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研究报告

Biocatalytic synthesis of *N*-methyl-L-phenylalanine by phenylalanine ammonia lyase from galanthamine-producing *Lycoris radiata*

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Abstract: [Background] *N*-Methyl-L-phenylalanine, an *N*-alkylated aromatic amino acid, is a valuable chiral building block/intermediate/ingredient presented in many specialized metabolites that are very important in pharmaceutical, nutraceutical, and agrochemical industries. The synthesis and preparation of *N*-alkylated aromatic amino acids from aromatic *α*,*β*-unsaturated carboxylic acid remain challenging. **[Objective]** Herein we report a one-step biologically catalyzed *N*-methylamination of *trans*-cinnamic acid by LrPAL3, a phenylalanine ammonia lyase (PAL) from galanthamine-producing *Lycoris radiata*, to generate *N*-methyl-L-phenylalanine. **[Methods]** HPLC-DAD and HRESIMS analyses revealed that *N*-methyl-phenylalanine was produced when incubated *trans*-cinnamic acid and methylamine using the whole *Escherichia coli* BL21(DE3) cells expressing LrPAL3 as catalyst. **[Results]** The ^{1}H-NMR data and optical rotation of the enzymatic bioconversion product are in agreement with those of the authentic *N*-methyl-L-phenylalanine, which demonstrated the LrPAL3-catalyzed one-step regio- and enantioselective *N*-methylamination product of *trans*-cinnamic acid is *N*-methyl-L-phenylalanine. **[Conclusion]** This work provides an alternative biocatalyst for the asymmetric synthesis of valuable chiral *N*-methyl-L-phenylalanine. It paves a way to biologically synthesize *N*-alkylated amino acids through metabolic engineering and direct protein evolution of LrPAL3.

Keywords: biocatalysis, *N*-alkylamination, *N*-methylamination, phenylalanine ammonia lyase, *N*-methyl-L-phenylalanine

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红花石蒜苯丙氨酸解氨酶催化合成 *N*-甲基-L-苯丙氨酸

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摘 要:【背景】*N*-甲基-L-苯丙氨酸是一种 *N*-烷基化芳香氨基酸，是重要的手性合成单元/中间体/组成成分，在医药、农业、食品等领域有重要应用价值的代谢产物中广泛存在。*N*-烷基化芳香氨基酸的合成与制备仍具有巨大的挑战。【目的】在研究加兰他敏的生物合成过程中，我们从产加兰他敏的红花石蒜中克隆并表征苯丙氨酸解氨酶 LrPAL3。LrPAL3 催化区域及对映选择性的氢胺化反应得到 L-苯丙氨酸。通过生物信息学分析，推测 LrPAL3 可能催化反式-肉桂酸的一步 *N*-甲基胺化反应得到 *N*-甲基-L-苯丙氨酸。【方法】将反式-肉桂酸与甲胺，以及表达 LrPAL3 的大肠杆菌全细胞一起孵育。HPLC-DAD 及 HRESIMS 分析表明，上述反应产物为 *N*-甲基-苯丙氨酸。为确定该产物的立体构型，将上述催化反应放大，通过分离纯化得到该酶催化反应产物。【结果】该化合物的氢谱数据及比旋光数据与 *N*-甲基-L-苯丙氨酸标准品的数据一致。由此说明，LrPAL3 能够催化反式-肉桂酸和甲胺发生 *N*-烷基胺化反应，区域和立体专一性地生成 *N*-甲基-L-苯丙氨酸。【结论】本研究为手性 *N*-烷基氨基酸的不对称合成提供了一种全新的绿色、高效生物催化剂。通过对 LrPAL3 的蛋白质定向进化及代谢工程，将会进一步扩展 LrPAL3 的催化反应范围，以多种 *N*-烷基胺类及取代的苯基丙烯酸为底物，实现手性 *N*-烷基-芳基氨基酸的高效区域及立体选择性生物合成。

关键词: 生物催化，*N*-烷基胺化，*N*-甲基胺化，苯丙氨酸解氨酶，*N*-甲基-L-苯丙氨酸

1 Introduction

N-Alkylated amino acids are highly valuable chiral building blocks/intermediates/ingredients for the pharmaceutical, nutraceutical, and agrochemical industries[1-3]. For instance, obyanamide (Figure 1A), a marine cyclic depsipeptide containing an *N*-methyl-L-phenylalanine moiety, was shown to have strong cytotoxic activity[4]. Two *N*-methyl-L-phenylalanine moieties are present in cycloaspeptide E5 (Figure 1A) that displayed interesting insecticidal activity [5]. Beauvericin (Figure 1A) contains three *N*-methyl-L-phenylalanine moieties and it was shown to have anticancer, antimicrobial, and insecticidal activities[6]. *N*-Methyl-L-phenylalanine-rich peptides (Figure 1B) were envisaged as promising highly versatile blood-brain barrier shuttle candidates for drug delivery to the brain[7]. *N*-Alkylated amino acids are fundamental materials and auxiliaries for the synthesis of biodegradable polymers, ligands for asymmetric catalysis, and amphoteric surfactants[8].

Chemical strategies for the synthesis of *N*-functionalized amino acids usually rely on either *N*-alkylation processes which often require toxic reagents and highly expensive metal-based catalysts[9] or reductive amination which utilizes hazardous inorganic reductants and harsh reaction conditions that make traditional chemical processes costly and impractical[10]. Biologically catalytic approaches have been regarded as more efficient and sustainable alternative to prepare chiral *N*-alkylated amino acids[11]. The *N*-methyltransferases, nitrile hydratases and amidases, dehydrogenases, pyrroline-5-carboxylate reductases, and ammonia lyases (ALs) have been characterized and employed to synthesize chiral *N*-alkylated amino acids[11]. For instance, SAM-dependent *N*-methyltransferases are responsible for the *N*-methylation of a broad range of amino acids, which generate *N*-methylated amino acids (Figure 2A)[12]. The microbial whole cell catalyst containing nitrile hydratase and amidase had been employed to resolve racemic α-methylamino amides into the corresponding optically active (*S*)-(+)-*α*-methyl amino acids and (*R*)-(-)-*α*-methyl amino amides (Figure 2B)[13]. Reductive amination of *α*-keto acids by the enzymes combination of *N*-methyl amino acid

dehydrogenases, ketimine reductases, and pyrroline-5-carboxylate reductases from different species have emerged as powerful tools for the asymmetric synthesis of *N*-alkyl-L-phenylalanines (Figure 2C)[14]. Aspartate ammonia lyases, methylaspartate ammonia lyase and its variants have been proven to be excellent catalyst for the synthesis of *N*-alkylated aspartic acid (Figure 2D)[15-18]. Recently

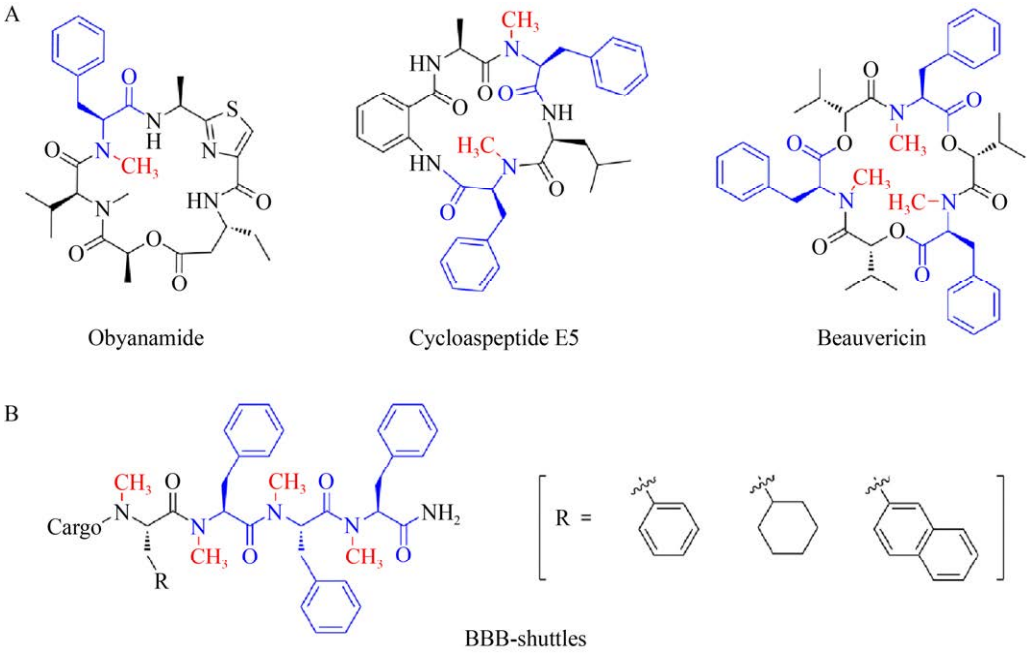

Figure 1 *N*-Methyl-L-phenylalanine-containing biologically active natural products (A) and synthetic blood-brain barrier (BBB)-shuttle peptide candidates (B)

图 1 含 *N*-甲基-L-苯丙氨酸的活性天然产物(A)和基于 *N*-甲基-L-苯丙氨酸设计合成的血脑屏障穿梭肽(B)

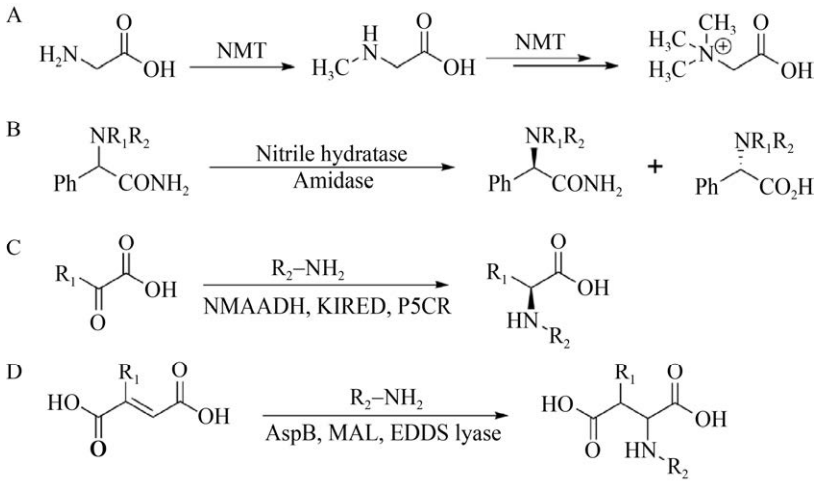

Figure 2 Biologically catalytic approaches to prepare *N*-alkylated amino acids and their derivatives

图 2 生物催化制备 *N*-烷基氨基酸及其衍生物

Note: A: Examples of *N*-methyltransferases catalyzed *N*-*α*-methylation of amino acids; B: Biocatalytic kinetic resolution of racemic *α*-alkylamino amides using nitrile hydratase and amidase; C: Biocatalytic reductive amination to *N*-alkylated amino acids using NMAADH, KIRED, and P5CR; D: Direct *N*-alkylamination of *α*,*β*-unsaturated carboxylic acids catalyzed by AspB, MAL, and EDDS lyase

注：A：*N*-甲基转移酶催化氨基酸的 *N*-*α*-甲基化；B：腈水合酶和酰胺酶催化消旋 *α*-烷基酰胺的动力学拆分；C：NMAADH、KIRED 和 P5CR 催化丙酮酸衍生物的还原胺化；D：C-N 裂解酶 AspB、MAL 和 EDDS 裂解酶催化 *α*,*β*-不饱和羧酸的直接 *N*-烷基胺化

ethylenediamine-*N*,*N*′-disuccinic acid lyase, a novel C-N lyase, had been engineered for the efficient asymmetric addition of challenging amines to fumarate to generate the *N*-alkylated aspartic acid (Figure 2D)[18-20]. However, no enzyme has been reported to catalyze direct biocatalytically asymmetric synthesis of *N*-alkylated aromatic amino acids from aromatic *α*,*β*-unsaturated carboxylic acid.

Phenylalanine ammonia lyases (PALs), a group member of ALs, have considerable potential as biocatalysts for the production of optically pure *α*- or *β*-phenylalanine derivatives[21]. PALs from *Petroselinum crispum*, *Anabaena variabilis*, *Rhodotorula glutinis*, and *Streptomyces maritimus* had been extensively studied and revealed to perform the regio- and stereoselective hydroamination of cinnamates for the synthesis of (*S*)-*α*-phenylalanine, (*R*)-*α*-phenylalanine, or (*S*)-*β*-phenylalanine derivatives[22-24]. In the course of the biosynthetic investigations of galanthamine, LrPAL3, a PAL from *Lycoris radiata*, had been demonstrated to exhibit highly regio- and enantioselective hydroamination of *trans*-cinnamic acid to give L-phenylalanine[25]. Herein, we report that LrPAL3 can recognize methylamine and catalyze the *N*-methylamination reaction with *trans*-cinnamic acid directly to generate *N*-methyl-L-phenylalanine with high regio- and stereoselectivity. The present work paves the way to direct *N*-alkylamination reaction of aromatic *α*,*β*-unsaturated carboxylic acid to afford *N*-alkylated phenylalanines with the help of metabolic engineering and directed protein evolution.

2 Materials and Methods

2.1 Materials

2.1.1 Strains and plasmids

pET28a-LrPAL3 recombinant plasmid and *E. coli* BL21(DE3) harboring pET28a-LrPAL3 recombinant strain were constructed and maintained in the author's laboratory[25].

2.1.2 Culture medium

The culture medium is Luria-Bertani (LB) broth (10 g/L of tryptone, 5 g/L of yeast extract, and 10 g/L of NaCl).

2.1.3 Reagents and instruments

Kanamycin, *β*-mercaptoethanol, phenylmethylsulfonyl fluoride (PMSF), and isopropyl *β*-D-1-thiogalactopyranoside (IPTG) were purchased from Sangon Biotech (Shanghai) Company, Limited. *trans*-cinnamic acid, *N*-methyl-L-phenylalanine, *N*-methyl-D-phenylalanine and methylamine were purchased from Aladdin reagent (Shanghai) Company, Limited. HPLC solvents were from J&K chemical Company, Limited. PCR amplification reactions were performed on an Eppendorf thermal cycler (Hamburg, Germany). High-speed refrigerated centrifugation was conducted on Xiang Yi H-2050R-1 centrifuge. HPLC-DAD analyses were conducted on SSI series 1500 HPLC equipment with an Altima C_{18} analytic column (250 mm × 4.6 mm, 5 μm). A Luna C_{18} column (250 mm × 10 mm, 10 μm) was employed for the semi-preparative HPLC separation.

2.2 Methods

2.2.1 Bioinformatics analysis and homology modeling of LrPAL3

Based on the previous report[25], bioinformatics characteristics of LrPAL3 were analyzed and compared with those of PcPAL from parsley (*Petroselinum crispum*)[26]. Molecular docking analysis was performed with AutoDock Vina 4.0 software (http://vina.scripps.edu/)[27]. The molecular docking results were displayed by using PyMOL molecular visualization system[28].

2.2.2 Heterologous overexpression of LrPAL3

According to the previous report[25], a single colony of the recombinant strain was inoculated into 5 mL of LB broth containing 50 μg/mL of kanamycin and incubated overnight at 37 °C, 200 r/min in a shaking incubator. The resulting bacterial culture (500 μL) was inoculated into 500 mL of LB broth supplemented with 50 μg/mL of kanamycin and incubated at 37 °C, 180 r/min. When the optical density at 600 nm of the culture reached 0.6−0.8, IPTG (1 mmol/L) was added into the culture to induce the overexpression of LrPAL3. The culture was shaken at 20 °C, 160 r/min for another 19 h. The cells were harvested by centrifugation at 4 000 r/min for 15 min at 4 °C, washed twice with PBS buffer (50 mmol/L NaH_2PO_4, 300 mmol/L NaCl, 2% glycerol, 10 mmol/L *β*-mercaptoethanol, pH 7.4, 4 °C). The overexpression of LrPAL3 was analyzed on 10% SDS-PAGE according to the previous report[25].

2.2.3 Whole-cell biotransformation assays

To 1 mL of methylamine aqueous solution

(3 mol/L, pH 10.0) containing 50 mmol/L of *trans*-cinnamic acid was added 400 mg of the abovementioned *E. coli* cells overexpressing LrPAL3. The reaction mixture was incubated at different reaction temperature (20, 30, 37, 45, and 50 °C), 250 r/min. A 200 μL of reaction mixture was sampled at different time point (18 h, 44 h, 60 h, 3 d and 4 d) and mixed with an equal volume of chill CH_3OH to quench the reaction. The resulting mixture was centrifuged at 12 000 r/min for 10 min. The supernatant was filtered through a 0.22 μm nylon membrane. The filtrate was subjected to HPLC-DAD analysis. The mobile phase consisted of solvent A (CH_3OH) and solvent B (H_2O containing 0.5% formic acid) followed a gradient elution program (0 min, 8% solvent A; 10 min, 8% solvent A; 30 min, 55% solvent A; 40 min, 95% solvent A; 50 min, 95% solvent A; 51 min, 8% solvent A) at a flow rate of 1 mL/min at 35 °C, monitored at 254 nm by a diode array detector. The control reactions were carried out in parallel with *E. coli* BL21(DE3) cells containing pET28a plasmid.

2.2.4 HRESIMS characterization of enzymatic reaction product

Comparison with the control experiments, the new product peak observed in LrPAL3-catalyzed *N*-methyl amination assays were collected and characterized by HRESIMS analysis. The pooled products were analyzed on a Bruker micrOTOF-Q mass spectrometer (Bremen, Germany) equipped with an electrospray ionization interface. The electrospray ionization mass spectrometer was operated in positive ion mode and the spectra were collected in the enhanced full mass scan mode from *m*/*z* 50−1 500.

2.2.5 Large scale enzymatic reaction and product preparation

The enzymatic reaction was scaled up to 20 mL of methylamine solution (1 mol/L, pH 10.0) containing *trans*-cinnamic acid (50 mmol/L) with 400 mg/mL of the afore-prepared *E. coli* cells overexpressing LrPAL3 as catalyst. The reaction mixture was incubated at 45 °C with stirring for 4 days. Three batches of the whole cell catalyst and substrate were supplied to the reaction system at every 24 h. To quench the reaction, an equal volume of chill CH_3OH was added to the reaction mixture. The mixture was centrifuged at 8 000 r/min for 30 min. The cell pellets were re-suspended in the mixture of CH_3OH and acetone (5:1). The resulting mixture was sonicated in an ice-bath at a 10 s interval until homogeneous and then the mixture was placed at room temperature overnight. The mixture was centrifuged and the supernatant was collected. All the above mentioned supernatants were combined and evaporated to dryness to give a crude residue. The residue was dissolved in H_2O and acidified to pH 3.0−4.0 with 2 mol/L of HCl solution to precipitate some solid. The precipitate was removed by filtration and the solution was evaporated to ~5 mL. The residue was separated by chromatography on reverse phase C_{18} silica gel eluting with the mixture of CH_3OH and H_2O containing 0.5% formic acid (3:17). The fraction containing the target product was separated by a preparative HPLC equipped with a Phenomenex Luna C_{18} column (250 mm×10 mm, 10 μm), monitored at 254 nm by a diode array detector. The fractions containing the target product were pooled and evaporated to remove solvents. The isolation yield of the final product is 3.9%.

2.2.6 ^{1}H-NMR spectroscopic and optical rotation analyses

The ^{1}H-NMR data were recorded on a Brucker Avance III 400 MHz NMR spectrometer. Optical rotations were determined on a PerkinElmer Model 341 polarimeter at 25 °C.

N-Methyl-L-phenylalanine: white powder; HRESIMS (positive ion mode): *m*/*z* 202.078 2 (calculated for $C_{10}H_{13}NO_2Na$, 202.083 8), error –5.7 ppm; ^{1}H-NMR (400 MHz, D_2O-NaOH): δ 7.43 (t, *J*=7.6 Hz, 2H), 7.39 (d, *J*=7.1 Hz, 1H), 7.32 (d, *J*=7.3 Hz, 2H), 3.87 (t, *J*=6.3 Hz, 1H), 3.24 (d, *J*=6.3 Hz, 2H), and 2.70 (s, 3H); $[\alpha]_D^{25}$ =+28.8 (*c* 0.1, 1 mol/L HCl).

3 Results and Discussion

3.1 Bioinformatics analyses and molecular docking

PALs are members of the 4-methylideneimidazol-5-one (MIO)-dependent enzyme family[29-30]. Most PALs, if not all, had been proven to catalyze the

conversion reactions of L-phenylalanine into *trans*-cinnamic acid and vice versa[29-30]. LrPAL3 had been characterized to catalyze not only the forward nonoxidative deamination of L-phenylalanine and its aromatic analogues to generate ammonia and *trans*-cinnamic acid and its aromatic analogues but also the reverse hydroamination of *trans*-cinnamic acid to form L-phenylalanine with high regio- and stereoselectivity[25]. Bioinfomatics analyses revealed that LrPAL3 shares 81% amino acid residue identity with PcPAL[26] (Figure 3A). The key amino acid residues E476, N479, and Q480 in LrPAL3 are identical to those of PcPAL, while A490, E494, and Q498 of LrPAL3 are different from the corresponding S498, S502, and E506 of PcPAL (Figure 3A). These key amino acid residues have already been demonstrated to be involved in the substrate amine binding in the catalytic pocket of PALs[26,31].

The crystal structure of PcPAL (PDB ID: 1W27)[26] was employed as a template by the SWISS-MODEL online software (http://www.swissmodel.expasy.org) to predict the 3-D structure of LrPAL3 that was constructed and refined by employing KoBaMIN web service. When superimposed the 3-D structures of LrPAL3 and PcPAL, no distinct difference was observed (*panel* I, Figure 3B). Molecular docking of *trans*-cinnamic acid into the catalytic cave of PALs indicated that the position of *trans*-cinnamic acid is identical in both LrPAL3 and PcPAL (*panel* II, Figure 3B)[31]. The aromatic phenyl group of *trans*-cinnamic acid was located at the hydrophobic binding pocket (highlighted in blue), while the hydrophilic carboxyl group was positioned at the orange carboxyl-binding pocket (*panel*s II and III, Figure 3B). The amino acid residues E476, N479, and Q480 may stabilize *trans*-cinnamic acid via hydrogen bond (*panel* III, Figure 3B). Based on the bioinformatics analyses and the catalytic mechanism of PAL-catalyzed forward deamination and revere amination (Figure 3C), we propose that LrPAL3 may recognize methylamine and catalyze *N*-methylamination of *trans*-cinnamic acid.

3.2 LrPAL3-catalyzed *N*-methylamination reaction

According to the reverse *N*-methylamination reaction mechanism (Figure 4A), the whole *E. coli* cells expressing LrPAL3 were incubated with *trans*-cinnamic acid and methylamine under the reported LrPAL3-catalyzed reverse biotransformation reaction conditions. A new peak with retention time at ~15.22 min was observed in the reaction mixture (*panel* III, Figure 4B), comparing with the control experiments using *E. coli* BL21(DE3) cells expressing pET28a vector as biocatalyst (*panel* II, Figure 4B). The aforementioned enzymatic reaction product shows identical retention time and UV profile with the standard *N*-methyl-phenylalanine (*panel* IV, Figure 4B). HRESIMS analyses revealed that the enzymatic product showed an $[M+Na]^+$ at *m*/*z* 202.078 2, which is identical to that of *N*-methyl-phenylalanine. These results indicated that LrPAL3 can recognize methylamine and catalyze its *N*-methylamination to *trans*-cinnamic acid to afford *N*-methyl-phenylalanine (Figure 4).

To determine the absolute configuration of the enzymatic *N*-methyl-phenylalanine, the LrPAL3-catalyzed reverse *N*-methylamination reaction was performed in preparative scale. The target product was obtained from preparative HPLC separation of the reaction mixture and its isolation yield is 3.9%. The ^{1}H-NMR spectroscopic data and the specific optical rotation of the enzymatic product are in perfect agreement with those of *N*-methyl-L-phenylalanine[32], which indicated the LrPAL3-catalyzed *N*-methylamination reaction product is *N*-methyl-L-phenylalanine.

N-Methyl-L-phenylalanine has been incorporated into various biologically active peptides and drugs. The development of sustainable and cost-effective methods is highly desirable. Herein, LrPAL3 was reported to catalyze *N*-methylamination of *trans*-cinnamic acid to produce *N*-methyl-L-phenylalanine with excellent regio- and stereoselectivity in one step. LrPAL3 is the first PAL that can recognize methylamine and catalyze its *N*-methylamination of *trans*-cinnamic acid. This biocatalytic methodology offers a useful alternative route to important chiral *N*-alkylated amino acids with the help of metabolic engineering and directed protein evolution.

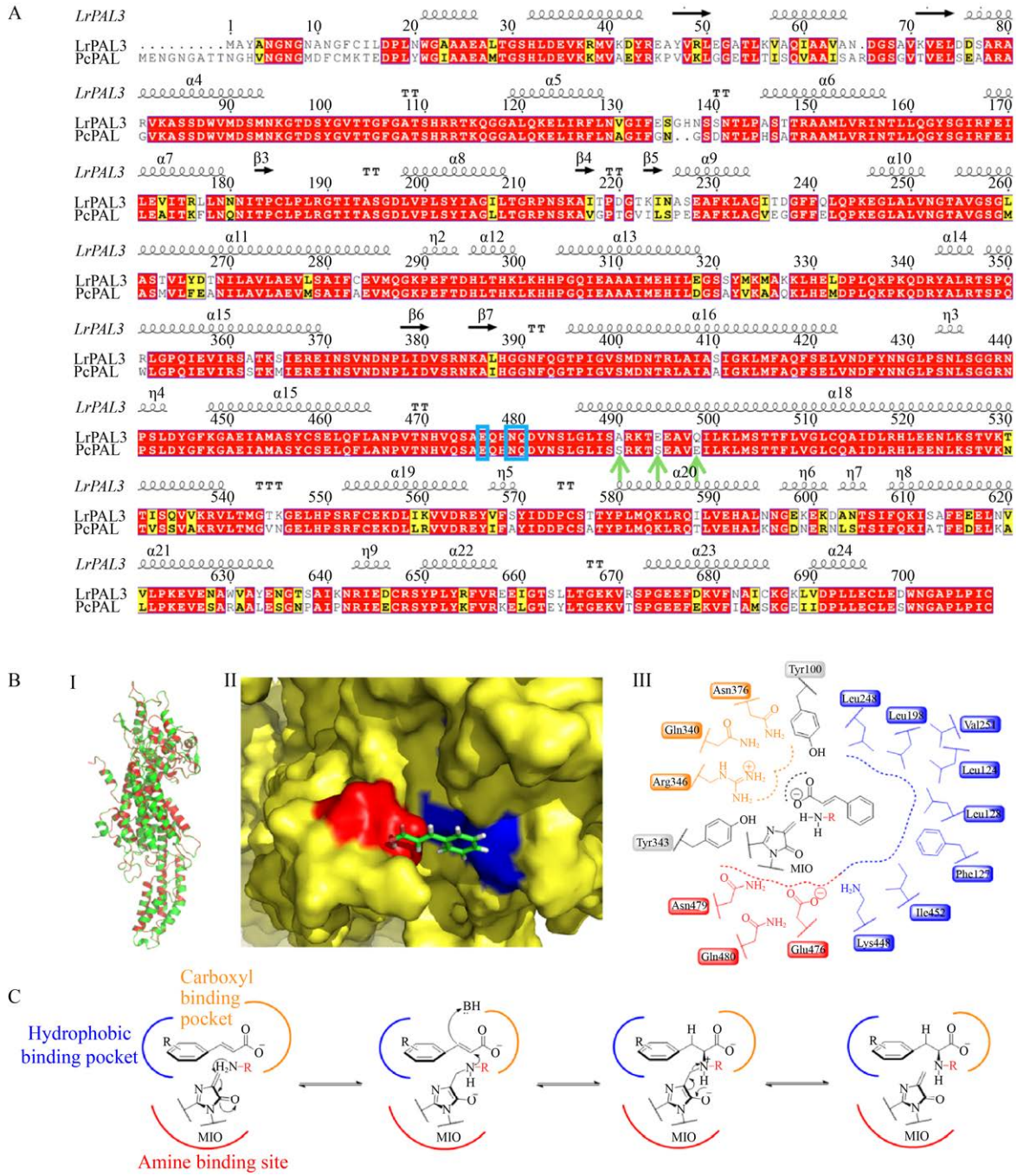

Figure 3 Bioinformatics characteristics of LrPAL3 and its catalytic mechanism

图 3 LrPAL3 的生物信息特性和催化反应机理

Note: A: Pairwise sequence alignment between LrPAL3 and PcPAL was constructed by ClustalX and the results were presented by using ESPript 3.0. B: Homology modeling and molecular docking of LrPAL3, I: Superimposition of the 3-D structures of LrPAL3 (red) and PcPAL (green); II: Molecular docking of *trans*-cinnamic acid into the active pocket of LrPAL3 (the amine-binding region was highlighted in red and the hydrophobic binding motif was in blue); III: The key amino acid residues involved in LrPAL3-catalyzed forward deamination and reverse amination. C: Mechanism of PAL-catalyzed forward deamination and reverse hydroamination

注：A：LrPAL3 和 PcPAL 的氨基酸序列比对结果。B：LrPAL3 的同源建模和分子对接，I：LrPAL3 (红色)与 PcPAL (绿色)的三维结构重叠分析；II：反式-肉桂酸在 LrPAL3 活性口袋的分子对接结果(绿色球棍结构为反式-肉桂酸，红色区域为氨基结合域，蓝色为疏水芳香结合域)；III：参与 LrPAL3 催化反应的氨基酸残基。C：LrPAL3 催化的正向脱氨与反向胺化反应机理

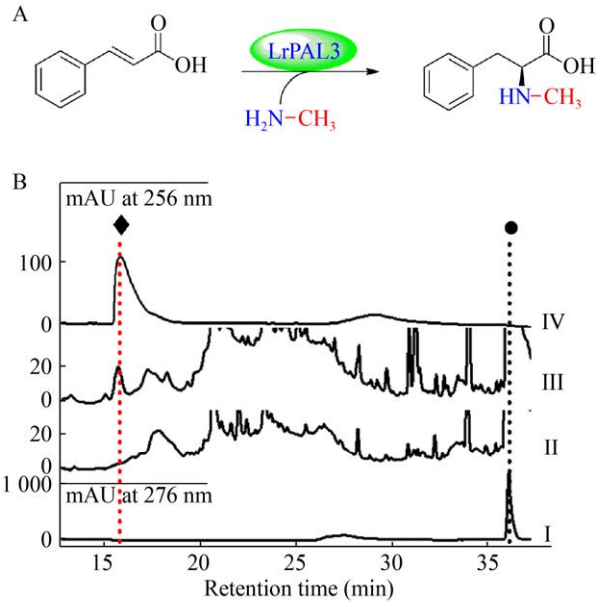

Figure 4 LrPAL3-catalyzed *N*-methylamination reaction of *trans*-cinnamic acid (A) and HPLC-DAD analysis (B)
图 4 LrPAL3 催化反式-肉桂酸的 *N*-甲基胺化反应(A)及 HPLC-DAD 分析(B)

Note: A: Schematic illustration of the *N*-methylamination reaction of *trans*-cinnamic acid catalyzed by LrPAL3. B: HPLC-DAD analyses of the standard *trans*-cinnamic acid (*panel* I) and *N*-methyl-L-phenylalanine (*panel* IV), the reaction mixture of the whole biotransformation reaction with whole *E. coli* cells expressing LrPAL3 as catalyst (*panel* III), and the control reaction mixture using whole *E. coli* cells expressing pET28a empty vector as catalyst (*panel* II). •: *trans*-cinnamic acid; ♦: *N*-methyl-L-phenylalanine

注：A：LrPAL3 催化反式-肉桂酸的 *N*-甲基胺化反应示意图。B：I：反式-肉桂酸标准品；II：以表达 pET28a 空载体的 *E. coli* 整细胞为催化剂的对照反应；III：以表达 LrPAL3 的 *E. coli* 整细胞为催化剂的 *N*-甲基胺化反应；IV：*N*-甲基-L-苯丙氨酸标准品；•：反式-肉桂酸；♦：*N*-甲基-L-苯丙氨酸

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