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HOMOGENEOUS STRICTOSIDINE SYNTHASE FROM CELL SUSPENSION CULTURES OF *RAUVOLFIA SERPENTINA*

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Key Word Index—*Rauvolfia serpentina*; Apocynaceae; immobilization; isolation; strictosidine synthase.

Abstract—Strictosidine synthase, the enzyme which catalyses the stereospecific condensation of secologanin and tryptamine to H-3α(*S*)-strictosidine, the key intermediate in monoterpenoid indole alkaloid biosynthesis, was purified to homogeneity from suspension cells of *Rauvolfia serpentina* (920-fold purification, 35% yield). The specific activity is 184 nkat/mg. The isolated enzyme is a single polypeptide, M_r 30000, possessing a 5.3% carbohydrate content. The enzyme has a pH-optimum at 6.5, a temperature optimum at 45°, isoelectric point at pH 4.5, and apparent K_M values both for tryptamine and secologanin of 4 mM. The enzyme was immobilized and has, in this form, a half-life of 100 days at 37°.

INTRODUCTION

Indole alkaloids represent, from a medical view point, one of the most important groups of plant derived natural products. The vast majority of these (up to now 1500 known) indole alkaloid structures are synthesized from the common and first intermediate of the pathway: strictosidine [1, for review see 2]. This gluco-alkaloid with H-3α(*S*)configuration is formed by the stereospecific condensation of tryptamine with secologanin (Fig. 1). The enzyme catalysing this condensation reaction was discovered and named strictosidine synthase by us [3]. It was purified from *Catharanthus roseus* cell cultures *ca* 50-fold and characterized [4].

This enzyme from periwinkle cell culture was subsequently purified to apparent homogeneity but was found to split into four distinct multiple forms which were difficult to characterize individually [5]. The occurrence of strictosidine synthase in a number of cell culture systems was subsequently verified [e.g. 4, 6–10]. The enzyme can most conveniently be assayed by the release of tritium from [2-^{3}H]tryptamine during the condensation reaction with secologanin [4]. The synthesis of the labelled substrate [4], however, obviously proved too tedious for biologists so that other assay systems for this enzyme were developed recently [11]. In an attempt to study the molecular biology of alkaloid biosynthesis and to learn about the organization of genes involved in secondary metabolism, it was necessary as a first step to purify strictosidine synthase to homogeneity. Because of the occurrence of multiple forms of the synthase in *Catharanthus* we purified the enzyme from *R. serpentina* suspension cultures which had previously been optimized to give large amounts (1.6 g/l medium) of indole alkaloids such as raucaffricine [12].

The present report describes the isolation of milligram amounts of strictosidine synthase from *R. serpentina*, in which species no isoenzymes or multiple forms of the synthase are observed.

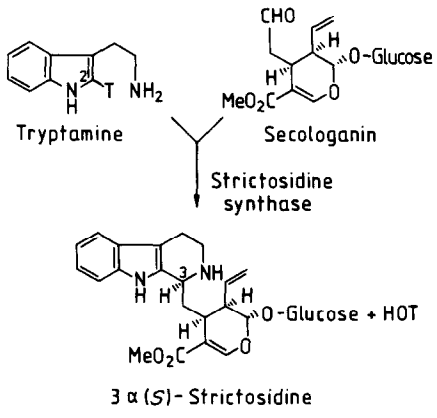

Fig. 1. Reaction sequence catalysed by strictosidine synthase, also indicating the assay principle employed during purification of the enzyme.

RESULTS

Purification of strictosidine synthase

Strictosidine synthase was found to occur in a number of our cell suspension cultures [4]. However, because of the high yield of *Rauvolfia* alkaloids synthesized in alkaloid production medium [12], *R. serpentina* cell suspension cultures were suspected to contain the highest yield of strictosidine synthase. This culture was therefore used to investigate the growth parameters and time course of enzyme formation. The enzyme is present in the inoculum (day 0) in only small amounts (Fig. 2). The activity increases slowly to peak at day 12. In contrast dry weight increase peaked at day 10 and maximal enzyme activity is therefore reached in the stationary growth phase. Suspension cultures were consequently harvested at day 12 of the growth cycle and cells of that age used for purification of the synthase. The purification of the enzyme from shock

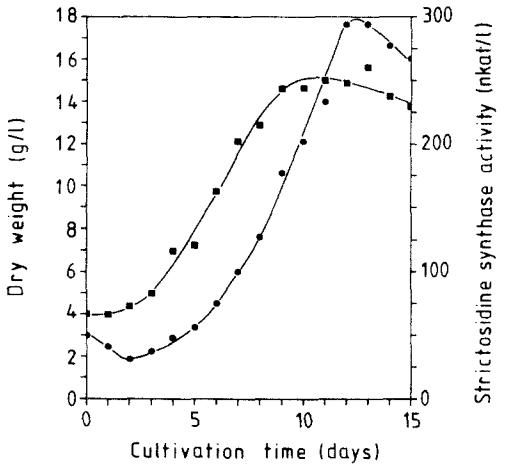

Fig. 2. Time course of strictosidine synthase activity (●) and cell dry weight (■) in a suspension culture of *R. serpentina* (1.5 l Fernbach flask containing 1.0 l medium).

frozen stored cells is summarized in Table 1. The enzyme so obtained has been purified 920-fold with a yield of 35%

Typically 1.9 mg of the synthase was obtained starting with 2.5 kg (fr. wt) of cells. In the course of this work and following this outline (with minor modifications) a total of 15 mg pure enzyme has been prepared. Three conventional purification steps and two powerful HPLC-steps rendered the enzyme homogeneous. Typical elution profiles of the final preparative ion exchange and gel permeation HPLC-steps are shown in Fig. 3. As can be noted, strictosidine synthase is desorbed from the IEC-column (DEAE-SP500) at 325 mM sodium acetate. Prior to GPC-HPLC the fractions containing the highest strictosidine synthase activity (fractions 12 and 13) were checked for the absence of unspecific glucosidases and the highly specific strictosidine glucosidase [13]. Both enzymes, in sequence, catalyse the first two steps in heteroyohimbine biosynthesis. To check the purity of the enzyme, the protein was subjected both to gel permeation chromatography and isoelectric focusing (Fig. 4). In both assay systems the enzyme travelled as a single band showing no major protein contamination. In the iso-

Table 1. Summary of purification procedure for strictosidine synthase from 2.5 kg (fr. wt) *R. serpentina* cells

Purification step	Total volume (ml)	Total protein (mg)	Specific activity (nkat/mg)	Yield (%)	Purification (-fold)
Supernatant of crude extract	6490	4840	0.2	100	1
DEAE-AH chromatography	800	2050	0.4	82	2
Phenyl-Sepharose CL-4B	75	470	1.5	68	7.5
Dialysis	80	460	1.4	65	7
DEAE (SP500) HPLC	24	16	35.0	55	175
TSK (G3000SW) HPLC + Amicon-Ultrafiltration	2.6	1.9	184	35	920

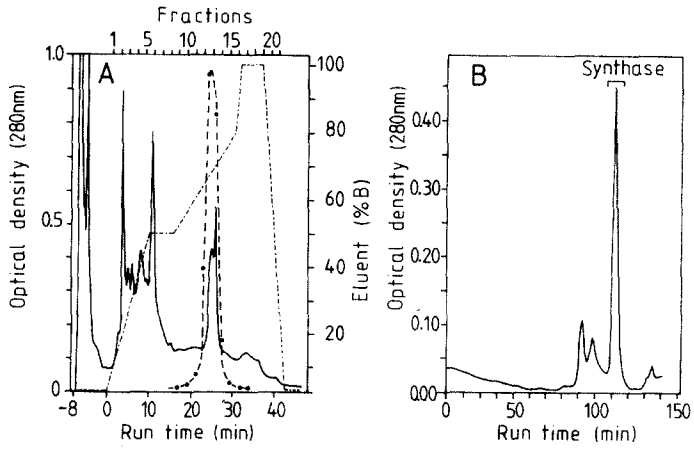

Fig. 3. Elution profiles of the final two HPLC purification steps. A: DEAE (SP500) ion exchange HPLC elution profile monitored by UV_{280} absorption (———). The non-linear eluent profile (- · - · - · -) is indicated. Fractions (1 ml) were analyzed for strictosidine synthase (● --- ●) and for the absence of specific strictosidine glucosidase and unspecific glucosidases. The peak maximum of the strictosidine synthase activity corresponds to 33 nkat/ml. B: Elution profile of the final gel permeation HPLC purification step on a TSK column (G3000SW) monitored by UV_{280} absorption (———).

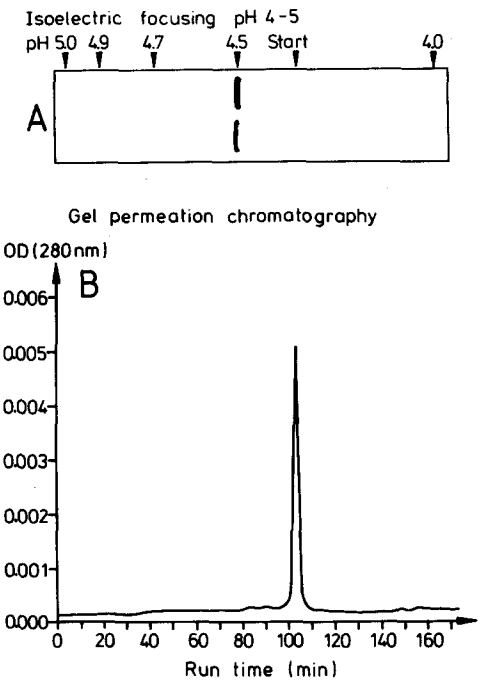

Fig. 4 Purity of strictosidine synthase after the final purification step by A: Isoelectric focusing in the range pH 4–5. Two separately prepared enzyme preparations were used. The position of reference proteins is as indicated. Proteins were visualized by staining with Serva Blue W. B: Analytical gel permeation chromatography (TSK G3000SW) with detection by UV_{280} absorption.

electric focusing system in the pH range 4–5, proteins with as little as a 1% difference in pH would have been resolved. We therefore can conclude that this purification procedure yields an apparently homogeneous enzyme preparation. The enzyme is present in the cells at about 0.1% of the total soluble protein.

Properties of strictosidine synthase

The pure enzyme from *Rauvolfia* showed a pH optimum at 6.5 in potassium phosphate buffer. 50% of the maximal enzyme activity was found at pH 5.0 and 8.0. The pH-optimum of the *Catharanthus* enzyme was previously determined to be at 6.8 [5]. The temperature optimum for the enzyme of *R. serpentina* was determined at 45°, and was only half as active at 30° regardless of whether 10 pkat of enzyme were allowed to react for 2 hr or 20 pkat for 1 hr. Most subsequent reactions were, however, run at 37° where the enzyme had 85% of its maximal activity. The activation energy for strictosidine synthesis was determined over the temperature range from 30–40° and found to be 11 kJ/mol. The isoelectric point of the homogeneous enzyme is at pH 4.5. The M_r of the enzyme was determined both by SDS gel electrophoresis in which system a M_r of 35 000 was determined. Gel permeation chromatography with a TSK G3000SW column and comparison with reference proteins yielded 30 000. We assume the latter value to be the correct one, since the gel permeation chromatography is less influenced by carbohydrate residues attached to the enzyme. As judged from SDS electrophoresis pattern the enzyme consists of only one polypeptide chain. Using Schiff's reagent, the presence of carbohydrates in the pure enzyme could be detected qualitatively. By using the anthrone colour reaction a carbohydrate moiety of 5.3% was determined (excluding any amino sugars which would not be detected by this reagent) using glucose oxidase (16.2% carbohydrates including 2% amino sugars) as standard. To determine the sugar composition 1 mg of protein was hydrolysed, derivatives prepared and subjected to GC. The carbohydrates were determined as mannose (2.6%), glucose (1.6%), and xylose (1.1%). The total carbohydrate content of the enzyme is therefore 5.3% (without hexosamines). When the enzyme was subjected to sodium periodate oxidation (20 mM, 1 hr, pH 5.5), the catalytic activity was retained to 100%. This fact suggests that the intact carbohydrate moiety of this enzyme is not necessary for catalytic activity. The enzyme is fully catalytically active in the presence of either 5% polyethyleneglycol, dimethylformamide, acetone, ethanol, dimethylsulphoxide. The half-life of the enzyme was determined at pH 6.5 under sterile conditions and found to be 36 days at 37°, 80 days at 20°, 300 days at 4°, and at least 1000 days at −20°. The product of the reaction using tryptamine and secologanin as substrates was identified rigorously and unequivocally as H-3α(*S*)strictosidine following the method of [14]. The K_M-values for both substrates were determined for the *Rauvolfia* strictosidine synthase. Keeping the secologanin concentration constant at 12 mM and varying tryptamine from 0.03 to 4 mM, a K_M for tryptamine was determined as 4 mM. At tryptamine concentrations higher than 0.9 mM a strong substrate inhibition was noticed. In order to circumvent this inhibition the tryptamine concentration was fixed at 0.9 mM and the secologanin concentration varied from 0.28 to 12 mM. In this way a K_M value for secologanin was determined to be also 4 mM. From the Lineweaver–Burk plot a positive cooperative effect in the case of secologanin was observed which was substantiated in the Hill plot. The Hill coefficient was determined as $n_H = 1.4$. The turnover number for the homogeneous enzyme was determined under standard conditions (37°) to be 270 mol of product formed per mol enzyme per minute (assuming a M_r of 30 000 for the enzyme). The high substrate specificity of the enzyme which was previously observed for the *Catharanthus* enzyme [4], was seen again also for the *Rauvolfia* enzyme. Thus neither L-tryptophan nor α-methyltryptamine served as substrate. The enzyme was also totally inactive with 5-hydroxytryptamine (serotonin), 4,5-dimethyl- or 4-methyl-5-methoxy-tryptamine as aromatic substrates.

Immobilization of the enzyme

Following the successful immobilization of strictosidine synthase from *Catharanthus* [14, 15], we attempted here also to fix the *Rauvolfia* enzyme onto water insoluble matrices. The soluble enzyme was exposed to activated carriers following the manufacturer's protocols. The resins were subsequently washed and immobilized enzyme analysed for catalytic activity at 37° with agitation (120 rpm). The following activity yields were observed: CNBr-activated Sepharose 4B: 38%, epoxy-activated Sepharose 6B: 31%, hydroxysuccinimidyl SP500: 18%, glutaraldehyde-activated controlled pore glass: 5%. Clearly, as observed before [14, 15], CNBr-activated

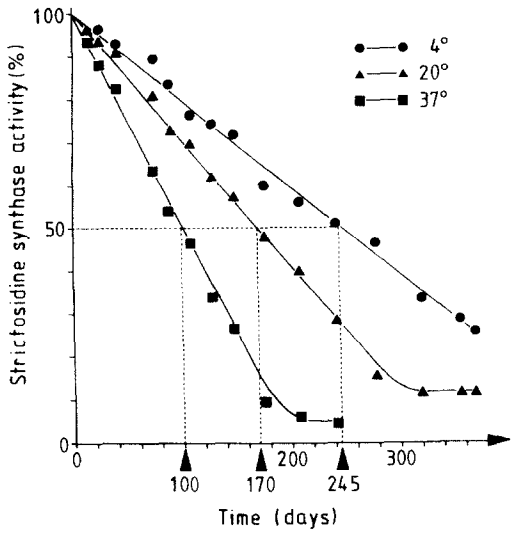

Fig. 5. Stability of Sepharose 4B immobilized strictosidine synthase at different storage temperatures: 4° (●); 20° (▲); 37° (■). 100% activity corresponds to 110 pkat enzyme activity.

Sepharose 4B was superior to all the other matrices tested. Subsequently homogeneous strictosidine synthase was immobilized on the carrier giving the highest yield and the enzyme activity measured at intervals given in Fig. 5. The immobilized enzyme was stored during these intervals at the temperature indicated using 0.02% NaN_3 as a preservative. As shown in Fig. 5, the enzyme is *ca* three-fold more stable as compared with the soluble enzyme showing a half-life of 100 days at 37°, and 170 days at 20°.

A total activity of 25 nkat Sepharose 4B-coupled strictosidine synthase was packed into a column (10 × 50 mm) and charged with an ice-cold unbuffered solution of 9 mM tryptamine and a 9 mM solution of secologanin each at pH 6.5 fed from separate reservoirs. Both solutions, in a ratio of 1:1, were mixed in the column head and the column operated at 37°. The mixture was passed through the column at 0.11 ml/min. The effluent was chilled to 0°, collected every 12 hr and the combined solutions, after a six day operation, freeze-dried to yield 5 g of pure strictosidine.

DISCUSSION

The occurrence of strictosidine synthase in *ca* 10 different species of the family Apocynaceae has been recognized [4]. In addition, this key enzyme catalysing the first step in monoterpenoid indole alkaloid biosynthesis was reported to also occur in one member of the family Rubiaceae, in suspension cultures of *Cinchona ledgeriana* [8]. Up to now the synthase was purified only from *Catharanthus roseus* cell cultures [4, 6], and its purification to homogeneity was reported from Scott's laboratory [6]. For biochemical and molecular experiments involving this interesting enzyme, however, large amounts of enzyme with high specific activity is required. We therefore chose to purify this enzyme from *Rauvolfia serpentina* cell cultures, a species which is the commercial source of reserpine, and ajmaline and which has been optimized in suspension culture to give the highest yield of indole alkaloids ever reported by this technique [12]. The crude enzyme extract from *R. serpentina* proved already to contain, by at least a factor of 20, more synthase than was reported from the *C. roseus* culture [6]. The final specific activity of the homogeneous enzyme differed between *Catharanthus* (5.9 nkat/mg [6]) and *Rauvolfia* (184 nkat/mg) by a factor of more than 30. Furthermore, the stability of the soluble *Rauvolfia* enzyme is considerable higher ($t_{1/2}$ = 36 days at 37°) as compared with the *Catharanthus* enzyme ($t_{1/2}$ = 5 hr at 37° [14]), and even in immobilized form the *Rauvolfia* enzyme is more stable by a factor of about 1.5 at 37° ($t_{1/2}$ = 100 days). The *Rauvolfia* enzyme, which has a turnover number of *ca* 270, is therefore the enzyme of choice for studies on the biotechnological application of secondary metabolism. On the other hand, there are large similarities of this enzyme from both sources regarding the pH (6.5 ± 0.3) and temperature (45°) optimum, K_M values for tryptamine and secologanin (2–4 mM), M_r (30 000 ± 10%) and isoelectric point (pH 4.5). Both enzymes must be similar in structure since antibodies directed against the synthase from *C. roseus* cross-react well with the enzyme from *R. serpentina* but are completely inactive with strictosidine synthases from Rubiaceae species [5]. The synthase activity increased considerably at the end of growth of the *Rauvolfia* cell cultures corresponding to the stationary phase of the culture which is in accordance with previous reports in the literature for *Catharanthus* [9]. Strictosidine synthase is the first enzyme of the biosynthetic pathway which later branches into a multitude of alkaloid structures. We and others [6] did not find any regulatory control of strictosidine synthase from Apocynaceae cell cultures. The synthesis of indole alkaloids seems to be influenced more by the primary substrate availability [12] rather than by stringent control of the first enzyme opening the pathway as is known from bacterial systems. The availability of the homogeneous strictosidine synthase from *Rauvolfia* should facilitate basic studies regarding the structure and the application of this polypeptide.

EXPERIMENTAL

Cell cultures. Cell suspension cultures of *R. serpentina* were grown in LS-medium [16] at 27° and 100 rpm in 1.5 l Fernbach flasks (Schott, Mainz, No. 21 511 62) on gyratory shakers. Cells were harvested by vacuum suction as soon as they reached maximum strictosidine synthase activity and then were shock frozen in liquid N_2. Tissue was stored frozen at −20° until use.

Preparation of crude extracts. Crude extracts were prepared by adding 2.5 kg shock frozen tissue to 2.5 l K-Pi buffer (25 mM, pH 6.5), and stirring at room temp. until a homogeneous suspension resulted. Cell debris was removed by filtration through three layers of cheese-cloth, followed by a continuous flow centrifugation at 7500 *g* at a flow rate of 15 ml/min (Labofuge 15000, Haereus) and a further filtration step through two layers of glass-fibre prefilters (Sartorius SM 13400) in a Sartorius SM 16268 device. To reduce alkaloid concentration in the crude extract a 30 × 150 mm Amberlite XAD-2 column (Serva) was directly connected to the outlet of the Sartorius filtration unit. Elimination of floating debris was reached by a final filtration step through a filtration cartridge type 250 (Whatman) mounted in a Gamma 12 in-line-filter (Whatman).

Enzyme isolation. To separate the fraction of acidic proteins, the crude extract was adsorbed to an ion-exchanger, Cellufine DEAE-AH (Amicon). The extract was pumped onto the equilibrated column (50 × 200 mm) at a flow rate of 5 ml/min at 4°. Flow-through was continuously monitored at 280 nm and the

effluent discarded. The loaded column was washed with starting buffer (25 mM K-Pi, pH 6.5) until the extinction was less than 0.1. The bound enzyme was eluted with starting buffer containing 1 M NaCl. The elution peak was collected until absorbance reached 10% of its maximum value. To prepare the eluate for hydrophobic interaction chromatography, solid NaCl was added to the protein soln to a final concn of 15% NaCl. To remove pptd material the soln was centrifuged at 13 000 *g* at 4° for 20 min (Sorvall RC-5B, GSA rotor, 9000 rpm). The soln was then pumped onto a column of phenyl-Sepharose CL-4B (Pharmacia) at a flow rate of 4 ml/min at 4°. The column was washed with starting buffer containing 15% NaCl until no material absorbing at 280 nm could be detected in the eluate. Bound material was desorbed with starting buffer containing no NaCl. The eluted protein peak was collected and dialysed overnight against 5 l HPLC-buffer (25 mM Tris–acetate, pH 6.5) at 4°. The dialysed protein soln can be stored over several months at −20° without any activity loss until use for enzyme isolation by the final two HPLC-steps. Ion exchange chromatography by HPLC was done on a 10 × 250 mm DEAE (SP500) column (Serva) at a flow rate of 1 ml/min at 4°. 15 ml of the concentrated protein soln (= 100 mg protein) were applied to the column per run. Adsorbed protein was eluted with a gradient varying from 0 to 500 mM NaOAc. The eluate was monitored at 280 nm and fractions of 2 ml each were collected. For further purification the active fractions (24 ml, 16 mg protein) were subjected to GPC-HPLC on a 21.5 × 600 mm TSK (G3000SW) column (LKB) at 4° at a flow rate of 1.5 ml/min. The chromatographic parameters (sample volume: 2 ml, protein content: 1.3 mg, flow rate: 1.5 ml/min) were selected according to Kato *et al.* [17, 18] to reach best resolution. The column eluate was monitored at 280 mm and automatically fractionated in steps of 2 min (3.0 ml). Strictosidine synthase containing fractions of 12 runs (72 ml) were pooled and concd to 2.6 ml by ultrafiltration in a 25 ml Amicon-cell.

Enzyme assays. Strictosidine synthase activity was measured by the published procedure [4] and by a slightly modified version [5]. Strictosidine glucosidase and unspecific glucosidase activities were determined according to ref. [13].

Analytical procedures. The purity of the isolated enzyme was controlled by analytical gel permeation chromatography HPLC and analytical isoelectric focusing electrophoresis. For analytical GPC-HPLC, 20 μl of the enzyme (15 μg) were rechromatographed at a flow rate of 1.5 ml/min under the conditions given above. Symmetry of the elution peak and absence of other peaks indicated homogeneity. For isoelectric focusing 10 μl of the enzyme preparation (7.5 μg) were applied to ultrathin polyacrylamide gels (Servalyt precotes, Serva, Heidelberg) and analysed in the ranges pH 3–10 and 4–5 on a thermostated Multiphore electrophoresis unit (LKB) at 8°. Separation parameters and protein staining procedure with Serva Blue W (Serva) were performed according to the manufacturer's instructions. As a reference 10 μl of a 3% soln of a protein test mixture according to [19] were co-analysed on each gel. For SDS gel electrophoresis the published [20] system was used. The method introduced by Bradford [21] was employed for the determination of protein concentrations with BSA as standard; for pure preparations the determination by 260/280 nm was employed. For Schiff-staining in ultrathin IEF-gels the protein was denaturated after the electrophoretic separation with 20% TCA for 10 min, washed in distilled water (10 sec) and equilibrated in ice-cooled 7.5% HOAc (5 min). Carbohydrate cleavage was achieved by submerging the gel for 5 min in 0.2% periodic acid at 4°. Then it was rapidly transferred to ice-cold Schiff's reagent and incubated for 10 min in an ice-bath in the dark followed by several 5 min washes in ice-cold 10% HOAc until the background was almost colourless. Stained gels were air-dried and remained unchanged for several months. To determine the carbohydrate content of the synthase the quantitative procedure described by Spiro [22] was employed using glucose oxidase and horseradish peroxidase as reference proteins. To identify the sugar components of the enzyme 1 mg strictosidine synthase was hydrolysed with 2 N trifluoroacetic acid for 90 min at 120°. After vacuum drying the hydrolysate was dissolved in 100 μl 1 M NH_3. 1 ml 2% $NaBH_4$ in DMSO was added and the mixture incubated at 4° for 90 min. Unreacted $NaBH_4$ was quenched with 100 μl HOAc. 2 ml Ac_2O and 200 μl 1-methylimidazole were added for acetylation. The reaction was stopped by addition of 20 ml ice-cold H_2O after 15 min. After an additional 15 min, the acetylated sugars were extracted with $CH_2Cl_2$and analysed on a GC-column GP 3% SP-2330 on 100/120 Supelcoport (Supelco) at 225° and 30 ml/min Ar with FID-detection.

Radioactive compounds. [7–^{3}H]Secologanin was synthesized and purified according to ref. [23] from [7-^{3}H]secologanol by Jones oxidation.

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