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Structural investigations on betacyanin pigments by LC NMR and 2D NMR spectroscopy

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Abstract

Four betacyanin pigments were analysed by LC NMR and subjected to extensive NMR characterisation after isolation. Previously, low pH values were applied for NMR investigations of betalains resulting in rapid degradation of the purified substances thus preventing extensive NMR studies. Consequently, up to now only one single ^{13}C NMR spectrum of a betalain pigment, namely that of neobetanin (= 14,15-dehydrobetanin), was available. Because of its sufficient stability under highly acidic conditions otherwise detrimental for betacyanins, this pigment remained an exemption. Since betalains are most stable in the pH range of 5–7, a new solvent system has been developed allowing improved data acquisition through improved pigment stability at near neutral pH. Thus, not only ^{1}H, but for the first time also partial ^{13}C data of betanin, isobetanin, phyllocactin and hylocerenin isolated from red-purple pitaya [*Hylocereus polyrhizus* (Weber) Britton & Rose, Cactaceae] could be indirectly obtained by gHSQC– and gHMQC–NMR experiments.

Keywords: Betalains; Betacyanin; Betanin; Isobetanin; Phyllocactin; Hylocerenin; Pitaya; *Hylocereus polyrhizus*; NMR spectroscopy; LC NMR spectroscopy

1. Introduction

Betalains are the nitrogenous vacuolar pigments of 13 families within the plant kingdom also accumulating in some members of the Basidiomycetes (Gill and Steglich, 1987; Gill, 1994; Clement and Mabry, 1996). They comprise two subgroups, the red-violet betacyanins and the yellow-orange betaxanthins (Steglich and Strack, 1990; Strack et al., 2003). Betalains are important chemotaxonomical markers and have never been found jointly with anthocyanins in the same plant (Stafford, 1994; Clement and Mabry, 1996). Besides their colouring function, betacyanins and betaxanthins exert diverse biological activities both in plants and in humans (Stintzing and Carle, 2004). Because of their high tinctorial strength and their colour hue remaining unchanged over a broad pH range from 3 to 7, betalains have attracted technological interest as natural food colourants (Von Elbe, 1975; Cai et al., 1998, 2001a; Cai and Corke, 1999; Stintzing et al., 2000, 2003).

The structures of betalains have mainly been studied to shed light on biochemical pathways (Hempel and Böhm, 1997; Schliemann et al., 1999; Kobayashi et al., 2000, 2001), but also to identify the colouring principles from plant tissues of various fruits and vegetables (Wyler and Dreiding, 1961; Glässgen et al., 1993; Schliemann et al., 1996, 2001; Cai et al., 2001a,b; Stintzing et al., 2002a,b).

The betacyanins from the red-violet cactus fruit [*Hylocereus polyrhizus* (Weber) Britton & Rose] also known as pitaya have been studied only recently by LC–MS and ^{1}H NMR investigations (Wybraniec et al., 2001; Stintzing et al., 2002b). However, no ^{13}C NMR data of the newly identified betacyanin, named hylocerenin, were provided (Wybraniec et al., 2001). Yet without exception highly acidic conditions were applied for NMR spectra acquisition of betacyanins (Strack et al., 1993; Strack and Wray, 1994) and betaxanthins (Strack et al., 1987a). Since the betalains are labile under these conditions (Schliemann et al., 1996; Kobayashi et al.,

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2000), degradation proceeds very quickly resulting in complex NMR spectra and in total loss of the analyte. This may explain the existence of only one report providing carbon-NMR data of a betalain pigment (Alard et al., 1985).

During NMR experiments in acidified solutions, besides signals associated with the genuine pigment, the spectrum is superposed by an array of additional structures that may be produced by configurational epimerisation at C-15 (Wilcox et al., 1965; Wyler et al., 1963) as well as *E*/*Z* double-bond isomerism at C-12, C-13 (Hilpert and Dreiding, 1984; Hilpert et al., 1985; Heuer et al., 1992). Therefore, one aim of the present study was to find a solvent system affording at the same time the chemical stability and avoiding rapid isomerisation of betalains thus permitting improved and prolonged data collection including ^{1}H–^{13}C NMR correlations. Since LC NMR experiments allow the rapid identification of natural products in extracts or pre-purified fractions without tedious isolation and especially the investigation of labile compounds (e.g. Bobzin et al., 2000; Bringmann et al., 2001; Cogne et al., 2003; Gavidia et al., 2002; Lindon et al., 2000; Setzer et al., 2003; Spring et al., 2001; Vogler et al., 1998), this technique was applied to the betalain enriched fraction of concentrated juice from red-violet pitaya *H. polyrhizus* (Weber) Britton & Rose in order to identify the individual betalains. Furthermore, the quality of the chromatographic separation for the subsequent isolation was proven.

2. Results and discussion

LC NMR and LC MS analysis of the betalain fraction of *Hylocereus polyrhizus* revealed the presence of six betalains. According to the HPLC elution order (Wybraniec et al., 2001; Stintzing et al., 2002b), compounds were identified as betanin (**1**), isobetanin (**2**), phyllocactin (**3**), hylocerenin (**4**), isophyllocactin (**5**) and isohylocerenin (**6**) (Fig. 1). Comparison of the on-line ^{1}H NMR spectra (Fig. 2, Table 1) of **1** and its stereoisomer **2** as well as **4** and **6** confirmed the findings that C-15 epimers do not unequivocally differ (Strack et al., 1993; Strack and Wray, 1994). In order to obtain ^{13}C information of the individual substances and to investigate structural differences between the respective betacyanins, compounds **1–4** were semi-preparatively isolated by HPLC–DAD (see Section 4.5). During the isolation process, slight degradation of the compounds was observed despite gentle concentration of the collected fractions with a rotavaporator at room temperature. As a consequence, direct lyophilisation of the semi-preparatively isolated eluates was found to be a prerequisite to prevent artefact generation.

For the NMR investigations in general, it has hitherto been postulated that a low pH ($\leqslant 2$) was a precondition for data acquisition in order to keep the respective betalain pigment in a stable zwitterionic state (Strack et al., 1993; Strack and Wray, 1994). Furthermore, except for neobetanin, no ^{13}C NMR spectrum has been reported so far (Alard et al., 1985). Neobetanin is exceptional as it exhibits a C-14/C-15 double bond and consequently does not produce C-15 epimers. Due to the symmetrical 1,4-dihydropyridyl moiety in neobetanin isomerisation of the C-12/C-13 double bond does not result in the formation of further stereoisomers as expected for common betacyanin structures. In addition, besides being a genuine pigment of red beet (Alard et al., 1985; Kujala et al., 2001) or cactus pear (Strack et al., 1987b), neobetanin has also been reported to be an artefact of sample treatment at low pH values (Wyler, 1986). Inversely, neobetanin is stable under acidic conditions allowing spectrum acquisition without progressing degradation. Actually, in previous investigations, the pH issue was found to be the most crucial parameter for the structural characterisation of betacyanins by NMR because not only did acidification of the most frequently used solvents DMSO-d_6 and methanol-d_4 by TFA or DCl ensure high solubility, but at the same time cause rapid and undesirable C-15 and C-12/C-13-isomerisations, and finally total betalain decomposition (Minale et al., 1966; Wyler and Dreiding, 1984; Hilpert et al., 1985; Schliemann et al., 1996; Kobayashi et al., 2000). Furthermore, in acidified methanol acylglucosides substituted with a dicarboxylic acid such as malonic acid may form methyl ethers (Fossen et al., 2001) and thus prevent structural elucidation of the respective genuine molecular structures.

For homo and especially heteronuclear 2D NMR experiments on quantities in the range of 1.5–2 mg pigment, pH conditions $\leqslant 2$ had to be avoided to ensure long-term stability. Additionally, screening for a optimum solvent system was essential to achieve high solubility of

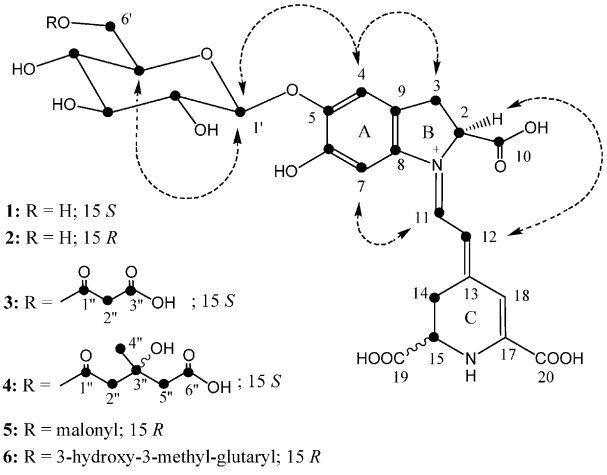

Fig. 1. Structures of betalains **1–6**. The carbons marked by (•) could be assigned by gHSQC and gHMQC NMR experiments. The unmarked carbons were not accessible. Important ROE's are shown by arrows.

betalains together with minimum overlapping of residual NMR solvent and analyte signals. As stability criteria, the time-dependent formation of additional ^{1}H NMR signals for H-4 and H-7 (Wyler and Dreiding, 1984; Hilpert and Dreiding, 1984; Hilpert et al., 1985) due to C-12/C-13-isomerism and the appearance of two additional anomeric protons arising from glucose released by hydrolytic betalain cleavage were monitored. Betacyanins were insoluble in organic solvents such as DMSO, ethanol, isopropanol and methanol unless the pH was set below 3. Although solvation in pyridine without pH adaption was possible if traces of water were added, betalain pigments degraded rapidly thereafter. Through the addition of water in trace amounts, the pigments were also soluble in *N*,*N*-dimethylacetamide and *N*,*N*-dimethylformamide. Since no overlapping of

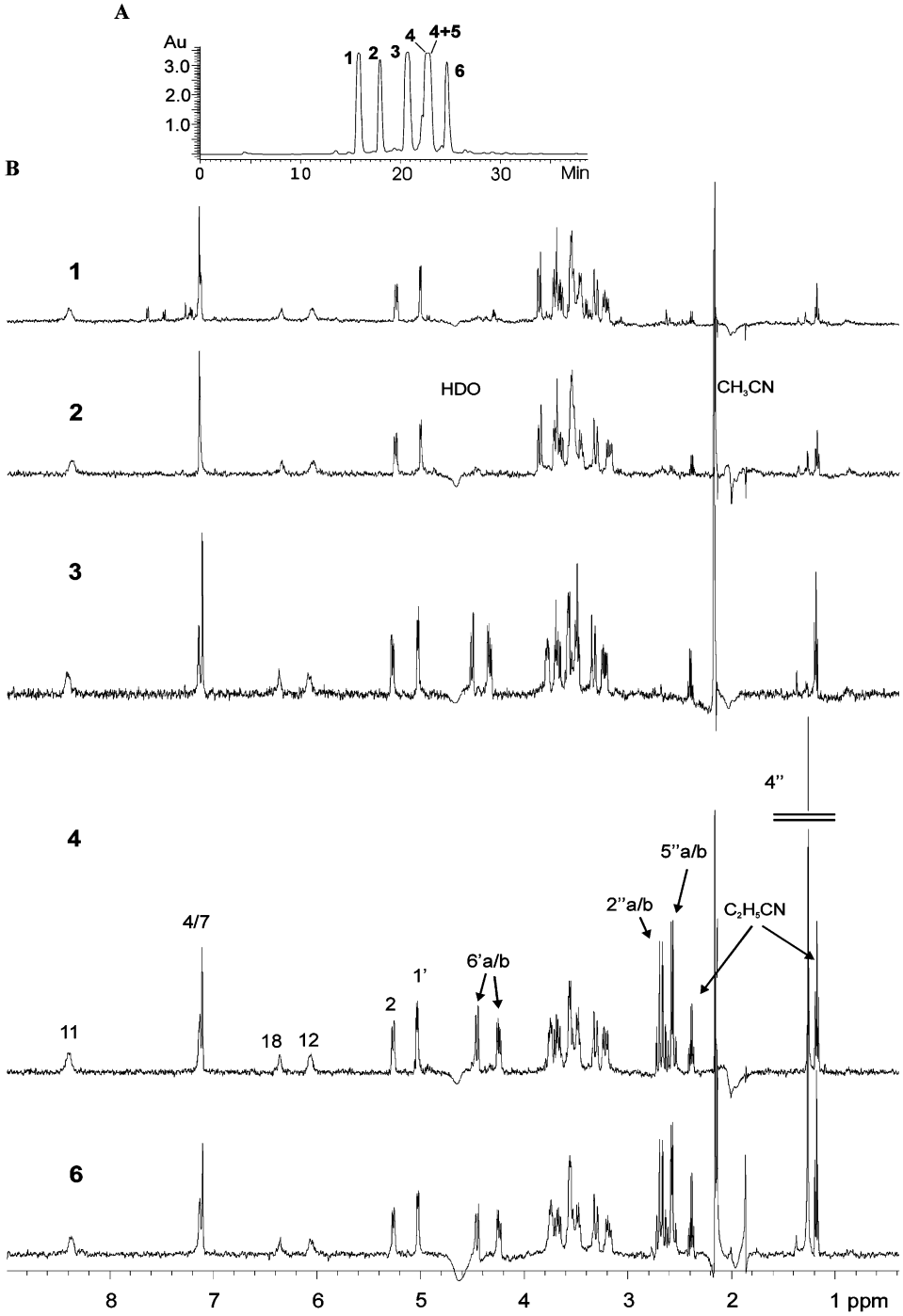

Fig. 2. Chromatogram (A) and stopped-flow ^{1}H NMR spectra (B) of betalains **1–6** except **5** which could not be totally separated from **4**. Typically 250–300 scans per ^{1}H NMR spectrum were acquired.

Table 1
LC–^{1}H NMR data of betanin (**1**), isobetanin (**2**), phyllocactin (**3**), hylocerenin (**4**), and isohylocerenin (**6**); MeCN/D_2O/0.05%TFA, δ (MeCN) = 2.0 ppm; ϑ = 25 °C; 500 MHz

	δ [ppm], mult, J [Hz]				
	1	**2**	**3**	**4**	**6**
2	5.23, *dd*, 2.0, 10.2	5.24, *dd*, 2.6, 10.7	5.26, *bd*, 10.6	5.26, *dd*, 2.1, 10.1	5.26, *dd*, 2.0, 9.8
3 a/b	3.66, *dd*, 10.4, 16.7	3.66, *dd*, 10.2, 16.5	3.67, *dd*, 10.6, 17.1	3.68, *dd*, 10.8, 16.9	3.68, *dd*, 10.2, 16.9
	3.31, *dd*, 2.0, 16.7	3.31, *dd*, 2.0, 16.9	3.32, *dd*, 2.0, 16.9	3.31, *dd*, 2.2, 17.0	3.31, *dd*, 2.0, 16.9
4	7.13, *s*	7.14, *s*	7.09, *s*	7.10, *s*	7.10, *s*
7	7.12, *bs*	7.14, *bs*	7.13, *bs*	7.13 *bs*	7.13, *bs*
11	8.38, *bd*, ≈11	8.37, *bs*	8.38, *bd*, ≈10	8.39, *bd*, ≈10	8.37, *bd*, 11
12	6.04, *bd*, ≈11	6.04, *bd*, ≈11	6.06, *bd*, ≈10	6.06, *bd*, ≈11	6.05, *bd*, 11
14 a/b	3.21, *dd*, 7.4, 17.2	3.18, *dd*, 7.7, 17.3	3.21, *dd*, 7.2, 17.2	3.21, *dd*, 7.5, 17.8	3.18, *dd*, 7.4, 17.2
	3.41–3.59 (overlap)	3.41–3.59 (overlap)	3.44–3.60 (overlap)	3.44–3.61 (overlap)	3.44–3.61 (overlap)
15	overlapped by D_2O	overlapped by D_2O	overlapped by D_2O	overlapped by D_2O	overlapped by D_2O
18	6.34, *bs*	6.34, *bs*	6.35, *bs*	6.35 *bs*	6.35, *bs*
1′	5.00, *d*, 6.9	5.00, *d*, 7.3	5.01, *d*, 7.1	5.03, *d*, 7.1	5.03, *d*, 6.7
2′	3.41–3.59 (overlap)	3.41–3.59 (overlap)	3.44–3.60 (overlap)	3.44–3.61 (overlap)	3.44–3.61 (overlap)
3′	3.41–3.59 (overlap)	3.41–3.59 (overlap)	3.44–3.60 (overlap)	3.44–3.61 (overlap)	3.44–3.61 (overlap)
4′	3.41–3.59 (overlap)	3.41–3.59 (overlap)	3.44–3.60 (overlap)	3.44–3.61 (overlap)	3.44–3.61 (overlap)
5′	3.41–3.59 (overlap)	3.41–3.59 (overlap)	3.76, *ddd*, 1.7, 6.1, 9.6	3.74, *ddd*, 1.6, 6.0, 9.4	3.74, *ddd*, 2.3, 6.2, 8.9
6′ a/b	3.86, *dd*, 1.4, 12.6	3.85, *dd*, 1.6, 12.7	4.49, *dd*, 1.7, 12.3	4.45, *dd*, 2.1, 12.3	4.45, *dd*, 1.8, 12.1
	3.70, *dd*, 5.5, 12.6	3.70, *dd*, 5.5, 12.7	4.32, *dd*, 6.0, 12.3	4.24, *dd*, 6.4, 12.4	4.24, *dd*, 6.1, 12.1
2″ a/b			3.28, *s*[a]	2.70, *d*, 14.4	2.71, *d*, 14.3
				2.64, *d*, 14.4	2.65, *d*, 14.3
4″				1.25, *s*	1.26, *s*
5″ a/b				2.59, *d*, 15.0	2.60, *d*, 14.9
				2.55, *d*, 15.0	2.55, *d*, 14.9

[a] After a total time of ca. 40 min for the HPLC and the stopped-flow NMR experiments a residual ^{1}H NMR signal of the malonyl side chain could be observed.

acetonitrile and pigment signals were observed in the LC NMR experiments, this solvent appeared to be promising. Thus, 100% acetonitrile or 100% acetone were also tested. However, only in mixtures of 30% water and 70% acetone or acetonitrile (v/v), betacyanins dissolved scarcely which was not sufficient for 2D NMR experiments. From these findings it was concluded that an aqueous system might be the best solution to achieve high solubility and acceptable stability. Subsequent experiments including divalent ions such as barium (as barium chloride) in 3- to 10-fold molar excess, phosphate buffer (50 mM Na_2HPO_4, pH 3), the ion pair reagent pentadecafluorooctanoic acid (1.5 mM, pH 3) or varying temperatures (5, 10, 15, 20 °C) did neither improve pigment stability nor the quality of the NMR spectra. Highest solubility and long term stability was achieved with a mixture of water and ethylene glycol (up to 4% ethylene glycol; v/v). However, the residual solvent peaks of the latter did not allow the unambiguous assignment of the protons H-2, H-15 and H-1′ due to extremely overlapping signals. In summary, of all tested variants, the most suitable solvent system was D_2O at 25 °C in the pH range 5–7 to achieve long-term stability, sufficient solubility and little overlapping of system and analyte signals. When NMR solvents and the sample tube were flushed with argon in order to remove oxygen prior to data acquisition betacyanin integrity could even be improved. This setup was used throughout the NMR investigations and required 1.5–2 mg pigment.

The individual ^{1}H-spin systems H-2 and H-3a/b; H-4; H-7; H-15 and H-14a/b; H-11 and H-12; as well as H-1′ to H-6′ (Fig. 1) of **1–4** were established by ^{1}H NMR, 1D TOCSY and gCOSY. Their directly bonded carbons were assigned by gHSQC for the first time (Table 2). In each case (**1–4**), a vicinal coupling constant H-1′, H-2′ of $J \approx 7$ Hz and a ^{1}H–^{13}C long range correlation (= gHMQC) between the anomeric proton H-1′ and the phenolic carbon at $\delta \approx 144$ ppm (C-5 or C-6) indicated the β-linkage of the glucopyranosyl moiety with the aglycon. The ROESY-correlation between H-1′ and H-4 established the substitution at C-5. gHMQC correlations of H-4, H-7, H-3a/b and H-2 with the respective carbons as well as ROE's between H-4 and H-3a/b allowed the complete assignment of rings A and B (Fig. 1). In each case, the coupling constant of $J \approx 12.5$ Hz between H-11 and H-12 which could be observed only after acidification to pH 1–2 together with the ROESY-correlations between H-7 and H-11 as well as H-2 and H-12 indicated the *s-trans* conformation of the dienyl-moiety (C-1, C-11; C-12, C-13; Fig. 1). The *E*-configuration of the C-12/C-13 double bond was

Table 2
^{1}H and ^{13}C NMR data of betanin (**1**), isobetanin (**2**), phyllocactin (**3**), and hylocerenin (**4**); D_2O, ref = 4.7 ppm; ϑ = 25 °C; 500 MHz

	^{1}H NMR δ [ppm], mult, J [Hz][a]				^{13}C NMR δ [ppm][b]			
	1	**2**	**3**	**4**	**1**	**2**	**3**	**4**
2	4.92, *dd*, 3.1; 10.3	4.97, *dd*, 2.7, 10.1	4.90, *dd*, 2.9; 10.1[c]	4.85, *dd*, 3.1, 10.5	65.0	64.7	64.9	64.6
3 a/b	3.53, *dd*, 11.5; 16.9[d]	3.51, *dd*, 11.6, 16.7[d]	3.57, *dd*, 11.4; 16.5[d]	3.56, *dd*, 11.6, 16.5[d]	32.7	33.5	33.0	32.9
	3.10, *dd*, 4.3; 16.8[d]	3.18, *bd*, 16.5	3.14, *dd*, 3.1; 16.7	3.11, *dd*, 4.3, 16.5				
4	7.06, *s*	6.93, *s*	7.03, *s*	7.00, *s*	113.9	113.9	113.9	113.9
5					144.0	144.1	143.9	143.9
6					146.1	146.6	146.2	146.7
7	6.98, *bs*	6.91, *bs*	6.98, *bs*	6.94, *bs*	100.0	100.3	100.1	99.9
8					137.4	137.9	137.9	138.0
9					124.1	124.3	124.1	124.3
10					175.8	176.1	176.5	176.5
11	8.19, *bs* (*d*, 12.6)[c]	8.11, *bs* (*d*, 11.6)[c]	8.19, *bs* (*d*, 9.8)[c]	8.14, *bs* (*d*, 11.8)[c]	144.4	144.6	144.3	143.7
12	5.84, *bs* (*d*, 12.6)[c]	5.84, *bs* (*d*, 12.46)[c]	5.84, *bs* (*d*, 10.5)[c]	5.80, *bs* (*d*, 12.6)[c]	106.9	–[e]	106.4	106.1
13					–[e]	–[e]	–[e]	–[e]
14 a/b	3.20, *bm*[d];	3.13, *bd*, 17.6	3.24, *bm*[d];	3.14, *bm*	26.5	26.4	26.7	26.4
	3.12, *bm*[d]	2.94, *bdd*, 7.0, 17.0	3.16, *bm*[d]					
15	4.40, *bt*, 7.1	4.31, *bs*	4.37, *bt*, 6.5	4.33, *bt*, 7.2	53.1	53.0	53.1	53.3
17					–[e]	–[e]	–[e]	–[e]
18	6.22, *bs*	6.18, *bs*	6.22, *bs*	6.20, *bs*	–[e]	–[e]	–[e]	–[e]
19					–[e]	–[e]	–[e]	–[e]
20					–[e]	–[e]	–[e]	–[e]
1′	4.98, *d*, 7.4	4.95, *d*, 7.1	4.98, *d*, 7.1	4.99, *d*, 7.2	101.4	101.0	101.4	101.1
2′	3.55 (overlap)	3.55 (overlap) [d]	3.56 (overlap)	3.56 (overlap)[d]	75.7[f]	73.4[f]	75.2[f]	75.2[f]
3′	3.55 (overlap)	3.55 (overlap)	3.57 (overlap)	3.56 (overlap)	73.9[f]	75.8[f]	72.8[f]	72.5[f]
4′	3.41 (overlap)	3.45, pt, 8.8	3.50, *t*, 9.2	3.47, *pt*, 9.51[d]	69.3[f]	69.7[f]	69.4	69.4
5′	3.52 (overlap)	3.51 (overlap)	3.76, *ddd*, 2.3, 5.6, 9.5	3.73, *ddd*, 1.9, 6.3, 9.4	76.2	76.6	73.8	73.5
6′ a/b	3.85, *dd*, 1.6, 12.3	3.86, *dd*, 1.4, 12.4	4.46, *dd*, 2.2, 12.3	4.45, *dd*, 1.6, 12.1	60.6	60.8	63.7	63.02
	3.70, *dd*, 5.3, 12.3	3.71, *dd*, 5.3, 12.4	4.33, *dd*, 5.6, 12.3	4.23, *dd*, 6.4, 12.2				
1″							170.1[g]	172.9
2″ a/b			3.34, *s*[g]	2.68, *d*, 14.4			43.3[g]	45.0
				2.64, *d*, 14.4				
3″							172.9[g]	69.9
4″				1.24, *s*				26.8
5″ a/b				2.56, *d*, 15.0				44.4
				2.52, *d*, 15.0				
6″								175.6

[a] Observed coupling constants were not averaged. Assignments based on gCOSY, gHSQC and gHMQC spectra.
[b] ^{13}C chemical shifts were derived from gHSQC and gHMQC.
[c] After acidification (TFA) to pH 2.
[d] Chemical shifts and coupling constants from 1D TOSCY.
[e] Chemical shifts were not observable.
[f] Assignments may be interchanged.
[g] Due to fast H/D exchange the NMR signals of the malonyl side chain could only be observed in H_2O/D_2O solution (9/1, v/v).

deduced by comparison of the ^{1}H chemical shifts of H-11, H-12 and H-18 with reported data from the literature (Hilpert et al., 1985; Hilpert and Dreiding, 1984; Wyler and Dreiding, 1984). Unfortunately, the direct proof of the *E*-configuration of the C-12/C-13 double bond by ROESY (H-12, H-18; H-11, H-14) as well as the assignment of the remaining carbons C-13, 17, 18, 19 and C-20 (Ring C) by gHMQC was not possible, as no ROESY- and gHMQC-correlations could be observed. This may be due both to characteristic structural features of betacyanins (Heuer et al., 1992) as well as to the respective pulse sequence applied (Claridge, 1999). The 3-hydroxy-3-methyl-glutaryl side chain in **4** was unambiguously assigned by ^{1}H NMR as well as gHSQC- and gHMQC-correlations. ^{1}H–^{13}C long range correlations of the protons H-2″a/b and the lowfield shifted glucopyranosyl protons H-6′a/b to the ester carbonyl carbon C-1″ established the structure of hylocerenin (**4**). The ^{1}H NMR spectrum of **3** displayed signals for the glucopyranosyl protons H-6′a/b at δ = 4.46/4.33 ppm that are shifted to lower field in comparison to betanin (**1**) δ = 3.87/3.70. In contrast to **4**, no additional signals for the H-2″a/b protons of **3** could be observed. However, the ESI–MS spectrum showed a pseudomolecular ion

$[M+H]^+$ at m/z 637 and daughter ions at m/z 551 (betanin) and 389 (betanidin). The mass difference of m/z 86 (637–551) suggested a malonyl-betanin structure. Due to the fast H/D exchange in malonyl derivatives in D_2O (Bridle et al., 1984; Horowitz and Asen, 1989), the gHSQC and gHMQC were re-run in a mixture of H_2O and D_2O (90/10, v/v). The analysis of these spectra clearly revealed the presence of 6′-*O*-malonyl-betanin (**3**, phyllocactin).

In summary, of all tested solvent setups, D_2O was found to be the most suitable system which afforded the best long-term stability required for gCOSY, gHSQC, and gHMQC experiments. Despite signal broadening due to a low protonation degree which needs to be accepted betacyanin hydrolysis could be prevented. However, after 70 h of data acquisition on 1.5–2 mg pigment, double bond isomerism at C-12, C-13 could be observed in the ^{1}H NMR spectra. The *E*/*Z* ratio of the two respective stereoisomers was about 80/20. Additionally, HPLC re-examination of each sample after 70 h showed the formation of 10–20% of the respective C-15 stereoisomer (data not shown). Nevertheless, distinct signal assignment in 2D experiments could be performed. For the first time, ^{13}C NMR data of betacyanin structures saturated at C-14 and C-15 were thus obtained, although complete assignment of carbons 13, 17, 18, 19, and 20 still remains to be resolved.

3. Experimental

3.1. Plant material

Hylocereus polyrhizus (Weber) Britton & Rose fruits were purchased from Israel and stored at −26 °C. After thawing at 4 °C, the pericarp was separated from the red-violet juicy pulp. The latter was pressed and the resulting juice was filtered, flushed with nitrogen and stored at −80 °C.

3.2. Solvents and reagents

Reagents and solvents were purchased from VWR (Darmstadt, Germany) and were of analytical or HPLC grade. NMR solvents were from Deutero GmbH (Kastellaun, Germany). Deionised water was used throughout.

3.3. Removal of pectic substances, colourless phenolics and sugars

In order to allow concentration and improved chromatographic performance during LC MS, LC NMR and preparative HPLC, pectic substances in the juice were precipitated as described earlier (Stintzing et al., 2002b). For removal of colourless phenolic compounds, the pitaya juice (1 part) was adapted to a pH of 1.5 by addition of TFA and three times fractionated against ethyl acetate (2 parts) before LC NMR experiments. The resulting betacyanin fraction was 8-fold concentrated by removal of water in vacuo and then applied to a 10 g C_{18} Sep-Pak cartridge (Waters Associates, Milford, MA, USA) in order to separate the sugars from the betacyanins as reported elsewhere (Stintzing et al., 2002b). This allowed a further 28-fold concentration of pigments by evaporation of water in vacuo.

3.4. NMR and LC NMR analyses

NMR and LC NMR spectra were recorded on a Varian Unity Inova 500 MHz NMR spectrometer. ^{1}H chemical shifts were referenced to the residual solvent signal at $\delta = 4.70$ ppm (D_2O) relative to TMS. All 1D (^{1}H, 1D TOCSY) and 2D NMR (gCOSY, TROESY, gHSQC, gHMQC, g = gradient enhanced) measurements were performed using standard Varian pulse sequences. For phyllocactin (**3**), additional NMR experiments (^{1}H, gHSQC, gHMQC) were carried out using a mixture of H_2O and D_2O (90/10, v/v), slightly acidified with 20 µl of an aqueous TFA solution (0.05% TFA, v/v). Solvent suppression was achieved by implementing a presaturation element in the pulse sequences. 2D NMR spectra were recorded with 1.5–2 mg of each sample.

LC NMR spectra were recorded in the stop-flow mode (200–300 scans per ^{1}H NMR spectrum) using an ID-PFG probe with a flow cell of 65 µl active volume. Solvent suppression was performed by WET (Smallcombe et al., 1995). The HPLC system consisted of a Varian 9012 pump and a Varian 9050 UV–Vis detector. A two solvent gradient system (A: 0.1% TFA in D_2O; B: MeCN/D_2O, 60/40, v/v) was used to separate the pre-concentrated betacyanin fraction (2–3 mg in 100 µl) on an analytical scale (250×4.6 mm i.d., 5 µm) AQUA C18 reversed-phase column fitted with a security guard C_{18} ODS (4×3.0 mm i.d., Phenomenex, Torrance, CA, USA). Beginning with 96% A for 4 min, a linear gradient was followed in 11 min to 86% A and then to 82% A in 15 min. The system was operated at room temperature, a flowrate of 0.8 ml/min and a pressure of 260 bar. Monitoring was performed at 578 nm.

3.5. Preparative HPLC

An HPLC-system (Bischoff, Leonberg, Germany) consisting of an LC-CaDI 22-14 control unit, two HPLC compact pumps, connected to a UV–Vis detector SPD 10 AV *VP* (Shimadzu, Tokyo, Japan), and a dynamic mixing chamber (Knauer, Berlin, Germany) equipped with Bischoff McDAcq 32 software were used for preparative isolation of betacyanin pigments from depectinized and 3-fold concentrated red-violet pitaya juice (see Section 3.3). Separation of betanin (**1**), iso-

betanin (2), phyllocactin (3) and hylocerenin (4) were achieved on a C18 AQUA column (250×21.2 mm i.d.; 5 μm; Phenomenex, Torrance, CA, USA) operated at room temperature, at a flow rate of 9 ml/min and a pressure of 70 bar.

The mobile phase consisted of 0.5% aqueous HCOOH (eluent A) and a mixture of MeCN and H_2O (50/50, v/v, eluent B). Starting with 84% A in B, a linear gradient was followed to 72% A in B at 14 min and then to 0% A in B in 2 min before re-equilibration to initial conditions. Monitoring was performed at 538 nm. Aliquots of 700 μl were injected and the effluents collected at 13.8–14.2 min (1), at 15.9–16.3 min (2), at 19.2–19.8 min (3) and at 20.9–21.2 min (4) and immediately cooled in an ice bath. The collected fractions were gently concentrated in vacuo at room temperature by repeated adding of purified water in order to reduce residual acid. Complete removal of water and acid was obtained by lyophilization. The resulting powders were stored in a sealed tube at −80 °C until analysis.

3.6. HPLC analyses

For checking the purity of semi-preparatively isolated betacyanins, the pigments were submitted to HPLC–DAD analyses as described earlier (Stintzing et al., 2002b). The purity of pigments as expressed by their peak area to the whole chromatogram area at 280 nm always exceeded 98%.

3.7. HPLC MS analyses

LC MS analyses were performed using an HPLC pump ProStar (Varian, Darmstadt, Germany) connected to a UV–Vis detector model ABI 785 (Applied Biosystems, Weiterstadt, Germany) in series with a TSQ 700 Mass Spectrometer (Finnigan MAT, San José, CA, USA) fitted with an ionspray source. Pigment separation was achieved on an AQUA C18 reversed-phase column (250×4.6 mm i.d., 5 μm) fitted with a security guard C_{18} ODS (4×3.0 mm i.d., Phenomenex, Torrance, CA, USA) operating at 25 °C and using 0.2% aqueous HCOOH (eluent A) and a mixture of MeCN and H_2O (80/20, v/v, eluent B). The elution was carried out following a linear gradient from 10% B in A to 27% B in A in 30 min. Monitoring was performed at 538 nm for betacyanins at a flow rate of 1 ml/min. Positive ion mass spectra of the eluting betacyanins were recorded setting the capillary temperature to 200 °C and keeping the ESI spray voltage at 4 kV. Spectra were monitored from m/z 10 to 700. 50 μl of the depectinised non-concentrated pitaya juice were applied per injection. Prior to NMR spectroscopy, all isolated substances were dissolved in purified water slightly acidified with 0.1% aqueous TFA and subjected to ESI mass spectrometry operating in the positive mode as described above.

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