Human Alkaloid Biosynthesis: Chemical Inducers of Parkinson's Disease?

by

Stavroula K. Hatzios

Submitted to the Department of Chemistry
in Partial Fulfillment of the Requirements
for the Degree of

Bachelor of Science

at the

Massachusetts Institute of Technology

June 2005

Signature of the Author.....
Department of Chemistry
April 29, 2005

Certified by.....
Sarah O'Connor
Latham Family Career Development Assistant Professor of Chemistry
Thesis Supervisor

Accepted by.....
Rick Danheiser
A.C. Cope Professor of Chemistry
Associate Department Head

HUMAN ALKALOID BIOSYNTHESIS:

CHEMICAL INDUCERS OF PARKINSON'S DISEASE?

by

STAVROULA K. HATZIOS

Submitted to the Department of Chemistry
on April 29, 2005 in partial fulfillment of the
requirements for the Degree of Bachelor of Science in
Chemistry

ABSTRACT

The occurrence of certain alkaloids in the human brain appears to be associated with the onset of Parkinson's disease (PD). Recently, a human protein bearing homology to an alkaloid synthase in plants was identified. This protein, termed BSCv, may catalyze alkaloid formation in humans. If such activity is confirmed, regulation of BSCv through the use of small molecule inhibitors could provide novel drug therapies for PD patients.

This paper describes the first heterologous expression and purification of this transmembrane protein and examines its biological function through a series of enzyme assays. The assays used to evaluate enzyme activity were modeled after the Pictet-Spengler condensation catalyzed by the plant enzyme. Substrates were selected based on their potential to form alkaloids previously identified in central nervous system tissue. Product formation was monitored via high-performance liquid chromatography.

Preliminary data suggest that BSCv does not function as an alkaloid synthase. However, further studies are needed to ascertain such conclusions. Alternative detergents should be evaluated to assess their influence on enzyme activity. The use of an expanded substrate pool may also provide insight into protein function since substrate specificity may have restricted product formation in the performed assays. Finally, incubation of BSCv with rat brain extract, which contains another species homologue of the protein, could provide insight into its natural substrates. If these studies are unsuccessful, consideration should be given to the possibility that BSCv may function as a receptor.

Once the mechanistic and structural properties of the plant enzyme are elucidated, it may be possible to take a more direct approach to the characterization of its human homologue.

Thesis Supervisor: Sarah O'Connor

Title: Latham Family Career Development Assistant Professor of Chemistry

Stavroula K. Hatzios was born in Radford, Virginia, on September 15, 1983. She graduated from Blacksburg High School in 2001 and subsequently began undergraduate studies at the Massachusetts Institute of Technology in Cambridge, Massachusetts. She will be graduating in 2005 with a B.S. in chemistry and a minor in music. She plans to pursue graduate studies in chemistry at the University of California, Berkeley. This thesis is dedicated to her father, the late Dr. Kriton K. Hatzios.

TABLE OF CONTENTS

INTRODUCTION

Parkinson's disease (PD), a common movement disorder among the elderly associated with tremors and muscle rigidity, results from a dopamine deficiency in the striatum region of the brain. The degeneration of dopamine-producing neurons in the brain is the probable cause of PD [1]. Two types of alkaloids, β-carbolines and tetrahydroisoquinolines (TIQ), have been identified as potential chemical inducers of this neurodegeneration (Figure 1) [2]. It is believed that these compounds are synthesized through Pictet-Spengler condensations involving stereoselective enzyme catalysts [3]. Identification of the enzymes involved in these biosynthetic pathways could lead to the development of inhibitors that may result in novel treatments for PD.

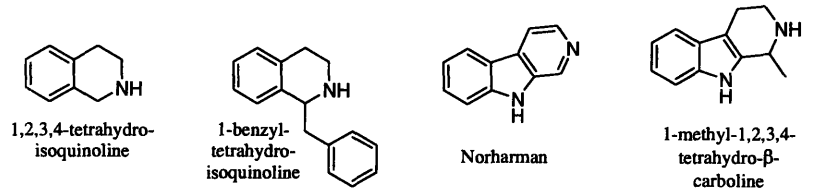


Figure 1 TIQ and β-carboline alkaloids implicated in PD [2].

Recently, a human protein bearing homology to strictosidine synthase (SS), an enzyme involved in plant β-carboline biosynthesis (Figure 2), was identified [2]. The gene that encodes this protein is known as *bscv* (C20orf3, accession #NP_065392) [4]. BSCv is predominantly expressed in the brain and bears some structural features of a membrane-bound protein (Figure 3) [2,5]. Importantly, it exhibits 39% similarity and 25% identity to SS (Figure 4) [6]. On the basis of this homology, it is hypothesized that BSCv functions as an endogenous synthase of human β-carbolines.

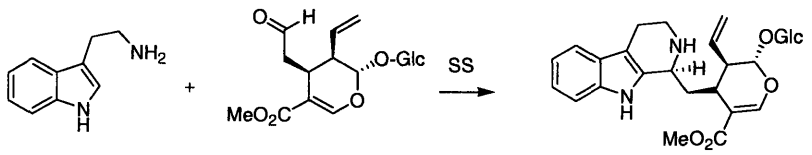


Figure 2 Stereoselective Pictet-Spengler condensation catalyzed by SS in *Catharanthus roseus* [2].

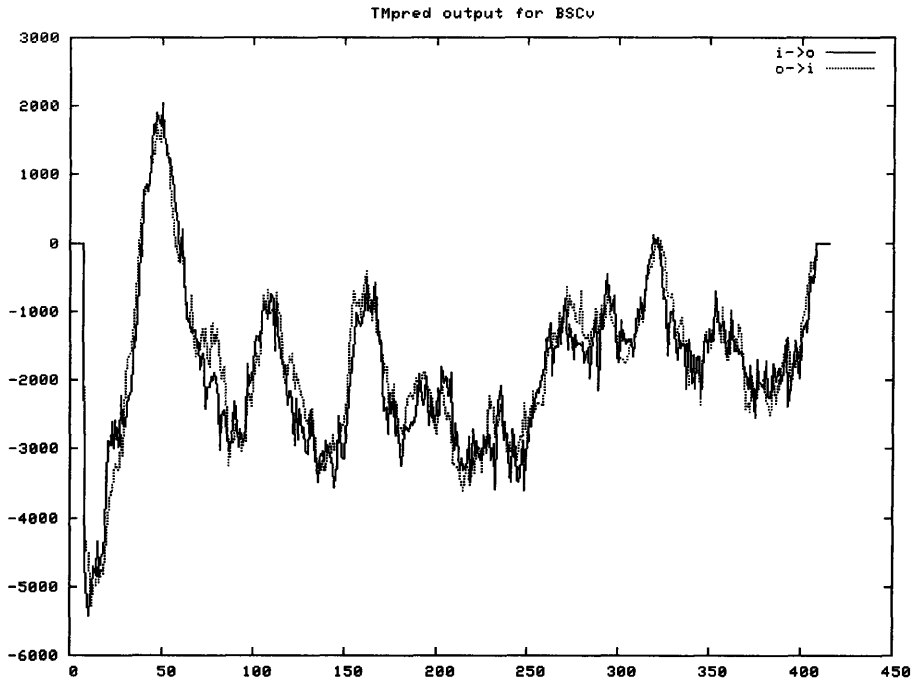


Figure 3 Transmembrane region prediction for BSCv, a 416-residue protein, generated using the TMpred algorithm. Sequence positions are plotted in increasing order along the x-axis. The TMpred output is plotted along the y-axis. Sequence regions generating a score over 500 have a significant probability of containing a transmembrane helix. The sequence region between residues 38 and 61 of BSCv produces a TMpred score of 2051, strongly suggesting the presence of a 24-residue, N-terminal transmembrane helix [5].

```
BSCv:  40   RVTFLMLAVSLTVPLLGAMMLLESPIDPQPLSFKEPPLLLGVLHPNTKLRQAERLFENQL  99
SS:    1    LSFFLFIALFLLISLLLYSLLTDASFPFKEYRLGPPPTLLGPLSFNFDLTGAEFYTVGQV  60

BSCv:  100  VGPESIAHIGD--VMFTGTADGRVVKLENGEIETIARFGSGPCKTRDDEPVCGRPLGIRA  157
SS:    61   QGPESIKYDGENNLIYTGIVDGAVIRVSSNDSLCD----GSPCDSVKTEPLCGRPLGLAF  116

BSCv:  158  G-PNGTLFVADAYKGLFEVNPWKREVKLLLSSETPIEGKNMSFVNDLTVTQDGRKIYFTD  216
SS:    117  DKQGGDLYIADAYLGLLKVGP---EGGKAELLTDEVDGVPFKFLNDLDVDPEGV-VYFTD  172

BSCv:  217  SSSKWQRRDYLLLVMEGTDDGRLLEYDTVTREVKVLLDQLRFPNGVQLSPAEDFVLVAET  276
SS:    173  SSSKYDRRDFIFAMLEGDPTGRLLKYDPSTKVTTVLLDELYFPNGLALSPDGSFVLVAET  232
```

```
BSCv:  277  TMARIRRVYVSGLMKGGADLFVENMPGFPDNIRPSSSGGYWVGMSTIRPNPGFSMLDFLS  336
SS:    233  PMARIRKYWLKGPKAGTSEVFAEGLPGYPDNIRRDGDGHFWVALVSHRS----TLWRLLM  288

BSCv:  337  ERPWIKRMIFKLFSQETVMKFVPRY-----SLVLELSDSGAFRRSLHDPDGLVATYISEV  391
SS:    289  SYPWVRKFLAKLLKLE-VLPLLPLNGKNPHAGVVKVDSDGNIIESLHDSTDKVGSLISEV  347

BSCv:  392  HEHDGHLYLGSFRSPFL  408
SS:    348  EEIDGHLYLGSVLNSYI  364
```

Figure 4 Sequence alignment of BSCv and SS. Conserved residues are shown in bold [6].

To investigate this possibility, an efficient expression system was established for the *bscv* gene and a series of *in vitro* enzyme assays were conducted to determine whether the resulting protein plays a role in β-carboline or TIQ biosynthesis. Characterization of BSCv through these studies may not only advance our understanding of PD, but also expand our limited knowledge of human alkaloid biosynthesis.

MATERIALS AND METHODS

Saccharomyces cerevisiae Expression Plasmid

The *bscv* gene was PCR-amplified from a commercially available Invitrogen clone (#I-3530962) containing all but the first 15 5' nucleotide residues [2]. The missing residues were added by PCR using the primer 5'-AAAAAAACATTATGCACGAGGTGGTTTCTGCGGGTG AGGCTGGCG- 3'. The recombinant construct was PCR-amplified using primers 5'-AAAAAA AAGCTTCACGAGGTGGTTTCTGCGGGTGAGGCTGGCGCCCGTACCATG-3' (*HindIII*) and 5'-TTTTTTCTCGAGCTAAACAGCCTGGAGGCTGAGTCTGCAGAGGAAGGGGGAC

CTGAAAGAGCCCAGGTACAGGTGCCCA-3' (*XhoI*). The resulting 1.29-kilobase (kb) fragment was digested with *HindIII* and *XhoI* and ligated with similarly digested pYES-His vector, a high copy plasmid with an inducible *GAL1* promoter, N-terminal His-tag, and *URA3* selection marker [7].

Expression of *bscv* in *S. cerevisiae*

The plasmid was transformed into the proteinase-deficient *S. cerevisiae* strain BJ5465, made competent with lithium acetate. Selective plates of yeast minimal medium minus uracil were used to cultivate the transformed yeast cells. A culture containing 20 mL of the same medium with 2% wt/vol glucose as the carbon source was innoculated with a single colony and grown for 18 hours at 30°C with shaking at 250 rpm. The culture was washed two times with the same volume of medium containing 2% raffinose as the carbon source. Four 2 L baffled Fernbach flasks each containing 500 mL of yeast minimal medium minus uracil with 2% raffinose were innoculated with 5 mL of the culture. After approximately 60 hours at 30°C with shaking at 200 rpm (OD_{600}=1.6-1.8), 10 g of solid galactose (2% wt/vol) were added to each flask. The yeast cells (approximately 4-6 g/L) were harvested after 6 hours using a Sorvall RC5C centrifuge (5000 rpm, 10 minutes, 25°C, SLC-4000 rotor) and stored at -80°C [7].

Purification of BSCv

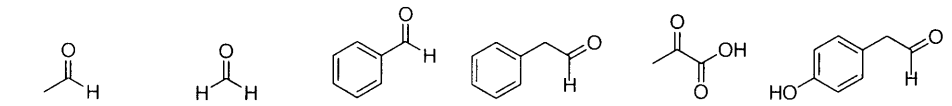
The harvested cells were thawed and suspended in 10 mL of breaking buffer (6 g sodium phosphate monobasic, 50 mL glycerol, and 200 mM sodium chloride dissolved in 900 mL water

and adjusted to pH 7) at 4°C. Protease inhibitors (pepstatin A and leupeptin) were added to a final concentration of 0.5 μg/mL. The cells were combined with 15 mL of 0.5 mm glass beads and lysed in a Biospec Bead Beater (Model 1107900) by beating and cooling the mixture on alternating one-minute intervals for a total of 10 minutes. The mixture was then incubated with detergent (0.5% Triton X-100, 1% lysophosphatidylglycerol, or 0.5% sodium deoxycholate) at 4°C for one hour with shaking [8,9]. The mixture was decanted from the beads and centrifuged for 30 minutes (15,000 rpm, 4°C, SS-34 rotor). Aliquots of the cell lysate and cell debris were set aside for future analysis by SDS-PAGE and Western Blot. The remaining cell lysate was incubated for 1 hour with shaking at 4°C with 1 mL of Talon cobalt metal affinity resin. The column was washed with 10 mL of breaking buffer, and BSCv was eluted using 2 mL of 500 mM imidazole. SDS-PAGE and Western Blot analysis using a His-specific antibody as a probe were used to analyze the column fractions. Purified protein was dialyzed against breaking buffer using a Slide-A-Lyzer Dialysis Cassette (10,000 MWCO) and stored at -80°C.

Enzyme Activity Assays

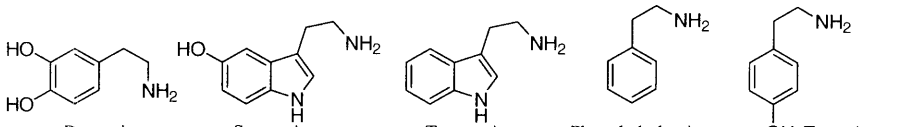
The reaction mixture (50 μL total volume) for each enzyme assay contained 900 μM amine substrate, 900 μM aldehyde substrate, and 30 μL of purified BSCv in 40 mM potassium phosphate buffer (pH 7.0). Aldehyde substrates included acetaldehyde, formaldehyde, benzaldehyde, phenylacetaldehyde, pyruvic acid, and 4-hydroxy-phenylacetaldehyde (Figure 5A). Amine substrates included dopamine, serotonin, tryptamine, phenylethylamine, and tyramine (Figure 5B). All possible combinations of substrates were tested.

A. Aldehyde Substrates



Acetaldehyde Exact Mass 44.03 | Formaldehyde Exact Mass 30.01 | Benzaldehyde Exact Mass 93.03 26.7 (254 nm) | Phenylacetaldehyde Exact Mass 120.06 19.6 (266 nm) | Pyruvic Acid Exact Mass 88.02 13.0 (254 nm) | 4-Hydroxy-phenyl-acetaldehyde Exact Mass 136.05 16.8

B. Amine Substrates



Dopamine Exact Mass 153.08 11.3 | Serotonin Exact Mass 176.09 13.7 | Tryptamine Exact Mass 160.10 16.4 | Phenylethylamine Exact Mass 121.09 15.2 (264 nm) | Tyramine Exact Mass 137.08 12.7

Figure 5 Aldehyde (A) and amine (B) substrates used in BSCv activity assays. The exact mass and HPLC retention time for each substrate is shown. Retention times are given in minutes and were calculated on a 0-70% acetonitrile gradient. HPLC traces for each substrate (data not shown) were analyzed at a wavelength of 280 nm except where noted. No HPLC data is available for acetaldehyde and formaldehyde (these substrates do not absorb in the UV range).

The reactions (Figure 6) were initiated by addition of BSCv. Each mixture was incubated for 1 hour at room temperature and then analyzed by HPLC in 0-70% acetonitrile in water with 0.1% trifluoroacetic acid over 25 minutes with a flow rate of 1 mL/min. HPLC was carried out using a System Gold HPLC (C18 reverse-phase column) from Beckman Coulter, Inc. Negative control assays were conducted using the cell lysate of a culture containing empty expression vector to rule out potential nonenzymatic reactions.

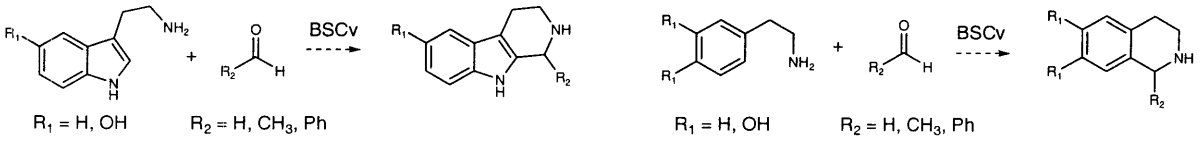


Figure 6 Sample enzyme assays.

Enzyme Activity Assays Using Radiolabeled Tryptamine as the Amine Substrate

The reaction mixtures were prepared following the above specifications. [2-^{14}C]-Tryptamine bisuccinate (50 mCi/mmol) purchased from American Radiolabeled Chemicals was combined with acetaldehyde, formaldehyde, benzaldehyde, and phenylacetaldehyde in separate reactions. After the 1 hour incubation period, the reactions were spotted on 2.5 x 6.5 cm silica gel 60 (250 μM layer) plates and monitored by thin-layer chromatography (TLC) in a butanol:acetic acid:water (4:1:1) solvent system [10]. The plates were exposed to x-ray film, stored at -80°C, and developed after two days. Control assays were conducted using the cell lysate of a culture containing empty expression vector.

Synthesis of Authentic Standards

All reagents and solvents were obtained from Aldrich and were used without further purification. Thin-layer chromatography (TLC) using 2.5 x 6.5 cm silica gel 60 (250 μM layer) plates with ninhydrin detection was used to monitor the synthesis of authentic standards. Each compound was concentrated by rotary evaporation, dissolved in a suitable solvent mixture, and centrifuged for 2 minutes at room temperature in a Sorvall Biofuge pico benchtop centrifuge (13,000 rpm) to remove particulates. The supernatant was passed through an Acrodisc syringe filter with a 0.2 μM HT Tuffryn membrane. A small aliquot was diluted fifty-fold in distilled water for analysis by HPLC. Possible product peaks were collected, concentrated on a Savant Speedvac Concentrator, and dissolved in a 5:1 water-ethanol mixture for analysis by TOF electrospray mass spectrometry (Mariner Applied Biosystems mass spectrometer, positive-ion

mode). Once the product peak was identified, the remaining sample was purified using preparative HPLC. A solvent gradient of 10-60% acetonitrile in water with 0.1% trifluoroacetic acid over 25 minutes with a flow rate of 9 mL/min was used. The combined fractions were concentrated by rotary evaporation and the resulting product dissolved in enough water to give an approximately 2.5 mg/mL solution. All standards were stored at -20°C.

1-Methyl-1,2,3,4-tetrahydro-β-carboline was prepared by dissolving tryptamine (100 mg, 0.625 mmol), acetaldehyde (70 µL, 1.25 mmol), and trifluoroacetic acid (116 µL, 1.5 mmol) in dichloromethane (1 mL) and stirring the reaction mixture at room temperature for 4 hours. A 4:1 dichloromethane-methanol TLC solvent system was used to monitor the progress of the reaction. The mixture was combined with an additional 10 mL of dichloromethane and washed with 1 mL of saturated sodium bicarbonate solution. Solvent was evaporated from the organic layer to isolate the product [11]. Orange solid; HPLC retention time (min): 16.9, 0-70% acetonitrile gradient; MS (m/z): MH^+ of 187.1; expected mass: 186.12 (Figures 7 and 8).

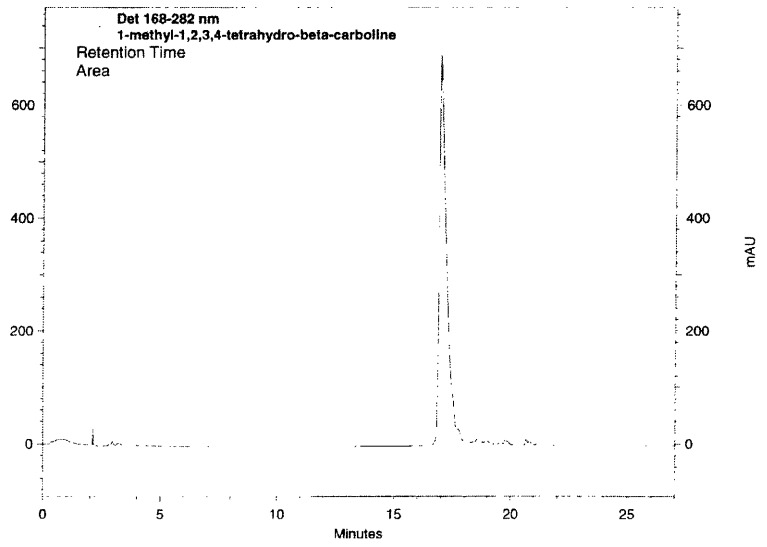


Figure 7 HPLC data for 1-methyl-1,2,3,4-tetrahydro-β-carboline.

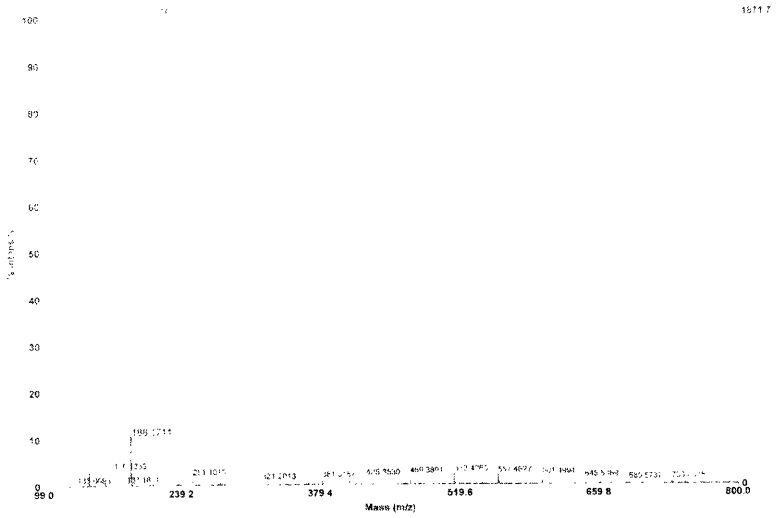


Figure 8 MS data for 1-methyl-1,2,3,4-tetrahydro-β-carboline.

5-Hydroxy-methtryptoline was prepared following the same procedure used for 1-methyl-1,2,3,4-tetrahydro-β-carboline, starting from serotonin hydrochloride (20 mg, 0.094 mmol), acetaldehyde (10 μL, 0.19 mmol), and TFA (8.7 μL, 0.113 mmol). The mixture was dissolved in a solution of methanol and dichloromethane. The crude product was isolated by rotary evaporation without prior extraction [11]. Pink solid; HPLC retention time (min): 15.6, 0-70% acetonitrile gradient; MS (m/z): MH^+ of 203.1; expected mass: 202.11 (Figures 9 and 10).

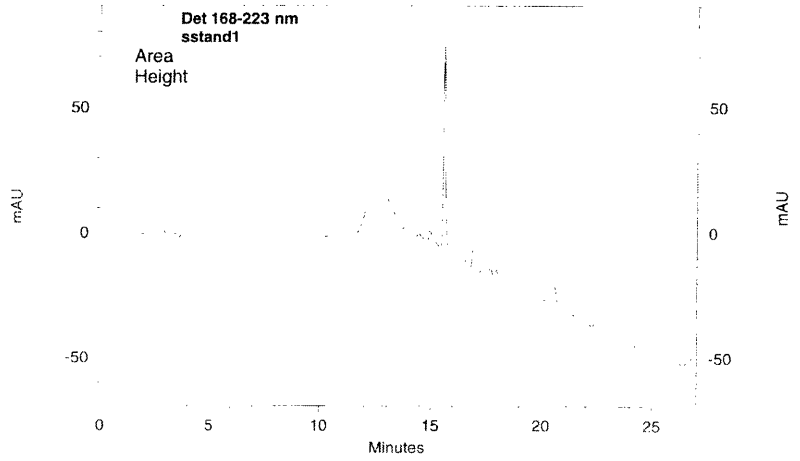


Figure 9 HPLC data for 5-hydroxy-methtryptoline.

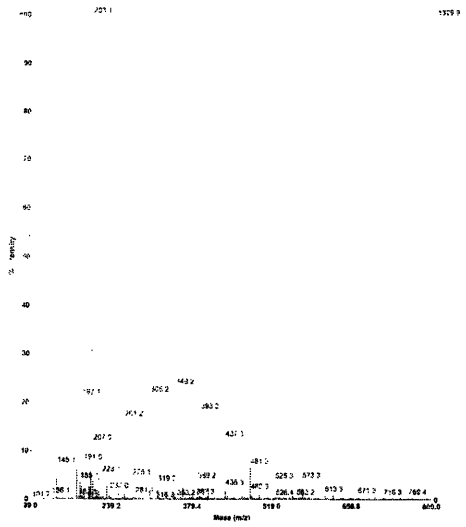


Figure 10 MS data for 5-hydroxy-methtryptoline.

Salsolinol was prepared by dissolving dopamine hydrochloride (100 mg, 0.530 mmol) in methanol (2 mL) and adding 8 equivalents of acetaldehyde (240 µL, 4.22 mmol) to the reaction mixture. The mixture was adjusted to pH 6 by addition of TFA and stirred for 24 hours at room temperature. The product was isolated by rotary evaporation [12]. Two product isomers were obtained. ^{1}H-NMR and ^{13}C-NMR were used to distinguish the correct isomer (data not shown). Preparative HPLC was used to purify the desired product. White solid; HPLC retention time (min): 13.7, 0-70% acetonitrile gradient; MS (m/z): MH^{+} of 180.1; expected mass: 179.09 (Figures 11 and 12).

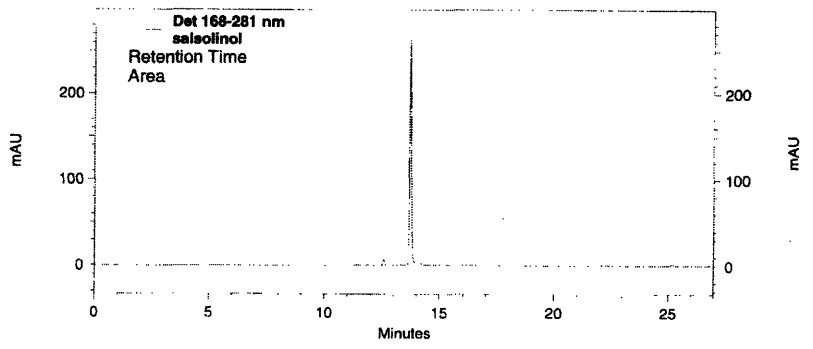


Figure 11 HPLC data for salsolinol.

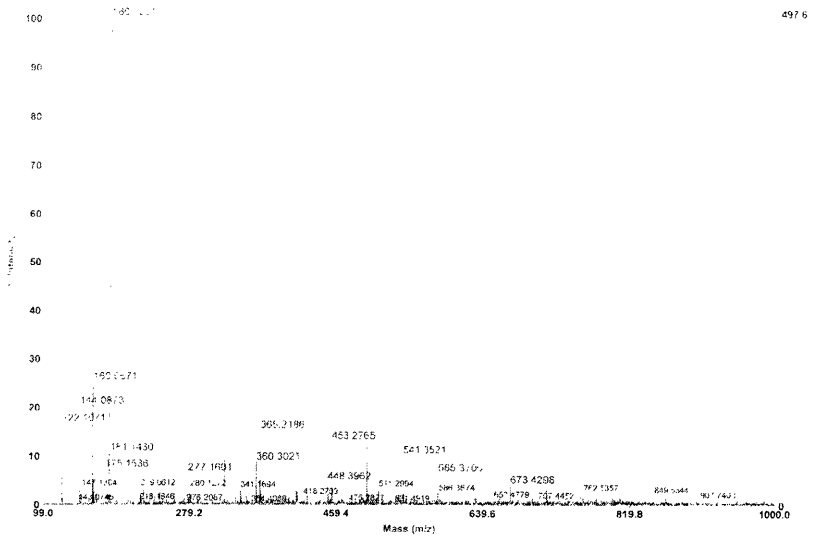


Figure 12 MS data for salsolinol.

1-Benzyl-1,2,3,4-tetrahydro-isoquinoline-6,7-diol was prepared by following the same procedure used for salsolinol, starting from dopamine hydrochloride (100 mg, 0.530 mmol) and phenylacetaldehyde (472 µL, 4.22 mmol). The reaction mixture was adjusted to pH 3 by addition of TFA. The product was isolated by rotary evaporation [12]. Yellow solid; HPLC retention time (min): 17.2, 0-70% acetonitrile gradient; MS (m/z): MH^+ of 256.2; expected mass: 255.13 (Figures 13 and 14).

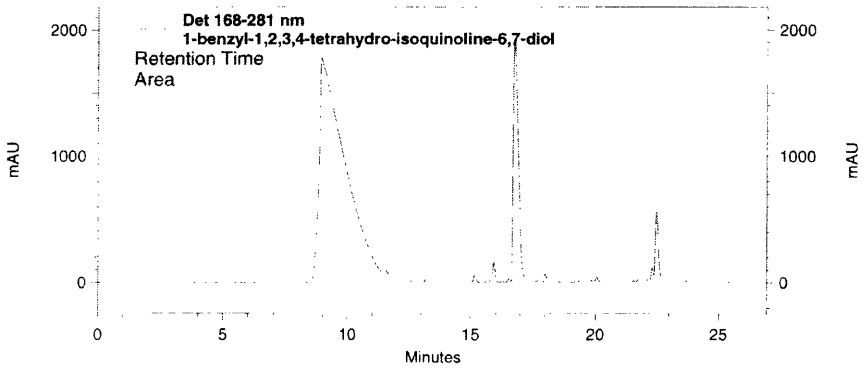


Figure 13 HPLC data for 1-benzyl-1,2,3,4-tetrahydro-isoquinoline-6,7-diol. This standard was not subjected to further purification by preparative HPLC and contains substrate impurities at retention times of 8.9 minutes (dopamine) and 22.5 minutes (phenylacetaldehyde).

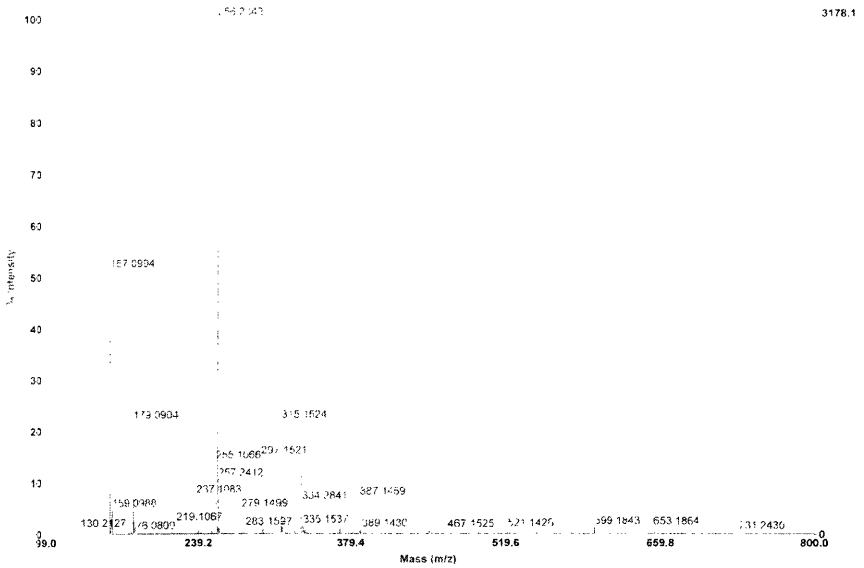


Figure 14 MS data for 1-benzyl-1,2,3,4-tetrahydro-isoquinoline-6,7-diol.

RESULTS

Expression of *bscv* in *S. cerevisiae*

The expression levels of *bscv* in *S. cerevisiae* were optimized by galactose induction at OD_{600} values at approximately 1.6. The protein was expressed with a N-terminal $(His)_6$ tag to facilitate purification. An uninduced culture was prepared following the specifications described in *Materials and Methods* to monitor expression activity. An additional culture containing empty expression vector was similarly prepared and retained for use in negative control assays.

Purification of BSCv

The addition of detergent to the lysed *S. cerevisiae* cells was necessary to procure soluble protein. Purification attempts in which no detergent was added resulted in the localization of all protein to the cell debris. Triton X-100 successfully solubilized the protein and facilitated purification by cobalt-affinity chromatography (Figures 15 and 16). Protein prepared in this manner was used in subsequent enzyme assays. Lysophosphatidylglycerol (LPG) was also successful at solubilizing BSCv, but impeded binding to the cobalt resin (Figures 17 and 18). Sodium deoxycholate failed to solubilize BSCv.

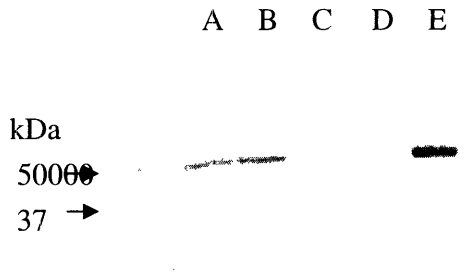


Figure 15 Western Blot analysis of purified BSCv, a 49 kDa protein with a N-terminal $(His)_6$ tag. A) Protein localized to cell debris; B) Protein suspended in cell lysate; C) Column flow-through; D) Column wash; E) 500 mM imidazole elution.

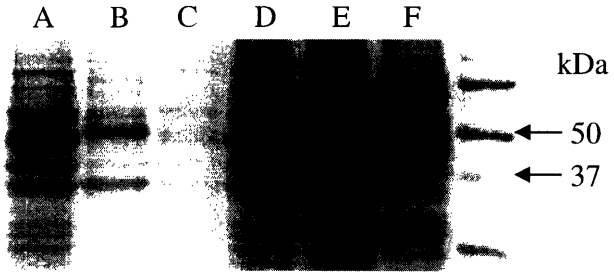


Figure 16 SDS-PAGE analysis of purified BSCv. A) Uninduced cell lysate; B) 500 mM imidazole elution; C) Column wash; D) Column flow-through; E) Cell lysate; F) Cell debris.

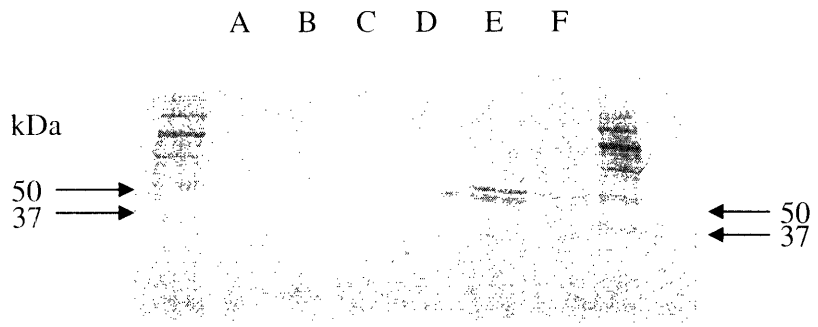


Figure 17 Western Blot analysis of BSCv solubilized with LPG and purified using cobalt-affinity chromatography. A) Second 500 mM imidazole elution; B) First 500 mM imidazole elution; C) Column wash; D) Column flow-through; E) Cell lysate; F) Cell debris. Proteolysis may explain the presence of two bands in lanes E and F.

Figure 18 SDS-PAGE analysis of BSCv solubilized with LPG and purified using cobalt-affinity chromatography. A) Cell debris; B) Cell lysate; C) Column flow-through; D) Column wash; E) First 500 mM imidazole elution; F) Second 500 mM imidazole elution.

Enzyme Activity Assays

The aldehyde and amine substrates used in BSCv activity assays were selected based on their potential to form β-carboline and TIQ structures that have previously been identified in central nervous system tissue [3,9]. Reaction conditions were drawn from related studies involving known enzymes of similar function.

Thus far, no enzyme activity has been observed among the assayed substrates. The assays have produced HPLC traces identical to those produced by control assays in which the cell lysate of a culture containing empty expression vector was substituted for BSCv (Figure 19).

Additional control assays in which the reaction substrates were individually substituted with water and the enzyme with breaking buffer have corroborated these findings.

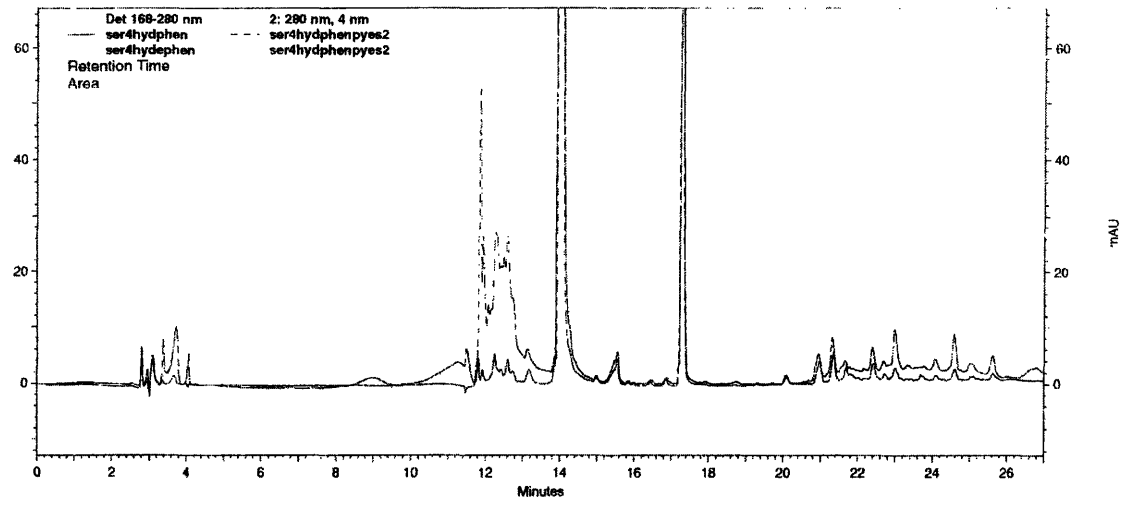


Figure 19 HPLC traces of a BSCv assay using serotonin and 4-hydroxy-phenylacetaldehyde as substrates. The solid-line trace represents the assay under experimental conditions (i.e. with the addition of enzyme). The dashed-line trace represents a control assay in which the enzyme was substituted with the cell lysate of a yeast culture expressing empty pYES2 vector.

Enzyme Activity Assays Using Radiolabeled Tryptamine as the Amine Substrate

Radioassays using [2-^{14}C]tryptamine bisuccinate as the amine substrate were employed to provide a more sensitive test of enzyme activity. TLC analysis failed to yield any promising results. The chromatograms of the experimental assays were identical to those of control assays in which cell lysate containing empty expression vector was substituted for BSCv (Figure 20).

Figure 20 Enzyme Assays Using [2-^{14}C]Tryptamine bissuccinate as the substrate. A-D: Control assays using cell lysate with empty expression vector; E-H: BSCv assays. A), E) Tryptamine + acetaldehyde; B), F) Tryptamine + formaldehyde; C), G) Tryptamine + benzaldehyde; D), H) Tryptamine + phenylacetaldehyde. Note: The solvent front of samples E-H was higher than that of samples A-D, which accounts for the discrepancy in sample migration.

Synthesis of Authentic Standards

In order to assess product formation in HPLC-monitored assays, it was necessary to synthesize authentic standards of Pictet-Spengler condensation products lacking commercial availability. These standards were used to determine product retention times and to validate potential products in a given reaction mixture. The four synthetic standards were isolated to varying degrees of purity. Mass spectrometry was used to ascertain the identity of the standards. At this preliminary stage, it was not deemed necessary to perform complete NMR characterization of these compounds. The chemistry is well established. In the event of enzymatic product formation, the authentic standards would be more rigorously characterized.

DISCUSSION

Isoquinoline derivatives and β-carbolines are widely regarded as endogenous neurotoxins [1,13-16]. Moreover, a number of these compounds appear to stimulate the onset of PD through oxidative stress [1,17]. Recent years have seen the isolation and characterization of several synthases involved in the production of such compounds in rats [3,9]. The discovery of a comparable human enzyme could revolutionize our approach to the study and treatment of PD.

Two significant findings support the existence of endogenous alkaloid synthases in humans. First, a SS gene family has been identified that spans a wide range of species, among them mouse and human. Interestingly, the human gene (*bscv*) is predominantly expressed in the brain, where alkaloid-induced neurodegeneration occurs [18]. Second, a recent study by *Poeaknapo et al.* indicates that human cells are able to synthesize morphine [19]. This finding supports the existence of at least one human alkaloid biosynthetic pathway and reinforces the notion that other alkaloids may be produced endogenously.

To clarify whether BSCv plays a role in alkaloid formation, a series of enzyme assays modeled after the SS Pictet-Spengler condensation were conducted. Substrates were selected based on their relevance to central nervous system tissue and product formation was monitored via HPLC. These assays showed no indication of enzyme activity. The HPLC traces produced under experimental conditions were identical to those produced by control reactions lacking BSCv. The limited number of assays performed using radiolabeled tryptamine yielded similar results. These initial studies appear to contradict the hypothesis that BSCv possesses catalytic activity.

Importantly, hydropathy data suggest that BSCv contains a hydrophobic region at its N-terminus and bears some structural features of a membrane-bound protein (Figure 3). A previous study involving the isolation of a membrane-bound isoquinoline synthase from rat brain indicates that the use of nonionic detergents such as Triton X-100 to solubilize the protein interferes with enzyme activity [9]. These findings prompted the application of milder ionic detergents to the solubilization of BSCv. Sodium deoxycholate, the detergent used to successfully purify the aforementioned rat isoquinoline synthase, failed to liberate BSCv from the cell debris. However, a second detergent, lysophosphatidylglycerol (LPG), shown previously to solubilize the Cystic Fibrosis transmembrane conductance regulator (CFTR) from yeast cells, was successful in solubilizing BSCv [8]. Unfortunately, this detergent appears to impede purification via cobalt-affinity chromatography. The evaluation of additional detergents may lead to more promising agents of BSCv purification.

In addition to exploring different detergents, the successful design of a soluble version of BSCv may facilitate its characterization. To this end, a truncated version of *bscv* was generated. Using the protein's TMpred plot, the presumed transmembrane region was identified and selectively removed. Primers designed to omit the first 297 base pairs of DNA were used during PCR amplification, eliminating the first 99 residues of the protein. The shortened version of the gene, termed *sbscv*, was cloned into the pYES-His vector, transformed into yeast, and expressed as described previously (see *Materials and Methods*). Subsequent purification and analysis of sBSCv by Western Blot and SDS-PAGE indicated that the protein was retained in the cell pellet following lysis (data not shown). This suggests that the protein is still membrane-associated. Because it is often challenging to predict the location of membrane-associated sequences with

precision and to anticipate the impact of their removal on protein folding and function, this may not be the most efficient approach to purification of active enzyme.

Despite the attractive possibility that disruptive protein purification is responsible for abolishing the alkaloid synthase activity of BSCv, other factors may explain its failure to elicit alkaloid formation in the described assays. Substrate specificity, for example, may impede enzyme activity. Substrates used here were selected based on their relevance to potential alkaloid products. The aldehydes and amines used in the assays may not resemble the natural substrates of BSCv enough to react with the enzyme. One approach to this problem would be to expand the pool of substrates. 3,4-Dihydroxy-phenylacetaldehyde, utilized in morphine biosynthesis, and histamine are two possibilities. Secologanin, the aldehyde substrate in the SS reaction, is another consideration. While physiologically insignificant, it may provide insight into the structural properties of the protein's natural aldehyde substrate. Alternatively, a less stochastic approach to substrate determination would be to immobilize BSCv on cobalt resin and then incubate it with rat brain extract to see if any small molecules bind the protein. After eluting BSCv with imidazole, ethanol or acetone could be used to precipitate BSCv. Any remaining small molecules in solution could then be separated by HPLC and analyzed by mass spectrometry. The use of a rat model system should be effective in this study as BSCv and SS homology extends to this species (BSCv exhibits 78% identity and 82% similarity to the rat protein) [5].

Clearly, additional studies are needed to assess the enzymatic capabilities of BSCv. Alternative purification protocols and substrate screens may facilitate this process. If a soluble version of the protein is successfully prepared, structural studies could be used to compare BSCv to its known homologues as their structures become available. Once the catalytically essential

residues of SS become clear, structural comparisons may help elucidate the function of the human protein. Characterization of other SS homologues may also suggest how accurate sequence homology is in predicting activity within this gene family. Since the structural properties of BSCv suggest that it is membrane-bound, an alternative possibility exists that BSCv may act as a receptor. Ligand-binding assays may confirm this hypothesis. Until these avenues are thoroughly explored, the role of BSCv in human alkaloid biosynthesis may remain an enigma.

ACKNOWLEDGEMENTS

I am very grateful to Dr. Sarah E. O'Connor for her invaluable guidance, vision, and support. I would also like to thank the members of the O'Connor lab for many insightful conversations. The pYES-His expression plasmid and BJ5465 yeast strain used in these studies were gifts from Deborah Perlstein. I would like to thank Ms. Perlstein and the Stubbe group at the Massachusetts Institute of Technology for helpful guidance on the topic of yeast genetics and related technical assistance. Most importantly, I would like to thank my family and friends for their constant support. This work was funded in part by the Undergraduate Research Opportunities Program (UROP) at the Massachusetts Institute of Technology.

REFERENCES

1. Nagatsu, T. (1997) Isoquinoline neurotoxins in the brain and Parkinson's disease. *Neurosci. Res.* **29**, 99-111.

2. O'Connor, S. (2003). Human biosynthesis of alkaloids: chemical inducers of Parkinson's disease. Grant Proposal.

3. Naoi, M., Maruyama, W., Dostert, P., Kohda, K., and Kaiya, T. (1996) A novel enzyme enantio-selectively synthesizes (*R*)salsolinol, a precursor of a dopaminergic neurotoxin, N-methyl(*R*)salsolinol. *Neurosci. Lett.* **212**, 183-186.

4. Morita, M., Hara, Y., Tamai, Y., Arakawa, H., and Nishimura, S. (2000) Genomic construct and mapping of the gene for *CMAP* (leukocystatin/cystatin F, CST7) and identification of a proximal novel gene, *BSC*v (C20orf3). *Genomics* **67**, 87-91.

5. http://www.ncbi.nlm.nih.gov/BLAST/

6. http://searchlauncher.bcm.tmc.edu/seq-search/struc-predict.html

7. Nguyen, H.T., Ge, J., Perlstein, D.L., and Stubbe, J. (1999) Purification of ribonucleotide reductase subunits Y1, Y2, Y3, and Y4 from yeast: Y4 plays a key role in diiron cluster assembly. *Proc. Natl. Acad. Sci. USA* **96**, 12339-12344.

8. Huang, P., Liu, Q., and Scarborough, G.A. (1998) Lysophosphatidylglycerol: A novel effective detergent for solubilizing and purifying the Cystic Fibrosis transmembrane conductance regulator. *Analytical Biochemistry* **259**, 89-97.

9. Yamakawa, T. and Ohta, S. (1997) Isolation of 1-methyl-1,2,3,4-tetrahydroisoquinoline-synthesizing enzyme from rat brain: a possible Parkinson's disease-preventing enzyme. *Biochem. Biophys. Res. Commun.* **236**, 676-681.

10. McIsaac, W.M., Ho, B.T., Estevez, V., and Powers, D. (1967) Chromatography of β-carbolines. *J. Chromatog.* **31**, 446-454.

11. Jiang, W., Zhang, X., and Sui, Z. Potassium superoxide as an alternative reagent for winterfeldt oxidation of β-carbolines: supplemental material.

12. Bates, H.A., Bagheri, K., and Vertino, P.M. (1986) Effect of pH on the regioselectivity of pictet-spengler reactions of 3-hydroxyphenethylamines with formaldehyde and acetaldehyde. *J. Org. Chem.* **51**, 3061-3063.

13. Matsubara, K., Gonda, T., Sawada, H., Uezono, T., Kobayashi, Y., Kawamura, T., Ohtaki, K., Kimura, K., and Akaike, A. (1998) Endogenously occurring β-carboline induces Parkinsonism in nonprimate animals: a possible causative protoxin in idiopathic Parkinson's Disease. *J. Neurochem.* **70**, 727-735.

14. Naoi, M., Maruyama, W., and Nagy, G.M. (2004) Dopamine-derived salsolinol derivatives as endogenous monoamine oxidase inhibitors: occurrence, metabolism and function in human brains. *NeuroToxicology* **25**, 193-204.

15. Yamakawa, T. and Ohta, S. (1999) Biosynthesis of a Parkinsonism-preventing substance, 1-methyl-1,2,3,4-tetrahydroisoquinoline, is inhibited by Parkinsonism-inducing compounds in rat brain mitochondrial fraction. *Neurosci. Lett.* **259**, 157-160.

16. Foppoli, C., Coccia, R., Blarzino, C., Cini, C., and Rosei, M.A. (2002) Tetrahydroisoquinoline derivatives of enkephalins: synthesis and properties. *Biochem Pharmacol* **63**, 1885-1892.

17. Sango, K., Maruyama, W., Matsubara, K., Dostert, P., Minami, C., Kawai, M., and Naoi, M. (2000) Enantio-selective occurrence of (*S*)-tetrahydropapaveroline in human brain. *Neurosci. Lett.* **283**, 224-226.

18. Fabbri, M., Delp, G., Schmidt, O., and Theopold, U. (2000) Animal and plant members of a gene family with similarity to alkaloid-synthesizing enzymes. *Biochem. Biophys. Res. Commun.* **271**, 191-196.

19. Poeaknapo, C., Schmidt, J., Brandsch, M., Dräger, B., and Zenk, M.H. (2004) Endogenous formation of morphine in human cells. *Proc. Natl. Acad. Sci. USA* **101**, 14091-14096.