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Identification of Hydrolysable Tannins in the Reaction Zone of *Eucalyptus nitens* Wood by High Performance Liquid Chromatography–Electrospray Ionisation Mass Spectrometry

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The first detailed analysis of the phenolic constituents of the reaction zones (tissue of antimicrobial defence) from the sapwood of a *Eucalyptus* spp. is presented. Plantation-grown *Eucalyptus nitens* trees with stem decay resulting from pruning wounds were sampled and extracts were prepared from healthy sapwood and from reaction zone tissue. Analysis by HPLC with ESI-MS revealed that a diverse range of hydrolysable tannins are present in both healthy sapwood and in reaction zone extracts, including over 30 gallotannins, ellagitannins and phenols. Eight tannins were unequivocally identified, including the gallotannins tri-*O*-galloyl-*β*-D-glucose, tetra-*O*-galloyl-*β*-D-glucose and penta-*O*-galloyl-*β*-D-glucose, and the ellagitannins pedunculagin, tellimagrandin I, casuarinin, casuarictin and tellimagrandin II. The phenols gallic acid, ellagic acid and catechin were also identified. The ellagitannins (particularly pedunculagin) are considerably more abundant in the reaction zone than in the healthy sapwood and may contribute to the effectiveness of the reaction zone as an antimicrobial barrier.

Keywords: HPLC; electrospray MS; tannin; ellagitannin; gallotannin; antimicrobial; *Eucalyptus* sp.

INTRODUCTION

The term hydrolysable tannin refers to both ellagitannins and gallotannins. Ellagitannins are esters of hexahydroxydiphenoyl (HHDP) groups with a sugar core (usually glucose) and often contain galloyl groups. Gallotannins consist of a sugar substituted only with galloyl groups. For reviews of hydrolysable tannins see Haslam (1989) and Okuda *et al.* (1995).

A role in antimicrobial defence has often been implicated for hydrolysable tannins; for example, durable eucalypt and oak heartwoods are rich in ellagitannins (Scalbert, 1992). Hillis (1987) states that gallotannins are found in the heartwood of *Castanea* and some *Quercus* species, while large amounts of ellagitannins exist in the heartwoods of some *Eucalyptus* and *Quercus* species. Approximately 20 ellagitannins have been reported from the heartwood of *Eucalyptus* species (e.g. Seikel and Hillis, 1970; Hillis and Yazaki, 1973; Hart and Hillis, 1974; Yazaki *et al.*, 1993). Other studies have noted the presence of numerous ellagitannins in eucalypt branch wood but have not identified individual compounds (Conde *et al.*, 1995; Cadahia *et al.*, 1997). Some heartwood ellagitannins show anti-fungal activities in bioassays, including ellagitannin D-6 and D-13 from *Eucalyptus* species (Hart and Hillis, 1974). Tannin toxicity to micro-organisms has been explained by reference to the characteristic ability of tannins to bind proteins (Field and Lettinga, 1992; Kawamoto *et al.*, 1997) or metals (Mila *et al.*, 1996) and their antioxidant nature (Okamura *et al.*, 1993; Hagerman *et al.*, 1998).

Reaction zones are a major component of active antimicrobial defence in the secondary xylem of woody plants (Shain, 1979; Pearce 1996). The reaction zone forms at the interface between living sapwood and fungal infection, and is able to restrict or slow fungal decay. This function has been attributed to the deposition of phenolics and polyphenolics, as well as to micro-environmental factors (Boddy and Rayner, 1983; Yamada *et al.*, 1988; Pearce, 1996). The reaction zone in *Eucalyptus nitens* is purple-blue in colour, is rich in polyphenols and appears to be resistant to colonisation by decay fungi (Barry *et al.*, 2000). The composition of phenolics in the reaction zone has never been studied in any *Eucalyptus* spp.

The study of hydrolysable tannins has developed substantially in the past two decades (Haslam, 1989; Okuda *et al.*, 1995). Molecular weight information has been obtained by fast-atom bombardment MS of purified compounds (Okuda *et al.*, 1995). The majority of tannins are beyond the range of GC analysis, and HPLC has been necessary for the determination of complex tannin extracts. Combining HPLC with MS has recently provided a powerful tool in the analysis of polyphenolics

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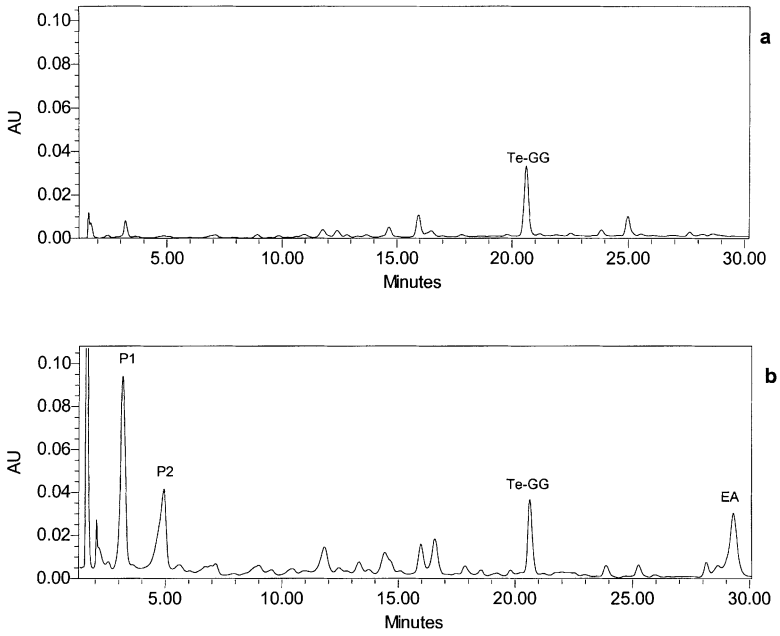

Figure 1. HPLC chromatograms of extracts (70% acetone) of wood of *Eucalyptus nitens* detected at 280 nm showing (a) healthy sapwood, and (b) reaction zone. Key to peak identities: P1 and P2, anomers of pedunculagin; Te-GG, tetra-galloylglucose; EA, ellagic acid: acetone co-elutes with P1. (For chromatographic protocols see Experimental section.)

from crude and purified extracts by detecting negative ions produced by electrospray ionisation (ESI) (Nawwar *et al.*, 1997; Puech *et al.*, 1999). However, little MS-MS detail of hydrolysable tannins has yet been provided.

The present study describes the application of HPLC-ESI-MS to elucidate the major components of the complex extract from the reaction zone of *E. nitens*. By reference to authentic standards of phenols, gallotannins and ellagitannins, 11 compounds have been unequivocally identified in crude extracts.

EXPERIMENTAL

Sample preparation. A number of plantation trees of *Eucalyptus nitens* between 5 and 7 years old were felled at various sites in Tasmania and those with decay columns interfaced with reaction zones were sampled. Extracts were prepared from fresh wood by obtaining chisel shavings (100–500 mg) of the reaction zone and of healthy sapwood tissue. Samples were extracted with 70% aqueous acetone for 24 h in the dark at 4°C. The extract was then transferred to a microcentrifuge tube, centrifuged to remove solids and then appropriately diluted for LC-MS. Samples were stored at −20°C and analysed as soon as possible (within a week). Injections of 2 μL sample were made, with pairs of extracts at equivalent concentrations (based on the weight of wood tissue) where semi-quantitative measurements were to be made. Typical yields of extract (as a percentage of fresh weight) were approximately 6% for reaction zone extracts and 1.5% for healthy sapwood extracts.

Authentic standards. The tannin standards were pedunculagin, pentagalloylglucose, corilagin, ellagitannins D-6 and D-13, 1,2,6-tri-*O*-galloyl-*β*-D-glucose, 1,2,3,6-tetra-*O*-galloyl-*β*-D-glucose, tellimagrandin I and II, casuarictin, casuarinin, and rugosin A and B [generous gifts from T. Yoshida (CSIRO, Melbourne, Australia) and from Y. Yazaki (Okayama University, Japan)]. The phenol standards were gallic acid, ellagic acid and catechin (Sigma, St Louis, MO, USA). Standards were accurately weighed and dissolved in 95% aqueous acetone at concentrations of 0.10 mg/100 μL. For later use, standards were dried under a stream of nitrogen at room temperature, stored at −20°C and reconstituted when further required.

HPLC-ESI-MS. HPLC separations were carried out on a Waters Alliance 2690 chromatograph employing a Waters Nova Pak C_{18} column (150 × 3.9 mm i.d.). The mobile phase consisted of solvent A (water:acetic acid, 98:2) and solvent B (methanol:acetic acid, 98:2); initial conditions were 95% A and 5% B followed by a linear gradient to 46% A and 54% B over 40 min at a flow-rate of 0.8 mL/min, before returning to initial conditions with 12 min re-equilibration between samples. Compounds were detected using a Waters model 996 photodiode array detector monitoring over the range 240–400 nm at a resolution of 1.2 nm.

MS was carried out on a Finnigan LCQ (San Jose, CA, USA) with an electrospray ion source, using LCQ Navigator version 1.2 software. The instrument was operated in the negative ion mode, scanning from *m/z* 125 to 1500, with an AGC target value of 2×10^7 and maximum ion injection time of 100 ms. Operating conditions were: sheath gas, 90 psi; auxiliary gas, 50 psi; ESI needle voltage, 4.5 kV; capillary temperature, 270°C; capillary voltage, −30 V. Data-dependent MS-MS spectra were routinely acquired from the most intense ion in the spectrum with a default collision energy of 30% and a peak isolation width of 3 amu.

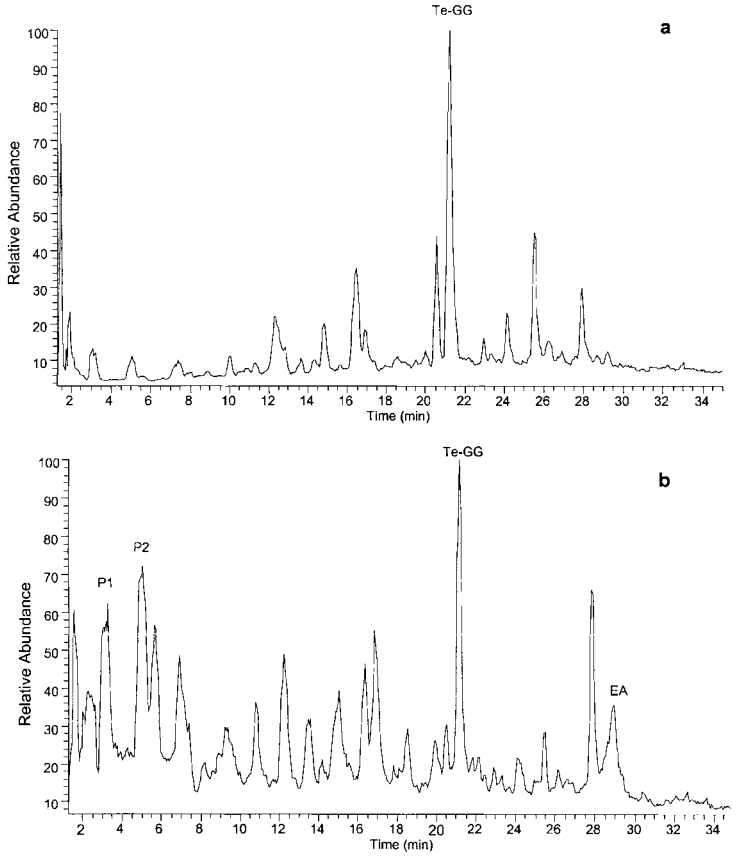

Figure 2. Total ion current chromatograms of extracts (70% acetone) of wood of *Eucalyptus nitens* showing (a) healthy sapwood, and (b) reaction zone. (Refer to the legend of Fig. 1 for key to peak identities and chromatographic protocols).

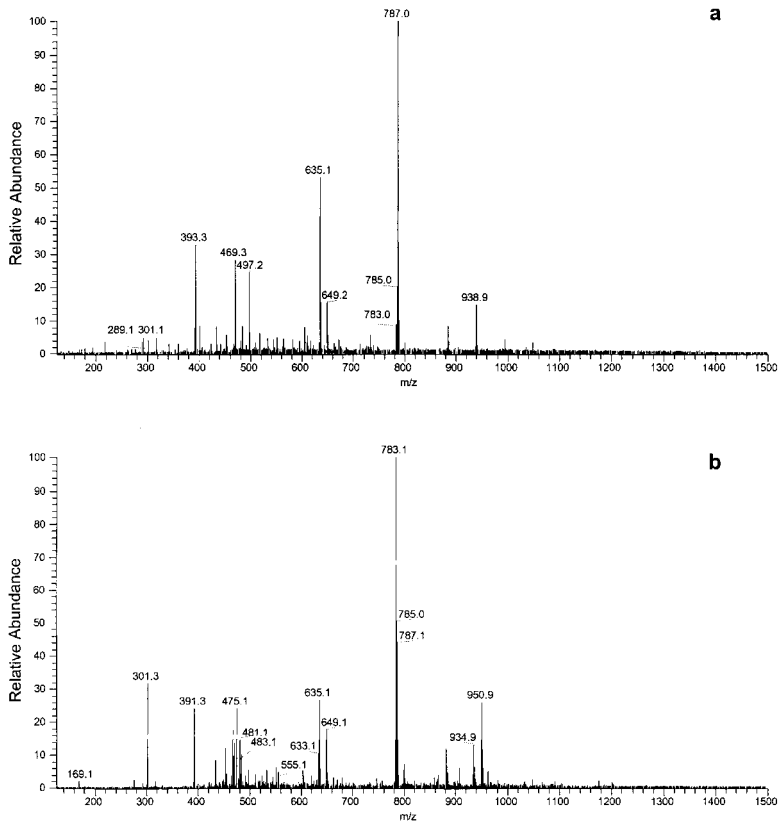

Figure 3. The summed mass spectra in the range of *m/z* 125–1500 for extracts (70% acetone) of wood of *Eucalyptus nitens* showing (a) healthy sapwood, and (b) reaction zone.

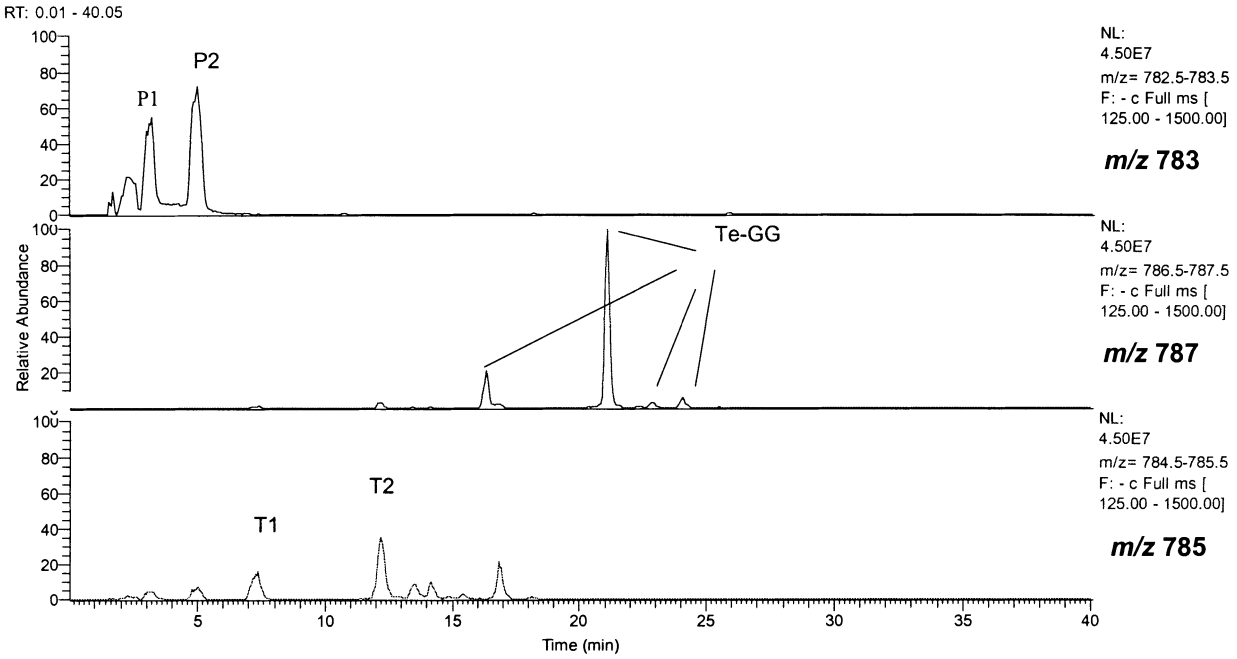

Figure 4. Mass chromatograms of three selected $[M\text{-}H]^-$ ions in the extract of the reaction zone of *Eucalyptus nitens*. Key to peak identities: P1 and P2, anomers of pedunculagin; Te-GG, tetra-*O*-galloyl-β-D-glucose isomers; T1 and T2, tellimagrandin I anomers. (For chromatographic protocols see Experimental section.)

RESULTS AND DISCUSSION

Methodology and interpretation

The polar nature and complexity of the extracts from the reaction zone of *E. nitens* limited successful HPLC resolution. The difficulty in obtaining good HPLC separation of wood tannins is evidenced from previous studies (e.g. Scalbert *et al.*, 1988; Yazaki *et al.*, 1993). However, long and time-consuming HPLC programs (e.g in excess of 80 min) can be employed to improve separation (Cadahia *et al.*, 1997). Trials of a range of HPLC programs did not substantially improve the separation of the extracts from *E. nitens*, precluding peak identification based on retention times and UV spectra alone.

Figure 1(a) and (b) shows that the HPLC chromatogram (detected at 280 nm) of the extract of the reaction zone is more complex than that of the healthy sapwood. Figure 2(a) and (b) presents the corresponding total ion chromatograms from the HPLC-MS analyses of these extracts, and indicates that the mass range employed (up to *m/z* 1500) is appropriate for the components detected by UV as similar peaks are observed in both spectra. MS revealed that many compounds in the extract co-elute and that peaks cannot be interpreted by UV detection alone using current HPLC systems.

In order to gain an overview of the molecular weight range of the major compounds present in each extract, a summed MS was determined between *m/z* 125 and 1500 [Fig. 3(a) and (b)]. Of the major ions present, those representing molecular weights corresponding to the tannin and phenolic standards available were selected and the retention times and negative ion ESI-MS characteristics were determined. Compounds were unequivocally identified based on matching mass chromatograms generated for specific $[M - H]^-$ ions (e.g. Fig. 4), on MS of the $[M - H]^-$ ions at specific retention times (e.g. Fig. 5)

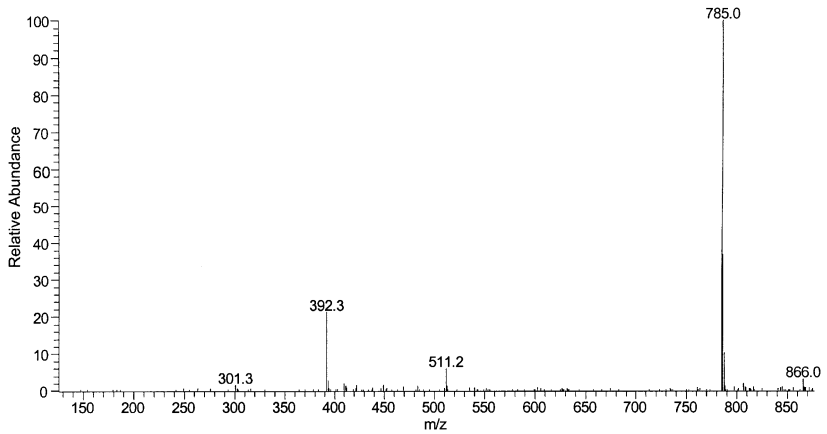

Figure 5. Mass spectrum of tellimagrandin I ($[M - H]^- = 785$; $[M - 2H]^{2-} = 392$) from the extract of the reaction zone of *Eucalyptus nitens*.

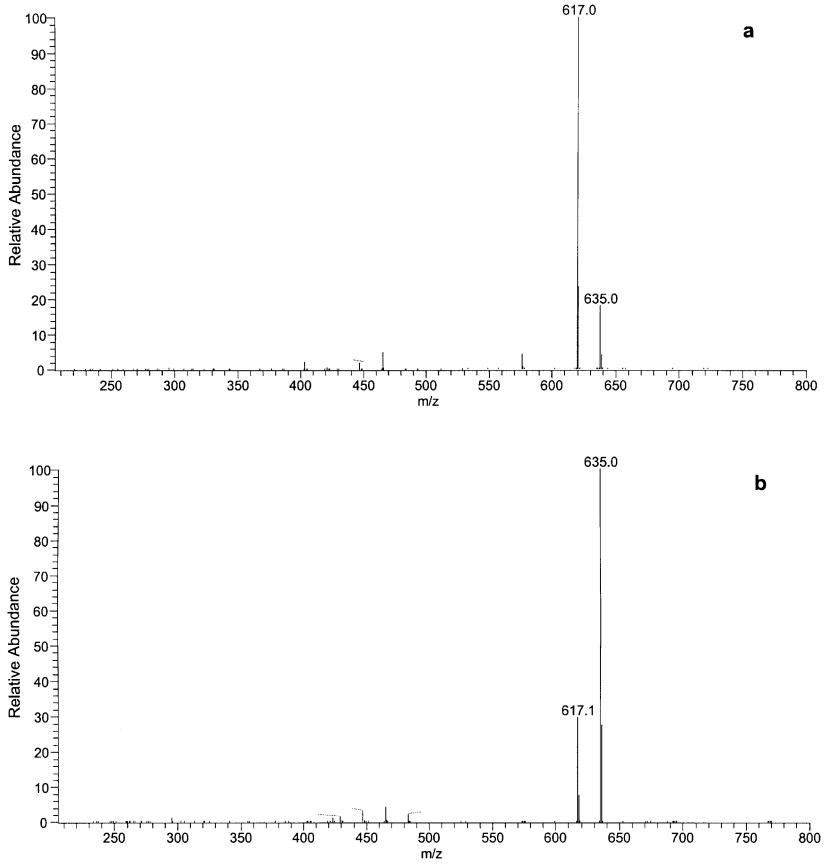

Figure 6. MS-MS daughters of the [M – H]-ion at *m/z* 787, (a) from the 1,2,3,6-tetra-galloyl- β-D-glucose standard at 21.08 min, and (b) from the reaction zone extract peak at 21.08 min.

and, subsequently, on MS-MS of daughter ions from a selected parent ion [e.g. Fig. 6(a)]. The assignment of numerous other peaks as positional isomers (e.g. various di-, tri- and tetra-galloylglucoses) was also possible. Furthermore, the degree of substitution of HHDP and galloyl groups in unknown compounds could be assigned. Interpretation of MS-MS data from first principles indicated the presence of galloyl groups (losses of 152 and 170 from the $[M - H]^-$ ion), and/or HHDP groups (loss of 302 from the $[M - H]^-$ ion and the presence of an ion at *m/z* 301). Loss of 44 from the $[M - H]^-$ ion was characteristic of a free carboxyl (e.g. on a trisgalloyl group) and abundant losses of 18 (water) from the $[M - H]^-$ ion was characteristic of C-glucosidic ellagitannins.

The characteristic trait that a free anomeric hydroxyl group at C1 of the glucose core of an ellagitannin molecule results in two chromatographic peaks was also a feature for identification (Hatano *et al.*, 1988). Addition of sodium borohydride to aqueous extracts reduces tannins with anomeric hydroxyls, resulting in a single peak (Hatano *et al.*, 1988) which also presents a tool for identification. Another characteristic of the ESI-MS of most tannins is that doubly-charged ions (i.e. $[M - 2H]^{2-}$) are also formed (e.g. Fig. 5).

Compound identification

Table 1 outlines some 30 major and minor compounds discriminated from the *E. nitens* extracts, showing relative proportions (based on MS peak area) which have been classified arbitrarily as described. The sensitive MS system used in the present study also detected a multitude of smaller peaks which have not been analysed. Five ellagitannins (pedunculagin, tellimagrandin I and II, casuarinin and casuarictin), three gallotannins (tri-galloylglucose, tetra-galloylglucose and penta-galloyl-glucose) and three phenols (catechin, gallic acid and ellagic acid) were unequivocally identified. The structure of an abundant ellagitannin (pedunculagin) is shown in Fig. 7 and a variety of gallotannins are illustrated in Fig. 8. A number of assumed positional isomers and unidentified compounds are also presented in Table 1 and are discussed below.

Treatment of reaction zone extracts of *E. nitens* (prepared as aqueous solutions) with sodium borohydride revealed that at least three prominent ellagitannins that form anomers are present, since three prominent "new" reduction product peaks were detected (K. M. Barry, unpublished results). Two of these anomer-forming ellagitannins are pedunculagin and tellimagrandin I, and the third may be a compound with $[M - H]^-$ at *m/z* 951.

The general substitution pattern of HHDP and galloyl groups for a number of compounds in Table 1 has been determined from molecular weight information and characteristic MS-MS daughter ions. Where molecular weights of hydrolysable tannins differ by two, it can be related to the difference between either an HHDP group or two galloyl groups. For example, by coupling two adjacent galloyl groups of the four from tetra-galloylglucose (788) by intramolecular oxidation, tellimagrandin I (786) would be formed. Coupling of the two remaining

Table 1. Major and minor hydrolysable tannins present in extracts of wood of *Eucalyptus nitens* as determined by HPLC-ESI-MS

			Relative abundance[a]	
$[M-H]^-$	Retention time (min)	Compound	Healthy sapwood	Reaction zone
169	2.79	Gallic acid[b]	tr	+
289	10.03	Catechin[b]	+	tr
301	28.93	Ellagic acid[b]	+	++
481	1.55	HHDP-glucose	tr	+++
483	8.83	Di-galloylglucose[v]	tr	tr
	10.67	Di-galloylglucose[w]	tr	tr
	12.00	Di-galloylglucose[x]	tr	tr
	13.59	Di-galloylglucose[y]	tr	tr
	14.49	Di-galloylglucose[z]	tr	tr
633	9.61	HHDP-GG	tr	++
635	12.46	Tri-galloylglucose[w b]	+	+
	14.75	Tri-galloylglucose[x b]	++	++
	16.23	Tri-galloylglucose[y b]	++	++
	16.39	1,2,6- Tri-galloylglucose[b]	tr	tr
	16.79	Tri-galloylglucose[z b]	+	+
649	27.82	Methyl-(tri-galloylglucose)	++	+++
783	2.40	Di-HHDP-glucose	tr	++
	3.16/5.00	Pedunculagin[b]	+	++++
785	7.27/12.19	Tellimagrandin I[b]	++	+++
	13.52	HHDP-di-galloylglucose[x]	tr	++
	14.19	HHDP-di-galloylglucose[y]	+	++
	16.85	HHDP-di-galloylglucose[z]	+	++
787	16.29	Tetra-galloylglucose[w b]	+	+
	21.08	Tetra-galloylglucose[x b] + 1,2,3,6-tetra-galloylglucose	++	+++
	22.86	Tetra-galloylglucose[y b]	tr	tr
	24.04	Tetra-galloylglucose[z b]	tr	tr
935	9.32	Casuarinin	0	+
	15.02	Casuarictin	0	+
	23.10	Di-HHDP-galloylglucose	tr	+
937	16.91	HHDP-tri-galloylglucose[x]	tr	+
	18.02	Tellimagrandin II[b]	tr	+
	19.24	HHDP-tri-galloylglucose[y]	tr	+
	32.95	HHDP-tri-galloylglucose[z]	+	0
939	25.42	Penta-galloylglucose[b]	tr	+
951	5.64/10.84	(Trisgalloyl)-HHDP-glucose[y]	tr	+++
	6.88	(Trisgalloyl)-HHDP-glucose[z]	0	+++

[a] Relative scale where 0 = none detected; tr (trace amount) = 0–1; + = 1–10; ++ = 10–50; +++ = 50–100; ++++ = >100.

[b] Unequivocal identification.

[v,w,x,y,z] Symbols used to discriminate putative individual isomers (see Results and discussion section).

galloyl groups from tellimagrandin I would produce pedunculagin (784) (Fig. 7). The loss of two hydrogen atoms resulting from bonding of galloyl groups may occur similarly for other structures. The strong $[M-H]^-$ at *m/z* 481 in the reaction zone samples has been assigned as an HHDP-glucose, based on molecular weight and the presence of an intense daughter ion at *m/z* 301. The compound with $[M-H]^-$ ion at *m/z* 633, abundant in the reaction zone samples, has been assigned as an HHDP-

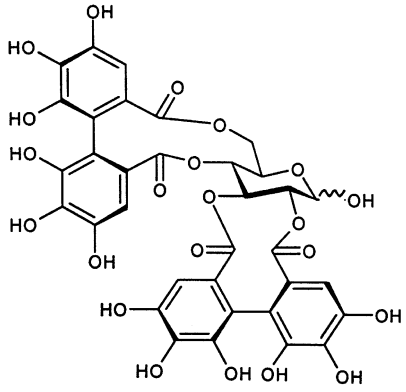

Figure 7. The structure of pedunculagin (2,3; 4,6-di-HHDP-glucose).

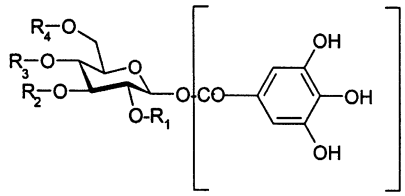

Figure 8. Examples of gallotannins with various numbers of galloyl groups (G; substituent shown in brackets) linked to the glucose core. *β*-Glucogallin *(1)*, $R_1 = R_2 = R_3 = R_4 = H$; 1,6-Di-*O*-galloyl-*β*-D-glucose *(4)*, $R_1 = R_2 = R_3 = H$, $R_4 = G$; 1,2,6-Tri-*O*-galloyl-*β*-D-glucose *(6)*, $R_1 = R_4 = G$, $R_2 = R_3 = H$; 1,2,3,6-Tetra-*O*-galloyl-*β*-D-glucose *(4)*, $R_1 = R_2 = R_4 = G$, $R_3 = H$; 1,2,3,4,6-Penta-*O*-galloyl-*β*-D-glucose *(1)*, $R_1 = R_2 = R_3 = R_4 = G$. The number of *β* positional isomers expected for each gallotannin is in brackets.

galloylglucose (Table 1), from the MS-MS evidence of intense ions at *m/z* 301 and 463. The minor $[M - H]^-$ ions at *m/z* 483 have been assigned as di-galloylglucoses.

Casuarinin and casuarictin (both with an $[M - H]^-$ ion at *m/z* 935) could be distinguished by their retention times and also by their MS-MS spectra. The MS-MS daughters of the casuarinin $[M - H]^-$ ion showed a strong ion (30% relative abundance) resulting from a loss of water, owing to its open glucose ring structure (*C*-glucoside rather than *O*-glucoside). For casuarictin this ion was only 0.2% relative to the strongest ion corresponding to the loss of 302 as discussed above. The peak eluting at 2.40 min prior to the two pedunculagin anomers at *m/z* 783 (Fig. 4) also showed a strong ion (20% relative abundance) resulting from a loss of water from the $[M - H]^-$ ion and therefore may be the C-glucoside version of pedunculagin.

Based on molecular weight and MS-MS data, the three abundant peaks with $[M - H]^-$ at *m/z* 951 in the reaction zone samples (Table 1) would appear to be tannins with one HHDP group and one trisgalloyl group (e.g. valoneoyl, tergalloyl, macaronyl). All three peaks lose 44 from the $[M - H]^-$ ion, consistent with a free carboxyl group, as found in a compound such as praecoxin A. As mentioned, the two peaks at *m/z* 951 eluting at 5.64 and 10.84 min (Table 1) may be anomeric forms of one compound, therefore suggesting a free hydroxyl at the glucose C1. The single peak at 6.88 min may be a variation on this which involves bonding at the C1 position.

The main peak of tetra-galloylglucose at 21.08 min in the *E. nitens* extracts (see Fig. 4) is likely to represent two isomers. The 1,2,3,6-tetra-*O*-galloyl-β-D-glucose standard eluted at this time and its MS-MS spectrum [Fig. 6(a)] showed two major daughter ions of which that at *m/z* 617 was 5-fold more abundant than the ion at *m/z* 635. However, the MS-MS spectrum of the *E. nitens* peak at 21.08 min [Fig. 6(b)] revealed that the daughter ions were in reverse proportion and, therefore, came largely from a different isomer. This latter ratio was consistent in all reaction zone and healthy sapwood extracts analysed. Four major tetra-galloylglucose isomers were detected in the *E. nitens* extracts (Fig. 4 and Table 1). As the possible number of β isomers for tetra-galloylglucose is four (see Fig. 8), it would be surprising if the 1,2,3,6-isomer were not present, and therefore it may be making a small contribution to the main peak in the *E. nitens* extracts. The number of possible positional isomers of the other gallotannins (Fig. 8) correlates similarly, but not exactly, to the number of peaks representing each gallotannin isomer in the wood extracts (Table 1).

Tannin levels and biological significance

For simplicity, compound abundance has been classified into groups based on MS peak areas (from a typical set of samples) as detailed in Table 1. The abundance of pedunculagin was found to be increased approximately 50-fold in the reaction zone compared to the healthy sapwood. Many other ellagitannins were also present at greatly increased levels in the reaction zone. However, there was at most only a slight increase in the levels of the gallotannins in the reaction zone samples. This suggests that the ellagitannins are more important in the antimicrobial effectiveness of the reaction zone. Gallotannins such as penta-*O*-galloyl-β-D-glucose may be important as ellagitannin precursors (Hatano *et al.*, 1986; Haslam, 1989). Interestingly, preliminary HPLC-MS studies of *E. nitens* leaves have shown that a range of gallotannins is present in light stressed leaves compared to normal leaves, but few/no ellagitannins (D. C. Close and N. W. Davies, unpublished results).

Haslam (1989) has classified plants with penta-*O*-galloyl-β-D-glucose and one or more of tellimagrandin II, tellimagrandin I, casuarictin, potentillin and pedunculagin as having "group 2B" phenolic metabolites. Metabolites from the wood of *E. nitens* therefore fall within this grouping, as has been found for the Myrtaceae family in general (Haslam, 1989). The range of closely related hydrolysable tannins and isomers found in *E. nitens* wood is a common occurrence and may prove advantageous for defence (Zucker, 1983). For example, tannin–protein binding may be an important mechanism to halt fungal metabolism, and a range of tannin structures may promote a variety of specific tannin–protein interactions.

As casuarictin and casuarinin (and a compound at *m/z* 951 eluting at 6.88 min) were not detected in the *E. nitens* healthy sapwood, these compounds may be produced *de novo* in the reaction zone from pedunculagin (Okuda *et al.*, 1995). All other tannins are detectable in at least trace amounts in the healthy sapwood (Table 1). Increases in the concentration of tannins of the magnitude found in the *E. nitens* reaction zone would be expected to confer biological activity: for example, Hart and Hillis (1972) found that even a 2-fold increase of white oak heartwood extracts resulted in antifungal inhibition to *Poria monticola* in bioassay.

Catechin has not been previously detected in *Eucalyptus* sapwood (Y. Yazaki, personal communication), and this may be because of a higher detection sensitivity in this study, or it may represent a novel finding for *E. nitens*. The decreased levels of catechin in the reaction zone may be due to the formation of condensed tannins, and the purple/blue colour of the zone is suggestive of condensed tannins. However these compounds were not analysed in this study and there was no evidence of condensed tannins such as epicatechin gallate.

In conclusion, HPLC-MS provides a powerful and relatively quick technique with which to elucidate the complex nature of crude wood extracts. It provides a solid basis for identification with reference to retention times, unequivocal molecular weight assignment and characteristic daughter ions. At least 30 hydrolysable tannins (including structural isomers) were detected in the wood extracts of *E. nitens*. Eight of the hydrolysable tannins and three phenols were unequivocally identified by comparison with standards. The ellagitannins were orders of magnitude more abundant in the reaction zone compared to healthy sapwood. The ellagitannin pedunculagin is particularly concentrated in the reaction zone and may play an important role in its antimicrobial function.

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