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Preparative Isolation and NMR Characterization of Carboxypyranoanthocyanins

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Three pyranoanthocyanins, the 3-glucosides of 5-carboxypyranodelphinidin (**2**), 5-carboxypyranopetunidin (**4**), and 5-carboxypyranomalvidin (**6**), were produced by nucleophilic addition of pyruvic acid to a purified extract of black beans (*Phaseolus vulgaris*) containing a mixture of the 3-glucosides of delphinidin (**1**), petunidin (**3**), and malvidin (**5**). A Sephadex LH-20 column was used for preparative separation of both pyranoanthocyanins and anthocyanins. The optimum solvent used for isocratic separation was 20% methanol containing 0.5% trifluoroacetic acid. The chromatographic method applied on the pigment mixture yielded in a one-step separation pigments **1**−**6**, with purities up to 98, 89, 99, 87, 55, and 81%, respectively. The structures of **2** and **4**, which previously have been tentatively identified mainly by mass spectrometric data acquired from pigment mixtures in wine samples or modified blueberry extract, were confirmed in the present study by two-dimensional nuclear magnetic resonance spectroscopy.

KEYWORDS: Pyranoanthocyanins; 5-carboxypyranodelphinidin 3-glucoside; 5-carboxypyranopetunidin 3-glucoside; preparative Sephadex LH-20; ^{13}C NMR; black beans

INTRODUCTION

The anthocyanins constitute a major flavonoid group which is responsible for cyanic colors ranging from salmon pink through red and violet to dark blue of most flowers, fruits, and leaves of angiosperms (*1*). These pigments are nowadays regarded as important nutraceuticals mainly due to their possible antioxidant effects, and they have been given a potential therapeutic role related to some cardiovascular diseases, cancer treatment, inhibition of certain types of virus including the human immunodeficiency virus type 1 (HIV-1), and improvement of visual acuity (*2*−*14*). The literature on the occurrence of anthocyanins and other flavonoids in foods, their possible dietary effects, bioavailability, metabolism, pharmacokinetic data, and safety have recently been reviewed by several authors (*15*−*20*). There is also a worldwide interest in further use of food colorants from natural sources as a consequence of perceived consumer preferences as well as legislative action in connection with synthetic dyes. However, a major problem with most anthocyanins has been insufficient stability in water solutions when the pH is above 3 (*21*).

Pyranoanthocyanins have been discovered in small amounts in wines and grape pomace (*22*, *23*), petals of *Rosa hybrida* cv. 'M'me Violet (*24*), black carrot (*Daucus carota*) juice (*25*), and blood orange (*Citrus sinensis*) juice (*26*). Four reported methylpyranoanthocyanins from black currant seeds (*27*) were later shown to be the oxidative cycloaddition products of the acetone extraction solvent and the natural anthocyanins (*28*). Pyranocyanin C and D and pyranodelphinidin C and D were also isolated by the same group from an extract of black currant seeds (*29*). These pigments were absent in fresh extracts, and their levels increased gradually with time. Their formation was likely to be from the reaction of the anthocyanins and *p*-coumaric acid in the extracts. Recently, analogous pigments have been isolated from strawberry and raspberry juices after addition of cinnamic acids (*30*). Among the carboxypyranoanthocyanins, vitisin A and acetylvitisin A were identified as the 3-glucoside and the 3-acetylglucoside of malvidin containing an additional $C_3H_2O_2$ unit linking the C-4 and the C-5 hydroxyl group (*31*, *32*). More recently, glucosides of carboxypyranocyanidin have been isolated from red onion (*33*), and carboxypyranopelargonidin 3-glucoside has been isolated from strawberry (*34*) extracts. Analogous delphinidin and petunidin derivatives have been indicated in various pigment mixtures (*35*−*45*).

Although several methods have been developed for separating anthocyanins, even on a preparative scale (*46*), no method has addressed isolation of individual pigments in mixtures of carboxypyranoanthocyanins. Recently, high-speed countercurrent chromatography (HSCCC) (*43*), cation exchange chromatography in the absence and presence of excess bisulfite (*47*), and column chromatography with Toyopearl HW-40F (*40*) have been used to separate pigments in red wines into simpler mixtures, including, however, the whole family of carboxypyranoanthocyanins in the same fraction. Vitisin A and acetylvitisin A have been isolated on a preparative scale by HSCCC; however, further purification steps by preparative HPLC were required to obtain pure vitisin standards (*48*). Individual

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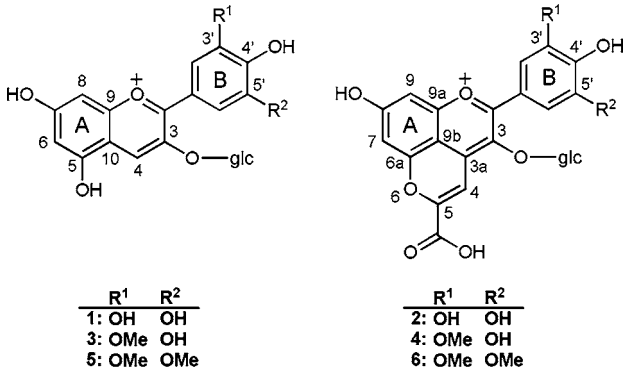

Figure 1. Structures of the 3-glucosides of delphinidin (**1**), petunidin (**3**), and malvidin (**5**), 5-carboxypyranodelphinidin (**2**), 5-carboxypyranopetunidin (**4**), and 5-carboxypyranomalvidin (**6**).

carboxypyranoanthocyanins in mixtures have been detected during analytical HPLC (*39*) and capillary zone electrophoresis (*42*).

A method for isolation of both carboxypyranoanthocyanins and anthocyanins on a preparative scale has now been developed. The method involves large-scale production of carboxypyranoanthocyanins produced by the known reaction between pyruvic acid and anthocyanins (*35*) applied to a partly purified anthocyanin extract of black beans (*Phaseolus vulgaris*). The potential of the method has been indicated by separation of various 5-carboxypyranoanthocyanins (**2**, **4**, and **6**) followed by structure elucidation by NMR spectroscopy. The two former pigments have previously been tentatively identified by UV/vis and mass spectra acquired from pigment mixtures in wine samples (*35*−*44*) or from a modified blueberry extract (*45*).

MATERIALS AND METHODS

Plant Material. Dried black beans (*P. vulgaris*) were purchased from a local food shop, Helios (Bergen, Norway). On-line HPLC analysis of the crude extract showed three major anthocyanins, corresponding to delphinidin 3-glucoside (**1**), petunidin 3-glucoside (**3**), and malvidin 3-glucoside (**5**) (*49*).

Extraction and Purification of the Anthocyanins. Dried black beans (3 kg) were soaked in approximately 3.5 L of water containing 0.5% trifluoroacetic acid (Merck, Darmstadt, Germany) at 4 °C for 24 h. The beans were then extracted four times with 3.5 L of methanol containing 0.5% TFA for 24 h. After concentration under reduced pressure at 28 °C, the combined concentrates were diluted with water to a total volume of 0.5 L before partitioning against ethyl acetate (4 × 0.5 L) (Fisons, Loughborough, U.K.). The water layer containing the anthocyanins was concentrated to 100 mL and subjected to an Amberlite XAD-7 (70 × 5 cm; Sigma, St. Louis, MO) column chromatography. The column was first washed with 2.0 L of distilled water. Then at neutral pH, the anthocyanins were eluted using methanol containing 0.5% TFA (1 L) and dried under reduced pressure followed by freeze-drying, giving a 17.4 g sample.

Hemisynthesis of 5-Carboxypyranoanthocyanins. 5-Carboxypyranoanthocyanins were produced by mixing the Amberlite XAD-7 purified anthocyanins (10 g) dissolved in ethanol (100 mL) containing 2 mL of TFA with pyruvic acid (100 g) (Fluka, Germany) dissolved in distilled water (900 mL) (*21*). The mixture was kept at 45 °C, and the synthesis was monitored by on-line HPLC (Agilent 1100 Series) after 5 min, 2 h, 5 h, 9 h, and 23 h. Relative proportions of pigments **1**−**6** (**Figure 1**) during the reaction are presented in **Figure 3A**. The reaction was terminated after 23 h by placing the reaction bottle in a refrigerator (4 °C). After termination of the synthesis, the ethanol in the reaction mixture was removed under reduced pressure, before the aqueous solution was applied to an Amberlite XAD-7 column (70 × 5 cm). Excess pyruvic acid was washed away with water (2 L), before the pigment mixture was eluted using methanol containing 0.5% TFA (1 L). The sample was then dried under reduced pressure, and out of the 9.4 g sample 6.5 g was subjected to preparative Sephadex LH-20 column chromatography. Using similar conditions, however including vigorous stirring, the hemisynthesis was repeated (**Figure 3B**). This time 7.0 g of the pigment mixture was separated by Sephadex LH-20 column chromatography.

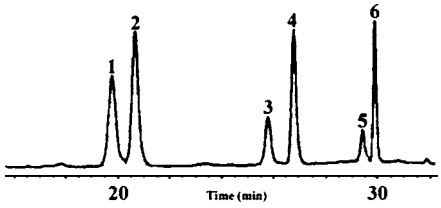

Figure 2. HPLC profile detected at 520 ± 20 nm recorded after 23 h hemisynthesis of the 3-glucosides of 5-carboxypyranodelphinidin (**2**), 5-carboxypyranopetunidin (**4**), and 5-carboxypyranomalvidin (**6**) from the corresponding 3-glucosides of delphinidin (**1**), petunidin (**3**), and malvidin (**5**).

Chromatography. The preparative column (i.d. 9.5 cm) was packed with Sephadex LH-20 (Amersham Bioscience, Sweden) to a height of 70 cm, using 20% methanol with 0.5% TFA. Approximately 6.5 L was eluted prior to the first colored band. The flow rate was 1200 mL/h, and individual fractions (500−2000 mL) were monitored by thin-layer chromatography (TLC) and analytical HPLC. Chromatography was performed on a 14 × 5 cm Toyopearl HW-40F (Tosoh Bioscience) column using 20% methanol with 0.5% TFA as eluent. The analytical HPLC system (Agilent 1100 Series) was equipped with a HP 1050 diode-array detector (Hewlett-Packard), a 20 μL loop, and a 200 × 4.6 mm i.d. 5 μm ODS Hypersil column (Supelco, USA). Two solvents, (A) water (0.5% TFA) and (B) acetonitrile (0.5% TFA), were used for elution. The elution profile for HPLC consisted of initial conditions with 90% A and 10% B followed by gradient elution for 10 min (14% B), isocratic elution 10−14 min, and the subsequent gradient conditions of 18 min (16% B), 22 min (18% B), 26 min (23% B), 31 min (28% B), and 32 min (40% B), isocratic elution 32−40 min, gradient elution for 3 min (10% B), and final linear elution 43−46 min (10% B). The flow rate was 1.0 mL/min, and aliquots of 15 μL were injected with a Micro Autosampler (Agilent 1100 Series). The UV/vis absorption spectra were recorded on-line during HPLC analysis over the wavelength range 240−600 nm in steps of 2 nm. The quantitative amount of anthocyanins **1**, **3**, and **5** in the purified extract used for the hemisynthesis of **2**, **4**, and **6** where determined from a standard curve based on delphinidin 3-glucoside (**1**), without taking into account variation of molar absorption coefficients of individual pigments. Purity determinations of isolated compounds (**1**−**6**) were based on integration data obtained from HPLC profiles monitored at 280 nm ± 10 nm, without taking into account the different molar absorption coefficients of the compounds and potential impurities without adsorption in this UV-range. The purity of **2**, **4**, and **6** (89, 87, and 81%, respectively) were included in the calculations of the yield of these compounds after hemisynthesis and isolation. TLC was carried out on 0.1 mm cellulose F (Merck) with the solvent FHW (HCO_2H−concentrated HCl−H_2O; 25:24:51, v/v/v) (**Table 1**).

NMR Spectroscopy. The NMR experiments on **2**, **4**, and **6** were carried out at 400.13 and 100.61 MHz for ^1H and ^{13}C, respectively, on a Bruker Avance DMX 400 spectrometer equipped with a multinuclear inverse probe for one-dimensional ^1H and two-dimensional heteronuclear single quantum coherence (^1H-^{13}C HSQC), heteronuclear multiple bond correlation (^1H-^{13}C HMBC), and double quantum filtered correlation (^1H-^1H DQF-COSY). The compensated attached proton test (CAPT) experiment was performed with a ^1H/^{13}C BBO probe. The temperatures were stabilized at 25 °C. The samples were prepared by dissolving individual 5-carboxypyranoanthocyanins in CD_3OD:CF_3-COOD (19:1 v/v). As secondary references, the deuteriomethyl ^{13}C and the residual ^1H signal of the solvent were used (δ 49.0 and δ 3.4 from TMS for ^{13}C and ^1H, respectively).

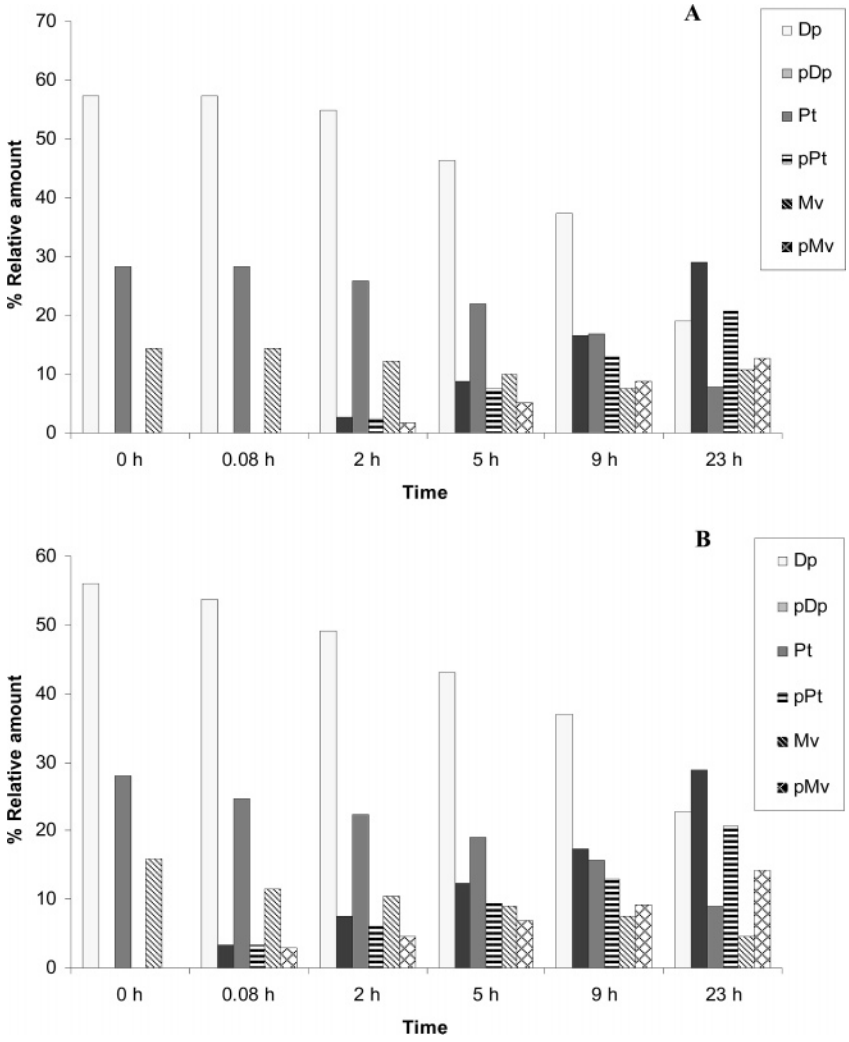

Figure 3. Relative proportions of individual anthocyanins and 5-carboxypyranoanthocyanins monitored at various time intervals during hemisynthesis. The proportions were based on integration data obtained from HPLC profiles monitored at 520 nm ± 20 nm, without taking into account the different molar absorption coefficients of the pigments. Dp = delphinidin 3-glucoside; Pt = petunidin 3-glucoside; Mv = malvidin 3-glucoside; pDp = 5-carboxypyranodelphinidin 3-glucoside; pPt = 5-carboxypyranopetunidin 3-glucoside; pMv = 5-carboxypyranomalvidin 3-glucoside. Parts **A** and **B** reveal the outcome of two different syntheses performed under similar conditions.

Table 1. Chromatographic and Spectroscopic Data of the Major Anthocyanins (**1**, **3**, **5**) in Black Beans (*Phaseolus vulgaris*) and Their 5-Carboxypyrano Derivatives (**2**, **4**, **6**)[a]

	on-line HPLC		TLC (FHW)
pigment	t_R (min)	vis_{max}	hR_F
1	19.81	525	16.5
2	20.69	510	57.5
3	25.82	527	26.3
4	26.79	508	66.3
5	29.43	524	37.5
6	29.88	507	78.8

[a] See **Figure 1** for structures.

RESULTS AND DISCUSSION

Hemisynthesis of 5-Carboxypyranoanthocyanins. Three 5-carboxypyranoanthocyanins (**2**, **4**, **6**) were produced by nucleophilic addition of pyruvic acid to a purified extract of black beans (*P. vulgaris*) containing a mixture of the 3-glucosides of delphinidin (**1**), petunidin (**3**), and malvidin (**5**) (**Figure 1**). The reaction was monitored by on-line HPLC and terminated after 23 h, when both the original anthocyanins (**1**, **3**, and **5**) as well as the synthesized 5-carboxypyranoanthocyanins (**2**, **4**, and **6**) occurred in considerable amounts (**Figure 2**). Using similar conditions during a second workout, however including stirring during the reaction, yielded similar results with respect to the relative proportions of the individual pigments involved (**Figure 3**).

Separation with Sephadex LH-20. During separation of the pigment mixture on a Sephadex LH-20 column a total of 17 fractions were collected manually on the basis of band colors, and the pigment content was monitored by analytical HPLC and TLC. Altogether six bands with mauve to red colors were chromatographically separated. The column separated both the 5-carboxypyranoanthocyanins and the anthocyanins according to their molecular masses. Thus, the 5-carboxypyranomalvidin 3-glucoside (**6**) (band 1) was eluted prior to the corresponding pyruvic adducts of petunidin 3-glucoside (**4**) (band 2) and delphinidin 3-glucoside (**2**) (band 3) followed by the 3-glucosides of malvidin (**5**) in band 4, petunidin (**3**) (band 5), and delphinidin (**1**) (band 6). Bands 3 and 4 were difficult to observe on the column as two separate bands. However, they were separated manually on the basis of the slightly darker color of band 3. This method applied on a 6.5 g sample of the pigment mixture from the first synthesis yielded in a one-step separation 376, 325, 376, 165, 163, and 140 mg of **1**−**6** with purities of

Table 2. ^{1}H (400.13 MHz) and ^{13}C (100.61 MHz) NMR Data for 5-Carboxypyranodelphinidin 3-*O*-β-Glucopyranoside (**2**), 5-Carboxypyranopetunidin 3-*O*-β-Glucopyranoside (**4**), and 5-Carboxypyranomalvidin 3-*O*-β-Glucopyranoside (**6**)[a]

	2 ^{1}H δ (ppm), *J* (Hz)	**4** ^{1}H δ (ppm), *J* (Hz)	**6** ^{1}H δ (ppm), *J* (Hz)	**2** ^{13}C δ (ppm)	**4** ^{13}C δ (ppm)	**6** ^{13}C δ (ppm)
aglycone[b]						
2				166.45	166.15	165.68
3				136.25	136.18	136.08
3a (4)				149.41	149.70	c
4	8.12 s	8.08 s	8.07 s	107.40	107.44	c
5				155.68[d]	155.66[d]	156.43[d]
–COOH				161.33	161.11	161.48
6a (5)				154.39	154.55	154.52
7 (6)	7.29[e] d, 1.9 Hz	7.25[e] d, 1.9 Hz	7.27[e] d, 1.9 Hz	101.34[e]	101.92[e]	101.98[e]
8 (7)				169.39	169.68	169.68
9 (8)	7.24[e] d, 1.9 Hz	7.34[e] d, 1.9 Hz	7.41[e] d, 1.9 Hz	101.75[e]	101.58[e]	101.82[e]
9a (9)				154.34[d]	154.50[d]	154.56[d]
9b (10)				110.71	110.94	111.01
1′				120.21	120.23	120.23
2′	7.65 s	7.82 d, 2.2 Hz	7.81 s	112.12	108.66	110.08
3′				147.24	149.93	149.56
4′				143.38	143.93	144.95
5′				147.24	147.31	149.56
6′	7.65 s	7.62 d, 2.2 Hz	7.81 s	112.12	113.45	110.08
–OMe		3.99 s			57.24	57.26
3-*O*-β-glucopyranoside						
1″	4.81 d, 7.7 Hz	4.81 d, 7.8 Hz	4.79 d, 7.8 Hz	105.73	105.47	105.36
2″	3.74 dd, 7.7 Hz, 9.3 Hz	3.71 dd, 7.8 Hz, 9.2 Hz	3.70 dd, 7.8 Hz, 9.2 Hz	75.43	75.64	75.71
3″	3.47 m	3.46 m	3.45 m	77.65	77.75	77.79
4″	3.35 dd, 9.0 Hz, 8.8 Hz	3.32 dd, 9.0 Hz, 8.7 Hz	3.32 dd, 9.1 Hz, 8.9 Hz	71.39	71.62	71.61
5″	3.24 ddd, 9.0 Hz, 6.8 Hz, 1.9 Hz	3.24 ddd, 9.0 Hz, 6.8 Hz, 1.9 Hz	3.24 ddd, 9.1 Hz, 6.8 Hz, 1.9 Hz	78.93	79.11	79.12
6A″	3.82 dd, 11.7 Hz, 1.9 Hz	3.82 dd, 11.7 Hz, 1.9 Hz	3.81 dd, 11.6 Hz, 1.9 Hz	62.77	62.85	62.83
6B″	3.48 dd, 11.7 Hz, 6.8 Hz	3.48 dd, 11.7 Hz, 6.8 Hz	3.48 dd, 11.6 Hz, 6.8 Hz	62.77	62.85	62.83

[a] The samples are dissolved in CF_3COOD–CD_3OD (5:95, v/v), and data are recorded at 25 °C. s, singlet; d, doublet; dd, double doublet; ddd, double double doublet; m, multiplet. [b] Numbers in parentheses represent anthocyanin positions (see **Figure 1**). [c] Signal is missing. [d,e] Assignments may be reversed. [f] Overlapped by another signal.

up to 98, 89, 99, 87, 55, and 81%, respectively. The low purity of pigment **5** (55%), which was eluted in band 4, was due to coelution with another phenolic compound detected at 280 nm. This impurity was removed by chromatography on a Toyopearl HW-40F column. The yield of the hemisynthesis of pigments **2**, **4**, and **6**, including their isolation by Sephadex LH-20 chromatography, was 20%, 19%, and 30%, respectively. Similar results were obtained when the Sephadex LH-20 column chromatography separation was repeated with a 7.0 g sample from the second hemisynthesis of 5-carboxypyranoanthocyanins. Individual pigments, **1**−**6**, were subjected to NMR analysis. Some chromatographic and spectroscopic data on pigments **1**−**6** are presented in **Table 1**.

Identification of 5-Carboxypyranoanthocyanins by Homo- and Heteronuclear NMR. The structures of the 5-carboxypyranoanthocyanins (**2**, **4**, and **6**) were confirmed by assignments of ^{1}H and ^{13}C NMR data obtained by one- and two-dimensional NMR experiments. In the ^{1}H NMR spectrum of **4**, five signals were located in the aromatic region—a singlet at δ 8.08 (H-4), two 2H *meta*-coupled protons at δ 7.25 (d, 1.9 Hz; H-7) and δ 7.34 (d, 1.9 Hz; H-9), and at δ 7.62 (d, 2.2 Hz; H-6′) and δ 7.82 (d, 2.2 Hz; H-2′)—revealing a 4-substituted anthocyanin having an asymmetrically substituted B-ring (**Table 2**). The signals of H-9 and H-7 may be reversed. The cross-peaks at δ 8.08/161.1 (H-4/COOH), δ 8.08/136.2 (H-4/C-3), δ 8.08/155.7 (H-4/C-5), and δ 8.08/110.9 (H-4/C-9b), and the $^1J_{CH}$ correlation at δ 8.08/107.4 (H-4/C-4) in the HMBC spectrum of **4** were used to assign COOH, C-3, C-5, C-9b, and C-4. Furthermore, C-2 was identified by its long-range correlation with H-2′ and H-6′ (δ 7.82/166.2 and δ 7.62/166.2). The anthocyanidin A-ring carbon signals were identified by the cross-peaks at δ 7.34/169.7 (H-9/C-8), δ 7.34/154.5 (H-9/C-9a), δ 7.34/110.9 (H-9/C-9b), δ 7.34/101.9 (H-9/C-7), δ 7.25/169.7 (H-7/ C-8), δ 7.25/154.6 (H-7/C-6a), δ 7.25/110.9 (H-7/C-9b), and δ 7.25/101.6 (H-7/ C-9), respectively. The carbons belonging to the anthocyanidin B-ring were assigned by the cross-peaks at δ 3.99/149.9 (OCH_3/C-3′), δ 7.82/143.9 (H-2′/C-4′), δ 7.62/143.9 (H-6′/C-4′), δ 7.82/149.9 (H-2′/C-3′), δ 7.62/147.3 (H-6′/C-5′), δ 7.82/113.5 (H-2′/C-6′), δ 7.62/108.7 (H-6′/C-2′), and δ 7.82/120.2 (H-2′/C-1′). There were no obvious cross-peaks in the HMBC spectrum involving C-3a; however, a resonance at δ 149.70 in the CAPT spectrum was assigned to this carbon. Thus, the aglycone of **4** was in agreement with 5-carboxy-2-(3,4-dihydroxy-5-methoxyphenyl)-3,8-dihydroxy-pyrano[4,3,2-*de*]-1-benzopyrylium, 5-carboxypyranopetunidin.

The sugar region of **4** showed the presence of only one sugar unit. The ^{1}H and ^{13}C resonances of the monosaccharide were assigned by a combination of the 1D ^{1}H NMR, CAPT, HSQC, and HMBC experiments (**Table 2**). The ^{1}H-^{1}H coupling constants and the six ^{13}C resonances in the sugar region of the ^{13}C spectrum of **4** were in accordance with β-glucopyranose (*50*). The cross-peak at δ 4.81/136.2 (H-1″/C-3) in the HMBC spectrum confirmed the linkage between the aglycone and the sugar unit to be at the 3-hydroxyl.

The assignments of the chemical shifts in the NMR spectra of 5-carboxypyranodelphinidin 3-*O*-β-glucopyranoside (**2**) and 5-carboxypyranomalvidin 3-*O*-β-glucopyranoside (**6**) were performed in a manner similar to that for 5-carboxypyranopetunidin 3-*O*-β-glucopyranoside (**4**) (**Table 2**).

It has previously been shown that anthocyanins with 4-substituted aglycones such as 5-carboxypyranoanthocyanins have favorable properties such as higher resistance to bleaching by sulfur dioxide, higher color intensity, and restricted formation of the unstable colorless equilibrium forms under weakly

acidic−neutral solution conditions compared to analogous anthocyanidin 3-glucosides (*32*, *34*, *51*, *52*). The carboxypyranoanthocyanins may thus be used as color additives in food, or as antioxidants in human fluids, etc. In this context it is interesting that the same anthocyanins (**1**, **3**, and **5**) from the same source (black beans) as used in the present paper have been considered to inhibit cancer cell growth as well as being the reason for other beneficial pharmaceutical effects (*53*). Carboxypyranoanthocyanins, prepared synthetically or isolated from natural sources, have previously been available only in the low milligram scale, which has limited their potential applications. The present hemisynthetical conversions of anthocyanidin 3-glucosides to their corresponding carboxypyranoanthocyanins performed on a partly purified plant extract of black beans followed by preparative isolation of individual pigments indicate a route for commercial utilization of carboxypyranoanthocyanins.

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