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Biotechnology Letters **26:** 793–798, 2004.

Alkaloid accumulation in *Catharanthus roseus* cell suspension cultures fed with stemmadenine

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Received 3 February 2004; Revisions requested 9 February 2004; Revisions received 8 March 2004; Accepted 8 March 2004

Key words: alkaloid accumulation *Catharanthus roseus*, cell culture condylocarpine, stemmadenine, tabersonine-catharanthine pathway

Abstract

Feeding stemmadenine to *Catharanthus roseus* cell suspension culture resulted in the accumulation of catharanthine, tabersonine and condylocarpine. Condylocarpine is not an intermediate in the pathway to catharanthine or tabersonine when it is fed to the cultures. The results support the hypothesis that stemmadenine is an intermediate in the pathway to catharanthine and tabersonine.

Introduction

The tropical plant Madagaskar Periwinkle [*Catharanthus roseus* (L.) G.Don] is a rich source of indole alkaloids which have a high medicinal and economic value, especially the anti-tumor alkaloids, vinblastine and vincristine, and the anti-hypertensive compound, ajmalicine (Creasey 1994, Verpoorte *et al.* 1997). Terpenoid indole alkaloid biosynthesis starts with the condensation of tryptamine and secologanin to form the key intermediate strictosidine. Strictosidine is then hydrolyzed by strictosidine β-glucosidase producing cathenamine as the main product. Besides cathenamine, the related carbinolamine and epi-cathenamine have been detected by NMR in the reaction mixture (Stevens 1994). Other studies proved indirectly that an equilibrium exists between cathenamine and 4,21-dehydrogeissoschizine (Heinstein *et al.* 1979). As cathenamine is the main product of strictosidine conversion by the enzyme strictosidine β-glucosidase, Stevens (1994) concluded that this equilibrium was more favored towards cathenamine rather than 4,21-dehydrogeissoschizine. At some point in this reaction equilibrium, routes diverge into different indole alkaloid pathways. Lounasmaa & Hanhinen (1998) presented the *'La Ronde'* scheme for the interconversion of cathenamines in three steps: cyclization, epimerization and isomerization. The intermediate branch-points leading to the different classes of alkaloids, though, were not as well-defined. Brown *et al.* (1971), suggested that geissoschizine could be converted to stemmadenine or akuammicine through some intermediates such as formylstrictamine and indolenine. Qureshi & Scott (1968a) reported the first evidence for formation of secodine as an intermediate when stemmadenine was refluxed in glacial acetic acid for 34 h yielding a mixture of tabersonine (12%), (±)catharanthine (9%) and pseudocatharanthine (16%). The same authors (Qureshi & Scott 1968b) claimed that tabersonine, after refluxing in glacial acetic acid for 16 h was converted into catharanthine and pseudocatharanthine. However, this work was not reproducible and contradictory results have been reported by Brown *et al.* (1969): when tabersonine was treated under the same conditions it failed to produce catharanthine or pseudocatharanthine. Battersby & Hall (1969) reported that, corynanthine aldehyde and geissoschizine fed to *Catharanthus roseus* plants were incorporated into catharanthine. From these experiments, the pathway was suggested as going from

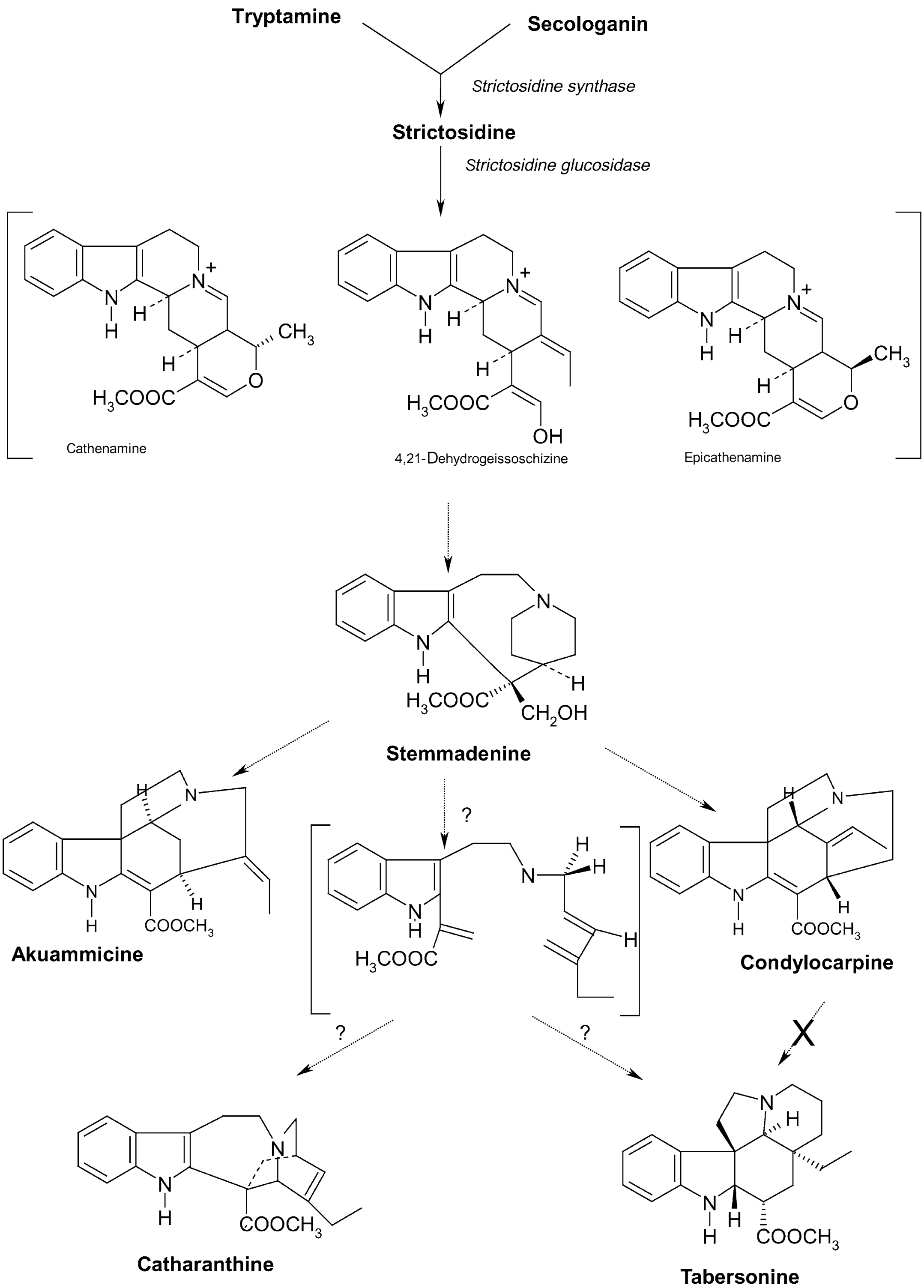

Fig. 1. Proposed pathway leading to catharanthine and tabersonine.

strictosidine via 4,21-dehydrogeissoschizine, stemmadenine and dehydrosecodine (Figure 1). None of the enzymes involved in this proposed pathway have been identified. The aim of the present study is to determine whether *C. roseus* cells are capable of producing tabersonine and catharanthine upon feeding with stemmadenine. Cells capable of this could serve for further studies to identify the enzymes and genes involved in this pathway.

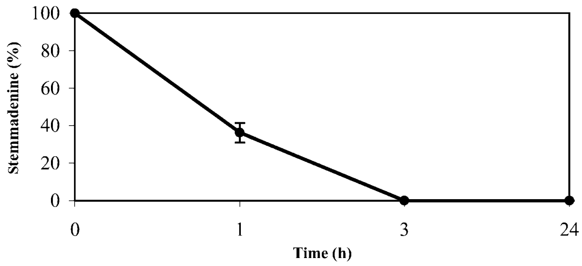

Fig. 2. Concentration availability of stemmadenine in the medium after feeding followed for 24 h. Two mg at time 0 is 100% and (n = 2).

Materials and methods

Cell line and culture conditions

Catharanthus roseus cell suspension culture line (CREG) accumulating serpentine was established from leaf explant of the Egyptian cultivar (No. 20000003) on B5 medium. The medium contained B5 salts (Gamborg *et al.* 1968), 100 mg myo-inositol l^{-1}, 10 mg thiamine l^{-1}, 1 mg pyridoxine l^{-1}, 1 mg nicotinic acid l^{-1}, 1.86 mg naphthalene acetic acid l^{-1} and 20 g sucrose l^{-1}. The friable callus was then transferred to the liquid medium under the same conditions to initiate the cell suspension and sub-cultured every two weeks. Cultures were shaken at 110 $\pm$ 5 rpm and at 25 $\pm$ 1 °C, with 24 h light (14–25 μ mol m^{-2} s^{-1}). For the experiment, 1.5 g filtered cells were transferred to a 150 ml Erlenmeyer flask containing 20 ml medium under sterile conditions. The experiment was performed in duplicate.

Addition of precursor and time course sampling

Stemmadenine, in distilled water, after filter sterilization was added at the fourth day after inoculation to the cultures to give a concentration of 2 mg per flask. The controls received the same amount of distilled water. For sampling, the treated flasks were placed in a laminar flow cabinet to settle the cells and 2 ml of the medium containing cells from the bottom of the flask were aseptically removed and filtered in order to separate them from the medium. For the control flasks, sampling was done at the beginning and the end of the experiment.

Sample extraction and HPLC analysis of alkaloids

Frozen biomass (125 mg in a 1.5 ml microtube) was extracted using a pestle and 50 mg sea sand with 350 μl of 0.1% trifluoroacetic acid. The vial was then sonicated for 30 min, centrifuged at 13 000 *g* for 15 min and the supernatant used for alkaloid determination. 100 μl of cell extract supernatant or centrifuged medium was analyzed by HPLC using a Waters 991 photo-diode array detector. A C18 RP Vydac column (no. 218 MS54, 4.6 $\times$ 250 mm, USA) with a guard column (Vydac, Hesperia, CA) was used with an isocratic elution system of trifluoroacetic acid/acetonitrile/water (0.1:21:79, by vol.) at 1 ml min^{-1}.

Peak identification was based on a comparison of retention time and UV spectra with authentic standards of catharanthine and tabersonine.

Detection of alkaloids in the cells

The stemmadenine-fed and control cells were visualized under a Nikon fluorescence microscope to detect tabersonine using a UV excitation filter UV330~380 nm that gives a main wavelength of 365 nm. The cell appeared transparent against a dark background limited by its cell wall while fed cells showed an emission of a blue color characteristic for the presence of tabersonine. For other alkaloids, the cells were stained with Dragendorff's reagent and visualized under a normal light microscope.

NMR spectroscopy

^{1}H-NMR (400 MHz), ^{1}H-^{1}H-COSY, and HMBC were recorded on Bruker AV-400 FT-NMR spectrometer with reference to the residual solvent signal.

Results and discussion

Uptake of stemmadenine by cell culture

The cell culture was fed with stemmadenine and the alkaloid content of the media and cells determined

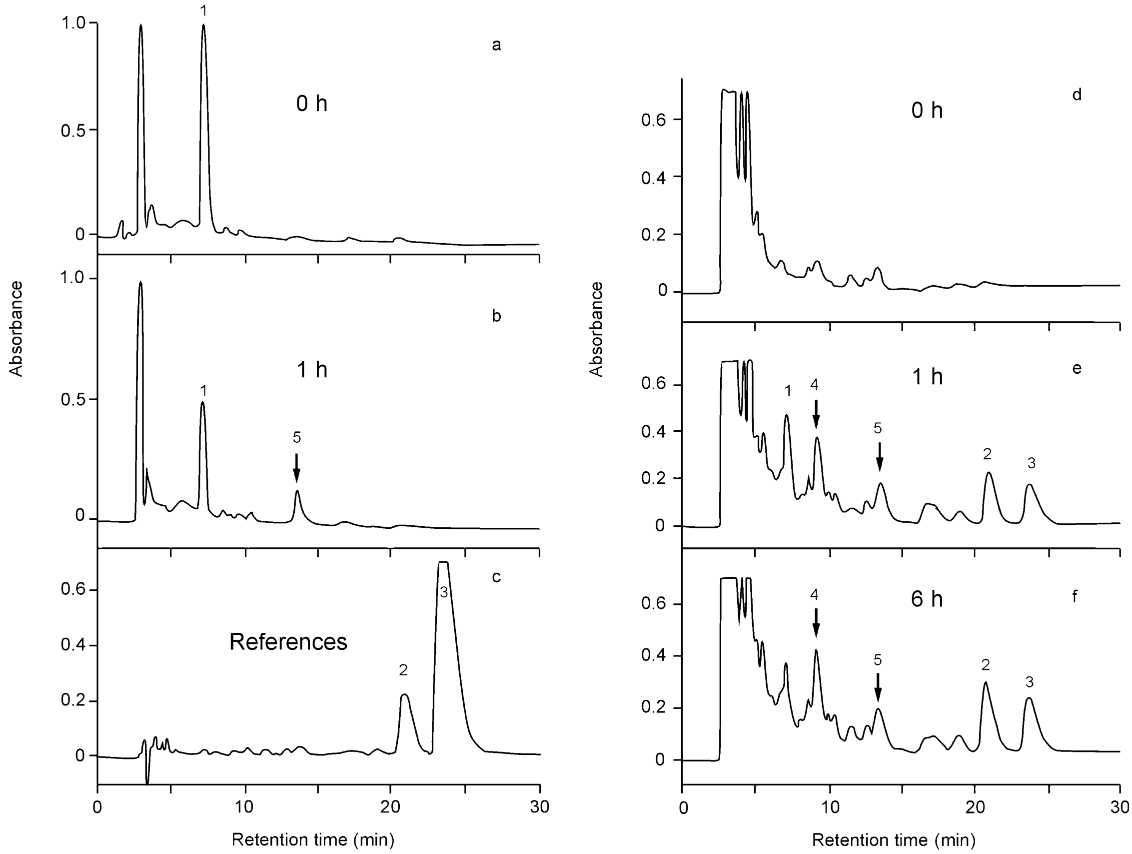

Fig. 3. HPLC chromatograms of media (a,b), Reference samples (c) and cells at different point in time (d–f) at 280 nm. Peak 1: stemmadenine, peak 2: catharanthine, peak 3: tabersonine, peaks 4 and 5 are unknown products after feeding.

at different points in time. A rapid depletion of the fed stemmadenine from the medium took place within an hour after feeding, completely disappearing after 3 h (Figure 2). Already 1 h after feeding stemmadenine, one new HPLC peak could be observed in the medium, possibly an oxidation product, which could have a more conjugated system, resulting in a UV-spectrum with absorption maxima at around 250 and 330 nm (Figure 3b). After 6 h no alkaloid peak could be detected in the medium. The peak observed in the medium was also found in the cells. For identification of the peak in the medium, the cell suspension system was fed with more stemmadenine and alkaloids were extracted from the medium and cells with ethyl acetate. This concentrated alkaloid extract was applied to a preparative TLC plate and eluted with a mobile phase consisting of *n*-butanol/acetic acid/water (4:1:1, by vol.). Ten bands were separated: the third one from the baseline with Rf 0.51 was identified as the compound of interest by HPLC. Further purification of this product was carried out using the HPLC system described previously. Its ^{1}H-NMR spectrum was in accordance with it being an indole alkaloid and showed signals due to four aromatic resonances such as H-9 at δ 7.38 (d, J = 7.2 Hz), H-10 at δ 6.96 (t, J = 7.2 Hz), H-11 at δ 7.2 (t, J = 7.6 Hz), H-12 at δ 6.95 (d, J = 7.2 Hz). In addition to these aromatic signals, H-15 at δ 4.1 (m), H-18 at δ 1.69 (d, J = 6.8 Hz), H-19 at δ 5.65 (q, J = 6.8 Hz), and H-22 at 3.76 (s) were assigned to ^{1}H-NMR signals of condylocarpine. These assignments were confirmed by HMBC correlations.

This sample was also analyzed by mass spectroscopy (ESI) which showed a molecular ion $[M+H]^{+}$ at *m/z* 323. From these data, the product was identified as condylocarpine. The final confirmation was done by HPLC, in which the product (peak) and condylocarpine showed the same retention time and UV spectrum.

At first, condylocarpine was thought to be an intermediate to catharanthine and tabersonine but feeding

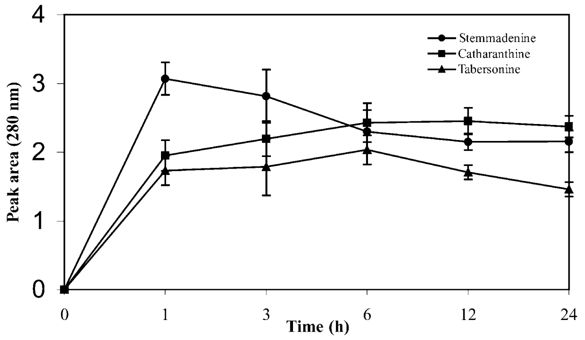

Fig. 4. Time course alkaloid accumulation in *Catharanthus roseus* cells after feeding stemmadenine. Data present the peak area of each compound at different time points calculated from the HPLC output chromatogram ($n = 2$).

condylocarpine to the cell suspension system did not result in the formation of either catharanthine or tabersonine. This suggests a non-specific conversion of stemmadenine to condylocarpine.

In the cells, stemmadenine could be observed after 1 h feeding and, at the same time, catharanthine and tabersonine were observed. After 6 h no further conversion of stemmadenine was observed until the end of the experiment. The total alkaloid levels detected at 24 h being the same as those detected at 6 h (Figure 4).

Microscopical detection of alkaloids showed that some cells did not produce tabersonine as no blue emission could be observed. On the other hand, all control and fed cells that were stained with Dragendorff's reagent were orange but with more intensity in the fed cells indicating that all cells were able to take up stemmadenine.

Figures 3d–f show the HPLC chromatograms of analyzed control and fed cells. Catharanthine and tabersonine HPLC peaks were clearly observed even in the early period (1 h) after feeding stemmadenine. Comparing the control (time 0) cell extract (Figure 3d) with that of cells fed for 1 h (Figure 3e), many more peaks appeared in the second one including one found in the medium suggesting that either more than one intermediate was involved in this pathway or other end products were formed. Van der Heijden *et al.* (1988) reported that incubation of stemmadenine with enzyme extract from cultured cells of *Tabernaemontana divaricata* resulted in formation of stemmadenine *N*-oxide, vallesamine, condylocarpine and its *N*-oxide in addition to an unidentified compound. Suggesting that the first step is formation of stemmadenine *N*-oxide, Van der Heijden *et al.* (1988) repeated the experiment replacing stemmadenine with its *N*-oxide but vallesamine was not formed and the incubation resulted in formation of condylocarpine and its *N*-oxide. Sandoval *et al.* (1962), stated that the formation of condylocarpine from stemmadenine is mechanistically straightforward and appears to involve initial introduction of a double bond next to the piperidine nitrogen followed by expulsion of formaldehyde and bond formation between the β-position of the indole system and carbon atom α to the piperidine nitrogen. Also Scott & Wei (1974), reported that through a series of chemical reactions starting with stemmadenine, condylocarpine and precondylocarpine were formed and the later was able to be converted to tabersonine after further reactions.

Conclusion

The *Catharanthus roseus* cell culture is capable of taking up and transforming stemmadenine within a few hours resulting in the accumulation of catharanthine and tabersonine. It seems that more than one conversion step is involved in this pathway. The culture showed two types of cells, one of them was able to convert stemmadenine to tabersonine. Identification of the products in this pathway requires further study as well as the enzymes involved in the bioconversion.

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