

EXCLUSIVE ACCUMULATION OF Z-ISOMERS OF MONOLIGNOLS AND THEIR GLUCOSIDES IN BARK OF *FAGUS GRANDIFOLIA*

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Abstract—In addition to Z-coniferyl and Z-sinapyl alcohols, bark extracts of *Fagus grandifolia* also contain significant amounts of the glucosides, Z-coniferin, Z-isoconiferin (previously called faguside) and Z-syringin. The corresponding E-isomers of these glucosides do not accumulate to a detectable level. The accumulation of the Z-isomers suggests that either they are not lignin precursors or that they are reservoirs of monolignols for subsequent lignin biosynthesis; it is not possible to distinguish between these alternatives. The co-occurrence of Z-coniferin and Z-isoconiferin demonstrate that glucosylation of monolignols can occur at either the phenolic or the allylic hydroxyl groups.

INTRODUCTION

It is widely accepted that the biogenesis of lignins occurs exclusively via the random dehydrogenative polymerization of the E-monolignols, *p*-coumaryl 1, coniferyl 2 and sinapyl 3 alcohols. Freudenberg, on the basis of his studies, concluded that these monolignols are stored and transported as their glucosides, e.g. E-coniferin 4, E-syringin 5. At the site of lignification, enzymic hydrolysis of the glucosides regenerates the E-monolignols which subsequently undergo a free-radical polymerization catalysed by peroxidase–hydrogen peroxide [1]. E-Coniferin (4) is not ubiquitously accumulated in woody plants and moreover, a study has shown that E-coniferin turnover in *Picea abies* does not completely account for the quantities of lignin produced [2].

We have recently demonstrated that American beech (*Fagus grandifolia*) bark contains detectable amounts of only the Z-isomers and not the E-isomers of coniferyl and sinapyl alcohols [3]. Furthermore, the transition from an E- to a Z-compound occurs at the level of the monolignol and not at an earlier stage, e.g. hydroxycinnamic acid [4]. Whereas it is very easy to bring about a photochemical isomerism of the cinnamic acids, we have been unable to demonstrate a similar photoisomerism for the monolignols. This suggests that the isomerism is enzyme-mediated.

Z-Coniferyl alcohol 7 also occurs as a glucoside 9, in which the allylic alcohol rather than the phenolic hydroxyl is involved in a β -glucosyl linkage in European beech (*Fagus sylvatica*) [5]. This isomer of coniferin has been called faguside, but we have called it Z-isoconiferin to make clearer its relationship to coniferin. It is likely that similar iso-glucosides will be found in other species.

E-Isoconiferin 6 has been identified tentatively in *Forstia ovata* [6].

In the present study we have identified the major monolignol glucosides of *Fagus grandifolia* bark and have speculated briefly on their role, if any, in lignin formation.

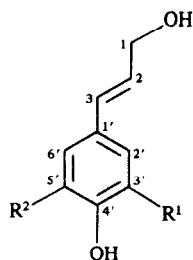
RESULTS AND DISCUSSION

Monolignol glucosides (I–III) were isolated by column chromatography or TLC from ethanolic extracts. ¹H NMR spectroscopy revealed that several of the resonances in the first two of the three compounds isolated bore striking resemblance to those of Z-coniferyl alcohol 7 whereas the third resembled Z-sinapyl alcohol 8 [4]. The stereochemistry about the olefinic double bond in each case was ~11.8 Hz, indicating a Z-configuration [7]. Whilst FABMS, ¹³C and ¹H NMR suggested that the associated monosaccharide was glucose, its identity and the nature of the glycosidic linkage needed verification. Treatment of each compound with β -glucosidase afforded D-glucose and the corresponding monolignol in all three cases.

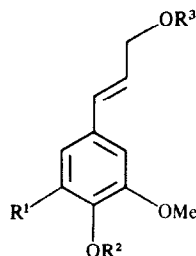
The amounts of glucose liberated for compounds I, II and III were 100, 81 and 86 mol % respectively, following incubation with β -glucosidase at 30° for 19 hr. Further, the relative initial rates of the β -glucosidase reaction with compounds I, II and III were 100:116:9. Additionally, the glucose moiety of these unknowns was also confirmed by acid hydrolysis and analysis of the products using paper chromatography. These experiments verified that the monosaccharide was glucose and that the linkage through C-1 was β .

In order to determine whether the monolignols were bound via phenolic or allylic hydroxyl groups, each glucoside was converted to its pentaacetate. Acetylation of I gave a pentaacetate having four overlapping acetate

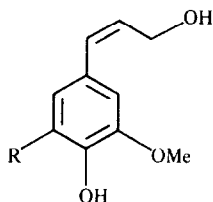
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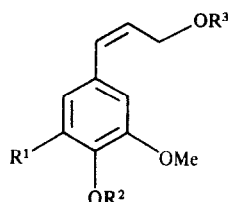
- 1** $R^1, R^2 = H$
2 $R = OMe, R^2 = H$
3 $R^1, R^2 = OMe$



- 4** $R^1 = R^3 = H, R^2 = Glc$
5 $R^1 = OMe, R^2 = Glc, R^3 = H$
6 $R^1 = R^2 = H, R^3 = Glc$



- 7** $R = H$
8 $R = OMe$



- 9** $R^1, R^2 = H, R^3 = Glc$
10 $R^1, R^3 = H, R^2 = Glc$
11 $R^1 = OMe, R^2 = Glc, R^3 = H$
12 $R^1 = H, R^2 = Ac, R^3 = Glc (Ac)_4$
13 $R^1 = H, R^2 = Glc (Ac)_4, R^3 = Ac$
14 $R^1 = OMe, R^2 = Glc (Ac)_4, R^3 = Ac$

resonances at $\delta 2.01$ – 2.06 and a fifth at $\delta 2.32$. The last resonance clearly indicated the presence of a phenolic acetate [8]. Thus this glucoside is fagusine or *Z*-isoconiferin **9** as we have preferred to call it. Acetylation of compounds **II** and **III** gave products with overlapping acetate resonances at $\delta 2.01$ – 2.08 , i.e. acetylated phenolic groups were not present in either compound. These glucosides are therefore *Z*-coniferin **10** (compound **II**) and *Z*-syringin **11** (compound **III**).

Thus in beech bark not only do the corresponding Z-monolignols accumulate, but so do their 'storage' glucosides, *Z*-coniferin, *Z*-isoconiferin and *Z*-syringin. Are these compounds to be considered as lignin precursors or are they important in some other metabolic sequences? *Z*-Coniferyl alcohol is a substrate for DHP lignin formation [4] with peroxidase but its ability to become incorporated into lignin *in vivo* remains to be determined.

EXPERIMENTAL

Z-Isoconiferin (fagusine) 9, Z-coniferin 10 and Z-syringin 11 from beech bark. Fresh beech bark, collected from branches of a mature tree, was immediately cut into narrow strips, frozen (liq. N_2) and freeze-dried. The resulting material was ground, by means of a Wiley Mill, to a fine powder (40 mesh). The powdered bark (10 g) was suspended in EtOH (100 ml, 95%) in the dark at room temp. for 24 hr and then filtered. Water (10 ml) was added to the EtOH solubles, and this mixture was then sequentially extracted with *n*-hexane (10 ml, 4 \times), Et₂O (10 ml, 4 \times) and EtOAc (10 ml, 4 \times). The resulting aqueous solubles were brought to dryness, under reduced pressure, to afford an oil

(0.76 g). The residual bark tissue (after EtOH extraction) was steeped in boiling H₂O (35 ml, 3 \times , 5 min duration) to give, after filtration and freeze-drying, a dark brown powder (0.38 g).

The combined water-soluble fractions (1.14 g) were applied to a silica gel column (10 cm \times 17 mm) and carefully eluted with Me₂CO–EtOAc (1:1). Selected fractions were combined and evaporated to give as major constituents, compound **I**, *Z*-isoconiferin **9** (62.44 mg), compound **II**, *Z*-coniferin **10** (175.42 mg) and compound **III**, *Z*-syringin **11** (130.99 mg). The crude glucosides were further purified on prep. silica gel 60 F₂₅₄ TLC plates (20 \times 20 cm, 0.25 mm thick) eluted with Me₂CO–EtOAc–H₂O (10:10:1) to afford pure *Z*-isoconiferin **9** (8.53 mg), *Z*-coniferin **10** (39.02 mg) and *Z*-syringin **11** (40.34 mg). *Z*-Isoconiferin **9**: ¹H NMR [(CD₃)₂CO]: δ 3–3.9 (10 H, *m*, glucose), 3.82 (3H, *s*, OMe), 4.36 (2H, *d*, $J = 7.6$ Hz, CH₂), 4.64 (1H, *dd*, $J_1 = 6.2$ Hz, $J_2 = 1.6$ Hz, HC₁ glucose), 5.71 (1H, *dt*, $J_1 = 11.78$ Hz, $J_2 = 6.2$ Hz, =CHCH₂OGlc), 6.47 (1H, *d*, $J = 11.86$ Hz, CH = CHCH₂OGlc), 6.72 (1H, *dd*, $J_1 = 1.8$ Hz, $J_2 = 8.1$ Hz, H-6'), 6.79 (1H, *d*, $J = 8.1$ Hz, H-5'), 6.89 (1H, *d*, $J = 1.7$ Hz, H-2'); ¹³C NMR (CD₃OD): δ 56.63 (OMe), 62.61 (C₆ glucose), 66.08 (CH₂OGlc), 71.50 (C₄ glucose), 74.93 (C₂ glucose), 77.65 (C₅ glucose), 77.93 (C₃ glucose), 103.61 (C₁ glucose), 113.77 (C-2'), 116.03 (C-5'), 123.08 (C-6'), 126.94 (=CHCH₂OGlc), 129.87 (C-1'), 132.96 (CH=CHCH₂OGlc), 146.99 (C-4'), 148.55 (C-3'); IR ν_{max}^{KCl} cm⁻¹: 3430–3330, 2900, 1640, 1590, 1510; UV λ_{max}^{MeOH} nm (log ϵ): 288 (sh), 260 (3.92), 206 (4.32); EIMS m/z (%): 342 [M⁺] (5), 180 [C₁₀H₁₂O₃] (20), 163 [C₁₀H₁₁O₂] (44), 131 [M⁺ – C₆H₁₁O₅, CH₂O] (100). Calculated for C₁₆H₂₂O₈: 342.131468. Found 342.131302; [α]_D^{33.5} – 27.6° (MeOH; *c* 0.129).

Z-Coniferin **10**. ¹H NMR [(CD₃)₂CO]: δ 3.3–4 (10 H, *m*, glucose), 3.81 (3H, *s*, OMe), 4.38 (2H, *dd*, $J_1 = 1.76$ Hz, $J_2 = 6.4$ Hz,

CHCH₂OH), 4.91 (1H, *d*, *J* = 6.97 Hz, HC₁ glucose), 5.76 (1H, *dt*, *J*₁ = 11.92 Hz, *J*₂ = 6.4 Hz, CHCH₂OH), 6.48 (1H, *d*, *J* = 11.82 Hz, CH=CHCH₂OH), 6.77 (1H, *dd*, *J*₁ = 1.86 Hz, *J*₂ = 8.4 Hz, H-6'), 6.89 (1H, *d*, *J* = 1.87 Hz, H-2'), 7.17 (1H, *d*, *J* = 8.34 Hz, H-5'); ¹³C NMR (CD₃OD): δ 56.93 (OMe), 59.91 (C₆ glucose), 62.60 (CH₂OH), 71.46 (C₄ glucose), 74.96 (C₂ glucose), 77.90 (C₅ glucose), 78.22 (C₃ glucose), 102.93 (C₁ glucose), 114.72 (C-2'), 117.97 (C-5'), 123 (C-6'), 131.01 (=CHCH₂OH), 131.97 (CH=CHCH₂OH), 133.33 (C-1'), 147.29 (C-4'), 151 (C-3'); IR $\nu_{\text{max}}^{\text{KCl}}$ cm⁻¹: 3410–3280, 2925, 2850, 1640, 1580, 1505; UV $\lambda_{\text{max}}^{\text{MeOH}}$ nm (log ϵ): 287 (sh), 254 (4.25), 209 (4.44); FABMS *m/z* (%): 365 [M + Na]⁺ (3), 180 [C₁₀H₁₂O₃] (15), 163 [M⁺ – C₆H₁₁O₅, OH] (20). Calculated for C₁₆H₂₂O₈Na: 365.1212377. Found 365.118958; $[\alpha]_{\text{D}}^{20}$ – 51.35° (MeOH, *c* 0.074).

Z-Syringin 11 (mp. 166–168°); ¹H NMR [(CD₃)₂CO]: δ 3.3–4 (10H, *m*, glucose), 3.84 (6H, *s*, 2 × OMe), 4.28 (2H, *dd*, *J*₁ = 1.55 Hz, *J*₂ = 6.5 Hz, =CHCH₂OH), 4.80 (1H, *d*, *J* = 6.5 Hz, HC₁ glucose), 5.81 (1H, *dt*, *J*₁ = 11.72 Hz, *J*₂ = 6.6 Hz, =CHCH₂OH), 6.50 (1H, *d*, *J* = 11.75 Hz, CH=CHCH₂OH), 6.58 (2H, *s*, H-2', H-6'); ¹³C NMR (CD₃OD): δ 57.19 (OMe), 59.78 (C₆ glucose), 62.65 (CH₂OH), 71.45 (C₄ glucose), 75.77 (C₂ glucose), 77.86 (C₅ glucose), 78.34 (C₃ glucose), 105.41 (C₁ glucose), 108.27 (C-2', C-6'), 131.39 (=CHCH₂OH), 132.57 (CH=CHCH₂OH), 134.78 (C-1', C-4'), 154.03 (C-3', C-5'); IR $\nu_{\text{max}}^{\text{KCl}}$ cm⁻¹: 3450–3350, 2900, 1650, 1580, 1505; UV $\lambda_{\text{max}}^{\text{MeOH}}$ nm (log ϵ): 258 (3.98), 218 (4.44); FABMS *m/z* (%): 395 [M + Na]⁺ (7), 193 [C₁₁H₁₃O₃] (15). Calculated for C₁₇H₂₄O₉Na: 395.131802. Found: 395.131699; $[\alpha]_{\text{D}}^{20}$ – 20.95° (MeOH, *c* 0.053).

Acetate derivatives 12–14

Z-Isoconiferin pentaacetate 12. ¹H NMR (CDCl₃): δ 2.01, 2.02, 2.03, 2.06 (12H, 4 *s*, 4 × OAc), 2.32 (3H, *s*, ArOAc), 3.64–3.69 (1H, *m*, H-C₅ glucose), 3.84 (3H, *s*, OMe), 4.06–4.39 (2H, *m*, CH₂, C₆ glucose), 4.56 (2H, *d*, *J* = 8.1 Hz, CH₂OGlc(Ac)₄), 5.01–5.24 (4H, *m*, HC_{1–4} glucose), 5.80 (1H, *dt*, *J*₁ = 11.72 Hz, *J*₂ = 6.5 Hz, =CHCH₂OGlc(Ac)₄), 6.61 (1H, *d*, *J* = 11.72 Hz, CH=CHCH₂OGlc(Ac)₄), 6.77 (1H, *dd*, *J*₁ = 1.8 Hz, *J*₂ = 8.18 Hz, H-6'), 6.83 (1H, *d*, *J* = 1.6 Hz, H-2'), 7.00 (1H, *d*, *J* = 8.0 Hz, H-5'); FABMS *m/z* (%): 553 [MH]⁺ (0.5).

Z-Coniferin pentaacetate 13. ¹H NMR (CDCl₃): δ 2.03, 2.04, 2.06, 2.07, 2.08 (15H, 5 *s*, 5 × OAc), 3.80 (1H, *m*, H-C₅ glucose), 3.82 (3H, *s*, OMe), 4.14–4.31 (2H, *m*, CH₂, C₆ glucose), 4.82 (2H, *dd*, *J*₁ = 6.71 Hz, *J*₂ = 1.48 Hz, =CHCH₂OAc), 4.96–5.31 (4H, *m*, HC_{1–4} glucose), 5.79 (1H, *dt*, *J*₁ = 11.7 Hz, *J*₂ = 6.67 Hz, CH=CHCH₂OAc), 6.61 (1H, *d*, *J* = 11.65 Hz, CH=CHCH₂OAc), 6.72–6.79 (2H, *m*, H-2', H-6'), 7.09 (1H, *d*, *J* = 8.1 Hz, H-5'); FABMS *m/z* (%): 553 [MH]⁺ (0.5).

Z-Syringin pentaacetate 14. ¹H NMR (CDCl₃): δ 2.00 (6H, 2 *s*, 2 × OAc), 2.01 (3H, *s*, OAc), 2.02 (3H, *s*, OAc), 2.06 (3H, *s*, OAc), 3.46 (1H, *m*, HC₅ glucose), 3.79 (6H, *s*, 2 × OMe), 4.11–4.22 (2H, *m*, CH₂, C₆ glucose), 4.80 (2H, *dd*, *J*₁ = 6.76 Hz, *J*₂ = 0.92 Hz, =CHCH₂OAc), 5.05–5.29 (4H, *m*, HC_{1–4} glucose), 5.78 (1H, *dt*, *J*₁ = 11.59 Hz, *J*₂ = 6 Hz, CH=CHCH₂OAc), 6.42 (2H, *s*, H-2', H-6'), 6.57 (1H, *d*, *J* = 11.58 Hz, CH=CHCH₂OAc); FABMS *m/z* (%): 584 [M + 2H]⁺ (0.2).

Enzymatic hydrolysis of glucosides 9–11 [9]. Glucosides 9–11 (10.00 mM) were individually dissolved in NaOAc–HOAc buffer (0.2 M, pH 5.3) and centrifuged (5 min, 8160 *g*). 200 μl aliquots of each were then incubated with 16.7 nKat (1 unit) of β-glucosidase (Sigma Type II, 4 units/mg protein/40 μl) for 19 hr at 30° in the dark, except for Z-syringin 11 which required 3 units. The rate and extent of enzymatic hydrolysis was determined using a Sigma diagnosis kit for glucose, containing glucose-6-phosphate dehydrogenase (*Leuconostoc mesenteroides*, 16.7 nKat/ml), hexokinase (yeast, 16.7 nKat/ml), ATP (1.0 mM), NAD⁺ (1.5 mM), Mg²⁺ (2.1 mM) and buffer (pH 7.5). The assay was carried out by taking 10 μl of the hydrolysate and incubating it with 1 ml of the glucose assay mixture. The reaction was monitored spectrophotometrically at 340 nm by measuring the change in absorbance due to the formation of NADH. Blank and standard glucose determinations were also carried out; sample glucose concentrations were calculated from the corrected absorbances of sample to standard, multiplied by the concentration of the standard glucose solution (10 mM).

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