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Betalains from Christmas cactus

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Abstract

The presence of 14 betalain pigments have been detected by their characteristic spectral properties in flower petals of Christmas cactus (*Schlumbergera* x *buckleyi*). Along with the known vulgaxanthin I, betalamic acid, betanin and phyllocactin (6′-*O*-malonylbetanin), the structure of a new phyllocactin-derived betacyanin was elucidated by various spectroscopic techniques and carbohydrate analyses as betanidin 5-*O*-(2′-*O*-β-D-apiofuranosyl-6′-*O*-malonyl)-β-D-glucopyranoside. Among the more complex betacyanins occurring in trace amounts, the presence of a new diacylated betacyanin {betanidin 5-*O*-[(5″-*O*-*E*-feruloyl)-2′-*O*-β-D-apiofuranosyl-6′-*O*-malonyl)]-β-D-glucopyranoside} has been ascertained. Furthermore, the accumulation of betalains during flower development and their pattern in different organs of the flower has been examined.

Keywords: Christmas cactus (*Schlumbergera* x *buckleyi*); Cactaceae; Flowers; Betalains; Phyllocactin; Betanidin 5-*O*-(2′-*O*-β-D-apiofuranosyl-6′-*O*-malonyl)-β-D-glucopyranoside

1. Introduction

The red–violet betacyanins and the yellow betaxanthins are a class of water-soluble pigments, the betalains, which are characteristic for certain members of the plant order Caryophyllales and some higher fungi (Steglich and Strack, 1990). Analogous to anthocyanins, they occur in various coloured plant parts and are as such responsible for the bright coloration of flowers and fruits of the Cactaceae. Recently, the structure of the dopamine-derived 6′-*O*-malonyl-2-descarboxy-betanin has been elucidated (Kobayashi et al., 2000). Its fragmentation pattern in LC-MS had to be compared with that of the known 6′-*O*-malonylbetanin (phyllocactin). Thus, an extract from flower petals of Christmas cactus (*Schlumbergera* spec., formerly *Epiphyllum*, *Phyllocactus*, *Zygocactus*; Bachthaler, 1992), a known source of phyllocactin (Piattelli and Minale, 1964a, 1964b; Wyler, 1969; Strack et al., 1981), was analysed, which revealed the presence of 14 betalain pigments, most of them unknown. Among these, the less polar pigments seem to represent betacyanins as complex as anthocyanins (Strack and Wray, 1989; Harborne, 1994). First evidence for the occurrence of a betacyanin with higher electrophoretic mobility than betanin was observed by Piattelli and Minale (1964a, 1964b) which identified its structure as 6′-*O*-malonylbetanin (phyllocactin) by chemical derivatization and degradation (Minale et al., 1965, 1966). Screening of 34 members of the Cactaceae revealed that phyllocactin is in most cases the main pigment in flowers and fruits of Cactoideae, whereas in the Pereskioideae and Opuntioideae it is present in low amounts or is completely absent (Piattelli and Imperato, 1969). The linkage of the malonyl moiety in phyllocactin to the 6′-*O*-

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position of glucose was determined previously by permethylation and analysis of the permethyl glucoses after hydrolysis (Minale et al., 1966). However, these authors could not exclude that an acyl migration during the betacyanin purification and derivatization process might occur. This uncertainty has been eliminated in the present study by ^{1}H-NMR spectroscopy. The major components of the flower pigments were elucidated, giving in addition evidence for the occurrence of heteropolyacylated structures. Furthermore, the accumulation of betalains during flower development and their organ specificity has been examined.

2. Results and discussion

HPLC analysis of an extract prepared from flower petals of Christmas cactus [(*S.* x *buckleyi* T. Moore) Tjaden] (Fig. 1) revealed the presence of 14 betalains (Fig. 2; structure scheme). As yet, only betanin (**3**) and phyllocactin/isophyllocactin (**5**, **5′**) have been assigned (Minale et al., 1966; Strack et al., 1981). Compounds **1**–**3** were readily identified by UV-VIS spectroscopy and co-chromatography (HPLC) with authentic or synthetic standards (Schliemann et al., 1999) as vulgaxanthin I (**1**), betalamic acid (**2**) and betanin (**3**). The identification was corroborated by LC-MS of the purified compounds giving the expected protonated molecular ions $[M + H]^+$ (**1**, *m/z* 341; **3**, *m/z* 551) (Table 1). The main betacyanin of the extract (R_t 24.8 min; λ_{max} 539 nm) was, as expected, the known phyllocactin (**5**, 6′-*O*-malonylbetanin), which was confirmed by LC-MS, ^{1}H-NMR spectroscopy and hydrolysis experiments. In the positive ion mode, the $[M + H]^+$ ion of **5** was observed at *m/z* 637 [550 (betanin) + 86 (malonyl) + H]$^+$, which gave a daughter ion at *m/z* 389, corresponding to [betanidin + H]$^+$. In the negative ion mode, the successive loss of four CO_2 (two from betalamic acid, one from *cyclo*-Dopa and the fourth from the acyl moiety) was demonstrated, which indicates the presence of a dicarboxylic acid as acyl residue.

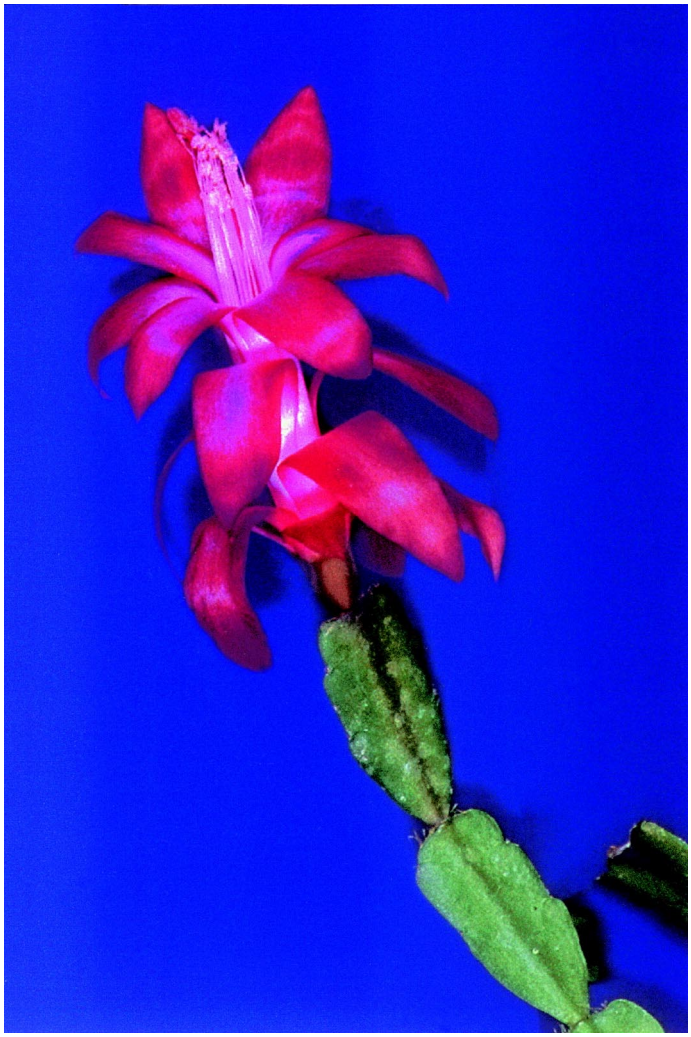


Fig. 1. Flowering Christmas cactus.

The characteristic signals of **5** in the 1D and 2D ^{1}H-NMR data confirmed the presence of the aglycone, glucose and malonyl moieties. In keeping with the previous data (Heuer et al., 1994), the small chemical shift difference between H-4 and H-7 of 0.15 ppm is characteristic of substitution at the hydroxyl group at C-5 of betanidin, as opposed to that at C-6 where differences of *ca* 0.8 ppm are to be expected. Likewise, the low field chemical shifts of H-6′A/H-6′B 4.60 and 4.33 ppm, respectively, are clear evidence that the malonyl system is bound to C′-6 of the glucose moiety.

Further evidence for the structure of **5** as 6′-*O*-malonylbetanin came from hydrolysis experiments. Treat-

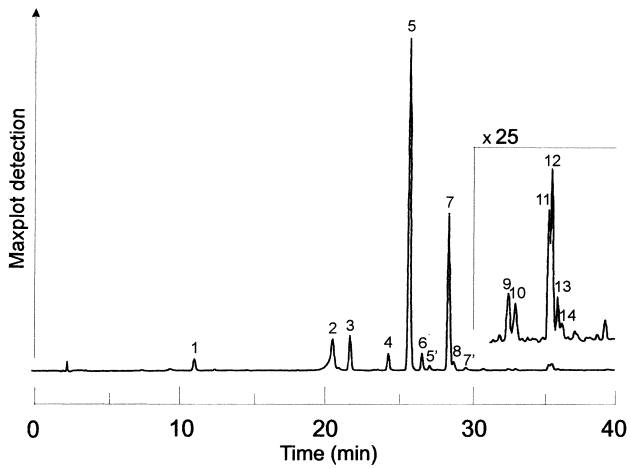


Fig. 2. HPLC elution profile of betalains (maxplot detection: 400–650 nm) from flower petals of the Christmas cactus. Peak numbers correspond to the numbers in Table 1, Fig. 3, and the structure scheme.

ment with 1 N HCl at room temperature and kinetic analysis by HPLC of the appearing reaction products showed both, the conversion of **5**–**3** and the racemization of **5**–**5′**, thus confirming the identity of the minor compound **5′** in the extract as isophyllocactin. The racemization proceeded after a 2-h-lag phase linearly in a time-dependent manner (2.3% h^{-1}) (data not shown) to the same extent as with betanin (2.2% h^{-1}) (Schliemann and Strack, 1998). These reactions are accompanied by hydrolysis of the aldimine bonds in **5** and **3** leading to betalamic acid (**2**), which is unstable under acidic conditions, the major compound *cyclo*-Dopa 5-*O*-(6′-*O*-malonyl)-β-D-glucoside (R_t 10.4 min; λ_{max} 282 nm) and traces of *cyclo*-Dopa 5-*O*-β-D-glucoside (R_t 4.1 min; λ_{max} 283 nm). The latter corresponded with the data of Wyler et al. (1984) for *cyclo*-Dopa 5-*O*-β-D-glucoside (λ_{max} 283 at pH 2.75).

The second major betacyanin **7** (R_t 27.6 min; λ_{max} 540 nm) showed a protonated molecular ion at *m*/*z* 769 and its daughter ion at *m*/*z* 389 using positive ion mode LC-MS. In the negative ion mode the loss of four CO_2 was observed as found with **5** indicating the presence of a malonyl moiety. The mass difference between **7** (*m*/*z* 769) and **5** (*m*/*z* 637) suggested the presence of an additional pentose moiety, although **7** eluted 2.8 min after **5** in the reversed phase HPLC.

Carbohydrate compositional analysis of the new malonylated betacyanin **7** confirmed the presence of glucose and the less common pentose, apiose (Schliemann et al., 1996) in a ratio of about 1:1. The identity of the pentose residue was unambiguously confirmed in comparison with an authentic sample of apiin containing terminal apiose. The linkage between the two sugar moieties was established by methylation analysis (Jansson et al., 1976). The detection of 1,4-di-*O*-acetyl-2,3,5-tri-*O*-methylapitol by GC-MS, identified by its characteristic fragmentation pattern (Wagner and Demuth, 1972), clearly showed the terminal position of this pentose, which is bound to C-2′ of the glucose as indicated by the detection of 1, 2, 5-tri-*O*-acetyl-3,4,6-tri-*O*-methylglucitol. All acyl-linked organic acids are lost under the basic conditions of the derivatization procedure.

In spite of the inherent instability of betacyanins in solution (in the presence of traces of DCl), the 1D and 2D ^{1}H-NMR spectra afforded at 600 MHz sufficient data to complement and confirm the mass spectrometric findings that completed the structural elucidation of **7**. Analogous to **5**, sugar substitution at C-5 of betanidin was confirmed by the small chemical shift difference between H-4 and H-7 of 0.08 ppm. In addition, the data are compatible with the presence of glucose and apiose units, found from the mass spectral data, in the 6-acylated β-glucose and free terminal apiofuranose forms. The latter is evident from the similarity of the ^{1}H shifts of H-1‴–H-4‴ A/B and high field shifts of H-5‴ A/B compared to the those of the previously described acylated derivatives (Schwind et

Table 1
Retention time, HPLC-PDA and MS data of betalains from flower petals of the Christmas cactus

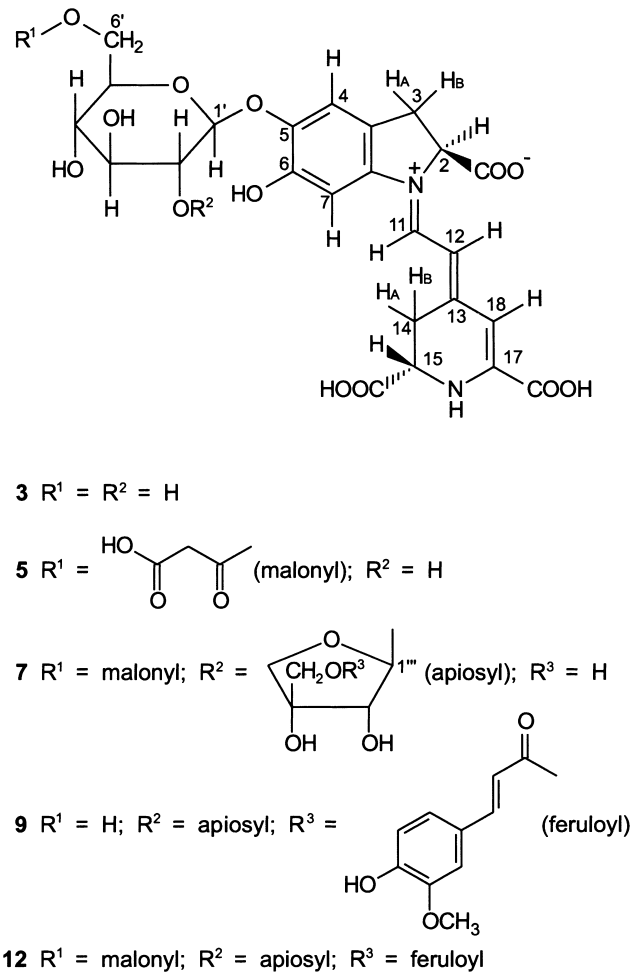
Peak	R_t (min)	HPLC-PDA (λ_{max}, nm)			LC-MS $[M + H]^+$	Betalain
		I: UV (HCA)[a]	II: VIS (BX/BC)[b]	Ratio A II:I		
1	11.0	–	468	–[c]	341	Vulgaxanthin I
2	20.3	–	405	–	n.d.[d]	Betalamic acid
3	21.4	–	537	–	551	Betanin
4	24.0	–	540	–	a.d.[e]	Unknown betacyanin
5	25.4	–	539	–	637	Phyllocactin
6	26.2	–	537	–	a.d.[e]	Unknown betacyanin
5′	26.8	–	538	–	n.d.[d]	Isophyllocactin
7	28.0	–	538	–	769	2′-Apiosyl-phyllocactin
8	28.3	–	537	–	n.d.[d]	Unknown betacyanin
7′	29.2	–	538	–	n.d.[d]	Iso-2′-apiosyl-phyllocactin
9	32.0	329	548	1 : 0.51	859[f]	5″-*O*-*E*-feruloyl-2′-apiosyl-betanin
10	32.5	–	544	–	n.d.[d]	Unknown betacyanin
11	34.7	331	551	1 : 0.48	n.d.[d]	Unknown HCA-betacyanin
12	34.9	328	549	1 : 0.46	945	5″-*O*-*E*-feruloyl-2′-apiosyl-phyllocactin
13	35.3	314	549	1 : 1.60	n.d.[d]	Unknown HCA-betacyanin
14	35.6	323	546	1 : 0.47	n.d.[d]	Unknown HCA-betacyanin

[a] λ_{max} of hydroxycinnamoyl moiety (HCA/I), –, no absorbance band.
[b] λ_{max} of betaxanthins or betacyanins in the visible range (II).
[c] Ratio of absorbance at λ_{max} (Vis) and at 320 nm is *ca* 1 : 0.1 (–).
[d] n.d., not determined.
[e] a.d., ambiguous data.
[f] From a previous publication (Schliemann et al., 1996).

al., 1990; Schliemann et al., 1996). Although in general, the magnitude of $^3J(1–2)$ of the apiose system is insufficient to define conclusively the relative configuration of the anomeric proton, the similarity of the coupling found here (3.9 Hz) as well as formerly in a betacyanin from *Phytolacca americana* (Schliemann et al., 1996) and that of the apiose moiety, e.g. in xeranthin (2.8 Hz) (Schwind et al., 1990), together with similarities of their chemical shifts, is suggestive of a β-configuration of the glycosidic linkage. As in **5**, the low field chemical shifts of H-6′A/H-6′B in **7** provides definitive evidence that the malonyl system is bound to C′-6 of the glucose moiety. Hence, the combination of LC-MS data, composition and linkage analysis of sugars as well as the results of the NMR measurements, identified **7** as betanidin 5-*O*-(2′-*O*-β-D-apiofuranosyl-6′-*O*-malonyl)-β-D-glucopyranoside. Treatment of **7** with 1 N HCl caused racemization and degradation as observed for **5** (data not shown); thus, the identification of the minor component **7′** as isobetanidin 5-*O*-(2′-*O*-β-D-apiofuranosyl-6′-*O*-malonyl)-β-D-glucopyranoside was possible.

For the minor pigments **4**, **6** and **8**, MS gave no conclusive results, but from their UV-VIS spectra and retention times (Table 1) acylation of these betacyanins with hydroxycinnamic acids could be excluded. Co-chromatographical analysis (solvent system 1, gradient 2) showed that **4** is not identical with gomphrenin I (betanidin 6-*O*-β-D-glucopyranoside), but eluted between isobetanin and gomphrenin I.

Besides the major malonylated derivatives **5** and **7** in the petal extract, minor less polar betacyanins (**9**–**14**) have been detected (Fig. 2), most of which contain a hydroxycinnamoyl moiety indicated by an absorption at 320 nm (Table 1). This substitution leads to a bathochromic shift of λ_{max} in the visible absorption (Heuer et al., 1994; Schliemann and Strack, 1998). Although the amounts of **9**–**14** were very low, some reliable structural information could be obtained. Compound **9** was found to be identical (R_t, UV-VIS) with 5″-*O*-*E*-feruloyl-2′-*O*-β-D-apiosyl-betanin, a betacyanin identified from *Phytolacca americana* (Schliemann et al., 1996) and used for co-chromatography (HPLC). For the less polar compound **12**, a similar structure was suggested from the almost identical UV and VIS spectral data, but in LC-MS the protonated molecular ion occurred at *m/z* 945. The mass difference of 86 between **12** and **9** (*m/z* 859) is indicative of the presence of an additional malonyl residue which further decreases the polarity. Although final proof of the structure by ^{1}H-NMR spectroscopy and sugar linkage analysis was not possible due to limitation of material,



Scheme 1.

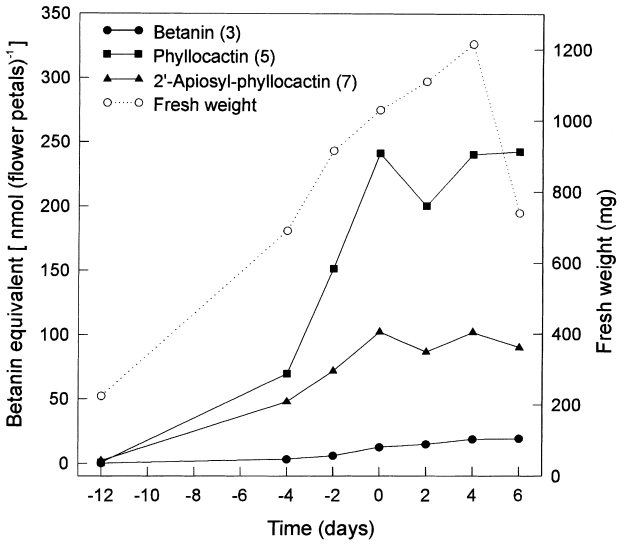


Fig. 3. Accumulation time course of the major betacyanins during flower development of the Christmas cactus. (Day 0 is the time of flower opening. The decrease in fresh weight at day 6 is caused by wilting.) Compound numbers correspond to the numbers in Fig. 2, Table 1, and the structure Scheme 1.

the most plausible new structure is betanidin 5-*O*-[(5″-*O*-*E*-feruloyl)-2′-*O*-β-D-apiofuranosyl-6′-*O*-malonyl]-β-D-glucopyranoside (5″-*O*-*E*-feruloyl-2′-*O*-β-D-apiosyl-phyllocactin) (**12**) (Scheme 1). The assignment of this structure is furthermore based on the fact that the linkage of the malonyl residues in both **5** and **7** was found in the 6′-*O*-position of the glucose and the linkage of the feruloyl residue in **9** is in the 5″-*O*-position of the apiose (Schliemann et al., 1996) found by ^{1}H-NMR. Compound **12** seems to be the first betacyanin containing both an aliphatic and an aromatic (hydroxycinnamoyl) acyl residue. This type of acylation has frequently been found in complex anthocyanins (Strack and Wray, 1989; Harborne, 1994). In pigments from bracts of *Bougainvillea glabra*, the attachment of two hydroxycinnamoyl residues to one betacyanin molecule has already been reported (Heuer et al., 1994).

As the malonyl derivatives **5** and **7** are the main pigments in the petals (Fig. 2), the question concerning the time point of betacyanin acylation during the flower development arose. Therefore, closed flowers at different developmental stages and material after flower opening were harvested and analysed (Fig. 3). Four days before flower opening, **5** and **7** are the major betacyanins which indicate that the malonylation is an early reaction. Then the amount of **5** strongly increased concomitantly with the fresh weight, and only a small portion of non-acylated **3** was left. During flowering (day 0–day 6) there was no further change of the betacyanin content. It is assumed that betacyanin acylation is the final biosynthetic step, but, as in the case of the glucosylation, the acylation on the *cyclo*-Dopa 5-*O*-glucoside level cannot be excluded from these experiments. The HPLC pattern (at 280 nm) of the petal extracts gave no evidence for the presence of *cyclo*-Dopa 5-*O*-(6′-*O*-malonyl)-β-D-glucoside and of *cyclo*-Dopa 5-*O*-β-D-glucoside. It is reasonable to suggest that the attachment of the apiose moiety is catalysed by a corresponding putative apiosyltransferase using betanin as a substrate, but this has not been proven yet. In any case, the malonylation of the resulting diglycoside must proceed rapidly as the direct precursor of **7** (non-acylated) is not found. The HPLC pattern (at 320 nm) of the petal extracts showed the presence of two hydroxycinnamic acid derivatives (R_t 30.9 and 37.5 min, both λ_{max} 329 nm) which did not coelute with the less polar acylated betacyanins, but flavonoids were not found.

Table 2
Betacyanin patterns in different organs of the Christmas cactus flower

Flower part	Total betacyanin content	Relative content (% of total betacyanins)				
	[μg betanin equivalents (g fresh weight)$^{-1}$]	**3**	**5**	**7**	**9/10**	**11/12**
Petals	594	6.7	59.9	21.5	0.3	0.9
Carpels	1013	19.1	47.3	9.8	2.7	5.5
Stamens	28	0.8	12.8	15.3	18.1	41.4

The analysis of carpels and stamens for betacyanin composition revealed marked quantitative differences between the flower organs (Table 2). Whereas the dark-red carpels contained nearly the two-fold amount of total betacyanins [expressed as betanin equivalents (g fresh weight)$^{-1}$] as compared with petals and slightly increased percentages of the less polar feruloyl-betacyanins (**9**–**12**) which are trace compounds in the petals, the latter are the predominant components in the extract from stamens. Finally, a comparative pigment analysis of a petal extract from *Schlumbergera truncata* (formerly *Zygocactus truncatus*) revealed almost the same betalain pattern (data not shown) as that in petals of *S.* x *buckleyi*.

In summary, the presented data describe the complete pattern of petal pigments of *Schlumbergera* x *buckleyi*. The ^{1}H-NMR data of **5** proved unequivocally the linkage of the malonyl residue at the 6′-*O*-position of **3**. The structure of a new betacyanin was elucidated as betanidin 5-*O*-(2′-*O*-β-D-apiofuranosyl-6′-*O*-malonyl)-β-D-glucopyranoside, a phyllocactin modified with the less common branched pentose, apiose, in 2′-*O*-position. Furthermore, the new malonyl conjugate of betanidin 5-*O*-[(5″-*O*-*E*-feruloyl)-2′-*O*-β-D-apiofuranosyl]-β-D-glucopyranoside containing two different sugars (hexose and pentose) and acyl moieties (aliphatic and hydroxycinnamoyl) represents a first analogue to the already known complex anthocyanins (Strack and Wray, 1989; Harborne, 1994). The analysis of betacyanins during flower development revealed that four days before flower opening both phyllocactin and its 2′-*O*-β-D-apiofuranosyl derivative are the predominant betacyanins of flower petals which indicates that there is an early concomitant expression of all enzymes involved in the formation of these complex betacyanins.

3. Experimental

3.1. Plant material

S. x *buckleyi* (T. Moore) Tjaden — Christmas cactus and *S. truncata* (Haworth) Moran were cultivated in a greenhouse and the flowers (petals without stamens and carpels) were harvested after full opening. Closed flowers of different size were harvested to study the betacyanin composition during flower development. At the same time, the development of closed flowers with identical sizes was followed until flower opening was

monitored. For the analysis of betalain composition of different flower parts, petals, stamens (filaments with anthers) and carpels were harvested separately after flower opening.

3.2. Isolation and purification of betalains

The flower petals (48 g) were frozen in liquid N_2, homogenised in a mortar and extracted with 150 ml 80% aq. MeOH containing 50 mM ascorbate for 30 min. After centrifugation at 14,000 g for 10 min at 4°C, the supernatant was removed and its betacyanin content was quantified photometrically at 540 nm (yield: 16.3 μmol betanin equivalents) using the molar extinction coefficient for betanin (62×10^6 cm^2 mol^{-1}; Wyler et al., 1959). The extract was concentrated in vacuo at 30°C. The presence of 14 pigments in various concentrations was detected by analytical HPLC (solvent system 1, gradient 1). The compounds were purified in a two-step procedure. After prep. HPLC with a linear gradient, the main betacyanin fractions were repurified by an additional prep. HPLC with isocratic elution. The minor betalains were separated by semi-prep. HPLC (solvent system 2) on an analytical column.

3.3. Racemization and degradation of phyllocactin

The initial content of phyllocactin (2.7 nmol betanin equivalents in 110 μl H_2O) was analysed by HPLC (10 μl injection, 0.25 nmol betanin equivalents). To start the racemization, 100 μl 1 N HCl was added to the injection vial 30 min after start of the HPLC run. The progress of racemization was monitored kinetically by automatic injection of 20 μl (0.25 nmol betanin equivalents). Racemization and degradation were calculated from peak areas and experiments were performed in duplicate. The racemization of **7** was performed as with **5**.

3.4. Betacyanin composition during flower development

Closed flowers of different sizes (1.5, 3.5 and 4.5 cm long), which correspond to 12, 4 and 2 days before flower opening, and flower petals (0, 2, 4, 6 days old) were extracted and the total betacyanin content was determined as described above. The betacyanin composition was obtained by analytical HPLC (solvent system 1, gradient 1). Petals, stamens and carpels were processed and analysed in the same way. The experiments were performed in duplicate.

3.5. HPLC

Analytical and semi-preparative HPLC was performed with a system from Waters (Waters, Milford, MA, USA), including the separation module 2690. The liquid chromatograph was equipped with a 5-μm Nucleosil C_{18} column (250 × 4 mm i.d.; Macherey-Nagel, Düren, Germany) and the following solvent and gradient systems were used. Solvent system 1: A: 1.5% aq. H_3PO_4; B: acetonitrile; gradient 1: linear gradient from 100% A to 76% A in (A + B) within 40 min; gradient 2: linear gradient from 90% A to 77.5% A in (A + B) within 10 min; the flow rate was 1 ml min^{-1}. Solvent system 2: A: 1% aq. HOAc; B: acetonitrile; gradient 1 as in solvent system 1. The betalains were detected at 540, 475 and 405 nm or by maxplot detection between 400 and 650 nm (photodiode array detection). The injection volume was 20 or 50 μl in analytical (solvent system 1) and 100 μl in semi-prep. work (solvent system 2). For preparative HPLC, the liquid chromatograph (System Gold; Beckman Instruments, München, Germany) was equipped with a 10 μm-Nucleosil 100–10 C_{18} column (VarioPrep; 250 × 40 mm i.d.; Macherey-Nagel, Düren, Germany). The separation of the concentrated extract from flower petals of the Christmas cactus was performed with a linear gradient within 120 min from 100% solvent A (aq. HCO_2H, pH 3) to 40% solvent A in solvent B (MeOH) at a flow rate of 10 ml min^{-1} (injection volume: 2 ml; detection at 475 and 540 nm; PDA-detection: 250–600 nm). **1**: R_t 49–52 min; **3**: R_t 75–78 min; **5**: R_t 86–87 min; **7**: R_t 88–92 min; **9/10**: R_t 100–104 min; **11-14**: R_t 106–111 min. The main compounds **5** and **7** were repurified under isocratic conditions [70% solvent A (aq. 0.6% HOAc, pH 3) and 30% solvent B (MeOH)]. To remove MeOH, the pooled fractions were concentrated in vacuo at 30°C and the aqueous residues were lyophilised (yield: **5**, 0.52 mg; **7**, 0.46 mg). The minor betacyanin fractions were concentrated and purified by semi-prep. HPLC (solvent system 2).

3.6. LC-MS

Positive and negative ion electrospray mass spectra were recorded on a Finnigan MAT TSQ 7000 instrument [electrospray voltage 4.5 kV (positive ions), 3.5 kV (negative ions); capillary 220°C; sheath gas : N_2] coupled with a Micro-Tech Ultra-Plus Micro LC system equipped with a 4-μm C_{18} column (100 × 1 mm i.d, ULTRASEP). For LC, a gradient system starting from 10% B (0.2% aq. AcOH in acetonitrile) in 90% A (0.2% aq. AcOH) to 50% B in (A + B) within 10 min was used, followed by 10 min isocratic elution at a flow rate of 70 μl min^{-1} (injection volume: 2 μl). The negative ion ES-MS spectra were recorded using an atmospheric pressure ionisation collision-induced dissociation (APICID) offset voltage of 10 V. The collision-induced dissociation (CID) mass spectra during the LC run were obtained under the following

conditions: collision energy (collision cell) −40eV (positive ions); collision gas: argon; collision pressure: 1.8 mT. All mass spectra were averaged and background subtracted.

Compound **1** (vulgaxanthin I), (positive ion mode) (*m*/*z*, rel. int.): 341 $[M + H]^+$ (100). Compound **3** (betanin), (positive ion mode) (*m*/*z*, rel. int.): 551 $[M + H]^+$ (100). Compound **5** [phyllocactin, betanidin 5-*O*-(6′-*O*-malonyl)-β-D-glucopyranoside], (positive ion mode) (*m*/*z*, rel. int.): 637 $[M + H]^+$ (100); daughter ion scan mode of *m*/*z* 637: 389 [betanidin + H]$^+$ (100); (negative ion mode) (*m*/*z*, rel. int.): 635 $[M - H]^-$ (90), 591 $[M - H - CO_2]^-$ (100), 547 $[M - H - 2CO_2]^-$ (88), 503 $[M - H - 3CO_2]^-$ (61), 459 $[M - H - 4CO_2]^-$ (18). Compound **7** [betanidin 5-*O*-(2′-*O*-β-D-apiofuranosyl-6′-*O*-malonyl)-β-D-glucopyranoside], (positive ion mode) (*m*/*z*, rel. int.): 769 $[M + H]^+$ (100); daughter ion scan mode of *m*/*z* 769: 389 [betanidin + H]$^+$ (100); (negative ion mode) (*m*/*z*, rel. int.): 767 $[M - H]^-$ (27), 723 $[M - H - CO_2]^-$ (81), 679 $[M - H - 2CO_2]^-$ (100), 635 $[M - H - 3CO_2]^-$ (97), 591 $[M - H - 4CO_2]^-$ (70). Compound **12** (5″-*O*-*E*-feruloyl-2′-apiosyl-phyllocactin), (positive ion mode) (*m*/*z*, rel. int.): 945 $[M + H]^+$ (30), 756 (100).

3.7. NMR

1D and 2D ^{1}H (COSY)-NMR spectra were recorded at 300 K on a Bruker AVANCE DMX 600 NMR spectrometer locked to the major deuterium resonance of the solvent, CD_3OD, containing a trace of DC1. All chemical shifts are given in ppm relative to TMS and coupling constants in Hz. Compound **5** (phyllocactin, betanidin 5-*O*-(6′-*O*-malonyl)-β-D-glucopyranoside) ^{1}H-NMR (CD_3OD/DCl) $\delta = 8.70$ [*d*, H-11, J(11–12) 12.3], 7.38 [*s*, H-7], 7.23 [*s*, H-4], 6.47 [*s*, H-18], 6.23 [*d*, H-12], 5.46 [*dd*, H-2, *J*(2-3A) 9.7], 4.85 [*d*, H-1′, *J*(1′–2′) 7.6], 4.63 [*dd*, H-15, *J*(15–14A) 5.3, *J*(15–14B) 7.4], 4.60 [*dd*, H-6′A, *J*(6′A-5′) 2.0, *J*(6′A–6′B) 12.0], 4.33 [*dd*, H-6′B, *J*(6′B-5′) 6.6], 3.74 [*dd*, H-3A, *J*(3A–3B) 16.3], 3.73 [*m*, H-5′], 3.72 [*dd*, H-14A, *J*(14A–14B) 17.2], 3.56 [*m*, H-3′], 3.55 [*m*, H-2′], 3.49 [*dd*, H-3B], 3.46 [AB system, H-2″], 3.42 [*m*, H-4′], 3.31 [*dd*, H-14B]. Compound **7** [betanidin 5-*O*-(2′-*O*-β-D-apiofuranosyl-6′-*O*-malonyl)-β-d-glucopyranoside] ^{1}H-NMR (CD_3OD/DCl) $\delta = 8.71$ [*d*, H-11, *J*(11–12) 12.3], 7.38 [*s*, H-7], 7.30 [*s*, H-4], 6.47 [*s*, H-18], 6.23 [*d*, H-12], 5.45 [*dd*, H-2, *J*(2–3A) 9.9, *J*(2-3B) 2.1], 5.36 [*d*, H-1‴, *J*(1‴-2‴) 3.9], 4.87 [*d*, H-1′, *J*(1′–2′) 7.4], 4.65 [*dd*, H-6′A, *J*(6′A–5′) 2.0, *J*(6′A–6′B) 11.9], 4.63 [*dd*, H-15, *J*(15–14A) 5.4, *J*(15–14B) 7.3], 4.32 [*dd*, H-6′B, *J*(6′B–5′) 6.6], 4.17 [*d*, H-4A‴], 4.03 [*d*, H-2‴], 3.84 [*d*, H-4B‴], 3.73 [*dd*, H-3A], 3.72 [*dd*, H-14A, *J*(14A–14B) 17.2], 3.70 [*m*, H-5′], 3.65–3.56 [*m*, H-2′, H-3′, H-5‴A/B], 3.47 [AB system, H-2″], 3.46 [*dd*, H-3B], 3.42 [*m*, H-4′], 3.31 [*dd*, H-14B].

3.8. Sugar composition analysis

Monosaccharides were analysed as the corresponding Me glycosides after methanolysis and trimethylsilylation (Chaplin, 1982) using a Finnigan gas chromatograph equipped with a 30-m DB5 capillary column connected to a Finnigan GCQ ion-trap mass spectrometer running in the electron-impact mode. Apiin [apigenin 7-*O*-(2′-*O*-β-D-apiofuranosyl)-β-D-glucopyranoside] from Roth (Karlsruhe, Germany) was used as standard.

3.9. Carbohydrate methylation analysis

For methylation analysis, the glycoconjugate was permethylated, hydrolysed, reduced, and peracetylated as described (Anumula and Taylor, 1992). Separation and identification of partially methylated alditol acetates was performed using the same GC/MS system described above.

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