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A sustainable route to produce the scytonemin precursor using *Escherichia coli*†

Sailes Malla* and Morten O. A. Sommer*†

Scytonemin is an indolic–phenolic natural product with potent pharmaceutical activities and possible application as a sunscreen. However, the productivity of the existing synthesis systems restrains its applications in medicine and cosmetics. In this paper, we report the generation of the monomer moiety of scytonemin from tryptophan and tyrosine in *Escherichia coli*. We heterologously expressed the biosynthetic pathway from *Nostoc punctiforme* and discovered that only three enzymes from *N. punctiforme* are required for the in vivo production of the monomer moiety of scytonemin in *E. coli*. We also found that the constructed recombinant *E. coli* strains are capable of producing novel alkaloids as shunt products. The recombinant *E. coli* strain expressing the putative scytonemin biosynthetic gene cluster produced 4.2 mg L\(^{-1}\) (2.46 µg mg\(^{-1}\) dry cell weight) of the monomer moiety of scytonemin without supplementation of extracellular substrates whereas upon supplementation with 1 mM of the substrates to the *E. coli* strain harboring scyABC genes, 8.9 mg L\(^{-1}\) (4.56 µg mg\(^{-1}\) dry cell weight) of the monomer moiety of scytonemin was produced in 5 days. Combining this cell factory with the previously described chemical dimerization process will contribute to a sustainable production of semi-synthetic scytonemin.

Introduction

Alkaloids, a diverse group of nitrogen-containing natural products, are produced by a large variety of organisms including bacteria, fungi, insects, plants and animals. Numerous alkaloids are pharmacologically well characterized and are used as clinical drugs, ranging from chemotherapeutics to analgesic agents.\(^1\) Studies on plant alkaloids suggest that they are involved in the defense mechanism against herbivores, insects and pathogens.\(^2\) Since alkaloids are toxic, they are usually produced in small quantities by their native producer organisms. Scytonemin is an alkaloid pigment consisting of a symmetrical dimeric carbon skeleton composed of fused heterocyclic units with conjugated double-bond distribution (Fig. 1) synthesized by numerous cyanobacteria.\(^3\) Scytonemin is the first described small molecule that inhibits human polo-like kinase 1 (PLK1).\(^4\) PLK1 has multiple functions during mitosis and plays a significant role in maintaining the genomic stability.\(^5\) Furthermore, PLK1 is highly expressed in a broad spectrum of cancer cells, indicating its possibility of being involved in carcinogenesis.\(^6\) Scytonemin (at 3–4 µM concentration) can inhibit cell growth by cell cycle arrest in multiple myeloma cells and renal cancer cells through specific down-regulation of the PLK1 activity.\(^7,8\) Scytonemin is not cytotoxic (up to 10 µM) to non-proliferating cells, highlighting its possible application in medicine.\(^9,10\) In addition to kinase inhibitory activities, scytonemin also acts as a natural microbial sunscreen by effectively minimizing cellular damage caused by UV (315–400 nm) exposure.\(^11\) Scytonemin also exhibits a radical-scavenging activity\(^12\) and its synthesis was enhanced by oxidative stress in cyanobacteria.\(^13\)

The putative scytonemin biosynthetic gene cluster from *Nostoc punctiforme* ATCC 29133 consists of 18 unidirectional open reading frames (orfs) (Fig. 2). Native expression of this gene cluster is triggered by exposure to UV light, resulting in extracellular pigment accumulation. Once scytonemin has reached sufficient quantities in the extracellular slime layer to block the incoming UVA, the gene expression returns to background levels and halts further scytonemin synthesis.\(^14,15\) Due to the potent UV light absorption of scytonemin, the accumulated scytonemin concentration is low (~1.3 µg mg\(^{-1}\) of dry cell weight (DCW)) in currently characterized cyanobacterial strains under laboratory culture conditions\(^16\) whereas naturally growing colonies of a terrestrial cyanobacterium *N. commune* contained only 0.4 µg mg\(^{-1}\) of DCW of scytonemin.\(^17\) Consequently, direct extraction from natural producers is unfeasible on a large scale. Another route to produce scytonemin is through chemical synthesis. The total synthesis of scytonemin has been reported from 3-indole acetic acid through a process...
comprising nine chemical steps resulting in approximately 4% conversion to the final product. Accordingly, more effective approaches are desired for the continuous, rapid and cost effective production of scytonemin.

Microbial cell factories offer extensive opportunities for the industrial production of complex biomolecules for cost effective biological synthesis. Furthermore, microbial fermentation often reduces the need for energy intensive reaction conditions, toxic organic solvents, heavy metal catalysts, and strong acids/bases, which are widely utilized in chemical synthesis routes. Among the microbial cell factories designed, the Gram-negative bacterium *Escherichia coli* has become one of the most promising hosts, with a highly tractable genetic system and favorable fermentation conditions for production purposes. Indeed, plant based alkaloid compounds have been successfully produced from the engineered *E. coli* strains. For example, 46 mg L\(^{-1}\) of the plant benzylisoquinoline alkaloid, (S)-reticuline, is produced from fermentation of metabolically engineered *E. coli* by utilizing simple carbon sources such as glucose or glycerol. Similarly, production of indole, a signaling molecule, from exogenous tryptophan in *E. coli* has been extensively studied. Yields up to 6 mM of indole have been achieved from *E. coli* by supplementation of enough tryptophan in culture media. In the present study, we described the construction of an *E. coli* cell factory for bio-based production of the key pharmaceutical intermediate, the monomer moiety of scytonemin (compound 4 in Fig. 1).

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**Materials and methods**

**Bacterial strains, plasmids, cultured conditions and chemicals**

All strains, vectors and plasmids used in this study are listed in Table 1. All DNA manipulations were carried out by following standard protocols. *E. coli* strains were routinely cultured in Luria–Bertani (LB) broth or on agar supplemented with the appropriate amount of antibiotics (ampicillin 100 \(\mu\)g mL\(^{-1}\), chloramphenicol 25 \(\mu\)g mL\(^{-1}\), streptomycin–spectinomycin 50 \(\mu\)g mL\(^{-1}\) and kanamycin 35 \(\mu\)g mL\(^{-1}\)) when necessary. M9 minimal medium was used for production of intermediates and derivatives of scytonemin. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs (Hertfordshire, UK) and Fermentas (Denmark). The DNA sequence was determined on an automated DNA sequence analyzer. The authentic scytonemin standard was kindly provided by Professor Jerker Mårtensson (Chalmer University of Technology, Sweden).

**Plasmid construction**

The construction of recombinant plasmids pCDF-ScyA, pCDF-ScyAC, pCDF-ScyACD, pACYC-ScyB, pET-ScyEF, pRSF-TyrP-DsbA, pC-ScyABC-ScyDEF, pE-GlAroB-TrpEC and pA-TrpAB-TrpDU is described below. All PCR primers used in this study are described in Table 2.

Based on pCDF-Duet-1, the expression recombinant plasmid pCDF-ScyACD was constructed, which allowed the
sequence along with the performed which allowed the amplification of the T7
ScyD_F
recombinant plasmid. Finally, using the primer pair
Kpn
pCDF-ScyA plasmid to get the pCDF-ScyAC recombinant expression plasmid. Similarly, the PCR product of scyF was cloned into pRSF-ScyE excised with Ndel/BglII sites to construct the pET-ScyEF expression recombinant plasmid.

To express the putative scytonemin gene cluster (Fig. 2), recombinant plasmids pC-ScyABC-ScyDEF, pEGTAroB-TrpEC and pA-TrpAB-TrpDU were constructed based upon pCDF-Duet-1, pET-Duet-1 and pACYC-Duet-1 expression vectors, respectively. The primer pairs ScyA_F/ScyC_R_BamHI, ScyD_F/ScyF_R, GT-AroB_F/GT-AroB_R, TrpEC_F/TrpEC_R, TrpAB_F/TrpAB_R, and TrpDU_F/TrpDU_R were used for amplification of scyABC, scyDEF, Gt-tyrA-dsbA-aroB, trpE-trpC, trpA-tryp-trPB, and trpD-aroG-NpR1259 regions of the putative scytonemin gene cluster from the genomic DNA of N. punctiforme, respectively. The PCR product of scyABC was cloned into the Ncol/BamHI (MSC1) sites of pC-ScyABC recombinant expression plasmid. Further, the PCR product of scyDEF was cloned into the Ndel/BglII (MSC2) of the pC-ScyABC plasmid to construct pC-ScyABC-ScyDEF. Similarly, the PCR products of Gt-tyrA-dsbA-aroB and trpE-trpC were cloned into Ncol/BamHI (MCS1) and Ndel/BglII (MSC2) of PET-Duet-1 vector, respectively, to construct the pE-GTAroB-TrpEC recombinant plasmid. Finally, the PCR products of trpA-tryp-trPB and trpD-aroG-NpR1259 were cloned into Ncol/BamHI (MSC1) and Ndel/BglII (MSC2) of the pACYC-Duet-1 vector, respectively, to construct the pA-TrpAB-TrpDU recombinant plasmid.

In all cases, construction of recombinant plasmids was verified by both restriction mapping and direct nucleotide sequencing of respective genes in the recombinant plasmids.

**Recombinant protein expression, whole-cell biotransformation, product isolation and determination of biomass**

E. coli BL21(DE3) harboring recombinant plasmids were precultured into 3 mL of LB liquid media with appropriate anti-
bacteria and incubated at 37 °C with 220 rpm overnight. The following day 200 μL of preinoculum was transferred into 4 mL of LB liquid media (with antibiotics) and cultured at 37 °C until the optical density at 600 nm (OD600 nm) reached approximately 0.6. Then isopropyl-β-D-thiogalactopyranoside (IPTG) was added at a final concentration of 1 mM and the culture was incubated at 30 °C for 20 h. The cells (~5 × 10^8 cells) were harvested by centrifugation, washed with 1 mL of phosphate buffer (pH 7.0) and then resuspended in 100 μL of phosphate buffer. The recombinant protein was released by following six cycles of the freeze–thaw method and checked by SDS-PAGE (ESI Fig. S1†). For freeze–thaw cycles, the cell

### Table 1  Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strains/plasmids</th>
<th>Description</th>
<th>Source/reference</th>
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<td>ompT hsdS (rB− mB−) gal (DE3)</td>
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<td>SM3</td>
<td>BL21(DE3) carrying pCDF-ScyB and pET-ScyEF</td>
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<td>SM4</td>
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<td>This study</td>
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<tr>
<td>pA-TrpAB-TrpDU</td>
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### Table 2  Oligonucleotides used in this study

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<tr>
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<td>TryP_R</td>
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* Restriction sites are indicated by underline and *italics.*
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Results and discussion

Heterologous expression of the putative scytonemin gene cluster

Recent studies have shown that *N. punctiforme* genes have been well expressed and functional in *E. coli*.^30,31^ Accordingly, we chose to construct our recombinant pathway in *E. coli* using the native genes of *N. punctiforme*. For the expression of the putative scytonemin biosynthetic gene cluster, the recombinant plasmids pC-ScyABC-ScyDEF, pE-GtAroB-TrpEC and pA-TrapAB-TrpDU were constructed, and they were transformed into *E. coli* BL21 to create the strain *E. coli* STN. Upon IPTG induction, the cultures of *E. coli* expressing the putative scytonemin biosynthetic gene cluster (*E. coli* STN strain) in M9 minimal media turned yellow whereas the uninduced cultures did not have any color (data not shown). The metabolites produced by *E. coli* STN strain were analyzed by HPLC and mass analysis. The *E. coli* STN strain did not produce scytonemin upon IPTG induction. However, the monomer of scytonemin (compounds 4) and a new alkaloid derivative (compound 7) were produced as the dominant products from the endogenous amino acids (Fig. 3).

Despite the production of compound 4, the absence of scytonemin in the metabolites from STN was either due to the lack of dimerization enzyme(s) in the putative scytonemin gene cluster or inactive putative dimerization enzyme(s) during heterologous expression in *E. coli*. Genome analysis and comparison among several cyanobacterial strains for the conserved localization in the scytonemin clusters revealed a five-gene satellite cluster, oriented in the same transcriptional direction in *N. punctiforme*.^15^ Of five genes in the cluster, two genes are annotated as unknown hypothetical proteins, and three genes are annotated as putative metal-dependenthydro-
E. coli and STN strains. (i) metabolites from \([M + H]^+\) mass of compound 8, (ii) metabolites from E. coli SM4 without supplementation of substrates (control), (iii) metabolites from E. coli STN with IPTG induction, and (iv) metabolites from E. coli SM4 supplemented with tryptophan and tyrosine as precursors. (B) LC/ESI-MS analysis of metabolites from E. coli SM4 in the positive mode: (i) exact mass of compound 8 \([M + H]^+\) (276.10229), (ii) exact mass of compound 7 \([M + H]^+\) (393.16049), compound 6 \([M + H]^+\) (393.16022), compound 5 \([M + H]^+\) (296.11301), compound 4 \([M + H]^+\) (278.11810), compound 3 \([M + H]^+\) (294.11301, found: 294.11344), compound 2 \([M + H]^+\) (275.11783), found: 275.11780), and (vii) exact mass of compound 9 \([M + H]^+\) (537.21747). All of these five new alkaloid derivatives have very similar UV-absorption spectra to that of compound 3 (ESI Fig. S3†). The structure of compounds 4, 6, 7, 8, and 9 was confirmed by NMR analysis (\(^1^H, ^{13}C, HSQC, HMBC\) (Table 3, S1–S4 and ESI Fig. S5–S10†).

The absence of scytonemin in the bioconversion products of both SM4 and STN strains indicates that the final dimerization step is the major bottleneck in E. coli. Structural elucidation of the new alkaloid derivatives (shunt products) revealed that all five compounds were produced from the oxidation of intermediate compound 3, i.e., either by the formation of C–C bond with indole or dimerization of compound 3. To gain more information about these new derivatives such as their synthetic origin and plausible bioactivities, we searched the literature to ascertain whether any of these compounds have been previously reported. An anti-inflammatory drug target IκB kinase inhibitor, PS1145, and a proteasome inhibitor, Nostodione A, are structurally similar to the monomer moity of scytonemin. \(^16\) Hence, it was predicted that besides the putative gene cluster shown in Fig. 2, this satellite five-gene cluster might be involved during scytonemin biosynthesis. However, due to unclear annotations and lack of biochemical characterization, the role of this satellite cluster is still ambiguous.

**Expression of structural core biosynthetic genes**

Comparative genomic analysis of the scytonemin gene cluster from various cyanobacterial strains revealed that six gene products of ScyA-F are anticipated to produce the monomer moiety of scytonemin, and the final reaction, i.e., dimerization step was predicted to catalyze by tyrosinase (TyrP) and/or oxidoreductase (DsbA) (Fig. 1). \(^15\) Accordingly, we constructed the recombinant plasmids pACYC-ScyB, pCDF-ScyAB, pRSF-ScyEF and pET-TyrP-DsbA and introduced them into E. coli BL21 (DE3). The resulting strain was designated as E. coli SM4. The in vivo isotope labeling studies in cyanobacterial strains showed that both labeled tryptophan and tyrosine were incorporated into the scytonemin structure during its biosynthesis. \(^12\) So, we supply tryptophan and tyrosine as precursor substrates during the biotransformation of E. coli SM4. The culture broth of E. coli SM4 strain supplemented with these precursors turned yellow and the yellowish product was primarily accumulated in the cell pellet (ESI Fig. S2†). HPLC and mass analysis of the bioconversion products of SM4 strain upon supplementation of 500 μM of tryptophan and tyrosine accumulated compound 2 \((C_{18}H_{15}NO_3)\) calculated \([M + H]^+\): 296.12866, found: 296.12876 and calculated \([M – H]^–\): 294.11301, found 294.11344), compound 3 \((C_{14}H_{15}NO_2)\) calculated \([M + H]^+\): 278.11810, found: 278.11783), compound 4 \((C_{18}H_{13}NO_3)\) calculated \([M + H]^+\): 276.10245, found: 276.10229) along with new alkaloid derivatives compound 5 \((C_{26}H_{20}N_2O_2)\) calculated \([M + H]^+\): 393.16030, found: 393.16022), compound 6 \((C_{26}H_{20}N_2O_2)\) calculated \([M + H]^+\): 393.16030, found: 393.16049), compound 7 \((C_{36}H_{24}N_2O_4)\) calculated \([M + H]^+\): 533.21733, found: 533.21837), compound 8 \((C_{36}H_{24}N_2O_4)\) calculated \([M + H]^+\): 595.22329, found: 595.22323) and compound 9 \((C_{36}H_{24}N_2O_4)\) calculated \([M + H]^+\): 537.21781, found: 537.21747) (Fig. 3A and 3B and ESI Fig. S4†).
of scytonemin. Nostodione A is generated upon ozonolysis of the reduced form of scytonemin, and this compound has been isolated from N. commune and a fresh water cyanobacterium, Scytonema hofmanni. Similarly, the three new scytonemin derivatives, dimethoxyscytonemin, tetramethoxyscytonemin and scytonin, have been identified from the organic extracts of Scytonema sp. These compounds do not possess cytotoxic effects even at 10 µM and also did not inhibit the growth of Gram positive, Gram negative and fungi at the concentration of 1 µM. All of these previously reported derivatives are derived from the scytoneman skeleton of scytonemin. To the best of our knowledge, all the shunt products we found in this study have not been reported yet from any cyanobacterial strains including N. punctiforme. So, it is plausible that these shunt oxidation pathways are catalyzed by E. coli endogeneous enzyme(s) consuming the accumulated compound in the cell.

**Structural elucidation of compound 4**

The $^1$H and $^{13}$C-NMR signals of compound 4 are given in Table 3 whereas the COSY, HMBC, HSQC and NEO spectrum are given in ESI (ESI Fig. S5†). In the $^1$H-NMR spectrum, the low field singlet signals at 10.03 ppm and 10.93 ppm correspond to phenyl hydroxyl and indole amide groups, respectively, the signals between 7 ppm and 8 ppm correspond to typical aromatic phenyl and indole rings and the signal at 3.51 ppm corresponds to an aliphatic signal. The COSY spectrum confirmed the proton observations and revealed a correlation between the amide and one of the terminal protons of the indole proton system (4 bonds apart), allowing a sequential assignment of the proton spectrum (in fact this seems to be a 5 bond correlation from the NH to the opposite side of the indole proton network). The HSQC correlated these proton signals to their respective carbons, permitting the firm assignment of all non-quaternary carbons. The HMBC allowed the assignment of some quaternary signals and the observation of a correlation between the aliphatic signal and a resonance at 204.85 ppm (only ketones resonate at this frequency). Due to the scarcity of protons in this molecule, the fact that HMBC signals can correlate to 2, 3 or 4 bonds apart and the cyclic nature of the molecule, sequential assignment and structural confirmation of the 5 membered ring becomes virtually impossible. The presence of an indole, a phenyl and a ketone group is indisputable, however their position could not be ascertained so six structures as shown in Fig. 4A were possible.

At this stage a NOE spectrum was acquired. The NOE spectrum revealed a correlation between the amide proton and signals of the phenyl group suggesting only possible structures (i) and (vi) in Fig. 4A. Also, a signal was observed between the aliphatic group and a proton on the indole ring but not with the phenyl ring and the amide group, which strongly suggests that the possible structure for compound 4 is structure (i) in Fig. 4A.

![Fig. 4](image_url)

(4) Six possible structures for compound 4 compatible with proton, carbon, COSY, HSQC and HMBC NMR analysis. (B) Structure of the compound 4 with atom numbering. (C) HPLC analysis of purified compound 4 from biotransformation of E. coli SM4 at 360 nm.
verts L-tryptophan to indole-3-pyruvic acid, which is coupled by enzymes of the scytonemin gene cluster proved that ScyB catalyzes deamination of tyrosine was similar to the control strain (ESI Fig. S2†). Unlike the yellowish culture broth of SM4, and both of these strains accumulated compounds 2a and 2b.44 On the other hand, in the presence of ScyC, this non-enzymatic decarboxylation reaction is suppressed in favor of an intramolecular cyclization followed by dehydration and irreversible decarboxylation to produce compound 3a.44 Although the in vitro studies on scyC only accumulated 3a,44 we found that in vivo production of a monomer moiety of scytonemin (compound 4) in E. coli can be achieved by expression of only three genes, scyABC, from N. punctiforme. This indicates that the endogenous enzyme(s) from the E. coli host catalyze the oxidation reaction to convert compound 3 into compound 4. Furthermore, the dimerization reaction for the generation of compounds 7, 8 and 9 are also likely catalyzed by the E. coli endogenous enzyme(s) instead of TyrP/DsbA from N. punctiforme as all five shunt products were also accumulated in the SM2 strain harboring only scyABC genes.

**Comparison of compounds 4 and 7 yields**

The production of the monomer moiety of scytonemin and shunt dimer compound 7 from E. coli strains SM2 and SM4 was analyzed by supplementing tryptophan and tyrosine in M9 minimal medium at 5 days of reaction time. Utilizing endogenous tryptophan and tyrosine, the strains can produce compounds 4 and 7 upon IPTG induction. However, the yields of these compounds are higher upon supplementation of tryptophan and tyrosine. The biotransformation of strain SM2 supplemented with 500 μM of substrates produced 5.0 mg L⁻¹ of compound 4 and 46.9 mg L⁻¹ of compound 7 whereas at 1 mM of substrate supplementation 7.3 mg L⁻¹ of compound 4 and 77.0 mg L⁻¹ of compound 7 were produced. Likewise, the strain SM4 produced 6.1 mg L⁻¹ of compound 4 and 46.3 mg L⁻¹ of compound 7 at 500 μM substrate supplementation whereas at 1 mM of substrate supplementation 8.9 mg L⁻¹ of compound 4 and 87.1 mg L⁻¹ of compound 7 were produced (Fig. 5). On the other hand, upon IPTG induction the strain STN produced 4.2 mg L⁻¹ of compound 4 and 39.2 mg L⁻¹ of compound 7, respectively, in M9 minimal media at 5 days.

![Graph showing production of compounds 4 and 7 by E. coli recombinant strains SM2 and SM4 with/without supplementation of tryptophan and tyrosine and strain STN with/without IPTG induction.](https://example.com/fig5)
The biomass (DCW) of IPTG induced substrate supplemented (1 mM of each) SM2 and SM4 strains were 1.84 g L\(^{-1}\) and 1.94 g L\(^{-1}\) at 5 days whereas those of the control strains were 1.87 g L\(^{-1}\) and 1.81 g L\(^{-1}\), respectively. Similarly, upon IPTG induction STN strain had 1.70 g L\(^{-1}\) of DCW, whereas in the absence of induction this strain had 1.86 g L\(^{-1}\) of DCW at 5 days. This showed the yield of 2.46 µg mg\(^{-1}\) DCW, 3.96 µg mg\(^{-1}\) DCW, and 4.56 µg mg\(^{-1}\) DCW of the compound 4 by STN, SM2 and SM4 strains, respectively.

Conclusions

Following our work, the final dimerization step remains a major hurdle for the complete production of scytonemin in *E. coli*. Yet commercially, many drugs such as an anticancer drug, paclitaxel (Taxol),\(^45\) and an antimalarial drug, artemisinin\(^46\) have been produced by combining the biosynthetic and chemical synthetic approaches, highlighting the advantageous features of the bio-chemical approach for production of complex compounds. Our construction of a cell factory producing the monomer moiety of scytonemin could facilitate such production when combined with the already described chemical synthetic dimerization step.

Upon supplementation of 1 mM of tryptophan and tyrosine, ca. 158 µM of compound 7 (i.e., 316 µM of the equivalent substrate concentration), ca. 32 µM of the monomer moiety of scytonemin, and comparable amounts of other derivatives (compounds 2, 3, 5, 6, 8, and 9) to that of compound 4 were produced. This indicates that nearly half of the supplemented substrates were utilized by the heterologously expressed scytonemin pathway in the constructed *E. coli* strain. This *E. coli* cell factory has a 3.5 fold higher yield of the scytonemin monomer moiety as compared to the scytonemin produced by the native producer *N. punctiforme*. Accordingly, our work represents an important milestone towards a green scytonemin process. However, the industrial applicability of this system requires a maximal conversion of substrates into the targeted product without (or low) the production of side products. Several techniques could possibly be applied for further optimization of this strain and biotransformation systems to enhance production. For example, inactivation of the targeted gene(s) could facilitate the production yields by preventing metabolic flux through undesired branch pathways.\(^47,48\) Furthermore, expression level optimizations of heterologous pathway enzymes could be achieved by altering the plasmid copy number\(^49\) and promoter strength\(^50\) and engineering the ribosome binding sites (RBS).\(^51\) Similarly, adaptive laboratory evolution (ALE) strategies have been broadly applied in metabolic engineering of *E. coli* for improving fitness, yield, production rate and cost-effectiveness. The ALE techniques are greatly effective for non-native pathway optimization which allows the selection of beneficial mutations in the production strains in an unbiased fashion.\(^52\) Likewise, immobilization of enzymes or whole cells has been successfully applied in numerous scientific and industrial processes.\(^53\) Enzyme properties such as stability, activity, specificity, selectivity, etc. have been greatly improved by enzyme immobilization and multi-enzyme co-localization.\(^54,55\) During biotransformation, supplementation of high substrate concentration may have a tendency to change the pH, osmotic pressure, etc. of culture media (or reaction conditions), thus limiting the bioconversion process. However, immobilization of the enzyme could increase resistance to such changes and it may also increase the enzyme concentration, which favors supplementation of higher substrate concentrations and hence increase the product yield. Immobilized technology has been extensively used in bioreactors for significant improvement of the yields in fermentation.\(^56\) In addition, systematic and careful design in bioreactor and optimization of physical parameters such as cultivation conditions (temperature, dissolved oxygen and RPM), pH condition, media composition, etc. has a great impact in the bioconversion process.\(^57\)

Further in-depth studies to better understand the shunt pathway B is essential as a majority of compound 3 was consumed by this pathway. Likewise, compound 3 was also consumed by forming an adduct with the indole moiety through a shunt pathway A. Since tryptophanase is responsible for degradation of L-tryptophan into indole, pyruvate and ammonia,\(^58\) the prevention of tryptophan degradation as well as the effect of shunt pathway A could be abolished by inactivation of chromosomal tryptophanase (*tnaA*) in *E. coli*. These strains could be further metabolically engineered for the overproduction of endogenous tryptophan and the tyrosine pool.\(^59,60\) For example, overexpression of branch pathway genes from chorismate to L-tyrosine and L-tryptophan can overproduce these amino acids.\(^61\) Hence, studies on the dimerization reaction for the complete synthesis of scytonemin in *E. coli* along with pathway optimizations to improve the yield of compound 4 will be the focus of future investigations.

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