used; *e.g.*, *m/e* $554-4\times CH_2CO$ gives *m/e* 386 and $408-CH_2CO+2\,H_2O$ gives *m/e* 402. By this procedure, the ions in both series were reduced to 402 and 386, respectively. Three (of five) accurate mass values were used to calculate an average so that the elemental composition of *m/e* 402 could be assigned without ambiguity.

B-5. *m/e* 322: 322.0692 (0.34 mmu err.), $C_{15}H_{14}O_8$; *m/e* 224: 224.0672 (1.20 mmu err.), $C_{11}H_{12}O_5$.

Results and Discussion

Sinapoylcholine (B-1, MW: 327, $C_{16}H_{24}NO_5^+OH^-$) *and 1-sinapoylglucose* (B-2, MW: 386, $C_{17}H_{22}O_{10}$)

In the 90 MHz ^1H NMR spectrum of B-1, the signals of sinapic acid (Table II) and those of the choline moiety (Table III) were found as expected. The most abundant ion in FDMS is *m/e* 310 (Fig. 1), which represents the quarternary ammonium cation of the salt. The anion of sinapoylcholine present in the cotyledons has not been investigated and the OH^- in B-1 was assumed to be produced by chromatographic procedures.

Table I. Elemental composition of some ions in the EI mass spectrum of B-4 acetate (peakmatching with PFK as reference).

m/e	Error [mmu]	Elemental composition		
570.1398	−0.9			$C_{25}H_{30}O_{13}S$
554.1646	1.1	$C_{25}H_{30}O_{14}$	$-CH_3COOH$	
542.1462	0.4		$-HO$	$C_{24}H_{30}O_{12}S$
537.1603	−0.1	$C_{25}H_{29}O_{13}$	$-CH_3COOH$	
528.1305	0.3		$-(CH_3COOH)_2$	$C_{23}H_{28}O_{12}S$
496.1555*	−2.6	$C_{23}H_{28}O_{12}$	$-CH_3COOH$	
494.1430	0.5	$C_{23}H_{26}O_{12}$	$-CH_3COOH$	
436.1364	−0.6	$C_{21}H_{24}O_{10}$	$-(CH_3COOH)_2$	
408.0887	0.8			$C_{19}H_{20}O_8S$
392.1111	0.3	$C_{19}H_{20}O_9$	$-CH_2CO$	
375.1090	1.1	$C_{19}H_{19}O_8$	$-(CH_3COOH)_2$	
Calculated				
402.0984	0.0			$C_{17}H_{22}O_9S$
386.1219	0.4	$C_{17}H_{22}O_{10}$		

* *m/e* 494 was taken as reference.

The structure of B-2 was confirmed by ^1H NMR and the mass spectrum of its TMS derivative (Fig. 2). The signal for the molecular ion appears at *m/e* 746. The fragmentation pattern is the pattern of a silylated glycoside, but it should be mentioned that the method described for positional analysis of fatty acid glucose esters [8] leads to an incorrect assignment in this case. Obviously, the ratio of the ions *m/e* 204 and *m/e* 217 is influenced by factors different from those in the spectra of silylated fatty acid esters. The influence of the aromatic moiety on the stability of fragment ions may be responsible for this behaviour.

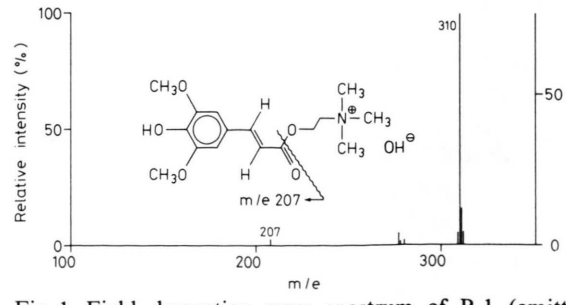

Fig. 1. Field desorption mass spectrum of B-1 (emitter temperature 12 mA).

Table II. Chemical shift of protons of sinapic acid moieties in ppm (δ) (int. standard: TMS).

Compound	Solvent	OCH_3 (s)	$H_{arom.}$ (s)	$H_{olef.}$	(d, J_{tr} = 16 Hz)	$H_{phen.}$	$H_{acetate}$
B-1	CD_3OD/DCl	3.90	6.97	6.47	7.72		
B-2	DMSO-D_6 *	3.82	7.05	6.51	7.65		
B-3	CD_3OD	3.90/3.87	6.90/6.95	6.48	7.64/7.73		
B-3 Ac	$CDCl_3$	3.88/3.90	6.86/6.95	6.44/6.49	7.66/7.75		2.33
B-4	DMSO-D_6 *	3.80	7.03	6.52	7.56	8.97	
	CD_3OD	3.90	6.95	6.44	7.65		
B-4 Ac	$CDCl_3$	3.90	6.91	6.57	7.69		2.34
B-5	CD_3OD	3.86	6.90	6.41	7.46		

* Int. standard: DMSO = 2.50 ppm.

Table III. Chemical shift of protons in the ester moieties in ppm (δ) (int. standard: TMS).

Compound	Solvent	Chemical shifts
B-1	CD_3OD/DCl	$-CH_2-OR$: 4.58–4.80, m; $-CH_2-N$: 3.80–3.90, m; $-N(CH_3)_3$: 3.32, s (+ solv.)
B-2	DMSO-D_6 *	H-1: 5.48, m; H-2–H-6: 2.90–4.00, unresolved
B-3	CD_3OD	H-1: 5.58, d; RO–CH–: 5.53, d; other: 3.30–5.00, unresolved
B-3 Ac	$CDCl_3$	H-1: 5.73, d; H-3,3′,4′: 5.33–5.79; H-2,4: 4.78–5.19 H-5,6,1′,5′,6′: 4.17–4.53, unresolved CH_3COO-: 1.90 s; 1.99, s; 2.08, s; 2.12, s (3)
B-5	CD_3OD	RO–CH–: 5.52, m; $-CH_2-COOH$: 2.85, m

* Int. standard: DMSO = 2.50 ppm.

The positional analysis and the stereochemistry of the glycosidic bond in B-2 are therefore based on the ^1H NMR spectrum. The doublet for the anomeric proton appears downfield at 5.48 ppm (δ) (Table III), which is similar to the value given for the proton of the β-glycosidic bound of *p*-coumaric acid [9].

These data support the identification of B-1 and B-2 in ref. [1].

6-Sinapoyl-α-D-glucopyranosyl-(1-2)-β-D-(3′-sinapoyl)-fructofuranose (B-3, MW: 754, $C_{34}H_{42}O_{19}$)

The molecular weight of B-3 is 754 mu as shown by FDMS (MH^+: 755 (1.6%), MNa^+: 777 (100%), MK^+: 793 (6.5%)). The location of one sinapic acid at each sugar unit its indicated by the fragments observed in FDMS: the signal at *m/e* 369 (6.3%) is derived from sinapoylhexose – H_2O and the signal at *m/e* 530 (7.7%) corresponds to sinapoyldisaccharide; a signal derived from disinapoylhexose is absent.

The EIMS data are of some interest. The elemental composition of the ion at *m/e* 574 is $C_{28}H_{30}O_{13}$ and could correspond to a product formed by migration of one sinapic acid to the pyranose ring, followed by elimination of the furanose to give *m/e* 592 (base peak). Subsequent loss of H_2O leads to *m/e* 574. The reason for this unexpected behaviour seems to be the 3′-acyl group in the furanose.

The fact that silylation or acetylation yields octasilyl or octaacetyl derivatives with molecular ions at *m/e* 1330 (TMS derivative) or *m/e* 1090 (acetate) confirms the findings described above (Fig. 3 and 4).

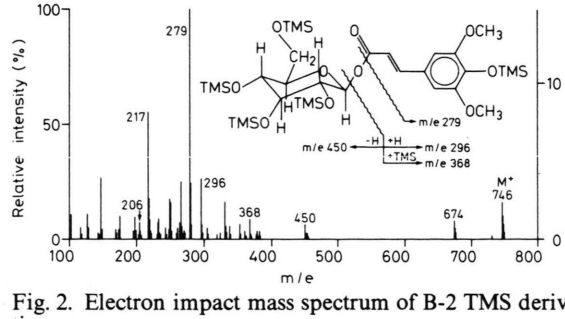

Fig. 2. Electron impact mass spectrum of B-2 TMS derivative.

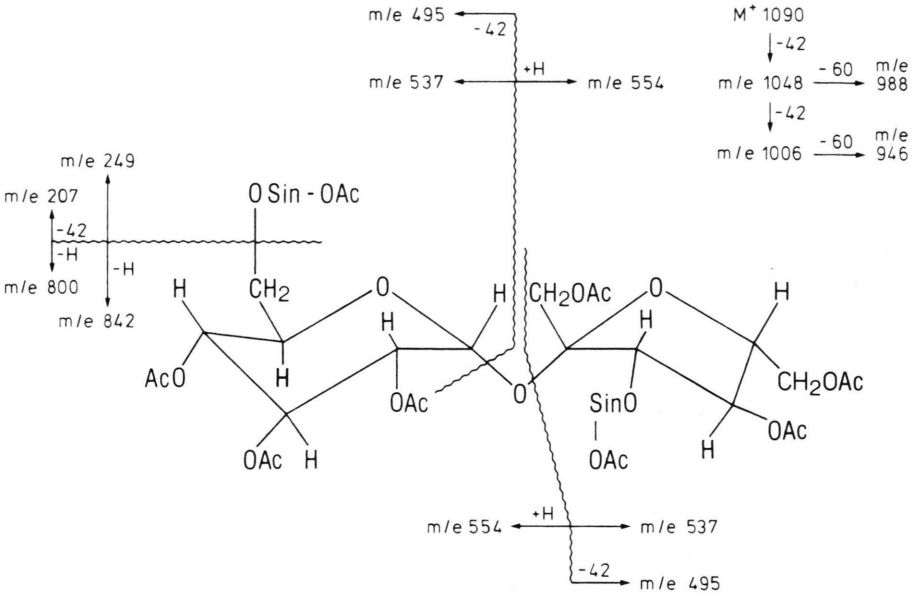

Fig. 3. Fragmentation pattern from the electron impact mass spectrum of B-3 acetate.

The ^{1}H NMR spectrum allows the assignment of the ester locations in the sugar system. In addition to the signals of sinapic acid (Table II) and the unresolved signals of the sucrose between 3.3 and 5.0 ppm, two doublets at 5.53 (J=6.0 Hz) and at 5.58 ppm (J=3.0 Hz) were observed (Table III). The doublet with the smaller coupling constant is due to the anomeric proton in the glucopyranose of sucrose (α-glycosidic bond) [10] and the second doublet is presumed to be the signal of the proton at C-3′; this resonance is split into a doublet and the chemical shift and the coupling constant are very similar to the values reported for the octaacetate of sucrose [10]. No further downfield signal (> 5.0) is observed and this leads to the conclusion that the second ester is attached to one of the primary hydroxyl functions. Since only one ester bond per sugar unit is possible – as discussed above – the second sinapic acid is attached to C-6 of the glucose moiety.

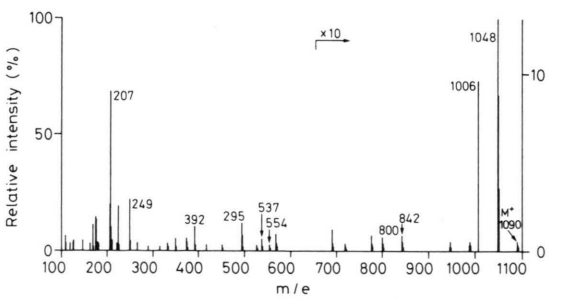

Fig. 4. Electron impact mass spectrum of B-3 acetate.

The ^{1}H NMR data for the peracetate of B-3 are useful for confirming the presence of eight acetyl groups in the derivative – two as phenolacetates (Table III) – and for a comparison of the chemical shifts given in the literature of the octaacetate of sucrose [10].

Results obtained from hydrolytic and chromatographic procedures substantiate the structure reported for B-3. Hydrolysis with 1 N HCl (100 °C for 30 min) yields, in addition to free sinapic acid, two esters present in equal amounts (1:0.9). Fig. 5 shows an HPLC analysis of the hydrolysis mixture, to which authentic 1-sinapoylglucose was added. Cleavage of the isolated esters with 1 N NaOH (room temperature for 30 min) gives sinapic acid and glucose (peak 2) or sinapic acid and fructose (peak 3). Alkaline hydrolysis of the intact B-3 produces sinapic acid and sucrose, contaminated with relatively high amounts of free glucose and fructose. The latter is taken as additional evidence for a 3′-acylation, increasing the reactivity of the α-glycosidic bond towards alkaline treatment. The hydrolytic products were identified by direct chromatographic comparison with authentic compounds as described in ref. [1, 9]. Detection of sugars was achieved by spraying with ammoniacal silver nitrate and heating at 100 °C.

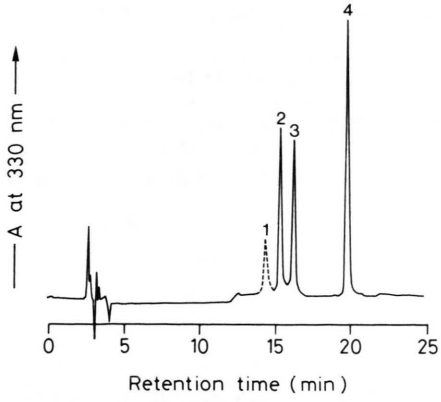

Fig. 5. HPLC chromatogram of acid hydrolysis products from B-3 to which intact B-2 (dashed line) is added. Peak identification: 1 = 1-sinapoylglucose; 2 = 6-sinapoylglucose; 3 = 3-sinapoylfructose; 4 = free sinapic acid. Conditions: linear gradient elution on LiChrosorb RP-8 with 2% acetic acid in water to methanol within 40 min (1 ml/min).

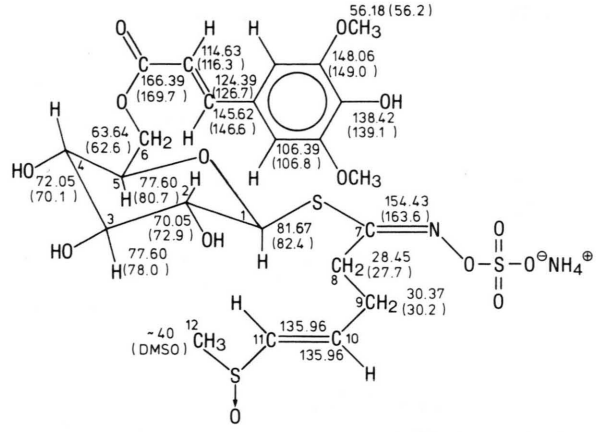

Fig. 7. Structure of B-4 including ^{13}C NMR data in comparison with data taken from literature [12–14].

6-Sinapoyl-β-D-1-thioglucoside of 4-methylsulfinyl-3-butenyl-isothiocyanate (6-sinapoylglucoraphenine) (B-4, MW: 658, $C_{23}H_{30}NO_{14}S_3^-NH_4^+$)

The structure of B-4 can be determined from the following results.

Because no FD data could be obtained in B-4, the first estimation of the molecular weight of the ester was determined *via* quantitative UV spectroscopy. With sinapoylcholine as reference (molar absorptivity in CH_3OH at 326 nm was determined to be 1.5×10^4), the MW was found to be ~658 mu.

After hydrolysis with HCl, a sulfate anion was detectable with $BaCl_2$ solution.

High resolution EIMS data of the acetate of B-4 (Fig. 6) reveals sinapoyl-thiohexose ($C_{17}H_{22}O_9S$) as a part of the molecule. To find the elemental composition, the exact masses of several ions were determined by peakmatching and reduction to their "central unit", following the usual fragmentation pathway of acetylglycosides [11] (for details see Experimental section). Hydrolysis of the thioglycosidic bond during heating of the sample (probably caused by the presence of sulfate) may produce the ion at *m/e* 554, which represents sinapoylhexose.

In Fig. 7 the chemical shifts of the carbons in the ^{13}C NMR spectrum are given, together with the reference data for sinapic acid [12], thioglucose and the carbon of the thioaldoximic acid function [13]. The carbons in the mustard oil moiety have been

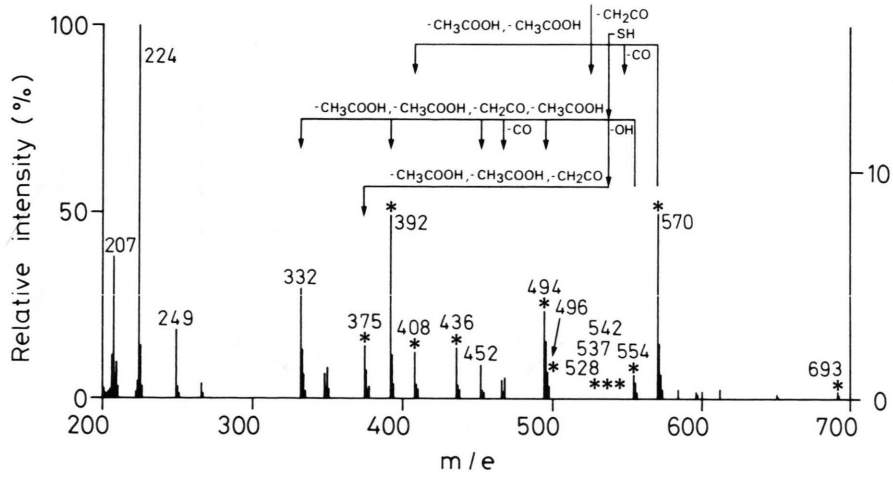

Fig. 6. Electron impact mass spectrum of B-4 acetate. The elemental composition of signals with an asterisk have been evaluated; the intensity of some peaks varies strongly with probe temperature and the given spectrum displays not all of them.

Table IV. 360 MHz ^{1}H-NMR data of B-4 with DMSO-D_6 (1) and CD_3OD (2) as solvents. From the spectrum of its acetate in $CDCl_3$ (3) only the glucose data are given.

		H-1	H-2	H-3	H-4	H-5	H-6,6′	OH	H-8	H-9	H-10	H-11	H-12*
(1)	ppm (δ)	4.88	3.10	3.31	3.18	3.35	4.12/4.46	5.30/5.36/5.58	2.74	2.50	6.31	6.53	2.46
	$J_{A,B}$ (Hz)	9.5	9	9	9	7.2/1.5	12.0	6.0/5.0		6.5	15.0		
(2)	ppm (δ)	4.92	3.31–3.44			3.68	4.26/4.63	4.86	2.87	2.68	6.48–6.49		2.59
	$J_{A,B}$ (Hz)	9.5			9	7.2/1.5	12.0						
(3)	ppm (δ)	5.66	5.00	5.31	5.20	4.14	4.43/4.56						
	$J_{A,B}$ (Hz)	9	9	9	9	9	12.0						

* For numeration of carbons see Fig. 7.

tentatively assigned, but calculations with increments [14] support the assignments, particularly of the sp^3-carbons.

The two ^{1}H NMR spectra of B-4 were taken in DMSO-D_6 and CD_3OD in order to resolve different areas of overlapping signals. The almost complete resolution of signals with DMSO as solvent shows the signal of the methyl group at 2.46 ppm. The sequence $-CH=CH-CH_2-$ is established by coupling constants and multiplicity [15]. As a result of the absolute exclusion of moisture during sample preparation, the signals of the three sugar-OH are resolved (Table IV, Fig. 8).

A small quantity of isomeric products not separated by our chromatographic procedures is detectable in the NMR spectrum. This is due to the presence of e/z isomeric double bonds and isomeric sulfoxides [16].

The structure of the B-4 acetate is still under investigation. We have not been able to isolate the

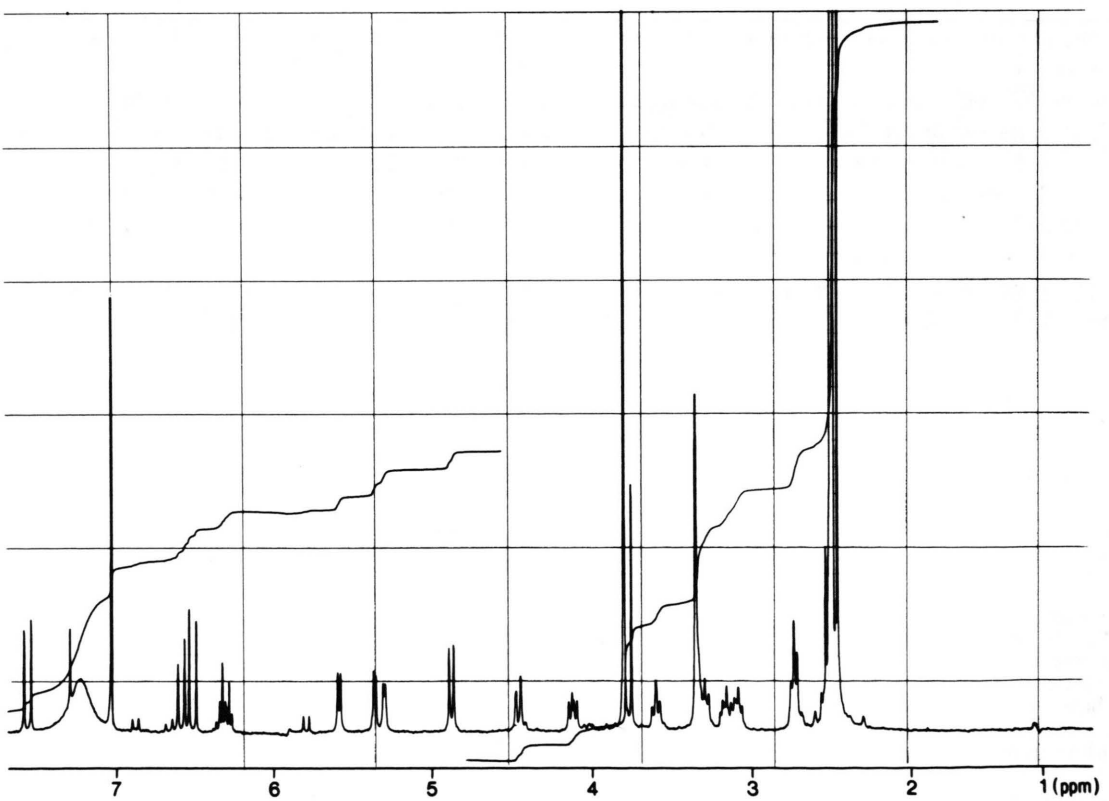

Fig. 8. 360 MHz ^{1}H NMR spectrum of B-4 in DMSO-D_6.

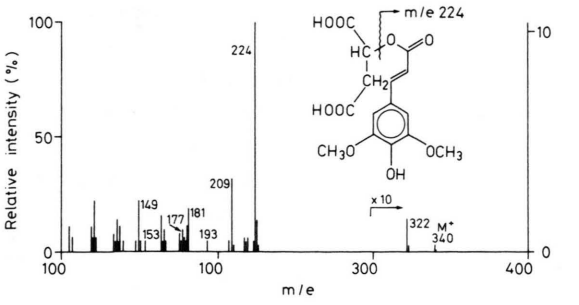

Fig. 9. Electron impact mass spectrum of B-5.

expected tetraacetate [17] because it appears that the sulfoxide undergoes a rearrangement of a vinylogous Pummerer type under the conditions used for acetylation [18–20]. Despite this fact, the main product could be used for confirming the structure of the sugar moiety [13, 21, 22] and for carrying out mass spectroscopy.

In conclusion, the data described above reveal a structure in which the known glucoraphenine [23, 24] is esterified with sinapic acid at C-6 of the thioglucose.

Sinapoylmalic acid (B-5, MW: 340, $C_{15}H_{16}O_9$)

FDMS of B-5 shows the M^+ signal at *m/e* 340, and higher emitter temperatures enhance the loss of H_2O. The EIMS displays the same signal at low intensity, and the elemental composition of *m/e* 322 was found to be $C_{15}H_{14}O_8$ (Fig. 9).

Methylation of B-5 with diazomethane yields a mixture of dimethyl and trimethyl products. The two carboxylic functions are readily methylated, whereas methylation of the phenol is not complete under these conditions.

The ^1H NMR spectrum contains two signals in addition to those of sinapic acid (Table III); with a double resonance experiment and integration, the structure of malic acid, esterified at its OH-group, could be established.

Metabolism of sinapic acid esters

Quantitative HPLC analyses revealed marked changes in concentration of the individual sinapic acid esters in developing *Raphanus* cotyledons. Sinapoylcholine was analyzed isocratically on LiChrosorb Si 60 with dichloromethane-methanol-H_2SO_4 (85:15:1) with a flow-rate of 2 ml/min (t_R = 450 sec). The other esters were chromatographed on LiChrosorb RP-8 with the following elution system: 50 min from solvent A (H_2O/CH_3OH/-CH_3COOH, 90:5:5) to 70% solvent B (H_2O/-CH_3OH/CH_3COOH, 5:90:5) in A+B at a flow-rate of 1 ml/min (t_R=B-2, 953; B-4, 1104; B-5, 1538; B-3, 1797 sec). The results are shown in Fig. 10.

Whereas the amount of disinapoylsucrose decreases only slightly during cotyledon growth, the seed constituents sinapoylglucoraphenine and sinapoylcholine are rapidly metabolized in early stages of germination. The degradation kinetics of these two compounds exhibit a significant difference. Following a 20–24 h lag-phase after sowing, sinapoylcholine is almost totally degraded within 24 h. At that time, sinapoylglucoraphenine remains at a constant level and its degradation starts one day later. In 4-day-old seedlings, both derivatives are no longer detectable.

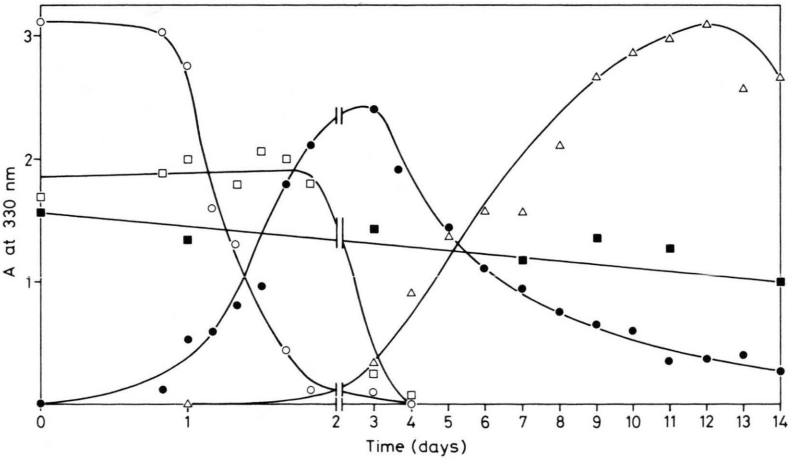

Fig. 10. Sequential quantitative changes of sinapic acid esters in growing cotyledons of *Raphanus sativus*. Each point represents the mean of three experiments. Ordinate: absorbance/ml/pair of cotyledons. The dry seed contains approx. 160 nmoles sinapoylcholine. –■– disinapoylsucrose; –□– sinapoylglucoraphenine; –○– sinapoylcholine; –●– sinapoylglucose; –△– sinapoylmalate.

The sinapic acid derived from sinapoylcholine degradation is most likely reesterified to 1-sinapoylglucose that reaches its highest concentration in 3-day-old cotyledons. This pathway is also proposed to occur in other Brassicaceae seedlings [3, 25]. The fate of the sinapic acid from sinapoylglucoraphenine is probably to serve as a precursor for several derivatives present in low concentrations. Degradation of the acid can be excluded since the total amount of sinapic acid in growing cotyledons remains unchanged and *in vivo* inhibition of L-phenylalanine ammonia-lyase activity does not affect the metabolism of sinapic acid esters [3].

Sinapoylglucose seems to be the precursor of a second reaction [1], resulting in the accumulation of sinapoylmalate. In late stages of *Raphanus* germination (up to 14 days), sinapoylmalate is the major sinapic acid derivative and sinapoylglucose reaches a low level in concentration.

At present we cannot establish with certainty that sinapoylmalate is the end-product. In the first study [1], done with classical methods, sinapoylmalate (B-5) seemed to be metabolized.

In conclusion, we believe that in growing cotyledons of *Raphanus sativus* the following interconversion sequence proceeds:

sinapoylcholine $\xrightarrow{1}$ 1-sinapoylglucose $\xrightarrow{2}$ sinapoylmalate

The first reaction step is catalyzed by a specific sinapoylcholine esterase [4] and UDPG:sinapic acid glucosyltransferase [5]. In the second reaction step, leading to sinapoylmalate, an enzyme which might be classified as 1-sinapoylglucose:L-malate sinapoyltransferase was shown to be involved [6].

Acknowledgements

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