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ENZYMATIC BIOSYNTHESIS OF RAUMACLINE

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Key Word Index—*Rauwolfia serpentina*; Apocynaceae; cell suspension cultures; indole alkaloids; ajmaline-feeding; raumacline biosynthesis; 21-hydroxyraumacline-/perakine-reductase; enzyme purification.

Abstract—The indole alkaloid, raumacline, is biosynthesized from ajmaline, when the latter was fed to cell suspensions of *Rauwolfia serpentina*. Formation in catalysed by two enzymes, a cell wall-bound peroxidase followed by a $NADPH_2$-dependent reductase. The first enzyme cleaves ajmaline oxidatively leading to a C-21 epimeric mixture of 21-hydroxyraumacline, a novel alkaloid, which, in turn, is reduced under formation of raumacline. The peroxidase reaction is not specific for *Rauwolfia* cells whereas the reductase is present exclusively in *Rauwolfia* cell suspensions. The reductase has been purified to homogeneity. The enzyme is highly substrate specific, only accepting 21-hydroxyraumacline and perakine, the latter leading to raucaffrinoline by reduction.

INTRODUCTION

The monoterpenoid indole alkaloid raumacline (**1**) has been isolated and identified together with its $N\beta$-methyl derivative (**2**) only recently for the first time [1]. Both alkaloids have been detected after administration of ajmaline (**3**) to a cell suspension culture of the Indian medicinal plant, *Rauwolfia serpentina*. A further search of alkaloids arising from ajmaline-feeding by the same process of biotransformation resulted finally in the detection of a whole alkaloid group consisting presently of a total of six alkaloids which possess the raumacline skeleton [2–4]. In addition, a novel sarpagan alkaloid, provisionally named alkaloid G (**4**), as well as the well known *Rauwolfia* alkaloid suaveoline (**6**) could be identified from the ajmaline-treated cells [4]. For the entire group of raumaclines, chemical syntheses have been recently developed which made all of these alkaloids available for further research [4, and referencees therein]. Raumacline (**1**) itself could be synthesized on the gram scale by a 'one-pot' reaction [5] based on a flavine-mediated, photooxidation of dihydrochanoajmaline (**5**), available in high yield by borohydride reduction of ajmaline (**3**) [6]. Because the flavine-light induced formation of raumacline (**1**) could not been shown to be enzyme dependent, the exact *in vivo* mechanism of the transformation of **3** remained unclarified. We have, therefore, searched the cultivated *Rauwolfia* cells for appropriate enzymes involved in the formation of **1** for a final delineation of the biosynthesis leading to the raumacline skeleton (Fig. 1).

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Dedicated to Professor Horst Rimpler on the occasion of his 60th birthday.

RESULTS AND DISCUSSION

In vivo *formation of raumacline* (1)

Comparison of the amounts and the structural features of the raumaclines formed after administration of ajmaline (**3**) to cell suspensions of *Rauwolfia* suggests, that the substituted raumaclines, like $N\beta$-methylraumacline (**2**), its 19-hydroxy-derivative (**7**), 6α-hydroxy- (**8**) or 6α-methoxyraumacline (**9**) are generated from raumacline (**1**). The latter alkaloid is the major alkaloidal consitituent of the ajmaline-treated cells and might serve as a precursor of the above derivatives.

When the kinetics of the alkaloid formation during a feeding experiment with ajmaline (**3**) were measured, it became clear, that raumacline (**1**) was the first alkaloid to be synthesized. After six days of ajmaline-feeding, compound **1** showed clearly an optimum of accumulation (Fig. 2). At this time, only a small increase of $N\beta$-methylated raumacline (**2**) occurred. This situation changed between the 8th and 14th day, a time-period where a significant decrease in the raumacline (**1**) content on one hand and a comparable increase of **2** on the other hand could be determined. Moreover, the amount of the hydroxylated raumaclines, **7** and **8**, also increased at the end of the experiment, so that these *in vivo* data would indeed point to an initial conversion of ajmaline (**3**) into raumacline (**1**) which in turn is transformed by later reactions into its derivatives. In fact, one of these late reactions

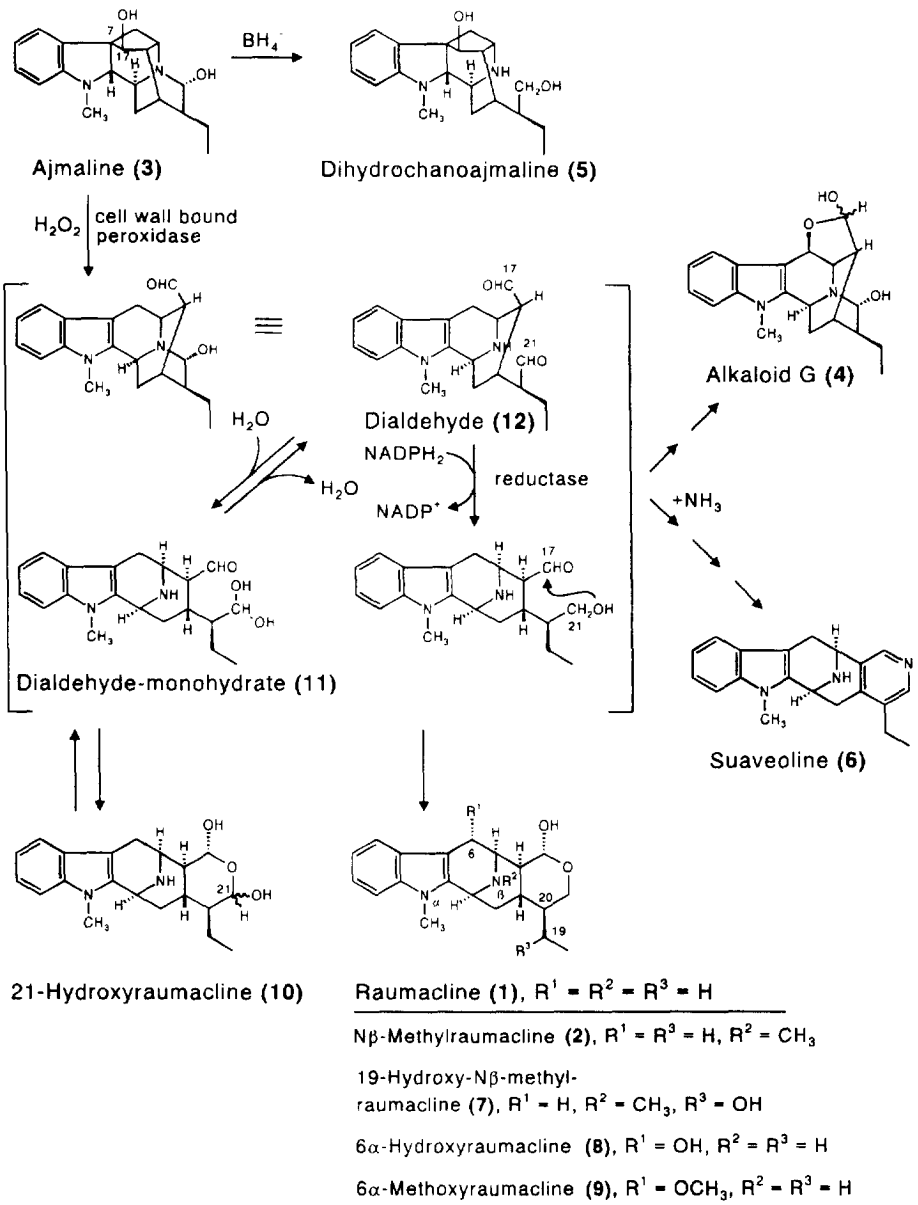


Fig. 1. Biosynthesis of raumacline (**1**) from ajmaline (**3**) in cell suspension cultures of *Rauwolfia serpentina*, catalysed by a cell wall-bound peroxidase and a soluble reductase.

could be proved *in vivo* by the feeding of [^{13}C-*Nβ*-methyl]dihydrochanoajmaline to the *Rauwolfia* cell suspension indicating a conversion of the fed precursor into 15% *Nβ*-methylraumacline (**2**) and 6% 19-hydroxy-*Nβ*-methylraumacline (**7**). This conversion clearly proved that dihydrochanoajmaline (**5**) can serve *in vivo* as a biogenetic progenitor of the raumacline skeleton and that the *Nβ*-methyl group is metabolically rather inert. The transformation of only 1% of the applied precursor **5** into raumacline (**1**) suggests that *Nβ*-demethylation is not an important reaction for the biosynthesis of **1**. This observation is in line with the above mentioned experiment in which the *Nβ*-methylated alkaloids appear late after feeding of ajmaline (**3**) and after the formation of raumacline (**1**). In the here discussed *Rauwolfia* system, a demethylation or migration of the methyl group as described for some aporphine and protoberberine alkaloids [7] or like the preferred demethylation found for nicotine in *Nicotiana* cells [8], does not take place. But, in contrast, the late hydroxylation at C-19 which leads to 19-hydroxy-*Nβ*-methylraumacline (**7**) is observed.

From a biosynthetic point of view, the formation of the carbon-skeleton of **1** would, however, be much more interesting to investigate than later reactions in the biosynthesis, like *Nβ*-methylation or hydroxylations. Raumacline biosynthesis must include oxidative ring-

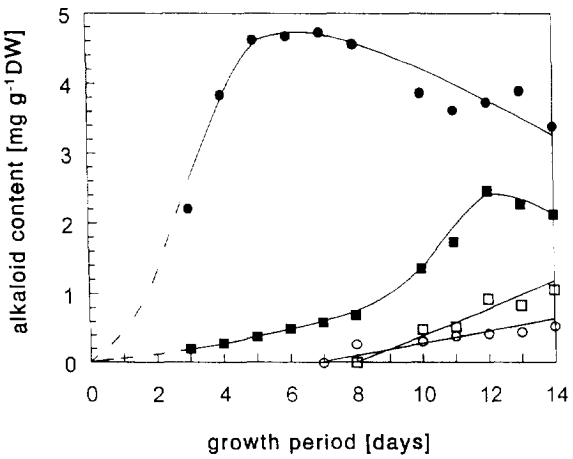


Fig. 2. Kinetics of alkaloid formation in cell suspension culture of *Rauwolfia serpentina*: 1 g ajmaline (**3**) l^{-1} AP-medium was added before autoclaving the medium. ●, Raumacline (**1**); ■, *Nβ*-methylraumacline (**2**); □, 19-hydroxy-*Nβ*-methylraumacline (**7**); ○, 6α-hydroxyraumacline (**8**).

opening of the ajmalan to the sarpagan skeleton. It was therefore advisable to search for those enzymes which participate in this oxidative reaction. The isolation and characterization of these enzymes would allow the clarification of the steps involved in the cell-free formation of the raumaclines. In order to apply a relatively fast and sensitive enzyme assay, HPLC separation of raumacline (**1**) from ajmaline (**3**) and dihydrochanoajmaline (**5**) was used to screen for enzymes metabolizing **3** or **5**. When these putative substrates were incubated under various conditions with a range of cofactors at different pH, and in the presence of crude soluble enzyme preparations from *Rauwolfia* cells, the cell-free formation of **1** could neither be measured under oxidative nor under reductive conditions. This observation excluded participation of soluble proteins in the first biosynthetic step leading to raumacline (**1**).

Since plant oxidases are frequently attached to cell walls, for example, phenol oxidases [9] or peroxidases [9–11], in a second series of experiments cell wall preparations were used. When **3** or **5** was incubated with such preparations in the presence of H_2O_2, both alkaloids were converted. Whereas **3** yielded an unknown product, **5** was enzyme-dependently transformed into raumacline (**1**), which favoured the biogenetic sequence **3** → **5** → **1** (Fig. 1). Unfortunately, all our attempts failed to verify the cell-free conversion of **3** into **5** which would be a simple reduction of ajmaline (**3**). Although this reaction can be easily performed chemically by BH_4^--reduction [6], we were not able to obtain any evidence for the appropriate enzymatic reduction in *Rauwolfia* cells. Obviously, the step **3** → **5** is not expressed in *Rauwolfia* and, therefore, the above proposed sequence starting first with a reduction followed by peroxidase-catalysed oxidation is not the one responsible for the formation of raumacline (**1**). The fact that dihydrochanoajmaline (**5**) has never been isolated from *Rauwolfia* plants [12] or from its cell suspension cultures [13], would also be in contrast to the above sequence of steps involving the reduction of ajmaline (**3**). However, the reverse sequence starting first with an oxidation of ajmaline (**3**) followed by a reduction would be an alternative route which could also lead to raumacline (**1**).

Structural determination of 21-*hydroxyraumacline* (**10**)

In this context, it was important to identify the unknown alkaloid which was formed from ajmaline (**3**) by the cell wall-bound peroxidase. Because this compound could be the substrate of the second putative enzyme, its structure and its behaviour in the presence of appropriate reducing enzymes would eventually allow direct conclusions to be drawn about raumacline (**1**) biosynthesis. Isolation and identification of the unknown component was possible from an appropriate large incubation experiment containing 280 ml of cell wall preparation from 250 g frozen *Rauwolfia* cells (≈ 11 g dry wt), 100 mg ajmaline (**3**) and H_2O_2. After extraction and purification of the alkaloidal mixture, 10 mg of the pure unknown compound remained, which showed the same UV absorptions as those from raumacline alkaloids (227, 284 and 290 nm). This result indicated the conversion of an indoline (ajmalan-type) into an indole (sarpagan-type) alkaloid, for instance, by oxidative splitting of the C-7/C-17 bond of ajmaline (**3**). In order to prove this suggestion more directly, detailed mass and NMR spectroscopic analyses were necessary. EI-mass spectrometric measurement of the isolated alkaloid revealed a fragment of *m/z* 183 which is characteristic of the raumacline group; the data also indicated an apparent $[M]^+$ at *m/z* 324. The latter, however, was shifted after acetylation of the sample to *m/z* 468. Such a shift would correspond to three acetyl groups and an additional *m/z* 18, which would suggest that the true $[M]^+$ of the non-acetylated sample should be *m/z* 342 instead of *m/z* 324. FD- and FAB-mass spectra indeed confirmed this suggestion and indicated ions at *m/z* 342.2 and *m/z* 343.2 $[M + H]^+$, respectively. Based on the shift of the fragment ion from *m/z* 183 to *m/z* 225 after acetylation, the alkaloid derivative should possess a *Nβ*-acetylated carboline structure and two additional acetylated groups, preferentially OH-groups, which are not part of the involved β-carboline system. A hydroxylated raumacline structure for the unknown alkaloid would at this point fit best to the described data here. ^1H NMR analysis performed in DMSO-d_6 supported such a structure. The overall pattern of signals in the region between δ1 and 3 was very similar to raumacline (**1**), but in the remaining part of the spectrum several signals appeared to be double, indicating a 1 : 1 mixture of the unknown alkaloid. In comparison to raumacline (**1**), the typical C-21α and C-21β protons were missing in the spectrum and suggested, therefore, hydroxylation on C-21. Indeed, in the region of the signal of the 17-OH-group of **1**, two further signals were found, resonating as doublets at δ6.22 and 6.01, respectively. These doublets (J = 6.7 and 4.4 Hz, respectively)

collapsed after D_2O-treatment to a singlet and demonstrated that this new alkaloid, which is formed from ajmaline (**3**) by the cell wall peroxidase, is indeed an epimeric mixture of 21α- and 21β-hydroxyraumacline (**10**). The presented structure and the occurrence of such an anomeric mixture is further substantiated by signal pairs for H-21, C-17 OH- or the C-18 methyl groups, as well as by the complete set of ^{13}C-data.

The double hemiacetal structure of **10** is a very rare structural element of natural products. There are some examples known with a modified double hemiacetal as for instance, the acetylated form or as a double acetal. Six isomers of the sesquiterpene hymenoxon, however, exhibit the same double hemiacetal structure as **10** [14–17].

Formation of the hemiacetal is most probably not enzyme-catalysed but an artificial process leading to the isolated 1 : 1 mixture of the C-21 epimers of **10**. The C-21 hydrate **11** of a C-17,C-21-dialdehyde **12** would be a good candidate for hemiacetal formation (see Fig. 1). In fact, such a dialdehyde structure has been discussed earlier [16] and an appropriate terpenoid dialdehyde could be isolated after saponization of the corresponding diacetate [18]. Our attempts to isolate the free dialdehyde **12** have so far, been unsuccessful.

21-*Hydroxyraumacline* (**10**) *as a precursor of raumacline alkaloids*

The suggested role of 21-hydroxyraumacline (**10**) as a biosynthetic progenitor of raumacline alkaloids could indeed be confirmed under *in vivo* and *in vitro* conditions, respectively. When alkaloid **10** was added *in vivo* to a *Rauwolfia* cell suspension its conversion into Nβ-methyl-raumacline (**2**) could clearly be demonstrated by EI-mass spectrometry. Transformation did not exceed, however, 1% of the starting product. After 4 days of feeding **10** to *Rauwolfia* cells, **10** was still the major alkaloid of the cells pointing to a relatively slow conversion. It remained, therefore, questionable whether **10** is actually the immediate precursor of the raumacline-skeleton or whether it must be transformed first to the direct progenitor of **1**. In order to get a more detailed insight into the nature of the transformation of **10** into **1**, we searched the *Rauwolfia* cells for the appropriate enzyme catalysing this process.

When **10** was incubated in the presence of a 1 : 1 mixture of $NADPH_2$ and $NADH_2$, and a crude enzyme preparation from cell wall fragments, microsomal protein or soluble enzymes ($(NH_4)_2SO_4$-precipitated protein), the latter enzyme preparation only showed formation of raumacline (**1**). This reaction was clearly dependent on $NADPH_2$ and active protein. When heat-denatured enzyme was used or the reducing cofactor was omitted, raumacline (**1**) biosynthesis could not be detected.

The obtained *in vivo* and *in vitro* data now strongly suggest that the biosynthesis of the skeleton of raumacline alkaloids is catalysed first by a cell wall-bound peroxidase followed by a soluble, $NADPH_2$-dependent reductase. It is therefore quite obvious, that during the feeding of ajmaline (**3**) to *Rauwolfia* cells the alkaloid is first transformed at the cell wall into 21-hydroxyraumacline (**10**), which in turn is taken up by the cells and then reduced to raumacline (**1**).

Both enzymes involved are therefore of a special interest and it was necessary to find out whether their properties would support our present knowledge on raumacline (**1**) biosynthesis. Because peroxidases are a broadly distributed class of enzymes [19] occurring often as a mixture of isoenzymes [20, 21] it was interesting to investigate whether the enzyme described herein is specific for the observed process and which properties are characteristic for this peroxidase. When the pH optimum of the conversion was determined for alkaloids **3** and **5**, the pH dependency was remarkable narrow with an optimum at pH 4.9 and pH 4.67, respectively, showing half optima around pH 4.4 and 5.6. These properties, e.g. the substrate-dependent pH optimum, has been frequently observed for plant peroxidases [22, 23]. Although peroxidases also occur as soluble proteins, the *Rauwolfia* enzyme exists almost exclusively in a bound form at the cell wall. Only about 1% of the oxidase activity was associated with microsomal protein and soluble protein did not contain any of the enzyme activity. This finding correlates precisely with our assumption, that ajmaline added to the cell culture is first oxidized at the cell wall. The reason for this process must, however, remain unsolved, although oxidative degradation of the alkaloids **3** and **5** could be a mechanism of protection for the cell. Two further observations would support this idea; firstly, cultivation of *Rauwolfia* cells in the presence of ajmaline (**3**) increases peroxidase activity up to 240% and, secondly, the enzyme has a broad taxonomic distribution, indicating it be involved in non-specific oxidation reactions. When an additional five cell cultures belonging to four different plant families *not* producing indole alkaloids were tested, enzyme activities transforming **3** into 21-hydroxyraumacline (**10**) were even higher than those found in *Rauwolfia* cells (Table 1). This result is in sharp contrast with the observation that all the enzymes of *Rauwolfia* cells which are involved in the biosynthesis of ajmaline (**3**) are highly specific and taxonomically restricted to ajmalan/sarpagan-containing plants or cells [13]. The cell wall-bound peroxidase is, in fact, not a specific enzyme of alkaloid metabolism but belongs to the general class of peroxidases. In this context, it is also important to note that the oxidation of **3** is also catalysed by horseradish peroxidase (data not shown). This result arises the question of whether the biosynthesis of raumacline (**1**) is a process which is specific for *Rauwolfia*. The properties of the second enzyme involved in the raumacline (**1**) formation should be important in answering this particular question.

Properties of the 21-*hydroxyraumacline-reductase*

In contrast to the cell wall-bound peroxidase, the 21-hydroxyraumacline-reductase shows a broad pH dependency with an optimum at pH 8.4. Other reductases from *Rauwolfia* also show broad pH optima but at lower values, for example, the vellosimine-reductase at pH 7.6.

Table 1. Taxonomic distribution of cell wall-bound peroxidase and soluble reductase involved in formation of raumacine (1)

Cell suspenion cultures	Enzyme activity			
	Cell wall-bound peroxidase		Reductase	
	nkat l^{-1} medium	Relative activity (%)	nkat l^{-1} medium	Relative activity (%)
Apocynaceae				
Rauwolfia serpentina (L.) Benth. ex Kurz	21.5	100	15.7	100
R. serpentina (L.) Benth. ex Kurz × *Rhazya stricta* Decaisne	23.8	111	6.7	43
Catharanthus roseus (L.) G. Don	31.6	147	n.d.*	n.d.
Solanaceae				
Nicotiana tabacum L.	39.2	182	n.d.	n.d.
Solanum marginatum L.	28.5	133	n.d.	n.d.
Fabaceae				
Phaseolus mungo L.	62.3	290	n.d.	n.d.
Caprifoliaceae				
Lonicera tatarica L.	28.7	133	n.d.	n.d.
Poaceae				
Agrostis tenuis Sibth.	39.8	185	n.d.	n.d.

* n.d., not detectable; detection limit 5% relative activity.

Table 2. Summary of purification of reductase from 1.3 kg fr. wt of *Rauwolfia serpentina* cells

Purification step	Volume (ml)	Protein (mg)	Total activity (pkat)	Specific activity (pkat mg^{-1})	Yield (%)	Purification (-fold)
Crude extract	2100	1575	630	0.4	100	1
$AmSO_4$ (30–70%)	180	990	495	0.5	79	1.3
DEAE-Sepharose	100	200	400	2	63	5
AcA 54	10	12	180	15	29	38
FPLC-Mono Q	3	0.9	56.7	63	9	158
FPLC-Mono P	1.5	0.11	27.3	248	4.3	620
FPLC-Superdex 75	0.5	0.003	2.25	750	0.4	1875

Reductases from other cell suspension systems show more basic pH optima, for example, the salutaridine: NADPH 7-oxidoreductase from *Papaver somniferum* at pH 9.0–9.5 [24]. The *Rauwolfia* reductase is exclusively located in the soluble protein fraction. Microsomal protein or cell wall preparations did not show any reductase activity. This observation again supports the biogenetic sequence **3** → **10** → **1**, including the oxidation of **3** at the cell wall, uptake of the intermediate **10** and intracellular reduction under formation of raumacline (**1**). It could be proved, that the reduction is a *Rauwolfia*-specific process. Investigation of the taxonomic distribution of the enzyme, as carried out for the above-discussed peroxidase, demonstrated the enzyme is up to now exclusively detectable in cell culture systems of *R. serpentina* and in a previously generated hybrid culture of *R. serpentina* × *Rhazya stricta* (Table 1) which has been characterized recently [25–27]. In this context, it is also noteworthy that in the hairy root cultures of *R. serpentina* and *R. vomitoria* this particular reductase activity could not be detected.

A six-step protocol of enzyme purification was elaborated, including besides $(NH_4)_2SO_4$-precipitation, a combination of chromatography steps like DEAE-Sepharose anion-exchange, AcA 54 gel filtration and FPLC-methods for Mono Q ion-exchange, Mono P chromatofocussing and Superdex 75 gel filtration. Purification of the enzyme resulted in a final enrichment of more than 1800-fold with 0.4% total recovery and 750 pkat mg^{-1} specific activity (Fig. 3, Table 2). The purity of this enzyme preparation was checked by SDS-PAGE (Fig. 4). In this denaturing system, the reductase migrated as a single

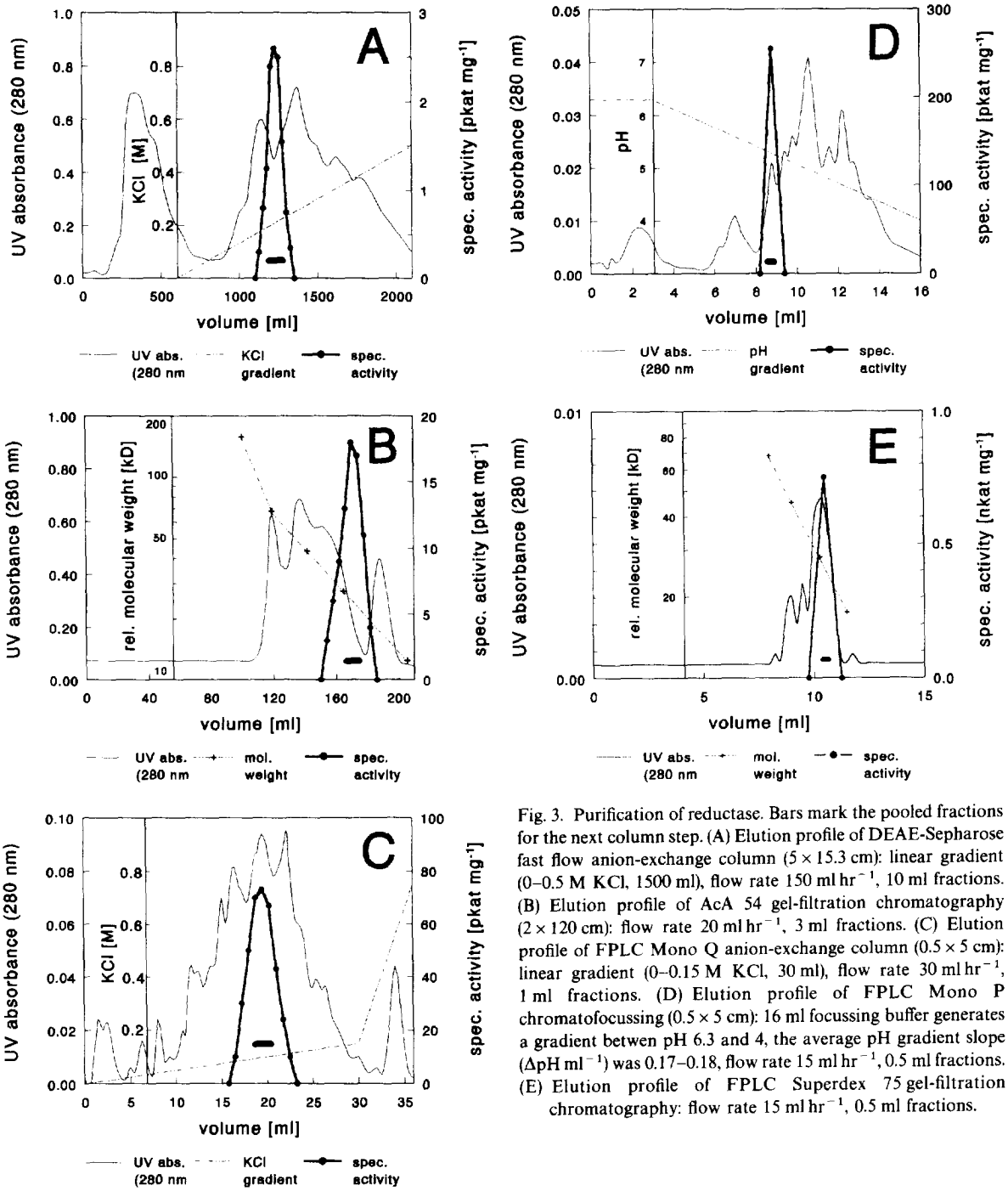


Fig. 3. Purification of reductase. Bars mark the pooled fractions for the next column step. (A) Elution profile of DEAE-Sepharose fast flow anion-exchange column (5 × 15.3 cm): linear gradient (0–0.5 M KCl, 1500 ml), flow rate 150 ml hr^{-1}, 10 ml fractions. (B) Elution profile of AcA 54 gel-filtration chromatography (2 × 120 cm): flow rate 20 ml hr^{-1}, 3 ml fractions. (C) Elution profile of FPLC Mono Q anion-exchange column (0.5 × 5 cm): linear gradient (0–0.15 M KCl, 30 ml), flow rate 30 ml hr^{-1}, 1 ml fractions. (D) Elution profile of FPLC Mono P chromatofocussing (0.5 × 5 cm): 16 ml focussing buffer generates a gradient betwen pH 6.3 and 4, the average pH gradient slope (ΔpH ml^{-1}) was 0.17–0.18, flow rate 15 ml hr^{-1}, 0.5 ml fractions. (E) Elution profile of FPLC Superdex 75 gel-filtration chromatography: flow rate 15 ml hr^{-1}, 0.5 ml fractions.

band at M_r 27000 ± 10% showing only slight protein contamination (Fig. 4). This result indicates that the reductase is a monomeric enzyme. The isoelectric point of the enzyme could be determined by FPLC-chromatofocussing on a Mono P column to be between pH 5.3 and 5.5. This protein is obviously relatively stable. When a 150-fold purified enzyme fraction was stored at −25° for six months the original activity was completely retained.

The reductase accepts exclusively $NADPH_2$ as a cofactor, which is in agreement with other *Rauwolfia* reductases, like the vellosimine-reductase [28]. Besides 21-hydroxyraumacline (**10**), only the indolenine alkaloid perakine (**13**) has been found as substrate for a 620-fold-enriched enzyme preparation. Perakine (**13**) is reduced to raucaffrinoline (**14**) (Fig. 5), exhibiting the same specific activity as determined for the reduction of **10**. The conversion from **13** $([M]^+$ m/z 350) into **14** $([M]^+$ m/z 352) could clearly be demonstrated by EI-mass spectrometry and UV analysis after TLC purification, in comparison with the borohydride reaction product of **13**.

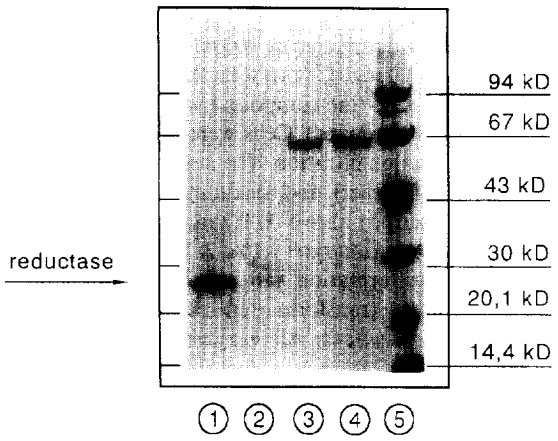
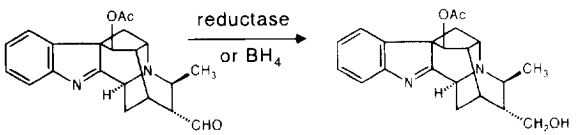


Fig. 4. Purity check of 1800-fold enriched reductase on SDS-PAGE. Lane 1, concentrated Superdex 75 eluate, fraction 17; lane 2, Superdex 75 eluate, fraction 16 (unsuccessful step); lane 3, 0.5 μg; lane 4, 1 μg BSA; lane 5, M_r standards (LMW-kit, Pharmacia).



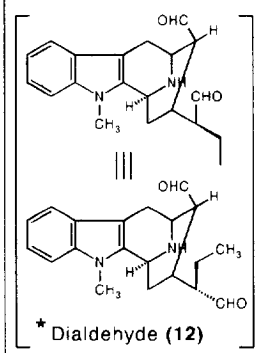
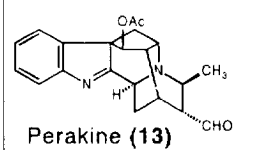
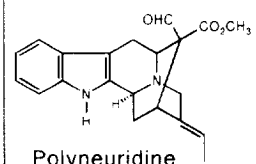
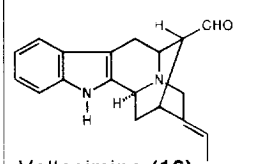
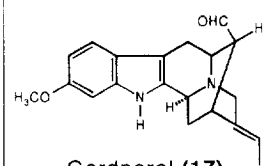
Perakine (**13**) Raucaffrinoline (**14**)

Fig. 5. Transformation of perakine (**13**) into raucaffrinoline (**14**) by reductase or by borohydride.

The UV spectrum showed the same absorptions as known from indolenine alkaloids (λ_{max}: 260, 220 nm). No reaction occurred with sarpagan-type alkaloids containing an aldehyde structure, like polyneuridine aldehyde (**15**), vellosimine (**16**) and gardneral (**17**). In addition, no transformation was observed when the purified enzyme was incubated with the substrates, cinnamic aldehyde (**18**), *p*-anisaldehyde (**19**) or benzaldehyde (**20**), indicating that the isolated reductase is a highly specific enzyme of indole alkaloid biosynthesis and not a common alcohol dehydrogenase-type enzyme. However, it should be noted that a crude enzyme extract from cultivated *Rauwolfia* cells indeed accepts **18**–**20** as substrates and therefore also contains unspecific reductase activities (data not presented). This result is in accordance with other enzyme transformations in *Rauwolfia*, like the ester hydrolysis of polyneuridine aldehyde (**15**) by the PNA-esterase. This substance (**15**) is only transformed by this enzyme and not by 'unspecific', esterases of the primary metabolism catalysing the hydrolysis of nitrophenyl acetate, for instance.

From the obtained results, it would be difficult to decide, whether **10** or **13** is indeed the natural substrate of the enzyme, which means that **10** should also be an endogenous alkaloid of *Rauwolfia*. But so far, there is no

Table 3. Substrate specificity of reductase from *Rauwolfia serpentina* cells under standard assay conditions

Substrate	Enzyme activity: Relative activity (%)	Enzyme activity: pkat mg^{-1}
 * Dialdehyde (**12**)	100	234
 Perakine (**13**)	102	240
 Polyneuridine aldehyde (**15**)	n.d.	n.d.
 Vellosimine (**16**)	n.d.	n.d.
 Gardneral (**17**)	n.d.	n.d.
Cinnamic aldehyde (**18**) p-Anisaldehyde (**19**) Benzaldehyde (**20**)	n.d.	n.d.

*Incubated as double hemiacetal, 21-hydroxyraumacline (**10**).
†n.d., not detectable; detection limit 5% relative activity.

evidence for the natural existence of 21-hydroxyraumacline (**10**) in *Rauwolfia* cell suspensions or other *Rauwolfia* systems, for instance whole plants, hairy roots or callus tissue. Thus would appear that **10** is formed exclusively during ajmaline-feeding. Therefore, perakine (**13**) remains at the present as the only naturally occurring compound which is reduced by the described novel reductase. In fact, structural comparison of the dialdehyde (**12**) with perakine (**13**) shows significant similarities (Table 3), which would explain their role as a substrate. It is now our firm belief, that **13** is indeed the endogenous substrate of the newly discovered enzyme, which we provisionally name perakine-reductase.

In conclusion, the biosynthesis of raumacline (**1**) from ajmaline (**3**) should involve the formation of a dialdehyde (**12**) which might be transformed through its monohydrate (**11**) into the stable 21-hydroxyraumacline mixture (**10**). This double hemiacetal is, *in vivo* and *in vitro*, the progenitor of **1**. Preferentially, **10** is in equilibrium with the monohydrate **11** and the dialdehyde **12** which might be the immediate substrate of the reductase. In addition, it can be expected that alkaloid G (**4**) and suaveoline (**6**) are biosynthesized from the intermediates proposed in Fig. 1. The *in vivo* and *in vitro* system described in this paper, therefore offers for the first time an opportunity to investigate the formation of suaveoline (**6**), an alkaloid isolated several times before from natural sources [29, 30].

EXPERIMENTAL

Cell suspension cultures. Cell suspension cultures of *R. serpentina* (L.) Benth. ex Kurz and other plants were grown at 23° in 1 l Erlenmeyer flasks on gyratory shakers (100 rpm) in the light. Inoculum of cells to 250 ml LS-medium [31] was *ca* 60 g fr. wt. For enzyme assays, cultures were harvested after 4 days by suction filtration, shock-frozen with liquid N_2 and stored at −25° until used. Feeding expts with *R. serpentina* cell cultures were performed in AP-medium [32].

Dynamics of in vivo *formation of raumacline alkaloids.* A cell suspension culture was grown in 1 l AP-medium in the presence of 1 g ajmaline (**3**) for 14 days. The inoculum of cells was 240 g fr. wt. Ajmaline was added before the medium was autoclaved. From the 3rd to 14th day of growth (except for day 9), every day a sample of 2 ml was withdrawn from the flask under sterile conditions. Cells were separated from the medium and extracted with MeOH. The content of **1**, **2**, **7** and **8** was measured by HPLC using ext. standards. HPLC conditions were similar to those described earlier [3].

TLC solvent systems. A: $CHCl_3$–MeOH–NH_3 (25%), 90:10:0.2. B: $CHCl_3$–MeOH–NH_3 (25%), 80:20:0.2. C: EtOAc–MeOH–H_2O–NH_3 (25%), 70:20:10:0.2.

Synthesis and feeding of [^{13}C-Nβ-methyl]dihydrochanoajmaline. Synthesis: 220 mg ajmaline (675 μmol) was stirred with 1 g [^{13}C]-MeI (7 mmol, 99% [^{13}C]) in 10 ml $CHCl_3$ for 5 hr. The crude mixt. was evapd to dryness, dissolved in MeOH and reduced by addition of 300 mg $NaBH_4$ (7.9 mmol). The product (**5**) was purified by MPLC [4] and TLC solvent A. The yield was 158 mg (461 μmol, 68%). Feeding: a suspension culture (26 g inoculum) was cultivated in 100 ml AP-medium in the presence of 100 mg [^{13}C-Nβ-methyl]dihydrochanoajmaline for 14 days (the compound was added before the medium was autoclaved). Cells were separated from the medium by suction filtration. The amount of **1**, **2** and **7** in cells (MeOH extract) and medium was examined by HPLC as described above. **1**: 0.2 mg (cells), 0.9 mg (medium), transformation rate: 1.1%; **2**: 7.6 mg (cells), 7.8 mg (medium), transformation rate: 15.4%; **7**: 2.4 mg (cells), 3.8 mg (medium), transformation rate: 6.2%.

Enzyme preparations. All enzyme prepns were performed at 0–4°.

Preparation of cell wall-bound enzymes. Frozen cells (20 g) were ground with liquid N_2 for 30 sec in a mill (M 20, IKA). To the powdered cells, 10 ml of H_2O was added. After thawing, the homogenous suspension was centrifuged for 5 min at 500 *g*. The sediment was washed ×3 with 4 ml of H_2O and centrifuged. The cell wall fragments were finally resuspended in H_2O to give a vol. of 10 ml.

Crude soluble enzyme preparation. Frozen cells (12 g) were ground as described above. Powdered cells were mixed with 12 ml of 0.1 M K-Pi buffer (pH 8) and 10 mM mercaptoethanol (MSH), and stirred for thawing. After filtration through cheesecloth to removing cell debris, the soln was centrifuged for 30 min at 13 700 *g*. $(NH_4)_2SO_4$ was then added to the supernatant (crude enzyme mixt.) within 30 min to give a final concn of 70%. After another 30 min stirring, the suspension was centrifuged (40 min, 10 000 *g*). The pptd protein was dissolved in 1–2 ml of K-Pi buffer (pH 8, 10 mM MSH) and again centrifuged at 10 000 *g* for 40 min. The supernatant, containing the sol. proteins, was dialysed for 15 hr against 0.5 l of 0.02 M K-Pi buffer (pH 8).

Microsomal enzymes. Frozen cells (100 g) were ground as described above. Powdered cells were added to 100 ml of 0.1 M K-Pi buffer (pH 7.5, 10 mM KCl, 20% sucrose (w/v)) and stirred for 30 min. The mixt. was filtered through cheese cloth and centrifuged for 30 min at 13 700 *g*. To the supernatant, 1 M $MgCl_2$-soln was added dropwise within 30 min to give a final concn of 50 mM $MgCl_2$. After another 30 min stirring, the suspension was centrifuged (1 hr, 39 000 *g*). The pellet was resuspended in 6–7 ml buffer (as above) and the concn of $MgCl_2$ was again adjusted to 50 mM. After 30 min stirring, the mixt. was again centrifuged (1 hr, 39 000 *g*) and the pellet resuspended in 0.1 M K-Pi buffer (pH 7.5).

Protein determination. Protein concentrations in the sol. enzyme prepn and the microsomal fr. were determined by the method of ref. [33] using BSA.

Cell-free preparation of 21-hydroxyraumacline (**10**). Cell wall prepn (280 ml) were obtained from 250 g cells following the above described procedure. Ajmaline (**3**) (100 mg dissolved in 50 ml EtOH), 5 ml H_2O_2 (3%) and 200 ml 0.1 M Na citrate buffer (pH 4.8) were added to the cell wall suspension. The mixt. was incubated on a gyratory shaker (75 rpm) at 30° for 19 hr. Cell wall fragments were separated by centrifugation, the supernatant ad-

justed to pH 9.3 (KOH) and extracted $\times 3$ with CH_2Cl_2. The evapd organic phase yielded 88 mg of a crude alkaloid mixt. This extract was purified by TLC in solvent B. The compound at R_f 0.19 was isolated and repurified by TLC in solvent C (R_f 0.25). The yield of pure alkaloid **10** was 10 mg (10%).

21-Hydroxyraumacline (**10**). $C_{20}H_{26}N_2O_3$. UV λ_{max} (MeOH) nm: 227, 284, 290. EI-MS 70 eV, *m/z* (rel. int.): 324 $[M - H_2O]^+$ (19), 225 (10), 211 (10), 183 (100), 168 (23), 157 (14), 144 (14). FD-MS: 356.2 $[M + CH_2]^+$ (4.6), 342.2 $[M]^+$ (15.8), 324.2 $[M - H_2O]^+$ (100). FAB-MS in glycerol: 357.2 $[M + H + CH_2]^+$ (53), 343.2 $[M + H]^+$ (51), 329.1 (18), 289.1 (15), 183.0 (34), 176.0 (41), 154.0 (75), 137.0 (100), 136.0 (95). The alkaloid was obtained as a 1:1 mixt. of the 21-hydroxy-epimers. In cases where ^{1}H- and ^{13}C-NMR signals of 21α-hydroxyraumacline differ from those of 21β-hydroxyraumacline, data of 21α-hydroxyraumacline are marked by an apostrophe. ^{1}H NMR (400 MHz, DMSO-d_6): δ7.42 (1H, *d*, $J_{9,10} = 7.7$ Hz, H-9), 7.39 (1H, *d*, $J_{12,11} = 8.0$ Hz, H-12), 7.10 (1H, *dd*, $J_{11,10} = 7.1$ Hz, $J_{11,12} = 8.0$ Hz, H-11), 7.00 (1H, *dd*, $J_{10,9} = 7.7$ Hz, $J_{10,11} = 7.1$ Hz, H-10), 6.46 (1H, *d*, $J_{OH17',17} = 7.0$ Hz, OH-17'), 6.22 (1H, *d*, $J_{OH21',21\beta'} = 6.7$ Hz, OH-21'), 6.17 (1H, *d*, $J_{OH17,17} = 7.5$ Hz, OH-17), 6.01 (1H, *d*, $J_{OH21,21\alpha} = 4.4$ Hz, OH-21), 4.99 (1H, *dd*, $J_{21\alpha,OH21} = 4.4$ Hz, $J_{21\alpha,20} = 3.1$ Hz, H-21α), 4.94 (1H, *dd*, $J_{17,OH17} = 7.5$ Hz, $J_{17,16} = 8.4$ Hz, H-17), 4.51 (1H, *dd*, $J_{17',OH17'} = 7.0$ Hz, $J_{17',16} = 8.1$ Hz, H-17'), 4.43 (1H, *dd*, H-3), 4.31 (1H, *dd*, $J_{21\beta',OH21'} = 6.7$ Hz, $J_{21\beta',20'} = 8.5$ Hz, H-21β'), 3.64 (3H, *s*, Nα-Me), 3.64 (1H, H-5, hidden by Nα-Me-peak), 2.85 (1H, *dd*, $J_{6\alpha,6\beta} = 16.5$ Hz, $J_{6\alpha,5} = 6.9$ Hz, H-6α), 2.63 (1H, *d*, $J_{6\beta,6\alpha} = 16.5$ Hz, H-6β), 2.62 (1H, *d*, $J_{6\beta',6\alpha'} = 16.5$ Hz, H-6β'), 1.82 (1H, *ddd*, $J_{14\beta,14\alpha} = 12.7$ Hz, H-14β), 1.57 (1H, *ddd*, $J_{16,17} = 8.4$ Hz, $J_{16,15} \approx 13.5$ Hz, H-16), 1.45 (1H, *ddd*, $J_{14\alpha,14\beta} = 12.7$ Hz, H-14α), 1.30 (1H, *m*, H-19), 1.30 (2H, *m*, H-19'), 1.16 (1H, *m*, $J_{15,16} \approx 13.5$ Hz, H-15), 1.16 (1H, *m*, $J_{20,21\alpha} = 3.1$ Hz, H-20), 0.95 (1H, *m*, $J_{20',21\beta'} = 8.5$ Hz, H-20'), 0.83 (1H, *m*, H-19), 0.76 (3H, *t*, $J_{18,19} = 6.8$ Hz, H-18), 0.51 (3H, Hz, $J_{18',19'} = 7.5$ Hz, H-18'). ^{13}C NMR (100 MHz, DMSO-d_6): δ136.3 (C-13), 126.1 (C-8), 120.4 (C-11), 118.5 (C-10), 118.4 (C-10'), 117.6 (C-9'), 117.5 (C-9), 109.3 (C-12), 106.9 (C-7), 106.6 (C-7'), 95.0 (C-21'), 93.8 (C-17'), 91.9 (C-21), 89.7 (C-17), 50.0 (C-16), 49.6 (C-16'), 47.0 (C-20'), 46.2 (C-20), 45.8 (C-5'), 45.6 (C-5), 45.1 (C-3), 32.6 (C-14), 28.8 (Nα-Me), 28.4 (C-15), 21.2 (C-6), 20.1 (C-19), 11.2 (C-18), 9.8 (C-18').

In vivo *feeding of 21-hydroxyraumacline* (**10**). Cells (6 g) were cultivated in 25 ml AP-medium in the presence of 2.5 mg 21-hydroxyraumacline (**10**) for 5 days. **10** was dissolved in 1 ml EtOH and added by sterile filtration after the medium was autoclaved. Cells were harvested and alkaloids extracted by acidic/alkaline fractionation. *N*β-methylraumacline (**2**) was purified by 2D-TLC in solvents B (R_f 0.49) and C (R_f 0.45). EI-MS 70 eV, *m/z* (rel. int.): 340 $[M]^+$ (28), 198 (17), 197 (100), 196 (22), 182 (25), 181 (12), 167 (13), 129 (16), 111 (18).

Cell-free transformation of 21-hydroxyraumacline (**10**) *into raumacline* (**1**). From 30 g cells, 75 mg of sol. protein was prepd as described above. To this soln, 12 mg of **10** (dissolved in 5 ml EtOH), 84 mg $NADPH_2$ and 0.1 M K-Pi buffer (pH 7.5) were added to a final vol. of 100 ml. The soln was incubated for 10 hr at 37°. During this time, a further 62 mg $NADPH_2$ was added portion-wise. The protein soln was adjusted to pH 9.2 (KOH) and extracted with CH_2Cl_2. The enzymatic product was purified by TLC in solvent B. The compound at R_f 0.29 was isolated and yielded 3 mg of pure alkaloid **1** (yield 25%). MS and ^{1}H NMR data were in accordance with those published for raumacline [1].

Characteristics of cell wall-bound peroxidase. The standard enzyme assay for cell wall-bound peroxidase contained 250 µl cell wall prepn, 300 nmol (0.6 mM) ajmaline and 440 nmol of H_2O_2 (0.88 mM) in 0.1 M sodium citrate buffer (pH 4.8); the total vol. was 500 µl. Assays were incubated for 1 hr at 30° with shaking (1000 min^{-1}). Incubation was terminated by the addition of 500 µl MeOH, followed by centrifugation. The supernatant was submitted to HPLC analysis [5]. R_t values for **3**, **10**, **5**, and **1** were 4 min 54 sec, 6 min, 5 min 18 sec, 7 min 36 sec, respectively. Quantitation was performed with an ext. standard. The pH optimum was determined using the standard assay and 0.1 M Na citrate buffers in the range pH 4.1 and 5.75. Incubation time was 2 hr. A max. activity of 1.29 nkat g^{-1} dry wt was determined at pH 4.9 (substrate was **3**), half max. activity at pH 4.3 and 5.6, respectively. The pH-optimum measured for **5** was 4.67. Cellular localization was determined by the standard assay, using alternatively cell wall prepn, sol. enzyme or microsomes as enzyme sources. In assays containing sol. protein or microsomes instead of cell wall fragments, protein concn was 1 mg ml^{-1}. Peroxidase activity in assays containing cell wall fragments was 23.8 nkat l^{-1} medium. In assays with sol. protein no peroxidase activity could be detected. In assays with microsomes, peroxidase activity was 0.3 nkat l^{-1} medium, corresponding to 1.3% relative activity referring to the activity of the cell wall prepn. The taxonomic distribution of peroxidase was examined using cell wall prepns of seven additional plant cell suspension cultures in the standard assay. Cell cultures were grown under identical conditions in LS-medium [31].

21-Hydroxyraumacline-reductase. The standard enzyme assay contained 0.5–1 pkat enzyme activity, 30 nmol 21-hydroxyraumacline (**10**) and 60 nmol $NADPH_2$ in 0.1 M K-Pi buffer (pH 8.4). The total vol. of the assay was 100 µl. Assays were incubated for 2 hr at 35° under shaking (1000 min^{-1}). Protein was pptd by addition of 200 µl MeOH and removed by centrifugation. The supernatant was submitted to HPLC analysis as described under cell wall bound peroxidase, using ext. standards.

Substrate specificity of reductase. HPLC and TLC assay. Alkaloids **13**, **15**, **16**, **17** were tested with the standard enzyme assay containing 2 pkat 620-fold purified enzyme and 30 nmol of tested alkaloid instead of **10**. After a prolonged reaction time (4 hr), the assay was followed by HPLC analysis [3]. In comparison with R_t values of the corresponding $NaBH_4$-reduced alkaloid

sample, only the enzymatic reduction of **13** into **14** could be detected. In this case, the enzyme assay was scaled up by 20-fold. After 240 min reaction time, the protein soln was adjusted to pH 9.2 (KOH) and extracted $\times 2$ with CH_2Cl_2. This extract was purified by TLC in solvent B. The compound at R_f 0.41 was isolated and submitted to UV and EI-MS analysis. For measuring the activity of **18**, **19** and **20**, an optical test was applied. For this purpose, 250 nmol $NADPH_2$, 150 nmol substrate and 5 pkat 620-fold purified enzyme were incubated in 0.1 M K-Pi buffer (pH 8.4) in a total vol. of 1 ml. The reaction was started by addition of enzyme and monitored for 4 hr by the decrease in A at 340 nm. Assays were conducted at 30°. Employing this assay, the transformation rates of **10** and **13** into **1** and **14**, respectively, were also determined.

Purification of reductase. Crude enzyme extracts were prepd by adding 1.3 l buffer A (0.1 M Tris–HCl pH 7.5, 20 mM MSH, 1 mM EDTA) to 1300 g of frozen cells, grown for 7 days as a cell suspension in LS-medium [31]. The mixt. was thawed at 35° until it was just ice-free, then homogenized for 2 min with an Ultraturrax-mixer, pressed through cheesecloth and centrifuged at 10 000 *g*. The supernatant was subjected to $(NH_4)_2SO_4$ ppt (30–75%), the ppt taken up in 150 ml buffer B (20 mM Tris–HCl, 10 mM MSH, pH 8) and desalted by dialysis overnight against 10 l of buffer B. This soln was applied to a DEAE-Sepharose fast flow column (5×15.3 cm) which was equilibrated with buffer B. The column was washed with 300 ml buffer B and elution of enzyme was achieved with a linear KCl-gradient from 0–50% buffer C (20 mM Tris–HCl, 10 mM MSH, 1 M KCl, pH 8) at 150 ml hr^{-1}. Enzyme activity was found at 0.2 M KCl. Major active frs were pooled and concd to 5 ml by ultrafiltration. This soln was added to an AcA 54 column (2×120 cm) which was equilibrated with buffer D (20 mM Tris–HCl pH 8, 10 mM MSH, 0.1 M KCl). The column was operated at a flow rate of 20 ml hr^{-1}. The main enzyme activity eluted after a total vol. of 170 ml. The frs were pooled and concd to 1 ml and desalted by repeated ultrafiltration against buffer B (pH 7.5). The desalted enzyme was applied to a Mono Q column (0.5×5 cm) in FPLC-mode equilibrated with buffer B (pH 7.5). Elution of enzyme was achieved with a flat KCl-gradient from 0–15% buffer C (pH 7.5) at 30 ml hr^{-1}. Enzyme activity was found at 0.1 M KCl. Major active frs were pooled and concd to 0.5 ml and desalted by repeated ultrafiltration with buffer E (25 mM bis-tris-iminodiacetic acid, 10 mM MSH, pH 6.3). This protein soln was pumped (0.25 ml min^{-1}) onto a Mono P column (0.5×5 cm) in FPLC-mode, pre-equilibrated with buffer E. Enzyme was eluted with 100% elution buffer, consisting of 10% Polybuffer 74 (Pharmacia) adjusted with HCl to pH 4 and 10 mM MSH, at 15 ml hr^{-1}. Activity eluted after a total vol. of 9 ml, correlating with an isoelectric point of pH 5.3–5.5. For the final purification, active frs were concd to 25 μl and applied to a Superdex 75 column (1×30 cm) in FPLC-mode, equilibrated in buffer D. The column was operated at a flow rate of 15 ml hr^{-1}. Enzyme activity eluted after a total vol. of 10.5 ml, which correlates to a M_r of 27 000 $\pm$ 10%. The fr. containing the highest enzyme activity was collected (0.5 ml, 3 μg protein). Purity of enzyme was substantiated by SDS-PAGE according to ref. [34]. Final CC yielded a nearly homogeneous enzyme from this particular fr.

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