SUBSTRATE SPECIFICITY OF VINORINE HYDROXYLASE, A NOVEL MEMBRANE-BOUND KEY ENZYME OF *RAUWOLFIA* INDOLE ALKALOID BIOSYNTHESIS

Heike Falkenhagen [a], Leo Polz [a], Hiromitsu Takayama [b], Mariko Kitajima[b], Shin-ichiro Sakai [b], Norio Aimi *[b], and Joachim Stöckigt *[a]

a) Institute of Pharmacy, Johannes Gutenberg-Universität Mainz, Staudinger Weg 5, 55099 Mainz, Germany

b) Faculty of Pharmaceutical Sciences, Chiba University, 1-33 Yayoi-cho, Inage-ku, Chiba 263, Japan

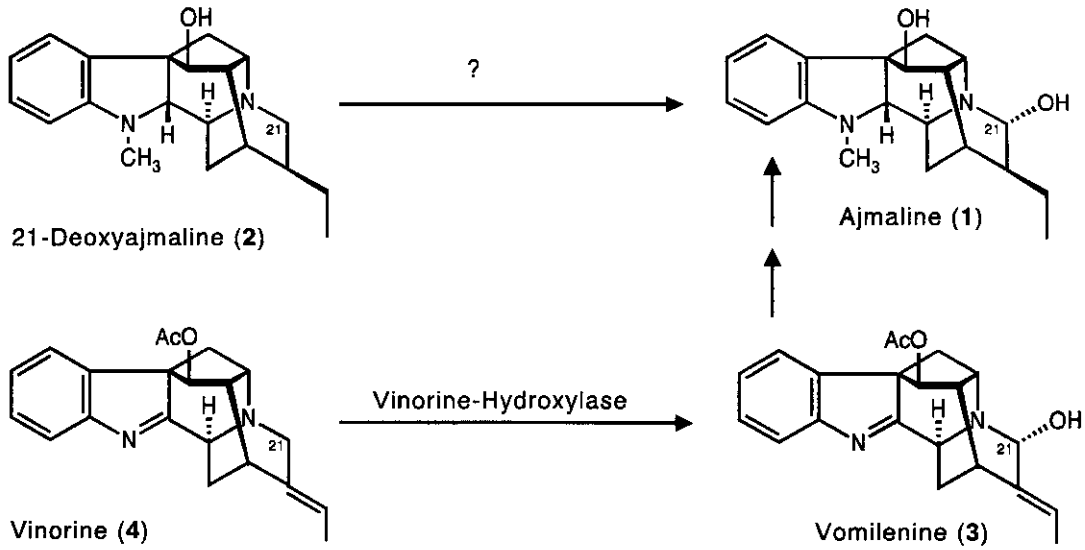
Abstract - The novel membrane bound enzyme vinorine hydroxylase exhibits an extraordinary high substrate specificity which points to its key function in the biosynthesis of monoterpenoid indole alkaloids of the ajmaline type in cell suspension cultures of the Indian medicinal plant *Rauwolfia serpentina*.

The monoterpenoid indole alkaloid ajmaline (**1**), a major constituent of plants belonging to the genus *Rauwolfia*, is biosynthesized by a large number of enzymes [1, 2]

Although a good number of these proteins is well known, an important reaction step remained unsolved up to recently. This reaction concerns the introduction of oxygen into position 21 of ajmaline (**1**), which has attracted much interest already several decades ago.

On one hand, during the earliest stages of the investigation of basic principles of the biosynthesis of **1**, feeding experiments of ^{3}H-labelled 21-deoxyajmaline (**2**) to plants of *Rauwolfia verticillata* lead to a low but significant incorporation of the deoxyalkaloid **2** into ajmaline (**1**) [3] suggesting the C-21 hydroxylation to be the final step in the biosynthesis of **1**. On the other hand with the increasing number of identified alkaloids from *Rauwolfia* species several isolations of vomilenine (**3**) have been reported.[4 - 8] This compound together with its C-21-deoxy derivative vinorine (**4**) turned out to be more typical *Rauwolfia*

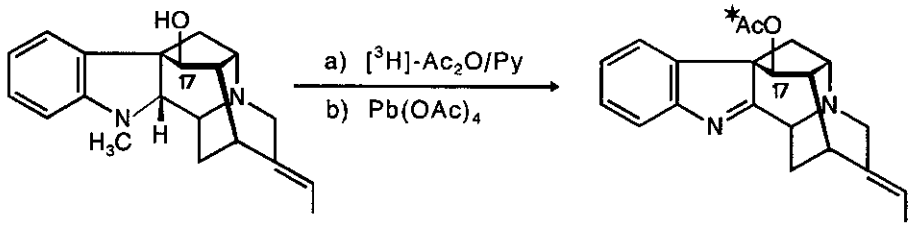
alkaloids because vinorine (**4**) too has been frequently isolated from *Rauwolfia* material, like *R. balansae*, *R. sevenetii* or *R. spathulata* plants, *R. serpentina* "hairy roots" or *R. serpentina* cell suspensions. In each case co-occurrence of **3** and **4** was observed. These findings would allow to assume another sequence of steps in which hydroxylation takes place earlier in the pathway e. g. at the stage of **4**, which would be transformed into the alkaloid(**3**).In fact, crude enzyme extracts from cultivated *Rauwolfia* cells converted vomilenine (**3**) into ajmaline (**1**), [9] which could be measured by a high specific radio immuno assay for **1**. This result proved the precursor role of **3** and made vinorine (**4**) attractive as a direct progenitor of its C-21 hydroxylated derivative(**3**)(Scheme 1)



Scheme 1: Role of biogenetic precursors of the ajmaline (**1**) biosynthesis in *Rauwolfia*.

Recent enzymatic studies demonstrated the occurrence of a cytochrome P450 dependent membrane bound enzyme which hydroxylates **4** in presence of NADPH and oxygen. This enzyme was named vinorine hydroxylase (vinorine 21-monooxygenase; EC 1.14 13 -) [10] To judge on the biosynthetic significance of this novel enzyme detailed studies on its substrate acceptance were indispensible. In order to find optimum conditions for substrate conversion the pH optimum of the hydroxylase needed to be known A sensitive enzyme assay was developed using labelled [^{3}H-acetyl]-vinorine (**4**) as substrate, which could be synthesized in a two step reaction from tetraphyllicine (**5**) by acetylation with [^{3}H]-acetic anhydride followed by $Pb(OAc)_4$ oxidation[11] (Scheme 2) Employing the labelled vinorine (**4**) as substrate, the pH dependency of its enzyme catalyzed hydroxylation was rather narrow

The optimum could be determined at pH 8.1 and half maximum activity was still measured at pH 7.0 and 8.5, respectively.



Tetraphyllicine (**5**) [^{3}H-Acetyl]-Vinorine (**4**)

Scheme 2: Synthesis of radioactive labelled vinorine (**4**).

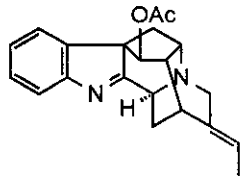
At pH 8.1 a number of putative substrates, especially alkaloids structurally related to **4**, were tested in comparison to vinorine (**4**) (100 % rel. enzyme activity).

The most interesting structures would be those of **5** and (*Z*)-tetraphyllicine (**6**) which belong, like **4**, to the ajmalan-group and exhibit the 19, 20-double bound which would allow a C-21 allylic hydroxylation as known for vinorine (**4**). However, both alkaloids were not transformed by the vinorine hydroxylase under optimum conditions. The detection limit was about 0.5 % compared to vinorine hydroxylation. Gardnerine (**7**), an alkaloid belonging to the ajmalan-related sarpagan-group exhibiting also the 19, 20-double bond as structural feature and humantenine (**8**), an oxindole, were not accepted as enzyme substrate. It was therefore not surprising and was in line with these observations, that the Corynanthé type compounds geissoschizine-methyl ether (**9**) and hirsuteine (**10**) do also not act as substrates for the vinorine hydroxylase (Scheme 3)

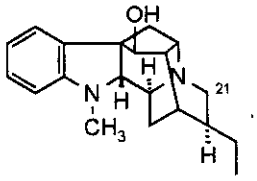
Because of the above mentioned *in vivo* experiments with 21-deoxyajmaline (**2**) it was important to include this alkaloid into the substrate studies too. But again under conditions of vinorine hydroxylation, **2** could not be hydroxylated *in vitro*. The detection limit of the enzymatic transformation was lower than 1 2 % which also excludes alkaloid **2** as an important candidate for the vinorine hydroxylase

Finally the influence of the acetyl group at O-17 on the substrate acceptance of the enzyme should be investigated. For this purpose the acetates of **2** and **5** were tested: 17-O-acetyl-tetraphyllicine (**11**) and 17-O-acetyl-21-deoxyajmaline (**12**) Especially the latter was interesting with regard to the above mentioned feeding experiments with 21-deoxyajmaline (**2**) since the detection of labelled ajmaline (**1**) might be explained by the following hypothetical enzymatic pathway. 21-deoxyajmaline (**2**) would *in vivo* be

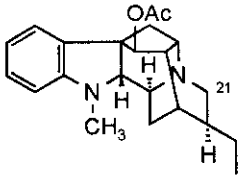
acetylated first, afterwards hydroxylated forming 17-O-acetylajmaline and finally hydrolyzed by an esterase forming ajmaline (**1**). But for 17-O-acetyl-21-deoxyajmaline (**12**) as well as for **2** hydroxylation could not be observed, detection limits being the same 1 2 % of the optimal turnover of vinorine (**4**) 17-O-Acetyl-tetraphyllicine (**11**) hydroxylation could be excluded above 7 % relative enzyme activity. Both experiments show that the acetyl group at O-17 does not influence enzyme activity and that both the indolenine and the 19,20 double bond are structural features which are required for hydroxylation.



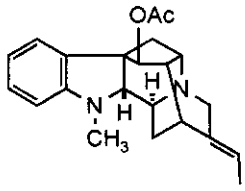
Vinorine (**4**)
[100]



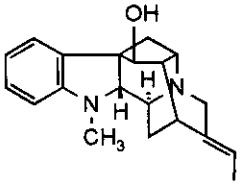
21-Deoxyajmaline (**2**)
[< 1.2]



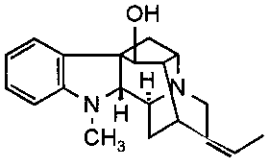
17-O-Acetyl-21-Deoxyajmaline (**12**)
[< 1.2]



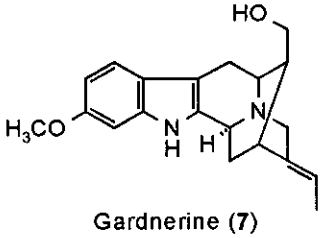
17-O-Acetyl-Tetraphyllicine (**11**)
[< 7]



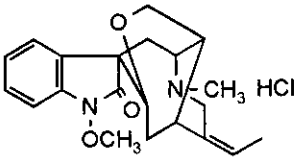
Tetraphyllicine (**5**)
[< 0.5]



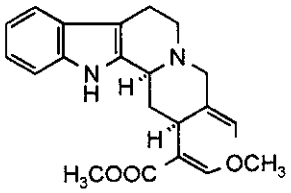
Z-Tetraphyllicine (**6**)
[< 0.5]



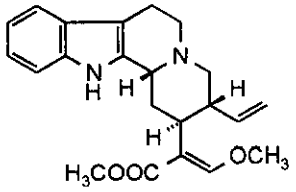
Gardnerine (**7**)
[< 0.5]



Humantenine HCl-Salt (**8**)
[< 0.5]



Geissoschizine-Methyl Ether (**9**)
[< 0.5]



Hirsuteine (**10**)
[< 0.5]

Scheme 3: Substrate specificity of the vinorine hydroxylase from *Rauwolfia serpentina* cell suspension cultures [rel enzyme activity, %].

The here discussed results now clearly indicate that this enzyme has an extraordinary substrate specificity accepting of a range of structurally related compounds exclusively the indolenine alkaloid (**4**). So far, it seems that vinorine (**4**) is the only natural substrate of this enzyme and that the introduction of oxygen into the C-21 position takes place earlier in the biosynthesis of ajmaline (**1**) at the indolenine stage.

The very narrow substrate specificity of vinorine hydroxylase is also characteristic for other plant mixed function oxygenases discribed by different authors. One example is known from monoterpene biosynthesis: (-)-limonene, which is, as well as vinorine (**4**) a substrate for an allylic hydroxylation, cannot be replaced by saturated derivatives. Also in (-)-limonene the double bonds are absolutely necessary for the acception of the substrate by the specific hydroxylase.[12] Furthermore there are also examples for highly selective monooxygenases in alkaloid biosynthesis. Cytochrome P450 dependent enzymes catalyzing the forming of a methylenedioxy bridge or an oxidative phenol coupling accepted from a number of putative substrates exclusively one compound.[13, 14]

In this feature monooxygenases of higher plants stand obviously in contrast to those of mammalian liver which are involved in the detoxification of exogenous substrates These mammalian cytochrome P450 enzymes possess a far broader substrate range to accomplish their physiological purpose to protect the organism from the multiplicity of toxic influences. But interestingly even in this xenobiotic metabolism plants may show several distinct cytochrome P450 isoenzymes each with a rather high specificity towards a single toxic substance. This topic has just recently been discussed in a review on this enzyme group.[15]

Purification and reconstitution of cytochrome P450 systems of higher plants have only sporadically been achieved and therefore very few investigators have tested the substrate specificity of homogenous enzymes in reconstituted systems However, there is evidence that sometimes to the main substrate structurally related compounds can be accepted by an enzyme but that they show very low turnovers which may not have been detected in enzyme assays with the crude microsomal preparation. An example is a purified cytochrome P450 enzyme catalyzing the generation of the bisbenzylisoquinoline alkaloid skeleton *via* oxidative phenol coupling.[16] The reconstituted enzyme system exhibited a dramatic increase of activity with different coclaurine derivatives that were metabolized in microsomal preparations only to about 5 % of the maximum activity. Consequently, it would be interesting to verify the here presented results with a purified vinorine hydroxylase, a work which is now in progress.

EXPERIMENTAL

Plant cell suspension cultures of *R. serpentina* were routinely grown under standard conditions as described previously.[17] For efficient enzyme isolation, cells were cultivated in LS-nutrition medium [18] and were harvested after 4 days of growth. For the preparation of microsomal protein from cultivated cells a procedure was applied as reported earlier.[19]

Synthesis of [^{3}H-acetyl]-vinorine (4):

Tetraphyllicine (**5**) (38.5 mg, 125 µmol) was dissolved in dry pyridine (0.8 ml) and acetylated with [^{3}H]-acetic anhydride (185 MBq, 100 µmol) for 48 h in a closed apparatus. After removal of the solvent, the remaining crude reaction mixture was oxidized as described[11] with lead tetraacetate (70 mg, 158 µmol) in presence of toluene (2 ml) for 1 h. The dried residue was then exaustively purified by silica gel thin layer chromatography in solvent system $CHCl_3$-MeOH-NH_3 (4:1:0.02) and the purity checked by tlc-scanning of the labelled vinorine (**4**) in solvents EtOAc-MeOH-H_2O-NH_3 (7 2:1 0 02) The isolated [^{3}H-acetyl]-vinorine (**4**) showed a purity of ca. 98 % (total yield 24 %).

Optimization of the enzyme assay:

For the determination of the pH optimum of the hydroxylase the assay contained in a tot. vol. of 250 µl Tris-HCl buffer (0.1 M) with various pH, 20 % sucrose, 10 mM KCl, 0.3 mM NADPH, 0.5 mg microsomal protein and 7 4 KBq [^{3}H-acetyl]-vinorine (**4**). After 1 h of incubation at 35 °C, the enzymatic reaction was terminated by extraction with 100 µl ethyl acetate. The organic layer was subjected to tlc analysis and quantitated by scanning the radioactivity employing solvent system $CHCl_3$-cyclohexane-diethylamine (6:3 1); R_f vinorine (**4**) 0.49, R_f vomilenine (**3**) 0.2. The optimum pH value was pH 8.1.

Substrate specificity study:

Typical incubation mixtures for substrate study contained in a tot. vol. of 10 ml Tris-HCl-buffer (pH 8.1) with the above mentioned concentrations of sucrose and KCl, 1 2 mM NADPH, microsomal protein (20 mg) and the putative substrate (1 mg) All substrates were authentic alkaloids of a purity > 98 % After incubation at 35 °C (1 h) the reaction mixture was extracted with ethyl acetate, the organic layer was evaporated and the residue analyzed for the formation of hydroxylated derivatives by method a), b) or c). Method a) was applied for analysis of incubation with vinorine (**4**), tetraphyllicine (**5**), its (Z)-isomer (**6**), gardnerine (**7**), humantenine (**8**), hirsuteine (**10**) and 17-O-acetyl-tetraphyllicine (**11**) (investigated in a seperate experiment with half of the substrate concentration) and was performed after acetylation by gc-ms analysis.

21-Deoxyajmaline (**2**) and 17-O-acetyl-21-deoxyajmaline (**12**) were analyzed by method b) 150 nmol of the putative substrate were incubated under the above conditions but in presence of 1.2 mM NADPH and 1 mg microsomal protein. After extraction with ethyl acetate, evaporation and acetylation of the residue (pyridine/acetic anhydride 1:1) hplc was used for the separation of acetylated alkaloids. Detection limit corresponds to < 1 2 % of vinorine hydroxylation (= 100 %)

Geissoschizine-methyl ether (**9**) incubation was analyzed by method c): After extraction, the residue was reduced by an excess of $NaBD_4$ and then analyzed by gc-ms as outlined in the following.

GC-MS-Analysis:

A quadrupole ms (MAT 44 S, Finnigan) was coupled to a capillary gaschromatograph (Varian 3700) equiped with a Durabond DB-1 column (30 m x 0.32 mm, film thickness 0.25 mm, J u W. Scientific/Fison). Temperature program was linear with 2 °C min^{-1} from 280 - 320 °C. Detection limit was about 0 5 % rel. enzyme activity compared to vinorine hydroxylation. In the experiment with 17-O-acetyl-tetraphyllicine the limit of detection was 7%.

HPLC Analysis

A Merck-Hitachi hplc system was applied, coupled to a RP select B Hibar LiChrosorb column (4 x 125 mm, 5 µm). A gradient with solvent A (acetonitrile) / B (10 mM $(NH_4)_2 CO_3$), 30 % · 68 % from 0 - 6 min and 60 % : 40 % from 6.1 - 12 min, was used. Flow rate was 1.25 ml/min and detection at 260 nm

ACKNOWLEDGEMENTS

Our thanks are due to the Deutsche Forschungsgemeinschaft (Bonn - Bad Godesberg), the Fonds der Chemischen Industrie (Frankfurt/Main) and to the Ministry of Education, Science and Culture, Japan (International Scientific Research Program Joint Research: No. 06044035 and a Grant-in-Aids for Scientific Researches: No. 06453187).

REFERENCES

1. J. Stöckigt, in "The Alkaloids", ed. by G.A. Cordell, Academic Press, 1995, in press.
2. J. Stöckigt, A Lansing, H. Falkenhagen, S Endreß and C.M. Ruyter, in "Plant Cell Culture for Breeding and Formation of Phytochemicals", ed. by K. Oono, T. Hirabayashi, S Kikuchi, H. Handa, K. Kajiwara, NIAR, Japan, 1992, pp 277-292

3. D.H.R. Barton, G.W. Kirby, R.H. Prager, and E M.J. Wilson, J. Chem Soc., 1965, 3990.

4. F. Libot, N. Kunesch, and J. Poisson, Phytochemistry, 1980, **19**, 989.

5 A. Hofmann and A J. Frey, Helv. Chim. Acta, 1957, **40**, 1866.

6. W.I. Taylor, A.J. Frey, and A. Hofmann, Helv. Chim Acta, 1962, **45** , 611.

7 J. Stöckigt, A. Pfitzner, and J. Firl, Plant Cell Reports, 1981, **1**, 36.

8. H. Falkenhagen, . I.N Kuzovkina, I.E. Alterman, L A. Nikolaeva, and J. Stöckigt, Nat. Prod. Letters, 1993, **2**, 107.

9. J. Stöckigt, A. Pfitzner, and P J. Keller, Tetrahedron Lett., 1983, **24**, 2485.

10 H. Falkenhagen and J Stöckigt, Z. Naturforsch., 1995, **50c**, 45.

11. A.K. Kiang, S.K. Loh, M. Demanczyk, C.W. Gemenden, G.J. Papariello, and W.I. Taylor, Tetrahedron, 1966, **22**, 3293.

12. F. Karp, C.A. Mihaliak, J.L. Harris and R. Croteau, Arch. Biochem. Biophys., 1990, **276**, 219.

13. W. Bauer and M H. Zenk, Phytochemistry, 1991, **30**, 2953

14. R. Gerardy and M H Zenk, Phytochemistry, 1993, **32**, 79

15. G.P. Bolwell, K. Bozak and A. Zimmerlin, Phytochemistry, 1994, **37**, 1491.

16. R. Stadler and M H. Zenk, J. Biol Chem., 1993, **268**, 823

17. R. Lutterbach and J. Stöckigt, Helv. Chim. Acta, 1992, **75**, 2009

18. E.M. Linsmaier and F Skoog, Physiol. Plant , 1965, **18**, 100.

19. H. Diesperger, C R. Müller, and H. Sandermann, FEBS Letters, 1974, **43**, 155.

Received, 3rd April, 1995