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4,4′-DIHYDROXYTRUXILLIC ACID AS A COMPONENT OF CELL WALLS OF *LOLIUM MULTIFLORUM*

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Key Word Index—*Lolium multiflorum*; Poaceae; cell walls; phenolic acids; dimers; *cis* and *trans*-*p*-coumaric acid; *cis* and *trans*-ferulic acid; 4,4′-dihydroxytruxillic acid; photodimerization.

Abstract—4,4′-Dihydroxytruxillic acid was released from the cell walls of *Lolium multiflorum* by treatment with sodium hydroxide. Combined gas chromatography–mass spectrometry indicated that other similar dimers, probably resulting from the photodimerization of *p*-coumaric acid or ferulic acid, were also released from the cell walls. The possible role of the dimers in cross-linking cell wall heteroxylans is discussed.

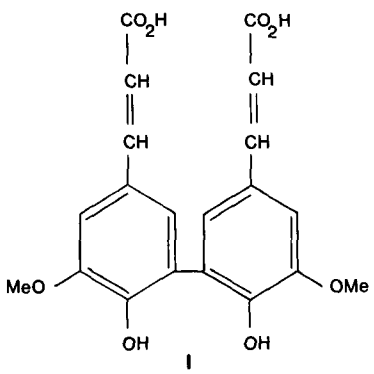
INTRODUCTION

The phenolic acids, ferulic, *p*-coumaric and diferulic, are covalently bound to the cell-wall polysaccharides of the Poaceae and a number of other angiosperm families [1–8]. The ferulic and *p*-coumaric acids are present mainly in the *trans* configuration and the diferulic acid (Formula 1) mainly in the *trans, trans* configuration. Recent work has shown that in the Poaceae, the ferulic and *p*-coumaric acids are bound to heteroxylans. The acids are esterified via their carboxyl group to the C(O)5 hydroxyl of single α-L-arabinofuranosyl residues linked through C(O)3 of the xylose residues in the heteroxylan backbone. This was shown by determining the structures of water-soluble compounds released by treating sugar cane bagasse [9], maize cell walls [10] and barley straw cell walls [11] with a mixture of polysaccharide hydrolases. These compounds were identified as *O*-[5-*O*-(*trans*-feruloyl)-α-L-arabinofuranosyl](1→3)-*O*-β-D-xylopyranosyl-(1→4)-D-xylopyranose (FAXX) [9–11] and *O*-[5-*O*-(*trans*-*p*-coumaroyl)-α-L-arabinofuranosyl]-(1→3)-*O*-β-D-xylopyranosyl-(1→4)-D-xylopyranose [11]. FAXX is also released from isolated barley aleurone layers after treatment with gibberellic acid [12].

It seems likely that in the cell walls of the Poaceae, the two carboxyl groups of diferulic acid are esterified to different heteroxylan molecules, so forming cross-links [13]. Such cross-links may have an important influence in limiting the digestibility of cell walls by ruminants. In this paper we report the presence of the phenolic dimer, 4,4′-dihydroxytruxillic acid (Fig. 1) in the cell walls of Italian ryegress (*Lolium multiflorum*).

RESULTS AND DISCUSSION

The tetra-TMSi derivative of 4,4′-dihydroxytruxillic acid synthesized from *trans*-*p*-coumaric acid by photodimerization (Fig. 1) had a R_t (GC-MS) of 69.3 min compared to 32.7 and 38.2 min for *cis* and *trans*-*p*-coumaric acid and 37.5 and 43.2 min for *cis* and *trans*-ferulic acids. The mass spectrum of the tetra-TMSi derivative of 4,4′-dihydroxytruxillic acid [major ions, *m/z* (rel. int.) 308 (M − 308, 45), 293 (35), 249 (17), 219 (38), 75



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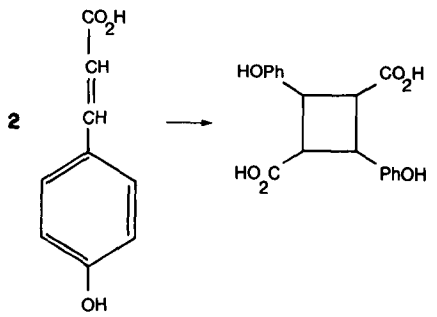


Fig. 1. Photodimerization of *trans*-*p*-coumaric acid to 4,4′-dihydroxytruxillic acid.

(15), 73 (100), 45 (15)] contained the same major ions as the mass spectrum of the bis-TMSi derivative of *trans-p*-coumaric acid [major ions, *m/z* (rel. int.) 308 (M, 17), 293 (29), 249 (26), 219 (38), 75 (27), 73 (100), 45 (29)]. In addition the spectrum of the tetra-TMSi derivative of 4,4′-dihydroxytruxillic acid had minor ions at *m/z* 616 (M) and 601 (M − 15). These results are consistent with the symmetrical splitting of this compound (Fig. 2); two fragments each equivalent to the bis-TMSi derivative of *p*-coumaric acid are produced [14, 15]. Cohen *et al.* [20] have provided evidence indicating that the synthesized dimer is 4,4′-dihydroxy-α-truxillic acid [2α,4β-bis(4-hydroxyphenylcyclobutane)-1α,3β-dicarboxylic acid] [Formula 2].

Treatment of cell walls of *L. multiflorum* with NaOH followed by silylation and GC/MS of the liberated phenols gave a major peak with the same R_t and major ions in the mass spectrum as the tetra-TMSi derivative of synthesized 4,4′-dihydroxytruxillic acid. A second compound had a similar mass spectrum but a lower R_t (67.7 min) suggesting that it is probably another isomer resulting from the photodimerization of *p*-coumaric acid. In theory 12 isomers can be obtained from the photodimerization of *p*-coumaric acid, depending on whether head-to-head or head-to-tail dimerizations take place with *syn* or *anti* and with *cis* or *trans* ring junctions [16]. All tetra-TMSi derivatives of the head-to-tail dimers (isomers of 4,4′-dihydroxytruxillic acid) will split symmetrically on electron impact (Fig. 2). However, tetra-TMSi derivatives of the head-to-head dimers (isomers of 4,4′-dihydroxytruxinic acid) may split symmetrically and asymmetrically (Fig. 3), but the two types of splitting may not occur to the same extent [14, 15]. Hence the second

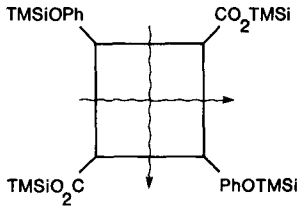
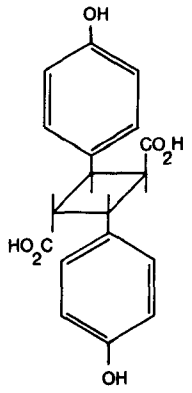


Fig. 2. Symmetrical splitting (mass spectrometry) of the tetra-TMSi derivative of 4,4′-dihydroxytruxillic acid.



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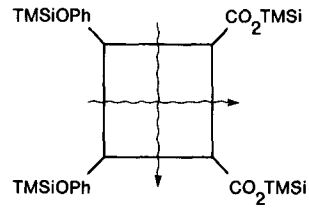

Fig. 3. Asymmetrical splitting (mass spectrometry) of the tetra-TMSi derivative of 4,4′-dihydroxytruxinic acid.

compound (R_t 67.7 min) is probably derived from another head-to-tail dimer but it is possible that it is a head-to-head dimer in which asymmetrical splitting was not detected. No dimers were found after *trans-p*-coumaric acid was treated with NaOH in the same way as the cell walls indicating that the dimers were not artefacts produced during isolation of phenolic acids from the walls.

No dimers of ferulic acid or mixed dimers of ferulic acid and *p*-coumaric acid could be synthesized using the same conditions as for the synthesis of 4,4′-hydroxytruxillic acid. However, five further compounds (R_t 68.5, 71.1, 71.4, 71.9 and 72.7 min) were detected in the silylated extract from the cell walls of *L. multiflorum*. These compounds had similar mass spectra and contained the same major ions as the mass spectrum of the bis-TMSi derivative of *trans*-ferulic acid [major ions, *m/z* (rel. int.) 338 (M, 38), 323 (26), 308 (29), 293 (20), 249 (25), 75 (31), 73 (100), 45 (38)]. The mass spectrum relating to the largest peak (R_t 72.7 min) was as follows: major ions, *m/z* (rel. int.) 339 (20), 338 (M − 338, 85), 323 (32), 308 (15), 293 (11), 249 (30), 219 (12), 75 (12), 73 (100), 45 (12). Hence these peaks probably represent isomers resulting from the photodimerization of ferulic acid in a manner analogous to *p*-coumaric acid. No dimers were found after *trans*-ferulic acid was treated with NaOH in the same way as the cell walls.

It seems likely that the 4,4′-dihydroxytruxillic acid component of cell walls is synthesized by the photodimerization of *p*-coumaric acid residues esterified to adjacent heteroxylan molecules. The photodimerization of the esters, rather than the free acid, may also lead to the formation of the second dimer of *p*-coumaric acid (R_t 67.7 min). Photodimerization of esters may also account for the presence of dimers of ferulic acid in cell walls, analogous to the dimers of *p*-coumaric acid. Such dimers could not be synthesized from *trans*-ferulic acid *in vitro*. 4,4′-Dihydroxytruxillic acid and analogous dimers of ferulic acid may thus form cross-links between heteroxylan molecules, in a way similar to that postulated for diferulic acid. Such cross-links may have important effects on the mechanical properties of the cell walls and limit their digestibility by the ruminant.

EXPERIMENTAL

All manipulations of solns of phenolic acids and their TMSi derivatives were carried out in 'white' fluorescent light to avoid UV radiation which causes *cis–trans* isomerization [17, 18].

Plant material. Primary growth of shoots of Italian ryegrass (*Lolium multiflorum* Lam. cv RvP) were harvested before inflorescence emergence, freeze dried, ground and cell walls extracted as previously described [19].

Treatment of cell walls with NaOH. Cells walls (100 mg) were shaken with 1 M NaOH (5 ml) under N_2 (containing < 5 ppm

O_2) at 20° for 20 hr. The mixture was filtered (No. 1 porosity glass sinter) and the filtrate acidified with 6 M HCl to pH 2.5 and extracted with Et_2O (3 × 10 ml). The combined Et_2O extracts were dried over dry Na_2SO_4, evapd and dried over silica gel. As controls, *trans-p*-coumaric acid (1 mg) and *trans*-ferulic acid (1 mg) were treated with NaOH in the same way, acidified and extracted with ether as described above.

Silylation. The dry residue from NaOH extracts of cell walls and authentic *trans-p*-coumaric acid (1 mg) and *trans*-ferulic acid (1 mg) were silylated by incubating with BSTFA [*N,O*-bis(trimethylsilyl)trifluoroacetamide] (100 μl) for 20 hr at 37°.

Preparation of authentic 4,4'-dihydroxytruxillic acid. This was prepared by irradiating *trans-p*-coumaric acid as described in ref. [20]. The product was extracted with Et_2O in a Soxhlet apparatus until all the *p*-coumaric acid had been removed; removal of the acid was monitored by TLC [4].

Combined GC/MS. GC/EIMS was carried out using a Finnigan MAT 1020B fitted with a 50 m × 0.3 mm i.d. OV-1, vitreous silica (FSOT) column (Hewlett Packard). The capillary column was interfaced with the ion source via a separator oven at 280°. The GC oven was held at 80° for 2 min, then programmed from 80 to 300° at 3°/min and held at the final temp. for 5 min. Sample (0.5 μl) were injected using the splitless mode; helium at 2.0 ml/min was the carrier gas. Compounds that eluted from the GC column were detected by EIMS at 70 eV using the total ion current (reconstructed ion chromatogram, RIC) by scanning from *m/z* 40 to 650 in 1.0 nominal sec.

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