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

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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–CH₂ linkage. NMR showed the presence of a 6–8 (ethylene-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 $M_1$ of 606 ([$M+H]^+$, $m/z = 607$, [M−H]$^-$, $m/z = 605$) corresponding to two catechin units linked by an

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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 I (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 I 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 I units (I and II). It was established unambiguously that a CH–CH3 bridge existed between the two A cycles of the two I units; the δ 1H of the CH group was characteristic of a proton located between two deshielding groups, like the A cycle of the I 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–flavylum 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 2H 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 1J[H–13C] correlation peaks between the proton linked to C-2 of the C cycle and C-8a of the two I 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 1J[H–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; J₂₃ coupling constants allowed discrimination between the equatorial or axial conformation (B ring) on each I unit in solution [17]. The measured constants implied an equatorial configuration for each catechol moiety (B ring). Inter-
Table 1. $^1$H and $^{13}$C assignments of compound 3

<table>
<thead>
<tr>
<th>No.</th>
<th>$\delta^1$H (I)</th>
<th>$\delta^1$H (II)</th>
<th>$\delta^{13}$C (I)</th>
<th>$\delta^{13}$C (II)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH</td>
<td>5.18</td>
<td>24.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CH$_2$</td>
<td>1.55</td>
<td>18.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>4.60</td>
<td>4.15</td>
<td>81.5</td>
<td>81.7</td>
</tr>
<tr>
<td>3</td>
<td>2.93</td>
<td>3.85</td>
<td>67.5</td>
<td>68.1</td>
</tr>
<tr>
<td>4</td>
<td>2.67; 2.52</td>
<td>2.92; 2.44</td>
<td>26.7</td>
<td>28.6</td>
</tr>
<tr>
<td>4a</td>
<td>—</td>
<td>—</td>
<td>100.1</td>
<td>100.5</td>
</tr>
<tr>
<td>5</td>
<td>—</td>
<td>—</td>
<td>154.4</td>
<td>153.9</td>
</tr>
<tr>
<td>6</td>
<td>5.96</td>
<td>—</td>
<td>95.9</td>
<td>111.0</td>
</tr>
<tr>
<td>7</td>
<td>—</td>
<td>—</td>
<td>154.3</td>
<td>154.2</td>
</tr>
<tr>
<td>8</td>
<td>—</td>
<td>5.98</td>
<td>109.9</td>
<td>96.0</td>
</tr>
<tr>
<td>8a</td>
<td>—</td>
<td>—</td>
<td>153.2</td>
<td>153.0</td>
</tr>
<tr>
<td>1'</td>
<td>—</td>
<td>—</td>
<td>131.4</td>
<td>131.4</td>
</tr>
<tr>
<td>2'</td>
<td>6.70</td>
<td>6.81</td>
<td>114.0</td>
<td>114.4</td>
</tr>
<tr>
<td>3'</td>
<td>—</td>
<td>—</td>
<td>144.9</td>
<td>145.0</td>
</tr>
<tr>
<td>4'</td>
<td>—</td>
<td>—</td>
<td>145.0</td>
<td>145.1</td>
</tr>
<tr>
<td>5'</td>
<td>6.65</td>
<td>6.75</td>
<td>115.3</td>
<td>114.9</td>
</tr>
<tr>
<td>6'</td>
<td>6.47</td>
<td>6.60</td>
<td>118.7</td>
<td>119.4</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Values (Hz)</th>
<th>Unit I</th>
<th>Unit II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Constants $^3$J</td>
<td>Measured MM3*</td>
<td>Measured MM3*</td>
</tr>
<tr>
<td>$^3$J$_{1,3}$</td>
<td>6.6</td>
<td>9.0</td>
</tr>
<tr>
<td>$^3$J$_{1,4}$</td>
<td>7.2</td>
<td>10.6</td>
</tr>
<tr>
<td>$^3$J$_{1,4}^{+}$</td>
<td>5.3</td>
<td>5.7</td>
</tr>
</tbody>
</table>

† $4'$ is the more deshielded proton.

Interestingly, 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-I interacts by means of π-π stacking with the benzo moiety of I-I (distance of the centers: 4.61 Å). In this conformation, the proton on the C-2 monomer I-I is in close proximity to the 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 \((d = 3.86 \text{ Å, Fig. 5})\) 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 \(-4^\circ\) 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\(^\circ\).

*HPLC analysis.* The column \((25 \times 0.46 \text{ cm})\) used was a 5 \(\mu\)m ODS2. The two solvents used were H\(_2\)O (A) and MeOH (B) both containing 5% HOAc. All gradient steps were linear. The composition of solvent A during the programme was:

\[
\begin{array}{lllllllllll}
\text{Time (min)} & 0 & 1 & 59 & 74 & 75 & 88 & 112 & 120 \\
A (vol \%) & 100 & 95 & 62 & 56 & 48 & 45 & 0 & 100
\end{array}
\]

*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 \(^1\)H and \(^13\)C, respectively. All spectra were recorded using 3 mg protein dissolved in 0.6 ml of CD\(_3\)OD in a 5 mm tube. \(^1\)H and \(^13\)C chemical shifts are given in ppm relative to TMS.

*1D spectra.* \(^1\)H spectra were recorded for both frs, with a spectral-width of 3600 Hz and a pulse-width of 7 \(\mu\)s (which corresponds to a nutation angle of 90\(^\circ\)). 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 \(^13\)C 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 \(\mu\)s (90\(^\circ\) 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 \(^1\)H–\(^1\)H shift correlated 2D COSY spectra of both frs were obtained using the COSY-90 pulse sequence. For each \(t_1\) increment, 16 scans were accumulated. The F1 and F2 spectral-widths were 3600 Hz and the initial \((t_1, t_2)\) matrices of 256 \times 1024 real data-points were zero-filled to 1024 \times 1024 to give a final resolution of 3.6 Hz point. The \(^1\)H–\(^1\)H 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 Qine multiplication of \(\times 3\) was used in the two dimensions. A 100 ms spin-lock mixing-time was used for each expt. \(^1\)H–\(^1\)H NOESY expts were recorded in the phase-sensitive mode with time-proportional phase-incrcementation according to the pulse sequence of ref. [20]. The acquisition and processing parameters were the same as in the COSY expt, except that a Qine multiplication of \(\times 2\) was used in the two dimensions, before the double Fourier transformation. One bond \(^1\)H–\(^13\)C chemical shift correlation HMQC were obtained for both isomers according to the Bax sequence [21], using B\(_0\) gradient-pulses for the selection of \(^1\)H coupled to \(^13\)C carbons. For each \(t_1\) increment, 64 scans were accumulated. The F1 and F2 spectral-widths were 17600 and 3600 Hz, respectively. The initial \((t_1, t_2)\) matrices of 256 \times 1024 real data-points were zero-filled to 1024 \times 1024, to give a final resolution of 68.8 Hz point\(^{-1}\) in the \(^13\)C dimension (F1) and 3.6 Hz point\(^{-1}\) in the proton dimension (F2). \(^1\)H-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, \(B_0\) gradient-pulses were applied in order to select \(^1\)H coupled to \(^13\)C 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-diy1) di(+) catechin. MeCHO (4.5 \times 10^{-3} mol l\(^{-1}\)) and I (8.6 \times 10^{-3} mol l\(^{-1}\)) were placed in model sohn at 35\(^\circ\) in the darkness for 6 hr. The resulting polymers were isolated using LH-20 with H\(_2\)O 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 I was collected and freeze-dried. Purity of this product was estimated by HPLC as 95\%. It was then ready for MS and NMR analysis.

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