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1,2-DISINAPOLYLGLUCOSE ACCUMULATED IN COTYLEDONS OF DARK-GROWN *RAPHANUS SATIVUS* SEEDLINGS

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Key Word Index—*Raphanus sativus*; Cruciferae; phenylpropanoid metabolism; etiolation; hydroxycinnamic acid ester; glucose ester; 1,2-disinapoylglucose.

Abstract—A new sinapic acid ester has been isolated and characterized as 1(*E*),2(*E*)-di-*O*-sinapoyl-β-D-glucopyranoside from cotyledons of dark-grown red radish (*Raphanus sativus*) seedlings. Its structure was elucidated by negative ion fast atom bombardment mass spectrometry, ^1H and ^{13}C NMR spectra and enzymatic determination of the glucose moiety. A possible biosynthetic mechanism for the formation of this new ester is discussed in which the energy-rich acyl glucoside 1-*O*-sinapoyl-β-D-glucose acts as the acyl donor in a sinapoyl transfer to the hydroxyl group at C-2 of the glucose moiety of another molecule of 1-*O*-sinapoyl-β-D-glucose ('disproportionation').

INTRODUCTION

Seeds and seedlings of red radish (*Raphanus sativus*) contain a variety of sinapic acid esters, which have been shown to be metabolically active [1–3]. The seed constituents sinapine (sinapoylcholine) and 6-sinapoylglucoraphenine are rapidly hydrolysed during early stages of seedling development. Although the fate of the metabolized glucosinolate conjugate has not been elucidated, it was shown that the liberated choline moiety from sinapine is consumed in the biosynthesis of phosphatidylcholine [4], whereas sinapic acid is re-esterified to give 1-sinapoylglucose. The latter compound is transiently accumulated and in later stages of development it is transacylated to yield sinapoylmalate. This reaction is catalysed by a 1-sinapoylglucose: L-malate sinapoyltransferase.

Red radish seeds contain a third major sinapic acid ester, the disaccharide derivative 6,3′-di-*O*-sinapoylsucrose, the concentration of which decreases only slightly during seedling development [2]. Recently, another sinapic acid disaccharide ester, disinapoylgentiobiose, was reported to occur in Sakurajima radish seedlings [5]. It was suggested that this conjugate together with some other compounds could be involved in a light-induced growth inhibition [6–8].

In a previous study on the metabolism of sinapic acid esters in seedlings of red radish [1] it was shown that in dark-grown, etiolated seedlings the quantitative changes of the esters are altered considerably. The transacylation reaction from 1-sinapoylglucose to sinapoylmalate was markedly affected: the amount of 1-sinapoylglucose failed to drop as rapidly as in light-grown seedlings and as a consequence sinapoylmalate was produced in smaller quantities.

This paper reports the accumulation of a new sinapic acid ester in etiolated cotyledons of radish seedlings. We describe the isolation and structure elucidation of the new compound and discuss its possible involvement in the metabolism of radish sinapic acid esters.

RESULTS AND DISCUSSION

Dark-grown (etiolated) seedlings of red radish exhibit a considerable alteration in the metabolism of sinapic acid esters in the cotyledons, compared to light-grown ones. Figure 1 illustrates this difference by HPLC analyses of the

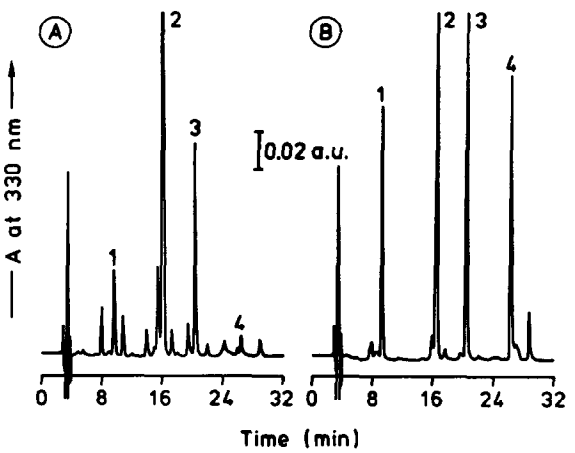

Fig. 1. HPLC separation of sinapic acid esters from crude extracts of 6-day-old red radish cotyledons. Peak identification: 1 = 1-sinapoylglucose; 2 = sinapoylmalate; 3 = 6,3′-disinapoylsucrose; 4 = 1,2-disinapoylglucose. Development: linear gradient elution within 30 min from 20 to 50% B (1% H_3PO_4, 25% HOAc, 50% MeCN in H_2O) in solvent A (1% H_3PO_4 in H_2O) at a flow-rate of 0.8 ml/min. (A) Light-grown (14-hr-day); (B) dark-grown.

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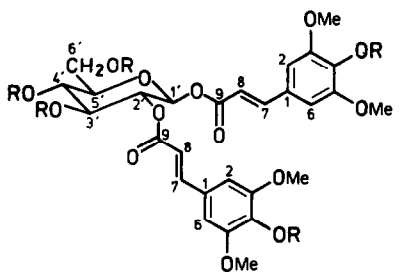
pattern of phenolic secondary compounds, among which sinapic acid esters are the main constituents. In 6-day-old cotyledons of light-grown seedlings sinapoylmalate (peak 2) is the predominant product of a transacylation reaction with 1-sinapoylglucose (peak 1) as acyl donor [3]. In etiolated cotyledons this reaction appears to be markedly reduced, whereas another pathway, leading to 1(*E*),2(*E*)-di-*O*-sinapoyl-β-D-glucopyranoside (peak 4) is strongly stimulated.

For isolation and structure elucidation the crude methanol-extracts from etiolated cotyledons of red radish were pre-fractionated on polyamide (perlon) columns. The 60% aqueous methanol-fractions, containing 1,2-disinapoylglucose together with 6,3'-disinapoylsucrose, were chromatographed on microcrystalline cellulose in BAW. The separated 1,2-disinapoylglucose was eluted and rechromatographed on a polyamide column. The compound was obtained in a 95% pure form after successive column chromatography on LiChroprep RP-8 (gravity flow) and Sephadex LH-20. It exhibited similar UV-spectroscopic properties as the sinapic acid esters, described previously in radish [1, 5]. Addition of aqueous sodium hydroxide gave the expected strong bathochromic shift of λ_{max}^{MeOH} from 323 to 393 nm. The alkaline hydrolytic products showed chromatographic identity with sinapic acid and glucose [1].

Both the ^1H and ^{13}C spectra of **1** show a doubling of the signals associated with the aromatic moiety, the intensity of which indicate the presence of two aromatic residues per sugar residue. The identity and the assignment of the ^{13}C signals of the aglycone follows from the known spectrum of 3,5-dimethoxy-4-hydroxycinnamic acid (*trans*-sinapic acid) [9]. The six remaining signals have shifts characteristic of a hexose. The molecular ion $(C_{28}H_{32}O_{14}-H)^-$ at *m/z* 591 *d* and the fragmentation pattern in the negative ion FAB mass spectrum of **1** confirms the ratio of two sinapic acid residues per hexose residue. A disubstituted hexose is indicated by the absence of any ions associated with a directly bonded disinapoyl moiety which thus excludes the molecule with the sequence glucose–sinapic acid–sinapic acid.

The couplings observed for the signals of H-1' and H-2' of the hexose moiety of **1** and of H-1', H-2', H-3' and H-4' of its peracetyl derivative (**2**) indicate that a β-sinapoyl residue is present at C-1' and that the sugar moiety is glucose. The position of the attachment of the second sinapoyl residue at C-2' is indicated by the low field shift of H-2' in **1**. An alternative substitution pattern is excluded by the shifts after acetylation. The signals of H-3' and H-4' show characteristic downfield shifts (1.6–1.8 ppm) upon acetylation, while H-2' is essentially unaffected. Hence the second sinapoyl residue is attached at C-2'.



1 R = H **2** R = Ac

Quantitative enzymatic glucose determination (UV-method) through conversion of hydrolytically liberated glucose to gluconate-6-phosphate by the aid of hexokinase and glucose-6-phosphate dehydrogenase gave a molar ratio of sinapic acid:glucose of 1.9:1 and substantiated the results discussed above.

Detailed quantitative HPLC analyses gave an indication of the possible metabolic source of this new compound. Whereas in light-grown seedlings the metabolic fate of 1-sinapoylglucose is a transacylation reaction leading to sinapoylmalate, in dark-grown seedlings a considerable portion of it is channelled into a 'disproportionation' reaction leading to the 1,2-disinapoylglucose. The accumulation kinetics of the two products, followed over a period of 8 days (determination of 24 hr intervals), suggest that the two pathways are stoichiometrically correlated to each other (data not shown). Table 1 shows a representative quantitative ratio of the compounds in question in extracts of light- and dark-grown 8-day-old seedlings. Figure 2 depicts a tentative scheme of this metabolism, which starts by degradation of the seed constituent sinapine and leads via 1-sinapoylglucose to sinapoylmalate and 1,2-disinapoylglucose.

Table 1. Amount (nmol/pair of cotyledons)* of sinapic acid esters, formed in cotyledons of light- and dark-grown 8-day-old red radish seedlings

Compound	Light-grown	Dark-grown
1-Sinapoylglucose	2	25
Sinapoylmalate	110	51
1,2-Disinapoylglucose	7	22
Sum of bound sinapic acid	126	120

*Mean of six determinations.

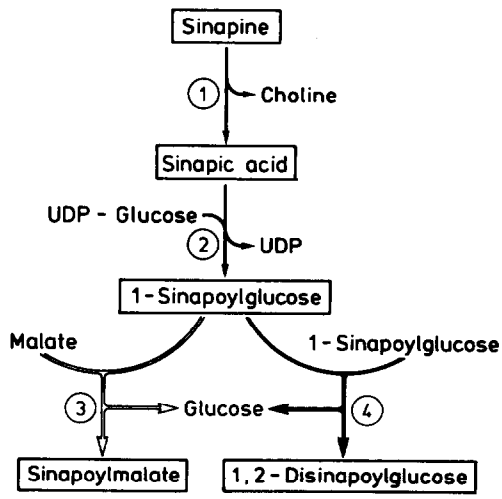


Fig. 2. Tentative scheme for the metabolism of sinapic acid esters during development of red radish. (1) Sinapine esterase; (2) UDP-glucose: sinapic acid glucosyltransferase; (3) 1-sinapoylglucose: L-malate sinapoyltransferase; (4) hypothetical: '1-sinapoylglucose: 1-sinapoylglucose sinapoyltransferase'.

Obviously the quantitative ratio of the two products is determined by photomorphogenetic processes. The pivotal point of regulation, however, is not yet known. The possibility of photoisomerization of the new product, resulting in its low concentration in light-grown seedlings, seems unlikely, as we could not detect any transacylation products during our analytical procedure. It might be that the metabolic fate of 1-sinapoylglucose is determined by the concentration of the available pool of free malate—extractable concentrations of malate are reduced from *ca* 1.3 μmol in 8-day-old light-grown cotyledons to *ca* 0.09 μmol in dark-grown ones—and/or by differentiating activities of two enzymes: firstly 1-sinapoylglucose: L-malate sinapoyltransferase and secondly a transacylase which might be classified as '1-sinapoylglucose: 1-sinapoylglucose sinapoyltransferase'. (We have preliminary results of *in vitro* studies [Dahlbender and Strack, unpublished] suggesting that such a 'disproportionation' is indeed realized in dark-grown cotyledons of radish.)

In summary the data indicate that the structure of the compound, accumulated in etiolated red radish cotyledons together with *O*-sinapoyl-L-malate is 1,2-di-*O*-sinapoyl-β-D-glucose. This compound seems to be synthesized by a 'disproportionation' reaction from 1-*O*-sinapoyl-β-D-glucose.

EXPERIMENTAL

Plant material and growing conditions. Source of red radish (*R. sativus* L. var. *sativus*) and standardization of the phytotron were the same as described previously [3] with the exception of the substratum. Seedlings were grown in quartz sand, moistened with a modified Knop-medium as nutrient soln. Details will be published elsewhere.

Extraction and separation. Etiolated cotyledons were treated with an Ultra-Turrax homogenizer in 80% aq. MeOH. The filtrate was concd to small vol. under red. pres. at 40° and fractionated on a polyamide column (CC 6-perlon, 3 × 30 cm) using (1) H_2O (1-sinapoylglucose), (2) 60% aq. MeOH (1,2-disinapoylglucose and 6,3'-disinapoylsucrose), (3) MeOH and (4) 0.035% NH_4OH in MeOH (sinapoylmalate). The 60% aq. MeOH-fraction was concd and chromatographed on microcrystalline cellulose in BAW (*n*-BuOH–HOAc–H_2O, 6:1:2). 1,2-Disinapoylglucose (R_f 0.77), separated from the sucrose ester (R_f 0.41), was detected under UV (366 nm, changing from dark blue to dark green fluorescence when treated with NH_3 vapour), scraped off and eluted with MeOH. After rechromatography on a polyamide column, the compound was purified by successive CC on LiChroprep RP-8 (40–63 μm; 31 × 2.5 cm; H_2O–MeOH gradient, containing 1% HOAc; gravity flow) and Sephadex LH-20 (85 × 2 cm; MeOH). Elution was monitored continuously by UV absorption at 254 nm. TLC on microcrystalline cellulose in $CHCl_3$–HOAc (3:2, H_2O satd), R_f 0.9, on silica gel in $CHCl_3$–MeOH (4:1), R_f 0.66.

Hydrolysis and identification of products. This was carried out as described previously [1]. Enzymatic quantitative glucose determinations were performed according to an instruction of Boehringer, Mannheim, West Germany [10] using a test-combination for spectrophotometric analysis (formation of NADPH). Quantification of the intact ester was performed with 1-sinapoylglucose as standard.

HPLC. The liquid chromatograph, detector and computing integrator are described elsewhere [11]. The chromatographic column (250 × 4 mm) was prepacked with LiChrosorb RP-8 (5 μm). The elution system is described in the legend to Fig. 1. The sample size was 20 μl out of 10 ml 80% aq. MeOH-extract from cotyledons of 25 seedlings. Prior to injection the extract was diluted with H_2O to give 40% MeOH. Quantitative data were obtained using 1-sinapoylglucose as standard.

Acetylation. The compound was treated with Ac_2O–pyridine for several hr at room temp. The product was purified on Sephadex LH-20, eluting with MeOH.

NMR and MS. ^1H and ^{13}C NMR spectra were recorded at ambient temp., at 400 and 100 MHz, respectively, on a spectrometer locked to the major deuterium resonance of the solvent, CD_3OD. Chemical shifts are reported in δ values (ppm) relative to TMS. A negative ion FAB MS was recorded on a Kratos spectrometer; glycerol was used as matrix.

1(E),2(E)-*Di*-O-*sinapoyl*-β-D-*glucopyranoside* (**1**). ^1H NMR (CD_3OD): δ 7.669, 7.660 [*d* × 2; 2 × H-7; *J* (7-8) = 15.8, 15.8 Hz], 6.910, 6.879 (*s* × 2; 2 × H-2, H-6), 6.446, 6.364 (*d* × 2; 2 × H-8), 5.849 [*d*; H-1'; *J* (1'-2') = 8. Hz], 5.122 [*d*, *d*; H-2'; *J* (2'-3') = 9.7 Hz]; 3.946 [*d*, *d*; $H\text{-}6'_A$; $J(6'_A\text{-}6'_B) = (-)$ 12.2, $J(6'_A\text{-}5')$ = 1.6 Hz]; 3.888, 3.869 [*s* × 2; 2 × Me-10, 11], 3.8–3.9 [$H\text{-}6'_B$ hidden under OMe signals], 3.775 [*d*, *d*; H-3'; *J* (3'-4') = 9.8 Hz]; 3.50–3.60 (*m*, H-4' and H-5'). ^{13}C NMR (CD_3OD): δ = 168.31, 167.09 (*s* × 2; 2 × C-9), 149.51, 149.49 (*s* × 2; 2 × C-3, C-5), 148.96, 147.82 (*d* × 2; 2 × C-7), 140.16, 139.85 (*s* × 2; 2 × C-4), 126.57, 126.39 (*s* × 2; 2 × C-1); 115.55, 114.66 (*d* × 2; 2 × C-8), 107.33, 107.10 (*d* × 2; 2 × C-2, C-6), 94.09 (*d*; C-1'), 79.18, 76.06, 71.36 (*d* × 3; C-3', C-4', C-5'), 74.43 (*d*; C-2'), 62.35 (*t*; C-6'), 56.92, 56.88 (*q* × 2; 2 × C-10, C-11). FAB MS *m/z*: 1183 $[2M-H]^-$, 683 $[M-H+\text{glycerol}]^-$, 591 $[M-H]^-$, 385 $[M-C_{11}H_{11}O_4]^-$, 223 $[C_{11}H_{11}O_5]^-$, 165, 151.

Peracetyl 1(E),2(E)-*di*-O-*sinapoyl*-β-D-*glucopyranoside* (**2**). ^1H NMR (CD_3OD): δ 7.757, 7.693 [*d* × 2; 2 × H-7; *J* (7-8) = 15.9, 16.2 Hz], 6.989, 6.944 [*s* × 2; 2 × H-2, H-6]; 6.577, 6.548 [*d* × 2; 2 × H-8), 6.097 [*d*; H-1'; *J* (1'-2') = 8.2 Hz], 5.562 [*d*, *d*(*t*); H-3' or H-4', *J* (3'-4') = 9.5 Hz], 5.361 [*d*, *d*; H-2'; *J* (2'-3') = 9.6 Hz], 5.219 [*d*, *d*(*t*); H-4' or H-3'; *J* (4'-5') = 9.6 Hz], 4.383 [*d*, *d*; $H\text{-}6'_B$; *J* ($6'_B\text{–}6'_A$) = (−) 12.5 Hz, *J* ($6'_B\text{–}5'$) = 4.2 Hz], 4.15–4.25 (*m*, $H\text{-}6'_A$, H-5'), 3.855, 3.840 (*s* × 2; 2 × Me–10, 11); 2.290, 2.286 (*s* × 2; Arom. OCOMe), 2.111, 2.087, 2.023 (*s* × 3; sugar OCOMe).

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