Contributions to Betalain Biochemistry

New structures, condensation reactions, and vacuolar transport

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Publications

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Betalains from Christmas cactus (*Schlumbergera* x *buckleyi*)
XXth International Conference on Polyphenols, Freising-Weihenstephan, September 11-15, 2000

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SCHLIEMANN, W., KOBAYASHI, N., STEINER, U., STRACK, D.
Non-enzymic steps in betalain biosynthesis
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Enzymic and molecular analysis of betalain biosynthesis
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Metabolic formation and occurrence of dopamine-derived betacyanins
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A new biosynthetic pathway leading to dopamine-derived betacyanins
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KOBAYASHI, N., SCHMIDT, J., NIMTZ, M., WRAY, V., SCHLIEMANN, W.
Betalains from *Schlumbergera* x *buckleyi*
Meeting „Biosynthesis and Accumulation of Secondary Products", Martin-Luther- University Halle-Wittenberg, Halle (Saale), September 24-27, 2000

SCHLIEMANN, W., KOBAYASHI, N., STEINER, U.S., VOGT, T., STRACK, D.
Advances in betalain biosynthesis of higher plants
Meeting „Biosynthesis and Accumulation of Secondary Products", Martin-Luther- University Halle-Wittenberg, Halle (Saale), September 24-27, 2000

Content

Abbreviations

ABC transporter	ATP-binding cassette transporter
AIP	2-Aminoindan 2-phosphonic acid
ATP	Adenosine 5'-triphosphate
AU	Absorbance unit
BSA	Bovine serum albumin
2,4-D	2,4-Dichloro-phenoxyacetic acid
2-D-*cyclo*-D	2-Descarboxy-*cyclo*-Dopa
Dopa	3,4-dihydroxyphenylalanine
DNB-GS	Dinitrobenzene glutathione
DTT	DL-Dithiothreitol
EDTA	Ethylenediamine tetraacetic acid
EGTA	Ethylene glycol bis (2-aminoethyl)-tetraacetic acid
ESI	Electrospray ionization
FW	Fresh weight
GS	Glutathione
Hepes	*N*-(2-Hydroxyethyl)-piperazine-*N*'-2-ethane sulfonic acid
HPLC	High performance liquid chromatography
HRC	Hairy root culture
KPi	Potassium phosphate buffer
LC-MS	Liquid chromatography-mass spectrometry
MS	Mass spectrometry
Mes	2-[*N*-Morpholino]-ethane sulfonic acid
β-NADP	β-Nicotinamide adenine dinucleotide phosphate
NMR	Nuclear magnetic resonance
PAL	Phenylalanine ammonia-lyase
PDA	Photodiode array
pers. commun.	Personal communication
Prep. HPLC	Preparative HPLC
rpm	Revolution per minute
R_t	Retention time
ThiaPro	(*S*)-4-Thiaproline
Tris	Tris (hydroxymethyl)-aminomethane
UDPG	Uridindiphosphate-glucose

1 Introduction

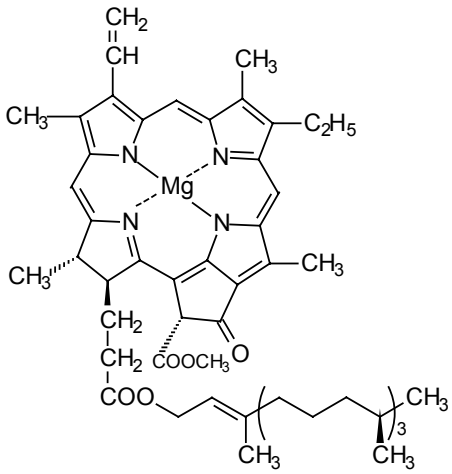
1.1 Plant pigments

The different colours which can be observed in nature are due to the presence of pigments. The pigments occur in leaves, flowers and fruits; they are also present in skin, eyes and other animal structures; and in bacteria and fungi. Natural and synthetic pigments are used in medicines, foods, clothes, furniture, cosmetics and in other products. However, natural pigments, such as chlorophylls, carotenoids, hemoglobin, myoglobin, flavonoids, quinones and melanins, have important functions and are produced by living organisms such as plants, animals, fungi, and microorganisms. They can be classified by their structural characteristics as follows: tetrapyrrole derivatives (chlorophylls and heme colours), isoprenoid derivatives (carotenoids), *N*-heterocyclic compounds different from tetrapyrroles (purines, pterins, flavins, phenazines, phenoxazines and betalains), benzopyran derivatives (anthocyanins and other flavonoid pigments), quinones (benzoquinone, naphthoquinone, anthraquinone), melanins (Fig. 1.1) (Delgado-Vargas *et al*., 2000). Important plant pigments are anthocyanins, flavonoids, carotenoids and betalains for the coloration of flowers and fruits. The physiological functions of plant pigments are antioxidant, UV protection, pollinator attraction and seed distribution. In contrast to well-known biosynthetic pathways of anthocyanins and carotenoids, there are open questions in the biosynthesis of betalains.

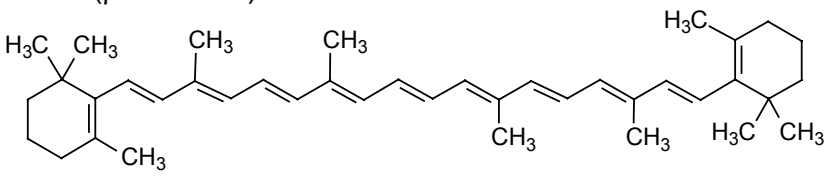
1.2 Betalains

Betalains consisting of the red-violet betacyanins and the yellow betaxanthins, are characteristic water-soluble, nitrogen-containing pigments which accumulate in flowers, fruits and occasionally in vegetative tissue of most families of the plant order Caryophyllales with the exception of the Caryophyllaceae and the Molluginaceae (Steglich and Strack, 1990). Thus the order Caryophyllales is divided into two suborders: The plants of the suborder Chenopodiineae contain betalains, but not anthocyanins, whereas in the suborder of Caryophyllineae anthocyanins occur but betalains are lacking. Table 1.1 shows the classification of Chenopodiineae and Caryophyllineae. There is a mutual exclusion of betalains and anthocyanins in the plant kingdom (Kimler *et al*., 1970). The Caryophyllales accumulating the betalains is one of the most prominent examples of the chemotaxonomic relevance of plant secondary products. In contrast to anthocyanins, betalains were also detected in some fungi, e.g. *Amanita muscaria* (Steglich and Strack, 1990), whereas anthocyanins occur in all species of the Angiosperms.

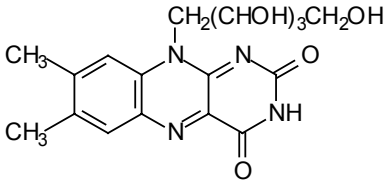
Tetrapyrrole derivatives
(Chlorophyll a)



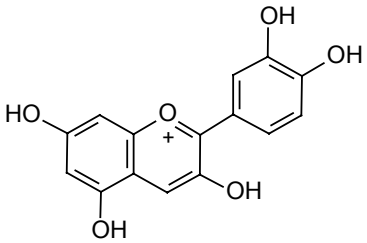
Isoprenoid derivatives
(β-Carotene)



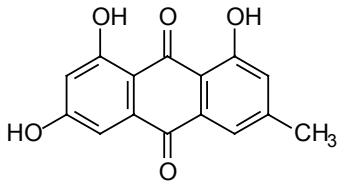
N-Heterocyclic compounds
different from tetrapyrroles
(Riboflavin)



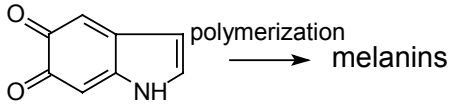
Benzopyran derivatives
(Cyanidin)



Quinones
(Emodin)



Melanins



indole 5,6-quinone

Fig. 1.1 Pigment groups and typical representatives (Delgado-Vargas *et al*., 2000)

Table 1.1 Classification of Caryophyllales (Clement and Mabry, 1996)

Suborder	Family	Examples of genus
Chenopodiinae (Betalain-producing anthocyanin-free taxa)	Achatocarpaceae	*Achatocarpus*
	Aizoaceae	*Dorotheanthus*
		Mesembryanthemum
	Amaranthaceae	*Amaranthus, Celosia, Gomphrena*
	Basellaceae	*Basella*
	Cactaceae	*Mammillaria, Opuntia, Schlumbergera*
	Chenopodiaceae	*Beta, Chenopodium, Salicornia, Spinacia*
	Didiereaceae	*Decaryia, Didierea*
	Halophytaceae	*Halophytum*
	Hectorellaceae	*Hectorella*
	Nyctaginaceae	*Bougainvillea, Mirabilis*
	Phytolaccaceae	*Gisekia, Phytolacca*
	Portulacaceae	*Claytonia, Portulaca*
	Stegnospermatacea	*Stegnosperma*
Caryophyllineae (Betalain-free anthocyanin -producing taxa)	Caryophyllaceae	*Dianthus, Melandrium, Silene*
	Molluginaceae	*Mollugo, Pharnaceum*

The basic structure of betalains was elucidated in the 1960s by chemical means. Wyler *et al.* (1963) identified betanidin and Piattelli *et al.* (1964) indicaxanthin as immonium derivatives of betalamic acid with *cyclo*-dopa (betacyanin) and proline (betaxanthin), respectively (Fig. 1.2).

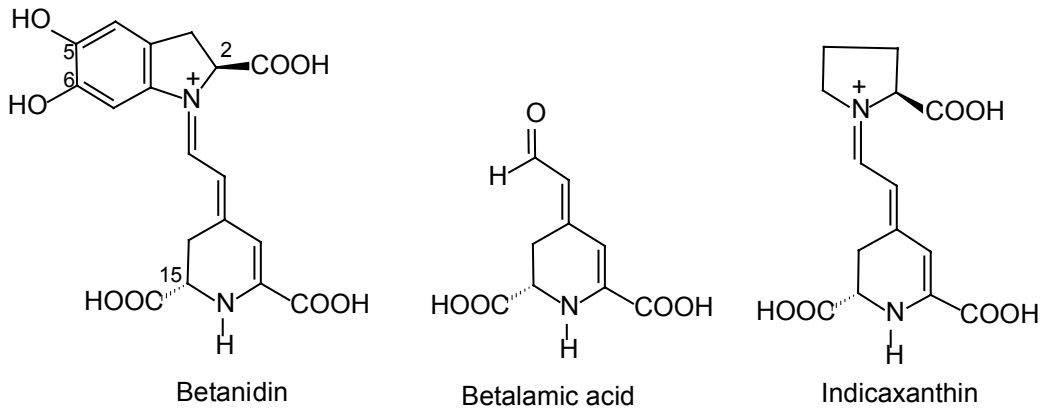


Fig. 1.2 Structures of betanidin, betalamic acid and indicaxanthin

As outlined in a recent betalain biosynthesis review (Strack, 1999), the initial reactions of betalain biosynthesis were elucidated in the 1960s by feeding experiments with isotopically-labelled tyrosine and dopa. Using [^{14}C, ^{15}N]-labelled tyrosine, Liebisch *et al.* (1969) proved

that the entire C_6C_3-skeleton of this amino acid is incorporated into betalamic acid and *cyclo*-dopa. Fig. 1.3 shows a current scheme of the betalain biosynthetic pathway, which includes

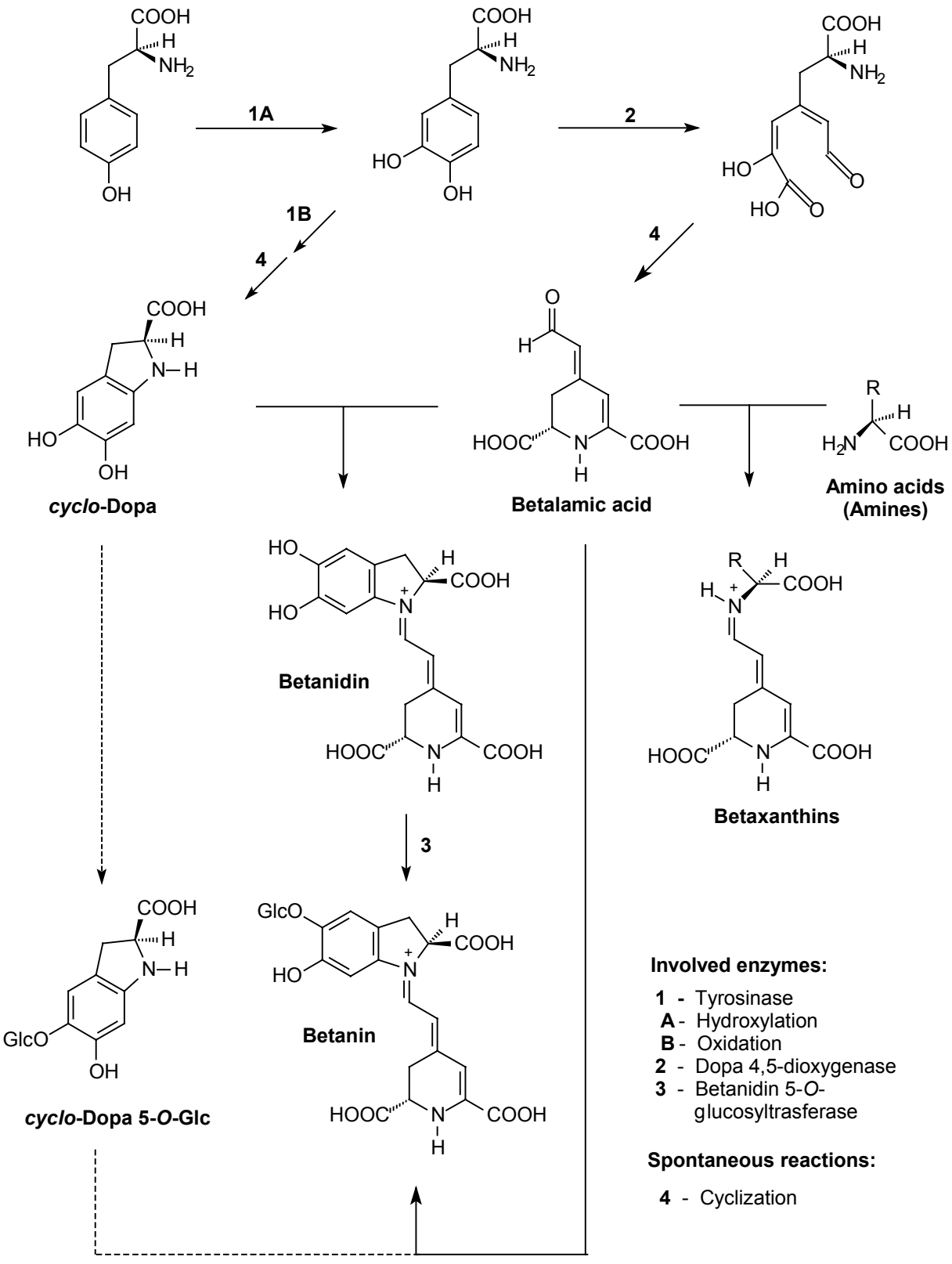


Fig. 1.3 Proposed current scheme of betalain biosynthesis

three enzymes, a tyrosinase, a dopa 4,5-dioxygenase and a betanidin glucosyltransferase. The bifunctional tyrosinase catalyses the formation of dopa and dopaquinone, the dioxygenase the formation of 4,5-*seco*-dopa. The subsequent cyclisations yield *cyclo*-dopa and betalamic acid, and the condensations of betalamic acid with *cyclo*-dopa and amino acids or amines lead to betacyanins and betaxanthins. Betanidin is transformed to betanin by a UDPG-dependent glucosyltransferase. Further glycosylation and acylation, mostly with hydroxycinnamic acids, may lead to acylated oligoglycosides of betanidin.

The involvement of oxidase activity in betalain biosynthesis had been suggested by Constabel and Haala (1968) who observed in betalain-producing callus cultures of *Beta vulgaris* cv. *crassa* higher phenol oxidase activities as compared to non-producing cultures. Endress (1979) assumed the involvement of a tyrosinase, indicated by the effects of enzyme cofactors and inhibitors on the betacyanin accumulation in *Portulaca grandiflora*. Joy *et al*. (1995) detected PPO transcripts in developing fruits of *Phytolacca americana* which correlated with the betacyanin accumulation. Mueller *et al.* (1996) described a tyrosinase from the betalain-accumulating pileus of *Amanita muscaria.* Recently, Steiner *et al.* (1996, 1999) showed with a protein preparation from callus cultures of *Portulaca grandiflora* that both the formation of dopa (by hydroxylation of Tyr) and its oxidation to dopaquinone are catalysed by a bifunctional betalain-specific tyrosinase. The highly reactive dopaquinone cyclises spontaneously to form *cyclo*-dopa. The tyrosinase belongs to the copper-binding polyphenoloxidase (PPO) family, inhibited by copper-chelating agents such as diethyldithiocarbamate or phenylthiocarbamide. The tyrosinase activity correlated with the betalain accumulation in different organs and in hypocotyls of growing seedlings of *Beta vulgaris* (Steiner *et al.*, 1999).

Dopa dioxygenase catalyses the transformation of dopa to 4,5-*seco*-dopa and 2,3-*seco*-dopa which recyclise spontaneously to betalamic acid and muscaflavin, respectively. Betalamic acid is the essential chromophore of all betalains. Dopa dioxygenase activity has been detected, isolated and characterized only from *Amanita muscaria* by Girod and Zryd (1991a) and Terradas and Wyler (1991). The gene encoding the fungal dopa dioxygenase was cloned from *Amanita muscaria* (Hinz *et al.*, 1997). Nothern-blot analysis of extracts from *Amanita muscaria* showed that dioxygenase is regulated at the transcript level. The specific mRNA accumulated mainly in the coloured tissue (Hinz *et al.*, 1997). The gene encoding the fungal dopa dioxygenase was expressed in *Escherichia coli* (Mueller *et al.*, 1997a). The recombinant protein catalysed both the 4,5- and the 2,3-extradiol cleavage of dopa. All attempts to detect dopa dioxygenase activity from betalain-forming higher plants have failed so far. Mueller *et al.* (1997) were unable to detect the plant enzyme by using antibodies directed against the

Amanita muscaria dioxygenase. Dopa dioxygenase from *Amanita muscaria* was expressed in white petals of *Portulaca grandiflora* after particle-bombardment and led to the formation of betalains, thus, betalain biosynthesis could be complemented by insertion of the fungal dioxygenase (Mueller *et al.*, 1997a).

Betalamic acid condenses either with *cyclo*-dopa to produce betacyanins or with amino acids or amines to produce betaxanthins. This condensation reaction forms an aldimine bond, but the nature of its formation (enzymic or spontaneous) was unknown. From crossing experiments with different *Portulaca grandiflora* lines, the existence of genes involved in betalain biosynthesis was deduced (Trezzini, 1990; Trezzini and Zryd, 1990). Hempel and Böhm (1997) suggested a spontaneous betaxanthin formation using hairy root cultures of *Beta vulgaris*.

The first enzymatic work on glucosyltransferases in betalain biosynthesis described two regiospecific UDPG- and betanidin-dependent enzymes from *Dorotheanthus bellidiformis* cell cultures, the UDPG:betanidin 5-*O*- and 6-*O*-glucosyltransferases (5-GT and 6-GT), leading to betanin (betanidin 5-*O*-glucoside) and gomphrenin I (betanidin 6-*O*-glucoside), respectively (Heuer and Strack, 1992; Heuer *et al*., 1996). Both enzymes have been purified to near homogeneity and characterized (Vogt *et al*., 1997). The enzymes catalyse the indiscriminate transfer of glucose from UDPG to hydroxy groups of betanidin, flavonols, flavones and anthocyanins, but discriminate between individual hydroxy groups of the respective acceptor compounds. A full-length cDNA encoding the 5-GT was obtained from a cDNA library of *Dorotheanthus bellidiformis* and expressed in *Escherichia coli* (Vogt *et al*., 1999). The recombinant protein displayed the same specificity towards betanidin and flavonoids as compared to the native enzyme. The glucosyl residue in betanin is linked with glucuronic acid in the case of amaranthin from *Amaranthus tricolor* (Mabry *et al*., 1967) or with apiose in betacyanins from *Phytolacca americana* (Schliemann *et al.*, 1996). Many betacyanins are conjugated with hydroxycinnamic acids (Minale *et al.*, 1966). A mixture of different betacyanins, mono- and di-ester of 4-coumaric acid and caffeic acid, occurs in bracts of *Bougainvillea glabra* (Nyctaginaceae) (Heuer *et al*., 1994). Enzymes responsible for acylation of betanidin glycosides have been described from eight members of four different families within the Caryophyllales (Bokern *et al.*, 1992).

1.3 Aim of the work

The main target of this study was the characterization of the condensation reaction between betalamic acid and amino acids/amines, the last step of betalain biosynthesis. For this pur-

pose, mainly hairy root cultures of yellow beet (*B. vulgaris* L. subsp. *vulgaris*) were used as the experimental system. Most of the experiments were repeated with seedlings of fodder beet (*B. vulgaris* L. subsp. *vulgaris*) to confirm the results. The main interest was the question whether this condensation reaction was a spontaneous or an enzymatic process. To achieve characterization of the biosynthetic capacity of the experimental system, the betalain pattern of hairy root cultures had to be analysed and the pigments had to be identified, supplemented by precursor feedings to the analogous fodder beet hypocotyls system. From crossing experiments with common portulaca (*Portulaca grandiflora*), the existence of the gene loci R, C and I were deduced and at this time a model for the betalain biosynthesis was proposed (Trezzini, 1990; Trezzini and Zryd, 1990): The products of gene R and C are responsible for the formation of *cyclo*-dopa and betalamic acid, respectively. The product of gene I modifies or inhibits membrane proteins involved in transport of betalamic acid into vacuoles. In the derived model, the biosynthesis of betacyanin takes place in the cytoplasm, but it is stored in the vacuole. On the other hand, betalamic acid is transported into the vacuole where it condenses spontaneously with amino acids to form betaxanthins. To make a contribution to verify this hypothesis, investigation of the transport of precursors and end products of betalain biosynthesis into vacuoles are a possible approach. In addition to the biochemical characterization of the experimental plants, microscopic and microspectrophotometric analyses were included to observe the betalain localization in intact beet plants.

2 Materials

2.1 Plants

2.1.1 Beets

The recent proposed classification of cultivated forms of beet has been used for the nomenclature of *Beta* (Lange *et al*., 1999).

Different fodder beet plants [*Beta vulgaris* L. subsp. *vulgaris* (Fodder Beet Group) 'Altamo', 'Brigadier' and 'Fumona'] and red and yellow beet plants [*B. vulgaris* L. subsp. *vulgaris* (Garden Beet Group) 'BV61243', 'Cylindra', 'Renova' and 'Golden Beet Geel'] were grown from seeds in a greenhouse and cultivated in soil. For feeding experiments and extract analyses from beet plants, 3-4 week-old seedlings and for studies of uptake into isolated vacuoles, 6 month-old red beet ('Cylindra') hypocotyls were used. The suppliers of the beet seed materials are described in Table 2.1.

Table 2.1 Suppliers of the beet seeds

Beets	Suppliers
Fodder Beet Group	
'Altamo'	Saaten-Zentrum Schöndorf, Weimar
'Brigadier'	
'Fumona'	
Garden Beet Group	
'Cylindra'	Dom Samen GmbH, Kevelaer
'Renova'	Saatzucht Quedlinburg GmbH, Quedlinburg
'BV 61243'	Bundesanstalt für Züchtungsforschung an Kulturpflanzen, Braunschweig
'Golden Beet Geel'	

2.1.2 Cactaceae

Schlumbergera x *buckleyi* (T. Moore) Tjaden - Christmas cactus - and *Schlumbergera truncata* (Haworth) Moran were cultivated in a greenhouse and the flowers (petals without stamens and carpels) were harvested after full opening.

Materials from other different cacti were provided by Ralf Dehn (Botanical Garden of the Martin-Luther-University, Halle, Germany) and harvested on September, 24, 1999. Extract from fruits and flowers of *Stenocereus queretaroensis* (A. Weber) F. Buxb. was obtained from Eugenia Lugo (CIATEJ, Guadalajara, Mexico). The list of cacti and the organs that were used in the experiments are compiled in Tab. 2.2.

Table 2.2 List of Cactaceae used in betacyanin screening

Cactaceae	Organs
Schlumbergera x *buckleyi* (T. Moore) Tjaden	Petals, Carpels, Stamens
S. truncata *(Haworth) Moran*	Petals
Epiphyllum hybrid	Petals
Gymnocalycium achirasense H. Till et S. Schatzl	Petals
G. quehlianum (F. A. Haage jr.) Berger var. *zantnerianum* Schick	Petals
Mammillaria duoformis Craig et Parson	Petals
M. huajuapensis Bravo	Petals, Fruits
M. muehlenpfordtii Foerster	Petals, Fruits
M. multiceps Saim-Dyck	Petals, Fruits
M. perbella Hildm.	Petals, Fruits
M. pitcayensis Bravo Repp. 766	Petals
M. saxicola REPP. ML 73	Petals
M. spec. camella	Petals
Rebutia flavistyla Ritt. FR 756	Petals
R. friedrichiana Rausch WR 646	Petals
R. tarvitaensis Ritt.	Petals
R. spec. KG 728	Petals
Stenocereus queretaroensis (A. Weber) F. Buxb.	Petals, Fruits

2.1.3 Plants

Broad beans (*Vicia faba* L. 'Fribo') and peas (*Pisum sativum* L. 'Belinda') were grown from seeds in a greenhouse and cultivated in soil.

2.2 Cell cultures

2.2.1. Hairy root cultures of beets

Hairy root cultures from yellow beet plants [*Beta vulgaris* L. subsp. *vulgaris* (Garden Beet Group) 'Golden Beet' line 5A, 5D and 7] were established and provided by H. Böhm (Deutsches Institut für Ernährungsforschung, Bergholz-Rehbrücke, Germany). Liquid cultures of these hairy root lines were maintained at a light intensity of 65 µmol/m^2/s under a photo-period of 16 h light/8 h dark at 25 °C on a shaker (120 rpm) and subcultivated on every 7th day by transferring root tips (*ca* 1 cm, 0.3 g fresh weight) into 30 ml modified 2,4-D-free B5 fresh liquid medium (Gamborg *et al*., 1968) containing 30 g/l sucrose, 18.6 mg/l Na_2EDTA and 13.8 mg/l $FeSO_4 \cdot 7\ H_2O$ in 100 ml Erlenmeyer flasks.

The hairy root culture from red beet plants [*B. vulgaris* L. subsp. *vulgaris* (Garden Beet Group) 'Egyptian Flatround'] was established by I. Kuzovkina (K. A. Timiryasev Institute of Plant Physiology, Russian Academy of Sciences, Moscow, Russia). The red hairy root culture

in the liquid medium was maintained in the dark at 25 °C on a shaker (120 rpm). Root tips (*ca* 1cm, 0.3 g fresh weight) were transferred on every 14th day into 30 ml modified hormone- and glycine-free ½ Murashige-Skoog fresh liquid medium (Murashige and Skoog, 1962) containing 20 g/l sucrose, 660 mg/l $CaCl_2 \cdot 2\ H_2O$, 80 mg/l *myo*-inositol, 0.1 mg/l pyridoxine · HCl and 0.1 mg/l thiamine · 2 HCl in 100 ml Erlenmeyer flasks.

The surface culture of this hairy root was maintained in the dark at 25 °C and subcultivated every 14th day by transferring root tips onto 30 ml modified Mugnier-Mosse fresh agar medium (Mugnier and Mosse, 1987) containing 20 g/l sucrose, 88 mg/l $CaCl_2 \cdot 2\ H_2O$, 1.0 mg/l *myo*-inositol, 0.2 mg/l pyridoxine · HCl, 0.2 mg/l thiamine · 2 HCl and 8 g/l Bacto®-agar (Difco, Detroit, USA) in 9 cm petri dishes.

2.2.2 Cell cultures

Suspension cultures from red beet plants [*B. vulgaris* L. subsp. *vulgaris* (Garden Beet Group)] were maintained at a light intensity of 65 µmol/m^2/s under a photo-period of 16 h light/8 h dark at 25 °C on a shaker (120 rpm). Cells were subcultivated on every 14th day by transferring cells into 30 ml modified hormone-free Murashige-Skoog fresh liquid medium (Murashige and Skoog, 1962) containing 30 g/l sucrose, 21.1 mg/l Na_2EDTA and 15.7 mg/l $FeSO_4 \cdot 7\ H_2O$ in 200 ml Erlenmeyer flasks.

Suspension cultures from *Dorotheanthus bellidiformis* (Burm. f.) N.E.Br. were maintained at a light intensity of 65 µmol/m^2/s under a photo-period of 16 h light/8 h dark at 25 °C on a shaker (120 rpm). Every 5th day cells were transferred into 30 ml Linsmaier-Skoog fresh liquid medium (Linsmaier and Skoog, 1965) containing 20 g/l sucrose, 0.5 mg/l pyridoxine · HCl, 0.1 mg/l thiamine · 2 HCl, 0.2 mg/l kinetin and 0.5 mg/l nicotinic acid in 200 ml Erlenmeyer flasks. These two cultures came from the Institute of Pharmaceutical Biology, Technical University, Braunschweig, Germany (D. Strack).

Callus cultures of *Portulaca grandiflora* Hook., line K64 (red) obtained from H. Böhm (Deutsches Institut für Ernährungsforschung, Bergholz-Rehbrücke, Germany), were maintained at a light intensity of 65 µmol/m^2/s under a photo-period of 16 h light/8 h dark at 25 °C. Cells were subcultivated on every 14th day by transferring cells onto 30 ml modified Linsmaier-Skoog fresh agar medium (Linsmaier and Skoog, 1965) containing 20 g/l sucrose, 2 mg/l 2,4-D, 0.2 mg/l kinetin and 8 g/l Bacto®-agar in 100 ml Erlenmeyer flasks.

2.3 Chemicals

The suppliers of the chemicals are described in Table 2.3. 2-Aminoindan 2-phosphonic acid (AIP) was provided by N. Amrhein (Eidgenössische Technische Hochschule Zürich, Switzerland) and (*S*)-thiaproline was obtained from A. Baumert (Leibniz-Institut für Pflanzenbiochemie, Halle, Germany).

Table 2.3 Suppliers of the chemicals

Chemicals	Companies
Poly (methylphenylsiloxane), 550® fluid	Aldrich (Milwaukee, USA)
(*S*)-[2,6-$^{3}H_2$]Phe, (*S*)-[U-^{14}C]Tyr, Tritiated water	Amersham Life Science (Braunschweig, Germany)
Percoll	Amersham Pharmacia Biotech (Sweden)
(*R*)-[1-^{14}C]Phe	Biotrend (Köln, Germany)
Glucose-6-phosphate	C. F. Boehringer & Soehne GmbH (Mannheim, Germany)
Tyramine, Betaine·H_2O, Potassium D-gluconate, 4-Nitrophenyl-α-D-mannopyranoside	Fluka (Neu-Ulm, Germany)
(*S*)-Phe, Ammonium chloride, D-(-)-Mannitol, Calcium chloride·2 H_2O, di-Potassium hydrogenphosphate·3 H_2O, Magnesium sulfate·7 H_2O, *ortho*-Phosphoric acid, Citric acid·H_2O , Tris (hydroxymethyl)-aminomethane	Merck (Darmstadt, Germany)
(*S*)-Ala, (*S*)-Arg, (*S*)-Asp, (*S*)-Asn, (*S*)-Glu, (*S*)-His, (*S*)-Hyp, (*S*)-Leu, (*S*)-Lys, (*S*)-Pro, (*S*)-Ser, (*S*)-Thr, (*S*)-Trp, (*S*)-Val, (*R*)-Val	Reanal (Budapest, Hungary)
Lyophilized red beet juice, D-Sorbit, EGTA, Methanol, Acetic acid	Roth (Karlsruhe, Germany)
Cellulase Y-C, Pectolyase Y-23	Seishin (Tokyo, Japan)
(*S*)-Met, (*S*)-Dopa, (*S*)-Leu, (*S*)-Tyr, Hepes, Bovine Serum Albumin , Triton X-100	Serva (Heidelberg, Germany)
(*R*)-Ala, (*R*)-Leu, (*R*)-His, (*S*)-Gln, Gly, Dopamine·HCl, β-Glucosidase, Ethylendiamine, Putrescine, Octanediamine, Cadaverine, Spermine, Spermidine, Mes, Neutral red, Sodium *ortho*-vanadate, Bis-tris propane, DL-DTT, ATP, Phospho-creatine, Creatine phosphokinase, Bafilomycin A1, β-NADP	Sigma (St. Louis, USA)
(*R*)-Phe	VEB Berlin-Chemie (Berlin, Germany)

2.4 Equipments

The supplies of equipment and material that were used in individual experiments are described at first description in the method chapter.

3 Methods

3.1 Isolation and purification of betalains

3.1.1 Isolation and purification of dopamine-derived betacyanins

Forty-eight hours after dopamine feeding, the hypocotyls (25.8 g) were harvested, frozen in liquid N_2, homogenized with a mortar and pestle and extracted with 100 ml 80 % aqueous methanol, containing 50 mM ascorbic acid, for 30 min. After centrifugation at 14,000*g* for 10 min at 4 °C, the supernatant was removed and its betacyanin content was photometrically quantified at 540 nm according to Wyler *et al*., 1959. The extract was concentrated *in vacuo* at 30°C to 10 ml.

The crude extract was purified by prep. HPLC (*solvent system 4*) in a two step procedure and subsequently by semi-prep. HPLC (*solvent system 2*) on analytical HPLC. After semi-prep. HPLC, the pooled fractions were concentrated *in vacuo* at 30°C and the aqueous residues were lyophilized.

3.1.2 Isolation and purification of betalains from Christmas cactus

Flowers (48 g) of Christmas cactus were frozen in liquid N_2, homogenized with a mortar and pestle and extracted with 150 ml 80 % aqueous methanol, containing 50 mM ascorbic acid, 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 using the extinction coefficient of betanin (Wyler *et al*., 1959). The extract was concentrated *in vacuo* at 30 °C to 10 ml. The compounds were purified in a two-step procedure. After prep. HPLC (*solvent system 4*) with a linear gradient, the main betacyanin fractions were re-purified by an additional prep. HPLC with isocratic elution (*solvent system 6*). To remove methanol, the pooled fractions were concentrated *in vacuo* at 30°C and the aqueous residues were lyophilized. The minor compounds were separated by semi-prep. HPLC (*solvent system 2*) on an analytical column.

3.1.3 Isolation of miraxanthin V (dopamine-betaxanthin) and betalamic acid

The hypocotyls (100 g) of fodder beet plants (4 weeks old) were frozen in liquid N_2, homogenized with a mortar and pestle and extracted with 300 ml 80 % aqueous methanol containing 50 mM sodium ascorbate. After centrifugation at 14,000*g* for 15 min at 4 °C, the supernatant was removed and concentrated *in vacuo* at 30 °C to 10 ml. By analytical HPLC (*solvent system 1*) betalamic acid and miraxanthin V were detected. From the concentrated extract, both

fractions of betalamic acid (R_t 27-33 min) and miraxanthin V (R_t 41-46 min) were obtained by prep. HPLC (*solvent system 7*). Subsequently, 100 ml of the betalamic acid fraction was concentrated *in vacuo* at 30 °C to 70 ml and lyophilized. The miraxanthin V fraction was concentrated *in vacuo* at 30 °C to dryness. For the preparation of the substrate solutions for vacuole uptake experiments, 500 µl of 500 mM sorbitol, 30 mM K-gluconate, 20 mM Hepes/KOH, pH 7.2, were added to the miraxanthin V fraction and the same solution at pH 8.2 to betalamic acid. Both solutions were centrifuged at 14,000*g* for 10 min. The supernatants were analysed by analytical HPLC (*solvent system 3*).

3.1.4 Partial synthesis of (*R*)-Phe-betaxanthin and vulgaxanthin I [(*S*)-Gln-betaxanthin]

The hypocotyls (1 kg) of red beet plants (6 months old) were harvested, sliced and frozen in liquid N_2, homogenized with a mortar and pestle and subsequently with a blender and extracted with 3 l of 80 % aqueous methanol containing 50 mM ascorbic acid. After centrifugation at 14,000*g* for 15 min, the supernatant was removed and concentrated *in vacuo* at 30 °C to 100 ml. 50 ml of the concentrated extract was passed through Sephadex LH-20 and eluted by 50 % aqueous methanol. The betacyanin fraction was collected and concentrated *in vacuo* at 30 °C to 10 ml. 2 ml of the concentrated betacyanin fraction was injected to prep. HPLC (*solvent system 8*) and the betanin fraction (R_t 36-51 min) was obtained and concentrated *in vacuo* 30 °C to 6 ml. After centrifugation at 14,000*g* for 15 min, aqueous NH_4OH (25 %) was added to the betanin solution to adjust to pH 11.0. After hydrolysis for 30 min at room temperature, the mixture was divided into two equal parts and 10 mmol (*R*)-Phe and (*S*)-Gln was added immediately. The mixtures were concentrated *in vacuo* at 30 °C to dryness and dissolved in 20 ml water. (*R*)-Phe-betaxanthin (R_t 55-60 min) or (*S*)-Gln-betaxanthin (R_t 35-38 min) was purified by prep. HPLC (*solvent system 8*). The fraction was pooled and concentrated *in vacuo* at 30 °C to dryness. For uptake experiments the residues were dissolved in 500 µl 500 mM sorbitol, 30 mM K-gluconate, 20 mM Hepes/KOH, pH 7.2. After centrifugation at 14,000g for 10 min at 4 °C, the supernatants were analysed by analytical HPLC (*solvent system 2*).

Unless otherwise stated, the following experiments were performed in at least duplicate.

3.2 Accumulation and occurrence of betalains

3.2.1 Short-term dopamine feeding experiment

For the pulse experiment, 5 ml 10 mM dopamine · HCl was fed to five de-rooted 28-day-old fodder beet plants ('Altamo'). After 0, 1 and 2 h, the hypocotyls (0.5 g) were extracted and analysed by HPLC (s*olvent system 1*). For the chase experiments, after 2 h of dopamine feeding (pulse phase), the dopamine solution was replaced by water. After 0, 1, 2, 4, 8 and 12 h the hypocotyls (0.5 g) were extracted, the extracts were analysed by HPLC (*solvent system 1*) and then the betacyanins were quantified photometrically. The peak areas of 2-descarboxy-betanin, 2-descarboxy-betanidin and 6'-*O*-malonyl-2-descarboxy-betanin were added up and the concentration of the individual compounds were expressed as the percentage of the sum.

3.2.2 Identification and quantification of dopamine-derived betacyanins by co-injection analyses

Fodder beet plants [*B. vulgaris* L. subsp. *vulgaris* (Fodder Beet Group) 'Altamo' and *B. vulgaris* L. subsp. *vulgaris* (Fodder Beet Group) 'Fumona'], red beet plants [*B. vulgaris* L. subsp. *vulgaris* (Garden beet Group) 'Renova'], hairy root cultures of yellow beet [*B. vulgaris* L. subsp. *vulgaris* (Garden Beet Group) 'Golden Beet'], line 5A, 5D and 7, a hairy root and suspension cultures of red beet [*B. vulgaris* L. subsp. *vulgaris* (Garden Beet Group)] and callus cultures of *Portulaca grandiflora* Hook., line K64 (red), were extracted. To 70 µl of the extracts, 5 µl solutions of 2-descarboxy-betanin, 2-descarboxy-betanidin and 6'-*O*-malonyl-2-descarboxy-betanin were added and 50 µl of the mixtures were analysed by HPLC (*solvent system 1*). To control samples, 5 µl water were added instead of the standards. After HPLC analysis, the peak areas were compared with those of the controls.

3.2.3 Betacyanin compositions during flower development and in different flower organs of Christmas cactus

Closed flowers of different sizes (1.5, 3.5 and 4.5 cm long), corresponding to 12, 4, and 2 days before flower opening, and flower petals (0, 2, 4 and 6 days old) were harvested to study the betacyanin compositions during flower development. After harvesting of the different stages of the buds and flowers, the plant materials were extracted and the betacyanin patterns were analysed by HPLC (*solvent system 1*). For the analysis of betalain compositions of dif-

ferent flower parts, petals, stamens and carpels were harvested separately after flower opening. They were extracted, analysed by HPLC (*solvent system1*) and quantified photometrically.

3.2.4 Betacyanin compositions in fruits and flowers from different species of Cactaceae

To compare the betacyanin compositions in fruits and flowers from different cacti with those of the flowers of Christmas cactus, the materials in Table 2.2 were extracted, analysed by HPLC (*solvent system 1*) and quantified photometrically.

3.3 Feeding experiments

3.3.1 Feeding of amino acids and amines to hairy root cultures of yellow beet and seedlings of fodder beet

(*S*)- and (*R*)-amino acids and polyamines [Gly, (*S*)-Ala, (*S*)-Val, (*S*)-Leu, (*S*)-Ile, (*S*)-Met, (*S*)-Pro, (*S*)-Phe, (*S*)-Trp, (*S*)-Ser, (*S*)-Thr, (*S*)-Asn, (*S*)-Gln, (*S*)-Lys, (*S*)-Arg, (*S*)-His, (*S*)-Asp, (*S*)-Glu, (*S*)-Orn, (*S*)-Hyp, (*S*)-4-thiaproline, (*R*)-Ala, (*R*)-Leu, (*R*)-His, (*R*)-Val, (*R*)-Phe, ethylendiamine, putrescine, cadaverine, octanediamine, spermine, spermidine [2 ml for (*S*)-Asp and (*S*)-Glu, 1 ml for the rest, final concentration: 2 mM)] were dissolved in water (Milli-Q plus, Millipore GmbH, Eschborn) and fed by sterile filtration to hairy root cultures (line BVL 5A) of yellow beet [*B. vulgaris* L. subsp. *vulgaris* (Garden Beet Group) 'Golden Beet'] on the 7th day after subcultivation. After 24 h, the hairy roots were harvested, extracted and analysed by HPLC (*solvent system 1*).

The same amino acids and polyamines as described above and, in addition, (*S*)-tyrosine, (*S*)-Dopa, tyramine and dopamine · HCl (5 ml, final concentration 10 mM) were fed to ten de-rooted fodder beet plants [*B. vulgaris* L. subsp. *vulgaris* (Fodder Beet Group) 'Altamo', 23 days old] via the hypocotyls. After 48 h, the hypocotyls were extracted and analysed by HPLC (*solvent system 1*). As a control, water was fed to hairy root cultures or de-rooted fodder beet plants instead of amino acids and amines.

In a large scale dopamine feeding experiment, 470 fodder beet 'Altamo' plants were fed with 250 ml 10 mM dopamine · HCl. After 48 h, the hypocotyls were extracted and the 2-descarboxy-betacyanins were purified by prep. HPLC (*solvent system 5*) and semi-prep. HPLC (*solvent system 2*).

3.3.2 Feeding of (*S*)-Phe, (*R*)-Phe and (*S*)/(*R*)-Phe to different cultures and fodder beet seedlings

For the competition experiments, (*S*)-Phe and (*R*)-Phe alone and together (2 ml, final concentration: 2 mM) were fed to hairy root cultures of yellow beet under the same condition as 3.3.1.

To compare the betaxanthin formation of different hairy root and cell cultures, (*S*)-Phe and (*R*)-Phe alone and together (2 ml, final concentration: 2 mM) were fed to hairy root and suspension cultures of red beet [*B. vulgaris* L. subsp. *vulgaris* (Garden Beet Group) 'Egyptian Flatround'] and to a suspension culture of *Dorotheanthus bellidiformis* (Burm. f.) N.E.Br under the same conditions as 3.3.1. After 24 h, the cell materials were harvested, extracted and analysed by HPLC (*solvent system 1*).

(*S*)-Phe and (*R*)-Phe (10 ml, 10 mM) were applied alone to fodder beet plants ('Altamo') under the same conditions as 3.3.1. After 48 h, the hypocotyls were harvested, extracted and analysed by HPLC (*solvent system 1*).

As a control, water was fed to hairy root and suspension culture or de-rooted fodder beet plants instead of amino acids.

3.3.3 Uptake kinetics of (*S*)-Phe, (*R*)-Phe and (*S*)/(*R*)-Phe by hairy root cultures of yellow beet

To study the competition in the uptake of (*R*)-Phe in the presence of (*S*)-Phe (final concentration: 2 mM), ^{3}H-labelled (*S*)-Phe (20 µl, 0.74 MBq) and ^{14}C-labelled (*R*)-Phe (50 µl, 0.185 MBq) were applied alone and together to hairy root cultures on the 7th day after subcultivation. To monitor the uptake of labelled (*S*)-Phe, (*R*)-Phe and (*S*)/(*R*)-Phe at 0, 1, 2, 4, 8, 12 and 24 h after the application, 50 µl of the nutrition solution were analysed by liquid scintillation counting and the results were used for the calculation of the ^{3}H/ ^{14}C ratio.

3.3.4 Feeding of amino acids and $(NH_4)_2SO_4$ to hairy root cultures of yellow beet

To study the metabolite pattern in dependence on the different concentrations of the fed precursor, (*S*)-Ala, (*R*)-Ala and (*S*)-Thr (2 ml, final concentrations: 2, 5, 10, 20 and 50 mM) and ammonium sulphate (2 ml, final concentrations: 3, 6, 11, 21, and 51 mM) were fed to hairy root cultures under the same conditions as 3.3.1. The hairy roots were harvested after 24 h, extracted and analysed by HPLC (*solvent system 1*).

(*S*)-Leu (2 ml, final concentration: 5 mM) was fed daily from the 4th to the 8th day to hairy root cultures. Every 24 hours after the application from the 4th to the 8th day, the hairy roots

were harvested, extracted and analysed by HPLC (*solvent system 1*). Water was fed as a control under the same conditions as described above.

3.3.5 Feeding of 2-aminoindan 2-phosphonic acid (AIP) to hairy root cultures of yellow beet and seedlings of fodder beet

2 ml AIP (final concentration: 0.25 µM) was fed daily from the 4th to the 7th day to hairy root cultures and on the 8th day the hairy roots were harvested, extracted and analysed by HPLC (*solvent system 1*). Controls were treated with water instead of AIP. 50 µl AIP in 5 ml 0.1 M potassium phosphate buffer pH 7.0 (final concentration: 0.25 µM) were also fed to three de-rooted fodder beet plants ('Altamo', 5 weeks old), which were harvested, extracted and analysed by HPLC (*solvent system 1*) after 24 h. Controls were treated with 5 ml 0.1 M potassium phosphate buffer pH 7.0.

3.3.6 Feeding of betalamic acid to broad bean and pea seedlings

Betalamic acid in 0.1 M potassium phosphate buffer pH 6.8 (1.4 ml, final concentration: 0.29 mM) was fed to the de-rooted 14-day-old broad bean (*Vicia faba* L. 'Fribo') and pea (*Pisum sativum* L. 'Belinda') plants via the hypocotyls, which were extracted after 24 h for HPLC (*solvent system 1*) analysis. Controls were treated with 0.1 M potassium phosphate buffer, pH 6.8, under the same conditions.

3.4 Extraction of betalains

After harvesting hairy roots, suspension-cultured cells or hypocotyls, the material was washed briefly with distilled water, blotted dry between filter paper, frozen in liquid N_2 and homogenized in a mortar. The betalains were extracted with 80 % aqueous methanol containing 50 mM ascorbate at tissue:solvent ratio of 1 g/3 ml. After centrifugation at 15,000*g* for 10 min at 4 °C, the supernatants were removed. Two aliquots of 20 µl were diluted to 1 ml with water and the absorbance was measured photometrically and quantified using the mean molar extinction coefficients for betanin (Wyler *et al*., 1959) and betaxanthin (Girod and Zryd, 1991b). Two samples (50 µl) were analysed by HPLC (*solvent system 1*).

3.5 Preparation of protein extracts and assays for the condensation reaction of betalamic acid with amino acids

Protein extracts from hairy root cultures of yellow beet were prepared according to Steiner *et al.*, (1996) (ammonium sulphate precipitation), De-Eknamkul *et al.* (1997) and Terradas and Wyler (1991) (60 % acetone precipitation).

For ammonium sulphate precipitation, 10 g hairy roots were frozen in liquid N_2, homogenized with a mortar and pestle and extracted with a three-fold volume of buffer (v/w) (0.1 M potassium phosphate buffer, pH 7.5, containing 50 mM ascorbate) with stirring for 30 min at 0 °C. The homogenate was filtered through Miracloth and centrifuged at 10,000*g* for 15 min at 4 °C. The supernatants were passed through PD-10 columns and eluted with 10 mM potassium phosphate buffer, pH 7.5. The eluates were precipitated at 80 % ammonium sulphate saturation, separated by centrifugation, dissolved by 10 mM potassium phosphate buffer, pH 7.5, and desalted with the PD-10 column. The eluted solution was used as a crude extract. For 60 % acetone precipitation, 5 g hairy roots were frozen in liquid N_2 and homogenized with 2.5 g Polyclar AT in a mortar. To the homogenate, acetone (- 20 °C) was added and stirred at 0 °C for 30 min. After centrifugation at 15,000*g* for 10 min at 4 °C, 3 ml diethylether were added to the pellet and dried *in vacuo* for 5 min. The pellet was dissolved with 0.1 M potasium phosphate buffer, pH 6.5, containing 50 mM ascorbate buffer and 100 µl protease inhibitor and stirred for 30 min at 0 °C. After centrifugation at 15,000*g* for 20 min at 4 °C, the supernatant was passed through a PD-10 column. The eluted solution was used as a crude extract. The protein content was determined by the Bradford method (Bradford, 1976) using human serum albumin as a standard.

In the enzyme assay for the condensation of betalamic acid with amino acids, the substrate solution (total volume 150 µl: 0.1 M potassium phosphate buffer, pH 7.5, with 50 mM ascorbate, 0.5 mM (*S*)-Phe or 1 mM (*S*)-Gln, 0.2 mM betalamic acid) was pre-incubated for 5 min at 30 °C in an Eppendorf thermomixer (900 shakes/min) and the reaction was started by the addition of 50 µl protein extract. The enzyme reaction was stopped after 30 min by boiling for 5 min. After centrifugation for 10 min at 14,000*g*, 50 µl of the supernatant were analysed by HPLC (*solvent system 1*) or quantified photometrically at 470 nm using the betaxanthin extinction coefficient (Girod and Zryd, 1991b). Controls contained heat-denatured protein.

3.6 Uptake of betaxanthins to red beet vacuoles

3.6.1 Preparation of protoplasts and vacuoles from red beet hypocotyls

The preparation of protoplasts and vacuoles was performed according to E. Martinoia (pers. commun.). Hypocotyls (30 g) of red beet plants (6 months old) were harvested, peeled and sliced by a razor into 5 x 5 x 0.5 mm pieces. The slices of red beet hypocotyls were pooled in 100 ml 800 mM sorbitol, 1 mM $CaCl_2$, 10 mM Mes/KOH, pH 5.6, 2 mM DTT and 0.1 % (w/v) BSA for 30 min at room temperature for plasmolysis. After filtration of the plasmolysis solution through eight layers cotton mesh, the cell walls were digested by incubation of 7.5 g slices of red beet hypocotyls in 50 ml 500 mM sorbitol, 1 mM $CaCl_2$, 20 mM Mes/KOH, pH 5.6, 1 mM DTT, 0.1 % (w/v) BSA, 1 % (w/v) cellulase Y-C and 0.2 % (w/v) pectolyase Y-23 on a shaker (150 rpm) for 1.5 h at room temperature. After filtration of the protoplast solution through eight layers cotton mesh and one layer nylon mesh (60 μm^2), the protoplasts were collected at 2,000*g* for 15 min by centrifugation on a 2 ml cushion of 500 mM sorbitol and 20 mM Mes dissolved in Percoll. They were further purified by centrifugation at 2,000*g* for 15 min through a step gradient prepared in the following order: protoplast solution with 500 mM sorbitol, 20 mM Mes dissolved in Percoll (lower phase, 15 ml); 25 % (v/v) Percoll, 500 mM sorbitol, 30 mM K-gluconate, 20 mM Hepes/KOH, pH 7.2, 0.1 % (w/v) BSA (middle phase, 20 ml); 400 mM betaine · H_2O, 30 mM K-gluconate, 20 mM Hepes/KOH, pH 7.2, 0.1 % (w/v) BSA (upper phase, about 5 ml). Pure protoplasts were obtained at the upper interphase.

Vacuoles were isolated from protoplasts according to Klein *et al.*, 1998, with some modifications. Red beet vacuoles were liberated from the protoplasts by a combination of destabilisation of the plasma membrane by EGTA, osmotic and pH shock. One part of concentrated protoplasts was mixed gently with one part of 5 mM EGTA, 20 mM Hepes/KOH, pH 8.5, and six parts of 150 mM K_2HPO_4, pH 8.5, 0.1 % (w/v) BSA at room temperature. After 15 min of gentle shaking the vacuoles were purified by centrifugation at 800*g* for 5 min through a step gradient prepared in the following order: vacuole solution (lower phase, about 10 ml); 5.5 % (v/v) Percoll, 500 mM sorbitol, 30 mM K-gluconate, 20 mM Hepes/KOH, pH 7.2, 0.1 % (w/v) BSA (middle phase, about 10 ml); 400 mM betaine · H_2O, 30 mM K-gluconate, 20 mM Hepes/KOH, pH 7.2, 0.1 % (w/v) BSA (upper phase, 5 ml). The vacuoles were collected at the upper interphase. All steps except for the lysis step were performed on ice and surveyed microscopically.

3.6.2 Marker enzymes

To check the contamination of the isolated vacuoles by cytosolic constituents, α-mannosidase, located only in the vacuoles, and the cytosolic glucose 6-phosphate dehydrogenase were used as marker enzymes. The contamination was determined by calculation of the protoplast : vacuole ratio of glucose 6-phosphate dehydrogenase/α-mannosidase in both the protoplast and vacuole preparations.

To measure α-mannosidase activity in red beet protoplast and vacuole preparations, 200 µl of protoplasts and vacuoles were added to 800 µl 100 mM citric acid · H_2O, pH 5.0, 0.05 % (w/v) Triton X-100, 0.1 % (v/v) BSA and 2.5 mM 4-nitrophenyl-α-D-mannopyranoside. After incubation for 0, 10, 20, 30, 40 min at 30 °C, the mixtures (100 µl) were added to 900 µl 1 M Na_2CO_3, centrifugated at 14,000*g* for 5 min and the optical density measured photometrically at 400 nm.

For measurement of glucose 6-phosphate dehydrogenase activity in the red beet protoplast and vacuole preparations, 200 µl of protoplasts and vacuoles were added to 800 µl 100 mM Tris/HCl, pH 7.5, 0.05 % (w/v) Triton X-100, 0.1 % (v/v) BSA, 0.65 mM β-NADP and 0.75 mM glucose 6-phosphate. After incubation of 0, 10, 20, 30, 40 min at 30 °C, the mixtures (1 ml) were centrifugated at 14,000*g* for 5 min and measured photometrically at 366 nm.

3.6.3 Transport studies

Studies of the uptake of betaxanthins into red beet vacuoles were performed as described by Rentsch and Martinoia (1991). Unless stated otherwise, for each time point and various experiments, 70 µl of a solution containing 30 % (v/v) percoll, 400 mM sorbitol, 30 mM K-gluconate, 20 mM Hepes/KOH, pH 7.2, 0.1 % (w/v) BSA, 3.7 kBq ^3H_2O and further solutes as indicated in figures and a table, were pipetted into 6 polyethylene microcentrifugation tubes (0.4 ml capacity). Uptake was started by adding 30 µl of concentrated vacuole suspension. The samples were rapidly overlayered with 200 µl of poly (methylphenylsiloxane), 500® fluid oil and 60 µl of water. The incubation was terminated by flotation of the vacuoles (12,000g, 10 sec, Primo Biofuge, Kendro, Germany). The aqueous phases of two tubes (50 µl) were pooled in one tube and centrifugated at 14,000*g* for 10 min. The supernatant which contained the vacuolar extract was used to measure the radioactivity of ^3H_2O by liquid scintillation counting for the vacuolar volume determination and to determine the transported substrates by peak areas of HPLC (*solvent system 3*). 10 µl of the aqueous supernatants were suspended in 4 ml scintillation cocktail and radioactivity was determined by liquid scintillation counting. The aqueous supernatants (50 µl) were analysed by HPLC (*solvent system 3*). The

betalains were quantified by the mean molar extinction coefficients for betaxanthins (Girod and Zryd, 1991b). Unless otherwise stated, uptake rates of each substrate were calculated as the mean of triplicates by subtracting the 4-min values of incubation from corresponding 24-min values.

3.6.4 Preparation of mini-protoplasts and uptake experiments

Mini-protoplasts were obtained according to Hörtensteiner *et al.* (1992) with some modifications. A 1 ml aliquot of purified red beet protoplasts was layered on a medium containing 3 ml 500 mM mannitol, 20 mM Hepes/KOH, pH 7.2, and 50 mM $CaCl_2$, dissolved in Percoll and overlayered with 0.5 ml 650 mM betaine · H_2O, 20 mM Hepes/KOH, pH 7.2, 50 mM $CaCl_2$ and 0.1 % (w/v) BSA. After centrifugation at 924,000*g* for 35 min (Beckman LE-80 Ultracentrifuge, USA), the complete evaculated mini-protoplasts were obtained at the upper interphase. The mini-protoplasts were separated from cell debris by centrifugation at 2,000*g* for 5 min through 500 mM sorbitol and 20 mM Mes dissolved in 40 % (v/v) Percoll and 500 mM sorbitol, 1 mM $CaCl_2$, 20 mM Mes/KOH, pH 5.6 as the upper phase. The evacuolated mini-protoplasts were collected at the interphase.

Mini-protoplasts were incubated in 2 ml 2 mM (*R*)-Phe, 500 mM sorbitol, 10 mM Mes/KOH, pH 5.6, 1 mM $CaCl_2$ with 4 mM ATP for 15 min at room temperature. The mini-protoplasts, containing (*R*)-Phe, were washed three times by centrifugation at 2,000*g* for 5 min through 2 ml 500 mM sorbitol, 10 mM Mes, Percoll and 1 ml 500 mM sorbitol, 10 mM Mes/KOH, pH 5.6, at the upper phase. The (*R*)-Phe containing mini-protoplasts were collected at the interphase. To examine the formation of (*R*)-Phe-betaxanthin in the mini-protoplasts, the uptake of 5.2 µM betalamic acid into the (*R*)-Phe containing mini-protoplasts was performed in the same way as vacuolar uptake as described above.

3.7 High performance liquid chromatography (HPLC)

Analytical and semi-preparative HPLC were performed with a Waters system (Waters, Milford, Mass., USA) including the separation module 2690. The liquid chromatograph was equipped with a 5-µm Nucleosil C_{18} column (250 x 4 mm, i.d.; Macherey-Nagel, Düren, Germany) and different solvent and gradient systems were used as Table 3.1 . The flow rate was 1 ml/min. Compounds were detected at 540, 475 and 405 nm or by maxplot detection between 400 and 650 nm or between 200 and 650 nm (photodiode array detection). Injection volume was 20 or 50 µl for analytical and 100 µl for semi-prep. HPLC.

For prep. 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) and the following solvent and gradient systems were used (Table 3.1). The flow rate was 10 ml/min. Compounds were detected at 470, 540 and 410 nm. Injection volume was 2 ml.

Table 3.1 Solvent and gradient systems of HPLC

Solvent system	Solvents	Gradient
Analytical or semi-preparative HPLC		
1	A: 1.5 % *ortho*-phosphoric acid B: 100 % acetonitrile	100 % A to 76 % A in (A+B) within 40 min
2	A: 1% acetic acid B: 100% acetonitrile	100 % A to 76 % A in (A+B) within 40 min
3		95 % A to 82 % A in (A+B) within 10 min
Preparative HPLC		
4	A: 1 % acetic acid B: 100 % methanol	100 % A to 10 % A in (A+B) within 90 min
5		100 % A to 40 % A in (A+B) within 120 min
6		70 % A / 30 % B isocratic for 60 min
7		80 % A / 20 % B isocratic for 60 min
8		100 % A to 10 % A in (A+B) within 60 min

3.8 Quantification of betalains

From the supernatants of the plant and cell culture extracts, 20 µl was diluted to 1 ml with water and the absorbance was measured at 475 nm for betaxanthins and 540 nm for betacyanins with a photometer (Shimadzu, Columbia, MD). For quantification of the compounds, the mean molar extinction coefficient for betaxanthins ($48 \cdot 10^6$ cm^2 mol^{-1}, Girod and Zryd, 1991b) and for betanin ($62 \cdot 10^6$ cm^2 mol^{-1}, Wyler *et al*., 1959) was used. Betalamic acid was quantified by HPLC using a purified standard [1 nmol betalamic acid = $1.206 \cdot 10^6$ peak area at 405 nm].

3.9 Radioactivity measurement

The solutions containing radioactivity (10 or 50 µl) were added to 4 ml scintillation cocktail (Ultima Gold™ MV, Packard, The Netherlands), mixed and measured for 2 min in a scintillation counter (LS 6000 TA, Beckman, USA).

3.10 Amino acid analyses

Amino acid analyses were performed by M. Kiess (GBF, Braunschweig, Germany). The mixture of the supernatants of betalain extraction and the re-extracted solution from pellets was partitioned with 5 ml of $CHCl_3$ until the $CHCl_3$ fractions were colourless. The aqueous upper phases were concentrated to dryness *in vacuo* and dissolved in water. Aliquots were used for amino acid analyses (ABI 420A, Applied Biosystems, Foster City, CA) with the amino acid standards. For Dopa analysis, extraction was carried out in the presence of ascorbate (100 mM) and the extract was analysed by HPLC as described in Steiner *et al.* (1996).

3.11 Chemical and spectroscopic identification of betalains

3.11.1 Enzymatic hydrolysis of 2-descarboxy-betanin

2-Descarboxy-betanin (20 nmol betalain equivalents in 200 µl 0.1 M potassium phosphate buffer, pH 5.0) was added to 200 µl β-glucosidase (almond) (2 mg/ml in 0.1 M potassium phosphate buffer pH 5.0) at 37 ^{o}C. After 0, 10, 20, 30 and 60 min, two 25 µl aliquots of the hydrolysis mixture were removed and added to 50 µl methanol for protein precipitation. After centrifugation at 15,000*g* for 5 min, the supernatant was analysed by HPLC (*solvent system 1*). A control was stopped by the addition of 50 µl methanol immediately after the addition of β-glucosidase.

3.11.2 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, *solvent system 1*). To start the racemization, 100 µl of 1 N HCl was added to the phyllocactin solution and the progress of racemization was monitored after the injection of 20 µl of the mixture (0.25 nmol betanin equivalents). Racemization and degradation were calculated from peak areas and experiments were performed in duplicate.

3.11.3 Liquid chromatography-mass spectrometry (LC-MS)

LC-MS was performed by J. Schmidt (Institut für Pflanzenbiochemie, Halle, Germany). Positive and negative electrospray mass spectra were obtained using a Finnigan MAT TSQ 7000 instrument [electrospray voltage 4.5 kV (positive ions), 3.5 kV (negative ions); capillary 220 ^{o}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 % aqueous acetic acid in acetonitonile) in 90 % A (0.2 % acetic acid) to 50 % B in (A+B) within 10 min was used, followed by 10 min of further isocratic elution at a flow rate of 70 µl/min (injection volume: 2 µl). The negative ion ESI-MS spectra were recorded using an atmospheric pressure ionization collision-induced dissociation (APICID) offset voltage of 10 V. The collision-induced dissociation (CID) mass spectra were obtained during LC analysis under the following conditions: collision energy (collision cell) - 40 eV (positive ions); collision gas: argon; collision pressure: 1.8 mTorr. All mass spectra are averaged, with the background subtracted.

3.11.4 Nuclear magnetic resonance (NMR) spectroscopy

NMR spectroscopy was performed by V. Wray (GBF, Braunschweig, Germany). 1-D and 2-D ^{1}H (COSY and TOCSY with a mixing time of 70 msec) 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 tetramethylsilane (TMS) and coupling constants in Hz.

3.11.5 Sugar composition and carbohydrate methylation analysis

Monosaccharides were analysed by M. Nimtz (GBF, Braunschweig, Germany) as the corresponding methyl glycosides after methanolysis and trimethylsilylation on a Carlo Erba Mega Series gas chromatograph incorporating a 30-m DB1 capillary column (Chaplin, 1982). Apiin [apigenin 7-*O*-(2'-*O*-*β*-D-apiofuranosyl)-*β*-D-glucopyranoside] (Roth, Karlsruhe, Germany) was used as standard. For methylation analysis the glycoconjugate was permethylated, hydrolysed, reduced and peracetylated as described (Anumula and Taylor, 1992).

3.11.6 Microscopy

The hairy roots of yellow beet, the hypocotyls of fodder beet and red beet plants and the hypocotyls after 48 h feeding of 10 mM dopamine to the de-rooted fodder beet plants were hand cut as 0.5 mm cross sections. The sections were observed using light microscopy (OPTIPHOT-2 Nikon, Japan) and photographed by MICROFLEX UFX-DX (Nikon, Japan). The spectra were measured by using the microspectral microscope Zeiss, MPM 800 (Zeiss, Germany).

To check the intactness of the vacuoles, 50 µl of vacuoles were treated by 2 µl 0.025 % (w/v) neutral red dissolved in 500 mM sorbitol, 20 mM Hepes/KOH, pH 7.2 for 5 min and checked

by light microscopy. The numbers of protoplasts and vacuoles from red beet plants were counted under the light microscope using a Thoma-hemocytometer.

4. Results

4.1 Dopamine-derived betacyanins

4.1.1 HPLC patterns of betalains in hairy root culture and seedlings of dopamine fed fodder beet

The presence of nine betalain pigments (**1**-**9**) in hairy root cultures of yellow beets has been detected by their characteristic spectral properties in HPLC analysis (*solvent system 1*) (Fig. 4.1 A). Betalamic acid (**1**), betanin (**2**), isobetanin (15*R*-isobetanin, **2'**), miraxanthin V (**3**) and 2-descarboxy-betanidin (**7**) have been first identified (Schliemann *et al*., 1999). Because of the high concentration of dopamine in hairy root cultures of yellow beet (*B. vulgaris* L.) and considering that the main betaxanthin (miraxanthin V, **3**) and the major betacyanin (2-descarboxy-betanidin, **7**) are both dopamine-derived betalains, similar structures for the minor betacyanins were assumed. The minor betacyanins in the hairy root cultures of yellow beet could not be obtained insufficient amounts due to their low concentrations. The feeding of the precursors of betalains (tyrosine and derived compounds) to this hairy root culture led to melanin formation because of high tyrosinase activity (Steiner *et al.* 1999). Therefore, fodder beet seedlings were used. HPLC analysis (*solvent system 1*) of the hypocotyl extract after feeding of dopamine to de-rooted fodder beet plants (Fig. 4.1 B) showed the presence of three major betacyanins (**5**, **7**, **8**) corresponding in retention times and UV-VIS data to the peaks of the hairy root extract (Fig. 4.1 A). Therefore, a large scale feeding of dopamine to seedlings of *B. vulgaris* was performed which gave a sufficient amount for isolation (yield: 2.8 µmol betanin equivalents). By analytical HPLC (*solvent system1*) four betacyanins (**5**, **7**, **8**, **9**) were detected. By prep. HPLC compound **5** and a mixture of **7**/**8**/**9** were obtained (compound **5**, R_t 61-63 min, 0.27 µmol betanin equivalents; compound **7**/**8**/**9**, R_t 66-70 min, 0.52 µmol betanin equivalents; *solvent system 4*). Subsequently, final purification of **5** and separation of **7**, **8** and **9** was achieved by semi-prep. HPLC on an analytical column (*solvent system 2*). Compounds **5** and **8** were obtained in 0.29 mg and 0.31 mg yield, respectively. The purified compounds were characterized by enzymatic hydrolysis, analytical HPLC, LC-MS and ^{1}H NMR analyses.

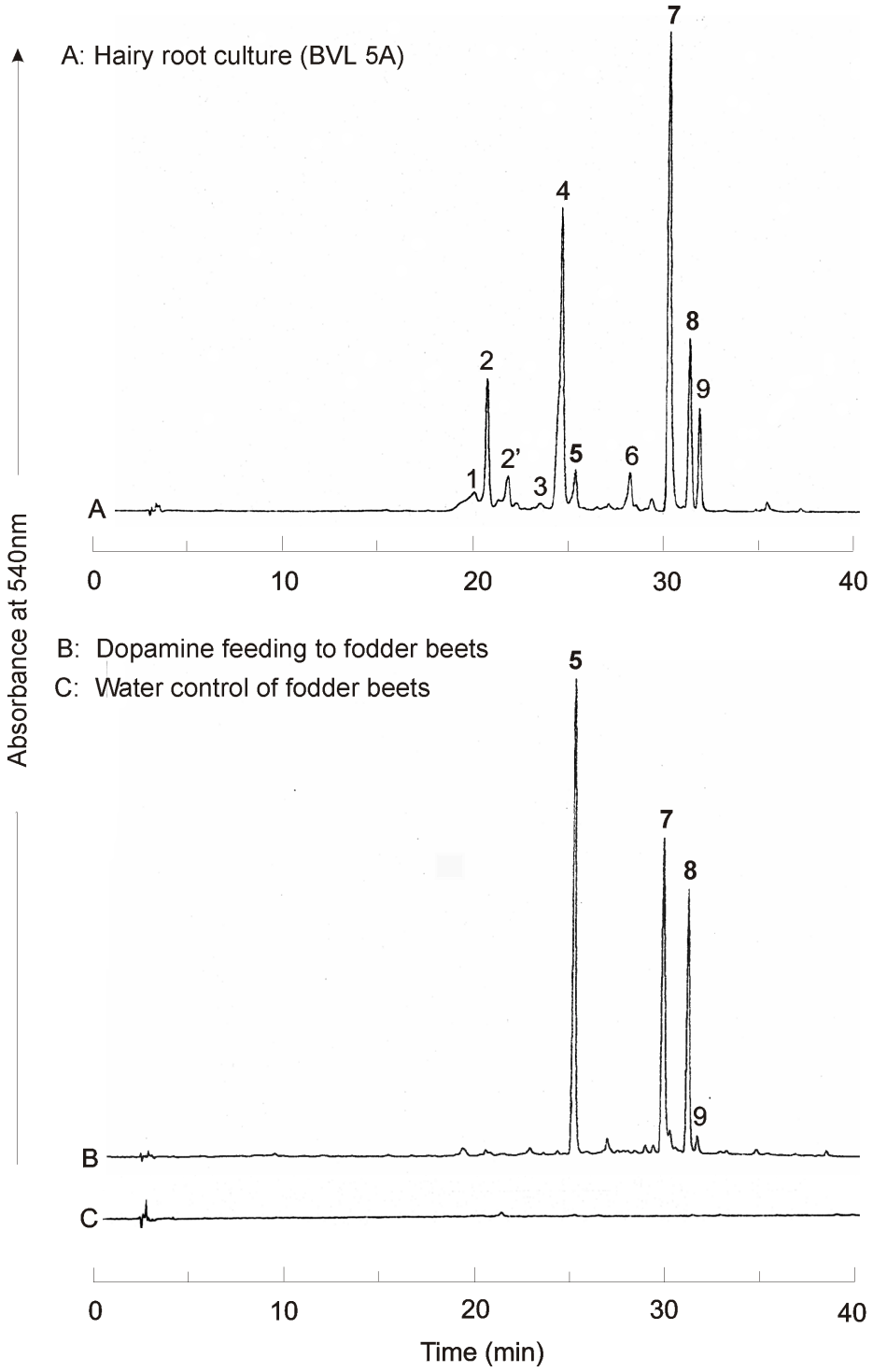


Fig. 4.1 HPLC profiles of betacyanins in *Beta vulgaris*. A: Hairy root culture (BVL 5A), B: Hypocotyls of fodder beet plants ('Altamo') (28 days old) after feeding of dopamine for 48 h, C: water control (for B); Full scale of A_{540}, A: 0.2 AU; B and C: 0.1 AU. (Note: Peaks **1** and **3** correspond to betalamic acid and miraxanthin V, respectively, in hairy root cultures, but are scarcely detectable at 540 nm). Peak numbers correspond to those in Table 4.1.

4.1.2 Retention time, HPLC-PDA, LC-MS and ^{1}H NMR data of betalains from hairy root cultures of yellow beet

Table 4.1 lists the analytical and spectroscopic data of betalains from hairy root cultures of *Beta vulgaris*.

Table 4.1 Data of betalains from hairy root cultures of *Beta vulgaris* L. subspec. *vulgaris* (Garden Beet Group) 'Golden Beet', line 5A. Compound numbers correspond to those in Fig. 4.1.

Compound	R_t (min)	HPLC-PDA λ_{max} (nm)	M_r	LC-MS $[M+H]^+$	Betalain
1	19.5	405	220	-[a]	betalamic acid
2	21.8	537	550	551	betanin
2'	22.3	536	550	-[a]	isobetanin
3	23.6	457	346	347	miraxanthin V
4	24.8	539	636	637	phyllocactin
5	25.5	532	506	507	2-descarboxy-betanin
6	28.5	536	522	523	unknown betacyanin
7	30.6	533	344	345	2-descarboxy-betanidin
8	31.8	535	592	593	malonyl-2-descarboxy-betanin
9	32.3	533	650	651	dopamine-derived betacyanin

[a] not determined

The presence of betalamic acid (**1**) was confirmed by co-chromatography with authentic standard at R_t 19.5 min and λ_{max} 405 nm.

Peak **2** was identified by co-chromatography with the standard as betanin (betanidin 5-*O*-*β*-D-glucoside) at R_t 21.8 min and λ_{max} 537 nm, and the $[M + H]^+$ ion was determined at *m/z* 551 (rel. int. 100) in positive ion mode of LC-MS. Peak **2'** was identified as isobetanin (isobetanidin 5-*O*-*β*-D-glucoside) at R_t 22.3 min and λ_{max} 536 nm by co-chromatography.

The presence of miraxanthin V (**3**, dopamine-betaxanthin) was confirmed by co-chromatography with the standard at R_t 23.6 min and λ_{max} 457 nm, and the $[M + H]^+$ ion was determined at *m/z* 347 (rel. int. 100) in positive ion mode of LC-MS. Collision-induced dissociation of the parent ion at *m/z* 347 yielded successive loss of the carboxyl groups, *m/z* 303 ($[M+H-CO_2]^+$, 18), *m/z* 301 ($[M+H-CO_2\ H]^+$, 16), *m/z* 257 ($[M+H-CO_2-HCO_2H]^+$, 16) and *m/z* 255 ($[M+H-2HCO_2H]^+$, 17). Furthermore, prominent ions at m/z 211 (30) [betalamic acid+H]$^+$ and *m/z* 137 (100, base peak) appeared. The ion at *m/z* 137 represents the deaminated component corresponding to dopamine.

Peak **4** was characterized as phyllocactin [betanidin 5-*O*-(6'-*O*-malonyl-*β*-D-glucoside)] by co-chromatography with the standard isolated from *Schlumbergera* x *buckleyi* at R_t 24.8 min and λ_{max} 539 nm, and the $[M + H]^+$ ion was determined at *m/z* 637 (rel. int. 100) in positive ion mode of LC-MS. The fragmentation in negative ion mode gave $[M-H]^-$ *m/z* 635 (95), [M-

H-CO_2]$^-$ *m/z* 591 (100), [M-H-2CO_2]$^-$ *m/z* 547 (88), [M-H-3CO_2]$^-$ *m/z* 503 (67) and [M-H-4CO_2]$^-$ *m/z* 459 (20). The loss of four CO_2 was typical for phyllocactin detecting a dicarboxylic acid (malonic acid) as acyl moiety (Fig. 4.10, p. 40). As a standard phyllocactin was isolated from flower petals of Christmas cactus (*Schlumbergera x buckleyi*). The data of LC-MS and ^{1}H NMR are described in detail at the section of 4.2 Betalains from Christmas cactus.

Compound **5** was observed at R_t 25.5 min and λ_{max} 532 nm by HPLC. The [M+H]$^+$ ion was *m/z* 507 (100) in positive ion mode and its daughter ion was *m/z* 345 [2-descarboxy-betanidin (**7**) + H]$^+$ (100). From the mass difference (162) between **5** (*m/z* 507) and **7** (*m/z* 345), the presence of an additional hexose was suggested in agreement with the shorter R_t of **5** in HPLC. This assumption was confirmed by treating **5** with β-glucosidase resulting in almost complete hydrolysis within 10 min and led to the liberation of the aglycone **7**. Thus, the presence of glucose in **5** connected in a β-glucosidic linkage with **7** was established. Table 4.2 and Fig. 4.2 show the chemical shifts and coupling and spectra of compound **5** from ^{1}H NMR.

Table 4.2 Chemical shifts and couplings of compound **5** from ^{1}H NMR (600 MHz, CD_3OD/DC1)

δ (ppm)	Assignment
8.67	1H, *d*, $J_{10\text{-}11}$ = 11.9 Hz, H-10
7.36	1H, *s*, H-7
7.30	1H, *s*, H-4
6.49	1H, *s*, H-17
6.39	1H, *d*, $J_{11\text{-}10}$ = 12.0 Hz, H-11
4.87	H-1’
4.59	1H, *dd*, $J_{14\text{-}13A}$ = 7.0, $J_{14\text{-}13B}$ = 5.6, H-14
4.40	2H, *t*, $J_{2A/B\text{-}3A/B}$ ~ 7 Hz, H-2A/B
3.96	1H, *dd*, $J_{6'A\text{-}5'}$ = 2.0 Hz, $J_{6'A\text{-}6'B}$ = 12.1 Hz, H-6’A
3.75	1H, *dd*, $J_{6'B\text{-}5'}$ = 5.3 Hz, $J_{6'B\text{-}6'A}$ = 12.0 Hz, H-6’B
3.66*	H-13A
3.56	1H, *dd*, $J_{2'\text{-}1'}$ ~ 8 Hz, $J_{2'\text{-}3'}$~ 9 Hz, H-2’
3.50*	H-5’
3.33*	H-3A/B
3.29*	H-13B
3.6-3.3	*m*, H-3’, H-4’

The chemical shifts of the signals marked * were determined from the cross peaks in the 2D COSY spectrum.

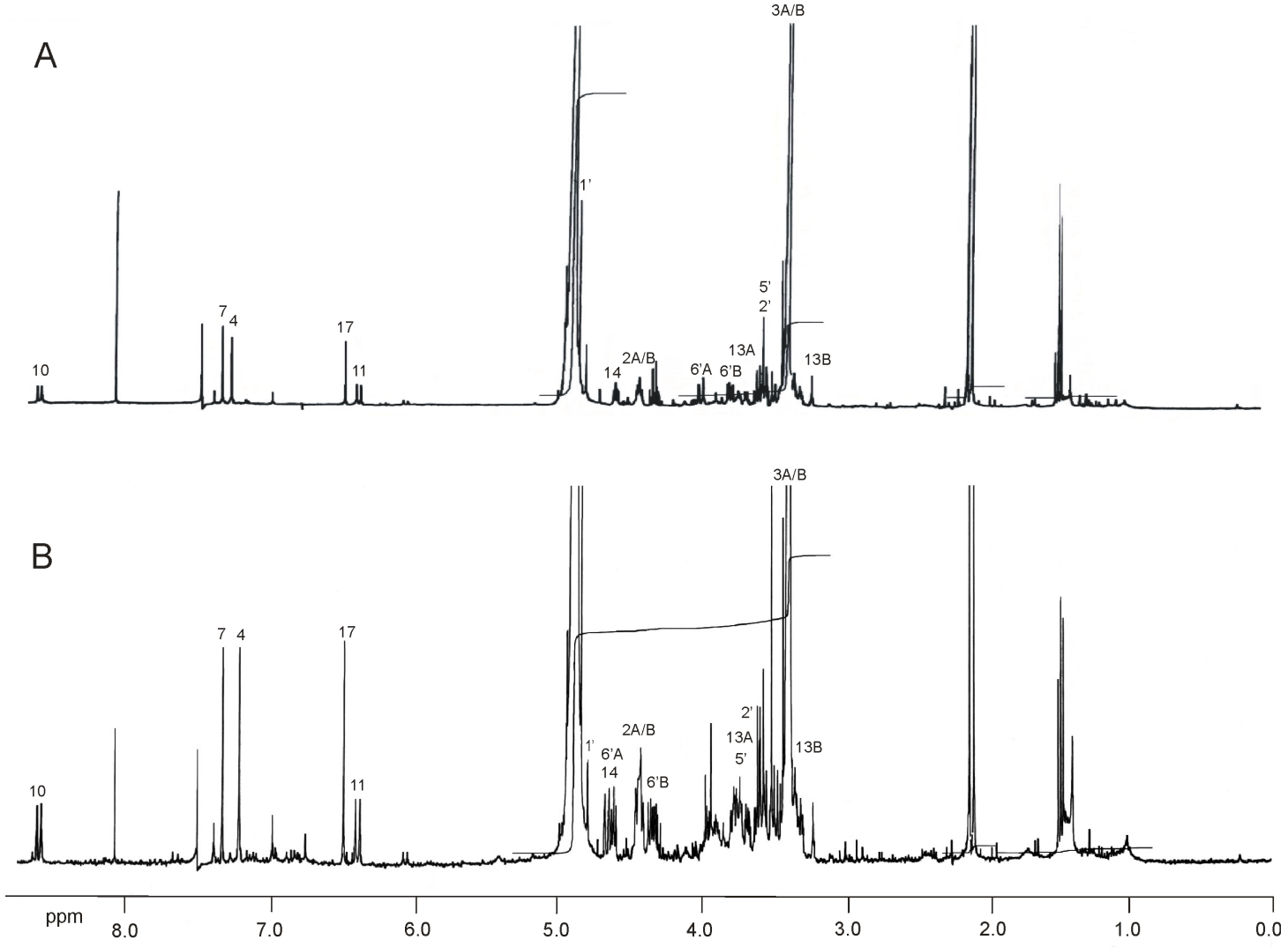


Fig. 4.2 ^{1}H NMR spectra of 2-descarboxy-betanin (**5**) and 6'-*O*-malonyl-2-descarboxy-betanin (**8**). A, 2-descarboxy-betanin; B, 6'-*O*-malonyl-2-descarboxy-betanin.

Thus, LC-MS, enzymatic hydrolysis and ^{1}H-NMR spectral data establish that compound **5** is 2-descarboxy-betanidin 5-*O*-*β*-D-glucoside (2-descarboxy-betanin, Fig. 4.3).

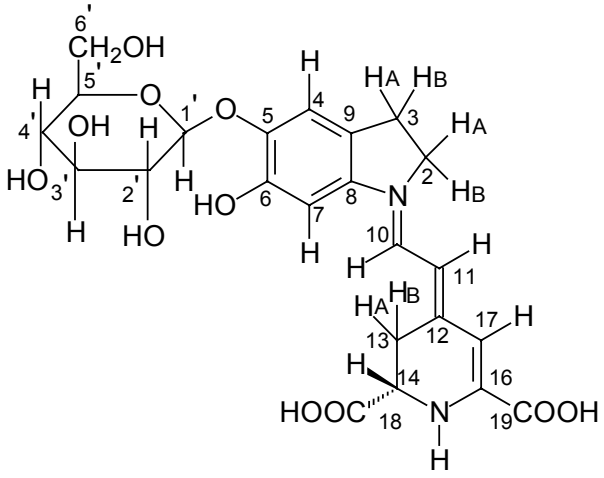


Fig. 4.3 Structure of 2-descarboxy-betanin (**5**)

Compound **6** gave R_t 28.5 min and λ_{max} 536 nm by HPLC and a $[M+H]^+$ ion at *m/z* 523 (100) by LC-MS which had a mass difference (16) between **6** (*m/z* 523) and **5** (*m/z* 507). This molecular ion yielded in the daughter ion scan mode neither a daughter ion at *m/z* 345 ([2-descarboxy-betanidin+H]$^+$, **7**) nor at *m/z* 389 ([betanidin+H]$^+$), but at *m/z* 361, an unknown betacyanin aglycone.

The presence of 2-descarboxy-betanidin (**7**; R_t 30.6 min, λ_{max} 533 nm) was confirmed by co-chromatography with a partial synthetic standard (Schliemann *et al*., 1999) in hairy roots of *B. vulgaris* and by LC-MS analysis giving the correct protonated molecular ion $[M+H]^+$ at *m/z* 345 (*m/z*, rel. int. 100).

Compound **8** (R_t 31.8 min, λ_{max} 535 nm) was less polar than 2-descarboxy-betanin (**5**; R_t 25.5 min, λ_{max} 532 nm) indicating the possible presence of an acyl moiety (Heuer *et al*., 1992). LC-MS analysis of **8** showed a $[M+H]^+$ ion at *m/z* 593 (100), which, in a daughter-ion scan, resulted in an intense peak at *m/z* 345 (rel. int. 100) [2-descarboxy-betanidin + H]$^+$, indicating that **8** belongs to the dopamine-derived betacyanins. The fragmentation in negative ion mode gave $[M-H]^-$ *m/z* 591 (73), $[M-H-CO_2]^-$ *m/z* 547 (73), $[M-H-2CO_2]^-$ *m/z* 503 (100) and $[M-H-3CO_2]^-$ *m/z* 459 (79). From the mass difference (86) between **8** (*m/z* 593) and **5** (*m/z* 507) the presence of a malonyl residue can be deduced. In fact, the presence of a dicarboxylic acid as acylating residue was confirmed by detection of three consecutive losses of CO_2 (two from the betalamic acid part and one from the acyl group) in LC-MS (negative ion mode) (Fig. 4.4). Similar fragmentation pattern (loss of four CO_2) was also observed using phyllocactin

isolated from flower petals of Christmas cactus (*Schlumbergera* x *buckleyi*) and analysed by LC-MS under the same conditions (Fig. 4.10, p. 40).

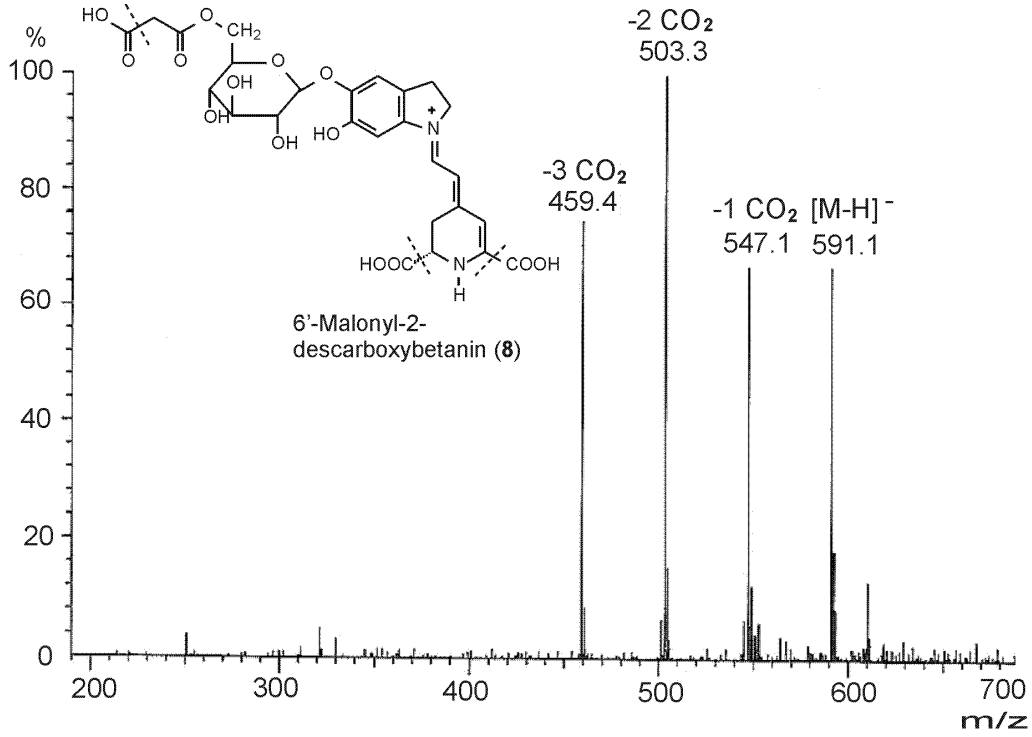


Fig. 4.4 MS spectrum of 6'-malonyl-2-descarboxybetanin (**8**) in negative ion mode

Table 4.3 Chemical shifts and couplings of compound **8** from ^{1}H NMR (600 MHz, CD_3OD/DC1)

δ (ppm)	Assignment
8.67	1H, *d*, $J_{10\text{-}11}$ = 12.1 Hz, H-10
7.36	1H, *s*, H-7
7.24	1H, *s*, H-4
6.49	1H, *s*, H-17
6.39	1H, *d*, $J_{11\text{-}12}$ = 12.3 Hz, H-11
4.88*	H-1'
4.62	1H, *dd*, $J_{6'A\text{-}5'A}$ = 1.9 Hz, $J_{6'A\text{-}5'B}$ = 12.0 Hz, H-6'A
4.57	1H, *dd*, $J_{14\text{-}13A}$ = 5.7 Hz, $J_{14\text{-}13B}$ = 6.8 Hz, H-14
4.40	2H, *t*, $J_{2A/B\text{-}3A/B}$ = 7.5 Hz, H-2A/B
4.32	1H, *dd*, $J_{6'B\text{-}5'A}$ = 6.7 Hz, $J_{6'B\text{-}5'B}$ = 11.9 Hz, H-6'B
3.73*	H-5'
3.67*	H-13A
3.57*	H-2'
3.36*	H-3A/B
3.27*	H-13B
3.7-3.3	*m*, H-3', H-4'

The chemical shifts of the signals marked * were determined from the cross peaks in the 2D COSY spectrum. A second betanidin spin system (~18%) was evident from signals at 8.70 and 6.05 ppm that presumably belong to the 11*Z*-isomer.

The linkage of the malonyl residue to the 6'-*O*-position of glucose in phyllocactin had been verified by ^{1}H NMR (see the section of 4.2 Betalains from Christmas cactus). Therefore, the site of malonyl attachment in **8** is most likely the same as in phyllocactin (**4**). This was confirmed by the ^{1}H NMR spectrum of **8** where the low field chemical shifts of H-6'A/H-6'B (4.62 and 4.32 ppm), respectively, provide definitive evidence that the acyl system is bound to C-6' of the glucose moiety (Table 4.3 and Fig. 4.2).

Thus, the combination of LC-MS and ^{1}H-NMR data identified **8** as 6'-*O*-malonyl-2-descarboxy-betanin [2-descarboxy-betanidin 5-*O*-(6'-*O*-malonyl-β-D-glucoside)] (Fig. 4.5).

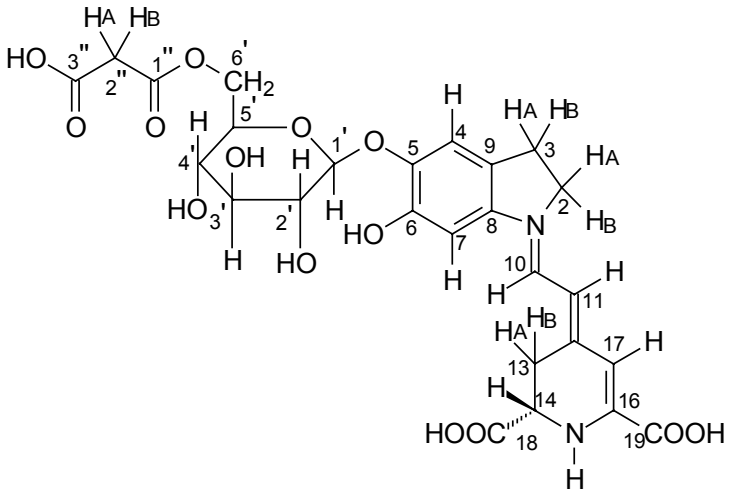


Fig. 4.5 Structure of 6'-*O*-malonyl-2-descarboxy-betanin (**8**)

Compound **9** (R_t 32.3 min, λ_{max} 533 nm), occurring in very low concentration in the extract from dopamine fed fodder beet hypocotyls, was also found in hairy root cultures. As **9** is a result of dopamine feeding and showed a characteristic daughter ion at *m/z* 345, it belongs unequivocally to the group of dopamine-derived betacyanins, but the $[M+H]^+$ ion at *m/z* 651 (100) did not fit with any plausible structural proposal.

4.1.3 Short-term dopamine feeding experiments

To follow the sequence of the formation of dopamine-derived betacyanins in fodder beets, a short-term dopamine feeding experiment was performed (Fig. 4.6). After feeding of dopamine for 2 h, the fate of the 2-descarboxy-betacyanins were followed for 12 h. 2-Descarboxy-betanidin amounted to nearly 80% of the total betacyanins at the end of the pulse phase (2 h). The chase phase is characterized by a decrease of 2-descarboxy-betanidin with a complementary increase of 2-descarboxy-betanin and malonyl-2-descarboxy-betanin.

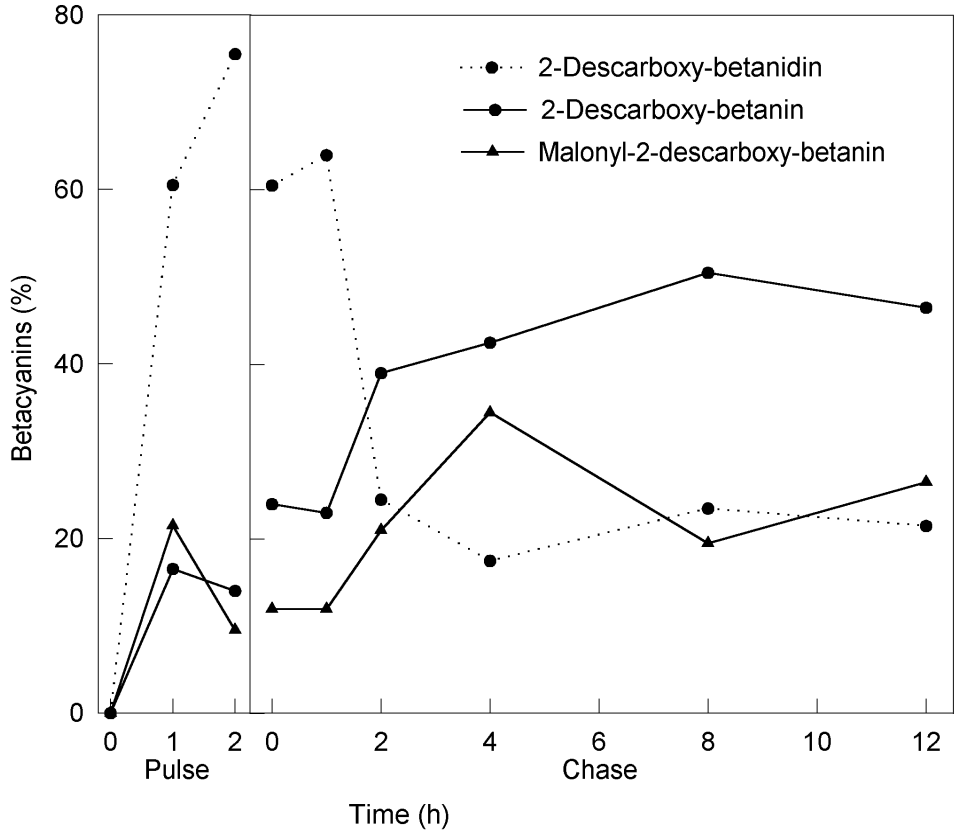


Fig. 4.6 Betacyanin levels of short-term dopamine administration experiment with hypocotyls of fodder beet plants ('Altamo') (28 days old)

4.1.4 Occurrence of dopamine-derived betacyanins

Using the purified dopamine-derived betacyanins as standards, the occurrence of these compounds was screened in different plants, cell and hairy root cultures by analytical HPLC (*solvent system 1*) and co-injection experiments (Table 4.4). Only in hairy root cultures derived from yellow beets, 2-descarboxy-betanidin is the prevailing betacyanin and followed by 6'-*O*-malonyl-2-descarboxy-betanin and 2-descarboxy-betanin. The occurrence of these dopamine-derived betacyanins is accompanied by a high amount of the dopamine-derived betaxanthin miraxanthin V. In red beet hairy root cultures, in hypocotyls of beet plants (Garden and Fodder Beet Group) and its cell suspension cultures, the concentrations of these betacyanins are either very low or not detectable. Likewise, callus cultures of *Portulaca grandiflora* Hook. did not contain any dopamine-derived betacyanin, but did contain a low amount of miraxanthin V.

Table 4.4 Occurrence of dopamine-derived betacyanins in hypocotyls, hairy root and cell cultures of *Beta vulgaris* and callus culture of *Portulaca grandiflora*. Each value is the average of duplicate samples.

Plant material	Total betacyanin (BC) content (nmol/g fw)[a]	2-Descarboxy-betanin (**5**) [% of total BC]	2-Descarboxy-betanidin (**7**) [% of total BC]	6'-*O* -Malonyl --2-descarboxy-betanin (**8**) [% of total BC]	Miraxanthin V (**3**) (nmol/g fw)
Beta vulgaris L. subsp. *vulgaris* (Garden Beet Group) 'Golden Beet'					
Hairy roots (7-d-old)					
BVL5A	97	3	37	10	546
BVL5D	133	5	56	6	1219
BVL7	96	3	31	15	925
BV red[b]	308	3	1	6	51
Hypocotyls (28-d-old)					
(Garden Beet Group)					
'Golden Beet Geel'	45	n.d.[c]	0.5	1.0	321
'Renova'	857	0.3	0.3	0.2	32
'BV 61243'	1426	n.d.	0.9	n.d.	51
(Fodder Beet Group)					
'Altamo'	3.4	n.d	0.5	n.d.	41
'Fumona'	1.9	n.d.	n.d.	n.d.	19
'Brigadier'	6.1	n.d.	0.2	n.d.	81
Cell suspension culture (14-d-old)					
BV[d]	198	1.5	n.d.	1.1	1.0
Portulaca grandiflora					
Callus culture (14-d-old)					
PG K64	34	n.d.	n.d.	n.d.	3.2

[a]In betanin equivalents; [b]*B. vulgaris* L. subsp. *vulgaris* (Garden Beet Group) 'Egyptian Flat Round'; [c]not detectable; [d]*B. vulgaris* L. subsp. *vulgaris* (Garden Beet Group) 'Rote Kugel'.

4.1.5 Feeding of Tyr, dopa, tyramine and dopamine to seedlings of fodder beet

Feeding of Tyr, dopa, tyramine and dopamine to hypocotyls of fodder beets ('Altamo') shows different metabolic flows (Fig. 4.7). In extracts from untreated hypocotyls, HPLC (*solvent system 1*) with maxplot detection (400-650 nm) shows that betalamic acid was the major compound (80 %), whereas betaxanthins were minor (19%), but betacyanins were not detected. After feeding of Tyr and dopa, betanin and the corresponding betaxanthins [Tyr-betaxanthin (portulacaxanthin II)], dopa-betaxanthin (dopaxanthin) occur, but not dopamine-derived betacyanins. In contrast, after tyramine and dopamine feeding, dopamine-derived be-

tacyanins amounts to 16 and 81%, respectively, and betalamic acid was in the latter case almost consumed, but betanin was hardly detectable.

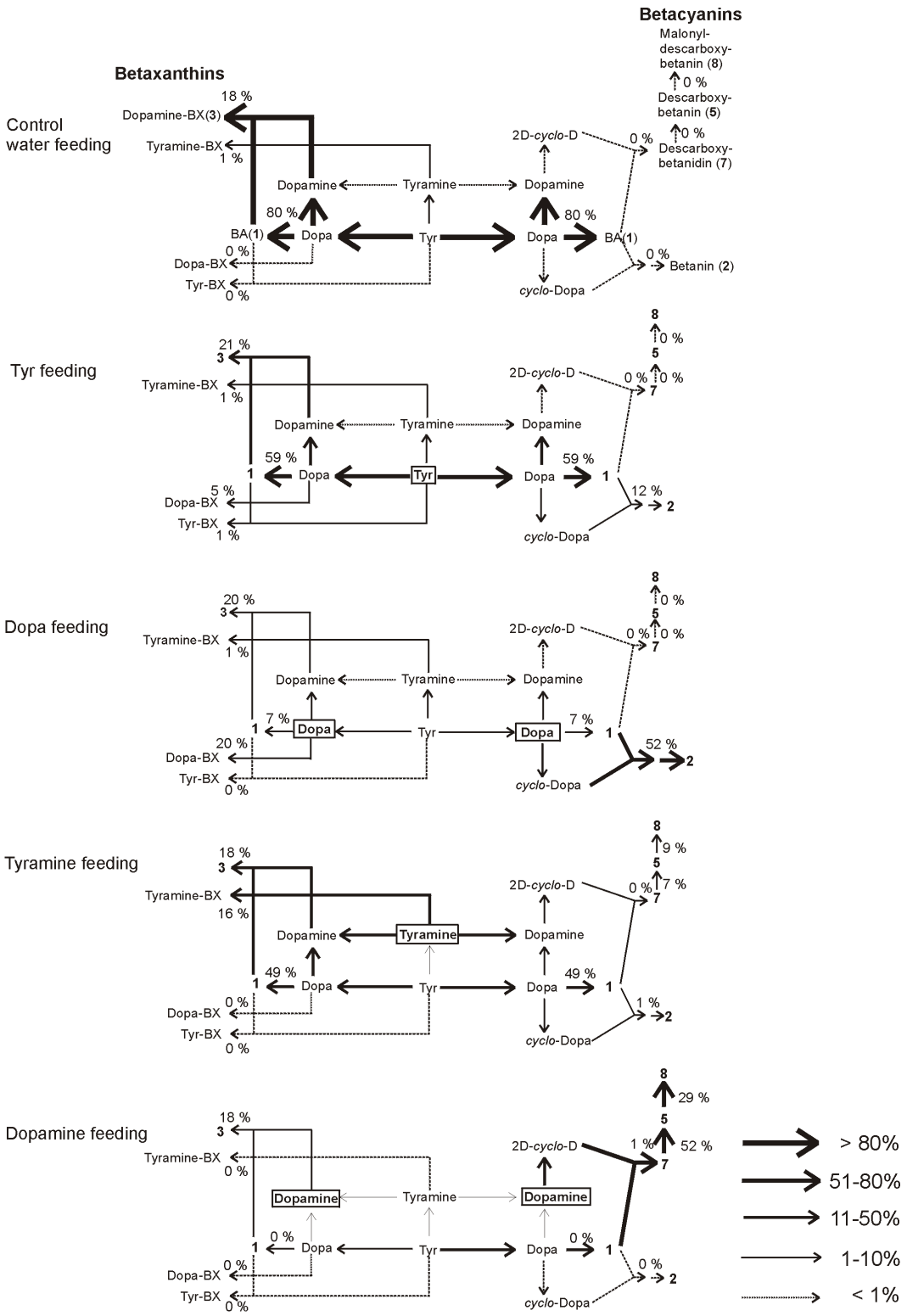


Fig. 4.7 Betalain formation after feeding of Tyr, Dopa, tyramine and dopamine to fodder beets ('Altamo')

4.2 Betalains from Christmas cactus

For identification of 6'-*O*-malonyl-descarboxy-betanin a comparison of its MS fragmentation pattern with phyllocactin (6'-*O*-malonyl-betanin) was necessary and therefore it was isolated together with the other unknown pigments from Christmas cactus.

Christmas cactus [*Schlumbergera x buckleyi* (T. Moore) Tjaden] (Fig. 4.8) is a hybrid of *Schlumbergera truncata* (Haworth) Moran and *Schlumbergera russeliana* (Hooker) Britton & Rose (Bachthaler, 1992).

Fig. 4.8 Flower of Christmas cactus [*Schlumbergera x buckleyi* (T. Moore) Tjaden]

4.2.1 HPLC pattern of betalains from Christmas cactus

HPLC analysis (*solvent system 1*) of an extract from flower petals of Christmas cactus revealed the presence of fourteen betalains (Fig. 4.9). After prep. HPLC with a linear gradient (**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; *solvent system 5*), vulgaxanthin I (**1**), betalamic acid (**2**) and betanin (**3**) were purified by semi-prep. HPLC (*solvent system 2*) and identified by co-chromatography (HPLC, *solvent system 1*) with authentic or synthetic compounds (Schliemann *et al.*, 1999). Phyllocactin/isophyllocactin (**5**, **5'**) were assigned by Minale *et al.* (1966) and Strack *et al.* (1981). Compound **5** and **7** (**5**, 5.7 µmol and **7**, 5.9 µmol betanin equivalents) were obtained in 0.52 mg and 0.46 mg, respectively. The further confirmation and identification were performed by HPLC-PDA, LC-MS, ^{1}H NMR spectroscopy and hydrolysis experiments.

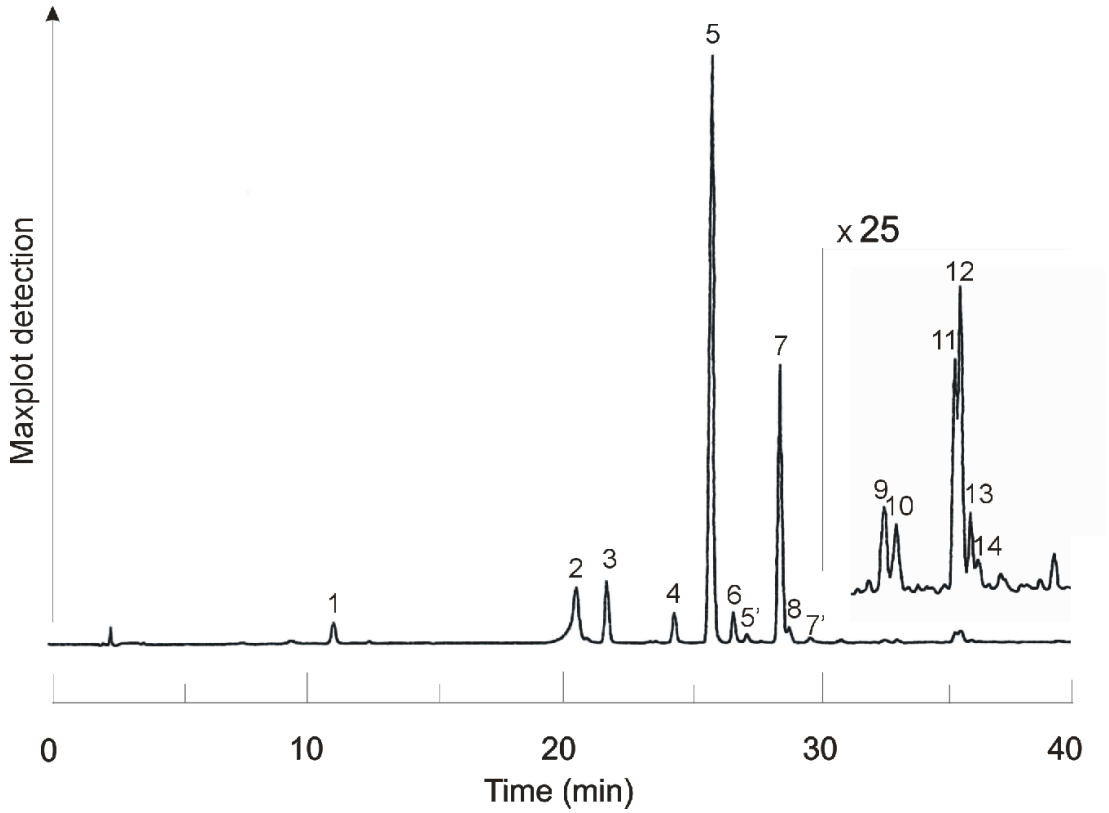


Fig. 4.9 HPLC pattern of betalains from flower petals of the Christmas cactus [*Schlumbergera x buckleyi* (T. Moore) Tjaden] (maxplot detection: 400 - 650 nm). Peak numbers correspond to the numbers in Table 4.

4.2.2 Retention time, HPLC-PDA, LC-MS and ^{1}H NMR data of betalains from Christmas cactus

Table 4.5 shows analytical spectroscopic data of betalains from the petals of Christmas cactus.

The presence of vulgaxanthin I (**1**, Gln-betaxanthin) was confirmed by co-chromatography with a standard at R_t 11.0 min (λ_{max} 468 nm). The $[M+H]^+$ ion was found at *m/z* 341 (rel. int. 100) by LC-MS (positive ion mode).

The presence of betalamic acid (**2**) was confirmed by co-chromatography with a standard at R_t 20.3 min (λ_{max} 405 nm).

Compound **3** was identified by co-chromatography with a standard at R_t 21.4 min (λ_{max} 537 nm) as betanin. The $[M+H]^+$ ion was found at *m/z* 551 (100) by LC-MS (positive ion mode).

Compound **4** was detected by HPLC-PDA at R_t 24.0 min and λ_{max} 540 nm. The $[M+H]^+$ ion could not be conclusively detected. From the UV-VIS spectrum by HPLC-PDA compound **4** must be a betacyanin but its structure could not be determined. Co-chromatographical analy-

sis showed that **4** is not identical with gomphrenin I (betanidin 6-*O*- β-D-glucopyranoside), but eluted between isobetanin and gomphrenin I.

Table 4.5 Retention time, HPLC-PDA and LC-MS data of betalains from flower petals of the Christmas cactus [*Schlumbergera x buckleyi* (T. Moore) Tjaden]

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).

The main betacyanin was phyllocactin (**5**, 6'-*O*-malonylbetanin, R_t 25.4 min, λ_{max} 539 nm) 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 (100) [550 (betanin)+86 (malonyl)+H]$^+$ which gave a daughter ion at *m/z* 389 (100), corresponding to [betanidin+H]$^+$. In the negative ion mode the following ions were assigned: *m/z* 635 $[M - H]^-$ (90); *m/z* 591 $[M - H - CO_2]^-$ (100); *m/z* 547 $[M - H - 2CO_2]^-$ (88); *m/z* 503 $[M - H - 3CO_2]^-$ (61) and *m/z* 459 $[M - H - 4CO_2]^-$ (18). The successive loss of four CO_2 (two from betalamic acid, one from *cyclo*-Dopa and the fourth from the malonylated moiety) was demonstrated, which indicates the presence of a dicarboxylic acid as acyl residue (Fig. 4.10).

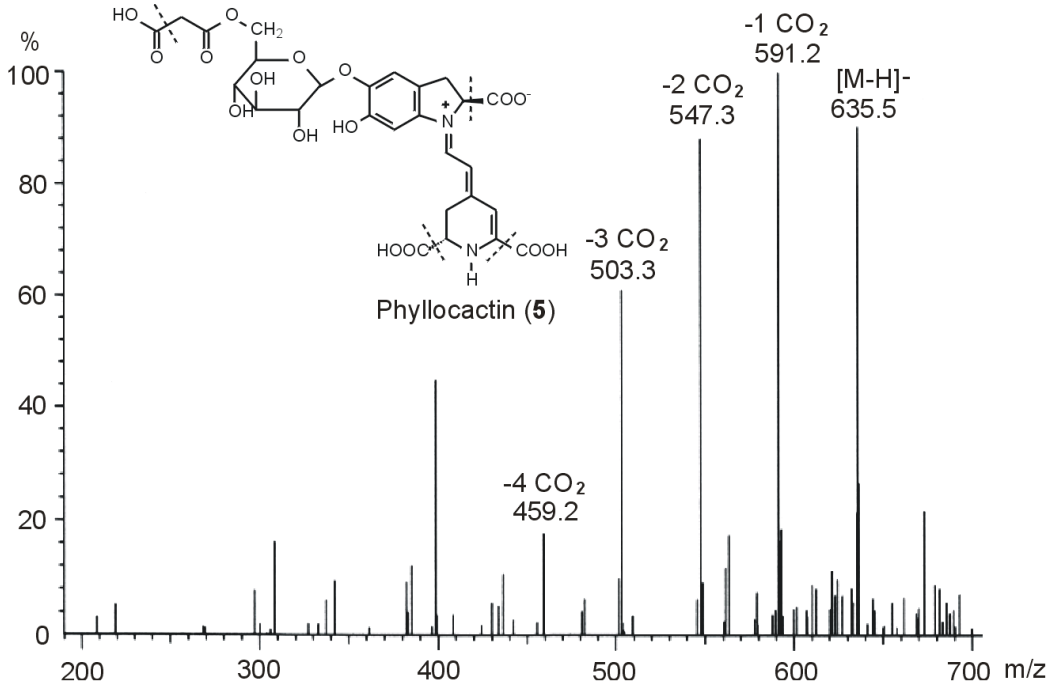


Fig. 4.10 LC-MS spectrum of phyllocactin (**5**) at negative ion mode

The characteristic signals of **5** in the 1D and 2D ^{1}H NMR data confirmed the presence of the aglycone, glucose and malonyl moieties (Table 4.6 and Fig. 4.14).

Table 4.6 Chemical shifts and couplings of compound **5** from ^{1}H NMR (600 MHz, CD_3OD/DC1)

δ (ppm)	Assignment
8.70	1H, *d*, $J_{11\text{-}12}$ = 12.3 Hz, H-11
7.38	1H, *s*, H-7
7.23	1H, *s*, H-4
6.47	1H, *s*, H-18
6.23	1H, *d*, H-12
5.46	1H, *dd*, $J_{2\text{-}3A}$ = 9.7 Hz, H-2
4.85	1H, *d*, $J_{1'\text{-}2'}$ = 7.6, H-1’
4.63	1H, *dd*, $J_{15\text{-}14A}$ = 5.3 Hz, $J_{15\text{-}14B}$ = 7.4 Hz, H-15
4.60	1H, *dd*, $J_{6'A\text{-}5'}$ = 2.0 Hz, $J_{6'A\text{-}6'B}$ = 12.0 Hz, H-6’A
4.33	1H, *dd*, $J_{6'B\text{-}5'}$ = 6.6 Hz, H-6’B
3.74	1H, dd, $J_{3A\text{-}3B}$ = 16.3 Hz, H-3A
3.73	*m*, H-5’
3.72	1H, *dd*, $J_{14A\text{-}14B}$ = 17.2 Hz, H-14A
3.56	*m*, H-3’
3.55	*m,* H-2’
3.49	1H, *dd*, H-3B
3.46	AB system, H-2’’
3.42	*m,* H-4’
3.31	1H, *dd*, H-14B

To characterize compound **5**, its fate under acidic conditions was analysed (Fig. 4.11). Treatment with 1 N HCl at room temperature and kinetic analysis by HPLC of the appearing reaction products showed both the conversion of **5** to **3** and the racemization of **5** to **5'**, thus confirming the identity of the minor compound **5'** in the extract as isophyllocactin (R_t 26.2 min and λ_{max} 537 nm).

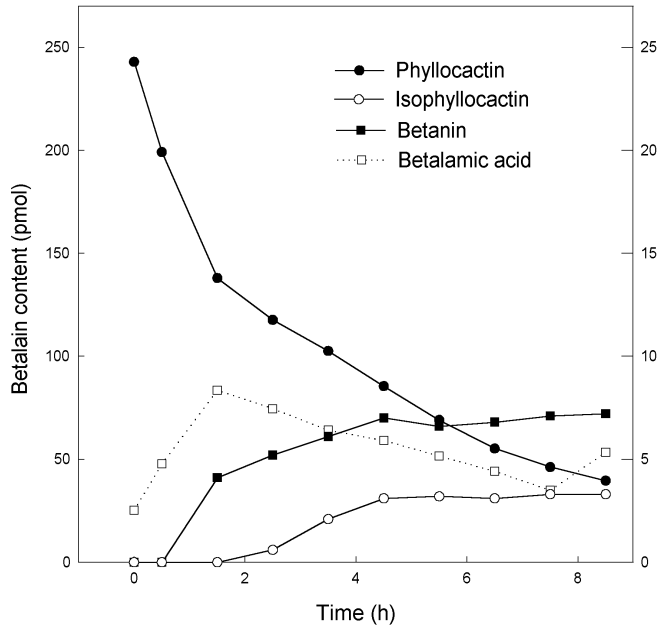


Fig. 4.11 Degradation and racemization of phyllocactin (**5**) by HCl treatment

Thus, compound **5** and **5'** was confirmed as betanidin 5-*O*-(6'-*O*-malonyl)-*β*-D- glucopyranoside (phyllocactin) and isobetanidin 5-*O*-(6'-*O*-malonyl)-*β*-D-glucopyranoside (isophyllocactin) (Fig. 4.12).

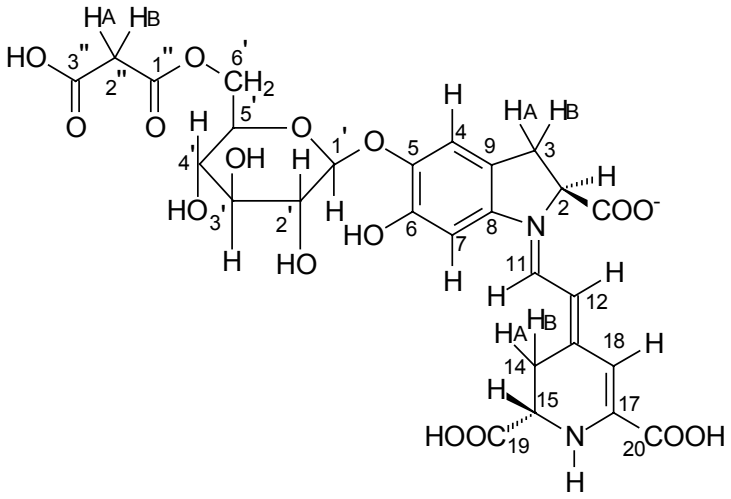


Fig. 4.12 Structure of phyllocactin (**5**)

Compound **6** was detected by HPLC-PDA at R_t 26.2 min and λ_{max} 537 nm. An unambiguous $[M+H]^+$ ion could not be determined. From the UV-VIS spectrum compound **6** must be a betacyanin but its structure could not be determined.

The second major betacyanin **7** (**7**, R_t 28.0 min, λ_{max} 538 nm; isoform **7'**, R_t 29.2 min, λ_{max} 537 nm) showed a protonated molecular ion at *m/z* 769 (100) and its daughter ion at *m/z* 389 (100) using positive ion mode LC-MS. In the negative ion mode the following ions were assigned: *m/z* 767 $[M - H]^-$ (27), *m/z* 723 $[M - H - CO_2]^-$ (81), *m/z* 679 $[M - H - 2CO_2]^-$ (100), *m/z* 635 $[M - H - 3CO_2]^-$ (97) and *m/z* 591 $[M - H - 4CO_2]^-$ (70). 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 later than **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 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 & 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** (Table 4.7 and Fig. 4.14).

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 (Fig. 4.13). Treatment of **7** with 1 N HCl caused racemization and degradation as observed for **5**; 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.

Table 4.7 Chemical shifts and couplings of compound **7** from ^{1}H NMR (600 MHz, CD_3OD/DC1)

δ (ppm)	Assignment
8.71	1H, *d*, $J_{11\text{-}12}$ = 12.3 Hz, H-11
7.38	1H, *s*, H-7
7.30	1H, *s*, H-4
6.47	1H, *s*, H-18
6.23	1H, *d*, H-12
5.45	1H, *dd*, $J_{2\text{-}3A}$ = 9.9 Hz, $J_{2\text{-}3B}$ = 2.1 Hz, H-2
5.36	1H, *d*, $J_{1'''\text{-}2'''}$ = 3.9 Hz, H-1'''
4.87	1H, *d*, $J_{1'\text{-}2'}$ = 7.4 Hz, H-1'
4.65	1H, *dd*, $J_{6'A\text{-}5'}$ = 2.0 Hz, $J_{6'A\text{-}6'B}$ = 11.9 Hz, H-6'A
4.63	1H, *dd*, $J_{15\text{-}14A}$ = 5.4 Hz, $J_{15\text{-}14B}$ = 7.3 Hz, H-15
4.32	1H, *dd*, $J_{6'B\text{-}5'}$ = 6.6 Hz, H-6'B
4.17	1H, *d*, H-4'''A
4.03	1H, *d*, H-2'''
3.84	1H, *d*, H-4'''B
3.73	1H, *dd*, H-3A
3.72	1H, *dd*, $J_{14A\text{-}14B}$ = 17.2 Hz, H-14A
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	1H, *dd*, H-3B
3.42	*m*, H-4'
3.31	1H, *dd*, H-14B

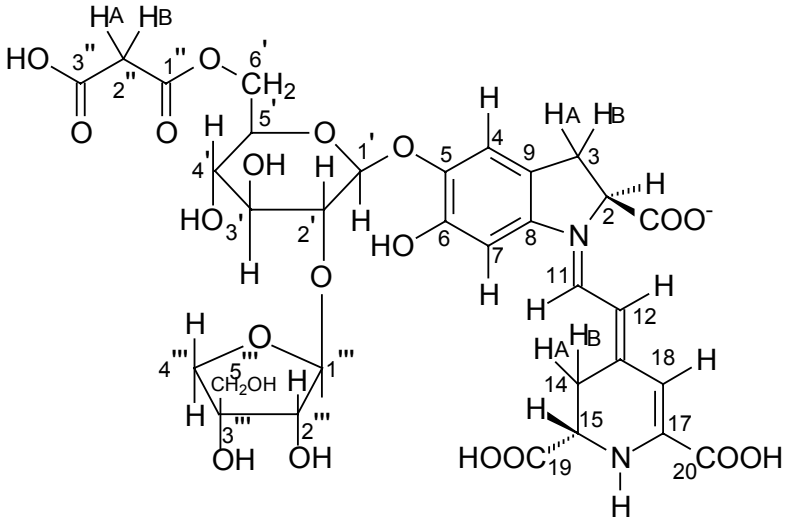


Fig. 4.13 Structure of 2'-apiosyl-phyllocactin (**7**)

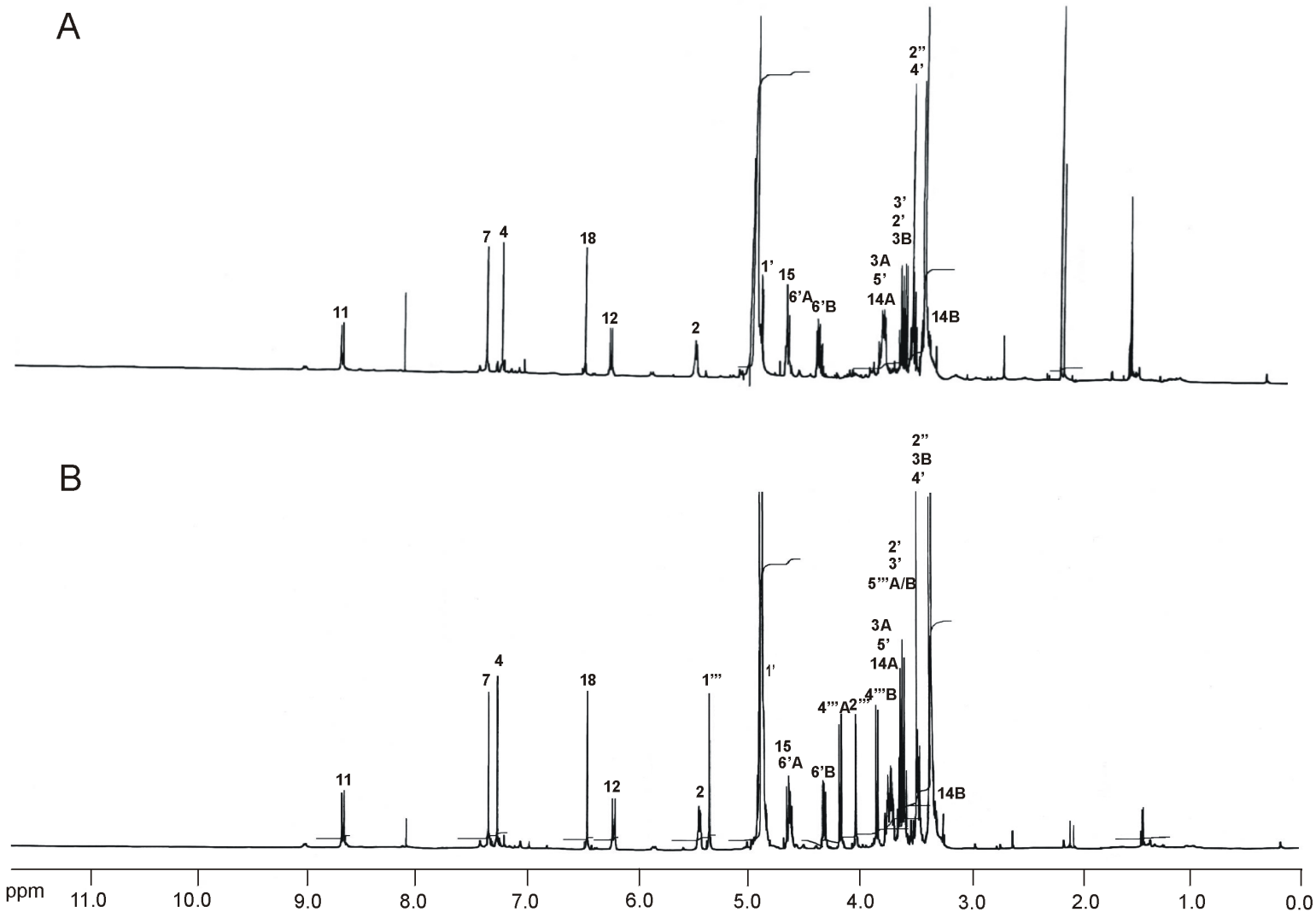


Fig. 4.14 ^{1}H NMR spectra of phyllocactin (**5**) and 2'-apiosyl-phyllocactin (**7**). A, phyllocactin; B, 2'-apiosyl-phyllocactin.

Compound **8** was detected by HPLC at R_t 28.3 min and λ_{max} 537 nm. The $[M+H]^+$ ion could not be detected. From the UV-VIS spectrum compound **8** must be a betacyanin, but its structure could not be determined.

Besides the major malonylated derivatives **5** and **7** in the petal extract, minor less polar betacyanins (**9-14)** have been detected (Fig. 4.9) and contains mostly a hydroxycinnamoyl moiety indicated by an absorption at 320 nm (Table 4.5). This substitution leads to a bathochromic shift of λ_{max} in the visible absorption (Heuer *et al*., 1994; Schliemann & Strack, 1998). Although the amounts of **9-14** were very low, some reliable structural information could be obtained.

The identity of compound **9** was confirmed by co-chromatography (HPLC) at R_t 32.0 min, λ_{max} 329/548 nm as 5''-*O*-*E*-feruloyl-2'-*O*-β-D-apiosyl-betanin which had been already identified from *Phytolacca americana* (Schliemann *et al*., 1996).

Compound **10** was detected by HPLC at R_t 32.5 min and λ_{max} 544 nm. The $[M+H]^+$ ion could not be determined. From the UV-VIS spectrum compound **10** must be a betacyanin but do not contain a hydroxycinnamoyl moiety.

Compound **11** was detected by HPLC at R_t 34.7 min and λ_{max} 331/551 nm. The $[M+H]^+$ ion could not be determined. From the UV-VIS spectrum compound **11** must be a betacyanin acylated with hydroxycinnamic acid, but its structure could not be determined.

The less polar compound **12** was detected by HPLC at R_t 34.9 min and λ_{max} 328/549 nm. In LC-MS the protonated molecular ion occurred at *m/z* 945 (rel. int. 30) and in daughter ion mode at *m/z* 756 (100). The mass difference of 86 between **12** (*m/z* 756) and **9** (*m/z* 859) is indicative for 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, 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-apiosylphyllocactin) (**12**) (Fig. 4.15).

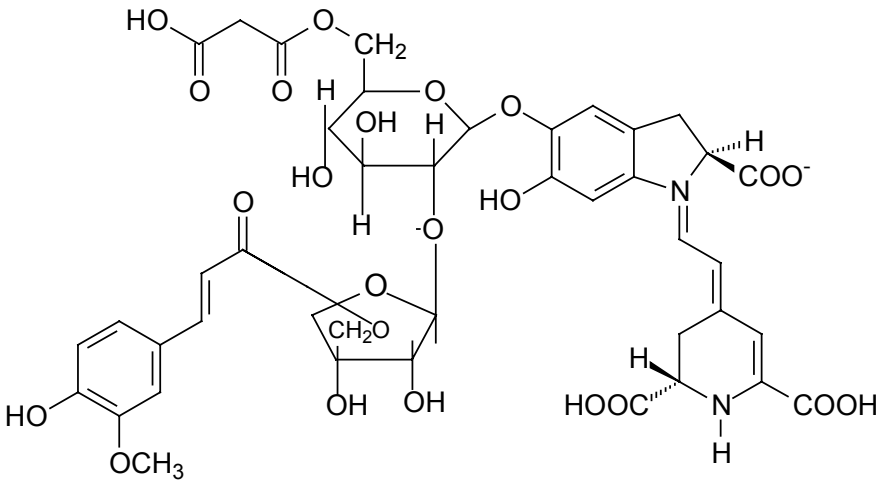


Fig. 4.15 Structure of 5''-*O*-*E*-feruloyl-2'-*O*-β-D-apiosyl-phyllocactin (**12**)

Compound **13** was detected by HPLC at R_t 35.3 min and λ_{max} 314/549 nm. The $[M+H]^+$ could not be determined. From the UV-VIS spectrum compound **13** must be a betacyanin acylated with hydroxycinnamic acid, but its structure could not be determined.

Compound **14** was detected by HPLC at R_t 35.6 min and λ_{max} 323/546 nm. The $[M+H]^+$ could not be determined. From the spectrum compound **14** must be a betacyanin acylated with hydroxycinnamic acid, but its structure could not be determined.

4.2.3 Betacyanin accumulation during flower development of Christmas cactus

As the malonyl derivatives phyllocactin and 2'-apiosyl-phyllocactin are the main pigments in the petals (Fig. 4.9), the question concerning the time points of betacyanin acylation during the flower development arose. Therefore, the flowers at different developmental stages were harvested and analysed (Fig. 4.16).

4.2.4 Betacyanin distribution in different organs of Christmas cactus

The comparative analysis of carpels, stamens and petals for betacyanin composition revealed quantitative differences (Table 4.8). The dark-red carpels contained nearly the 2-fold amount of total betacyanins [expressed as betanin equivalents/g fresh weight] as compared with petals and 36-fold higher amount than in stamens. Phyllocatin (**5**) is the main betacyanin in the petals and the carpels, while in the stamens contain low amounts. The less polar feruloylbetacyanins (**9**-**12**) are trace compounds in the petals and the carpels. In contrast, feruloylbetacyanins (**9**-**12**) are the predominant components in the extract from stamens (59.5%). Finally, a com-

parative pigment analysis of a petal extract from *Schlumbergera truncata* (formerly *Zygocactus truncatus*) revealed almost the same betalain pattern as that in petals of *S. buckleyi.*

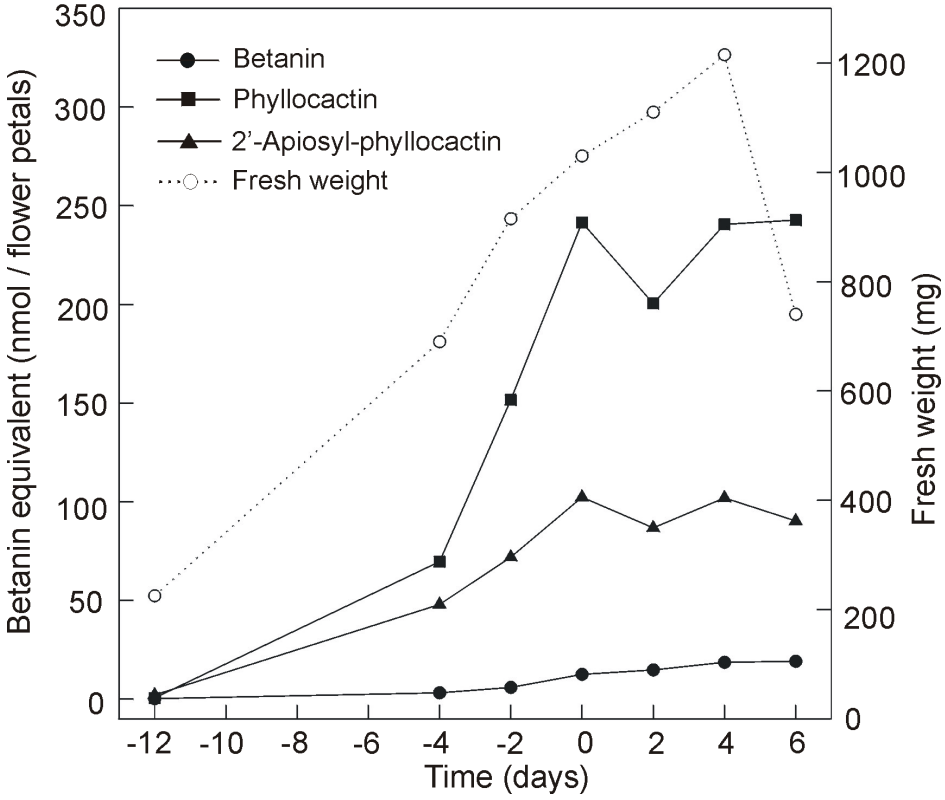


Fig. 4.16 Time course of the major betacyanins during flower development of the Christmas cactus [*Schlumbergera x buckleyi* (T. Moore) Tjaden]. (Day 0 is the time of flower opening. The decrease in fresh weight at day 6 is caused by wilting).

Table 4.8 Betacyanin patterns in different organs of the flowers of Christmas cactus flower. The number corresponds to peak numbers in Fig. 4.9 and the numbers in Table 4.5.

Flower organs	Total betacyanin content (µg betanin equivalents/ g fresh weight)	Relative content of [% of total betacyanins]				
		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

4.2.5 Betacyanins in flowers and fruits of Cactacea

The results of a comparative analysis of the betacyanin composition of petals and fruits of different Cactaceae is summarized in Table 4.9.

Table 4.9 Betacyanins in flowers and fruits of different cactus species

Plant material	Total beta-cyanin (BC) (nmol/g fw)	Betanin **3**	Phyllocactin **5**	2'-Apiosyl-phyllocactin **7** [% of total BC]	**9-10**	**11-14**
Schlumbergera. truncata						
Petals	305	7.5	61.2	16.1	0.5	2.4
Epiphyllum hybrid						
Petals	327	6.4	50.6	3.3	1.0	2.0
Gymnocalycium achirasense H. Till et S. Schatzl						
Petals	7	-	-	1.8	33.8	61.8
G. quehlianum (F. A. Haage jr.) Berger var. *zantnerianum* Schick						
Petals	19	6.2	6.5	5.2	32.2	49.9
Mammillaria duoformis Craig et Parson						
Petals	162	21.2	22.7	6.6	2.2	2.8
M. huajuapensis Bravo						
Petals	39	13.1	22.4	-	-	-
M. multiceps Salm-Dyck						
Petals	2	-	8.1	-	45.1	-
Fruits	97	11.3	-	-	-	-
M. muehlenpfordtii Först. (syn. *M. celsiana*)						
Petals	77	4.3	49.2	-	-	1.5
Fruits	68	8.0	3.9	-	-	0.4
M. perbella Hildm.						
Petals	31	3.7	3.9	-	-	1.2
M. pitcayensis Bravo Repp. 766						
Petals	94	9.2	22.8	3.5	3.8	5.1
M. saxicola Reppenhagen ML 73						
Petals	12	5.1	38.6	16.4	1.2	6.3
M. spec. camella						
Petals	29	27.9	48.3	1.5	-	-
Rebutia flavistyla Ritt. FR 756						
Petals	29	41.2	13.0	18.7	-	-
R. friedrichiana Rausch WR 646						
Petals	58	28.8	33.7	8.6	1.5	3.7
R. tarvitaensis Ritt.						
Petals	68	36.2	26.3	11.3	-	0.4
R. spec. KG 728						
Petals	121	28.8	31.9	13.4	0.5	1.0
Stenocereus queretaroensis						
Petals	72	5.9	14.3	4.2	12.2	30.8
Fruits	42	1.1	88.1	2.2	-	-

[a] in betanin equivalents; fw, fresh weight.

4.3 Condensation reaction between betalamic acid and amino acids

4.3.1 Experiments to catalyse the condensation reaction by protein extracts

Due to the occurrence of specific betaxanthin patterns in plants, the condensation reaction was assumed to be enzyme-catalysed. Therefore, protein extracts from hairy root cultures of yellow beets prepared according to Steiner *et al.* (1996) [$(NH_4)_2SO_4$ preparation], Eknamkul *et al.* (1997) and Terradas & Wyler (1991) (Acetone powder) were used in a photometric assay (at 470 nm) to monitor the betaxanthin formation from glutamine or phenylalanine and betalamic acid. No absorbance increase was found. Alternatively, the assay mixtures were analysed by HPLC, but betaxanthin formation could not be detected.

4.3.2 Hairy root cultures of yellow beet and seedlings of fodder beet

To analyse the betaxanthin formation *in vivo*, hairy root cultures of yellow beets were used as the experimental system. Hairy roots of yellow beets are orange coloured (Fig. 4.17). They contain as major compounds yellow betaxanthins and as minor compounds red betacyanins. The pigments of hypocotyls of fodder beet ('Altamo') at 28th day (Fig. 4.17) are qualitatively the same, but with trace amounts of betacyanins. For the following feeding experiments, the roots of fodder beets were removed and compounds were administered to the de-rooted seedling.

Fig. 4.17 Hairy root culture (BVL 5A) of yellow beets and fodder beet seedlings

4.3.3 HPLC pattern of betaxanthins from hairy root cultures of yellow beet

HPLC analysis of an extract from hairy roots of yellow beets revealed that the betalains mixture consisted predominantly of betaxanthins, a major (**2**) and a minor one (**1**) together with a lower portion (< 30 %) of different betacyanins (Fig. 4.18). Hempel and Böhm (1997) described the betaxanthins as portulacaxanthin II [(*S*)-Tyr-betaxanthin] and vulgaxanthin I [(*S*)-Gln-betaxanthin]. Whereas the latter (R_t 12.0 min; λ_{max} 468 nm) was confirmed as vulgaxanthin I by co-injection analysis, the major (**2**) betaxanthin (R_t 24.7 min; λ_{max} 457 nm) did not match synthetically prepared (*S*)-Tyr-betaxanthin (R_t 25.2 min; λ_{max} 469 nm; Schliemann *et al*., 1999).

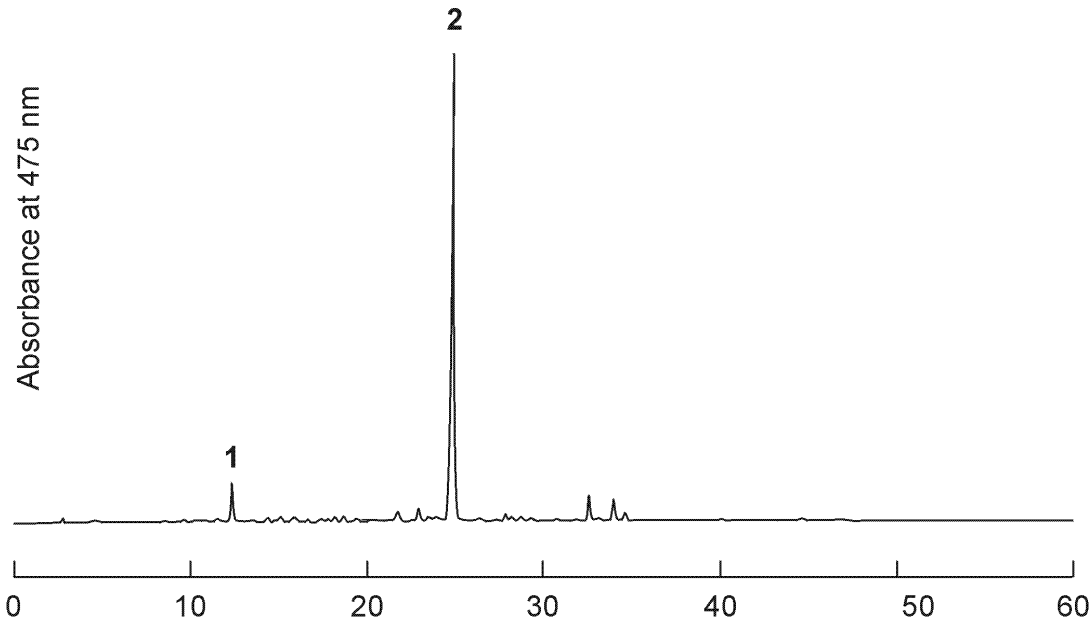


Fig. 4.18 HPLC profile of betalains in hairy root culture of yellow beets

Therefore, hairy root material was extracted and compound **2** was purified by conventional anion-exchange chromatography on Dowex 1 x 8 column (Strack *et al*., 1993) and by semi-preparative and analysed by HPLC-PDA, LC-MS and co-chromatography with synthetic dopamine-betaxanthin. Thus, compound **2** was identified as miraxanthin V (dopamine-betaxanthantin) (Fig. 4.19). A detailed identification is described in 4.1 Dopamine-derived betacyanins.

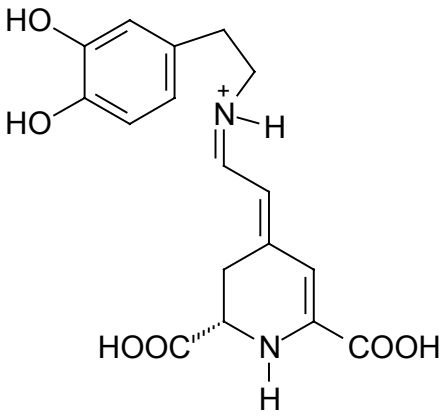


Fig. 4.19 Structure of miraxanthin V (**2**)

4.3.4 Betaxanthin accumulation and growth of yellow beet hairy roots

Both hairy root culture lines (5A and 7) of yellow beets showed the most intensive fresh weight increase between 7th and 9th day, which was paralleled by a steep increase in miraxanthin V content (Fig. 4.20). Vulgaxanthin I was a minor betaxanthin in hairy root culture of yellow beets.

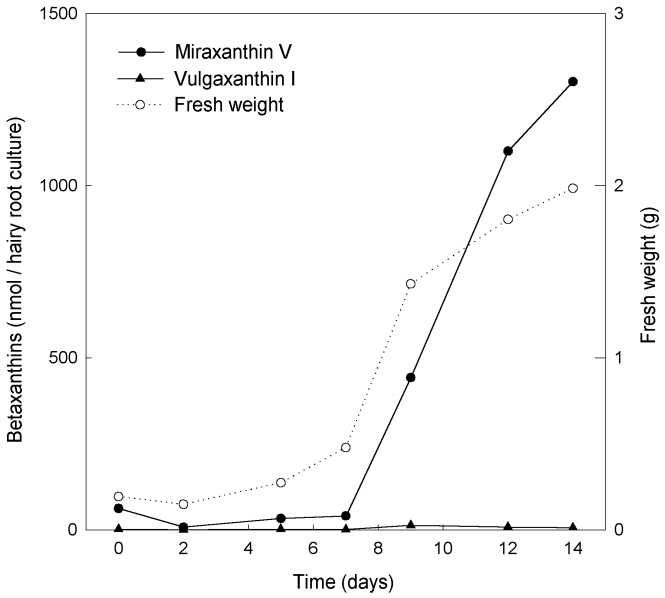


Fig. 4.20 Time course of growth (fresh weight) and betaxanthin content [miraxanthin V and vulgaxanthin I] in hairy root cultures of yellow beets

4.3.5 Retention time and HPLC-PDA data of synthetic betaxanthin standards and betaxanthins after feeding of amino acids and polyamines to hairy root cultures of yellow beet

Betaxanthin standards were necessary for the identification of the betaxanthins of amino acid and amine feedings to hairy root cultures and plants from *Beta*. According to Schliemann *et*

al. (1999) the betaxanthin standards were prepared by the hydrolysis of commercial lyophilised red beet juice (containing racemic betanin) with aqueous ammonia solution. After the acidification with HCl to the hydrolysed extract (pH 2), betalamic acid was extracted with ethyl acetate. After concentration, betalamic acid was reextracted with water. This solution was added to different amino acids and amines and gave after concentration directly the diastereoisomeric betaxanthins ([2*S/S*]- and [*2S/R*]-forms). These standards were compared with corresponding betaxanthins after feeding of amino acids (Table 4.10 and 4.11).

Table 4.11 Retention time and HPLC-PDA data of stereoisomeric betaxanthins (derived from Gly, (*S*)-amino acids and amines), betalamic acid and betanin (*Solvent system 1*)

Amino acid /Amine	Synthetic betaxanthin standards			Betaxanthins after feeding	
	R_t		HPLC-PDA[a]	R_t	HPLC-PDA
	2 *S/S*	2 *S/R*		2 *S/S*	
	min		λ_{max} *(nm)*	*min*	λ_{max} *(nm)*
His	8.6	8.4	472	9.3	472
Lys	9.5		468	10.0	468
Asn	10.6		469	10.7	468
Ser	11.1		466	11.4	466
Arg	11.5		468	12.2	468
Gln	12.0		468	12.7	468
Asp	12.5	12.3	467	12.8	469
Hyp	12.6		480	13.0	480
Gly	12.7[b]		465	12.9	465
Thr	13.5	13.3	468	13.9	467
Glu	14.9	14.7	468	14.7	467
Ala	17.4	16.7	465	18.4	475
Pro	19.8	19.3	477	20.1	475
(Betalamic acid)	21.8		405	21.8	405
Dopa	22.3	22.1	470	22.5	470
CDG[c] (betanin:isobetanin)	22.7	24.1	538	23.0	538
Dopamine	24.7[c]		457	25.7	457
Tyr	25.2	24.8	469	26.6	470
Val	26.4	25.0	467	25.8	466
Met	26.4	25.3	468	26.5	468
Tyramine	29.3[c]		459	29.7	458
Ile	33.0	31.5	466	32.2	466
Leu	34.0	32.7	468	33.0	467
Phe	34.5	33.3	472	35.4	471
Trp	37.3	36.8	472	37.5	470
Thiaproline	20.5		475	20.4	475

[a] λ_{max} of the (2 *S/S*)-form; [b] only S- and R-isomers of the BA moiety; [c] CDG, *cyclo*-Dopa 5-*O*-glucoside.

Table 4.11 Retention time and HPLC-PDA data of betaxanthins (derived from polyamines) (*Solvent system 1*)

Polyamines	Synthetic betaxanthin standards		Betaxanthin after feeding to seedlings	
	R_t (min)	HPLC-PDA [λ_{max} (nm)]	R_t (min)	HPLC-PDA [λ_{max} (nm)]
Ethylendiamine	5.2	466	5.1	467
Putrescine	5.0	465	-	-
	8.9	455	9.0	456
Cadaverine	10.9	471	-	-
	11.5	454	11.0	458
Octanediamine	22.5	453	22.0	460
	25.2	464	-	-
Spermidine	1.9	468	-	-
	2.6	472	-	-
	3.7	462	-	-
	4.1	459	-	-
Spermine	3.4	474	-	-
	5.4	467	-	-
	6.7	454	-	-

4.3.6 Feeding of amino acids and amines to hairy root cultures of yellow beet and seedlings of fodder beet

The results of (*S*)-amino acid feeding to a hairy root culture summarized in Table 4.12 showed that all amino acids were accepted in the formation of the corresponding betaxanthins, but to a different extent. Also (*S*)-4-thiaproline, a synthetic amino acid, led to formation of the respective betaxanthin.

The feeding of these amino acids and amines to fodder beet seedlings showed also the same patterns as those of hairy root cultures of yellow beets (Fig. 4.21).

In the case of polyamine feeding to hairy root cultures, the formation of corresponding betaxanthins could not be detected. In similar experiments with seedlings of fodder beet 'Altamo', only ethylendiamine, putrescine, cadaverine and octanediamine gave the respective betaxanthin, but in trace amounts (Table 4.14).

Table 4.12 Feeding of amino acids to hairy root cultures of yellow beet, line 5A

Amino Acid	Formed Betaxanthins		Constitutive Betaxanthins			
			Miraxanthin V		Betalamic acid	
(2 mM)	nmol/HRC	%Incorporation	nmol/HRC	% Control	nmol/HRC	% Control
d-7 Extract	-	-	998	-	327	-
Control	-	-	1457	100	345	100
Gly	98	0.15	1560	107	328	95
(*S*)-Ala	87	0.14	2477	170	431	124
(*S*)-Ser	210	0.34	2188	150	229	87
(*S*)-Thr	497	0.80	1471	101	209	61
d-7 Extract	-	-	1015	-	311	-
Control	-	-	2297	100	521	100
(*S*)-Leu	946	1.53	1543	67	265	43
(*S*)-Ile	603	0.97	1373	60	264	43
(*S*)-Val	426	0.69	1857	81	267	43
d-7 Extract	-	-	761	-	290	-
Control	-	-	1417	100	372	100
(*S*)-Gln	197	0.32	1672	118	343	92
(*S*)-Asn	162	0.26	1520	107	243	65
(*S*)-Glu	35	0.06	2448	172	329	88
(*S*)-Asp	28	0.05	1812	128	260	70
d-7 Extract	-	-	1289	-	312	-
Control	-	-	2271	100	319	100
(*S*)-Lys	128	0.21	2890	127	192	69
(*S*)-Arg	285	0.46	2184	96	142	45
(*S*)-Orn	84	0.14	1967	87	86	27
(*S*)-Met	479	0.77	1775	78	98	31
(*S*)-Trp	245	0.40	1792	79	92	29
d-7 Extract	-	-	896	-	446	-
Control	-	-	2958	100	272	100
(*S*)-Phe	646	1.04	1423	48	109	24
(*S*)-His	1204	1.94	2375	80	96	22
(*S*)-Pro	300	0.84	3127	106	255	57
(*S*)-Hyp	877	1.41	2184	74	93	21
Control	-	-	2326	100	520	100
(*S*)-ThiaPro	701	1.13	1192	51	465	89

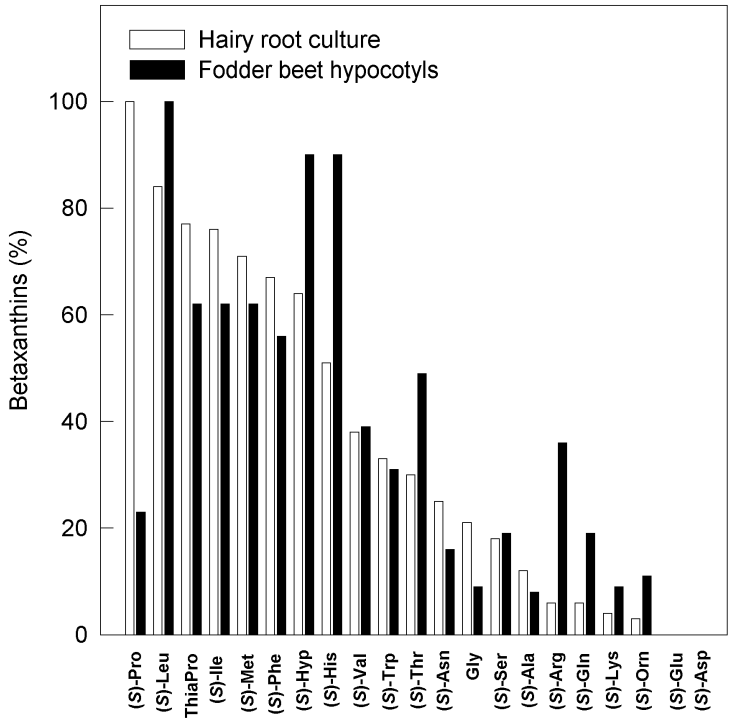


Fig. 4.21 Betaxanthin contents of feeding of amino acids to hairy root cultures of yellow beet and to seedlings of fodder beet 'Altamo'

Table 4.13 Feeding of (*S*)- and (*R*)-amino acid to hairy root cultures of yellow beet, line 5A

Amino Acid (2 mM)	Formed Betaxanthins		Constitutive Betaxanthins			
			Miraxanthin V		Betalamic acid	
	nmol/HRC	%Incorporation	nmol/HRC	% Control	nmol/HRC	% Control
d-7 Extract	-	-	764	-	268	-
Control	-	-	1444	100	392	100
(*S*)-Ala	105	0.17	2691	186	567	145
(*R*)-Ala	104	0.17	1308	91	189	48
(*S*)-His	769	1.24	1082	75	151	39
(*R*)-His	295	0.48	1686	117	304	78
d-7 Extract	-	-	n.d.[a]	-	n.d.	-
Control	-	-	1754	100	598	100
(*S*)-Leu	673	1.09	1464	83	232	39
(*R*)-Leu	744	1.20	1709	97	221	37
d-7 Extract	-	-	1141	-	71	-
Control	-	-	2117	100	57	100
(*S*)-Phe	566	0.90	1533	72	35	61
(*R*)-Phe	661	1.07	1698	80	33	58
d-7 Extract	-	-	342	-	n.d.	-
Control[b]	-	-	976	100	n.d.	n.d.
(*S*)-Val[b]	111	0.18	200	20	n.d.	n.d.
(*R*)-Val[b]	115	0.19	1047	107	n.d.	n.d.

[a] n.d., Not determined; [b] Hairy root culture, line 7, was used

Table 4.14 Feeding of polyamines to fodder beet 'Altamo'

Amine	Formed Betaxanthins	Constitutive Betaxanthins			
		Miraxanthin V		Betalamic acid	
(10 mM)	nmol/g fw	nmol/g fw	% Control	nmol/g fw	% Control
Control	-	114	100	231	100
Ethylendiamine	19	57	50	n.d.[a]	n.d.
Putrescine	6	53	46	44	19
Cadaverine	4	69	60	19	8
Octanediamine	4	11	10	n.d.	n.d.
Spermidine	-	27	24	12	5
Spermine	-	20	18	12	5

[a]n.d., not determined

4.3.7 Feeding of (*S*)-Phe and (*R*)-Phe to different cell cultures and fodder beet seedlings

Simultaneous application of (*S*)- and (*R*)-Phe to hairy root cultures unexpectedly yielded a (*S*)-Phe-betaxanthin/(*R*)-Phe-betaxanthin ratio of 10:1 (Fig. 4.22). The same pattern could also be observed in hairy root and suspension cultures of red beet and fodder beet 'Altamo' plants.

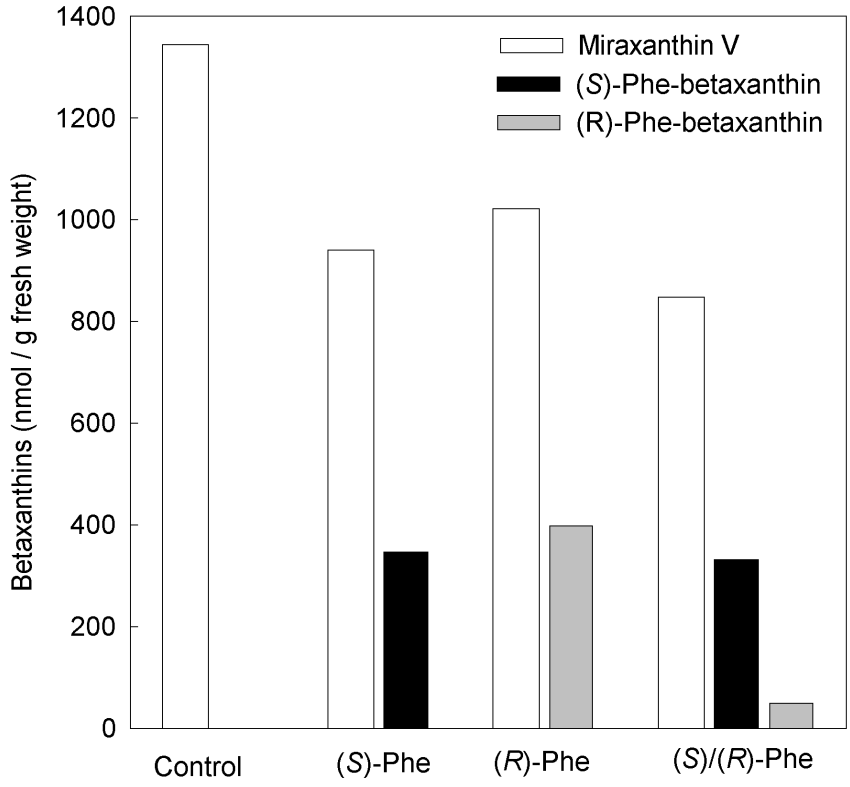


Fig. 4.22 Betaxanthin contents of feeding of (S)- and (R)-Phe alone and (*S*)/(*R*)-Phe to hairy root cultures, line 5A

This unexpected result could be clarified by uptake studies using (*S*)-[2,6-$^{3}H_2$]Phe/(R)-[1-^{14}C]Phe mixtures. The ^{3}H/^{14}C ratio of the compounds decreased in the nutrition solution within the feeding time (24h) from 4.4 to 0.75 (Fig. 4.23).

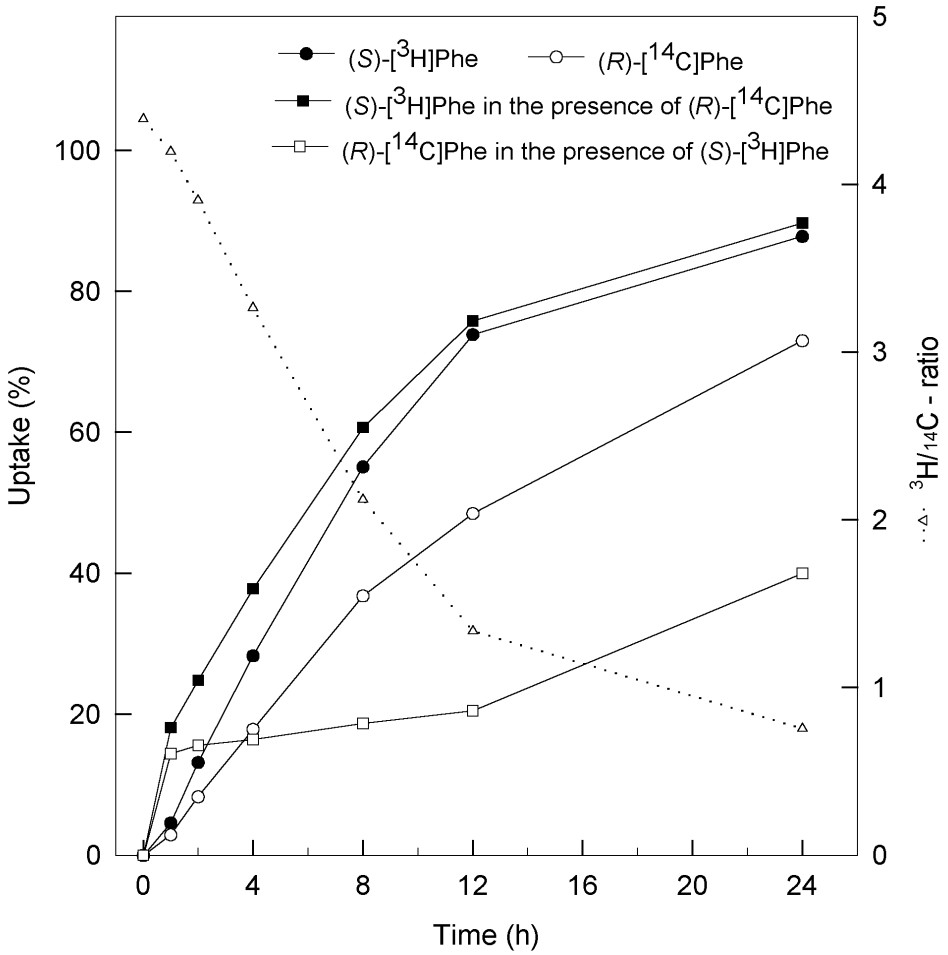


Fig. 4.23 Time course of uptake of ^{3}H-labeled (*S*)-Phe and ^{14}C-labelled (*R*)-Phe alone and both together by hairy root cultures of yellow beet at 7th day and ^{3}H/^{14}C ratio in the nutrition solution

4.3.8 Feeding of (*S*)-Thr, (*S*)-Ala, (*S*)-Leu and $(NH_4)_2SO_4$ to hairy root cultures of yellow beet

Feeding of (*S*)-Thr, an amino acid of high solubility, with final concentrations up to 50 mM in the nutrition solution to hairy root culture at 7th day, led to an increased (*S*)-Thr-betaxanthin formation (optimum, 10 mM (*S*)-Thr), with simultaneous decreased betalamic acid and miraxanthin V levels compared to the control (Fig. 4.24).

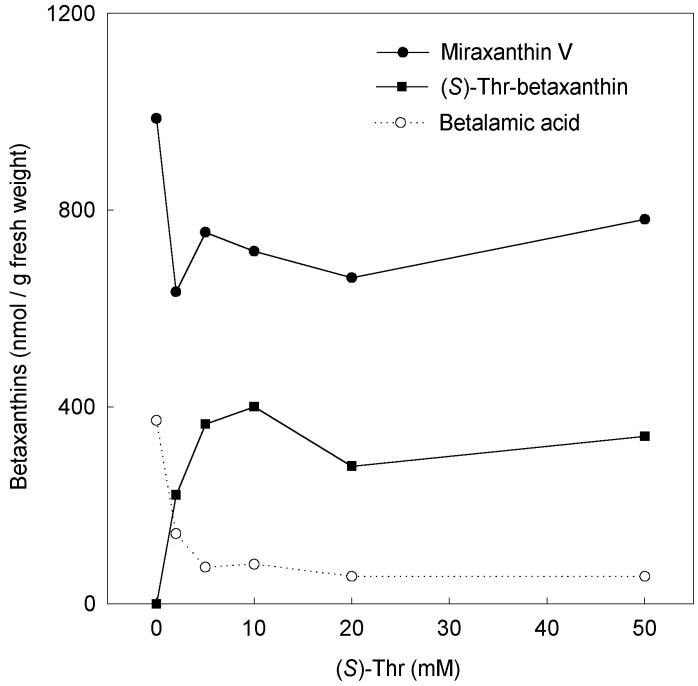


Fig. 4.24 Betaxanthin contents of feeding of (*S*)-Thr in increasing concentrations (final: 2-50 mM) to hairy root cultures of yellow beet

Feeding of (*S*)-Ala in increasing concentrations (2-50 mM) led, in addition to the formation of (*S*)-Ala-betaxanthin, to the appearance of an additional betaxanthin, the (*S*)-Gln-derived vulgaxanthin I, but not in the case of increasing concentrations of (*R*)-Ala (Fig 4.25)

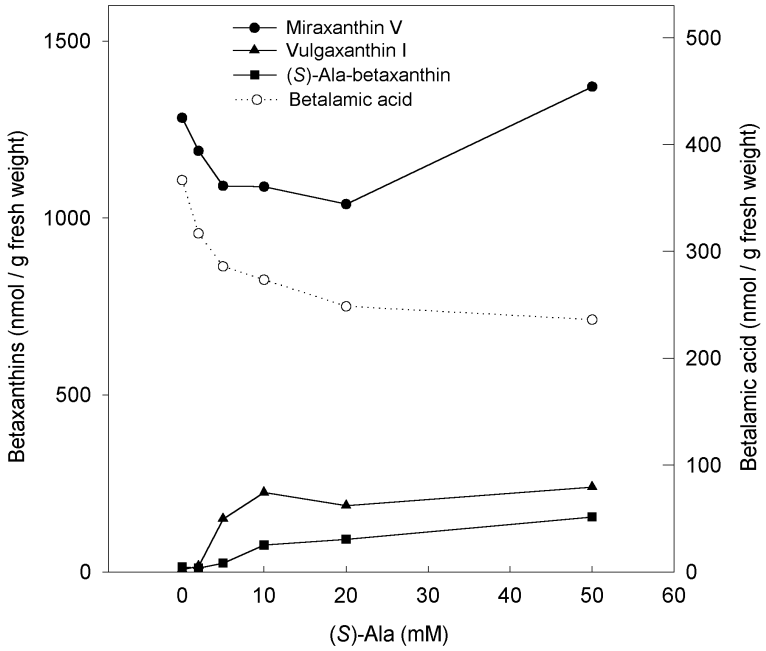


Fig. 4.25 Betaxanthin contents of feeding of (*S*)-Ala in increasing concentrations (final: 2-50 mM) to hairy root cultures of yellow beet.

The unexpected increase in vulgaxanthin I level was also induced by feeding of increasing concentration of $(NH_4)_2SO_4$ (Fig. 4.26).

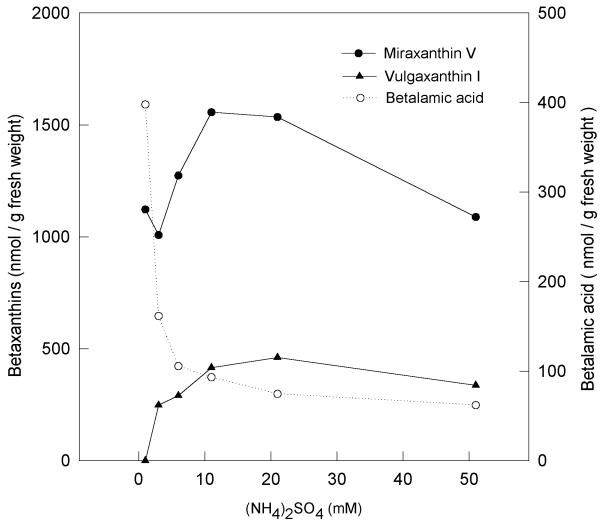


Fig. 4.26 Betaxanthin contents of feeding of $(NH_4)_2SO_4$ in increasing concentrations (final: 3-51 mM) to hairy root cultures of yellow beet

To suppress the miraxanthin V formation more efficiently, high amounts of (*S*)-Leu (5mM) were given daily to the hairy root culture between 4th and 8th day, and hairy roots were harvested 24 h after each addition. The strong increase of the miraxanthin V and betalamic acid content seen in the controls was totally suppressed, with a simultaneous increase in the (*S*)-Leu-betaxanthin level (Fig. 4.27).

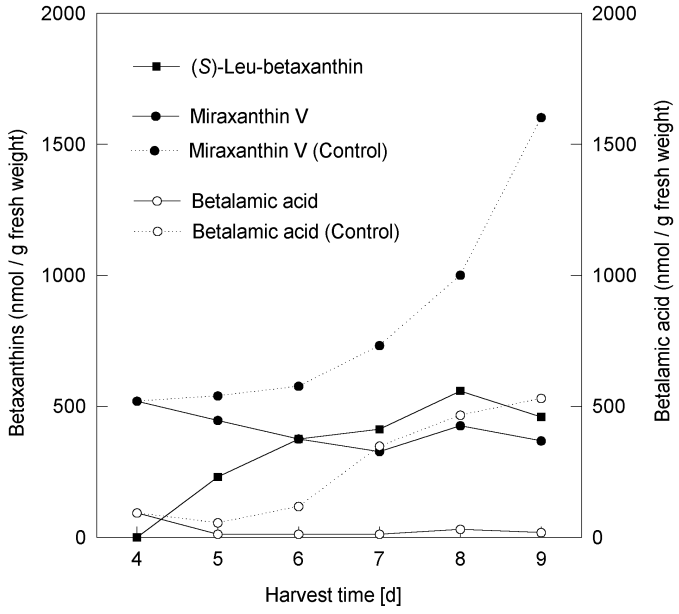


Fig 4.27 Betaxanthin contents of daily feeding of (S)-Leu (5mM) to hairy root cultures of yellow beet from 4th to 8th day

4.3.9 Feeding of 2-aminoindan 2-phosphonic acid (AIP) to hairy root cultures of yellow beet

Betaxanthin formation was affected indirectly without amino acid feeding. The addition of AIP, a strong inhibitor of PAL (EC 4.3.1.5) (Zon and Amrhein, 1992), to a hairy root culture, led to an increase of endogenous (*S*)-Phe level and, subsequently, (*S*)-Phe-betaxanthin. In the control culture (*S*)-Phe-betaxanthin was missing (Fig. 4.28). As AIP itself is an amino acid and could result in the formation of a derived betaxanthin, the AIP-betaxanthin was synthesized as the standard, but no ATP-betaxanthin was found in the extract after AIP feeding.

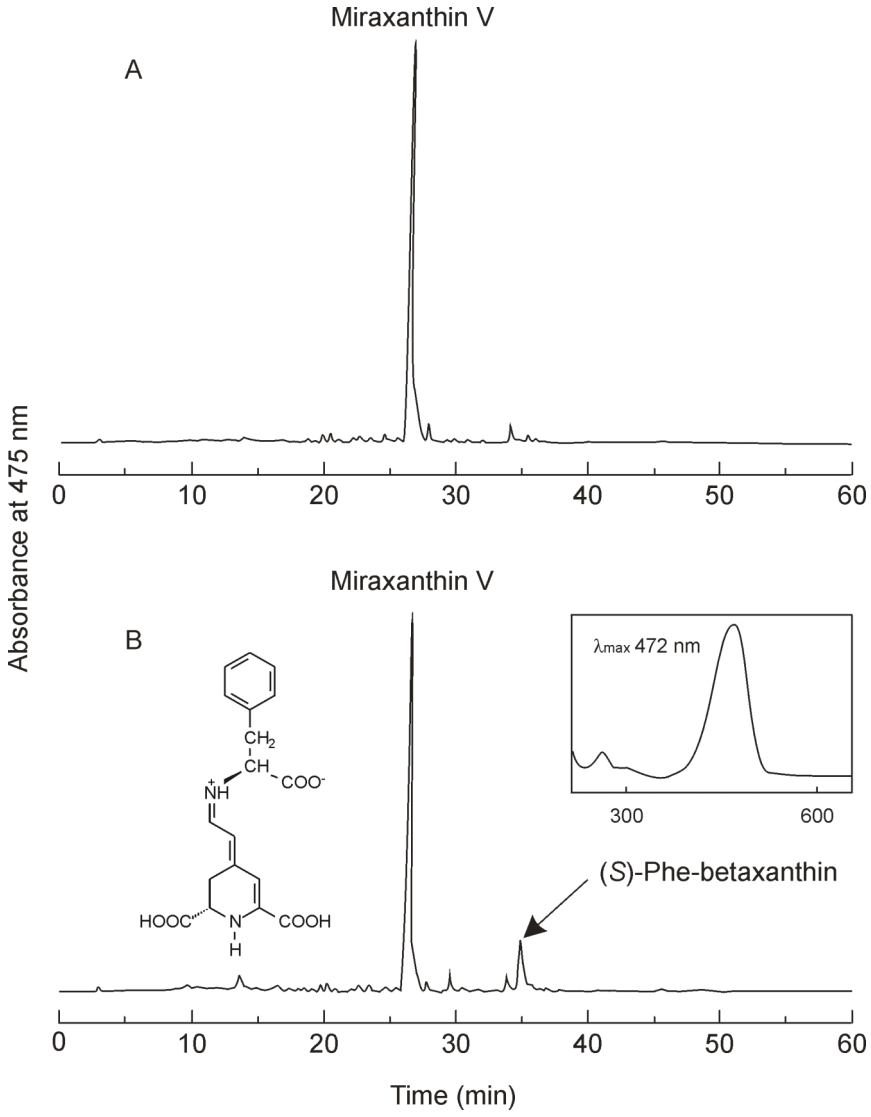


Fig. 4.28 HPLC profiles of the betaxanthins after daily feeding of AIP to hairy root culture of yellow beets from 4th to 7th day. A, Control; B, AIP feeding. Full scales of A_{475} 0.6 absorbance units. The insets in B are the PDA spectrum and the structure of the newly formed (*S*)-Phe-betaxanthin.

4.3.10 Feeding of betalamic acid to plants that do not belong to the Caryophyllales

Betalamic acid isolated from fodder beet hypocotyls and purified by preparative HPLC was fed in phosphate-buffered solution, pH 6.8, for 24 h to 2-week-old de-rooted broad bean and pea seedling via the hypocotyls (Schliemann et al., 1999). Although the uptake was low, HPLC analysis of the hypocotyl extracts of both plants showed the presence of betaxanthins, identified by their characteristic UV/VIS spectra. The major betaxanthin from the broad bean experiment (Fig. 4.29) had been identified as dopaxanthin (λmax 470 nm) by comparison with a synthetic standard. Amino acid analysis of hypocotyl extracts of broad bean seedlings revealed that Dopa was present at the highest concentration of all amino acids determined (Table 4.15).

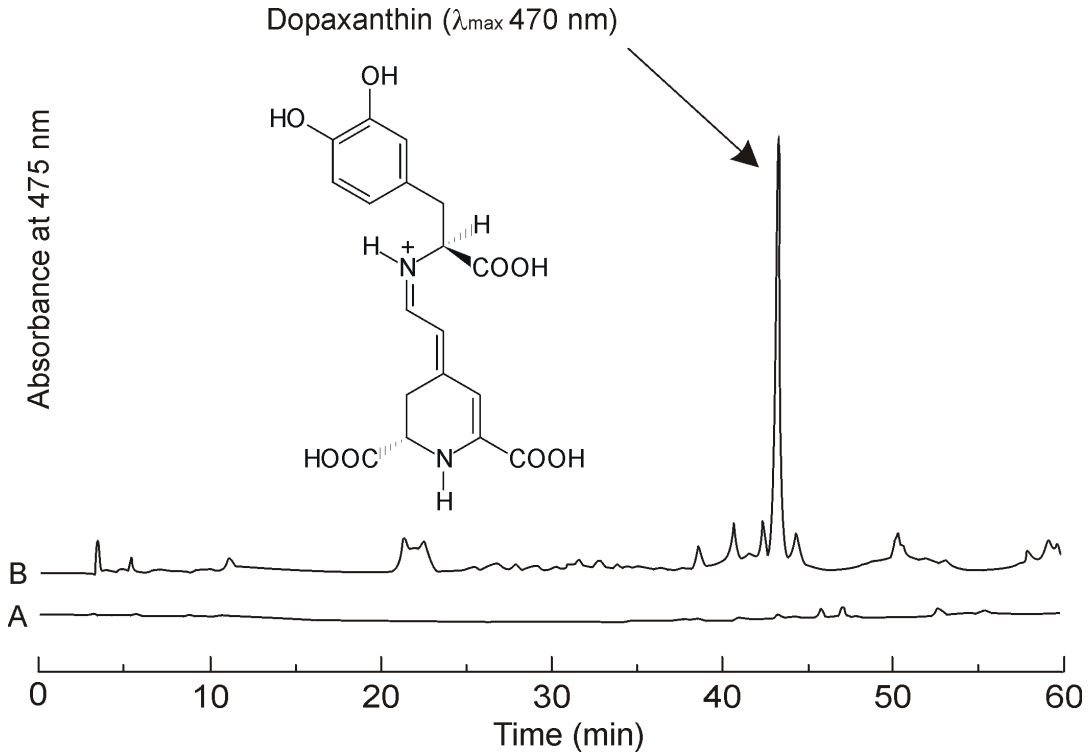


Fig. 4.29 HPLC profiles of betaxanthins after feeding betalamic acid to two de-rooted broad bean plants (14 days old) via the hypocotyls. A, Control; B, betalamic acid feeding. A_{475} (full scale 0.07 absorbance units) are the same in A and B (Schliemann *et al.* 1999).

Table 4.15 Amino acid analysis of extracts from hypocotyls of broad bean plants used in betalamic acid feeding experiments (Schliemann *et al.* 1999).

(*S*)-Dopa	(*S*)-Asp	(*S*)-Glu	(*S*)-Asn	(*S*)-Gln	(*S*)-His
		µmol/g fresh weight			
23.0	1.1	1.6	21.5	1.0	1.4

4.4 Transport of betaxanthins into red beet vacuoles

4.4.1 Microscopic analysis of red beet vacuoles

Vacuoles isolated from red beet hypocotyls were analysed microscopically (Fig. 4.30). The vacuoles contained mainly red betacyanins but also low amounts of betaxanthins. In the case of a mixture of both they appear orange. After 5 min neutral red treatment of red beet vacuoles they became dark red.

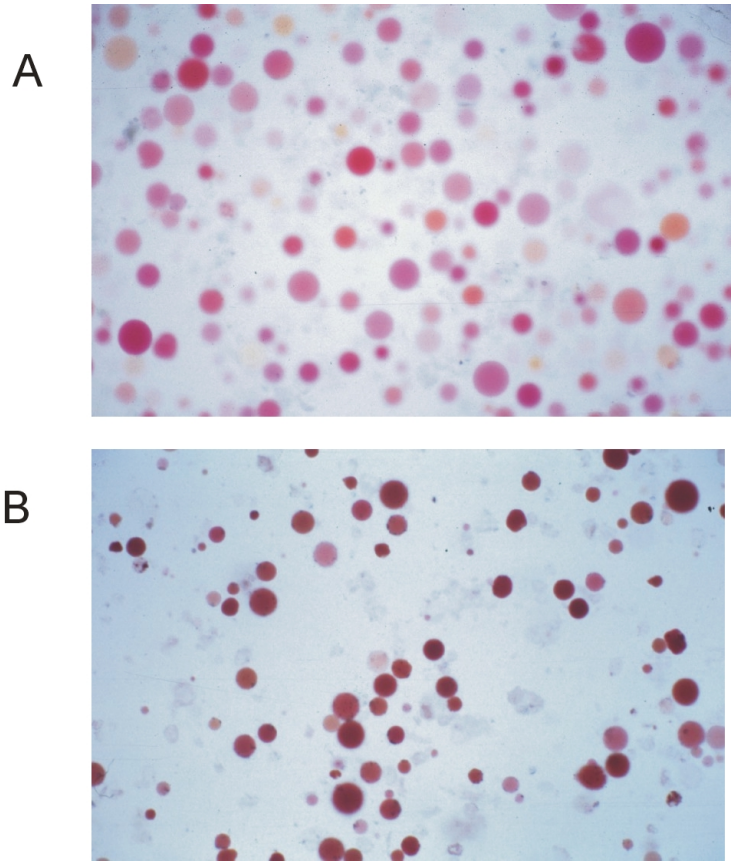


Fig. 4.30 Intact vacuoles isolated from red beet hypocotyls (A) and the neutral red treated vacuoles (B)

4.4.2 HPLC patterns of betalains in vacuoles and hypocotyls of red beet

HPLC analyses of extracts from hypocotyls and vacuoles of red beets (Fig. 4.31) revealed the presence of vulgaxanthin I (**1**, R_t 10.7 min, λ_{max} 468 nm), betalamic acid (**2**, R_t 18.4 min, λ_{max} 405 nm), betanin/isobetanin (**3**, R_t 20.1 min, λ_{max} 539 nm; **3'**, R_t 20.8 min , λ_{max} 536 nm), betanidin (**4**, R_t 21.8 min , λ_{max} 540 nm), miraxanthin V (**5**, R_t 23.3 min, λ_{max} 458 nm) and neobetanin (**6**, R_t 26.3 min, λ_{max} 474 nm) (*solvent system 1*).

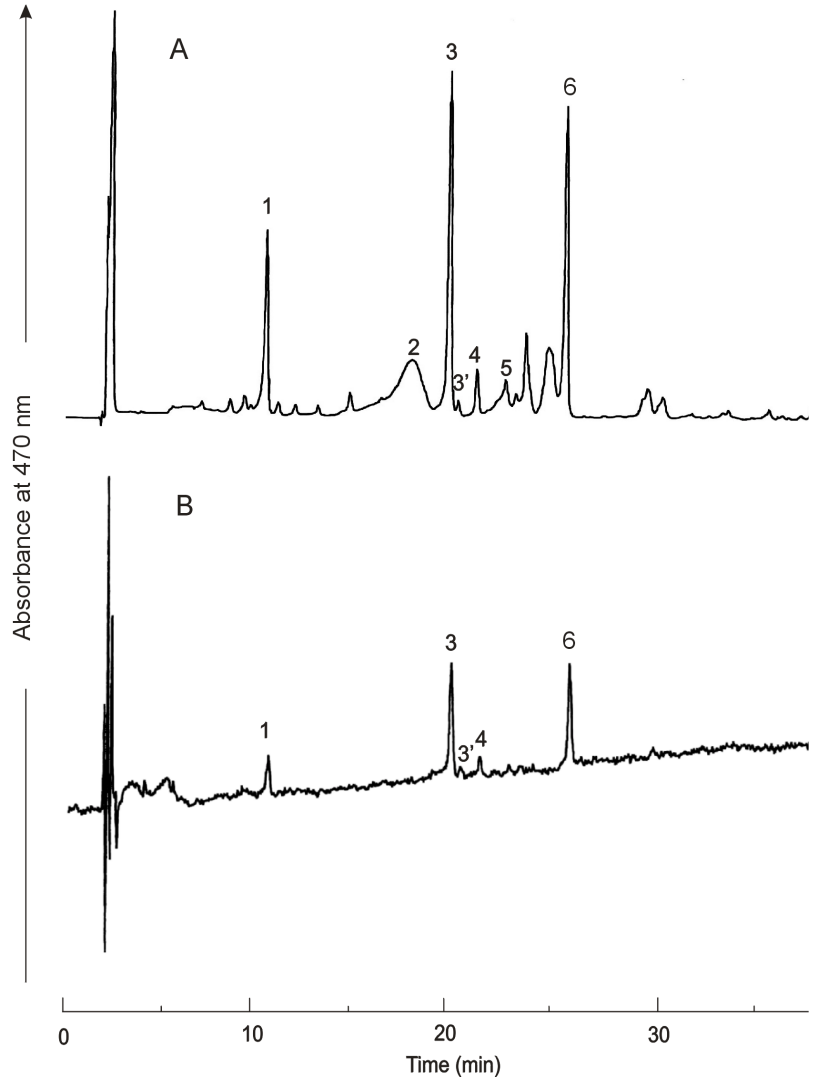


Fig. 4.31 HPLC profiles of betalains from red beet hypocotyls and vacuoles (detection at 470 nm). A, Betalains from hypocotyls; B, Betalains from vacuoles. Peak numbers: **1**, vulgaxanthin I; **2**, betalamic acid; **3**, betanin; **3'**, isobetanin; **4**, betanidin; **5**, miraxanthin V; **6**, neobetanin. Full scale: 0.0025 absorbance units. Note: Due to the detection at 470 nm betanin (λ_{max} 540 nm) under this condition do not appear as the strongly dominating compound.

4.4.3 Marker enzymes

To examine the contamination of the vacuole fraction, α-mannosidase localised in vacuoles and glucose 6-phosphate dehydrogenase localized in the cytosol but not in vacuoles, were used. α-Mannosidase and glucose 6-phosphate dehydrogenase were measured in both protoplasts and vacuoles (Fig. 4.32). The contamination of vacuoles by the cytosol fraction was *ca.* 9.1 %.

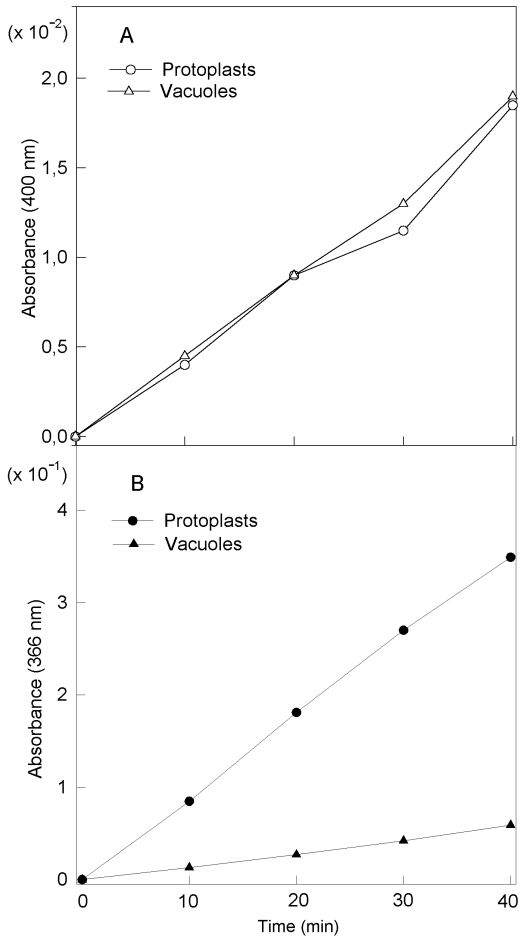


Fig. 4.32 Time course of α-mannosidase (A) and glucose 6-phosphate dehydrogenase (B) activities in red beet preparation of protoplasts and vacuoles

The number of protoplasts and vacuoles were counted under the microscope by using Thoma-hemocytometer. Protoplasts were 1047500 protoplasts/ml and vacuoles were 138750 vacuoles/ml.

4.4.4 Identification of miraxanthin V and (*R*)-Phe-betaxanthin after uptake into red beet vacuoles

After the uptake of miraxanthin V and (*R*)-Phe-betaxanthin, obtained at 0.5 µmol and 0.4 µmol, respectively, into red beet vacuoles the supernatants were analysed by HPLC (*solvent system 3*) and were identified by comparison of their retention times and spectra with authentic compounds. After 20 min of incubation, miraxanthin V was taken up in the presence of MgATP and eluted at 9.4 min with λ_{max} 466 nm, but not in the absence of MgATP (Fig. 4.33 A). (*R*)-Phe-betaxanthin was also taken up preferentially in the presence of MgATP and eluted at 26.3 min with λ_{max} 472 nm (Fig. 4.33 B). In the absence of MgATP (*R*)-Phe-betaxanthin was only slightly transported.

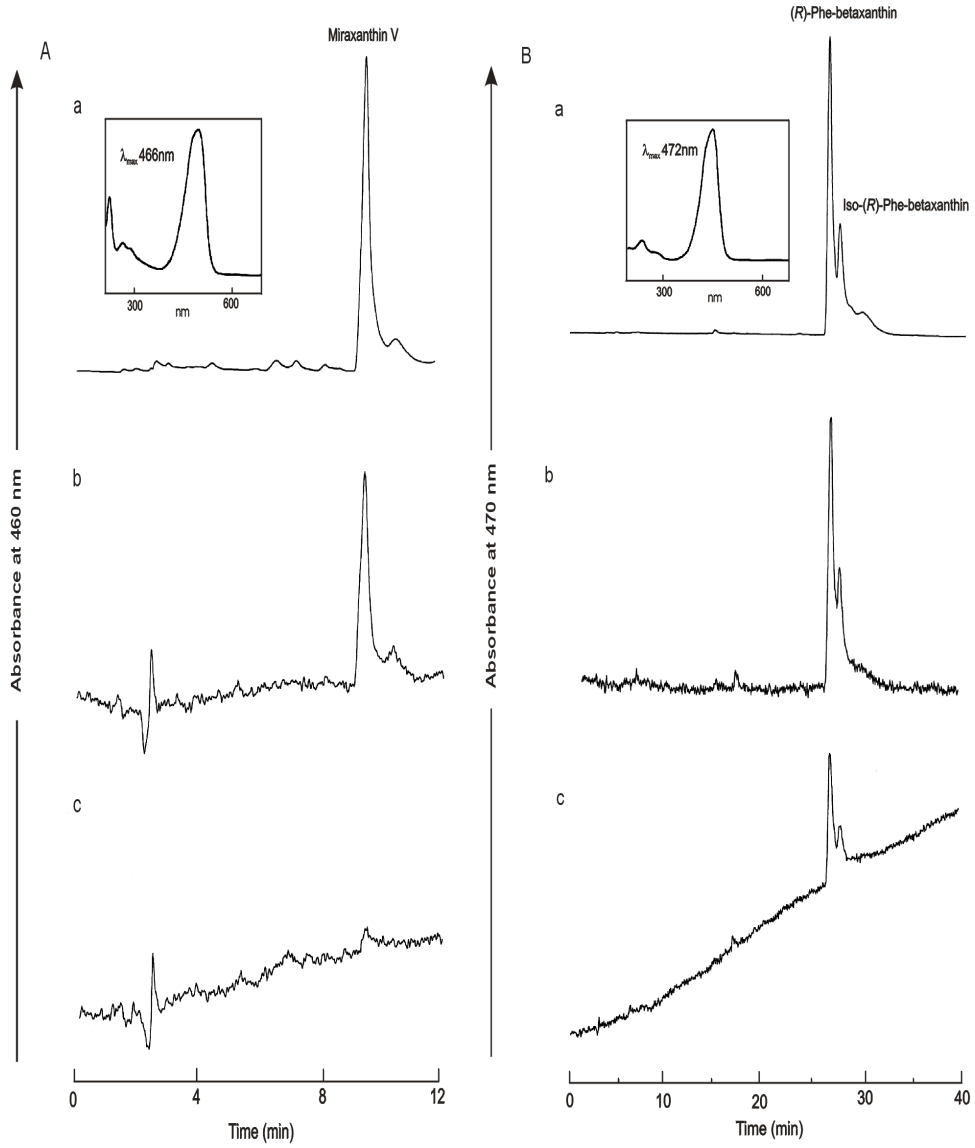


Fig. 4.33 HPLC profiles of vacuolar extracts after the incubation of 68 µM miraxanthin V (A) and 58 µM (*R*)-Phe-betaxanthin (B) with red beet vacuoles. Authentic standards (a); in the presence of 4 mM ATP and 5 mM Mg^{2+} (+ ATP) (b); 1 mM Mg^{2+} (- ATP) (c).

4.4.5 Uptake of miraxanthin V and (*R*)-Phe-betaxanthin in the presence of MgATP or ATP

To consider the possibility of an amino acid transport system, which is dependent on ATP but not on MgATP with a broad amino acid-specificity (Martinoia and Ratajczak, 1999), the uptake of miraxanthin V and (*R*)-Phe-betaxanthin were also performed in the presence of free ATP instead of MgATP (Fig. 4.34). The uptake of both miraxanthin V (A) and (*R*)-Phe-betaxanthin (B) was lower in the presence of 4 mM ATP (4) than in controls [time 0 (1) and

without MgATP (2)]. The uptake of miraxanthin V and (*R*)-Phe-betaxanthin was 2-3 times higher in the presence of MgATP (3) than in the presence of ATP (4) alone.

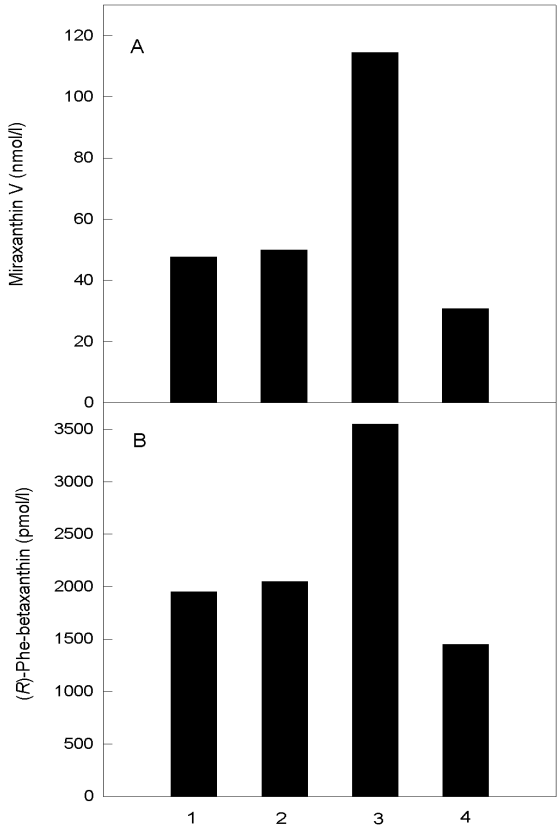


Fig. 4.34 Incubation of 68 µM miraxanthin V (A) and 58 µM (*R*)-Phe-betaxanthin (B) with red beet vacuoles in the presence of 1 mM Mg^{2+} for 4 min (1), 1 mM Mg^{2+} for 24 min (2), 4 mM ATP and 5 mM Mg^{2+} for 24 min (3) and 4 mM ATP for 24 min (4).

4.4.6 Kinetics of miraxanthin V and (*R*)-Phe-betaxanthin uptake into red beet vacuoles

The time-dependent uptake of miraxanthin V and (*R*)-Phe-betaxanthin into red beet vacuoles was strongly increased in the presence of 4 mM MgATP (Fig. 4.35). The uptake of miraxanthin V (A) in the presence and absence of MgATP increased for about 20 min. A lag phase of a first few minutes was observed and a saturation reached at 20 min. The uptake rate of miraxanthin V was 1.03 nmol/l/min in the presence of MgATP, while the rate in the absence of MgATP was 0.24 nmol/l/min. In contrast, the uptake of (*R*)-Phe-betaxanthin steadily increased up to 25 min in the presence and absence of MgATP. After 25 min the vacuoles were destroyed as checked microscopically, which made the further measurements impossible. The (*R*)-Phe-betaxanthin uptake rate in the presence of MgATP was 27.8 pmol/l/min, whereas the uptake rate was 12.4 pmol/l/min in the absence of MgATP. The lag phases in the uptake of miraxanthin V and (*R*)-Phe-betaxanthin might be due to the slow mixing of the reaction mixture and vacuoles because of high Percoll concentrations.

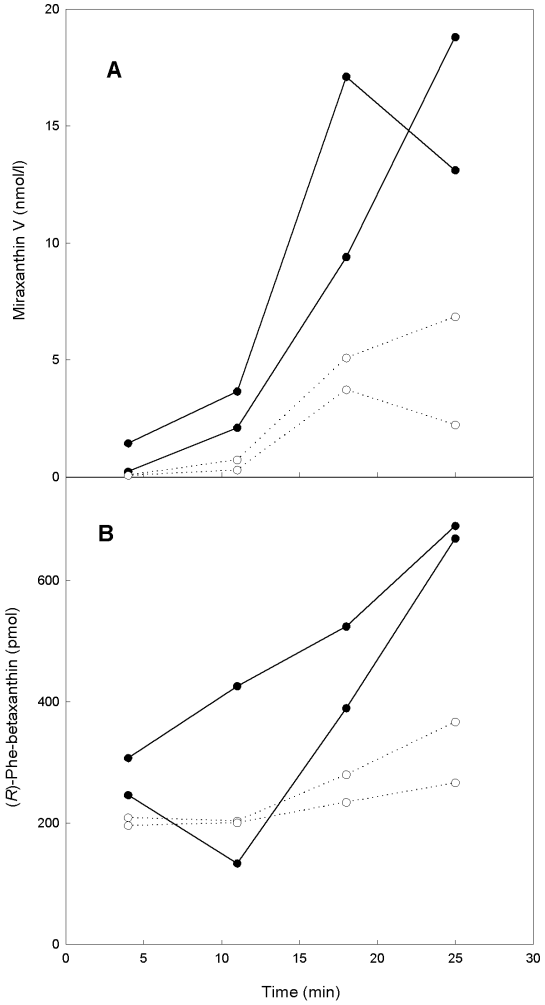


Fig. 4.35 Time-dependent uptake of 35 µM miraxanthin V (A) and 37 µM (*R*)-Phe-betaxanthin (B) into red beet vacuoles in the presence of 4 mM ATP and 5 mM Mg^{2+} (+ ATP, ●) and 1 mM Mg^{2+} (- ATP, ○). The experiments were performed in duplicate.

4.4.7 Inhibition of betaxanthins uptake by different inhibitors

Differences in the transport systems between miraxanthin V and (*R*)-Phe-betaxanthin could also be observed under treatment with different inhibitors in the presence of MgATP (Table 4.16). Vanadate, an inhibitor which acts as a phosphate analogue on P-type ATPase and specifically inhibits ABC-type transport, strongly inhibited the uptake of miraxanthin V (43 %) and vulgaxanthin I (73 %) but not that of (*R*)-Phe-betaxanthin (3 %). Bafilomycin A1, a highly specific inhibitor of the vacuolar membrane H^+-ATPase, does not inhibit an ABC-type transporter and strongly inhibited the uptake of (*R*)-Phe-betaxanthin (89 %) but not that of miraxanthin V (0 %) and vulgaxanthin I (0 %). NH_4Cl, a protonophore dissipating the pH-gradient across the tonoplast, inhibited the uptake of (*R*)-Phe-betaxanthin (75 %) but not that of miraxanthin V (27 %) and vulgaxanthin I (30 %). Vulgaxanthin I showed essentially the same inhibition characters as miraxanthin V.

Table 4.16 Effect of different inhibitors on the uptake of miraxanthin V, vulgaxanthin I and (*R*)-Phe-betaxanthin into red beet vacuoles. Red beet vacuoles were incubated in the presence of miraxanthin V (I, 68.4 µM; II, 67.3 µM; III, 65.7 µM), vulgaxanthin I (I, 53.0 µM; II, 57.2 µM; III, 71.1 µM) and (*R*)-Phe-betaxanthin (I, 48.8 µM; II, 47.8µM; III, 53.9 µM), 4 mM ATP, 5 mM Mg^{2+} (+ ATP), 1mM Mg^{2+} alone (- ATP) and the inhibitor as described in the table. The values of vulgaxanthin I transport are corrected by subtraction of the endogenous vulgaxanthin I content.

Experiments		Miraxanthin V (pmol)				Vulgaxanthin I (pmol)				(*R*)-Phe-betaxanthin (pmol)			
		I	II	III	Mean%	I	II	III	Mean%	I	II	III	Mean%
- ATP	1	33.8	50.2	5.9		119.1	13.4	50.8		15.1	22.5	26.0	
	2	19.9	47.3	8.2		118.9	9.6	34.6		8.2	10.1	33.6	
Mean (%)		26.9 (37)	42.1 (67)	7.1 (11)	38%	119.0 (75)	11.5 (8)	42.7 (39)	40%	11.7 (22)	16.3 (8)	29.8 (27)	19%
+ ATP	1	73.3	66.5	74.9		172.7	140.5	95.1		57.4	197.8	140.6	
	2	70.8	69.8	55.0		146.6	155.1	125.1		48.0	192.0	83.1	
Mean (100%)		72.1	61.3	65.0	100%	159.7	147.8	110.1	100%	52.7	194.9	111.9	100%
+ ATP + vanadate	1	46.8	29.3	38.2		13.6	42.1	36.7		47.3	161.0	164.6	
(1 mM)	2	34.9	30.6	45.5		20.4	45.5	47.4		75.2	104.7	76.3	
Mean (%)		40.9 (57)	30.0 (49)	41.9 (64)	57%	17.0 (11)	43.8 (30)	42.1 (40)	27%	61.2 (116)	132.9 (68)	120.4 (108)	97%
+ATP +bafilomycinA1	1	42.1	75.2	72.9		132.3	117.2	162.3		4.1	19.8	19.4	
(0.1 µM)	2	72.6	70.0	89.5		137.8	176.5	113.7		9.8	10.7	9.0	
Mean (%)		57.4 (80)	62.9 (103)	81.2 (124)	102%	132.8 (83)	146.9 (99)	138.0 (125)	102%	7.0 (13)	15.3 (8)	14.2 (13)	11%
+ ATP + NH_4Cl	1	47.6	52.9	25.6		117.2	114.7	97.1		10.8	58.0	30.5	
(5 mM)	2	43.4	72.0	42.8		82.7	103.5	61.5		7.0	42.7	38.5	
Mean (%)		45.5 (63)	62.3 (102)	34.2 (53)	73%	99.9 (63)	109.1 (74)	78.3 (73)	70%	8.9 (17)	50.4 (26)	34.5 (31)	25%

4.4.8 Formation of (*R*)-Phe-betaxanthin in red beet evacuolated mini-protoplasts

In order to examine the localization of the condensation reaction, red beet mini-protoplasts were used (Fig. 4.36). For sequential uptake of (*R*)-Phe and betalamic acid, mini-protoplasts are prepared from protoplasts by ultra-centrifugation.

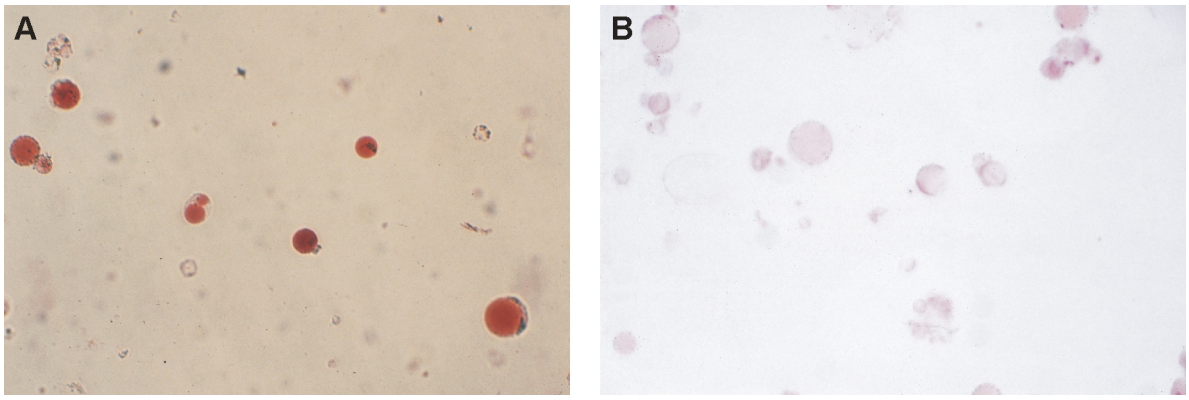


Fig. 4.36 Protoplast (A) and mini-protoplasts (B) from red beet hypocotyls after 5 min neutral red treatment

The extract of the mini-protoplasts which had been treated with 2 mM (*R*)-Phe were analysed by HPLC and the presence of (*R*)-Phe (R_t 3.7 min, λ_{max} 225/278 nm; *solvent system 2*) was detected. After uptake of betalamic acid (0.04 nmol) into these mini-protoplasts in the presence of 4 mM MgATP, (*R*)-Phe-betaxanthin was detected at R_t 25.9 min, λ_{max} 472 nm. The compound was identified by comparison of its retention time and UV-VIS spectrum with the standard. However, its formation could not be detected in the absence of MgATP.

4.5 Microspectrophotometric analyses of beet hypocotyls

During the transport studies, the interest arose to get information of the pigment localization in intact beet plants. Therefore, sections of hypocotyls from beet plants were analysed by microspectral microscopy. In the sections of red beet hypocotyls, the outer small cells were intensive red (λ_{max} 540 nm) and contained only betacyanins. The middle large cells were pink, and exhibit λ_{max} 535 nm and λ_{max} 421 nm. The latter was identical with betalamic acid standard under the microscope. These cells contained both betacyanins and betalamic acid. The inner small cells were red and showed λ_{max} 536 nm, which contained only betacyanins (Fig. 4.37). The localization of individual betacyanins is unknown.

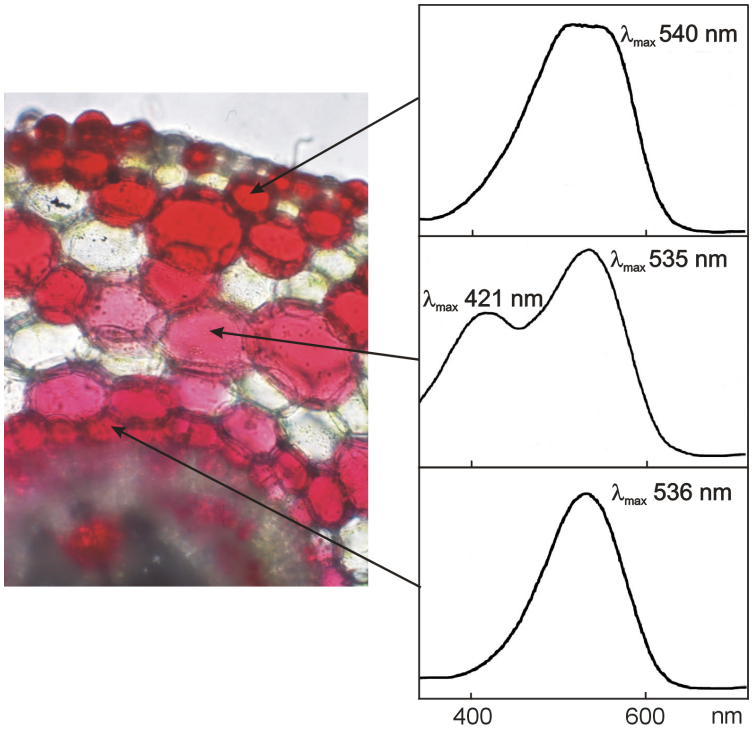


Fig. 4.37 Spectra of pigments in outer, middle and inner cells of red beet hypocotyls

Three different spectra could be observed from the cross section of fodder beet hypocotyls (Fig. 4.38). The outer small cells were intensive yellow, the maximal absorption was at λ_{max} 472 nm which indicate the presence of betaxanthins. From HPLC data this betaxanthin was mainly miraxanthinn V. The middle layer cells were large and pale yellow (λ_{max} 421 nm with a shoulder). These cells contained betalamic acid mainly and betaxanthins. The inner cells were large and pale yellow (λ_{max} 419 nm) corresponding to betalamic acid.

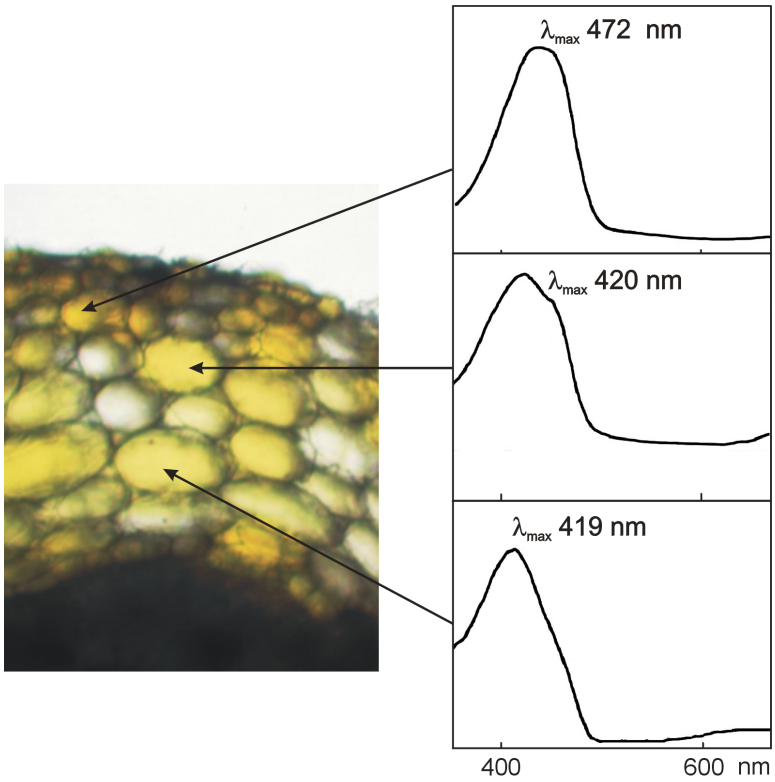


Fig. 4.38 Spectra of pigments in outer, middle and inner cells of fodder beet hypocotyls

After feeding of dopamine to fodder beet hypocotyls, three different spectra could be observed from the cross section of the hypocotyls (Fig. 4.39). The cells contained orange (λ_{max} 420/535 nm), yellow (λ_{max} 472 nm) and red (λ_{max} 423/524 nm) pigments, which correspond to betalamic acid (λ_{max} 420 nm), betacyanins (λ_{max} 535 nm) and betaxanthins (λ_{max} 472 nm) (Fig 4.39 A). From the cross section of hairy roots at 14th day, the outer layer cells contained betacyanins, the middle cells contained the mixture of betacyanins, betalamic acid and betaxanthins, and the inner cells contained betaxanthins (Fig. 4.39 B). In the young hairy roots (7th day) the red pigmentation of outer layer cells was very weak, but intensive orange middle layer cells were present (Fig. 4.39 C).

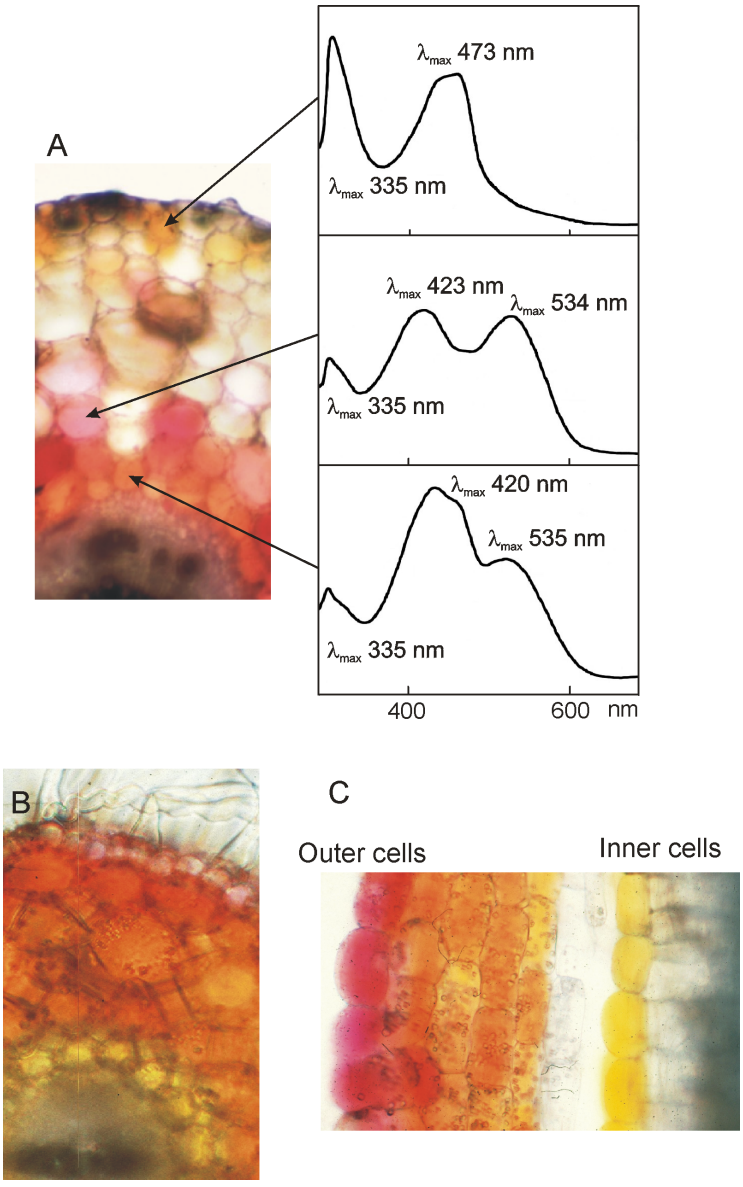


Fig. 4.39 Spectra of pigments in outer, middle and inner cells layers of dopamine fed fodder beet hypocotyls (A), cross section (B) and longitudinal sections (C) of hairy roots

5 Discussion

5.1 Betalain biosynthesis

In contrast to the well-characterized genes and enzymes involved in anthocyanin biosynthesis (Heller and Forkmann, 1993), there are only two enzymes known to be involved in betalain biosynthesis: tyrosinase, which is responsible for the formation of dopa and *cyclo*-dopa (Mueller *et al*., 1996; Steiner *et al*., 1996, 1999) and dopa dioxygenase, which catalyses the dopa extradiol cleavage, leading to the formation of the chromophor betalamic acid (Girod and Zryd, 1991a; Terradas and Wyler, 1991; Hinz *et al*., 1997; Mueller *et al*., 1997a, b) (Fig. 1.2). Two further steps had to be clarified. (1) the condensation reaction between betalamic acid and amino acids (including *cyclo*-dopa) and amines (i.e. aldimine formation) and (2) the possible glucosylation of *cyclo*-dopa before the condensation with betalamic acid as an alternative to the glucosylation of betanidin (Heuer and Strack, 1992; Heuer *et al*., 1996; Vogt *et al*., 1997).

5.2 Dopamine-derived betacyanins

For a complete characterization of our experimental system, the betalains have been isolated and structurally elucidated. Due to the relatively low concentrations of these compounds, the feeding of dopamine to fodder beet seedlings was used, which led to the formation of betacyanins with retention time and UV-VIS spectral properties in HPLC identical with those of the minor betacyanins in hairy roots. In considering the fact that the main betaxanthin (miraxanthin V) and the major betacyanin (2-descarboxy-betanidin) in hairy roots of yellow beet (*B. vulgaris* L.) are both dopamine-derived betalains (Schliemann *et al*., 1999), the occurrence of similar structures for the minor betacyanins was also suggested.

In hairy root cultures of yellow beet nine betalains were detected (Fig. 4.1). Compound **1-3** have been already known as betalamic acid **1**, betanin **2** and miraxanthin V **3**. Compound **4** was shown to be phyllocactin by comparison with an authentic standard (R_t, UV-VIS, LC-MS and ^{1}H-NMR) isolated from *S. buckleyi*. Compound **7** is the aglycon of compound **5** and **8**. The structure elucidation of 2-descarboxy-betanidin **7** from hairy roots of *Beta vulgaris* (Schliemann *et al*., 1999) was based on comparison with synthetic material (Schliemann *et al*., 1999). This compound was formerly found as a minor betacyanin pigment in flowers of *Carpobrotus acinaciformis* (L.) L. Bol. (Aizoacea) (Piattelli and Impellizzeri, 1970), a xerophilous plant native in South Africa. The possibility that this compound was an artefact of isolation due to decarboxylation of betanidin was excluded by former proving that during heat

treatment of betanidin the carboxy group was not split off from C-2, but from C-15 with an additional migration of the C-17/C-18 double bond to the C-14/C-15-position (Minale & Piattelli, 1965; Dunkelblum *et al*., 1972) leading to 15-descarboxy-betanidin. In 2-descarboxy-betanin **5**, the β-glucosidic linkage is possible at the hydroxyl group at C-5 or at C-6 of the aglycone, 2-descarboxy-betanidin. On the basis of its retention characteristics, betanidin 5-*O*-glucoside (betanin) was found to be more polar than betanidin 6-*O*-glucoside (gomphrenin I) (Heuer *et al.*, 1992) and the fact that in *Beta* species only betanidin derivatives glucosylated at the 5-*O*-position have been found, 2-descarboxy-betanin **5** is assumed to be a 5-*O*-linked betacyanin. The characteristic signals of 2-descarboxy-betanin in 1D and 2D ^{1}H (COSY) NMR spectroscopic analyses confirmed it. From the previous data (Heuer *et al*., 1994), the small chemical shift between H-4 and H-7 of 0.06 ppm is characteristic of substitution at the hydroxy group at C-5 of the aglycone (Table 4.2 and Fig. 4.2), as opposed to that at C-6 where difference 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 indicate the absence of the carboxyl group at the C-2 position corresponding with the incorporation of dopamine into 2-descarboxy-betanin.

It had been questioned whether the condensation reaction (aldimine formation) with betalamic acid takes place before or after glucosylation of the *cyclo*-dopa moiety in betacyanin biosynthesis. The betanidin 5-*O*-glucosyltransferase (Vogt *et al*., 1997) and the corresponding recombinant enzyme, expressed in *Eschericha coli* (Vogt *et al*., 1999), exhibited no activity towards *cyclo*-dopa, thus favouring the first alternative in *Dorotheanthus bellidiformis* (Aizoaceae). By analysis of betanin and *cyclo*-dopa 5-*O*-glucoside levels in red beet hypocotyls it was shown (Schliemann, pers. commun.) that betanin increased in parallel with the fresh weight increase whereas *cyclo*-dopa 5-*O*-glucoside was a trace compound. This result is in contrast to previous data of Wyler *et al*. (1984), but in accordance with recent studies (Vogt *et al*. pers. commun.) that a glucosyltransferase from red beet accepts betanidin, but not *cyclo*-dopa as a substrate. Therefore, the possibility of glucosylation of *cyclo*-dopa before the condensation with betalamic acid seems to be excluded. To prove which alternative is used for the formation of dopamine-derived betacyanins in fodder beets, a short-term dopamine feeding experiment was performed (Fig. 4.6). The results indicate that the condensation reaction with betalamic acid with 2-descarboxy-*cyclo*-dopa takes place prior to the glucosylation step and that the chase phase is characterized by a direct precursor-product relationship confirming the former assumption. The reaction sequence (Fig. 5.1) in fodder beets obviously

holds true for the hairy root system, as compound **7** is there the main betacyanin component (Fig. 4.1).

The analysis of the distribution of dopamine-derived betacyanins in different cultures and plants (Tabel 4.4) shows the involvement of dopamine in betaxanthin biosynthesis. Very recently the trace of 2-descarboxy-betanidin was found in yellow *Celosia* varieties (Schliemann *et al*., 2001). From these data, and a former study in which tyrosinase activity was correlated with the betalain content (Steiner *et al*., 1999), it can be concluded that the formation of these betacyanins only takes place when both the dopamine level and the tyrosinase activity are high. Under these conditions 2-descarboxy-*cyclo*-dopa does compete with dopamine in the condensation reaction with betalamic acid, which leads to a simultaneous formation of 2-descarboxy-betanidin and miraxanthin V. While miraxanthin V is a biosynthetic end product, 2-descarboxy-betanidin can be further conjugated with glucose giving 2-descarboxy-betanin and can finally be acylated to form the malonyl-2-descarboxy-betanin, the last two identified in nature for the first time. Furthermore, phyllocactin, often the main pigment in flowers and fruits of Cactaceae (Piattelli and Imperato, 1969) was detected in hairy root extracts. Its occurrence was formerly also observed in leaves of *Kochia scoparia*, stems of *Salsola soda* and petioles of *Spinacia oleracea* (all Chenopodiaceae as *B. vulgaris*) (Steglich and Strack, 1990).

The feeding of Tyr, dopa, tyramine and dopamine to fodder beet plants shows (Fig. 4.7) that Tyr and dopa feeding lead to dopa-derived betacyanins, while dopamine (and tyramine partly) feeding leads to dopamine-derived betacyanins. In all these cases the tyrosinase of the cut surface of the hypocotyls transforms the amino acid and mainly betacyanins or 2-descarboxy-betacyanins were formed, but only low amounts of the corresponding betaxanthins. In contrast, tyramine-betaxanthin and 2-descarboxy-betacyanins are major products of tyramine feeding.

The results of the structure elucidation and the dopamine short-term feeding experiment are summarized in a pathway of the biosynthesis of dopamine-derived betalains in hairy root cultures (Fig. 5.1). By combination of different enzymatic and spontaneous reactions, the characteristic pattern of the predominant dopamine-derived betalains in hairy root cultures is realized, in addition to the low amount of co-occurring betanin, which is otherwise the prevailing betacyanin pigment in *Beta* species.

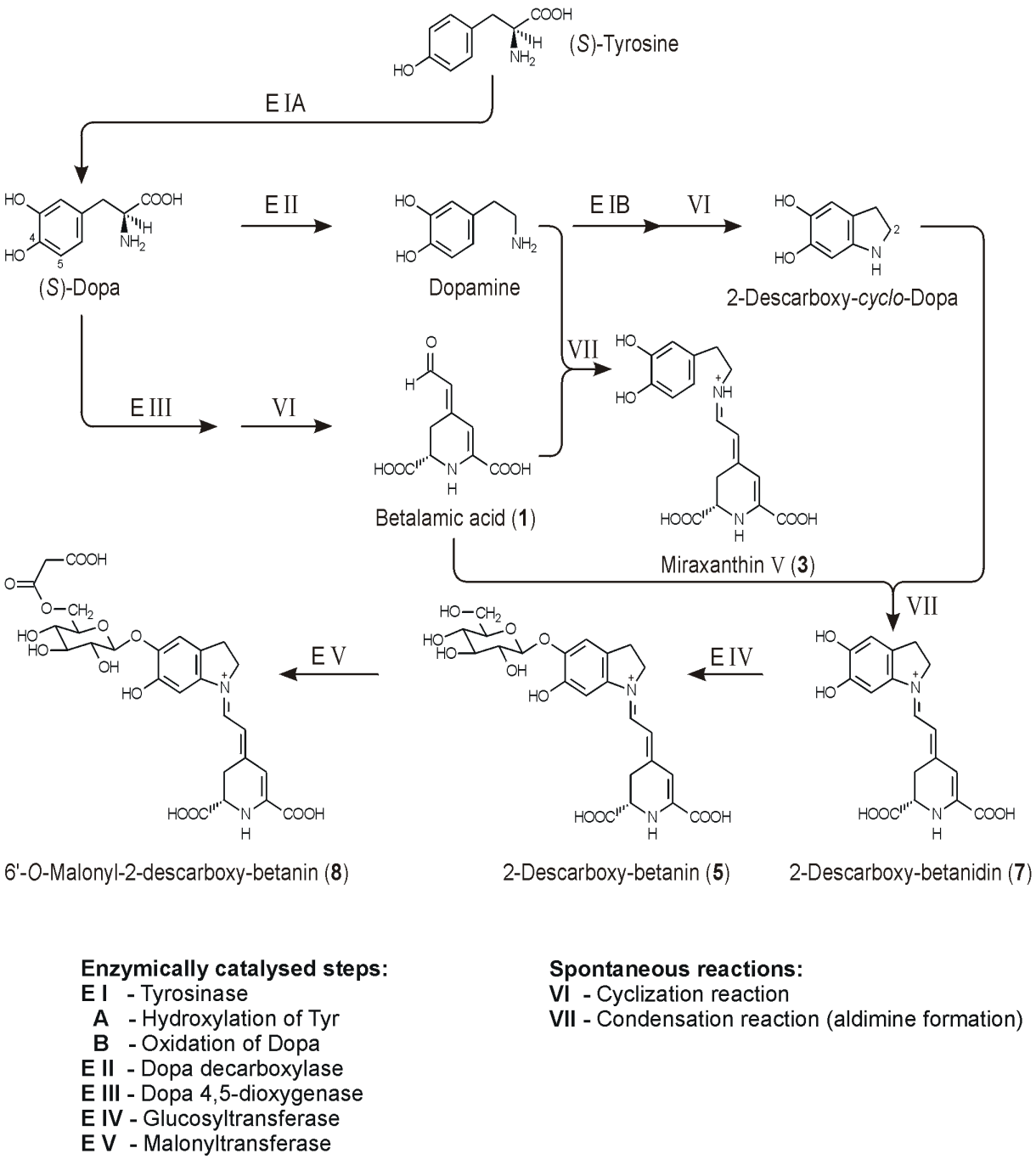


Fig. 5.1 Proposed biosynthetic pathway of dopamine-derived betalains in *Beta vulgaris* (hairy root culture). Compound numbers of this pathway correspond to the numbers in Table 4.1 and Fig. 4.1.

5.3 Betalains from Christmas cactus

Betalains are also responsible for the bright coloration of flowers and fruits of the Cactaceae. During the structure elucidation of dopamine-derived betacyanins, the fragmentation pattern of the new 6'-*O*-malonyl-2-descarboxy-betanin in LC-MS should be compared with that of the known 6'-*O*-malonyl-betanin, phyllocactin (Fig. 5.2).

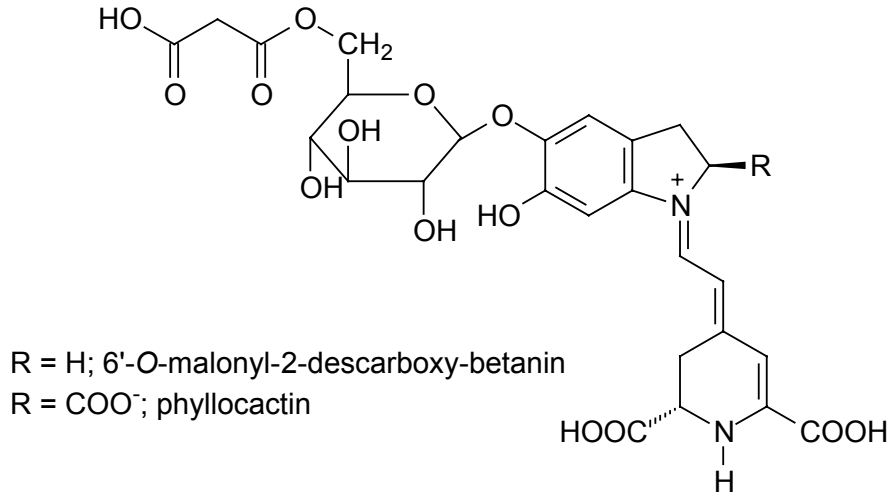


Fig. 5.2 Structures of 6'-*O*-malonyl-2-descarboxy-betanin and phyllocactin

For this reason, an extract from petals of Christmas cactus [*Schlumbergera* x *buckleyi* (T. Moore) Tjaden] (Bachthaler, 1992) which contains phyllocactin as a major compound, was used as a source. First evidence for the occurrence of a betacyanin with higher electrophoretic mobility than betanin was observed by Piattelli and Minale (1964 a, b) which identified its structure as 6'-*O*-malonyl-betanin (phyllocactin) by chemical derivatization and degradation (Minale *et al*., 1965, 1966). The linkage of the malonyl moiety in phyllocactin to the 6'-*O*-position of glucose was determined previously by permethylation and analysis of the per-methyl glucoses after hydrolysis (Minale *et al*., 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 completely absent (Piattelli and Imperato, 1969). 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.

In isolating phyllocactin from petals of Christmas cactus (Fig. 4.9), fourteen betalains were detected, but most of them were unknown. The known vulgaxanthin I, betalamic acid and betanin were confirmed by UV-VIS spectroscopy and co-chromatography with authentic and synthetic compounds (Schliemann *et al*., 1999, Table 4.5) and the main compound was characterized as phyllocactin as assigned by Minale *et al*. (1966) and Strack *et al*. (1981). In keeping with 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, is evidence that the malonyl system is bound to C'-6 of the glucose moiety (Fig. 4.14). Treating

phyllocactin with 1N HCl, the racemization proceeded after a 2-h-lag phase linearly in a time-dependent manner (2.3% h^{-1}) to the same extent as with betanin (2.2% h^{-1}) (Fig. 4.11, Schliemann & Strack, 1998) and led to the formation of isophyllocactin by isomerization and betanin by hydrolysis.

The structure of compound **7** was elucidated as betanidin 5-*O*-(2'-*O*-β-D-apiofuranosyl-6'-*O*-malonyl)-β-D-glucopyranoside (2'-apiosyl-*O*-phyllocactin) by HPLC-PDA, LC-MS and ^{1}H NMR spectroscopy and carbohydrate analyses. Analogous to phyllocactin, 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. As in phyllocactin, the low field chemical shifts of H-6'A/H-6'B in 2'-apiosyl-*O*-phyllocactin provides definitive evidence that the malonyl system is bound to C'-6 of the glucose moiety. Among the more complex betacyanins occurring in trace amounts, compound **9** was identified as 5''-*O*-*E*-feruloyl-2'-*O*-apiosyl-betanin which had been already identified from the fruits of *Phytolacca americana* (Schliemann *et al*., 1996). The assignment of this structure is furthermore based on the fact that the linkage of the malonyl residues in both phyllocactin and 2'-apiosyl-*O*-phyllocactin was found in the 6'-*O*-position of the glucose and the linkage of the feruloyl residue in 5''-*O*-*E*-feruloyl-2'-*O*-apiosyl-betanin is in the 5''-*O*-position of the apiose (Schliemann *et al*., 1996). The presence of a new diacylated betacyanin was ascertained as 5''-*O*-*E*-feruloyl-apiosyl-phyllocactin **12** from MS data and by comparison with 5''-*O*-*E*-feruloyl-2'-apiosyl-betanin. 5''-*O*-*E*-Feruloyl-apiosyl-phyllocactin seems to be the first betacyanin containing two different sugars, glucose and apiose, and both an aliphatic (malonyl) and an aromatic (feruloyl) acyl residue. This type of acylation has frequently been found in complex anthocyanins (Strack & 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). For the minor pigments **4**, **6** and **8**, MS gave no conclusive results, but from their UV-VIS spectra and retention times (Table 4.5) acylation of these betacyanins with hydroxycinnamic acids could be excluded. Less polar minor betacyanins (**9-11, 13, 14**) contained hydroxycinnamoyl moieties indicated by an absorption at 320 nm, but for the further identification the amounts of these compounds were too low.

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) was not found. Therefore, it is assumed that betacyanin acylation is the final biosynthetic step. 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 co-elute with the less polar acylated betacyanins, but flavonoids were not found. The analysis of betacyanins during flower development (Fig. 4.16) 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 the malonylation is an early reaction and then the amount of phyllocactin strongly increased concomitantly with the fresh weight: There is a similar expression pattern of all enzymes involved in the pathway of these complex betacyanins.

From the analysis of betalains in different organs phyllocactin is dominating in petals and carpels whereas in stamens its content is relatively low (Tabl 4.8). In contrast hydroxycinnamoyl betacyanins **9-12** are very low in petals and in carpels, but in stamens they are the major components. The physiological meaning of this results remain to be studied.

To see if the betacyanin pattern in *S. buckleyi* also occur in other Cactaceae, flowers and fruits of different species were analysed (Table 4.9), which shows that some cactus, *Rebutia, Gymnocalycium*, *M. multiceps* Salm-Dyck and *Stenocereus queretaroensis,* contains different ratio of betanin, phyllocactin, 2'-apiosyl-phyllocactin and hydroxycinnamoyl betacyanins **9-14**. In general, phyllocactin is the major betacyanin, betanin is present in all extracts as a minor betacyanin, but also the newly discovered compound, 2'-apiosyl-phyllocactin, was frequently detected. In *Rebutia* spec. phyllocactin and betanin occur in equal amounts. It can be summarized that betanin, phyllocactin and 2'-apiosyl-phyllocactin are present in all flowers and fruits of Cactaceae analysed, confirming that malonylation of betacyanins is typical for cacti.

5.4 The spontaneous reaction in betalain biosynthesis

It was not clarified whether the aldimine formation, the last step in betalain biosynthesis, depends on an enzyme or is a non-enzymatic reaction. The aldimine bond formation proceeds in two steps: the nucleophilic addition of the amino group at the aldehyde group leads to an intermediate that eliminates water and forms the aldimine bond (Fig. 5.3).

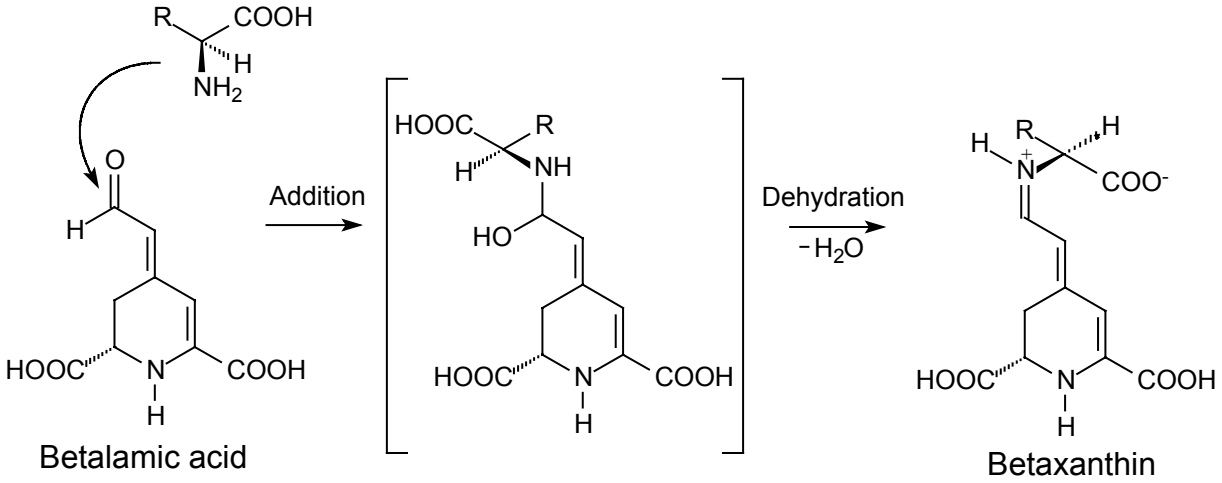


Fig. 5.3 The formation of the aldimine bond in betaxanthin biosynthesis

De-Eknamkul *et al*. (1997) found enzyme-catalysed condensation of dopamine with the iridoid aldehyde secologanin, including aldimine formation. It was known that *cyclo*-dopa condenses with increasing rates at decreasing pH values spontaneously with betalamic acid to give betanidin (Schliemann *et al.*, 1999). In the physiologically relevant pH range above 6.0, the spontaneous reaction was negligible. This was a prerequisite for all of the enzymatic attempts to show enzymatic betaxanthin formation. Using protein extracts from hairy root cultures, neither by the photometric assay nor by HPLC, could enzyme-catalysed betaxanthin formation from betalamic acid and glutamine or phenylalanine be shown.

In benzylisoquinoline biosynthesis, the reaction of an amine with an aldehyde is an enzyme catalysed step (dopamine with 4-hydroxyphenylacetaldehyde or 3,4-dihydroxyphenylacetaldehyde), which leads to the cyclised intermediates norcoclaurine and noraudanosoline, respectively (Rueffer and Zenk, 1987). Similarly, the condensation of dopamine with the aldehyde secologanin was found to be catalysed by cell-free extracts of *Alangium lamarckii* (De-Eknamkul *et al*., 1997). This cyclisation proceeded, in contrast to the betaxanthin formation, directly to tetrahydroisoquinoline derivatives (*R*- and *S*-form), which spontane-ously cyclised further in lactam formation. The first reaction steps can also proceed non-enzymatically at pH 5.0 (Itoh *et al*., 1995).

The involvement of non-enzymatic steps in the biosynthesis of secondary compounds is observed rarely; for example, transformation of neopinone to codeinone (Gollwitzer *et al*., 1993) in morphine biosynthesis; intermolecular cyclisation of γ-methylaminobutyraldehyde to *N*-metyl pyrrolinium cation, and its coupling with acetoacetic acid giving hygrine (Endo *et al*., 1998); Michael-type addition of (*S*)-kynurenine to *N*-β-alanyldopamine quinone methide leads

to papiliochrome II, a yellow pigment of butterflies (Saul and Sugumaran, 1991); hydration at the 6-position of protein-bound dopaquinone to form 6-hydroxy-dopa (Topa), the precursor of topaquinone that was identified as an essential co-factor of copper amine oxidase (Mure and Tanizawa, 1997) and late biosynthetic steps in the formation of antibiotics (Mayer and Thiericke, 1993).

Cross-breeding of different coloured lines of large-flowered purslane (*Portulaca grandiflora* Hook.) suggested the involvement of three genes in the control of betalain biosynthesis (Adachi *et al*., 1985), disregarding a gene responsible specifically for betaxanthin formation. Later, a hypothetical model was proposed that included transport of betalamic acid into the vacuole, where under acidic conditions condensation between betalamic acid and amino acids or amines proceeds spontaneously (Trezzini, 1990; Trezzini and Zryd, 1990). Feeding of some amino acids to seedlings and hairy root cultures of yellow beet (*B. vulgaris* L. subsp. *vulgaris* 'Golden Beet'), which resulted in the formation of the corresponding betaxanthins, were in accordance with this model (Böhm *et al*., 1991; Hempel and Böhm, 1997). On the other hand, feeding of dopa to cotyledons of different *Amaranthus* species stimulates amaranthin biosynthesis and leads to the formation of some betaxanthins, but not dopaxanthin (French *et al.*, 1974; Guidici de Nicola *et al*., 1975; Bianco-Colomas, 1980). Induction of betaxanthin (mainly vulgaxanthin I) and betalamic acid formation has also been observed in some cases after dopa feeding to betalain-forming inflorescences and petals of different plants (Rink and Böhm, 1985).

Further experiments were aimed to confirm the evidence for a spontaneous character of the condensation reaction. Therefore, nearly all proteinogenic amino acids, including some (*R*)-forms were fed to hairy root cultures of yellow beet used as experimental system.

Characterizing the betalain pattern in hairy roots of yellow beets, the identity of the minor betaxanthin, compound **1** (Fig. 4.18) was confirmed as (*S*)-Gln-betaxanthin (vulgaxanthin I) by comparison and co-chromatography with the synthetic vulgaxanthin I standard confirming Hempel and Böhm (1997). In contrast to the results of Hempel and Böhm (1997), the major betaxanthin, compound **2** was identified as dopamine-betaxanthin (miraxanthin V, Fig. 4.19) by LC-MS and co-chromatography in HPLC with synthetic dopamine-betaxanthin (Schliemann *et al*., 1999). This betaxanthin was first isolated as miraxanthin V from flowers of *Mirabilis jalapa* (Piattelli *et al*., 1965) and also from callus cultures of *B. vulgaris* (Girod and Zryd, 1991b).

During the logarithmic phase of hairy root growth the fresh weight increased in parallel with miraxanthin V content (Fig. 4.20). The relation between growth and betaxanthin accumulation in hairy roots of yellow beet had also investigated by Hamill *et al.* (1986).

As a prerequisite for the analysis of the products expected from the amino acid/amine feedings, a simple method for the synthesis of stereoisomeric betaxanthins and their analytical separation was elaborated (Table 4.11, Schliemann *et al*., 1999). Betaxanthins can occur in four different diastereoisomers (2*S/S*, 2*S/R*, 2*R/S*, 2*R/R*). The separation experiments (Schliemann *et al*. 1999) revealed that the 2*S/S*- and 2*R/R*-isomers had identical retention times, likewise, the 2*S/R*- and 2*R/S*-derivatives could not be separated. As in feeding experiments, only the endogenous (*S*)-betalamic acid may react with (*S*)- and (*R*)-amino acids, the possible metabolites can be easily separated in most cases with our analytical tools.

The detection of a protein-catalysed condensation between betalamic acid and glutamine or phenylalanine failed *in vitro*. To analyse the betaxanthin formation *in vivo* amino acid feeding experiments were undertaken. The results of amino acid feeding to hairy root cultures summarized in Table 4.12 show that all amino acids were used in the formation of the corresponding betaxanthins, but to different extents. Thus, no amino acid specificity was observed. As noted previously by Hempel and Böhm (1997), (*S*)-Glu did not give the expected vulgaxanthin II, but yielded vulgaxanthin I. There is no clear trend in betaxanthin formation concerning the polarity and charge of the amino acids. Polar or basic amino acids as (*S*)-Hyp and (*S*)-His resulted in equally high incorporation rates as the nonpolar neutral amino acids [(*S*)-Leu and (*S*)-Phe]. Feeding of (*S*)-Tyr, (*S*)-dopa, tyramine and dopamine to hairy root cultures of yellow beets could not be evaluated, because of the high tyrosinase activity in hairy roots which turned the cultures rapidly black (melanin formation) after feeding of these amino acids or amines. By comparison of the increase of miraxanthin V levels at 7^{th} day with those at 8^{th} day (harvest day) in the controls and the amino acid feeding experiments, the amino acids compete with endogenous dopamine for betalamic acid, resulting in a lower miraxanthin V content together with a lower betalamic acid level (Table 4.12). Deviations from this trend might be caused by remarkably higher fresh weight of the hairy roots in the fed plants than in water controls. (*S*)-4-Thiaproline, a synthetic sulphur analogue of (*S*)-Pro, was accepted as a precursor and yielded a high incorporation into the corresponding betaxanthin (Table 4.12). It may be argued that the results of the amino acid feeding experiments with the highly artificial hairy root cultures may not be representative. Therefore, the same amino acids feeding were fed to fodder beet seedlings, resulting in similar pattern of formed betaxanthins (Fig. 4.21). In the case of polyamine feeding, the corresponding polyamine-betaxanthins were not detected

in hairy roots, but only in trace amounts after feeding of ethylendiamine, putrescine, cadaverine and octanediamine to fodder beet seedlings (Table 4.14). The reason for these unexpected results is unknown.

To examine the stereoselectivity of the condensation reaction, feeding of (*S*)- and (*R*)-amino acids to hairy root cultures showed that both isomers were similarly accepted (Table 4.13). Obviously, the aldimine bond formation proceeds without any stereoselectivity, which is further indicative of a spontaneous process. The apparent stereoselectivity for the (*S*)-isomer in simultaneous feeding of (*S*)- and (*R*)-Phe mixture (Fig. 4.22) was proven to be caused by an inhibited uptake of (*R*)-Phe in the presence of the (*S*)-isomer, as shown in a double-labelling experiment (Fig. 4.23). The decrease of the (*S*)-[2,6-$^{3}H_2$]Phe/(*R*)-[1-^{14}C]Phe ratio in the nutrition solution demonstrated a preferential uptake of (*S*)-Phe in the presence of (*R*)-Phe. (*S*)- and (*R*)-Phe were also taken up by de-rooted fodder beet plants and incorporated into the corresponding betaxanthins in the same way and to the same extent as in the hairy root culture without any stereoselectivity. The condensation of different amino acids and amines with betalamic acid in hairy root cultures and fodder beet seedlings showed neither an amino acid/amine specificity nor a stereoselectivity. But in the case of suspension cultures of red beet and *D. bellidiformis*, which form mainly betacyanins, the formation of a betaxanthin derived from (*S*)- or (*R*)-Phe was not observed, whereas in a hairy root cultures of red beet (*S*)- and (*R*)-Phe-betaxanthin could be detected after feeding, although in low amounts. This may indicate that the betalamic acid metabolism in these systems is controlled in a different way as compared to mainly betaxanthin-forming cultures, i.e. that betalamic acid is completely channelled into betacyanins.

Saturation experiments (2-50 mM amino acids) were performed to determine whether amino acids applied exogenously in increasing concentrations can compete with the endogenous dopamine in betaxanthin formation. Feeding of (*S*)-Thr to hairy root cultures show that the (*S*)-Thr-betaxanthin levels increased in increasing concentrations of (*S*)-Thr (up to 10 mM) with a simultaneous decrease in the betalamic acid and miraxanthin V levels compared with water controls (Fig. 4.24). An interruption of miraxanthin V formation was achieved by daily application of (*S*)-Leu (5 mM) from 4th to 8th day (Fig. 4.27). Due to the constant high supply of (*S*)-Leu (final concentration in the hairy roots was found to be 30 mM), the miraxanthin V level did not increase because the synthesised betalamic acid was rapidly consumed in the condensation reaction with (*S*)-Leu and was then unavailable for condensation with endogenous dopamine. Increasing concentrations (up to 50 mM) of (*S*)-Ala showed a relatively low rate of incorporation into (*S*)-Ala-betaxanthin (Table 4.12) and also showed a concentration-

dependent increase of another betaxanthin, (*S*)-Gln-betaxanthin (vulgaxanthin I, Fig. 4.25). This phenomenon can be explained by the action of L-alanine : 2-oxoglutarate aminotransferase (EC 2.6.1.19), which leads to the formation of pyruvate and glutamate. Gln is then formed by ammonia fixation via glutamine synthetase (EC 6.3.1.2) and serves as a direct precursor for vulgaxanthin I (Fig. 5.4).

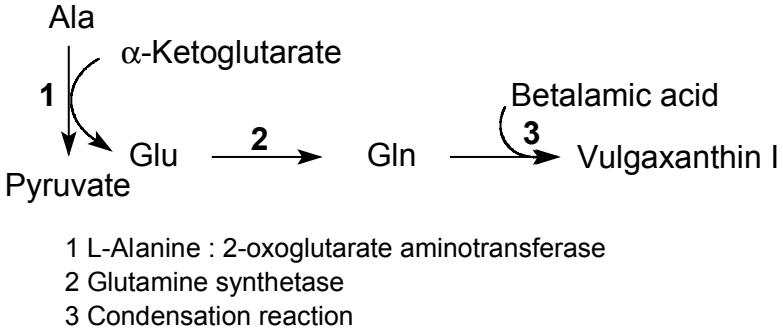


Fig. 5.4 Increased vulgaxanthin I formation by alanine feeding

To determine whether vulgaxanthin I formation is indeed dependent on increased ammonia fixation, $(NH_4)_2SO_4$ was added to the standard nutrition solution in increasing concentrations (3-51 mM, Fig. 4.26). Indeed, vulgaxanthin I accumulated dependent on the fed $(NH_4)_2SO_4$ concentration with the optimum at 20 mM. Furthermore, (*R*)-Ala feeding (2-50 mM) did not result in the formation of vulgaxanthin I, but only in increased (*R*)-Ala-betaxanthin levels, because (*R*)-Aln can not be used as the substrate of aminotransferase. Thus, it could be shown that betaxanthin biosynthesis can be regulated *in vivo* not only by amino acid feeding, but also by substances indirectly involved in the biosynthesis.

It may be argued that the increase of intracellular amino acid levels by feeding from outside may disturb the normal betalain biosynthetic pathway, therefore, an increase in endogenous phenylalanine levels was induced by application of AIP, a strong inhibitor of PAL (EC 4.3.1.5). AIP was added daily over 4 days to hairy root cultures of yellow beet. Although the content of phenylpropanoids was relatively low in the hairy roots, AIP treatment increased the (*S*)-Phe level and (*S*)-Phe-betaxanthin was subsequently formed (Fig. 4.28). In the water control (*S*)-Phe-betaxanthin formation could not be detected. Thus, by endogenous increase of the concentration of a selected amino acid, the spontaneous formation of the corresponding betaxanthin derived from this amino acid could be induced. In the same way, the application of AIP to the de-rooted fodder beet plants led to an increase in the (*S*)-Phe level and in the formation of (*S*)-Phe-betaxanthin, although to a smaller extent than in hairy roots.

To examine whether spontaneous condensation is specific for betaxanthins, *cyclo*-dopa, the intermediate of betacyanin formation, was fed to de-rooted fodder beet seedlings (Schliemann *et al.*, 1999). After less than 1 h, a red coloration of the hypocotyls was observed. HPLC analyses proved that betanidin was formed and was accompanied by a low amount of betanin. By analysing the betalain pattern, it could be seen that betanidin seemed to be formed by consuming free betalamic acid. The results of the comprehensive amino acid feedings including *cyclo*-Dopa suggest that the condensation reaction in both betaxanthin and betacyanin biosynthesis proceeds according to the same mechanism, a spontaneous reaction.

To find additional evidence for the spontaneous character of the condensation reaction, betalamic acid was fed to broad bean and pea seedlings (Schliemann *et al*., 1999), which do not belong to the betalain-forming Caryophyllales. The analyses of both extracts after betalamic acid feeding showed the presence of betaxanthins, in contrast to an extract of controls (Fig. 4.29). The major betaxanthin from broad beans after feeding of betalamic acid was identified as dopaxanthin on the basis of the R_t , UV-VIS spectra and co-injection analysis. Amino acid analysis and dopa determination of hypocotyl extracts revealed that the Dopa concentration was higher than that of all the other amino acids (Table 4.15). Although the Asn concentration showed the same level as that of dopa, Asn-betaxanthin was not detected. This may indicate different localizations for different amino acids, leading to a more facilitated access of betalamic acid to dopa than to Asn. The pattern of distribution and concentration of amino acids in the vacuole is similar to that in the cytoplasm, but quite different from that in the chloroplast (Mimura *et al*., 1990), the site of synthesis of many amino acids in higher plants. Because broad bean hypocotyls do not contain betalains and therefore do not have an enzyme catalysing the condensation reaction, the dopaxanthin formation must result from a non-enzymic spontaneous process.

These results indicate that the betalain formation proceeds most likely in a spontaneous rather than an enzymatic reaction without any amino acid specificity and setereoselectivity (Fig. 5.5).

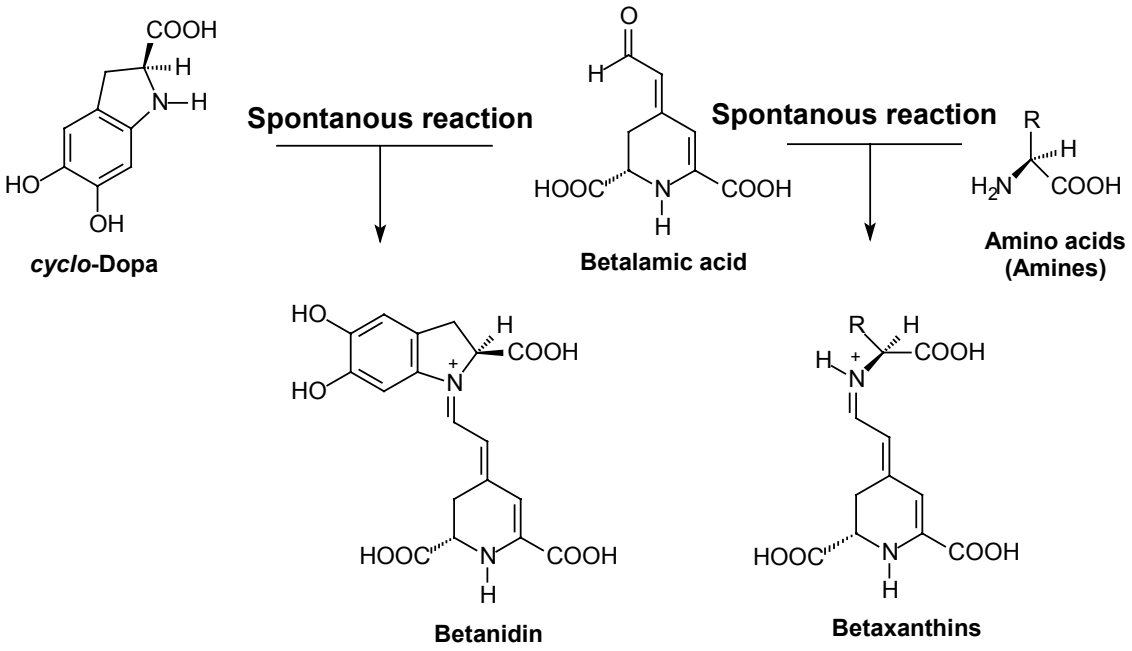


Fig. 5.5 Spontaneous condensation reaction in betalain biosynthesis

5.5 Transport of betaxanthins into red beet vacuoles

In plants, many water-soluble pigments such as flavonoids, anthocyanins and betalains are accumulated in the central vacuole occupying up to 90 % of the total cell volume and responsible for the bright coloration in flowers and fruits. It is known that species-specific betalains accumulate in the vacuoles, e.g. betanin and vulgaxanthin I in red beet plant, miraxanthin V and descarboxy-betacyanins in hairy roots of yellow beets and phyllocactin in Christmas cactus (Steglich and Strack, 1990; Schliemann *et al*., 1999; Piattelli and Minale, 1964a,b). The hypothesis of Trezzini 1990 and Trezzini & Zryd 1990 concerning the localization of the betalain biosynthesis can be illustrated as in Fig. 5.6. They proposed that the betacyanin formation takes place in the cytosol, whereas the betaxanthins are formed after transport of betalamic acid into vacuoles.

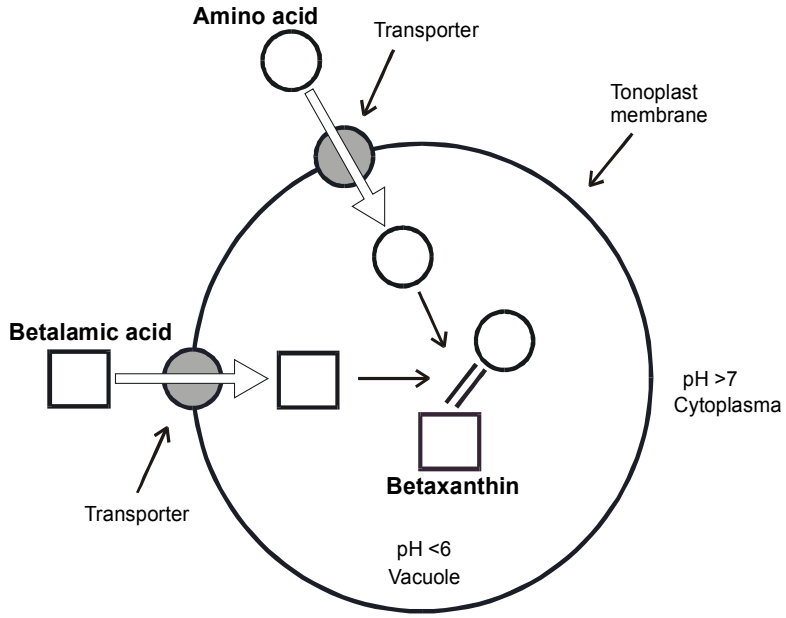


Fig. 5.6 Vacuolar model of betaxanthin formation

Many secondary metabolites such as phenolics, alkaloids, flavonoids and anthocyanins are accumulated vacuoles, which show species-specific uptake of the corresponding substrates (Deus-Neumann and Zenk, 1984, 1986; Hopp and Seitz, 1987; Werner and Matile, 1985; Martinoia and Ratajczak, 1997). Uptake of secondary metabolites is often coupled with the pH gradient between vacuoles and cytosol. Apigenin 7-*O*-(6'-*O*-malonyl)-glucoside, a vacuolar pigment, is trapped in parsley vacuoles as a result of conformational changes at acidic pH (Matern *et al*., 1986). Anthocyanins are transported by a selective carrier and trapped by a pH-dependent conformational change under the acidic condition of the vacuoles from the carrot cell culture (Hopp and Seitz, 1987). Coumaric acid glucosides are transported into vacuoles from sweet clover leaves under the acidic pH (Rataboul *et al*., 1985). Some secondary metabolites are transported into vacuoles by other transporter mechanisms. Esculin and coumaric acid glucosides are suggested to be transported into barley vacuoles by a proton antiport mechanism, although neither substance is produced in barley (Werner and Matile, 1985). Comparison of the barley vacuolar uptake of isovitexin, a glucosylated flavonoid occurring in barley, and that of hydroxyprimisulfuron glucoside, a detoxification product of the herbicide primisulfuron, showed two different glucoside transport mechanisms (Klein *et al*., 1996): a proton antiport system for the uptake of isovitexin, and a directly ATP-energized transport mechanism for that of hydroxyprimisulfuron glucoside. In rye vacuoles containing luteolin glucuronides as major compounds, these glucuronides are transported by a directly

energized transport mechanism (Klein *et al*., 2000). Furthermore, saponarin (the main flavonoid of barley) is taken up by a proton antiport mechanism in barley but by a direct energization mechanism in *Arabidopsis,* which does not contain this flavonoid (Martinoia *et al*., 2000). Until now, two transport mechanisms of different compounds have been known: a) Two proton pumps that are driven by a H^+-ATPase and H^+-PPiase generating an electrochemical gradient and inhibited by protonophores but not by vanadate; b) A directly energized transport mechanism that is directly energized by MgATP, insensitive towards protonophores and strongly inhibited by vanadate. The second transporter belongs to the family of widely described ABC-type transporters. In plants, a vacuolar ABC-type transporter has already been identified (Martinoia *et al*., 1993, Li *et al.*, 1995, Klein *et al*., 1996, 2000). The typical ABC-type transporter requires the following conditions: 1. Direct energization by MgATP, 2. Insensitivity to the transmembrane H^+-electrochemical potential difference, but sensitivity to vanadate and 3. Lack of activity toward GSH, but stimulation by GS-conjugates (Rea *et al*., 1998).

The transport studies with red beet vacuoles were restricted by the relatively low yield, despite intensive optimization. Contamination of the vacuolar preparation with other cell constituents was *ca*. 9 % (Fig. 4.32). The hypocotyls of red beets were chosen for vacuole preparation because the yields from fodder beets and hairy root cultures were even lower.
According to the hypothesis that the GS-conjugates stimulate uptake, betalamic acid uptake experiments in the presence and absence of DNB-GS into red beet vacuoles were performed, but both failed. This may be caused by the instability and the low concentration of betalamic acid. Thereafter, the uptake experiments with miraxanthin V and vulgaxanthin I, both endogenous betaxanthins and with (*R*)-Phe-betaxanthin as an unnatural substrate (Fig. 5.7) were performed. Because of the difficulties to isolate or synthesize ^{14}C-labelled substrates with high specific radioactivity, non-labelled betaxanthins were used as substrates for all vacuolar uptake experiments. Miraxanthin V (dopamine-betaxanthin) (Fig. 5.7) was chosen as a substrate of endogenous betaxanthins. Since the red beet vacuoles contain only trace amounts of this compound endogenously, it does not disturb the detection of the miraxanthin V transported into the vacuoles. In addition, vulgaxanthin I (Gln-betaxanthin) was also used as an endogenous substrate (Fig. 5.7). (*R*)-Phe-betaxanthin, whose retention time and UV-VIS spectrum from HPLC has been already known (Schliemann *et al*. 1999), was used as an unnatural betaxanthin (Fig. 5.7).

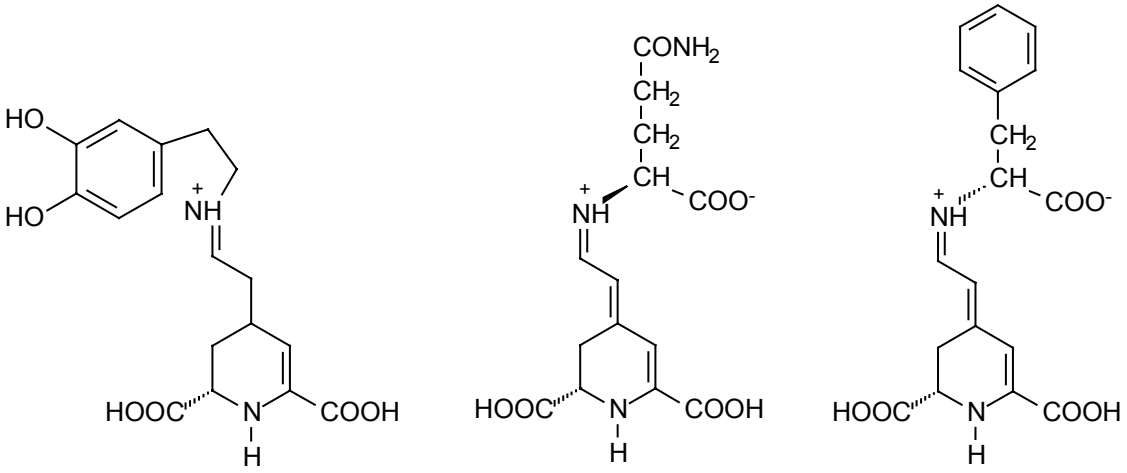


Fig. 5.7 Structures of miraxanthin V, vulgaxanthin I and (*R*)-Phe-betaxanthin

The results show that the uptake of both miraxanthin V and (*R*)-Phe-betaxanthin is favoured by MgATP (Fig. 4.33).

The uptake of miraxanthin V and (*R*)-Phe-betaxanthin in the presence of free ATP was lower than that of controls and in the presence of MgATP (Fig. 4.34). This indicates that an amino acid transport system transporting the substrates with free ATP but not MgATP is not responsible for the transport of miraxanthin V and (*R*)-Phe-betaxanthin.

Time-dependent uptakes of miraxanthin V and (*R*)-Phe-betaxanthin show different trends (Fig. 4.35). The uptake of miraxanthin V is 2-fold higher in the presence of MgATP than in the absence of MgATP. In contrast the uptake of (*R*)-Phe-betaxanthin stimulated only slightly in the presence of MgATP. This result suggest that two different transport systems exist for miraxanthin V and (*R*)-Phe-betaxanthin.

By using the inhibitors for different types of transporters, the transport mechanisms for miraxanthin V, vulgaxanthin I and (*R*)-Phe-betaxanthin were studied (Table 4.16). The uptakes of miraxanthin V and vulgaxanthin I are inhibited by 1 mM vanadate, an inhibitor of ABC-transporter, but not bafilomycin A1 (0.1 µM) and NH_4Cl (5 mM). In contrast, the relatively low uptake of the non-endogenous (*R*)-Phe-betaxanthin was not inhibited by vanadate, but by bafilomycine A1 and NH_4Cl. The large deviations after the uptake of all the substrates might be due to the following reasons: 1. The red beet vacuoles were isolated at very small amount and seemed to be more unstable than the barley vacuoles. 2. If ^{14}C-labelled betaxanthins would be available, a higher number of repetitions with better precision would be possible. 3. Because long HPLC runs had to be used, and the substrates are not very stable, fewer repetitive measurements could be done, leading to higher deviations. Therefore, the results of the uptake of these substrates into red beet vacuoles can only cautiously be interpreted. However,

the experiments with every substrate (Table 4.16) show the same trend in inhibition pattern: the inhibition of the MgATP-stimulated uptake of the beet specific miraxanthin V and vulgaxanthin I by 1 mM vanadate indicates the participation in an ABC-like directly-energized transport mechanism, whereas the uptake inhibition of the unnatural (*R*)-Phe-betaxanthin by 0.1 µM bafilomycin A1 and 5 mM NH_4Cl is compatible with a H^+/antiport system.

To test the necessity of vacuoles in betaxanthin formation, evacuolated mini-protoplasts from red beets were prepared and loaded with (*R*)-Phe and subsequently with betalamic acid. The formation of (*R*)-Phe-betaxanthin could be detected, but in very low amounts. This is in accordance with the apparent energy-dependent uptake of betaxanthins into the vacuoles which suggest the localization of betaxanthin formation in the cytosol rather than in the vacuoles. Due to the highly artificial mini-protoplast system, a direct conclusion to the biological system of plants is hypothetical. This result and the uptake inhibition data are not definitive proof of an extravacuolar localization of the last step of betalain biosynthesis.

5.6 Microspectrophotometric analyses of beet hypocotyls

The cross sections of red and fodder beet hypocotyls show differently pigmented cells; betalamic acid alone or mixtures of betalamic acid and betacyanins and betaxanthins are in inner cells, betaxanthins or betacyanins alone are in outer cells (Fig. 4.37 and 4.38). The cross sections of dopamine fed fodder beet hypocotyls showed that inner cells contain both betalamic acid and betacyanins and outer cells are only the end product, betaxanthin (Fig. 4.39 A). Feeding of dopamine to de-rooted fodder beet seedlings showed the red or orange cells, instead of cells containing excess amount of miraxanthin V. The sections from old hairy roots showed clearer difference than those of young hairy roots (Fig. 4.39 B,C). In the cells of *Beta* plants, the pigmented bodies that were found in cells of some cactaceous species (Iwashina *et al*., 1988) could not be observed. From these results, further conclusions on the course of betalain biosynthesis could not be drawn.

6 Summary

The aim of the presented work was to determine wheather the last biosynthetic step in betalain biosynthesis, the condensation between betalamic acid and amino acids/amines, is an enzymic or a spontaneous reaction.

As experimental systems betalain-forming hairy root cultures of yellow beets and hypocotyls of fodder beets (*Beta vulgaris*) were used. For the characterization of the biosynthetic capacity of the transgenic hairy roots of yellow beets, the betalain pattern during growth was analysed and the components structurally elucidated. Besides the already known betanin, betalamic acid and miraxanthin V (dopamine-betaxanthin), the dopamine-derived betacyanins (2-descarboxy-betanidin, its 5-*O*-glucoside and the corresponding 6'-*O*-malonyl conjugate) have been identified for the first time and a pathway of biosynthesis of these betacyanins was proposed. A screening of different cell cultures for the occurrence of these new compounds showed a strong correlation between the occurrence of dopamine-derived miraxanthin V and high tyrosinase activity. During the structure elucidation of 2-descarboxy-betanidin 5-*O*-(6'-*O*-malonyl)-β-D-glucoside, a comparison with the fragmentation pattern of betanidin 5-*O*-(6'-*O*-malonyl)-β-D-glucoside (phyllocactin) was necessary. Phyllocactin isolated from Christmas cactus flowers showed the same sequential elimination of CO_2 in ESI-MS. The subsequent complete analysis of Christmas cactus flower pigments led to the identification of new malonylated apiosyl derivatives of phyllocactin. A betalain screening of flowers and fruits of different cactus species showed a wide distribution of occurrence of the newly discovered betacyanins.

Due to the occurrence of specific betaxanthin patterns in plants, the condensation reaction was assumed to be enzyme-catalyzed. Assays designed to detect *in vitro* a protein-catalysed condensation between betalamic acid and amino acids (glutamine/phenylalanine) failed. To analyse the betaxanthin formation feeding of different amino acids (both *S*- and *R*-forms) to hairy root cultures was carried out, which led to the formation of the corresponding betaxanthins. These data were completely confirmed by analogous feeding experiments using hypocotyls of fodder beets. The results show that the betaxanthin formation reaction exhibits neither an amino acid specificity nor a stereoselectivity. Feeding an inhibitor of the phenylalanine ammonia-lyase, 2-aminoindan 2-phosphonic acid, to both experimental systems resulted in the formation of the phenylalanine-derived betaxanthin due to an inhibitor-induced increase of the endogenous phenylalanine level. Feeding of (*S*)-alanine in increasing concentrations (2-50

mM) led, in addition to the formation of (*S*)-Ala-betaxanthin, to an increase of the glutamine-derived vulgaxanthin I level. This result could be explained by the increased availability of ammonia provided by (*S*)-Ala as a similar induction could be mimicked by feeding of $(NH_4)_2SO_4$, but not by increasing concentrations of (*R*)-Ala. Feeding of *cyclo*-Dopa, the building block of most betacyanins, to fodder beet hypocotyls led in less than 1 h to the formation of betanidin indicating that the final step in the formation of both betacyanins and betaxanthins proceeds according to the same mechanism. An additional argument for an obvious spontaneous character of the condensation reaction was the finding that feeding of betalamic acid to hypocotyls of plants [e.g. broad bean (*Vicia faba* L.) seedlings] which do not belong to the betalain-synthesizing Caryophyllales led to the formation of betaxanthins. The only major betaxanthin from the broad bean experiment was identified as dopaxanthin derived from Dopa, an amino acid occurring in concentrations higher than all other amino acids analysed, but betaxanthins derived from all other amino acids were not found. All experimental results together indicate that the condensation of betalamic acid with amino acid/amines (including *cyclo*-Dopa) is most probably *in planta* a spontaneous rather than an enzyme-catalyzed reaction. A literature search showed that the involvement of nonenzymic steps in the biosynthesis of secondary compounds is a rare but important phenomenon.

To deal with the questions how do betaxanthin-forming plants achieve the accumulation of specific betaxanthin patterns, most likely irrespectively of the pattern of amino acids in these plants, and in which subcellular compartment the aldimine formation is located, the transport of precursors and endproducts of betalain biosynthesis into red beet vacuoles was studied. The transport studies were strongly hampered by the failure to synthesize ^{14}C-labelled substrates with high specific activity, therefore HPLC had to be used for the quantification of uptake. Furthermore, the preparation of vacuoles of red beet gave low yields despite intensive optimisation and due to the inherent chemical instability of betalamic acid no reproducible results with this substrate were obtained. The results of the uptake experiments with the beet-specific miraxanthin V (dopamine-betaxanthin) and vulgaxanthin I as well as the unnatural synthetic (*R*)-Phe-betaxanthin into red beet vacuoles showed large variability despite triple determination and can only cautiously be interpreted. As a cause of the variability of the uptake results in different experiments, the heterogeneity of the vacuole populations could be discussed. At least a trend in the experiments to impair the vacuolar betaxanthin uptake by inhibitors can be seen: the inhibition of the MgATP-stimulated uptake of the beet-specific miraxanthin V and vulgaxanthin I by 1 mM vanadate points to the participation of an ABC-like directly-ener-

gized transport mechanism, whereas the uptake inhibition of the unnatural (*R*)-Phe-betaxanthin by 0.1 µM bafilomycin A1 and 5 mM NH_4Cl is compatible with a H^+/antiport system. By sequential uptake of (*R*)-Phe and betalamic acid into evacuolated miniprotoplasts, the formation of (*R*)-Phe-betaxanthin could be detected in trace amounts. This result and the uptake inhibition data are not definitive proof of an extravacuolar localization of the last step of betalain biosynthesis.

Summarizing all biochemical results it can be stated that the investigation presented led to the characterization of the last betalain biosynthetic step as a most probably spontaneous reaction.

7 Zusammenfassung

Das Ziel der Arbeit war die Klärung der Frage, ob der letzte Schritt der Betalainbiosynthese, die Kondensation der Betalaminsäure mit Aminosäuren/Aminen (die Aldiminbildung), eine enzymatisch katalysierte oder eine spontan ablaufende Reaktion ist.
Als experimentelle Systeme dienten Betalain-bildende Hairy-Root-Kulturen der Gelben Rübe und Hypokotyle der Futterrübe (*Beta vulgaris*). Um das experimentelle System umfassend zu charakterisieren, wurden die Betalainmuster der transgenen Hairy-Roots der Gelben Rübe während des Wachstums analysiert und seine Komponenten strukturell aufgeklärt. Neben den bereits bekannten Verbindungen Betanin, Betalaminsäure und Miraxanthin V konnten die Dopamin-abgeleiteten Betacyane (2-Descarboxy-betanidin, sein 5-*O*-glucosid und das entsprechende 6'-*O*-Malonylkonjugat) erstmalig identifiziert und damit ein neuer Zweig der Betalainbiosynthese nachgewiesen werden. Ein Screening verschiedener Zellkulturen auf das Vorkommen dieser neuen Verbindungen zeigte eine enge Korrelation dieser Verbindungen mit dem Vorkommen des ebenfalls Dopamin-abgeleiteten Miraxanthins V und hoher Tyrosinaseaktivitäten. Während der Strukturermittlung des 2-Descarboxy-betanidin-5-*O*-(6'-*O*-malonyl)-glucosids war der Vergleich seines massenspektrometrischen Fragmentationsmusters mit dem des Betanidin-5-*O*-(6'-*O*-malonyl)-glucosids (Phyllocactin) notwendig. Phyllocactin wurde dazu aus Blüten des Weihnachtskaktus isoliert und zeigte ebenfalls eine sequenzielle Abspaltung der Carboxylgruppen in der ESI-MS. Die begleitende komplette Analyse der Blütenpigmente des Weihnachtskaktus führte zur Identifizierung neuer malonylierter Apiosylderivate des Phyllocactins. Ein Screening von Blüten und Früchten verschiedener Kaktusarten zeigte eine weite Verbreitung des Vorkommens dieser neu entdeckten, offensichtlich kaktusspezifischen Betacyane.

Auf Grund des Vorkommens spezifischer Betaxanthinmuster in Pflanzen lag die Vermutung nahe, dass die Kondensationsreaktion ein Enzym-katalysierter Vorgang sein könnte. Mittels photometrischer und HPLC-Enzymassays nach verschiedenen Proteinextraktionsverfahren konnte *in vitro* der Nachweis einer protein-katalysierten Kondensation zwischen Betalaminsäure und Aminosäuren (Glutamin/Phenylalanin) nicht erbracht werden. Um die Betaxanthinbildung *in vivo* zu analysieren, wurden verschiedene Aminosäuren (sowohl *S*- als auch *R*-Form) an Hairy-Root-Kulturen appliziert, die zur Bildung der entsprechenden abgeleiteten Betaxanthine führte. Diese Ergebnisse konnten komplett durch analoge Fütterungsexperimente an Hypokotyle von Futterrüben bestätigt werden. Selbst nach Gabe einer ungewöhnli-

chen Aminosäure [(*S*)-4-Thiaprolins] wurde ein Einbau in das entsprechende Betaxanthin beobachtet. Die Daten belegen, dass die Betaxanthinbildungsreaktion weder eine Aminosäurespezifität noch eine Stereoselektivität zeigt. Die Applikation von 2-Aminoindan-2-phosphonsäure, ein effektiver Inhibitor der Phenylalaninammoniak-Lyase, an beide experimentelle Systeme führte zur Bildung des vom Phenylalanin-abgeleiteten Betaxanthins auf Grund der Inhibitor-induzierten Erhöhung des endogenen Phenylalaninspiegels. Die Fütterung von (*S*)-Alanin in steigenden Konzentrationen (2 -50 mM) an Hairy-Root-Kulturen führten, zusätzlich zur erwarteten (*S*)-Ala-Betaxanthinbildung, zu einem Anstieg des Gehaltes an endogenem Vulgaxanthin I [(*S*)-Gln-Betaxanthin]. Dieses Ergebnis konnte durch eine aus der (*S*)-Alanin-Fütterung herrührende erhöhte Verfügbarkeit von Ammonium erklärt werden, da eine gleichartige Vulgaxanthin I-Erhöhung durch $(NH_4)_2SO_4$-Zugabe erreicht werden konnte, nicht aber durch ansteigende Konzentrationen von (*R*)-Alanin. Die Bildung von Betanidin nach Fütterung von *cyclo*-Dopa, dem Strukturelement fast aller Betacyane, zeigte an, das der letzte Schritt in der Bildung der Betacyane und der Betaxanthine nach dem gleichen Mechanismus verläuft. Ein zusätzliches Argument für den offensichtlich spontanen Charakter der Konden-sationsreaktion war die Tatsache, dass die Fütterung von Betalaminsäure an Hypokotyle von Pflanzen [z. B. Keimlinge der Ackerbohne (*Vicia faba* L.)], die nicht zu den Betalain-synthetisierenden Caryophyllales gehören, zur Bildung von Betaxanthinen führte. Das einzige Hauptbetaxanthin des Ackerbohnenexperiments erwies als Dopaxanthin, abgeleitet von Dopa, die in höherer Konzentration als alle anderen Aminosäuren in Kontrollpflanzen vorkam, aber Betaxanthine abgeleitet von allen anderen Aminosäuren wurden nicht gefunden. Alle experimentellen Daten zusammengenommen weisen daraufhin, dass die Kondensation der Betalaminsäure mit Aminosäuren/Aminen (einschließlich *cyclo*-Dopa) *in planta* sehr wahrscheinlich eine spontane, und nicht eine Enzym-katalysierte Reaktion ist. Nach einer Literaturrecherche ist die Beteiligung von nichtenzymatischen Schritten in der Biosynthese sekundärer Pflanzenstoffe ein zwar seltenes, aber dennoch wichtiges Phänomen.

Um auf die Fragen weiter einzugehen, wie die Betaxanthin-bildenden Pflanzen es erreichen, ein spezifisches Muster an Betaxanthinen trotz der spontanen Bildungsreaktion zu akkumulieren, und in welchem Kompartiment die Aldiminbildung abläuft, wurde der Transport von Präkursoren und Endprodukten der Betalainbiosynthese in Vakuolen Roter Beete untersucht. Da die Synthese ^{14}C-markierter Substrate mit hoher spezifischer Radioaktivität und Reinheit nicht realisiert werden konnte, waren die Transportexperimente stark erschwert und die HPLC musste zur Quantifizierung der Aufnahme herangezogen werden. Auch waren die Vakuolen-

ausbeuten aus Roten Rüben trotz intensiver Optimierungsversuche vergleichsweise gering und die chemische Instabilität der Betalaminsäure ließ keine reproduzierbaren Ergebnisse zu. Die Ergebnisse der Aufnahme der Rüben-spezifischen Betaxanthine Miraxanthin V (Dopamin-Betaxanthin) und Vulgaxanthin I wie auch des artifiziellen (*R*)-Phe-Betaxanthins in die Vakuolen Roter Rüben zeigten trotz Dreifachbestimmungen eine erhebliche Variabilität und können deshalb nur eingeschränkt interpretiert werden. Ein Grund für die Variabilität der Aufnahmeresultate in verschiedenen Experimenten könnte in der unvermeidbaren Heterogenität der Vakuolenpopulationen liegen. Dennoch kann aus den Daten der vakuolären Betaxanthintransportes, die bei Verwendung verschiedener Inhibitoren erhalten wurden, folgender Trend diskutiert werden: die Hemmung des MgATP-stimulierten Transportes der Rüben-spezifischen Betaxanthine Miraxanthin V (Dopamin-Betaxanthin) und Vulgaxanthin I in die Vakuolen durch 1 mM Vanadat weist auf die Beteiligung eines direkt-energisierten ABC-Transporters hin, während die Hemmung der Aufnahme des unnatürlichen (*R*)-Phe-Betaxanthins durch 0,1 µM Bafilomycin A1 und 5 mM NH_4Cl mit einem H^+/Antiport-System kompatibel ist. Nach sequenzieller Aufnahme von (*R*)-Phenylalanin und Betalaminsäure in Miniprotoplasten, die keine Vakuolen enthalten, konnte die Bildung von (*R*)-Phe-Betaxanthin, wenn auch nur in Spuren, nachgewiesen werden. Dieses Ergebnis und die Daten der Vakuolentransporthemmung stellen dennoch keinen eindeutigen Nachweis für die extravakuoläre Lokalisation des letzten Schrittes der Betalain-Biosynthese dar.

Alle biochemischen Resultate zusammenfassend kann festgestellt werden, dass die vorgelegte Arbeit den letzten Schritt der Betalain-Biosynthese als eine höchst wahrscheinlich spontane Reaktion charakterisiert.

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Erklärungen

Ich erkläre hiermit, dass ich mich mit der vorliegenden wissenschaftlichen Arbeit erstmals um die Erlangung des Doktorgrades bewerbe.

Ich habe diese Arbeit selbständig und ohne fremde Hilfe verfasst, nur die von mir angegebenen Quellen und Hilfsmittel benutzt und den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht.

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