

(+)-CATECHIN-ACETALDEHYDE CONDENSATION PRODUCTS IN RELATION TO WINE-AGEING

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(Received in revised form 24 February 1997)

Key Word Index—Catechin; acetaldehyde; condensation; wine; NMR; molecular mechanics.

Abstract—Dimers resulting from acetaldehyde-flavanol condensation were studied in an acidic hydroalcoholic medium (12% ethanol pH 3.2) in order to simulate the conditions of wine tannin-transformation during the wine-ageing process. One of the dimers was isolated after hemisynthesis and studied by mass spectrometry, NMR and molecular mechanics. Mass spectrometric analysis was in accordance with a dimeric structure with a CH-CH₃ linkage. NMR showed the presence of a 6–8 (ethane-1,1-diyl)di(+)-catechin. The carbon atoms, C-6 and C-8, involved in the linkage, have an asymmetric conformation, with the two catechols in an equatorial position. © 1997 Elsevier Science Ltd

INTRODUCTION

Procyanidins extracted from grapes are responsible for the astringency of young red wines. They consist of polymers of (+)-catechin (**1**) and (–)-epicatechin (**2**) units (Fig. 1) with C-4–C-6 or C-4–C-8 linkages. During wine-ageing, acid-catalysed cleavage of interflavan bond is likely to occur [1]. At the same time, condensation reactions may also occur. Among these reactions, a Baeyer acid-catalysed condensation, involving acetaldehyde, has for a long time been proposed [2, 3]. The occurrence of this reaction products affects the taste [4] and colloidal stability [5] of wine. It may also be important for the colour of red wine, when anthocyanins are involved [6–9].

Recent mass spectroscopic studies have enabled the identification of condensed products of acetaldehyde with **1** in a model solution [10–12] and in red wine [13]. Nevertheless, complete structural and conformational analysis of such products require NMR measurements and molecular mechanics calculations. In the present work, we have studied the dimers obtained by the reaction of **1** with acetaldehyde in a model solution (cf. **3**, Fig. 1).

RESULTS AND DISCUSSION

The HPLC chromatogram recorded at 280 nm for the solution after 10 days of reaction at 20° and pH

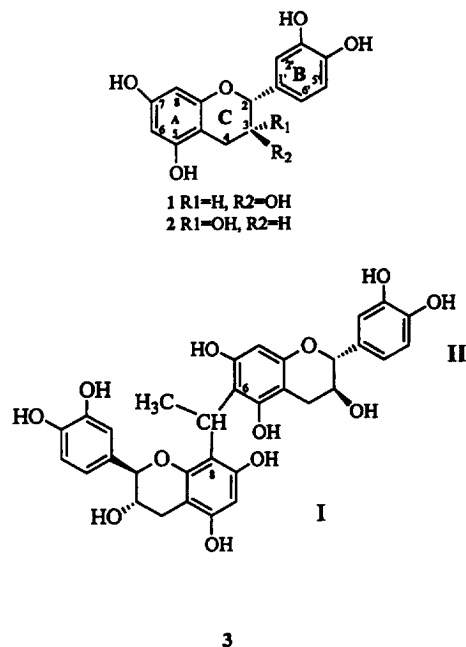


Fig. 1. Elemental structure of procyanidin monomer (catechin and epicatechin) and one of the possible dimers resulting from catechin-acetaldehyde condensation.

3.2 is shown in Fig. 2. The first peak is catechin and the four other ones (a–d) are potentially condensed products of catechin with acetaldehyde. Mass spectrometric analysis revealed that they all had a *M_r* of 606 ($[M+H]^+$, $m/z = 607$, $[M-H]^-$, $m/z = 605$) corresponding to two catechin units linked by an

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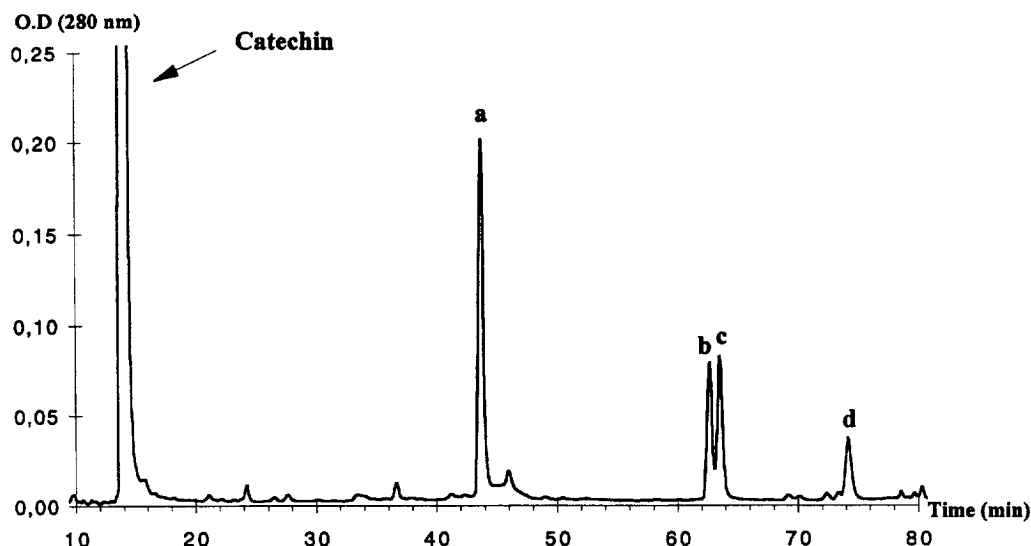


Fig. 2. HPLC (280 nm detection) showing the presence of reaction products of acetaldehyde-(+)-catechin reaction.

ethyl-bridge. Although these results are in accordance with previous studies [10, 11], the number of dimer peaks found was four and not three, due to separation improvement; these peaks should correspond to the four expected isomers (6-6, 8-8, 6-8 and 8-6) due to two more reactive sites on **1** (6 and 8) and the asymmetric carbon in the linkage. In order to study these compounds by NMR it was necessary to purify them. Unfortunately, conventional preparative HPLC was unsuccessful, because the isomers underwent spontaneous cleavage and rearrangement [12, 14]. Consequently, we used low pressure TSK HW 40-(s) gel chromatography with MeOH as eluent. The first fraction eluted after catechin contained only dimer **a**. It was then possible to investigate its structure and conformation by mass spectrometry, NMR and molecular mechanics.

In order to obtain mass spectral fragments, the voltage of the electrospray source was raised to 90 V (absolute value). In addition to the $[M + H]^+$, intense fragment peaks corresponding with vinyl-catechin and catechin were also observed (Fig. 3). These results are in accordance with previous studies with LSI mass spectrometry [5]. Another fragment was also observed, especially in the negative mode, which corresponded with a retro-Diels-Alder (RDA) fragmentation of the dimer. Such fragmentations (RDA and linkage-cleavage) are usually seen with flavonoid compounds, such as procyanidins [15, 16].

All the NMR spectra recorded for this fraction suggested that only one compound containing **1** units was present in a dimeric form, as suggested by mass spectrometry. First, it was important to demonstrate the nature of the linkage existing between the two **1** units (I and II). It was established unambiguously that a CH-CH₃ bridge existed between the two A cycles of the two **1** units; the δ ¹H of the CH group was characteristic of a proton located between two deshielding groups, like the A cycle of the **1** moiety

(quadruplet at 5.18 ppm). On the contrary, the δ ¹³C of the CH group was not influenced by the presence of such deshielding groups (24.7 ppm). Concerning the CH₃ group, a doublet at 1.55 ppm was observable in the proton spectrum, this resonance being coupled to the CH group proton; the δ ¹³C was typical of an alkyl group (18.7 ppm). Such results are in accordance with previous studies on acetaldehyde-flavylium coupling reactions [9].

Secondly, the question of isomerism was addressed—is it the 6-6, one of the two 6-8 or the 8-8 isomer? Since we observe a pair of distinct chemical shifts in the ¹H as in the ¹³C NMR spectra, and because the only asymmetric isomer is the 6-8 one [4], we can assign this isomer to **3**. Nevertheless, other proof is necessary. Consequently, all the ¹H and ¹³C resonances were assigned using 2D techniques, such as COSY, TOCSY, HMQC and HMBC, giving us the ability to assign each catechin unit of the studied molecule (Table 1). The chemical shift of 2-H on the C-ring of unit I is in good agreement with the one in free catechin in the same solvent (δ 4.57, [17]). This indicates that the methine carbon of the ethyl bridge is attached to C-6 of unit I [18] and, consequently, to C-8 of unit II. Nevertheless, the presence of a correlation peak in the HMBC experiment between the proton of the CH group of the ethyl link and carbon I 8a demonstrated that the ethyl bridge is attached to C-8 of unit I. Indeed, the two quaternary carbons I 8a and II 8a are assigned without ambiguity by the presence of ³J[¹H-¹³C] correlation peaks between the proton linked to C-2 of the C cycle and C-8a of the two **1** units, I and II (Fig. 3). Finally, all the results confirm the presence of a 6-8 (ethane-1,1-diyl)di(+)-catechin dimer **3**.

In order to study the spatial conformation of **3**, we used both NMR and molecular mechanics. The ¹H NMR spectrum enables the determination of ³J[¹H-¹H] coupling constants (Table 2). These allow the

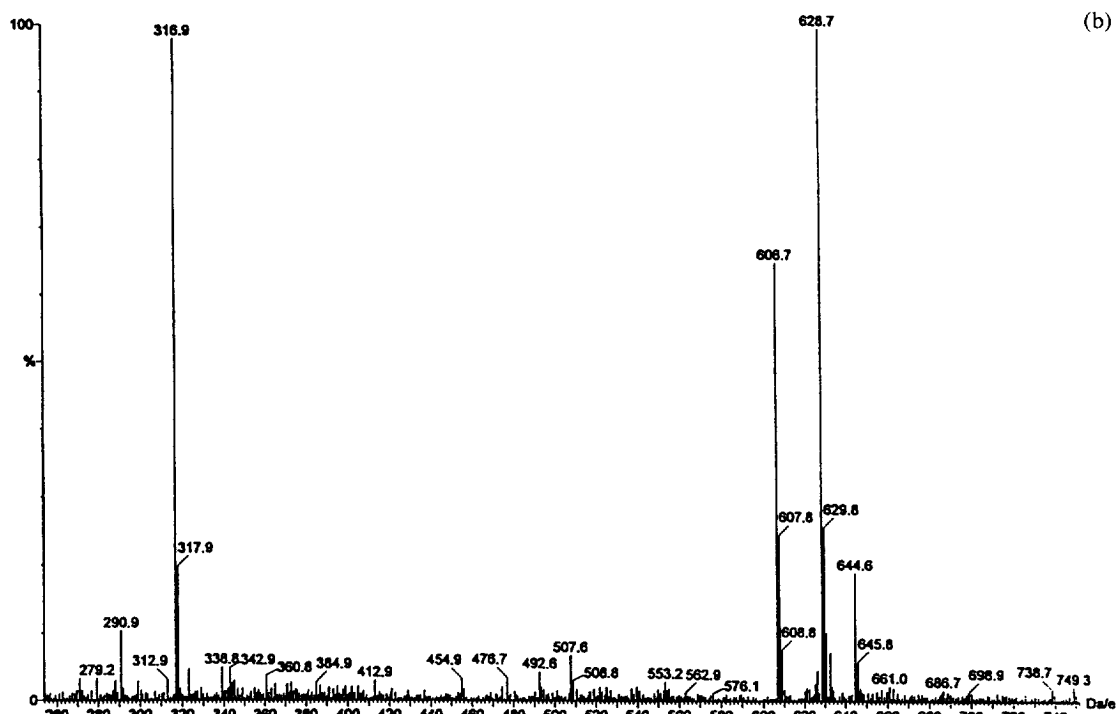
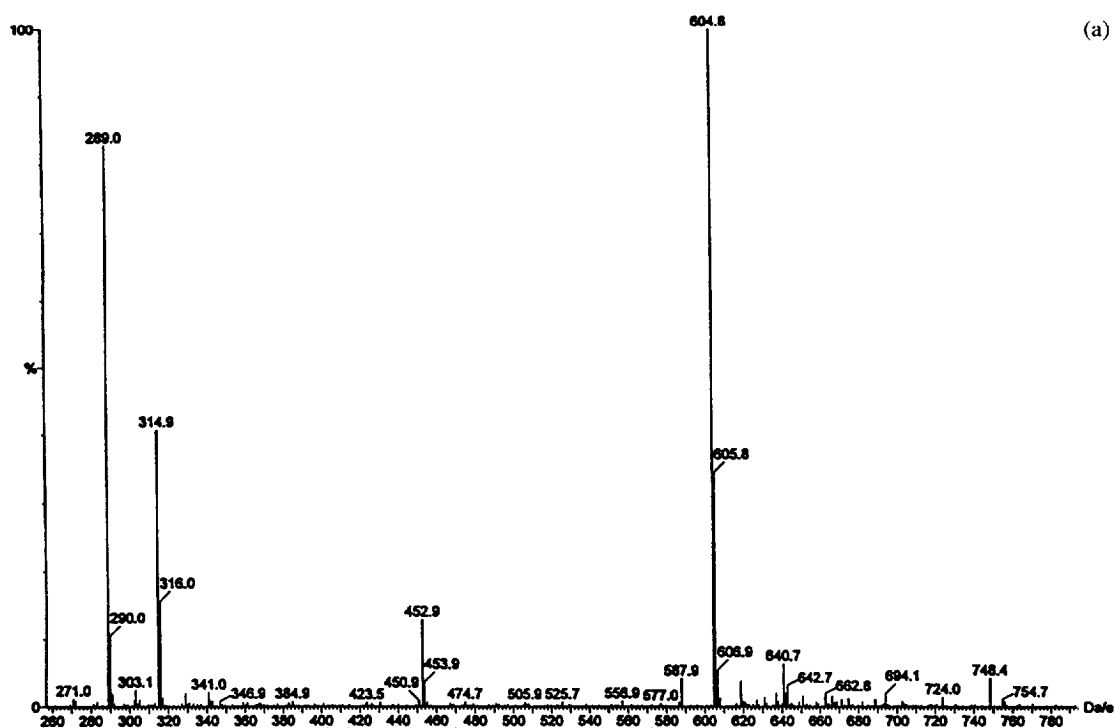


Fig. 3. Mass spectra obtained in electrospray of dimer 'a' in negative (a) and positive (b) mode. Catechin ($[M-H]^-$: m/z 289, $[M+H]^+$: m/z = 291) and vinyl-catechin ($[M-H]^-$: m/z = 315, $[M+H]^+$: m/z = 317) fragments are present. Other intense peaks are the RDA fragment ($[M-H]^-$: m/z = 453) in the negative mode and a sodium adduct in the positive mode ($[M+H]^+$: m/z = 629).

determination of the stereochemistry of the heterocyclic pyran (C) ring; $J_{2,3}$ coupling constants allowed discrimination between the equatorial or axial con-

formation (B ring) on each 1 unit in solution [17]. The measured constants implied an equatorial configuration for each catechol moiety (B ring). Inter-

Table 1. ^1H and ^{13}C assignments of compound 3

No.	$\delta^1\text{H}$ (I)	$\delta^1\text{H}$ (II)	$\delta^{13}\text{C}$ (I)	$\delta^{13}\text{C}$ (II)
CH	5.18		24.7	
CH_3	1.55		18.7	
2	4.60	4.15	81.5	81.7
3	3.93	3.85	67.5	68.1
4	2.67; 2.52	2.92; 2.44	26.7	28.6
4a	—	—	100.1	100.5
5	—	—	154.4	153.9
6	5.96	—	95.9	111.0
7	—	—	154.3	154.2
8	—	5.98	109.9	96.0
8a	—	—	153.2	153.0
1'	—	—	131.4	131.4
2'	6.70	6.81	114.0	114.4
3'	—	—	144.9	145.0
4'	—	—	145.0	145.1
5'	6.65	6.75	115.3	114.9
6'	6.47	6.60	118.7	119.4

Table 2. ^1H - ^1H 3J coupling constants of compound 3 expressed in Hz

Constants 3J	Values (Hz)			
	Unit I	Unit II	Unit I	Unit II
	Measured	MM3*	Measured	MM3*
$^3J_{2-3}$	6.6	9.0	8.3	9.0
$^3J_{3-4}^\dagger$	7.2	10.6	9.1	10.8
$^3J_{3-4}^\ddagger$	5.3	5.7	5.7	5.4

† 4' is the more deshielded proton.

estingly, the conformational search using MM2* or MM3* gave very different results. A Monte-Carlo conformational search was used, followed by a cluster analysis with XCluster; 3000 steps were run within a 15 kJ mol⁻¹ energy-range, resulting in 77 and 35 conformers (respectively, with MM2* and MM3*). With MM2*, all low energy conformers were axial-equatorial or bi-axial. The first bi-equatorial was found 10.1 kJ mol⁻¹ higher in energy (no. 24). On the contrary, with MM3*, the lowest energy conformer was bi-equatorial. The first axial-equatorial conformer found was no. 9 ($\Delta E = 4.7$ kJ mol⁻¹). No bi-axial conformers were found among the lowest energy part of the conformers.

As the experimental data clearly show that all compounds are bi-equatorial, it seems that, as already noticed in the polyphenol series [19], MM3* gives better results than MM2*. The selected conformer (Fig. 5) shows several interesting features. The structure is first stabilized by two hydrogen bonds between the OH of I-I (positions 5,7) with the phenolic OH (position 7) and the pyranic oxygen of I-II. Secondly, the catechol moiety from I-II interacts by means of π - π stacking with the benzo moiety of I-I (distance of the centres: 4.61 Å). In this conformation, the proton on the C-2 monomer I-I is in close proximity to the

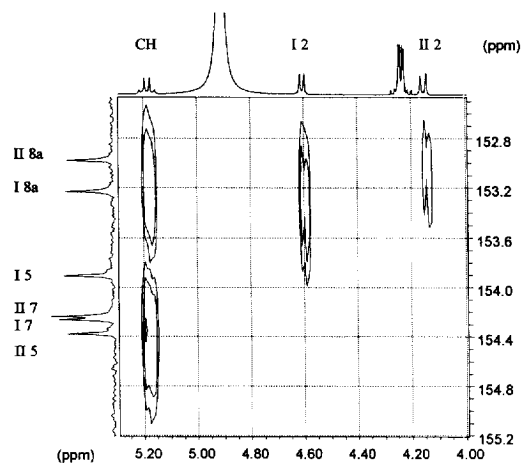


Fig. 4. Part of HMBC spectrum of compound 3, showing the linkage involving C-6 and C-8 of the two I units.

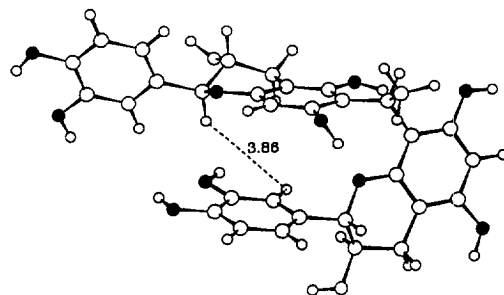


Fig. 5. Conformer of compound 3 calculated using MM3*.

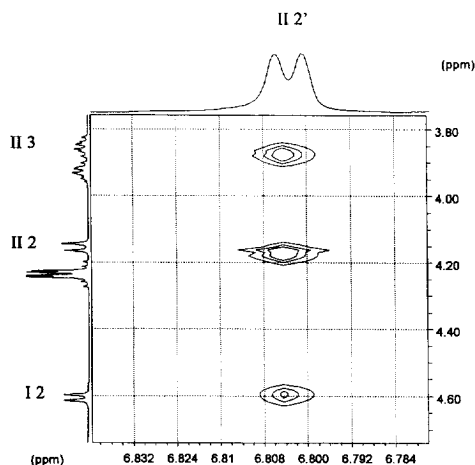


Fig. 6. Part of NOESY spectrum of compound 3.

protons 2',5' and 6' of the catechol from I-II. It was interesting to check this using NOESY experiments. The NOESY spectrum was obtained under qualitative conditions (a long mixing-time, 800 ms, and a short relaxation delay, 1.5 s). This experiment gave mainly correlation peaks between the proximal protons of ring B and C of the same I unit. Only one correlation peak is of interest in terms of tertiary structural information, a correlation peak between the proton I 2 and the proton II 2' (Fig. 6); this is in good agreement

with the conformational analysis ($d = 3.86 \text{ \AA}$, Fig. 5) of dimer **3**.

EXPERIMENTAL

Model solution and reagents. All chemicals were of reagent grade. The model wine soln used was 12% EtOH buffered on pH 3.2 (5 g l⁻¹ tartaric acid, N NaOH for pH = 3.2). Acetaldehyde was mixed at -4° and its concn estimated by assuming a density of 0.78. Starting concs of catechin and acetaldehyde were 6.9 10⁻³ mol l⁻¹ and 2 10⁻² mol l⁻¹, respectively. The temp. was 20°.

HPLC analysis. The column (25 × 0.46 cm) used was a 5 μm ODS2. The two solvents used were H₂O (A) and MeOH (B) both containing 5% HOAc. All gradient steps were linear. The composition of solvent A during the programme was:

Time (min)	0	1	59	74	75	88	112	120
A (vol %)	100	95	62	56	48	45	0	100

Mass spectrometry. MS was performed on a quadrupole instrument with an electrospray source, in positive and negative mode with a cone voltage of 25 V. Conditions of sepn were identical to those in ref. [13]. The flow rate was reduced to 0.1 ml min⁻¹ using a post-column split. For dimer analysis, the cone voltage was raised to 90 V in order to induce fragmentations.

NMR. 1D and 2D NMR expts were performed on a spectrometer equipped with an inverse 5 mm broadband probe at 400 and 100 MHz for ¹H and ¹³C, respectively. All spectra were recorded using 3 mg product dissolved in 0.6 ml of CD₃OD in a 5 mm tube. ¹H and ¹³C chemical shifts are given in ppm relative to TMS.

1D spectra. ¹H spectra were recorded for both frs, with a spectral-width of 3600 Hz and a pulse-width of 7 μs (which corresponds to a nutation angle of 90°). A scan number of 32 and an interpulse delay of 14.56 s (4.56 s for acquisition time and 10 s for relaxation delay) were used. Processing, which was done without any multiplication, was carried out with a 16 K data-points. The proton-decoupled ¹³C spectra of each fr. were recorded with a spectral-width of 18 000 Hz with 32 K data-points and a pulse-width of 9.5 μs (90° nutation angle). A scan number of 10 000 and an interpulse delay of 3.86 s (1.86 s for acquisition time and 2 s for relaxation delay) were used. Exponential weighting with a line-broadening factor of 1 Hz was applied before Fourier transformation.

2D spectra. The ¹H-¹H shift correlated 2D COSY spectra of both frs were obtained using the COSY-90 pulse sequence. For each t₁ increment, 16 scans were accumulated. The F1 and F2 spectral-widths were 3600 Hz and the initial (t₁, t₂) matrices of 256 × 1024 real data-points were zero-filled to 1024 × 1024 to give a final resolution of 3.6 Hz point. The ¹H-¹H total correlation TOCSY spectra of both frs were obtained using the basic phase-sensitive TOCSY sequence using

MLEV-17 mixing-pulse. The acquisition and processing parameters were the same as in the COSY expt, except that a Qsine multiplication of ×3 was used in the two dimensions. A 100 ms spin-lock mixing-time was used for each expt. ¹H-¹H NOESY expts were recorded in the phase-sensitive mode with time-proportional phase-incrementation according to the pulse sequence of ref. [20]. The acquisition and processing parameters were the same as in the COSY expt, except that a Qsine multiplication of ×2 was used in the two dimensions, before the double Fourier transformation. One bond ¹H-¹³C chemical shift correlation HMQC were obtained for both isomers according to the Bax sequence [21], using B₀ gradient-pulses for the selection of ¹H coupled to ¹³C carbons. For each t₁ increment, 64 scans were accumulated. The F1 and F2 spectral-widths were 17 600 and 3600 Hz, respectively. The initial (t₁, t₂) matrices of 256 × 1024 real data-points were zero-filled to 1024 × 1024, to give a final resolution of 68.8 Hz point⁻¹ in the ¹³C dimension (F1) and 3.6 Hz point⁻¹ in the proton dimension (F2). ¹H-detected heteronuclear multiple bond correlation spectra (HMBC) were recorded using the pulse sequence of ref. [22], involving a low-pass J-filter (3.8 ms) and a delay for the long-range coupling (60 ms). As in the HMQC expt, B₀ gradient-pulses were applied in order to select ¹H coupled to ¹³C nuclei. Except for the sequence and the delays mentioned, all parameters were the same as in the HMQC expt.

Isolation of 6-8 (ethane-1,1-diyl) di(+)-catechin. MeCHO (4.5 × 10⁻¹ mol l⁻¹) and **1** (8.6 × 10⁻³ mol l⁻¹) were placed in model soln at 35° in the darkness for 6 hr. The resulting polymers were isolated using LH-20 with H₂O and MeOH. The MeOH fr., containing phenolic compounds, was evapd and freeze-dried. Polymers were then purified using TSK HW 40(s) gel with MeOH as eluant. The second fr. eluting after **1** was collected and freeze-dried. Purity of this product was estimated by HPLC as 95%. It was then ready for MS and NMR analysis.

Acknowledgements—This work was supported by CNRS and Conseil Régional d'Aquitaine.

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