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Phenylalanine Aminomutase-Catalyzed Addition of Ammonia to Substituted Cinnamic Acids: a Route to Enantiopure α- and β-Amino Acids

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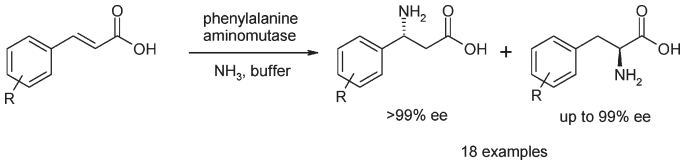

An approach is described for the synthesis of aromatic α- and β-amino acids that uses phenylalanine aminomutase to catalyze a highly enantioselective addition of ammonia to substituted cinnamic acids. The reaction has a broad scope and yields substituted α- and β-phenylalanines with excellent enantiomeric excess. The regioselectivity of the conversion is determined by substituents present at the aromatic ring. A box model for the enzyme active site is proposed, derived from the influence of the hydrophobicity of substituents on the enzyme affinity toward various substrates.

Introduction

There is a large and growing interest in the synthesis and application of optically pure β-amino acids and β-peptides.[1,2] The importance of these compounds resides mainly in their pharmaceutical potential.[3] The unique features of β-peptides, when compared to their α-analogues, are increased metabolic stability,[4] higher structural diversity,[5] and formation of well-defined secondary structures.[6] Because of these features, β-peptides are important peptidomimetics with various bioactivities.[7] β-Amino acids are components of many bioactive natural and synthetic products, such as the antitumor agents Taxol[8] and cryptophycin 1,[9] the antibiotic Adda,[10] the insecticidal and antifungal agent jasplakinolide,[11] and the aminopeptidase inhibitor bestatin.[12] Synthetic β-amino acids are also precursors of bioactive β-lactams.[13]

Because of the increasing demand for β-amino acids, different synthetic routes have been explored.[14−18] Biocatalytic routes toward β-amino acids, although offering certain advantages, are mostly limited to kinetic resolutions of racemic precursors,[19] including esters,[20] nitriles,[21] amides,[22]

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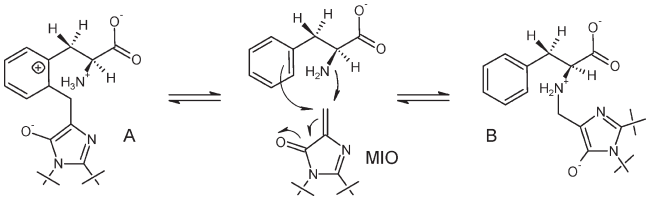

FIGURE 1. Initial stages of two proposed catalytic mechanisms of PAM.

β-lactams,[23] and dihydrouracils.[24] Methods which are free of the typical limitations connected with kinetic resolutions, such as low efficiency and purification issues, are scarce and rely on the use of aspartases[25] and aminotransferases.[26] The use of an aminomutase for the partial biotransformation of α-phenylalanine and its derivatives into their respective β-isomers has recently been described.[27]

Phenylalanine aminomutase (PAM) from *Taxus chinensis* is a recently discovered enzyme that catalyzes the conversion of α-phenylalanine to β-phenylalanine in the biochemical route leading to the side chain of Taxol.[28] Unlike the aminomutases that require external cofactors,[29] the activity of PAM depends on a protein-derived internal cofactor, 5-methylene-3,5-dihydroimidazol-4-one (MIO, Figure 1).

Two different catalytic mechanisms have been proposed for PAM-catalyzed amination (Figure 1), in which either the aromatic ring (Figure 1, A)[30] or the amino group (Figure 1, B)[31] of the α-amino acid adds to MIO. In both cases, the elimination of ammonia from the MIO adduct is facilitated, and (*E*)-cinnamic acid is the product of this transformation. Recently published crystal structures of a related enzyme, tyrosine aminomutase (TAM), with covalently bound inhibitors present in the active site,[31] together with our observations that PAM-catalyzed readdition of ammonia to cinnamic acid in the second step of the PAM reaction is MIO-dependent,[32] support the hypothesis that the MIO-adduct is formed by a reaction with the amine group of the substrate (Figure 1, B).

It has been confirmed experimentally that both (*E*)-cinnamic acid and β-phenylalanine are formed during the PAM-catalyzed conversion of α-phenylalanine.[32] This observation indicates that PAM exhibits ammonia lyase activity and encourages the exploration of the reverse lyase reaction to synthesize α- and β-amino acids (**2** and **3**, respectively) (Scheme 1). Recently, we have observed that PAM may catalyze the addition of ammonia to substituted (*E*)-cinnamic acids.[32] Here we describe the factors that govern the kinetic parameters and selectivity of PAM-catalyzed reactions, demonstrating that a broad range of enantiopure α- and β-amino acids can be produced and defining the substrate scope of this novel biocatalytic system.

Results and Discussion

Phenylalanine aminomutase (PAM) from *Taxus chinensis* was obtained as described in our previous communication[32] with high purity (>95% as judged by SDS-PAGE with Coomassie staining).

In order to determine the substrate scope of PAM, a series of substituted cinnamic acids was prepared in very good yields (usually >90%) by the Knoevenagel−Doebner procedure.[33] The obtained library of compounds was tested in the enzyme-catalyzed addition of ammonia to the activated olefinic bond (Scheme 1). For every substrate, the kinetic parameters of the reaction (k_{cat} and K_M) were determined in order to estimate the structural features that influence the affinity and catalytic rate. The ratio of the initial rates of formation of the isomers **2** and **3** (α/β ratio, Scheme 1) was also determined, aiming at understanding the factors that govern the regioselectivity of the enzymatic reaction. Finally, the enantiomeric excess of the products was determined.

Addition of ammonia to cinnamic acid (Table 1, entry 1) yielded a 1:1 mixture of α- and β-phenylalanine, with excellent enantioselectivity (>99% ee for both isomers). For compounds **1f** (Table 1, entry 6) and **1g** (Table 1, entry 7), no detectable activity was observed. Substrates with fluoro, chloro, bromo, and methyl substituents are accepted by PAM (Table 1, entries 2−5), but the presence of any of these substituents at the *ortho* position results in almost exclusive formation of α-phenylalanine analogues. This phenomenon is likely to be caused by steric shielding of the β-position. The affinity of PAM toward these substrates seems to be influenced by the lipophilicity of the substituent, as the lowest K_M value (corresponding to the highest affinity) was observed for the bromo-substituted substrate, while for the compound with the most hydrophilic substituent (fluoro) the affinity is the lowest. This observation suggests the existence of hydrophobic side chains of amino acids in the active site of PAM around the *ortho* positions of the substrate.

The PAM-catalyzed addition of ammonia to *meta*-substituted cinnamic acids yields a mixture of α- and β-amino acids. In case of halogen substituents (Table 2, entries 1−3) the α isomer **2** is dominating, while the use of 3-methylcinnamic acid (**1k**) as a substrate leads to preferential formation of 3-methyl-β-phenylalanine (**3k**). A possible explanation of this effect of the ring substituent on the regioselectivity is provided in the section on *para*-substituted cinnamic acids (*vide infra*). For 3-methoxycinnamic acid (Table 2, entry 5) and 3-hydroxycinnamic acid (Table 2, entry 6) no detectable enzymatic activity was observed. Due to the limited data obtained it is difficult to draw conclusions on the factors

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SCHEME 1. PAM-Catalyzed Addition of Ammonia to Substituted Cinnamic Acids

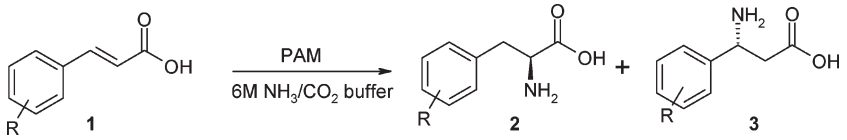
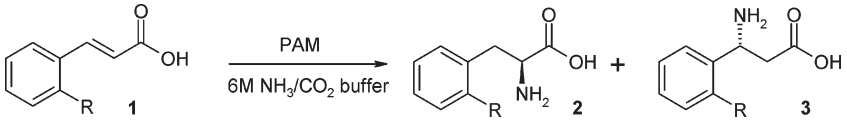


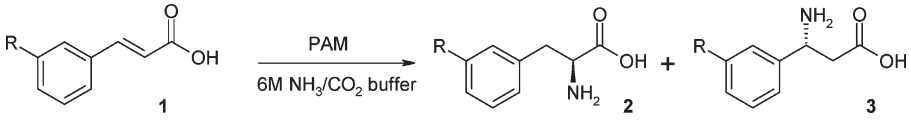
TABLE 1. PAM-Catalyzed Addition of Ammonia to *Ortho*-Substituted Cinnamic Acids[a]



entry	1	R	k_{cat} (s^{-1}) × 10^3	K_M (mM)	k_{cat}/K_M ($s^{-1} \times mM^{-1}$) × 10^3	initial **2**:**3** ratio	ee of **2**[b] (%)	ee of **3**[b] (%)
1	**1a**	H	24 ± 1	1.8 ± 0.1	13 ± 1	51:49	> 99 (*S*)	> 99 (*R*)
2	**1b**	F	226 ± 11	13 ± 1	17 ± 2	98:2	> 99 (*S*)	nd
3	**1c**	Cl	359 ± 20	8.6 ± 1.0	42 ± 7	> 99:1	> 99 (*S*)	nd
4	**1d**	Br	145 ± 11	6.9 ± 1.0	21 ± 5	99:1	> 99 (*S*)	nd
5	**1e**	Me	110 ± 8	9.3 ± 1.4	12 ± 3	> 99:1	> 99 (*S*)	nd
6	**1f**	MeO			< 0.1			
7	**1g**	OH			< 0.1			

[a]All reactions were performed in 6 M aqueous ammonium carbonate buffer at pH = 10. [b]Determined by HPLC when the initial ratio of formation for the given isomer was not lower than 20% of the ratio for the other one; nd, not determined.

TABLE 2. PAM-Catalyzed Addition of Ammonia to *Meta*-Substituted Cinnamic Acids[a]



entry	1	R	k_{cat} (s^{-1}) × 10^3	K_M (mM)	k_{cat}/K_M ($s^{-1} \times mM^{-1}$) × 10^3	initial **2**:**3** ratio	ee of **2**[b] (%)	ee of **3**[b] (%)
1	**1h**	F	68 ± 2	5.6 ± 0.5	10 ± 1	86:14	92	nd
2	**1i**	Cl	111 ± 6	9.4 ± 1.1	12 ± 2	94:6	> 99 (*S*)	nd
3	**1j**	Br	nd[c]		20 ± 4	96:4	> 99 (*S*)	nd
4	**1k**	Me	10 ± 2	7.6 ± 2.9	1.3 ± 0.8	20:80	> 99 (*S*)	> 99 (*R*)
5	**1l**	MeO			< 0.1			
6	**1m**	OH			< 0.1			

[a]All reactions were performed in 6 M aqueous ammonium carbonate buffer at pH = 10. [b]Determined by HPLC when the initial ratio of formation for the given isomer was not lower than 20% of the ratio for the other one; nd, not determined. [c]The kinetic parameters for 3-bromocinnamic acid were not determined because of the insufficient solubility of this substrate in the reaction medium.

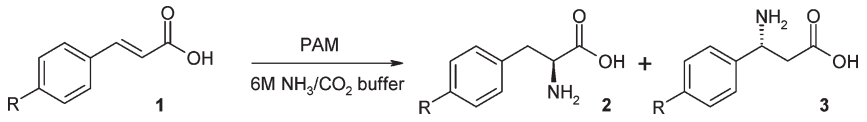
influencing the kinetic parameters for the reactions with *meta*-substituted cinnamic acids.

Results obtained for *para*-substituted cinnamic acids are shown in Table 3. The catalytic rates (k_{cat}) for compounds with substituents at the *para* position are generally lower than for the *ortho*- and *meta*-substituted substrates, as can be seen by comparing cinnamic acids with methyl and halogen substituents (Table 3, entries 1−4, compare to Tables 1 and 2). However, they seem to fit better into the active site of PAM, as K_M values are generally lower, and compound **1r** (Table 3, entry 5) is accepted by the enzyme, in contrast to its analogues **1f** and **1l**. These observations prompted us to further investigate the activity of PAM with this class of compounds, and to include an additional set of substrates (Table 3, entries 7−11).

The affinity of PAM for *para*-substituted cinnamic acids is influenced both by hydrophobicity and by steric effects of the substituents. Lower K_M values are generally observed for compounds with lipophilic groups (**1q**, **1t**, **1u**), whereas the presence of hydrophilic substituents (fluoro, nitro; Table 3, entries 1 and 10) results in a lower affinity of PAM toward the substrate. However, this trend is disturbed by steric effects, as shown by the changes of K_M in the series of homologues substituted with methyl (**1q**), ethyl (**1t**), *n*-propyl (**1u**), *iso*-propyl (**1v**), and *tert*-butyl groups (**1x**) (Table 3, entries 4, 7, 8, 9, and 11). In this series the affinity initially rises, reaching a maximum for the propyl substituent, but with the introduction of bulkier, branched groups the trend is reversed, and *tert*-butylcinnamic acid is not accepted by PAM at all.

The results obtained for this set of molecular probes allow an estimation of the size of the hydrophobic pocket which exists close to the *para* position in the active site. The length of this pocket is sufficient to incorporate an *n*-propyl substituent (*n*-butyl was not tested due to its very poor solubility in the reaction medium). However, it seems to be very narrow, as PAM has a low affinity toward compounds with branched aliphatic groups at the *para* position of the

TABLE 3. PAM-Catalyzed Addition of Ammonia to *Para*-Substituted Cinnamic Acids[a]



entry	1	R	k_{cat} (s^{-1}) x 10^3	K_M (mM)	k_{cat}/K_M ($s^{-1} \times mM^{-1}$) $\times 10^3$	initial **2**:**3** ratio	ee of **2**[b] (%)	ee of **3**[b] (%)
1	**1n**	F	37 ± 1	2.5 ± 0.2	15 ± 2	35:65	> 99 (*S*)	> 99 (*R*)
2	**1o**	Cl	46 ± 1	0.40 ± 0.02	115 ± 8	41:59	> 99 (*S*)	> 99 (*R*)
3	**1p**	Br	29 ± 1	0.18 ± 0.02	161 ± 23	52:48	85 (*S*)	> 99 (*R*)
4	**1q**	Me	35 ± 1	0.89 ± 0.06	39 ± 4	4:96	> 99 (*S*)	> 99 (*R*)
5	**1r**	MeO	27 ± 1	0.79 ± 0.04	34 ± 2	14:86	> 99 (*S*)	> 99 (*R*)
6	**1s**	OH			< 0.1			
7	**1t**	Et	38 ± 3	0.44 ± 0.10	86 ± 26	12:88	nd	> 99[c]
8	**1u**	Pr	18 ± 1	0.11 ± 0.01	164 ± 24	9:91	nd	> 99[c]
9	**1v**	*i*-Pr	46 ± 3	2.1 ± 0.3	22 ± 5	9:91	nd	> 99[c]
10	**1w**	NO_2	133 ± 1	9.9 ± 0.6	13 ± 1	98:2	> 99 (*S*)	nd
11	**1x**	*t*-Bu			< 0.1			

[a]All reactions were performed in 6 M aqueous ammonium carbonate buffer at pH = 10. [b]Determined by HPLC when the initial ratio of formation for the given isomer was not lower than 20% of the ratio for the other one; nd, not determined. [c]Absolute configuration was not determined.

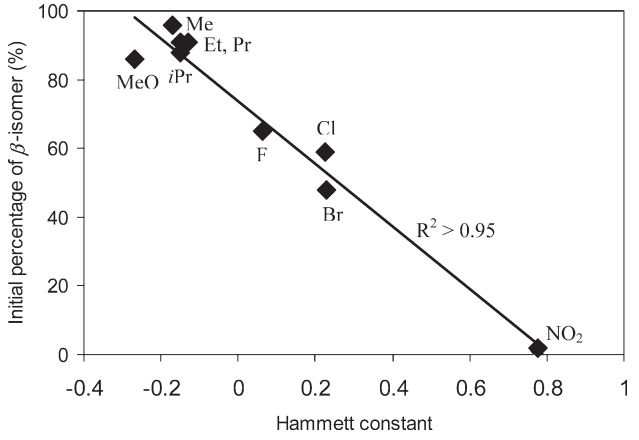


FIGURE 2. Correlation between the initial percentage of β-isomer (**3**) and the Hammett constant of the substituent at the *para* position of the substrate.

substrate. The existence and nature of this binding site is an important feature and distinguishes PAM from a similar MIO-dependent enzyme, tyrosine aminomutase (TAM), which catalyzes the reversible addition of ammonia to *p*-hydroxycinnamic acid (**1s**). The crystal structure of TAM was recently solved[31] and revealed the existence of a histidine residue close to the hydroxyl group at the *para* position of **1s**, which can form a hydrogen bond with it. Compound **1s** is not a substrate for PAM, which suggests that the respective histidine residue is missing in PAM and additional space is available for linear, lipophilic substituents.

The ratio of the initial rates of formation of the isomers **2** and **3** is governed by electronic properties of the substituents on the aromatic ring. Figure 2 shows that there is a good correlation ($R^2 > 0.95$) between the initial percentage of β-isomer **3** (over the first 30 min of reaction) and the Hammett constant (σ_p) of the substituent in the *para* position.[34] A similar observation can be made with compounds that carry substituents at the *meta* position.

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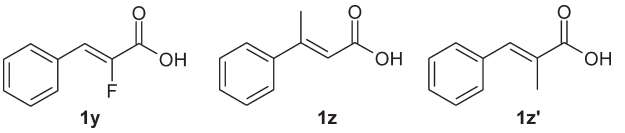

FIGURE 3. Additional set of substrates.

Both the carboxylate group and the aromatic ring can activate the double bond for an addition of ammonia. The regioselectivity of the reaction is therefore influenced by the substituents present at the aromatic ring, which explains the correlation presented in Figure 2. Cinnamic acid derivatives with electron-donating groups (alkyl and methoxy; Table 3, entries 4, 5, 7, 8, and 9) are converted predominantly to β-amino acids, which can be explained by a decreased ability of the aromatic system to accept electrons. The cinnamic acid derivative with a strong electron-withdrawing nitro group (Table 3, entry **10**) is transformed almost exclusively to an α-amino acid, which suggests that the electron-deficient aromatic ring is the dominant moiety responsible for the olefinic bond activation in this case.

For all the obtained β-amino acids the formation of only one enantiomer was observed (ee > 99%). Most α-amino acids also have excellent enantiomeric excesses of > 99%; only products **2h** and **2p** have slightly lower ee's.

An additional set of substrates was tested in order to further investigate the size and properties of the active site (Figure 3). This included cinnamic acid analogues with a fluoro (**1y**) and methyl (**1z**, **1z′**) substituent at the olefinic bond. No conversion was observed in PAM-catalyzed reactions of compounds **1y**, **1z**, and **1z′**, which suggests further limitations to the size and structure of substrates (vide infra).

An analysis of the factors influencing the affinity of the enzyme for various substrates provides information about the nature of different parts of the active site. The empirically derived geometrical model is presented in Figure 4. This method of visualization, based on literature precedence,[35]

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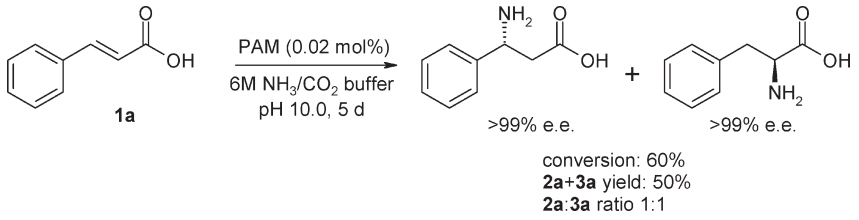
SCHEME 2. PAM-Catalyzed Conversion of (*E*)-Cinnamic Acids into a Mixture of α- and *β*-Phenylalanine



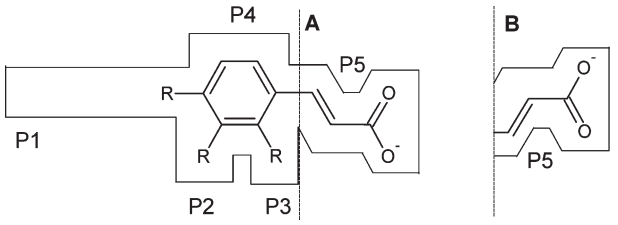


FIGURE 4. Proposed model of the active site of PAM.

allows us to avoid claims on specific amino acids present in the active site.

Correlations between the Michaelis constants and the hydrophobicity suggest important hydrophobic interactions with substituents at the *ortho* position of the substrate (Figure 4, P3) and the existence of a larger, hydrophobic groove around the *para* position (Figure 4, P1), which is able to interact with nonbranched, aliphatic chains. The higher affinity for compounds with hydrophilic substituents at the *meta* position suggests the presence of a polar environment close to this part of the substrate (Figure 4, P2).

In general, the active site of PAM seems narrow and deep, as longer substituents are accommodated only at the *para* position. The introduction of methyl substituents at the olefinic bond (as in compounds **1z** and **1z′**), or the use of (*Z*)-cinnamic acid as substrate[32] also results in complete loss of activity, indicating that the active site is narrow in this region (Figure 4, P5), although the conformation of the substrate within the active site (Figure 4, parts **A** and **B**) remains unclear. A lack of activity for compounds **1z** and **1z′** can also be explained by the fact that the methyl substituent increases the electron density at the olefinic bond, making it less prone to undergo a nucleophilic attack by the MIO−NH_2 adduct. However, we also observed no conversion for cinnamic acid substituted with the electron-withdrawing fluoro group (**1y**), which further confirms the hypothesis that the active site is narrow around the carbon−carbon double bond.

The turnover numbers (k_{cat}) were measured for 17 substrates. The highest values were obtained in cases where the α-isomer was predominantly formed (ammonia addition to compounds **1b**−**1d**, **1h**, **1i**, **1w**). Without further insight in the kinetics of individual steps, no further conclusions can be drawn about the factors influencing k_{cat}. This lack of correlation between the structure of substrate and the turnover number was also observed for the previously reported mutase reaction (equilibration between α- and *β*-amino acids) of PAM.[27]

A comparison of the results obtained for the mutase reaction[27] and the ammonia addition to substituted cinnamic acids (this study) reveals a higher affinity of PAM for α-amino acids (K_M in the range of 35−950 μM)[27] than for cinnamic acids (K_M in the range of 0.2−10 mM, this work). This suggests that during the course of the mutase reaction the exchange of the intermediate ((*E*)-cinnamic acid) between the active site and the solvent is prevented, facilitating the readdition of ammonia from the MIO−NH_2 adduct. This feature distinguishes PAM from phenylalanine ammonia lyase (pcPAL). A similar conclusion was drawn based on the comparison of the crystal structures of tyrosine aminomutase (TAM) and tyrosine ammonia lyase (TAL).[36] A further comparison of PAM and PAL shows that the substrate scopes of both enzymes are similar, although PAL was shown to accept compounds with hydroxy substituents on the aromatic ring.[37]

Finally, we decided to investigate the practical applicability of PAM-catalyzed conversion by performing the reaction on preparative scale (Scheme 2).

After 5 days, the conversion reached a value of 60%, and a roughly 1:1 mixture of enantiopure compounds **2a** and **3a** was obtained in 50% yield, demonstrating the potential usefulness of PAM for the preparation of α and *β*-phenylalanines. Research on the practical separation of regioisomers prepared in this reaction is underway in our group.

Summary and Conclusions

Our data show that a wide range of substituted cinnamic acids can be directly aminated in PAM-catalyzed reactions. For all *β*-amino acids that were produced, the ee values exceeded 99%, and for most of the α-isomers the enantioselectivity of the enzymatic reaction is also excellent (ee > 99%). In most of the reactions both α- and *β*-isomers are formed. For the synthesis of *β*-amino acids, the best results were obtained with cinnamic acid derivatives with electron-donating (alkyl and alkoxy) groups at the *para* position, as in these cases the desired isomers are formed in excess (> 90%) with excellent enantioselectivity (> 99%).

Considering the easy way in which the substrates can be obtained in a one step reaction from the corresponding aldehydes, and the high enantioselectivity of phenylalanine aminomutase, this catalytic system provides new possibilities for the synthesis of enantiopure aromatic *β*-amino acids.

Experimental Section

Methods for the preparation of cinnamic acids (**1**) and racemic reference α-amino acids (**2**) and *β*-amino acids (**3**) are presented in the Supporting Information.

(36) Christianson, C. V.; Montavon, T. J.; Van Lanen, S. G.; Shen, B.; Bruner, S. D. *Biochemistry* **2007**, *46*, 7205.
(37) Poppe, L; Rétey, J. *Curr. Org. Chem.* **2003**, *7*, 1297.

Determination of Kinetic Parameters for the Amination Activity of PAM. UV-Vis spectroscopy was used to determine the kinetic parameters of the PAM-catalyzed ammonia addition reaction. A 6 M ammonia solution was prepared, and the pH was adjusted to 10 by bubbling CO_2 through the solution. In a typical assay, (*E*)-cinnamic acid or a derivative was incubated at various concentrations with 0.06 mg of purified PAM in ammonia solution (300 μL). The reaction mixture was incubated at 30 °C. The ammonia addition activity was monitored by UV-Vis spectroscopy. The initial rates were plotted against the substrate concentration, and these data were fitted to the Michaelis−Menten equation to obtain the kinetic constants.

Stereochemical Analysis of the Phenylalanine Products by Chiral HPLC. Purified PAM (0.02 mg) was added to 5 mM of (*E*)-cinnamic acid or a derivative in ammonia solution (6 M, pH 10, 200 μL). The reaction mixture was incubated for 24 h at 30 °C. Subsequently, a 20-μL portion was taken, and the reaction was quenched by heating for 5 min at 99 °C. A 40-μL portion of 2 M aqueous NaOH was added to remove the excess of ammonia. The sample was then frozen in liquid nitrogen. Subsequently, the sample was lyophilized and dissolved in 55 μL of 2 M aqueous $HClO_4$. Analysis was carried out by chiral HPLC. Details are provided in the Supporting Information.

Preparative-Scale Conversion of (*E*)-Cinnamic Acid into a Mixture of α- and *β*-Phenylalanine. A 6 M ammonia solution was prepared, and the pH was adjusted to 10 by bubbling CO_2 through the solution. (*E*)-Cinnamic acid (30 mg, 0.2 mmol) was added, followed by PAM (2.0 mg), and the reaction mixture was left at room temperature. The conversion was monitored by UV measurement at 291 nm. After 48 h, another portion of the enzyme (1.0 mg) was added. After 5 days, the conversion reached 60%. The reaction mixture was lyophilized, redissolved in 1 N aq HCl (10 mL), and loaded on a column packed with ion-exchange resin (4.0 g of Dowex 50 × 8, 50−100 mesh, pretreated subsequently with 2 M NH_4OH, 1 M HCl, and water). The column was washed with water, and the product was eluted with 2 M aq NH_4OH. Ninhydrin-positive fractions were collected and lyophilized to yield 17 mg (50% isolated yield) of a roughly 1:1 mixture of α- and *β*-phenylalanine. The enantiomeric excess for both amino acids was determined to be >99% (HPLC, see the Supporting Information for details).

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Supporting Information Available: Experimental procedures and full spectroscopic data for all new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.