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ENZYMATIC FORMATION OF INTERMEDIATES IN THE BIOSYNTHESIS OF AJMALICINE: STRICTOSIDINE AND CATHENAMINE

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Key Word Index—*Catharanthus roseus*; Apocynaceae; cell suspension culture; cell-free synthesis; strictosidine; cathenamine; enzymes of biosynthesis; indole alkaloids.

Abstract—Pre-purified enzymes isolated from *Catharanthus roseus* suspension cultures synthesize strictosidine and cathenamine from tryptamine and secologanin. Whereas strictosidine showed metabolic activity, cathenamine accumulates during the cell-free incubations in the absence of reduced pyridine nucleotides. In the presence of δ-D-gluconolactone (0.1 M), strictosidine accumulates in a yield of *ca* 50%. Optimum conditions for its accumulation in crude extracts were found to be at pH 4.1, 0.25 mM tryptamine and 1.25 mM secologinin. Strictosidine synthase is stable for more than 1.5 months at 4°. The optimum conditions for the enzymatic synthesis of cathenamine are 1.54 mM tryptamine and 7.7 mM secologanin at pH 7.5. In the presence of NH_4^+, the formation of the latter alkaloid decreases due to the synthesis of unidentified compounds.

INTRODUCTION

Formerly, pathways of indole alkaloid formation in higher plants were elucidated *in vivo* only by tracer methods. However, the rapid development of cell suspension culture techniques in recent years has provided the basis for the reinvestigation of alkaloid biosynthesis at the cell-free level. Thus, in combination with the radioimmunoassay technique [1], it was possible to establish cell suspension cultures of *Catharanthus roseus* (syn. *Vinca rosea*) which were capable of synthesizing substantial amounts of the *Corynanthe* alkaloids, ajmalicine **10** and serpentine [2]. Using cell strains selected for a high content of ajmalicine **10** as an enzyme source, the precursor role of tryptamine **1** and secologanin **2** in the biosynthesis of the former and related alkaloids was confirmed *in vitro* [3]. Similar results were obtained for the formation of **10** using callus tissue or seedlings of *C. roseus* [4].

Recently, the main steps in the cell-free formation of ajmalicine **10**, 19-epi-ajmalicine **11** and tetrahydroalstonine **12** were suggested (Scheme 1). The first intermediate in the pathway is the glucoalkaloid strictosidine **3** (isovincoside) formed by the condensation of the biosynthetic precursors tryptamine **1** and secologanin **2** [5–7]. This reaction is catalysed by the enzyme strictosidine synthase, which specifically controls the C-ring closure mechanism. By the action of a second enzyme, a glucosidase, strictosidine is converted via highly reactive intermediates **4–6** to 4,21-dehydrocorynantheine aldehyde **7** (D-ring closure) [8]. This aldehyde in turn cyclizes (E-ring formation), probably after isomerization to **8**, to the next stable intermediate cathenamine **9** (20,21-didehydroajmalicine) [9]. Finally, the NADPH-dependent cathenamine reductase catalyses the synthesis of the *Corynanthe*-type alkaloids **10–12**.

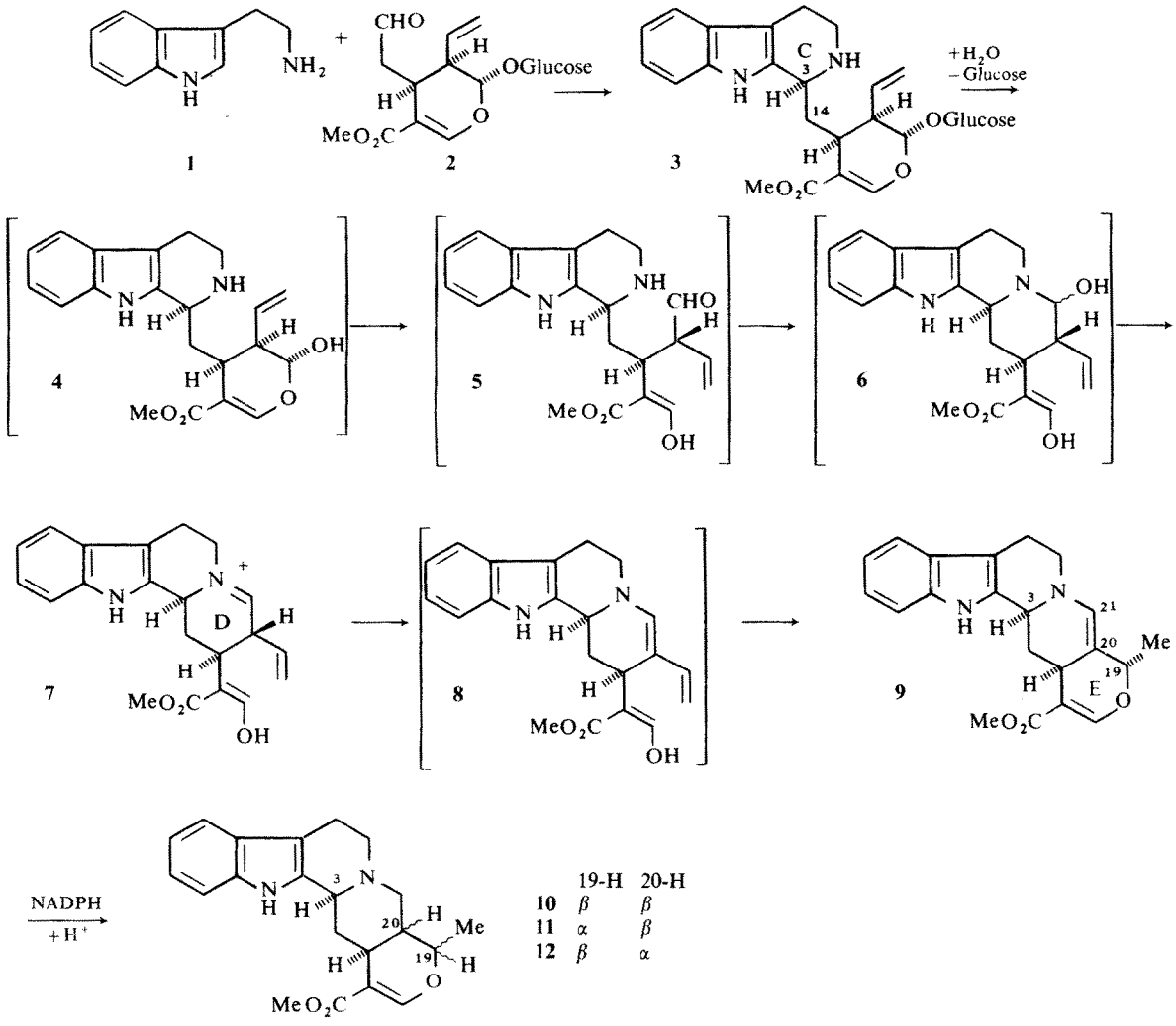
In view of the central intermediacy of strictosidine **3** [6, 10] and cathenamine **9** in this biosynthesis as well as in the chemical synthesis of heteroyohimbine alkaloids [11, 12], I wish to report the optimization of the enzymic synthesis and the identification and structure elucidation of both compounds.

RESULTS AND DISCUSSION

Formation and identification of strictosidine 3

The first indication of an enzyme which catalyses the coupling between tryptamine **1** and the monoterpenoid secologanin **2** with the resulting formation of the first stable intermediate, an alkaloidal glucoside, could be observed by time course investigations using a cell-free extract of *C. roseus* cell suspension cultures capable of synthesizing ajmalicine **10**.

In order to accumulate material for identification purposes, the overall reaction leading to the *Corynanthe*-type alkaloids from the substrates **1** and **2** was blocked by omitting the reduced pyridine nucleotides from the enzyme incubation, which are necessary for the final step of the alkaloid synthesis [3]. Analysis of the incubation mixture (standard conditions; see Experimental) after different reaction times (Fig. 1) showed during the first 20 min of a 60 min incubation the accumulation of a very polar fraction which remains at the origin on Si gel TLC plates developed with Me_2CO–petrol–diethylamine. This polar compound(s) reached a maximum accumulation at this incubation time (yield 23%), whereas the concentration of the starting material (tryptamine-[2-^{14}C]) rapidly decreased during this time. Forty min later the amount of this unknown product(s) decreased to only 3%. This indicates a rapid metabolism



Scheme 1. Cell-free biosynthesis of ajmalicine **10**, 19-epi-ajmalicine **11** and tetrahydroalstonine **12** [8].

and is indicative of its intermediacy in the biosynthetic pathway to ajmalicine **10** and its isomers **11**, **12**.* However, the low yield of this fraction prevented its isolation and complete structure elucidation. The system was therefore further optimized.

Since the synthesis of the alkaloids **10**–**12** was strongly sensitive to δ-D-gluconolactone, an inhibitor of plant glucosidases [13], an enzyme with glucosidase activity was expected to be involved in the overall reaction leading to **10**–**12** [14]. Therefore the glucoalkaloid should accumulate if the glucosidase is inhibited. We extended these experiments for the quantification of the polar fraction and found an increase in formation of more than 50% at 0.1 M inhibitor concentration (Fig. 2). This accumulation would allow an enzymatic synthesis in substantial amounts. The large inhibitor concentration, however, might interfere with the purification of the glucoalkaloid, so that other means were investigated to optimize this cell-free synthesis.

Since a series of enzymes should be involved in the biosynthetic pathway and one could expect different pH optima for each step, the pH dependency of the condensation reaction was studied. Using citrate buffer at pH 4.1, most of the cell-free incubations showed at least a 60–80% conversion of tryptamine **1** to the polar compound(s). At a pH lower than 4.0, the concentration of product decreased rapidly due to denaturation of the protein. At pH values between 4.0 and 6.0 (citrate buffer), and 6.0 and 7.5 (K-Pi buffer), 50 and 30% respectively of product accumulated which indicates a wide pH tolerance of the enzyme mixture and a further metabolism of the product at higher pH values.

Further optimization of the glucoalkaloid production as a function of the secologanin **2** concentration (pH 4.1) showed higher rates of formation (*ca* 85%) at 0.25 mM of **2** (substrate ratio 1:2 = 1:5).† Under these optimal conditions (pH 4.1, 1.25 mM secologanin **2**, 0.25 mM tryptamine **1**), the end point of the reaction was usually achieved after 90 min of incubation. Since no substantial

* The formation and disappearance of the polar fraction was absolutely dependent on the presence of a functional enzyme indicating that a chemical condensation of **1** and **2** does not occur under these conditions.

† Similar results were obtained for the cell-free ajmalicine **10** synthesis at pH 7.0 [14].

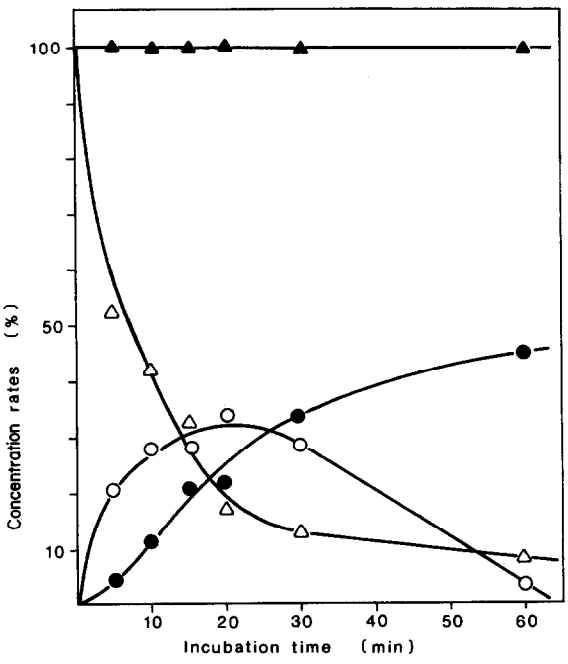


Fig. 1. Time course of the formation of the polar fraction (○), cathenamine **9** (●) and the conversion of tryptamine-[2-^{14}C] (△), either no or heat denatured enzyme (▲), by a cell-free extract of *Catharanthus roseus* suspension cultures under standard conditions (final volume of 0.5 ml contained: 50 μmol K-Pi, pH 7.6, 125 nmol tryptamine-[2-^{14}C] (0.2 μCi), 625 nmol secologanin and 1.0 mg protein, 29°).

decrease of the enzyme activity was observed after storage at 4° in 0.1 M borate buffer (pH 7.6, 20 mM β-mercaptoethanol) for 1.5 months, this enzyme provides the means for a simple, high yielding synthesis of the condensation product.

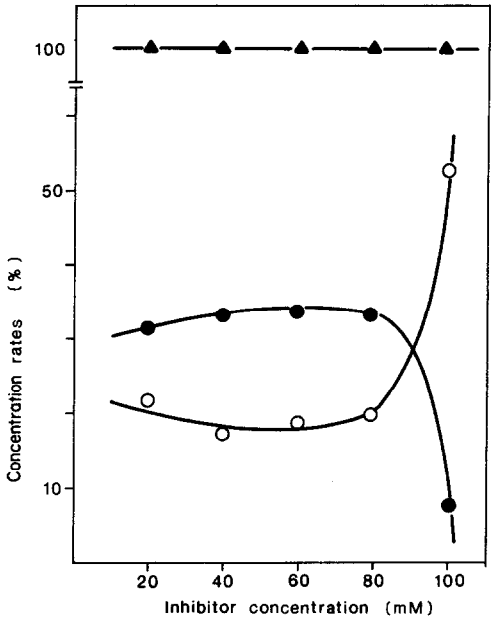


Fig. 2. Enzymatic formation of the polar fraction (○) and cathenamine **9** (●) as a function of the glucosidase inhibitor δ-D-gluconolactone. Standard conditions, incubation time 90 min, (▲) control with heat denatured protein.

For the identification of the condensation product, its chromatographic behaviour was tested by TLC (Si gel). Scanning for radioactivity showed only one main compound (besides the starting material) which migrated together with a mixture of the unlabelled 3α- and 3β-glucoalkaloids, strictosidine **3** and vincoside, in a neutral solvent system I (R_f 0.35). The same R_f value (0.53) as strictosidine **3** was observed in a basic system III, which quantitatively converts the more unstable vincoside to its lactam (R_f 0.44) and therefore allows a simple separation for product identification. After isolation and saponification (Na_2CO_3) of the unknown compound, TLC showed identity with strictosidine lactam (I, 0.46). This indicated that the enzymatically synthesized product could be the 3α-alkaloidal glucoside, strictosidine **3**. Secondly, acetylation of the radioactive compound and TLC comparison in four solvents (IV–VII) with the unlabelled pentaacetates of both epimers, strictosidine **3** and vincoside, showed that the radioactivity coincided exclusively with the 3α-derivative **3**. However, in view of the assumed precursor role of vincoside in the ajmalicine **10** biosynthesis [15], more rigorous proof that the enzymatic product is strictosidine **3** was necessary.

Isotope dilution analysis for vincoside and **3** should be sensitive enough to decide whether both or only one epimer is enzymatically formed. Three independent enzyme mixtures were incubated with tryptamine-[2-^{14}C] (standard conditions) and after 60 min diluted with the unlabelled chemically synthesized epimers. Isolation and purification of a series of their corresponding derivatives showed that the specific activities (Table 1) were at least constant by successive conversion to three derivatives of strictosidine **3**, whereas the vincoside derivatives were found to be close to background. The same results were found for the analyses Nos. 2 and 3 and therefore the enzymatic product must be the 3α-glucoalkaloid.

However, direct evidence as to its structure came from spectroscopic analysis. In large scale incubations (74 ml), the compound was formed under optimum conditions in a yield of 65%. After acetylation and purification, 13.7 mg of a pentaacetate were obtained and analysed by physical methods. The UV spectrum was identical to that described for the acetates of vincoside and strictosidine [15].

The IR spectrum showed NH absorption at 3400 cm^{-1}, acetyl group and amide bands at 1755 and 1705 cm^{-1}. The MW and basic structure of the compound were determined by MS showing M^+ at *m/e* 740 the loss of the glucose-$(OAc)_4$ part at *m/e* 331 and the base peak at *m/e* 213 from fragmentation of the C_3-C_{14} bond. Deacetylation of this fragment leads to *m/e* 171 and, together with *m/e* 169, these fragments indicated the expected β-carboline structure. These data are in agreement with the structure of pentaacetyl vincoside or strictosidine. For the determination of the C-3 configuration, the CD curves of authentic pentaacetyl vincoside and strictosidine samples were compared. As shown in Fig. 3, the curves were different. The 3β-epimer (vincoside derivative) showed a positive $\Delta\varepsilon = +10.0$ at 217 nm, at 271 nm and around 290 nm, a negative value of $\Delta\varepsilon = -2.35$ and -2.0 resp. By comparison, the 3α epimer (strictosidine derivative) exhibited at 217 nm a $\Delta\varepsilon = -8.5$, around 275 nm nearly zero and at 290 nm a value of $+0.45$. These differences permit the determination of the C-3 stereochemistry of the enzymatically

Table 1. Identification of enzymatically formed strictosidine 3 by isotope dilution analysis

Expt No.	Vincoside derivatives Specific activity (dpm/μmol)		Strictosidine derivatives Specific activity (dpm/μmol)
1	Pentaacetate	(8)	Pentaacetate (2.57×10^3)
	—		18,19-Dihydro-pentaacetate (2.34×10^3)
	—		18,19-Dihydro-*N*-Acetyl-strictosidine (2.35×10^3)
2	Pentaacetate	(25)	Pentaacetate (2.62×10^3)
	N-Acetylvincoside	(3)	*N*-Acetyl-strictosidine (2.50×10^3)
3	Lactam	(37)	Lactam (7.82×10^3)

formed product. The CD data of the acetylated product were superimposable with those found for strictosidine pentaacetate and furnish definite proof that the cell-free formed glucoalkaloid is strictosidine 3.

In addition, the analysis of a further derivative, obtained by saponification with Na_2CO_3, acetylation and TLC purification of the enzymatic product, showed the corresponding lactam tetraacetate by IR and MS spectra. IR data revealed the presence of NH (3400 cm^{-1}) and CO (1745 and 1655 cm^{-1}). MS peaks at *m/e* 666 (M^+) indicated a tetraacetate of the expected lactam and the base peak at *m/e* 169 corresponded to the β-carboline moiety. However, the final proof that the stereochemistry is 3α came from ^1H NMR analysis. Besides signals at 7.95 ppm (NH), 7.41–7.05 ppm (aromatic H), the four acetyl groups gave 4 singlets, three very close to 2.0 ppm (2.06, 1.98, 1.87 ppm) and a well separated one at 1.23 ppm which was the direct evidence for the 3α configuration [16] of the product. All these data are in full agreement with the structure **3** and partly published values for strictosidine-lactam tetraacetate [17].

Formation and identification of cathenamine **9**

The enzymatic synthesis of ajmalicine **10** and its isomers **11** and **12** is completely dependent on the presence

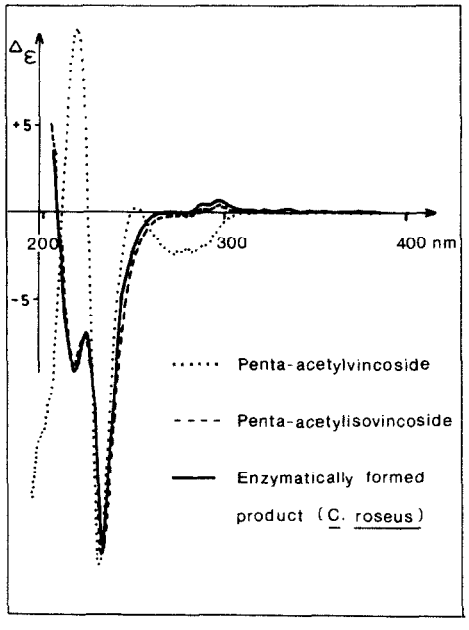


Fig. 3. Identification of enzymatically formed strictosidine 3 as pentaacetate by CD.

of NAD(P)H [3]. Under non-reductive conditions, a new compound accumulated [3]. In order to optimize this reaction, we investigated some of the characteristics of its enzymatic synthesis.

Time course experiments (Fig. 1) showed under standard conditions (see Experimental) an accumulation of this product upto 30% during the first 30 min. However, the compound continued to accumulate after 30 min and the end-point of the reaction was not reached until after 60 min. Further conversion to other compounds, as in the case of strictosidine **3**, was not observed. Therefore reaction times were extended upto 4 hr and it was found that the maximum accumulated between 90 and 120 min. The average formation of the desired product was found to be *ca* 50% (±20%) in 20 experiments. Usually the enzyme mixtures were dialysed for 120 min before use to avoid a large excess of NH_4^+, since these ions can affect the enzymatic synthesis of the desired compound by formation of an artefact (most likely the 18-N analog of **9** [18]). A dialysed enzyme preparation produced about 6% of this byproduct; however, in the presence of an estimated 5% $(NH_4)_2SO_4$ formation of the artefact reached 26%.

For a further optimization of the enzymatic process, the influence of the substrate ratios (**1**:**2**) and the end product concentration were tested. For ajmalicine **10** [14] as well as strictosidine **3** synthesis, the substrate ratio (**1**:**2**) was found to be optimal in the range of 1:5; the same results were obtained for the formation of the new compound. When the tryptamine **1** concentrations were increased, the formation of the product decreased; a substrate ratio of 2:5 showed a 15% decrease and a ratio of 4:5, a 45% decrease in synthesis. Therefore as in ajmalicine and strictosidine syntheses, the 1:5 ratio gave the optimal yields. To test whether the end product concentration has an influence on its own synthesis, from small scale preparations (final volume 10 ml, 10–20 mg protein) the isolated compound was incubated with the enzyme in the presence of **1** and **2** under standard conditions. At a concentration of 0.2 mM an inhibition of its synthesis was not observed. At higher concentrations 0.5–1.0 mM, the product was mostly insoluble but did not show an inhibition effect. Therefore an accumulation of 0.35 mg of the compound per ml incubation mixture should be possible.

An increase of the substrate concentration corresponding to 1.54 mM tryptamine **1** at the optimum substrate ratio resulted in a corresponding increase in product formation. Thus, under these optimal conditions, 55 mg of the unknown compound were synthesized per 200 ml incubation volume in the presence of 400 mg protein. Using these optimal conditions, the product was separa-

ted from the incubation mixtures (200 ml, 400 mg protein) by extraction with ethyl acetate (quantitative) and purified by PLC in solvent system VIII(R_f 0.78). When kept in nitrogen in the dark at −18°, the product was stable for several weeks. This product was identical in TLC behaviour (solvents VIII–XII) to 20,21-didehydroajmalicine, which was isolated from *Guettarda eximia* (Rubiaceae) [18]. However, spectroscopic structure elucidation was done for unambiguous identification. The UV data (λ_{max} 228, 274, 280, 290) indicated a compound with an aryl-unsubstituted indole. The MS showed a molecular ion at *m/e* 350 and peaks at *m/e* 349 ($M^+ - H$), 170, 169 and 156 which were in accord with a β-carboline skeleton. The presence of a methyl ester group was indicated by the loss of CH_3 (*m/e* 335) and the base peak at *m/e* 249 could be explained by a retro-Michael addition followed by a McLafferty rearrangement ($M^+ - C_4H_5O_3$). However, the evidence that the compound under investigation was 20,21-didehydroajmalicine (cathenamine **9**) came from NMR analysis and derivatization. The NMR spectrum revealed signals at 1.42 ppm as a doublet (J = 6 Hz) corresponding to the methyl group at C-19, a quartet at 4.63 ppm J = 6 Hz) for C-19-H by coupling with C-19-CH_3, a methyl ester group at 3.73 ppm and a singlet at 6.18 ppm (C-21-H). Four aromatic H's of the indole moiety were shown between 7.05 and 7.50 ppm, whereas the C-17-H and NH corresponded to signals at 7.55 and 8.02 ppm, respectively. These data, together with those of its reduction product tetrahydroalstonine **12**, showed that the C-3 and C-19-CH_3 stereochemistry was α, and were in full agreement with structure **9**.

EXPERIMENTAL

TLC was carried out on Si gel plates with fluorescence indicator (Polygram Sil G/UV_{254}; Macherey-Nagel), with solvent systems I, $CHCl_3$–MeOH (4:1); II, petrol–Me_2CO–$NHEt_2$ (7:2:1); III, Me_2CO–petrol–$NHEt_2$ (7:1:1); IV, EtOAc–C_6H_6 (2:1); V, EtOAc–*n*-hexane (7.5:5); VI, C_6H_6–Me_2CO–petrol (5:2:3); VII, HCO_2Et–Et_2O–*n*-hexane (5:1:1); VIII, $CHCl_3$–EtOH (90:5); IX, Me_2CO–petrol–CCl_4–*n*-hexane (3.5:2:2:3); X, *n*-hexane–$CHCl_3$–Me_2CO (7:2.5:2); XI, $CHCl_3$–EtOH (30:0.6); XII, $CHCl_3$–EtOH (30:1).

C. roseus cell suspension cultures and enzyme isolation. Cell suspension cultures of high ajmalicine-producing strains were grown in a modified MS medium [2, 19] at 30° in 30 l. airlift fermenters. After an average growth period of 21 days, cells were harvested, frozen with liquid N_2, stored at −18° and used as an enzyme source. The crude enzyme preparations were obtained by crushing the frozen cells (20 g) in a BIO-X press and subsequent treatment of the extract with 50 ml 0.1 M borate buffer pH 7.6 containing 20 mM β-mercaptoethanol (buffer A). After addition of 20 g PVP, the mixture was stirred for 20 min at 0° then filtered through cheese-cloth, the filtrate was centrifuged at 48000 *g* for 15 min. The protein in the supernatant was pptd between 35 and 50% $(NH_4)_2SO_4$ for strictosidine **3** formation, between 35 and 70% $(NH_4)_2SO_4$ for cathenamine production, and redissolved in 4 ml buffer A. After stirring this soln with 0.5 g anion exchange resin Dowex AG 1-X4, 100–200 mesh, borate form (BIO-RAD) for 10 min, the filtrate was freed of low MW contaminants by addition of 5 mg/ml charcoal coated with dextran, centrifuged at 48000 *g* and the supernatant dialysed for 120 min against 3 × 100 vol. of buffer A. This enzyme soln was immediately used for incubation experiments. The average protein content was *ca* 5 mg/ml.

Optimization of product formation of strictosidine **3** *and cathenamine* **9**. *Time course investigations.* The enzyme soln was incubated for 60 min under standard conditions (Fig. 1). The standard assay mixture contained in a total vol. of 0.5 ml: 50 μmol K-Pi buffer pH 7.6 (buffer B), 125 nmol tryptamine-[2-^{14}C] 1 (0.2 μCi), 625 nmol secologanin **2**, 1 mg protein, 29°. After 5, 10, 20, 30 and 60 min, 1/10 of the incubation mixture was spotted on Si gel plates, chromatographed in system II and the zones which contained the labelled starting material (tryptamine-[2-^{14}C]) (R_f 0.45), the polar fraction (R_f 0.0) and cathenamine **9** (R_f 0.60) were counted for radioactivity. Control experiments were carried out with heat denatured enzyme (10 min at 100°).

Influence of δ-D-gluconolactone on the synthesis of the polar fraction and cathenamine **9** (*Fig.* 2). Standard conditions were used as described above, incubation time was 90 min. The cell-free synthesis of the polar fraction and cathenamine **9** was measured at 20, 40, 60, 80 and 100 mM δ-D-gluconolactone (Merck) concns, TLC in II and counting the radioactive zones.

Strictosidine **3** *formation as a function of pH and secologanin concn.* The strictosidine **3** formation at different pH was quantitated in the same manner; standard conditions, incubation time 90 min, solvent system III, buffer B between 6.0 and 7.5 (0.5 intervals) and 0.1 M citrate buffer containing 20 mM β-mercaptoethanol (buffer C) between pH 4.0 and 6.0 (0.5 intervals). At pH 4.1 (buffer C) the influence of the secologanin **2** concn was tested in the same way; solvent system III.

Influence of the $(NH_4)_2SO_4$ *concn on the synthesis of cathenamine.* After incubation of the dialysed enzyme soln in the presence of different $(NH_4)_2SO_4$ concns under standard conditions, the formation of cathenamine **9** was determined as above. TLC II, unknown product R_f 0.33.

Dependency of cathenamine formation on substrate ratio and concn, and on its own concn. Under standard conditions, substrate ratios of (**2**:**1**); (5:1, 5:2, 5:4) were used and the incubation mixtures, after TLC (system II) analysed for radioactivity. In the same manner, the influence of the substrate concns (ratio was 5:1) was tested. After isolation and purification by TLC, **9** was obtained from prep. scale incubations (final vol. 10 ml protein content between 10 and 20 mg) and the influence of its cell-free synthesis tested at different concns in the standard assay.

Identification of enzymatically formed strictosidine **3** *and cathenamine* **9** *by TLC.* Solvent system, R_f for **3**: I, 0.35; III, 0.53. Identification by derivatives: pentaacetate of **3**; standard incubations were evapd at 30° or the purified **3** (system III), were dried over $CaCl_2$ *in vacuo*, acetylated with Ac_2O–Py, 20°, for 12 hr and the reaction mixture analysed [15]. TLC of acetylated **3**: IV, 0.48; V, 0.61; VI, 0.15; VII, 0.48 (in systems V and VI plates were developed twice). The lactamization of **3** (for procedure see isotope dilution analysis) gave one main compound, strictosidine lactam, system I, R_f 0.46. For isolation and identification of cathenamine **9**, incubation mixtures were purified in II (**9** R_f 0.60) or extracted with EtOAc (3 × 1 ml) and rechromatographed in VIII, 0.78 (PLC, Si gel); R_f values: IX, 0.81; X, 0.86; XI, 0.4; XII, 0.59. All R_f values were identical with those of authentic samples by co-chromatography.

Isotope dilution analysis for enzymatically formed vincoside and strictosidine **3**. Three incubation mixtures (3 different enzyme preparations, one tissue batch, standard conditions) were analysed after dilution with a synthesized [15] mixture of vincoside and **3** starting from 150 mg (762 μmol) **1** and 300 mg (773 μmol) **2**. Expt Nos. 1 and 2 contained 0.5 μCi **1**-^{14}C; No. 3 was performed with 1.45 μCi **1**-^{14}C in a 1 ml incubation mixture. No. 1 was acetylated as described above, the epimeric pentaacetates were twice purified by PLC 1.5 mm (Si gel PF_{254},

Merck), eluted with $CHCl_3$–EtOAc–MeOH (1:1:1) and the eluate evapd: 172 mg (232 μmol) vincoside pentaacetate (spec. act. 8 dpm/μmol) and 127.7 mg (172 μmol) pentaacetate of **3** (spec. act. 2.57×10^3 dpm/μmol) as a foam were isolated. 120 mg (162 μmol) pentaacetate of **3** were hydrogenated in MeOH (20 ml) on 40 mg Pd/C (10%, Merck) for 20 min at 20°; filtration and evapn afforded a foam, which was crystallized from MeOH (Uvasol), 55 mg needles 18,19-dihydro-pentaacetate of **3**, mp 117–20°; 120–1° [15], spec. act. 2.37×10^3 dpm/μmol. After recrystallization from MeOH, mp 118–20°, spec. act. 2.34×10^3 dpm/μmol. 24 mg (32 μmol) of this compound were *O*-deacetylated [15] in 2.5 ml dry MeOH with 0.15 ml NaOMe (0.1 N at 20° for 30 min). The reaction mixture was neutralized with Dowex 50 W × 8 (H phase), filtrated and evapd. The residue was purified (TLC, system I, R_f 0.64) and gave 13.3 mg (23 μmol) *N*-acetyl-18,19-dihydro-strictosidine as a foam, spec. act. 2.35×10^3 dpm/μmol. Expt. No. 2 was carried out as No. 1 for acetylation and the pentaacetates were twice purified by PLC: vincoside pentaacetate 92.3 mg (124.7 μmol) with a spec. act. 25 dpm/μmol, pentaacetate of **3** 49.6 mg (67 μmol spec. act. 2.62×10^3 dpm/μmol Both compounds were converted to the corresponding *N*-acetyl compounds as described for the 18,19-dihydro derivative; *N*-acetyl-vincoside 50 mg (87.5 μmol) needles from MeOH showed mp 180–81.5°; 180–181° [15], spec. act. 3 dpm/μmol; *N*-acetyl-strictosidine 34 mg (59.5 μmol) amorphous, spec. act. 2.5×10^3 dpm/μmol. In expt No. 3, the incubation mixture, reference vincoside and **3** were dried and saponified with 5 ml Na_2CO_3 (10%) at 80° for 90 min in N_2. The reaction soln was satd with NaCl, extracted with EtOAc (7 × 10 ml), the organic layer evapd and the residue purified by PLC (system VI, vincoside-lactam 0.36, strictosidine-lactam R_f 0.46); after elution with MeOH and rechromatography, 98 mg (196.5 μmol) vincoside-lactam, pale yellow needles from MeOH, mp 197–99°; 201–2° [15], spec. act. 37 dpm/μmol and 68 mg (136.3 μmol) strictosidine-lactam, amorphous, 7.82×10^3 dpm/μmol, were obtained. All derivatives gave MS spectra as described [15].

Large scale preparation, chemical and spectroscopic identification of strictosidine **3** *and cathenamine* **9**. Strictosidine **3** was formed in an incubation mixture (total vol. 74 ml) of 0.608 mM **1**, 3.04 mM **2**, 82 mg protein, 0.122 mM citrate buffer, pH 4.1 (at 29° for 120 min), and isolated after derivatization for structure elucidation.

Isolation as strictosidine pentaacetate. The incubation mixture was evapd to dryness after an incubation time of 130 min (accumulation of *ca* 65% of **3**), dried for 4 hr *in vacuo* over $CaCl_2$ and acetylated with 14 ml Ac_2O–Py (v/v) for 12 hr with shaking. The reaction mixture was decanted from pptd salts and evapd to dryness, hydrolysed with 1.5 ml H_2O, dried, redissolved in MeOH and purified on PLC (1.5 mm) in EtOAc–C_6H_6 (1.5:1). After rechromatography (TLC, system IV), 13.7 mg (yield 41%) strictosodine pentaacetate were obtained as a glass. UV λ_{max}^{MeOH} nm: 229, 273, 281, 289. IR ν_{max}^{KBr} cm^{-1}: 3400 (NH), 1755 (C=O), 1705 (C=O), 1630, 1430, 1370, 1224. MS *m/e* (rel. int.): 740 (M^+, 3), 697 (21), 409 (5), 393 (5), 331 (4), 214 (46), 213 (100), 171 (55), 169 (55), 127 (15), 115 (10), 109 (40). CD (acetonitrile) λ_{max} ($\Delta\varepsilon$): 217 (−9.0), 233 (−18.4), *ca* 275 (0), 288 (+0.45), 296 (+0.52). CD data for authentic strictosidine-pentaacetate; λ_{max} ($\Delta\varepsilon$): 217 (−8.5), 233 (−18.9), *ca* 275 (0), 288 (+0.4), 296 (+0.65) and vincoside-pentaacetate; λ_{max} ($\Delta\varepsilon$): 217 (+10.0), 231 (−19.8), 271 (−2.35), 281 (−2.3), 289 (−2.0).

Isolation as strictosidine-lactam tetraacetate. The incubation mixture was alkalized with Na_2CO_3 (final concn 10%), heated for 2 hr at 70–80°, filtered and the formed strictosidine-lactam isolated by TLC in system I. Elution with MeOH and evapn gave a slightly yellow residue which was dried over KOH and P_2O_5; 6 mg (yield 27%) strictosidine-lactam. After acetylation (0.25 ml A_2O–Py), 5 hr, 20° and purification by TLC (system IV), 4.2 mg (94%) of strictosidine-lactam tetraacetate were obtained as a glass. UV λ_{max}^{MeOH} nm: 229, 273, 281, 289. IR ν_{max}^{KBr} cm^{-1}: 3400 (NH), 1745 (C=O), 1655 (C=O), 1600 (C=C). MS *m/e* (rel. int.): 666 (M^+, 15), 335 (8), 331 (9), 319 (11), 289 (5), 271 (7), 267 (6), 266 (8), 265 (12), 264 (5), 236 (12), 235 (17), 211 (10), 171 (9), 170 (14), 169 (100), 149 (19), 145 (10), 144 (14), 143 (24), 139 (11), 127 (27), 109 (80). ^1H NMR (90 MHz, $CDCl_3$, TMS = 0): δ 1.23 (3H, *s*, −OCOMe), 1.87 (3H, *s*, −OCOMe), 1.98 (3H, *s*, −OCOMe), 2.06 (3H, *s*, −OCOMe), 2.55–3.10 (4H, *m*), 3.70 (1H, *m*), 4.10–4.25 (2H, *m*), 4.70–5.60 (11H, *m*), 7.05–7.41 (5H, *m*), 7.95 (1H, *s*, >NH).

Cathenamine **9**. **9** was isolated from an enzyme incubation mixture (200 ml total vol.) containing 77.0 mM buffer B, 1.54 mM tryptamine **1**, 7.7 mM secologanin **2** and 400 mg protein (20°, 90 min) by extraction with EtOAc (3 × 350 ml), evapn of the solvent and purification of the residue in VIII. The product (R_f 0.78) was eluted with $CHCl_3$–MeOH (7:3) and the solvent evapd, slightly yellow glass of pure **9** 55.5 mg (158 μmol) in a yield of 51%. UV λ_{max}^{EtOH} nm: 228, 274, 280, 290 (shoulder). MS *m/e* (rel int.) 350 (M^+, 65) 335 (9), 332 (15), 331 (18), 330 (12), 329 (21), 322 (20), 321 (24), 289 (12), 250 (25), 249 (100), 248 (10), 247 (17), 193 (18), 170 (41), 169 (47), 168 (23), 167 (35), 156 (46). ^1H NMR (240 MHz, $CDCl_3$, TMS = O): δ 1.42 (3H, *d*, *J* = 6Hz, C-19-CH_3), 3.73 (3H, *s*, $-CO_2Me$), 4.63 (1H, *q*, *J* = 6Hz, C-19-H), 6.18 (1H, *s*, C-21-H), 7.05–7.5 (4H, *m*, aromatic H), 7.55 (1H, *s*, C-17-H), 8.02 (1H, *s*, N–H).

Reduction of **9** *to tetrahydroalstonine* **12**. 10 mg (28.6 μmol) of **9** were dissolved in 5 ml MeOH and reduced with 25 mg $NaBH_4$ at 20° for 20 min. The reaction mixture was diluted with 25 ml H_2O, extracted with 3 × 50 ml Et_2O and the organic layer evapd. The residue was crystallized from MeOH, 5.5 mg (55% yield) white needles mp 230°; 230–1° [20]. UV, IR, MS and ^1H NMR data were identical with those of an authentic sample.

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