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The Interaction of Heteroaryl-Acrylates and Alanines with Phenylalanine Ammonia-Lyase from Parsley

Csaba Paizs,[a, b] Adrian Katona,[a] and János Rétey*[a]

Abstract: Acrylic acids and alanines substituted with heteroaryl groups at the β-position were synthesized and spectroscopically characterized (UV, HRMS, ^{1}H NMR, and ^{13}C NMR spectroscopy). The heteroaryl groups were furanyl, thiophenyl, benzofuranyl, and benzothiophenyl and contained the alanyl side chains either at the 2- or 3-positions. While the former are good substrates for phenylalanine ammonia-lyase (PAL), the latter compounds are inhibitors. Exceptions are thiophen-3-yl-alanine, a moderate substrate and furan-3-yl-alanine, which is inert. Possible reasons for these exceptions are discussed. Starting from racemic heteroaryl-2-alanines their D-enantiomers were prepared by using a stereodestructive procedure. From the heteroaryl-2-acrylates, the L-enantiomers of the heteroaryl-2-alanines were prepared at high ammonia concentration. These results can be best explained by a Friedel–Crafts-type electrophilic attack at the aromatic part of the substrates as the initial step of the PAL reaction.

Keywords: biosynthesis · enantioselectivity · enzyme catalysis · Friedel–Crafts

Introduction

Phenylalanine ammonia-lyase (PAL) is an important plant enzyme, as its product, (*E*)-cinnamic acid is the precursor of lignines, coumarins, and flavonoids. Its mechanism of action has been the subject of great interest.[1]. PAL and HAL (histidine ammonia-lyase) contain the unique superelectrophilic prosthetic group 5-methylene-3,5-dihydroimidazol-4-one (MIO) which is able to attack the phenyl or imidazolyl group of their substrates. Thereby, the β-protons of the amino acids are activated allowing their abstraction by an enzymatic base, a step, which is followed by elimination of ammonia. This mechanism is initiated by a Friedel–Crafts-type attack (Scheme 1).

The X-ray structure of HAL was solved in 1999;[2] however, the structure of PAL has only recently published.[3] The more highly resolved structure of PAL obtained from parsley (*Petroselinum crispum*)[4] is consistent with the Friedel–Crafts like attack of MIO at the phenyl group of phenylalanine.[5]

Another interesting property of PAL is its ability to react with a large number of arylalanines, which makes it a useful enantiospecific biocatalyst.[6] Originally it was believed that the ammonia eliminations both by PAL and HAL were irreversible. In this case, enantioselectivity for the L-amino acids would allow preparation of the D-amino acids by a stereodestructive procedure. However, at a surprisingly high ammonia concentration (~6 M) both the PAL and HAL reactions can be run in the reverse direction, that is, the enantioselective addition of ammonia can be achieved on a preparative scale. Thus the pure L-enantiomers of various halogen-substituted phenylalanines (Cl, F), pyridyl and pyrimidyl alanines etc. have been prepared by the reverse PAL reaction.[7] Here we describe the synthesis of various acrylic acids, substituted with heterocyclic aromatic groups, and the corresponding amino acids both in the racemic and enantiomerically pure form. They are either substrates or inhibitors of PAL.

Results

Synthesis of the heteroaryl- acrylic acids and racemic amino acids: The starting materials for the synthesis of the acrylic

[a] Dr. C. Paizs, A. Katona, Prof. Dr. J. Rétey
Institute of Organic Chemistry and Biochemistry
University of Karlsruhe, Richard-Willstätter-Allee
76128 Karlsruhe (Germany)
Fax: (+49) 721-608-4823
E-mail: janos.retey@ioc.uka.de

[b] Dr. C. Paizs
Department of Biochemistry and Biochemical Engineering
Babes-Bolyai University, Arany János str. 11
400028 Cluj-Napoca (Kolozsvár) (Romania)

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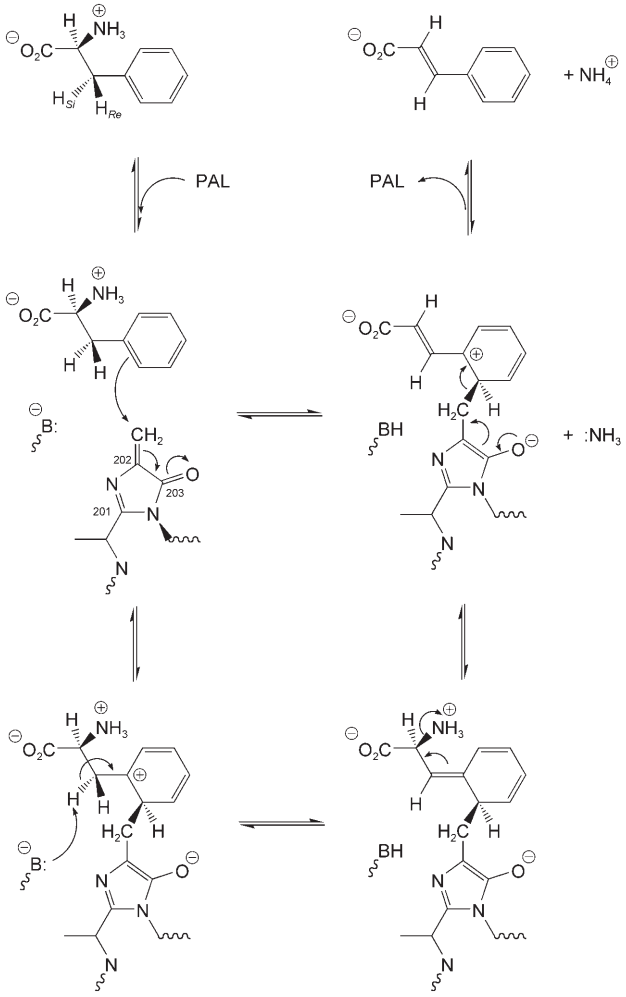
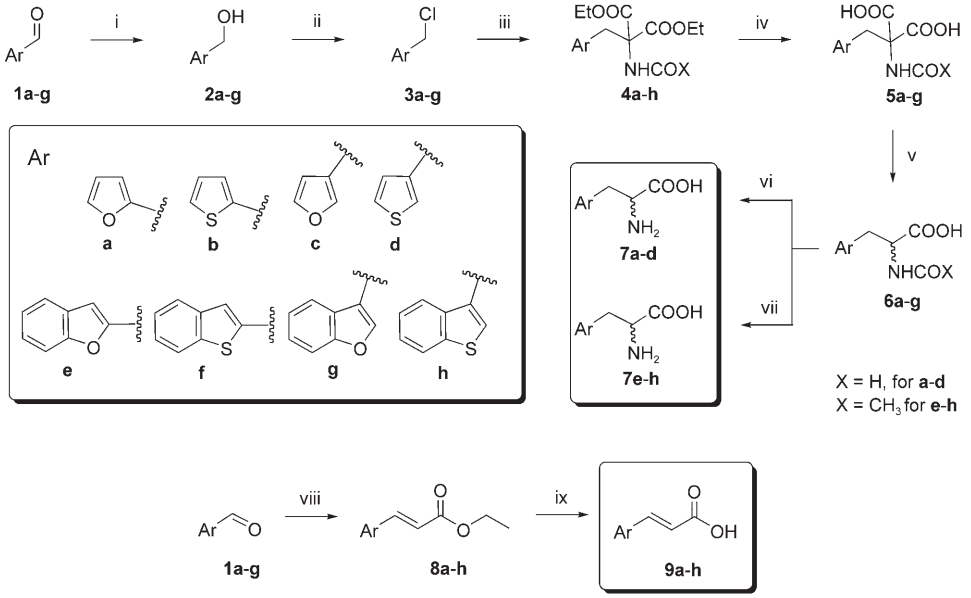


Scheme 1. Mechanism of the PAL-mediated reaction involving a Friedel–Crafts-type attack of the MIO group at the phenyl moiety of phenylalanine.

and amino acids were the corresponding heteroaryl aldehydes. These compounds were transformed with triphenyl-λ^5-phosphanilidene ethyl acetate into the corresponding acrylic acid esters, which were subsequently hydrolyzed in presence of KOH to yield the desired cinnamates. Alternatively, the aldehydes were transformed into chloromethylene derivatives; this was followed by their coupling with *N*-protected diethyl amino malonate. Mild basic hydrolysis of the diethyl esters formed produced *N*-protected amino malonic acids, which were decarboxylated in boiling toluene. In a final step the protecting group was removed and the phenylalanine analogues were isolated by precipitation at their isoelectric point (Scheme 2).

Determination of the kinetic constants for the heteroaryl alanines: The kinetic measurements are based on the UV spectrophotometrical determination of the concentration of the acrylates formed. First, their extinction coefficients (ε) were determined in Tris-buffer (0.1 M, pH 8.8) at wavelengths (λ) for which the corresponding amino acids do not show absorption. The enzymatic assays were carried out in 1 mL cuvettes in the above mentioned buffer at 30 °C in presence of 25 μg of PAL with the substrate concentration varying between 0.5–2.5 mM. In Table 1 the maximal velocity (V_{max}) values relative to that of phenylalanine and the values of the Michaelis constant (K_m) are listed. For amino acids in which the alanine group is attached to the heteroaromatic ring at the 2-position, PAL could catalyze their transformation into acrylates; however, the reaction rates generally were lower than for the parent compound, only *rac*-thiophen-2-yl alanine reacts approximately at the same rate as phenylalanine.

The behavior of the amino acids in which the alanyl portion is attached to the heteroaromatic ring at position 3 was different. Only *rac*-thiophen-3-yl-alanine (**7d**) was a poor substrate for PAL. In addition, we investigated the interaction of the nonsubstrate amino acids with PAL, by kinetic measurements on the parent reaction, monitoring the formation of cinnamate at 290 nm. While *rac*-furan-3-yl-alanine (**7c**) appeared not to interact with PAL, *rac*-benzofuran- (**7g**) and *rac*-benzothiophen-3-yl alanines (**7h**) proved to be strong competitive inhibitors. Moreover, L-**7g** and L-**7h** were



Scheme 2. Synthesis of the racemic arylamino acids and acrylic acids. i) $NaBH_4/CH_3OH$; ii) $SOCl_2$, benzotriazole/CH_2Cl_2; iii) NaH, $XCONH(CO_2Et)_2$/DMF, 60 °C; iv) 10% KOH, 20 h; v) toluene/xylene, reflux, 2 h; vi) 10% LiOH, 60 °C, 2.5 h; vii) 18% HCl, reflux, 4 h; viii) $Ph_3P{=}CHCO_2Et$/toluene, reflux, 2 h; ix) 10% KOH, reflux.

Table 1. Kinetic constants for the PAL-mediated reactions.

Substrate	K_m [μM]	K_i [μM]	$V_{max}/V_{max\ Phe}$
L-Phe	32.01 ± 2	–	1
rac-**7a**	76.39 ± 3	–	0.34
rac-**7b**	134.96 ± 3	–	1.01
rac-**7d**	18.24 ± 1	–	0.16
rac-**7e**	48.38 ± 2	–	0.49
rac-**7f**	116.63 ± 4	–	0.14
rac-**7g**	–	25 ± 3	1
L-**7g**	–	13 ± 2	1
rac-**7h**	–	16 ± 3	1
L-**7h**	–	7 ± 2	1

approximately doubly as strong inhibitors as the racemates, indicating that the D-amino acids do not interact with the enzyme.

Difference UV spectroscopy: The difference UV spectra of wild-type- and the MIO-less mutant Ser202Ala-PAL were recorded in the absence and in the presence of the competitive inhibitor *rac*-**7g**. Earlier, it was shown that the difference UV spectra of the above mentioned protein pair exhibits a discrete maximum between 305 and 310 nm, which was assigned to the cross-conjugate double bond system of MIO.[8] This maximum also appeared unaltered when the difference spectra were recorded in the presence of *rac*-**7g** (Figure 1a). In addition, the difference spectra of wild-type PAL in presence of *rac*-**7g**, with wild-type PAL as blank, were identical to the UV spectra of *rac*-**7g** (Figure 1b).

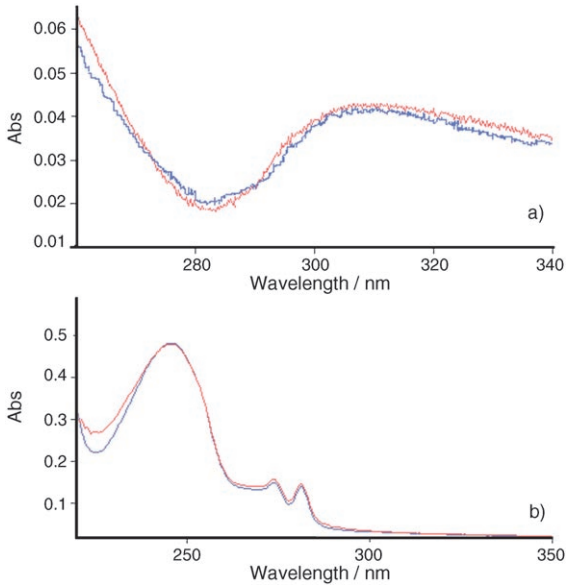


Figure 1. The UV difference spectra show no interaction between inhibitor *rac*-**7g** and MIO. a) Blue trace: wt-PAL in Tris-HCl buffer/S202A-PAL in Tris-HCl buffer as the blank; red trace: *rac*-**7g** and wt-PAL in Tris-HCl buffer/*rac*-**7g** and S202A-PAL in Tris-HCl buffer as the blank. b) Blue trace: *rac*-**7g** and wt-PAL in Tris-HCl buffer/wt-PAL in Tris-HCl buffer as a blank; red trace: *rac*-**7g** in Tris-HCl buffer/Tris-HCl buffer as a blank.

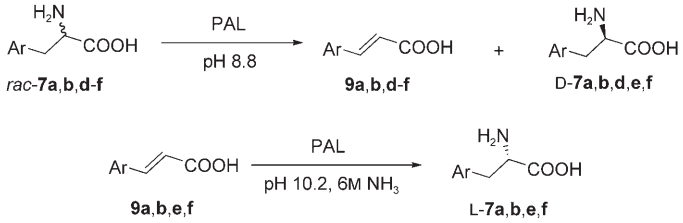
Table 2. Preparative enantioselective synthesis of heteroaryl amino acids catalyzed by PAL.

Substrate	Product[a]	Yield [%][b]	PAL [iU]	Time [days]	$[\alpha]^D$
(*E*)-**9a**	L-**7a**	66	6	3	−41.8° in H_2O, 25 °C (−41.8° in H_2O, 25 °C)[11]
(*E*)-**9b**	L-**7b**	89	6	2	−30.3° in H_2O, 20 °C (−30.3° in H_2O, 20 °C)[12]
(*E*)-**9e**	L-**7e**	54	6	4	−14.5° in CH_3CO_2H, 20 °C
(*E*)-**9f**	L-**7f**	49	6	4	−23.8° in CH_3CO_2H, 20 °C
rac-**7a**	D-**7a**	44	2.5	4	+41.7° in H_2O, 25 °C (+41.7° in H_2O, 25 °C)[11]
rac-**7b**	D-**7b**	45	2	2	+31.8° in H_2O, 25 °C (+31.8° in H_2O, 25 °C)[13]
rac-**7d**	D-**7d**	45	7.5	7	+42.6° in H_2O, 25 °C (+42.6° in H_2O, 25 °C)[12]
rac-**7e**	D-**7e**	43	6	3	+14.4° in CH_3CO_2H, 20 °C
rac-**7f**	D-**7f**	44	6	3	+23.8° in CH_3CO_2H, 20 °C

[a] > 98 % *ee* for all the compounds (*ee* = enantiomeric excess). [b] Yields are given for the isolated compounds.

Biocatalysis: There are some examples for using PAL or *Rhodotorula graminis* and *Rhodotorula glutinis* cells in the preparative scale production of L-amino acids starting from the corresponding acrylates. As far as we know PAL was not used for the preparation of D-amino acids by enantioselective kinetic resolution of the racemates. Exploiting the results obtained by the kinetic measurements, we performed the synthesis of D-**7a**,**b**,**d**–**f** by incubating the corresponding racemates with different amounts of PAL at 30 °C (Table 2, Scheme 3) in Tris-buffer (0.1 M, pH 8.8). The progress of the reaction was monitored by HPLC with a Chirobiotic-T column (Figure 2). When the L-amino acids were completely transformed into acrylates the reaction was stopped by adjusting the pH to 1.5 and the acrylates were filtered off. The enzyme was precipitated by heating the mixture to 90 °C, followed by filtration of the turbid solution. The D-amino acids were isolated and purified from the filtrate by ion exchange chromatography.

For the production of the L-amino acids, acrylic acids **9a**,**b**,**e**,**f** were incubated with PAL (Table 2, Scheme 3) in ammonia (6 M) buffered at pH 10.2 by bubbling CO_2; the reaction and the workup procedure were conducted as described above (Figure 3).



Scheme 3. PAL-mediated reactions for the preparation of enantiopure D- and L-heteroaryl alanines.

Discussion

The synthesis of the acrylic acids substituted with heteroaryl groups and the corresponding racemic amino acids was

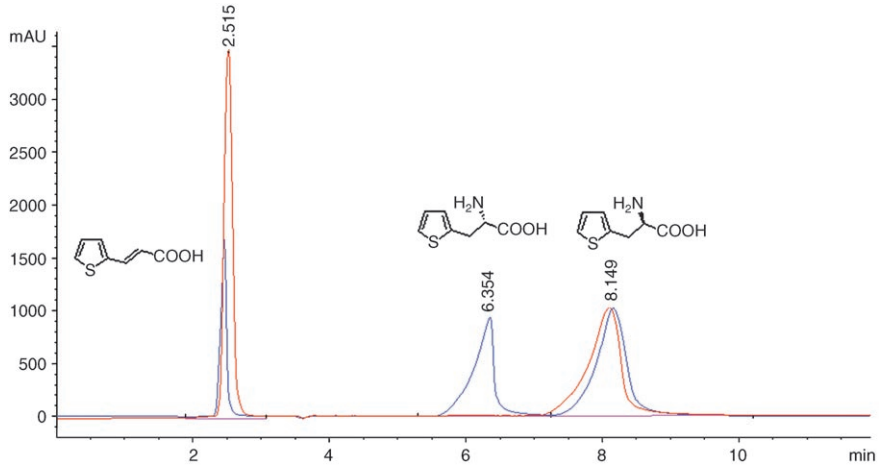


Figure 2. The elution diagram for PAL-catalyzed enantiodestructive synthesis of D-thiophen-2-yl-alanine. PAL and *rac*-amino acid, Tris-HCl buffer (pH 8.8): blue trace: after 30 min; red trace: after 2 days.

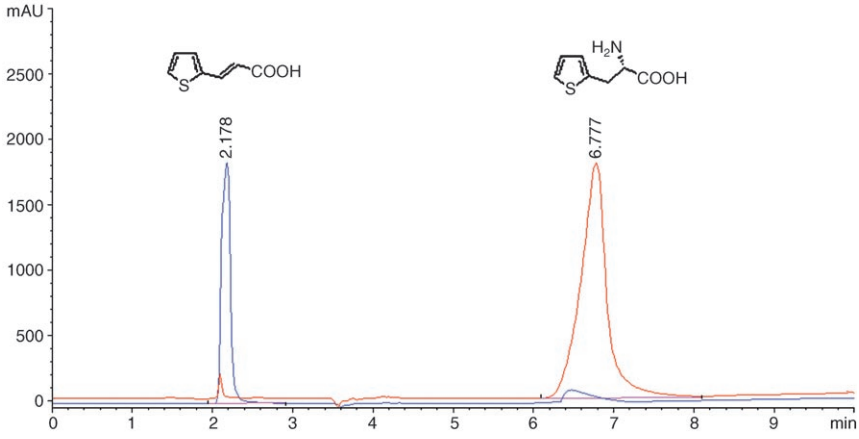


Figure 3. Elution diagram for the synthesis of L-thiophen-2-yl-alanine.

rather conventional. Some of them had been described in the early literature (see references in the Supporting information), but their characterization by modern spectroscopic methods (UV, high resolution ^{1}H- and ^{13}C-NMR spectroscopy, and MS) was not available. This is now provided in the Supporting Information.

To test whether the electrophilic attack of MIO at the aromatic ring is a prerequisite for the acceptance by PAL, the alanyl side-chain was attached at the 2- or 3-position of the heteroaryl group. Most of the latter isomers were inhibitors rather than substrates of PAL. One exception is thiophen-3-yl-alanine (**7d**) which is, in contrast to furan-3-yl alanine (**7c**), a reluctant substrate of PAL. This may be due to the decreased electronegativity of sulphur compared with oxygen. In other words, sulphur is similar to carbon and **7d** can be bound by the active site in a conformation in which the attack by MIO is possible (Figure 4a and b). By contrast, the furanyl group of **7c** seems to be too polar to bind in the alternative conformation. Therefore **7c** is neither a substrate nor an inhibitor. Also in a whole cell mediated biocatalytic reaction with *Rhodotorula graminis* cells resuspended in a buffered 9 M ammonia solution, it was found that while furan-3-yl-acrylate is inert, thiophen-2-yl-acrylate is a good substrate for the preparation of the corresponding L-amino acid.[9]

The binding pocket of PAL is more selective for the benzofuran- and benzothiophenyl alanines. The 2-yl isomers (**7e** and **7f**) are good substrates, while the 3-yl isomers (**7g** and **7h**) are strong competitive inhibitors. For the 2-yl isomers, MIO can be attached to C-3 in the aromatic ring (Figure 4c) and ammonia elimination follows. In contrast, both *ortho* positions with respect to the alanyl group of the 3-yl isomers were not accessible by MIO. The quaternary C-4 is sterically hindered to a Friedel–Crafts-type attack, while C-2 is too far from the prosthetic group (Figure 4d). Difference UV spectroscopy also indicated that the latter isomers can bind strongly, but only in the conformation that cannot be attacked by MIO.

The stereodestructive preparation of enantiopure D-aryl-amino acids by PAL is possible because the reaction is only reversible at extremely high ammonia concentrations (>4 M). The K_m for ammonia for the reverse reaction has been determined to be 4.4 M at pH 8.8 and 2.6 M at pH 10.[10] Thus, about 90% of the possible yields of the D-amino acids were achieved (Table 2). This is the first time that these D-amino acids were prepared by the PAL reaction.

Four L-aryl amino acids could be prepared by conducting the PAL reaction in the reverse direction with a 6 M concentration of ammonia. As shown in Figure 3 and Table 2, the yields were good to moderate and the L-amino acids were enantiomerically pure. The latter could be determined by using a chiral column and/or by a comparison of their $[\alpha]^{D}$ values with those in the literature[11–13] (Figures 2 and 3, and Table 2). In contrast to the whole cell procedure using *Rhodotorula glutinis* cells[14] for PAL from parsley, L-**7d** was a poor substrate. However, it could not be prepared by the reverse PAL reaction, but *rac*-**7d** was used to obtain the D-enantiomer by the stereodestructive forward reaction.

In conclusion, furanyl, thiophenyl, benzofuranyl, and benzothiophenyl groups attached to acrylyl and alanyl side

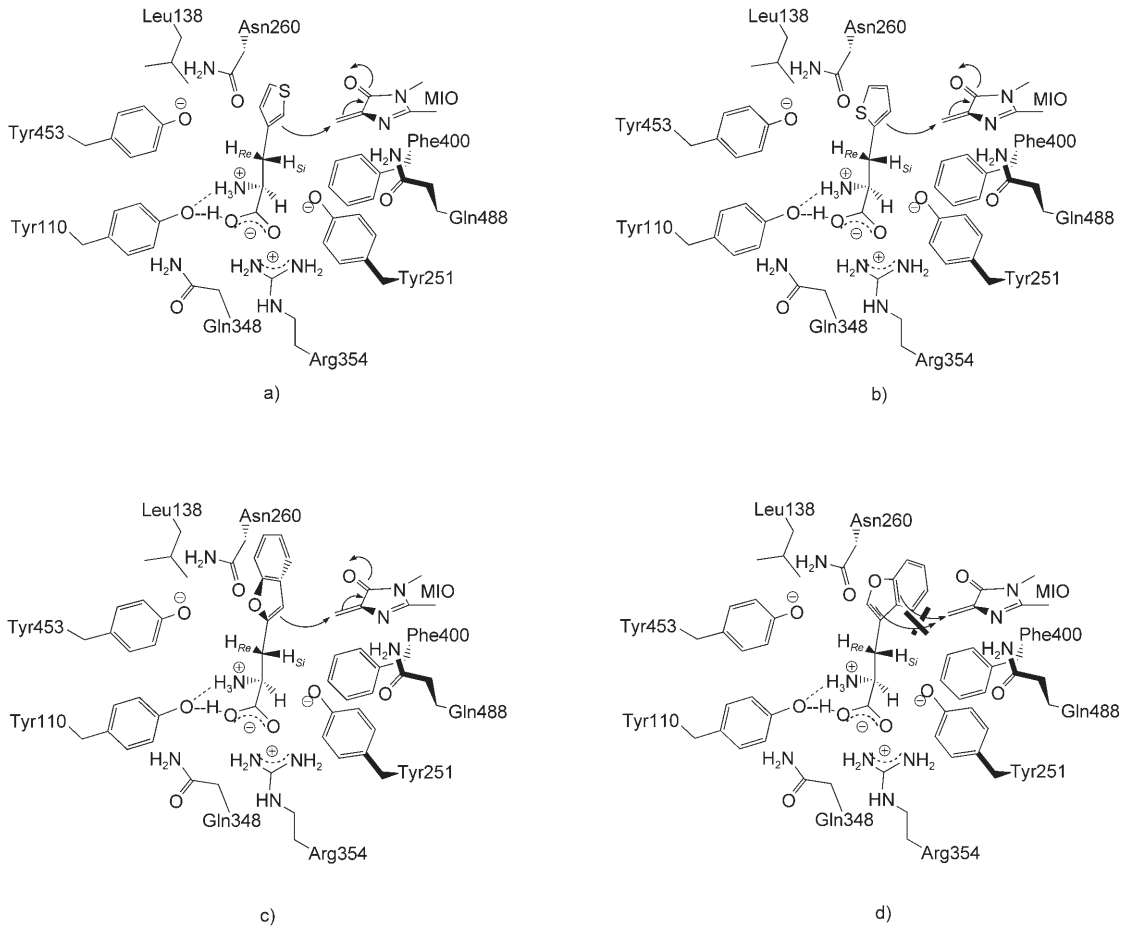


Figure 4. Model of the active site of PAL with various arylalanines arranged in accordance with the Friedel–Crafts-like mechanism.

chains were examined as substrates or inhibitors of PAL. The results support the mechanism involving a Friedel–Crafts attack as the first step. In addition the usefulness of PAL as biocatalyst for the preparation of enantiopure heterocyclic arylamino acids is shown.

Experimental Section

Analytical methods: The ^{1}H- and ^{13}C NMR spectra were recorded on a Bruker spectrometer operating at 400 and 100 MHz, respectively, at 25 °C. EI-MS were taken on a VG 7070E mass spectrometer operating at 70 eV. HPLC analyses were conducted with a HP 1050 instrument by using a Chirobiotic-T column (0.46 × 25 cm) and a mixture of methanol and water as the eluent at 1 mL min^{-1} flow rate. Retention times of the enantiomers and the used eluents are presented in the Supporting Information. For kinetic measurements, a Varian Cary 3E UV-VIS spectrophotometer was used. TLC was carried out by using Merck Kieselgel $60F_{254}$ sheets. Spots were visualized by treatment with 5% ethanolic phosphomolybdic acid solution and heating. Preparative chromatographic separations were performed by using column chromatography on Merck Kieselgel 60 (0.063–0.200 μm). Melting points were determined by a hot plate method and are uncorrected. Optical rotations were determined on a Perkin–Elmer 201 polarimeter and $[\alpha]_D$ values are given in units of 10^{-1} deg cm^2 g^{-1}.

Reagents and solvents: Furan-2-carbaldehyde (**1a**), thiophene-2-carbaldehyde (**1b**), furan-3-carbaldehyde (**1c**), thiophene-3-carbaldehyde (**1d**), benzofuran, benzothiophene, *ortho*-hydroxyacetophenone, ethylchloroacetate, triphenyl-λ^5-phosphanilidene ethyl acetate, all inorganic reagents, and solvents were products of Aldrich or Fluka. All solvents were purified and dried by standard methods as required.

Recombinant PAL: The wild-type- and mutant Ser202Ala phenylalanine ammonia-lyases were overexpressed in *E. coli* and purified, first according to the method described by Schuster and Rétey[15] and later by using the improved method described by Baedeker and Schulz.[16] Kinetic measurements and enzymatic reactions were performed at 30 °C.

Kinetic measurements

Determination of V_{max} and K_m values for substrate amino acids: The kinetic constants were determined by using a 1 mL cuvette and were recorded at intervals of 1 min over 5 min. The UV absorptions of the acrylates produced were recorded at wavelengths for which the corresponding amino acids show only low absorption. After incubation of the enzyme (25 μg) in 0.1 M Tris-HCl buffer at pH 8.8 and 30 °C for 5 min, various amounts of amino acids were added with the substrate concentration of the enzymatic assays varied between 05–2.5 mM.

Determination of K_i for inhibitor amino acids: Following the procedure described above, the PAL reaction was monitored by measuring the absorption of the produced cinnamate at intervals of 1 min at 290 nm. The phenylalanine concentration was varied with three different concentrations of the inhibitors (0.1, 0.2, and 0.5 mM). The kinetic parameters are shown in Table 1.

Difference UV spectroscopic measurements: UV difference spectra were measured at an enzyme concentration of 0.5 mg × mL^{-1} and inhibitor concentration of 0.5 μM in 0.1 M Tris-HCl buffer at pH 8.8 and 30 °C from 260 to 350 nm with 1 mL quartz cuvettes.

Enzymatic synthesis of enantiopure amino acids

Kinetic resolution of racemic amino acids catalyzed by PAL: PAL was added to a solution of one of the amino acids *rac*-**7a**,**b**,**d**–**f** (0.5 mmol) in Tris-HCl buffer (0.1 M, pH 8.8, 40 mL), and the reaction mixture was stirred under argon at 30 °C. The progress of the reaction was followed by HPLC. When the L-enantiomer was completely consumed, the pH of the solution was adjusted to 1.5 with 5% HCl (filtered in case of **7e** and **f**), and then the solution was heated to 90 °C for 10 min, cooled to room temperature, filtered, and applied to a Dowex 50X8 cation exchange resin column. The elution of the pure D-enantiomer of the amino acids was achieved by using 2 M ammonia solution. Reaction times, quantity of the enzyme in each case, and the yields of the products are presented in Table 2.

Synthesis of L-amino acids from cinnamates by PAL-mediated enantioselective ammonia addition: CO_2 was bubbled into a solution of one of the cinnamates **9a**,**b**,**d**,**e** (0.675 mmol) in half-concentrated ammonia solution (20 mL) until the pH of the solution was adjusted to 10.2. After the addition of PAL, the reaction mixture was stirred under argon at 30 °C. The progress of the reaction was followed by HPLC and when the formation of the L-amino acids had stopped, the reaction mixture was degassed under reduced pressure. The pH was adjusted to 1.5 with 5% HCl, filtered, and the solution was then heated to 90 °C for 10 min, cooled to room temperature, filtered again, and applied to a Dowex 50X8 cation exchange resin column. Elution of the pure L-enantiomer of the amino acids was achieved by using 2 M ammonia solution. Reaction times, quantity of the enzyme in each case, and the yields of the products are presented in Table 2.

Acknowledgements

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