163. Enzymatic Formation of Raucaffricine, the Major Indole Alkaloid of *Rauwolfia serpentina* Cell-Suspension Cultures

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Dedicated to Prof. *Leopold Horner* on the occasion of his 80th birthday

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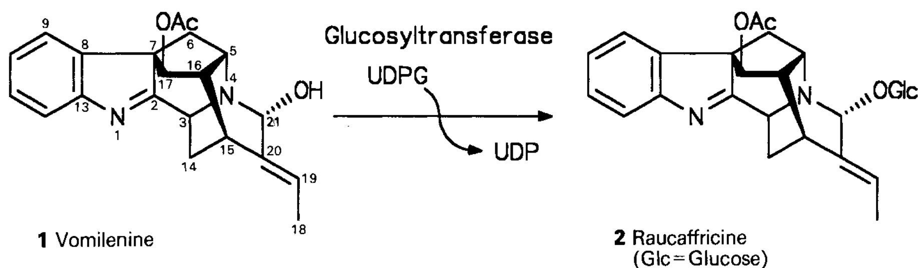
The major alkaloid from *Rauwolfia serpentina* cell-suspension cultures, the glucoalkaloid raucaffricine (**2**), was enzymatically formed from vomilenine (**1**) and UDPG in presence of microsomal-bound enzyme. This glucosyltransferase exhibits a relatively high substrate specificity with strong preference for **1** and UDPG. The apparent K_m values for **1** and UDPG were 40 µM and 0.8 mM, respectively, for raucaffricine formation. Optimum transferase activity was observed at 50° and pH 6.3. The taxonomic distribution of this enzyme seems to be very limited because the transferase can be exclusively detected in raucaffricine-producing plant cells.

Introduction. – Within the vast group of monoterpenoid indole alkaloids consisting now of nearly 2000 different structures, a very small class of about 30 glycoalkaloids exists [1] [2]. In general, these glycoalkaloids were isolated only as trace compounds from plant material, and their biosynthesis was not investigated in detail. None of the involved enzymes were so far isolated, although glycosylating enzymes from plants have often been described [3], and the number of newly detected glycosyltransferases is permanently increasing.

The richest source for enzymes involved in the biosynthesis of indole alkaloids proved to be the cell-suspension culture of the Indian medicinal plant *Rauwolfia serpentina* (L.) BENTH. producing the antiarrhythmic alkaloid ajmaline [4]. This culture generates, under optimum conditions, the glucoalkaloid raucaffricine (= vomilenine 21-β-D-glucoside; **2**) in amounts of up to 1.6 g/l nutrition medium [5], a value which is 67 times higher than that recently observed in roots of *R. serpentina* [6]. This enhanced raucaffricine accumulation requires an efficient biosynthesis of the glucoalkaloid. Previously, we had already characterized a specific soluble glucosidase (EC 3.2.1.125) converting raucaffricine (**2**) into its aglycone vomilenine (**1**) [7]. After the detection of this novel enzyme, we attempted to give evidence for the reverse reaction – the raucaffricine synthesis. In this paper, we report on the isolation and on the properties of the new enzyme synthesizing specifically raucaffricine (**2**) from vomilenine (**1**) and UDPG[1]) (*Scheme*).

[1]) *Abbreviations:* ADP, adenosin 5′-diposphate; CDP, cytidine 5′-diphosphate; GDP, guanosine 5′-diphosphate; TDP, thymidine 5′-diphosphate, UDPG (= UDP-Glc), uridine 5′-diphosphateglucose; Glc (or G), glucose; BSA, bovine serum albumin; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]propane-1-sulfonate; DTE, dithioerythritol; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); EDTA, ethylenediaminetetraacetic acid; NEM, *N*-ethylmaleimide; SH-, mercapto-; d. wt., dry weight.

Scheme. *Enzymatic Formation of Raucaffricine* (**2**) *from Vomilenine* (**1**)



Results. – *Isolation and Properties of UDPG: Vomilenine 21-β-D-Glucosyltransferase.* From cell-suspension cultures of *R. serpentina* which were grown in *Linsmaier* and *Skoog* medium [8], the highest amounts of active microsomal UDPG: vomilenine 21-β-D-glucosyltransferase (32.4 nkat from 100 g d.wt. tissue, 96.2 pkat/mg microsomal protein) could be isolated using a general procedure for microsome isolation described previously [9]. This novel enzyme, EC 2.4.1, catalyzed the glucosylation of vomilenine (**1**) in presence of UDPG (*Scheme*). The enzyme product raucaffricine (**2**) was unambiguously identified by TLC and formation of its tetra-*O*-acetyl- and 1,2-dihydro derivative. Moreover, after optimization of the cell-free synthesis, enzymatically formed **2** could be isolated from large-scale incubations in the lower mg range (3.3 mg) and was characterized by mass and ^{1}H-NMR spectroscopy. For the determination of the transferase activity, a fast HPLC-based assay was developed showing good separation characteristics.

The transferase reaction was absolutely dependent on the presence of active protein and gave normal *Michaelis-Menten* kinetics for **1** (*Fig. 1*) and UDPG (*Fig. 2*). For both substrates, the double-reciprocal plots of tranferase activity and substrate concentration gave a linear relationship (*Figs. 1* and *2*). The apparent K_m values were found to be 0.04 mM for **1** (V_{max} = 17 pkat) and 0.8 mM for UDPG (V_{max} = 14 pkat). When the enzyme activity of the transferase was determined under standard conditions (see *Exper. Part*) at different pH, a clear pH optimum was observed at pH 6.3. The enzyme tolerated a broad pH range showing half-maximal activity at pH 4.8 and 7.6 (*Fig. 3*). The temperature optimum of the enzymatic reaction was 50°. The stability of the transferase was increased by adding SH-protecting agents like 2-mercaptoethanol, DTE[1]), or ascorbic acid. In presence of 50% glycerol, the enzyme could be stored at −20° for 3 weeks without loosing catalytic activity. The enzyme was solubilized to an extent of 20% of the total activity when the zwitterionic detergent CHAPS was applied in a final concentration of 0.5% to the microsomes.

Substrate Specificity of the Transferase. The substrate acceptance of the enzyme was examined using HPLC (detection of 0.2 pkat enzyme activity (30 pmol of **2**)) and was applied to a variety of ajmalan-type alkaloids bearing a 21α-OH group. The best substrate was found to be vomilenine (**1**; 100% rel. activity). Under these conditions, exclusively the derivative 1,2-dihydrovomilenine with (2α*S*)-configuration was glucosylated (3%), whereas ajmaline, *N*α-norajmaline, 17-*O*-acetylajmaline and 17-*O*-acetyl-*N*α-norajmaline did not act as a substrate (detection limit 0.05% rel. activity). Of different UDP monosaccharides, exclusively UDP-Glc (= UDPG) was accepted as a cofactor; UDP-glucuronic acid, -galactose, -mannose, or -xylose were inactive with

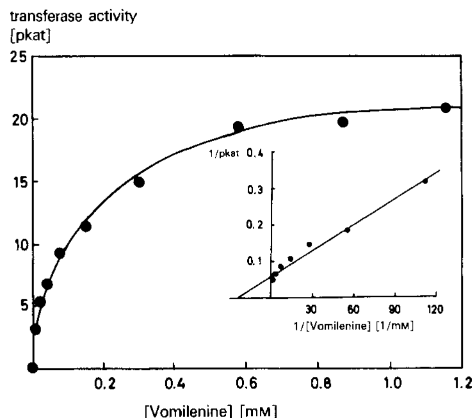


Fig. 1. *The effect of the concentration of vomilenine* (**1**) *on the reaction rate of UDPG: vomilenine β-D-glucosyltransferase.* Standard incubation mixture, $K_m = 0.04$ mM, $V_{max} = 17$ pkat.

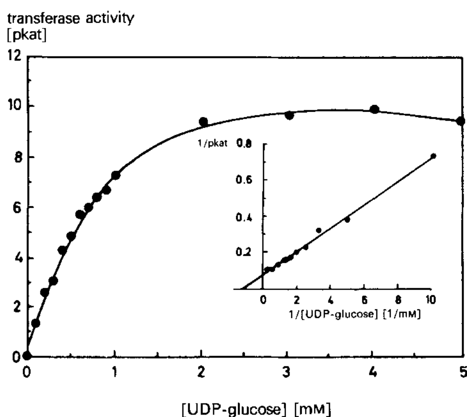


Fig. 2. *The effect of the concentration of UDPG on the reaction rate of the transferase.* Standard incubation mixture, $K_m = 0.8$ mM, $V_{max} = 14$ pkat.

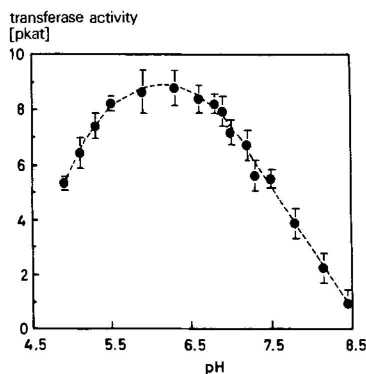


Fig. 3. *The effect of pH on the transferase activity.* Enzyme activity was measurd under standard conditions in presence of 0.2M *Tris*-HCl buffer.

respect to the catalytic activity of the enzyme. When the nucleotide part was varied, a broader acceptance was observed (nucleotide glucose (% rel. activity)): UDP-Glc (100), CDP-Glc (16), ADP-Glc (9), TDP-Glc (5), and GDP-Glc (4).

Transferase Dependency on Mineral Salts and Enzyme Inhibitors. At 1 mM concentrations, $CuSO_4$, $ZnCl_2$, and $HgCl_2$ inhibited the enzyme to an extent of 80, 94, and 100%, respectively. Complete inhibition of the enzyme was monitored at 10 mM concentrations of $FeCl_3$, $CuSO_4$, $ZnCl_2$, $ZnSO_4$, and $HgCl_2$. Strong inhibition of the transferase was also measured in presence of the following inhibitors (mM, % inhibition): DTNB[1]) (1.25, 100), (phenylmethyl)sulfonyl fluoride (10, 100), *p*-hydroxybenzaldehyde (5, 40), 1,10-phenanthroline (5, 70 or 10, 100), EDTA (5, 96).

When the taxonomic distribution of the transferase was investigated, we detected the enzyme exclusively in cell systems able to synthesize the enzyme product **2**. Whereas *R. serpentina* cell suspensions contained 0.32 nkat enzyme activity per g d.wt. (6 nkat/l

medium), in cultivated *R. canescens* cells, < 0.1 nkat/g d.wt. were determined. In a variety of cell suspensions of the plant family Apocynaceae and Solanaceae which do not produce **2**, this enzyme could not be detected (see below).

Discussion. – The phytochemical characterization of cell-suspension cultures of *Rauwolfia serpentina* BENTH. resulted so far in the isolation and identification of 31 different monoterpenoid indole alkaloids, among them 5 glucoalkaloids [10]. Whereas most of these glucosidic alkaloids occur in trace amounts, the major alkaloid raucaffricine (**2**; vomilenine glucoside) and the antiarrhythmic active ajmaline accumulate in amounts of up to 2.3 and 0.6%, respectively, of the dried cells. In the differentiated plant, however, **2** was detected only as a trace compound (0.04% of the d. wt.) and ajmaline as one of the main alkaloids 0.1% [11]. Obviously, raucaffricine synthesis is a more important reaction in the cell-culture system as compared to the intact plant. Raucaffricine (**2**) biosynthesis can be assumed to be a one-step glucosylation of vomilenine (**1**). This process might limit a higher ajmaline production in cultivated cells, because **1** is a late intermediate located directly on the pathway leading to ajmaline. The isolation and determination of the properties of this important enzyme, trapping **1** by glucosylation and thus forming **2**, were now achieved.

Since all our efforts failed in the past to detect the glucosyltransferase in soluble protein fractions of *Rauwolfia* cells, we analyzed microsomal fractions successfully for transferase activity. Control experiments of **1** with denatured protein clearly established the formation of **2** to be enzyme-catalyzed.

The determination of the enzyme characteristics using six ajmaline type alkaloids bearing a 21α-OH group indicates the high substrate specificity (for **1**) of the transferase. The relatively low K_m value of **1** (40 μM) points to a high affinity of the enzyme towards this alkaloid which is, however, different for the cofactor UDPG exhibiting a significant higher K_m (800 μM). The vomilenine-converting enzyme is much more susceptible to changes in the saccharide part than to the variation of the nucleotide part of the cofactor. The high cofactor specificity of the transferase (exclusively for UDP-Glc) also found for other glucosyltransferases, *e.g.* the UDPG:indole-3-acetate 1-β-D-glucosyltransferase (EC 2.4.1.121) from *Zea mays* which also did not accept UDP-galactose or -xylose [12]. That the here described enzyme is not very pretentious to the nucleotide part of different activated glucoses (UDP-Glc was the best cosubstrate, but CDP-, ADP-, TDP-, and GDP-Glc were also effective, with conversion rates of 16–4% (rel. activity)), is similar to results obtained for the enzymatic biosynthesis of steryl glucosides [13] [14].

For optimum isolation of the enzyme activities, SH-protecting agents had to be applied (DTE, 2-mercaptoethanol), suggesting that free SH groups are necessary for full enzyme activity. The enzyme inhibition by iodoacetamide, NEM, and DTNB or by Hg^{2+}, Zn^{2+}, and Cu^{2+} ions is in full agreement with the above suggestion. The isolation of highest enzyme activities from *R. serpentina* (L.) BENTH. cells (6 nkat/l medium) and low activity (*ca.* 1 nkat/l) from *R. canescens* L. cell cultures is in sharp contrast with the results from cell cultures of the Apocynaceae *Catharanthus roseus* (L.) G. DON and *Allamanda cathartica* L. or *Nicotiana plumbaginifolia* L. (Solanaceae) or *Beta vulgaris* L. (Amaranthaceae) which did not show any transferase activity. This observation, coupled with the striking substrate and cofactor specificity, indicates that vomilenine glucosyltransferase is indeed a very specific enzyme. Because this protein is responsible for the excellent

raucaffricine production rates in cultivated *Rauwolfia* cells, this membrane-bound enzyme will be of a special interest, *e.g.* for inhibition studies, to increase the ajmaline biosynthesis in cultivated *Rauwolfia* cells.

Experimental Part

Plant Cell Cultures. Plant cell-suspension cultures of *R. serpentina* were grown at 25° in 1-l *Erlenmeyer* flasks on a gyratory shaker at 100 rpm. Cells were cultivated for 7 days in 250 ml *LS* medium [8], harvested by suction filtration, stored at −25°, and used for the isolation of the microsomal enzyme fraction.

Materials. UDP-[^{14}C]glucose (7.4 GBq·mmol^{-1}) was purchased from *Amersham* (Braunschweig). Tris(hydroxymethyl)aminomethane-hydrochloride (= 2-amino-2-(hydroxymethyl)propane-1,3-diol; *Tris*-HCl), CHAPS, UDPG, UDP-galactose, and UDP-glucuronic acid were obtained from *Boehringer Mannheim* (Mannheim). BSA, (phenylmethyl)sulfonyl fluoride, and $NaBH_3CN$ were from *Serva* (Heidelberg); ADPG, CDPG, DTNB, GDPG, iodoacetamide, NEM, TDPG, UDP-mannose, and UDP-xylose were purchased from *Sigma* (München-Deisenhofen). Other chemicals were from *Merck* (Darmstadt).

Isolation of Microsomal Protein. Of the frozen cells, 100 g were homogenized with a '*Braun*-Starmix' for 2 min in 200 ml of *Tris*-HCl buffer (0.1M, pH 8.5, 10 mM KCl, 1 mM EDTA, 20 mM BME). After stirring this mixture for 30 min, cell debris were removed by filtration through cheese cloth and centrifugation at 3000 × *g* for 20 min. To the supernatant, 1M $MgCl_2$ was added (per 100 ml, 5 ml of $MgCl_2$ soln.), and after stirring for 15 min at 4°, microsomes were obtained by centrifugation at 20000 × *g* for 1 h. The residue was dialyzed each for 12 h against 20 mM citrate-phosphate buffer (pH 5.5, 1M NaCl, 10 mM $HSCH_2CH_2OH$) and 20 mM *Tris*-HCl (pH 7.5, 10 mM $HSCH_2CH_2OH$).

Protein Determination. Protein concentrations were determined by the method of *Bradford* [15] using a calibration curve obtained with BSA.

Assay for UDPG: Vomilenine Glucosyltransferase. Under standard condition, 2 mM UDPG was incubated in presence of 1.14 mM vomilenine (**1**) in a volume of 250 µl of 0.1M *Tris*-HCl buffer at pH 7.5. After incubation, the enzyme was precipitated with 750 µl of MeOH. After centrifugation at 14000 rpm (*Eppendorf* centrifuge), the supernatant was submitted to HPLC analysis (*Spectra Physics* (Darmstadt) instrument, *SP8440* UV/VIS detector, *SP4100* computing integrator, *SP8700* solvent delivery system, *SP8780XR* autosampler; *Lichrospher*® *60 RP*-select *B* column (4 × 125 mm, 5 µm, *Merck* (Darmstadt))). With a flow rate of 1 ml/min, a gradient of system *A* (MeCN/KPi (6.66 g of KH_2PO_4, 2.8 ml of H_3PO_4 (85%), ad 1 l) 1:9) and system *B* (MeCN/KPi 6:4) was applied: *A*/*B* 8:2, then 5 min *A*/*B* 7:3, 7.5 min *B*, 9.5 min *B*, and 10 min *A*/*B* 8:2. Detection at 258 nm, t_R (**2**) 4.2 min. The detection limit of this assay corresponds to 30 pmol of **2**.

TLC Chromatography. Purification and identification of alkaloids was performed on silica gel TLC plates G/UV_{254} (0.25 mm, *Macherey* and *Nagel* (Düren)) with the following solvent systems: *A*, $AcOEt/MeOH/H_2O/NH_3$ (25%) 7:2:1:0.02; *B*, $CHCl_3/MeOH/NH_3$ (25%) 9:1:0.02; *C*, $CHCl_3/MeOH/NH_3$ (25%) 4:1:0.02; *D*, petroleum ether/acetone/Et_2NH 7:2:1; *E*, $CHCl_3$/cyclohexane/Et_2NH 6:3:1.

Identification of the Enzyme Product Raucaffricine (**2**). UDP-[^{14}C]glucose (5920 Bq, 5 nmol), microsomes (50 µg protein), and 57 nmol of **1** were incubated at 30° for 90 min in a total volume of 100 µl. [^{14}C]Raucaffricine ([^{14}C]-**2**) was obtained and identified as follows: the incubation mixture was submitted to TLC in solvent system *A*, R_f 0.44 (identical to authentic **2**). Further identity was found with system *B* (R_f 0.09), *C* (R_f 0.32), and *D* (R_f = 0). The purified [^{14}C]-**2** was acetylated and showed identity with unlabelled raucaffricine tetraacetate in system *B* (R_f 0.88) and *E* (R_f 0.61). When [^{14}C]-**2** was reduced with $NaBH_3CN$, the formed (2α*S*)-1,2-dihydroraucaffricine was chromatographically identical with a reference sample. For a final identification, 138 mg of microsomal protein was isolated from 1.8 kg of cell suspension and used for the cell-free formation of 3 mg of **2** which showed the same MS and ^{1}H-NMR data as described in [16].

Properties of the Transferase. Substrate Specificity. Under standard conditions were incubated for 30 min at 37° microsomal protein (0.29 mg), **1** (1.14 mM), and different nucleotide sugars (10 mM). Samples were monitored by HPLC for glucoalkaloid formation. For the investigation of the alkaloid acceptance of the enzyme, different alkaloids (100 nmol) were incubated for 120 min with protein (0.1 mg) in presence of UDP-[^{14}C]glucose (4600 Bq, 3.89 nmol) in a total volume of 0.1 ml. Samples were analyzed by TLC and scanning of radioactivity (*Bertholdt* TLC scanner *LB2722-2* (Wildbad)) for glucoalkaloid synthesis.

Inhibition of the Transferase. A variety of mineral salts was added to the assay, in final concentrations of 0.1, 1, and 10 mM; nucleotides were applied in amounts of 0.1, 1, 5, and 10 mM under the same conditions. Enzyme inhibitors were added in varying concentrations.

Solubilization of the Enzyme. Microsomes (1.5 ml, 11 mg of protein) were suspended in 20 mM *Tris*-HCl buffer (20% glycerol, 20 mM $HSCH_2CH_2OH$) at different concentrations of CHAPS. H_2O was added up to 2 ml, and the mixture was stirred for 30 min at 4°. After centrifugation at 105000 × *g* (ultracentrifuge *L*-2, *Beckman* (München)), the protein concentration and the enzyme activity were measured in the supernatant and the residue.

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