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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

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Phenylalanine ammonia-lyase (PAL), found 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 demonstrated that PAL can catalyze ammonia elimination from the acyclic propargylglycine (PG) to yield (*E*)-pent-2-ene-4-ynoate. This highlights new opportunities to extend MIO enzymes towards acyclic substrates. As PG is acyclic, its deamination cannot involve a Friedel–Crafts-type attack at an aromatic ring. The reversibility of the PAL reaction, 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 with the QM/MM models of the *N*-MIO intermediates from L-PG and L-Phe in PAL show similar arrangements within the active site, thus supporting a mechanism via the *N*-MIO intermediate.

In nature, phenylalanine, tyrosine, and histidine ammonia-lyases (PAL,[1] TAL,[2] and HAL,[3] respectively) catalyze 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 an electrophilic prosthetic group in both. X-ray crystallographic studies of HAL[5] revealed the 5-methylene-3,5-dihydro-4*H*-imidazol-4-one (MIO) structure as the electrophilic prosthetic group.[6] MIO was later identified in crystal structures of PALs from yeasts,[7,8] plants,[9] and bacteria,[10,11] and in TAL from bacteria,[12] as well as in L-phenylalanine and L-tyrosine 2,3-aminomutases (PAM[13,14] and TAM,[15] respectively). PAL has potential in enzyme replacement therapy of phenylketonuria,[11,16] and as a 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 "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 *"conserved motif"?*■■ 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 play crucial roles. One of the catalytically essential Tyr residues (Tyr_A) sits in a mobile, lid-like loop (Tyr110 in *Pc*PAL, Tyr60 in *Rs*TAL, Tyr54 in *Pp*HAL);[5,9,12,23] the other (Tyr_B) is buried inside the active site close to the methylidene moiety of MIO (Tyr351 in *Pc*PAL, Tyr300 in *Rs*TAL, Tyr281 in *Pp*HAL).[5,9,12]

Based on extensive biochemical, structural and computational studies, three significantly different mechanisms were sug-

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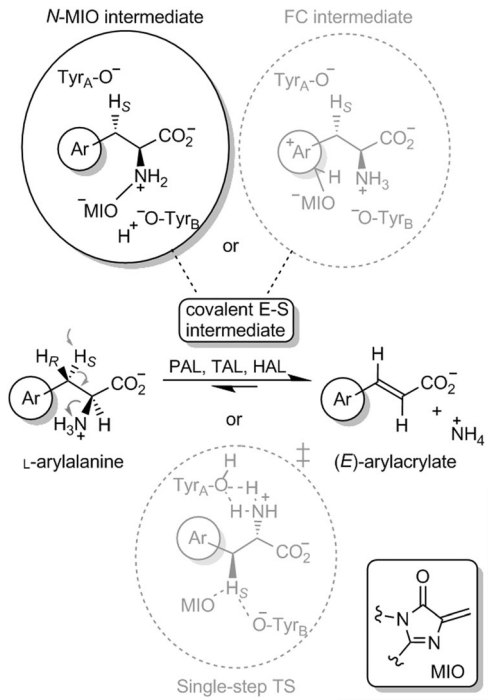


Scheme 1. Alternative pathways for the reaction catalyzed by the MIO-containing aromatic ammonia lyases (PAL, TAL, and HAL).

gested for the reactions catalyzed by HAL,[24,25] PAL,[26–28] 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 facilitates the ammonia-lyase reaction by generating a better leaving group (*N*-MIO intermediate in Scheme 1). For HAL[26] and PAL,[28] elimination was assumed to be a stepwise process via a carbanion intermediate (E_1cB), but a concerted process for TAL[19].

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

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

The correct mechanistic proposal has remained controversial.[31] Although it was demonstrated that *Pc*PAL accepts several cycloalkenylalanine substrates, such as DL-3-(cycloocta-1,3,5,7-tetraen-1-yl)alanine[17b] and L-3-(cyclohexa-1,4-dien-1-yl)alanine,[28,32] these reactions could still be rationalized by an FC-like or a single-step mechanism. On the other hand, the constitutional isomer L-3-(cyclohexa-2,5-dien-1-yl)alanine[32] and the saturated version DL-β-cyclohexylalanine[28] were not accepted, but rather proved to be moderate inhibitors of PAL.

Acceptance of a non-aromatic acyclic amino acid as a PAL substrate strongly disfavors the FC mechanism. Therefore, we decided to test PAL with DL-propargylglycine (DL-PG, Figure 1).

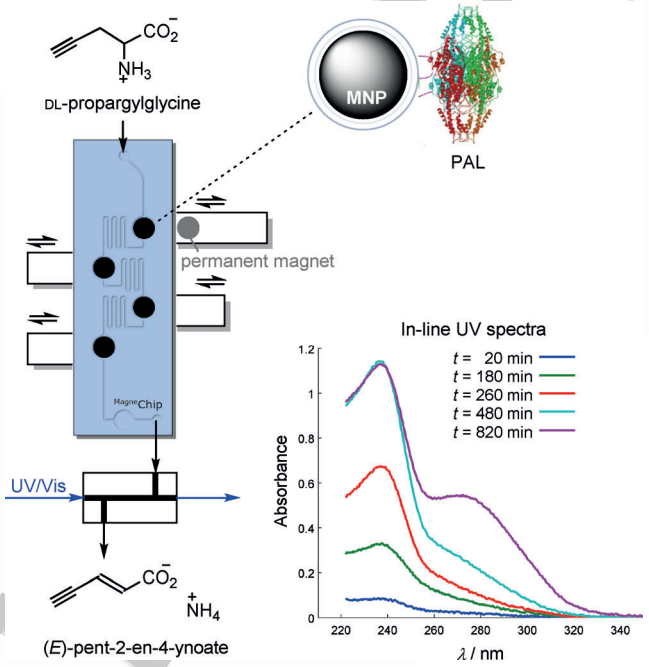


Figure 1. Ammonia elimination from DL-propargylglycine in a light-protected microfluidic reactor with multiple magnetic cells filled with PAL immobilized on MNPs and equipped with an in-line UV/Vis detector (reaction in D_2O at pD 8.8, 37 °C). The progress of the reaction was followed in the full UV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

To detect the forming (*E*)-pent-2-en-4-ynoate, even in very small amounts, we used an efficient novel microfluidics-based test system with PAL-coated magnetic nanoparticle and in-line UV/Vis detection. Application of microfluidic systems for systems biocatalytic and bioanalytical purposes is an efficient ■■new *?([33a] from 2006)*■■ solution.[33] The major advantages of fluidic systems with reduced dimensions for analytical and catalytic applications are the minute quantities (picoliters) of sample and reagents, the comparatively fast reaction times (because of the short molecular diffusion lengths), and the large surface-to-volume ratios.

Immobilization of proteins is another efficient method for systems biocatalysis in a variety of applications.[34] On nanoparticles or magnetic nanoparticles (MNPs), the immobilized proteins are spread on a large surface and are free of a diffusion barrier.[35] Suspensions of MNPs are free from sedimentation, and this approximates the behavior of homogeneous liquids. Furthermore, separation of MNPs from the liquid phase is simple.[36] For use in the novel microfluidic reactor the established epoxy chemistry[37] was applied to bind PAL onto magnetic nanoparticles (MNP-PAL in Figure 1).

In microfluidic systems the protein-coated MNPs can either flow together with the liquid, or be anchored by a magnet at

definite site(s) while the free-flowing fluid passes the particle regions.[33b,38] 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.[39] To the best of our knowledge, no microfluidic system has exploited multiple magnetic cells for biotransformation, analysis, or protein biochemistry studies ("Magne-Chip" in Figure 1). ■■*Is this name confusing with Millipore's "Magna-Chip"? Please add reference if Magne-Chip is an established tool, or use a phrase like "which we here term" if novel*■■

The 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, (*E*)-pent-2-en-4-ynoic acid (Scheme S2 in the Supporting Information) was chemically synthesized to determine the extinction coefficient ($\lambda_{max} = 242$ nm, $\varepsilon = 10.4\ mM\ cm^{-1}$, water, pH 8.8) of the product (Figures S5 and S6). The magnetic LoC device with in-line UV detection enabled detection of the formation of (*E*)-pent-2-en-4-ynoate at 242 nm, and produced measurable quantities of the product for recording ^1H NMR spectra without work-up. Besides the significant increase of the UV signal ($A = 1.2$ at 242 nm) in the on-line UV cell (Figure 1), the appearance of olefin hydrogen signals in the ^1H NMR spectrum of the reaction mixture ($\delta = 6.34$ (d) and 6.85 ppm (d)) indicated unambiguously the formation of (*E*)-pent-2-en-4-ynoate. A further UV signal at 274 nm indicated the formation of other by-product(s) (Figures 1 and S3).

After verifying that DL-propargylglycine was a substrate of PAL, its kinetic parameters were determined with *Pc*PAL and compared to the values with L-phenylalanine (Table 1). The K_m of L-PG (30-times higher than for L-Phe) indicated significantly 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-times lower than that for L-Phe, thus indicating that electronic effects in the elimination step were not significantly different between the two substrates.[40] The twofold-higher K_m and similar k_{cat} for DL-PG compared to L-PG indicate negligible inhibition of PAL by D-PG.

Table 1. Kinetic constants of L-phenylalanine and L- and DL-propargylglycine with *Pc*PAL (30 °C, ■■Tris buffer (0.1 M, pH 8.8), *OK?*■■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 MIO enzymes catalyze ammonia elimination/abstraction by similar mechanisms.[18,19] Experiments have 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 substrates.[41] Moreover, the H89F mutation[12,42] in *Rs*TAL resulted in conversion into full PAL activity, and mutation F144H in PAL1 from *Arabidopsis thaliana*[42] led to full TAL activity. Therefore, computational studies on TAL[19,29] should be relevant for the PAL reaction.

A detailed quantum mechanics/molecular mechanics (QM/MM) study on TAL revealed that ammonia elimination can proceed through a covalent *N*-MIO intermediate (Scheme 1) followed by a concerted elimination step.[19] This study suggested that in the reactive state, both Tyr_A and Tyr_B are deprotonated and that the L-Tyr substrate is present as a zwitterion. A recent study, by independent QM/MM calculation, confirmed the possibility of the TAL reaction via the *N*-MIO intermediate;[29] the authors 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] 1) It is unlikely to have two tyrosine residues simultaneously deprotonated in the same active center without any strong charge compensation, and 2) the calculated activation energy between the lowest energy *N*-MIO intermediate and the elimination product (26.3 or 28.5 kcal mol^{-1})[19,29] is rather high. For the alternative route through a single TS,[29] a 16.6 kcal mol^{-1} barrier was calculated for the elimination. Hence, 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" conforms with the experimentally observed irreversibility.[29]

In fact, a reactive state with both Tyr_A and Tyr_B deprotonated and the zwitterionic L-Tyr substrate for PAL (or TAL[19]) reaction can be easily formed at ~pH 9 if the amino group of anionic L-Phe (or L-Tyr) 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: 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 (−5.5 to −21.6 kcal mol^{-1}, even in the study favoring the single-step mechanism)[29] indicate that the real *N*-MIO–product barrier might be lower than the disputed values[19,29] of 26.3 or 28.5 kcal mol^{-1}. Moreover, all structural data agree only with the *N*-MIO route. X-Ray structural studies on various MIO enzymes revealed that several substrates and substrate analogues bind covalently at their *N*-atoms to the MIO group in the 2-aminoindan-2-phosphonate in the *Rs*TAL mutant H89F,[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 *Streptomyces globisporus* TAM,[43] L-Tyr in the Y63F (Tyr_A) mutant of *Sg*TAM,[44] and (*S*)-3-amino-2,2-difluoro-3-phenylpropanoic acid in ■■*Taxus? Tsuga?*■■ *canadensis* PAM.[45] Our ■■high level *"detailed"?*■■ QM/MM calculations also indicate quite similar *N*-MIO intermediate structures for the reaction of PAL with L-propargylglycine and with L-phenylalanine (Figures 2 and S16).

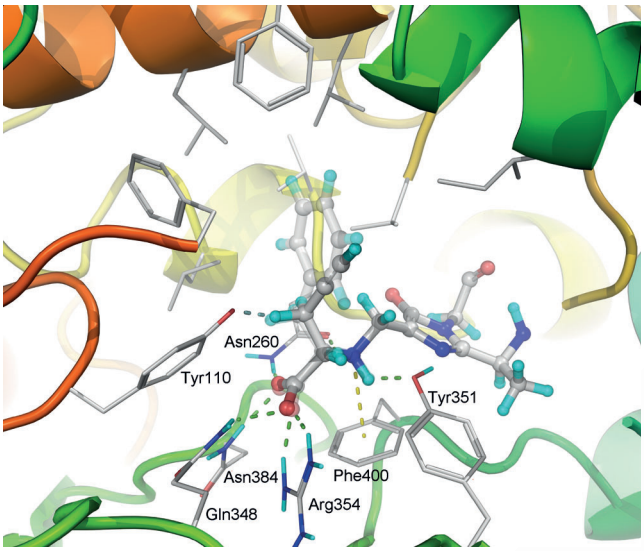
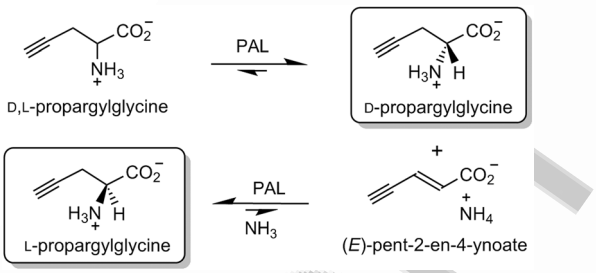


Figure 2. Model of the *N*-MIO intermediate of PAL with L-propargylglycine (solid stick model) compared to the model of *N*-MIO intermediate of PAL with its natural L-phenylalanine substrate (transparent stick model). The models were obtained by ONIOM (ωB97XD/6-31g(d):AMBER; http://www.gaussian.com).

The much higher Gibbs energies calculated for the FC-type covalent complexes relative to the substrate-binding states (166.7 kcal mol^{-1} for L-Phe, 9.5 kcal mol^{-1} for L-PG) and their distorted, unreactive structures (Figure S17 A and B) strongly disfavor the FC route ("FC intermediate" in Scheme 1) as the 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 for the *N*-MIO route calculations because of differences in atom number and total charge.[19,29] Nevertheless, the single-step proposal strongly conflicts with a number of experimental results on the PAL reaction with L-Phe because 1) negligible isotope effects were found for the atoms that should be involved in the single-step TS (1.0021 (^{15}N) and 1.15 (D) for Phe dideuterated at C_3),[28] 2) cinnamate product was released prior to ammonia in the catalytic sequence,[46] and 3) the PAL reaction was reversible at elevated ammonia concentration (> 5 M).[47]

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 (0.1, 0.5, or 1.0 M ammonium carbonate (pH 8.8) with CO_2). Equilibria were almost reached in three days (Supporting Information) from both directions, as indicated by the (*E*)-cinnamate/L-phenylalanine ratios (7.6 in elimination and 10.8 in addition, in 0.1 M $(NH_4)_2CO_3$; 2.8 in both elimination and addition, in 0.5 M $(NH_4)_2CO_3$; 1.2 in elimination and 1.1 in addition, in 1.0 M $(NH_4)_2CO_3$). This allowed us to evaluate an apparent equilibrium constant of 2.5 (at pH 8.8; average for equilibrations in 0.5 and 1 M $(NH_4)_2CO_3$). Taking all these serious contradictions into account, the single-step route ("single-step, TS" in Scheme 1) cannot be considered a valid pathway for the PAL (or TAL) reaction.



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.

Finally, preparative biotransformations were performed with *Pc*PAL to obtain enantiopure D- and L-PG (Scheme 2). Because of the high enantioselectivity of *Pc*PAL in the ammonia elimination from DL-PG, enantiopure D-PG was readily isolated. 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.

Conclusion

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 synthesis and high-level■■ QM/MM calculations, confirmed for the first time that the acyclic, nonaromatic propargylglycine could be reversibly transformed by PAL into (*E*)-pent-2-ene-4-ynoate. This reaction strongly disfavored a mechanism involving an FC-type attack at an aromatic ring of the substrate. Our experimental evidence contradicts the recently proposed highly exothermic single-step mechanism: 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 to yield enantiopure L-propargylglycine also proved 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 support a 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

Sources of materials and enzymes, characterization of immobilized MNP-PAL, details of synthetic and computational procedures with Gaussian structure files for the calculated hypothetical ligand–PAL complexes are in the Supporting Information.

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; Supporting Information) and the four magnetic cells of the Magne-Chip were filled with MNP-PAL (Sup-

porting Information). A thermostated chip holder ensured a constant 35 °C in the Magne-Chip. The DL-propargylglycine solution (40 mM in D_2O with Na_2CO_3 buffer; pD 8.8) 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. Formation of the product (followed at 240 nm on the recorded UV spectrum) 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 stored at 4 °C until the ^{1}H NMR measurement (Figure S3).

D-Propargylglycine obtained by PAL-catalyzed ammonia elimination from DL-propargylglycine. PAL solution (5 mg mL^{-1} in phosphate buffer (1 mL 50 mM, pH 7.5)) was added to DL-propargylglycine (30 mg, 0.27 mmol) in Tris buffer (9 mL, 100 mM, pH 8.8), and the mixture was shaken (200 rpm) for six 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 HCl (10%)) and extracted with EtOAc (3×15 mL). The aqueous phase was loaded onto a small Amberlyst 15 cation-exchange column. The product was eluted in 2 M NH_3, and the elute was concentrated in vacuum to yield D-propargylglycine as a white solid (11 mg, 37%). $[\alpha]_D^{27>} = +32$ ($c = 1$, H_2O); *ee* > 98% (HPLC, Figure S9), [ref. [48]: $^{25>}_{D} = +32.9$ ($c = 1$, H_2O)].

L-Propargylglycine synthesis by PAL-catalyzed ammonia addition to (*E*)-pent-2-ene-4-ynoic acid. Aqueous NH_3 (10 mL, 6 M, adjusted to 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 (200 rpm) for seven days in the dark at 30 °C. Work-up as described above yielded L-propargylglycine as a white solid (7.5 mg, 14%). $^{27>}_{D} = -32.9$ ($c = 1$, H_2O); *ee* > 98% (HPLC, Figure S11), [ref. [48]: $^{25>}_{D} = -31.1$ ($c = 1$, H_2O)].

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Keywords: enzyme catalysis · magnetic nanoparticles · microreactors · phenylalanine ammonia lyase · reaction mechanisms

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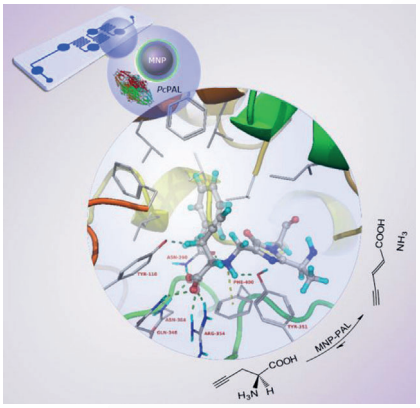
Solution of the PAL mystery: Novel tools, like magnetic nanoparticle immobilization and a magnetic cell microchip, proved that phenylalanine ammonia-lyase accepts the non-aromatic, acyclic substrate L-propargylglycine. This is by a covalent intermediate with a bond between the amino group of the substrate and the MIO prosthetic group.



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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

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