Phytochemistry, Vol. 31, No. 4, pp. 1179–1183, 1992
Printed in Great Britain.

0031-9422/92 $5.00+0.00
Pergamon Press plc

CINNAMIC ACID BRIDGES BETWEEN CELL WALL POLYMERS IN WHEAT AND PHALARIS INTERNODES

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(*Received in revised form* 14 *August* 1991)

Key Word Index—*Triticum aestivum*; *Phalaris aquatica*; Gramineae; lignin; polysaccharide; *p*-coumaric acid; ferulic acid; esterified cinnamic acid; etherified cinnamic acid.

Abstract—A method has been devised for the quantitative determination of cinnamic acids participating in ester–ether bridges between cell wall polymers based on the different reactivities of free carboxylic acids and their esters towards borohydride reductants and the different susceptibilities of cinnamic acid ester and benzyl ether linkages to alkaline treatments. Lignin–polysaccharide containing fractions extracted with dioxane–H_2O from cell walls of wheat (*Triticum aestivum*) and phalaris (*Phalaris aquatica*) internodes are hydrogenated using a Pd/C catalyst at room temperature to convert cinnamic acids to their corresponding dihydrocinnamic acids. The sample is subsequently reduced with $LiBH_4$ in ether–toluene to convert ester-linked dihydrocinnamates to their corresponding alcohols, hydrolysed with 4 M NaOH at 170°, and the dihydrocinnamic acid derivatives released from their etherified forms determined by GC. Using model compounds it was shown that these reactions proceeded quantitatively. The results indicate that all of the etherified ferulic acid in the dioxane–H_2O-soluble fractions of walls of wheat and phalaris internodes is also ester-linked. It has been calculated that there are nine to 10 ferulic acid ester–ether bridges for every 100 C_6–C_3 lignin monomers. *p*-Coumaric acid is not involved in ester–ether bridges.

INTRODUCTION

A number of covalent interactions between cell wall polysaccharides and lignin, involving either direct glycosidic linkages, direct ether linkages, or direct ester linkages, have been described [1–3]. Interactions between polysaccharides through dehydro-diferulic acid dimers [4, 5] and cyclobutane-type dimers, related to truxillic and truxinic acids [6, 7], have also been proposed. In addition, interactions between polysaccharides and lignin through cinnamic acid ester–ether bridges have been postulated for walls of grasses [8–10] and indirect evidence provided that most of the etherified ferulic acid in wheat internodes is involved in ester–ether bridges [10].

We describe a novel procedure for the direct analysis of the proportion of cinnamic acids in wall fractions that are solely ester-linked, solely ether-linked or both ester- and ether-linked. This analysis is based on the different reactivities of carboxylic acids and their esters to borohydride reagents, the observation that cinnamic acids in benzyl ether linkage are released by alkaline treatment at high temperature [10], and the lability of ester linkages to alkaline hydrolysis at room temperature.

RESULTS AND DISCUSSION

Reduction of model compounds

A series of cinnamic acids and their esters were used to determine if it was feasible to measure separately free and esterified forms based on the differences in their reactivity with borohydride reductants. It was expected that cinnamic acids with a free carboxylic acid would not be reduced, but that esterified cinnamic acids would be reduced quantitatively to their corresponding alcohols. In preliminary experiments, cinnamic acids (**1–4**) and their methyl esters (**5–8**) were treated with several borohydride reductants ($NaBH_4$–MeOH [11], $NaBH_4$–$AlCl_3$–diglyme [12], $NaBH_4$–$CaCl_2$–THF [13], $LiBH_4$–$LiEt_3BH$–Et_2O [14], $LiBH_4$–toluene–Et_2O [15]) under various conditions according to the literature. Unexpectedly, however, the methyl esters were not reduced by any of the reductants tested under any of the conditions used. This was due to the presence of the α,β-unsaturation in the propyl side chain conjugated with the phenolic ring. After hydrogenation of the cinnamic acids and their esters to give saturated derivatives (**9–16**), it was found that the dihydrocinnamic acid esters were quantitatively reduced by $LiBH_4$.

The cinnamic acids and their methyl esters (**1–8**) were easily hydrogenated using a 10% Pd/C catalyst in methanol. The yields of products (**9–16**) were shown to be quantitative by UV, ^1H and ^{13}C NMR spectral analyses and GC. The hydrogenated acids and their corresponding esters (**9–16**) were reduced with $LiBH_4$. The ^1H and ^{13}C NMR spectral analyses and GC-MS confirmed that the esters (**8–16**) were reduced quantitatively to their corresponding alcohols (**17–20**). On the other hand, compounds with free carboxyl groups (**9–12**) were recovered unchanged. The results of experiments with model compounds show that these procedures are able to distinguish esterified cinnamic acids from cinnamic acids with free carboxyl groups. Thus, the three types of cinnamic acids (ester-only, ether-only and ether–ester bridge) can be distinguished and quantified using the scheme shown in Fig. 1.

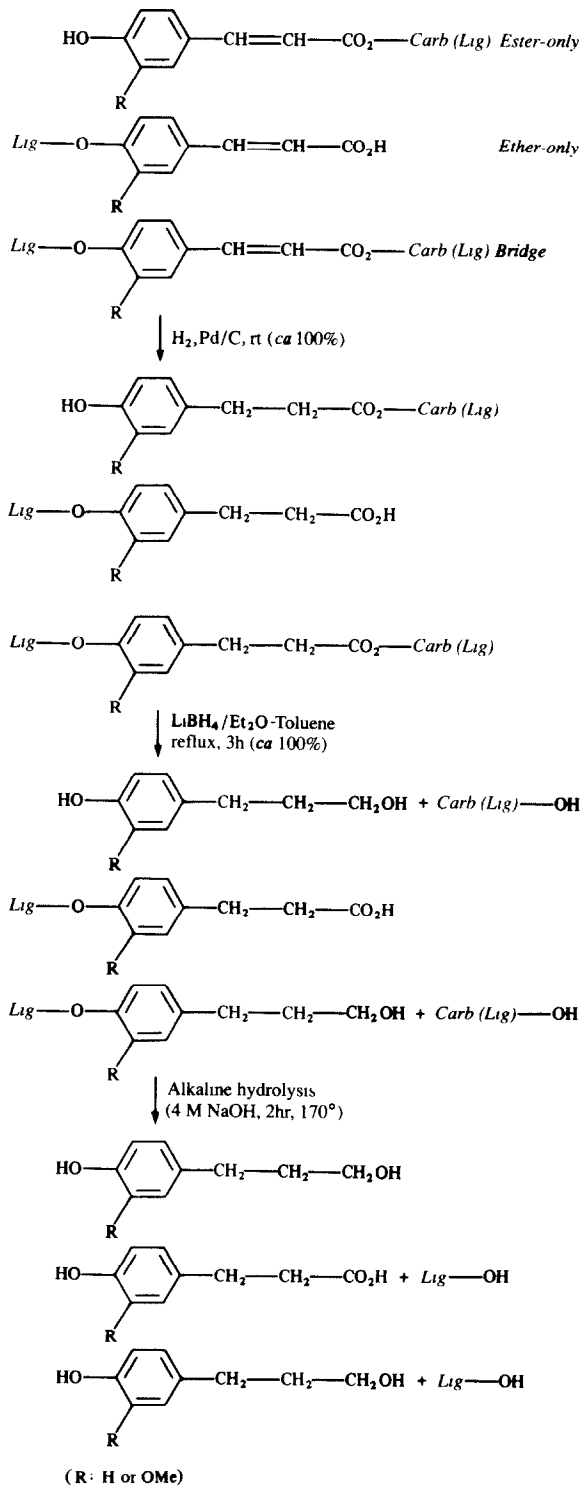

Fig. 1. Analytical scheme for determination of cinnamic acids, in ester-only, ether-only or ester–ether linkages (*Carb*, carbohydrate; *Lig*, lignin).

Application of the analytical procedure to lignin–polysaccharide samples

Since the rate of the hydrogenation reaction in lignin–polysaccharide samples was much slower than with the model compounds, complete hydrogenation as judged by UV spectral analysis was achieved only after repeated treatments. The $LiBH_4$ reduction was also repeated twice. The small shoulder at 305–310 nm in the UV spectrum of the final product may be contributed by conjugated carbonyl groups in the lignin [16], as it disappeared completely after reduction with $LiBH_4$. To avoid possible losses of sample due to cleavage of lignin–polysaccharide bridges following reduction, subsequent alkaline hydrolysis at 170° to release ether-linked cinnamic acid derivatives was conducted directly on the

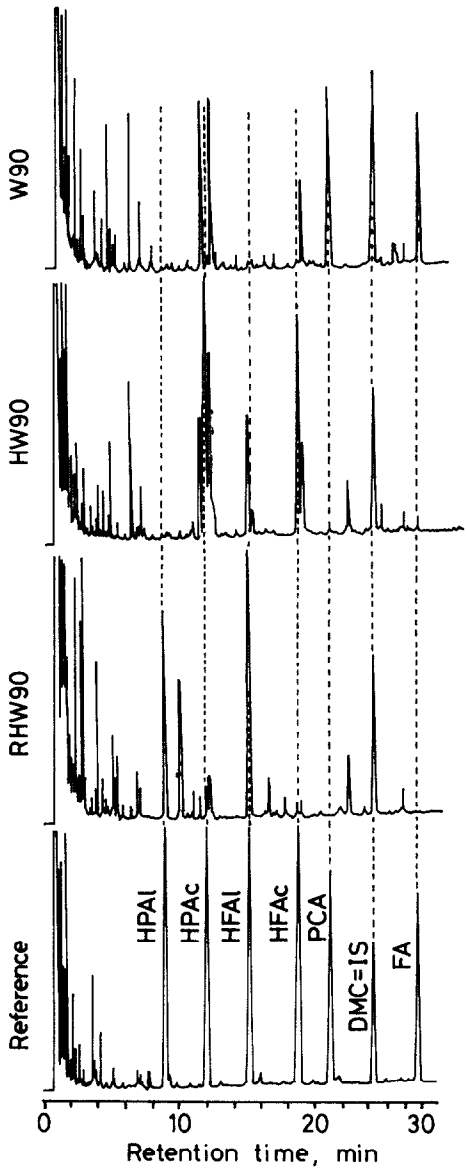

Fig. 2. GC of products of alkaline hydrolysis at 170° for 90% (v/v) dioxane–H_2O soluble wheat internode fractions (W90), hydrogenated W90 (HW90), and borohydride-reduced HW90 (RHW90). HPAl: 3-(4-hydroxyphenyl)-1-propanol (**17**), HPAc: 3-(4-hydroxyphenyl-)-propionic acid (**9**), HFAl: 3-(3-methoxy-4-hydroxyphenyl)-1-propanol (**18**), HFAc: 3-(3-methoxy-4-hydroxyphenyl)-propionic acid (**10**), PCA: (*E*)-*p*-coumaric acid (**1**), FA: (*E*)-ferulic acid (**2**), DMC = IS: (*E*)-3,4-dimethoxycinnamic acid (**4**) as an int. standard.

reaction mixture. Gas chromatography of the products of alkaline hydrolysis of the hydrogenated-reduced lignin–polysaccharide samples is shown in Fig. 2 (W90 series) and Fig. 3 (P90 series). No (*E*)-PCA (**1**) or (*E*)-FA (**2**) was detected in the hydrolysates of the hydrogenated samples showing that the hydrogenation had proceeded completely. However, appreciable amounts of HFAl (**18**) and HPAl (**17**) arising from bridged cinnamic acids were detected. On the other hand, much smaller amounts of HPAc (**9**) and HFAc (**10**) arising from cinnamic acids with free carboxyl groups, i.e. etherified-only, were found (RHW90 in Fig. 2, and RHP90 in Fig. 3).

Esterified and etherified cinnamic acids in lignin–polysaccharide fractions of wheat and phalaris internodes were determined separately (Table 1). 4 M NaOH treatment was used as a check on the results of the sequential treatment. The ratios of bridged cinnamic acids to etherified cinnamic acids were calculated from the above values and the ratios of alcohols (i.e. HFAl or HPAl) which were produced by alkaline hydrolysis at 170° of hydrogenated-reduced samples to total cinnamic acids (i.e. HFAl plus HFAc or HPAl plus HPAc) (Table 1).

We have previously suggested [10] from considerations of the changes in solubility of lignin fractions from wheat internode walls, before and after saponification, and the accompanying changes in esterified and etherified ferulic acids in these fractions, that most of the ferulic acid in ether-linkage is also esterified in the original lignin fraction. The results presented here show directly that all the etherified ferulic acid in wheat and phalaris internode fractions is also ester-linked. The results also confirm that ferulic acid, but not *p*-coumaric acid, is involved in ester–ether linkage (Table 2).

These results do not show which wall polymers are bridged by the ester–ether linked ferulic acid. However, it is most likely that the ferulic acid forms bridges between wall polysaccharides and lignin. An experiment with wheat internode fractions [10] showed that the content of ferulic acid was low in the Björkman lignin fraction, which had a low polysaccharide content, but that lignin fractions rich in polysaccharide also contained high levels of esterified and etherified ferulic acid. These fractions lost polysaccharide on treatment with dilute alkali that would have cleaved the ester linkages involving ferulic acid bridges between polysaccharides and lignin. Furthermore, although the ether linkages of ferulic acid to lignin would be easily formed non-enzymatically through a quinone methide intermediate during lignin biosynthesis [17], the formation of an ether linkage between ferulic acid and carbohydrate is not possible by chemical reaction under *in vivo* conditions, nor have enzymes catalysing this reaction been identified.

The wheat and phalaris internode cell walls used in these experiments contain 14.2 and 14.4% lignin, respectively, and their etherified FA contents were 1.3% for wheat and 1.3% for phalaris. If all the etherified ferulic acid is in bridge form then it can be calculated that there are nine to 10 ester–ether bridges for every 100 phenylpropane lignin monomers in the whole wheat and phalaris internode cell walls. This is the first quantitative estimation of a lignin–polysaccharide association in plant cell walls. This novel analytical procedure can be applied to determine ester–ether bridges in lignified walls of other species and to follow changes in these associations during plant growth and after chemical or biological treatments.

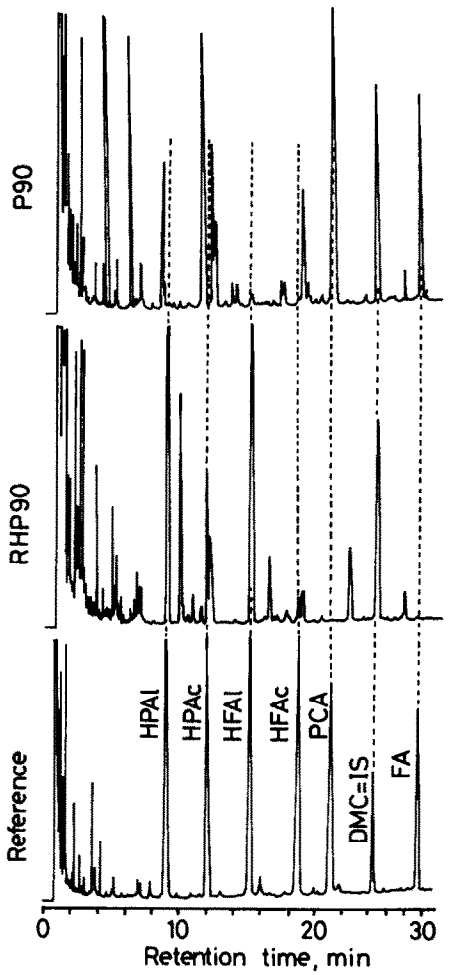

Fig. 3. GC of products of alkaline hydrolysis at 170° of 90% (v/v) dioxane–H_2O soluble phalaris internode fractions (P90) and hydrogenated-reduced P90 (RHP90). Abbreviations are shown in Fig. 2.

EXPERIMENTAL

Materials. Maturing terminal (upper) internodes of wheat (*Triticum aestivum* L. cv Millewa) and phalaris (*Phalaris aquatica* L. cv Cirosa) plants grown at the Victorian Crops Research Institute, Horsham and farmland at Alexandra, Victoria, respectively, in the 1989 season were harvested just after anthesis and immediately frozen and freeze-dried. The dried internode was ground in a Wiley mill to pass a 420 μm sieve and extracted with boiling 80% (v/v) aq. EtOH ×3 for 1 hr followed by extraction with H_2O at 40° under toluene for 18 hr. Yields of extract-free internodes of wheat and phalaris were 73.7 and 83.7%, respectively, based on original dry matter. The ground, extracted internodes were dried in a vacuum oven at 40° for 1 week, and further ground for 50 hr in a stainless steel vibrating ball mill without solvent and kept below 35°. The finely ground internodes were next successively extracted with 90 and 50% (v/v) dioxane–H_2O at room temp. for 1 week. These lignin–polysaccharide fractions contained both esterified and etherified cinnamic acids and were designated W90 [wheat internodes extracted with 90% (v/v) dioxane–H_2O (yield: 2.3% w/w based on dry wall)]; P90 [phalaris internodes extracted with

Table 1. Cinnamic acid content and ratio of bridge to total etherified cinnamic acids in wheat and phalaris lignin–polysaccharide fractions

	Cinnamic acid (CA) in lignin–polysaccharide fraction (wt %)				Total esterified CA‡		Bridged CA§	
	Esterified-only*		Etherified-only plus ester–ether bridge†		Total CA		Total etherified CA	
Sample	PCA	FA	PCA	FA	PCA	FA	PCA	FA
W90	1.13	0.69	0.31	1.41	0.78	1.00	0.00	1.00
P90	2.09	0.71	0.89	0.65	0.70	0.96	0.00	0.92
P50	0.38	0.31	0.07	0.51	0.58	1.00	0.00	1.00

Results are based on duplicate or triplicate analyses of each fraction.

*Determined from original lignin–polysaccharide sample by room temperature alkaline hydrolysis.

†Calculated from the difference between total cinnamic acids determined by alkaline hydrolysis at 170°, and esterified only cinnamic acids determined as in*.

‡Calculated from the contents of alcohol and acid derivatives of cinnamic acids released from hydrogenated-reduced, lignin–polysaccharide samples by alkaline hydrolysis at 170°. [HPAl/(HPAl + HPAc) and HFAl/(HFAl + HFAc)].

§The amounts of bridged cinnamic acids (X) were calculated from the values in columns 1, 2 and 3, e.g. for PCA in W90; (total esterified CA)/(total CA) = $(1.13 + X)/(1.13 + 0.31) = 0.78$, thus $X = 0$ and (bridged CA)/(total etherified CA) = 0.

Abbreviations used are defined in the text and in the legend to Fig. 2.

The W50 fraction could not be analysed due to a technical problem in satisfactorily separating the activated carbon used in the hydrogenation reaction.

Table 2. Values of each type of cinnamic acid associated with wheat and phalaris lignin–polysaccharide fractions

	PCA, wt % on sample			FA, wt % on sample		
Sample	Ester-only	Ether-only	Ester–ether bridge	Ester-only	Ether-only	Ester–ether bridge
W90	1.13 (78.5)	0.31 (21.5)	0.00 (0.00)	0.69 (32.9)	0.00 (0.00)	1.41 (67.1)
P90	2.09 (70.1)	0.89 (29.9)	0.00 (0.00)	0.71 (52.2)	0.05 (3.7)	0.60 (44.1)
P50	0.38 (84.4)	0.07 (15.6)	0.00 (0.00)	0.31 (37.8)	0.00 (0.00)	0.51 (62.2)

( ): Weight percentage of each type of cinnamic acid.
Abbreviations are as shown in Table 1.

90% (v/v) dioxane–H_2O (yield: 2%)]; W50 [W90-residue extracted with 50% (v/v) dioxane–H_2O (yield: 6.0%)]; P50 [P90 residue extracted with 50% (v/v) dioxane–H_2O (yield: 4.3%)] respectively. (*E*)-*p*-Coumaric acid (PCA, **1**), (*E*)-ferulic acid (FA, **2**), (*E*)-PCA Me ether (**3**) and (*E*)-FA Me ether (**4**) were obtained from Sigma. The corresponding Me esters (**5–8**) were prepd by refluxing **1–4** (1 mmol) with MeOH (100 ml) containing 0.1 ml 12 M HCl for 6 hr. The reaction mixtures were extracted twice with EtOAc and then shaken with 3% (w/v) $NaHCO_3$ aq. soln until all the free acid was removed. The purity was confirmed by GC as TMS deriv. using the same procedure as described below.

Hydrogenation. Model compounds **1–8** were hydrogenated in the presence of 10% Pd/C in MeOH at room temp. for 30 min under 1 atm H_2. At the end of the treatment, Pd/C was removed by filtration and the solvent evapd *in vacuo*. The hydrogenated products corresponding to **1–8**, 3-(4-hydroxyphenyl)-propionic acid (HPAc; **9**), 3-(3-methoxy-4-hydroxyphenyl)-propionic acid (HFAc; **10**), 3-(4-methoxyphenyl)-propionic acid (**11**), 3-(3,4-dimethoxyphenyl)-propionic acid (**12**), methyl-3-(4-hydroxyphenyl)-propionate (**13**), methyl-3-(3-methoxy-4-hydroxyphenyl)-propionate (**14**), methyl-3-(4-methoxyphenyl)-propionate (**15**), and methyl-3-(3,4-dimethoxyphenyl)-propionate (**16**), were identified by UV (solvent MeOH), ^1H and ^{13}C NMR spectroscopy (solvent CD_3OD) and by GC-MS (as TMS deriv.) under the same conditions as the GC described below.

The lignin–polysaccharide samples from wheat and phalaris internodes were dissolved in 90 or 50% (v/v) dioxane–H_2O and hydrogenated under the conditions described. The hydrogenation was repeated until the UV maximum or shoulder at 324 nm disappeared. The product was freeze-dried.

Reduction with $LiBH_4$. The hydrogenated compounds (**9–16**) (50 mg) were dissolved in dry Et_2O containing (3 ml) $LiBH_4$ (20 mg), toluene (4 ml) was added and the reaction mixture boiled under reflux for 15 min. Et_2O was completely removed in a stream of dry N_2, the mixture was refluxed for a further 3 hr and the borate complex decomposed with dry MeOH. Solvents were removed by evapn *in vacuo*. The resulting products were suspended in H_2O, the mixture adjusted to pH 1 with 6 M HCl and extracted into EtOAc. Analyses by ^1H NMR, ^{13}C NMR and GC-MS showed that the free acids (**9–12**) were recovered unchanged, and that Me esters (**13–16**) were completely converted to corresponding alcohols, 3-(4-hydroxyphenyl)-1-propanol (HPAl; **17**), 3-(3-methoxy-4-hydroxyphenyl)-1-propanol (HFAl; **18**), 3-(4-methoxyphenyl)-1-propanol (**19**), and 3-(3,4-dimethoxyphenyl)-1-propanol (**20**) respectively, without any side-products.

The hydrogenated lignin–polysaccharide samples were reduced with $LiBH_4$ under the conditions described. Complete reduction was judged by GC analysis using the procedure described below, to confirm minimum production of HPAc and HFAc from unreduced parts of the sample. This was achieved after two treatments, giving trace amounts of HFAc as shown in Fig. 2. After decomposition of excess $LiBH_4$ the product was dried under red. pres.

Alkaline hydrolysis of ether-linked cinnamic acids. The reduced and hydrogenated lignin–polysaccharide samples were hydrolysed with 4 M NaOH at 170° for 2 hr. (*E*)-3,4-Dimethoxycinnamic acid (**4**) in MeOH was added as int. standard. The reaction products were trimethylsilylated using bis-*N*,*N*-trimethylsilylacetamide, and analysed by GC using a BP-1 bonded-phase (layer thickness: 0.25 μm), vitreous silica capillary column (25 m × 0.25 mm i.d.). The GC conditions were: isothermal, 150° for 10 min, then temp. programmed to 180° at 1.5° min^{-1}; detector, FID; He at 45 kPa. The quantity of each product was calculated using a survival factor (0.70–0.91) which was determined by identical treatment of model compounds.

Spectral data. 3-(4-Hydroxyphenyl)-propionic acid (**9**): UV λ_{max}^{MeOH} nm: 276; MS *m/z* (%) as TMS deriv.: 310 ($[M]^+$, 49), 295 (17), 192 (98), 179 (100); ^{13}C NMR (CD_3OD): δ31.1 (C_γ), 37.0 (C_β), 116.2 (C-2, C-6), 130.1 (C-3, C-5), 132.9 (C-1), 156.3 (C-4), 177.1 (C_α). 3-(3-methoxy-4-hydroxyphenyl)-Propionic acid (**10**): UV λ_{max}^{MeOH} nm; 280; MS *m/z* (%) as TMS deriv.: 340 ($[M]^+$, 100), 325 (36), 310 (36), 222 (23), 209 (75); ^{13}C NMR (CD_3OD): δ31.5 (C_γ), 36.9 (C_β), 56.3 (OMe-3), 113.0 (C-2), 116.1 (C-5), 121.6 (C-6), 133.6 (C-1), 145.6 (C-4), 148.7 (C-3), 176.9 (C_α). 3-(3,4-dimethoxyphenyl)-Propionic acid (**12**): UV λ_{max}^{MeOH} nm: 278; ^{13}C NMR (CD_3OD): δ31.6 (C_γ), 37.1 (C_β), 56.4 (OMe-3), 56.6 (OMe-4), 113.3 (C-2), 113.6 (C-5), 121.5 (C-6), 135.2 (C-1), 148.8 (C-4), 150.3 (C-3), 177.0 (C_α). Methyl-3-(4-hydroxyphenyl)-propionate (**13**): UV λ_{max}^{MeOH} nm: 276; ^{13}C NMR (CD_3OD): δ30.9 (C_γ), 36.8 (C_β), 51.9 (OMe_α), 116.1 (C-2, C-6), 130.0 (C-3, C-5), 132.5 (C-1), 156.4 (C-4), 175.1 (C_α). Methyl-3-(3-methoxy-4-hydroxyphenyl)-propionate (**14**): UV λ_{max}^{MeOH} nm: 280; ^{13}C NMR (CD_3OD): δ31.2 (C_γ), 36.6 (C_β), 51.8 (OMe_α), 56.2 (OMe-3), 112.7 (C-2), 115.9 (C-5), 121.4 (C-6), 133.2 (C-1), 145.5 (C-4), 148.4 (C-3), 174.8 (C_α). Methyl-3-(3,4-dimethoxyphenyl)-propionate (**16**): UV λ_{max}^{MeOH} nm: 278; ^{13}C NMR (CD_3OD): δ31.5 (C_γ), 36.7 (C_β), 51.9 (OMe_α), 56.4 (OMe-3), 56.5 (OMe-4), 113.3 (C-2), 113.6 (C-5), 121.5 (C-6), 134.8 (C-1), 149.0 (C-4), 150.4 (C-3), 174.9 (C_α). 3-(4-hydroxyphenyl)-1-Propanol (**17**): UV λ_{max}^{MeOH} nm: 276; MS *m/z* (%) as TMS deriv.: 296 ($[M]^+$, 16), 281 (16), 206 (100), 191 (60); ^{13}C NMR (CD_3OD): δ32.1 (C_γ), 35.5 (C_β), 62.4 (C_α), 116.1 (C-2, C-6), 130.2 (C-3, C-6), 134.3 (C-1), 156.2 (C-4). 3-(3-methoxy-4-hydroxyphenyl)-1-Propanol (**18**): UV λ_{max}^{MeOH} nm: 280; MS *m/z* (%) as TMS deriv.: 326 ($[M]^+$, 96), 311 (35), 236 (36), 206 (100), 205 (50); ^{13}C NMR (CD_3OD): δ32.5 (C_γ), 35.5 (C_β) 56.3 (OMe-3), 62.2 (C_α), 113.1 (C-2), 116.0 (C-5), 121.7 (C-6), 134.9 (C-1), 145.3 (C-4), 148.6 (C-3). 3-(3,4-dimethoxyphenyl)-1-Propanol (**20**): UV λ_{max}^{MeOH} nm: 278; ^{13}C NMR (CD_3OD): δ32.4 (C_γ), 35.2 (C_β), 56.6 (OMe-3), 56.7 (OMe-4), 62.2 (C_α), 113.7 (C-2), 114.0 (C-5), 121.6 (C-6), 136.4 (C-1), 148.7 (C-4), 150.4 (C-3).

Acknowledgements—This work was conducted under a grant from the Wool Research Trust Fund. We thank Drs R. T. C. Brownlee and M. Sadek and Mr I. Thomas (Department of Chemistry, La Trobe University) for NMR analyses.

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