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Deoxysarpagine Hydroxylase — A Novel Enzyme Closing a Short Side Pathway of Alkaloid Biosynthesis in *Rauvolfia*

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Abstract—Microsomal preparations from cell suspension cultures of the Indian plant *Rauvolfia serpentina* catalyze the hydroxylation of deoxysarpagine under formation of sarpagine. The newly discovered enzyme is dependent on NADPH and oxygen. It can be inhibited by typical cytochrome P450 inhibitors such as cytochrome *c*, ketoconazole, metyrapone, tetcyclacis and carbon monoxide. The CO-effect is reversible with light (450 nm). The data indicate that deoxysarpagine hydroxylase is a novel cytochrome P450-dependent monooxygenase. A pH optimum of 8.0 and a temperature optimum of 35 °C were determined. K_m values were 25 μM for NADPH and 7.4 μM for deoxysarpagine. Deoxysarpagine hydroxylase activity was stable in presence of 20% sucrose at −25 °C for >3 months. The analysis of presence of the hydroxylase in nine cell cultures of seven different families indicates a very limited taxonomic distribution of this enzyme.

Introduction

The biosynthesis of the monoterpenoid indole alkaloid ajmaline, which has been in use as an antiarrhythmic drug for about four decades,[1–3] has been previously elucidated at the enzymatic level in much detail.[4,5] Cell suspension cultures of the Indian medicinal plant *Rauvolfia serpentina* Benth. ex Kurz are an efficient source for the isolation of the particular enzymes, because the cells also produce a great variety of indole alkaloids of different types and especially of the Sarpagan and Ajmalan types.[6] Although the biosynthetic pathway leading to ajmaline is now well known, side routes emerging directly from this pathway still need further exploitation. Such knowledge might become very important in the near future for the metabolic regulation of the cellular alkaloid formation in *Rauvolfia*, which is presently unknown.

Despite several not deeply investigated examples of such side sequences, like the biosynthesis of the alkaloid groups tetraphyllicines, of the rauglucines and of the raumaclines, the formation of the typical *Rauvolfia* alkaloid sarpagine, also a side product of ajmaline biosynthesis, is in principle well known. Recently published in vivo NMR experiments at 800 MHz performed without labeling[7] clearly demonstrated that the final biosynthetic step in sarpagine biosynthesis must be the hydroxylation of deoxysarpagine at carbon position 10 (Scheme 1). Although these results marked an important step in understanding the sarpagine formation, they did not allow a final conclusion concerning the type of reaction, the mechanism of reaction or the nature of the involved enzyme.

In this communication, we describe the in vitro biosynthesis of sarpagine from 10-deoxysarpagine which is catalyzed by a novel NADPH- and cytochrome P450-dependent *Rauvolfia* enzyme named deoxysarpagine hydroxylase (DOSH).

Results and Discussion

The alkaloid sarpagine is a typical *Rauvolfia* constituent and has been isolated as one of the first examples of this alkaloid group a long time ago.[8] Its biological activities were not pronounced very much and the alkaloid did not become a commercially applied drug. Sarpagine deserves, however, attention in the light of biosynthesis, especially what the enzyme was concerned introducing oxygen into the aromatic ring. Therefore, we concentrated on searching for the enzyme which would hydroxylate 10-deoxysarpagine leading to sarpagine. Hydroxylation reactions at carbon atoms are usually

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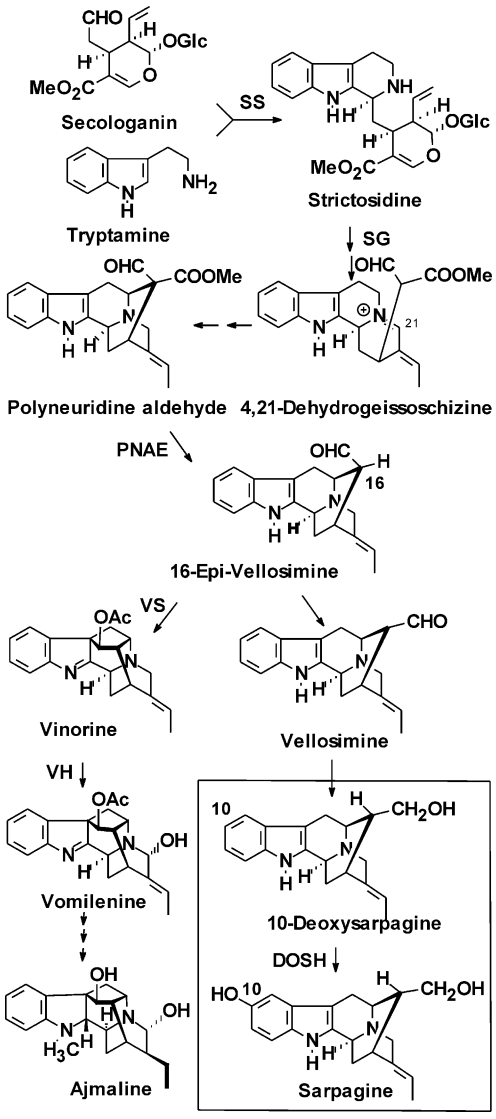

Scheme 1. Major steps of the biosynthetic pathways of sarpagan- and ajmalan-type alkaloids in cell suspension culture of *Rauvolfia serpentina*. The reaction catalyzed by the deoxysarpagine hydroxylase (hydroxylation to sarpagine at carbon position 10) is boxed (DOSH, deoxysarpagine hydroxylase; PNAE, polyneuridine aldehyde esterase; SG, strictosidine glucosidase; SS, strictosidine synthase; VH, vinorine hydroxylase; VS, vinorine synthase).

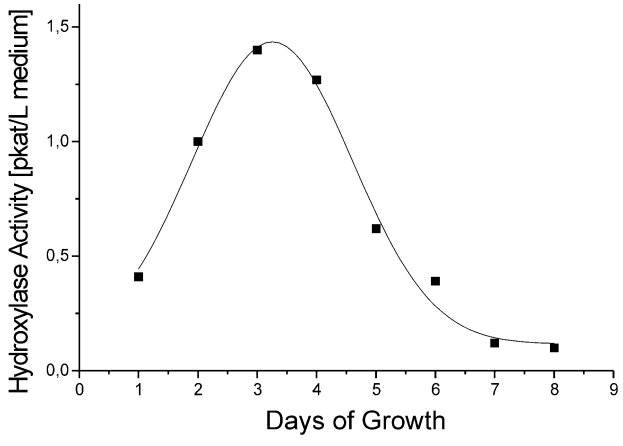

Figure 1. Changes of deoxysarpagine hydroxlase activity of *R. serpentina* cell cultures during a cultivation period of 8 days,.

catalyzed by either cytochrome P450-dependent enzymes[9] or 2-oxoglutarate-utilizing dioxygenases.[10,11] The presumptive substrate, 10-deoxysarpagine, was easily separated by HPLC from the putative enzyme product sarpagine, and thus it was possible to establish a simple and fast HPLC-based enzyme assay. Microsomal fractions of *Rauvolfia* cells prepared by a standard method[12] were incubated with 10-deoxysarpagine in the presence of NADPH. Based on HPLC analysis this incubation mixture showed the cell-free synthesis of sarpagine. In detail, when a crude cell-free extract or a microsomal preparation of *R. serpentina* cultured cells was incubated with 0.1 mM 10-deoxysarpagine and NADPH at pH 8.0 for 4 h, a new peak at R_t 7.0 min was detected by HPLC. The isolated compound showed an UV spectrum identical to that of sarpagine (data not shown). It also had the same behavior pattern on TLC. In addition, EI–MS analysis revealed the same fragmentation pattern, indicating that the enzyme product indeed was sarpagine. Since no conversion was observed with cell-free extracts or microsomal preparations which had been denatured by heating, the sarpagine formation was clearly enzyme-catalyzed. We named this enzyme deoxysarpagine hydroxylase (DOSH).

As the cell-free conversion was not high yielding, we screened for optimum enzyme activity during the whole period of cell growth (Fig. 1). The maximum enzyme activity was detected on the third day of cell growth which is much earlier than observed for other enzymes in ajmaline biosynthesis[4] and then decreased. Enzymatic sarpagine synthesis in the microsomal fraction obtained from these cells was more than 10 times higher compared to that in the crude cell-free extract, suggesting that the enzyme activity was membrane-associated. The transformation catalyzed by the hydroxylase showed an optimum at pH 8.0 in Tris–HCl buffer. This pH is higher than reported for most other enzymes in ajmaline biosynthesis (Scheme 1) despite two exceptions, viz. vinorine synthase (pH 8.5) and vinorine hydroxylase (pH 8.3)[5,13,14] which suggests different subcellular enzyme localization. The optimum temperature for DOSH reaction is 35 °C. Under these conditions the catalyzed reaction was distinctly dependent on NADPH and oxygen. NADPH could not be replaced by NADH, indicating a remarkable cofactor specificity of DOSH. The appropriate Michaelis–Menten kinetics showed an apparent K_m value for NADPH of 25 μM (Fig. 2) demonstrating a high affinity of the enzyme towards the cofactor. The low K_m value of 7.4 μM for 10-deoxysarpagine (Fig. 3) let us conclude that 10-deoxysarpagine is most probably the natural substrate of the isolated hydroxylase. Because of the membrane-association and the necessity for NADPH for the enzymatic activity, we suggested that the reaction is cytochrome P450-dependent. Since $NADP^+$ is a typical competitive inhibitor of this class of enzymes,[15] we measured the hydroxylase activity at different NADPH/$NADP^+$ ratios. Indeed, the results showed a distinct inhibition of the hydroxylation in presence of the oxidised cofactor. A Lineweaver–Burk plot for three different $NADP^+$ concentrations proved the competitive inhibition of the enzyme by $NADP^+$ (Fig. 4).

Cytochrome *c* is another typical P450 inhibitor.[16] When 10 μM cytochrome *c* was included to the reaction mixture, a 45.7% inhibition was observed, whereas nearly complete inhibition was found at 20 μM cytochrome *c* (Table 1). This result strongly suggested the cytochrome P450 character of the hydroxylase. We also tested a series of typical cytochrome P450 inhibitors[17,18] and all showed significant inhibition of the reaction, which indicated that DOSH belongs to the class of cytochrome P450 monooxygenases. The strongest inhibitor is BAS 110W which resulted in complete inhibition at 1 mM and 83.3% inhibition at 0.1 mM concentration. Further studies showed that the hydroxylase could also be inhibited by carbon monoxide. In particular, the reversal of the inhibition by light with a maximum at 450 nm is regarded as the most reliable proof for a cytochrome P450 containing enzyme.[19,20] In fact, the activity of deoxysarpagine hydroxylase was reduced 30% in dark when compared to that in light in the presence of a nitrogen/oxygen mixture, indicating that light can increase the hydroxylase activity (Table 2). The enzyme activity decreased to ~30% when the gas mixture was changed from N_2/O_2 to CO/O_2 in light. In the dark the conversion was reduced from ~70 to ~20%. Under the same conditions light at 450 nm can in principle reverse the reaction, but this was not observed at 700 nm. Thus, the hydroxylase was finally confirmed as a member of the class of cytochrome P450 enzymes and most probably was a new member. When a selection of cell suspension cultures of different plant families and genera was tested for hydroxylase activity it turned out that exclusively *R. serpentina* cells contained DOSH indicating a specific occurrence of the enzyme in this particular cell system producing sarpagine (Table 3).

Sarpagine is one of the major indole alkaloids in root bark of *Rauvolfia* species[8] but occurring also in other plant parts,[21] *Rauvolfia* cell cultures[6] and *Rauvolfia* hybride systems.[22] It might be a late alkaloid in the biosynthesis of alkaloids of this particular plant or cell systems. The enzymatic formation of sarpagine described here is obviously a reaction terminating one of the side routes of alkaloid metabolism emerging from ajmaline biosynthesis.

Table 1. Influence of typical cytochrome P450 inhibitors on 10-deoxysarpagine hydroxylase activity of cultivated *Rauvolfia* cells[a]

Inhibitor	Concentration (mM)	Rel. inhibition (%)
Ancymidole	1.0	48.8
	0.1	45.5
BAS 110W	1.0	100.0
	0.1	83.3
BAS 111W	1.0	83.6
	0.1	35.2
Cytochrome *c*	0.02	90.8
	0.01	45.7
Ketoconazole	1.0	69.2
	0.1	26.3
LAB 150978	1.0	74.1
	0.1	35.7
Metyrapone	1.0	83.1
	0.1	57.8
Tetcyclacis	1.0	45.3
	0.1	30.1

[a]Standard incubation mixture; 100% enzyme activity = 0.45 nkat.

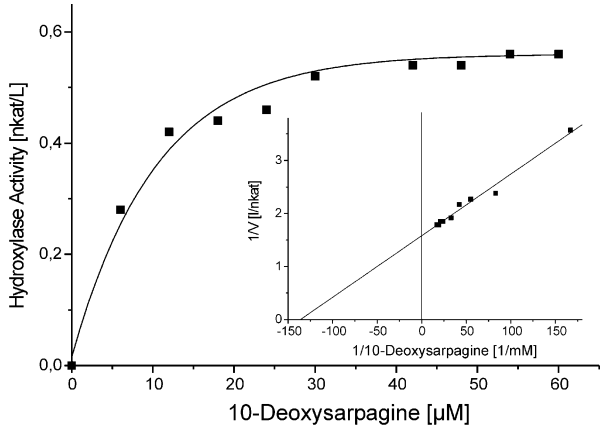

Figure 3. The effect of 10-deoxysarpagine concentration on deoxysarpagine hydroxylase activity (standard incubation mixture, $K_m = 7.4$ μM, $V_{max} = 0.55$ nkat). The insert shows the double-reciprocal plot.

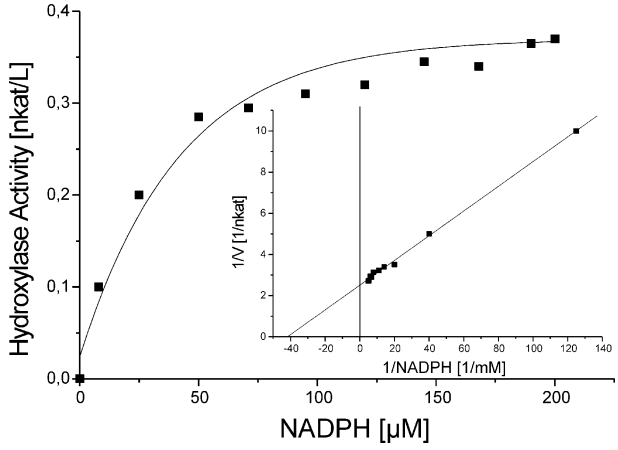

Figure 2. The effect of NADPH concentration on deoxysarpagine hydroxylase activity (standard incubation mixture, $K_m = 25$ μM, $V_{max} = 0.36$ nkat). The insert shows the double-reciprocal plot.

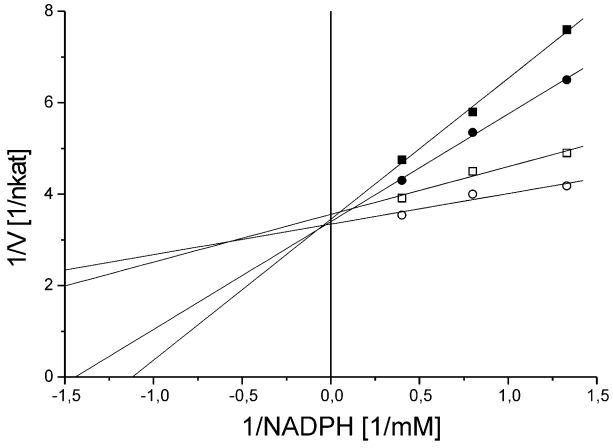

Figure 4. Competitive inhibition of deoxysarpagine hydroxylase by $NADP^+$ in presence of 0.75, 1.25, 2.5 mM NADPH (Lineweaver–Burk plot: ○, without $NADP^+$; □, 0.5 mM $NADP^+$; ●, 1.0 mM $NADP^+$; ■, 1.5 mM $NADP^+$).

Table 2. Effect of light and carbon monoxide on deoxysarpagine hydroxylase activity assayed with microsomes isolated from 3 days old *R. serpentina* cell cultures[a]

Gas mixture	Illumination	Deoxysarpagine hydroxylase activity (%)
N_2/O_2 9:1	Light	100
N_2/O_2 9:1	Dark	72.1
CO/O_2 9:1	Dark	19.8
CO/O_2 9:1	Light	29.2
CO/O_2 9:1	Blue (450 nm)	23.4
CO/O_2 9:1	Red (700 nm)	19.1

[a]Standard incubation mixture in a special apparatus for varying illumination and different gas mixtures as described previously;[13] 100% enzyme activity = 0.25 nkat.

Table 3. Taxonomic distribution of deoxysarpagine hydroxylase in various cell suspension cultures[a]

Cell culture	Family	Sarpagine synthase (rel. act.%)
Rauvolfia serpentina	Apocynaceae	100
Agrostis tenuis.	Poaceae	[b]
Catharanthus roseus	Apocynaceae	[b]
Euphorbia peplus	Euphorbiaceae	[b]
Lonicera morrowii	Caprifoliaceae	[b]
Nicotiana tabacum	Solanaceae	[b]
Ophiorrhiza pumila	Rubiaceae	[b]
Rhazya stricta	Apocynaceae	[b]
Saponaria officinalis	Caryophyllaceae	[b]

[a]Standard growth conditions and harvest on day 3.
[b]No detectable activity in HPLC, detection limit < 1% rel. act., 100% activity = 0.45 nkat.

The obtained results extend our knowledge on enzymatic *Rauvolfia* alkaloid biosynthesis. They also allow us to study the appropriate cDNA coding for the sarpagine synthesizing enzyme after its purification, Cytochrome P450 enzymes are also an interesting class of enzymes from the point of view of organic synthesis. They hydroxylate a variety of compounds in a regio- and stereoselective manner which is difficult to achieve by synthetic methods. For that reason this type of enzyme might become very important in future times when a high yielding heterologous expression becomes available. Therefore, the purification of the novel DOSH is now under further investigation.

Materials and Methods

Plant cell material and culture method

Cell suspension cultures of *R. serpentina* were grown as previously described[23] under constant illumination on gyratory shakers (100 rpm) in 1-L Erlenmeyer flasks containing 400 mL LS-medium (Table 3).[24] For enzyme preparation 3 days old cultured cells were harvested by suction filtration. The cell material was frozen with liquid nitrogen and stored at −25 °C until required for isolation of microsomes.

Biochemicals

NADPH, $NADP^+$ and NADH were purchased from AppliChem (Darmstadt, Germany). Ancymidole, cytochrome *c*, ketoconazole, metyrapone and sarpagine were obtained from Sigma-Aldrich (Taufkirchen, Germany). Tetcyclacis, LAB 150978, BAS 110W and BAS 111W were kindly provided by BASF (Ludwigshafen, Germany). All other commercially available materials were of highest purity. Vellosimine was isolated from *R. serpentina* hairy root cultures by a method which was earlier described for the alkaloid vinorine.[14]

Subsequently, 10-deoxysarpagine was prepared from vellosimine: vellosimine (1 mg) was dissolved in the mixture of 0.6 mL MeOH and 0.6 mL citrate–buffer (1 M, pH 5.0). An excess of $NaBH_4$ was added and the reaction lasted for 1 h and then the reaction mixture was extracted with EtOAc three times (2 mL each). The organic layers were combined, concentrated, and chromatographed on TLC (silica gel 60 F_{254}; Merck Darmstadt, Germany) with solvent system $CHCl_3$/MeOH/NH_3 90:10:0.1. The R_f value of 10-deoxysarpagine (R_f 0.54) was identical with that of a reference compound. After elution the product was measured by EI–MS (Finnigan MAT 44S quadrupole instrument) at 70 eV. Fragmentation pattern were identical to a reference sample of 10-deoxysarpagine.

Enzyme preparations

All enzyme isolations were carried out at 4 °C. The used buffers were: Buffer A: 100 mM Tris–HCl, pH 8.0, 10 mM KCl, 20% sucrose and 10 mM β-mercaptoethanol. Buffer B: buffer A with additional 0.1 mM EDTA. The deep-frozen cell material (200 g) of *R. serpentina* was pulverized in the presence of liquid nitrogen, homogenized by stirring for 0.5 h in 200 mL buffer A and filtered through four layers of cheese-cloth. This crude protein extract was centrifuged at 13,000*g* for 30 min. From the supernatant the microsomes were prepared by Mg^{2+} precipitation as described.[12] The microsomal pellets were washed twice with 40 mL buffer B, then suspended in 16 mL buffer B and homogenized with a Potter homogenisator (8.0×0.5 cm, Xinghua Company, Shanghai, China). The obtained suspension was used as microsomal fraction and was employed directly for enzyme assays or stored at −25 °C. Protein concentrations were determined according to a method described by Bradford.[25]

Enzyme assay

Deoxysarpagine hydroxylase was assayed in an incubation mixture comprising 10 μL 10-deoxysarpagine (1 mM), 100 μL NADPH (1.2 mM) and 200 μL microsomes (1 mg protein) in buffer B (total volume 500 μL). Incubation was performed for 4 h at 35 °C with shaking (400 rpm) in open reaction vials. The reaction was initiated by the addition of NADPH to the incubation mixture and terminated by adding 500 μL of MeOH. The mixture was subsequently centrifuged at 14,000*g* for 5 min and 40 μL of the supernatant was assayed by RP-HPLC.

HPLC analysis

The amount of sarpagine was determined by RP-HPLC using a Merck LiChrosolv RP select B column [250 × 4 mm, Merck (Darmstadt, Germany)]; gradient 68% 20 mM $(NH_4)_2CO_3$/32% MeCN for 5 min, 40% $(NH_4)_2CO_3$/60% MeCN for 7 min, monitoring the absorption at 278 nm. The quantities of sarpagine were calculated from the peak area at 278 nm recorded using a Chromato-integrator (Hitachi, Japan). Retention times of 10-deoxysarpagine and sarpagine were 11 and 7 min, respectively.

Identification of the reaction product

The identification of the product was carried out on the basis of UV and MS-measurement. In an open 100 mL flask 200 μL 10-deoxysarpagine (1.0 mM), 2 mL NADPH (1.2 mM), 20 mL microsomes (20 mg protein) and 20 mL buffer B were incubated while shaking (100 rpm) at 35 °C for 4 h. The appropriate control experiment was performed without substrate. MeOH (20 mL) was added to the mixture to terminate the reaction. Then the reaction solution was three times extracted with EtOAc (each 30 mL). The organic fractions were combined and evaporated. The residue was dissolved in 2 mL $CHCl_3$ and added to TLC (silica gel 60 F_{254}, Merck, Damstadt, Germany). Chromatography was performed with the solvent system $CHCl_3$ / MeOH 4:1. The appropriate band (R_f 0.43) was cut off and eluted with acetone. Then the product was measured by UV and EI–MS. EI–MS: 310 $[M]^+$ (93), 309 $[M-1]^+$ (96), 295 (6), 279 (31), 265 (6), 198 (14), 185 (98), 184 (100).

pH-Optimum determination and oxygen dependence

Oxygen dependence experiments were performed as described previously.[13] The determination of pH optimum of the hydroxylase was carried out as described above, but using the following two buffer solutions: 100 mM potassium phosphate buffer (pH 5.0–7.5), 100 mM Tris–HCl buffer (pH 7.0–9.0); each with 10 mM KCl, 20% sucrose, 0.1 mM EDTA and 10 mM β-mercaptoethanol.

Inhibition experiments

The P450 inhibitors, ancymidole, cytochrome *c*, ketoconazole, metyrapone, tetcyclacis, LAB 150978, BAS 110W and BAS 111W were tested. Metyrapone and cytochrome *c* were dissolved in H_2O and others were dissolved in Buffer B. The concentrations of inhibitors were adjusted to 1 and 0.1 mM in the reaction solution except for cytochrome *c* (20 and 10 μM). The inhibition experiments were carried out using the standard assay described above.

Determination of kinetic parameters

The apparent Michaelis constant (K_m) and maximal reaction velocity (V_{max}) for NADPH and sarpagine were determined by Lineweaver–Burk plots (Figs. 2 and 3).

Taxonomic distribution of the hydroxylase

For the determination of the taxonomic distribution of DOSH the isolation of microsomes from cell suspension cultures of different plant families and genera and incubation conditions were as described above.

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