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HYDROXYCINNAMIC ACID ESTERS FROM BROCCOLI FLORETS

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Key Word Index—*Brassica oleracea*; Cruciferae; broccoli; florets; gentiobiose ester; sinapic acid; ferulic acid.

Abstract—Four conjugates of gentiobiose were isolated, from fresh, raw broccoli florets, using mild extraction conditions and preparative HPLC. From spectroscopic evidence the structures were characterized as two novel conjugates of gentiobiose: 1,2′-disinapoyl-2-feruloylgentiobiose and 1-sinapoyl-2-feruloylgentiobiose and, two compounds previously reported to be present in the fruits of *Boreave orientalis*, a member of the Cruciferae, 1,2,2′-trisinapoylgentiobiose and 1,2-disinapoylgentiobiose.

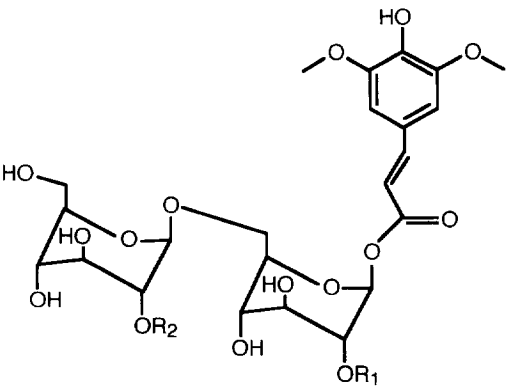
INTRODUCTION

Broccoli is becoming increasingly popular as a fresh vegetable and is a significant source of several classes of biologically active dietary components such as the flavonol glycosides [1] and sulphur containing compounds such as the glucosinolates [2]. Another class, the hydroxycinnamic acids, is attracting interest as a group with protective activity against degenerative diseases [3]. The isolation and identification of four compounds in this class and novel to broccoli is the subject of this communication. Two of these compounds have been reported present in *Boreave orientalis* seed [4].

RESULTS AND DISCUSSION

Fresh broccoli florets were freeze-dried and extracted with aqueous methanol to minimize any enzymic or autolytic degradation. The extract was treated and fractionated as described in the Experimental to yield compounds **1**–**6** as colourless oils, although only **1**–**4** were isolated in sufficient quantity for full characterization.

The acid hydrolysate of **1** was partitioned between ethyl acetate and water. The ethyl acetate fraction contained four compounds identified from HPLC retention times and UV spectra as sinapic acid and ferulic acid in a molar ratio of 2:1, and their corresponding methyl esters which are known artefacts of the acid hydrolysis procedure. The aqueous fraction, following reduction, acetylation and GC gave one component containing 2 moles of glucose. Mass spectrometry in the positive ion mode gave a quasi-molecular ion at m/z 930 of low abundance, and major fragment ions at m/z 913, $[M-18+H]^+$, 707 and 369 suggesting the presence of a diglucoside moiety ester linked to both a ferulic acid and 2 sinapic acids. Enzymic hydrolysis with β-glucosidase yielded products which were identified as free sinapic acid (by HPLC and UV) present in the ether fraction, demonstrating the presence of sinapic acid conjugated at the 1-C



	R_1	R_2
1	feruloyl	sinapoyl
2	feruloyl	H
3	sinapoyl	sinapoyl
4	sinapoyl	H

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Table 1. ^{1}H NMR spectral data (400 MHz) of **1**–**4** in DMSO-d_6

Sugar	H	1	2	3	4
	1	5.72 *d* (8.4)	5.77 *d* (8.8)	5.71 *d* (8.4)	5.78 *d* (8.8)
	2	4.82 *dd* (8.4, 9.2)	4.89 *dd* (8.8, 8.8)	4.83 *dd* (8.4, 9.2)	4.91 *dd* (8.8, 8.8)
	3	3.50 *m*	3.59 *m*	3.49 *m*	3.59 *m*
	4	3.23 *dd* (8.8, 8.8)	3.38 *dd* (8.8, 8.8)	3.43 *dd* (8.8, 8.8)	3.40 *dd* (8.8, 8.8)
	5	3.45 *m*	3.58 *m*	3.46 *m*	3.58 *m*
	6a	3.67 *m*	3.64 *m*	3.67 *m*	3.64 *m*
	6b	3.96 *d* (11.6)	4.04 *d* (11.2)	3.96 *d* (11.2)	4.05 *d* (10.4)
	1′	4.54 *d* (8.0)	4.19 *d* (8.0)	4.54 *d* (8.0)	4.21 *d* (8.0)
	2′	4.64 *dd* (8.0, 8.8)	2.97 *dd* (0.0, 8.8)	4.64 *dd* (8.0, 8.8)	2.99 *dd* (8.0, 8.8)
	3′	3.37 *dd* (8.8, 8.8)	3.13 *dd* (8.4, 8.8)	3.7 *dd* (8.8, 8.8)	3.14 *dd* (8.0, 8.8)
	4′	3.18 *m*	3.05 *m*	3.19 *dd* (8.8, 8.8)	3.08 *m*
	5′	3.15 *m*	3.07 *m*	3.15 *m*	3.08 *m*
	6′a	3.47 *m*	3.44 *dd* (5.2, 11.2)	3.48 *m*	3.45 *dd* (5.2, 11.6)
	6′b	3.69 *m*	3.66 *m*	3.69 *m*	3.67 *m*
Acyl	2,6	6.96 *s*	6.98 *s*	6.95 *s*	6.98 *s*
	2′,6′	6.98 *s*		6.96 *s*	7.00 *s*
	2″,6″			6.98 *s*	
	7	7.49 *d* (16.0)	7.52 *d* (16.0)	7.49 *d* (16.0)	7.54 *d* (16.0)
	7′	7.60 *d* (16.0)		7.53 *d* (16.0)	7.56 *d* (16.0)
	7″			7.60 *d* (16.0)	
	8	6.39 *d* (16.0)	6.44 *d* (16.0)	6.43 *d* (16.0)	6.46 *d* (16.0)
	8′	6.55 *d* (16.0)		6.44 *d* (16.0)	6.51 *d* (16.0)
	8″			6.55 *d* (16.0)	
	OMe	3.76 *s*	3.77 *s*	3.76 *s*	3.78 *s*
	2‴	7.25 *d* (2.0)	7.27 *d* (2.0)		
	5‴	6.75 *d* (8.0)	6.75 *d* (8.0)		
	6‴	7.04 *dd* (2.0, 8.0)	7.05 *dd* (2.0, 8.0)		
	7‴	7.53 *d* (16.0)	7.54 *d* (16.0)		
	8‴	6.43 *d* (16.0)	6.44 *d* (16.0)		
	OMe	3.78 *s*	3.78 *s*		

All assignments were made from 2D COSY experiments [coupling constants are in parentheses (*J*)].

position of glucose, and two more-polar compounds in the aqueous phase, one containing ferulic the other sinapic acid as shown by HPLC and UV measurements, the ferulic acid giving a characteristic absorption maximum at 290 nm. This mixture suggests the sinapic acid and the ferulic acid were each conjugated at non-reducing positions on each of the glucose moieties. Detailed analysis of the ^{1}H and ^{13}C NMR spectra (Tables 1 and 2) determined the ester positions and the mode of linkage between the 2 glucose units and confirmed the identification of the acid moieties. In the ^{1}H spectrum two anomeric protons were observed in the β form (8 Hz) and confirmed by the ^{13}C resonance and C/H shift correlation. The upfield shift for the anomeric carbon at 91.4 ppm suggests the position of the ester group is C-1 [4] and the sugar linkage is β 1- > 6 as shown by the resonance of C-6 at 66.8 ppm. The ^{1}H NMR spectra (Table 1) confirms the presence of three hydroxycinnamoyl groups by the six olefinic resonances with coupling constant of 16 Hz (*E*-configuration). Also, the singlets at 6.96–6.98 ppm are indicative of the H-2,6 of two sinapic acid moieties with the characteristic strong singlet at 3.76 ppm equivalent to four aromatic methoxyl groups. The feruloyl group was confirmed by the appearance of ABX-type aromatic proton signals at δ 6.75 (1H, *d*, J = 8 Hz), 7.04 (1H, *dd*, J = 2.0, 8.0 Hz) and 7.25 (1H, *d*, J = 2.0 Hz), with the singlet at 3.78 ppm for the methoxyl group. The ester linkages are in the C-1, C-2 positions as shown by the proton resonance upfield shift for H-1 and H-2. The proton resonance shifts as reported by other workers [4, 5] showed the third acyl group to be bonded at the C-2′ from the strong downfield effect of 2 ppm for H-2′. The position of the ferulic acid is shown to be at C-2 from the two-dimensional NOESY spectrum and the enzymatic hydrolysis. Finally study of the two-dimensional homo and hetero shift correlations allowed complete assignment. Therefore **1** is, 1,2′-disinapoyl-2-feruloylgentiobiose, a novel natural product.

Acid hydrolysis of **2** yielded in the ethyl acetate fraction four compounds identified from HPLC retention times and UV spectra as sinapic acid and ferulic acid but in equimolar proportions and their corresponding methyl esters. The aqueous fraction following reduction, acetylation and GC gave one component identified as 2 moles of glucose. Mass spectrometry in the positive ion mode gave a quasi-molecular ion at m/z 725 of low abundance and fragment ions at m/z 707 $[M-18+H]^+$, 501 and 339 sug-

Table 2. ^{13}C NMR spectral data (100 MHz) of **1**–**4** in DMSO-d_6

Sugar	C	1	2	3	4
	1	91.4	91.4	91.4	91.4
	2	73.0	72.9	73.0	73.0
	3	73.3	73.3	73.3	73.3
	4	69.0	69.0	69.0	69.0
	5	76.6	76.2	76.6	76.2
	6	66.8	67.3	66.8	67.3
	1′	100.1	102.6	100.1	102.6
	2′	72.0	72.1	71.9	72.1
	3′	73.8	75.9	73.8	75.9
	4′	69.7	69.5	69.7	69.5
	5′	76.7	76.4	76.7	76.4
	6′	60.2	60.5	60.2	60.5
Acyl	1	123.5	123.5	123.5	123.7
	1′	124.1	—	123.7	123.5
	1″	—	—	124.1	—
	2	105.6	106.1	105.6	105.7
	2′	106.0	—	105.7	106.1
	2″	—	—	106.0	—
	3,3′,3″	147.5	147.5	147.5	147.5
	4	137.6	138.3	137.6	137.9
	4′	138.3	—	137.8	138.3
	4″	—		138.3	
	5,5′,5″	147.5	147.5	147.5	147.5
	6	105.6	106.0	105.6	105.7
	6′	106.0	—	105.7	106.1
	6″	—		106.0	—
	7	144.7	146.8	144.7	145.4
	7′	146.8	—	145.3	146.8
	7″	—		146.8	
	8	112.9	113.0	112.8	113.0
	8′	114.8		113.9	114.0
	8″	—		114.8	
	9	165.0	165.1	164.3	164.3
	9′	165.2	—	165.0	165.1
	9″	—	—	165.2	—
	OMe	55.4	55.6	55.4	55.5
	OMe′	55.6	—	55.5	55.6
	OMe″	—	—	55.5	—
	1‴	124.9	124.9		
	2‴	110.5	110.5		
	3‴	147.4	147.4		
	4‴	148.9	148.9		
	5‴	114.9	114.9		
	6‴	122.7	122.8		
	7‴	145.0	145.0		
	8‴	113.5	113.6		
	9‴	164.4	164.4		
	OMe	55.1	55.1		

All assignments were made from 2D C H shift correlations.

gesting the presence of a diglucoside moiety ester linked to both ferulic acid and sinapic acid. Enzymic hydrolysis products were identified as free sinapic acid present in the ether fraction, demonstrating the presence of sinapic acid conjugated at the 1-C position of glucose, and, a more polar compound containing ferulic acid present in the aqueous layer. The latter suggesting the ferulic acid to be conjugated at a non-reducing position on 1 of the glucose moieties. The ^1H and ^{13}C NMR spectra were similar to that of **1** except for the lack of substitution at the C-2′ position, see Tables 1 and 2. Therefore **2** is 1-sinapoyl-2-feruloylgentiobiose, a novel natural product.

Acid hydrolysis of **3** yielded in the ethyl acetate fraction two compounds identified from HPLC retention times and UV spectra as sinapic acid and its corresponding methyl ester. The aqueous fraction following reduction, acetylation and GC gave one component identified as 2 moles of glucose. Mass spectrometry in the positive ion mode gave a quasi-molecular ion of low abundance at *m/z* 961 and fragment ions at *m/z* 943 $[M-18+H]^+$, 737 and 575 suggesting the presence of a diglucoside moiety ester linked to three sinapic acids. The ^1H and ^{13}C NMR spectra determined the ester positions and the mode of linkage between the two glucose units as for compound **1**, see Tables 1 and 2. Therefore, **3** is 1,2,2′-trisinapoylgentiobiose, a product previously reported in the seed of *Boreave orientalis*.

Acid hydrolysis of **4** again yielded in the ethyl acetate fraction two compounds identified from HPLC retention times and UV spectra as sinapic acid and its corresponding methyl ester. The aqueous fraction following reduction, acetylation and GC gave one component containing 2 moles of glucose. Mass spectrometry in the positive ion mode gave a quasi-molecular ion of low abundance at *m/z* 755 and fragment ions at *m/z* 737 $[M-18+H]^+$, 531 and 369 suggesting the presence of a diglucoside moiety ester linked to two sinapic acids. The ^1H and ^{13}C NMR spectra determined the ester positions and the mode of linkage between the 2 glucose units as for **2**, see Tables 1 and 2. Therefore **4** is 1,2-disinapoylgentiobiose, a product also previously reported in the seed of *Boreave orientalis* [3] and an isomer of the 1-β,4-di-*O*-sinapoylgentiobiose reported as a new growth inhibitor in light exposed Sakurajima radish [6].

Compounds **5** and **6** were only subjected to mass spectral studies indicating them to be isomeric and of similar polarity to **2** and **1**, respectively. This would suggest the ferulic acid in each compound is substituted in either the second glucose molecule of the gentiobiose or in position 3 or 4 of the first glucose. Any of these cases would result in the existence of novel structures.

EXPERIMENTAL

^1H NMR spectra were obtained using a 400 MHz instrument and ^{13}C NMR spectra using a 100 MHz instrument with the samples dissolved in DMSO-d_6; chemical shifts are given in δ relative to TMS as internal standard. APCI spectra were obtained by flow injection in positive ion mode using a mobile phase of H_2O–MeCN (3:2) at a flow of 200 μl/min with corona 3.00 kV, high voltage lens 0.10 kV, cone 10 V, source temp. 140 and APCI probe temperature at 600 .

Material. The florets of broccoli were taken from

freshly harvested plants of cv Marathon grown under commercial conditions in the U.K.

Isolation. The fresh material (4.7 kg) was immersed in liquid nitrogen, lyophilized and ground to a powder in a domestic blender. The dry tissue (500 g) was extracted twice with cold MeOH (4 l and 3 l, 5 min for each) with a Pro400 homogenizer, filtered and the combined filtrates reduced at $<40^{\circ}$ to 600 ml and defatted by extracting twice with hexane (2 × 300 ml). The aqueous layer was passed through a polyamide column (50 g), prewashed with H_2O (500 ml) under low pressure (10 psi) and washed with water (300 ml). The compounds of interest were eluted from the column with MeOH (1 l). The MeOH eluate was evapd to dryness under red. pres. (2.3 g) and the residue (1.8 g) was dissolved in aqueous MeOH and fractionated on prep. HPLC using reversed silica (SiO_2-C18) packing and a H_2O–MeCN gradient elution. Six fractions (**1**–**6**) containing hydroxycinnamic acid derivatives were collected, four of which, were of sufficient purity and quantity for complete chemical characterization as **1**, **2**, **3** and **4**. Relative retention times (by reversed phase HPLC) for **1**–**6** were 0.98, 0.81, 0.90, 0.76, 0.85, 1.00, respectively.

High pressure liquid chromatography

Preparative HPLC. A Dynamax reversed phase (C18) silica column 250 × 21.2 mm at a flow rate of 5 ml/min was used with a solvent gradient comprising 15%B increasing to 30%B over 45 min, where solvent A was 0.1% TFA in H_2O and solvent B was MeCN. The eluate was monitored at 270 nm and fractions collected using a Gilson fraction collector.

Analytical HPLC. **1**–**4** each gave a single peak on analytical HPLC carried out using a Hewlett Packard 1050 system comprising autosampler and quaternary pump coupled to a diode array detector and controlled by Chemstation software. A solvent gradient of A (H_2O–THF–TFA 98:2:0.1) and B (MeCN) used in the proportion of 17%B for 2 min increasing to 25%B after 5 min, to 35%B after a further 8 min and to 50%B after 5 min. A column clean-up stage was used by increasing B to 90% after a further 5 min and finally re-equilibration for 20 min at 17%B. The column was 250 mm by 4.6 mm i.d. packed with Dynamax reversed phase (ODS) silica (8 μm) and the eluate was monitored at 270 nm and 20 μl of each sample injected.

Thin layer chromatography. **1** **4** each gave a single spot on normal phase TLC (silica gel) and eluted with a solvent comprising the lower layer of a mixture of 65:35:10 $CHCl_3$–MeOH–H_2O.

6-O-β-D-(2′-O-*Sinapoyl*)*glucopyranosyl*-β-D-(1-O-*sinapoyl*, 2-O-*feruloyl*)*glucopyranose* (**1**). Oil, 90 mg. APCI-MS *m/z*: 913 $[M-18+H]^+$, 5%; 707 $[MH-18-$sinapic acid$]^+$, 72%; 369 $[MH-18-$sinapic acid–ferulic acid–glucose$]^+$, 100%. TLC R_f = 0.61. HPLC R_t = 14.0 min. UV λ_{max} nm: 256, 268, 355. ^1H and ^{13}C NMR data see Tables 1 and 2.

6-O-β-D-*Glucopyranosyl*-β-D-(1-O-*sinapoyl*,2-O-*feruloyl*)*glucopyranose* (**2**). Oil, 110 mg. MS *m/z*: 707 $[M-18+H]^+$, 12%; 501 $[MH-18-$sinapic acid$]^+$, 100%; 339 $[MH-18-$sinapic acid–glucose$]^+$, 37%. TLC R_f = 0.43. HPLC R_t = 12.0 min. UV λ_{max} nm: 223, 241, 330. ^1H and ^{13}C NMR data see Tables 1 and 2.

6-O-β-D-(2′-O-*Sinapoyl*)-*glucopyranosyl*-β-D-(1-O-*sinapoyl*,2-O-*sinapoyl*)*glucopyranose* (**3**). Oil, 75 mg. MS *m/z*: 943 $[MH-18]^+$, 6%; 737 $[MH-18-$sinapic acid$]^+$, 76%; 575 $[MH-18-$sinapic acid × 2$]^+$, 12%; 369 $[MH-18-$sinapic acid × 2 − glucose$]^+$, 100%. TLC R_f = 0.64. HPLC R_t = 13.4 min. UV λ_{max} nm: 223, 240, 330. ^1H and ^{13}C NMR data see Tables 1 and 2.

6-O-β-*Glucopyranosy*-β-D-(1-O-*sinapoyl*,2-O-*sinapoyl*)*glucopyranose* (**4**). Oil, 55 mg. MS *m/z*: 737 $[MH-18]^+$, 12%; 531 $[MH-18-$sinapic acid$]^+$, 100%; 369 $[MH-18-$sinapic acid glucose$]^+$. TLC R_f = 0.45. HPLC R_t = 11.3 min. UV λ_{max} nm; 227, 240, 262, 330. ^1H and ^{13}C NMR data see Tables 1 and 2.

Acidic hydrolysis of **1**, **2**, **3** *and* **4**. Compounds **1**–**4** (10 mg) were each hydrolysed in methanolic HCl (1:1 2 M HCl–MeOH, 2 ml) for 1 hr under reflux in N_2. The reaction mixts were evapd to dryness under red. pres. and over NaOH under vacuum, redissolved in water (5 ml) and extracted twice with EtOAc (5 ml). The EtOAc layers were dried over anhydrous Na_2SO_4, filtered, evapd to dryness and analysed by HPLC as described above. Peaks in the chromatograms of **1** and **2** at 10.3, 10.6, 18.8 and 19.4 min were identified by comparison with standards as sinapic acid and ferulic acid and their corresponding methyl esters. Peaks at 10.3 and 18.8 min for **3** and **4** demonstrated the presence of only sinapic acid and its methyl ester. UV spectra of the sinapic acid and its methyl ester gave λ_{max} nm: 222, 238, 320; for the ferulic acid and its ester λ_{max} nm: 222, 238, 290, 320.

Reduction and acetylation of hydrolysates from **1**–**4**. The aqueous layers from the hydrolysis of **1**–**4** were freeze dried and each redissolved in H_2O (1 ml) for reduction, acetylation and GC analysis for sugar identification. Ammonia (50 μl, concentrated) was added to 300 μl of each extract then $NaBH_4$ (100 μl of 150 mg in 3 M ammonia) added immediately. An internal standard of deoxyglucose (100 μl of 12 mg/ml) added and the reaction mixt. held at room temp. for 1 hr, after which 2 × 50 μl glacial acetic acid added. Acetic anhydride (3 ml) was added to reaction mixt. (300 μl), precooled in ice, and 1-methyl imidazole added (0.45 ml). Reaction mixt. was mixed, cooled in ice (5 min), warmed to room temp. (300 min) and cooled in ice with the addition of H_2O (4.5 ml). The mixtures were each extracted twice with dichloromethane (5 ml), centrifuged and the organic layer evapd to dryness under nitrogen. The residues were redissolved in dichloromethane for GC analysis.

Gas chromatographic analysis of hydrolysates of **1**–**4**. Analysis was carried out using a Hewlett Packard 5890 chromatograph with a 15 m, 0.25 μm capillary

column coated with OV225 0.32 mm thick at 55° increasing to 145° at 45°/min and to 220° at 2°/min. A single peak was obtained for each extract corresponding to glucose (R_t = 36.7 min, RR_t = 1.23). The molar ratios of internal standard to glucose indicated 2 moles of glucose were released per mole for each of the compounds **1**–**4**.

Enzymic hydrolysis of **1** *and* **2**. Compounds **1** and **2** were hydrolysed with β-glucosidase (isolated from almonds, Sigma) to ascertain the relative positions of the ferulic acid and sinapic acids on the reducing sugar in the 1,2 positions. Glucosidase (1 mg = 20 units) dissolved in MES buffer (50 mM, pH 5.1, 1 ml) added to each compound (1 mg) and incubated at 37° for 30 min. Reaction mixts were acidified with HCl (5 M, 0.1 ml) and extracted twice with ether (2 × 2 ml). Both the bulked ether layers and the aqueous layers were evapd to dryness in N_2 and each redissolved in MeOH (20 μl) for HPLC analysis. The ether layers from both **1** and **2** (containing the acid moiety released from the 1-position) produced a single peak by HPLC coeluting with sinapic acid and having an identical spectrum. The aqueous layer from **2** (containing the reducing sugar substituted at the 2 position) produced a single peak by HPLC (9.8 min) with a UV spectrum identical to ferulic acid, whilst the aqueous layer from **1** (containing two glucose units both substituted at the 2 position) produced two peaks (8.4 + 9.6 min) with UV spectra identical to sinapic acid and ferulic acid corresponding to 2-*O*-sinapoylglucose and 2-*O*-feruloylglucose, respectively.

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