Improved Expression of His_6-Tagged Strictosidine Synthase cDNA for Chemo-Enzymatic Alkaloid Diversification

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Strictosidine synthase (STR1) catalyzes the stereoselective formation of 3α(*S*)-strictosidine from tryptamine and secologanin. Strictosidine is the key intermediate in the biosynthesis of 2,000 plant monoterpenoid indole alkaloids, and it is a key precursor of enzyme-mediated synthesis of alkaloids. An improved expression system is described which leads to optimized His_6-STR1 synthesis in *Escherichia coli*. Optimal production of STR1 was achieved by determining the impact of co-expression of chaperones pG-Tf2 and pG-LJE8. The amount and activity of STR1 was doubled in the presence of chaperone pG-Tf2 alone. His_6-STR1 immobilized on Ni-NTA can be used for enzymatic synthesis of strictosidines on a preparative scale. With the newly co-expressed His_6-STR1, novel 3α(*S*)-12-azastrictosidine was obtained by enzymatic catalysis of 7-azatryptamine and secologanin. The results obtained are of significant importance for application to chemo-enzymatic approaches leading to diversification of alkaloids with novel improved structures.

Introduction. – The universal role of the glucoalkaloid strictosidine as a precursor of *ca.* 2,000 structurally diverse monoterpenoid indole alkaloids, some being of high commercial value, has been unequivocally demonstrated [1][2]. The enzyme responsible for strictosidine synthesis, which occurs by way of a stereoselective *Pictet–Spengler* reaction [3] of tryptamine with secologanin, has been discussed in detail [4–6] and is named strictosidine synthase (STR1, EC 4.3.3.2). Isolated from *Catharanthus roseus* cell-suspension cultures and immobilized, the enzyme exhibits excellent stability rendering it able to enzymatically produce strictosidine on a preparative scale [7]. The major limiting factor in the application of this process is the availability of the enzyme, since tryptamine is commercially available, and secologanin is readily attainable in large quantities through a simple isolation procedure, previously described and subsequently modified [8][9]. Biotechnological production of STR1, such as that performed by fermenter-grown recombinant bacteria, could potentially lead to nearly 'unrestricted' quantities of the enzyme, and requires the successful expression of the cDNA of STR1 in *E. coli* [10]. One drawback, however, is the low solubility of STR1 when produced in *E. coli*, which is attributable to the formation of inclusion bodies.

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Heterologous fusion expression is a popular strategy widely used in the production of genetically engineered proteins [11–13]. Purification by affinity chromatography using various fusion tags, cloned and expressed as a part of the protein of interest, often allows single-step column purification of the proteins: this technique may improve yields by increasing solubility or may positively affect the catalytic activity of the target protein [14]. On the other hand, His_6-tag, as described here, allows simple immobilization of the enzyme on Ni-NTA columns and synthesis of the enzyme product strictosidine or its analogs in preparative amounts.

Moreover, we examined the influence of molecular chaperones on the solubility of the His_6-STR1 in *E. coli* strains M15 and BL21(DE3), and describe here the resulting, improved strategy for expressing higher levels of STR1, which is important for future fermenter-based production of this synthase. The resulting STR1 or its engineered mutants will be used in 3D X-ray analyses of the enzyme, but especially for generation of strictosidine analogs and for application of the enzyme to chemo-enzymatic approaches, leading to novel and diverse alkaloids with optimized structural frameworks [15]. The strategy is summarized in *Fig. 1*.

Results. – *Construction of Expression-Plasmid pET28a-His_6-STR1 and pET28a-His_6-STR1-His_6.* The STR1 polymerase chain reaction (PCR) amplicon obtained (see *Exper. Part*) was digested with *Bam*HI and *Sac*I, and cloned into a T7 promoter-driven fusion expression vector pET28a, to yield pET28a-His_6-STR1 (*Fig. 2*) or pET28a-His_6-STR1-His_6.

Construction and Comparison of Different Expression Systems. By transformation of the STR1-harboring vectors and the molecular chaperones into *E. coli* M15 strain and BL21(DE3) strain, respectively, nine different expression systems, **A**–**I** (see *Table 1*) were established. Their co-expression with the chaperones was then analyzed for production of His_6-STR1 in soluble form, and the results are shown in *Table 1*.

Table 1. *Construction and Comparison of Nine Different Expression Systems,* **A**–**I**, *Used Vectors, Chaperones,* E. coli *Strains and Growth, Production of Ni-NTA-Purified STR1 per Liter* Luria Bertani (*LB*) *Medium, and Enzyme Activity*

Express. system[a])	Vector	Chaperone	*E. coli* strain	Growth [g FW/l]	STR1 [mg/l]	nKat/l
A	pQE-2	–	M15	2.92 ± 0.03	0.341	33.83
B	pQE-2	pG-Tf2	M15	2.72 ± 0.01	0.658	67.15
C	pQE-2	pG-LJE8	M15	2.28 ± 0.03	0.318	31.34
D	pET28a	–	BL21(DE3)	2.95 ± 0.01	0.152	14.29
E	pET28a	pG-Tf2	BL21(DE3)	2.65 ± 0.02	0.345	31.41
F	pET28a	pG-LJE8	BL21(DE3)	2.25 ± 0.05	0.181	17.07
G	pET28a	–	BL21(DE3)	2.80 ± 0.04	0.423	25.45
H	pET28a	pG-Tf2	BL21(DE3)	2.29 ± 0.01	0.727	44.29
I	pET28a	pG-LJE8	BL21(DE3)	2.16 ± 0.03	0.502	30.72

[a]) **A**–**F** Coding for His_6-STR1, **G**–**I** for His_6-STR1-His_6.

After IPTG induction of *E. coli* transformed with the pQE-2-His_6-STR1 vector (known expression system **A**), SDS-PAGE showed an extra band around 35 kDa (*Fig. 3*), which was consistent with the expected molecular mass of His_6-STR1. The

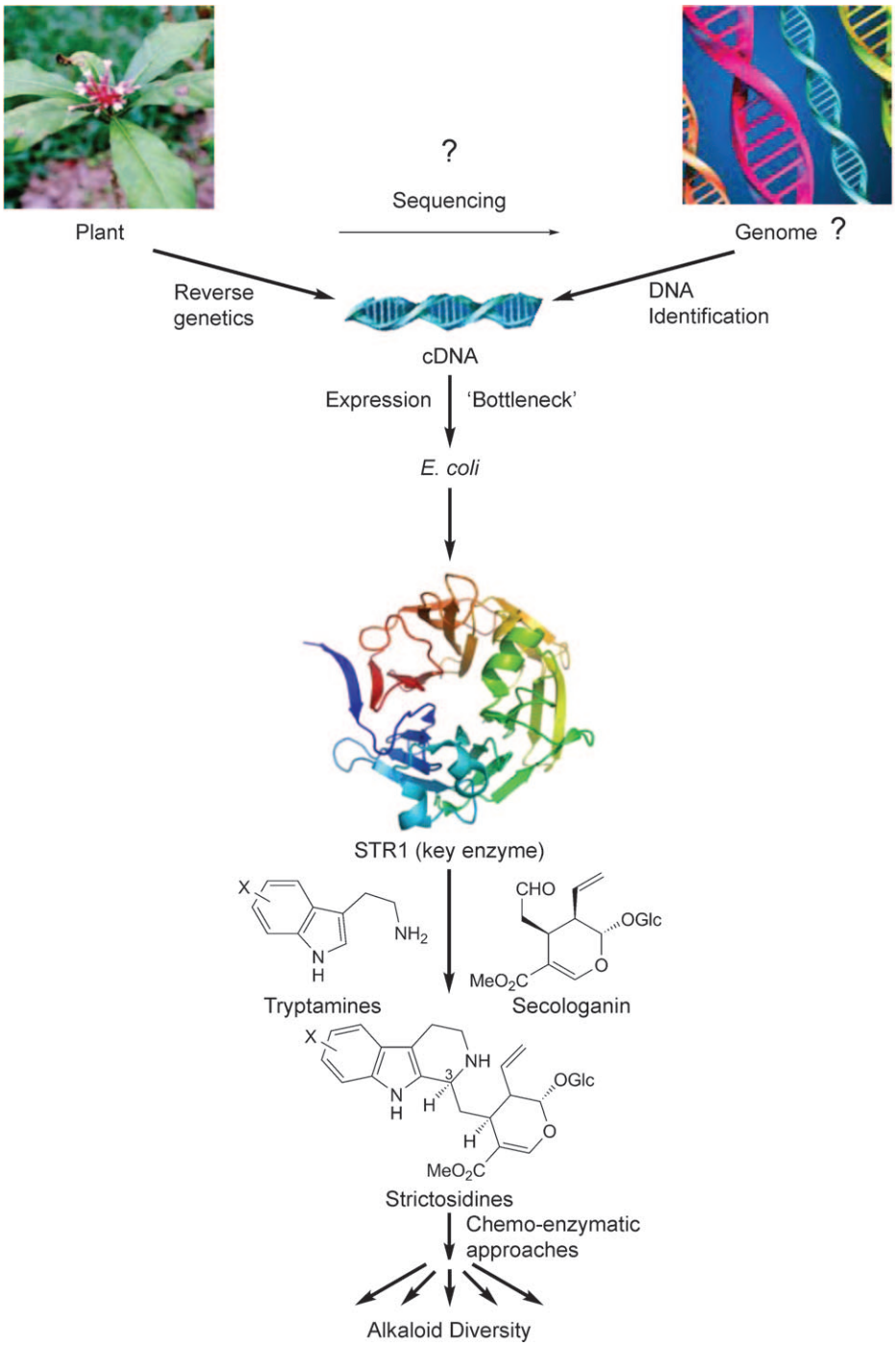


Fig. 1. *Heterologous expression of strictosidine synthase cDNA in* E. coli *as a 'bottleneck' in the generation of alkaloid diversity by enzymatic and chemo-enzymatic approaches*

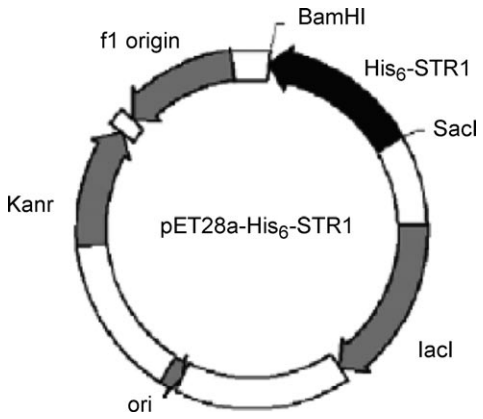


Fig. 2. *Vector diagram of the constructed pET28a-His$_6$-STR1 plasmid used for the production of His$_6$-tagged strictosidine synthase* (His$_6$-STR1) *in* E. coli

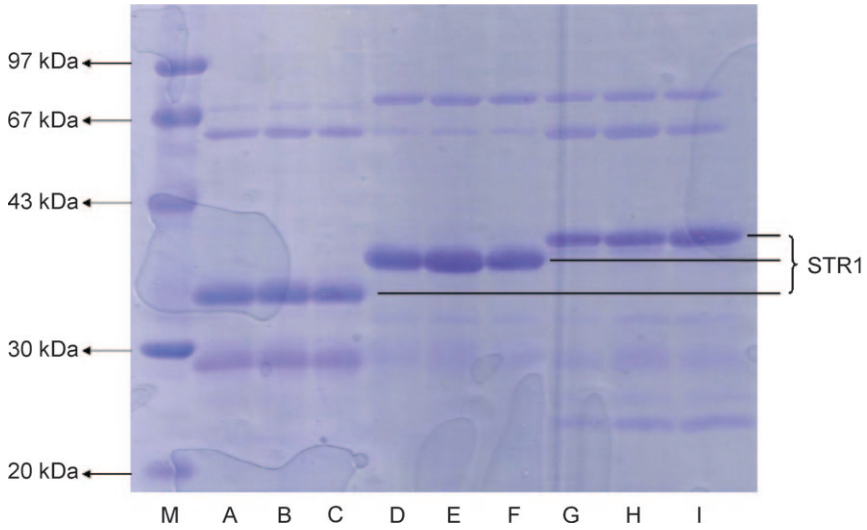


Fig. 3. *Purity test by SDS-PAGE of Ni-NTA pre-purified STR1 enzyme samples obtained from nine different expression systems* (M: Marker proteins; **A**–**I**: according to the nine different expression systems shown in *Table 1*). The calculated size of expressed His-tagged fusion proteins are: **A**, **B**, and **C**, 36.857 KDa; **D**, **E**, and **F**, 38.714 KDa; and **G**, **H**, and **I**, 40.679 KDa.

production of recombinant His$_6$-STR1 accounted for an estimated 30% of total cellular protein; however, most of His$_6$-STR1 (70%) was present as inclusion bodies.

To improve the production of soluble, catalytically active His$_6$-STR1, co-expression with two different molecular chaperones, pG-Tf2 and pG-LJE8, was employed respectively. This is illustrated in *Table 1* together with the *E. coli* growth results and amounts of Ni-NTA-purified STR1 obtained from 1 l of culture broth.

The data clearly indicate that optimal cell growth (from 2.76 to 2.96 g FW/l) was observed without chaperone co-expression (*i.e.*, **A**, **D**, and **G**). Lowest cell growth (from 2.13 to 2.31 g FW/l) was seen under co-expression with chaperone pG-LJE8 (system **C**, **F**, and **I**). In the presence of pG-Tf2, growth rate was found to lie between these values, and the enzyme activity per liter of bacterial culture was significantly higher in **B**, **E**, and **H** compared to the remaining systems.

Purification and Catalytic Activity of His_6-STR1. Recombinant His_6-STR1 fusion proteins were purified by one-step Ni-NTA affinity chromatography. The purity of the recombinant STR1 is shown in *Fig. 3*. From 1 l culture, the highest value of *ca.* 0.73 mg His_6-STR1 was obtained with system **H**, followed by **B** (0.66 mg).

According to the enzyme-activity assay employed (see *Exper. Part*), the catalytic activity of STR1, isolated using the nine different expression systems, **A**–**I**, summarized in *Table 1*, indicates that two systems, **B** and **H**, were superior to system **A** which had been routinely applied earlier in our research. The remaining systems **C**, **E**, and **I** yielded similar results when compared to the previously used system **A**. However, **D**, **F**, and **G** showed significantly lower STR1 activities (14.29–25.45 nKat/l *LB*).

Synthesis of 12-Azastrictosidine by Immobilized His-Tagged STR1 and Its Lactam Tetraacetate. After optimizing expression of cDNA for His_6-STR1 formation, the enzyme could be prepurified and immobilized in one step on Ni-NTA. Unbound and slightly bound proteins were removed by washing the Ni-NTA material with 20 mM and 50 mM imidazole solution, and His_6-STR1 remained bound on the resin (the target protein can be obtained by 250 mM imidazole elution).

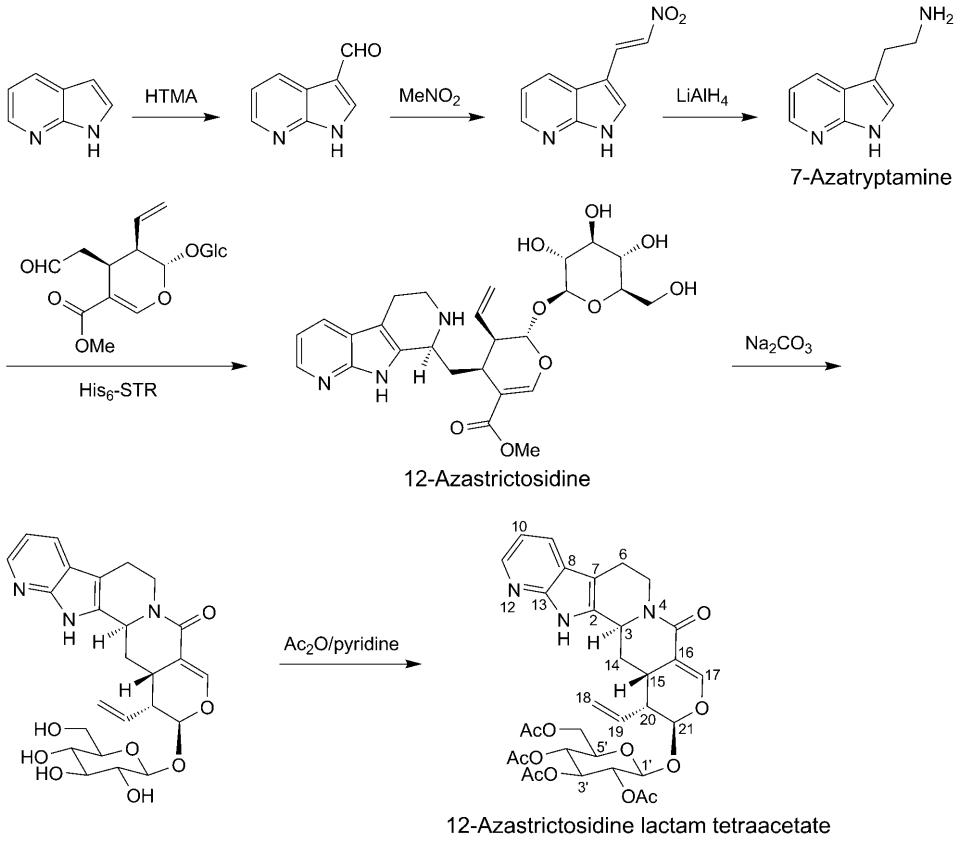
12-Azastrictosidine was prepared from 7-azatryptamine (mimic synthesis as reported) [16] and secologanin (isolated from *Lonicera tartaric*) by enzymatic catalysis with immobilized His_6-STR1 (*Scheme*; see *Exper. Part*). With overnight running, 0.7 g 12-azastrictosidine (yield 50.2%) with ESI-MS 531 was obtained. This immobilized resin-bound STR1 column can be used for one week and re-used after treatment of antibacterial agent NaN_3. This fast and simple strategy can easily be applied to a small-scale preparation of 12-azastrictosidine in which 10 g of target compound can be obtained in two weeks by 15 runs.

To determine the configuration at C(3), the 12-azastrictosidine lactam tetraacetate was also prepared by acetylation of the lactam, which was the mild basic-catalysis product of 12-azastrictosidine. The high-field shift of the AcO group at 1.32 ppm (*Table 2*) indicates the 3α(*S*)-configuration of the enzymatically formed 12-azastrictosidine, which is consistent with that reported by *Loris et al.* using the acetylated 10-methylstrictosidine lactam as supporting evidence [15].

Discussion. – Satisfying the requirement for novel, naturally derived pharmaceuticals is a general aim for drug research and development, although interest by the pharmaceutical industry in such products may slowly decline, as discussed recently [17][18].

Starting with the detection of morphine, the last two centuries have focused on the isolation and identification of secondary metabolites, of which the alkaloid family, consisting of *ca.* 20,000 members, still plays a dominant role [19][20]. In recent years, new techniques are being developed to expand structural diversity of alkaloids, with the hope of finding more valuable compounds exhibiting new biological activities or better

Scheme. *Chemo-enzymatic Synthesis of 12-Azastrictosidine by Immobilized His_6-STR1 Enzymatic Catalysis and 12-Azastrictosidine Lactam Tetraacetate*



pharmacological profiles. A number of highly promising strategies are based on the understanding of alkaloid biosynthesis, and detailed knowledge of the participating enzymes and of the genes that encode the latter. In this respect, by combining the necessary physiological, biochemical, genetic, and chemical methodologies, modern medicinal-plant research occupies an extraordinary and integrative position.

Genome sequencing of medicinal plants is not yet routine. In the past, the most successful strategy employed for detecting cDNA of plant alkaloid biosynthetic pathways consisted of the 'reverse genetics' approach. This technique consists of an initial enzyme purification, followed by sequence determination and finally isolation of the coding cDNA *via* the plant's mRNA pool. In most cases, plant cell culture systems, rather than the differentiated plant, were the key source for enzyme detection and isolation, followed by cDNA cloning.

Heterologous expression of these cDNAs for the generation and application of key biosynthetic enzymes may represent a critical step in the enzymatic and chemo-enzymatic approaches to generating alkaloid diversity. A prime example is strictosidine

Table 2. *^{13}C- and ^{1}H-NMR Data of 12-Azastrictosidine Lactam Tetraacetate*

	δ(C)	δ(H)
C(2)	136.2	
H–C(3)	55.1	5.09 (*m*)
CH_2(5)	43.9	3.13 (*td*, *J* = 12.6, 4.2), 4.89–4.92 (*m*)
CH_2(6)	21.7	2.60–2.69 (*m*)
C(7)	109.6	
C(8)	121.9	
H–C(9)	127.6	7.83 (*dd*, *J* = 7.7, 1.4)
H–C(10)	116.7	7.05 (*dd*, *J* = 7.7, 4.9)
H–C(11)	143.0	8.14 (br. *s*)
C(13)	149.7	
CH_2(14)	26.7	2.00 (*td*, *J* = 14.0, 6.3), 2.52 (*ddd*, *J* = 14.0, 4.2, 2.1)
CH–(15)	25.3	2.92 (*m*)
C(16)	110.1	
H–C(17)	148.4	7.36 (*s*)
CH_2(18)	121.1	5.37 (*dd*, *J* = 16.8, 1.4), 5.31 (*dd*, *J* = 10.5, 2.1)
H–C(19)	133.4	5.61 (*dt*, *J* = 16.8, 9.8)
H–C(20)	44.8	2.60–2.69 (*m*)
H–C(21)	96.8	5.25 (*d*, *J* = 1.4)
C(22)	166.8	
H–C(1′)	96.2	4.60 (*d*, *J* = 9.8)
H–C(2′)	73.5	4.89–4.92 (*m*)
H–C(3′)	73.2	5.14 (*t*, *J* = 9.8)
H–C(4′)	71.7	4.89–4.92 (*m*)
H–C(5′)	69.6	3.86 (*dd*, *J* = 9.8, 4.2, 2.1)
CH_2(6′)	62.8	4.22 (*dd*, *J* = 12.6, 4.2), 4.10 (*dd*, *J* = 11.9, 2.1)
*Ac*O–C(2′)	172.3 (CO), 20.6 (Me)	2.02 (*s*)
*Ac*O–C(3′)	171.4 (CO), 20.5 (Me)	1.94 (*s*)
*Ac*O–C(4′)	171.2 (CO), 20.4 (Me)	1.87 (*s*)
*Ac*O–C(6′)	167.0 (CO), 19.8 (Me)	1.32 (*s*)

synthase (STR1), which is involved in the enzymatic biosynthesis of monoterpenoid indole alkaloids, significant amounts of which may be required in the near future [15].

However, an important limitation in reaching this objective is the low solubility of STR1 when over-expressed in *E. coli*. Previously, when His-tagged forms of wild-type STR1 were produced in *E. coli*, the major part of the enzyme (*ca.* 70%) was found in inclusion bodies, the insoluble protein fraction. Since purification of active and high-yielding STR1 from its insoluble form involves complex renaturation processes [21], optimization of over-expression of the soluble enzyme remained the preferred method.

In the present paper, twofold production of soluble STR1 was achieved by investigating chaperone co-expression with a T7-driven expression plasmid pET28a. The T7 system is well-known for its high production level of heterologously expressed proteins, mainly due to strong transcriptional initiation activity of the T7 promoter and T7 RNA polymerase. The enhanced mRNA level resulted in improved production of target protein, but also led to the formation of inclusion bodies due to incorrect protein folding, usually caused by high translation rate and incorrect hydrophobic interaction of nascent peptides. To reduce the amount of inclusion bodies and to obtain higher

yields of soluble His_6-STR1, STR1 was co-expressed with chaperones in *E.coli* at low temperature (25°). Under these conditions, the translation rate decreased resulting in a reduction of incorrect folding of His_6-STR1, while co-expressed chaperones participated in the nascent His_6-STR1, resulting in correct folding and inhibition of incorrect hydrophobic interaction of nascent peptides. These factors taken together facilitated over-expression of soluble His_6-STR1, finally resulting in higher yields of the enzyme.

Using the pET28a-STR1 construct expressing both the N- and C-terminal His-tag in the BL21(DE3) strain expression system, a higher expression could be observed, compared to both the previously applied pQE-2-STR1 system in M15 cells, and the construct pET28a-STR1 with N-terminal His-tag in the BL21(DE3) expression system. The highest amount of recombinant STR1 obtained (0.727 mg/l of culture) resulted from STR1 being fused to two His-tag residues, one at both the N- and the C-terminals of the enzyme (system **H**, *Fig. 4*). In this case, the enzyme formed can bind to the Ni-NTA matrix more tightly, and, therefore, support higher enzyme yields during the purification procedure. Although co-expression with molecular chaperone pG-Tf2 results in more soluble enzyme compared to chaperone pG-LJE8 (system **I**), the enzyme activity was moderate, most probably due to the influence of the two His-tag residues.

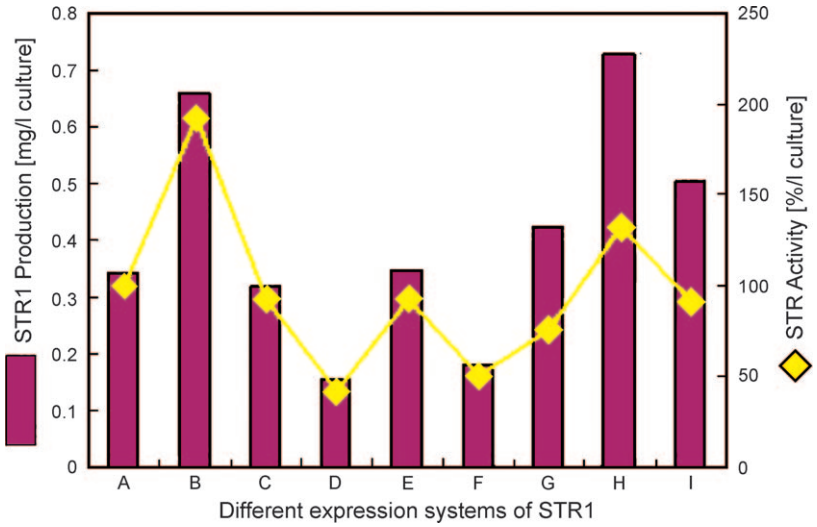


Fig. 4. *Production and activity results of STR1 in* E. coli *for the different expression systems,* **A**–**I**. **A** was set to 100% STR1 activity.

Plasmid pQE-2-STR1 co-expressed with molecular chaperone pG-Tf2 in *E. coli* M15 (system **B**) at lower temperature (25°) currently appears to be the most efficient system for large-scale His_6-STR1 production, followed by expression system **H**.

In conclusion, the application of different expression systems has led to the development of a novel and more efficient way of producing functional STR1. Higher amounts of the enzyme may not only facilitate further studies of its structure–function relationship by 3D X-ray analysis, but they are also expected to allow large, fermenter–

scale production of catalytically active STR1 for enzymatic synthesis of a broad range of alkaloids, including the design and generation of novel alkaloid libraries which might present a promising resource of new pharmaceuticals [15].

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Experimental Part

General: Silica gel (GF_{254}). Column chromatography (CC): silica gel *H* (10–40 μm). All of the silica gel GF_{254} and silica gel *H* were purchased from *Qingdao Marine Chemical Factory*, P. R. China. Primers were synthesized by *MWG Biotech AG* (D-Ebersberg). Expression vector pET28a and strain BL21(DE3) were purchased from *Novagen* (D-Darmstadt). pGEM-T Easy vector and dNTPs were obtained from *Promega* (D-Mannheim). Molecular chaperone expression plasmids were from *TaKaRa* (D-Potsdam). M15[pREP4] and Ni-NTA superflow were purchased from *Qiagen* (D-Hilden). TOP10 Strain was obtained from *Invitrogen* (D-Karlsruhe). *NucleoSpin Plasmid* kit and *NucleoSpin Extract* kit were purchased from *Macherey-Nagel* (D-Düren). Restriction enzymes *Bam*HI and *Sac*I were from *New England Biolabs* (D-Frankfurt/Main). *Pfu* Turbo DNA polymerase and Taq-DNA polymerase were obtained from *Stratagene* (La Jolla, USA), and IPTG was from *Gerbu* (D-Gaiberg). Other reagents were obtained from standard commercial sources and were of anal. reagent grade. ^{1}H-NMR Spectra: *Varian INOVA 400* spectrometer with TMS as an internal standard and CD_3OD as solvent. ESI-MS: *Bruker Esquire 3000+* spectrometer.

Construction of Expression Plasmid pET28a-His$_6$-STR1 and pET28a-His$_6$-STR1-His$_6$. STR1 cDNA (GenBank: Y00756.1) from *Rauvolfia serpentina* was amplified by polymerase chain reaction (PCR) using an STR1-gene-containing plasmid pQE-2 as template [22]. PCR was performed by using *Pfu* Turbo DNA polymerase. PCR Primers were: primer 1: 5′-GGATCCCCAATCTTGAAAGAG-3′ and primer 2: 5′-GAGCTCTTAATGACTTGAAAC-3′ (one N-terminal His-tag); primer 3: 5′-GAGCTCCCATGACTTGAAAC-3′ (one N-terminal His-tag and one C-terminal His-tag). PCR Cycles were as following: following a hot-start at 95° for 1 min, 1 μl of *Pfu* Turbo DNA polymerase was added to the sample, then denaturation at 94° for 0.5 min, annealing at 52° for 1 min, and extension at 72° for 3 min for 31 cycles, and extension at 72° for additional 10 min, addition of 1 μl Taq-DNA polymerase, followed by extension at 72° for another 10 min. Following agarose-gel analysis, the DNA was extracted using the *NucleoSpin Extract* kit. The PCR amplicon obtained was ligated with pGEM-T easy vector for sequence confirmation. The plasmid was then digested with *Bam*HI (the recognition site is underlined in primer 1) and *Sac*I (the recognition site is underlined in primer 2 and 3), and inserted between the same sites of *E. coli* expression vector pET28a.

Recombinant pET28a-His$_6$-STR1 and pET28a-His$_6$-STR1-His$_6$ Production. His$_6$-STR1 Fusion protein expression-plasmid pET28a-His$_6$-STR1 and pET28a-His$_6$-STR1-His$_6$, resp., were transformed into *E.coli* BL21(DE3), and a single colony was inoculated into fresh *Luria Bertani* (*LB*) medium containing 50 mg/l kanamycin at 25°. When the OD_{600} was between 0.6–0.8, IPTG was added to final concentration of 0.6 mM to induce the production of target protein. After 24 h, bacteria were collected, and protein was extracted and purified by single-step Ni-NTA affinity chromatography, and analyzed by 11% SDS-PAGE.

Co-Expression of His$_6$-STR1 with Chaperones. Plasmid pG-Tf2, which could express TF and GroEL-GroES together under the tetracycline-inducible promoter Pzt-1, harbored a chloramphenicol resistance gene and was compatible with plasmid containing ColE1 origin [23][24]. The plasmid pET28a-His$_6$-STR1 was transformed into *E. coli* BL21(DE3) together with plasmid pG-Tf2, and positive clones were selected on solid *LB* medium containing kanamycin (50 mg/l) and chloramphenicol (34 mg/l). A culture from a single colony was grown at 25° overnight, and inoculated into 5 l *LB* medium with kanamycin

(50 mg/l) and chloramphenicol (34 mg/l). When the OD_{600} reached *ca.* 0.6, tetracycline was added to induce chaperone expression at a concentration of 20 μg/l. After 30 min, IPTG (final concentration 0.6 mM) was added to induce expression of the recombinant His_6-STR1. The culture was incubated for an additional 24 h, and bacteria were harvested by centrifugation (10,000 rpm for 10 min), and proteins were purified (see *Protein Purification*).

The same procedure was applied for pG-LJE8 and also for both chaperone plasmids with pET28a-His_6-STR1-His_6. System **A** (pQE-2-His_6-STR1) was expressed in *E. coli* M15 and with molecular chaperone plasmid pG-Tf2 and pG-LJE8, resp. The concentrations of antibiotics were: ampicillin (50 mg/l in H_2O), kanamycin (25 mg/l in H_2O), and chloramphenicol (34 mg/l in EtOH).

Protein Purification. The purification of His_6-STR1 and His_6-STR1-His_6 was performed according to the manufacturer's instruction by Ni-NTA superflow. Cell pellets were re-suspended in 50 ml of 50 mM sodium phosphate (pH 8.0), containing 0.3M NaCl, 10 mM 1*H*-imidazole, 1 mg/ml lysozyme, and were lysed by ultrasonic treatment (*Sonoplus HD*, 3 × 10 sec, 75 W) for 30 min. The supernatant was collected by centrifugation at 25,000 rpm for 20 min. The resulting supernatant was loaded onto a Ni-NTA affinity column (1 cm × 5 cm), which was equilibrated with equilibration buffer (50 mM sodium phosphate, 0.3M NaCl, 10 mM 1*H*-imidazole, pH 8.0). The column was then washed with 50 ml of wash-buffer (50 mM sodium phosphate, 0.3M NaCl, 50 mM 1*H*-imidazole, pH 8.0) and then eluted with the elution buffer (50 mM sodium phosphate, 0.3M NaCl, 250 mM 1*H*-imidazole, pH 8.0) into 10 tubes, 1 ml each. Dialysis was then performed against buffer 50 mM KPi (pH 7.0, 1 mM EDTA, 10 mM 2 (sulfanylethanol) to remove imidazole.

Protein concentrations of all fractions during the purification process were measured using *Bradford*'s method with bovine serum albumin (BSA) as reference [25].

Enzyme Assay. To determine enzyme activity, the incubation mixture consisting of a total volume of 50 μl contained 0.2 μg of enzyme, 1 mM secologanin, 2 mM tryptamine, 50 mM KPi buffer, pH 7.0. The mixture was incubated for 30 min at 28° with shaking (450 rpm). The reaction was terminated by addition of 100 μl of MeOH, followed by centrifugation (20,000 rpm × 5 min). An aliquot (50 μl) of each assay mixture was subjected to HPLC analysis. For HPLC, a *Merck Hitachi* instrument and a *Lichrospher® 60* RP-select *B* column (125 × 4 mm, 5 μm) (*Merck*, D-Darmstadt) were used: injection volume 50 μl; flow rate 1 ml/min; absorption measured at 250 nm. For separation, MeCN/H_2O (pH 2.3, adjusted by conc. H_3PO_4) was used as solvent system, gradient 10 : 90 → 50 : 50 within 8 min → 80 : 20 within 3 min → 10 : 90 within 0.5 min → 10 : 90 for 3.5 min.

Synthesis of 12-Azastrictosidine by Immobilized STR1 and Its Lactam Tetraacetate. Prepurified His_6-STR1 was dissolved in 50 mM KPi buffer (pH 7.0), immobilized on the Ni-NTA column. 7-Azatryptamine (0.4 g, 2.5 mmol) was dissolved, and 1.1 g of secologanin (88% purity, 2.5 mmol) were dissolved in KPi buffer (50 mM, 200 ml) separately. Both solns. were pumped through the column at a speed of 0.5 ml/min at low temp. (4–6°). After overnight running, the combined aq. soln. was collected and freeze-dried to yield the dry powder which was washed with MeOH to remove inorg. salt. The org. phase was evaporated under reduced pressure, and the crude product (1.3 g) was subjected to prep. HPLC to afford pure 12-azastrictosidine (0.7 g, yield: 50.2%).

12-Azastrictosidine (100 mg) was dissolved in 5% Na_2CO_3 (10 ml), and the mixture was stirred at 70° for 1 h. AcOEt was used for extraction (10 ml × 3), and the combined org. phase was washed with H_2O and brine separately, and dried (Na_2SO_4) overnight. The solvent was removed, and the concentrated solid (74 mg) was directly dissolved in 1.5 ml of Ac_2O and 0.5 ml of pyridine. The mixture was then stirred at r.t. for 2 h. It was poured into cold H_2O (4°), and $CHCl_3$ was used for extraction (10 ml × 3). The combined org. phase was washed with 2M HCl (10 ml × 1) and 5% Na_2CO_3 (10 ml × 1), and dried (Na_2SO_4) overnight. The solvent was removed, and the concentrated crude product was subjected to CC with $CHCl_3$/AcOEt 5 : 1 to afford 73 mg of 12-azastrictosidine lactam tetraacetate (yield: 57.7%).

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