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CHARACTERISTICS OF VELLOSIMINE REDUCTASE, A SPECIFIC ENZYME INVOLVED IN THE BIOSYNTHESIS OF THE RAUWOLFIA ALKALOID SARPAGINE

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Abstract: A plant enzyme - vellosimine reductase - has been isolated from *Rauwolfia* cell suspension cultures. This new enzyme has been purified (110-fold) and characterized. The reductase is a specific enzyme of the sarpagine pathway catalyzing the NADPH dependent conversion of vellosimine into 10-deoxysarpagine. The latter alkaloid is the immediate biogenetic precursor of sarpagine as shown by its high *in vivo* incorporation rate (86%) into sarpagine.

Thirty years ago Stoll and Hofmann isolated the monoterpenoid *Rauwolfia* alkaloid sarpagine for the first time[1]. Since then 46 alkaloids belonging to the sarpagine group have been identified. In recent years the biosynthesis of this alkaloid type has attracted intense interest from the theoretical[2] and the experimental[3] point of view. At present 7 new plant enzymes are well established as being involved in the biogenesis of both the sarpagine alkaloids and the structurally related group of ajmaline alkaloids. In this paper we report on the characterization of vellosimine reductase, an enzyme of the sarpagine route, which catalyzes the formation of 10-deoxysarpagine (syn. vellosimol, normacusine B). This alkaloid serves as the immediate biogenetic precursor of sarpagine.

Results

Isolation and partial purification of vellosimine reductase. Extraction of 0.5 kg *Rauwolfia* cells with 1 l (20mM) KPi buffer (10 mM BME†), pH 7 yielded 1.064 g protein containing the reductase (spec. act. 0.35 nkat/mg). When this crude extract was subjected to an ammonium sulfate precipitation an 80 % loss of enzyme activity was observed. Therefore the reductase was applied directly to 4 chromatographic steps which resulted in a 110-fold purification (Table I). The enzyme activity in the crude extract could be enriched 5.3-fold by ion exchange chromatography on DEAE DE 52 cellulose using a KCl gradient (0 -0.3 M). The enzyme was eluted at 125 mM KCl. After dialysis against 10 mM KPi buffer the enzyme solution was "filtrated" on Hydroxylapatite with minimal loss of total activity and a 6.9-fold purification resulted. Polyneuridine aldehyde esterase†, which is an earlier enzyme in the pathway, is quantitatively removed by this procedure. After concentration of the enzyme with a Berghoff cell gelchromatography on Ultrogel AcA 54 showed a 17.8-fold enrichment of reductase activity. During this procedure about 50% of the total enzyme activity was lost. For the final purification step affinity chromatography on Matrex Gel Green A was chosen and the enzyme was eluted with 5 mM $NADP^+$. When the elution procedure immediately followed the binding of the enzyme on Matrex Gel, less than 1% of the enzyme activity was recovered. However, when the $NADP^+$ solution was left

†BME = β-mercaptoethanol

†PNA-esterase

on the column for 12 hrs, vellosimine reductase was obtained in about 40% yield with a 110-fold purification. The elution profile of the Matrex Gel column is illustrated in Figure I.

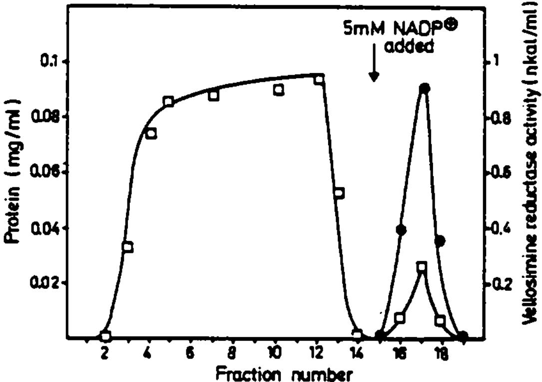

Figure I: Purification of vellosimine reductase by affinity chromatography on Matrex Gel Green A. Proteins were eluted with KPi buffer (pH 7) followed by addition of 5mM $NADP^+$. Fractions of 2 ml were collected, analysed for protein content (□–□) and assayed for reductase activity (●–●).

Enzyme assay. For the quantitation of enzyme activity two methods can be employed, an optical assay to follow the consumption of NADPH (decrease of the extinction at 340 nm) and a TLC-test for determination of the NADPH dependent conversion of [aryl-^{3}H]-vellosimine into [aryl-^{3}H]-deoxysarpagine. The optical test is usable with enzyme preparations which do not contain measurable levels of NADPH oxidase activity. In general, oxidase activity is absent in extracts of the plant cell cultures tested. The TLC assay can be used as a cross-check of the results obtained from cell cultures and it is the method of choice for enzyme extracts containing high oxidase activity. We have found that crude cell-free extracts of differentiated plants can only be assayed by the TLC assay.

Enzyme properties. The conversion of vellosimine into 10-deoxysarpagine was dependent on enzyme and NADPH, and exhibited a typical saturation curve. The effect of substrate concentration on the reaction rate is presented in Figure II.

The 110-fold purified reductase showed normal Michaelis-Menten kinetics for the substrate vellosimine, apparent K_m of 17 µM and V_{max} = 24 pkat. In contrast the Menten kinetics for the co-substrate NADPH have a sigmoid relationship (appa-

Table I: Purification of vellosimine reductase isolated from 0.5 kg(fresh weight) cultured R.serpentina cells.

Purification step	Total volume ml	Total protein mg	Total activity nkat	Specific activity nkat/mg	Yield %	Purification -fold
1.Supernatant of the crude extract	1400	1064	369	0.35	100	-
2.DEAE DE 52 chromatography	100	115	211	1.54	57	5.3
3.Hydroxylapatite chromatography	82	71.3	173	2.43	46.8	6.9
4.Ultrogel AcA 54 chromatography	120	13.2	82.3	6.23	22.3	17.8
5.Matrex Gel Green A	36	0.86	33.2	38.5	9	110

rent K_m for NADPH was 20 µM, V_{max} = 28 pkat).

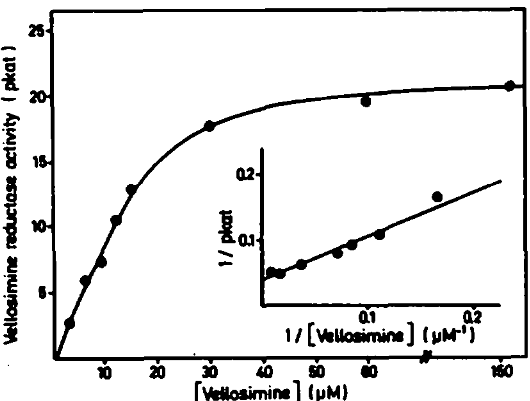

Figure II: Effect of the concentration of vellosimine on the reaction rate. Standard incubation mixtures contained 24 pkat vellosimine reductase and 200 µM NADPH, K_m = 17 µM, V_{max} = 24 pkat.

The enzymatic reaction is inhibited by the oxidized form of the co-substrate (Figure III). The Lineweaver-Burk plot (Figure IV) demonstrates that a non-competitive inhibition of the reductase takes place by $NADP^+$ when vellosimine is the substrate.

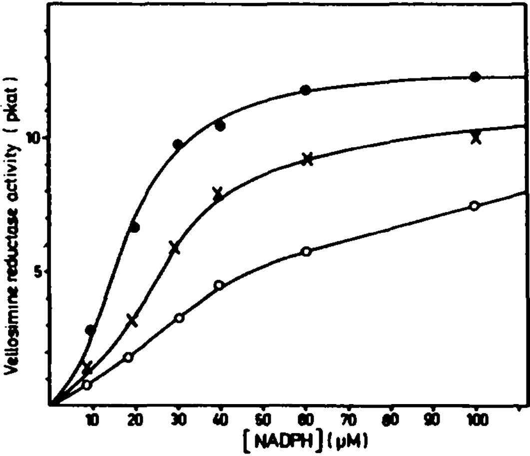

Figure III: Effect of the concentration of NADPH on the reaction rate (●—●) and the inhibitory influence of $NADP^+$ (100 µM x—x , 200 µM o—o). Standard incubations with 17 pkat reductase and 45 µM vellosimine.

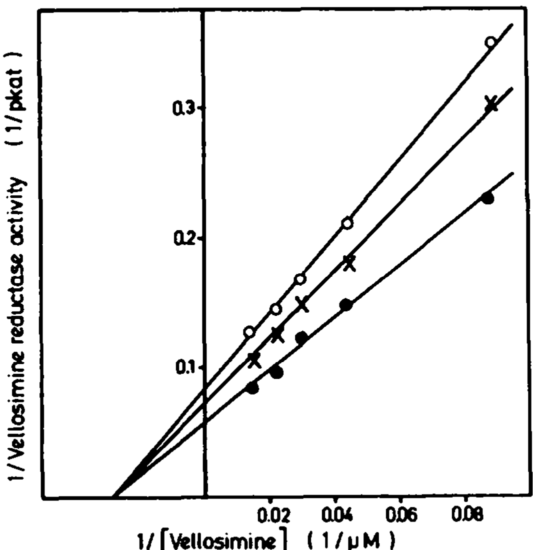

Figure IV: Inhibition of vellosimine reductase by $NADP^+$. Standard incubation contained 17 pkat reductase and 100 µM NADPH (●—● without $NADP^+$, x—x with 200 µM $NADP^+$, o—o with 400 µM $NADP^+$).

Vellosimine reductase is also inhibited by complexing reagents. Concentrations of 1 mM EDTA, 1,10-phenanthroline and neocuproin showed 77%, 71% and 65% inhibition, respectively. Reconstitution of full enzyme activity could not be achieved by addition of divalent cations e.g. Mg^{2+}, Zn^{2+}, Mn^{2+} in concentration of 0.02 - 2 mM. The reductase is highly instable. In crude extracts, half-life of enzyme activity is 25 hrs at 4°C. After 7 days only 0.25% of the activity is retained. The instability remarkably increases with purification. Enzyme activity is dependent on the presence of BME. After dialysis against buffer without BME, the activity is irreversibly lost. The substrate specificity of the enzyme was examined using 12 structurally different aldehydes. Enzyme activity was tested in a range of protein (20 nkat/mg) between 0.5 µg and 0.5 mg. As illustrated in Table II, a broad variety of compounds is accepted by the enzyme, with the exception of secologanin and picralinal. Except for geissoschizal and anthirinal, all other substrates are of the sarpagine class. Within this class, the enzyme shows a higher affinity for

Table II: Substrate specificity of vellosimine reductase.

Substrates "exo-group"	K_m (µM)	V_{max} (pkat)	Substrates "endo-group"	K_m (µM)	V_{max} (pkat)
Vellosimine	17	24	16-epi-Vellosimine	120	22.5
Geissoschizal	110	19	Gardneral	125	19.2
Deoxyajmalal B	42	9.2	N_a-Methylgardneral	167	21.8
Akuammidine aldehyde	17	0.7	Polyneuridine aldehyde	31	0.6
Anthirinal	15	0.5	Voachalotine aldehyde	133	1.2
Secologanin	–	<0.1	Picralinal	–	<0.1

the alkaloids with an exo configurated aldehyde function as observed with the "endo-aldehydes". K_m-values of the first group are significantly lower compared with those obtained for substrates belonging to the "endo-group". Vellosimine exhibited the lowest K_m-value and the highest V_{max} (24 pkat). The enzyme is absolutely specific for NADPH; V_{max} = 0 for NADH. As determined with a calibrated AcA 54 column the enzyme has a relative molecular weight of 42 000 ± 8%. The isoelectric point was found to be at pH 5,0. The influence of the pH on vellosimine reduction is depicted in Figure V.
The enzyme has a half maximal activity at pH 6.8 and 8 with optimum at pH 7.6.

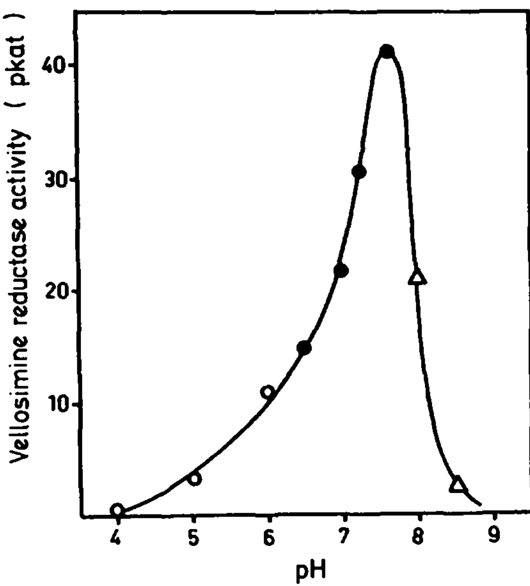

Figure V: Effect of pH on the reductase activity. The assay was done in 0.1 M citrate/KPi (o—o), KPi (●—●) and borate (△—△) buffers.

Table III: Survey of vellosimine reductase activities in plant cell suspension cultures and leaves+ (detection limit 0.04 pkat).

Species	Tribe	Family	Vellosimine Reductase pkat / mg Protein
Rauwolfia serpentina	Rauwolfieae	Apocynaceae	380
Rauwolfia verticillata	Rauwolfieae	Apocynaceae	320
Rauwolfia vomitoria	Rauwolfieae	Apocynaceae	210
Alstonia scholaris	Alstonieae	Apocynaceae	0
Catharanthus longifolius	Alstonieae	Apocynaceae	0.8
Catharanthus roseus	Alstonieae	Apocynaceae	260 (10+)
Rhazya stricta	Alstonieae	Apocynaceae	0.6
Vinca herbacea	Alstonieae	Apocynaceae	3
Vinca minor	Alstonieae	Apocynaceae	10
Tabernanthe iboga	Tabernaemontaneae	Apocynaceae	0
Stemmadenia tomentosa	Tabernaemontaneae	Apocynaceae	0
Voacanga africana	Tabernaemontaneae	Apocynaceae	0
Carissa macrocarpa	Carisseae	Apocynaceae	0
Picralima nitida	Carisseae	Apocynaceae	0
Malus sylvestris	-	Rosaceae	0
Coleus blumei	-	Labiatae	0
Nicotiana sylvestris	-	Solanaceae	0

Distribution of the enzyme within different plant cell suspension cultures. A survey of the distribution of vellosimine reductase in plant cultures is outlined in Table III. The enzyme was found exclusively in cultures belonging to the family *Apocynaceae*. In other plant families e.g. *Rosaceae*, *Labiatae* or *Solanaceae* enzyme activity could not be detected. Within the *Apocynaceae* the reductase was observed in species of the tribes *Rauwolfieae* and *Alstonieae*, but not in the tribes *Tabernaemontaneae* and *Carisseae*. The highest activities were measured in *Rauwolfia* species (spec. act. between 0.2 and 0.4 nkat/mg protein). Comparison of reductase activities in cell suspension cultures and differentiated plants (leaves) of *C.roseus* showed a 26-fold higher specific activity in cultured cells.

Precursor role of the enzymatic product 10-deoxysarpagine. The product of vellosimine reductase is 10-deoxysarpagine, which is a putative biogenetic precursor of sarpagine[4]. When 17-^{3}H labelled deoxysarpagine (15 μCi, spec. act. 720 μCi/ μmol) was fed to *R. serpentina* cell suspensions or a leaf for 3 days, about 97% was taken up by the plant material. Of this amount, 86% was incorporated into deoxysarpagine using the cell suspension culture. The incorporation rate in the "leaf experiment" was determined to be 9%. No further metabolism of the precursor occured in cultured cells. The labelled sarpagine formed could be quantitatively acetylated yielding diacetylsarpagine with the same spec. act. (3.7 μCi/ μmol) as determined for sarpagine.

Discussion

In the biosynthesis of the monoterpenoid indole alkaloid, sarpagine, the NADPH assisted reduction and the arylhydroxylation of alkaloidal aldehydes can be expected to be late stage reactions. Our recent work on sarpagine biosynthesis, conducted with isolated enzymes from cell suspension cultures of *R. serpentina* Benth., led to the detection of the new plant enzyme, named vellosimine reductase (EC 1.1.1-)[5]. This enzyme catalyzes the reduction of vellosimine with the formation of 10-deoxysarpagine as shown in the scheme. We had previously proposed the latter alkaloid to be the direct biogenetic precursor of sarpagine[6]. Here we report on the partial purification and characterization of the reductase involved in this biogenetic sequence. Because most of the reductase activity was lost during ammonium sulfate fractionation, the crude enzyme extract was chromatographed in 4 steps. This procedure resulted in a 110-fold purification of the vellosimine reductase. At this stage of purification, the reductase was free of other enzymes which are presently known to play a role in the sarpagan/ajmalan biosynthesis, e. g. PNA-esterase, vinorine synthase[7]. The absence of the esterase in vellosimine reductase preparations is absolutely necessary for the determination of the substrate specificity of the reductase. Because the esterase would interfer with those studies carried out with polyneuridine aldehyde or akuammidine aldehyde as substrates by formation of 16-epivellosimine or vellosimine, resp. Investigation of the substrate specificity of the reductase would give definitive information regarding the involvement of this enzyme in the cell-free biosynthesis of all the sarpagan alkaloids possessing a CH_2OH-function at C-16. It is interesting to note, that in contrast to other *Rauwolfia* enzymes i.e. PNA-esterase or vinorine synthase, the 110-fold purified reductase shows a relatively low substrate specificity. Of the 12 different aldehydes tested only two are not accepted as substrate; the monoterpene secologanin and the alkaloid picralinal. The remaining aldehydes are reduced to the corresponding alcohols in presence of NADPH and enzyme. Except for geissoschizal and anthirinal, the other substrates tested can be divided into two groups. Alkaloids of the first group show the aldehyde function in the *exo*-configuration ("*exo*-group"), where-

Vellosimine — Vellosimine Reductase (NADPH → NADP⊕) → 10-Deoxysarpagine — in vivo 86% → Sarpagine

Scheme

as in the second group, the compounds have an *endo*-configurated CHO group ("*endo*-group"). Comparison of the apparent K_m-values of these substrates indicates that the reductase exhibits a higher affinity to alkaloids of the "*exo*-group" as observed for aldehydes which belong to the "*endo*-group". Obviously binding of aldehydes of the latter class at the reductase is more hindered than those with *exo*-configuration. In this context it is attractive to speculate, whether this fact is important for the regulation of sarpagine/ajmaline biosynthesis. Sarpagan type aldehydes of 16(S)-configuration might be, from the biosynthetic point of view, too valuable for a simple reduction step leading to less reactive alkaloidal alcohols. This type of alkaloid has a very restricted occurrence and there exist only a few examples of compounds derived from these "*endo*-alcohols"[2]. Aldehydes with 16(S)-configuration however are required for the biosynthesis of ajmalan alkaloids and the epimerization of 16(R)- into 16(S)-configuration has never been reported. Therefore since the reductase has a high K_m-value for the "*endo*-aldehydes"these compounds might be preserved for the ajmalan pathway.

The optimum substrate of vellosimine reductase is vellosimine (K_m= 17 µM, V_{max}= 24 pkat), which also occurs in the *Rauwolfia* culture used as the enzyme source. Since the product of the enzyme reaction is 10-deoxysarpagine, one of the most widely distributed sarpagan alkaloids, this also suggests that vellosimine is the best substrate of the reductase.

In contrast to the relatively broad substrate specificity, the catalytic activity of the reductase is entirely dependent on NADPH (V_{max} for NADH = 0). Whereas normal Michaelis-Menten kinetics were determined for vellosimine, the co-substrate gave a sigmoid relationship which suggests that the enzyme must be activated by NADPH. The oxidized form of the cofactor leads to a non-competitive inhibition of the reductase. Thus even over a broad range of incubation conditions the reverse reaction, the $NADP^+$ dependent oxidation of 10-deoxysarpagine, cannot be detected. Apparently this sarpagine derivative is primarily synthesized as precursor of sarpagine.

The importance and central role of vellosimine reductase in sarpagine formation is substantiated by its distribution in different plant families, tribes and genera. As it has already been found for other *specific* enzymes of alkaloid metabolism[6,8,9], the occurrence of the reductase is also in full accord with the general expectation, that the particular enzyme is easily dectable when cells produce the appropriate alkaloid. In cell suspensions derived from plant families which do not synthsize monoterpenoid indole alkaloids e.g. *Rosaceae*, *Labiatae* and *Solanaceae*, vellosimine reductase is *not* present. In the cases where cell cultures were established from plants bearing indole alkaloids, the enzyme was found only when cellular production of sarpagan alkaloids was detectable.

The key position of this enzyme is further supported by the fact that the enzymatic product, 10-deoxysarpagine, is an excellent biosynthetic precursor of sarpagine. Using cultured *Rauwolfia* cells which were synthesizing a whole set of sarpagan/ajmalan alkaloids[10],the deoxycompound was incorporated into sarpagine to an extent of 86% under standard *in*

vivo conditions. In addition, the precursor did not show any further metabolism, notably, there was no oxidation to vellosimine.
Moreover, the corresponding in vivo experiment with a leaf of R. serpentina resulted also in an excellent incorporation of 10-deoxysarpagine into sarpagine (9% incorporation rate).
Thus far, these results strongly substantiate our hypothesis that 10-deoxysarpagine is a final biogenetic intermediate of sarpagine formation and we conclude vellosimine reductase to be a sarpagan-pathway specific enzyme.

Experimental

Plant material. For the described investigation, plant cell suspension cultures of Rauwolfia serpentina Benth. were used. The tissues were grown on Linsmaier and Skoog (LS)-medium[11] under conditions previously reported[3].
Substrates. Vellosimine and 16-epivellosimine were enzymatically synthesized from polyneuridine aldehyde and 65-fold purified polyneuridine aldehyde esterase as we had recently reported[3]. Geissoschizal was prepared according to Rapoport et al.[12]. Akuammidine aldehyde, polyneuridine aldehyde, voachalotine aldehyde and anthirinal were obtained according to Corey's method[13] starting with the appropriate alkaloidal alcohols. The remaining substrates were kind gifts of Prof. S.Sakai, Chiba, Prof. J.N.Smith, Manchester, Dr. Zeugner and Dr. Kehrbach, Hannover. The synthesis of [aryl-^{3}H] - vellosimine was performed as described by Muccino and Serico for similar compounds[14].
Enzyme purification. Vellosimine reductase activity was extracted from 0.5 kg R. serpentina cells by stirring the crushed cells for 60 min at 4°C in 1 l KPi buffer (20 mM, pH 7) containing 10 mM ß-mercaptoethanol (BME). The resulting suspension was filtered through cheese cloth, the filtrate was centrifuged for 5 min at 25 ooo x g and the supernatant was dialyzed against 10 l of the extraction buffer. This crude enzyme extract was then subjected to the following 4 purification steps:
Step 1: The crude protein solution was added to a DEAE DE 52 column (2 x 23 cm) equilibrated with extraction buffer and the column was eluted with a KCl gradient (0 - 0.3 M). The flow rate was 70 ml/hr. 3 ml fractions were collected. Vellosimine reductase was eluted at 125 mM KCl and fractions 90-120 were pooled.
Step 2: The enzyme solution was then dialyzed against 10 mM KPi (10 mM BME) and chromatographed on a Hydroxylapatite column (1.5 x 9 cm). The flow rate was 50 ml/hr and the first 82 ml were concentrated using a Berghoff cell.
Step 3: The concentrated enzyme solution was applied to AcA 54 gel chromatography (column size 2.3 x 92 cm, pH 7,50 mM KPi buffer with 10 mM BME, flow rate 20 ml/hr). Fractions of 3 ml were collected and fractions 60-74 were combined.
Step 4: The pooled, dialyzed enzyme solution was put onto a Matrex Gel column (Green, 1 x 5 cm). The oolumn was washed with 72 ml KPi buffer (pH 7.5, 10 mM BME) at a flow rate of 4 ml/hr. 5 ml of 5 mM NADP$^+$ solution, in the same buffer, was applied to the column and left for 12 hrs. The reductase then was eluted with the same buffer. Fractions of 2 ml were collected and the enzyme containing tubes were combined. Protein was determined as outlined by Bradford[15].
Enzyme assay.
Assay 1: The reaction mixture contained 200 µM NADPH, 50 µM substrate and up to 30 pkat of enzyme in a total volume of 1 ml of 100 mM KPi buffer, pH 7. The reaction was incubated at 30°C and reductase activity was measured by monitoring a decrease in absorbance at 340 nm.
Assay 2: For enzyme preparations from differentiated plants the following TLC-assay was employed: In 0.1 ml KPi buffer (50 mM, pH 7, 10 mM BME) the substrate, 16-epivellosimine, was generated from 3,5 nMol [aryl-^{3}H]-polyneuridine aldehyde (3.37 x 10^4 dpm) with 4.2 pkat PNA-esterase. After one hr at 30°C the reductase preparations were added. This mixture was incubated for 2 hrs in presence of 0.2 mM NADPH. Extraction of the incubation mixture with 0.4 ml ethyl aco-

tate, TLC in chloroform: methanol: ammonia = 90: 10: 0.1 and scanning for radioactivity were employed for quantitative analyses.

Feeding experiments. [^{3}H]10-Deoxysarpagine (15 μCi, spec. act. 720 μCi/ μmol) was fed to cell suspensions (6 days old) or one leaf of Rauwolfia serpentina for 70 hrs. The plant material was then "harvested", 1 mg (3.2 μmol) sarpagine was added and the material was extracted 3 times with ethyl acetate. The crude alkaloid mixture was chromatographed on TLC (Sil Gel plates, Macherey and Nagel) using the above solvent system. After determination of the specific activity, the isolated sarpagine was acetylated (50 μl pyridine: Ac_2O = 1: 1, 12 hrs) and purified on TLC with acetone: petroleum ether: diethylamine = 2: 7: 1 as solvent system.

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