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Enzymatic Synthesis of Enantiopure α- and β-Amino Acids by Phenylalanine Aminomutase-Catalysed Amination of Cinnamic Acid Derivatives

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The phenylalanine aminomutase (PAM) from Taxus chinensis *catalyses the conversion of α-phenylalanine to β-phenylalanine, an important step in the biosynthesis of the* N*-benzoyl phenylisoserinoyl side-chain of the anticancer drug taxol. Mechanistic studies on PAM have suggested that (*E*)-cinnamic acid is an intermediate in the mutase reaction and that it can be released from the enzyme's active site. Here we describe a novel synthetic strategy that is based on the finding that ring-substituted (*E*)-cinnamic acids can serve as a substrate in PAM-catalysed ammonia addition reactions for the biocatalytic production of several important β-amino acids. The enzyme has a broad substrate range and a high enantioselectivity with cinnamic acid derivatives; this allows the synthesis of several non-natural aromatic α- and β-amino acids in excellent enantiomeric excess (*ee *> 99%). The internal 5-methylene-3,5-dihydroimidazol-4-one (MIO) cofactor is essential for the PAM-catalysed amination reactions. The regioselectivity of amination reactions was influenced by the nature of the ring substituent.*

Introduction

Over the past decades, the preparation of optically pure β-amino acids has received increasing attention because of their occurrence in a range of bioactive compounds and natural products.[1,2] This interest resulted in the development of several chemical methods for the preparation of these compounds, by employing either a chiral pool approach[3] or asymmetric protocols.[4–7] In contrast to the range of chemical methods that is available, biocatalytic routes towards β-amino acids are scarce and rely mostly upon the kinetic resolution of racemic derivatives.[8] Enzyme-catalysed asymmetric addition of ammonia to activated double bonds by ammonia lyases would be a very attractive synthetic strategy for the production of chiral β-amino acids. However, this strategy is limited by the narrow substrate range and strict regioselectivity (preference for ammonia addition to the α-position) of ammonia lyases.[9,10]

Phenylalanine aminomutase (PAM) from *Taxus chinensis* is a recently discovered enzyme that catalyses the conversion of α-phenylalanine to β-phenylalanine; this is the first committed step in the biosynthesis of the *N*-benzoyl phenylisoserinoyl side-chain of the anticancer drug taxol.[11] Unlike the aminomutases that require external cofactors,[12] PAM relies on a protein-derived cofactor, 5-methylene-3,5-dihydroimidazol-4-one (MIO), which is formed autocatalytically in the active site from the internal tripeptide Ala-Ser-Gly.[11,13] The MIO group is present in a family of sequence-related enzymes, including histidine ammonia lyase (HAL), phenylalanine ammonia lyase (PAL) and tyrosine aminomutase (TAM).[14] Since the initial discovery of MIO in the X-ray crystal structure of HAL,[15] the mechanistic role of this electrophile has been extensively investigated and debated. In the literature, two different mechanisms have been proposed for the lyase reaction, in which MIO either reacts with the amino group or with the aromatic ring of the α-amino acid substrate.[16] When applied to PAM, the first mechanism suggests the formation of an amine–MIO adduct that facilitates the deprotonation of the substrate at the β-position by an enzyme general base (Scheme 1, path A). Subsequently, the carbon–nitrogen bond is broken while the MIO–NH_2 bond and the α,β-unsaturated carboxylic acid are formed. In the second part of the reaction, the latter is aminated by MIO-NH_2 at the β-position. Alternatively, PAM follows a Friedel–Crafts-type reaction in which a σ-complex is formed by reaction of MIO with the phenyl ring of the substrate (Scheme 1, path B). The electron-deficient ring renders the β-hydrogen of the substrate acidic, thus facilitating its abstraction. The amino group is eliminated, yielding the α,β-unsaturated carboxylic acid, which undergoes ammonia addition at the β-position, after which the bond between MIO and substrate is broken. One important difference between the two mechanisms is that in the

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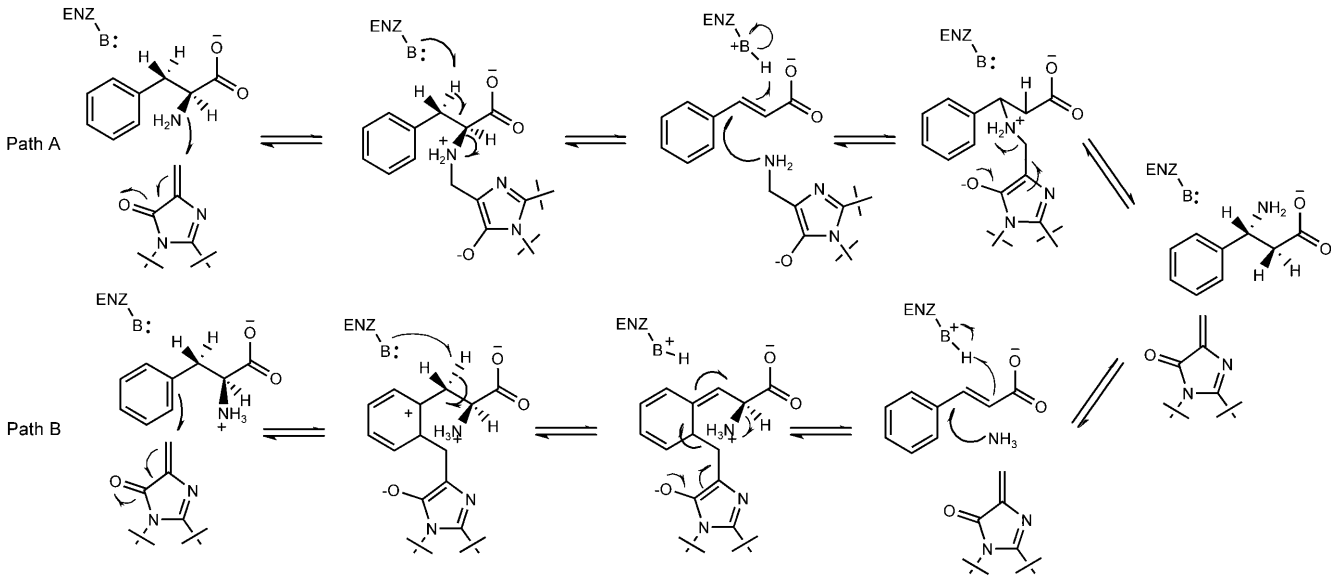


Scheme 1. Catalytic mechanisms that have been proposed for phenylalanine aminomutase (PAM).

reverse direction the nucleophile in path A (Scheme 1) is the MIO-bound amine, whereas free ammonia serves this function in path B. Earlier mechanistic studies on the role of MIO in the lyase reactions that are catalysed by PAL and HAL led to conflicting conclusions.[16–18] Recent elegant crystallographic experiments on the MIO-dependent tyrosine aminomutase have extended the knowledge of this enzyme family and shed light on the precise role of MIO.[19,20] TAM structures with covalently coupled substrate analogues were solved, and these strongly suggested that MIO reacts with the amino group of the substrate during the initial deamination step of the aminomutase reaction.[20]

The reversibility of the ammonia lyase reaction that is catalysed by MIO-dependent enzymes was recognised decades ago.[21] In the presence of a high concentration of ammonia, PAL[17,22] and HAL[23] can catalyse the α-addition of ammonia to aryl acrylic acids. In contrast to the ammonia lyases, aminomutases are expected to retain the α,β-unsaturated acid in their active sites and promote the re-addition of ammonia to the β-position. However, recent mechanistic studies on the aminomutases PAM and TAM revealed that 4-hydroxycinnamic acid (in the case of TAM)[24] and cinnamic acid (in the case of PAM)[13] are intermediates in the aminomutase reaction that can be released from the enzyme active site. These observations indicate that TAM and PAM might exhibit ammonia lyase activity and suggest the possibility of using the second half of the aminomutase reaction to synthesise β-amino acids from cinnamates. In the present paper, we describe the enantioselective synthesis of α- and β-phenylalanine derivatives by the PAM-catalysed amination of substituted cinnamic acids.

Results

Expression, purification and functional characterisation of PAM

The PAM gene, which encodes a protein of 687 amino acid residues with a calculated molecular mass of 78 kDa, was synthesised. The encoded amino acid sequence is identical to *T. chinensis* PAM (ExPASy ID: Q68G83_TAXCH), but the codon usage was optimised for *E. coli*. The PAM gene was cloned in the pBAD-His expression plasmid, and the recombinant enzyme was expressed upon induction with arabinose in *E. coli* TOP10 as an N-terminal hexahistidine fusion protein. Wild-type PAM was purified by using a Ni-based immobilised metal affinity chromatography procedure. SDS-PAGE analysis with Coomassie staining showed that the purity of the protein was more than 95%, and the apparent molecular mass was consistent with the calculated mass. The yield of purified protein from 1 liter of cell culture was 5–7 mg.

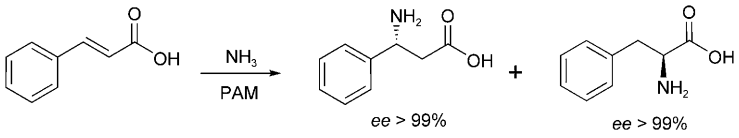
The mutase activity of the recombinant PAM was monitored by HPLC assays, and the absolute configurations of the products from the mutase reaction were assigned by chiral HPLC analysis. PAM specifically converted (*S*)-α-phenylalanine to (*R*)-β-phenylalanine (ee > 99%) according to Michaelis–Menten kinetics with a k_{cat} value of $0.013 \pm 0.0004\ s^{-1}$ and a K_m of $34 \pm 3\ \mu M$. The enzyme showed no detectable activity with (*R*)-α-phenylalanine as the substrate. The catalytic efficiency is similar to that of other MIO-dependent aminomutases (Table 1). The reverse mutase reaction was also studied. PAM exhibited high enantioselectivity when *rac*-β-phenylalanine was used as a substrate; only (*R*)-β-phenylalanine was isomerised to (*S*)-α-phenylalanine (ee > 99%). Furthermore, HPLC analysis showed that (*E*)-cinnamic acid was formed and released from the enzyme in the initial stage of the mutase reaction both with (*S*)-α-phenylalanine and (*R*)-β-phenylalanine as the substrate.

Table 1. Kinetic parameters for the mutase activity of *T. chinensis* PAM and two other aminomutases.

Organism	Enzyme	Substrate	K_m [mM]	k_{cat} [s^{-1}] $\times 10^3$	k_{cat}/K_m [$mM^{-1}s^{-1}$] $\times 10^3$	Ref.
T. chinensis	PAM	(*S*)-α-phenylalanine	34 ± 3	13 ± 0.4	380 ± 10	this work
T. cuspidata	PAM	(*S*)-α-phenylalanine	45 ± 0.8	15	440	[13]
St. globisporus	TAM	(*S*)-α-tyrosine	28 ± 2	10 ± 1	360 ± 44	[24]

Ammonia addition reaction and product identification

The formation of (*E*)-cinnamic acid as an intermediate or side product in the mutase reaction and its release from the enzyme active site prompted us to examine if (*E*)-cinnamic acid can be used as a substrate in a PAM-catalysed amination reaction. The kinetic assay was performed at pH 10 in the presence of a high concentration of ammonia (6 M) by following the decrease in absorbance at 290 nm, which results from the amination of (*E*)-cinnamic acid. Interestingly, we observed a small but significant activity of PAM with (*E*)-cinnamic acid. A k_{cat} of 0.024 s^{-1} and a K_m of 1.8 mM were found, which results in a k_{cat}/K_m of ~0.013 $mM^{-1}s^{-1}$ (Table 2). Control experiments showed that ammonia does not react with (*E*)-cinnamic acid in the absence of PAM.



Scheme 2. Synthesis of enantiomerically pure (*S*)-α-phenylalanine and (*R*)-β-phenylalanine by using PAM.

Mixtures containing PAM, ammonia and (*E*)-cinnamic acid were incubated, and samples were analysed by LC–MS to identify the products of the amination reaction. Two products were observed and identified as α-phenylalanine and β-phenylalanine, based both on retention time comparison and the detection of fragment ions with masses identical to those that were found with authentic standards. A comparison of the HPLC spectra that were obtained with samples from various times during conversion revealed that both α-phenylalanine and β-phenylalanine emerged simultaneously in the initial stage of the ammonia addition reaction at an α/β ratio of 51:49 (Table 2). The stereochemistry of the products was assigned by using chiral HPLC, with authentic samples for comparison. This analysis showed that the absolute configuration of the products was *S* for α-phenylalanine and *R* for β-phenylalanine. The *ee* values that were obtained with both products were higher than 99% (Scheme 2).

(*Z*)-Cinnamic acid was tested as a potential substrate or inhibitor of PAM. This compound was not processed by the enzyme. Furthermore, (*Z*)-cinnamic acid (1 mM) caused no inhibition of the amination activity of PAM when (*E*)-cinnamic acid (1 mM) was the substrate.

Role of MIO in the ammonia addition reaction

It has been shown that the internal MIO cofactor is essential for the mutase activity of PAM.[13] This observation prompted us to examine whether this cofactor is important for the β-ammonia addition activity, which can be considered as the second half reaction of the aminomutase. The hallmarks of the MIO-based mechanism are loss of catalytic activity when the internal cofactor is disrupted by site-directed mutagenesis and inactivation of the enzyme in the presence of cyanide.

To determine whether cyanide, which is assumed to react with the activated double bond of MIO, can cause inactivation of the amination activity of PAM,[13] the enzyme was treated with KCN (2 mM, 30 min) and the excess reagent was removed by ultrafiltration. When PAM was treated with KCN in this way, amination activity was almost completely lost (only 2% of the original activity was remaining). This result is indicative of covalent modification at the active site. Binding at the active site is further indicated by the observation that the substrate, either 5 mM (*E*)-cinnamic acid or 6 M NH_3, partially protects the enzyme against the inactivation by KCN (90% and 80%, respectively).

The second hallmark of the MIO-based mechanism, the formation of inactive enzyme after disruption of the cofactor by mutagenesis, was investigated by replacing Ser176, which is

Table 2. Kinetic parameters and regio- and enantioselectivity of PAM-catalysed amination reactions.

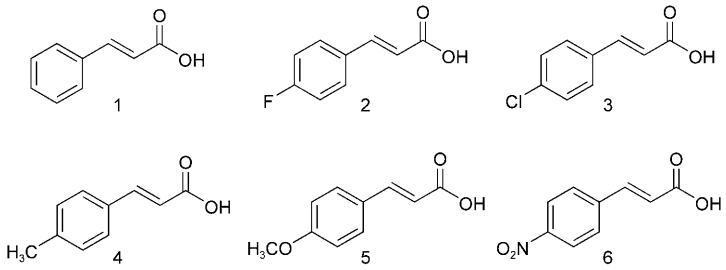
Substrate	k_{cat} [s^{-1}] $\times 10^3$	K_m [mM]	k_{cat}/K_m [$s^{-1}mM^{-1}$] $\times 10^3$	V_{rel}[a]	α [%]	ee_α[b]	ee_β[c]
1	24 ± 1	1.8 ± 0.1	13 ± 1	1.0	51	>99%	>99%
2	37 ± 1	2.5 ± 0.2	15 ± 2	1.5	35	>99%	>99%
3	46 ± 1	0.38 ± 0.02	115 ± 9	1.9	41	>99%	>99%
4	35 ± 1	0.89 ± 0.06	39 ± 4	1.4	4	>99%	>99%
5	27 ± 1	0.79 ± 0.04	34 ± 3	1.1	14	>99%	>99%
6	133 ± 1	9.9 ± 0.6	13 ± 1	5.6	98	>99%	–

[a] V_{rel} is the ratio between the k_{cat} for a particular substrate and the k_{cat} for cinnamic acid. [b] The (*S*)-α-phenylalanines were formed. [c] The (*R*)-β-phenylalanines were formed.

part of the Ala-Ser-Gly motif involved in the autocatalytic formation of MIO, with alanine. The S176A mutant was expressed and purified to homogeneity according to the procedures that were used for wild-type PAM. The structural integrity of the S176A mutant enzyme was assessed by circular dichroism, and the CD spectrum of this mutant was similar to that of wild-type PAM (data not shown); this indicates that the mutation did not result in any major conformational change. The activity of the S176A enzyme was tested in both the deamination (by using (*R*)-β-phenylalanine or 4-NO_2-α-phenylalanine as the substrate) and the amination (by using (*E*)-cinnamic acid or (*E*)-4-nitrocinnamic acid and ammonia as the substrates) directions. It was found that replacement of Ser176 with an alanine abolished all enzymatic activity. These observations provide support for an important catalytic role of MIO not only in deamination but also in the amination activity of PAM.

Substrate scope

After establishing that PAM can catalyse the addition of ammonia to (*E*)-cinnamic acid with high stereoselectivity, several (*E*)-cinnamic acid derivatives were tested as potential substrates. The results show that PAM converts (*E*)-4-fluorocinnamic acid, (*E*)-4-methylcinnamic acid, (*E*)-4-methoxycinnamic acid, (*E*)-4-nitrocinnamic acid and (*E*)-4-chlorocinnamic acid to amino acids (Scheme 3). A comparison of the kinetic parameters that were obtained with these compounds indicates that the turnover numbers (k_{cat}) for the five substituted (*E*)-cinnamic acids are somewhat higher than that observed with (*E*)-cinnamic acid, regardless of the electronic property of the substituent on the aromatic ring of the substrate (Table 2). In the absence of the enzyme, no addition of ammonia to these compounds was observed. Under identical assay conditions, (*E*)-4-hydroxycinnamic acid, (*E*)-3-hydroxycinnamic acid, (*E*)-3,4-dihydroxycinnamic acid, (*E*)-3-methoxycinnamic acid, (*E*)-2-methoxycinnamic acid, (*E*)-α-methylcinnamic acid and (*E*)-β-methylcinnamic acid did not serve as substrates for PAM.



Scheme 3. Chemical structures of compounds that can serve as substrates for PAM-catalysed amination reactions.

The products of these ammonia addition reactions were analysed by LC–MS. From the retention time and the observed fragment ions, substituted α- and β-phenylalanines were identified as the amination products of the corresponding substituted cinnamic acids. Similar to what was observed with (*E*)-cinnamic acid as the substrate, both α- and β-amino acids were formed in the initial stage of the reaction. Interestingly, the strongly electron-withdrawing nitro group promoted ammonia addition to the α-position, but the electron-donating methyl and methoxy group shifted the regioselectivity to the β-position. Chiral HPLC analysis showed that the enantioselectivity (all *ee* values $>99\%$) of the PAM-catalysed ammonia addition reactions was not influenced by the substituents on the aromatic ring. Hence, enantiopure (*S*)-α-amino acids and (*R*)-β-amino acids were identified as the sole products of the PAM-catalysed amination reactions.

Discussion

The work reported here provides an enzymatic system for the synthesis of enantiopure aromatic β-amino acids by ammonia addition to the double bond of cinnamic acid and its ring-substituted derivatives. The use of a reverse ammonia lyase reaction to synthesise aromatic α-amino acids has been recognised and investigated for many years. For instance, PAL from *Rhodotorula graminis* is regarded as a valuable biocatalyst for the enantioselective synthesis of (*S*)-α-phenylalanine and various substituted aryl α-alanines.[25] Although some of the MIO-containing ammonia lyases accept a broad range of substrates, only α-amino acids are formed by PAL and related enzymes. Other classes of enzymes that can catalyse ammonia addition to double bonds are the L-aspartate ammonia lyases (aspartases) and 3-methylaspartate ammonia lyases, which belong to the class II fumarase superfamily and the enolase superfamily of proteins, respectively. However, the substrate range of these enzymes is very restricted, because both carboxylates in the substrate molecule are essential for catalytic activity, which limits their application scope to formation of substituted aspartic acids.[9] The possibility to use PAM for ammonia addition to aromatic acrylic acids with formation of β-amino acids thus widely expands the usefulness of biocatalytic ammonia addition reactions.

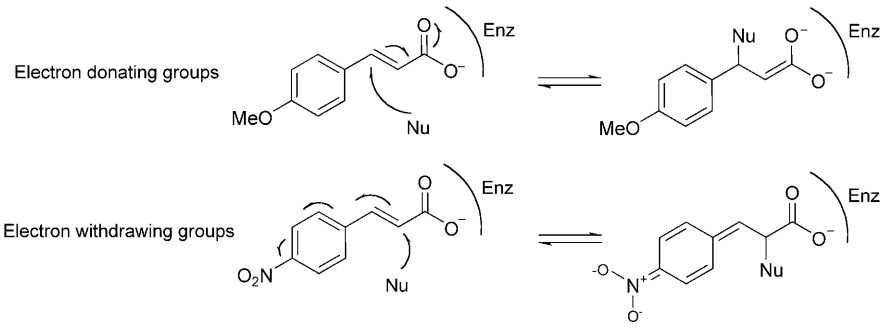
The difference between the PAM from *Taxus chinensis* described here and other MIO-dependent mutases is the high enantioselectivity towards β-amino acids exhibited by the *T. chinensis* enzyme. Previous work showed that TAM possesses β-tyrosine racemase activity and can catalyse the reversible conversion of (*S*)-β-tyrosine to (*R*)-β-tyrosine.[24] Similarly, PAM from *Taxus cuspidata* converted both enantiomers of β-phenylalanine to (*S*)-α-phenylalanine, and (*S*)-β-phenylalanine was found to be an even better substrate for the enzyme than (*R*)-β-phenylalanine, which is the natural product of the PAM reaction.[13] In contrast, the recombinant PAM from *T. chinensis* that is described here showed an extremely high enantioselectivity and only (*R*)-β-phenylalanine was accepted as a substrate or synthesised from the cinnamic acid by this enzyme. A comparison of amino acid sequences reveals that the only difference between PAM from *T. chinensis* and the corresponding enzyme from *T. cuspidata* is an eleven-residue segment at the C terminus that is missing in *T. chinensis* PAM. The corresponding C-terminal segment is also present in PAL, in

which it was hypothesised to be important for activity.[26] Whether the lack of acceptance of (*S*)-β-enantiomers by *T. chinensis* PAM is a consequence of the C-terminal truncation is presently unknown.

The catalytic mechanism of MIO-containing enzymes and the precise mechanistic role of the cofactor have been debated for years (see Introduction). The Friedel–Crafts mechanism, in which MIO reacts with the aromatic ring of the substrate (path B in Scheme 1 A), predicts that the MIO cofactor is essential for ammonia elimination, but, if applied to PAM, it is not essential for ammonia addition to the β-position. Interestingly, we found that when MIO is disrupted in *T. chinensis* PAM by chemical or genetic modification, the resulting enzyme is inactive both in ammonia elimination and addition reactions. This observation suggests that MIO is a key component for ammonia elimination from and addition to both the α- and β-positions of the substrate; this is in agreement with a mechanism in which MIO accepts and transfers the amine group in the aminomutase reaction (path A in Scheme 1 A). Strong evidence for this latter mechanism comes from X-ray crystallographic work on TAM that had been inactivated by tyrosine analogues in which the α-hydrogens are substituted with fluorine atoms.[20,27] The use of these compounds, which cannot undergo ammonia elimination resulted in the formation of covalent adducts between MIO and the amino group of the analogues. This suggests that MIO might be coupled with the amino group of the substrate in the initial stage of the aminomutase reaction.[20]

Studies on the overall stereochemical course of the *T. cuspidata* PAM reaction revealed that the enzyme shuttles both the amino group (from C2 of the substrate to C3 of the product) and the *pro*-3*S* hydrogen (to C2 of the β-isomer product) with retention of configuration. To explain the retention of configuration at both reaction centres, Walker and co-workers proposed that the substrate might bind in the PAM active site with the carboxylate group and the phenyl ring arranged in a *syn*-periplanar orientation, which positions the migrating NH_3 and hydrogen groups on the same side of the substrate molecule.[28] This orientation could be consistent with (*Z*)-cinnamic acid as an intermediate. Based on this assumption, we expected (*Z*)-cinnamic acid to be a substrate for the ammonia addition reaction. However, we observed that (*Z*)-cinnamic acid is neither a substrate nor an inhibitor of *T. chinensis* PAM, which suggests either that this compound can not enter the active site of the enzyme or that it is not a real intermediate in the PAM reaction, in which case the stereochemistry that is observed might be related to other factors, such as a substrate distortion or a rotation of the cinnamates relative to the general base expected to be present in the active centre.

PAM accepts a series of *para*-substitutions on the aromatic ring of the cinnamic acids that undergo amination (Table 2). Similar to the result of the kinetic study of different substrates in PAM-catalysed mutase reactions,[29] there was no correlation between the reaction rate and the electronic properties of the substituents. However, we observed a clear effect of the nature of the ring substituent on the regioselectivity of the addition reaction (Table 2, 6th column); this suggests that electronic effects dominate the regioselectivity. If both the carboxylate and aromatic ring that are attached to the substrate's olefinic group can act as an electron sink in a conjugate addition of ammonia, the tendency of the β- versus the α-carbon of the substrate to undergo this addition will be influenced by the substituents that are present at the aromatic ring. Indeed, cinnamic acids that posses electron-donating groups (Table 2, entries 4 and 5) are converted predominantly to β-amino acids, which can be explained by a lowered ability of the aromatic



Scheme 4. The electronic effect of aromatic ring substituents on the regioselectivity of PAM-catalysed amination reaction.

system to accept electrons (Scheme 4, upper part). On the other hand, the cinnamic acid derivative with the strongly electron-withdrawing nitro group (Table 2, entry 6) is transformed almost exclusively to its respective α-amino acid, which suggests that the electron-poor aromatic ring acts as an electron sink in this case (Scheme 4, lower part). Deviations from this trend might be due to steric or electrostatic effects that cause substituents at the *para*-position to cause different interactions between substrate and enzyme and influence the geometry of a Michaelis complex that undergoes amination, thereby influencing the α/β ratio.

In conclusion, we have shown that enantiopure (*S*)-α-phenylalanines and (*R*)-β-phenylalanines can be synthesised by PAM-catalysed ammonia addition reactions with cinnamic acids as substrates. To our knowledge, this is the first reported enzymatic synthesis of aromatic β-amino acids that uses a one-step ammonia addition reaction. We observed that the substrate scope of PAM is rather broad, which makes this enzyme an attractive candidate for the biocatalytic synthesis of non-natural aromatic β-amino acids. Challenging targets for optimisation are an enhanced catalytic rate and an increased selectivity towards β-addition.

Experimental Section

Materials: The compounds (±)-α-phenylalanine, (±)-β-phenylalanine, (*E*)-cinnamic acid, (*E*)-4-chlorocinnamic acid, (*E*)-4-fluorocinnamic acid, (*E*)-4-methylcinnamic acid, (*E*)-4-hydroxycinnamic acid were purchased from Acros organics. (*R*)-α-phenylalanine, (*S*)-α-phenylalanine, (*E*)-4-methoxycinnamic acid, (*E*)-4-nitrocinnamic acid, (*S*)-4-nitro-α-phenylalanine and (±)-4-nitro-α-phenylalanine were obtained from Sigma–Aldrich–Fluka. (*R*)-β-phenylalanine, (*S*)-β-phenylalanine, (*S*)-3-amino-3-(4-fluorophenyl)propionic acid, (*R*)-3-amino-3-(4-fluorophenyl)propionic acid, (*S*)-3-amino-3-(4-chlorophenyl)propionic acid, (*R*)-3-amino-3-(4-chlorophenyl)propionic acid, (*S*)-3-amino-3-(4-methylphenyl)propionic acid, (*R*)-3-amino-3-(4-methylphenyl)propionic acid, (*S*)-3-amino-3-(4-methoxyphenyl)-propionic acid, (*R*)-3-amino-3-(4-methoxyphenyl)propionic acid, (*S*)-4-fluoro-α-phenylalanine, (*R*)-4-fluoro-α-phenylalanine, (*S*)-4-chloro-α-phenylalanine, (*R*)-4-chloro-α-phenylalanine, (*S*)-4-methyl-α-phenylalanine, (*R*)-4-methyl-α-phenylalanine, (*S*)-4-methoxy-α-phenylalanine and (*R*)-4-methoxy-α-phenylalanine were synthesised by Peptech Corp (Burlington, USA). (±)-β-4-Nitrophenylalanine[30] and (*Z*)-cinnamic acid[31] were prepared according to literature procedures.

Construction of the PAM expression vector: The PAM gene (*T. chinensis*) with optimised codon usage for *E. coli* was synthesised by DNA 2.0 Inc (Menlo Park, USA) and provided as an insert that was cloned in the vector pJ-36. The PAM gene was excised from the provided pJ-36 vector by NdeI and HindIII digestion, separated from the vector DNA by agarose gel electrophoresis and purified by using the Qiagen gel extraction kit. The gene was then ligated into NdeI/HindIII-digested pBAD-His vector (Invitrogen). The PAM gene was expressed in *E. coli* TOP10 from the arabinose promoter, resulting in the production of an N-terminal hexahistidine fusion protein.

Plasmid preparation, PCR purification and DNA extraction were carried out by using commercial kits (Qiagen). Restriction enzymes were purchased from New England Biolabs. Phusion™ high-fidelity polymerase was purchased from Finnzymes (Espoo, Finland). The Quick-ligation kit was purchased from Roche Applied Science.

Construction of the S176A PAM mutant: The S176A mutant of PAM was generated by using the megaprimer method and the coding sequence for PAM in plasmid pBAD-His-PAM as the template. In the first stage of PCR, the megaprimer was made by using the primer 5′-CG TGG TTC TGT TTC TGC AGC CGG CGA TCT GAT TCC-3′, with the mutated codon underlined, and the primer 5′-AAC GGT AGA CGC AAC TGC AGT AG-3′ (designated PstI reverse primer). In the second stage of PCR, this mega-primer was used in combination with primer 5′-CGC GCG GCA GCC ATA TGG GTT TC-3′ (designated NdeI forward primer) to amplify the full-length mutant gene. When the second PCR was finished, DpnI (10 units) was added directly to the PCR mixture and the mixture was incubated at 37 °C for 2 h to remove the template DNA. The final PCR product was purified, digested with NdeI and PstI, and ligated into the NdeI/PstI-treated pBAD-His vector. The ligation mixtures were transformed into competent *E. coli* TOP10 cells and transformants were plated on LB plates with ampicillin. All mutant constructs were confirmed by sequencing the region between the NdeI and PstI sites. All primers were supplied by Sigma–Aldrich.

Expression and purification of PAM wild-type and the S176A mutant: The PAM enzyme, either wild-type or mutant, was produced in *E. coli* TOP10 by using the pBAD expression system (Invitrogen). Fresh TOP10 cells that contained the appropriate expression plasmid were used to inoculate LB medium (10 mL) containing ampicillin (50 μg mL^{-1}, LB/Amp medium). After overnight growth at 37 °C, the culture (10 mL) was used to inoculate LB/Amp medium (1 L). Cultures were grown at 37 °C to an OD_{600} of 0.5 with vigorous shaking and then induced with arabinose (0.002% (*w*/*v*) final concentration). Incubation was continued for 20 h at 17 °C. Cells were harvested by centrifugation at 5000 *g* for 20 min, washed with buffer A (50 mL, 20 mM Tris–HCl buffer, pH 8.0, 20 mM imidazole, 0.5 M NaCl) and suspended in the same buffer (50 mL). Cells were disrupted by sonication, after which unbroken cells and debris were removed by centrifugation (15000 *g*, 60 min). All steps were performed at 4 °C. PAM was purified by Ni-based immobilised metal affinity chromatography by using an AKTA system (Amersham). After washing with 20 mM and 60 mM imidazole, the bound protein was eluted with 200 mM imidazole. Fractions that contained PAM were desalted and concentrated to 8 mg mL^{-1} in storage buffer (20 mM Tris–HCl, 50 mM NaCl, 2 mM EDTA, 1 mM DTT, 25% glycerol, pH 8.0). The purified enzyme was flash-frozen in liquid N_2 and stored at −80 °C until further use. The purity of the protein was analysed by SDS-PAGE and the protein concentration was determined by the Bradford assay.

Determination of aminomutase activity: The mutase activity of PAM was determined by monitoring the production of β-phenylalanine upon incubation with α-phenylalanine. Incubation mixtures contained α-phenylalanine (0.02 mM to 0.2 mM), phosphate buffer (50 mM, pH 8.0), and purified PAM (0.08 mg). At different times, samples (34 μL) were taken from the reaction mixture and quenched by adding HCl (2 M, 18 μL) with cooling on ice for 5 min. The pH was readjusted to 8.0 by adding small aliquots of an aq KOH solution. The reaction mixture was directly loaded onto the HPLC system.

An Alltech (Lexington, USA) C18 3u column (3 μm, 100 mm × 4.6 mm) was used for analytical separation of α- and β-phenylalanine. The solvents were phosphate buffer (50 mM, pH 2.7) (eluent A) and HPLC pure CH_3CN (eluent B). The flow rate was 1 mL min^{-1}. The elution gradient was formed as follows: start with 100:0 for 10 min, in 5 min from 100:0 to 70:30, and from 70:30 to 100:0 from 20 min to 28 min. The analyses were carried out at 20 °C, with detection at 210 nm. The initial rates were plotted against the substrate concentrations and fitted to the Michaelis–Menten equation to obtain the kinetic parameters.

Determination of kinetic parameters of PAM by UV spectroscopy: NH_3 addition reactions were performed as follows. A 6 M NH_3 solution was prepared and the pH was adjusted to 10 by bubbling CO_2 into the solution. (*E*)-Cinnamic acid or a derivative was mixed at various concentrations with purified PAM (0.06 mg) in NH_3 solution (6 M, 300 μL). The reaction mixture was incubated at 30 °C. Conversion was followed by UV spectroscopy, monitoring the depletion of substrate absorbance at appropriate wavelengths [(*E*)-cinnamic acid: $\varepsilon_{290}=10000$ L cm^{-1} mol^{-1}, $\varepsilon_{298}=5100$ L cm^{-1} mol^{-1}, $\varepsilon_{302}=2600$ L cm^{-1} mol^{-1}; (*E*)-4-fluorocinnamic acid: $\varepsilon_{290}=10500$ L cm^{-1} mol^{-1}, $\varepsilon_{304}=2500$ L cm^{-1} mol^{-1}; (*E*)-4-chlorocinnamic acid: $\varepsilon_{301}=10200$ L cm^{-1} mol^{-1}, $\varepsilon_{306}=5500$ L cm^{-1} mol^{-1}; (*E*)-4-methylcinnamic acid: $\varepsilon_{301}=11000$ L cm^{-1} mol^{-1}, $\varepsilon_{308}=5000$ L cm^{-1} mol^{-1}; (*E*)-4-methoxycinnamic acid: $\varepsilon_{315}=10500$ L cm^{-1} mol^{-1}, $\varepsilon_{322}=7500$ L cm^{-1} mol^{-1}, $\varepsilon_{327}=2500$ L cm^{-1} mol^{-1}; (*E*)-4-nitrocinnamic acid: $\varepsilon_{375}=1000$ L cm^{-1} mol^{-1}, $\varepsilon_{383}=500$ L cm^{-1} mol^{-1}]. The initial rates were fitted to the substrate concentrations with the Michaelis–Menten equation to obtain the kinetic constants.

Product identification by LC–MS: Reaction mixtures contained (*E*)-cinnamic acid (5 mM) or a derivative, NH_3 (6 M, pH 10), and PAM (0.2 mg mL^{-1}). Reactions were initiated by the addition of enzyme,

incubated at 30 °C, and quenched by heating for 5 min at 99 °C. The reaction mixtures were directly loaded onto an Alltech C18 3u column (3 μm, 100 mm × 4.6 mm). LC–MS/MS analysis was performed by using an LC–MS ion-trapping system (Thermo Scientific, Waltham, USA). The solvent system consisted of 0.01 % formic acid (eluent A) and 0.07 % formic acid in 30 % CH_3CN (eluent B). The elution gradient was formed as follows: start with 100:0 for 10 min, in 5 min from 100:0 to 0:100, and from 0:100 to 100:0 from 20 min to 28 min. The analyses were carried out at 20 °C, with concomitant UV detection at 260 nm.

Analysis of the phenylalanine products by chiral HPLC: Purified PAM (0.02 mg) was added to (*E*)-cinnamic acid (5 mM) or a derivative in 6 M NH_3 solution (pH 10, 200 μL). The reaction mixture was incubated for 24 h at 30 °C. Subsequently, a portion (20 μL) was taken and the reaction was quenched by heating for 5 min at 99 °C. A portion of 2 M NaOH (40 μL) was added to remove the excess of NH_3 . The sample was then frozen in liquid N_2, lyophilised and dissolved in $HClO_4$ (2 M, 55 μL). Analysis was carried out on a Crownpak CR(+) (4 mm × 150 mm) column. Compounds were eluted isocratically with $HClO_4$ containing MeOH (15 %, pH 2.5) with UV detection at 210 nm. The flow rate was 0.3 mL min^{-1} and the temperature of the column was maintained at −5 °C.

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