Remodeling of Conformational Dynamics Enhances Catalytic Activities of M1 Zinc-metalloproteinases from Lanthipeptide Biosynthesis

chang zhao

Sichuan University

Wang-Jian Sheng

Nanjing University

ying wang

Nanjing University

Jie Zheng

Nanjing University

Xiangqian Xie

Nanjing University

Yong Liang

Nanjing University <https://orcid.org/0000-0001-5026-6710>

wanqing wei

Nanjing University

Rui Bao

Department of Laboratory Medicine, State Key Laboratory of Biotherapy and Cancer Center, West China Hospital, Sichuan University and Collaborative Innovation Center

Huan Wang (✉ wanghuan@nju.edu.cn)

Nanjing University

Article

Keywords: Lanthipeptides, biosynthesis, catalysis

Posted Date: September 14th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-860352/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Version of Record: A version of this preprint was published at Nature Chemical Biology on May 5th, 2022.
See the published version at <https://doi.org/10.1038/s41589-022-01018-2>.

Remodeling of Conformational Dynamics Enhances Catalytic Activities of M1 Zinc-metallopeptidases from Lanthipeptide Biosynthesis

Chang Zhao,^{1,3} Wangjian Sheng,^{2,3} Ying Wang,² Jie Zheng,² Xiangqian Xie,² Yong Liang,² Wanqing Wei,^{2*} Rui Bao^{1*} and Huan Wang^{2*}

¹Center of Infectious Diseases, State Key Laboratory of Biotherapy, West China Hospital, Sichuan University and Collaborative Innovation Center, Chengdu, 610093, China

²State Key Laboratory of Coordination Chemistry, Chemistry and Biomedicine Innovation Center of Nanjing University, Jiangsu Key Laboratory of Advanced Organic Materials, School of Chemistry and Chemical Engineering, Nanjing University, Nanjing, 210093, China

³These authors contributed equally: Chang Zhao, Wangjian Sheng

*e-mail: wanghuan@nju.edu.cn; baorui@scu.edu.cn; wanqwei@163.com

ABSTRACT

Lanthipeptides are an important group of natural products with diverse biological functions, and their biosynthesis requires the removal of N-terminal leader peptides (LPs) by designated proteases. LanP_{M1} enzymes, a subgroup of M1 zinc-metallopeptidases, are recently identified as bifunctional proteases with both endo- and aminopeptidase activities to remove LPs of class III and class IV lanthipeptides. Herein, we report the biochemical and structural characterization of EryP as the LanP_{M1} enzyme from the biosynthesis of class III lanthipeptide erythreapeptin. We determined X-ray crystal structures of EryP in three conformational states, the *open*, *intermediate* and *closed* states and identified a unique inter-domain Ca binding site as a regulatory element to modulate its domain dynamics and proteolytic activity. Inspired by the regulatory Ca binding, we develop a strategy to engineer LanP_{M1} enzymes for enhanced catalytic activities by strengthening inter-domain associations and driving the conformational equilibrium toward their *closed* forms.

INTRODUCTION

M1 family zinc-metallopeptidases are widely distributed in many species and regulate a diverse range of biological processes.¹ For instance, *Escherichia coli* (*E. coli*) aminopeptidase N (ePepN) is involved in ATP-dependent downstream processing during cytosolic protein degradation.^{2, 3} *Thermoplasma acidophilum* tricorn interacting factor F3 (F3) acts downstream of the proteasome to produce di- and tripeptides.⁴ Human endosomal insulin-regulated aminopeptidase (IRAP) is a key regulator in endocytic trafficking and signaling,⁵ while the endoplasmic reticulum aminopeptidases (ERAP1 and ERAP2) are responsible for antigen-trimming.^{6, 7} Recently, a new biological function of M1 metallopeptidases is revealed in the biosynthesis of bacteria-derived natural product lanthipeptides, a subfamily of ribosomally synthesized and

30 posttranslationally modified peptides (RiPPs).⁸ As the most studied and rapidly growing family of RiPPs, lanthipeptides
31 are featured with diverse structures and biological activities, including antimicrobial, anti-fungal, anti-virus and
32 antiallodynic properties.⁸ The precursor peptide of a lanthipeptide is usually composed of an N-terminal leader peptide and
33 a C-terminal core peptide, which undergoes enzymatic cyclization during biosynthesis. The N-terminal leader peptide
34 plays important roles during the biogenesis of lanthipeptides as recognition elements for modification enzymes, as
35 secretion signals during the exportation or for self-immunity.⁹ The removal of leader peptides is often the last but essential
36 step to produce the mature lanthipeptides. If additional modification is required on the N-terminus of lanthipeptides, such
37 as in the biosynthesis of lipolanthines and goadvionins,^{10, 11} precise and efficient removal of the N-terminal leader peptide
38 becomes even more essential.

39 A subgroup of M1 zinc-metallopeptidases, named LanP_{M1}, function as key proteases to remove the N-terminal leader
40 peptides (LPs) of class III and class IV lanthipeptides during their maturation.¹² AplP from the biosynthesis of class III
41 lanthipeptide NAI-112 represents the first example of LanP_{M1} proteases and removes the leader peptide of precursor
42 peptide AplA after cyclization and glycosylation at its core peptide.¹³ As M1 metallopeptidases usually prefer linear
43 peptides that are shorter than 20 amino acids,¹⁴ LanP_{M1} enzymes therefore represent a rare subgroup of M1
44 metallopeptidases that cleave large cyclic peptide substrates (> 50 amino acid long).^{12, 13, 15, 16} Although the endopeptidase
45 and exopeptidase activities of ePepN remain debatable,^{2, 17, 18} it is generally observed that M1 metallopeptidases only act
46 on the N-terminus of polypeptides and exhibit no endopeptidase activity. Intriguingly, LanP_{M1} proteases possess dual
47 enzymatic activities and remove the LP of precursor peptides through a two-stage process: first, LanP_{M1} cleaves a major
48 N-terminal portion of the LP as an endopeptidase; second, LanP_{M1} trims off the remaining overhang residues as an
49 aminopeptidase to produce the mature natural products.¹³ The mechanism that LanP_{M1} enzymes switch between these two
50 functions remains unknown.

51

Fig. 1 | LanP_{M1} enzymes in lanthipeptide biosynthesis. **a**, Bifunctional LanP_{M1} enzymes remove LP of lanthipeptides through a two-step process. **b**, EryP partially removes the LP of cyclized EryA peptide (EryA_{cyc}) by generating erythreapeptin congeners. The coding gene of EryP is located outside the erythreapeptin BGC.

LanP_{M1} enzymes are usually highly active as endopeptidases, but significantly less efficient as aminopeptidases during the processing of LP overhang residues.^{13, 16} The inefficient aminopeptidase activities of LanP_{M1} enzymes often result in incomplete LP removal both *in vitro* and *in vivo* (Fig. 1a), which impedes the heterologous production and bioengineering of corresponding lanthipeptides.^{9, 12} Thus, to understand the enzymology underlining the dual functionality of LanP_{M1} proteases and to engineer LanP_{M1} enzymes with improved aminopeptidase activities for lanthipeptide production are highly appealing. Herein, we report the biochemical and structural characterization of EryP, which is the LanP_{M1} enzyme from the biosynthesis of class III lanthipeptide erythreapeptin. We determined crystal structures of EryP in three conformational states, the *open*, *intermediate* and *closed* states, by X-ray diffraction and identified a unique inter-domain Ca binding site, which functions as a regulatory element by modulating domain dynamics and the activity of the enzyme. Inspired by the effect of the regulatory Ca binding, we have developed a practical strategy to acquire engineered LanP_{M1} mutants with enhanced aminopeptidase activities by strengthening inter-domain associations of LanP_{M1} enzymes and driving the conformational equilibrium toward their *closed* forms.

RESULTS

EryP is a bifunctional LanP_{M1} protease for leader peptide removal in erythreapeptin biosynthesis. To obtain mechanistic understanding of LanP_{M1} enzymes, we focused on EryP from the biosynthesis of class III lanthipeptide erythreapeptin, which is encoded in the genome of its producing strain *Saccharopolyspora erythraea* NRRL 2338 but

located far outside the erythreapeptin BGC (Fig. 1b, Supplementary Fig. 1).^{13, 19} EryP was expressed heterologously as a His₆-tagged fusion protein from *E. coli* BL21 (DE3) and purified by immobilized metal affinity chromatography as a monomer in solution (Supplementary Fig. 2). His₆-EryP cleaved EryA leader peptide (EryA_{LP}) by generating fragments EryA_{LP}(-15) and EryA_{LP}(-21) as major products, indicating its function as an endopeptidase (Fig. 2a). In addition, His₆-EryP exhibited aminopeptidase activity by hydrolyzing amino acid *para*-nitroanilide (*p*NA) derivatives, including Ala-*p*NA, Pro-*p*NA and Leu-*p*NA, therefore confirming the dual proteolytic activities of EryP for EryA_{LP} processing (Fig. 2b, Supplementary Fig. 3).

Fig. 2 | Bifunctional protease EryP processes both EryA_{LP} and AplA_{cyc} peptides. **a**, MS spectrum of EryA_{LP} peptide cleaved by EryP at multiple sites as an endopeptidase. EryA_{LP}: $M_{calc.}$ =2876.18 Da, $M_{obs.}$ =2876.60 Da; EryA_{LP}(-15): $M_{calc.}$ =1544.62 Da, $M_{obs.}$ =1544.76 Da; EryA_{LP}(-21): $M_{calc.}$ =2271.98 Da, $M_{obs.}$ =2272.13 Da. **b**, EryP hydrolyzes amino acid-*p*NA derivatives as an aminopeptidase. Error bars indicate standard deviation of three independent replicates. **c**, Schematic illustration of EryP removing the LP of AplA_{cyc} peptide through a two-stage process. **d**, EryP completely removed the leader peptide of AplA_{cyc} by generating AplA_{cyc}-CP as the final product. AplA_{cyc}-CP: $M_{calc.}$ *=2242.95 Da, $M_{obs.}$ *=2243.42 Da; AplA_{cyc}-CP(-3): $M_{calc.}$ *=2584.12 Da, $M_{obs.}$ *=2584.76 Da; AplA_{cyc}-CP(-6): $M_{calc.}$ *=2929.24 Da, $M_{obs.}$ *=2928.94 Da. Labels * and # represent sodium and potassium adducts of corresponding peptides, respectively.

Next, we examined the proteolytic activity of EryP toward cyclized lanthipeptide substrates. Since cyclized EryA peptide (EryA_{cyc}, Fig. 1b), the native substrate of EryP, was not accessible, we employed cyclized AplA, the precursor peptide of NAI-112, as an alternative substrate. Cyclized AplA (AplA_{cyc}) shares structural similarity with EryA_{cyc} in the leader peptide and the core peptide, therefore serving as a good substrate mimic (Fig. 2c, Supplementary Fig. 4). As expected, EryP cleaved AplA_{cyc} at multiple sites in the segment of AplA_{LP} peptide after 90 min incubation by generating AplA_{cyc}-CP(-6) as the detectable core peptide product (Supplementary Fig. 5). Prolonged incubation led to stepwise removal of overhang residues in AplA_{cyc}-CP(-6) and eventually yielded AplP_{cyc}-CP as the final product (Fig. 2d). These results indicated that EryP possesses relaxed substrate specificity and is capable of completely removing LPs of class III lanthipeptides other than its native substrate. It is noteworthy that as an endopeptidase, EryP exhibited distinct preference of cleavage sites in EryA_{LP} and AplP_{LP}: it cleaved EryA_{LP} at the N-terminus of ELQ and APN motifs but preferred the C-terminus of ELQ and S-A-(S/T) motifs towards AplA_{LP}, indicating that the site-selectivity of EryP for endoproteolytic cleavage might be substrate-dependent (Fig. 2a and Supplementary Fig. 5).

Peptides	$K_D/\mu\text{M}$		
	EryP	EryP _{E802R}	AplP
EryA _{LP}	40 ± 6	32 ± 14	N.D.
AplA _{cyc}	0.50 ± 0.11	0.57 ± 0.08	0.36 ± 0.18
AplA _{LP}	8.0 ± 2.7	4.0 ± 0.6	2.3±0.5
AplA _{cyc} -CP	720±18	N.D.	N.D.

Table 1 | Binding affinities of EryP, EryP_{E802R} and AplP with peptide substrates. N.D. = not determined

To understand the substrate recognition mechanism of EryP, we performed microscale thermophoresis (MST) to determine the binding affinity between EryP and peptide substrates. Results showed that EryP binds to EryA_{LP} with a K_D of (40.8±5.8) μM , whereas its binding with EryA_{LP(-15)} is too weak to measure (Table 1). EryP binds to full length AplA_{cyc} and AplA_{LP} with K_D values of (0.503±0.109) μM and (7.98±2.72) μM , respectively, indicating that the cyclized AplA core peptide contributes to binding with EryP. Indeed, EryP binds to AplA_{cyc}-CP with a weak but measurable K_D of (721±18) μM . Similarly, AplP binds to full length AplA_{cyc} with a K_D ~9-fold lower than that of AplA_{LP} (Table 1). Therefore, the substrate recognition of EryP and AplP primarily depends on their binding with leader peptides, whereas core peptides act as complementary binding elements.

Overall structure and active-site architecture of EryP. Three sets of EryP crystal structures were determined in distinct conformations, which are postulated as *closed*, *intermediate* and *open* states, with resolution limits from 1.80-2.66 Å (Fig. 3a). The structure of the *closed* conformation was solved by single isomorphous replacement with anomalous scattering from a Sederivative (SAD), and was used as a model to solve the other two structures by molecular replacement. Similar

114 to tricorner-interacting factor F3 (TIFF3) and ePepN of the M1 metalloprotease family, EryP folds into four distinct domains:
115 domain-I (residues 1-201), domain-II (residues 202–456), domain-III (residues 457-552) and domain-IV (residues 553-
116 860) (Fig. 3a). The barrel-like N-terminal domain-I is packed by a central 12-stranded β -sheet and two smaller antiparallel
117 β -sheets. The following catalytic domain-II adopts a thermolysin-like fold characterized by an α/β lobe on the top of an α -
118 helices lobe. A Zn ion binding motif, HEXXH(X)₁₈E, which is highly conserved among M1 metalloproteases, is located
119 at the deep groove between the two lobes (Supplementary Fig. 6). Domain-III presents a barrel-like structure formed by
120 eight β -strands. The C-terminal domain-IV consists of 16 α -helices that are organized in an antiparallel manner by forming
121 a bowl-shaped structure, which might regulate the accessibility of the catalytic pocket in domain-II.⁶

122 In the *closed* state of EryP, a zinc atom resides at the bottom of active-site cleft in catalytic domain and is coordinated
123 by NE2 atoms of two histidine residues (H306 and H310) and OD atoms of E329 (Fig. 3b, Supplementary Fig. 6).²⁰ A
124 catalytic water molecule lies immediately (2.0 Å) next to the Zn ion, resulting in a distorted trigonal-pyramidal
125 coordination network (Fig. 3b). The presence of a catalytic water molecule in the active site prior to peptide substrate
126 binding is consistent with previous studies of M1 metalloproteases, such as thermolysin, and is considered to be essential
127 for their enzymatic functions.²⁰ Residue E307 locates 3.3 Å from the water molecule and might participate in catalytic
128 solvent activation as a general base. The highly conserved Y392 resides in the catalytic pocket and might participate in the
129 proteolysis by stabilizing the tetrahedral intermediate formed by water addition to the substrate scissile amide bond.²¹ Both
130 endopeptidase and aminopeptidase activities of EryP_{E307Q} and EryP_{Y392F} were abolished, confirming the essential role of
131 these residues for catalysis (Supplementary Fig. 7).

132 The interactions between short peptide substrates and the reaction center were visualized by docking a dipeptide Leu-
133 Glu into the active site of EryP in the *closed* state (Supplementary Fig. 8). The highly conserved G₂₇₂AME₂₇₅N motif in
134 EryP binds to the dipeptide substrate through multiple hydrogen bonds in the docking model. In particular, residue E275
135 directly binds to the amino group at the N-terminus of the dipeptide with additional binding from residues E132 and E329
136 (Supplementary Fig. 8). Furthermore, this docking model suggests that both S1 and S1' pockets are spacious to
137 accommodate amino acid side chains of various sizes in peptide substrates, allowing the enzyme to sequentially cleave
138 various residues from the leader of EryA (Supplementary Fig. 8).

Fig. 3 | Overall structure and the metal ion binding sites of EryP. **a**, Three sets of crystal structures of EryP. The four domains of EryP are shown in forest green (domain-I), slate (catalytic domain-II), pink (domain-III), and orange (domain-IV). The catalytic Zn atom is shown as a yellow sphere, and the Ca atom is shown as a red sphere in the *closed* state. **b**, Superposition of the active site of EryP in *closed* (forest green), *intermediate* (slate) and *open* (orange) states. The Zn atom and the catalytic water molecule are shown in yellow and forest green, respectively, in the *closed* state. **c**, Superposition of *closed*, *intermediate* and *open* states of EryP in ribbon style. The interdomain angle θ is defined as the angle of the centers of mass of domain-I and domain-II, domain-III, and domain-IV. The torsion angle ϕ is measured between the center of mass of domain-I and domain-II, the residues 513 and 551 in domain-III, and the center of mass of domain-IV.²² The angles were measured in UCSF chimera.²³ **d**, The inter-domain Ca ion binding site in the *closed* state of EryP.

Comparative analysis of various conformations of EryP. Conformational flexibility is a common feature among four-domain M1 metalloproteases, including ePepN and ERAP1.²⁴⁻²⁶ Among the three sets of EryP crystal structures, domain-I and domain-II are well aligned and share an interface of 36.0-44.3 Å² in area (RMSD from 0.155-0.313 Å) (Fig. 3c). Domain-III serves as a hinge that allows the domain-IV to move toward or away from the Zn-binding active site in domain-

153 II. Scissor motions of domain-IV (0.76 to 1.72 degrees) around domain-III (0.61 to 11.70 degrees of rotation) were
154 observed in the *intermediate* and *open* states (Fig. 3c). In comparison with the *closed* conformation, the areas of domain
155 interface between domain-II with domain-III and domain-IV are reduced from 878.7 to 851.8-876.7 Å² and from 1334.8
156 to 1129.6-476.2 Å², respectively, in *intermediate* and *open* states. The EryP structure in the *open* state has an approximate
157 6852 Å³ internal cavity volume between domain-II and domain-IV as calculated by CASTp 3.0,²⁷ which is highly
158 accessible to solvent. This cavity of EryP is significantly larger than known M1 metallopeptidases in their open
159 conformations. For example, ERAP1 accepts long linear peptide substrates and has a cavity of 5080 Å³ between its domain-
160 II and IV in its open conformation.¹⁴ The exceptionally large internal cavity of EryP may facilitate the recruitment of large
161 cyclized peptide substrate during catalysis, as AplA_{cyc-CP} alone has an estimated volume of ~ 2300 Å³. In the *closed*
162 conformation, the volume of this cavity significantly shrinks to 1531 Å³, and the cavity is fully encompassed by the domain
163 rims in the *closed* state. Despite the significant conformation alteration among three EryP structural sets, critical active-
164 site residues did not display significant structural differences (Fig. 3b). Specifically, residues Y392 in the *closed* and *open*
165 states of EryP are in almost identical position. This is in contrast with several four-domain M1 metallopeptidases, such as
166 ERAP1, in which the catalytic tyrosine residue orients closer toward the Zn center to participate amide hydrolysis in its
167 *closed* conformation than that in its *open* conformation.¹⁴

168 **Calcium ion enhances inter-domain interactions and the aminopeptidase activity of EryP.** In the *closed* state of EryP,
169 a calcium ion is located about 12.1 Å from the Zn ion through coordination by residues E112, E132 and D135 from
170 domain-I, residues E384 from domain-II and E802 from domain-IV (Fig. 3d). Such a Ca ion is missing in the *open* and
171 *intermediate* states, which is in accordance with the shift of residue E802 away from E112 and D135 (Supplementary Fig.
172 9). In addition, the side chain of E384 orients in an opposite direction in the *open* state in comparison with that in the
173 *closed* state, and the loop of residues 384-390 could not be modeled in the *intermediate* conformation likely due to its high
174 flexibility (Supplementary Fig. 9). Together, these data suggest that the Ca ion binding site might function as a regulatory
175 element that mediates the domain dynamics, particularly relative positions of domain-IV to domain-I and II.

176 To understand the role of Ca ion in mediating the tri-domain interaction, we performed 200 ns classical molecular
177 dynamics (MD) simulations based on the crystal structure of EryP in the *closed* state with or without Ca²⁺ ion bound. Two-
178 dimensional descriptors were selected to describe the motion of domain-IV relative to domain-I and II: **d1** is the distance
179 between the CA atoms of E802 and E112, and **d2** is the distance between the CA atoms of E802 and E384 (Supplementary
180 Fig. 10). Considering that **d1** and **d2** in the *closed* state of EryP is 10.95 Å and 9.22 Å, respectively, snapshots are marked
181 as *closed* conformations if **d1** ≤ 10.95 Å and **d2** ≤ 9.22 Å; otherwise, conformational transformation is suggested to occur
182 from *closed* states to an *intermediate* or *open* state. Results indicate that with Ca²⁺ ion bound, EryP tends to adopt *closed*
183 conformations with domain-IV in close distance with domain-I and II (Fig. 4a). When the Ca²⁺ ion was removed from

184 EryP, a significantly wider distribution of conformations was observed, a portion of which deviated from the *closed*
185 conformation (Fig. 4a). When Leu-*p*NA, the favorite aminopeptidase substrate of EryP, was docked into the active site to
186 mimic the substrate-bound EryP, the binding of Ca ion showed an even stronger effect on locking EryP in the *closed*
187 conformations (Fig. 4b). Collectively, these data suggest that by bridging residues from domain-I, II and IV, the Ca ion
188 shifts the conformational equilibrium of EryP toward the *closed* conformation.

189 Next, we evaluated the impact of Ca ions on the proteolytic activity of EryP. Elevation of Ca²⁺ concentration from 0
190 to 1.0 mM in assay buffer increased catalytic efficiency of EryP toward amino acid-*p*NA derivatives by 3-5 folds (Fig. 4c,
191 Supplementary Fig. 3). In addition, both endopeptidase and aminopeptidase activities of EryP toward peptide substrates
192 EryA_{LP} and AplA_{cyc-CP(-4)}, respectively, were significantly improved by the addition of CaCl₂ in reaction buffers (Fig. 4d
193 and 4e), although the binding affinity was not altered (Supplementary Fig. 11). To confirm that the response of EryP activity
194 to Ca ions is resulted from the interdomain Ca²⁺ binding site, E384 was mutated into an Ala residue. EryP_{E384A} mutant
195 retains the same level of aminopeptidase activity toward Ala-*p*NA as EryP, however, was no longer responsive to the
196 change of Ca²⁺ concentration (Supplementary Fig. 12). Collectively, our results demonstrate that although not strictly
197 required for activity, the interdomain Ca²⁺ binding site provides a level adjusting mechanism to stabilize the *closed*
198 conformation of EryP, which consequently enhances its aminopeptidase activity.

199

Fig. 4 | The Ca ion binding site regulates the conformational equilibrium and catalytic activity of EryP. **a**, Distances **d1** and **d2** in EryP and EryP_{E802R} with or without Ca ions. **b**, Distances **d1** and **d2** in EryP-(Leu-*p*NA) and EryP_{E802R}-(Leu-*p*NA) complexes with or without Ca ions. Data were collected from 20000 snapshots during 200 ns MD simulations. **c**, Relative aminopeptidase activity of EryP in the presence or absence of Ca ions toward three amino acid-*p*NA derivatives. Error bars indicate standard deviation of three independent replicates. **d**, EryP displays enhanced aminopeptidase activity toward peptide substrate AplA_{cyc}-CP(-4) in the presence of CaCl₂. AplA_{cyc}-CP(-3): $M_{\text{calc.}}^*=2584.12$ Da, $M_{\text{obs.}}^*=2584.77$ Da; AplA_{cyc}-CP(-4): $M_{\text{calc.}}^*=2699.15$ Da, $M_{\text{obs.}}^*=2700.05$ Da. * and # represents the sodium and potassium adducts of peptides in MS, respectively. **e**, EryP displays enhanced endopeptidase activity toward EryA_{LP} in the presence of CaCl₂. EryA_{LP}: $M_{\text{calc.}}=2876.18$ Da, $M_{\text{obs.}}=2876.63$ Da; EryA_{LP}(-15): $M_{\text{calc.}}=1544.62$ Da, $M_{\text{obs.}}=1544.27$ Da; EryA_{LP}(-21): $M_{\text{calc.}}=2271.98$ Da, $M_{\text{obs.}}=2271.45$ Da.

Rational engineering of inter-domain interactions enhances aminopeptidase activities of EryP. The removal of LP overhang residues is the rate-limiting and often incomplete step during the biosynthesis of class III lanthipeptides, and

213 therefore LanP_{M1} proteases with enhanced aminopeptidase activities are highly desired.^{12, 15, 16} The correlation between
 214 structural dynamics and enzymatic activities of EryP provides an opportunity to create an engineered EryP mutant with
 215 improved aminopeptidase activity by stabilizing the tri-domain association. The Ca binding residues in EryP provide a
 216 promising engineering site to incorporate mutations for enhanced inter-domain network. Based on the *closed* structure of
 217 EryP, we replaced the Ca²⁺ coordinating residue E802 from domain-IV with an arginine (R) residue, which potentially
 218 forms charge-charge interactions with residues E112 and E384 to strengthen the association between domain-I, II and IV.
 219 To confirm the engineering effect, we determined the crystal structure of EryP_{E802R} in 1.77 Å resolution (Supplementary
 220 Fig. 13). In line with our expectation, the overall structure of EryP_{E802R} displays a more compact conformation than EryP
 221 (inter-domain angle $\theta=48.18$, and the torsion angle $\varphi=27.77$) mainly due to the newly formed interactions introduced by
 222 the E802R mutation (Fig. 5a). Specifically, residue R802 inserts into the cleft of domain II and forms a salt-bridge with
 223 residue E384, as well as cation- π interaction with residue F387 from domain II (Fig. 5b; Supplementary Fig. 13). Additional
 224 interaction between R802 and T133 from domain I was observed, which pulls domain I closer to domain IV and increases
 225 their domain interface from 58.2 to 352.3 Å² (Fig. 5a). MD simulation based on the structure of EryP_{E802R} showed that
 226 domain-IV of EryP_{E802R} is in significantly closer contact with domain-I and II than that of EryP, and conformations of
 227 EryP_{E802R} are largely maintained in the *closed* state (Fig. 4a, b). Together, these data support that the E802R mutation
 228 strengthens the interactions between domain IV and domain I & II and drives the conformational distribution of EryP_{E802R}
 229 toward a compacted *closed* state.

230 Despite the conformational changes altered by the E802R mutation, the majority of active site residues in the catalytic
 231 pocket of EryP_{E802R} reside almost identically as in EryP with the exception of Y392, which displays a significant shift with
 232 the phenolic oxygen atom approaching the Zn ion center, shortening their distance from 8.6 to 4.1 Å (Fig. 5c). Y392 is
 233 proposed to stabilize the tetrahedral intermediate during enzymatic amide hydrolysis (Supplementary Fig. 14), and a closer
 234 distance between the Zn ion and Y392 might facilitate the hydrolytic reaction.^{21, 26, 28} We next examined whether the
 235 proteolytic activity of EryP_{E802R} was improved by the conformational remodeling. By Michaelis-Menten analysis,
 236 EryP_{E802R} exhibited aminopeptidase activity toward amino acid-*p*NA substrates with k_{cat}/K_m values increased by ~800-fold
 237 and K_m values lowered by 7-60 folds, indicating that both substrate binding and catalytic efficiency are improved (Fig. 5d;
 238 Supplementary Fig. 15). Meanwhile, the catalytic activity of EryP_{E802R} is no longer responsive to the alteration of Ca ions
 239 in the reaction buffer, supporting the notion that the E802R mutation is a reasonable design which could replace the
 240 enhancement effect induced by Ca binding (Supplementary Fig. 15). Importantly, EryP_{E802R} showed significantly higher
 241 aminopeptidase activity towards peptide AplA_{cyc-CP(-4)} than EryP during the removal of the N-terminal Asp residue (Fig.
 242 5e). Together, our data showed that the E802R mutation stabilizes the overall structure of EryP_{E802R} in a more compact
 243 *closed* conformation than EryP, which leads to improved aminopeptidase activity.

244 To further validate the origin of the enhanced aminopeptidase activity of EryP_{E802R}, we performed MD analysis of the
 245 substrate binding of EryP and EryP_{E802R} to Leu-*p*NA (Supplementary Fig. 16). Typically, three major factors govern the
 246 course of amide bond hydrolysis: the activation of an amide bond by Zn ion, the nucleophilic attack by the hydrolytic
 247 water and the stabilization of the resulting tetrahedron intermediate.²⁹ MD analysis indicated that the average distance
 248 between the Zn ion and the amide oxygen of Leu-*p*NA bound in EryP_{E802R} was ~1.5 Å shorter than that in EryP, suggesting
 249 a stronger amide activation and stabilization of the tetrahedron intermediate (Supplementary Fig. 16). In addition, the
 250 hydrolytic water is ~0.5 Å closer to the amide α-carbon, which increases the likelihood of nucleophilic attack to occur.
 251 Finally, the phenolic oxygen of Y392 displays an average distance of 2.0 Å from the amide oxygen, which is ~2.0 Å shorter
 252 than that in EryP, leading to a significantly stronger stabilization effect of the tetrahedron intermediate. These results show
 253 that the active site constellation of EryP_{E802R} is more optimized for amide hydrolysis than that of EryP.

254

255 **Fig. 5 | The E802R mutation enhanced the inter-domain interaction and the catalytic activity of EryP.** **a**, Structural
 256 comparison between EryP and EryP_{E802R}. **b**, The remodeled inter-domain hydrogen bonding network introduced by the
 257 E802R mutation. **c**, Comparison of active site residues in EryP and EryP_{E802R}. **d**, The aminopeptidase activity toward Ala-
 258 *p*NA and Leu-*p*NA was significantly enhanced. Error bars indicate standard deviation of three independent replicates. **e**,
 259 The aminopeptidase activity toward AplA_{cyc}-CP(-4) peptide was significantly enhanced. Assay conditions: 20 mM Tris, pH
 260 8.0, 100 μM AplA_{cyc} peptide, 1.0 μM EryP or EryP_{E802R} at 37 °C for 90 min. AplA_{cyc}-CP(-3): $M_{calc.}^* = 2584.12$ Da,

261 $M_{\text{obs.}}^*=2584.69$ Da; $\text{AplA}_{\text{cyc-CP}(-4)}$: $M_{\text{calc.}}^*=2699.15$ Da, $M_{\text{obs.}}^*=2699.94$ Da. * and # represents the sodium and potassium
262 adducts of peptides in MS, respectively.

263 **Engineering of inter-domain interactions as a general strategy to modulate the activity of LanP_{M1} enzymes.** The
264 successful engineering of EryP by remodeling the inter-domain interaction prompted us to examine the general
265 applicability of this strategy for other LanP_{M1} enzymes. AplP, the protease from NAI-112 biosynthesis and the first example
266 of LanP_{M1} proteases, was selected as a model. Based on the high sequence similarities between EryP and AplP (49.5%),
267 we built a model of AplP using EryP structure (*closed*) as a template to analyze the potential domain interface. Referring
268 to the T133-E384-R802 inter-domain hydrogen bonding network in EryP_{E802R}, we speculated that the incorporation of two
269 Glu residues and an Arg residue at positions 98, 368 and 779 of AplP might also introduce charge-charge interactions and
270 strengthen the inter-domain association (Supplementary Fig. 17). Thus, we prepared AplP_{R98E-A368E-A779R} and evaluated its
271 catalytic activities. Gratifyingly, the aminopeptidase activity of AplP_{R98E-A368E-A779R} was enhanced by 2.6-fold compared
272 with AplP toward Ala-*p*NA (Supplementary Fig. 18). Furthermore, AplP_{R98E-A368E-A779R} displayed significantly higher
273 endopeptidase activity toward its native peptide substrate AplA_{cyc} than wild-type AplP, but with altered preference to
274 cleavage sites (Fig. 6a). During prolonged incubation, AplP generated AplA_{cyc-CP(-4)} as the major product but no further
275 trimming was detected, indicating that residue Glu(-4) is a challenging residue to remove (Fig. 6b). In contrast, AplP_{R98E-}
276 _{A368E-A779R} exhibited enhanced aminopeptidase activity by overcoming residue Glu(-4) and further produced AplA_{cyc-CP(-1)}
277 as a major product. Therefore, we have constructed an AplP mutant with improved proteolytic activity for the removal of
278 AplA leader peptide through rational introduction of bridging residues that strengthen the inter-domain interaction, which
279 might serve as a general strategy to engineer LanP_{M1} proteases for improved efficiency during leader removal of
280 corresponding lanthipeptides.

a. AplP_{R98E-A368E-A779R} cleaves AplA_{cyc} with improved endopeptidase activity

b. AplP_{R98E-A368E-A779R} displays improved aminopeptidase activity during prolonged incubation

Fig. 6 | AplP_{R98E-A368E-A779R} display improved efficiency in removing the LP of AplA_{cyc} peptide in comparison with AplP. **a**, AplP_{R98E-A368E-A779R} cleaved AplA_{cyc} with higher endopeptidase activity than AplP. Assay conditions: AplP or AplP_{R98E-A368E-A779R} (10 μ M) and AplA_{cyc} (100 μ M) were incubated in 20 mM Tris buffer, pH 8.0, at 37 °C for 1 h. **b**, AplP_{R98E-A368E-A779R} displayed higher aminopeptidase activity than AplP during 72 h incubation. * and # represents the sodium and potassium adducts of peptides in MS, respectively. Detailed MS data are provided in Supplementary Table 3&4.

DISCUSSION

A molecular ruler model has been proposed to explain the substrate preference toward peptides of certain length and sequence for four-domain M1 metalloproteases ERAP1/ ERAP2 and IRAP.^{14, 24, 30} The C-termini of peptide substrates

291 bind to a regulatory site in ERAP1 and induce allosteric activation of ERAP1 by shifting its conformation toward the
 292 closed form.^{14, 24} In contrast, the presence of peptide substrates has little impact on the hydrolytic efficiency of EryP toward
 293 Ala-*p*NA under assay conditions (Supplementary Fig. 19). In addition, the inter-domain cavity in the *closed* form of EryP
 294 is $\sim 1530 \text{ \AA}^3$, which is not sufficient to fully accommodate the cyclized core peptide of AplA peptide ($\sim 2300 \text{ \AA}^3$). These
 295 data implicate that EryP might have distinct mechanisms of substrate recognition and enzyme activation. Although EryP
 296 binds to full length EryA_{LP} and AplA_{LP} peptides (Table 1), it shows no measurable binding with endoproteolytic products
 297 EryA_{LP(-15)-(-1)} and AplA_{LP(-6)-(-1)} and displays no aminopeptidase activity toward them even after long time incubation
 298 (Supplementary Fig. 20). However, EryP could recognize and trim the C-terminal products with cyclized core peptide
 299 (AplA_{cyc-CP(-6)}), suggesting that the core peptide plays a critical role to facilitate substrate recognition by EryP during the
 300 second phase of leader removal. Incubation of EryP and AplA_{LP(-6)} *in trans* with AplA_{cyc-CP} peptide did not lead to improved
 301 aminoproteolytic removal of N-terminal residues from AplA_{LP(-6)}. Together, these data indicate that the core peptide
 302 AplA_{cyc-CP} functions as a substrate recognition unit to deliver the AplA_{LP(-6)} segment to EryP, instead of as an allosteric
 303 activator. Therefore, we propose that the apparent low aminopeptidase activity of EryP during the second phase of leader
 304 removal is primarily due to the low binding affinity to peptide substrates.

305 Three sets of EryP crystal structures, the *open*, *intermediate* and *closed* states, demonstrate that domain-IV are highly
 306 dynamic with large-scale motions relative to domain-I and II (Fig. 3a). The dynamic domain motion is a critical
 307 evolutionary adaptation for M1 aminopeptidases to act as efficient trimming enzymes and regulates the catalytic efficiency
 308 indirectly. A unique inter-domain Ca binding site was visualized in the *closed* state of EryP, which bridges coordinating
 309 residues from domain-I, II and IV, thereby stabilizing the inter-domain association. Unlike thermolysin,³¹ the Ca binding
 310 is not strictly required for the folding or proteolytic activity of EryP, and mutations of Ca binding site have no negative
 311 impact on its activity (Supplementary Fig. 12). However, the binding of Ca ions drives the conformational equilibrium of
 312 EryP toward the *close* state and enhanced its aminopeptidase activity. Thus, this Ca binding site appears to be the key
 313 regulatory element with direct impact on its aminopeptidase activity. The direct correlation between the dynamic
 314 conformational changes and enzymatic activity is further demonstrated by the EryP_{E802} mutant with a more compact and
 315 stabilized *closed* structure and aminopeptidase activity enhanced by over 700-fold compared with EryP. This result could
 316 be rationalized by considering that the long-range attractive electrostatic forces introduced by the E802R mutation
 317 facilitates the domain motion. Therefore, our observation strongly supports the proposal from previous studies that the
 318 conformational closure of M1 metallopeptidases is mandatory for optimal aminopeptidase activity, while the reciprocal
 319 open-close transition promotes the substrate binding and product release.^{14, 22, 26} Importantly, elucidation of the Ca binding
 320 site as the conformational regulatory element of LanP_{M1} enzymes allows their engineering for improved aminopeptidase
 321 activity. The successful construction of EryP_{E802R} and AplP_{R98E-A368E-A779R} with improved activities support our working

322 principle, providing a practical engineering strategy for improved LanP_{M1} enzymes for lanthipeptide production.

323 Metallopeptidases with endopeptidase activities, such as astacin and human matrix metalloproteinase-1, contain a
324 catalytic groove lying between the helices bundle and Greek key-like beta sheet to accommodate peptide substrates.³²
325 However, the N-terminal cleft of the substrate binding groove in M1 family metallopeptidases is largely blocked by domain
326 I, although preserves space to lodge the substrate N-terminus.^{25, 26, 33} In comparison to other M1 metallopeptidases, EryP
327 has a relatively elongated groove extending from the catalytic site with potential binding subsites to accommodate longer
328 N-terminal fragment of peptide substrates. This enlarged peptide binding site might facilitate EryP to achieve recognition
329 of leader peptides and perform endoproteolytic cleavage. The V(L/Q)E(I/L)(Q/L)EL motif in EryA_{LP} and AplA_{LP}, which
330 is highly conserved in class III lanthipeptide leader peptides for substrate recognition by lanthipeptide synthetases, appears
331 to be important for LanP_{M1} enzymes. Major endoproteolytic cleavage sites by EryP enzymes often reside in the middle or
332 to the C-terminal to this conserved site (Fig. 2a; Supplementary Fig. 5), suggesting that this motif might associate to the
333 substrate binding groove around the active site of EryP. Nevertheless, how LanP_{M1} enzymes switch functions between
334 endo- and aminopeptidases requires further investigation.

335 In conclusion, we report the biochemical and structural characterization of bifunctional M1 zinc-metallopeptidase
336 EryP. Crystal structures of EryP in different states provide insights into the conformational dynamics of the rate-limiting
337 protease LanP_{M1} enzymes in lanthipeptide biosynthesis. An unprecedented inter-domain Ca binding site is revealed as an
338 effector mechanism for inter-domain association and aminopeptidase activity. These results not only highlight the
339 importance of the conformation dynamics in M1 metallopeptidase function but also provide valuable insights with respect
340 to understanding their trimming mechanism. Importantly, strengthening the inter-domain interactions through rationally
341 designing bridging residues generates LanP_{M1} variants with improved aminopeptidase activities, which hold potential
342 application in the bioengineering and production of lanthipeptides. This successful engineering effort could potentially
343 guide site-specific protein engineering on other M1 metallopeptidases to improve their catalytic efficiency.

344 References

- 345 1. Rawlings, N.D., Barrett, A.J. & Bateman, A. MEROPS: the peptidase database. *Nucleic Acids Res.* **38**,
346 D227-233 (2010).
- 347 2. Chandu, D., Kumar, A. & Nandi, D. PepN, the major Suc-LLVY-AMC-hydrolyzing enzyme in
348 Escherichia coli, displays functional similarity with downstream processing enzymes in Archaea and
349 eukarya. Implications in cytosolic protein degradation. *J. Biol. Chem.* **278**, 5548-5556 (2003).
- 350 3. Chandu, D. & Nandi, D. PepN is the major aminopeptidase in Escherichia coli: insights on substrate
351 specificity and role during sodium-salicylate-induced stress. *Microbiology* **149**, 3437-3447 (2003).
- 352 4. Tamura, N., Lottspeich, F., Baumeister, W. & Tamura, T. The role of tricorn protease and its
353 aminopeptidase-interacting factors in cellular protein degradation. *Cell* **95**, 637-648 (1998).

- 354 5. Hermans, S.J. *et al.* Crystal structure of human insulin-regulated aminopeptidase with specificity for cyclic
355 peptides. *Protein Sci.* **24**, 190-199 (2015).
- 356 6. Nguyen, T.T. *et al.* Structural basis for antigenic peptide precursor processing by the endoplasmic
357 reticulum aminopeptidase ERAP1. *Nat. Struct. Mol. Biol.* **18**, 604-613 (2011).
- 358 7. Zervoudi, E. *et al.* Rationally designed inhibitor targeting antigen-trimming aminopeptidases enhances
359 antigen presentation and cytotoxic T-cell responses. *Proc. Natl. Acad. Sci. U S A* **110**, 19890-19895
360 (2013).
- 361 8. Montalban-Lopez, M. *et al.* New developments in RiPP discovery, enzymology and engineering. *Nat.*
362 *Prod. Rep.* **38**, 130-239 (2020).
- 363 9. Repka, L.M., Chekan, J.R., Nair, S.K. & van der Donk, W.A. Mechanistic Understanding of Lanthipeptide
364 Biosynthetic Enzymes. *Chem. Rev.* **117**, 5457-5520 (2017).
- 365 10. Kozakai, R. *et al.* Acyltransferase that catalyses the condensation of polyketide and peptide moieties of
366 goadivionin hybrid lipopeptides. *Nat. Chem.* **12**, 869-877 (2020).
- 367 11. Wiebach, V. *et al.* The anti-staphylococcal lipolanthines are ribosomally synthesized lipopeptides. *Nat.*
368 *Chem. Biol.* **14**, 652-654 (2018).
- 369 12. Hegemann, J.D. & Süssmuth, R.D. Matters of class: coming of age of class III and IV lanthipeptides. *RSC*
370 *Chem. Biol.* **1**, 110-127 (2020).
- 371 13. Chen, S. *et al.* Zn-dependent bifunctional proteases are responsible for leader peptide processing of class
372 III lanthipeptides. *Proc. Natl. Acad. Sci. U S A* **116**, 2533-2538 (2019).
- 373 14. Nguyen, T.T. *et al.* Structural basis for antigenic peptide precursor processing by the endoplasmic
374 reticulum aminopeptidase ERAP1. *Nat. Struct. Mol. Biol.* **18**, 604-613 (2011).
- 375 15. Ren, H., Shi, C., Bothwell, I.R., van der Donk, W.A. & Zhao, H. Discovery and Characterization of a
376 Class IV Lanthipeptide with a Non-overlapping Ring Pattern. *ACS. Chem. Biol.* **15**, 1642-1649 (2020).
- 377 16. Wiebach, V. *et al.* An Amphipathic Alpha-Helix Guides Maturation of the Ribosomally-Synthesized
378 Lipolanthines. *Angew. Chem. Int. Ed.* **59**, 16777-16785 (2020).
- 379 17. Lazdunski, C., Busuttil, J. & Lazdunski, A. Purification and properties of a periplasmic
380 aminoendopeptidase from Escherichia coli. *Eur. J. Biochem.* **60**, 363-369 (1975).
- 381 18. Mccaman, M.T. & Villarejo, M.R. Structural and Catalytic Properties of Peptidase-N from Escherichia-
382 Coli K-12. *Arch. Biochem. Biophys.* **213**, 384-394 (1982).
- 383 19. Voller, G.H. *et al.* Characterization of new class III lantibiotics--erythreapeptin, avermipeptin and
384 griseopeptin from Saccharopolyspora erythraea, Streptomyces avermitilis and Streptomyces griseus
385 demonstrates stepwise N-terminal leader processing. *Chembiochem* **13**, 1174-1183 (2012).
- 386 20. Cerda-Costa, N. & Gomis-Ruth, F.X. Architecture and function of metallopeptidase catalytic domains.
387 *Protein Sci.* **23**, 123-144 (2014).
- 388 21. Thompson, M.W., Archer, E.D., Romer, C.E. & Seipelt, R.L. A conserved tyrosine residue of
389 Saccharomyces cerevisiae leukotriene A4 hydrolase stabilizes the transition state of the peptidase activity.
390 *Peptides* **27**, 1701-1709 (2006).
- 391 22. Papakyriakou, A. & Stratikos, E. The Role of Conformational Dynamics in Antigen Trimming by
392 Intracellular Aminopeptidases. *Front. Immunol.* **8**, 946 (2017).

- 393 23. Pettersen, E.F. *et al.* UCSF Chimera--a visualization system for exploratory research and analysis. *J.*
394 *Comput. Chem.* **25**, 1605-1612 (2004).
- 395 24. Kochan, G. *et al.* Crystal structures of the endoplasmic reticulum aminopeptidase-1 (ERAP1) reveal the
396 molecular basis for N-terminal peptide trimming. *Proc. Natl. Acad. Sci. U S A* **108**, 7745-7750 (2011).
- 397 25. Kyrieleis, O.J., Goettig, P., Kiefersauer, R., Huber, R. & Brandstetter, H. Crystal structures of the tricorn
398 interacting factor F3 from *Thermoplasma acidophilum*, a zinc aminopeptidase in three different
399 conformations. *J. Mol. Biol.* **349**, 787-800 (2005).
- 400 26. Addlagatta, A., Gay, L. & Matthews, B.W. Structure of aminopeptidase N from *Escherichia coli* suggests
401 a compartmentalized, gated active site. *Proc. Natl. Acad. Sci. U S A* **103**, 13339-13344 (2006).
- 402 27. Tian, W., Chen, C., Lei, X., Zhao, J. & Liang, J. CASTp 3.0: computed atlas of surface topography of
403 proteins. *Nucleic Acids Res.* **46**, W363-W367 (2018).
- 404 28. Cadel, S., Darmon, C., Pernier, J., Herve, G. & Foulon, T. The M1 family of vertebrate aminopeptidases:
405 role of evolutionarily conserved tyrosines in the enzymatic mechanism of aminopeptidase B. *Biochimie*
406 **109**, 67-77 (2015).
- 407 29. Tholander, F. *et al.* Structure-based dissection of the active site chemistry of leukotriene A4 hydrolase:
408 implications for M1 aminopeptidases and inhibitor design. *Chem. Biol.* **15**, 920-929 (2008).
- 409 30. Sui, L. & Guo, H.C. ERAP1 binds peptide C-termini of different sequences and/or lengths by a common
410 recognition mechanism. *Immunobiology* **226**, 152112 (2021).
- 411 31. Eijssink, V.G., Matthews, B.W. & Vriend, G. The role of calcium ions in the stability and instability of a
412 thermolysin-like protease. *Protein Sci.* **20**, 1346-1355 (2011).
- 413 32. Bode, W., Gomis-Ruth, F.X., Huber, R., Zwilling, R. & Stocker, W. Structure of astacin and implications
414 for activation of astacins and zinc-ligation of collagenases. *Nature* **358**, 164-167 (1992).
- 415 33. Ito, K. *et al.* Crystal structure of aminopeptidase N (proteobacteria alanyl aminopeptidase) from
416 *Escherichia coli* and conformational change of methionine 260 involved in substrate recognition. *J. Biol.*
417 *Chem.* **281**, 33664-33676 (2006).

419 Acknowledgments

420 This work is supported by NSF of China (Grant 21922703 and 91953112 to H.W.; 81871615 and 81670008 to R. B.), the
421 Natural Science Foundation of Jiangsu Province (Grant BK20190004 and BK20200335), the Fundamental Research Funds
422 for the Central Universities (Grant 14380138 and 14380131 to H.W.), Ministry of Science and Technology of the People's
423 Republic of China (Grant No.2018ZX09201018-005) and National Mega-project for Innovative Drugs
424 (2019ZX09721001-001-001). We thank National Center for Protein Sciences Shanghai (NCPSS) beamlines BL18U and
425 BL19U allowance. We thank the staffs of National Center for Protein Sciences Shanghai (NCPSS) beamlines BL18U and
426 BL19U and Shanghai Synchrotron Radiation Facility (SSRF) BL17U, Shanghai, People's Republic of China, for assistance
427 during data collection. We thank the High Performance Computing Center (HPCC) of Nanjing University for the numerical
428 calculations on its blade cluster system.

429 **Author contributions**

430 H.W. and R.B. initiated and directed this study together with W.W., W.S., C.Z. and Y.W. prepared enzymes and peptides
431 and performed biochemical assays. C.Z. performed structural biology experiments. W.W. performed computational studies
432 on protein dynamics. All authors participate in the data analysis and manuscript preparation.

433 **Competing interests**

434 The authors declare no competing interests.

435 **Additional information**

436 Supplementary information is available for this paper online.

437 Correspondence and requests for materials should be addressed to H.W. or R.B.

438 **Data availability**

439 All crystal structures have been deposited in the PDB database under accession number 7V9N for EryP_{closed}, 7V9P for
440 EryP_{intermediate}, 7V9Q for EryP_{open} and 7V9O for EryP_{E802R}.

441

442 **Methods**

443 **General methods.** Polymerase chain reactions (PCR) were carried out on a C1000 Touch™ thermal cycler (Bio-Rad).
444 DNA sequencing was performed by the Genscript Biotech, using appropriate primers. Matrix-assisted laser
445 desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was carried out on Bruker UltraFlex™.
446 Liquid chromatography electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS) was carried out and
447 processed using a Triple TOF 4600 System (AB Sciex) equipped with a Prominence Ultra-Fast Liquid Chromatography
448 (UFLC) system (Shimadzu). UV-Vis spectrometry was recorded by Cary 300 (Agilent Technologies). Conditions for all
449 ESI-MS and MS/MS were set as follows: nebulizer gas: 55 psi; heater gas: 55 psi; curtain gas: 35 psi; drying temperature:
450 550 °C; ion spray voltage: 5500 V; declustering potential: 100 V; collision energy: 35 V (positive); collision energy spread:
451 10 V. The mass range and accumulation time are 400-4000 m/z, 250 ms for ESI-MS and 100-2000 m/z, 100 ms for MS/MS,
452 respectively. Collision-induced dissociation (CID) was performed for fragmentation of the respective peptide ions.
453 Calibration solutions purchased from AB SCIEX were used for instrument calibration, and high resolution was chosen in
454 the ESI+ mode.

455 **Materials.** All oligonucleotides were purchased from Genscript Biotech (Nanjing, China). Restriction endonucleases and
456 T4 DNA ligase were purchased from New England Biolabs (Ipswich, MA, USA). Phanta® Max Master Mix was purchased
457 from Vazyme Biotech (Nanjing, China). Media components for bacterial cultures were purchased from Thermo Fisher
458 (Waltham, MA, USA). Chemicals were purchased from Aladdin Reagent (Shanghai, China) or from Sigma-Aldrich
459 (Schnelldorf, Germany) unless noted otherwise. Endoprotease GluC was purchased from Roche Biosciences (Basel,
460 Switzerland). *E. coli* DH5α was used as the host for cloning and plasmid propagation, and *E. coli* BL21 (DE3) was used
461 as a host for expression of proteins and peptides.

462 **General nomenclature.** In this report, we use the standardized nomenclature recommended by the lanthipeptide
463 community in 2020 (*Nat. Prod. Rep.* **2020**, 38, 130-239). The numbering of residues in lanthipeptide precursor peptides
464 starts with the first residue of the core peptide. Residues in the leader peptide are indicated with negative numbers counting
465 back from the junction between the leader peptide and core peptide.

466 **Molecular cloning of *aplA*, *aplKC*, *aplP* and *eryP* genes.** Plasmids containing target genes were synthesized by Genscript
467 Biotech and PCR amplified by 30 cycles of denaturing (95 °C for 30 s), annealing (65 °C for 30 s), and extending (72 °C,
468 1 min/kb) using high fidelity Phanta® DNA Polymerase. Amplifications were confirmed by 2% agarose gel electrophoresis,
469 and the PCR products were purified using an Omega Biotech Cycle Pure Kit. The target DNA fragment and pRSFDuet-1
470 vector were digested in separate reactions containing 1X NEB buffer (New England Biolabs) with selected pair of
471 restriction enzymes for 2 h at 37 °C. The digested products were purified by agarose gel electrophoresis, and the DNA
472 fragments were extracted from the gel using an Omega Biotech Gel Extraction Kit. The resulting DNA products were

473 ligated at 16 °C for 12 h in 1X T4 DNA Ligase buffer with T4 DNA Ligase (0.7 U/ µL). *E. coli* DH5α cells were
474 transformed with 2.5 µL of the ligation product by heat shock, and cells were plated on LB-kanamycin agar plates and
475 grown for 15 h at 37 °C. Several colonies were picked and used to inoculate separate 5 mL cultures of LB-kanamycin
476 medium. The cultures were grown at 37 °C for 12 h, and plasmids were isolated using an Omega Biotech Plasmid Mini
477 Kit. The sequences of the resulting plasmid products were confirmed by DNA sequencing.

478 **Mutagenesis of AplP and EryP.** Mutagenesis of AplP and EryP was carried out by a two-stage protocol, which starts with
479 the generation of primers using two parallel asymmetric PCRs, followed by an exponential whole-plasmid amplification.
480 PCR amplification was performed by 30 cycles of denaturing (95 °C for 30 s), annealing (65 °C for 30 s), and extending
481 (72 °C, 1 min/kb) using high fidelity Phanta® DNA Polymerase and confirmed by 1% agarose gel electrophoresis. PCR
482 products were purified using an Omega Biotech Cycle Pure Kit. The target DNA fragment was digested by DpnI in NEB
483 buffer (New England Biolabs) for 3 h at 37 °C. *E. coli* DH5α cells were transformed with 2.5 µL of the digested product
484 by heat shock, plated on LB-agar plates and grown for 15 h at 37 °C. Single colonies were picked to inoculate separate 5
485 mL cultures of LB-chloramphenicol medium, which were grown at 37 °C for 12 h. Plasmids were isolated using an Omega
486 Biotech Plasmid Mini Kit. The sequences of the resulting plasmid products were confirmed by DNA sequencing.

487 **Overexpression and purification of AplA_{cyt} peptide.** *E. coli* BL21(DE3) cells were transformed with pRSFDuet-1-His₆-
488 AplA and pACYCDuet-1- His₆-AplKC and plated on a Luria broth (LB) agar plate containing 50 mg/L of kanamycin and
489 35 mg/L of chloramphenicol. A single colony was used to inoculate a 5 mL culture of LB supplemented with 50 mg/L of
490 kanamycin and 35 mg/L of chloramphenicol at 37°C for 12 h. The culture was used to inoculate 4 L of LB containing 50
491 mg/L of kanamycin and 35 mg/L of chloramphenicol. Cells were grown at 37 °C to OD₆₀₀~0.6-0.8, cooled to 16 °C before
492 IPTG was added to a final concentration of 0.2 mM. The resulting cultures were grown for another 20 h, harvested and
493 processed following the purification procedure described in the previous section.

494 **Overexpression and purification of EryP and AplP enzymes.** *E. coli* BL21 (DE3) cells were transformed with pET-28a
495 plasmids containing genes encoding target enzymes. A single colony transformant was used to inoculate a 30 mL culture
496 of LB supplemented with 50 µg/mL kanamycin. The culture was grown at 37 °C for 12 h and was used to inoculate 3 L of
497 LB containing 50 µg/mL kanamycin, and cells were grown at 37 °C to OD₆₀₀ ≈ 0.6. The culture was incubated at 4 °C on
498 ice for 20 min, then IPTG was added to a final concentration of 0.2 mM and the culture was incubated at 18 °C for 20 h.
499 Cells were harvested by centrifugation at 12,000 ×g for 15 min at 4 °C, and the pellet was resuspended in 30 mL of start
500 buffer (20 mM Tris buffer, pH 8.0, 500 mM NaCl, 1.0 mM TCEP, 10% glycerol) and stored at -80 °C. All protein
501 purification steps were performed at 4 °C. The cell paste was suspended in start buffer and the cells were lysed using a
502 high pressure homogenizer (Avestin, Inc.). Cell debris was pelleted via centrifugation at 23,700 ×g for 20 min at 4 °C. The
503 supernatant was loaded onto a 5 mL HisTrap HP IMAC column charged with Ni²⁺ and equilibrated with start buffer. The

column was washed with 50 mL of buffer A (30 mM imidazole, 20 mM Tris, pH 7.5, 300 mM NaCl), and the protein was eluted using a linear gradient of 0-100% buffer B (200 mM imidazole, 20 mM Tris, pH 7.5, 300 mM NaCl) over 40 min at a 2 mL/min flow rate. UV absorbance (280 nm) was monitored and fractions were collected and analyzed by SDS-PAGE. The fractions containing target proteins were combined and concentrated using an Amicon Ultra-50 Centrifugal Filter Unit (30 kDa MWCO, Millipore). Gel filtration purification was used to further purify target proteins. The concentrated protein sample was injected onto an FPLC system (ÄKTA) equipped with an XK16 16/60 (GE Healthcare Life Sciences) column packed with SuperDex 75 resin previously equilibrated in 20 mM HEPES (pH 7.5), 200 mM KCl. The protein was eluted with a flow rate of 1.0 mL/min. Both UV (280 nm) and conductance were monitored and fractions were collected. Misfolded/aggregated protein was efficiently separated from soluble, correctly folded protein and the desired fractions were combined and concentrated using an Amicon Ultra-15 Centrifugal Filter Unit. The resulting protein sample was stored at -80°C . Protein concentration was determined using a Bradford Assay Kit (Pierce).

Preparation of AplA_{cyc}-CP(-4) peptide. AplA_{cyc} (100 μM) was digested by GluC (1.0 μM) in 50 mM Tris buffer, pH 8.0, at 37°C for 12 h. The resulting AplA_{cyc}-CP(-4) peptide was purified by HPLC.

***In vitro* assay of EryP and EryP mutants cleaving EryA_{LP}, AplA_{cyc} and AplA_{cyc}-CP(-4) peptides.** Typically, EryP or a EryP mutant (10 μM) was incubated with a peptide substrate (100 μM) in 20 mM Tris buffer, pH 8.0, in a 37°C water bath. EryP enzymes were omitted in negative control experiments.

***In vitro* assay of EryP cleaving EryA_{LP}, AplA_{cyc} and AplA_{cyc}-CP(-4) in the presence of CaCl₂.** Typically, EryP or a EryP mutant (10 μM) was incubated with EryA_{LP} peptide (100 μM) in 20 mM Tris buffer, pH 8.0, with or without CaCl₂ (0.1 mM or 1.0 mM) in a 37°C water bath. EryP enzymes were omitted in negative control experiments.

Reaction kinetics of the hydrolysis of amino acid-*p*NA derivatives by EryP and AplP. Typically, AplP, EryP (1.0 μM), inactive EryP mutants (1.0 μM) or EryP_{E802R} (0.02 μM) was incubated with amino acid-*p*NA derivatives of varied concentrations in 20 mM Tris buffer, pH 8.0, at 37°C (assay samples). AplP or EryP enzymes were omitted in control samples. The formation of product *p*-nitroanilide was measured by the absorbance at 405 nm ($\text{Abs}_{p\text{NA}} = \text{Abs}_{405\text{nm_assay_sample}} - \text{Abs}_{405\text{nm_control_sample}}$). To investigate the impact of Ca ions on enzymatic activities, CaCl₂ of certain concentration was added in the assay and control buffers.

Fluorescence-labeling of peptides. FITC-labeling of AplA_{cyc} peptide was performed in conditions: 90 mM phosphate buffer, pH 8.4, 45 μM peptide and 450 μM FITC for 12 h at room temperature and in dark conditions. Fluorescence-labeling of EryA_{LP} and AplA_{LP} peptides was performed in conditions: 20 mM phosphate buffer (EtOH:H₂O=2:8, v/v), pH 7.4, 10 μM peptide, 20 μM fluorescein o-acrylate. The reaction was allowed to proceed for 1 h at room temperature in dark conditions. The desired FITC-peptide conjugates were purified by HPLC.

Measurement of protein-peptide interactions by MST. As a representative example, the binding affinity between EryP

535 and FITC-labeled EryA_{LP} was measured using Monolith NT.115 Pico (Nanotemper Technologies). The EryA_{LP} sample was
536 diluted using MST buffer (50 mM Tris, 150 mM NaCl, 0.05% Tween-20, 10 mM MgCl₂, 5% glycerol) until pretest
537 fluorescence excitation range from 200-2000 for the most optimized response (~1.0 μM). To perform the MST assay,
538 FITC-labeled EryA_{LP} sample (10 μL) was first incubated with EryP (10 μL) of 16 different serial dilutions in the assay
539 buffer for 5 min to allow binding. Samples were then loaded into Monolith NT.115 capillary (Nanotemper Technologies)
540 and measured using 20% (Auto-detect) Nano – BLUE as excitation power and medium MST power. The measurement
541 was repeated for three times. Data analysis was performed using Nanotemper affinity analysis software.

542 **Crystallization, data collection of EryP.** The purified protein was adjusted to about 9 mg/ml and subjected to screenings
543 of different crystallization conditions at 18 °C. For EryP in the *closed* state, crystals in the space group *P*4₃2₁2 were grown
544 from hanging drops with 3 μL of reservoir solution (0.1 M calcium acetate; 0.1 M sodium acetate, pH 4.5; 10% w/v PEG
545 4000). The condition for the growth of Se-Met-labeled crystals is the same as that for the native crystals in the closed state.
546 When co-crystallized with 2mM VLELQE peptide, which is conserved in the leader peptides of class III lanthipeptides,
547 crystals of EryP in the intermediate state that belongs to the space group *P*12₁1 appeared after two months under the
548 condition of 0.2 M NaCl, 0.1 M Tris, pH 8.0 and 20% w/v PEG 4000. When co-crystallized with 4mM cyclized-AplA,
549 which contains the leader peptide and the cyclized core peptide, the open form crystals in the space group *P*2₁2₁2₁ were
550 obtained by mixing 1.5 μL of sample solution and 1 μL of reservoir solution (0.1 M citric acid, pH 3.5; 25% w/v PEG3350)
551 using the sitting method. The crystals of EryP_{E802R} were grown in 0.2 M ammonium chloride, 20% w/v PEG 6000 and
552 MES, pH 6.0. Prior to data collection, all the crystals were transferred to cryoprotectant solutions (20% v/v glycerol in
553 corresponding crystallization solution) and flash-cooled in liquid nitrogen. X-ray data were collected at BL-17U1 or BL-
554 18U1 station of the Shanghai Synchrotron Radiation Facility (SSRF), China.

555 **Data processing and structure determination of EryP.** Diffraction images were integrated and scaled with the HKL2000
556 program package³⁴. The phase problem of Se-Met-labeled EryP was solved by using the single-wavelength anomalous
557 dispersion (SAD) method, and nine Se sites were found by using the AutoSol tool of PHENIX suite³⁵. The native structures
558 of EryP in different conformations were determined by molecular replacement using the Phaser-MR tool of PHENIX and
559 the structure of Se-Met-labeled EryP was selected as a template. The structures were rebuilt with COOT³⁶ and refined
560 using the Refinement tool of PHENIX or CCP4³⁷ for several rounds. Data collection and refinement statistics are shown
561 in Table S1. The structural figures were drawn with PyMOL (<https://pymol.org/2/>) and USCF Chimera X³⁸.

562 **Initial structural preparation for computational studies.** The initial structure of EryP with Ca²⁺ ion bound was based
563 on the *closed* structure of EryP (PDB ID: 7V9N). The Ca ion was manually removed from the EryP_{closed} structure to build
564 an EryP structure without Ca²⁺ bound. The starting coordinate of the EryP_{E802R} mutant were based on its crystal structure
565 (PDB ID: 7V9O). The protonation states of charged residues were determined at constant pH 7.5 based on *pK_a*

calculations via the PROPKA program³⁹ and the consideration of the local hydrogen bonding network. In models of EryP with/without Ca ion bound, residues His102, 182, 231, 249, 422, 489, 492, 512, 532, 659, 756, 774 and 797 were assigned as HIE; residues His306, 310 and 710 were set as HID; the rest His residues were HIP. In the model of EryP_{E802R}, residues His102, 182, 306, 310, 512, 756, 710, 756 and 797 were assigned as HIE; residues His115, 231, 249, 422, 489, 492, 532, 659 and 774 were set as HID; the rest His residues were HIP. In above three models, all Asp and Glu residues were set as deprotonated status except for Asp315, while Lys and Arg residues were all set as protonated status. The force field parameters for the Zn center active site were prepared using the Metal Center Parameter Builder (MCPB.py)⁴⁰ as implemented in Amber 16⁴¹, in which residues H306, H310, E329 and a catalytic water molecule were all treated in coordinate bonded way. Bond and angle force constants were derived using the Seminario method⁴², and point charge parameters for the electrostatic potentials were obtained using the ChgModB method. Each model was neutralized by adding Na⁺ ions and solvated into a truncated octahedron TIP3P⁴³ water box with a 10 Å buffer distance on each side. These three models consisted of 84549, 84547 and 88052 atoms for the EryP bound with or without Ca²⁺ and EryP_{E802R}, respectively.

Molecular docking. The GLIDE program⁴⁴ was used to perform molecular docking of EryP with the LE peptide. The crystal structure of EryP in the closed state was minimized by using the OPLS3 force field and the ligand was prepared with the ‘LigPrep’ module. All docking procedures were calculated in the “Standard Precision” (SP) mode with default parameters. The final scoring was performed on energy-minimized poses and displayed as Glide scores. The best-docked pose with the lowest Glide score value was selected for further analysis.

To dock Leu-*p*NA into the active sites of EryP enzymes, 50000 snapshots uniformly distributed at equal intervals from the last 100 ns MD simulation (with time intervals of 2 ps) were picked up and divided into ten groups using hierarchical agglomerative (bottom-up) approach⁴⁵. Leu-*p*NA, the favorite aminopeptidase substrate of EryP, was fully optimized at the B3LYP-D3/6-31+G(d) level of Gaussian 16 using the CPCM⁴⁶⁻⁴⁸ model in water, and then docked into the active site of one representative group snapshot to mimic the native substrate-EryP complex. Molecular docking was performed using the Lamarckian genetic algorithm local search method in the AutoDock 4.2 and AutoDockTools-1.5.6⁴⁹. The docking approach was employed on rigid-receptor conformation, while all the rotatable torsional bonds of Leu-*p*NA were set free. A grid box was centered on the Zn atom and its size was set to 40 Å × 40 Å × 40 Å points with a 0.375 Å spacing. A total of 500 independent docking runs were undertaken with a maximum energy evaluation of 2.5×10^7 . The obtained 500 docked conformations were clustered with 2.0 Å RMSD and ranked depending on an energy-based scoring function. The possible catalytically active binding modes were

595 selected as initial configurations to perform MD simulations of EryP and EryP_{E802R} in complex with Leu-*p*NA
596 according to scoring function and reasonable conformation.

597 **Molecular dynamics simulation.** All molecular dynamics (MD) simulations were performed by Amber 16
598 package⁴¹. The MD pre-equilibrated EryP and EryP_{E802R} structures and possible catalytic active binding modes of
599 Leu-*p*NA were used as the starting conformations for MD simulations on the protein-ligand complexes. The partial
600 charge of Leu-*p*NA was fitted with HF/6-31G(d) calculations and the restrained electrostatic potential (RESP)^{50, 51}
601 protocol implemented by the Antechamber module in Amber 16 package⁴¹. The force field parameters for Leu-*p*NA
602 were adapted from the standard general amber force field 2.0 (gaff2)⁵² parameters, while the standard Amber14SB
603 force field was applied to describe the protein. Here, the force field parameters for the Zn center active site were
604 treated in a hybrid bonded/restrained nonbonded mode in which H306, H310 and E329 were treated in bonded way
605 while a catalytic water molecule was in nonbonded way using MCPB.py⁴⁰ module. Each system was initially
606 neutralized with Na⁺ counter ions and solvated with explicit TIP3P⁴³ water in a truncated octahedron box with a 10
607 Å buffer distance. The resulting system contained 90788 (EryP bound with Ca²⁺ and Leu-*p*NA), 89937 (EryP bound
608 with Leu-*p*NA but no Ca²⁺) and 87661 (EryP_{E802R} bound with Leu-*p*NA but no Ca²⁺) atoms, respectively.

609 Each system was equilibrated with a series of minimizations interspersed by short MD simulations during which
610 restraints on the protein backbone heavy atoms were gradually released (with force constant of 10, 2, 0.1 and 0
611 kcal/(mol·Å²)), and then heated slowly from 0 to 300 K for 50 ps. Finally, the standard unrestrained 200 ns MD
612 simulation with periodic boundary condition at 300 K and 1 atm was carried out. The pressure was maintained at 1
613 atm and coupled with isotropic position scaling. The temperature was controlled at 300 K with Berendsen thermostat
614 method. Long-range electrostatic interactions were treated with particle mesh Ewald (PME)⁵³ method and 12 Å cutoff
615 was applied to both PME and van der Waals (vdW) interactions. Time step of 2 fs was employed along with SHAKE
616 algorithm for hydrogen atoms, and periodic boundary condition was used. Each system was checked for stability
617 (structure, energy, and temperature fluctuations) and convergence (root mean square deviations-RMSD of structures).

618 References

- 619 34. Otwinowski, Z. & Minor, W. Processing of X-ray diffraction data collected in oscillation mode. *Methods*
620 *Enzymol.* **276**, 307-326 (1997).
- 621 35. Adams, P.D. *et al.* PHENIX: a comprehensive Python-based system for macromolecular structure solution. *Acta*
622 *Crystallogr. D Biol. Crystallogr.* **66**, 213-221 (2010).

623 36. Emsley, P., Lohkamp, B., Scott, W.G. & Cowtan, K. Features and development of Coot. *Acta Crystallogr.* **66**,
624 486-501 (2010).

625 37. Winn, M.D. *et al.* Overview of the CCP4 suite and current developments. *Acta Crystallogr. D Biol. Crystallogr.*
626 **67**, 235-242 (2011).

627 38. Pettersen, E.F. *et al.* UCSF ChimeraX: Structure visualization for researchers, educators, and developers.
628 *Protein science : a publication of the Protein Society* **30**, 70-82 (2021).

629 39. Rostkowski, M., Olsson, M.H.M., Sondergaard, C.R. & Jensen, J.H. Graphical analysis of pH-dependent
630 properties of proteins predicted using PROPKA. *BMC Structural Biol.* **11**, 6-12 (2011).

631 40. Li, P. & Merz, K.M., Jr. MCPB.py: A python based metal center parameter builder. *J. Chem. Inf. Model.* **56**,
632 599-604 (2016).

633 41. Case, D.A. *et al.* (University of California, San Francisco; 2016).

634 42. Seminario, J.M. Calculation of intramolecular force fields from second-derivative tensors. *Int. J. Quant. Chem.*
635 **60**, 1271-1277 (1996).

636 43. Jorgensen, W.L., Chandrasekhar, J., Madura, J.D., Impey, R.W. & Klein, M.L. Comparison of simple potential
637 functions for simulating liquid water. *J. Chem. Phys.* **79**, 926-935 (1983).

638 44. Friesner, R.A. *et al.* Extra precision glide: docking and scoring incorporating a model of hydrophobic enclosure
639 for protein-ligand complexes. *J. Med. Chem.* **49**, 6177-6196 (2006).

640 45. Ward, J.H. Hierarchical Grouping to Optimize an Objective Function. *J. Am. Stat. Assoc.* **58**, 236-244 (1963).

641 46. Barone, V. & Cossi, M. Quantum calculation of molecular energies and energy gradients in solution by a
642 conductor solvent model. *J. Phys. Chem. A* **102**, 1995-2001 (1998).

643 47. Takano, Y. & Houk, K.N. Benchmarking the conductor-like polarizable continuum model (CPCM) for aqueous
644 solvation free energies of neutral and ionic organic molecules. *J. Chem. Theory Comput.* **1**, 70-77 (2005).

645 48. Cossi, M., Rega, N., Scalmani, G. & Barone, V. Energies, structures, and electronic properties of molecules in
646 solution with the C-PCM solvation model. *J. Comput. Chem.* **24**, 669-681 (2003).

647 49. Morris, G.M. *et al.* Automated docking using a Lamarckian genetic algorithm and an empirical binding free
648 energy function. *J. Comput. Chem.* **19**, 1639-1662 (1998).

649 50. Jakalian, A., Bush, B.L., Jack, D.B. & Bayly, C.I. Fast, efficient generation of high-quality atomic Charges.
650 AM1-BCC model: I. Method. *J. Comput. Chem.* **21**, 132-146 (2000).

651 51. Jakalian, A., Jack, D.B. & Bayly, C.I. Fast, efficient generation of high-quality atomic charges. AM1-BCC
652 model: II. Parameterization and validation. *J. Comput. Chem.* **23**, 1623-1641 (2002).

- 653 52. Wang, J.M., Wolf, R.M., Caldwell, J.W., Kollman, P.A. & Case, D.A. Development and testing of a general
654 amber force field. *J. Comput. Chem.* **25**, 1157-1174 (2004).
- 655 53. Darden, T.; York, D.; Pedersen, L., Particle mesh Ewald - an $n \cdot \log(n)$ method for Ewald sums in large systems.
656 *J. Chem. Phys.* **1993**, 98 (12), 10089-10092.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementrayfileNCB.pdf](#)
- [SupplementrayfileNCB.pdf](#)
- [Wangreportingsummary.pdf](#)
- [Wangeditorialpolicychecklist.pdf](#)
- [Validationreport7V9O.pdf](#)
- [Validationreport7V9Q.pdf](#)
- [Validationreport7V9P.pdf](#)
- [Validationreport7V9N.pdf](#)