Circular dichroism of anthocyanin 3-glucoside self-aggregates

Raquel Gavara, Vesselin Petrov, Alexandre Quintas, Fernando Pina

The circular dichroism spectra of the six most common anthocyanin 3-glucoside show the formation of left-handed aggregates compatible with dimers. The absorption bands of the monomer split by increasing concentration according to the formation of H and J aggregates. The angle and distance between the transition moments of the two monomers in the dimer was calculated from the splitting of the 0–0 absorption band. While the angle is similar for the series the distance changes dramatically. The intensity of the CD signal is proportional to the inverse of the square of the distance.

Highlights

1. The circular dichroism spectra of six common anthocyanins 3-glucosides were obtained. 2. Like 3,5-diglucoside analogs, they exhibit left-handed CD signals. 3. J and H aggregates are formed by concentration increasing. 4. The distance of the transition moments correlates with the intensity of the CD signal.
Circular dichroism of anthocyanidin 3-glucoside self-aggregates

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Abstract

Self-association constants for the flavilyum cations of the six most common anthocyanidin 3-glucosides were determined by circular dichroism (CD) and UV–Vis spectroscopy. Along with previous 1H NMR results, all measurements were consistent with a monomer–dimer model. The CD spectra of the anthocyanidin 3-glucosides were similar to the analogues 3,5-diglucosides. All dimers of the anthocyanidin 3-glucosides exhibited left-handed CD signals, with petunidin-3-glucoside and myrtiltin having the most intense signals. In addition, the magnitude of the molar ellipticity, [θ], was generally higher for the 3-glucosides than for the 3,5-diglucosides. For all six anthocyanins studied, the CD absorption spectra of their dimers showed evidence of the splitting of the monomer absorption into lower (J) and higher (H) energy bands. The angle and the distance between the dipolar moments of the two monomers comprising the dimer were obtained from the lower energy absorption band.

While the angle was more or less similar in all six dimers, the separation distance between the monomer dipole moments differed dramatically. The intensity of the CD signal displayed a linear dependence with the inverse square of the dipole moment distances.

Keywords:
Circular dichroism
Anthocyanins
J and H aggregates

1. Introduction

Anthocyanins are the pigments responsible for the beautiful red to blue colours of flowers and fruits. However, when their pH dependent equilibrium is studied in water at low concentrations, the red flavilyum cation is only stable at very acidic pH values, while the blue quinoidal base is a minor species (<5%) at moderately acidic conditions. Due to the fact that the pH of the vacuoles, where anthocyanins are located, changes roughly from 3 to 6 (Steward et al., 1975) some kind of interactions that permit to achieve colour in these conditions must take place. One solution found by Nature regarding the achievement of blue colour, is the formation of supramolecular structures involving metals and flavonoids, which are able to stabilize the blue quinoidal base (Yoshida et al., 1995, 2009).

In the case of red colour it was reported for raspberry the presence of high concentrations of cyanidin 3-glucoside (kuromanin, ca. 2.4 mg/g of fresh fruit) and other derivatives bearing different sugars in position 3 (Melo et al., 2000). Nature uses high concentrations of the anthocyanin to compensate for the fact that is not using the total colouring power of the flavilyum cation (for pH 3.1 found in raspberry extract, the mole fraction of flavilyum cation of the cyanidin 3-glucoside is only ca. 0.33). The use of high concentration of anthocyanins (Wu et al., 2006), in particular anthocyanin 3-glucosides, raises the question of self-aggregation and its influence on the colour definition.

In a series of papers Hoshino et al. (1982) demonstrated that anthocyanidin 3,5-diglucosides self-aggregate by stacking in a right-handed or left-handed screw axis. While quinoidal bases of cyanin and pelargonin lead to right-handed adducts, peonin, delphin and malvin form left-handed ones. On the other hand, all the respective flavilyum cations lead to aggregates exhibiting left-handed CD signals, due to a super-asymmetry imposed by the oligomeric species (Rodger and Nordén, 1997).

In a recent paper we have carried out an extended studied of the self-aggregation in the six most abundant anthocyanidin 3-glucosides (Leydet et al., in press), by means of 1H NMR and UV–Vis absorption. It was verified that the rate and equilibrium constants of the network of chemical reactions involving the dyes is dramatically dependent on the anthocyanin concentration. In this work we report the circular dichroism spectra of these compounds, Scheme 1, and correlate the magnitude of the CD signal with the angle and distance between the dipolar moments of the monomers in the dimer.

2. Experimental

Myrtiltin chloride, oenin chloride, kuromanin chloride and callistephin chloride were purchased from Extrasynthese; peonidin-3-glucoside chloride and petunidin-3-glucoside chloride were purchased from PhytoLab. All the reagents (≥95%) were used...
without any further purification. The solutions were prepared in Millipore water, and HCl 0.1 M was employed to acidify the samples at pH 1.

UV–Vis absorption spectra were recorded on Varian Cary 100 Bio, Varian Cary 5000i and Jasco V-530 spectrophotometers.

2.1. Circular dichroism analysis

Myrtillin chloride, oenin chloride, kuromanin chloride, callistephin chloride, peonidin-3-glucoside chloride and petunidin-3-glucoside chloride solutions at pH 1 were prepared 12 h before the experiments and kept at 4°C.

Circular dichroism were performed using near-UV and visible (350–700 nm) CD in a Jasco J810 spectropolarimeter equipped with a temperature control unit Julabo F25 using a range of concentration of the different 3-glucoside anthocyanins from 10⁻³ to 10⁻⁶ M. Near-UV and visible CD spectra were recorded with 0.01, 0.05, 0.1 and 1 cm (linear) path length quartz cuvette at 20°C, according to the measured high tension (HT) voltage measured in each sample. For each spectrum, three scans were averaged and anthocyanidin 3-glucosides concentration was checked by absorbance at the maximum in the visible region using the molar absorption coefficients of each flavylium cation under study (myrtillin chloride, oenin chloride, kuromanin chloride, callistephin chloride, peonidin-3-glucoside chloride and petunidin-3-glucoside chloride) on a UV–Vis spectrophotometer Jasco V-530.

Molar ellipticity for each compound has been calculated using the formulae:

\[ \theta = \frac{\theta_{\text{measured}}}{C \cdot l} \text{ deg cm}^2 \text{ dmol}^{-1} \]

where \( \theta \) is the ellipticity (mdeg), \( C \) the concentration (mol l⁻¹) and \( l \) the path length (cm).

CD spectra of the appropriate blanks (HCl 0.1 M) were recorded and subtracted from the anthocyanidin 3-glucosides spectra.

3. Results and discussion

3.1. Circular dichroism

The circular dichroism spectra representing the ellipticity of petunidin-3-glucoside at pH 1.0 is shown in Fig. 1A. The molar ellipticity at the maximum of the first and second cotton bands is represented in Fig. 1B. The same is shown for oenin (malvidin 3-glucoside) in Fig. 2A and B. Identical figures for the four remaining anthocyanins of Scheme 1, are reported in Supplementary material, Fig. S1–S4. At lower concentrations the molar ellipticity

![Scheme 1. The six anthocyanidin 3-glucosides.](image_url)
in the visible region is practically zero, indicating that the monomer does not exhibit signal. Signal rises when concentration increases due to the appearance of supramolecular chirality upon aggregation.

The fittings of the molar ellipticity of all the six anthocyanins were achieved using a monomer–dimer model (see below) and the dimerization constants previously calculated by $^1$H NMR, (Leydet et al., in press).

As shown in Figs. 1 and 2 (as well as Fig. S1–S4 in Supplementary material) the flavilium cation adducts of the six anthocyanidin 3-glucosides of Scheme 1, self-associate in a left-handed manner, as reported for the 3,5-diglucoside analogues (Hoshino, 1982). The CD strongest signal occurs for petunidin-3-glucoside [1982). The CD strongest signal occurs for petunidin-3-glucoside $^1$H NMR data of the six anthocyanidin 3-glucosides (Leydet et al., in press).]

### Table 1

**Ellipticity data for the six anthocyanidin 3-glucosides.a**

<table>
<thead>
<tr>
<th>Anthocyanin</th>
<th>$\theta_1$ First Cotton (deg cm$^2$ dmol$^{-1}$)</th>
<th>$\theta_2$ Second Cotton (deg cm$^2$ dmol$^{-1}$)</th>
<th>$\theta_{dimer}$/M$^{-1}$ cm$^{-1}$ ($\theta_{max}$)</th>
<th>Corrected CD intensity$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myrtillin</td>
<td>-76,610 (539 nm)</td>
<td>57,870 (452 nm)</td>
<td>28,600 (483 nm)</td>
<td>2.8</td>
</tr>
<tr>
<td>Oenin</td>
<td>-29,550 (550 nm)</td>
<td>16,830 (492 nm)</td>
<td>40,300 (514 nm)</td>
<td>3.3</td>
</tr>
<tr>
<td>Petunidin</td>
<td>-94,740 (539 nm)</td>
<td>69,820 (461 nm)</td>
<td>41,700 (497 nm)</td>
<td>1.1</td>
</tr>
<tr>
<td>Kuromanin</td>
<td>-55,130 (539 nm)</td>
<td>25,000 (452 nm)</td>
<td>22,700 (503 nm)</td>
<td>1.7</td>
</tr>
<tr>
<td>Peonidin</td>
<td>-28,300 (541 nm)</td>
<td>13,310 (466 nm)</td>
<td>41,100 (503 nm)</td>
<td>4.0</td>
</tr>
<tr>
<td>Calistephan</td>
<td>-19,080 (529 nm)</td>
<td>13,610 (414 nm)</td>
<td>56,220 (496 nm)</td>
<td>0.58</td>
</tr>
</tbody>
</table>

a Obtained from the fitting of Fig. 1B and the equivalent figures in supplementary material.

b Calculated as the sum of the maximum of the absolute value of the 1st and 2nd Cotton bands divided by the molar absorption coefficient of the dimer, see below.

### 3.2. $^1$H NMR

In a recent paper (Leydet et al., in press) we reported that the $^1$H NMR peaks of the protons $H_6$, $H_8$ and $H_9$ of the flavilium cation of anthocyanidin 3-glucosides are dramatically shifted to high field by increasing concentration. This is a result of the shielding of these protons upon aggregation, a phenomenon previously observed by other authors (Hoshino, 1992; Hoshino et al., 1982; Houbiers et al., 1998), see Supplementary material for the $^1$H NMR data. The difference between the chemical shift of the dimer and monomer, Table 2, suggests that protons $H_6$, $H_8$ and $H_9$ are more affected than proton $H_5$ by the interaction in the aggregate, and this should be an important clue for the determination of the dimer structure.

The $^1$H NMR data of the six anthocyanidin 3-glucosides (Leydet et al., in press) was treated with a monomer–dimer and isodesmic models (Dimicoli and Hélène, 1973) the last one considering the existence of higher order aggregates with the same association.
constant. In both cases good fittings have been obtained and the 1H NMR data was not able to discriminate between the two models.

### 3.3. Monomer–dimer model

Similarly to the data of the 1H NMR, the circular dichroism spectra presented in this work was also treated with a monomer–dimer model, as follows:

The monomer (M)–dimer (D) equilibrium is described by Eq. (1):

\[
M + M \leftrightarrow D
\]

and the dimeric constant (K\textsubscript{D}) is defined by Eq. (2):

\[
K_D = \frac{|D|\text{eq}}{|M|\text{eq}^2} = \frac{\chi^D}{2K M \chi^M}
\]

Considering the mass balance of the system given by Eq. (3):

\[
|M|\text{eq} + 2|D|\text{eq} = C_0
\]

\[
\chi^M + \chi^D = 1
\]

where the molar fractions (\(\chi\)) are defined according the Eqs. (4) and (5):

\[
\chi^M = \frac{|M|\text{eq}}{C_0} = -\frac{1 + \sqrt{1 + 8K M K_D}}{4K_D C_0}
\]

\[
\chi^D = \frac{2|D|\text{eq}}{C_0}
\]

Finally, the molar ellipticity of the system is defined as:

\[
[\theta] = [\theta]_M \chi_M + [\theta]_D \chi_D
\]

where [\(\theta\)] is the 1\textsuperscript{st} or 2\textsuperscript{nd} cotton bands signal [\(\theta\)]\textsubscript{M} the CD signal of the monomer (\(\approx 0\)) and [\(\theta\)]\textsubscript{D} the molar ellipticity of the dimer.

Fitting was achieved for the same set of constants calculated on the basis of the 1H NMR chemical shifts (Leydet et al., in press), see Table 3. However, good fittings are also obtained using the isodemic model which implies the formation of n-Mers with identical successive association constants. Consequently, it cannot be excluded the formation of higher order aggregates, but the accuracy of the data does not allow discrimination between monomer–dimer and alternative models. It is worth to comment that this conclusion cannot be extended, in principle, to the self-aggregation of the quinoidal base, where indirect evidence to the formation of higher order aggregates was obtained (Hoshino et al., 1981a, b). Regarding to the flavylum cation it is possible that its positive charge hinders the association of the dimer with additional monomers, because of the growing electrostatic repulsion.

The following discussion will be carried out using the monomer–dimer model.

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### Table 2

Variation of the chemical shifts from the dimer to the monomer obtained by 1H NMR (Leydet et al., 2011).

<table>
<thead>
<tr>
<th>Anthocyanin</th>
<th>H\textsubscript{4}</th>
<th>H\textsubscript{5}</th>
<th>H\textsubscript{8}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myrtilllin</td>
<td>1.06</td>
<td>0.96</td>
<td>0.54</td>
</tr>
<tr>
<td>Oenin</td>
<td>0.73</td>
<td>0.78</td>
<td>0.49</td>
</tr>
<tr>
<td>Pettunidin-3-glucoside</td>
<td>1.27</td>
<td>1.2</td>
<td>0.69</td>
</tr>
<tr>
<td>Kuromanin</td>
<td>0.75</td>
<td>0.7</td>
<td>0.4</td>
</tr>
<tr>
<td>Peonidin-3-glucoside</td>
<td>0.89</td>
<td>0.75</td>
<td>0.51</td>
</tr>
<tr>
<td>Callicrethrin</td>
<td>0.47</td>
<td>0.44</td>
<td>0.31</td>
</tr>
</tbody>
</table>

### Table 3

Angle and distance between the dipole moments of monomers in dimers (calculated from the 0–0 electronic absorption band).

<table>
<thead>
<tr>
<th>Anthocyanin</th>
<th>K\textsubscript{D} (\text{M}^{-1})</th>
<th>(\lambda_0) (nm)</th>
<th>(\lambda_1) (nm)</th>
<th>(\lambda_2) (nm)</th>
<th>Angle\textsuperscript{a} ((^\circ))</th>
<th>Distance\textsuperscript{b} (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myrtilllin</td>
<td>1240</td>
<td>529</td>
<td>572</td>
<td>497</td>
<td>–100</td>
<td>2.9</td>
</tr>
<tr>
<td>Oenin</td>
<td>976</td>
<td>532</td>
<td>562</td>
<td>519</td>
<td>–128</td>
<td>5.2</td>
</tr>
<tr>
<td>Pettunidin-3-glucoside</td>
<td>900</td>
<td>531</td>
<td>569</td>
<td>498</td>
<td>–97</td>
<td>2.5\textsuperscript{c}</td>
</tr>
<tr>
<td>Kuromanin</td>
<td>700</td>
<td>525</td>
<td>558</td>
<td>495</td>
<td>–103</td>
<td>3.2</td>
</tr>
<tr>
<td>Peonidin-3-glucoside</td>
<td>661</td>
<td>526</td>
<td>557</td>
<td>495</td>
<td>–114</td>
<td>3.2</td>
</tr>
<tr>
<td>Callicrethrin</td>
<td>404</td>
<td>508</td>
<td>529</td>
<td>475</td>
<td>–110</td>
<td>4.5</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Same constants of the 1H NMR studies (Leydet et al., in press).

\textsuperscript{b} Absorption maximum of the monomer.

\textsuperscript{c} Absorption maximum of the J-band.

\textsuperscript{d} Absorption maximum of the H-band.

\textsuperscript{e} Estimated error in the angle \pm 10\(^\circ\); distance \pm 0.2 Å; on the basis of the equations involved, the distance error increases when the angle approaches 90\(^\circ\) and this value could be underestimated.

\textsuperscript{f} The sign of the anthocyanidin 3-glucoside self-aggregates overlay angle is negative because the front molecule covers the rear molecule in an anti-clockwise turn around an axis coinciding with the direction of the molecules (Klyne and Prelog, 1960).

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### 3.4. UV–Vis absorption

The effect of the aggregation in the UV–Vis absorption spectra is clearly observed when the molar absorption coefficients \(\varepsilon\) (\text{M}^{-1} \cdot \text{cm}^{-1}) are represented as a function of the anthocyanin concentration, see Fig. 3A for petunidin-3-0-glucoside and Fig. 3B for oenin.

Inspection of Fig. 3 shows the splitting of the monomer absorption in two new red and blue shifted absorptions upon aggregation, see below. The individual spectra of the monomer and dimer in the visible region were obtained as reported previously (Cruz et al., 2010). Thus, a set of absorption spectra at several concentrations (the same range of concentrations employed in the CD measurements) was treated by FiNAl algorithm (Antonov and Petrov, 2002; Antonov et al., 1999). Taking into account the dimerization constant, this algorithm recovers the individual spectrum of the monomer and dimer and gives their contribution (mole fraction) for each concentration. The monomer and dimer absorption bands were additionally deconvoluted using Gaussian functions, see Fig. 4 for oenin as an illustrative example.

The absorption spectrum of the monomer, Fig. 4A, shows the existence of two transitions, the one of lower energy exhibiting a shoulder, and the other of higher energy exhibiting two vibrational bands (for example, in the case of oenin, the distance between the vibration maxima (532 and 505 nm) is 27 nm). Decomposition of the dimer of each anthocyanin was achieved using six bands, Fig. 4B. Each of the monomer absorption bands splits into new bands, corresponding to H and J aggregates (Spano, 2010). The situation is illustrated in Scheme 2.

In the present paper calculations of the distance and angle were performed on the 0–0 absorption band (532 nm for oenin in Fig. 4A), following the Kasha theory (Kasha et al., 1965). It is important to stress that the following calculations do not predict the relative position of the two monomers in the dimer, but the distance and angle between the respective transition dipole moments. Moreover, the position of the transition moment of the monomers...
in each anthocyanin could be slightly different, due to the different substitution pattern.

### 3.5. Calculations

The two H and J absorption bands of the dimer were obtained by spectral decomposition and the angle and distance between the transition moments can be interpreted through the exciton model using Eqs. (7) and (8), where the angle \( \alpha \) between the transition moments is defined by:

\[
\alpha = 2 \arctan \sqrt{\frac{f_H}{f_J}}
\]

and the distance (R in Å) between the monomers in the dimer is defined by the expression:

\[
R = \sqrt{\frac{2.14 \times 10^{16} \cos \alpha \cdot f_M}{\Delta \nu \cdot \nu_M}}
\]

\( \nu_X \) is position of the band X in cm\(^{-1}\), \( f_X = 1.3 \times 10^{-4} \Theta(n) \int_0^{\infty} c(v) dv \), \( \Theta(n) = \frac{n^2}{1.4 \times 10^{-2}} \) is factor of the environment, \( n \) is refraction index, \( \Delta \nu \) is the difference of the positions of the split bands (J and H) in cm\(^{-1}\).

The control of the spectral decomposition can be made by the coincidence, within experimental error, of the energy of the absorption maximum of the monomer and the semi sum of the identical energies of the H and J bands.

In Table 3, the results for the six anthocyanidin 3-glucosides are shown.
Inspection of Table 3 suggests that in the case of the 0-0 band the angle between the transition moments of the monomers in the dimer are similar and thus the distance should be the crucial parameter to account for the CD signal. According to the theory, the intensity of the CD signal is proportional to the inverse of the square of the distance (Berova and Di Bari, 2007) and such a relation was observed, as shown in Fig. 5A.

Calculations based on the exciton model have been questioned, in particular when distances lower than Van Der Waals (3.5 Å) are obtained (Arik and Onganer, 2003). However, the existence of hydrogen bonds could lead to shorter values. In addition, anthocyanins are not planar molecules and by consequence their dipole moments are predicted to be out of the plane formed by ring A and C. It is possible to conceive geometries where the two dipoles are closer than the atoms of the whole molecule, if the dimers interact through the same face.

In Fig. 5B the plot of the difference between the chemical shift of the monomer and dimer for protons H4 (●), H6 (□) and H8 (○) as a function of the corrected CD signal suggests, in spite of the great dispersion, that the increasing of the CD signal and the magnitude of the chemical shifts follow the same trend.

### 4. Conclusion

The self-aggregation of the flavylium cation of the six most common anthocyanidin 3-glucosides is responsible for the chemical shift variations observed in the $^1$H NMR spectra, appearance of a CD signal characteristic of a left-handed aggregation and split of the UV–Vis absorption band in two other bands (red and blue shifted). In terms of the consequences on the colour expression in plants, the splitting of the absorption bands upon aggregation leads to a slight modification of the colour tonality. Moreover, aggregation stabilizes the colour arising from the flavylium cation because the nucelophilic attack of water occurs at higher pH values. The three techniques (NMR, CD and UV–Vis) lead to self-consistent results and are compatible with a monomer–dimer aggregation type for the flavylium cation. One possible explanation for the apparent lack of high order aggregates is the positive charge of the flavylium cation, which prevents the addition of more monomers to the dimer, due to the increasing electrostatic repulsion.

### 5. Uncited references


### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytochem.2012.12.011.

### References


$^2$ We thank the referee who raised this question.


