Characterization of the metallo-dependent amidohydrolases responsible for “auxiliary” leucinyl removal in the biosynthesis of 2,2'-bipyridine antibiotics

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ABSTRACT

2,2'-Bipyridine (2,2'-BiPy) is an attractive core structure present in a number of biologically active natural products, including the structurally related antibiotics caerulomycins (CAEs) and collismycins (COLs). Their biosynthetic pathways share a similar key 2,2'-BiPy-L-leucine intermediate, which is desulfurated or sulfurated at C5, arises from a polyketide synthase/nonribosomal peptide synthetase hybrid assembly line. Focusing on the common off-line modification steps, we here report that the removal of the “auxiliary” L-leucine residue relies on the metallo-dependent amidohydrolase activity of CaeD or ColD. This activity leads to the production of similar 2,2'-BiPy carboxylate products that then receive an oxime functionality that is characteristic for both CAEs and COLs. Unlike many metallo-dependent amidohydrolase superfamily proteins that have been previously reported, these proteins (particularly CaeD) exhibited a strong zinc ion-binding capacity that was proven by site-specific mutagenesis studies to be essential to proteolytic activity. The kinetics of the conversions that respectively involve CaeD and ColD were analyzed, showing the differences in the efficiency and substrate specificity of these two proteins. These findings would generate interest in the metallo-dependent amidohydrolase superfamily proteins that are involved in the biosynthesis of bioactive natural products.

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1. Introduction

Polyketide synthases (PKSs) or non-ribosomal peptide synthetases (NRPSs) are large multimodular enzymes involved in biosynthesis of polyketides (PKs), non-ribosomal polypeptides (NRP)s and their hybrids. In general, modular PKSs or NRPSs appear to function as assembly lines, which program monomer polymerization/modification and chain tailoring/termination following a non-iteratively “one domain, one function” like co-linearity rule [1,2]. PKSs usually catalyze C–C bond formation using short carboxylic acids as monomers, whereas NRPSs incorporate amino acids through the formation of C–N bonds. Given the similarity in acyl transfer chemistry and catalytic logic, modular PKSs and NRPSs can be highly compatible. Their recombination results in a number of hybrid modular systems to incorporate both carboxylic acids and amino acids for production of various polyketide-peptide hybrid molecules, including the 2,2'-bipyridine natural products caerulomycins and collismycins in this study.

2,2'-Bipyridine (2,2'-BiPy) is an attractive core structure of a large class of chelating compounds that are able to form stable complexes with metal ions [3,4]. Several classes of biologically active natural products share this core structure, including caerulomycins (CAEs) [5–7], collismycins (COLs) [8], orellanine [9], camptothecin [10] and streptonigrinoids [11]. CAEs and COLs, the two groups of antibiotics that are structurally similar to each other, share the 2,2'-BiPy core structure in which a di or tri-substituted ring A conjugates an unmodified ring B. The major difference between them is located at the C5 of ring A, which has (for COLs) or
lacks (for CAEs) sulfur decoration (Fig. 1). Both CAEs and COLs exhibit a wide variety of biological properties, including antibacterial, antifungal and cytotoxic activities [7,8,12–18]. In addition, CAE-A can inhibit the mixed lymphocyte reaction, induce the generation of regulatory T cells, significantly suppress T cell responses, change the function of B cells and prolong the survival of allogeneic skin graft [19–22]. COL-A exhibits neuroprotective activity against oxidative stress in a zebrafish model [18,23,24].

Consistent with their high structural similarity, both CAEs and COLs are biosynthesized by an atypical hybrid PKS-NRPS system (Fig. 1a). This system serves as an assembly line to template the sequential incorporation of picolinic acid, malonyl-CoA, l-cysteine and l-leucine (l-Leu) monomers and programs the formation of a desulfurated (1, for CAEs) or sulfurated (2, for COLs) 2,2′-BiPy-l-leucine intermediate (Fig. 1b) [24–27]. Interestingly, isotope labeling experiments showed that the l-Leu is not involved in the atom construction of CAEs, but the gene caeA3/colA3 which is responsible for the up-load of l-Leu is essential and the intermediates 1 and 2 that are immediately individually off-load from the PKS-NRPS assembly lines contain an L-Leu residue [24–27]. This residue needs to be removed to release a carboxylate group, which is then tailored in several steps, including the reduction of the nascent carboxylate group to an aldehyde, transamination and the oxidation of the resulting terminal amino group to afford an oxime functionality that is common in both CAEs and COLs (Fig. 1b) [18,24,28–30]. In this study, we report that two metallo-dependent enzymes, CaeD from Actinoalloteichus cyanogriseus NRRL B-2194 and ColD from Streptomyces roseosporus NRRL 11379, perform the key reaction of removing the “redundant” Leu in the biosynthetic pathways of CAEs and COLs, respectively.

2. Material and methods

2.1. Materials, bacterial strains, plasmids and culture conditions

Chemicals and media were purchased from Sinopharm Chemical Reagent Co. Ltd. (China) or Oxoid Ltd. (UK) unless otherwise stated. Enzymes were purchased from Takara Biotechnology Co. Ltd. (China). The bacterial strains, primers and plasmids used in this study are summarized in Tables S1 and S2. Escherichia coli were grown at 37 °C in LB (1% tryptone (OXIOD), 0.5% yeast extract (OXIOD), and 1% NaCl) or on LB agar Miller (Biosharp, Japan). To select for plasmid-containing cells, 50 μg/mL ampicillin, 50 μg/mL kanamycin or 25 μg/mL chloromycetin were added to LB plates.

2.2. DNA isolation, manipulation and sequencing

DNA isolation and manipulation in E. coli, Actinoalloteichus and

![Fig. 1. a) Comparison of biosynthetic gene clusters of cae (from A. cueruleus B-2194) and col (from S. roseosporus NRRL 11379); b) Proposed routes in the biosynthesis of CAE-A and COL-A, respectively.](image-url)
Streptomyces strains were performed according to standard protocols [31,32]. PCR amplification was carried out in an Eppendorf AG 22331 thermal cycler (Eppendorf AG, Germany) using KOD FX or PrimeSTAR HS DNA polymerase for high-fidelity amplification. Primer synthesis and DNA sequencing were performed at Shanghai Sangon Biotech Co. Ltd. (China), and Shanghai Majorbio Biotech Co. Ltd. (China), respectively.

2.3. Sequence analysis

The proteins that were identified were compared with other known proteins in the databases using BLAST methods (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Amino acid sequence alignments were performed using the ClustalX program. Homology modeling of the CaeD structure was performed using the I-TASSER on-line server (http://zhanglab.ccmb.med.umich.edu/I-TASSER/).

2.4. Analytical methods

High-performance liquid chromatography (HPLC) was performed using Agilent 1200 and 1260 HPLC systems (Agilent Technologies Inc., USA). Electrospray ionization mass spectrometry (ESI-MS) was performed on a Thermo Fisher LTQ Fleet ESI-MS spectrometer (Thermo Fisher Scientific Inc., USA), and the data were analyzed using Thermo Xcalibur software. ESI-high resolution MS (ESI-HR-MS) analysis was performed on a 6230B Accurate-Mass TOF LC/MS System or a 6530 Accurate-Mass Q-TOF LC/MS System (Agilent Technologies Inc., USA), and the data were analyzed using Agilent MassHunter Qualitative Analysis software. Primer synthesis and DNA sequencing were performed at Shanghai Sangon Biotech Co. Ltd. (China), respectively.

The resulting plasmids were transformed into E. coli BL21 (DE3) cells that harbored the pGro7 (Takara) plasmid to increase yields. A 50 ml LB culture of E. coli was grown overnight with 25 μg/mL chloromycetin and 50 μg/mL kanamycin, diluted 100-fold in fresh medium and then incubated at 37 °C at 220 rpm for 3 h, which was followed by the addition of 0.5 mg/L l-arabinose (added when the OD600 reached 0.6). To induce protein expression, 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and 1 mM ZnCl2 were added into the culture 2 h later, and the cultures were incubated at 25 °C for 20 h. The cells were harvested via centrifugation and stored at −80 °C before lysis. The thawed cells were resuspended in lysis buffer (50 mM Tris-HCl, 300 mM NaCl, 2.5 mM imidazole, and 10% (v/v) glycerol, pH 8.0). After the cells were disrupted with an ultrahigh pressure homogenizer (FB-110X, Shanghai Litu Mechanical Equipment Engineering Co., Ltd, China or JN-02HC, JNBIO, China), the soluble fraction was collected, and the desired protein was purified using a HiTrap FF column (GE Healthcare, USA). The proteins were then dialyzed against buffer (50 mM Tris-HCl, 100 mM NaCl, 1 mM DTT, and 10% (v/v) glycerol, pH 7.5) and passed through a PD-10 Desalting Column (GE Healthcare, USA) according to the manufacturers’ protocols, to remove salts. The resulting proteins were concentrated and stored at −80 °C for in vitro assays. The purity of the proteins was examined via 10% SDS-PAGE analysis (Fig. S1), and the concentration was determined using a Bradford assay with bovine serum albumin (BSA) as the standard. The metal ion concentration of each protein sample was determined using a PE AAnalyst800 atomic absorption spectrometer (PerkinElmer, USA) (Table 1).

2.5. Plasmid construction for heterologous gene expression in E. coli

The caeD (1266 bp, from A. cyanogriseus NRRL B-2194) and colD (1242 bp, from the S. roseosporus NRRL 11379) genes were amplified using KOD FX DNA polymerase under the following conditions: 98 °C for 2 min and 35 cycles of 98 °C for 30 s, 60 °C for 10 s, and 68 °C for 2 min. After being digested by restriction enzymes, the caeD and colD gene fragments were inserted into pET-28a plasmids that had been digested with EcoRI and HindIII to construct pQL1071 and pQL1073, respectively.

2.6. Site-directed mutagenesis of CaeD

The plasmids pQL1074 containing a caeD variant (encoding an H61A mutation), pQL1075 (encoding an H63A mutation), pQL1076 (encoding an H227A mutation), and pQL1077 (encoding an H247A mutation), and pQL1078 (encoding a D317A mutation) were generated using PrimeSTAR HS DNA polymerase under the following conditions: 98 °C for 2 min and 20 cycles of 98 °C for 30 s, 60 °C for 10 s, and 68 °C for 7 min (using pQL1071 as the template). Then, 1 μL of DpnI enzyme was added to the PCR system to remove the template.

2.7. Protein expression and purification

The resulting plasmids were transformed into E. coli BL21 (DE3) cells that harbored the pGro7 (Takara) plasmid to increase yields. A 50 ml LB culture of E. coli was grown overnight with 25 μg/mL chloromycetin and 50 μg/mL kanamycin, diluted 100-fold in fresh medium and then incubated at 37 °C at 220 rpm for 3 h, which was followed by the addition of 0.5 mg/L l-arabinose (added when the OD600 reached 0.6). To induce protein expression, 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and 1 mM ZnCl2 were added into the culture 2 h later, and the cultures were incubated at 25 °C for 20 h. The cells were harvested via centrifugation and stored at −80 °C before lysis. The thawed cells were resuspended in lysis buffer (50 mM Tris-HCl, 300 mM NaCl, 2.5 mM imidazole, and 10% (v/v) glycerol, pH 8.0). After the cells were disrupted with an ultrahigh pressure homogenizer (FB-110X, Shanghai Litu Mechanical Equipment Engineering Co., Ltd, China or JN-02HC, JNBIO, China), the soluble fraction was collected, and the desired protein was purified using a HiTrap FF column (GE Healthcare, USA). The proteins were then dialyzed against buffer (50 mM Tris-HCl, 100 mM NaCl, 1 mM DTT, and 10% (v/v) glycerol, pH 7.5) and passed through a PD-10 Desalting Column (GE Healthcare, USA) according to the manufacturers’ protocols, to remove salts. The resulting proteins were concentrated and stored at −80 °C for in vitro assays. The purity of the proteins was examined via 10% SDS-PAGE analysis (Fig. S1), and the concentration was determined using a Bradford assay with bovine serum albumin (BSA) as the standard. The metal ion concentration of each protein sample was determined using a PE AAnalyst800 atomic absorption spectrometer (PerkinElmer, USA) (Table 1).

2.8. Biochemical assays of CaeD and ColD

The CaeD and ColD assays were performed at 30 °C in 50 mM Tris-HCl buffer (pH 8.0) that contained approximately 0.4 mM compound 1 (or 2) in the presence of 5 μM Caed (or ColD). After 2 h of reaction, an equal volume of MeCN was added into the assay to quench the reaction. After the supernatant was centrifuged (10 min at 12000 rpm), it was analyzed via HPLC or HPLC-ESI-MS on a Phenomenex column (Luna 5 μm C18 (2), 4.6 × 250 mm, Phenomenex, USA) and eluted at a flow rate of 1 mL/min over a 35 min gradient as follows: T = 0 min, 5% B; T = 2, 20% B; T = 7, 20% B; T = 17, 50% B; T = 22, 90% B; T = 27, 90% B; T = 30, 20% B; and T = 35, 20% B (solvent A, H2O + 0.1% HCOOH; solvent B, MeCN + 0.1% HCOOH). The absorbance of the products was detected at 315 nm.

2.9. Analysis of the kinetic properties of CaeD

The hydrolysis of 1 or 2 was detected by HPLC with monitoring at 315 nm to quantify the amount of 3 or 4 that was produced. The assay (final volume of 50 μL) was performed in a 1.5 mL Eppendorf tube that contained 50 mM Tris-HCl (pH 8.0) and 1 μM CaeD. The concentration of 1 or 2 was varied from 0.04 to 2 mM. After the reaction mixture was incubated at 30 °C for 1 min (1 as substrate) or 8 min (2 as substrate), an equal volume of MeCN was added to the assay to quench the reaction. After centrifugation (10 min at

| Proteins | Moles of metal ion per mole of proteins |
|----------|----------------------------------------|
|          | Zn²⁺ | Co²⁺ | Fe²⁺ | Ni²⁺ |
| WT       | 2.15 | –    | –    | –    |
| H61A     | 1.21 | –    | –    | –    |
| K186A    | 1.53 | –    | –    | –    |
| H227A    | 2.90 | –    | –    | –    |
| H247A    | 2.95 | –    | –    | –    |
| D317A    | 1.45 | –    | –    | –    |

The protein concentration was determined using a Bradford assay with bovine serum albumin as the standard, and the metal ion concentration of each protein sample was determined by inductively coupled plasma optical emission spectrometry (ICP-OES).

Table 1
Analysis of metal content of CaeD and its site-directed mutants.
2.10. Analysis of the kinetic properties of ColD

The kinetic parameters of ColD were determined using HPLC to quantify the amount of 3 or 4 that was produced. The assay (final column of 50 µL) was performed in a 1.5 mL quartz cuvette that contained 50 mM Tris-HCl (pH 8.0) and 1 µM ColD. The concentration of 1 or 2 was varied from 0.04 to 2 mM. After the reaction mixture was incubated at 30 °C for 20 min, an equal volume of MeCN was added to the assay to quench the reaction, which was then centrifuged (10 min at 12000 rpm); the absorption of the supernatant at 315 nm was measured via HPLC. The kinetic constants were calculated using a nonlinear regression fit to the Michaelis-Menten equation using GraphPad Prism 5 (Table 2, Fig. S3).

2.11. Chemical Synthesis of Intermediates 1 and 3 in the CAE biosynthetic pathway

These syntheses were performed according to a slightly modified version of a method that was previously described [26,33] (Fig. S2a). Compounds 1, 1–1 and 1–2 have been previously characterized [26,33].

2.11.1. Preparation of Synthetic Intermediate 1-1 (4-methoxy-2,2'-bipyridinyl N-oxide)

In total, 2.0 g of 4-nitro-2,2'-bipyridinyl N-oxide (9.2 mM, Langchem Inc., China) was suspended in 50 mL of MeOH, and 10 mL of sodium methoxide solution (5 M in MeOH) was slowly added into this MeOH suspension at 0 °C. The reaction mixture was stirred at 82 °C for 1 h. After the solvent was removed under reduced pressure at room temperature, 20 mL of H2O was added, and the resulting solution was subsequently extracted three times with 50 mL of CH2Cl2. The extracted organic fractions were combined, treated with anhydrous Na2SO4 overnight, filtered, and concentrated under reduced pressure to give compound 1-1 as a yellow solid (1.7 g, yield 93%). For the purified compound, 1H NMR (500 MHz, DMSO-d6) δ 8.88 (d, J = 8.1 Hz, 1H), 8.74 (dd, J = 3.8, 0.7 Hz, 1H), 8.25 (dd, J = 7.3 Hz, 1H), 7.93 (t, J = 7.7 Hz, 1H), 7.65 (d, J = 3.4 Hz, 1H), 7.49 (m, 1H), 7.11 (m, 1H), 3.88 (s, 3H). ESI-HRMS calcld. for C11H11N2O2 [M-H]−, found 203.0817.

2.11.2. Preparation of Synthetic Intermediate 1-2 (4-methoxy-2,2'-bipyridinyl-6-carbonitrile)

Compound 1-1 (1.7 g, 8.4 mM) was suspended in 17 mL of MeCN, and 1.4 mL of Et3N (10.1 mM) and 3.9 mL of PO(OEt)2CN (25.2 mM) were sequentially added to the MeCN suspension at room temperature. The reaction mixture was then stirred at 100 °C for 1.5 h before it was cooled to room temperature. The residue was filtered and then washed with 5 mL of MeCN at 0 °C, yielding compound 1-2 as a yellow, needle-like solid (1.3 g, yield 73%). For the purified compound, 1H NMR (500 MHz, DMSO-d6) δ 8.73 (d, J = 4.5 Hz, 1H), 8.34 (d, J = 7.9 Hz, 1H), 8.14 (d, J = 2.4 Hz, 1H), 7.99 (td, J = 7.8, 15.3 Hz, 1H), 7.78 (d, J = 2.4 Hz, 1H), 7.54 (dd, J = 7.1, 5.1 Hz, 1H), 4.00 (s, 3H). ESI-HRMS calcld. for C17H19N3O− 210.0824 [M-H]−, found 210.0818.

2.11.3. Preparation of Synthetic Intermediate 3 (4-hydroxy-2,2'-bipyridinyl-6-carboxylic acid hydrobromide)

Compound 1-2 (300 mg, 1.4 mM) was dissolved in 3 mL of 40% (wt/wt) aqueous hydrobromic acid at room temperature. The reaction mixture was stirred at 140 °C for 3 h, cooled and filtered. The resulting solid was washed with H2O (2 mL) and dried under reduced pressure at 70 °C to yield compound 3 as a white powder (430 mg, yield 95%). For the purified compound, 1H NMR (500 MHz, DMSO-d6) δ 8.95 (d, J = 5.1 Hz, 1H), 8.75 (d, J = 8.1 Hz, 1H), 8.51 (t, J = 7.7 Hz, 1H), 8.07 (d, J = 1.4 Hz, 1H), 7.99–7.91 (m, 1H), 7.64 (d, J = 1.3 Hz, 1H); and 13C NMR (125 MHz, DMSO-d6) δ 167.6, 164.7, 150.4, 148.9, 148.7, 145.3, 143.8, 126.8, 123.7, 113.6, 112.7. ESI-HRMS calcld. for C11H8N2O2 217.0613 [M+H]+, found 217.0609.

2.11.4. Chemical Synthesis of Intermediate 1

Fifty-six milligrams of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide dihydrochloride (EDCI, 0.29 mM), 44 mg of l-leucine methyl ester hydrochloride (0.24 mM), 40 mg of hydroxybenzotriazole (HOBT, 0.29 mM) and 240 µL of N,N-diisopropylethylamine (DIEA, 1.45 mM) were added to 50 mg of compound 3 that was suspended in 5 mL of anhydrous CH3Cl2. The reaction mixture was stirred at room temperature under an argon atmosphere for 48 h, and then washed with H2O and brine. The organic phase was dried over anhydrous Na2SO4 overnight, filtered and then concentrated to dryness under reduced pressure. The resulting crude was redissolved in 8 mL of THF and 2 mL of an aqueous solution containing 66 mg of LiOH·H2O (1.3 mM) and then stirred at 0 °C for 2 h. After the THF was removed and the pH was adjusted to 2 by the addition of 1 M HCl, the solution was extracted five times with 10 mL of MeTOAc. The combined organic phase was dried over anhydrous Na2SO4 overnight, filtered, concentrated under reduced pressure and then subjected to semi-preparative HPLC to give TFA-bound intermediate 1 as a yellow solid (48 mg). This semi-preparative HPLC was performed on an Agilent Zorbax column using a gradient elution of solvents A (H2O containing 0.1% TFA) and B (MeCN containing 0.1% TFA) at a flow rate of 3 mL/min over a 30-min period with the following program: t = 0 min, 5% B; t = 2.5% B; t = 20, 90% B; t = 25, 90% B; t = 26, 5% B and t = 30, 5% B (absorbance monitored at 254 nm). To remove the TFA, 10 mg of solid 1 was dissolved in 100 µL of MeCN. After this solution was mixed with 900 µL of 0.2 M phosphate buffer (pH 7.0), it was desalted via solid-phase extraction according to the previously described method [24]. For this purified compound, 1H NMR (500 MHz, DMSO-d6) δ 8.89 (d, J = 7.9 Hz, 1H), 8.67 (d, J = 4.0 Hz, 1H), 8.48 (d, J = 7.9 Hz, 1H), 7.96 (td, J = 7.7, 1.7 Hz, 1H), 7.91 (d, J = 2.4, 1H), 7.45 (d, J = 2.4 Hz, 1H), 7.44 (td, J = 4.8, 1.0 Hz, 1H), 4.24 (td, J = 7.9, 4.8 Hz, 1H), 1.76–1.57 (m, 2H), 0.92 (d, J = 6.0, 3.0 Hz), 0.89 (d, J = 6.2, 3.0 Hz). ESI-HRMS calcld. for C17H20N3O4 330.1454 [M+H]+, found 330.1450.

2.12. Chemical Synthesis of Intermediates 2 and 4 from the COL biosynthetic pathway

The synthesis was performed according to a slightly modified variant of the method that was previously described [26,33] (Fig. S2b). Compounds 4 and 2–2–2–6 have been previously characterized [18,24,33].

2.12.1. Preparation of Synthetic Intermediates 2-2 (sodium 4-methoxy-2,2'-bipyridinyl-6-carboxylate) and 2-3 (4-methoxy-2,2'-bipyridinyl-6-carboxylic acid diisopropylamide)

Compound 1-2 (1.2 g, 5.7 mM) was suspended in 7 mL of EtOH. To this solution at room temperature, 10 mL of a 10% aqueous NaOH solution was added. The resulting solution was stirred at 100 °C for 1.5 h and then cooled to 0 °C. After the solution was filtered, the residue was washed with 2.5 mL of EtOH and 1 mL of H2O and then dried under reduced pressure at 70 °C to give compound 2-2 as a white solid (1.25 g, yield 95%). ESI-HRMS calcld. for C17H19N3O4 231.0770 [M+H]+, found 231.0765. Then, 5.5 mL of thiouyl chloride was slowly added to 1.24 g of the obtained 2-2 (4.9 mM). After the
reaction mixture was stirred at 100 °C for 1.5 h, it was cooled and concentrated under reduced pressure. After the resulting solution was cooled to 0 °C, 12 mL of CH₂Cl₂ was added; then, 2.5 mL of HNPF₂ (17.7 mM) was added. After the suspension was stirred at room temperature for 16 h, it was diluted with 10 mL of H₂O and then extracted three times with 15 mL of CH₂Cl₂. The organic phase fractions were combined, dried over anhydrous Na₂SO₄ overnight, filtered and concentrated to dryness under reduced pressure to give compound 2–3 as a light-brown solid. (1.3 g, yield 85%). For purified 2–3, ¹H NMR (500 MHz, DMSO-d₆) δ 8.70 (ddd, J = 4.7, 1.8, 1H), 8.33 (d, J = 7.9 Hz, 1H), 7.97 (td, J = 7.7, 1.6 Hz, 1H), 7.94 (d, J = 2.4 Hz, 1H), 7.48 (ddd, J = 7.4, 4.9, 0.7 Hz, 1H), 7.04 (d, J = 2.4 Hz, 1H), 3.95 (s, 3H), 3.79 (dd, J = 13.5, 6.7 Hz, 1H), 3.62 (dd, J = 13.5, 6.7 Hz, 1H), 1.48 (d, J = 6.7 Hz, 6H), 1.16 (t, J = 9.3 Hz, 6H). ESI-HRMS calcd. for C₈H₆N₂O₃: 134.1869 [M+H]⁺, found 134.1863.

2.12.2. Preparation of Synthetic Intermediate 2–4 (4-methoxy-5-methylsulfanyl-2,2'-bipyridinyl-6-carboxylic acid disopropylamide) Compound 2–3 (810 mg, 2.6 mM) was dissolved in 10 mL of anhydrous THF, and the solution was stirred at –78 °C under an argon atmosphere; 3.3 mL of 1.6 M n-BuLi in hexane (5.2 mM) was then slowly assayed. After the solution was stirred for 1 h, 0.38 mL of MeSSA (4.2 mM) was added during a 5 min period at this temperature. The reaction mixture was then quenched by the addition of 1 mL of MeOH and concentrated at room temperature. The resulting residue was further purified via chromatography on silica gel using 1:1 n-hexane/EtOAc as the eluent to give compound 2–4 as a light-brown solid (659 mg, yield 71%). For purified 2–4, ¹H NMR (500 MHz, DMSO-d₆) δ 8.71 (d, J = 4.3 Hz, 1H), 8.30 (d, J = 7.9 Hz, 1H), 8.00 (s, 1H), 7.96 (td, J = 7.8, 14.1 Hz, 1H), 7.48 (ddd, J = 6.8, 5.2 Hz, 1H), 4.66 (s, 3H), 3.66–3.56 (m, 1H), 3.53–3.44 (m, 1H), 3.49 (dt, J = 13.3, 6.0 Hz, 1H), 2.33 (s, 3H), 1.50 (d, J = 6.7 Hz, 6H), 1.14 (br, 6H). ESI-HRMS calcd. for C₉H₁₄N₂O₅S⁺: 360.1746 [M+H]⁺, found 360.1740.

2.12.3. Preparation of Compound 2 Though Synthetic Intermediates 4–2–6 Compound 2–4 (618 mg, 1.7 mM) was dissolved in 10 mL of anhydrous THF. After the solution was cooled to –78 °C, 3.4 mL of 1.0 M DIBAL-H in heptane (3.4 mM) was slowly added. The reaction mixture was stirred at –78 °C for 1.5 h and was then quenched by the addition of 2 mL of MeOH. After the solution was mixed with 3 mL of EtOAc at –78 °C, it was warmed to room temperature, and another 10 mL of EtOAc was added. Then, the mixture was washed twice with a saturated aqueous solution of NaHCO₃. The organic phase was dried over anhydrous Na₂SO₄ overnight, filtered and concentrated to dryness under reduced pressure to give the crude compound 4, 4-methoxy-5-methylsulfanyl-2,2'-bipyridinyl-6-carboxaldehyde.

Compound 4 was suspended in 6 mL of H₂O and 6 mL of t-BuOH. Then, 12 mL of 2-methyl-2-buten-1.17 g of NaH₂PO₄+2H₂O (7.5 mM) and 675 mg NaClO₂ (7.5 mM) were added into this suspension, which was stirred at room temperature. The reaction was monitored via HPLC and completed in 2 h, at which point 10 mL of EtOAc was added. The aqueous phase was concentrated to dryness under reduced pressure. The resulting residue was dissolved in 10 mL of MeOH, filtered and concentrated to dryness. The obtained residue was redissolved in 10 mL of MeCN, which was then filtered and concentrated to dryness to give the crude compound 2–5, 4-methoxy-5-methylsulfanyl-2,2'-bipyridinyl-6-carboxylic acid. The obtained compound 2–5 was dissolved in 4 mL of 40% (wt/wt) aqueous hydrobromic acid at room temperature. The reaction mixture was stirred at 140 °C for 2 h before it was cooled to room temperature. The mixture was then neutralized with a saturated aqueous solution of NaHCO₃ and concentrated to dryness to give the crude compound 2–6, 4-hydroxy-5-methylsulfanyl-2,2'-bipyridinyl-6-carboxylic acid.

The obtained compound 2–6 was suspended in 20 mL of THF. Then, 760 mg of DCC (3.7 mM), 420 mg of l-leucine methyl ester hydrochloride (2.3 mM) and 340 mg of DMAP (2.8 mM) were added to this suspension at room temperature. After the reaction mixture was stirred for 12 h, it was filtered and concentrated under reduced pressure. The resulting solid was redisolved in 16 mL of THF and 4 mL of an aqueous solution containing 400 mg of LiOH·H₂O (9.5 mM), and the solution was stirred at 0 °C for 2 h before the THF was removed. After the pH was adjusted to 2 by 1 M HCl, the reaction mixture was extracted five times with 10 mL of EtOAc. The organic phase fractions were combined, dried over anhydrous Na₂SO₄ overnight, filtered, concentrated under reduced pressure and then subjected to semi-preparative HPLC to give TF-bound compound 2 (20 mg) as a yellow solid. This semi-preparative HPLC and the subsequent removal of TFA were performed according to the methods that were described for compound 1. For purified 2, ¹H NMR (500 MHz, DMSO-d₆) δ 8.68 (d, J = 6.0 Hz, 1H), 8.33 (d, J = 7.9 Hz, 1H), 7.97 (t, J = 7.4 Hz, 1H), 7.94 (s, 1H), 7.48 (t, J = 5.4 Hz, 1H), 4.48 (m, 1H), 2.33 (s, 3H), 1.90–1.76 (m, 1H), 1.76–1.65 (m, 1H), 1.63–1.53 (m, 1H), 0.94 (d, J = 5.0, 3H), 0.93 (d, J = 5.9, 3H). ESI-HRMS calcd. for C₉H₁₂N₂O₅S⁺: 376.1331 [M+H]⁺, found 376.1332.

3. Results

3.1. CaeD and ColD are metallo-dependent amidohydrolase superfamily proteins

Previous studies showed that the inactivation of caeD (also named crml) or colD (also named clmA) genes resulted in the accumulation of a 2,2'-BiPy-l-leucine intermediate, i.e., desulfurated 1 in the CAE-producing strain A. cyanogriseus WH1–2216–6 and sulfurated 2 in the COL-producing strain Streptomyces sp. CS40 [24,26]. In particular, the transformation of 1 to carboxylate 3 was observed in the cell homogenate of the recombinant S. coelicolor strain that expressed caeD [26], suggesting that CaeD, as well as its counterpart ColD, has proteolytic activity. Sequence analysis revealed that CaeD and ColD are members of subgroup A of the metallo-dependent amidohydrolase superfamily of proteins. Typically, the members in this subgroup have a conserved metal-binding site that is composed of four histidine residues. In the case of CaeD, these four histidine residues are H61, H63, H227, and H247, and they are joined by one aspartic acid residue, D317, to enable the chelation of two metal ions (e.g., Zn²⁺ or Ni²⁺) and form a binuclear metal center (Fig. 2a) [34,35]. A structural model of CaeD was constructed on the basis of the crystal structures of several homologous metallo-dependent amidohydrolase enzymes from the same superfamily, i.e., the microbial ochratoxinase [36] and the prolidases Sgx9260c and Cc2672 [37,38]. This model predicts that an additional carboxylated lysine residue (K186) in CaeD is involved in the formation of metal ion binding site. Consequently, if the metal ions at the active site are both zinc ions (Zn²⁺ and Zn²⁺), residues H61, H63, and D317 specifically bind to Zn²⁺, in contrast to residues H227 and H247, which bind to Zn²⁺, while the both ions could coordinate residue K186 (Fig. 2b and 2c).

3.2. Each molecule of CaeD contains two zinc ions

Despite our numerous attempts, the soluble expression of CaeD was very difficult to achieve in E. coli. Even in the co-expression system in which the plasmid pGro7, which enables the expression of two molecular chaperones known to cooperate in the folding process was added [39], little target protein product was observed in the soluble fraction. Since CaeD is essentially a metallo-
Fig. 2. a) Sequence alignments of CaeD and CoID with Cc2672 (PDB code 3MTW) [37,38]. b) The comparison of the structure model of CaeD (green) with SgX9620b (PDB code 3BE7, blue). The numbered residues in CaeD are proposed to bind the zinc ion [37,38]. c) The key residues of CaeD that bind the zinc ion based on the structural model.
dependent protein according to the above bioinformatics analysis, 1 mM ZnCl₂ was added to the co-expression system, which indeed led to the robust production of soluble CaeD (0.71 mM) (Fig. S1). We then examined the ability of CaeD to chelate different types and concentrations of ions using ICP-OES. We found that CaeD incorporates two zinc ions, rather than any Ni²⁺, Co²⁺ or Fe²⁺, per protein molecule (Table 1).

### 3.3. Chemical Synthesis of compounds 1–4

To confirm the metallo-dependent amidohydrolase activity of CaeD and ColD, we synthesized compounds 1 and 2 to act as substrates and compounds 3 and 4 to act as the product standards by slightly modifying the methods that were previously reported [33] (Fig. S2). Briefly, the synthesis of 3 started from the commercially available 4-nitro-2,2'-bipyridinyl N-oxide and proceeded with the replacement of the nitro group with a methoxy group using anhydrous sodium methoxide in methanol. Then, a nitrile group at the C6 position was introduced by using diethyl cyanophosphonate as a reagent. Finally, to give compound 3, the hydrolysis of the nitrile and methyl was performed in one step in 40% (wt/wt) aqueous hydrobromic acid. The conjugation of i-Leu was performed...
in one pot. The amide was formed by using \( l\)-leucine methyl ester hydrochloride as a reagent, then the methyl ester was hydrolyzed to afford TFA-bound intermediate \( 1 \). In contrast, the synthesis of \( 4 \) was branched from intermediate \( 1-2 \), and intermediate \( 2-5 \) was prepared from \( 1-2 \) by a four-step sequence that is known to introduce a methythio moiety at \( C5 \) \[33]\). In a second sequence of reactions, the new aldehyde group of \( 2-5 \) was oxidized to carboxyl by \( \text{NaClO}_2 \), and the methoxy group at position \( C4 \) was hydrolyzed in aqueous hydrobromic acid to give compound \( 4 \). To produce the target molecule \( 2, l\)-Leu was introduced under similar conditions as in the production of compound \( 1 \).

### 3.4. Biochemical characterization of CaeD

CaeD activity was examined by incubating the purified recombinant protein with compound \( 1 \), which was completely consumed in the presence of CaeD in a 2 h period (Fig. 3a). Analyzing the profile of the products revealed a new compound ([M + Na\(^+\)]/m/z: calcld. 239.0427 for \( C_{11}H_8N_2O_3Na \), found 239.0432). The identity of this product as compound \( 3 \) was validated by comparing it to the synthetic standard, thereby demonstrating that CaeD catalyzes the removal of the extended \( l\)-Leu of \( 1 \). The conversion to the carboxylate, \( 3 \), occurred in the absence of exogenous \( \text{ZnCl}_2 \), consistent with the above finding that the purified protein chelates ions to form an active center. To evaluate the metal ion-binding affinity of CaeD, the reaction mixture that contained CaeD was supplemented with the general ion chelator ethylenediaminetetraacetic acid (EDTA, to a final concentration of 10 or 20 mM) or the \( \text{Zn}^{2+} \)-specific chelator 1,10-phenanthroline (Phen, to a final concentration of 0.25, 0.5, 1, 2 or 5 mM). Intriguingly, none of these attempts affected the production of \( 3 \) and thus the amidohydrolase activity of CaeD (Fig. 3a).

#### 3.5. Validation of the residues necessary for the metal ion-binding site of CaeD

Based on the bioinformatics analysis, residues \( H61, H63, H227, H247, D317 \) and \( K186 \) are expected to form the metal ion-binding site of CaeD. To determine whether each of these residues fulfills this expectation, we mutated these residues to Ala, by expressing CaeD mutants using the method that was established to prepare the wild-type CaeD. Consequently, the mutants CaeD-H61A (0.64 mM), CaeD-K186A (0.10 mM), CaeD-H227A (1.05 mM), CaeD-H247A (1.30 mM), and CaeD-D317A (1.06 mM) were prepared, while the mutant CaeD-H63A did not solubly express (Fig. S1). The ICP-OES analysis showed that CaeD-H61A, CaeD-K186A and CaeD-D317A incorporated less zinc than the wild type did; in contrast, H227A and H247A incorporated more zinc than the wild type did (Table 1). Subsequently, we examined the amidohydrolase activity of these mutants. Only the K186A mutant retained the ability to catalyze the conversion of \( 1 \) to \( 3 \) (Fig. 3b), and like the wild-type activity, it was not sensitive to the addition of EDTA (20 mM) or Phen (5 mM), indicating that residue K186 is unnecessary for metal ion chelation and CaeD activity.

#### 3.6. Biochemical characterization of ColD

ColD, which shares 58% sequence identity to CaeD, was proposed to hydrolyze the C5-sulfurated 2,2′-BiPy-\( l\)-leucine intermediate \( 2 \) to carboxylate \( 4 \) in the biosynthetic pathways of COLs. ColD can form a metal ion-binding site with four histidine residues \( (H62, H64, H228, \text{and} H248) \), one aspartic acid residue \( (D318) \) and one lysine residue \( (K187) \) (Fig. 2a), consistent with the notion that ColD is functionally related to CaeD as a metallo-dependent amidohydrolase. Using the method that was established for CaeD preparation, we purified soluble ColD (0.046 mM) from \( E. coli \), albeit in much lower yield and quality than CaeD (Fig. S1). We then examined the amidohydrolase activity of ColD. During a 2 h incubation period, \( 2 \) was completely converted into \( 4 \) ([M + H]\(^+\)]/m/z: calcld. 263.0485 for \( C_{12}H_7N_2O_4S \), found 263.0490) in the presence of ColD (Fig. 3c). This conversion proceeds in the absence of exogenous \( \text{ZnCl}_2 \) or in the presence of the general ion-chelator EDTA (10 and 20 mM). However, the production of \( 4 \) clearly decreased when the concentration of the \( \text{Zn}^{2+} \)-specific chelator Phen (Fig. 3d) was increased, indicating that the ion-binding affinity of ColD is lower than that of CaeD.

#### 3.7. Kinetic properties of CaeD and ColD

Exploiting the available 2,2′-BiPy-\( l\)-leucine substrates \( 1 \) and \( 2 \), which differ from each other based on C5 functionalization, we determined the steady-state kinetic parameters of the reactions that catalyzed by CaeD and ColD, respectively (Table 2, Fig. S3). Both CaeD and ColD converted \( 1 \) and \( 2 \), indicating their promiscuity in amidohydrolase activity. Overall, the CaeD-catalyzed conversions were more efficient than the ColD-catalyzed conversions, in part because the recombinant CaeD product was of higher quality than the recombinant ColD product. CaeD clearly preferred the C5-desulfurated \( 1 \) as the substrate, while ColD preferred the C5-sulfurated \( 2 \) as the substrate. These findings are consistent with the specific roles that CaeD and ColD play in the biosynthetic pathways of CAEs and COLs, respectively.

### 4. Discussion

In this study, we demonstrated that CaeD and ColD are members of the metallo-dependent amidohydrolase superfamily of proteins and catalyze the removal of the “auxiliary” leucine residue from the 2,2′-BiPy-\( l\)-leucine intermediates during the biosynthesis of the structurally related 2,2′-BiPy antibiotics CAEs and COLs, respectively. The soluble production of ColD or CaeD in \( E. coli \) requires the addition of exogenous \( \text{ZnCl}_2 \), indicating that zinc ions play a crucial structural role in the formation of their active protein folds. Metallo-dependent enzymes typically lose their activity in the presence of metal chelators, as exemplified by the ochratoxinas from \( \text{Aspergillus niger} \), whose activity can be partially inhibited by the addition of the general chelator EDTA \[40\] or completely abolished by the addition of the \( \text{Zn}^{2+} \)-specific chelator Phen \[36\]. In contrast, CaeD binds zinc very strongly, as the addition of EDTA or Phen had no impact on its hydrolytic activity. The activity of ColD, the homolog of CaeD in the biosynthetic pathway of COLs, is relatively more sensitive to the presence of Phen, suggesting that this protein chelates zinc ions more poorly than CaeD does. Site-specific mutagenesis studies revealed that the residues \( H61, H63, H227, H247 \) and \( D317 \) of CaeD participate in the chelation of zinc ions and the formation of a functional binuclear metal center. The mutation of \( H61A, H227A, H247A \) and \( D317A \) could disrupt the metal ion incorporation and thereby abolish the amidohydrolase activity of CaeD.

### Table 2

| Proteins | Substrate | \( K_m \) (mM) | \( k_{cat} \) (min\(^{-1}\)) | \( k_{cat}/K_m \) (mM\(^{-1}\) min\(^{-1}\)) |
|----------|-----------|---------------|-----------------------------|---------------------------------|
| CaeD     | 1         | 1.51 ± 0.15   | 763.6 ± 46.72               | 506.4                           |
|          | 2         | 0.44 ± 0.06   | 23.60 ± 1.25                | 54.00                           |
| ColD     | 1         | 0.37 ± 0.03   | 1.95 ± 0.05                 | 5.31                            |
|          | 2         | 0.12 ± 0.01   | 0.79 ± 0.02                 | 6.64                            |

Six assays were performed in total. The data represents mean ± S.E.M. The \( k_{cat}/K_m \) value was calculated based on the mean value of \( K_m \) and \( k_{cat} \).

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\[ * \] A blank space may be inserted here to denote a figure or table that is not included in the text.
CaeD. The H63 mutation resulted in the failure of the preparation of the soluble CaeD-H63A recombinant protein, suggesting that this residue is indispensable for zinc ion chelation and the subsequent proper protein folding. To our knowledge, some of the metallo-dependent amidohydrolase superfamily proteins that have been characterized thus far, e.g., urease, dihydroorotase, ochratoxinases, and the prolidases Sgx9260c and Cc2672, are involved in degradative metabolism. The biochemical characterization of CaeD and ColD presented here should generate interest in the associated biosynthetic pathways of natural products, where the roles played by amidohydrolase homologs appear have been less appreciated.

In the biosynthetic pathways of CAEs and COLs, extensive isotope-labeling experiments have revealed that the functional association of Cae/ColA1 and Cae/ColA2 is enough to construct the 2,2'-BiPy backbone [25]. These findings raise an interesting question regarding the role of the Cae/ColA3-catalyzed Leu extension, which appears to be “auxiliary”; however, this extension has been confirmed to be indispensable for the generation of the 2,2'-BiPy intermediates (Fig. 1b) [24–27]. Over the past decade, modifications of growing acyl or peptidyl intermediates has long been known to be able to occur on modular PKSs or NRPSs in both cis and trans manners. The former depends on the activities of the domain(s) embedded in the module, whereas the latter involves the actions of protein(s) that are independent of the assembly line machinery [41]. As proposed here in 2,2'-BiPy biosynthesis, on-line enzymatic cyclization to achieve correct active conformation is no exception [42]. Such PKS or NRPS precedents involving new chemistry have increasingly been characterized, highlighting the promising recruitment ability of thioester assembly-line platforms, particularly when multiple internal and external activities are necessary for complex conversions.

Conflict of interest

The authors declare that they have no conflicts of interest with contents of this article.

Author contributions

MC and WL designed the study. MC performed most of the experiments, analyzed the primary data and wrote the draft of the manuscript. MC and WL supervised the whole research project and revised the manuscript. All authors expressed and assayed the site-directed mutants. WL supervised the experiments, analyzed the primary data and wrote the draft of the manuscript. J. Rodriguez-Martinez, M. Chen, G. Tan, and Y. Liu contributed to the primary data analysis. K. C. Wong Education Foundation and Chang-Jiang Scholars Program of China.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.sysbio.2017.07.002.

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