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PROANTHOCYANIDIN DIMERS AND POLYMERS FROM *QUERCUS DENTATA*

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Key Word Index—*Quercus dentata*; Fagaceae; proanthocyanidin dimers and polymers; proanthocyanidin gallates; gallotannins.

Abstract—In a chemical investigation of the bark of *Quercus dentata* gallic acid, (+)-catechin, (+)-gallocatechin, hexagalloyl glucose and five proanthocyanidin dimers namely catechin-(4α → 8)-catechin, gallocatechin-(4α → 8)-gallocatechin, gallocatechin-(4α → 8)-catechin, gallocatechin-(4α → 6)-catechin and 3-*O*-galloylepigallocatechin-(4β → 8)-catechin were identified. The proanthocyanidin polymers also isolated from the same extract have been shown to consist of monomeric and terminal units identifiable with the corresponding units of all the five co-occurring proanthocyanidin dimers.

INTRODUCTION

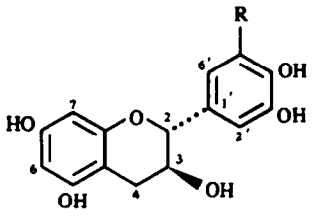
Quercus dentata Thunb. is one of a number of tree species which serve as a source of tanning materials for the leather industry in the People's Republic of China. This species is of limited distribution and is found mainly in the Province of Shangxi. The bark yields some 17% of tannins with a total annual output of several hundred tonnes. The nature of tanning materials in the bark has not been investigated in any detail although other *Quercus* species have been studied and shown to produce a wide spectrum of phenolic compounds including some novel hydrolysable tannins [1–3], catechin dimers and oligomers linked together via A- to B-ring biphenyl bonds [4–6]. As there is such a wide range of tannin compounds present in other oak species the chemical nature of the tannins in *Quercus dentata* is of great interest.

RESULTS AND DISCUSSION

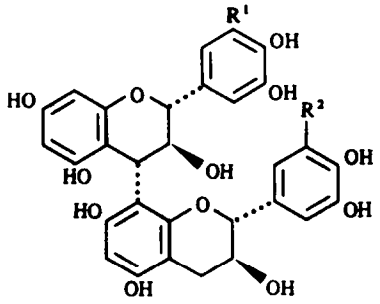
The ethyl acetate soluble fraction of the aqueous acetone extract of *Q. dentata* bark was fractionated by a combination of Sephadex LH-20, Fractogel and MCI gel chromatography. Evidence for the presence of both gallotannins and proanthocyanidins was the ready isolation of gallic acid, (+)-catechin and (+)-gallocatechin with catechin being about twice as abundant as its higher oxygenated counterpart. The biflavanoid fractions were more complex and multiple chromatographic treatments alternating between the three chromatographic systems and varying the eluting solvents afforded chromatographically pure procyanidin B3 or catechin-(4α → 8)-catechin (3) and four other biflavanoids (4–7).

The structure of 3 was derived from the analysis of its ^{13}C NMR spectrum and from degradation studies. It reacted with alcoholic hydrochloric acid to generate cyanidin and with phloroglucinol under acidic conditions to give catechin-(4α → 2)-phloroglucinol and catechin. Final confirmation of the structure was made by chromatographic and spectroscopic comparison with authentic procyanidin B3 obtained by reaction of leucocyanidin with catechin [7, 8]. Compound 4 was chromatographically more closely associated with 3 than the rest of the biflavanoids. Unlike 3 it yielded delphinidin on treatment with alcoholic hydrochloric acid. Examination of its ^{13}C NMR spectrum showed the intensity of the C-2 and C-3 chemical shifts of the monomeric and terminal units were comparable and therefore consistent with a biflavanoid constitution. The relative down-field position of the C-3 chemical shifts indicated that both the flavan units possessed the 2,3-*trans* configuration and was in full accord with its infrared spectrum [9]. Examination of the high field region of the chemical shifts of the aromatic carbons showed a strong peak at 108.7 ppm identifiable with the C-2′ and C-6′ of the pyrogallol B-ring in addition to the C-2′, C-3′ (115.0 and 116.3 ppm, respectively) and C-6′ (119.6 ppm) chemical shifts of the unsubstituted catechol B-ring [10]. The chemical shift evidence in conjunction with the anthocyanidin production suggested a biflavanoid constitution consisting of a gallocatechin upper unit linked to the C-6 or C-8 of a catechin lower unit. Further corroborative evidence for this flavanoid constitution was obtained by acid catalysed degradation of 4 with phloroglucinol which yielded gallocatechin-(4α → 2)-phloroglucinol and catechin as sole reaction products. The assignment of the structure gallocatechin-(4α → 8)-catechin to 4 [11] was made by consideration of its ^{13}C NMR data and comparison of the data with those of its procyanidin analogue (3). Subsequent identification of its C-4/C-6 linked regioisomer 5, which was the only other alternative structure, confirmed the structural assignment.

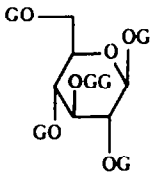
The novel compound 5 which was isolated in low yield was chromatographically very different from the two dimers so far described. Its isolation was more difficult and purification could only be achieved by repeated chromatography on Sephadex LH20 and MCI gel. Compound 5 gave delphinidin on reaction with alcoholic



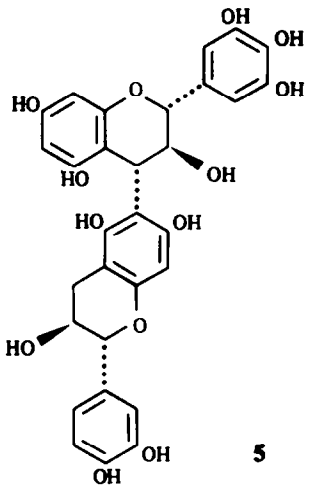
1 R = H
2 R = OH



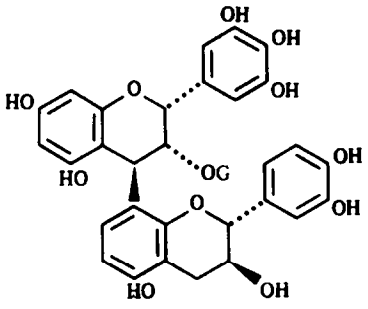
3 $R^1 = R^2 = H$
4 $R^1 = OH, R^2 = H$
6 $R^1 = R^2 = OH$



8 G = Galloyl



5



7 G = Galloyl

hydrochloric acid and yielded gallocatechin-(4α → 2)-phloroglucinol and catechin on treatment with phloroglucinol under acidic conditions. The ^{13}C NMR spectrum of **5** was consistent with a biflavanoid constitution and was confirmed by chemical degradation studies, which suggested a gallocatechin upper unit linked to the catechin terminal unit. Compound **5** was therefore an isomer of **4** with the former compound being the C-4/C-6 linked regioisomer based on ^{13}C NMR evidence. In addition to the chemical shifts, the peak broadening of the upper C-2 and the splitting of the upper C-4 chemical shifts which were probably due to restricted rotation about the inter flavanoid bond [12, 13] observed in the spectrum of **5** were similar in almost every respect to those observed for procyanidin B6. Also like procyanidin B6, **5** had a chemical shift (111.1 ppm) attributable to the substituted A-ring carbon at the point of interflavanoid linkage considerably downfield from its C-4/C-8 counterpart (107.1 ppm). The regioisomers **4** and **5** were isolated in a ratio of 10:1. The more restricted occurrence of the C-4/C-6 linked compound compared to the more common C-4/C-8 linked dimer was consistent with other reports [14, 15].

Compound **6** was again shown by its ^{13}C NMR spectrum to be an all 2,3-*trans* biflavanoid with both B-rings possessing a pyrogallol hydroxylation pattern. This gallocatechin dimer constitution was also corroborated by the reaction products gallocatechin-(4α → 2)-phloroglucinol and gallocatechin which were identified on treatment of **6** with phloroglucinol. The assignment of a C-4/C-8 linkage for this dimer was deduced from ^{13}C NMR chemical shift considerations and in particular the relative position (107.6 ppm) of the substituted A-ring carbon chemical shift at the point of interflavanoid bonding. This value was comparable to the substituted C-8 shift (107.4 ppm) of the prodelphinidin dimer, gallocatechin-(4α → 8)-catechin (**4**).

From ^{13}C NMR evidence, the new compound **7** also had a biflavanoid constitution but the chemical shifts particularly in the heterocyclic region were significantly

different from those of the previous dimers so far described. The presence of a carbonyl carbon chemical shift of 166.5 ppm, which was supported by infrared absorption at 1700 cm^{-1}, together with the observation of the chemical shift at 110.2 ppm, supported the presence of a gallate function. The downfield shift of the upper C-3 chemical shift (74.1 ppm) in conjunction with the upfield shift of the neighbouring C-4 at the point of interflavanoid linkage was consistent with the galloyl group being positioned on C-3 of the upper flavan unit [16]. The high field position of the C-2 caused in part by the presence of a neighbouring galloyl group, but more by the *γ-gauche* interaction with the substituent at C-4 [14] was clearly consistent with the substituents in the heterocyclic ring of the upper flavan unit being in a 2,3-*cis*-3,4-*trans* relationship. That the terminal unit was a catechin unit was deduced from the ^{13}C NMR data and confirmed by acid degradation studies. Comparison of the chemical shift of the A-ring carbon at the point of interflavanoid bond with that found in other dimers suggests the probable linkage in **7** to be 3-*O*-galloylepigallocatechin-[4$\beta \rightarrow$ 8]-catechin. The presence of the bulky galloyl group on the C-3 of the upper flavan unit apparently caused steric hindrance to acid catalysed degradation by benzylthiol or phloroglucinol as **7** had to be reacted for periods considerably longer than the other proanthocyanidin dimers before enough reaction products could be observed.

Hydrolysable tannins constituted only a minor component of the polyphenolic fraction. The hexagalloylglucose **8** was readily isolated from the proanthocyanidins by column chromatography on sephadex LH20 and purified on MC1 gel. The gallotannin constitution was confirmed by acid hydrolysis which yielded gallic acid and glucose. The 3-*O*-digalloyl-1,2,4,6-tetra-*O*-galloyl-β-D-glucose structure was deduced from the ^{13}C NMR spectrum and from consideration of the sugar carbon chemical shifts as detailed by Nishigawa and co-workers [17, 18]. The diagnostic feature which was characteristic of the gallotannin **8** was the observation of the downfield shift of the C-3 resonance of the glucose residue to coincide with the C-5 resonance.

The proanthocyanidin polymer from the aqueous phase was purified on sephadex LH20 as previously described [19]. The polymer had an average molecular weight (M_r) of 3706 as determined by VPO measurements which suggested an average number of flavan units of up to 12 depending on the frequency of galloylation on the 3-hydroxyl. Evidence for the presence of 3-*O*-gallate units was obtained from the ^{13}C NMR spectrum and indicated by the upfield position of the C-4 chemical shift at the point of interflavanoid linkage [16] in addition to the normal C-4 resonances [10] (see Fig. 1). This presence was further corroborated by the observation of the carbon chemical shifts at 110, 120, 139 ppm which were characteristic of the galloyl ring as well as the carbonyl carbon chemical shift at 166 ppm. The heterogeneity of the flavan B-ring was indicated by the occurrence of both the unsubstituted pyrogallol ring carbon at 107 ppm (C-2′, C-6′) and the catechol ring carbons at 116–117 ppm (C-2′, C-5′) with the former signals being about three times as intense as the latter. This approximation was supported by treatment of the polymer with alcoholic hydrochloric acid which yielded delphinidin and cyanidin in a ratio of about 3:1, respectively. Examination of the ^{13}C chemical

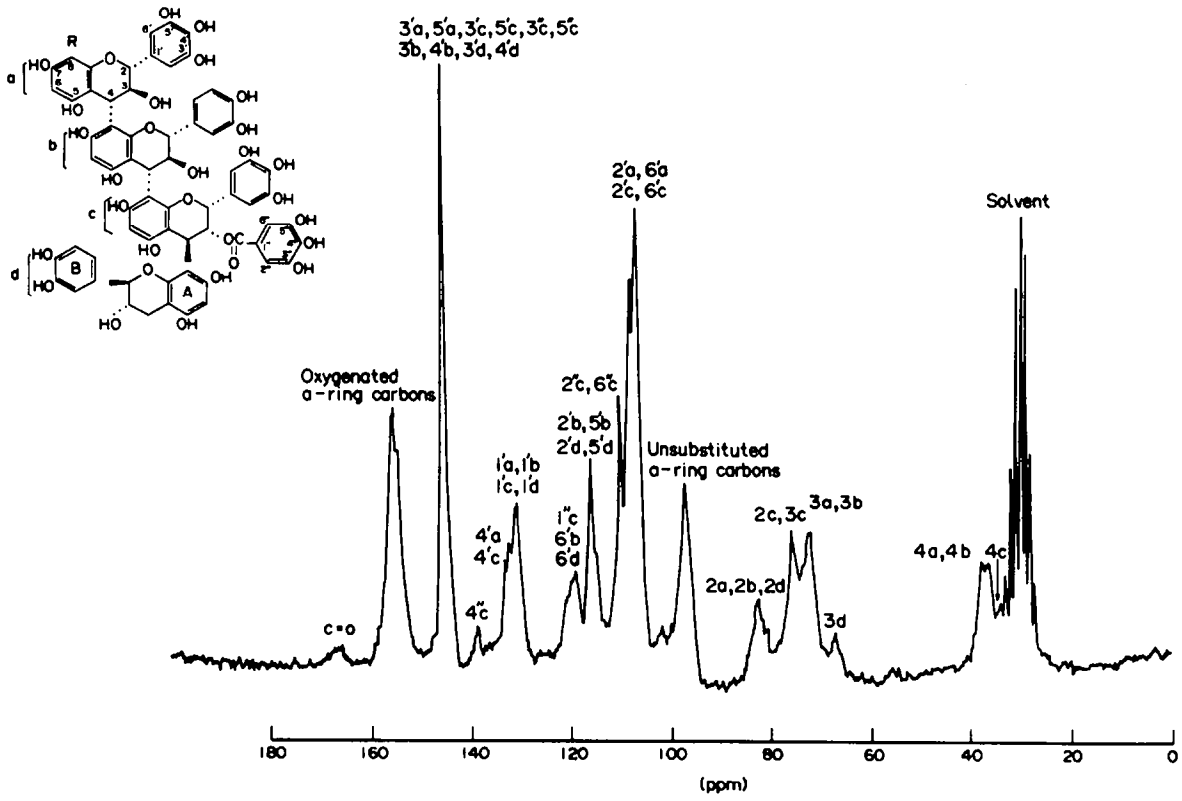


Fig. 1. ^{13}C NMR spectrum of the proanthocyanidin polymer from *Quercus dentata* bark.

shifts in the heterocyclic region showed resonances at 81–84 ppm and at about 76 ppm corresponding to the C-2 chemical shifts of flavans with 2,3-*trans* and 2,3-*cis* configurations, respectively. It was clear that this multiplicity of stereochemistry, the B-ring hydroxylation pattern and the presence or absence of galloyl groups could give rise to a large number of possible variations for the structure of the proanthocyanidin polymers of *Quercus dentata*. A closer definition of the flavan units in the polymers was obtained by chemical degradation with phloroglucinol and benzylthiol under acid conditions. Reaction of the polymers with phloroglucinol yielded catechin and gallocatechin in a ratio of about 3:1, respectively. In addition, catechin-(4α → 2)-phloroglucinol and gallocatechin-(4α → 2)-phloroglucinol were also detected by chromatographic comparison with authentic markers on 2D TLC. Other phloroglucinol addition products were not apparent in the reaction mixture and this could be due to flavan units with galloyl groups in close proximity to the interflavanoid bonds being more resistent to reaction with phloroglucinol on account of steric interference. This premise was supported by the observed relative resistance of 3-*O*-galloylepigallocatechin-(4β → 8)-catechin (7) to degradation with phloroglucinol compared with the remaining dimers. However, the reaction of the polymers with benzylthiol was more facile and not only confirmed the results of phloroglucinol degradation but also revealed the 3-*O*-galloylepigallocatechin unit as being a part of the polymer chain. This structural deduction was made from chromatographic comparison of the benzylthiol degradation products with the 4-thiobenzyl derivative of 7.

It is clear that the flavan-3-ols, namely catechin and gallocatechin, are readily identifiable with the terminating or bottom groups of co-occurring oligomeric proanthocyanidins as previously observed by Thompson *et al.* [20]. In addition, the proanthocyanidin or upper units of the dimers are also closely associated with the monomeric units of their higher molecular weight analogues. The observed restricted distribution of the 3-*O*-galloyl derivative to the epigallocatechin monomer units in the dimer and the polymers suggest that there is considerable enzymic control during the biosynthesis of these polymers.

EXPERIMENTAL

^{13}C NMR spectra were determined at 20 MHz on a Varian FT-80A spectrometer in Me_2CO-d_6–H_2O (1:1). TLC were carried out on Schleicher and Schull cellulose plates using *t*-BuOH–HOAc–H_2O (3:1:1, solvent A) and HOAc–H_2O (3:47, solvent B) and visualized by spraying with vanillin-HCl reagent.

Extraction and isolation: The bark (300 g) of *Quercus dentata* was finely milled and percolated with 50% aq. Me_2CO (3 × 1 l.). The combined extract was treated with excess NaCl and the resulting acetone layer was collected and concd under red. pres. To the residue was added H_2O (500 ml) and the aq. soln partitioned with EtOAc. The aq. fraction was separated from the upper EtOAc fraction, diluted with an equal vol. of MeOH and subjected to CC on Sephadex LH-20. The column was washed through with excess MeOH–H_2O (1:1) until no apparent colour was observed to come through with the solvent.

Proanthocyanidin polymers. Eluting the MeOH–H_2O washed column with Me_2CO–H_2O (1:1), concd under red. pres. at < 40° and freeze-drying the residue gave the polymers as a brown powder (20 g), $[\alpha]_{578}^{20}$ +64° (MeOH–H_2O, 1:1, *c* 0.1), λ_{max} (MeOH): 277 nm, ($E_{cm}^{1\%}$ 150); M_r 3706 (VPO) IR ν_{max}^{KBr} (cm^{-1}): 3100–3600, 1700 (w), 1620, 1520, 1535, 1450, 1350, 1210, 1110, 1040, 800 (w), 770 (w), 730 (w). ^{13}C NMR (MeCO-d_6–H_2O, 1:1, broad peaks ppm): 34.0, 37.0, 67.5, 72.5, 76.5, 83.0, 97.5, 102.0, 107.0, 108.5, 110.1, 116.0, 119.0, 131.5, 139.0, 146.0, 156.0, 155.0 (see Fig. 1).

Cleavage reactions with benzylthiol and phloroglucinol. The cleavage reactions of the dimers or polymers with benzylthiol or phloroglucinol were carried out in sealed vials in EtOH as previously described [19]. The identification of the degradation products were made by chromatographic comparisons with authentic markers on cellulose 2D TLC using solvents A and B.

The EtOAc portion (4.1 g) of the original extraction was subjected to CC on Sephadex LH20 eluting with MeOH–H_2O (1:19, 1 l.), (1:9, 1 l.), (1:4, 1 l.), (2:3, 1 l.) and (1:1, 1 l.) to furnish gallic acid (0.2 g) and 4 other main fractions: 1 (0.8 g), 2 (0.1 g), 3 (1.3 g) and 4 (0.5 g). Fraction 1 was rechromatographed over MCI gel eluting with MeOH–H_2O (3:7) to yield (+)-catechin (0.41 g) and (+)-gallocatechin (0.20 g).

Gallocatechin-(4α → 8)-gallocatechin (6). Chromatography of fraction 2 on MCI gel using MeOH–H_2O (3:7) gave gallocatechin-(4α 8)-gallocatechin (14 mg) $[\alpha]_{578}^{20}$ – 146 MeOH–H_2O (1:1, 0.05); R_f 0.20 (A), 0.48 (B), IR ν_{max}^{KBr} cm^{-1}: 3100–3600, 1620, 1520, 1530, 1460, 1350, 1210, 1150, 1090, 1070, 1040, 830, 730. For ^{13}C NMR see Table 1.

Gallocatechin-(4α → 8)-catechin (4). Chromatography of fraction 3 on MCI gel with MeOH–H_2O did not give homogeneous compounds and so was subjected to further CC on Sephadex LH20 using EtOH–H_2O (1:1) as solvent. This gave fractions: 3a (200 mg), 3b (200 mg) and 3c (90 mg). Chromatography of fraction 3a on MCI gel using MeOH–H_2O (3:7) gave gallocatechin-(4α → 8)-catechin (4), (99 mg), $[\alpha]_{578}^{20}$ – 247° (MeOH–H_2O, 1:1; *c* 0.07); R_f 0.28 (A); 0.48 (B). IR ν_{max}^{KBr} (cm^{-1}): 3100–3600; 1620, 1, 1525, 1460, 1350, 1240, 1210, 1150, 1093, 1068, 1037, 827, 780 (w), 770 (w), 730. For ^{13}C NMR see Table 1.

Catechin-(4α → 8)-catechin (3). Continued elution of the MCI gel column of fraction 3a gave catechin-(4α → 8)-catechin (50 mg), $[\alpha]_{578}^{20}$ – 185° (MeOH–H_2O, 1:1; *c* 0.09); R_f 0.45 (A); 0.50 (B), IR ν_{max}^{KBr} (cm^{-1}): 3100–3600, 1620, 1525, 1467, 1360, 1290, 1240, 1212, 1150, 1108, 1090, 1065, 1040, 825, 780 (w), 770 (w). For ^{13}C NMR see Table 1.

3-O-*Galloylepigallocatechin-(4β → 8)-catechin* (7). Chromatography of fraction 3b on MCI gel using MeOH–H_2O (1:9) and gradually increasing the MeOH ratio to 100% gave 3-*O*-galloylepigallocatechin-(4β → 8)-catechin, (37 mg); $[\alpha]_{578}^{20}$ + 2.8° (MeOH–H_2O, 1:1; *c* 0.07); R_f 0.60 (A) and 0.20 (B). IR ν_{max}^{KBr} (cm^{-1}): 3100–3600, 1700, 1620, 1523, 1535, 1450, 1350, 1240, 1150, 1120, 1040, 830, 770 & 740. For ^{13}C NMR see Table 1.

Gallocatechin-(4α → 6)-catechin (5). Fractionation of 3c on MCI gel was not successful and the mixture was rechromatographed on Fractogel TSK using MeOH to give gallocatechin-(4α → 6)-catechin (10 mg), $[\alpha]_{578}^{20}$ – 114° (MeOH–H_2O, 1:1; *c* 0.08); R_f 0.40 (A) and 0.48 (B). IR ν_{max}^{KBr} (cm^{-1}): 3100–3600, 1625, 1520, 1455, 1350, 1290, 1240, 1210, 1150, 1115, 1075, 1035, 828, 780 (w), 770 (w), 730.

3-*O*-*Digalloyl*-1,2,4,6-*tetra*-O-*galloyl*-β-D-*glucose* (8). Fraction 4 was subjected to chromatography on MCI gel using MeOH–H_2O (3:7 → 1.0) to yield 3-*O*-digalloyl-1,2,4,6-tetra-*O*-galloyl-β-D-glucose, $[\alpha]_{578}^{20}$ + 12° (MeOH–H_2O, 1:1; *c* 0.08), R_f 0.0 (A). IR ν_{max}^{KBr} (cm^{-1}): 3100–3600, 1710, 1620, 1540, 1455, 1355, 1210, 1100, 1040, 873, 767. ^{13}C NMR (Me_2CO-d_6–H_2O, 1:1; ppm): 62.8 (C-6 Glc), 69.5 (C-4 Glc), 71.9 (C-2 Glc), 73.5 (C-3, C-5 Glc), 110.6 (C-2′, C-6′ galloyl), 119.1, 119.7, 120.7 (C-1′ galloyl), 139.7, 140.1, 140.6, (C-4′ galloyl), 146.0 (C-3′, C-5′ galloyl), 166.0, 166.6, 166.8, 167.0 and 167.6 (carbonyl C).

Table 1. ^{13}C NMR chemical shifts of proanthocyanidin dimers*

	Heterocyclic carbons			A-Ring carbons		B-Ring carbons						Galloyl carbons					
	C-2	C-3	C-4	C-6	C-8	C-1′	C-2′	C-3′	C-4′	C-5′	C-6′	C-1″	C-2″C-6″	C-3″	C-4″	C-5″C=O	
Dimer 3																	
Upper	83.2	73.5	37.9	96.4	96.6	131.4	115.4	144.8	144.9	116.5	119.8						
Lower	81.4	68.2	†	97.2	108.6	132.1	116.2	145.0	145.4	116.6	120.8						
Dimer 4																	
Upper	83.5	73.3	37.9	96.7	96.3	131.3	108.7	146.0	133.7	146.2	108.7						
Lower	80.9	67.8	†	97.3	107.4	131.6	115.0	144.6	144.8	116.3	119.6						
Dimer 5																	
Upper	83.6	73.0	37.6	97.4	96.3	131.1	108.3	146.2	133.5	146.2	108.3						
Lower	81.6	67.7	†	111.1	96.3	131.1	115.4	145.4	145.5	116.5	120.3						
Dimer 6																	
Upper	83.9	73.2	37.9	97.6	96.3	131.6	108.1	146.1	133.4	146.0	108.1						
Lower	83.1	68.2	†	97.9	107.6	131.6	107.2	146.0	133.4	146.1	107.2						
Dimer 7																	
Upper	75.2	74.1	34.0	96.0	95.2	131.1	107.2	146.2	133.2	146.0	107.2						
Lower	81.7	67.4	†	97.1	107.9	131.3	115.8	145.4	145.6	116.8	120.6	120.5	110.7	145.0	139.3	145.0	166.8
Gallic acid												122.0	110.7	145.7	138.8	145.7	169.8

*Using Me_2CO–d_6–H_2O (1:1) as solvent.
†Signals obscured by solvent peaks.

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