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Structure determination and colour properties of a new directly linked flavanol–anthocyanin dimer

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Abstract—The structure of catechin-(4α→8)-malvidin 3-*O*-glucoside obtained by reaction of taxifolin and malvidin 3-*O*-glucoside following a protocol adapted from proanthocyanidin dimer synthesis was determined by NMR spectrometry. Incorporation of the anthocyanin moiety into a covalent linked flavanol–anthocyanin dimer did not modify its colour properties (i.e., hydration equilibrium constant and copigmentation).

Red wine is rich in phenolic compounds, which are responsible for its colour and astringency while also contributing to its flavour. During ageing and storage of red wine, many changes occur. Phenolic compounds in wines, particularly anthocyanins and proanthocyanidins (i.e., flavanol oligomers and polymers, also referred to as condensed tannins) are progressively converted by various reaction mechanisms to new pigment and tannin species during wine storage. These new pigments present different colour properties from those of their anthocyanin precursors (i.e., red grape pigments).[1]

Two mechanisms, leading, respectively, to anthocyanin–flavanol (A^+–F) and to flavanol–anthocyanin (F–A^+) adducts, are postulated for direct reactions between anthocyanins and flavanols. During the formation of A^+–F, the anthocyanin is in the flavylium form (A^+). Nucleophilic addition of the flavanol onto the flavylium cation leads to the colourless flavene (A–F), which can be oxidized to the red flavylium (A^+–F) and then to a xanthylium salt.[1–3] In the formation of F–A^+, proanthocyanidins (F–F) are affected by acid-catalyzed cleavage of their interflavanic bond, releasing the intermediate carbocation F^+,[4] which acts as an electrophile. Nucleophilic addition of the anthocyanin in its hydrated hemiketal form (AOH)[5] yields the colourless dimer (F–AOH), which dehydrates to the red flavylium form (F–A^+).

So far, flavanol–anthocyanin adducts in the flavylium form have been observed in wine[6] and recently been successfully synthesized[7] but only tentative identification based on mass spectrometry data was provided.

The objective of the work was to fully elucidate the structure of the F–A^+ dimer, and more specifically to unambiguously determine the position and configuration of linkage.

The dimer was synthesized according to previously published data.[7] During the reaction, catechin carbocation (catechin$^+$) was obtained from taxifolin after reduction into a flavan-3,4-diol followed by protonation and dehydration. The malvidin 3-*O*-glucoside (Mv3glc) added in its hydrated form reacted with catechin$^+$ to lead to two F–A^+ dimers. The major dimer was extracted from reaction medium by countercurrent chromatography. The presence of catechin–Mv3glc dimer was monitored by HPLC–ESI-MS. The mass spectrum showed a signal at $m/z = 781$ in the positive ion mode, which is in agreement with the dimeric structure. The characteristic fragmentations obtained by MS^2 and MS^3 experiments confirmed the postulated structure. In particular the loss of a 126 fragment indicated the flavanol to be in the upper position.[7]

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NMR experiments were performed at 298 K, using a Varian UNITY INOVA 500 MHz spectrometer equipped with a 3 mm reverse probe. The sample was dissolved in DMSO-d_6/TFA (9:1) to ensure that the F–A^+ dimer was under the flavylium form. Complete structure elucidation was achieved using both 1D ^{1}H and 2D homonuclear ^{1}H and heteronuclear ^{1}H–^{13}C NMR experiments, which allowed ^{1}H and ^{13}C chemical shift assignments (Table 1).

The NMR spectra of the dimeric compound showed one set of signals, which indicated the presence of only one compound.

Analysis of both ^{1}H and 2D ^{1}H–^{13}C HSQC spectra allowed to assign most of proton resonances and corresponding carbons. Characteristic singlet resonances of the malvidin 3-*O*-glucoside moiety were easily identified in accordance to previous works:[8–10] H4 (1H, 9.01 ppm), equivalent H2′ and H6′ (2H, 8.06 ppm), OCH_3 (6H, 3.39 ppm). Only one D ring proton of malvidin could be observed (1H, 6.74 ppm) suggesting that the interflavanic linkage implied either C6 or C8 position of malvidin D ring.

Table 1. ^{1}H (500 MHz) and ^{13}C (125.75 MHz) assignments of catechin–Mv3glc in DMSO/TFA (9:1) at 25 °C

Ring	Position	δ ^{1}H (ppm); m; *J* (Hz)	δ ^{13}C (ppm)
C	Catechin		
	2	4.43; d; *J* = 9.3	82.7
	3	4.21; m	71.0
	4β	4.82; d; *J* = 8.3	38.7
	4a	—	103.7
	8a	—	156.35
A	5	—	157.9
	6	5.77; s	96.0*
	7	—	156.8
	8	5.77; s	94.8*
B	1′	—	130.9
	2′	6.85; br s	115.3
	3′	—	145.5
	4′	—	145.7
	5′	6.73; m	115.3
	6′	6.67; dd; *J* = 8.2, 1.9	119.6
	Malvidin		
F	2		161.2
	3		143.9
	4	9.01; s	137.0
	4a	—	112.7
	8a		154.9
D	5	—	155.9
	6	6.74; m	102.9
	7	—	166.9
	8	—	110.5
E	1′	—	119.1
	2′; 6′	8.06; s	109.4
	3′	—	148.7
	4′	—	144.1
	5′	—	148.7
	OCH_3	3.93; s	56.6
	Glucose		
	1′	5.40; d; *J* = 7.6	103.0
	2′	3.45; m	73.7
	3′	3.39; m	76.7
	4′	3.21; m	69.9
	5′	3.47; m	78.0
	6″a	3.74; m	60.9
	6″b	3.51; m	60.9

* Could be inverted.

The anomeric proton H1′ of the glucose moiety showed a characteristic chemical shift at 5.40 ppm. The high coupling constant (*J* = 7.6 Hz) indicated the anomeric sugar conformation to be β. The remaining glucosyl protons gave resonance peaks in the upfield region of 1D ^{1}H spectrum (from 3.2 to 3.7 ppm), which were attributed with the help of 2D ^{1}H COSY spectrum.

Resonance peaks of the catechin moiety were typical of a flavanol unit substituted in the C4 position.[11–15] The remaining H4 proton appeared as a doublet (1H, 4.82 ppm) whereas C4 resonance was downfield shifted at 38.7 ppm. The H3 and H2 protons were unambiguously identified since they gave a broad multiplet (1H, 4.21 ppm) and a doublet (1H, 4.43 ppm), respectively. Aromatic protons H6 and H8 of flavanol units are known to rapidly exchange against deuterium. However, under our experimental conditions, they were still observable even if they gave weaker peak intensity than expected. Whereas their proton chemical shifts were degenerated (5.77 ppm), their ^{13}C chemical shifts could be distinguished (94.8 and 96 ppm). The three protons H2′, H5′ and H6′ of the other aromatic ring were easily identified (6.85, 6.73 and 6.67 ppm).

With the proton resonances having thus been assigned, the covalent linkage was known to imply both the C4 position of the catechin moiety (upper unit) and the C6 or C8 position of the D ring of Mv3glc moiety (lower unit). This result confirmed the compound to be F–A^+ dimer as deduced from mass spectroscopy data and expected from the hemisynthesis pathway.[7] However whether the junction is 4–8 or 4–6 remained to be determined as well as the stereochemistry of the linkage.

Different NMR strategies have been used to answer the question of junction positions between flavonoid units: ^{1}H–^{13}C heteronuclear long-range correlations, ^{1}H through space correlations, chemical shift parameters.

Even if some studies used chemical shift argument to base assignments and structural conclusions, it seems a little bit unwise since chemical shift positions may be solvent dependent and may change when units are implied in oligomeric structures.[13]

HMBC experiments often provide an efficient way to identify linkage positions between procyanidin units.[11,13,16] However it was no use in the case of a flavanol–anthocyanin dimer as illustrated in Figure 1. The H4C would indeed give correlations with both C7D and C8aD in the case of a C4–C8 linkage and with both C5D and C7D in the case of C4–C6 linkage. Since C8aD, C7D and C5D cannot be attributed accurately from other long-range correlations, no conclusion could be made.

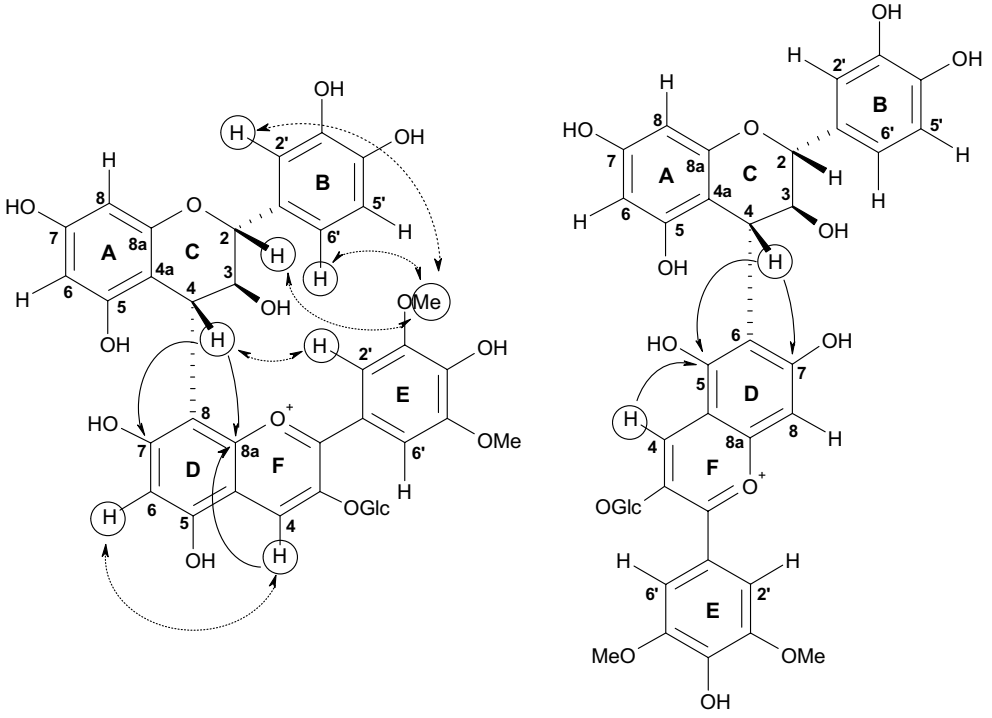

Figure 1. Structure of C4–C8 or C4–C6 TA dimers. (⟶) HMBC and ROESY (◀···▶) correlations.

In contrast, ^{1}H through space correlations gave conclusive evidences of linkage position. ROESY spectrum showed indeed strong correlations between E ring protons (H2′, H6′) and H4C, medium correlations between OCH_3 and both B ring protons (H2′, H6′) and H2C, and weak correlations between H4F and D ring proton and between OCH_3 and H4C. These correlations can only be fulfilled in the case of a C4–C8 linkage as illustrated in Figure 1. Consequently, the remaining D ring proton is H6.

After the H6 resonance had been assigned, the quaternary carbons were attributed from long-range HMBC experiment (Table 1). The HMBC spectrum showed a long-range correlation between the anomeric proton of glucose and C3F, confirming the linkage of the glucose unit at the C3 position of Mv3glc.

From a configuration point of view, the large coupling constant between protons H3 and H4 of C ring ($J_{3,4}$ = 8.3 Hz) indicates a 3,4-*trans* relative configuration. This allowed to deduce that the remaining proton of the catechin C4 position corresponded to 4Hβ and the subunit bond was a 4α linkage.

Conformational insights could also be obtained from NMR experiments. Flavonoid oligomers often exist as rotamers in solution. In a previous study dealing with the structure of flavanol–pelargonidin dimers,[17] duplication of NMR signals was observed and attributed to conformers resulting from a rotation around the interflavonoid bond. In contrast, our dimer composed with Mv3glc as anthocyanin unit gave only one set of sharp NMR resonances suggesting a rotational hindrance at the subunit linkage. This assumption was confirmed by molecular modelling. Two low energy conformers were found in which both aromatic rings B and E were close to each other and the planes defined by the long axes of the upper and lower monomers were approximately perpendicular to each other. The two conformations differ by the direction of the anthocyanin unit, which is either in front of or behind the plane defined by the long axis of catechin, leading to C3–C4–C8–C8a torsion angles (ϕ) of about 95° and −80°, respectively (Fig. 2). Only the former conformation is compatible with the observed ROEs. This result suggests that intra-molecular interactions stabilized one minimum energy conformation and the energy barrier to get the other conformation by rotation around the linkage was too large due to steric hindrance.

Moreover, the $^{3}J_{2,3}$ and $^{3}J_{3,4}$ coupling constants provide information upon the geometry of the heterocyclic ring C. The large values observed for both $^{3}J_{2,3}$ and $^{3}J_{3,4}$ (9.3 and 8.3 Hz, respectively) are characteristic of an heterocyclic ring of catechin closed to an half chair geometry with a slight distortion to a C-2c sofa as reported for other catechin units implied in oligomeric structures, the B ring being in a quasi-equatorial position.[11,12] This data also suggests that no interconversion at the heterocyclic ring occurred.

Colour properties of the catechin–Mv3glc dimer were studied through absorbance measurements in the UV–vis range.

UV–vis spectra of catechin–Mv3glc were recorded in buffers going from pH = 1 to pH = 7 obtained by mixing

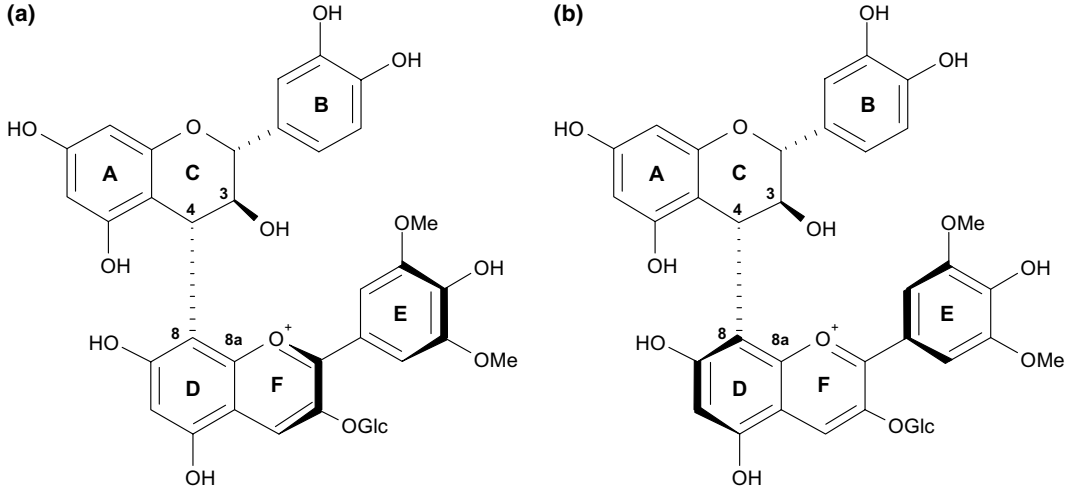

Figure 2. Two possible minimum energy conformers: (a) $\phi > 0$ and (b) $\phi < 0$.

two solutions (A and B). Solution A: HCl 0.1 M. Solution B: $Na_2HPO_4 \cdot 12H_2O$ 0.1 M. The F–A dimer reached maximum colour expression at pH = 1 and lost approximately 50% in intensity upon an increase in pH to pH = 2.6 as shown in Figure 3. The cat–Mv3glc dimer showed a similar behaviour towards hydration compared to monomeric Mv3glc.

Copigmentation experiments were also carried out using chlorogenic acid as copigment at wine pH (pH = 3.5). Changes in the vis spectra as a function of the chlorogenic acid concentration are shown in Figure 4. Both hyperchromic shift (171%) and bathochromic shift (14 nm) were observed upon addition of chlorogenic acid up to 1:100 molar ratio. Similar effects were observed by Eiro and Heinonen[18] using monomeric Mv3glc and chlorogenic acid. The hyperchromic effect may be due to a displacement of the hydration equilibrium towards the flavylium form whereas the bathochromic shift can be attributed to an increased proportion of quinonoidal bases in the pigment–copigment complexes as suggested by other authors.[19]

These results showed that the direct linkage of Mv3glc to a catechin unit does not change its colour properties.

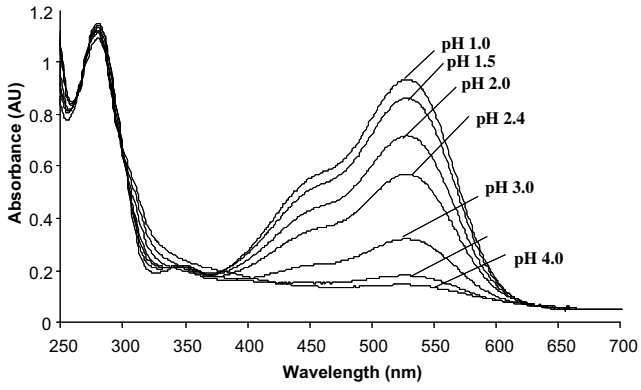

Figure 3. UV–vis of the cat–Mv3glc dimer at various pH.

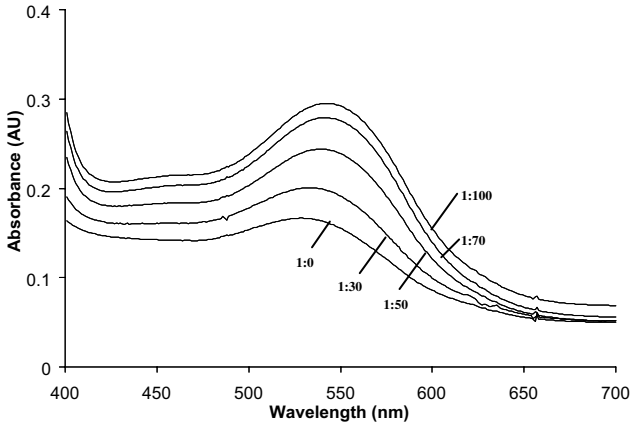

Figure 4. Visible spectra of the cat–Mv3glc dimer after addition of chlorogenic acid for different pigment/copigment molar ratios.

Thus, Mv3glc engaged in a direct linkage with catechin is not more protected against water attack than Mv3glc in a monomeric form, possibly due to the constraints of the dimeric structure.

This study confirmed the structure of a new type of pigment involving anthocyanins and flavanols directly linked, which has earlier been shown to occur in wine.[7] Studies of its colour properties allowed to rule out the assumption that flavanol–anthocyanin adducts are more resistant to hydration and sulfite bleaching than anthocyanins.

Studies on the antioxidant properties of the cat–Mv3glc dimer are under way.

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