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.