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BACTERIAL BIOTRANSFORMATION OF 3α(*S*)-STRICTOSIDINE TO THE MONOTERPENOID INDOLE ALKALOID VALLESIACHOTAMINE

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Abstract—3α(*S*)-Strictosidine produced by heterologously expressed strictosidine synthase from *Rauwolfia serpentina* was used in biotransformation experiments with a series of 22 bacterial strains. All strains tested were found to deglucosylate and rearrange the alkaloid to vallesiachotamine, thereby providing an example of how gene technology and microbial biotransformation can be combined for the biotechnological production of alkaloidal natural products.

INTRODUCTION

3α(*S*)-Strictosidine (**1**) is the central intermediate in the biosynthesis of over 1800 monoterpenoid indole alkaloids [1]. The enzyme strictosidine synthase [EC 4.3.3.2] responsible for the stereospecific condensation of tryptamine (**2**) and secologanin (**3**) to this first monoterpenoid indole alkaloid has been characterized [2] and purified [3, 4] from both *Catharanthus roseus* and *Rauwolfia serpentina*. Partial amino acid sequences of tryptic peptides prepared from the pure enzyme from *R. serpentina* were determined and facilitated the isolation of the cDNA clone encoding this enzyme [5]. This cDNA has been functionally expressed in *Escherichia coli*, *Saccharomyces cerevisiae* and in Sf9 cell culture of the insect *Spodoptera frugiperda* [6, 7]. The native enzyme is very stable in immobilized form and is therefore amenable to biotechnological exploitation for the production of strictosidine (**1**) [8, 9]. We demonstrate herein the synthesis of strictosidine using immobilized, heterologously expressed strictosidine synthase coupled to bacterial biotransformation of this alkaloidal intermediate to isomers of the monoterpenoid indole alkaloid vallesiachotamine (**4**) [10].

RESULTS AND DISCUSSION

Fifteen grams of strictosidine (**1**) were enzymatically synthesized using heterologously produced (in *S. frugiperda* Sf9 cells [7]) strictosidine synthase immobilized on cyanogen bromide-activated Sepharose 4B [9]. A glass column (1 × 15 cm) filled with 2 g of Sepharose 4B-immobilized enzyme (150 nkatal) was washed thoroughly with distilled H_2O. Unbuffered solns of tryptamine-HCl (**2**) and of secologanin (**3**) (15 mM each) were separately prepared and the pH adjusted to 6.5. The substrate solns were pumped separately onto the column containing immobilized enzyme at a flow rate of 3 ml/h at 37°. Mixing of the two substrate solns occurred in the column head prior to contact with the enzyme. In 12 h intervals, the accumulated eluate was lyophilized and an aliquot analyzed chromatographically for the presence of strictosidine (**1**).

Given this large amount of strictosidine (**1**), 22 bacterial strains (Table 1) were then tested for the ability to convert strictosidine to novel or known products. This presumably would proceed by cleavage of the glucose moiety of strictosidine (**1**) to form the labile dialdehyde (**5**), which could then undergo rearrangement to one or more indole alkaloids. In all cases tested, the bacteria were able to transform strictosidine into the same two products, as determined by TLC. In order to isolate and identify these products of bacterial biotransformation, 220 mg of strictosidine (**1**) were added to 12 ml of a culture of *Staphylococcus aureus* in a minimal medium. After incubation at 32° for 24 h, a yellow precipitate had formed. The solid was collected by centrifugation and was purified by TLC in two products with identical molecular masses, CI-MS *m/z*: $[M+1]^+$ (351). ^1H and ^{13}C NMR identified the presence of an aldehyde group in each prod-

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Table 1. Select bacterial strains tested for the ability to biotransform strictosidine (**1**)

Species	Growth medium
Aeromonas sp.	HD*
Brochothrix thermosphacta, B. campestris	HD
Bacillus licheniformis	HD
Cellulomonas uda	HD
Citrobacter freundii	LB
Enterobacter aerogenes, E. cloacae	LB
Escherichia coli, E. coli T	HD, LB
Klebsiella oxytoca, K. pneumoniae, K. terrigena	LB
Listeria grayi, L. innocus	BHI
Proteus mirabilis, P. vulgaris	LB
Salmonella typhimurium	LB
Serratia marcescens	LB
Staphylococcus aureus, S. epidermidis, S. carnosus	HD

* Media composition is provided in the Experimental.

uct (^{1}H NMR (360 MHz, $CDCl_3$): δ 10.3 (1H, *s*, CHO); 9.4 (1H, *s*, CHO)) and ascertained that the two products were of highly similar structure, likely isomers. The biotransformation products were, therefore, subsequently reduced with $NaBH_4$ to yield more stable derivatives for complete structure elucidation. Purification by TLC after reduction yielded slightly yellow solids in a ratio of 2:1. Analysis of the major product by MS and one- and two-dimensional NMR spectroscopy identified it as the alcoholic reduction product (**4a**) of vallesiachotamine (**4**) (Fig.1) [1]. The structure of the minor product was deduced to be the reduction product (**6a**) of isovallesiachotamine (**6**).

Vallesiachotamine (**4**) is a natural product that has been isolated from extracts of the Peruvian plants *Vallesia dichotoma* (Apocynaceae) [10]. The total synthesis of vallesiachotamine (**4**) and isovallesiachotamine (**6**) has been reported [11] and the $NaBH_4$ reduction of the crotonaldehyde moiety of these alkaloids has been demonstrated to yield the corresponding alcohols [1, 10], consistent with those results presented herein. The presence of non-specific glycosidases in all of the bacterial strains tested in this study that are capable of hydrolyzing the glucose moiety of a molecule of such complex structure as strictosidine (**1**) was unexpected. This lack of specificity is in sharp contrast to those glucosidases analyzed from the plant kingdom. Strictosidine (**1**) is transformed to the aglycone (**7**) in *C. roseus* and *R. serpentina* by highly substrate specific β-glucosidases [12, 13]. These specific strictosidine β-glucosidases [EC 3.2.1.105] have been found to occur only in those plant species that produce monoterpenoid indole alkaloids [13].

The biotechnological production of known and novel alkaloids is a field that in recent years shows much potential due to the successes in cloning the genes of alkaloid biosynthesis [14]. Through combinations of functional heterologous expression of alkaloid biosynthetic genes, microbial biotransformations and combinatorial chemistry, the production of potential new pharmaceuticals should be made possible.

EXPERIMENTAL

Enzyme production and immobilization

Strictosidine synthase was produced in *S. frugiperda* Sf9 cells and was partially purified exactly according to Ref. [7]. A soln of the enzyme in 20 mM K-Pi pH 7.0 (4 ml, 150 nkatal) was dialyzed against 100 mM $NaHCO_3$ buffer pH 8.5 that contained 500 mM NaCl for 20 h at 4°. The protein in the dialysate was then immobilized onto 2 g of CNBr-activated Sepharose 4B (Pharmacia Biotech) according to Ref. [9].

Microorganisms and culture conditions

Bacterial cultures were routinely maintained in either LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl, pH 7.5), HD medium (1% tryptone, 0.5% yeast extract, 0.5% glucose, 0.5% NaCl, pH 7.2), or BHI medium (Oxoid) as indicated in Table 1.

For the large scale isolation of the strictosidine biotransformation products, *S. aureus* was cultured for 1 month in a minimal medium of the following composition (1 l, pH 7.2, containing 1% salicin): 2 g KH_2PO_4, 1 g $(NH_4)_2SO_4$, 0.5 g $NaNO_3$, 0.05 g $MgSO_4$, 0.01 g $FeSO_4 \cdot 7H_2O$, 0.05 g $MnSO_4 \cdot 2H_2O$, 0.1 g NaCl, 0.01 g $CoCl_2$, 0.01 g $ZnSO_4$, 0.001 g $CuSO_4 \cdot 5H_2O$, 0.001 g H_3BO_3, 0.001 g $NaMoO_4 \cdot 2H_2O$, 0.0025 g $NiCl_2 \cdot 6H_2O$, 0.01 g $CaCl_2$. After 1 month, 220 mg strictosidine (**1**) was added to 12 ml of the above medium, without salicin. Bacteria were inoculated into this soln and incubated for 24 h.

All cultures were maintained in a gyratory shaker at 160 rpm and 32°.

Purification of metabolic products

Within 24 h after the addition of 220 mg strictosidine to an *S. aureus* culture, 15 mg of a yellow precipitate could be collected by centrifugation. This precipitate was resolved into two components (R_f 0.48 and 0.50) by analytical TLC (silica gel) with $CHCl_3$–MeOH (40:1) as the developing solvent. The yellow solid was then dissolved in 10 ml MeOH, 20 mg $NaBH_4$ was added and the suspension was stirred 2 h at room temperature and taken to dryness *in vacuo*. The residue was dissolved in MeOH and resolved into two bands (R_f 0.35 and 0.40) by preparative silica gel TLC using the above solvent system.

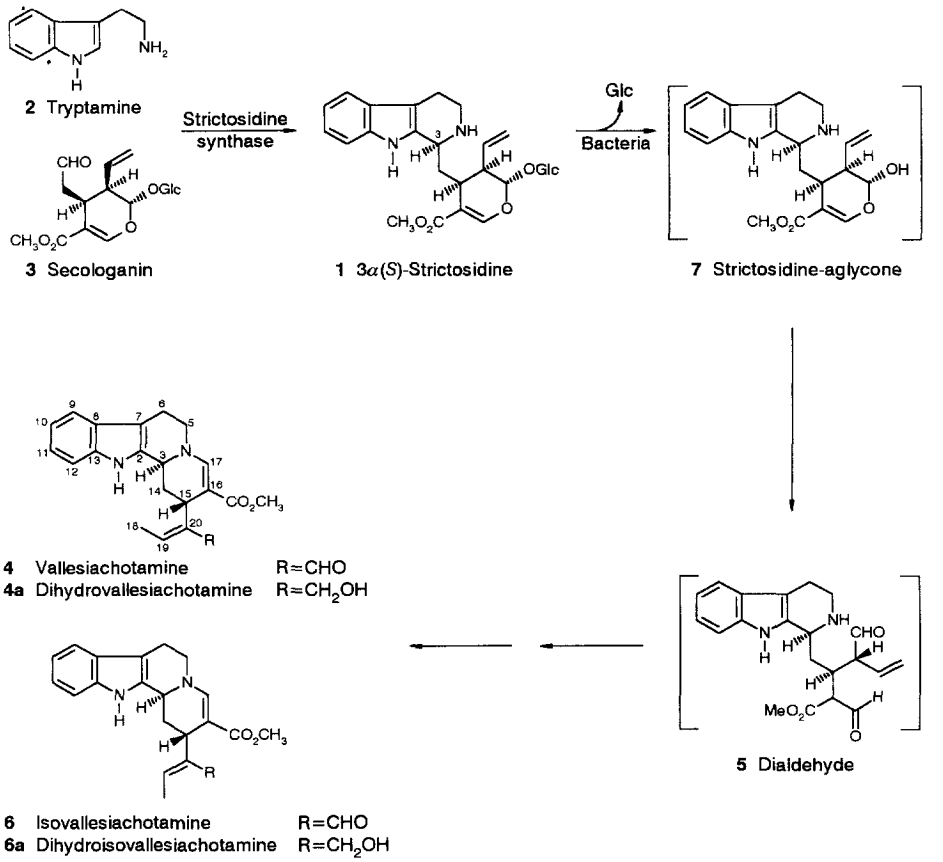

Fig. 1. Synthesis of vallesiachotamine (**4**) and isovallesiachotamine (**6**) from tryptamine (**2**) and secologanin (**3**) using a combination of immobilized, heterologously expressed strictosidine synthase, bacterial biotransformation, and chemical reduction.

NMR spectroscopy

^{1}H and ^{13}C NMR spectra were recorded at 22°C using a DRX 500 spectrometer from Bruker, Karlsruhe, Germany. ^{1}H detected experiments were performed using an inverse broadband probehead and ^{13}C detected experiments were performed using a dual ^{13}C/^{1}H probehead. The parameters of ^{1}H spectra were as follows: transmitter frequency, 500.13 MHz; 45° pulse, 3 μs; repetition time, 3 s; spectral width, 8.0 kHz; 32 K data set, zero-filled to a 64 K prior to Fourier transformation; Gaussian apodization. The parameters of ^{13}C NMR spectra were as follows: transmitter frequency, 125.77 MHz, 30° pulse, 3 μs; repetition time, 2 s; spectral width, 28.9 kHz; 64 K data set, zero-filled to 128 K; ^{1}H composite pulse decoupling (WALTZ 16) during relaxation and acquisition; Gaussian apodization. DEPT, two-dimensional double quantum filtered COSY, HMQC, HMBC and ROESY experiments were performed according to standard Bruker software. All of the ^{1}H NMR and ^{13}C NMR signals of dihydrovallesiachotamine were assigned unequivocally on the basis of homo- and heteronuclear correlation patterns in the two-dimensional NMR experiments.

Dihydrovallesiachotamine

CI-MS *m/z*: 353 ($M^+ + 1$); ^{1}H NMR (500.13 MHz, $CDCl_3$, TMS as int. standard): δ 1.80 (3H, *d*, *J* = 7.0 Hz, H-18), 1.87 (1H, *m*, H-14), 2.23 (1H, *m*, H-14′), 2.78 (1H, *m*, H-6), 2.90 (1H, *m*, H-6′), 3.63 (1H, *m*, H-5), 3.66 (3H, *s*, H-23), 3.70 (1H, *m*, H-5′), 3.84 (1H, *d*, *J* = 5.8 Hz, H-15), 3.96 (1H, *d*, *J* = 12.2 Hz, H-21), 4.05 (1H, *d*, *J* = 12.2 Hz, H-21′), 4.62 (1H, *d*, *J* = 11.5 Hz, H-3), 5.68 (1H, *q*, *J* = 7.0 Hz, H-19), 7.10 (1H, *m*, H-10), 7.16 (1H, *m*, H-11), 7.31 (1H, *d*, *J* = 8.0 Hz, H-12), 7.47 (1H, *d*, *J* = 7.6 Hz, H-9), 7.66 (1H, *s*, H-17), 7.80 (1H, *s*, NH); ^{13}C NMR (125.7 MHz, $CDCl_3$, TMS as int. standard): δ 169.0 (*s*, C-22), 147.1 (*d*, C-17), 143.3 (*s*, C-20), 136.2 (*s*, C-13), 132.8 (*s*, C-2), 126.9 (*s*, C-8), 124.4 (*d*, C-19), 122.3 (*d*, C-11), 119.9 (*d*, C-10), 118.2 (*d*, C-9), 111.0 (*d*, C-12), 108.5 (*s*, C-7), 94.9 (*s*, C-16), 67.4 (*t*, C-21), 51.1 (*t*, C-5), 50.8 (*q*, C-3), 49.0 (*d*, C-3), 35.3 (*t*, C-14), 31.0 (*d*, C-15), 22.1 (*t*, C-6), 13.6 (*q*, C-18).

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