Novel Fatty Acid Esters of *p*-Coumaryl Alcohol in Epicuticular Wax of Apple Fruit

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Hexane extracts of epicuticular wax from cv. Gala apples were noted to have an unusual, broad absorbance maximum at ~258 nm, which led us to isolate and identify the primary UV-absorbing compounds. Column and thin-layer chromatography yielded a fraction that gave a series of paired, 260-nm-absorbing peaks on C_{18} HPLC. These were shown to be a family of phenolic fatty acid esters, for which retention times increased with increasing fatty acid chain length, and paired peaks were esters of two related phenolics with the same fatty acid moiety. Alkaline hydrolysis of the esters released two water-soluble phenolics separable by C_{18} HPLC. Electrospray ionization mass spectrometry gave a molecular mass of 150 for both, and ^{1}H NMR plus UV absorbance spectra identified them as *E* and *Z* isomers of *p*-coumaryl alcohol. Alkaline cleavage of the fatty acid esters in the presence of methanol or ethanol resulted in partial derivatization of *E*-*p*-coumaryl alcohol to the corresponding γ-*O*-methyl or *O*-ethyl ether. Gradient HMQC NMR of the HPLC-purified stearate ester of *E*-*p*-coumaryl alcohol indicated that fatty acid esterification occurs at the γ-OH rather than at the 4-OH on the phenyl ring. This is the first report of fatty acid esters of monolignols as a natural plant product.

Keywords: *p-Coumaryl alcohol; fatty acid esters; monolignol; epicuticular wax; apple fruit; Malus domestica*

INTRODUCTION

We previously reported a family of phenolic fatty acid esters, with two broad UV absorbance maxima at about 258 and 206 nm, which were isolated from the epicuticular coating of cv. Gala apple fruit (*1*). Mild alkaline methanolysis of the esters yielded two major methanol/water-soluble free-phenolic constituents, with UV absorbance spectra very similar to those of the intact compounds, plus a series of mostly even-chained, *n*-alkyl, saturated fatty acid methyl esters ranging from C_{16} to C_{26}. On the basis of preliminary ^{1}H NMR data, UV absorbance maxima, and C_{18} HPLC retention times, it was proposed that the free phenolic moieties of the esters might be novel isoflavones. However, subsequent analysis by electrospray ionization-mass spectrometry (ESI-MS) clearly showed that these moieties are simple phenolics, with molecular masses in the range of 150−178, rather than the hypothesized isoflavones (molecular mass ≥ 270).

In this paper we present ^{1}H and ^{13}C NMR, ESI-MS, and UV spectral data that identify the phenolic esters from Gala apple wax as γ-OH fatty acid esters of *E* and *Z* isomers of *p*-coumaryl alcohol (4-hydroxycinnamyl alcohol). It was also determined that mild alkaline methanolysis or 80% ethanolic saponification of the fatty acid esters resulted in partial derivatization of the *E*-*p*-coumaryl alcohol moiety to the corresponding γ-*O*-methyl or *O*-ethyl ether, respectively. Recently, Griffiths et al. (*2*) were the first to identify long-chain fatty acid esters of cinnamyl alcohol as naturally occuring compounds in epicuticular wax from faba bean flowers. This is the first demonstration of γ-OH fatty acid esters of *p*-coumaryl alcohol, a monolignol, in the epicuticular material of plants.

MATERIALS AND METHODS

Plant Material and Tissue Sampling. Cv. Gala apple (*Malus domestica* Borkh. Rosaceae) fruit was harvested on August 27, 1998, from a commercial orchard in southern Pennsylvania at the preclimacteric stage (ethylene production was ≤0.1 μL g^{-1} of fresh weight and the climacteric rise in CO_2 had not yet begun). Apples were washed in warm tap water, rinsed with deionized water, and then allowed to dry overnight at 20 °C. After culling, 100 fruit were selected for uniformity of size and color. The skin and outer 2−3 mm of cortical tissue were excised from these fruit with a mechanical peeler and immediately frozen in liquid N_2. Total yield was ~1.5 kg of frozen peel tissue, which was stored in a large ziplock freezer bag at −80 °C until used for extraction.

Extraction and Fractionation of Apple Peel Constituents. A 500 g portion of frozen peel tissue was immersed in liquid N_2 for 2 min, sieved, and transferred to a 4-L Erlenmeyer flask. Hexane (1.5 L) and a large magnetic stir bar were

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added to the flask, which was then flushed with N_2 and sealed. Tissue was extracted with stirring at 4 °C for 2 h and at 22 °C for an additional hour. The hexane extract was decanted and filtered through a Büchner funnel fitted with two disks of Miracloth (Calbiochem, La Jolla, CA). It was dried by swirling with 30 g of anhydrous sodium sulfate, refiltered through a glass-fiber disk, and reduced to 100 mL by rotary evaporation.

The concentrated extract was applied to a 2.5-cm-diameter glass column containing 150 g of 100−200 mesh silicic acid in 2,2,4-trimethylpentane, followed by step-gradient elution with 50 mL of hexane, 105 mL of hexane/diethyl ether (20:1), 105 mL of hexane/diethyl ether (6:1), and 150 mL of hexane/diethyl ether (2:1). UV spectra indicated that the phenolic fatty acid esters eluted in the final (2:1) column fraction. Solvent was removed from this fraction by rotary evaporation and the residue dissolved in 50 mL of hexane/ethanol (1:1) with warming at 50 °C. After this mixture had been kept for 4 h at 20 °C, a precipitate largely free of phenolic esters was removed by filtration, affording a partial purification of the phenolic ester fraction. The solvent volume was then reduced to ∼10 mL by evaporation under a stream of N_2.

One-milliliter aliquots of the concentrated phenolic ester fraction were applied to 20 × 20 cm preparative silica gel GF thin-layer chromatography (TLC) plates (1-mm thickness; Aldrich, Milwaukee, WI) in a single 20-cm-long band. TLC plates were developed in heptane/2-propanol/ethanol (8:1:1) and then dried in a tank flushed with N_2. The phenolic ester band, with an R_f of ∼0.53, was visualized and marked under 312 nm UV light, then scraped and eluted with 40 mL of hexane/ethanol (3:1). After 3 min of centrifugation at 1000*g* to pellet the silica gel, the solvent was decanted and filtered, and a phase separation was effected by adding 10 mL of deionized water. The upper hexane phase containing the phenolic esters was transferred to a new tube and the solvent evaporated under a stream of N_2. TLC-purified phenolic esters were dissolved in 2 mL of hexane/ethanol (1:1) and stored at −20 °C until used for HPLC separation and analysis of molecular species and free phenolic moieties derived from the esters by alkaline cleavage.

HPLC Separation and Analysis of Phenolic Esters and Free Phenolics. Phenolic ester molecular species were separated by C_{18} HPLC with UV detection at 260 and 210 nm as previously described (*1*) except that the column used was a 25 cm × 4.6 mm diameter 5μ Luna C18 from Phenomenex (Torrence, CA). TLC-purified phenolic esters dissolved in methanol/ethanol (1:1) were injected in 80 μL portions, and the separated components were collected manually as they eluted. Final purification of individual esters was performed using a 25 cm × 4.6 mm diameter 5μ Luna C18(2) column in a Hewlett-Packard 1100 series HPLC system with photodiode array detection and HP ChemStation software. The tertiary mobile phase gradient was programmed as follows: methanol/acetonitrile/water (90:6:4) at 1 mL min^{-1} at time 0, increased linearly to 1.2 mL min^{-1} at 14 min; solvent mix and flow rate changed linearly over 14−20 min to methanol/acetonitrile (90:10) at 1 mL min^{-1}; solvent mix returned linearly to methanol/acetonitrile/water (90:6:4) at 1 mL min^{-1} over 20−25 min and then held constant to 30 min. Mobile phase was evaporated from the HPLC-purified phenolic esters with a stream of N_2, after which they were dissolved in the appropriate solvent for analysis by mass spectrometry (MS) or nuclear magnetic resonance spectroscopy (NMR).

Initially, free phenolics were obtained from the esters as previously described (*1*) by alkaline methanolysis (0.6 M KOH in methanol at 35 °C with shaking for 2 h). In an effort to improve the yield and efficiency, several phenolic ester aliquots were subsequently saponified with 1 M KOH in 80% ethanol for 1 h at 88 °C. When it became evident that alkaline cleavage of phenolic fatty acid esters in the presence of alcohols resulted in the formation of the corresponding phenolic ethers as reaction products, two samples were hydrolyzed with 0.5 M aqueous KOH at 100 °C for 1 h. Regardless of which alkaline medium was used to cleave the esters, after the reaction the mixture was acidified slightly with HCl, fatty acids and/or fatty acid methyl esters were extracted with hexane, and the free phenolic products were partially purified by loading on a Waters C_{18} Sep-Pak and then washing with water and eluting with acetonitrile (*1*). The same HP 1100 series system and Luna C18(2) column used to purify individual phenolic esters were used for separation and analysis of free phenolics. A binary mobile phase gradient was programmed as follows: water/acetonitrile (90:10) at 1 mL min^{-1} for 0−5 min; solvent mix changed linearly to water/acetonitrile (45:55) over 5−15 min and then held constant at 1 mL min^{-1} to 25 min; solvent mix returned linearly to water/acetonitrile (90:10) at 1 mL min^{-1} over 25−30 min and then held constant to 32 min. Mobile phase was evaporated from HPLC-purified phenolics with a stream of N_2, after which they were dissolved in the appropriate solvent for MS or NMR analysis.

Mass Spectrometry. All samples were analyzed by ESI-MS in the negative ion mode on a PE-Sciex API III triple-quadrupole mass spectrometer (Concord, ON, Canada). Free phenolics (synthetic *E*-*p*-coumaryl alcohol, FP-1A, FP-1B, FP-2A-Me, and FP-2A-Et) dissolved in methanol were injected into a 25 μL min^{-1} flow of acetonitrile/water (50:50) including 10 mM ammonium acetate. Phenolic fatty acid esters (PE-3a, PE-3b, PE-4a, and PE-4b) dissolved in 2-propanol were injected into a 25 μL min^{-1} flow of 2-propanol including 10 mM ammonium acetate. For phenolic ester samples, the orifice potential was set to −40 V to increase the proportion of acetate adducts of the parent compounds. MS-MS spectra were obtained by selecting the parent ions, $[M - H]^-$, with the first quadrupole, channeling these into the second quadrupole where collision with argon/nitrogen (9:1) gas caused fragmentation, and then scanning the fragment ions with the third quadrupole.

Nuclear Magnetic Resonance Spectroscopy. ^{1}H NMR spectra of synthetic *E*-*p*-coumaryl alcohol and free phenolics FP-1A, FP-1B, FP-2A-Me, and FP-2A-Et (∼1−3 mg) dissolved in 0.8 mL of CD_3OD were acquired, deuterium locked at 25 °C, using a Bruker QE 300 MHz NMR spectrometer. Chemical shift values were assigned relative to the frequencies of residual nondeuterated water and methanol externally referenced to TMS. Gradient heteronuclear multiple quantum coherence (HMQC) experiments comparing ^{1}H and ^{13}C NMR spectra of *E*-*p*-coumaryl alcohol, *E*-*p*-coumaryl alcohol diacetate, and PE-3a (*E*-*p*-coumaryl alcohol stearate ester) dissolved in 0.4 mL of acetone-d_6/D_2O (9:1) were performed on a Bruker DRX 360 MHz digital instrument. The 5 mm probe had inverse electronics (proton coils closest to the sample) and was equipped with three-axis pulsed field gradients.

RESULTS

HPLC Separation and UV Spectra of Phenolic Esters and Free Phenolics. Phenolic fatty acid esters in the hexane extract of cv. Gala apple peel tissue were partially purified by column and thin-layer chromatography and then analyzed and separated by C_{18} HPLC with UV detection. The two HPLC chromatograms obtained with UV monitoring at 260 and 210 nm were very similar (Figure 1, left panels); they showed an apparent family of related compounds eluting in pairs, with the first peak of each pair (PEs 1a−6a) ∼2−3-fold greater than the second (PEs 1b−6b). Two pooled HPLC fractions including only the PE-a and only the PE-b components had different UV absorbance spectra (Figure 1, right panels). The second maximum in the spectra of the two ester fractions in ethanol was at 265 nm for PE-a and at 259 nm for PE-b. Also, the PE-a spectrum had distinct small shoulders at about 298 and 304 nm, which were absent from the PE-b spectrum. Individual phenolic ester molecular species in HPLC peaks 3a, 3b, 4a, and 4b (Figure 1, upper left panel) were collected and repurified by C_{18} HPLC with a gradient of methanol/acetonitrile/water ranging from 90:6:4 to 90:10:0 (see Materials and Methods) prior to analysis by ESI-MS and

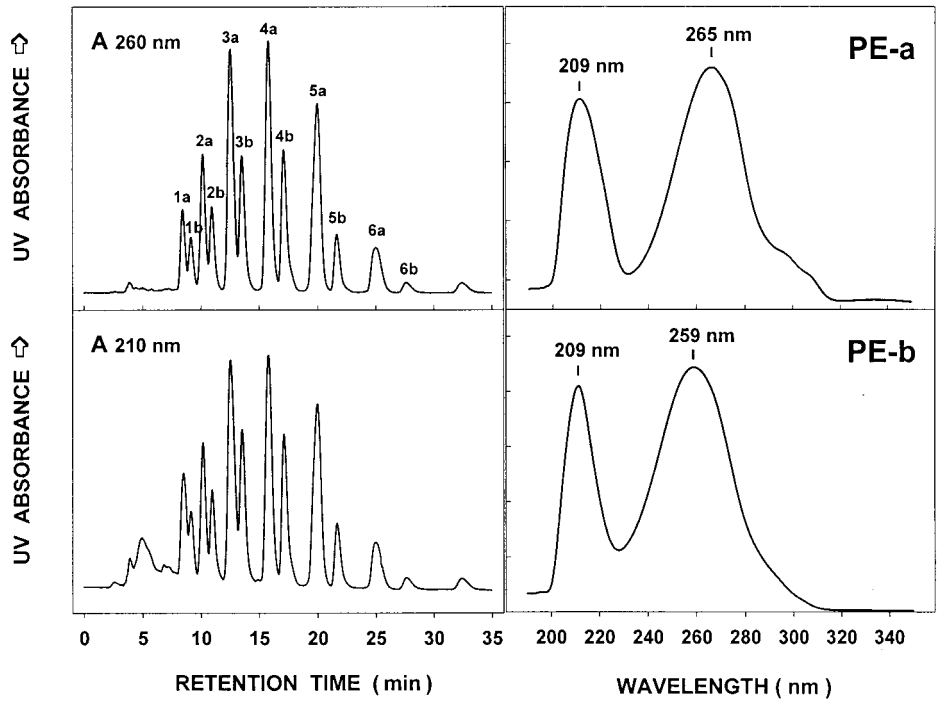


Figure 1. (Left panels) C_{18} HPLC chromatograms of TLC-purified phenolic fatty acid esters from Gala apple wax with UV absorbance monitoring at 260 nm (upper) and 210 nm (lower). (Right panels) UV absorbance spectra of pooled PE-a (upper) and PE-b (lower) molecular species separated by C_{18} HPLC (shown in upper left panel). Pooled PE-a and PE-b fractions were dissolved in ethanol.

NMR. Retention times of PE-3a, PE-3b, PE-4a, and PE-4b under these conditions were 17.6, 19.8, 23.5, and 25.6 min, respectively.

Previously, C_{18} HPLC separation of free phenolics derived from the TLC-purified esters by mild alkaline methanolysis gave two peaks with UV absorbance spectra similar to that of the parent compounds (*1*). Subsequently, changing the mobile phase from water/acetonitrile 30:70 to 80:20 afforded separation of the first peak into two components, designated FP-1A and FP-1B. Three major phenolics were also obtained when TLC-purified esters were saponified with 1 M KOH in 80% aqueous ethanol. It was noted that the HPLC retention time of the third component, FP-2, was longer than that of FP-2 obtained with alkaline methanolysis, suggesting that FP-2 might be an artifact of alkaline cleavage of the esters in the presence of alcohols. In accord with this, alkaline hydrolysis of TLC-purified esters with 0.5 M aqueous KOH yielded only FP-1A and FP-1B. UV spectra of the FP-2s produced under methanolic and ethanolic conditions were nearly identical and were quite similar to that of FP-1A; hence, they were designated FP-2A-Me and FP-2A-Et, respectively. Moreover, the UV spectra of FP-1A, FP-2A-Me, and FP-2A-Et (second maximum at 262, 263, and 263 nm, respectively) all closely resembled the spectrum of the PE-a fraction (Figure 1) with a slight hypsochromic shift, whereas the spectrum of FP-1B (second maximum at 257 nm) was a slightly blue-shifted analogue of the PE-b spectrum (Figure 1). C_{18} HPLC of the four free phenolics, FP-1A, FP-1B, FP-2A-Me, and FP-2A-Et, eluted with a gradient of increasing acetonitrile in water from 10:90 to 55:45 (see Materials and Methods), gave retention times of 15.6, 15.9, 18.7, and 20.2 min, respectively.

Mass Spectra of Free Phenolics and Phenolic Esters. ESI-MS of FP-1A, FP-1B, FP-2A-Me, and FP-2A-Et in the negative ion mode with 10 mM ammonium acetate in the delivery solvent indicated that the phenolic moieties of cv. Gala apple phenolic esters are phenylpropenoids rather than isoflavones as was previously proposed (*1*). For both FP-1A and FP-1B, the major ion was *m/z* 149, representing $[M - H]^-$, as confirmed by presence of the acetate adduct $[M + CH_3COO]^-$, *m/z* 209. The molecular mass of 150 led us to suppose that FP-1A and FP-1B might be isomers of *p*-coumaryl alcohol (4-hydroxycinnamyl alcohol).

A sample of synthetic *E-p*-coumaryl alcohol (kindly provided by Dr. John Ralph, USDA, ARS, and University of Wisconsin, Madison, WI) had a UV absorbance spectrum and an HPLC retention time identical to those of FP-1A and also gave prominent negative ions at *m/z* 149 and 209 on ESI-MS with 10 mM ammonium acetate. FP-2A-Me and FP-2A-Et produced major ions at *m/z* 163 and 177 ($[M - H]^-$), respectively, and at *m/z* 223 and 237 ($[M + CH_3COO]^-$), respectively, suggesting that they are methylated and ethylated derivatives of FP-1A.

MS-MS analysis of the parent ions, $[M - H]^-$, from *E-p*-coumaryl alcohol and FP-1A, FP-1B, FP-2A-Me, and FP-2A-Et gave similar daughter ion profiles. The profiles were nearly identical for *E-p*-coumaryl alcohol, FP-1A, and FP-1B, with *m/z* 130 and *m/z* 102 as the predominant fragment ions derived from *m/z* 149. These two ions were also prominent in the MS-MS spectra of *m/z* 163 from FP-2A-Me and *m/z* 177 from FP-2A-Et. However, FP-2A-Me gave a major ion at *m/z* 31, $[OCH_3]^-$, and FP-2A-Et gave a major ion at *m/z* 45, $[OCH_2CH_3]^-$, which were completely absent in the MS-MS spectra of *E-p*-coumaryl alcohol, FP-1A, and FP-1B. These data are consistent with FP-2A-Me and FP-2A-Et being *O*-methyl and *O*-ethyl ethers, respectively, of FP-1A.

ESI-MS of phenolic ester molecular species PE-3a, PE-3b, PE-4a, and PE-4b (Figure 1) was also performed in the negative ion mode with 10 mM ammonium

Table 1. ^{1}H NMR Data for *E*-*p*-Coumaryl Alcohol and Free Phenolics Derived by Alkaline Cleavage of Phenolic Fatty Acid Esters from the Epicuticular Coating of Gala Apple Fruit

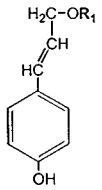
compound	proton(s)	δ, ppm (multiplicity)	J, Hz
E-*p*-coumaryl alcohol (synthetic)	α	6.50 (d)	15.9
	β	6.15 (dt)	15.9, 6.1
	γ	4.17 (d)	6.1
	2,6	7.22 (d)	8.7
	3,5	6.71 (d)	8.7
FP-1A[a] (*E*-*p*-coumaryl alcohol, **1a**)	α	6.51 (d)	15.9
	β	6.15 (dt)	15.9, 6.1
	γ	4.17 (d)	6.1
	2,6	7.22 (d)	8.7
	3,5	6.71 (d)	8.7
FP-1B (*Z*-*p*-coumaryl alcohol, **2a**)	α	6.43 (d)	11.6
	β	5.66 (dt)	11.6, 6.3
	γ	4.33 (d)	6.3
	2,6	7.06 (d)	8.5
	3,5	6.76 (d)	8.5
FP-2A-Me (*E*-*p*-coumaryl alcohol methyl ether, **1b**)	α	6.51 (d)	15.9
	β	6.09 (dt)	15.9, 6.4
	γ	4.04 (d)	6.4
	2,6	7.24 (d)	8.3
	3,5	6.72 (d)	8.3
	O-**CH$_3$**	3.34 (s)	
FP-2A-Et (*E*-*p*-coumaryl alcohol ethyl ether, **1c**)	α	6.47 (d)	15.9
	β	6.07 (dt)	15.9, 6.1
	γ	4.03 (d)	6.1
	2,6	7.19 (d)	7.2
	3,5	6.67 (d)	7.2
	O-[CH$_2$]**CH$_3$**	3.49 (d)	6.1
	O-**CH$_2$**[CH$_3$]	1.15 (t)	6.1

[a] Free phenolics were separated and purified by C_{18} HPLC. All phenolics were dissolved in CD_3OD.

acetate present. For both PE-3a and PE-3b, the intense molecular ion, $[M - H]^-$, was *m*/*z* 415, with the corresponding acetate adduct, $[M + CH_3COO]^-$, at *m*/*z* 475 and another abundant ion at *m*/*z* 283. For PE-4a and PE-4b, the $[M - H]^-$ ion was *m*/*z* 443 and the $[M + CH_3COO]^-$ adduct ion was *m*/*z* 503, with a third major ion at *m*/*z* 311. MS-MS of the parent ion, $[M - H]^-$, from PE-3a and PE-3b (*m*/*z* 415) and from PE-4a and PE-4b (*m*/*z* 443) gave single, intense fragment ions at *m*/*z* 283 and 311, respectively. Collectively, the data support the conclusion that PE-3a and PE-3b are stearate esters and PE-4a and PE-4b are arachidate esters of *p*-coumaryl alcohol isomers.

NMR Spectra of Free Phenolics and PE-3a. Samples of the *E*-*p*-coumaryl alcohol standard, FP-1A, FP-1B, FP-2A-Me, and FP-2A-Et dissolved in CD_3OD were analyzed by ^{1}H NMR (Table 1). Spectra of the synthetic standard and FP-1A were identical, confirming that FP-1A is the *E* isomer of *p*-coumaryl alcohol (Figure 2, **1a**). The spectrum of FP-1B differed substantially from that of FP-1A. Most notably, resonances of the two γ-protons were shifted downfield by ~0.16 ppm, whereas resonance of the β-proton was shifted upfield by ~0.49 ppm. Also, the coupling constants $J_{\alpha\beta}$ and $J_{\beta\alpha}$ were both 11.6 Hz for FP-1B and 15.9 Hz for FP-1A. According to Lewis et al. (*3*), these data indicate that FP-1B is the *Z* isomer of *p*-coumaryl alcohol (Figure 2, **2a**). The intense singlet at 3.34 ppm in the ^{1}H NMR spectrum of FP-2A-Me and the doublet at 3.49 ppm plus triplet at 1.15 ppm in the spectrum of FP-2A-Et are consistent with *O*-methylation and *O*-ethylation, respectively, of these two derivatives of FP-1A. Furthermore, the upfield shift of ~0.14 ppm for the two γ-protons of FP-2A-Me and FP-2A-Et relative to those of FP-1A indicates that methylation and ethylation occur at the γ-OH rather than at the phenyl-4-OH (Figure 2, **1b** and **1c**).

1 *E*-*p*-Coumaryl Alcohol



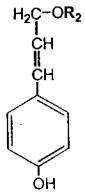
a R_1 = H (FP-1A)

b R_1 = CH_3 (FP-2A-Me)

c R_1 = CH_2CH_3 (FP-2A-Et)

d R_1 = $CO(CH_2)_nCH_3$ (PE-a) [n = 14–24]

2 *Z*-*p*-Coumaryl Alcohol



a R_2 = H (FP-1B)

b R_2 = $CO(CH_2)_nCH_3$ (PE-b) [n = 14–24]

Figure 2. Structures of phenolic fatty acid esters from epicuticular wax of Gala apple fruit and free phenolics liberated by alkaline cleavage of TLC-purified phenolic esters. FP-1A and FP-1B, the bona fide phenolic moieties of Gala phenolic esters, were identified as *E*-*p*-coumaryl alcohol (**1a**) and *Z*-*p*-coumaryl alcohol (**2a**). Free phenolics produced only when esters were cleaved in the presence of methanol (FP-2A-Me) or ethanol (FP-2A-Et) were identified as the γ-*O*-methyl and *O*-ethyl ethers of *E*-*p*-coumaryl alcohol (**1b** and **1c**), respectively. PE-a and PE-b molecular species were shown to be γ-OH fatty acid esters of *E*- and *Z*-*p*-coumaryl alcohol (**1d** and **2b**), respectively. Fatty acid moieties were largely even-chained, *n*-alkyl, saturated species ranging from C_{16} to C_{26} (*1*).

Table 2. ^{13}C and ^{1}H NMR Data from Gradient HMQC Analysis of *E*-*p*-Coumaryl Alcohol, *E*-*p*-Coumaryl Alcohol Diacetate, and PE-3A (*E*-*p*-Coumaryl Alcohol Stearate Ester)

	E-*p*-coumaryl alcohol		*E*-*p*-coumaryl alcohol diacetate		PE-3a	
C/H position	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H
γ	63.4[a]	4.19	65.2	4.69	65.3	4.64
3,5	116.2	6.78	122.8	7.08	115.9	6.77
2,6	128.3	7.25	128.3	7.47	128.5	7.25

[a] Values represent δ (ppm).

A sufficient quantity of one phenolic ester molecular species, PE-3a, was purified to enable correlation of ^{13}C and ^{1}H NMR data by gradient HMQC analysis (Table 2). To determine the position at which the fatty acid is esterifed in the cv. Gala apple phenolic esters, δ values of the γ-, the 3,5-, and the 2,6-carbons and hydrogens were compared for PE-3a and synthetic *E*-*p*-coumaryl alcohol and *E*-*p*-coumaryl alcohol diacetate (acetylated at both the γ-OH and phenyl-4-OH) dissolved in acetone-d_6/D_2O (9:1). Values for the γ-carbon and hydrogens of PE-3a and *E*-*p*-coumaryl alcohol diacetate were very similar and were shifted substantially downfield relative to the corresponding values for unesterified *E*-*p*-coumaryl alcohol. In contrast, values for the 3,5- and 2,6-carbons and hydrogens of PE-3a and *E*-*p*-coumaryl alcohol were very similar or identical and were (except for those of the 2,6-carbons) substantially upfield of the corresponding values for *E*-*p*-coumaryl alcohol diacetate. These data unequivocally establish that fatty acid

esterification occurs at the γ-OH of *p*-coumaryl alcohol (Figure 2, **1d** and **2b**).

DISCUSSION

On the basis of the combined ESI-MS, NMR, and UV spectral data presented here, we have identified the phenolic fatty acid esters present in the epicuticular wax of cv. Gala apple fruit as *E* and *Z* isomers of *p*-coumaryl alcohol with primarily long-chain, saturated fatty acids esterified to the γ-hydroxyl (Figure 2, **1d** and **2b**). In addition to *E*-*p*-coumaryl alcohol (FP-1A; **1a**) and *Z*-*p*-coumaryl alcohol (FP-1B; **2a**), the γ-*O*-methyl and *O*-ethyl ethers of *E*-*p*-coumaryl alcohol, FP-2A-Me (**1b**) and FP-2A-Et (**1c**), were among the free phenolics released from the Gala phenolic esters by alkaline cleavage. It was determined that these ether derivatives were formed as a consequence of alkaline cleavage of the esters in the presence of the corresponding alcohols (i.e., methanol and ethanol). This reaction was apparently specific for the *E*-*p*-coumaryl alcohol fatty acid esters because no *Z*-*p*-coumaryl alcohol methyl or ethyl ethers were recovered. Identification of FP-1A as *E*-*p*-coumaryl alcohol was facilitated by the availability of, and confirmed by comparison with, a synthetic standard (*4*). FP-1B had a slightly longer retention time on C_{18} HPLC and a slightly blue-shifted second UV absorbance maximum relative to FP-1A. These data combined with the ^{1}H NMR olefinic resonances at 5.66 and 6.43 ppm, and $J_{\alpha\beta}$ and $J_{\beta\alpha}$ coupling constants at 11.6 Hz, confirmed that FP-1B is *Z*-*p*-coumaryl alcohol (*3*).

Cinnamyl alcohol and its acetate ester often occur as aromatic constituents in plant essential oils (*5*, *6*), and recently a family of cinnamyl alcohol esters of saturated C_{16}−C_{24} fatty acids was found in the epicuticular wax extracted from faba bean flowers (*2*), the first report of such natural products. Two γ-OH acetate ester derivatives of sinapyl alcohol were identified in bergamot essential oil (*7*), and γ-OH benzoate esters of coniferyl and *p*-coumaryl alcohols occur in benzoin gum (*8*). However, after an extensive search of the literature, this appears to be the first report of γ-OH long-chain fatty acid esters of a monolignol (*p*-coumaryl alcohol) in plants.

Free monolignols are typically found only in trace amounts in plant tissues and are largely utilized in lignin biosynthesis (*9*), but they are also sequestered as phenyl-4-*O*-glucosides (*10*) and serve as precursors to lignans and neolignans, two classes of defense compounds (*11*). Pomace from processed apples includes lignin as a major component (*12*); hence, apple fruit tissues clearly have all of the requisite enzymes and abundant substrates for monolignol synthesis. With few exceptions, *E*-monolignols are predominant in plants, and it appears that isomerization to *Z*-monolignols occurs enzymatically as a late step in the pathway (*9*, *13*). It is possible that *E*-*p*-coumaryl alcohol fatty acid esters in the epicuticular wax of apple fruit are photochemically converted to the corresponding *Z* isomers by UV light (*3*), but the fact that phenolic esters from cv. Granny Smith apples are nearly devoid of *Z* isomers would argue against this (B. D. Whitaker, unpublished observation). At present, nothing is known about the fatty acid transferase that catalyzes fatty acid esterification of the γ-OH on *p*-coumaryl alcohol. Conceivably, it could be an enzyme that participates in suberin biosynthesis, but so far this complex process remains largely uncharacterized. Possible functions of phenolic esters in the epicuticular wax of apple fruit include a role as feeding repellents (*14*) or as antifungal compounds (*15*), among others. It remains to be determined why these monolignol fatty acid esters are present at high concentration in the epicuticular coating of fruit from a few apple cultivars such as Gala but are essentially absent in fruit of other cultivars such as Empire.

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