Amino Acid Synthesis

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# Stereospecific Biosynthesis of β-Methyltryptophan from L-Tryptophan Features a Stereochemical Switch**

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Nonproteinogenic amino acids, especially β-methyl amino acids, are important building blocks in the assembly of bioactive natural products.[1] For example, β-methyl aspartic acid is a biosynthetic precursor of friulimicin,[2] nikkomycin,[3] streptolydigin,[4] and vicenistatin,[5] (2*S*,3*S*)-β-methylphenylalanine is a precursor of mannopeptimycin,[6] and the lipopeptide antibiotics daptomycin, calcium-dependent antibiotic (CDA), and A54145 contain a (2*S*,3*R*)-β-methyl glutamate unit.[7] Intriguingly, two different diastereoisomers of β-methyl tryptophan (β-MeTrp) have been found to contribute to nonribosomally synthesized peptides and tryptophan-derived alkaloids: maremycin A and B (MARs)[8] and FR900452[9] contain the (2*S*,3*S*)-β-MeTrp (**5**) unit, while chaetoglobisin K,[10] indolmycin,[11] and telomycin[12] contain the (2*S*,3*R*)-β-MeTrp (**4**) moiety (Figure 1). This moiety is also proposed to be the biosynthetic precursor of both streptonigrin[13] and lavendamycin.[14] Stereospecific alkyl functionalization of amino acids at the relatively unreactive β-position is a synthetic challenge which has attracted considerable interest in understanding the enzymology of stereospecific biosynthesis of β-MeTrp from L-tryptophan.

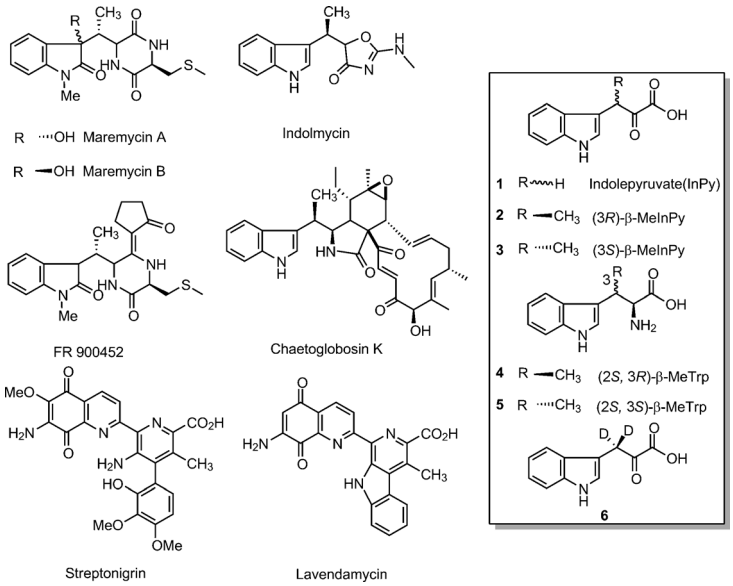


***Figure 1.*** Chemical structures of maremycins, FR900452, indolmycin, chaetoglobosin K, streptonigrin, lavendamycin, and compounds **1**–**6**.

To investigate the biosynthesis of **5** in MARs, the biosynthetic gene cluster of MARs has been cloned and identified by genome sequencing of *Streptomyces* sp. B9173,[8a] the MARs-producing strain, which consists of about a 21 kb DNA sequence and 17 open reading frames (see Table S3 in the Supporting Information). Within this cluster, a three-gene cassette, *marG-marH-marI*, was revealed to encode a pyridoxal 5′-phosphate (PLP)-dependent aminotransferase, a cupin-fold protein, and an *S*-adenosylmethionine (SAM)-dependent C-methyltransferase (Figure 2). Previous studies have demonstrated a two-step conversion of α-keto acids into β-methyl amino acids catalyzed by a methyltransferase and an aminotransferase.[15] Therefore, we hypothesized that MarG and MarI might be involved in furnishing β-MeTrp for the biosynthesis of MARs.

To solidify the link between the *marG-marH-marI* cassette and the biosynthesis of **5**, these enzymes were characterized in vitro. MarI was overexpressed and purified from *E. coli* BL21(DE3)pLysS as an N-terminal $His_6$-tagged fusion protein ($His_6$-MarI; see Figure S1 a). The MarI-catalyzed β-methylation was measured by using either indolepyr-

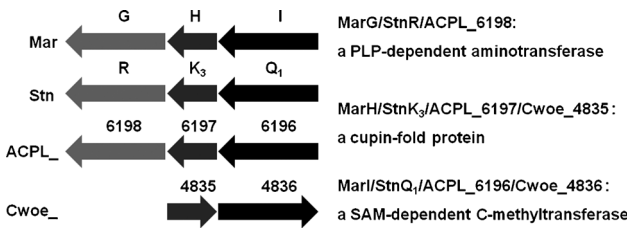


***Figure 2.*** Putative gene cassettes for the conversion of L-tryptophan into β-MeTrp.

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uvate (InPy; **1**) or L-Trp as the substrate. When **1** was incubated with MarI and SAM, a new peak was observed and identified as β-methyl indolepyruvate (β-MeInPy; **2**; *m/z* 218.1 $[M+H]^+$) by LC-MS analysis (Figure 3a, trace 2), while MarI was unable to directly install a methyl group onto the β-carbon atom of L-Trp under the same assay conditions (Figure S1b).

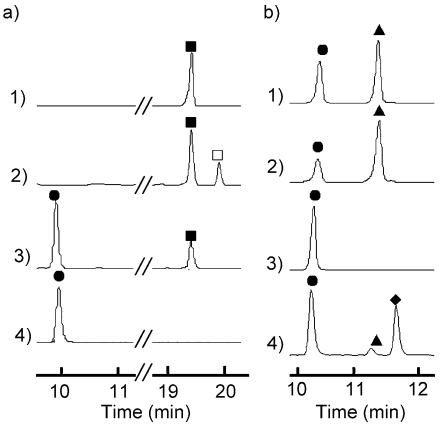



***Figure 3.*** Characterization of MarG, MarI, and MarH in vitro. LC-MS profiles (UV 280 nm) of biochemical assays of MarG, MarI, and MarH (flow rate is 0.6 mL min$^{-1}$. ● L-Trp, ■ **1**, □ **2**, ▲ **4**, ◆ **5**). a: 1) **1** (standard); 2) MarI incubated with **1** and SAM; 3) MarG incubated with L-Trp and αKG; 4) L-Trp (standard). b: 1) MarG/I incubated with L-Trp, SAM and αKG; 2) MarG/I incubated with L-Trp and SAM; 3) MarG/I incubated with L-Trp and αKG; 4) MarG/H/I incubated with L-Trp and SAM.

Next, $His_6$-MarG was overexpressed and purified for testing its activity in vitro (see Figure S2a). UV/Vis spectroscopy shows that MarG was purified with the cofactor PLP (Figure S2b),[16] so no exogenous PLP was added to the assays of MarG. When MarG was incubated with L-Trp and α-ketoglutarate (αKG), a new peak was observed and confirmed to be **1** by comparison with a standard of **1** using HPLC analysis (Figure 3a, trace 3). This result confirmed that MarG functions as a L-Trp aminotransferase, which is crucial for the supply of **1** (the substrate of MarI) during the biosynthesis of β-MeTrp.

Furthermore, a transamination is required to convert the β-MeInPy into β-MeTrp. Within the *mar* cluster, *marG* is the only identified aminotransferase gene (see Table S3), so MarG would be the candidate to catalyze the second round transamination. MarG was added to the previous MarI-catalyzed methylation reaction of **1** with a commonly used amino donor (L-Glu or $NH_4^+$). However, the expected products were not detected (see Figure S2c). Further assays with other natural amino acids as putative amino donors showed that L-Trp is the best amino donor for conversion of β-MeInPy into β-MeTrp by MarG (Figure S2c). MarG was also able to utilize L-tyrosine, L-phenylalanine, L-histidine, and L-methionine as the amino donor, albeit in low efficiency (Figure S2c).

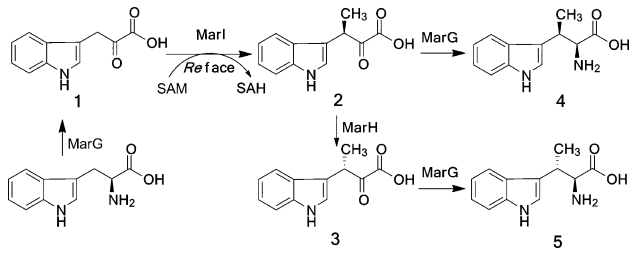
Subsequently, a coupled deamination/methylation/transamination enzymatic assay was developed using MarG/I to covert L-Trp into β-MeTrp. When MarG and MarI were incubated with L-Trp, αKG, and SAM, the expected β-MeTrp (*m/z* 219.1 $[M+H]^+$) was observed upon LC-MS analysis (Figure 3b, trace 1). Assays without the exogenous αKG or SAM established that SAM, but not αKG, is an essential cofactor for the MarG/I-coupled reaction (Figure 3b, traces 2 and 3). Therefore, the aminotransferase MarG and methyltransferase MarI are necessary and sufficient for the biosynthesis of β-MeTrp from L-Trp.

To determine whether the enzymatic product is the desired **5**, the configuration of the α-carbon atom of the β-MeTrp was first identified as 2*S* based on the L(s)-specificity of MarG. No formation of β-MeTrp was observed from D-Trp in the MarG/I-catalyzed coupled reaction (see Figure S3a). The stereospecificity of MarI was determined by identifying the configuration of the β-carbon atom of the β-MeTrp. β-MeTrp was prepared by biotransformation of L-Trp in recombinant *E. coli* BL21(DE3) overexpressing both *marG* and *marI* (see the Supporting Information), and proved to be identical to the product of the MarG/I-catalyzed in vitro reaction as judged by HPLC analysis (Figure S3b). The identity of the β-MeTrp was confirmed by NMR spectroscopy (Figure S3c). Unexpectedly, its optical rotation value ($[\alpha]_D^{27}$ −33.9°, *c* 1.70, 0.1M HCl) is close to that reported for **4**.[17] This value indicates that the β-MeTrp formed by the MarG/I-catalyzed reaction is **4** rather than the desired **5**. To corroborate the assignment of the 3*R* configuration, a Δ*marI* mutant that abolished the production of MARs (see Figures S4a,b, and c, trace iii) was constructed for carrying out feeding experiments. The compound **4** was unable to restore the production of MARs when it was fed to the Δ*marI* mutant strain (Figure S4c, trace iv). This result confirmed the *3R* configuration, and suggested that **4** must be epimerized prior to incorporation into the nonribosomal peptide synthetase assembly line proposed for MARs.

Thus, we turned our attention to the gene *marH* which encodes a small protein (129 aa). Although sequence analysis shows MarH of unknown function, the secondary structure prediction indicates that MarH possesses a cupin fold[18] (see Figure 2 and Figure S5a). The cupin superfamily is a group of proteins with diverse functions including epimerases and isomerases catalyzing isomerizations of sugars involved in the biosynthesis of cell-wall carbohydrates in bacteria.[18] Therefore, MarH was proposed to be involved in the biosynthesis of **5** by catalyzing a stereochemical inversion of the β-methyl group. To test this hypothesis, purified $His_6$-MarH (Figure S5b) was added to the MarG/I-catalyzed coupled reaction with SAM and L-Trp as substrates. A new peak was eluted with a retention time that was different from that of **4**, but had the same ion peak at *m/z* 219.1 for $[M+H]^+$ (Figure 3b, trace 4). To elucidate its structure, it was prepared by the biotransformation of L-Trp in the recombinant *E. coli* BL21-(DE3) overexpressing *marG/H/I* (Figure S5c). The NMR spectra confirmed this new peak to be β-MeTrp (Figure S5d). It is exciting that its optical rotation value ($[\alpha]_D^{29}$ +46.5°, *c* 1.70, 0.1M HCl) is almost identical to that reported for **5**.[17] When the Δ*marI* mutant was fed with **5**, the production of MARs was perfectly restored (see Figure S4c, trace v), thus confirming that the product from the MarG/H/I-catalyzed

coupled reaction is the biosynthetic precursor (**5**) of MARs. Taken together, these results suggest that MarI is *R* specific and introduces the methyl group from the Re face onto the β-carbon atom of **1** to form (*R*)-β-MeInPy (**2**) (Scheme 1).




***Scheme 1.*** Pathways for the MarG/I- and MarG/H/I-catalyzed biosynthesis of **4** and **5** from L-Trp via the intermediates **1**, **2**, and **3**.

MarH is necessary for formation of **5** by catalyzing the stereochemical inversion. A time course study shows that the biosynthesis of both **4** and **5** is time dependent (see Figure S6).

MarG and MarI catalyze the formation of **4** from L-Trp with **1** and **2** as intermediates. However, the addition of MarH to the MarG/I-catalyzed coupled reaction led to the formation of **5**, thus raising the question of when and how MarH inverts the configuration of the β-methyl group. It is reasonable to propose that MarH-catalyzed stereochemical inversion is most likely to occur with **2**, which is epimerized to **3**, with subsequent transamination by MarG to eventually form **5** (Scheme 1).

To examine this hypothesis, we synthesized [3,3-$^2H_2$]InPy (**6**; Figure 4b, trace 1; see also the Supporting Information), and used MarI to generate the monodeuterated **2** as a substrate for MarH. The monodeuterated **2** (synthesized in situ; see the Supporting Information for details) was incubated with MarG and with or without MarH for 1 hour. In contrast to the MarH-free reaction that produced **4**, the reaction with MarH generated **5** (Figure 4a). Surprisingly, both loss (*m/z* 219.1128 $[M+H]^+$) and retainment (*m/z* 220.1191 $[M+H]^+$) of deuterium were observed in **5** at a ratio of 4.40:1 (Figure 4b, traces 2 and 3). These observations

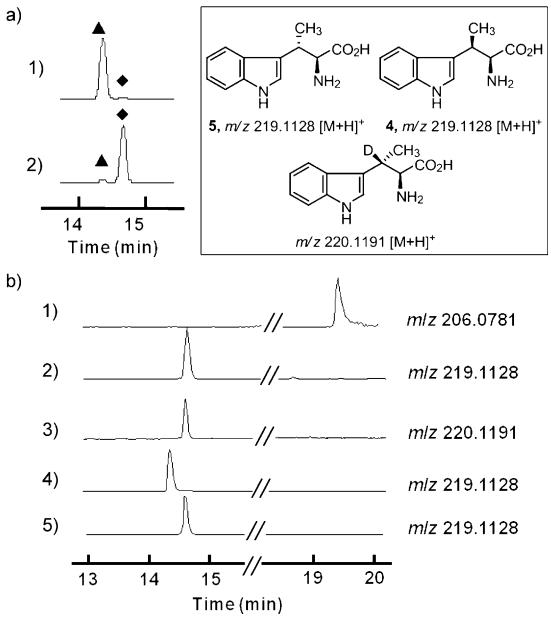
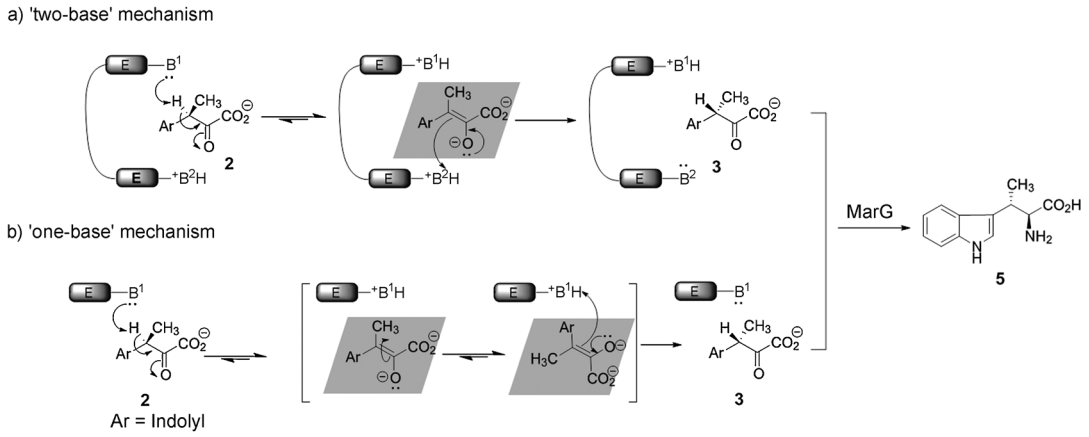



***Figure 4.*** Timing and mechanism of MarH-catalyzed stereochemical inversion. a) HPLC profiles (UV 280 nm and the flow rate of 0.5 mL min$^{-1}$. ▲ **4**, ◆ **5**) of **2** incubated with MarG alone (1) and with MarG and MarH (2). b) Extracted ion chromatograms of *m/z* $[M+H]^+$ 219.1128 and 220.1191 corresponding to unlabeled and monodeuterium-labeled **5**. 1) **6** (standard); 2) unlabeled **5**; 3) monodeuterium-labeled **5**; 4) **4** (standard); 5) **5** (standard).

suggest that two possible mechanisms may be involved in the epimerization of **2** into **3** (Scheme 2). In the one-base mechanism, the deuterium on the β-carbon atom should be retained, while in the two-base mechanism, the hydrogen in the newly formed C–H bond is expected to be solvent derived.[19]

To differentiate whether the MarH-catalyzed epimerization follows the one-base or two-base mechanism, a series of site-directed mutageneses of MarH were performed. Mutiple sequence alignments reveal that MarH possesses two conserved motifs (HxHxxxE and PxGxxH) which are characteristic of the cupin superfamily of proteins (see Figure S5a).[18]




***Scheme 2.*** Two possible mechanisms of MarH-catalyzed epimerization of **2** to **3**. In the two-base mechanism (a), a catalytic base deprotonates the β proton of **2** to form an enolate intermediate which is then reprotonated by the acid form of a second base. In the one-base mechanism (b), a single base is used in both deprotonation and reprotonation.

Given that His and Glu or Asp usually act as a general-base dyad for formation of an enolate intermediate in most epimerases,[19] the mutants H62A, H64A, E68A, and H107A were constructed (see Figure S8a). The H62A and H64A mutants have no effect on the activity of MarH (Figure 5a,

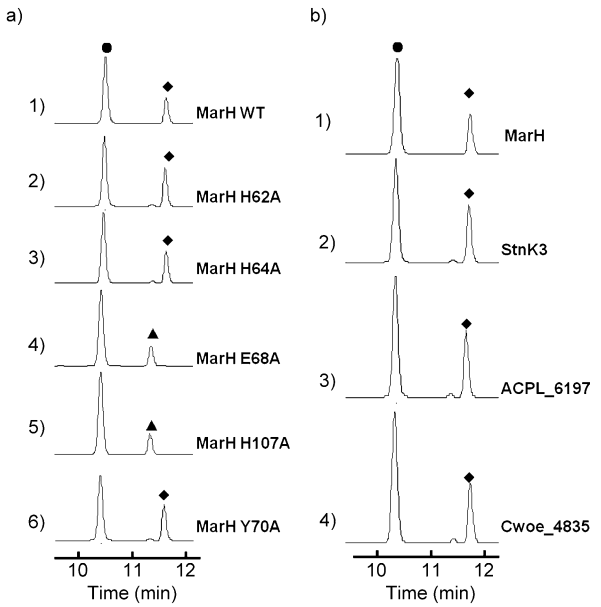



***Figure 5.*** HPLC profiles (UV 280 nm and the flow rate of 0.6 mL min$^{-1}$. ● L-Trp, ▲ **4**, ◆ **5**) of the activity assays of MarH mutants (a) and MarH homologous proteins (b) by coupling with MarG/I.

traces 2 and 3), and contrary to the typical cupin superfamily of proteins in which two His residues of the motif 1 are essential for activity by acting as metal ligands.[18] However, both E68A and H107A mutants completely lost the activity (Figure 5a, traces 4 and 5), thus confirming that E68 and H107 are essential for MarH activity and may act as the dyad for deprotonation.

The only difference between the one-base and two-base models is the existence of the second base for reprotonation for the latter (Scheme 2). Several typical two-base epimerases such as the epimerization (E) domain in nonribosomal peptide synthetases[20] and dTDP-4-keto sugar epimerases[21–24] have been confirmed to employ Tyr for reprotonation. Therefore, the only conserved and putative proton donor, Tyr70, was mutated to Ala (see Figure S5a). The resulting mutant remained active (Figure 5a, trace 6), thus indicating that MarH most likely catalyzes epimerization of the β-stereocenter by the one-base mechanism, rather than by the two-base mechanism (Scheme 2).

However, the washout of deuterium from the basic residue, His, of MarH may be due to the fact that the formed conjugated anionic enolate intermediate has a longer residence time on the protein surface so that the base can exchange with the media (Scheme 2b).[19] To examine this hypothesis, we carried out an antiparallel study. The compound **2** was synthesized in situ by the MarI-catalyzed methylation of **1** instead of **6**, and incubated with MarH and MarG under deuterated conditions (see the Supporting Information for details). Complementing the result obtained with **6**, the deuterated ($m/z$ 220.1191 $[M+H]^+$) and non-deuterated ($m/z$ 219.1128 $[M+H]^+$) forms of **5** were observed at a ratio of 4.42:1 (see Figure S9).

Notably, three homologues of MarH were identified and annotated as hypothetical proteins [StnK3 from the biosynthetic pathway of streptonigrin[25] (similarity/identity, 86%/82%), ACPL_6197 from the genome of *Actinoplanes* sp. SE50/110 (82%/75%), and Cwoe_4835 (57%/41%) from the genome of *Conexibacter woesei* DSM 14684; see Figures 2 and S5a]. *stnK3* and *ACPL_6197* were cloned, and *Cwoe_4835* was synthesized (see the Supporting Information for details). These three homologues were also overexpressed and purified from *E. coli* BL21(DE3)pLysS (see Figure S8b). When they were individually added to the MarG/I-catalyzed coupled reactions, all of them entirely complement the activity of MarH to allow the biosynthesis of **5** (Figure 5b). A C-methyltransferase (the homologue of MarI) and an aminotransferase (the homologue of MarG) were found to be clustered with this cupin-fold protein as MarG/H/I except for *C. woesei* DSM 14684 (Figure 2). Phylogenetic analysis indicated that MarG/H/I and their homologues formed a separate clade from the aminotransferases, cupin-fold epimerases, and methyltransferases, respectively (Figures S10–S12). The recognition of such enzymatic cassettes and biochemical characterization of MarG/H/I suggest a common biosynthetic strategy for the formation of (2*S*,3*R*)- and (2*S*,3*S*)-β-MeTrp in the biosynthesis of the β-MeTrp-derived natural products.

In conclusion, a three-gene cassette, *marG-marH-marI*, was identified in the biosynthetic gene cluster of MARs from *Streptomyces* sp. B9173 by genome sequencing and bioinformatic analyses. It was biochemically demonstrated that MarG/I specifically catalyzes the biosynthesis of (2*S*,3*R*)-β-MeTrp from L-Trp, while MarG/H/I catalyzes the specific formation of (2*S*,3*S*)-β-MeTrp from L-Trp with MarH as a stereochemical switch. By using isotope-labeled substrate and protein mutageneses, MarH-catalyzed stereochemical inversion has been demonstrated to occur on (3*R*)-β-MeInPy putatively through the one-base mechanism with H107-E68 acting as a base dyad for deprotonation. Three analogues of MarH were also biochemically characterized and show the same function as MarH. To unveil the detailed mechanism of this conversion, efforts are underway to solve the structure of MarH by X-ray crystallography and NMR spectroscopy.

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