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 (Rf, UV-VIS, LC-MS and 1H-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 1H (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 (Table 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.
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).
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 permethyl 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
Discussion

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, Schlie- 

The structure of compound 7 was elucidated as betanidin 5-O-(2’-O-β-D-apiofuranosyl-6’-O-malonyl)-β-D-glucopyranoside (2’-apiosyl-0-phylllocactin) by HPLC-PDA, LC-MS and ¹H 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-phylllocactin 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’-0-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-phylllocactin 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-phylllocactin 12 from MS data and by comparison with 5’’-O-E-feruloyl-2’-apiosyl-betanin. 5’’-O-E-Feruloyl-apiosyl-phylllocactin 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 $\lambda_{max}$ 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-$\beta$-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-dihydroxyphenylacet-aldehyde), 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 spontaneously 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-methyl pyrrolium 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 ama-
ranthin 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 be-
taxanthin (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 (2S/S, 2S/R, 2R/S, 2R/R). The separation experiments (Schliemann et al. 1999) revealed that the 2S/S- and 2R/R-isomers had identical retention times, likewise, the 2S/R- and 2R/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 7th day with those at 8th 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 ethylenediamine, putrescine, cadaverine and octanediadamine 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)-[2,6-3H2]Phe/(R)-[1-14C]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).

![Biological pathway diagram]

Fig. 5.4 Increased vulgaxanthin I formation by alanine feeding

To determine whether vulgaxanthin I formation is indeed dependent on increased ammonia fixation, (NH₄)₂SO₄ was added to the standard nutrition solution in increasing concentrations (3-51 mM, Fig. 4.26). Indeed, vulgaxanthin I accumulated dependent on the fed (NH₄)₂SO₄ 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 stereoselectivity (Fig. 5.5).
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.
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 $\text{\textit{(R)}}$-Phe-betaxanthin as an unnatural substrate (Fig. 5.7) were performed. Because of the difficulties to isolate or synthesize $^{14}\text{C}$-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). $\text{\textit{(R)}}$-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).
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 NH₄Cl (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 NH₄Cl. 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 ¹⁴C-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 \( \mu \)M 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-protopect 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.