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Phenylalanine ammonia-lyase catalyzed deamination of an acyclic amino acid - Enzyme mechanistic studies aided by a novel microreactor filled with magnetic nanoparticles

--Manuscript Draft--

Manuscript Number:	
Article Type:	Communication
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Keywords:	enzyme catalysis; magnetic nanoparticles; microreactor; phenylalanine ammonia-lyase; reaction mechanism
Manuscript Classifications:	Enzyme catalysis; Enzyme models; Microreactors; Nanoparticles; Reaction mechanisms
Suggested Reviewers:	Nicolas Turner, PhD Professor, The University of Manchester nicholas.turner@manchester.ac.uk Prof. Turner is one of the most renown experts utilizing ammonia-liases among other enzymes for biocatalysis
	Uwe T Bornscheuer, PhD Prof., University of Greifswald bornsche@uni-greifswald.de Prof. Bornscheuer is one of the most renown experts in enzyme chemistry and he also published a paper in Angewandte Chemie on phenylalanine ammonia-lyase
Opposed Reviewers:	
Abstract:	Phenylalanine ammonia-lyase (PAL), in many organisms, catalyzes the deamination of L-phenylalanine (Phe) to (E)-cinnamate by the aid of its MIO prosthetic group. By using PAL, immobilized on magnetic nanoparticles and fixed in a microfluidic reactor with an in-line UV detector, we first demonstrated that PAL can catalyze the ammonia elimination from the acyclic propargylglycine (PG) to yield (E)-pent-2-ene-4-ynoate indicating new opportunities to extend the MIO-enzyme toolbox towards acyclic substrates. Deamination of PG, being acyclic, cannot involve a Friedel-Crafts-type attack at an aromatic ring. The reversibility of the PAL-reaction, also demonstrated by the ammonia addition to (E)-pent-2-ene-4-ynoate yielding enantiopure L-PG, contradicts the proposed highly exothermic single-step mechanism. Computations on the QM/MM models of the N-MIO intermediates from L-PG and L-Phe in PAL, showing similar arrangements within the active site, support a mechanism via the N-MIO intermediate.

Author Comments:	Dear Editor, Please find attached our manuscript entitled "Phenylalanine ammonia-lyase catalyzed deamination of an acyclic amino acid - Enzyme mechanistic studies aided by a novel microreactor filled with magnetic nanoparticles" which is a revised version of the manuscript submitted to Angewandte Chemie, International Edition (ANIE-S-15-08600) under the title "Systems Biocatalysis Tools for Enzyme Mechanistic Studies - Phenylalanine Ammonia-lyase Catalyzed Deamination of an Acyclic Amino Acid". I hope you will find eligible our revised manuscript for publication in ChemBioChem. Our paper demonstrates for the first time, by using phenylalanine ammonia-lyase (PAL), immobilized on magnetic nanoparticles and fixed in a microfluidic reactor with an in-line UV detector, that PAL can catalyze the ammonia elimination from an acyclic amino acid (propargylglycine) indicating new opportunities to extend the MIO-enzyme toolbox towards acyclic substrates. In addition, we answered a long discussed question about the possible mechanistic routes for aromatic ammonia-lyases and the s.c. MIO-enzymes which might justify the publication of our paper.
Section/Category:	
Additional Information:	
Question	**Response**
Dedication	
Submitted solely to this journal?	No
Please give details of any related work. as follow-up to "Submitted solely to this journal?"	The first version of this manuscript was submitted originally to Angewandte Chemie.
Has there been a previous version?	Yes
Please state previous 1) Manuscript ID and 2) journal. 3) If the paper was reviewed, please include a point-by-point response to the reviewer comments. as follow-up to "Has there been a previous version?"	This submission is a corrected version of the manuscript ANIE-S-15-08600, submitted originally to Angewandte Chemie and transferred to ChemBioChem upon the suggestion of the Reviewers and Editor. Reviewer 1: ... With this study broad the possibilities to another aminoacids however I am not sure that this enzyme can act over other aliphatic aminoacids and can be of utility in organic synthesis. Response: The authors agree that this enzyme cannot act on aliphatic amino acids, but acceptance of a wide range of unnatural olefinic and acetylenic amino acids as substrates are foreseen by computations. Reviewer 2: (i) the term 'systems biocatalysis' … has a completely different meaning …, its appearance in the title and in the conclusions should be eliminated. Response: The term 'systems biocatalysis' has been eliminated from title and conclusions. This is the reason why this submission has a title different from the previous one. All changes in the novel submission are marked by typesetting the changed content in red. (ii) scheme 1: in order to enhance the readability of the graphic, it is suggested to incorporate the structure of the MIO-cofactor to top left and the bottom intermediate. for the anti-elimination pathway, a base is missing in the L-alanine structure, which deprotonates Hs. Response: The structure of the MIO-cofactor is now included in Scheme 1. The second statement is related to the ‘single-step’ proposal which we also criticize. However, in this case TyrB-O(-), which is in the close neighborhood of Hs, can act as a base. (iii) overall, the paper is too long to fit into Angew. if publication in Angew. is desired, it is suggested to drastically shorten the sections on the mechanistic debate and the § describing the immobilization and the advantages of MNPs. if this would disrupt the

	flow of arguments, ChemBioChem would be a better alternative. Response: We accepted the transfer of our manuscript to ChemBioChem. (iv) a serious point of criticism arises from the use of L-Phe, but rac-PropargylGly as substrates for kinetic measurements. I would not dare to compare kcat and KM values derived from an enantiopure and a racemic substrate, because using the racemate, inhibition by the D-enantiomer must be taken into account. these values should be re-measured by using L-PropargylGly (which the authors have prepared during the course of this study). Response: We fully agree with the criticism of Reviewer 2. Therefore, we performed the kinetics with L-phenylalanine (L-Phe), L-propargyglycine (L-PG) and DL-PG at identical high enzyme concentration as required for the reaction with PG. The content of the original Table 1 was replaced with these data (the corresponding kinetic curves in the Supplementary Information were also replaced). The small section above the Table 1 in the main article was changed accordingly (changed content is indicated by red typesetting). (v) references: a number of references show issue numbers, which should be deleted to comply with the style of Angew.; journal name abbreviation of ref. 42 should read Plant Physiol. according to the recommendations of CASSI. Response: All required corrections were performed in the reference list.
Animal/tissue experiments?	No

COMMUNICATION

Phenylalanine ammonia-lyase catalyzed deamination of an acyclic amino acid – Enzyme mechanistic studies aided by a novel microreactor filled with magnetic nanoparticles

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Abstract: Phenylalanine ammonia-lyase (PAL), in many organisms, catalyzes the deamination of L-phenylalanine (Phe) to (*E*)-cinnamate by the aid of its MIO prosthetic group. By using PAL, immobilized on magnetic nanoparticles and fixed in a microfluidic reactor with an in-line UV detector, we first demonstrated that PAL can catalyze the ammonia elimination from the acyclic propargylglycine (PG) to yield (*E*)-pent-2-ene-4-ynoate indicating new opportunities to extend the MIO-enzyme toolbox towards acyclic substrates. Deamination of PG, being acyclic, cannot involve a Friedel-Crafts-type attack at an aromatic ring. The reversibility of the PAL-reaction, also demonstrated by the ammonia addition to (*E*)-pent-2-ene-4-ynoate yielding enantiopure L-PG, contradicts the proposed highly exothermic single-step mechanism. Computations on the QM/MM models of the *N*-MIO intermediates from L-PG and L-Phe in PAL, showing similar arrangements within the active site, support a mechanism via the *N*-MIO intermediate.

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Supporting information for this article is given via a link at the end of the document.

In nature, phenylalanine, tyrosine and histidine ammonia-lyases (PAL, [1] TAL [2] and HAL, [3] respectively) catalyze the ammonia elimination[4] from their corresponding substrates L-phenylalanine (L-Phe), L-tyrosine (L-Tyr) and L-histidine. Early biochemical studies[4] on PAL[1] and HAL[3] identified in both an electrophilic prosthetic group. First, X-ray crystallography of HAL[5] revealed the 5-methylene-3,5-dihydro-4*H*-imidazol-4-one (MIO) structure as the electrophilic prosthetic group.[6] In addition to HAL, MIO was later identified in crystal structures of PALs from yeasts,[7,8] plant[9] and bacteria, [10, 11] in TAL from bacteria [12] and also in L-phenylalanine and L-tyrosine 2,3-aminomutases (PAM[13,14] and TAM, [15] respectively). PAL has a potential in the enzyme replacement therapy of phenylketonuria[11,16] and as biocatalyst in the preparation of aromatic L- and D-α-amino acids.[4b,17]

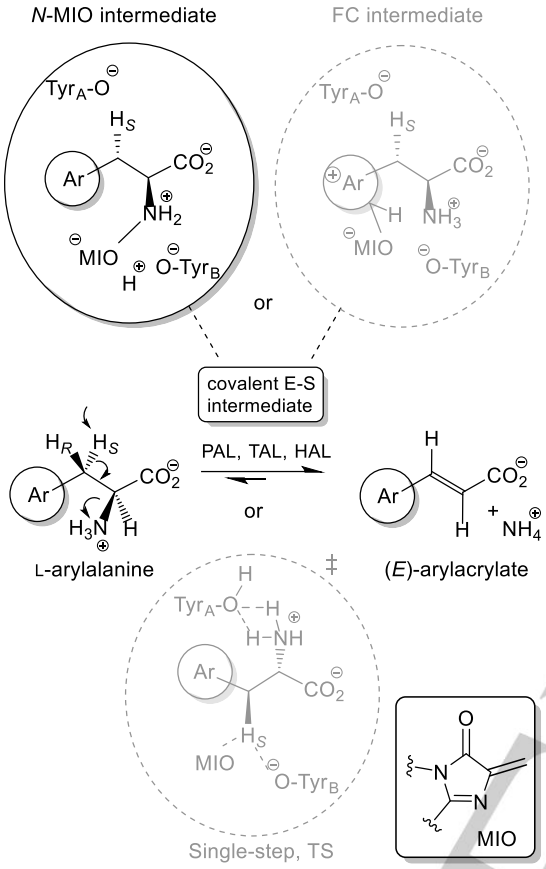
In spite of their divergent properties, based on the identity of their prosthetic group, it was believed that catalysis by the so called MIO-enzymes involves similar mechanisms.[18,19] Active site mutagenesis studies on PAL, [20] TAL, [21] and HAL [22] also demonstrated their similarity. Besides the Ala-Ser-Gly triad as the precursor of the MIO prosthetic group, Tyr110 / Tyr351 in PAL from *Petroselinum crispum* (*Pc*PAL),[20] Tyr60 / Tyr300 in TAL from *Rhodobacter sphaeroides* (*RsTAL*)[21] and Tyr54 / Tyr281 in HAL from *Pseudomonas putida* (*Pp*HAL)[22] all proved to play a crucial role, too. One of the catalytically essential Tyr residues sits in a mobile, lid-like loop (Tyr_A: Tyr110, Tyr60 and Tyr54 in *Pc*PAL, *Rs*TAL and *Pp*HAL, respectively), [5, 9,12,23] while the other one is buried inside the active site close to the methylidene moiety of MIO (Tyr_B: Tyr351, Tyr300 and Tyr281 in *Pc*PAL, *Rs*TAL and *Pp*HAL, respectively).[5, 9,12]

Based on extensive biochemical, structural and computational studies, three significantly different mechanisms were suggested for the reactions catalyzed by HAL,[24,25] PAL[26,27,28] and TAL[29] (Scheme 1).[4]

First, it was suggested that an interaction between the amino group of the substrate and the electrophilic prosthetic group of the enzyme facilitated the ammonia-lyase reaction by generating a better leaving group (in Scheme 1: *N*-MIO intermediate). For HAL[26] and PAL[28] elimination was assumed to be a stepwise process *via* a carbanion intermediate (E_1cB) while for TAL[19] a concerted process.

By using L-Phe, stereospecifically deuterated at C_3, it has been shown that the PAL-catalyzed reaction proceeded with the loss of the *pro-S* β-proton.[30] Because abstraction of the non-acidic *pro-S* β-proton was considered to be difficult by an enzymic base in the course of ammonia elimination, an alternative mechanism has been proposed involving a Friedel-Crafts (FC) type attack at the aromatic ring of the substrates by the electrophilic prosthetic group which acidifies the *pro-S* β-hydrogen by generating a positive charge at the FC-complex (in Scheme 1: FC intermediate).[25,27]

Recently, a third, single-step mechanism was proposed for the TAL reaction assuming a single transition state (TS) for the deamination without the formation of a covalent bond between the substrate and the MIO group (in Scheme 1: Single-step, TS).[29]



Scheme 1. Alternative pathways for the reaction catalyzed by the MIO-containing aromatic ammonia-lyases (PAL, TAL and HAL).

Decision among the above mechanistic proposals has remained up to this date controversial.[31] Although it was demonstrated that *Pc*PAL accepts as substrates several cycloalkenylalanines such as DL-3-(cycloocta-1,3,5,7-tetraen-1-yl)alanine[32] and L-3-(cyclohexa-1,4-dien-1-yl)alanine,[28, 33] these reactions could still be rationalized by a Friedel-Crafts-like or a single-step mechanism. On the other hand, the constitutional isomer L-3-(cyclohexa-2,5-dien-1-yl)alanine[33] and the saturated version DL-β-cyclohexylalanine[28] were not accepted as substrates but proved to be moderate inhibitors for PAL.

Acceptance of a non-aromatic acyclic amino acid as a substrate of PAL strongly disfavors the FC mechanism. Therefore, we decided to test PAL with DL-propargylglycine (DL-PG, Figure 1). To detect the forming (*E*)-pent-2-en-4-ynoate, even in very small amounts, an efficient novel microfluidics-based test system using PAL-coated magnetic nanoparticle and in-line UV-Vis detection capabilities was used.

Application of microfluidic systems for systems biocatalytic and bioanalytical purposes is an efficient new solution.[34] The most important advantages of using fluidic systems of reduced dimensions for analytical and catalytic applications are[34] *(i)* minute quantities (down to picoliters) of sample and reagents are required, *(ii)* comparatively fast reaction times due to short molecular diffusion lengths, and *(iii)* large surface-to-volume ratios.

Immobilization of proteins is another efficient tool for systems biocatalysis enabling a variety of applications.[35] On nanoparticles or magnetic nanoparticles (MNPs), the immobilized proteins are spread on a large surface and free of a diffusion barrier.[36] Suspensions of MNPs are free from sedimentation and approximates the behavior of homogeneous liquids. Furthermore, separation of MNPs from the liquid phase is simple.[37] For use in the novel microfluidic reactor the established epoxy chemistry[38] was applied to bind PAL onto magnetic nanoparticles (MNP-PAL in Figure 1).

In microfluidic systems the protein-coated MNPs can *(i)* either flow together with the liquid, or *(ii)* can be anchored by a magnet at definite site(s) while the free flowing fluid passes the region(s) where the particles are fixed.[34b,39] It was demonstrated recently that enzyme-coated MNPs can be fixed at a certain position of a Lab-on-a-Chip (LoC) system by using a permanent magnet.[40] To the best of our knowledge, no microfluidic system was built so far exploiting multiple magnetic cells for biotransformation, analysis or protein biochemistry studies (Magne-Chip in Figure 1).

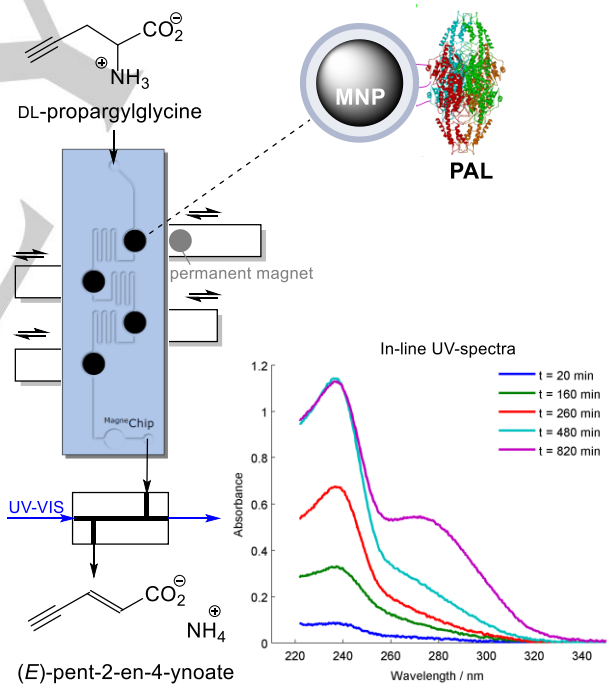


Figure 1. Ammonia elimination from DL-propargylglycine in a light protected microfluidic reactor with multiple magnetic-cells filled with PAL immobilized on MNPs and equipped with in-line UV-Vis detector (reaction in D_2O at pD 8.8, 37 °C). The progress of the reaction was followed by full UV-spectra.

This Magne-Chip was used for the microscale biotransformation of DL-propargylglycine in sodium carbonate-buffered D_2O at pD 8.8. For quantification of the product formation in the Magne-Chip device by in-line UV detection and in later enzyme kinetics studies, the chemical synthesis of (*E*)-pent-2-en-

4-ynoic acid (Scheme S2 in SI) was performed to determine the extinction coefficient (λ_{max}= 242 nm, ε= 10.4 mM cm^{-1}, water, pH 8.8) of the product (see Figures S5 and S6 in SI). This magnetic LoC-device with in-line UV-detection enabled to detect the formation of (*E*)-pent-2-en-4-ynoate at 242 nm and produced measurable quantities of the product for recording ^{1}H-NMR spectra without any work-up. Besides the significant increase of the UV-signal at 242 nm (up to A= 1.2) in the on-line UV-cell (Figure 1), the appearance of olefin hydrogen signals in the ^{1}H-NMR spectrum of the reaction mixture [at δ= 6.34 (*d*) and 6.85 (*d*) ppm] indicated unambiguously the formation of (*E*)-pent-2-en-4-ynoate. On the other hand, emergence of the UV signal at 274 nm during the process indicated the formation of further by-product(s) apart from (*E*)-pent-2-en-4-ynoate (Figure 1 and Figure S3).

After verifying that DL-propargylglycine was a true substrate of PAL, its kinetic parameters were determined with *Pc*PAL and compared to the values with L-phenylalanine (Table 1). The 30 times higher K_m of L-PG as compared to L-Phe indicated significantly much weaker binding of the non-aromatic, acyclic substrate than the natural one to the active site of *Pc*PAL. The turnover number (k_{cat}) of L-PG was only six time lower than that of L-Phe indicating that electronic effects involved in the elimination step were not significantly different for the two substrates.[41] The two times higher K_m and similar k_{cat} with DL-PG as with L-PG indicated negligible inhibition of PAL by D-PG.

Table 1. Kinetic constants of L-phenylalanine, L- and DL-propargylglycine with *Pc*PAL (30 °C, pH 8.8, TRIS buffer, 0.1M, 120 μg mL^{-1} *Pc*PAL).

Substrate	K_m (mM)	k_{cat} (s^{-1})
L-Phenylalanine	0.52	2.28
L-Propargylglycine	16.0	0.37
DL-Propargylglycine	32.9	0.34

It was assumed that the MIO-containing enzymes catalyze their ammonia elimination-abstraction by similar mechanisms.[18,19] Experiments indicated that at least the TAL and the PAL catalyzed reactions should have the same mechanism, because maize PAL accepts both L-Phe and L-Tyr as substrate.[42] Moreover, the H89F mutation[12,43] in *Rs*TAL resulted in conversion to full PAL activity and a F144H mutation in PAL1 from *A. thaliana* [43] led to full TAL activity. Therefore, conclusions drawn from computational studies on TAL[19,29] should be relevant for the PAL-reaction as well.

A detailed quantum mechanics/molecular mechanics (QM/MM) study found that ammonia elimination may proceed through a covalent *N*-MIO intermediate ("*N*-MIO intermediate" in Scheme 1) followed by a concerted elimination step in TAL.[19] This study suggested that in the reactive state, both Tyr_A and Tyr_B are deprotonated and the L-Tyr substrate is present as a zwitterion. A recent study, by independent QM/MM calculations, confirmed the possibility of the TAL reaction via the *N*-MIO intermediate[29] and at the same time hypothesized an alternative route for the ammonia elimination through a single transition state (TS) without forming a covalent bond between the substrate and the MIO prosthetic group ("single-step, TS" in Scheme 1).

Two arguments were raised against the route through an *N*-MIO intermediate in TAL:[29] *(i)* it is unlikely to have two tyrosine residues simultaneously deprotonated in the same active center without any strong charge compensation; and *(ii)* the calculated activation energy between the lowest energy *N*-MIO intermediate and the elimination product (26.3 kcal mol^{-1} or 28.5 kcal mol^{-1})[19,29] is rather high. For the alternative route through a single transition state (TS)[29] for the elimination a barrier of 16.6 kcal mol^{-1} was calculated. According to these calculations, the single-step reaction is quite exothermic with a barrier of 64.7 kcal mol^{-1} for the reverse reaction [i.e. ammonia addition to (*E*)-coumarate]. Therefore it was stated, that the "single-step route" is in conformity with the experimentally observed irreversibility.[29]

In fact, a reactive state with both Tyr_A and Tyr_B as deprotonated and the L-Tyr substrate as zwitterionic for the PAL (or TAL[19]) reaction can be easily formed at ~ pH 9 if the amino group of anionic L-Phe (or L-Tyr) substrate deprotonates Tyr_A–OH when entering into the active site containing only Tyr_B in a deprotonated state. This would perfectly agree with the analysis of the pH-profile of the PAL reaction showing that to be able to react the amino group of the substrate must not be protonated but in turn, there should be a group with a p*K* of 9 on the enzyme which must be protonated.[28] This eliminates the first argument raised against the calculated *N*-MIO route.

The calculated relative energies for the *N*-MIO intermediate varying between -5.5 and -21.6 kcal mol^{-1} even in the study favoring the single-step mechanism[29] indicated that the real *N*-MIO – product barrier may be lower than the disputed values[19,29] of 26.3 kcal mol^{-1} or 28.5 kcal mol^{-1}. Moreover, all structural data so far agree only with the *N*-MIO route. X-Ray structural studies on various MIO-enzymes revealed that several substrates and substrate analogues were found to be covalently bound via their *N*-atoms to the MIO-group in the of [2-aminoindan-2-phosphonate in H89F mutant of *Rs*TAL;[12] (3*R*)-3-amino-2,2-difluoro-3-(4-hydroxyphenyl)propanoic acid and (3*R*)-3-amino-2,2-difluoro-3-(4-methoxyphenyl)propanoic acid in *S. globisporus* TAM;[44] L-Tyr in the Y63F (Tyr_A) mutant of *Sg*TAM;[45] and (*S*)-3-amino-2,2-difluoro-3-phenylpropanoic acid in *T. canadensis* PAM[46]]. Our high level QM/MM calculations also indicated quite similar *N*-MIO-intermediate structures for the reaction of PAL with L-propargylglycine and with L-phenylalanine (Figure 2 and Figure S16).

The much higher Gibbs energies calculated for the Friedel-Crafts-type covalent complexes relative to the substrate-binding states (166.7 kcal mol^{-1} from L-Phe and 9.5 kcal mol^{-1} from L-PG) and their distorted, non-reactive structures (Figure S17 A and B) strongly disfavor the FC route ("FC intermediate" in Scheme 1) as possible pathway for the PAL (or TAL) reaction.

The initial energy for the calculations supporting the single-step mechanism[29] cannot be compared directly to that of the *N*-MIO-route calculations due to differences in atom numbers and total charges.[19,29] Nevertheless, the single-step proposal strongly contradicts to a number of experimental results on the PAL reaction with L-Phe because *(i)* negligible isotope effects were found for the atoms which should be involved in the single-step TS (^{15}N of 1.0021 and D of 1.15 for Phe dideuterated at C_3);[28] *(ii)* cinnamate as product was found to be released prior to ammonia in the catalytic sequence[47] and *(iii)* the PAL reaction was found to be reversible at elevated (>5 M) ammonia concentration.[48]

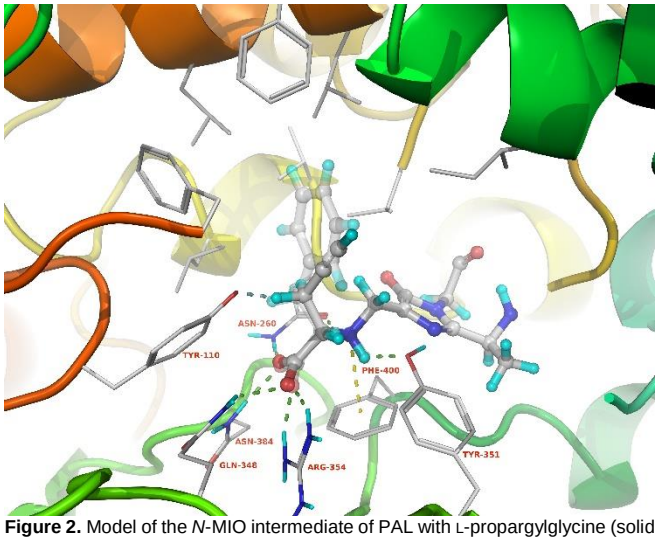


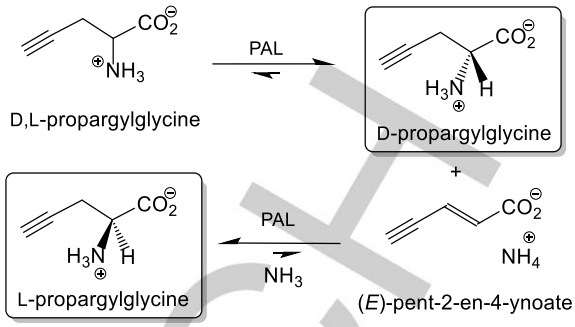
Figure 2. Model of the *N*-MIO intermediate of PAL with L-propargylglycine (solid stick model) compared to the model of *N*-MIO intermediate of PAL with its natural L-phenylalanine substrate (transparent stick model). The models were obtained by ONIOM (ωB97XD/6-31g(d):AMBER) calculations.

Moreover, our equilibration studies with PAL starting from both substrate and product at much lower ammonia concentrations indicated that ammonia addition to (*E*)-cinnamate proceeded easily even at quite low ammonia concentration (in 0.1 M, 0.5 M or 1.0 M ammonium carbonate buffered to pH 8.8 with CO_2). Equilibria were almost reached in 3 days (see SI) from both directions as the (*E*)-cinnamate / L-phenylalanine ratio values indicated [in 0.1 M $(NH_4)_2CO_3$ it was 7.6 in elimination and 10.8 in addition; in 0.5 M $(NH_4)_2CO_3$ it was 2.8 both in elimination and in addition; in 1.0 M $(NH_4)_2CO_3$ it was 1.2 in elimination and 1.1 in addition]. This allowed us to calculate – as the average for equilibrations in 0.5 M and 1 M $(NH_4)_2CO_3$ – the apparent equilibrium constant as 2.5 (at pH 8.8). Taking all these serious contradictions into account, the single-step route ("single-step, TS" in Scheme 1) cannot be considered as a real pathway for the PAL (or TAL) reaction.

Finally, preparative biotransformations were performed with *Pc*PAL resulting in enantiopure D- and L-PG (Scheme 2). Due to the high enantiomer selectivity of *Pc*PAL in the ammonia elimination from DL-PG, enantiopure D-PG could be readily isolated from the reaction mixture. The formation of enantiopure L-PG in the *Pc*PAL-catalyzed ammonia addition to (*E*)-pent-2-en-4-ynoic acid proved unambiguously the reversibility and exclusive stereoselectivity of the PAL-catalyzed reaction with L-PG.

Conclusions

Novel tools, such as PAL immobilization on magnetic nanoparticles (MNPs) and its use in a microfluidic reactor with in-line UV detection, combined with chemoenzymatic syntheses and high-level QM/MM calculations, confirmed for the first time that the acyclic, non-aromatic propargylglycine could be reversibly transformed by PAL to (*E*)-pent-2-ene-4-ynoate. This reaction strongly disfavored a mechanism involving a Friedel-



Scheme 2. Preparation of enantiopure D-propargylglycine by PAL-catalyzed ammonia elimination from DL-propargylglycine and enantiopure L-propargylglycine by PAL-catalyzed ammonia addition to (*E*)-pent-2-ene-4-ynoate.

Crafts-type attack at an aromatic ring of the substrate. As an experimental evidence contradicting the recently proposed highly exothermic single-step mechanism, the reversibility of the PAL-reaction was demonstrated for the natural substrate L-Phe at an ammonium carbonate concentration as low as 0.1 M. Ammonia addition by *Pc*PAL to (*E*)-pent-2-ene-4-ynoate yielding enantiopure L-propargylglycine proved also the reversibility of the PAL-reaction with L-propargylglycine. The similar arrangements of the *N*-MIO intermediates from L-PG and L-Phe in the active site in the calculated QM/MM models of *Pc*PAL supported the mechanism via the *N*-MIO intermediate. Our findings open up new opportunities for the application of the MIO-enzyme toolbox towards non-aromatic acyclic substrates.

Experimental Section

Source of materials and enzymes, characterization of immobilized MNP-PAL, the details of synthetic and computational procedures and Gaussian structure files for the calculated hypothetical ligand-PAL complexes are provided as supplementary information (SI).

Detection of ammonium (*E*)-pent-2-ene-4-ynoate formation from DL-propargylglycine by MNP-PAL in a microfluidic reactor with multiple magnetic-cells. *Pc*PAL was immobilized on epoxy-MNPs (MagneCat-250GP14, see SI) and the four magnetic cells of the Magne-Chip were filled with MNP-PAL (see SI). A thermostated chip holder ensured the constant temperature of the Magne-Chip (35 °C). The DL-propargylglycine solution (40 mM in D_2O at pD= 8.8 with Na_2CO_3 buffer) was driven from an 1 mL dark glass syringe through the thermostated and light protected Magne-Chip equipped with an in-line UV-cell (Figure 1). A flow rate of 0.6 µL min^{-1} (resulting in a residence time of 6 min) was maintained for 24 h. The formation of the product (constantly followed at 240 nm on the recorded UV spectra) reached a stationary state after 4 h. In the following 20 h, the product was collected in an ice-cooled and light protected 1 mL amber glass vial. The product solution collected during this period (720 µL) was kept at 4 °C until the ^{1}H-NMR measurement (Figure S3).

D-Propargylglycine obtained by PAL-catalyzed ammonia elimination from DL-propargylglycine. To a solution of DL-propargylglycine (30 mg, 0.27 mmol) in Tris buffer (9 mL, 100 mM, pH 8.8) was added PAL solution (5 mg mL^{-1} in 1 mL of phosphate buffer, 50 mM, pH 7.5) and mixture was shaken at 200 rpm for 6 days in the dark at 30 °C. After addition of activated carbon (~10 mg), the mixture was heated to 80 °C for 15 min and

filtered on a 0.22 µm filter. The filtrate was acidified (pH 1 with 10 % HCl) and extracted with EtOAc (3 ×15 mL). The aqueous phase was loaded to a small Amberlyst 15 cation-exchange column. The product was eluted from the column by 2 M NH_3 solution and the eluted solution was concentrated in vacuum to yield D-propargylglycine as a white solid (11 mg, 37 %). $[\alpha]_D^{27}$= +32 (c 1, water), E.e. > 98 % (HPLC, see Figure S9), {lit.[49]: $[\alpha]_D^{25}$= +32.9 (c 1, water)}.

L-Propargylglycine synthesis by PAL-catalyzed ammonia addition to (*E*)-pent-2-ene-4-ynoic acid. Aqueous NH_3 (10 mL, 6 M, adjusted pH 10 with CO_2) containing PAL (0.5 mg mL^{-1}) and (*E*)-pent-2-en-4-ynoic acid (50 mg, 0.52 mmol) was shaken at 200 rpm for 7 days in the dark at 30 °C. Work up as described above yielded L-propargylglycine as a white solid (7.5 mg, 14 %). $[\alpha]_D^{27}$= -32.9 (c 1, water), E.e. > 98 % (HPLC, see Figure S11), {lit.[49]: $[\alpha]_D^{25}$= -31.1 (c 1, water)}.

Acknowledgements

The authors thank the support from COST Action CM1303 (*SysBiocat*). *LP* and *BGV* thank for financial support from Hungarian OTKA Foundation (NN-103242 and K109486), and from ICGEB CRP/HUN-14-01. *LP, BGV, ASz* and *BG* thank for financial support from the New Hungary Development Plan (TÁMOP-4.2.1/B-09/1/KMR-2010-0002 and TÁMOP-4.2.2.B-10/1–2010–0009). *CP* thanks for financial support from the Romanian National Authority for Scientific Research, CNCS – UEFISCDI (PN-II-IDPCE-2011-3-0799). *LCB* thanks for the financial support from the Sectoral Operational Program for Human Resources Development 2007-2013, co-financed by the European Social Fund, under the project POSDRU/159/1.5/S/137750. *LP* and *GB* are grateful to Schrödinger LLC for multiple licenses and allowance to publish the results. We thank Prof. Mihály Nógrádi (Budapest) for helpful discussions.

Keywords: Enzyme catalysis • magnetic nanoparticles • microreactor • phenylalanine ammonia-lyase • reaction mechanism

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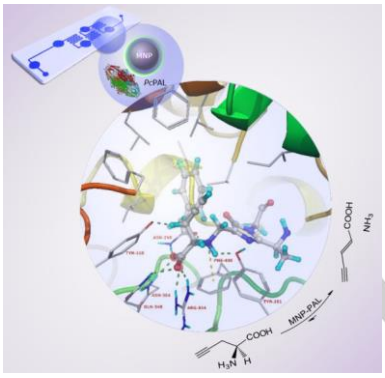
COMMUNICATION

Solution of the PAL mystery: Systems biocatalysis tools like magnetic nanoparticle immobilization and magnetic cell microchip helped to prove that phenylalanine ammonia-lyase accepts the non-aromatic, acyclic L-propargylglycine as substrate. This is possible only through a covalent intermediate with a bond between the amino group of the substrate and the MIO prosthetic group.



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