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NMR and molecular modeling: application to wine ageing

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RÉSUMÉ

Le vin rouge contient des polyphénols appelés tanins qui sont très importants pour son goût et sa longévité. Il s'agit principalement de polymères de catéchine et d'épicatéchine. Durant le vieillissement du vin, des réactions de condensation interviennent lentement et conduisent à de nouvelles structures. Parmi les réactions possibles, nous avons plus spécialement étudié la polymérisation par pontage avec l'éthanal. La catéchine a été utilisée comme modèle de tannin et mise en présence d'éthanal en milieu acide proche du vin. Deux fractions de produits de réaction ont été isolées par chromatographie liquide. La spectrométrie de masse a révélé la présence de dimères. La RMN (1D et 2D) et la modélisation moléculaire ont ensuite été utilisées pour déterminer la structure et la conformation de ces produits. La première fraction a été identifiée comme étant un dimère de deux unités catéchines reliées par un pont éthyle par leur carbones 6 et 8. La seconde fraction isolée est un mélange de deux dimères (50/50). Les mesures RMN montrent qu'il pourrait s'agir de dimères symétriques reliés par le même carbone (6-6 et 8-8).

mots clés : vin rouge, tanin, catéchine, éthanal, RMN, modélisation moléculaire

ABSTRACT

Red wine contains polyphenols called tannins which are very important for its taste and longevity. These polymers consist in repeating units of catechin and its epimer epicatechin. During ageing, slow condensation reactions take place which lead to new chemical structures. Among the possible reactions, we have focused our attention on acetaldehyde cross-linking. Catechin was used as a model for the production of polymers with acetaldehyde. Two reaction product fractions have been

isolated by liquid chromatography. Mass measurement indicated that these fractions contain dimers. NMR (1D and 2D) and molecular modelling were then used to study the structure and conformations of these products. The first product consist in a pure dimer with the two catechin moieties connected with an ethyl bridge on the carbon 6 and 8. The second fraction was a mixture of two dimers (50/50). NMR measurements showed that it could be two symmetrical dimers involving the same carbon for each catechin moiety (6 or 8).

keywords: red wine; catechin; tannin ; acetaldehyde; NMR; molecular modelling.

INTRODUCTION

Polyphenols of red wines are responsible for their different taste and colours. These compounds are extracted from the solid parts of grape during wine making. The most abundant compounds are condensed tannins which are very important for the taste and longevity of the wine [1] They are polymers of flavan-3-ols like catechin (scheme) with C_4-C_6 or C_4-C_8 bonds. These polymers interact with salivary proteins [2] to promote astringency, which is a tactile sensation rather than a taste like sweetness or bitterness.

During wine ageing, slow condensation and oxidation reactions occur, leading to the transformation and precipitation of polyphenols. Among all possible reactions, the one involving acetaldehyde has for a long time retained the attention of researchers [3,4,5,6,7]. Indeed, acetaldehyde is produced in wine by yeasts [8] during wine-making and also by oxidation of ethanol during ageing [9]. Recently, LC-MS was used to study the reaction mechanism [10] and to prove the occurrence of these compounds in wine [11]. Nevertheless, the complete identification and structural elucidation of these products requires NMR and molecular modelling. Here, we study the reaction products obtained by hemisynthesis in the catechin-acetaldehyde reaction.

EXPERIMENTAL

-Isolation of the dimers : Catechin and acetaldehyde were mixed in solution for 7 hours as described elsewhere [12]. The resulting products were purified on Toyopearl TSK HW40 (s) with methanol as eluent with a 40*1.6cm column at 1ml/min flow rate. The two fractions eluting after catechin were then recovered and freeze-dried.

-HPLC and mass spectrometry : HPLC was performed with a H_2O / MeOH gradient using an ODS2 column (25*0.46 cm, 5mm). Mass experiments were performed on a quadrupole instrument with an electrospray source in positive mode. Conditions used were identical to previous studies [12].

-NMR experiments : Spectra were recorded on a Bruker 400 MHz, using 5mm tubes with CD_3OD for solvent. Conditions for 1D ^1H and ^{13}C and 2D (COSY, HMBC and HMQC) were identical to [12].

-Molecular Mechanics : MM2* and MM3* force fields were used in the conditions of [13].

RESULTS AND DICUSSION

The HPLC chromatogram depicted in figure 1 shows the products obtained during catechin-acetaldehyde reaction. Mass analysis reveals that products **a**,**b**,**c**,**d** all have a mass in accordance with a dimeric structure ($[M+H]^+ = 607$). As HPLC shows a good separation, it would seem natural to use semi-preparative HPLC to isolate these products. Unfortunately, these dimers undergo spontaneous cleavage and rearrangements during isolation [10]. As an alternative, we used low pressure gel chromatography with TSK gel and pure methanol (without acid) as eluent. Several fractions were obtained. We focus here on the two fractions eluting after catechin which was the first compound eluted.

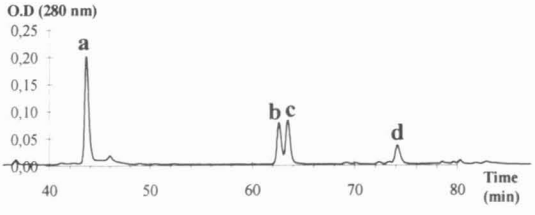
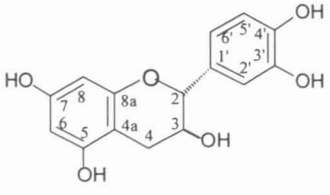


Figure 1 : HPLC chromatogram (280nm) of catechin-acetaldehyde reaction



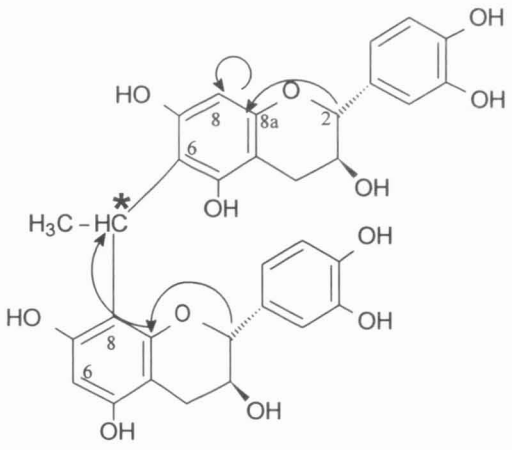
3s: (+)-Catechin
3r: (-)-Epicatechin

Figure 2 : Structure and labelling of catechin units

HPLC revealed that the first fraction contained only the dimer **a**, while the second one contained the two dimers **b** and **c** (see fig.1) in a 50/50 proportion. The first point was then to see the kind and the number of linkage existing in each fraction.

One CH- CH_3 was seen in the first fraction while two were present in the second one, which was in accordance with the HPLC results. In the ^1H spectrum the

quadruplets of the CH groups were characteristic of a proton located between two deshielding groups like the A cycle of catechin (scheme). Indeed, their d ^{1}H were 5.18 for the first fraction and 4.78 and 4.80 for the second one. Each CH was coupled with one CH_3 which had δ ^{13}C typical from an alkyl group (18.7 ppm for the first fraction and 17.43 and 17.68 for the second one). Such a result suggests that the catechin units are linked with a $CHCH_3$ bridge as expected during wine tannin ageing.



6-8

Fig.3. HMBC correlations seen on the first fraction, showing the presence of a 6-8 isomer.

At this stage, the crucial problem was to assign each isomer. In regard to the reactivity of the catechin, the carbon 6 and 8 are expected to be more reactive. Four isomers are then expected (6-6, 8-8, and two 6-8 due to the asymmetrical carbon of the $CHCH_3$ bridge). Indeed four dimeric products, were seen (Fig. 1) indicating that all possible dimer are formed. These results contrast with strong regiospecificity which occur with similar compounds : when catechin reacts with anthocyanins, only two products are formed [7].

The first step was to assign each catechin unit present in the fractions. This was achieved mainly by COSY, HMBC and HMQC experiments. In the first fraction two distinct catechin units were present (Table I). As one linkage is present and as the

only asymmetrical dimer were the 6-8 ones, the presence of the 6-8 isomer is highly suspected. In the second fraction, two groups of two catechin units with very close d ^{13}C and d ^{1}H were seen. As two linkages were present, two isomers are attempted in this fraction.

In order to assign correctly the isomers, it is necessary to seek correlations that prove the connectivity of the CH-CH3 with the catechin units. HMBC showed decisive $^{3}J[^{1}H - ^{13}C]$ correlations between the CH proton of the linkage and some carbons presents on A cycle of the catechin units :

- for the first fraction (Fig 3) each H2 was correlated with C8a from the same unit. Then, correlation between the CH proton of the linkage and C8a of unit I only was a proof that C8 of unit I was involved in the linkage. Other proofs are correlations between the CH proton and C5 and C7 from unit II, which indicates a linkage in C6 for this unit. Moreover, I C7 correlates with the CH proton but not I C5. All these results confirm the presence of a 6-8 type isomer [12].

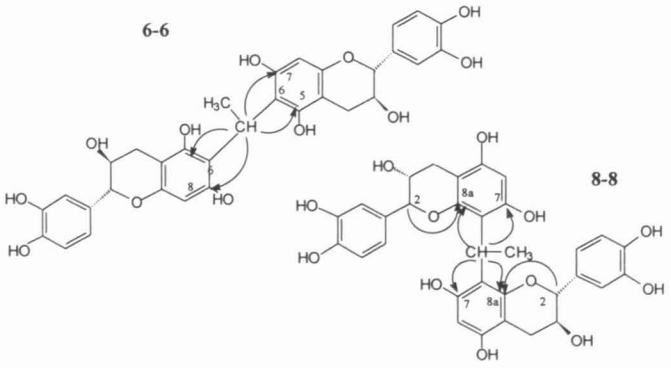


Fig 4. Correlations seen in HMBC experiment of the second fraction permitting the assignments of the linkages

- for the second fraction, the H2 protons were again very important because they permit to assign two groups of carbons C8a of each isomer. Then, it could be seen that the CH of the linkage showed correlations with the two C8a of one isomer, and with carbons C5 and C7 of the second one (Fig 4.). This last point was not so obvious to determine since, in one hand, the CH of the two isomers have very close d ^{1}H, and, in the other hand, 12 different carbons resonate close to 154 ppm.

Unfortunately, the chromatographic separation of these two isomers is very difficult because they have very close polarity as can be seen in fig.1.

We conclude that the isomer 8-8 and 6-6 were presents. It is noteworthy that the two catechin units of 6-6 and 8-8 isomers have different ^{1}H and ^{13}C. Indeed, these results were not awaited in regard to their symmetrical structure. One explanation could be the presence of rotational isomerism which occurs with procyanidins [14]. In this type of tannins, the catechin units are directly connected with C4-C6 or C4-C8 bonds. This induces an energy barrier which permits to see the different rotamers in the NMR spectrum. In our case, the rotation is facilitated by the ethyl bridge. So, the occurrence of rotamers may be questionable. Other experiments will be necessary to fully understand the ^{1}H and ^{13}C spectrum lineshapes of the 6-6 and 8-8 isomers. The separation of these two isomers could certainly make all assignments more clear.

TABLE I : NMR chemical shifts in the ^{1}H and ^{13}C

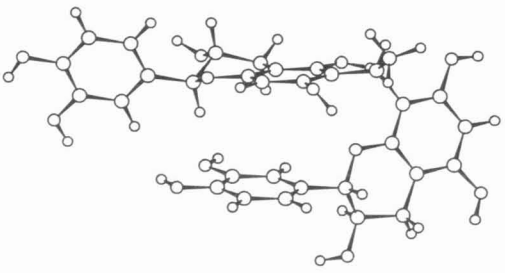
N° C	*$\delta^{1}H$ (8-6)*	*$\delta^{1}H$ (8-8)*	*$\delta^{1}H$ (6-6)*	*$\delta^{13}C$ (8-6)*	*$\delta^{13}C$ (8-8)*	*$\delta^{13}C$ (6-6)*
Linkage	5.18	4.78	4.80	24.7	25.48	25.54
«	1.55	1.66	1.67	18.7	17.43	17.68
2	4.60 ;4.15	4.69	4.55	81.5 ;81.7	83.09 ; 83.19	81.64 ; 81.66
3	3.93 ;3.85	4.09	3.96	67.5 ;68.1	67.04 ; 67.18	67.79 ; 67.88
4a	2.67 ;2.92	2.57	2.49	26.7 ;28.6	28.21	27.65 ; 27.76
4b	2.52 ;2.44	2.97 ; 2.98	2.81 ; 2.82			
4a	-	-	-	100.1 ;100.5	100.85 ; 100.99	101.44 ; 101.52
5	-	-	-	153.9 ;154.4	154.58	~153.6
6	5.96 ; -	6.05 ; 6.06	-	95.9 ;111.0	96.56 ; 96.80	111.21; 111.51
7	-	-	-	154.3 ;154.2	~154	~154
8	- ; 5.98	-	5.907;5.910	109.9 ;96.0	109.56 ; 109.79	95.6 ; 95.7
8a	-	-	-	153.2 ;153.0	152.21 ; 152.57	153.59
1'	-	-	-	131.4 ;131.4	129.72 ; 129.74	131.24
2'	6.70 ;6.81	6.91 ; 6.94	6.83	114.0 ;114.4	114.63 ; 114.73	114.07 ; 114.14
3'	-	-	-	144.9 ;145.0	145.2	145.2
4'	-	-	-	145.0 ;145.1	145.8 ; 145.7	145.4 ; 145.3
5'	6.65 ;6.75	~6.82	6.77	115.3 ;114.9	115.23 ; 115.29	115.05
6'	6.47 ;6.60	~6.82	6.70	118.7 ;119.4	119.55 ; 119.67	118.89 ; 119.00

In order to have spatial information we used both ^{3}J coupling constant measurements and molecular modelling . Among the measured constant (Table II), only the $^{3}J_{2\text{-}3}$ [H-H] coupling constant was of interest to study the conformation of the molecules. Indeed, this constant permits to know the stereochemistry of the heterocylclic ring in solution [15] and the catechol conformation. In our case, the relatively high values obtained for J_{23} coupling constants (table II) imply that all isomers contains (+)-catechin units in equatorial conformation.

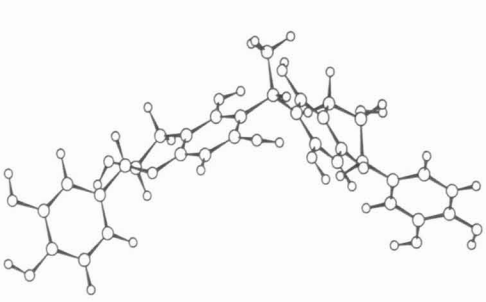
TABLE II : Coupling constants measured by NMR for each isomer

	Coupling constants (Hz)		
	8-6	6-6	8-8
$^3J_{2-3}$	6.6 ;8.3	8.1	7.2
$^3J_{3-4}$	7.2 ;9.1	7.9	8.5
$^3J_{3-4'}$	5.3 ;5.7	7.8	6.0

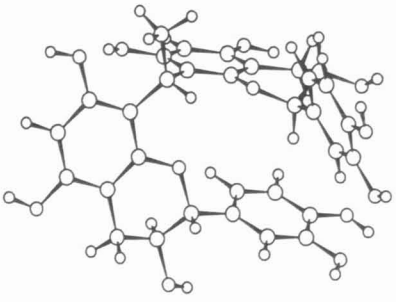
Molecular modelling was used to calculate the most probable conformation for each isomer. All the lowest energy conformers found with MM3* force field were indeed bi-equatorial (Fig.5). The presence of C8 linkage induces a folding in the shape of the dimer : for example the 8-8 isomer is much more compact compared to the 6-6 one. The two possible 6-8 isomers are not represented since they have very similar conformations which mainly differ by the position of the methyl and methine group of the linkage.



6-8 (a)



6-6 (b or c)



8-8 (b or c)

Fig.5. Conformers obtained with MM3 force field*

CONCLUSION

Acetaldehyde-catechin reaction in acid medium leads to ethyl-bridged catechin units. The carbons involved in the linkage could be determined by using 1D and 2D NMR, and particularly HMBC. Three isomers could be assigned with these techniques. The $^{3}J_{2\text{-}3}$ [H-H] coupling constant measurements showed that all catechol cycles are in equatorial conformation as seen in MM3* forcefield calculations results. In fact, the conformation and the stereochemistry of the catechins are little influenced by the polymerization process. However, the different isomers have very different conformations according to the carbons involved in the linkage. The linkage in C8 results in more folded structures than the ones in C6.

Further experiments are necessary to separate all these dimers in order to rely their conformations to their different hydrophobic behaviours. This may explain some differences in taste [6] and longevity of red wines as different solubility and protein affinity are expected with the different conformations.

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