Biosynthesis

International Edition: DOI: 10.1002/anie.201910659
German Edition: DOI: 10.1002/ange.201910659

Characterization of a Citrulline 4-Hydroxylase from Nonribosomal Peptide GE81112 Biosynthesis and Engineering of Its Substrate Specificity for the Chemoenzymatic Synthesis of Enduracididine

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Abstract: *The GE81112 tetrapeptides are a small family of unusual nonribosomal peptide congeners with potent inhibitory activity against prokaryotic translation initiation. With the exception of the 3-hydroxy-L-pipecolic acid unit, little is known about the biosynthetic origins of the non-proteinogenic amino acid monomers of the natural product family. Here, we elucidate the biogenesis of the 4-hydroxy-L-citrulline unit and establish the role of an iron- and α-ketoglutarate-dependent enzyme (Fe/αKG) in the pathway. Homology modelling and sequence alignment analysis further facilitate the rational engineering of this enzyme to become a specific 4-arginine hydroxylase. We subsequently demonstrate the utility of this engineered enzyme in the synthesis of a dipeptide fragment of the antibiotic enduracidin. This work highlights the value of applying a bioinformatics-guided approach in the discovery of novel enzymes and engineering of new catalytic activity into existing ones.*

Identified in 2006 through a high-throughput in vitro screening, the GE81112s (**1**) are a small family of three tetrapeptide congeners (A, B and B1) that display prokaryotic-specific initiation inhibition (Figure 1 A).[1] Antibacterial profiling has shown that each congener displays effective growth inhibition against several Gram-positive and Gram-negative pathogens.[2] Recent studies indicated that GE81112 B stalls initiation in the unlocked 30S pre-initiation complex state and impedes its transition to the corresponding initiation complex.[3] This process represents a unique mechanism of action relative to other antibiotics that target the ribosome.[4] Thus, the GE81112 family represents an intriguing scaffold to further optimize as an antibacterial drug candidate.

Structurally, the GE81112s contain several highly unusual amino acid monomers, including 3-hydroxy-L-pipecolic acid, 4-hydroxy-L-citrulline, *O*-carbamoyl-α-amino-dihydroxyvaleric acid, 2-amino-L-histidine, and β-hydroxy-2-chloro-L-histidine. Biosynthetic studies have traced the production of these peptides to a nonribosomal peptide synthetase (NRPS).[5] In addition, the biosynthetic gene cluster of **1** contains several genes encoding tailoring enzymes, including two iron- and α-ketoglutarate-dependent enzymes (Fe/αKGs), GetF and GetI

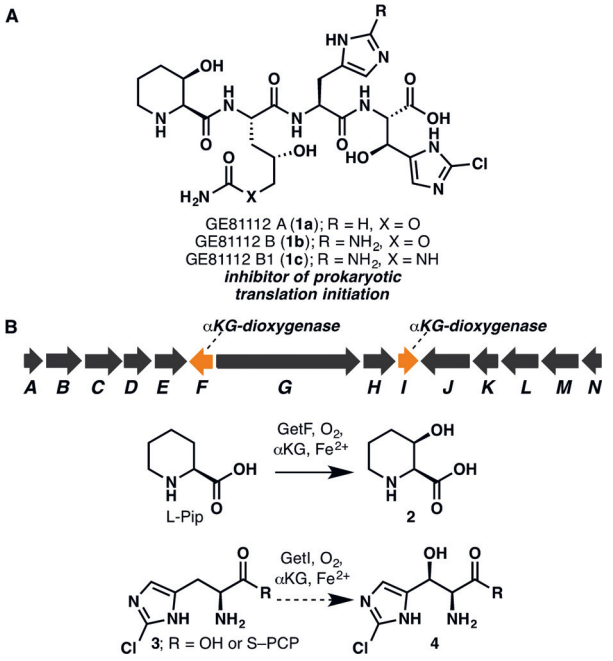

Figure 1. A) Structures of GE81112 A, B, and B1 peptide antibiotics. B) The presence of two Fe/αKGs in the biosynthetic gene cluster of GE81112.

(Figure 1 B). GetF was recently characterized as an L-pipecolic acid hydroxylase responsible for the production of the 3-hydroxy-L-pipecolic acid monomer (**2**).[6] GetI was initially proposed to catalyze the β-hydroxylation of 2-chloro-L-histidine, either as the free or peptidyl carrier protein (PCP)-bound amino acid (**3**).[5] Given the potential utility of GetI in the production of novel noncanonical amino acids, we became interested in its functional characterization and exploration of its biocatalytic utility.[7]

GetI is annotated as a member of clavaminate synthase-like protein (InterPro family IPR014503) in Uniprot. BLAST analysis revealed that GetI is 45 % and 51 % identical to VioC and OrfP, two arginine hydroxylases from the capreomycidine[8] and streptolidine[9] biosynthetic pathways, respectively. Furthermore, sequence alignment (Figure 2 A and Figure S2 in the Supporting Information) shows conservation of α-amino and carboxylate binding residues in GetI, VioC, and OrfP (Q124 and R322 in GetI, Q137 and R334 in VioC, Q123 and R321 in OrfP). Given this observation, GetI seems more likely to act on a free amino acid than on one that is bound to a PCP. At lower levels of sequence identity (28–40 %), several hydroxylases that act on free-standing amino acids[10] could

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Supporting information (including experimental details) and the ORCID identification number(s) for the author(s) of this article can be found under: https://doi.org/10.1002/anie.201910659.

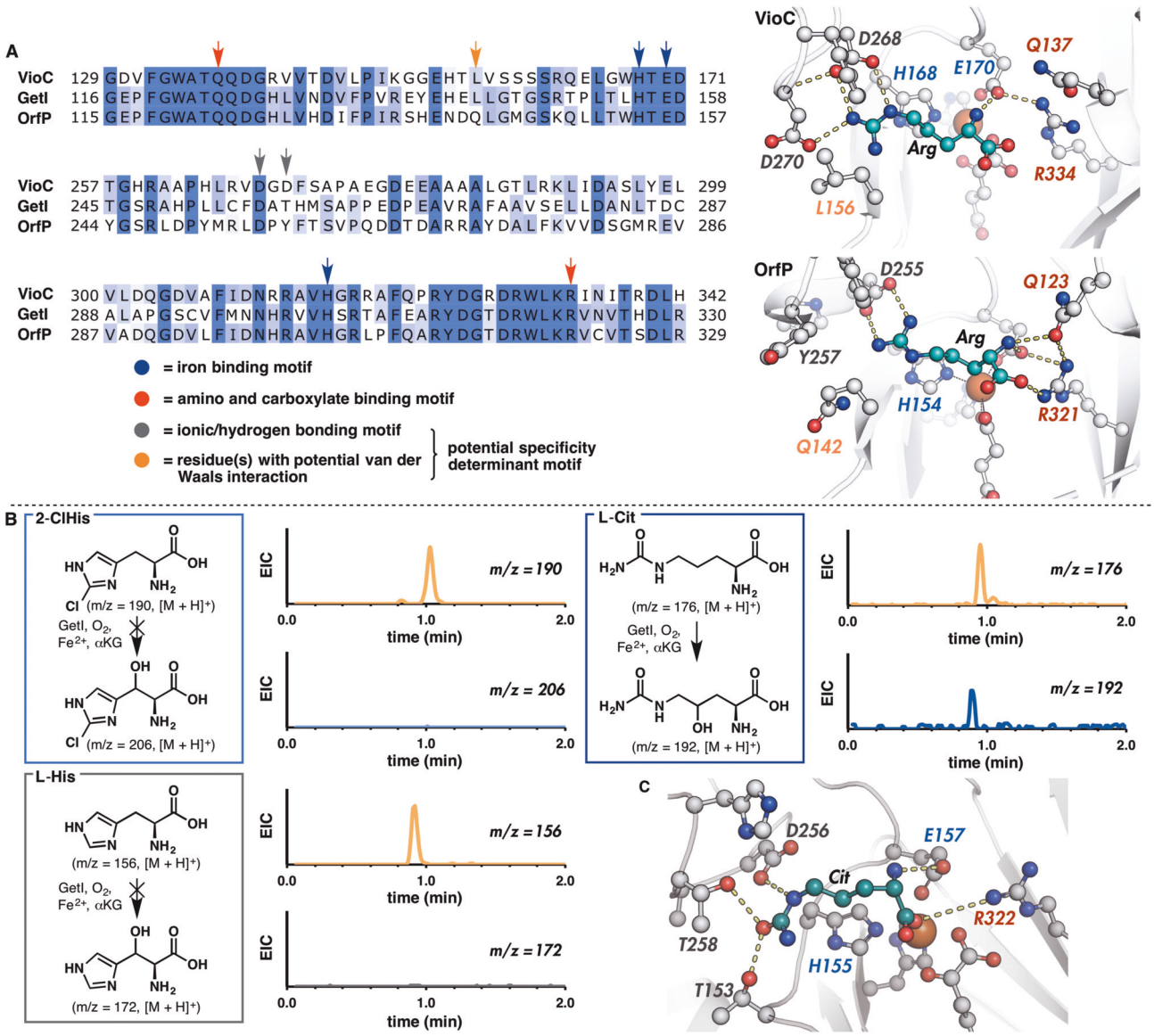


Figure 2. A) Sequence alignment of VioC, GetI, and OrfP, highlighting key residues that are involved in iron and amino acid substrate binding. B) LC–MS profile of hydroxylation of select amino acids with GetI, suggesting a role of GetI in the biogenesis of the 4-hydroxy-L-citrulline monomer. C) Homology model of GetI using a solved crystal structure of OrfP as the template (PDB ID: 4M26).

also be located and no hydroxylases that act on (PCP)-bound amino acid could be identified in the BLAST analysis. Interestingly, neither 2-chloro-L-histidine nor L-histidine provided any desired hydroxylation product when subjected to reaction with GetI, αKG, O_2, and Fe^{2+} at various different pH values (Figure 2B). A control experiment showed that the *S*-*N*-acetylcysteamine (SNAc) derivative of L-histidine is not accepted as a substrate by GetI either. These results led us to suspect that GetI might be involved in the biogenesis of a different monomer. At this stage, we realized that the origins of the 4-hydroxy-L-citrulline and *O*-carbamoyl-α-amino-dihydroxyvaleric acid were unaccounted for in the original biosynthetic proposal of GE81112. Given its high sequence identity to arginine hydroxylases, it seemed likely that GetI would act on structurally related δ-carbamoyl amino acids. Indeed, treatment of L-citrulline (Cit) with Fe^{2+}, O_2, and αKG in the presence of GetI led to the formation of a hydroxylated product as judged by LC–MS. Subsequent ^{1}H NMR analysis confirmed the C4 selectivity of the hydroxylation reaction, thus suggesting that GetI is responsible for the production of the 4-hydroxy-L-citrulline monomer prior to its loading to the NRPS assembly line. A similar outcome could also be observed when α-amino-δ-carbamoylhydroxyvaleric acid was employed as a substrate. Prior to this work, an Fe/αKG from polyoxin biosynthesis, PolL, was previously characterized as a α-amino-δ-carbamoylhydroxyvaleric acid 4-hydroxylase.[11] However, this enzyme is classified as a member of the PF10014 family, shares only minimal sequence identity with GetI, and affords product with an opposite stereochemical configuration at C4 to GetI. Finally, several other polar and charged amino acids were also tested for reaction with GetI (See Table S1 in the Supporting

Information), but among those tested, only L-arginine (Arg) yielded low levels of hydroxylation activity.

To rationalize this outcome, we constructed a homology model of GetI using a solved crystal structure of OrfP as the template and performed virtual docking of Cit into the predicted active site of the enzyme (Figure 2C). Our model suggested that several hydrogen bonding and ionic interactions are potentially in play for Cit recognition. We propose that the α-amino and carboxylate groups of Cit form salt bridges with E157 and R322 in the active site. With respect to side-chain engagement, T153 and T258 are predicted to act as hydrogen-bond donors to the δ-carbamoyl group and D256 is predicted to serve as a hydrogen-bond acceptor to the ε-nitrogen of Cit. This binding mode stands in stark contrast to what was previously observed in Arg binding to VioC and OrfP, whereby aspartate residues (D268 and D270 in VioC, and D255 in OrfP, respectively) are involved in salt-bridge formation with the guanidine side chain. This observation also raises the possibility of a "specificity determinant" loop in the active site of clavaminate-synthase-like amino acid hydroxylases that serves to govern their substrate and/or reaction pathway specificity. A similar phenomenon has been observed previously in NRPS adenylation domains, whereby critical active-site residues serve as a specificity determinant to govern substrate recognition in the active site.[12] This observation has led to the development of a predictive model for substrate specificity and forms the basis for existing NRPS predictor tools.[13]

Based on the hypothesis that the substrate specificity of clavaminate-synthase-like amino acid hydroxylases is governed by their specificity determinant loops, we asked whether the substrate specificity of GetI could be altered through a simple loop-grafting procedure. Given its low levels of hydroxylation activity on Arg, we targeted the conversion of GetI to an Arg-specific hydroxylase. To date, no dedicated Arg C4-hydroxylase has been identified: VioC hydroxylates exclusively at C3 and OrfP provides predominantly dihydroxylation at C3 and C4. A biocatalyst that can catalyze a selective C4 oxygenation of Arg could find useful application in the chemoenzymatic synthesis of L-enduracididine or L-*allo*-enduracididine,[14] which is a key motif in enduracidin,[15] teixobactin,[16] and mannopeptimycin[17] antibiotics. Additionally 4-hydroxyarginine can also be found in several peptide antibiotics, such as argimicins A and B,[18] and K-582.[19] To this end, we performed sequential site-directed mutagenesis to incrementally incorporate the guanidine binding sequence of VioC or OrfP into GetI (Figure 3). Towards a VioC-like enzyme, mutation T258D ("DAD") led to a 2-fold reduction in total turnover number (TTN) for Cit hydroxylation and a marginal increase in TTN for Arg hydroxylation. Interestingly, introduction of A257G mutation into this variant ("DGD") resulted in complete abolition of Cit hydroxylation activity and 1.4-fold increase in TTN for Arg hydroxylation. Finally, the triple mutant A257G/T258D/H259F ("DGDF") was found to catalyze the C4 hydroxylation of Arg with 87 TTN. However, this reaction was also accompanied by appreciable dihydroxylation (ca. 4:1 monohydroxylation at C4: dihydroxylation at C3 and C4 by ^{1}H NMR). Given the sub-optimal site-selectivity of this

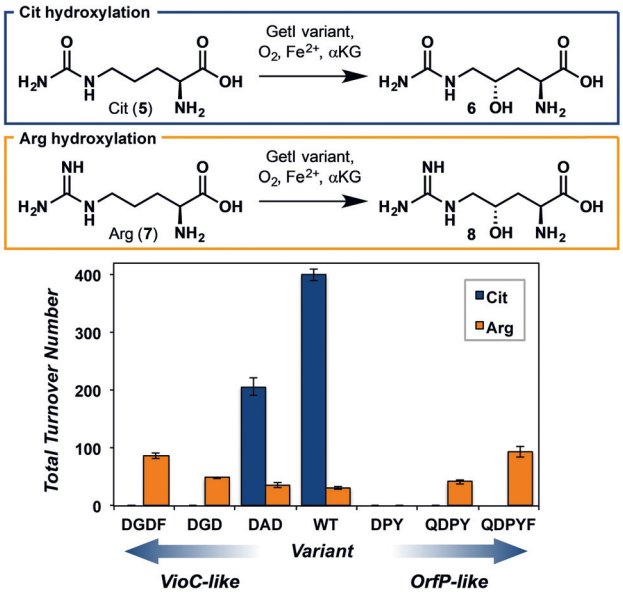


Figure 3. Rational engineering of GetI to convert its substrate specificity from L-citrulline to L-arginine. Conditions for total turnover number (TTN) measurements: free amino acid (20 mM, 1 equiv), αKG (60 mM, 3 equiv), sodium ascorbate (10 mM, 0.5 equiv), $FeSO_4$ (1 mM, 0.05 equiv), GetI variant (0.1–0.2 mol%), kPi buffer (pH 8.0, 50 mM, 3.0 mL total volume), 12 h at 20°C. TTNs were determined by ^{1}H NMR analysis of the reaction conversion.

engineered enzyme, we next investigated the conversion of GetI into an OrfP-like enzyme. The double mutant A257P/T258Y ("DPY") was found to be completely unreactive towards Arg and Cit. However, introduction of L143Q mutation into this variant ("QDPY") rescued hydroxylation activity towards Arg (TTN = 42). One additional mutation, H259F, afforded the variant "QDPYF", which is able to catalyze selective C4-hydroxylation of Arg with 94 TTN without any observable activity on Cit. Steady-state kinetic analyses revealed an apparent K_M of 4.8 ± 1.5 mM and k_{cat} of 21 ± 2.3 min^{-1} for hydroxylation of Arg with GetI QDPYF. In contrast, wild-type GetI shows a slightly lower apparent K_M for Cit (2.1 ± 0.41 mM) and a much higher k_{cat} (69 ± 5.3 min^{-1}). The loop-grafting approach therefore results in reduced substrate affinity but is also accompanied by an even larger decrease in turnover efficiency. This observation suggests the presence of non-obvious secondary interactions that contribute to accelerating the various elementary steps in the catalytic cycle. We anticipate that further directed evolution of GetI QDPYF could result in the identification of an enzyme variant with improved catalytic efficiency.

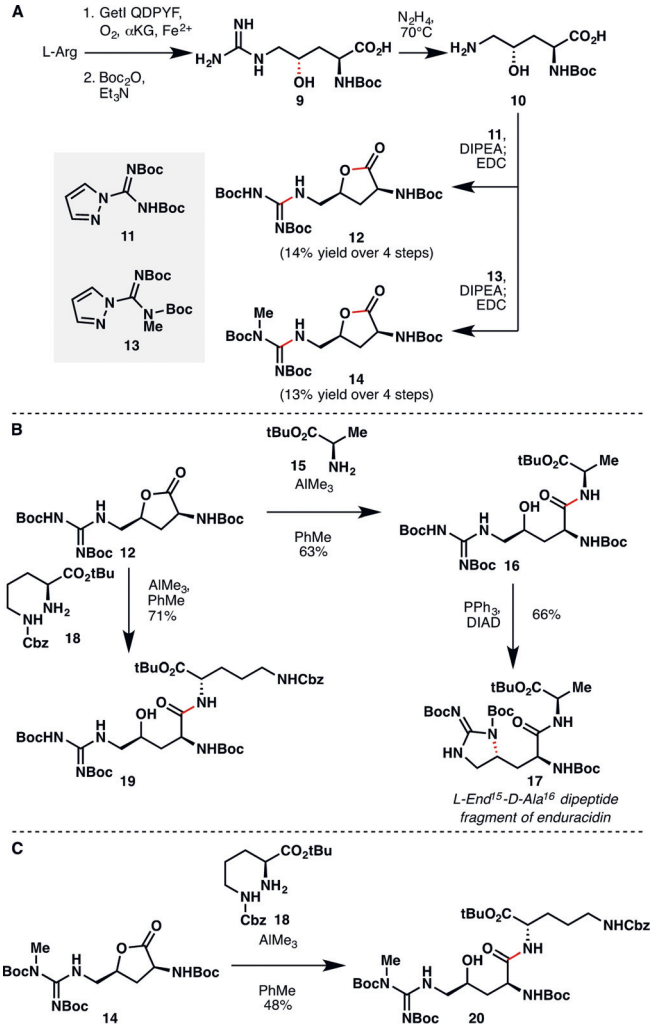
Notwithstanding the modest TTN, GetI QDPYF was able to catalyze C4 hydroxylation of Arg to full conversion when the reaction was conducted in unclarified cell lysate (pre-lysis $OD_{600} = 30$). Encouraged by this observation, we next pursued chemoenzymatic synthesis of a dipeptide fragment of enduracidin starting from 4-hydroxyarginine (**8**). We initially focused on global Boc protection[20] of the α-amino and guanidine side chain of **8**. However, attempts to effect this transformation were plagued by low conversion and yield, as well as formation of an NBoc regioisomer on the side chain.

This mixture proved to be problematic to carry forward for subsequent manipulations. As a workaround, we elected to first selectively protect the α-amino group as the corresponding Boc derivative (Scheme 1 A). Treatment[19] of **9** with N_2H_4 afforded clean removal of the guanidine side chain to provide N^2-Boc-4-OH-L-ornithine (**10**). In contrast, these conditions proved unreactive for removing the ureido group of Cit. Despite giving an increased step count, this sequence resulted in superior material throughput and higher overall yield of **10** relative to the initial global Boc protection approach. Furthermore, the sequence enables the most direct access to 4-OH-L-ornithine to date[21] and could also be adapted to the formation of alkylated guanidine side chain by simply switching the coupling partner (e.g., to **13**).

In our previous work,[7] we found that 4 and 5-hydroxyacids could be activated for subsequent peptide coupling through intramolecular lactonization, followed by treatment with the appropriate amine nucleophile. In the same vein, the free 4-hydroxyacids from **10** were lactonized by treatment with EDC. Lactone opening of **12** with H-Ala-OtBu (**15**) was carried out in the presence of $AlMe_3$ to afford alcohol **16** in 63% yield (Scheme 1 B).[22] Finally, use of Mitsunobu conditions[23] (DIAD, PPh_3) on **16** effected an intramolecular displacement of the secondary alcohol by the pendant guanidine to complete the synthesis of the L-enduracididine[15]-D-alanine[16] dipeptide fragment of enduracidin (**17**). Our chemoenzymatic route compares favorably to previous synthetic approaches[14] to L-enduracididine (see Table S2 for comparison), which typically result in poor stereocontrol at C4. Highly diastereoselective and high-yielding syntheses[24] of L-*allo*-enduracididine from *N*-Boc-*trans*-4-hydroxy-L-proline have recently been developed. However, these approaches require at least eight steps and their adaptation to the synthesis of L-enduracididine would necessitate the use of the more expensive *N*-Boc-*cis*-4-hydroxy-L-proline diastereomer ($\$15\,g^{-1}$) as the starting material.

To test the versatility of our method, **12** was subjected to the same $AlMe_3$-assisted coupling conditions with H-L-Orn-(*Z*)-OtBu (**18**) as the reaction partner. Smooth formation of dipeptide **19** was realized in 71% yield of isolated product. The reaction was highly chemoselective, since no side products arising from attack by δ-amino group of **18** were observed. Lactone **14** was also found to be an able coupling partner in this reaction, affording dipeptide **20** in 48% yield when **18** was used as the nucleophile (Scheme 1 C). Thus, we believe that this method could be adapted to the preparation of enduracidin analogues and related 4-hydroxyarginine-containing peptides.

Selective remote oxidation of amino acids remains an unmet challenge in the field of C–H functionalization. Although the hydroxylation activity of the Fe/αKGs was known as early as 1966, many Fe/αKG amino acid hydroxylases[25] have remained uncharacterized to date. By relying on bioinformatics analysis and chemical intuition, we have performed the first functional characterization of GetI and revised its functional annotation from chlorohistidine hydroxylase to citrulline hydroxylase. Further sequence similarity analysis allowed us to predict GetI's substrate recognition ensemble (specificity determinant) and facilitated its rational engineering to become a specific 4-arginine hydroxylase with just four mutations. The utility of this engineered enzyme is highlighted by the concise chemoenzymatic synthesis of several novel dipeptides related to enduracidin. This work expands the catalytic repertoire of the IPR014503 family and lays the groundwork for the rational discovery of novel enzymatic reactions within this family through further phylogenetic and sequence-similarity-network analysis.[26]



Scheme 1. A) Chemoenzymatic synthesis of lactones **12** and **14** featuring regioselective C4 hydroxylation of L-arginine with GetI QDPYF. B) Conversion of lactone **12** into the L-End[15]-D-Ala[16] dipeptide fragment of enduracidin and related dipeptide **19** through $AlMe_3$-mediated lactone opening. C) Utility of $AlMe_3$-mediated lactone opening in the conversion of **14** into dipeptide **20**.

Acknowledgements

Financial support for this work was generously provided by The Scripps Research Institute and the National Institutes of Health (grant GM128895). We thank Dr. Hajeung Park for assistance in homology modelling and in silico docking. We acknowledge the Shen lab and the Roush lab for generous access to their instrumentation.

Conflict of interest

The authors declare no conflict of interest.

Keywords: biosynthesis · biocatalysis · citrulline hydroxylase · enzyme engineering · non-heme dioxygenases

How to cite: *Angew. Chem. Int. Ed.* **2019**, *58*, 18854 – 18858
Angew. Chem. **2019**, *131*, 19030 – 19034

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Manuscript received: August 20, 2019
Revised manuscript received: October 6, 2019
Accepted manuscript online: October 14, 2019
Version of record online: November 11, 2019

