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HYDROLYSABLE TANNINS OF *EUCALYPTUS DELEGATENSIS* WOOD

MARGARET K. SEIKEL* and W. E. HILLIS

Division of Forest Products, C.S.I.R.O., Melbourne, Australia

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Abstract—Five ellagitannins have been isolated from the wood of *Eucalyptus delegatensis* by fractionation with Sephadex and characterized by hydrolysis reactions and NMR spectra. They include 2,3- and 4,6-(hexahydroxydiphenoyl)-glucose, a di-(hexahydroxydiphenoyl)-glucose known as pedunculagin, and two more complex tannins of incompletely determined structure. Two new chromatographic sprays for distinguishing between ellagitannins have been developed.

INTRODUCTION

THE HEARTWOODS of the low-density, pale-coloured eucalypts contain up to 25 per cent or more of extractives.[1] The monomeric components of the extractives are largely composed of hydrolysable tannins[1-5] which, together with the associated ellagic and gallic acids and polymers, are responsible for several problems encountered in the manufacture of pulp and paper from eucalypts.[1,5-7] An earlier examination[2] of the extractives of *Eucalyptus delegatensis* R. T. Baker (syn. *E. gigantea* Hook; Family Myrtaceae) "Alpine Ash", revealed a complex mixture of ellagitannins, which yielded ellagic acid (and sometimes gallic acid) and glucose on hydrolysis. One of the components was identified chromatographically as juglanin.[8] Very few compounds which yield the above hydrolytic products have been reported so far.[9,10] This paper describes the separation of several ellagitannins by means of different grades of Sephadex dextran gels and characterization of them by two newly developed chromogenic sprays, by hydrolytic studies, and by NMR spectral studies. Attention was given to the latter technique as it avoids the harsh conditions sometimes used by classical methods for the examination of these labile compounds.

* This collaboration was done when the late Dr. M. K. Seikel was a visiting scientist on leave from Forest Products Laboratory, Forest Service, U.S.D.A., Madison, Wisconsin 53705, U.S.A.

[1] J. A. F. GARDNER and W. E. HILLIS, in *Wood Extractives* (edited by W. E. HILLIS), p. 367, Academic Press, New York (1962).
[2] W. E. HILLIS and A. CARLE, *Biochem. J.* **74**, 607 (1960).
[3] W. E. HILLIS and A. CARLE, *Holzforschung* **12**, 136 (1958).
[4] D. F. BOWMAN and P. F. NELSON, *Appita* **19**, 8 (1965).
[5] G. J. LEES and P. F. NELSON, *Appita* **20**, 113 (1967).
[6] W. E. HILLIS and A. CARLE, *Appita* **13**, 74 (1959).
[7] W. E. HILLIS and T. SWAIN, in *Wood Extractives* (edited by W. E. HILLIS), p. 405, Academic Press, New York (1962).
[8] L. JURD, *J. Am. Chem. Soc.* **80**, 2249 (1958).
[9] L. JURD, in *Wood Extractives* (edited by W. E. HILLIS), p. 229, Academic Press, New York (1962).
[10] E. HASLAM, *Chemistry of Vegetable Tannins*, Academic Press, London (1966).

RESULTS

Paper Chromatographic (PC) Examination and Colour Reactions

The best PC resolution of the ellagitannins was achieved by a two-dimensional method with butanol–acetic acid–water and dilute acetic acid. At least twenty-one ellagitannins were revealed not including possible polymers and those present in very small amounts.[11] They were detected under u.v. light (254 nm) and by spraying with ferric chloride–potassium ferricyanide[12] or with a newly developed but less sensitive spray of a buffered alkaline solution at pH 9·5–10 (base mixture). Under basic conditions the ellagitannins were air-oxidized at different rates producing distinctive colours which greatly assisted their identification (Table 1). This pH was chosen as the colours developed at a moderate rate, and the changes could be followed before the formation of the final yellow to brown colour. Of other oxidizing sprays, iodate,[10] although insensitive, gave some characteristic colours, and a very weakly

TABLE 1. CHROMATOGRAPHIC PROPERTIES OF ELLAGITANNINS

Tannin	R_f × 100* BAW	R_f × 100* 6HA	Colour ($u.v._{254}$)†	Colours with sprays: Base mixture‡	Colours with sprays: (KIO_3)§	Colours with sprays: Gibbs'‖ basic PbOAc
D-1 (2,3-HHDP-glucose)	30	76	Purple	Violet → bright violet	Light green	Violet-grey
D-2 (pedunculagin)	30	57	Purple	Violet → violet-pink (rose if conc.)	Grey-brown	Violet-grey
D-3	23	21	Light blue	Yellow —(at once)→ yellow-brown	Pink-purple	Green-blue
D-4 (4,6-HHDP-glucose)	23	73	Faint blue	Bright pink, stable	Slowly green-grey	Light brown
D-13, *a*	9	60	Light blue	Grey → dark grey		Grey-brown
b	7	61	Dark blue	Violet → violet-grey, fades soon		
3,6-HHDP-glucose	37	81		Violet → pink tan		
Corilagin	41	43		Faint pink → yellow tan		Green-blue
Gallic acid	63	41	Pink, blue	Slowly yellow-green → green		Green-blue
Ellagic acid	46	2	Faint violet	Light yellow		Yellow

* R_f values were measured from leading edge of spot and those in BAW were variable.

† Under some conditions all tannins are absorbing in u.v. (254 nm); under others they fluoresce as listed.

‡ Strong spots are necessary (except for D-4 which is more sensitive to the spray than to u.v. light). The colours change through grey and tan shades to yellow-orange or brown-orange within 3–20 min except for the persistent pink colour with D-4. Each series of colour changes is characteristic, but exact shades depend somewhat on concentration. In u.v. (366 nm) the final colours are all bright orange or yellow-brown.

§ All colours change quickly to brown or grey-brown.

‖ Colours seen after 5 min and retained for hours. Initial colours are either all grey or reminiscent of colours with base mixture. Gallotannins give blue or blue-grey generally.

[11] W. E. HILLIS, *Appita* **23**, 89 (1969).

[12] H. G. C. KING and T. WHITE, *J. Soc. Leath. Trades Chem.* **41**, 368 (1957).

basic Gibbs' reagent appeared to distinguish ellagitannins with galloyl groups from simpler ones.

Qualitative Analysis of Various Extracts

Four different samples of the extractives of *Eucalyptus delegatensis* wood were examined (see Experimental). The ellagitannins that were resolved chromatographically constituted about 10 per cent of the "ethanolic extractives" that were prepared at room temperature from freshly cut wood. The extractives contained outstanding amounts of D-1 (the simple hexahydroxydiphenoyl (HHDP) tannin, namely 2,3-HHDP-glucose, Fig. 1) and almost none of its 4,6-isomer, D-4. The other extracts had undergone more handling and higher temperatures. The "crude ellagitannins" were richer in D-3 and the "pressed" and "Asplund" liquors were richer in D-6, D-12, and D-13.

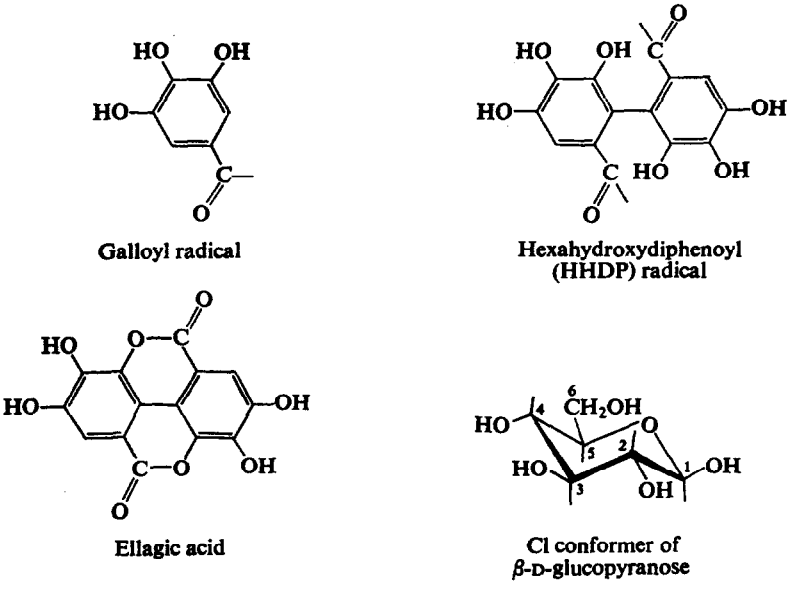

FIG. 1. FORMULAE OF UNITS OF HYDROLYSABLE TANNINS.

Isolation of Ellagitannins with Sephadex

When the "pressed" and Asplund liquors were freeze-dried and then extracted with dry acetone most of the monomeric ellagitannins were removed. A method for their separation, based on various types of Sephadex, was developed. Aqueous solutions of the ellagitannins were successively fractionated on G-50, G-25, sometimes G-15, and G-10 Sephadex with water as the eluant. The resulting crude fractions were rechromatographed on longer columns for isolation and purification of individual compounds. G-50 Sephadex removed polymeric material quickly, G-25 was the best for most fractionations, but G-10 was necessary for separating the two mono-HHPD-glucoses (D-1 and D-4).

When the eluates of the columns were freeze-dried, the ellagitannins were obtained as white or almost white amorphous residues. These were chromatographically pure but have not yet been crystallized. By these methods five compounds have been isolated in solid form (0·04–1·0 g): D-1, D-2, D-3, D-4, and D-13.

Characterization of Ellagitannins

Only two methods were employed for studying the ellagitannins, namely, hydrolysis and NMR spectra.

By hydrolysis. A variety of weakly acidic and milder hydrolytic methods showed that (Table 2): *a.* D-1, D-2, D-4, and D-13 consist solely of HHDP residues (yielding ellagic acid,

TABLE 2. HYDROLYSIS OF ELLAGITANNINS

Compounds*	Conditions†	Products‡
D-1 (2,3-HHDP-glucose)	Hot water (4–50 hr)	D-p, D-q (?D-13 or D-14), ellagic acid
	N H_2SO_4 (0·3–13 hr)	Ellagic acid, glucose and traces of other components
D-3	Hot water (4–? hr)	Ellagic and gallic acids
	N H_2SO_4 (0·2–6 hr)	D-r, D-s, D-t, ellagic acid (0·2 hr), gallic acid (1 hr), glucose, polymers
	Tannase (0·1–66 hr)	D-7, gallic acid
D-13	Hot water (0·7–24 hr)	Ellagic acid
	N H_2SO_4 (0·2–6 hr)	D-14, D-a, D-b, D-c, D-d, ellagic acid, glucose, polymers
D-4 (4,6-HHDP-glucose)	Room-temp. water	(Stable for 18 days)
	Hot water (0·2–14 hr)	Ellagic acid, other traces
	N H_2SO_4 (0·2–4 hr)	Ellagic acid, glucose
D-2 (pedunculagin)	Cold water (1 month)	D-1, D-x, (?D-5b), ellagic acid
	Room-temp. water (2 weeks)	D-1, D-x (?D-5b), ellagic acid
	Hot water (0·1–24 hr)	D-1, D-17, D-z (?D-7), ellagic acid, polymers
	N H_2SO_4 (0·2–4 hr)	D-1, D-x (?D-5b), ellagic acid, glucose polymers
Corilagin	Room-temp. water (2 weeks)	Gallic acid, 3,6-HHDP-glucose
	Hot water (0·7–10 hr)	Gallic acid (0·7 hr), 3,6-HHDP-glucose, ellagic acid (3 hr)
	N H_2SO_4 (5–30 min)	Gallic acid (5 min), 3,6-HHDP-glucose (gone 8 hr) ellagic acid

* In order of decreasing stability.

† The times are respectively those at which the first hydrolysis products are visible on PC and at which the original spot is no longer visible; or a single time of complete hydrolysis.

‡ Numbered and named spots identified by co-chromatography; lettered spots indicate as yet unidentified degradation products seen on chromatograms. No attempt was made to detect glucose as a product of the hot-water reactions.

Fig. 1) and glucose, but D-3 has a galloyl group in addition; *b.* with all compounds, except D-4, a series of intermediate products of varying stability are formed; and *c.* the compounds vary considerably in their stability to water at various temperatures. Some of the intermediates were identified or postulated by PC methods as components of the original extractives; others (identified merely by letter in Table 2) are listed merely to show the number seen. The compound most sensitive to hydrolysis was D-2 (pedunculagin, a di-HHDP-glucose). It was

TABLE 3. NUCLEAR MAGNETIC RESONANCE SPECTRAL DATA AND ASSIGNMENTS*

	Aromatic hydrogens						Glucose hydrogens							Spacings of glucose hydrogens (Hz)								
	HHDP hydrogens on glucose					Gallic																
Compound	1	2	3	4	6	1	H-1	H-2	H-3	H-4	H-5	H-6	H-6′	$J_{1,2}$	$J_{2,3}$	$J_{3,4}$	$J_{4,5}$	$J_{5,6}$	$J_{5,6'}$	$J_{6,6'}$	Other	Postulated conformation
D-1 (2,3-HHDP-glucose)		6·66	6·56				5·34	4·94	4·65	(all between 3·50–3·90)				0	8	10					$J_{1,5}=2$	3B
D-4 (4,6-HHDP-glucose)				6·66	6·56		α5·16	3·51	†	4·73‡	4·34‡	5·08‡	3·78‡	4	9	10	10	6	0	13		C1
							β4·55	3·29	†	4·78‡	†	5·10‡	3·70‡	8	9	9	9	6	0	12		
D-2 (pedunculagin)	6·65	6·58	6·53		6·48		6·30	5·44	~5·30	4·16§	4·80	¶	3·80**	0	2	7	9	8	0	13		B1
										4·56‖	††	††	3·75**									
D-13 (in DMSO)	(6·48,	6·36,	?6·34)				?6·34															
D-3	(6·73,	6·51,	6·44)			(7·08)	~5·33	~5·33	~5·33	5·59	4·65	4·74	4·00				4			13		B3
(in DMSO)	(6·60,	6·32,	6·26)			(6·96)																
Corilagin††			6·81		6·67	7·09	6·35	4·06	4·83	4·42	4·54	5·00	4·08	0	2	0	0	0	8	11	$J_{2,4}=4$	B3 (or 1C)

* The δ values had been measured in acetone-d_6 solutions unless noted. Unassigned shifts are given in parentheses.
† In multiplet between 3·50 and 4·00 ppm.
‡ Assignment of these shifts to α and β epimers is based on a newly determined NMR spectrum of pure 4,6-(HHDP)-β-glucose in acetone-d_6 by Jochims and Schmidt (personal communication) which at first showed only one set of signals; after slow epimerization in solution, both sets were present.
§ Integrates for ½H; assigned to folded form.
‖ Integrates for ½H; assigned to extended form.
¶ In multiplet 4·90–5·30 ppm.
** Together integrate for 1H.
†† OH-2 shift at 4·80 ppm before adding D_2O, $J_{2\text{-OH},2}=>1$.

converted to D-1 when an aqueous solution of it stood in the refrigerator and resembled corilagin (1-galloyl-3,6-HHDP-glucose, see Fig. 1) in its instability in water solution. D-4 and D-13 were stable in water at room temperature but not in hot water, while D-1 and D-3 required heating in water for several hours before degradation products appeared.

By NMR spectra. The NMR spectra of the ellagitannins (Fig. 2) were generally different in the aromatic region (8·0–6·5 ppm in acetone-d_6) and characteristic in the pattern of the shifts of the glucose hydrogens (6·4–3·0 ppm). The aromatic region gave clear-cut evidence for the number of galloyl groups by two proton singlets and for the number of HHDP groups by the slightly upfield pairs of one-proton singlets. These shifts (Table 3) agreed in general with those recently published by Haslam[13,14] and by Schmidt and co-workers.[15] The glucose hydrogens showed either a sharp pattern (as D-4) or a more or less envelope pattern. Table 3 presents the partial analyses of this region of the spectra based on the magnitude of the shifts, integration, and decoupling studies, interpreted by reference to published work on acetylated sugars by Lemieux's group,[16–19] Perlin,[20] Arndt,[21] and Tulloch and Hill,[22] and on ellagitannins by Schmidt and co-workers.[15] Whether or not the anomeric hydroxyl was aroylated could generally be determined by a prominent downfield signal over 6·00 ppm for H-1, for the so-called "aroylated H-1" or under 5·40 for the "non-aroylated H-1".

Identification of Ellagitannins

D-1 was shown to be a mono-HHDP derivative of glucose. Since no "aroylated H-1" signal appeared in the NMR spectrum and since the compound was also derivable by hydrolysis from pedunculagin (D-2) (Table 2), it was compared chromatographically and found to be identical with the cleavage product from a tannase hydrolysis of pedunculagin.[23] This cleavage product is 2,3-(hexahydroxydiphenoyl)-glucose.[23]

D-2 was shown to be a di-HHDP-glucose. When co-chromatographed with known ellagitannins, it resembled both pedunculagin (a di-HHDP- glucose from the galls of *Quercus pedunculata*[23]) and juglanin (a compound from walnut pellicles reputed to be a monogalloyl-mono-HHDP-glucose[8]). Similar NMR spectra from all three samples proved their identity. The spectrum of the juglanin sample indicated that the galloyl residue was present in far less than an equimolar amount and it was probably derived from the impurities seen on PC. Therefore, the earlier report of the presence of juglanin in *E. delegatensis*[2] should be corrected to read pedunculagin. For comments on structure see "Discussion".

D-4 was shown to be isomeric with D-1, with similar aroyl hydrogen signals in its NMR spectrum but with a very different pattern of glucose hydrogens. Comparison with the published NMR shifts of 4,6-(hexahydroxydiphenoyl)-glucose[15] suggested their identity; this was proven by PC with, and an NMR spectrum of, an authentic sample. Note that this

[13] E. HASLAM, *J. Chem. Soc.* (C), 1734 (1967).
[14] E. HASLAM and M. UDDIN, *J. Chem. Soc.* (C), 2381 (1967).
[15] J. C. JOCHIMS, G. TAIGEL and O. T. SCHMIDT, *Ann.* **717**, 169 (1968).
[16] R. U. LEMIEUX, R. K. KULLNIG, H. J. BERNSTEIN and W. G. SCHNEIDER, *J. Am. Chem. Soc.* **80**, 6098 (1958).
[17] R. U. LEMIEUX and J. D. STEVENS, *Can. J. Chem.* **43**, 2059 (1965).
[18] R. U. LEMIEUX and A. R. MORGAN, *Can. J. Chem.* **43**, 2205 (1965).
[19] R. U. LEMIEUX and J. D. STEVENS, *Can. J. Chem.* **44**, 249 (1966).
[20] A. S. PERLIN, *Can. J. Chem.* **44**, 539 (1966).
[21] O. ARNDT, *Ann.* **695**, 175 (1966).
[22] A. P. TULLOCH and A. HILL, *Can. J. Chem.* **46**, 2485 (1968).
[23] O. T. SCHMIDT, L. WÜRTELE and A. HARRÉUS, *Ann.* **690**, 150 (1965).

compound from both sources exists as a mixture of α and β anomers, which fact increases the complexity of the NMR spectrum.

D-3, D-6, and D-13 are not identical with any known ellagitannins, and their structures have not been completely deduced. See "Discussion".

Ellagic and gallic acids were observed by PC to be present in considerable amounts in the extractives of *E. delegatensis*.

DISCUSSION

Refinement of chromatographic techniques and the development of weakly basic sprays which produced characteristic colours with each component revealed a larger number of ellagitannins in *Eucalyptus delegatensis* wood than was previously shown. The twenty-one compounds discovered in various extracts plus several additional hydrolysis products suggest that great complexity exists in the combinations of glucose, 4,4',5,5',6,6'-hexahydroxydiphenic acid (HHDP), and in some cases gallic acid. Whether some of this is due to the presence of still other acid residues derived from HHDP acid by degradation, such as Schmidt and co-workers found with the Myrobalan and Algarobilla tannins,[24] has not yet been determined. Models show that a bridging HHDP group, while restricted to certain positions of attachment for any given conformation of glucose, might stabilize less-stable conformations (witness the variety of conformations for ellagitannins recently proposed by Jochims, Taigel and Schmidt[15]).

Application of modern methods has greatly facilitated separating and identifying the ellagitannins. Fractionating the mixtures by Sephadex, freeze-drying the eluates, and studying the amorphous, but chromatographically pure products by NMR, subjected the labile compounds to very mild and non-destructive conditions. The fractionation by Sephadex depends on its adsorptive powers, not on gel filtration. The smaller molecules eluted first; this phenomenon has been noticed recently for other phenolics.[25] As might be expected from the R_f values in 6 per cent acetic acid (PC), D-3 was the most strongly adsorbed, and the mono-HHDP compounds, D-1 and D-4, eluted first. The intermediate compounds have been far more difficult to isolate in pure form since a large number exist with very similar adsorptivity.

The ease with which the ellagitannins are hydrolysed by water alone, some even with ice-cold water, emphasizes the care with which they must be handled in the laboratory. The least-stable compounds have an esterified anomeric hydroxyl. The presence of relatively large amounts of the most labile D-2 in all the extracts studied, particularly in the Asplund liquor, is remarkable. D-3 and D-13, when hydrolysed, produced much more polymeric material than D-1, D-2, D-4, and corilagin. Therefore this material does not arise principally from the simple constituent parts (glucose, gallic, ellagic and HHDP acids) but from some active intermediate.

NMR spectra have been very useful but not the complete answer in the study of ellagitannins. They are more satisfactory than analyses for determining the constituent parts of an unknown. Used alone, however, they cannot solve new structures at present although with increased data this situation may improve. For example, correlation of NMR shifts associated with the aryl hydrogens of the HHDP group with the position of the esterified hydroxyls in the glucose molecule (such as was postulated by Haslam for the gallotannins[13])

[24] O. T. SCHMIDT and co-workers, *Ann.* **706**, 131, 154, 169, 180, 187, 198, 204 (1967).
[25] J. B. WOOF and J. S. PIERCE, *J. Chromatog.* **28**, 94 (1967).

from our data and from that recently published by Schmidt and co-workers[15] has been impossible. D-1 and D-4 illustrate the situation well since the HHDP hydrogens show identical

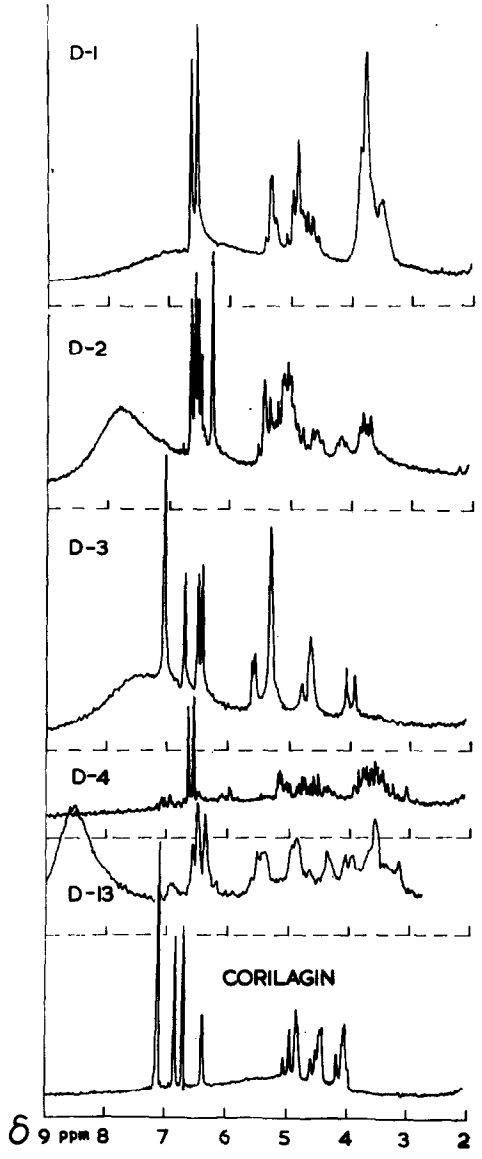

FIG. 2. NMR SPECTRA OF ELLAGITANNINS (TMS $\delta = 0$).

D-1, D-2, D-3, D-4 in acetone-d_6, D-13 in dimethylsulphoxide-d_6 after nine scans, corilagin in acetone-d_6 and D_2O.

shifts although the position of esterification and the probable conformations of the glucose are quite different. The shifts of the hydrogens of the bridging HHDP groups always appear upfield from the galloyl hydrogens and Dreiding models clearly show the reasons: 1, possible decreased deshielding by the benzoyl system due to lack of planarity between the phenyl and

carbonyl groups; and 2, increased shielding when the aryl hydrogen is situated more or less above a carbonyl plane. The same two effects, coupled with different conformations of the glucose core, account for the twinned signals produced by the HHDP groups; due to adjustments to restraints placed on the molecule by the diphenyl bridge, the aryl hydrogens of even a single HHDP group are seldom in similar environments. The position assumed by the HHDP bridge probably favours planarity or near planarity of one or both of the two aryl ester systems.[26,27] As an example, a Dreiding model of D-1 shows that in either the C1 or 3B conformations (Fig. 3) *trans*-2,3-di-equatorial links of the HHDP bridge allow both ester systems to be nearly planar. Thus, too many interacting factors prevent a strict relationship between shift and position of aroylation and limit structural deductions from NMR data.

Similar interactions prevent easy identification in ellagitannins of the positions of the glucose hydrogens and their attachments to carbon atoms bearing free hydroxyl or esterified groups, although in general the assignments made show some regularity. Decoupling and consideration of the magnitude of the coupling constants (or rather observed spacings[19]) in relation to the conformation of the pyranose ring, as applied by Schmidt and co-workers,[15] was most helpful in interpreting the spectra in this region.

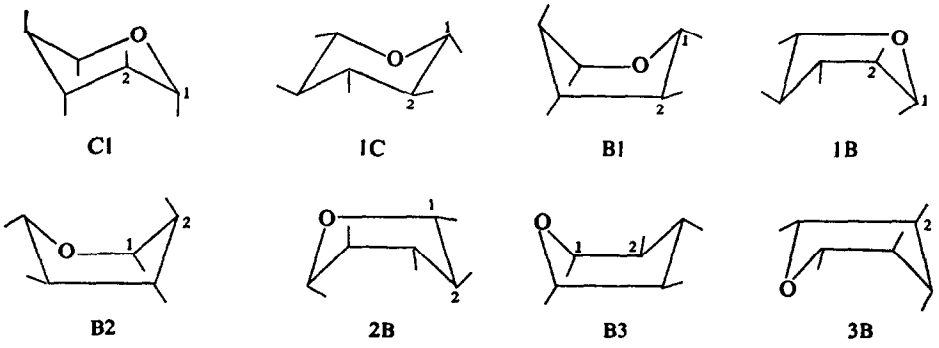

FIG. 3. CONFORMERS OF GLUCOSE.

The striking difference between the NMR spectra of D-4 and the remainder suggests a fundamental structural difference. D-4 appears to exist in the C1 conformation with a rigid pyranose ring with axial hydrogens which give sharp signals, while the more-or-less envelope spectra of the others denote more labile molecules.[28] This suggests B conformations. That the solvent can make a difference in the preferred conformation is illustrated by corilagin. In dimethylsulphoxide-d_6 (DMSO) it exhibits a 1B⇄B3 (or 1C) conformation[15] (Fig. 3) with $J_{1,2} = 6{\cdot}8$ Hz while in acetone-d_6 a slightly perturbed B3 (or 1C) conformation is indicated since H-1 gives a sharp singlet. [Note that brevilagin 2 with a 2B⇄B3 (or 1C) conformation shows a singlet H-1 signal[15].] It is also interesting that in acetone-d_6 corilagin showed a sharp signal for the glucose 2-hydroxyl hydrogen (as in DMSO where the 4-hydroxyl hydrogen is also discernible),[15] whereas no traces of similar signals were found for the other ellagitannins studied.

The published structure of pedunculagin is $\overline{2,3}$; $\overline{4,6}$-di-(HHDP)-glucose[23] but some of our data is not consistent with that conclusion. We wish to postulate that the original

[26] C. C. J. CULVENOR, *Tetrahedron Letters* 1091 (1966).
[27] A. M. MATHIESON, *Tetrahedron Letters* 4137 (1965).
[28] C. C. J. CULVENOR, personal communication.

compound is $\overline{1,6}$; $\overline{2,3}$-di-(HHDP)-glucose (Fig. 4) and in support of that view present the following evidence. Pedunculagin is hydrolysed by water, acid, or tannase[23] to 2,3-(HHDP)-glucose, the structure of which was proven by methylation experiments and is substantiated here by NMR spectrum (no signal above 5·34 ppm). The NMR spectrum with the sharp downfield signal at δ 6·30 (s, 1 H) and the extremely easy hydrolysis, both very similar to corilagin, point to aroylation of the anomeric hydroxyl group. With one exception (chebulinic acid, H-3 doublet)[15] no signal for any other "acetylated" or "unacylated" hydrogen has been found in the literature above 6·00 ppm in any solvent while most "acylated" H-1 signals fall below this value.[15,17–22] Aroylation of the 6-hydroxyl in combination with the anomeric hydroxyl instead of the 4-hydroxyl is sterically much more possible although it has not been previously reported among the ellagitannins. Schmidt and co-workers[23] have methylated pedunculagin and prepared the 1 methyl glucosides. However their difficulties in methylation[23] are in contrast to the reported ease of methylation of the anomeric hydroxyl of glucose by diazomethane.[29] In addition pedunculagin gave only a very weak aniline phthalate test.[23] The question arises as to whether acyl migration occurred during methylation.

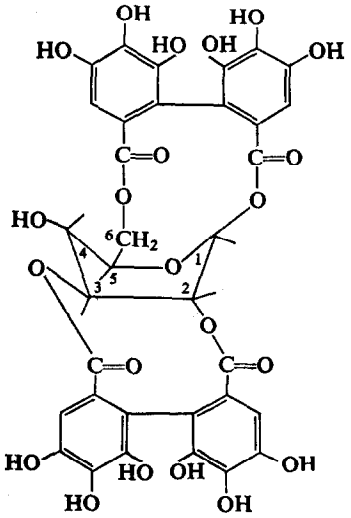

FIG. 4. PROPOSED STRUCTURE FOR PEDUNCULAGIN WITH A GLUCOSE B1 CONFORMATION.

Another structural problem arose in interpreting the NMR spectrum of D-2 because of the two unsharp triplets (or quartets) at δ 4·16 and 4·56, each representing ½H. In addition the upfield multiplet near δ 3·7 (equivalent to 1 H) analysed on decoupling as two overlapping doublets. The spectra obtained at temperatures up to 85° did not show any significant changes in these regions. We suggest the following interpretation (Fig. 5). With a B1 conformation of the sugar, the $\overline{1,6}$-HHDP bridge exists in two relatively stable positions. In the extended form the 6-benzoyl moiety of the bridge extends linearly away from the glucose ring. In the folded form this same group lies above the plane of the ring so that the phenyl ring strongly shields the "flagpole" H-4; an upfield shift of 0·40 ppm results. Since H-1 is a sharp singlet, the position in space of the 1-benzoyl moiety of the $\overline{1,6}$-bridge must be essentially the same in both forms or the effects on H-1 of the change must cancel. Models show that the 6-benzoyl moiety may be flipped over with little change in the position of the 1-benzoyl

[29] R. KUHN and H. H. BAER, *Chem. Ber.* **86**, 724 (1953).

moiety. The multiplet near 3·7 ppm can be interpreted as two doublet signals of H-6′ in the folded and extended forms, coupled only to the appropriate H-6, not to H-5. This B1 conformation contrasts with the C1 conformation with an α configuration proposed earlier for acetylated pedunculagin;[15] the latter seems to be a less probable interpretation of the data on the unacetylated material. Further study of D-2 is required.

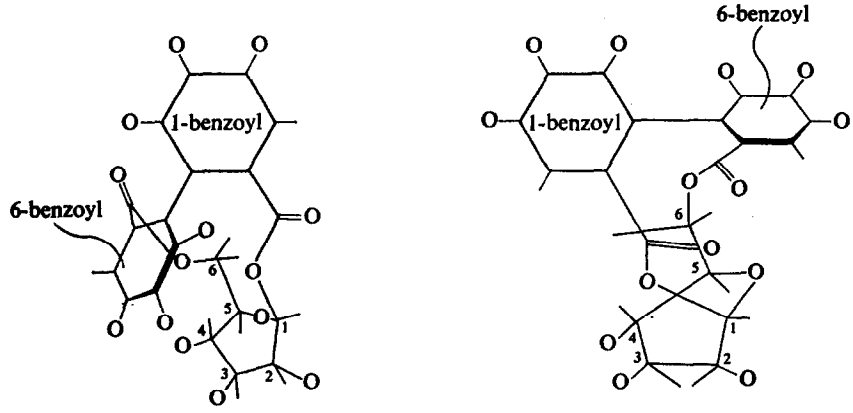

FIG. 5. TWO POSITIONS FOR THE PROPOSED $\overline{1,6}$-BRIDGE IN PEDUNCULAGIN: *a*, FOLDED, *b*, EXTENDED (DRAWN FROM PHOTOGRAPHS). THE HYDROGENS HAVE NOT BEEN SHOWN.

The structure and even the component parts of D-3 are uncertain. Simple hydrolysis revealed only the final products glucose, gallic, and ellagic acid, and NMR spectra show their equimolar ratio. On the other hand the low R_f value in dilute acetic acid, the one hydrogen singlet at 6·73 ppm in the NMR spectrum, and in the i.r. spectrum the prominent shoulder at 1714 cm^{-1} in the carboxylic acid or ester region and the extra band at 1089 cm^{-1} in the alcoholic hydroxyl region (neither seen in the very similar spectra of D-1 and D-2) suggest a heavier molecule. An aroyl residue such as the degradation product of HHDP found in chebulinic acid[14] would satisfy the spectral data. Preliminary chromatographic studies failed to find chebulic acid or any other acid, but work is continuing. From another approach, the hydrolysis and NMR results indicate an unesterified anomeric hydroxyl group, and one galloyl and one HHDP would esterify three of the remaining four hydroxyls of glucose. That leaves only one position open for an extra (unidentified) acyl residue. Perhaps an extra HHDP residue links two aroylated glucose molecules as shown in Fig. 6. The magnitude of the shift of the extra hydrogen (6·73 ppm) fits this idea slightly better as the chebulic-related residues show the extra hydrogen signal over 7·00 ppm in chebulic and neochebulinic acid.[14]

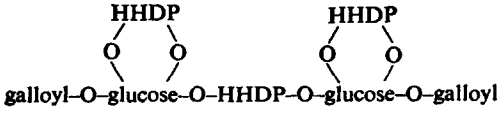

FIG. 6.

D-13 appeared to be a different type of ellagitannin, namely one with several glucose residues to one HHDP group. Integration of the NMR spectrum suggested a 3:1 ratio, but

this is unreliable. No coupling was found, so the observed broad pairs (Fig. 2) may be slightly offset signals from two or three different glucose residues. Decreased solubility in acetone supports this interpretation.

In conclusion, *E. delegatensis* wood has been found to contain simpler ellagitannins than have been isolated elsewhere, namely the two isomeric mono-HHDP-glucoses as well as the recently discovered[23] di-HHDP derivative pedunculagin. In addition a mixture of related compounds and hydrolysis products are present.

EXPERIMENTAL*

1. *Paper Chromatographic Examination (PC)*

Whatman No. 2 chromatographic paper was used. The two principal developing solvents were 6% acetic acid (6HA) and 1-butanol–27% acetic acid (1:1, BAW). Since examination showed that a saturated mixture of BAW was requisite for good separation of the slow-running ellagitannins, water was added dropwise to ensure saturation of the equi-volume mixture. The best definition of crude mixtures was frequently obtained by preparing two-dimensional chromatograms, first with 6HA then BAW. Generally the solvents were used in reverse order. Two-dimensional co-chromatograms were prepared by offsetting known and unknown spots by a short distance. The same solvents were used routinely for one-dimensional chromatograms and co-chromatograms. Other solvents for co-chromatographic identification were: ethyl acetate–acetic acid–water (3:1:1); benzyl alcohol–*tert*-butyl alcohol–*iso*propyl alcohol–water (3:1:1:1) with the addition of 1·8% (w/v) of 99% HCOOH;[30] 1-butanol–ethanol–water (4:1:5 and homogeneous 2:1:2·5); and 3% NaCl solution. Sugars were examined with 1-butanol–pyridine–water (10:3:3).

The chromatograms were viewed under short-wave u.v. light (254 nm). Chromogenic sprays were: $FeCl_3$ (1%)–$K_3Fe(CN)_6$ (0·3%) (1:3) for detecting weak spots; a "base mixture" of saturated $NaHCO_3$–20% Na_2CO_3 (2:1, pH 9·5–10) for characterizing ellagitannins; freshly prepared saturated aq. KIO_3 for galloyl and HHDP esters and gallic acid;[31] $SbCl_3$ (2% in $CHCl_3$) for ellagic acid;[32] Gibbs' reagent (0·1% methanolic 2,6-dichloroquinone-4-chloroimide) oversprayed with lead subacetate (basic lead acetate) solution[33] diluted with methanol (1:5); $ArN_2^+A^-$, oversprayed with 20% Na_2CO_3 for detecting other polyphenolics in fresh extractives, namely, pNA (fresh 0·1% stabilized diazotized *p*-nitroaniline) and dSA (0·4% sulfanilic acid, 30% H_2SO_4, 0·34% $NaNO_2$ (10:1:2·2)); aniline phthalate for sugars.

2. *Materials Examined*

a. Ethanolic extractives. Chips of undried *Eucalyptus delegatensis* heartwood were extracted by repeatedly soaking in EtOH at room temp.; the extract was concentrated *in vacuo* at 40° and then freeze-dried. Heartwood that had been air-dried for several months yielded (with aqueous acetone) the same constituents in the same proportion except for an increase in polymeric material.

b. Crude ellagitannins. A conc. MeOH extract of fresh heartwood was filtered and the aqueous filtrate extracted with ethyl acetate for 1 month in a liquid–liquid extractor with frequent changes of solvent. Evaporation of the extract yielded the crude ellagitannins which were stored for several years.

c. Cold pressed liquor.† Fresh heartwood chips were pressed at room temp. and the expressed liquor stored at 4° for 6 weeks and then freeze-dried.

d. Liquor from Asplund defibrator.† The expressed liquor was collected from the defibrator when steam-heated chips of *E. delegatensis* were being treated before the preparation of fibre board. It was stored and dried as in *c*.

3. *Column Chromatography on Sephadex*

The following grades of Sephadex (Pharmacia Fine Chemicals AB, Sweden) were used as shown:

* HHDP = hexahydroxydiphenoyl.

† Kindly provided by Mr. C. H. Turner, Assoc. Pulp Paper Manuf., Burnie, Tasmania.

[30] D. E. HATHWAY, *Biochem. J.* **67**, 445 (1957).
[31] E. HASLAM, *Chemistry of Vegetable Tannins*, p. 95, Academic Press, London (1966).
[32] D. J. SESSA and J. J. RACKIS, *J. Chromatog.* **23**, 487 (1966).
[33] C. S. GIBSON and E. MATTHEWS, *J. Chem. Soc.* 596 (1928).

Type	*Application*
G-50	Preliminary separation of monomeric ellagitannins from polymeric material; D-1 and D-4 often separated from others.
G-25 (fine)	*a.* Separation of three groups of ellagitannins, namely the fast-running D-1 and D-4, the intermediate materials D-2, D-13 and similar, and the slow-running D-3. *b.* Separation of D-2 and similar materials. *c.* Purification of D-3 on short columns (10–20 cm). *d.* Purification of D-2, D-6 and D-13 on long columns (26–33 cm).
G-15	Separation of D-1 in fresh ethanolic extractives from the other ellagitannins (which remain adsorbed) and from other types of polyphenolics (visualized by diazonium sprays).
G-10	*a.* Separation of D-1 and D-4 on long columns (40–55 cm). *b.* Purification of D-1 and D-4 on long columns.

The special Sephadex columns had diameters of 0·9 and 2·5 cm. The adsorbent was prepared essentially as recommended by the supplier. It was recovered after each use by washing out the residual adsorbed material with 0·02 N NaOH followed by H_2O, very dilute HCl, and H_2O until neutral (an operation completed in 1 day); it was re-used repeatedly.

For most separation on G-25 or G-50, 10–20 mg of sample/ml of swollen adsorbent gave the best results although as much as 30 mg/ml was used. On G-15 100 mg/ml of the crude ethanolic extractives (10 g on a large column) were quite well separated in a very short time. On G-10, good separation of D-1 from D-2 and low-molecular-weight phenolics was obtained with a loading of 40–60 mg/ml. Separation of D-1 from D-4 was only partial even with loadings as low as 2·8–13 mg/ml.

The material for application to the column was dissolved in water (10–20 ml/g), allowed to stand for an hour, and any precipitated ellagic acid removed and weighed. Toward the end of the study the dark polymeric materials insoluble in dry acetone were removed before solution in water. The columns were developed and eluted with water, the eluates examined by PC, and the combined fractions freeze-dried. The total recovery from purified materials was 75–95%. Ellagic acid, which contaminated all fractions at first, was slowly removed during repeated column chromatography.

4. *Additional Methods of Purification*

Attempts to crystallize the ellagitannins failed. The purified freeze-dried samples were treated with small volumes of dried, MeOH-free spectraquality acetone (Mallinkrodt), and dark insoluble material removed. The soluble material was refreeze-dried from water. D-3 and D-13, however, retained some colour. The D-1 samples obtained from the crude ethanolic extractives and eluted from G-10 Sephadex was contaminated with an artefact.* The latter was removed from concentrated aqueous solutions of the sample by extracting ten times with large (2–3 ×) volumes of ethyl acetate, but some D-1 was lost.

5. *Spectra*

The NMR spectra were determined with a Varian HA-100 spectrophotometer, with acetone-d_6 as the routine solvent. Addition of D_2O yielded little extra information because of the massive spreading H_2O signal. Integration and decoupling experiments were also done. Dimethylsulfoxide-d_6 (DMSO) was used as the solvent in an unsuccessful attempt to reveal the alcoholic hydroxyls of the sugar core of D-3, but it was not used routinely because of difficult recovery.

I.r. spectra were run on a Perkin-Elmer 457 Grating Infrared Spectrophotometer.

6. *Hydrolysis of Ellagitannins*

a. Methods. The following reagents and conditions were employed:

Cold water. 0·5–2·0% aqueous solutions of ellagitannins were kept in a refrigerator (0–5°) for several months.

Room-temp. water. Aqueous solutions of various strengths were kept in stoppered containers in the laboratory (15–30°) for 1–3 weeks.

Hot water. 0·5–1·0% solutions were heated, sometimes intermittently, on a steam bath (90–95°) in closed tubes at constant volume for many hours.

N Sulfuric acid. 1·0% solutions in acid were heated as above for several hours.

Tannase. 5 mg of D-3 in 0·8 ml H_2O was treated at room temp. successively with 0·4, 0·2, and 0·1 ml of tannase prepared by growing *Aspergillus niger* on tannic acid.[34,35]

* Tannins from oak and eucalypts, when treated with cold alcoholic solvents, both produce a similar artefact which gives a YO color with pNA. The oak artefact has been identified as resorcinol.

[34] L. KNUDSON, *J. Biol. Chem.* **14**, 159 (1913).
[35] E. HASLAM, personal communication.

b. Analysis. Time studies were carried out at appropriate intervals (from 5 min to several days) by analysing the reaction mixtures by PC, with 6HA routinely and when necessary with BAW. Frequently the complex mixture of products required two-dimensional PC. Final products and stable intermediates were identified by co-chromatography in several solvents.

c. Results. See Table 2.

7. Characterization of Individual Ellagitannins

D-1; 2,3-(4,4',5,5',6,6'-*hexahydroxydiphenoyl*)-*glucose*. The freeze-dried powder was colourless and gave only faintly yellow solutions. Its i.r. spectrum (KCl disc) showed peaks at 3380s, 1738s, 1615s, 1511w, 1435m, 1350–1305 (plateau), 1230s, 1185s, 1160–1135 (shoulder), 1060–1080 (sh), 1040s 970–950 (sh), 875w, 830w, 735w cm^{-1} and in DMSO a single C=O peak at 1742 cm^{-1}; both spectra were very similar to those of D-2. Its NMR spectrum is given in Fig. 2 and Table 3. A solution in DMSO became red on the surface in 2 hr and completely red in 1 day. The compound had R_f values and colour reactions identical with those of 2,3-HHDP-glucose (kindly provided by O. T. Schmidt).

D-2: *Pedunculagin*. The freeze-dried powder was colourless and gave only faintly yellow solutions. Its i.r. spectrum (KCl disc) showed no significant difference from that of D-1 (see above), and in DMSO its single C=O peak was at 1747 cm^{-1}. Its NMR spectrum is given in Fig. 2 and Table 3. A solution in DMSO became red only on the surface in 1 day. The compound had the same R_f values, colour reactions, and NMR spectrum as pedunculagin[23] (kindly provided by O.T. Schmidt).

Ellagitannin D-3. The best samples were still faintly tan when freeze-dried and gave brown solutions in acetone. Its i.r. spectrum (KCl disc) showed peaks at 3380s, 1725m, 1615m, 1510–1480 (plateau), 1445m, 1350–1305 (plateau), 1220–1200 (sh), 1175s, 1089m, 1040m, 985–970 (sh), 865w, 760w cm^{-1}. In DMSO the single C=O peak at 1739 had a strong shoulder around 1714 cm^{-1}. Its NMR spectrum is given in Fig. 2 and Table 3. A solution in DMSO became weak brown in 1 day.

D-4: 4,6-(4,4',5,5',6,6'-*Hexahydroxydiphenoyl*)-*glucose*. Passage through several columns failed to separate this minor constituent completely from tannin and non-tannin impurities, although it was obtained colourless. Its NMR spectrum is shown in Fig. 2 and Table 3. The compound gave the same R_f values, colour reactions, and NMR spectrum as 4,6-HHDP-glucose, kindly provided by O. T. Schmidt.

Ellagitannin D-6. This compound, isolated from the Asplund liquor, could not be separated completely from D-2, D-8, D-9, and D-12 by Sephadex.

Ellagitannin D-13. This compound, isolated from the Asplund liquor, was freed from gallic acid by extraction with ethyl acetate, but despite repeated column chromatography some color remained and one fraction was slightly contaminated with D-12, another with D-2 and D-8. Its i.r. spectrum (KBr disc) showed peaks at 3400s, 1735m, 1614m, 1500w, 1445m, 1314m, 1176s, 1040m, 1000m, 895w, 858w, 820w, 775w, 548w. Its NMR spectrum is given in Fig. 2 and Table 3. This compound is much less soluble in acetone than the others.

Acknowledgements—We thank Professor O. T. Schmidt and Dr. L. Jurd for samples of ellagitannins, Dr. J. C. Jochims for new work on the NMR spectrum of 4,6-(HHDP)-glucose, Dr. A. J. Michell (Division of Forest Products, C.S.I.R.O.) for the i.r. spectra, R. I. Willing (Chemical Research Laboratories, C.S.I.R.O.) and M. F. Wesolowski (Forest Products Laboratory, U.S.D.A.) for the NMR spectra, Marilyn J. Effland (Forest Products Laboratory, U.S.D.A.) for the sugar analyses, and Donald Lang and Andrew Rozsa for help with some of the experimental work.