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**Biosynthesis**

# Widely Distributed Bifunctional Bacterial Cytochrome P450 Enzymes Catalyze both Intramolecular C–C Bond Formation in *cyclo*-L-Tyr-L-Tyr and Its Coupling with Nucleobases

*Jing Liu[+], Lauritz Harken[+], Yiling Yang, Xiulan Xie, and Shu-Ming Li**

**Abstract:** Tailoring enzymes are important modification biocatalysts in natural product biosynthesis. We report herein six orthologous two-gene clusters for mycocyclosin and guatyromycine biosynthesis. Expression of the cyclodipeptide synthase genes $gymA_1$–$gymA_6$ in *Escherichia coli* resulted in the formation of *cyclo*-L-Tyr-L-Tyr as the major product. Reconstruction of the biosynthetic pathways in *Streptomyces albus* and biochemical investigation proved that the cytochrome P450 enzymes $GymB_1$–$GymB_6$ act as both intramolecular oxidases and intermolecular nucleobase transferases. They catalyze not only the oxidative C–C coupling within *cyclo*-L-Tyr-L-Tyr, leading to mycocyclosin, but also its connection with guanine and hypoxanthine, and are thus responsible for the formation of tyrosine-containing guatyromycines, instead of the reported tryptophan-nucleobase adducts. Phylogenetic data suggest the presence of at least 47 GymB orthologues, indicating the occurrence of a widely distributed enzyme class.

## Introduction

2,5-Diketopiperazines (DKPs) and their derivatives are widespread in nature and represent an important class of secondary metabolites.[1,2] They display a broad spectrum of biological and pharmacological activities, such as antibacterial, antifungal, antitumor, anti-plasmodial, and anti-Alzheimer properties.[3–8] Apart from these effects, they are also involved in quorum sensing and might be used in future as drug delivery systems due to a high cell penetration.[9–11] All these features make them attractive molecules for drug discovery and development. In nature, the heterocyclic DKP core of cyclodipeptides (CDPs) is usually formed by condensation of two α-amino acids in two manners. This reaction is either catalyzed by nonribosomal peptide synthetases (NRPSs), mainly found in fungi, or by cyclodipeptide synthases (CDPSs), commonly occurring in bacteria.[12–14] In comparison to NRPSs, CDPSs are small proteins of approximate 30 kDa and use activated aminoacyl-tRNAs from the ribosomal machinery as substrates.[15] The formation of a DKP as backbone exhibits an increased stability against proteolysis compared to acyclic dipeptides, which enables tailoring enzymes to modify the heterocycle or the side chains.[16] In the CDPS-dependent pathways, such enzymes include cytochrome P450 enzymes, cyclodipeptide oxidases, $Fe^{II}$/2-oxoglutarate-dependent oxygenases, prenyltransferases, methyltransferases, and terpene cyclases.[17–22]

Members of the P450 enzyme superfamily occur ubiquitously in living organisms and play vital roles in the metabolism of xenobiotics as well as the biosynthesis of natural products.[23–25] Due to different lengths of the polypeptide chains, shapes of the binding pockets, and electron transfer partners, they can catalyze a large variety of transformations.[25] The catalytic activities of P450 enzymes in CDPS-associated pathways include hydroxylation, e.g. BcmD in the formation of bicyclomycin,[18,26] multistep oxidation as CypX in the biosynthesis of pulcherriminic acid,[27] dimerization in the production of naseazine C and tetratryptomycins,[28–30] the formation of an intramolecular C–C bond by CYP121 in the mycocyclosin biosynthesis,[31] and transfer of nucleobases, e.g. guanine and hypoxanthine in the biosynthesis of guanitrypmycins, purincyclamide, and guatrypmethines (Figure 1A).[32–34]

Bioinformatic analysis revealed the presence of a widely distributed family of two-gene clusters from *Streptomyces* species, termed *gym* clusters, each coding for a CDPS GymA and a P450 enzyme GymB. Herein, we report the identification of six of these clusters by heterologous expression in *E. coli* and *Streptomyces albus* (*S. albus*) as well as by biochemical characterization. Our results demonstrated that $GymA_1$–$GymA_6$ assemble *cyclo*-L-Tyr-L-Tyr (cYY, **1**) as main and *cyclo*-L-Tyr-L-Phe (cYF, **2**) as side product. The P450 enzymes $GymB_1$–$GymB_6$ catalyze the formation of mycocyclosin (**3**) by oxidative intramolecular C–C coupling and the transfer of a nucleobase, guanine or hypoxanthine, onto different positions of **1** for the formation

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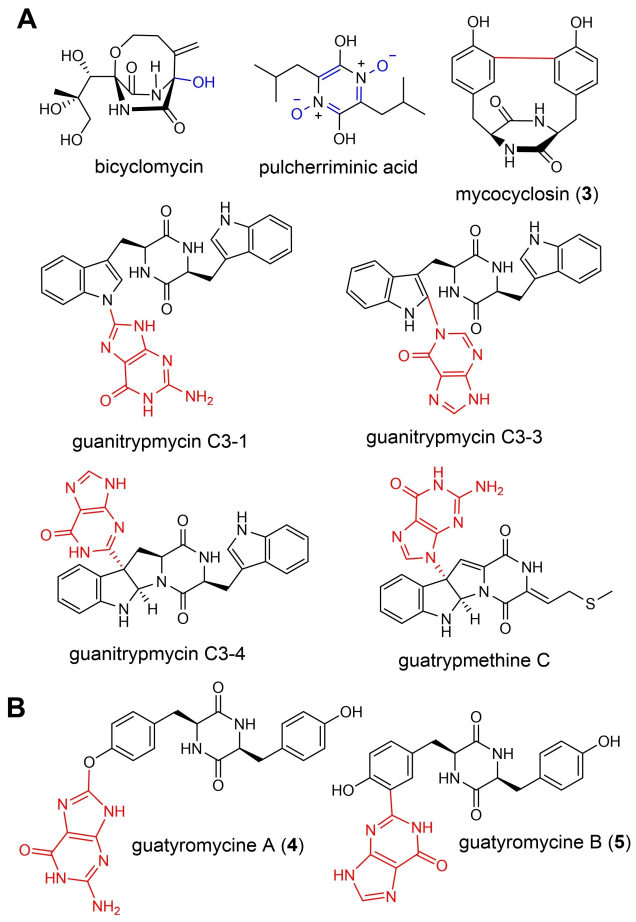


***Figure 1.*** Known (A) and new (B) secondary metabolites from biosynthetic pathways containing CDPS and P450 enzymes. P450 enzymes catalyze novel CDP modifications including hydroxylation/oxidation (depicted in blue), formation of the intramolecular C–C bond in mycocyclosin and the nucleobase transfer reactions in guanitrypmycins and guatrypmethines (depicted in red).

of guatyromycines A (**4**) and B (**5**, Figure 1B), respectively. Thus, GymBs represent the first P450 members in CDPS-related pathways that can catalyze both intra- and intermolecular coupling reactions.

## *Results and Discussion*

In recent years, the number of microbial genomic sequences in the public databases, especially those of bacteria and fungi, has increased exponentially.[35] The available sequence data made us aware that the number and diversity of metabolites from a candidate organism is far greater than those reported. Among the tremendous number of putative gene clusters containing *cdps* genes, only a dozen CDPS-related biosynthetic pathways have been characterized yet, implying that a great potential still remains unexplored.[15,17,19] In order to identify more *cdps-P450*-containing gene clusters, we used the sequences of the functionally characterized CDPSs as probes to search for

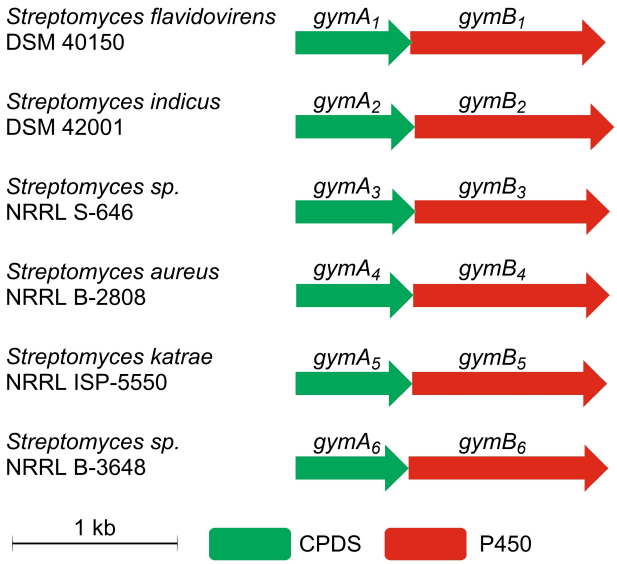


***Figure 2.*** Genetic organization of the *gym* clusters from six *Streptomyces* strains.

their homologs by using BLAST® and analyzed their neighboring genes coding for tailoring enzymes, especially for P450 enzymes. Phylogenetic analysis of known and putative CDPS and associated P450 sequences indicated the presence of a large group of unknown *cdps-P450* gene clusters (Figures S1 and S2). Phylogenetic analysis also revealed that the reported P450 enzymes responsible for nucleobase transfer and CDP dimerization are clustered in two distinct clades (Figure S2). It is noteworthy that CYP121, which catalyzes the intramolecular oxidative C–C coupling of *cyclo*-L-Tyr-L-Tyr,[31] is located closest to the largest clade containing 47 unknown P450 homologs. Thus, we selected one representative from each of the subclades, in total six different *cdps-P450* gene clusters, for detailed investigation. These clusters were identified in *Streptomyces flavidovirens* DSM 40150 ($gymA_1B_1$), *Streptomyces indicus* DSM 42001 ($gymA_2B_2$), *Streptomyces sp.* NRRL S-646 ($gymA_3B_3$), *Streptomyces aureus* NRRL B-2808 ($gymA_4B_4$), *Streptomyces katrae* NRRL ISP-5550 ($gymA_5B_5$), and *Streptomyces sp.* NRRL B-3648 ($gymA_6B_6$) (Figure 2, Table S2). These candidate P450s share approximately 60 % sequence identities with CYP121 encoded by *rv2276* on the amino acid level (Table S1).

Alignments of the six CDPSs from the selected clusters with AlbC from *Streptomyces noursei* (Figure S3)[36] and prediction of their substrate specificities[14,37] revealed that both binding pockets are specific for L-Tyr-tRNA, so that *cyclo*-L-Tyr-L-Tyr can be expected as their product (Table S3). To investigate the *cdps* function, the coding sequences of $gymA_1$–$gymA_6$ were cloned into pET28a(+) for heterologous expression in *E. coli* BL21(DE3) (Tables S2 and S4). LC-MS analysis of the bacterial cultures bearing the *gymA* genes revealed similar metabolite profiles and the presence of one predominant product with a $[M+H]^+$ ion at *m/z* 327.134 ± 0.005 and a minor peak with a $[M+H]^+$ ion at *m/z* 311.139 ± 0.005 (Figure S4). Comparison of

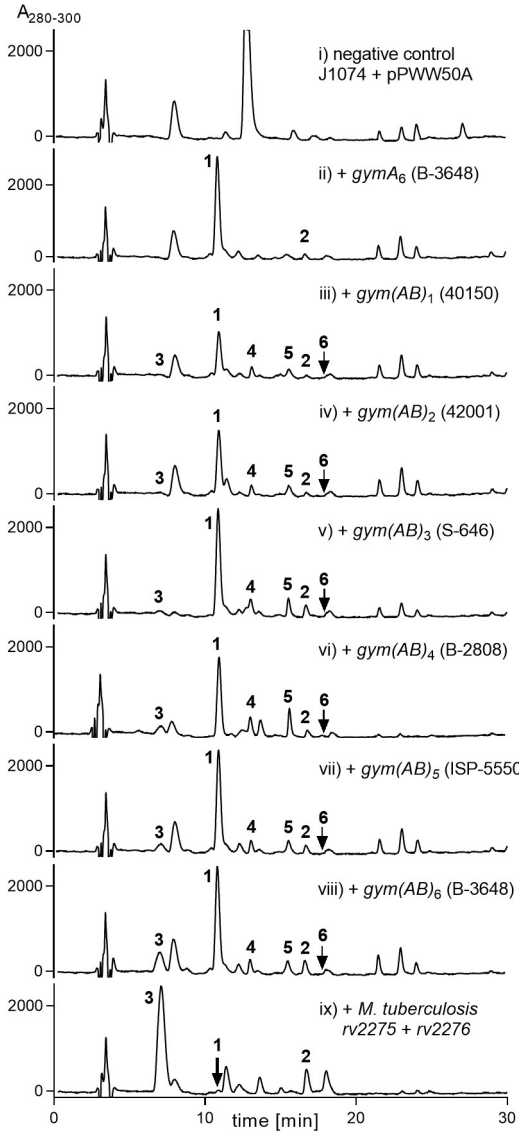


***Figure 3.*** HPLC analysis of the extracts from *S. albus* transformants. The dominant peak at 13 min in the negative control was strongly reduced in the transformants, as observed in other expression studies.[22,34] Therefore, it is unlikely related to the introduced *gym* genes.

the retention times, $MS^2$ data, UV-spectra (Figure S5), and NMR data (Table S6, Figures S8 and S9) with authentic standards led to the identification of predicted *cyclo*-L-Tyr-L-Tyr (cYY, **1**) as the main product (Table S3) and *cyclo*-L-Tyr-L-Phe (cYF, **2**) as the minor one. Production of two CDPs by one CDPS is in agreement with the published substrate promiscuity of other characterized enzymes.[38,39]

Heterologous expression of microbial biosynthetic gene clusters in well-investigated host strains has proven to be an effective approach to discover new natural products.[40] Having determined the function of the backbone enzymes $GymA_1$–$GymA_6$ as cYY and cYF synthases, we decided to heterologously express the two-gene clusters in *S. albus* J1074 to identify the cluster products.[41] Since $GymB_6$ was located closest to CYP121 in the phylogenetic tree (Figure S2), the $gymA_6B_6$ cluster from *Streptomyces sp.* NRRL B-3648 was taken as the first candidate. We first reproduced the expression of $gymA_6$ in J1074 under control of the constitutive *ermEp** promotor by using the replicative vector pPWW50A.[42] The transformant was cultivated in MR5 media (Table S2) and extracted with EtOAc. LC-MS analysis revealed that, in comparison to the negative control with pPWW50A (Figure 3i), the $gymA_6$ transformant produced both **1** and **2** as CDPS products (Figure 3ii), corresponding very well to those identified by expression in *E. coli* (Figure S4).

The $gymA_6B_6$ cluster was then cloned into pPWW50A and expressed in *S. albus.* LC-MS analysis of the cultural extract displayed three new peaks (Figure 3viii) with distinct UV absorptions (Figure S5). Peak **3** has a $[M+H]^+$ ion at *m/z* 325.1180, corresponding well to that of mycocyclosin.[31] The $[M+H]^+$ ion of **4** (*m/z* 476.1840) is 149 Da larger than that of **1**, suggesting an additional guaninyl moiety on it. The $[M+H]^+$ ion of **5** at *m/z* 461.1719 is 134 Da larger than that of **1**, indicating the attachment of a hypoxanthinyl residue.

In order to elucidate the structures of the newly accumulated products, analytically pure compounds **3**–**5** were obtained by semi-preparative HPLC and subjected to NMR analysis. Comparison of the $^1H$ NMR spectrum with that published previously supported **3** to be mycocyclosin (see Figure 1A for structure, Table S6, Figure S10).[31] Detailed inspection of the NMR data of **4**, including $^1H$, $^{13}C$, $^1H$–$^1H$ COSY, HSQC, and HMBC (Table S7, Figures S11–S15, see Supporting Information for detailed structure elucidation), suggested a cYY derivative with a guanine residue attached to a hydroxyl group (Figure 1B). Structure elucidation of **5** confirmed our assumption that a hypoxanthine is transferred via its C-2′ to C-10 of the cYY residue. (Table S8, Figures S16–S20). Cultivation of *S. albus* transformant harboring $gymA_6B_6$ in medium with $^{15}NH_4Cl$ and subsequent LC-MS analysis of the cultural extract demonstrated that at least two $^{15}N$ atoms are incorporated into in **3**, seven in **4**, and six in **5** (Figure S21), confirming the structures of **3**, **4**, and **5** (Figure 1). Based on the fact that **4** and **5** are derived from L-tyrosine and guanine/hypoxanthine, they are therefore termed as guatyromycines A and B, respectively.

In analogy to $gymA_6B_6$ cluster from *Streptomyces sp.* NRRL B-3648, we performed co-expression of the genes for CDPS and P450 enzyme from the other five candidates (Figure 2) in *S. albus.* The plasmids (Table S4) were constructed as described in the Supporting Information and transferred into *S. albus* by conjugation. The extracts of the corresponding transformants were analyzed by LC-MS (Figure 3), which revealed the presence of very similar product profiles as described for NRRL B-3648. Together with their precursor **1** and the side product **2**, all three products **3**, **4**, and **5** were clearly observed by UV and EIC detections (Figure 3iii–3vii, Figure S6). Their structures were further confirmed by comparison of their retention times, UV-spectra, and MS data including $MS^2$ fragmentation pattern with those of **3**, **4**, and **5** isolated in this study. As compound **3** is also the product of CYP121 from *Mycobacterium tuberculosis*, we decided to express the responsible cluster

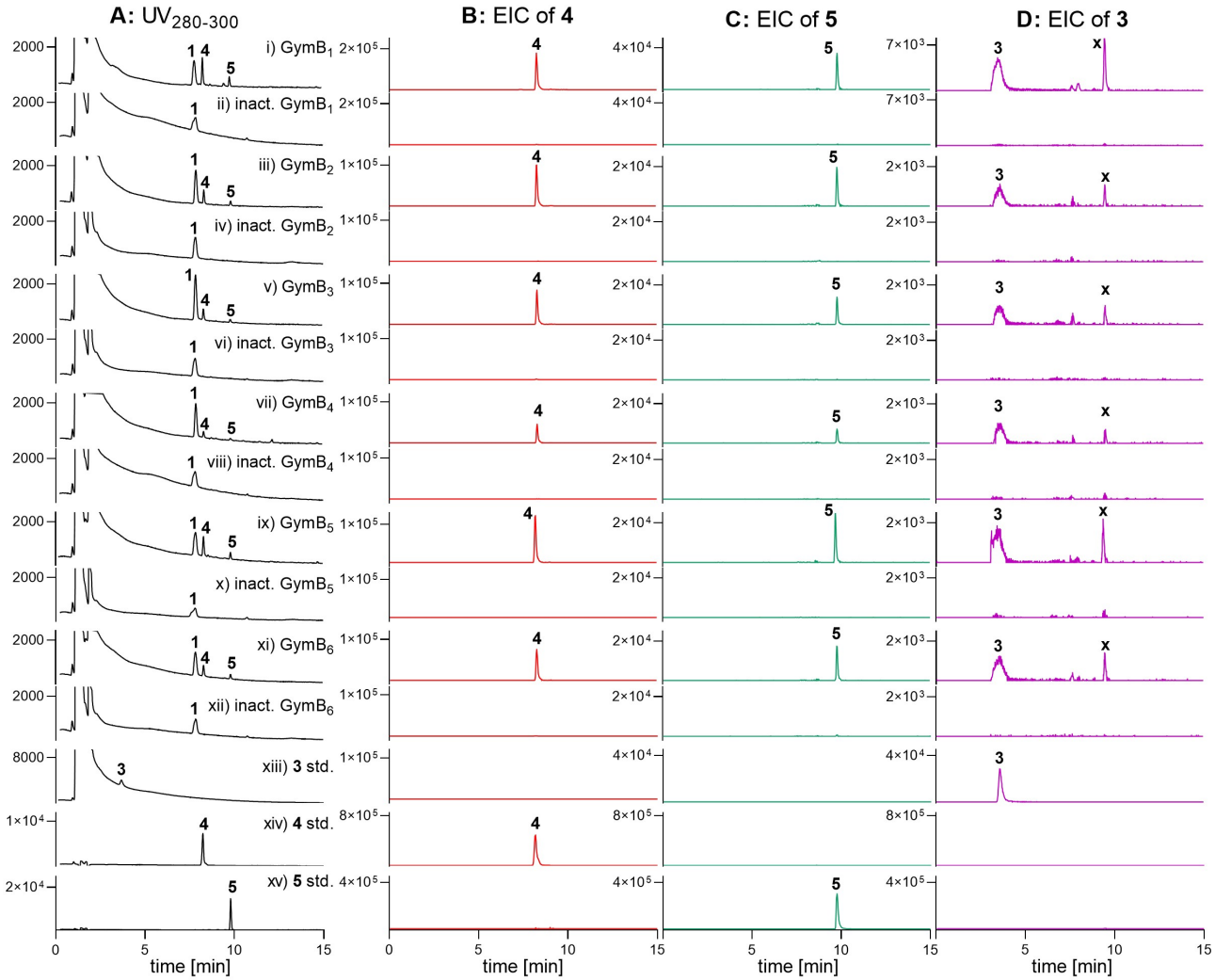


***Figure 4.*** LC-MS chromatograms of cYY assays with $GymB_1$–$GymB_6$ in the presence of guanine and hypoxanthine. EICs of **3**, **4**, and **5** refer to $[M+H]^+$ ions at *m/z* 325.118, 476.184, 461.172 with a tolerance range of ±0.005. Peak **x**: unknown mycocyclosin isomer. inact.: heat-inactivated; std.: standard.

with two genes *rv2275* and *rv2276* as well.[31] In comparison, the cluster from *M. tuberculosis* mainly produced **3** with a product yield of 53.9 ± 2.8 mg $L^{-1}$ (Table S5), accompanied by **1** and **2** as minor peaks (Figure 3ix, Figure S6), confirming that CYP121 merely catalyzes the formation of **3**.[31] Quantification of the productivity of the different transformants (Table S5) showed that **4** and **5** were accumulated in comparable product yields of 2.3–5.5 mg $L^{-1}$, while the contents of **3** varied significantly from 1.6 ± 0.2 mg $L^{-1}$ for $gymA_2B_2$ to 24.0 ± 1.3 mg $L^{-1}$ for $gymA_6B_6$.

An intramolecular coupling product of cYF (**2**) or its adduct with hypoxanthine were detected neither by UV nor by $[M+H]^+$ ion detection in all of the transformants described in this study (Figure S7). In contrast, a clear peak **6** at 17.8 min was detected with a *m/z* of 460.173 ± 0.005, corresponding to the $[M+H]^+$ ion of **2** with guanine, in the transformants carrying $gymA_1B_1$–$gymA_6B_6$. However, this peak is so weak that it's almost not visible by UV detection (Figure 3). Due to the low quality, its structure could not be elucidated in this study.

From these results, it can be postulated that the CDPSs $GymA_1$–$GymA_6$ use tyrosyl-tRNA and phenylalanyl-tRNA as substrates to assemble cYY (**1**) as the major and cYF (**2**) as the minor product. cYY serves then as the substrate of the cytochrome P450 enzymes $GymB_1$–$GymB_6$ for intramolecular oxidative C–C bond formation and coupling with guanine and hypoxanthine via different atoms and positions of cYY, resulting in mycocyclosin (**3**), guatyromycines A (**4**) and B (**5**), respectively (Figure 1). It is likely that **2** was also used by $GymB_1$–$GymB_6$ as the substrate for coupling with guanine.

To prove the activities of $GymB_1$–$GymB_6$ in vitro, their coding sequences were cloned into pET28a(+) for overproduction in *E. coli* BL21(DE3) (Table S4). The recombinant $His_6$-tagged $GymB_1$–$GymB_6$ were purified on Ni-NTA agarose (Figure S22) and used for in vitro assays with **1**, guanine, and hypoxanthine in the presence of ferredoxin,

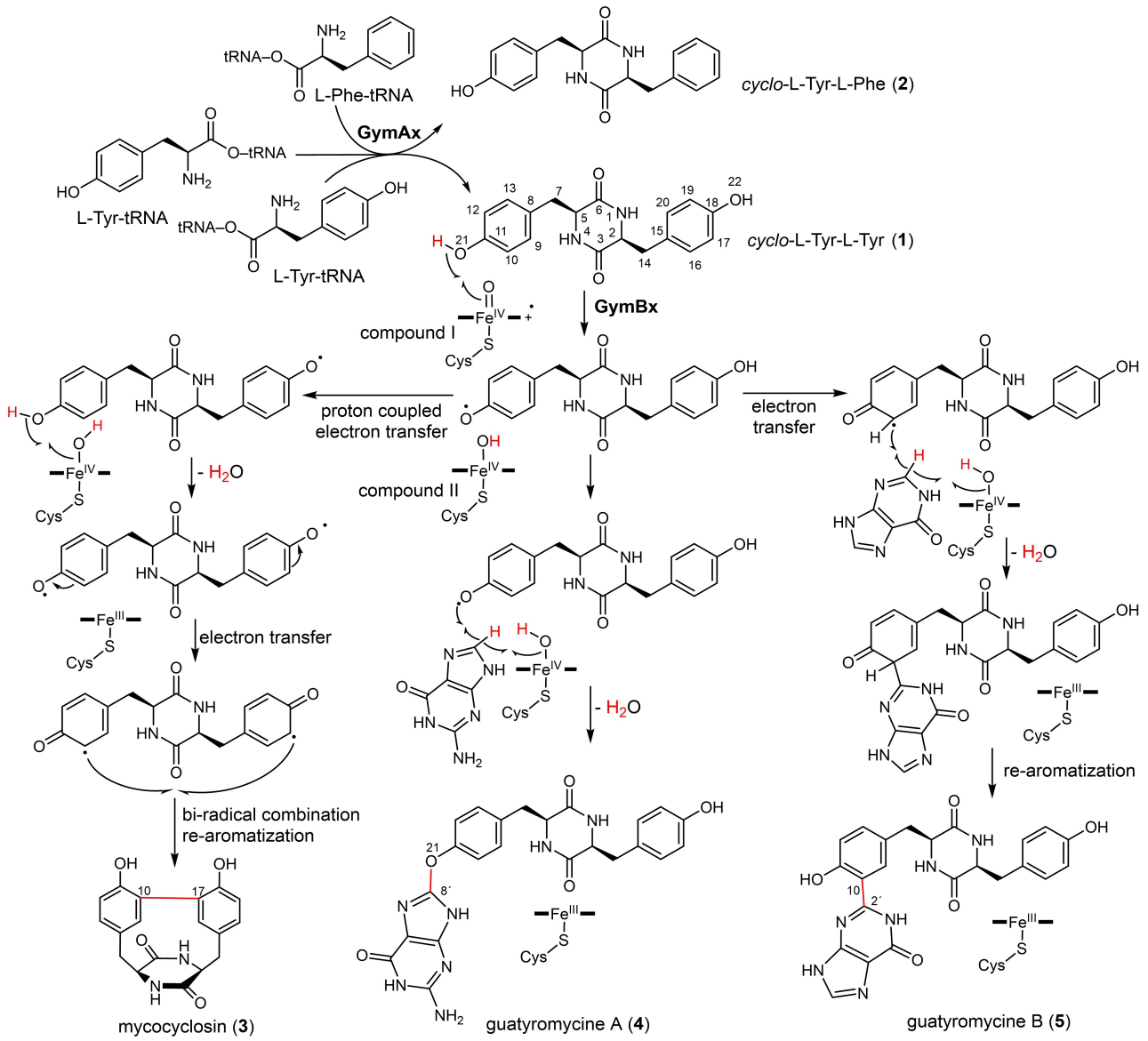


***Figure 5.*** Biosynthetic pathway of **3**, **4**, and **5** and mechanism of the GymB-catalyzed reactions.

ferredoxin-reductase, and NADPH. Heat-inactivated proteins served as negative controls. After incubation at 30 °C for 16 h, the reactions were quenched with MeOH and analyzed by LC-MS. As shown in Figure 4, **4** was detected in all the assays with active enzymes as the major product, followed by **5** and **3**. These results confirmed unequivocally that $GymB_1$–$GymB_6$ catalyze both the nucleobase transfer reactions and the intramolecular C–C bond coupling. They act therefore as bifunctional enzymes. In the absence of guanine and hypoxanthine, $GymB_1$–$GymB_6$ converted **1** to **3** with much higher yields than with nucleobases (Figure S23). A peak "**x**" at 9.5 min was detected in EIC for **3** of some assays with and without nucleobases (Figures 4 and S23), indicating the presence of a mycocyclosin isomer. Due to the trace product amount, its structure could not be determined in this study.

It can be postulated that the abstraction of one hydrogen from OH at C-11 by the $Fe^{IV}$=O species (compound I) leads to the formation of a radical at O-21, which acts as a central intermediate for the formation of mycocyclosin (**3**)[43] and guatyromycines A (**4**) and B (**5**) (Figure 5). Transfer of a hydrogen from the second hydroxyl group to $Fe^{IV}$–OH (compound II) and release of one molecule $H_2O$ will result in the formation of a diradical intermediate. Electron migration to C-10 and C-17, radical combination and subsequent rearomatization will release product **3** from the GymBx template. Direct reaction of the radical at O-21 with C-8′ of guanine, transfer of one hydrogen to $Fe^{IV}$–OH and water elimination lead to the formation of guatyromycine A (**4**). In the case of the formation of guatyromycine B (**5**), the radical at O-21 would first undergo electron migration, resulting in a radical at C-10, which couples with hypoxanthine at its C-2′. Water elimination and rearomatization

complete the formation of **5** (Figure 5). The $Fe^{III}$ species will be reduced to $Fe^{II}$ by reduction partners ferredoxin/ferredoxin reductase/NADPH and is ready for binding of $O_2$ for the next reaction cycle.[25,44] It seems that C-2′ in hypoxanthine is preferred to react with C-10 of cYY in analogy to the formation of mycocyclosin, which has been investigated in detail.[43] The connection of guanine with cYY is likely caused by the amino substitution at C-2′ of the purine skeleton and a resulting steric hindrance in the binding pocket of the enzymes.

## Conclusion

In this study, we demonstrated by heterologous expression the presence of a widely distributed family of two-gene clusters coding for a CDPS and a cytochrome P450 enzyme in actinobacteria (Figures S1 and S2), which produce both mycocyclosin (**3**) by intramolecular oxidative C–C coupling of cYY (**1**) and guatyromycines A (**4**) and B (**5**) by transfer of a nucleobase onto different positions of **1**. Biochemical investigations by using recombinant P450 enzymes $GymB_1$–$GymB_6$ proved the conversion of **1** to **3**, **4**, and **5**. To the best of our knowledge, the GymBs are the first described CDPS-associated P450s that function as unique bifunctional enzymes for both intramolecular coupling and nucleobase transfer onto a tyrosyl moiety, which is different from those reported previously.[31,32,34,45,46]

Cultivation of the six *gymAB*-bearing *Streptomyces* species given in Figure 2 in two different media and LC-MS analysis of the extracts did not lead to detection of any products mentioned above, indicating the presence of silent genes in the native strains (data not shown).

Mycocyclosin (**3**) has been proven to be the product of a gene cluster with a CDPS gene *rv2275* and the gene *rv2276* for the cytochrome P450 enzyme CYP121 from *M. tuberculosis*.[31] No adduct of **1** with nucleobases was reported. We confirmed in this study these results by heterologous expression of *rv2275/rv2276* in *S. albus*. A product yield of 53.9 $mg\,L^{-1}$ was achieved for **3**. This is two- to twenty-fold of the transformants carrying *gym* clusters (Table S5). It could be speculated that *gymBs* and their potential orthologues evolved from *rv2276*. Phylogenetic analysis revealed a coherence of the product yields for **3** in the respective transformants and their sequence proximity. For example, $GymB_{4-6}$ are located closely to CYP121 and the *S. albus* transformants with their clusters produced **3** at 9.4–24.0 $mg\,L^{-1}$, significantly higher than 1.6 $mg\,L^{-1}$ in the $gymA_2B_2$ and 2.3 $mg\,L^{-1}$ in the $gymA_1B_1$ transformant.

In summary, this study demonstrates again the power of the combinational approaches of genome mining in the available databases, heterologous expression in well-established hosts and biochemical characterization with recombinant proteins for the discovery of new secondary metabolites and intriguing enzymes. It can be expected that more novel natural product structures and enzymes will be discovered in the future by unravelling the biosynthetic potential hidden behind the silent/cryptic genes in the tremendous available genome sequences.

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## Conflict of Interest

The authors declare no conflict of interest.

## Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

**Keywords:** Biosynthesis · Cyclodipeptide Synthase · Cytochrome P450 Enzymes · Natural Products · Nucleobase Transferase

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