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COUMAROYL-, CAFFEOYL- AND FERULOYLTARTRONATES AND THEIR ACCUMULATION IN MUNG BEAN

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Key Word Index—*Vigna radiata* (*Phaseolus aureus*); Leguminosae; mung bean; hydroxycinnamic acid; tartronic acid; ester; phenylpropanoid metabolism; accumulation.

Abstract—Three new plant constituents were isolated from the primary leaves of *Vigna radiata* (= *Phaseolus aureus*) and their structures elucidated and characterized with the aid of negative-ion fast atom bombardment mass spectrometry (FAB MS), ^{1}H NMR and UV spectroscopy, thin-layer, gas–liquid and high performance liquid chromatography. The new conjugates are (*E*)-*p*-coumaroyl-, (*E*)-caffeoyl- and (*E*)-feruloyltartronic acids. Their structures were unequivocally confirmed by comparison with synthetic material. The metabolism of the new hydroxycinnamic acid conjugates in young plants of *Vigna radiata* is described.

INTRODUCTION

The mung bean, a plant which is well established in plant biochemistry and physiology (e.g. refs. [1–3]), has received considerable attention, for example in studies on isoflavonoid phytoalexins [4–6] and on developmental processes [7, 8]. This plant is also of economic importance as an essential human food crop [6].

The structural elucidation of the major soluble secondary (natural) products of *Vigna radiata* (= *Phaseolus aureus*) is at present incomplete. Two flavonoids have been identified, the anthocyanin delphinidin 3-glucoside [9] and the flavonol glycoside rutin [10], while in a recent publication on *ortho*-hydroxylation of phenolic compounds in mung been seedlings [11] caffeic acid was determined by HPLC to be a major hydroxycinnamic acid, liberated by acid hydrolysis.

In continuation of our studies on the metabolism of hydroxycinnamic acid conjugates (e.g. ref. [12] and literature cited therein), our attention was drawn to young plants of *V. radiata*. HPLC analyses revealed that the primary leaves rapidly accumulated large amounts of hydroxycinnamic acid conjugates, which could also be detected in all overground organs of the young plant. The objectives of our studies were to identify the phenolic constituents and to quantify and characterize the accumulation of these phenolic compounds.

In this paper we report on the structural elucidation of caffeic, *p*-coumaric and ferulic acid tartronic acid esters, their accumulation in the primary leaves, and their distribution in different organs of young *V. radiata* plants.

RESULTS AND DISCUSSION

Figure 1 shows a typical HPLC elution profile of the phenolic compounds from a methanolic extract from the primary leaves of 10-day-old plants of mung bean. The predominant constituent (peak 1) was identified as (*E*)-caffeoyltartronic acid. Peaks 2 and 3 represent (*E*)-*p*-

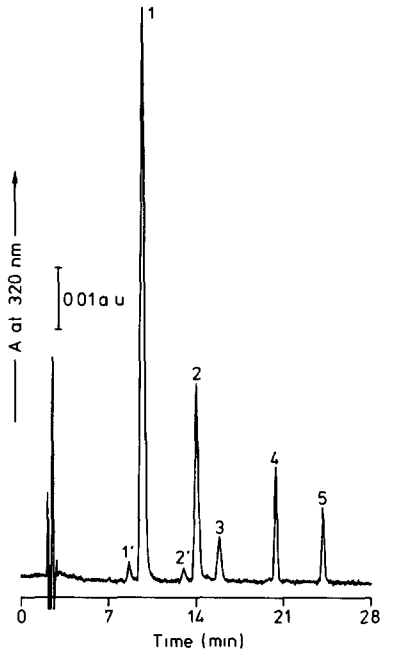

Fig. 1 HPLC separation of phenylpropanoid products from a crude extract of 10-day-old mung bean primary leaves. Peak identification: (1) caffeoyltartronate; (2) *p*-coumaroyltartronate; (3) feruloyltartronate; (4) rutin; (5) nicotiflorin; (1′ and 2′) *cis*-configuration of caffeoyl- and *p*-coumaroyltartronates. Development: linear gradient elution within 30 min from 30 to 90% solvent B (1% H_3PO_4, 20% HOAc, 25% MeCN in H_2O) in solvent A (1% H_3PO_4 in H_2O) at a flow rate of 1 ml/min

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coumaroyl- and (*E*)-feruloyltartronic acid, respectively. The known flavonoid constituent rutin [10, 11] eluted as peak 4, followed by another flavonol glycoside (peak 5), identified as nicotiflorin (kaempferol 3-rutinoside).

For isolation and structural elucidation, the crude methanol extract from the primary leaves of mung bean was pre-fractionated on a polyamide (Perlon) column. The 80% aqueous methanol fraction contained rutin and nicotiflorin. Both flavonoids were identified by direct chromatographic comparison (TLC on microcrystalline cellulose in CAW, BAW and 10% HOAc and HPLC as shown in Fig. 1). The ammonium hydroxide–methanol fraction contained the hydroxycinnamic acid esters, which were separated into three constituents on microcrystalline cellulose in CAW. The separated esters were eluted with methanol and re-chromatographed on polyamide columns. The compounds were obtained in a pure form after column chromatography on Sephadex LH-20 with methanol. The chromatographic behaviour and detection of the esters on TLC under UV (350 nm) with and without treatment of ammonia vapour gave the first indication as to the nature of the compounds as caffeic, *p*-coumaric and ferulic acid esters. UV spectroscopic analyses showed that the addition of sodium hydroxide gave strong bathochromic shifts of λ_{max}^{MeOH} (caffeoyl ester from 325 to 368 nm, *p*-coumaroyl ester from 309 to 356, and feruloyl ester from 322 to 370 nm).

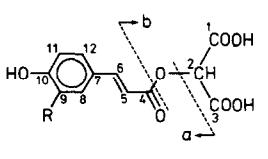
The nature and substitution pattern of the aromatic ester moieties were characterized from the ^{1}H NMR spectra, which indicated the presence of both *cis* and *trans* configurations of the double bond. However, the *trans* configuration is definitely the naturally occurring one, as was clearly indicated by HPLC (Fig. 1). (We observed an increasing portion of the *cis* form during isolation of the compounds.) The presence of the tartronic acid residue in each compound was detected by singlet signals at δ 5.4 and its nature was clearly evident from the fragmentation in the FAB mass spectra (Scheme 1).

GC comparison of the products from alkaline hydrolysis of **1** with standard compounds (*p*-coumaric acid and tartronic, malonic, malic, *meso*- and L-tartaric acid) showed identity with *p*-coumaric and tartronic acids, which were found to be in a quantitative ratio of *ca* 1:1. As tartronic acid rarely occurs in nature, the absolute configuration of **1** was confirmed by comparison of the chromatographic behaviour (TLC and HPLC) and spectroscopic data with that of synthetic (*E*)-*p*-coumaroyltartronic acid. It was found that the naturally occurring and synthetic esters were identical.

To our knowledge, this is the first example of the occurrence of tartronic acid accumulating as a major plant constituent in the form of a conjugating moiety in the metabolism of hydroxycinnamic acids. So far, this acid has been sporadically found in living systems. Its presence has been established in sugar refinery liquor [13] and it was identified as a product of bacterial metabolism [14, 15]. Alkyl-tartronic acid was detected in the uropygial gland secretion of birds [16]. According to Rieben and Hastings [17], tartronic acid is formed in various plants, and Wesson [18] reported on the tartronic acid in tissues of celery, lettuce, apple, potato and walnut. Low concentrations of free tartronic acid were also found in olive pulp [19]. Stafford [20] reported on dehydrogenase activity of tartronate and related acids in plants.

Detailed quantitative HPLC revealed a rapid accumulation of large amounts of caffeoyltartronate as the major soluble secondary product of phenylpropanoid metabolism of the primary leaves. The highest concentration of *ca* 200 nmol per leaf (3.2 μmol/g fr. wt) was reached on the seventh day of culture (Fig. 2). *p*-Coumaroyltartronate accumulated up to *ca* 75 nmol (1.2 μmol/g fr. wt) and feruloyltartronate reached *ca* 5 nmol (80 nmol/g fr. wt). The flavonoids rutin and nicotiflorin, minor secondary products in the primary leaf, reached concentrations of *ca* 25 and 10 nmol (403 and 161 nmol/g fr. wt), respectively. Further studies will have to show whether these compounds, especially the tartronic acid esters, are end-products or are subject to further metabolism. We observed a rapid decrease in the concentration of the caffeoyltartronic acid when detached primary leaves were allowed to stand for up to 30 min at room temperature before extraction.

We examined all the organs of the young plant and found that the phenylpropanoid products accumulated in the primary leaves were present in the epicotyl, cotyledons and the hypocotyl. Compared to the primary leaf, however, the caffeoyl conjugate was markedly reduced and



		[M-H]$^-$	a	b
1	R=H	265	163	119
2	R=OH	281	179	119
3	R=OCH$_3$	295	193	119

Scheme 1. Mass fragmentation pattern of hydroxycinnamoyltartronic acids.

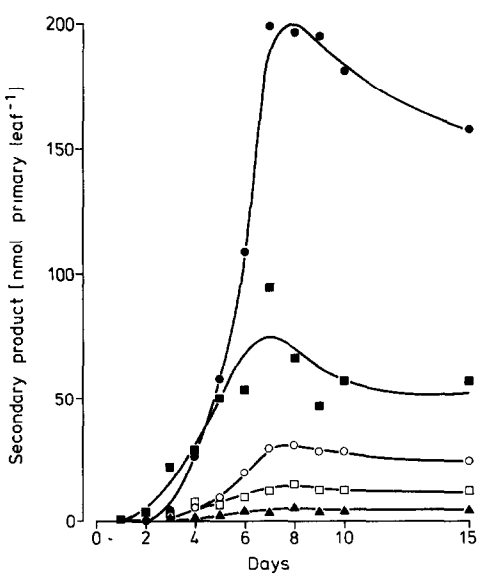


Fig. 2. Kinetics of the formation of caffeoyltartronate (●), *p*-coumaroyltartronate (■), feruloyltartronate (▲), rutin (○) and nicotiflorin (□) in growing mung bean primary leaves. Each point represents the mean value from two independent determinations.

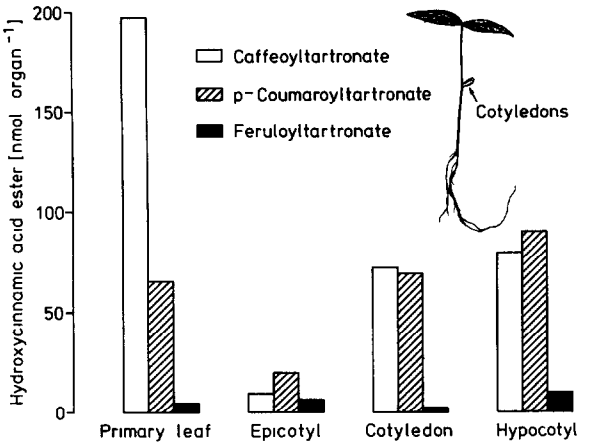


Fig. 3. Pattern of tartronic acid esters of hydroxycinnamic acids in different organs of the young plant (8 days old) of mung bean, at the stage when the primary leaf has reached *ca* 5 cm^2.

reached levels similar to those of the *p*-coumaroyl conjugate. The feruloyltartronate was always found in small quantities. Figure 3 illustrates the distribution of the hydroxycinnamic acid tartronic acid esters in the different organs of the young plant of *V. radiata*. The dry seed contained small quantities of caffeoyl- and *p*-coumaroyltartronate. One of the tartronic acid esters, *p*-coumaroyltartronate, was also most likely present in the root.

In summary, our studies show that mung bean accumulates large amounts of tartronic acid esters of hydroxycinnamic acids. The quantitative ratio of the caffeoyl and *p*-coumaroyl conjugates seems to be organ-specific. It would be of great interest to isolate the enzymes involved in the catalysis of tartronic acid ester formation and to study their involvement and regulation in the general metabolism of the mung bean. In a previous study on *ortho*-hydroxylation of phenolic compounds in mung bean seedlings [11] it was suggested that *p*-coumaric acid hydroxylase is not involved in the formation of caffeic acid in this plant. Thus it would also be of interest to pursue the possible hypothesis that cinnamic or *p*-coumaric acid becomes conjugated with tartronic acid before the subsequent hydroxylation to caffeoyltartronic acid, as is suggested for the biosynthesis of chlorogenic acid in potatoes [21].

EXPERIMENTAL

Plant material. Young plants of *Vigna radiata* (L.) Wilczek (= *Phaseolus aureus* Roxb.) ("Katjang Idjoe", Conimex Baarn, The Netherlands) were grown in a defined soil (type T, Balster, Fröndenberg, West Germany, mixed 1:1 with peat) in a phytotron under fluorescent light (*ca* 10000 lx) with a 14 hr day at 25° and 70% relative humidity.

Preparative extraction and separation. Primary leaf material of fresh 7-day-old *V. radiata* (320 g) was extracted with 2 l. 80% aq. MeOH with an Ultra-Turrax homogenizer (10 min) After filtration and extensive washing of the residue, the extract was concd under vacuum to a vol. of *ca* 70 ml and extracted with $CHCl_3$. The aq. phase was concd to 4 ml, to which 100 ml MeOH was added. This was allowed to stand for 12 hr at −20°. Denatured protein was eliminated by centrifugation (25 min at 3500 *g*). The supernatant was concd to 10 ml and fractionated on a polyamide column (CC-6 Perlon, 3.5 × 78 cm) using H_2O (1.2 l.), 80% aq. MeOH (1.4 l.), MeOH (1 l.), and 0.035% NH_4OH in MeOH (1.3 l.). The 80% aq. MeOH and the NH_4OH–MeOH fractions were concd and kept for further analyses. The 80% aq. MeOH fraction contained rutin and nicotiflorin and was kept for co-chromatography. The NH_4OH–MeOH fraction contained the hydroxycinnamoyltartronic acids and was chromatographed on microcrystalline cellulose layers (20 × 40 cm) in CAW ($CHCl_3$–HOAc, 3:2, H_2O saturated) and separated into three constituents (caffeoyltartronate, R_f 0.13; *p*-coumaroyltartronate, R_f 0.23; feruloyltartronate R_f 0.30), which were detected under UV at 350 nm. The caffeoyl conjugate changed from blue to bright greenish-blue fluorescence when treated with NH_3 vapour, the *p*-coumaroyl conjugate from dark absorbing to dark blue and the feruloyl conjugate from blue to dull greenish-blue. The esters were scrapped off and eluted with MeOH. After re-chromatography on polyamide columns (2 × 30 cm), the compounds were purified on Sephadex LH-20 columns (2 × 88 cm; MeOH). Caffeoyl-, *p*-coumaroyl- and feruloyltartronates exhibited the following TLC R_f values on microcrystalline cellulose in BAW (*n*-BuOH–HOAc–H_2O, 6:1:2): 0.29, 0.46, 0.40; in 10% aq. HOAc 0.64, 0.76, 0.85.

Analytical extraction and separation. Individual organs from 25 plants at different stages of development were separately homogenized in an Ultra-Turrax homogenizer in 50 ml 80% aq. MeOH for 4 min. The resulting mixture was filtered and concd to dryness under vacuum at 30°. The residue was redissolved in 5 ml 50% aq. MeOH. After centrifugation, the clear supernatant was kept for further analysis. Extracts from primary leaves, epicotyl, cotyledons and hypocotyl were co-chromatographed (TLC in CAW, BAW and 10% aq. HOAc and HPLC as shown in Fig. 1). For quantitative determinations, the extracts were analysed by HPLC. The liquid chromatograph, detector and computing integrator have been described elsewhere [22]. The chromatographic column (250 × 4 mm) was prepacked with LiChrosorb RP-8 (5 μm). The elution system is described in the legend of Fig. 1. The sample size was 20 μl. Quantitative data were obtained using 1-sinapoylglucose isolated from seedlings of *Raphanus sativus* [23] and nicotiflorin (Roth) as standards.

GC. Hydrolysed (1 ml 1 N NaOH, room temp., 2 hr) compound **1** was dissolved in 10 ml H_2O and chromatographed on Dowex Bio-Rad AG 50 W-X 8 (100–200 mesh, H^+). The eluate was evapd to dryness under vacuum at 50°. The residue was silylated using 100 μl MSTFA (*N*-methyl-*N*-trimethylsilyltrifluoroacetamide) in 400 μl dry pyridine. Samples (1 μl) were injected onto an OV-101 column (25 m) (100° for 1 min, then programmed at 6°/min at 250°). Compound **1** gave peaks at R_t 6.85 and 23.45 min, identical to tartronic and *p*-coumaric acid, respectively.

NMR and MS. ^{1}H NMR spectra were recorded at ambient temp. at 400 MHz with the spectrometer locked to the major deuterium resonance of the solvent, CD_3OD. Chemical shifts are reported in ppm relative to TMS. Negative-ion FAB MS were recorded in glycerol as matrix.

(E)-p-*Coumaroyltartronic acid* (**1**). ^{1}H NMR (CD_3OD): δ7.776 (*d*, H-6, *J* (6–5) = 15.9 Hz), 7.537 ('*d*', H-8, H-12, *J* (8–9) + *J* (8–11) = 8.6 Hz), 6.854 ('*d*', H-9, H-11), 6.514 (*d*, H-5), 5.489 (*s*, H-2). FAB MS *m/z*: 265 $[M-H]^-$, 163 $[C_9H_7O_3]^-$, 119 $[C_3H_3O_5]^-$.

(E)-*Caffeoyltartronic acid* (**2**). ^{1}H NMR (CD_3OD): δ7.702 (*d*, H-6, *J* (6–5) = 15.9 Hz), 7.124 (*d*, H-8, *J* (8–12) = 2.0 Hz), 7.017 (*dd*, H-12, *J* (12–11) = 8.2 Hz), 6.822 (*d*, H-11), 6.447 (*d*, H-5), 5.444 (*s*, H-2). FAB MS *m/z*: 281 $[M-H]^-$, 179 $[C_9H_7O_4]^-$, 119 $[C_3H_3O_5]^-$.

(E)-*Feruloyltartronic acid* (**3**). ^{1}H NMR (CD_3OD): δ7.772 (*d*,

H-6, J (6–5) = 15.9 Hz), 7.276 (*d*, H-8, J (8–12) = 1.9 Hz), 7.149 (*dd*, H-12, J (12–11) = 8.2 Hz), 6.860 (*d*, H-11), 6.535 (*d*, H-5), 5.447 (*s*, H-2), 3.945 (*s*, 9-OMe). FAB MS *m/z*: 295 $[M-H]^-$, 193 $[C_{10}H_9O_4]^-$, 119 $[C_3H_3O_5]^-$.

Synthesis of (E)-p-*coumaroyltartronic acid.* *p*-Coumaric acid (200 mg) was dissolved in 4 ml dry pyridine and 2 ml Ac_2O was added to produce the *O*-acetyl derivative, which was then evapd to dryness under vacuum at 60°. The acid chloride was produced by dissolving the compound in 5 ml oxalyl chloride. This was evapd to dryness and treated, with stirring in the dark, with tartronic acid (250 mg) in 10 ml dry pyridine. Deacetylation of the product was performed by refluxing it with 0.12 N HCl in 75% aq. dioxane. Purification of the (*E*)-*p*-coumaroyl tartronic acid was carried out by polyamide CC, prep. TLC and Sephadex LH-20 CC, as described for the isolation of the naturally occurring esters. Co-chromatography of the naturally occurring and the synthetic tartronic acid esters (TLC in CAW, BAW, 10% HOAc and HPLC), and ^{1}H NMR spectroscopy and negative-ion FAB MS spectrometry revealed that both compounds were identical.

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