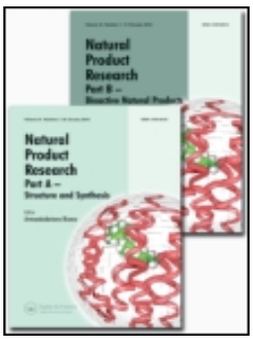

Natural Product Research

ISSN: 1478-6419 (Print) 1478-6427 (Online) Journal homepage: http://www.tandfonline.com/loi/gnpl20

Construction and expression of a dual vector for chemo-enzymatic synthesis of plant indole alkaloids in Escherichia coli

Joachim Stöckigt , Bodo Hammes & Martin Ruppert

To cite this article: Joachim Stöckigt , Bodo Hammes & Martin Ruppert (2010) Construction and expression of a dual vector for chemo-enzymatic synthesis of plant indole alkaloids in Escherichia coli , Natural Product Research, 24:8, 759-766, DOI: 10.1080/14786410903247304

To link to this article: http://dx.doi.org/10.1080/14786410903247304

Published online: 29 Apr 2010.

Natural Product Research
Vol. 24, No. 8, 10 May 2010, 759–766

Taylor & Francis
Taylor & Francis Group

Construction and expression of a dual vector for chemo-enzymatic synthesis of plant indole alkaloids in *Escherichia coli*

Joachim Stöckigt[ab]*, Bodo Hammes[b] and Martin Ruppert[b]

[a]*College of Pharmaceutical Sciences, Institute of Materia Medica, Zhejiang University, Zijingang Campus, 388 Yu Hang Tang Road, Hangzhou 310058, P.R. China;* [b]*Department of Pharmaceutical Biology, Institute of Pharmacy, Johannes Gutenberg-University Mainz, Staudinger Weg 5, D-55128 Mainz, Germany*

(*Received 20 May 2009; final version received 10 August 2009*)

A dual vector (pQE-70-STR1-SG) containing coding regions of strictosidine synthase (STR1, EC 4.3.3.2) and strictosidine glucosidase (SG, EC 3.2.1.105) from the Indian medicinal plant *Rauvolfia serpentina* was constructed. Functional expression of the vector in *Escherichia coli* cells (M15 strain) was proven by isolation of prepurified enzyme extracts, which show both STR1 and SG activities. Incubation of the enzyme in the presence of tryptamine and secologanin delivered the indole alkaloid cathenamine, demonstrating functional co-expression of both STR1- and SG-cDNAs. Cathenamine reduction by sodium borohydride leading to tetrahydroalstonine revealed the chemo-enzymatic indole alkaloid synthesis.

Keywords: *Rauvolfia serpentina*; dual vector pQE-70-STR1-SG; engineered *Escherichia coli*; chemo-enzymatic synthesis; indole alkaloids

1. Introduction

The increasing number of well-known biosynthetic pathways, the concomitant knowledge of participating enzymes and the availability of the relevant genes (cDNAs) have led to new opportunities for applying these to future production strategies of natural products: for example, alkaloids (Kutchan, 1995; Loris et al., 2007; Ruppert, Ma, & Stöckigt, 2005). In this context, the heterologous co-expression of plant genes in microbial hosts, such as *Escherichia coli*, may play an important role (Chemler & Koffas, 2008; Minami et al., 2008). This article describes the construction and the functional expression in *E. coli* of a dual vector harbouring both cDNAs of the enzymes strictosidine synthase (STR1) (Kutchan, Hampp, Lottspeich, Beyreuther, & Zenk, 1988) and strictosidine glucosidase (SG) (Gerasimenko, Sheludko, Ma, & Stöckigt, 2002) from *Rauvolfia serpentina* (L.) Benth. ex Kurz. Both the gene products can be used for the chemo-enzymatic formation of indole alkaloids.

*Corresponding author. Email: joesto2000@yahoo.com

ISSN 1478–6419 print/ISSN 1029–2349 online

DOI: 10.1080/14786410903247304
http://www.informaworld.com

2. Material and methods

2.1. *Bacterial strains and culture conditions*

Escherichia coli cultures were cultivated in Luria–Bertani (LB) medium (Bertani, 1951, 2004) and, dependent on the strain, supplemented with ampicillin and/or kanamycin at 37°C (100 rpm). TOP10 (Invitrogen, Karlsruhe, Germany) strains were used for cloning experiments and M15 (Qiagen, Hilden, Germany) bacteria were used for enzyme expressions.

2.2. *Cloning and expression of STR1 cDNA*

Restriction enzymes were purchased from New England Biolabs (Frankfurt am Main, Germany) and the T4 DNA ligase was bought from Promega (Mannheim, Germany). The template used for primer design was the cDNA sequence of STR1 (GenBank: Y00756.1) from *R. serpentina* (Kutchan et al., 1988). pQE-70 (Qiagen, Hilden, Germany) was selected as the host vector as it contains the *Sph*I and *Bam*HI restriction sites. Polymerase chain reaction (PCR) was carried out with primers (MWG, Ebersberg, Germany) STR1for*Sph*I (5′-GCA TGC ATG GCC AAA CTT TCT GAT TCG CAA AC-3′, forward) and STR1rev*Bam*HI (5′-GGA TCC TTA ATG ACT TGA AAC AAA AGA ATT TCC-3′, reverse) in Mastercycler Personal (Eppendorf, Hamburg, Germany) with Advantage® Polymerase (Clontech, Heidelberg, Germany) under the following conditions: 94°C for 1 min, followed by 30 cycles of 94°C for 30 s, 58°C for 1 min, 72°C for 2 min, then held at 72°C for 10 min.

After ligation into pGEM-T vector (Promega), correct cDNA orientation was confirmed by using *Sph*I. As the STR1 cDNA contains an internal restriction site for *Sph*I, the resultant pattern of the fragments shows either one line (403 bp, correct orientation) or two lines (403 and 658 bp, false orientation) on a 1% agarose gel. Because of the internal restriction site, a partial digestion was necessary in order to obtain the 1035 bp fragment. The cDNA was therefore double digested with *Sph*I and *Bam*HI (30 min, 37°C). The designated fragment was detected after 10 min incubation and extracted with the NucleoSpin Extract kit (Macherey & Nagel, Düren, Germany).

The fragment was then ligated into the *Sph*I and *Bam*HI sites of the expression vector pQE-70 and transformed into strain TOP10 in order to prove its presence. The vector including the STR1 cDNA was then transformed into M15 cells for expression tests. For activity tests, 12 mL of overnight culture was inoculated into 200 mL of LB-medium containing 50 mg L^{-1} ampicillin and 25 mg L^{-1} kanamycin and incubated at 37°C up to $OD_{600} = 0.5$. The production of STR1 was induced by adding 1 M stock solution of isopropyl β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.6 mM for 16 h at 24°C and 100 rpm. A control culture was incubated containing only vector pQE-70.

2.3. *Enzyme assay for STR1*

Bacteria were harvested after 16 h by centrifugation (10 min at 5000 × *g*, 4°C) and then resuspended in 2.5 mL of lysis buffer (50 mM potassium phosphate buffer (KPi) pH 7.0; 20 mM mercaptoethanol (MSH); 1 mg mL^{-1} lysozyme) per vial.

After cracking with sonication (Sonoplus HD 2070, 3×10 s, 75 W), the suspension was centrifuged again (30 min, 12,000 $\times g$, 4°C). The supernatant was taken for enzyme assays.

An assay (100 µL) contained 6.25 µg tryptamine HCl, 12 µg secologanin and 81.75 µL (500 µg) enzyme solution. Incubation was for 30 min at 30°C and 500 rpm. The reaction was stopped by adding 100 µL MeOH. Following centrifugation (5 min at 14,000 $\times g$, 4°C), the supernatant was analysed by high-performance liquid chromatography (HPLC) using a LiChroCART® 250-4 LiChrospher® 60 RP-select B (5 µm) column (Merck, Darmstadt, Germany), solvent system : acetonitrile/H_2O pH 2.3 (adjusted with concentrated H_3PO_4), gradient $10:90 \rightarrow 50:50$ within 8 min, $\rightarrow$ $80:20$ within 3 min, $\rightarrow 10:90$ within 0.5 min, $\rightarrow 10:90$ for 4.5 min, flow rate 1.5 mL min^{-1}, detection at 250 nm, 40 µL injection volume. Retention times (R_t) were 4.7 min for tryptamine HCl, 5.8 min for secologanin and 8.0 min for strictosidine.

2.4. *Construction of the dual vector pQE-70-STR1-SG*

2.4.1. *Introduction of an additional multiple cloning site*

The initial system for construction of the gene cluster was the pQE-70 vector containing STR1 cDNA. In order to potentiate a vector system that can harbour more than one gene, the pQE-70-STR1-system was supplemented with new restriction sites. Therefore, a portion of the multiple cloning site (MCS) from the pSE280 vector (Invitrogen) was subjected to a restriction digest with *Bam*HI and *Hind*III. In between these two sites, there are 28 other possible restriction sites that can be used for ligation of additional cDNAs.

Isolation of the MCS from the pSE280 vector was carried out by digesting the plasmid extract with *Hind*III and *Bam*HI for 1.5 h at 37°C. The resulting 253 bp fragment was isolated from agarose gel (NucleoSpin Extract kit) and ligated into *Bam*HI and *Hind*III restriction sites of pQE-70-STR1. Following the digestion of the plasmid extract, the presence of the MCS adjacent to the STR1 cDNA in pQE-70-STR1 was verified.

2.4.2. *Cloning of strictosidine β-D-glucosidase cDNA with a ribosome binding site*

A PCR template pTYB1 vector harbouring the SG cDNA (Gerasimenko et al., 2002) was extracted from *E. coli* strain ER2566 by the use of the NucleoSpin Plasmid kit (Macherey & Nagel). Primer design was based on the SG sequence (GenBank: AJ302044.1) from *R. serpentina* (Gerasimenko et al., 2002). In order to ensure that the enzyme was also expressed after its ligation upstream of the STR1 gene, the sequence of the ribosome binding site (RBS) from pQE-70 vector was transferred into the SG forward primer. PCR was performed under the following conditions: 94°C for 1 min, followed by five cycles of 94°C for 30 s, 45°C for 1 min, 72°C for 2.5 min, 25 cycles of 94°C for 30 s, 58°C for 1 min, 72°C for 2.5 min then held at 72°C for 10 min. By using the primers SG + RBSfor*Bam*HI (5′-GGA TCC GAA TTC ATT AAA GAG GAG AAA TTA ATG GAC AAT ACT CAA GCT G-3′) and SGrev*Kpn*I (5′-GGT ACC TTA GGT TTT TTG CCT CTT GAC TAA CTC-32), a 1633 bp fragment was obtained.

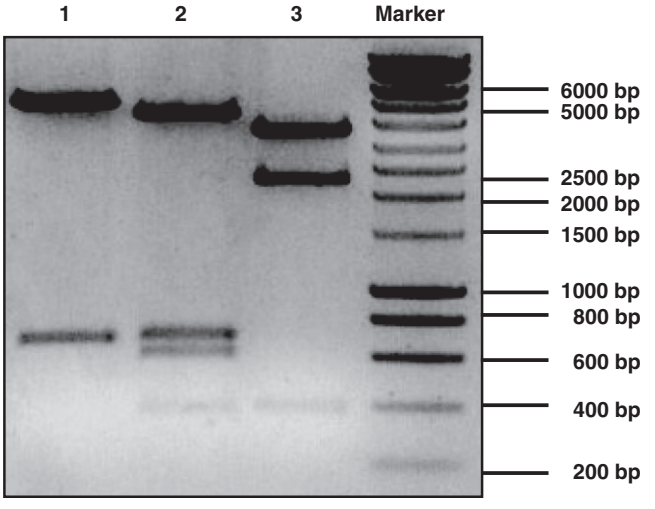

Figure 1. Agarose gel (1%) after digestion of pQE-70-STR1-SG to prove for successful ligation of SG cDNA with the RBS into pQE-70-STR1.
Notes: Line 1: Digestion with *Bam*HI, fragments with the size of 725 and 5500 bp appeared; line 2: digestion with *Sph*I and *Bam*HI, fragments with the size of 404, 635, 725 and 4461 bp are visible; line 3: digestion with *Sph*I and *Kpn*I, resulting in fragments of 404, 2268 and 3553 bp; marker: SmartLadder 200 (Eurogentec, Köln, Germany).

2.4.3. *Completion of the dual vector pQE-70-STR1-SG*

The pQE-70-STR1 vector could be completely digested with *Kpn*I and *Bam*HI at 37°C for 2 h. The resulting band of 4592 bp was extracted and then used as a host for ligation of SG cDNA. The linearised SG cDNA was digested with *Kpn*I (2 h at 37°C) followed by partial digestion with *Bam*HI for 5, 10, 15, 20, 30 and 45 min (37°C, tot. vol. 150 µL). The desired band of the 1633 bp fragment appeared in each incubation, even though the intensity decreased with incubation time, and was extracted from gel.

The presence of SG cDNA as well as the short MCS was proved by three digestions containing different combinations of restriction endonucleases:

(1) 10 U *Bam*HI with expected fragments of 725/5500 bp.
(2) 10 U *Bam*HI + 10 U *Sph*I with expected fragments of 404/635/725/4461 bp.
(3) 10 U *Sph*I + 10 U *Kpn*I with expected fragments of 404/2268/3553 bp.

After incubation for 1.5 h at 37°C, each digestion yielded the expected fragments (see Figure 1). Additionally, 100 mL control cultures containing pQE-70 and pQE-70-STR1 vectors were treated analogously.

2.5. *Product identification*

The alkaloids formed were identified by R_f values (TLC), R_t (HPLC), mass spectrometry (Finnigan MAT44S quadrupole instrument, 70 eV) and derivatisation (reduction of cathenamine), and comparison with standard samples.

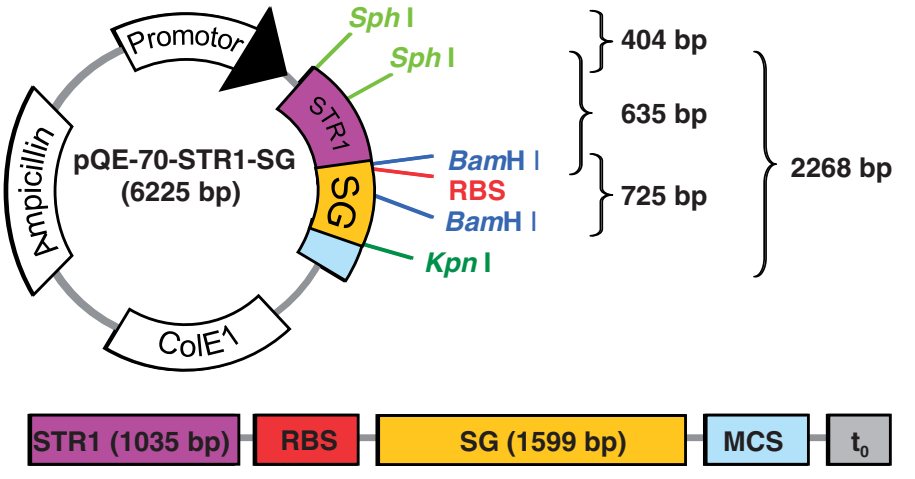

Figure 2. Expression vector map of the pQE-70-STR1-SG plasmid with recognition sequences for the restriction enzymes *Sph*I, *Bam*HI and *Kpn*I (colour online).
Notes: The new cloned cDNAs and MCS are also shown linearised. Light green: *Sph*I recognition sequence; blue: *Bam*HI recognition sequence; dark green: *Kpn*I recognition sequence; purple: STR1; orange: SG; red: RBS; light blue: MCS; t_0: stop codons.

3. Results and discussion

3.1. *Functional co-expression of STR1 and SG cDNAs*

In order to test the enzyme activity of the protein synthesised by the vector construct pQE-70-STR1-SG (see Figure 2), the construct was transformed into *E. coli* strain M15. When an overnight culture of M15 cells (6×10 mL) containing the vector construct was inoculated into 6×600 mL LB-medium (50 mg L^{-1} ampicillin and 25 mg L^{-1} kanamycin) and incubated at 37°C up to $OD_{600} = 0.5$, expression of the two enzymes was induced by addition of IPTG to a final concentration of 0.6 mM (100 rpm, 25°C).

3.2. *Assay for functional co-expression of STR1 and SG cDNAs*

Following a 22 h incubation (25°C), *E. coli* transformants were harvested by centrifugation (10 min at 5000 $\times g$ and 4°C). Following resuspension of each pellet in 4 mL lysis buffer (50 mM KPi buffer pH 6.0; 20 mM MSH; 1 mg mL^{-1} lysozyme), the cells were cracked by sonication (10 s, 75 W). After incubation on ice (30 min), bacteria were cracked again by sonication (3×10 s, 75 W). Cell fragments were separated (30 min at 12,000 $\times g$, 4°C) and the supernatant was taken for precipitation between 35 and 70% $(NH_4)_2SO_4$. After centrifugation (30 min, 9000 $\times g$, 4°C) the pellet containing the expressed enzymes was resuspended in 1 mL 50 mM KPi buffer (pH 6.0, 150 mM NaCl, 20 mM MSH) and the solution was centrifuged as before. The supernatant was purified on a HiTrap desalting column (General Electric, Freiburg, Germany) to remove molecules < 5000 Da. After centrifugation, the supernatant was used for activity tests. Assays were performed with enzyme preparations obtained from pQE-70 vectors without insert, pQE-70-STR1 and pQE-70-STR1-SG.

Incubations carried out to determine the single activity of STR1, as well as the detection of both enzyme activities in the dual expression system, were carried out as described for STR1-activity above. Assays for testing the single activity of SG contained 8.5 µg strictosidine and 91.5 µL (729 µg) protein solution. Each reaction was incubated at 35°C. Reactions were terminated after 1.5 h by addition of MeOH (0.1 mL), followed by centrifugation (14,000 × *g*, 5 min). HPLC analysis (same column as above) was performed using the following solvent system (Gao, von Schumann, & Stöckigt, 2002): acetonitrile/25 mM K_2HPO_4 pH 2.3 (adjusted with conc. H_3PO_4), gradient 28 : 72 → 30 : 70 within 4 min, → 35 : 65 within 2 min, → 28 : 72 within 0.1 min, → 28 : 72 for 3.9 min, flowrate 1.68 mL min^{-1}, detection at 280 nm, 60 µL injection volume.

HPLC analyses showed that single enzymes as well as the enzymes obtained by co-expression of the dual vector were active. STR1 and SG activities detected under described conditions indicated cathenamine to be the major product (R_t 8.1 min) of the co-expression system. Strictosidine as an intermediate could, however, not be detected, due to its immediate SG-catalysed conversion into cathenamine.

3.3. *Identification of cathenamine as a major product of STR1-SG-co-expression by dual vector*

In order to ascertain that cathenamine was the reaction product of co-expressed STR1 and SG (see Figure 3), a tenfold incubation (pH 6.9) was made (tot. vol. 1 mL). Previous tests to determine the occurrence of by-products based on long-term incubations (0.5–3.0 h) demonstrated that additional peaks in HPLC-chromatograms were caused by degradation of cathenamine (data not shown). Enzyme reactions for identification of cathenamine were therefore incubated for 15 min at 35°C. After adjusting the pH to 8.0 with 1 M KPi buffer (pH 8.0) reaction products were extracted with 2 × 0.6 mL ethyl acetate followed by evaporation under nitrogen at room temperature. The dried yellow residue was dissolved in 50 µL mixture of $CHCl_3$/MeOH (9 : 1) and examined via TLC (solvent system : petrolether/acetone/diethylamine 7 : 2 : 1, TLC plates (Merck)). The major product (absorption at 254 nm, R_f 0.46) co-migrated with an authentic cathenamine sample.

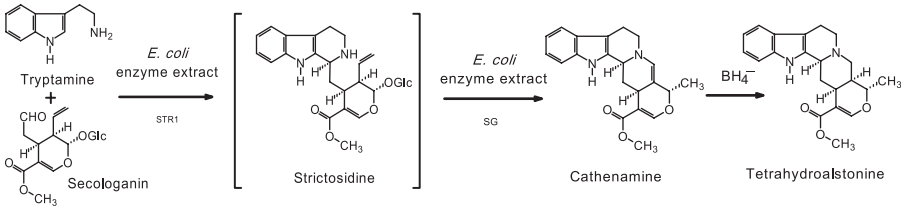

Figure 3. Chemo-enzymatic synthesis of tetrahydroalstonine from tryptamine and secologanin.

Notes: An enzyme extract from *E. coli* harbouring the dual vector pQE-70-STR1-SG expressing STR1 and SG followed by chemical reaction with borohydride was used. Due to simultaneous expression of STR1 and SG, the intermediate strictosidine does not accumulate, but is immediately converted into cathenamine.

For MS analysis, the product was eluted from the TLC-plate and could clearly be identified as cathenamine, showing identical mass fragmentation (major fragments: 350, 249, 225, 169 m/z) compared to a reference cathenamine. In order to further identify cathenamine produced by the enzyme extract of the engineered bacteria and also to extend the enzymatic synthesis to a chemo-enzymatic approach, reduction with borohydride was performed, delivering the alkaloid tetrahydroalstonine (THA).

3.4. *Identification of THA as a reduction product*

After enzymatic formation of cathenamine (see Figure 3) from tryptamine and secologanin using the dual vector, incubation mixtures were adjusted to pH 9–10, and extracted twice with 0.6 mL ethyl acetate. Ethyl acetate fractions were pooled, evaporated to dryness and resolved in 0.15 mL MeOH followed by incubation at room temperature for 15 min after supplementing with ~1 mg $NaBH_4$. The reduction was terminated by adding 20 µL acetone and the reaction mixture analysed by TLC, solvent system (1)–(3): (1) petrolether/acetone/diethylamine 7 : 2 : 1. Other solvent systems confirmed THA as product; (2) *n*-hexane/diethylether/xylol/ethyl acetate 9 : 8 : 3 : 1, R_f 0.35; (3) *n*-hexane/ethyl acetate/diethylether R_f 0.29. All R_f values coincided with those of the authentic THA sample.

Moreover, elution of the compound at R_f 0.48 from TLC and its measurement by direct-inlet EI-MS gave the characteristic mass spectrum of THA (352 M^+, 351, 337, 223, 184, 169, 156, 143 m/z) and identical fragmentation pattern of reference THA, which finally allowed verification of its overall chemo-enzymatic synthesis from tryptamine and secologanin through strictosidine and cathenamine based on co-expression of STR1 and SG using the newly constructed dual vector pQE-70-STR1-SG.

4. Conclusions and prospects

Expression of STR1 and SG cDNAs using the newly constructed vector pQE-70-STR1-SG in *E. coli* M15 cells allowed enzymatic formation of cathenamine from tryptamine and secologanin and proved the functionality of this dual vector construct. The combination with chemical reduction (borohydride) led to the chemo-enzymatic synthesis of the alkaloid THA. Thanks to the structure-based mutation of STR1 (Loris et al., 2007) and to the insertion of additional restriction sites, cDNAs encoding other alkaloid-biosynthesising enzymes, for example cathenamine reductase, may extend the use of the vector for multi-step *in vitro* enzymatic and chemo-enzymatic approaches of indole alkaloid synthesis. Such a strategy will provide an important future tool for developing appropriate *in vivo* production strategies for pharmacologically interesting indole alkaloids.

Acknowledgements

The authors are grateful to the Deutsche Forschungsgemeinschaft (Bad-Godesberg, Germany), Fonds der Chemischen Industrie (Frankfurt/Main, Germany) and Zhejiang University K.P. Chao Foundation (Hangzhou, China) for support. The original STR1 cDNA-clone was kindly provided by Prof. Kutchan, T.M. (Danforth Center, St Louis, USA).

References

Bertani, G. (1951). Studies on lysogenesis: I. The mode of phage liberation by lysogenic *Escherichia coli*. *Journal of Bacteriology, 62*, 293–300.

Bertani, G. (2004). Lysogeny at mid-twentieth century: P1, P2, and other experimental systems. *Journal of Bacteriology, 186*, 595–600.

Chemler, J.A., & Koffas, M.A.G. (2008). Metabolic engineering for plant natural product biosynthesis in microbes. *Current Opinion in Biotechnology, 19*, 597–605.

Gao, S., von Schumann, G., & Stöckigt, J. (2002). A newly-detected reductase from *Rauvolfia* closes a gap in the biosynthesis of the antiarrhythmic alkaloid ajmaline. *Planta Medica, 68*, 906–911.

Gerasimenko, I., Sheludko, Y., Ma, X., & Stöckigt, J. (2002). Heterologous expression of a *Rauvolfia* cDNA encoding strictosidine glucosidase, a biosynthetic key to over 2000 monoterpenoid indole alkaloids. *European Journal of Biochemistry, 269*, 2204–2213.

Kutchan, T.M. (1995). Alkaloid biosynthesis – The basis for metabolic engineering of medicinal plants. *Plant Cell, 7*, 1059–1070.

Kutchan, T.M., Hampp, N., Lottspeich, F., Beyreuther, K., & Zenk, M.H. (1988). The cDNA clone for strictosidine synthase from *Rauvolfia serpentina*: DNA sequence determination and expression in *E. coli*. *FEBS Letters, 237*, 40–44.

Loris, E.A., Panjikar, S., Ruppert, M., Barleben, L., Unger, M., Schübel, H., et al. (2007). Structure-based engineering of strictosidine synthase: Auxiliary for alkaloid libraries. *Chemistry & Biology, 14*, 979–985.

Minami, H., Kim, J.-S., Ikezawa, N., Takemura, T., Katayama, T., Kumagai, H., et al. (2008). Microbial production of plant benzylisoquinoline alkaloids. *Proceedings of the National Academy of Science USA, 105*, 7393–7398.

Ruppert, M., Ma, X.Y., & Stöckigt, J. (2005). Alkaloid biosynthesis in Rauvolfia – cDNA cloning of major enzymes of the ajmaline pathway; Thematic issue 'Recent Progress in Alkaloid Chemistry'. *Current Organic Chemistry, 9*, 1431–1444.