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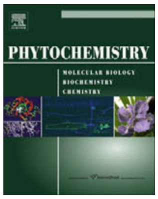

Extraction, structural characterisation and evaluation of hydroxycinnamate esters of orchard grass (*Dactylis glomerata*) as substrates for polyphenol oxidase

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ABSTRACT

Polyphenol oxidase (PPO) activity has been reported in orchard grass (*Dactylis glomerata*); however, to date, no endogenous substrates have been identified. In the present study, we report the isolation and structural elucidation of PPO substrates in this species. The free phenol fraction was extracted, separated by reverse-phase chromatography and six potential substrates, including two hydroxycinnamate esters, were identified by UV spectrometry, electrospray ionisation-tandem mass spectrometry (LC–ESI-MS^n) and 1D and 2D NMR analyses (^{1}H NMR, ^{13}C NMR, DEPT, COSY, HMQC and HMBC). Furthermore, three caffeoylquinic acids (3-CQA, 4-CQA and 5-CQA) were identified by comparison of their spectral data (ESI-MS) with those of known compounds and literature data. Five of these compounds were demonstrated to be substrates for orchard grass PPO.

1. Introduction

Orchard grass (*Dactylis glomerata*) is a common perennial grass that is frequently found in woodland edges, rough grassland, meadows and permanent pastures. In the UK and continental Europe, orchard grass, together with other native species, including fescues, occupies around 30% of pastoral land (Xu et al., 2006). Recently, high levels of polyphenol oxidase (PPO) activity have been reported in this temperate grass (Lee et al., 2006).

PPOs, also known as catechol oxidase, catecholase, diphenol oxidase, *o*-diphenolase, phenolase and tyrosinase (Martinez and Whitaker, 1995; Mayer, 2006) are copper-containing enzymes that can catalyse either one or two reactions involving molecular oxygen. In the first reaction, PPO catalyses the hydroxylation of monophenols, resulting in the formation of *o*-dihydroxyaryl compounds. In the second reaction, PPO catalyses the oxidation of the *o*-dihydroxyaryl compounds to *o*-quinones. Quinones are highly electrophilic and bind rapidly to nucleophilic sites on other compounds (e.g., phenols, amino-acids and proteins) and condense with phenols to form brown-, black- or red-coloured polymers (melanins).

Almost all higher plants contain PPOs, including food crops such as tomato (Newman et al., 1993), wheat (Hatcher and Kruger, 1993), potato (Hunt et al., 1993) and pear (Siddiq et al., 1994), as well as seeds such as cocoa (Wong et al., 1990). PPOs have also been reported in forage crops other than orchard grass, including red clover (Sullivan and Hatfield, 2006) and a range of temperate grass species (Marita et al., 2005). PPOs are strongly implicated in discoloration of fruit and vegetables, which results in shortened shelf-life and consequent commercial losses. Nevertheless, PPOs can also be advantageous as it has been proposed that PPO activity can reduce loss of proteins in red clover and alfalfa forage crops during ensilage (Sullivan and Hatfield, 2006) and lower plant-mediated lipolysis in red clover (Lee et al., 2006).

Families of phenolic compounds found in plants include benzoic acids (*i.e.* 4-hydroxybenzoic acid **1**, protocatechuic acid **2**, vanillic acid **3**, syringic acid **4**, salicylic acid **5** and gentisic acid **6**, Fig. 1), cinnamic acids (i.e. *p*-coumaric acid **7**, caffeic acid **8** and ferulic acid **9**), flavanols (i.e. kaempferol **10**, quercetin **11** and myricetin **12**), tannin 'precursors' (i.e. catechin **13**, gallocatechin **14** and epicatechin **15**) and anthocyanidins (i.e. cyanidin **16**, peonidin **17**, delphinidin **18**, petunidin **19** and malvidin **20**) (Martinez and Whitaker, 1995). A wide range of 1,2-dihydroxyarenes can potentially serve as PPO substrates; these are readily oxidised because of the *ortho* positioning of the OH groups (Martinez and Whitaker, 1995). Recently, the PPO substrate (−)-epicatechin **15** has been isolated from *litchi* pericarp (Sun et al., 2006), whilst one of the major PPO substrates in grapes is reported to be caftaric acid (Sarni-Manchado et al., 1997). In timothy (*Phleum pratense*), meadow fescue (*Festuca pratensis*) and Italian (*Lolium multiflorum*), perennial (*Lolium perenne*) and hybrid ryegrasses (*L. multiflorum* x

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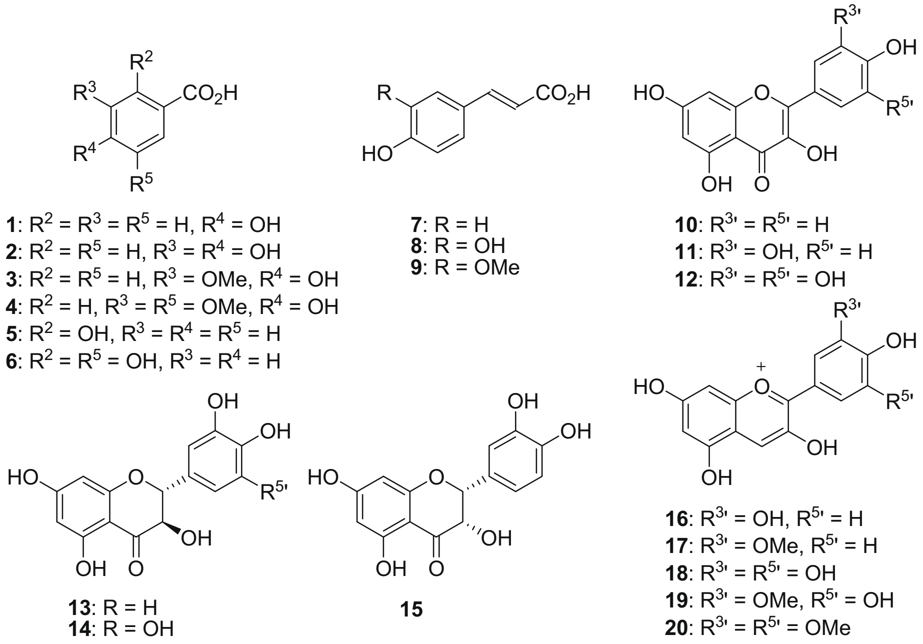


Fig. 1. Structures of examples of known phenolic compounds found in plants.

Lolium perenne), the major PPO substrate is chlorogenic acid (Winters et al., 2006).

At present, little work has been carried out to identify substrates of the enzyme PPO in grass species, although PPOs themselves have been studied in various grass species. In this study, we report on the isolation and structural elucidation of substrates of the enzyme PPO in orchard grass, using nuclear magnetic resonance (NMR) spectroscopy and liquid chromatography-electrospray ionisation-tandem mass spectrometry (LC–ESI-MS^n).

2. Results and discussion

Free phenols were obtained from the methanol extract of fresh leaves of orchard grass. An initial screening of the grass extract identified thirteen phenolic constituents with characteristic UV shapes and UV absorptions (240–340 nm) typical of hydroxycinnamate esters. Nine of these compounds (**21–29**) were separated and isolated using preparative HPLC (Fig. 2). The mass spectra and retention times of the phenols were compared with those of standards including; coumaric acid, ferulic acid, cinnamic acid, caffeic acid and chlorogenic acid (5-caffeoylquinic acid). The patterns of fragmentation observed for three of the compounds, **21**, **22** and **23**, by MS^2 (Table 1) and order of elution following separation by C_{18} reverse-phase chromatography under defined conditions enabled these to be identified as three isomers of caffeoylquinic acid (3-CQA (**21**), 4-CQA (**22**) and 5-CQA (**23**)) (Fig. 3) (Clifford et al., 2006). Extensive studies have reported the separation of caffeoylquinic acid isomers by HPLC, using reverse-phase packings with elution in aqueous alcohol or acetonitrile (Crozier, 2003). The elution patterns of isomers in the extract of grass under conditions reported by Clifford et al. (1989) were consistent with the above assignments. Five of the cinnamate esters (two of which were the major components in orchard grass), for which standards were not available, were collected and structurally identified by 2D NMR and ESI-MS spectra.

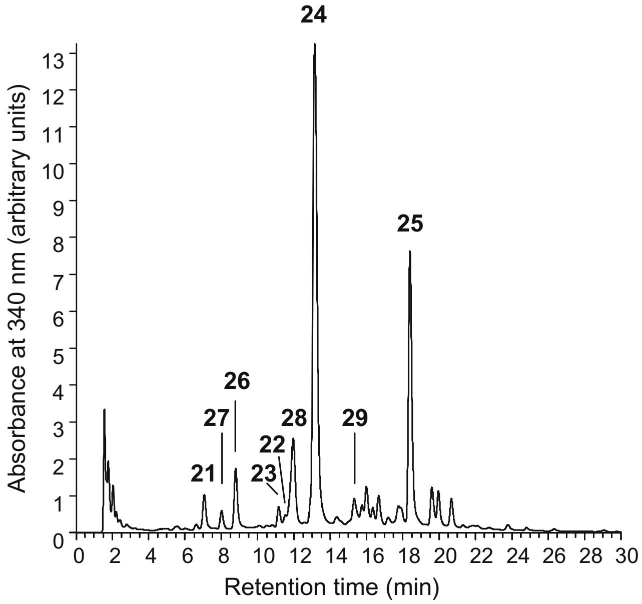


Fig. 2. HPLC/UV (340 nm) chromatogram of phenolic components in orchard grass (*Dactylis glomerata*) extract.

Compound **24** was obtained as pale yellow crystals, with a retention time (t_R) of 13.14 min, following separation by C_{18} reverse-phase HPLC under conditions described here. Its UV absorbance maximum was 328 nm, consistent with a 3,4-dioxygenated cinnamate chromophore. A molecular formula of $C_{15}H_{14}O_{10}$ was confirmed by an ESI-MS spectrum, supported by counts of ^1H and ^{13}C signals in the appropriate NMR spectra. The predominant ions in the full MS negative-ionisation mode were *m/z* 353 [M−H] and 707 [2 M−H] (Table 1). MS^2 of the *m/z* 353 ion gave two fragments, *m/z* 191 [M−caffeoyl] and 173 [M−(caffeoyl, H_2O)] (Fig. 4) In the MS^3 experiment, fragment *m/z* 173 gave ions *m/z* 155 [M−(caffeoyl, 2 H_2O)] and 111 [M−(caffeoyl, 2 H_2O, CO_2)]. Further fragmentation of *m/z* 191 gave *m/z* 173 [isocitric acid−(H_2O, H)] and *m/z* 111 [isocitric acid−(2 H_2O, CO_2)]. These data suggest that **24** contains a dihydroxycinnamate (caffeoyl) unit and the loss of CO_2 during fragmentation points to the presence of at least one carboxylic acid. Full MS in the positive-ionisation mode

Table 1
HPLC/ESI-MS/MS characterisation of hydroxycinnamate ester components in orchard grass (*Dactylis glomerata*)

Peak/ Cpd. No.	HPLC t_R (min)	*m/z* Fragment ion (negative-ion mode)	*m/z* Fragment ion (positive-ion mode)	λ_{max} (nm)
21	7.06	353 [M−H], 191 [M−caffeoyl], 179 [caffeic acid−H], 173 [M−caffeic acid], 162 [M−(quinic acid, H)], 135 [caffeic acid−CO_2H]	377 [M + Na], 355 [M + H], 337 [M−OH], 163 [M−quinic acid], 145 [caffeic acid−(H_2O, OH)]	325
22	11.57	353 [M−H], 191 [M−caffeoyl], 179 [caffeic acid−H], 173 [M−caffeic acid], 162 [M−(quinic acid, H)], 135 [caffeic acid−CO_2H]	377 [M + Na], 355 [M + H], 337 [M−OH], 163 [M−quinic acid], 145 [caffeic acid−(H_2O, OH)]	326
23	11.16	353 [M−H], 191 [M−caffeoyl], 179 [caffeic acid−H], 173 [M−caffeic acid], 162 [M−(quinic acid, H)], 135 [caffeic acid−CO_2H]	377 [M + Na], 355 [M + H], 337 [M−OH], 163 [M−quinic acid], 145 [caffeic acid−(H_2O, OH)]	325
24	13.14	707 [2 M−H], 353 [M−H], 191 [M−caffeoyl], 179 [caffeic acid−H], 173 [isocitric acid−(H_2O, H)], 173 [M−(caffeic acid)], 155 [M−(caffeic acid, H_2O)], 111 [isocitric acid−(2 × H_2O, CO_2H)], 111 [M−(caffeic acid, H_2O, CO_2]	377 [M + Na], 215 [isocitric acid + Na], 197 [isocitric acid + Na−H_2O], 163 [caffeic−OH], 145 [caffeic−(H_2O, OH)]	329
25	18.39	735 [2 M−H], 367 [M−H], 205 [M−caffeoyl], 187 [M−caffeic acid], 179 [caffeic acid−H], 173 [M−(caffeoyl, CH_3OH)], 155 [M−(caffeic acid, CH_3OH)], 135 [caffeic acid−CO_2H], 111 [M−(caffeic acid, CH_3OH, CO_2)]	391 [M + Na], 229 [isocitric acid methyl ester + Na], 211 [isocitric acid methyl ester + Na−H_2O], 163 [caffeic acid−OH], 145 [caffeic acid−(H_2O, OH)]	330
26	8.79	595 [2 M−H], 297 [M−H], 179 [caffeic acid−H], 135 [M−caffeoyl], 117 [M−caffeic acid]	299 [M + H], 163 [caffeoyl−OH], 145 [caffeoyl−(H_2O, OH)], 136 [threonic acid + H], 117 [threonic acid−H_2O]	327
27	8.01	595 [2 M−H], 297 [M−H], 179 [caffeic acid−H], 135 [M−caffeoyl], 117 [M−caffeic acid]	299 [M + H], 163 [caffeoyl−OH], 145 [caffeoyl−(H_2O, OH)], 136 [threonic acid + H], 117 [threonic acid−H_2O]	325
28	11.96	369 [M−H], 207 [M−caffeoyl], 189 [M−caffeic acid], 179 [caffeic acid−H], 171 [M−(caffeic acid, H_2O)], 135 [caffeic acid−CO_2H], 127 [M−(caffeic acid, H_2O, CO_2)]	371 [M + H], 209 [hydroxycitric acid + H], 191 [hydroxycitric acid−OH], 163 [caffeic acid−OH], 145 [caffeic acid−(H_2O, OH)]	328
29	15.32	383 [M−H], 189 [M−ferulic acid], 171 [M−(ferulic acid, H_2O)], 127 [M−(ferulic acid, H_2O, CO_2)]	385 [M + H], 209 [hydroxycitric acid + H], 195 [feruloyl + H], 191 [hydroxycitric acid−OH], 177 [ferulic acid−OH]	328

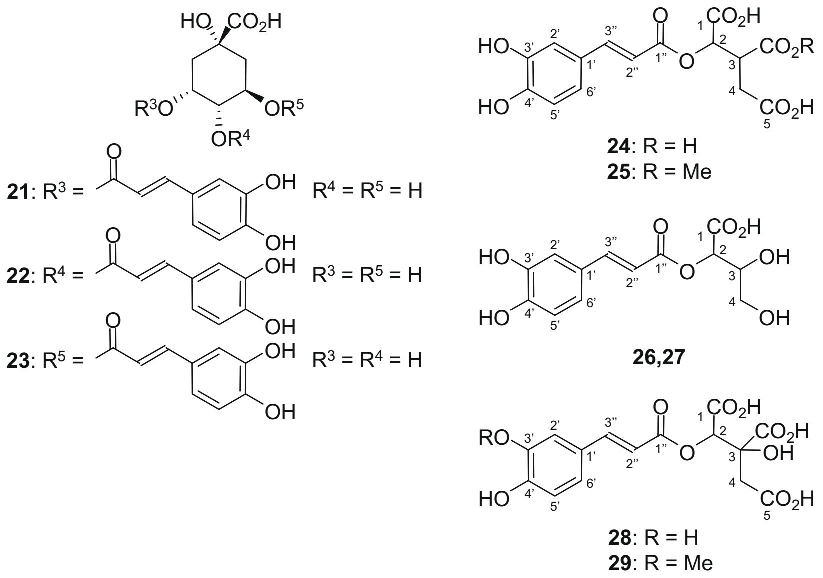


Fig. 3. Structures of hydroxycinnamate esters isolated from *Dactylis glomerata*.

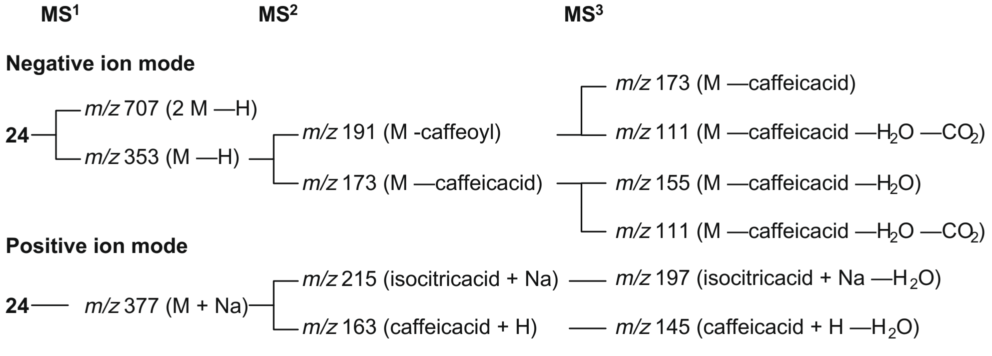
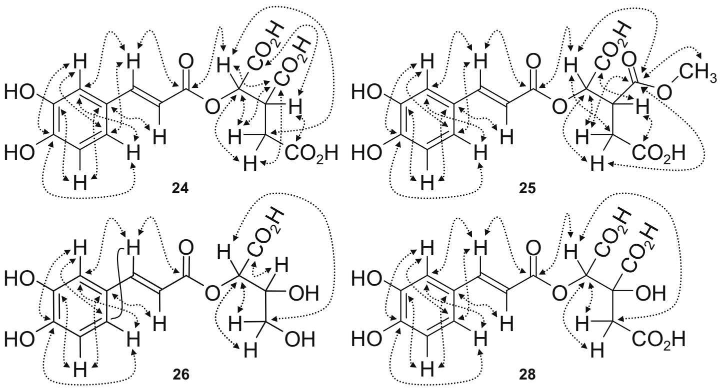


Fig. 4. Principal fragmentations of 24 in tandem MS.

resulted predominantly in an ion of *m/z* 377 [M + Na]. This Na-adduct (*m/z* 377) was fragmented in MS^2 to give two ions, *m/z* 215 [isocitric acid + Na] and *m/z* 163 [caffeic acid + H] (Fig. 2). MS^3 of fragment *m/z* 215 gave *m/z* 197, whilst fragment *m/z* 163 gave an *m/z* 145 ion, which corresponded to a loss of a water molecule from the isocitric acid and caffeic acid ions, respectively (Fig. 4, Table 1). In the aliphatic region of the ^{1}H NMR spectrum of **24** in CD_3OD, signals at δ_H 5.43 (d, *J* = 3.8 Hz), δ_H 3.53 (multiplet) and δ_H 2.82 (dd, *J* = 17.1 and 9.1 Hz) and 2.60 (dd, *J* = 17.1 and 5.1 Hz) suggested the presence of an isocitric acid (3-carboxy-2-hydroxypentanedioic acid) moiety. The latter two signals corresponded to a diastereotopic CH_2. A COSY spectrum showed that the δ_H 5.43 signal was coupled to that at δ_H 3.53 and that the latter was further coupled to the CH_2 signals at δ_H 2.82 and δ_H 2.60. The CH adjacent to the ester oxygen appeared at low field (δ_H 5.43) due to the deshielding influence of the ester O–C=O and C=O functionalities. In the aromatic part of the ^{1}H NMR spectrum, signals for three protons, a broad singlet at δ_H 7.06 and two doublets at δ_H 6.97 (*J* = 8.2 Hz) and δ_H 6.78 (*J* = 8.2 Hz) were observed as an AMX system, corresponding to a 1,3,4-trisubstituted benzene where the 3- and 4-substituents were RO—. Also observed were two olefinic doublets (*J* = 16.1 Hz, *trans*) at δ_H 7.61 and δ_H 6.33, corresponding to a cinnamoyl group. Taken together, these data indicate the presence of a caffeoyl (3,4-dihydroxycinnamyl) group (Strack et al., 1987). The connectivity between the caffeoyl and the isocitric acid units was established by consideration of the chemical shift of the 2-H in the ^{1}H NMR spectrum and by the HMBC spectrum. The 2-H signal appeared at δ 5.43, a chemical shift much more appropriate for HO_2C-C*H*-Oacyl, rather than for HO_2C-C*H*-OH. The HMBC spectrum allowed full assignment of the signals for the four carbonyls and showed a cross-peak between δ_H 5.43 and δ_C 168.6 (1″-C, the ester carbonyl) (Fig. 5). Therefore, the structure of **24** was assigned as 2-*O*-caffeoylisocitric acid.

Compound **25** was obtained as pale yellow crystals, with a t_R of 18.39 min, following separation by HPLC as described above, and showed a UV absorbance maximum at 330 nm, closely analogous to that of **24** above. Ions of *m/z* 367 [M−H] and *m/z* 735 [2 M−H], observed in spectra following full MS in the negative-ion mode, corresponded to the molecular formula $C_{16}H_{16}O_{10}$ (Table 1). MS^2 of the molecular ion (*m/z* 367) gave two fragments, *m/z* 179 and *m/z* 205, which corresponded to a caffeoyl ion and an isocitric acid monomethyl ester moiety, respectively. MS^3 of *m/z* 179 gave the *m/z* 135 ion, indicating the loss of CO_2 from the caffeoyl ion, whilst the *m/z* 205 fragment yielded several ions at *m/z* 187, 173, 155 and 111. The *m/z* 187 ion corresponded to the loss of a H_2O from the isocitric ion, whilst the ion at *m/z* 173 indicated the loss of methanol and suggested the presence of a methyl ether or methyl ester group in the structure. The ^{1}H NMR spectrum of **25** was similar to that of **24**, except for the addition of one OCH_3 signal observed downfield at δ_H 3.70 (integrating for 3 protons) and at δ_C 52.40. These chemical shifts suggest a methyl ester, rather than a methyl ether. Using the HMBC technique, the methyl ester was located at 6-C, the central carboxylic group of the isocitric acid. A strong correlation signal was observed at δ_C 173.45 and δ_H 3.70, which corresponds to a three-bond H-C coupling (Fig. 5). A weak four-bond HMBC correlation was also observed between the methyl carbon signal at δ_C 52.40 and the 3-H proton signal at δ_H 3.58, providing additional evidence for the methyl ester being located at the central carboxyl group. Owing to the limited amounts of material available, the relative and absolute configuration of the isocitric acid unit could not be confirmed. The structure of **25** was, therefore, elucidated as 2-*O*-caffeoylisocitric acid 6-methyl ester.

Compound **26** was obtained as pale yellow crystals, with a HPLC t_R of 8.79 min (following separation by HPLC as described above) and a UV absorbance maximum at 327 nm, corresponding again to a caffeoyl ester. Negative-ion ESI-MS showed a molecular ion of *m/z* 297 [M−H] and *m/z* 595 [2 M−H], indicating the molecular formula $C_{13}H_{14}O_8$ (Table 1). MS^2 of the molecular ion *m/z* 297 gave *m/z* 179 [caffeoyl−H] and *m/z* 135 ion [threonic acid−H]. Further fragmentation of the *m/z* 179 ion in negative-ion mode gave the *m/z* 135 [caffeoyl−CO_2H] ion, whilst in the positive mode the *m/z* 179 ion gave the characteristic *m/z* 163 ion, corresponding to loss of a hydroxy group from caffeic acid. The second fragment (*m/z* 135) in MS^3 gave the *m/z* 117 ion, which corresponded to a lactone formed from the loss of a water molecule. The ^{1}H NMR spectrum of **26** showed signals for the protons of the *E*-olefinic double bond of the side chain at δ_H 7.64 and δ_H 6.39 (*J* = 15.7 Hz) and for three aromatic protons at δ_H 7.07 (singlet), and two doublets at δ_H 6.96 and δ_H 6.78 (*J* = 8.2 Hz); these were typical of a caffeoyl moiety, as noted above. The signals coupled to each other (COSY) at δ_H 5.12 (d, *J* = 3.8 Hz), δ_H 4.11 (multiplet) and δ_H 3.62 (multiplet) and the carbonyl signal at δ_C 168.9 suggested that **26** was a derivative of threonic acid (*threo*-2,3,4-trihydroxybutanoic acid) (Hahn and Nahrstedt, 1993). The MS and NMR data thus indicate that **26** comprises a threonic acid moiety with one of the three alcohols esterified with a caffeoyl group. Unfortunately, although appropriate HMBC correlations were observed for the individual caffeoyl and threonic acid units (Fig. 5), there were no cross-peaks to identify which alcohol of the threonic acid was esterified. However, the location of the ester was established by consideration of the ^{1}H NMR chemical shifts and comparison with related *O*-acyl threonic acids reported in the literature. Lee et al. (2000) isolated 4-*O*-caffeoyl-L-threonic acid **31** (Fig. 6) from *Cornus controversa* and reported that the 4-H_2 resonated as a doublet at δ 4.12, with the 3-H signal at δ 4.05 and the 2-H signal at δ 4.09. The 4-H_2 of **26**



24 **25** **26** **28**

Fig. 5. Principal diagnostic correlations in the HMBC NMR spectra of 24–6 and 28.

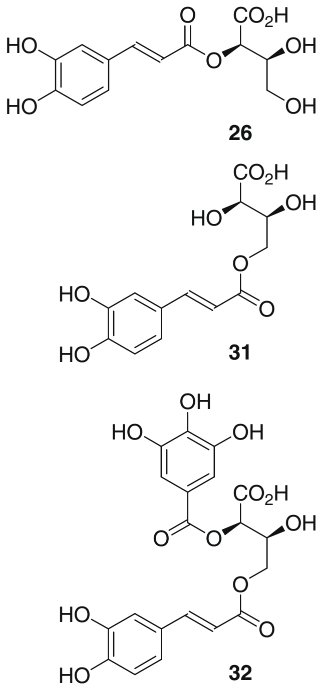


Fig. 6. Comparison of structures of 26 and 4-*O*-acyl- and 2,4-di(*O*-acyl)-threonic acids 30,31 isolated from *Cornus controversa* (Lee et al., 2000).

resonates as a multiplet at δ 3.62, the 3-H at δ 4.11 and the 2-H at δ 5.12. Thus the 4-H_2 signal of **26** is 0.50 ppm upfield of the corresponding resonance of 4-*O*-caffeoyl-L-threonic acid, the 3-H signal is 0.06 ppm downfield and the 2-H signal is 1.03 ppm downfield. When a gallate (3,4,5-trihydroxybenzoate) ester is present at the 2-*O* (in **31**; Fig. 6) (Lee et al., 2000), the chemical shift of the 2-H moves downfield to δ 5.33. These comparisons confirm that the caffeoyl group of **26** is esterified at the 2-OH of the threonic acid; the structure of **26** was identified as 2-*O*-caffeoylthreonic acid. Again, the paucity of material did not allow assignment of the configuration, but it is tentatively assigned as *L* as only this stereoisomer of threonic acid (and derivatives) has been found as a constituent of plants (Lee et al., 2000).

Interestingly, a very small but distinct peak (**27**) in the HPLC analysis (t_R 8.01 min) showed very similar properties, with a UV absorbance maximum at 325 nm (3,4-dihydroxycinnamate) and a molecular ion *m/z* 297 [M−H] in ESI-MS (negative-ion mode). A peak at *m/z* 595 [2 M−H] was also detected. MS^2, MS^3 and MS^4 of the *m/z* 297 ion and its corresponding fragment ions showed very similar fragmentation patterns to those of **26**. The very low abundance of this compound **27** precluded extensive NMR studies but the observation of a doublet (integrating for 1 H) at δ_H 5.25 indicated that it was a 3,4-dihydroxy-2-(3,4-dihydroxycinnamyloxy)butanoic acid. Thus **27** is likely to be a diastereoisomer of **26**, as it has a different t_R.

Compound **28** was obtained as pale yellow crystals, with a HPLC t_R of 11.96 min (following separation by HPLC as described above) and a UV absorbance maximum at 328 nm. Negative-ion ESI-MS showed a molecular ion of 369 [M−H] and corresponded to a molecular formula $C_{15}H_{14}O_{11}$ (Table 1). MS^2 of the molecular ion gave *m/z* 179 [caffeoyl−H] and 207 [M−caffeoyl]. Further fragmentation of the *m/z* 179 ion in the negative-ion and positive-ion modes was consistent with a caffeoyl moiety. MS^2 of the *m/z* 207 ion in the negative mode gave a *m/z* 189 fragment which corresponded to loss of a water molecule. MS^3 of the *m/z* 189 ion gave *m/z* 171 [M−(caffeic acid, H_2O)] and *m/z* 127 [M−(caffeic acid, H_2O, CO_2)]. Positive-ion ESI-MS gave *m/z* 371 [M + H] and *m/z* 191 [hydroxycitric acid−OH], respectively. The aromatic/alkene/carbonyl region of the ^1H and ^{13}C NMR spectra of **28** showed all the signals characteristic of a caffeoyl ester. The ^1H NMR spectrum showed additional signals at δ 5.48 (1 H, s), δ3.01 (1 H, d, J = 15.5 Hz) and δ 2.90 (1 H, d, J = 18.3 Hz), and the ^{13}C NMR spectrum at δ 174.5, δ173.8 and δ 171.1 (3 CO_2H), δ77.2 (CH), δ76.9 (quaternary C) and δ 42.0 (CH_2) was consistent with a hydroxycitric acid moiety. The positions of substitution of hydroxycitric acid were determined by HMBC as C-1, C-3 and C-5 (3 CO_2H) and C-2 (caffeoyl) and the carbonyl carbon signal at δ_C 168 (Fig. 5). 2*S*,3*S*-2-Caffeoyloxycitric acid (2*S*,3*S*-2-*O*-caffeoyl-hydroxycitric acid) has been isolated (Ozawa et al., 1977) from *Zea mays* and the diastereoisomeric 2*S*,3*R*-2-caffeoyloxycitric acid (2*S*,3*R*-2-*O*-caffeoyl-hydroxycitric acid) has been reported (Corthout et al., 1992) from *Spondias mombin*. However, the reported ^1H NMR spectra of these diastereoisomers were not sufficiently different to assign confidently the relative configuration of **28**. The structure of **28** was, therefore, elucidated as a 2-*O*-caffeoylhydroxycitric acid.

Analysis of the ESI-MS showed a small peak at t_R 15.32 min (following separation by HPLC as described above), with a UV absorbance maximum at 325 nm and a molecular ion 383 [M−H] corresponding to a molecular formula $C_{16}H_{16}O_{11}$ (Table 1). Further fragmentation of the molecular ion gave *m/z* 189, 171 and 127 ions, which was characteristic for the hydroxycitric acid moiety. In the positive-ion mode, *m/z* 195 [M + H] and 177 [feruloyl + H] were observed, which was consistent with a ferulic acid moiety (Swatsitang et al., 2000). Further structural elucidation was not possible because of its low abundance of this compound. The structure of **29** was, therefore, proposed to be a 2-*O*-feruloyl-hydroxycitric acid. 2*S*,3*S*-2-Feruloyloxycitric acid (2*S*,3*S*-2-*O*-feruloyl-hydroxycitric acid) has been reported by Ozawa et al. (1977) as a component of *Zea mays*.

Structural elucidation of compounds **21-29** demonstrates that these are potential PPO substrates with a 3,4-dihydroxycinnamic structure. Typical relative abundances of hydroxy-cinnamate esters in crude orchard grass extract on a µmol basis were as follows: **21** (2.0%), **22** (0.73%), **23** (1.09%), **24** (39.51%), **25** (38.57%), **26** (6.33%), **27** (1.7%), **28** (9.58%) and **29** (0.48%), respectively. Fig. 7 shows changes in abundances of compounds **24** and **25** over a growing season. Levels of these compounds showed fluctuation possibly related to the effects of changing environmental conditions. Compound **25** probably directly results from methylation of compound **24**. Orchard grass PPO showed optimal activity at neutral pH (Fig. 8) and all substrates were assayed at pH 7. Table 2 shows a comparison of apparent K_m and V_{max} values for these compounds as substrates for orchard grass PPO.

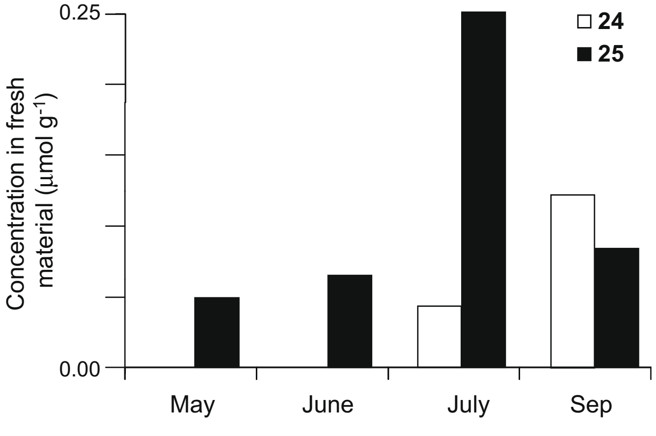


Fig. 7. Changes in abundance of 24 and 25 in *D. glomerata* during a growing season.

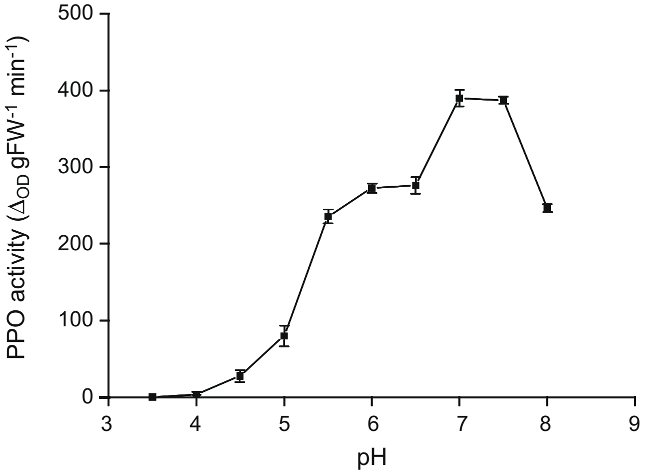


Fig. 8. The effect of pH on orchard grass PPO activity with 10 mM 4-methylbenzene-1,2-diol as substrate in citrate-P$_i$ buffer.

Table 2
K$_m$ and V$_{max}$ values for orchard grass (*Dactylis glomerata*) PPO for a range of substrates

Compound	Apparent K_m (mM)	V_{max} (μmol product /min/g fresh weight)
Chlorogenic acid **23**	3.47	222.2
2-Caffeoylisocitric acid **24**	0.37	50.5
2-Caffeoylisocitric acid 6-methyl ester **25**	0.82	76.3
4-Methylbenzene-1,2-diol	2.62	1.68

Orchard grass PPO showed a very high affinity for compounds **24** and **25** and a very high rate of activity with chlorogenic acid. Compounds **26** and **28** were also shown to be PPO substrates; however, there was insufficient material for analysis of kinetic parameters. The enzyme was not active at low pH and was inhibited by the presence of SDS in contrast with many other PPOs reported in the literature (Table 3) (Mayer, 2006). Results for analyses of monophenolase activity are also shown in Table 3. The enzyme did not demonstrate monophenolase activity with *p*-coumaric acid and tyrosine as substrates. Other workers have reported that monophenolase activity is activated by the presence of diphenols (Gowda and Paul, 2002). Enzyme was incubated with tyrosine in the presence of low concentrations (0.5 mM) of 4-methylbenzene-1,2-diol (4-methylcatechol) and chlorogenic acid to test the effect of diphenol activation. 4-Methylcatechol and chlorogenic acid showed no effect on activity in the presence of *p*-coumaric acid (data not presented); however, when included with tyrosine at a concentration of 0.5 mM, activity was higher than with 4-methylcatechol (or chlorogenic acid alone (9.6 *vs.* 4.1 and 33.9 *vs.* 20.4 respectively). While this measured increase in activity was low, the occurrence of monophenolase activity was apparent from the reddish colour formed with tyrosine in the presence of *o*-diphenols due to oxidation of 3′,4′-dihydroxy-L-phenylalanine (L-DOPA). A similar coloured product was formed when the enzyme was incubated with L-DOPA. These findings are consistent with those of Gowda and Paul, (2002) in studies with *Dolichos lablab* PPO where they demonstrated activation of monophenolase activity in the presence of *o*-diphenols.

Table 3
Relative PPO activity of orchard grass (*Dactylis glomerata*) protein extracts with a range of substrates

Compound	δ OD/g FW/min
4-Methylbenzene-1,2-diol[c] (10 mM)	132.4 (± 0.21)
4-Methylbenzene-1,2-diol (10 mM) (pH 4.5)	1.2 (± 0.27)
4-Methylbenzene-1,2-diol (10 mM) + SDS (0.05%)	1.5 (± 0.20)
Chlorogenic acid (10 mM)	345.3 (± 4.92)
[c]Tyrosine (20 mM)	0 (± 0.0)
[c]Tyrosine (20 mM) + 4-Methylbenzene-1,2-diol (0.5 mM)	9.6 (± 0.42)
[c]Tyrosine (20 mM + Chlorogenic acid (0.5 mM)	33.9 (± 1.99)
[c]4-Methylbenzene-1,2-diol (0.5 mM)	4.1 (± 0.10)
[c]Chlorogenic (0.5 mM)	20.4 (± 0.73)

All compounds were tested at pH 7.0 and reactions were monitored by increase in absorbance at 420 nm unless otherwise stated.
[a]Values in brackets represent SEM for 3 reps.
[b]a.k.a 4-methylcatechol.
[c]Reactions were monitored for increase in absorbance at 500 nm.

3. Conclusions

Extraction of the free phenols with MeOH, HPLC chromatography and ESI-MS^n proved to be an efficient method for the isolation of PPO substrates from orchard grass. The presence of hydroxycinnamate esters in some plants have previously been reported (Strack et al., 1987; Ozawa et al., 1977; Corthout et al., 1992; Hahn and Nahrstedt, 1993;). Compounds **25** and **26** are new natural products, whilst other closely related compounds **21–23** (*Hemerocallis*) (Clifford et al., 2006), **24** (*Amaranthus cruentus*) (Strack et al., 1987), **28** (*Chelidonium majus*) (Hahn and Nahrstedt, 1993) and **29** (*Zea mays*) (Ozawa et al., 1977) have previously been identified. This is the first study to report on the isolation and structural elucidation of endogeneous PPO substrates in orchard grass.

4. Experimental

4.1. General Experimental Procedures

Melting points were determined on a MEL-TEMP® capillary melting point apparatus with a thermocouple attached to a digital thermometer (Sigma–Aldrich, U.K.) and are uncorrected. ^{1}H, ^{13}C and two-dimensional NMR spectra were recorded on a Bruker DRX500 instrument operating at 500 MHz (for ^{1}H) and 125 MHz (for ^{13}C), using CD_3OD as the solvent and $SiMe_4$ as the internal standard. Chemical shifts (δ) are given in ppm relative to $SiMe_4$.

4.2. Plant materials

Orchard grass (*D. glomerata*), cv. Abertop, was sown in experimental plots (1.25 m 1.25 m plots in September 2003 and 1.2 m 20 m plots in September 2005) at the Institute of Grassland and Environmental Research (IGER), Aberystwyth, UK. Plant material was harvested in a vegetative state, *ca.* 2 cm above soil level, in July 2006 and stored at −80 °C for subsequent enzyme studies. For the purpose of monitoring free phenol content throughout the growing season, approximately 5 g of vegetative plant material was cut *ca.* 2 cm above soil level (three replicates sampled at random points) mid-morning at monthly intervals, between 22 May and 5 September, 2006. Bulk vegetative plant material was harvested on March 2007 and stored at −20 °C for phenol extraction, purification and analysis.

4.3. Extraction and purification of phenol substrates

Free phenols were extracted by adding fresh orchard leaf material (approximately 300 g) to purified boiling H_2O (500 mL) for 10 min. This fraction was retained and after cooling, the phenols were extracted with cold HPLC-grade MeOH (Aldrich, Dorset, UK); the fractions were combined and filtered and the solvent was evaporated under reduced pressure to yield a brown residue (27.54 g). A sample (439 mg) of this residue, in purified H_2O

(1.0 mL), was partially purified on a Waters Sep-Pak (500 mg) C_{18} reverse-phase extraction cartridge, using gradient elution with water-acetic acid (5%)/methanol (100% H_2O, 95:5, 90:10, 85:15, 80:20, 75:25, 70:30, 65:35, 60:40, 55:45, 50:50, 100% MeOH) to give twelve fractions. The solvents were evaporated from each fraction, the residues were dissolved in 30% aq. MeOH and centrifuged for 2 min at 10000*g*, the supernatant was then decanted and sample was subjected to HPLC analysis.

4.4. Preparative HPLC

A Waters HPLC system (Waters Corporation, USA) consisted of an auto-sampler, a photodiode array detector coupled with an analytical workstation. The column configuration consisted of a Waters C_{18} reversed-phase Nova-Pak column (4 µm, 8 mm 100 mm). The sample injection volume was 10 µL.

The detection wavelength was set at 240–400 nm, the flow rate was 1.0 mL min^{-1}, the auto-sampler tray temperature was kept at 4 °C and the column was at room temperature. The mobile phase consisted of purified water-acetic acid (A; 95:5, v/v) and HPLC grade methanol (B). The initial condition was A:B (100:0, v/v), the percentage of mobile-phase B increased linearly to 70% over the 35 min run. Phenols were detected with a PDA detector at two wavelengths ($\lambda_{\max}$ 280 and 340 nm) and individual peaks were collected and solvents rotary evaporated. The software Empower (Waters Corporation, USA) was run on a Pentium III personal computer.

4.5. LC-ESI-MS^n analysis

A Thermo Finnigan HPLC/MS^n system (Thermo Electron Corporation, USA) comprised an on-line degasser, an auto-sampler, a column temperature controller and photodiode array detector coupled to an analytical workstation. The column configuration consisted of a Waters C_{18} reversed-phase Nova-Pak column (4 µm, 3.9 mm 100 mm). The sample injection volume was 10 µL.

The detection wavelength was set at 240–400 nm, the flow rate was 500 µL min^{-1}, with 50 µL min^{-1} going to the mass spectrometer. The auto-sampler tray temperature was kept at 4 °C and the column temperature was maintained at 20 °C. The mobile phase consisted of purified water-formic acid (A; 100:0.1, v/v) and HPLC grade methanol (B). The initial condition was A:B (100:0, v/v); the percentage of mobile-phase B increased linearly to 70% over the 35 min run. The software Xcaliber 1.4, 2003 (Thermo Electron Corporation, USA) was run on a Pentium IV personal computer and used to process the chromatographic data.

The instrumentation and chromatographic conditions of HPLC for LC–MS^n was the same as that described above. The mass spectra were acquired using a Finnigan LTQ linear ion trap instrument with an ESI source (Thermo Electron Corporation, USA). N_2 was used as the sheath and auxiliary gas and He was used as the collision gas. For the phenolic acids, the ionisation mode in negative and the interface and MSD parameters were as follows: sheath gas, 30 arbitrary units; auxiliary gas, 10 units; spray voltage, 4 KV; capillary temperature 320 °C; capillary voltage, −31 V; tube lens offset, −63 V. The ionisation mode in positive for the phenolic acids and the interface and MSD parameters were as follows: sheath gas, 30 arbitrary units; auxiliary gas, 5 units; spray voltage, 4.0 KV; capillary temperature 320 °C; capillary voltage, 23 V; tube lens offset, 50 V.

*4.6. E-3-Carboxy-2-(3-(3,4-dihydroxyphenyl)prop-2-enoyloxy)pentanedioic acid/2-O-caffeoylisocitric acid (**24**)*

Compound **24** was obtained as a pale yellow oil: ^{1}H NMR δ 2.60 (1 H, *dd*, *J* = 17.1, 5.1 Hz, H-4B), 2.82 (1H, *dd*, *J* = 17.1, 9.1 Hz, H-4A), 3.53 (1H, m, 3-H), 5.43 (1H, *d*, *J* = 3.8 Hz, 2-H), 6.33 (1H, *d*, *J* = 16.1 Hz, 2”-H), 6.78 (1H, *d*, *J* = 8.2 Hz, 5’-H), 6.97 (1H, *d*, *J* = 8.2 Hz, 6’-H), 7.06 (1H, s, 2’-H), 7.61 (1H, *d*, *J* = 16.1 Hz, 3″-H); ^{13}C NMR δ 33.6 (4-C), 44.7 (3-C), 73.7 (2-C), 114.3 (2″-C), 115.2 (2′-C), 116.5 (5′-C), 123.3 (6′-C), 127.7 (1′-C), 147.9 (3″-C), 148.2 (4′-C), 148.7 (3′-C), 168.6 (1″-C), 173.3 (6-C), 173.5 (1-C), 174.5 (5-C).

*4.7. E-2-(3-(3,4-dihydroxyphenyl)prop-2-enoyloxy)-3-(methoxycarbonyl)pentanedioic acid/2-O-caffeoylisocitric acid 6-methyl ester (**25**)*

Compound **25** was obtained as pale yellow crystals: mp 160.2 °C; ^{1}H NMR δ 2.64 (1H, *dd*, *J* = 17.0, 5.4 Hz, H-4B), 2.83 (1H, *dd*, *J* = 17.0, 9.1 Hz, H-4A), 3.58 (1H, m, H-3), 3.70 (3H, s, CH_3), 5.46 (1H, *d*, *J* = 3.5 Hz, 2-H), 6.32 (1H, *d*, *J* = 15.7 Hz, 2”-H), 6.79 (1H, *d*, *J* = 8.2 Hz, 5′-H), 6.97 (1H, *d*, *J* = 8.2 Hz, 6’-H), 7.07 (1H, s, 2′-H), 7.61 (1H, *d*, *J* = 15.7 Hz, 3″-H); ^{13}C NMR δ 33.3 (4-C), 44.4 (3-C), 52.4 (CH_3), 73.1 (2-C), 114.1 (2″-C), 115.2 (5′-C), 116.5 (2′-C), 123.3 (6′-C), 127.8 (1′-C), 148.2 (3″-C), 146.8 (4′-C), 148.9 (3’-C), 168.6 (1′″-C), 173.3 (5-C), 173.5 (6-C), 175.8 (1-C); HRMS (ESI −ve) 368.0699 (M−H) ($^{13}C_1{}^{12}C_{15}H_{15}O_{10}$ requires 368.0699), 367.0656 (M−H) ($C_{16}H_{15}O_{10}$ requires 367.0665), 205.0337 (M−caffeoyl) ($C_7H_9O_7$ requires 205.0295), 180.0365 ($(HO)_2C_6H_3CH{=}CHCO_2^-$) ($^{13}C_1{}^{12}C_8H_7O_4$ requires 180.0378), 179.0342 ($(HO)_2C_6H_3CH{=}CHCO_2^-$) ($C_9H_7O_4$ requires 179.0344), 135.0428 (caffeoyl−CO_2^-) ($C_8H_7O_2$ requires 135.0446).

*4.8. E-2-(3-(3,4-dihydroxyphenyl)prop-2-enoyloxy)-3,4-dihydroxybutanoic acid/2-O-caffeoylthreonic acid (**26**)*

Compound **26** was obtained as pale yellow crystals: mp 154.8 °C; ^{1}H NMR δ 3.62 (2H, m, 4-H_2), 4.11 (1H, m, H-3), 5.12 (1H, *d*, *J* = 3.8 Hz, 2-H), 6.39 (1H, *d*, *J* = 15.7 Hz, 2″-H), 6.78 (1H, *d*, *J* = 8.2 Hz, 5′-H), 6.96 (1H, *d*, *J* = 8.2 Hz, 6′-H), 7.07 (1H, s, 2′-H), 7.64 (1H, *d*, *J* = 15.7 Hz, 3″-H); ^{13}C NMR δ 64.1 (4-C), 73.9 (3-C), 75.0 (2-C), 115.4 (2′-C), 115.4 (2″-C), 116.5 (5′-C), 123.0 (6′-C), 127.6 (1′-C), 146.5 (3″-C), 146.9 (4′-C), 147.0 (3′-C), 168.9 (1″-C), 173.9 (1-C); HRMS (ESI−ve) 617.1122 (2 M−2 H + Na) ($C_{26}H_{26}NaO_{16}$ requires 617.1119), 298.0637 (M−H) ($^{13}C_1{}^{12}C_{12}H_{13}O_8^-$ requires 298.0644), 297.0593 (M−H) ($C_{13}H_{13}O_8^-$ requires 297.0610), 237.0390 (M−$OHCCH_2OH$) ($C_{11}H_9O_6$ requires 237.0399), 179.0352 ($(HO)_2C_6H_3CH{=}CHCO_2^-$) ($C_9H_7O_4$ requires 179.0344).

*4.9. E-3-Carboxy-2-(3-(3,4-dihydroxyphenyl)prop-2-enoyloxy)-3-hydroxypentanedioic acid/2-O-caffeoylhydroxycitric acid (**28**)*

Compound **28** was obtained as pale yellow crystals: mp 161 °C; ^{1}H NMR δ 2.90 (1H,*d*, *J* = 18.3 Hz, H-4B), 3.01 (1H, *d*, *J* = 18.3 Hz, H-4A), 5.48 (1H, s, 2-H), 6.37 (1H, *d*, *J* = 15.7 Hz, 2″-H), 6.79 (1H, *d*, *J* = 8.2 Hz, 5′-H), 6.98 (1H, *d*, *J* = 8.2 Hz, 6′-H), 7.08 (1H, s, 2′-H), 7.67 (1H, *d*, *J* = 15.7 Hz, 3″-H); ^{13}C NMR δ 42.0 (4-C), 77.2 (2-C), 76.9 (3-C), 113.5 (2″-C), 115.2 (2′-C), 116.5 (5′-C), 123.2 (6′-C), 126.8 (1′-C), 144.9 (3′-C), 148.1 (3″-C), 147.3 (4′-C), 168.8 (1″-C), 174.5, 173.8, 171.1 (1,5,6-C_3); HRMS (ESI−ve) 370.0519 (M−H) ($^{13}C_1{}^{12}C_{14}H_{13}O_{11}$ requires 370.0491), 369.0476 (M−H) ($C_{15}H_{13}O_{11}$ requires 369.0458), 189.0044 (hydroxycitric acid lactone−H) ($C_6H_5O_7$ requires 188.9982), 179.0355 ($(HO)_2C_6H_3CH{=}CHCO_2^-$) ($C_9H_7O_4$ requires 179.0344), 135.0418 (caffeoyl−CO_2^1) ($C_8H_7O_2$ requires 135.0446).

4.10. Enzyme preparation and PPO assays

Leaf material (approx. 0.5 g) was extracted at 0 °C in 2 mL of McIlvaine buffer, pH 7, containing 50 mM ascorbic acid to inhibit

PPO activity. Extracts were centrifuged at 15000*g* for 10 min at 4 °C and desalted by passage through Bio-gel P6DG (Biorad, Hertfordshire, UK) prepared in McIlvaine buffer pH 7.0, as described by Lee et al.[2] This fraction was used in enzyme studies. PPO activity was determined spectrophotometrically at 420 nm with the appropriate substrate. These included chlorogenic acid, caffeic acid, 4-methylbenzene-1,2-diol (4-methylcatechol), *p*-coumaric acid, tyrosine and a range of compounds isolated from orchard grass. Standard reactions were carried out in a volume of 1.5 mL containing McIlvaine buffer, pH 7.0 with a range of substrate concentrations. For determination of pH optimum, reactions were carried out with McIlvaine buffer with a pH range of 3.5–8 and 10 mM 4-methylbenzene-1,2-diol (4-methylcatechol) as substrate. Reactions were initiated by the addition of 20 µL of the enzyme preparation and the initial rate of increase in absorbance at 420 nm was recorded. Activity was recorded as the rate of product formed or increase in optical density (OD) /min/g fresh weight.

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