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Biochemical and genetic characterization of fungal proline hydroxylase in echinocandin biosynthesis

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Abstract

An intriguing structural feature of echinocandins is the incorporation of hydroxylated amino acids. Elucidation of the machinery and the mechanism responsible for this modification is critical to generate new echinocandin derivatives with enhanced antifungal activity. In our present study, we biochemically characterized the α -ketoglutarate/ Fe^{2+} -dependent proline hydroxylase (HtyE) from two *Aspergillus* species, *Aspergillus pachycristatus* and *Aspergillus aculeatus*, in the respective echinocandin B and aculeacin A biosynthetic gene clusters. Our results showed that both Ap- and Aa-HtyE converted L-proline to *trans*-4- and *trans*-3-hydroxyproline, but at different ratios. Both enzymes also effectively hydroxylated C-3 of 4R-methyl-proline, L-pipecolic acid, and D-proline. Our homology modeling and site-directed mutagenesis studies identified Leu182 of Ap-HtyE as a key residue in determining the regioselectivity of Ap-HtyE. Notably, we found that the efficiency in C-3 hydroxylation of 4R-methyl-proline has no direct correlation with the ratio of *trans*-4-hydroxyproline to *trans*-3-hydroxyproline catalyzed by HtyE. Deletion of *Ap-htyE* abolished *A. pachycristatus* anti-*Candida* activity and the production of echinocandin B, demonstrating that HtyE is the enzyme responsible for the hydroxylation of L-proline and 4R-methyl-proline in vivo and is essential for the anti-*Candida* activity of echinocandin B. Our present study thus sheds light on the biochemical basis for the selective hydroxylation of L-proline and 4R-methyl-proline and reveals a new type of biocatalyst with potential for the custom production of hydroxylated proline and pipecolic acid derivatives.

Keywords *Aspergillus pachycristatus* · *Aspergillus aculeatus* · Echinocandins · Proline hydroxylase · L-proline · 4R-methyl-proline

Introduction

Echinocandins are a type of fungal lipidated cyclic hexapeptide, possessing excellent antifungal activity (Stan et

al. 2014). Their semisynthetic derivatives have been developed as commercial antifungal drugs including cilofungin, anidulafungin, micafungin, and caspofungin (Balkovec et al. 2014). Echinocandins have been proven to inhibit fungal cell wall synthesis through noncompetitive inhibition of 1,3- β -glucan synthase, inducing cell wall lysis and death (Denning 2003). Echinocandin B, the precursor molecule of anidulafungin, is synthesized by an *Aspergillus pachycristatus* nonribosomal peptide synthetase (NRPS) by incorporating six amino acids (Supplementary Fig. S1) (Stack et al. 2007). Among these amino acids, four are nonproteinogenic, 4R,5R-dihydroxyl-L-ornithine, 4R-hydroxyl-L-proline (*trans*-4-Hyp), 3S,4S-dihydroxyl-L-homotyrosine, and 3S-hydroxyl-4S-methyl-L-proline (4-Me-3-Hyp) at the first, third, fourth, and sixth position of the hexapeptide, respectively (Cacho et al. 2012). These unusual amino acids are derived from the modification of natural proteinogenic amino acids before or after incorporation into the final peptidyl product. Incorporation of nonproteinogenic amino acids represents one of the hallmark mechanisms that contribute to the structural diversity and hence extensive biological activity of peptidic

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natural products (Li et al. 2015). Thus, elucidation of the machinery involved in modifying these amino acids is critical for the engineering of new echinocandin derivatives of interest for the pharmaceutical industry.

A single coherent gene cluster was identified to be responsible for the biosynthesis of echinocandin in both *Aspergillus pachycristatus* NRRL 11440 and *Aspergillus nidulans* NRRL 8112 (Hüttel 2017; Hüttel et al. 2016). In this cluster, *ecdA* encodes a six-module NRPS synthetase with each module consisting of a condensation (C), adenylation (A), and thiolation (T) domain (Singh et al. 2017). The *hty* subcluster containing *htyA*, *htyB*, *htyC*, and *htyD* genes has been shown to be responsible for L-homotyrosine (L-homoTyr) biosynthesis (Hüttel et al. 2016). Of note, synthesis of echinocandin B involves a cascade of hydroxylation reactions during the maturation process of the peptide scaffold. Five hydroxylase genes (*ecdG*, *ecdH*, *ecdK*, *htyE*, and *htyF*) are present in the echinocandin biosynthetic gene cluster and mediate hydroxylation of the respective amino acids. Whereas *EcdH*, a predicted P450 type of heme protein monooxygenase, has been shown to iteratively hydroxylate C5 and C4 of L-ornithine after the formation of the cyclic peptide, the *ecdG* gene encodes an α -ketoglutarate (α -KG)-dependent mononuclear, nonheme iron oxygenase which has been proven to hydroxylate free L-homoTyr to generate 3-hydroxyl-L-homoTyr (Jiang et al. 2013). Another α -KG-dependent nonheme iron oxygenase *EcdK* is otherwise involved in iteratively hydroxylating C5 of leucine to produce 4R-methyl-proline (4-Me-Pro) (Luesch et al. 2003). Although *HtyE* has been proposed to be responsible for the hydroxylation of proline and 4-Me-Pro (Houwaart et al. 2014), its detailed biochemical and genetic characterization has been lacking.

Pneumocandins belong to the echinocandin family lipopeptide and are synthesized by *Glarea lozoyensis* as the starting metabolites for caspofungin (Chen et al. 2013). Among the six amino acids of pneumocandin A₀ (the major product synthesized by *G. lozoyensis*), five are incorporated in the same order as those in echinocandin B with the exception of the fifth threonine being replaced by hydroxyl-L-glutamine (Supplementary Fig. S1) (Schwartz et al. 1992). Analogously, amino acids in pneumocandins A₀ including proline and 4-Me-Pro display the same hydroxylation pattern as that of echinocandin B (Bills et al. 2014). Petersen et al. first reported the presence of proline-4- and proline-3-hydroxylase activities converting free proline into *trans*-4- and *trans*-3-Hyp, respectively, in the crude extracts of *Glarea lozoyensis* (Petersen et al. 2003). Houwaart et al. further identified the proline hydroxylase GloF in the *G. lozoyensis* pneumocandin biosynthetic gene cluster, which was shown to be an α -KG/ Fe^{2+} -dependent nonheme dioxygenase capable of transforming free proline to both *trans*-4- and *trans*-3-Hyp with a ratio of 8: 1 (Houwaart et al. 2014). Notably, the sequence similarity of GloF with other known bacteria proline hydroxylases is low

(15–20% sequence identity). GloF thus represents a new type of α -KG/ Fe^{2+} -dependent nonheme dioxygenase (Hausinger 2004), and its orthologs in other echinocandin-producing fungi merit a comparative study. Besides pneumocandin, aculeacin A produced by *Aspergillus aculeatus* ATCC 16872 contains the same six amino acids as those of echinocandin B and differs only in the fatty acid side chain attached to the backbone via the amino group of L-ornithine (Supplementary Fig. S1) (Bills et al. 2014). In the present study, the GloF orthologs in the echinocandin B and aculeacin A biosynthetic gene clusters were identified as Ap-HtyE and Aa-HtyE by pairwise sequence alignment. These enzymes were characterized biochemically and their proline hydroxylase activities were confirmed. In particular, the regioselectivity of Ap-HtyE was investigated by site-directed mutagenesis and its relevance to the echinocandin B biosynthesis is discussed. Finally, Ap-HtyE was proven to be essential for the synthesis of echinocandin B by targeted gene deletion.

Materials and methods

Cloning of Ap-htyE and Aa-htyE

The proline hydroxylase genes were cloned by PCR using Primer star polymerase from the *Aspergillus pachycristatus* ATCC 58397 and *Aspergillus aculeatus* CPCC 400035 cDNA, respectively. The primers for the cloning of Ap-htyE and Aa-htyE were provided in Table S1. The PCR fragments were ligated into plasmid pGEX-4T-1 digested with *Bam*HI and *Not*I to express GST-tagged Ap-HtyE and Aa-HtyE proteins, respectively.

Expression and purification of Ap-HtyE and Aa-HtyE

Escherichia coli BL21 (DE3) strains with the appropriate expression plasmid were cultivated at 37 °C until an OD₆₀₀ of 0.4–0.5 was attained. The induction of recombinant proteins was achieved by adding isopropyl- β -D-thiogalactopyranoside (IPTG) to a final concentration of 0.2 mM, and the culture was continued for 16 h at 18 °C.

The GST-tagged enzymes were purified by glutathione sepharose 4B (GE Healthcare, Sigma) and eluted with a buffer containing 50 mM Tris-HCl (pH 8.0) and 10 mM reduced glutathione. The reduced glutathione was removed via HiTrap desalting column (GE Healthcare, Marlborough, USA). GST-tag-free Ap-HtyE and Aa-HtyE proteins were produced by thrombin digestion according to the manufacturer's instructions (GE Healthcare). GST-tag exerted no obvious effect on the activity and the ratio of *trans*-4- to *trans*-3-Hyp produced by Ap-HtyE and Aa-HtyE proteins. In the present study, GST-tagged Ap-HtyE and Aa-HtyE proteins were thus used. The concentration of the purified recombinant

enzymes was measured by the Bradford protein assay with bovine serum albumin (BSA) as a standard.

Enzyme activity assays

Enzyme activities of Ap-HtyE and Aa-HtyE were determined by measuring the products formed. The products were quantified by HPLC analysis using their respective standards. One unit of enzyme activity was defined as the release of 1 nmol of corresponding products per minute. Reaction samples were incubated for 1 h and stopped by adding acetonitrile to a final concentration of 20% (v/v). The effects of pH on hydroxylation activity of Ap-HtyE and Aa-HtyE were tested at 20 °C using 5 µM of purified enzyme in various buffers ranging from pH 5.0 to 9.0. The buffers used include sodium acetate (pH 5.0), MES (pH 6.0), HEPES (pH 7.0 and 8.0), and sodium borate (pH 9.0). The effects of temperature on hydroxylation activity were determined in 50 mM HEPES buffer pH 8.0 by varying the temperature from 10 to 50 °C. The effects of α -KG, L-ascorbate, and Fe^{2+} on hydroxylation activity were determined using different concentrations of α -KG (0, 6, and 12 mM), L-ascorbate (0, 0.5, 1, 2, 4, and 8 mM), and Fe^{2+} (0, 0.05, 0.1, 0.2, 0.4, 0.8, and 1.6 mM), respectively.

For the kinetic analysis of Ap-HtyE and Aa-HtyE, the recombinant enzymes (5 µM) were incubated with substrates (L-Pro, 4-Me-Pro, and L-Pip), α -KG (6 mM), L-ascorbate (2 mM), and Fe^{2+} (0.1 mM for Ap-HtyE and 0.2 mM for Aa-HtyE) in 50 mM HEPES buffer pH 8.0 in a total volume of 500 µL at their respective optimum temperature. Activities at varying concentrations of different substrates from 0.1 to 40 mM were determined and plotted against concentrations of substrates. The kinetic parameters were determined by fitting the data with Michaelis-Menten model using SigmaPlot software. All enzyme activity assays were measured in triplicates.

Amino acid derivatization and HPLC detection

Amino acids in samples were derivatized with 9-fluorenylmethoxycarbonyl chloride (9-FMOC-Cl) as previously described (Klein and Hüttel 2011). Briefly, 40 µL of sodium borate buffer (0.5 M, pH 7.7) and 80 µL of 9-FMOC-Cl (1.5 mM in acetone) were added into a 40-µL reaction sample, and the mixture was vortexed for 90 s. Afterwards, 100 µL of 1-adamantanamine [40 mM in acetone and sodium borate buffer pH 7.7 (1:1, v/v)] was added, and the mixture was vortexed for 90 s.

The derived samples were analyzed by HPLC on an Acclaim C18 5-µm column (4.6 × 250 mm) using a gradient of solvent A (H_2O , 0.1% TFA) and solvent B (acetonitrile, 0.1% TFA). The gradient program used was as follows: buffer B—0–25 min, 40–75%; 25–27 min, 75–95%; 27–32 min, 95%; 32–35 min, 95–40%; and 35–45 min, 40%. The injection volume was 10 µL and the flow rate was 1 mL/min.

Derivatized amino acids were detected at 265 nm using the UV detector.

Homology modeling

Homology model of the Ap-HtyE protein structure was modeled with the Phyre server (Kelley et al. 2015) using the normal search mode. The homology model of Ap-HtyE was superimposed with the crystal structure of *cis*-4-proline hydroxylase from *Mesorhizobium loti* (MIP4H, PDB: 4P7X), in which Co^{2+} , α -KG, and L-Pro were bound (Koketsu et al. 2015). Figures were prepared using PyMOL (The PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC).

Construction of the mutants

The active site amino acids were mutagenized by fusion PCR using the *Ap-htyE* gene template. The primers used are listed in Supplementary material Table S1. Fused PCR fragments containing the desired mutations were ligated into plasmid pGEX-4T-1 digested with *Bam*HI and *Not*I. Mutations were verified by sequencing. Mutant enzymes were expressed and purified as described above.

Gene knockout by homologous recombination in *Aspergillus pachycristatus* ATCC 58397

To construct the *Ap-htyE* gene knockout cassette, the 1.9-kb genomic fragments up- and downstream of the *Ap-htyE* gene were amplified from genomic DNA of *Aspergillus pachycristatus* ATCC 58397 (Supplementary Fig. S2) and digested with *Sgs*I/*Mss*I and *Spe*I/*Not*I, respectively. The digested fragments were ligated into pMD-19T with a pyrithiamine resistance gene *ptrA* maker yielding the deletion plasmid pMD-19T- Δ *htyE* (Kubodera et al. 2000) (Supplementary Fig. S2). The linearized pMD-19T- Δ *htyE* was transformed into the protoplasts of *Aspergillus pachycristatus* ATCC 58397 by polyethyleneglycol-mediated transformation. Transformants were selected on minimal medium plates (glucose 20 g/L, NaNO_3 8 g/L, KH_2PO_4 4 g/L, D-sorbitol 218 g/L, and agar 1.5 g/L, pH 6.0) containing 0.25 µg/mL pyrithiamine hydrobromide. Anchored PCR (Supplementary Fig. S3) was performed to confirm the proper knockout of *Ap-htyE* using the primers listed in Table S1.

Extraction and characterization of echinocandins in wild-type and *Ap-htyE* knockout strains

The wild-type *A. pachycristatus* ATCC 58397 and *Ap-htyE* knockout strains were grown in a fermentation medium (glucose 25 g/L, $(\text{NH}_4)_2\text{SO}_4$ 5 g/L, KH_2PO_4 9 g/L, starch

10 g/L, CaCO₃ 2 g/L, pH 6.0) at 28 °C for 7 days. An equal volume of methanol was added to the mycelia harvested by filtration, and the mixture was shaken at 16 °C for 1 h. The methanol extract was collected by filtration and was evaporated to dryness using a rotary evaporator. The dried methanol extracts were redissolved in methanol and the insoluble pellets were removed by centrifugation (4 °C, 14,000 rpm). The supernatant (10 µL) was injected into an Acclaim C18 5-µm column (4.6 × 250 mm) using a gradient elution program over 40 min (solvent A, 0.1% TFA/H₂O; solvent B, 0.1% TFA/acetonitrile) at the flow rate of 1 mL/min. The gradient program used was as follows: buffer B—0–25 min, 30–100%; 25–30 min, 100%; 30–32 min, 100–30%; and 32–34 min, 30%. Echinocandins were monitored using a UV detector at 227 nm.

Anti-Candida assay

Anti-*Candida* activity of the wild-type and the *Ap-htyE* knockout strain extracts was tested by plate inhibition assay against *Candida albicans* CPCC 360003. *C. albicans* CPCC 360003 was cultured in the Sabouraud dextrose broth overnight. Then, 200 µL of *C. albicans* CPCC 360003 cells was spread onto the surfaces of Sabouraud dextrose plates. The culture extract from the wild type and two independent *htyE* deletion mutants ($\Delta htyE$ -1 and $\Delta htyE$ -2) were spotted onto paper discs placed on the *C. albicans* CPCC 360003 plates. The plates were incubated at 30 °C for 20 h and the inhibition zone was observed.

Phylogenetic analysis of proline hydroxylation enzymes

Phylogenies of biochemically characterized propyl hydroxylases, proline hydroxylases, and pipercolic acid hydroxylases in bacteria and fungi were separately reconstructed using MEGA version 7.0. Using the maximum likelihood method based on the JTT matrix model, the phylogenetic tree was constructed. The positions containing alignment gaps and missing data were partially deleted. The statistical confidence of the inferred phylogenetic relationships was determined by conducting 1000 bootstrap replicates. The GenBank accession numbers of the sequences used in the phylogenetic analysis were included in the phylogenetic tree.

NCBI accession codes

The gene sequences of *Ap-htyE* and *Aa-htyE* are available at GenBank under the accession JX421685.1 and MH243072.1, respectively.

Results

Cloning and expression of Ap-HtyE and Aa-HtyE

Pairwise sequence alignments readily identified Ap-HtyE and Aa-HtyE as GloF orthologs in the echinocandin biosynthetic gene clusters of *Aspergillus pachycristatus* and *Aspergillus aculeatus*, which shared 63.83 and 63.33% sequence identity with GloF, respectively. Ap-HtyE and Aa-HtyE both comprise 329 amino acids and share a relatively high (69.60%) sequence identity. Conserved domain analysis revealed that both Ap-HtyE and Aa-HtyE belong to the α -KG/Fe²⁺-dependent oxygenase superfamily (Pfam 03171). No signal peptide sequence was predicted for Ap-HtyE and Aa-HtyE, indicating their intracellular location.

To facilitate their expression, *Ap-htyE* and *Aa-htyE* were cloned into pGEX-4T-1 and expressed in *E. coli* BL21 (DE3) as GST-tag fusion proteins. Under the growth and induction conditions used, a high level of protein expression was observed in the soluble fraction and a total of ~20 mg pure proteins per liter of culture was obtained following glutathione sepharose 4B affinity purification for both enzymes. SDS-PAGE analysis estimated that the molecular weight of both recombinant Ap-HtyE and Aa-HtyE proteins is about 65 kDa (Supplementary Fig. S4), which is in agreement with their predicted molecular weights.

Identification and biochemical characterization of the proline hydroxylase activity of Ap-HtyE and Aa-HtyE

The proline hydroxylase activities of Ap-HtyE and Aa-HtyE were initially tested with L-proline (L-Pro) as substrate. HPLC analysis showed that both Ap-HtyE and Aa-HtyE converted L-Pro into two product peaks with retention time at 10.3 and 11.7 min (Fig. 1a), which coincided with authentic standards *trans*-4-Hyp and *trans*-3-Hyp, respectively. LC-MS analysis revealed that the molecular ions of the two products were both detected at *m/z* 132.06 [M+H]⁺ as predicted (Supplementary Fig. S5a). Furthermore, the fragmentation patterns of the two products were identical to authentic standards *trans*-4-Hyp and *trans*-3-Hyp, respectively. Our results thus showed that Ap-HtyE and Aa-HtyE hydroxylated L-Pro in a *trans*-selective manner at both C-4 and C-3 position. Interestingly, the ratio of *trans*-4-Hyp to *trans*-3-Hyp produced by Ap-HtyE (*trans*-4-Hyp/*trans*-3-Hyp ≈ 2.5:1) was much lower than that the products from Aa-HtyE (*trans*-4-Hyp/*trans*-3-Hyp ≈ 7.2:1) as observed in HPLC analysis (Fig. 1a).

Using L-Pro as substrate, the effect of pH and temperature on the catalytic activity of Ap-HtyE and Aa-HtyE was determined. Ap-HtyE and Aa-HtyE showed relatively stable

Fig. 1 HPLC analysis of the products generated by incubation of Ap-HtyE and Aa-HtyE with L-Pro (**a**), 4-Me-Pro (**b**), D-Pro (**c**), and L-Pip (**d**). Samples were derived with 9-fluorenylmethoxycarbonyl chloride (9-FMOC-Cl) and detected at 265 nm using UV detector

activity within a broad range of pH (6.0 to 9.0) and displayed maximal activities at pH 8.0 (Fig. 2). The optimal temperature of Ap-HtyE and Aa-HtyE was observed at 30 and 20 °C, respectively (Fig. 2). Their activities dropped dramatically when the temperature was above 30 °C. Although the activities of Ap-HtyE and Aa-HtyE were affected by different pH values and temperatures, the ratio of *trans*-4-Hyp to *trans*-3-Hyp in the product mixture was not significantly affected.

In the absence of α -KG, no activity was observed for Ap-HtyE and Aa-HtyE, confirming that α -KG is absolutely required for the hydroxylase activities. Two different concentrations of α -KG were tested for its effect on the activity. The

activities of Ap-HtyE and Aa-HtyE with 12.0 mM α -KG (9.32 and 10.38 U/mg, respectively) were slightly reduced compared to those with 6.0 mM α -KG (11.85 and 14.47 U/mg). The optimal concentrations of Fe^{2+} were 0.1 and 0.2 mM for Ap-HtyE and Aa-HtyE (Fig. 3), respectively. As reported previously (Klein and Hüttl 2011), whereas low concentrations of Fe^{2+} significantly reduced the activities, increased concentrations of Fe^{2+} only slightly decreased their activities. Proline hydroxylation activities of Ap-HtyE and Aa-HtyE were still observed even in the absence of Fe^{2+} though at much lower levels (51 and 47% of activity at their optimum Fe^{2+} concentration, respectively), indicating that trace amount of

Fig. 2 Effects of pH and temperature on the activity of Ap-HtyE (**a**, **c**) and Aa-HtyE (**b**, **d**) were assayed under standard conditions with varied pH values (5.0 to 9.0) and temperatures (10 to 50 °C)

Fe^{2+} may be already bound by these enzymes during the expression and purification. Adding the chelating agent EDTA to the reaction strongly inhibited the activity to 5.0 and 8.0% (Table 1), respectively, confirming the requirement of Fe^{2+} for Ap-HtyE and Aa-HtyE activities. The effects of other metal ions on the activity of Ap-HtyE and Aa-HtyE were also tested. Drastic inhibition or inactivation of Ap-HtyE and Aa-HtyE activity was observed with copper, nickel, zinc, and cobalt ions, probably due to their competition binding to enzymes with ferron ions. Ferron (III) and manganese ions showed moderate inhibition, while no effect was observed with magnesium ion.

L-ascorbate has been reported to be essential for prolyl hydroxylases but not for proline hydroxylases (Hara and Kino 2009; Katz et al. 1979). However, inclusion of L-ascorbate was found to be in favor of reducing iron (III) to iron (II) and thus stimulate the activity of proline

hydroxylases. The optimal concentration of L-ascorbate was observed at 2 mM for both Ap-HtyE and Aa-HtyE. Further increasing the concentration of L-ascorbate slightly impaired their activities, as reported by Kleina and Hüttel (2011). Again, the ratio of *trans*-4-Hyp to *trans*-3-Hyp in the products was not significantly affected under all the tested conditions.

Hydroxylation of 4-Me-Pro, D-Pro, and L-Pip by Ap-HtyE and Aa-HtyE

In addition to *trans*-Hyps, 4-Me-3-Hyp is another structural element of echinocandins. Considering HtyE-catalyzed production of *trans*-3-Hyp from L-Pro, hydroxylation of 4-Me-Pro by Ap-HtyE and Aa-HtyE was tested as well. The results showed that both enzymes converted 4-Me-Pro into a major product with $[\text{M}+\text{H}]^+$ at m/z 146.08 (Fig. 1b; Supplementary Fig. S5b), suggesting the hydroxylation of 4-Me-Pro. Another

Fig. 3 Effects of concentrations of Fe^{2+} and L-ascorbate on the activity of Ap-HtyE (a, b) and Aa-HtyE (c, d) were assayed under standard conditions with varied concentrations of Fe^{2+} and L-ascorbate being added

minor product with $[\text{M}+\text{H}]^+$ at m/z 162.07 was also detected (Fig. 1b; Supplementary S5b), implicating the double hydroxylation of 4-Me-Pro. Due to the lack of their respective standards, the identity of these products was not confirmed. Our observation was, however, consistent with recently reported data (Mattay et al. 2018), wherein they confirmed that the major product catalyzed by Ap-HtyE was 4-Me-3-Hyp while the minor product was 3-hydroxy-4-hydroxymethyl-L-proline (4-HyMe-3-Hyp).

D-proline was also tested for the hydroxylation by Ap-HtyE and Aa-HtyE. Our results showed that Ap-HtyE and Aa-HtyE transformed D-proline into two products with m/z 132.06 $[\text{M}+\text{H}]^+$ (Fig. 1c; Supplementary Fig. S5c), suggesting the hydroxylation of D-proline. In view of the regioselectivity of Ap-HtyE and Aa-HtyE, we assumed that these two products were *trans*-4-D-proline and *trans*-3-D-proline, respectively. However, the exact

identity of these two products was not confirmed due to the lack of corresponding authentic standards. Nevertheless, the ratio of these products was significantly different from that of the two products from L-Pro. Only a very small amount of the second product with a longer retention time (assumed to be *trans*-3-D-proline) was produced from D-proline (Fig. 1c).

Previously, L-pipecolic acid (L-Pip), a six-membered ring homolog of proline, has been shown to be hydroxylated by bacterial *trans*-P4H and *cis*-P3H (Shibasaki et al. 1999b). In the case of Ap-HtyE and Aa-HtyE, L-Pip was also accepted as a substrate and was converted regioselectively into a single product (Fig. 1d). The retention time, molecular ions m/z 146.08 $[\text{M}+\text{H}]^+$, and fragmentation pattern of this product were identical to the authentic standard *trans*-5-hydroxyl-pipecolic acid (*trans*-5-Hypip, Supplementary Fig. S5d).

Table 1 Effects of the chelating agent EDTA and different metal ions on the activities of Ap-HtyE and Aa-HtyE

Additives	Relative activity (%) ^a	
	Ap-HtyE	Aa-HtyE
None	100	100
EDTA	8	5
MgSO ₄	98	98
MnSO ₄	66	77
FeCl ₃	69	72
CoCl ₂	26	27
ZnSO ₄	0.2	0.2
CuSO ₄	0	0
NiSO ₄	14	13

^a Effects of the chelating agent EDTA and different metal ions on the activities of Ap-HtyE and Aa-HtyE were determined under standard assay conditions; 2 mM of EDTA or other metal ions was added. Relative activity was calculated as the percentage of activity under optimal conditions

Kinetic properties of Ap-HtyE and Aa-HtyE with L-Pro, 4-Me-Pro, and L-Pip

To provide insight into the catalytic properties of Ap-HtyE and Aa-HtyE with different substrates, the kinetic parameters were determined under their respective optimal conditions using varying concentrations of L-Pro, 4-Me-Pro, or L-Pip. When the reaction velocities were plotted against the substrate concentrations, all the six reactions were demonstrated to follow the Michaelis-Menten model without apparent substrate inhibition (Supplementary Fig. S6). K_m and k_{cat} values of Ap-HtyE for L-Pro were determined to be 2.91 mM and $6.72 \times 10^{-2} \text{ s}^{-1}$ (Table 2), respectively. As for 4-Me-Pro, the Michaelis constant (K_m) of Ap-HtyE was about two fold lower, while its k_{cat} was four times higher than that for L-Pro (Table 2), thus displaying a significantly higher catalytic efficiency (k_{cat}/K_m) for 4-Me-Pro than L-Pro. Aa-HtyE showed very similar K_m and k_{cat} values for L-Pro and 4-Me-Pro to those of Ap-HtyE. In the case of L-Pip, Ap-HtyE and Aa-HtyE also showed comparable k_{cat} values; however, K_m of

Table 2 Kinetic parameters of Ap-HtyE and Aa-HtyE for L-Pro, 4-Me-Pro, and L-Pip

Enzyme	Substrate	K_m [mM]	k_{cat} [s^{-1}]	k_{cat}/K_m [$\text{mM}^{-1} \text{ s}^{-1}$]
Ap-HtyE	L-Pro	2.91	6.72×10^{-2}	2.31×10^{-2}
	4-Me-Pro	1.49	2.59×10^{-1}	1.74×10^{-1}
	L-Pip	4.89	1.22×10^{-1}	2.49×10^{-2}
Aa-HtyE	L-Pro	2.83	7.34×10^{-2}	2.59×10^{-2}
	4-Me-Pro	1.81	2.61×10^{-1}	1.44×10^{-1}
	L-Pip	3.12	1.34×10^{-1}	4.29×10^{-2}

Ap-HtyE (4.89 mM) was slightly higher than that of Aa-HtyE (3.12 mM). In addition, whereas Ap-HtyE converts L-Pip at a comparable efficiency to that of L-Pro, the conversion of L-Pip is faster than L-Pro in Aa-HtyE catalysis (Table 2).

Identification of amino acids affecting the regioselectivity of proline hydroxylase by site-directed mutagenesis

Proline hydroxylases from fungi represent a distinct group of α -KG/ Fe^{2+} -dependent nonheme dioxygenases, sharing very low homology with bacterial proline hydroxylases (approximately only 15–20% identity). Currently, no crystal structure of a fungi proline hydroxylase is available. In order to identify amino acid residues critical for the recognition of the L-Pro substrate and hence for its regioselectivity, a homology model of Ap-HtyE was constructed with the Phyre server. The best scoring structural template was that of anthocyanidin synthase (PDB: 1GP6) from *Arabidopsis thaliana* (Wilmouth et al. 2002), which, however, displays a low sequence identity (22%) with Ap-HtyE. Of the 329 amino acids, 89% was modeled with 100% confidence. The homology model of Ap-HtyE was superposed with the crystal structure of the *cis*-4-proline hydroxylase from *Mesorhizobium loti* (MIP4H, PDB: 4P7X), in which Co^{2+} , α -KG, and L-Pro were bound (Koketsu et al. 2015). Similar to the structure of *cis*-4-proline hydroxylase from *M. loti*, the active site of Ap-HtyE is composed of a distorted jelly roll β -sheet core (Fig. 4a). However, the N- and C-terminal α -helix domains differed significantly. Twenty amino acid residues located within a radius $< 4.5 \text{ \AA}$ around L-Pro and α -KG were identified (Fig. 4b). These include two histidine residues (His201 and 261) and an aspartate residue (Asp203) that coordinate the Co^{2+} , and comprise the HxD/H motif conserved in α -KG/ Fe^{2+} -dependent nonheme dioxygenase (Feig and Lippard 1994). Arg280 is also conserved in α -KG/ Fe^{2+} -dependent nonheme dioxygenases and is assumed to form a salt bridge with α -KG (Koketsu et al. 2015). We firstly employed the Ala scanning strategy to evaluate the effects of mutating these amino residues on the activity and regioselectivity of Ap-HtyE. In addition, among these 20 residues, Aa-HtyE only differs by one amino acid residue (Gln101) from Ap-HtyE (Arg101) (Supplementary Fig. S7), whereas GloF differs by two amino acid residues (Thr101, Asn182) from Ap-HtyE (Arg101, Leu182). In light of their clearly different product ratios, we mutated the residues in Ap-HtyE accordingly (R101Q, R101T, and L182N). In total, 23 mutants were constructed in the first round of mutagenesis. The relative activities of these mutants varied from 0 to 106.52% (Table 3). The activities of H201A, D203A, H261A, and R280A mutants were severely impaired, resulting in 0, 4.43, 17.67, and 14.06% of WT activity, respectively, confirming their importance for catalysis. Activities of E178A, F286A, F204A, T208A, and R197A were also

Fig. 4 Homology model of Ap-HtyE. L-Pro, α -KG, and Co^{2+} were taken from the superposition with the crystal structure of the *cis*-4 proline hydroxylase from *Mesorhizobium loti* (MIP4H, PDB: 4P7X). **a** The α -helices, β -sheets, and loops of the Ap-HtyE model are colored red, blue, and light gray, respectively. **b** Amino acid residues located within a radius $< 4.5 \text{ \AA}$ around L-Pro and α -KG. The conserved residues H201, D203, and H261 in the HxD/H motif and R280 are shown in stick

Table 3 The relative activities and the product ratios (*trans*-4-Hyp/*trans*-3-Hyp) of Ap-HtyE-derived mutants in the first round of mutation

	Relative activity ^a with L-Pro	The ratio of <i>trans</i> -4- and <i>trans</i> -3-Hyp ^b	Relative activity ^a with 4-Me-Pro	Relative activity ^a with L-Pip
Ap-HtyE	100%	2.5:1	100%	100%
R101T	98.91%	2.4:1	104.35%	111.32%
V263A	42.45%	2.4:1	54.04%	49.32%
L182A	98.13%	2.5:1	104.43%	106.50%
R101A	99.63%	2.6:1	104.01%	87.16%
F204A	6.34%	2.6:1	92.12%	77.08%
R101Q	97.24%	2.6:1	109.28%	108.40%
E178A	3.36%	2.6:1	31.94%	11.59%
Y184A	60.45%	2.6:1	106.76%	75.37%
F286A	0.68%	2.7:1	16.85%	1.27%
R180A	20.57%	2.8:1	12.77%	14.47%
S282A	101.03%	2.8:1	109.30%	103.47%
L210A	68.26%	2.8:1	93.41%	78.02%
L218A	106.52%	3.0:1	103.86%	90.76%
T208A	1.98%	3.8:1	ND	17.94%
N243A	102.33%	4.0:1	104.38%	85.68%
D203A	4.43%	4.0:1	27.04%	8.01%
H261A	17.67%	4.4:1	66.52%	48.81%
I198A	26.62%	4.5:1	99.79%	50.67%
R280A	14.06%	5.3:1	87.13%	35.20%
L182N	101.36%	7.4:1	104.33%	92.86%
A284L	ND	ND	9.75%	ND
R197A	ND	ND	11.10%	3.16%
H201A	ND	ND	16.85%	1.01%

ND not detected

^a The activities of all mutant enzymes with L-Pro, 4-Me-Pro, and L-Pip were relative to those of wild-type Ap-HtyE (100%), respectively

^b The data represent the ratios of *trans*-4-Hyp to *trans*-3-Hyp produced by wild-type Ap-HtyE and its derived mutant enzymes

drastically reduced. These amino acid residues are located close to either substrate L-Pro or α -KG with the exception of Glu178. Regarding the regioselectivity, 13 mutants displayed no change on the product ratio. In contrast, T208A, N243A, D203A, H261A, I198A, and R280A increased the ratio of *trans*-4- to *trans*-3-Hyp from 2.5 (WT) up to 5.3 (Table 3). Interestingly, mutation of Leu182 to Asn significantly increased the ratio to 7.4:1 (*trans*-4-Hyp/*trans*-3-Hyp) (Table 3; Supplementary Fig. S8), which is very close to that of GloF (8.0:1). However, mutation of Arg101 to Gln and Thr revealed no effect. Notably, L182N retained the wild-type Ap-HtyE activity (Table 3) in spite of the significantly changed regioselectivity. In the second round of mutagenesis, saturation mutagenesis targeting the amino acid residue of Leu182 was performed considering its obvious effect on the regioselectivity. Among the 17 mutants constructed, 14 mutants showed obvious changes in the *trans*-4-Hyp/*trans*-3-Hyp ratio (Table S2). However, none of the mutants showed better regioselectivity than L182N, and the activity of these 17 mutants with L-Pro was also affected to a different extent (Table S2). In the third round of mutagenesis, combinatory mutagenesis was performed by incorporating other effective mutations of the first round of mutagenesis (N243A, D203A, H261A, I198A, L218A, and R280A) into the L182N mutant. Of the six double mutants constructed (Table 4), four mutants (L182N/D203A, L182N/I198A, L182N/N243A, and L182N/L218A) displayed further increase in the *trans*-4-Hyp/*trans*-3-Hyp ratio, while L182N/H261A slightly decreased the product ratio compared to L182N (Table 3). In particular, L182N/D203A showed the most dramatic improvement in the regioselectivity and produced over 93% of *trans*-4-Hyp despite its severely impaired activity (1.6%). In contrast, L182N/N243A had a *trans*-4-Hyp/*trans*-3-Hyp ratio of 11:1 while maintaining an activity even slightly higher than that of wild type (Table 4). No preferable production of *trans*-3-Hyp over *trans*-4-Hyp was observed in all the mutants constructed. The effect of these mutations on the hydroxylation of 4-Me-

Pro and L-Pip was also determined (Tables 3, 4, and S2). The catalytic conversion of both substrates seemed to be predominantly affected by mutations interfering with the catalytic efficiencies with L-Pro over those affecting the regioselectivity. Therefore, the majority of mutations which compromised the catalytic activity with L-Pro simultaneously decreased the activity with 4-Me-Pro or L-Pip to different extents. However, there does not seem to exist an apparent correlation between the regioselectivity for L-Pro and the catalyzed hydroxylation of its congeners for HtyE. Of note, there were quite a few mutations, e.g., F204A, which almost crippled the activities with L-Pro but still maintained much higher activities with 4-Me-Pro and L-Pip (92.12 and 77% of WT activity, respectively) (Table 3). On the other hand, whereas L182C and L182V had hardly any effect on the activities with L-Pro, their conversion of L-Pip was reduced to 59 and 65%, respectively.

Deletion of *Ap-htyE* abolished the production of echinocandin B in *Aspergillus pachycristatus*

In order to investigate the in vivo function of *Ap-htyE* in echinocandin B biosynthesis, the *Ap-htyE* gene was replaced by pyrithiamine resistance gene *ptrA* using homologous recombination. Our results revealed that two independently obtained *Ap-htyE* deletion mutants ($\Delta htyE$ -1 and $\Delta htyE$ -2) lost anti-*Candida* activity (Fig. 5). HPLC analysis showed that, whereas wild-type *A. pachycristatus* strain mainly produced echinocandin B and also minor echinocandin C with molecular ions $[M+H]^+$ 1060.58 and 1044.58, respectively (Supplementary Fig. S9), *Ap-htyE* deletion abolished the production of echinocandin B and C but instead produced two new products with molecular ions at $[M+H]^+$ 1028.59 and $[M+H]^+ [-H_2O]$ 994.58, corresponding to the loss of L-Pro and 4-Me-Pro hydroxylation in the echinocandin B and C (Supplementary Fig. S9), respectively. The data suggest that HtyE is the enzyme responsible for the hydroxylation of L-Pro

Table 4 The relative activities and the product ratios (*trans*-4-Hyp/*trans*-3-Hyp) of Ap-HtyE-combined mutants

	Relative activity ^a with L-Pro (%)	The ratio of <i>trans</i> -4- and <i>trans</i> -3-Hyp ^b	Relative activity ^a with 4-Me-Pro (%)	Relative activity ^a with L-Pip (%)
Ap-HtyE	100	2.5:1	100	100
L182N/H261A	14.30	5.4:1	45.41	25.88
L182N/R280A	2.28	6.0:1	63.08	20.59
L182N/L218A	11.50	10.3:1	11.26	46.98
L182N/N243A	114.61	11.0:1	104.37	67.77
L182N/I198A	36.67	11.0:1	95.54	54.86
L182N/D203A	1.67	14.7:1	13.26	6.54

^a The activities of all mutant enzymes with L-Pro, 4-Me-Pro, and L-Pip were relative to those of wild-type Ap-HtyE (100%), respectively

^b The data represent the ratios of *trans*-4-Hyp and *trans*-3-Hyp produced by wild-type Ap-HtyE and its derived mutant enzymes

Fig. 5 Anti-*Candida* activity test of the culture extract from the wild-type *A. pachyristatus* strain and two independent *htyE* deletion mutants (Δ *htyE*-1 and Δ *htyE*-2)

and 4-Me-Pro in vivo, which is essential for the anti-*Candida* activity of the *A. pachyristatus* extracts.

Discussion

Echinocandins are a family of antifungal cyclic lipohexapeptide synthesized by NRPS (Yue et al. 2015). A structural feature of echinocandins is the incorporation of nonproteinogenic amino acids, most of which are hydroxylated. Engineering the hydroxylation pattern of echinocandin hexapeptide offers an intriguing approach to expand their structural diversity and to generate new echinocandin derivatives with enhanced antifungal activity (Li et al. 2015). Elucidation of the amino acid modification machinery is therefore critical in this aspect. In echinocandin B and aculeacin A, precursor molecules of anidulafungin, L-Pro, and 4-Me-Pro were hydroxylated at C-4 and C-3, resulting in *trans*-4-Hyp and 4-Me-3-Hyp, respectively. However, the enzymes responsible for these hydroxylated reactions remain uncharacterized.

In the present study, we biochemically characterized two α -KG/ Fe^{2+} -dependent proline hydroxylases, Ap-HtyE and Aa-HtyE, encoded in their respective biosynthetic gene clusters, and provided the biochemistry basis behind the hydroxylation of L-Pro and 4-Me-Pro. Our results showed that Ap-HtyE and Aa-HtyE are both capable of converting L-Pro to *trans*-3- and *trans*-4-Hyp, but at very different ratios. Moreover, both Ap-HtyE and Aa-HtyE were shown to effectively hydroxylate C-3 of 4-Me-Pro in vitro, confirming their involvement in the generation of both *trans*-4-Hyp and 4-Me-3-Hyp. During the preparation of this manuscript, Mattay et al. independently published the characterization of Ap-HtyE and reported some of the results discussed above (Mattay et al. 2018). We further demonstrated that Ap-HtyE and Aa-HtyE

are also capable of hydroxylating D-proline though with relatively lower efficiency. We further showed that *A. pachyristatus* ATCC 58397 *htyE* knockout strain lost the anti-*Candida* activity and the production of echinocandin B, demonstrating that HtyE is the enzyme responsible for the hydroxylation of L-Pro and 4-Me-Pro in vivo, and more importantly, their hydroxylations are essential for the anti-*Candida* activity of echinocandin B.

In nature, different diastereomers of hydroxylproline, namely *cis*-4-hydroxylproline (*cis*-4-Hyp), *cis*-3-hydroxylproline (*cis*-3-Hyp), *trans*-4-Hyp, and *trans*-3-Hyp, are abundantly present in collagen, gelatin, plant cell wall proteins, and microbial secondary metabolites (Kuttan and Radhakrishnan 1973; Yi et al. 2014). Hydroxylation of proline is catalyzed by the family of α -KG-dependent nonheme iron dioxygenases belonging to different groups (Fig. 6). In the mammalian system, prolyl-4-hydroxylase and prolyl-3-hydroxylase regio- and stereoselectively catalyze the posttranslational hydroxylation of L-Pro residues in procollagen strands for the generation of *trans*-3- and *trans*-4-Hyp, respectively. However, they do not accept free L-Pro as substrate. In contrast, microbial proline hydroxylases hydroxylate free L-Pro but not L-Pro in the peptides. In bacteria, regiostereoselective proline hydroxylases producing three different distereoisomers of hydroxylproline are found (Hara and Kino 2009; Mori et al. 1996; Shibasaki et al. 1999a). Surprisingly, the characterized proline hydroxylases from fungi, including Ap-HtyE and Aa-HtyE in the present study, have been found to catalyze the formation of two distereoisomers *trans*-4-Hyp and *trans*-3-Hyp. It is worth noting that although prolyl hydroxylases from mammal as well as proline hydroxylases from bacteria and fungi all belong to the family of α -KG-dependent nonheme iron dioxygenase and catalyze the hydroxylation of L-Pro, they share very low sequence identity to each other. Nonetheless, these enzymes share the commonly distorted jelly roll β -sheet catalytic core and employ the same catalytic chemistry mechanism, implying their convergent evolution pathways with different overall folds (Clifton et al. 2001; Koketsu et al. 2015).

One assumption about the cryptic activity of fungal proline hydroxylases for the production of *trans*-3-Hyp is that this dual regioselectivity may facilitate the generation of *trans*-4-Hyp and 4-Me-3-Hyp simultaneously with the same enzyme. Our present study indeed showed that both Ap-HtyE and Aa-HtyE are able to hydroxylate 4-Me-Pro. Our kinetic analysis further revealed that both Ap-HtyE and Aa-HtyE are more efficient in the catalytic hydroxylation of 4-Me-3-Hyp than L-Pro in vitro. However, the results of mutagenesis showed that the efficient C-3 hydroxylation of 4-Me-Pro has no direct correlation with the ratio of *trans*-4-Hyp to *trans*-3-Hyp produced with the L-Pro as substrate. This also explains the 1:1 presence of *trans*-4-Hyp and 4-Me-3-Hyp in echinocandin B and aculeacin A despite the obviously different regioselectivity displayed by Ap-HtyE and Aa-HtyE. The reason for the dual

Fig. 6 Representative enzymes of different sources in nature that are involved in the hydroxylation of L-Pro and L-Pip. The proline hydroxylases and pipecolic acid hydroxylases have been found both in bacteria and fungi (**a**). The propyl hydroxylases (**b**) catalyze the posttranslational hydroxylation of L-Pro residues in protocollagen strands for the generation of *trans*-3- and *trans*-4-Hyp, respectively. The phylogenetic tree of propyl hydroxylases and proline hydroxylases and pipecolic acid

hydroxylases found in prokaryotes were constructed separately due to their very low sequence identity. The evolutionary history was inferred by using the maximum likelihood method based on the JTT matrix-based model. The bar represents a genetic distance of 0.5 substitution per position. Each sequence is labeled with its GenBank accession number, corresponding enzymes, and bacterial origin

regioselectivity of fungal proline hydroxylase remains unknown, probably resulting from an evolutionary relief for HtyE which is otherwise taken on by NRPS in echinocandin biosynthesis (Mattay et al. 2018).

Hydroxyprolines represent excellent starting building blocks for the synthesis of a large diversity of chiral molecules for the pharmaceutical industry, such as carbapenem antibiotics (Shibata et al. 1986). Microbial fermentation processes for the production of *trans*-4-Hyp, *cis*-4-Hyp, and *cis*-3-Hyp have been established making use of microbial proline hydroxylases (Johnston et al. 2009; Thi et al. 2013; Zhang et al. 2018). Up to now, characterized fungal proline hydroxylases have been found to generate two products *trans*-4-Hyp and *trans*-3-Hyp at different ratios (Houwaart et al. 2014). Whereas the regioselectivity of fungal proline hydroxylases remains to be exploited, these hydroxylases with dual activity for the production of *trans*-4- and *trans*-3-Hyp provide the opportunity for the construction of a *trans*-3-proline hydroxylase. In the present study, we found that the ratio of *trans*-4-Hyp to *trans*-3-Hyp produced by Ap-HtyE and Aa-HtyE was not affected by the reaction conditions, suggesting that their regioselectivity is an intrinsic property of Ap-HtyE and Aa-HtyE probably determined by the configuration of their active catalytic pockets. By site-directed mutagenesis, we found that Leu182 of Ap-HtyE is a hot spot for the regioselectivity of

Ap-HtyE. Considering the proximity of Leu182 to α -KG, it is probably involved in the coordination of the binding mode of α -KG, hence in determining the regioselectivity of Ap-HtyE. Other mutations targeting residues either located close to L-Pro (Thr208, Asp203, and Asn243) or α -KG (Ile198, Leu218, His261, and Arg280) also showed remarkable improvement in the regioselectivity of Ap-HtyE. The combination of these effective mutations with L182N further increased the regioselectivity of Ap-HtyE. Thus, our mutagenesis study demonstrated that the interplay of residues surrounding either L-Pro or α -KG probably determines the proximity of different C-H positions to the reactive center of proline hydroxylases, hence their regioselectivities. The hydroxylation ratio could thus be changed by mutations of the amino acid residues within the active site, which probably alter the orientation and binding mode of L-Pro and α -KG in the active site. However, mutant enzymes with enhanced production of *trans*-3-Hyp have not been obtained yet. Nevertheless, detailed biochemical and mutagenesis study of more kinds of fungal proline hydroxylases would provide the opportunity and guiding knowledge for the mining and engineering of new proline hydroxylases.

All the reported proline hydroxylases including prolyl hydroxylases are also capable of catalyzing the hydroxylation of the six-membered ring proline homolog L-Pip. The catalyzed production of *trans*-5-Hypip by both Ap-HtyE and Aa-HtyE

has also been reported for *trans*-4-proline hydroxylase from *Dactylosporangium* sp. strain RH1 (Klein and Hüttel 2011; Shibasaki et al. 1999b). On the other hand, a distinct group of α -KG/Fe²⁺-dependent L-Pip *trans*-4-hydroxylase from fungi has been reported to convert L-Pip into *trans*-4-Hypip (Hibi et al. 2016). Biochemical characterization revealed that they also have proline hydroxylase activity although L-Pip was determined to be the preferred substrate over L-Pro. This results contrasts with Ap- or Aa-HtyE which hydroxylates L-Pip at an efficiency comparable or even slightly higher than L-Pro. Considering the relatively higher sequence identity (>40%) between fungal L-Pip and the proline hydroxylases characterized in the present study than bacterial L-Pip and L-Pro hydroxylases, fungal L-proline hydroxylases were probably co-evolved with fungal L-Pip hydroxylase, sharing the same ancestor enzyme. Since Hypips are also useful chiral building blocks in the synthesis of novel pharmaceutical drugs, such as β -lactamase inhibitor MK-7655 (Miller et al. 2014) and fungal L-proline hydroxylases are able to catalyze the formation of optically pure *trans*-5-Hypip, they represent a novel type of biocatalyst with high potential for the preparative production of *trans*-5-Hypip.

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Compliance with ethical standards

This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest The authors declare that they have no conflict of interest.

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