Phytochemistry, Vol. 27, No. 3, pp. 785–789, 1988.
Printed in Great Britain.

0031-9422/88 $3.00 + 0.00

BIOSYNTHESIS OF 3-DEOXYANTHOCYANINS WITH FLOWER EXTRACTS FROM *SINNINGIA CARDINALIS*

K. STICH and G. FORKMANN*

(*Received* 29 *May* 1987)

Institut für Chemie der Universität für Bodenkultur, Gregor-Mendel-Strasse 33, A-1180 Wien, Austria; *Institut für Biologie II der Universität, Auf der Morgenstelle 28 D-7400 Tübingen, F.R.G.

Key Word Index—*Sinningia cardinalis*; 3-deoxyanthocyanidin biosynthesis; flavanone 4-reductase; flavan-4-ol.

Abstract—Soluble enzyme preparations from flowers of *Sinningia cardinalis* catalyse a NADPH-dependent reduction of (2*S*)-naringenin to the respective flavan-4-ol apiforol (leucoapigeninidin) and of (2*S*)-eriodictyol to luteoforol (leucoluteolinidin). NADPH can be substituted to a great extent by NADH. The reaction has a pH optimum around 6.0 and is inhibited about 50% by *p*-chloromercuribenzoate. The significance of the reaction for the 3-deoxyanthocyanin pathway and the relationship of flavanone 4-reductase and dihydroflavonol 4-reductase, which was also found to be active in the flower extracts, is discussed.

INTRODUCTION

In contrast to the common anthocyanins, which possess a hydroxyl group at the 3-position, the 3-deoxyanthocyanins are of rare occurrence in nature. They have been found in mosses and ferns and in some other plants, but they are in particular present in the flowers of the new World species of the Gesneriaceae [1].

During biosynthesis of the common anthocyanins, the hydroxyl group in the 3-position is introduced by flavanone 3-hydroxylase, which converts flavanones to dihydroflavonols [2–6]. Subsequent reduction of dihydroflavonols catalysed by dihydroflavonol 4-reductase leads to the flavan-3,4-*cis*-diols (leucoanthocyanidins) which were recently shown to be intermediates in anthocyanin biosynthesis [7, 8].

With regard to biosynthesis of 3-deoxyanthocyanins, however, the reduction step most likely proceeds with flavanones such as naringenin or eriodictyol. This reaction leads to the flavan-4-ols apiforol and luteoforol which, by analogy with the flavan-3,4-diols, can be regarded as the direct precursors of the respective 3-deoxyanthocyanidins apigeninidin and luteolinidin (Fig. 1). The concomitant occurrence of flavan-4-ols with the respective anthocyanidins in *Sorghum* supports this assumption [9–11].

To elucidate the biosynthetic pathway of 3-deoxyanthocyanidins we used flowers of *Sinningia* (syn. *Rechsteineria*) *cardinalis* which are known to contain 3-deoxyanthocyanins. In this paper we report on the enzymatic reduction of flavanones to flavan-4-ols with flower extracts of this plant.

RESULTS

Chemical studies

Only the aglycones of the flavonoids present in the scarlet flowers of *Sinningia cardinalis* were identified. In agreement with an earlier report [1] the flowers were found to contain the 3-deoxyanthocyanidins, apigeninidin and luteolinidin along with the related flavones apigenin and luteolin. With regard to intermediates naringenin was found to be present, but dihydroflavonols

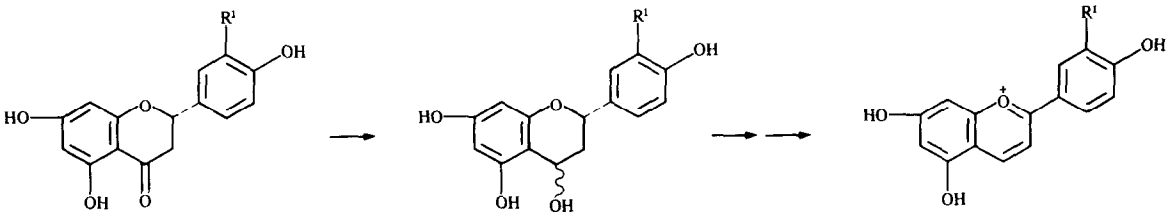

Fig. 1. Structures of the flavonoids involved in 3-deoxyanthocyanidin biosynthesis of *Sinningia cardinalis*.
1: $R^1 = H$, naringenin, $R^1 = OH$, eriodictyol
2: $R^1 = H$, apiforol; $R^1 = OH$, luteoforol
3: $R^1 = H$, apigeninidin; $R^1 = $ luteolinidin

could not be observed. The related flavonols were also absent.

Enzymatic reduction of (2S)-*naringenin to apiforol*

When (2*S*)-(^{14}C)naringenin was incubated with an enzyme preparation from the scarlet flowers of *S. cardinalis* in the presence of NADPH, the radiochromatogram of the reaction mixture in solvent system 1 showed a new radioactive compound with a broad peak around R_f 0.46 (Fig. 2). The R_f value (Table 1) and the broad shape of the peak corresponded to synthetic apiforol. With longer incubation time some radioactivity streaks from the start-line as far as the main product, indicating the formation of polymeric material (Fig. 2). Moreover, small amounts of the flavone apigenin were sometimes found to be formed. With boiled enzyme extracts (5 min, 95°) no product was formed.

Naringenin and apiforol as well as the radioactive substrate and product were not separated sufficiently in a range of usual solvent systems. In the solvent systems 2 and 3, however, separation of the radioactive peaks of naringenin and the main product were considerably improved by addition of synthetic apiforol to the EtOAc extracts of the reaction mixture before chromatography. In both solvent systems and in solvent system 4, where apiforol has a slightly smaller R_f than naringenin, the product of the enzymatic reaction corresponded to synthetic apiforol (Table 1). Moreover with two-dimensional chromatography using solvent 2 in the first and solvent system 1 in the second dimension the radioactive product clearly comigrated with synthetic apiforol. Separation of 2,4-stereoisomers similar to that of the 3,4-isomer of flavan-3,4-diols in solvent system 4 [8, 12] was not observed. The identity of apiforol and the radioactive product was further confirmed by its chemical conversion to the respective anthocyanidin. After co-chromatography with an authentic sample in four solvent systems, radioactivity was found to be localized to a great extent in the apigeninidin band.

Properties of reductase preparation

At 25° the synthesis of apiforol from naringenin was linear with protein concentration up to 17 μg protein (Sephadex G-25 eluate). Linearity with time was observed for at least 60 min. The reaction had an optimum at about pH 6.0 with half maximal activities at pH 4.7 and 6.9. When the substrate (naringenin) concentration exceeded 100 μM, an inhibition of the reaction was observed. Under standard conditions (50 μM naringenin), the reaction rate amounted to about 15 μkat $\times$ kg^{-1} protein. From two independent experiments K_M values of 21 and 23 μM were estimated. Addition of potassium cyanide, EDTA, diethyldithiocarbamate, phenanthroline or *N*-ethylmaleimide (2 mM each) to the enzyme assays had no or only a slightly reducing effect on enzyme activity. Clear inhibition (about 50%) was observed with 0.5 mM *p*-chloromercuribenzoate.

When flowers were frozen in liquid nitrogen and stored at −80° for several weeks, the extractable enzyme activity somewhat increased compared to fresh flowers. Sephadex G-25 eluates, but not crude extracts, could be stored under the same conditions without loss of enzyme activity. Addition of 10% glycerol to the buffer used for enzyme preparation completely destroyed reductase activity. Addition of glycerol or sucrose (20%) to the Sephadex G-25 eluates before storage also reduced reductase activity drastically even if these compounds were again removed by gel filtration after storage.

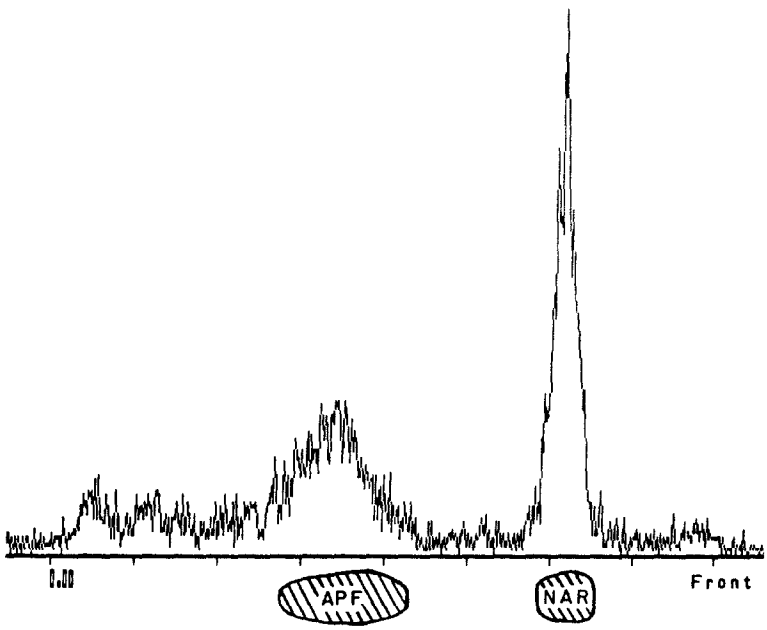

Fig. 2. Radioscan of TLC on cellulose with solvent system 1 from incubation of (^{14}C)naringenin with enzyme extracts from *S. cardinalis* flowers in presence of NADPH after 30 min incubation time. NAR: naringenin; APF: apiforol.

Cofactor dependence

The enzymatic reduction of naringenin was clearly dependent on NADPH. In incubation without NADPH, no enzyme activity was observed. Saturation of the reaction was found with 5 mM NADPH per assay. NADPH could be substituted to a great extent by NADH. With 5 mM NADH per assay about 77% of the reaction rate found with NADPH at the same concentration was observed. FMN, FAD or tetrahydropterin had no effect on enzyme activity whether or not NADPH was present.

Substrate specificity

Besides naringenin, eriodictyol served as substrate for the reductase leading to the formation of luteoforol, which was identified chromatographically as described above. Similarly for naringenin and apiforol, clear separation of eriodictyol and luteoforol was only achieved with solvent system 1 (Table 1). Under standard conditions (50 μM eriodictyol) the rate of luteoforol formation amounted to about 90% of the rate of apiforol formation from naringenin. When [^{14}C] 5,7,3′,4′,5′-pentahydroxyflavanone was used as substrate, a product with R_f 0.06 was observed in solvent system 1. Although no authentic sample was available, this product is most likely 5,7,3′,4′,5′-pentahydroxyflavan-4-ol.

Apart from flavanones, dihydroflavonols were tested as substrates. Dihydrokaempferol as well as dihydroquercetin and dihydromyricetin were found to be reduced by the enzyme preparations from flowers of *S. cardinalis*. The 3,4-*cis*-diols of (+)-leucopelargonidin, (+)-leucocyanidin and (+)-leucodelphinidin were identified as products of the enzymatic reaction by two-dimensional co-chromatography with the synthetic compounds, as described earlier [8].

DISCUSSION

With flower extracts from defined genotypes of *Matthiola incana*, it was recently proved that dihydroflavonol 4-reductase, catalysing the conversion of dihydroflavonols to flavan-3,4-*cis*-diols, is involved in the biosynthesis of the common anthocyanins [8]. Using enzyme preparations from *S. cardinalis* flowers, which contain 3-deoxyanthocyanins, we now demonstrate an enzyme which reduces the carbonyl group of flavanones. The products formed are the respective flavan-4-ols which, in analogy to the flavan-3,4-diols, can be regarded as intermediates in 3-deoxyanthocyanidin formation. Therefore, the reductase activity of *S. cardinalis* flowers is most likely an enzyme which is specifically involved in the 3-deoxyanthocyanidin pathway.

In agreement with the presence of high amounts of both apigeninidin and luteolinidin 5-glucosides in the flowers, the flavanones naringenin and eriodictyol are substrates for the reduction to apiforol and luteoforol, respectively. Although tricetinidine derivatives are not formed in *S. cardinalis* flowers, 5,7,3′,4′,5′-pentahydroxyflavanone also seems to be reduced. Similarly, the dihydroflavonol 4-reductase from *Matthiola* flowers, which naturally lack delphinidin derivatives, reduce dihydromyricetin to leucodelphinidin [8].

The stereochemistry of the 3,4-diols is known and methods are available to synthesize and separate the 3,4-*trans*- and the 3,4-*cis*-isomer [7, 8, 12–14]. Less information, however, is available about the occurrence and stereochemistry of flavan-4-ols. Apiforol and luteoforol are assumed to be present as monomeric flavanols in *Sorghum* tissue [9–11]. But they have not been isolated and characterized from plants as yet. Moreover, chemical synthesis of luteoforol by borohydride reduction of eriodictyol resulted in a rather unstable product, which could only be characterized by further transformation to anthocyanidin [10, 15]. We observed a similar high instability of both the synthetically and the enzymatically produced flavan-4-ols in the presence of acid. But they were found to be stable during chromatography on cellulose in water and during elution from the plates with MeOH. This allowed the purification of the synthetic compounds in amounts sufficient for spectral analysis and cochromatography with the enzymatically formed product.

The Gesneriaceae is a highly advanced plant family. Here, the 3-deoxyanthocyanidins are assumed to be synthesized in response to selection for scarlet flower colour, which is favoured by bird pollinators [1]. Provided that the same set of enzymes catalyse the conversion of flavan-3,4-diols and flavan-4-ols to the respective anthocyanidins, an enzyme for the reduction of flavanones had to be developed during this selection process. In this context, it is of special interest that the properties of the reductase from *S. cardinalis* flowers are similar to those of the dihydroflavonol 4-reductase from *Matthiola* flowers [8]. Both enzymes catalyse the reduction of the carbonyl group of flavonoids, the reactions are dependent on NADPH, which can be replaced to a great extent by NADH, and the greatest activity is found around pH 6.0. Moreover, the enzyme preparations from *S. cardinalis* flowers reduce not only flavanones to flavan-4-ols but also dihydroflavonols to flavan-3,4-*cis*-diols. These results suggest that the enzyme reducing the flavanones in *S. cardinalis* flowers might have been derived from a dihydroflavonol 4-reductase or that the reduction of flavanones and dihydroflavonols is catalysed by one and the same enzyme.

Although dihydroflavonols are also reduced by enzyme extracts of *S. cardinals* flowers, 3-deoxyanthocyanidins are formed in this plant. This fact can be explained by the observation that flavanones but not dihydroflavonols are naturally present in the flowers. Preliminary results show that this lack of dihydroflavonols is most likely caused by a deficiency of flavanone 3-hydroxylase activity. Thus, for the exclusive formation of 3-deoxyanthocyanins two events are probably necessary: development of a flavanone-specific reductase and loss of flavanone 3-hydroxylase.

EXPERIMENTAL

Plant material. The scarlet flowers of the commercial strain 'Feuerschein' (Fa. Walz, Stuttgart) of *Sinningia cardinalis* were used for the investigations. The plants were cultivated in a greenhouse.

Chemicals and synthesis of substrates. Naringenin, eriodictyol, dihydroquercetin and apigeninidin were obtained from Roth (Karlsruhe, F.R.G.). 5,7,3′,4′,5′-pentahydroxyflavanone, dihydrokaempferol, dihydromyricetin, luteolinidin and 4-coumaroyl-CoA were from our laboratory collection. The flavan-3,4-*trans*-diols were kind gifts from W. Heller (Neuherberg, F.R.G.) and L. Britsch (Freiburg, F.R.G.). The respective *cis*-isomers were prepared according to Stafford and Lester [12].

(2-^{14}C)Malonyl-CoA (2.22GBq/mmol) was obtained from Amersham–Buchler (Braunschweig, F.R.G.) and diluted to 1.03 GBq/mmol with unlabelled material from Sigma. (4a,6,8-^{14}C)Naringenin (3.09 GBq/mmol) was prepared from (^{14}C)malonyl-CoA and 4-coumaroyl-CoA with enzyme preparation from parsley (5, 16). Hydroxylation of labelled naringenin to dihydrokaempferol and hydroxylation of both compounds in 3′-and 3′,5′-position was achieved with partially purified enzyme preparations from *Petunia hybrida* as described earlier [8, 17, 18].

Buffers. The following buffers were used: (A) 0.1 M Tris–HCl, pH 7.5, 20 mM Na ascorbate; (B) 0.05 M McIlvaine, pH 5.8, 20 mM Na ascorbate. For the determination of the pH optimum buffer B was used in a range from pH 3.0 to pH 7.0. The pH was adjusted after addition of Na ascorbate. Buffers were made O_2-free by boiling for 5 min and subsequent cooling in an ice bath in N_2 before addition of Na ascorbate.

Preparation of crude extracts. All steps were carried out at +4°. 4 g flowers (1.5–2 cm long, top still closed) were homogenized in a prechilled mortar together with 2 g Dowex 1 × 2, 1 g quartz sand and 8 ml buffer A. The homogenate was centrifuged twice for 3 min each at 10000 *g*. The supernatant of the second centrifugation was subjected to buffer exchange by gel filtration on Sephadex G-25 with buffer B. The eluate containing the protein fraction served as enzyme source.

Standard enzyme assay. The incubation mixture contained in a total volume of 50 μl: 167 Bq (4*a*,6,8-^{14}C)naringenin, 2.5 nmol unlabelled naringenin, 250 nmol NADPH in 5 μl H_2O, and 1–17 μg protein in buffer B. Incubation was carried out for 30 min at 25°. The mixture was immediately extracted twice (50 and 30 μl) with EtOAc and the extract chromatographed on a cellulose plate with solvent system 1. Radioactivity was localized by scanning the plates (TLC analyser, Berthold, Wildbad, F.R.G.) and enzyme activity was determined by integration of the peak areas of naringenin used as substrate and of apiforol formed.

Determination of pH optimum. Crude extract was subjected to buffer exchange by gel filtration on Sephadex G-25 using buffer B with pH between 3.0 and 7.0. The enzyme assays were carried out in mixtures of 20 μl enzyme solution and 25 μl buffer B both with the respective pH and 5 μl NADPH (50 mM) solution in H_2O.

Analytical methods. TLC was performed on precoated cellulose plates (Merck, Darmstadt, F.R.G.) with (1) $CHCl_3$–HOAc–H_2O (10:9:1), (2) H_2O, (3) 6% HOAc and (4) *n*-BuOH satd with 0.01 M Pi buffer, pH 6.8. Flavanones were detected by reduction with borohydride and subsequent exposure to HCl fumes [20]. Dihydroflavonols were detected by treatment of the plates with Zn dust and subsequent spraying with 6 M HCl [21]. Flavan-4-ols and respective polymeric material could be made visible by exposure of the plates to HCl fumes or spraying with *n*-BuOH–HCl (7:3). The flavonoids were also detected by spraying the plates with 0.1% aqueous fast blue B salt and subsequent exposure to NH_3 vapours.

Apiforol and luteoforol were prepared from naringenin and eriodictyol (Fig. 1), respectively, by borohydride reduction [10, 15]. After acidification, the reaction mixture was extracted with Et_2O. Chromatographic separation of the Et_2O phase revealed beside the flavan-4-ol and unreduced flavanone the presence of appreciable amounts of polymeric material forming streaks from the start-line. Best separation was achieved with solvent system 1, but during chromatography and/or drying of the plates the flavan-4-ols rapidly polymerized. They were only found to be stable in the complete absence of acid. Thus, after separation of the Et_2O extracts in H_2O, apiforol and luteoforol could readily be eluted with MeOH. Their chromatographic data are summarized in Table 1. Both apiforol and luteoforol have single peaks in the UV at 275 and 278 nm, respectively, in MeOH. When heated in 2 M HCl for 15 min the product formed from naringenin (apiforol) became yellow and that from eriodictyol (luteoforol) cherry red. Both the spectral data (λ_{max} = 485 and 495 nm in MeOH) and the chromatographic data correspond to those for apigeninidin and luteolinidin, respectively [19]. Moreover, both reduction products formed in *n*-BuOH/HCl (7:3) at room temperature an anthocyanindin-like pink pigment (λ_{max} about 550 nm) [10, 11]. The flavan-4-ols formed by enzymatic reduction of labelled flavanones were identified by co-chromatography with the synthetic compounds. Apiforol was also identified by 2D chromatography with solvent 2 in the first and solvent 1 in the second dimension. In that case radioactivity was localized using the Betacamera (Berthold, Wildbad, F.R.G.). The reaction product apiforol was also transformed to the respective anthocyanidin apigeninidin. Because a transformation rate of only 15% was expected [22] the standard enzyme assay was performed with 1167 Bq (^{14}C) naringenin as substrate. After extraction of the reaction mixture, the EtOAc phase was treated with 10 μl 3M HCl and the mixture kept at 100° for 60 min. The apigeninidin formed was extracted with 50 μl *n*-BuOH and co-chromatographed with an authentic sample on cellulose plates in the solvent systems BAW, HOAc–HCl and Forestal and on silica gel in HOAc–HCO_2H–H_2O–HCl (85:6:8:1). R_f values of apigeninidin were 0.77, 0.16, 0.83 and 0.50, respectively. Standard methods [19] were used for analysis of the flavonoids in the flowers. Protein was determined by a modified Lowry procedure as described earlier [8].

Table 1. R_fs (× 100) of substrates and products on cellulose TLC plates

Compound	Solvent systems 1	2	3	4
Naringenin	83	16	20	97
Eriodictyol	61	10	14	93
Apiforol	46	23	26	93
Luteoforol	20	16	19	88

For solvent key, see Experimental.

Acknowledgements—The authors thank Prof. H. Grisebach, Dr W. Heller and Dr L. Britsch for parsley enzyme preparation, containing chalcone synthase and chalcone isomerase and for the flavan-3,4-*trans*-diols. The studies were supported by the 'Fond zur Förderung der wissenschaftlichen Forschung', Austria.

REFERENCES

1. Harborne, J. B. (1966) *Phytochemistry* **5**, 589.
2. Forkmann, G. (1980) *Z. Naturforsch.* **35c**, 691.
3. Forkmann, G. and Stotz, G. (1981) *Z. Naturforsch.* **36c**, 411.
4. Forkmann, G. and Stotz, G. (1984) *Planta* **161**, 261.
5. Britsch, L., Heller, W. and Grisebach, H. (1981) *Z. Naturforsch.* **36c**, 742.
6. Britsch, L. and Grisebach, H. (1986) *Eur. J. Biochem.* **16**, 1.
7. Heller, W., Britsch, L., Forkmann, G. and Grisebach, H. (1985) *Planta* **163**, 191.
8. Heller, W., Forkmann, G., Britsch, L. and Grisebach, H. (1985) *Planta* **165**, 284.
9. Stafford, H. A. (1965) *Plant Physiol.* **40**, 184.

10. Bate-Smith, E. C. (1969) *Phytochemistry* **8**, 1803.
11. Watterson, J. J. and Butler, L. G. (1983) *J. Agric. Food. Chem.* **31**, 41.
12. Stafford, H. A. and Lester, H. H. (1984) *Plant Physiol.* **76**, 184.
13. Porter, L. J. and Foo, L. Y. (1982) *Phytochemistry* **21**, 2947.
14. Kristiansen, K. N. (1986) *Carlsberg, Res. Commun.* **51**, 51.
15. Bate-Smith, E. C. and Rasper, V. (1969) *J. Food. Sci.* **34**, 203.
16. Britsch, L. and Grisebach, H. (1985) *Phytochemistry* **24**, (9), 1975.
17. Stotz, G. (1983) Ph.D. Thesis, Tübingen.
18. Froemel, S., de Vlaming, P., Stotz, G., Wiering, H., Forkmann, G. and Schram, A. W. (1985) *Theor. Appl. Genet.* **70**, 561.
19. Harborne, J. B. (1967) *Comparative Biochemistry of the Flavonoids*. Academic Press, London.
20. Eigen, E., Blitz, M. and Gunsberg, E. (1957) *Arch. Biochem. Biophys.* **16**, 1.
21. Barton, G. M. (1968) *J. Chromatogr.* **34**, 562.
22. Krishnamurty, H. G., Krishnamoorthy, V. and Seshadri, T. R. (1962) *Phytochemistry* **2**, 47.