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β-Styryl- and β-Aryl-β-alanine Products of Phenylalanine Aminomutase Catalysis

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β-Amino acids are emerging as an important class of compounds that are present in pharmaceutically important natural products, such as the antineoplastic agent Taxol,[1] from *Taxus*, and the aminopeptidase inhibitor bestatin,[2] from *Streptomyces*. Single β-aryl-β-alanines have shown anti-epileptogenesis activity,[3] while other amino acids have been used as building blocks toward the synthesis of complex bioactive molecules, including β-lactams and β-peptides as mimics of α-peptide hormones and as antimicrobial compounds.[4]

Several synthetic strategies are used for the synthesis of variously substituted β-amino-β-arylpropionic acids that include multistep processes[5] and one-pot procedures such as the Knoevenagel condensation of benzaldehyde and malonic acid in the presence of an amine source (e.g., NH_4OAC).[6] However, there are presently no reports on biosynthetic strategies toward the production of asymmetric β-aryl-β-alanines from the corresponding α-isomers. The *Taxus* phenylalanine aminomutase (PAM)[7] that catalyzes the stereospecific isomerization of (*S*)-α-phenylalanine to (*R*)-β-phenylalanine (Scheme 1) provides a potential alternative route toward scalable production of novel β-amino acids as drug intermediates.

The PAM enzyme requires substrate with 2*S* stereochemistry and a phenyl ring at the β-carbon of the propionate chain (Scheme 1) but requires no external cofactors.[7] This aminomutase shows high amino acid sequence homology (56% similarity) to a family of ammonia lyases that contain a signature Ala-Ser-Gly motif,[7,8] which cyclizes to a 3,5-dihydro-5-methylidene-4*H*-imidazol-4-one (MIO) prosthesis group within the active site.[9] This MIO facilitates the elimination of NH_3/H^+ from the arylalanine substrate to form an aryl acrylate product, purportedly either via formation of a σ-complex by electrophilic attack on the *ortho*-carbon of the aromatic ring or by direct electrophilic activation of the amino group (Figure 1). Similar MIO involvement is proposed in the aminomutase reaction mechanism where, instead, vicinal NH_3/H^+ intramolecular interchange and rebound complete the reaction cycle to the β-amino acid product.[7,8,10]

Despite the wealth of information on the substrate specificity of the MIO-based reactions of the ammonia lyase family,[11] there remains a paucity of investigation on the scope of the substrate specificity of the PAM enzyme. The potential of the mutase enzyme to catalyze the production of novel β-amino acids prompted a survey of several commercially available (*S*)-α-amino arylpropanoic acids as substrates to examine specificity.

Escherichia coli BL21(DE3) cells transformed to express PAM were grown for 16 h in 6 L of minimal growth phosphate media at 18 °C with ampicillin selection and isopropyl-D-thiogalactopyranoside induction. The cells were harvested, lysed by sonication, and clarified by centrifugation. The crude soluble fraction was partially purified by anion exchange and Ni-affinity chromatographies[7] to yield PAM at ∼25 ng/mL as assessed by SDS-PAGE and Coomassie Blue staining.[7]

Prior to kinetics analysis, each "unnatural" α-amino acid substrate, at apparent saturation (2−3 mM), was assayed by in vitro incubation with ∼25 ng of PAM to identify productive substrates. After establishing linearity with respect to a fixed protein concentration and time using the natural substrate, surrogate substrates demonstrating sufficient turnover from the preliminary screen were incubated under steady-state conditions for 2 h (31 °C) at 0.01, 0.05, 0.1, 0.25, 0.5, 1, 2, and 3 mM with PAM (∼25 ng) in duplicate single stopped-time assays. The enzymatically derived β-amino acids were derivatized to their *N*-acetyl (or *N*-benzoyl) methyl esters, and each sample was quantified by capillary gas chromatography/electron impact-mass spectrometry (GC/EI-MS).[7] Linear regression analysis was used to convert the abundance of the base peak fragment ion produced by the product in each sample by corresponding the abundance of the base peak ion of authentic sample, derivatized similarly, to the concentration of each standard in a dilution series from 0 to 30 μM. The initial rates (v_o, nmol·h^{-1}) were plotted against substrate concentration [S] on KALEIDAGRAPH (Synergy Software, Reading, PA) to determine kinetic constants (K_M and V_{max}) of PAM by nonlinear regression analysis (R^2 was typically >0.98). (*R*)-β-Amino acids were commercially available, except only 4′-*tert*-butyl-(*RS*)-β-phenylalanine was available and styryl-(*R*)-β-alanine was synthesized by modification of a described procedure.[12] The stereochemistry of the unnatural β-amino acids made biosynthetically, and derivatized as described,[7] was judged to be *R* by chiral GC/MS analysis (Chirasil-D-Val column, Varian).

The steric flexibility of the PAM active site was investigated with 2′-, 3′-, and 4′-methyl- and 4′-*tert*-butyl-α-phenylalanines. The catalytic efficiency at ∼0.02 nmol·h^{-1}·μM^{-1} of PAM for the 3′- and 4′-methyl-α-phenylalanine substrate (**7** and **5**, respectively) is nearly equal to the value for the natural substrate **6** (Table 1). However, noticeably higher V_{rel} values of 8.9 and 2.3 for **7** and **5**,

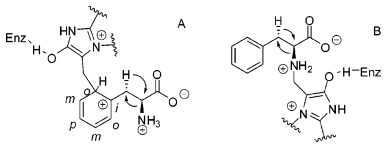

Figure 1. Two hypothetical MIO-based pathway intermediates in the PAM reaction. Path A: MIO attacked by aromatic ring electrons. Removal of β-hydrogen ($_\beta H^+$) quenches the σ-complex carbocation at the *ipso*-carbon. Path B: MIO attacked by amino group. The amine/MIO complex facilitates NH_3/H^+ removal. For both pathways, collapse of the MIO complexes and NH_3/H^+ exchange and rebound yields product. The (*i*)*pso*-, (*o*)*rtho*-, (*m*)-*eta*-, and (*p*)*ara*-positions indicated. Enz: enzyme.

Scheme 1. Isomerization of (*S*)-α-Arylalanines to (*R*)-β-Arylalanines by Catalysis of a *Taxus* Phenylalanine Aminomutase

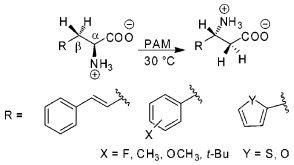

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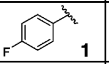
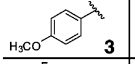
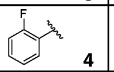
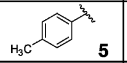
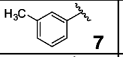
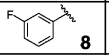
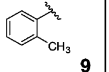
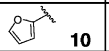
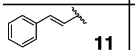
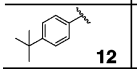
Table 1. Kinetic Parameters of PAM with Various Substrates[a]

Substrate (R)	K_M (μM)	V_{max} (nmol•h^{-1})	V_{max}/K_M (nmol•h^{-1}•μM^{-1})	V_{rel}
 **1**	75 (17)	180 (10)	2.4 (0.6)	95 (7)
 **2**	35 (8)	26 (1)	0.74 (0.17)	14 (1)
 **3**	410 (46)	48 (1)	0.12 (0.01)	25 (1)
 **4**	330 (65)	14 (1)	0.042 (0.01)	7.4 (<1)
 **5**	160 (39)	4.4 (0.1)	0.028 (0.007)	2.3 (0.1)
 **6**	97 (6)	1.9 (0.1)	0.020 (0.002)	1.0 (0.1)
 **7**	940 (180)	17 (1)	0.018 (0.004)	8.9 (0.7)
 **8**	560 (60)	3.2 (0.1)	5.7×10^{-3} (0.001)	1.7 (0.1)
 **9**	50 (5)	0.11 (0.01)	2.2×10^{-3} (3.0×10^{-4})	0.058 (0.006)
 **10**	410 (60)	0.030 (0.001)	7.3×10^{-5} (1.0×10^{-5})	0.016 (0.001)
 **11**	780 (71)	0.078 (0.001)	1.0×10^{-4} (1.0×10^{-5})	0.041 (0.002)
 **12**	~10^3	<0.01	<<10^{-5}	<<0.001

[a] V_{rel} is the ratio of V_{max} of a particular substrate and V_{max} of phenylalanine. Catalytic efficiency is defined by V_{max}/K_M. Refer to Scheme 1 for position of R substituent. Standard deviation in parentheses.

respectively, were observed (Table 1), while a V_{rel} at 0.058 and catalytic efficiency at 0.0022 nmol•h^{-1}•μM^{-1} for the isomerization of the 2′-methyl regioisomer **9** was substantially lower. Interestingly, the K_M (50 μM) for **9** is lowest in the methyl series, indicating that substrate binding is likely not the rate-determining factor of the methylated species. Thus, the *ortho*-methyl group (cf. Figure 1) may restrict conformational dynamics of reaction intermediates that are less affected by the *meta*- and *para*-methyls during reaction progress.

The V_{max} of PAM for 4′-*tert*-butyl-(*RS*)-α-phenylalanine (**12**) was slower ($V_{rel} \ll 0.001$) than that of **6**; however, the modest isomerization of **12** reveals that the PAM active site can accommodate a relatively bulky, aliphatic substituent at the *para*-position of the substrate (cf. Figure 1). However, the nine −C−C−H bond extensions of the *tert*-butyl group likely place **12** ($K_M \approx 1000$ μM) near the steric limits of the active site and affect binding compared to the smaller 4′-methyl homologue **5** ($K_M = 160$ μM).

Phenylalanine analogues bearing electron-withdrawing or electron-donating groups on the aromatic ring were used to investigate the effects of electron induction on PAM at steady-state. The catalytic efficiency of PAM catalysis for the 4′-fluoro-α-regioisomer (**1**) at 2.4 nmol•h^{-1}•μM^{-1} was greatest of all of the substrates tested. The 2′-fluoro isomer **4** was less efficiently catalyzed (0.027 nmol•h^{-1}•μM^{-1}) by ~60-fold compared to **1**, while the 3′-fluoro isomer **8** was even less efficiently isomerized (0.0057 nmol•h^{-1}•μM^{-1}) by ~400-fold (Table 1). The turnover of each fluorinated substrate was greater than that of phenylalanine. In general, an electron-withdrawing fluoro on the ring of the substrate may increase the acidity of the $_\beta$H of the substrate (cf. Figure 1), thus increasing the overall reaction rate, and therefore supports pathway B in Figure 1. In contrast, however, substrate **3** with an electron-donating 4′-methoxy group also demonstrated greater efficiency (0.12 nmol•h^{-1}•μM^{-1}) and superior turnover ($V_{rel} = 25$) compared to that of **6** (Table 1), thus supporting pathway A in Figure 1. Surprisingly, the turnover of the 3′-methoxy isomer was below detection limits, considering that the weakly electron-donating 3′-methyl substrate **7** was converted to its β-isomer nearly 4 times faster and with greater efficiency than the 4′-methyl isomer **5**.

Thien-2-yl-(*S*)-α-alanine (**2**) was converted to β-amino acid product much faster ($V_{rel} = 14$) than the furan-2-yl analogue **10** ($V_{rel} = 0.02$, Table 1). The efficiency of PAM with **2** was second to that of the 4′-fluoro species **1** and was also isomerized ~40 times more efficiently than natural substrate **6**. The K_M value for **2** (35 μM) compared to that of **10** (410 μM) indicates that **2** likely has better binding affinity.

Intriguingly, (*S*)-styrylalanine (**11**) was also a productive substrate of PAM with a $V_{rel} = 0.041$ compared to **6** (Table 1), and its K_M was similar to the *meta*-substituted analogue **7**, although the catalytic efficiency ($V_{max}/K_M \ll 0.0001$ nmol•h^{-1}•μM^{-1}) was much lower. Notably, PAM was unable to convert the saturated styrylalanine analogue ((*S*)-2-amino-5-phenylpentanoic acid) to its β-isomer, suggesting that an extended conjugated allyl π-system next to the α-carbon, bearing the migrating amino group, is required for isomerization.

In conclusion, it is evident that the native PAM can accept arylalanine substrates with various substituents on the phenyl ring. However, no definitive trend in the catalytic rate emerged in parallel with strong electron-withdrawing or -donating substituents at steady-state. In this scenario, substrate release or rotation dynamics during the reaction may be rate-limiting and thus masks cryptic inductive effects associated with the MIO mechanism. Single turnover kinetic analysis of PAM is currently being developed to further dissect the mechanism.

As the properties of the phenylalanine aminomutase mechanism are better understood, the integration of amino acid isomerase chemistry into custom asymmetric synthesis becomes practical. Engineering PAM to broaden its substrate specificity for the production of novel nonpeptidic β-amino acids could provide building blocks for constructing second-generation compounds.

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Supporting Information Available: Experimental procedures, mass spectral analysis (Figure 1S), and the enantiopurity of the biosynthetic products (Figure 2S). This material is available free of charge via the Internet at http://pubs.acs.org.

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