Mammillarinin: A New Malonylated Betacyanin from Fruits of *Mammillaria*

SŁAWOMIR WYBRANIEC*,† AND BARBARA NOWAK-WYDRA‡

Department of Chemical Engineering and Technology, Institute C-1, Faculty of Analytical Chemistry, Cracow University of Technology, ul. Warszawska 24, 31-155 Cracow, Poland, and Faculty of Chemistry, Adam Mickiewicz University, ul. Grunwaldzka 6, 60-780 Poznań, Poland

A new betacyanin endogenously occurring in fruits of nine *Mammillaria* species, i.e., *M. roseo-alba* (Boedecker), *M. donatii* (Berge), *M. coronata* (Scheidweiler), *M. karwinskiana* (Martius), *M. gummifera* (Engelmann), *M. infernillensis* (Craig), *M. centricirrha* (Lemaire), *M. krameri* (Muehlenpfordt), and *M. magnimamma* (Haworth), was studied by means of spectroscopic techniques. The betacyanin was identified as betanidin 5-*O*-(6′-*O*-malonyl)-β-sophoroside for which the trivial name mammillarinin is proposed. In some *Mammillaria* species this compound was reported as a dominating pig-ment. Except for its epimer, two other isomers of mammillarinin were tentatively identified as betanidin/isobetanidin 5-*O*-(4′-*O*-malonyl)-β-sophoroside present in the fruits as acyl migration products.

KEYWORDS: *Mammillaria*; Cactaceae; acylated betacyanins; betalains; mammillarinin; phyllocactin; acyl migration

INTRODUCTION

Mammillaria is the largest and most morphologically variable genus of the Cactaceae family, with about 200 species found mainly in Northern and Central America (*1, 2*). The stems of these low-growing, usually globular or sometimes columnar cacti are covered by a system of tubercles and a crown composed of small pink or white flowers. The bright red or violet juicy fruit berries protrude beyond the tubercles and are usually edible. Having a strawberry taste, some *Mammillaria* fruits are called "chilitos" because of their little chili-pepper shape (**Figure 1**).

It has been known that the fruits of *Mammillaria*, like other cacti species, are pigmented by betalains which are characteristic for plants of the order Caryophyllales (*3*). Betalains, a group of red-violet betacyanins and yellow betaxanthins, have been one of the most frequently investigated plant pigments during the past decade (*4, 5*). Furthermore, betalains are used in various applications in the food industry due to their colorant properties (*6*).

Our research on new betalain structures in fruits of cacti (*7, 8*) has continued on the fruits of *Mammillaria* containing a bunch of polar betacyanins which were detected during our preliminary investigations. Recently, some of the pigments (mainly betanin and phyllocactin) were identified in fruits, and other acylated betacyanins were noticed in petals of *Mammillaria* species after chromatographic comparison to Christmas cactus in the study of Kobayashi (*9*). Very recent studies on *M. candida* callus were

Figure 1. A photograph of *Mammillaria coronata* with protruding fruit berries.

performed to achieve cultures with higher pigmentation (*10*); however, no pigment identification was accomplished. This contribution reports on a new malonylated betacyanin which is probably a dominating pigment in the *Mammillaria* genus.

MATERIALS AND METHODS

Plant Material. The fruits of *M. roseo-alba*, *M. donatii*, *M. coronata*, *M. karwinskiana*, *M. gummifera*, *M. infernillensis*, *M. centricirrha*, *M. krameri*, and *M. magnimamma* as well as the flowers of *Bougainvillea*

* Corresponding author. Phone: +48-12-628-2707. Fax: +48-12-628-2036. E-mail: swybran@chemia.pk.edu.pl.
† Cracow University of Technology.
‡ Adam Mickiewicz University.

Table 1. Total Contents and Relative Concentrations of Major Betacyanins Identified in Nine *Mammillaria* Species

species/peaks	relative betacyanin concentration (%)[a]							total pigment content[a,b] [mg/g]
	1 / 1′	3 / 3′	4 / 4′	6 / 6′	7 / 7′	8 / 8′	9 / 9′	
M. centricirrha	4.0 ± 0.31	26.6 ± 2.4	7.4 ± 0.65	0.13 ± 0.021	26 ± 2.3	0.52 ± 0.067	0.52 ± 0.078	0.080 ± 0.0065
	2.1 ± 0.24	15.2 ± 1.3	3.5 ± 0.43	0.070 ± 0.0091	13 ± 1.2	0.64 ± 0.098	0.32 ± 0.044	
M. roseo-alba	26.1 ± 2.2	9.9 ± 0.87	18.8 ± 1.6	0.27 ± 0.023	6.2 ± 0.57	0.92 ± 0.12	0.47 ± 0.56	0.048 ± 0.0031
	15.9 ± 1.2	6.1 ± 0.55	11.2 ± 1.2	0.15 ± 0.023	3.6 ± 0.41	0.16 ± 0.023	0.23 ± 0.041	
M. donatii	30.3 ± 2.6	2.2 ± 0.25	34.7 ± 2.9	0.28 ± 0.029	3.5 ± 0.41	0.19 ± 0.032		0.172 ± 0.0098
	12.1 ± 0.91	0.66 ± 0.073	14.1 ± 1.2	0.11 ± 0.024	0.66 ± 0.087	1.2 ± 0.21		
M. coronata	18.9 ± 1.4	5.1 ± 0.56	40.2 ± 3.4	0.23 ± 0.037	6.7 ± 0.77	0.24 ± 0.044	0.11 ± 0.023	0.181 ± 0.0092
	6.5 ± 0.53	1.0 ± 0.15	18.1 ± 1.5	0.12 ± 0.033	1.7 ± 0.22	1.1 ± 0.25		
M. karwinskiana	27.1 ± 2.5	1.7 ± 0.11	40.7 ± 3.9	0.35 ± 0.045	1.8 ± 0.21	1.1 ± 0.24		0.017 ± 0.0013
	9.6 ± 0.91	0.75 ± 0.082	16.1 ± 1.3	0.16 ± 0.042	0.64 ± 0.082			
M. magnimamma	0.61 ± 0.057	34.3 ± 0.31	0.70 ± 0.091		29.2 ± 2.1	1.9 ± 0.31	0.32 ± 0.051	0.066 ± 0.0047
	0.31 ± 0.035	17.8 ± 1.5	0.050 ± 0.0071		13.7 ± 1.4	0.80 ± 0.21	0.31 ± 0.051	
M. gummifera	3.6 ± 0.33	13.1 ± 0.11	25.8 ± 2.2	0.15 ± 0.022	40.3 ± 3.4	4.2 ± 0.52	2.4 ± 0.27	0.016 ± 0.0012
	1.5 ± 0.17	1.4 ± 0.16	4.0 ± 0.42	0.05 ± 0.0074	3.5 ± 0.043			
M. infernillensis	28.1 ± 2.4	3.3 ± 0.041	24.2 ± 2.6	0.30 ± 0.047	3.7 ± 0.32		0.48 ± 0.056	0.058 ± 0.0032
	18.1 ± 1.4	1.7 ± 0.19	18.6 ± 1.6	0.12 ± 0.025	1.4 ± 0.027			
M. krameri	4.1 ± 0.31	32.1 ± 3.1	4.2 ± 0.47	0.020 ± 0.0038	13.1 ± 1.6	0.56 ± 0.074	0.51 ± 0.079	0.051 ± 0.0029
	3.2 ± 0.39	27.8 ± 2.9	4.3 ± 0.41	0.01 ± 0.0031	9.7 ± 0.89	0.23 ± 0.037	0.17 ± 0.035	

[a] Relative concentrations were expressed as percentage of total peak area. Average of three measurements. For all peak assignments, see **Table 2**. [b] In betanin equivalents.

glabra Choisy were obtained from the Botanical Garden of Jagiellonian University Institute of Botany during the seasons of 2005 and 2006. The Christmas cactus [*Schlumbergera* x *buckleyi* (T. Moore) Tjaden] flowers were harvested from a plant of the local market.

Fast Betacyanin Screening in the Fruits. The betacyanins from fresh *Mammillaria* fruits (kept at 0 °C after harvesting for a maximum of 2 h) (0.1–0.2 g) were extracted with 1.5 mL of water during 3 min of fruit grinding in a mortar, followed by centrifugation, and immediate HPLC analysis (gradient system 2) without any purification. Only pigments with higher concentration could be analyzed this way because of the higher detection limit. For the measurement of a total concentration of the pigments, 4 mL extracts were prepared according to a similar procedure and analyzed on a UV-160A UV–vis spectrophotometer (Shimadzu, Japan) (**Table 1**). The total concentration was expressed as mg betanin equivalents/g of fresh fruit, taking $E_{538}(1\%) = 1120$ for betanin after spectrophotometric calculations. Three fruits per species were analyzed according to this procedure.

Pigment Extraction. For the semipreparative pigment isolation, the fruits of *M. coronata*, *M. roseo-alba*, *M. donatii*, *M. karwinskiana*, *M. gummifera*, and *M. infernillensis* (200 g each) were separately extracted three times with 300 mL of 80% aq. MeOH and subsequently filtered through a 0.2 μm i.d. pore size filter (Millipore, Bedford, MA). The extract was concentrated using a rotary evaporator under reduced pressure at 25 °C and diluted with water before being freeze–dried. For the coinjection experiments, the extracts of *Hylocereus polyrhizus* fruits and Christmas cactus flowers from the previous study (*8*) as well as the *Bougainvillein glabra* flowers were processed by a similar procedure.

Pigment Purification. For the isolation, the pigment extract was chromatographically concentrated by solid-phase extraction on C18 cartridges (Merck, Darmstadt, Germany) according to the procedure of Stintzing et al. (*21*). The betacyanin fraction was eluted with acidified methanol (methanol/TFA acidified water at pH 2, 95:5, v/v) after rinsing with water and acetonitrile. The eluates were pooled and concentrated using a rotary evaporator under reduced pressure at 25 °C and freeze–dried. The freeze–dried residue was subjected to semipreparative HPLC for isolation of betacyanins.

Semipreparative HPLC. For the semipreparative isolation of betacyanins from the purified extracts, a Gynkotek HPLC system with a UVD170S, Gynkotek HPLC pump series P580, and thermostat (Gynkotek Separations, H.I. Ambacht, The Netherlands) was used. The semipreparative column used was a 250 mm × 10 mm i.d., 10 μm Luna C18(2) (Phenomenex, Torrance, CA) under the following gradient system (system 1): 6% A in B at 0 min; gradient to 10% A in B at 30 min (solvent A, acetonitrile; solvent B, 4% HCOOH in H₂O). In each case the injection volume was 100 μL and the flow rate was 3 mL/min. Detection was generally performed at 538, 505, 480, and 310 nm

with a DAD UV–vis detector. The columns were thermostated at 30 °C. All obtained fractions diluted with water were submitted to freeze-drying and analysis.

Carbohydrate Linkage Analysis. The linkage between the carbohydrate moieties was established by GC-MS analysis of partially methylated alditol acetates prepared by permethylation of betacyanins in basic conditions (dispersed NaOH in DMSO) using methyl iodide with subsequent hydrolysis, reduction, and peracetylation as described by Anumula and Taylor (*15*). Any existing acyl-linked organic residues are lost under these conditions. The GC-MS analyses were performed on a Finnigan gas chromatograph equipped with a 30 m × 0.25 mm i.d., 0.25 μm film thickness, Rtx-5 capillary column (Restek Co., Bellefonte, PA) connected to a Finnigan GCQ ion-trap mass spectrometer (ThermoFinnigan, San Jose, CA) running in the 70 eV electron-impact mode with both the transfer line and the ion source temperature at 200 °C. The gas chromatographic program used was 80 °C held for 1 min then increased at 10 °C/min to 300 °C. The analytes were identified by their retention time and characteristic fragmentation patterns (*16*).

Deacylation Experiments. For the identification of deacylated betacyanins, a modified procedure of Minale et al. (*22*) of alkaline hydrolysis of betacyanins in deoxygenated 0.1 N NaOH for 10 min in an ice-bath was applied. Subsequent acidification of the generated mixture with 0.1 M HCl resulted in recovery of an epimerized mixture of deacylated betacyanins.

For the determination of the type of aliphatic acyl groups attached to the carbohydrate moieties, acid hydrolysis of betacyanins was performed according to Donner et al. (*23*) in a mixture of 2 N HCl and MeOH under a N₂ atmosphere for 1 h in boiling water. The acids were determined as methyl esters by GC-FID after their extraction with chloroform and drying with sodium sulfate. The analyses were performed on a GC 6000 Vega series 2 gas chromatograph (Carlo Erba Instruments, Milan, Italy) equipped with a 30 m × 0.32 mm i.d., 0.25 μm film thickness, DB-FFAP or DB-1701 capillary column (J&W Scientific, Folsom, CA). As standards, dicarboxylic acids were subjected to the same methylation procedure and run by GC.

Analytical HPLC. In general, for the separation and quantification of betacyanins, the analytical HPLC system was the same as in the semipreparative mode except for the analytical column, which was a 100 mm × 4.6 mm i.d. ONYX monolithic column (Phenomenex, Torrance, CA), the gradient system (system 2) (from 5% A in B at 0 min to 20% A in B at 35 min (A, acetonitrile; B, 10% HCOOH in H₂O)), an injection volume of 10 μL, and a flow rate of 0.5 mL/min. In addition, for a better separation of decarboxylated betacyanins, this gradient system was modified (system 3) by changing the composition of eluent B (4% HCOOH in H₂O).

Table 2. Chromatographic, Spectroscopic, and Mass Spectrometric Data of the Analyzed Pigments Found in the *Mammillaria* Fruits

no.	compound	R_t [min]	$\lambda_{max}{}^a$ [nm]	m/z [M + H]$^+$	m/z from MS/MS of [M + H]$^+$
1	betanidin 5-*O*-β-sophoroside	5.5	538	713	551; 389
l′	isoindicaxanthin	6.2	475	309	
l	indicaxanthin	6.4	475	309	
2	17-decarboxylated **1**	6.4	505	669	507; 345
1′	isobetanidin 5-*O*-β-sophoroside	6.8	538	713	551; 389
3	betanin	7.2	537	551	389
2′	17-decarboxylated **1′**	7.9	505	669	507; 345
3′	isobetanin	8.6	537	551	389
4	betanidin 5-*O*-(6′-*O*-malonyl)-β-sophoroside	8.8	539	799	755; 713; 637; 551; 389
5	17-decarboxylated **4**	10.0	505	755	669; 593; 507; 345
4′	isobetanidin 5-*O*-(6′-*O*-malonyl)-β-sophoroside	10.2	539	799	755; 713; 637; 551; 389
6	betanidin 5-*O*-(4′-*O*-malonyl)-β-sophoroside^b	10.4	538	799	755; 713; 637; 551; 389
7	phyllocactin	11.2	538	637	619; 593; 551; 389
6′	isobetanidin 5-*O*-(4′-*O*-malonyl)-β-sophoroside^b	11.4	539	799	755; 713; 637; 551; 389
5′	17-decarboxylated **4′**	11.7	505	755	669; 593; 507; 345
8	4′-*O*-malonyl-betanin	12.1	538	637	619; 593; 551; 389
7′	isophyllocactin	13.0	538	637	619; 593; 551; 389
8′	4′-*O*-malonyl-isobetanin	14.0	538	637	619; 593; 551; 389
9	2′-*O*-apiosyl-phyllocactin	14.3	539	769	683; 551; 389
10′	2′-decarboxylated **4′**	15.0	533	755	669; 593; 507; 345
10	2-decarboxylated **4**	15.4	533	755	669; 593; 507; 345
9′	2′-*O*-apiosyl-isophyllocactin	15.8	538	769	683; 551; 389

^{a} λ_{max} of betaxanthin or betacyanins in the visible range. ^{b} Tentatively identified.

LC-ESI-MS/MS and LTQ FT-ICR Analysis. The positive ion electrospray mass spectra (LC-ESI-MS) and collision-induced-dissociation (CID) spectra (MS/MS mode) were recorded on a ThermoFinnigan LCQ Advantage (electrospray voltage, 4.5 kV; capillary temperature, 250 °C; sheath gas, N_2) coupled to a ThermoFinnigan LC Surveyor pump utilizing the HPLC gradient system 2. The MS was controlled as well as the total ion chromatograms and mass spectra were recorded using the ThermoFinnigan Xcalibur software (San Jose, CA). The relative collision energies for the CID experiments were set at 30% (according to a relative energy scale). Helium was used to improve trapping efficiency and as the collision gas for the CID experiments. The positive LTQ FT-ICR (linear trap quadrupole Fourier transform ion cyclotron resonance) high-resolution mass spectra were recorded on a ThermoFinnigan LTQ FT-ICR (Waltham, MA) mass spectrometer (electrospray voltage, 2.5 kV; capillary temperature, 250 °C; sheath gas, N_2) with direct injection of the sample (5 μL/min). Compound **4** (mammillarinin, betanidin 5-*O*-(2′-*O*-β-D-glucopyranosyl-6′-*O*-malonyl)-β-D-glucopyranoside): LTQ FT-ICR, m/z 799.2057 (calcd for $C_{33}H_{39}N_2O_{21}$, 799.2040, Δ = 1.7 mmu).

NMR Experiments. The NMR spectra of **4** (1.5 mg) isolated from the fruits of *M. coronata* (**Figure 1**) were recorded on a Bruker Avance 600 MHz instrument in D_2O at 300 K. The reference for the ^1H chemical shifts was the residual solvent signal at δ = 4.70 ppm (D_2O) relative to TMS. All 1D (1H, 1D TOCSY) and 2D NMR (gCOSY, gHSQC, gHMBC, *g* = gradient enhanced) measurements were performed using standard Bruker pulse sequences. All chemical shifts are given in ppm relative to TMS, and coupling constants are in Hz. Compound **4** (mammillarinin, betanidin 5-*O*-(2′-*O*-β-D-glucopyranosyl-6′-*O*-malonyl)-β-D-glucopyranoside): ^1H NMR (600 MHz, D_2O) δ = 8.18 (1H, bs, H-11), 7.24 (1H, s, H-7), 7.05 (1H, s, H-4), 6.20 (1H, bs, H-18), 5.83 (1H, bs, H-12), 5.04 (1H, d, $J_{1''-2'} = 7.0$ Hz, H-1″), 4.99 (1H, d, $J_{1'-2'} = 7.0$ Hz, H-1′), 4.92 (1H, dd, $J_{2-3A} = 10.0$ Hz, H-2), 4.40 (1H, bt, $J_{15-14B} = 7.3$ Hz, H-15), 4.38 (1H, dd, $J_{6'A-6'B} = 12.5$ Hz, H-6′A), 4.27 (1H, dd, H-6′B), 3.86 (1H, dd, $J_{6''A-6''B} = 12.6$ Hz, H-6″A), 3.69 (1H, m, $J_{5'-6'A} = 2.0$ Hz, $J_{5'-6'B} = 6.5$ Hz, H-5′), 3.63 (1H, dd, H-6″B), 3.58 (1H, dd, $J_{3A-3B} = 15.6$ Hz, H-3A), 3.57 (1H, m, $J_{5''-6''A} = 2.1$ Hz, $J_{5'-6'B} = 6.5$ Hz, H-5″), 3.52 (1H, m, H-2″), 3.51 (1H, m, H-2′), 3.50 (1H, m, H-3″), 3.48 (1H, m, H-3′), 3.43 (1H, m, H-4′), 3.38 (1H, m, H-4″), 3.27 (due to a fast D/H exchange on the malonyl side chain, the NMR signals were observed in H_2O/D_2O solution (9/1, v/v)) (2H, s, H-2A/B‴), 3.24 (1H, bs, H-14A), 3.15 (1H, dd, $J_{2-3B} = 3.3$ Hz, H-3B), 3.13 (1H, bs, H-14B); ^{13}C NMR (150 MHz, D_2O) δ = 175.2 (C-10), 168.8 (C-1‴), 146.8 (C-6), 145.1 (C-5), 144.0 (C-11), 138.3 (C-8), 127.0 (C-9), 115.0 (C-4), 106.3 (C-12), 101.8 (C-7), 101.3 (C-1″), 100.9 (C-1′), 76.4 (C-5″), 75.3 (C-3′), 73.6 (C-5′),

75.0 (C-2′), 72.8 (C-3″), 72.4 (C-2″), 69.4 (C-4″), 69.3 (C-4′), 64.4 (C-2), 63.7 (C-6′), 60.4 (C-6″), 52.7 (C-15), 37.4 (due to a fast D/H exchange on the malonyl side chain, the NMR signals were observed in H_2O/D_2O solution (9/1, v/v)) (C-2‴), 33.1 (C-3), 26.5 (C-14).

RESULTS AND DISCUSSION

Screening of the Pigments in the Fruits. Our first LC-MS survey conducted on the *Mammillaria* fruit pigments in some species grown in a botanical garden resulted in the discovery of an unusual pattern of relatively polar betacyanins, in comparison to more hydrophobic acylated pigments of *Bougainvillein glabra* flowers (*11–13*) in all the fruits (**Tables 1** and **2**). The process of sample extraction and purification can change the starting concentration ratio between the pigments (e.g., by epimerization or deacylation); therefore, in order to keep the betacyanin profile as stable as possible before the chromatographic analysis, the fresh fruits were processed within a few minutes of extraction and centrifugation and analyzed without any purification on a monolithic HPLC column. On the basis of the chromatographic results obtained, a decision was made on the fruit choice for further semipreparative isolation of the pigments. The HPLC data of decarboxylated betacyanins and indicaxanthin were not processed this way because of their different spectroscopic properties (**Table 2**). The highest total concentration of betacyanins which was close to their concentration in fruits of some *Hylocereus* species (*14*) was found in *M. donatii* and *M. coronata* (~0.18 mg/g).

The most interesting observation was the presence of a new pigment characterized by the same retention times (**4/4′**) existing in relatively high concentration in *M. roseo-alba*, *M. donatii*, *M. coronata*, *M. karwinskiana*, *M. gummifera,* and *M. infernillensis*. Therefore, these fruits were collected during a 2 year period, and their extracts were semipreparatively fractionated for the isolation of **4/4′** which were used for further studies on acyl migration (unpublished results) and decarboxylation. For the NMR analysis, pigment **4** was isolated from the fruits of *M. coronata* (**Figure 1**), because of its highest content in the sample (**Table 1**).

The chromatogram in **Figure 2** depicts a typical betacyanin profile in *M. roseo-alba* samples which were purified and

Mammillarinin: A New Betacyanin

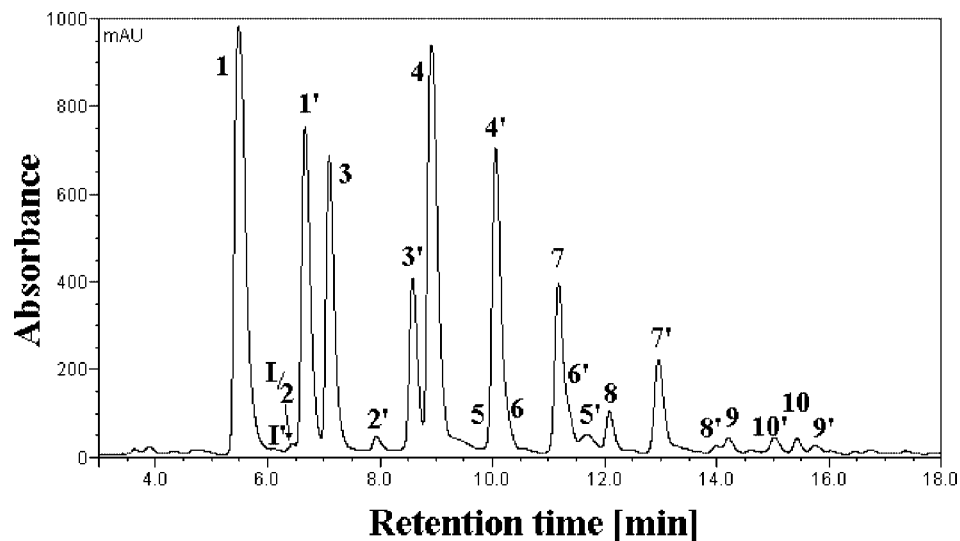*J. Agric. Food Chem.*, Vol. 55, No. 20, 2007 **8141**

Figure 2. HPLC elution profile of betacyanins (λ = 538 nm) in purified and concentrated fruit extract of *Mammillaria roseo-alba*.
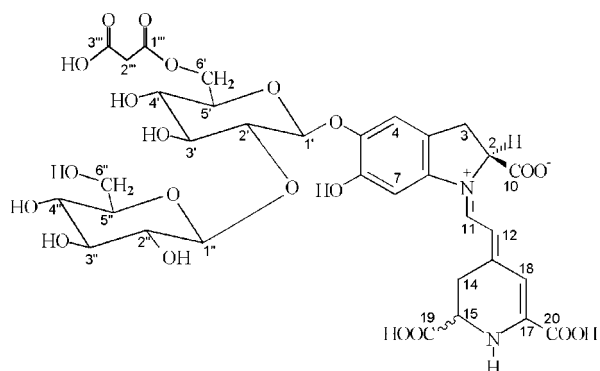

Figure 3. Chemical structure of betanidin 5-*O*-(6′-*O*-malonyl)-β-sophoroside (mammillarinin), a new pigment found in *Mammillaria* fruits.

concentrated. The presence of the known betalains (indicaxanthin **I**, betanidin 5-*O*-β-sophoroside **1**, betanin **3**, phyllocactin **7**, 4′-*O*-malonyl-betanin **8**, and 2′-*O*-apiosyl-phyllocactin **9**) as well as their 15R-isoforms (**Figure 3**) was confirmed by their characteristic spectroscopic properties (**Table 2**) and coelution experiments with the authentic pigments from a recent study (*8*). In contrast to the fruits of *Hylocereus* species, hylocerenin was not present in the analyzed *Mammillaria* fruits. In addition, it should be mentioned that **8** exists in the fruits in equilibrium with phyllocactin **7** as an acyl migration product (*8*). The structures of other compounds were investigated as well as the structure of **4** which was identified by the NMR experiments.

Malonylated Betanidin 5-*O*-β-Sophoroside (Mammillarinin). The major betacyanin **4** showed a protonated molecular ion at *m/z* 799 and its daughter ion fragments at *m/z* 713, 637, 551, and 389 using positive ion mode LC-MS/MS. The mass and the fragmentation pattern suggested the presence of malonylated (mass difference of 799 – 713 = 86) dihexose (713 – 389 = 2 × 162). The position of the malonyl residue on the first hexose unit was suggested by the loss of one hexose (799 – 637 = 162). By LTQ FT-ICR, the protonated molecular ion was found at *m/z* 799.2057 (calcd for $C_{33}H_{39}N_2O_{21}$, 799.2040) supporting the conclusion that **4** could be a malonylated dihexosyl of betanidin. From the ratio of the absorbances at 537 and 327 nm (1:0.16), the presence of hydroxycinnamoyl residues as acylating moieties in **4** could be excluded (*12*). The GC-FID analysis of the methylated hydrolysate of **4** resulted in identification of dimethyl malonate ester, supporting the presence of the malonyl residue.

Furthermore, no any other acylated moieties were found. The carbohydrate system was indicated by the alkaline deacylation of **4** and subsequent acidification of the resulting mixture with HCl. The liberation of a mixture of **1/1′** analyzed chromatographically suggested the presence of the 5-*O*-β-sophorosyl system in the structure of **4**. The linkage between the two sugar moieties was established by methylation analysis (*15*) under basic conditions, in which all acyl residues are lost, with subsequent detection of 1,2,5-tri-*O*-acetyl-3,4,6-tri-*O*-methyl-glucitol and 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-glucitol by GC-MS, as in the case of recently analyzed **1** (*8*), identified by their characteristic fragmentation patterns (*16*). The analysis showed the terminal position of the second glucopyranosyl bound to C-2′ of the first glucopyranosyl moiety.

Recent reports provided the first ^{13}C NMR data of C14-C15 saturated betacyanins (*8, 17*), decarboxy-betacyanins (*18*), and betaxanthins (*19*). The long-term experiments (e.g., gCOSY, gHSQC, and gHMBC) required good stability of betalains dissolved in an appropriate solvent. In highly acidic conditions, betalains were too labile for such experiments; therefore, the NMR spectra acquisitions were performed in D_2O without acid additions despite signal broadening due to a low degree of protonation (*17*).

The characteristic NMR signals of the aglycone and glucose moieties confirmed the presence of a betanin derived compound (*8, 17*). The individual coupled [1]H-spin systems of the aglycone (H-2, H3a/b, H-4; H-7; H-11, H-12; H-14a/b, H-15) and of the hexose moieties were assigned in the ^{1}H NMR, 1D TOCSY, 1D NOESY, and gCOSY spectra. The dihydroindolic system was assigned by gHSQC correlations of H-2, H-3a/b, H-4, and H-7 with their respective carbons. The correlations of C-5 to H-4 and H-7, C-6 to H-4, C-8 to H-4, C-9 to H-7, and H-3A/H-3B as well as C-10 to H-3A/H-3B were determined by gHMBC.

The other ^{13}C chemical shifts for carbons directly bound to protons were assigned by gHSQC correlations. The attachment positions of the first and the second hexose moieties were indicated by the gHMBC correlations of the anomeric proton H-1′ to the phenolic carbon C-5 and the second hexose proton H-1″ to the first hexose carbon C-2′, respectively. In addition, the β-linkage between the aglycone and glucopyranosyl moiety was supported by the three-bond vicinal proton coupling constant $(3)J_{1'-2'} \sim 7$ Hz. The low field chemical shift of H-6′A/H-6′B provided definitive evidence that the malonyl moiety was bound to C-6′. The gHMBC correlations of C-1‴ to H-6′A and

8142 *J. Agric. Food Chem.,* Vol. 55, No. 20, 2007

Wybraniec and Nowak-Wydra

H-6′B confirmed this linkage position. The gHMBC and gHSQC experiments were repeated in a mixture of H_2O and D_2O (90/10, v/v) in order to detect the malonyl moiety with H-2‴A and H-2‴B protons not being exchanged with deuterium, completing the structure identification of **4** as betanidin 5-*O*-(6′-*O*-malonyl)-β-sophoroside.

Betanidin 5-*O*-(4′-*O*-Malonyl)-β-sophoroside: An Acyl Migration Product. Two small additional peaks at *m/z* 799 for **6/6′** were detected when monitoring with an MS detector, eluting after the corresponding **4/4′**, respectively. Compounds **6/6′** coeluted with other peaks with the gradient system 3 applied (**6** coeluted with **4′**); however, increasing the concentration of formic acid in eluent B allowed the separation of **4′** and **6** (data not shown). These isomeric pigments were tentatively identified in analogy to phyllocactin isomers as acyl migration products of **4/4′**. Therefore, it is likely that the malonyl migration in **6** proceeds from the C′-6 to the C′-4 carbon, and that compound **6** is betanidin 5-*O*-(4′-*O*-malonyl)-β-sophoroside.

The phenomenon of acyl migration in betacyanins has been recently noticed (*8*), and further studies supported this finding (unpublished results). The interconversion between purified **4** and **6** as well as between **4′** and **6′**, reaching an equilibrium within minutes, was observed in alkaline solutions (pH 10) but also proceeded within a few days in neutral solutions (unpublished results). In each case, the resulting equilibrated pigment composition favored the 6′-*O*-malonylated forms (**4/4′**). The formation of an intermediate strainless cyclic ortho ester structure between the glucosidic *O*-4′ and *O*-6′ hydroxyls, which was reported frequently in many cases of acyl migration in acylated β-D-glucosides, can also be responsible for the interconversion between **4** and **6** (*24*).

Decarboxylated Betacyanins. Closer inspection of the LC-MS spectrometric data revealed two peaks at *m/z* 669 for **2/2′** and four peaks at *m/z* 755 for **5/5′** and **10′/10**. This data (**Table 2**) and the mass difference between **1** and **2** as well as between **4** and **5/10** suggested the decarboxylation of the corresponding betacyanins (**1** and **4**) at the C-2 or C-17 carbon. Coelution experiments with prepared standards by decarboxylation of the isolated pigments **1/1′** and **4/4′** in ethanolic and aqueous solutions according to Wybraniec et al. (*18, 20*) confirmed these suggestions; therefore, **2** and **5** are the 17-decarboxylated derivatives of **1** and **4**, respectively, and **10** is the 2-decarboxylated derivative of **5**. In addition, the isoform of **10′** should elute earlier than **10** according to Wybraniec et al. (*20*).

The presence of decarboxy-betacyanins in the samples can be an indicator of decarboxylation occurring usually during the extraction and cleanup process at elevated temperatures (*20*); however, this could be excluded, since the artifacts of betanin and phyllocactin did not appear in the same samples. It should also be considered that the decarboxylation of the pigments, if any, could occur between the harvesting and freezing period.

The *Mammillaria* fruits contain a new betacyanin identified as betanidin 5-*O*-(6′-*O*-malonyl)-β-sophoroside which is a dominating pigment in some *M.* species. Two pigments were detected as the possible acyl migration products of mammill-arinin and phyllocactin. The other betacyanins are betanidin 5-*O*-β-sophoroside, betanin, phyllocactin, and 2′-*O*-apiosyl-phyllo-cactin. All these pigments constitute a group of relatively polar betacyanins. In the case of acylated compounds in the analyzed *Mammillaria* fruits, the malonyl is the only acylating moiety. On the basis of the studies on the nine *Mammillaria* species, it can be stated that the fruits are characterized by exceptional betacyanin profiles in comparison to edible fruits of other very well-known cacti species (*Hylocereus* and *Opuntia*) and the

concentration of these pigments in the fruits of some of the species is comparable to that of the *Hylocereus* fruits, confirming that they are another rich source of these potentially chemo-preventive compounds.

LITERATURE CITED

(1) Pilbeam, J. *Mammillaria*; Nuffield Press: Oxford, U.K., 1999.
(2) Butterworth, C. A.; Wallace, R. S. Phylogenetic studies of *Mammillaria* (Cactaceae) - insights from chloroplast sequence variation and hypothesis testing using the parametric bootstrap. *Am. J. Bot.* **2004**, *91*, 1086–1098.
(3) Piattelli, M.; Imperato, F. Betacyanins of Family Cactaceae. *Phytochemistry* **1969**, *8*, 1503–1507.
(4) Strack, D.; Vogt, T.; Schliemann, W. Recent advances in betalain research. *Phytochemistry* **2003**, *62*, 247–269.
(5) Carle, R.; Schieber, A.; Stintzing, F. C. *Pigments in Food -A Challenge to Life Sciences*; Shaker Verlag: Aachen, Germany, 2006.
(6) Henry, B. S. Natural Food Colours. In *Natural Food Colorants*; Hendry, G. A. F., Houghton, J. D., Eds.; Blackie Chapman & Hall: London, U.K., 1996; pp 40–79.
(7) Wybraniec, S.; Platzner, I.; Geresh, S.; Gottlieb, H. E.; Haimberg, M.; Mogilnitzki, M.; Mizrahi, Y. Betacyanins from vine cactus *Hylocereus polyrhizus*. *Phytochemistry* **2001**, *58*, 1209–1212.
(8) Wybraniec, S.; Nowak-Wydra, B.; Mitka, K.; Kowalski, P.; Mizrahi, Y. Minor betalains in fruits of *Hylocereus* species. *Phytochemistry* **2007**, *68*, 251–259.
(9) Kobayashi, N. Contributions to Betalain Biochemistry: New structures, condensation reactions, and vacuolar transport, Martin-Luther-Universität Halle-Wittenberg, Mathematisch-Naturwissen-schaftlich-Technische Fakultät, Halle (Saale), Germany, 2002.
(10) Santos-Diaz, M. S.; Garcia, Y. V.; Chavez, M. M. G. Producción de pigmentos por callos de *Mammillaria candida* Scheidweiler (Cactaceae). *Agrociencia* **2005**, *39*, 619–626.
(11) Piattelli, M.; Imperato, F. Betacyanins from *Bougainvillea*. *Phytochemistry* **1970**, *9*, 455–458.
(12) Heuer, S.; Richter, S.; Metzger, J. W.; Wray, V.; Nimtz, M.; Strack, D. Betacyanins from bracts of *Bougainvillea glabra*. *Phytochemistry* **1994**, *37*, 761–767.
(13) Kugler, F.; Stintzing, F. C.; Carle, R. Characterisation of betalain patterns of differently coloured inflorescences from *Gomphrena globosa* L. and *Bougainvillea* sp. by HPLC-DAD-ESI-MS[n]. *Anal. Bioanal. Chem.* **2006**, *387*, 637–648.
(14) Wybraniec, S.; Mizrahi, Y. Fruit flesh betacyanin pigments in *Hylocereus* cacti. *J. Agric. Food. Chem.* **2002**, *50*, 6086–6089.
(15) Anumula, K. R.; Taylor, P. B. A comprehensive procedure for preparation of partially methylated alditol acetates from glycoprotein carbohydrates. *Anal. Biochem.* **1992**, *203*, 101–108.
(16) Biermann, C. J.; McGinnis, G. D. *Analysis of Carbohydrates by GLC and MS*. CRC Press: Boca Raton, FL, 1989.
(17) Stintzing, F. C.; Conrad, J.; Klaiber, I.; Beifuss, U.; Carle, R. Structural investigations on betacyanin pigments by LC NMR and 2D NMR spectroscopy. *Phytochemistry* **2004**, *65*, 415–422.

Mammillarinin: A New Betacyanin

J. Agric. Food Chem., Vol. 55, No. 20, 2007   **8143**

(18) Wybraniec, S.; Nowak-Wydra, B.; Mizrahi, Y. ^{1}H and ^{13}C NMR spectroscopic structural elucidation of new decarboxylated betacyanins. *Tetrahedron Lett.* **2005**, *47*, 1725–1728.

(19) Stintzing, F. C.; Kugler, F.; Carle, R.; Conrad, J. First ^{13}C-NMR assignments of betaxanthins. *Helv. Chim. Acta* **2006**, *89*, 1008–1016.

(20) Wybraniec, S.; Mizrahi, Y. Generation of decarboxylated and dehydrogenated betacyanins in thermally treated purified fruit extract from purple pitaya (*Hylocereus polyrhizus*) monitored by LC-MS/MS. *J. Agric. Food Chem.* **2005**, *53*, 6704–6712.

(21) (a) Stintzing, F. C.; Schieber, A.; Carle, R. Betacyanins in fruits from red-purple pitaya*Hylocereus polyrhizus* (Weber) Britton& Rose. *Food Chem.* **2002**, *77* (1), 101–106. (b) Stintzing, F. C.; Schieber, A.; Carle, R. Betacyanins in fruits from red-purple pitaya, *Hylocereus polyrhizus* (Weber) Britton & Rose. *Food Chem.* **2002**, *77* (4), 517 (Corrigendum).

(22) Minale, L.; Piattelli, S.; De Stefano, S.; Nicolaus, R. A. Pigments of Centrospermae VI. Acylated betacyanins. *Phytochemistry* **1966**, *5*, 1037–1052.

(23) Donner, H.; Gao, L.; Mazza, G. Separation and characterization of simple and malonylated anthocyanins in red onions *Allium cepa* L. *Food Res. Int.* **1997**, *30*, 637–643.

(24) Haines, A. H. Relative reactivities of hydroxyl groups in carbohydrates. *Adv. Carbohydr. Chem. Biochem.* **1976**, *33*, 11–109.