Genetic Control of Flavanone 3-Hydroxylase Activity and Flavonoid 3′-Hydroxylase Activity in *Antirrhinum majus* (Snapdragon)

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In flower extracts of defined genotypes of *Antirrhinum majus*, two different hydroxylases were found catalysing the hydroxylation of naringenin and eriodictyol in the 3-position and of naringenin in the 3′-position. The 3-hydroxylase is a soluble enzyme and belongs according to its cofactor requirement to the 2-oxoglutarate-dependent dioxygenases. Investigations on different genotypes revealed a clear correlation between block of the anthocyanin pathway by recessive alleles of the gene *inc* and a complete lack of 3-hydroxylase activity. Chemogenetic studies on different genotypes suggested that the 3′-hydroxyl group of the B-ring of flavonoids is introduced at the stage of C_{15} intermediates. The corresponding 3′-hydroxylase was found to be localized in the microsomal fraction and required NADPH as cofactor. In confirmation of the chemogenetic studies, a strict correlation was found between 3′-hydroxylase activity and the gene *eos* which is known to control the hydroxylation of flavones, flavonols and anthocyanins in the 3′-position. These results are similar to those previously obtained with *Matthiola incana*.

Introduction

Recently, the first successful correlation between a gene in *Matthiola incana* regulating introduction of the 3′-hydroxyl group of anthocyanins and enzymatic 3′-hydroxylation was reported [1]. A NADPH-dependent microsomal 3′-hydroxylase was detected which catalysed hydroxylation of naringenin (**1**) to eriodictyol (**2**) and of dihydrokaempferol (**3**) to dihydroquercetin (**4**), respectively (Fig. 1). Furthermore, a soluble enzyme was described which catalysed hydroxylation of flavanones in the 3-position to dihydroflavonols [1]. The gene controlling 3-hydroxylation of flavanones in *M. incana* has, however, not yet been identified. A correlation between genotype and 3-hydroxylase activity could therefore not be established in this plant.

In *Antirrhinum majus*, however, genes which seem to control the conversion of flavanones to dihydroflavonols and the 3′-hydroxylation of flavonoids are known (Fig. 1). We now report on the first successful correlation between a genotype of *A. majus* and enzymatic 3-hydroxylation of flavanones. Furthermore, chemogenetic evidence is presented that introduction of the 3′-hydroxyl group occurs at the flavanone stage. A hydroxylase catalysing this reaction was detected only in flowers with wild-type alleles of the gene *eos*.

Abbreviations: K-Pi, potassium phosphate; PVP, polyvinylpyrrolidone.

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Materials and Methods

Plant material

The investigations included six defined genotypes of *A. majus* (Table I). In this plant, the gene *niv* controls the activity of chalcone synthase [2]. Flavonoids can only be formed in presence of wild-type alleles of the gene *niv*. The gene *sulf* concerns aurone production in the flowers. The formation of aurones is known to be suppressed by dominant alleles of this gene [3]. The actions reported for the genes *inc* and *eos* were of special interest for our investigations (Fig. 1). Chemogenetic studies and supplementation experiments on white flowering mutants indicated that the gene *inc* is responsible for the conversion of flavanones to dihydroflavonols. Recessive genotypes (*inc*/*inc*) were found to accumulate flavones and flavanones [4]. Furthermore, anthocyanin synthesis could readily be initiated in the white flowers of recessive genotypes by administration of dihydroflavonols, but not by administration of flavanones [5].

The gene *eos* concerns the B-ring hydroxylation of flavonoids in 3′-position. Recessive genotypes

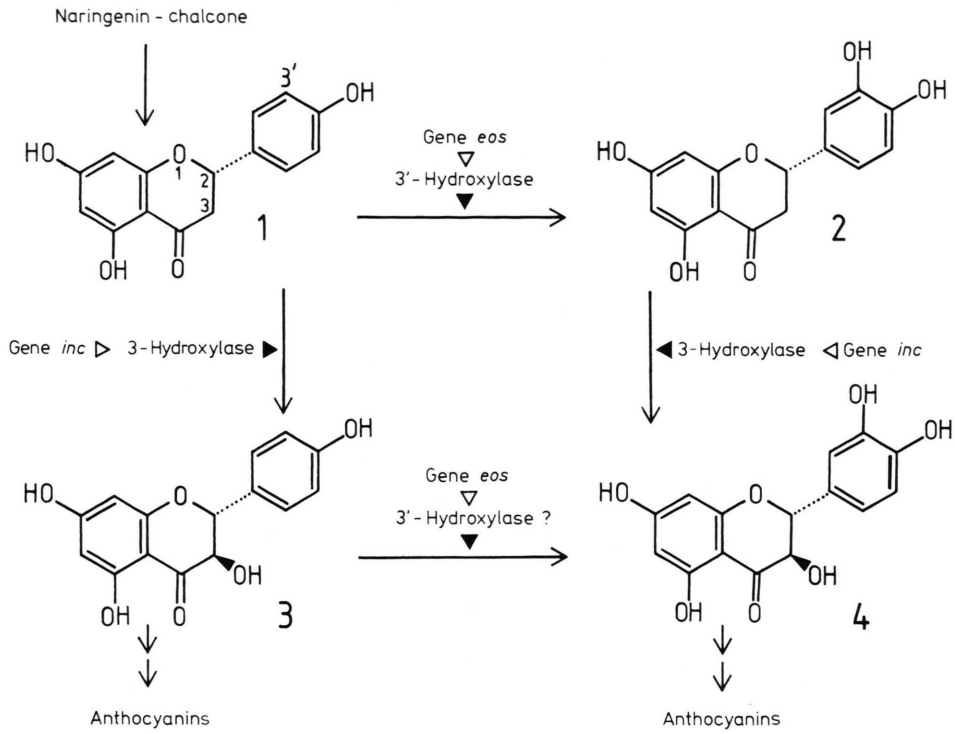

Fig. 1. Structural formulas of substrates and products and the genetic control of two different hydroxylases in *Antirrhinum majus*. Naringenin (**1**); eriodictyol (**2**); dihydrokaempferol (**3**); dihydroquercetin (**4**).

(*eos/eos*) are known to produce apigenin, kaempferol and pelargonidin in the flowers, whereas under the influence of wild-type alleles (*Eos*) the corresponding 3′,4′-hydroxylated compounds were found.

The plant material was cultivated during the summer months of 1980 in the experimental garden of our institute.

Chemogenetic studies

Only the aglycones of flavanones and dihydroflavonols present in the flowers of *A. majus* were identified. The flowers were twice extracted with ethyl acetate at 4 °C. The pooled extracts were subjected to acid or enzymatic (*β*-glucosidase, Serva) hydrolysis [6] and the aglycones extracted from the reaction mixture with ether. The flavanones and dihydroflavonols present in the ether phase were identified by co-chromatography with authentic samples on 0.1 mm cellulose TLC plates (Schleicher and Schüll) in three different solvent systems (Table II). Flavanones were detected by reduction with borohydride and subsequent exposure to HCl fumes [7] and dihydroflavonols by the zinc-HCl test [8].

Chemicals and synthesis of substrates

[2-^{14}C]Malonyl-CoA (60 Ci/mol) was obtained from Amersham Buchler and diluted to 26 Ci/mol with unlabelled material from Sigma. [4a,6,8-^{14}C]-Naringenin, [4a,6,8-^{14}C]eriodictyol and [4a,6,8-^{14}C]dihydrokaempferol were prepared enzymatically [1]. Naringenin, hesperitin, dihydroquercetin, kaempferol and quercetin were obtained from Roth (Karlsruhe). Dihydrokaempferol was isolated from petals of *M. incana* [9]. Eriodictyol was prepared by demethylation of hesperitin by a BBr_3 treatment (Kho, personal communication).

Preparation of crude extract and microsomal fraction

1 g flowers (without anthers and pistils) were homogenized at 4 °C in a prechilled mortar together with 0.5 g PVP, 0.5 g quartz sand and 4 ml 0.1 M Tris-HCl buffer, pH 7.5, containing 28 mM 2-mercaptoethanol. The homogenate was transfered to Micro Test Tubes (Eppendorf) and centrifugated for 5 min at about $10000 \times g$. The supernatants were pooled and centrifugated again as described above.

Table I. Chemogenetic characterisation of six flower colour types of *A. majus* and the genetic control of 3-hydroxylase and 3′-hydroxylase activity.

Genotype (homozygous)	Flower colour	Flavonoid aglycones in flowers		Hydroxylating activities	
		End products	Intermediates	3-Hydroxylase	3-′Hydroxylase
Niv Sulf Inc eos	pink	Ap, Ka, Pg	NAR, DHK	high	not detectable
Niv Sulf Inc Eos	magenta	Ap, Lu, Ch, Qu, Cy	NAR, ERI, DHK, DHQ	high	high
Niv Sulf inc Eos	ivory	Ap, Lu, Ch	NAR, ERI	not detectable	high
Niv sulf inc eos	yellow	Au, Ap	not investigated	not detectable	not detectable
Niv sulf Inc eos	orange	Au, Ap, Ka, Pg	not investigated	high	not detectable
niv Sulf Inc Eos	white	no flavonoids present		high	high

Au = Aurones, Ap = Apigenin, Lu = Luteolin, Ch = Chrysoeriol, Ka = Kaempferol, Qu = Quercetin, Pg = Pelargonidin Cy = Cyanidin, NAR = Naringenin, ERI = Eriodictyol, DHK = Dihydrokaempferol, DHQ = Dihydroquercetin.

Table II. R_f-values (× 100) on cellulose plates (Schleicher and Schüll) of the intermediates present in the flower extracts and of the substrates and products.

Compound	Solvent systems			
	HOAc	CAW	BAW	TBA
Naringenin	42	85	92	90
Eriodictyol	37	60	87	86
Dihydrokaempferol	59	65	90	88
Dihydroquercetin	56	37	85	74
Kaempferol	06	–	87	80
Quercetin	03	–	75	60
Luteolin	08	45	83	81

HOAc = 15% acetic acid, CAW = chloroform/acetic acid/water (10:9:1), BAW = *n*-butanol/acetic acid/water (6:1:2), TBA = *tert.*-butanol/acetic acid/water (3:1:1).

The clear supernatant of the second centrifugation served as enzyme source for flavanone 3-hydroxylase.

The preparation of the crude extract and microsomal fraction for the determination of flavonoid 3′-hydroxylase activity was performed according to ref. [1].

Enzyme assays

The enzyme assays for 3-hydroxylase and 3′-hydroxylase, and the chromatographic separation and identification of the reaction products were performed as described earlier [1].

Determination of pH optimum

The enzyme assays were carried out in mixtures of 180 µl buffer (between pH 6.5 and 8.5) and 20 µl crude extract (3-hydroxylase) or microsomal fraction (3′-hydroxylase).

Determination of protein

The method of Bradford [10] was used for both, crude extract and microsomal fraction.

Results

The investigations revealed two hydroxylating enzyme activities in the flower petals of *A. majus*. One enzyme catalysed hydroxylation of flavanones in the 3-position to dihydroflavonols; the second enzyme catalysed the hydroxylation of naringenin in the 3′-position to eriodictyol. These enzymes could be investigated separately by genetic and biochemical methods. Besides the two hydroxylases a further enzyme activity was found for conversion of flavanones to flavones (Stotz and Forkmann, unpublished).

3-Hydroxylation

To characterize the flavanone 3-hydroxylase reaction extracts of pink flowers were used (Table I). This genotype is homozygous recessive for the gene *eos* and was therefore expected to lack flavonoid 3′-hydroxylase activity.

When a crude extract prepared with PVP was incubated with [^{14}C]naringenin (**1**), the radiochromatograms of the reaction mixture showed a new radioactive product which corresponded to dihydrokaempferol (**3**) in three different solvent systems (Table II). The formation of dihydrokaempferol was further confirmed by oxidation of the reaction product to kaempferol with sodium metabisulfite [12]. Incubations with [^{14}C]eriodictyol (**2**) as substrate led to the formation of dihydroquercetin (**4**) which was identified similarly.

As in *M. incana*, where 2-oxoglutarate, ascorbate and ferrous ions were found to be cofactors for the 3-hydroxylase reaction [1], the addition of 2-oxoglutarate and ascorbate to the enzyme assays clearly stimulated the 3-hydroxylase activity (Table III). Furthermore, after treatment of the extracts with Dowex 1 × 2 or after gel filtration, only very low enzyme activity could be observed. In this case enzyme activity could be restored by addition of a combination of these cofactors, whereas no pronounced effect was found upon addition of any of the cofactors alone (Table III). In contrast to *M. incana*, however, highest restoration of enzyme activity was achieved without addition of ferrous ions (Table III). NADPH showed no stimulatory effect on enzyme activity in PVP preparations but led to a slightly lower product yield. Because in presence of NADPH apigenin was formed from naringenin in addition to dihydrokaempferol (Stotz and Forkmann, unpublished), this result can probably be explained by competition of these two reactions for the same substrate.

The activity of 3-hydroxylase from flowers of *A. majus* was found to be dependent on the type of buffer. Highest enzyme activity was observed when the crude extract was prepared with Tris-HCl buffer in presence of ascorbic acid (Table III). In contrast to *M. incana*, no enzyme activity could be demonstrated in flower extracts of *A. majus* with K-Pi buffer. With this buffer enzyme activity could not be restored by addition of ascorbate and 2-oxoglutarate (Table III). In preparations of the microsomal fraction by Mg^{2+}-precipitation or by ultracentrifugation, the activity remained in the supernatant. The reaction was linear with protein concentration up to 40 µg protein per assay and with time to about 20 min. Highest conversion of naringenin to dihydrokaempferol was found at about pH 7.5. The reaction was strongly inhibited by KCN, EDTA and diethyldithiocarbamate, whereas *p*-chloromercuribenzoate and diethylpyrocarbonate had no appreciable effect (Table IV). The latter reagent was found to inhibit flavonoid 3′-hydroxylase [1] and chalcone isomerase [13].

Tests with enzyme preparations of the other genotypes investigated revealed that activity for hydroxylation is clearly controlled by the gene *inc* (Table I). Enzyme activity could only be demonstrated in flower extracts of all genotypes with wild-type alleles of this gene. In mixtures containing 3-hydroxylase and extracts from flowers with the recessive allele *inc*, no inhibition of enzyme activity was found.

Table III. Dependence of 3-hydroxylase activity on enzyme preparation and on cofactors added.

Enzyme source	Cofactor added	3-Hydroxylation (cpm in dihydrokaempferol) [a]
Tris-HCl buffer with 10 mM ascorbic acid, PVP preparation (A)	none	15 560
	ascorbate and 2-oxoglutarate	31 675
	NADPH	11 865
A after gel filtration (G-50)	none	927
	ascorbate	1 327
	2-oxoglutarate	2 927
	Fe^{2+}	782
	ascorbate and 2-oxoglutarate	31 318
	ascorbate and 2-oxoglutarate and Fe^{2+}	20 264
Tris-HCl buffer, PVP preparation	none	8 042
	ascorbate and 2-oxoglutarate	22 510
	NADPH	6 221
Tris-HCl buffer, Dowex preparation	none	1 858
	ascorbate and 2-oxoglutarate	8 150
K-Pi buffer, PVP preparation	none	0
	ascorbate and 2-oxoglutarate	0

[a] Product formed with 100 µg protein.

Table IV. Effect of inhibitors on 3-hydroxylation of naringenin.

Additions	3-Hydroxylation activity [%]
none	100
1 mM EDTA	24
2 mM EDTA	21
2 mM KCN	28
5 mM KCN	14
2 mM Diethyldithiocarbamate	16
0.5 mM Diethylpyrocarbonate	78
0.1 mM Chloromercuribenzoate	81

3′-Hydroxylation

Chromatographic studies proved that flavanones and dihydroflavonols occured in addition to the end products in extracts of cyanic flowers. In flower extracts of genotypes blocked by recessive alleles of

the gene *inc* only flavanones were present. The correlation between the B-ring hydroxylation pattern of these intermediates and the genetic state of the gene *eos* should give clues at what stage in the pathway introduction of the 3′-hydroxyl group occurs.

Only the aglycones of the intermediates were identified. Because part of the intermediates were found to be glycosylated, the flower extracts were subjected to enzymatic or acid hydrolysis and the aglycones analyzed by co-chromatography with authentic samples on cellulose TLC plates in three different solvents (Table II). In the cyanic genotype with recessive alleles of the gene *eos* only the 4′-hydroxylated intermediates naringenin (**1**) and dihydrokaempferol (**3**) were found (Table I). In contrast, in genotypes with wild-type alleles of the gene *eos*, both 4′-hydroxy and 3′,4′-dihydroxy intermediates were present. Thus, in the magenta genotype (*Inc/Inc Eos/Eos*) naringenin (**1**), eriodictyol (**2**), dihydrokaempferol (**3**) and dihydroquercetin (**4**) could be demonstrated and in the acyanic mutants (*inc/inc Eos/Eos*) naringenin and eriodictyol were found (Table I).

From these results, it can be concluded that in *A. majus* the 3′-hydroxyl group is introduced at the stage of C_{15} intermediates. Consequently, a corresponding enzyme should be demonstrable in flower extracts of genotypes with wild-type alleles of the gene *eos*. In order to exclude enzyme activity for 3-hydroxylation, the crude extracts for the determination of 3′-hydroxylase activity were prepared from a genotype blocked by recessive alleles of the gene *inc* (Table I). After incubation of [^{14}C]naringenin with crude flower extract and NADPH, the reaction mixture showed three new radioactive products in solvent system CAW. One product was identified as eriodictyol (**2**) by co-chromatography with the authentic flavanone (Table II). This result was confirmed by enzymic 3-hydroxylation of the product to dihydroquercetin and by enzymic oxidation of eriodictyol to luteolin (Stotz and Forkmann, unpublished). The other products were identified as apigenin and luteolin (Stotz and Forkmann, unpublished).

The 3′-hydroxylase activity was present in the microsomal pellet prepared by Mg^{2+}-precipitation or by centrifugation at $90000 \times g$ (Table V). Hydroxylation in 3′-position was strictly dependent on NADPH. Substitution of NADPH by NADH gave a much lower product yield (Table V). Flavone formation was also found to be located in the microsomal fraction and required NADPH as cofactor (Stotz and Forkmann, unpublished). Separate investigation of 3′-hydroxylation could not be achieved by addition of several inhibitors. The dependence of the 3′-hydroxylase reaction on protein concentration and time, and the influence of some inhibitors on enzyme activity could therefore not be determined precisely. The pH optimum of the 3′-hydroxylase reaction was found to be at about pH 7.5. With [^{14}C]dihydrokaempferol as substrate, no clear 3′-hydroxylation to dihydroquercetin could be observed although dihydrokaempferol is known to be readily converted to cyanidin in supplementation experiments [14].

Table V. Cofactor requirement and subcellular localisation of 3′-hydroxylase in flowers of *A. majus*.

Enzyme source	Cofactor added	3′-Hydroxylation (cpm in eriodictyol)	
		Mg^{2+}-Precipitation[a]	Ultracentrifugation[a]
Crude extract	none	0	0
	NADPH	4 476	1 244
Supernatant of microsomal pellet	none	0	0
	NADPH	3 086	0
Microsomal pellet	none	0	0
	NADPH	12 678	10 550
	NADH	2 384	–

[a] Product formed with 100 µg protein at two different enzyme preparations.

Tests with enzyme preparation from other genotypes of *A. majus* showed clearly that the 3′-hydroxylase activity is controlled by the gene *eos*. Enzyme activity could only be demonstrated in flower extracts of all genotypes with wild-type alleles of this gene. In mixtures containing 3′-hydroxylase and extracts from flowers with the recessive allele *eos*, no inhibition of enzyme activity was found.

Discussion

Earlier chemogenetic studies [4] and supplementation experiments [5] with acyanic mutants of *A. majus* suggested that in flowers of this plant the gene *inc* most probably controls the conversion of flavanones to dihydroflavonols. A enzyme catalysing this reaction was recently demonstrated for the first time in flower extracts of *M. incana* [1]. But in this plant it

could not be proved definitely whether the 3-hydroxylase activity found in the *in vitro* experiments actually catalyses this important step *in vivo*, because the gene controlling this special step is not yet known.

3-Hydroxylase activity could now also be demonstrated in flower extracts of defined genotypes of *A. majus*. In confirmation of the genetic work, 3-hydroxylase activity was detected only in flower extracts prepared from genotypes with wild-type alleles of the gene *inc*. The block of the anthocyanin pathway by recessive alleles of this gene is therefore clearly due to a lack of flavanone-3-hydroxylase activity. These results establish for the first time a correlation between genotype and enzymatic 3-hydroxylation. This correlation proves that the enzyme activity measured in the *in vitro* assays is definitely responsible for 3-hydroxylation of flavanones *in vivo*.

In accordance with the enzyme of *M. incana* the 3-hydroxylase of *A. majus* is a soluble enzyme and belongs to the 2-oxoglutarate dependent dioxygenases [15]. Furthermore, the 3-hydroxylases of both plants have similar pH optima and behave similarily towards several enzyme inhibitors. From these results it can be concluded that in both in *A. majus* and *M. incana* the conversion of flavanones to dihydroflavonols is catalysed by the same type of enzyme.

The determination of the substitution pattern of the B-ring of flavonoids was investigated chemogenetically and enzymatically. The chemogenetic studies showed that the 3′-hydroxyl group is introduced by hydroxylation of the C_{15} skeleton and is not due to the incorporation of caffeic acid into the flavonoid skeleton [11]. The gene *eos* therefore does not code for a specific chalcone synthase but rather controls a hydroxylase.

These results are compatible to those obtained in similar studies with flowers of defined genotypes of *M. incana* [16]. In this plant a NADPH-dependent microsomal 3′-hydroxylase was found [1]. In *A. majus* 3′-hydroxylase activity was also found to be localized in the microsomal fraction and the enzyme required the same cofactor. In confirmation of the chemogenetic studies a strict correlation was found between 3′-hydroxylase activity and the wild-type allele *Eos*. The presence of only 4′-hydroxylated flavonoids in the flowers of recessive genotypes (*eos/eos*) of *A. majus* is therefore due to the complete lack of 3′-hydroxylase activity. These results prove that the 3′-hydroxyl group is introduced at the flavanone stage by action of a specific hydroxylase which is controlled by the gene *eos*.

Flavanones are intermediates for the formation of flavones, flavonols and anthocyanins [11]. Introduction of the 3′-hydroxyl group at the flavanone stage is therefore in full agreement with the fact that in *A. majus* the gene *eos* not only controls the B-ring hydroxylation pattern of anthocyanins and flavonols but also of flavones (Table I). This result also explains the presence of some apigenin besides 3′-4′-hydroxylated flavones in genotypes with wild-type alleles of this gene (Table I). This incomplete dominance is due to the presence of naringenin and eriodictyol which can be used both for the formation of flavones.

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[1] G. Forkmann, W. Heller, and H. Grisebach, Z. Naturforsch. **35 c,** 691–695 (1980).
[2] G. Forkmann, Hoppe-Seyler's Z. Physiol. Chem. **362,** 12 (1981).
[3] H. S. A. Sherratt, J. Genet. **56,** 1 (1958).
[4] T. A. Geissman, E. C. Jorgensen, and B. L. Johnson, Arch. Biochem. Biophys. **49,** 368 (1954).
[5] B. J. Harrison and R. G. Stickland, Heredity **33,** 112–115 (1974).
[6] T. J. Mabry, K. R. Markham, and R. B. Thomas, The Systematic Identification of Flavonoids. Springer, New York.
[7] E. Eigen, M. Blitz, and E. Gunsberg, Arch. Biochem. Biophys. **68,** 501 (1957).
[8] G. M. Burton, J. Chromatogr. **34 c,** 562 (1968).
[9] G. Forkmann, Phytochemistry **18,** 1973–1975 (1979).
[10] M. M. Bradford, Analytical Biochemistry **72,** 248–254 (1976).
[11] K. Hahlbrock and H. Grisebach, Biosynthesis of Flavonoids. The flavonoids (J. B. Harborne, T. J. Mabry, H. Mabry, eds.), p. 866–915, Chapman and Hall, London 1975.
[12] K. F. F. Kho, J. H. Bennink, and H. Wiering, Planta **127,** 271–279 (1975).
[13] A. Mühlrad, G. Hegyi, and M. Horányi, Biochim. Biophys. Acta **181,** 184–190 (1969).
[14] R. G. Stickland and B. J. Harrison, Heredity **33,** 108–112 (1974).
[15] M. T. Abbott and S. Undenfriend, Molecular Mechanism of Oxygen Activation (O. Hayaishi, ed.), p. 167–214, Academic Press, New York 1974.
[16] G. Forkmann, Planta **148,** 157–161 (1980).