Phenylalanine Ammonia-Lyase: The Use of Its Broad Substrate Specificity for Mechanistic Investigations and Biocatalysis—Synthesis of L-Arylalanines

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Abstract: Several fluoro- and chlorophenylalanines were found to be good substrates of phenylalanine ammonia-lyase (PAL/EC 4.3.1.5) from parsley. The enantiomerically pure L-amino acids were obtained in good yields by reaction of the corresponding cinnamic acids with 5 M ammonia solution (buffered to pH 10) in the presence of PAL. The kinetic constants for nine different fluoro- and chlorophenylalanines do not provide a rigorous proof for but are consistent with the previously proposed mechanism comprising an electrophilic attack of the methylidene-imidazolone cofactor of PAL at the aromatic nucleus as a first chemical step. In the resulting Friedel–Crafts-type σ complex the β-protons are activated for abstraction and consequently the *pro-S* is abstracted by an enzymic base. Results from semi-empirical calculations combined with a proposed partial active site model showed a correlation between the experimental kinetic constants and the change in polarization of the *pro-S* C_β–H bond and heat of formation of the σ complexes, thus making the electrophilic attack at the neutral aromatic ring plausible. Furthermore, while 5-pyrimidinylalanine was found to be a moderately good substrate of PAL, 2-pyrimidinylalanine was an inhibitor.

Keywords: chemoenzymatic synthesis · enzyme inhibitors · enzyme mechanism · halogenated L-phenylalanines · lyases

Introduction

Phenylalanine ammonia-lyase (PAL) catalyzes the reversible conversion of L-phenylalanine to *trans*-cinnamic acid. PAL is the key enzyme in the metabolism of phenylpropanoids in plants, since cinnamic acid is the precursor of lignins, flavonoids, and coumarins.[1, 2] Early biochemical evidence indicated a catalytically essential electrophilic group at the active sites of PAL and the related enzyme histidine ammonia-lyase (HAL). Inactivation by radiolabelled nucleophiles ($K^{14}CN$ and NaB^3H_4) followed by total hydrolyses of the proteins afforded radioactive products which could be derived from a dehydroalanine residue being the electrophilic prosthetic group.[3, 4, 5]

Its precursors have been found by site-directed mutagenesis to be Ser202[6] and Ser143[7] in PAL and HAL, respectively. Since both enzymes have been overexpressed in *E. coli* cells in active forms, the post-translational modification takes place autocatalytically.[8, 9]

Recently, the 3D structure of HAL has been elucidated by X-ray analysis which revealed that the electrophilic prosthetic group is methylidene-imidazolone (MIO).[10] MIO can be regarded as a modified dehydroalanine. The ring structure and the geometry dictated by the protein conformation prevents the lone pairs of the imidazole nitrogens to delocalize into the α/β-unsaturated carbonyl system, thus enhancing the electrophilicity of the latter. The driving force for the formation of MIO from the tripeptide Ala142Ser143Gly144 is unknown but not without precedence. The chromophoric imidazolone of the green fluorescent protein[11, 12, 13] must be formed by a similar autocatalytic process.

The discovery of the electrophilic prosthetic group was followed by the proposal that it reacts with the amino group of the substrate in a type of Michael addition.[14] It was suggested that this reaction would enhance the leaving ability of the amino group, that is facilitate the elimination of ammonia, but it leaves the question open how the "non-acidic β-proton" could be abstracted by an enzymic base.

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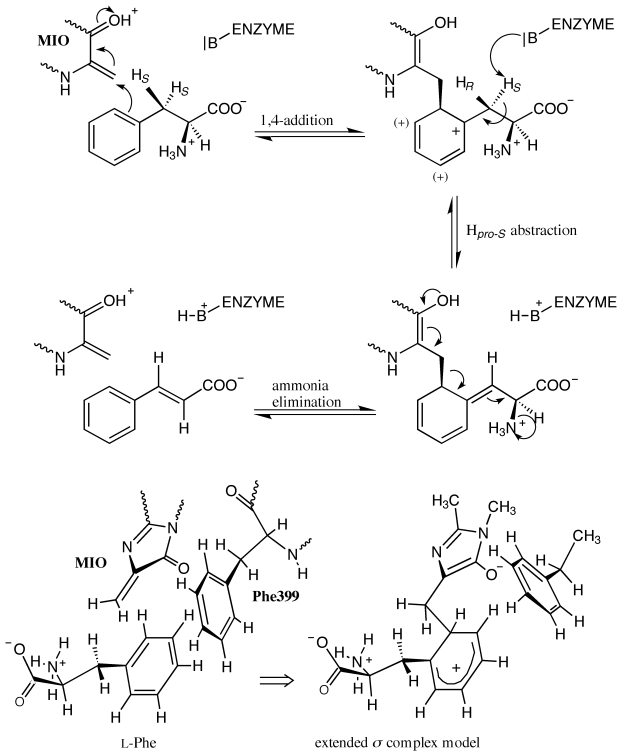
Recently, experimental evidence accumulated in favor of an electrophilic attack at the aromatic nucleus as shown in Scheme 1 for the PAL reaction.[15, 16] In the Friedel–Crafts-type σ complex the β-protons of the side chain are activated for abstraction by an enzymic base. The proton transfer is then followed by ammonia elimination concomitant with restoration of the prosthetic group and the aromaticity of the six-membered ring.



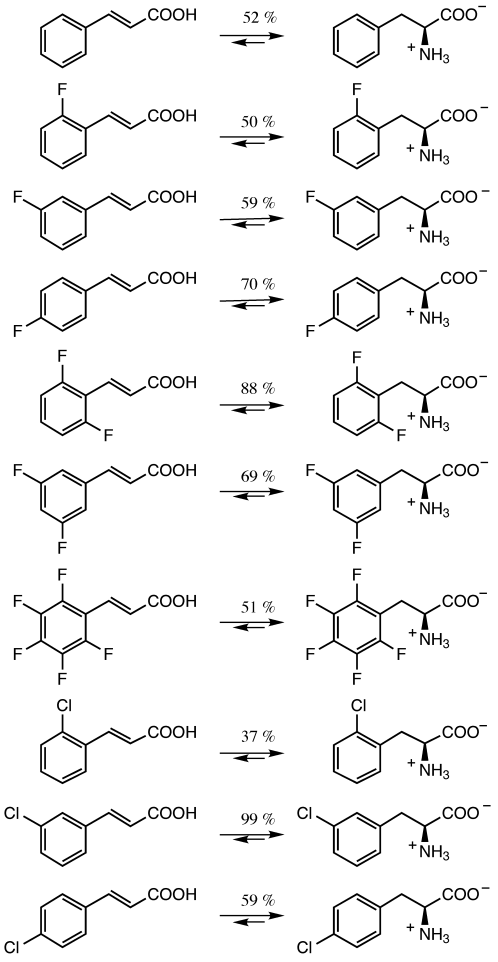
Scheme 1. Postulated mechanism, partial putative active site/substrate, and active site/σ-complex models for the reaction catalyzed by phenylalanine ammonia-lyase.

The question is legitimate, why the β-proton is abstracted leading to an exocyclic double bond while abstraction of the ring proton would straightforwardly lead to re-aromatization. Although the latter would preferentially take place in solution, PAL prevents this reaction by excluding any basic group in the binding site for the phenyl group. Simultaneously, a basic group is correctly positioned to abstract the *pro-S* β-proton of the substrate.

In the present communication we describe the enantiospecific synthesis of various fluoro- and chlorophenylalanines by reversal of the PAL reaction. Kinetic measurements with these new substrates and the results from theoretical calculations and a proposed partial active site model are discussed in terms of the Friedel–Crafts-type mechanism of the PAL reaction.

Results and Discussion

Enantioselective synthesis of L-phenylalanines halogenated in the ring: Contrary to early reports that the PAL reaction is irreversible[17] it can be reversed by applying high concentrations of ammonia.[18] For preparative conversions the halogenated cinnamic acids were solved in half concentrated ammonia solution whose pH was brought to 10 by bubbling CO_2 into the solution. The reaction was started by addition of recombinant PAL (1–2 iU). After incubation overnight at 37 °C the enzyme was removed by boiling and acidification (pH 1.5) followed by filtration. Chromatography on an acidic cation exchange column afforded the enantiomerically pure substituted phenylalanines in moderate to excellent yields. Their *ee* was determined by chromatography on a chiral column to be more than 99%. The separability of the enantiomeric phenylalanine derivatives was checked also on chiral thin layer plates. The halogenated cinnamic acids and phenylalanines were characterized by their NMR-, and UV spectra, as well as their R_f values. Their structures together with the yields of the isolated phenylalanines are shown in Scheme 2.



Scheme 2. Preparative conversion of various halogenated phenylalanines by reversal of the phenylalanine ammonia-lyase reaction.

Determination of the kinetic constants for the halogenated phenylalanines: The prerequisits for the quantitative measurement of the concentrations of the cinnamic acid products was the determination of their extinction coefficients (ε) at the wavelengths at which a maximal difference existed between those of the arylalanines and the corresponding cinnamic acids (see Experimental Section). The values were

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measured in 0.1M Tris-buffer pH 8.8. These were the conditions also for the kinetic measurements. In columns 4 and 5 of Table 1 the V_{max} values relative to that of phenylalanine and the K_m values, respectively, are listed.

Inspection of the relative V_{max} values reveals that phenylalanines halogenated in the 3′-position react on PAL significantly faster than the parent compound. This is consistent with an electrophilic attack of MIO at the aromatic nucleus. A halogen substituent in 3′-position facilitates such an attack in the *ortho*- or *para* position by stabilisation of the cationoid transition state. Both positions are available for the attack by the sterically fixed MIO due to free rotation of the phenyl ring before substrate binding. This effect is similar to that previously found with a 3′-hydroxyphenylalanine (*m*-tyrosine) as substrate.[15] Halogen substituents in the phenyl ring may also influence the acidity of the β-protons of the side chain. While this effect should activate the β-protons in the neutral ground state, the opposite effect is expected for the cationoid intermediate. In the latter, delocalization of the positive charge to the halogen atoms will decrease electron deficiency in the ring and hence diminish the acidity of the side chain β protons. The acidifying effect of the halogen substituents is, however, much less than that of a nitro group. Apart from that, the β-proton abstraction does not seem to be the rate determining step for good substrates as shown by deuterium labelling.[19] While the attack by MIO is partially rate determining, another rate-limiting step seems to be product release. The kinetic significance of these two steps is substrate-dependent. The K_m values vary only moderately, but roughly increase with increasing numbers of the halogenic substituents.

Theoretical calculations: To demonstrate the feasibility of the electrophilic attack by MIO at the halogenated aromatic rings semiempirical calculations for the heat of formation and for the activating effect on the β-protons by the positive charge in the ring were carried out. To facilitate the problem, semiempirical calculations were applied to simplified models of the putative σ complexes containing the substrates in their zwitterionic form. In these studies two types of models were investigated. Simple models contained a methyl group which corresponds to the carbon of the methylidene moiety of MIO (Scheme 3), while the more sophisticated models were derived from a putative partial active site model (Scheme 1). Conformational analysis of the simple model from L-Phe indicated that the most decisive conformation factor for the molecular properties is the dihedral angle (Φ) between the $C_{1'}-C_{2'}$ and the *pro-S* C_β–H bonds (Scheme 3). Results from semiempirical calculations are compiled in columns 2 and 3 of Table 1.

With respect to the σ complex formation and scissible bond polarization the 2′F, 2′Cl and 4′Cl substituted compounds, particularly the B-type sigma complexes are similar to the unsubstituted L-Phe. Accordingly, their kinetic behavior is similar to that of the natural substrate.

The putative active site model suggests that the aromatic ring of the substrate is sandwiched between MIO and a phenyl ring of Phe399. The arrangement as shown in Scheme 1 allows maximal overlap between the π system of MIO and the aromatic C_2 position. The 2′ H of the aromatic moiety might be stabilized in a charge transfer or π complex type manner without the possibility of rearomatization by its removal. The conserved nature of the analogous phenylalanine in all PAL and HAL sequences (e.g., the GGN**F**H segment is present in the HAL enzymes of microorganisms such as *P. putida* or *B. subtilis* as well as in mammalian HAL enzymes like mice, rat or human) supports the importance of this amino acid.

Inspection and comparison of data with the simple σ-complex models (Scheme 3, Table 1) and partial active site/σ-complex models (Scheme 1, Table 1) reveals some important

Table 1. Kinetic constants for the various phenylalanine analogues and calculated properties of their active complex models.

Substrate (σ complex model)		$\Delta\Delta H_{\sigma\text{-}g}$[a] [kcal mol^{-1}][b]	$\Delta\Delta H_{\sigma\text{-}g}$[a] [kcal mol^{-1}][c]	$\Delta\Delta C_\beta$–$H_{\sigma\text{-}g}$[d] [M.c.u]	$V_{max}/V_{max\text{-}Phe}$[e]	K_m [mM]
L-Phe		0.0	0.0	0.184	1.00	0.033
2′F-L-Phe	(A)	15.7	12.4	0.135	1.14	0.065
	(B)	7.8	3.6	0.129		
3′F-L-Phe	(A)	3.1	3.9	0.109	2.09	0.079
	(B)	1.5	1.8	0.116		
4′F-L-Phe		7.6	3.1	0.128	0.56	0.010
2′,6′F_2-L-Phe		21.9	14.6	0.160	0.85	0.085
3′,5′F_2-L-Phe		4.8	4.1	0.037	2.72	0.159
2′,3′,4′,5′,6′F_5-L-Phe		29.9	15.0	0.141	0.16	0.076
2′Cl-L-Phe	(A)	7.4	23.0	0.117	1.03	0.050
	(B)	5.2	11.0	0.115		
3′Cl-L-Phe	(A)	−1.7	15.6	0.100	2.01	0.094
	(B)	−4.2	−0.1	0.102		
4′Cl-L-Phe		3.2	1.9	0.126	0.82	0.045
β-(5-pyrimidinyl)-D,L-Ala		25.8	10.0	0.229	0.80	4.2
β-(2-pyrimidinyl)-D,L-Ala		−24.0	18.8	−0.314	0.00	(K_i = 7 mM)

[a] The difference for heat of formation calculated for the active complex model for L-Phe and the ground state L-Phe substrate was substracted from the corresponding difference for the substituted models and substrates. [b] Values calculated for simple models (fixed $\Phi = -90^\circ$ torsion angle, compare Scheme 3) using PM3 method[25] in vacuum. [c] Values for the partial active site models (c.f. Scheme 1) were obtained using MM+ optimization (with fixed position of the methyl groups of the MIO and Phe399 models and carboxylate of the substrate)[26] followed by single point PM3 calculation of the optimized structure.[25] [d] The difference in Mulliken charges on the C_β–H atoms in the substrate substracted from the corresponding difference in the simple models (fixed $\Phi = -90^\circ$ torsion angles, compare Scheme 3). [e] Relative V_{max} values of the analogues compared with V_{max} with L-Phe.

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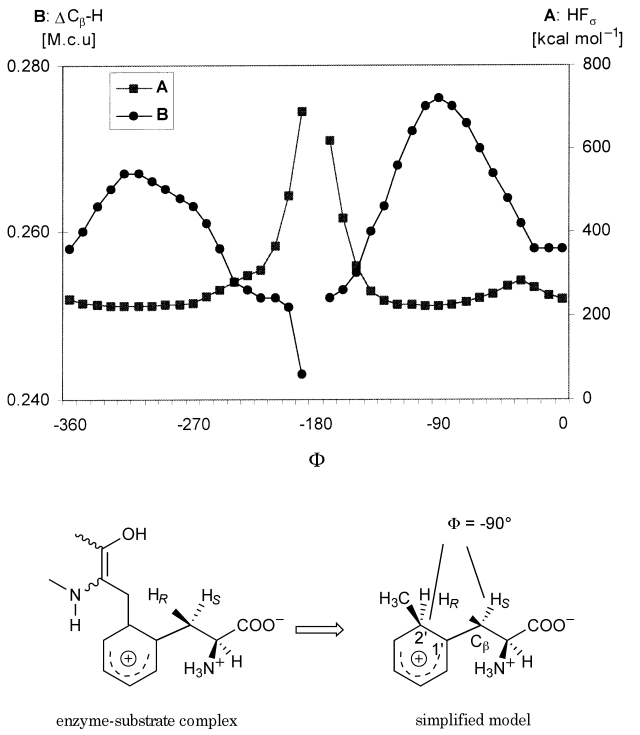
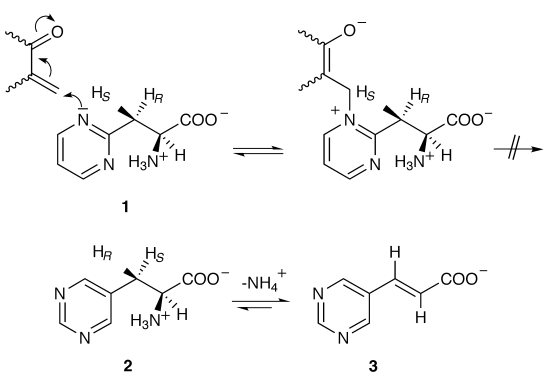


Scheme 3. Conformational properties of the simplified model and the postulated σ complex as intermediate of the phenylalanine ammonia-lyase reaction.

differences. The phenyl ring of Phe399 in these σ complexes adopts nearly perpendicular arrangement to the C_{sp^3}–H bond axis and the hydrogen points toward the central point of the ring indicating strong interaction with the aromatic π-electrons. For the 2′-halogen substituted analogues the B-type intermediates are substantially favored, especially in the 2′-chloro case. The presence of the Phe399 phenyl ring substantially lowers the relative energy of the intermediate cations for the polyfluorinated analogues, especially for 2′,6′-F_2-L-Phe and 2′,3′,4′,5′,6′-F_5-L-Phe. Thus, the maximal energy requirement (2′,3′,4′,5′,6′-F_5-L-Phe: 29.9 kcal mol^{-1}) calculated in vacuum in the absence of Phe399 for formation of the σ complex intermediates substantially dropped when the aromatic "protective group" was present (2′,3′,4′,5′,6′-F_5-L-Phe: 15.0 kcal mol^{-1}). In addition, the *pro-S* C_β–H bond in the ground state of the 3′,5′F_2 substituted compound is significantly more polarized (ca. 0.040 Mulliken charge units) then in all the other models.

For the 5-pyrimidinyl compound relatively high energy is required for formation of the σ complex but the polarization of the scissible *pro-S* C_β–H is relatively strong. Accordingly, a high K_m value but a normal V_{max} is observed. The inhibition observed with the 2-pyrimidinyl compound is a consequence of the stability of the cationic pyrimidinium complex and the unfavorable proton abstraction from the negatively polarized *pro-S* C_β–H bond (Scheme 4).

All these correlations are consistent with the intermediacy of the Friedel–Crafts-type σ complexes and the kinetic significance of their formation as partially rate-determining step. Further support for our mechanism was provided by recent publications.[20, 21]



Scheme 4. The postulated unreactive pyrimidinium complex with 2-pyrimidinylalanine **1** and the reaction with 5-pyrimidinylalanine **2**. (Although for the experiments the racemic compounds were used, only the L-enantiomers are shown here which are presumed to be the enzymatically active species).

Synthesis of and kinetic measurements with pyrimidinylalanines: The synthesis of β-(2-pyrimidinyl)-D,L-alanine (**1**) was carried out in six steps following the procedure of Haggerty et al.[22] A chemoenzymatic strategy was applied to the preparation of β-(5-pyrimidinyl)-L-alanine (**2**). First, β-(5-pyrimidinyl)-acrylic acid (**3**) was synthesised starting from 5-bromo-pyrimidine and *tert*-butylacrylate under Heck conditions. Treatment of the product with trifluoro acetic acid afforded the free acid **3**, which was converted in 57% yield into the corresponding enantiomerically pure L-alanine **2** using PAL as a catalyst.

While the 5-pyrimidinyl analogue **2** turned out to be a moderately good substrate of PAL, its 2-pyrimidinyl counterpart **1** was a competitive inhibitor. The corresponding kinetic constants are shown in Table 1. The K_m value of **2** is comparable to the K_i value of **1**.

These results support the postulated *ortho*-attack of MIO at the aromatic nucleus. When both *ortho*-positions are occupied by a nitrogen atom such an attack leads to a pyrimidinium ion which is so stable that no further reaction occurs (see Scheme 4). The competitive nature of the inhibition requires, however, that the binding of **2** is reversible.

Experimental Section

Recombinant PAL: The lyase was overexpressed in *E. coli* and purified as described, first according to Schuster and Rétey[6] and later using the improved method of Baedeker and Schulz.[23]

Chloro- and fluoro-L-phenylalanines: Chlorocinnamic acids and fluorocinnamic acids were purchased from Fluka and from Lancaster. Reaction of the chloro- and fluorocinnamic acids with ammonia catalyzed by PAL afforded the corresponding chloro- and fluoro-L-phenylalanines. A concentrated aqueous ammonia solution (10 mL) was diluted with water (10 mL). The pH of the solution was adjusted to pH 10 by bubbling CO_2 through the solution. Cinnamic acid (100 mg, 0.675 mmol) and recombinant wtPAL (1 U) (*Petroselinum crispum*) were added to this solution. The reaction mixture was agitated overnight at 37 °C. The solution was acidified with 5% HCl to pH 1.5, heated to boiling, filtered, and applied to a Dowex 50 cation exchange resin column. The elution occurred with diluted ammonia solution. The crude product was purified with HPLC [Nucleosil 100 C18, 7 μm, 250 × 20 mm (Macherey–Nagel); flow rate: 5 mL min^{-1}; load: 20 mg; mobile phase: 0–15 min: 99.9% H_2O/0.1% TFA, 15–90 min: linear increasing gradient to 99.9% CH_3CN/0.1% TFA, retention times:

L-phenylalanine: 41.7 min, 2′-fluoro-L-phenylalanine: 42.4 min, 3′-fluoro-L-phenylalanine: 44.1 min, 4′-fluoro-L-phenylalanine: 43.9 min, 2′,6′-difluoro-L-phenylalanine: 42.7 min, 3′,5′-difluoro-L-phenylalanine: 46.5 min, 2′,3′,4′,5′,6′-pentafluoro-L-phenylalanine: 48.4 min, 2′-chloro-L-phenylalanine: 45.3 min, 3′-chloro-L-phenylalanine: 48.0 min, 4′-chloro-L-phenylalanine: 48.7 min]. For the determination of the enantiomeric excess we used a chiral column from astec/ict (Handels-GmbH, 61352 Bad Homburg, Germany) (Chirobiotic T, 5 μm, 250 × 4.6 mm; mobile phase: 70% H_2O/30% EtOH; flow rate: 0.8 mL min^{-1}; load: 5 μg; retention times: L-phenylalanine: 7.22 min, 2′-fluoro-L-phenylalanine: 6.87 min, 3′-fluoro-L-phenylalanine: 6.98 min, 4′-fluoro-L-phenylalanine: 7.20 min, 2′,6′-difluoro-L-phenylalanine: 6.42 min, 3′,5′-difluoro-L-phenylalanine: 6.75 min, 2′,3′,4′,5′,6′-pentafluoro-L-phenylalanine: 5.89 min, 2′-chloro-L-phenylalanine: 9.06 min, 3′-chloro-L-phenylalanine: 8.22 min, 4′-chloro-L-phenylalanine: 9.25 min). In all cases enantiomeric excess over 99% was determined. The D-enantiomers were eluted between 1 and 2 min later, as described in the astec/ict prospect.

***β*-(2-Pyrimidinyl)-D,L-alanine (1)**: Preparation as reported by W. J. Haggerty, R. H. Springer, and C. C. Cheng.[22]

***β*-(5-Pyrimidinyl)-L-alanine (2)**: *β*-(5-Pyrimidinyl)-acrylic acid (**3**) obtained by Heck coupling between 5-bromopyrimidine and *tert*-butyl acrylate, followed by ester-cleavage in TFA.[24]

A concentrated, aqueous ammonia-solution (10 mL) was diluted with distilled water (10 mL). The pH was adjusted to 10.0 by bubbling CO_2 into the solution. Acrylic acid **3** (64.3 mg, 0.428 mmol) and PAL (1 U) were added to this solution. After 24 h agitation at 37 °C the enzyme was denatured by heat and removed by filtration. The clear solution was applied to a Dowex 50 cation exchange resin column. The elution occurred with diluted ammonia solution. The crude product was purified with HPLC (Nucleosil 100 C18, 7 μm, 250 × 20 mm; Macherey–Nagel, flow rate: 5 mL min^{-1}; load: 20 mg; mobile phase: 0–15 min: 99.9% H_2O/0.1% TFA, 15–90 min: linear increasing gradient to 99.9% CH_3CN/0.1% TFA, retention times: 23.5 min). The solvent was carefully removed under reduced pressure to give a white solid (50 mg, 0.246 mmol, 57%).

Determination of V_{max}- and K_m values: The kinetic constants were determined by measuring the UV absorption of the produced acrylates (*trans*-cinnamic acid: $\varepsilon_{290} = 10000$ L cm^{-1} mol^{-1}; 2′-fluorocinnamic acid: $\varepsilon_{280} = 12550$ L cm^{-1} mol^{-1}; 3′-fluorocinnamic acid: $\varepsilon_{280} = 13850$ L cm^{-1} mol^{-1}; 4′-fluorocinnamic acid: $\varepsilon_{280} = 15320$ L cm^{-1} mol^{-1}; 2′,6′-difluorocinnamic acid: $\varepsilon_{290} = 3960$ L cm^{-1} mol^{-1}; 3′,5′-difluorocinnamic acid: $\varepsilon_{290} = 5060$ L cm^{-1} mol^{-1}; 2′,3′,4′,5′,6′-pentafluorocinnamic acid: $\varepsilon_{280} = 7910$ L cm^{-1} mol^{-1}; 2′-chlorocinnamic acid: $\varepsilon_{285} = 10770$ L cm^{-1} mol^{-1}; 3′-chlorocinnamic acid: $\varepsilon_{285} = 10680$ L cm^{-1} mol^{-1}; 4′-chlorocinnamic acid: $\varepsilon_{290} = 15790$ L cm^{-1} mol^{-1}; *β*-(5-pyrimidinyl)-acrylic acid: $\varepsilon_{270} = 11110$ L cm^{-1} mol^{-1}) using 0.05–10 mM of the corresponding amino acid as substrate. Conditions: 0.05 mM–10 mM substrate in 0.1 M Tris-HCl buffer pH 8.8 at 30 °C. The reaction was initiated by addition of 20 μg (0.02 U) PAL. ^{1}H-NMR (250 MHz; D_2O, 25 °C): L-phenylalanine: $\delta = 7.28–7.43$ (m, 5H), 3.96 (t, 1H), 3.27 (dd, 1H), 3.08 (dd, 1H), 2′-fluoro-L-phenylalanine: $\delta = 7.34$ (m, 2H), 7.17 (m, 2H), 4.26 (t, 1H), 3.40 (dd, 1H), 3.20 (dd, 1H), 3′-fluoro-L-phenylalanine: $\delta = 7.38$ (m, 1H), 7.08 (m, 3H), 4.19 (t, 1H), 3.32 (dd, 1H), 3.16 (dd, 1H), 4′-fluoro-L-phenylalanine: $\delta = 7.26$ (m, 2H), 7.10 (m, 2H), 4.28 (t, 1H), 3.29 (dd, 1H), 3.15 (dd, 1H), 2′,6′-difluoro-L-phenylalanine: $\delta = 7.36$ (m, 1H), 6.99 (m, 2H), 4.28 (t, 1H), 3.37 (dd, 1H), 3.26 (dd, 1H), 3′,5′-difluoro-L-phenylalanine: $\delta = 6.90$ (m, 3H), 4.32 (t, 1H), 3.35 (dd, 1H), 3.17 (dd, 1H), 2′,3′,4′,5′,6′-pentafluoro-L-phenylalanine: $\delta = 4.01$ (t, 1H), 3.26 (dd, 1H), 3.19 (dd, 1H), 2′-chloro-L-phenylalanine: $\delta = 7.48$ (m, 1H), 7.34 (m, 3H), 4.05 (t, 1H), 3.45 (dd, 1H), 3.16 (dd, 1H), 3′-chloro-L-phenylalanine: $\delta = 7.36$ (m, 3H), 7.21 (m, 1H), 4.20 (t, 1H), 3.31 (dd, 1H), 3.16 (dd, 1H), 4′-chloro-L-phenylalanine: $\delta = 7.40$ (d, 2H), 7.26 (d, 2H), 3.97 (t, 1H), 3.23 (dd, 1H), 3.10 (dd, 1H).

Theoretical calculations

Simple σ-complex models for L-phenylalanine, the halogenated L-phenylalanine, and pyrimidinyl-L-Ala derivatives were calculated in vacuo using AM1 and PM3 methods.[25, 26] Conformational analysis showed two favored states for zwitterionic structures with antiperiplanar *pro-S* H_β–NH_3^+ and zig–zag arrangement (Scheme 3). All further calculations refer to the arrangement with a fixed $\Phi = -90°$ torsion angle. Since the parallel results from the two methods were similar, values derived from PM3 calculations are indicated only. In the cases where two distinct σ complex models may arise by rotation of the substrate prior to the active complex formation, model A represents the structure where the halogen substituent is closer to the sp^3 center in the σ complex.

The *putative active site model* was built from a sequence segment of PAL (SP: P24481) containing the ASGDL active site motif and the F399 by homology-modelling[27] using HAL structure (PDB: 1B8F[10]) as folding template, followed by Gromos[27] and Amber[26] energy minimizations. The ASG structure of the model was finally replaced by MIO taken from the 1B8F HAL structure (less than 0.4 Å deviations in the corresponding atomic positions).

Partial active site/σ *complex models* (c.f. Scheme 1) were obtained using MM+ optimization (with fixed position of the methyl groups of the MIO and Phe399 models and carboxylate of the substrate)[26] followed by single point PM3 calculation of the optimized structure.[25]

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