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POLYPHENOLS ISOLATED FROM THE BARK OF *CASTANEA SATIVA* Mill. CHEMICAL STRUCTURES AND AUTO-ASSOCIATION

IN HONOUR OF PROFESSOR G. H. NEIL TOWERS 75TH BIRTHDAY

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Key Word Index—*Castanea sativa*; Fagaceae; chestnut; bark; ellagitannins; galloyl esters; phenol glycosides; hamamelitannins; intermolecular associations; NMR spectroscopy; mass spectrometry

Abstract—Eight phenolic compounds (castalin, castalagin, vescalagin, kurigalin, 5-*O*-galloylhamamelose, (3′, 5′-dimethoxy-4′-hydroxyphenol)-1-*O*-β-D-(6-*O*-galloyl)glucose, chestanin, and acutissimin A) were isolated from chestnut bark and their structures elucidated on the basis of spectroscopic analyses. Evidence for strong intermolecular associations of 5-*O*-galloylhamamelose is presented on the basis of NMR and mass spectroscopic data. © 1998 Elsevier Science Ltd. All rights reserved

INTRODUCTION

Most barks are rich in polyphenol extractives which contribute to protect trees against predators and pathogens. In many angiosperm and gymnosperm species, the main polyphenols found in bark are proanthocyanidins [1]. Examination of the bark of various Fagaceae spp. such as *Castanea sativa* [2], *Castanea crenata* [3, 4], *Quercus robur* [5], *Quercus petraea* [6], *Quercus acutissima* [7–9] and *Quercus stenophylla* [10–15] has led to the identification of various phenol glucosides and galloyl and hexahydroxydiphenoyl esters. Here we report the structural study of several ellagitannins, galloyl esters and phenol glycoside gallates from the bark of *Castanea sativa* Mill., one of the main European forest species. Evidence for autoassociation of a galloyl ester is also presented.

RESULTS AND DISCUSSION

The polyphenols from chestnut bark were extracted with aq. MeOH and the water-soluble materials were separated on a Sephadex LH20 column. Fractions presenting a major peak by analytical HPLC were further purified by preparative reversed-phase HPLC. Eight compounds were recovered (Table 1) and seven of them were identified by comparison of their ^{1}H and ^{13}C NMR and mass spectra with those of the literature. These compounds are castalin **1** [16], vescalagin **2**, castalagin **3** [17], acutissimin A **4** [8], kurigalin **6** [3], (3,5-dimethoxy-4-hydroxyphenol)-1-*O*-β-D-(6-*O*-galloyl)glucose **7** [7] and chestanin **8** [18]. Only partial assignments of the ^{1}H NMR spectra have been reported for compounds **6** and **7** which contain second order spin systems. The complete proton assignments were obtained through spectral simulations (Table 2).

The remaining compound **5** was obtained as a 40/60 mixture of two isomers. NMR spectroscopic data (Tables 2–3) showed that they contained one galloyl substituent and one sugar residue. Two narrow singlets were observed at 5.43 and 5.31 ppm for the anomeric protons of the carbohydrate moiety and the corresponding signals of the anomeric carbons resonated at high field (96.57 and 101.77 ppm) suggesting that the sugar residue was not glycosylated. From the homonuclear coupling constants, it could be seen that **5** also contained an -HC(OH)-HC(OH)-H_2C(OR) fragment and an isolated hydroxymethylene group (tight AB spin systems with no other coupling).

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Table 1. Phenolic compounds isolated from *Castanea sativa* Mill bark.

Compound	HPLC t_R	Name	Mol. wt.	Ref.
1	5.9	Castalin	632	16
2	7.9	Vescalagin	934	17
3	8.8	Castalagin	934	17
4	11.4	Acutissimin A	1206	8
5	11.5	5-*O*-Galloylhamamelose	332	4
6	14.0	Kurigalin	636	3
7	15.1	(3,5-Dimethoxy-4-hydroxyphenol)-1-*O*-*β*-D-(6′-*O*-galloyl)-glucoside	484	7
8	18.6	Chestanin	938	18

Table 2. 400.13 Mhz ^{1}H NMR spectroscopic data of compounds **5–7** extracted from chestnut bark referenced to Me_2CO-d_6 (δ 2.2 ppm; 1 drop of D_2O was added in the case of **5**).

Compounds	**5**		**6**		**7**
	α (^{3}J)‡	β (^{3}J)‡	α (^{3}J)‡	β (^{3}J)‡	β (^{3}J)‡
Carbohydrate					
1	5.43	5.31	5.44	5.47	5.04† (7.8)†
2					3.61† (9.3)†
3	4.10† (8.3)†	4.37† (7.8)†	4.09† (8.0)†	4.38† (7.5)†	3.69† (9.3)†
4	4.28† (2.9, 5.8)†	4.26† (3.3, 6.3)†	4.36† (3.0, 6.4)†	4.32† (3.2, 6.4)†	3.66† (9.7)†
5a	4.63† (11.7)†	4.60† (11.7)†	4.66† (12)†	4.62† (11.5)†	3.98† (1.9, 5.8)†
5b	4.405†	4.415†	4.43†	4.43†	
6a					4.79† (11.7)†
6b					4.54†
2′a	3.70 (11.2)	3.89 (10.8)	4.59 (11.5)	4.46 (12.4)	
2′b	3.69	3.86	4.56	4.40	
Galloyl					
2″, 6″	7.285 (×2)	7.31 (×2)	7.30,7.29,7.28,7.27§		7.28 (×2)
Aglycone					
2′, 6′					6.57 (×2)
Methyl					3.87 (×6)

† values confirmed by simulation with PANIC on AM400 Brüker.
‡ coupling constant in Hertz ($^3J_{(i,i+1)}$, $^3J_{(i,i+2)}$).
§ not assigned.

Selective long-range heteronuclear correlation spectra (INAPT [19]) acquired with selective excitation of the methylene protons of the former three-carbon fragment contained a single resonance corresponding to the carbonyl carbon of the galloyl moiety. This revealed that the aromatic substituent was attached to this methylene group. Two quaternary carbons were detected at 76.77 (major isomer) and 78.80 (minor isomer) ppm in the J-modulated ^{13}C spectrum. The INAPT spectrum obtained with selective excitation of the low-field anomeric proton contained a correlation with the highly-substituted carbon which resonated at 76.77 ppm. It could be concluded that the carbohydrate moiety was hamamelofuranose (a quaternary carbon at the 2-position substituted by both a hydroxymethyl group, C2′, and a OH group). Comparison of the ^{13}C and ^{1}H chemical shifts of **5** with that of **6** showed that the NMR data of the two compounds were analogous. The only major differences in the ^{1}H and ^{13}C spectra were the signals of H2′a and H2′b and the C2′ carbons which resonate at lower field in the case of kurigalin ($\Delta\delta_H$ +0.5–0.9 ppm; $\Delta\delta_C$ +2.5–3.3 ppm) due to the galloyl substituents at C2 and C2′. The ^{13}C chemical shifts of **5** were very similar to those

Table 3. 100.6 MHz carbon chemical shifts of compounds **5–8** extracted from chestnut bark referenced to Me_2CO-d_6 (δ_{Me} 28.1 ppm; 1 drop of D_2O was added to solubilize samples **5** and **8**).

Compound	**5**		**6**		**7**	**8**	
	α	β	α	β	β	β	
Carbohydrate							
1	96.57	101.77	96.76	100.69	101.29	105.62, 105.64	
2	76.77	78.80	75.49	78.48	73.27	72.57 (×2)	
3	70.16	71.33	71.12	72.31	75.66	76.35 (×2)	
4	78.27	79.30	78.09	79.45	69.26	68.45 (×2)	
5	63.20	64.79	63.64	64.92	72.69	75.21, 75.19	
6					62.97	59.79 (2)	
2′	62.44	62.27	65.09	65.55			
Gallic acid						**a**	**b**
1	119.94	120.07	119.3–119.9§		119.65	119.12*	113.36*
2	108.19	108.25	108.02, 108.08,108.15§		107.99	109.99*	141.54*
3	144.30	144.25	144.17, 144.26§		144.30	146.29*	135.21*
4	137.08	137.01	137.01–137.28§		137.13	138.34*	138.14*
5	144.30	144.25	†		144.30	144.62*	138.34*
6	108.19	108.25	‡		107.99	107.71*	105.74*
7	164.87	164.95	165.19, 165.30, 165.56§		165.26	164.82, 163.63§	
Aglycone							
1					149.84		
2, 6					94.43 (×2)		
3, 5					146.88 (×2)		
4					130.38		
Methyl					54.76 (×2)		
Phenolic group							
1						133.67	133.23
2, 6						106.19	105.98
3, 5						149.15	148.99
4						132.06	132.04
7						64.65	64.48

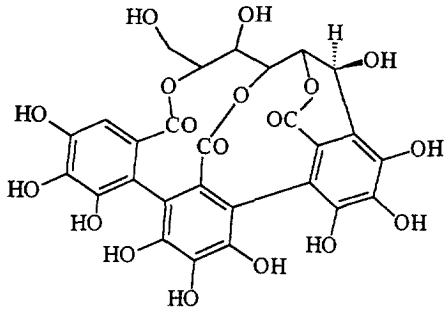
* assigned with empirical substituents increments of benzene derivatives.
† see values of 3 because of the symmetry of the galloyl.
‡ see values of 2 because of the symmetry of the galloyl.
§ not assigned.

reported for 5-*O*-galloylhamamelose [4] in the same solvent mixture (acetone-d_6 + D_2O) and the α and β furanosyl ring forms for the sugar residues of the major and minor isomers respectively, have been assigned in accordance with these data and those of **6**. A $^3T_2$ ring form for hamamelose in phenolic derivatives **5** and **6** could be proposed from the large $^3J_{H3,H4}$ coupling constants (7.5–8.3 Hz) using a Karplus-type equation optimized for carbohydrates [20].

On the basis of the NMR spectroscopic data it was very tempting to conclude that compound **5** was 5-*O*-galloylhamamelose. However, in the INAPT spectra acquired with selective excitation of the low-field anomeric proton, a long-range correlation between H1 (major isomer) and the meta carbon of the galloyl moiety (C3″, 144.30 ppm) was detected and this signal suggested that **5** contained a glycosidic linkage between the anomeric carbon and C3″ of the galloyl moiety. However, as stated previously, the chemical shifts of the anomeric carbons were not shifted to low field as would have been expected for a glycoside. Moreover, singlets integrating for two protons were observed for galloyl H2″ and H6″ (at 7.29 and 7.31 ppm for the α and β isomers, respectively) indicating that the phenolic moiety most likely possessed an axis of symmetry which was not compatible with substitution at C3″.

In an attempt to establish sugar-galloyl spatial proximities, a phase-sensitive NOESY spectrum was acquired with a 1s mixing time. Strong negative crosspeaks were observed for several sugar proton pairs (e.g., H5a/H5b and H3/H4) but no crosspeaks could be detected between the aromatic protons and the glycosyl ones. In order to optimize sensitivity, steady-state NOE difference spectra were also recorded but again only very weak effects were observed between the aromatic protons and the carbohydrate moiety.

The existence of a glycosidic linkage was definitively disproved by degradation of **5** with tannase which only gave gallic acid and hamamelose (see experimental section) and by the formation of tri-*O*-methylbenzoic acid as main product under degradation in



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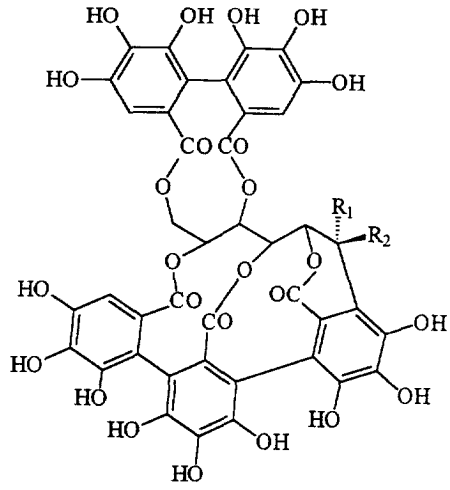
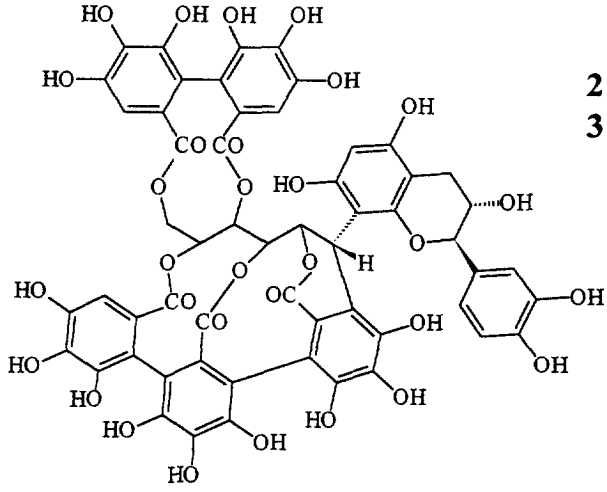


2 $R_1 = H, R_2 = OH$

3 $R_1 = OH, R_2 = H$



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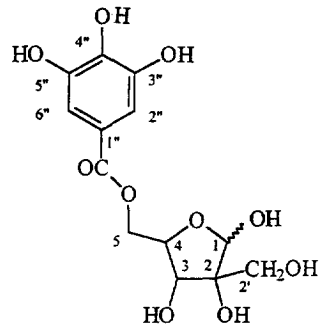

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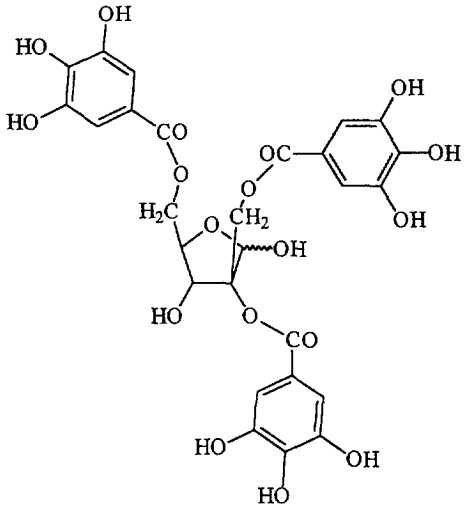
EtOH/water/TFA 1:6:3 (100°C, 1 hr) of the permethylated (diazomethane) compound. The heteronuclear long-range correlation between H1 and C3″ (meta carbon) of the galloyl moiety of 5-*O*-galloylhamamelose may well be an example of through-space coupling [21] which is quite common for nuclei other than hydrogen.

The electrospray-ionization (ESI) mass spectrum of compound **5** showed a major ion peak at *m/z* 355 corresponding to the cationized species $[M+Na]^+$. The molecular weight of 332 was also confirmed by the presence in the spectrum of an other ion peak, at *m/z* 687, which was attributed to the dimeric $[2M+Na]^+$ ion.

Further investigation of the ion $[M+Na]^+$ by high-energy collision-induced dissociation (CID) and tandem mass spectrometry (MS/MS) led to the observation of structurally significant diagnostic fragment



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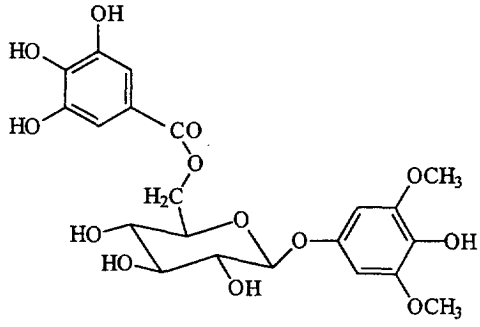

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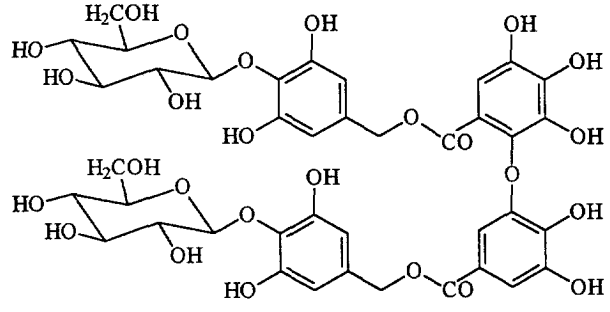

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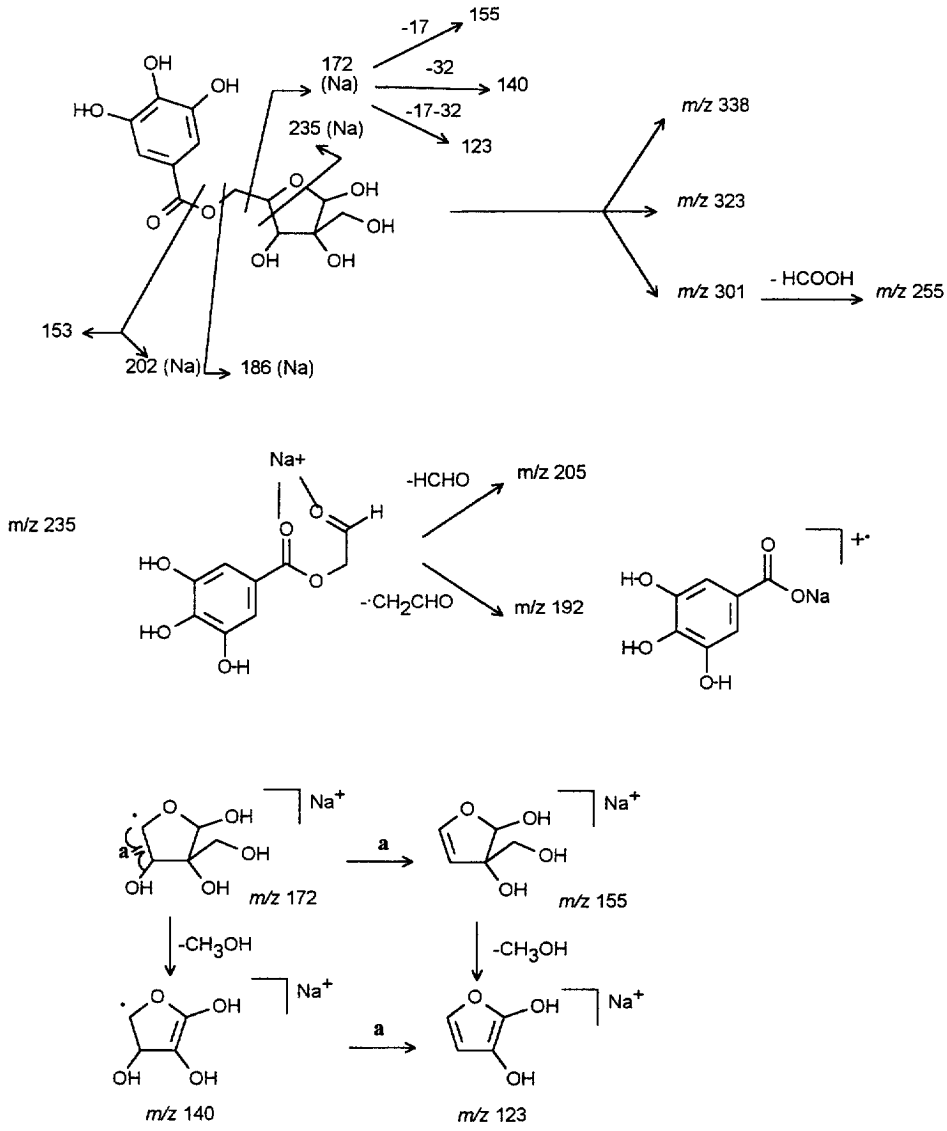
ions. Besides the loss of small neutral species from the precursor ions, such as OH, MeOH and MeONa, the ESI-MS/MS spectrum of $[5+Na]^+$ displayed several structurally significant ion peaks which were indicative of the galloyl moiety location on the hamamelose skeleton. In particular, a characteristic cleavage across the sugar ring allowed a fragment ion at *m/z* 235 incorporating the C5-O1 bond (Scheme 1). Successive decompositions of this ion and a series of fragments arising from consecutive dissociations of the hamamelose diagnostic fragment at *m/z* 172 (schema) unambiguously confirmed the 5-*O*-galloyl-hamamelose structure of **5**.

It is noteworthy that even-electron ion species were accompanied, in the MS/MS spectrum of $[5+Na]^+$ by odd-electron (radical) fragments, as exemplified by the ion peak at *m/z* 172. Such a feature, previously observed in the case of fatty acid derivatives [22], seems to be a characteristic of the high-energy collision-induced dissociation of precursor ions generated by electrospray ionization [22, 23].

As dimeric species were detected by electrospray mass spectrometry in case of compound **5** and of *β*-glucogallin, a galloyl ester of glucose [24], it appeared reasonable to question whether the signals observed in the ^1H and ^{13}C spectra of **5** corresponded to monomeric, dimeric or higher order auto-associated species.

Indeed, the NMR spectra of **5** evolved with time (a couple of months in acetone-d_6) as four new sets of signals appeared in the regions of the original ones (Figure 1). This transformation proved to be reversible as when the solvent (acetone-d_6) was evaporated and replaced with acetone-d_6 or MeOH-d_4 only the two original sets of signals corresponding to the *α* and *β* isomers were detected. As four out of the six signals might reflect an equilibrium between open-chain and furanosyl forms of hamamelose (the pyranose form could be neglected as O5 was substituted by the galloyl



Scheme 1. Fragmentation of the precursor ion $[5+Na]^+$ under high-energy CID conditions and tandem mass spectrometry.

group), a sample of commercially available hamamelitannin (2′,5-di-*O*-galloyl hamamelose) was also investigated under similar conditions. This sample which contained both α and β isomers as a 45/55 mixture did not give multiple signals upon standing. This infers an open-chain/furanosyl equilibrium for hamamelose. However, the hydroxyl protons, which were not initially observed due to fast exchange with the residual water, were detected after a couple of weeks. The signals originating from aliphatic hydroxyl protons were well-resolved doublets (for example $^3J_{OH1\alpha,H1\alpha}$ and $^3J_{OH1\beta,H1\beta}$ were 4.1 and 7.3 Hz, respectively) whereas the phenolic hydroxyl proton resonances were broad. This strongly suggests that hydrogen-bonded associated species were slowly formed. Exchange rates of hydrogen-bonded protons can be sufficiently slow to allow their detection even in aqueous solution.

Considering that the hamamelose ring of **5** is much less sterically hindered than that of hamamelitannin, it is not surprising that the associated species have different characteristics. Detection of six sets of chemical shifts in the NMR spectra of **5** may be due to the existence of monomeric,dimeric and higher order complexes or it may stem from the formation of tightly bound dimeric species whose chemical shifts are sensitive to the configuration at the anomeric center. It should be recalled that detection of strong negative intra-ring correlation peaks for the sugar protons in the homonuclear relaxation experiments indicated a fairly low molecular weight for the initial form of **5** compatible with rapid molecular tumbling.

Complexation of anthocyanins with other polyphenols has been extensively studied and is recognised as one of the major factors affecting the color of plant tissues or plant extracts [25]. Auto-association of other

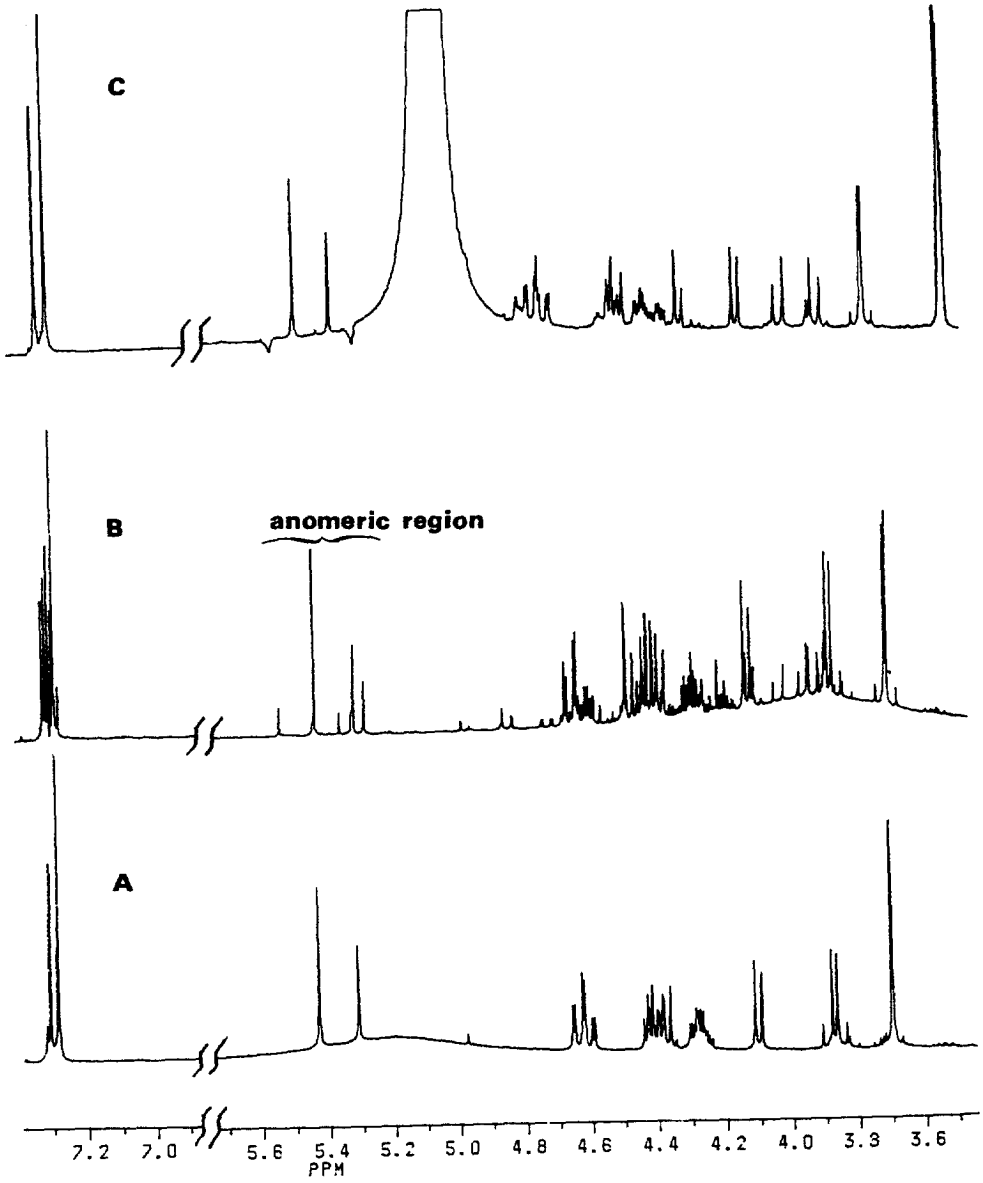


Fig. 1. 400 Mhz ^{1}H NMR spectra of 5-*O*-galloylhamamelose, **5**, recorded at different times: (A) in acetone-d_6 upon isolation, (B) in acetone-d_6 six months later and (C) same as B after removal of acetone-d_6 and addition of MeOH-d_4. The anomeric region has been indicated.

phenolic compounds has been comparatively poorly investigated although several authors have underlined its putative importance in biology or technology. Molecular weight values measured on chinese gallotannins by osmometry varies according to the solvent used [26]. Values were four times higher when measured in water as compared to acetone and increased with the tannin concentration, indicating the formation of complexes in concentrated solution. Auto-association of penta-*O*-galloyl-*β*-D-glucose was also evidenced by a dependence of aromatic proton chemical shifts upon concentration [27]. Calculation of the dissociation constants showed that the affinity of the polyphenol for itself was even stronger than that of the same polyphenol for a peptide. 1,3,6,8-Tetrahydroxyxanthylium chloride, a polyphenolic dye, was shown to form a non-covalent dimer in aqueous solution by UV-visible spectroscopy [28].

Such an auto-association of polyphenols explains some artefacts observed in reversed-phase HPLC analysis of ellagitannins [29] or flavonol glycosides: they give two sets of multiple peaks when injected as solutions in methanol or acetone which disappear when a sufficient amount of water in added to the solution. It may also explain the formation of highly concentrated solutions of tannins in water solutions,

a phenomenom early described as "mutual solubilisation" [30]. This may facilitate the accumulation in the vacuole of the high concentrations of polyphenols required for protecting the plant against stress, without damaging the plant cell [31, 32]. It may also limit rainwater leaching of polyphenols in protective tissues such as bark [33].

EXPERIMENTAL

Extraction and isolation of polyphenols. Chestnut (*Castanea sativa* Mill.) bark was harvested from a 50-year-old tree two months after the tree was felled. The air-dried bark, ground in a Retsch mill SM1 (particle size less than 60 mesh; 400 g) was extracted with MeOH/H_2O 4:1 at room temperature for 2 hrs. The extract, after removal of MeOH in a rotary evaporator, was fractionated successively into Et_2O (2.7 g), EtOAc (2.2 g) and H_2O (23.4 g) soluble frs. The H_2O soluble fr. (20 g) was chromatographed on a Sephadex LH20 (Pharmacia) column (39 cm × 50 mm i.d.) and successively eluted by 2 l H_2O, 4 l H_2O/EtOH (9:1), 2 l H_2O/EtOH (7:3) and 2 l H_2O/EtOH (5:5). Each fr. (50–200 ml) was analysed by high performance cellulose thin layer chromatography using 6% acetic acid and *s*-butanol/acetic acid/water (14:1:5) as eluents. Identical frs were pooled and their purity assessed by HPLC. The fractions were further purified by preparative HPLC (see infra). The following compounds were successively eluted: castalin (8 mg), 5-*O*-galloylhamamelose (12 mg) with H_2O; (3,5-dimethoxy-4-hydroxyphenol)-1-*O*-β-D-(6′-*O*-galloyl)glucoside (11.5 mg), vescalagin (45.6 mg) and kurigalin (5.6 mg) with H_2O/EtOH (9:1); castalagin (71.8 mg) and chestanin (4.8 mg) with 2 l H_2O/EtOH (7:3); acutissimin A (29.7 mg) with H_2O/EtOH (5:5). Vescalagin and castalagin were identified by comparison with authentic samples (HPLC and NMR spectra). The structures of the other compounds were established by NMR and mass spectroscopic analysis and comparison of the spectra with those previously published when available.

Degradation with tannase. Compound 5 (1 mg/ml in water, 1 ml) was treated by tannase (ICN Biomedicals Inc., 20,000 units/g, 2 mg/ml, 15 μl) at 37°C during 23 hrs. The enzyme was removed by ultrafiltration. Gallic acid was the only product observed by reversed-phase HPLC (detection: 280 nm). The products were further freeze-dried, solubilised in dioxane (0.5 ml), silylated with BSTFA (50 μl) and pyridine (5 μl) at room temperature during four hours and analysed by GC–MS on a fused silica capillary column coated with polydimethylsiloxane (30 m × 0.32 mm I.D., film thickness 0.25 μm, SE30 Supelco) with helium as a carrier gas (inlet pressure 0.6 bar) and with a temperature program from 140 to 210°C at 5°C/min. Detection was carried out with a Nermag R10-10B quadrupole spectrometer operating in the electron impact mode (70 eV). Five peaks were observed. One corresponds to the 4-O-TMS-derivative of gallic acid (tr 15.33 min.) and the remaining four were sugar derivatives (*m/z* 73, 103, 117, 129, 147, 191, 204, 207, 217, 319) originating from hamamelose (the same peaks with tr 11.26, 1.49, 12.03 and 13.08 min. were produced by hydrolysis of commercial hamamelitannin with tannase). It was also checked on rutin and dihydroquercetin-3′-O-glucoside that the tannase preparation contained no glycosidase activity.

Analytical HPLC. The Sephadex frs. were analysed on a Merck Lichrospher RP 18e (5 μm) column (25 cm × 4 mm i.d.) with the following eluents: solvent A, H_2O/H_3PO_4 (990:1), solvent B, MeOH, linear gradient from 0 to 90% solvent B in 30 min; flow rate: 1 ml.min^{-1}; detection: 280 nm.

Preparative HPLC. Samples (20–170 mg) dissolved in H_2O (2 ml) were chromatographed on a Hibar Lichrospher RP-18, 7 μm column (25 cm × 25 mm i.d.) (Merck) in an isocratic mode with the solvents used for analytical HPLC. Various percentages of solvent B (2–20% B) were used according to the polarity of the compound to be purified. The flow-rate was 15 ml/min and the detection was carried out at 280 nm. The collected fractions were concentrated under reduced pressure to eliminate methanol, and adsorbed on a Sephadex LH-20 column (5 cm × 1.5 cm i.d.) to eliminate phosphoric acid [29]. Pure compounds were eluted with H_2O, and dried under reduced pressure and in a freeze-drier.

Mass spectrometry. All compounds were subjected to chemical ionization (NH_3) on a quadrupole R10-10C Nermag spectrometer (70 eV). In the case of 5-*O*-galloylhamamelose, the absence of the expected $[M+H]^+$ ion peak prompted us to use electrospray ionization mass spectrometry. The mass spectrometer was a Zabspec-T five-sector tandem instrument (Micromass, Manchester, U.K.). The sample was dissolved in water at a concentration of approximately 10^{-5} M. The solution was infused continuously in the ion source at a flow rate of 10 μL/min using a syringe pump (Model 11, Harvard apparatus, South Natick, MA). The electrosprayed ions were introduced in the mass analyzer at 4 keV kinetic energy. Helium was used as collision gas for the MS/MS experiments (70% attenuation of the main beam) and the collision energy was set at 2 keV by floating the collision cell. MS/MS signals were detected according to a previously published procedure [34].

NMR spectroscopy. NMR spectra were obtained for solutions of tannins in acetone-d_6 and MeOH-d_4 at 296K. The digital resolution of the ^{1}H spectra was 0.5Hz/point and the acquisition time was 2 s. J-Values are given in Hz. ^{13}C spectra were recorded with J-modulation and complete proton decoupling, an acquisition time of 1.11 s, digital resolution of 0.9 Hz/point, and a recycle time of 4 s. INAPT [19] spectra were acquired under similar conditions (10.000–15.000 scans) by using a 5 Hz filter for polarization transfer. A phase-sensitive NOESY [35] spectrum was recorded for a mixing time of 1 s and a recycle time of 5 s. Steady-state NOE experiments

were performed by applying low-power irradiation at the offset frequency of the saturated spin for 20 s (recycle time of 40 s).

Compound **5**. CI^+-MS (NH_3, direct inlet probe) 70 eV, *m/z* (rel. int.): 315 ($[M+H-H_2O]^+$). FAB-MS (glycerol matrix), *m/z*: 315 ($[M+H-H_2O]^+$), 333 ($[M+H]^+$). ESI-MS: see text. ^1H NMR: Table 2. ^{13}C NMR: Table 3.

Compound **6** CI^+-MS (NH_3, direct inlet probe) 70 eV, *m/z* (rel. int.): 636 $[M]^+$ (1). ^1H NMR: Table 2. ^{13}C NMR: Table 3.

Compound **7** CI^+-MS (NH_3, direct inlet probe) 70 eV, *m/z* (rel. int.) 485 $[MH]^+$ (12), 502 $[M+18]^+$ (15). ^1H NMR: Table 2. ^{13}C NMR: Table 3.

Compound **8** CI^+-MS (NH_3, direct inlet probe) 70 eV, *m/z* (rel. int.): 488 $[M/2+18+H]^+$ (15). Methylated **8**: CI-MS (NH3, probe) 200eV, *m/z* (rel. int.): 980 $[M+3Me]^+$ (8). ^1H NMR: Table 2. ^{13}C NMR: Table 3.

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