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Consecutive Enzymatic Modification of Ornithine Generates the Hydroxamate Moieties of the Siderophore Erythrochelin

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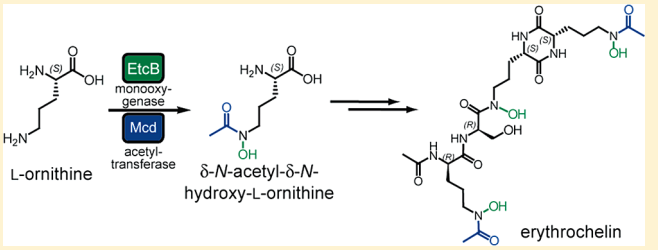
Supporting Information

ABSTRACT: Biosynthesis of the hydroxamate-type siderophore erythrochelin requires the generation of δ-*N*-acetyl-δ-*N*-hydroxy-L-ornithine (L-haOrn), which is incorporated into the tetrapeptide at positions 1 and 4. Bioinformatic analysis revealed the FAD-dependent monooxygenase EtcB and the bifunctional malonyl-CoA decarboxylase/acetyltransferase Mcd to be putatively involved in the generation of L-haOrn. To investigate if EtcB and Mcd constitute a two-enzyme pathway for the biosynthesis of L-haOrn, they were produced in a recombinant manner and subjected to biochemical studies *in vitro*. Hydroxylation assays employing recombinant EtcB gave rise to δ-*N*-hydroxy-L-ornithine (L-hOrn) and confirmed the enzyme to be involved in building block assembly. Acetylation assays were carried out by incubating L-hOrn with recombinant Mcd and malonyl-CoA as the acetyl group donor. Substrate turnover was increased by substituting malonyl-CoA with acetyl-CoA, bypassing the decarboxylation reaction which represents the rate-limiting step. Consecutive enzymatic synthesis of L-haOrn was accomplished in coupled assays employing both the L-ornithine hydroxylase and Mcd. In summary, a biosynthetic route for the generation of δ-*N*-acetyl-δ-*N*-hydroxy-L-ornithine starting from L-ornithine has been established *in vitro* by tandem action of the FAD-dependent monooxygenase EtcB and the bifunctional malonyl-CoA decarboxylase/acetyltransferase Mcd.



Siderophores are a class of structurally diverse natural products that bind iron extracellularly with high affinities. After reimportation into the cells, iron is released from the chelator complex and subsequently channeled to the intracellular targets.[1−3] Siderophores are assembled by either nonribosomal peptide synthetases (NRPSs) (e.g., erythrochelin) or NRPS-independent (NIS) (e.g., aerobactin). Characteristics of aerobactin that is produced by *Escherichia coli* K-12 are hydroxylated and acetylated lysine residues. Therefore, this natural product belongs to the class of so-called hydroxamate-type siderophores (Figure 1).[4,5] Biosynthesis of aerobactin involves the FAD-dependent monooxygenase IucD, which catalyzes the hydroxylation of the ε-amino group of L-lysine as the first step. The following acetylation reaction is mediated by the acetyltransferase IucB to generate *N*-ε-hydroxy-*N*-ε-acetyl-L-lysine (L-haLys) whose hydroxamate functionalities are crucial for biological activity. As the last steps in biosynthesis, citric acid is subsequently condensed with two L-haLys residues by the NIS synthetases IucA and IucC (Figure S1).[5,6] Another example of hydroxamate-type siderophores is erythrochelin from *Saccharopolyspora erythraea* (Figure 1).[7−9] The *etc* gene cluster is one of seven NRPS-containing gene clusters in *S. erythraea*, and it has been shown by specific deletion of the NRPS-gene *etc*D that this gene cluster encodes the proteins needed for erythrochelin production.[9,10] The *etc* gene cluster contains 11 coding sequences that encode among others typical transporter elements and one tetramodular NRPS EtcD. Each module contains the essential condensation (C), adenylation (A), and peptidyl carrier protein (PCP) domains. In addition, two epimerization (E) domains are located within modules 1 and 2, and the *C*-terminal module contains a C-domain instead of a thioesterase domain commonly responsible for product release. A model for erythrochelin biosynthesis assumes that it follows a linear enzymatic logic, in which each module of EtcD is responsible for recognition, activation, and incorporation of one building block. Erythrochelin is composed of α-*N*-acetyl-δ-*N*-acetyl-δ-*N*-hydroxy-D-ornithine$_1$, D-serine$_2$, δ-*N*-hydroxy-L-ornithine$_3$ (L-hOrn), and δ-*N*-acetyl-δ-*N*-hydroxy-L-ornithine$_4$ (L-haOrn), whereas L-haOrn is also activated by the A-domain of module 1, and the following α-*N*-hydroxylation is likely to be carried out by the C$_1$-domain located at the *N*-terminus of EtcD. After peptide assembly, it is proposed that C$_5$-domain catalyzed cyclorelease of erythrochelin takes place through diketopiperazine (DKP) formation of L-hOrn$_3$ and L-haOrn$_4$.[8]

The hydroxamate moieties are crucial for iron coordination, and therefore the evaluation of building block biosynthesis starting with L-ornithine (L-Orn) is of great interest. The gene product of *etc*B, which is located upstream of *etc*D, shares significant sequence identity with FAD- and NADPH-dependent monooxygenases like CchB from *Streptomyces coelicolor*, IucD

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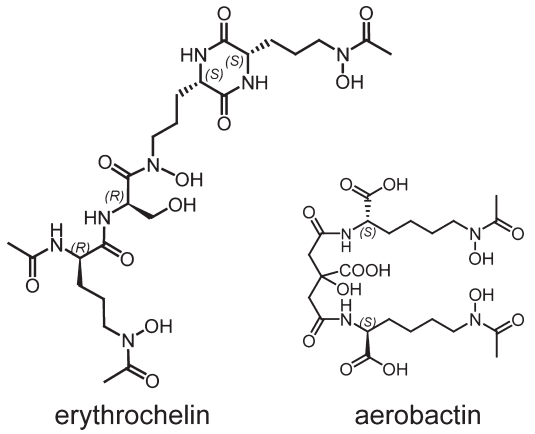

Figure 1. Chemical structures of the siderophores erythrochelin and aerobactin that contain δ-*N*-acetylated residues.

from *Escherichia coli*, or VbsO from *Rhizobium leguminosarum*. All these enzymes catalyze *N*-hydroxylation in the presence of molecular oxygen of an ornithine or lysine side chain to generate hydroxylated compounds.[5,11,12] This leads to the assumption that EtcB is involved in δ-*N*-hydroxylation of ornithine during erythrochelin biosynthesis.

Interestingly, the *etc* gene cluster does not contain a gene encoding an *N*-acetyltransferase required for the biosynthesis of L-haOrn as it is the case in coelichelin assembly in *Streptomyces coelicolor* where the formyltransferase CchA is integrated into the biosynthetic gene cluster.[13] Therefore, a BLAST search of the complete genome sequence of *S. erythraea* was made using the IucB protein sequence, as this enzyme is a member of the *N*-acetyltransferase protein family often found in the biosynthetic gene clusters of NIS.[9,14,15] The only significant hit was the *mcd* gene that is located in the gene cluster *nrps1* nearly 2 Mbp away from the *etc* cluster on the chromosome.[9] The *mcd* gene was disrupted in wild-type *S. erythraea*, and erythrochelin production can be fully restored by the addition of chemically synthesized L-haOrn.[9,16] This result excludes online δ-*N*-hydroxylation and δ-*N*-acetylation of the NRPS-bound substrates as seen in the hydroxylation of PCP-bound glutamic acid in kutzneride biosynthesis.[17] It has been shown previously that the biotin-independent decarboxylation of malonyl-coenzyme A (m-CoA) by the bidomain protein McD is crucial for the production of the siderophore erythromycin in *S. erythraea*.[10,18] The sequence of the C-terminal domain of Mcd (residues 243−417) resembles that of the GCN5-like acetyltransferase (GNAT) domain found in the loading module of some type I polyketide synthases (PKSs) (Figure S2A).[19,20] The loading module of the polyketide synthase of curacin A from *Lyngbya majuscule* contains for example a $GNAT_L$ domain that bears bifunctional decarboxylase/*S*-acetyltransferase activity, and a similar loading module also occurs in the biosynthetic pathway for onnamide A from *Theonella swinhoei* (Figure S2B,C).[19−21]

Concerning the proposed biosynthesis of the erythrochelin building block L-haOrn, two mechanistic routes are conceivable. One pathway describes the so-called "hydroxylation-first" mechanism in which hydroxylation of L-Orn by the FAD-dependent monooxygenase EtcB precedes acetylation by Mcd. Contrary to this, another biosynthesis model describes the "acetylation-first" mechanism. L-Orn is acetylated by the action of the *N*-acetyltransferase Mcd, and the second tailoring enzyme EtcB uses δ-*N*-acetyl-L-ornithine as substrate (Figure S3).

In this study, we produced the FAD-dependent monooxygenase EtcB and the bifunctional malonyl-CoA decarboxylase/acetyltransferase Mcd as recombinant proteins and performed biochemical characterizations *in vitro*. The substrate specificities of the enzymes were investigated, and kinetic parameters for the L-Orn modification reactions were determined. With the help of the obtained results we could confirm the proposed functions of EtcB and Mcd. Additionally, we were able to establish a consecutive enzymatic synthesis of L-haOrn as a building block for erythrochelin assembly.

EXPERIMENTAL PROCEDURES

Preparation of Genomic DNA. Liquid cell cultures of *S. erythraea* (5 mL) were harvested by centrifugation (13 000 rpm, 3 min). Cell pellets were washed with water (1 mL) and resuspended in 500 μL of lysis buffer (100 mM TRIS, 50 mM EDTA, 1% (w/v) SDS, pH 8.0). Acid-washed glass beads were added to the suspension to obtain a final volume of 1.25 mL. The suspension was vortexed for 2 min, and the liquid was separated from the glass beads. Subsequently, 275 μL of 7 M ammonium acetate solution (pH 7.0) was added, and the solution was incubated for 5 min at 65 °C. After 5 min incubation on ice, 500 μL of chloroform was added, and the solution was shaken gently. Centrifugation (13 000 rpm, 5 min) allowed the transfer of the aqueous phase to a new reaction tube. The genomic DNA was precipitated by addition of 1 mL of isopropyl alcohol and incubation at RT for 5 min. The DNA pellet obtained by centrifugation (13 000 rpm, 5 min) was washed twice (200 μL, 70% EtOH (v/v)), dried for 5 min at RT, and solubilized in 200 μL of ddH_2O. Genomic DNA was stored at 4 °C.

Amplification of Genes and Construction of Expression Vectors. The genes *etcB* and *mcd* were amplified from genomic DNA of *S. erythraea* using the primers listed below (restriction sites are underlined):

5′ *etcB* (*Nde*I) GGAATTCCATATGACGCGGTATGACGACTCGAA

3′ *etcB* (*Hind*III) TTTTAAGCTTCTACTGCGCCACGCGCAGC

5′ *mcd* (*Nhe*I) TTTTTGCTAGCATGGCGGGCGACGTGGAACTC

3′ *mcd* (*Xho*I) TTTTTCTCGAGTCAGACCCGGGAGAAGACCATCAGA

The amplification was carried out using the Phusion High-Fidelity DNA Polymerase (Finnzyme) following the manufacturer's instructions for GC-rich DNA templates. The amplicons were digested using corresponding endonucleases (New England Biolabs) and ligated into the vectors pCB28a(+) (*etcB*) or pET28a(+) (*mcd*) following established protocols. After verification of DNA-fidelity via sequencing (GATC-Biotech), *E. coli* BL21 (DE3) (Invitrogen) were transformed with the plasmids via electroporation for heterologous production of the corresponding protein.

Heterologous Production of Recombinant Proteins. Expression of the constructs encoding either EtcB or Mcd was carried out by inoculating 500 mL of prewarmed (37 °C) LB medium, supplemented with kanamycin (50 μg/mL) in a 2 L baffled shaker flask with 5 mL of an overnight culture of the *E. coli* BL21 (DE3). The cells were grown to an optical density (OD) of 0.6 (λ = 600 nm) at 37 °C and 250 rpm, induced with 0.1 mM IPTG and cultivated for ∼18 h at 20 °C. The cells were harvested by centrifugation (6000 rpm, 4 °C, 30 min), resuspended in

40 mL of buffer (50 mM HEPES, 250 mM NaCl, pH 7.5), and either directly processed or stored at −20 °C.

Purification of Recombinant EtcB and Mcd. The purification of recombinant His_6-tagged proteins was initiated by disruption of the cells using either an EmulsiFlex-C5 high pressure homogenizer (Avestin) or a French pressure cell (SLM Aminco). Cell debris and insoluble components were precipitated by centrifugation (17 000 rpm, 4 °C, 30 min), the supernatant containing the protein was separated, and the protein was purified by Ni-NTA (Qiagen) affinity chromatography using a FPLC system 250 (Amersham Pharmacia Biotech). The column was equilibrated with buffer A (50 mM HEPES, 250 mM NaCl, pH 7.5). The supernatant was applied onto the column with a flow rate of 0.7 mL/min until the absorption of the flowthrough at 280 nm (A_{280}) reached baseline level. Selective elution of the immobilized protein was performed by application of following solvent gradient of buffer A and buffer B (50 mM HEPES, 250 mM NaCl, 250 mM imidazole, pH 7.5) at a flow rate of 1 mL/min: linear increase from 0% to 50% B within 35 min followed by a linear increase to 95% B in 5 min, holding B for an additional 5 min. The wavelength chosen for detection of the protein was 280 nm, and the fraction size was 2 mL. Protein-containing fractions were identified via qualitative Bradford assays and analyzed by SDS-PAGE.[22,23] Fractions containing the recombinant protein were combined, concentrated with Amicon Ultra-15 concentrators (Millipore), and subjected to buffer exchange utilizing HiTrap Desalting columns (GE Healthcare) and a dialysis buffer (20 mM TRIS, 100 mM NaCl, pH 7.2). Buffers for recombinant EtcB were supplemented with glycerol (20% (v/v), pH 8.0). Protein solutions were subsequently aliquoted, flash-frozen in liquid nitrogen, and stored at −80 °C.

Protein Concentration Determination. Protein concentrations were determined spectrophotometrically using calculated extinction coefficients after thawing of the corresponding protein solution. The coefficients were calculated by analysis of the primary sequence with Protean (DNAstar). The determination was carried out on a Nanodrop ND-1000 (PEQLab) spectrophotometer (λ = 280 nm). Absorption spectra of EtcB (50 μM) were recorded to analyze the cofactor content of the recombinant protein between 300 and 600 nm.

EtcB-Mediated Hydroxylation Assay. The FAD-dependent monooxygenase EtcB catalyzes the hydroxylation of the δ-amino functionality of L-Orn in presence of the cosubstrate NADPH. Hydroxylation assays were carried out by incubating recombinant EtcB (20 μM) with L-Orn (1 mM), the cosubstrate NADPH (1 mM), and the cofactor FAD (20 μM) in assay buffer (20 mM TRIS, 100 mM NaCl, pH 8.0) for 4−8 h at 25 °C. The reactions were stopped by addition of 10 μL of 4% (v/v) TFA. Hydroxylation assays were analyzed by reversed phase(RP)-LCMS (Agilent) on a Hypercarb 100 × 2.1 mm column (Thermo) utilizing the following solvent gradient of solvent A (20 mM NFPA) and solvent B (acetonitrile) at a flow rate of 0.4 mL/min: linear increase from 0% to 15% B within 20 min followed by a linear increase to 100% B in 2 min, holding B for an additional 5 min. The wavelength chosen for detection was 215 nm, and the column temperature was 20 °C. Substrate specificity assays were performed by employing the same conditions stated above with different amino acids as substrates and an incubation time of 8 h. Specificity assays were analyzed by RP-LCMS (Agilent) on a Nucleodur C_{18}(ec) 125 × 2 mm column (Macherey & Nagel) utilizing the following solvent gradient of water/0.1% TFA (solvent A) and acetonitrile/0.1% TFA (solvent B) at a flow rate of 0.2 mL/min: linear increase from 0% to 100% B within 30 min holding B for an additional 5 min. The column temperature was 45 °C. Reaction kinetics were determined spectrophotometrically on an Ultrospec 3100 pro spectrophotometer (Amersham Biosciences). Concentration of L-Orn was varied between 75 μM and 20 mM. Calculation of initial reaction rates was based on the decrease of NADPH absorbance (ε = 6300 M^{-1} cm^{-1}), and the determination of kinetic parameters was carried out with the Enzyme Kinetics Module for SigmaPlot 8.0 (Systat Software Inc.).

Mcd-Mediated Acetylation Assay. Acetylation of the δ-amino functionality of L-hOrn is mediated by the bifunctional malonyl-CoA decarboxylase/acetyltransferase Mcd, as shown via *in vitro*-acetylation assays. Acetylation assays were carried out by incubating recombinant Mcd (50 μM) with L-hOrn (1 mM) and the cosubstrates m-CoA (1 mM) or acetyl-CoA (ac-CoA) (1 mM) in assay buffer (20 mM TRIS, 100 mM NaCl, pH 7.2) for 4−8 h at 25 °C. The reaction was stopped by addition of 10 μL 4% (v/v) TFA. Acetylation assays were analyzed by RP-HPLC (Agilent) on a Hypercarb 100 × 2.1 mm column (Thermo) utilizing the following solvent gradient of solvent A (20 mM NFPA) and solvent B (acetonitrile) at a flow rate of 0.4 mL/min: linear increase from 0% to 15% B within 20 min followed by a linear increase to 100% B in 2 min, holding B for an additional 5 min. The wavelength chosen for detection was 215 nm, and the column temperature was 20 °C.

Substrate specificity studies employing alternative acyl donors were carried out and analyzed as described above by substituting m-CoA with methylmalonyl-CoA (mm-CoA) or acetoacetyl-CoA (aa-CoA) (1 mM each). Substrate specificity analysis of the acyl acceptor was performed by employing the same conditions stated above with different amino acids as substrates and an incubation time of 8 h. Specificity assays were analyzed by RP-LCMS (Agilent) on a Nucleodur C_{18}(ec) 125 × 2 mm column (Macherey & Nagel) with a column temperature of 45 °C utilizing the following solvent gradient of water/0.1% TFA (solvent A) and acetonitrile/0.1% TFA (solvent B) at a flow rate of 0.2 mL/min: linear increase from 0% to 100% B within 30 min holding B for an additional 5 min.

Kinetic parameters for acetylation of L-hOrn in the presence of m-CoA were determined by incubating 15 μM Mcd with 1.5 mM m-CoA and substrate concentrations between 25 and 600 μM in assay buffer (20 mM TRIS, 100 mM NaCl, pH 7.2) for 1 min at 25 °C. Additionally, the kinetic parameters were determined in presence of ac-CoA. For this purpose, 1 μM Mcd was incubated with 1.5 mM ac-CoA and substrate concentrations between 25 and 600 μM in assay buffer (20 mM TRIS, 100 mM NaCl, pH 7.2) for 3 min at 25 °C. After stopping the reactions by addition of 4% (v/v) trichloroacetic acid, they were analyzed by RP-LCMS (Agilent) on a Hypercarb 100 × 2.1 mm column (Thermo) using the conditions described above. On the basis of integration of the corresponding substrate and product signals, the initial reaction rates were determined and kinetic parameters were calculated with the help of the Enzyme Kinetics Module for SigmaPlot 8.0 (Systat Software Inc.).

Coupled Hydroxylation and Acetylation Assays. Coupled hydroxylation and acetylation assays were carried out to establish a biosynthetic route for the generation of L-haOrn. Coupled assays were conducted by incubating recombinant EtcB (20 μM), Mcd (50 μM), L-Orn (1 mM), the cosubstrates NADPH (1 mM) and ac-CoA (1 mM), and the cofactor FAD

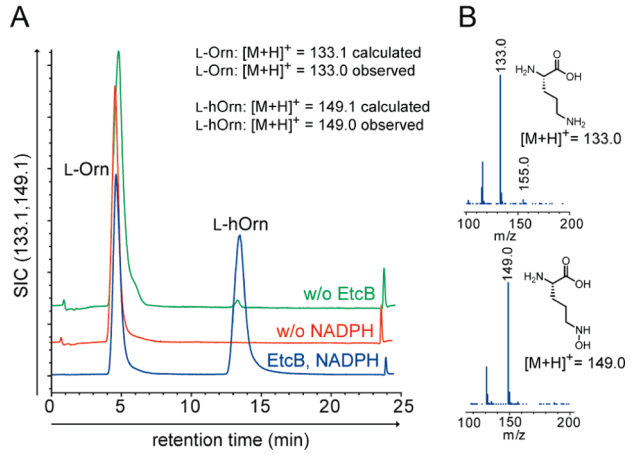

Figure 2. δ-*N*-Hydroxylation of L-Orn catalyzed by EtcB. (A) LCMS traces of the hydroxylation assays are shown as selected ion chromatograms (SIC). Incubation of the substrate L-Orn (t_R = 4.8 min) with EtcB and the cosubstrates gives rise to the product L-hOrn (t_R = 13.7 min) (blue trace). The control lacking the reducing cosubstrate NADPH is shown in red, and the control lacking EtcB is shown in green. The calculated and observed m/z ratios are given in the inset. (B) Chemical structures and the observed ESI-MS spectra of the substrate L-Orn (m/z = 133.0 $[M + H]^+$) and the product L-hOrn (m/z = 149.0 $[M + H]^+$).

(20 μM) in assay buffer (20 mM TRIS, 100 mM NaCl, pH 8.0) for 8 h at 25 °C. The reaction was quenched by addition of 10 μL of 4% (v/v) TFA. Product identities were analyzed by RP-HPLC (Agilent) on a Hypercarb column as mentioned above.

HRMS Analysis. High-resolution mass spectrometric (HRMS) analysis of products of enzymatic reactions was performed with an LTQ-FT instrument (Thermo Fisher Scientific) connected to a microbore Agilent 1100 HPLC system. The compounds were analyzed on a Nucleodur C_{18}(ec) 125 × 2 mm column utilizing the following solvent gradient: 0−30 min, 0%−100% acetonitrile into water, both supplemented with 0.1% TFA. The column temperature was 45 °C, and the flow rate was 0.3 mL/min.

RESULTS AND DISCUSSION

Biochemical Characterization of the Monooxygenase EtcB Catalyzing the First Step in the Generation of L-haOrn. Erythrochelin features three ornithine residues, of which two are δ-*N* acetylated and δ-*N* hydroxylated and one is solely δ-*N* hydroxylated. These groups contribute to the iron-chelating properties of erythrochelin and are involved in the octahedral coordination of ferric iron. Biosynthesis of the hydroxylated ornithine residue would require a monooxygenase mediating the δ-*N*-hydroxylation of L-Orn. Bioinformatic analysis of the *etc* gene cluster revealed the gene *etcB* to encode a FAD-dependent monooxygenase putatively involved in the generation of L-hOrn from L-Orn. Multiple sequence alignments of the enzyme with the characterized FAD- and NADPH-dependent monooxygenases CchB, IucD, PvdA, and VbsO confirmed EtcB to share a high degree of sequential identity to this class of enzymes, containing the three highly conserved motifs typical for these monooxygenases (Figure S4). EtcB itself displays 64% sequential identity and 78% similarity to the characterized FAD-dependent monooxygenase CchB from *S. coelicolor*, involved in coelichelin biosynthesis.[11]

To obtain insights into the formation of L-hOrn during erythrochelin biosynthesis, *etcB* was amplified from the chromosomal DNA of *S. erythraea* NRRL 23338 and the monooxygenase was heterologously produced and isolated as a *C*-terminally His_6-tagged *holo*-protein (Figure S5A). Final protein yield per liter culture after concentration and dialysis was 6.6 mg. UV/vis spectroscopic analysis confirmed the recombinant protein to be loaded with the proposed cofactor FAD (Figure S5B). To investigate if EtcB is catalyzing the δ-*N*-hydroxylation of L-Orn, it was incubated with the substrate L-Orn and the reducing cosubstrate NADPH in the presence of molecular oxygen. RP-LCMS analysis of the assays after 4 h revealed 50% conversion of L-Orn (t_R = 4.8 min, m/z = 133.0 $[M + H]^+$ observed, m/z = 133.1 $[M + H]^+$ calculated) to L-hOrn (t_R = 13.7 min, m/z = 149.0 $[M + H]^+$ observed, m/z = 149.1 $[M + H]^+$ calculated) in the presence of the enzyme (Figure 2). Substrate turnover could not be detected in the absence of EtcB or NADPH. HRMS analysis confirmed the product to be a hydroxylated ornithine species (m/z = 149.0922 $[M + H]^+$ observed, m/z = 149.0921 $[M + H]^+$ calculated). Determination of substrate conversion was based on the measured selected ion chromatogram (SIC) integrals and a calibration curve of L-hOrn. The identity of the product was confirmed by comparison with synthetically obtained L-hOrn.[24] The product of the enzymatic assay and the synthetic standard displayed the same retention times and MS spectra, when subjected to RP-LCMS analysis. Interestingly, the utilization of NADH instead of NADPH as the reducing cosubstrate did not lead to the formation of hOrn (Figure S6). This specificity toward the cofactor and the reducing cosubstrate has also been observed for CchB and PvdA, as both enzymes solely exhibit activity in the presence of FAD and NADPH.[11,25] In contrast, IucD mediates hydroxylation of L-Lys in the presence of FAD and NADPH or NADH as reducing cosubstrates.[26]

To evaluate the substrate specificity of EtcB, the recombinant enzyme was incubated with a set of alternative substrates representing the different classes of amino acids. In addition, the D-isomer of L-Orn was employed as well as α-*N*-acetyl-L-ornithine (L-acOrn) that represents a possible precursor of the α-*N*-acetyl-δ-*N*-acetyl-δ-*N*-hydroxy-L-ornithine$_1$ building block. Assays were carried out according to the conditions described in the Experimental Procedures section and were analyzed via RP-LCMS. Hydroxylation was exclusively limited to L-Orn as shown in Table S1. Similar results were obtained during the characterization of FAD-dependent monooxygenases involved in siderophore biosynthesis. IucD specifically hydroxylates L-Lys during aerobactin biosynthesis in *Escherichia coli*, whereas hydroxylation activity of CchB, of PvdA from *Pseudomonas aeruginosa*, or of VbsO during vicibactin biosynthesis is exclusively limited to L-Orn.[5,11,12,27,28]

Kinetic parameters for EtcB-catalyzed δ-*N*-hydroxylation of L-Orn were determined spectrophotometrically by measurement of the initial rates of the assay employing different substrate concentrations. The calculation of kinetic parameters was carried out with the Enzyme Kinetics Module for SigmaPlot 8.0 using Michalis−Menten and Lineweaver−Burk equations (Figure S7). The kinetic parameters for EtcB-mediated δ-*N*-hydroxylation of L-Orn were calculated to an apparent K_M of 0.286 ± 0.035 mM and k_{cat} = 19.6 ± 0.03 min^{-1}, leading to a catalytic efficiency of k_{cat}/K_M = 68.5 min^{-1} mM^{-1}. These parameters are given with the kinetic parameters of several other FAD-dependent monooxygenases for comparison in Table 1. The value for K_M with 0.286 ± 0.035 mM is for example in the range of the values

determined for VbsO, PvdA, and IucB but is lower than the K_M of CchB, being indicative of a higher affinity of EtcB toward the cognate substrate.

In summary, EtcB represents a typical member of FAD-dependent monooxygenases involved in the *δ*-*N*-hydroxylation of L-Orn or L-Lys, employing a common catalytic mechanism (Figure S8). Similar results were obtained for the FAD-dependent monooxygenase Sace_1309, encoded in the *nrps1* gene cluster (data not shown). Taking the results together, one can imply that EtcB can provide the L-hOrn building block required for NRPS-based erythrochelin assembly. L-hOrn itself represents a branching point in erythrochelin biosynthesis as it can be directly incorporated into the tetrapeptide siderophore at position 3 or be subjected to an additional tailoring step yielding L-haOrn.

Biochemical Characterization of the Bifunctional Enzyme Mcd. Bioinformatic analysis revealed the gene *mcd* to display 33% sequential identity and 49% sequential similarity to the *N*-acetyltransferase IucB, catalyzing the acetylation of *ε*-*N*-hydroxy-L-lysine during the biosynthesis of the mixed citrate/hydroxamate-type siderophore aerobactin.[5,9,30] As seen in multiple sequence alignments, Mcd also shows homology to other putative *N*-acyltransferases encoded in the biosynthetic operons for the production of mycobactin (MbtK, *Mycobacterium tuberculosis*) or vicibactin (VbsA, *Rhizobium leguminosarum*) (Figure S9).[28,31] Sequence analysis of Mcd revealed the enzyme to contain two putative activities: on the one hand, a m-CoA decarboxylation activity to afford ac-CoA and an acetyltransferase activity to catalyze the acetylation of L-Orn or L-hOrn. To investigate if Mcd catalyzes the decarboxylation of m-CoA and subsequent acetyltransfer, it was heterologously produced and isolated as a *C*-terminally His_6-tagged protein (Figure S10). Final protein yield per liter culture after concentration and dialysis was 9.6 mg.

Subsequently, it was incubated with the substrates L-Orn and m-CoA. RP-LCMS analysis of the assay after 8 h showed that L-Orn is not the cognate substrate for Mcd-mediated acetylation as no substrate turnover was observed. In contrast, incubation of Mcd with L-hOrn and m-CoA as the acetyl donor afforded acetylation of the substrate. RP-LCMS analysis of the assays after 4 h revealed 38% conversion of L-hOrn (t_R = 12.1 min,

Table 1. Kinetic Parameters of EtcB-Mediated Hydroxylation of L-Orn in Comparison with Kinetic Parameters of FAD-Dependent Monooxygenases Involved in Siderophore Biosynthesis

enzyme	substrate	K_M (mM)	k_{cat} (min^{-1})	k_{cat}/K_M (min^{-1} mM^{-1})
EtcB	L-Orn	0.286 ± 0.035	19.6 ± 0.03	68.5
CchB[11]	L-Orn	3.6 ± 0.58	17.4 ± 0.87	4.83
VbsO[12]	L-Orn	0.305 ± 0.024	108 ± 2	354.1
PvdA[27]	L-Orn	0.593 ± 0.012	26.4 ± 0.4	44.52
IucD[29]	L-Lys	0.11	n.d.[a]	n.d.[a]

[a] n.d.: not determined.

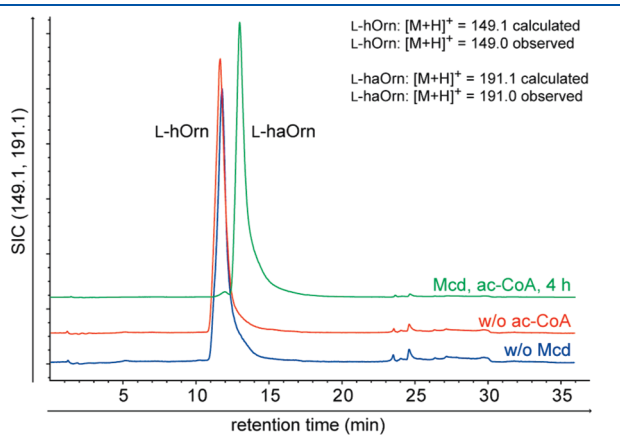

Figure 4. Mcd-mediated acetylation of L-hOrn. LCMS traces of the acetylation assays are shown as selected ion chromatograms (SIC). Incubation of the substrate L-hOrn (t_R = 12.1 min) with Mcd and ac-CoA gives rise to the product L-haOrn (t_R = 13.2 min) (green trace). The control lacking Mcd is shown in blue, and the control lacking ac-CoA is shown in red. The calculated and observed *m*/*z* ratios are given in the inset.

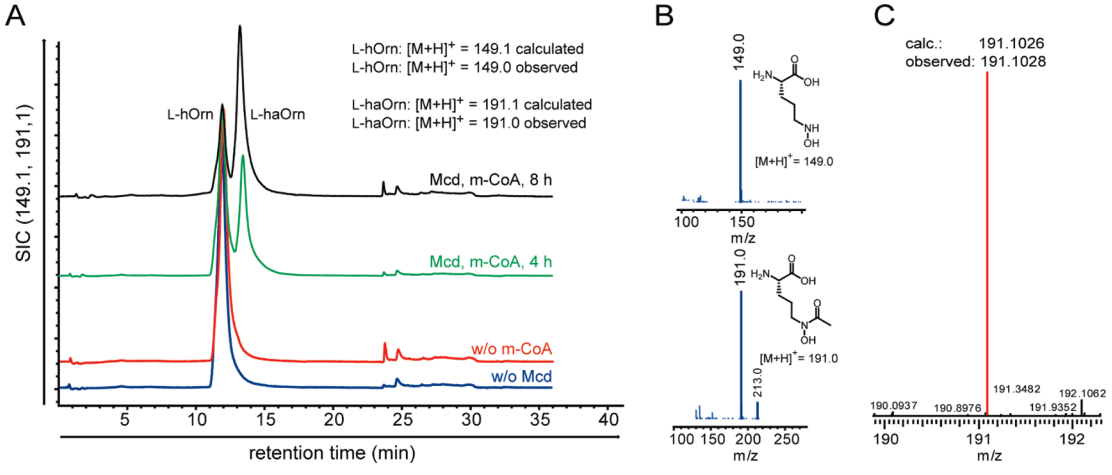

Figure 3. Mcd-mediated acetylation of L-hOrn. (A) LCMS traces of the acetylation assays are shown as selected ion chromatograms (SIC). Incubation of the substrate L-hOrn (t_R = 12.1 min) with Mcd and m-CoA gives rise to the product L-haOrn (t_R = 13.5 min, green and black trace). The control lacking Mcd is shown in blue and the control lacking m-CoA is shown in red. The calculated and observed *m*/*z*-ratios are given in the inset. (B) Chemical structures and the observed ESI-MS spectra of the substrate L-hOrn (*m*/*z* = 149.0 $[M + H]^+$) and the product L-haOrn (*m*/*z* = 191.0 $[M + H]^+$). (C) HRMS analysis of L-haOrn (*m*/*z* = 191.1028 $[M + H]^+$ observed, *m*/*z* = 191.1026 $[M + H]^+$ calculated).

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m/z = 149.0 $[M + H]^+$ observed, m/z = 149.1 $[M + H]^+$ calculated) to L-haOrn (t_R = 13.5 min, m/z = 191.0 $[M + H]^+$ observed, m/z = 191.1 $[M + H]^+$ calculated) in the presence of the enzyme (Figure 3A,B). Substrate conversion was increased to 79% after 8 h. Determination of substrate conversion was based on the measured SIC integrals and a calibration curve of L-hOrn. Substrate turnover could not be detected in the absence of Mcd or m-CoA. HRMS-analysis of the product confirmed it to be the acetylated hydroxyornithine species (m/z = 191.1028 $[M + H]^+$ observed, m/z = 191.1026 $[M + H]^+$ calculated) (Figure 3C).

Acetylation of the cognate substrate L-hOrn by Mcd requires the decarboxylation of m-CoA, which precedes the acetyltransfer. In addition, substrate channelling of the resulting ac-CoA from the decarboxylase domain to the acetyltransferase domain has to occur simultaneously to the binding of L-hOrn.[20] In order to circumvent the decarboxylation reaction and to enable direct acetyltransfer, ac-CoA was employed as an alternative acetyl group donor. Analysis of the assays revealed total substrate turnover after 4 h (Figure 4). Substrate turnover could not be detected in the absence of Mcd or ac-CoA. This result demonstrated Mcd to govern quantitative acetylation of L-hOrn in the presence of the acetyl group donor ac-CoA.

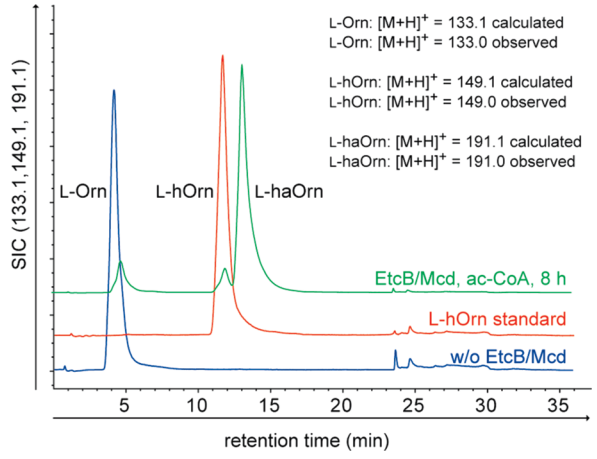

Figure 5. Consecutive enzymatic synthesis of L-haOrn. The LCMS traces of the coupled assays are shown as selected ion chromatograms (SIC). Incubation of the substrate L-Orn (t_R = 4.5 min) with EtcB, Mcd, and ac-CoA gives rise to the products L-hOrn (t_R = 12.2 min) and L-haOrn (t_R = 13.3 min) (green trace). Utilization of ac-CoA as acetyl group donor enhances the generation of L-haOrn, resulting in almost complete substrate turnover. The control lacking both enzymes is shown in blue, and the L-hOrn standard is shown in red. The calculated and observed m/z ratios are given in the inset.

As malonyl decarboxylation and subsequent substrate channeling appeared to be the rate-limiting step of L-hOrn acetylation, kinetic parameters for L-hOrn acetylation employing m-CoA or ac-CoA were determined by RP-LCMS analysis of assays using different substrate concentrations. The kinetic parameters were calculated assuming Michaelis−Menten behavior and using the Enzyme Kinetics Module for SigmaPlot 8.0 (Figure S11). The kinetic parameters of Mcd in presence of the cosubstrate m-CoA were determined to an apparent K_M of 0.025 ± 0.008 mM and a k_{cat} of 2.3 ± 0.15 min^{-1}, leading to a catalytic efficiency of k_{cat}/K_M = 92 min^{-1} mM^{-1}. Concerning the use of ac-CoA as cosubstrate for Mcd, the following kinetic parameters were calculated: an apparent K_M of 0.103 ± 0.033 mM^{-1}, a k_{cat} of 47.4 ± 5 min^{-1}, and a k_{cat}/K_M of 460 min^{-1} mM^{-1}.

Substrate specificity of Mcd regarding the acetyl group acceptor was evaluated by incubating the recombinant enzyme with a set of alternative substrates representing different classes of amino acids. Assays were carried out using both m-CoA and ac-CoA as the acetyl group donor and were analyzed via RP-LCMS. Acetylation was exclusively limited to L-hOrn as shown in Table S2. In addition, the substrate tolerance of Mcd toward the acyl group donor was evaluated *in vitro* by substituting m-CoA or ac-CoA with aa-CoA or mm-CoA. In both cases the formation of the corresponding acylated hydroxyornithine species could be observed via RP-HPLC analysis (Figure S12). The identities of *δ-N*-acetoacetyl-*δ-N*-hydroxy-L-ornithine (L-haaOrn) and *δ-N*-propionyl-*δ-N*-hydroxy-L-ornithine (L-hpOrn) were also confirmed via HRMS analysis (Figure S13).

Taking the results together, one can imply that Mcd exhibits a high degree of substrate specificity since acetylation was exclusively limited to L-hOrn. In contrast, Mcd displays promiscuity toward the acyl-group donor. *In vitro* characterization of CurA also demonstrated a high degree of substrate specificity toward the acetyl group acceptor.[20] Substitution of the naturally occurring ACP phosphopantetheine group by loading the CurA ACP with amino-CoA revealed only trace amounts of acetyl-NH-ACP. The determination of kinetic parameters for *S*-acetylation by CurA using m-CoA and ac-CoA revealed similar k_{cat} values for both substrates. In addition, the k_{cat} value for acetyl-group transfer was 780-fold lower than the k_{cat} for the decarboxylation of m-CoA, suggesting that decarboxylation and acetyltransfer are separated by conformational changes leading to an effective

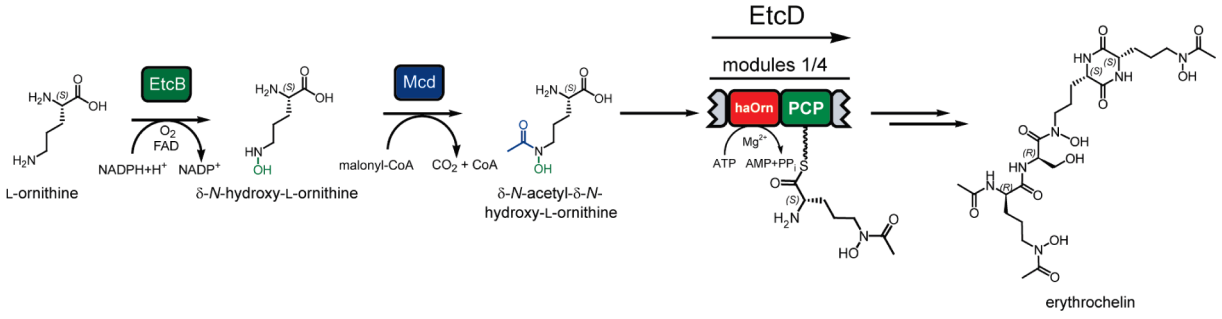

Figure 6. Summarizing overview of the biosynthesis of L-haOrn by tandem action of EtcB and Mcd and subsequent incorporation of the building block into erythrochelin. *δ-N*-Hydroxylation of L-Orn is mediated by the FAD-dependent monooxygenase EtcB giving rise to L-hOrn. Subsequent *δ-N*-acetylation is governed by the bifunctional malonyl-CoA decarboxylase/*N*-acetyltransferase Mcd. Selective A-domain-catalyzed activation of L-haOrn followed by covalent immobilization on the adjacent PCP ensures productive incorporation of the building block into the tetrapeptide siderophore erythrochelin.

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coordination of the ACP phosphopantetheine arm in the acceptor site.[20] Mcd itself is a GNAT-like protein catalyzing *N*-acetylation instead of *S*-acetylation. In contrast to CurA, the k_{cat} value of Mcd for acetylation of L-hOrn in presence of ac-CoA is 20-fold higher than the k_{cat} when m-CoA is provided. In addition, the catalytic efficiency of Mcd is 5-fold higher when ac-CoA is used as acetyl group donor. This leads to the conclusion that the decarboxylation of m-CoA and the subsequent substrate channeling are the rate-limiting steps of this acetylation reaction and that the enzyme is able to function more efficiently when ac-CoA is employed as donor substrate. Comparison of the kinetic parameters of Mcd with those of the *N*-acyltransferase VbsA from the vicibactin biosynthetic operon reveals kinetic parameters within the same range for both enzymes. VbsA has an apparent K_M of 0.018 ± 0.002 mM and a k_{cat} of 504 ± 12 min^{-1} for the acylation of L-hOrn in presence of ((*R*)-3-hydroxybutyryl)-CoA as acyl group donor. Interestingly, this enzyme is also able to acylate D-hOrn with the following kinetic parameters: an apparent K_M of 0.319 ± 47 mM and a k_{cat} of 1960 ± 98 min^{-1}.[12] However, VbsA is more efficient (k_{cat}/K_M of 28 000 min^{-1} mM^{-1} for acylation of L-hOrn) than Mcd, either in the presence of m-CoA or in the presence of ac-CoA.

Consecutive Enzymatic Synthesis of *δ*-*N*-Acetyl-*δ*-*N*-hydroxy-L-ornithine. Biosynthesis of the building block L-haOrn requires the tandem action of two distinct enzymes. In the biosynthetic scheme based on the results obtained it is postulated that the first step is L-Orn hydroxylation catalyzed by EtcB. This hydroxylated ornithine species is subsequently acetylated by Mcd, which utilizes either m-CoA or ac-CoA as the acetyl group donor. As acetylation of L-Orn could not be observed *in vitro*, the alternative biosynthetic scheme in which acetylation of ornithine precedes hydroxylation was ruled out. To establish a biosynthetic route for the generation of L-haOrn, a coupled assay employing both the FAD-dependent monooxygenase EtcB and the bifunctional malonyl-CoA decarboxylase/acetyltransferase Mcd was carried out. In these assays ac-CoA served as cosubstrate for Mcd-mediated acetyl transfer. RP-LCMS analysis of the assay with EtcB, Mcd, and ac-CoA revealed an almost quantitative conversion of L-Orn to L-hOrn and L-haOrn [L-Orn (t_R = 4.5 min, m/z = 133.0 $[M + H]^+$ observed, m/z = 133.1 $[M + H]^+$ calculated), L-hOrn (t_R = 12.2 min, m/z = 149.0 $[M + H]^+$ observed, m/z = 149.1 $[M + H]^+$ calculated), L-haOrn (t_R = 13.1 min, m/z = 191.0 $[M + H]^+$ observed, m/z = 191.1 $[M + H]^+$ calculated)] after 8 h (Figure 5).

In summary, it can be concluded that EtcB in combination with Mcd guarantees for the proliferation of L-haOrn, with EtcD A_1 and A_4 recognizing and activating the fully tailored building block to ensure erythrochelin production *in vivo* (Figure 6).

■ ASSOCIATED CONTENT

Supporting Information. The postulated pathway for aerobactin biosynthesis and the two mechanistic routes for the biosynthesis of L-haOrn; sequence comparisons of EtcB and Mcd with other members of the corresponding enzymes families; detailed information concerning the substrates used for enzyme activity determination and the putative mechanism of EtcB catalysis; additional LCMS chromatograms presented to complete the obtained results. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS

aa-CoA, acetoacetyl-coenzyme A; ac-CoA, acetyl-CoA; ACP, acyl carrier protein; A-domain, adenylation domain; C-domain, condensation domain; E-domain, epimerization domain; FAD, flavin adenine dinucleotide; GNAT, GCN5-like acetyltransferase; L-acOrn, α-*N*-acetyl-L-ornithine; L-haaOrn, *δ*-*N*-acetoacetyl-*δ*-*N*-hydroxy-L-ornithine; L-haLys, *ε*-*N*-acetyl-*ε*-*N*-hydroxy-L-lysine; L-haOrn, *δ*-*N*-acetyl-*δ*-*N*-hydroxy-L-ornithine; L-hOrn, *δ*-*N*-hydroxy-L-ornithine; L-hpOrn, *δ*-*N*-propionyl-*δ*-*N*-hydroxy-L-ornithine; m-CoA, malonyl-CoA; mm-CoA, methylmalonyl-CoA; NADH, nicotinamide adenine dinucleotide; NADPH, nicotinamide adenine dinucleotide phosphate; NIS, NRPS-independent siderophore; NRPS, nonribosomal peptide synthetase; PCP, peptidyl carrier protein; PKS, polyketide synthase.

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