Two-dimensional Nuclear Overhauser Enhancement NMR Experiments on Pelargonidin-3-glucopyranoside, an Anthocyanin of Low Molecular Mass

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Pelargonidin-3-glucoside has been isolated from the acidified methanolic extract of strawberries (*Fragaria anannassa* variety Corona) by successive application of an ion-exchange resin, droplet-counter chromatography and gel filtration. The pigment in acidified methanolic solution was studied by means of the two-dimensional nuclear Overhauser enhancement NMR technique, and the sugar unit was found to be attached to the 3-position on the aglycone. At +20 °C the pigment was found to be in the extreme narrowing limit where the NOESY cross-peaks are negative. However, at −20 °C this low-mass anthocyanin could be studied in the slow motion regime where the NOESY cross-peaks are positive. With a mixing time of 0.3 s, the glucose H1″–H4″ proton pair was measured in the initial cross-relaxation rate and their cross-peak volume corresponded to the H1″–H4″ distance found in a $^4C_1$ chair conformation.

Several chromatographic and spectroscopic methods have been used to gain structural information about anthocyanins.[1,2] When suitable amounts of material are available, the use of ^1H and ^{13}C NMR techniques can provide an exceptionally large amount of information regarding the aglycone skeleton and the nature and number of substituents.[1-5] Even though H_2O_2 oxidation and UV–VIS spectroscopy have been extensively used to determine the linkages between the aglycone and the sugar(s),[6-8] only nuclear Overhauser enhancement (NOE) measurements have provided similar information in complex anthocyanins.[1,9-11] For many purposes the 1D-NOE approach is sufficient, especially when the amount of sample is limited. However, more reliable quantitation of NOE measurements can be achieved by the use of two-dimensional NOE spectroscopy (NOESY).

The present work describes applications of the NOESY NMR method in the structure elucidation of the anthocyanin pelargonidin-3-glucoside (1). In order to show how 'relationships through space' between hydrogen atoms can be used to give information about the conformation of the sugar part, the NOESY data were subjected to distance calculations. Previously, we have shown how intermolecular association behaviour of anthocyanin of relative large molecular mass can be detected by the use of quantitative NOESY experiments.[5,12] In this paper we demonstrate how both positive and negative (depending on the temperature) NOESY cross-peaks are detected in a pigment with a molecular mass as low as 433.4 amu.

Experimental

Extraction. Ripe fruits of strawberries (*Fragaria anannassa* variety Corona) were purchased in Bergen (Norway) in July 1991 and stored at −20 °C. The fruits were extracted twice with MeOH containing 0.07 % conc. HCl. The filtered extracts were combined and concentrated under reduced pressure.

Chromatography. Thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), gel filtration with Sephadex LH-20 and chromatography on Amberlite XAD-7 resin were performed according to published procedures.[2,11] UV–VIS absorption spectra were recorded with a photo-diode array detector during HPLC analysis. Droplet counter-current chromatography (DCCC), was carried out with the upper layer of 1-butanol–acetic acid–water (BAW; 4:1:5, v/v) as the mobile phase. The chromatograph (Eyela Tokyo Rikakikai, Model A DCC) was fitted with 300 glass capillaries (40 cm × 2.0 mm l.d.).

NMR techniques. The ^1H NMR spectra were obtained at 400.13 MHz on a Bruker AM-400 instrument. The 1D-^1H NMR spectrum was recorded at +20 °C. The residual ^1H signal (3.4 ppm from TMS) of the solvent (CF_3COOD–CD_3OD; 8:92 v/v) was used as secondary reference for the chemical shifts. For all experiments, 24.9 mg of the pigment were dissolved in 0.5 ml of the solvent.

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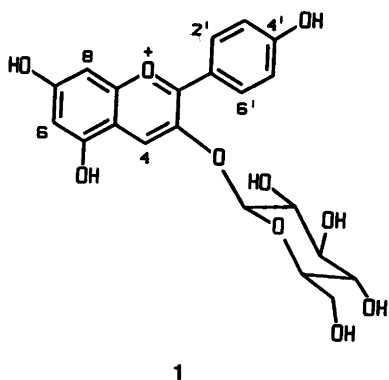
NOESY experiments. Three NOESY experiments were collected; two at −20 and one at +20 °C using the method of time-proportional phase increments. The two experiments at −20 °C were collected into 2K complex data points, a spectral width of 2994 Hz, and mixing times of 0.6 and 0.3 s, respectively. The experiment with 0.6 s mixing time was acquired with 64 scans for each of the 775 t_1-slices. The experiment with 0.3 s mixing time was acquired with 32 scans for each of the 810 t_1-slices. The experiment at +20 °C was obtained with a spectral width of 3500 Hz, 32 scans for each of the 800 t_1-slices and a mixing time of 0.6 s into 2K complex data points. The acquired NOESY data were transferred to a Silicon-Graphics SGI-4D/25 work-station and processed with the NMR processing software FELIX (Hare Research, Inc., Woodinville, WA, USA). All spec-

tra were processed with an exponential function increasing the linewidth by 2 Hz in the t_2-dimension before the spectra were phased and baseline corrected. In the t_1-dimension the data were lightly apodized by a sinebell-squared function, shifted 90 degrees and dropped to zero at the last t_1-incremented free-induction decay acquired in each of the three experiments.

Distance measurements from the nuclear Overhaüser effect. In the slow motion regime ($\omega \tau \gg 1$) the zero-quantum relaxation term dominates over the one-quantum and two-quantum terms in the absence of local motion at the NMR frequency.[13] In a two-spin system under these conditions, the magnetically excited spin i relaxes by dipolar magnetization transfer to spin j at the relaxation rate given in eqn. (1) were γ is the magnetogyric ratio, τ the rotational

$$R_{ij} = -\frac{\gamma^4 \cdot \hbar^2 \cdot \tau}{10 \cdot r_{ij}^6} \qquad (1)$$

correlation time and r_{ij} is the distance separating the two cross-relaxing protons. The relaxation rates of two protons pairs R_{ij} and R_{kl} can be found from the corresponding cross-peak volumes; the spins k and l are separated by a known fixed distance r_{kl}. The distance r_{ij} can then be calculated from eqn. (2). This equation is valid for isotropically
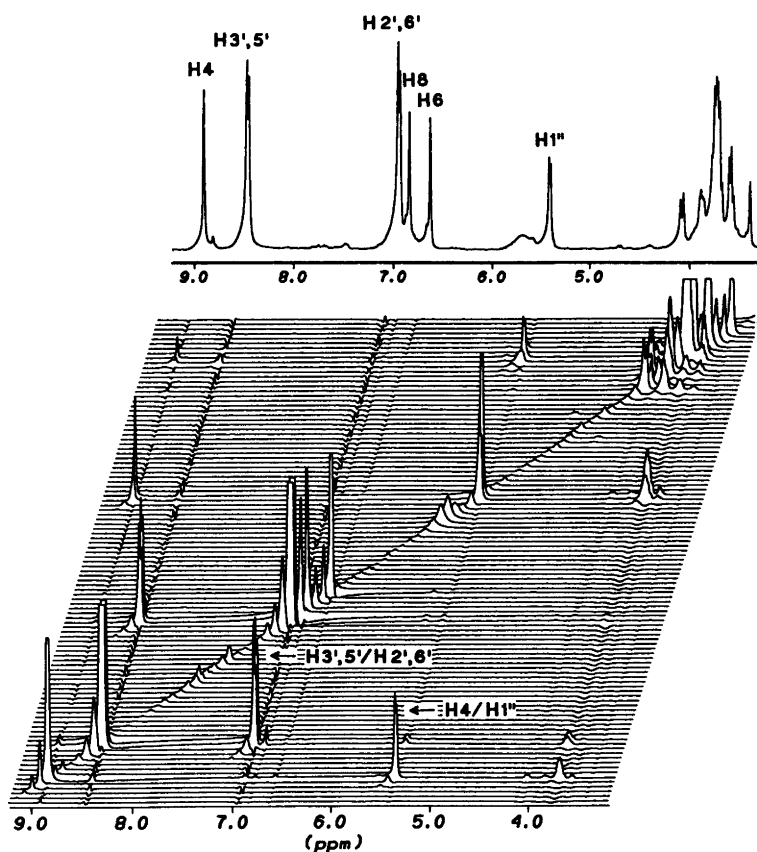

Fig. 1. Top: one-dimensional ¹H NMR spectrum of pelargonidin-3-glucoside (1) at −20 °C; bottom: stacked plot of the NOESY experiment on 1 with 0.6 s mixing time at −20 °C. The cross-peak labelled H4/H1″ shows that the glucose unit is attached to the aglycone 3-position. See the text for details regarding the other labelled cross-peak (H3′,5′/H2′,6′).
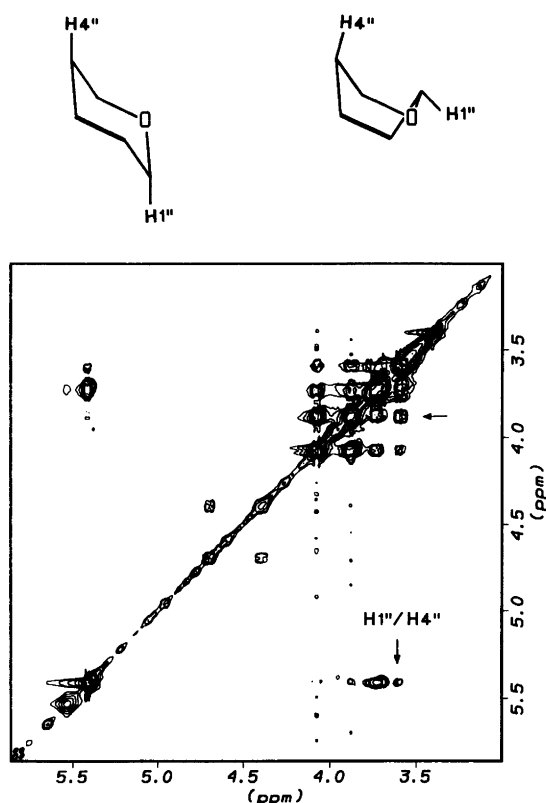
Fig. 2. Top: two conformations (left, chair $^4C_1$; right, boat) of modified glucopyranose. The $-CH_2OH$ and $-OH$ groups are not shown, and only H4″ and H1″ are labelled; bottom: sugar region of a NOESY spectrum (contour plot) of pelargonidin-3-glucoside (1) recorded at $-20\,^\circ C$ with 0.3 s mixing time. The volume of the cross-peak labelled H1″/H4″ corresponds to the distance of 3.6 Å between H-1″–H-4″ in the chair conformation of the sugar. The horizontal arrow points to a cross-peak that corresponds to a single proton pair separated by 2.8 Å. See the text for details.

$$\frac{R_{ij}}{R_{kl}} = \frac{r_{kl}^6}{r_{ij}^6} \qquad (2)$$

tumbling molecules in the absence of differential local motion and spin diffusion.[14]

Results and discussion

Compound 1 was isolated from the strawberry (Fragaria anannassa var. Corona). The UV–VIS spectrum of 1 taken during on-line HPLC showed a visible maximum at 504 nm with A_{440}/A_{504} of 0.43 %, indicating the presence of a 3-glycoside with a pelargonidin nucleus.[15] The downfield part of the ^1H NMR spectrum of 1 (Fig. 1) showed a 4 H AA′XX′ system at 8.61 ppm ['d', H-2′, H-6′, J (2′–3′) + (6′–5′) = 9.1 Hz], and 7.10 ppm ('d', H-3′, H-5′) and three broad singlets at 6.96 ppm (H-8), 6.74 ppm (H-6) and 9.10 ppm (H-4).

The anomeric ^1H signals in the ^1H NMR spectrum of 1 appear considerably downfield of the other sugar resonances,[16] and thus the doublet at 5.37 ppm [J(1″–2″) 7.6 Hz,

Fig. 1] together with the integration data defined the pelargonidin:sugar ratio as 1:1. The ^1H signals at 4.02, 3.82, 3.78, 3.67, 3.65 and 3.56 ppm in the ^1H NMR spectrum of 1 were in accordance with published data on cyanidin-3-glucoside[2] assigned to H-6A″, H-6B″, H-2″, H-5″, H-3″ and H-4″, respectively, of a glucose unit.

The problem of assigning the point of attachment between the aglycone and the sugar of 1 was addressed by NOESY experiments. In the stacked plot (Fig. 1) based on the NOESY experiment with a 0.6 s mixing time at $-20\,^\circ C$, the prominent cross-peaks between the H-4 and the H-1″ (anomeric proton) confirmed that the sugar was attached to C-3. This two-dimensional experiment demonstrates that pelargonidin-3-glucoside was monitored in the slow motion regime by the presence of positive cross-peaks. However, the small H4 to H6A″, H6B″, H3″, H5″, H2″ and H4″ cross-peaks due to secondary cross-relaxation indicate that the 0.6 s mixing time is too long to obtain initial ^1H pair cross-relaxation.

In order to obtain more quantitative distance information, a second NOESY experiment was acquired at the same temperature ($-20\,^\circ C$) with the shorter mixing time of 0.3 s. A contour plot of the sugar ^1H region of this experiment is shown in Fig. 2. With the exception of the anomeric ^1H, the sugar ^1H are found in the rather narrow chemical shift range of 3.65 to 4.15 ppm. The crowding of this portion of the spectrum limits accurate single cross-peak volume integration. However, the anomeric ^1H (5.37 ppm) displays a well-resolved cross-peak to H4″ and the integrated volume of this cross-peak is converted by the r^6 relationship (between cross-relaxation rate and distance between the cross-relaxing protons) into a H1″–H4″ distance.

By comparing the cross-peak volume of the two 'yardstick' ^1H pairs in the molecule, i.e. the aglycone H2′–H3′ (also H5′–H6′) and the glucose H6A″–H6B″ pairs which are separated by fixed distances of 2.49 and 1.8 Å, respectively. ^1H pairs separated by 2.49 Å or more can be approximated to be in the initial (cross-relaxation) rates. Their cross-peak volumes can thereafter be converted to yield a distance. The two geminal H6A″ and H6B″ cross-relax very rapidly, and this pair is beyond the initial rate when a NOESY mixing time of 0.3 s is used. This is reflected by the fact that the H6A″–H6B″ distance is calculated to be 2.1 Å when the H2′–H3′ pair is used as a reference, i.e. taking half the H3′, 5′/H2′, 6′ cross-peak volume to represent a distance separation of 2.49 Å, the H6A″–H6B″ cross-peak volume is calculated to represent a ^1H pair separated by 2.1 Å, while the actual distance is 1.8 Å. However, ^1H pairs separated by 2.49 Å or more, can be approximated to be in the initial rate at a NOESY mixing time of 0.3 s. Therefore, using half the H3′, 5′/H2′, 6′ cross-peak volume (Fig. 1) to represent a ^1H pair separated by 2.49 Å, the H1″–H4″ cross-peaks in Fig. 2 correspond to a calculated H1″–H4″ distance of 3.6 Å.

As indicated by the boat and chair ($^4C_1$) conformations of the modified glucopyranose given in Fig. 2, the H1″–H4″

Fig. 3. Sugar regions of the two NOESY spectra of pelargonidin-3-glucoside (1) recorded at two different temperatures. A) 0.3 s mixing time at −20 °C. The arrow points to the positive H6A″/H6B″ cross-peak. B) 0.6 s mixing time at +20 °C. The arrow points to the negative H6A″/H6B″ cross-peak.
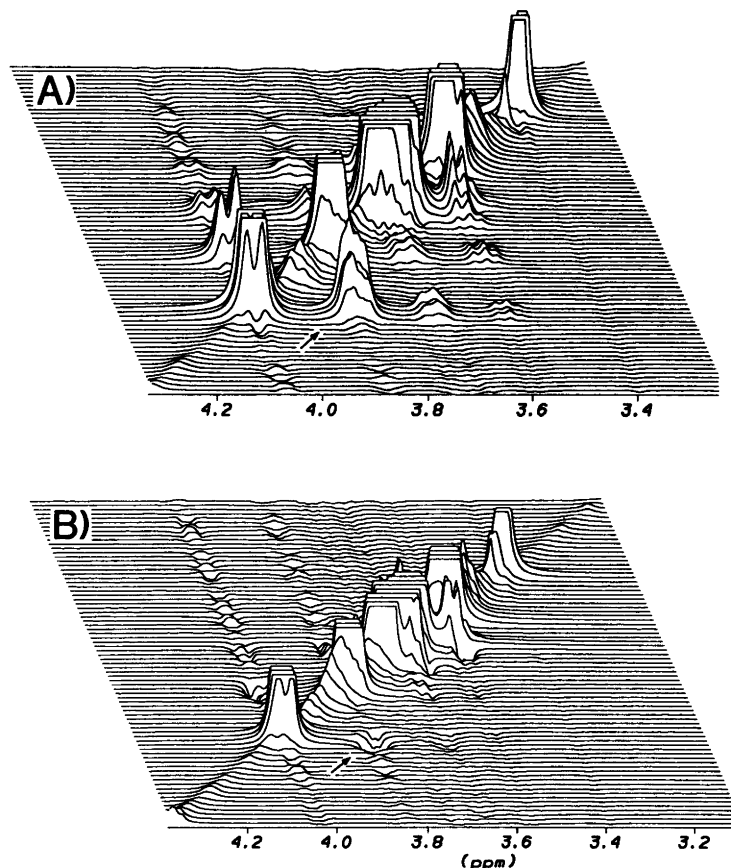
distances in these two conformations are different. From a representative model, these distances were found to be ca. 3.0 Å (boat) and ca. 3.6 Å (chair). Thus, the H1″–H4″ distance (3.6 Å) corresponding to the cross-peaks in the NOESY spectrum (Fig. 2) indicates that the glucose of pelargonidin-3-glucoside is a glucopyranoside in the $^4C_1$ conformation. This result was confirmed by a comparison of other sugar cross-peaks volumes. The cross-peak labelled with a horizontal arrow in Fig. 2 has a volume integral of about four times the H-1″/H-4″ cross-peak and corresponds to a single proton-pair separated by 2.8 Å. A proton-pair separated by 3.0 Å, as calculated for the H-1″–H-4″ distance in the boat conformation (Fig. 2), corresponds to three quarters of the size of the cross-peak labelled with the horizontal arrow in Fig. 2. Earlier reports of pelargonidin-3-glucoside isolated from strawberry[17–19] or any other source, have not discussed the sugar conformation of this pigment.

The NOESY NMR technique has found widespread use in structural studies of proteins and nucleic acids.[20] These macromolecules have been studied in the slow motion regime where the NOESY cross-peaks are positive. Fig. 3 shows how pelargonidin-3-glucopyranoside (1) can be studied in both the slow motion regime and in the extreme narrowing limit by changing the sample temperature. Therefore, when it is certain that the molecule in question is studied in the slow motion regime, then valuable struc-

tural information can be obtained from NOESY experiments on even low-molecular-mass compounds.

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