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DNA triplex stabilization property of natural anthocyanins

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Abstract

The DNA triplex stabilization property of seven natural anthocyanins (five monoglucosides and two diglucosides) has been measured by the mean of triplex thermal denaturation experiments. We have noticed a difference between the diglucosides that do not modify this melting temperature and the monoglucosides (namely 3-*O*-β-D-glucopyranoside of malvidin, peonidin, delphinidin, petunidin and cyanidin) which present a weak but significant stabilizing effect. It appears clearly that the difference between the two series could be due to the supplementary sugar moiety at the 5 position for the diglucosylated compounds, that would make them too crowded to allow interaction with the triplex. Among the monoglucoside series, the most active compounds are the only ones to embody a catechol B-ring in their structure that could be important for such an interaction. The need to have pure and fully characterized compounds to run these measurements, made it possible for us to unambiguously assign the ^{1}H and ^{13}C NMR spectra with the help of 2D NMR experiments. Thus, missing data of compounds not totally described earlier, are provided herein.

Keywords: Glucosylated anthocyanins; *Vitis vinifera*; *Malva sylvestris*; *Rosa gallica*; DNA triplex intercalators; Thermal denaturation experiments; ^{13}C NMR

1. Introduction

The regulation of gene expression by nucleic acids is a strategy that has the potential to be used for the treatment of genetic-based diseases (Hélène & Toulmé, 1990; Uhlmann & Peyman, 1990; Murray & Crockett, 1992; Crooke & Lebleu, 1993). Oligonucleotides may halt translation by specific recognition and binding to messenger RNA (antisense approach) (Stein & Cheng, 1993). They may also impede transcription by binding to the major groove of double-stranded DNA (antigene approach) (Thuong & Hélène, 1993; Radhakrishnan & Patel, 1994). In the latter case, a (T, C)-containing third strand (pyrimidine motive) runs parallel to the mandatory oligopurine strand of a duplex, through Hoogsteen hydrogen bonds, with the formation of T.A*T and C.G*C^{+} triplets (the dots represent the Watson–Crick appariements while the asterisks are for the Hoogsteen ones) (Le Doan et al., 1987; Moser & Dervan, 1987). Alternatively, a (G, A)-containing third strand can bind in an antiparallel orientation with respect to the oligopurine target strand through reverse Hoogsteen hydrogen bonds, leading to the formation of C.G*G and T.A*A triplets (Beal & Dervan, 1991; Pilch, Levenson & Shafer, 1991). The (G, T)-containing oligonucleotides bind in

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either a parallel or an antiparallel orientation depending on the sequence (i.e. length of G and T tracts and number of GpT/TpG steps) (de Bizemont, Duval-Valentin, Sun, Bisagni, Garestier & Héléne, 1996).

At the present time, there are several limitations to the development of this strategy. Except for a few cases (Svinarchuk, Paoletti & Malvy, 1995), the stability of the triple-helical complexes is usually weaker than that of double-helical complexes. In addition, the requirement for cytosine protonation in the pyrimidine third strand limits triplex stability at neutral pH. A means to improve this stability is to covalently attach DNA intercalators to the third strand (Héléne, 1989; Sun et al., 1989; Giovannangeli, Rougée, Garestier, Thuong & Héléne, 1992). Acridine is an intercalator that is commonly used for this purpose due to its well-known ability to significantly stabilize triple helices in such a case (Cassidy, Strekowski, Wilson & Fox, 1994). Recently, anthraquinone sulfonamide derivatives (Kan, Armitage & Schuster, 1997), naphtyl quinoline (Cassidy, Strekowski & Fox, 1996) and benzopyridoindole derivatives (Kim et al., 1997) have proved to selectively stabilize triplex DNA sequences.

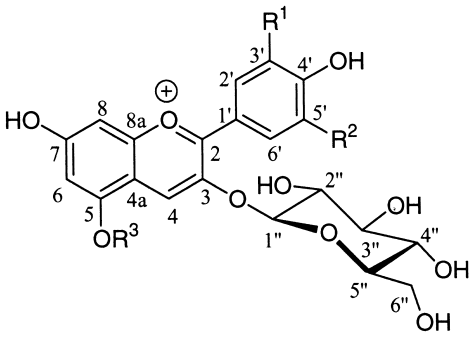
Anthocyanins, the most important group of water-soluble plant pigments (Harborne & Grayer, 1988; Strack & Wray, 1994), are structurally very closely-related to several potent intercalators. These anthocyanins are natural aromatic compounds, and all derive from the flavylium cation (2-phenyl-benzopyrylium), with variation according to the hydroxylation and/or methoxylation patterns of the A and B-rings as well as to the possible glycosylation of the hydroxyl group at the 3 and/or 5 positions. Their most evident property in nature is their color that varies from pink to purple: this can be due to discrepancies in their structures as well as to the presence of natural copigments (Brouillard & Dangles, 1994). Among them, purines such as caffeine, but also adenine and adenosine, exert a strong copigmentation effect (Brouillard, Wigand, Dangles & Cheminat, 1991; Mistry, Cai, Lilley & Haslam, 1991). This phenomenon is due to the formation of molecular complexes involving π-stacking interactions. Nucleic acids such DNA and RNA have also been proved to act as strong effective copigments for natural anthocyanins (Mistry et al., 1991). In this case, the copigmentation effect was attributed to an intercalating phenomenon between two adjacent base pairs, the intercalation providing proper sites for π–π hydrophobic stacking. All these physical and structural characteristics cited above, and inherent to these cationic polyphenols, allowed us to envision that they could exhibit strong stabilizing property towards DNA triplexes. Therefore, we decided to evaluate natural anthocyanins, under their monoglucosylated (**1**–**5**) or their diglucosylated (**6** and **7**) forms (Fig. 1) with respect to this property.

Having at hand these pure compounds, ^{1}H and ^{13}C NMR spectra of compounds not totally described earlier were recorded, and all signals were unambiguously assigned with the help of 2D NMR experiments. The results are presented below.

2. Results and discussion

2.1. DNA triplex stabilization properties

The ability of anthocyanins to stabilize triple-helical complexes was measured by using the thermal denaturation method. In the model DNA triplex chosen, the third strand is bridged by a pentanucleotide loop to the first strand of the double-helical target, leading to bimolecular complexes allowing fast kinetics for its formation (Kool, 1996; Wang, Friedman & Kool, 1995). This triple-stranded complex is constituted by a *homopyrimidine.homopurine*homopyrimidine* motif in



Compound	Name*	R^1	R^2	R^3
1	Mal †	OMe	OMe	H
2	Peo †	OMe	H	H
3	Del †	OH	OH	H
4	Pet †	OMe	OH	H
5	Cy †	OH	H	H
6	Mal ‡	OMe	OMe	β-D-glc
7	Cy ‡	OH	H	β-D-glc

Fig. 1. Substitution sites and atomic numbering of the seven isolated anthocyanins (for the diglucosides **6** and **7**, the carbon atoms of the second sugar will be numbered with ‴ or designed as 5-*O*-β carbons). Abbreviations for general name of aglycones: Mal = malvidin; Peo = peonidin; Del = delphinidin; Pet = petunidin; Cy = cyanidin; † 3-*O*-β-D-glucopyranoside; ‡ 3,5-di-*O*-β-D-glucopyranoside.

which the third Hoogsteen strand is in a parallel orientation relative to the purine target strand (Fig. 2). Its formation occurs by base pairing of the two oligonucleotides **8** and **9** in a suitable buffer (see Section 3).

Thermal denaturation of such complexes, monitored by UV spectroscopy, generally occurs in a single transition from triplexes to fully dissociated strands. This dissociation generates a hyperchromic effect in the absorption spectrum of the medium ($\lambda_{max}^{H_2O}$ nm: 260), due to the transition of nucleic acid bases from a stacked to an unstacked state. The melting temperature (T_m) is defined as the temperature at which half of the complex is dissociated. With these kinds of model triple helical complexes, determination of the T_m values (which correspond, in all cases, to the only inflection point of the curve) is easier and unambiguous (Giovannangeli, Montenay-Garestier, Rougée, Chassignol, Thuong & Héléne, 1991; Kool, 1996).

The melting curves of the triple-stranded complex actually show a single transition from the bound to the dissociated state, as expected. Its melting temperature is equal to 47.5°C in the buffer used (see more detailed composition in Section 3). The T_m of the duplex obtained with the complementary oligonucleotides **9** and **10** (Fig. 3) in the same buffered solution is lower than those of the triple helical complex, with a value equal to 29.5°C (Table 1). The difference between these two melting temperatures, $\Delta = +18$°C more for the triplex, clearly indicates a third-strand contribution to the overall complex stability, and unambiguously attests to its formation. Identical

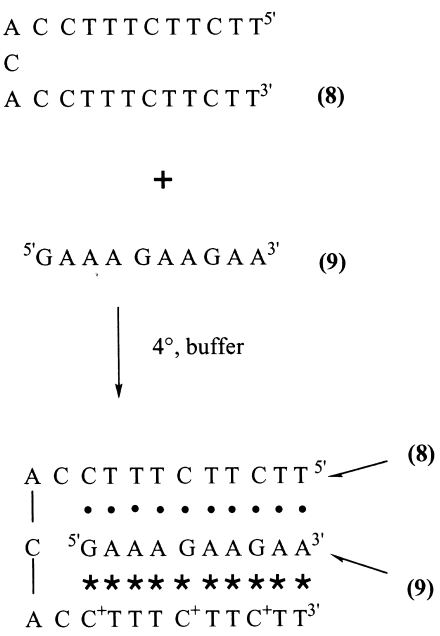


Fig. 2. Structure and formation of the triple-stranded complex studied (dots: Watson–Crick interactions; asterisks: Hoogsteen interactions).

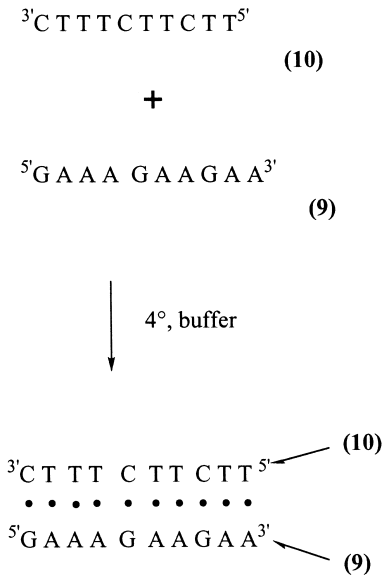


Fig. 3. Structure of the duplex (dots: Watson–Crick interactions).

proofs were also obtained with closely-related DNA sequences, which are now well known to afford obviously triple helical complexes (Michel, Gueguen, Vercauteren & Moreau, 1997; Godde, Toulmé & Moreau, 1998).

An increase of the melting temperature of such complexes, occurring in the presence of a derivative, indicates that this latter compound possesses stabilizing properties. The anthocyanins investigated were all obtained from natural sources: the monoglucosides from *Vitis vinifera* grapes (Bakker & Timberlake, 1985; Castagnino, 1996), and the diglucosides from petals of *Malva sylvestris* and *Rosa gallica* (Hayashi, 1962), respectively. Three independent buffered solutions of every compound in the presence of the triplex were then prepared, and each experiment was carried out two or three times. The melting curves obtained with every anthocyanin solution were collected, and the differences (ΔT_m) between the average value for each derivative and the average value obtained in the absence of this derivative are reported in Table 2. The mean values of experimental results are expressed, together with standard deviations from the mean. Comparative statistical analysis of groups was performed using Student's unpaired t test. This analysis

Table 1
Comparison of the melting temperature of the duplex and triplex

Complexes	Duplex	Triplex
T_m (°C)	29.5	47.5
Oligonucleotides[a]	9 + 10	8 + 9

[a] see Figs. 2 and 3.

shows that values superior to 0.5°C are significant (Table 2). Examples of melting curves, one corresponding to the thermal denaturation of the triplex alone (reference) and one obtained in the presence of compound **4**, are shown in Fig. 4.

The ΔT_m values clearly indicate that one series of these flavylium derivatives (compounds **1**–**5**) stabilizes DNA triple-helical complexes: positive ΔT_m values higher than 0.5°C with a maximum value reaching 2.1°C were obtained.

Stabilizing effects are meanwhile slightly lower than those expected. One could argue that anthocyanins do not remain in their native form in the medium in which the tests are performed. It is well known that these derivatives undergo transformations towards quinonoidal bases, hemiacetal and, eventually, chalcone forms in such aqueous solutions (Brouillard & Delaporte, 1977; Brouillard & Dubois, 1977). However, all these transformations are reversible; the ratio of each form depends on the pH of the solution and the cationic form is the only one existing when the pH is lower than 2. Thus, the overall equilibrium could be shifted towards the stabilizing form, even if it is not the major one at the beginning of the experiment, as it is known to occur in the copigmentation phenomenon (Brouillard et al., 1994).

Taking a closer look at the results, we may note that the compounds examined can be divided into two classes: the monoglucosides that possess stabilizing properties and the diglucosides that do not. The difference between these two groups is due to the presence of the sugar at the 5 position for compounds **6** and **7**, in place of a hydrogen for compounds **1**–**5**. So, we can conclude that the absence of stabilizing effects for the diglucosides is possibly due to the second sugar moiety, making the diglucosides too crowded to allow interactions with the triplex.

As good intercalators, in such experimental conditions, would show a $\Delta T_m > 5.0$°C (**BgPI**, a positive reference (Kim et al., 1997), give a ΔT_m equal to 5.2°C), the monoglucosides **1**–**5** have to be considered as only weak stabilizers of triplex. Here again, the sugar remaining at the 3 position could preclude efficient interactions of these compounds with the triplex. Within this group, however, the most active compounds, **3**, **4** ($\Delta T_m = 2.1$°C) and **5**, are the only ones to embody a catechol B-ring, which could play a major role in their better stabilizing properties.

Thus, these anthocyanins have proved useful to stabilize DNA triple-helical complexes, albeit relatively weak. This encourages us to synthesize non-glycosylated flavylium salts embodying or lacking a catechol B-ring, in order to assess whether this property could be optimized. Natural salts, such as apigeninidin and luteolinidin chlorides, as well as other fully-synthesized compounds that would have the advantage of being more easily bonded to the oligonucleotide, are currently under investigation.

Table 2
T_m values and ΔT_m obtained in the presence of different indicated anthocyanins

Compound	T_m (°C)	Standard deviation	ΔT_m (°C)[a]	Student's t test
None	47.5	0.5 (6)[b]	–	
1	48.5	0.4 (5)	+1.0	$p < 0.01$
2	48.2	0.3 (6)	+0.7	$p < 0.05$
3	49.2	0.6 (6)	+1.7	$p < 0.001$
4	49.6	0.5 (6)	+2.1	$p < 0.001$
5	48.9	0.5 (6)	+1.4	$p < 0.01$
6	47.3	0.5 (4)	−0.2	NS[c]
7	47.6	0.4 (5)	+0.1	NS
BgPI[d]	48.5	0.4 (5)	+1.0	$p < 0.001$

[a] Differences between the average value for each derivative and the average value without any derivative.
[b] Values in parentheses are the number of measurement.
[c] NS: non significant values ($p > 0.05$).
[d] Known intercalating agent used as positive reference; **BgPI**: benzo[g]pyrido[4,3-b]indole (Kim et al., 1997).

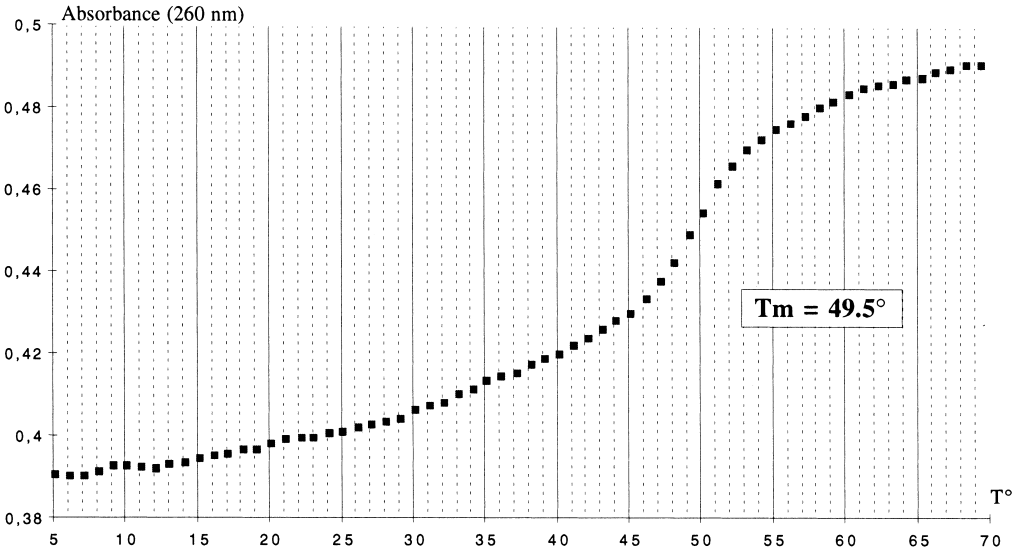
2.2. Complete NMR data for compounds *1*, *3*, *4* and *7*

The structure of each isolated compound was confirmed by comparing their NMR data to those reported in literature. Both ^{1}H and ^{13}C NMR spectra of compounds **2** (Van Calsteren, Cormier, Do & Laing, 1991), **5** and **6** (Van Calsteren et al., 1991; Andersen, Viksund & Pedersen, 1995) were identical to those previously reported, but only the ^{1}H NMR spectra of compounds **1** (Pale, Nacro, Vanhaelen & Vanhaelen-Fastré, 1997), **3** (Andersen, 1988; Pale et al., 1997), and **4** (Pale et al., 1997), showed acceptable agreement. The slight discrepancies, observed for compound **3** in ^{13}C NMR spectrum with literature data ($\Delta\delta$ 5 ppm max) (Andersen, 1988), were attributed to the fact that the authors recorded the spectrum in DMSO-d_6–HCl while we recorded it in CD_3OD–TFA. In the case of compounds **1**, **3** and **4**, and of compound **7** (which had never been described earlier by NMR) we carried out complete 1D and 2D NMR analysis (Balas & Vercauteren, 1994), in order to unambiguously assign their ^{13}C chemical shifts.

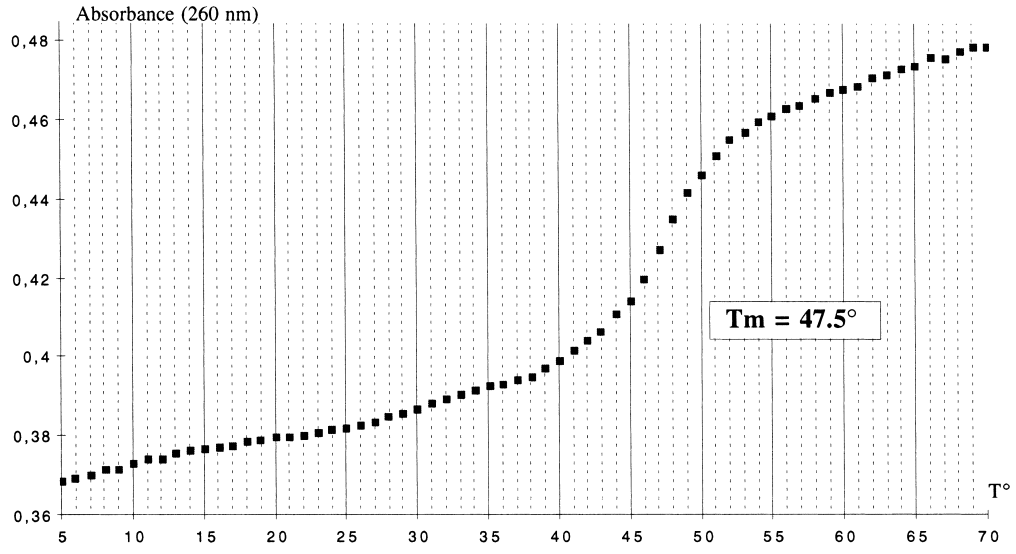
Anthocyanins are compounds that bear a few protons on their aglycone moiety, and this makes the interpretation of their NMR spectra difficult. In particular, H-6 and H-8 signals are not easily distinguished by ^{1}H NMR (Pedersen & Andersen, 1995). In order to overcome this problem, the authors based their structural assignments on the observation of a long-range coupling between H-4 and H-8 ($^5J_{H-H} = 0.7$ Hz) (Johansen, Andersen, Nerdal & Aksnes, 1991). However, in our hands, this coupling was not always

observed, even under high-resolution conditions. In fact, only nuclear Overhauser effects revealed the required information. The NOE measurement is most reliably achieved, whatever the field strength is, by the use of the "Rotating frame" two-dimensional experiment (ROESY (Bax & Davis, 1985)). Thus, the correlation between H-8 and H-2′ plus H-6′ leads to the unambiguous assignment of the H-8 proton signal (Fig. 5) and, by deduction, of the H-6 signal. From these signals, directly-bonded C-8 and C-6 were assigned via the HMQC (Bax & Subramanian, 1986) experiment, and remote atom signals (C-4a, C-5, C-7 and C-8a, namely) via the HMBC (Bax & Summers, 1986) correlation (Fig. 5).

The heterosidic linkages were determined through the oxygen at C-3 by the three-bond correlation of C-3/H-1″ in all cases (HMBC experiments), and at C-5, in addition, in the case of **7**, by the C-5/H-1‴ corre-



A: Example of curve obtained in the presence of compound **4**



B: Example of curve obtained with the triplex alone (reference)

Fig. 4. Examples of melting curves. (A): one of the curves obtained in the presence of **4**; (B): one of the curves obtained with the triplex alone (reference).

lation (Fig. 5). These linkages were further proved by the ROESY correlation between H-4 and anomeric proton(s).

Chemical shifts of these four compounds are listed in Table 3 (^{13}C) and in Section 3 (^{1}H).

3. Experimental

3.1. Spectroscopy

UV–Vis absorption spectra were recorded on a Hitachi U-2000 spectrophotometer in solution in 0.1 M HCl in H_2O. Fast Atom Bombardment (FAB+) mass spectra were obtained from glycerol suspension with a FINIGAN MAT TSQ 700 mass spectrometer in the positive mode. All NMR spectra were recorded on a Bruker AMX 500 spectrometer using ca. 10 mg of anthocyanidin dissolved in 0.6 ml of CD_3OD–TFA in a 5 mm tube. ^{1}H and ^{13}C measurements were carried out at 500.13 and 125.77 MHz, respectively. The temperature was maintained at 303 K. Chemical shifts (δ) in ppm are calibrated against the signals of CD_3OD ($\delta = 49.0$ and 3.3 from TMS). The two-dimensional experiments were run on an inverse-probe equipped with the Z-gradient coil for homo-spoil pulses. The $^{1}J_{^{1}H-^{13}C}$ experiments in the inverse mode (HMQC) were optimized for 130 Hz one-bond couplings while the inverse ^{2}, $J_{^{1}H-^{13}C}$ experiments (HMBC) were optimized for 7 Hz long-range couplings and suppression of direct residual correlation was obtained by a low-pass *J*-filter (3.8 ms).

3.2. HPLC conditions

Two solvents were used for elution: A (H_2O, TFA 0.15%) and B (H_2O–CH_3CN (70:30), TFA 0.15%). Detection was performed via an UV–Vis ICS detector. Prior to injection, all samples were filtered through a 0.45 μm Millipore membrane filter.

Analytical mode: analytical HPLC was performed on an ICS module system using an Ultrasep RP18 column (25 × 0.46 cm, 5 μm) equipped with a Lichrosorb RP18 precolumn. The elution gradient was linear from 65:35 to 0:100 ratio of A–B over 60 min. The flow rate was 1 ml min^{-1}.

Preparative mode: preparative HPLC was performed on an ICS module system using an Ultrasep ES100RP18 column (25 × 2 cm, 6 μm) equipped with a Lichrosorb RP18 precolumn. The elution

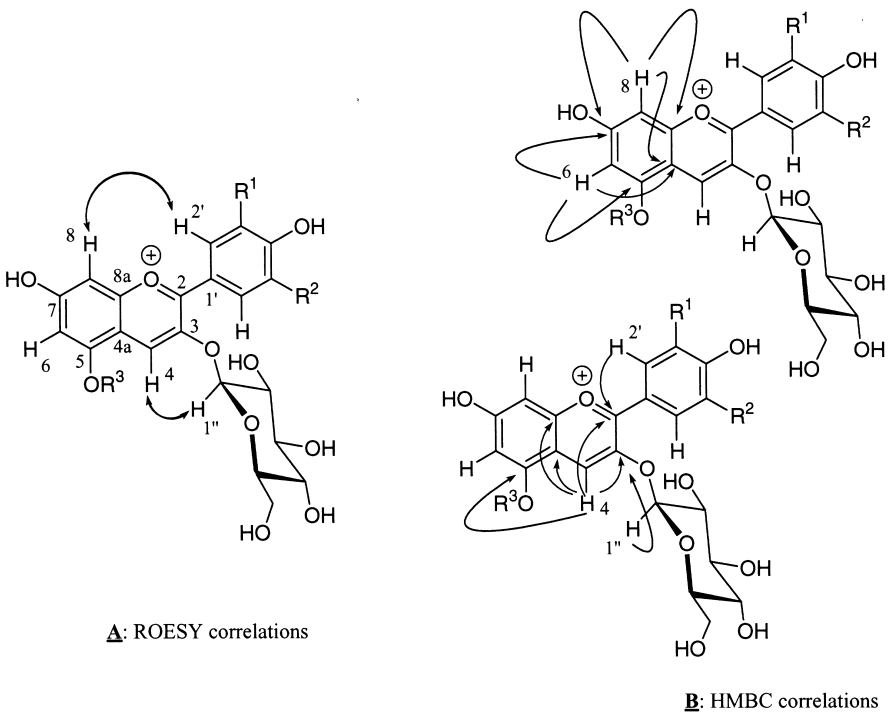


Fig. 5. Principal ROESY (A) and HMBC (B) correlations appearing for monoglucosylated compounds **1**–**5**. These correlations allowed reliable determination of the H-8 signal and point of attachment of the sugar, leading to total and unambiguous assignment of all the chemical shifts (the same can be performed for the 3,5-diglucosides compounds **6** and **7**).

gradient was linear from 65:35 to 0:100 ratio of A–B over 120 min. The flow rate was 7 ml min^{-1}.

3.3. 3-O-β-D-glucopyranoside of malvidin (*1*)

This compound was isolated from an aqueous extract of *Vitis vinifera* grapes by HPLC at a preparative scale from fractions eluted from 45 to 60 min. Retention time and purity (analytical mode): 24.8 min and 98.9%. UV–Vis λ_{max} nm 520, 274. MS *m/z* 493 for $M^+ = C_{23}H_{25}O_{12}^+$. ^{1}H NMR δ 3.43 (1H, *t*, *J* = 9.2 Hz, H-4″), 3.55 (2H, *m*, H-3″ and H-5″), 3.62 (1H, *dd*, *J* = 8.9, 7.6 Hz, H-2″), 3.72 (1H, *dd*, *J* = 12.0, 5.9 Hz, H-6″$_B$), 3.91 (1H, *dd*, *J* = 12.0, 1.9 Hz, H-6″$_A$), 3.94 (6H, *s*, OCH_3), 5.30 (1H, *d*, *J* = 7.6 Hz, H-1″), 6.58 (1H, *s*, H-6), 6.83 (1H, *s*, H-8), 7.84 (2H, *s*, H-2′ and H-6′), 8.91 (1H, *s*, H-4).

3.4. 3-O-β-D-glucopyranoside of peonidin (*2*)

This compound was isolated in the same manner as compound **1** from 39 to 44 min fractions. Retention time and purity (analytical mode): 23.6 min and 98.2%. UV–Vis λ_{max} nm 510, 278. MS *m/z* 463 for $M^+ = C_{22}H_{23}O_{11}^+$. ^{1}H NMR δ 3.44 (^{1}H, *t*, *J* = 9.3 Hz, H-4″), 3.56 (2H, *m*, H-3″ and H-5″), 3.65 (1H, *dd*, *J* = 9.1, 7.7 Hz, H-2″), 3.73 (1H, *dd*, *J* = 12.2, 6.0 Hz, H-6″$_B$), 3.93 (1H, *dd*, *J* = 12.2, 2.2 Hz, H-6″$_A$), 3.97 (3H, *s*, OCH_3), 5.28 (1H, *d*, *J* = 7.7 Hz, H-1″), 6.62 (1H, *d*, *J* = 1.9 Hz, H-6), 6.84 (1H, *d*, *J* = 1.9 Hz, H-8), 6.99 (1H, *d*, *J* = 8.7 Hz, H-5′), 8.12 (1H, *d*, *J* = 2.2 Hz, H-2′), 8.16 (1H, *dd*, *J* = 8.7, 2.2 Hz, H-6′), 8.96 (1H, *s*, H-4).

Table 3
^{13}C NMR spectral data for the 3-*O*-β-D-glucopyranosides of malvidin (**1**), delphinidin (**3**) and petunidin (**4**), and for the 3,5-di-*O*-β-D-glucopyranosides of cyanidin (**7**) in acidified CD_3OD at 303 K

	1 δ (ppm)	**3** δ (ppm)	**4** δ (ppm)	**7** δ (ppm)	
Aglycone	Mal	Del	Pet	Cy	
2	164.1	164.3	163.6	164.3	
3	146.2	148.7	145.7	147.2	
4	137.5	137.0	136.5	136.9	
4a	114.2	113.4	113.4	113.5	
5	160.3	157.7	159.7	156.2	
6	104.0	103.5	103.5	103.8	
7	172.2	170.4	170.6	170.5	
8	95.9	99.8	95.2	95.1	
8a	158.6	153.9	157.6	155.6	
1′	120.3	120.1	119.9	119.7	
2′	111.2	113.47	109.3	118.1	
3′	150.6	147.6	149.8	146.6	
R^1	57.6	–	57.2	–	
4′	147.3	149.0	145.2	155.8	
5′	150.6	147.6	147.4	118.5	
R^2	57.6	–	–	–	
6′	111.2	113.4	113.7	128.2	
Glucose	3-*O*-β	3-*O*-β	3-*O*-β	3-*O*-β	5-*O*-β
1″	104.5	103.7	103.7	104.1	102.8
2″	75.5	75.0	74.9	74.9	74.4
3″	79.3	79.2	78.8	78.8	77.7
4″	71.6	71.5	71.1	71.3	71.1
5″	78.8	78.5	78.2	79.1	78.6
6″	62.7	62.4	62.4	62.5	62.2

3.5. 3-O-β-D-glucopyranoside of delphinidin (*3*)

This compound was isolated in the same manner as compound **1** from 20 to 25 min fractions. Retention time and purity (analytical mode): 14.8 min and 99.2%. UV–Vis λ_{max} nm 518, 276. MS *m/z* 465 for $M^+ = C_{21}H_{21}O_{12}^+$. ^{1}H NMR δ 3.46 (1H, *t*, *J* = 9.3 Hz, H-4″), 3.55 (2H, *m*, H-3″ and H-5″), 3.70 (1H, *dd*, *J* = 9.2, 7.8 Hz, H-2″), 3.73 (1H, *dd*, *J* = 12.1, 5.7 Hz, H-6″$_B$), 3.91 (1H, *dd*, *J* = 12.1, 2.2 Hz, H-6″$_A$), 5.29 (1H, *d*, *J* = 7.8 Hz, H-1″), 6.64 (1H, *s*, H-6), 6.84 (1H, *s*, H-8), 7.75 (2H, *s*, H-2′ and H-6′), 8.95 (1H, *s*, H-4).

3.6. 3-O-β-D-glucopyranoside of petunidin (*4*)

This compound was isolated in the same manner as compound **1** from 32 to 37 min fractions. Retention time and purity (analytical mode): 19.7 min and 98.8%. UV–Vis λ_{max} nm 517, 275. MS *m/z* 479 for $M^+ = C_{22}H_{23}O_{12}^+$. ^{1}H NMR δ 3.46 (1H, *t*, *J* = 9.3 Hz, H-4″), 3.58 (2H, *m*, H-3″ and H-5″), 3.67 (1H, *dd*, *J* = 9.1, 7.8 Hz, H-2″), 3.75 (1H, *dd*, *J* = 12.2, 5.9 Hz, H-6″$_B$), 3.92 (1H, *dd*, *J* = 12.2, 2.2 Hz, H-6″$_A$), 3.95 (3H, *s*, OCH_3), 5.31 (1H, *d*, *J* = 7.8 Hz, H-1″), 6.62 (1H, *d*, *J* = 1.9 Hz, H-6), 6.82 (1H, *d*, *J* = 1.9 Hz, H-8), 7.71 (1H, *d*, *J* = 2.2 Hz, H-6′), 7.86 (1H, *d*, *J* = 2.2 Hz, H-2′), 8.92 (1H, *s*, H-4).

3.7. 3-O-β-D-glucopyranoside of cyanidin (*5*)

This compound was isolated in the same manner as compound **1** from 29 to 31 min fractions. Retention time and purity (analytical mode): 17.9 min and 97.9%. UV–Vis λ_{max} nm 511, 276. MS *m/z* 449 for $M^+ = C_{21}H_{21}O_{11}^+$. ^{1}H NMR δ 3.44 (1H, *t*, *J* = 9.3 Hz, H-4″), 3.54 (2H, *m*, H-3″ and H-5″), 3.67 (1H, *dd*, *J* = 9.2, 7.8 Hz, H-2″), 3.71 (1H, *dd*, *J* = 12.1, 6.0 Hz, H-6″$_B$), 3.91 (1H, *dd*, *J* = 12.1, 2.0 Hz, H-6″$_A$), 5.27 (1H, *d*, *J* = 7.8 Hz, H-1″), 6.65 (1H, *d*, *J* = 1.9 Hz, H-6), 6.87 (1H, *d*, *J* = 1.9 Hz, H-8), 7.01 (1H, *d*, *J* = 8.7 Hz, H-5′), 8.05 (1H, *d*, *J* = 2.3 Hz, H-2′), 8.22 (1H, *dd*, *J* = 8.7, 2.3 Hz, H-6′), 9.01 (1H, *s*, H-4).

3.8. 3,5-di-O-β-D-glucopyranoside of malvidin (*6*)

This compound was extracted from dried petals of malva (*Malva sylvestris*) by a classical method (Haya-

shi, 1962). Retention time and purity (analytical mode): 16.1 min and 98.5%. UV–Vis λ_{max} nm 516, 270. MS m/z 655 for $M^+ = C_{29}H_{35}O_{17}^+$. ^{1}H NMR δ 3.39 (1H, J = 9.2 Hz, H-4″), 3.45 (1H, t, J = 9.2 Hz, H-4‴), 3.55 (3H, m, H-3″, H-3‴ and H-5‴), 3.65 (3H, m, H-2″, H-2‴ and H-5″), 3.73 (1H, m, H-6″$_B$), 3.75 (1H, m, H-6‴$_B$), 3.96 (2H, m, H-6″$_A$ and H-6‴$_A$), 4.00 (6H, s, OCH_3), 5.16 (1H, d, J = 7.7 Hz, H-1‴), 5.32 (1H, d, J = 7.7 Hz, H-1″), 7.06 (1H, d, J = 1.9 Hz, H-6), 7.14 (1H, d, J = 1.9 Hz, H-8), 8.01 (2H, s, H-2′ and H-6′), 9.13 (1H, s, H-4).

3.9. 3,5-di-O-β-D-glucopyranoside of cyanidin (7)

This compound was extracted from dried petals of rose (*Rosa gallica*) by the same method as for **6**. Retention time and purity (analytical mode): 10.0 min and 99.0%. UV–Vis λ_{max} nm 508, 272. MS m/z 611 for $M^+ = C_{27}H_{31}O_{16}^+$. ^{1}H NMR δ 3.40 (1H, J = 9.3 Hz, H-4″), 3.44 (1H, J = 9.3 Hz, H-4‴), 3.54 (3H, m, H-5‴, H-3″ and H-3‴), 3.61 (1H, m, H-5″), 3.67 (2H, m, H-2″ and H-2‴), 3.71 (1H, m, H-6″$_B$), 3.74 (1H, m, H-6‴$_B$), 3.95 (2H, m, H-6″$_A$ and H-6‴$_A$), 5.14 (1H, d, J = 7.8 Hz, H-1‴), 5.29 (1H, d, J = 7.8 Hz, H-1″), 7.02 (1H, d, J = 8.8 Hz, H-5′), 7.05 (1H, s, H-6), 7.07 (1H, s, H-8), 8.05 (1H, d, J = 2.3 Hz, H-2′), 8.34 (1H, dd, J = 8.8, 2.3 Hz, H-6′), 9.13 (1H, s, H-4).

3.10. Thermal denaturation studies

Melting curves were recorded on a Varian Cary 1E spectrophotometer with a temperature controller unit. For each, purified oligonucleotides of commercial source (oligonucleotides $^{5'}$TTCTTCTTTC-CACAC-CTTTCTTCTT$^{3'}$ (**8**) and $^{5'}$GAAAGAAGAA$^{3'}$ (**9**) for the triplex, and oligonucleotides $^{5'}$GAAAGAAGAA$^{3'}$ (**9**) and $^{5'}$TTCTTCTTTC$^{3'}$ (**10**) for the duplex), 0.25 nmol were dissolved in 0.5 ml of the appropriate buffer and boiled for 2 min. The buffer used was: 10 mM sodium cacodylate (pH 6), 100 mM sodium chloride, 10 mM magnesium acetate, 1 mM spermine. Then, 20 μl of a solution of anthocyanin 1.25×10^{-4} M (2.5 nmol) were added to the medium. Samples were kept for at least 30 min at 4°C and were then heated from 4°C to 80°C at a rate of 0.5°C min^{-1}. The absorbance at 260 nm was measured every 2 min. T_m values were then obtained by graphic measurement of the inflection point of the curves.

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