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Different types of microsomal enzymes catalyze *ortho*- or *para*-hydroxylation in the biosynthesis of carnation phytoalexins

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Cell suspension cultures of carnation (*Dianthus caryophyllus* L.) accumulate, upon challenge with crude fungal elicitor, various dianthramide phytoalexins, all of which derive from *N*-benzoylanthranilate. In vitro, microsomes from the elicited carnation cells hydroxylated *N*-benzoylanthranilate in the 4- and/or 2′-positions to yield the hydroxyanthranilate and/or salicyloyl derivatives. 2′-Hydroxylation was shown to precede 4-hydroxylation in the formation of *N*-salicyloyl-4-hydroxyanthranilate, and both these activities depended strictly on NADPH and molecular oxygen. 4-Hydroxylation was shown to be catalyzed by cytochrome *P*-450-dependent monooxygenase(s), whereas the 2′-hydroxylating activity appeared to be due to a novel class of enzymes, also responding synergistically to NADH in combination with NADPH and showing apparent inhibition by cytochrome *c* but not by carbon monoxide. The difference in type of 4- and 2′-hydroxylases was corroborated by the exclusive inhibition of either activity in imidazole vs. MOPS buffers as well as their differential heat sensitivities. In the course of these studies, low concentrations of *N*-salicyloylanthranilate turned out to inhibit the cytochrome *P*-450-dependent 4-hydroxylation more strongly than any of the commercial inhibitor chemicals tested, while neither the substrate, *N*-benzoylanthranilate, nor the final product, *N*-salicyloyl-4-hydroxyanthranilate, exhibited such significant inhibition. In addition, 2′-hydroxylation activity was affected much less by *N*-benzoylanthranilate, *N*-salicyloylanthranilate or by inhibitor chemicals. The results demonstrate the requirement of two different classes of hydroxylase activities that appear to introduce the antimycotic quality to the dianthramides for phytoalexin defense.

Dianthus caryophyllus cell culture; carnation phytoalexin; ortho- and parahydroxylations

1. INTRODUCTION

Cell cultures derived from the petal tissue of carnation, *Dianthus caryophyllus* L. cv. Eleganz, synthesize dianthramides B (mostly MDB) and S (mostly MDS) (Fig. 1) as well as dianthalexin after treatment with a crude cell wall elicitor from phytopathogenic fungi or commercial yeast extract [1,2]. All the dianthramides, which had been identified previously as phytoalexins from carnation plants [3,4], originate from anthranilate which is initially *N*-benzoylated by the action of an elicitor-responsive transferase [2] and using benzoyl-CoA as the acyldonor. A first report on the transferase [2] documented its narrow specificity for anthranilate, ruling out 3- or 4-hydroxyanthranilate as alternative substrates. *N*-Benzoylanthranilate is then hydroxylated in the 4- and/or 2′-position to yield the phytoalexins HDB, DS and HDS (Fig. 1), which may be subsequently methylated to MDB and MDS. Cyclization of HDB may also occur to yield the heterocyclic dianthalexin [5].

4-Hydroxylation of aromatic acids in plants is usually catalyzed by cytochrome-P-450-dependent monooxygenases [6], which require NADPH and molecular oxygen for catalytic activity and commonly exhibit narrow substrate specificity. Such an enzyme activity could be expected to hydroxylate the anthranilic moiety of dianthramides. It had been observed before that *N*-acylanthranilate rather than anthranilate was the substrate for hydroxylation, which altogether complements the narrow substrate specificity of the *N*-benzoyltransferase for anthranilic acid [2]. Concerning the *ortho*-hydroxylation of the benzoyl moiety, however, no reliable precedent exists in the literature, and the reaction is particularly interesting not only in the phytopathological context. *o*-Hydroxylation of cinnamic and coumaric acids in coumarin biosynthesis has been claimed to be catalyzed by chloroplasts from *Melilotus* [7], *Petunia* [8] and *Hydrangea* [9], but all attempts to repeat these results have failed [10]. The 2′-hydroxylation of isoflavone by a microsomal cytochrome *P*-450-dependent monooxygenase from chickpea, which has been reported recently in the course of pterocarpan phytoalexin formation [11,12], may serve as another, albeit not directly related, example.

Abbreviations: DB, (dianthramide B) *N*-benzoylanthranilate; HDB, (hydroxydianthramide B) *N*-benzoyl-4-hydroxyanthranilate; MDB, (methoxydianthramide B) *N*-benzoyl-4-methoxy-anthranilate; DS, (dianthramide S) *N*-salicyloylanthranilate; HDS, (hydroxydianthramide S) *N*-salicyloyl-4-hydroxyanthranilate; MDS, (methoxydianthramide S) *N*-salicyloyl-4-methoxyanthranilate; DP, (dianthramide P) *N*-(*para*-coumaroyl)anthranilate. The buffers ACES, BES, BIS-Tris, HEPES, HEPPSO, MOPS, MOPSO and PIPES refer to the commercial acronyms. Pmg, *Phytophthora megasperma* f. sp. *glycinea*.

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This report describes the in vitro sequence of 2′- and 4-hydroxylations of *N*-benzoylanthranilate in the course of hydroxydianthramide biosynthesis and the inducibility of these enzyme activities by elicitor treatment of cells. The data demonstrate that the 2′-hydroxylation, in contrast to the 4-hydroxylation, is catalyzed by a novel microsomal enzyme species that does not depend on cytochrome *P*-450 and may instead be related to the bacterial flavine-dependent hydroxylases. 2′- and 4-Hydroxylations appear to increase the antimycotic potential of the dianthramides and hence their phytoalexin quality.

2. MATERIALS AND METHODS

2.1. *Chemicals and enzymes*

Glucose oxidase, catalase, cytochrome *c*, and cofactors were purchased from Boehringer-Mannheim. By definition, 1 unit of glucose oxidase or catalase turns over 1 μM of substrate/min at 25°C and pH 7.0. All buffers were from Sigma, Deisenhofen or Serva, Heidelberg. Reference samples of HDB, HDS and DS were kindly provided by P. Ricci, Antibes, France. Commercial cytochrome *P*-450-specific inhibitors were kindly supplied by BASF, Ludwigshafen [13]. Technical gas was from Messer, Griesheim, and TLC and HPLC materials were purchased from Merck, Darmstadt. Cinnamoyl-CoA and trans-4-coumaroyl-CoA were from our collection.

2.2. *Elicitation of cell cultures*

Carnation cell cultures were propagated and induced with crude elicitor from the cell walls of *Phytophthora megasperma* f. sp. *glycinea* (4 mg/40 ml culture) as described previously [2]. Alternatively, Difco yeast extract (Difco, Michigan) (100 mg/40 ml culture) was used for elicitation. The cells were harvested after various time intervals by filtration, frozen in liquid nitrogen and stored at −70°C until use.

2.3. *Microsomes*

The microsomal fraction was isolated by ultracentrifugation (100 000 × *g* for 1 h) in 0.1 M potassium phosphate buffer, pH 7.5, containing 10% sucrose and 14 mM 2-mercaptoethanol, as described elsewhere [13] and stored at −70°C until use.

2.4. *Labelled substrates*

[carboxyl-^{14}C]Benzoyl-CoA (207 MBq/mmol) was synthesized from [carboxyl-^{14}C]benzoic acid (Amersham-Buchler, Braunschweig) similar to Stöckigt and Zenk [14]. *trans*-4-[^{14}C]Coumaric acid (17.1 GBq/mmol) was prepared enzymatically from L-[U-^{14}C]tyrosine (R.E. Kneusel, Freiburg) and [carboxyl-^{14}C]anthranilic acid (455 MBq/mmol) was purchased from Amersham-Buchler, Braunschweig. *N*-[carboxyl- ^{14}C]benzoylanthranilate (DB) (207 MBq/mmol) was isolated by HPLC with an overall yield of 60% from incubations of anthranilate and [carboxyl-^{14}C]benzoyl-CoA, employing partially purified benzoyl-CoA:anthranilate *N*-benzoyltransferase from elicited carnation cells [2] in 0.1 M imidazole-HCl buffer, pH 7.0. *N-trans*-Cinnamoyl-[carboxyl-^{14}C]anthranilate (455 MBq/mmol) and *N*-(*trans*-coumaroyl)-[carboxyl-^{14}C]anthranilate (455 MBq/mmol) were prepared enzymatically from [carboxyl-^{14}C]anthranilate and cinnamoyl- or 4-coumaroyl-CoA as outlined above, purified by silica TLC in solvent I and eluted from silica with methanol (overall yield of 50%). *N*-[carboxy-^{14}C]benzoyl-4- hydroxyanthranilate (HDB) (207 MBq/mmol) and *N*-[carboxy-^{14}C]salicyloyl anthranilate (DS) (207 MBq/mmol) were produced by incubations of labelled DB with microsomes from elicited carnation cells (12 h) and NADPH in MOPS and Tris buffers, respectively, and recovered by HPLC.

2.5. *Enzyme assays*

Microsomal fractions were isolated from cells that had been induced for 12 h by Pmg elicitor. The standard assay for 2′- and/or 4-hydroxylation of DB contained *N*-[carboxyl-^{14}C]benzoylanthranilate (13.4 M), NADPH (1 mM) and up to 50 μg of microsomal protein in 0.1 M Tris-HCl buffer, pH 7.5 (total volume 0.1 ml). After an incubation period of 15 min at 20°C, the reaction was terminated by addition of 20 μl 25% HCl, and the dianthramides were extracted subsequently with ethyl acetate (170 μl) and separated by RP_{18}-TLC in solvent I. Distribution of radioactivity on the plates was determined by an LB 2832 automatic linear analyzer (Berthold, Wildbad). For qualitative confirmation of 4-hydroxylation of DS, synthetic DS (0.2 mM) was incubated for 4 h at 20°C in 0.1 M Tris-HCl buffer, pH 7.5, in the presence of NADPH (0.5 mM) and using 400 μg of microsomal protein derived from cells after 10 h of elicitation with Pmg-elicitor. For separate determination of K_m-values, Tris-HCl buffer in the standard assay was replaced by imidazole-HCl (2′-hydroxylation) or MOPS-NaOH buffer (4-hydroxylation).

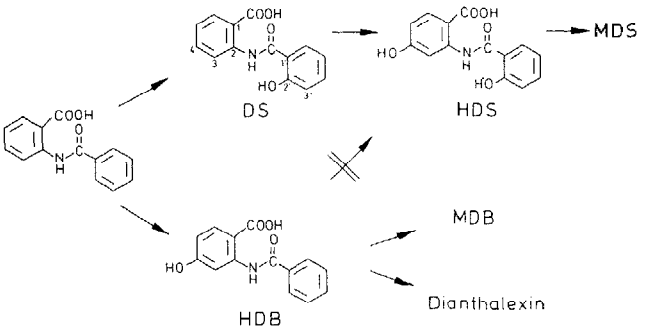

Fig. 1. Pattern of hydroxylations in the biosynthesis of phytoalexins from dianthramide B (DB) in carnation cells. Methoxydianthramide S (MDS) represents the final product in the series of *ortho*-hydroxylated dianthramides (DS, dianthramide S; HDS, hydroxydianthramide S; MDS, 4-methoxydianthramide S), whereas methoxydianthramide B (MDB) and dianthalexin originate from *para*-hydroxylation to hydroxydianthramide B (HDB).

2.6. *Chromatography*

TLC was carried out on RP_{18} plates in solvent I, methanol/water/acetic acid 80:19:1 (v/v/v) (R_{fHDB} 0.52, R_{fHDS} 0.45, R_{fDS} 0.28), on cellulose plates in solvent II, 1 M sodium borate buffer, pH 7.0 (R_{tDS} 0.51), and on silica plates in solvent III, trichloromethane/methanol 80:20, or solvent IV, *n*-hexane/ethyl acetate/methanol 60:40:1 (R_{fHDB} 0.11, R_{fHDS} 0.05). Isocratic HPLC was performed on Lichrosorb RP_{18} (5 μm, 250 × 4 mm, Knauer, Bad Homburg) in solvent V, methanol/water/acetic acid 70:29.4:0.6, at two flow rates (0.5 and 0.75 ml/min). The elution was monitored by absorbance at 260 nm, and when necessary, the HPLC separation of incubation products was monitored on line with a Waters 900 photodiode array spectrometer and/or a Ramona radiomonitor (Raytest, Wildbad). The following retention times were determined: HPLC at a flow rate of 0.5 ml/min; R_{tHDB} 8.5 min, R_{tHDS} 11 min, R_{tDB} 12.5 min. HPLC at a flow rate of 0.75 ml/min; R_{tDS} 14.7 min. HDB: UVλ_{max},MeOH nm: 251, 275, 304. HDS: UVλ_{max},MeOH nm: 253, 316.

2.7. *Inhibition studies*

Mixed incubations with the various inhibitor chemicals as well as the inhibition studies with carbon monoxide, employing mixtures of either 10% oxygen in nitrogen or 10% oxygen in carbon monoxide, were carried out as described elsewhere [15].

2.8. *Protein determination*

Protein was measured according to Bradford [16] using BSA as a standard.

3. RESULTS AND DISCUSSION

3.1. *Hydroxylase activities*

Microsomes isolated from carnation cultures that had been treated for 10 h with Pmg elicitor and incubated in Tris-HCl buffer, pH 7.5, in the presence of NADPH catalyzed the conversion of *N*-[carboxyl-^{14}C]benzoylanthranilate to three products (R_fs 0.52, 0.45 and 0.28 on RP_{18}-TLC in solvent I). A minor proportion of [carboxyl-^{14}C]benzoic acid (R_f 0.61) was released from the substrate in a time-dependent fashion by amidase activity intrinsic to the microsomes irrespective of elicitation. The three products were identified further as HDB, DS and HDS (Fig. 1) by co-chromatography with authentic samples on cellulose and silica TLC in solvents II or III and IV, as well as by RP_{18}-HPLC in solvent V in line with photodiode array spectroscopy.

Formation of all three hydroxylation products from DB was strictly dependent on molecular oxygen (Table I) plus NADPH and was competitively inhibited by $NADP^+$. At a NADPH:$NADP^+$ ratio of 1:2 the inhibition of 2′-hydroxylase was 53% and the inhibition of 4-hydroxylase was 30%. Apparent K_m values of 13 and 20 μM DB or 15 and 9 μM NADPH were determined for the formation of DS and HDB, respectively. Broad pH optima were observed between pH 7.0–7.6 in potassium phosphate buffer. Hydroxylation proceeded best at 20°C, particularly narrow for formation of HDB, and the catalytic activities of microsomes were gradually and irreversibly lost at temperatures beyond 30°C. Boiling the microsomes for 5 min abolished all the activities. Aliquoted microsomes preserved their hydroxylase activities for at least 3 months upon storage at −70°C and were used only once for incubation, since the activities suffered from repeated freezing and thawing. Under standard assay conditions (20°C, 1 mM NADPH, 13.4 μM *N*-[carboxyl-^{14}C]benzoylanthranilate), the rate of product formation was linear with time between 10–50 μg of microsomal protein for up to 25 min. Long-term incubations yielded HDB and HDS only and, depending on the batch of microsomes, the ratio of the two dianthramides varied considerably. Synthetic DS or DS isolated from short-term microsomal incubation was converted efficiently to HDS upon re-incubation with fresh microsomes and NADPH. In contrast, the microsomes failed to metabolize HDB any further, which clearly indicates the sequential action of individual hydroxylases, as outlined in Fig. 1, and their narrow substrate specificities. Moreover, the results pinpoint the microsomes as the catalytic site in carnation cells where DB is hydroxylated either to HDB (4-hydroxylation) or via DS to HDS (2′-hydroxylation followed by 4-hydroxylation) (Fig. 1). It cannot be distinguished at present, whether 4-hydroxylation of DB and/or DS is brought about by one or different enzyme species.

Table I

Dependency of *N*-benzoylanthranilate hydroxylase activities on oxygen

Addition to standard assay	2′-Hydroxylase activity[a] (%)	4-Hydroxylase activity[b] (%)
None	100 (48/52)*	100
Glucose/glucose oxidase/catalase (40 mM/5 U/10 U)	15 (11/4)	28
Glucose/glucose oxidase (40 mM/5 U, boiled for 5 min)	90 (45/45)	105

*Relative ratio of DS/HDS formation is indicated in brackets.
[a]Data based on formation of DS and HDS from DB (see Fig. 1) in 0.1 M Tris-HCl buffer, pH 7.5.
[b]Data based on formation of HDB from DB in 0.1 M Tris-HCl buffer, pH 7.5.

Incubations employing *N*-(*para*-coumaroyl)anthranilate (DP) instead of DB as a substrate suggested that DP was hydroxylated by the microsomes analogous to DB, although no such products have been isolated from infected carnation tissue, in contrast to DP [4]. Three products were preliminarily distinguished from these incubations by TLC and radiodetection, but their molecular structures have not yet been unequivocally assigned. It is noteworthy in this context, however, that neither *N*-cinnamoyl anthranilate nor benzoic, cinnamic or *para*-coumaric acids were accepted as substrates under the conditions of the standard assay.

3.2. *Induction of hydroxylase activities*

Significant catalytic activities for both 4- and/or 2′-hydroxylation of DB were present in dark-grown carnation cultures (on the average 3 μkat/kg), although the actual levels varied considerably with the batch of cells used. Specific hydroxylase activities nevertheless increased about 4-fold in response to elicitation of the cells, showing one broad transient maximum at 6–10 h (4-hydroxylation, determined solely from HDB) and 8–15 h (2′-hydroxylation, determined from DS and HDS, Fig. 1). The time course of induction resembled the induction response of *N*-benzoyltransferase activity, which provides the substrate for the hydroxylase activities. In case of the transferase, the elicitation of carnation cells with commercial yeast extract (100 mg/40 ml culture) had turned out as efficient as that with crude Pmg elicitor (4 mg/40 ml culture). Hydroxylase activities, however, responded better to elicitation with Pmg which was used exclusively further on in this study.

3.3. *Characterization of hydroxylase activities*

The synergistic effect of NADH in combination with NADPH observed during the initial investigation of cofactor requirements suggested the involvement of cytochrome *P*-450 in all the hydroxylations. Both hydroxylase activities were increased up to 25% at a NADH:NADPH ratio of 2:1. Furthermore, the addition of cytochrome *c* caused the apparent inhibition of

Table II

Apparent inhibition of *N*-benzoylanthranilate hydroxylations by cytochrome *c*

Cytochrome *c* (μM)	Inhibition of 2-'hydroxylase activity[a] (%)	Inhibition of 4-hydroxylase activity[b] (%)
0	0	0
0.5	61 (55/68)*	26
1	76 (67/86)	47
10	86 (86/100)	79

*Relative inhibition of DS/HDS is indicated in brackets.
[a]Data based on formation of DS and HDS from DB in 0.1 M Tris-HCl buffer, pH 7.5.
[b]Data based on formation of HDB from DB in 0.1 M Tris-HCl buffer, pH 7.5.

both 4- and 2'-hydroxylase activities (Table II). Subsequent hydroxylation assays in various buffer systems (Table III), however, gave a first indication of the different nature of the 2'-hydroxylase. Whereas 4-hydroxylation was completely abolished in 0.1 M imidazole-HCl buffer, as would be expected for cytochrome *P*-450-dependent monooxygenases, 2'-hydroxylation was not diminished (Table III) under these conditions as compared to the assay in Tris-HCl buffer and considering DS rather than the sum of DS and HDS as the measure for catalytic turnover. Incubations in MOPS or MOPSO buffer, on the other hand, inhibited 2'-hydroxylation completely while the 4-hydroxylation was even greatly stimulated (Table III). To monitor the inhibitory effects of carbon monoxide, synthetic inhibitors, and dianthramides (Tables IV–VI) on 4-hydroxylase activity incubations were carried out in MOPS buffer to suppress the pathway DB-DS(-HDS) via 2'-hydroxylase. All the other buffers used for assay (Table III) modulated only slightly the rates of hydroxylation. Subsequent inhibition experiments with carbon monoxide (Table IV) corroborated the distinction of 2'- and 4-hydroxylases. Whereas 4-hydroxylation was significantly inhibited by carbon monoxide and this effect was partly reversed upon irradiation of the incubation at 450 nm, 2'-hydroxylation was hardly inhibited under these conditions (Table IV). The reduction of enzyme activities observed in the irradiated assays (Table IV) was due to warming of the incubation mixture, to which 4-hydroxylation is particularly sensitive (data not shown). Inhibition experiments with commercial, synthetic inhibitor chemicals for cytochrome *P*-450-dependent monooxygenases, which revealed ketoconazole and tetcyclacis as potent inhibitors for 4-hydroxylation (Table V), supported the differential classification of the enzymes. 4-Hydroxylation, in the course of carnation phytoalexin biosynthesis, is therefore catalyzed by cytochrome *P*-450-dependent monooxygenase(s), whereas 2'-hydroxylation appears to belong to a different category of hydroxylases. The addition of EDTA failed to

Table III

Effect of assay buffer on *N*-benzoylanthranilate hydroxylase activities

Buffer[a]	Relative 2'-hydroxylase activity[b] (%)	Relative 4-hydroxylase activity[c] (%)
Tris-HCl	100 (33/67)*	100
HEPES-NaOH[d]	69 (26/43)	88
Imidazole-HCl	39 (39/0)	0
MOPS-NaOH[e]	0	157

*Relative ratio of DS/HDS is indicated in brackets.
[a]Buffers were used at pH 7.5 and 0.1 M concentration.
[b]Data based on formation of DS and HDS from DB.
[c]Data based on formation of HDB from DB.
[d]Equivalent results were obtained in ACES, BES, BIS-Tris, HEPPSO phosphate and PIPES buffers at pH 7.0.
[e]2'-Hydroxylase activity was also completely inhibited in MOPSO buffer, pH 7.5.

reduce the latter activity in a concentration-dependent manner (Table V), which argues against a metal-dependency of 2'-hydroxylation, nor was any additional cofactor, i.e. ascorbate or oxoglutarate, required for the activity. Although the mechanism of catalysis is unknown at present, our data clearly distinguish the carnation 2'-hydroxylase also from the cytochrome *P*-450-dependent enzyme activity reported to catalyze isoflavone 2'-hydroxylation in chickpea [11,12]. The lack of precedent still leaves an option for a flavoenzyme in analogy to such hydroxylases from bacterial and fungal sources [17,18].

3.4. *Significance of 2'-hydroxylation*

During the initial incubations with different preparations of microsomes, a peculiar and almost inverse relationship was observed between the formation of HDB and DS, even though different hydroxylases were engaged (Fig. 1). Therefore, DB, HDB, DS and HDS were included in the inhibition studies (Table VI), which, for the sake of clarity, were carried out in different buffers.

Table IV

Effect of carbon monoxide on *N*-benzoylanthranilate hydroxylations

Gas mixture (v/v)	Irradiation at 450 nm	Relative 2'-hydroxylase activity[a] (%)	Relative 4-hydroxylase activity[b]
N_2/O_2 9/1	–	100 (17/83)*	100
	+	98 (75/23)	49
CO/O_2 9/1	–	84 (70/14)	26
	+	61 (50/11)	42

*Relative ratio of DS/HDS formation is indicated in brackets.
[a]Data based on formation of DS and HDS from DB in 0.1 M Tris-HCl buffer, pH 7.5.
[b]Data based on formation of HDB from DB in 0.1 M MOPS-NaOH buffer, pH 7.5. Neither DS no HDS are formed under these conditions.

While DB, being the substrate, and HDB (data not shown) at low concentration hardly influenced either one of the hydroxylations, DS strongly inhibited the 4-hydroxylation of DB and DS which depends on cytochrome *P*-450 (Tables IV and VI). Inhibition was so extensive that DS turned out to be far more effective than the most potent of the commercial synthetic inhibitors. The inhibitory quality was completely lost, however, upon further hydroxylation to HDS. Synthetic inhibitors for cytochrome *P*-450-dependent monooxygenases, i.e. ketoconazole, have been developed and applied for antimycotic medicinal therapy as well as for plant protection against fungal diseases [19]. It is conceivable that 2′-hydroxylation of dianthamides is a crucial measure in the strategy of carnations to cope with fungal pathogens. This is supported by validations of the phytoalexin character of dianthramides [20,21], which ascribes an important role to hydroxylations.

4. CONCLUSIONS

This report presents the first example of a novel type of microsomal hydroxylase that is involved, together with cytochrome *P*-450-dependent monooxygenase(s), in the biosynthesis of carnation phytoalexins. This 2′-hydroxylase is not inhibited by carbon monoxide, although its apparent inhibition by cytochrome *c* suggests a reductase-mediated electron transfer during catalysis. Since no additional cofactor is required, we consider the possibility that 2′-hydroxylation may belong to the flavoenzyme hydroxylases as described for hydroxylations of salicylate [22], melilotate [23], *para*-hydroxybenzoate [24], phenol [17] or anthranilate [18]. However, these flavine-dependent hydroxylases are soluble, microbial enzymes and require, for mechanistic reasons [18], a hydroxyl- or amine-substitution *ortho* or *para* to the ring-position to be hydroxylated. If carnation 2′-hydroxylase belongs to this group of enzymes, it would represent the first such enzyme from a plant source and the first example of such an enzyme associated with microsomes.

Hydroxylation of DB yields potent phytoalexins [20,21], which underlines the important function of the inducible 2′- and 4-hydroxylase activities for the defense against widespread fungal pathogens such as *Phytophthora parasitica* [25] or *Fusarium oxysporum* [26]. It has been reported that treatment of carnation cuttings with salicylic acid prior to infection significantly suppresses the induction of phytoalexin accumulation and concomitantly lowers the level of resistance [25,27] against these fungi. The phenomenon is supported by our in vivo inhibition studies, which document the inhibitory effect of salicylate on the hydroxylations, while the transferase activity forming *N*-benzoylanthranilate is

Table V

Effect of synthetic inhibitors on *ortho*- and/or *para*-hydroxylation of *N*-benzoylanthranilate

Inhibitor	Concentration (μM)	Inhibition of 2′-hydroxylase activity[a] (%)	Inhibition of 4-hydroxylase activity[b] (%)
None		0	0
Ketoconazole	10	8 (0[c]/37)*	76
	100	67 (42/100)	100
Metyrapone	10	0 (0/4)	10
	100	11 (0/38)	50
Ancymidol	10	21 (27/12)	0
	100	35 (25/47)	34
Tetcyclacis	10	28 (0/100)	100
	100	76 (74/100)	100
BAS 111	10	0 (0/17)	42
	100	7 (0/65)	90
BAS 110	10	15 (0/38)	4
	100	44 (15/81)	76
LAB 978	10	6 (0/46)	46
	100	15 (0/100)	100
KSCN	10	0 (0/0)	0
	100	28 (0/38)	18
	1000	30 (22/39)	24
EDTA	10	43 (31/57)	24
	100	54 (41/72)	62
	1000	50 (34/85)	72

*Relative ratio of inhibition for DS/HDS formation is given in brackets.

[a]Data based on formation of DS and HDS from DB in 0.1 M Tris-HCl buffer, pH 7.5.

[b]Data based on formation of HDB from DB in 0.1 M MOPS-NaOH buffer, pH 7.5.

[c]Zero % inhibition does not rule out a relative increase in DS formation.

Table VI

Inhibition of *N*-benzoylanthranilate hydroxylase activities by dianthramides, salicylate or coumarate

Inhibitor	Concentration (μM)	Inhibition of 2′-hydroxylase activity[a] (%)	Inhibition of 4-hydroxylase activity[b] (%)
DS	0.5	1.5 (0[c]/51)*	50
	1	10 (0/67)	58
	0.5	44 (4/100)	100
	10	50 (15/100)	100
	100	94 (90/100)	100
HDS[d]	100	32 (15/52)	28
Salicylate	10	42 (29/57)	46
	100	49 (22/83)	73
	1000	50 (23/80)	73
trans-4-Coumarate	10	36 (22/56)	18
	100	28 (4/63)	53
	1000	36 (11/70)	100

*Relative ratio of inhibition for DS/HDS formation is given in brackets.

[a]Data based on formation of DS and HDS from DB in 0.1 M Tris-HCl buffer, pH 7.5.

[b]Data based on formation of HDB from DB in 0.1 M MOPS-NaOH buffer, pH 7.5.

[c]Zero inhibition does not rule out a relative increase in DS formation.

[d]No inhibition of 2′- or 4-hydroxylase observed at concentrations in the range 0.5–10 μM HDS.

not affected at concentrations below 1 mM (data not shown). This again claims a pivotal role for the newly described 2′- and 4-hydroxylase activities in the survival of infected carnations.

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