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(*S*)-Styryl-α-alanine Used To Probe the Intermolecular Mechanism of an Intramolecular MIO-Aminomutase

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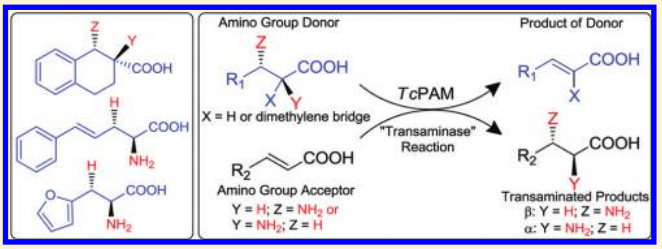
Supporting Information

ABSTRACT: A *Taxus canadensis* phenylalanine aminomutase (*Tc*PAM) catalyzes the isomerization of (*S*)-α- to (*R*)-β-phenylalanine, making (*E*)-cinnamate (~10%) as a byproduct at steady state. A currently accepted mechanism for *Tc*PAM suggests that the amino group is transferred from the substrate to a prosthetic group comprised of an amino acid triad in the active site and then principally rebinds to the carbon skeleton of the cinnamate intermediate to complete the $\alpha-\beta$ isomerization. In contrast, when (*S*)-styryl-α-alanine is used as a substrate, *Tc*PAM produces (2*E*,4*E*)-styrylacrylate as the major product (>99%) and (*R*)-styryl-β-alanine (<1%). Comparison of the rates of conversion of the natural substrate (*S*)-α-phenylalanine and (*S*)-styryl-α-alanine to their corresponding products (k_{cat} values of 0.053 ± 0.001 and 0.082 ± 0.002 s^{-1}, respectively) catalyzed by *Tc*PAM suggests that the amino group resides in the active site longer than styrylacrylate. To demonstrate this principle, inhibition constants (K_I) for selected acrylates ranging from 0.6 to 106 μM were obtained, and each had a lower K_I compared to that of (2*E*,4*E*)-styrylacrylate (337 ± 12 μM). Evaluation of the inhibition constants and the rates at which both the α/β-amino acids (between 7 and 80% yield) and styrylacrylate were made from a corresponding arylacrylate and styryl-α-alanine, respectively, by *Tc*PAM catalysis revealed that the reaction progress was largely dependent on the K_I of the acrylate. Bicyclic amino donor substrates also transferred their amino groups to an arylacrylate, demonstrating for the first time that ring-fused amino acids are productive substrates in the *Tc*PAM-catalyzed reaction.



A *Taxus canadensis* phenylalanine aminomutase (*Tc*PAM) requires no cofactors to intramolecularly transfer the amino group of (*S*)-α-phenylalanine to form (*R*)-β-phenylalanine, utilizing the same carbon skeleton.[1] This aminomutase activates the amino group of the substrate as an alkylammonium leaving group via ligation with a methylidene imidazolone (MIO) prosthetic group, formed by autocatalytic post-translational condensation of active site residues (A-S-G).[2] This MIO purportedly serves as an electrophilic sink (a 1,4-Michael acceptor) and is nucleophilically attacked by the amino group of the substrate to form the alkylammonium complex to facilitate Hofmann-like elimination.[2] The *pro*-3*S* hydrogen and an alkylamine are transiently eliminated to form a cinnamate intermediate and then rebound to interchanged positions on the phenylpropenoid skeleton. Under steady-state reaction conditions, *Tc*PAM catalyzes the production of a significant proportion of (*R*)-β-phenylalanine compared to (*E*)-cinnamate, which is ordinarily between 10 and 20 times slower.[1]

A recent study showed that when *Tc*PAM was co-incubated with both [^{15}N]-(*S*)-α-phenylalanine (**1**) and [ring,3-$^{2}H_6$]-(*E*)-cinnamate (**2**) (each at 10 mM), the [^{15}N]amino group incorporated (97%) intramolecularly into (*R*)-β-phenylalanine. However, slight intermolecular amino group transfer occurred, as well. Analysis of the isolated β-phenylalanine revealed a crossover reaction, in which the amino group of **1** was incorporated partially into precursor **2** to form [^{15}N,$^{2}H_6$]-β-phenylalanine (**5**) in ~3% yield (Scheme 1).[1] Thus, the unlabeled cinnamate intermediate **3** derived from [^{15}N]-α-phenylalanine did not appreciably dissociate from the active site or exchange with exogenous ^{2}H-labeled cinnamate (an only 3% occurrence), before the amino group of the $^{15}NH_2$−MIO complex rebounded. A few labeled cinnamate molecules did, however, competitively bind the active site, accounting for the dearth of doubly labeled β-amino acid product observed.

The foregoing intermolecular data are supported by an earlier study demonstrating that a tyrosine aminomutase (TAM) transferred the amino group from 3′-chlorotyrosine to 4-hydroxycinnamate to form 3′-chloro-4′-hydroxycinnamate and a mixture of α- and -β-tyrosine.[3] The results of the latter intermolecular process were paradoxically used to explain that the TAM reaction proceeds intramolecularly. This argument was further thought to be supported by the faster transfer of the amino group in the cross reaction compared to the rate when exogenous NH_4^+ was used as the amino group source. Notably, the high molar concentrations of ammonia used in the catalysis of the reverse reaction likely approached conditions typically

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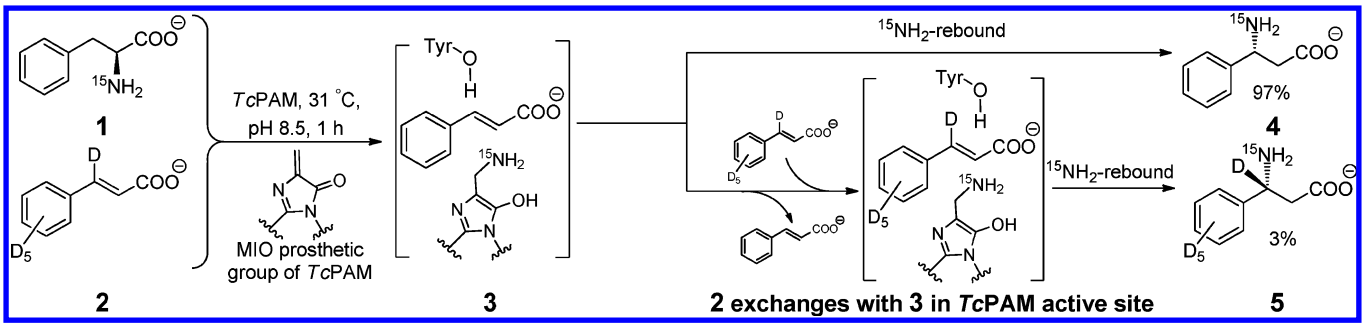
Scheme 1. Mechanism of the Transaminase Reaction Catalyzed by *Tc*PAM with Its Natural Substrate



used to precipitate and denature proteins,[4] and thus affected the catalytic efficiency of the ammonia transfer reaction. In this earlier study, it also remained unclear whether the amino group removed from α-tyrosine was then transferred intramolecularly to the same 4′-hydroxycinnamate reaction intermediate en route to β-tyrosine, on the natural reaction pathway.[3]

Moreover, a homologous phenylalanine aminomutase (PAM) isolated from *Taxus chinensis*, in an earlier study, was incubated with an exogenous supply of 6 M NH_4^+ salts (pH 10) to provide ammonia to the reaction. The ensuing NH_2−MIO complex (or other amine complex) likely formed, and the hydrogen and amino group (from the MIO complex) were transferred to various (*E*)-arylacrylates to form a mixture of the corresponding enantiopure α- and β-amino acids in the reverse reaction.[5,6] This latter study was identical in technique to another earlier work demonstrating that mechanistically similar MIO-dependent enzymes also catalyzed their reverse reactions. This earlier compendium of work includes a description of how a phenylalanine ammonia lyase added a hydrogen/amino group pair from 6 M NH_4^+ salts (pH 10) to (*E*)-arylacrylates to produce non-natural (*S*)-α-amino acids,[7] while a tyrosine aminomutase analogously added the same pair from NH_4^+ salts to 4-hydroxycinnamate to form a mixture of α- and β-tyrosine.[3]

Furthermore, while *Tc*PAM converts (*S*)-α-phenylalanine principally to (*R*)-β-phenylalanine at a rate of 0.053 ± 0.001 s^{-1}, it also converts (*S*)-styryl-α-alanine to (2*E*,4*E*)-styrylacrylate as the major product (99%) at approximately the same rate (0.082 ± 0.002 s^{-1}).[1] Consequently, the styrylacrylate is released from the active site before the amino group can rebound appreciably to form styryl-β-alanine. These kinetic data along with the proposed MIO-dependent mechanism suggest that when (*S*)-styryl-α-alanine is converted to (2*E*,4*E*)-styrylacrylate, the transient amino group remains as the NH_2−MIO complex, likely for the same duration as the α-β-phenylalanine isomerization reaction.

A hypothesis emerged from the aforementioned intra- and intermolecular mechanistic evaluations[1] and observations.[3,5−7] Conceptually, a weakly binding acrylate (AC1) intermediate derived from an amino acid (AA1) in the *Tc*PAM reaction was replaced in the active site with a tighter binding, competitive acrylate (AC2). The amino group removed from AA1, but now in complex with the enzyme, could rebind to AC2 and form α- and β-amino acids (AA2) different from those derived from AA1. Thus, guided by the partial intermolecular process observed for *Tc*PAM[1] and by the details of the of kinetic parameters described herein, we explored four non-natural amino acids as amino group donor substrates. The amino group of these donor substrates was transferred intermolecularly to another arylacrylate skeleton by *Tc*PAM catalysis to form an α- and β-arylalanine mixture.

MATERIALS AND METHODS

Chemicals. (*R*)-β-Phenylalanine, 3′-methyl-(*S*)-α-, 3′-methyl-(*R*)-β-, 4′-methyl-(*S*)-α-, 4′-methyl-(*R*)-β-, 4′-fluoro-(*S*)-α-, 4′-fluoro-(*R*)-β-, and 3′-fluoro-(*R*)-β-phenylalanine and (*S*)-styryl-α-, (*S*)-2′-thienyl-α-, (*R*)-2′-thienyl-β-, and (*S*)-2′-furyl-α-alanine were purchased from Peptech Inc. (Burlington, MA). (1′*S*)-Camphanoyl chloride, (*S*)-α-phenylalanine, (*S*)-2-aminotetralin-2-carboxylic acid, (3*R*)-3-aminotetralin-(2*R*)-2-carboxylic acid, (DL)-4′-chloro-α-phenylalanine methyl ester, (DL)-4′-chloro-β-phenylalanine, (*E*)-2′-methyl- and (*E*)-4′-methylcinnamate, (*E*)-2′-furyl- and (*E*)-2′-thienylacrylate, and (*E*)-cinnamic acid were purchased from Sigma-Aldrich-Fluka (St. Louis, MO). (*E*)-4′-Chloro-, (*E*)-3′-methyl-, and (*E*)-4′-fluorocinnamate and (2*E*,4*E*)-styrylacrylate were acquired from Alfa Aesar (Ward Hill, MA). All chemicals were used without further purification, unless noted.

Instrumentation. The GC oven (Agilent, model 6890N) conditions were as follows. The column temperature was programmed from 70 to 250 °C at a rate of 10 °C/min (18 min total run time); splitless injection was selected, and helium was used as the carrier gas (1.2 mL/min). The GC oven was coupled to a mass selective detector (Agilent, model 5973 *inert*) in ion scan mode from 100 to 300 atomic mass units at a 70 eV ionization voltage.

Expression of the *tcpam* cDNA. Codon-optimized *tcpam* cDNA was previously ligated into expression vector pET28a-(+), and the recombinant plasmid encoded an N-terminal His_6 tag.[8] The *tcpam* clone was overexpressed in six 1 L cultures of *Escherichia coli* BL21(DE3) cells by induction with isopropyl β-D-thiogalactopyranoside. The overproduced protein was isolated from the bacteria and purified to 95% by Ni affinity chromatography to yield 5 mg of protein, as described previously.[8] Routine assays for assessing enzyme function were conducted with (*S*)-α-phenylalanine at saturation (1 mM) and *Tc*PAM (100 μg, 1.3 nmol) in 50 mM phosphate buffer (pH 8.5) in 1 mL assays.

Identification of an Amine Donor Substrate. (*S*)-Styryl-α-alanine (**6**), 2′-furyl-(*S*)-α-alanine (**15**), (3*R*)-aminotetralin-(2*R*)-carboxylic acid (**16**), and (*S*)-2-aminotetralin-2-carboxylic acid (**17**) (each at 1 mM) were incubated separately with *Tc*PAM (250 μg, 3.3 nmol) and 3′-methylcinnamate (**14**) (1 mM) in 5 mL assays at 31 °C. To calculate the initial steady-state rates, aliquots (1 mL) were withdrawn at 10, 20, 30, and 40 min from the reaction mixture containing **6**, at 15, 30, 45, and 60 min from the reaction mixture containing **15**, and at 1,

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2, 3, and 4 h from the reaction mixture containing **16** or **17**. An internal standard (*R*)-3′-fluoro-*β*-phenylalanine (20 nmol) was added; the reactions were quenched by increasing the pH to 10 (6 N NaOH), and the amino acids were immediately *N*-carbonylated by adding ethyl chloroformate (100 μL). After 10 min, the reaction mixtures were acidified to pH 2 (6 N HCl), the derivatives were extracted with diethyl ether (2 × 1 mL), and the solvent was removed in vacuo. To the remaining residue was added a trimethylsilyl diazomethane solution dissolved in an ethyl acetate/methanol mixture (3:1, v/v) (methanol was used to liberate the diazomethane), until the yellow color of diazomethane persisted, to convert the *N*-acyl amino acids to their methyl esters.

Assessing the Optimal Concentration of Amino Group Donor 6. After compound **6** had been identified as an amino group donor, **6** was incubated at 400, 600, 800, 1000, 2000, and 3000 μM in the presence of *Tc*PAM (50 μg, 0.7 nmol) in 1 mL reaction mixtures for 30 min at 31 °C. An aliquot (1 mL) was withdrawn from the reaction mixture and added to a 1.5 mL polystyrene cuvette (General Laboratory Supply, Pasadena, TX) and analyzed by UV−visible spectroscopy (Beckmann DU 640, Beckmann Coulter, Brea, CA) with A_{305} monitoring of the sample to quantify the product (2*E*,4*E*)-styrylacrylate (**7**). The absorbance values obtained from the samples were compared against those of a concentration series (ranging from 0.3 to 80 μM) made from authentic (2*E*,4*E*)-styrylacrylate dissolved in 50 mM phosphate buffer (pH 8.5), analyzed by the same method. A sample blank of **6** (λ_{max} = 275 nm; A_{305} = 0.007 at 0.1 mM) was used to subtract the background absorbance. A nonlinear regression plot of the steady-state production rate of **7** versus the concentration of **6** was used to calculate the maximal steady-state velocity (V_{max}) and to assess which concentrations of **6** were at V_{max}.

Calculation of the Inhibition Constants of Various Acrylates in the *Tc*PAM Reaction. Kinetic parameters and inhibition constants for various substrates and inhibitors in the *Tc*PAM reaction were acquired by first establishing the linearity of enzyme activity with respect to both time and protein concentration for the substrate without inhibitor. (2*E*,4*E*)-Styrylacrylate (at 50, 100, and 200 μM) (inhibitor) was incubated with *Tc*PAM (30 μg, 0.4 nmol) containing 4′-methyl-(*S*)-*α*-phenylalanine as the substrate at 10, 20, 40, 80, 150, 300, 500, 750, and 1000 μM (in separate dilution series) in 50 mM phosphate buffer (pH 8.5, 1 mL) for 30 min. To each reaction mixture were added 3′-fluoro-(*R*)-*β*-phenylalanine and (*E*)-2′-methylcinnamic acid (at final concentrations of 20 μM) as internal standards at 0 °C. The reaction mixtures were basified to pH 10 (6 N NaOH). Ethyl chloroformate (100 μL) was added. After 5 min, the pH of the samples was readjusted to 10. A second batch of ethyl chloroformate (100 μL) was added, and the solution was mixed for 5 min. The samples were acidified to pH 2 (6 N HCl), and the arylacrylic acids and *N*-ethoxycarbonyl amino acids were extracted into diethyl ether (2 × 1 mL). The organic fractions were combined, and the solvent was removed in vacuo. To the remaining residue was added a trimethylsilyl diazomethane solution dissolved in an ethyl acetate/methanol mixture until the yellow color of diazomethane persisted to produce the methyl esters of the *N*-ethoxycarbonyl amino acids and arylacrylates. The derivatized samples were quantified by GC−EI-MS, wherein the relative abundances of the base peak fragment ions of the amino acid derivatives present in the samples were compared to those of authentic standards at various concentrations. From the Lineweaver−Burk plots of the data, the Michaelis−Menten constants, inhibition constants, and rates were calculated.

(*E*)-Cinnamate (**3**), (*E*)-4′-chloro-, (*E*)-4′-methyl-, (*E*)-4′-fluoro-, and (*E*)-3′-methyl-cinnamate (**9**−**11** and **14**, respectively), and (*E*)-2′-thienylacrylate (**13**) (each at 50, 100, 150, 300, 500, 1000, and 2000 μM in separate dilution series) were incubated with *Tc*PAM (50 μg, 0.7 nmol) containing **6** (1 mM) as a cosubstrate in 50 mM phosphate buffer (pH 8.5, 1 mL) for 30 min. The reaction mixtures in each series were placed on ice and acidified to pH 2 (6 N HCl); (*E*)-2′-methylcinnamic acid (at a final concentration of 20 μM) was added to each as an internal standard, and the carboxylic acids were extracted into diethyl ether (2 × 1 mL). The organic fractions were combined, and the solvent was removed in vacuo. To the remaining residue was added a trimethylsilyl diazomethane solution dissolved in an ethyl acetate/methanol mixture until the yellow color of diazomethane persisted to produce the methyl esters of the acrylate. The derivatized samples were quantified by GC−EI-MS, wherein the relative abundances of the base peak fragment ions of methyl styrylacrylate in the samples were compared to those of an authentic standard at various concentrations. From the Lineweaver−Burk plots of these data, the Michaelis−Menten constants and rates were calculated.

Time Course Assays for Intermolecular Amino Group Transfer. *Tc*PAM (300 μg, 3.9 nmol) was incubated separately with various acrylates at 50, 100, 150, 300, 500, 1000, and 2000 μM and **6** (1 mM) in 6 mL assays in 50 mM phosphate buffer (pH 8.5). Aliquots (1 mL) were withdrawn from each reaction mixture at 0.5 h intervals over 1 h, and then at 2, 4, 8, and 12 h. Internal standards (*E*)-2′-methylcinnamic acid and 3′-fluoro-(*R*)-*β*-phenylalanine (each at 20 μM) were added to each aliquot, and the reactions were quenched by increasing the pH to 10 (6 N NaOH). The amino acids and acrylates were derivatized, as described earlier, and quantified by GC−EI-MS.

Assessing the Absolute Stereochemistry of *α*- and *β*-Phenylalanine Product (8f) by the *Tc*PAM Transaminase Pathway. (*E*)-4′-Fluorocinnamate (500 μM) was incubated with *Tc*PAM (200 μg, 2.6 nmol) and **6** (1 mM) in 1 mL assays in 50 mM phosphate buffer (pH 8.5) at 31 °C. After 3 h, the amino acids were derivatized to their *N*-[(1′*S*)-camphanoyl] methyl esters. The derivatized *α*- and *β*-amino acids were identified by GC−EI-MS analysis and compared against the retention time and mass spectrometry fragmentation of authentic *N*-[(1′*S*)-camphanoyl]-4′-fluoro-(2*S*)-*α*- and -4′-fluoro-(3*R*)-*β*-phenylalanine methyl esters.

Assessing the Effects of Maintaining the Steady-State Conversion of 6 to 7 on the Production of 8f. To a solution of **6** (31 μmol) in 50 mM phosphate buffer (pH 8.5, 30 mL) were added (*E*)-3′-methylcinnamic acid (**14**) (31 μmol) and *Tc*PAM (1.5 mg, 20 nmol) at 31 °C. The reaction mixture was shaken slowly in a water bath. An aliquot (20 μL) was withdrawn from the reaction flask every hour and diluted to 200 μL in 50 mM phosphate buffer. The entire sample was added to a 450 μL cuvette (Quartz, Hellma GmbH & Co. KG, Müllheim, Germany) and analyzed by UV−visible spectroscopy (Beckmann DU 640, Beckmann Coulter) with A_{305} monitoring of the sample to quantify the product (2*E*,4*E*)-styrylacrylate (**7**) by comparison against a concentration series (ranging from 1 to 100 μM) made from authentic (2*E*,4*E*)-styrylacrylate dissolved in 50 mM phosphate buffer (pH 8.5), analyzed by the same method. A sample blank of **6** (λ_{max} = 275 nm; A_{305} = 0.007 at

0.1 mM) was used to subtract the background absorbance. Every hour, amino group donor **6** was added in an amount equal to that of **7** produced, to keep **6** at a concentration of ~1 mM during reaction.

Aliquots (100 μL) were withdrawn at 0, 4, 7, 10, 15, and 20 h, and to each were added internal standards 2′-methylcinnamate and 3′-fluoro-(*R*)-*β*-phenylalanine (100 nmol of each) and 6 N HCl, until the pH was 2. The samples were extracted with diethyl ether (2 × 1 mL). The organic fractions were combined. The solvent was evaporated, and the remaining residue was treated with a dilute diazomethane solution to convert the acrylic acids to their methyl esters. Methyl esters of the biosynthetically derived **7** and the unused acrylates **14** in the reaction mixture were quantified by GC−EI-MS. The abundances of their base peak fragment ions were compared to those of the same fragment ions generated from authentic samples of the methyl esters of **7** and **14**, analyzed by an identical method. The remaining aqueous fraction was basified to pH 10 (6 N NaOH), and the amino acids were derivatized to their *N*-(ethoxycarbonyl) methyl ester analogues by reaction with ethyl chloroformate and subsequently with a dilute diazomethane solution, as described before. The esters were quantified by GC−EI-MS analysis where the abundance of the base peak fragment ion derived from each ester was compared to that of the same ion generated by identically analyzed authentic samples of *N*-acyl 3′-methyl-(*S*)-*α*- and (*R*)-*β*-phenylalanine methyl esters at concentrations between 1 and 100 μM.

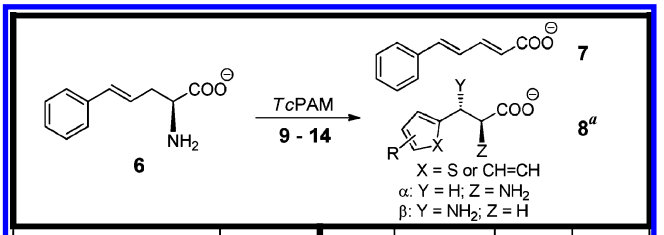
Biosynthesis and Characterization of a 3,4-Dihydronaphthalene-2-carboxylic Acid (16-Acr) from 16. (1*R*)-1-Amino-1,2,3,4-tetrahydronaphthalene-2-carboxylic acid (**16**) (5 mM) was incubated with *Tc*PAM (1 mg, 13 nmol) in 50 mM phosphate buffer (20 mL, pH 8.5) at 31 °C. After 3 days, the reaction mixture was acidified to pH 2 (6 N HCl), and the acrylate derived by deamination of **16** (designated **16-Acr**) was extracted into diethyl ether (3 × 20 mL). The ether layer was dried ($NaSO_4$) and evaporated to dryness. The residue (~10 mg) was recrystallized with a 0.1 M HCl/ethanol mixture (10:1, v/v), and the solvent was decanted and removed in vacuo. The resulting crystals were characterized by ^1H and ^{13}C NMR and GC−EI-MS analyses. The exact mass was assessed on a Q-TOF Altima API mass spectrometer (Waters, Milford, MA): ^1H NMR (500 MHz, $CDCl_3$) δ 7.65 (bs, 1H, H−C═C, olefin), 7.22 (m, 4H, aromatic-H), 2.88 (t, 2H, $^3J_{HH}$ = 8.5 Hz, CH_2, benzylic), 2.61 (dt, 2H, $^3J_{HH}$ = 8.5 Hz, $^4J_{HH}$ = 1.0 Hz, CH_2, allylic); ^{13}C NMR (125 MHz, $CDCl_3$) δ 172 (C═O), 138.8 (H−C═C−CO_2H), 126.8 (H−C═C−CO_2H), 137.2, 132.3, 129.9, 128.8, 128.4, 127.7 (aromatic-C), 27.5 (CH_2 benzylic), 21.8 (CH_2, allylic); exact mass $[M - H]^-$ obsd 173.0599, calcd 173.0603 for $C_{11}H_9O_2$.

RESULTS

Overproduction of *Tc*PAM. Codon-optimized *tcpam* was overexpressed from a pET vector by induction with isopropyl *β*-D-thiogalactopyranoside in six 1 L cultures of *E. coli* BL21(DE3) cells engineered to express *tcpam*. The overproduced *Tc*PAM was isolated from the bacteria as an N-terminal His_6 fusion, purified to ~95% by nickel affinity chromatography, as described previously.[8] A 1 mL stock of this enzyme at 5 mg/mL was used as the source of *Tc*PAM in the assays described herein.

Calculation of K_I for Various Acrylates and Time Course Studies for Optimal Amino Transfer. Preliminary guidelines were established to examine the intermolecular transfer of the amino group from (*S*)-styryl-*α*-alanine (**6**) to an arylacrylate catalyzed by *Tc*PAM. First, under steady-state conditions and in the absence of an inhibitor, **6** was optimally converted to **7** at V_{max} when the concentration of **6** was 1 mM, but conversely, the rate plummeted when the concentration of **6** exceeded 1 mM (data not shown), likely because of substrate inhibition by a so far unknown mechanism. Therefore, 1 mM **6** was used in reaction mixtures with *Tc*PAM to assess the dynamics of the transamination reaction in the presence of various arylacrylates at varying concentrations. Next, arylacrylates (**9**−**14**) were each separately incubated with *Tc*PAM during the catalysis of (*S*)-4′-methyl-*α*- (**8b**/***α***) to (*R*)-4′-methyl-*β*-phenylalanine (**8b**/***β***) to calculate the competitive dissociation constants [$K_{I(A)}$] of **9**−**14**. The $K_{I(A)}$ values were lower for arylacrylate inhibitors **9**−**14** (Table 1 and Figure S1 of the Supporting Information) than for (2*E*,4*E*)-styrylacrylate (**7**) (K_I = 337 ± 12 μM) (Figure S6 of the Supporting Information), suggesting that in the amine exchange reactions, **9**−**14** would bind *Tc*PAM better than the styrylacrylate product **7**, derived from **6**.

Table 1. Kinetic Parameters of Various Arylacrylates and Their Conversion to Amino Acids Using (*S*)-Styryl-*α*-alanine in the *Tc*PAM Reaction



Arylacrylate	$K_{I(A)}$ (μM)[b]	nmol[c]	8 (nmol)	8 (%)[d]	8(α:β)
9	0.60 (± 0.04)	150	72 (**8a**)	48 (**8a**)	49:51
10	1.70 (± 0.05)	150	120 (**8b**)	80 (**8b**)	17:83
11	12.0 (± 0.5)	150 (8 h)	120 (**8c**)	80 (**8c**)	33:67
12	23.0 (± 0.7)	300	170 (**8d**)	57 (**8d**)	41:59
13	47.0 (± 2.6)	1000	73 (**8e**)	7.3 (**8e**)	75:25
14	106 (± 8)	1000	200 (**8f**)	20 (**8f**)	25:75

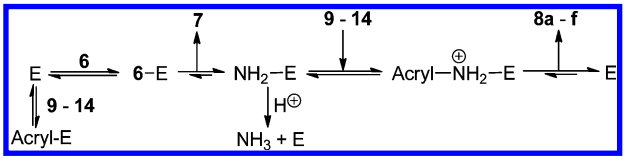
[a]R substituents are inferred from structures. [b]*Tc*PAM catalyzed reaction converting **8b**/***α*** to **8b**/***β*** was inhibited for the calculation of K_I values. [c]Initial concentration of acrylate in the 12 h transamination assays containing *Tc*PAM (0.1 mg/mL). [d]Yield is with respect to the arylacrylate substrate. The standard deviations are shown and were calculated from triplicate assays.

Amino donor substrate **6** at 1 mM (initial concentration) was incubated with each acrylate (**9**−**14**) between 50 and 2000 μM and co-incubated with *Tc*PAM (50 μg, 0.7 nmol) to evaluate the steady-state rate parameters and to assess the point at which **6** could outcompete **9**−**14** for the active site. The production of arylalanines (**8a**−**f**) for each reaction was measured over time,

and the yield was found to be maximal at ~12 h, typically when the corresponding arylacrylate precursor was between 150 and 1000 μM (Table 1 and Figure S2 of the Supporting Information). Then **8a**−**f** were converted to their *N*-ethoxycarbonyl methyl ester derivatives and verified by GC−EI-MS analysis (Figure S3 of the Supporting Information). The stereochemistry of **8c** was assessed, as an example, by converting the amino acids to their *N*-[(1′*S*)-camphanoyl] methyl ester derivatives. The retention times and mass spectrometry fragment ions determined by GC−EI-MS analysis of the derivatized biosynthetic amino acid diastereomers were compared to those of diastereomerically pure authentic compounds; the stereochemistries of the biosynthetic α- and β-amino acids were identified as 2*S* and 3*R*, respectively (Figure S4 of the Supporting Information). These stereochemical data supported earlier findings reported elsewhere for the *Tc*PAM reaction.[8,9]

Relationship between the Rates of Formation of 7 and 8 and the K_I values of 9−14. The approximate steady-state production rate of **8a**−**f** (designated as v_o→**8**), reflecting the overall reaction flux after 1 h, was compared to that of the formation of the NH_2−MIO complex, estimated by the observed rate at which **6** (at 1 mM) was converted to **7** (designated as v_o→**7**) (Scheme 2 and Figure S5 and Table S1 of the Supporting Information). The (v_o→**8**)/(v_o→**7**) ratio was charted versus the inhibition constants for each acrylate (**9**−**14**) [$K_{I(A)}$], yielding a logarithmic relationship (Figure 1) (see eqs 7−9 of the Supporting Information). This set of arylacrylates **9**−**14** was selected for this study because the dissociation constants (0.6−106 μM) of these inhibitors were wide-ranging, thus allowing the logarithmic dependence between K_I and reaction rates to be observed. Theoretically, if $K_{I(A)}$ were increased to 438 μM, the (v_o→**8**)/(v_o→**7**) ratio would approach zero as v_o→**8** was slowed because of the poor binding acrylate (amine acceptor). Meanwhile, v_o→**7** would effectively approach the maximum rate at which **6** can be converted to **7**; i.e., the conditions could resemble those as if the acrylate were absent. By contrast, as $K_{I(A)}$ hypothetically approached 0 μM (representative of an irreversibly bound acrylate), the (v_o→**8**)/(v_o→**7**) ratio mathematically approached a maximum of ~3.8 under steady-state conditions (cf. eq 7 of the Supporting Information). Thus, an amine acceptor that bound tightly and nonproductively to *Tc*PAM (**E** in Scheme 2) yet also bound productively to the acryl−$^+NH_2$−E complex (Scheme 2) would theoretically increase the overall reaction rate (v_o→**8**) ~3.8-fold relative to v_o→**7**. Understandably, the rate of production of **8a**−**f** cannot exceed the rate at which the amine complex is formed in *Tc*PAM (estimated by v_o→**7**). Therefore, v_o→**8** can never exceed v_o→**7**, and consequently, (v_o→**8**)/(v_o→**7**) approaches 1 as the $K_{I(A)}$ approaches 0 μM (Figure 1) in the ping-pong-like reaction mechanism (Scheme 2). This was evident when the steady-state production rate of **8a** [v_o→**8a** = (1.7 ± 0.1) × 10^{-4} s^{-1}] was compared to that of **7** [v_o→**7** = (1.7 ± 0.1) × 10^{-4} s^{-1}] (Table S1 of the Supporting Information). These data indicated that the rate of transfer of an amino group to acrylate **9**, producing **8a**, matched the amination rate for formation of the NH_2−E complex (Scheme 2), suggesting that 100% of the amino group was transferred from the enzyme to the acrylate.

Scheme 2. Reaction Profile of the Transaminase Reaction[a]



[a]Abbreviations: E, *Tc*PAM; Acryl-E, acrylate−*Tc*PAM complex; NH_2-E, NH_2−MIO complex within *Tc*PAM; Acryl-$^+NH_2$-E, acrylate−$^+NH_2$−MIO complex within *Tc*PAM.

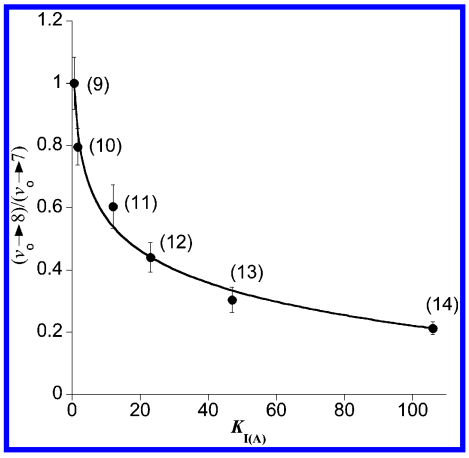


Figure 1. Ratio [(v_o→**8**)/(v_o→**7**)] of the steady-state rates for the conversion of acrylates **9**−**14** to **8** and of the formation of the NH_2−MIO complex plotted vs $K_{I(A)}$ for **9**−**14** (shown in parentheses).

Titration of the Amino Group Donor 6 To Maintain Steady-State Conversion to 7. Also observed during the time course study, when a mixture of **6** and **14** (each at 1 mM) and *Tc*PAM (300 μg, 3.9 nmol in 6 mL of buffer) were incubated (12 h), the production of **8f** was maximal at ~20% converted yield (36 ng). Beyond 12 h, **6** was depleted, and consequently, the rate of production of **8f** rapidly approached equilibrium (Figure 2A). Thus, as an alternative, the conversion of **6** to **7** was monitored by UV spectroscopy at A_{305} during the reaction, and **6** was added accordingly to maintain its concentration at 1 mM in the presence of **14** (5 mg, 31 μmol) and *Tc*PAM (1.5 mg, 20 nmol). The production of **8f** was measured over 20 h and was obtained at 42% converted yield (2.3 mg, 13 μmol) with respect to **14** (Figure 2B). Therefore, when the reaction mixture was titrated with **6**, v_o→**7** was apparently kept at the steady-state rate over a longer time frame, resulting in the greater production yields of **8f**.

As mentioned previously, 6 M ammonium salts were used in previous studies[10] to provide a hydrogen/amino group pair that

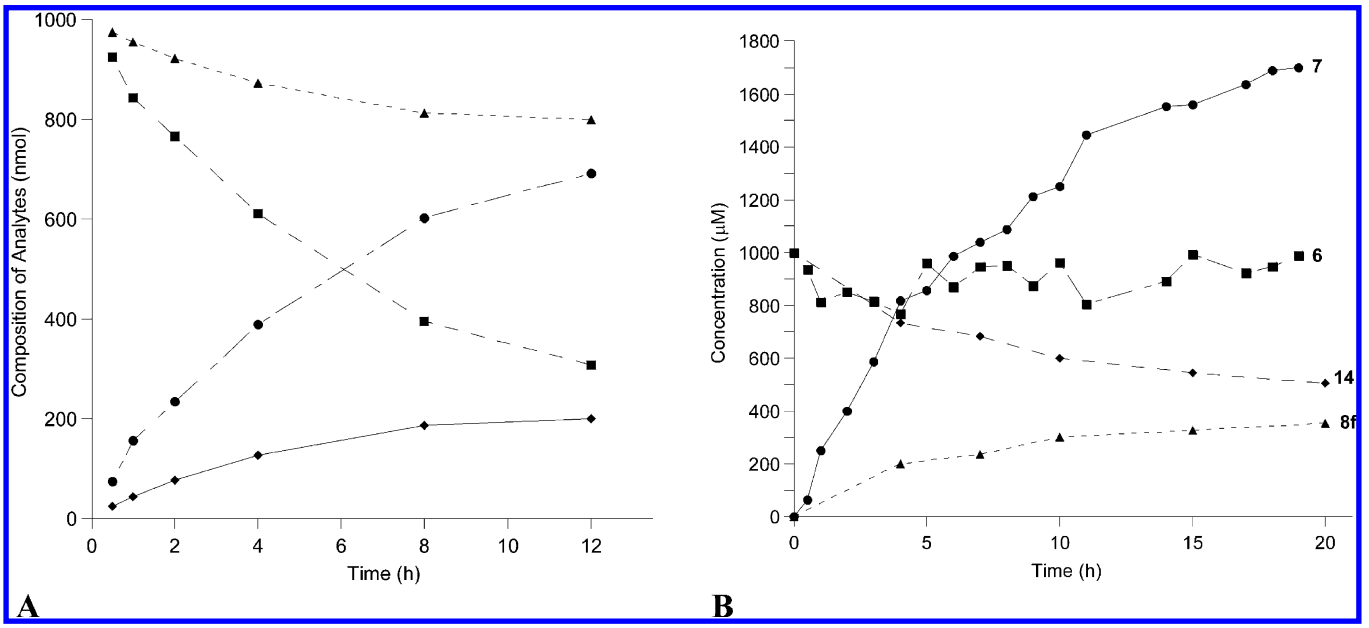
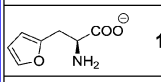
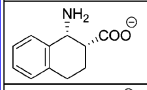
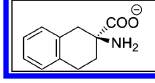


Figure 2. (A) Time course assay. Amounts of (*S*)-styryl-α-alanine (■, **6**), (2*E*,4*E*)-styrylacrylate (●, **7**), (*S*)-3′-methyl-α- and (*R*)-3′-methyl-β-phenylalanine (◆, **8f-α** and **8f-β**), and (*E*)-3′-methylcinnamate (▲, **14**) in an aminotransferase reaction catalyzed by *Tc*PAM over 12 h. (B) Steady-state conversion of **6** to **7** of **8f/α** and **8f/β** by transfer of an amino group from **6** to **14** (5 mg) by *Tc*PAM catalysis: (2*E*,4*E*)-styrylacrylate (**7**, ●), (*S*)-styryl-α-alanine (**6**, ■), (*E*)-3′-methylcinnamate (**14**, ◆), and total of α- and β-isomers of 3′-methylphenylalanine (**8f**, ▲).

was added across the double bond of an acrylate substrate in reverse reactions catalyzed by various MIO-dependent enzymes. These reverse reactions were typically conducted at pH 10 at high ammonium salt concentrations, which likely affected the catalytic activity. In this study, the metered addition of the amino group donor **6** likely allowed the pH of the reaction to remain optimal at 8.5 and, more importantly, prevented an excessive surplus of ammonium ions from accumulating in the reaction mixture that could potentially affect the rate of catalysis.

Other Amino Donor Substrates. Non-natural amino acids, (*S*)-2′-furyl-α-alanine (**15**), (3*R*)-aminotetralin-(2*R*)-carboxylic acid (**16**), and (*S*)-2-aminotetralin-2-carboxylic acid (**17**), were incubated in separate assays to assess their utility as amino group donors. These amino acids were chosen because, like **6**, they were nearly exclusively converted by *Tc*PAM to their corresponding acrylates with only minor (<10%), if any, isomeric amino acid made (Table 2). This suggested that the acrylates from **15**−**17** were likely derived by a route mechanistically similar to that of **7** (i.e., the acrylates dissociate from *Tc*PAM and leave the NH_2−MIO enzyme complex behind). Compounds **15**−**17** indeed transferred their amino group to **14**, but **6** did so faster (0.56 ± 0.02 nmol/min) (Table 2). To simplify the kinetic evaluation, the rate of transfer of an amino group to **14** was considered identical in each reaction; thus, the different steady-state rates of production of **8f** by use of "sacrificial" substrates **6** and **15**−**17** were reflective of the NH_2−MIO complex loading rate, e.g., 2.5-fold faster with **6** (0.56 ± 0.02 nmol of **8f**/min) than with **15** (0.22 ± 0.01 nmol of **8f**/min). This difference paralleled the 2-fold difference in catalytic efficiency (k_{cat}/K_M) between **6** (0.020 ± 0.001 min^{-1} μM^{-1}) and **15** (0.010 ± 0.001 min^{-1} μM^{-1}). By contrast, the catalytic efficiencies of tetralins **16** (0.005 ± 0.001 min^{-1} μM^{-1}) and **17** (0.0020 ± 0.0003 min^{-1} μM^{-1}) were only 4- and 10-fold lower, respectively, than that of **6**, whereas the transamination rates differed 56- and 140-fold, respectively (Table 2). Apparently, the catalytic efficiencies of *Tc*PAM with amino donors **6** and **15**−**17** trend with the transamination rate, yet the two kinetic parameters are not directly proportional. The identity of the 3,4-dihydronaphthalene-2-carboxylic acid (designated **16-Acr**) biosynthesized by *Tc*PAM from both **16** and **17**, in the amino group transfer reactions, was compared to an authentic standard derived biosynthetically in a large-scale reaction that converted **16** to **16-Acr**. NMR and mass spectrometry analysis verified the product as authentic 3,4-dihydronaphthalene-2-carboxylic acid.

Table 2. Relative Steady-State Rates of Transfer of an Amino Group from Non-Natural Amino Acids (6 and 15−17) to 3′-Methylcinnamate (14) by *Tc*PAM Catalysis

NH_2 Donor	k_{cat} (min^{-1})[a]	K_M (μM)[a]	Acryl : β-A.a.[b]	$v_o \times 10^2$ (nmol 8f •min^{-1})[c]
6	4.9 (± 0.1)	250 (± 4)	99:1	56 (± 2)
 **15**	1.3 (± 0.1)	130 (± 6)	91:9	22 (± 1)
 **16**	1.7 (± 0.3)	341 (± 6)	100:0[c]	1.0 (± 0.3)
 **17**	0.7 (± 0.1)	352 (± 9)	100:0	0.4 (± >0.1)

[a]Acryl, acrylate derived from elimination of H/NH_2 from the amino donor; β-A.a., β-amino acid made from either **6**, **15**, or **17** via *Tc*PAM catalysis. [b]Compound **14** was used as the amino group acceptor. [c]This ratio represents the proportion of acrylate to α-amino acid. Supplied arylacrylates **9**−**14** were the sole means of amino group exchange to produce (*S*)-α- and (*R*)-β-arylalanine. Standard deviations are in parentheses and were calculated from triplicate assays.

DISCUSSION

Overall, the intermolecular transamination reaction catalyzed by *Tc*PAM required that the amino donor substrate yield an acrylate intermediate with a binding affinity for the active site

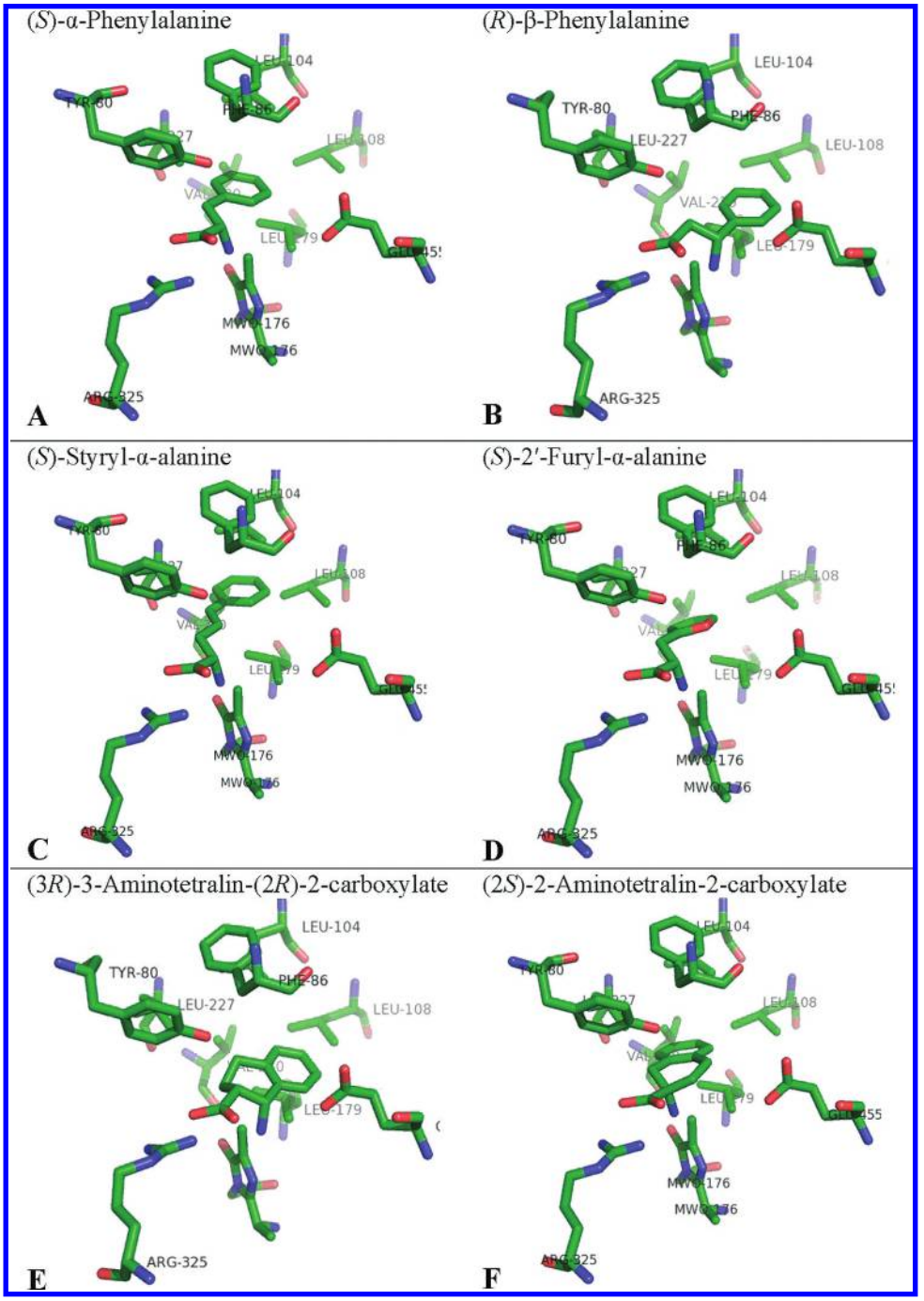


Figure 3. Modeled in the *Tc*PAM active site are natural substrates (A) (*S*)-*α*-phenylalanine and (B) (*R*)-*β*-phenylalanine for reference, (C) (*S*)-styryl-*α*-alanine, (D) (*S*)-2′-furyl-*α*-alanine, (E) (3*R*)-3-aminotetralin-(2*R*)-2-carboxylate, and (F) (*S*)-2-aminotetralin-2-carboxylate. PyMOL (Schrödinger LLC, Cambridge, MA) was used for the substrate modeling by preserving the key interactions with the active site residues.

lower than that of the acrylate serving as the amino acceptor. (*S*)-Styryl-*α*-alanine served as a suitable amino donor for probing the kinetic parameters of the transaminase reaction catalyzed by *Tc*PAM in the presence of a series of arylacrylates, with varying enzyme binding affinities, that served as amino group acceptor substrates and as inhibitors in a quasi-ping-pong exchange mechanism. These results suggested that the nearly exclusive intramolecular transfer process observed for *Tc*PAM and its natural substrate[1] represented an extraordinary balance between both the retention of the cinnamate reaction intermediate and the migratory amino group in the active site. Moreover, the transamination reaction followed a course on which **6** and acrylates **9**−**14** engaged in a sequential ping-pong exchange in the active site. Because **9**−**14** competitively inhibited the catalysis of the conversion of **6** to **7** in the first step in which modified *Tc*PAM (the NH_2−MIO complex) was produced, the transaminase mechanism accordingly deviated from true ping-pong (double-displacement) exchange. Generally, the substrate of the second step does not inhibit the first reaction step in a ping-pong mechanism.[11] Thus, strong inhibition of the first step in the *Tc*PAM transaminase reaction by a tight binding acrylate (amino group acceptor) resulted in a slower than expected reaction flux to the *α*- and *β*-arylalanine products **8** as the amount of acrylate substrate was increased.

Interestingly, the *Tc*PAM active site can apparently accommodate the bicyclic tetralin amino acids **16** and **17** in an orientation that produces the corresponding dihydronaphthalene derived from each. The structure of *Tc*PAM in complex with cinnamate was determined in a previous report[1] and is used here to approximate the trajectory of each non-natural amino acid substrate used as an amino group donor (Figure 3). Key substrate docking interactions occur between the natural substrate phenylalanine and active site residues. The carboxylate and the aromatic ring of the substrate form a salt bridge with Arg325 and a hydrophobic contact with Leu104 of *Tc*PAM, respectively.[1] The amino group of (*S*)-α-phenylalanine is held above and proximate to the methylidene carbon of the methylidene imidazolone (MIO) group by a hydrogen bond interaction between the amino group of the substrate and the hydroxyl of Tyr322 (not shown) in the active site (Figure 3A). The trajectory of the natural substrate phenylalanine essentially traces the carbon skeleton of the cocrystallized cinnamate scaffold, and the β-phenylalanine traces the carbon configuration of the presumed cinnamate rotamer needed to position C_β for the amino group rebound (Figure 3B).[1] *Tc*PAM seemingly accommodates (*S*)-styryl-α-alanine (Figure 3C) and (*S*)-2′-furyl-α-alanine (Figure 3D) in a conformation similar to the trajectory of the modeled natural substrate (Figure 3A), and these substrates exhibited rates of amino group transfer higher than those of the amino tetralins. The furyl ring (Figure 3D) is oriented so that the heteroatom is pointed at Glu455 and toward the solvent-exposed entry point of the active site, where it could engage in hydrogen bonding.

By contrast, the dimethylene bridge of (3*R*)-3-aminotetralin-(2*R*)-2-carboxylate (Figure 3E) causes the aromatic ring of the substrate to reside at a displaced angle compared to the β-phenylalanine congener model (Figure 3B); this displacement of the aromatic ring is more pronounced in the model for (*S*)-2-aminotetralin-2-carboxylate [the presumed productive enantiomer that is analogous to the (2*S*)-antipode of the natural substrate] (Figure 3F). The distorted docking conformations of the latter likely contribute to their poor catalytic efficiencies (k_{cat}/K_M) in the transamination reaction catalyzed by *Tc*PAM. In addition, on the basis of the structure of a *Tc*PAM−cinnamate complex,[1] the dimethylene bridge likely prevented the tetralins from adopting an optimal conformation for binding to the active site and transferring their amino group. Further, the bridged **16** and **17** likely also, in part, precluded the reversible $\alpha-\beta$ interchange via a two-bond rotation of the dihydronaphthalene intermediate, as proposed in previous accounts.[1,12,13]

CONCLUSION

*Tc*PAM was employed as an amino acid:arylacrylate transaminase, and an interesting mechanistic property of the reaction was elucidated. The enzyme was originally characterized as principally producing its natural product (*R*)-β-phenylalanine from (*S*)-α-phenylalanine with nearly exclusive retention of the amino group and the carbon skeleton during the reaction.[1] A distinguishing feature of the *Tc*PAM reaction was revealed in this study when (*S*)-styryl-α-alanine (**6**) was used as a substrate. The release of the ensuing **7** from *Tc*PAM was apparently significantly faster than the release of the amino group from the enzyme. Thus, a significant proportion of *Tc*PAM existed as the NH_2−MIO enzyme complex, and intermolecular transfer of the amino group to the exogenously supplied arylacrylates to produce α- and β-arylalanines was observed as the principal route of amino group transfer. In addition, it was demonstrated that under steady-state conditions, an appreciable amount of the amino group is lost nonproductively, likely as NH_3, from the NH_2−MIO complex to reset *Tc*PAM for the next round of catalysis. This loss of the amino group was observed to occur prevalently when the exogenously supplied acrylate (amino group acceptor) bound the enzyme more weakly in the reaction mixture. With an improved understanding of the mechanism of *Tc*PAM and knowledge of how to employ surrogate substrates in examining cryptic aspects of the aminomutase chemistry and kinetics, it may become feasible to measure the rate at which the amino group is released from the enzyme complex.

In addition, bicyclic tetralin amino acids **16** (a bicyclic β-amino acid) and **17** (a bicyclic α-amino acid) were shown for the first time to function as surrogate substrates in the *Tc*PAM reaction, or in any MIO-dependent enzyme-catalyzed reaction, to the best of our knowledge. The product pools derived from substrates **16** and **17** were comprised exclusively of the same corresponding acrylate (**16-Acr**). This suggested that a so far unknown impediment of the reaction stalled the progress of $\alpha-\beta$ isomerization, such as the ring fusion of the substrates preventing access to a productive rotamer of the dihydronaphthalene intermediate. Moreover, the amino acids produced during the transamination reaction were made concurrently with significant (2*E*,4*E*)-styrylacrylate (**7**) derived from **6** at >99% de.[1] Thus, *Tc*PAM could be a tractable resource of conjugated dienic carbonyl derivatives such as **7** (and its analogues). Dienes of this type are typical structural subunits for the synthesis of natural products and useful as synthetic precursors.[14]

ASSOCIATED CONTENT

Supporting Information

Equations, NMR profiles, mass spectrometry profiles, and time course plots for the amino transfer reaction. This material is available free of charge via the Internet at http://pubs.acs.org.

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ABBREVIATIONS

AA1, hypothetical amino acid that serves as an amino group donor in the double-displacement reaction catalyzed by *Tc*PAM; AA2, hypothetical amino acid derived from the amination of an exogenously supplied arylacrylate in the double-displacement reaction catalyzed by *Tc*PAM; AC1, hypothetical arylacrylate derived from an amino acid after

elimination of ammonia in the double-displacement reaction catalyzed by *Tc*PAM; AC2, hypothetical exogenously supplied arylacrylate in the double-displacement reaction catalyzed by *Tc*PAM; GC−EI-MS, gas chromatography−electron impact mass spectrometry; MIO, 3,5-dihydro-5-methylidene-4*H*-imidazol-4-one; TAM, tyrosine aminomutase; *Tc*PAM, phenylalanine aminomutase from *T. canadensis*.

REFERENCES

(1) Feng, L., Wanninayake, U., Strom, S., Geiger, J., and Walker, K. D. (2011) Mechanistic, mutational, and structural evaluation of a *Taxus* phenylalanine aminomutase. *Biochemistry 50*, 2919−2930.

(2) Christianson, C. V., Montavon, T. J., Van Lanen, S. G., Shen, B., and Bruner, S. D. (2007) The structure of L-tyrosine 2,3-aminomutase from the C-1027 enediyne antitumor antibiotic biosynthetic pathway. *Biochemistry 46*, 7205−7214.

(3) Christenson, S. D., Wu, W., Spies, M. A., Shen, B., and Toney, M. D. (2003) Kinetic analysis of the 4-methylideneimidazole-5-one-containing tyrosine aminomutase in enediyne antitumor antibiotic C-1027 biosynthesis. *Biochemistry 42*, 12708−12718.

(4) Creighton, T. E. (1984) *Proteins. Structure and Molecular Principles*, 2nd ed., pp 515, W. H. Freeman and Co., New York.

(5) Szymanski, W., Wu, B., Weiner, B., de Wildeman, S., Feringa, B. L., and Janssen, D. B. (2009) Phenylalanine aminomutase-catalyzed addition of ammonia to substituted cinnamic acids: A route to enantiopure α- and β-amino acids. *J. Org. Chem. 74*, 9152−9157.

(6) Wu, B., Szymanski, W., Wietzes, P., de Wildeman, S., Poelarends, G. J., Feringa, B. L., and Janssen, D. B. (2009) Enzymatic synthesis of enantiopure α- and β-amino acids by phenylalanine aminomutase-catalysed amination of cinnamic acid derivatives. *ChemBioChem 10*, 338−344.

(7) Takac, S., Akay, B., and Ozdamar, T. H. (1995) Bioconversion of *trans*-cinnamic acid to L-phenylalanine by L-phenylalanine ammonia-lyase of *Rhodotorula glutinis*: Parameters and kinetics. *Enzyme Microb. Technol. 17*, 445−452.

(8) Cox, B. M., Bilsborrow, J. B., and Walker, K. D. (2009) Enhanced conversion of racemic α-arylalanines to (*R*)-β-arylalanines by coupled racemase/aminomutase catalysis. *J. Org. Chem. 74*, 6953−6959.

(9) Klettke, K. L., Sanyal, S., Mutatu, W., and Walker, K. D. (2007) β-Styryl- and β-aryl-β-alanine products of phenylalanine aminomutase catalysis. *J. Am. Chem. Soc. 129*, 6988−6989.

(10) Turner, N. J. (2011) Ammonia lyases and aminomutases as biocatalysts for the synthesis of α-amino and β-amino acids. *Curr. Opin. Chem. Biol. 15*, 234−240, (and references cited therein).

(11) Fersht, A. (1985) *Enzyme structure and mechanism*, 2nd ed., pp 475, W. H. Freeman and Co., New York.

(12) Christianson, C. V., Montavon, T. J., Festin, G. M., Cooke, H. A., Shen, B., and Bruner, S. D. (2007) The mechanism of MIO-based aminomutases in β-amino acid biosynthesis. *J. Am. Chem. Soc. 129*, 15744−15745.

(13) Ratnayake, N. D., Wanninayake, U., Geiger, J. H., and Walker, K. D. (2011) Stereochemistry and mechanism of a microbial phenylalanine aminomutase. *J. Am. Chem. Soc. 133*, 8531−8533.

(14) Lipshutz, B. H., Ghorai, S., and Boskovic, Z. V. (2008) Tandem olefin metathesis-elimination reactions. A new route to doubly unsaturated carbonyl derivatives. *Tetrahedron 64*, 6949−6954, (and references cited therein).