First ^{13}C-NMR Assignments of Betaxanthins

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Dedicated to Senator e.h. e.h. Dr. Dr. h.c. *Hermann Eiselen*, Ulm, on the occasion of his 80th birthday

Due to their inherent liability towards highly acidic conditions previously considered to be a prerequisite for data acquisition, betaxanthin structure dereplication by NMR spectroscopy has been scarcely reported and was, hitherto, exclusively based on ^{1}H-NMR data interpretation. Applying only slightly acidic conditions, we herein report the first ^{13}C-NMR data of two betaxanthins, *i.e.*, indicaxanthin (**1**), isolated from yellow-orange cactus pear fruits (*Opuntia ficus-indica* [L.] MILL. cv. 'Gialla'), and of miraxanthin V (**2**) from yellow Swiss chard petioles (*Beta vulgaris* L. ssp. *cicla* [L.] ALEF. cv. 'Bright Lights'), as derived by gHSQC- and gHMQC-NMR experiments and inverse detection.

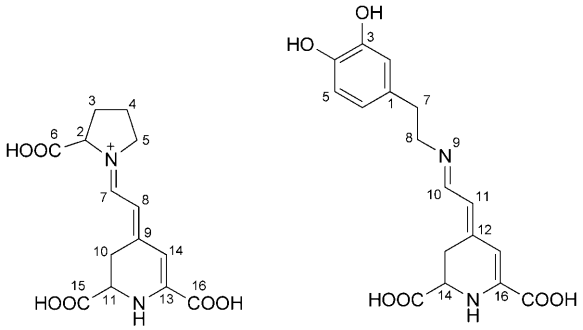
Introduction. – The betalains are water-soluble N-heterocyclic pigments imparting a wide range of hues to various flowers, fruits, and vegetables from most family members of Caryophyllales, but also to some higher fungi [1]. The red-purple betacyanins represent immonium conjugates of betalamic acid with 'cyclo-Dopa' derivatives[1]), while the yellow-orange colored betaxanthins are condensation products of betalamic acid and various amino compounds, respectively [2].

Acidification has been considered a prerequisite for appropriate signal detection during NMR measurement [3][4]. However, due to the inherent liability of betacyanins [5][6] and betaxanthins [7–9] under acidic conditions, ^{13}C-NMR measurements requiring prolonged data acquisition have been exclusively reported for neobetanin [10] and, just recently, for acylated and non-acylated betacyanins under neutral conditions [11]. Apart from an earlier report by *Wyler* and *Dreiding* [12] acquiring NMR signals for indicaxanthin over two years in neat CF_3CO_2D (TFA), only few ^{1}H-NMR data for betaxanthins exist [7–9][12–15]. And even more so, a literature review [2–4] revealed that ^{13}C-NMR data for betaxanthins are completely lacking.

Indicaxanthin (**1**) is the predominant betalain in fruits from the Cactaceae family [16][17], and was the first betaxanthin reported and characterized by ^{1}H-NMR at 60 MHz [8]. According to *Terradas* and *Wyler* [18], C(2) of the proline moiety in natural indicaxanthin is (*S*)-configured. Furthermore, treatment of synthetic indicaxanthin

[1]) The term 'cyclo-Dopa' refers to (2*S*)-2,3-dihydro-5,6-dihydroxy-1*H*-indole-2-carboxylic acid, a cyclized variant of 3-hydroxytyrosine (DOPA).

with dilute alkali induces epimerization at C(11) to yield genuine (2*S*,11*S*)-indicaxanthin and (2*S*,11*R*)-isoindicaxanthin [19–21]. Unfortunately, these diastereoisomers could not be differentiated by NMR spectroscopy [7], but have been distinguished by HPLC separation [19][22]. Miraxanthin V (**2**) was first identified in flowers of *Mirabilis* species, and its structure was elucidated by ^{1}H-NMR analysis on a 60-MHz spectrometer [9]. According to more-recent reports, this betaxanthin has also been detected in common cockscomb, feathered amaranth, yellow beet, and Swiss chard [16][23][24].



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Due to the general lack of thorough structural assignments on betaxanthins, the present study aimed at complete ^{1}H- and ^{13}C-NMR assignments of the major indicaxanthin and miraxanthin-V stereoisomers through extensive 1D- and 2D-NMR experiments in slightly acidified D_2O and D_2O/H_2O solutions.

Results and Discussion. – Indicaxanthin (**1**) isolated from yellow-orange cactus pear fruit (*Opuntia ficus-indica* [L.] MILL. cv. 'Gialla') and miraxanthin V (**2**) from yellow Swiss chard stems (*Beta vulgaris* L. ssp. *cicla* [L.] ALEF. cv. 'Bright Lights') were isolated as described in the *Exper. Part*. Analytical HPLC [16][17][22][24] established the sole occurrence of the respective (11*S*)-**1** and (14*S*)-**2** epimers. For NMR measurements, the compounds were dissolved both in D_2O and in D_2O/H_2O 1:4, each containing 0.01% (*v*/*v*) of TFA.

The ^{1}H-NMR spectra of **1** and **2** displayed three sets of signals each consisting of four signals in the chemical-shift region of the 1,7-diazaheptamethinium substructure (δ(H) 8.6–7.5, 6.5–5.8, and 6.0–5.2) suggesting an equilibrium mixture of the four indicaxanthin and miraxanthin V stereoisomers, respectively. The observation of strong EXSY cross-peaks (chemical exchange) in the NOESY spectra of **1** and **2** between the respective H-atoms indicated a dynamic system of the exchanging stereoisomers (*e.g.*, H–C(8) of **1a**–**1d**; *Fig. 1*). Similar saturation-transfer effects on NOE irradiation in betacyanins had been observed before [25]. Integration of the NMR signals yielded a ratio of 52:33:9:6 for the stereoisomers **1a**–**1d**, and of 49:36:8:7 for **2a**–**2d**, respectively.

NMR Assignment of Indicaxanthin (**1**). The structures of the two major stereoisomers of indicaxanthin, **1a** and **1b** (*Fig. 2*), were elucidated as follows: the ^{1}H spin systems

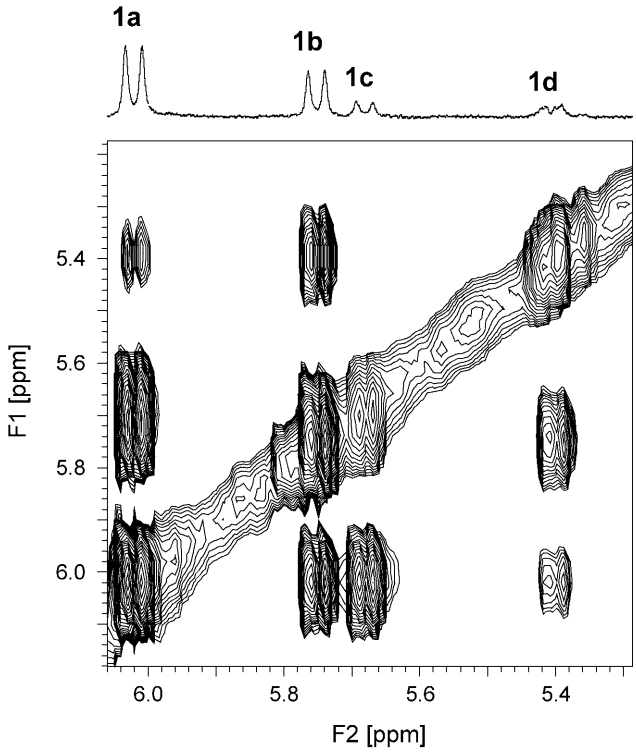

Fig. 1. *The H–C(8) Resonances in the NOESY spectrum of the Four Stereoisomers* **1a**–**d**. All cross-peaks originate from chemical exchange.

H–C(2) to CH_2(5), H–C(7) and H–C(8), CH_2(10) and H–C(11), and H–C(14) were determined by ^{1}H-NMR, gCOSY, TOCSY, and their directly bonded C-atoms by gHSQC correlations (*Table 1*). ^3J(C,H) Long-range correlations between H–C(7) and both C(2) and C(5) clearly showed that the proline moiety is linked to the betalamic substructure *via* N(1). The C(6) and C(15) position of the two COOH groups at δ(C) *ca.* 175 were established by HMBC correlations between H–C(2) as well as CH_2(3) and C(6), and between both H–C(11) and CH_2(10) and C(15), respectively. Due to both time- and solvent-dependent H/D exchange of the H-atoms H–C(8) and H–C(14) [11][15], the ^{13}C-NMR chemical shifts of C(8), C(9), C(13), C(14), and C(16) could only partially be obtained by indirect detection (gHSQC, gHMQC) in the inverse mode. By performing the experiments in acidified (0.01% TFA) D_2O/H_2O 1:4 (*v*/*v*), and by applying slightly modified pulse sequences with an implemented presaturation element, the remaining ^{13}C-NMR resonances for **1a** and **1b** could be identified, and the structure of the betalamic acid moiety was unambiguously assigned (*Table 1*).

Due to the low concentrations of the minor stereoisomers **1c** and **1d** in the equilibrium mixture, no ^{13}C-NMR information, not even of the well-separated signals for H–C(7), H–C(8), and H–C(14) in the ^{1}H-NMR spectrum, could be obtained. Consid-

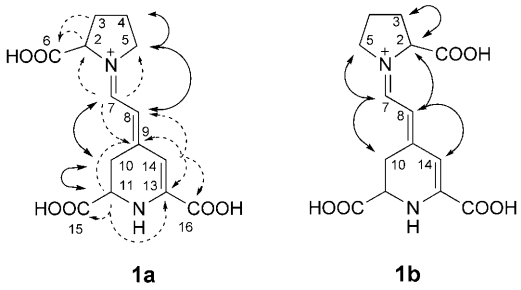

Fig. 2. *Major Stereoisomers of Indicaxanthin* (**1**) *in Aqueous Solution.* Arrows show key NOESY (↔) and HMBC correlations (----). The latter are identical for **1a** and **1b**, resp.

ering the 1,7-diazaheptamethinium substructure of indicaxanthin, a total of eight stereoisomers are theoretically possible due to double-bond isomerism at N(1)=C(7) and C(8)=C(9), as well as based on s-*cis*/s-*trans* conformations of the dienyl moiety caused by rotation about the C(7)–C(8) bond [3][12].

NOESY Correlations and chemical-shift considerations allowed the unambiguous identification of the major stereoisomers **1a** and **1b**. NOEs between H–C(7) and CH_2(10), as well as between H–C(8) and H–C(14) indicated (*E*)-configuration for C(8)=C(9) and s-*trans* conformation for the dienyl moiety N(1)=C(7)–C(8)=C(9) in both stereoisomers. Comparison of the ^{13}C-NMR chemical shifts of **1a** and **1b** revealed major differences only in positions C(2) and C(5) of the proline moiety, suggesting double-bond isomerism at N(1). NOESY Correlations of H–C(2) and H–C(8), and of H–C(5) and H–C(7) for **1b**, and of H–C(5) and H–C(8) for **1a**, allowed us to establish the (1*Z*)- and a (1*E*)-configuration for **1b** and **1a**, respectively.

Our experiments showed a strong solvent impact on the isomer equilibrium, as reported earlier for betacyanins [3][11]. In aqueous solution, indicaxanthin mainly undergoes double-bond isomerization at N(1)=C(7). In contrast, in neat TFA, only (8*E*)- and (8*Z*)-indicaxanthin were reported, in a ratio of 65:35 [12]. By comparing the ^{1}H-NMR chemical shifts and shift differences of especially H–C(7), H–C(8), and H–C(14), assignment of the two diastereoisomers based on anisotropy effects was achieved [12]. However, no (*E*/*Z*)-stereoisomerism at the partial double bonds N(1)=C(7) and C(7)=C(8) could be observed due to fast rotation and steric conditions in TFA, respectively [12].

Considering the above-mentioned anisotropy and ^{1}H-NMR chemical-shift differences, the structures of the two minor stereoisomers **1c** and **1d** were tentatively assigned in the present study. For the (8*E*)- and (8*Z*)-isomers, shift differences of $\Delta\delta(H) = -0.29$ for H–C(7), +0.29 for H–C(8), and −0.26 for H–C(14) were reported earlier [12]. Comparison of the magnitude of these values with the respective shift differences of (1*E*,8*E*)-**1a** and **1c** ($\Delta\delta$(**1a**–**1c**): −0.32 for H–C(7), +0.33 for H–C(8), −0.30 for H–C(14)), as well as for (1*Z*,8*E*)-**1b** and **1d** ($\Delta\delta$(**1b**–**1d**): −0.32 for H–C(7), +0.34 for H–C(8), −0.36 for H–C(14)) suggest the (1*E*,8*Z*)- and (1*Z*,8*Z*)-configurations for **1c** and **1d**, respectively.

NMR Assignment of Miraxanthin V (**2**). The structure elucidation of the two major stereoisomers of miraxanthin V, **2a** and **2b** (*Fig. 3*), was performed in analogy to that of

Table 1. *^{1}H- and ^{13}C-NMR Data* (500/125 MHz) *of the Stereoisomers of Indicaxanthin* (**1**). Solvent: D_2O or D_2O/H_2O 1:4 each containing 0.01% TFA; $T = 298$ K. For the minor isomers **1c** and **1d**, only clearly separated ^{1}H-NMR signals are given (see footnotes). For details, see *Exper. Part.* Chemical shifts δ in ppm, coupling constants J in Hz; asterisks (*) mark overlapping signals.

Atom	δ(H)		δ(C)[a]	
	1a	**1b**	**1a**	**1b**
H–C(2)	4.63 (*dd*, *J* = 3.5, 8.4)	4.53 (*dd*, *J* = 4.3, 8.6)	67.6	63.1
H_a–C(3)	2.32*	2.39–2.46 (*m*)	29.6	30.5
H_b–C(3)	2.19*	2.18*		
H_a–C(4)	2.03–2.10 (*m*)	1.96–1.98*	23.1	23.5
H_b–C(4)	2.00*	1.96–1.98*		
H_a–C(5)	3.62–3.71*	3.83–3.93*	49.8	55.6
H_b–C(5)	3.62–3.71*	3.83–3.93*		
C(6)	–	–	175.6	175.6
H–C(7)[b])	8.19 (*d*, *J* = 12.2)	8.29 (*d*, *J* = 12.3)	156.8	156.2
H–C(8)[c])	6.02 (*d*, *J* = 12.2)	5.76 (*d*, J = 12.3)	107.7	107.4
H–C(9)	–	–	163.7	163.5
H_a–C(10)	3.28*	3.28*	26.4	26.4
H_b–C(10)	3.04*	3.04*		
H–C(11)	4.43 (*t*, *J* = 6.5)	4.43 (*t*, *J* = 6.5)	52.5	52.5
H–C(13)	–	–	153.0	152.9
H–C(14)[d])	6.15 (*s*)	6.10 (*s*)	103.6	103.6
C(15)	–	–	175.5	175.5
C(16)	–	–	166.6	166.5

[a]) Chemical shifts derived from gHSQC and gHMQC experiments at 500 MHz in the inverse mode. No data are available for the minor stereoisomers present in the mixture. [b]) ^{1}H-NMR data for **1c** and **1d**: 8.51 (*d*, *J* = 12.0) and 8.61 (*d*, *J* = 12.4), resp. [c]) ^{1}H-NMR data for **1c** and **1d**: 5.69 (*d*, *J* = 12.0) and 5.42 (*d*, *J* = 12.4), resp. [d]) ^{1}H-NMR data for **1c** and **1d**: 6.45 (*s*) and 6.46 (*s*), resp.

indicaxanthin. The NMR data are compiled in *Table 2*. HMBC Correlations between CH_2(8) and C(10), and between H–C(10) and C(8) allowed unambiguous establishment of N(9) as the linkage between the dopamine and betalamic acid moieties. NOEs between H–C(10) and CH_2(13), as well as a coupling constant of *J ca.* 12 Hz between H–C(10) and H–C(11), indicated (*E*)-configuration for C(11)=C(12) and s-*trans* conformation of the dienyl moiety N(9)=C(10)–C(11)=C(12) in both stereoisomers.

Comparison of the ^{13}C-NMR data (*Table 2*) of **2a** and **2b** revealed major differences of $\Delta\delta$(C) 4–5 ppm only for C(8) and C(10), suggesting double-bond isomerization at N(9)=C(10). NOE Correlations between H–C(10) at δ(H) 7.48 and CH_2(8) at δ(H) *ca.* 3.6 clearly showed that the major stereoisomer **2a** has (9*E*)-configuration, thus representing the (9*E*,11*E*)-stereoisomer. The (9*Z*)-configuration of **2b** was deduced by comparison of the ^{13}C-NMR data of **2a** and **2b**, and relative to those of indicaxanthin, since a direct proof *via* NOE was impossible in this case. Due to the small overall quantity (*ca.* 0.5 mg) of miraxanthin V available, and/or because of dynamic processes within the equilibrium **2a**–**d**, the NOESY spectrum did not reflect the expected correlation between H–C(11) and CH_2(8). From careful inspection of the ^{13}C-NMR data of **1a**,**b**

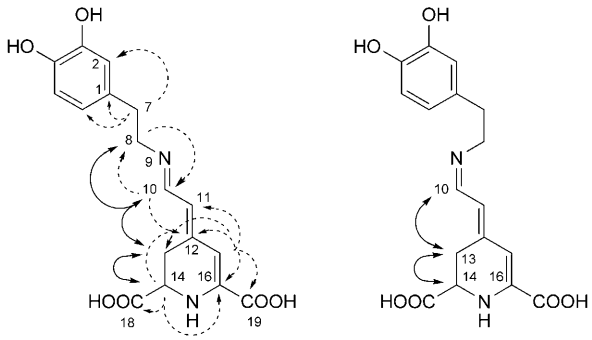

Fig. 3. *Major Stereoisomers of Miraxanthin V* (**2**) *in Aqueous Solution*. Arrows show key NOESY (↔) and HMBC correlations (----→). The latter are identical for **2a** and **2b**, resp.

Table 2. *^{1}H- and ^{13}C-NMR Data* (500/125 MHz) *of the Stereoisomers of Miraxanthin V* (**2**). Solvent: D_2O or D_2O/H_2O 1:4 each containing 0.01% TFA; $T = 298$ K. For the minor isomers **2c** and **2d**, only clearly separated ^{1}H-NMR signals are given (see footnotes). Chemical shifts δ in ppm, coupling constants J in Hz; asterisks (*) mark overlapping signals.

Atom	δ(H)		δ(C)[a])	
	2a	**2b**	**2a**	**2b**
H–C(1)	–	–	130.7	131.1
H–C(2)	6.68 (*d*, *J* = 2.0)	6.71 (*d*, *J* = 1.8)	117.5	117.0
H–C(3)	–	–	144.0	143.0[b])
H–C(4)	–	–	142.8	143.8[b])
H–C(5)	6.79 (*d*, *J* = 8.2)	6.75 (*d*, *J* = 8.1)	116.6	116.5
H–C(6)	6.61 (*dd*, *J* = 2.0, 8.2)	6.63 (*dd*, *J* = 1.8, 8.1)	121.9	121.8
H_a–C(7)	2.78*	2.77*	35.1	33.6
H_b–C(7)	2.75*	2.77*		
H_a–C(8)	3.63 (*dt*, *J* = 6.0, 13.7)	3.59*	51.2	45.3
H_b–C(8)	3.60*	3.59*		
H–C(10)[c])	7.48 (*d*, *J* = 11.9)	7.83 (*d*, *J* = 12.6)	160.3	156.5
H–C(11)[d])	5.80 (*d*, *J* = 11.9)	5.66 (*d*, *J* = 12.6)	106.2	103.8
H–C(12)	–	–	162.8	164.3
H_a–C(13)	2.75*	2.88 (*dd*, *J* = 7.0, 17.0)	27.3	27.0
H_a–C(13)	2.82*	2.97 (*dd*, *J* = 8.0, 17.0)		
H–C(14)	4.03 (*t*, *J* = 7.4)	4.09 (br. *t*, *J* = 7.4)	54.3	54.1
H–C(16)	–	–	153.4	154.1
H–C(17)[e])	5.86 (*s*)	5.88 (*s*)	101.3	102.4
H–C(18)	–	–	176.9	176.8
H–C(19)	–	–	167.4	167.2

[a]) Chemical shifts derived from gHSQC and gHMQC experiments at 500 MHz in the inverse mode. No data are available for the minor stereoisomers present in the mixture. [b]) Assignments may be interchanged. [c]) ^{1}H-NMR data for **2c** and **2d**: 7.80 (*d*, *J* = 12.0) and 8.14 (*d*, *J* = 12.5), resp. [d]) ^{1}H-NMR data for **2c** and **2d**: 5.46 (*d*, *J* = 12.0) and 5.24 (*d*, *J* = 12.5), resp. [e]) ^{1}H-NMR data for **2c** and **2d**: 6.03 (*s*) and 6.20 (*s*), resp.

(*Table 1*), it turned out that a change of (*E*)- to (*Z*)-configuration for N(1)=C(7) causes a highfield shift of *ca.* $\Delta\delta$(C) 4–6 ppm of the respective C-atom of the (*E*)-isomer. For example, while in the case of **1a** the C(2) resonance was shifted by $\Delta\delta$(C) 63.1–67.6, C(5) of **1b** was shifted by 49.8–55.6 ppm. Based on these findings and a calculated highfield shift of $\Delta\delta$(C) 5.9 for C(8) between **2a** and **2b**, the (9*Z*)-configuration was derived for **2b**.

The structures of the minor stereoisomers **2c** and **2d** were tentatively assigned according to the method used for the identification of **1c** and **1d**. The ^{1}H-NMR shift differences between (9*E*,11*E*)-**2a** and **2c** [$\Delta\delta$(**2a**–**2c**): –0.31 for H–C(10), +0.34 for H–C(11), –0.17 for H–C(17)] as well as between (9*Z*,11*E*)-**2b** and **2d** [$\Delta\delta$(**2b**–**2d**): –0.31 for H–C(10), +0.42 for H–C(11), –0.32 for H–C(17)] suggest (9*E*,11*Z*)- and (9*Z*,11*Z*)-configuration for **2c** and **2d**, respectively.

Conclusions. – For the first time, the ^{13}C-NMR data and complete ^{1}H-NMR assignment by 2D-NMR of the major stereoisomers of indicaxanthin (**1**), the proline-betaxanthin, and of miraxanthin V (**2**), the dopamine-betaxanthin, have been resolved. Based on NMR assignments, strong solvent dependencies on the formation of the preferred stereoisomers as well as the presence of dynamic equilibria of at least four detectable stereoisomers of **1** and **2** in aqueous solutions were clearly observed.

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Experimental Part

1. *General.* Reagents and solvents were purchased from *VWR* (Darmstadt, Germany), and were of anal. or HPLC grade. NMR Solvents were obtained from *Deutero GmbH* (Kastellaun, Germany). Deionized H_2O was used throughout.

2. *Plant Material.* Yellow-colored Swiss chard (*Beta vulgaris* L. ssp. *cicla* [L.] ALEF. cv. 'Bright Lights'; Chenopodiaceae) was purchased from a local grower (*Pommerenke*, Steinheim am Albuch, Germany) in summer 2003. After washing, the leaf material was separated from the stems, and the latter were sealed in polyacrylamide-polyethylene bags under reduced pressure before being stored at –19° until pigment extraction. Cactus pear (*Opuntia ficus-indica* [L.] MILL. cv. 'Gialla'; Cactaceae) was purchased from Italy, and stored at –19°. After thawing, cactus fruits were manually squeezed, and the filtered juice was stored at –26° until pigment isolation.

3. *Extraction and Purification*. 3.1. *Cryogenic Grinding of Swiss Chard Stems and Pigment Extraction.* Liquid N_2 was added to the yellow petioles during grinding in a blender (*Waring 38BL41*; *Waring Products*, Torrington, CT, USA) additionally equipped with an outlet for pressure release at the top. The resulting powder was stored at –26° until pigment extraction. About 5 kg of yellow Swiss chard petioles were ground for the isolation of **2**. For pigment extraction, 150 g of the yellow powder was extracted by addition of 600 ml of 60% aq. MeOH containing 100 mM sodium ascorbate. After stirring for 10 min, the colored soln. was separated from the plant material by filtration through folded filter paper *595 A1/2* (*Schleicher & Schuell*, Dassel, Germany). The extract was concentrated under reduced pressure at 30°, re-suspended in H_2O (50 ml), and stored at –26°.

3.2. *Removal of Pectic Substances and Sugars.* Pectic substances were precipitated by addition of i-PrOH (100 ml) to cactus pear juice (50 ml) or aq. Swiss chard extract, resp. Precipitated pectic substances were removed after 15 min by filtering through folded filter paper *595 A1/2*, and the filtrate was subsequently concentrated *in vacuo* at 30° to *ca.* 10 ml. The depectinized pigment extract (10 ml) was then

applied to an *XAD 16-HP* column (830×40 mm) conditioned with H_2O (3 l) acidified with TFA (pH 3). Sugars were removed from the samples by rinsing with 17 l of TFA-acidified H_2O (pH 3). Subsequently, the betaxanthins were eluted with 1.2 l of MeOH containing 5% of TFA-acidified H_2O (pH 2). The collected betaxanthin eluates were adjusted to pH 5 by addition of 4% aq. NH_4OH to prevent acid hydrolysis, concentrated *in vacuo* at 30°, and directly purified by HPLC (see below).

3.3. *HPLC Purification*. 3.3.1. *Semi-Preparative HPLC.* The pre-treated extracts containing **1** and **2** were first purified on a semi-prep. HPLC (*Bischoff*, Leonberg, Germany) consisting of an *LC-CaDI 22–14* control unit, two HPLC compact pumps, an *SPD-10-AV-VP* UV/VIS detector (*Shimadzu*, Tokyo, Japan), and a dynamic-mixing chamber (*Knauer*, Berlin, Germany) equipped with the software Bischoff McDAcq 32. Separation was achieved on a semi-prep. *C_{18} Aqua* column (250 mm×21.2 mm, 5 μm; *Phenomenex*, Torrance, CA, USA) operating at r.t., a flow rate of 9 ml/min, and a pressure of 70 bar. As described earlier [11][24], the mobile phase consisted of 0.5% (*v*/*v*) aq. HCO_2H (solvent *A*) and MeCN/H_2O 1:1 (solvent *B*). Indicaxanthin (**1**) was isolated from the depectinized and desalted cactus pear juice starting with 84% of *A* in *B*, followed by a linear gradient to 72% *A* in *B* at 14 min, and then to 0% *A* in *B* within 2 min, with the latter kept const. for 2 min, before re-equilibration to starting conditions. Miraxanthin V (**2**) was isolated from depectinized and desalted Swiss chard extract applying the same gradient as used for the isolation of **1**, except for the step from 72% *A* in *B* to 0% *A*, which took 5 instead of 2 min. Upon isolation, aliquots of 700 μl of the respective pigment extracts were injected, and monitoring was performed at 470 nm for **1** (t_R 16.0 min) or **2** (22.0 min). The collected effluents were immediately cooled in an ice bath, and then gently concentrated under reduced pressure at 30°. Residual HCO_2H was removed by repeated addition of deionized H_2O.

3.3.2. *Analytical HPLC of* **1**. Further purification of **1** was achieved on a *Merck* (Darmstadt, Germany) anal. HPLC system equipped with an *L-7200* autosampler, a *D-7000* interface module, an *L-7100* pump, an *L-7350* column oven with a *Peltier* cooling module, an *L-7400* UV/VIS detector, and an *Atlantis dC_{18}* reversed-phase column (250×4.6 mm, 5 μm; *Waters*, Wexford, Ireland) fitted with a *C_{18}-ODS* security-guard column (4×3.0 mm). The system was operated at 30° and a flow rate of 1 ml/min. The mobile phase consisted of 1% aq. HCO_2H (solvent *A*) and MeCN/H_2O 4:1 (solvent *B*). Starting isocratically with 100% *A* for 2 min, linear gradients were followed from 0 to 9% *B* in 28 min, and from 9 to 100% *B* in 1 min, before re-equilibration to starting conditions. Aliquots of 90 μl of an aq. indicaxanthin soln. were injected, and monitoring of **1** (t_R 27.0 min) was performed at 470 nm. The eluted indicaxanthin fractions were immediately frozen at −26°. After thawing, they were pooled and dried by centrifugal evaporation on a *SPD-111 V SpeedVac* (*Thermo Electron*; Waltham, MA, USA).

3.3.3. *Final Purification of* **2**. For the removal of colorless phenolics and colored components, the pre-purified aq. solns. of **2** were applied on a *Sephadex LH-20* column (650×30 mm) conditioned with one bead vol. of deionized H_2O. Pigment elution was performed with H_2O. Purity checks of the aq. pigment solns. after chromatography showed that even further purification steps were required. Thus, the aq. solns. of **2** were repeatedly subjected to semi-prep. HPLC: 92% *A* in *B*, followed by a linear gradient to 86% *A* in *B* at 14 min, then to 77% *A* in *B* at 16 min, then 77% *A* in *B* for 7 min (isocratic), before re-equilibration to starting conditions. For distinction between phenolic compounds and the almost co-eluting **2**, monitoring was performed at 280 nm (t_R 22.0 min). After evaporation under reduced pressure at 30°, the aq. soln. of **2** was again purified by *Sephadex LH-20* chromatography, as described above. The purity of **2** was checked by HPLC-DAD at 280 nm, indicating a peak area of at least 90% of the total area. Finally, the aq. soln. of **2** was lyophilized.

3.3.4. *Purity Determination by HPLC/MS.* Purity checks of the pigments **1** and **2** were performed by anal. HPLC on an *Agilent 1100* apparatus (*Agilent*, Waldbronn, Germany) equipped with the ChemStation software, a degasser model *G1322A*, a binary gradient pump model *G1312A*, an autosampler model *G1329/1330A*, a column oven model *G1316A*, a diode-array detector model *G1315A*, and an *Atlantis dC_{18}* reversed-phase column (250×4.6 mm), 5 μm; *Waters*, Wexford, Ireland), fitted with a C_{18}-*ODS* (4×3.0 mm) security-guard column operated at 30° and a flow rate of 1 ml/min. 1% Aq. HCO_2H (solvent *A*) and MeCN/H_2O 4:1 (solvent *B*) were used, starting isocratically with 100% *A* for 2 min, followed by a linear gradient from 0 to 20% *B* within 60 min, and a subsequent linear gradient from 20 to 100% *B* in 5 min. The HPLC system was connected in series with an *Esquire 3000+* ion-trap mass spectrometer

(*Bruker*, Bremen, Germany) fitted with an electrospray-ionization (ESI) source operated in the pos. mode. N_2 was used as dry gas at a flow rate of 12 l/min and a pressure of 70 psi. The nebulizer temp. was set to 365°.

4. *NMR Analyses.* NMR Spectra were recorded at 298 K on a *Varian Unity Inova 500* spectrometer at 500 (^{1}H) or 125 MHz (^{13}C) using a 3-mm and/or a 5-mm PFG ID probe. ^{1}H-NMR Chemical shifts were referenced to the residual solvent signal at δ(H) 4.70 ppm (DOH) rel. to Me_4Si. 2D-NMR Spectra were recorded with *ca.* 0.5 mg of **2** and 1 mg of **1**, resp., both in D_2O and D_2O/H_2O 1:4 soln., each containing 0.01% of TFA. All 1D- (1D-^{1}H-TOCSY) and 2D-NMR experiments (gCOSY, TOCSY, NOESY, gHSQC, gHMQC)[2]) measurements were performed with standard *Varian* pulse sequences. For H_2O/D_2O mixtures, water suppression was achieved by implementing a pre-saturation element in the respective pulse sequences.

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[2]) The prefix 'g' refers to gradient-enhanced.