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Title: Formation and Modification of Enduracididine, A Nonproteinogenic Amino Acid.

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Molecular genetic and enzymological techniques have been employed to study antibiotic biosynthesis. In this thesis, we studied the formation and modification of the nonproteinogenic amino acid enduracididine (End), which exists in two important antibiotics, mannopeptimycins (MPPs) and enduracidin.

Sequence analysis of the MPP gene cluster revealed that the product of *mppO* belongs to His-3 variant of non-heme iron, α-ketoglutarate dependent oxygenase superfamily. The *mppO* gene was subcloned and heterologously expressed in *E. coli*. Enzyme activity assays showed that MppO stereospecifically catalyzes hydroxylation of the β-carbon of L-End and results in the formation of 3S-hydroxy-L-End. MppO is the first known enzyme that catalyzes the β-hydroxylation of a nonproteinogenic amino acid.

The formation of enduracididine was also studied in the enduracidin

biosynthesis pathway. Three genes in the end cluster, *endP*, *endQ* and *endR* are predicted to be involved in the formation of L-End. The gene products of *endP* and *endQ* are proposed to be pyridoxal phosphate (PLP)-dependent enzymes. These genes were subcloned and expressed in *E. coli*. A fragment containing the whole *endPQR* operon was introduced into *S. lividans* and *S. fungicidicus*. Two possible mechanisms of enduracididine formation were proposed with β-OH-L-Arg or γ-OH-L-Arg as precursor, respectively.

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Formation and Modification of Enduracididine,
A Nonproteinogenic Amino Acid

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TABLE OF CONTENTS

Page

I. GENERAL INTRODUCTION……………………………………………….1

ANTIBIOTIC HISTORY AND RESISTANCE…………………………1

ANTIBIOTIC MECHANISMS OF ACTION…………………………...2

ORIGINS OF ANTIBIOTICS……………………………………………6

NONPROTEINOGENIC AMINO ACIDS………………………………7

NEW TRENDS AND TECHNIQUES…………………………………..10

REFERENCES…………………………………………………………..12

II. CHARACTERIZATION OF MPPO, AN OXYGENASE

INVOLVED IN FORMATION OF β-HYDROXYENDURACIDIDINE

IN MANNOPEPTIMYCIN BIOSYNTHESIS…………………………16

INTRODUCTION………………………………………………………16

RESULTS AND DISCUSSIONS………………………………………..22

MATERIALS AND METHODS………………………………………...34

REFERENCES…………………………………………………………..38

III. STUDIES ON THE FORMATION OF ENDURACIDIDINE

IN ENDURACIDIN BIOSYNTHESIS………………………………40

INTRODUCTION………………………………………………………40

RESULTS AND DISCUSSION…………………………………………47

MATERIALS AND METHODS……………………………………………...59

REFERENCES…………………………………………………………..60

IV. CONCLUSIONS……………………………………………………………69

BIBLIOGRAPHY………………………………………………………………71

LIST OF FIGURES

Figure Page

1.1 Antibiotics block cell wall synthesis……………………………………4

2.1 Chemical structure of mannopeptimycin α, β, γ, δ, ε……………………..18

2.2 Sequence alignment of MppO and VioC…………………………………23

2.3 ClustalW sequence alignment of MppO and related enzymes……………24

2.4 SDS-PAGE analysis of the expression and purification of His_6-MppO….26

2.5 SDS-PAGE analysis of the expression and purification of His_6-MppO…..26

2.6 RP-HPLC analysis of the MppO assay with D,L-End as substrate……....28

2.7 RP-HPLC analysis of the MppO assay with dideoxy-MPP γ…………..…29

2.8 RP-HPLC analysis of MppO activity affected by EDTA and α-KG.……..30

2.9 RP-HPLC analysis of the substrate stereospecificity of MppO…….…….31

3.1 Structure of enduracididin and ramoplanin A1……………..……………..44

3.2 ClustalW sequence alignment of EndP and related proteins….…………..49

3.3 ClustalW sequence alignment of EndQ and related proteins…………….…50

3.4 SDS-PAGE analysis of the EndP and EndQ overexpression in

Rosetta DE3 cells with IPTG induction…..……………….……………….…..53

3.5 Western blot analysis of soluble EndP and EndQ……………….….…..54

3.6 SDS-PAGE analysis of the endPQR expression in *S. lividans*……..……..55

LIST OF SCHEMES

Scheme Page

1.1 Conversion of an α-amino acid to a β-amino acid by an aminomutase.…...7

1.2 Formation of β-hydroxytyrosine by NovH and NovI………….……..…..9

1.3 β-Hydroxyarginine as an intermediate in the formation of
capreomycidine………………………………………………………..9

1.4 Proposed biosynthesis of enduracididine
and β-hydroxyenduracididine……………………………………...…...10

2.1 Reaction mechanism of non-heme iron, α-KG dependent oxygenases.…..11

2.2 Clavaminate synthase catalyzed reactions in clavulanic acid biosynthesis
pathway…………………………………………………………….....22

2.3 Dansylation of amino acid for UV detection…………………………..….27

2.4 Preparation of L-End and D-End using D-amino acid oxidase and
L-amino acid oxidase…………………………….……….………………31

2.5 Overall reaction catalyzed by MppO……………………………………..32

3.1 PLP and substrate binding mechanism……………………………………45

3.2 Proposed mechanism for the VioD-catalyzed formation of
2S,3R-capreomycidine……………………………...…………………..46

3.3 Proposed mechanism of L-End formation through β-OH-L-Arg……..…..57

3.4 Proposed mechanism of L-End formation through γ-OH-L-Arg………..58

LIST OF TABLES

Table Page

2.1 Antibacterial activity of MPPs α-ε……………………………….….……17

2.2 His-2 and His-3 motif represented enzymes and sequence….……………21

3.1 PCR primers used to amplify endP, endQ and endR………….………..60

ABBREVIATIONS

α-KG	α-ketoglutarate
2,4 D	2,4-dichlorophenoxyacetate monooxygenase
AstK	alkysulfatase
BSA	bovine serum albumin
CarC	carbapenem synthase
CS	clavaminate synthase
ddH_2O	double deionized water
DNS-Cl	dansyl chloride
DPG	3,5-dihydroxyphenylglycine
DTT	dithiothreitol
ED_{50}	50% effective dose
EDTA	ethylenediaminetetraacetic acid
End	enduracididine
ESI	ion electrospray ionization
G-BBH	γ-butyrobetaine hydroxylase
HPG	4-hydroxyphenylglycine
IPTG	isopropyl-beta-D-thiogalactopyranoside
MIC	minimum inhibitory concentration
MOPS	3-N-morpholinopropanesulfonic acid

ABBREVIATIONS (Continued)

MPPs	mannopeptimycins
MRSA	methicillin-resistant *Staphylococus aureus*
MS	mass spectrum
NRP	nonribosomal peptide
NRPS	nonribosomal peptide synthase
PCP	peptidyl carrier
PCR	polymerase chain reaction
PG	peptidoglycan
PLP	pyridoxal phosphate
RP-HPLC	reverse phase high performance liquid chromotography
SAM	S-adenosylmethionine
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TauD	taurine dioxygenase
TE	thioesterase
TWB	talon wash buffer
VRE	vancomycin resistant enterococci

Formation and Modification of Enduracididine, a Nonproteinogenic Amino Acid

Chapter 1

General Introduction

Antibiotic History and Resistance

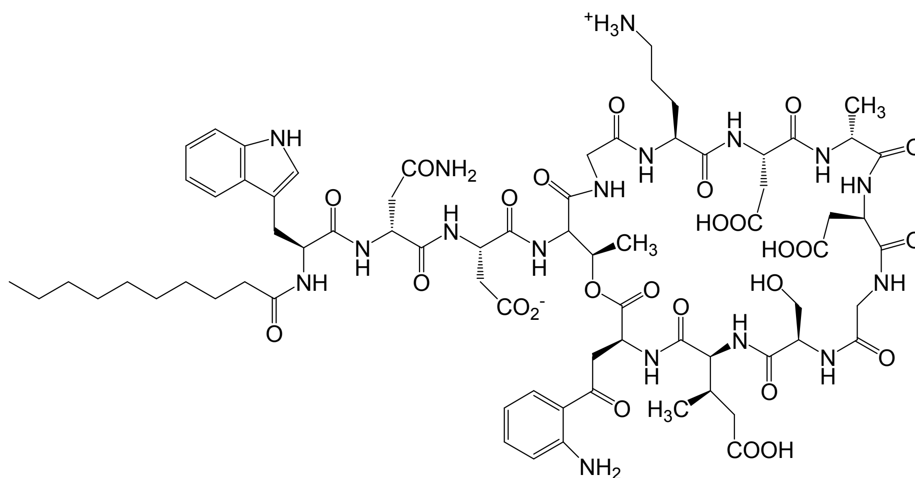
The appearance of multidrug resistant bacteria is primarily caused by wide use and misuse of antibiotics. In 1928, Alexander Fleming discovered the first natural product antibiotic, penicillin. That was a monumental achievement in the fight against bacterial infections. Before the discovery of penicillin, common bacterial infections, such as pneumonia, were virtually untreatable. Early antibiotics helped saving millions of lives during and after World War II and lowered the mortality rate among world populations[1]. The demand for more effective antibiotics with fewer side effects and broader spectrum kept the pharmaceutical companies focused on developing new antibiotics. However, a growing number of big pharmaceutical companies withdrew or shrunk antibiotic research and development in the last 20 years due to economic reasons[1]. Only three new classes of antibiotics entered the market since 1985 and they are: mupirocin (1985), linezolid (2000) and daptomycin (2003)[2]. Meanwhile, multidrug resistant bacteria such as methicillin-resistant *S. aureus* (MSRA) evolved and spread worldwide due to improper use of antibiotics[3]. Vancomycin was long regarded to be the last line of defense against antibiotic resistant Gram-positive bacteria. However, vancomycin-resistant strains emerged clinically in *Enterococci* spp. and resistance

was passed to *S. aureus*, both of which are important Gram-positive bacteria associated with hospital-acquired infections[4]. New antibiotics are required to combat the future threat of multidrug resistant bacteria.

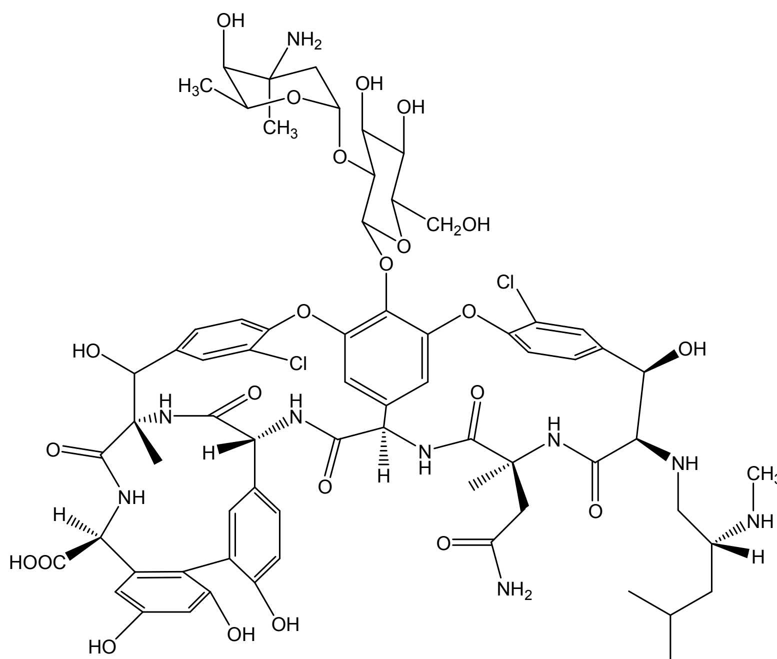
Antibiotic Mechanisms of Action

Antibiotics have different mechanisms of action to combat bacteria. Common mechanisms involve inhibiting or preventing cell wall biosynthesis, protein biosynthesis, DNA replication and repair, folate coenzyme biosynthesis, aromatic amino acid biosynthesis, cell division, two component signal transduction, fatty acid biosynthesis, isoprenoid biosynthesis and tRNA synthetases[5, 6]. Inhibition of cell wall biosynthesis is the most common and effective mechanism of action against bacteria propagation. Mannopeptimycins (MPPs) and enduracidin inhibit cell wall synthesis and will be discussed throughout this thesis. The bacterial cell wall is composed of a cross-linked peptidoglycan (PG) layer in which glycan strands are covalently cross-linked through peptide bonds. There are three stages during bacterial cell wall biosynthesis that can be targeted by antibiotics. The first stage involves the formation of UDP-N-acetylmuramyl pentapeptide and is catalyzed by a series of enzymes, MurA-MurF, starting with UDP-N-acetylglucosamine (UDPGlcNAc). Fosfomycin, a clinically used antibiotic, inhibits MurA and is effective in this stage[7, 8]. In the second stage MraY transfer UDP-N-acetylmuramyl pentapeptide to an undecaprenylphosphate carrier in the cell membrane, forming Lipid I. MurG then catalyzes the addition of UDPGlcNAc to the MurNac residue to form Lipid II[9, 10]. Amphomycin and

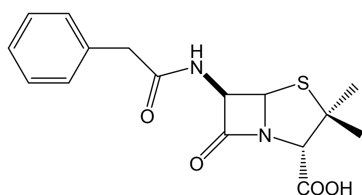
muraymycins inhibit MraY[11-13], therefore, they are effective at stopping the second stage. In the final stage Lipid II on the exterior of the cell is the substrate for forming the PG chain in a process called transglycosylation. The formation of crosslinks between peptide chains is called transpeptidation. MPPs, vancomycin, moenomycin and ramoplanin inhibit transglycosylation while penicillins and vancomycin inhibit transpeptidation[14]. The structures of daptomycin, vancomycin, penicillin, fosfomycin, moenomycin, ramoplanin, and muraymycin are illustrated in Figure 1.1. The structures of MPPs and enduracidin will be discussed in Chapter 2 and Chapter 3.



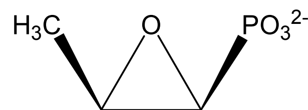
Daptomycin



Vancomycin

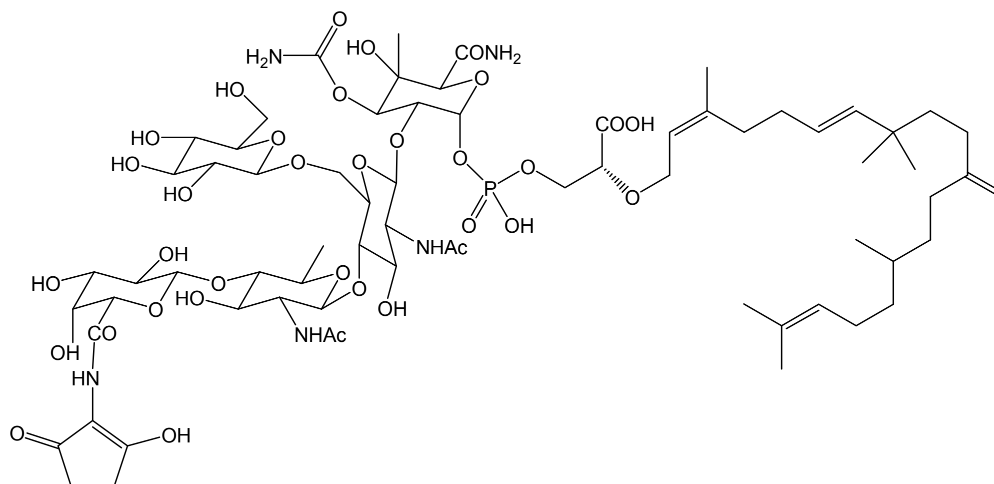


Penicillin G

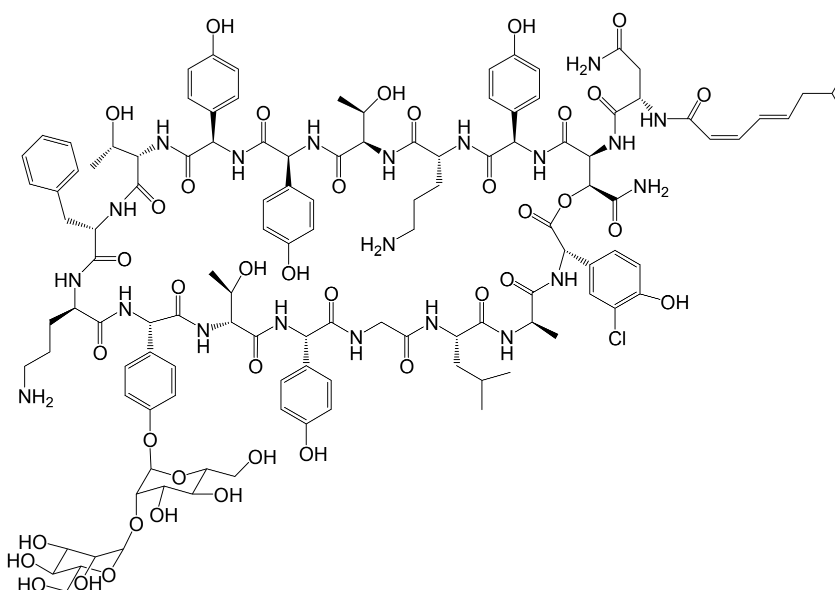


Fosfomycin

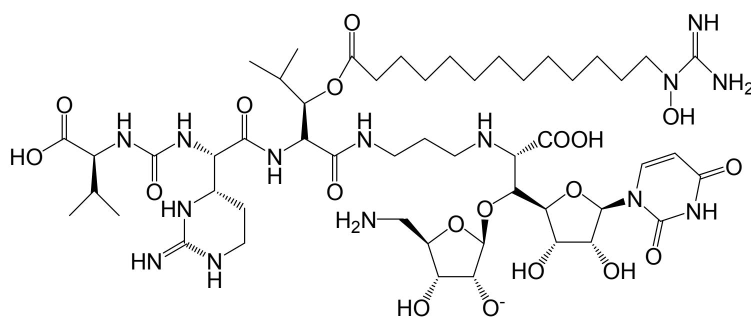
Figure 1.1 Antibiotics block cell wall synthesis



Moenomycin A



Ramoplanin A2



Muraymycin A1

Figure 1.1 (cont'd) Antibiotics blocking cell wall synthesis

Origins of antibiotics

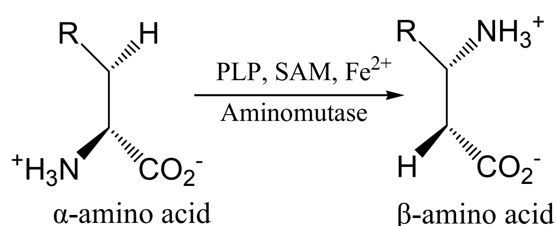
Most of the clinical antibiotics are natural products or result from natural product leads[15]. They have more complicated structures and extraordinary specificity and potency compared to synthesized compounds[16]. Soil bacteria produce antibiotic compounds to eliminate bacteria that compete for space and nutrients. During the long evolutionary process, those biologically active compounds which aim at specific targets were screened and optimized with well defined structures[16].

Many antibiotics are nonribosomal peptides (NRP) and polyketides (PK). Mannopeptimycins and enduracidin discussed in Chapter 2 and Chapter 3 are both NRP-derived compounds. NRPs are small peptide molecules containing 3 to 22 amino acid residues and synthesized by a family of modular enzymes called nonribosomal peptide synthetases (NRPS)[17]. Each module generally includes three catalytic domains: an adenylation (A) domain, a peptidyl carrier (PCP) domain which is also known as the thiolation (T) domain, and a condensation domain (C). Amino acids are first selected and activated by the A domain and covalently attached to the PCP domain. Peptide bond formation is catalyzed by the C domain. Generally, the first module lacks a C domain and the last module contains a thioesterase (TE) domain which cyclizes and releases the new peptide. In addition, an epimerization (E) domain is present in some modules that catalyze the racemization of L-amino acids[5, 18-20]. Moreover, the NRPs are often modified through methylation, hydroxylation, heterocyclization, oxidative crosslinking and attachment of sugars by tailoring enzymes[21].

Nonproteinogenic Amino Acids

The diversity of NRPs comes from not only the late stage modifications but also from the building blocks, which can include nonproteinogenic amino acids. Nonproteinogenic amino acids are unusual amino acids that normally do not integrate into ribosomal peptides. Numerous nonproteinogenic amino acids are integrated into NRPs and are a distinguishing structure feature of NRPs. In addition, they are also involved in target recognition and further modification and play important roles in antibiotics activity.

β-Amino acids are a large group of nonproteinogenic amino acids that include β-alanine, β-leucine, β-arginine, β-phenylalanine, β-tyrosine and β-lysine[23]. The formation of β-amino acids has been elucidated in two pathways. β-Alanine can be formed from uracil or aspartic acid[24, 25], whereas other β-amino acids are usually formed from α-amino acids that are modified by specific aminomutases. Lysine aminomutase is the best studied example and uses pyridoxal phosphate (PLP), S-adenosylmethionine (SAM) and iron as cofactors (Scheme 1.1) [23, 26, 27].

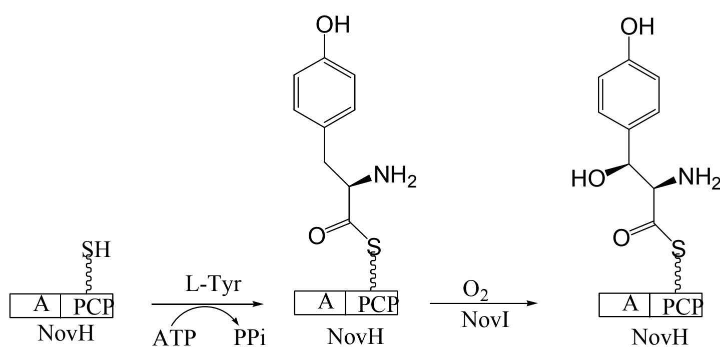


Scheme 1.1 Conversion of an α-amino acid to a β-amino acid by an aminomutase

Amino acids can undergo hydroxylation at different sites and result in different hydroxylated forms such as hydroxyphenylglycines, β-hydroxyamino acids and γ-hydroxyamino acids. The hydroxyl group may activate the carbon and can

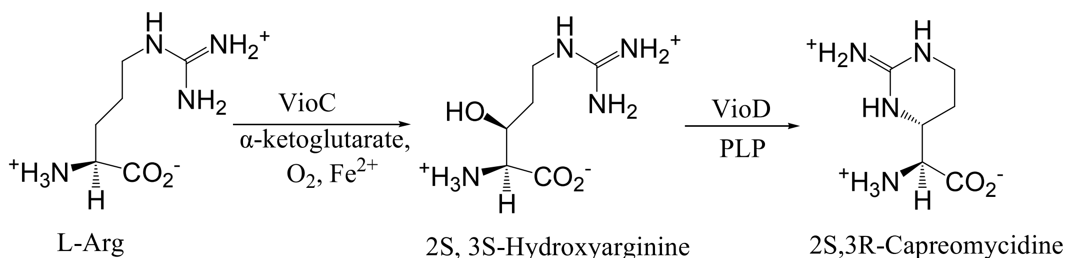
facilitate further reactions like glycosylation, oxidation, retro-aldol cleavage and macrolactonization[28]. 4-Hydroxyphenylglycine (HPG) and 3,5-dihydroxyphenylglycine (DPG) are two residues in the vancomycin class of antibiotics. Although they have similar structures, their biosyntheses are totally unrelated. HPG originates from chorismate through the shikimate pathway[29], while DPG is produced by a polyketide synthase from malonyl-CoA[30-32].

β-Hydroxyamino acids widely exist in various antibiotics such as β-OH-Tyr in vancomycin[33] and β-OH-Asn in ramoplanin[34] and bleomycin[35]. β-Hydroxy amino acids also serve as intermediates in biosyntheses such as β-OH-Arg in streptothricin [36] and viomycin[37, 38] biosynthesis, β-OH-His in nikkomycin biosynthesis[39], and β-OH-Tyr in novobiocin biosynthesis[40]. γ-Hydroxyarginine (γ-OH-Arg) is found in antibiotic K-582[41]. The mechanism of β-hydroxylation was revealed in two different pathways while the biosynthesis of γ-OH-Arg is still unresolved. One mechanism for β-hydroxylation occurs in a PCP-bound manner such as in the novobiocin and nikkomycin biosynthesis pathways[39, 40]. Two enzymes, NovH and NovI, were found to carry out the β-hydroxylation in novobiocin biosynthesis. NovH is a two-domain protein, which contains A and PCP domains. NovI is a P450 monooxygenase. L-Tyr is first loaded in NovH to form a L-Tyr-S-PCP intermediate then NovI introduces the hydroxyl group to the Tyr β-carbon (Scheme 1.2)[42].



Scheme 1.2 Formation of β-hydroxytyrosine by NovH and NovI.

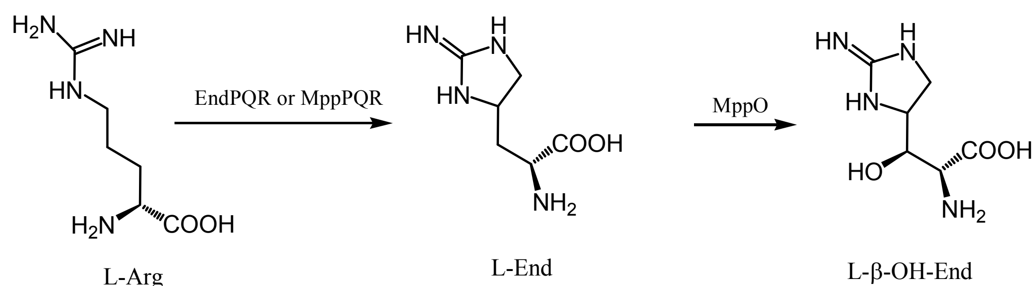
The other mechanism was first found in the viomycin biosynthesis pathway. Arginine is hydroxylated by a non-heme iron, α-ketoglutarate dependent oxygenase, VioC, to form β-hydroxy-L-arginine. A PLP-dependent enzyme, VioD, catalyzes the ring formation in capreomycidine, which is also a nonproteinogenic amino acid (Scheme 1.3)[37, 38].



Scheme 1.3 β-Hydroxyarginine as an intermediate in the formation of capreomycidine.

Enduracididine (End) is another nonproteinogenic amino acid similar to capreomycidine. End was first found in enduracidin, an antibiotic produced by *Streptomyces fungicidicus*[43]. In the antibiotic mannopeptimycins, two stereoisomers of β-hydroxyenduracididine were discovered[44]. Feeding experiments with radiolabeled compounds revealed that the End residues in enduracidin are derived from L-arginine[45]. End undergoes β-hydroxylation to form the

nonproteinogenic amino acid β-hydroxyenduracididine (β-OH-End) (Scheme 1.4). The formation and β-hydroxylation of End will be discussed in Chapter 2 and Chapter 3.



Scheme 1. 4 Proposed biosynthesis of enduracididine and β-hydroxyenduracididine.

New Trends and Techniques

Like most natural product work, antibiotic research used to focus solely on isolation, purification and structure determination. The biosynthesis pathways were usually elucidated by isotope precursor feeding experiments. The rapid development of molecular biology opened another door for studying antibiotic biosynthesis. In 1984, the first whole antibiotic biosynthesis pathway was cloned[46]. Since then, hundreds of pathways have been cloned and many have been heterologously expressed. Genetic manipulation and enzymology in the study of biosynthesis pathway may help increase the yield of original antibiotics and can also produce novel compounds with improved efficacy and new applications. The whole sequences of many bacterial genomes have revealed new drug targets as well as "silent" secondary metabolite pathways[47]. Furthermore, the emerging field of metagenomics provides genetic information on the 99.8% of soil microbes that are not culturable in the laboratory[48]. Many more "unnatural" products can be

generated through combinatorial chemistry and screened for new biological activity. The progress of bioinformatics provides another powerful method in linking and analyzing all the data[49].

In this thesis, two antibiotic biosynthesis pathways were studied by molecular genetics and enzymology. The β-modification of the nonproteinogenic amino acid enduracididine was elucidated and research on formation of enduracididine was carried out.

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Chapter 2
Characterization of MppO, an Oxygenase Involved in Formation of β-Hydroxyenduracididine in Mannopeptimycin Biosynthesis

Introduction

Introduction of Mannopeptimycins

Mannopeptimycins (MPPs) are a series of metabolites, including mannopeptimycin α, β, γ, δ, and ε, which are produced by *Streptomyces hygroscopicus,* LL-AC98 and discovered in 1958 by scientists at Wyeth[1]. MPPs are active against Gram-positive bacteria but not Gram-negative bacteria. Recently, MPPs were reexamined in a program searching for new classes of antibiotics to combat multidrug resistant pathogens. Amazingly, MPPs exhibited *in vitro* activity against clinically important pathogens like methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *enterococci* (VRE)[2]. This prompted further studies to investigate the structures of the MPPs, their pharmacological activities and mechanisms, biosynthesis and chemical modifications.

Mannopeptimycin α-ε are cyclic glycopeptide antibiotics characterized by two stereoisomers of the nonproteinogenic amino acid β-hydroxyenduracididine (β-OH-End). All MPPs share a cyclic nonribosomal peptide core with a mannosyl monosaccharide group attached to the D-β-OH-End. This structure is also named Mpp-β. The hydroxyl group of D-tyrosine in MPP α, γ, δ, ε is modified with a dimannosyl moiety (Figure 2.1) [3]. MPP γ, δ and ε all show moderate to good antibacterial activities. The MPP ε is most active of the family against MRSA and

VRE (Table 2.1). It is indicated that the dimannosyl moiety is required for MPPs activity and the isovaleryl group on the mannose affects the activity[4]. The minimum inhibitory concentrations (MICs) are 2-4 μg/ml for staphylococci including MRSA, 2-4 μg/ml for streptococci and 4-32 μg/ml for enterococci including VRE. Mannopeptimycin γ and δ demonstrate two to four fold less activity than mannopeptimycin ε (Table 2.1)[2].

Table 2.1 Antibacterial activity of MPPs α-ε

MPPs	MIC (μg/ml)			ED_{50} (i. v., mg/kg)
	S. aureus	*E. faecalis*	*E. faecium*	*S. aureus*
α	128	>128	>128	20
β	64	128	32-128	nt
γ	8	64-128	16-64	3.5
δ	4-8	64	8-64	2.6
ε	4	16-32	4-32	0.6

nt: not tested.

MPPs block bacteria cell wall synthesis by inhibiting peptidoglycan formation[5]. Later research on the cellular target of MPPs revealed that MPPs inhibit transglycosylase by binding to its substrate lipid II[6], which is similar to the mechanism of ramoplanin[7] and vancomycin[8]. MPPs don’t bind to the D-Ala-D-Ala terminus of lipid II which is the target of vancomycin and explains why mannopeptimycin has antibacterial activity against vancomycin-resistant bacteria [6, 9].

The MPP biosynthesis gene cluster was cloned and sequenced by Wyeth Research and the sequence was deposited in NCBI GenBank (assession number AY735112). The MPP gene cluster contains a gene (*mppO*) whose product has high similarity to non-heme iron, α-ketoglutarate dependent oxygenases, such as

VioC (58% similarity, 42% identity) and clavaminate synthase (46% similarity, 31% identity)[10, 11].

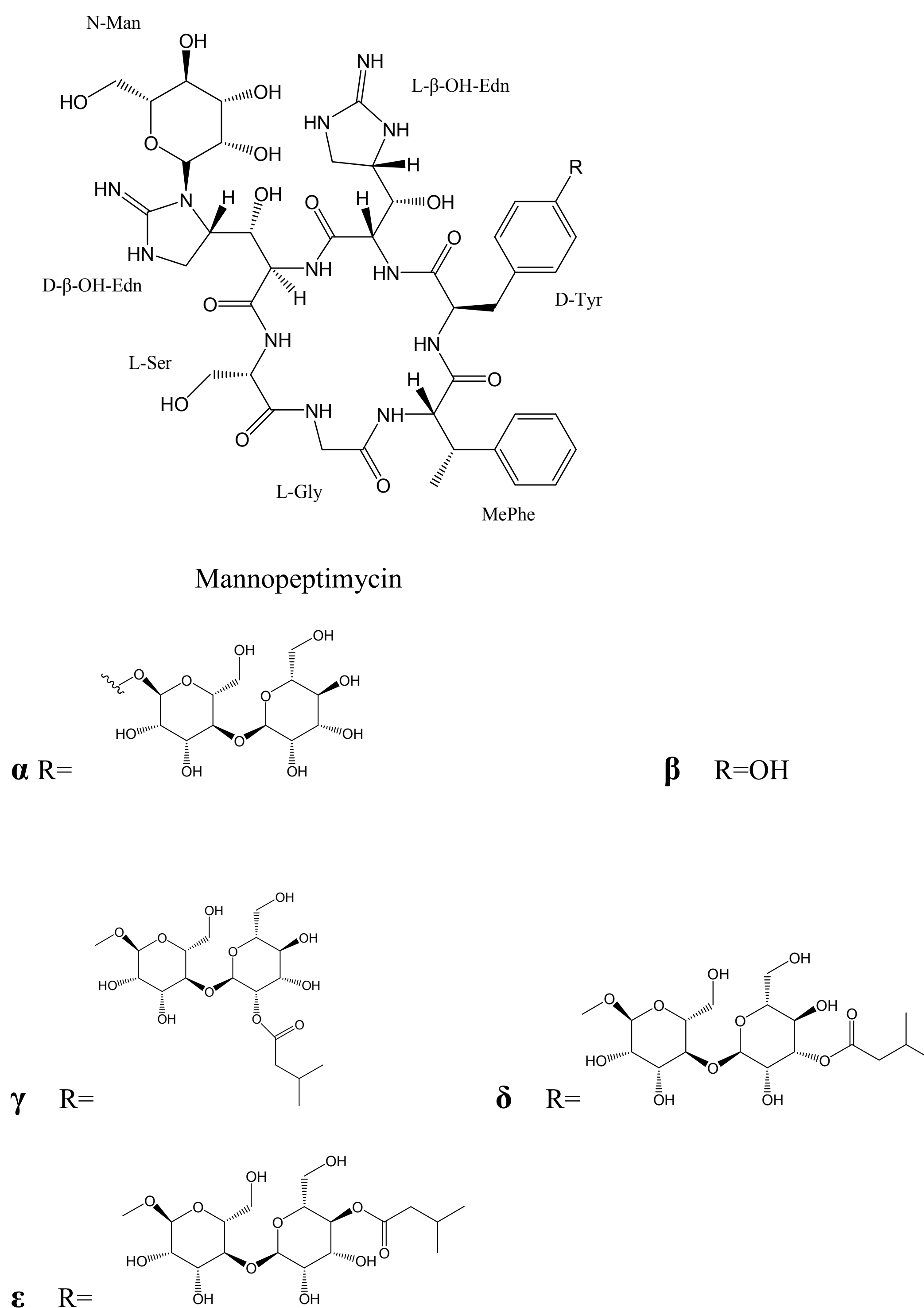
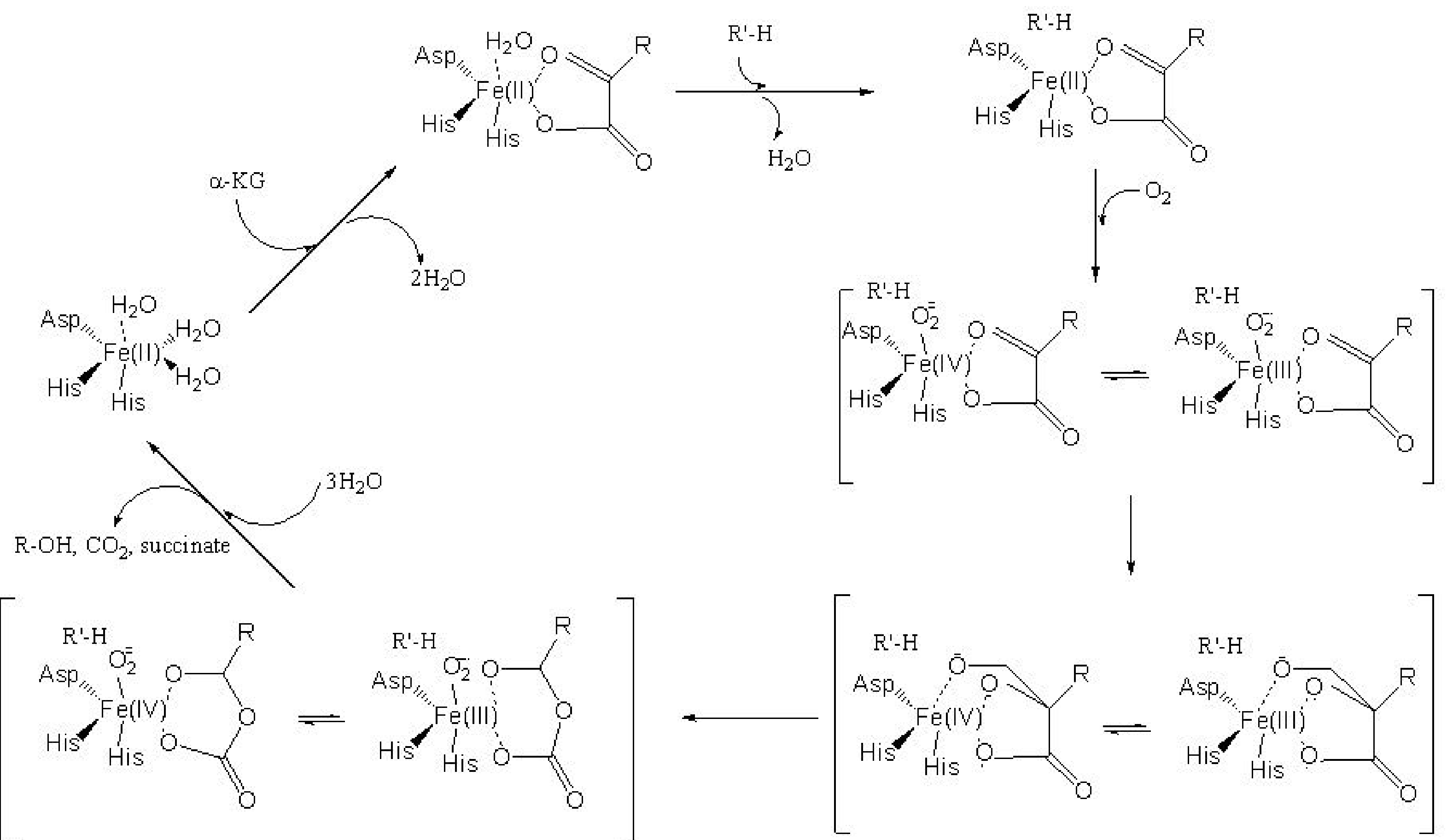


Figure. 2.1 Chemical structure of mannopeptimycin α, β, γ, δ, and ε

Non-heme iron, α-ketoglutarate dependent oxygenases

Non-heme iron, α-ketoglutarate (α-KG) dependent oxygenases are a superfamily of enzymes that catalyze a wide range of reactions including protein modifications, alkylated DNA/RNA reparation, antibiotics and plant product biosynthesis, lipid metabolism and biodegradation of herbicides[12]. They share a common 2-His-1-carboxylate (His-X-Asp/Glu-Xn-His) motif to weakly bind Fe(II) and have a jelly roll structure[12]. α-KG plays an important role by chelating iron through the C-1 carboxylate and C-2 keto-group. The C-5 carboxylate of α-KG is stabilized by a salt bridge to an arginine residue or by ionic interaction with a lysine side chain[12, 13]. The generally accepted reaction mechanism of these oxygenases was proposed over 20 years ago (Scheme 2.1)[14]. Firstly, the six-coordinates of Fe(II) are occupied by three molecules of H_2O, two His residues and one Asp residue (or Glu in some cases). Then α-KG binds to Fe(II) through C-1 carboxylate and C-2 carbonyl moieties and replaces two H_2O molecules. In the next step, the substrate molecule approaches the iron center to cause the leaving of the last water molecule. In consequence, the unsaturated iron is bound to oxygen and oxidized to a ferryl Fe(IV)-peroxo or Fe(III)-superoxo form that attacks the α-KG carbonyl group, resulting in insertion of an oxygen atom into the C1-C2 carbon-carbon bond of α-KG. The resulting active Fe(IV)-oxo species inserts oxygen into the target C-H bond of the substrate by hydrogen atom transfer and oxygen rebound to restore the Fe(II) state of the enzyme[14, 15]. The Fe(IV)-oxo intermediate was confirmed in TauD, taurine dioxygenase from *E. coli*[16, 17]. The last step in the reaction is similar to heme-containing oxygenases[18].



Scheme 2.1 Reaction mechanism of non-heme iron, α-KG dependent oxygenases[11]

Recently, a new His-3 motif variation on the 2-His-1-carboxylate structure theme was found in clavaminate synthase (CS), taurine dioxygenase (TauD), carbapenem synthase (CarC), γ-butyrobetaine hydroxylase (G-BBH), 2, 4-dichlorophenoxyacetate monooxygenase (2,4 D) and alkylsulfatase (AtsK). In the His-3 variation, the separation of the His-1 and His-3 is 145±10 residues instead of 55-59 residues between His-1 and His-2 motif (Table 2.2)[19]. X-ray crystallography confirmed the involvement of His-1 and His-3 motifs in binding Fe(II)[20-23].

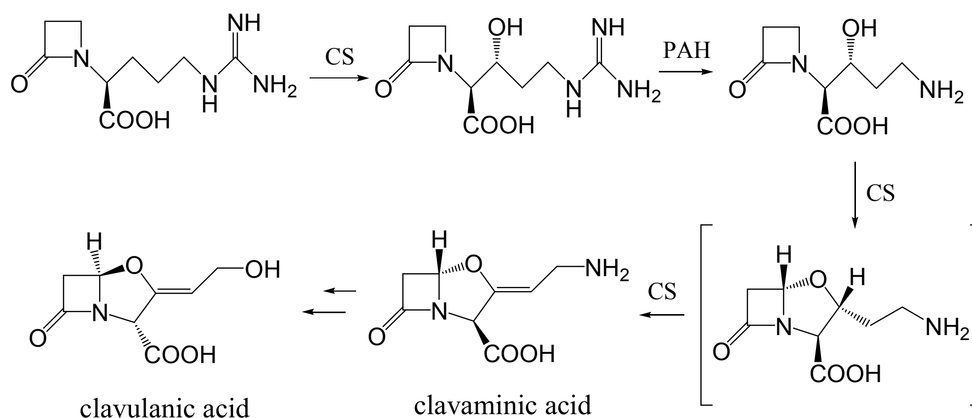
Table 2.2 His-2 and His-3 motif represented enzymes and sequence.

Type	**Enzyme**	**Sequence motif**
His-2 variant	ANS	$HXDX_{53}H$
	DAOCS	$HXDX_{57}H$
	FIH-1	$HXDX_{77}H$
	Proline 3—hydroxylase	$HXDX_{48}H$
His-3 variant	CS	$HXEX_{132}H$
	CarC	$HXDX_{147}H$
	TauD	$HXDX_{156}H$
	AtsK	$HXDX_{153}H$
	G-BBH	$HXDX_{147}H$
	2,4-D	$HXDX_{150}H$

ANS, anthocyanidin synthase from *Arabidopsis thaliana*; DAOCS, deacetoxycephalosporin C synthase from *S. clavuligerus*; FIH-1, factor-inhibiting hypoxia-inducible factor-1 from *Homo sapiens*; CS, clavaminate synthase from *S. clavuligerus*; CarC, carbapenem synthase from *Erwinia carotovora*, TauD, Taurine dioxygenase from *E. coli*; AtsK, alkysulfatase from *Pseudomonas putida*; G-BBH, γ-butyrobetaine hydroxylase from *Pseudomonas* sp. AK1; 2,4 D, 2, 4-dichlorophenoxyacetate monooxygenase from *Alcaligenes eutrophus*.

Clavaminate synthase (CS) is a well-studied non-heme iron, α-KG dependent oxygenase in the biosynthesis of clavulanic acid, a clinically used inhibitor of serine β-lactamases[24, 25]. CS performs three distinct oxidations including hydroxylation, oxidative cyclization and desaturation using a single ferrous active site (Scheme 2.2)[26]. His-144, Glu 146 and His-279 are involved in binding Fe(II)[11].

VioC is another non-heme iron, α-KG dependent oxygenase which is already discussed in Chapter 1[26-29]. Based on the significant similarities between MppO, VioC and CS, we proposed that MppO catalyzes one or more reactions in the conversion of L-Arg to β-OH-L-End.



Scheme 2.2 Clavaminate synthase catalyzed reactions in clavulanic acid biosynthesis pathway. CS, clavaminate synthase; PAH, Pro-clavaminate amidinohydrolase.

Results and Discussions

Sequence Analysis

The gene *mppO* codes for a 342 amino acid protein with a calculated molecular weight of 38.3 kDa. *mppO*m is a mutant of *mppO* with a single point mutation (C→T) at nucleotide 449, which causes an alanine at position 150 to be mutated to a valine (Brad Haltli, personal communication). MppO contains the 2-His-1-carboxylate motif that is common to non-heme iron, α-KG-dependent oxygenase superfamily[12]. Furthermore, MppO contains the characteristic His-1 (HXE) and His-3 (DNXXXXH) motifs of the His-3 variant subset of the

superfamily, and the spacing (134 residues) between the two motifs is approximately in the range (145±10 residues)[19]. Based on these facts, MppO most likely belongs to the His-3 variant subset of non-heme iron oxygenases. Sequence alignment of just MppO and VioC is shown in Figure 2.2. ClustalW Sequence alignment of MppO, VioC, SttL and CS is shown in Figure 2.3.

```
MppO   -----------------------MLTLHLQDDDVAAIDAVADELSRRYDSVESTEFQAESRLYADE
VioC   MTESPTTHHGAAPPDSVATPVRPWSEFRLTPAEAAAAAAALAARCAQRYDETDGPEFLLDAPVIAHE
Cons                             ::*   :.**  *:* . ::***..:..**  :: : *.*

MppO   LPRRVRRALHEYRSTEKSGILVVTGLPVDDSALGATPADRRHKPVPSTSLRQDIAFYLIANLLGDP
VioC   LPRRLRTFMARARLDAWPHALVVRGNPVDDAALGSTPVHWRTARTPG-SRPLSFLLMLYAGLLGDV
Cons   ****:*  : . *    .  *** * ****:***:**.. *   .*. *   .: : * *.****

MppO   IGWATQQDGFIMHDVYPVQGFEHEQIGWGSEETLTWHTEDAFHPLRTDYLGLMCLRNPDGVETTAC
VioC   FGWATQQDGRVVTDVLPIKGGEHTLVSSSSRQELGWHTEDAFSPYRADYVGLLSLRNPDGVATTLA
Cons   :******** :: ** *::* **  :. .*.: * ******* * *:**:**:.******* ** .

MppO   DIADVEIDDETRETLSQERFRILPDDAHRIHGKAPGDESARESALRERSRQRVASALESPDPVAVL
VioC   GVPLDDLDERTLDVLFQERFLIRPDDSHLQVNNSTAQQGRVEF-------EGIAQAADRPEPVAIL
Cons   .:.  ::*:.* :.* **** * ***:*   .::..::.  *        : :*.* : *:***:*

MppO   FGDRDDPYLRIDPHYMQGVQGETEQRA-LETIGAAIDDAMSGVVLSPGDIVFIDNYRVVHGRKPFR
VioC   TGHRAAPHLRVDGDFSAPAEGDEEAAAALGTLRKLIDASLYELVLDQGDVAFIDNRRAVHGRRAFQ
Cons    *.*  *:**:* .:   .:*: *  * * *:   ** ::  :**. **:.**** *.****:.*:

MppO   ARFDGTDRWLRRLNIARDLRKSREARLAATTRVIY--
VioC   PRYDGRDRWLKRINITRDLHRSRKA-WAGDSRVLGQR
Cons   .*:** ****:*:**:***::**:*  *. :**:
```

Figure 2.2 Sequence alignments of MppO and VioC. Residues that are conserved are indicated with an asterisk. Residues that are conservative are indicated with a colon. Residues that are semiconservative are indicated with a period. VioC is from *S. vinaceus*.

```
VioC MTESPTTHHGAAPPDSVATPVRPWSEFRLTPAEAAAAAALAARCAQRYDETDGPEFLLDA 60
SttL MSNLTDQST---------------SDYSLTAEEASAIAALSLELADSYPSFNDPVLLRDA 45
MppO -----------------------MLTLHLQDDDVAAIDAVADELSRRYDSVESTEFQAES 37
CS1  ----------------------MTS--VDCTAYGPELRALAARLP-RTPRADLYAFLDAA 35
CS2  ----------------------MASPIVDCTPYRDELLALASELP-EVPRADLHGFLDEA 37
                                    *:: . .     :  :   :

VioC PVIAHELPRRLRTFMARARLDAWP-HALVVRGNPVDDAALGSTPVHWRTARTPGS-RPLS 118
SttL PRLAARLPEGVQRFLREFKLTDHE-GHAVIRGHEFDQQRIGPTPDDWRGRQRPGPEFPEE 104
MppO RLYADELPRRVRRALHEYRSTEKS-GILVVTGLPVDDSALGATPADRRHKPVPSTSLRQD 96
CS1  HTAAASLPGALATALDTFNAEGSEDGHLLLRGLPVEADADLPTTPSSTPAPEDRSLLTME 95
CS2  KTLAARLPEGLAAALDTFNAVGSEDGYLLLRGLPVD-DSELPETPTSTPAPLDRKRLVME 96
       * **  :   :  .       :: *  .:    . .             .
                                            His-1
VioC FLLMLYAGLLGDVFGWATQQDGRVVTDVLPIKGGEHTLVSSSSRQELGWHTEDAFSPYRA 178
SttL LLLMLYAALLGEPFGWATQQDGHLVHDIFPIRQHENDQLGMGSKELLTWHTEDAFHPYRS 164
MppO IAFYLIANLLGDPIGWATQQDGFIMHDVYPVQGFEHEQIGWGSEETLTWHTEDAFHPLRT 156
CS1  AMLGLVGRRLGLHTGYRELRSGTVYHDVYPSPG-AHHLSSETSETLLEFHTEMAYHRLQP 154
CS2  AMRALAGRRLGLHTGYQELRSGTVYHDVYPSPG-AHYLSSETSETLLEFHTEMAYHILQP 155
        * . **   *:  :.* :  *: *    :   . *.  * :*** *:  :.

VioC  DYVGLLSLR-NPDGVATTLAG---VPLDDLDERTLDVLFQERFLIRPDDSHLQVNN---- 230
SttL  DYLILGALR-NPDRVPTTLGG---LDVASLSAEDIDILFEPRFSIAPDESHLPKNNT--- 217
MppO  DYLGLMCLR-NPDGVETTACD---IADVEIDDETRETLSQERFRILPDDAHRIHGKAPGD 212
CS1   NYVMLACSRADHERTAATLVASVRKALPLLDERTRARLLDRRMPCCVDVAFR-------- 206
CS2   NYVMLACSRADHENRAETLVGSVRKALPLLDEKTRARLFDRKVPCCVDVAFR-------- 207
     :*: * . * : :   *          :. .   * : :.   * :.   ..:...

VioC  STAQQGRVEFEG---IAQAADRPEPVAILTGHRAAPHLRVDGDFSAPAEGDEEAAAALGT 287
SttL  ITGEEEEARFAT---IQRMIDERPLGPLLYGSRLDPYMRLDPYFTSVPEGDTDARRAYDA 274
MppO  ESARESALRERSRQRVASALESPDPVAVLFGDRDDPYLRIDPHYMQGVQGETEQR-ALET 271
CS1   -----GGVDDPG---------AIAQVKPLYGDADDPFLGYDRELLAPE--DPADKEAVAA 250
CS2   -----GGVDDPG---------AIANVKPLYGDANDPFLGYDRELLAPE--DPADKEAVAH 251
     :. .       .     .      * *   *.:  *     ..:     *
                           His-3
VioC  LRKLIDASLYELVLDQGDVAFIDNRRAVHGRRAFQPRYDGRDRWLKRINITRDLHR--SR 345
SttL  LYKLVDAGMREVVADQGDVLFIDNHRAVHGRLPFKAHYDGTDRWLKRVCVTADLRR--SR 332
MppO  IGAAIDDAMSGVVLSPGDIVFIDNYRVVHGRKPFRARFDGTDRWLRRLNIARDLRK--SR 329
CS1   LSKALDEVTEAVYLEPGDLLIVDNFRTTHARTPFSPRWDGKDRWLHRVYIRTDRNGQLSG 310
CS2   LSQALDDVTVGVKLVPGDVLIIDNFRTTHARTPFSPRWDGKDRWLHRVYIRTDRNGELSG 311
     :   :*    :   **: ::** *..*.* .* .::** ****:*: :  * .  *

VioC  KAWAGDS-RVLGQR 358
SttL  EMRATAATRLLG-- 344
MppO  EARLAATTRVIY-- 341
CS1   GERAGDVVAFTPRG 324
CS2   GERAGDTISFSPRR 325
```

Figure 2.3 ClustalW Sequence alignment of MppO and related enzymes. VioC from *S. vinaceus*; SttL from *S. rochei* F20; CS1 and CS2 from *S. clavuligerus*. Invariant residues are indicated with an asterisk. Positions with conservative substitutions are indicated with a colon. Positions with semiconservative substitutions are indicated with a period. Residues involved in His motifs are shaded. The residue affect activity of MppO is in bold type.

Heterologous Expression of mppO and mppOm in E. coli

The genes *mppO* and *mppO*m were amplified with the same PCR primers from pBWA30a or pBWA30b (gifts from Wyeth Research) and cloned into the pET28a expression vector to form pET28a-*mppO* or pET28a-*mppO*m. The primers introduced an *Nde*I site overlapping the start codon and created an *EcoR*I restriction site downstream of the stop codon. The amplified DNA fragments were cloned and sequenced before transferring to *E. coli* (DE3) Rosetta cells for expression. The Rosetta cell line contains extra copies of genes for rare *E. coli* tRNAs and allows for improved expression of GC rich genes. Heterologous expression in *E. coli* resulted in N-terminal His_6-tagged recombinant proteins with calculated molecular weights of 39.1 kDa. MppO and MppOm were purified by Co^{2+} affinity chromatography and the efficiency of purification was verified by SDS-PAGE (Figure 2.4 and Figure 2.5).

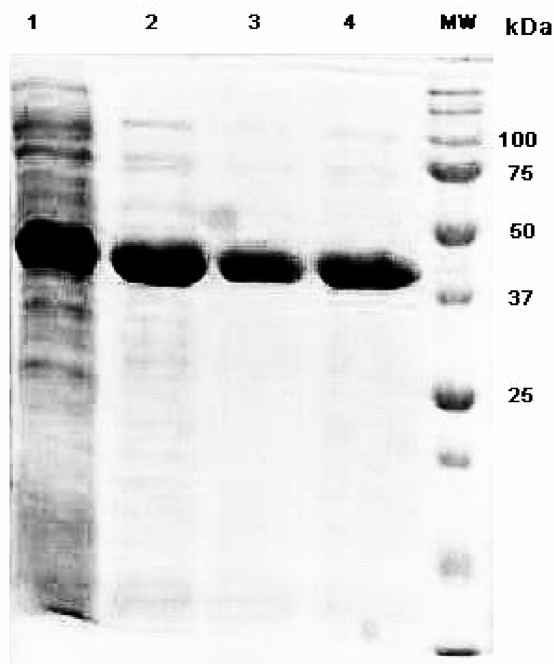


Figure 2.4 SDS-PAGE analysis of the expression and purification of His_6-MppO. Lane 1, soluble proteins; lane 2, protein eluted from Co^{2+} affinity column in 150 mM imidazole; lane 3, protein eluted from Co^{2+} affinity column in 250 mM imidazole; lane 4, protein combined from 2 and 3 for enzyme assay. The calculated MW of His_6-MppO is 39.1 kDa.

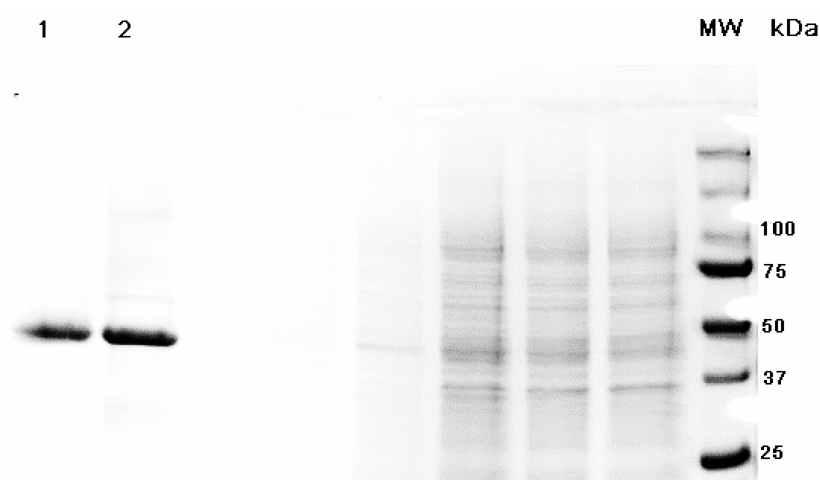
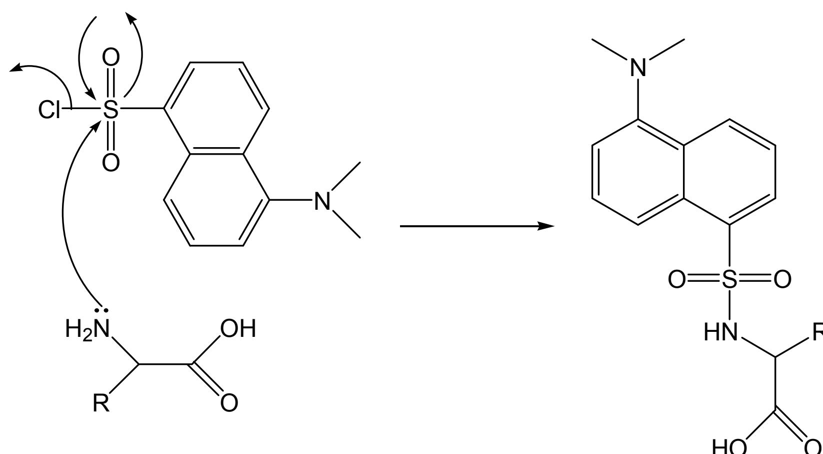


Figure 2.5 SDS-PAGE analysis of the expression and purification of His_6-MppOm. Lane 1, protein eluted from Co^{2+} affinity column in 150 mM imidazole; lane 2, protein eluted from Co^{2+} affinity column in 250 mM imidazole; The calculated MW of His_6-MppOm is 39.1 kDa.

Oxygenase Activity Assay and Analysis

Based on the high similarity of MppO and VioC, we proposed that MppO catalyzes the β-hydroxylation of enduracididine. Previous radiolabeled feeding experiment showed that enduracididine was derived from L-arginine[29]. Arginine is also a possible substrate for MppO and may undergo several oxidations to form enduracididine, similar to CS[25]. In addition, dideoxy-mannopeptimycin (dideoxy-MPP) can not be ruled out as a substrate because MppO may function as a tailoring enzyme after NRPS assembly[30, 31]. We performed MppO assays with several different substrates including enduracididine, L-arginine and dideoxy-MPP γ. Reaction mixtures were treated with dansyl chloride to allow for the detection of the substrate and product by HPLC (Scheme 2.3) [32].



Scheme 2.3 Dansylation of amino acid for UV detection

When D, L-End was used as substrate for MppO, a new peak appeared at 3.9 min (Figure 2.6). The product was confirmed to be β-OH-End by coinjection analysis of authentic β-OH-End obtained from the acid hydrolysis of MPP.

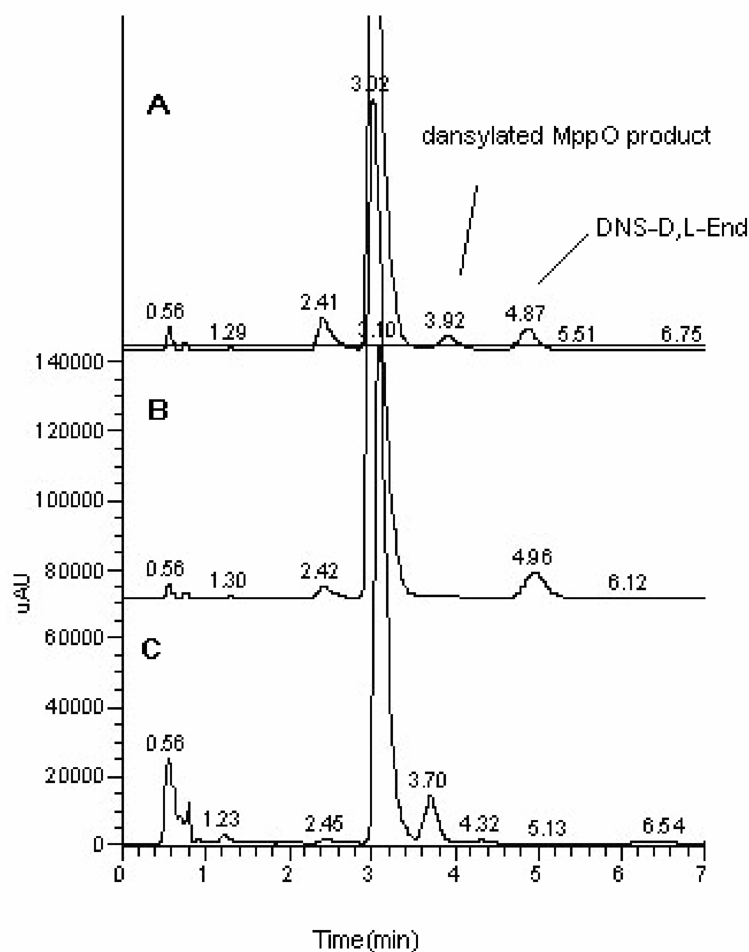


Figure 2.6 RP-HPLC analysis of the MppO assay with D,L-End as substrate. A, complete MppO assay; B, assay with boiled MppO; C, 1 mM DNS-D,L-End standard.

There was no detectable product when L-Arg was used as substrate for MppO. Similarly, VioC does not use D,L-End as substrate. The activities of these two enzymes are extremely specific though they have highly similar sequences, very similar substrates and enclose the same His-1 and His-3 motifs.

MppO also did not exhibit activity with dideoxy-MPP-γ as substrate, which confirmed that MppO is involved in β-OH-End biosynthesis rather than being a tailoring enzyme in the whole MPP biosynthesis pathway (Figure 2.7).

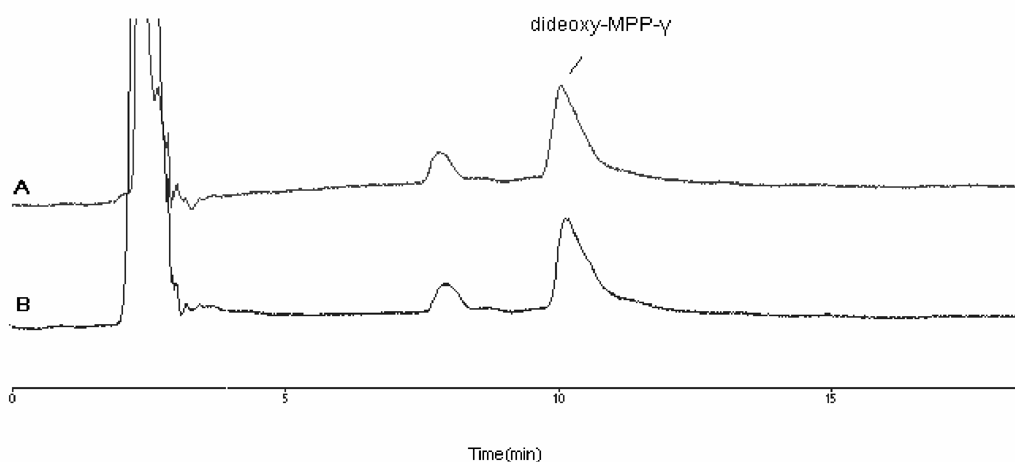


Figure 2.7 RP-HPLC analysis of the MppO assay with dideoxy-MPP γ. A, MppO assay with dideoxy-MPP-γ; B, assay with boiled MppO.

When MppOm was assayed, there was no new peak in the HPLC chromatogram.The mutation in MppOm results in a conservative change wherein an invariant alanine at position 150 is changed to valine. This mutation probably does not directly affect residues in the predicted active site, but it is proximal to the essential residues of the His-1 motif (H146 and E148), and the added bulk may hinder the ability to bind Fe(II).

β-OH-End was not observed in HPLC chromatograms of MppO assay when EDTA was added to chelate the iron or when α-KG was omitted from the reaction, confirming that α-KG and Fe(II) are necessary for MppO activity (Figure 2.9).

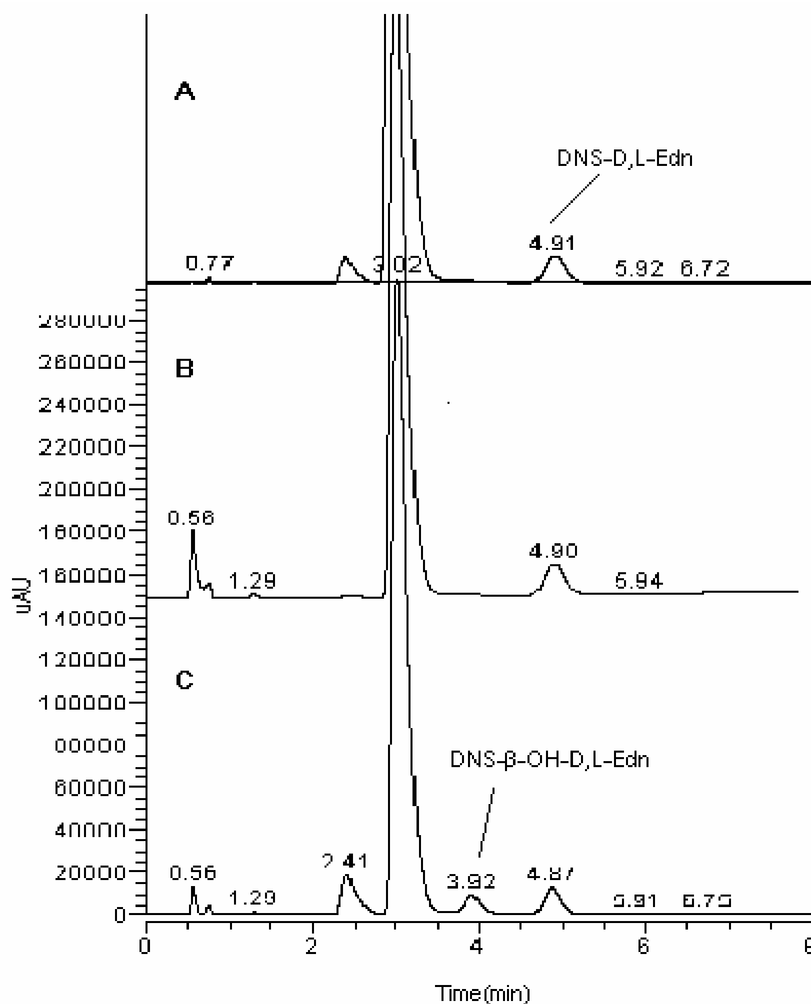
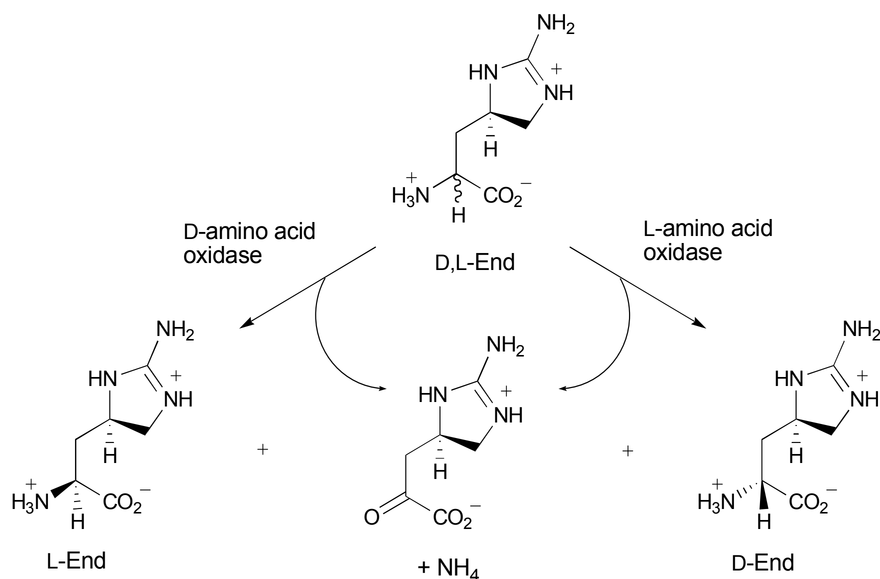


Figure 2.8 RP-HPLC analysis of the MppO activity affected by EDTA and α-KG. A, MppO assay with EDTA adding to the mixture; B, MppO assay without α-KG; C, complete MppO assay

Stereospecificity of MppO

Because the enduracididine used as substrate in the above assays was obtained by hydrolysis of dideoxy-MPP, which is produced by a *mppO* mutant of *S. fungicidicus*, both D and L forms were present. To examine the stereospecificity of MppO, samples of D, L-End were preincubated with either D-amino acid oxidase (D-AAO) or L-amino acid oxidase (L-AAO) to destroy the D or L isomers, respectively (Scheme 2.4).



Scheme 2.4 Preparation of L-End and D-End using D-amino acid oxidase and L-amino acid oxidase

Preincubation of D, L-End with L-AAO resulted in a significant decrease in the amount of β-OH-End formed (Figure 2.10). A small amount of β-OH-End was still detected and was probably due to incomplete elimination of L-End. Preincubation with D-AAO had no effect on product formation. Hence, MppO specifically hydroxylates only L-End. The D-β-OH-End residue in the MPPs structure must come from L-β-OH-End epimerization. Based on the above results, MppO stereospecifically catalyzes hydroxylation of L-End and results in the formation of 3S-hydroxy-L-End (Scheme 2.5).

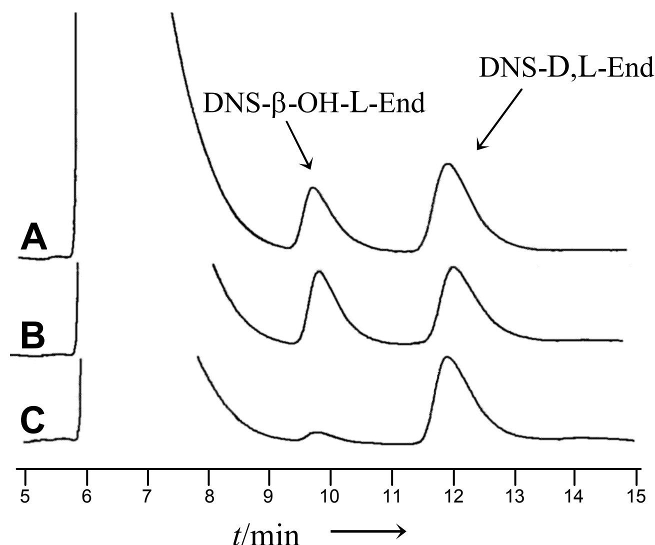
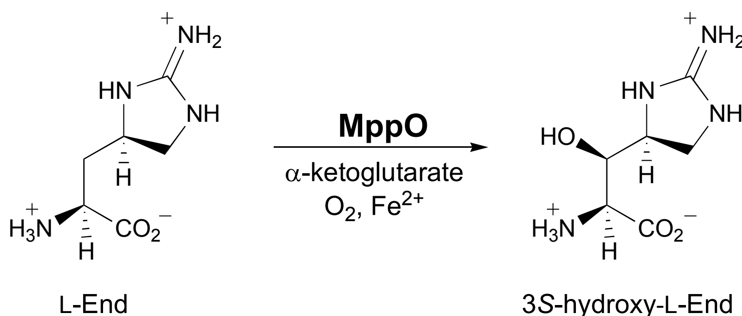


Figure 2.9 RP-HPLC analysis of the substrate stereospecificity of MppO. A, control MppO assay with untreated D,L-End; B, MppO assay in which the D, L-End was preincubated with D-amino acid oxidase for 1 hr; C, MppO assay in which the D,L-End was incubated with L-amino acid oxidase for 1 hr .



Scheme 2.5 Overall reaction catalyzed by MppO

MppO, CS and VioC all introduce a hydroxyl group at the β carbon of L-Arg or a derivative of Arg. Unlike CS and VioC, MppO oxidation does not facilitate subsequent cyclization involving the β carbon. However, the γ carbon must be activated for cyclization to enduracididine which is similar to capreomycidine biosynthesis[10, 26, 33]. Following *mppO* in the MPP biosynthesis gene cluster are *mppP*, *mppQ* and *mppR*. MppP and MppQ are assumed PLP-dependent enzymes.

Additionally, MppP (24% identity, 41% similarity) and MppQ (29% identity, 44% similarity) show moderate similarity with VioD[33]. Sequence analysis of the enduracidin gene cluster (unpublished results, Xihou Yin) revealed three ORFs *endP*, *endQ* and *endR*, whose products have remarkable similarity with MppP, MppQ and MppR. Thus we propose that the formation of End may involve a PLP-dependent-elimination/replacement reaction through MppP and/or MppQ. MppR may function as a regulatory factor. The cloning and heterologous expression of *endP*, *endQ* and *endR*, is discussed in chapter 3.

Significance

Nonproteinogenic amino acids play an important role in nonribosomal peptide diversity. Among them, common amino acids with β-hydroxylation modifications are a large subset. β-Hydroxylation facilitates further alteration and these hydroxyls are involved in a variety of biological functions such as target recognition, peptide glycosylation, or as sites of macrolactone cyclization. To the best of our knowledge, MppO is the first enzyme that catalyzes the β-hydroxylation of a nonproteinogenic amino acid. This study further expands our understanding of the formation of these key residues in numerous bioactive peptides.

Materials and Methods

General

Routine molecular biology procedures including DNA manipulation, plasmid construction, transformation, growth and maintenances of *E. coli*, competent cell preparation and SDS-PAGE analysis were conducted according to standard techniques[34]. Restriction enzymes, T4 DNA ligase and Taq polymerase were purchased from various suppliers and used according to the manufacturer's protocol. QIAprep® spin miniprep and QIAquick® gel extraction kits (Qiagen, Valencia, CA) were used for DNA purification. Sequencing of vector inserts was conducted by the Center for Genome Research and Biocomputing at Oregon State University. Chemicals were purchased from Sigma-Aldrich, Fisher Scientific and VWR International.

Subcloning MppO and MppOm for Heterologous Expression

PCR primers were designed to amplify the *mppO* gene from cosmid pBWA30a containing a segment of the mannopeptimycin gene cluster. The same primers were used to amplify the mutant *mppO* from pBWA30b which contains a point mutation (C→T) at nucleotide 449. The two cosmids are gifts from Wyeth

Research. PCR was carried out in a total volume of 50 µl containing 10 ng template DNA, 1× $(NH_4)_2SO_4$ buffer (Invitrogen), 2.5 mM $MgCl_2$, 5% DMSO, 0.4 mM each dNTP, 50 pmol forward (5'-GGACAT<u>ATGCTG</u>ACGCTCCACCTG-3',

*Nde*I site is underlined) primer and reverse primer (5'-CAAGAATTCTCAGTAGATGAC-3', *Eco*RI site is underlined).

PCR products were purified by gel electrophoresis and digested with *Nde*I and *Eco*RI before being ligated into *Nde*I and *Eco*RI digested pET28a. The resulting plasmid was called pET28a-*mppO* and pET28a-*mppO*m. The two plasmids were sequenced and the correct constructions were confirmed.

Expression and Purification of Recombinant MppO and MppOm

Plasmid pET28a-*mppO* and pET28a-*mppO*m were transformed into *E. coli* (DE3) Rosetta cells (Novagen). A single colony was picked to inoculate 5 ml LB seed culture containing 50 µg/ml kanamycin. After overnight culture at 30 ºC, the seed cultures were used to inoculate 500 ml LB containing 50 µg/ml kanamycin. Cells were grown at 20 ºC till the A_{600}=0.6-0.9, then IPTG (isopropyl-β-D-thiogalactopyranoside) was added to the medium at final concentration of 0.1 mM. Cells were cultured for an additional 5 hours at 20 ºC, then harvested by centrifugation at 3000 ×g for 10 min at 4 ºC, and washed with TWB (Talon Wash Buffer, 50 mM NaH_2PO_4, 300 mM NaCl, pH7.5). The precipitated cells were stored at -80 ºC. Frozen cells were thawed on ice, resuspended in TWB and lysed by sonication in a Microson ultrasonic cell disruptor (six 10 s bursts at six watts output with 10 s cooling on ice between bursts). The lysate was centrifuged at 18,000 ×g for 30 minutes at 4 ºC and the supernatant was used for enzyme purification. The His_6-MppO was purified with BD Talon™ Metal Affinity Resins (BD Biosciences) according to the

manufacturer's instructions. His_6-MppO was eluted with elution buffer (150mM or 250 mM imidazole, 50 mM NaH_2PO_4, 300 mM NaCl, pH7.0). Efficiency of purification was verified by SDS-PAGE. Fractions containing MppO were pooled and dialyzed against TWB to remove imidazole. Protein concentration was determined by Bradford analysis using BSA as standard.

Oxygenase Activity Assay

Assays were conducted in 50 mM MOPS pH 7.0 and included 2mM D,L-enduracididine, 1mM L-arginine, or 1 mM dideoxy-mannopeptimycin as substrate and 1mM α-ketoglutarate, 25 μM $FeSO_4$, 0.5 mM DTT, 0.1 mM ascorbate and appropriately diluted enzymes in a total volume of 200 μl. Reactions were initiated by adding enzyme and incubated at 30°C for 6 hours. Protein was precipitated with cold ethanol, and the supernatant was decanted and stored at -20 °C prior to derivatization with dansyl chloride (DNS-Cl) to permit HPLC analysis with UV detection (note: the reaction that contained dideoxy-mannopeptimycin was not dansylased because dideoxy-mannopeptimycin and mannopeptimycin are UV active). Dansylation reactions were conducted by mixing 50 μl reaction mixture with 50 μl of 80mM Li_2CO_3, pH 10 followed by 50 μl of DNS-Cl in MeCN (1.5 mg/ml). The reaction mixtures were votexed briefly and kept in the dark at room temperature for 1 hr. Then the reactions were quenched with 20 μl of 2% aqueous ethylamine. Samples were filtered through 0.45 μm syringe filter before HPLC analysis.

Stereospecificity of MppO

Assays were conducted in a buffer system containing 40 mM Tris and 80 mM KCl, pH 8.5 with 25 mM D,L-enduracididine, 0.2 U porcine kidney D-amino acid oxidase or 0.8 U L-amino acid oxidase from *Crotalus adamanteus* venom in a total volume of 40 µl. Reactions were initiated by adding of the substrate and incubated at 37 °C for 1 hr. The reaction mixtures were centrifuged at 18,000 ×g for 1 min and 20 µl of the supernatant was removed and used as substrate in later MppO assays. MppO assays were conducted under the same condition as described above.

HPLC Analysis of Dansylated Reaction Mixtures

Reversed phase HPLC analysis (Beckman Ultrasphere C18 column, 5 µm; 4.6 × 250 mm) was performed on a Thermofinnigan Surveyor system using photodiode array detection or a Beckman Gold system using UV detection at 254 nm. Analysis of the reactions that included L-Arg or D, L-End as substrate were conducted under isocratic conditions of 80% 50 mM NH_4OAc, pH 5.5:20 % MeCN. Analysis of the reaction that included dideoxymannopeptimycin γ as substrate was conducted using a linear gradient from 10% A (0.05% aqueous formic acid) to 50% B (MeCN with 0.05 % formic acid) in 25 minutes.

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Chapter 3
Studies on the Formation of Enduracididine in the Enduracidin Biosynthesis

Introduction

Introduction of Enduracidin

Enduracidin, an antibiotic active against Gram-positive bacteria, was first isolated from a strain of the soil bacterium *Streptomyces fungicidicus* B5477 in 1968[1]. In the following five years, a series of studies were conducted on its antibacterial activity[2, 3], mechanism of action[4] and structure[5]. However, enduracidin was largely ignored until ramoplanin, a structure similar antibiotic, was discovered in 1984[6, 7]. Ramoplanin exhibits excellent activity against multidrug resistant Gram-positive bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococci spp.* (VRE), and is currently in Phase III clinical trials[8, 9]. Enduracidin, like ramoplanin, is a promising antibiotic and worthy of further research.

Enduracidin and ramoplanin share similar peptide cores containing 17 amino acids with chlorinated Hpg residues (Figure 3.1). The main differences between them are the length of acyl chain attached to an aspartate or asparagine residue, two D-mannose units in ramoplanin and the two arginine-derived enduracididine residues in enduracidin[10, 11]. The region of Hpg3 to Orn10 in ramoplanin recognizes and binds to the MurNAc-Ala-γ-D-Glu pyrophosphate region of Lipid II, a precursor of peptidoglycan, and results in fibril formation[12]. A nearly identical motif is conserved in enduracidin. The structure and 3-dimensional conformation

of enduracidin showed that it contains two antiparallel β-strands including residues 5-7 and 10-12 connected by a turn including residues 8 and 9[13]. Hydrogen bonding exists between Thr5 and Ser12, Hpg7 and Orn10 and Hpg7 and Cit9. Ramoplanin demonstrates similar overall structural characteristics which may contribute to their similar activity[13, 14].

Enduracidin showed antibacterial activity against Gram-positive bacteria both *in vitro* and *in vivo*, but not against Gram-negative bacteria except *Neisseria gonorrhoeae*. The MICs of enduracidin range between 0.048-0.19 μg/ml against various strains of MRSA[15]. The 50% effective does (ED50) of enduracidin for mice with *S. aureus* infections is 2.27 mg/kg[3]. Enduracidin also showed activity in inhibiting avian myeloblastosis virus reverse transcriptase[16], hepatitis B virus *in vitro*[17], and prolyl endopeptidase[18].

Enduracidin and ramoplanin are cell wall synthesis inhibitors. Early studies reported that bacteria exposed to these antibiotics accumulate UDP-N-acetylmuramyl pentapeptide by interfering with the utilization of this cell wall precursor[4, 19]. New research results revealed that enduracidin inhibits transglycosylase by binding to Lipid II with a ratio of enduracidin: Lipid II = 2:1, which is the same mechanism of action for ramoplanin. Although enduracidin will also binds to Lipid I and inhibit MurG, it is too big to penetrate the cell membrane and bind to Lipid I which is only found inside the cell[7].

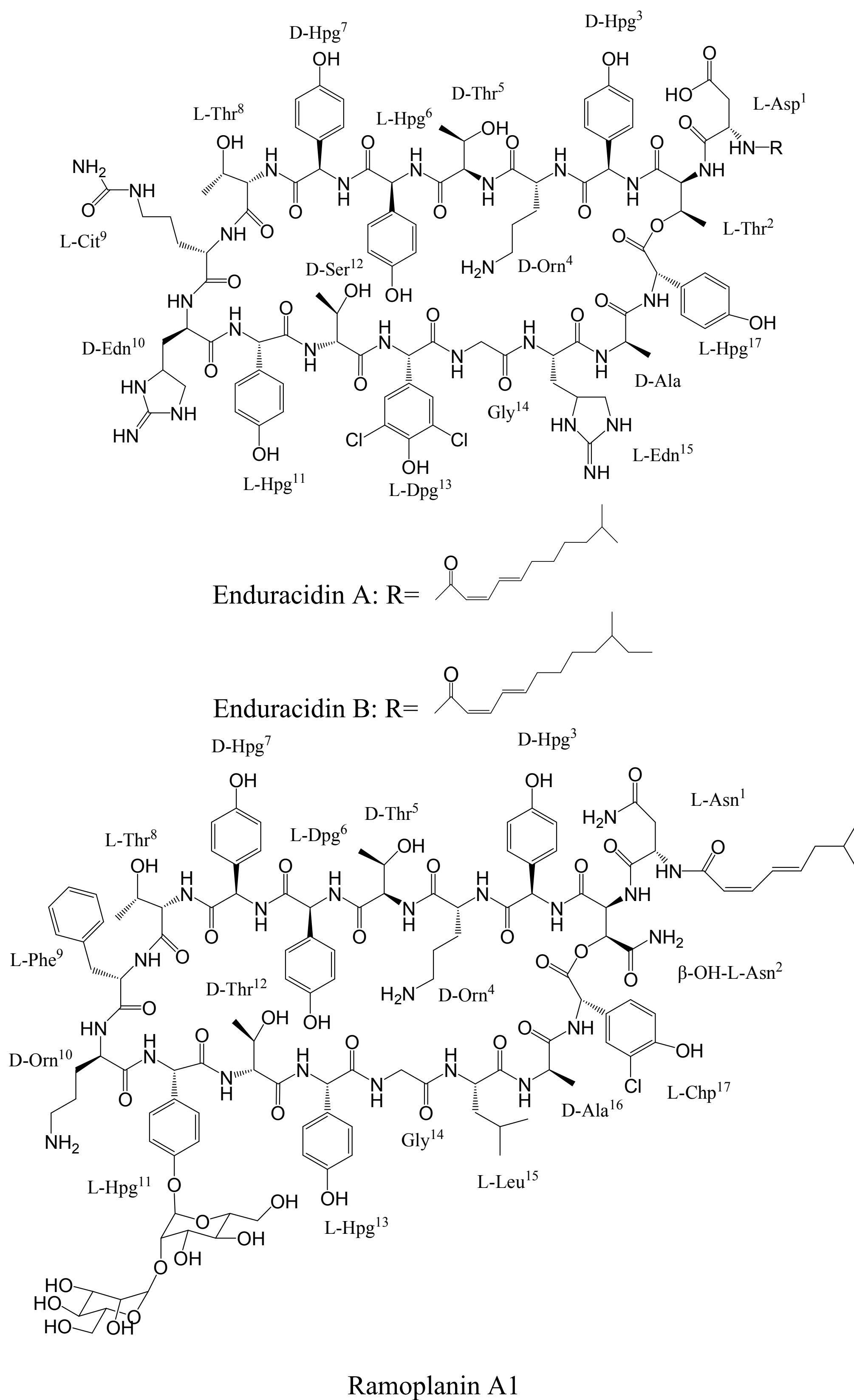


Figure 3.1 Structures of Enduracidin and Ramoplanin A1

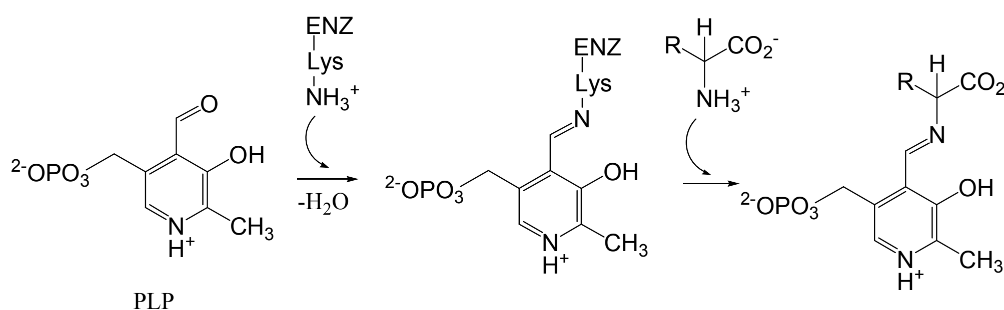
The entire enduracidin biosynthesis gene cluster has been cloned and sequenced in our laboratory (Xihou Yin, unpublished results). Three genes were found to form an operon with high similarity to the *mpp*PQR operon in the mannopeptimycin cluster. Blast search analysis showed that the putative EndP and EndQ are PLP-dependent enzymes. Sequence similarity searches with EndR returned hits with two acetoacetate decarboxylases.

Pyridoxal Phosphate (PLP)-dependent Enzymes

The pyridoxal-5'-phosphate (PLP, also known as vitamin B_6)-dependent enzymes are a superfamily of enzymes that are found in all organisms. They are involved in a wide range of metabolic reactions related to amino acids, including synthesis, degradation and interconversion of amino acids[20].

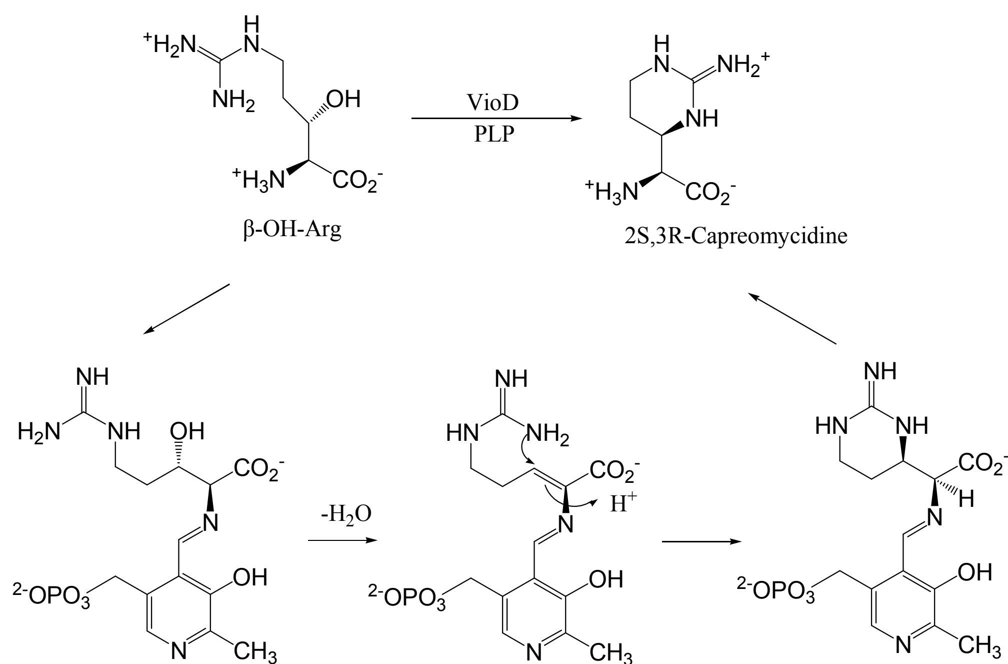
PLP-dependent enzymes are divided into five distinct groups by their structure and mechanism characteristics. Among them, type I has the most common structure and includes many of the well-characterized PLP-enzymes. Type I is the aspartate aminotransferase family, including a range of aminotransferases, decarboxylases and enzymes that catalyze α-, β-, and γ-eliminations. Type II is the tryptophan synthase family which is similar to type I. They mostly catalyze β-elimination reactions. Type III is the alanine racemase family which differs from other PLP enzymes because of its $(\beta/\alpha)_8$ barrel structure. Type IV is the D-amino acid aminotransferase family which is superficially similar to type I and type II. Type V is the glycogen phophorylase family, also including starch phosphorylase[21, 22].

All PLP-dependent enzymes have a common mechanism in binding PLP. First, PLP is bound through a Schiff's base linkage between the aldehyde group of PLP and the ε-amino group of an active-site lysine residue. Then the amino group of the substrate replaces the ε-amino and forms a coenzyme/substrate complex (Scheme 3.1). PLP plays a role as an electron sink which stores and dispenses electron and facilitates a variety of reactions[23, 24].



Scheme 3.1 PLP and substrate binding mechanism[23, 24]

VioD is a PLP-dependent enzyme in the viomycin biosynthesis pathway involved in formation of 2S, 3R-capreomycidine from β-hydroxyarginine[25, 26]. VioD catalyzes a β-elimination and replacement reaction described in Scheme 3.2.



Scheme 3.2 Proposed mechanism for the VioD-catalyzed formation of 2S, 3R-capreomycidine[25]

Based on similarity and Blast search results, we propose that the formation of enduracididine may involve PLP-dependent-elemination/replacement reactions through EndP and/or EndQ. The function of EndR is unclear.

Results and Discussion

Sequence Analysis

Three open reading frames (ORFs) *end*P, *end*Q and *end*R in the enduracidin gene cluster existed in an operon organization with overlapped start and stop codons. They code for three separate proteins. The three enduracidin proteins have high similarity with the mannopeptimycin proteins MppP (identity=80%, similarity=86%), MppQ (identity=67%, similarity=75%) and MppR (identity=76%, similarity=86%), respectively. As described in Chapter 2, MppO was revealed to be an amino acid β-hydroxylase, like VioC, but catalyzes the formation of β-hydroxy-L-enduracididine (β-OH-L-End) from L-enduracididine (L-End). MppP, MppQ and MppR are proposed to be involved in the cyclization of L-Arg to form L-End. A PSI-Blast search with EndP and EndQ showed that they belong to the PLP-dependent superfamily, and are most similar to the type I aminotransferase family. ClustalW sequence alignments of EndP and EndQ with related PLP-dependent enzymes showed a conserved lysine residue in both enzymes. This conserved lysine residue also aligns with VioD Lys230 residue which may be involved in binding PLP[25] (Figure 3. 2 and Figure 3.3).

```
COG0436            MSTQTSAPRVSDRAGLVQPFAAMGIVAKVQEMSRNGRDTIAMCLGEPTQGAPGPVRRRAA 60
Aminotran_1_2      -------MRVS-RRGAVDPFIVMDVMEQARTLEAAGRSIIHMEVGQPGTPAPAGARAALA 52
Cys_Met_Meta_PP    -------MRSATAPRSKLPDVGTTIFTVIGQLAAQ-HDALNLSQGAPNFAPDPSLVEGVA 52
EndP               ------------------------------------------------------------
HisC               -----------------------------------------MIYIDKNESPVTPLDEKTM 19

COG0436            EVVTDGTNLGYSPIFGIPELRTAIAGHYRDWYGVEVP-AERIAITTGSSGAFQTAFLTCF 119
Aminotran_1_2      RAMEAGP-LGYTVALGLPELRKGIADLYRRWYGVELD-PNRVVVTAGSSSAFLLAFTALF 110
Cys_Met_Meta_PP    RAMRDGH-NQYAPMAGVMALRERLAEKTEALYGTHYDPATEITVIASASEGLYAAISALV 111
EndP               -----------------------------------------VLSCYSSSVAMEILSRALA 19
HisC               TSIISATPYNLYPDAAYEQFKEAYAKFYG---------LSPEQIIAGNGSDELIQKLMLI 70
                                                              . .

COG0436            DAGDRVALARPGYGAYKNILAALNCEIVELDCGGDHGFQPTVELLEQVHAQAPLKGLMLA 179
Aminotran_1_2      EAGDRVALGEPGYPSYRQILRALSLEPVGIPTREENRLQPVPEDLEGV---ADLAGLIVA 167
Cys_Met_Meta_PP    HPGDEVIYFEPSFDSYAPIVRLQGATPVAIKLSPEHFRVNWDEVAAAIN--PRTRMIIVN 169
EndP               SVTDSVALVHPTFDNIADLLRGNRLGLVPLEEDPLHADDLPAELLESVG------CVFVT 73
HisC               MPEGPALTLNPDFFMYQAYAAQVNREIAFVDAG-SDLTFDLETILTKID-EVQPSFFIMS 128
                      . .   .* :             .  :     .          :        .::

COG0436            SPANPTGTMISGEHLGELISWCAEHGVQVISDEIYHGISYIGTRGETAL---AHDDNAIV 236
Aminotran_1_2      SPGNPSGTMLSQEALAGLTGHCADRAIAFISDEIYHGLDY-GTRAVSAL---EITDDVYV 223
Cys_Met_Meta_PP    TPHNPTATVFSADDLERLAQLTRDTGIVILSDEVYEHVVFDGAQHQSMARHRELAERSVI 229
EndP               TPNNPTGRVVSAERLRRLAGQCARHGVILALDTSFRGFDTRAQYDHYEILD-ASDVRWVV 132
HisC               NPHNPSGKQFDTAFLTAIADKMKALNGYFVIDEAYLDYGTAYDVELAP--------HILR 180
                   .* **:.  ..   *  :          .  *  :

COG0436            ISSFSKYWAMTGWRLGWAILPESLASAAQNVTGNLSLCAPVPAQYAAVAAFTEESYAECE 296
Aminotran_1_2      INSFSKYFSMTGWRLGWLVVPEAHVRPIERLAQNMFICPPHASQIAALAAL--DCAEELE 281
Cys_Met_Meta_PP    VSSFGKSFHVTGWRVGHCVAPAELMDEIRKVHQFMVFAADTPMQVAFAEIL---ARPDSY 286
EndP               IEDTGKLWPTLDLKVGMLVHSENLGLPVEKIYSDILLG-VSPLILGMVRRFSEDAAAGGL 191
HisC               MRTLSKAFGIAGLRLGVLISTAGTIKHIQKIEHPYPLN-VFTLNIATYIFRHREETRQFL 239
                   :   .* :   . ::*  : .       ..:    :    .   .

COG0436            TAVESFARARKHVLDATADLGFSDMAPPDGAFYMYARIDEILQRSDAGIDTATDWCQSLL 356
Aminotran_1_2      ANRIVYAENRRLMLEGLPKAGFTRFAPPDGAFYVYADVSDLT-------DDSLAFAAEIL 334
Cys_Met_Meta_PP    LGLSAFYQAKRDLLARELADSRFELLPSEGSFFMLARFRHFSD------ESDSDFVLRLI 340
EndP               TELHEFIAAQRSVVRAGLADLSTTGVPDPD---SRASVERVLIR-----HLTGTEVWEAL 243
HisC               TMQRQLAEQLKQIFDTHVADKMSVFPSNANFVLTKGSAAQQLG----------QYVYEQG 289
                        : :.           .  .     .

COG0436            ETTGVALAPGDDFDSLN---GPRSVRLSLAVGADKTAEALDRILDFMR---- 401
Aminotran_1_2      REAGVAVTPGLDFDPVR---GARTLRFSYARATEDIVEGLRRLEAFMAACRG 383
Cys_Met_Meta_PP    RDARVATIPLSAFYTDGT--DAGVIRLSFSKDDATLVEGARRLRSL------ 384
EndP               REHHVYALPCRAFYWADPARGDRTLRLALARASAPLAQCVRALRHVLTPR-- 293
HisC               FKPRFYDEPVMKGYVRYS--IATASQLKQLEEIVKEWSAKYDLSKTTKHS-- 337
                       .   *               ::          .   :
```

Figure 3.2 ClustalW Sequence alignments of EndP and related proteins. COG0436: aspartate/tyrosine/aromatic aminotransferase, Aminotran_1_2: aminotransferase class I and II, Cys_Met_Meta_PP: Cys/Met metabolism PLP-dependent enzyme , HisC: histidinol-phosphate/aromatic aminotransferase and cobyric acid decarboxylase. Invariant residues are indicated with an asterisk. Positions with conservative substitutions are indicated with a colon. Positions with semiconservative substitutions are indicated with a period. Conserved lysine residues are shaded.

```
EndQ               GLVRDAYARALEEYGAAALGYGHDPGALPLRAELAARATVRGRSPCGPE--HVVVTAGTS 109
Aro8               DEFAELINRVLKRDRGYAFEYQDSRGYKPLRESILKFVRLYG-IETGVE--NIQVISGGQ 177
Aminotran_1_2      AGARAALARAMEAG---PLGYTVALGLPELRKGIADLYRRWYGVELDPN--RVVVTAGSS 99
COG0436            ALIRDAAAAALDGLAS----YPATAGTDALRDTVARWLERRYGLPAIDATTQVLAASGSR 105
HisC               KTMTSIISATPYNLYP-------DAAYEQFKEAYAKFYGLSP---------EQIIAGNGS 60
                      .       :     .   ::                .    ..

EndQ               QALHLLATTLARPGDT------------VLVEGLGYDLGQRILGDCALRLRRVALDASGM 157
Aro8               QGIDVVSKALINFGDT------------IVVERPTYSWALASFQSRGADILEVNLNKDGI 225
Aminotran_1_2      SAFLLAFTALFEAGDR------------VALGEPGYPSYRQILRALSLEPVGIPTREENR 147
COG0436            EALFSLAQAVIDSSPRENGATQDGERPIVLCPNPFYQIYEGAALLAGAAPYFVNSDPARN 165
HisC               DELIQKLMLIMPEGPA-------------LTLNPDFFMYQAYAAQVNREIAFVDAGSDLT 107
                   . :     :  .            :     :              :

EndQ               VPEALRRALAGTARGGEGGTGRTAFVYLTPTHHNPTGATMPLERRLRLLEAAAEHGVLVV 217
Aro8               DIEDLEDKLK---------KFKPKFIYVMPNFHNPTGILYSDEKKEKLVGLAEKYETYLL 276
Aminotran_1_2      LQPVPEDLEG----------VADLAGLIVASPGNPSGTMLSQEALAGLTGHCADRAIAFI 197
COG0436            FAPDYSSVPAD--------VWARTQLVYVCSPGNPTGAVLTLDDWRELFALSDEHGFVIA 217
HisC               FDLETILTKID---------EVQPSFFIMSNPHNPSGKQFDTAFLTAIADKMKALNGYFV 158
                                                 .  **:*        :        .

EndQ               EDDAYGELGLTDGPPAPPPLAALAGH----RGVVRLGSFSKTL-GPGLRLGWLVTEPALA 272
Aro8               EDDFAIELSFTETDVFP--LKAFDKY----DRVIYLKSFSKVH-MPGLRLGFIIAPEKLV 329
Aminotran_1_2      SDEIYHGLDYGTR-----AVSALEIT----DDVYVINSFSKYFSMTGWRLGWLVVPEAHV 248
COG0436            SDECYSEIYFDETKPPLGGLEAAHRLGRDFTRLVMLSSLSKRSNVPGMRSGFVAGDAALL 277
HisC               IDEAYLDYGTAYD-------VELAPH------ILRMRTLSKAFGIAGLRLGVLISTAGTI 205
                    *:             :          :  : ::**   .* * * :

EndQ               ERIASHGLFRSGGSLNHITSLAVAGLLSDGGYDRHLEMLRAGLRARRDALLDALREAADL 332
Aro8               SSFLKA-KYVTDLTTSGLMQRAFDLYLRENIWKKHIEEVKGVMRERFEKMKEGLTQLKSY 388
Aminotran_1_2      RPIERLAQNMFICPPHASQIAALAALD----CAEELEANRIVYAENRRLMLEGLPKAGFT 304
COG0436            KRFLLYRTYHGAALSPVWQKASVAAWG----DEAHVRENRALYAQKFATVTPMLAEVLDV 333
HisC               KHIQKIEHPYPLNVFTLNIATYIFRHR---EETRQFLTMQRQLAEQLKQIFDTHVADKMS 262
                   :                .         ..   :    .  :

EndQ               PVRISRPEGGFFLWLRCGTGLGEDELLARAER--AGVRVTAG----SRFGGTREPS--VR 384
Aro8               -FEFDIPKGGFYYWVKLKDNWKAVDFYQKCLE--RGLLVVPG----DMFFGIKKEDNFLR 441
Aminotran_1_2      --RFAPPDGAFYVYADVSDLTDDSLAFAAEILREAGVAVTPG----LDFDPVRGAR-TLR 357
COG0436            ----RLPDAAFYLWANVARTGLSDTEFARRLYADYNVTVLPGSYLARDAHGANPGRDFVR 389
HisC               ---VFPSNANFVLTKGSAAQQLGQYVYEQGFK--------------PRFYDEPVMKGYVR 305
                    . . ... *                     : :                :*

EndQ               LAYSFN-PPPLLERAARRLTQAWSGGPPDRQIGGNP- 419
Aro8               LSFASC-DVQEIEKGIEILRQVLSEGQNENEMYLPII 477
Aminotran_1_2      FSYAR--ATEDIVEGLRRLEAFMAACRG--------- 383
COG0436            IALVAG-TAECVEGAQRIVDFCRGLAR---------- 415
HisC               YSIATASQLKQLEEIVKEWSAKYDLSKTTKHS----- 337
                   :       :   .
```

Figure 3.3 ClustalW Sequence alignments of EndQ and related proteins. ARO8: transcriptional regulators containing a DNA-binding HTH domain and an aminotransferase domain, Aminotran_1_2: aminotransferase class I and II, COG0436: aspartate/tyrosine/aromatic aminotransferase, HisC: histidinol-phosphate/aromatic aminotransferase and cobyric acid decarboxylase. Invariant residues are indicated with an asterisk. Positions with conservative substitutions are indicated with a colon. Positions with semiconservative substitutions are indicated with a period. Conserved lysine residues are shaded.

The Blast search with EndR returned hits with two acetoacetate decarboxylases (E value=4e-41/5e-41, identity=37%/40%). The function of EndR in the formation of enduracididine is not obvious.

Subcloning of endP, endQ, endR for Heterologous Expression

In order to express *end*P, *end*Q and *end*R in pET28a and pBADN3 vectors, PCR primers were designed to amplify these genes and introduce appropriate restriction enzyme sites. While *end*P and *end*Q were successfully amplified by PCR, *end*R was extremely hard to amplify using numerous approaches. One reason may be that *end*R has very high GC content (GC% = 75%), which is a common characteristic of *Streptomyces* DNA. The GC content of *end*R is higher than *end*P (GC% = 71%) but not *end*Q(GC% =76%). Another reason may be 10 consecutive G/C nucleotides existing in the region upstream of the reverse primer site, which is a key region for polymerase binding. PCR was conducted with different buffer systems (Invitrogen or Promega) and different templates (pXYFD16 or *Eco*RI digested pXYFD16 fragment containing the *end*PQR operon). The effect of $MgCl_2$ concentration and annealing temperature were also tested. In addition to the normal three steps (denaturation, annealing and elongation) PCR, two-step "hot" PCR also was conducted[27]. Annealing and elongation were combined in one step at a temperature above 70 °C. This method is often effective for high GC DNA amplification, but also failed to produce amplified *end*R. A final approach was conducted to amplify *end*R starting downstream of the stop codon so the polymerase will not intermediately encounter the 10 consecutive GC sequences

when it starts. This reaction produced a longer product which contains an incomplete *end*R gene in both directions. Also, an earlier attempt to amplify the whole *end*PQR operon generated a product missing 18 bp from *end*R (Xihou Yin, unpublished results). All of these results suggested that the 10 consecutive GC sequence upstream of *end*R may account for the difficulty of PCR amplification.

Heterologous Expression and Purification of endP and endQ in E. coli

Both *end*P and *end*Q were amplified by PCR and ligated into the pBADN3 and pET28a expression vectors (pBADN3 was a gift from Dr. Philip Proteau) . pBADN3-*end*P (*Nde*I-*Hind*III) and pBADN3-*end*Q (*Nde*I-*Hind*III) were used to transform Top10 cells and induced by arabinose. Plasmid pBADN3-*end*P and pBADN3-*end*Q encode native proteins while plasmid pET28a-*end*P and pET28a-*end*Q encode N-terminal His_6-tagged recombinant proteins. No expression of *end*P or *end*Q was detected in the pBADN3/Top10 system. Plasmid pET28a-*end*P(*Nde*I-*Xho*I) and pET28a-*end*Q(*Nde*I-*Xho*I) were transformed into Rosetta (DE3) or BL21 (DE3) pLysS cells and induced with IPTG. Both cell lines contain extra copies of genes for rare *E. coli* tRNAs (AGA, AGG, AUA, CUA, GGA, CCC, and CGG) and allows for improved expression of GC rich genes. The Top10 cell line does not have rare tRNA genes which may have hindered *end*P and *end*Q expression. The expressions were very strong in pET28a/Rosetta (Figure 3.4) and pET28a/BL21 pLysS system. However most of the overexpressed proteins are in inclusion bodies, which is a common problem in over-expression of heterologous proteins. In order to get more soluble protein, expression was carried

under a series of different conditions (decrease incubation temperature, decrease or omit IPTG, replace IPTG by glucose). However, none of these approaches produced enough soluble proteins for purification. Western blot analysis showed that the His_6-tagged EndP and EndQ existed in the supernatant but only in very small amounts (Fig 3.5). Leaky expression (no IPTG induction) in Rosetta (DE3) and expression in BL21 (DE3) pLysS produced more soluble proteins. Non-specific binding dominates the purification process and results in unsuccessful purification even though we increase the culture volume.

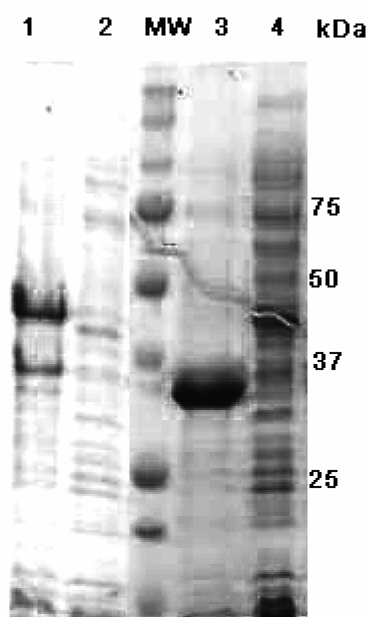


Figure 3.4 SDS-PAGE analysis of the EndP and EndQ overexpression in Rosetta DE3 cells with IPTG induction. Lane 1, pellet of Rosetta/pET28a-*end*Q; lane 2, supernatant of Rosetta/pET28a-*end*Q; lane 3, pellet of Rosetta/pET28a-*end*P; lane 4, supernatant of Rosetta/pET28a-*end*P. The calculated MW of His_6-EndP is 33.0 kDa, His_6-EndQ is 45.0 kDa.

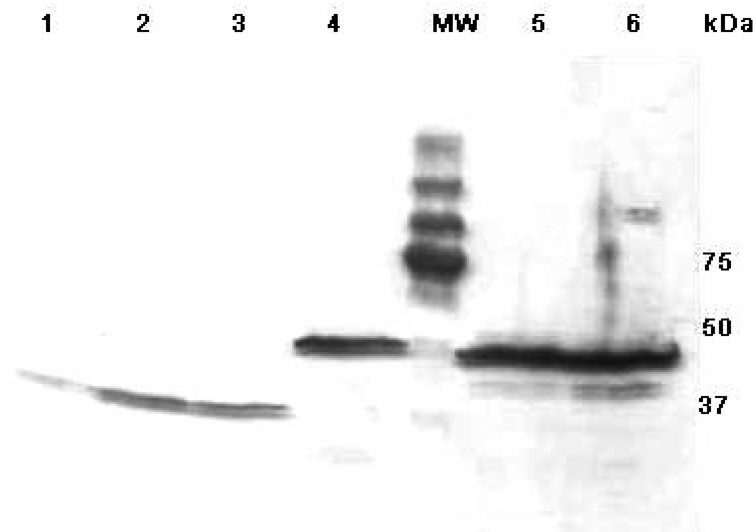


Figure 3.5 Western Blot analysis of soluble EndP and EndQ. lane 1, supernatant of Rosetta/pET28a-*end*P with IPTG induction; lane 2, supernatant of Rosetta/pET28a-*end*P without IPTG induction; Lane3, supernatant of BL21 pLysS/pET28a-*end*P with IPTG induction; lane 4, supernatant of Rosetta/pET28a-*end*Q with IPTG induction; lane 5, supernatant of Rosetta/pET28a-*end*Q without IPTG induction; lane 6, supernatant of BL21 pLysS/pET28a-*end*Q with IPTG induction. The calculated MW of His_6-EndP is 33.0 kDa, His_6-EndQ is 45.0 kDa. The MW of EndP and EndQ was determined by compare with another identical gel.

Attempts at Heterologous Expression of endP and endQ in S. lividans

Because expression in *E. coli* failed to produce enough soluble protein, we constructed pXY200-*end*P and pXY200-*end*Q for expression in *S. lividans*. pXY200 is an *E. coli*-*Streptomyces* shuttle plasmid (developed by Dr. Xihou Yin in our laboratory). Plasmid pXY200-*end*P and pXY200-*end*Q code for N-terminal His_6-tagged EndP and EndQ. Studies with pXY200-*end*P and pXY200-*end*Q to transform *S. lividans* by protoplast transformation are underway.

Expression of the endPQR operon in S. lividans

The inability to get a PCR product for *end*R and get adequate soluble EndP and EndQ in *E. coli* led us to take another approach to assign the function of these genes. When pXYFD16 was digested with *Eco*RI, a 4.1 kb fragment was obtained that contained the whole *end*PQR operon with part of *end*O and *end*S. This fragment was ligated into two *E. coli*-*Streptomyces* shuttle vectors, pSET152[28] and pXY248 (developed by Dr. Xihou Yin in our laboratory) and form pSET152-*end*PQR and pXY248-*end*PQR. Plasmid pSET152 integrates into the *Streptomyces* chromosome while pXY248 maintains high copy number in *Streptomyces* cells and ensures the high level expression of proteins. Both plasmids harbor an origin of transfer (oriT) site that is necessary to transfer DNA between bacteria. The plasmids were transformed into *E. coli* S17-1 cell which carries an integrated derivative of RP4, a mobilization element also necessary for conjugation[28, 29]. Then the plasmids were transformed to *S. lividans* by intergeneric conjugation.

From SDS-PAGE analysis it is unclear if EndP, EndQ and EndR have been expressed in *S. lividans*, although differences exist between the wilde type and transformant (Figure 3.6). LC-MS analysis of broth and mycelium of *S. lividans*/pXY248-*end*PQR and *S. lividans*/pSET152-*end*PQR did not detect enduracididine in either of the two transformants. We presume that *S. lividans* may not produce the necessary precursor of enduracididine, which may be a derivative of arginine.

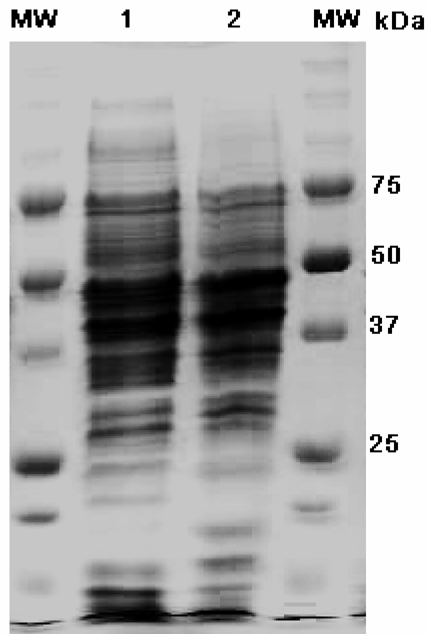


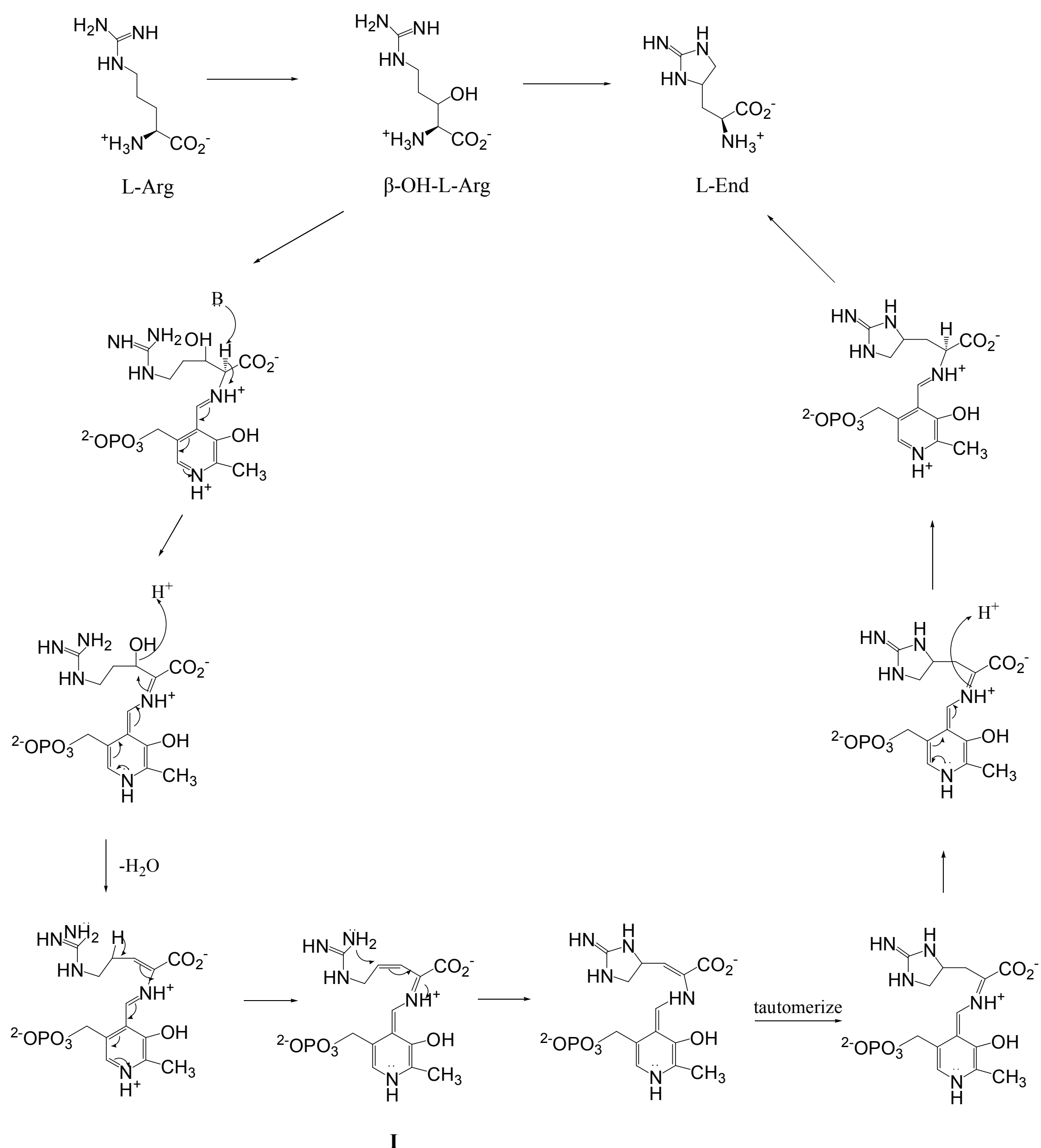
Figure 3.6 SDS-PAGE analysis of the *end*PQR expression in *S. lividans*. Lane 1, wild type S. lividans; lane 2, *S. lividans*/pXY248-*end*PQR. The calculated MW of EndP is 32.2 kDa, EndQ is 44.2 kDa, EndR is 29.5 kDa.

Expression of endPQR operon in S. fungicidicus

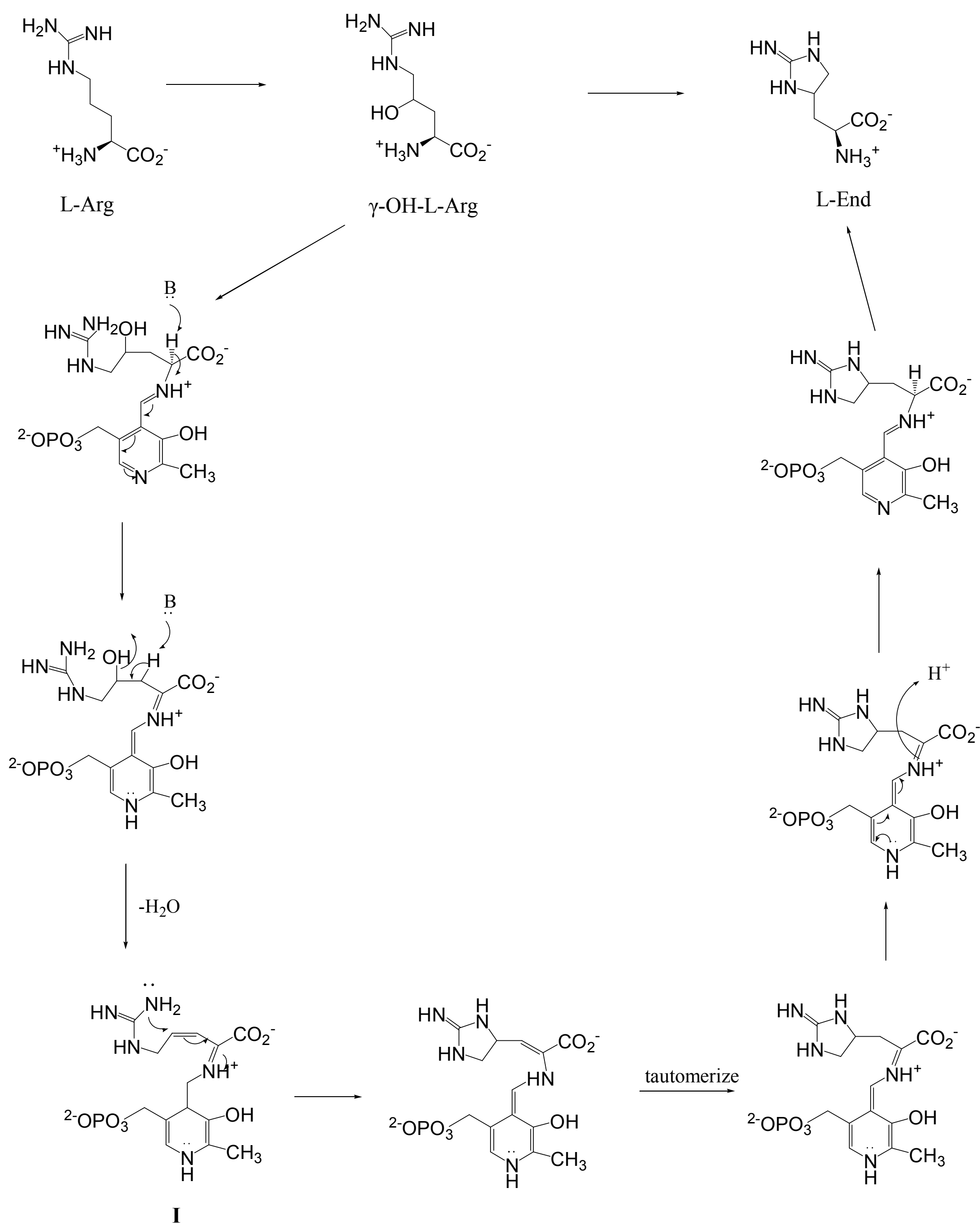
The results from introducing the *end*PQR operon into *S. lividans* led us to take an alternative approach. The operon was introduced into *S. fungicidicus*, the enduracidin producer to solve the apparent problem of precursor supply. Surprisingly, neither enduracididine nor enduracidin was detected in cultures of *S. fungicidicus*/pXY248-*end*PQR even though we detected these compounds in wild type cultures. The possible reason is that EndR may function as a negative regulatory protein. If EndR blocks the enduracididine biosynthesis pathway, this would result in significantly decreased levels of enduracididine and, consequently, the final product enduracidin.

Proposed Enduracididine Biosynthesis Pathway

Based on the high sequence similarity between *end*PQR and *mpp*PQR together with the Blast analysis and above experiment results, we propose that either β-OH-L-Arg or γ-OH-L-Arg could be the precursor of enduracididine. Either arginine derivative could serve as substrate for a PLP catalyzed cyclization by EndP or EndQ to form enduracididine. The key intermediate from both precursors is the conjugated imine species (I) in scheme 3.3 and 3.4 that can be attacked by the guanidine to form the enduracididine side chain.



Scheme 3.3 Proposed mechanism of L-End formation through β-OH-L-Arg. I, conjugated imine species.



Scheme 3.4 Proposed mechanism of L-End formation through γ-OH-L-Arg. I, conjugated imine species.

Materials and Methods

General

Routine molecular biology procedures including DNA manipulation, plasmid construction, transformation, growth and maintenances of *E. coli*, competent cell preparation and SDS-PAGE analysis were conducted according to standard techniques[30]. *S. lividans* and *S. fungicidicus* were maintained according to standard *Streptomyces* techniques[28]. Restriction enzymes, T4 DNA ligase and Taq polymerase were purchased from various suppliers and used following the manufacturer's protocol. All PCRs were conducted in an Eppendorf Mastercycler gradient PCR thermal cycler. QIAprep spin miniprep and QIAquick gel extraction kits (Qiagen, Valencia, CA) were used for DNA purification. Sequencing of vector inserts was conducted by the Center for Genome Research and Biocomputing at Oregon State University. Chemicals were purchased from Sigma-Aldrich, Fisher Scientific and VWR International.

Subcloning of endP, endQ, endR for Heterologous Expression

PCR primers were designed to amplify the *endP, endQ and endR* genes from fosmid pXYFD16 containing part of the enduracidin biosynthesis gene cluster (Xihou Yin, unpublished results). PCR was carried out in a total volume of 30 µl containing 10 ng template, 1× buffer A (AccurePrime™ GC-rich polymerase system, Invitrogen), 50 pmol forward primer and 50 pmol reverse primer, 5 units FastStart Taq DNA Polymerase (Roche), or in a total volume of 50 µl containing

10 ng template, 1× PCR buffer with $(NH_4)_2SO_4$ (Fermentas), 5% dimethyl sulfoxide (DMSO), 1.5-3.0 mM $MgCl_2$, 0.4 mM each dNTP together with the same primers and polymerase.

PCR products were gel purified and ligated into pGEM®-T easy vector (Promega). The correct construction was confirmed by sequencing and digestion by appropriate restriction enzymes. The targeted fragments were restricted with appropriate enzymes and ligated into expression vector.

Table3.1 PCR primers used to amplify *end*P, *end*Q and *end*R.

*end*P	*Nde*I(pf)	5'-GGCCATATGCTGAGCTGCTACTCCTGGT-3'
	*Hin*dIII(pr)	5'-AGCAAGCTTCAGCGGGGTGTCA-3'
*end*Q	*Nde*I(pf)	5'-CCGCATATGACCGCTGCCGGCGA-3'
	*Hin*dIII(pr)	5'-GGTAAGCTTCATGGGTTCCCTCCGA-3'
	*Xho*I(pr)	5'-TGGCTCGAGTGGGTTCCCTCCGAT-3'
*end*R	*Nde*I(pf)	5'-GAACATATGACGGCCACCACCGGGCA-3'
	*Xho*I(pr)	5'-CGCACTCGAGCCCCCAGAGGAC-3'
	*Hin*dIII(pr)[a]	5'-TGAAAGCTTCGTCGTCGCGGCGGTTC-3'

Restriction enzyme sites are underlined. a: reverse primer corresponding to downstream of stop codon.

Expression and purification of EndP and EndQ in E. coli

The plasmids pBADN3-*end*P and pBADN3-*end*Q were transformed into *E. coli* Top10 cells (Invitrogen). Single colonies were picked up and used to inoculate 5 ml seed culture of LB medium containing ampicillin100 µg/ml. After incubation at 37 ºC overnight, the seed cultures were used to inoculate 50 ml LB containing ampicillin100 µg/ml. Cells were grown at 37 ºC until the A_{600}=0.6-0.9, then arabinose was added to the medium to a final concentration of 2%. Cells were cultured for an additional 3-5 hours at 37 ºC and harvested by centrifugation at

3000 ×g for 10 min at 4 ºC, and washed with TWB (Talon Wash Buffer, 50 mM NaH_2PO_4, 300 mM NaCl, pH 7.5). The expression level was monitored by SDS-PAGE.

Plasmid pET28a-*end*P and pET28a-*end*Q were transformed into *E. coli* Rosetta (DE3) cells or *E. coli* BL21 (DE3) pLysS cells (Novagen). Single colonies were picked to inoculate 5 ml LB seed cultures containing kanamycin 50 μg/ml. After 37 ºC overnight culture, the seed cultures were used to inoculate 500 ml LB containing 50 μg/ml kanamycin. Cells were grown at 20 ºC until the A_{600}=0.6-0.9, then IPTG (isopropyl-β-D-thiogalactopyranoside) was added to the medium to a final concentration of 0.1 mM. Cells were cultured for an additional 3-5 hours at 20 ºC. For leaky expression, cells were grown at 20 ºC for 36 hours without adding IPTG. Cells were harvested by centrifugation at 3000 ×g for 10 min at 4 ºC, and washed with TWB. The precipitated cells were stored at -80 ºC. Frozen cells were thawed on ice, resuspended with TWB and lysed by sonication in a Microson ultrasonic cell disruptor (six 10 s bursts at 6 watts output with 10 s cooling on ice between bursts). The lysate was centrifuged at 18,000 ×g for 30 minutes at 4 ºC to obtain a supernatant. The solubility of both proteins was monitored by SDS-PAGE and Western blot. The His_6-EndP and His_6-EndQ were purified with BD Talon™ Metal Affinity Resins (BD Biosciences) according to the manufacturer's directions. Efficiency of purification was checked by SDS-PAGE.

Protoplast transformation of S. lividans

Approximately $2x10^7$ *S. lividans TK24* spores were used to inoculate 300 ml YEME medium, containing 0.5% glycine, in a 2 L baffled flask. Protoplasts were prepared from 48 hours culture grown at 30 ºC and 300 rpm. Mycelia were harvested by centrifugation at 3000 rpm and 10 ºC for 10 min on a Beckman GPR swinging bucket centrifuge. Harvested mycelia were washed once in 10.3% sucrose and twice in Buffer P (10.3% sucrose, 44 mM K_2SO_4, 41 mM $MgCl_2$, 0.2% *Streptomyces* trace element solution, 0.005% KH_2PO_4, 0.368% $CaCl_2$, and 0.573% TES, pH 7.2). Mycelia were then resuspended in 50 ml sterile Buffer P supplemented with 1 mg/ml lysozyme (Sigma) and incubated at 30 ºC for 20-40 min. Incubation times varied and protoplast formation was monitored by microscopic examination. After the majority of mycelia had been converted to protoplasts, the mycelia were passed through a sterile protoplast filter on ice. Protoplasts were gently pelleted from the filtrate by centrifuging for 10 min at 10 °C and 2500 rpm. Harvested protoplasts were washed twice by gently resuspending in 10 ml 4 °C Buffer P followed by centrifugation and removal of supernatant. Finally, protoplasts were resuspended in 1.2 ml 4 °C Buffer P and 120 µl aliquots were transferred to sterile 1.5 ml microcentrifuge tubes. Protoplasts were frozen slowly by first placing in fresh ice at 20 °C overnight and were then transferred to a -80 °C freezer, still in ice, to allow for slow freezing. Before use, protoplasts were thawed rapidly by placing tubes into running warm water.

Protoplasts were transformed using the method of Hopwood *et al*[28]. In a 1.5 ml microcentrifuge tube, 5 µl pXY200-*end*P or pXY200-*end*Q was combined with

120 μl protoplasts at room temperature followed by adding 250 μl 25% PEG1000 in Buffer P. The solution was mixed gently. Following incubation at room temperature for 2 min, 250 μl Buffer P was added quickly to the protoplasts. The solution was transferred to a 12×75 mm sterile culture tube. An additional 3.5 ml Buffer P was added and the suspension was mixed gently. Protoplasts were harvested by centrifugation at 10 °C for 7 min at 2500 rpm. The supernatant was removed and the protoplasts were resuspended in 600 μl Buffer P and plated on two R2YE plates. The plates were incubated at 30 °C for 18-24 h. On the second day, soft nutrient agar containing appropriate selection antibiotic was added to the plates. The plates were then incubated at 30 °C for several days to one week to allow transformants to grow.

Cloning and Expression of the endPQR fragment in S. lividans and S. fungicidicus

The *Eco*RI fragment that contains the whole *end*PQR operon was excised from pXYFD16 and gel purified. The 4.1 kb fragment was ligated with *Eco*RI cut pSET152 or pXY248. The latter plasmid was treated with alkaline phosphatase (Invitrogen) prior to ligation. Correct plasmid construction was confirmed by *Eco*RI digestion. Plasmids pSET152-*end*PQR and pXY248-*end*PQR were transformed to S 17-1 *E. coli* cells and then transformed into *S. lividans* or *S. fungicidicus* by conjugation. *S. lividans*/pSET152-*end*PQR and *S. lividans*/pXY248-*end*PQR were grown in liquid TSB medium containing thiostrepton 5 μg/ml. *S. fungicidicus*/pXY248-*end*PQR was first grown in 50 ml TSB seed culture, then the seed culture was inoculated into enduracidin production

medium (3% corn steep liquor, 1% soy bean flour, 0.5% NaCl, 1% $CaCO_3$, 2% Glucose, 3% soluble starch, pH 7.0).The protein expression in *S. lividans*/pXY248-*end*PQR and *S. fungicidicus*/pXY248-*end*PQR was monitored by SDS-PAGE.

Transformation of S. lividans and S. fungicidicus by conjugation

Transformation by conjugation was conducted according to methods of Hopwood[28]. Plasmid pSET152-*end*PQR and pXY248-*end*PQR were used to transform *E. coli* S 17-1 competent cells. Single colonies were picked and used to inoculate 5 ml LB medium containing appropriate antibiotics and incubated overnight in a shaker at 37 °C and 250 rpm. The overnight cultures were diluted 10 times to inoculate 5 ml LB medium containing appropriate antibiotics. Cells were grown at 37 °C until A_{600}=0.6 then harvested by centrifugation at 4 °C and 3500 rpm for 5 minutes. Cells were washed twice with 2 ml 2×YT medium and resuspended in 0.2 m 2×YT medium. Cells were kept on ice until preparation of spores was complete.

Fresh *S. lividans* or *S. fungicidicus* spores were harvested by adding 5 ml ddH_2O onto plates and then scratching with pipet tips. The spore suspension was removed by pipeting and centrifuged for 5 minutes at 3500 rpm. The pellet was washed with 5 ml 0.05M TES pH 8.0 and resuspended in 0.5 ml TES pH 8.0. The spores were incubated at 50 °C for 10 min then cooled to room temperature by tap water. The spore suspension was then added into 2 ml of 2x pregermination buffer (1% Difco yeast extract, 1% casaminoacids, and 10 mM $CaCl_2$) and incubated in a shaker at

37 °C and 300 rpm for 3 hours. The spores were votexed once per hour to keep the spores suspended during incubation. After pregermination, spores were harvested by centrifugation at 3000 rpm for 10 minutes and resuspended in 1 ml 2×YT.

Different volumes of spores (1 μl, 5 μl, 50 μl and 100 μl) were added to prepared *E. coli* cells and plated on AS-1 (1 g/L yeast extract, 0.2 g/L L-alanine, 0.2 g/L L-arginine, 0.5 g/L L-asparagine, 5 g/L dextrin, 2.5 g/L NaCl, 10 g/L Na_2SO_4 and 20 g/L agar) or ISP4 plates. After incubation at 30 °C for 16-20 hours, soft nutrient agar with nalidixic acid and appropriate antibiotic was added on top of the plates. Plates were incubated at 30 °C for one to two weeks.

HPLC-MS Analysis of Transformants Metabolites

Wet mycelia from 500 ml cultures were washed with ddH_2O and centrifuged at 2,000 ×g for 15 min. Cell pellet was resuspended in 300 ml of 70% methanol and sonicated at 18 watts for 3 min. The extraction continued for 2 hr by continuous stirring using an orbital shaker at 230 rpm at room temperature. After 2 hr, the sample was centrifuged at 2,000 ×g for 20 min. The supernatant was recovered and reduced to almost dryness by rotary evaporation at 37 ^oC. The contents left in the flask were resuspended in 10 ml of ddH_2O and the solution was centrifuged at 4,500 ×g for 10 min. The supernatant was used for dansyl chloride derivatization according to the protocol described in Chapter 2.

The dansylated samples were filtered through a 0.45 μm syringe filter before being injected on to the HPLC-MS column. Reversed phase HPLC analysis (Gemini 5 μm, 150 × 4.6 mm I.D. with guard column) was performed on a

ThermoFinnigan LCQ Advantage LC-MS system, consisting of a solvent pump, an autosampler, a PDA detector and an ion trap mass spectrometer detector. The system was controlled by a PC running Xcalibur 1.3 software. Isocratic elution was carried out using 30% acetonitrile - 70% 50mM ammonium acetate, pH 5.5 at a flow rate of 0.8 ml/min. The injection volume was 20 µl. The effluent was monitored at 254 nm and meanwhile scanned from 200 nm to 350 nm with the PDA detector.

Positive ion electrospray ionization (ESI+) was used for MS detection. The capillary temperature was 300 ^{o}C and the capillary voltage was 15 Volts. The sheath gas flow was 60 units. Mass range from m/z 150 to 500 was scanned in full scan mode.

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Chapter 4

Conclusions

Combating multidrug resistance bacteria is an important issue throughout the world. It calls for scientists to discover new antibiotics and antibiotic targets and to modify existing antibiotics. Research on antibiotic biosynthesis pathways is also helping to achieve the latter goal. Nonproteinogenic amino acids play an important role in nonribosomal peptide antibiotic diversity and activity. In this thesis, we studied the formation and modification of the nonproteinogenic amino acid enduracididine (End), which exists in two important antibiotics, mannopeptimycins (MPPs) and enduracidin.

MppO was characterized *in vitro* and shown to catalyze the β-hydroxylation of L-End. Sequence analysis revealed that the gene product of *mpp*O belongs to the His-3 variant of non-heme iron, α-ketoglutarate dependent oxygenase superfamily. The *mpp*O gene was subcloned and heterologously expressed in *E. coli*. Enzyme activity assays showed that MppO stereospecifically catalyzes β-hydroxylation of L-End and results in the formation of 3S-hydroxy-L-End. MppO is the first known enzyme that catalyzes the β-hydroxylation of a nonproteinogenic amino acid.

Enduracididine formation was studied in the enduracidin biosynthesis pathway. Three genes in the End cluster, *end*P, *end*Q and *end*R, exists in one operon and their gene products are predicted to be involved in the formation of L-End. The prediction is primarily based on their sequence similarity with gene products from

the mannopeptimycin pathway. The gene products of *end*P and *end*Q are proposed to be PLP-dependent enzymes. These genes were subcloned and expressed in *E. coli*, however, both recombinant proteins are mostly insoluble. Their expression in *S. lividans* is underway. A fragment containing the whole *end*PQR operon was introduced in *S. lividans* and *S. fungicidicus*. However, no detectable enduracididine or increased enduracidin production was observed. Two possible mechanisms of enduracididine formation were proposed with β-OH-L-Arg or γ-OH-L-Arg as precursor, respectively.

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