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Review

# Recent advances in betalain research

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Dedicated to Meinhart H. Zenk on the occasion of his 70th birthday

**Abstract**

Betalains replace the anthocyanins in flowers and fruits of plants of most families of the Caryophyllales. Unexpectedly, they were also found in some higher fungi. Whereas the anthocyanin-analogous functions of betalains in flower and fruit colouration are obvious, their role in fungi remains obscure. The nature of newly identified betalains as well as final structure elucidation of earlier putatively described compounds published within the last decade is compiled in this report. Recent advances in research on betalain biosynthesis is also covered, including description of some 'early' reactions, i.e. betalain-specific dopa formation in plants and fungi and extradiolic dopa cleavage in fungi. Work on betalain-specific glucosyltransferases (GTs) has given new insights into the evolution of secondary plant enzymes. It is proposed that these GTs are phylogenetically related to flavonoid GTs. It was found that the decisive steps in betalain biosynthesis, i.e. condensation of the betalain chromophore betalamic acid with *cyclo*-dopa and amino acids or amines in the respective aldimine formation of the red-violet betacyanins and the yellow betaxanthins, are most likely to be non-enzymatic. Betalains have attracted workers in applied fields because of their use for food colouring and their antioxidant and radical scavenging properties for protection against certain oxidative stress-related disorders.


*Keywords:* Aldimine formation; Dopa dioxygenase; Glucosyltransferase; Polyphenoloxidase; Spontaneous reaction; Structure elucidation; Tyrosinase

**Contents**



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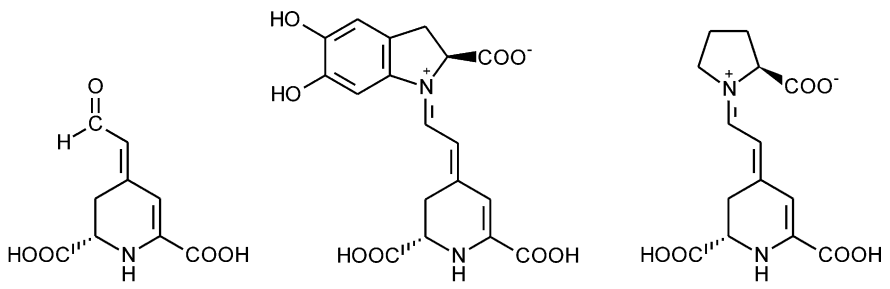
## 1. Introduction

Betalains are water-soluble nitrogen-containing pigments, which comprise the red-violet betacyanins and the yellow betaxanthins. They are immonium conjugates of betalamic acid with *cyclo*-dopa and amino acids or amines, respectively. The structure scheme shows betalamic acid (**1**), the chromophore of all betalains, betanidin (**2**) (Wyler et al., 1963), the aglycone of most of the betacyanins, and indicaxanthin (**3**) (Piattelli et al., 1964), a proline-containing betaxanthin. These were the first betalains identified by chemical means. Since then, betaxanthins with various other amino acids and amines as well as various betanidin conjugates (glycosides and acylglycosides) were identified by spectroscopic techniques (Strack et al., 1993; Strack and Wray, 1994a).

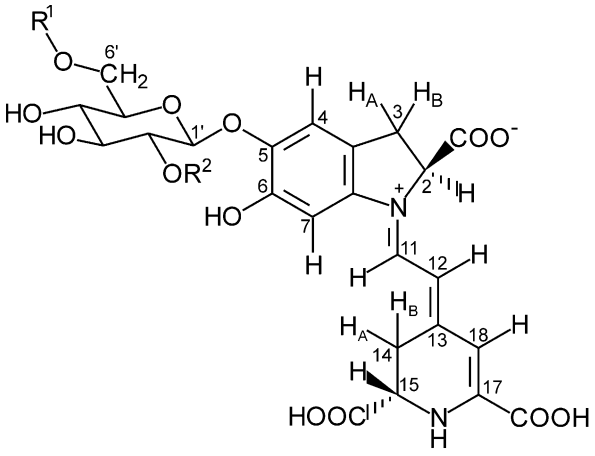
Within the last decade, a series of publications has described new structures and complete identification of some putative ones as well as elucidation of biosynthetic reactions (Roberts and Strack, 1999). Some early enzymatic reactions in betalain biosynthesis were characterized, polyphenoloxidase (PPO)-type tyrosinase and extradiolic dopa dioxygenase. Furthermore, the decisive steps in the biosynthesis of both betanidin and betaxanthins by aldimine formation were recently identified to proceed non-enzymatically. Finally, betalain-specific glucosyl- and hydroxycinnamoyltransferases were characterized.

Betalains accumulate in flowers, fruits and occasionally in vegetative tissues of plants belonging to most families of the Caryophyllales (Steglich and Strack, 1990). However, there are two exceptions: the Caryophyllaceae and the Molluginaceae accumulate anthocyanins instead, flavonoid-derived pigments occurring in all other families of flowering plants. The Caryophyllales-specific occurrence of betalains is a prominent example of the chemotaxonomic relevance of plant secondary products. Gain and loss of anthocyanins and betalains during plant evolution still remain a mystery (Clement and Mabry, 1996). Molecular studies are needed to elucidate the evolutionary mechanisms leading to the mutual exclusion of the betalain and anthocyanin pathways in flowering plants (Kimler et al., 1970; Stafford, 1994). Betalains were also detected in some higher fungi (Steglich and Strack, 1990), for example in the fly agaric (*Amanita muscaria*). Whereas the functions of betalains in plant flower and fruit colouration are obvious, their role in fungi is unknown.

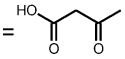
There is growing interest in the use of natural pigments for food colouring, since synthetic dyes are becoming more and more critically assessed by the consumer. In food processing, betalains are less commonly used than anthocyanins and carotenoids, although these water-soluble pigments, stable between pH 3 and 7, are well suited for colouring low acid food. The most important source of betanin (**4**) as colouring agent is the red beet (*Beta vulgaris* subsp. *vulgaris*) root. The corresponding cell cultures (Leathers et al., 1992; Trejo-Tapia et al., 1999; Akita et al., 2000) and hairy roots (Mukundan et al., 1998a,b; Berzin et al., 1999) cannot compete with the plant's root (50-60 t/ha with ca. 0.5 g betanin/kg) (Stintzing et al., 2000) with respect to betanin accumulation. Besides betanin from red beet, also betacyanins from plants of the Amaranthaceae were tested concerning colour properties and pigment stability in model food systems (Cai et al., 1998; Cai and Corke, 1999, 2000).

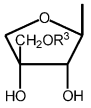


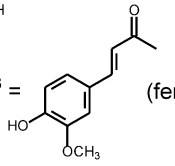

Betalamic acid (**1**) Betanidin (**2**) Indicaxanthin (**3**)



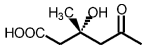
$R^1 = R^2 = H$ Betanin (**4**)

$R^1$ =  (malonyl) $R^2 = H$ Phyllocactin (**5**)

$R^1$ = malonyl $R^2$ =  (apiosyl) $R^3 = H$ 2'-*O*-Apiosylphyllocactin (**6**)

$R^1 = H$ $R^2$ = (apiosyl) $R^3$ =  (feruloyl) 2'-(5''-*O*-*E*-Feruloylapiosyl)betanin (**7**)

$R^1$ = malonyl $R^2$ = apiosyl $R^3$ = feruloyl 2'-(5''-*O*-*E*-Feruloylapiosyl)phyllocactin (**8**)

$R^1$ =  Hylocerenin (**9**)

Betacyanins are a class of compounds with antioxidant and radical scavenging activities (Pedreno and Escribano, 2000; Escribano et al., 1998; Kanner et al., 2001). It is known that they prevent oxidative processes, which contribute to the onset of several degenerative diseases in human. Since betanin exerts a good bioavailability, red beet products may provide protection against certain oxidative stress-related disorders (Kanner et al., 2001).

## 2. Chemistry

### 2.1. Isolation and structure elucidation

The methods recommended for analytical characterization, preparative isolation, photometric quantification and structure elucidation of betalains, the latter mainly by MS- and NMR-techniques, are comprehensively summarized in two reviews (Strack et al., 1993; Strack and Wray, 1994a).

Extraction of betalains from plant tissues or cell cultures is commonly performed with aqueous methanol; however, the addition of ascorbic acid (ca. 50 mM) in the extraction medium is recommended (Schliemann et al., 1999). It leads to a slightly acidic pH, which stabilizes betacyanins and inhibits possible oxidation by PPOs. When high tyrosinase activities and certain betaxanthins, e.g. miraxanthin V, are present as in case of hairy root cultures of yellow beets (Steiner et al., 1999), the addition of ascorbic acid is absolutely necessary. Otherwise, a fast and almost complete loss of miraxanthin V occurs, accompanied by the appearance of artefacts (Willibald Schliemann, unpublished). Conventional anion exchange column chromatography on Dowex 1X8, using a gradient of aqueous formic acid, is recommended for purification of highly concentrated betacyanin extracts (Strack et al., 1993). But this may result in complete degradation of unstable betacyanin conjugates as found in the isola-tion of the betacyanins from *Phytolacca americana* (Schliemann et al., 1996). In such cases gel filtration on Sephadex LH-20 can be

used, despite its low capacity and separation performance. Recently, high-speed countercurrent chromatography was applied to isolate betalains from red beet extracts (Degenhardt and Winterhalter, 2001).

Electrospray ionization tandem mass spectrometry (ESI-MS–MS) is the method of choice to identify structurally related pigments in mixtures (Heuer et al., 1992; Gläβgen et al., 1993). The protonated molecular ions $[M + H]^+$ of (iso)betanidin derivatives can be collectively identified by parent ion scans selecting the [betanidin + H]$^+$ fragment ion ($m/z$ 389) as daughter ion (Heuer et al., 1992). Although the parent ion scan technique is usually well-suited for the analysis of crude extracts (Gläβgen et al., 1993), a better signal-to-noise ratio is obtained after separation by HPLC coupled with MS–MS detection. In recent years, micro-HPLC systems using electrospray ionization and QTOF mass detection (Perkin Elmer Sciex, Foster City, USA) were introduced giving access to accurate masses ($\Delta < 5$ mDa) of molecular ions and fragments from which the elemental composition can be deduced. This technique has recently been used for the identification of betacyanins and betaxanthins from different *Celosia* species (Schliemann et al., 2001). Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR–MS) gives ultra-high-mass resolution (Amster, 1996) and could be very useful for the structure elucidation of betalains.

## 2.2. Betacyanins

### 2.2.1. Betanin group

*2.2.1.1. Phyllocactin (**5**).* This betacyanin (6′-*O*-malonyl-betanin) is known as the characteristic pigment in flowers and fruits of the Cactaceae (Piattelli and Imperato, 1969). The linkage of the malonyl moiety in phyllocactin to the 6-*O*-position of the glucose was deduced from permethylation analysis (Minale et al., 1966). This has recently been proven by $^1$H NMR of phyllocactin isolated from flowers of Christmas cactus (*Schlumbergera×buckleyi*) flowers (Fig. 1) (Kobayashi et al., 2000). Besides characteristic signals in the 1D and 2D $^1$H NMR data, confirming the presence of betanin, the low field chemical shifts of H-6′A/H-6-′B to 4.60 and 4.33 ppm, respectively, are clear evidence that the malonyl system is bound to C-6′ of the glucose moiety.

*2.2.1.2. 2′-Apiosyl-phyllocactin (**6**).* The second major betacyanin isolated from Christmas cactus flowers showed in LC-MS a protonated molecular ion at $m/z$ 769 in comparison to $m/z$ 637 for phyllocactin. The mass difference (132) suggested the presence of an additional pentose moiety. Carbohydrate compositional analysis of this new malonylated betacyanin confirmed the presence of glucose and the less common branched pentose, apiose, in a ratio of about 1:1. The linkage

Fig. 1. Flower of Christmas cactus (*Schlumbergera×buckleyi*).

between the two sugar moieties was established by methylation analysis according to 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. As in phyllocactin, the low field chemical shifts of H-6′A/H-6′B provides definitive evidence that the malonyl system is bound to C-6 of the glucose moiety. Hence, the combination of LC–MS data, sugar composition and linkage analysis as well as the results of the NMR measurements, identified this new cactus betacyanin as betanidin 5-*O*-(2′-*O*-β-D-apiofuranosyl-6′-*O*-malonyl)-β-D-glucopyranoside (**6**) (Kobayashi et al., 2000). Besides the two major malonylated betacyanins in the petal extract, trace amounts of less polar betacyanins have been detected, most of which contain a hydroxycinnamoyl moiety indicated by an absorption at 330 nm. One component was found to be identical (HPLC and UV–vis spectral data) with 5″-*O*-*E*-feruloyl-2′-*O*-β-D-apiosyl-betanin (**7**), a betacyanin identified earlier from *Phytolacca americana* (Schliemann et al., 1996). For an additional less polar compound, a similar structure as **7** was suggested from the almost identical spectral data, but in LC–MS the protonated molecular ion occurred at *m*/*z* 945 with a mass difference of 86 from **7** (*m*/*z* 859) indicative of the presence of an additional malonyl residue. Although final proof of the structure by $^1$H NMR spectroscopy and sugar linkage analysis was not possible due to limited material, the most plausible new structure is betanidin 5-*O*-[(5″-*O*-*E*-feruloyl)-2′-*O*-β-D-apiofuranosyl-6′-*O*-malonyl]-β-D-glucopyranoside (**8**) (5″-*O*-*E*-feruloyl-2′-*O*-β-D-apiosyl-phyllocactin). The assignment of this structure is also based on precedents: the fact that the linkage of the malonyl residues in both **5** and **6** was found to the 6-*O*-position of the glucose and the linkage of the feruloyl residue in **7** was found to the 5-*O*-position of the apiose (Schliemann et al., 1996) by $^1$H NMR. This new compound **8** 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) (see gomphrenin group).

*2.2.1.3. 2′-(5″-O-E-Feruloylapiosyl)-betanin (7).* In fruit extracts of *P. americana* (pokeberry) (Fig. 2) **4**, prebetanin (6′-*O*-hemisulphate of **4**), gomphrenin I (**10**) and lampranthin II (6′-*O*-*E*-feruloyl-betanin) were found, accompanied by a pair of isomeric acyl betacyanins in low amounts which were characterized by typical absorptions at 330 nm as hydroxycinnamoyl derivatives (Schliemann et al., 1996). Interestingly, these derivatives are the main betacyanins of the extract from epidermal layers of the stems, but their concentrations in the total extract are not sufficient for isolation and characterization. Cell cultures (callus and cell suspension) initiated from stem explants aquired the ability to produce these betacyanins as the main pigments (Sakuta et al., 1986). Therefore, extracts from suspension cultures were used for structure elucidation.

LC–MS of the purified betacyanins showed the presence of a protonated molecular ion ($[M+H]^+$) at *m*/*z* 859 with a characteristic mass difference of 132 from lampranthin II (6′-*O*-*E*-feruloyl-betanin) (*m*/*z* 727) indicating the presence of an additional pentose. Alkaline hydrolysis yielded ferulic acid, but hydrolysis under acidic conditions (50% formic acid) gave no trace of lampranthin II. Therefore the ferulic acid moiety should be attached to the proposed additional pentose. These assumptions have been completely confirmed by NMR and sugar analysis. Sugar composition and linkage analysis as described above detected apiose linked to C-2′ of glucose. The low field chemical shifts of H-5″A and H-5″B of the apiose moiety gave definite evidence that the *E*-feruloyl system is bound to C-5″ of this pentose.

Fig. 2. Fruit-bearing pokeberry (*Phytolacca americana*).

All data together identify the new betacyanins from *Phytolacca americana* as betanidin 5-*O*-[(5″-*O*-*E*-feruloyl-2′-*O*-β-D-apiofuranosyl]-β-D-glucopyranoside (**6**) and its 15*R*-isoform.

The iceplant (*Mesembryanthemum crystallinum*) (Fig. 3) was used as a model system to analyse UV-light induced betacyanin formation and simultaneous formation of flavonol conjugates (Ibdah, 2002; Ibdah et al., 2002). Besides the known **4** and lampranthin II, complex betacyanins (mono- and diferuloyl betanidin 5-*O*-diglucosides and diferuloyl betanidin 5-*O*-triglucoside) were found to accumulate after light stress in epidermal layers (Vogt et al., 1999b), but they were not further characterized concerning the attachment of the feruloyl moieties to the sugar molecules and the linkage of the glucoses in the di- and trisaccharides.

*2.2.1.4. Hylocerenin (9).* Some cacti form edible fruits, which are used for colouring drinks and ice cream. The pigments of the commercially used vine cactus *Hylocereus polyrhizus* have now been structurally elucidated (Wybraniec et al., 2001). The main betacyanins from fruit pulp were characterized as **4** and **5**, but the data obtained from the third pigment did not match with those from any other known betacyanin. In LC–MS, the protonated molecular ion was found at *m*/*z* 695, giving daughter ions of *m*/*z* 551 [betanin + H]$^+$ and *m*/*z* 389 [betanidin + H]$^+$. The mass difference of 144 to betanin indicated the presence of an additional residue possibly connected to the glucose moiety. The $^1$H NMR data were virtually identical to those of phyllocactin, except of the absence of the malonyl protons $H_2$-2″ at $\delta = 3.46$ ppm. Instead, an extra singlet of a methyl group at $\delta = 1.57$ ppm and two new methylene groups at $\delta = 2.89$ ppm (singlet of H-4″) and 2.71 ppm (AB quartet of H-2″A/H-2″B) were detected. The small chemical shift differences between H-7 and H-4 and the low field shift of H-6′A/H-6-′B are indicative for the presence of a 3-hydroxy-3-methylglutaryl moiety at C-6′ of betanin. Hence, the MS and $^1$H NMR data identified the new pigment as betanidin 5-*O*-[6′-*O*-(3″-hydroxy-3″-methylglutaryl)-β-D-glucopyranoside (**9**), called hylocerenin. A similar compound, amaranthin (2′-*O*-β-D-glucuronopyranosyl-betanin), acylated at C-6′ with 3-hydroxy-3-methylglutaric acid (iresinin I), has been isolated from *Iresine herbstii* (Amaranthaceae) (Minale et al., 1966)

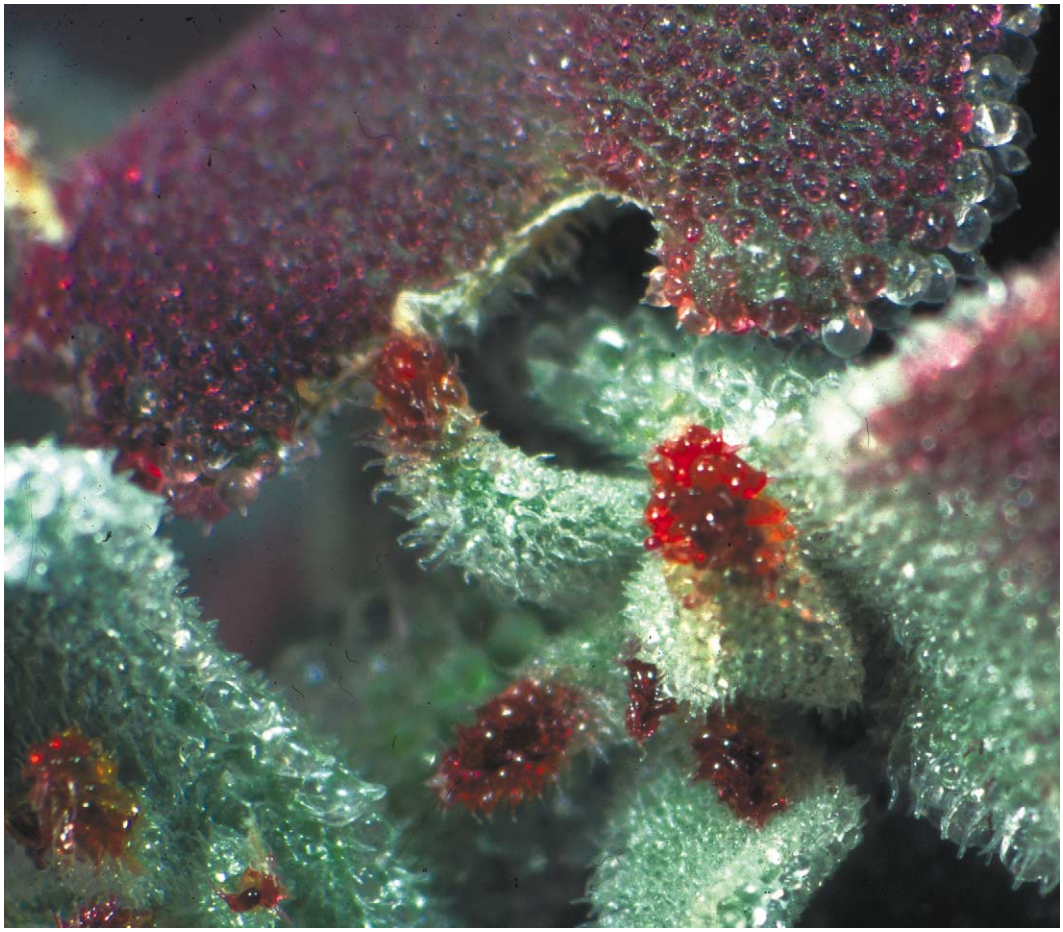


Fig. 3. Micrograph of bladder cells located on fully expanded leaves and leaf tips of the iceplant (*Mesembyanthemum crystallinum*) after irradiation with high light intensities.

and recently reinvestigated by LC–MS ($m/z$ 871, $[M+H]^+$) (Cai et al., 2001). Upon β-glucuronidase treatment, iresinin I yielded glucuronic acid and degradation products (Minale et al., 1966) containing most probably betanidin 5-*O*-[6′-*O*-(3″-hydroxy-3″-methylglutaryl)-β-D-glucopyranoside, an artificially formed compound that was now been shown to occur endogenously in *H. polyrhizus* (Wybraniec et al., 2001). In a parallel investigation on the same fruit material (red-purple pitaya) the presence of **4** and **5** as well as a betacyanin with the MS characteristics of **9** was shown (Stintzing et al., 2002a), but insufficient structural evidence was obtained for further betacyanins [betanidin 5-*O*-β-sophoroside, betanidin 5-*O*-(6′-*O*-3-hydroxybutyryl)-β-D-glucoside].

In contrast, in fruit juice of a cultivated Mexican cactus (*Stenocereus queretaroensis*) only **4**, **5** and **6** in addition to **3** were found (Eugenia Lugo and Willibald Schliemann, unpublished).

### 2.2.2. Amaranthin group

*2.2.2.1. Amaranthin.* This compound, the characteristic pigment in the Amaranthaceae, was recently analysed in extracts of red inflorescences of common cockscomb (*Celosia argentea* var. *cristata*) and orange-red inflorescences of feathered amaranth (*C. argentea* var. *plumosa*). The identification was corroborated by LC–MS of the purified compound giving the expected protonated molecular ion $[M + H]^+$ ($m/z$ 727). Furthermore, the collision induced dissociation (CID) mass spectrum of $m/z$ 727 gave the main fragments $m/z$ 551 [betanin + $H]^+$ and $m/z$ 389 [betanidin + $H]^+$ indicative for the presence of amaranthin. Furthermore, high-resolution LC-ESI-TOF–MS yielded $m/z$ 727.1913 ($C_{30}H_{35}N_2O_{19}$, calculated mass: 727.1829) completing the structure elucidation (Schliemann et al., 2001). In a survey on the distribution of free and acylated betacyanins in the Amaranthaceae, 37 species of eight genera were investigated (Cai et al., 2001). The pigments were characterized by HPLC and LC-ESI–MS as amaranthin (protonated molecular ion) ($m/z$ 727), iresinin I ($m/z$ 871), celosianin I ($m/z$ 872) and celosianin II ($m/z$ 903). Some cultivated species contained a higher percentage of acylated betacyanins than wild species, representing a potential new source of these pigments as natural colorants.

### 2.2.3. Gomphrenin group

*2.2.3.1. Gomphrenin I (**10**) II and III.* A group of closely related betacyanins, the gomphrenins, known as structural isomers of betanin, are the main pigments from the violet inflorescences of *Gomphrena globosa* L. (Piattelli and Minale, 1964; Minale et al., 1967). They have been completely characterized by NMR spectroscopy and MS techniques (Heuer et al., 1992). The structure of gomphrenin I was confirmed as betanidin 6-*O*-β-D-glucopyranoside, whereas the further pigments were shown to be the 6′-*O*-*E*-4-coumaroyl and 6′-*O*-*E*-feruloyl derivatives of gomphrenin I accompanied by the corresponding isoforms (15*R*). To simplify matters they were renamed as gomphrenin II [betanidin 6-*O*-(6′-*O*-*E*-4-coumaroyl]-β-D-glucopyranoside] and gomphrenin III [betanidin 6-*O*-(6′-*O*-*E*-feruloyl)-β-D-glucopyranoside] (Heuer et al., 1992). An interesting feature is that the $\lambda_{max}$ value of gomphrenin I (543 nm) changed to 550 nm for gomphrenin II and III. This bathochromic shift is larger than that observed between betanin ($\lambda_{max}$ 539 nm) and lampranthin II ($\lambda_{max}$ 543 nm). In NMR significant chemical shift differences, in particular for H-2 and H-14A/B, were observed between gomphrenin I and gomphrenin II/III. Such alterations cannot arise from through-bond effects caused by substitution at C-6 of the glucose moiety, but originate most likely from through-space intramolecular stacking interactions (Heuer et al., 1992). Effects of a similar magnitude have been reported for intramolecular copigmentation in monoacylated anthocyanins (Gläβgen and Seitz, 1992). The consequences of the intramolecular association between the betanidin and feruloyl moieties were studied by comparing the stability and racemization behaviour of betanin and amaranthin with those of the corresponding feruloyl derivatives, lampranthin II and celosianin II, under acidic conditions (Schliemann and Strack, 1998). Both acylbetacyanins showed a reduced racemization velocity. Celosianin II exhibits in addition an enhanced stability, the latter most likely caused by intramolecular stacking.

A similar set of compounds found in *G. globosa* were readily identified as gomphrenin I/II/III directly from fresh fruit juice of *Basella rubra* (Basellaceae) using ESI-MS-MS (Gläβgen et al., 1993). The presence of gomphrenins in fruits of this plant might be of chemotaxonomic interest, since so far betacyanins with C-6 substitution are rare and have only be identified from *G. globosa* (Amaranthaceae) (Heuer et al., 1992; Cai et al., 2001) and *Bougainvillea* (Nyctaginaceae) (Heuer et al., 1994).

*2.2.3.2. Betanidin 6-O-(6-O-hydroxycinnamoyl)-β-sophoroside derivatives.* The most complex pattern of betacyanins observed in plants containing more than 30 structures was detected by HPLC in methanolic extracts from bracts of *Bougainvillea glabra* (Fig. 4), and is composed mainly of hydroxycinnamoyl conjugates of **10** and derived compounds (Heuer et al., 1994). Electrospray tandem mass spectrometry (ESI-MS–MS) of crude betacyanin mixtures (direct inlet) and separation by HPLC coupled with MS–MS (parent ion mode) detected 16 precursor ions of $m/z$ 389 [betanidin + $H]^+$ which was in agreement with the HPLC results. Due to the complexity of the betacyanin pattern and the lability of the highly conjugated components, it was not

possible to obtain fractions containing only one compound, most of which were present in two isomeric forms each (15*S* and 15*R*). Nevertheless, 1D and in most cases 2D NMR identified the betanidin system from the low and high field doublet signals of H-11 and H-12, two three-spin systems corresponding to H-2/H-3A,B and H-14A,B/H-15 and finally three singlets corresponding to H-4, H-7 and H-18 (Heuer et al., 1992). The shift differences between H-4 and H-7 of ca 0.8 ppm indicated the presence of the sugar substituent at the C-6 hydroxyl of the betanidin system. As expected, signals for H-11 and H-12 of the 12 *Z*-isomer (<20%) were detected at lower and higher field, respectively, from those of the major 12 *E*-isomer. An unequal doubling of the 12 *E*-isomer signals arose from the presence of both normal (2*S*, 15*S*) and unusual isoforms (2*S*, 15*R*) of the betanidin moiety (Strack et al., 1988). In these hydroxycinnamic acid conjugates the aromatic systems and the *trans* olefinic protons gave characteristic signals. The 4-coumaroyl and the caffeoyl systems were located at C-6 of the glucose systems in all compounds. The linkage between sugar units was determined by GC–MS after permethylation showing that the only sugar identified was glucose, and that 1,2-linkages are present between all glucose units. Summarizing these data, the betacyanins were identified as **10**, betanidin 6-*O*-β-sophoroside (**11**), betanidin 6-*O*-(6′-*O*-*E*-caffeoyl)-β-sophoroside (**12**), betanidin 6-*O*-(6″-*O*-*E*-4-coumaroyl)-β-sophoroside (**13**), betanidin 6-*O*-(6′-*O*-*E*-4-coumaroyl)-β-sophoroside (**14**), betanidin 6-*O*-{2″-*O*-β-sophorosyl[(6′-*O*-*E*-caffeoyl)-(6″-*O*-*E*-4-coumaroyl)]}-β-sophoroside (**18**), betanidin 6-*O*-{2″-*O*-β-glucosyl[(6′-*O*-*E*-caffeoyl)-(6″-*O*-*E*-4-coumaroyl)]}-β-sophoroside (**17**), betanidin 6-*O*-[(2″-*O*-β-glucosyl)(6′,6″-di-*O*-*E*-4-coumaroyl)]-β-sophoroside (**16**), and betanidin 6-*O*-(6′,6″-di-*O*-*E*-4-coumaroyl)-β-sophoroside (**15**). The main features of the betacyanins from *Bougainvillea glabra* are the attachment of sophorose and oligomers at the C-6 hydroxyl of betanidin and the acylation with 4-coumaric and caffeic acids (mono- and diester linkages). Interestingly, other varieties e.g. *Bougainvillea* 'Mrs Butt' accumulate similarily acylated 5-*O*-β-sophorosides (Piattelli and Imperato, 1970), but the pigments could not be completely identified at that time.

Fig. 4. Flowering *Bougainvillea glabra*.

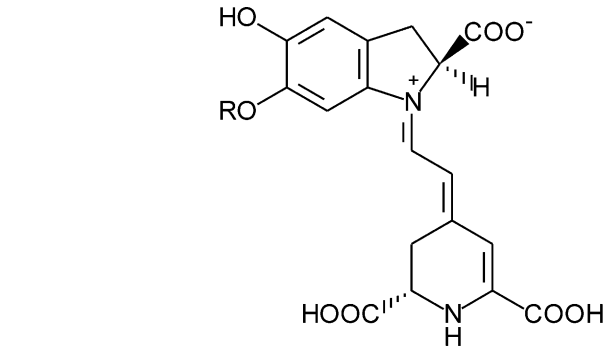
### 2.2.4. 2-Descarboxy-betanin group

*2.2.4.1. 2-Descarboxy-betanidin (**19**).* Betacyanins usually derive from *cyclo*-dopa. The only exception until now was the dopamine-derived **19**, a minor pigment in flowers of *Carpobrotus acinaciformis* (Aizoaceae) (Piattelli and Impellizzeri, 1970), a xerophilous plant native to South Africa. Recently, **19** has also been found in hairy root cultures from yellow beet (*Beta vulgaris* subsp. *vulgaris*; Garden Beet Group, 'Golden Beet') (nomenclature of cultivated plants according to Lange et al., 1999) (Schliemann et al., 1999) (Fig. 5). The identification was based on co-chromatography (HPLC) with a standard compound synthesized by tyrosinase-catalysed oxidation of dopamine to 2-descarboxy-*cyclo*-dopa and its condensation with betalamic acid. Furthermore, the identity as **19** ($\lambda_{max}$ 533 nm) was further confirmed with LC–MS analysis which showed the correct protonated molecular ion $[M+H]^+$ at *m*/*z* 345 (base peak) (Kobayshi et al., 2001).

*2.2.4.2. 2-Descarboxy-betanin (**20**).* The other co-occurring minor betacyanins of hairy root cultures from yellow beet had not been identified at that time (Schliemann et al., 1999) due to their low abundance. While administration of the precursors of betalains (tyrosine and derived compounds) to this hairy root culture led to melanin formation due to high tyrosinase activity (Steiner et al., 1999), the administration of dopamine to fodder beet seedlings led to the formation of betacyanins with retention and UV–vis spectral properties identical with those of the minor betacyanins in hairy roots. Feeding high amounts of dopamine to *Beta vulgaris* seedlings yielded sufficient amounts of betacyanins for isolation by preparative HPLC. A compound more polar than **19** was shown by LC–MS to be 2-descarboxy-betanin (**20**)

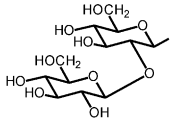
([M + H]$^+$ ion at $m/z$ 507). By NMR, the small chemical shift difference between H-4 and H-7 of 0.06 ppm was characteristic of substitution at the hydroxyl group at C-5 of the aglycone, as opposed to that at C-6 where differences of ca. 0.8 ppm are to be expected. The double doublet of H-2 at 5.46 ppm of betanidin was not detected, but was replaced by a two proton triplet at 4.40 ppm, which indicated the absence of the carboxyl group at the C-2 position as a consequence of the incorporation of dopamine into **20** (Kobayashi et al., 2001).

*2.2.4.3. 6′-O-Malonyl-2-descarboxy-betanin (**21**).* A further betacyanin from hairy root cultures from yellow beet was found to be less polar than **20**, indicating the possible presence of an acyl moiety. LC–MS analysis showed a [M + H]$^+$-ion at $m/z$ 593, which, in a daughter-ion scan, resulted in an intense peak at $m/z$ 345, indicating that the compound belongs to the dopamine-derived betacyanins. From the mass difference (86) ($m/z$ 593−507) the presence of a malonyl residue could be deduced. In fact, the presence of a dicarboxylic acid as acylating residue was confirmed by detection of three

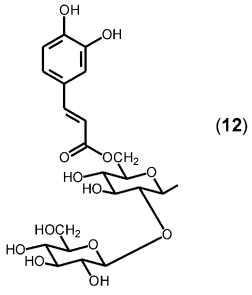



R =

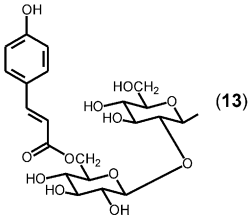
Betanidin 6-*O*-glucoside (Gomphrenin I) (**10**)



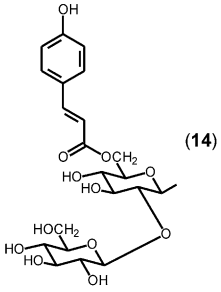
Betanidin 6-*O*-sophoroside (**11**)



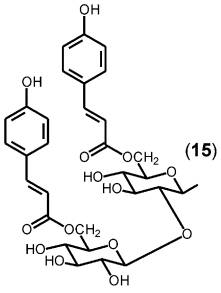
(**12**)



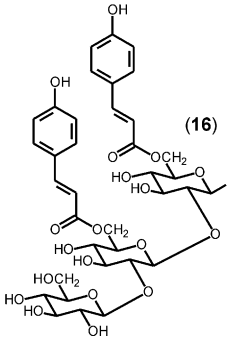
(**13**)



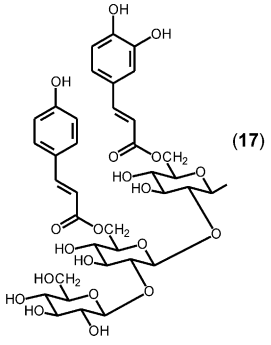
(**14**)



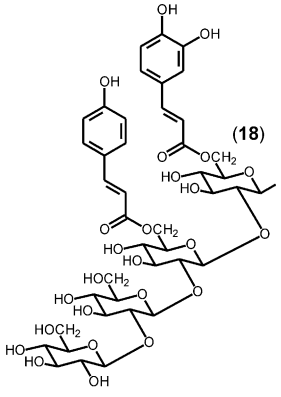
(**15**)



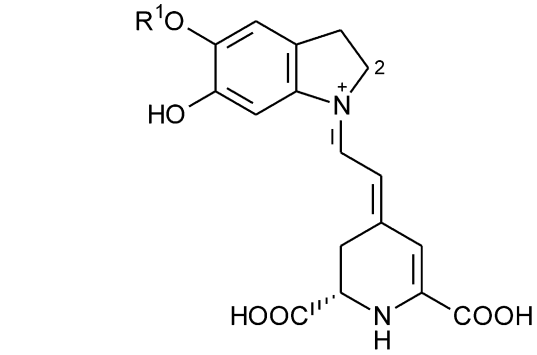
(**16**)



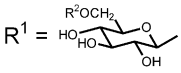
(**17**)



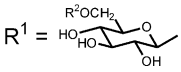
(**18**)

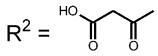


$R^1$ = H 2-Descarboxy-betanidin (**19**)



$R^2$ = H 2-Descarboxy-betanin (**20**)





6'-*O*-Malonyl-2-descarboxy-betanin (**21**)

consecutive losses of $CO_2$ (two from the betalamic acid part and one from the acyl moiety) in its ESI–MS (negative ion mode). The linkage of the malonyl residue at the 6-*O*-position of glucose was confirmed by the low field chemical shifts of H-6′A/H-6′B (4.62 and 4.32 ppm), respectively, which proves that the acyl system is bound to C-6′ of the glucose moiety. Hence, the combination of LC–MS and NMR data identified the pigment as 6′-*O*-malonyl-2-descarboxy-betanin [2-descarboxy-betanidin 5-*O*-(6′-*O*-malonyl-β-D-glucoside)] (**21**) (Kobayashi et al., 2001). Additionally, **5**, often the main pigment from flowers and fruits of Cactaceae (Piattelli and Imperato, 1969), was shown to co-occur in hairy root cultures of yellow beet (as evidenced by HPLC co-chromatography and LC–MS analysis). In earlier studies its occurrence was also observed in other members of the Chenopodiaceae, in leaves of *Kochia scoparia*, stems of *Salsola soda* and petioles of *Spinacia oleracea* (Steglich and Strack, 1990).

Fig. 5. Longitudinal section of a hairy root (one half) of yellow beets (*Beta vulgaris*). Note the different localization of the red betacyanins and yellow betaxanthins.

### 2.3. Betaxanthins

#### 2.3.1. Amino acid-derived conjugates

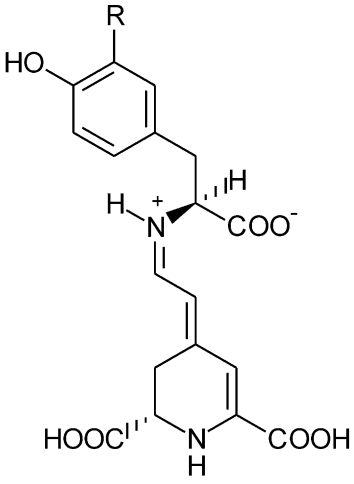
*2.3.1.1. Portulacaxanthin II (**22**).* Common portulaca (*Portulaca grandiflora*) (Fig. 6) accumulates betalains in stems and flowers. A variety of phenotypes exists with petal colours ranging from white to pale yellow, deep yellow, orange, red and red-violet. Portulacaxanthin (hydroxyproline-containing betaxanthin) (Piattelli et al., 1965) is accompanied in yellow flowers by a dozen other betaxanthins including vulgaxanthin I, dopaxanthin and miraxanthin V (Adachi and Nakatsukasa, 1983; Trezzini and Zryd, 1991b; Böhm and Böhm, 1996), of which two were additionally characterized. The major compound coeluted with semisynthetic tyrosine-betaxanthin (**22**) in HPLC and gave tyrosine on acid hydrolysis. Final proof was provided by $^1$H NMR. For this new betaxanthin the trivial name portulacaxanthin II was proposed (Trezzini and Zryd, 1991b). Although Piattelli changed the spelling of portulacaxanthin to portulax-

anthin (Piattelli, 1981), we propose the use of portulacaxanthin I for portula(ca)xanthin to avoid confusion.

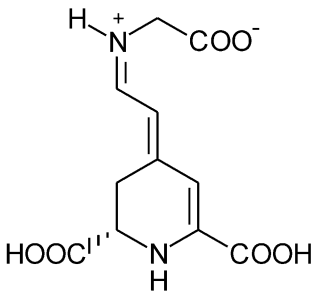
At the same time, **22** besides vulgaxanthin I, vulgaxanthin II, dopaxanthin (**23**) and miraxanthin V (**25**) was found to occur preferentially in orange callus cultures of *Beta vulgaris* var. bikores monogerm (Girod and Zryd, 1991b).

*2.3.1.2. Portulacaxanthin III (24).* A minor pigment from common portulaca flower petals coeluted with semisynthetic glycine-betaxanthin (**24**) in HPLC. The identity of the amino acid was confirmed by acid hydrolysis of the purified pigment giving glycine identified as phenylisocyanate derivative. In analogy, this new pigment was termed portulacaxanthin III (**24**) (Trezzini and Zryd, 1991b). The concentrations of **22** and **24** in deep yellow petals were determined to be 2.4 and 0.4 mg per g dry weight, respectively.

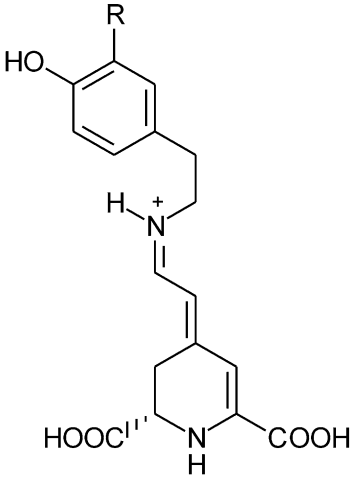
*2.3.1.3. Tryptophan-betaxanthin (27).* Until recently, there was a complete lack of knowledge about the betaxanthins in plants of the Amaranthaceae. Comparative analyses of the pigments of yellow and orange inflorescences of *C. argentea* var. *cristata* (common cockscomb) and *Celosia argentea* var.*plumosa* (feathered amaranth) (Fig. 7) gave identical betalain patterns with the presence of three betaxanthins, two of them being new structures (Schliemann et al., 2001). Besides the main compound **25**, a minor betaxanthin exhibiting an unpolar character eluted very late in RP-HPLC suggesting its structure might resemble that of the most unpolar betaxanthin, tryptophan-betaxanthin,



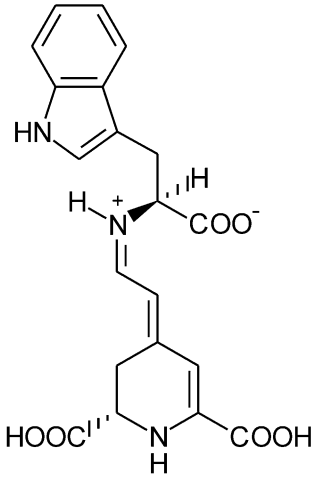
R = H Portulacaxanthin II (**22**) [(*S*)-Tyrosine-betaxanthin]
R = OH Dopaxanthin (**23**)



Portulacaxanthin III (**24**) (Glycine-betaxanthin)



R = OH Miraxanthin V (**25**) (dopamine-betaxanthin)
R = $OCH_3$ 3-Methoxytyramine-betaxanthin (**26**)



(*S*)-Tryptophan-betaxanthin (**27**)

synthesized as a reference compound (Trezzini and Zryd, 1991b; Schliemann et al., 1999; Stintzing et al., 2002b). The comparison of the endogenous compound with synthetic tryptamine-betaxanthin ($R_t$ 37.8 min; $\lambda_{max}$ 217/262/456 nm) and tryptophan-betaxanthin ($R_t$ 34.7 min; $\lambda_{max}$ 218/264/471 nm) revealed a complete match with the latter. Co-injection experiments and LC–MS fragmentation pattern confirmed the structure proposal. By ESI-TOF–MS the protonated molecular ion was found at $m/z$ 398.1327 ($C_{20}H_{20}N_3O_6$, calculated mass: 398.1347) completing the structure identification as tryptophan-betaxanthin (**27**), a new betaxanthin from plants.

*2.3.2. Amine-derived conjugates*

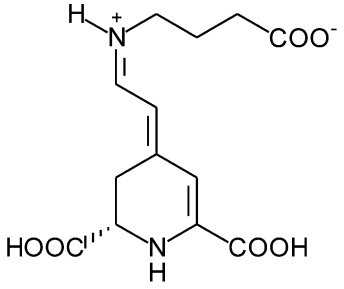
*2.3.2.1. 3-Methoxytyramine-betaxanthin (**26**).* The prevailing betaxanthin from yellow and orange inflorescences of common cockscomb and feathered amaranth, identified as miraxanthin V (dopamine-betaxanthin) (**25**), was accompanied by a less polar pigment with identical UV–vis spectral properties which indicates a similar structure. This lower polarity observed could be caused by the lack of a hydroxyl group in the aromatic amine moiety (tyramine instead of dopamine) or by modification of a hydroxyl group with an acyl or alkyl residue. The first proposal could be disproved since synthesized miraxanthin III (tyramine-betaxanthin) ($R_t$ 26.6 min; $\lambda_{max}$ 262/457 nm) did not coelute with the endogenous pigment. Subsequently, the betaxanthins derived from 4-methoxy-3-hydroxy-phenylethylamine and 3-methoxy-4-hydroxy-phenylethylamine (3-methoxytyramine) were prepared and used as reference compounds in co-injection experiments. Both compounds were identical with respect to UV–vis data ($\lambda_{max}$ 261/457 nm). Whereas 4-methoxy-3-hydroxy-phenylethylamine-betaxanthin eluted somewhat later than the endogenous betaxanthin, the 3-methoxy-4-hydroxy-phenylethylamine-betaxanthin (3-methoxytyramine-betaxanthin) showed complete co-elution. This chromatographical identity was supported by comparison of the mass spectral data of the standard compound with that of the endogenous compound. Both pigments showed in LC–MS a protonated molecular ion at $m/z$ 361 ($[M+H]^+$) indicative for a methyl derivative of miraxanthin V ($m/z$ 347). In ESI-TOF–MS the protonated molecular ion was found at $m/z$ 361.1417 ($C_{18}H_{21}N_2O_6$, calculated mass: 361.1394) confirming the structure elucidation of the *Celosia* pigment as 3-methoxytyramine-betaxanthin (**26**), the first methylated betaxanthin (Schliemann et al., 2001). Concerning its biosynthesis the question arose as to whether 3-methoxytyramine-betaxanthin is formed by methylation of the main compound **25** or by methylation of a biosynthetic intermediate. An indication for the latter option would be the detection of 3-methoxytyramine in the extract. The analysis of the extract from yellow inflorescences of common cockscomb with respect to the precursors of the identified betaxanthins showed that all three precursors, dopamine, 3-methoxytyramine and Trp could be detected. 3-Methoxytyramine was found to be present at low concentration, but in the same

Fig. 6. Flowering common portulaca (*Portulaca grandiflora*) with different petal colours.

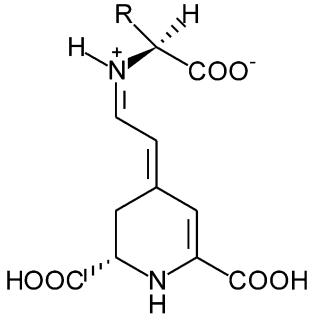
Fig. 7. Flowering common cockscomb (*Celosia argentea* var. *cristata*) (above) and feathered amaranth (*Celosia argentea* var. *plumosa*) (below).

order of magnitude as the derived **26**. Therefore, it is most probable that the methylation takes place at the catecholic level rather than at the betaxanthin level. In contrast to the relatively low concentration of 3-methoxytyramine and tryptophan, the precursor of **25**, dopamine, occurs in thousand-fold higher concentration than that of 3-methoxytyramine. Due to its various biological actions, these high dopamine concentrations found may be toxicologically relevant when yellow inflorescences are consumed as a vegetable or used as material in traditional Chinese medicine.

*2.3.2.2. Betaxanthins from cactus pear and yellow beets.* In fruits of cactus pear (*Opuntia ficus-indica*) seven pigments, besides the already known **3** and **4**, were identified by comparison with semisynthetic betaxanthin standards and LC–MS as immonium conjugates of betalamic with aspartic acid, γ-aminobutyric acid (**28**), serine (**29**), valine (**30**), leucine (**31**), isoleucine (**32**) and phenylalanine (**33**), the latter six were new in plants (Stintzing et al., 2002b). The same set of betaxanthins were found to occur in hypocotyls of the yellow beet (*Beta vulgaris* subsp. *vulgaris*; Garden Beet Group, 'Bejo Zaden'); additionally, **25**, already known from hairy root cultures of yellow beets (Schliemann et al., 1999) and **27**, previously identified in different *Celosia* species (Schliemann et al., 2001) were assigned. Through removal of both pectic substances and sugars by solid-phase extraction with $C_{18}$ sorbents a 40-fold enrichment of the betalain mixtures was possible with minor compounds reaching a sufficient concentration for MS detection (Stintzing et al., 2002b). The betaxanthin patterns of the original extracts were compared with the patterns in the concentrated extracts to rule out artificial



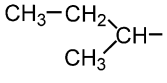
γ-Aminobutyric acid-betaxanthin (**28**)

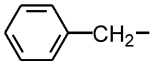


R = HO–$CH_2$– (*S*)-Serine-betaxanthin (**29**)

R = $(CH_3)_2CH$– (*S*)-Valine-betaxanthin (**30**)

R = $(CH_3)_2CH$–$CH_2$– (*S*)-Leucine-betaxanthin (**31**)

R =  (*S*)-Isoleucine-betaxanthin (**32**)

R =  (*S*)-Phenylalanine-betaxanthin (**33**)

betaxanthin formation. Using this methods, a more complex profile of betaxanthins in yellow beets and cactus pear than previously reported could be identified.

### 2.3.3. Semisynthetic structures

A first approach to identify betaxanthins is a comparison of their HPLC retention time and online UV–vis spectral data with those of authentic betaxanthins prepared by partial synthesis. Purified betanin was used as starting material for the partial synthesis of betaxanthins. After hydrolysis with 1.5 M $NH_4OH$ a 10- to 25-fold molar excess of the desired amino acid or amine was added, the solution was adjusted to pH 6 with HCl, concentrated to dryness and the formed betaxanthin purified by HPLC (Trezzini and Zryd, 1991a; Schliemann et al., 1999; Stintzing et al., 2002b) (Table 1). The simplest and most rapid procedure is the hydrolysis of commercial lyophilized red beet juice (containing racemic betanin) by aqueous ammonia solution (pH 11.3 for 30 min at room temperature), ethyl acetate extraction of the liberated racemic betalamic acid after acidification (by 5 N HCl under ice-cooling, pH 2) and its addition to different amino acids yielding the diastereoisomeric betaxanthins ([2*S,S*]- and [2*S,R*]-forms) (Schliemann et al., 1999). Twenty-one of 25 isomer pairs could be separated by two HPLC solvent systems. However, due to the racemic nature of the starting material, an isomer assignment of the separated peaks was not possible. Starting the partial synthesis with purified betanin:isobetanin mixtures (2:1), the betaxanthin isomers were obtained in the same ratio. The larger later eluting peaks of the isomer pairs corresponded to the (2*S,S*)-forms. When (*R*)-amino acids were used in parallel with the natural (*S*)-forms in betaxanthin biosynthesis, all four possible diastereoisomers (2*S,S*, 2*S,R*, 2*R,S*, 2*R,R*) were accessible. The separation experiments revealed that (2*S,S*)- and (2*R,R*)-isomers had identical retention times and, likewise, the (2*S,R*)- and (2*R,S*)-derivatives could not be separated. These standards were prepared to analyse amino acid feeding experiments, when endogenous (*S*)-betalamic acid reacts with the applied (*S*)- and (*R*)-amino acids giving distinct 2*S*,*S*- and 2*R*,*S*-betaxanthin HPLC signals (Schliemann et al., 1999) (Table 1). A further method for partial synthesis of betaxanthins includes the isolation of betalamic acid from fodder beet hypocotyl extracts as exemplified for the synthesis of reference compounds for the identification of betaxanthins from *Celosia* species (Schliemann et al., 2001) (Table 1).

## 3. Biochemistry

The biosynthetic steps involved in betalain biosynthesis are summarized in Fig. 8. While some 'early' and 'late' reactions are enzymatically catalysed, the intermediate steps (cyclizations, **X**–**XIII**; aldimine formation, **XIV**–**XVIII**) are assumed to proceed spontaneously, i.e. formation of *cyclo*-dopa via dopaquinone, betalamic acid via 4,5-*seco*-dopa, muscaflavin via 2,3-*seco*-dopa and the condensations of betalamic acid with *cyclo*-dopa (betanidin formation) or amino acids/amines (betaxanthin formation).

Early reactions are catalysed by the bifunctional tyrosinase (**EIA**, **EIB**) and the dopa 4,5- or 2,3-dioxygenase (**EII**, **EIII**), and late reactions by glucosyl-(**EIV**, **EV**), hydroxycinnamoyl-(**EVI**) and malonyltransferases (**EVII**). In addition, there are two rare enzymatic steps (decarboxylation and methylation, **EVIII**, **EIX**) leading to dopamine-derived betalains.

### 3.1. Tyrosinase

Tyrosinases are copper-containing PPO-type bifunctional enzymes, which catalyse hydroxylation of phenols to *o*-diphenols (EC 1.14.18.1; monophenol: monooxygenase) and their subsequent oxidation to *o*-quinones (EC 1.10.3.1.; *o*-diphenol:oxygen oxidoreductase) (Kaim and Rall, 1996). A tyrosinase involved in betalain biosynthesis has been described from common portulaca (*Portulaca grandiflora*) (Fig. 9) and red beet (*Beta vulgaris* subsp. *vulgaris*) (Steiner et al., 1996, 1999). The enzyme (**EIA**) hydroxylates tyrosine to dopa and oxidizes (**EIB**) dopa to dopaquinone. Subsequent cyclization (**X**) of dopaquinone to *cyclo*-dopa proceeds spontaneously. Analogous reactions (**EIB** and **XI**) are assumed to occur from dopamine to 2-descarboxy-*cyclo*-dopa. A tyrosinase from the pileus of fly agaric (*Amanita muscaria*) (Fig. 10) was also assumed to catalyse the early steps in the fun-gus-specific betalain biosynthesis (Mueller et al., 1996).

The involvement of tyrosinase activity in betalain biosynthesis was also indicated by detection of PPO transcripts correlating with betacyanin accumulation in *Phytolacca americana* fruits (Joy et al., 1995). Both deduced amino acid sequences contained the typical copper-binding domains characterized by histidine-rich regions. Based on the presence of a transit peptide sequence, one of the deduced polypeptides was assumed to be targeted to the internal lumen of plastidic thylakoids.

A betalain-specific tyrosinase has been partially purified from *P. grandiflora* by hydroxyapatite chromatography and gel filtration (Steiner et al., 1999). The enzyme appeared to be a monomer with an apparent molecular mass of 53 kDa. Highest hydroxylation activities were found when high salt concentrations (500 mM NaCl), 10 mM ascorbate and 10 μM $CuCl_2$ were included in the extraction buffer, 50 mM ascorbate and 0.1 mg per ml catalase were added to the assay mixtures and the solution saturated with $O_2$. The enzyme was highly specific for (*S*)-tyrosine with its (*R*)-isomer only accepted to about 20% of the rate with (*S*)-tyrosine.

Other potential monophenolic substrates were not hydroxylated.

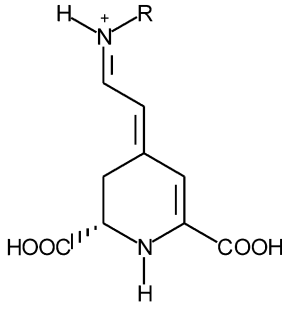
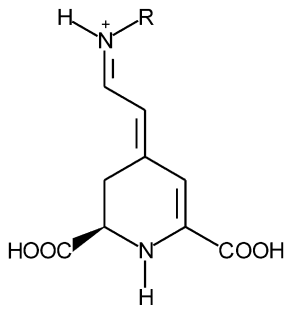
Interestingly, there are recent reports on a bifunctional PPO catalysing hydroxylation and oxidation of chalcones leading to aurones in snapdragon (*Antirrhinum majus*) flowers (Nakayama et al., 2000, 2001; Sato et al., 2001). Thus, bifunctional PPOs may be involved in the biosynthesis of two completely different classes of plant pigments, aurones and betalains (Strack and Schliemann, 2001). Earlier work on these tyrosinases had focused on their roles in the formation of complex polyphenols in bacteria, fungi, plants and animals and their putative role in defense reactions.

### 3.2. Dopa dioxygenase

The formation of betalamic acid in plants and muscaflavin in fungi demands extradiolic 4,5- and 2,3-cleavage of dopa, catalysed by dioxygenases (**EII** and **EIII**). The products, 4,5- and 2,3-*seco*-dopa, are assumed to recyclize spontaneously (**XIII**, **XII**) to betalamic acid and muscaflavin, respectively. So far, the 4,5-dopa dioxygenase in plants could not be described. However, the dopa dioxygenase in fungi has been detected in *Amanita muscaria* by two independent groups (Girod and Zryd, 1991a; Terradas and Wyler, 1991a). The fungal dioxygenase was cloned (Hinz et al., 1997) and functionally expressed in *Escherichia coli* (Mueller et al., 1997b). Unexpectedly, the 26 kDa enzyme catalysed both the 2,3- and the 4,5-cleavage. In an earlier publication (Kishima et al., 1992) the correlation of a 27 kDa protein on SDS-PAGE with colour formation in petals of near isogenic *Portulaca* lines was reported. No sequence information, however, was obtained, and speculations that this might be a candidate for the plant 4,5-dopa dioxygenase have to await more molecular evidence.

A cDNA clone encoding the fungal dopa dioxygenase was expressed in white *P. grandiflora* petals, using the particle bombardment technique (Mueller et al., 1997a). The appearance of red-violet (betacyanins) and yellow (betaxanthins/muscaflavin) cells proved that the recombinant protein maintained its specificity for 4,5- and 2,3-cleavage in the plant cell environment indicating

Table 1
Semisynthetic betaxanthins and isobetaxanthins

| | Amino acids/amines | References |
|---|---|---|
| Betaxanthins | | |
|  | Gly, β-alanine, γ-aminobutyric acid | Trezzini and Zryd, 1991a; Schliemann et al., 1999; Stintzing et al., 2002b |
| | *(S)-Amino acids* | |
| | His, Lys, Asn, Ser, Arg, $(Cys)_2$, Gln, Asp, Hyp, Thr, Glu, Ala, Pro, dopa, Tyr, Val, Met, Ile, Leu, Phe, Trp, Orn, citrulline, 4-thiaproline, methioninsulfoxide, | Trezzini and Zryd, 1991a; Schliemann et al., 1999; |
| | 5-hydroxytryptophan, norvaline, norleucine | Stintzing et al., 2002b |
| | *Amines* | |
| | dopamine, tyramine, (*S*/*R*)-octopamine, histamine, epinephrine, norepinephrine, serotonin, phenylethylamine, 3-methoxytyramine, 4-methoxy-3-hydroxyphenyl- ethylamine, tryptamine, taurine | Schliemann et al., 1999, 2001; Stintzing et al., 2002b |
| | *(R)-Amino acids* | |
| | Glu, Val, Phe, Trp | Schliemann et al., 1999 |
| *Isobetaxanthins* | | |
|  | *(S)-Amino acids* | |
| | His, Lys, Asn, Ser, Arg, $(Cys)_2$, Gln, Asp, Hyp, Thr, Glu, Ala, Pro, dopa, Tyr, Val, Met, Ile, Leu, Phe, Trp, citrulline | Schliemann et al., 1999 |
| | *(R)-Amino acids* | |
| | Glu, Val, Phe, Trp | Schliemann et al., 1999 |

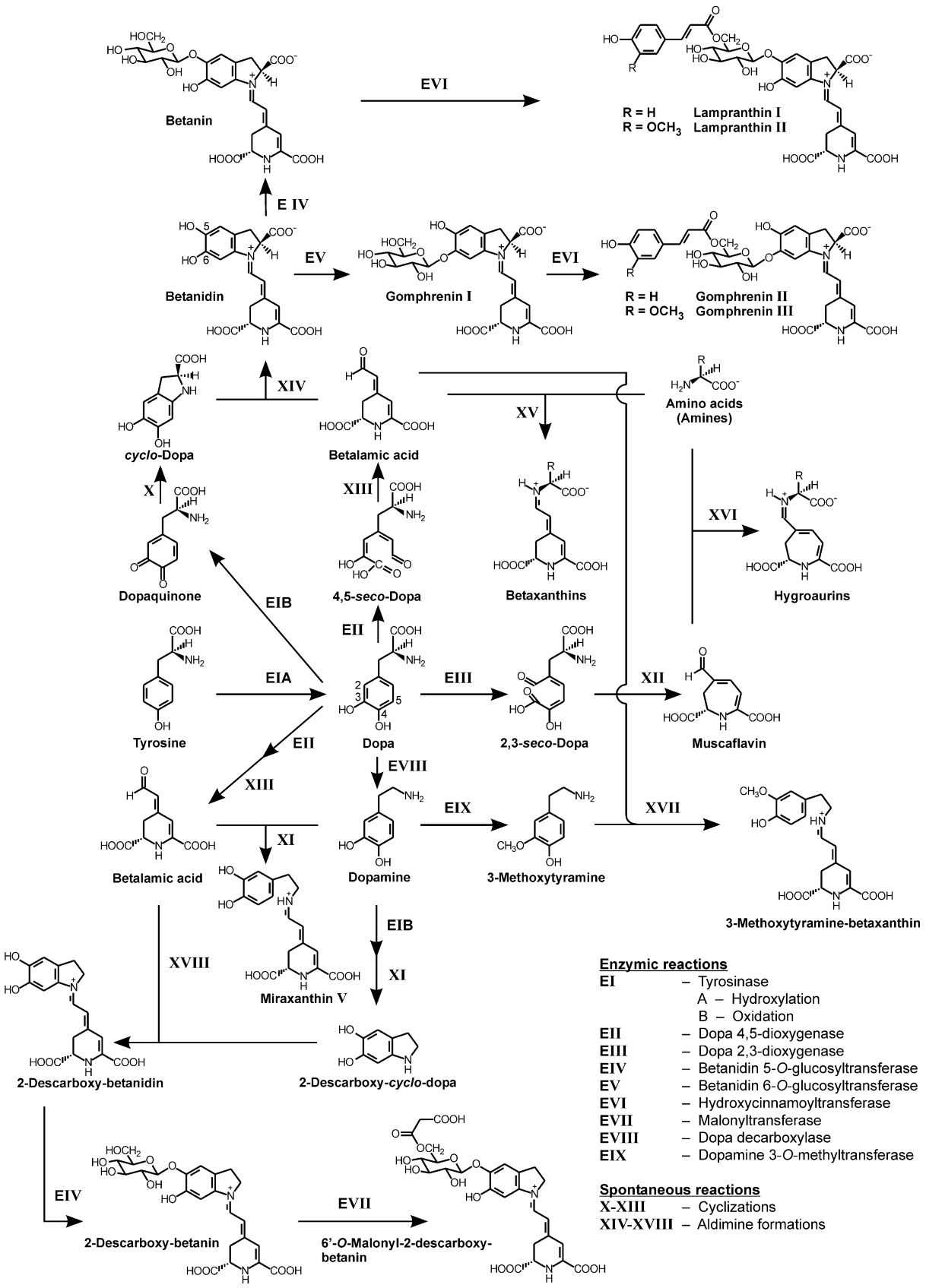



Fig. 8. Biosynthetic scheme of betacyanin and betaxanthin formation.

the difference in the specificity of the fungal and plant enzymes. Both proteins are most likely encoded by completely different genes.

The fungal dioxygenase is encoded by a single copy gene, which does not share any significant sequence similarities to other dioxygenases. Attempts to detect the plant enzyme by using antibodies directed against the fungal dioxygenase failed. The corresponding plant gene could therefore also not be identified by homology screening or by a substractive library approach from a variety of tissues (Zaiko, 2000). Failure to detect the plant dopa dioxygenase gene by molecular approaches and the different dopa cleaving activity of the fungal enzyme may indicate an early and independent evolution of the plant enzyme, apart from the respective fungal activity.

Fig. 9. Flowering common portulaca (*Portulaca grandiflora*) and a derived callus culture.

In a two-step model assay system, with dopa as substrate, using a dioxygenase preparation from *A. muscaria* and the tyrosinase from *P. grandiflora* callus subsequently, the formation of betanidin could be demonstrated in vitro (Schliemann et al., 1998). 2,3- and 4,5-*Seco*-dopas were shown to occur as natural pigments in *Hygrocybe conica* and *A. muscaria* (Terradas and Wyler, 1991b). Their identification was based on direct HPLC comparison with a mixture of authentic standards generated enzymatically from dopa and confirmed by their conversion to their cyclized successors, muscaflavin and betalamic acid, respectively.

### 3.3. Dopa decarboxylase

The occurrence of dopamine-derived betacyanins (**19**–**21**) and betaxanthins (**25**, **26**) implies the involvement of enzymes catalysing the decarboxylation of either tyrosine (with subsequent hydroxylation) or dopa. In protein extracts from cell cultures of red beet, an enzyme (**EVIII**) was detected that decarboxylates dopa to dopamine (pH 7.0, 4 mM dopa, 40 μM pyridoxal phosphate) (Terradas, 1989). In similar experiments with crude protein extracts from hairy root cultures of yellow

Fig. 10. Fly agaric (*Amanita muscaria*).

beets, a high conversion of dopa to dopamine was found, but tyrosine was not accepted as a substrate pointing to the presence of a dopa decarboxylase (Alfred Baumert and Willibald Schliemann, unpublished).

### 3.4. Aldimine formation

In extensive studies in our laboratory, all attempts to detect enzyme activity catalysing the decisive step in betalain biosynthesis, aldimine formation, failed. But a series of amino acid feeding experiments (Schliemann et al., 1999) supported the suggestion of spontaneous in vivo betaxanthin and betanidin formation (**XIV**–**XVIII**) (Trezzini and Zryd, 1990; Hempel and Böhm, 1997).

(i) Feeding *S*- and *R*-forms of various amino acids to hairy roots of yellow *Beta vulgaris*, which accumulates high amounts of betalamic acid, resulted in the formation of the respective betaxanthins. Thus, betaxanthin synthesis is neither amino acid specific nor stereoselective to *S*- and *R*-forms This was also observed by feeding unnatural amino acids, such as (*S*)-4-thiaproline.

(ii) Spontaneous betaxanthin formation could also be demonstrated by raising the endogenous concentration of amino acids in hairy roots of *Beta vulgaris*. Increasing the phenylalanine level by feeding the phenylalanine ammonia-lyase (PAL) inhibitor 2-aminoindan 2-phosphonic acid (Zoń and Amrhein, 1992) led to accumulation of the phenylalanine-derived betaxanthin. Increasing the glutamine level after feeding ammonium sulfate or alanine led to the formation of glutamine-derived betaxanthin, as a consequence of the actions of glutamine synthetase and alanine:2-oxoglutarate aminotransferase, respectively.

(iii) Feeding betalamic acid to de-rooted broad bean seedlings, a dopa-accumulating plant which does not belong to the Caryophyllales, resulted in the formation of dopaxanthin, the condensation product of betalamic acid with dopa.

(iv) Additional evidence that betanidin is formed spontaneously came from feeding experiments with *cyclo*-dopa to seedlings of yellow *Beta vulgaris*. Betalamic acid accumulating in this plant reacts with the administered *cyclo*-dopa to form betanidin, which is glucosylated to betanin. Whether there is also spontaneous aldimine formation (**XVI**) in fungi (e.g. synthesis of hygroaurines) awaits future studies.

Trezzini and Zryd (1990) postulated from a genetic approach that spontaneous betaxanthin formation occurs in the plant vacuole but betanidin formation in the cytoplasm. They described a gene locus that correlates with inhibition of betaxanthin formation, and was suggested to be related to transport of betaxanthins into the vacuole. However, vacuolar aldimine formation raises the question, how plants achieve specific betaxanthin patterns (Steglich and Strack, 1990), irrespectively of the presence of high amounts of individual soluble amino acids and amines (Willibald Schliemann and Dieter Strack, unpublished).

### 3.5. Glucosyltransferases

There are two regiospecific betanidin glucosylating enzymes, identified from *Dorotheanthus bellidiformis* cell cultures (Fig. 11) (Heuer and Strack, 1992; Heuer et al., 1996). UDP-glucose:betanidin 5-*O*- and 6-*O*-glucosyltransferases (5-GT and 6-GT) (**EIV**, **EV**) catalyse the formation of the isomeric **4** and **10**. Both enzymes were partially purified and shown to catalyse the indiscriminate transfer of glucose from UDP-glucose to hydroxyl groups of betanidin, flavonols and anthocyanidins, but these enzymes discriminate between individual hydroxyl groups of the respective substrates (Vogt et al., 1997). Besides betanidin, the 5-GT catalyses the glucose transfer to the C-4′ hydroxyl group of quercetin, and the 6-GT to the C-3 hydroxyl group of cyanidin with high efficiency.

It has been proposed that **4** could be formed by GTs acting at the *cyclo*-dopa level, followed by condensation with betalamic acid. This was indicated by the detection of considerable amounts of *cyclo*-dopa glucoside (up to 46% relative to the betanin content) in young red beet plants (Wyler et al., 1984). However, all attempts in our laboratory to reproduce this finding were unsuccessful (Willibald Schliemann, unpublished). Only traces of *cyclo*-dopa 5-*O*-glucoside were found, liberated from betanin that is in equilibrium with its hydrolytic products, *cyclo*-dopa glucoside and **1**. In addition, it was not possible to detect an enzyme or a recombinant gene product that catalyses the glucosylation of *cyclo*-dopa (Judith Hans and Thomas Vogt, unpublished). Thus, the glucose transfer reaction in betacyanin biosynthesis is most likely the C-5 or C-6 glucosylation of betanidin.

The unexpected flavonoid acceptor specificity of the betanidin 5-GT and 6-GT raises an interesting hypothesis. It indicates that these enzymes might be phylogenetically related to flavonoid GTs (Vogt et al., 1999a), and one might speculate that the 5-GT and the 6-GT are derived from distinct yet unknown ancestors with a broad substrate specificity, but a precise regiospecificity for particular hydroxyl groups of flavonoids and/or other hydroxylated phenylpropanoids. Whereas the 5-GT may have been derived from a cluster of enzymes glycosylating vicinal dihydroxyl groups, e.g. present in the B-ring of quercetin or luteolin, the 6-GT may be regarded as a member of a new cluster of anthocyanidin 3-*O*-GTs.

Fig. 11. Flowering Livingstone daisy (*Dorotheanthus bellidiformis*) and a derived suspension culture.

The two betanidin GTs show only 15% amino acid sequence identity (Vogt, 2002), much less than would be expected if both enzymes were derived from a common ancestor. In a phylogenetic dendrogram, based on the CLUSTAL W multiple sequence alignment (Thompson et al., 1994) both GTs are grouped into two different poorly characterized GT clusters within the large GT superfamily (Fig. 12). It is interesting to note that the 6-GT in this cluster is not correlated with the specific anthocyanidin/flavonoid 3-*O*-GT cluster, but is most homologous to a group of enzymes from *Nicotiana tabacum* with a broad specificity for flavonols, like kaempferol and a variety of xenobiotics (Taguchi et al., 2001). Identities on the amino acid level within each of these four clusters are around 50%, supporting the idea of conserved position specificity, but also suggesting that the betanidin GTs have evolved separately. The origin of both GTs can therefore be described as paraphyletic. This implies that betacyanins appeared later in evolution than the anthocyanins. In an ongoing debate on the origin of betalain accumulation (Stafford, 1994), the recent data on betanidin GTs corroborate the suggestion of Clement and Mabry (1996), that both classes of pigments have co-occurred in hypothetical ancestors.

### 3.6. Hydroxycinnamoyltransferases

Acylation (**EVI**) of betanidin glycosides with hydroxycinnamic acids has been described from eight members of four different families within the Caryophyllales to proceed via hydroxycinnamoylglucoses (β-acetal esters) as acyl donors (Bokern et al., 1992). This is supported by the finding that hydroxycinnamoyl betacyanins are always accompanied by their respective 1-*O*-acylglucosides (Strack et al., 1990). It has not yet been possible to show the acceptance of the alternative acyldonors, the hydroxycinnamoyl-CoA thioesters, in betacyanin acylation. This is in contrast with studies on the common thioester-dependent hydroxycinnamoyl transfer reactions involved in flavonoid biosynthesis (Strack and Wray, 1994b; Fujiwara et al., 1998; Yonekura-Sakakibara et al., 2000). However, the β-acetal esters may also be accepted as substrates in flavonoid biosyntheses as shown for the formation of cyanidin hydroxycinnamoyl-triglycosides in *Daucus carota* (Gläβgen and Seitz, 1992).

Some recent papers revealed that 1-*O*-acylglucoside-dependent acyltransferases might have evolved from serine carboxypeptidases. This was reported for the synthesis of dibutyrylglucose in *Lycopersicon pennellii*

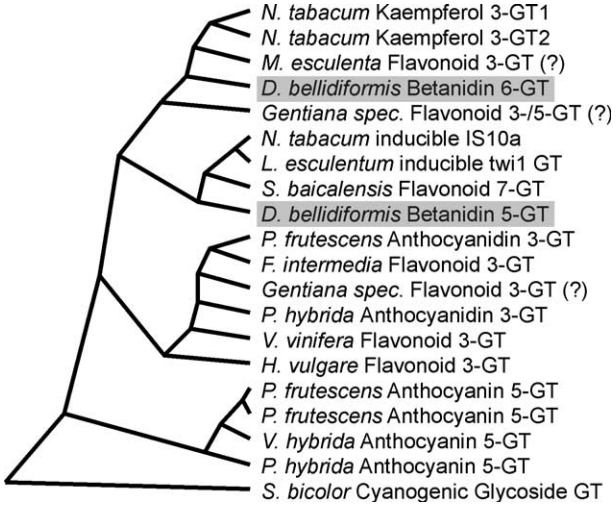



Fig. 12. Unrooted cladogramm of betanidin 5- and 6-GT among a variety of related amino acid sequences from different plant species. The *Sorghum bicolor* dhurrin forming GT (Jones et al., 1999) is used as an outgroup to illustrate the close similarity of the other selected sequences [*Sorghum bicolor* Cyanogenic glycoside GT (AAF17077); *Petunia hybrida* Anthocyanin 5-GT (AB027455); *Verbena hybrida* Anthocyanin 5-GT (BAA36423); *Perilla frutescens* Anthocyanin 5-GT1 (AB013596); *Perilla frutescens* Anthocyanin 5-GT2 (AB013597); *Hordeum vulgare* Flavonoid 3-GT (X15697); *Vitis vinifera* 3-GT (AF000371); *Petunia hybrida* Anthocyanidin 3-GT (AB027454); *Gentiana* spec., Flavonoid 3-GT(?) (D85186); *Forsythia intermedia* Flavonoid 3-GT (AAD21086); *Perilla frutescens* anthycyanidin 3-GT (AB002818); *Dorotheanthus bellidiformis* Betanidin 5-GT (Y18871); *Scutellaria baicalensis* Flavonoid 7-GT (BAA83484); *Lycopersicon esculentum* inducible twi 1 GT (X85138); *Nicotiana tabacum* inducible Is10a (U32643); *Gentiana* spec. Flavonoid 3-/5-GT (?) (E15917); *Dorotheanthus bellidiformis* Betanidin 6-GT (AF374004); *Manihot esculenta* Flavonoid 3-GT (?) (X 77459); *Nicotiana tabacum* Kaempferol 3-GT1 (AB052557); *Nicotiana tabacum* Kaempferol 3-GT2 (AB052557)].

(Li and Steffens, 2000) and sinapoylmalate in *Arabidopsis thaliana* (Lehfeldt et al., 2000; Hause et al., 2002). Thus, it will be an interesting task to clone some of the hydroxycinnamoyltransferases involved in betalain biosynthesis, which may possibly also be related to serine carboxypeptidase-like proteins.

### 3.7. Degradation

In contrast to the progress in studies of betalain biosynthetic enzymes, conclusive proof of the nature of betalain degrading enzymes is lacking. The first substantial evidence for the enzymatic decolouration of betanin came from studies with protein fractions from red beet (Soboleva et al., 1976). A membrane-associated fraction degraded betanin in an oxygen-dependent reaction (pH optimum 3.4); its inhibition by metal chelators suggested the involvement of a PPO. Later it was shown that this activity (same source) also decolourises betaxanthins (Shih and Wiley, 1981; Zakharova et al., 1988, 1995; Zakharova and Petrova, 1996). On the other hand, cell wall fractions from red beet catalysed the $H_2O_2$-stimulated decolouration of betanin suggesting the participation of a peroxidase (Wasserman and Guilfoy, 1983, 1984).

These decolourising enzyme activities are not restricted to red beets, but were also detected in *Amaranthus* species (Elliott et al., 1983; Zakharova et al., 1995) and in pokeweed leaves (*Phytolacca americana* L.) (Kumon et al., 1990). It has been shown that the betanin decolourising enzyme activity of callus cultures of *Portulaca grandiflora* was inhibited by ascorbic acid, which blocks the oxidising activity of PPOs, as well as the β-glucosidase inhibitor β-gluconolactone. Therefore, it was proposed that a hitherto uncharacterized betacyanin decolourising enzyme is composed of a betanin hydrolysing β-glucosidase and a PPO. Only one of two detected β-glucosidases from this plant material exhibited high betanin deglucosylating activity (Willibald Schliemann, unpublished). Recently, β-glucosidases have been detected in roots and leaves of red beet catalysing the hydrolysis of betanin (Zakharova and Petrova, 2000). Although the involvement of catabolic enzymes in regulation of betalain metabolism is still an open question, their presence must be taken into consideration when studying betalain metabolism and preparing betalain extracts.

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