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Enhanced Conversion of Racemic α-Arylalanines to (*R*)-*β*-Arylalanines by Coupled Racemase/Aminomutase Catalysis

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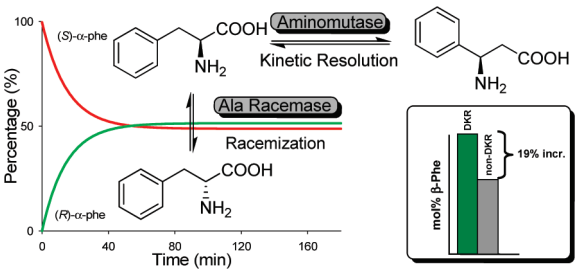

The *Taxus* phenylalanine aminomutase (PAM) enzyme converts several (*S*)-α-arylalanines to their corresponding (*R*)-*β*-arylalanines. After incubating various racemic substrates with 100 *μ*g of PAM for 20 h at 31 °C, each (*S*)-α-arylalanine was enantioselectively isomerized to its corresponding (*R*)-*β*-product. With racemic starting materials, the ratio of (*R*)-*β*-arylalanine product to the (*S*)-α-substrate ranged between 0.4 and 1.8, and the remaining nonproductive (*R*)-α-arylalanine became enriched. To utilize the (*R*)-α-isomer, the catalysis of a promiscuous alanine racemase from *Pseudomonas putida* (KT2440) was coupled with that of PAM to increase the production of enantiopure (*R*)-*β*-arylalanines from racemic α-arylalanine substrates. The inclusion of a biocatalytic racemization along with the PAM-catalyzed reaction moderately increased the overall reaction yield of enantiopure *β*-arylalanines between 4% and 19% (depending on the arylalanine), which corresponded to as much as a 63% increase compared to the turnover with the aminomutase reaction alone. The use of these biocatalysts, in tandem, could potentially find application in the production of chiral *β*-arylalanine building blocks, particularly, as refinements to the process are made that increase reaction flux, such as by selectively removing the desired (*R*)-*β*-arylalanine product from the reaction mixture.

Introduction

Enantiomerically pure *β*-amino acids are emerging as an important class of compounds due to the frequent occurrence of *β*-amino acid substructure motifs in pharmaceutically important natural products.[1] In addition, single *β*-arylalanines have shown antiepileptogenesis activity,[2] while other optically active *β*-amino acids serve as useful chiral scaffolds for the synthesis of *β*-peptides, *β*-lactams, and biologically active natural products.[3] Therefore, efficient methods for the production of enantiopure *β*-amino acids would benefit novel drug synthesis and development. A recently applied method for the synthesis of primarily racemic mixtures of *β*-amino acids proceeds from *N*-carbamate α-amino acids. This process involves radical scission of carbon dioxide from the α-amino acid by treatment with (diacetoxyiodo)benzene, iodine, and visible light. The resulting transient acyliminium ion is then quenched by the addition of a silyl ketene to yield an *N*-acyl-*β*-amino acid methyl ester.[4]

In a biosynthetic context, a phenylalanine aminomutase (PAM), isolated from *Taxus* plants, catalyzes the stereospecific isomerization of (*S*)-α-phenylalanine to the single isomeric product (*R*)-*β*-phenylalanine.[5] In the host plant, the

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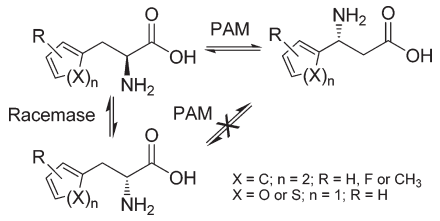
SCHEME 1. Biocatalytic Resolution of Racemic α-Arylalanines to Enantiopure β-Arylalanines



latter is the biosynthetic precursor of the phenylisoserine side chain of the antineoplastic drug paclitaxel (Taxol), which has application in the treatment of heart disease[6−8] and cancer.[9−11] The equilibrium constant of the reaction catalyzed by the heterologously expressed PAM showed that the formation of (*R*)-β-product is slightly favored over the (*S*)-α-phenylalanine substrate.[12] Moreover, the substrate specificity of PAM was remarkably flexible as demonstrated by the conversion of a homologous series of non-natural 2′-, 3′-, or 4′-substituted (*S*)-α-arylalanines, (*S*)-β-styryl-α-alanine, and (*S*)-β-heterole-α-alanines to their corresponding (*R*)-β-isomers by the aminomutase.[5] Since PAM is specific for the (*S*)-enantiomer of the aryl α-amino acid substrates,[13] the proportion of (*S*)-α-arylalanine to (*R*)-β-arylalanine at equilibrium in a PAM-catalyzed reaction, in which the substrate is racemic α-arylalanine, is theoretically limited by the equilibrium constant between the (*S*)-α- and (*R*)-β-isomers; consequently, the nonproductive (*R*)-α-arylalanine isomer accumulates. Conceptually, the yield of (*R*)-β-arylalanine in the PAM catalyzed reaction however could be increased above the theoretical value by establishing resolution conditions where the (*R*)-α-isomer remains in dynamic equilibrium with the productive (*S*)-enantiomer (Scheme 1).

Described herein is the discovery of the promiscuous function of a pyridoxal-5′-phosphate-dependent alanine racemase from *Pseudomonas putida*[14] used to maintain an (*R*,*S*)-α-arylalanine racemate in the reaction mixture. This catalyst was coupled to the resolution reaction catalyzed by PAM, and the in situ coupled enzyme system enriched the availability of the productive (*S*)-substrate of the α-arylalanine racemate mixture, and notably increased the production of the (*R*)-β-arylalanines catalyzed by the enantioselective PAM reaction.

Experimental Section

PAM Enzyme Preparation. The cDNA of the phenylalanine aminomutase, isolated from *Taxus canadensis*,[15] was synthesized with codon optimization by DNA 2.0 (Menlo Park, CA) for expression in *E. coli*. The gene was PCR amplified with mutagenic primers that encoded *NdeI* (5′-CGGCATCCATATGGGTTTTGCTGTTGAATCT-3′) and *Bam*HI (5′-CGCGGATCCTTATTATGCAGATTTGTTCCAAAC-3′) restriction sites at the sequence termini. The resulting amplicon was cut with *Nde*I and *Bam*HI and ligated in-frame into the expression vector pET28a(+) that was digested with the same restriction enzymes. The recombinant pET28a(+) plasmid that encoded an N-terminal His_6-tag on the PAM cDNA was verified by DNA sequencing and used to transform *E. coli* BL21(DE3) by standard methods.[16]

E. coli BL21(DE3) cells transformed to express the phenylalanine aminomutase were grown at 37 °C for 12 h in 100 mL of Luria−Bertani medium. Separate aliquots (5 mL) of this inoculum culture were then added to each of six 1-L cultures of Luria−Bertani medium supplemented with kanamycin (50 μg/mL). The cells were incubated at 37 °C until OD_{600} = 0.7. Isopropyl-D-thiogalactopyranoside (500 μM) was added to the cultures with expression conducted at 16 °C. After 16 h, the cells were harvested by centrifugation at 5,000*g* (15 min), diluted in 100 mL of resuspension buffer (50 mM sodium phosphate containing 5% (v/v) glycerol and 300 mM NaCl, pH 8.0), and lysed by brief sonication [five 1-min bursts at 30% power with 1 min intermittent intervals using a Branson Sonifier (Danbury, CT)], and the cellular debris was removed by centrifugation at 15,000*g* (30 min) followed by high-speed centrifugation at 40,000*g* (45 min) to remove light membrane debris. The resultant crude aminomutase in the soluble fraction was purified by nickel-nitrilotriacetic acid (Ni-NTA) affinity chromatography according to the protocol described by the manufacturer (Qiagen, Valencia, CA); PAM was eluted in 250 mM imidazole. Fractions containing active soluble PAM (78 kDa) were combined and loaded into a size-selective centrifugal filtration unit (Centriprep centrifugal filter units, 30,000 MWCO). The protein solution was concentrated to 1 mL and diluted several cycles until the imidazole and salt concentrations were <1 μM. The quantity of PAM and purity of the concentrated enzyme were assessed by SDS−PAGE with Coomassie Blue staining[17] using Kodak 1D image analysis software (version 3.6.3) to integrate the relative intensities of the scanned protein bands with concentration standards. A Bradford assay was used to confirm the quantity of total protein.

Alanine Racemase Enzyme Preparation. The putative alanine racemase (accession number AE015451 range 4,245,041−4,246,270) was PCR amplified from *Pseudomonas putida* KT2440 (American type Culture Collection (ATCC) (Manassas, VA)) genomic DNA using primers that encoded *NdeI* (5′-AATCCATATGCCCTTTCGCCGTACCCT-3′) and *Bam*HI (5′-CGCGGATCCTCAGTCGACGAGTATCTT-3′) restriction sites at the cDNA termini. The amplicon was digested with the appropriate restriction enzymes and subcloned into an identically digested expression vector pET28a(+) that encoded an N-terminal His_6-tag. Transformed *E. coli* BL21(DE3) cells expressing the racemase were grown, harvested, lysed, and clarified, as described above for the aminomutase, to give the soluble enzyme preparation. The crude soluble racemase was partially purified by Ni-NTA affinity chromatography, and the total protein concentration and purity were determined by the methods described earlier.

Derivatization and Quantification of Amino Acids. The amino acids in all assays described were derivatized generally as

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follows. To each mixture was added 0.5 N NaOH to adjust the pH to >9, and then ethyl chloroformate (200 equiv, 100 μL) was added to *N*-acylate the arylalanines. After 10 min, the solutions were again basified (pH > 9), and a second batch of ethyl chloroformate (200 equiv, 100 μL) was added. After derivatization, the mixture was acidified to pH 2−3 with 6 N HCl and extracted with ethyl acetate (2 × 0.75 mL). The organic solvent was evaporated in vacuo, the residue was dissolved in ethyl acetate/methanol (3:1, v/v) (200 μL) [methanol was used to liberate diazomethane in the following step], and the solution was treated with excess (trimethylsilyl)diazomethane (~5 μL) to make the methyl ester of the *N*-acyl amino acid.

To assess the level of the arylalanines in this study, concentrations were calculated by coupled gas chromatography/electron-impact mass spectrometric (GC/EI-MS) analysis, and the analytes were separated on a Chirasil-D-Val column (0.25 mm i.d. × 25 m, 0.08-μm film thickness, Varian, Palo Alto, CA). A 1-μL aliquot of the derivatized material was loaded onto the column mounted in the GC (model 6890N, Agilent, Santa Clara, CA) coupled to a mass analyzer (model 5973 *inert*, Agilent, Santa Clara, CA) in ion scan mode from 100−400 atomic mass units. The GC conditions were as follows: column temperature was held at 100 °C for 3 min and then increased linearly at 10 °C/min to 180 °C with a 3 min hold, followed by a 20 °C/min linear ramp to 200 °C with a 3 min hold. Splitless injection was selected, and helium was used as the carrier gas. The relative amounts of each α-arylalanine enantiomer at equilibrium were determined by linear regression analysis of the area of the base peak ion of the derivatized α-arylalanines generated in the EI-MS. The peak area was converted to concentration by solving the corresponding linear equation, derived by plotting the area of the base peak ion (produced by the corresponding authentic standard) against concentration ranging from 0 to 1.5 mM. GC/EI-MS analysis of equimolar concentrations of derivatized (*R*)-α-, (*S*)-α-, and (*R*)-β-phenylalanine revealed that the abundances of the diagnostic base peak ion for each amino acid were equal.

Assessing the Racemization Rate of α-Arylalanines by the Alanine Racemase. The partially purified (>90%) alanine racemase (500 μg) was added to 2.5 mL of 50 mM phosphate buffer (pH 8.0) containing 5% (v/v) glycerol and one different (*S*)-α-arylalanine (1.5 mM) in separate assays; the mixtures were incubated at 31 °C. An aliquot was withdrawn from each reaction at five time points (0.5, 10, 30, 60, and 360 min). A similar time course experiment was conducted with (*R*)-α-phenylalanine.

Cofactor Dependency of the Alanine Racemase. To assess whether sufficient PLP, made by the *E. coli* BL21(DE3) expression host, remained bound to the functional PLP-dependent racemase for the duration of the assay, partially purified enzyme (400 μg) was added to each of two 2-mL assays that contained (*S*)-α-phenylalanine (1.5 mM). To one assay, exogenous PLP (50 μM) was added, and to the other, no cofactor was added. After 6 h, the contents of the reaction vial without PLP added were divided into two fractions (0.5 mL); to one fraction was added PLP (50 μM), and the other fraction remained unchanged. Additional (*S*)-α-phenylalanine was added to a final concentration of 1.5 mM in all three assays and incubated for 14 h. After complete equilibration was noted at 20 h, another aliquot of (*S*)-α-phenylalanine (1.5 mM) was added to the assay, and the substrate/product ratio reached equilibrium at 24 h.

Evaluation of the Effect of (*R*)-β-Phenylalanine on Racemase Activity. The partially purified alanine racemase (400 μg) in 50 mM phosphate (2 mL, pH 8.0) containing 5% (v/v) glycerol buffer and (*S*)-α-phenylalanine (0.75 mM) was incubated in separate assays with or without (*R*)-β-phenylalanine (0.75 mM) added. Aliquots (0.5 mL) of each reaction mixture were withdrawn from each assay at three time points (30, 60, and 360 min). The amino acids in each fraction were derivatized and analyzed by chiral GC/EI-MS as described previously. The mol % of (*S*)-α-, (*R*)-α-arylalanine, and (*R*)-β-arylalanine in each sample were assessed by comparing the abundance of the diagnostic base peak fragment ion (*m*/*z*) derived by GC/EI-MS fragmentation for each of the corresponding analytes.

Assessment of Time to Equilibrium for the PAM Reaction with α- and β-Arylalanines. Purified PAM (250 μg) was added to 2.5 mL of 50 mM phosphate buffer (pH 8.0) containing 5% (v/v) glycerol and one different (*S*)-α-arylalanine (0.4 mM) or (*R*,*S*)-α-arylalanine racemate, in separate assays, and the mixtures were incubated at 31 °C. An aliquot was withdrawn from each reaction at five time points (0, 3, 6, 9, and 20 h). The ratio of (*R*)-β-arylalanine to (*S*)-α-arylalanine in each aliquot was calculated by GC/EI-MS fragmentation analysis, and the abundances of the base peak fragment ions of the amino acid derivatives were compared as described earlier. The relative amount of each α-arylalanine and β-arylalanine at equilibrium was determined by linear regression analysis of the area of the base peak ion of the derivatized α- and β-arylalanines generated in the EI-MS. The peak area was converted to concentration of product (or substrate) by solving the corresponding linear equation, derived by plotting the area of the base peak ion (produced by the corresponding authentic standard in the mass spectrometer) against concentration ranging from 0 to 1.5 mM.

Evaluation of the Effect of (*R*)-α-Phenylalanine on PAM Activity. Phenylalanine aminomutase (100 μg) was added to 1 mL of 50 mM phosphate buffer (pH 8.0) containing 5% (v/v) glycerol at 31 °C containing either (*S*)-α-phenylalanine or (*R*)-β-phenylalanine at a range of concentrations (0.05, 0.10, 0.20, 0.40, 0.75, 1.5, and 3 mM). The reactions were incubated at 31 °C for 90 min under steady-state conditions. Identical series of assays were run in parallel for the (*S*)-α- or (*R*)-β-phenylalanine as substrates, except 0.2 mM and 0.75 mM of (*R*)-α-phenylalanine were added separately to each series. Double reciprocal plots of velocity (v_0) and concentration were constructed for the forward and reverse PAM reaction data sets. The equation of the best-fit line ($R^2 \approx 0.98$) was determined to calculate the apparent K_M and K_i for the appropriate reaction.[18]

Coupled Enzyme Reaction. The coupled enzyme reaction mixture incubated at 31 °C contained partially purified alanine racemase (200 μg) and PAM (100 μg) in 1 mL of 50 mM phosphate buffer (pH 8.0) containing 5% (v/v) glycerol. Each of the aryl α-amino acids (0.4 mM) were individually added to a separate assay tube containing the coupled enzyme mixture. After 20 h, the reactions were terminated by basification to pH 10−12 (with a 0.5 N NaOH solution), and the amino acids were derivatized for quantification by chiral GC/EI-MS analysis as described previously.

Synthesis of 3-(2-Furanyl)-(*R*,*S*)-α-alanine and 3′- and 4′-Methyl-(*R*,*S*)-α-phenylalanine. 2-Furanyl-(*S*)-α-alanine, 3′-methyl-(*S*)-α-phenylalanine, and 4′-methyl-(*S*)-α-phenylalanine (0.5 mg, 3 μmol) were separately incubated with 400 μg of alanine racemase (43 kDa) at 31 °C in 2 mL of 50 mM phosphate buffer (pH 8.0) containing 5% (v/v) glycerol. The reaction was incubated for 2 h, a 0.5-mL aliquot was withdrawn from each assay, the amino acids were derivatized as described before, and the racemization was judged complete by chiral GC/EI-MS analysis. The racemic products were separated from the enzyme by size-selective centrifugal filtration (Centriprep centrifugal filter units, 10,000 MWCO); the protein solution was concentrated to 10 μL, and the filtrate (~1.5 mL) was collected. The recovery of the racemic α-amino acids was quantitative as determined by linear regression analysis of the area of the base peak ion of the derivatized (*S*)-α-arylalanine. The

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isolated yield of the racemic mixtures was between 0.35 and 0.4 mg (2.2−2.4 μmol) at 90−99% based on the material remaining after 25% of the reaction volume was withdrawn earlier for chiral GC/EI-MS analysis.

Results and Discussion

Racemization of α-Arylalanines by Alanine Racemase Activity. An alanine racemase was cloned from the genome of *Pseudomonas putida* (KT2440) and was chosen for the present investigation on the basis of its amino acid similarity to a previously reported racemase with broad specificity for naturally occurring aromatic amino acids, including but not limited to histidine ($V_{rel} = 1$), phenylalanine ($V_{rel} = 0.18$), and tryptophan ($V_{rel} = 0.004$).[19] Although not previously described, this racemase was anticipated to epimerize the non-natural aryl amino acids used in this investigation. The acquired racemase cDNA was heterologously expressed in *E. coli* BL21(DE3), and the resultant enzyme (43 kDa) was His_6-affinity purified to >90% purity and was deemed suitable for use in the assays described. Notably, on the basis of the reported enzyme mechanism of other pseudomonad racemases,[20] the pyridoxal-5′-phosphate (PLP) is likely a cofactor of the racemase used herein; the natural reaction of this catalyst converts (*S*)-alanine into (*R*)-alanine, a key component of bacterial peptidoglycan.[21] The cellular concentration of unbound PLP in *E. coli* is estimated at ∼120 μM,[22] and therefore, this cofactor was likely available to covalently bind and activate the alanine racemase during recombinant expression in the bacterial host strain in this study. No change in the reaction rate of the purified racemase was observed with or without PLP supplementation in the racemization assays containing 1.5 mM α-arylalanines. This result suggested that sufficient *E. coli*-derived PLP remained bound in the active site of the functional racemase during the course of the assay, likely through formation of an internal Schiff-base aldamine with a conserved lysine residue (Lys-75) during protein expression.[20,23] In addition, prior to using the racemase to increase the amount of the productive antipode of the substrate, (*R*)-β-phenylalanine (the product of the PAM reaction) was shown to not affect the V_{max} of the racemase during the 20 h assay, nor was the (*R*)-β-amino acid a substrate of the racemase.

A necessary condition to increase the biosynthesis of the product through biocatalytic resolution of α-arylalanine substrates to their β-isomers in the present study was to keep the substrates racemized in the presence of the reaction catalyzed by the aminomutase. In individual assays, the parameters of the racemase were assessed to establish equilibrium conditions by incubating one of each α-arylalanine, known to be a substrate of the *Taxus* phenylalanine aminomutase.[5] Each substituted-ring- or heterole-(*S*)-α-arylalanine substrate at 1.5 mM was incubated with 500 μg of the racemase in 2.5 mL assays. Aliquots were withdrawn from the reactions at designated time points over 3 h, and the amino acids were derivatized as their *N*-ethylcarbamate methyl esters for quantitation by GC/EI-MS analysis. Both *N*-mono- (90−99 mol %) and *N*,*N*-dicarbamate (1−10 mol %) derivatives were observed for each α-arylalanine substrate, and the area of the base peak fragment ion for each *N*-mono-/*N*,*N*-dicarbamate pair were added together to account for the total mol % of each α-arylalanine enantiomer. Each α-arylalanine was found to be completely racemized by the alanine racemase within 60 min. These data indicated that the alanine racemase rapidly epimerized the non-natural α-arylalanines to dynamic equilibrium and had broad substrate specificity, making this enzyme favorable to enhance production yields in a one-pot reaction containing PAM. In addition, the racemase remained active for the duration of the 20-h assay, as evidenced by the complete racemization (within 4 h) of each (*S*)-α-arylalanine added to a racemase assay at 20 h, the stop-point of the coupled enzyme assays described herein.

Effects of (*R*)-β-Phenylalanine on Racemase Activity. Since the alanine racemase would ultimately be added to a reaction in which PAM catalyzed the production of (*R*)-β-amino acids, the effect of β-arylalanines on the racemase activity was studied prior to conducting the coupled enzyme assay. In general, the racemase activity on 1.5 mM of (*S*)-α-arylalanine was not affected by any of the β-arylalanines at 1.5 mM within the time allotted for the coupled reactions. Furthermore, (*S*)-β-arylalanines were not detected in any of the assays, indicating that the β-amino acids were not substrates of the racemase.

Effect of the Non-natural (*R*)-α-Phenylalanine Enantiomer on PAM activity. A previous investigation demonstrated that PAM was stereospecific for (*S*)-α-phenylalanine, whereas the (*R*)-α-isomer was nonproductive;[13] however, the inhibitory effects of the (*R*)-α-amino acid were not assessed.[13] Therefore, in the present investigation, to determine if the non-natural (*R*)-α-phenylalanine isomer affected the kinetics of PAM, the aminomutase was incubated with (*S*)-α-phenylalanine in the presence of 0.2 and 0.75 mM (*R*)-α-phenylalanine, and the kinetic constants were calculated. (*R*)-α-Phenylalanine did not significantly inhibit the PAM reaction; the apparent Michaelis constant (apparent K_M at 0.75 mM inhibitor) for the forward reaction was 1.0 mM for the substrate tested, and the inhibition constant (K_i) was 3.0 mM, without an evident change in V_{max} (0.1 μmol min^{-1} mg^{-1}) relative to the rate when no inhibitor was present. Comparably, in the absence of (*R*)-α-phenylalanine, the K_M of PAM was 0.8 mM, which was similar to the value previously reported.[24] Moreover, the reverse reaction was not inhibited by (*R*)-α-phenylalanine (data not shown). Overall, the inhibition data suggests that the (*R*)-stereoisomer, which increased the K_M slightly without changing V_{max}, is at most a weak competitive inhibitor of PAM in the forward reaction. When PAM was incubated in the presence of 0.2 mM (*R*)-α-phenylalanine, the V_{max} and K_M of the catalysis were virtually unaffected.

Dynamic Equilibration of α- and β-Arylalanines by the Aminomutase. It is known that the PAM-catalyzed reaction converts (*S*)-α-arylalanine substrates to their respective, enantiopure (*R*)-β-arylalanine products (>99% ee).[5] In the

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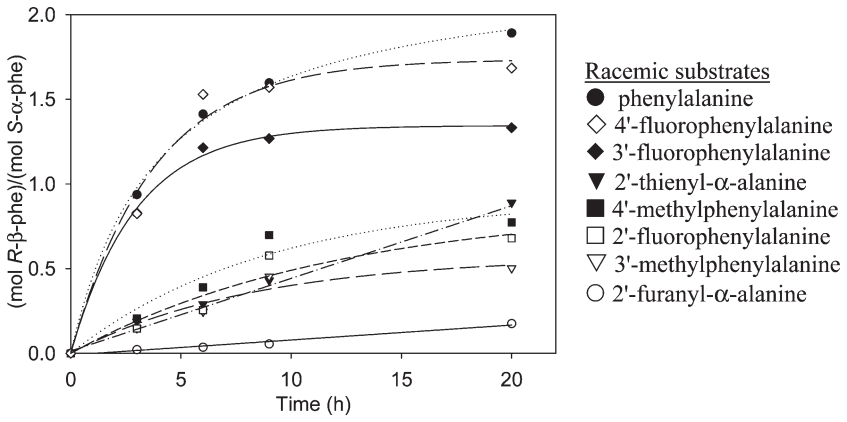

FIGURE 1. Assessment of the equilibrium of the PAM reaction with (*R*)-*β*- and (*S*)-α-arylalanines.

present study, the enantioselective PAM catalysis provided the necessary step to resolve the racemic substrate, while the racemase kept the enriched, nonproductive substrate enantiomer at dynamic equilibrium with its antipode.[25] Parameters were established pertaining to PAM concentration and reaction time needed for each (*S*)-α-arylalanine of an (*R*,*S*)-racemic mixture to reach dynamic equilibrium with its *β*-isomeric product. Each amino acid substrate at 0.4 mM was incubated for 20 h at 31 °C with 250 *μ*g of PAM in 2.5 mL of phosphate buffer. Aliquots were withdrawn from the assays at specific time intervals, and the amino acids in the reaction mixture were derivatized as their *N*-ethylcarbamate methyl esters for analysis by GC/EI-MS. Under these conditions, all of the (*R*)-*β*-arylalanine products approached maximum accumulation except for the heteroaromatic amino acid substrates, 2′-furanyl-α-alanine and 2′-thienyl-α-alanine (Figure 1).

PAM catalysis maintained the ratio of the heterole-*β*-arylalanines to their counterpart heterole-α-arylalanines ((*S*)-2-thienyl-α-alanine or (*S*)-2-furanyl-α-alanine) at steady state (Figure 1), indicating that the reactions containing these amino acids were slower and therefore had not yet reached equilibrium. The *β*-product to α-substrate ratio for these heterole arylalanines was at 0.71 and 0.41, respectively, after 20 h, and reflected that the steady-state turnover rate (v_0) at the stop-point for the conversion of (*S*)-2-thienyl-α-alanine and (*S*)-2-furanyl-α-alanine to their respective *β*-isomers by PAM was greater for the former compared to the latter (Figure 1). This finding was consistent with kinetics data reported in a previous study for these heterole aromatic amino acids.[5] Presently, the basis for this difference in turnover rate of the heterole-α-arylalanines by PAM remains unknown. Furthermore, the variability of the K_{eq} values for various substrates is intriguing; however, it is presently unclear whether inductive or steric effects are influencing the position of the equilibrium for the various substituted-arylalanine regioisomers.

Curiously, during PAM catalysis of the conversion of α-phenylalanine to (*R*)-*β*-arylalanine the K_{eq} for the forward reaction was calculated in this study at 1.8, which was ~1.6-fold greater than calculated ($K_{eq} = 1.1$) at the same temperature in a previous study.[5] This increase might reflect the higher enzyme (100 *μ*g/mL) and substrate (400 *μ*M) concentrations used in each assay, in this study, which represented an approximate order of magnitude increase in concentration of both parameters compared to the 10 *μ*g/mL enzyme and 10 *μ*M substrate used in the previous investigation.[13] The elevated concentrations used herein increased the yield of the biosynthetic product and thus likely improved the signal-to-noise of the GC/EI-MS analysis of the derivatized amino acids isolated from the assay mixtures.

Production of Enantiopure *β*-Arylalanines in Coupled Racemase/Aminomutase Assays. The enzyme reaction conditions described above established equilibrium parameters needed to increase the production of (*R*)-*β*-arylalanines. Assays were conducted to directly compare the conversion of α-arylalanines to *β*-arylalanines in the presence and absence of racemase. In the first set of assays, each (*R*,*S*)-α-arylalanine (0.4 mM) racemate was separately incubated with 200 *μ*g of PAM in 2 mL of phosphate buffer for 20 h to establish dynamic equilibrium. Synthetic derivatization of the amino acids to their carbamate methyl esters, followed by GC/EI-MS analysis of these products showed that between 12 and 30 mol % of the various (*R*)-*β*-arylalanines were produced relative to the α-arylalanine racemate (Figure 2). The relative ratio of (*R*)-*β*/(*S*)-α-arylalanine pairs was consistent with the equilibrium constants for the PAM reaction established earlier in this study; the mole percent of the (*R*)-α-arylalanines in each assay remained virtually unchanged, as these enantiomers were not isomerized by PAM.

In a separate complementary set of assays, each racemic α-arylalanine at 0.4 mM was separately mixed with 400 *μ*g of the alanine racemase and 200 *μ*g of PAM in 2 mL assays to increase the product yields. After incubating for 20 h, the amino acids were derivatized for GC/EI-MS analysis, as described previously. When the alanine racemase and aminomutase activities were coupled in the same reaction, examination of the analytes showed that the production of various (*R*)-*β*-arylalanines was between 16 and 49 mol % (Figure 2). This represented an increase in percent yield of 4−19% (depending on the substrate) from the conversion of (*R*,*S*)-α-arylalanines to (*R*)-*β*-arylalanines in the coupled enzyme reaction compared to the same reaction excluding the racemase. These experimentally derived values obtained from the coupled assays corresponded to modest increases in yield, but they largely adhered to the theoretical yields, which were calculated on the basis of the equilibrium constants

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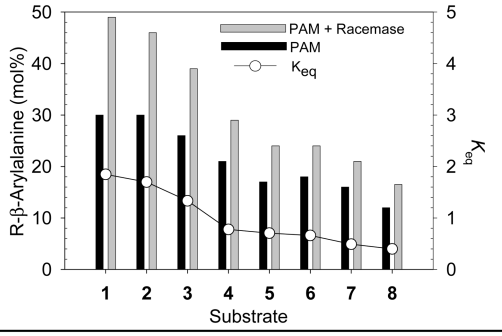

Substrate	Arylalanine Mol% Distribution PAM alone			PAM and racemase			%Increase Arylalanine (*R*-β)	Increase in %Yield
	R-α	*S*-α	*R*-β	*R*-α	*S*-α	*R*-β		
(**1**)	53 (50)	17 (18)	30 (32)	26 (26)	25 (26)	49 (48)	63	19
(**2**)	52 (50)	18 (19)	30 (31)	27 (27)	27 (27)	46 (46)	53	16
(**3**)	54 (50)	20 (22)	26 (28)	31 (31)	30 (31)	39 (38)	50	13
(**4**)	53 (50)	26 (28)	21 (22)	36 (36)	35 (36)	29 (28)	38	8
(**5**)	60 (50)	23 (29)	17 (21)	38 (37)	36 (37)	25 (26)	41	8
(**6**)	54 (50)	27 (30)	18 (20)	39 (38)	37 (38)	24 (24)	33	6
(**7**)	54 (50)	31 (34)	16 (16)	39 (40)	40 (40)	21 (20)	31	5
(**8**)	63 (50)	26 (36)	12 (14)	42 (42)	42 (42)	16 (16)	33	4

FIGURE 2. Percent yield of biosynthetic β-arylalanine by PAM reaction or by the PAM reaction coupled with alanine racemase catalysis in the assay. Mole percent distribution values of arylalanines produced biocatalytically are included. Theoretical mol % values are included in parentheses and are calculated on the basis of the equilibrium constants calculated for the racemase and PAM with each of the arylalanines. Standard error was ±2%.

separately assessed for racemase and the aminomutase with the various arylalanine compounds (Figure 2). The natural product β-phenylalanine showed the largest mol % increase (19%) from α-phenylalanine (followed by the 4′-fluoro- (16%), 3′-fluoro- (13%), 4′-methyl- (8%), 2′-thienyl- (7%), 2′-fluoro- (6%), 3′-methyl- (5%), and 2′-furanyl- (4%) α-arylalanines) compared to the conversion of the α-arylalanines under kinetic resolution conditions with PAM alone (Figure 2). When the racemates of heteroaromatic substrates (entries 5 and 8) (2-thienyl-α-alanine and 2-furanyl-α-alanine, respectively) were incubated with PAM alone, the distribution of compounds after a 20-h incubation revealed that ∼60% of the nonproductive (*R*)-α-arylalanine remained. The nonproductive isomers were expected at 50%, which makes this 10% increase over the theoretical value intriguing. In contrast, reactions containing the racemase, aminomutase, and compounds **5** or **8** revealed a substrate/product distribution that was aligned with theoretical values. It is difficult to speculate at this time why the heteroaromatic amino acids displayed unusual substrate/product distribution at equilibrium in reactions containing the aminomutase catalyst alone, compared to the theoretically agreeable distribution when the racemase was coupled with the aminomutase (cf. Figure 2).

Conclusion

Coupling a broad-spectrum racemase with a promiscuous yet enantioselective aminomutase in the enhanced resolution reactions described herein was key toward moderately increasing the conversion yields of (*R*)-β-arylalanines (at >99% ee) from racemic α-arylalanines. The principal enzymes in this investigation are easily accessible by overexpression in *E. coli* and purification by His-tag affinity chromatography, which are necessary for their biocatalytic application. The relatively incremental increase in β-arylalanine production in the biocatalytic system, described herein, is notably dependent on the position of equilibrium between the substrate and product in the PAM reaction. However, the impact of this investigation centers firmly on employing racemic α-arylalanine substrates in the production of β-aryl-β-amino acids, instead of utilizing only the natural (*S*)-antipode in the processing scheme. The inclusion of the racemase, which kept the substrates in dynamic equilibrium, can thereby theoretically double the product yield under dynamic kinetic resolution conditions. The latter condition is potentially achievable by including a provision to drive the reaction to completion by, for example, selective amidation of the various β-amino acids by amidase/acylase catalysis.[26,27] Isolation of the derivatized product from the reaction pool and repeat incubation of the remaining α-amino acid with the racemase and aminomutase could effectively increase flux through the reaction forward. Moderate to significant increases in the production yields of enantiomerically enriched products by enzymatic resolution and semibiochemical dynamic kinetic resolution experiments are finding a niche in asymmetric synthesis. Generally, the ultimate objective of these methods is to develop innovative strategies that exploit the enantioselectivity of

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enzymatic catalysis to optimize the resolution and/or yields of products.[28−32] The capacity to obtain one enantiomer (or diastereomer) from a racemic mixture is attractive especially if the product equilibrium can be further increased. Thus, future work toward biosynthesizing (*R*)-β-arylalanines related to those in the present investigation includes developing a facile, selective process to remove the β-arylalanine product from a one-pot reaction mixture containing the aminomutase and the alanine racemase. Moreover, the coupled enzyme assay developed here provides a critical first step toward potentially making β-amino acid scaffolds to construct bioactive chiral molecules principally via biocatalysis.

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Supporting Information Available: Listing of chemicals and reagents used. This material is available free of charge via the Internet at http://pubs.acs.org.

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