JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

J. Agric. Food Chem. XXXX, XXX, 000–000 A
DOI:10.1021/jf900759q

Structural Properties of Anthocyanins: Rearrangement of *C*-Glycosyl-3-deoxyanthocyanidins in Acidic Aqueous Solutions

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Seven *C*-glycosyl-3-deoxyanthocyanidins were made from their corresponding *C*-glycosylflavones. The structures of their rearrangement products, which were formed in acidic aqueous solutions, were elucidated. Rotameric conformers were detected for all of the 8-*C*-glycosyldeoxyanthocyanidins but were absent for their isomeric 6-*C*-analogues in acidified methanolic NMR solvent. A correlation method based on HPLC-DAD and NMR integration of similar samples made it possible for the first time to determine accurately the proportions of two isomeric 6-*C*- and 8-*C*-glycosylflavonoids occurring in mixtures. Each of the *C*-glycosyldeoxyanthocyanidins established fixed equilibrium proportions with their corresponding A-ring isomer in aqueous solutions, even under relatively strong acidic conditions (pH ~1), whether one started with pure 6-*C*- or 8-*C*-glycosyl-3-deoxyanthocyanidin. The nature of the aglycone, *C*-glycosyl moiety, and temperature were found to affect the equilibrium proportions. Increased water content (to a certain level) and temperatures were shown to increase the isomerization rates. The flavylium cations were the only equilibrium forms present at detectable quantities. The significance of rotation of the A-ring during isomerization was confirmed by lack of rearrangement of both 6-*C*- and 8-*C*-glycosyl-3-deoxy-5-carboxypyranoanthocyanidins. The intermediary C-ring open forms of the *C*-glycosyldeoxyanthocyanidins experience fast ring closure to their cyclic forms, which may reduce irreversible degradation reported for open chalcone forms of the common anthocyanins. The stable *C*-glycosyl-3-deoxyanthocyanidins may thus attract interest as possible colorants in the food industry, etc.

KEYWORDS: Anthocyanidin *C*-glycosides; 6-*C*- and 8-*C*-glycosyl-3-deoxyanthocyanidins; *C*-glycosyl-3-deoxy-5-carboxypyranoanthocyanidins; rotamers; isomerization reaction; equilibrium forms; HPLC-DAD/NMR integration method; diagnostic UV−vis spectra

INTRODUCTION

Anthocyanins (*1*) are responsible for most red to blue colors of plants. The various nuances are determined by a range of factors including pigment structure, in vivo pH, and copigmentation mechanisms (*2*). Evidence collected during mainly the past two decades indicates that adequate fruit and vegetable consumption has a role in maintaining health and preventing various diseases. Some of these protective effects seem to be caused by the content of anthocyanins and other flavonoids or their degradation products (*3*−*7*). There is worldwide interest in extended use of anthocyanins as color additives.

Anthocyanins are rather unique compounds as they actually represent a range of different equilibrium forms strongly dependent on the pH of their solvent as well as other factors (*2*, *8*). However, among the various equilibrium forms of the different anthocyanins, only the flavylium cation (*9*) and, in a few cases, their 2-OH hemiketal forms (*10*−*12*) have been completely characterized. Most anthocyanins are associated with restricted stability, including loss of *O*-glycosyl moieties by hydrolysis or irreversible degradation caused initially by opening of the heterocyclic anthocyanin C-ring. In the latter case, some *trans*-chalcones as well as A-ring and B-ring fragments such as phloroglucinaldehyde and various benzoic acid derivatives have been identified (*13*, *14*). It has recently been shown that a chalcone possessing a hydroxyl group in the 2-position (chalcone nomenclature) cyclizes to form flavylium salt in acidic media, this reaction being reversible under neutral−basic conditions (*14*). When 2′-hydroxyflavylium tetrafluoroborate was dissolved in an aqueous alcoholic solution followed by adjustment of the pH, it was possible to precipitate both the corresponding *trans*-2,2′-dihydroxychalcone and the 2′-hydroxyflavanone, as yellow and white solids, respectively (*14*).

Whereas opening of the heterocyclic C-ring may lead to flavonoid degradation, it may, if certain criteria are satisfied, also result in rearrangement. Depending on the flavonoid type and aglycone substitution pattern, cleavage of the bond between the heterocyclic oxygen of the C-ring and C-2 may result in several isomerization/rearrangement reactions caused by rotation of the open form (**Figure 1**): (i) epimerization at C-2, (ii) ring closure involving the hydroxyl groups attached to C-5 (5-OH) of the original aglycone and C-2 leading to 6/8 rearrangement of the A-ring, or (iii) ring closure involving the hydroxyl groups attached to C-2′ (2′-OH) of the original aglycone and C-4 resulting in a more obscure A−B-ring rearrangement (*15*). In the following cases these rearrangements are not observed: identical substituents at C-6 and C-8 of the A-ring, absence of a

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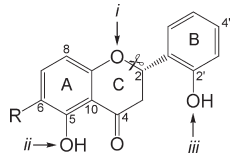


Figure 1. Possible (theoretical) rearrangements resulting in (i) epimerization, (ii) 6/8 (A-ring) rearrangement, and (iii) A-B ring rearrangement.

free hydroxyl group at C-5 (or occasionally C-2′), or absence of a stereocenter at C-2 (*15*). However, when rearrangements are observed, they seem to be influenced by the A-ring substituents; 8-hydroxyanthocyanidins rearrange completely within a few hours to the corresponding 6-hydroxyanthocyanidins, whereas the reverse rearrangement has never been observed (*16*−*19*).

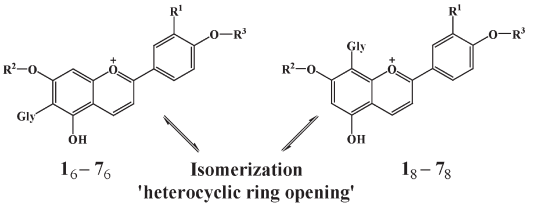
3-Deoxyanthocyanins have attracted interest as possible food colorants (*20*, *21*). Recently we have reported a new class of 3-deoxyanthocyanins when describing the reductive synthesis of two 6,8-di-*C*-glycosyldeoxyanthocyanidins and seven mono-*C*-glycosyldeoxyanthocyanidins (**1**−**7**) by the Clemmensen reduction of the structurally analogous *C*-glycosylflavones (*22*). A new type of anthocyanins with special properties was thus achieved by adding the relatively high color stability of 3-deoxyanthocyanidins (*23*) to the relatively high stability of the *C*-glycosyl linkage against acid hydrolysis, compared to the stability of most natural anthocyanidin *O*-glycosides (*22*). Analysis of the reaction products by HPLC-DAD and LC-DAD-MS showed that $\mathbf{1}_6$−$\mathbf{4}_6$ and $\mathbf{5}_8$−$\mathbf{7}_8$ were during the purification steps separately transformed partly into other forms, $\mathbf{1}_8$−$\mathbf{4}_8$ and $\mathbf{5}_6$−$\mathbf{7}_6$. The major aims of the present study are to elucidate properly the structures of these latter forms, to describe the isomerization reactions of $\mathbf{1}_6$−$\mathbf{7}_6$ into $\mathbf{1}_8$−$\mathbf{7}_8$ and vice versa (**Figure 2**), including depiction of factors influencing these rearrangements. It was evident that these isomerization reactions were continuous even at pH ∼1. A new experimental approach enabled for the first time accurate determination of the existence and proportions of the two involved isomeric flavonoids in mixtures during analyses and monitoring of this type of A-ring isomerization.

MATERIALS AND METHODS

Hemisynthesis of *C*-Glycosyl-3-deoxyanthocyanidins. The *C*-glycosyl-3-deoxyanthocyanidins $\mathbf{1}_6$−$\mathbf{4}_6$ and $\mathbf{5}_8$−$\mathbf{7}_8$ were synthesized by Clemmensen reduction of the corresponding *C*-glycosylflavones isolated from various plant sources according to a previously published procedure (*22*). The crude reaction products were purified by Sephadex column chromatography and/or preparative HPLC (*22*). The isomeric *C*-glycosyl-3-deoxyanthocyanidins, $\mathbf{1}_8$−$\mathbf{4}_8$ and $\mathbf{5}_6$−$\mathbf{7}_6$, were formed from $\mathbf{1}_6$−$\mathbf{4}_6$ and $\mathbf{5}_8$−$\mathbf{7}_8$, respectively, during the workup procedure of the reaction mixture whenever acidified aqueous solvents were applied.

Hemisynthesis of *C*-Glycosyl-5-carboxypyrano-3-deoxyanthocyanidins. The *C*-glycosyl-3-deoxy-5-carboxypyranoanthocyanidins, $\mathbf{CP3}_6$ and $\mathbf{CP3}_8$, were synthesized from approximately 23 mg of a mixture of the 6-*C*- and 8-*C*-β-(2″-*O*-β-glucopyranosyl)-glucopyranosyls of 7-*O*-methylapigeninidin (**Figure 3**) according to published procedures (*21*, *24*, *25*). The reaction was terminated after 20 h. Three milligrams of pure $\mathbf{CP3}_6$ was isolated from the reaction mixture after separation by preparative HPLC. Two milligrams of pure $\mathbf{CP3}_8$ was obtained by further purification of fractions from preparative HPLC on a Sephadex LH-20 column using CH_3OH/H_2O (1:4; v/v) containing 0.5% CF_3COOH as eluent.

High-Performance Liquid Chromatography (HPLC). Preparative HPLC (Gilson 305/306 pump equipped with an HP-1040A detector) was performed using an Econosil C18 column (250 mm × 22 mm; length × i.d., 10.0 μm). Mixtures of 6-*C*- and 8-*C*-glycosyl-3-deoxyanthocyanidins were separated into pure 6-*C*-glycosyl-3-deoxyanthocyanidin and pure 8-*C*-glycosyl-3-deoxyanthocyanidin using isocratic elution conditions: $H_2O/CH_3CN/CF_3COOH$ (79.5:20.0:0.5; v/v); flow rate = 14 mL min^{-1}. Pure pigments were injected into the analytical HPLC system immediately after isolation on preparative HPLC. The analyses were performed with the same solvent $H_2O/CH_3CN/CF_3COOH$ (79.5:20.0:0.5; v/v) that was used for isolation of pure compounds by preparative HPLC;



	Gly	R^1	R^2	R^3
1	Glc	H	CH_3	H
2	Glc	OH	CH_3	H
3	2GlcGlc	H	CH_3	H
4	2GlcGlc	H	CH_3	CH_3
5	Glc	H	H	H
6	2RhaGlc	H	H	H
7	2(4Ac)RhaGlc	H	H	H

Figure 2. *C*-Glycosyl-3-deoxyanthocyanidins $\mathbf{1}_6$−$\mathbf{7}_6$ and $\mathbf{1}_8$−$\mathbf{7}_8$. The 6-*C*-glycosyl derivatives are under specific solvent conditions transformed into their analogous 8-*C*-glycosyl derivatives and vice versa due to heterocyclic ring opening. $\mathbf{1}_6$ = 6-*C*-β-glucopyranosyl-7-*O*-methylapigeninidin, $\mathbf{1}_8$ = 8-*C*-β-glucopyranosyl-7-*O*-methylapigeninidin, $\mathbf{2}_6$ = 6-*C*-β-glucopyranosyl-7-*O*-methylluteolinidin, $\mathbf{2}_8$ = 8-*C*-β-glucopyranosyl-7-*O*-methylluteolinidin, $\mathbf{3}_6$ = 6-*C*-β-(2″-*O*-β-glucopyranosyl)glucopyranosyl-7-*O*-methylapigeninidin, $\mathbf{3}_8$ = 8-*C*-β-(2″-*O*-β-glucopyranosyl)glucopyranosyl-7-*O*-methylapigeninidin, $\mathbf{4}_6$ = 6-*C*-β-(2″-*O*-β-glucopyranosyl)glucopyranosyl-7,4′-di-*O*-methylapigeninidin, $\mathbf{4}_8$ = 8-*C*-β-(2″-*O*-β-glucopyranosyl)glucopyranosyl-7,4′-di-*O*-methylapigeninidin, $\mathbf{5}_6$ = 6-*C*-β-glucopyranosylapigeninidin, $\mathbf{5}_8$=8-*C*-β-glucopyranosylapigeninidin, $\mathbf{6}_6$ = 6-*C*-β-(2″-*O*-α-rhamnopyranosyl)glucopyranosylapigeninidin, $\mathbf{6}_8$ = 8-*C*-β-(2″-*O*-α-rhamnopyranosyl)glucopyranosylapigeninidin, $\mathbf{7}_6$ = 6-*C*-β-(2″-*O*-α-(4‴-*O*-acetylrhamnopyranosyl)glucopyranosylapigeninidin, $\mathbf{7}_8$ = 8-*C*-β-(2″-*O*-α-(4‴-*O*-acetylrhamnopyranosyl)glucopyranosylapigeninidin. Glc = glucosyl, Rha = rhamnosyl, Ac = acetyl.

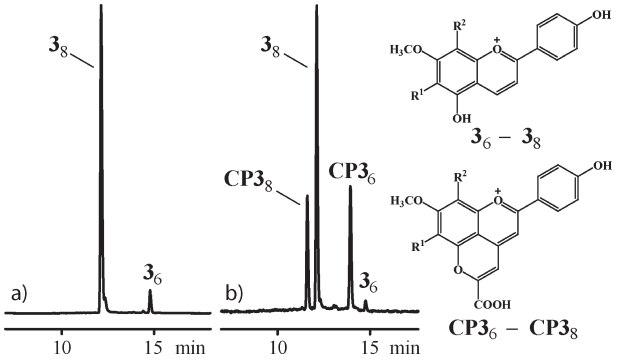


Figure 3. HPLC chromatograms recorded at 475 ± 20 nm of a mixture of 6-*C*-sophorosyl ($\mathbf{3}_6$) and 8-*C*-sophorosyl ($\mathbf{3}_8$) of 7-*O*-methylapigeninidin recorded (**a**) prior to addition of pyruvic acid and (**b**) after addition of pyruvic acid followed by 20 h of reaction time at 45 °C. $\mathbf{CP3}_6$ and $\mathbf{CP3}_8$ correspond to the formed 6-*C*-sophorosyl and 8-*C*-sophorosyl of 3-deoxy-5-carboxypyrano-7-*O*-methylapigeninidin, respectively. Each chromatogram is scaled to its highest peak. $\mathbf{3}_6$ and $\mathbf{CP3}_6$, R^1 = 2-glcglc, R^2 = H; $\mathbf{3}_8$ and $\mathbf{CP3}_8$, R^1 = H, R^2 = 2-glcglc); glc = glucosyl.

however, the analytical HPLC was equipped with an ODS-Hypersil column (20 × 0.5 cm, 5 μm); flow = rate 1.0 mL min^{-1}. When the isomerization experiments were performed with different solvent systems, or when several parallel experiments requiring the same concentration were needed, the samples were divided into fractions and evaporated under reduced pressure prior to dissolution of the pigments in the solvents used during the analysis. Temperature studies were performed by keeping the samples in closed vials in thermostated water baths at the various temperatures. For NMR studies the solutions of pure compounds were evaporated to dryness immediately after isolation by preparative HPLC.

Spectroscopy. UV−vis absorption spectra were recorded online during isocratic HPLC analysis using a photodiode array detector (HP 1050, Hewlett-Packard). All samples were dissolved in the same solvent as used for isocratic HPLC analysis, H_2O/CH_3CN/CF_3COOH (79.5:20.0:0.5; v/v). Spectral measurements were made over the wavelength range from 240 to 600 nm in steps of 2 nm. The relative quantitative data were based on the average values of the absorptions on every second nanometer between 455 and 495 nm.

The NMR experiments (1D ^{1}H, 2D ^{1}H−^{13}C HMBC, ^{1}H−^{13}C HSQC, ^{1}H−^{1}H COSY, ^{1}H−^{1}H TOCSY, ^{1}H−^{1}H ROESY, ^{1}H−^{1}H NOESY, and 1D ^{13}C CAPT) were obtained at 600.13/500.13 and 150.90/125.76 MHz for ^{1}H and ^{13}C, respectively, on a Bruker Biospin AV-600 MHz instrument equipped with a TCI ^{1}H−^{13}C/^{15}N CryoProbe and a Bruker Ultrashield Plus AV-500 MHz instrument. All experiments were recorded at 298 K. Chemical shift values were set relative to the deuteriomethyl ^{13}C signal and the residual ^{1}H signal of the solvent, at δ 49.0 and δ 3.4 for CD_3OD (containing CF_3COOD). 1D ^{1}H NMR experiments for determination of isomerization of $\mathbf{1}_6$ and $\mathbf{1}_8$ were performed with H_2O/CD_3CN/CF_3COOH (79.5:20.0:0.5; v/v) as solvent. The chemical shift values were set relative to the residual ^{1}H signal of CD_3CN, δ 1.94. Water suppression was achieved using excitation sculpting methodology (*26*).

High-resolution LC−electrospray mass spectrometry (ESI/TOF) in positive ion mode spectra were recorded using a JEOL AccuTOF JMS-T100LC in combination with an Agilent Technologies 1200 series HPLC system. A Zorbax SB-C18 (50 mm × 2.1 mm, length × i.d., 1.8 μm) column was used for separation, and combinations of two solvents were used for elution: A, H_2O containing 0.5% CF_3COOH (v/v), and B, CH_3CN containing 0.5% CF_3COOH (v/v). The following solvent composition was used: 0−1 min, 5% B (isocratic); 1−3 min, 5−13% B (linear gradient); 3−6 min, 13% B (isocratic); 6−8 min, 13−30% B (linear gradient); 8−14 min, 30−40% B (linear gradient). The flow rate was 0.4 mL min^{-1}.

Determination of Relative Molar Absorption Coefficients of *C*-Glycosyl-3-deoxyanthocyanidins. Relative molar absorption coefficients were determined for isomeric pairs using the following procedure: A 1D ^{1}H NMR spectrum was recorded for a mixture of $\mathbf{1}_6$ and $\mathbf{1}_8$, in which equilibrium proportions of these isomers were established on the basis of integration of their H-4 resonances. An aliquot of the same sample was subjected to isocratic HPLC-DAD analysis directly after the 1D ^{1}H NMR spectrum was recorded, and the areas of the two isomers were integrated. Thereafter, a new 1D ^{1}H NMR spectrum was recorded to confirm that the relative proportions of the isomers were unchanged prior to and after HPLC injection. The relative molar absorptivity coefficient for the isomeric pair, *k*, was established according to the equation given in **Figure 4**. An identical procedure was followed to determine the analogous coefficients for the isomeric pairs $\mathbf{2}_6$/$\mathbf{2}_8$ and $\mathbf{5}_6$/$\mathbf{5}_8$, respectively.

RESULTS AND DISCUSSION

Identification of *C*-Glycosyldeoxyanthocyanidin Rearrangement Products. Structural identification of seven *C*-glycosyl-3-deoxyanthocyanidins, $\mathbf{1}_6$−$\mathbf{4}_6$ and $\mathbf{5}_8$−$\mathbf{7}_8$, synthesized by the Clemmensen reduction of analogous *C*-glycosylflavones has recently been described (*22*). The yields of the individual reductions were between 14 and 32%. During purification in acidic aqueous-methanolic solutions each of the pigments $\mathbf{1}_6$−$\mathbf{4}_6$ and $\mathbf{5}_8$−$\mathbf{7}_8$ were transformed into isomeric forms, $\mathbf{1}_8$−$\mathbf{4}_8$ and $\mathbf{5}_6$−$\mathbf{7}_6$, respectively, which were not characterized properly (*22*). **Table 1** shows MS characteristics of $\mathbf{1}_8$−$\mathbf{4}_8$ and $\mathbf{5}_6$−$\mathbf{7}_6$, which are similar to those previously reported for the corresponding 3-deoxyantho-

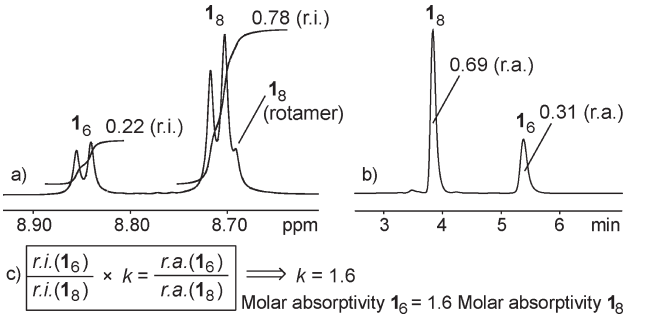


Figure 4. (**a**) Region of the ^{1}H NMR spectrum of an equilibrium mixture of 6-*C*-β-glucosyl-7-*O*-methylapigeninidin ($\mathbf{1}_6$) and its isomer 8-*C*-β-glucosyl-7-*O*-methylapigeninidin ($\mathbf{1}_8$) dissolved in H_2O/CD_3CN/CF_3COOH (79.5:20.0:0.5; v/v) showing the two integrated H-4 resonances. (**b**) Integrated HPLC chromatogram of the same mixture of $\mathbf{1}_6$ and $\mathbf{1}_8$ at isocratic solution conditions with the solvent H_2O/CH_3CN/CF_3COOH (79.5:20.0:0.5; v/v) detected at 475 ± 20 nm. (**c**) Equation for determination of the relative molar absorption coefficient, *k*. Analogous coefficients were determined similarly for the two pairs of isomers, $\mathbf{2}_6$/$\mathbf{2}_8$ and $\mathbf{5}_6$/$\mathbf{5}_8$.

Table 1. High-Resolution Electrospray MS Data[a] and Chromatographic (HPLC) and UV−Vis Spectral Data[b] Recorded for the *C*-Glycosylanthocyanidins, $\mathbf{1}_8$−$\mathbf{4}_8$, $\mathbf{5}_6$−$\mathbf{7}_6$, $\mathbf{CP3}_6$, and $\mathbf{CP3}_8$ (See **Figures 1** and **2** for Structures)

pigment	$[M + H]^+$ (obsd)	$[M + H]^+$ (calcd)	mol formula	t_R (min)	$\lambda_{UV\text{-}max}$ nm	$\lambda_{VIS\text{-}max}$ nm
$\mathbf{1}_8$	431.1352	431.1342	$C_{22}H_{23}O_9$	3.84	279, 324	419, 481
$\mathbf{2}_8$	447.1290	447.1291	$C_{22}H_{23}O_{10}$	3.71	283, 325s[c]	428s, 496
$\mathbf{3}_8$	593.1907	593.1870	$C_{28}H_{33}O_{14}$	3.69	279, 325	421, 482
$\mathbf{4}_8$	607.1986	607.2027	$C_{29}H_{35}O_{14}$	4.96	279, 325	422, 481
$\mathbf{5}_6$	417.1196	417.1186	$C_{21}H_{21}O_9$	4.63	278, 325	473
$\mathbf{6}_6$	563.1747	563.1765	$C_{27}H_{31}O_{13}$	4.80	279, 326	477
$\mathbf{7}_6$	605.1853	605.1870	$C_{29}H_{33}O_{14}$	4.96	280, 327	480
$\mathbf{CP3}_6$	661.1739	661.1769	$C_{31}H_{33}O_{16}$	4.55	266, 300s,[c] 353	477
$\mathbf{CP3}_8$	661.1742	661.1769	$C_{31}H_{33}O_{16}$	3.23	266s,[c] 298s,[c] 360	486

[a] In H_2O/TFA (99.5:0.5 v/v) and CH_3CN/TFA (99.5:0.5 v/v); gradient solvent conditions. [b] In H_2O/CH_3CN/TFA (79.5:20:0.5 v/v); isocratic solvent conditions. See Materials and Methods for more details. [c] Weak shoulder.

Table 2. ^{1}H NMR Chemical Shift Values (Parts per Million) and Coupling Constants (Hertz) for the *C*-Glycosylanthocyanidins, $\mathbf{1}_8$−$\mathbf{4}_8$ and $\mathbf{5}_6$−$\mathbf{7}_6$, Dissolved in 5% CF_3COOD in CD_3OD, v/v at 25 °C (See **Figure 1** for Structures)[a]

position	$\mathbf{1}_8$	$\mathbf{2}_8$	$\mathbf{3}_8$	$\mathbf{4}_8$	$\mathbf{5}_6$	$\mathbf{6}_6$	$\mathbf{7}_6$
3	8.19 d 8.8	8.11 d 8.8	8.13 d 8.7	8.21 d 8.7	8.18 d 8.9	8.15 d 8.8	8.18 d 8.7
	8.20 d 8.9	8.13 d 8.9	8.13 d 8.7	8.25 d 8.7			
4	9.24 d 8.8	9.19 d 8.8	9.21 d 8.7	9.31 d 8.7	9.23 d 8.8, 0.9	9.19 d 8.8	9.22 d 8.7
		9.16 d 8.9	9.19 d 8,8	9.29 d 8.7			
6	6.96 s	6.94 s	6.92 s	6.95 s			
	6.97 s	6.95 s	6.96 s	6.99 s			
8					7.13 d 0.8	7.13 s	7.20 s
2′	8.52 d 9.0	8.01 d 2.3	8.50 d 8.9	8.61 d 9.1	8.41 d 9.0	8.34 d 9.0	8.45 d 8.9
	8.40 d 9.1	7.83 d 2.3	8.37 d 8.9	8.48 d 9.2			
3′	7.17 d 9.0		7.17 d 8.9	7.35 d 9.1	7.18	7.18 d 9.0	7.19 d 8.9
	7.19		7.18 d 8.9	7.39 d 9.2			
5′	7.17 d 9.0	7.13 d 8.6	7.17 d 8.9	7.35 d 9.1	7.18	7.18 d 9.0	7.19 d 8.9
	7.19	7.15 d 8.6	7.18 d 8.9	7.39 d 9.2			
6′	8.52 d 9.0	8.07 dd 8.6, 2.3	8.50 d 8.9	8.61 d 9.1	8.41 d 9.0	8.34 d 9.0	8.45 d 8.9
	8.40 d 9.1	7.8 dd 8.6, 2.3	8.37 d 8.9	8.48 d 9.2			
7-MeO	4.18 s	4.17 s	4.15 s	4.18 s			
	4.17 s	4.17 s	4.19 s	4.22 s			
4′-MeO				4.09 s			
position	**8-*C*-Glc**	**8-*C*-Glc**	**8-*C*-Glc**	**8-*C*-Glc**	**6-*C*-Glc**	**6-*C*-Glc**	**6-*C*-Glc**
1″	5.15 d 10.0	5.14 d 10.0	5.23 d 10.0	5.24 d 10.1	5.17 d 9.8	5.20 d 9.6	5.21 d 10.1
	5.23 d 9.9	5.23 d 9.8	5.32 s br	5.35 d 10.0			
2″	4.11 dd 10.0, 8.9	4.10 dd 10.0, 8.9	4.34 m	4.35 dd 10.1, 8.6	3.79 dd 9.9, 9.0	4.05	4.07
	4.39 dd 9.9, 8.9	4.39 dd 9.8, 8.8	4.5	4.53			
3″	3.68 t 8.9	3.68 t 8.9	3.88 m	3.89 t br 8.6	3.66 t 9.0	3.62	3.6
	3.67	3.70	3.9				
4″	3.87 dd 8.9, 9.8	3.89 dd 9.8, 8.9	3.88 m	3.92 t br 8.6	3.73 dd 9.8, 8.9	3.63	3.7
	3.51 t (br) 9.3	3.52 dd 9.7, 9.0	3.8				
5″	3.59 ddd 9.8, 5.0, 2.3	3.62 ddd 9.8, 5.6, 2.4	3.59 m	3.58 m	3.62	3.39	3.7
	3.65	3.70	3.6				
6″A	4.04 dd 12.1, 2.3	4.08 dd 12.2, 2.4	4.04 dd 11.9, 1.7	4.02 dd 12.2, 2.4	3.98	3.98	4.0
	4.04	4.05	4.0 m	4.01			
6″B	3.94 dd 12.1, 5.0	3.99 dd 12.2, 5.6	3.94 dd 11.9, 4.9	3.97 dd 12.2, 4.6	3.98	3.95	3.9
	3.76	3.77	3.9	3.76			

position	**2″-*O*-Glc**	**2″-*O*-Glc**	**2″-*O*-Rha**	**2″-*O*-Rha**
1‴	4.40 d 7.7	4.42 d 7.7	5.40 d 1.9	5.31
	4.46 d 7.9	4.46		
2‴	2.95 m	2.94 dd 9.3, 7.7	3.95	4.0
	3.0	2.98		
3‴	3.23 t 9.0	3.22 t br 9.0	3.42	nd
	3.1			
4‴	2.95 m	2.91 m	3.19	4.7
	3.0			
5‴	2.86 m	2.84 ddd 9.8, 6.2, 2.2	2.35	2.5
	2.9	3.01		
6‴A	3.35 dd 11.4, 1.9	3.37 dd 11.3, 2.2	0.66 d 6.3	0.59 d 6.3
6‴B	2.95 m	2.91 m		
	3.4			

position	**4‴-Ac**
2‴′	2.1 s

[a] Duplicated sets of signals for $\mathbf{1}_8$−$\mathbf{4}_8$ correspond to two rotamers: major (top) and minor (bottom). s = singlet, d = doublet, dd = double doublet, t = triplet, m = multiplet, br = broad, Glc = glucoside, Rha = rhamnoside, Ac = acetyl.

cyanin ($\mathbf{1}_6$−$\mathbf{4}_6$ and $\mathbf{5}_8$−$\mathbf{7}_8$, respectively) from which they were formed (*22*). However, the chromatographic retentions of $\mathbf{1}_8$−$\mathbf{4}_8$ and $\mathbf{5}_6$−$\mathbf{7}_6$, respectively, on reversed phase C_{18} material were significantly different from those of their original forms (**Table 1**), which allowed preparative HPLC separation of each pair of 8-*C*-glycosyl- and 6-*C*-glycosyldeoxyanthocyanidins using customized isocratic solution conditions. The structure of each anthocyanin, $\mathbf{1}_8$−$\mathbf{4}_8$ and $\mathbf{5}_6$−$\mathbf{7}_6$ (**Figure 2**), was elucidated by high-resolution MS (**Table 1**) and one- and two-dimensional NMR spectroscopic techniques (**Tables 2** and **3**) in a similar way as described in the next paragraph for $\mathbf{1}_8$. In accordance with previous observations (*22*), rotameric conformers were detected for all of the 8-*C*-glycosyldeoxyanthocyanidins, $\mathbf{1}_8$−$\mathbf{4}_8$, but were absent for the structurally analogous 6-*C*-glycosyldeoxyanthocyanidins, $\mathbf{5}_6$−$\mathbf{7}_6$, in deuterated acidified methanolic NMR solvent (**Tables 2** and **3**).

Clemmensen reduction of 6-*C*-glucosyl-7-*O*-methylapigenin (swertisin) isolated from iris provided the corresponding 3-deoxyanthocyanin, 6-*C*-*β*-glucopyranosyl-7-*O*-methylapigeninidin ($\mathbf{1}_6$) (*22*), which during purification partly rearranged

Table 3. ^{13}C NMR Data (in Parts per Million) for the *C*-Glycosylanthocyanidins, $\mathbf{1}_8-\mathbf{4}_8$ and $\mathbf{5}_6-\mathbf{7}_6$, Dissolved in 5% CF_3COOD in CD_3OD, v/v at 25 °C (See **Figure 1** for Structures)[a]

position	$\mathbf{1}_8$	$\mathbf{2}_8$	$\mathbf{3}_8$	$\mathbf{4}_8$	$\mathbf{5}_6$	$\mathbf{6}_6$	$\mathbf{7}_6$
2	173.79	174.02	174.12	173.6	172.5	172.51	172.9
	173.50	173.6					
3	111.10	111.36	111.45	111.4	110.8	111.11	109.5
	111.17	111.4	112.00	110.9			
4	150.05	149.69	149.96	150.4	149.2	149.5	148.7
	150.20	149.6					
5	160.76	160.70	160.54	160.4	157.5	160.26	159.0
	161.08	160.8	160.0				
6	98.38	98.26	98.26	98.0	114.1	114.64	114.7
	99.25	99.1	99.3	99.0			
7	170.14	170.00	169.94	170.1	169.4	170.84	170.3
	171.35	171.0	171.2				
8	107.85	107.86	107.93	107.8	96.0	96.8	96.5
	107.31	107.3	107.86				
9	156.81	156.92	157.05	156.9	158.4	158.76	157.2
	157.04	157.0	157.2				
10	113.48	113.46	113.21	113.8	113.5	113.76	113.4
	112.97	112.8	113.0				
1′	121.34	122.03	121.86	123.0	120.7	121.39	119.8
		121.6	122.6				
2′	134.40	117.44	134.34	133.6	133.2	133.32	133.6
	133.84	116.3	133.61	133.0			
3′	118.53	148.44	118.38	116.6	118.2	118.65	118.5
	118.6	148.3	116.8	116.8			
4′	168.03	157.05	167.65	168.0	167.0	167.55	168.1
	168.0	157.2	167.7				
5′	118.53	117.92	118.38	116.6	118.2	118.65	118.5
	118.6	118.2	116.8	116.8			
6′	134.40	126.43	134.34	133.6	133.2	133.32	133.6
	133.84	126.2	133.61	133.0			
7-MeO	58.00	57.95	57.93	57.7			
	57.73	57.1	58.00	57.5			
4′-MeO				56.4			
position	**8-*C*-Glc**	**8-*C*-Glc**	**8-*C*-Glc**	**8-*C*-Glc**	**6-*C*-Glc**	**6-*C*-Glc**	**6-*C*-Glc**
1″	74.82	74.91	73.45	73.2	76.4	74.19	74.4
	75.14	75.1	73.5	73.1			
2″	73.05	73.05	79.47	79.0	73.9	76.77	76.2
	72.0	72.1	81.9	82.0			
3″	80.06	80.13	80.11	79.8	79.0	80.66	81.5
	80.47	80.2	79.8				
4″	72.03	72.16	71.77	71.3	70.6	71.1	71.3
	72.22	72.2	70.1				
5″	83.14	83.26	83.15	82.8	82.5	82.8	83.0
	83.30	82.9	82.6				
6″	62.55	62.89	62.47	62.0	61.3	61.7	61.6
	63.45	63.5	61.8	63.0			
position		**2″-*O*-Glc**	**2″-*O*-Glc**		**2″-*O*-Rha**	**2″-*O*-Rha**	
1‴		104.22	103.9		102.8	101.9	
		106.2	105.7				
2‴		75.73	75.4		73.05	72.9	
		75.9	75.6				
3‴		77.74	77.5		72.1	nd	
		77.7					
4‴		71.46	71.2		71.9	76.1	
		70.9					
5‴		77.49	77.3		69.81	68.7	
		77.3	77.5				
6‴		62.52	62.2		17.85	18.0	
		62.0					
position						**4‴-Ac**	
1⁗						172.2	
2⁗						20.2	

[a] Signals with two and one significant decimals are recorded from ^{13}C CAPT and heteronuclear experiments, respectively. Duplicated signals of $\mathbf{1}_8-\mathbf{4}_8$ correspond to two rotamers: major (top) and minor (bottom). Glc = glucoside, Rha = rhamnoside, Ac = acetyl, nd = not detected.

into $\mathbf{1}_8$. Isomers $\mathbf{1}_6$ and $\mathbf{1}_8$ were isolated by preparative HPLC. Isomer $\mathbf{1}_8$ was dissolved in CD_3OD containing 5% CF_3COOD (v/v), a NMR solvent that provided no significant conversion of $\mathbf{1}_8$ to other compounds during storage. The aromatic region of the 1D ^1H NMR spectrum of $\mathbf{1}_8$ revealed a 2H AX system at δ 9.24 (d, 8.8 Hz, H-4) and δ 8.19 (d, 8.8 Hz, H-3), an AA′XX′ system at δ 8.52 (d, 9.0, H-2′,6′) and δ 7.17 (d, 9.0, H-3′,5′), and a 1H singlet at δ 6.96. The latter singlet was identified as H-6 by the $^1J_{CH}$ correlation at δ 6.96/98.4 (H-6/C-6) observed in the $^1H-^{13}C$ HSQC spectrum and the $^3J_{CH}$ correlations observed at δ 6.96/107.9 (H-6/C-8), δ 6.96/113.5 (H-6/C-10) and δ 6.96/160.8 (H-6/C-5) in the $^1H-^{13}C$ HMBC spectrum, corresponding to an 8-*C*-substituted 3-deoxyanthocyanidin with a symmetrically substituted B-ring. A 3H singlet at δ 4.18 (OMe) belonging to the aglycone was confirmed to be at the 7-position by the crosspeak at δ 4.18/170.1 (OMe/C-7) in the long-range $^1H-^{13}C$ HMBC spectrum, in accordance with 7-*O*-methylapigeninidin. All of the sugar proton resonances were assigned by the 2D $^1H-^1H$ DQF-COSY experiment (**Table 2**), and the corresponding ^{13}C resonances (**Table 3**) were then identified by the 2D $^1H-^{13}C$ HSQC and 1D ^{13}C CAPT experiments. The anomeric shift value δ 5.15 (d 10.0 Hz, H-1″), together with the six ^{13}C resonances between 62 and 83 ppm, were in accordance with a *C*-β-glucopyranosyl unit. The crosspeaks at δ 5.15/107.9 (H-1″/C-8), δ 5.15/170.1 (H-1″/C-7) and δ 5.15/156.8 (H-1″/C-9), in the HMBC spectrum of $\mathbf{1}_8$, confirmed the *C*−*C* linkage between the sugar and the aglycone at the 8-position. A molecular ion $[M]^+$ at *m*/*z* 431.1352, corresponding to the molecular formula $C_{22}H_{23}O_9$ (calcd 431.1342), in the HR-ESMS spectrum, confirmed the structure of $\mathbf{1}_8$ to be 8-*C*-β-glucopyranosyl-7-*O*-methylapigeninidin.

UV−Vis Spectroscopic Properties of Isomeric 6-*C*- and 8-*C*-Glycosyl-3-deoxyanthocyanidins. Online UV−vis spectra of isomeric 6-*C*- and 8-*C*-glycosyl-3-deoxyanthocyanidins in the same solvent, $H_2O/CH_3CN/CF_3COOH$ (79.5:20.0:0.5; v/v), were recorded during HPLC separation at isocratic solution conditions. In general, bathochromic shifts of $\lambda_{Vis\text{-}max}$ were observed for 8-*C*-glycosyl-3-deoxyanthocyanidins compared with similar values of their analogous 6-*C*-glycosyl-3-deoxyanthocyanidins (**Figure 5**). These shift differences were most pronounced for the *C*-glycosyl-3-deoxyanthocyanidins with a 7-OMe (10−13 nm), compared with more modest differences (2−4 nm) observed for *C*-glycosyl-3-deoxyanthocyanidins with 7-OH substituents. The shoulder around 419−428 nm on the visible absorption band of the 8-*C*-glycosyl-3-deoxyanthocyanidins was absent in the UV−vis spectra of the analogous 6-*C*-glycosyl-3-deoxyanthocyanidins (**Figure 6**). Comparison of UV−vis spectra of pigments $\mathbf{5}_6-\mathbf{7}_6$ and $\mathbf{5}_8-\mathbf{7}_8$, which were based on the same aglycone and differed by the complexity of their glycosyl substituents (**Figure 2**), showed that increased $\lambda_{Vis\text{-}max}$ values correlated with increased bulkiness of the *C*-glycosyl substituent (**Figure 5**).

Determination of Molar Proportions of Individual *C*-Glycosyldeoxyanthocyanidins in Mixtures. New methodology was developed to determine the molar proportions of individual *C*-glycosyldeoxyanthocyanidins in equilibrium mixtures. The same equilibrium mixtures of isomeric 6-*C*- and 8-*C*-glycosyldeoxyanthocyanidins were compared by both HPLC-DAD detection and ^1H NMR integration. Correlated integration data giving relative molar absorption coefficients and different HPLC retentions of the two isomers made it thereafter possible for the first time for any of this type of flavonoid A-ring rearrangement to determine accurately during monitoring by HPLC-DAD the existence and ratio of the two involved isomeric flavonoids in sample mixtures.

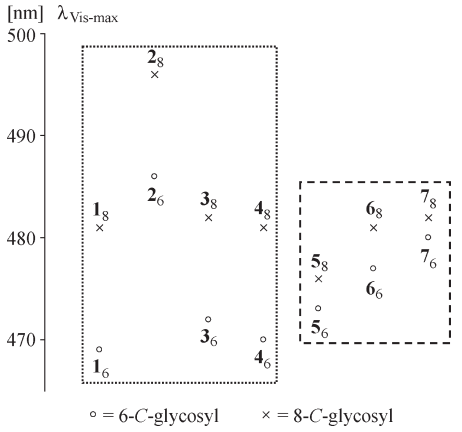


Figure 5. $\lambda_{Vis\text{-}max}$ values for various *C*-glycosyl-3-deoxyanthocyanidins ($\mathbf{1}_6-\mathbf{7}_6$, $\mathbf{1}_8-\mathbf{7}_8$; **Figure 1**), dissolved in $H_2O/CH_3CN/CF_3COOH$ (79.5:20.0:0.5; v/v) at 25 °C. Pigments are grouped according to key substituents; 7-OMe-substituted *C*-glycosyl-3-deoxyanthocyanidins (left box), 7-OH-substituted *C*-glycosyl-3-deoxyanthocyanidins (right box).

Comparative DAD-HPLC and NMR analyses were performed on the reference compounds $\mathbf{1}_6/\mathbf{1}_8$, $\mathbf{2}_6/\mathbf{2}_8$, and $\mathbf{5}_6/\mathbf{5}_8$ using nearly the same solvents; $H_2O/CH_3CN/CF_3COOH$ (79.5:20.0:0.5; v/v). The only difference here was that the CH_3CN component of the HPLC solvent was replaced with CD_3CN in the NMR solvent. After recording of a 1D ^1H NMR spectrum of the equilibrium mixture of $\mathbf{1}_6$ and $\mathbf{1}_8$ that was achieved after 50 h, an aliquot of this sample was subjected to isocratic HPLC-DAD analysis giving an integrated HPLC profile (**Figure 4**). For control reasons, a new 1D ^1H NMR spectrum of the sample was directly thereafter recorded. Integration of the ^1H NMR signals recorded prior to and after HPLC analysis revealed that the relative proportions of $\mathbf{1}_6$ and $\mathbf{1}_8$ remained constant. However, the integrated relative proportions of $\mathbf{1}_6$ and $\mathbf{1}_8$ detected by HPLC-DAD at 475 ± 20 nm were different from the relative proportions determined by NMR by a factor of 1.6. Therefore, the relative molar absorption coefficient of $\mathbf{1}_6$ around $\lambda_{Vis\text{-}max}$ (475 ± 20 nm) was established to be 1.6 times that of its isomer, $\mathbf{1}_8$. On the basis of similar comparisons the relative molar absorption coefficient of $\mathbf{2}_6$ was determined to be 1.4 times that of $\mathbf{2}_8$, whereas the molar absorption coefficient of $\mathbf{5}_6$ was virtually equal to that of its isomer, $\mathbf{5}_8$. Thus, the differences between the molar absorption coefficients were most pronounced for the *C*-glycosyl-3-deoxyanthocyanidins with a 7-OMe ($\mathbf{1}_6/\mathbf{1}_8$ and $\mathbf{2}_6/\mathbf{2}_8$) compared with similar values of $\mathbf{5}_6/\mathbf{5}_8$ with 7-OH substituents.

The difference in the relative molar absorption coefficients of $\mathbf{3}_6$ and $\mathbf{3}_8$ around $\lambda_{Vis\text{-}max}$ (475 ± 20 nm) was assumed to be equal to that of $\mathbf{1}_6$ and $\mathbf{1}_8$ (1.6) due to the similarities of the UV−vis spectra of $\mathbf{1}_6/\mathbf{3}_6$ and $\mathbf{1}_8/\mathbf{3}_8$.

Importance of Restricted Rotation of the A-Ring for Rearrangement of *C*-Glycosyldeoxyanthocyanidins. To confirm the importance of rotation of the deoxyanthocyanidin A-ring during the rearrangement of 6-*C*- to 8-*C*-glycosyldeoxyanthocyanidins, and vice versa, it was decided to make and examine analogous *C*-glycosyl-3-deoxy-5-carboxypyranoanthocyanidins. These compounds have an extra D-ring involving the 5-oxygen covalently connected to C-4 of the C-ring through a −C=C− bridge, which prevents rotation of the A-ring relative to the C-ring. The known reaction between pyruvic acid and anthocyanins for production of carboxypyranoanthocyanins (*21*, *24*, *25*) was thus applied to produce two *C*-glycosyl-3-deoxy-5-carboxypyranoanthocyanidins ($\mathbf{CP3}_6$ and $\mathbf{CP3}_8$) for the first time. **Figure 3** shows the HPLC chromatograms detected around 475 nm of a mixture of 6-*C*-sophorosyl- ($\mathbf{3}_6$) and 8-*C*-sophorosyl-7-*O*-methylapigeninidin

Table 4. ^{1}H and ^{13}C NMR Data for 6-*C*- and 8-*C*-Sophorosyl-5-carboxypyrano-7-*O*-methylapigeninidin (**CP3$_6$** and **CP3$_8$**, Respectively) Dissolved in 5% CF_3COOD in CD_3OD (v/v) at 25 °C

	CP3$_6$		**CP3$_8$**	
position[a]	^{1}H δ *J* (Hz)	^{13}C δ [b]	^{1}H δ *J* (Hz)	^{13}C δ [b]
3	7.91 s	104.0	7.869 s	103.6
5	7.82 s	109.7	7.867 s	nd
6 (*7*)		[c]	7.59 s	97.5
8 (*9*)	7.73 s	97.4		[c]
2′	8.36 d 9.0	132.3	8.43 d 8.9	133.2
3′	7.17 d 9.0	117.8	7.16 d 8.9	117.9
5′	7.17 d 9.0	117.8	7.16 d 8.9	117.9
6′	8.36 d 9.0	132.3	8.43 d 8.9	133.2
7-OCH_3	4.24 d	58.0	4.22 d	58.2
position	**6-*C*-β-Glc**		**8-*C*-β-Glc**	
1″	5.23 d 10.0	72.2	5.31 d 10.0	73.4
2″	4.60 dd 10.0, 8.9	79.8	4.31 dd 10.0, 8.4	79.0
3″	3.83 t 9.1	79.4	3.89 t 8.9	79.8
4″	3.73 t 9.3	71.1	3.86 t 9.1	71.5
5″	3.56 m	82.0	3.60 m	82.9
6(A)″	4.00	62.9	4.04 m	62.3
6(B)″	3.80 dd 11.8, 6.3		3.94 dd 12.2, 5.2	
position	**2″-*O*-β-Glc**		**2″-*O*-β-Glc**	
1″	4.54 d 7.8	104.6	4.42 d 7.8	104.1
2″	3.03 dd 9.2, 7.8	75.4	2.95 dd 9.3, 7.7	75.3
3″	3.29 t 9.2	77.5	3.22 t 9.1	77.5
4″	2.98 t 8.8−9.4	70.7	2.94 t 9.1	70.8
5″	2.94 m	77.4	2.86 ddd 9.8, 5.4, 2.1	77.3
6(A)″	3.33	62.0	3.29 m	62.2
6(B)″	3.08 dd 11.6, 5.4		3.05 dd 11.5, 5.6	

[a] Positions in italics are according to nomenclature for 5-carboxypyranoanthocyanidins. s = singlet, d = doublet, dd = double doublet, t = triplet, m = multiplet, nd = not detected. Glc = glucoside. [b] From ^{1}H−^{13}C HSQC. [c] Data not recorded.

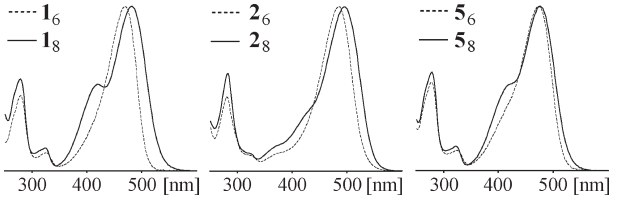


Figure 6. UV−vis spectra recorded for the 6-*C*-glucosyl (dashed line) and 8-*C*-glucosyl (solid line) of 7-*O*-methylapigeninidin (**1**), 7-*O*-methylluteolinidin (**2**), and apigeninidin (**5**) dissolved in $H_2O/CH_3CN/CF_3COOH$ (79.5:20.0:0.5; v/v) at 25 °C.

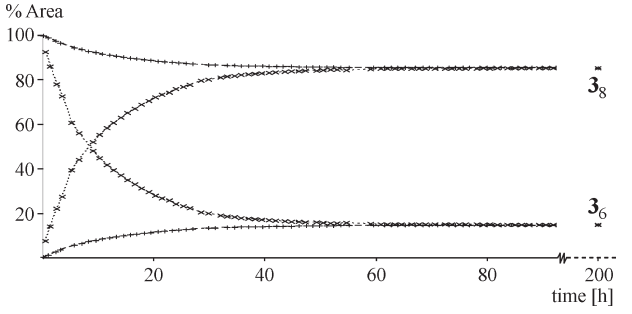


Figure 7. Isomerization of either 6-*C*-sophorosyl-7-*O*-methylapigeninidin (**3$_6$**) or its isomer 8-*C*-sophorosyl-7-*O*-methylapigeninidin (**3$_8$**) ending with the same equilibrium proportions after storage in the same solvent ($H_2O/CH_3CN/CF_3COOH$, 79.5:20.0:0.5; v/v) at 25 °C.

(**3$_8$**) prior to and after addition of pyruvic acid. The occurrence of two extra peaks (**CP3$_6$** and **CP3$_8$**) was revealed in the chromatogram after 20 h of reaction time at 45 °C. After isolation of **CP3$_6$** and **CP3$_8$** by preparative HPLC, structure elucidation by NMR and MS (**Table 1** and **4**) showed that they corresponded to 6-*C*-sophorosyl- and 8-*C*-sophorosyl-5-carboxypyrano-7-*O*-methylapigeninidin (anthocyanidin numbering), respectively. When **CP3$_6$** and **CP3$_8$** were dissolved in acidic aqueous solutions, no rearrangement was indeed observed for any of these anthocyanins. In comparison, **3$_6$** and **3$_8$** (having no D-ring) dissolved individually in the same solvent as **CP3$_6$** and **CP3$_8$**, rearranged both into each other (**Figure 7**).

Various Effects on Rearrangement of *C*-Glycosyldeoxyanthocyanidins. Isomerization of **3$_6$** into its isomer, **3$_8$**, and the opposite reactions ended up with the same equilibrium proportions after storage in the same solvent ($H_2O/CH_3CN/CF_3COOH$, 79.5:20.0:0.5; v/v) at 25 °C (**Figure 7**). The effect of the nature of the *C*-glycosyl moiety on the rearrangement process was examined by subjecting 6-*C*-β-glucopyranosyl-7-*O*-methylapigeninidin (**1$_6$**) and 6-*C*-β-(2″-*O*-β-glucopyranosyl)glucopyranosyl-7-*O*-methylapigeninidin (**3$_6$**) to the same aqueous solvent, $H_2O/CH_3CN/CF_3COOH$ (79.5:20.0:0.5; v/v). At 25 °C the rearrangement of **3$_6$** into its 8-*C*-glycosyl isomer (**3$_8$**) was found to proceed nearly twice as rapidly as the corresponding rearrangement of **1$_6$** into **1$_8$**. Equal amounts of the 6-*C*-glycosyl and 8-*C*-glycosyl isomers were obtained after ∼9.4 and ∼18.5 h for the bioside (**3$_6$** and **3$_8$**) and the monoside (**1$_6$** and **1$_8$**), respectively. At established equilibrium the molar ratios between the 6-*C*- and 8-*C*-glycosyl isomers were 14:86 and 17:83, respectively, for the bioside and the monoside, respectively.

The nature of the aglycone also affected the relative equilibrium proportions of the isomeric *C*-glycosyldeoxyanthocyanidins.

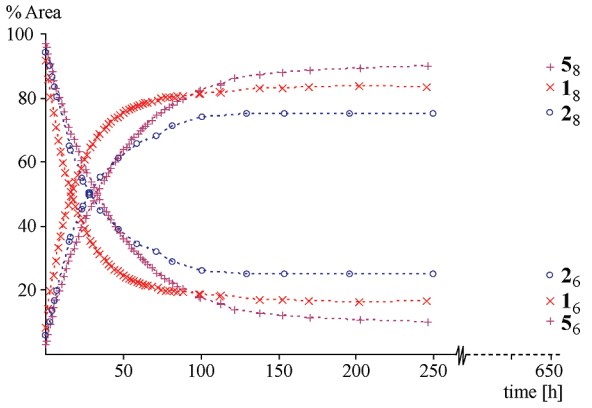


Figure 8. Effect of different *C*-glycosyldeoxyanthocyanidin aglycones on isomerization rates and equilibrium proportions during rearrangements of three 6-*C*-glycosyl-3-deoxyanthocyanidins ($\mathbf{1}_6$, $\mathbf{2}_6$, and $\mathbf{5}_6$) into their 8-*C*-glycosyl isomers ($\mathbf{1}_8$, $\mathbf{2}_8$, and $\mathbf{5}_8$) at 25 °C. The solvent was $H_2O/CH_3CN/CF_3COOH$ (79.5:20.0:0.5; v/v). See **Figure 2** for structures.

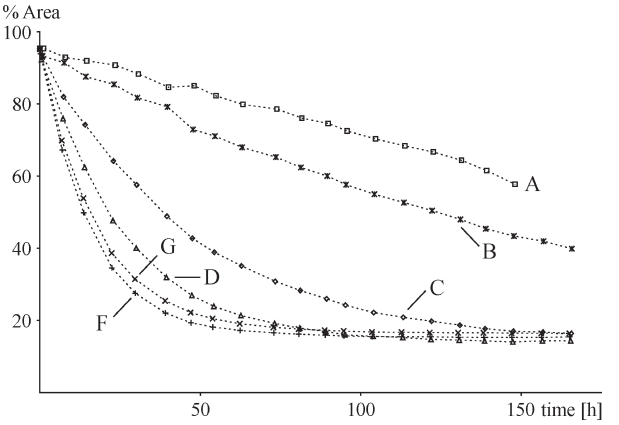


Figure 9. Effect of water content in methanolic solvents on the rearrangements of 6-*C*-β-glucosyl-7-*O*-methylapigeninidin ($\mathbf{1}_6$) into its 8-*C*-glycosyl isomer ($\mathbf{1}_8$) at 25 °C. The curves represent the corrected relative HPLC area % of $\mathbf{1}_6$ during storage in the various solvents, which are described by their mole fraction of water, χ_{H2O}: ~0 (A), 0.02 (B), 0.08 (C), 0.20 (D), 0.43 (E), 0.69 (F), and 0.90 (G). The curve showing $\mathbf{1}_6$ dissolved in solvent E (not shown) was identical with the curve showing $\mathbf{1}_6$ dissolved in solvent F.

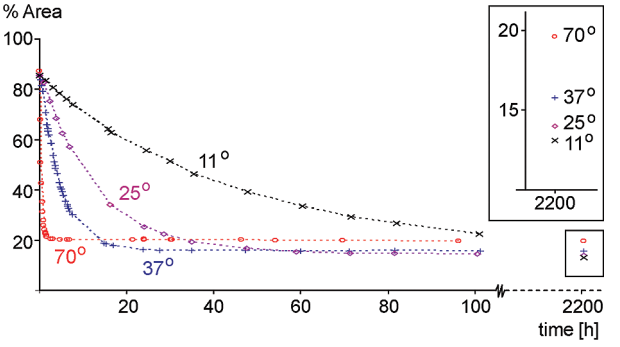


Figure 10. Effect of four different temperatures on isomerization rates and equilibrium proportions during rearrangements of 6-*C*-sophorosyl-7-*O*-methylapigeninidin, $\mathbf{3}_6$, into its 8-*C*-glycosyl isomer, $\mathbf{3}_8$, in $H_2O/CH_3CN/CF_3COOH$ (79.5:20.0:0.5, v/v). The expanded box shows the equilibrium proportions of $\mathbf{3}_6$ at the various temperatures.

When the 6-*C*- and 8-*C*-glucosyldeoxyanthocyanidins of apigeninidin ($\mathbf{5}_6$ and $\mathbf{5}_8$), 7-*O*-methylapigeninidin ($\mathbf{1}_6$ and $\mathbf{1}_8$), and 7-*O*-methylluteolinidin ($\mathbf{2}_6$ and $\mathbf{2}_8$) were examined, the equilibrium proportions were found to be 10:90, 17:83, and 24:76, respectively, in the same solvent ($H_2O/CH_3CN/CF_3COOH$; 79.5:20.0:0.5; v/v) at 25 °C (**Figure 8**). In comparison, lack of the 7-*O*-methyl group ($\mathbf{5}_6$ and $\mathbf{5}_8$) indicates an increase in the proportions of the 8-*C*-glycosyl isomer, whereas two hydroxyl groups in ortho position to each other on the B-ring ($\mathbf{2}_6$ and $\mathbf{2}_8$) indicate an increase in the proportions of the 6-*C*-glycosyl isomer.

To reveal the effect of the solvent for the rate of rearrangement of *C*-glycosyldeoxyanthocyanidins, a sample containing $\mathbf{1}_6$ (~95%) and $\mathbf{1}_8$ (~5%) was first dissolved in methanol containing 0.5% trifluoroacetic acid and equally distributed in seven test tubes before the solvent was removed under nitrogen flux. Various proportions of water and methanol were then added to the test tubes, giving the same concentration of $\mathbf{1}_6$ (1.2 mM) before the content of each sample during storage was monitored by injecting aliquots at regular time intervals into the HPLC system. The proportions of $\mathbf{1}_6$ (and $\mathbf{1}_8$) were normalized by the correlated NMR and HPLC analyses as described above (**Figure 9**). In methanol containing only traces of water, $\chi_{H2O} \ll 0.02$ (A) and $\chi_{H2O} = 0.02$ (B), the rearrangements proceeded relatively slowly. However, when the molar fraction of water increased to 0.08 (C) and further to 0.20 (D) and 0.43 (E), the rearrangement rates increased considerably, in accordance with increased water concentration. A further increase of the molar fraction of water to 0.69 (F) gave no further increase in the rearrangement rate, whereas a molar fraction of 0.90 (G) actually decreased the rearrangement rate slightly compared to the rate observed for solvent E. At this high water concentration, $\chi_{H2O} = 0.90$ (G), increased hydrogen bonding between the water molecules giving a more ordered system (*27*) may account for the reduced rearrangement rate.

The reaction temperature had a profound effect on the rearrangement rate of *C*-glycosyldeoxyanthocyanidins. When samples containing $\mathbf{3}_6$ (~86%) and $\mathbf{3}_8$ (~14%) were dissolved in $H_2O/CH_3CN/CF_3COOH$ (79.5:20.0:0.5; v/v), a 1:1 molar ratio between $\mathbf{3}_6$ and $\mathbf{3}_8$ was established in these solutions after 0.3, 3.5, 9, and 32 h at 70, 37, 25, and 11 °C, respectively (**Figure 10**). At the same temperatures equilibrium proportions of $\mathbf{3}_6$ and $\mathbf{3}_8$ were established after 3, 25, 70, and 200 h, respectively. The relative molar proportion of the 6-*C*-glycosyl isomer ($\mathbf{3}_6$) increased slightly from 13 to 14, 16, and 20% as the temperature increased from 11 to 25, 37, and 70 °C, respectively. The sample subjected to 70 °C showed signs of hydrolysis of the terminal *O*-glucosyl after 24−30 h, forming $\mathbf{1}_6$ and $\mathbf{1}_8$. The monitoring of this sample was ended after ~96 h, and the signal at 2200 h in **Figure 10** for this sample is stipulated.

The rearrangement rates and equilibrium proportions of 6-*C*- into 8-*C*-glucosyl-7-*O*-methylapigeninidin ($\mathbf{1}_6$ and $\mathbf{1}_8$) remained unchanged at sample concentrations of 0.12 and 1.75 mM, respectively. Similar observations showing a lack of concentration effects on the rearrangement of *C*-glycosyldeoxyanthocyanidins were also made for $\mathbf{2}_6$ and $\mathbf{2}_8$ in the same solvent.

Mechanism Involved in Rearrangement of *C*-Glycosyldeoxyanthocyanidins. Rearrangements of *C*-glycosyl-3-deoxyanthocyanidins such as $\mathbf{1}_6-\mathbf{7}_6$ and $\mathbf{1}_8-\mathbf{7}_8$ will most probably occur because of their ability to reside on multiple equilibrium forms. However, evidenced by isocratic HPLC and NMR spectroscopy, the flavylium cation forms of these *C*-glycosyldeoxyanthocyanidins were the only equilibrium forms present at detectable quantities, indicating that only trace amounts of eventual other forms might be present during the rearrangements.

The interesting observation that no rearrangement was observed for the *C*-glycosyl-3-deoxy-5-carboxypyranoanthocyanidins, $\mathbf{CP3}_6$ and $\mathbf{CP3}_8$, supports the importance of rotation of the

A-rings in the isomerization of *C*-glycosyl-3-deoxyanthocyanidins. The high correlation between increased rearrangement rate and increased water content in the solvent supports formation of an intermediate formed by nucleophilic attack by water on the flavylium cation forms. In pure methanol (A) the rearrangements proceeded relatively slowly, which may be due to traces of water or the ability of methanol to function as a nucleophile. A plausible explanation of the rearrangement mechanism might thus be outlined as follows: The initial step may involve nucleophilic attack by a solvent molecule to C-2 of the 3-deoxyanthocyanidin, leading to formation of 2-OH hemiketal equilibrium forms (*8*, *11*, *12*, *14*, *28*). The 2-OH hemiketal forms are prone to undergoing ring opening of the heterocyclic C-ring at C-2. As a consequence, rotation around the C-4−C-10 bond (anthocyanidin numbering) of the open C-ring form, probably a *cis*-chalcone, is now possible. Given that the *C*-glycosylanthocyanidin possesses a free 5-OH, which is the case for $\mathbf{1}_6$−$\mathbf{7}_6$ and $\mathbf{1}_8$−$\mathbf{7}_8$, two different hydroxyl groups located at the A-ring of the open C-ring form are available for ring closure by involving the hydroxyl group positioned either ortho or para to the *C*-glycosyl substituent on the A-ring. After a fast ring closure to give the cyclic form (s) and a subsequent dehydration, these equilibrium reactions are displaced toward the corresponding flavylium cation forms. As a consequence, the relative position of the *C*-glycosyl substituent may be changed from C-6 to C-8, alternatively from C-8 to C-6 in the latter flavylium cation forms.

As described above, different populations of the 6-*C*- and 8-*C*-*β*-glycosyldeoxyanthocyanidins were observed at equilibrium, with a predominance of the 8-*C*-*β*-glycosyldeoxyanthocyanins (76−90%). The nature of the glycosyl substituent as well as the aglycone influenced slightly the relative proportions of the isomeric *C*-glycosyldeoxyanthocyanidins at equilibrium under similar solvent and temperature conditions. These population differences might be partly a result of different conformations (rotamers) of the intermediary open C-ring forms with a steric hindrance resulting in restricted, however, different rotation around the C-4−C-10 bond linking the A-rings of the open forms to the remaining parts of the molecule. Rearrangement of the examined *C*-glycosyldeoxyanthocyanidins giving equilibrium proportions of the 6-*C*- and 8-*C*-*β*-glycopyranosyldeoxyanthocyanidin isomers proceeded considerably more quickly when the solution temperatures were increased from 11 to 70 °C. However, the molar equilibrium proportions of the two isomeric *C*-glycosyldeoxyanthocyanidins were only slightly affected (from 13:87 to 20:80) by this temperature increase (**Figure 10**).

Comparison of A-Ring Rearrangement of Different Flavonoid Types. The substituent pattern on the flavonoid A-ring and the composition of the solvent have been shown to influence Wessely−Moser rearrangements of flavones and flavanones. Whereas the rearrangements of flavones require boiling concentrated hydroiodic (or hydrochloric) acid, flavanones easily undergo A-ring rearrangements in mildly acidic or alkaline solutions (*15*). In general, when trisubstituted flavones and flavanones, having a free hydroxy group at C-5, are considered, the following results have been observed (*15*, *29*). When the substituent at C-7 is equal to its ortho substituent (−OH or $-OCH_3$), isomerization will occur toward the 5,6,7-configuration, whereas an ortho substituent (−OH, $-OCH_3$, or $-CH_3$) different from that at C-7 gives various outcomes; when C-7 is substituted by a OCH_3 group, the 5,7,8-configuration is dominant, whereas a 7-OH group implies formation of a mixture of the 5,6,7- and 5,7,8-isomers. Nonetheless, when 8-alkylamino-5,7-dimethoxyflavones underwent rearrangements to the 5,6,7-configuration in good yields when boiled in concentrated hydrochloric acid during demethylation, the corresponding 5,7-dimethoxy-8-nitroflavone (the NO_2 group being strongly electron-withdrawing) was not rearranged under the same conditions (*30*).

Jurd (*17*) has observed that the 5,7,8-trihydroxyflavylium salt rearranged to its corresponding 5,6,7-trihydroxyflavylium salt within 7 h under mildly acidic conditions (pH ∼2.6), apparently through a chalcone-mediated reaction sequence. In 1% aqueous HCl solution (pH ∼0.5) the same rearrangement required 3 days, possibly due to the shift of the flavylium−chalcone equilibrium more toward the flavylium cation form. Interestingly, the reverse rearrangement, starting with 5,6,7-trihydroxyflavylium salts, was not observed. These observations are in contrast to our observations of *C*-glycosyl rearrangements of *C*-*β*-glycopyranosyldeoxyanthocyanidins, where the equilibrium mainly resides on the 8-*C*-glycosyl isomer, whatever isomer is the starting point. Effects due to substituents, either inductive effects leading to different charge distributions on the oxygen connected to C-5 and C-9 or resonance effects within the A-ring, should therefore be considered when factors affecting rearrangement of *C*-glycosyldeoxyanthocyanidins are observed.

As described above, the transformation of 8-hydroxyanthocyanins to 6-hydroxyanthocyanins has previously been described (*16*−*19*). However, due to the equilibrium being greatly shifted to the formation of 6-hydroxyanthocyanins, hence the use of the word *transformation* in the literature, the experimental data obtained by TLC and UV−vis spectroscopy in these papers will not be sufficient to describe the situation at equilibrium. The precise determination of an established equilibrium in solution, as described in this work, may indicate that the conformations of the open ring intermediate may influence the final isomeric *C*-glycosyl-3-deoxyanthocyanidin ratios. Our observations show that the isomerization processes proceed in both directions, from the 6-*C*- to the 8-*C*-glycosyl-3-deoxyanthocyanidin and vice versa. This precise determination of an established equilibrium in solution may present a unique system for the study of heterocyclic ring opening of anthocyanins. This system has also the advantage that the mixtures of the isomeric 6-*C*- and 8-*C*-glycosyl-3-deoxyanthocyanidins at equilibrium may easily be separated into pure 6-*C*- and 8-*C*-glycosyl-3-deoxyanthocyanidins by preparative HPLC.

It is generally accepted that 3-deoxyanthocyanidins are more stable than anthocyanidins. Open chalcone forms of the common anthocyanins are assumed to be crucial in reactions leading to irreversible degradation of anthocyanins, particularly under weakly acidic to weakly alkaline solution conditions (*3*, *8*, *13*, *31*, *32*), which limits the application of most anthocyanins, for instance, as food colorants. Even in relatively highly acidified aqueous solutions after long-term storage, degradation of the common anthocyanidin *O*-glycosides can be observed (*31*, *32*). In contrast, judged by the relatively high stability of the *C*-glycosyl-3-deoxyanthocyanidins of this study, the forms with open C-rings seem to undergo fast ring closure back to their cyclic forms. After subsequent dehydration, the equilibrium reactions are displaced toward the corresponding flavylium cation forms, which may reduce irreversible degradation of this type of anthocyanin. The *C*-glycosyl-3-deoxyanthocyanidins may thus attract interest as possible food colorants.

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Received March 5, 2009. Revised manuscript received May 16, 2009. Accepted May 22, 2009. This work is part of Project 157347/I20, which receives financial support from the Norwegian Research Council.